Modification and Validation of a Semi-Automated *In vitro* Method for Predicting the Glycemic Index of Starchy Foods

by

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ABSTRACT

Modification and Validation of a Semi-Automated *In vitro* Method for Predicting The Glycemic Index of Starchy Foods

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The glycemic index (GI) can assist with determining the effect of food on blood glucose levels. The Englyst method is commonly used *in vitro* method to predict the GI, but it does not use food as eaten. The aim of this study was to establish and validate a modified semi-automated Englyst method that better estimates the GI of foods as consumed. The method was successfully established through a simulated upper gastrointestinal digestion protocol and compared with *in vivo* GI values. Predicted GI (pGI) was calculated from starch digestion profiles. *In vivo* GI was moderately correlated with pGI ($r(18) = 0.51$; $p < 0.05$), but highly correlated ($r(18) = 0.72$; $p < 0.05$) with rapidly digestible starch (RDS). Both pGI and RDS can be used as predictors for *in vivo* GI. This novel finding and the modified method can be used together to effectively predict the GI of starchy foods.

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
AMG	Amyloglucosidase
AUC	Area Under the Curve
CDA	Canadian Diabetes Association
CV	Coefficient of Variation
DM	Dry Mass
eGI	Estimated Glycemic Index
G	Mass of Glucose
GI	Glycemic Index
GOPOD	Glucose Oxidase-Peroxidase Enzyme Cocktail Mixture
HbA1c	Glycated Haemoglobin (A1c)
HI	Hydrolysis Index
iAUC	Incremental Area Under the Curve
LADA	Latent Autoimmune Diabetes in Adults
pGI	Predicated Glycemic Index
PHAC	Public Health Agency of Canada
RDS	Rapidly Digestible Starch
RS	Resistant Starch
SDS	Slowly Digestible Starch
TS	Total Starch
TV	Total Volume
WHO	World Health Organization

1 CHAPTER 1

Background

1.1 Diabetes:

1.1.1 Definition:

Diabetes is one of the most common chronic diseases in developed and developing countries. Globally, there were about 347 million people with diabetes and 1.5 million diabetes-related deaths in 2012 (World Health Organization (WHO), 2016). According to WHO (2006), diabetes is defined as "a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin that it produces. Lack of insulin synthesis by the pancreas or its ineffective use by the body leads to high blood sugar concentrations, known as 'hyperglycemia'. This can negatively affect the function of body systems, including the cardiovascular and nervous systems. Individuals with diabetes may have one or more symptoms, including unusual thirst, frequent urination, weight change (gain or loss), extreme fatigue or lack of energy, blurred vision, frequent or recurring infections, cuts and bruises that are slow to heal, tingling or numbness in the hands or feet, and in men, trouble getting or maintaining an erection (Canadian Diabetes Association (CDA), 2016).

1.1.2 Types of Diabetes:

There are three main types of diabetes: type 1, type 2 and gestational diabetes. Type 1, or 'insulin-dependent diabetes', represents about 5 to 10% of individuals with diabetes. Type 1 diabetes occurs mainly in childhood and adolescence; however, it can occur in adulthood as well. This type of diabetes is caused by cellular-mediated autoimmune destruction of the β-cells of the pancreas. The destruction of β-cells means that the pancreas either cannot produce insulin at all, or produces too little. Insulin shortages result in the accumulation of sugar in the bloodstream (rather than being used by cells for energy), which results in hyperglycemia. Type 1 diabetes

treatment depends on insulin, but meal planning is also important to manage blood sugar control. Latent Autoimmune Diabetes in Adults (LADA), another term used to describe type 1 diabetes, defines a small number of individuals who have type 2 diabetes symptoms. LADA patients also have an immune-mediated loss of pancreatic β-cells (CDA, 2016).

Type 2 diabetes is the most common type of diabetes, comprising approximately 90% of people with diabetes. In type 2 diabetes, the pancreas cannot produce sufficient amounts of insulin, or body cells are increasingly resistant to insulin. This defect leads to an accumulation of sugar in the bloodstream, leading to hyperglycemia. Predisposing factors for type 2 diabetes include being overweight and having a lack of physical activity; therefore, type 2 diabetes can be managed with a healthy diet, exercise, and/or medication (Health Canada, 2013). Lastly, gestational diabetes occurs during pregnancy and results in high blood sugar levels among women who did not have diabetes pre-pregnancy. Roughly 2-4% of pregnant women develop gestational diabetes. Gestational diabetes increases the risk for diabetes in the future for both the mother and the child (WHO, 2015).

1.1.3 Risk Factors, Complications, and Diagnosis of Diabetes:

According to WHO (2015), people have a higher likelihood of developing diabetes if they have one or more of the following risk factors: overweight or obesity (central obesity), family history of diabetes, African, Asian, Hispanic, or Aboriginal ethnicity, high blood pressure, high blood cholesterol, and/or hyperlipidemia. Diabetes-related complications can be serious and may lead to death if not treated properly. Examples of these complications include chronic renal disease, foot ulceration, non-traumatic lower limb amputation, retinopathy (which can lead to blindness), cardiovascular disease, heart attack, stroke, anxiety, damaged nerve ends, and in men,

erectile dysfunction (CDA, 2016). Individuals can be diagnosed with diabetes via blood testing. Individuals are diagnosed as diabetic if they have a fasting blood glucose 7.0 mmol/L or higher, a random blood glucose test 11.1 mmol/L or higher, or if their glycated hemoglobin test (A1C) is 6.5% or higher. Diabetes can also be diagnosed using an oral glucose tolerance test, where the individual consumes a glucose solution before performing the blood test. People with a blood glucose concentration of 11.1 mmol/L or higher 2 hours after drinking the solution are diagnosed with diabetes (CDA, 2016). These complications bring to the surface the importance of diabetes management and treatment, which is crucial to reducing the risk of developing diabetes-related complications.

1.1.4 Diabetes in Canada and Worldwide:

In Canada, there are more than 11 million people who live with diabetes or prediabetes. The prevalence of diabetes increased dramatically among Canadians in a ten-year period (1999 to 2009), with 70% more cases than in the previous decade. It is estimated that the prevalence of diabetes will increase to 44% in Canada by 2025 (CDA, 2016). Most people with diabetes are aged between 35 and 44 years. The prevalence of diabetes increases with age; more than 50% of Canadians with diabetes are between the ages of 25 and 64 years. The prevalence of diabetes in Canada is higher among males (7.2%) than females (6.4%) (Public Health Agency of Canada (PHAC), 2011). Every three minutes, there is one person diagnosed with diabetes worldwide, and diabetes is anticipated to be the seventh leading cause of mortality internationally by 2030 (WHO, 2014).

1.1.5 The Impact of Diabetes on Health and the Economy in Canada:

Diabetes is associated with several health complications. According to the CDA (2015), individuals with diabetes are three times more likely to be hospitalized with cardiovascular disease and twelve times more likely to have the end-stage renal disease than healthy people. Diabetes is also associated with other chronic disease risk factors. In Canada, 36% of individuals with diabetes have at least two chronic disease conditions, including high blood pressure, cardiovascular disease, mental health, and/or arthritis. One-third of the amputations conducted between 2011 and 2012 were in individuals with diabetes; foot ulcers affect between 15% to 25% of diabetes sufferers (Pelletier, Dai, Roberts, & Bienek, 2012). According to PHAC (2015), 30% of individuals with diabetes show symptoms of depression, while 60% of people with depression have a high risk of developing type 2 diabetes. Diabetes may also carry some stigma; 37% of Canadians with diabetes do not feel comfortable telling others about their illness. Importantly, the complications of diabetes can lead to death. In 2015, one out of ten Canadians with diabetes died due to complications related to diabetes (PHAC, 2015). In addition to the health impacts of diabetes, there are economic effects as well. Diabetes affects the Canadian economy on both an individual and a governmental level. Three-quarters of Canadians with diabetes do not follow their treatment regime appropriately. Diabetes is costly to the individual; diabetes medication, devices, and supplies cost patients more than 3% of their income, or more than $1,500 annually (CDA, 2015). At the governmental level, the Institute for Clinical Evaluative Sciences reported that Canadians with diabetes cost the healthcare system twice as much as people without diabetes, leading to billions of dollars spent on diabetes-related health care annually (Rosella et al., 2016). These statistics illustrate the importance of increasing awareness about diabetes and reducing its effect on public health and the economy.

1.1.6 Diabetes Management and Treatment:

Diabetes treatment and management mainly involve a change of lifestyle. Increasing physical activity to reduce bodyweight, and diet planning to control blood glucose have the strongest beneficial effects on the health of individuals with diabetes (CDA, 2016). In a recent report, the American Diabetes Association (ADA) recommended that individuals with diabetes increase their physical activity to improve their health and reduce the risk of complications (ADA, 2016). The report recommended that the exercise program should include at least 2.5 hours per week of moderate intensity exercise, with a 50% to 70% maximum heart rate and resistance exercise at least twice a week. Individuals with diabetes should also reduce their sedentary time by taking a break after 90 minutes of sitting. Dietary management is as important as physical activity in the treatment of diabetes. Many studies have shown that dietary intervention is the first line of defence in diabetes control; particularly in type 2 diabetes, in which diet plays an important role in the control of hyperglycemia (Würsch & Pi-Sunyer, 1997). A great deal of research has been conducted to investigate how food could improve the blood glucose profile in diabetes patients. Diabetes guidelines recommend consuming a balanced diet with a variety of vegetables, fruits, whole grains, complex carbohydrates, low-fat milk products, and meat/meat alternatives. Adding fats in small amounts and consuming smaller portion sizes to control bodyweight and blood cholesterol are also recommended (CDA, 2016). This varied diet helps individuals with diabetes control their blood glucose levels, lower or maintain their bodyweight, reduce blood lipids, decrease the risk of developing hypertension and prevent or delay diabetes complications.

1.2 The Importance of Carbohydrates in Diabetes Management:

Carbohydrates are one of the main components used in the dietary management of diabetes due to their influence on blood glucose level. There are three types of carbohydrates: starch (complex carbohydrates), sugars (simple carbohydrates), and fibre.

1.2.1 Fibre:

Fibre is an important element in controlling blood glucose, which is why research related to dietary fibre and its effect on blood glucose has increased in recent years. Some studies have found that dietary fibre can significantly reduce postprandial (i.e., post-meal) glucose concentrations (Anderson et al., 2009; Kaczmarczyk, Miller, & Freund, 2012). There exist two types of dietary fibre: soluble and insoluble. Recent studies have shown that soluble fibre can reduce postprandial blood glucose (ADA, 2016; Joslin Diabetes Center, 2014). Soluble fibre acts by increasing the viscosity of digested food and slowing the movement of food from the stomach to the intestines. This mechanism leads to a decrease in the sugar absorption from the small intestine and controls blood glucose levels (El Khoury, 2012). Beta-glucan is an example of soluble fibre that has many bioactive properties, and that shows promising effects on bodyweight, blood glucose, and hypercholesterolemia control (Daou & Zhang, 2012; El Khoury et al., 2012; Wolever et al., 2010). Foods that are high in fibre tend to have a low glycemic index (GI) and a positive impact on blood glucose control.

1.2.2 Starch:

Starch is another form of carbohydrate that plays an important role in managing blood glucose levels. Starches are classified as one of three types: rapidly digestible starch (RDS), slowly digestible starch (SDS), or resistant starch (RS) (Englyst et al., 1992). Diabetes guidelines recommend that people with diabetes should consume complex carbohydrates (i.e., starch) as their main source of carbohydrate and avoid consumption of simple sugars (ADA, 2013; CDA, 2016). Complex carbohydrates have a positive effect on blood sugar because they have slower digestion and absorption rates, which help in controlling blood glucose levels and in avoiding hyperglycemia after meals. Recently, the interest in studying SDS and resistant starch has increased. Some studies have found that foods high in SDS have a lesser effect on blood glucose than foods high in RDS (Englyst et al., 1992, 1999). Based on this concept, many researchers have found that foods with high levels of SDS and RS also have a low GI (Englyst et al., 1999; Goni et al., 1997). Low GI foods that are high in SDS and RS may be helpful in improving blood glucose levels among individuals with diabetes.

1.3 Glycemic Index (GI):

1.3.1 Definition:

The GI is an indicator used to measure the ability of carbohydrates to increase blood glucose after consuming food. The GI, which ranges from 0 to 100, ranks carbohydrates in foods based on their effect on blood glucose levels two hours after eating. The GI is classified into three groups. Foods with a low GI (below 55) are typically slowly digested and absorbed, leading to a gradual increase in blood glucose and insulin levels. Examples of low-GI foods are beans, small seeds, nuts, whole wheat, and most fruits and vegetables. Medium-GI foods (GI 56-

69) include pita bread, basmati rice, and bananas. High-GI foods have a GI of 70 or higher and are rapidly digested and absorbed, leading to a marked fluctuation in blood glucose. Examples include corn syrup, white bread, white rice, white potato, and Corn Flakes (Foster-Powell et al., 2002). Low-GI diets are proven to have many health benefits such as improving blood glucose and lipids, reducing insulin levels, and reducing insulin resistance for individuals with diabetes. Further, low-GI diets help control body weight by reducing the effect of appetite and delaying the feeling of hunger (The University of Sydney, 2016).

Recently, the application of GI measurements became an important tool to assist people with diabetes improve their health and control blood glucose, as there is strong evidence indicating that low-GI foods not only reduce the risk of developing diabetes (Chiu et al., 2011) but also help to improve the management of diabetes (Barclay et al., 2008). In 1998, WHO and the Food and Agriculture Organization of the United Nations (FAO) published a report about the importance of GI. The report encouraged developed countries to increase the amount of low-GI foods in the food guidelines in order to reduce the prevalence of chronic diseases, including diabetes, obesity, and cardiovascular disease. Around the same time, a study published by the Harvard School of Public Health found that chronic diseases such as cardiovascular disease and type 2 diabetes were significantly associated with the high consumption of high GI foods (Salmerón et al., 1997).

1.3.2 Measurement of Glycemic Index:

In 1981, Jenkins and colleagues developed a new method to measure the effect of food on blood glucose. They established a method based on the concept that foods high in simple carbohydrates are digested and absorbed quickly into the bloodstream and tend to have a high

GI; whereas, foods high in complex carbohydrates are digested and absorbed slowly into the bloodstream and tend to have a low GI. Thus, they ranked foods on a scale from 0 to 100 based on their effect on blood glucose and called it the GI of food. This scale could be used to advise people with diabetes to select appropriate foods for the management of their disease. Foods with a lower GI also have a lower insulin response and help with long-term control of blood glucose and blood lipids.

The GI of food is the ratio between the incremental area under the curve (iAUC) of the tested food and the iAUC of the reference food (glucose or white bread). To measure the GI value of food *in vivo*, volunteers who have been fasting overnight are served a starchy food that contains 50 g of available carbohydrates. Blood samples are taken at 15-30 minute intervals for two hours. The GI value measurement of tested food should be performed on at least two to three occasions. The iAUC is calculated from blood measurements of the tested food. The GI value is then calculated by dividing the iAUC of the tested food by the iAUC of the reference food and multiplied by 100. The results are ranked from 0 to 100 based on the GI value. GI measurements should be performed with the same amount of available carbohydrates in both the tested food and the reference food. The GI is calculated based on average results of at least ten different subjects before being published as the GI value for the specific food (Brouns et al., 2005).

Most current methods use glucose as the reference food when measuring GI, assigning it a glycemic value of 100. The advantage to using glucose is that it becomes a standard reference worldwide, with a maximum value of 100. However, some studies have used white bread as a reference because it is easier to use in locales where white bread is a basic food item. However, the disadvantage of using white bread is that the available carbohydrate content and portion size is not standardized (Foster-Powell et al., 2002).

1.3.3 The Importance of Glycemic Index to Health:

Interest in the GI value of food and its effect on human health has increased as of late. Recent studies have shown that low-GI foods have a positive impact on bodyweight and the prevention and control of certain chronic diseases. Chiu et al., (2011) showed that people who follow a long-term low-GI diet have a significantly lower chance of developing type 2 diabetes, cardiovascular diseases, and age-related diseases compared to people who do not follow a low-GI diet. High blood glucose levels after eating high-GI foods can increase the risk of developing systemic glycation stress and may also raise insulin levels (Uchiki et al., 2012). High-GI food is not only associated with high postprandial glucose and diabetes; several studies have also shown that high peak blood glucose is a risk factor for cardiovascular and renal disease (Balkau et al., 1998; Fox et al., 2012). One study found that people who consume white bread and high-sugar cereals for breakfast have a higher risk of developing diabetes, coronary disease, and cancer (Barclay et al., 2008). In 2007, Chui and colleagues found that age-related adult macular degradation (AMD), which leads to blindness in older individuals, was 42% higher among people on a high-GI diet compared with people on a low-GI diet. The researchers also found that AMD can be reduced by 20% when individuals consume a greater proportion of low-GI foods. A systemic review of several human trials found that a low-GI diet positively impacts pregnancy outcomes. Specifically, a low-GI diet reduced postprandial responses and the need for insulin during pregnancy. Additionally, the low-GI food was somewhat beneficial to the outcomes of overweight and obese pregnant women (Mohd Yusof et al., 2014). Low-GI foods can help reduce and control bodyweight. Recent studies on humans and animals have demonstrated the benefits of low-GI food on bodyweight. For example, in one study, a group of male mice was divided into two groups: one group was fed a high-GI diet, and the other group was fed a low-GI

diet for four months. The results showed that mice fed the high-GI diet were 71% more overweight and had 8% less lean mass compared to the group fed a low-GI diet. Further, the postprandial glucose and insulin levels were significantly higher in the mice fed a high-GI diet (Pawlak et al., 2004).

Regarding diabetes management, several studies have shown significant evidence that a low-GI diet can reduce postprandial glucose in individuals with diabetes. For example, Brand-Miller et al. (2003) performed a meta-analysis of 14 randomized controlled trials which included 356 participants with diabetes. The aim of the study was to investigate whether a low-GI diet can improve hyperglycemia in individuals with diabetes compared with a high GI-diet. The results showed that the low GI-diet resulted in a 0.43% reduction in HbA1c compared with the high-GI diet. The authors concluded that even though the effect of a low-GI diet was small, it is nevertheless important for long-term control of blood glucose in individuals with diabetes. In another randomized controlled trial, Jenkins and colleagues (2012) examined the effect of a low-GI diet (which included different kinds of legumes) on the blood glucose of 121 individuals with type 2 diabetes. The results illustrated that the low-GI legume diet reduced HbA1c by 0.5% compared with the control group. The study also demonstrated that a low-GI legume diet could play a significant role in controlling the blood glucose of people with diabetes. These findings show the importance of a low-GI diet in blood glucose control and the maintenance of health among individuals with diabetes.

1.3.4 Limitations of the *in vivo* Measurement of Glycemic Index:

Although GI has become an important tool for measuring the effect of carbohydrates on blood glucose, a lot of variation in GI values exists across studies. The main limitation of *in vivo*

GI is the inter- and intra-individual variation in glycemic response. This was demonstrated in a study by Vega-Lopez et al. (2007), which investigated the inter-individual variability and intra-individual reproducibility of GI values of commercial white bread. Twenty-three healthy volunteers each underwent two rounds of intervention: one with white bread and the other with glucose as a reference. After each intervention, blood samples were drawn, and the iAUC and GI were calculated. The results showed that there was significant variation within an individual's results as well as among individuals who consumed the same amount of available carbohydrate from commercial white bread (50 g). Another study conducted by Vrolix and Mensink (2009) aimed to assess the variability of the glycemic response to a single food product in healthy individuals. In this study, the researchers assessed the GI of eight different common foods by using 40 healthy volunteers. Volunteers were divided into four groups; each group was randomly assigned to consume two different foods plus glucose as the reference food. The results illustrated a significant difference in the coefficient of variation (CV) of the reference food. Variation was found in both inter-individual (13-38%) and intra-individual (33-80%) iAUCs. Another study by Zeev and colleagues (2015) aimed to assess the effects of the same food on blood glucose response. The study included 800 participants who were served a total of 46,898 meals throughout one week. Participants' post-prandial glucose levels were measured and iAUC was calculated. The results showed a significant variation in postprandial response among participants who consumed the same food. For example, foods such as bread had a high variation among participants, with a mean glycemic response of 44 (SD ± 31) mg/dL. These results demonstrate the high variability in results obtained with the *in vivo* method for GI measurement.

Although these GI studies found considerable variation within and among individuals, the source of this variability in GI values remained unclear. Certain factors may influence the

interpersonal postprandial glucose response, including: insulin resistance (Parillo et al., 1992), heredity (Carpenter et al., 2015), lifestyle (Dunstan et al., 2012), and exocrine pancreatic and glucose transporter activity levels (Gibbs et al., 1995). Another factor that may be involved is the gut microbiota. Pioneering work by Ley and colleagues (2006) showed that gut microbiota were associated with a propensity for obesity and its complications. Later works demonstrated that microbiota were also associated with glucose intolerance, type 2 diabetes, hyperlipidemia, and insulin resistance (Karlsson et al., 2013; Le Chatelier et al., 2013; Qin et al., 2012; Suez et al., 2014; Turnbaugh et al., 2006; Zhang et al., 2013).

1.4 *In vitro* Glycemic Index:

The *in vivo* measurement of GI has several inherent limitations, such as interpersonal variation, ethical concerns, and the fact that it is unsuitable for routine food analysis. GI measurement is also costly and time-consuming. As a result, there has been increased interest in developing an alternative *in vitro* method to measure GI value. In 2006 *ad hoc* members of the Glycaemic Carbohydrate Definition Committee (Brooks et al., 2006) expressed some concern about using an *in vivo* method to determine the GI value of food because of the inter- and intra-individual variation in glycemic response. Therefore, the committee recommended establishing a robust *in vitro* method that can mimic *in vivo* conditions to predict GI values.

1.4.1 Classification of *in vitro* Glycemic Index Methods:

Most of the recent GI *in vitro* methods were based on principles used to measure the amount of carbohydrate in food—including total carbohydrates, fibre, and resistance starch. Additionally, most *in vitro* GI studies were performed simultaneously with *in vivo* studies, using

the same foods. The aim of using the two methods concurrently is to compare the GI values of the new *in vitro* methods with the GI values of the standard method (i.e., the *in vivo* trial). A strong correlation between the two methods indicates that the new method is reliable and can be used as an alternative to the *in vivo* method.

In general, *in vitro* digestion methods are classified into two main groups. The first group is known as the 'unrestricted method', and essentially uses test tubes for food mixing and digestion. Examples of studies utilizing this approach are Englyst et al. (1992), Goni et al. (1997), Muri et al. (1992), and Mishra, Monro, & Hedderley (2008). The second group is called the 'restricted method' and uses a dialysis bag for food analysis. Researchers who used this method over the past two to three decades include Jenkins et al. (1984), Granfeldt et al. (1992) and Brennan et al. (1996).

The initial interest in using an *in vitro* method arose at the end of the 1960s. The first trial was conducted in 1969 by Southgate, who aimed to measure the available carbohydrates in food using the pullulanase and amyloglucosidase enzymes. This method had some limitations, primarily related to incomplete starch removal (Southgate, 1969). In 1982, Englyst and colleagues developed a new method based on the analysis of dietary fibre, which involved a more specific and more effective procedure for starch digestion. In this method, α-amylase and pullulanase were used to hydrolyze RDS, and amyloglucosidase was used for the more resistant starch portion. In the same year, Jenkins et al. (1982) conducted research examining the effect of food processing on the digestibility of lentils and the effects of this processing on the blood glucose response. In that method, Jenkins used human saliva for starch digestion; the results of this *in vitro* trial were positively correlated with *in vivo* results. In 1986, Berry established a method to measure resistant starch based on the Englyst method. In that method, Berry attempted

to mimic the physiological conditions of digestion by removing the first step of the Englyst method, which involved heating the sample to 100˚C. The result of this modification was an increased resistant starch value (Berry, 1986). Until that time, most established methods did not aim to simulate physiological digestion conditions, and there was some variation between *in vivo* and *in vitro* GI results.

By 1990s, many endeavours had been made to improve *in vitro* methods and to make them more similar to *in vivo* digestion conditions. Most of the *in vitro* methods being developed focused on mimicking physiological conditions of digestion to improve the correlation with *in vivo* GI results. With this objective in mind, Granfeldt and Bjorck (1991) established a new method that mimicked physiological digestion and involved three *in vivo* digestion stages: oral, gastric, and intestinal. Other methods using the three physiological digestion stages were published the following year (Brighenti et al. 1992; Englyst et al., 1992; Muir & O'Dea, 1992). Since then, many *in vitro* methods have been established based on physiological conditions. The following section provides an overview of the three main phases of the *in vitro* method: oral, gastric, and intestinal.

1.4.1.1 The Oral Phase:

The oral phase is the first stage in food digestion, and it consists of two processes: 1) mechanical degradation of the food into smaller fragments by chewing, and 2) partial chemical breakdown of the starch complex by the α-amylase contained in saliva. By the end of the oral phase, a bolus is formed which then descends into the stomach via the esophagus.

In some previous developed *in vitro* methods, researchers used an actual chewed sample in the oral phase. For example, Muir and his colleagues (1995) used volunteers to chew food

samples until just before swallowing. The samples were then taken and weighed; results indicated that they contained an average of 0.1 g of carbohydrates. The same process was followed by Akerberg et al. (1998) after the volunteers cleaned their mouths and brushed their teeth; they chewed samples containing approximately 1 g of available carbohydrate 15 times, and the samples were then used for *in vitro* digestion. However, a comparison of the *in vitro* methods indicated that chewing had some limitations, including variation in the number of chewing times, the amount of available carbohydrate in each sample, and the fact that chewing was very subjective and differed between participants. Other researchers used *in vitro* techniques to mimic the chewing process. For example, Karkalas (1985) and Brighenti et al. (1998) used sieves with different pore sizes, Englyst et al. (1999) and Araya et al. (2002) both used mincers with specific pore sizes which were then compared with actual chewed samples, while Brennan and his colleagues (1996) preferred to use a chopper to prepare the samples. Finally, other methods did not consider food particle size in sample preparation, instead of using samples that were ground, milled or homogenized (Champ, 1992; Goni et al., 1997; Weurding et al., 2001).

1.4.1.2 Variation in the Oral Phase:

There has been a lot of variation in the oral phase of various *in vitro* methods. In terms of the chemical process, in methods that used actual chewing, samples were already mixed with saliva, which allowed partial degradation of starch by salivary amylase (Granfeldt et al., 1992; Muri et al., 1992). Several other methods utilized saliva *in vitro* to mimic the conditions of the oral phase. For example, Brighenti et al. (1995) and Lebet et al. (1998) incubated prepared samples in human salivary α-amlyase at 37˚C with pH 6.9 for 5 and 15 minutes, respectively. Urooj and Puttraj (1999) incubated their samples in actual human saliva, while Monro used α-

amylase in his method (Mishra et al., 2008). Finally, some methods did not use any α-amylase or saliva during the oral phase (Englyst et al., 1992; Brennan et al., 1996; Goni et al., 1997).

