Effects of carbohydrase inhibiting polyphenols on glycaemic response *in vivo*

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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.

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Chapter 2 contains work which has also been used in the publication

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Chapter 5 contains work which has also been used in the publication

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Chapter 6 contains work which has also been used in the publication

Publication in preparation - Pomegranate polyphenols in a juice but not a supplement confer a lower glycemic response on a high glycemic index food.

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Abstract

Background and Objective: Diabetes is a global problem and high postprandial blood glucose is one of the risk factors for developing type 2 diabetes. *In vitro* studies have shown that polyphenols have inhibitory effects on carbohydrate-digesting enzymes and glucose transporters which could lead to reduced postprandial glucose *in vivo*. This study investigated the effects of a mixture of polyphenols (for maximum effect) capable of inhibiting the different stages of carbohydrate digestion on glycaemic response. Additionally, a single source of polyphenols in two different matrices was also examined.

Methods: *In vitro* inhibition assays were used to determine the inhibition potential of polyphenols from the polyphenol and fibre-rich food (PFRF) consisting of freeze-dried apple, blackberry, blackcurrant, strawberry and green tea as well as from a single source (pomegranate). Three randomised, crossover studies were conducted on healthy volunteers (n=16 for each study) to determine the effects of polyphenols on glycaemic response *in vivo* using PFRF, pomegranate capsules and juice as sources of polyphenols.

Results: Polyphenols found in PFRF dose-dependently inhibited α-amylase and α-glucosidase *in vitro* and gave rise to a decrease in postprandial glucose area under the curve by (IAUC) =-27.4±7.5 % (mean ± SD) $p<0.001$ and IAUC=-49.0±15.3 %, $p<0.001$) for the single and double dose respectively. Insulin IAUC was also attenuated by -46.9±13.4% (mean ± SD; $p<0.01$) for the double dose. Pomegranate polyphenols in juice dose-dependently inhibited α-amylase and α-glucosidase *in vitro* and gave rise to a decrease (-33.1±18.1 %, $p <0.01$) in postprandial glucose IAUC but did not show any effect when administered in the form of capsules.

Conclusions: Certain polyphenol-rich foods have the potential to be used in the risk prevention and management of type 2 diabetes since they inhibit carbohydrate digestive enzymes *in vitro* and reduce postprandial glycaemic response in healthy volunteers, but only in certain food matrices.

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List of abbreviations

IGT	Impaired glucose tolerance
IFG	Impaired fasting glucose
FBG	Fasting blood glucose
GI	Glycaemic index
RDS	Rapidly digestible starch
SDS	Slowly digestible starch
RS	Resistant starch
SGLT1	Sodium-dependent glucose transporter 1
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
IDDM	Insulin-dependent diabetes mellitus
NIDDM	Non-insulin dependent mellitus
WHO	World health organisation
AUC	Area under the curve
IAUC	Incremental area under the curve
PFRF	Polyphenol and fibre-rich food
HPLC	High performance liquid chromatography
EC	Epicatechin
EGC	Epigallocatechin
ECG	Epicatechin gallate
EGCG	Epigallocatechin gallate
RGE	Relative glycaemic response
CV	Coefficient variation
STOP-NIDDM	Study to prevent non-insulin dependent diabetes mellitus
IDDM	Insulin-dependent diabetes mellitus
NIDDM	non-insulin dependent diabetes mellitus
EDTA	Ethylenediaminetetracetic acid
ANOVA	Analysis of variance
HOMA	Homeostasis model assessment
HOMA-IR	Homeostasis model assessment for insulin resistance
HOMA-B	Homeostasis model assessment for β-cell function
mRNA	Messenger ribonucleic acid

