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Prediction of the glycaemic index of simple and composite dishes

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others. Except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others." Chapter 5 is based on work from jointly authored publications as follow:

S. Al Hamli, M. Holmes, S. Khokhar and C. Orfila. Oral presentation: Predicting glycaemic index in cereal and legume-based foods from macronutrient composition data. Proceedings of the Nutrition Society, 70th Summer Meeting, from plough through practice to policy, Reading, UK, July 2011.

In the oral presentation submitted for the Nutrition Society, 70th Summer Meeting, the prediction models and data used is directly attributed to my work and covered in chapter 5. Caroline Orfila and Melvin Holmes contributed to review the presentation.

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Abstract

Dietary carbohydrates play a crucial role in human nutrition. They are considered one of the major sources of energy and provide between 55-75 % energy of the human diet (FAO/WHO, 1998). In 1980, the glycaemic index (GI) concept was developed as a tool to compare foods for their ability to provide glucose to the blood circulation after ingestion and absorption in individuals. Epidemiological studies have shown a relationship between GI and non-communicable diseases such as type 2 diabetes using published GI values (Barclay *et al*., 2008b). However, measuring GI *in vivo* for every food used in the epidemiological field, for example, is time-consuming, expensive and requires the participation of human volunteers (Jenkins, 2007). The aim of the study is to develop methodology to estimate GI from the macronutrient composition of mixed foods, and the hypothesis is that GI can be predicted from composition data without the need for human volunteers.

Available carbohydrate (av.CHO) analysis of 16 foods from the cereal and legume groups were undertaken and values were used to generate the prediction models. The relationship between GI and macronutrient composition was investigated in the 16 foods using multiple regression analysis methods. The results indicate that starch and fat are the only macronutrients that correlate significantly with published GI values. Three foods were used to validate the prediction models using *in vitro* and *in vivo* measurements and these correlated significantly with the statistically predicted GI values. In conclusion, statistically predicted GI might be a useful approach to eliminate the need for human subject or blood analysis to measure GI in multi-component foods.

Publication

Conference presentations

S. Al Hamli, M. Holmes, S. Khokhar and C. Orfila. Oral presentation: Predicting glycaemic index in cereal and legume-based foods from macronutrient composition data. Proceedings of the Nutrition Society, 70th Summer Meeting, From Plough Through Practice to Policy, Reading, UK, July 2011.

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List of Abbreviations

3, 5-dinitrosalicylic acid assay	DNS
Adenosine triphosphate	ATP
Amyloglucosidase	AMG
Available carbohydrate	Av.CHO
Body mass index	BMI
Carbohydrate	CHO
Cholecytokinin	CCK
Degree of polymerization	DP
Gastric inhibitory peptide	GIP
Glucagon-like peptide	GLP
Glucose linked transporter	GLUT
Glucose or glycaemic response	GR
Glucose-6-phosphate dehydrogenase	G6PDH
Glycaemic index	GI
Glycaemic load	GL
Hexokinase assay	HK
High-performance anion exchange with pulsed amperometric detection	HPAE-PAD
Hydrolysis Index	HI

Incremental under curve area	iAUC
National Health and Nutrition Examination Survey Nutrient Database	NHNES
Nicotinamide adenine dinucleotide phosphate	NADP
Non-starch polysaccharides	NSP
Phenol sulphuric assay	PS
Phosphoglucose isomerase	PGI
Reference	ref
Resistant starch	RS
Room temperature	RT
Sodium-glucose linked transporter	SGLT
Tricarboxylic acid cycle	TCA

1 Chapter one: general introduction

1.1 Introduction

1.1.1 The structure of carbohydrates (CHO)

CHO are organic compounds that play essential roles in human physiology. They are found as individual molecules or combined with components such as protein, lipid or nucleic acid. Furthermore, CHO are abundant components in a number of staple foods including cereal grains, legumes (pulses), vegetables and fruits. They are also used as additives in many processed foods, contributing to their sweetness and textural characteristics.

CHO are composed of carbon, hydrogen and oxygen atoms and the molecular formula is $(CH_2O)_n$ where n can be three or more. They can be classified according to their degree of polymerization (DP) (Table 1.1), where monosaccharides are the simplest form of CHO that cannot be hydrolyzed. Monosaccharides may be linked by glycosidic bonds to form complex compounds with DP from 2 to >20000 molecules and can be hydrolyzed.

1.1.1.1 Properties of mono- and disaccharides

1.1.1.1.1 Monosaccharides

Monosaccharides are the simplest CHO units. The most common monosaccharides include glucose, fructose and galactose. Two types of carbonyl groups can be found in each monosaccharide: aldehyde or ketone. The carbonyl group is the same, but its position is different in aldoses or ketoses.

The minimum number of carbon atoms that made up monosaccharide is three when they are called trioses. Monosaccharides with four carbon atoms are tetroses, with five are called pentoses, and those with six carbon atoms are hexoses. Aldohexose, like glucose, is six

carbons with an aldehyde groups while ketohexose, like fructose, is six carbons with a ketone group. Monosaccharides form ring structures in solution. Monosaccharides with five membered rings are called furanoses while with six membered rings are called pyranoses.

Glucose, fructose and galactose are the most abundant sugars found naturally in food. Glucose is an aldohexose monosaccharide and forms a pyranose ring structure (Figure 1.1-A). Glucose is mildly sweet but not abundant in its simple form, instead it is usually found in combination with other sugars as in sucrose (glucose + fructose), lactose (glucose +galactose) and maltose (glucose +glucose), and it is one of the major sub units that made up the polysaccharides (Figure 1.3).

Fructose has the same number of carbon atoms as glucose but varies in the position of the carbonyl group and the number of carbon atoms in the ring structure (Figure 1.1-B). Fructose is a ketohexose and forms a furanose ring structure. It is the sweetest of the monosaccharides due to the 5-carbon atom ring structure (furanose) that stimulates the sweet region of the taste buds on the tongue. Fructose is the only monosaccharide that is abundant in its monosaccharide form, being naturally present in ripe fruits and honey. Fructose is also found in glucose-fructose syrups (GFSs) produced industrially by enzymatic conversion of glucose to fructose. GFSs are used widely in food and beverage production as sweetening ingredients.

Galactose also is an aldohexose and forms a pyranose ring structure but with different arrangements of hydroxyl groups attached to each carbon atom (Figure 1.1-C). Galactose is hardly sweet and rarely found as a free sugar in nature. It is usually found in milk and milk products as the disaccharide lactose (milk sugar).

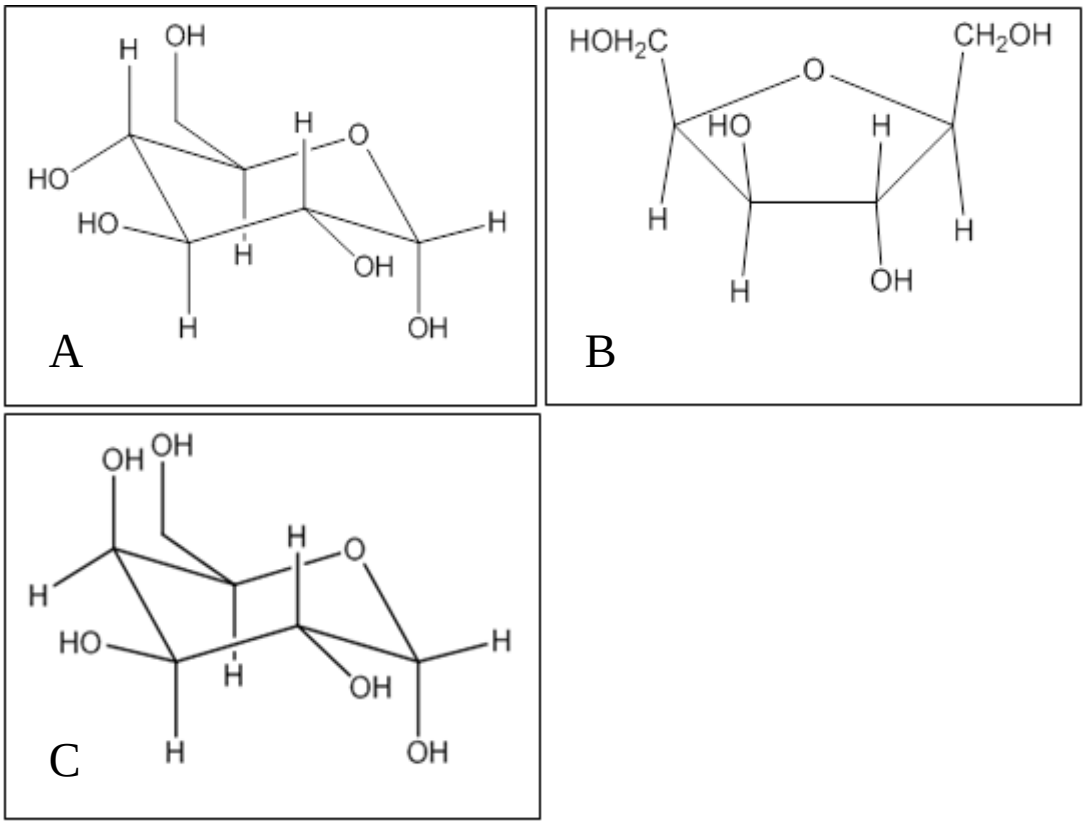


Figure 1.1: Monosaccharides chair structures A) glucose, B) fructose, and C) galactose.

1.1.1.1.2 Disaccharides

Disaccharides are composed of two sugars connected together by a glycosidic linkage through dehydration or condensation reactions, where a hydroxyl group from carbon number 1 is combined with the hydrogen atom on carbon number 4 or 6 from the other monosaccharide to release a molecule of water.

There are two types of disaccharides: reducing and non-reducing disaccharides. Reducing disaccharides like maltose are formed with one reducing monosaccharide that has a free hemiacetal unit, whereas non-reducing disaccharides like sucrose are formed with monosaccharides that have no free hemiacetal unit. Both mono- and disaccharides have a low molecular weight and tend to be highly soluble in water.

Sucrose (Figure 1.2-A) is a disaccharide that is commonly found in fruits and vegetables and is composed of glucose and fructose linked by α-(1, 4)-glycosidic linkages. Sucrose is the main component of table sugar, and it is a food commodity, added during cooking and processing to many foods and drinks. Lactose is a disaccharide that is found exclusively in milk and is composed of glucose and galactose linked by β-(1, 4)-glycosidic linkage. Maltose is a disaccharide that consists of two glucose units linked by an α-(1, 4)-glycosidic linkage and it is either found in germinating cereal seeds like barley or produced as a result of partial starch digestion.

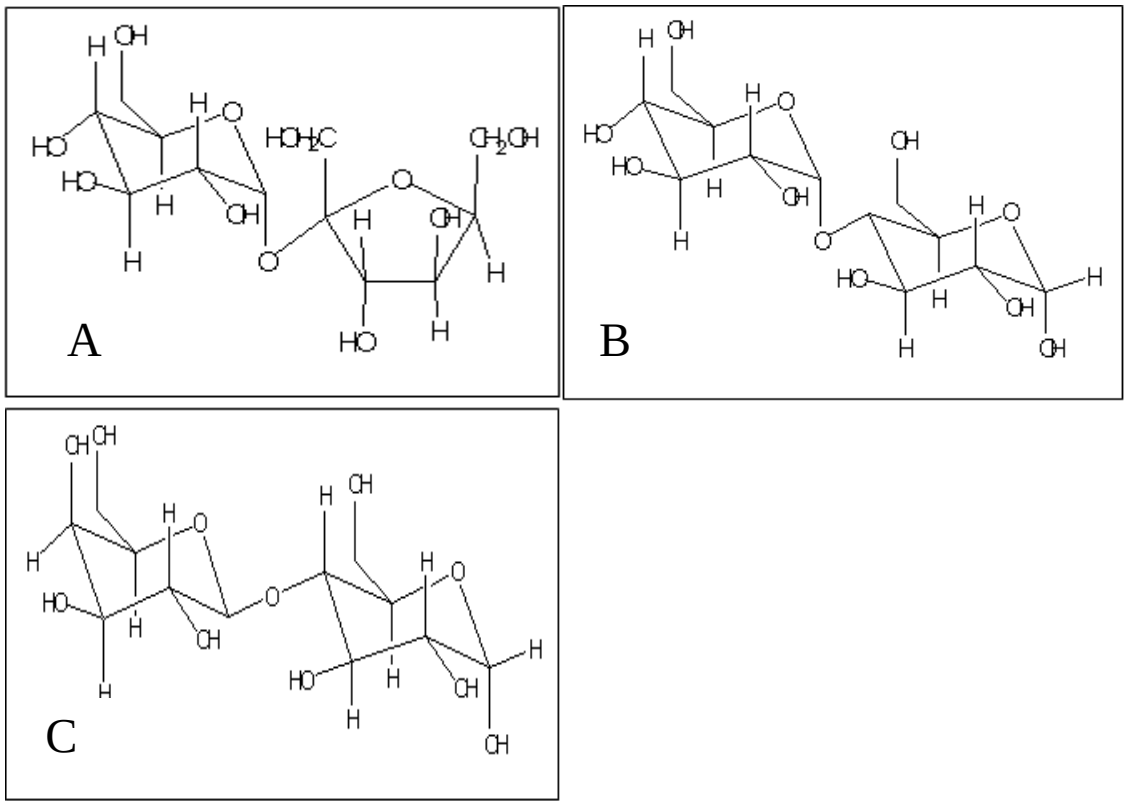


Figure 1.2: Disaccharide chair structures: A) Sucrose, B) Maltose, and C) Lactose.

1.1.1.1.3 Sugar alcohols

Sugar alcohols are organic compounds known as polyols and they are derivatives from sugars in which a hydroxyl group replaces the aldehyde or ketone group in the sugars

They are classified according to the number of monosaccharide units. They are water-soluble solids and are found abundantly in nature in fruits and vegetables. They are also produced commercially by hydrogenation of the formed sugar and widely used as thickeners or sweeteners. The most commercial sugar alcohols are sorbitol and mannitol.

1.1.1.2 Properties of oligosaccharides and polysaccharides

1.1.1.2.1 Oligosaccharides

Oligosaccharides have a DP of between 3-20 monosaccharide units joined by glycosidic linkages. They are less soluble in water than mono- and disaccharides and are 30-60% times less sweet than disaccharides. They used in food production and bulking agent (Mussatto and Mancilha, 2007).

Some are considered as prebiotics because they enhance the growth of the micro-flora in the gut (Mussatto and Mancilha, 2007), and the most important oligosaccharides are those which form from partially digested starch like malto-oligosaccharides or those which are found in legumes like raffinose, strachyose, and verbascose. Inulin, fructo, – and galactooligosaccharides are also other types of oligosaccharides that are found abundantly in vegetables such as onion, garlic, chicory and artichoke.

1.1.1.2.2 Polysaccharides

Polysaccharides have a DP above 20 up to several 100 monosaccharide units and have a high molecular weight (50 kDa-250 kDa).

The partial solubility of CHO in water is attributed to the presence of the hydroxyl groups attached to each carbon atom and to DP; therefore mono- and disaccharides are highly soluble in water, while polysaccharides vary in their solubility in water.

1.1.1.2.2.1 Starch

Starch is an α-glucan polysaccharide that is composed of many glucose units linked together by α-1-4 glycosidic linkages. Starch is the main storage polysaccharide in plants. It is the most important dietary CHO in the human diet, and it is found in many staple foods worldwide including cereals, legumes and vegetables. It is composed of two main polysaccharides: amylose (linear chains of α-1-4 glycosidic linkage) and amylopectin (linear backbone of α-1-4 linked glucose and α-1, 6-linked glucose branches) (Figure 1.3). The glucose monomers in amylose are packed closely together in a helical configuration, whereas in amylopectin, they are less tightly packed and the structure is more open. In the free form, amylopectin has a higher solubility in water compared to amylose. However, both molecules are arranged in intracellular and water-insoluble bodies known as starch granules. Starch granules have been classified into three types (A, B, and C types) using X-ray diffraction (Cummings and Englyst, 1995). Type B and C starches are usually found in tubers and legumes respectively, whereas type A is usually found in cereals (Englyst *et al*., 1992). The shape, size, arrangement of starch components, ratio of amylose/amylopectin, and molecular weight of these components are different in each crop. Each plant has its unique starch granular size and shape (Lindeboom *et al*., 2004).

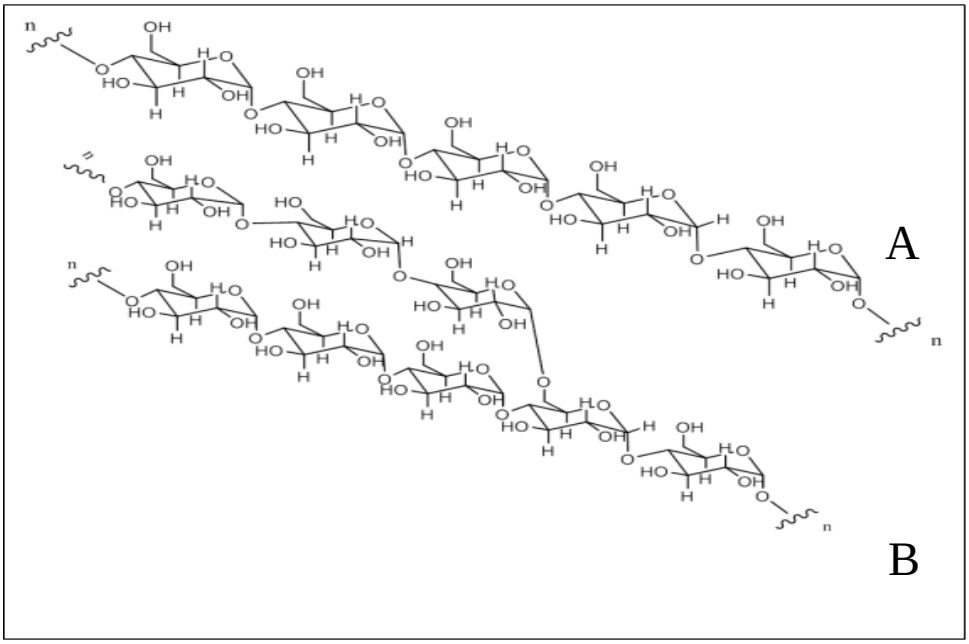


Figure 1.3: Structure of starch components; A) Amylose and B) Amylopectin

1.1.1.2.2.2 Glycogen

Glycogen is the main storage polysaccharide in human and other animals. It is similar in structure to starch. However, glycogen is more branched than starch. It is stored in the liver and muscle cells and serves as short term energy source and for glucose storage.

1.1.1.2.2.3 Resistant starch (RS)

RS is a type of starch that is resistant to digestion by amylolytic enzymes (Vosloo, 2005). RS can be classified into four types: RS1 is found in raw seeds and legumes and also found in unprocessed grains, RS2 is found in green banana and uncooked potato, RS3, also known as retrograded starch, is found in processed food such as cooked legumes and breakfast cereals, and RS4 is modified starch which is chemically modified and isn't found in nature (Vosloo, 2005).

1.1.1.2.2.4 Non-starch polysaccharides (NSP)

NSP are composed of a diverse family of polysaccharides and are mainly found in the plant cell wall. NSP comprises of various molecules with different forms, sizes, and water solubility. Cellulose, hemicellulose, arabinoxylan, xyloglucan, pectin, and other β-glucans are the most common types of NSP found in the structures of plant cell walls.

1.1.2 CHO metabolism

CHO are further classified into two groups: digestible CHO and non-digestible CHO according to their digestibility in humans. Digestible CHO are those that are digested like starch and disaccharides, while the non-digestible CHO are those that escape digestion in the small intestine and are rather fermented in the colon. In order to use energy produced from CHO, they have to be digested, absorbed and metabolized in the body. Table 1.1 represents the classification of CHO according to their digestibility and absorption in the body.

CHO (DP)	Subgroup	Examples	Gastrointestinal fate
Mono-disaccharides (1-2)	Monosaccharide	Glucose	Absorbed directly in the small intestine and utilized by the tissues
		Fructose	Absorbed directly in the small intestine and metabolized in the liver
		Galactose	
	Disaccharide	Sucrose	Digested and absorbed in the small intestine
		Lactose	
		Maltose	
	Sugar alcohol (polyols)	Sorbitol	Partly digested and/or partly absorbed into the small intestine
		Mannitol	
		Xylitol	
Oligosaccharides (3-9)	Malto-oligosaccharide	Maltodextrin	Digested and absorbed in the small intestine
	Fructo-oigosaccharide	Raffinoses	Fermented in the colon
Polysaccharides (>9)	Starch	Amylose	Digested and absorbed in the small intestine
		Amylopectin	
	Resistant starch (RS)	RS1	Fermented in the colon
		RS2	
		RS3	
		RS4	
	NSP Insoluble	Cellulose	Fermented in the colon
	Soluble	Pectin	
		Xylans	

Table 1.1:Classification and bioavailability of carbohydrates found in food (Englyst and Englyst, 2005)

1.1.2.1 Digestion of starch

Digestion of starch starts in the mouth by the salivary enzyme α-amylase/ptyalin which is secreted by the salivary gland to hydrolyze the glycosidic linkage to yield short oligosaccharides like maltose and maltotriose. Therefore a mild sweet taste may become evident upon masticating starch-based foods.

After swallowing, the partially digested starchy CHO reach the stomach where hydrochloric acid can chemically hydrolyze the CHO and the saliva (α-amylase) is inactivated due to the acidic environment. The passage of chyme from the stomach to the small intestine varies from person to person but the average time it takes is 6-8 hours. In the upper section of the small intestine (duodenum), first sodium bicarbonate ($NaHCO_3$) will be secreted to neutralize the acid from the stomach, and then pancreatic juice will be secreted for further CHO digestion. Pancreatic juice contains α-amylase to produce malto-oligosaccharides and maltose. Finally, disaccharides are hydrolyzed by enzymes at the brush border (e.g. maltase and sucrase) present at the brush border membrane lining the small intestine (Table 1.2). Table 1.3 summarizes the gastrointestinal hormones that contribute to food digestions.

Name	Substrate	Mode of action	Location
Salivary α amylase	Starch	Hydrolyzes the α-1-4 glycosidic linkages between glucose	Salivary gland in the mouth
Pancreatic α amylase	Starch	Hydrolyzes the α-1-4 glycosidic linkages between glucose	Lumen of the small intestine
Sucrase	Sucrose	Hydrolyzes the α-1-4 glycosidic linkages between glucose and fructose	Brush border
Isomaltase	Maltose and α-limited dextrin	Hydrolyzes polysaccharides at the α-1-6 glycosidic linkages to glucose	Brush border
Maltase-glucoamylase -	Maltose and β-limited dextrin	Hydrolyzes polysaccharides at the α -1-4 glycosidic linkages to glucose	Brush border
Lactose-phlorizin hydrolase	Lactose	Hydrolyzes the β -1-4 glycosidic linkages between glucose and galactose	Brush border

Table 1.2: Properties and locations of the CHO digestive enzymes produced in the digestive system.

Hormones	Location	Stimulator	Function	Comments
Glucagon-like peptide (GLP)	Small intestine	Glucose Amino acid Fatty acid	Suppress gastric secretion Suppress gastric emptying Stimulates satiety signals	Type of incretin hormone that affect the insulin secretion.
Gastric inhibitory peptide (GIP)	Small intestine	Glucose Amino acid Fatty acids	Suppress gastric emptying Suppress gastric secretion Stimulates fat metabolism	Type of incretin hormone that affect the insulin secretion.
Gastrin	Stomach	Amino acid	Stimulates gastric secretion	Secretion of gastrin is affected by the acidity of the stomach Secretion of gastrin is affected by secrtin
Secrtin	Small intestine	Low pH Amino acid Fatty acid	Suppress gastric emptying Suppress gastric secretion Improves the function of CCK Suppress the secretion of gastrin	Secretion of secrtin is affected by the acidity of the stomach
Cholecytokinin (CCK)	Small intestine	Amino acid Fatty acids	Suppress gastric emptying Suppress gastric secretion Stimulate pancreatic enzymes Satiety signals	Secretion on CCK is affected by the presence of nutrients in the lumen Secretion of CCK is affected by the acidity of the stomach

Table1.3: Properties and locations of the regulator gastrointestinal hormones

1.1.2.2 Absorption

The majority of ingested and generated monosaccharides are absorbed in the small intestine through the brush border epithelium covering the villi. Glucose and galactose are absorbed through sodium-dependent active transport involving SGLT1 transporter into the enterocyte across the apical membrane, while fructose is absorbed solely through sodium-independent facilitative diffusion involving GLUT5 (Williamson, 2013).

The three monosaccharides then diffuse out of the enterocyte through the basolateral membrane into the portal circulation via sodium-independent facilitated diffusion involving GLUT2 (Figure 1.4) (Williamson, 2013).

Available carbohydrate (av.CHO), are CHO that are digested like starch and disaccharides, absorbed as monosaccharide (glucose, fructose, and galactose) and further metabolized. Nevertheless, fructose is not absorbed as efficiently as glucose or galactose (Wolever, 2006).

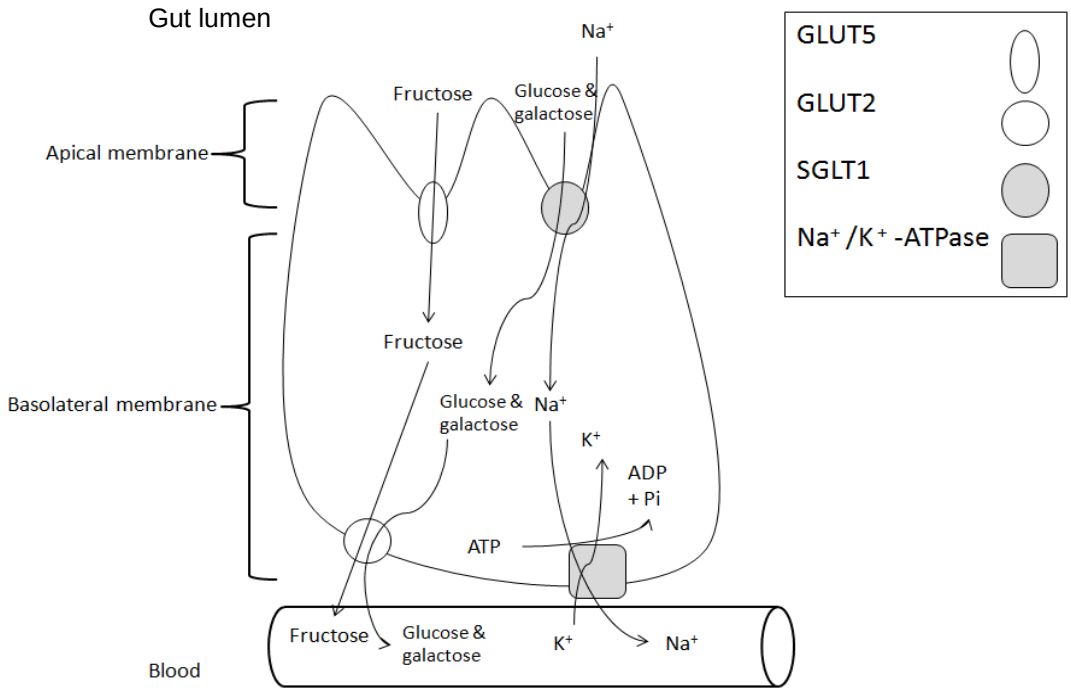


Figure 1.4: Sugar transporters on the enterocytes, showing the movement of sugars across the intestinal epithelium.

1.1.2.3 CHO metabolism

Dietary CHOs play a crucial role in human nutrition. They are considered one of the major sources of energy and provide between 55-75 % energy of the human diet (FAO/WHO, 1998). Energy is obtained largely from the oxidation of macronutrients including CHOs. After CHOs digestion glucose will be absorbed into the body. Glucose is the most important CHO for metabolism in all organisms. Glycolysis is a series of reactions that convert glucose to pyruvate and energy (ATP) and then pyruvate will enter the TCA cycle and acetyl coenzyme A will be produced. Whenever CHOs are entering into the bloodstream, energy will be provided in the form of glucose while fructose and galactose will be metabolized in the liver and converted to fructose-1-phosphate and galactose-1-phosphate respectively. In humans, glucose is the preferred fuel for the brain and it is

required for most of the tissues like blood and nerve cells. Also, extra glucose can be converted to glycogen by glycogenesis and stored in the liver and muscle cells and utilized later. Although glucose is an important nutrient, it considered as non-essential because it can be synthesized in the body through gluconeogenesis from non-CHO substance such as amino acids (Englyst and Englyst, 2005).

1.1.2.4 Homeostasis

In healthy humans, the concentration of fasting blood glucose ranges between 3.5 to 5.5 mmol/L. Glucose is regulated tightly by two hormones, insulin and glucagon, produced in the islets of Langerhans in the pancreas. Insulin is secreted from the β-cell in the islets of Langerhans in the pancreas, while glucagon, which is antagonistic to insulin, is secreted from the α-cell in the islets of Langerhans in the pancreas.

When glucose levels drop below 5 mmol/L after prolonged fasting periods or physical activity, glucagon will be released from the α cells of the pancreas to stimulate the liver to break down the glycogen to glucose (glycogeolysis) or stimulate the conversion of proteins to glucose by gluconeogenesis. In contrast, after a meal, insulin will be released to stimulate the uptake of glucose by cells and conversion of glucose to glycogen in the liver and the muscles (glycogenesis). The amount of hormones released depends on the level of glucose in the blood. Epinephrine (adrenaline) is another hormone that stimulates converting glycogen to glucose in the liver and increase the glucose level in the blood in case of stress, exercise or fight-or-flight response (Gibney *et al*., 2009).

Table 1.4 and 1.5 represent the major gastrointestinal hormones with their properties that contribute to the CHO homeostasis and the glucose response (GR) in the blood.

Hormones	Location	Stimulator	Function	Comments
Insulin	Pancreas	High glucose Amino acid Fatty acids GLP-1 GIP	Signals the removal of glucose in the blood Stimulates the glycogenesis Stimulates the uptake of amino acid Stimulates the protein synthesis Stimulates the uptake of fatty acid Stimulates the storage of fat	Antagonistic to glucagon
Glucagon-like peptide (GLP)	Small intestine	Glucose Amino acid Fatty acid	Induces insulin secretion Suppress glucagon secretion Improve insulin sensitivity	Type of incretin hormone that affect the insulin secretion.
Gastric inhibitory peptide (GIP)	Small intestine	Glucose Amino acid Fatty acids	Induces insulin secretion Stimulates fat metabolism Suppresses glucagon secretion	Type of incretin hormone that affect the insulin secretion.
Glucagon	Pancreas	Low glucose Amino acid Fatty acids	Signals the release of glucose to the blood Stimulates the gluconeogenesis Stimulate the glycogeolysis Suppresses the protein synthesis Suppresses the fat storage	Antagonistic to insulin

Table 1.4: Properties and functions of the major regulator gastrointestinal hormones produced from the small intestine and pancreas.

Hormones	Location	Stimulator	Function	Comments
Epinephrine	Adrenal gland	Stress Low glucose	Signals the release of glucose to the blood Stimulates the glycogeolysis Stimulates gluconeogenesis Stimulates lipolysis	Stimulated in case of stress, exercise, or fight-or-flight response
Cortisol	Adrenal gland	Stress Low glucose	Signals the release of glucose to the blood Stimulates the glycogeolysis Stimulates gluconeogenesis Stimulates lipolysis	Stimulated in case of stress, exercise, or fight-or-flight response
Growth hormones	Pituitary gland	Stress Low glucose	Signals the release of glucose to the blood Stimulates proteolysis Stimulates lipolysis	Stimulated in case of stress, exercise, or fight-or-flight response

Table 1.5: Properties and functions of the major regulator gastrointestinal hormones produced from the adrenal and pituitary glands.

1.1.3 Glycaemic index (GI)

Since blood sugar levels varied according to the food consumed, there has been interest from physiologists since the 1980's to categorize CHO by their ability to raise blood glucose levels and eliciting an insulin response (Jenkins et al., 1981). However, in 1981 Jenkins *et al.* introduced the concept of GI and classified CHO based on their physiological effects into two groups: glycaemic and non-glycaemic CHO (Jenkins *et al*., 1981). Glycaemic CHO elicits a GR, while non-glycaemic CHO (like NSP) do not (Jenkins *et al*., 1981).

GI can be defined as the "incremental area under the blood GR curve (iAUC) of a 50 g av.CHO portion of a test food over 120 min expressed as a percentage of the response to the same amount of av.CHO from a reference food taken by the same subject" (FAO/WHO, 1998), while GR is the blood glucose response toward the food.

Moreover, glycaemic load (GL) is a system to classify CHO in food based on the amount consumed. It is calculated as the GI multiplied by the total amount of CHOs consumed and divided by 100. GL is considered a more useful measurement than GI because it considers both the quality and the quantity of CHO consumed (Monro and Shaw, 2008). Also GL has a scale since the GL can be classified as low (≤ 10), medium (11-19) and high (≥ 20) (WHO/FAO, 1998).

1.1.3.1 GI protocol

In late 1990s the FAO/WHO established a standardized method for measuring GI *in vivo,* and in 2010 the international standard Organization published a "recognized scientific method" for measuring GI of the food (International Organization for Standardization,

2010). However, most of the studies have been done using different numbers of subjects, different reference foods, and different methods for collecting blood samples (Brouns *et al*., 2005, Wolever *et al*., 2008, Simila *et al*., 2011). Moreover, there is an international table of the GI and GL where the GI values are gathered from publications all over the world using different methodology (Atkinson *et al*., 2008).

Basically, GI methodology is conducted by collecting blood samples from volunteer subjects after fasting for at least 10 hours (baseline), then feeding subjects a 50 g portion of available CHO of either test food or a reference food (white bread or glucose), then collecting blood samples at different intervals of 15-30 minutes over a 2-3 hours period (FAO/WHO, 1998).

1.1.3.1.1 Procedural effects of GI protocol

Procedural variations frequently occur in GI trials which lead to a lack of accuracy and standardization.

1.1.3.1.1.1 Number of subjects

FAO/WHO (1998) stated, without giving any reason, that 6 subjects are enough for GI studies. Previous GI studies found that a significant difference in GR amongst two different groups (n~7) of subjects can be detectable (Coulston *et al*., 1984). Furthermore, Brouns *et al.* (2005) suggested that 10 people will provide "reasonable degree of power and precision" for most GI measurements.

1.1.3.1.1.2 Number of tests repetitions

Repeating the food test during GI measurements seeks to reduce potential intra-individual variation and improve the precision of the results (Williams *et al*., 2008) . Brouns *et al.* (2005) suggested that reduction of intra-individual variation can be tracked through the reference food. Therefore, Brouns *et al.* (2005) recommended taking the mean of at least two trials of the reference food for each subject during the GI measurements.

1.1.3.1.1.3 The importance and types of reference food

According to the WHO/FAO (1998) report, the common reference food that should be used for GI studies is glucose with an assigned value of 100. However, it has been found that using glucose may affect the GR by increasing the osmolarity of the stomach and leading to quick gastric emptying (Wolever *et al*., 1991). In addition subjects may experience nauseous and dizziness after high concentrations of glucose is ingested quickly after fasting, hence white bread has replaced glucose. White bread was selected because it is the most consumable staple globally and it is usually consumed with most of the meals (Almousa *et al*., 2013).

Moreover, the GI values obtained from white bread as a reference food should be converted to glucose because the response of white bread is 1.4 times the response of glucose (Brouns *et al*., 2005).

In the updated version of the international table of GI there were seven types of reference foods used in 2487 GI studies (glucose, white bread, rice, potato, wheat, arepa, and barley bread) (Atkinson *et al*., 2008). Brouns *et al.* (2005) found that any CHO-rich foods considered as staple can be used as a reference in GI studies, however more studies were

needed regarding these because there is limited information available. In addition, Wolever *et al.* (2008), tested two types of food in 28 laboratories from different countries (n=311). They did not find significant variations in AUC and GI regarding the type of reference food.

1.1.3.1.1.3.1 Amount of Av.CHO content in both reference food and test food

50g av.CHO portion of reference (glucose or white bread) is fed to the subjects and then GR is calculated every 15 min with healthy subjects or every 30 min with diabetic patients. 25g av.CHO portion size of reference food is acceptable and can be used in case of testing low CHO food like vegetables or legumes because the portion of 50 g av.CHO will be too large to be consumed by subjects (Brouns *et al*., 2005).

1.1.3.1.1.3.2 Measurements of the mount of av.CHO portions in both reference food and test food

The terminology of av.CHO does not provide enough description of their effects in the human body. Therefore, Jenkins *et al.* (1981) classified CHO based on its physiological impact on the GR. Amongst all the monosaccharides, only glucose has an effect on the GR, while fructose and galactose do not behave as av.CHO because they are not detected as glucose. As a result, measuring the amount of glucose present essential in GI studies because the GR in the blood depends on the type and the amount of CHO consumed in the portion size.

Food composition analysis of CHO does not appear to be standardised across the world. Most composition databases estimate the total amount of CHO ’by difference’ as shown in the equation below:

Total CHO = 100 - (protein + fat + water + ash+ NSP + alcohol)

Equation 1.1: Measuring the total CHO by difference in foods.

Total CHO are calculated by the summation of all weights of the other food constituents that have been analyzed separately, such as protein, fat, water, alcohol, fibre and ash, and then subtracted from the total weight of the food.

Studies have found that these methods are not sufficient for consumers since the physiological effect of each CHO fraction is different between constituents (Greenfield and Southgate, 2003, Menezes *et al*., 2009). Moreover, measuring CHO by difference may overestimate the carbohydrate content particularly in food rich in RS (Granfeldt *et al*., 2006) or due to the experimental errors of any of the other analyzed methods (protein, fat, water, alcohol, fibre, and ash). Consequently, measuring CHO directly is required for accurate analysis.

There are many analytical techniques for measuring the amount and type of CHO present in foods including colorimetric or enzymatic methods, or high performance liquid chromatography (HLPC).

1.1.3.1.1.4 Subject preparation

It is recommended that subjects also should avoid strenuous physical activity prior the test day as it may increase the uptake of glucose into the muscles on the next day and decrease the insulin concentration in the blood (Malkova *et al*., 2000).

1.1.3.1.1.5 Second meal effect

Subjects should consume a standardized evening meal the day before the study to avoid the so called "second meal effect" (Brouns *et al*., 2005, Granfeldt *et al*., 2006). Studies found that the effect of the second meal may extend overnight (Granfeldt *et al*., 2006, Nilsson *et al*., 2008). The GR in the morning of the test day after a low GI dinner was found to be lower than the GR after a high GI dinner (Wolever *et al*., 1988, Nilsson *et al*., 2008). Therefore, consumption of a low GI diet (rich in NSP) may reduce the amount of glucose absorbed and lower the GR on the next day (Brouns et al., 2005).

1.1.3.1.1.6 Time of the test conducted

Wolever and Bolognesi (1996) found that GR reported from afternoon tests were significantly different from those obtained in morning tests because GR differs throughout the day. Therefore, Brouns *et al.* (2005) recommended that all GI trials should be conducted in the morning after fasting for 10-14 hours to reduce the variation within the subjects.

1.1.3.1.1.7 Drinks served during the trial

In most GI trials, water is the only drink that is allowed to be consumed with both reference and test foods (Brouns *et al*., 2005). Coffee and tea contain polyphenols (chlorogenic acid and tannin) which may affect glucose digestion and absorption (section 1.1.4.1.7.1) (Williamson, 2013).

1.1.3.1.1.8 Blood collection and measurements

The main interest in blood glucose concentration is the one in the arterial blood because it is delivered from the heart to the tissues. There are two possible locations for blood collection: capillary blood and venous plasma from forearm.

Capillary blood is taken by puncturing the finger-tips, forearm, or abdominal tissues, while venous plasma is taken by either cannula or catheter from the forearm.

Although subjects sometimes do not feel happy by being pricked several times, still the capillary blood is considered as the most preferable and convenient method for taking blood whereas venous blood is regarded as less preferable by the subjects and requires special nursing and medical attention, plus unlike capillary blood, venous plasma cannot be taken by the subjects themselves.

Furthermore, the concentration of glucose in venous plasma is affected by insulin secretion after ingestion of the food therefore the variations within subjects are greater and the glucose concentration is more variable depending on the insulin sensitivity of the subjects. Also, the flow rate of venous plasma is influenced by room temperature which in turn will affect the glucose concentrations. Consequently, capillary blood is more preferable, is easy to obtain and provides a more sensitive response than venous plasma.

1.1.3.1.1.9 Calculating the area under Curve (AUC) of GR

A FAO/WHO (1998) report established a standard method for calculating the AUC to minimize the differences among laboratories, giving rise to the AUC value (Wolever, 1990, Jenkins, 2007).

The incremental area under GR curve (iAUC) is calculated by using the trapezoidal rule where all the areas of GRs collected during the two hours period are added together and the area beneath the baseline (fasting glucose curve) is ignored (Equation 1.2).

As seen in Figure 1.5, the partial area of segment 5 and the whole area of segment 6 are below the baseline which means they will be ignored according to the standardized iAUC method (FAO/WHO, 1998).

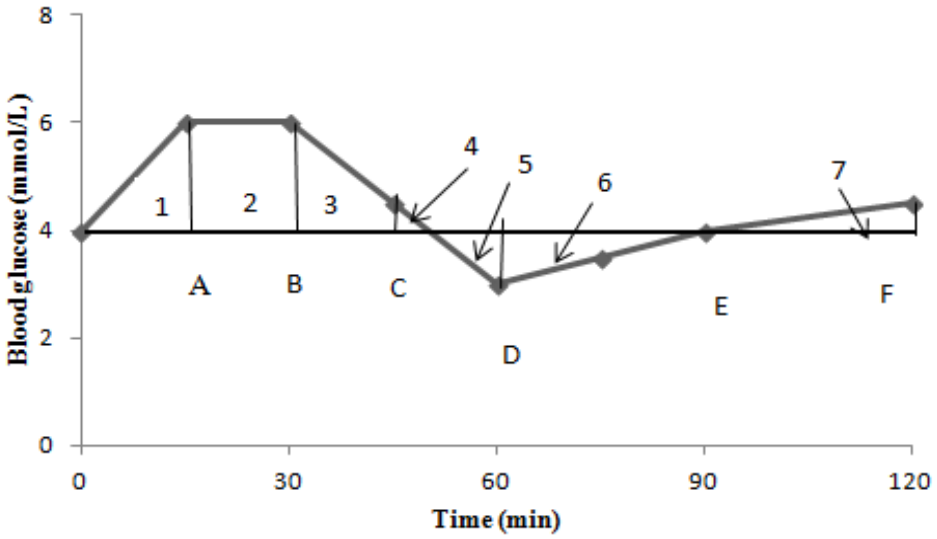


Figure 1.5: Calculation of incremental area under the curve (adapted from (Brouns et al., 2005).

Accordingly, the iAUC can be calculated using the following equations (Brouns *et al*., 2005):

For $A_1 = (G_1 - G_0) \times (t_1 - t_0) / 2$, as $G_1 > G_0$;

For A_2, $G_2 = G_1$, hence, $A_2 = G_1$ (or G_2) $\times (t_2 - t_1)$;

For A_3, $G_3 < G_2$, hence, $A_3 = (((G_3 - G_0) + (G_2 - G_0) \times (t_3 - t_2)) / 2$.

For A_4, = $((G_3 - G_0)^2 / (G_3 - G_4) \times (t_4 - t_3)) / 2$;

$iAUC = A_1 + A_2 + A_3 + A_4 + A_6$

Equation 1.2. Measuring the incremental area under curve of glucose response of certain food; G is the glucose concentration in mmol/L, **t** is the time in minutes, and **A** is the Area under curve of the glucose response to a certain food.

"Assuming that at time t_0, $t_1 \ldots t_6$ (equalling 0, 15 …120 min, respectively), the blood glucose concentration are, G_0, $G_1 \ldots G_6$, respectively, where A_x is the AUC for the X_{th} time interval, and the X_{th} time interval is the interval between times t_{x-1} and t_x" (Brouns *et al*., 2005).

1.1.4 Factors affecting GR

1.1.4.1 Food factors

The composition of the food has an effect on blood GR. Brouns *et al*. (2005) suggested that others factors which are not related to the composition of foods that also affect the GR include physical entrapment, particle sizes, cooking or food processing, and maturity of the fruits or vegetables.

Therefore, in 1998 WHO/FAO classified dietary GI into three categories (Table 1.6): low GI food (GI ≤55), moderate GI (55-69), and high GI food (GI ≥70) based on their effects on the glucose level in the blood (Brand-Miller *et al*., 2003).

Classification	GI range	Examples
Low GI	≤ 55	Legumes; seeds; whole grains; vegetables, most sweet fruits (peaches, strawberries, mangos) and; fructose
Medium GI	56–69	enriched wheat, unpeeled boiled potato, some of fruit juices like grape or cranberry juice, dried fruit, , sucrose, snickers bar, and banana
High GI	≥ 70	white bread , most white rice, extruded breakfast cereals, glucose, maltose, watermelon, and potato

Table 1.6: GI classification according to WHO/FAO 1998.

1.1.4.1.1 Type of CHO present in food

Monosaccharide, such as glucose, fructose, and galactose, present in food as free sugar are absorbed in the small intestine, however; only glucose raises the GR (Brand-Miller et al., 1996).

1.1.4.1.2 Type of starch

1.1.4.1.2.1 Amylose: amylopectin ratio

The ratio of amylose and amylopectin in food has an effect on the GR, in which amylose has a lower effect on the GR, compared to amylopectin (Brouns *et al*., 2005). The different effects of the starch components are due to the crystalline structure of amylose, in which glucose monomers in amylose are highly and closely packed together in a helical configuration, whereas in amylopectin they are less tightly packed and the structure is more open, therefore amylose is less accessible to amylolytic digestion (Vosloo, 2005).

1.1.4.1.2.2 The presence of resistant starch (RS)

The presence of RS will have a lowering effect on the GR because the starch is resistant to digestive enzymes and pass through the small intestine without being digested (Cummings and Englyst, 1995).

1.1.4.1.3 NSP content in food

NSP contribute to lowering the GR in the blood by stimulating the gastric emptying and slowing down the appearance of glucose in the blood (Nishimune *et al*., 1991, Lightowler and Henry, 2009). Gastric emptying is a process where food leaves the stomach to enter the small intestine for digestion by pancreatic enzymes (Wolever, 2006).

Nishimune *et al.* (1991), elucidated the five mechanisms for the role of NSP in lowering the blood GR: first NSP contributed to a slowdown in CHO digestion in the stomach, second, NSP stimulated gastric emptying, third, NSP affected the rate of digested CHO diffusion in the first section of the small intestine (duodenum), fourth NSP affected the digestion of CHO in the duodenum, and finally NSP slowed down the absorption of sugar after hydrolysis.

1.1.4.1.4 Fat content in food

Usually foods with added fat or high fat content have lower GR. Fat slows down the gastric emptying process by stimulation of gut hormone like CCK, GIP, GLP-1, and insulin (Henry *et al*., 2006, Wolever, 2006). Moreover, Henry *et al*. suggested that any types of fat (saturated, mono- or polyunsaturated) had a lowering effect on GR (Henry *et al*., 2008b).

Examples of food with high fat content are peanuts and potato chips which have low GI values (GI 33) and (GI 54) respectively (Foster-Powell *et al*., 2002), however in this case the low GI foods are not always the healthy choices which may be confusing to the consumers who may consume more fatty foods.

1.1.4.1.5 Protein content in food

The protein content of food or adding proteins to the food have a lowering effect on the GR by stimulation of the secretion of insulin, and also ingestion of protein delays gastric emptying by stimulating the gut hormone CCK, GIP and GLP-1 (Henry *et al*., 2006, Wolever, 2006). Protein may lower the bioavailability of the CHO by binding to them and forming a complex that affects the accessibility of the digestive enzymes (Bornet *et al*.,

1987). A study conducted with normal weight subjects confirmed low GRs after protein intake at breakfast (Esteves de Oliveira *et al*., 2011).

1.1.4.1.6 Acid content in food

The presence of organic acids in foods, such as acetic acid or citric acid will lower the GR (Guevarra and Panlasigui, 2000). Like NSP, fats and proteins, organic acid slow down the gastric emptying this slows down digestion and slows the rise in blood glucose. Many studies have indicated that the more acidic the food, the lower the GI (Leeman *et al*., 2005).

1.1.4.1.7 The presence of anti-nutrients

Anti-nutrients are natural compounds that are found in food and interfere with the bioavailability of the nutrients either by binding to these nutrients and preventing them from being available (polyphenols) or by acting as enzyme inhibitors (phytic acid and lectin). Amylase inhibitors are compounds that are found in many raw foods, e.g. banana, and are found in legumes too. They inhibit α amylases and prevent nutrients from being absorbed, but they are also destroyed by cooking.

1.1.4.1.7.1 Polyphenols

Phytochemicals like polyphenols may also affect the GR by inhibiting CHO digestion and absorption. They are found in wide ranges of foods including cereals, legumes, and vegetables (Williamson, 2013).

1.1.4.1.7.2 Phytic acid

Phytic acid (phytate) is a type of anti-nutrient that is found in plant seeds. Phytic acid can affect the bioavailability of the CHO by forming a complex and reducing CHO absorption, also inhibiting amylase activity, and reducing CHO digestibility (Lee *et al*., 2006).