A variety of techniques has also been used to simulate the chewing process during the oral phase of *in vitro* methods. The difference in chewing techniques resulted in differing food particle sizes depending on the process that was used—especially since there is no standard procedure for food preparation in the oral phase. Food particle size in both *in vivo* and *in vitro* methods plays a critical role in the amount of starch digested and GI values (Holt & Brand Miller, 1994; Kim et al., 2004; Read et al., 1986). Consequently, most *in vitro* methodologies aimed to standardise the particle size of the prepared food sample against food that was actually chewed. Many *in vitro* methods used alternatives to chewing, such as sieving, mincing or chopping, to standardise the oral phase. These techniques are easier to standardise and produce more accurate results (Brighenti et al.,1998; Champ, 1992; Weurding et al., 2001).

Woolnough and his colleagues (2008) compared sample preparation procedures among different *in vitro* methods and examined the effect of procedures on the final amount of starch digested and glucose released for the same food. They also compared results of the *in vitro* method with the actual chewed sample of the corresponding food. In the experiment, several common starchy foods were prepared for the oral phase via sieving, mincing or chopping, and underwent digestion *in vitro*. Results showed that the starch digestion amount of pasta and wheat differed depending on preparation method. Wheat that was prepared via artificial chewing (i.e., sieving, mincing, or chopping) underestimated the glucose release compared with the actual chewed samples. In addition, a significantly different amount of starch digested was observed in the first 60 minutes, depending on the method of preparation (Woolnough et al., 2008). These results demonstrated that the variation between preparation techniques—even for the same

foods—could affect the amount of starch digested and in turn, the final results. The study illustrated the importance of standardising the oral phase of *in vitro* methods to produce more accurate results that are more comparable with those of *in vivo* methods.

1.4.1.3 The Gastric Phase:

The second stage of food digestion is the gastric phase. In the human body, food digestion during the gastric phase depends on three components: the mechanical motion of the stomach; the acidity (i.e., pH); and the gastric enzyme (i.e., pepsin). These components are essential for food digestion in the gastric phase. The food digestion in the gastric phase is also affected by other factors, such as gastric emptying time, food quantity, and viscosity (Guyton & Hall, 2000; Turnbull et al., 2005).

Most of the *in vitro* methods use pepsin proteolytic enzymes to ensure simulation of the physiological digestion process. Pepsin is a fundamental factor in the completion of starch digestion, as it breaks down the protein-starch interaction in food. Three decades ago, Holm and colleagues (1985) established an *in vitro* method to increase the access of the α-amylase enzyme to wheat samples by preincubating the samples with pepsin. In 1986, Holm et al. improved the method by incubating cereal samples with pepsin at 37˚C with pH 1.5 for 60 minutes on a magnetic stirrer. Granfeldt et al. (1992) developed an *in vitro* method based on the method by Holm & Bjorck (1985), in which a chewed sample was mixed with pepsin in a beaker and incubated for 30 minutes at 37˚C with pH 1.5. These methods found that treating samples with pepsin degraded the starch-protein interaction and increased the susceptibility of α-amylase to starch. Further, based on the results of previous methods, Brighenti et al. (1992) treated a spaghetti sample with HCl pH 2.0 and incubated the mixture with pepsin for one hour. Similarly,

both Muir & O'Dea (1992) and Englyst (Silvester et al., 1995) included pepsin in their methods; samples were incubated with pepsin at pH 2.0 for 30 minutes in a shaking water bath. Throughout the 1990s, many other *in vitro* methods included pepsin in the gastric phase to mimic physiological conditions (Brennan et al., 1996; Goni et al., 1997; Lebet et al., 1998; Weurding et al., 2001). However, some *in vitro* digestion methods omitted the gastric phase (Araya et al., 2002; Brighenti et al., 1998; Urooj & Puttraj, 1999).

1.4.1.4 Variation in the Gastric Phase:

The previously mentioned studies indicate that the main source of variation in the gastric phase among *in vitro* methods was the use or absence of pepsin. To investigate the effect of using pepsin on starch digestion and GI, Woolnough et al. (2008) ran an *in vitro* experiment with and without pepsin in the gastric phase. In the experiment, the various starchy foods were either incubated with pepsin for 30, 60 minutes or without pepsin. A nonsignificant difference between the "pepsin" or "no pepsin" conditions was found for most samples tested. However, for pasta, which was higher in protein compared to other foods, the rate of starch digestion increased with time of incubation with pepsin. In other words, the digestion rate for samples incubated with pepsin for 60 minutes had a higher amount of starch digested than those incubated with pepsin for 30 minutes. These results suggest that incubating the samples with pepsin during the gastric phase is more important for foods that contain a significant amount of protein. Some protocols suggested standardising the pepsin for all *in vitro* digestion methods. However, it is important to note that the *in vivo* glycemic response happens within 10 minutes after eating and reaches its peak within 20 minutes. As such, the effect of pepsin on glucose release if incubated for more than 30 minutes remains unclear.

Viscosity is another important factor that affects the rate of starch digestion and absorption in the human intestine (Lifschitz et al., 2002; Turnbull et al., 2005). Viscosity affects the glycemic response via two mechanisms: first, slowing the gastric emptying time, and second, reducing the access of digestive enzymes to food. Viscosity is a difficult factor to mimic under *in vitro* conditions. As such, most *in vitro* methods do not account for the effect of viscosity on the amount of starch digested and GI. One exception was Englyst et al. (1999), whose method involved adding guar gum powder to the media in order to mimic the viscosity conditions of *in vivo* digestion. Englyst not only simulated the physiological conditions of viscosity by adding guar gum powder but also enhanced the suspension of enzymes in the media. Moreover, the viscosity of the guar gum reduced the mechanical effect of the beads that were used for mixing the food sample during digestion (Englyst et al., 1999). This step by Englyst was considered important in the improvement of *in vitro* methodology and enabled researchers to measure the effect of viscosity on food digestion rate and glucose release.

A final consideration of *in vitro* methods is the duration of the gastric phase, which typically varies between 10 minutes to 1 hour across studies. In the body (i.e., *in vivo),* gastric emptying time is affected by the quantity of food and its viscosity (Frost et al., 2003; Santangelo et al., 1998). In contrast, it is difficult to simulate gastric emptying time based on food quantity using *in vitro* methods. Further, food composition and structure also affect the incubation time of the gastric phase.

1.4.1.5 The Intestinal Phase:

The intestinal phase begins with the emptying of chyme into the duodenum. The pancreas secretes pancreatic juices, which contain pancreatic amylase that is mixed with the chyme. This mixture moves through the intestinal lumen via peristaltic contractions. Brush border enzymes (maltase, sucrase, and lactase) associated with the microvilli of the intestinal epithelium hydrolyse the starch into simple sugars, disaccharides (maltose, sucrose and lactose) and further to monosaccharides (glucose and fructose). These monosaccharides are then absorbed into the bloodstream, resulting in the glycemic response (Guyton & Hall, 2000).

Many techniques have been used to mimic *in vivo* conditions in the intestinal phase of *in vitro* methods. The intestinal phase of *in vitro* methods can be classified into two categories: the restricted *in vitro* method (using dialysis bags) and the unrestricted *in vitro* method (using test tubes). In 1984, Jenkins et al. were the first to use dialysis bags in *in vitro* starch digestion. In this method, 1 mL of human saliva was mixed with different starchy foods and placed in dialysis bags. The bags were then suspended in water and incubated for 3 hours with regular stirring. Next, 5 mL of the dialysate (which contains the glucose released from the food sample), was taken for glucose measurement. The concentration of glucose in the dialysate represented the digestibility of starch in the sample. The percentage of total digested starch was compared with the digested starch of the reference food, which was white bread. The results of the *in vitro* GI values were positively correlated with the *in vivo* GI results. In 1995, Brighenti and colleagues established a restricted *in vitro* method to measure the digestibility of starch based on botanical origin and type of processing. During the gastric phase, the samples were incubated for 1 hour and then transferred into dialysis bags. Pancreatin enzyme was added to the samples and incubated at 37˚C with 6.9 pH for 5 hours; meanwhile, the aliquots were taken from the dialysate

every 30 minutes. The amount of starch digested was measured as the amount of glucose released in the dialysate, which was in turn expressed as the amount of available carbohydrate in the food. The foods digested starch amount were compared with those of reference foods, resulting in a strong correlation between the GI values and the digestion rate at 150 minutes.

In contrast, Englyst et al. (1990) proposed his unrestricted method by using test tubes, and then improved upon this method in 1992. Briefly, minced foods were mixed with pancreatic enzymes (pancreatin, amyloglucosidase, and invertase). In order to mimic the viscosity of *in vivo* conditions, guar gum powder was added and mixed with glass beads in a shaking water bath at 37˚C and pH 5.2 for 2 hours. At 20 and 120 minutes, 0.5 mL aliquots were taken to measure glucose content. Englyst expressed the amount of starch that was digested in the first 20 minutes as RDS, while the starch digested between 20 and 120 minutes was defined as SDS. The remaining fraction that was left undigested after 120 minutes was called RS (Englyst et al., 1992). Englyst's method was considered a revolutionary step in the measurement of *in vitro* starch and has become one of the most commonly used methods to determine digestible starch and GI (Woolnough et al., 2008).

The buffer is another important element in the intestinal phase, and is used to mimic the optimal pH for pancreatic enzymes during this phase. In the *in vitro* method established by Goni et al. (1997), a Tris-Maleate buffer with 6.9 pH was added to adjust the pH of the intestinal phase. In this method, the food sample was mixed with α-amylase in a shaking water bath at 37˚C for 3 hours and aliquots were taken every 30 minutes. Next, the aliquots were mixed with 3 mL sodium acetate buffer and 60 µL amyloglucosidase to hydrolyse the starch to glucose. Goni et al. performed the method with several starchy foods and measured the glucose at different time points, expressing this measurement as the percentage of starch hydrolysis. The AUC of

digested food was calculated compared with the reference food (white bread) and expressed as the hydrolysis index (HI) of foods. The researchers then compared the HI of these foods with the *in vivo* glycemic response. The digestion rate of the *in vitro* method at 90 minutes showed a strong correlation with the *in vivo* GI of the same food (Goni et al., 1997).

1.4.1.6 Variation in the Intestinal Phase:

There has been some variation in the enzymes and pH of the intestinal phase of *in vitro* methods. Although most past methods used pancreatin (Englyst et al., 1999; Mishra et., 2008; Weurding et al., 2001) or α-amylase (Brennan et al., 1996; Muir et al., 1995; Granfeldt et al., 1992) as a primary hydrolytic enzyme, there was still variation in the concentration of these enzymes between methods. Additionally, methods differed in whether or not they used amyloglucosidase, and at which stage of digestion the amyloglucosidase should be added. The pH is another factor that varies between methods. The optimum pH for α-amylase and pancreatin is between 6 and 7, while the optimum pH for amyloglucosidase is between 4 and 5. Therefore, most methods have used amyloglucosidase with α-amylase or pancreatin incubated with a pH of approximately 5, so as not to affect the activity of either enzyme (Akerberg et al., 1998; Muir et al., 1995). The variation in pH can lead to an underestimate of the amount of starch digested which leads to different results among the *in vitro* methods. Therefore, to investigate the effect of pH on enzyme activity, Woolnough and colleagues (2008) used the most common *in vitro* methods to test the stability of pancreatin, incubating the samples at a pH ranging from 4-9. The amount of digested starch was comparable across methods, regardless of the pH value at the beginning of the experiment. Further, results indicated that a pH of 5-7 was sufficient to keep the pancreatin enzyme active during *in vitro* digestion.

Enzyme concentration and incubation time are other important variables to consider. Researchers have used different concentrations of α-amylase during the intestinal phase. For example, Akerberg et al. (1998) used a very low concentration of α-amylase and pancreatin (0.01%) to digest 11 g food samples. Brennan used an α-amylase solution with a concentration of 5.1% and a larger food sample (30 g) and ran the experiment for approximately 90 minutes to measure the amount of starch digested (Tudorica, Kuri, & Brennan, 2002). In the Englyst method (Englyst et al., 1992, 1999), the enzyme mixture used in starch digestion was composed of 3% pancreatin, 0.5% amyloglucosidase, and 1.3% invertase. This mixture was used to digest food samples weighing from 1 to 8 g. In general, using low concentrations of enzymes result in more variation in the amount of starch digested. Therefore, more accuracy is required when measuring small concentrations of enzymes in order to reduce variation. The duration of incubation is another factor that affects the starch digestion. Therefore, the incubation duration is critical especially with methods that measure RDS, SDS, and GI. Finally, in addition to the mentioned factors, the intestinal phase of *in vitro* methods is influenced by other factors, including the mixing approach (i.e., stirring versus shaking), temperature, and viscosity, all of which varied greatly between methods. This literature review demonstrates that there is a significant variation in the intestinal phase between methods, and indicates that it is important to establish a standard and robust method for the *in vitro* digestion of starch.

1.5 Conclusion:

The GI measurement is an important tool to assistant the individual with diabetes to improve their food choices. The traditional *in vivo* measurement method is variable, costly and time-consuming. Therefore, an alternative *in vitro* method was needed. Although the use of *in*

vitro digestion methods has increased in the last two decades, there remains a lot of variation between methods. Most recent methods have attempted to simulate the physiological digestion phases (i.e., oral, gastric and intestinal). However, there is no single robust, reliable and standard *in vitro* method. These variations in *in vitro* techniques lead to differences in the amount of starch digested and discrepancies *in vitro* versus *in vivo* GI values for a given food. Further investigation and systemic reviews are needed to establish a standard and robust *in vitro* method to measure the digestibility of starch and predict GI.

2 CHAPTER 2

Research Rationale, Objectives, and Hypotheses

2.1 Research Rationale

Food management intervention is the first line of diabetes treatment, particularly for type 2 diabetes, which accounts for 90% of diabetes cases. Recently, the diabetes nutrition guidelines recommended the intake of foods with high fibre and complex starch; foods with high amounts of SDS and RS are especially recommended (CDA, 2016). Foods that are high in fibre and complex starch usually have a lower GI, which means better blood glucose control. GI assessment of foods is essential to determine which foods are better choices for individuals with diabetes. The basic method to assess the GI of foods is the *in vivo* method, which involves testing food in at least ten participants. However, this method is complicated, highly variable, time-consuming, labour- and resource-intensive, and requires ethics approval. As such, there has been increased interest in developing an alternative *in vitro* method of GI assessment. Indeed, the Glycaemic Carbohydrate Definition Committee has recommended the development of an *in vitro* method to assess the GI of foods (Brooks et al., 2006). There are currently many *in vitro* methods that predict GI, the most common of which is the Englyst method. However, this method has some limitations, including uncertainty as to how to use food 'as eaten', and the fact that this method is similarly time- and labour-intensive. In sum, there is still no single robust, reliable and standard *in vitro* method to measure the GI of food. Therefore, the aim of this thesis was to establish and validate a semi-automated *in vitro* procedure based on the Englyst method, which will estimate the GI of foods as consumed. This method will be more reliable and applicable than previous methods, will be easier to perform, and will result in significant time savings. Further, this method will benefit individuals who suffer from diabetes and will help them make better dietary choices to improve their overall health and wellbeing.

2.2 Global Research Objectives and Hypotheses:

1. To adapt and modify the *in vitro* modified method based on the standard Englyst method to assess the amount of digestible starch.

Hypothesis: The amount of starch digested in the modified *in vitro* method will be statistically comparable with the amount of starch digested in the standard Englyst method.

2. To validate the modified *in vitro* method for digestible starch measurement by analyzing 20 lentil powdered samples and 17 yellow pea powdered samples and to compare results with the amount of digested starch of the same foods from the standard Englyst method.

Hypothesis: The correlation between the amount of digested starch in the modified method and the standard Englyst method will be significant, positive, and strong.

3. To validate the modified *in vitro* method for predicting the GI of foods by analyzing 20 starchy foods available in the Canadian market and comparing results with published derived *in vivo* GI values.

Hypothesis: The GI values that obtained from the *in vitro* modified method will be significantly correlated with the *in vivo* GI values. The modified *in vitro* method will represent a suitable alternative to the *in vivo* GI method to predict the GI value of starchy foods.

4. To determine the relationship between RDS values obtained from the modified *in vitro* method and *in vivo* GI values.

Hypothesis: The RDS will be significantly correlated with *in vivo* GI values and will be available for use as an indicator of the glycemic effect of starchy food.

3 CHAPTER 3

Modification and Adaptation of an Existing *in vitro* Digestion Method

3.1 Introduction

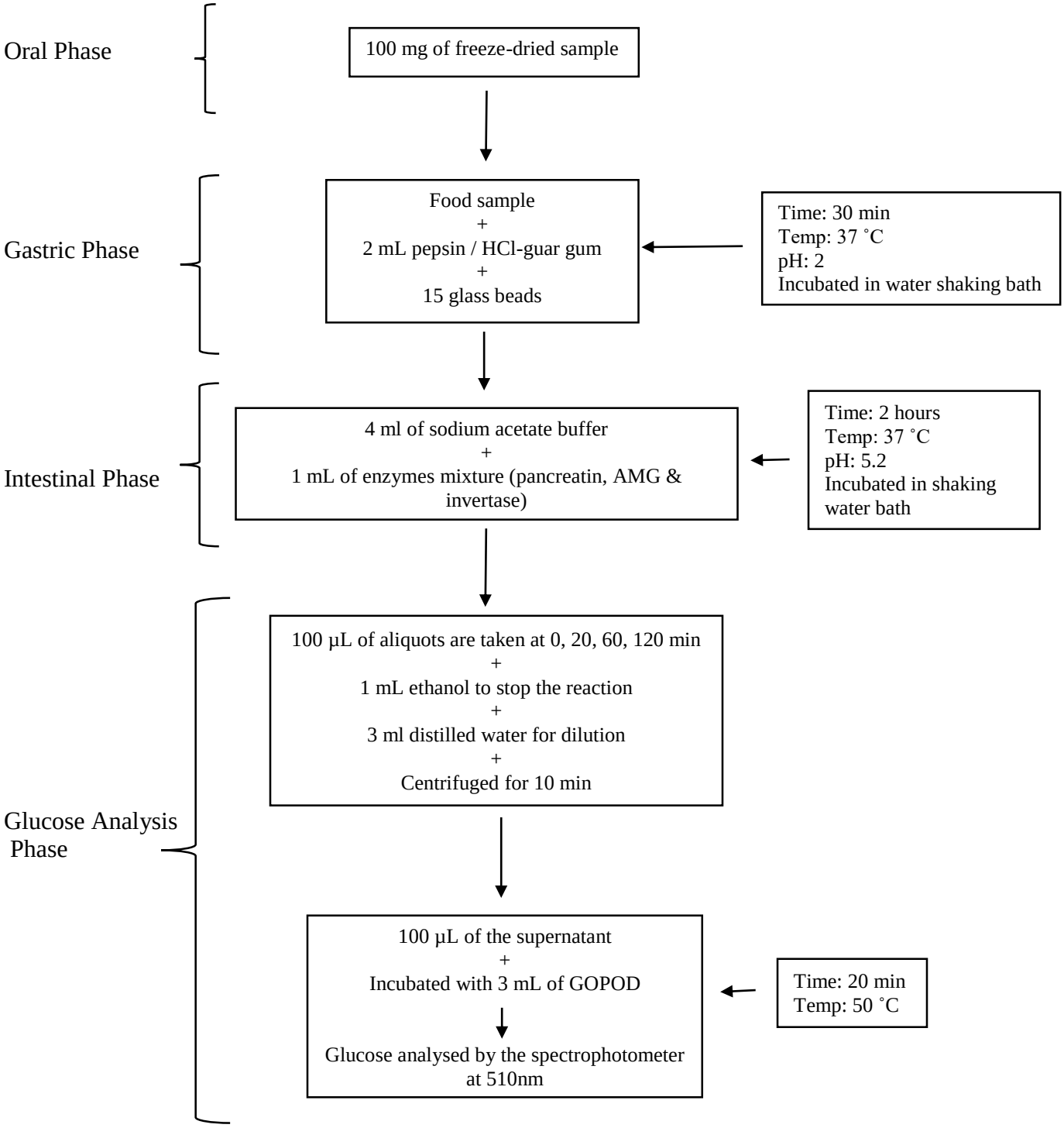
3.1.1 Diabetes and Dietary Management:

Diabetes is one of the most common chronic diseases worldwide, greatly impacting both public health and the economy (WHO, 2016). Dietary intervention is considered the first line of treatment in diabetes management. Low-GI starchy foods are important in the control of blood glucose for individuals with diabetes (ADA, 2016). Therefore, knowing the GI values of starchy foods is important in dietary planning for diabetes. Human studies using volunteers is the main method used to measure the GI value of foods *in vivo*. However, this method is variable, costly and time-consuming (Vega-Lopez et al., 2007). Interest in finding an alternative to the *in vivo* method has increased as of late. Currently, there are many studies using the *in vitro* method that mimic the *in vivo* digestion condition to measure digestible starch and GI of food. One of the most common is the Englyst method (Englyst et al., 1992). However, the Englyst method has some considerations, including the fact that it is unclear how foods were cooked and prepared for starch analysis in this method. Freeze-dried food samples were used for the digestible starch and GI measurement and do not represent food "as eaten". Further, the Englyst method procedure is complicated and time-consuming. To resolve the limitations of this method, a modified version of the Englyst method was established and validated for routine use.

3.1.2 Standard Englyst Method:

Recently, several *in vitro* methods have been used to measure the *in vitro* digestibility of starch in food. The Englyst method is one of the most common *in vitro* methods used to assess digestible starch and to predict GI. Early in the 1980s, Englyst started working on developing an *in vitro* method to measure the digestible starch in food. He established his first method in 1990

and then improved it in 1992 (Englyst & Kingman, 1990; Englyst et al., 1992). Englyst et al. (1992) ran an experiment to measure the starch fractions of 30 different starchy foods. The results showed that the RDS had the best correlation with *in vivo* GI values. As such, the study concluded that the RDS values might be used as a suitable predictor of GI values in starchy food, and an alternative to the *in vivo* GI method. Englyst suggested that this method could help people with diabetes make better choices when selecting starchy foods. In 1999, Englyst et al. modified and improved his method (Figure 3.1). The aim of this study was to assess the rapidly available glucose (RAG) and slowly available glucose (SAG) in different starchy foods and to compare the results with the glycemic response of eight volunteers consuming the same foods. Results indicated that there was a significant association ($r = 0.98$. $p > 0.001$) between the *in vitro* RAG and *in vivo* glycemic response (Englyst et al., 1999). In another study, Goni et al., (1997) established an *in vitro* method to assess the GI values of starchy foods. The researchers assessed the starch digestibility and the hydrolysis index of starchy foods and then compared them with *in vivo* GI values. Results indicated that the best association between the *in vivo* GI values and hydrolysis index occurred at 90 minutes ($r = 0.91$, $p \leq 0.05$). These differing results found by Englyst and Goni could be attributed to differences in procedure or the use of different equations to calculate GI values.



Figuer 3.1: Standard Englyst method Procedure

Abbrevtions: min = miuntes; Temp = temprature; AMG = Amyloglucosidase; GOPOD = Glucose oxidase-peroxidase enzyme cocktail mixture

3.1.3 Limitations of the Standard Englyst Method:

Although the standard Englyst method is now one of the most commonly used methods for *in vitro* starch digestion and GI prediction, some limitations remain. This is especially true regarding sample preparation; it was unclear how the food samples were prepared and whether this was done in a standardized manner. One of the Englyst studies used food samples 'as eaten' but how the samples were cooked and prepared was not described (Englyst et al., 1992). In another study, Englyst et al., (1999) used freeze-dried powder samples that were ground to a particle size of 250 µm. Moreover, most of the studies based on the Englyst method principle used freeze-dried samples to assess the digestible starch or GI value ((Mishra, Monro & Hedderley, 2008; Silvester, Englyst & Cummings, 2005). The rationale for using a powdered sample instead of normal food is that the sample food particles are not homogeneous and they could stick on the tip of the pipette during sample transfer. These factors are known to hinder the ability to take a representative sample. Importantly, the freeze-dried sample does not represent the food as it is eaten because the process breaks food particles down to a fine powder. This process makes the powdered sample easily accessible to enzymes, accelerating the amount of starch digested and giving the method a higher rate of GI prediction compared to normal *in vivo* digestion (Woolnough et al., 2008).

Other limitations of the standard Englyst method are that it is time-consuming, lengthy, laborious, and requires a user who is very skilled at pipetting. Further, a single, strong, standard *in vitro* method to predict the GI in food as eaten does not exist. Due to these limitations, establishing a valid and reproducible modified *in vitro* method based on the Englyst method was crucial. Specifically, a method that used cooked samples rather than freeze-dried powder was

required. For those reasons, our research group began to explore an alternative method that uses food ‘as eaten’, saves time, and is reproducible and accurate.

3.2 Study Objectives:

The objectives of this chapter were:

1. To adapt the standard Englyst method for use on a semi-automated GI analyser instrument.
2. To validate the modified method by analyzing 20 lentil powdered samples and 17 yellow pea powdered samples and comparing the amount of digested starch against the standard Englyst method.

3.3 First Stage: Establishing the Modified Method:

3.3.1 Method and Materials:

Materials:

Polyethylene cups (100 mL; Next Instruments Pty Ltd, Australia) were used to hold the samples, and 5 mm diameter glass ball beads (Pyrex®, Germany) were used to facilitate sample digestion. A NutraScan GI20 Glycemic Index Analyser instrument (Next Instruments Pty Ltd, Australia) was used for the *in vitro* starch digestion. The GI analyser is attached to a GL6 glucose analyser instrument (oxygen electrode-based instrument for glucose measurement), which was used for glucose analysis (Analox Instruments Ltd, UK). Dialysis bags (mwco 12000 – 14000, fw 45 mm) were used in food sample analysis (Spectrum Laboratories, Inc., USA).

Reagents:

All reagents used in the modified method were the same as those used in the standard Englyst method (Englyst et al., 1999), with appropriate adjustments for assay volumes. The enzymes used consisted of pepsin (Sigma P7125), porcine pancreatin (Sigma P7545), amyloglucosidase (AMG) (Megazyme E-AMGDF), and invertase (Sigma I4504).

Enzyme solution preparation involved the following steps. First, the pancreatin solution was prepared; 0.45 g of porcine pancreatin was weighed into a beaker and mixed with 4 mL of distilled water for 5 minutes at ~900 rpm by a magnetic stirrer. The mixture was centrifuged at 1,500 g for 10 minutes, and a portion of the cloudy supernatant was drawn off with a pipette and transferred into a small beaker. Second, the diluted AMG was prepared; 80 µL of distilled water was added to 320 µL of AMG and vortexed. Third, the enzyme solutions from the first and second step were mixed; 300 µL of diluted AMG was mixed with 2.7 mL of pancreatin supernatant, 2 mg of invertase, and 200 µL distilled water.