PON1	Paraoxinase 1
HDL	High density lipoprotein
SOD	Superoxide dismutase
PPAR	Peroxisome proliferator activated receptor
BMI	Body mass index
DMSO	Dimethylsulphoxide
LPO	Lipid peroxidation
LDL	Low density lipoprotein
HK	Hexokinase
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide plus hydrogen
UV	Ultraviolet
SD	Standard deviation
ELISA	Enzyme-linked immune sorbent assay
ABI	Antibody
TMB	Tetramethylbenzidine
GAE	Gallic acid equivalent
DAD	Diode array detector
RIP	Rat intestinal acetone powder
PBS	Phosphate buffer saline
SPE	Solid phase extraction
DNS	Dinitrosalicylic
SLC5A1	Solute carrier family 5 member 1
SLC2A2	Solute carrier family 2 member 2
NAOH	Sodium hydroxide
mAU	Milli absorbance unit
DW	Dry weight
FW	Fresh weight
NaH_2PO4	Sodium dihydrogen phosphate
Na_2HPO4	Disodium hydrogen phosphate
NaCl	Sodium chloride
IS	Internal standard

Chapter 1 . Introduction and Literature review

1.1 Introduction

Diabetes is a global problem with estimates indicating that by the year 2030, 439 million adults worldwide will have diabetes with a 69 and 20 % increase in prevalence in developing and developed countries between the years 2010 and 2030 (Shaw *et al*., 2010). In addition to diabetes, other related conditions include pre-diabetes (impaired glucose tolerance (IGT) and impaired fasting glucose (IFG)) as well as metabolic syndrome (obesity, hypertension and insulin resistance). Pre-diabetes and metabolic syndrome increases the risk of developing cardiovascular disease and diabetes mellitus (Coutinho *et al*., 1999).

Diet plays an important role in chronic diseases such as cardiovascular disease, type 2 diabetes, hypertension and obesity (Wolfe *et al.*, 2003). For type 2 diabetes, scientific evidence suggests that hyperglycaemia caused by frequent rise of blood glucose in humans has a major role to play in the development of the disease as it leads to the exhaustion of the β-cells of the pancreas and hinders the production of insulin to overcome the high blood glucose (WHO, 2016). This results in high blood glucose levels above the healthy range and develops into pre-diabetes and eventually type 2 diabetes. In the diet, carbohydrates are the major source of blood glucose. (Hanhineva *et al*., 2010). Dietary carbohydrate is important to maintain glycaemic homeostasis and is the major source of energy in the diets of most people. The control of blood glucose is a hormonal process and is very important for human physiology. Hormonal processes involve the

release of insulin from the β-cells of the pancreatic cells which stimulates the uptake of glucose after a meal, to other tissues either for utilisation (glycolysis) or to be stored in the liver as glycogen (glycogenesis). When blood glucose falls below normal, glucagon is secreted from the pancreatic α-cells and it promotes liver glucose production by inducing the generation of glucose from non-carbohydrate substrates such as amino acids, lactate and glycerol (gluconeogenesis) and the generation of glucose from glycogen (glycogenolysis) (Macdonald, 2014). When the glucose homeostatic hormonal control fails, it entails high blood glucose levels (postprandial hyperglycaemia) because insulin plays a major role in signalling the uptake of glucose from the blood .This can lead to metabolic syndrome which includes obesity, impaired glucose tolerance (IGT), hypertension and dyslipidaemia. Disturbance of glucose homeostasis can also lead to other symptoms such as inflammation and oxidative stress at the whole body level, disturbances of the functionality in several organs as well as diabetes and cardiovascular disease (Hanhineva *et al.*, 2010). Blood glucose concentration is one of the important criteria used to diagnose diabetes in addition to other signs and symptoms. According to the American Diabetes Association, ranges for normal or healthy, pre-diabetic and diabetic are fasting plasma glucose (FPG) levels of 3.9 to 5.6 mmol/L, 5.7 to 6.9 and ≥ 7 mmol/L respectively whereas the World Health Organisation provides a higher cut off of 6.1 mmol/L for the normal range of FPG. For screening purposes for the current study, an average of 5.9 will be used as the cut off for healthy range.