1.1.4.1.7.3 Lectins

Lectins are another type of anti-nutrient that affects the bioavailability of the CHO and are found in plants and mostly in legumes. Lectins are glycoproteins that bind to CHO and prevent CHO from being digested. However, lectins can be destroyed by cooking (Leeds *et al*., 1998).

1.1.4.1.8 Structural factors

1.1.4.1.8.1 Physical entrapment

Physical entrapment of carbohydrate lowers the GR by making the CHO less available to digestive enzymes. The fibrous coating around legumes, cereals and seeds act as physical barriers, which inhibits the action of enzymes on the food and decreases absorption (Asp and Bjorck, 1992).

1.1.4.1.9 Food processing

1.1.4.1.9.1 Mechanical processing

1.1.4.1.9.1.1 Particle size and disruption of the starch granule

Milling, beating, shearing or homogenizing of food is a process that decreases particle size and leads to water absorption and promotes digestion by enzymes. The GR of foods is

increased whenever the surface area of the food particles is increased (Foster-Powell and Miller, 1995).

1.1.4.1.9.2 Thermal processing

Highly processed foods have higher GR values if compared to their unprocessed equivalents. The more cooking, pounding, mixing, or grinding processes are applied to the foods, the higher the glucose in the blood and the higher GI values will be obtained. For example, cooking the food disturbs the crystalline arrangement of the starch (gelatinization) prompting easier digestion, while cooling the starch will re-crystallize the starch (retrogradation) enhancing resistance to digestive enzymes (RS3) (Burton and Lightowler, 2008).

1.1.4.2 Human factors affecting GR

1.1.4.2.1 Between-individual variations

Between-individual variations (inter-) can be defined as variations in GR between the subjects under standardized conditions. Demographic characteristics like ethnicity and life style factors like smoking, alcohol consumption, body mass index (BMI) and physical activity may affect the GR.

1.1.4.2.1.1 Demographic characteristics

1.1.4.2.1.1.1 Ethnicity

There were negligible differences in GR between ethnicity where 34 Europe subjects were recruited in UK and 13 Indian subjects in India (Henry *et al*., 2008a). Five test foods were tested in each groups and glucose was used as a reference food. Higher GR was recorded in

Indian subjects compared to UK subjects. Yet no significant differences in GI were found between the two group testing five foods, because GI is expressed as a ratio between the test and the reference food (Wolever *et al*., 1985)

Another recent study conducted to investigate whether a low GI diet can help in preventing diabetes and CVD using a high GI drink (glucose) and low GI drink (maltitol) in three different ethnic groups living in the UK (n=10 Europe, n=10 Indian, and n=10 Chinese). One of the outcomes of this study confirmed that there were no significant differences regarding ethnicity (Pratt *et al*., 2011).

1.1.4.2.1.2 Life style

1.1.4.2.1.2.1 Smoking

Smoking may lead to insulin resistance and elevated glucose levels in the blood (20% higher than non-smoking), however the mechanisms are still not clear (Bergman *et al*., 2012).

1.1.4.2.1.2.2 Alcohol

Since alcohol is mainly energy and cannot be stored in the body, once alcohol enters the body it will be metabolized and may affect the glucose homeostasis and reduce the GRs next morning (Hatonen *et al*., 2012).

1.1.4.2.1.2.3 Body mass index (BMI)

Consuming CHO-rich foods will elevate the blood glucose level rapidly (> 5.0 mmol/L) and induce the secretion of insulin and over a long time insulin sensitivity will decrease (Onyesom *et al*., 2013). Moreover, one insulin function is preventing fat oxidation; hence

fat will be stored (Wolever, 2006). As a result overweight or obesity may affect the GR through affecting the insulin sensitivity(Onyesom *et al*., 2013).

1.1.4.2.1.2.4 Physical activity

Physical activity improved the glycaemic control and insulin sensitivity, therefore the GR might be affected by the physical status of the subject (Mikus *et al*., 2012).

1.1.4.2.2 Within-individual variations

On the other hand, within-individual variations (intra-) can be defined as day to day variations in GR of the same subject under standardized conditions and after repetitive consumption of reference food (at least 2 times). Many studies found significant variations within the same subjects during GI trials when compared to variations between the group of subjects (Williams *et al*., 2008, Wolever *et al*., 2008, Vrolix and Mensink, 2010) which means that GI should be measured in a group of subjects rather than in one subject (Williams *et al*., 2008). Unfortunately, it is difficult to control the intra-individual variations during the GI even under standardized conditions because it depends on the subject him/herself (Brouns *et al*., 2005).

In conclusion, each food elicits GR differently in different subjects and the GR of each subject differs from one to another. As a result standardization of GI measurements seeks to reduce all the GR variations associated with food, methodology or human factors. However by expressing the results as GI (GR of rest food /GR of the Reference food) 50% of these variations will be eliminated (Wolever *et al*., 1985).

1.1.5 *In vitro* starch digestion to predict GI as a measure of the GI of the food

Measuring GI *in vivo* is time-consuming, expensive, and requires the participation of human volunteers. In addition the human body system is complex and variable and many factors may interfere with the GR even with a standardized method. Regardless the presence of standardized *in vivo* GI measurements, Jenkins *et al.* (1980) found that *in vitro* starch digestion can be used to predict the GR. As a result, as shown in Table 1.7, many studies have estimated the food GR via *in vitro* methods of either single food or mixed meals (Araya *et al*., 2002, Granfeldt *et al*., 2006, Chung *et al*., 2008). In 1997, Goni and colleagues concluded that GI can be estimated through an equation after measuring the rate of starch hydrolysis:

$$GI = 39.7 + (0.6 \times HI)$$

Equation 1.3: Equation created by (Goni et al., 1997) to estimate the GI after measuring the rate of starch hydrolyzed by pancreatic enzymes over 180 min. HI refers to Hydrolysis Index.

The *in vitro* method can be used to classify CHO into two groups: rapidly digestible CHO and slowly digestible CHO based on the rate of starch hydrolysis by a mixture of enzymes (microbial and pancreatic enzymes) (Englyst *et al*., 1999). *In vitro* starch digestion methods have two main variants: non-restricted and restricted. In non-restricted systems, samples are kept in a closed tube with digestive enzymes for 120 min, and then the amount of glucose released is measured, whereas in a restricted system, samples are digested in a dialysis bag (13 cm length, 12000-18000 KD) and the amount of glucose released from the bag is measured. The dialysis bag is a semi-permeable membrane allowing the passage of low

molecular weight molecules such as salts and sugars through the pores. According to Goni *et al.* (1997), restricted *in vitro* starch digestion cannot be used to mimic the actual human digestive system because sugars (except fructose) are absorbed by active transport rather than being diffused. *In vitro* methods would have the advantages of being less expensive, less time-consuming, and easier to conduct because they do not require subjects (Jenkins, 2007). Table 1.7 represents the *in vitro* starch digestion methods commonly referred to and conducted over the years.

Author(s)	Place	*In vitro* digestion	# of subjects	Reference & test food	Outcomes
(Jenkins *et al*. 1980)	UK	Human saliva & jejuna juice Water & Isotonic phosphate buffer Restricted system	6 healthy subjects 6 diabetic subjects	Whole-meal bread (ref) 3 test foods	✓ Results obtained from *in vitro* were closely to the *in vivo* results ✓ *In vitro* starch digestion can be used to estimate the GI of the food
(Granfeldt et al., 1992)	Sweden	Human saliva, pepsin, and pancreatic α-amylase Na, K phosphate buffer Restricted system	10 healthy subjects	Maltose (ref) 21 cereal and legume	✓ Results obtained from *in vitro* were closely to the *in vivo* results ✓ *In vitro* starch digestion can be used to estimate the GI of the food ✓ GI prediction equation was used to calculated the GI from the rate of starch hydrolysis = 18.8 +0.7 $HI_{180\ min}$.
(Goni *et al*., 1997)	Spain	Microbial AMG 2M KOH Non-restricted system	30 healthy subjects	White bread (ref) 11 tests foods	✓ Results of *in vitro* showed good but lower, linear correlation with *in vivo* results ✓ GI prediction equation was used to calculated the GI from the rate of starch hydrolysis = 39.7 +0.55 $HI_{90\ min}$.
(Englyst *et al*. 2003)	UK	Pepsin, pancreatin & invertase water Non-restricted system	11-17 healthy subjects	Glucose solution (ref) 23 cereals-based foods	✓ Strong correlation was found between GI and the amount of CHO digested ✓ Results show relationship between the chemical analysis, rate of digestion and GI

Author(s)	Place	*In vitro* digestion	# of subjects	Reference & test food	Outcomes
(Germaine *et al.*, 2008)	Australia	Human saliva, pepsin, and pancreatic α-amylase Na, K phosphate buffer Restricted and non-restricted system	6 healthy subjects were recuirted for the chewing phase only	White bread (ref) 2 cereal based foods 1 legume based food	✓ GI prediction equation was used (Goni, *et al*, 1997). ✓ Strong correlation was found between GI and the amount of CHO digested ✓ Non-restricted system showed good & potential to predict GI in grain food, ✓ GI values were from the the international table of GI and GL (Foster-Powell *et al*., 2002)
(Ballance *et al.*, 2013)	Norway	Human saliava (chewing) Gastric juice Pancreatic juice	12 healthy subjects	Glucose solution (ref.) 2 test foods	✓ Stimulation of *in vivo* GR is probable via *in vitro* starch digestion if the kinetics starch hydrolysis are known
(Kim and White, 2012)	USA	Pepsin, porcine pancreatin, & microbial AMG Water	No subjects were recruited	2 test foods	✓ Result of *in vitro* digestion give similar results to those of estimated GI values from (Goni *et al.* equation)

(ref) =reference food

Table 1.7: Studies regarding the relationship between *in vitro* starch digestion and GR from *in vivo* GI measurements.

1.1.6 Measuring the GI from mixed meals

The majority of GI and *in vitro* starch digestion studies have been done with single foods. However, the majority of the foods are eaten as a part of meal rather than as single foods. There are several food factors as mentioned previously in section 1.1.4 that affect the GI; therefore, it is important to find an approach to measure the GI of a complex mixed meal. Consequently, estimating GI of mixed meals using summation models has been investigated since the 80's (Wolever *et al*., 1985).

The summation models have been designed to estimate the total meal GI from the GI values of the component foods within the meal, taking into the account the amount of av.CHO. However, one of the main limitation of this approach is that food components such as fat, protein and fibre are not taken in consideration (Dodd *et al*., 2011).

Urooj and Puttaraj (2001) were the first to predict GI from nutrient composition using statistical analysis and compare them with *in vivo* and *in vitro* methods (Figure 1.6). The GI values of 6 cereal-based-south Asian mixed dishes were measured using three different approaches: *in vivo*, *in vitro*, and statistical prediction from nutrient content. *In vivo* measurements were conducted with healthy south Asian subjects (n=11) and with type II diabetic south Asian subjects, with normal range of BMI and using white bread as reference food. *In vitro* starch digestion was conducted using the method by Jenkins *et al.* (1980) (restricted system) with modifications. The six cereal-based-south Asian mixed dishes were: chapatti and dhal, dosai and chutney, idli and chutney, pongal, poori and potato palya, upittu and white bread as a reference food. Statistical analysis used to predict the GI from nutrient composition was a simple linear correlation. As shown in Figure 1.6, *in vitro* starch digestion and predicted GI were similar to those obtained by *in vivo* GI measurements in type II diabetic south Asian. Although the results of *in vitro*

starch digestion and predicted GI were close to the observed GI vales, they did not reach a significant level of correlation.

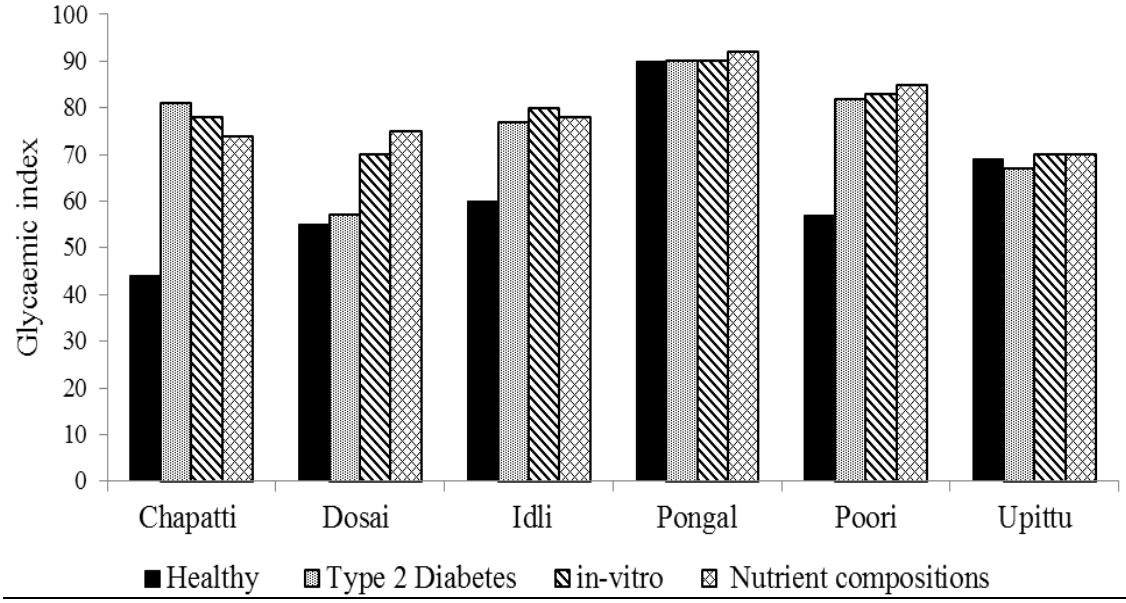


Figure 1.6: Four (GI) values obtained by different methods. First column *in vivo* GI values in healthy subjects, second column *in vivo* GI values in type II diabetic subjects, third column is *in vitro* estimated GI, and the last one is GI values predicted from nutrient composition using nutrient compositions in the six mixed dishes, data adapted from Urooj and Puttaraj (2000).

1.1.7 GI utilization

1.1.7.1 GI and food Databases

GI values from the international tables of GI and GL have been added to databases such as the National Health and Nutrition Examination Survey Nutrient Database (NHNES) (Lin *et al*., 2012), and food composition database (Martin *et al*., 2008) in the USA and food composition databases in the UK (Levis *et al*., 2011). Also, GI values from the international table of GI and GL were used for dietary assessment in USA (Schakel *et al*., 2008) and in Australia (Barclay *et al*., 2008a), or to assess food consumption in Europe (Aston *et al*., 2010).

Furthermore, epidemiological studies have used GI values from the international table of GI and GL to investigate the effect of GI on health or the association between GI and non-communicable diseases (Barclay *et al*., 2008b, Thomas and Elliott, 2009, Denova-Gutierrez *et al*., 2010).

In the international table of GI and GL, the same item can have different GI values because the table collects and gathers information from all the publications from all over the world (Atkinson *et al*., 2008). However, there is not an internationally agreed method for GI measurements, and the values have been obtained using different sample sizes, reference foods, and blood collection methods.

Therefore, it is crucial to standardize the GI protocol to enhance the application of GI values in epidemiology and clinical studies.

1.1.7.2 GI and food labelling

The concept of GI was first used to help diabetic patients regulate their GR, later it was adopted as a weight management tool by dietitians (Jenkins *et al*., 1981).

Since the epidemiological studies found that consuming low GI food might be associated with lowering the risk of non-communicable diseases (Barclay *et al*., 2008b), health professionals suggested the labelling CHO-containing food might be useful tool to promote healthy food choices for the consumers (Mitchell, 2008).

According to the dietary recommendations worldwide, the energy intake from CHO should be between 40 and 80% of total energy (Englyst and Englyst, 2005).

Therefore, several GI foundations in different parts of the world were established to provide people with information regarding low GI diets. The GI symbol considered as a reliable tool to help consumers choose healthy food during grocery shopping (Mitchell, 2008).

For example, Australia in 2002 established the GI Foundation and has launched a labelling system for low GI food; also in South Africa there is a GI Foundation to ensure the correct labelling system for both consumers and food industries. In UK the supermarkets introduced the low GI symbol on several items such as legumes (Mitchell, 2008).

However, low GI food can be misleading and encourage people to consume food with high fat content since high fat foods such as chocolates or fried potatoes have low GI values. Education about GI has been suggested to improve food selections (Candido *et al*., 2013).

1.1.8 GI and diet-related diseases

The role of CHO and low GI foods has been investigated systematically and intensively by researchers and health professionals. Based on recent epidemiological studies, it has been observed that low GI diets reduce the risk of developing chronic diseases such cardiovascular diseases, diabetes, and obesity (Barclay *et al*., 2008b, Thomas and Elliott, 2009, Denova-Gutierrez *et al*., 2010)

1.1.8.1 GI and type II diabetes

Diabetes Mellitus is a condition that occurs when the action of insulin is not sufficient or efficient. In the case of type I diabetes, there is no or little insulin produced in the body (Wolever, 2006), whereas in case of type II diabetes the insulin produced is not functioning efficiently. 90% of all diabetic cases belong to type II diabetes (Wolever, 2006). Over a long-term, repeatedly elevated glucose levels in the blood after consuming high available CHO meals (high GI diet) reduces the efficiency of insulin and reduces the uptake of glucose (Wolever, 2006).

High glucose levels in the blood (hyperglycaemia) will cause so-called glucose toxicity and will not only affect the sensitivity of insulin but also damage the tissues such as retina, nerves, and kidney tissues (Wolever, 2006). There is a strong evidence that consumption of low GI and GL diets may reduce the risk of developing type II diabetes (Thomas and Elliott, 2009). Another study showed that the consumption of low GI diets may significantly lower the risk for developing type II diabetes and improve insulin sensitivity in diabetic patients (Riccardi *et al*., 2008).

1.1.8.2 GI and coronary heart diseases (CHD) and cardiovascular (CVD)

Recent evidence shows that consumption of high GI diets may increase the risk for CHD (Hardy *et al*., 2010). In this study 13,051 patients (with ages ranging from 45-64) of African Americans and EU origin were recruited from the Atherosclerosis Risk in Communities (ARIC) database. It was found that high levels of glucose and insulin lead to hyperlipidemia, which is an abnormal elevation of lipid in the blood, and increased the risk of CVD.

Consuming high GI diets over the long term will lead to elevated glucose and insulin levels in the blood (hyperglycaemia and hyperinsulinism respectively) and hyperglycaemia and hyperinsulinism have a number of harmful effects such as increasing the food intake, preventing fat from being oxidized and more fat to be stored. Accumulation of fat and hyperlipidemia will affect the thickness of the blood vessel walls and lead to development of atherosclerosis plaque (Hardy *et al*., 2010).

1.1.8.3 GI and obesity

The prevalence of obesity is increasing rapidly all over the world (Nielsen *et al*., 2005). A study found a significant correlation between low GI diets and lower prevalence of over-weight and obesity in children (Nielsen *et al*., 2005). Another study found that the consumption of high GI food is related to obesity in obese women with polycystic ovaries syndrome (Ludwig *et al*., 2009). Another study found that GI and GL diet may reduce the weight of over-weight and obese subjects (Thomas *et al*., 2007). High levels of insulin after consuming high GI foods prevent fat oxidation and promote accumulation of fat but low GI (NSP-rich) foods may contribute to improving the level of insulin in the blood (Brand-Miller *et al*., 2009).

1.2 Aim

The aim of this research is to develop methodology to estimate GI from macronutrient composition of simple and composite dishes.

1.2.1 Objectives

1. Optimizing analytical methods for measuring av.CHO in cereals, legumes and composite foods.
2. Using restricted system to measure Av. CHO in cereals, legumes, and composite foods.
3. Applying statistical modelling to predict GI in foods from macronutrient composition cereals, legumes and composite foods.
4. Validating the prediction models with *in vivo* GI measurements and *in vitro* starch digestion.

1.2.2 Experimental hypothesis

The GI of simple and composite foods can be predicted from macronutrient composition and *in vitro* starch digestion.

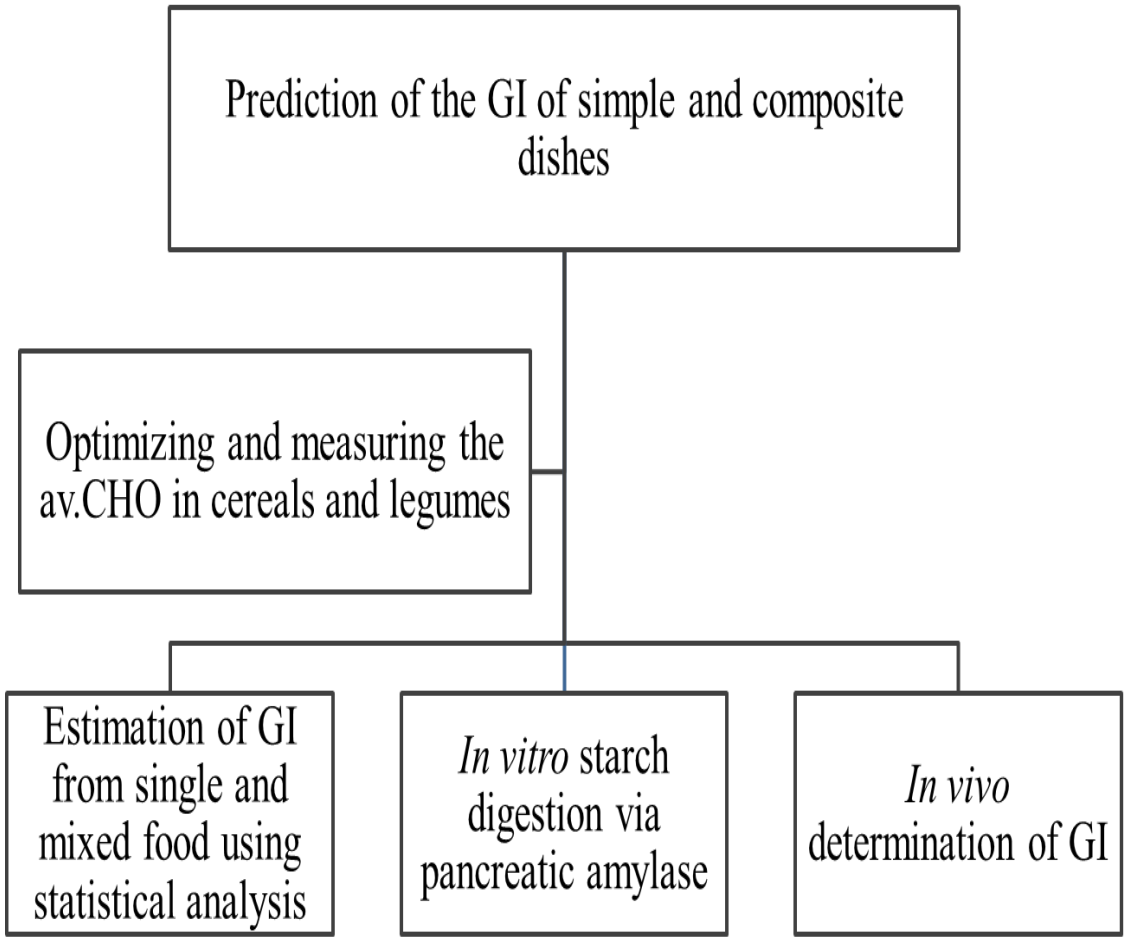


Figure 1.7: Thesis plan

2 Chapter two: general materials and methods

2.1 Materials used for the analysis of av.CHO

2.1.1 Foods

2.1.1.1 Cereals

Breakfast cereals and breads were purchased from a local supermarket in Leeds, UK. They included Cornflakes, Bran flakes, and Coco-Pops (all Kellogg's) and Weetabix (Weetabix), while the bread samples were white bread, brown bread, and whole meal bread and they were all from Warburton's. Cornstarch (Tesco's) was used as a positive control for CHO digestion. Samples were homogenised using a coffee grinder (Moulinex OPTILA ND 2000) prior to analysis.

2.1.1.2 Legumes

Dried legumes were purchased from a local supermarket in Leeds, UK, and they were processed before analysis. Samples were soaked overnight in deionized water (1:5g w/v) at room temperature, then they were drained and boiled in deionized water according to the UK food composition description in McCance and Widdowson's The Composition of Foods dataset (Food Standards Agency, 2002). Finally, samples were homogenised using a Moulinex OPTILA ND 2000 prior to analysis.

2.1.2 Equipment

The equipment listed below was used routinely in the experiments: Coffee mill (Moulinex OPTILA ND 2000), deionized and filtered water was obtained in these experiments using Millipore equipment (Milli-Q/Q-POD), boiling water bath (Grant SBB14), water bath (Grant GLS Aqua 12 plus), UV-Vis spectrophotometer (Super Aquarius 9000 series, Cecil Instrument Limited, UK) and HPAE-PAD (Dionex DX500 instrument equipped with a GP40 gradient pump, PAD system ED 40 electrochemical

detectors including gold working, silver (reference), (and titanium electrode, and a LC20 chromatography enclosure column oven).

2.1.3 Chemicals

Monosaccharide and disaccharide standards (D-glucose, D-fructose, L-fucose, D-sucrose and D-maltose) were purchased from Sigma Aldrich.

Phenol solution 80% (W/W) was purchased from Acros. Sulfuric acid 95+% 1.84 S.G, sodium hydroxide 98+% and 3, 5-dinitrosalicylic acid (DNS) 98% was purchased from Fisher Scientific. (2(*N*-morpholino) ethanesulfonic acid (MES), tris (hydroxymethyl) aminomethane (TRIS) maleic acid, potassium sodium tartrate tetrahydrate, acetic acid 99-100%, ethanol >=99.8% (GC), 50% w/w aqueous solution and sodium azide 50% sodium hydroxide (Fisher Scientific), potassium phosphate monobasic anhydrous, potassium phosphate dibasic anhydrous, sodium chloride, sodium bicarbonate, hydrochloric acid, were purchased from Sigma Aldrich.

2.1.4 Analytical methods for quantifications and av.CHO determinations

2.1.4.1 Hexokinase (HK) assay

The enzymatic essays are one of the most recommended methods for measuring CHO especially monosaccharide and disaccharides because of their specificity and selectivity (Southgate, 1976). HK assay was used because it is specific and selective where different of monosaccharides can be detected using this method (Southgate, 1976). HK is indirect method that measured av.CHO indirectly based on reduction of $NAPD^+$ to NAPDH. HK and glucose-6-phosphate dehydrogenase will phosphorylate the glucose to glucose-6-phosphate and fructose to fructose-6-phosphate. In the presence of ATP and $NADP^+$ and phosphoglucose isomerase, will detect the glucose and fructose respectively. This was performed using an av.CHO kit (K-ACHDF 09/11) suitable for performing 100 assays. This kit was purchased from Megazyme International (Bray, Ireland).

2.1.4.1.1 Reagents

2.1.4.1.1.1 Sodium maleate buffer (0.1 M, pH 6.2)

Maleic acid (11.6 g) was dissolved in 900 mL of deionized water and the pH was adjusted to 6.2 with 2M sodium hydroxide. 0.2 g of sodium azide was added as a preservative, and finally the volume was adjusted to1 L.

2.1.4.1.1.2 Imidazole buffer (11 mL, pH 7.6)

Imidazole buffer was supplied with the enzyme kit (av.CHO and total dietary fibre).

2.1.4.1.1.3 NADP+ (250 mg) plus ATP (500 mg) solution

NADP+ (250 mg) plus ATP (500 mg) solution was supplied with the enzyme kit (av.CHO and total dietary fibre). The entire bottle was dissolve with 12.5 mL deionized water.

2.1.4.1.1.4 Sucrase (200 U) plus β-galactosidase (8,000 U) solution:

Sucrase from yeast plus β-galactosidase from *Agrobacterium sp* were supplied with the enzyme kit. The freeze-dried powder was dissolved in 10.5 mL deionized water.

2.1.4.1.1.5 Hexokinase (HK) (425 U/mL) plus glucose-6-phosphate dehydrogenase (G6PDH) (212 U/mL) suspension, 2.25 mL from solution:

HK from *Escherichia coli* and G6PDH suspensions (2.25 mL) were supplied with the enzyme kit.

2.1.4.1.1.6 Phosphoglucose isomerase (PGI) (2.25 mL, 1,000 U/mL) solution:

PGI from *Saccharomyces Cerevisiae* was supplied with the enzyme kit.

2.1.4.1.1.7 D-Glucose and D-fructose standard solution (5 mL, 0.2 mg/mL of each sugar) solution:

D-Glucose and D-fructose standard solutions were supplied with the enzyme kit.

2.1.4.1.2 Protocol for measuring av.CHO using HK

One mL of extract was diluted 50 fold with sodium maleate buffer. 0.2 mL of diluted extract was transferred to a plastic cuvette, and 0.1 mL of a solution containing 95U sucrase and 152.4U of β-galactosidase was added to the sample. The mixture was incubated for 60min at room temperature. Then, 2.0mL of deionized water, 0.1mL of imidazol buffer and 0.1mL of NADPH/ATP solution were added to the cuvette. The

mixture was incubated for 3 min, then the absorbance (A1) was read at 340 nm. Then 0.02mL of 4U of HK and 2U of G6PD were added. The mixture was incubated for 5min at RT, and the absorbance (A2) was read at 340nm. Then 8.88U of PGI solution was added and the mixture incubated for 10min at RT, and the absorbance was read at 340nm (A3). The MES/Tris buffer was used as a blank, and sugar standard solution (glucose and fructose) was used as a positive control.

2.1.4.2 Phenol-sulphuric assay (PS)

PS method is indirect colorimetric method that detects the presence of CHO. And it was performed according to Dubois *et al*. (1956). PS was selected because it is a common, simple, rapid and considered as broad-spectrum method that detects various sugars (Southgate, 1976). Also, the advantages of this method are: inexpensive and the materials are widely available. The method can be specific since each sugar has different wavelength of absorbance (Dubois *et al*., 1956).

The principle of the detection using this method is based on converting the glucose and fructose to hydroxymethyl furfural which then will react with the phenol giving the yellowish to orange color (Dubois *et al*., 1956).

2.1.4.2.1 Reagents

Concentrated sulfuric acid and 80% phenol were added directly to the samples.

2.1.4.2.2 Protocol for measuring av.CHO using PS

A glucose standard was prepared by diluting 100 mg of D-glucose anhydrous in 10 mL deionized water (10 mg/mL) then further serial dilutions of glucose were prepared to contain 0, 5, 10, 15, 20, 25, 30, 35, 40 µg of glucose in 1 mL of deionized water . 10 µL of 80% (w/w) phenol was added to the sugar solutions. One ml of concentrated sulfuric acid was then carefully dispensed to the samples and shaken by vortexing. The solution was allowed to cool down for 20min before taking the absorbance reading at 490nm.

For the analysis of foods, 1.0 mL of extract was removed from the digestion mixture and diluted with 9 mL deionized water(1:10) and then 100 µL is diluted with 300 µL deionized water (1:4) in an Eppendorf tube (dilution dependent on CHO content).

2.1.4.3 3, 5-dinitrosalicylic acid assay (DNS)

This procedure was obtained from Miller (1959) with minor modification and it is indirect method for measuring CHO. DNS was selected because it is sensitivity, simplicity, and reproducibility (Hall, 2003). The detection is based on oxidizing the aldehyde group in glucose or the ketone group in fructose with 3,5-dinitrosalicylic acid and the reduction of DNS reagent to 3 amino and 5-salicilyic acid indicates the amount of CHO present in foods (Miller, 1959).

2.1.4.3.1 Reagents

Five gram of 3:5-DNS was dissolved in 250 mL of deionized water at 80˚C and 100 mL of 2 N NaOH was added. Finally, 150 g of potassium sodium tartarate-4-hydrate was added and the volume was completed with deionized water to 500 mL.

2.1.4.3.2 Protocol for measuring av.CHO using DNS

A glucose standard (2 mg/mL) was used, then serial dilutions were prepared (0, 0.5, 1, 2, 3, mg/ml). One mL of the digestion extract was diluted (1:5) (depending on CHO content) into a test tube. One mL of DNS reagent and 2 mL of deionized water were added to the tubes (final volume = 4.0 mL). Tubes were placed in a boiling water bath for 5 min to allow the reaction between reducing sugar and the DNS. Tubes were allowed to cool down, and then 16mL of deionized water were pipetted to stop the reaction immediately. The absorbance readings were taken at 540nm.

2.1.4.4 High performance anion exchange chromatography with pulsed amperometric detector (HPAE-PAD)

HPAE-PAD was selected because of the high sensitivity in which small amount (picomole) can be detected andspecificity in which different sugars can be identified and quantified. Samples do not need to be derivatized (Hall, 2003, Dean, 1978, Southgate *et al*., 1978). The CHOs are separated by anion-excahnge chromatography, and since CHOs are weak acids, the detection is based on amperometric detection which depends on the oxidation of CHO in the presence of alkali (NaOH) at the gold working electrode.

2.1.4.4.1 Reagents

2.1.4.4.1.1 200 mM sodium hydroxide

200 mM of sodium hydroxide was prepared by dissolving 21.8 mL of 50% sodium hydroxide from Fisher scientific into 2 L deionized water.

2.1.4.4.2 Protocol for measuring av.CHO using HPAE-PAD

Mixed sugar (glucose, fructose, sucrose, & maltose) were used as standard reference material (100 µM), then serial dilutions were prepared (0, 5, 10, 15, 20, 30, 40 µM). One mL of the extracted aliquot was removed and diluted 4 times with absolute ethanol then incubated in the fridge overnight. One mL from the extract was diluted in deionized water (1:500 for digestible CHO and 1:50 for soluble sugar). Samples and standards were spiked with internal standard (fucose 10 µM), samples were filtered through PTFE 0.2 µm membrane filters (Chromacol LTD, 100X17-SF-02(T), UK,) and analyzed by HPAE-PAD (Dionex DX500 instrument equipped with a GP40 gradient pump, PAD system ED 40 electrochemical detectors including gold working, silver

(reference) and titanium electrode, and a LC20 chromatography enclosure column oven). The analytical column used was Carbopac PA20 (Dionex, 3×150mm) with guard (3×30mm) with anion exchange capacities 65μeq/column. The mobile phase was 200 mM NaOH and the flow rate was 0.4 mL/min. Injections (10 μL) were made by using an AS 500 autosampler, and the maximum operating pressure was 3500 psi.

Elution after injection was as following: 30% of 200mM NaOH was used for 8 min to wash the columns, followed by increasing the concentration of 200mM NaOH to 70% for 17 min. Then concentration was reduced back to 60mM and washing was carried out for next 6 min. Therefore, the run times were 30 min for separation of CHOs.

2.1.4.5 Statistical analysis

Statistical analyses was conducted to *analyse* effect of the modification using one way ANOVA using IBM SPSS statistic 2007 for Windows.

2.1.5 Determination of av. CHO using microbial enzymes

2.1.5.1 Extraction of soluble sugars before starch digestion

Foods were ground to a fine powder using a coffee mill. The powder (0.5 g) was suspended in 0.1 M MES-TRIS buffer (20 mL, pH 6.5). The suspension was shaken for 2 min at RT using a vortex mixer. At the end of the incubation, the suspension was mixed with 4 volumes of absolute ethanol, kept at 4°C overnight. The ethanolic supernatant was filtered through Whatman filter paper (number 1) and was analyzed for CHO content using the 4 analytical methods.

2.1.5.2 Extraction of digestible CHO

2.1.5.2.1 Reagents

2.1.5.2.1.1 MES-TRIS buffer solution (0.05 M each, pH 8.0 at 23°C)

19.52g of 2(*N*-morpholino) ethanesulfonic acid (MES) and 14.2 g tris (hydroxymethyl) aminomethane (TRIS) were dissolved in 1.7 L deionized water. The pH was adjusted to 8.0 with 6.0 M NaOH at 23°C. Then the mixture was diluted to 2 L with water.

2.1.5.2.1.2 Thermostable α-amylase solution (6 mL, 3,000 U/mL):

Thermostable α-amylase (*Bacillus licheniformis*) was supplied with the enzyme kit (Table 2.3).

2.1.5.2.1.3 Protease solution (10 mL, ~350 U/mL) :

Protease (*Bacillus licheniformis*) was supplied with the enzyme kit (Table 2.3).

2.1.5.2.1.4 Amyloglucosidase (AMG) solution (20 mL, 3,300 U/mL):

AMG (*Aspergillus niger)* was supplied with the enzyme kit (Table 2.3).

2.1.5.3 Protocol for extraction of digestible CHO

The procedure for the CHO digestion was based on Lee *et al*. (1992), while the av.CHO analysis was adapted from McCleary (2007) with series of modifications to optimize the digestion.

2.1.5.3.1.1 Original procedure for the extraction of digestible CHO

One gram of the sample was weighed in 250 mL Duran® bottles with 40 mL MES/Tris (pH 6.5) buffer and a magnetic stirring bar was added to each bottle. Sample was stirred on a magnetic stirrer plate until the sample was completely dispersed in the solution. Approximately 50 µL of thermostable α-amylase (25U) was added to the sample and incubated in a shaking water bath with continuous agitation for 30min at 80°C. The sample was removed from the water bath to be allowed to cool down, and then 100µL of protease (3.5U) was incubated with continuous agitation for 30min at 60°C. The pH was adjusted to pH 4.1-4.5 with 5 mL of 3 M acetic acid, then 200 µL of AMG (33U) was added and the mixture incubated for 30 min at 60°C with continuous agitation. One mL of the extracted aliquot was removed and diluted 4 times with absolute ethanol. The supernatant was used for the determination of av.CHO.

2.1.5.3.1.2 Modified procedure 1 (Heat treatment prior to enzyme treatment)

This procedure was similar to the original method but with a modification in which samples were cooked prior to enzyme treatment either by incubation in the boiling bath for 120min or autoclaving for 30 min at 129°C and 10 psi.

One gram of the sample was weighed in 250 mL Duran® bottles with 40mL MES/Tris buffer. Bottles were either autoclaved for 30 min or were cooked for 120 min at 100°C. These two treatments were conducted before enzyme addition to enhance starch gelatinization. Then the amount of both enzymes (α-amylase and AMG) and incubation periods (30 min autoclaving and 2 h boiling) were conducted. Finally, 1mL of the extracted aliquot was removed and diluted 4 times with absolute ethanol for determination av.CHO.

2.1.5.3.1.3 Modified procedure 2 (Enzyme dosage)

In this procedure a series of dosages of thermostable α-amylase & AMG was investigated without changing the incubation periods of the enzymes (Table 2.1).

Protocol	**Enzymes (µL)**	
	α -amylase	**AMG**
Original Megazyme	50	200
Modification A	100	200
Modification B	50	400
Modification C	100	400

Table 2.1: Amount of amylolytic enzyme used in digestible CHO extraction and compared to the original Megazyme protocol.

2.1.5.3.1.4 Modified procedure 3 (Enzyme incubation period)

In this procedure, a series of incubation periods of the amylatic enzymes was conducted without changing the enzyme dosage of both enzymes (thermostable α-amylase & AMG) (Table 2.2).

Procedure	Incubation periods (min)	
	A-amylase	**AMG**
Original Megazyme	35	30
Modification A	120	30
Modification B	35	Over night
Modification C	35	240
Modification D	120	240

Table 2.2: Incubation periods of enzymes used in digestible CHO extraction and compared to the original Megazyme protocol.

2.1.5.4 Statistical analysis

Samples were run in triplicates and expressed as g/ 100 g. Statistical analysis was conducted to analyse effect of the modification using T-Test and one way ANOVA using IBM SPSS statistic 2007 for Windows.

Name	Source	Substrate	Specific Activity Unit/Portion	Optimum pH	Stability pH	Optimum Temperature	Stability Temperature
α-Amylase	*Bacillus licheniformis*	*p*-nitrophenyle maltoheptaoside	25 units/50 µL	6.0-6.5	4.5-8.0	75°C	< 80°C
Protease		Tyrosine	3.5 units/ 100 µL	7.0-7.5	5.5-10.0	60°C	< 60°C
AMG	*Aspergillus niger*	Soluble starch & reducing sugar.	33 units/ 200 µL	4.0	4.-5.5	70°C	< 60°C
Sucrase	Yeast	Sucrose	95 units/ 200 µL	6.4	6.8	40°C	< 40°C
ß-Glucosidase	*Agrobacterium sp.*	Lactose	152.4 units/200 µL	6.5	7.0	50°C	Unstable above 50oC
HK	Yeast	D-glucose	4 units/20 µL	7.6	40°C	-	-
G6PDH	*Escherichia coli*	Glucose 6-phosphate	2 units/20 µL	7.6	25°C	-	-
PGI	*Saccharomyces. Cerevisiae*	Fructose 6-Phosphate	8.88 units/ 20 µL	7.6	40°C	-	-

Table2.3: The enzymes and their properties used for in extraction av.CHO and CHO analysis

2.1.6 *In vitro* restricted starch digestion using pancreatic enzymes

This method was adapted from Germaine *et al*. (2008) with modifications.

2.1.6.1 Reagents

2.1.6.1.1 Sodium potassium phosphate buffer (0.05M, pH 6.9) (extraction buffer)

1 M potassium phosphate monobasic (18.1 mL) and 31.9 mL of 1M potassium phosphate dibasic were mixed and made to 100 ml deionized water. 5M sodium chloride (30 ml) was added to the precious mixture and then made to 1 L deionized water.

2.1.6.1.2 Pepsin from porcine gastric mucosa solution (powder, ≥250 units/mg solid)

50 mg (53200 U) of pepsin was dissolved in 6 mL 0.05 M sodium potassium phosphate buffer and pH adjusted to 1.5 by 1 M hydrochloric acid.

2.1.6.1.3 A-Amylase from porcine pancreas (Type VI-B, ≥10 units/mg solid) /AMG solution (20 mL, 3,300 U/mL)

0.015 mg (1100U) of α-amylase was dissolved in 10 ml potassium phosphate buffer, and pH was adjusted to 5 with 3 M acetic acid. AMG (0.1 ml, 330 U) solution was added to the mixture.

2.1.6.2 Protocol for *in vitro* restricted starch digestion using pancreatic enzymes

2.1.6.2.1 Gastric phase

One gram of grounded food sample was weighed into tubes containing 6 mL pepsin plus potassium phosphate buffer (pH 1.5 adjusted with 1M HCl) and incubated at 37 ˚C for 120 min and gentle shaking (100 RPM) in the water bath. It was assumed that digesting in the stomach takes 1-2 hours (Gibson *et al*., 2011), After incubation the pH adjusted to 6 with 1 M $NaHCO_3$.

2.1.6.2.2 Intestinal phase

One ml of α-amylase /AMG solution was added to the sample then incubated for 120 min at 37 ˚C with shaking. Duplicate aliquots 1.0 mL of the sample were removed at 15 min interval, from time=0, and at 15, 30, 45, 60, 90, and 120 min. These aliquots were placed in a tube at 100°C for 5 min and were energically shaken for 5 min to inactivate the enzyme. All digestible samples were diluted 4 times in ethanolic solution for CHO analysis (PS, DNS, or HPLC-PAD).

2.1.7 *In vitro* restricted starch digestion using microbial enzymes

2.1.7.1 Protocol for *in vitro* restricted starch digestion using microbial enzymes

This method is similar to the method describe in 2.1.6 but with using microbial enzyme to investigate the differences between the two approaches.

2.1.7.2 Statistical analysis

Statistical analyses were conducted to analyse effect of the modifications using one way ANOVA using IBM SPSS statistic 2007 for Windows.

Name	Source	Substrate	Specific Activity Unit/Portion	Optimum pH	Stability pH	Optimum Temperature	Stability Temperature
α-Amylase	porcine pancreas (Type VI-B, ≥10 units/mg solid	α(1 -> 4) glucan linkages	1100 units/10 mL	6.0-6.5	4.5-8.0	75°C	< 80°C
Pepsin	porcine gastric mucosa	Tyrosine	53200 units/6 mL	7.0-7.5	5.5-10.0	60°C	< 60°C
AMG	*Aspergillus niger*	Soluble starch & reducing sugar.	33 units/200 µL	4.0	4.-5.5	70°C	< 60°C

Table 2.4: The enzymes and their properties used for *in vitro* restricted starch digestion

2.1.8 Estimation of GI from single and mixed food using statistical analysis

2.1.8.1 Food selection

The method used in this study was adapted from Urooj and Puttaraj (2000) with modification. Information of nutrient composition and description of the food were extracted from McCance and Widdowson's the composition of foods integrated dataset (Food Standards Agency, 2002) and nutritional information on the label. Total CHOs, soluble sugar, and digestible CHO were measured experimentally for selected breakfast cereals (n=7) and legumes (=10) using four analytical methods: enzymatic assay, 2 colorimetric assays, and HPLC (details in section 2.1.4).

2.1.8.2 GI selection

The GI values were extracted from the international table of GI and GL and the values were selected against glucose reference (Atkinson *et al*., 2008) and from the official website of the GI database created by Brand-Miller (http://www.glycemicindex.com) (Brand-Miller and Holt, 2004).

Forty food samples (cereals =24 & legumes =16) were investigated in this study to assess the GI prediction models.

Food (Cereal)	Published GI
All-Bran	44
Bagels, white & plain	69
Bran Flakes	62
Brown rice, boiled	63
Coco pops	77
Corn Flakes	81
Crunchy Nut Corn Flakes	74
Frosties	55
Fruit 'n Fibre	65
Hamburger buns	62
Muesli, Swiss style	57
Nutri-Grian	66
Pita bread, white	68
Pita bread, wholemeal	56
Puffed Wheat	80
Rice Krispies	95
Spaghetti, white, boiled	47
Spaghetti, wholemeal, boiled	47
Special K	62
Weetabix	71
White bread, average	85
White bread, toasted	60
White rice, easy cook, boiled	49
White rice, glutinous, boiled	91
Wholemeal bread, average	70

Table 2.5: List of cereals (n=25) with corresponding GI values.

Food (Legume)	Published GI
Baked beans, canned in tomato sauce	40
Baked beans, canned in tomato sauce, re-heated	57
Black-eye beans, dried, boiled in unsalted water	41
Butter beans, canned, re-heated, drained	34
Butter beans, dried, boiled in unsalted water	26
Chick peas, canned, re-heated, drained	38
Chick peas, whole, dried, boiled in unsalted water	36
Haricot beans, dried, boiled in unsalted water	35
Red kidney beans, canned, re-heated, drained	40
Red kidney beans, dried, boiled in unsalted water	51
Lentils, green and brown, whole, dried, boiled in salted water	33
Lentils, red, split, dried, boiled in unsalted water	21
Marrowfat peas, canned, re-heated, drained	47
Mung beans, dahl, whole, dried, boiled in unsalted water	37
Chick peas, split, dried, boiled in unsalted water	25
Broad beans, canned, re-heated, drained	63
Peas, frozen, boiled in unsalted water	51

Table 2.6: List of legumes (n=17) with corresponding GI values

2.1.8.3 Pearson correlation

The Pearson correlation coefficient is a statistical measure that determines the strength of the linear relationship between two continuous variables (e.g. GI & macronutrient) assuming that both variables have finite variances. A linear relationship can be demonstrated by drawing a straight line on a scatterplot between the two variables. The value of the correlation provides information regarding the nature and the strength of the relationship with the correlations ranging between -1.0 and 1.0.

The sign of the correlation represents the direction of the relationship in which a positive sign indicates there is a direct relationship, whereas a negative sign indicates that there is an inverse relationship (Data Analysis in SPSS).

The value of the correlation represents the strength of the relationship which exists between the numerica values of the variables with a value of 1.0 indicating a perfect positive association, a value of zero no association and a value of -1,0 indicating a perfect negative association(Data Analysis in SPSS).

Pearson correlation (Bivariant, two tailed) (PASW Statistic version 17, Chicago) was applied to analyse the relationships between the GI values and the macronutrients present in the 42 foods per 100 g.

2.2 Multiple Linear regression

Linear and multiple linear regression are statistical models which are used to explain the relationships between a dependent scalar variable and one or more explanatory independent variables. Assuming that the dependent variable is linearly associated with the sum of the individual independent variables. In this study, linear regression was adopted to provide prediction models of GI values from the explanatory macronutrient contents in 42 foods. Each macronutrient is associated with a specific coefficient in the linear regression equation which quantifies the extent of the relationship between that macronutrient and the GI value (Data Analysis in SPSS).

2.2.1 *In vivo* determination of GI

2.2.1.1 Ethics

An intervention design was chosen to investigate the effect of single or mixed foods on the GR in healthy individuals. The study has been reviewed and approved by the Ethics Committee of the Faculty of Mathematics and Physicals Science, University of Leeds. [MEEC 11-027] (Appendix A).

2.2.1.2 Sample size and subjects

Sample size calculation was based on a previous study conducted by William *et al.* (2008) where a sample size of 30 subjects allowed measurement of differences of 10 GI units with 80% power at a level 95% confidence interval. According to Brouns *et al.* (2005) 10 subjects is enough to predict the GI of the foods. Therefore, 30 healthy adults were recruited in this study following a health screening.

2.2.1.3 Recruitment

Advertisements were put on the School of Food Science and Nutrition notice boards and in different places at the University of Leeds (Appendix E). Anyone (including students or staff) were eligible to participate in the study, provided they met the inclusion criteria (18-35 years; not allergic to any food, not pregnant or lactating, not diagnosed with chronic diseases such as diabetes, cancer, cardiovascular or digestive diseases, and not taking any medication that might affect the GR).