The HCl-guar gum solution was made by mixing 0.05 M HCl solution with 5 mg/mL guar gum. Pepsin/HCl-guar gum solution was prepared fresh before analysis by mixing 5 mg of pepsin with 4 mL of HCl-guar gum. The buffer was 0.5 M sodium acetate solution (pH 5.2) with 20 mM $CaCl_2$ (28.7 mL glacial acetic acid and 2.94 g $CaCl_2 \cdot 2H_2O$ per litre).

3.3.2 Sample Preparation:

Cooked, freeze-dried Greenland lentil powder was used as a reference food for the establishment phase. The Greenland lentil was rinsed in deionized water and cooked in boiling deionized water (35-70 minutes). Cooked samples were dried in a FreeZone® Plus 12-liter Cascade freeze-dry system (Labonco Corp., USA), ground into fine powder using a M-20

universal mill (IKA Works Inc., USA), and then passed through a 250 µm (No. 60) sieve and stored in a desiccator at room temperature until required. The Greenland lentil was chosen as a quality control material for monitoring the reproducibility of the assays because of our research group's long-standing experience and familiarity with this variety and the fact it has shown to yield results by the standard Englyst method (Englyst et al., 1999). Therefore, Greenland lentil powder was used as the reference food in the establishment of a modified method.

3.3.3 Method:

A semi-automated instrument (i.e., NutraScan GI20 Glycemic Index Analyser) was used for measuring the GI of starchy foods in house. This instrument is comprised of a hot plate and a magnetic stirrer with 20 wells which can hold up to 20 sample cups. The GI analyser is attached to an oxygen electrode-based instrument for glucose measurement (Analox Instrument). The glucose analyser was calibrated before each experiment with two glucose standards (90 mg/mL and 144.1 mg/mL) at least twice to ensure the accuracy of the glucose measurement. A robotic arm at the top of the instrument automatically takes an aliquot from the cup that contains the sample and injects it into the Analox Instrument for glucose analysis. The instrument's reading reflects the glucose concentration (mg/dL), which later can be expressed as a rate of starch digestion by using a specific equation.

The objective of this study was to adapt the GI Analyser Instrument to the standard Englyst method. However, several obstacles were encountered. The main dilemma faced by our research group was how to use the GI Analyser Instrument to accommodate the standard Englyst method to use the food 'as eaten'. The method modification required systematically examining the variables incorporated in the standard Englyst method; these factors included whether to use

the restricted method or non-restricted method in starch digestion, optimum buffer volume, optimum pH, optimum number of beads that should be used, optimum temperature, and the ideal concentration of enzymes.

3.3.4 Step 1: Using the Restricted Method in the Establishment of the Modified Method:

The first step of this project was to find a technique to use food 'as eaten' for glucose analysis without interfering with the food particles during sample pipetting. Therefore, the restricted method was applied as modification to separate the analysed glucose from other food sample components during *in vitro* food digestion. As such, the initial approach explored was the use of dialysis bags as previously used by other researchers (Jenkins et al., 1982; Granfeldt et al., 1992; Brighenti et al., 1992).

Step 1 Aim:

To assess the effect of using of dialysis bag for *in vitro* starch digestion of lentil powder and to compare results the amount of the digested starch in the modified method against the standard Englyst method.

Step 1 Methods:

A Greenland lentil powder sample (cooked, freeze-dried and ground to 250 µm, as previously described) weighing 400 mg was mixed with 8 mL of pepsin/ guar gum solution and placed along with a magnetic stir bar inside a dialysis bag. The dialysis bag was then suspended in the digestion cup containing 50 mL of sodium acetate buffer and stirred for 30 minutes; this represented the gastric phase of simulated *in vitro* digestion. To simulate the intestinal phase, 4 mL of enzyme mixture was added to the sample mixture inside the dialysis bag; the bag was then resealed and left in the digestion cup. During 2-hour of the intestinal phase, aliquots were taken

from the dialysate for glucose analysis at different time points (5, 10, 20, 30, 60, 90, and 120 minutes). The amount of starch digested was calculated based on the glucose that was released from the sample in the dialysate and expressed as a percentage of the total starch content.

Calculations, Data Analysis and Statistics:

Digestible Starch Calculations:

% Digestible Starch = G x (TV/0.5) x (100/DM) x (162/180), Where:

G = mass of glucose (µg) calculated from the standard curve= (absorbance - y-intercept)/slope

TV = Total Volume

TV/0.5 = volume correction for aliquot removed at different hydrolysis times (note: the total volume of solution is reduced by 0.5 mL at each time point)

DM = dry mass of sample (mg) = (sample mass × % moisture)/100

100/DM = factor to express digestible starch as a % of dry sample mass

162/180 = factor to convert free glucose (as it was measured) into anhydro-glucose, as it occurs in starch (0.9).

Statistics:

Unless otherwise stated, experimental data for each modification step were statistically analysed as outlined here using SPSS version 24 (IBM SPSS Statistics, IBM Corporation, Armonk, NY, USA). Differences between the two methods were tested using one-way analysis of variance (ANOVA), and the Tukey-Kramer *post hoc* test was used to test mean differences between the amount of starch digested at each time point. The level of concordance between the amount of starch digested in the two methods (i.e., standard versus modified method) was

analyzed using Pearson's correlation coefficient (Pearson's r). All results are presented as means ± SEMs; statistical significance was defined as $p < 0.05$.

Results and Discussion of Step 1:

Results showed that the digested amount in the modified method was lower than that of the standard Englyst method. There was almost no glucose released during the first 10 minutes. At all the time points, the difference between the two methods in the amount of starch digested was significant ($p < 0.05$), at approximately 60% (13% in the dialysis modified method and 73% in the standard Englyst method, respectively). This variation between the two methods decreased to 26% at 120 minutes but remained significant ($p < 0.05$). The level of concordance between the starch digestion rates of the two was nonsignificant at each time point ($r(4) = 0.74$, $p = 0.85$; Figure 3.2).

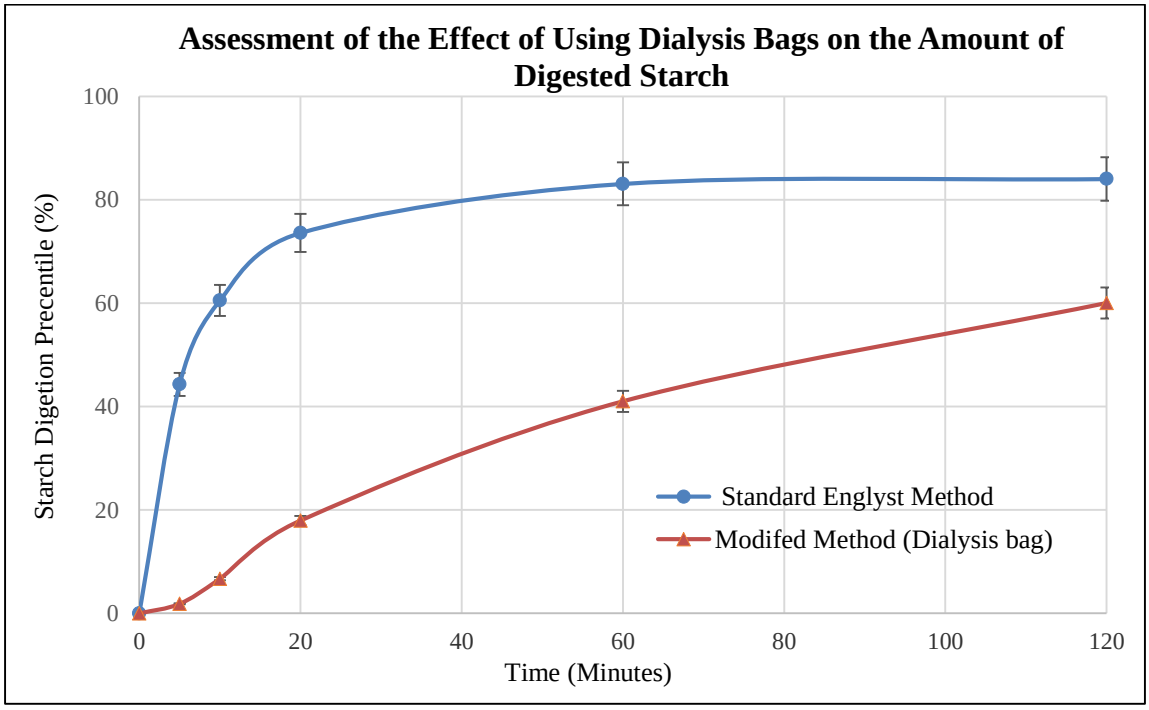


Figure 3.2: Comparison Between the Englyst Method and Modified Method after Using Dialysis bags in Greenland Lentil Starch Digestion

Data are means ± SEM (n = 4). The level of concordance between the two methods was not significant $r(4) = 0.75$ ($p = 0.85$)

The aim of this step was to use the restricted method as an alternative technique to digest food as eaten. After running the experiment for two hours, the results of the two methods did not show statistical agreement. Although the sample was in powder form, the amount of released glucose in dialysate was low compared with the standard Englyst method. This low quantity of released glucose was expected due to presence of the dialysis bag, which suppressed the flow of glucose from the sample mixture to the dialysate. Further, using the dialysis bag was unsuitable and difficult to handle. Due to the difference in quantity of digested starch between the standard Englyst and modified methods, the dialysis bag was omitted in subsequent stages.

Conclusion of Step 1:

Using a dialysis bag in the modified method led to significantly less glucose release compared to the standard method. Therefore, the dialysis bag was excluded in subsequent steps.

3.3.5 Step 2: Determining the Optimum Dilution Volume for the Modified Method:

Due to the omission of dialysis bags in the previous step, the modification made in this step was to mix all the materials (i.e., 400 mg sample, 4 mL enzymes, and 16 mL buffer) directly in the sample cup. It was assumed that the glucose concentration would be higher than the standard Englyst method prior to running the assay because the sample was not diluted. To confirm this assumption, an experiment was run with all the materials and reagents (samples, enzymes, and buffer) placed directly in the cups and mixed. The result of this step indicated that the released glucose concentration was higher than the standard Englyst method and that it fluctuated (data not shown). This result can be explained by the fact that there was no dilution in this experiment; whereas, in the standard Englyst method, the sample mixtures were diluted

more than 40 times before the glucose analysis and starch assessment were performed (Englyst et al., 1992). Therefore, the dilution step was deemed necessary before starch digestion and glucose analysis.

Step 2 Aim:

To optimize the assay dilution volume in order to reduce glucose concentration prior to sample analysis, and to compare results with the standard Englyst method.

Step 2 Methods:

The assay was run in triplicate on two separate occasions using Greenland lentil samples weighing 400 mg. At the gastric phase, the sample was mixed with 8 mL of pepsin/guar-gum solution for 30 minutes. In the intestinal phase, 16 mL of sodium acetate and 4 mL of enzyme mixture were added. At each time point (0, 5, 10, 20, 60, and 120 minutes), a 500 µl aliquot was taken from each sample and diluted with 1 mL of water before centrifuging for 30 seconds. Finally, the supernatant of the centrifuged aliquot was taken and injected in the Analox Instrument for glucose analysis.

Results and Discussion of Step 2:

The glucose readings resulting from this step were still higher than the standard Englyst method and fluctuated greatly (Figure 3.3). The three replicates were not significantly correlated ($p > 0.05$). Further, there were significant differences between the modified method and the standard Englyst method at both 60 and 120 minutes ($p < 0.05$).

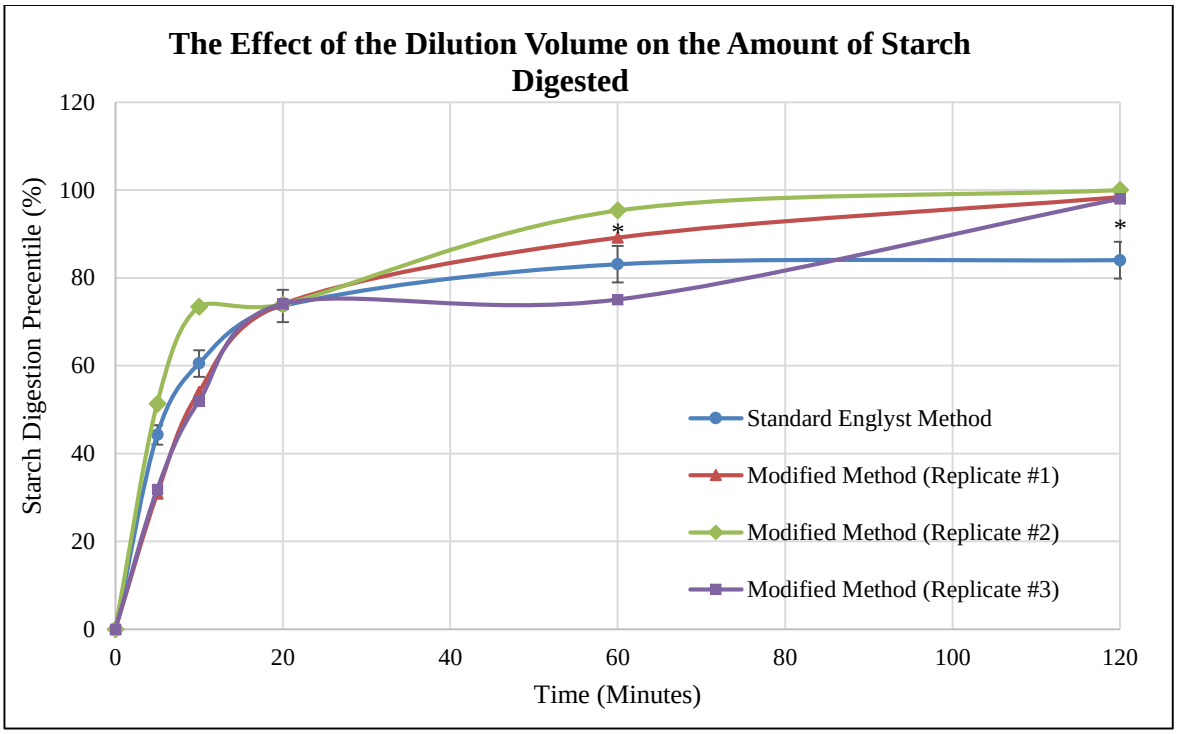


Figure 3.3: Comparison Between Englyst Method and Modified Method after Diluting the Aliquots with Distilled Water

Data are means ± SEM (n = 4). There was no agreement between the three replicates in digestion rate of the modified method and the standard method ($p > 0.05$). * Significant differences were found at 60 and 120 minutes ($p < 0.05$)

The present step (Step 2) involved two consecutive experiments. The first experiment was run by adding 1 ml of distilled water to each aliquot before glucose analysis. Results showed a higher amount of digested starch (approximately 100%) in the three replicates at 120 minutes compared with the standard method (84%). Further, results indicated a high variability in the amount of starch digested at the majority of time points. Moreover, there was no concordance between the standard Englyst method and the standard method (Figure 3.3). This variability and high concentration of glucose could be due to the fact that the dilution was insufficient and that the aliquot was not thoroughly mixed with the added water. Given these issues, finding an alternative method with which to dilute the sample was needed.

To resolve the dilution issue, it was found that it was preferable to dilute the whole system rather than solely the aliquots, in order to ensure that all materials were well diluted and homogeneous. A second experiment was performed by adding 72 ml of distilled water to the entire system, which consisted of a food sample, 4 mL enzyme mixture, 8 mL HCL/guar gum solution, and 16 mL of sodium acetate buffer (total volume = 100 mL). After two hours of running the assay, results showed improvement in the amount of starch digested, where the glucose concentration was lower than in previous steps and no variability was observed. The amount of starch digested was 20% and 44% at 5 and 10 minutes, respectively compared with 56% and 72% in the previous experiment. However, the amount of digested starch remained high at 120 minutes, where it was approximately 100% for all replicates (Figure 3.4).

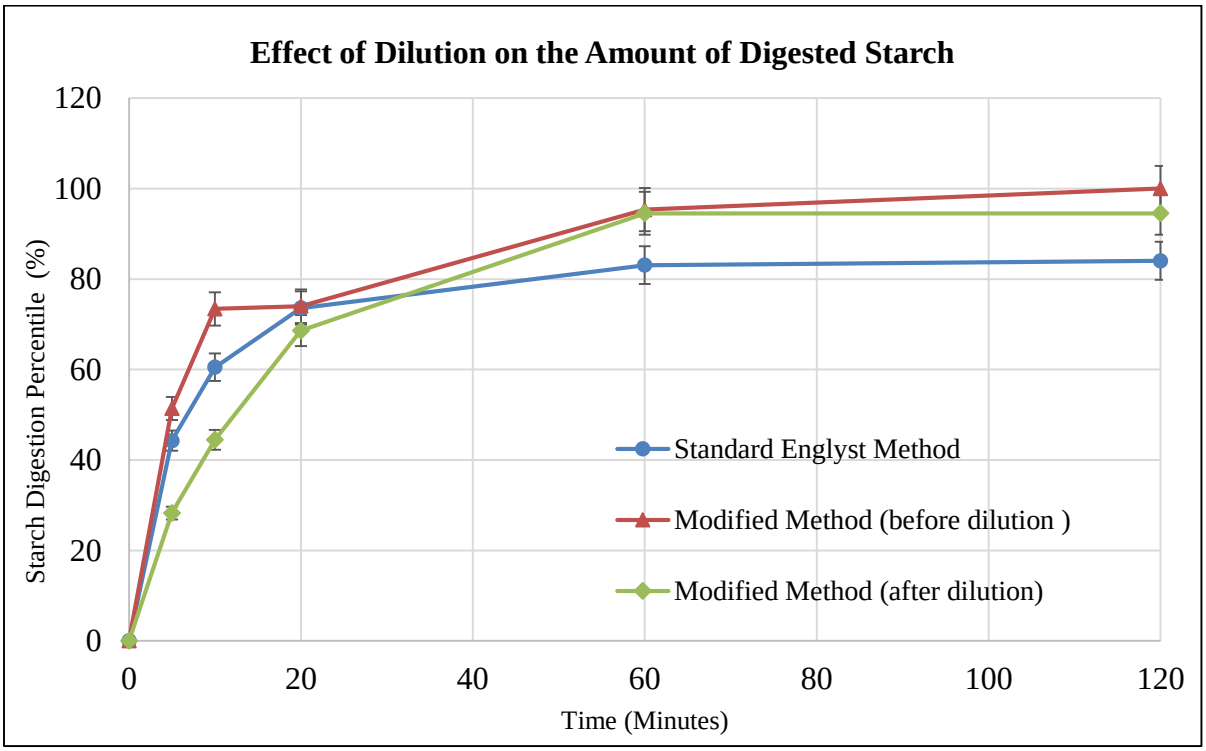


Figure 3.4: Comparison Between Englyst Method and Modified Method before/after Diluting the Whole System

Data are means ± SEM (n = 4). Although there was no fluctuation and lower amount of the digestion compared with the previous step, the amount of starch digested amount still higher than the standard Englyst method

Conclusion of Step 2:

The previous step indicated that diluting the aliquot was ineffective and produced considerable variation. However, after diluting the entire system with distilled water, the results improved. Less fluctuation was observed compared to the previous experiment, in which each aliquot was diluted individually. However, the concentration of released glucose in the modified method remained higher than that of the standard Englyst method, which needed to be addressed in the next step. Another concern that remained was that the use of water for dilution might affect the final pH of the assay, which in turn could affect the rate of the reaction.

3.3.6 Step 3: Determining the Optimum Dilution pH for the Modified Method:

The pH is another critical variable in starch digestion that needed to be considered for *in vitro* digestion. The pH plays an important role in enzyme activity (Reed, 2012; Whitehurst &Van Oort, 2010). In the standard Englyst method, the pH value rises from approximately pH 2 in the gastric phase to approximately pH 5 in the intestinal phase (Englyst et al., 1992). In the previous stage, diluting the assay using 72 mL of water was required to obtain reproducible glucose concentrations that fell within the linear range of the glucose analyzer. However, it was assumed using that this volume of water might disturb the mixture's pH capacity. Therefore, the question remaining was whether water or another buffer would be preferable for performing this dilution.

Step 3 Aim:

To compare the effect of using water or buffer (sodium acetate; pH 5.2) as a diluter during the intestinal phase, in order to determine the optimal diluter for the modified method.

Step 3 Methods:

The assay was run in duplicate using the same procedure described in the previous step. The only modification was that 88 mL of 0.5 M sodium acetate buffer with pH 5.2 was added to one of the assays (16 mL from the previous step plus 72 mL added in the current modification). Another assay was run by adding 72 mL of distilled water as described above; the total volume in both assays was 100 mL. The assays were run for two hours, and aliquots were taken at different time points (0, 10, 20, 60, 120 minutes).

Results and Discussion of Step 3:

The water and buffer produced somewhat similar results. There was no significant difference in the amount of digested starch when using buffer versus distilled water in the modified method ($p > 0.05$). Accordingly, the correlation between use of water and buffer was highly significant $r(2) = 0.99$ ($p < 0.001$). However, the amount of starch digested in the assay that was run with buffer was slightly closer to the standard method at 60 and 120 minutes. The level of concordance between the two assays of the modified method and standard Englyst method was significant $r(2) = 0.95$ ($p < 0.05$). Nevertheless, the two assays run in the modified method had a significantly lower digestion rate in the first 20 minutes than the Englyst method, $F(2,11) = 35.19$ $p < 0.001$ (Figure 3.5).

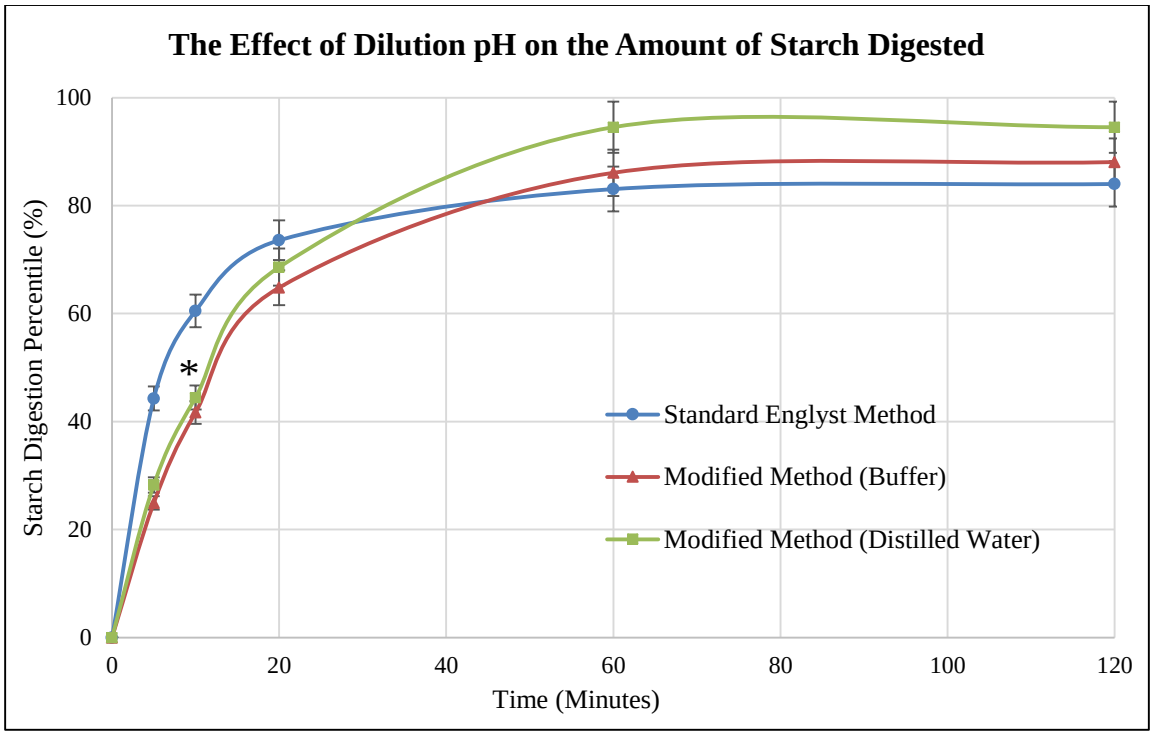


Figure 3.5: Comparison Between the Englyst Method and Modified Method Using Two Different Diluters to Assess the pH Effect on the Amount of Starch Digested

Data are means ± SEM (n = 4). *The two assays of the modified method had a significantly lower digestion rate in the first 20 minutes compared to the Englyst method ($p < 0.05$). The level of concordance between the using buffer and distilled water was high ($r(2) = 0.99$, $p < 0.001$).

The pH of the diluter in the gastric phase is an important element for the function of enzymes. This is especially true given that the pH rises from 2 to 5.2 between the gastric and intestinal phases, representing the optimum pH for the pancreatic and intestinal enzyme activity (Whitehurst, & Van Oort, 2010; Reed, 2012). Although the dilution with distilled water in the last step resulted in a better correlation compared with previous experiments, it was important to investigate the effect of adding 72 mL of water on the pH of the solution in the intestinal phase. In the standard Englyst method, 4 mL of sodium acetate buffer was added to a mixture of 3 mL of enzymes and guar gum solution (Englyst et al., 1992). The amount of the buffer in the standard Englyst method accounts for approximately 57% of the entire assay volume. In the

modified method, after adding 72 mL of distilled water to the entire system, the buffer comprised only 16% of the assay volume. Therefore, it was unclear if this dilution with water would affect the pH of the whole system and the enzyme activity. Additionally, in the standard Englyst method, the dilution happened after the aliquots were taken and the reaction was stopped, so it did not influence the system pH and the enzyme activity (Englyst et al., 1992). In the current step, it was important to consider the pH after adding the distilled water when conducting the modified method. To overcome this issue, the experiment was run with two assays: one with water and another with sodium acetate buffer (pH 5.2) as diluters. After two hours, the results indicated no significant difference between the two assays. However, the sodium acetate buffer assay resulted in a slightly higher correlation with the standard method compared with the distilled water assay. This result can be explained by the use of sodium acetate buffer as the diluter, which resulted in the preservation of the optimum pH for enzyme function. This, in turn, may have led to an improved digestion rate and a higher correlation with the standard Englyst method.

Conclusion of Step 3:

Using the sodium acetate buffer as a diluter resulted in a slightly lower amount of starch digestion compared with the distilled water. Therefore, sodium acetate was used as a diluter in subsequent experiments.

3.3.7 Step 4: Determining the Optimum Number of Beads for the Modified Method:

In the standard Englyst method, beads play an important role in food sample digestion. Besides facilitating food digestion, the beads also work as mechanical tools to break down the food samples into small particles, allowing the pancreatic enzymes to access the food sample.