As a dietary intervention, the glycaemic index (GI) was originally proposed with the aim of managing diabetes. However, recent studies have shown that the GI has potential in the prevention of type 2 diabetes as well as in the treatment of metabolic syndrome. High GI diets are associated with a number of abnormalities like increased metabolic syndrome and insulin resistance (McKeown *et al*., 2004, Scaglioni *et al*., 2004) and hence

increases the risk of developing type 2 diabetes (Steven *et al*., 2002, Hodge *et al*., 2004,). On the contrary, low GI diets have shown to have the beneficial effects of improving insulin sensitivity (Frost *et al*., 1996) but it was not certain whether this was as a result of improved insulin sensitivity, improved insulin secretion or due to reduced rate of glucose absorption. Therefore, low glycaemic index diets or any intervention that can lower the glycaemic index of foods can play a major role in the prevention and management of type 2 diabetes and contribute in lessening the burden of the diabetes prevalence of which type 2 diabetes accounts for >90 % of diabetes cases (Kahn, 1998).

Research using animal models, as well as a limited number of human studies, have shown that polyphenols and polyphenol-rich foods or beverages have the potential to affect postprandial glycaemic responses and fasting glycaemia as well as improve acute insulin secretion and sensitivity (Hanhineva *et al.*, 2010). Other possible mechanisms include pancreatic β-cells stimulation to secrete insulin as well as activation of insulin receptors, modulation of the release of glucose from the liver as well as of intracellular signalling pathways and gene expressions (Hanhineva *et al.*, 2010). A recent review (Williamson, 2013) concluded that it is very possible that the effects of polyphenols in the diet will affect glycaemic index of foods as well as postprandial glucose responses in humans and highlighted that inhibition of starches/disaccharides metabolising enzymes as well as transporters are potential mechanisms by which this can be achieved. Therefore, the greatest effect would be observed when more than one of the suggested pathways was inhibited (Williamson, 2013).

1.2 Polyphenols

Polyphenols are one of the major groups of phytochemicals. Phytochemicals are natural biochemical compounds found in plant-derived foods like tea, coffee, fruits and vegetables. Other groups of phytochemicals include carotenoids (found in carrots, yam and apricots) and allyl sulphides (found in garlic and onions) (Wolfe *et al*., 2003). It is assumed that plants produce phytochemicals for defence purposes whereas emerging evidence suggests that phytochemicals can also protect humans against diseases (Slavin and Lloyd, 2012). Fruits and vegetables contain many compounds that are responsible for the protective health effects and they include; fibre, carotenoids and the vitamins E and C (Wolfe *et al*., 2003, Liu, 2013). Polyphenols are non-nutritive plant chemicals that are produced by plants as secondary metabolites whose chemical structure has more than one aromatic ring and at least one hydroxyl group. They are widely distributed in various plant-based foods in varying amounts (Perez-Jimenez *et al*., 2010) and types and they are further divided into different groups based on their structures. Depending on the source of polyphenols, some can be consumed unprocessed like in fruits and some vegetables whereas mostly polyphenols are consumed in the processed forms in plant-based food products which include tea, wine, fruit juices, canned fruits and jams. Approximately 1 g/day is the estimated intake of dietary polyphenols (Scalbert and Williamson, 2000). Polyphenols are divided into phenolic acids, flavonoids, stilbenes, lignans and tannins with flavonoids being an essential sub-group of polyphenols which have received the most attention in research. Flavonoids have a basic structure of a three ringed molecule having the carbon 6-carbon 3-carbon 6 (C6-C3-C6) general structural backbone (fig 1-1).