Volunteers who expressed an interest were given information about the study (Appendix D) and two consent forms. A two signed copy was retained by volunteer and the other by the researcher (Appendix C). Volunteers had the opportunity to ask questions and two weeks to

decide whether to take part. Interested subjects were asked to complete a health questionnaire on food allergies to further assess their eligibility (Appendix B).

2.2.1.4 Study protocol

2.2.1.4.1 Reference and test food

Four types of foods (2 references and 2 tests) were selected for this study. They were: three slices of white bread (~50 g av.CHO portion), one slice and a half white bread (~25 g av.CHO portion), homemade lentil soup (~25 g av.CHO portion) and homemade lentil soup meal (with 1.5 slices of white bread) (~50 g av.CHO portion). White bread (Warburtons Medium Sliced 800g) and lentils (Tesco Red Split Lentils 500g) were purchased from a local store (Tesco). Bread was served fresh without toasting or heating, while the lentil soup was prepared by measuring out 150 g of dry lentils and washing with water, adding 300g of DI. water to fully submerge the lentils, microwaving on full power for 5min initially, mixing thoroughly and then heating for another 5min, after which, the lentils were ground/mashed using a standard mechanical potato masher forming a homogenized soup which was further stirred thoroughly, at which point salt and cumin (flavoring) were added according to taste (~5 g).

White bread (3 slices & 1.5 slices) was used as a reference food as recommended by WHO/FAO, 1998. Each of the four foods were served as 50g/25g of av.CHO per portion and av.CHO in foods was analyzed experimentally (see section 2.1.4). The other information about chemical composition of the food was taken from McCance and Widdowson's the composition of foods integrated dataset (Food Standards Agency, 2002).

2.2.1.4.2 Subject preparations

The subjects were asked to arrive at the School of Food Science and Nutrition following overnight fasting (10-14 h). All the subjects were asked to remain on their usual diets, but fasted overnight before the study. Upon arrival, the subjects were made comfortable.

Antiseptic wipes (Boots Pharmaceuticals Antiseptic Wipes) were used to sanitize the finger before and after collecting blood and the finger was rubbed gently using a gloved hand before pricking to stimulate the blood flow. The baseline blood glucose level then was measured from a finger-prick blood sample (lancet Accu-Chek Softclix) Roche diagnostic Limited, UK. Glucometers were calibrated against glucose reference supplied be the manufacturer.

A calibration test was conducted to check whether the meter and test strips were working appropriately and provided reliable results. Two calibration glucose solutions were supplied by the manufacturer; the acceptable range of glucose concentration was printed on the test strip container label provided by the manufacturer (solution 1 = 1.7-3.3 mmol/L and solutions 2 = 14.1-19.1 mmol/L). The glucometer was calibrated by applying a small drop of solution 1 to the test strip until the meter flashed with L. The test strip was not removed until another small drop of solution 2 was added; if the glucometer displayed "OK" the glucometer was calibrated.

The extracted blood was inserted into a glucometer (Accu-Chek Compact Plus, Model GT, Roche) diagnostic Limited, UK. The blood droplet was placed on the test strip (Accu-Chek Compact 17-Drum Test Strips) to measure the blood glucose concentration in mmol/L.

Baseline fasting blood glucose was taken at time 0 min, then the subjects were randomly allocated a specified portion of a food (white bread, lentil soup or white bread and lentil soup), to be consumed in a period between 15-20 min. Only water was permitted to be consumed during the tests.

The blood glucose analysis was repeated 15, 30, 45, 60, 90 and 120 minutes after the ingestion of the test food. Following the last analysis (120 min), the subjects were provided with a light breakfast. Also, weight, height, and activity level were collected from the subjects. Subjects were asked to repeat the procedure above another 7 times, to replicate the analysis with each food (e.g. 4x white bread, 2 x lentil soups, and 2 x white bread with lentil soup). The data were analyzed using paired t-test and Pearson correlation for each individual.

2.2.1.5 GR calculation (AUC)

The incremental area under the blood GR curve (iAUC), ignoring area beneath the baseline, was calculated as recommended by (FAO/WHO, 1998). The mean, standard deviation and coefficient of variation of the iAUC of each subject's repeated reference food trials was calculated. The GI for each food was expressed as a percentage of the mean iAUC of the tested food over the mean iAUC for the reference food taken by the same subject.

2.2.1.5.1 Statistical analysis

Statistical analyses were conducted to investigate the effect of factors (non-dietary and dietary) factors on GR using Pearson correlation, Spearmen correlation and one way ANOVA using IBM SPSS statistic 2007 for Windows.

3 Chapter three: determination of av.CHO using microbial enzymes

3.1 Introduction

3.1.1 Starch gelatinization and digestion

Starch is the main storage polysaccharide in plants. It is one of the most important dietary CHO in the human diet, and it is found in many staple foods worldwide including cereals, legumes and vegetables. In the presence of water and heat, starch granules will enlarge and swell because the arrangement of amylose and amylopectin is disturbed, allowing water to diffuse into the granules. Continued heating will lead the starch granules to burst and starch to be released. This process is known as starch gelatinization and results in the shear thickening of the solution. Gelatinization also facilitates the access of amylolytic enzymes to its starch substrate. This process is very common in cooking (Alsaffar, 2011).

The gelatinization process depends on many factors such as type of the starch, proteins, lipid, non-starch polysaccharides and polyphenols (Singh *et al*., 2010).

3.2 Aim

The aim is to optimize the measurement for av.CHO in cereals foods. This was done by optimizing (systematically) the enzymatic digestion of starch and the detection of released CHO using different analytical methods.

3.2.1 Objectives

To optimize the enzymatic starch digestion through manipulation of different conditions such as preheating before enzyme treatment, and enzymes doses and incubation period.

3.3 Results

3.3.1 Determination of av. CHO using microbial enzymes

3.3.1.1 Original procedure for the extraction of digestible CHO

Av.CHO was measured using Megazyme commercial kit (available CHO and dietary fibre (K-ACHDF 09/11), Megazyme International, Ireland) and the method for starch digestion was adapted from Lee *et al.* (1992) and the av.CHO analysis was modified from McCleary, (2007). HK was used rather than glucose oxidase due to its specificity and selectivity where glucose and fructose can be detected (Sonowane *et al*., 1976, McCleary, 2007).

Using the manufacturer's instructions, it was found that the amount of av.CHO measured was considerably less than that reported in McCance and Widdowson's the composition of foods integrated dataset (Figure 3.1). Around 50 % of total av.CHO was recovered from both Weetabix and cornstarch, which implied that either the av.CHO was not digested completely or that the digested CHO was not detected fully.

However, the analysis of the standard (pure glucose and fructose) which was supplied with the enzyme kit as positive control at a final concentration of 0.2 mg/ml, showed detection 92% (Figure 3.2). Therefore, a series of modifications took place to enhance starch digestion.

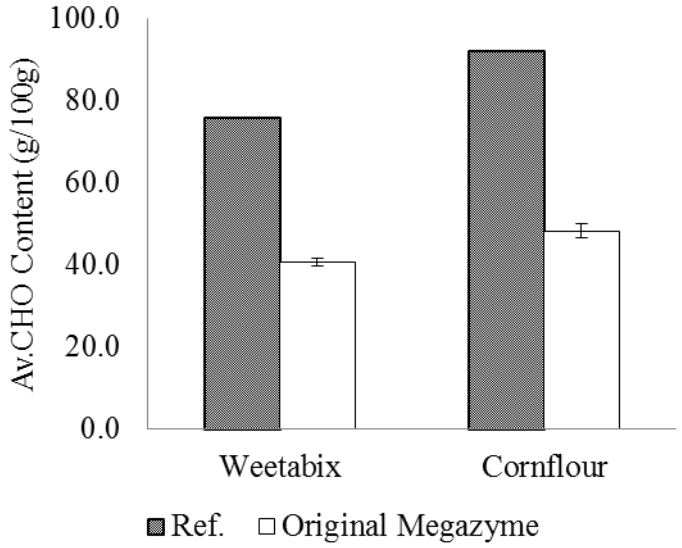


Figure 3.1: Amount of av.CHO detected by HK using the Megazyme original protocol. av.CHO in Weetabix and cornstarch reported by McCance and Widdowson's the composition of foods integrated dataset (first bar), compared to the amount of av.CHOcontent measured by HK (second bars). Data is expressed as g/100g of food (n=3) and error bars are the standard error of the mean.

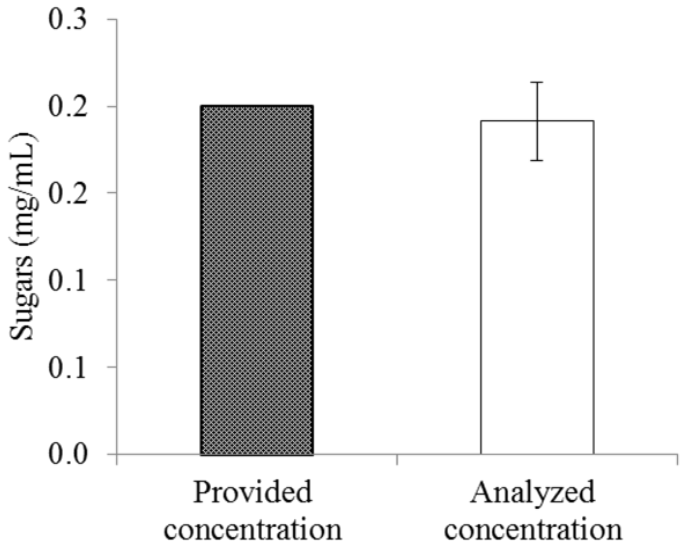


Figure 3.2: The concentration of sugars detected by HK using the Megazyme original protocol. The first bar represents the sugar concentration as supplied by the manufacture and compared to the second bar which represents the sugar concentration analyzed by the Megazyme protocol. Data is expressed as mg/ml of std. (n=3) and error bars are the standard error of the mean.

3.3.1.2 Pre-heating before enzyme treatment

The first modification was gelatinizing the samples prior to enzyme treatment to increase the yields of av.CHO by making starch more available to the hydrolysis enzymes and to denature any protein might be surrounding the starch granules or present in the food .

Samples were incubated in the boiling bath for 120 min at 100 ºC or were autoclaved for 30 min at 129 ºC and 10 psi to enhance starch gelatinization. Only 48% and 50% of total av.CHO were recovered from Weetabix and cornstarch respectively using autoclaving for 30 min, while 45% of total av.CHO from both Weetabix and cornstarch was recovered when samples were cooked for 120 min at 100 ºC. The concept of moisture heat treatment (boiling) is to enhance starch gelatinization and allow water penetration for easy enzyme hydrolysis; however results suggested that autoclaving increased the digestion yields slightly (4%), more than cooking the samples, yet the modification did not improve the CHO yield (Figure 3.3).

A t-test was used to investigate the effect of the modification on the yield of the av.CHO; any significance level will be $P \leq .0.05$. Therefore, no significant differences were found in the total av.CHO from Weetabix between the original protocols and autoclaving (P=0.25) and the boiling method (P =0.07), and in cornstarch no significant differences were found between the original protocols and autoclaving (P =0.78) and boiling methods (P =0.28).

Moreover, the results suggest that either the heat treatment was not sufficient and did not gelatinize the starch completely; or the presence of inhibitors like protein or polyphenols may affect the activity of the digestive enzymes. Also, excess maltose from Weetabix and cornstarch may have saturated the digestive enzymes and non-competitively limited the amount of starch digested. Moreover, the results suggested that the detection method (HK) might be inhibited by the food matrix in Weetabix; it is possible that protein or polyphenols

may inhibit HK and or excess substrates may have saturated the detection enzymes limiting the amount of sugars detected.

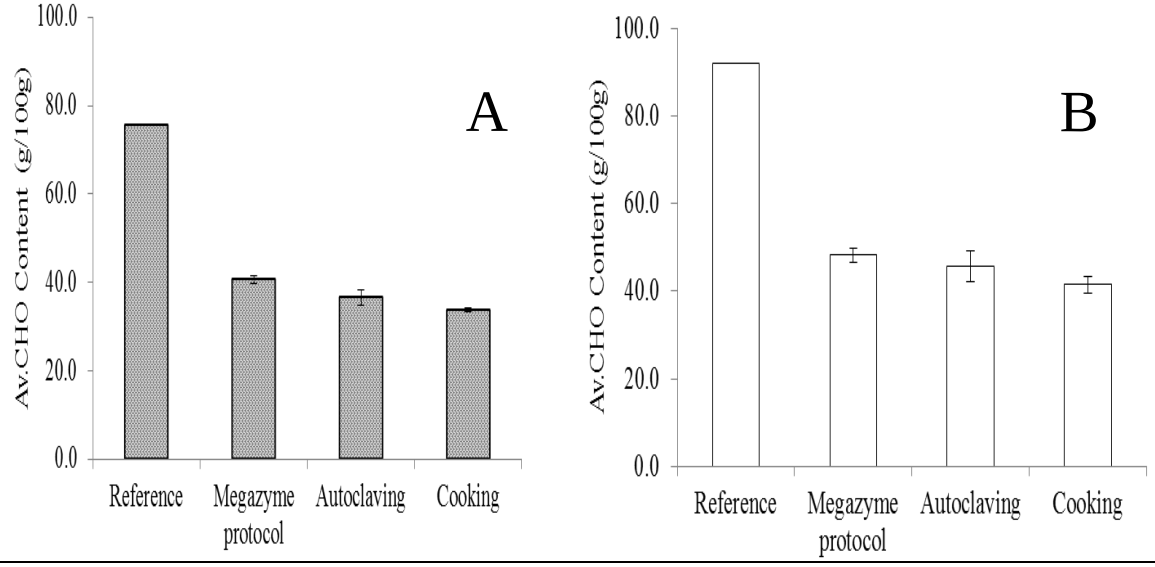


Figure 3.3: Amount of av.CHO detected by HK after pre-enzyme treatment. Av.CHO content measured in A) Weetabix and B) cornstarch, where the Reference is the amount of av.CHO reported in McCance and Widdowson's the composition of foods integrated dataset, the Megazyme protocol is the original method, autoclaving is for 30 min 129 ºC and 10 psi and boiling for 120 min at 100 ºC. Data is expressed as g/100g (n=4) and the error bars are the standard error of the mean.

3.3.1.3 Enzyme dosages

Further modifications were carried out in this study to test whether increasing the amount of enzymes could increase the amount of starch digestion in the period. Table 3.1 represented the dosage used in this part to increase the CHO yields.

As shown in Table 3.1, 47% and 48% of av.CHO were recovered from Weetabix and cornstarch respectively after increasing the dose of α-amylase only. The amount of av.CHO recovered from both Weetabix and cornstarch was 44% of av.CHO of both foods after increasing the dose of AMG. Finally 46 and 45% of av.CHO was recovered from Weetabix and cornstarch after increasing the dose of both amylolytic enzymes.

Food	Av.CHO g/100g
Weetabix	
Reference *	75.5
Megazyme Protocol (α-amylase 50 µL & AMG 200 µL)	36.5± 4.2
Modification A (α-amylase 100 µL & AMG 200 µL)	33.2± 4.0
Modification B (α-amylase 50 µL & AMG 400 µL)	29.79± 0.7
Modification C (α-amylase 100 µL & AMG 400 µL)	31.1± 0.5
Cornstarch	
Reference *	92.0
Megazyme Protocol (α-amylase 50 µL & AMG 200 µL)	43.4± 6.3
Modification A (α-amylase 100 µL & AMG 200 µL)	39.7± 2.2
Modification B (α-amylase 50 µL & AMG 400 µL)	36.1± 2.4
Modification C (α-amylase 100 µL & AMG 400 µL)	37.6± 1.5

*** Reference is the amount of CHO reported in McCance and Widdowson's the composition of foods integrated dataset.**

Table 3.1: Amount of av.CHO in Weetabix and cornstarch obtained after different dosage of enzyme treatments used in starch digestion. Data expressed as mean g/100 g ± SD (n=4).

One way ANOVA was used to assess the significance between the modifications. Significant differences were found in the results obtained using the original protocols and increasing the dose of the amylolytic enzymes (α-amylase P=0.05, AMG P=0.00, & both enzymes P=0.03) in Weetabix, whereas in cornstarch no significant differences were found in the results obtained using the original protocols and the dose of α-amylase and the dose of both enzymes (α-amylase P=0.18 & both enzymes P=0.15). Significant differences were found between the original protocol and the dose of AMG (P=0.01).

3.3.1.4 Enzyme incubation period

Moreover, in this study increasing the incubation periods was investigated to enhance starch digestion. The incubation period of digestive enzymes are listed in Table 3.2. Forty three

percent of av.CHO was recovered from Weetabix, while 49% of av.CHO was recovered from cornstarch by increasing the incubation period of α-amylase only (2 h). On the other hand, 44% of av.CHO was recovered from Weetabix, while 47% of av.CHO was recovered from cornstarch by incubating the AMG overnight.

Also, 42% of av.CHO was recovered from Weetabix, while 47% of av.CHO was recovered from cornstarch by incubating the AMG for 4h. The amount of av.CHO detected in Weetabix and cornstarch were ~17% less than the total av.CHO obtained by the Megazyme protocol. The results suggested that manipulating the incubation periods does not improve the yield of CHO or the CHO released is not detected.

Like the enzyme dose modification, one way ANOVA was used to assess the significance between the modifications. Significant differences were found in the results obtained using the original protocols and increasing the incubation period of the amylolytic enzymes (α-amylase at 2h P=0.00, AMG at overnight P=0.00, AMG at 4h p=0.00 & both enzymes P=0.00) in Weetabix, whereas in cornstarch no significant differences were found in the results obtained using the original protocols and the incubation periods of α-amylase, AMG at overnight, and AMG at 4h (P=0.58, 0.44, and 0.19 respectively). Significant differences were found between the original protocol and increasing the incubation period of both enzymes (P=0.03).

Food	Av.CHO g/100g
Weetabix	
Reference *	75.5
Megazyme Protocol (α-amylase 35 min @ 80 Cº and AMG 30 min at 60 Cº)	40.6 ± 4.2
Modification A (α-amylase 2h at 100 Cº and AMG 30 min at 60 Cº)	32.8 ± 5.3
Modification B (α-amylase 35 min at 80 Cº and AMG overnight at 40 Cº)	33.4 ± 4.0
Modification C (α-amylase 35 min at 80 and AMG 4h at 60 Cº)	31.4 ± 3.4
Modification D (α-amylase 2h at 100 Cº and AMG 4h at 60 Cº)	31.6 ± 2.8
Cornstarch	
Reference *	92.0
Megazyme Protocol (α-amylase 35 min at 80 Cº and AMG 30 min at 60 Cº)	48.2 ± 6.3
Modification A (α-amylase 2h at 100 Cº and AMG 30 min at 60 Cº)	44.7± 1.6
Modification B (α-amylase 35 min at 80 Cº and AMG overnight at 40 Cº)	42.9 ± 4.3
Modification C (α-amylase 35 min at 80 and AMG 4h at 60 Cº)	43.2 ± 6.5
Modification D (α-amylase 2h at 100 Cº and AMG 4h at 60 Cº)	40.5 ± 5.7
*** Reference is the amount of CHO reported in McCance and Widdowson's the composition of foods integrated dataset.**	

Table 3.2: Amount of av.CHO in Weetabix and cornstarch obtained after different enzyme incubation period. Data expressed as mean g/100 g ± SD (n=3).

3.3.1.5 Applying all the modification at the same time

Since the modifications tested so far did not increase the yields of av.CHO, samples were treated with the combination of all the modifications mentioned above to increase starch gelatinization and digestion. Therefore, 1 g of food samples were cooked for 120 minutes in the boiling bath and the amount of both amylolytic enzymes were doubled (α-amylase =100µL and AMG =400 µL) and the incubation of the both amylolytic enzymes were also increased (α-amylase 60 min and AMG = 4 h). One way ANOVA was used to assess the significance levels between the modifications and only 46% of av.CHO was recovered from Weetabix, while in cornstarch no significant differences (P=0.28) were found in the results obtained using the original method and all the modifications, where in 47% of av.CHO was recovered (Figure 3.4). Non-significant (P=0.06) differences in the results obtained using the original method and all the modifications.

The results suggested that the modifications did not improve the CHO yield and the low detection may be due to several reasons such as food factors affecting the starch gelatinization or inhibiting the digestive and detection enzymes. Moreover, saturation of the assay enzymes could be the cause of low detection in food sample rich in CHO. Indicating that the modifications may have decreased CHO detected, or properly CHO have been degraded by the high temperature.

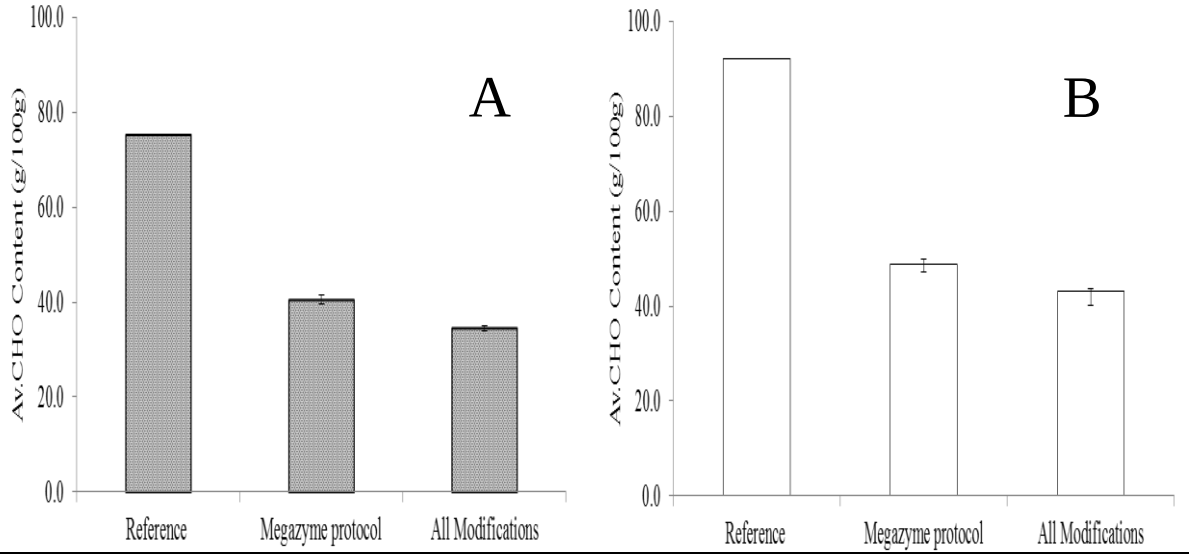


Figure 3.4: Amount of av.CHO detected by HK after treatment with all modifications. Av.CHO content measured in A) Weetabix and B) cornstarch, where the reference is the amount of av.CHO reported in McCance and Widdowson's the composition of foods integrated dataset, Megazyme protocol, and all modifications (boiled for 2 h @ 100 Cº and both amylolytic enzymes dose and incubation were increased (αamylase 100 µL for 2h @ 100 Cº, & AMG 400 µL for 4h @ 60 Cº. Data express as g/100g (n=4) and the error bars are the standard error of the mean.

3.3.2 Determination of av.CHO with alternative method

3.3.2.1 Megazyme protocol

After applying all these modification still the CHO content was around 50% lower than the amount reported by McCance and Widdowson's the composition of foods integrated dataset. Consequently, another analytical approach was investigated in this study. Phenol sulfuric assay (PS) was used for measuring av.CHO in Weetabix and cornstarch as an alternative detection method. Figure 3.5 shows the differences between the amounts of CHO detected by PS was higher than HK (Weetabix 25%, and Cornstarch 15%). However, still the amount recovered is lower than the amount reported by McCance and Widdowson's the composition of foods integrated dataset.

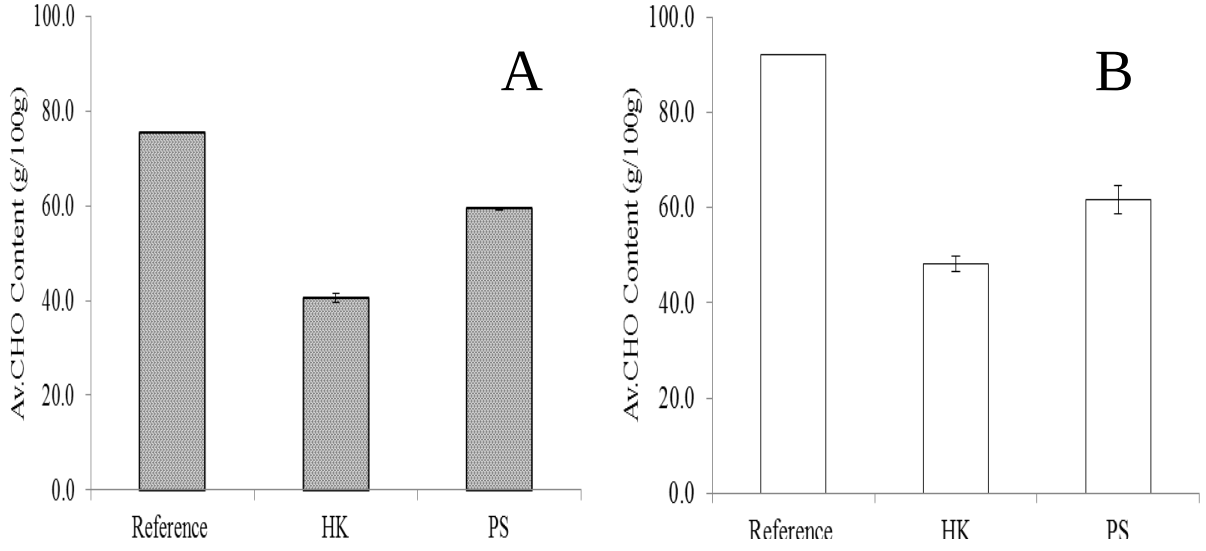


Figure3.5: Amount of av.CHO detected by HK compared to the amount of av.CHO detected by PS. Av.CHO content measured in A)Weetabix and B)cornstarch, where the reference is the amount of av.CHO reported in McCance and Widdowson's the composition of foods integrated dataset, av.CHO detected by HK (n=3), and av.CHO detected by PS (n=4). Data expressed as g/100g and the error bars are the standard error of the mean.

3.3.2.2 Pre-heating before enzyme treatment

As shown in Figure 3.6 when samples were autoclaved for 30 min at 129 Cº the amount of av.CHO recovered from Weetabix using PS was 78%, and from cornstarch 67% of CHO. Seventy four percent of CHO was recovered using PS from Weetabix when samples were cooked for 120 min at 100 Cº and 60% of CHO from cornstarch was recovered using PS.

However, still the amount of CHO detected was30-40% less than the amount reported by McCance and Widdowson's the composition of foods integrated dataset (Food Standards Agency, 2002).

A t-test was used to assess the significance between the modifications and no significant differences were found between Weetabix results obtained using the original protocols and autoclaving (P=1.0) and the boiling method (P=0.45). In cornstarch no significant differences were found in the results obtained using the original protocol and autoclaving (P=1.0) and the

boiling method (P=0.45). Moreover, the results suggest that the heat treatment still did not improve the starch gelatinization.

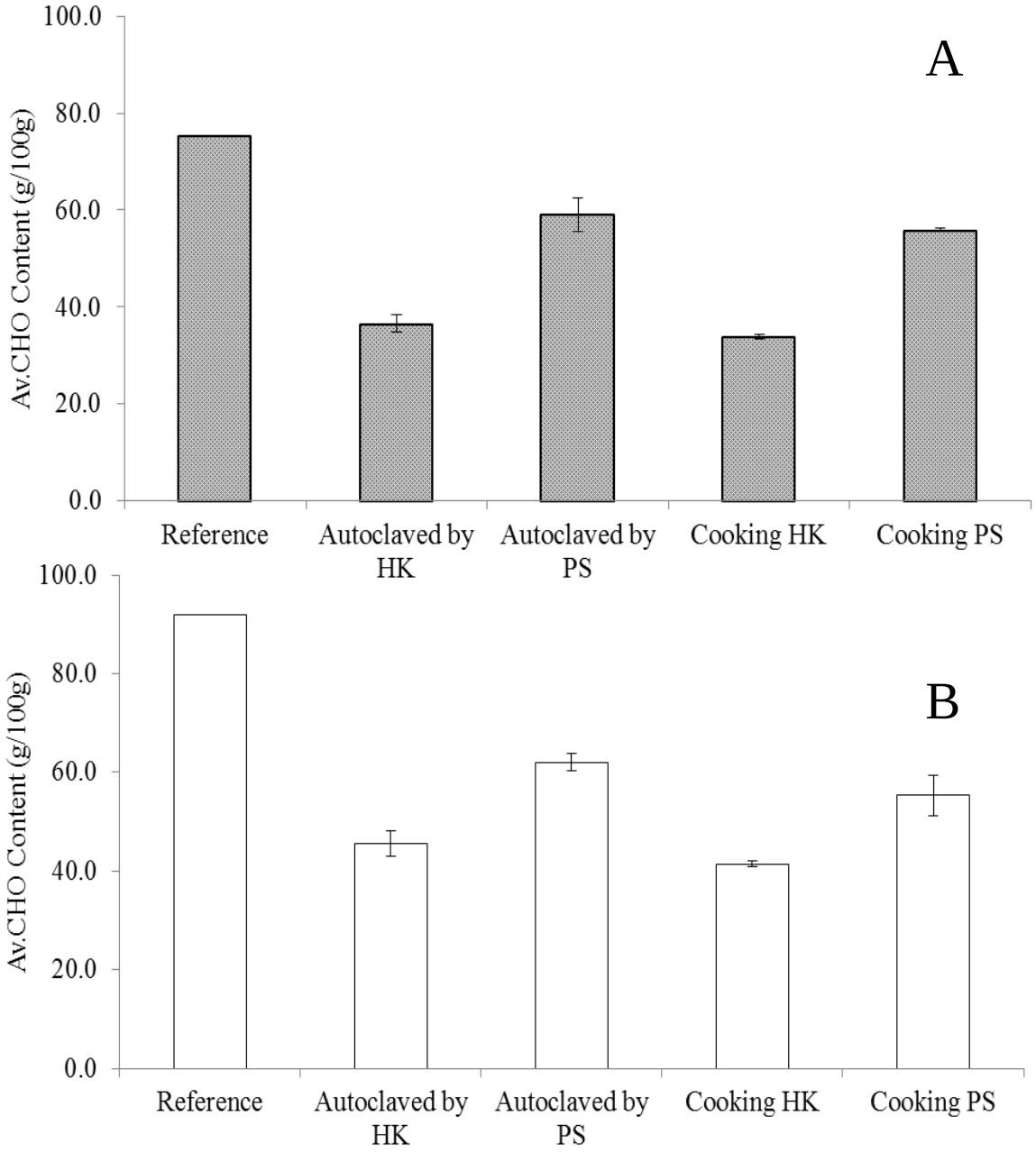


Figure 3.6: Amount of av.CHO detected by HK compared to the amount of av.CHO detected by PS. Av.CHO content measured in A) Weetabix and B) cornstarch, where the reference is the amount of av.CHO reported in McCance and Widdowson's the composition of foods integrated dataset, av.CHO detected by HK, and av.CHO detected by PS; the effect of cooking prior enzyme treatment (HK; n=4, & PS; n=4). Data expressed as g/100g and the error bars are the standard error of the mean.

3.3.2.3 Enzyme dosages

The enzyme dosage was modified from the original protocol according to Table 3.1, and likewise, more av.CHO was recovered from Weetabix and cornstarch using PS when manipulating the enzyme dosages as shown in Figure 3.6.

Eighty percent of av.CHO was recovered from Weetabix, while 95% was recovered from cornstarch after increasing the dose of α-amylase only. On the other hand, after increasing the dose of AMG only the amount of av.CHO recovered from both Weetabix and cornstarch were 100% compared to the reference, while when both enzyme dosages were increased 97% of total av.CHO was recovered from both Weetabix and cornstarch.

One way ANOVA was used to assess the significance levels between the modifications. In Weetabix no significant difference was found in the results obtained using the original protocols and increasing the dose of α-amylase only (P=0.87), while significant differences were found in the results obtained using original protocol and increasing AMG (P=0.00, and both enzymes P=0.00). In cornstarch significant differences were found in the results obtained using the original protocols and the dose of the amylolytic enzymes (α-amylase P=0.00, AMG P= 0.00 & both enzymes P=0.00). However, still the amount of CHO detected by this modification was slightly lower (8%) than the amount reported by McCance and Widdowson's the composition of foods integrated dataset. The results suggested that this modification did not improve the amount of CHO detected but further modifications need to increase the yields of CHO.

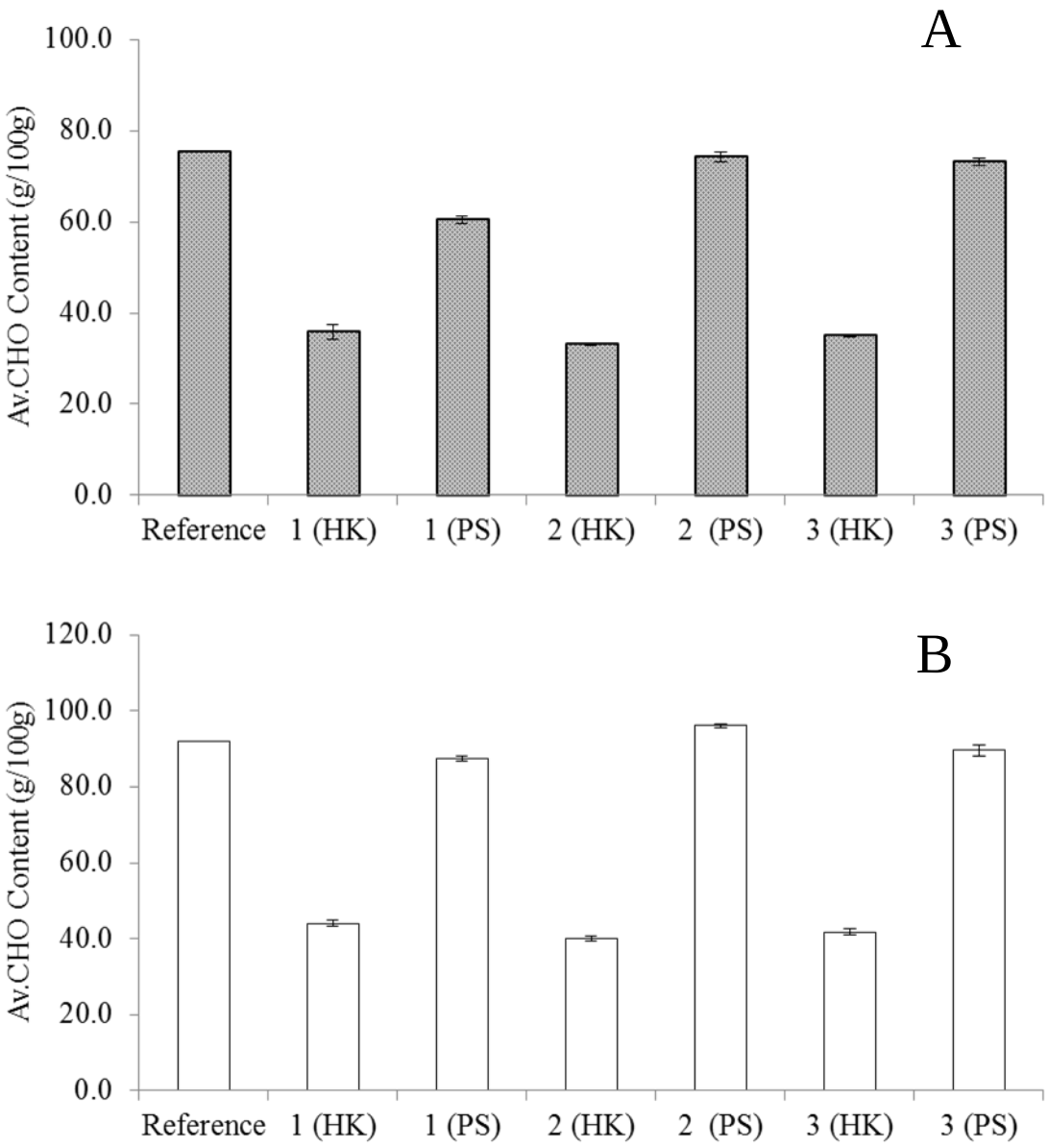


Figure3.7: The effect of enzymes dose and amount of av.CHO detected by HK compared to the amount of av.CHO detected by PS. Av.CHO content measured in A) Weetabix and B) cornstarch, where the Reference is the amount of av.CHO reported in McCance and Widdowson's the composition of foods integrated dataset, av.CHO detected by HK (n=3), and av.CHO detected by PS (n=4). 1) Increasing the dose of α-amylase, 2) increased the dose of AMG, and 3) increasing the dose of both enzymes Data express as g/100g and the error bars are the standard error of the mean.

3.3.2.4 Enzyme incubation periods

Likewise HK, the incubation period of the enzymes dosage was modified according to Table 3.2. The average yield of detected of CHO in Weetabix with PS was 44% higher than CHO detected with HK when incubation period of the digestive enzymes was manipulated, whereas the average yield of detected CHO in cornstarch with PS was 50% higher than CHO detected with HK (Figure 3.7).

One way ANOVA was used to assess the significance levels between the modifications. In Weetabix no significant differences were found in the av.CHO between the original protocols and increasing the incubation period of α-amylase to 2h and AMG to 4h (P=0.09, and 0.00) respectively, while significant difference were found between the original protocol and incubation the AMG overnight or manipulating the incubation periods of both enzymes (AMG overnight P=0.01 or 0.01).

In cornstarch significant differences were found in the av.CHO between the original protocols and manipulating the incubation periods of the digestive enzymes (α-amylase P=0.00, AMG at overnight P=0.01, AMG at 4h P=0.00, and both enzymes P= 0.00 respectively). Still the amount of CHO detected by PS was again slightly less (8-9%) than the total av.CHO reported by McCance and Widdowson's the composition of foods integrated dataset.

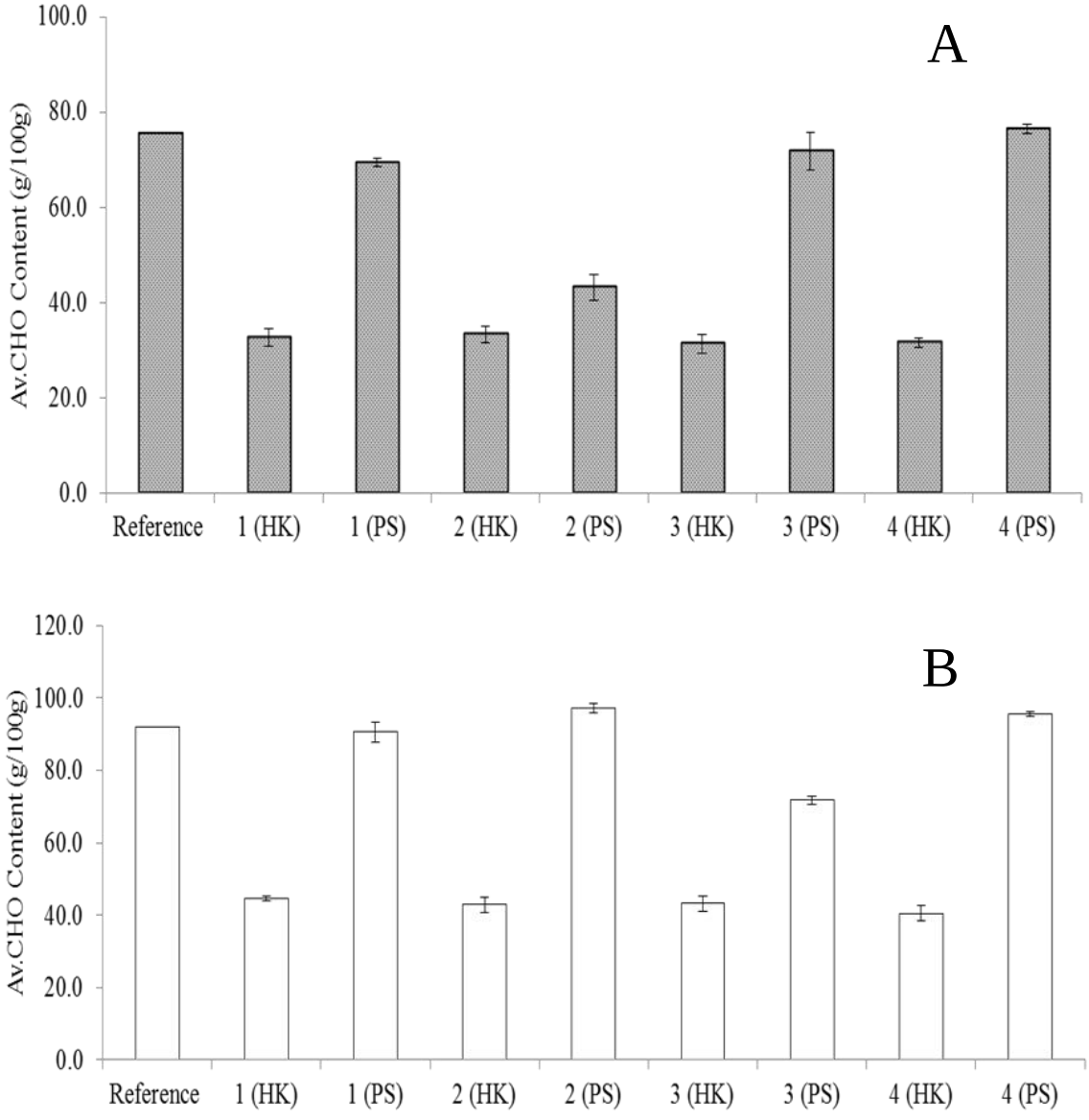


Figure 3.8: The effect of enzymes incubation period and the amount of av.CHO detected by HK compared to the amount of av.CHO detected by PS. Av.CHO content measured in A) Weetabix and B) cornstarch, where the Reference is the amount of av.CHO reported in McCance and Widdowson's the composition of foods integrated dataset, av.CHO detected by HK, and CHO detected by PS (HK; n=4, PS; n=4). 1) Increasing the incubation of α-amylase for 2 h, 2) increasing the incubation of AMG overnight, 3) increasing the incubation of AMG for 4h, and 4) increasing the incubation of both enzymes. Data expressed as g/100g and the error bars are the standard error of the mean.

3.3.2.5 Applying all the modification

After applying all the modification mentioned above, the yields of CHO detected by PS have increased by 54, and 59% higher than those detected by HK in both Weetabix and cornstarch respectively. 100% of total CHO was recovered from both Weetabix and cornstarch (Figure 3.9). There was significant differences between the CHO detected by HK and PS.

The results suggested that the problem was with the detection method (HK), although each modification per se did not improve the CHO yield.

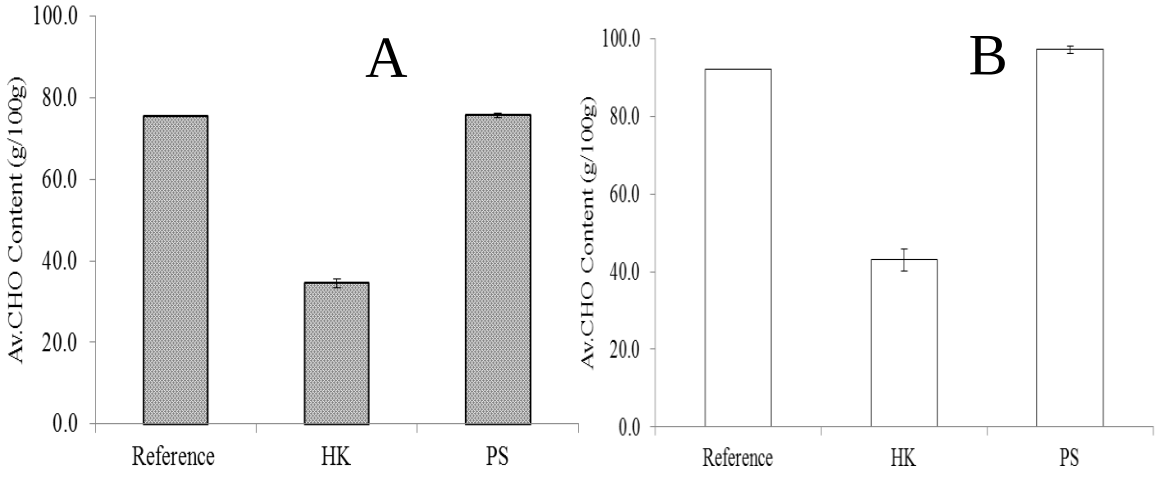


Figure 3.9: Amount of av.CHO detected by HK and compared to the amount of av.CHO detected by PS. Av.CHO content measured in A) Weetabix and B) cornstarch, where the Reference is the amount of av.CHO reported in McCance and Widdowson's the composition of foods integrated dataset, av.CHO detected by HK(n= 4), and av.CHO detected by PS (n= 4) after applying all the modifications (cooking for 2 h @ 100 Cº and increasing both amylolytic enzymes doses and incubations (α-amylase 100 µL for 2 h @ 100 Cº, & AMG 400 µL for 4 h @ 60 Cº . Data expressed as g/100g and the error bars are the standard error of the mean.

3.4 Discussion

3.4.1 Issues with Megazyme commercial kit (HK)

Food samples were incubated with amylolytic and protolytic enzymes and the amount of av.CHO were detected by HK. Weetabix was used to optimize the digestion and cornstarch was used as positive control.

It is well known that enzymatic essays are one of the most recommended methods for measuring CHO especially monosaccharide and disaccharides because of their specificity and selectivity (Southgate, 1976). As a result, enzymatic assay were chosen because different monosaccharides can be detected using this assay (Southgate, 1976).

The results showed that the amount of av.CHO in both Weetabix and cornstarch obtained by HK was around 50% less than the amount of av.CHO reported by McCance and Widdowson's the composition of foods integrated dataset, while the analysis of the standard (pure glucose and fructose) provided by the manufacturer (Megazyme) was nearly fully detected (92%), which means the kit did not allow complete detection of av.CHO. Therefore, a series of modifications took place in this study to enhance starch digestion and to increase the yield of av.CHO.

3.4.1.1 Pre-heating before enzyme treatment

The protocol originally was used in the present study was to determine the total dietary fibre in food (Lee *et al*., 1992), therefore it might be not suitable for measuring av.CHO as suggested by the manufacturer (Megazyme).

The first modification was manipulating the incubation period and temperature of the samples prior to enzyme treatments. Nevertheless still the amount of av.CHO recovered was lower than the reference.

It is well known that native starch is indigestible because it is encapsulated inside a starch granule and through the heating in water, starch granule will swell due to the amylose/amylopectin crystallinity distribution (Alsaffar, 2011). Besides, studies suggested that heating most of the foods, including legumes, for various times (mean= 2 h and 30 min) at 100 ºC is more than enough to gelatinize starch completely (Dona *et al*., 2010, Guzel and Sayar, 2010). Also, pressure cooking (30 min) may gelatinize starch completely by making the starch granule enlarge abnormally and become disrupted in a short time (Ezeogu *et al*., 2005, Yadav *et al*., 2010). According to the results, the amount recovered by autoclaving is slightly higher (~6%) than cooking which supported the study conducted recently by Yadav *et al*. (2010), however, no significant difference in av.CHO yield between the two approaches, and the amount recovered did not improve the yields of CHO in both approaches which suggested that gelatinization is not the main issue.

3.4.1.1.1 Enzyme dosages and incubation periods

After starch is gelatinized, amylolytic enzymes will attack and hydrolyze starch however the rate of the hydrolysis depend on many factors. One of the main factors is the enzyme kinetics in which there is a direct relationship between the concentration of the digested starch and the rate of hydrolysis, until the concentration of the starch reaches the maximum then the level decreases due to inhibition of the amylolytic activity (Singh *et al*., 2010). Maltose released after starch digested by α-amylase and will inhibit the amylolytic activity by binding to the enzyme either non-competitively or uncompetitively because maltose and starch have the same affinity to the amylolytic enzymes hence the rate of starch digestion will be decreased (Dona *et al*., 2010, Singh *et al*., 2010). Moreover, others found that maltose may affect the viscosity of the solution and affect the velocity of the hydrolysis reaction (Singh *et al*., 2010).

In addition, the rate of the hydrolysis also depend other factors such as the source of the starch (Vosloo, 2005), the presence of proteins, fibres, and polyphenols. Protein may form a complex with the granule's surface while fibres may affect the hydration of the matrix and retain the water then preventing starch from being gelatinized and being accessible to the digestive enzymes or might interfere with the HK enzyme (Ezeogu *et al*., 2005). On the other hand, polyphenols are considered as α-amylase inhibitors and can slow down the digestion rate and HK (Vosloo, 2005). However, increasing the enzyme dosage did not improve the amount of av.CHO yields.

3.4.1.1.2 Applying all the modification

After applying all the modification and still the yield of CHO did not improve, this suggested that there were some difficulties with the detection method using HK and not the digestion protocol. This conclusion was reached because cornstarch was behaving similarly to Weetabix, even though cornstarch was a pure starch and free of any nutrient that may interfere with HK. Weetabix is considered as a complex matrix that may contain many interferences like polyphenols, phytic acid, protein or lipids (Southgate, 1976). According to Southgate (1976) that may be due to the presence of protein or polyphenol since they are important interference that may affect the results, but unfortunately the amount of interferences that affect the method is not identified (Southgate, 1976). Therefore, HK was excluded from the evaluation and PS was an alternative method and replaced HK in this study.