The beads were another variable that needed to be added in the modified method to simulate the standard Englyst method. In the standard Englyst method, 15 glass beads were used as a mechanical tool to facilitate food digestion (Englyst et al., 1999). Therefore, in the modified method, it was important to add the optimal number of beads that would result in the highest correlation with the standard Englyst method.

Step 4 Aim:

To determine the optimal number of beads required in the modified method to produce the strongest correlation with the standard method.

Step 4 Methods:

The assay was run with the same procedure described in the previous stage, including the use of 88 mL of sodium acetate as a buffer to regulate the pH of the assay medium. The assay was performed in triplicate with varying numbers of beads; the first assay had five beads, the second assay had ten beads, and the third assay had 15 beads. The experiment was run for two hours, and aliquots were taken at different time points (0, 10, 20, 60, 120 mins).

Results and Discussion of Step 4:

The correlation between the amount of starch digested in the three assays was high and significant ($p < 0.001$) for all. Also, the results showed that the amount of starch digested was slightly positively correlated with the number of beads in the first 20 minutes. The highest amount of starch digested was observed in the 15-bead condition (58 %), while the lowest rate was observed in the 5-bead condition (54 %). However, results indicated that the quantities of starch digested in the first 20 minutes of the three assays were still significantly lower than the standard Englyst method at the same time points ($p < 0.05$; Figure 3.6).

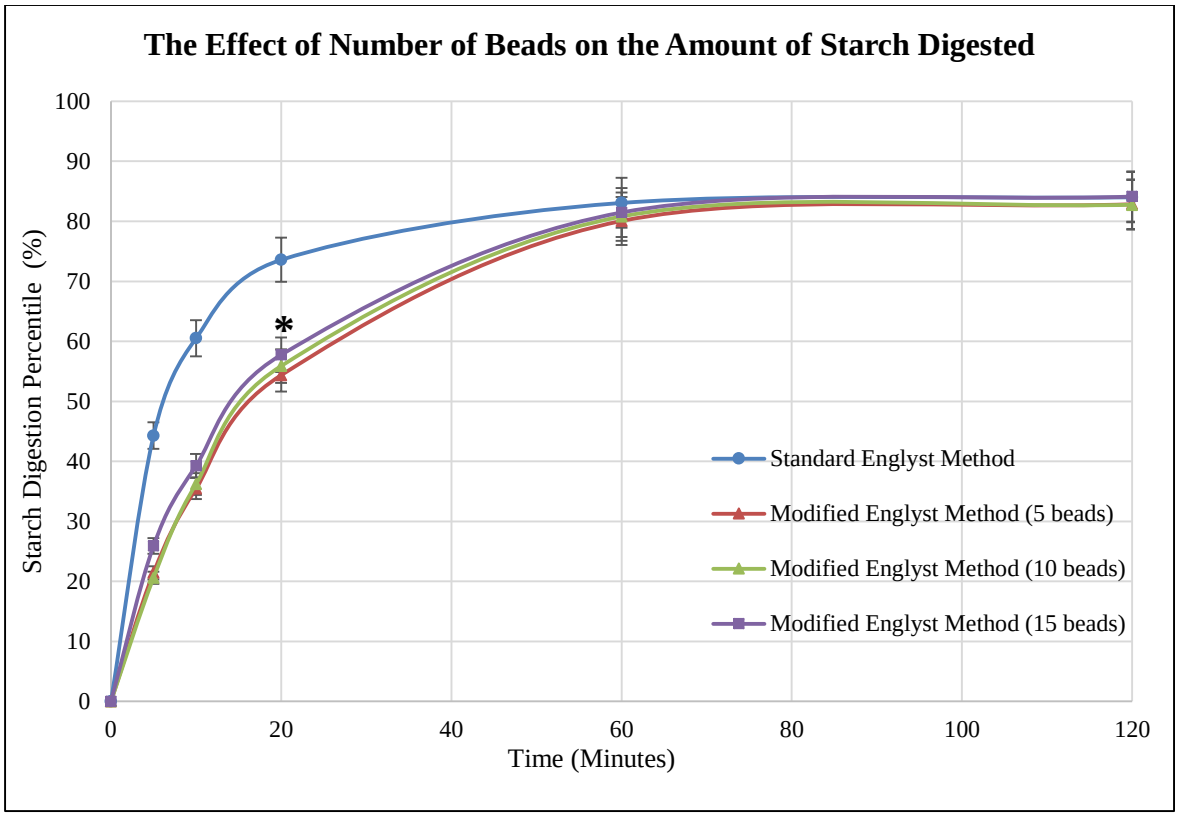


Figure 3.6: Comparison Between the Englyst Method and Modified Method Using Different Numbers of Beads

Data are means ± SEM (n = 4). There was an excellent concordance among the amount of starch digested s of the 3 experiments (r (n) = 0.99, $p < 0.001$), indicating no difference between different numbers of beads. However, a significant difference was found between the Englyst method and the modified method in the first 20 minutes ($p < 0.05$).

In this stage, the experiment was run with three assays with different numbers of beads (5, 10, and 15, consecutively). Results indicated that the highest number of beads (i.e., 15) resulted in the best correlation compared with the standard method. Also, due to the effect of the mechanical breakdown of beads in the modified method, the assay with 15 beads resulted in more homogenous samples compared with cups holding 5 or 10 beads. Based on these results, 15 beads were used in subsequent stages of the study. It was noted that the reaction rate differed during the first 20 minutes, but it was unclear whether this was related to the number of beads. Thus, alternative reasons for this difference were investigated in subsequent steps.

Conclusion of Step 4:

Although the difference between the three assays was nonsignificant, the assay with 15 beads was selected for use in subsequent experiments. The assay with 15 beads resulted in a well-homogenised sample in the assay, which led to a more representative sample for glucose analysis. However, results suggested that an issue remained in the first 20 minutes, wherein the amount of starch digested was still lower than the standard Englyst method. Therefore, further investigation was required in the next step of the study.

3.3.8 Step 5: Determining the Optimal Temperature for the Modified Method:

The results from the previous step showed, for the first time, a strong and significant correlation between the modified method and the standard Englyst method at 60 and 120 minutes. However, the amount of starch digested in the first 20 minutes was significantly lower in the modified method (57%) compared to the standard Englyst method (73%). Therefore, step 5 focused on improving the amount of starch digested in the first 20 minutes.

Temperature is one of the most important variables involved in *in vitro* digestion, since it plays a crucial role in enzyme activity (Reed, 2012; Whitehurst, & Van Oort, 2010). The optimal temperature for enzyme function according to most *in vitro* methods is 37˚C (Akerberg et al., 1998; Englyst et al., 1992; Granfeldt et al.,1992; Muir et al., 1995). In the standard Englyst method, the sodium acetate buffer used for the assay was stored in the fridge at 4˚C. In the intestinal phase, 4 mL of buffer was added into the digestion tube and was left for 5 minutes in the shaking water bath at 37˚C to equilibrate the temperature. This amount of time was sufficient to raise the buffer temperature from 4˚C to 37˚C (Englyst et al., 1999). However, in the modified method, 88 mL of the buffer (removed from the fridge at 4˚C) was used in the assay and was left

for 5 minutes for equilibrium. It is unlikely that time was sufficient to raise the temperature of that volume of buffer from 4˚C to 37˚C. Even after 15 minutes of running the assay, the temperature remained below 25˚C. For that reason, it was important to determine an alternative technique to increase the entire system temperature to the reach the optimal temperature before adding the digestion enzymes.

Step 5 Aim:

To raise the buffer temperature to 37˚C before it was used in the assay to produce the optimal temperature for enzyme activity.

Step 5 Methods:

The buffer was incubated in a separate water bath at 37˚C before being used in the assay. The assay was run in duplicate according to the procedure described previously, but without using the beads to reduce the number of variables in the assay. At the intestinal phase, the first run was performed with 88 mL of the preheated sodium acetate buffer at 37˚C, while the second run was performed with buffer from the fridge. The assay was run for two hours, and the aliquots were taken at different time points (0, 10, 20, 60, 120 minutes).

Results and Discussion of Step 5:

As a result of this modification, the level of concordance between the amount of digested starch in the standard Englyst method and modified method was improved after adjusting the buffer temperature ($r(2) = 0.97$, $p < 0.001$). In addition, the amount of starch digested in the first 20 minutes of the modified method was significantly higher after adjusting the assay temperature (65%) than before the adjusting temperature (54%) ($p < 0.05$). However, the assay with the adjusted temperature remained significantly lower than the standard Englyst method (73.59%) ($p < 0.05$; Figure 3.7).

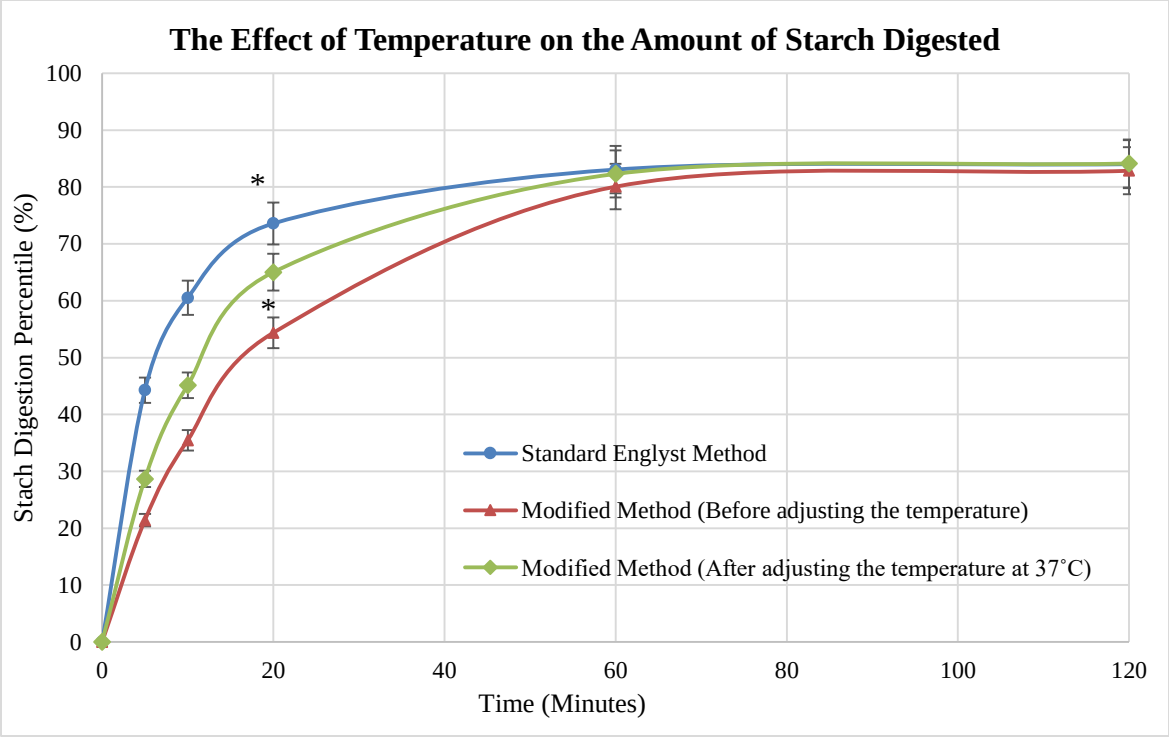


Figure 3.7: Comparison Between the Englyst Method and Modified Method After Adjusting the Temperature

Data are means ± SD (n = 4). A significant correlation was observed between the Englyst method and modified method ($r = 0.97$, $n = 4$, $p < 0.001$). The amount of digested starch in the first 20 min was significantly higher in the adjusted temperature ($p < 0.001$) compared to the unadjusted temperature. Note: this experiment was run without beads.

The optimal temperature for the function of pancreatic enzymes in *in vivo* as well as in *in vitro* is 37˚C (Akerberg et al., 1998; Englyst et al., 1992; Granfeldt et al.,1992; Muir et al., 1995). Therefore, the GI analyser instrument was set at 37˚C since the first step of the modified method establishment. However, after running the modified method several times, the temperature in the intestinal phase was found to be approximately 25˚C, implying that the optimal temperature for digestive enzyme activity was not achieved. This temperature issue was likely to be the main cause of the low quantity of digested starch in the previous stages. To solve this temperature issue, the sodium acetate was placed in a 37˚C water bath before running the assay. The assay was rerun with preheated buffer and results showed a significantly improved amount of starch digested in the first 20 minutes. Moreover, this procedure resulted in the highest correlation

between the two methods observed since the first experiment. Unfortunately, it remained significantly lower than the standard Englyst method in the first 20 minutes. This lower amount of starch digested in the first 20 minutes could be due to the fact that the beads were omitted in this experiment. This omission was made deliberately in order to reduce the number of variables in the assay, allowing for the determination of the effect of buffer temperature on the assay without the interference of other variables.

Conclusion of Step 5:

After using the preheated buffer, the amount of starch digested was significantly improved in the first 20 minutes compared with previous experiments, although it remained lower than the standard Englyst method. This preheating process was used for all subsequent stages of the experiment.

3.3.9 Step 6: Determining the Optimal Enzyme Concentration for the Modified Method:

Although a significant improvement in the digestion rate was observed in the first 20 minutes, it remained lower than that of the standard Englyst method. Two factors could explain this low amount of digested starch. First, the reduced amount of starch digested in the first 20 minutes might be due to the fact that beads were not used in the previous experiment. Second, it could be attributed to the lower ratio of enzymes to available starch in the sample. Therefore, it was important to determine the optimal enzyme concentration for the modified method.

Step 6 Aim:

To assess whether enzyme concentration was sufficient to hydrolyse all the digestible starch in the food sample in the modified method.

Step 6 Methods:

At this stage of the experiment, the modified method was run in duplicate using the same procedure described in step 5, including the adjusted temperature. After the first 60 minutes of the intestinal phase, 1 mL of the same enzyme activity mixture was added to one of the assays. The aliquots were taken at different time points, as described above.

Results and Discussion of Step 6:

Although there was a slight difference in the amount of starch digested before and after adding the enzyme at 60 minutes, it was non-significant ($p > 0.05$). Results showed a strong, significant agreement between the amount of starch digested in the two assays in the modified method ($r(2) = 0.99$, $p < 0.05$). However, the amount of starch digested at the first 20 minutes in the modified method once again remained below that of the standard Englyst method (Figure 3.8).

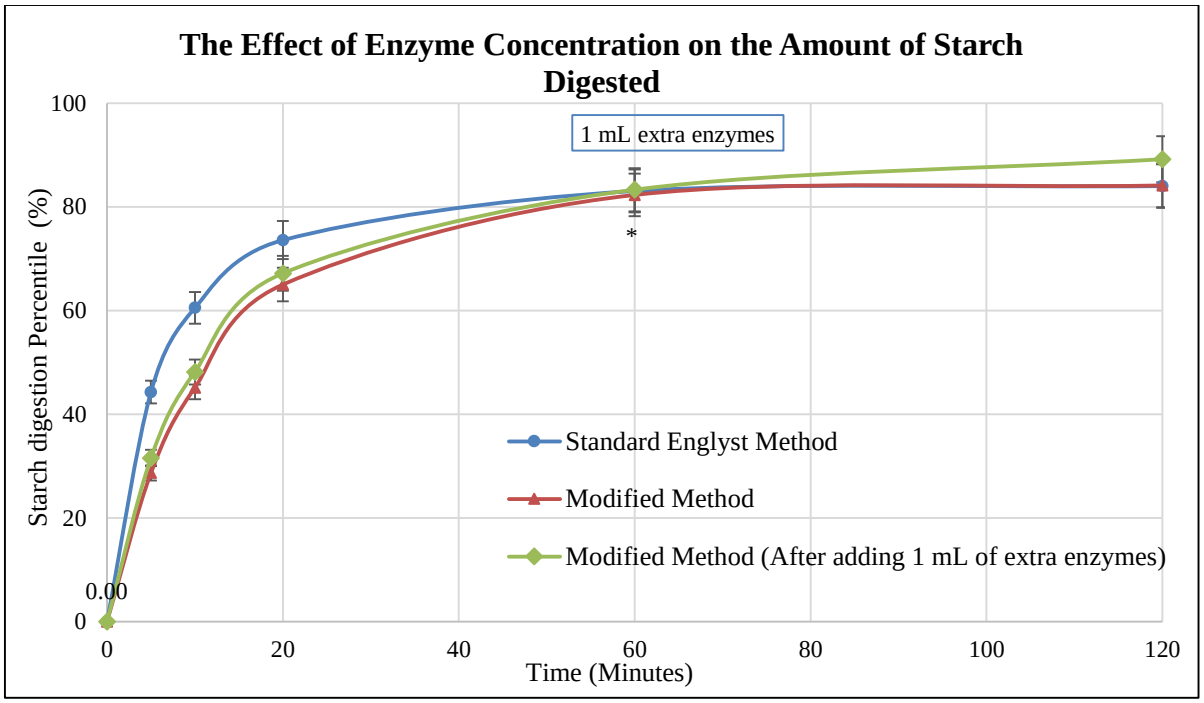


Figure 3.8: Comparison Between the Englyst Method and Modified Method after Adding Extra Enzymes to Assess Enzyme Concentration in the Modified Method

Data are means ± SEM (n = 4). The level of concordance between the modified method before and after adding the enzymes was strong and significant ($r = 0.99$, $n = 4$, $p < 0.05$).

Enzyme concentration is another important element in starch digestion. The enzyme concentration varied from method to method (Akerberg et al., 1998; Granfeldt et al.,1992; Muir et al., 1995). In the standard method, 1 mL of enzyme mixture was sufficient to analyse the digestible starch in 100 mg of the powdered sample (Englyst et al., 1999). However, because of the larger cup size used in the modified method, all materials were increased by a factor of four and the entire system volume was increased from 7 to 100 mL. Due to this increase in the system volume in the modified method, it was necessary to ensure that the enzyme concentration was sufficient to analyse 100% of the digestible starch in the sample. The experiment was run with two assays; an additional 1 mL of the same enzyme activity solution was added to one of the assays 1 hour after the intestinal phase. Results showed no significant difference between the two

assays. This result confirmed that the enzyme volume (4 mL) in the modified method was enough to digest all the available starch in the lentil sample. Although the correlation between the standard method and modified method was improved, a slight difference remained in the first 20 minutes of the starch digestion—which might be a result of omitting the beads in this stage. As described below, the beads were included in the final stage of the experiment after all modifications were performed.

Conclusion of Step 6:

The volume of enzyme mixture that had been used in the previous stages (4 mL) was sufficient to digest all the starch in the sample.

3.3.10 Step 7: Stopping the Enzyme Activity:

Another issue that required addressing was how to halt the digestion reaction in order to quantify the glucose released at the various time points. In order to obtain an accurate glucose reading at a given time point, the enzyme activity needed to be stopped immediately. In the standard Englyst method, 3 mL of ethanol was used to stop the reaction at a specific time point (Englyest et al., 1999). However, in the modified method, this technique could not be used because it might affect the performance of the glucose analyser instrument. To overcome this issue, two techniques were applied to stop enzyme activity in the current study: boiling and freezing. Additionally, rapid centrifugation of sediment food particles followed by direct glucose analysis was assessed (Reed, 2012; Whitehurst, & Van Oort, 2010; Wong & Whitesides, 1994). In the boiling technique, the aliquot was taken at a given time point and placed in a boiling water bath for 2 minutes at 100˚C. It was then centrifuged at 12000 rpm for 30 seconds, and the

supernatant was analysed for glucose content. In the freezing technique, the aliquot was taken at a given time point and placed in a freezing ice bath at -18˚C for 3 minutes. It was then centrifuged and the supernatant was analysed by the glucose measurement.

The results of these techniques suggested that neither boiling nor freezing was effective in stopping enzyme activity (data not shown). However, it was found that immediate centrifuging of the aliquots for 30 seconds at 12000 rpm was more efficient compared to the other two techniques. Furthermore, centrifuging was the most effective, easiest and quickest procedure to determine glucose concentration in the aliquots.

3.3.11 The Final Modified Method:

The final experiment included all modifications from previous stages. This experiment aimed to obtain the best correlation between the modified method and the standard Englyst method. In this step, the Greenland lentil sample was used in duplicate for two consecutive days, as described below.

3.3.11.1 Procedure for the Final Modified Method:

In the final method, 400 ± 5 mg of Greenland lentil sample (cooked and freeze-dried powder) was accurately weighed directly into a cup with 15 glass beads (5 mm diameter) and a magnetic stir bar. Then, 8 mL of the 5 mg/mL pepsin/HCl-guar gum solution was added to each cup and vigorously vortexed. The cups were transferred to the GI analyser instrument and placed into the hot plate wells at 37°C with continuous stirring (~300 rpm) for 30 minutes. Next, 88 mL of preheated (37˚C) 0.5 M sodium acetate buffer (pH 5.2) was added to each cup, mixed, and returned to the wells of the hot plate. The cups were incubated for approximately 5 minutes to

allow the contents to equilibrate to 37°C. After the 0-time aliquot was taken, 4 mL of enzyme solution were added to each cup. At each time point (5, 10, 20, 60, and 120 minutes) a single aliquot (500 µL) was removed into a microcentrifuge tube and centrifuged immediately at 12000 rpm for 30 seconds. Once the aliquot was centrifuged, 10 µL of the supernatant was taken and injected into the glucose analyser (Analox Instrument) to the measure the digestible starch in the samples

Calculations and Statistics:

Digestible Starch Calculations:

% Digestible Starch = G x (TV/0.5) x (100/DM) x (162/180) Where:

G = mass of glucose (µg) calculated from the standard curve= (absorbance - y-intercept)/slope

TV = Total Volume

TV/0.5 = volume correction for aliquot removed at different hydrolysis times (note: the total volume of solution is reduced by 0.5 mL at each time point)

DM = dry mass of sample (mg) = (sample mass × % moisture)/100

100/DM = factor to express digestible starch as a % of dry sample mass

162/180 = factor to convert free glucose (as it was measured) into anhydro-glucose, as it occurs in starch (0.9)

Statistics:

SPSS version 24 (IBM Corporation) was used for statistical analyses. A statistically significant comparison between the two methods was investigated by one-way ANOVA, and the Tukey-Kramer post hoc test was used to describe mean differences between the amount of starch

digested at each time point. The level of concordance between the amount of starch digested in the two methods (the standard Englyst and the modified method) were performed by using Pearson's correlation coefficient. All results are presented as means ± SEM. Statistical significance was concluded at $p < 0.05$.

3.3.11.2 Results and Discussion:

After running the final assay, results showed a strong, significant correlation between the amount of starch digested in the modified and standard Englyst methods $r(2) = 0.90$, ($p < 0.05$). Further, there was no significant difference in the amount of starch digestion between the two methods in the first 20 minutes ($p > 0.05$; Figure 3.9).

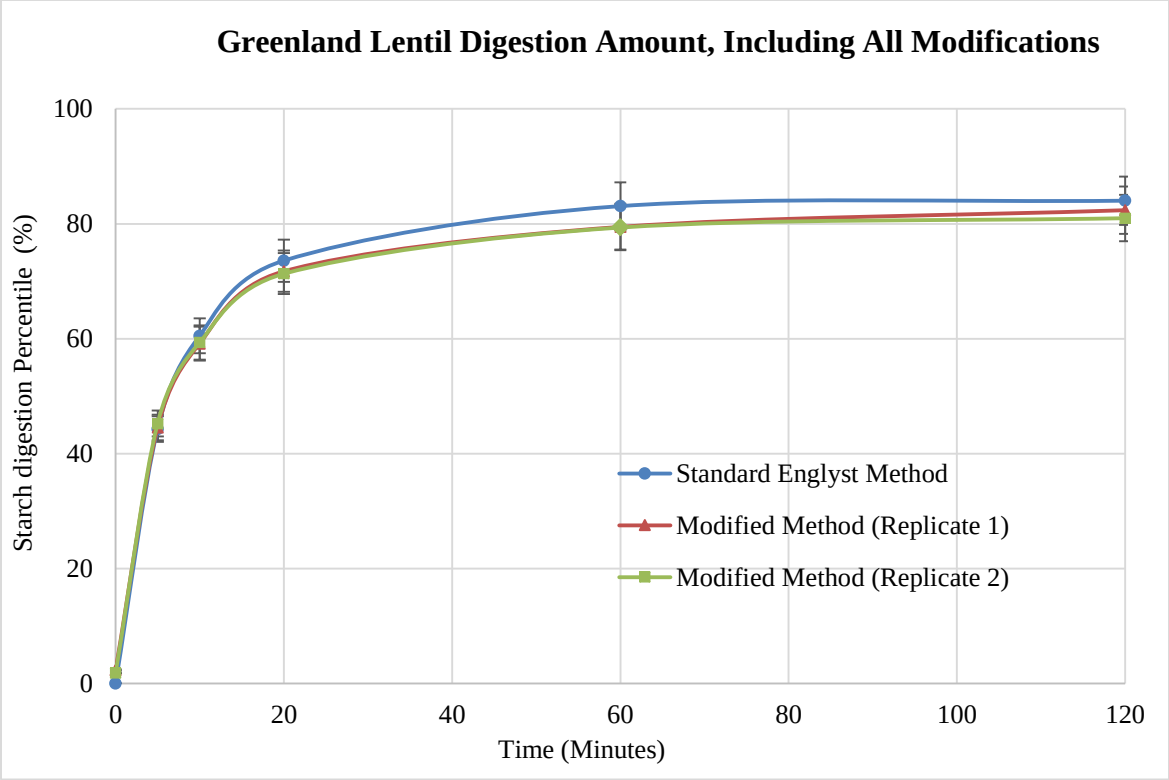


Figure 3.9: Comparison between the Englyst Method and Modified Method after Including All Modifications

Data are means ± SEM (n = 4). The correlation between the Englyst method and modified method after all modifications was positive, strong and significant ($r = 0.90$, $n = 4$, $p < 0.05$).

The results from the final run were promising and confirmed that our modified method and the standard method were highly correlated. However, the two methods were compared using only one sample (i.e., Greenland lentil powder). Therefore, in order to ensure reproducibility and reliability of the modified method, it was essential to run the modified method with a greater number and different varieties of samples. In the second stage of this study (described below), results from the modified method were compared with those obtained by the standard method (using the same foods), in order to produce the best possible correlation and to validate the method.

3.4 Second Stage: Modified Method Validation:

After the modified method had been established, the next step was to validate it. The objective of this stage was to validate the modified method by running 37 different samples and comparing the results of the modified method with those from the standard Englyst method which were previously obtained in-house. All samples used in this stage were freeze-dried powders (i.e., the same food format used previously in the standard Englyst method).

3.4.1 Stage 2 Aim:

To validate the modified method by analyzing 37 powdered lentil and yellow pea samples, and then to compare the results with the standard Englyst method.