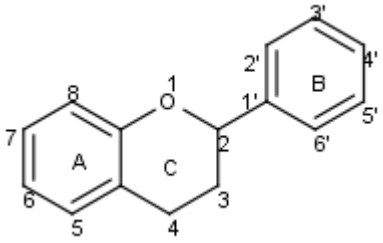
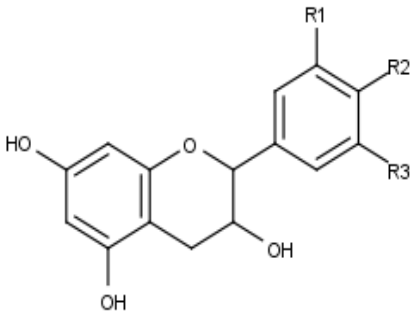


Figure 1-1: Basic structure of flavonoids

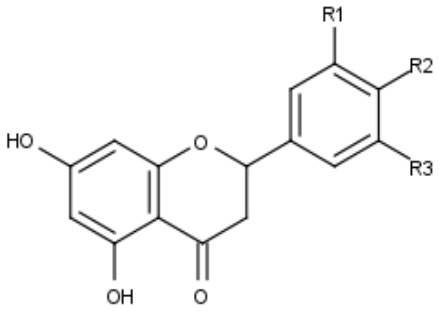
The two C6 units (ring A and ring B) are of phenolic nature whereas the chromane ring (ring C) may differ and may have different hydroxylation patterns. The variations in the chromane ring give rise to flavonoids being divided into different sub-groups which are flavanols, flavones, isoflavones, flavanones, flavonols and anthocyanidins (fig 1-2). Although the basic structures of flavonoids are aglycones, they mostly exist as glycosides in plants. Their biological activities depend on the glycosylation patterns as well as the structural differences (Tsao, 2010).

Flavanols



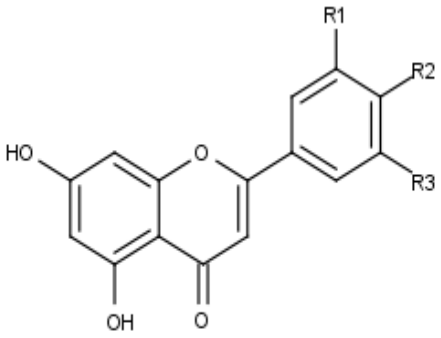
R1=R2=OH, R3=H Catechins
R1=R2=R3=H Gallocatechin

Flavanones



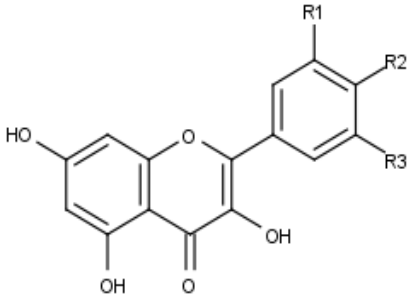
R1=H R2=OH, Naringenin
R1=R2=OH, Enodictyol
R1=OH, R2=OCH_3 Hesperitin

Flavones



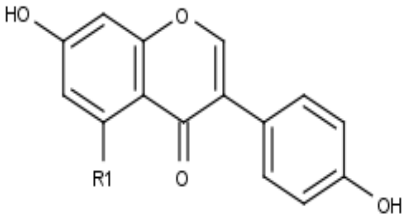
R1= H, R2=OH Apigenin
R1=R2= OH Luteolin

Flavonols



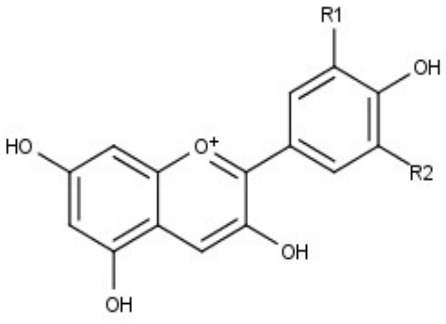
R2=OH, R1=R3=H Kaempferol
R1=R2=OH,R3=H Quercetin
R1=R2=R3=OH Myricetin

Isoflavones



R1=H Daidzein
R1=OH, Genistein

Anthocyanidins



Cyanidin R1-OH, R2- H
Delphinidin R1-OH, R2-OH
Pelargonidin R1-H, R2-H

Figure 1-2: Flavonoids sub-classes and their structures.