3.4.2 Determination of av.CHO with alternative method

Another analytical method was used which is phenol sulfuric assay (PS) because is simple and broad spectrum. The amount detected by PS was ~20% higher than HK in both foods, but

still did not recover the amount of av.CHO. Therefore same the modifications to enzyme conditions were carried out but using PS as detection method.

3.4.2.1 Pre-heating before enzyme treatment

Like using the HK method, this modification did not improve the av.CHO yield but the amount of av.CHO detected by PS was ~24% higher than HK in autoclaved food whereas the amount of av.CHO detected by PS was 15% higher than HK in both foods. In addition

The results, supported by the study conducted recently by Yadav *et al.* (2010) in which the amount of av.CHO recovered by autoclaving is slightly higher (~5%) than cooking though the differences in av.CHO yield between the two approaches is not significant, which confirming that that gelatinization is not the main issue.

3.4.2.2 Enzyme dosage and incubation

The optimum CHO recovered was achieved by manipulating the incubation period before the enzyme treatments and manipulating both enzymes (α-amylase & AMG) dosages and incubation periods (2h & 4h) in which 100% of CHO was recovered from Weetabix while 106% was recovered from cornstarch. From the results, increasing the amount of both enzymes might be more advantageous since α-amylase hydrolyze starch into small fractions (maltose, maltodextrine) but not glucose and as mentioned earlier these fractions may affect the viscosity of the solution and then affect the velocity of the hydrolysis (Singh *et al*., 2010). Nevertheless, this approach did not reach the amount reported by McCance and Widdowson's the composition of foods integrated dataset.

3.4.2.2.1 Applying all the modification

After applying all the modification mentioned above, av.CHO were fully recovered (100%) in both foods which means that heating treatment enzymes dosage and incubation all together are important factors that determine the amount of av.CHO released from the starch.

The yields of CHO detected by PS are higher by 56% than HK in both foods and there were significant differences in the amount of av.CHO recovered between the two detection methods. The results suggested that although each modification *per se* did not improve the av.CHO yield, there was a problem with the HK detection method.

Moreover, PS yielded better results, but due to the wide specificity of the PS detection assay, it was decreased recovery to test other methods.

3.5 Conclusion

Systematic investigation was conducted throughout a serial of modifications to enhance starch gelatinization and increasing the yield of av.CHO using microbial enzymes, but still the results showed that the amount of av.CHO in both samples was ~ 50% less than what was reported by McCance and Widdowson's the composition of foods integrated dataset and the reduction in yields indicated that there was difficulty with the detection method (HK) not the digestion protocol because of the low detection associated even with the positive control (pure cornstarch). Although enzymatic essays are recommended methods for measuring av. CHO because of their specificity and selectivity, in this study it is not recommended because of the low av.CHO detection and HK was excluded from the present study. Moreover, neither the method of heat treatment before the addition of digestive enzymes nor the enzyme dosage and incubation alone had improved the yield of av.CHO, nevertheless the combination of all in one go boosted up the amount of av.CHO released from the digested starch.

Finally, this enzymatic protocol will be applied to the rest of the food samples for measuring av.CHO in of cereal and legume foods, and other detection methods will also be investigated.

4 Chapter four: evaluation of different methods for av.CHO analysis

4.1 Introduction

4.1.1 Cereals grains

Cereals grains are the edible portion of the grasses and they are composed of endosperm, germ, and bran. They are the major energy supplier in diets all over the world. They are considered one of the most harvested crops and contribute to more than 60% of the world food production (Charalampopoulos *et al*., 2002). They are rich in CHO and are the staple foods in many cultures. Cereals include: rice, corn, wheat, barley, rye, and oat. Wheat is one of the first cereals to be known to the human race (Caldwell and Fast, 2000). Cereal grains are composed of an average of 10.1% proteins, 1.2% fat, 8.8% NSP and total 72.2 % starch (Yadav *et al*., 2010). Among the cereals, breakfast cereals and breads are considered one of the important sources of CHO in human diet (Dewettinck *et al*., 2008). Breakfast cereals and breads provide around 30% of dietary energy. In UK breakfast cereals contributed to 7% while breads contributed to 21% to total CHO intake (Henderson et al., 2003, Rufian-Henares and Delgado-Andrade, 2009).

4.1.2 Legumes

Legumes belong to the family of leguminosae and the well-known legumes include: peas, beans, lentils. They are considered one of the important food staples especially in developing countries (McCrory *et al*., 2010). They are composed of an average of 18.8% proteins, 2.3% fat, 20.7% NSP and 54.3% starch (Yadav *et al*., 2010). Legumes are classified under vegetables and they provide around 7% of dietary energy.

Legumes are known by their unique compositional and structural properties which make them difficult to digest by humans. The compositional properties include the presence of indigestible oligosaccharides like raffinose, stachyose and verbascose. These types of sugar

escape the digestion in the small intestine and are fermented by micro-flora in the gut (Apata, 2008). Also, the presence of anti-nutrients like polyphenols and phytic acid, and high amylose/amylopectin ratios are compositional properties that make legumes difficult to be digested in the human gut. The structural properties are the thick cell wall they possess and type of crystalline polymorphic (B type) forms of starch which are difficult to digest (Hoover and Zhou, 2003).

4.1.3 Av. CHO analysis

Calculation of av.CHO 'by difference' is a common method in food composition databases (Food Standards Agency, 2002).

Studies have found that the difference method is not sufficient for consumers since the physiological effect of each carbohydrate fraction is different between constituents (FAO/WHO, 1998, Greenfield and Southgate, 2003, Menezes *et al*., 2009). Furthermore, measuring carbohydrates by this procedure may overestimate the amount of av.CHO particularly in food rich in resistant starch (Granfeldt *et al*., 2006). In addition, the calculation may be inaccurate due to the experimental errors of any of the other analytical methods used to determine protein, fat, water, alcohol, fibre, and ash. Consequently, measuring carbohydrates directly is required for accurate determination. Colorimetric methods can be used to estimate carbohydrates (Dubois *et al*., 1956). Two colorimetric methods are widely used to determine the content of carbohydrates present in foods, which are: the phenol-sulfuric acid (PS) assay (Dubois *et al*., 1956) and 3, 5-dinitrosalicylic acid assay (DNS) (Miller, 1959). All types of carbohydrate can be detected based on color developed due to the reaction between sugars and reagents. The intensity of the developed color is related to the concentration of the carbohydrates present in the food samples. Chromatographic techniques are considered the most powerful method for quantification and identification carbohydrates

in foods and include high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) (Jager *et al*., 2007). HPAEC-PAD can provide selective and sensitive separation, direct quantification of carbohydrates at a very small concentration (picogrames) without any derivatization (Jager *et al*., 2007).

4.2 Aim

The aim is to develop a reliable laboratory method to measure soluble sugars and av.CHO in cereals and legumes as test foods using an optimized digestion method in the previous chapter.

4.2.1 Objectives

To measure soluble sugars in cereals and legumes using three different methods: PS, DNS, and (HPAEC-PAD).

To measure av.CHO optimized previously in chapter 3 in cereals and legumes using three different methods.

To evaluate the sensitivity and reproducibility of the three methods by using pure sugar standards.

To choose the method of av.CHO analysis by statistical analysis one-way ANOVA.

4.3 Result

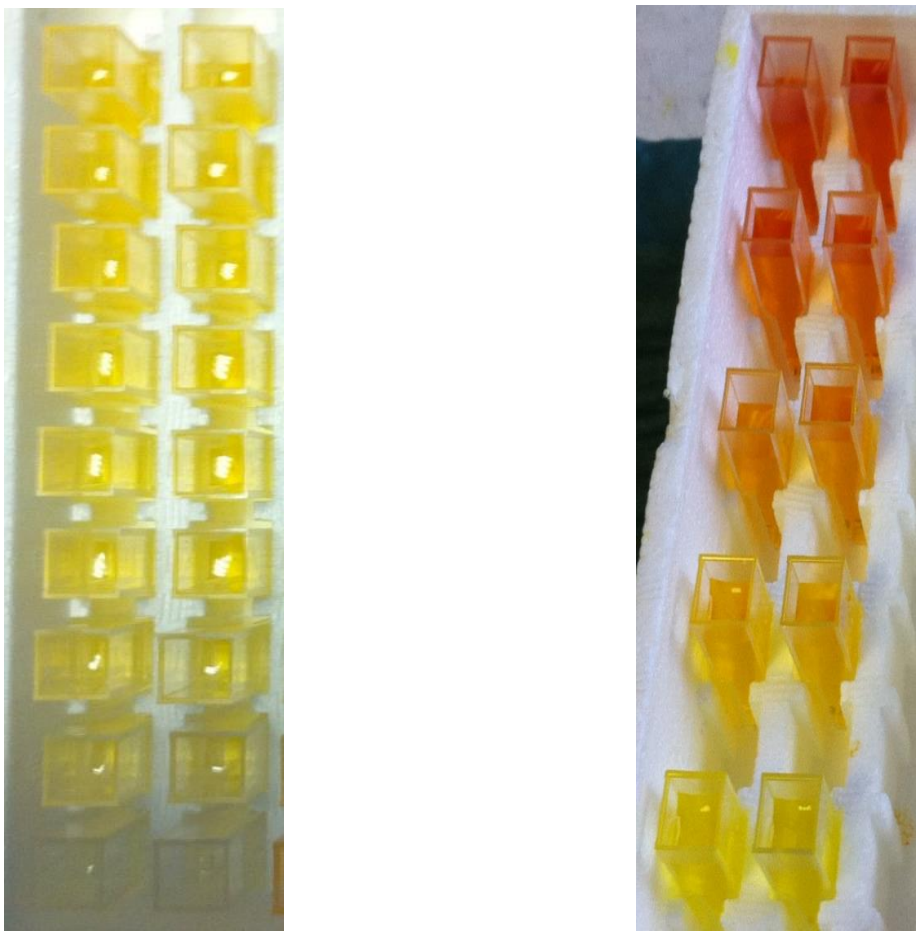
4.3.1 Sugar standards and sugar ranges used for av.CHO detection

Four different detection methods were investigated in this study: HK, PS, DNS, and HPAE-PAD to measure CHO in cereals and legumes foods. Cereals samples (n=7) were divided into two groups, breakfast cereals (n=4) and breads (n=3), while nine cooked legumes samples were used in this study to evaluate the CHO measurements.

For the colorimetric methods, different ranges of glucose standard (glucose ranges for PS= 0 were used for PS and DNS. The intensity of the colour depended on the reaction between the sugars and sulphuric acid in the PS method and DNS reagent in the case of DNS method. As seen in Figure 4.1 the darker the colour the more sugars detected.

For the HPAE-PAD, a calibration of sugar standards was prepared by injecting different ranges of mixed sugars plus 100 µl of fucose as the internal standard. The peak area is proportional to the concentration of the sugar in food samples. As shown in Figure 4.2, the order of elution of each sugar was 2.86 min for fucose, 5.05 min for glucose, 5.51 min for fructose, 7.51 min for sucrose, and 14.73 min for maltose.

Figure 4.3 represents the three typical glucose standard curves for PS, DNS and HPAE-PAD respectively that have been used in this study to measure the amount of soluble sugars and av. CHO.



A B

Figure 4.1: Glucose Standard. A) PS glucose standard and the ranges from 0-0.04 mg/ml measured at 340 nm, B) DNS glucose standard and the ranges from 0 to 3.0 mg/ml measured at 540 nm.

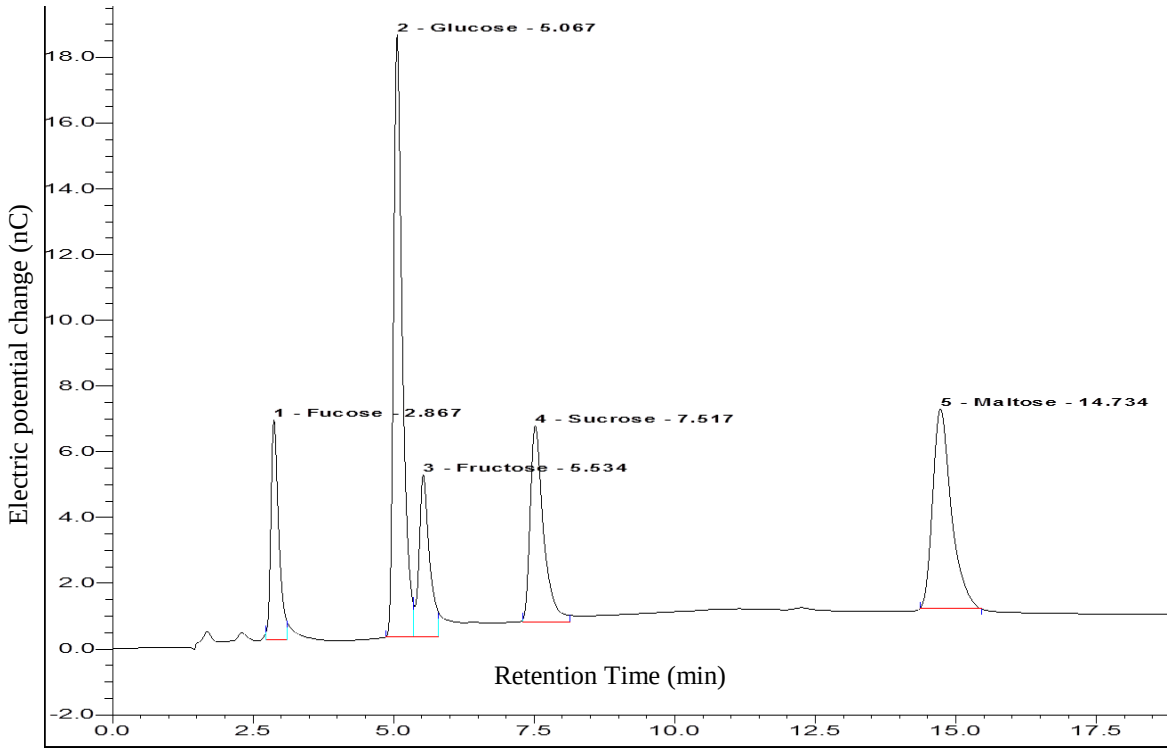


Figure 4.2: Sugar standard chromatogram. standard ranges from 0-0.1 mM level analyzed using HPAE-PAD and 1) Fucose was used as internal standard, and 2) glucose, 3) fructose, 4) sucrose, and 5) maltose were the sugar standard.

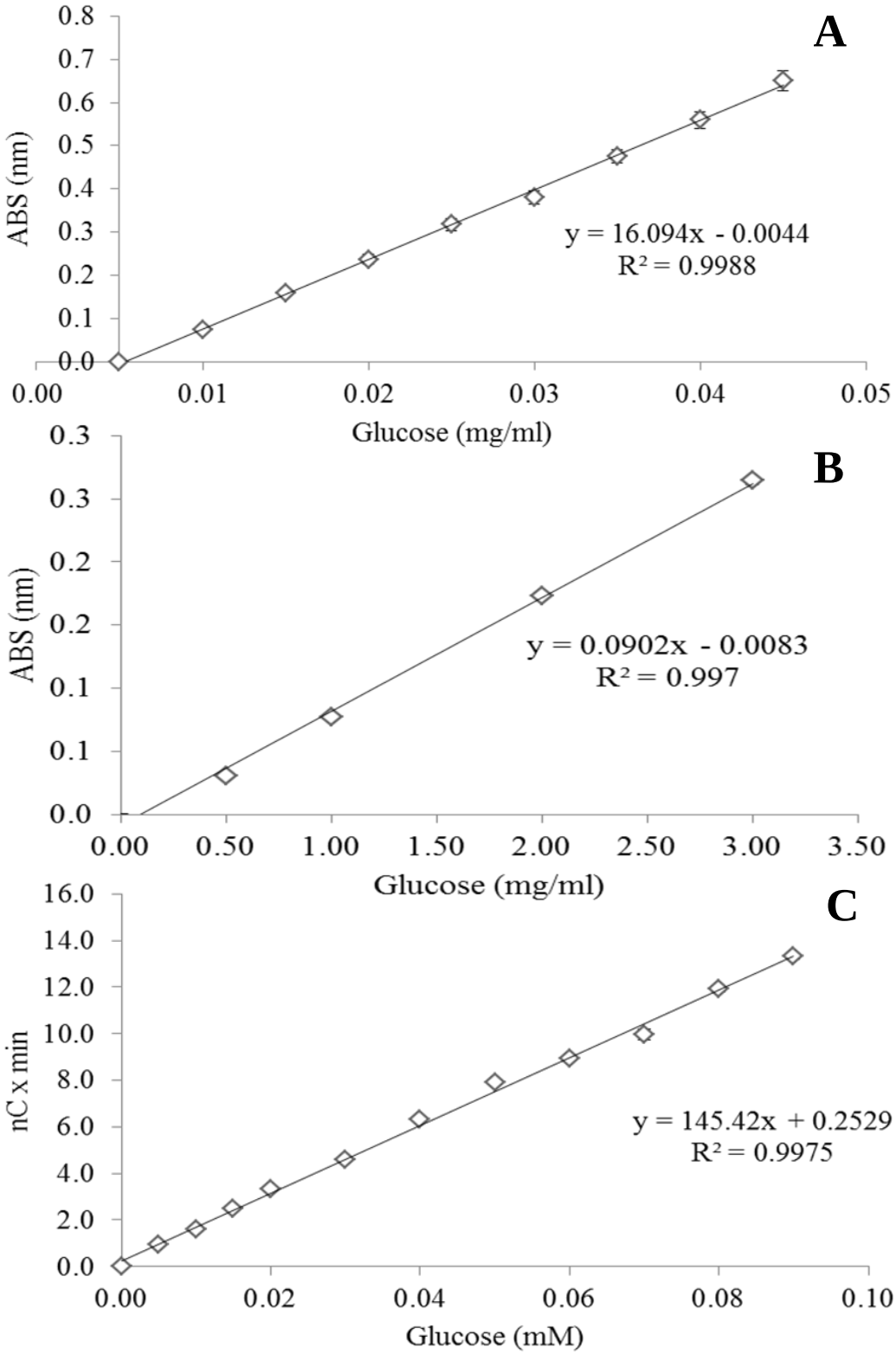


Figure 4.3: Three standard curves for measuring CHO content in food using glucose solution as standard. A) Glucose detected by PS (n=21) at 340 nm, B) glucose detected by DNS (n=18) at 540 nm and glucose detected by HPAE-PAD detector (n=11). Error bars represent the standard error of

4.3.2 Evaluation of soluble sugars and av.CHO analysis in cereals and legumes

4.3.2.1 Extraction of soluble sugars content

The soluble sugars in this study were extracted with 80% ethanol and did not undergo starch digestion. Cereals were divided into two groups: breakfast cereals and breads. The amount of soluble sugars of breakfast cereals (n=4) and breads (n=3), are presented in Figure 4.4, while cooked legumes were divided into three groups beans (n= 3), peas (n=3) and lentils (n=3) and they are presented in Figure 4.5.

The soluble sugar content in breads and legumes were present in low quantities per 100 g because they are soluble sugars present naturally, unlike breakfast cereals where sugars are usually added.

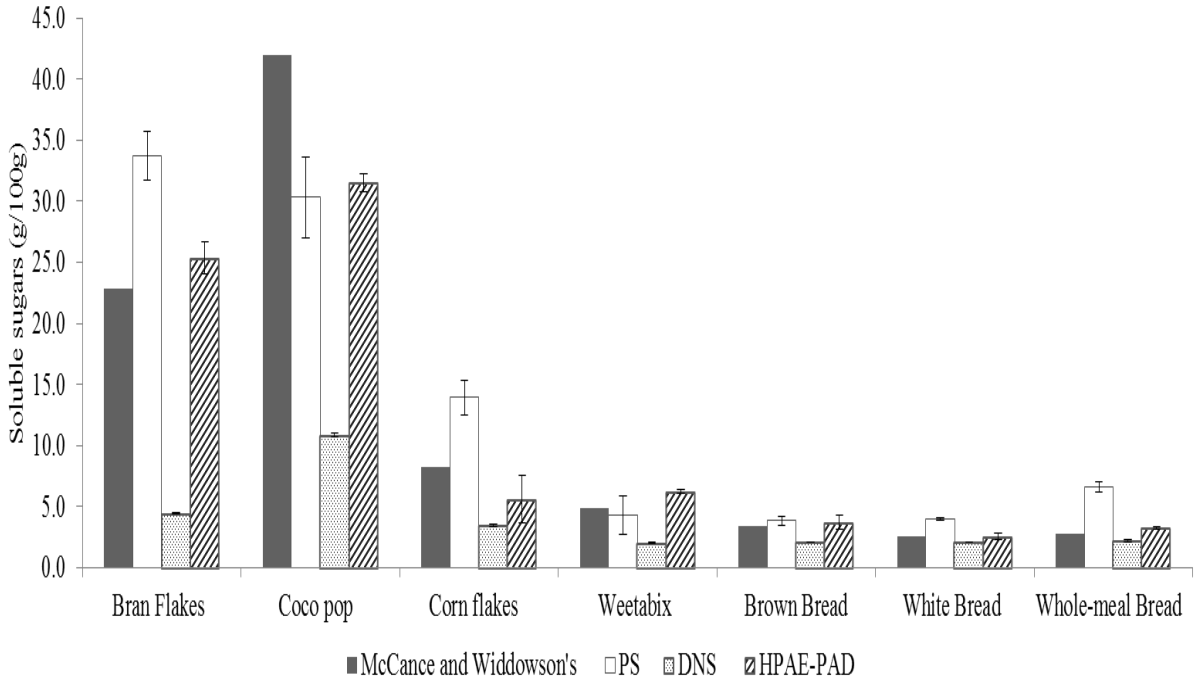


Figure 4.4: Amount of soluble sugars in cereals using three different methods and compared to a reference from McCance and Widdowson's the composition of foods integrated dataset. First bars are the amount of free sugar reported by McCance and Widdowson's the composition of foods integrated dataset, second bars are the amount of soluble sugar detected by PS (n=4), third bars are the amount of soluble sugars detected by DNS (n=4), and the last bars are the amount of soluble sugars detected by HPAE-PAD (n=4). Data expressed as g/100g and the error bars are the standard error of the mean.

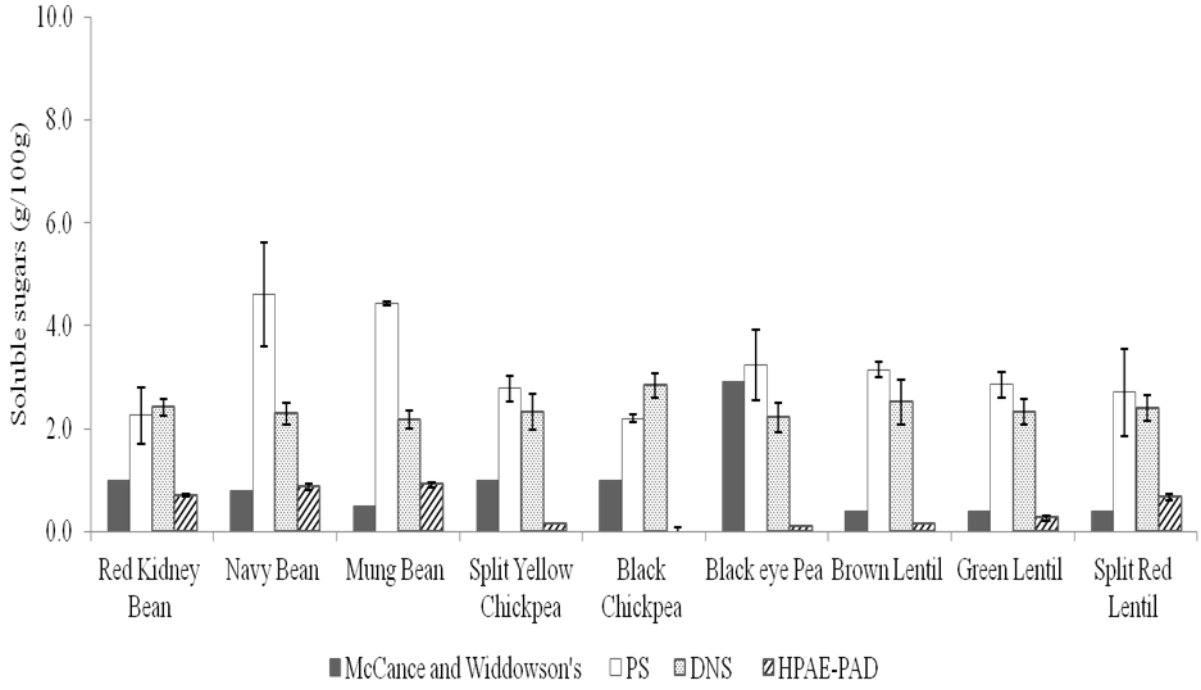


Figure 4.5: Amount of soluble sugars in legumes using three different methods and compared to a reference from McCance and Widdowson's the composition of foods integrated dataset. First bars are the amount of free sugar reported by McCance and Widdowson's the composition of foods integrated dataset, second bars are the amount of soluble sugar detected by PS (n=4), third bars are the amount of soluble sugars detected by DNS (n=4), and the last bars are the amount of soluble sugars detected by HPAE-PAD (n=4). Data expressed as g/100g and the error bars are the standard error of the mean.

4.3.2.2 Extraction of av.CHO

Extracted av.CHO was optimized in the previous chapter using a mixture of heating and enzyme treatments to recover the maximum amount of av.CHO. The amount of av.CHO content of cereals (n=7) and legumes (n=9) are presented in Figure 4.6 and 4.7.

Av.CHO in McCance and Widdowson's the composition of foods integrated dataset were calculated rather being analyzed.

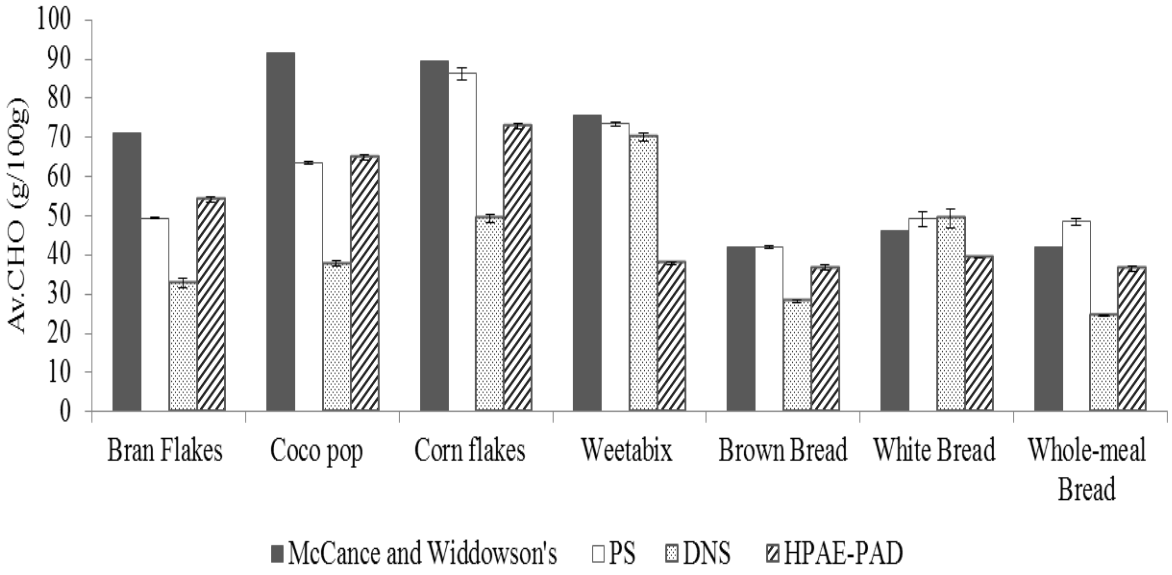


Figure 4.6: Amount of av.CHO in cereals using three different methods and compared to a reference from McCance and Widdowson's the composition of foods integrated dataset. First bars are the amount of av.CHO reported by McCance and Widdowson's the composition of foods integrated dataset, second bars are the amount of av.CHO detected by PS (n=4), third bars are the amount of av. CHO detected by DNS (n=4), and the last bars are the amount of av.CHO detected by HPAE-PAD (n=4). Data expressed as g/100g and the error bars are the standard errors of the samples.

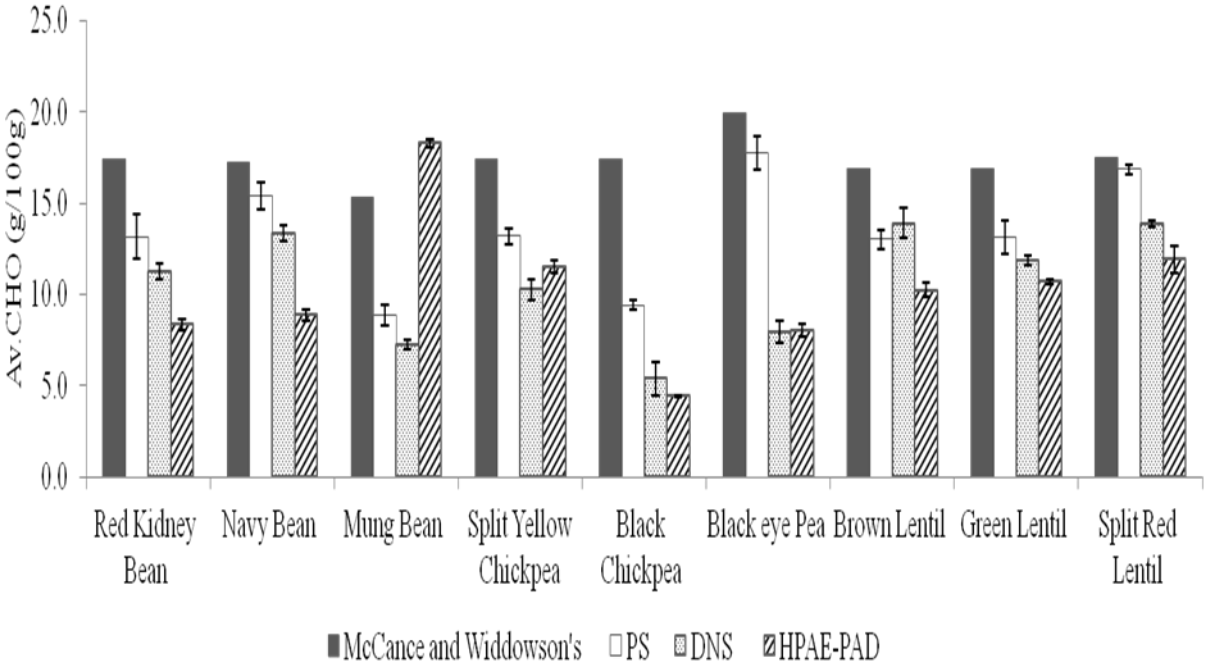


Figure 4.7: Amount of CHO in legumes using three different methods and compared to a reference from McCance and Widdowson's the composition of foods integrated dataset. First bars are the amount of free sugar reported by McCance and Widdowson's the composition of foods integrated dataset, second bars are the amount of soluble sugar detected by PS (n=4), third bars are the amount of soluble sugars detected by DNS (n=4), and the last bars are the amount of soluble sugars detected by HPAE-PAD (n=4). Data expressed as g/100g and the error bars are the standard error of the mean.

4.3.2.2.1 Phenol sulphuric assay (PS)

4.3.2.2.1.1 Soluble sugars

In cereals, the amount of soluble sugars detected was 140% above what was reported in McCance and Widdowson's the composition of foods integrated dataset except for Coco Pops and Weetabix (72 and 87 % sugars recovered). The average soluble sugars recovered were 20.6±4.1 g/100g from breakfast cereals and 4.8±0.6g/100g from breads.

Like the cereals, the amount of soluble sugars measured in legumes found to be over 100% compared to the compositional data and the average soluble sugars found in legumes were: beans = 3.8 ±1.3, peas = 2.7 ±0.5 and lentils 2.9 ±0.2 g/100g.

A recovery range test for each sugar (glucose, fructose, sucrose, and maltose) was conducted. Around 88 % of glucose, 83%, of fructose, and 89% of maltose were recovered while only 48% of sucrose was recovered.

4.3.2.2.1.2 Av.CHO

In cereals, PS seemed to give slightly higher levels than the other methods most of the time but with breakfast cereals only 83% was detected. PS provided better detection in breads. The average av.CHO was found in breakfast cereals was 68.2 ±145.6 g/100g, while the average av.CHO in breads 38.8 ±15.8 g/100g.

On the other hand, the amount of CHO detected in legumes by PS was lower than the amount of CHO reported by McCance and Widdowson's the composition of foods integrated dataset and only 79% was recovered from CHO in legumes. The average av.CHO measured in legumes was: beans = 12.5 ±3.3, peas = 13.5 ±4.2 and lentils 14.3 ±2.2 g/100g.

4.3.2.2.2 3, 5 Ditrosalicylic acid (DNS)

4.3.2.2.2.1 Soluble sugars

In cereals, the lowest detection of soluble sugars was found with this approach and the results were 50% below the amount reported in McCance and Widdowson's the composition of foods integrated dataset.

The amount of soluble sugars recovered in breakfast cereal was 32%, whereas 73% of sugars were recovered from bread samples. The average soluble sugars measured in cereal were 5.2 ±3.9 g/100g from breakfast cereals and 2.1 ±0.1g/100g in breads.

In legumes, the amount of sugars measured with DNS was found to be higher than the sugars values reported by McCance and Widdowson's the composition of foods integrated dataset, and more than 100% of soluble sugars were recovered. The average soluble sugars in legumes were: beans = 2.3 ±0.1, peas = 2.5 ±0.3 and lentils 2.4 ±0.1 g/100g.

Range test was also conducted with DNS for each sugar (glucose, fructose, sucrose, and maltose) to check the accuracy. Around 83 % of glucose, 15%, of fructose, and 65% of maltose were recovered while no sucrose was recovered.

4.3.2.2.2.2 Av.CHO

In cereals, the amount of av.CHO recovered was 67% of what was reported by McCance and Widdowson's the composition of foods integrated dataset. The average av.CHO found in breakfast cereals was 47.5 ±16.5 g/100g, while the amount measured in breads was 29.6 ±14.0 g/100g.

DNS showed lower detection of CHO in legumes among the other methods and only 61% of CHO was found in legumes. The average av.CHO measured in legumes was: beans = 10.6 ±3.1, peas = 7.9 ±2.5 and lentils 13.2 ±1.2 g/100g.

4.3.2.2.3 High performance anion exchange with pulsed amperometric detector (HPAE-PAD)

4.3.2.2.3.1 Soluble sugars

The amount of soluble sugars recovered from cereals was more than 100% than what was reported by McCance and Widdowson's the composition of foods integrated dataset. The amount of sugars analyzed by HPAE-PAD in breakfast cereals was 17.2 ±13.2 g/100g whereas the amount in breads was 3.2 ±0.6 g/100g.

The amount of sugars recovered from HPAE-PAD in legumes was 84% below the amount of sugars obtained from McCance and Widdowson's the composition of foods integrated dataset except for mung beans and red split lentils in which more than 100% was recovered. The average soluble sugars were: beans = 0.8 ±0.1, peas = 0.1 ±0.1 and lentils 0.4 ±0.3 g/100g.

Moreover a soluble sugars profile was provided for both food samples as shown in Table 4.1 and 4.2. The amount of sucrose was higher in breakfast cereals than breads and legumes whereas maltose is the dominant sugar in breads only. The amount of soluble sugar detected in this study for both types of foods was slightly higher than the amount reported by the McCance and Widdowson's the composition of foods integrated dataset.

Soluble sugars profile in breakfast cereals (mean g/100g ±SD)								
	Glucose		Fructose		Sucrose		Maltose	
	Ref.[1]	Det.[2]	Ref.[1]	Det.[2]	Ref.[1]	Det.[2]	Ref.[1]	Det.[2]
Breakfast cereals								
Bran flakes	1.0	1.9±0.1	3.0	2.6±0.3	17.3	20.2±2.1	Tr.*	0.6±0.1
Coco pop	0.5	0.04±0.0	0.5	0.1±0.0	41.0	27.4±1.3	Tr*.	4.04±0.2
Corn flakes	1.5	0.7±0.1	1.5	2.1±1.5	4.2	1.9±2.01	1.0	0.9±0.3
Weetabix	0.7	0.6±0.0	0.7	0.8±0.0	2.6	3.7±0.2	0.8	1.1±0.1
Breads								
Brown Bread	Tr.*	0.2±0.1	0.3	0.2±0.1	Tr.*	0.2±0.1	3.0	3.2±0.9
White Bread	Tr.*	0.1±0.0	0.2	0.3±0.0	Tr.*	0.0±0.0	3.2	2.9±0.1
Whole-meal Bread	0.2	0.5±0.1	0.5	0.4±0.1	Tr.*	0.6±0.04	2.2	1.5±0.2

[1] Ref = Reference provided by the McCance and Widdowson's the composition of foods integrated dataset.

[2] Det = Determine by HPAE-PAD.

*** Tr. = traces**

Table 4.1: Soluble sugars profile in breakfast cereals and breads obtained by HPAE-PAD. Data expressed as g/100g (n=4).

Soluble sugars profile in legumes (mean g/100g ±SD)								
	Glucose		Fructose		Sucrose		Maltose	
	Ref.[1]	Det.[2]	Ref.[1]	Det.[2]	Ref.[1]	Det.[2]	Ref.[1]	Det.[2]
Beans								
Red Kidney Bean	0.1	0.2±0.04	Tr.*	0.0±0.0	0.8	0.5±0.0	0.0	0.1±0.1
Navy Bean	0.1	0.3±0.0	Tr.*	0.0±0.0	0.7	0.5±0.03	0.0	0.1±0.1
Mung Bean	0.2	0.2±0.02	0.3	0.0±0.0	1.0	0.4±0.03	0.0	0.3±0.1
Peas								
Split Yellow Chickpea	Tr.*	0.2±0.0	0.1	0.0±0.0	0.9	0.0±0.0	0.0	0.0±0.0
Black Chickpea	Tr.*	0.0±0.0	0.1	0.0±0.0	0.9	0.0±0.0	0.0	0.0±0.0
Black eye Pea	1.0	0.1±0.1	Tr.*	0.0±0.0	0.1	0.0±0.0	Tr.*	0.0±0.0
Lentils								
Brown Lentil	Tr.*	0.2±0.01	0.1	0.0±0.0	1.1	0.0±0.0	0.0	0.0±0.0
Green Lentil	Tr.*	0.2±0.02	0.1	0.0± 0.0	1.1	0.0± 0.0	0.0	0.1±0.1
Split Red Lentil	Tr.*	0.2±0.01	0.1	0.0±0.0	0.7	0.4±0.2	0.7	0.1±0.1

[1] Ref = Reference provided by the McCance and Widdowson's the composition of foods integrated dataset.

[2] Det = Determine by HPAE-PAD.

*** Tr. = traces**

Table 4.2: Soluble sugars profile in legumes obtained by HPAE-PAD. Data expressed as g/100g (n=4)

4.3.2.2.3.2 Av.CHO

In cereals, 77% of total CHO was found using this approach of what was reported in McCance and Widdowson's the composition of foods integrated dataset. The amount of recovered av.CHO analyzed in breakfast cereals was 57.5 ±15.1 g/100g whereas the amount in breads was 32.0 ±11.3 g/100g.

The amount of av.CHO was recovered from HPAE-PAD in legumes was 61% of the amount reported in McCance and Widdowson's the composition of foods integrated dataset except for mung beans in which more than 100% was recovered. The average of soluble sugars found in legumes was: beans = 11.8 ±5.6, peas = 8.0 ±3.6 and lentils 10.9 ±0.9 g/100g.

4.3.3 Statistical analysis

One way ANOVA was conducted to evaluate whither the av.CHO analysis methods had an effect on soluble sugars and av.CHO contents. In cereals it found that no significant differences were found among the methods in soluble sugars (P=0.21). In contrast measuring soluble sugars in legumes showed significant differences between the three methods (P=0.01).

No significant differences were found among the methods in av. CHO analysis (P= 0.15) for the cereals similarly, no significant differences were found when measuring total CHO in legumes (P = 0.10).

4.4 Discussion

Breakfast cereals were selected because the they are consumed frequently in UK (Henderson et al., 2003) and the av.CHO content in McCance and Widdowson's the composition of foods integrated dataset was calculated by difference rather than analysed (Food Standards Agency, 2002).

4.4.1 Evaluation of soluble sugars and av.CHO analysis in cereals and legumes

4.4.1.1 Extraction of soluble sugars content

Soluble sugars are present in the food naturally in very small quantities or they are added to improve the palatability of the foods. Soluble sugars are still presented on nutritional labels as sugars, but measuring total soluble sugar in foods may not be useful since each sugar has different metabolic response in the human body (Southgate *et al*., 1978)

In this study soluble sugars were extracted using 80% ethanol to extract the maximum amount of sugars and to remove any interference substances may present in samples. In general, the ranges of soluble sugars in breakfast cereals reported by Southgate (1976) were 48-62% and the results of this study obtained by PS and HPEA-PAD came within this range, while the ranges of soluble sugars in breads reported by same author were 1-2% and they were more than the ranges obtained by all the methods in this study.

However, samples varieties must be taken into consideration because in the present study only one batch of food was tested whereas in McCance and Widdowson's the composition of foods integrated dataset different food batches and different varieties were analyzed. This may explain some of the differences and that may be reason for the fluctuation in the results used in this study.

4.4.1.2 Extraction of Av.CHO

Av.CHO was optimized in the previous chapter using a mixture of heating and enzymes treatments then they were extracted using 80% ethanol to extract the maximum amount of sugars and to remove any interference present in samples.

4.4.1.3 Phenol sulphuric (PS)

Despite the low selectivity, PS was selected because it is a common, simple, and considered as broad-spectrum method that detects various sugars (Southgate, 1976). Also, the advantages of this method are: it is inexpensive and the materials are widely available. The method can be specific since each sugar has different wavelength of absorbance (Dubois *et al*., 1956).

The disadvantages are using harsh chemicals like phenol and sulfuric acid, the hydrolysis of other substances may present in the food and affect the results. Further sugars like raffinose and NSP may react with PS, leading to overestimation of sugars. The presence of substances like polyphenols may interfere with the colour complex formation, since both cereals and legumes are high in polyphenols (Wei *et al*., 2011).

Lastly, PS may not be suitable for soluble sugar analysis because many types of sugars are present in the food, and each type of sugar has a different wavelength of absorbance. On the other hand, PS may be appropriate for detecting av.CHO in both types of food. Furthermore, glucose, which is the main sugar released after starch digestion using α-amylase and AMG, is well detected by PS

4.4.1.4 3,5 Dinitrosalicylic acid (DNS)

DNS was also investigated and it was selected because of the many advantages such as sensitivity, simplicity, and reproducibility (Hall, 2003). However, one of the main disadvantage is that this method only works with reducing sugars like glucose and fructose,

and did not detect the non-reducing sugars like sucrose in case of cereals or raffinose in case of legumes (Miller, 1959). Non-reducing sugars are these type of sugars that formed with monosaccharide and have no hemiacetal unit and they lack the ability to reduce oxidizing agent like Benedict's reagent and DNS (Southgate, 1976).

In this study, the amount of soluble sugars and av.CHO were found to be low in cereals especially breakfast cereals where sucrose is added to improve the sensory properties (Southgate, 1976). DNS may not be suitable method for soluble sugar analysis and av.CHO analysis in both cereals and legumes because of the presence of sucrose and raffinoses, while without explanation DNS might be appropriate for detecting soluble sugar in legumes, despite that they are known for having raffinoses in their composition (McCrory *et al*., 2010).

The result suggested the DNS might be unpredictable method and might be not suitable for measuring soluble sugars and av.CHO in cereals and legumes used in this study.

4.4.1.5 High performance anion exchange with pulsed amperometric detector (HPAE-PAD).

HPAE-PAD has advantages like high sensitivity in which small amount (picomole) can be detected, selectivity where in many sugars can be analyzed, robust in which many samples can be analyzed, specificity in which different sugars can be identified and quantified. Simplicity in samples preparation in which samples do not need to be derivatized, whereas the disadvantage of this method is the high cost, complexity, samples need to be diluted a lot and that might be create an error and finally technical issues that may happened suddenly (Dean, 1978, Southgate *et al*., 1978, Hall, 2003).

HPAE-PAD seemed to be suitable for the detection of soluble sugars in cereals because the values agreed with the amount reported by McCance & Widdowson’s The Composition of

foods dataset. On the other hand, low detections were associated with legumes due to technical issues with the machine that affected the intensity of sugars detection.

The amount of soluble sugars in beans and chickpeas in this study were slightly high compared to the reported values in by Menezes *et al.* (2009), while the lentils and white bread were in agreement with the same study although same method of detection was used HPAE-PAD but different column (Carbopac PA1), lower eluent concentration (NaOH 18nM) and higher flow rate more than 0.4 ml/min.

The values obtained with HPAE-PAD for cereals were in agreement with McCance & Widdowson's The Composition of foods dataset. As mentioned previously, poor detection was found in legumes due to issues with the HPLC.

4.4.2 Calculating av.CHO in compositional database

Determination of CHO by difference is a common method in food composition databases. In McCance and Widdowson's the composition of foods integrated dataset the av.CHO and starch content of the foods were calculated in breakfast cereals by difference rather being directly analysed. Whereas for the other food the av.CHO was calculated to by summation the analyzed total starch and the analyzed total soluble sugar in foods.

Nevertheless, it is important to quantify av.CHO in food experimentally and calculation methods may lead to accumulation of experimental errors arising from the determination of the other food components (Menezes *et al*., 2009). For instance, calculation may overestimate the av.CHO content particularly in food rich in RS (Granfeldt *et al*., 2006). Moreover, studies found that the calculation methods are not sufficient for consumers since the physiological effect of each carbohydrate fraction is different between constituents

(FAO/WHO, 1998, Greenfield and Southgate, 2003, Menezes *et al*., 2009)

4.4.3 Statistical analysis

One way ANOVA was conducted to compare the three methods. There were not significant differences between the three methods for measuring the av.CHO. Hence the selection was based on the lowest standard error and in this case HPAE-PAD was the selected method for measuring CHO in foods. However, because there was an issue with detection using this approach, PS was the second option for av.CHO because it is simple and non-reducing sugars can be detected.

4.4.4 Limitations

The amount of soluble sugars and av.CHO especially in legumes were poorly detected due to issues with the machine. Because of the limited time more food need to be used to evaluate the av.CHO and more food matrixes like mixed food need to be tested to find out the appropriate methods for certain foods. In this study, the amount of soluble sugars and av.CHO were measured only in one batch or in one variety so may explain the disagreement in the literatures (Food Standards Agency, 2002, Menezes *et al*., 2009).

4.5 Conclusion

Due to the various analytical methods available to analyse av.CHO, it is important to standardize the method to measure the av.CHO extraction (FAO/WHO, 1998). The optimized av.CHO conducted in the previous chapter was suitable for cereals as well as legumes.

It has been found that regardless the advantageous outcomes from using HPAE-PAD, the PS colorimetric method in this study provided better and more robust detection of soluble sugars and av.CHO. Composition information on soluble sugars and av.CHO from this chapter will be used in the next chapters.

5 Chapter five: estimation of GI from single and mixed foods using statistical analysis

5.1 Introduction

In 1981 Jenkins *et al.* introduced the concept of GI and classified CHO based on their physiological effects (Jenkins *et al*., 1981). Blood sugar response varies according to the type of food consumed and there are many factors affecting the blood sugar response. These factors include: types of CHO, fat, protein, and processing which food has been subjected to (e.g. extrusion) (see Section 1.1.4).

With the increased number of foods consumed by the population due to increased product development it is unfeasible to create a complete food database, and in particular those that contains GI values (Schakel *et al*., 2008, Aston *et al*., 2010, Dodd *et al*., 2011). The measurement of GI for every single food is not practical because it is time-consuming, costly, and requires human subjects with consideration of ethical and logistical factors. Additionally, variation within and across individuals of different population will pose further considerations.

Moreover, using the international table for GI and GL might be problematic because the information is gathered from different publications and from different countries (Atkinson *et al*., 2008). There is no internationally agreed standard protocol used in compiling the table with most of the studies using different numbers of subjects and different food references including: glucose, white bread, rice, potato, wheat, arepa, and barley bread (Atkinson *et al*., 2008). Finally and most importantly there is no standard method for measuring av.CHO in foods.

In addition, in a normal consumption pattern most foods are eaten as a meal rather than single foods such as sandwiches, soups and breads, with limited understanding in testing all the effects of all components together due to the difficulties in measuring GI *in vivo.*

On the other hand, *in vitro* starch digestion seeks to mimic the human digestive system and simulate the starch digestion with comparable rate and amount of glucose released and it is time-saving, cost-effective and does not require human subjects, however these studies are mostly for single food items and not multi-component meals (Germaine *et al*., 2008, Monro *et al*., 2010, Ballance *et al*., 2013).

Summation models are another approach established in the 80s to calculate total GI of a meal through GI values of the component foods within the meal taking into account the amount of its av.CHO content (Wolever *et al*., 1985), the equation bellow illustrate the calculation.

$$\text{Mean GI of the meal} = \frac{(\sum \text{of GI}_{\text{FOOD (n)}} \times \text{amount of av.CHO}_{\text{FOOD (n)}})}{\text{Total amount of av.CHO of the meal}}$$

Equation 5.1: summation model used to calculate the GI of the multi-component food. (n) represents the number of foods.