3.4.2 Stage 2 Method and Materials:

3.4.2.1 Food Samples:

In this experiment, there were 20 different varieties of lentil and 17 different varieties of yellow peas. All samples were freeze-dried powders; preparation followed the same procedure described in the previous stage. The percent moisture and total starch of the samples are displayed in Table 3.1.

Table 3.1: Lentils and Yellow Peas Moisture and Total Starch Content

Lentils	Moisture %	Total Starch %	**Yellow Peas**	Moisture %	Total Starch %
3592_13	3.73	42.18	**Abrath**	3.26	47.25
3959_6	3.66	44.3	**Agassiz**	3.39	45.89
Astrerix	4.12	44.25	**Amarillo**	3.24	45.83
Cherie	3.39	44.67	**Ardill**	4.68	42.31
Dazil	3.62	44.55	**Argus**	3.7	45.69
Greenland	4.06	43.94	**Barhead**	3.64	44.46
Greenstar	2.96	43.21	**Canstar**	2.79	45.48
Ibc-479	4.34	43.08	**Eralysatr**	4.58	45.47
Ibc-550	3.61	44.43	**Golden**	3.39	43.74
Imax	3.87	44.38	**Hornet**	3.91	45.18
Imigreen	3.45	44.81	**Lcombe**	3.35	44.56
Impala	3.38	43.09	**Midas**	4.21	46.27
Impower	4.07	45.6	**Peace River**	3.5	43.68
Impress	3.45	45.89	**Prosper**	3.78	44.58
Improve	3.36	45.17	**Srento**	3.53	45.18
Imvincible	3.55	43.93	**Teasure**	3.39	43.74
Redberry	3.61	44.54	**Thunderbird**	4.5	45.89
Rosie	3.87	43.82			
Scarlet	3.93	43.09			
Viceory	3.59	45.24			

The moisture and total starch were obtained from unpublished data from Ramdath et al.; % = percentage based on dry weight.

3.4.2.2 Materials:

The same materials and reagents described above in the final stage of the modified method (section 3.3.11) were used for validation experiments. However, to save on materials and cost, the reagents were reduced to half the amount used in the initial stage. Therefore, the sample weight became 200 mg, the number of beads was reduced to 5, the sodium acetate buffer became 44 mL, the pepsin/ HCL-guar gum solution became 4 mL, and the pancreatic enzyme mixture was reduced to 2 mL, resulting in a total volume of 50 mL rather than 100 mL.

3.4.2.3 Method:

The procedure was based on the newly established modified method previously described (section 3.3.11). All samples were run in duplicate for two consecutive days to account for any day-to-day variation within the method and to ensure that the modified method achieves reproducible results that are comparable to the standard Englyst method. At the end of each second day, a set of four glucose readings was obtained for each sample. The average of these four readings was calculated and compared with previous results from the standard Englyst method.

3.4.2.4 Calculations, Statistics, and Data Analysis:

Calculations and Statistics:

As above, SPSS version 24 was used for statistical analyses. A statistically significant comparison between the two methods was tested using one-way ANOVA, and the Tukey-Kramer post hoc test was used to describe mean differences among the food samples at each time points. The correlation between the amount of starch digested in the standard and the modified

method were performed using Pearson's correlation coefficient. All results are presented as means ± SEMs; statistical significance was defined as $p < 0.05$. In addition, Bland Altman plots were used to determine the correlation between the AUC of the two methods.

Data Analysis:

Normality testing was performed on the data obtained from the two analytical methods of *in vitro* digestion and found that neither was normally distributed. Logarithmic transformation of the variables did not improve the distribution. Therefore, untransformed data were used in the analysis.

The difference between the AUC values obtained by the two methods (standard vs. modified) and the mean of those values were calculated in SPSS. A one-sample *t*-test was performed with "difference" as the test variable and test value = 0.

A scatterplot was developed in SPSS with the "difference" AUC in the y-axis and "mean" AUC in the x-axis. The y-axis reference line (position = mean = 0.70) was added to the plot. The limits of agreement (95% confidence interval) were then calculated:

Upper limit= mean + (1.96*standard deviation)

Lower limit= mean- (1.96*standard deviation)

Y-axis lines at these two positions were then added to the plot, creating a Bland Altman plot which displays trends observed in the dataset.

A linear regression between the AUC in the two methods was performed to test for the existence of proportionality bias. If the significance value of the coefficient was greater than or equal to 0.05.

3.4.3 Results of Stage 2:

3.4.3.1 Starch Fractions:

Lentils:

Overall, the results of the lentil assays showed that most of the digestible starch was analysed in the first 20 minutes (RDS). IBC-550 and Scarlet had the highest RDS value (86%). In contrast, Cherie and Redberry lentils had the lowest RDS value (74%). For the rest of the lentil samples, the RDS values ranged from 77% to 85%. The one-way ANOVA test showed a significant difference among the lentils' RDS values, $F(19,60) = 7.44$, $p < 0.001$. A Tukey *post hoc* test indicated that the Scarlet and Impower were significantly different from the other varieties of lentils ($p < 0.05$), while RDS values for IBC-479 and imigreen were not significantly different from any other lentil varieties' RDS values ($p > 0.05$). Regarding SDS, Cherie and Redberry lentils had the highest values (14%, 13%), while Greenland, Dazil, Imvincible and 3592-13 had the lowest (4%). The remaining lentils' SDS values ranged from 5% to 11%. The one-way ANOVA test for the SDS did not show any significant difference between the varieties ($F(19,60) = 1.23$, $p = 0.26$). RS values ranged from 6 % to 13 %. RS values were significantly different among the lentil varieties ($F(19,60) = 16.87$, $p < 0.001$). Scarlet was significantly different across thirteen varieties ($p < 0.05$), while Cherie had a significant difference with only two lentil varieties ($p < 0.05$; Table 3.2). Overall, results showed that the lentil varieties had significantly different RDS and RS values, which meant that the starch content of these varieties were different. Further, this variability in results indicated that the method was sensitive to differences in food starch content.

Table 3.2: Lentil Starch Fractions (RDS, SDS, and RS) Determined by the Modified Method

Lentil	RDS %	SDS %	RS %
3592-13	80.12 ± 0.36 ^{abc}	6.62 ± 0.36	13.26 ± 0.57 ^{gh}
3959-6	80.72 ± 0.42 ^{abcdef}	6.70 ± 0.70	12.58 ± 0.59 ^{fgh}
Astrerix	84.94 ± 0.49 ^{efg}	5.97 ± 0.51	9.08 ± 0.21 ^{abcd}
Cherie	80.28 ± 1.31 ^{abcd}	8.35 ± 0.77	11.37 ± 0.61 ^{cdefgh}
Dazil	78.39 ± 0.43 ^{a}	8.01 ± 0.36	13.60 ± 0.37 ^{h}
Greenland	79.89 ± 0.99 ^{abc}	7.94 ± 0.82	12.17 ± 0.91 ^{efgh}
Greenstar	78.63 ± 0.44 ^{ab}	7.16 ± 0.49	14.22 ± 0.20 ^{h}
IBC-479	82.95 ± 1.19 ^{abcdefg}	7.92 ± 1.41	9.13 ± 0.79 ^{abcd}
IBC-550	84.17 ± 1.04 ^{cdefg}	9.16 ± 0.43	6.67 ± 0.70 ^{ab}
Imax	84.77 ± 0.27 ^{defg}	5.81 ± 0.37	9.42 ± 0.29 ^{bcde}
Imigreen	82.51 ± 1.43 ^{abcdefg}	8.27 ± 1.81	9.22 ± 0.57 ^{bcde}
Impala	83.00 ± 1.15 ^{abcdefg}	8.14 ± 0.96	8.85 ± 0.22 ^{abcd}
Impower	85.16 ± 0.12 ^{fg}	6.22 ± 0.20	8.62 ± 0.10 ^{abc}
Impress	79.22 ± 0.55 ^{ab}	7.42 ± 0.55	13.36 ± 0.26 ^{gh}
Improve	81.67 ± 0.54 ^{abcdefg}	7.89 ± 0.98	10.45 ± 0.65 ^{cdefg}
Imvincible	83.11 ± 1.74 ^{bcdefg}	6.80 ± 0.80	10.09 ± 1.08 ^{cdef}
Redberry	80.47 ± 0.57 ^{abcde}	6.95 ± 0.34	12.58 ± 0.26 ^{fgh}
Rosie	79.29 ± 0.50 ^{ab}	7.21 ± 0.78	13.50 ± 0.86 ^{h}
Scarlet	86.09 ± 0.91 ^{g}	7.76 ± 0.86	6.15 ± 0.27 ^{a}
Viceory	80.14 ± 0.86 ^{abc}	8.21 ± 0.59	11.65 ± 0.65 ^{defgh}

Abbreviations used: RDS = Rapidly Digestible Starch; SDS = Slowly Digestible Starch; RS = Resistant Starch; SD = Standard Deviation
% = percentage based on the total starch. Within each column values without the same superscript are significantly different
Note, there was no any significant difference between the samples in the SDS ($p = 0.26$).

Yellow Peas:

Most of the digestible starch of the yellow pea samples was also analysed in the first 20 minutes (RDS). Barhead yellow peas had the highest RDS value (88%) while Agassiz yellow peas had the lowest RDS value (79%). The RDS values for the other yellow peas ranged between 82% and 86%. The one-way ANOVA test showed there was a significant difference among the yellow pea varieties $F(16,51) = 3.90$, $p < 0.001$. Lcombe was significantly different from five different varieties of yellow peas ($p < 0.05$), whereas most other yellow peas had nonsignificant differences or one significant difference compared to other yellow peas. Regarding SDS, Peace River had the highest value (6%), while Amarillo and Argus had the lowest (2%). In the rest of the yellow peas, SDS ranged from 3% to 5%. A one-way ANOVA illustrated that the Amarillo was the only variety that significantly differed from Peace River and Lcombe. The RS values ranged from 2% to 10 % of total starch. A one-way ANOVA indicated that there was a highly significant difference among the RS of yellow pea varieties, $F(16,51) = 6.90$, $p < 0.001$ (Table 3.3).

Table 3.3: Yellow Pea Starch Fractions (RDS, SDS, and RS) Determined by the Modified Method

Yellow Peas	RDS %	SDS %	RS %
Abrath	86.84 ± 0.71 ^{bc}	7.19 ± 0.69 ^{ab}	5.96 ± 0.10 ^{abc}
Agassiz	84.10 ± 0.30 ^{abc}	6.36 ± 0.31 ^{ab}	9.55 ± 0.15 ^{cd}
Amarillo	87.42 ± 0.22 ^{bc}	5.08 ± 0.36 ^{a}	7.50 ± 0.5 ^{bcd}
Ardill	85.74 ± 1.78 ^{abc}	7.30 ± 0.62 ^{ab}	6.96 ± 1.31 ^{abcd}
Argus	87.15 ± 0.61 ^{bc}	6.89 ± 0.66 ^{ab}	5.96 ± 0.40 ^{abc}
Barhead	87.99 ± 0.19 ^{bc}	6.96 ± 0.03 ^{ab}	5.05 ± 0.20 ^{ab}
Canstar	84.40 ± 0.57 ^{abc}	7.10 ± 0.67 ^{ab}	8.50 ± 0.41 ^{bcd}
Eralysatr	86.70 ± 1.57 ^{abc}	7.35 ± 1.15 ^{ab}	5.94 ± 0.53 ^{abc}
Golden	87.22 ± 0.78 ^{bc}	7.81 ± 0.26 ^{ab}	4.97 ± 0.83 ^{ab}
Hornet	82.69 ± 0.71 ^{ab}	7.46 ± 0.78 ^{ab}	9.85 ± 0.24 ^{cd}
Lcombe	80.98 ± 2.20 ^{a}	8.83 ± 0.71 ^{b}	10.19 ± 1.59 ^{cd}
Midas	83.03 ± 1.80 ^{ab}	6.55 ± 0.51 ^{ab}	10.43 ± 1.41 ^{d}
Peace river	83.56 ± 1.83 ^{ab}	9.27 ± 0.57 ^{b}	7.17 ± 1.51 ^{bcd}
Prosper	84.50 ± 0.72 ^{abc}	6.44 ± 0.96 ^{ab}	9.06 ± 0.30 ^{bcd}
Srento	83.89 ± 1.28 ^{abc}	6.06 ± 0.35 ^{ab}	10.04 ± 1.21 ^{cd}
Teasure	89.62 ± 0.48 ^{c}	7.83 ± 0.82 ^{ab}	2.55 ± 0.94 ^{a}
Thunderbird	84.78 ± 0.34 ^{abc}	6.18 ± 0.37 ^{ab}	9.04 ± 0.12 ^{bcd}

Abbreviations used: RDS = Rapidly Digestible Starch; SDS = Slowly Digestible Starch; RS = Resistant Starch; SD = Standard Deviation % = percentage based on the total starch. Within each column values without the same superscript are significantly different.

3.4.3.2 Agreement Between Two Methods:

Analysis by Time Point:

The procedure described above was followed herein. However, the data were analyzed separately at each time point (beginning at t = 5 minutes, as t = 0 minute observations existed only in the standard method). Regression analysis at 60 minutes and 120 minutes showed significance values greater than 0.05 (suggesting no trends or proportionality bias). For other time points, significance values were < 0.05 (i.e., there was some trend/bias observed).

When a trend is observed between variables being tested, it is generally suggested that the analysis is performed on transformed variables (natural logarithm) rather than the original variables. Therefore, natural logarithms of the observations in the standard and modified methods were calculated in SPSS (for t = 5, 10 and 20 minutes). Difference and mean variables were calculated from the transformed standard and modified observations. The previously described procedure was followed. A trend or bias was still observed (as significance values in the linear regression analysis were less than 0.05).

Analysis by AUC:

The level of agreement between the two methods was also tested using the AUC of all food samples in the two methods. Results showed there was no significant difference between the two methods, $F(1, 35) = 0.50$, $p = 0.49$ (Figure 3.10). Further, in this dataset, the significance value was greater than 0.05, suggesting no proportionality bias (i.e., there was no relationship or trend observed between the data points above and below the mean line).

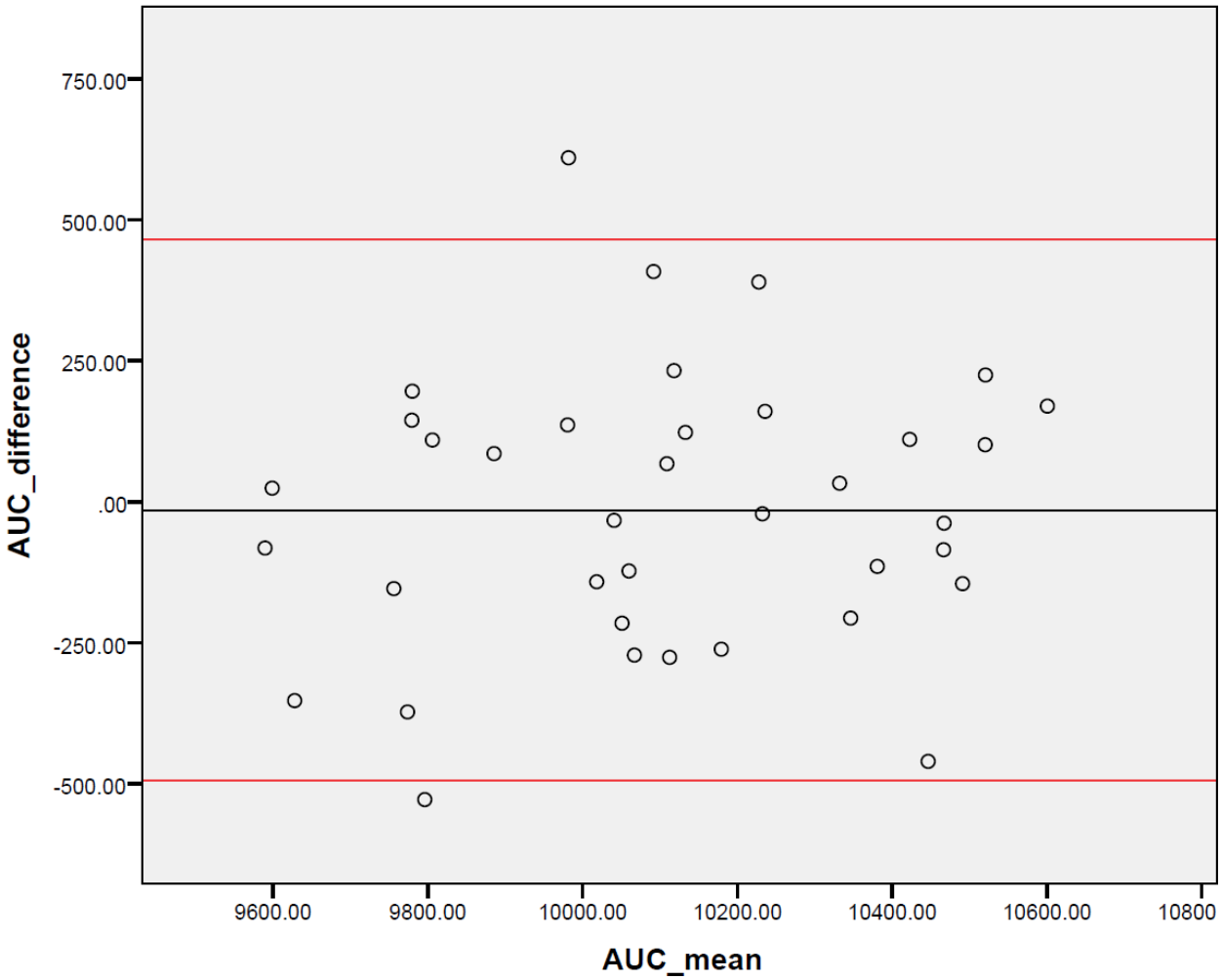


Figure 3.10: The Level of Agreement Between the AUC Values of the Modified Method and the Standard Englyst Method Using the Bland Altman Test.

Correlation and Regression Analysis:

At each time point, correlation and regression analysis were performed between the AUC in the standard method and the modified method for all samples. The equation of the regression line and the significance of the regression model were obtained in SPSS along with the correlation coefficient. A scatter plot of the regression line and the 95% confidence intervals of

the AUC mean were plotted. Results showed a significant regression between the AUC values of the two methods, $F(1, 35) = 23.22$, $p < 0.001$ with $R^2 = 0.48$ (Figure 3.11).

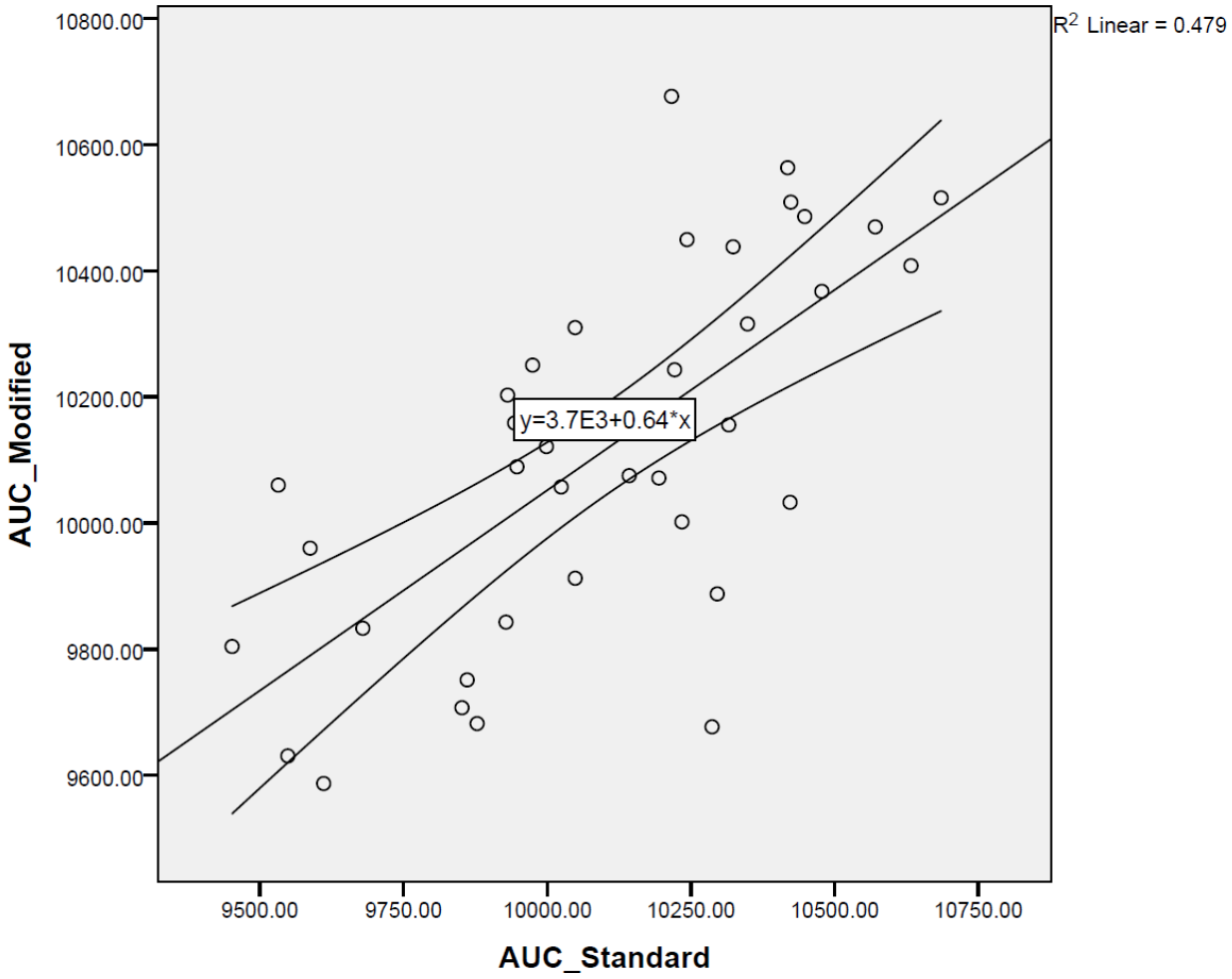


Figure 3.11: Linear Regression Between the AUC of the Modified Method and the Standard Englyst Method

Pearson's correlation was used to compare the individual samples in both methods. The starch digestion values of each variety of lentils and yellow peas in the modified method were compared with the values of the corresponding variety previously obtained using the standard method. The correlation was positive and strong for all samples; the majority of the 37 samples

had correlations between the two methods greater than r = 0.90 (n = 4, p < 0.05). A typical digestion profile is shown in Figure 3.12 for one lentil and one yellow pea sample, where the correlation between the amount of starch digested in the two methods was 0.99 and 0.98, respectively.

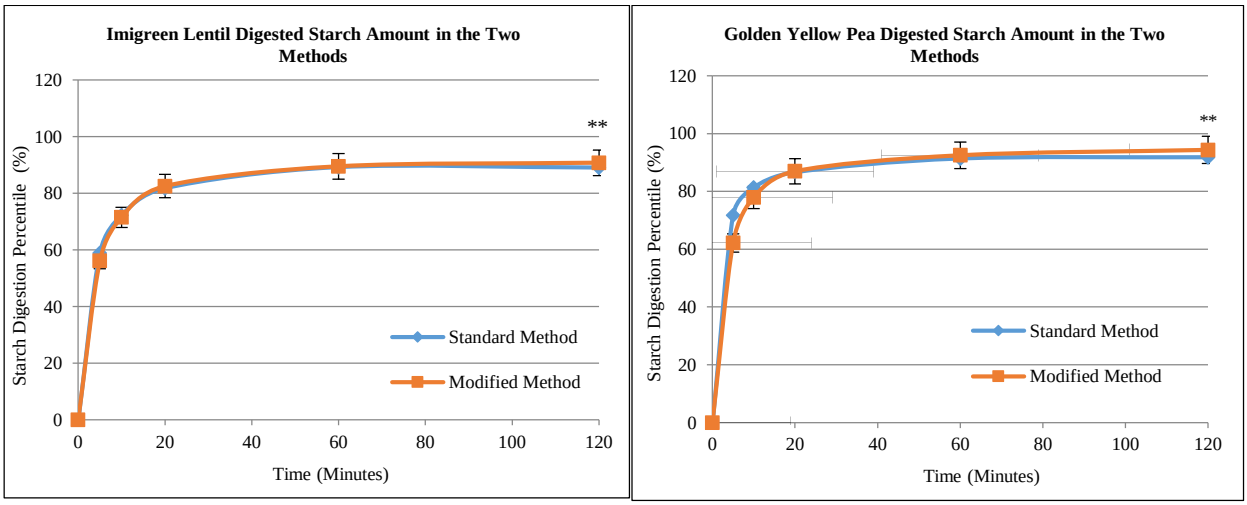


Figure 3.12: Examples of Lentil and Yellow Pea Varieties Show High Correlation Between the Modified and Standard Method

Data are means ± SEM (n = 4). Strong correlation between the two methods after using the Imigeen and Golden as simple, * r = 0.99 (n = 4, p < 0.001), ** r = 0.98 (n = 4, p < 0.001)

The overall level of agreement between the two methods was also assessed. All analysed glucose values at different time points for the 37 samples in the modified method were compared with glucose values of the standard method for the same samples, wherein each sample had four replicates for each time point. Pearson's correlation was used to compare 888 glucose readings with those of the standard method. The correlation between the two methods was positive and strong (r (886) = 0.98, p < 0.05). Figure 3.12 shows the agreement between the standard method and the modified method in the starch fractions for all lentil samples, while Figure 3.13 shows the same agreement between all yellow pea samples.