1.2.1 Polyphenol bioavailability

Knowledge about the bioavailability of polyphenols *in vivo* is important in order to understand whether the polyphenols are absorbed, metabolised and reach their target sites in amounts that are required for them to exert beneficial effects. Polyphenols are absorbed and metabolised in the human body (Scalbert and Williamson, 2000, Williams *et al*., 2004) and this varies greatly between individuals and depends on a number of factors such as differences in microbial populations as well as the influences of other dietary components present together with the polyphenols. Even though most polyphenols are absorbed, the bioavailability of different classes depends on the food source, type of polyphenol and especially due to the nature of the sugar attached (Hollman *et al.*, 1999, Williamson and Manach, 2005). Studies have shown that polyphenols are much lower in the human body and rarely exceed nM concentrations in plasma after normal dietary intake and that after consumption of 10-100 mg of a single phenolic compound, the maximum plasma concentration rarely exceeds 1 μM without accounting for any metabolites that may be formed (Scalbert and Williamson, 2000, Del Rio *et al*., 2010).

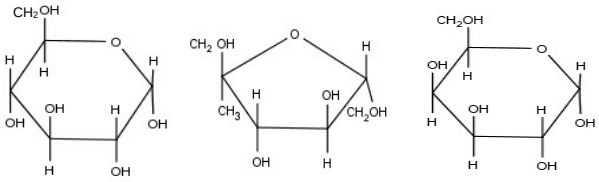
For polyphenols that have been reported to be poorly absorbed from the small intestine such as proanthocyanidins, anthocyanins and galloylated tea catechins, it is suggested that their action is thus restricted to the intestine (Manach *et al*., 2004, Manach *et al*., 2005). This agrees with the *in vitro* studies that have shown that anthocyanins and galloylated catechins have the potential to inhibit energy metabolism as they would be in high amounts in the intestine where they can inhibit digestion enzymes and glucose transporters. For the purpose of this research, polyphenol concentrations that have been shown to inhibit carbohydrate digestion and absorption as shown in table1-1, are achievable in the intestinal lumen (Scalbert and Williamson, 2000) which justifies one

of their mechanism of action (inhibition of digestive enzymes and glucose transporters). A recent review also points out that the gut lumen is the prime likely target for polyphenol effects on carbohydrates metabolism as it contains the highest concentration of polyphenols post-prandially (Williamson, 2013).

1.3 Carbohydrate digestion and absorption

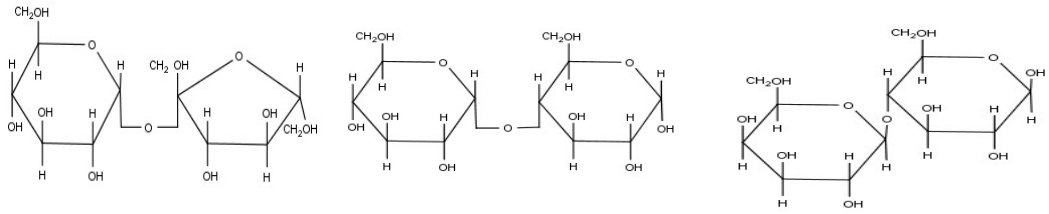
Dietary carbohydrates are macronutrients that have a range of physical and chemical properties. They are important in the human diet as they are the principal substrate of energy metabolism, blood glucose and insulin among other functions (Cummings and Stephen, 2007). Dietary carbohydrates are classified according to the degree of polymerization or number of monomeric units (single sugars). They are classified as mono, di, oligo and polysaccharides (fig 1-3). The simplest is glucose and polysaccharides are the most complex with more than 10 glucose units and they include amylose, amylopectin, modified starches, cellulose and hemicelluloses.