However, one of the main limitations is that food components such as fat and fibre are not taken into consideration (Dodd *et al*., 2011).Therefore, it is crucial to have standardized, reproducible approaches to determine the GI of multi-component foods.

5.2 Aim

The aim of this study is to assess the prediction of the GI in cereals, legumes and mixed dishes from macronutrient composition using statistical analysis (Pearson correlation and linear regression).

5.2.1 Objectives

1. Investigate the correlation between GI and published macronutrients in food obtain from food compositional table using Pearson correlation.
2. Generate prediction models from published macronutrient contents of the food using linear regression.
3. Investigating the correlations between the GI and previously measured av.CHO content of the foods
4. Apply the prediction models to predict GI of the unknown food.

5.3 Results

5.3.1 Food composition and description

Cereals /100g (n=24)				Legume /100g (n=16)		
	Mean	**Max**	**Min**	**Mean**	**Max**	**Min**
GI[2]	65.0±14.0	95.0	40.0	38.9±11.2	63.0	21.0
Protein	7.7±3.8	15.2	0.9	7.2±1.2	8.8	4.8
Fat	2.3±1.9	8.4	0.2	0.8±0.7	2.9	0.2
CHO	59.2±24.0	94.6	21.1	16.6±3.9	27.4	8.6
Soluble Sugar	12.2±14.0	82.5	21.0	13.4±3.4	18.0	4.7
Starch	46.9±17.2	44.0	0.0	2.3±1.9	5.9	0.4
NSP[4]	3.9±5.2	24.5	0.1	4.2±1.5	6.7	1.0

Table 5.1: Mean, maximum, minimum GI and macronutrients content of foods per 100g.

Forty starchy food (24 cereals & 16 legumes) samples were used to assess the prediction of GI from food composition tables using statistical models. Table 5.1, shows the mean, maximum, and minimum GI and associated published macronutrients obtained from Food Standards Agency (2002). The average GI for the cereal foods was 65 and rang from 40-95. According to the FAO/WHO (1998) the average GI therefore is categorized as medium GI. The average protein and fat content in cereals were 7.3 g and 2.3 g respectively per 100 g while the average total content of CHO, starch and soluble sugar were found to be 59.2, 46.9, and 12.3 g respectively. The average NSP content was 3.9 g.

The average GI for the legume foods was 39 ranged from 36-21. According to the (FAO/WHO, 1998) the average GI is therefore categorized as low. The average protein and fat content in legumes were 7.2g and 0.8g respectively per 100g, while the average total content of CHO, starch and soluble sugar were found to be 16.6, 13.4, and 2.3g respectively and the average NSP content was 4.2g.

Also, nutri-grain was excluded from the generating the prediction models because the amount of av.CHO is calculated incorrectly (shaded row in table 5.2).

5.3.2 GI selection

The GI values were extracted from the international table of GI and GL and the values were selected against glucose reference (Atkinson *et al*., 2008) .

Food/ 100g	GI	Protein (g)	Fat (g)	CHO (g)	Starch (g)	soluble sugar (g)	NSP (g)
Cereals							
All-Bran	44	13	4	*48.5*	*28.6*	19.9	24.5
Bagels, white & plain	69	10	1.8	**57.8**	51.3	6.5	2.4
Bran Flakes	62	10.2	2.5	*71.2*	*48.4*	22.8	13
Brown rice, boiled	63	2.6	1.1	**32.1**	31.6	0.5	0.8
Coco pops	77	4.5	2.5	*91.5*	*49.5*	42.0	0.6
Corn Flakes	81	7.9	0.9	*89.6*	*81.4*	8.2	0.9
Crunchy Nut Corn Flakes	74	7.4	3.5	*91.6*	*53.9*	37.7	0.8
Frosties	55	5.3	0.6	*94.6*	*50.6*	44	0.6
Fruit 'n Fibre	65	9	5	*72.5*	*49.5*	23	7
Hamburger buns, Whole-meal	62	9.1	5	**48.8**	46.6	2.2	1.5
Muesli, Swiss style	57	9.8	5.9	*72.2*	*46.0*	26.2	6.4
Nutri-grian	66	4.1	8.4	*71.5*	*35.0*	30.7	3.0
Pita bread, white	68	9.1	1.3	**55.1**	52.2	3.0	2.4
Puffed Wheat	80	14.2	1.3	**67.3**	67.0	0.3	5.6
Rice Krispies	95	6.1	1	**92.9**	82.5	10.4	0.7
Rice noodles, cooked	40	0.9	0.2	**24.9**	23.9	0.6	0.8
Spaghetti, white, boiled	47	3.6	0.7	**22.2**	21.7	0.5	1.2
Spaghetti, wholemeal, boiled	47	4.7	0.9	**23.2**	21.9	1.3	3.5
Special K	62	15.3	1	**81.6**	63.8	17.8	2
Weetabix	71	11.2	2.7	**75.5**	70.6	4.9	9.7
White bread, average	85	8.4	1.9	**49.3**	46.7	2.6	1.5

Food/ 100g	GI	Protein (g)	Fat (g)	CHO (g)	Starch (g)	soluble sugar (g)	NSP (g)
White bread, toasted	60	9.7	2	**56.2**	52.1	4.1	2.3
White rice, easy cook, boiled	49	2.6	1.3	**30.9**	30.9	0.0	0.1
White rice, glutinous, boiled	91	2.0	0.2	**21.1**	21.0	0.1	0.8
Wholemeal bread, average	70	9.4	2.5	**42**	39.3	2.8	5
Legumes							
Baked beans, canned in tomato sauce	40	4.8	0.6	**15.1**	9.3	5.8	3.5
Baked beans, canned in tomato sauce, re-heated	57	5.2	0.6	**15.3**	9.4	5.9	3.7
Blackeye beans, dried, boiled in unsalted water	41	8.8	0.7	**19.9**	18	1.1	3.5
Butter beans, canned, re-heated, drained	34	5.9	0.5	**13**	10.9	1.1	4.6
Butter beans, dried, boiled in unsalted water	30	7.1	0.6	**18.4**	15.6	1.5	5.2
Chick peas, canned, re-heated, drained	23	7.2	2.9	**16.1**	15.1	0.4	4.1
Chick peas, whole, dried, boiled in unsalted water	38	7.7	2.1	**27.4**	15.9	4.8	4.3
Haricot beans, dried, boiled in unsalted water	35	6.6	0.5	**17.2**	15.8	0.8	6.1
Red kidney beans, canned, re-heated, drained	38	6.9	0.6	**17.8**	12.8	3.6	6.2
Red kidney beans, dried, boiled in unsalted water	37	8.4	0.5	**17.4**	14.5	1	6.7
Lentils, green and brown, whole, dried, boiled in salted water	33	8.8	0.7	**16.9**	15.9	0.4	3.8
Lentils, red, split, dried, boiled in unsalted water	21	7.6	0.4	**17.5**	16.2	0.8	1.9
Marrowfat peas, canned, re-heated, drained	47	6.9	0.8	**17.5**	13.9	2	4.1

Food/ 100g	GI	Protein (g)	Fat (g)	CHO (g)	Starch (g)	soluble sugar (g)	NSP (g)
Mung beans, dahl, whole, dried, boiled in unsalted water	37	7.8	1.1	**15.3**	14.1	2.8	3.0
Broad beans, canned, re-heated, drained	63	8.3	0.7	**12.7**	11.9	0.6	5.2
Peas, frozen, boiled in unsalted water	51	7.7	0.2	**8.6**	4.7	3.8	1.0

Table 5.2: GI, Portion size, and macronutrient content of foods per 100 g food. Values in *italic* were calculated by difference, whereas values in **bold** are calculated by adding (starch +sugars).

5.3.3 The effect of macronutrients content on GI

The nutrient composition of the foods and the amounts of each food contained in meals are shown in Table 5.2. In order to evaluate the relationship between GI and macronutrient content in food the Pearson correlation coefficient was determined for all foods in the group consisting of cereals & legumes. The correlation coefficient was calculated for macronutrients present in food per 100g in food; any p value ≥ 0.05 indicates that there is no correlation between the macronutrient and the GI. The Table 5.3 **(shaded and bold**) showed the relationship between macronutrient content in food of GI. Protein, fat, and NSP contents in food were the only components that did not correlate significantly with GI (Pearson coefficient= 0.11, 0.23 and --0.19, p= 0.51, 0.14, and 0.23) respectively.

Significant positive correlations were found between the GI and starch and soluble sugars content (Pearson coefficient =0.74 and 0.31 p= 0.00, 0.05 respectively) in mixed foods (Figure 5.1). For the cereals (Table 5.4), starch was the only macronutrient that correlated significantly with GI (Pearson coefficient= 0.57, p= 0.03) respectively. Similarly, for the legumes (Table 5.5) only starch correlated significantly with GI Pearson coefficient = -0. 56, p=0.24) (Figure 5.2 and 5.3).

Correlations of mixed foods (n=40)		
		GI
GI	Pearson Correlation	1
	Sig. (2-tailed)	
Protein	Pearson Correlation	0.11
	Sig. (2-tailed)	0.51
Fat	Pearson Correlation	0.23
	Sig. (2-tailed)	0.14
Total starch	**Pearson Correlation**	**0.74**
	Sig. (2-tailed)	**0.00**
Soluble sugars	**Pearson Correlation**	**0.31**
	Sig. (2-tailed)	**0.05**
NSP	Pearson Correlation	-0.19
	Sig. (2-tailed)	0.23

Table 5.3: Pearson correlation between GI and macronutrients content of mixed foods per 100g (n=40)

Correlations of cereal foods (n=24)		
		GI
GI	Pearson Correlation	1
	Sig. (2-tailed)	
Protein	Pearson Correlation	0.10
	Sig. (2-tailed)	0.62
Fat	Pearson Correlation	-0.07
	Sig. (2-tailed)	0.73
Starch	**Pearson Correlation**	**0.57**
	Sig. (2-tailed)	**0.00**
Soluble sugars	Pearson Correlation	0.01
	Sig. (2-tailed)	0.96
NSP	Pearson Correlation	-0.28
	Sig. (2-tailed)	0.16

Table 5.4: Pearson correlation between GI and macronutrients content of cereal foods per 100g (n=24).

Correlations legumes foods (n=16)		
		GI
GI	Pearson Correlation	1
	Sig. (2-tailed)	
Protein	Pearson Correlation	-0.08
	Sig. (2-tailed)	0.76
Fat	Pearson Correlation	-0.31
	Sig. (2-tailed)	0.24
Starch	**Pearson Correlation**	**-0.56**
	Sig. (2-tailed)	**0.02**
Soluble sugars	Pearson Correlation	0.39
	Sig. (2-tailed)	0.13
NSP	Pearson Correlation	-0.02
	Sig. (2-tailed)	0.95

Table 5.5: Pearson correlation between GI and macronutrients content of legume foods per 100g (n=16).

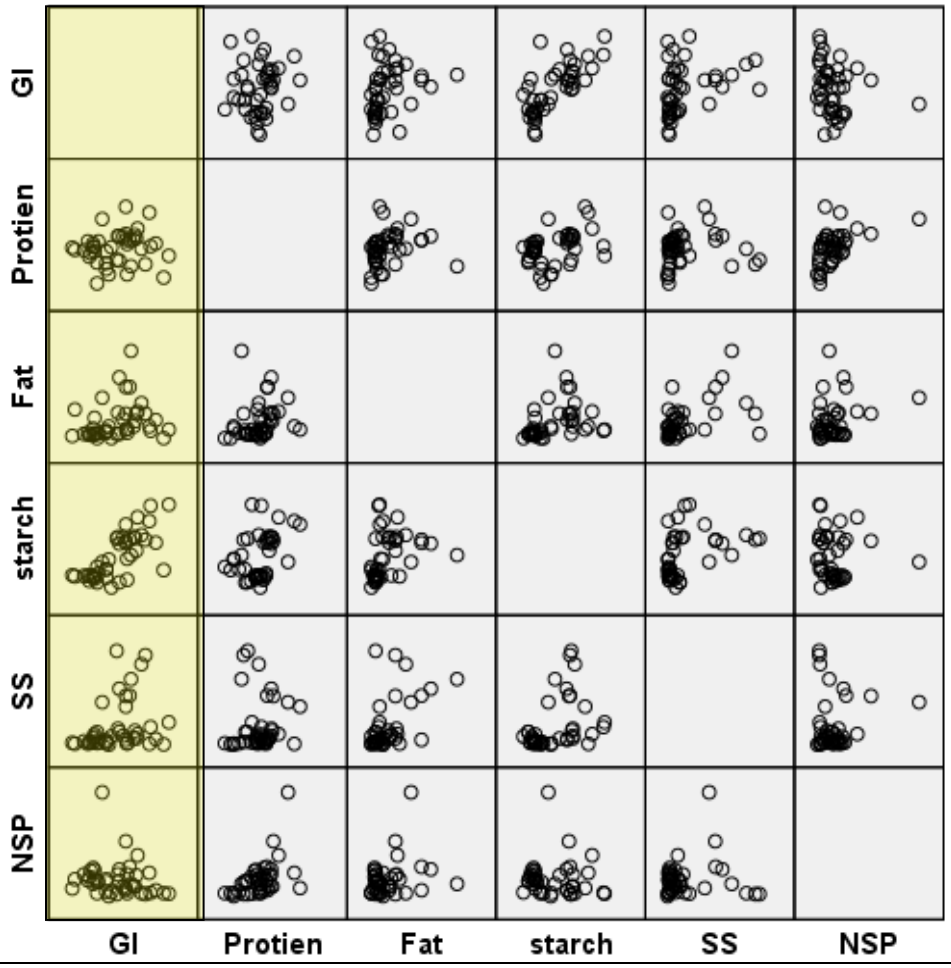


Figure 5.1: scatter plot of the relationship of protein, fat, starch, soluble sugars (SS) Soluble sugar, and NSP content to GI per 100g in 40 mixed foods.

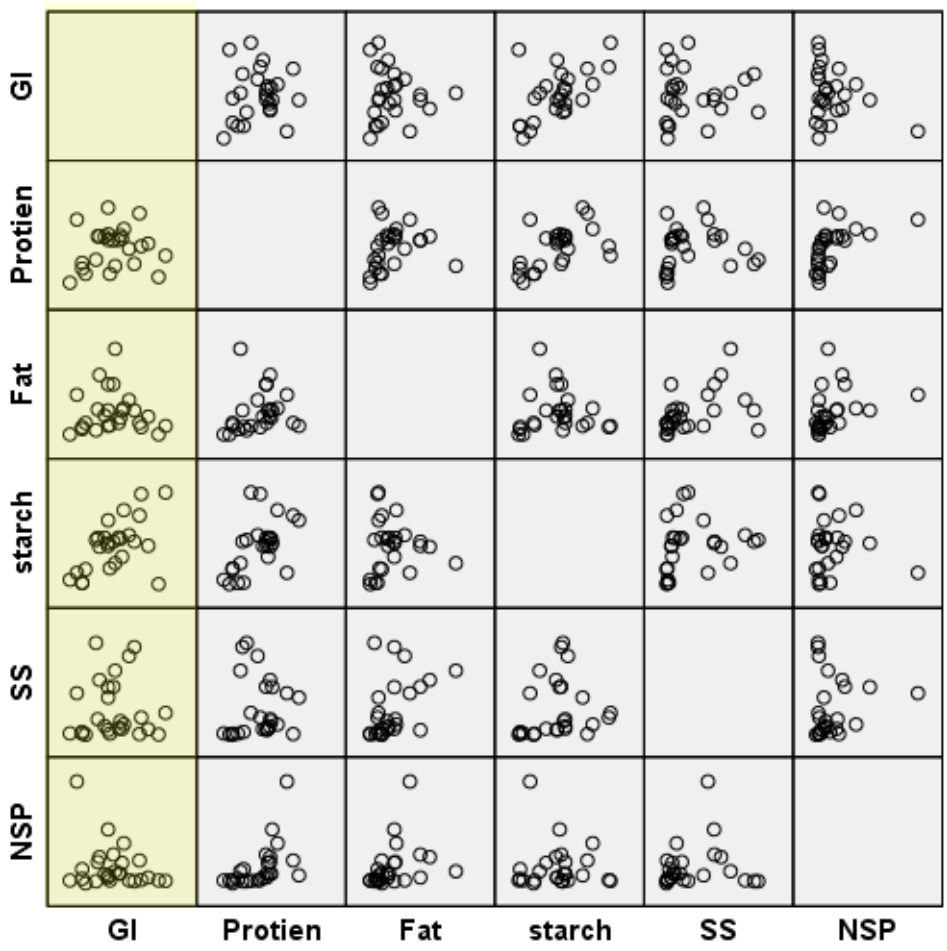


Figure 5.2: scatter plot of the relationship of protein, fat, starch, soluble sugars (SS) Soluble sugar, and NSP content to GI per 100g in 24 cereal foods.

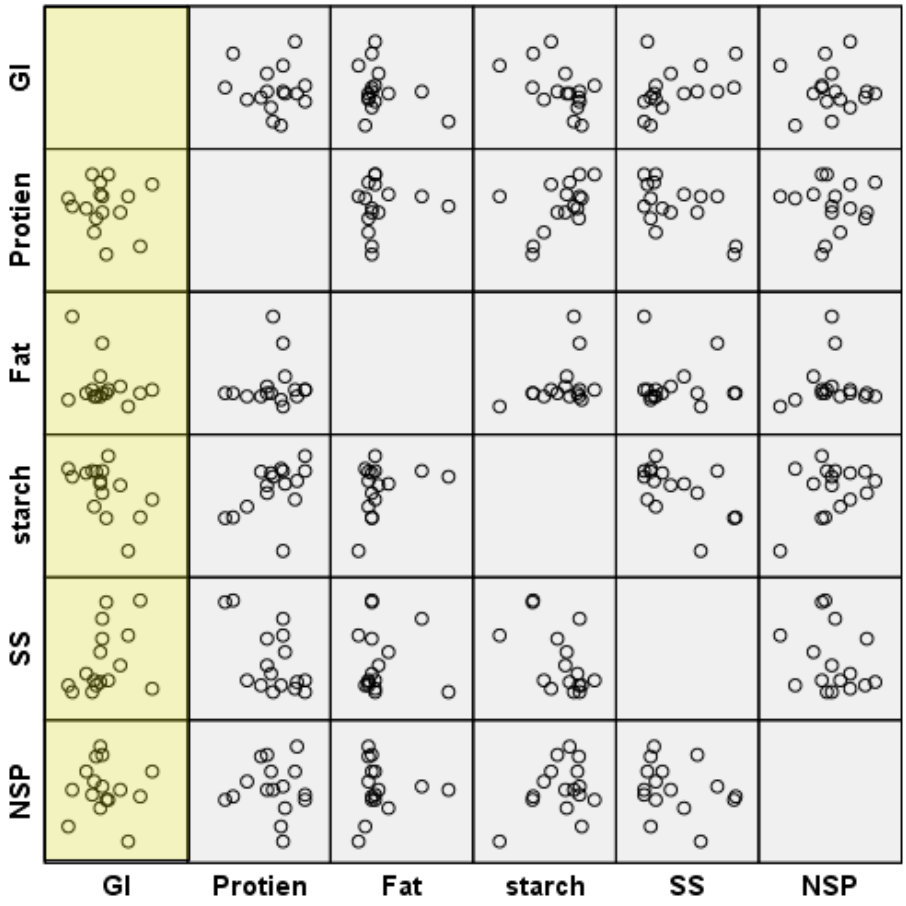


Figure 5.3: scatter plot of the relationship of protein, fat, starch, soluble sugars(SS) Soluble sugar, and NSP content to GI per 100g in 16 legume foods.

5.3.3.1 GI prediction models generation

The prediction models were divided into three groups: mixed foods (cereal + legume foods), cereals, and legumes models, and multiple linear regression was applied to generate the prediction models. Four prediction models were generated using 42 mixed food samples (Table 5.6). Protein and starch were found to be always present in the entire models which mean they are the only significant predictors for GI (bold font). The constant seems to be similar in all the models (homogeneous).

On the other hand, 26 cereal foods were used to generate 5 prediction models where starch only was found to be always present in all the models and is the only significant predictor for GI (bold font), and like the mixed food models the constant seems to be similar in all the models (homogeneous) (Table 5.7).

16 legume foods were used to generate 5 prediction models. Like the cereal model, starch was found to be always present in models and it was the only significant predictor (bold font), but the constant in legume models seems to be varied in the entire models (heterogeneous) (Table 5.8). As shown in the prediction models starch is the main contributor to GI predictions.

Prediction models mixed foods (n=40)			
	Model	**Coefficients**	***P* value.**
1	**Constant**	41.01	0.00
	Protein	-1.21	0.17
	Fat	0.42	0.76
	Starch	0.70	0.00
	Soluble sugar	-0.04	0.85
	NSP	-0.22	0.72
2	**Constant**	40.94	0.00
	Protein	-1.18	0.17
	Fat	0.31	0.80
	Starch	0.69	0.00
	NSP	-0.23	0.69
3	**Constant**	41.13	0.00
	Protein	-1.20	0.15
	Starch	0.70	0.00
	NSP	-0.19	0.74
4	**Constant**	**41.18**	**.00**
	Protein	**-1.35**	**.05**
	Starch	**0.71**	**.00**

Table 5.6: Prediction models for mixed food (n=40). *P values* represent the significance of the corresponding coefficients.

Prediction models for cereal foods (n=24)			
	Model	**Coefficients**	***P* value.**
1	**Constant**	45.88	0.00
	Protein	-0.823	0.45
	Fat	0.60	0.69
	Starch	0.58	0.01
	Soluble sugar	-0.11	0.58
	NSP	-0.36	0.59
2	**Constant**	46.85	0.00
	Protein	-0.76	0.47
	Starch	0.56	0.01
	Soluble sugar	-0.08	0.67
	NSP	-0.32	0.625
3	**Constant**	46.52	0.00
	Protein	-0.67	0.51
	starch	0.54	0.01
	NSP	-0.39	0.53
4	**Constant**	45.49	0.00
	Protein	-1.10	0.15
	starch	0.60	0.00
5	**Constant**	**43.27**	.000
	Starch	**0.47**	.003

Table 5.7: Prediction models for cereal foods (n=24). *P values* represent the significance of the corresponding coefficients.

Prediction models for legume foods (n=16)			
Model		**Unstandardized Coefficients**	***P* value**
1	**Constant**	21.69	0.41
	Protein	4.43	0.14
	Fat	-2.38	0.55
	Starch	-2.05	0.07
	Soluble sugar	2.25	0.23
	NSP	2.29	0.24
2	**Constant**	22.53	0.38
	Protein	4.47	0.13
	Starch	-2.28	0.03
	Soluble sugar	2.09	0.25
	NSP	2.38	0.21
3	**Constant**	46.52	0.00
	Protein	-0.67	0.51
	Starch	0.54	0.01
	NSP	-0.39	0.53
4	**Constant**	45.49	0.00
	Protein	-1.10	0.15
	Starch	0.60	0.00
5	**Constant**	**43.27**	**0.00**
	Starch	**0.47**	**0.00**

Table 5.8: Prediction models for legume foods (n=16). *P values* represent the significance of the corresponding coefficients.

5.3.4 Application of prediction models with calculated CHO

Evaluation of the GI prediction models was conducted using two foods with known GI and was not involved in model generation while white bread was used as reference food, these food compared to the published ones from the international table of GI and GI (Atkinson *et al*., 2008). As shown in Table 5.9. The predicted GI for both foods was within the acceptable range of differences (differences ≤10) (Brouns *et al*., 2005).

Food	Published GI	Predicted GI	Differences units (%error)
Lentil soup meal	57	56[1]	1 (2%)
Lentil soup	40	38[2]	2 (5%)
White bread	71	76[3]	5 (%)

1 GI predicted from model 4 for the mixed foods =

41.18 - (1.35 Protein) + (0.71 Starch)

2 GI predicted from model 5 for the legume foods =

43.27 + (0.47 Starch)

3 GI predicted from model 1 for the cereals foods =

63.49 –(1.83 Starch)

Table 5.9:Comparison between predicted GI values and published GI values (Atkinson *et al*., 2008). Data are calculated from the equations above.

5.3.5 Generation of the prediction models using measured CHO

The content of total CHO in McCance and Widdowson's the composition of foods integrated dataset was calculated as mentioned previously. The soluble sugar and av.CHO content were analysed in the previous chapter and PS was the selected method for measuring av.CHO.

5.3.5.1 GI prediction models using measured data (CHO)

Five prediction models were generated using multiple linear regression and 16 mixed food samples (Table 5.10). Fat and starch were found the only significant predictors for GI (**shaded bold**). The constant seems to be similar in all the models (homogeneous).

On the other hand, 7 cereal foods were used to generate just 2 models but the fat was found to be the only significant predictors of GI (**shaded bold**), and like the mixed food models the constant seems to be similar in all the models (homogeneous) (Table 5.11).

9 legume foods were used to generate 5 prediction models. Unlike the other models, NSP was found to be the only significant predictor (**shaded bold**) although NSP did not significantly correlate with GI; also the constant in legume models seem to be varied in the entire models (heterogeneous) (Table 5.12).

Prediction models mixed foods (n=16)			
	Model	**Coefficients**	***P* value.**
1	**Constant**	12.81	0.55
	Protein	1.45	0.67
	Fat	9.51	0.03
	Starch	0.68	0.00
	Soluble sugar	0.23	0.64
	NSP	-1.0	0.51
2	**Constant**	21.56	0.00
	Fat	9.94	0.01
	Starch	0.72	0.00
	Soluble sugar	0.08	0.81
	NSP	-0.47	0.59
3	**Constant**	21.11	0.00
	Fat	10.25	0.01
	Starch	0.73	0.00
	NSP	-0.44	0.59
4	**(Constant)**	**19.65**	**0.00**
	Fat	**9.57**	**0.00**
	Starch	**0.75**	**0.00**

Table 5.10: Prediction models for mixed foods using measured av.CHO (n=16). *P values* represent the significance of the corresponding coefficients.

Prediction models cereal foods (n=7)			
	Model	**Coefficients**	***P* value.**
1	**Constant**	-148.66	0.15
	Protein	30.13	0.08
	Fat	-0.94	0.72
	Starch	-0.18	0.19
	Soluble sugar	3.41	0.09
	NSP	-13.45	0.08
2	**Constant**	**-160.62**	**0.02**
	Protein	**31.27**	**0.01**
	Starch	**-0.16**	**0.02**
	Soluble sugar	**3.53**	**0.01**
	NSP	**-13.93**	**0.01**

Table 5.11: Prediction models for cereal foods using measured av.CHO (n=7). *P values* represent the significance of the corresponding coefficients.

Prediction models for legume foods (n=9)			
Model		**Coefficients**	***P* value**
1	**Constant**	7.76	0.89
	Protein	0.91	0.89
	Fat	2.37	0.75
	Starch	0.77	0.62
	Soluble sugar	-8.23	0.72
	NSP	3.24	0.26
2	**Constant**	14.89	0.26
	Fat	1.54	0.67
	Starch	0.92	0.32
	Soluble sugar	-10.95	0.27
	NSP	3.42	0.12
3	**Constant**	15.33	0.20
	Starch	1.04	0.20
	Soluble sugar	-13.25	0.09
	NSP	3.63	0.06
4	**Constant**	28.91	0.00
	Soluble sugar	-7.00	0.22
	NSP	2.16	0.13
5	**Constant**	26.97	0.00
	NSP	1.88	0.19

Table 5.12: Prediction models for legume foods using measured av.CHO (n=9). *P values* represent the significance of the corresponding coefficients.

5.3.6 Application of prediction models with measured CHO

GI of the three tested foods was predicted using the models generated from the macronutrient content and analysed av.CHO and soluble sugars. One way ANOVA was used to investigate the differences between the two approaches compared to the published GI and there were no significant differences between them (Figure 5.4).

Moreover, GI of 16 food was predicted using the same models for the analysed CHO from chapter 4, and as shown in Figure 5.4 the measured av.CHO data improved the predicted GI values and the differences were within the acceptable range (≤10), plus there were no significant associations between the predicted GI values using analysed av.CHO and the published GI values (P=0.01) (Figure 5.5).

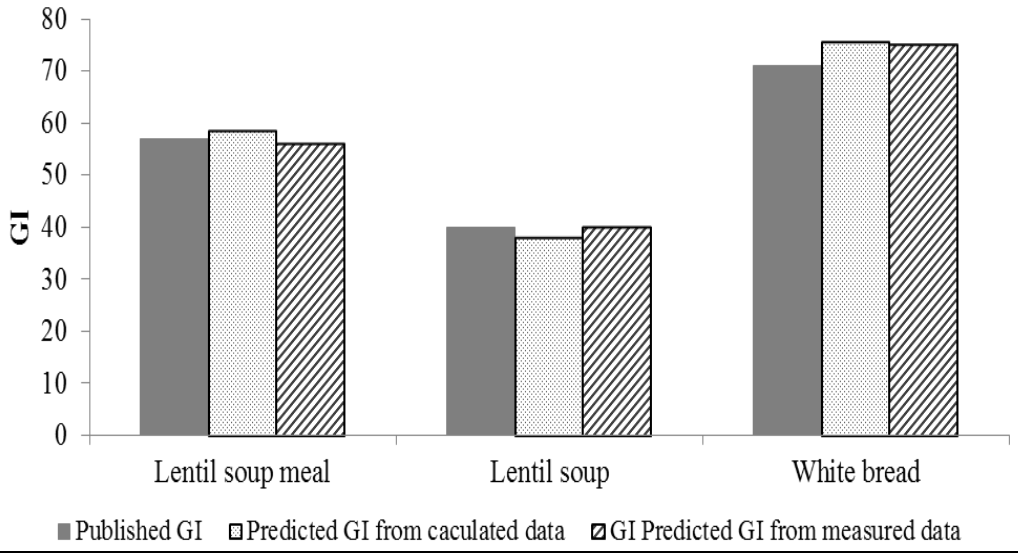


Figure5.4: GI values predicted in foods using analyzed av.CHO and calculated av.CHO from McCance and Widdowson's the composition of foods integrated dataset. The first bars are published GI values from the international GI and GL, the second bars are predicted GI values using av.CHO from McCance and Widdowson's the composition of foods int1egrated dataset, and the ;last bar are predicted GI values using analyzed av.CHO.

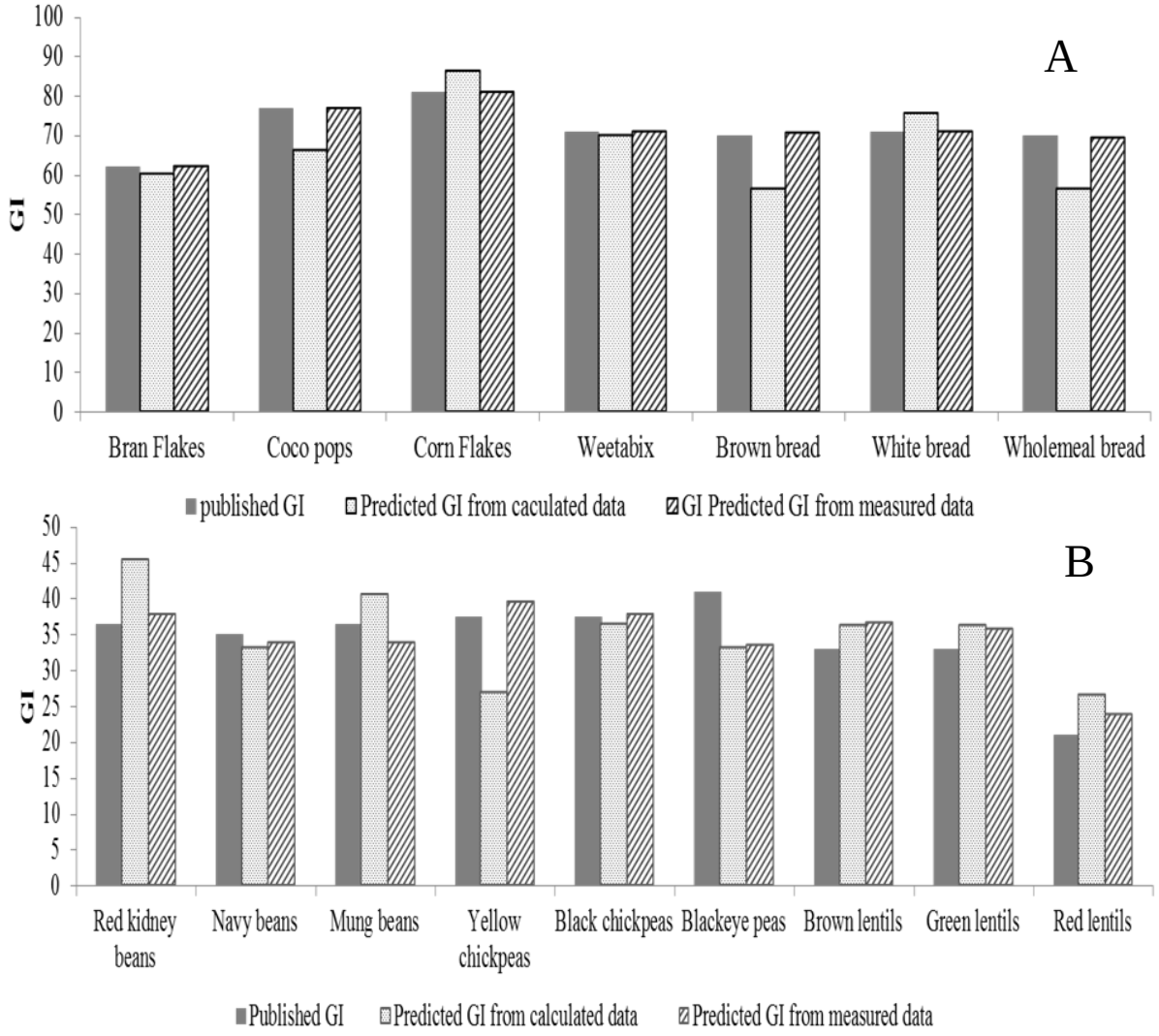


Figure5.5: GI values predicted in cereals (A) and legumes (B) using analyzed av.CHO and calculated av.CHO from McCance and Widdowson's the composition of foods integrated dataset. The first bars are published GI values from the international GI and GL, the second bars are predicted GI values using av.CHO from McCance and Widdowson's the composition of foods integrated dataset, and the ;last bar are predicted GI values using analyzed av.CHO.

5.4 Discussion

5.4.1 The effect of macronutrients content on GI

Pearson correlation was conducted to measure the linear correlation between two variables were value between +1 indicate positive correlation, 0 indicate no correlation and −1 indicate negative correlation, also any significant level will be $P \leq .0.05$.

A number of investigations showed that macronutrient contents in the foods are factors affecting the GI. Three positive relationships were found between total CHO, starch and soluble sugars, starch and GI, the results for the total soluble sugars are in agreement with Jenkins *et al.* (1981) who found that sugar contents are related to the GR. Even though sugars (e.g. glucose, fructose, and galactose) differ in their absorption in the small intestine; to our knowledge glucose is the only sugar that affects the GR positively unlike fructose and galactose.

In this study, the result did not agree with Wolever (1990), Trout *et al.* (1993) and Widanagamage *et al.* (2009) and NSP did not correlate significantly with GI except with legumes. The NSP are known for their role in lowering the GR by increasing the gastric emptying (Nishimune *et al*., 1991). Although the results did not show significant correlation between protein and GI, they contributed to the prediction models of mixed foods and supported the fact that protein affect GR and GI negatively in the human gastrointestinal track through stimulation several hormones (GLP, GIP-1, CCK and insulin) as mentioned previously in section 1.1.4 (Wolever, 2006).

Like the protein, fat is also considered a macronutrient that has been shown to lower GI and the GR in the blood through delaying the gastric emptying as a result of stimulation

several hormones mentioned in section 1.1.4 (Flint *et al*., 2004, Widanagamage *et al.*, 2009).

However, the no significant correlation between these two components might be due to the lower content per portion used in the prediction (100 g) in which the average protein content in the foods used in this study was 8.3 g while the average fat content was 2.6 g per 100 g. Wolever and Bolognesi (1996) have shown that the amount of protein and fat should be larger than (50 g) in a normal meal to have an impact upon the GR in the blood.

5.4.1.1 GI prediction models using multiple linear regression

The present study was adapted from Urooj and Puttaraj (2000) with modification such as the selection of foods. In Urooj and Puttaraj (2000) study 6 multi-component south Asian foods where used to assess the prediction of GI from nutrient composition. To our knowledge, the use of food that is specific for certain populations might be useful by reducing the uncertainty levels (low data extrapolation), however, it also may limit the application of the study worldwide (Lin *et al*., 2012). Therefore the present study was not population specific. Also the present study assessed the predictions using 40 multi-component foods of calculated (av.CHO) data and 16 measured (av.CHO) multi-component foods.

Also in the Urooj and Puttaraj (2000) study, the energy (KJ) was involved in the generation of the prediction model; however soluble sugars were not analyzed nor involved in their study. It is remain unclear whether energy may affect the GR or not therefore more investigation regarding the effect of energy on GR must be conducted.

According to Jenkins *et al.* (1981), soluble sugars are could have an effect on GR. In the present study starch and fat were the significant predictors that present in all models.

The number of foods consumed by the population keep increasing and even with a large food database it is not feasible for data (especially for GI database) to cover the entire range of foods (Schakel *et al*., 2008). The aim of this study was to find a practical approach regarding measuring GI using nutrient compositions rather than using human subjects and blood samples. These models may provide deeper understanding of the effects of macronutrients on GI particularly, the effect of cooking, and storage, especially when the food component is measured.

The present study may indicate that GI of food could be predicted from its nutrient content and agreeing with published data calculated by using a variety of independent analysis. Additionally, evaluation of reported values indicates difference methodology regarding measuring CHO in which measured CHO values are important in the predicted GI.

5.5 Limitations

We found it difficult to compare this work with Urooj and Puttaraj (2000) because their study was population-specific, unlike the present study.

Moreover, more food samples need to be tested that to improve the prediction models. Because of the limited time analysis of food component was not conducted and only av.CHO was analyzed. Analysis of the anti-nutrients (e.g. polyphenols) might be useful and may provide deeper understanding of their effect on GR.

5.6 Conclusion

The results indicated that measuring the av.CHO improved the prediction models and provided better estimation. Also, the results indicated that the models might be a useful approach for measuring GI without using human subjects. The models were within the acceptable ranges, nevertheless, more unknown foods (multi-component) need to be tested and *in vitro* validation is required to make sure that the models are robust.

6 Chapter six*: in vitro* starch digestion using pancreatic amylase

6.1 Introduction

Multi-component foods were used in the previous chapter to generate models that can estimate GI statistically without using human subjects or collecting blood samples. However, these models need to be validated with *in vitro* experiments to ensure the usefulness of their estimations.

The *in vitro* method can be used to classify carbohydrates into two groups: rapidly digestible carbohydrates and slowly digestible carbohydrates based on the rate of starch hydrolysis (Englyst *et al*., 1999). *In vitro* starch digestion can be conducted in two ways: non-restricted and restricted. In non-restricted systems, samples are kept in a closed the tube with digestive enzymes for 120 min, then the amount of glucose released is measured, whereas in the restricted system, samples are digested in a dialysis bag (13 cm length, 12000-18000 KD) and the amount of glucose diffused from the bag is measured. The dialysis bag is made of a semi-permeable membrane allowing the passage of low molecular weight molecules like salts and sugars through the pores.

In vitro methods have the advantages of being less expensive, cost-effective, and easy to conduct because they do not require subjects and ethical approval compared to *in vivo* methods (Germaine *et al*., 2008).

6.2 Aim

To verify the GI values predicted in the previous chapter (chapter 5).

6.2.1 Objective

To analyze the rate of *in vitro* starch digestion in non-restricted system of single and multi-components foods using pancreatic α-amylase.

To explore the variation of *in vitro* starch digestion using α-amylases from different origin.

To correlate the results from *in vitro* with the GI values predicted from the previous chapter.

6.3 Results

6.3.1 Non-restricted *in vitro* starch digestion

Non-restricted *in vitro* starch hydrolysis was adapted from (Germaine *et al*., 2008) with modifications. Starch was hydrolyzed with a mixture of enzymes (α-amylase, AMG) in sodium phosphate buffer at 37 ºC to mimic the digestion in the gastrointestinal tract and the amount of glucose released was measured by PS.

Moreover, the rate of non- restricted *in vitro* starch digestion using microbial enzymes (bacterial proteases, themostable α-amylase and fungal AMG) in MES/Tris buffer at 60º and 100 ºC respectively was also explored to find out the variation that may exist.

The two test foods that were used in this study are: lentil soup, lentil soup meal (lentil soup + white bread) and one reference food which is white bread. Av.CHO content of all the foods was analyzed experimentally in chapter 4 using PS (Table 6.1). The amount of starch digested was multiplied by 0.9 conversion factor (converted to anhydrous glucose) and data expressed as an average of four replicates and ± standard deviation. Figure 6.1 and 6.2 illustrate the steps of the non- restricted *in vitro* using pancreatic and microbial enzymes respectively.

Amount of av.CHO in foods (n=3) mean ±SD			
	Lentil soup	**Lentil soup meal[1]**	**White bread**
Av.CHO g/100g	17.4 ± 2.2	28.2 ±7.2	50.0± 2.9

[1] lentil soup and white bread (50:50 ratio of av.CHO in soup to bread)

Table 6.1: Av.CHO content in food used in this study. Data expressed as mean of the food (n=4) ±SD.

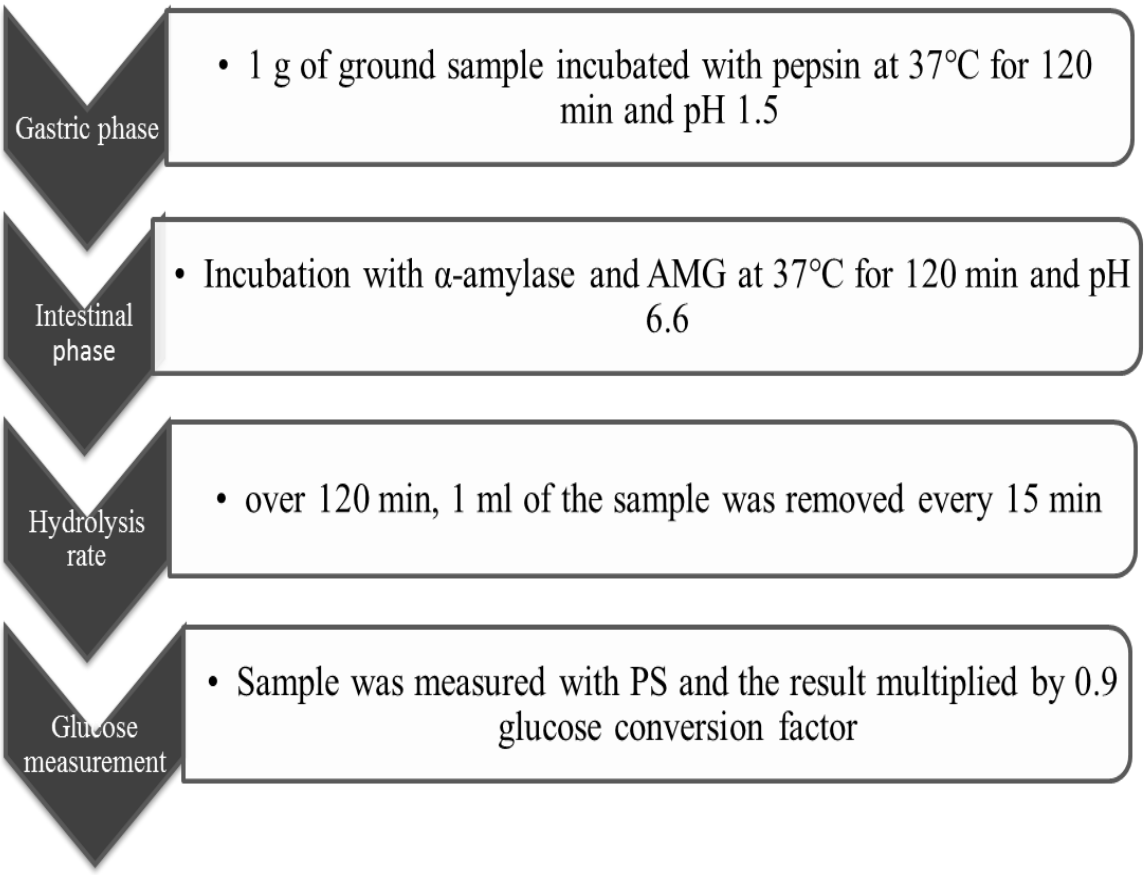


Figure 6.1: Flow chart illustrates the steps of the non-restricted *in-vitro* starch digestion method adapted from Germaine *et al*.(2008) using pancreatic enzymes in sodium phosphate buffer at body temperature.

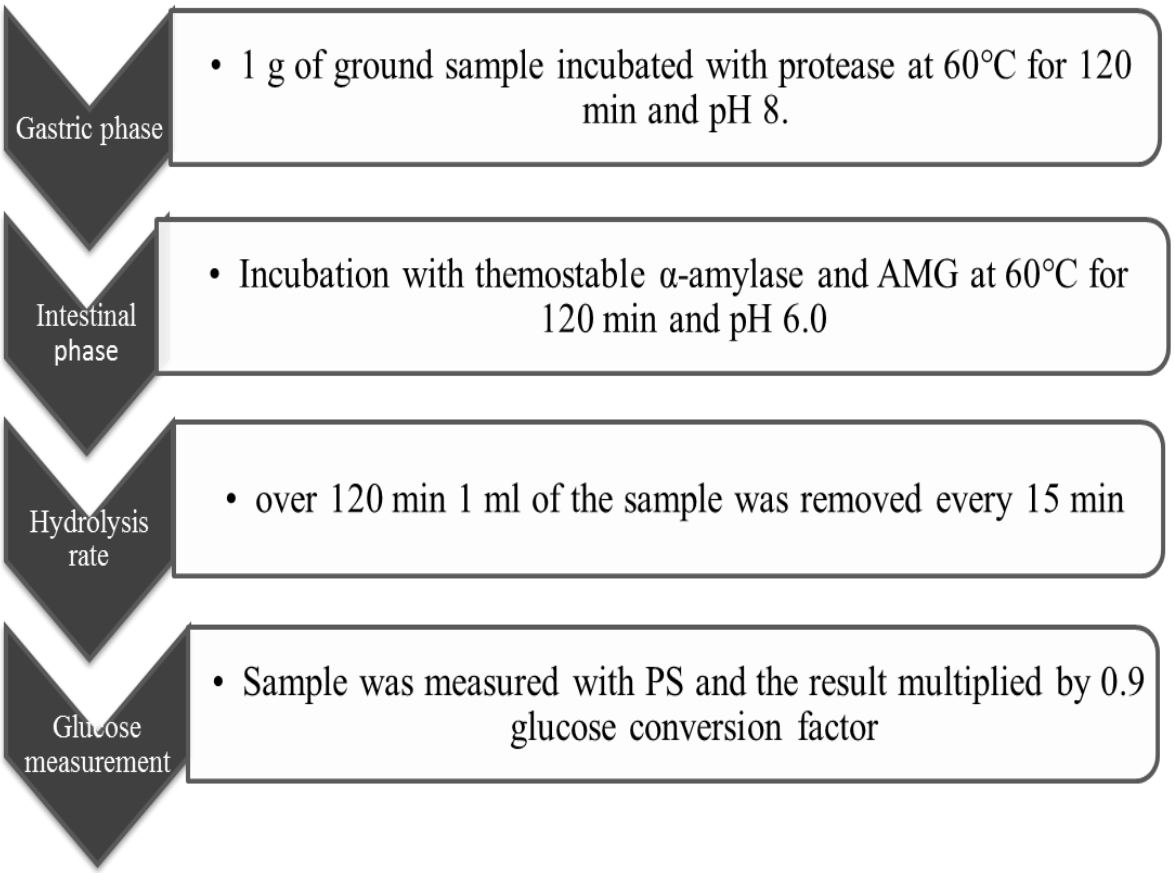


Figure 6.2: Flow chart illustrates the steps of non-restricted *in-vitro* starch digestion method adapted from Germaine *et al*. (2008) using microbial enzymes in and MES/Tris Buffer and high temperature.