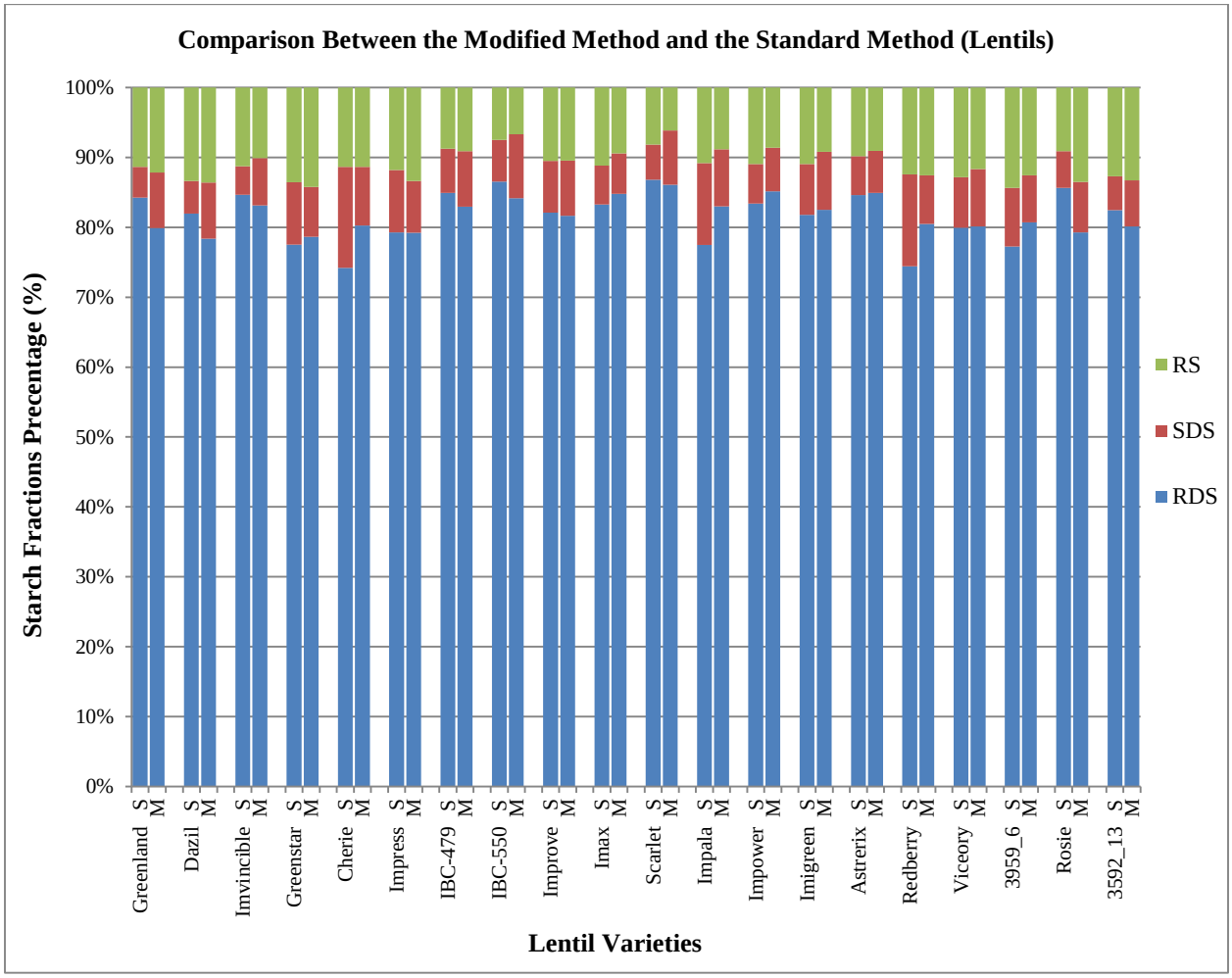


Figure 3.13: Comparison Between Modified and Standard Method Including All Lentil Samples

Abbreviation used: RDS = Rapidly Digestible Starch; SDS = Slowly Digestible Starch; RS = Resistant Starch; S = standard method; M = modified method

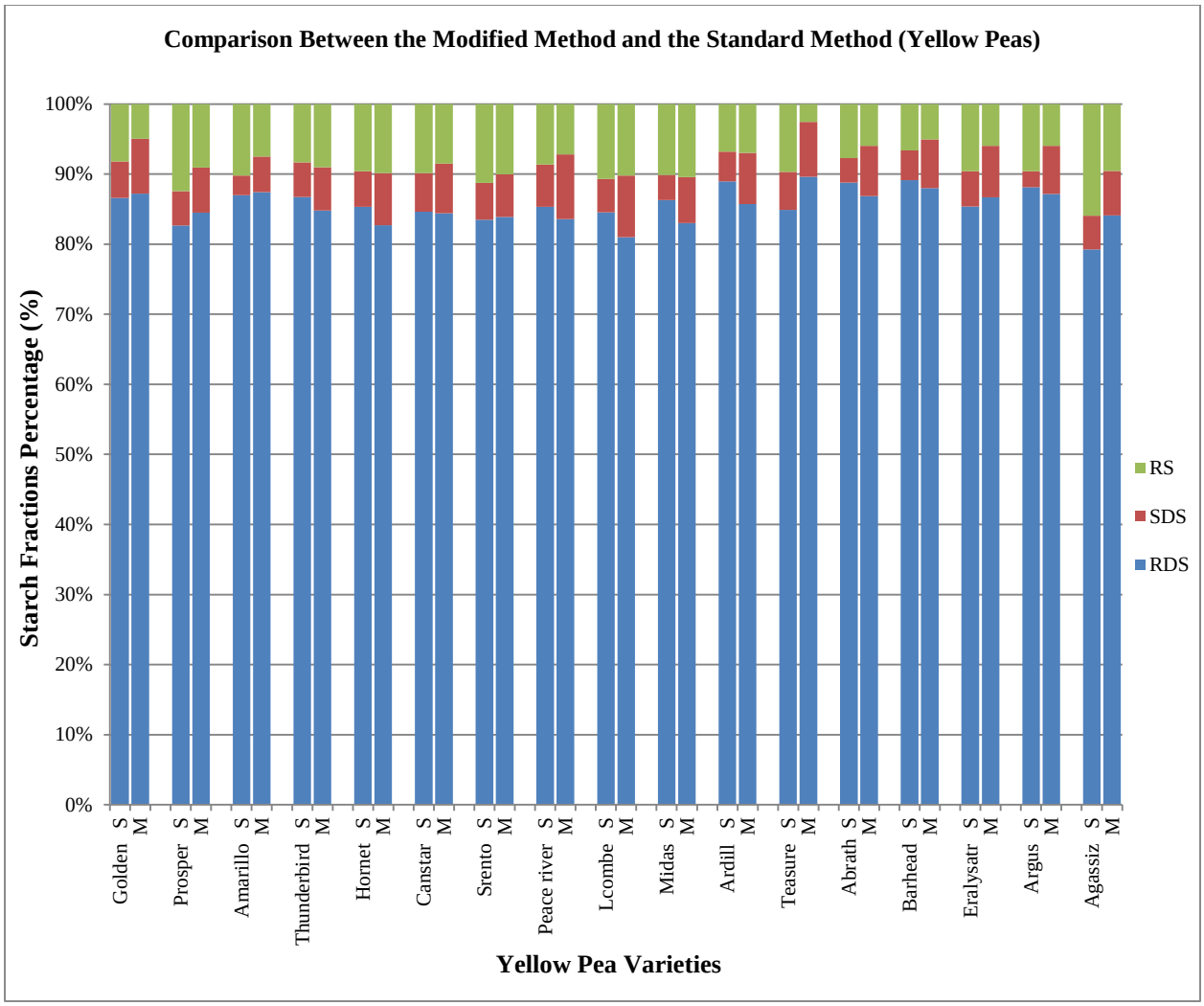


Figure 3.14: Comparison between Modified Method and Standard Method Including All Yellow Pea Samples

Abbreviation used: RDS = Rapidly Digestible Starch; SDS = Slowly Digestible Starch; RS = Resistant Starch; S = standard method; M = modified method

3.4.4 Discussion of Stage 2:

The results from this stage showed a strong correlation between the standard Englyst method and the modified method. This agreement between the two methods confirmed the sensitivity of the new modified method to different starchy food contents and the reproducibility of the method. These results were also supported by previous studies that used the Englyst

method to measure the digestibility of starch (Mishra, Monro & Hedderley, 2008; Silvester, Englyst & Cummings, 2005). For example, a study by Weurding et al. (2001) aimed to assess the correlation between the *in vitro* starch digestion and *in vivo* rate and the extent of the effect of starch digestion in broiler chickens. A modified method based on the Englyst method was used. Results showed strong agreement between the *in vitro* starch digestion measurement in the modified Englyst method and the amount and extent of starch digested in the broiler chickens. Several other studies used the Englyst method to show the correlation between the *in vitro* amount of starch digested and the *in vivo* glycemic response. For example, Araya et al., (2002) examed the effects of a few starchy foods (rice, pasta, potatoes, and some legumes) on glycemic response using the Englyst method. The enzymes used in this *in vitro* method were based on the Englyst method. The results showed a high and significant correlation between the rapid carbohydrate digestion assessed by the Englyst method and the *in vivo* glycemic response. Another study by Anderson et al.,(2010) used the Englyst method as an *in vitro* method to estimate the relation between *in vitro* cornstarch digestibility and glycemic response. Results demonstrated that the *in vitro* digestible starch estimated by the Englyst method could be used to predict the effect of the starch fraction on the *in vivo* glycemic response. However, despite the fact that most of the previous studies that were based on the Englyst method or that used a modified version of the Englyst method showed a high correlation with the *in vivo* method, none of these studies highlighted the limitations of the standard Englyst method and how this might affect their results. Therefore, this project aimed to focus on these limitations to obtain a more efficient method to assess starch digestion and to predict GI values. Results from previous studies also supported the aim of the next stage of this study, which was to validate the new modified method against the *in vivo* method.

3.5 Conclusion of Stage 2:

In conclusion, the final results of this stage illustrated a strong correlation between the two methods. Further, they demonstrated that the modified method had a constant, reliable, and reproducible outcome. The aim of this chapter was achieved, and the results showed that the modified method could be a suitable alternative to the standard Englyst method. However, the experiments in this stage used powdered samples only. In order to establish the modified method as an alternative to *in vivo* GI measurement, validation with food samples 'as eaten' was required.

4 CHAPTER 4

Validation of a Modified *In vitro* Digestion Method Using *In vivo* GI Values

4.1 Introduction:

The previous chapter successfully modified and validated an *in vitro* digestion method using a semi-automated instrument. The modified method was reproducible and was adapted for measurement of freshly cooked foods. Therefore, it was necessary to compare the results from a new established *in vitro* method with the *in vivo* method to ensure that the modified method was comparable, reproducible and could be used as an alternative to the *in vivo* method. Several previous studies have used the *in vivo* method to validate the *in vitro* method. For example, Englyst et al. (1999) used the *in vitro* method to measure the RDS in eight different starchy meals and compared results with the glycemic response for the same foods in eight volunteers. The researchers found a highly significant correlation between the two methods ($r = 0.98$, $p < 0.001$). Another study conducted by Goni et al. (1997) established a new *in vitro* method to assess the GI values in starchy food. The *in vitro* method was conducted using different starchy foods, and the results were compared with the glycemic response of 30 subjects who consumed the same foods. Results showed a high correlation between the glycemic response and the *in vitro* amount of starch digested at 90 minutes ($r= 0.91$, $p \leq 0.05$). These studies illustrated the importance of validating a new established *in vitro* method using the results of the *in vivo* method. However, the methods established by Englyst and Goni compared the glycemic response of freshly cooked feeds with *in vitro* methods that used the same foods, which were cooked, freeze-dried, and milled. The concern regarding these previous studies was that it was unclear how food sample processing might have affected the assay results and the *in vitro* GI values.

4.2 Rationale and Objectives:

In the previous chapter, the modified method was compared with the standard Englyst method by comparing the results of 37 different freeze-dried samples of lentils and yellow peas. The results were very promising, with a strong, positive correlation between the amount of starch digested in the modified method and standard method ($r(886) = 0.98$, $p < 0.05$). However, one of the main limitations of the standard Englyst method was the use of freeze-dried samples, which is different from food 'as eaten'. In other words, freeze-dried samples do not mimic the physiological processes of food digestion in the human body. The freeze-dried food used in the standard Englyst method is ground into a very fine powder, leading to both a higher digestion rate and GI. The aim of this chapter was to validate the modified method by using food 'as eaten'. Validating the modified method involved two stages: first, the modified method was conducted with fresh food ('as eaten'); and second, results of the modified Englyst method were compared with published *in vivo* GI results to ensure that the modified method was comparable and reproducible.

4.3 Method and Materials:

4.3.1 Materials:

The materials and reagents were identical to those mentioned in the previous chapter. In addition, a meat grinder attachment with two different pore sizes (4.5 mm and 8 mm) was attached to 5.5 Quart Stand Mixer (Cuisinart®, USA). The grinder was used for sample preparation. The Ro-Tap sieve shaker instrument (W.S. Tyler, USA) was used to separate the food samples based on particle size.

4.3.2 Food List:

To validate the modified method, a variety of starchy foods with different, but known *in vivo* GI values was required. The starchy food list was chosen from the International Tables of Glycemic Index and Glycemic Load Values (Atkinson, Foster-Powell, & Brand-Miller, 2008)**.** The International Table is a systemic review study that includes published and reliable studies of GI. The International Tables study comprises a pool of international human GI values for more than 200 studies published between 1981 and 2007. Most of the GI values for foods listed in these tables consisted of mean values of many studies conducted with the same foods. This review listed 2500 foods in different categories, such as legumes, vegetables, and fruits (Atkinson, et al., 2008). Because the International Table is considered a reliable source for GI values, the food list for the current study was based on these tables. Food selections were made based on foods that were previously examined in Canada and that are readily available in the local market. Twenty different starchy foods were selected based on availability in the marketplace in Guelph, Ontario. The food list includes bread, rice, pasta, cereals, legumes, potatoes, crackers, and barely.

4.3.3 Estimating Total Starch:

The total starch of most foods used in this study was calculated based on the Canadian Nutrient File (CNF) (Health Canada, 2015). The total starch (available carbohydrate) is estimated by subtracting the fibre from the carbohydrate of each food with consideration of moisture content. For yellow pea, the total starch value was obtained from a previous study

(Marinangeli, Kassis, & Jones, 2009). The total starch for foods not available in CNF was calculated based on the Nutrition Facts Table listed on the food package (Table 4.1).

Table 4.1: Starchy Food List Profile and Preparation

Food	Brand & Origin	GI value[+]	Moisture %	Available CHO	Total starch %	Fibre %	Free sugar %	Cooking and preparation	Sample wt (mg)
Barley (4486)	Barley, pot, Blue Menu, President's Choice Canada	37	68.8	25.72	82.44	2.5	0.28	Rinse barley and drain. Place around 100 g of barley in a medium pot, cover with cold water and bring it to a boil. Reduce heat, cover, and cook for 40 minutes	600
Bread (4066)	White, Wonder Canada	73	37.34	45.91	73.27	3.3	7.62		350
Cheerios (1475)	General Mills Canada	74	3.99	61.83	64.40	10.9	4.85		200
Chickpeas (7061)	Canned chickpeas President's Choice® Blue Menu™, Canada	38	66.87	16.57	50.02	6.3	4		650
Corn Flakes (1242)	Kellogg's Canada	93	3.4	82.98	85.90	4.2	8.57		200
Cracker (6792)	President's Choice® Blue Menu™ Ancient Grains snack crackers Canada	65	2.59	64.69	66.41	10.9	1.22		200
Digestive	McVitie's UK	59	2.5	63.19	64.81[*]	2.6	18.42		200
English muffin (3902)	Whole Grain Multigrain, President's Choice® Blue Menu™, Canada	45	40.2	43.5	72.74	2.8	0.81		360
Granola *	Granola Clusters, Original, low fat, President's Choice® Blue Menu™ Canada	63	3.2	69.1	71.38	12.72	21.82		200
Green Peas (4212)	Green Giant Canada	51	79.52	9.86	48.14	4.4	4.65	In a medium pot place 1 cup of green peas with 50 ml of water, cover and bring it to a boil. Reduce heat and simmer 3 to 5 minutes. Drain off excess water before grinding.	1000
Pasta (4464)	100% durum semolina spaghetti, Unico Canada	41	62.13	29.06	76.74	1.8	0.56	94 g of pasta, bring 1-litre of water to boil in medium pot. Add the pasta, stirring for 7 to 9 minutes.	500

Pasta (4469)	Barilla, Canada	58	62.13	29.06	76.74	1.8	0.56	Bring 1 liter of water to a boil in a medium pot and add 100 g of pasta. Boil for 8 minutes, stirring. Drain excess water before grinding.	600
Pasta (4517)	Whole Wheat Spaghetti President's Choice® Blue Menu™ Canada	45	67.15	23.34	71.05	3.2	0.8	Bring 1 liter of water to a boil in a medium pot and add 100 g of pasta. Boil for 10 to 12 minutes, stirring. Drain extra water before grinding.	600
Potato (2422)	White Canada	73	77.46	18.61	82.56	1.4	0.85	One medium white potato peeled and cut into cubes ~ 2.5 cm in size, put in water and bring to a boil for 12 minutes. Drain excess water.	800
Potato (5708)	Red potato without skin. Canada	89	76.67	17.79	76.25	1.8	1.43	One medium red potato peeled and cut into cubes ~ 2.5 cm in size, then put in water and bring it to boil for 12 minutes. Then drain extra water	1000
Potato (5708)	Red potato without skin, overnight fridge Canada	56	76.67	17.79	76.25	1.8	1.43	One medium red potato peeled and cut into cubes ~ 2.5 cm in size, then put in water and bring it to boil for 12 minutes. Then drain extra water and kept it in fridge overnight	1000
Rice (4497)	Brown, long grain, President's Choice®	66	73.09	21.46	79.75	1.5	0.35	In rice cooker place half cup of rice and 1 cup of water. Let stand for 5 minutes after cooking.	600
Rice (4523)	Basmati, President's Choice Canada	69	68.44	27.77	87.99	0.4	0.05	In rice cooker place half cup of rice and 1 cup of water and turn on the rice cooker. Let stand for 5 minutes after cooking.	600
Rice (4523)	white, long grain, No name Canada	72	68.44	27.77	87.99	0.4	0.05	In rice cooker place half cup of rice and 1 cup of water. Let stand for 5 minutes after cooking.	800
Yellow Peas **	Whole yellow pea Canada	32**	66.37	-	43.74**	-	-		600

Abbreviation used: CHO=carbohydrates; wt = sample weight; Number between the brackets is the food Food code in Canadian Nutrient File (CNF); * Data based on Nutrition Facts Table; ** Published value; + *In vivo* published GI values from the International GI Tables

4.3.4 Sample Cooking:

All foods were prepared in the lab on the same day as the experiment. Foods that required cooking were prepared based on manufacturer instructions as listed on the package label. Cooking instructions are listed in Table 4.1.

4.3.5 Sample Preparation: Comparison between Chewing and Mincing:

In order to assess the amount of digested starch in the various starchy foods *in vitro*, an appropriate preparation method that mimics physiological food digestion in the oral phase was required. In previous studies, a variety of techniques were used to mimic chewed food, including grinding, sieve blending, and mincing (Brighenti et al.,1998; Champ, 1992; Weurding et al., 2001). In the Englyst method, a food processor with pore size 4 mm was used to compare with the actual chewed sample of the same food and results showed a significant correlation between the minced and the chewed samples (Englyst et al., 1992). In the current study, mincing was chosen for sample preparation. In order to investigate whether the mincing was comparable with actual chewing, samples of lentils and yellow peas were used. Two techniques were performed to assess the correlation between the minced and chewed samples. The first technique was based on the amount of starch digestion and the second was based on food particle size.

4.3.5.1 Step 1: Comparison between Mincing and Chewing Based on the Amount of Starch Digested:

The first technique was a comparison between the minced and chewed based on the starch digestion profile. The rationale was that if mincing and chewing were comparable, they should produce comparable results in terms of the amount of digested starch in the starch

analysis. In the standard Englyst method, a grinder with 4 mm was used in sample preparation (Englyst et al., 1992). In this experiment, two pore sizes (4.5 mm and 8 mm) were used to assess which one was more comparable to chewing.

In this method, all samples consisted of cooked Imigreen lentils. The sample was divided into two different groups: the first sample group was minced using a meat grinder into two different sizes (4.5 mm and 8 mm), and 1 g of the minced sample was taken for the *in vitro* digestion; the second group was the reference group, in which the food samples were chewed by three volunteers. Each volunteer was served with a tablespoon of the cooked sample on three different occasions and each sample was chewed ten times. Next, three samples from each volunteer were mixed and 1 g of the chewed sample was taken for starch analysis. Finally, all samples were run for two hours using the modified Englyst method and based on the procedure described above.

Results indicated that there was a high level of agreement between the amount of digested starch in the two minced samples ($r(2) = 0.99$, $p > 0.05$). Furthermore, in the comparison between the minced and chewed samples, there was a significant correlation in the amount of starch digested between the two minced samples (4.5 mm and 8 mm) and chewed samples ($r(2) = 0.97$, $p < 0.05$) for both correlations. However, the only difference between the minced and chewed samples was the amount of digested starch at 20 minutes ($p = 0.02$; Figure 4.1).

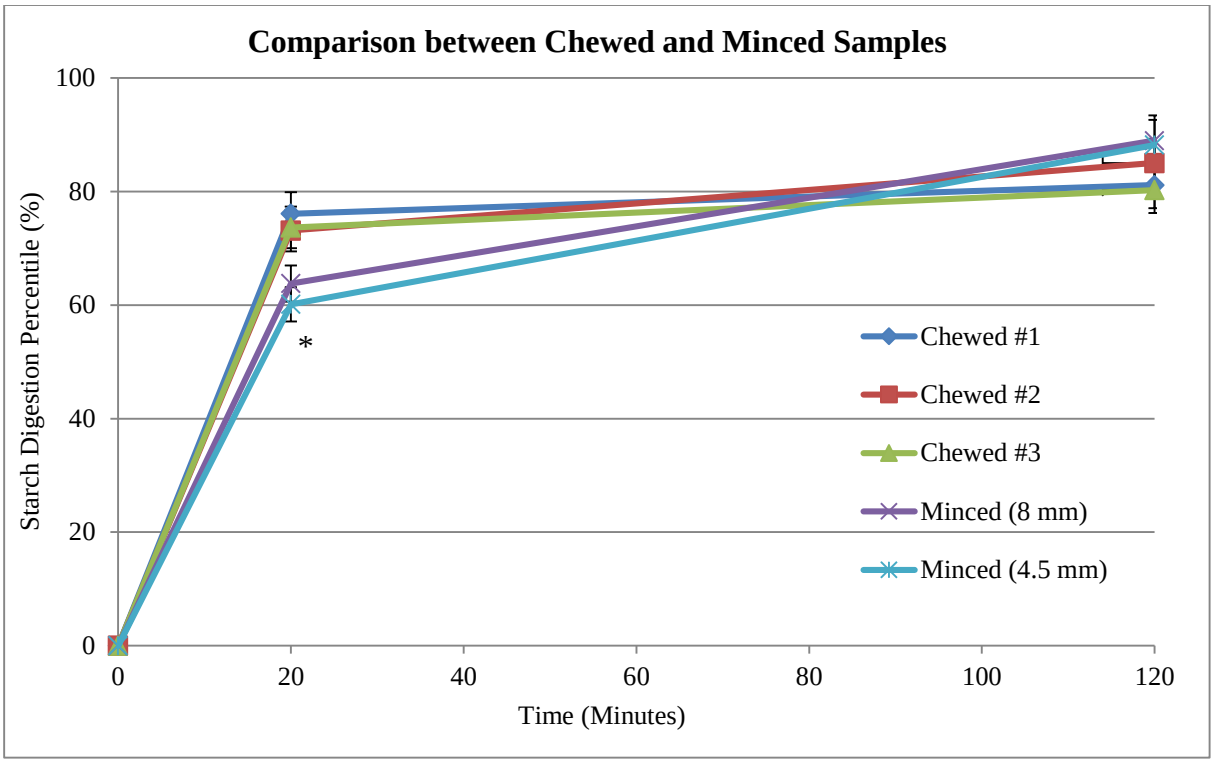


Figure 4.1: Comparison Between Chewed and Minced Samples Based on Amount of Starch Digested

The level of concordance between the minced sample and chewed sample was high ($p < 0.05$)

4.3.5.2 Step 2: Comparison between Mincing and Chewing Based on Food Particle Size:

The second technique was to compare between the minced and chewed samples based on particle size using sieve shaker instrument. The aim of this step was to assess the difference between the chewed and minced food samples based on particle size.

In this method, cooked Imigreen lentil samples were divided into two groups: in the first group two samples were passed through 4.5 mm and 8 mm pore-sized mincers, then one tablespoon was taken from each minced sample for oven drying. In the second group ($n = 3$), each volunteer was served one tablespoon of the sample on three different occasions and chewed it ten times. Next, all minced and chewed food samples were dried in a dry oven overnight at

30˚C. On the second day, each food sample was processed in a particle-size analyser comprising a sieve shaker with seven different sieve sizes (0.25, 0.35, 0.50, 0.85, 1.40, 1.70, 2.36 mm). The sample particles trapped at each sieve layer were removed and weighed. The proportion of trapped food at each level was then compared with the proportion of chewed sample at the same level. Pearson's correlation was performed to assess the level of concordance between the two minced samples (4.5 mm and 8 mm) and the chewed sample at each layer of the sample based on the proportion weight. The results of this experiment indicated that both minced food samples (4.5 mm and 8 mm) were highly correlated with chewed food samples ($r(2) = 0.98$, $p < 0.05$ and $r(2) = 0.97$, $p < 0.05$, respectively; Figure 4.2).

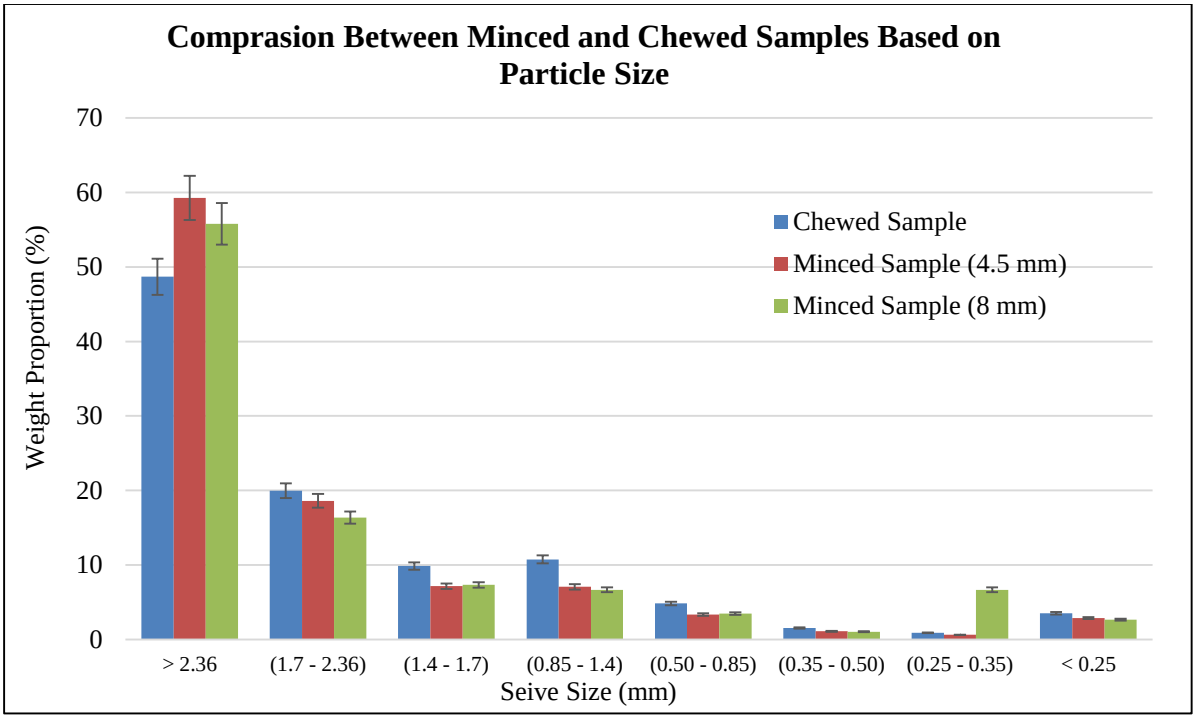


Figure 4.2: Comparison Between Chewed and Minced Samples Based on Food Particle Size

The correlation between mincing and chewing was highly significant ($p < 0.05$).