Monosaccharides



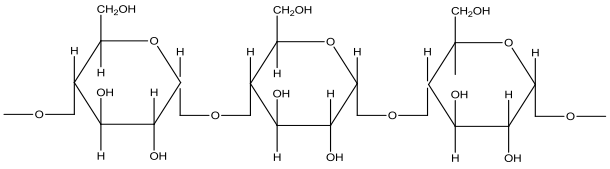
α –D-Glucose Fructose Galactose

Disaccharides

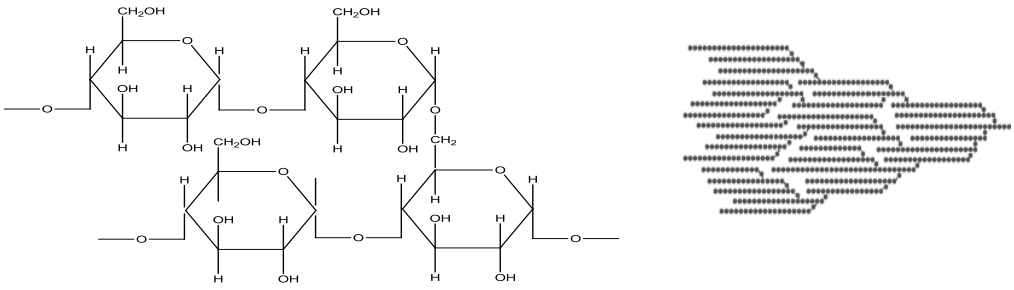


Sucrose Maltose Lactose

Polysaccharides



Amylose – More than 10 glucose molecules in a straight line



Amylopectin – more than 10 glucose molecules but branched

Figure 1-3: Chemical structures of some monosaccharides, disaccharides and polysaccharides amylose and amylopectin.

In the diet, polysaccharides which are most represented by starch play a major role as the source of energy supply in the human diet. Starch is made up of amylose and amylopectin polymers of glucose linked by α-1, 4 glycosidic bonds together with alpha-1, 6-glycosidic bonds which form branch points mainly in amylopectin. It has to be broken down during digestion to produce glucose which is the main carbohydrate that is usually absorbed through the intestinal wall. Starch in foods is classified into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). RDS is the starch that is digested to glucose rapidly (approximately 20 min) and SDS is digested into glucose much slower (approximately 2 h). The total of RDS and SDS makes up what is known as available carbohydrate and is defined as the starch that is digested to give glucose plus any glucose already present in the food. This is achieved by a series of enzymes with the upper gastrointestinal tract being the main site of digestion (Hanhineva *et al*., 2010). Salivary and pancreatic α-amylases catalyse the endo-hydrolysis of α-1, 4 glycosidic bonds giving mainly maltose, maltotriose and related α-1, 6-oligomers which contain 1, 6-linked glucose units from amylopectin. The final step in the digestion of carbohydrates takes place in the small intestinal brush boarder where α-glucosidases catalyse the hydrolysis of a single glucose residue from disaccharides and oligosaccharides by acting on the terminal α-1, 4 bonds in addition to the cleavage of α-1, 2 bonds found in sucrose and α-1, 6 bonds in iso-maltose (fig 1-3) (Quezada-Calvillo *et al*., 2008).

When glucose is produced in the gut lumen, it enters the blood stream through absorption through the gut wall. Active transport is responsible for the absorption of glucose via the sodium-dependent glucose transporter 1 (SGLT1) and by facilitated sodium-independent glucose transporter 2 (GLUT2). This is achieved by conformation changes that allow glucose to bind to the transporters. When two sodium ions binds to SGLT1 on the luminal