6.3.1.1 *In vitro* starch hydrolysis with different enzymes origin

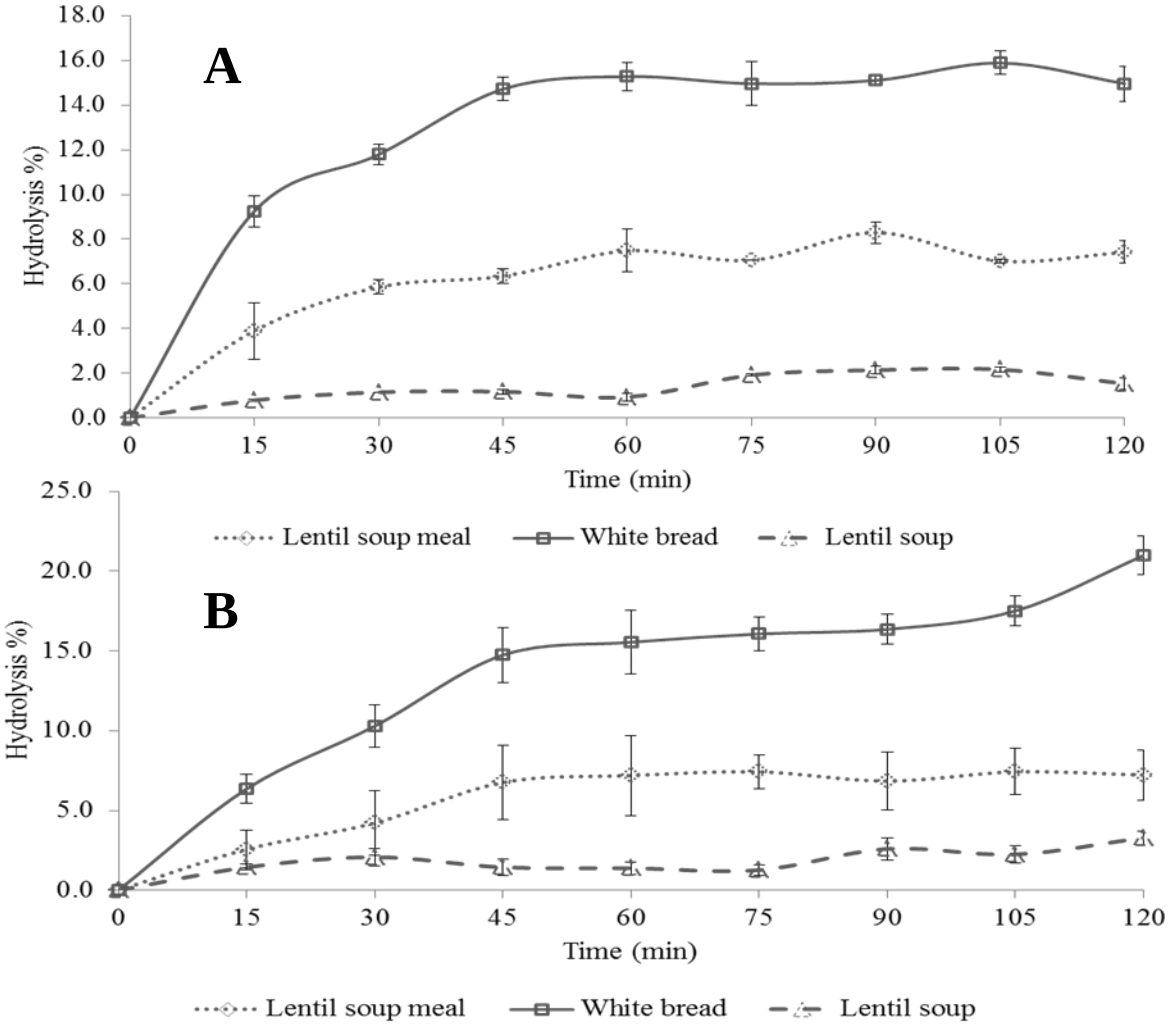


Figure 6.3; Starch hydrolysis curve after using pancreatic enzymes (A) and microbial enzymes (B) over 120 min. (…) represent the glucose concentration curve after *in vitro* starch hydrolysis of lentil soup meal, (__) represent the glucose concentration curve after *in vitro* starch hydrolysis of white bread, (---) represent the glucose concentration curve after *in vitro* of lentil soup. Data expressed as the average % of starch hydrolysis and the error bars are the standard error of the mean (n=4).

Figure 6.3 illustrates the hydrolysis rates of each food tested with different enzymes origin in this study. The rate of starch hydrolysis in the three foods was behaved somehow similarly but the hydrolysis with the microbial enzymes (Figure 6.3-B) tends to be slightly higher. Therefore, one way ANOVE using Tukey analysis was conducted to investigate the variations in the AUC of the foods between the two approaches and there was no significant difference between the two as shown in the table below:

% of AUC of starch hydrolysis calculated in food (n=4)			
	AUC of starch hydrolysis with pancreatic enzymes[1]	**AUC of starch hydrolysis with microbial enzymes[1]**	***P****
Lentil soup meal	745.1 ±58.9	691.1 ±196.7	0.67
White bread	1566.8 ±58.2	1610.7 ±279.0	0.96
Lentil soup	166.0 ±21.8	212.0 ±93.9	0.63

[1] AUC= area under the glucose concentration curve (g.min/100g)
***Significance value using ANOVA**

Table 6.2: Variation of between the percentage AUC of the of starch hydrolysis over 120 incubations with different enzyme origins. Data expressed as mean (n=4) ± SD.

6.3.1.2 Starch hydrolysis rate of the test foods

White bread shows a higher rate of starch digestion than the other foods, while lentil soup shows the lowest, and lentil soup meal shows moderate digestion rate. The time of the maximum rate of starch hydrolysis in white bread and lentil soup meal was at 60 min for pancreatic enzymes and 45 min for microbial enzymes. The low rate for lentil made it difficult to estimate.

6.3.2 Hydrolysis rate and hydrolysis index

The area under the glucose concentration curves was calculated for each sample and the hydrolysis index (HI) was calculated the by the equation below:

$$HI = \frac{\text{AUC of av.CHO released of the test food}}{\text{AUC of av.CHO released of the reference food}} \times 100$$

Equation 6.1: Hydrolysis Index (HI) calculation.

White bread was used as a reference food with HI =100, whereas the HI for lentil soup was 42.0 ± 5.0 and HI for lentil soup meal was 86.0 ±8.0.

GI was estimated using Equation 6.2 created by Goni *et al.* (1997) then they were multiplied by 0.7 conversion factor to convert them to $GI_{glucose}$ equivalence as recommended by (Wolever, 2006). Table 6.3 represents the comparison between the GI values obtained by the two approaches. According to the WHO/FAO (1998), the GI value of lentil soup was classified as low GI (GI=35) while white bread was classified as high (GI=70), and lentil soup meal was medium (GI=57).

$$GI = 39.71 + (0.549 \times HI)$$

Equation 6.2: Equation created by Goni *et al*.(1997) to estimate the GI after measuring the rate of starch hydrolyzed by pancreatic enzymes. HI refers to Hydrolysis Index.

GI values of the foods (n=4)			
	GI[1]	**Predicted GI[2]**	**Difference unit (%)**
Lentil soup meal	60± 6.0	58	2 (3%)
Lentil soup	34± 3.0	40	-6 (15%)
White bread	70± 0.0	75	-5 (7%)

[1] GI calculated by Goni *et al.* (1997) equation and converted to glucose equivalence (n=4)

[2] GI values obtained from prediction models in chapter 5

Table 6.3: Calculated GI and predicted GI values of the three food samples. Data express as a percentage ±SD.

Estimated GI from the *in vitro* digestion (pancreatic enzymes) verified the statistically predicted GI from previous chapter and the difference units were within the acceptable ranges (difference unit≤ 10) as suggested by Brouns *et al.* (2005). Also there were not significant differences between the two approaches (P=0.46).

Moreover, Figure 6.4 represents the comparison between the two approaches and published data. Although the results from *in vitro* digestion (pancreatic enzymes) were overestimated in case of white bread and lentil soup meal, they were underestimated for lentil soup, the differences in GI values were not significant (P=0.92).

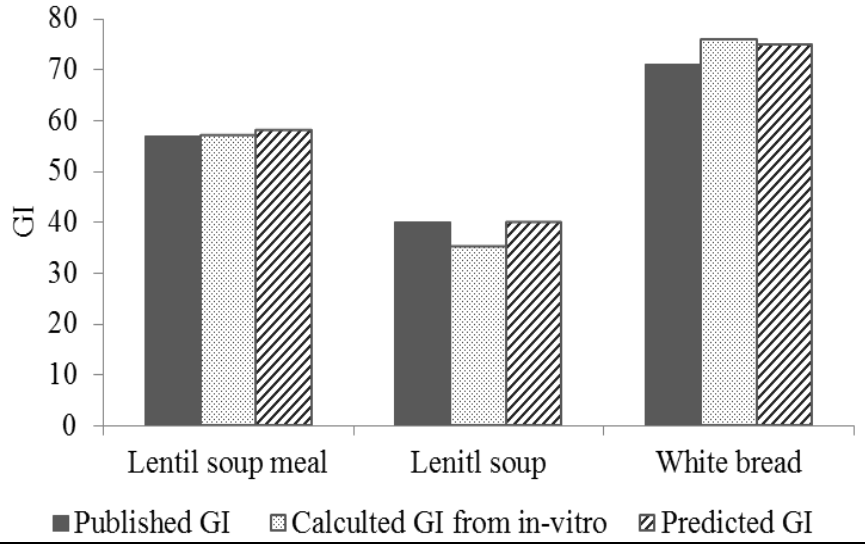


Figure6.4: comparison between the three (GI) values. The first bar represents published GI values (Atkinson et al., 2008), the second bar represent the GI values calculated from non-restricted *in vitro* digestion (n=3), the last bar represent the GI values predicted from macronutrient composition using statistical analysis . Data expressed as percentage and error bars represent the standard errors of the mean.

6.4 Discussion

6.4.1 Non-restricted *in vitro* starch digestion

In this study, non-restricted *in vitro* starch hydrolysis was conducted instead of restricted *in vitro* starch hydrolysis (dialysis), because according to Goni, *et al.* (1997) it is not recommended to use the restricted digestion system. Also, the sugars in the gastrointestinal tract (except fructose) are absorbed by active transport rather than being diffused and in the restricted digestion the amount of av.CHO diffused is measured (Lindeboom *et al*., 2004). In addition, restricted hydrolysis may not be convenient regarding routine testing or screening purposes because of its complexity (Germaine *et al.*, 2008).

According to Germaine *et al.* (2008), the addition of salivary α-amylase does not have an impact on the *in vitro* digestion and can be omitted therefore; the oral phase consisted of grinding of the samples without the addition of salivary α-amylase in present study.

6.4.1.1 *In vitro* starch hydrolysis with different enzymes origin

Types of food and the origin of the enzymes are one the main factors that affect the rate of *in vitro* starch digestion (Hur *et al*., 2011). Usually thermostable α-amylase isolated from bacteria is used in the *in vitro* starch hydrolysis to gelatinize and hydrolyze at the same time (Hur *et al*., 2011). In this study, non-restricted *in vitro* starch digestion using pancreatic and bacterial enzymes was investigated. The results showed non-significant differences in the rate of hydrolysis using these two approaches. This might be due to the similar composition of the two enzymes with both enzymes having similar 3D structures and amino acid sequence and they only differ in the optimum temperature (Colonna *et al*., 1992). However, many studies have been conducted to measure the rate of starch hydrolysis *in vitro* using a mixture of enzymes from different origin (bacterial, fungal, or animal) and different types of food, which make difficult to makes a comparison (Hoover and Zhou, 2003, Hur *et al*., 2011).

6.4.1.2 Starch hydrolysis rate of the test foods

Cereals and legumes represent the most staple consumed food all over the world, and 50-80% of their content is starch (Sonia *et al*., 2013).

Legumes were chosen in this study because of their constituent starch which has type B crystallinity and thick cell wall surrounding the legume cell which is difficult to digest.

Lentils are a type of pulse and are mostly found in the Mediterranean diet but in UK they are consumed on a small scale (Schneider, 2002).

To our knowledge, food processing like cooking and grinding will affect the starch digestibility and increase the rate of starch digested by facilitating the access to the substrate (Ranawana *et al*., 2011). However, in present study, the rate of starch hydrolysis of lentil soup was the slowest among the other food samples; even though lentil soup was microwaved for 10 min at maximum power then the soup was mashed. It is known that the microwave contributes to more heat penetration and enhances starch gelatinization (Singh *et al*., 2010). Therefore, low starch digestibility might be due to insufficient time for cooking; the rate of starch hydrolysis is increased with the increasing of cooking period (Traianedes and O'Dea, 1986) or may be due to the presence of several factors such as type of starch crystallinity, protein-starch interaction, lipid-starch interaction, the presence of indigestible oligosaccharides like raffinoses and the presence of amylase inhibitors like polyphenols and phytic acid (Cummings and Englyst, 1995).

On the other hand, white bread was chosen because it is the most consumed staple food globally and it is consumed with most meals (Brouns *et al*., 2005, Almousa *et al*., 2013). The rate of starch hydrolysis of white bread was the highest among the other food samples, it contains rapidly digestible starch and has a porous structure that facilitates the access by the digestive enzymes (Zabidi and Aziz, 2009).

Foods are eaten as mixed; lentil soup and white bread were mixed together and showed moderate effects on the rate of the starch hydrolysis. The hydrolysis curve was as one step.

6.4.2 Hydrolysis rate and hydrolysis index

HI is the AUC of the av.CHO released from *in vitro* digestion of the test food over the AUC of the av.CHO released from *in vitro* digestion of the reference food HI can be used to predicted the GI of the food, as a result HI values were applied to an equation to estimate the value of GI. Granfeldt *et al*. (1992) and Hettiaratchi *et al.* (2012) reported higher HI values (63±4, 52±4) respectively for lentil, however, the disagreement could be due to several reasons: both studies were using similar enzymes, enzyme dosages, and buffer but different type of *in vitro* starch digestion (dialysis), whereas Germaine *et al.* (2008) found that the rate of restricted starch digestion is significantly higher than non-restricted digestion. Furthermore, the higher values could due to the botanical variation among the lentils used in each of studies. Most other studies also have used restricted *in vitro* starch digestion which makes it difficult to compare between the two approaches (Tovar *et al*., 2003).

Unfortunately, information regarding legumes in a mixed food is limited (Hutchins *et al*., 2012) and there is only one recent study that predicted GI in mixed food, mainly south Asian food, using restricted *in vitro* starch hydrolysis (Hettiaratchi *et al*., 2012). The HI value for their lentil soup meal was 81 and it was slightly lower than what was reported in this study (HI= 86.0 ±8.0), despite the differences in the type of starch digestion method used in Germaine *et al.* (2008) study. Yet, the lower value might be due to the type of bread they used in their study which was whole meal bread and it is known that whole meal bread considered a good source of NSP and NSP has a lowering effect on the rate of the starch hydrolysis (Lightowler and Henry, 2009).

The aim of this study was to validate the predicted GI values mentioned in chapter 5 via non-restricted *in vitro* starch digestion. The GI values of lentils reported in Germaine *et al.* (2008) study using non-restricted *in vitro* starch was close (estimated GI=32±1) to what was reported in this study (estimated GI= 34± 3.0). This indicates that the statistically predicted GI (predicted GI= 38) are close to those predicated by *in vitro* starch digestion. Both statistical and *in vitro* starch digestion approach might be useful to limit the need of human subjects and blood sample analysis.

6.5 Limitations

More complex multi-component foods need to be analyzed using non-restricted *in vitro* starch hydrolysis to improve assessing the prediction models. Further investigations on the uses of microbial rather than pancreatic enzymes are needed to be undertaken. Information regarding legumes in mixed food is limited and further investigation of each type of legume consumed in a mixed meal is needed. Analysis of the anti-nutrients (e.g. polyphenols) might be useful and may provide deeper understanding on their effect on the rate of starch hydrolysis. Finally, *in vivo* GI measurement is needed for further validation.

6.6 Conclusion

In this study, it has been found that pancreatic or microbial digestive enzymes will have the same effect on the rate of digestion, but more intensive investigations are required.

Also, the present study validated the statistically predicted GI values from macronutrient content generated in the previous chapter. A good correlation and non-significant differences between the two approaches was absorbed. Finally, *in vivo* GI measurement is

essential to provide further validation and accuracy and this will be described in the next chapter.

7 Chapter seven: *in vivo* determination of GI

7.1 Introduction

GI was predicted in chapter 5 from macronutrient content using statistical analysis, then in chapter 6, *in vitro* starch digestion was conducted to verify the predicted values and good correlations were found between these two approaches. However, for further validation, *in vivo* GI measurement is needed.

In vitro starch digestion measured the rate of starch digestion and measured the variation between the foods only, whereas *in vivo* GI measurements determine the variation between the foods as well as between and within -individual variations among the subjects.

Between and within-individual variations may have an impact on the measured GR in the blood. Between-individual variations are the differences between subjects, for example; the health status of the subject, smoking, age, gender body mass index (BMI), physical status, stress, ethnicity and daily total energy and CHO intake, while within-individual variations are day-to-day variations in GR of the same subject under standardized conditions and after repetitive consumption of a reference food (at least 2 times and preferably 3 times).

Despite the growing interest in GI and GL, there are insufficient studies that investigate the effect of ethnicity of GR (Kataoka *et al*., 2013). Moreover, the majority was concentrating on the GR of Asian (e.g. Chinese, Thai, Indian, etc.) subjects comparing to Europe subjects using single food (Dickinson *et al*., 2002, Venn *et al*., 2010, Pratt *et al*., 2011, Kataoka *et al*., 2013, Henry *et al*., 2008a). On the other hand, few studies regarding

the GR of African population compared to Europe subjects were conducted (Walker and Walker, 1984).

In addition, there are limited studies regarding GR of Arab ethnicity and the GI of Arabic foods. Studies which are available investigated the GI of few foods like different varieties of dates (Ahmed, 2002, Miller *et al*., 2002, Alkaabi *et al*., 2011) or different varieties of breads (Almousa *et al*., 2013, Takruri and Alkurd, 2008). Only two studies measured the GI in mixed meals, these include: Arabic coffee with dates (Al-Mssallem and Brown, 2013), and yogurt with dates (Miller *et al*., 2003).

Furthermore, there is only one study that measured the effect of low GI foods on GR, appetite, and food intake in young females (Zafar *et al*., 2011). To our knowledge, there is only one study that recruited Arabic subjects and compared the iAUC, GR and ethnic groups using white bread (Dickinson *et al*., 2002).

7.2 Aim

To investigate the GR of two types of food, white bread (a low fibre cereal based food) and lentil soup (a high fibre legume based food provided single or in combination) in healthy human subjects of different backgrounds and to assess the validity of the GI values of single and mixed foods predicted by the mathematical models (chapter 5).

7.2.1 Objective

1. To measure the GR in human volunteers after consuming a set portion of four combinations of food (3 slices of white bread, 1.5 slices of white bread, bowl of lentil soup, and lentil soup meal (a bowl of soup +1.5 slices of white bread).
2. To explore the effect of demographic characteristic like: age, gender, BMI, physical activity status and ethnicity on the GR of the subjects Pearson correlation, Spearman correlation and one way ANOVA.
3. To explore the effect of stress on the GR using Pearson correlation.
4. To explore the effect of dietary factor likes the daily energy and CHO intake on the GR using Pearson correlation.
5. To explore the effect of wash out period on GR (wash out period) using Pearson correlation.
6. To evaluate the correlation of GI prediction models from the previous chapters (chapter 5 and 6) with *in vivo* measurements using one way ANOVA.

7.3 Results

7.3.1 Ethics

Ethical approval for this study was granted by MAPS and Engineering Ethical Committee at University of Leeds (MEEC 11-027). The research was carried out at the School of Food Science and Nutrition at the University of Leeds (Appendix A).

7.3.2 Data collection , recruitment and dietary survey

7.3.2.1 Demographic characteristics

Questionnaires used in this study were adapted from the National Health and Nutrition Examination Survey (NHNAES) and modified to reflect the cultural and socio-economic specificities of the recruitment (Appendix B).

Table 7.1 shows the recorded characteristics of the subjects; 17 healthy subjects were recruited from different backgrounds (n=5 from Arab countries, n=5 form Europe, and n=7 from Asian countries) with age ranging from 18-48 years.

The mean BMI of the Europe subjects and Asian countries subjects were 23.3±1.3 kg/m^2 and 19.8±3.0 kg/m^2 respectively and they were within the normal weight ranges (18.5-24.9 kg/m^2), whereas the mean BMI of the Arab countries subjects was 27.7±2.2 kg/m^2 and the categorized as over weight (25-29.9 kg/m^2). Thirty five percent of the subjects were males (n=7) while 65% (n=10) were females. Among the 17 subjects, 1 subject was a smoker. Forty one percent were alcohol consumers. Sixty five percent reported that they performed physical activity at least 32±19 min daily and the physical ranges reported were walking, cycling, and swimming.

Subjects were asked to recall all the foods and drinks consumed during the last 24 hours before each session.

7.3.2.2 Recruitment

GI was measured in 13 out of 17 subjects, four subjects were excluded because of noncompliance with the study in which; 2 of the subjects were working in the lab most of the time, one of the subject was drinking tea before coming to the tests, and the last one had the a very low incremental area under the GR curve after consuming 50g av.CHO reference food (participant #2) because of the lack of sleep and in some trials he was fasting for more than 20h.

#	Age (year)	Ethnicity	BMI[1] (kg/m^2)	Gender	Smoking	Alcohol consumption	Physical activity
1	27	Europe	22.9	Male	No	No	No
4	46	Europe	25.4	Male	No	No	No
6	19	Europe	22.6	Female	No	No	Yes
3	48	Europe	23.5	Male	No	Yes	No
7	37	Arab countries	31.5	Male	No	No	Yes
9	23	Arab countries	26.3	Male	Yes	No	Yes
5	38	Arab countries	26.7	Female	No	No	Yes
8	25	Asian countries	22.1	Male	No	No	Yes
11	20	Asian countries	18.7	Female	No	Yes	No
13	24	Asian countries	25.4	Female	Yes	No	Yes
14	23	Asian countries	17.3	Female	No	No	Yes
13	23	Asian countries	18.5	Female	No	Yes	No
16	22	Asian countries	20.0	Female	No	Yes	No
17	23	Asian countries	16.9	Female	No	No	Yes
			Excluded subjects				
2	28	Europe	22.2	Male	No	No	Yes
10	19	Europe	22.6	Female	No	No	Yes
12	37	Arab countries	26.1	Female	No	No	Yes
15	30	Arab countries	28.0	Female	No	No	Yes

[1] Body Mass Index (BMI) ranges:

Underweight 18.5 or below — Normal 18.5-24.9

Overweight 25-29.9 — Obese 30 and over

Table 7.1: Characteristics of the subjects (n=17)

7.3.2.3 Dietary survey

Table 7.2 shows the staple food, the average daily intake of energy (kcal), and the average intake of CHO (g) that were calculated from the 24h recall before each test. Wheat and rice were the most common staple foods among the subjects. The average energy and CHO was above the daily recommendation (male 2550 kcal/day, female 1940 kcal/day, and 230g of CHO/day) (Department of Health, 1991). Although most of them were within the normal BMI range, in which the male in this study consumed 3086 ±677 kcal/day, while the females consumed 3666 ±1356 kcal/day. On the other hand the average daily intake of CHO was 435 ±210.7g/day (Department of Health, 1991).

The highest daily total energy and CHO intakes were associated with subjects #8 (female and Asian subject) (6205 ±3557 Kcal, 1066.1 ±606.0 g) respectively, while the lowest daily energy intake was associated with subject #4 (male and Europe subject) 1995 ±480 Kcal and the lowest total CHO intake was 205.6 ±57.7 g.

#	Staple food	Total energy(kcal)/ day	Total CHO g / day
1	Wheat, rye, rice, potato	3476 ±640	444.5± 75.0
4	Wheat, rye, rice, potato	1995 ±480	261.0 ±77.5
6	Wheat, rye, rice, potato	3590.3 ±135	342.1 ±981.4
3	Wheat, rice	2475 ±1011	330.4 ±145.9
7	Wheat, rice	3769 ±1294	465.1 ±166.0
9	Wheat, rice	4019 ±335	539.4 ±64.9
5	Rice	2754 ±757	205.6 ±57.7
8	Rice	6205 ±3557	1066.1 ±606.0
11	Rice	2577 ±1441	302.4 ±148.4
13	Rice	4284 ±1845	455.7 ±260.7
14	Rice	3181 ±1260	367.0 ±146.5
16	Rice	3963 ±1276	445.3 ±132.0
17	Rice	2704 ±1050	247.6 ±143.5
	Excluded subjects		
2	Wheat, rye, rice, potato	3538 ±716	489.1 ±120.0
10	Wheat, rye, rice, potato	2595 ±917	425.5 ±145.5
12	Wheat, rice	5298 ±793	758.5 ±183.9
15	Wheat, rice	1838 ±606	256.3 ±80.7

Table 7.2: Staple food the energy and CHO intake of the participants taking over 8 days. Data express as the mean + SD (n=17)

7.3.3 GI protocol

The *in vivo* GI method listed in this study was adapted from (FAO/WHO, 1998, Jenkins *et al*., 1981). Av.CHO was measured experimentally with PS. The preparation of foods is described in Table 7.3. Subjects were divided into three ethnic group (n=3 Arab countries, n=3 Europe, n= 7 Asian countries). Figure 7.1, represents the soup portion served to the subjects.

	Lentil soup	Lentil soup meal	White bread	White bread
Av.CHO portion	25g	50g	25g	50g
Test portion	Bowl of lentil soup 1:2 (v/w)	Bowl of lentil soup 1:2 (v/w) + 1.5 slices	1.5 slices	3 slices
Energy/ portion	181 kcal	325 kcal	144 kcal	288 kcal

Table 7.3: Av.CHO portion and test portion in food used in this study

Figure 7.1: A bowl of lentil soup 25g av.CHO portion as served to the subjects.

Table 7.4 shows the fasting glucose level in the blood and AUC in response to the 50g av.CHO reference food portion (three slices of bread). The fasting period was within the recommended period by Brouns, *et al*. (2005) (10-14 h) to reduce the intra- individual variations. The fasting blood level for the participants was within the normal ranges (<0.7 mmol/L) (Badran and Laher, 2012). The average iAUC after consuming 50g av.CHO reference food portion (three slices of bread) was 134.0 ±66.2 mmol.min/L. The highest iAUC was associated with participants 13 (female and Asian subjects) 244.1 ±132.1 mmol.min/L, whereas the lowest was associated with participant 7 (male and Arab subject) 10.9 ±75.8 mmol.min/L).

#	Fasting time (min)[1]	FBG (mmol/L)[2]	iAUC (mmol.min/L)[3]
1	14.0 ±2	5.3± 0.2	138.8 ±97.4
4	13.0 ±2	4.9±0.6	103.5 ±62.5
6	13.0 ±3	5.3± 0.5	196.1 ±21.2
3	14.0 ±2	6.1± 0.6	157.9 ± 20.7
7	14.0 ±2	4.9± 0.1	91.5 ±42.0
9	14.0 ±2	4.7± 0.4	167.0 ±32.42
5	13.0 ±2	5.7± 0.4	159.0 ±9.8
8	13.0 ±1	5.2±0.8	142.3 ± 118.9
11	14.0 ±2	5.6± 0.1	105.0 ±64.5
13	13.0 ±2	5.5± 0.5	225.8 ±125.2
14	13.0 ±2	5.3± 1.1	244.1 ±132.1
16	13.0 ±3	5.6± 0.1	183.8 ±23.5
17	13.0±4	5.0±0.1	187.7 ±23.6
	Excluded subjects		
2	14.0 ±3	5.0± 0.3	10.9 ± 75.8
10	13.0±2	5.6± 0.3	49.5 ±41.7
12	14.0 ±1	5.3± 0.1	43.1 ±4.4
15	14.0±2	5.3± 0.4	72.7 ±35.2

[1] Mean of fasting time before conducting reference tests (n=2)

[2] mean fasting blood glucose (n=2)

[3] Incremental Area Under Curve (iAUC) (n=2)

Table 7.4: Blood sugar levels of the participants of 50g av.CHO reference food portion. Data express as the mean + SD (n=17)

7.3.4 GR and GI of different foods

Figure 7.2 and 7.3 represent the mean values of blood glucose concentrations of the 13 subjects after ingestion of 50 or 25g av.CHO portion foods over a 120 min period. Single foods like white bread and the lentil soup alone had the highest and lowest blood sugar responses iAUC (163.4 ±42.2 and 90.8± 5.3 mmol.min/L) respectively, while mixing the two foods together (lentil soup meal) shows a moderate response.

Moreover, 25 and 50g av.CHO portion of white bread and lentil soup meal have the same glucose peak at 45 min unlike the lentil soup which has glucose maximum peak at 30 min then the glucose levels remain flat for the rest of the test. The glucose level of lentil soup meal after 60 min kept increasing slightly, in a similar way to lentil alone. As shown in the Figures 7.2 and 7.3, 120 min may not be enough for GR of lentil soup and lentil soup meal to reach back the baseline again.

As shown in Figures 7.2 and 7.3, the GR curve for 50g av.CHO portion of the white bread, lentil soup, and lentil soup meal did not reach the baseline unlike the GR curve for 25g av.CHO portion of the white bread.

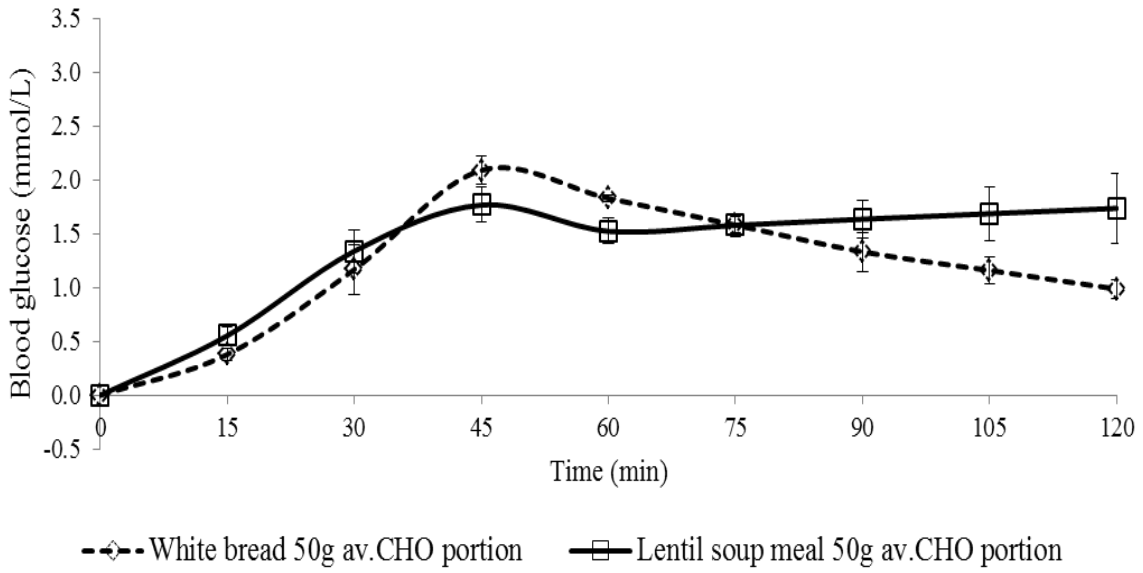


Figure 7.2: The mean blood glucose response curves after ingestion of 50g av. CHO of foods over 120 min. (__) represents the blood glucose curve of lentil soup meal and (---) represents the blood glucose curve of white bread (3 slices. Data expressed as the amount of blood glucose in mmol/L and the error bars represent the standard error of the mean (n=13).

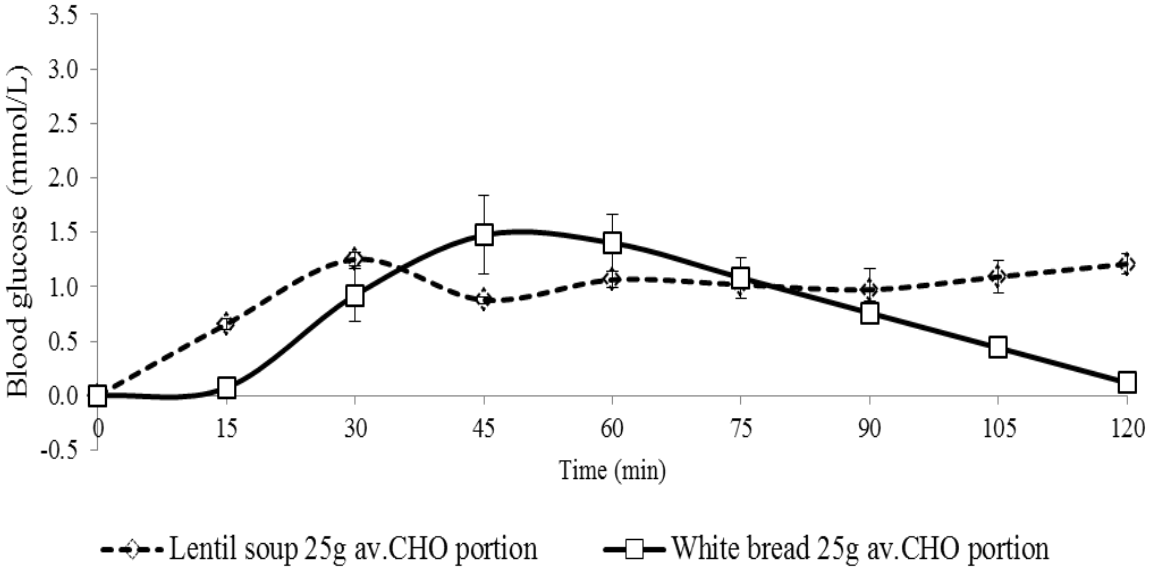


Figure7.3: The mean blood glucose response curves after ingestion of 25g av. CHO of foods over 120 min. (…) represents the blood glucose curve of lentil soup, and (-..-) represents the glucose curve of white bread (1.5 slices). Data expressed as the amount of blood glucose in mmol/L and the error bars represent the standard error of the mean (n=13).

7.3.5 Dose response of 25/50 g av.CHO reference portion

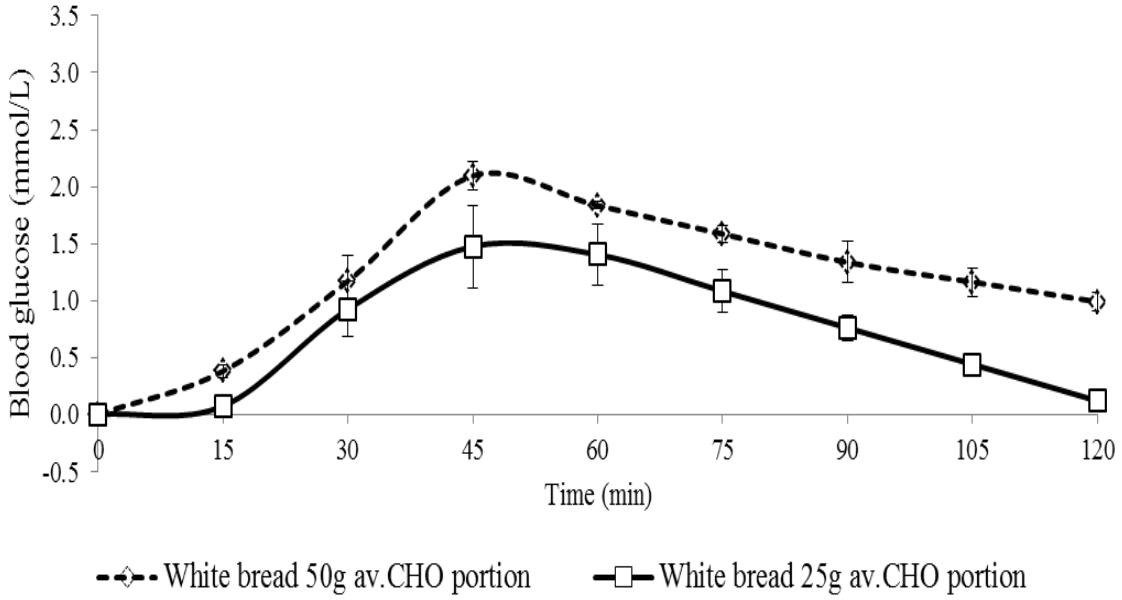


Figure 7.4: The effect of dose response after ingestion 50/25g av. CHO of foods on GR over 120 min. (__) represents the blood glucose curve of white bread (3 slices), and (---) represents the blood glucose curve of white bread (1.5 slices). Data expressed as the amount of blood glucose in mmol/L and the error bars represent the standard error of the mean (n=13).

Figure 7.4 represents the dose response of different portion of reference food. The response of 50g av.CHO for the white bread was around 50% higher than for the 25 g av.CHO of the same food, indicate the dose response. Also the 50 g av.CHO portion of white bread may require more than 120 min to reach the baseline level again. Furthermore, the GR curve for 50g av.CHO portion of the white bread did not reach the baseline unlike the GR curve for 25g av.CHO portion of the white bread.

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7.3.6 Inter-individual variation in GR and the dietary and non-dietary factors affecting GR

One way ANOVA using Tukey analysis was conducted to investigate the dietary and non-dietary variation between the subjects that may affect the GR and there were not any significant differences in fasting blood glucose FBG, and iAUC after ingestion 50g av.CHO portion of reference food between three ethnic groups (Table 7.5).

	Europe	**Arab**	**Asian**	***P*[4]**
Age	33.6 ±12.7	33.0±6.5	22.9 ±1.6	0.71
Gender	1F:4M [1]	3F:2M	6F:1M	-
BMI[2]	23.3±1.3	27.7±2.2	19.8±3.0	0.54
Daily energy intake (kcal)	3135±551	3536± 1312	3342±757	0.54
Daily CHO intake (g)	392.4 ±90.8	469.9 ±195.7	441.4 ±291.1	0.64
FBG[3] (mmol/L)	5.2 ±0.3	5.2 ± 0.6	5.4 ±0.3	0.51
AUC[4] (mmol.min/L)	115.9 ±55.0	90.3 ±69.4	178.8 ±47.9	0.59

[1] F:M = # Female: # Male

[2] Body Mass Index

[3] Fasting blood glucose

[4] Area under curve after ingestion 50 g av.CHO portion reference food

[5] Significance value (significant $P \leq 0.05$)

Table 7.5: Characteristics of the participants. Data express as the mean + SD **(**n=13).

7.3.6.1 Non-dietary factors affect GR

7.3.6.1.1 Demographic factors and GR

Subject #6 was the oldest among the other subjects (48 years) and the iAUC of (196.1±21.2 mmol.min/L) was 30 % higher than subject #8 who was the youngest (20 years).

Moreover the number of the males recruited in this study was 6 and the average iAUC was 138.4 ±37.8 mmol.min/L, whereas 7 females were recruited and the average iAUC was slightly higher (34%) than the males (181.8 ± 45.6 mmol.min/L)

However, there was no significant association between the iAUC of subjects and age, nor gender (respectively) (Pearson correlation coefficient =-0.08, and 0.29, P= 0.71, and P= 0.26).

7.3.6.1.2 Life style factors and GR

Subject #3 had the highest BMI (31.5 kg/m^2) and the iAUC of (157.9 ±20.7 mmol.min/L) was 26 % lower than subject #17 who had the lowest BMI (16.9 kg/m^2) and the iAUC (187.7 ±187.7 mmol.min/L).

Furthermore, subjects # 13 and 7 had the highest and the lowest iAUC respectively (244.1 ±132.1, 91.5 ±42.1 mmol.min/L) were both physically active.

Overall, there was no significant relation between the iAUC of subjects and BMI, nor physical activity (respectively) (Pearson correlation coefficient =-0.39, and 0.39, P= 0.12, and P= 0.34).

7.3.6.1.3 Effects of stress

Five subjects among the 13 claimed that they were stressed due to upcoming examinations and projects. Blood glucose curves of the stressed subjects were below and shifted forward compared to the blood glucose curve of the non-stressed subjects. As shown in Figure 7.5, iAUC of stressed subjects were lower than those who claimed not to be stressed (iAUC of stressed subjects 134.1 ±24.3 mmol.min/L and non-stressed subjects =67.0 ± 32.7 mmol.min/L). Nevertheless, the lower response of the stressed subjects was not significant (Pearson correlation coefficient= -0.39, *P*= 0.24).

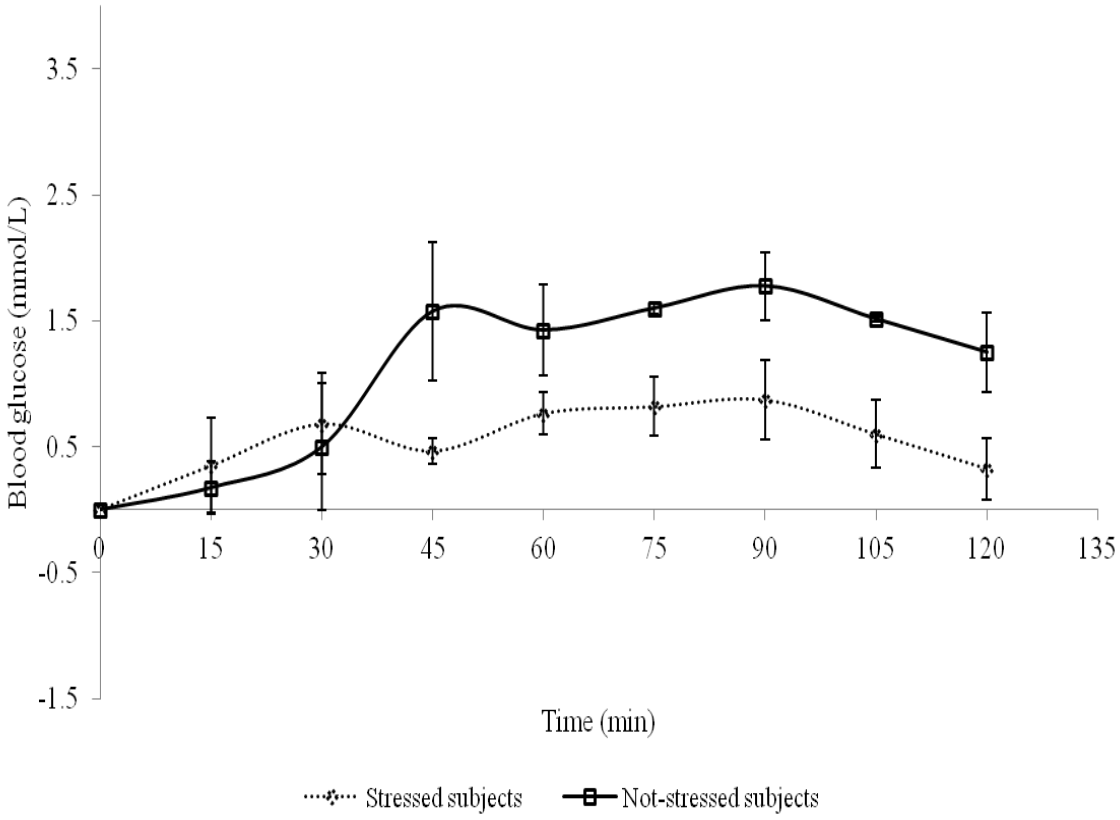

Figure 7.5: The mean blood glucose response curves of subjects claiming stress after ingestion of 50g av.CHO portion reference food (3 slice f bread). (__) represent the blood glucose curve of the stressed subjects, and (…) represent the blood glucose curve of the not-stressed subjects. Data expressed as the amount of blood glucose in mmol/L and the error bars represent the standard error of the mean (n=5).

7.3.6.2 Dietary factors affecting GR

7.3.6.2.1 Effect of energy and CHO intake on GR

Table 4.2 shows the average daily energy and CHO intake of the subjects before the test period using their 24-hour food recall diaries and analyzed with WinDiets. It can be seen that subject # 4 had the lowest daily energy intake (1995 ±480 Kcal) and the iAUC (103.5 ±62.5 mmol.min/L) was 48% lower than subject # 3 (iAUC =167.0 ±32.42 mmol.min/L) who the highest energy intake (6205 ±3557 Kcal).

However, subject # 3 had the highest average daily intake of CHO among the subjects and had 10 % lower iAUC (142.3 ±118.9 mmol.min/L) than subject #5 (iAUC=159.0 ±9.8 mmol.min/L) which had the lowest CHO intake (205.6 ±57.7g).

So, it can be noticed that there is no correlation between the iAUC and energy intake nor total CHO intake during the 24 hours before the test and the AUC values achieved through the blood tests. The Pearson correlation coefficient was -0.15 and 0.02 (P= 0.56, and P= 0.95) respectively.

7.3.6.2.2 Demographic factors and GR

7.3.6.2.2.1 Effects of ethnicity on GR and GI

Although ethnicity is considered one of the non-dietary and demographic factors that may affect the GR and GI, this factor was explored separately and more deeply than the others because of the limited and inconsistent information published previously (Kataoka *et al*., 2013). Table 7.6 represents the iAUC (mmol.min/L) observed in each ethnic group.

	Arabs (n=3)	Europe (n=3)	Asian (n=7)	P^3
25g av.CHO reference food[1]	116.4 ±21.9	98.0 ±34.1	165.3 ±31.4	0.37
50g av.CHO reference food[2]	127.9 ±43.5	132.5 ±46.5	176.8 ±52.1	0.40
Lentil soup	126.5 ±8	86.5 ±12.0	108.2+36.7	0.42
Lentil soup meal	158.1 ±63.9	113.6 ±29.1	213.0 ±70.5	0.47

[1] 25g av.CHO portion = 1.5 slices of bread
[2] 50g av.CHO portion = three slices of bread
[3]Significance value (significant $P \leq 0.05$)

Table 7.6: iAUC (mmol.min/L) observed in the subjects from different background after ingestion a set of portion foods. Data expressed as mean of the iAUC ± SD.

As shown in Figure 7.6 the GR of 50g av.CHO portion reference food for the Asian subjects was the highest (iAUC = 176.8 ± 52.1 mmol.min/L) while the iAUC of the Arab countries and the Europe subjects were (iAUC = 127.9 ±43.5, 132.5 ±46.5 mmol.min/L respectively).

The iAUC of the Arabs after ingestion 50g av.CHO reference foods was 4% higher than the Europe subjects and 28% lower than the iAUC of Asian subjects. However, the differences in iAUC of the 50g av.CHO reference were not significant between the three ethnic groups (P=0.40).

Moreover, the maximum glucose peak of 50g av.CHO portion reference food for Asian subjects was the highest (2.63 mmol/L) at 45 min, while the maximum peak for Europe subjects was (2.04 mmol/L) at 45 min. Arab-subject peaked at 90 min (1.82 mmol/L). However, there was no significant differences among the groups (P= 0.21).

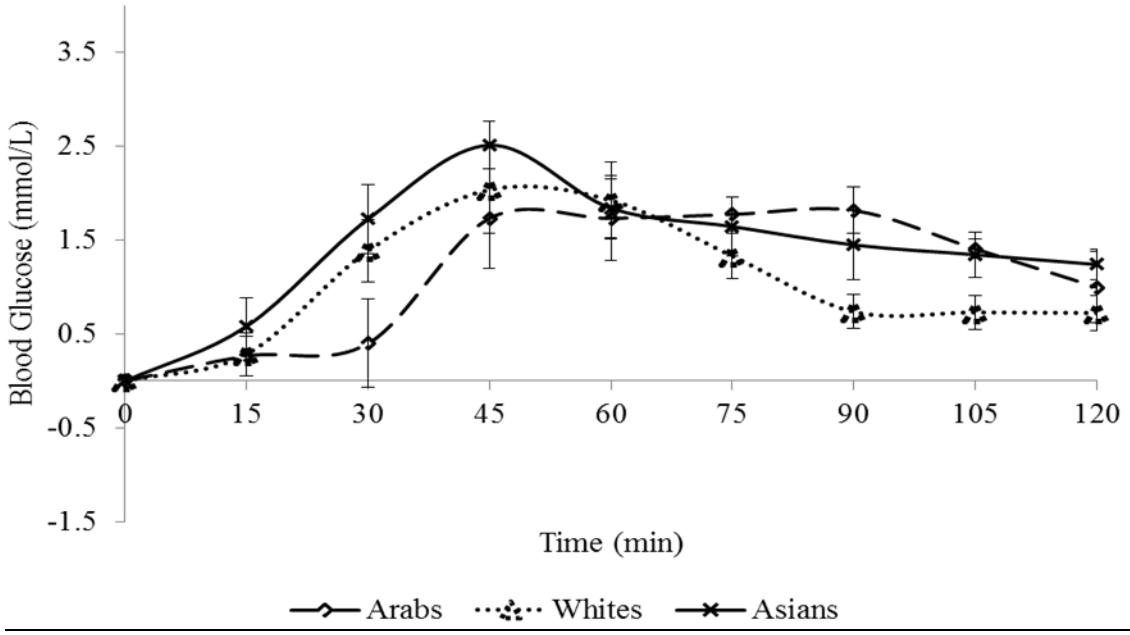

Figure7.6: The mean blood glucose response curves after ingestion of 50g av.CHO reference food (3 slices of bread) over 120 min of the three groups. (__) represent the blood glucose curve of the Arabs, (…) represent the blood glucose curve of the white, and (---) represent the blood glucose curve of Asian. Data expressed as the amount of blood glucose in mmol/L and the error bars represent the standard error of the mean (n=13).

The GR of the Arabs subjects to lentil soup was the highest as shown in Figure 7.7. The iAUC for the Arab subjects was 126.5 ±8 mmol.min/L, whereas the iAUC Europe and for Asian-countries subjects were 86.5 ±12.0 and 108.2+36.7 mmol.min/L respectively.

The iAUC of the Arabs after ingestion 25g av.CHO portion of lentil soup was 44% higher than the Europe subjects and 17% higher the iAUC of Asian subjects and no significant differences were found between the three iAUC (P= 0.42).

The Europe and the Asian subjects maximum glucose peak was at 30 min (0.8 and 1.3 mmol/L) respectively, whereas the Arabs subjects maximum glucose peak was at 90 min

(1.5 mmol/L). In addition no significant differences were found between the three groups regarding the glucose peak (*P*= 0.53).