The two techniques showed a high level of agreement between mincing and chewing. Results illustrated that mincing could be used as an alternative technique to chewing in sample preparation. However, the food processor with a 4.5 mm pore size was selected for use in this study because the minced food samples were more homogeneous in texture, producing a representative sample and reduced variation between samples**.**

4.3.6 Method: *in vitro* Measurement of RDS and SDS Using the Modified Method:

This experiment was performed based on the procedure described in the previous chapter. The food samples were cooked and prepared on the same day as the experiment. All samples were run in duplicate for two consecutive days to ensure that results were reproducible and constant and an internal quality control sample was included in all assays. The sample weight was 200 ± 5 mg based on each sample's dry weight, where the moisture content of freshly cooked foods was considered. For example, for foods that contained 80% moisture, a 1000 mg sample was used, equalling 200 mg of dry weight.

4.3.7 Calculation and Statistics:

RDS and SDS, RS calculations:

The rate of starch digestion was expressed as the percentage of total starch digestion at different times (0, 20, 60, and 120 min). The RDS was obtained based on the amount of starch digested at 20 minutes while the SDS was calculated by subtracting the amount of starch digested at 120 minutes from that of 20 minutes.

Digestible Starch Calculations:

% Digestible Starch = G x (TV/0.5) x (100/DM) x (162/180) Where:

G = mass of glucose (µg) calculated from the standard curve= (absorbance - y-intercept)/slope

TV = Total Volume

TV/0.5 = volume correction for aliquot removed at different hydrolysis times (note: since the total volume of solution is reduced by 0.5 mL at each time point.

DM = dry mass of sample (mg) = (sample mass × % moisture)/100

100/DM = factor to express digestible starch as a % of dry sample mass

162/180 = factor to convert free glucose (as it was measured) into anhydro-glucose, as it occurs in starch (0.9).

GI Calculation:

The AUC was calculated based on the starch hydrolysis amount at each time point (0, 20, 60, and 120 minutes) by using the SigmaPlot (Systat Software Inc., UK). The HI was calculated as the ratio of the AUC for the tested food and the AUC for a reference food (white bread or glucose). The GI value was then estimated based on HI using one of two equations:

1- Granfeldt equation:

eGI_{Hi} = 0.862× calc HI + 8.198 (Granfeldt et al., 1992)

2- Goni equation:

eGI = 39.21 + 0.803(H_{90}) (Goni et al., 1997)

Abbreviation: eGI = estimated glycemic index, HI = hydrolysis index

Statistics:

SPSS version 24 (IBM Corporation) was used for statistical analyses. A statistically significant correlation between the two methods was followed-up using one-way ANOVA, and the Tukey-Kramer post hoc test was used to test mean differences between the starch fraction for the tested food. The level of concordance between the GI values in the two methods (*in vivo* versus *in vitro*) was performed using Pearson's correlation coefficient. All results are presented as means ± SEMs. Statistical significance was concluded with $p < 0.05$.

4.4 Results:

4.4.1 RDS, SDS, and RS Values:

After all foods samples were run in duplicate for two consecutive days, the mean of the four glucose readings was calculated. The results showed that there was some variation in the amount of starch digestibility among the tested foods. For example, foods such as Corn Flakes, pasta, brown rice, Cheerios, and white rice had the highest amount of digested starch after two hours of running the experiment, wherein the amount of digested starch at 120 minutes was between 83% and 88% of available starch. In contrast, green peas had the lowest digested starch amount (40%) and barley had the second lowest digested starch amount at 58% of available starch. The rest of the foods ranged between 64% and 77% (Figure 4.3). Most of the foods had high RDS values, meaning that the majority of starch in the food was digested in the first 20 minutes. However, there was a wide range of RDS values among the foods. For example, Corn Flakes and Cheerios had the highest RDS values, at approximately 82% of available starch, while green pea and barley had the lowest RDS values at 37% and 38%, respectively. The RDS

values of the remaining foods ranged between 41% and 70%. A one-way ANOVA showed a significant difference in the RDS values among all tested foods, $F(19,60) = 73.88$, $p < 0.001$.

The SDS values were lower for all foods compared with RDS. For example, Corn Flakes and English muffins had the lowest SDS values at 0.77% and 0.65%, respectively, while foods such as brown rice and white rice had the highest SDS values, at approximately 28% (Table 4.2). A one-way ANOVA showed a significant difference in the SDS values of all tested foods, $F(19, 60) = 31.88$, $p < 0.001$. The RS values also were varied among the food samples, wherein RS was highest in green peas (61%) and lowest in brown rice and whole wheat pasta (~12%). Finally, a one-way ANOVA also indicated a significant difference in the RS values of all tested foods, $F(19, 60) = 91.99$, $p < 0.001$. This significant variation in RDS, SDS, and RS among the tested foods reflects the starch variability among the tested foods. This variability in the results was important to demonstrate the modified method's sensitivity and reproducibility.

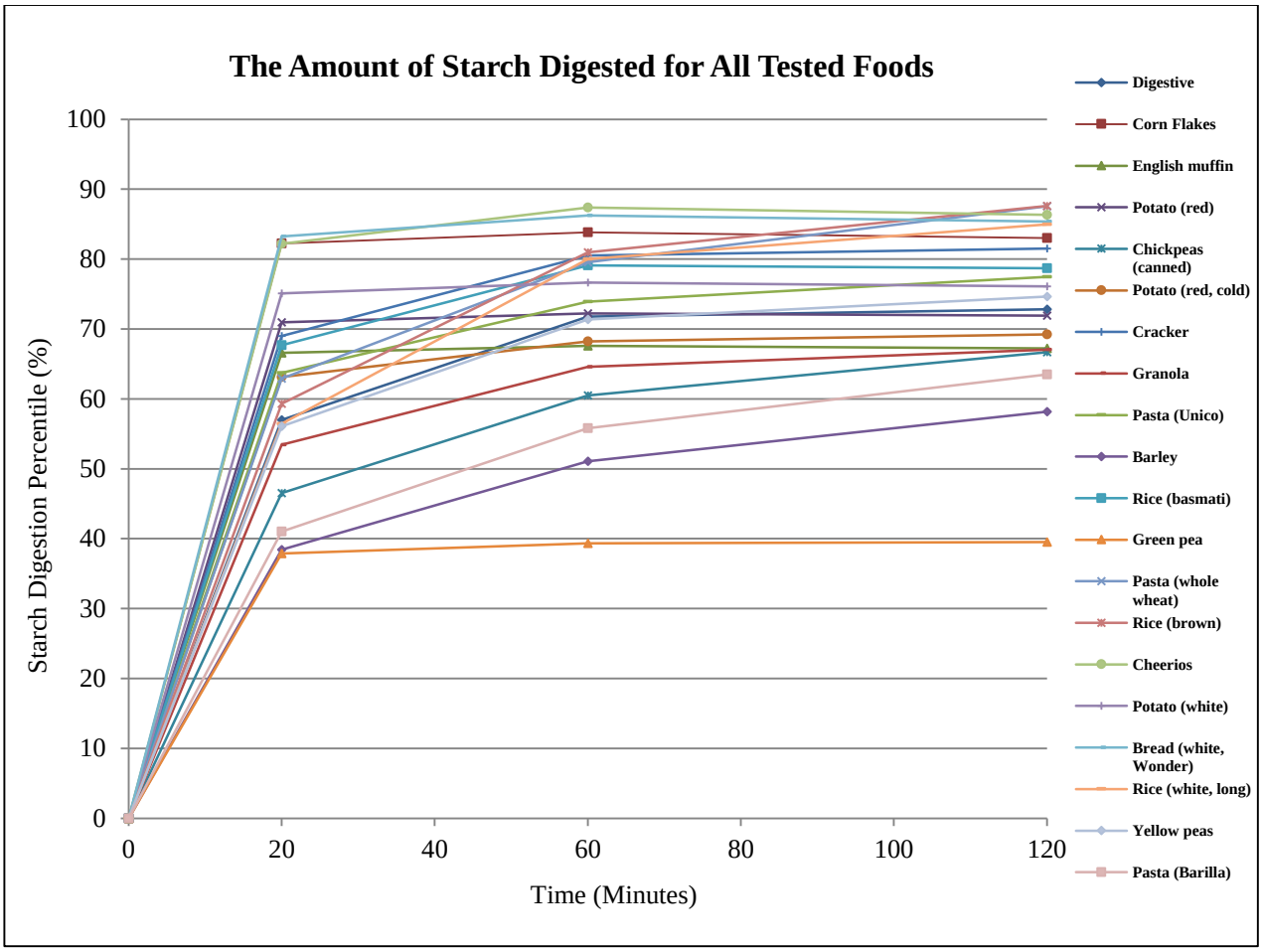


Figure 4.3: Amount of Starch Digested for All Food Samples

Table 4.2: Starch Fractions (RDS, SDS, and RS) of Test Foods Determined by the Modified Method

Food	RDS %	SDS %	RS %
	Mean ± SE	Mean ± SE	Mean ± SE
Barley	38.41 ± 0.68 [a]	19.75 ± 0.30 [efg]	41.84 ± 0.30 [j]
Bread (white)	83.26 ± 0.78 [k]	2.12 ± 0.97 [a]	14.63 ± 0.97 [ab]
Cheerios	81.15 ± 2.14 [jk]	5.27 ± 0.88 [ab]	13.58 ± 0.88 [ab]
Chickpeas (canned)	46.55 ± 0.56 [b]	20.14 ± 0.87 [efg]	33.31 ± 0.87 [hi]
Corn Flakes	82.20 ± 0.69 [k]	0.77 ± 0.67 [a]	16.97 ± 0.67 [abc]
Cracker	68.98 ± 0.67 [ghi]	12.55 ± 0.54 [bcde]	18.47 ± 0.54 [bcd]
Digestive biscuit	56.99 ± 0.44 [cde]	15.85 ± 0.47 [def]	27.16 ± 0.47 [fg]
English muffin	66.59 ± 0.42 [fgh]	0.65 ± 0.94 [a]	32.76 ± 0.94 [hi]
Granola	53.45 ± 0.88 [c]	13.61 ± 0.94 [cde]	32.94 ± 0.94 [hi]
Green peas	37.52 ± 0.35 [a]	1.56 ± 0.40 [a]	60.92 ± 0.40 [k]
Pasta (Barilla)	41.01 ± 0.20 [ab]	22.50 ± 1.51 [fgh]	36.49 ± 1.51 [i]
Pasta (Unico)	63.74 ± 1.77 [efgh]	13.75 ± 0.63 [cde]	22.52 ± 0.63 [cdef]
Pasta (whole wheat)	62.89 ± 1.82 [defg]	24.68 ± 0.74 [gh]	12.43 ± 0.74 [a]
Potato (white)	75.08 ± 2.31 [ij]	1.00 ± 2.33 [a]	23.92 ± 2.33 [def]
Red potato	70.96 ± 0.25 [hi]	0.96 ± 0.38 [a]	28.08 ± 0.38 [fgh]
Red Potato (fridge)	63.14 ± 1.63 [defg]	6.08 ±1.51 [abc]	30.78 ± 1.51 [ghi]
Rice (basmati)	67.68 ± 1.61 [gh]	10.99 ± 1.27 [bcd]	21.33 ± 1.27 [cde]
Rice (brown)	59.30 ± 3.23 [cdef]	28.32 ± 2.04 [h]	12.39 ± 2.04 [a]
Rice (long grain)	56.46 ± 1.20 [cde]	28.53 ± 0.46 [h]	15.01 ± 0.46 [ab]
Yellow peas	56.06 ±1.32 [cd]	18.59 ± 0.91 [defgs]	25.35 ± 0.91 [efg]

Abbreviations: RDS, rapidly digestion starch; SDS, slowly digestible starch; RS, resistant starch; SE, Standard Error; n = 4; % = percentage of the total starch.
Within each column values without same superscript are significantly different

4.4.2 *In vivo* GI Values:

As previously mentioned, *in vivo* GI values were obtained from the International Tables of GI for starchy foods previously tested in Canada and available in Canadian Supermarkets (Table 4.3). However, the English muffin value in the International GI table was unusually low (GI = 46) compared with previously published *in vivo* studies (GI = 78; Neuhouser et al., 2006; GI = 86; Wolever et al., 1994). Therefore, the mean (GI = 82) of the published English muffin GI values was used in the current study.

4.4.3 *In vitro* GI Values:

In vitro GI values were calculated as mentioned above based on two GI estimating equations: the Granfeldt equation (eGIHi = 0.862× calc HI + 8.198; Granfeldt et al., 1992) and the Goni equation (eGI = 39.21 + 0.803(H90; Goni et al., 1997) (Table 4.3).

Table 4.3: *In vivo* and *In vitro* GI Values for All Tested Foods

Food	*In vivo* Values*		*In vitro* Values**		
	GI/glucose	GI/bread	HI	GIa	GIb
Barley	37	53	79.96	53.99	83.61
Bread (white)	72	103	79.75	94.40	83.49
Cheerios	74	74	90.70	87.40	89.50
Chickpeas (canned)	38	54	46.36	56.97	65.16
Corn Flakes	93	114	90.10	84.83	89.17
Cracker	65	93	84.34	80.90	86.01
Digestive biscuit	59	84	72.29	68.22	79.40
English muffin	45	64	91.02	89.91	89.68
Granola	63	90	66.43	64.87	76.18
Green peas	51	73	46.17	43.82	65.06
Pasta (Barilla)	58	83	76.66	74.55	81.80
Pasta (Unico)	41	58	96.83	74.55	92.87
Pasta (whole wheat)	45	65	91.81	79.40	90.11
Potato (white)	73	105	100.00	88.21	94.61
Red potato	89	126	80.61	77.68	83.96
Red Potato (fridge)	56	79	74.47	72.39	80.59
Rice (basmati)	69	99	94.51	89.67	91.60
Rice (brown)	66	94	85.66	82.03	86.74
Rice (long grain)	73	41	34.97	76.94	58.91
Yellow peas	32	32	72.53	81.40	79.53

Abbreviations used: HI=Hydrolysis Index; GI a= calculated based on the Goni equation; GI b= calculated based on Granfeldt equation; GI/glucose; glycemic index values calculated based on glucose as reference; GI/bread; glycemic index values calculated based on bread as reference; * The GI values based on the GI International Tables, calculated based on glucose as reference; ** The GI value estimated based on the *in vitro* modified method

4.4.4 Comparison Between the Modified Method and the *In vivo* Method:

4.4.4.1 *In vivo* vs. *In vitro* GI Values:

To validate the modified method against the *in vivo* method, a correlation between *in vitro* GI values and *in vivo* GI values was performed. The first comparison was between the absolute *in vitro* GI values (obtained from the modified method and calculated based on the Goni equation) and the *in vivo* GI values (obtained from the International GI Tables, based on white bread as a reference). Pearson's correlation showed a medium and significant level of agreement between the two methods ($r(18) = 0.51$, $p < 0.05$; Figure 4.4). The linear correlation between the *in vivo* and *in vitro* GI values was $R^2 = 0.26$, $n = 20$ (Figure 4.5).

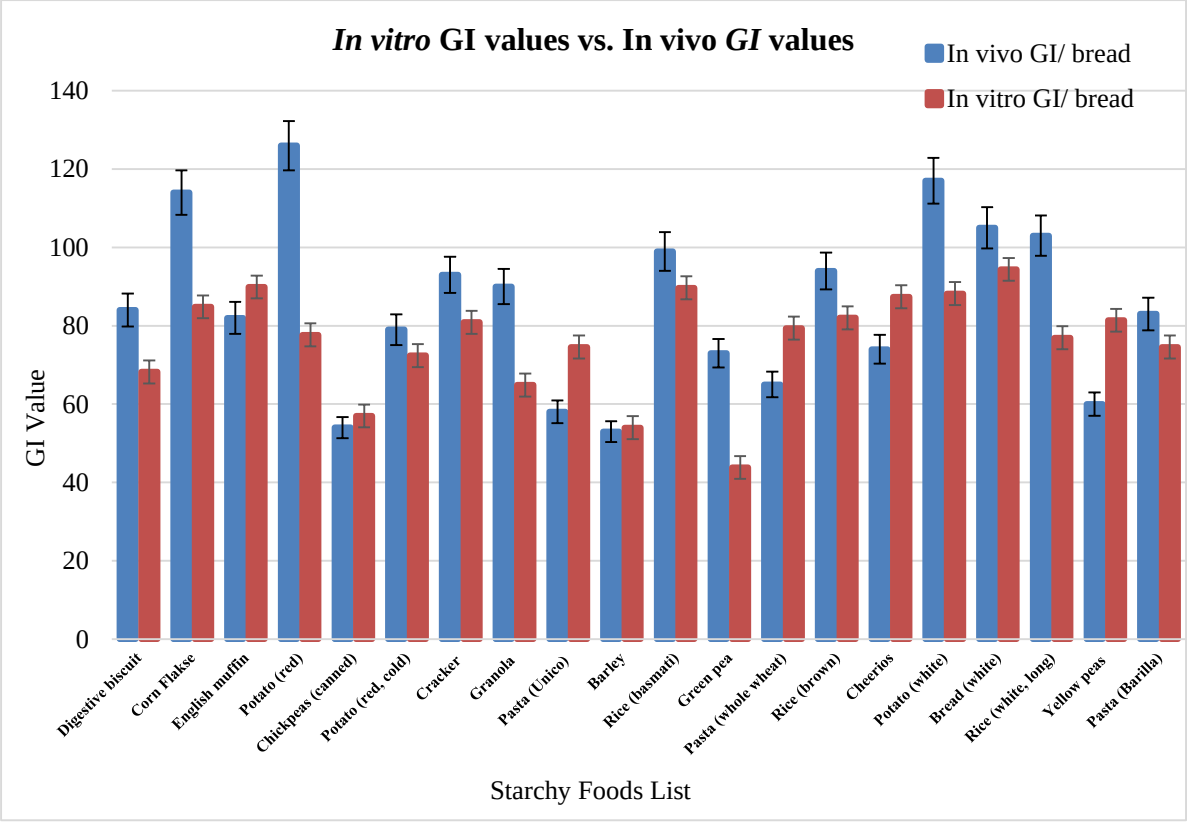


Figure 4.4: Comparison Between *in vitro* and *in vivo* GI Values of All Food Samples

Abbreviation: GI = glycemic index

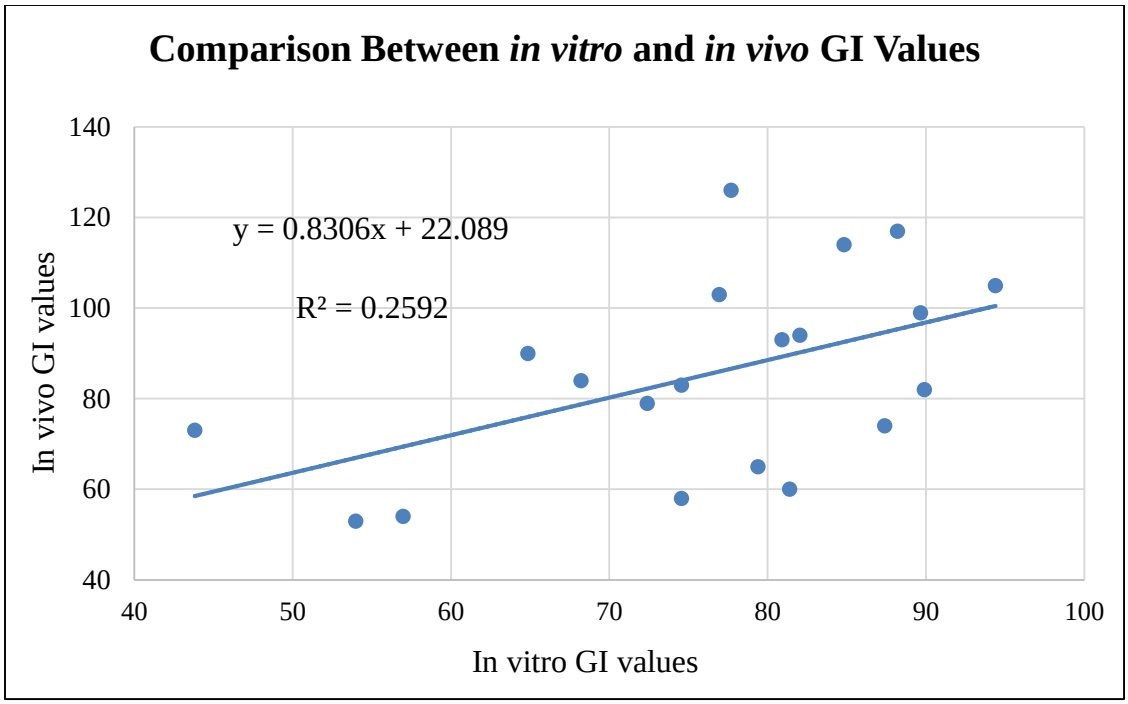


Figure 4.5: Linear Correlation Between *in vitro* and *in vivo* GI values

Abbreviation: RDS = GI = glycemic index. $R^2 = 0.26$ ($n = 20$, $p < 0.05$)

In general, there was a moderate association between the *in vitro* GI values and the *in vivo* GI values. As shown in Figure 4.4 the two methods were comparable for some foods, while this was not the case for other foods. In previous studies, it was found that RDS had a major effect on the amount of starch digested (Englyst et al., 1992, 1996). From a physiological perspective, the glycemic response curve for the human body (i.e., *in vivo*) is different from the *in vitro* starch digestion curve. In the *in vivo* glycemic response curve, the glucose level reaches its peak in the first 20-30 minutes and then decreases gradually over a 2-hour period. In contrast, in the *in vitro* starch digestion curve, the digestion rate reaches its peak in the first 20 minutes and then remains at that level for 2 hours, and does not decline (Barclay, Brand-Miller, & Wolever, 2005). Figure 4.6 shows the difference between the *in vivo* glycemic response and the

in vitro starch digestion of white beard (Englyst et al., 1999). Despite the difference in the two hydrolysis curve patterns, both curves hit their peak in the first 20 minutes, at which point most of the starch was digested. This could explain the agreement between the *in vivo* GI values and *in vitro* RDS values in the previous studies. Therefore, in the following steps of the experiment, it was decided that the RDS values would be compared with the *in vivo* GI values rather than the *in vitro* GI values.

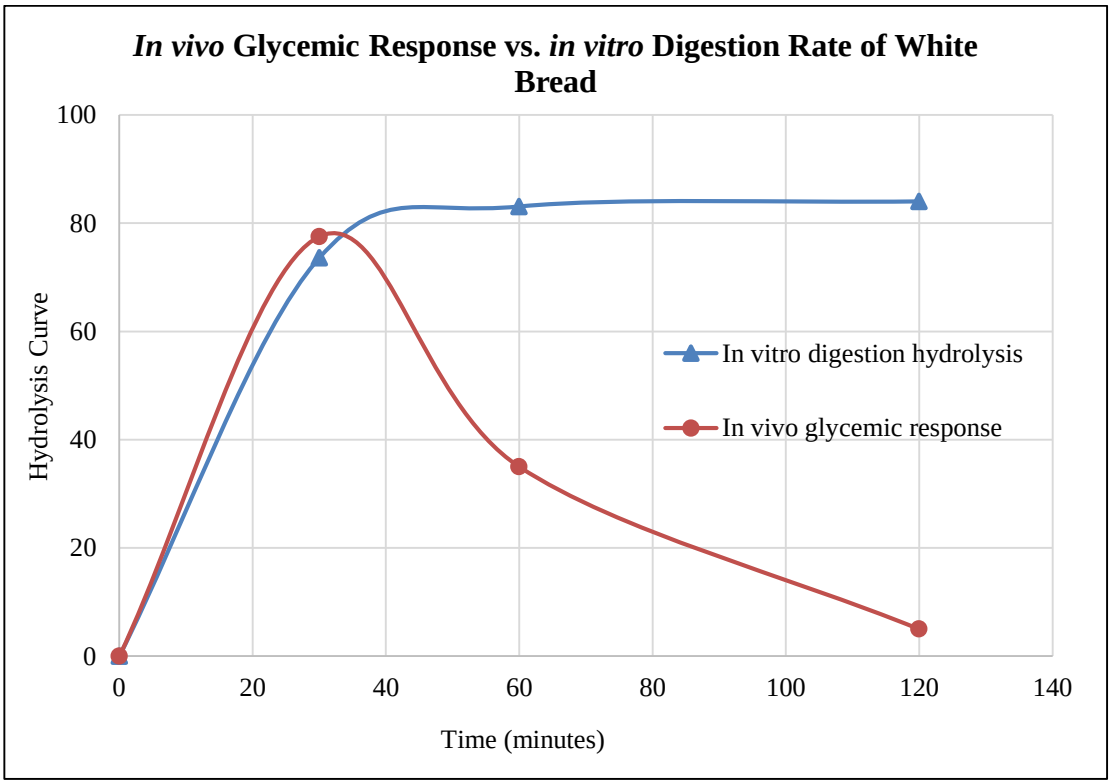


Figure 4.6: Comparison Between the *in vivo* Glycemic Response Curve and *in vitro* Digestion Curve

(Englyst et al., 1999)

4.4.4.2 Rapidly Digestible Starch vs. *in vivo* GI

To assess the agreement between the *in vitro* and *in vivo* methods, another Pearson's correlation test was run using RDS values that were obtained from the modified method and *in vivo* GI values based on glucose as a reference. The results showed an improved level of concordance between the two methods, wherein the association was higher than the previous step and significant ($r(18) = 0.72$, $p < 0.05$; Figure 4.7). In addition, the linear correlation improved to $R^2 = 0.51$ (Figure 4.8).

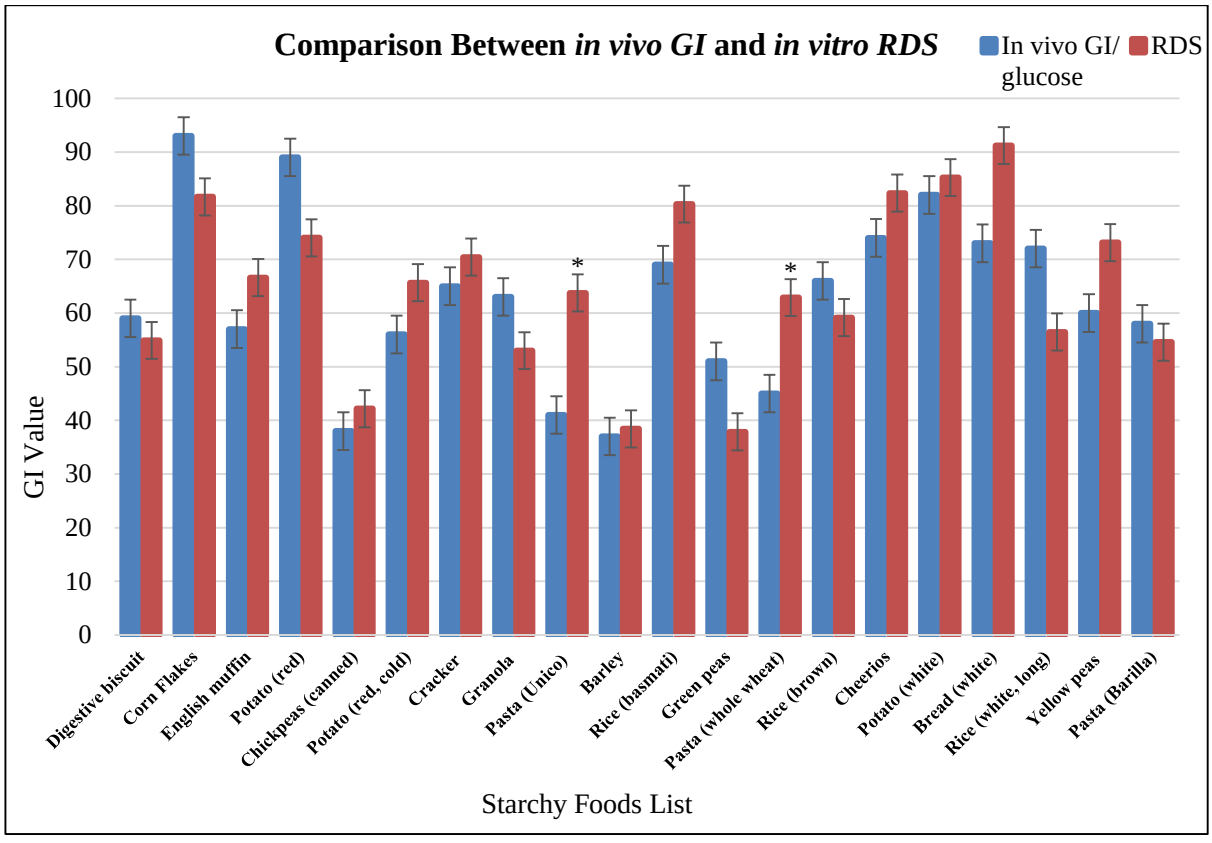


Figure 4.7: Comparison Between RDS Values and *in vivo* GI Values for All Food Samples

Abbreviation: RDS = rapidly digestible starch; GI = glycemic index.
*significantly different at $p < 0.05$

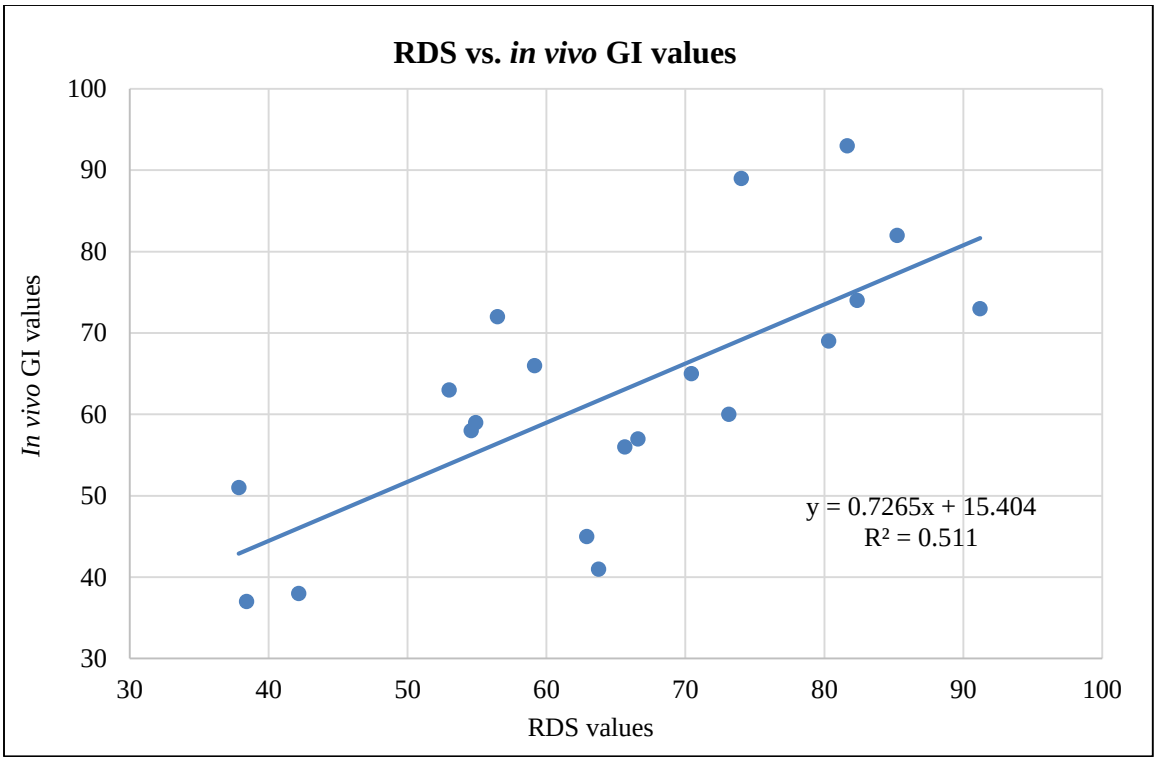


Figure 4.8: The Linear Correlation Between RDS Values and *in vivo* GI Values

Abbreviation: RDS = rapidly digestible starch; GI = glycemic index.
$R^2 = 0.51$ ($n = 20$, $p < 0.05$)

Results indicated that the RDS values had a higher correlation with *in vivo* GI values than *in vitro* GI values. However, not all foods in the assay showed the same level of agreement between the two methods; the RDS and *in vivo* GI values of some foods differed between the two methods. For example, the *in vitro* GI value (63) of two kinds of pasta (Unico pasta and whole wheat pasta) was higher than their respective *in vivo* values (41 and 45, respectively). To assess the level of agreement between the two methods for each food, a Pearson's correlation test was repeated using the RDS and *in vivo* GI values of 18 starchy foods (excluding the two kinds pasta) (Figure 4.7). The association between the two methods was improved ($r(16) = 0.78$, $p < 0.05$) compared to the value before the pastas were excluded ($r = 0.72$; Figure 4.10). The linear correlation also improved to $R^2 = 61$ (Figure 4.10).

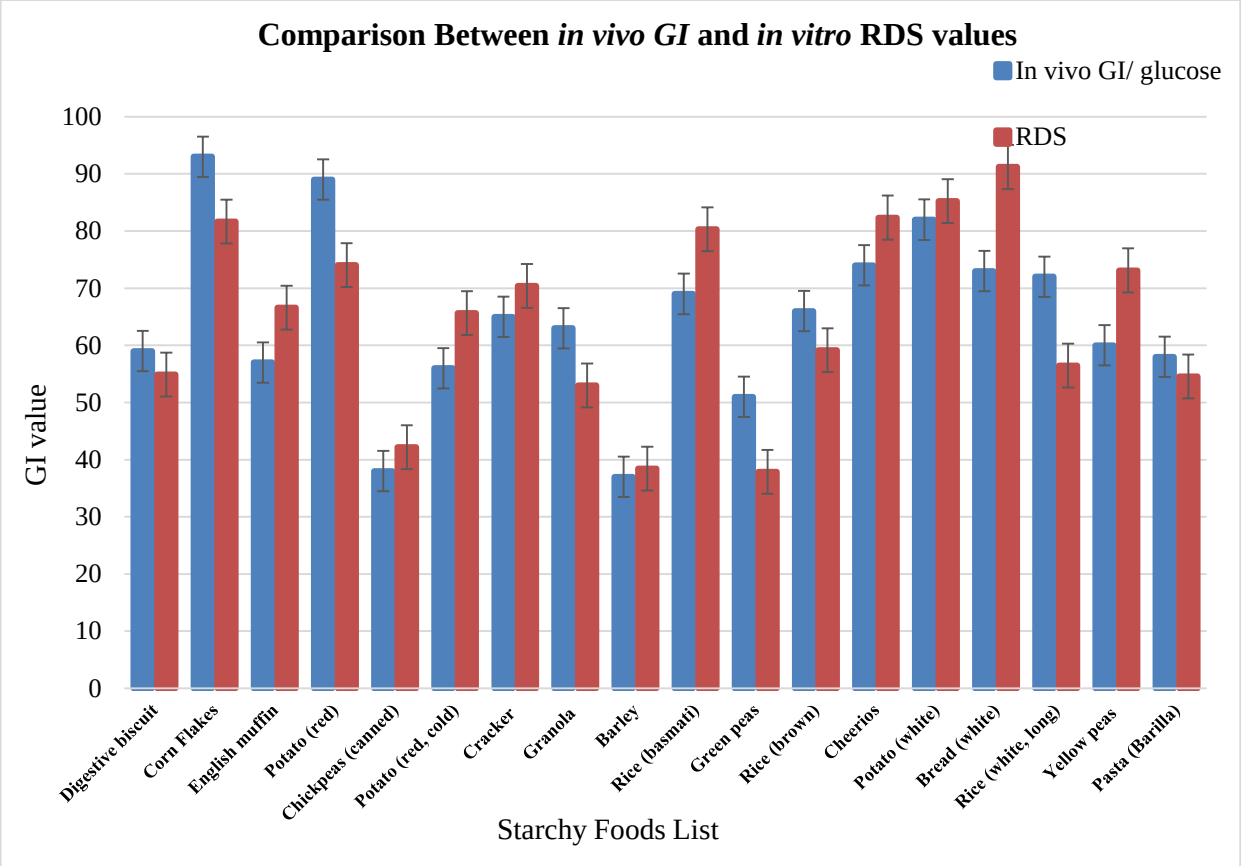


Figure 4.9: Comparison Between *in vivo* GI Values and RDS Values (n = 18)

Abbreviation: RDS = rapidly digestible starch; GI = glycemic index.
The correlation between the two methods was positive and significant, $r = 0.78$, $p < 0.05$

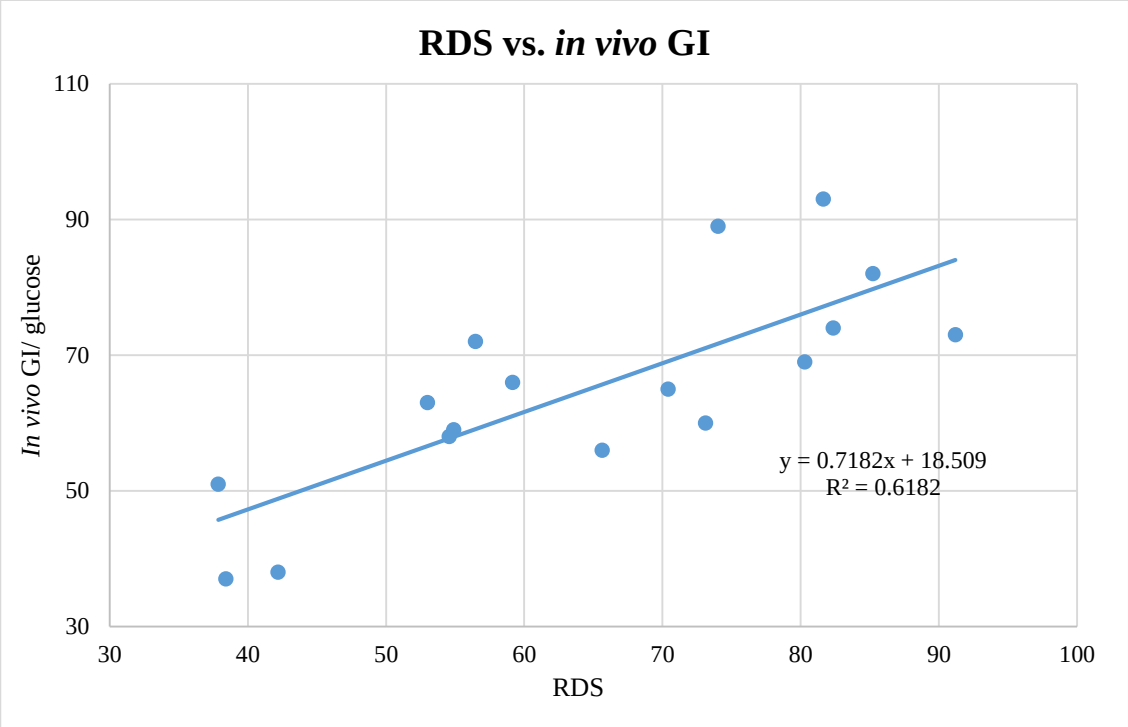


Figure 4.10: The Linear Correlation Between *in vivo* GI Values and RDS Values

$R^2 = 0.62$, $p < 0.05$

Alougth the correlation between the two methods was improved after excluding the two types of pastas, there was no strong evidence to exclude them. There was no clear explanation as to why the *in vitro* method yielded a higher GI for the two pastas than did the *in vivo* method. This variation might be due to different pasta manufacturers, differences in the preparation procedure, and/or cooking time of the food samples in the International GI Tables. However, as a result of this unexplained variation between the two methods, the two types of pasta were not excluded from further analysis. The final result thus included all 20 food samples, which also illustrated the GI values variety of food samples in the *in vitro* method.

4.5 Discussion:

The results from the final modified *in vitro* digestion method showed high correlation with the *in vivo* method and suggested that the *in vitro* method could be used to predict *in vivo* GI. This project achieved its main objective, which was to develop an alternative method to the standard Englyst method, using food 'as eaten' to predict the GI of starchy food.

The first objective of this chapter was to validate a new established *in vitro* method by comparing the *in vitro* method with the *in vivo* method. Most of the previous *in vitro* starch digestion methods were validated using *in vivo* results as a reference point. For example, Englyst et al. (1999) assessed ten different starchy foods and compared the results with the blood glucose response in eight volunteers using the same foods. Similarly, Goni et al. (1997) followed the same principle, comparing the results of a new method with the glycemic response of 30 subjects. Both Englyst and Goni's methods showed a strong correlation with the *in vivo* method. Therefore, it was important to validate our method against the *in vivo* method. Based on this principle, 20 different starchy foods that were available in the local market were chosen from the International GI Tables.

The second objective of this stage was to establish an *in vitro* method of sample preparation that would mimic chewing and have a lesser effect on food structure characteristics. Previous studies varied widely in terms of sample preparation methods. Some studies used actual chewed samples, wherein volunteers were given food samples and asked to chew a specific number of times (Akerberg et al., 1998; Granfeldt et al., 1992; Muir & O'Dea, 1992). However, chewing in food preparation is subjective; there was interpersonal variation in results even when volunteers chewed the same food, as well as a wide variation in the number of chewing times and the size of the sample across methods. Therefore, the use of chewing to prepare the samples

was omitted in this project, and an alternative method of preparing the samples for *in vitro* digestion was sought.

In other digestion methods, *in vitro* techniques to mimic chewing were used in sample preparation, including sieving, grinding, and homogenization (Brighenti et al.,1998; Champ, 1992; Weurding et al., 2001). In the standard Englyst method, a mincer was used to prepare the food sample (Englyst et al., 1999). In the present study, using the Englyst technique (i.e., mincing) in food preparation was an option. However, to ensure that minced samples would be comparable to *in vivo* oral conditions, minced samples with two pore sizes (8 mm and 4.5 mm) were compared with actual chewed samples. Findings indicated that both were comparable to chewed samples. However, the grinder with pore size 4.5 mm was chosen to prepare the samples for subsequent stages of the experiment because the samples that were prepared with the 4.5 mm pore size were more homogeneous, which in turn led to more representative samples and reduced inter-sample variation.

The third step of the study involved assessing the amount of starch digested in 20 different starchy foods using the new modified method. The estimated GI value was derived by calculating the AUC of the amount of starch digested and applying the result in a predictive equation. These predicted GI values were compared with *in vivo* GI values from the International GI Tables. Findings indicated a medium level of agreement between the two methods. However, the result was improved after comparing the *in vivo* GI values with RDS, indicating a high level of concordance between the two methods.

These results support previous studies showing that RDS could be used as a better predictor than AUC for the GI values of starchy food. This association between the *in vivo* GI value and RDS value was explained in a study by Englyst et al. (1996), in which the GI value of

food and its effect on the blood glucose response was found to depend on the quality of starch and not the quantity of available carbohydrates in food. Study results showed a highly significant association between RDS and RAG (which includes RDS and free sugar) of 39 foods with the *in vivo* GI values of the same foods. Another study by Englyst et al. (1999) confirmed these results, finding that the percentage increase in RAG was significantly correlated with the same percentage increase in the glycemic response, wherein RAG explained 70% of the glycemic response variance.

4.6 Conclusion:

In conclusion, the new modified method used herein responded differently to various starchy foods, suggesting that this method is sensitive to the starch content of each food. The RDS and GI values from the modified method were comparable with *in vivo* GI values. RDS produced more predictive values for the *in vivo* GI values than AUC. As such, this method could represent a novel way with which to predict the *in vitro* GI value of foods.

5 CHAPTER 5

Integrated Discussion

Diabetes is one of the most common chronic diseases worldwide, negatively impacting human health and placing a burden on the economy (WHO, 2015). Treatment interventions and the prevention of disease risk factors are important in reducing the prevalence of diabetes. Dietary intervention has been recently considered the first line of treatment for individuals with diabetes. High-fibre foods and complex carbohydrates are the best choices for the control of blood glucose among individuals with diabetes. These foods generally have a low GI, which translates to a smaller impact on the blood glucose response. However, measuring the effect of carbohydrates on blood glucose is complicated and expensive, requiring a minimum of ten subjects, not to mention a great deal of time and financial resources. In addition, assessing the effect of carbohydrates on blood glucose is often influenced by inter-subject variation (Vrolix & Mensink, 2009; Zeev et al., 2015). In order to address the limitations of the *in vivo* method, interest in developing an alternative *in vitro* method has increased in recent years. Several *in vitro* methods have been developed to predict the GI values of food by mimicking *in vivo* digestion conditions. However, there is currently no one standard, robust and reliable method to assess the GI of food. One of the most common existing *in vitro* methods is the Englyst method, which has been used for a number of years by researchers in the field (Anderson et al., 2010; Englyst et al., 1992, 1996, 1999). Although the Englyst method is one of the most common *in vitro* methods, it has inherent limitations including the fact that the food sample is not in the form 'as eaten' (i.e., some Englyst studies used a ground powder sample). The method also requires highly-trained personnel and is quite time-consuming. Given these limitations, the current project aimed to modify the Englyst method to assess the GI of starchy foods using a food sample 'as eaten'.

The *in vitro* GI method is based on the principle that the body's glycemic response to food depends on the starch hydrolysis rate. The present project was based on a concept developed by Englyst et al. (1999). Specifically, the methodology involved measuring the amount of starch digested at 20 minutes (RDS) and 120 minutes (SDS) to draw the starch hydrolysis curve, which could then be used to predict GI.

There were several differences between the present and previous methods. Firstly, the methodology used in this project was unique in that it was validated twice: first, it was validated against the standard Englyst method, using a powdered sample to ensure that the modified method could be used as an alternative to the standard method. The results of this first stage showed a strong, positive association between the standard Englyst method and the *in vitro* method ($r(886) = 0.98$, $p < 0.05$). The second stage involved validating the modified method against published *in vivo* GI values; results indicated a significant correlation between the *in vitro* modified method and *in vivo* GI method ($r(18) = 0.72$, $p < 0.001$).

Secondly, preparation of the sample and use of food 'as eaten' varied greatly between previous *in vitro* methods. Some studies used actual chewed samples, wherein volunteers were given food samples and instructed to chew them a specific number of times (Akerberg et al., 1998; Granfeldt et al., 1992; Muir & O'Dea, 1992). However, chewing is subjective, and variation between participants existed even when they chewed the same food. Further, there was wide variation between methods in the number of chews and size of the food sample (Muir & O'Dea, 1992). Therefore, chewing to prepare the samples was omitted in the current project, which instead sought to develop an alternative method of sample preparation.

In other studies, *in vitro* techniques aiming to mimic chewing for sample preparation included sieving, grinding, or homogenization (Brighenti et al.,1998; Champ, 1992; Weurding et

al., 2001). In the standard Englyst method, a ground powder sample was used. However, other studies indicated that the manner of processing food can affect the rate of starch digestion and the GI of foods (Goni et al., 1997). The oral phase of the present project aimed to develop an *in vitro* method to mimic chewing with a lower effect of processing on the starch structure during sample preparation. In this project, it was found that preparing the food sample with a 4.5 mm pore size mincer was comparable with chewing. In addition, the samples were more homogeneous, resulting in more representative samples and reduced variability between samples. This result was also supported by a previous study which illustrated that using a mincer with a specific pore size for sample preparation was significantly correlated with chewed samples (Englyst et al., 1999).

Thirdly, this project aimed to determine the source of *in vivo* GI values. To validate the modified method against the *in vivo* method, a published list of GI values list was needed. This food list was required to include a wide range of GI values and different types of starchy foods. In the previous studies, the number and type of starchy foods varied among the studies. For example, Englyst et al. (1992) used approximately 30 different starchy foods, while Goin et al. (1997) used ten different starchy food samples to validate their method. Yet another study by Englyst et al. (1999) used only four samples; whereas, Granfeldt et al. (1992) used 21 different starchy foods. Most of the previous studies used starchy foods including cereal, pasta, bread, legumes, rice, biscuits, and starchy vegetables. In the current study, 20 starchy foods were chosen from the International GI Tables, based on their GI value and availability in the local market. International GI Tables are considered a reliable source for *in vivo* GI values, as they include most of the GI studies published worldwide (Foster-Powell, Holt, & Brand-Miller, 2002).

Fourthly, a novel aspect of this project was that it involved a simpler procedure and saved time compared with previous studies. Specifically, in the standard Englyst method, sample preparation and procedure take approximately three hours in addition to the two hours required for glucose measurement, which utilizes the GOOPD technique (Englyst et al., 1999). The standard Englyst method is therefore quite time-consuming (lasting approximately five hours in total). In the current study, the starch digestion procedure was shorter and simpler. In addition to decreased complexities during sample digestion, the glucose analysis step was easier, involving a simple injection of the sample aliquot into the glucose analyser (Analox Instrument) at specific time points, providing immediate results. This significant modification in the modified method saved time and reduced labour.

On the other hand, there were some similarities between the current modified method and previous methods; namely, the reagents and enzymes used in the current project were the same as those used in the standard Englyst method. One advantage of the standard Englyst method is using a HCl/pepsin and guar-gum solution in the assay. By using this solution, Englyst considered three important variables in the *in vivo* digestion conditions: (1) the pH, by adding HCl to mimic the pH in the gastric phase; (2) adding pepsin to hydrolyze the protein that may associate with starch; and (3) adding guar gum to simulate the viscosity of the stomach contents (Englyst et al., 1999). In fact, the Englyst method is the only *in vitro* method that considered viscosity in starch digestion. Given these careful considerations made by Englyst, we chose to use the same reagents and enzymes in the current project.

The new modified method used herein was developed by adapting the GI analyser to the standard Englyst method. In this project, the objective was to combine the semi-automated GI analyser (which utilizes the simpler procedure) with the commonly used Englyst method

reagents and enzymes. Based on this combination, a modified method was developed that has a less complicated procedure and more accurate results due to the use of food samples 'as eaten'. To develop this modified method, the establishment phase included two validation stages. The first stage validated the modified method against the standard method, and the second validated the modified method against the *in vivo* method.

In the first validation stage, after establishing the method (including all modifications), a strong association between the newly established method and the standard method was found using green lentil powder. However, the main objective of this project was to use food 'as eaten' in GI measurement. Thus, the second stage of the project aimed to use food 'as eaten' to validate the modified method. After, the experiment was run (including all the food samples 'as eaten'), the results showed that the correlation between the *in vitro* GI values and *in vivo* GI values was moderate. The correlation was then re-calculated, using the RDS values instead of the *in vitro* GI values. The correlation between the *in vivo* GI values and the RDS was thus improved, resulting in a stronger association. The association was further strengthened after adjusting the data by omitting two foods that had different GI values (i.e., outliers). However, because there was no strong justification for excluding these two foods, they were included in the final results. The strong association between the RDS and the *in vivo* GI values was unsurprising given that previous studies have shown a strong correlation between the *in vivo* GI and the starch hydrolysis amounts during the first 20-30 minutes (Bornet et al., 1989; Englyst et al., 1996; Snow & O'Dea, 1981).

This project was not without limitations. First, the list of foods used in the second stage was limited to 20 items. It would have been preferable to include a greater number of starchy foods in the validation stage, such as a greater variety of beans, as well as more vegetables and

fruit. However, the food list was limited by availability in the local market. In addition, the foods tested in the International GI Tables were likely to be from a different geographic area and possibly subjected to different processing techniques, leading to further differences between the two sources. Secondly, running this study in conjunction with an *in vivo* study would have been beneficial. In this parallel study, volunteers would ideally be served the same foods that were used in the *in vitro* digestion experiment, allowing for a more accurate comparison between the two methods. This was not done due to time and financial constraints, and represents a potential avenue for future research. Thirdly, the published *in vivo* GI values showed wide variability. This variability may have been due to various factors, including intra- and inter-variability between subjects, differences in the physical and chemical nature of the same food product (e.g., type of starch, dietary fibre content), food processing effects (e.g., cooking times, temperature and methods), gelatinization (the process of rendering starch water soluble), and retrogradation (a realignment of starch molecules during cooling and storage). Each of these factors has a critical effect on the consumed food and leads to potential variability in the glycemic response and GI measurement. As such, these factors likely explain differences in the GI values of certain foods in the two methods used in this project.

In conclusion, the new modified method developed herein met the objective of establishing an *in vitro* method to predict the GI of foods 'as eaten'. This method showed sensitivity to the starch content of various foods and demonstrated a high level of accuracy compared with the *in vivo* method. Overall, it is less complicated and reduces time and labour compared with previous methods. This novel methodology could be used in the future to predict GI values with less time and expense, representing a quick tool with which to assess the GI of foods. The new modified method could be used among individuals who suffer from diabetes

and/or obesity, helping them to make healthier dietary choices, and could also be used in food inspection centres to quickly detect the GI values of food products. Finally, the findings of this project could be applied within industry sectors for food labeling purposes, ultimately serving to improve the health and wellbeing of the general population.

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