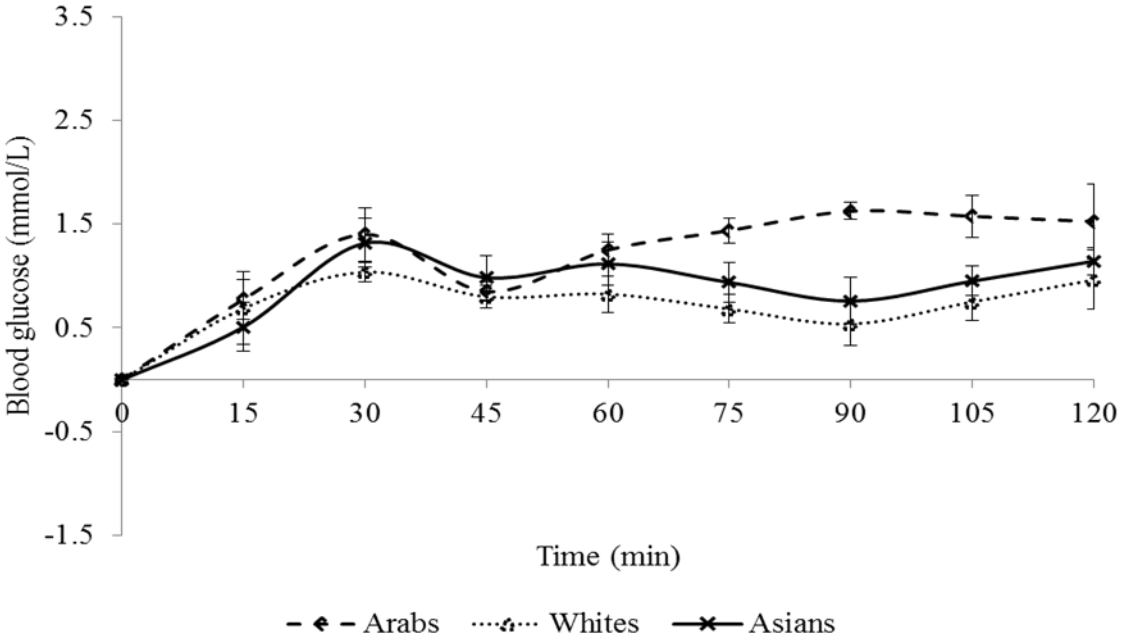

Figure7.7: The mean blood glucose response curves after ingestion of lentil soup over 120 min of the three groups. (__) represent the blood glucose curve of the Arabs, (…) represent the blood glucose curve of the white, and (---) represent the blood glucose curve of Asian. Data expressed as the amount of blood glucose in mmol/L and the error bars represent the standard error of the mean (n=13).

The GR of the lentil soup meal for the Asians shows the highest iAUC observed (213.0 ±70.5 mmol.min/L), while Europe subjects had the lowest iAUC 113.6 ±29.1 mmol.min/L and the Arab countries subjects iAUC was between 158.1 ±63.9 mmol.min/L.

The iAUC of the Arabs after ingestion 50g av.CHO portion of lentil soup meal was 40% higher than the Europe subjects and 26% lower the iAUC of Asian subjects and there was no significant differences in the iAUC between the three groups (*P* =0.47).

The glucose peak for lentil soup meal of all the subjects was at 45 min except for the Arab subjects where GR kept increasing after 60 min unexpectedly (Figure 7.8). However, similarly, for the 50g av.CHO portion reference food (3 slices white bread), the maximum glucose peak of lentil soup meal was the highest among the Asian subjects (2.6 mmol/L), while the Europe and Arab subjects were the same at (1.3 mmol/L). No significant differences were found between the maximum glucose peaks of the three group (P=0.47).

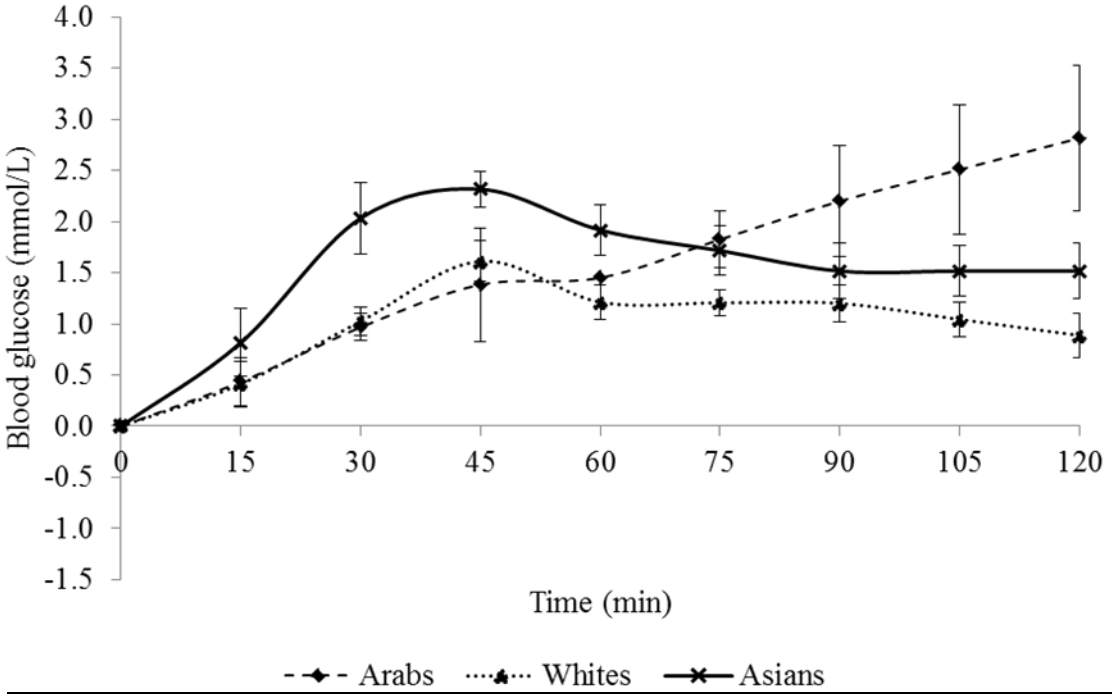

Figure 7.8: The mean blood glucose response curves after ingestion of lentil soup meal over 120 min of the three groups. (__) represent the blood glucose curve the Arabs, (…) represent the blood glucose curve the white, and (---) represent the blood glucose curve Asian. Data expressed as the amount of blood glucose in mmol/L and the error bars represent the standard error of the mean (n=13).

GI was calculated using the equation below (Equation 7.1) and the *in vivo* GI values were expressed as mean values of 13 subjects.

$$GI = \frac{\text{iAUC of GR of test food}}{\text{iAUC of GR of the reference food}} \times 100$$

Equation 7.1: Glycaemic Index (GI) calculation

Table 7.7 represents the GI values of the test food used in this study according to ethnic groups. The lentil soup elicited low responses in the three groups and the GI calculated was classified as low GI. There were no significant differences between the GI of either lentil soup meal or lentil soup amongst the three ethnic groups.

	GI of the subjects (n=13)			
	Arabs (n=3)	**Europe (n=3)**	**Asian (n=7)**	***P***
Lentil soup	39± 13	44± 16	38± 15	0.84
Lentil soup meal	65± 9	60± 5	70± 8	0.47

Table 7.7: GI of the participants from different backgrounds. Data expressed as mean of the GI of the subjects in each group ± SD.

7.3.7 Within-individual variation

7.3.7.1 Effect of wash out period

In this study two set of reference foods portions (50 or 25g av.CHO) were tested on two separate occasions: after two days and after four days. The iAUC of the 50 g av.CHO reference tests conducted after two days showed no significant difference when compared to the iAUC of the tests conducted after four days (Pearson correlation coefficient =0.49, *P*=0.09) (Figure 7.9).

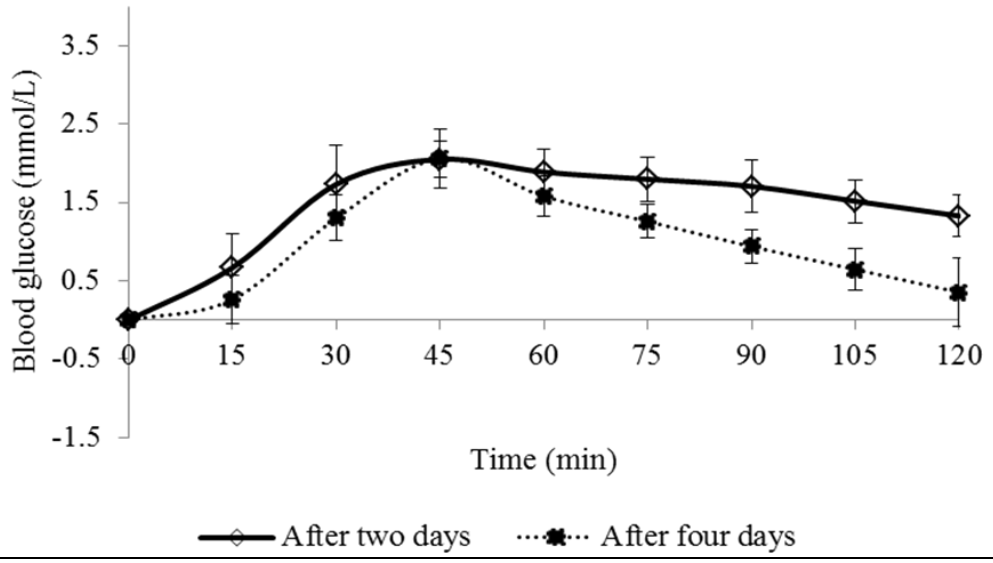

Figure 7.9: The mean blood glucose response curves of trials conducted on different occasions after consuming of 50 av.CHO reference food (3 slices of bread). (__) represent the blood glucose of the reference trails conducted after 2d, and (…) represent the blood glucose curve of the reference trails conducted after 4d. Data expressed as the amount of blood glucose in mmol/L and the error bars represent the standard error of the mean (n=13).

7.3.8 Variation within individuals

Figure 7.10 represents the individual variations in the iAUC of the 50 and 25g av.CHO reference food portion. The mean (CV%) for the variation within the subject for both reference (25g and 50g) were 24% and 33% respectively.

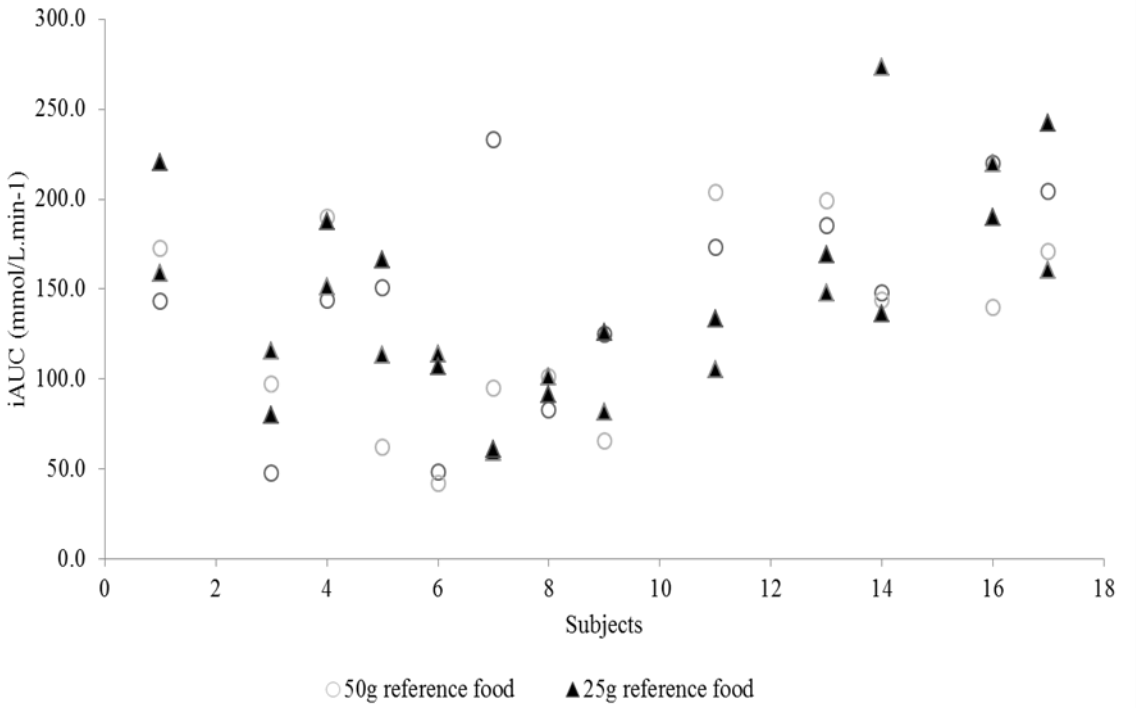

Figure7.10: Within – subjects iAUC variation elicit by consuming of 25g av.CHO reference foods on different occasions. (○) represents the iAUC after 2d, and (▲) represents the iAUC after 4d. Data expresses as the iAUC mmol/L (n=13).

The coefficient of variation (CV) showed in Table 7.8 represents the within-individual variations. CV was calculated for each food repeated twice in each group. The within-individual variation (CV) for the Europe subjects was the highest (CV= 35%) among the other group for the 25/ 50g av.CHO portion of the reference food. Asian subjects show high within-individual variation (CV=34%) for the lentil soup while Arab subjects show high CV (40%) for the lentil soup meal.

	Arabs (n=3)	Europe (n=3)	Asian (n=7)
50g av.CHO reference food[1]	34%	35%	29%
25g av.CHO reference food[2]	19%	35%	19%
Lentil soup	18%	14%	34%
Lentil soup meal	40%	26%	33%

[1] 50g av.CHO portion = 3 slices of bread

[2] 25g av.CHO portion = 1.5 slices of bread

Table 7.8: Coefficient of variation (CV) observed in the subjects from different ethnicities after ingestion a set of portion foods.

7.3.9 Application to the prediction models

In the previous chapters (5 and 6), GI values were predicted statistically from macronutrient content and from non-restricted *in vitro* starch hydrolysis respectively and these values have been validated with the *in vivo* GI from this chapter as shown in this Table 7.9.

Food	Published GI[1]	Measured GI[2]	Estimated GI[3]	Predicted GI[4]
Lentil soup	40	38 17	34± 3	40
Lentil soup meal	57	65± 17	60± 6	58
White bread	71	70 ± 0	70± 0	75

[1] GI published in the international table of GI and GL by (Atkinson *et al*., 2008)

[2]GI measured *in vivo* using (n=13)

[3]GI estimated in chapter 6 using non-restricted *in vitro* starch hydrolysis (n=4)

[4] GI obtained from the prediction models in chapter 5

Table 7.9: Comparison between GI values obtained by different approaches. Data express as mean of the GI± SD).

White bread was a reference food and by definition should be = 100 but it was multiply by 0.7 to converted as $GI_{glucose}$ equivalence as suggested by Wolever (2006).

The SD in the estimated GI from *in vitro* was lower than the SD in the *in vivo* measured GI. However, the difference units between the GI values were within the acceptable ranges (10 GI units) as defined by Brouns *et al.* (2005), and for more conformation, one way ANOVA using Tukey analysis was conducted and there were no significant differences between the approaches used to predicted GI of lentil soup meal (p=0.86) and lentil soup (p=0.86).

7.4 Discussion

So as detailed earlier, the study protocol used here was based on standardized methods described by WHO/FAO (1998) except that av.CHO was measured experimentally rather than calculated. Set portion of four foods with different av.CHO amounts were used in this study to assess the measuring the GI *in vivo.* To ensure as much subject consistency as possible, healthy subjects were selected i.e. with no long-term health problems such as diabetes or cardiovascular disease. It was tried to ensure they remained as stress free as possible throughout the test periods but occasionally a number of subjects had to attend to lab duties. In order to improve reliability in the study the enforcement and adherence to the standardized protocol is very important. For example, with regards to the reasoning behind the method of blood sampling chosen, capillary blood was chosen as opposed to venous blood, because it has been shown to have a lower coefficient of variation (CV = standard deviation/mean) therefore, more consistent readings are obtained (Hätönen *et al*., 2006). Regardless being pricked several times, capillary blood is the preferred method of sampling (FAO, 1998, Wolever, 2006).

7.4.1 GR and GI of different foods

As mentioned in the previous chapter legumes have gained extra interest from nutritionist recently because of their low GI; however there is limited information regarding legumes in a mixed meal (Hutchins *et al.*, 2012).

Lentils are classified as low GI food because they are digested and absorbed slowly; they contain slowly digestible CHO, NSP, vegetable protein, indigestible oligosaccharides, and polyphenols (Hoover and Zhou, 2003, Sonia *et al*., 2013). The GR of lentil soup was low but sustained over 120 min and may need to measure blood GR for longer. In mixed meal, this is still observed

The result of this chapter agreed with the previous chapter in which lentil soup elicited the lowest response despite being microwaved and mashed. White bread elicited the highest GR and lastly the GR of the lentil soup meal was in between the two foods

Moreover, the GR for the white bread peaked at 45 min and it was similar to those reported by Wolever *et al*. (2008), Burton and Lightowler (2008) and Wolever *et al*. (2009) but the average iAUC (134.0 ± 66.2 mmol.min/L) was below what was reported by Burton and Lightowler (2008) and Wolever *et al*. (2008). The differences in the iAUC are might be due to subject variations. In addition, bread varieties or manufacturer differences might also contribute to the differences in the iAUC (Tovar *et al*., 2003).

7.4.2 Dose response of 25/50 g av.CHO reference portion

The amount of av.CHO in the reference foods influenced the GR in which the GR of 50 g av.CHO portion was double the GR of 25 g av.CHO portion of white bread. According to Wolever (2006), 25 g av.CHO portion of food is used because the amount

of av.CHO in lentil soup is small and we found that 50 g av.CHO potion of lentil soup was too much to be consumed by the subjects.

Also, this finding may indicate the importance of measuring av.CHO in food precisely and any overestimation may influence the GR by providing unreliable responses which may later affect the GI.

Therefore in present study av.CHO was measured with evaluated method (chapter 4) in foods rather than expressed as total of analysed sugars and starch together (summation) or calculated by differences.

The GR of 50 g av.CHO portion of reference white bread did not reach the baseline after 2 hours, this may be might be due to the complex composition of white bread that may affect the GR of the participants, as explained earlier in section 1.1.4, unlike glucose reference which is pure sugar, as shown in Burton *et al.* (2008). However, using glucose drink as reference food my not be desirable for the subjects because it may lead to nauseas after taking a glucose drink in the morning after an overnight fasting, as suggested by Brouns *et al*. (2005).

7.4.3 Inter-individual variation in GR and the dietary and non-dietary factors affecting GR

7.4.3.1 Non-dietary factors affecting GR

7.4.3.1.1 Demographic factors and GR

It has been shown that age is positively correlated to the GR, in which GR increases with increase in age (Tuomilehto *et al*., 2003), however in this study different age groups were recruited and did not significantly correlate with the iAUC. In this study

genders did not affect the GR too. This is most likely might be due to the small sample size.

7.4.3.1.2 Life style factors and GR

BMI has been shown to correlate negatively with the GR because overweight subjects tend to be insulin resistant (Wolever, 2006). In this study there was no association between the BMI and GR and that might be due to the sample samples size (Kataoka *et al*., 2013).

It has been shown that physically active subjects tend to have lower GI because of improved the glycaemic control and insulin sensitivity (Wolever, 2000, Mikus *et al*., 2012).

Despite the number of subject in present study was less (n=13) than two previous studies (n= 24, n=34) (Mettler *et al*., 2007, Perala *et al*., 2011) , the result came in agreement with them in which no significant correlation was found between the iAUC and the BMI (P= 0.121), nor physical activities status of the subjects (P= 0.337) after consuming reference food.

7.4.3.2 Effect of stress on GR

Stress has a major role in the metabolic activity in which it stimulate the secretion of epinephrine (adrenaline) (Surwit *et al*., 1992). Epinephrine (adrenaline) is a hormone that stimulates the conversion of glycogen to glucose in the liver and increase the glucose level in the blood. In addition, craving on CHO rich food is a "common phenomena" in stressed subjects (Mwamburi *et a*l., 2011), therefore the iAUC of stressed subjects was higher than others but the level was not significantly higher between the group. The results of this study agree with a previous study that investigated the coefficient of variation within the subjects using 30 subjects and stress was one of the factors that have been investigated in this study and stress did not showed significant effect on GR (Campbell *et al*., 2002, Wolever, 2006).

7.4.3.3 Dietary factors affecting GR

7.4.3.3.1 Effect of energy and CHO intake

It has been shown that high intake of CHO rich food will increase the stimulation the insulin secretion, and dietary pattern with consumption of CHO rich foods over a long period of time will affect the insulin sensitivity, and prevent fat oxidation and lead to hyperinsulinemia (high insulin level in the blood) (Wolever, 2000, Radulian *et al*., 2009, Peters *et al*., 2011).

One of the objectives of this study was to explore the effect of the energy and CHO intake (dietary pattern) on GR and it was found that there is neither significant correlation between the energy nor the CHO intake and the calculated iAUC respectively. However, the lack of significance again might be due to the small sample size.

7.4.3.3.2 Demographic factors and GR

7.4.3.3.2.1 Effect of ethnicity

Because of the limited studies regarding the Arab-countries group, one of our objectives was to explore the GR of these subjects and compared them to other ethnic groups. Moreover, to our knowledge there are only two previous studies that recruited subjects from Middle East: one on 2003 recruited two subjects while the other one recruited one subject on 2008, however both studies grouped them with other ethnic subjects (Wolever *et al*., 2003, Wolever *et al*., 2008). Therefore, this study is the first that investigated this group using complex foods other than dates or breads (Almousa *et al*., 2013) or single foods like chickpeas or potato (Zafar *et al*., 2011), also this is the first study that compared the GR and GI of this group with other ethnic groups.

According to Kataoka *et al.* (2013), they suggested the food used to investigate the GI of different ethnic groups should be within the staple food of the selected subjects and to our knowledge lentils are not the commonly consumed food in Asian-countries especially in China, whereas in Europe it is consumed on small scale (Schneider, 2002). However in this study there was no significant difference in GI toward the lentil soup between the three groups.

In this study the Arabic-countries subjects did not show any significant difference in GI from the Europe and the Asian-countries subjects after testing the reference and test foods despite the different shapes of GR curves in each group. Also the iAUC of the Arabs was around 24% lower than the iAUC of the Asians but there were not statistically significantly different from each other.

iAUC is the GR of the food and each food elicits GR differently in each subjects while GI is express as a ratio between the response to the tested and the reference food. According to Wolever *et al.* (1985) expressing the results as GI will eliminate the intra-individual variation by 50% (Wolever *et al*., 1985).

However, the results here support the finding by in recent study (Pratt *et al*., 2011), wherein no significant differences were found in the GR nor GI between the three ethnic groups (south Asian n=10, Chinese n= 10, and Europe n=10). Nevertheless, the coefficient of variation (CV) of the groups was higher than the recommended CV (≤30) suggested by Wolever *et al.* (2008).

7.4.4 Within-individual variation

7.4.4.1 Effect of wash-out period

To our knowledge this is the first study that explored the effect of the length of washout period on GR. The iAUC and GR observed for the two days trial were not significantly different than those obtained after four days. Nevertheless, 2 days wash-out might be enough to avoid influence of the food from previous trial on the GR (Brouns *et al*., 2005). However, more foods need to be tested to confirm the results suggested in this study.

7.4.5 Variation within individuals

Standardized conditions for GI measurements seek to reduce potential variation within subjects. Brouns *et al.* (2005) suggested that selecting healthy subject will improve the GI results and reduce the variation within the subjects. Furthermore, repeating tests will allow characterization of variations within the subjects (Williams *et al*., 2008). In the present study, the CV within the subjects for the 25g av.CHO portion of reference food was below (CV= 28) the recommended values as suggested by Wolever *et al.* (2008) (CV≤ 30). While the CV for the 50g of av.CHO portion of reference food was slightly higher (CV=31) which may consider reliable results.

7.4.6 Application to the prediction models

Four foods were used to assess and validate the predicted and *in vitro* GI values from the previous chapters. The av.CHO content in each food was determined experimentally rather than calculated. *In vivo* measured GI correlates significantly with the predicted values and the measured GI values. Although the *in vivo* measured GI was significantly correlated to the *in vitro* estimated GI and statistically predicted GI values, the *in vivo* measured GI values are more variable than other approaches.

Moreover, although the CV of *in vivo* GI was within the recommendation by Wolever *et al*. (2008), the CV of *in vivo* GI was close to the CV values of *in vitro* starch digestion approach (except for white bread) as shown in Table 7.10.

Despite the SD values were within the acceptable ranges suggested by Wolever *et al.* (2003), this may affect the application of the *in vivo* approach to comply nutrients databases, in food labeling, or in the epidemiological fields by providing variable information.

	***In vivo* GI (n=13)**	***In vitro* starch digestion (n=4)**
50g av.CHO reference food[1]	28%	17%
Lentil soup	30%	31%
Lentil soup meal	28%	28%

[1] 50g av.CHO portion = 3 slices of bread

[2] 25g av.CHO portion = 1.5 slices of bread

Table 7.10: Coefficient of variation (CV) observed in *in vivo* GI measurement and *in vitro* starch digestion

As a result, prediction of GI values from macronutrient content using statistical analysis might be a practicable approach and may considered as a robust method for measuring GI without using human subjects and might be an appropriate approach to be used by researcher, dietitians, nutritionists and professionals.

Also, the statistically predicted and *in vitro* starch digestion might be useful in food labeling and epidemiology fields, instead of using the international table of GI and GL that gathered the information from all over the world

This approach might be useful to limit the need for human subjects and blood sample analysis.

7.4.7 Limitations

Small sample size is considered one of the weaknesses in this study, although novel information was gathered such as: using Arabic subjects and the effect of the washout period length. Therefore, recruiting more subjects would improve the statistical confidence. Finally, using four foods to assess the GI is small; more complex foods should be tested.

7.5 Conclusion

The present study successfully validated the *in vitro* estimated GI and the predicted GI values with *in vivo* GI values. Nevertheless, the result of the *in vivo* measured GI values were more variable than the other two approaches which may confirms the usefulness of a prediction approach as a tool to predict GI of mixed foods without using human subjects nor analyzing blood glucose.

Moreover, to get more reproducible and reliable results more subjects of different dietary habits and different ethnic groups should be recruited and more complex foods should be tested.

8 Chapter eight: general discussion

8.1 General discussion

The *in vivo* GI measurement for every single food is time-consuming, costly, and requires human subjects with consideration of ethical and logistical factors. In addition, variation within and across subjects of different populations may pose further considerations.

Therefore, this study investigated whether the GI of mixed foods can be predicted from macronutrient composition, and we investigated the prediction of GI of multi-component foods from macronutrient composition using different approaches. Several stages were carried out to develop a methodology to estimate the GI of simple and mixed dishes from macronutrient composition.

The first stage of the present study was the analytical methods for starch digestion and measuring av.CHO in cereals and legumes.

Determination of av.CHO by difference is a common method in food composition databases like those produced by the USDA. In the UK food composition tables the av.CHO and starch content were calculated in certain foods (breakfast cereals) by difference, while the av.CHO in the others (e.g. bread) were calculated by summation of the analyzed total starch and total soluble sugar together (Food Standards Agency, 2002). Moreover, the amount of av.CHO in legumes reported in McCance & Widdowson's The Composition of Foods dataset included the oligosaccharides, and to our knowledge, the oligosaccharides in legumes are neither digested nor absorbed in the small intestine and do not contribute to the GR, they are rather fermented by the micro-flora in the colon. Therefore, there is no standard method to measure av.CHO in food for composition purposes.

Furthermore, CHOs are known for being one of the main dietary nutrients, therefore it is important to quantify av.CHO in food experimentally to avoid unreliable results arising from accumulation of experimental errors (Menezes *et al*., 2009).

The Megazyme kit is sold for composition purpose and relies on the breakdown of starch by microbial enzymes to glucose, following by breakdown of sucrose to glucose and fructose, and finally determination of glucose using a hexokinase assay (HK) as suggested by McCleary (2007).

However, the protocol of starch digestion suggested by the Megazyme kit was not appropriate for the samples used in present study because it was originally intended to determine the soluble and insoluble total dietary fibre in food not av.CHO (Lee *et al*., 1992). Therefore a series of modifications were made to increase the yields of the av.CHO after starch digestion.

Despite many attempts to increase the yields of the av.CHO, the HK was only able to detect around 50% of the expected av.CHO due to several factors, such as: the presence of interference substances like protein, polyphenols or lipids in the samples (in the case of food samples) and secondly is the enzyme kinetics in which concentration of av.CHO maybe higher than the concentration of the HK enzymes which lead to substrate saturation and these sugars (e.g. maltose) might bind to the enzyme either non-competitively or uncompetitively because of their same affinity to the detection enzymes (Dona *et al*., 2010, Singh *et al.,* 2010). The difficulties mentioned above indicate that the HK is not suitable for measuring av.CHO in food. To our knowledge, no papers that we can find have used the Megazyme kit except two (McCleary, 2007, Mettler *et al*., 2007).

Several other methods of CHO detection were tested. Two colorimetric methods were investigated in this study, PS and DNS, because they were common, simple, inexpensive, reproducible and considered as broad spectrum methods where varied sugars can be detected and samples do not need to be diluted most of the time (Southgate, 1976, Hall, 2003, Jeong *et al*., 2010), whereas HPAE-PAD was investigated because of its high sensitivity, selectivity and only a small amount of sugar is needed to be detected (Southgate, 1976, Hall, 2003, Jeong *et al*., 2010).

Selection of the analytical method must be based on the purpose of the analysis and the type of sugar present in the samples, where in each method has its own interference, time of the analysis, availability and cost of the chemicals and apparatus used in the method. Additionally, specificity, sensitivity, reproducibility, accuracy, and most importantly precision are the major elements for choosing a method for CHO analysis. Therefore, phenol sulfuric assay (PS) was found to be the most suitable for av.CHO detection following starch digestion. Moreover, the modified starch digestion protocol (chapter3) was suitable for food of different matrices.

Linear regression models were generated statistically using the macronutrient composition then they were applied to predict GI in multi-component foods, as the main aim of this stage. As mentioned previously, regardless the harmonization conducted the measurement of GI (FAO/WHO, 1998, International Organization for Standardization, 2010), there is no internationally agreed standard protocol . Several procedural variations are still seen in GI measurements: the number of subjects (≤10), the blood sampling method (capillary blood versus venous plasma), the selection of the reference food, repetition of experiments (2-3 times), and measurement of av.CHO (International

Organization for Standardization, 2010, Simila et al., 2011) (mentioned earlier in section 1.1.3.1.1). Also, there are debates regarding the variations that take place within and between the subjects, even though 50% of these variations will be diminished after expressing the GR as GI values as suggested previously (Wolever *et al.*, 1985), also with increased numbers of foods consumed and the availability of the international of GI and GI that cover more than 2,400 GI values it is not feasible to cover the entire list of foods consumed (Schakel *et al*., 2008) and measuring GI every single food is not practical too.

For this reason a practicable approach is required for measuring GI like predicting GI of multi-component foods using information regarding nutrient content.

In this stage, the method was adapted from Urooj and Puttaraj (2000) with modifications regarding the food selections, in which the food that was selected was not country-specific. The food used in the study of Urooj and Puttaraj (2002) was specific to south Asians, whereas in the present study 40 starchy foods samples (24 cereals & 16 legumes) were used to assess the prediction of GI from food composition tables (Food Standards Agency, 2002) using statistical models.

Three prediction models (mixed, cereal, and legume prediction models) were generated and starch was the main predictor present in all the models. The predicted GI values were significantly correlated ($P \leq 0.05$) to the published GI values in the international table of GI and GI. However, since the starch contents were calculated in the breakfast cereals (Food Standards Agency, 2002), the amount of measured av.CHO previously substituted the calculated values and the predicted values were even more improved.

The findings in present study disagreed with Urooj and Puttaraj (2002) and Flint *et al.* (2004) but might be due to several reasons such as the study design; the study of Urooj and Puttaraj was for south Asian subjects using south Asian mixed food, whereas Flint *et al.* (2004) used breakfast cereals that were commonly consumed in Europe. Also, sample size in the previous (Urooj and Puttaraj, 2000) study was 4.5 times higher than the present study. Finally, they generated the prediction models from proteins, fat, energy (kJ), fibre, and starch, whereas soluble sugars were involved in this study and only starch and fat were contributing to the prediction models.

The findings in the present study may provide evidence that GI of food could be predicted from its nutrient content using statistical analysis. For more certainty, validation of models via non-restricted *in vitro* starch digestion and *in vivo* GI measurements in multi-component foods were conducted.

Validation of the predicted GI using non-restricted *in vitro* starch hydrolysis was carried out. Furthermore, the origin of the digestive enzymes was investigated too. Many studies (Monro *et al*., 2010) conducted to measure the rate of starch hydrolysis *in vitro* using a mixture of enzymes from different origin (bacterial, fungal, or animal). The results from pancreatic enzymes were significantly correlated with the microbial enzymes. This factor might be considered the first that has been investigated in this study and may suggest the possibility of replacing pancreatic enzymes with microbial enzymes for *in vitro* digestion because microbial enzymes may be easier to obtain and might be less expensive than pancreatic enzymes. However, more research is required to give deeper understanding regarding this factor (Hoover and Zhou, 2003, Hur *et al*., 2011).

Moreover, the results from (non-restricted *in vitro* starch digestion) correlated significantly with the statistically GI predicted unlike Urooj and Puttaraj (2000). The disagreement might be due to the type of the *in vitro* starch digestion, in which they used restricted *in vitro* starch digestion using dialysis bag and the sugars released were analyzed by the glucose oxidase method.

Also, Urooj and Puttaraj (2000) used a multi-component south Asian dishes (n=6) while in the present study three foods were used to assess the validation (lentil soup, lentil soup meal and white bread) and increasing the food samples or using more complex dishes may improve the results even further. The findings might provide evidence that GI of food can be predicted from its nutrient content using statistical analysis when validated with non-restricted *in vitro* starch digestion.

The aim of the *in vivo* GI measurement (chapter 7) was to further validate the estimated *in vitro* GI (chapter 6) and the statistically predicted GI (chapter 5). However, several factors that affecting the GR were explored at this stage too, such as the effect of demographic factor like ethnicity.

To our knowledge there are limited studies regarding the Arab-countries group, therefore, this study considered the first that explored the GR of this group using realistic complex foods rather than dates with coffee or with yogurt (Miller *et al*., 2003, Al-Mssallem and Brown, 2013), just varieties of dates (Alkaabi *et al*., 2011, Ahmed, 2002, Miller *et al*., 2002), types of breads (Takruri and Alkurd, 2008, Almousa *et al*., 2013), or single food like chickpeas or potato (Zafar *et al*., 2011). Also, this is the first study that compared this group with other subjects from different backgrounds. There are studies that compare

Arabic group with other ethnic groups but not in testing the GI (Dickinson *et al*., 2002). Dickinson *et al.* (2002) investigated the metabolic biomarkers like GR and insulin response after ingestion 75 g of av.CHO portion of white bread. Despite the different responses elicited after consuming the test and references foods in each ethnic group, the Arabic-countries subjects did not show any significant difference in GR from other subjects after testing the reference, agreeing with (Dickinson *et al*., 2002). Moreover, there were no significant differences in GI values between the three ethnic groups, despite the different shapes of GR. This confirmed that GR is the property of the subjects while the GI is the property of the food and the human variation will diminish once GR is expressed as GI (Wolever *et al*., 1985).

According to Brouns *et al.* (2005) there was not any study that investigates the effect of the length of washout period on the GR and in this study it has been explored for the first time. The results suggested that GR obtained after 2 days might be enough to avoid this influence of the food and may improve the GR.

In vivo GI measurement was conducted using four foods with different CHO contents and 13 subjects according the method suggested by WHO/FAO (1998) and Brouns *et al.* (2005). Unlike other studies av.CHO content of the food was measured.

Although, *in vivo* measured GI values were higher than *in vitro* predicted values, there were no significant differences between the three approaches and the differences were within the standard error of the *in vivo* results (Table 8.1). The *in vivo* measured GI values were more variable, having a high standard deviation (SD), this is due to inter and intra individual variation. Variation may affect the application of the *in vivo* approach to

compile nutrients databases, in food labeling, or in the epidemiological fields by providing misleading information.

Food	Published GI[1]	Measured GI[2]	Estimated GI[3]	Predicted GI[4]
Lentil soup	40	38 17	34± 3	40
Lentil soup meal	57	65± 17	60± 6	58
White bread	71	70 ± 0	70± 0	75

[1] GI published in the international table of GI and GL by (Atkinson *et al*., 2008)

[2]GI measured *in vivo* using (n=13)

[3]GI estimated in chapter 6 using non-restricted *in vitro* starch hydrolysis (n=4)

[4] GI obtained from the prediction models in chapter 5

Table 8.1: Comparison between GI values obtained by different approaches. Data expressed as mean of the GI± SD).

As a result, by using prediction of GI values from macronutrient content using statistical analysis and associated with the non-restricted *in vitro* starch hydrolysis might be a practicable approach for measuring GI without using human subjects or blood analysis and might be appropriate approach to be used in research and industry instead of using the international table of GI and GL.

Moreover, the disagreement might be because that the study of Urooj and Puttaraj (2000) validated their prediction model using unrestricted *in vitro* starch digestion and this method is not recommended since glucose is transported not diffused in the body.

8.1.1 GI and major issues

GI concept attracted the attention of many researchers and investigators to measure the usefulness application of GI in complying food databases or in the clinical and epidemiological fields (Foster-Powell and Miller, 1995).

The international table for GI and GL was established in 1995 by Foster-Powell and brand-Miller in Australia and the purpose was to gather all the GI values to be used by researcher, health professional and etc. The last up-to-date edition was published in 2008 with 2,480 food items gathered with same principle as the previous editions (Atkinson *et al*., 2008). In addition, in 2004 an electronic GI database which can be access through the internet was created in University of Sydney by Brand-Miller (http://www.glycemicindex.com).

Majority of databases have been using the GI values from international table of GI and GL to compile their own food composition database, some studies have documented the methodology for adding the GI values to their database such as (Martin *et al*., 2008, Schakel *et al*., 2008, Aston *et al*., 2010). The food survey research group of the USDA used the international table to compile their food composition tables (Martin *et al*., 2008). In UK, the MRC human nutrition research group also used the international tables of GI and GL (http://www.mrc-hnr.cam.ac.uk/research/gi-database). Cork university hospital in the republic of Ireland updated their nutrition analysis software package through the international table of GI and GL to Levis *et al.* (2011).

Moreover, several studies used the international table of GI and GL for their dietary surveys like the National Health and the Nutrition Examination Survey Nutrient Database

(NHNES) (Lin *et al.*, 2012) and for dietary assessment purposes such as Schakel *et al.* (2008) in USA, Barclay *et al.* (2008a) in Australia, and Aston *et al.* (2010) in Europe.

The role of CHO and low GI foods have been investigated systematically and intensively by researchers and health professional, it has been observed that low GI diet reduces the risk of developing chronic diseases such cardiovascular diseases, diabetes, and obese subjects and obesity (Thomas *et al*., 2007, Barclay *et al*., 2008b, Thomas and Elliott, 2009).

The GI information used in these studies was pulled either from previous cohort studies (Barclay *et al*., 2008b, Hardy *et al*., 2010, Dong *et al*., 2011), from previous literatures (Thomas *et al*., 2007, Livesey *et al*., 2008, Thomas and Elliott, 2009) or from the international table of GI and GL GI and food labelling (Barclay *et al*., 2008a).

Since the previous epidemiological studies found that consuming low GI food might be associated with lowering the risk of non-communicable diseases (Barclay *et al*., 2008b), health professionals suggested the labelling CHO-containing food might be useful tool to promote healthy food choices for the consumers (Mitchell, 2008).

The aim of labelling is to provide the consumers with nutrient composition of the food to guide their selections. Therefore, several GI foundations (Australia and South Africa) were established to provide people with information regarding low GI diets through food labeling (Mitchell, 2008). Moreover, consumers must be educated regarding the certain low GI foods such as food high with fat like chocolate (Mitchell, 2008). Foods will not be GI labelled unless they were CHO-containing foods (≥10 av.CHO/100g) or providing 40-50% of energy from av.CHO, tested with standardized method in accredited laboratories

using at least 10 healthy subjects and a detailed and clear description regarding the methodology (number, statues of the subjects, reference food, AUC calculation, and SD) (Mitchell, 2008).

To our knowledge, using the international table of GI and GL for labelling might be confusing because of the methodological issues mentioned previously (Schakel *et al*., 2008) and measuring every single food for GI labeling as suggested is not applicable (Brand-Miller *et al*., 2003, Mitchell, 2008). Therefore, the GI modelling in the present study might be useful for diet planning, developing food composition data or food labelling.

8.2 The obstacles and limitations of the study

The amount of soluble sugars and av.CHO especially in legumes were poorly detected due to issues with the machine. Because of the limited time, more food need to be used to evaluate the av.CHO and more food matrixes need to be tested to find out the appropriate methods for certain foods, and analysis of other macronutrients like protein and fat for example was not conducted. In addition, analysis of the anti-nutrients (e.g. polyphenols or phatic acid) might be useful and may provide deep understanding on their effect on GR.

Because of the limited number of enzymes commercially available the effect of the enzymes origin on the rate of starch digestion was only tested with amylases (bacterial and pancreatic origins).

Moreover, the sample size for the human study was suitable for GI validation but was too small to reach any significant levels regarding the factors affect the GR and GI. The study

may provide preliminary results and might be useful for further studies to test specific hypothesis.

Information regarding legumes in mixed food is limited and more investigation of each type of legume consumed in a mixed meal is needed.

The effect of more complex and multi-component foods on the rate of starch digestion using non-restricted *in vitro* starch hydrolysis may improve the prediction models and can be achieved by testing more food samples.

Moreover, more food samples need to be tested so that an improvement in assessing the prediction models may be achieved.

In summary, because of limited time the weakness of present study was the low number of samples and subjects used to assess the prediction models which meant that the results of the present study might be only valid for the samples and subjects tested and with increase of the sample size the result might be improved.

8.3 Usefulness of the study

After 30 years of the establishment of the GI concept by Jenkins *et al*, still there are challenges in using GI for the development of food database, in the epidemiological studies, or labelling because of the methodological variation mentioned earlier.

It has been suggested that "more food should be tested for GI" (Brand-Miller *et al*., 2003), and according to Mitchell (2008) regarding CHO-containing foods labelling that GI should be analyzed using standardized method in accredited laboratories in at least 10 healthy subjects and a detailed and clear description regarding the methodology should be

provided with labelling. However, this is unrealistic due to the large number of food product and the complexity of diet, therefore a statistical approach would be more practical.

This approach might be useful in the development of food databases, for the epidemiological studies, or GI food labelling with the understanding that GI is highly variable and therefore not only health indicator. Furthermore, education might be useful for consumers to understand GI labelling of food.

8.4 Conclusion

In conclusion measuring av.CHO in food by difference rather than experimentally is not adequate in terms of GI because it may overestimate the av.CHO content especially in food rich in resistant starch, and measuring av.CHO by difference do not provide detailed information about each individual sugar, which may have different GR.

The role of using different digestive enzymes origin on the rate of starch digestion does not show any significant differences and *in vitro* starch digestion is highly correlated with *in vivo* which measures the robustness of using microbial over porcine pancreatic enzymes.

The statistically predicted GI values may offer an alternative to *in vitro* and *in vivo* GI methodology. Since GI might be useful as consumer guide for food selection, the present study found that with GI modelling might be useful for diet planning, developing food composition database or food labelling, whereas *in vitro* starch hydrolysis might useful in case of predicting GI in single food and both approaches together may reduce the need for human subject or blood analysis for measuring GI in food.

8.5 Future work

Because of time limitation, more reproducible and reliable results might be achieved with bigger sample size and more tested foods to enhance the validity of the approaches. Also, it would be useful for the generation the GI prediction models to analyze other component like protein, fat or polyphenols.

Moreover, in late 90's Kuwait Institute for scientific research (KISR) has established the first edition of the food composition of Kuwaiti composite dishes (Sawaya *et al*., 1998), the CHO content in this database was calculated by differences rather than analyzed. As a result, CHO content of the Kuwaiti composite dishes will be analyzed using the suitable analytical methods for measuring CHO. In addition, applying the prediction models using Kuwaiti food might be useful for the composition databases.

Lately Diabetes mellitus considered one of the most important threats to human, the number of people with type II diabetes is globally and largely increase, including the Arabic countries since some of them have the highest incidence of type II diabetes, due to several reasons such development, aging, urbanization, obesity and lack of physical activity (Badran and Laher, 2012). The Arab Gulf countries, which are oil-producing countries, in particular known for having the highest prevalence of type II diabetes due to high income, using cheap immigrant labor, high food consumption and obesity, and physical inactivity (Badran and Laher, 2012). Kuwait considered the first country with the highest prevalence of type II diabetes among the Gulf countries (Badran and Laher, 2012), also 80 % of Kuwait population considered as either obese or over weight.

Moreover, there is only one study conducted in Kuwait with Kuwaiti females testing single foods mainly (Zafar *et al*., 2011), plus there is limited information regarding the GR of subjects from Arabic countries, investigating the GR in this group would be useful using wide selection of foods and applying this approach might be helpful for reducing the development of type II diabetes since there is a correlation between consuming low GI food (food rich with NSP) and the prevalence of type II diabetes (Thomas and Elliott, 2009).

References

REFERENCES

AHMED, M. 2002. Glycemic index of 3 varieties of dates. *Saudi Medical Journal,* 23, 1426-1426.

AL-MSSALLEM, M. Q. & BROWN, J. E. 2013. Arabic coffee increases the glycemic index but not insulinemic index of dates. *Saudi Medical Journal,* 34, 923-8.

ALKAABI, J. M., AL-DABBAGH, B., AHMAD, S., SAADI, H. F., GARIBALLA, S. & AL GHAZALI, M. 2011. Glycemic indices of five varieties of dates in healthy and diabetic subjects. *Nutrition Journal,* 10, 59-68.

ALMOUSA, A., THOMAS, M., SIDDIEG, H., VARGHESE, S. & ABUSNANA, S. 2013. The Glycemic Index of Traditional Types of Bread in UAE. *Nutrition and Food,* 3, 1-3.

ALSAFFAR, A. A. 2011. Effect of food processing on the resistant starch content of cereals and cereal products - a review. *International Journal of Food Science and Technology,* 46, 455-462.

APATA, D. F. 2008. Effect of cooking methods on available and unavailable carbohydrates of some tropical grain legumes. *African Journal of Biotechnology,* 7, 2940-2945.

ARAYA, H., CONTRERAS, P., ALVINA, M., VERA, G. & PAK, N. 2002. A comparison between an in vitro method to determine carbohydrate digestion rate and the glycemic response in young men. *European Journal of Clinical Nutrition,* 56, 735-739.

ASP, N.-G. & BJORCK, I. 1992. Resistant starch. *Trends in Food Science and Technology,* 3, 111-114.

ASTON, L. M., JACKSON, D., MONSHEIMER, S., WHYBROW, S., HANDJIEVA-DARLENSKA, T., KREUTZER, M., KOHL, A., PAPADAKI, A., MARTINEZ, J. A., KUNOVA, V., VAN BAAK, M. A., ASTRUP, A., SARIS, W. H. M., JEBB, S. A. & LINDROOS, A. K. 2010. Developing a methodology for assigning glycaemic index values to foods consumed across Europe. *Obesity Reviews,* 11, 92-100.

ATKINSON, F. S., FOSTER-POWELL, K. & BRAND-MILLER, J. C. 2008. International tables of glycemic index and glycemic load values: 2008. *Diabetes Care,* 31, 2281-2283.

BADRAN, M. & LAHER, I. 2012. Type II Diabetes Mellitus in Arabic-Speaking Countries. *International Journal of Endocrinology,* 2011, 11 pages.

BALLANCE, S., SAHLSTROM, S., LEA, P., NAGY, N. E., ANDERSEN, P. V., DESSEV, T., HULL, S., VARDAKOU, M. & FAULKS, R. 2013. Evaluation of gastric processing and duodenal digestion of starch in six cereal meals on the associated glycaemic response using an adult fasted dynamic gastric model. *European Journal of Nutrition,* 52, 799-812.

BARCLAY, A. W., FLOOD, V. M., BRAND-MILLER, J. C. & MITCHELL, P. 2008a. Validity of carbohydrate, glycaemic index and glycaemic load data obtained using a semi-quantitative food-frequency questionnaire. *Public Health Nutrition,* 11, 573-80.

BARCLAY, A. W., PETOCZ, P., MCMILLAN-PRICE, J., FLOOD, V. M., PRVAN, T., MITCHELL, P. & BRAND-MILLER, J. C. 2008b. Glycemic index, glycemic load, and chronic disease risk - a metaanalysis of observational studies. *American Journal of Clinical Nutrition,* 87, 627-637.

BERGMAN, B. C., PERREAULT, L., HUNERDOSSE, D., KEREGE, A., PLAYDON, M., SAMEK, A. M. & ECKEL, R. H. 2012. Novel and reversible mechanisms of smoking-induced Insulin resistance in humans. *Diabetes,* 61, 3156-3166.

BORNET, F. R., COSTAGLIOLA, D., RIZKALLA, S. W., BLAYO, A., FONTVIEILLE, A. M., HAARDT, M. J., LETANOUX, M., TCHOBROUTSKY, G. & SLAMA, G. 1987. Insulinemic and glycemic indexes of six starch-rich foods taken alone and in a mixed meal by type 2 diabetics. *American Journal of Clinical Nutrition,* 45, 588-95.

BRAND-MILLER, J., FOSTER-POWELL, K. & COLAGIURI 1996. *The GI factor: The glycaemic index solution.,* Rydelmere. Hodder & Stoughton.

BRAND-MILLER, J., HAYNE, S., PETOCZ, P. & COLAGIURI, S. 2003. Low-glycemic index diets in the management of diabetes. *Diabetes Care,* 26, 2261-2267.

BRAND-MILLER, J. & HOLT, S. 2004. Testing the glycaemic index of foods: in vivo, not in vitro. *European Journal of Clinical Nutrition,* 58, 700-701.

BRAND-MILLER, J., MCMILLAN-PRICE, J., STEINBECK, K. & CATERSON, I. 2009. Dietary Glycemic Index: Health Implications. *Journal of the American College of Nutrition,* 28, 446s-449s.

BROUNS, F., BJORCK, I., FRAYN, K. N., GIBBS, A. L., LANG, V., SLAMA, G. & WOLEVER, T. M. S. 2005. Glycaemic index methodology. *Nutrition Research Reviews,* 18, 145-171.

BURTON, P. & LIGHTOWLER, H. J. 2008. The impact of freezing and toasting on the glycaemic response of white bread. *European Journal of Clinical Nutrition,* 62, 594-599.

CALDWELL, E. & FAST, R. (eds.) 2000. Breakfast cereals and how they are made

St. Paul, Minnesota, USA: American Association of Cereal Chemists, inc.

CAMPBELL, J. E., GLOWCZEWSKI, T. & WOLEVER, T. M. S. 2002. Controlling subjects' prior diet and activities does not reduce within-subject variation of glycemic responses. *Journal of Federation of American Societies for Experimental Biology,* 16, A631-A631.

CANDIDO, F. G., PEREIRA, E. V. & ALFENAS, R. D. G. 2013. Use of the glycemic index in nutrition education. *Revista De Nutricao-Brazilian Journal of Nutrition,* 26, 89-96.

CHARALAMPOPOULOS, D., WANG, R., PANDIELLA, S. S. & WEBB, C. 2002. Application of cereals and cereal components in functional foods: a review. *International Journal of Food Microbiology,* 79, 131-141.

CHUNG, H. J., SHIN, D. H. & LIM, S. T. 2008. In vitro starch digestibility and estimated glycemic index of chemically modified corn starches. *Food Research International,* 41, 579-585.

COLONNA, P., LELOUP, V. & BULEON, A. 1992. Limiting Factors of Starch Hydrolysis. *European Journal of Clinical Nutrition,* 46, S17-S32.

COULSTON, A. M., HOLLENBECK, C. B., LIU, G. C., WILLIAMS, R. A., STARICH, G. H., MAZZAFERRI, E. L. & REAVEN, G. M. 1984. Effect of source of dietary carbohydrate on plasma glucose, insulin, and gastric inhibitory polypeptide responses to test meals in subjects with noninsulin-dependent diabetes mellitus. *American Journal of Clinical Nutrition,* 40, 965-70.

CUMMINGS, J. H. & ENGLYST, H. N. 1995. Gastrointestinal effects of food carbohydrate. *American Journal of Clinical Nutrition,* 61, 938S-945S.

DEAN, A. C. 1978. Method for Estimation of Available Carbohydrate in Foods. *Food Chemistry,* 3, 241-250.

DECOSTER, J. (2004). Data Analysis in SPSS. [Access date: 27.03.2014] (Downloaded from http://www.stat-help.com/notes.html.)

DENOVA-GUTIERREZ, E., HUITRON-BRAVO, G., TALAVERA, J. O., CASTANON, S., GALLEGOS-CARRILLO, K., FLORES, Y. & SALMERON, J. 2010. Dietary glycemic index, dietary glycemic load, blood lipids, and coronary heart disease. *Journal of Nutrition and Metabolism,* 2010, 1-8.

DEPARTMENT OF HEALTH 1991. *Dietary Reference Values for Food Energy and Nutrients in the United Kingdom. Report on health and social subjects,* Norwich, The Stationer Office.

DEWETTINCK, K., VAN BOCKSTAELE, F., KUHNE, B., DE WALLE, D. V., COURTENS, T. M. & GELLYNCK, X. 2008. Nutritional value of bread: Influence of processing, food interaction and consumer perception. *Journal of Cereal Science,* 48, 243-257.

DICKINSON, S., COLAGIURI, S., FARAMUS, E., PETOCZ, P. & BRAND-MILLER, J. C. 2002. Postprandial hyperglycemia and insulin sensitivity differ among lean young adults of different ethnicities. *Journal of Nutrition,* 132, 2574-2579.

DODD, H., WILLIAMS, S., BROWN, R. & VENN, B. 2011. Calculating meal glycemic index by using measured and published food values compared with directly measured meal glycemic index. *American Journal of Clinical Nutrition,* 94, 992-996.

DONA, A. C., PAGES, G., GILBERT, R. G. & KUCHEL, P. W. 2010. Digestion of starch: In vivo and in vitro kinetic models used to characterise oligosaccharide or glucose release. *Carbohydrate Polymers,* 80, 599-617.

DONG, J. Y., ZHANG, L. J., ZHANG, Y. H. & QIN, L. Q. 2011. Dietary glycaemic index and glycaemic load in relation to the risk of type 2 diabetes: a meta-analysis of prospective cohort studies. *British Journal of Nutrition,* 106, 1649-1654.

DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. 1956. Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry,* 28, 350-356.

ENGLYST, H. N., KINGMAN, S. M. & CUMMINGS, J. H. 1992. Classification and Measurement of Nutritionally Important Starch Fractions. *European Journal of Clinical Nutrition,* 46, S33-S50.

ENGLYST, K. N. & ENGLYST, H. N. 2005. Carbohydrate bioavailability. *British Journal of Nutrition,* 94, 1-11.

ENGLYST, K. N., ENGLYST, H. N., HUDSON, G. J., COLE, T. J. & CUMMINGS, J. H. 1999. Rapidly available glucose in foods: an in vitro measurement that reflects the glycemic response. *American Journal of Clinical Nutrition,* 69, 448-454.

ESTEVES DE OLIVEIRA, F. C., PINHEIRO VOLP, A. C. & ALFENAS, R. C. 2011. Impact of different protein sources in the glycemic and insulinemic responses. *Nutrición Hospitalaria,* 26, 669-76.

EZEOGU, L. I., DUODU, K. G. & TAYLOR, J. R. N. 2005. Effects of endosperm texture and cooking conditions on the in vitro starch digestibility of sorghum and maize flours. *Journal of Cereal Science,* 42, 33-44.

FAO 1998. Carbohydrates in Human Nutrition: Report of a Joint FAO/WHO Expert Consultation. *Food and Agriculture Organisation.* Rome: FAO Food and Nutrition Paper

FAO/WHO 1998. Carbohydrates in human nutrition. *Report of a joint FAO/WHO expert consultation.* April 14-18, 1997 ed. Rome: FAO Food and Nutrition Paper, FAO.

FLINT, A., MOLLER, B. K., RABEN, A., PEDERSEN, D., TETENS, I., HOLST, J. J. & ASTRUP, A. 2004. The use of glycaemic index tables to predict glycaemic index of composite breakfast meals. *British Journal of Nutrition,* 91, 979-989.

FOOD STANDARDS AGENCY (ed.) 2002. *McCance & Widdowson's The Composition of Foods,* Cambridge: Royal Society of Chemistry.

FOSTER-POWELL, K., HOLT, S. H. A. & BRAND-MILLER, J. C. 2002. International table of glycemic index and glycemic load values: 2002. *American Journal of Clinical Nutrition,* 76, 5-56.

FOSTER-POWELL, K. & MILLER, J. B. 1995. International tables of glycemic index. *American Journal of Clinical Nutrition,* 62, 871S-890S.

GERMAINE, K. A., SAMMAN, S., FRYIRS, C. G., GRIFFITHS, P. J., JOHNSON, S. K. & QUAIL, K. J. 2008. Comparison of in vitro starch digestibility methods for

predicting the glycaemic index of grain foods. *Journal of the Science of Food and Agriculture,* 88, 652-658.

GIBNEY, M., LANHAM-NEW, S., CASSIDY, A. & VORSTER, H. (eds.) 2009. *Introduction to Human Nutrition,* Oxford, UK: Blackwell Publishing.

GIBSON, N., SCHONFELDT, H. C. & PRETORIUS, B. 2011. Development of a rapid assessment method for the prediction of the glycemic index. *Journal of Food Composition and Analysis,* 24, 750-754.

GLYCEMIC INDEX: http://www.glycemicindex.com [access date: 14.12.13]

GONI, I., GARCIAALONSO, A. & SAURACALIXTO, F. 1997. A starch hydrolysis procedure to estimate glycemic index. *Nutrition Research,* 17, 427-437.

GRANFELDT, Y., BJORCK, I., DREWS, A. & TOVAR, J. 1992. An in vitro procedure based on chewing to predict metabolic response to starch in cereal and legume products. *European Journal of Clinical Nutrition* 46, 649-60.

GRANFELDT, Y., WU, X. & BJORCK, I. 2006. Determination of glycaemic index; some methodological aspects related to the analysis of carbohydrate load and characteristics of the previous evening meal. *European Journal of Clinical Nutrition,* 60, 104-112.

GREENFIELD, H. & SOUTHGATE, D. 2003. *Food composition data: production, management, and use,* Rome, Food and Agriculture Organization

GUEVARRA, M. T. B. & PANLASIGUI, L. N. 2000. Blood glucose responses of diabetes mellitus type II patients to some local fruits. *Asia Pacific Journal of Clinical Nutrition,* 9, 303-308.

GUZEL, D. & SAYAR, S. 2010. Digestion profiles and some physicochemical properties of native and modified borlotti bean, chickpea and white kidney bean starches. *Food Research International,* 43, 2132-2137.

HALL, M. B. 2003. Challenges with nonfiber carbohydrate methods. *Journal of Animal Science,* 81, 3226-3232.

HARDY, D. S., HOELSCHER, D. M., ARAGAKI, C., STEVENS, J., STEFFEN, L. M., PANKOW, J. S. & BOERWINKLE, E. 2010. Association of glycemic index and glycemic load with risk of incident coronary heart disease among Whites and African Americans with and without type 2 diabetes: the Atherosclerosis Risk in Communities study. *Annals of Epidemiology,* 20, 610-6.

HÄTÖNEN, K. A., SIMILÄ, M. E., VIRTAMO, J. R., ERIKSSON, J. G., HANNILA, M.-L., SINKKO, H. K., SUNDVALL, J. E., MYKKÄNEN, H. M. & VALSTA, L. M. 2006. Methodologic considerations in the measurement of glycemic index: glycemic response to rye bread, oatmeal porridge, and mashed potato. *The American Journal of Clinical Nutrition,* 84, 1055-1061.

HATONEN, K. A., VIRTAMO, J., ERIKSSON, J. G., PERALA, M. M., SINKKO, H. K., LEIVISKA, J. & VALSTA, L. M. 2012. Modifying effects of alcohol on the

postprandial glucose and insulin responses in healthy subjects. *American Journal of Clinical Nutrition,* 96, 44-49.

HENDERSON, L., GREGORY, J., IRVING, K. & SWAN, G. 2003. The National Diet & Nutrition Survey: adults aged 19 to 64 years energy, protein, carbohydrate, fat and alcohol intake. *National Statistics and Medical Research Council Human Nutrition Research,* 2, 1-106.

HENRY, C. J. K., LIGHTOWLER, H. J., KENDALL, F. L. & STOREY, M. 2006. The impact of the addition of toppings/fillings on the glycaemic response to commonly consumed carbohydrate foods. *European Journal of Clinical Nutrition,* 60, 763-769.

HENRY, C. J. K., LIGHTOWLER, H. J., NEWENS, K., SUDHA, V., RADHIKA, G., SATHYA, R. M. & MOHAN, V. 2008a. Glycaemic index of common foods tested in the UK and India. *British Journal of Nutrition,* 99, 840-845.

HENRY, C. J. K., LIGHTOWLER, H. J., NEWENS, K. J. & PATA, N. 2008b. The influence of adding fats of varying saturation on the glycaemic response of white bread. *International Journal of Food Sciences and Nutrition,* 59, 61-69.

HETTIARATCHI, U. P. K., EKANAYAKE, S. & WELIHINDA, J. 2012. Prediction of glycaemic indices (GI) of meals by starch hydrolysis indices. *International Food Research Journal,* 19 1153-1159

HOOVER, R. & ZHOU, Y. 2003. In vitro and in vivo hydrolysis of legume starches by alpha-amylase and resistant starch formation in legumes - a review. *Carbohydrate Polymers,* 54, 401-417.

HUR, S. J., LIM, B. O., DECKER, E. A. & MCCLEMENTS, D. J. 2011. In vitro human digestion models for food applications. *Food Chemistry,* 125, 1-12.

HUTCHINS, A. M., WINHAM, D. M. & THOMPSON, S. V. 2012. Phaseolus beans: impact on glycaemic response and chronic disease risk in human subjects. *British Journal of Nutrition,* 108, S52-S65.

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION 2010. Food products - Determination of the glycaemic index (GI) and recommendation for food classification. *International Standard Organization.* Switzerland.

JAGER, A. V., TONIN, F. G. & TAVARES, M. F. 2007. Comparative evaluation of extraction procedures and method validation for determination of carbohydrates in cereals and dairy products by capillary electrophoresis. *Journal of Separation Science,* 30, 586-94.

JENKINS, A. L. 2007. The glycemic index: Looking back 25 years. *Cereal Foods World,* 52, 50-53.

JENKINS, D. J. A., WOLEVER, T. M. S., TAYLOR, R. H., BARKER, H., FIELDEN, H., BALDWIN, J. M., BOWLING, A. C., NEWMAN, H. C., JENKINS, A. L. & GOFF, D. V. 1981. Glycemic indes of foods - a physiological-basis for carbohydrate exchange. *American Journal of Clinical Nutrition,* 34, 362-366.

JEONG, W. H., HARADA, K., YAMADA, T., ABE, J. & KITAMURA, K. 2010. Establishment of new method for analysis of starch contents and varietal differences in soybean seeds. *Breeding Science,* 60, 160-163.

KATAOKA, M., VENN, B. J., WILLIAMS, S. M., TE MORENGA, L. A., HEEMELS, I. M. & MANN, J. I. 2013. Glycaemic responses to glucose and rice in people of Chinese and European ethnicity. *Diabetic Medicine* 30, e101-7.

KIM, H. J. & WHITE, P. J. 2012. In vitro digestion rate and estimated glycemic index of oat flours from typical and high beta-glucan oat lines. *Journal of Agricultural and Food Chemistry* 60, 5237-42.

LEE, S. C., PROSKY, L. & DEVRIES, J. W. 1992. Determination of total, soluble, and insoluble dietary fiber in foods - enzymatic gravimetric method, Mes-Tris Buffer - collaborative Study. *Journal of AOAC International,* 75, 395-416.

LEE, S. H., PARK, H. J., CHUN, H. K., CHO, S. Y., CHO, S. M. & LILLEHOJ, H. S. 2006. Dietary phytic acid lowers the blood glucose level in diabetic KK mice. *Nutrition Research,* 26, 474-479.

LEEDS, A., BRAND-MILLER, J., FOSTER-POWELL, K. & COLAGUIRI, S. 1998. *The GI Factor*, London. Hodder & Stoughton.

LEEMAN, M., OSTMAN, E. & BJORCK, I. 2005. Vinegar dressing and cold storage of potatoes lowers postprandial glycaemic and insulinaemic responses in healthy subjects. *European Journal of Clinical Nutrition,* 59, 1266-1271.

LEVIS, S. P., MCGOWAN, C. A. & MCAULIFFE, F. M. 2011. Methodology for adding and amending glycaemic index values to a nutrition analysis package. *British Journal of Nutrition,* 105, 1117-32.

LIGHTOWLER, H. J. & HENRY, C. J. K. 2009. Glycemic response of mashed potato containing high-viscocity hydroxypropylmethylcellulose. *Nutrition Research,* 29, 551-557.

LIN, C. S., KIMOKOTI, R. W., BROWN, L. S., KAYE, E. A., NUNN, M. E. & MILLEN, B. E. 2012. Methodology for Adding Glycemic Index to the National Health and Nutrition Examination Survey Nutrient Database. *Journal of the Academy of Nutrition and Dietetics,* 112, 1843-1851.

LINDEBOOM, N., CHANG, P. R. & TYLER, R. T. 2004. Analytical, biochemical and physicochemical aspects of starch granule size, with emphasis on small granule starches: A review. *Starch-Starke,* 56, 89-99.

LIVESEY, G., TAYLOR, R., HULSHOF, T. & HOWLETT, J. 2008. Glycemic response and health - a systematic review and meta-analysis: relations between dietary glycemic properties and health outcomes. *American Journal of Clinical Nutrition,* 87, 258s-268s.

LUDWIG, A. K., GOHARIAN, L. G., DIETZE, T., TAUCHERT, S., RUDOLF, S., DIEDRICH, K., SCHWEIGER, U. & OLTMANNS, K. M. 2009. Impact of glycemic variations on the regulation of androgen metabolism in obese women with polycystic ovary syndrome. *Fertility and Sterility,* 92, 271-6.

MALKOVA, D., EVANS, R. D., FRAYN, K. N., HUMPHREYS, S. M., JONES, P. R. M. & HARDMAN, A. E. 2000. Prior exercise and postprandial substrate extraction across the human leg. *American Journal of Physiology-Endocrinology and Metabolism,* 279, E1020-E1028.

MARTIN, C. L., MURPHY, S. P. & AU, D. L. M. 2008. Compiling glycemic index and glycemic load values for addition to a food composition database. *Journal of Food Composition and Analysis,* 21, 469-473.

MCCLEARY, B. V. 2007. An integrated procedure for the measurement of total dietary fibre (including resistant starch), non-digestible oligosaccharides and available carbohydrates. *Analytical and Bioanalytical Chemistry,* 389, 291-308.

MCCRORY, M. A., HAMAKER, B. R., LOVEJOY, J. C. & EICHELSDOERFER, P. E. 2010. Pulse Consumption, Satiety, and Weight Management. *Advances in Nutrition,* 1, 17-30.

MENEZES, E. W., GIUNTINI, E. B., DAN, M. C. T. & LAJOLO, F. M. 2009. New information on carbohydrates in the Brazilian Food Composition Database. *Journal of Food Composition and Analysis,* 22, 446-452.

METTLER, S., LAMPRECHT-RUSCA, F., STOFFEL-KURT, N., WENK, C. & COLOMBANI, P. C. 2007. The influence of the subjects' training state on the glycemic index. *European Journal of Clinical Nutrition,* 61, 19-24.

MIKUS, C. R., OBERLIN, D. J., LIBLA, J. L., TAYLOR, A. M., BOOTH, F. W. & THYFAULT, J. P. 2012. Lowering Physical Activity Impairs Glycemic Control in Healthy Volunteers. *Medicine and Science in Sports and Exercise,* 44, 225-231.

MILLER, C. J., DUNN, E. V. & HASHIM, I. B. 2002. Glycemic index of 3 varieties of dates. *Saudi Medical Journal,* 23, 536-538.

MILLER, C. J., DUNN, E. V. & HASHIM, I. B. 2003. The glycaemic index of dates and date/yoghurt mixed meals. Are dates 'the candy that grows on trees'? *European Journal of Clinical Nutrition,* 57, 427-30.

MILLER, G. L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry,* 31, 426-428.

MITCHELL, H. L. 2008. The glycemic index concept in action. *American Journal of Clinical Nutrition,* 87, 244S-246S.

MONRO, J. A., MISHRA, S. & VENN, B. 2010. Baselines representing blood glucose clearance improve in vitro prediction of the glycaemic impact of customarily consumed food quantities. *British Journal of Nutrition,* 103, 295-305.

MONRO, J. A. & SHAW, M. 2008. Glycemic impact, glycemic glucose equivalents, glycemic index, and glycemic load: definitions, distinctions, and implications. *American Journal of Clinical Nutrition,* 87, 237S-243S.

MUSSATTO, S. I. & MANCILHA, I. M. 2007. Non-digestible oligosaccharides: A review. *Carbohydrate Polymers,* 68, 587-597.

MEDICAL RESEARCH COUNCIL: http://www.mrc-hnr.cam.ac.uk/research/research-sections/nh/gi-database: [access date: 14.12.13]

MWAMBURI, D. M., LIEBSON, E., FOLSTEIN, M., BUNGAY, K., TUCKER, K. L. & QIU, W. Q. 2011. Depression and glycemic intake in the homebound elderly. *Journal of Affective Disorders,* 132, 94-98.

NIELSEN, B. M., BJORNSBO, K. S., TETENS, I. & HEITMANN, B. L. 2005. Dietary glycaemic index and glycaemic load in Danish children in relation to body fatness. *British Journal of Nutrition,* 94, 992-7.

NILSSON, A. C., OSTMAN, E. M., GRANFELDT, Y. & BJORCK, I. M. 2008. Effect of cereal test breakfasts differing in glycemic index and content of indigestible carbohydrates on daylong glucose tolerance in healthy subjects. *American Journal of Clinical Nutrition,* 87, 645-54.

NISHIMUNE, T., YAKUSHIJI, T., SUMIMOTO, T., TAGUCHI, S., KONISHI, Y., NAKAHARA, S., ICHIKAWA, T. & KUNITA, N. 1991. Glycemic response and fiber content of some foods. *American Journal of Clinical Nutrition,* 54, 414-419.

ONYESOM, I., OWEH, O. T., ETUMAH, O. S. & IFIE, E. J. 2013. Correlation between body mass index and blood glucose levels among some Nigerian undergraduates. *Herbet Open Acess Journal Biology (HOAJ Biology),* 2, 4 pages.

PERALA, M. M., HATONEN, K. A., VIRTAMO, J., ERIKSSON, J. G., SINKKO, H. K., SUNDVALL, J. & VALSTA, L. M. 2011. Impact of overweight and glucose tolerance on postprandial responses to high- and low-glycaemic index meals. *British Journal of Nutrition,* 105, 1627-1634.

PETERS, H. P. F., RAVESTEIN, P., VAN DER HIJDEN, H. T. W. M., BOERS, H. M. & MELA, D. J. 2011. Effect of carbohydrate digestibility on appetite and its relationship to postprandial blood glucose and insulin levels. *European Journal of Clinical Nutrition,* 65, 47-54.

PRATT, M., LIGHTOWLER, H., HENRY, C. J., THABUIS, C., WILS, D. & GUERIN-DEREMAUX, L. 2011. No observable differences in glycemic response to maltitol in human subjects from 3 ethnically diverse groups. *Nutrition Research,* 31, 223-228.

RADULIAN, G., RUSU, E., DRAGOMIR, A. & POSEA, M. 2009. Metabolic effects of low glycaemic index diets. *Nutrition Journal,* 8, 1-5.

RANAWANA, V., CLEGG, M. E., SHAFAT, A. & HENRY, C. J. 2011. Postmastication digestion factors influence glycemic variability in humans. *Nutrition Research,* 31, 452-459.

RICCARDI, G., RIVELLESE, A. A. & GIACCO, R. 2008. Role of glycemic index and glycemic load in the healthy state, in prediabetes, and in diabetes. *American Journal of Clinical Nutrition,* 87, 269S-274S.

RUFIAN-HENARES, J. A. & DELGADO-ANDRADE, C. 2009. Effect of digestive process on Maillard reaction indexes and antioxidant properties of breakfast cereals. *Food Research International,* 42, 394-400.

SAWAYA, W., AL-AWADHI, F., EID, N. & DASHTI, B. 1998. *Food Composition, Kuwaiti composite dishes as first edition KISR Book,* Kuwait, KISR Press

SCHAKEL, S., SCHAUER, R., HIMES, J., HARNACK, L. & VAN HEEL, N. 2008. Development of a glycemic index database for dietary assessment. *Journal of Food Composition and Analysis,* 21, S50-S55.

SCHNEIDER, A. V. C. 2002. Overview of the market and consumption of pulses in Europe. *British Journal of Nutrition,* 88, S243-S250.

SIMILA, M. E., VALSTA, L. M., KONTTO, J. P., ALBANES, D. & VIRTAMO, J. 2011. Low-, medium- and high-glycaemic index carbohydrates and risk of type 2 diabetes in men. *British Journal of Nutrition,* 105, 1258-64.

SINGH, J., DARTOIS, A. & KAUR, L. 2010. Starch digestibility in food matrix: a review. *Trends in Food Science & Technology,* 21, 168-180.

SONIA, G. S., JUSCELINO, T., VICTOR, M. Z. & LUIS, A. B. 2013. Starch digestibility and predicted glycaemic index (pGI) in starchy foods consumed in Mexico. *Starch - Stärke,* 66, 1-11.

SONOWANE, M., SAVORY, J., CROSS, R. E., HEINTGES, M. G. & CHESTER, B. 1976. Kinetic measurement of glucose with a centrifugal analyzer - hexokinase and glucose oxidase procedures compared. *Clinical Chemistry,* 22, 1100-1101.

SOUTHGATE, D. A. T. 1976. *Determination of food carbohydrates,* London, UK, Applied Science Publisher LTD.

SOUTHGATE, D. A. T., PAUL, A. A., DEAN, A. C. & CHRISTIE, A. A. 1978. Free Sugars in Foods. *Journal of Human Nutrition,* 32, 335-347.

SURWIT, R. S., SCHNEIDER, M. S. & FEINGLOS, M. N. 1992. Stress and diabetes mellitus. *Diabetes Care,* 15, 1413-22.

TAKRURI, H. R. & ALKURD, R. A. A. 2008. The glycemic index of a new bread brand (biobread). *Jordan Medical Journal,* 42, 117-123.

THOMAS, D. & ELLIOTT, E. J. 2009. Low glycaemic index, or low glycaemic load, diets for diabetes mellitus. *Cochrane Database of Systematic Reviews,* 21, 1-34.

THOMAS, D. E., ELLIOTT, E. J. & BAUR, L. 2007. Low glycaemic index or low glycaemic load diets for overweight and obesity. *Cochrane Database of Systematic Reviews,* 18, 1-43.

TOVAR, J., SAYAGO-AYERDI, S. G., PENALVER, C., PAREDES-LOPEZ, O. & BELLO-PEREZ, L. A. 2003. In vitro starch hydrolysis index and predicted glycemic index of corn tortilla, black beans (Phaseolus vulgaris L.), and Mexican "taco". *Cereal Chemistry,* 80, 533-535.

TRAIANEDES, K. & O'DEA, K. 1986. Commercial canning increases the digestibility of beans in vitro and postprandial metabolic responses to them in vivo. *American Journal of Clinical Nutrition,* 44, 390-7.

TUOMILEHTO, J., LINDSTROM, J., KEINANEN-KIUKAANNIEMIE, S., HILTUNEN, L., KIVELA, S. L., GALLUS, G., GARANCINI, M. P., SCHRANZ, A., BOUTER, L. M., DEKKER, J. M., HEINE, R. J., NIJPELS, G., PAJAK, A., CASTELL, C., LLOVERAS, G., TRESSERRAS, R., DE PABLOS-VELASCO, P. L., MARTINEZ-MARTIN, F. J., RODRIGUEZ-PEREZ, F., GABRIEL, R., SERRANO-RIOS, M., PLADEVALL, M., MUNIZ, J., ELIASSON, M., STEGMAYR, B., LUNDBERG, V., UNWIN, N., GEORGE, K., ALBERTI, M. M., HAYES, L., BORCH-JOHNSEN, K., ERIKSSON, J., QIAO, Q., HU, G., BALKAU, B. & DECODE STUDY GRPJOUSILAHTI, P. 2003. Age- and sex-specific prevalences of diabetes and impaired glucose regulation in 13 European cohorts. *Diabetes Care,* 26, 61-69.

UROOJ, A. & PUTTARAJ, S. 2000. Glycaemic responses to cereal-based Indian food preparations in patients with non-insulin-dependent diabetes mellitus and normal subjects. *British Journal of Nutrition,* 83, 483-488.

VENN, B. J., WILLIAMS, S. M. & MANN, J. I. 2010. Comparison of postprandial glycaemia in Asians and Caucasians. *Diabetic Medicine,* 27, 1205-1208.

VOSLOO, M. C. 2005. Some factors affecting the digestion of glycaemic carbohydrates and the blood glucose response. *Journal of Family Ecology and Consumer Sciences,* 33, 1-9.

VROLIX, R. & MENSINK, R. P. 2010. Variability of the glycemic response to single food products in healthy subjects. *Contemporary Clinical Trials,* 31, 5-11.

WALKER, A. R. P. & WALKER, B. F. 1984. Glycemic Index of South-African Foods Determined in Rural Blacks - a Population at Low-Risk of Diabetes. *Human Nutrition-Clinical Nutrition,* 38C, 215-222.

WEI, X. L., XI, X. G., WU, M. X. & WANG, Y. F. 2011. A novel method for quantitative determination of tea polysaccharide by resonance light scattering. *Spectrochimica Acta Part a-Molecular and Biomolecular Spectroscopy,* 79, 928-933.

WIDANAGAMAGE, R. D., EKANAYAKE, S. & WELIHINDA, J. 2009. Carbohydrate-rich foods: glycaemic indices and the effect of constituent macronutrients. *International Journal of Food Sciences and Nutrition,* 60, 215-223.

WILLIAMS, S. M., VENN, B. J., PERRY, T., BROWN, R., WALLACE, A., MANN, J. I. & GREEN, T. J. 2008. Another approach to estimating the reliability of glycaemic index. *British Journal of Nutrition,* 100, 364-372.

WILLIAMSON, G. 2013. Possible effects of dietary polyphenols on sugar absorption and digestion. *Molecular Nutrition & Food Research,* 57, 48-57.

WOLEVER, T. 2006. *The Glycaemic Index A physiological Classification of Dietary Carbohydrate,* Oxford, UK, CABI Publishing.

WOLEVER, T. M. 1990. The glycemic index. *World Review of Nutrition and Dietetics,* 62, 120-85.

WOLEVER, T. M., JENKINS, D. J., OCANA, A. M., RAO, V. A. & COLLIER, G. R. 1988. Second-meal effect: low-glycemic-index foods eaten at dinner improve subsequent breakfast glycemic response. *American Journal of Clinical Nutrition,* 48, 1041-7.

WOLEVER, T. M. S. 2000. Dietary carbohydrates and insulin action in humans. *British Journal of Nutrition,* 83, S97-S102.

WOLEVER, T. M. S., BRAND-MILLER, J. C., ABERNETHY, J., ASTRUP, A., ATKINSON, F., AXELSEN, M., BJORCK, I., BRIGHENTI, F., BROWN, R., BRYNES, A., CASIRAGHI, M. C., CAZAUBIEL, M., DAHLQVIST, L., DELPORT, E., DENYER, G. S., ERBA, D., FROST, G., GRANFELDT, Y., HAMPTON, S., HART, V. A., HATONEN, K. A., HENRY, C. J., HERTZLER, S., HULL, S., JERLING, J., JOHNSTON, K. L., LIGHTOWLER, H., MANN, N., MORGAN, L., PANLASIGUI, L. N., PELKMAN, C., PERRY, T., PFEIFFER, A. F. H., PIETERS, M., RAMDATH, D. D., RAMSINGH, R. T., ROBERT, S. D., ROBINSON, C., SARKKINEN, E., SCAZZINA, F., SISON, D. C. D., SLOTH, B., STANIFORTH, J., TAPOLA, N., VALSTA, L. M., VERKOOIJEN, I., WEICKERT, M. O., WESELER, A. R., WILKIE, P. & ZHANG, J. 2008. Measuring the glycemic index of foods: interlaboratory study. *American Journal of Clinical Nutrition,* 87, 247s-257s.

WOLEVER, T. M. S., JENKINS, D. J. A., JENKINS, A. L. & JOSSE, R. G. 1991. The glycemic index - methadology and clinical implications. *American Journal of Clinical Nutrition,* 54, 846-854.

WOLEVER, T. M. S., NUTTALL, F. Q., LEE, R., WONG, G. S., JOSSE, R. G., CSIMA, A. & JENKINS, D. J. A. 1985. Prediction of the Relative Blood-Glucose Response of Mixed Meals Using the White Bread Glycemic Index. *Diabetes Care,* 8, 418-428.

WOLEVER, T. M. S., VORSTER, H. H., BJORCK, I., BRAND-MILLER, J., BRIGHENTI, F., MANN, J. I., RAMDATH, D. D., GRANFELDT, Y., HOLT, S., PERRY, T. L., VENTER, C. & WU, X. M. 2003. Determination of the glycaemic index of foods: interlaboratory study. *European Journal of Clinical Nutrition,* 57, 475-482.

YADAV, B. S., SHARMA, A. & YADAV, R. B. 2010. Resistant starch content of conventionally boiled and pressure-cooked cereals, legumes and tubers. *Journal of Food Science and Technology-Mysore,* 47, 84-88.

ZABIDI, M. A. & AZIZ, N. A. A. 2009. In vitro starch hydrolysis and estimated glycaemic index of bread substituted with different percentage of chempedak (Artocarpus integer) seed flour. *Food Chemistry,* 117, 64-68.

ZAFAR, T. A., KABIR, Y. & GHAZAII, C. 2011. Low glycemic index foods suppress glycemic response, appetite and food intake in young Kuwaiti Females. *Kuwait Journal of Science & Engineering,* 38, 111-123.

Appendices

8.6 Appendix A: ethical approval

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UNIVERSITY OF LEEDS

Sarah Al Hamli
Food Science and Nutrition
University of Leeds
Leeds, LS2 9JT

MEEC Faculty Research Ethics Committee
University of Leeds

22 February 2012

Dear Sarah

Research title **Glycaemic response to cereal and legume based foods (a pilot study)**
Ethics reference **MEEC 11-027**

I am pleased to inform you that the application listed above has been reviewed by the MaPS and Engineering joint Faculty Research Ethics Committee (MEEC FREC) and following receipt of your response to the Committee's initial comments, I can confirm a favourable ethical opinion as of the date of this letter. The following documentation was considered:

Document	*Version*	*Date*
Response to MEEC 11-027 Committee Provisiona .doc	1	21/02/12
MEEC 11-027 Sarah Al-Hamli Ethical_Review_Form corrected 21.02.12.doc	2	21/02/12
Appendix 1 invetation sheet Poster Study.doc	1	27/01/12
Appendix 2 Health Screening Questionnaire.doc	2	21/02/12
Appendix 4 new Consent_form_(GLycaemic index).doc	1	27/01/12
Appendix 5 new Participant_information_sheet_(Glycaemic_response).doc	2	21/02/12

Please notify the committee if you intend to make any amendments to the original research as submitted at date of this approval. This includes recruitment methodology. All changes must be ethically approved prior to implementation.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. There is a checklist listing examples of documents to be kept which is available at http://researchsupport.leeds.ac.uk/index.php/academic_staff/good_practice/other_information_nhs_sites in the '*Other useful documentation*' section.

Yours sincerely

Jennifer Blaikie
Senior Research Ethics Administrator, Research & Innovation Service
On behalf of Professor Gary Williamson, Chair, MEEC FREC

CC: Student's supervisor(s)

8.7 Appendix B: health questionnaire

Date: / / Participants Code……

INTRODUCTION:

Thank you for making time to answer the questions. My name is Sarah F. AL-Hamli, a PhD student of the University of Leeds. I am conducting a study on the impact of ethnicity on the glycaemic response of food among adult aged 18 years and over. Any provided information will be treated with the utmost confidentiality. Completed questionnaires will be number coded and you will not be identified by name. Please tick your answer(s) or provide additional information in the spaces on the form. It will take you about **less than 10** minutes to complete the questionnaire.

A. Personal Information

Please provide brief information about yourself by circling the appropriate answer (s) or provide additional information where necessary.

1**. Full Name** **……**

2. **Age (years)** **……**

3. **Gender** o Male o Female

4. **Height** **.....**

5. **Weight** **.....**

6. o United Kingdom o EU o Other**.....** **Nationality**

7. **Ethnicity**

- o Asian or Asian British – Bangladeshi
- o Asian or Asian British - Indian
- o Asian or Asian British - Pakistani
- o Chinese
- o Irish Traveller
- o Other Ethnic Background
- o White - British
- o White - Irish
- o White – Scottish
- o Mixed – White and Black African
- o Mixed – White and Asian
- o Asian Other
- o Other Mixed Background
- o Other Black Background
- o Mixed – White and Black Caribbean
- o Other White Background
- o Black or Black British – Caribbean
- o Black or Black British – African

B. Current Health Status

8. **Would you say your health in general is...?**

o excellent o very good o good, o fair o poor o Don't know

9**. Would you consider yourself to be in good health?**

o Yes o No o Do not know

10. **During the past 30 days, for how many days was your mental health not good (mental health includes: stress, depression, and problems with emotions (Put # of days)?.....**

11. **During the past 30 days, for about how many days (have you/has SP) felt worried, tense, or anxious (Put # of days)? .....**

C. Health History

12. **Have you ever received surgery on your stomach, small intestine or colon**?

o Yes o No o Do not know

13. **Do you have any history allergy?**

In yes can you specify which type of allergy and from which type of food?

……

14. **Do you have any history of metabolic disease**?

o Yes o No o Do not know

15. **Other than during pregnancy have you been told by a doctor or other health professional that you have diabetes?**

o Yes o No o Do not know

16. **How old were you when a doctor or other health professional first told you that you had diabetes (Put age in year)?.....**

17. **Have you ever been told by a doctor or other health professional that you have any of the following: prediabetes, impaired fasting glucose, impaired glucose tolerance, borderline diabetes or that your blood sugar is higher than normal but not high enough to be called diabetes or sugar diabetes?**

○ Yes ○ No ○ Do not know

18**. Have you ever been told by a doctor or other health professional that you have health conditions or a medical or family history that increases your risk for diabetes?**

○ Yes ○ No ○ Do not know

19. **Have you had a blood test for high blood sugar or diabetes within the past three years?**

○ Yes ○ No ○ Do not know

20. **Which type of diabetes do you have?**

○ Type I diabetes ○ Type II diabetes
○ Gestational diabetes (diabetes during pregnancy) ○ Do not know

21**. Are you currently taking any medication for the diabetes (whether tablets or insulin)?**

(*Insulin is a chemical used in the treatment of diabetes. Typically, insulin is administered with a syringe by the patient.)*

○ Diabetic Pills (Oral Agent) ○ Insulin ○ No ○ Do not know

For how long (have you/has SP) been taking this medication (enter number of year or months?.....

23. **How often do you check your blood sugar level (include times when checked by a family member or friend, but do not include times when checked by a doctor or other health professional nor urine test)?**

o Never o Daily o Weekly o Monthly o Annually o Don't know

24**. Are you taking any routine medication other than diabetes**?

o Yes o No o Don't Now

If yes, which kind of medication are you taking**?**

.....

25. **Have you been diagnosed in the past with the following?**

High blood pressure ( ) Yes (specify)**……** ( ) No ( ) Do not know

Food allergies ( ) Yes (specify)**…** ( ) No ( ) Do not know

Nutrient deficiency ( ) Yes (specify)**……** ( ) No ( ) Do not know

Heart disease ( ) Yes (specify)**……** ( ) No ( ) Do not know

Mental health problem ( ) Yes (specify)**……** ( ) No ( ) Do not know

Kidney disease ( ) Yes (specify)**……** ( ) No ( ) Do not know

Liver disease ( ) Yes (specify)**……** ( ) No ( ) Do not know

Respiratory diseases ( ) Yes (specify)**…….....** ( ) No ( ) Do not know

Stroke ( ) Yes (specify)**……** ( ) No ( ) Do not know

High cholesterol ( ) Yes (specify)**……** ( ) No (c) Do not know

List any other condition (s) you have (a)**……** (b)**……..**

D. Life Style

26. **Are you currently on any type of diet either to lose weight or health-related reason?**

o Yes (specify .....) o No o Do not know

27. **Are you Vegetarian / Vegan**?

o Yes Vegetarian o Yes Vegan o No o Don't know

28. **in the past 30 days, have you used or taken any vitamins, minerals, herbals or other dietary supplements (Include prescription and non-prescription supplements.)?**

o Yes o No o Do not know

29. **During the past 7 days, on how many days were you physically active for a total of at least 60 minutes per day(Add up all the time spent in any kind of physical activity that increased your heart rate , made you breathe hard some of the time and sweat.)?**

o 0 days o 1 day o 2 days o 3 days o 4 days
o 5 days o 6 days o 7 days o Don't know

30. **Does your work involve vigorous-intensity activity that causes large increases in breathing or heart rate like carrying or lifting heavy loads, digging or construction work for at least 10 minutes continuously?**

o Yes o No o Do not know

31. **Does your work involve moderate-intensity activity that causes small increases in breathing or heart rate such as brisk walking or carrying light loads for at least 10 minutes continuously?**

○ Yes ○ No ○ Do not know

32. **Do you do any vigorous-intensity sports, fitness, or recreational activities that cause large increases in breathing or heart rate like running or basketball for at least 10 minutes continuously?**

○ Yes ○ No ○ Do not know

33**. Do you do any moderate-intensity sports, fitness, or recreational activities that cause a small increase in breathing or heart rate such as yoga, brisk walking, bicycling, swimming, or golf for at least 10 minutes continuously?**

○ Yes ○ No ○ Do not know

34. **During your life, on how many days have you had at least one drink of alcohol (including: beer, wine, wine coolers, and liquor such as rum, gin, vodka, or whiskey. This does not include drinking a few sips)**

○ Never ○ 1 or 2 days ○ 3 to 9 days ○ 10 to 19 days
○ 20 to 39 days ○ 40 to 99 days ○ 100 or more days ○ Don't know

35. **Do you smoke (not include cigars or other types)**?

○ Every day ○ Sometimes ○ Not at all ○ Refused ○ Don't know

36. **How old were you when you first started to smoke cigarettes fairly regularly (put age in year)...**

37. **How long has it been since you quit smoking cigarettes (Put age in year)?.....**

38. **Is there any reason that you consider yourself to be unable to fully participate in the study?**

.....

.....

Note: I am looking for volunteers to interview about their food preparation methods. Please tick this box and provide your contact details if you are happy to take part.

I wish to take part ☐ **Telephone ……..** **OR** **Email……...**

Thank you for your time.

8.8 Appendix C: medical questionnaire

Name:	**Nationality:**
Age:	**Gender :**
Height:	**Weight:**
Contact (telephone number or e-mail):	

1. Do you consider yourself to be in good health today?

a)Yes. b) No. Other:

2. Do you come to school today?

a) By any transportation other than bicycle

b) By bicycle

c) By walking

3. Are you taking any medication before coming to school?

a)Yes. b) No

If yes, what is it? .....

4. Do you feel stress or depressed today?

Yes. No.

5. Do you have any history of allergy?

Yes. No.

If Yes, which kind of allergy do you have? .

.....

6. What is the stable food of you in usual (e.g. Rice, wheat, corn, potato)?.....

7. Timetable for the blood collection

Time	0 Min	15 Min	30 Min	45 Min	60 Min	90 Min	120 Min

Thank you for your time

8.9 Appendix D: participant information sheet

Name of center: School of Food Science and Nutrition, University of Leeds

Project title: Glycaemic response to cereal and legume based foods (a pilot study)

You are invited to take part in a research study. It is entirely up to you to decide whether or not to take part. You can choose not to take part without having to give a reason and without penalty. Please take time to read this information carefully before making a decision. Feel free to discuss it with your friends and family. If you have any questions or require further clarification, you can contact the researcher (see contact details on page 2). You have two weeks to decide whether to take part.

What is the purpose of this study?

The purpose of this study is to find out the impact of cereal and legume based dish on the glycaemic response as single or mixed meal. Glycaemic response (GR) of food can be defined as the ability of a food to raise the sugar level in the blood, while, glycaemic index is a way of measuring the glycaemic response of food compared to a reference food. Many factors can affect the glycaemic response such as portion size, fibre, fat and protein content, and food processing.

The research will involve feeding adults with white bread and lentil soup for 1-3 days. The glucose/sugar levels will be measured before and after feeding, up to 2 hours at an interval of 15 -30 minutes. Measuring glycaemic responses from foods have been shown to be beneficial to health by explaining its association with nutrition-related diseases such as type 2 diabetes.

Researchers have performed some mathematical predictions of GI of cereals and legumes based on their composition. The modelling has predicted that cereals and legumes also differ in their in vivo digestibility. However, the mathematical modelling is not able to predict the interaction between the two food groups when consumed as part of a meal. The information obtained will help to better understand the interaction between two types of food, white bread (a low fibre cereal based food) and lentil soup (a high fibre legume based food), on the glyceamic response in healthy human adult subjects.

Who is doing the study?

The study is being carried out by a PhD research student Sarah Alhamli at the School of Food Science and Nutrition, University of Leeds, towards the award of PhD.

Who is being asked to participate?

I am looking for males and females aged 18 -35 years, of different ethnic origins, who is non-allergic to food (nuts, gluten; legumes), pregnant, and lactating women , not

diagnosed with other chronic diseases such as diabetes, cancer, cardiovascular diseases, or digestives system disease, and who are not taking any medication that effect the glucose response, and living in Yorkshire & Humber.

What will be involved if I take part in this study?

If you decide to take part, you will be involved in the study for one day at 8 occasions, over a period of 4 weeks. You will be asked to attend a briefing session (about 30 minutes or less with the researcher), you will be asked to complete a health questionnaire, consume white bread, lentil soup or both provided by the researcher, and your blood glucose measured by finger prick for a drop of blood.

The briefing session will take place at the University campus. At this session, the researcher will explain the study in details, what it will involve, ethical considerations, and answer any questions you may have.

After this briefing, the research student will ask for some general information about you and your health. Health questionnaire will be completed to enable the researcher to assess whether you may be allergic to the test foods.

On a test day, you will be asked to fast overnight and to refrain from strenuous exercise for 24 hours and then your blood glucose measured in the morning. Then you will be asked to consume white bread provided by the researcher and your blood glucose measured again for up to 2 h at 15-30 minutes interval. You will also be asked to abstain from all foods (you can drink water) during these two hours. A total of seven finger prick readings will be taken from you on each test day. The procedure will be conducted on weekdays only with test food alternatively.

What are the advantages and disadvantages of taking part?

There are no direct benefits to you for taking part. However, the information obtained from this study will allow for better understanding of the role of Glycaemic index in the prevention of nutrition-related diseases and whether different food component may have an affect the glycaemic response on people..

Can I withdraw from the study at any time?

Participation is voluntary and you can withdraw at any stage without having to give a reason, this will not affect your statutory rights.

Will the information I give be kept confidential?

Any information you provide will be treated with the utmost confidentiality and handled according to the Data Protection Act 1998. All completed food diaries will be number coded and you will not be identified as having taken part. Completed diaries will be securely locked in a cabinet at the School of Food Science and Nutrition, University of Leeds. Only the researcher and the Dr. Caroline Orfila will have access to the cabinet.

What will happen to the results of study?

The information obtained from the study will allow for better understanding of the role of Glycaemic index in the prevention of diet-related diseases and whether different food component may have an affect the glycaemic response on people.

Who has reviewed this study?

The study has been reviewed by the Ethics Committee of the Faculty of Mathematics and Physicals Science, University of Leeds. [**MEEC 11-027**]

If you agree to take part, would like more information or have any questions please contact:

Sarah Alhamli

PhD Research student

School of Food Science and Nutrition

University of Leeds

LS2 9JT

TEL:

E-mail: fs08sah@leeds.ac.uk

Or

If you require further information, you may also contact **(Dr. Caroline Orfila)** on **Tel:** 0113 343 2966 or by **E-mail:** C.Orfila@leeds.ac.uk

If you have no questions and you agree to take part, please complete the participant consent form, and send it to the researcher using the stamped addressed envelope provided.

Thank you for making the time to read this information

8.10 Appendix E: invitation sheet

Volunteers needed for a study investigating the glucose response toward white bread and lentil soup

You are being invited to take part in this research project.

We are looking for:

- ***Healthy men or women, aged from 18-55 years***
- ***Able to attend 8 2-hour sessions over four weeks***
- ***Willing to have a blood test (finger prick) every 15 min over a 2 hours period during each session***

After each visit, a light breakfast will be provided, plus you will be compensated for your time with a £5 shopping voucher.

Many thanks for your participation.

If you need further information about this study, you can contact **Sarah Alhamli** by an e-mail fs08sah@leeds.ac.uk.

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8.11 Appendix C: consent form

UNIVERSITY OF LEEDS

School of Food Science and Nutrition

Participant Consent Form

Project Title: Glycaemic response to cereal and legume based foods (a pilot study)

	Please confirm the statements below
I have read and understood the participant information sheet	
I have had the opportunity to ask questions and discuss this study	
I understand that my participation is voluntary and I am free to withdraw from the study:- 1 At any time 2 Without having to give a reason for withdrawing	
I understand that any information I provide, including personal details, will be confidential, stored securely and only accessed by those carrying out the study.	
(When relevant) I understand that any information I give may be included in published documents but my identity will be protected by the use of pseudonyms	
I agree to take part in this study Yes/No	
Participant Signature …… Date.....	
Name of Participant	
Home Address of participant..... Tel..... Email.....	
Researcher Signature …… Date.....	
Name of Researcher	

Thank you for agreeing to take part in this study