side of the intestinal brush boarder membrane, the conformation of SGLT1 changes and allows glucose to bind to it. Another conformational change occurs which then allows glucose and sodium ions to enter the enterocyte. GLUT2 found in the basolateral membrane releases glucose from the enterocyte into the circulation (Hanhineva *et al*., 2010). This results in an increase in blood glucose concentration which stimulates insulin release. Insulin is a hormone produced by the pancreas and is secreted by the β-cells of the pancreas which is part of the digestive system and is located behind the stomach, next to the duodenum. The exocrine cells of the pancreas release digestive enzymes in the gut and the islets of Langerhans release hormones like insulin and glucagon when required in glucose regulation. The primary function of insulin is to regulate blood glucose to avoid hyperglycaemia (too high) or hypoglycaemia (too low). The presence of insulin in the blood then stimulates the uptake of glucose to other tissues either to be utilised for inhibition of lipolysis in adipose tissue or stored in the liver as glycogen (glycogenesis) and in this way bringing the concentration of glucose back to normal within 1-3 h. Similarly, when blood glucose levels fall below normal, insulin release is inhibited and glucagon is secreted from the pancreatic α-cells and it promotes liver glucose production by breaking down glycogen to glucose (glycogenolysis) and the production of glucose from other sources other than carbohydrates (gluconeogenesis) raising the glucose level to normal and hence maintaining glucose homeostasis (Hanhineva *et al*., 2010, Williamson, 2013, Macdonald, 2014). Insulin is very important because its absence or the inability of the body to respond to it can both lead to hyperglycaemia and diabetes (Mueller and Jungbauer, 2009). Fasting insulin concentration > 62.5 pM is regarded to identify pre-diabetes as reported by Johnson *et al*. (2010). In insulin-dependent diabetes mellitus (IDDM) there is a severe reduction of insulin concentrations whereas in non-insulin dependent diabetes mellitus (NIDDM), insulin levels are raised. In the absence

of hindrances from other food components, carbohydrate digestion in the gut occurs quite rapidly although this may vary depending on the source and physical state of the starch. The carbohydrates that provide glucose for metabolism after digestion are said to be glycaemic carbohydrates and those which do not are non-glycaemic carbohydrates. Most unprocessed foods contain both glycaemic and non-glycaemic carbohydrate (Cummings and Stephen, 2007). Ingestion of digestible carbohydrates leads to a sharp rise of postprandial blood glucose and these foods are classified as 'high glycaemic index' foods.

1.3.1 Concept of Glycaemic index

Glycaemic index was originally defined as "The indexing of the glycaemic response to a fixed amount of available carbohydrates from a test food to the same amount of available carbohydrates from a standard food consumed by the same subject (glucose or white bread) (Jenkins *et al*., 1981). A practical definition by (FAO/WHO, 1998) says that glycaemic index is the increase in the area under the blood response curve of a 50 g carbohydrate portion of a test food over 2 hours. This is then expressed as the percentage of the response of the test food, to the response of a standard food of the same amount consumed by the same person. The glycaemic index of glucose is set at 100 and other foods are presented relative to this.

Classification of carbohydrates based on mono, oligo and polysaccharides is not sufficient to determine how they affect blood glucose (glycaemic response). Different carbohydrates have different glycaemic indices and can therefore be classified into high and low glycaemic index foods depending on their postprandial glucose as well as insulin response (Jenkins *et al.*, 2002). Carbohydrates that are digested and absorbed slowly, are said to be low GI foods and those that are digested and absorbed rapidly are said to be

high GI foods and it is this high digestion and absorption rate that may elevate blood glucose levels which induces many imbalances that may affect health and disease (Bornet *et al.*, 2007). Investigations have shown that consumption of low GI foods or having components in the diet that can lower the GI of foods, can lower the risk of certain chronic diseases such as cardiovascular diseases and diabetes (Aston, 2006, Barclay *et al.*, 2008). Similarly, consumption of high glycaemic index foods plays a role in the development of chronic diseases like type 2 diabetes, obesity and cardiovascular disease (Ludwig, 2002).

1.3.2 Carbohydrates metabolism and disease

The control of blood glucose is a hormonal process and it is very important to human physiology. When this control fails, it can lead to metabolic syndrome which is a major contributing factor to the development of diabetes and increasing the risk of cardiovascular disease. Metabolic syndrome consists of a number of conditions such as obesity, hypertension and insulin resistance whereas high postprandial blood glucose and high fasting blood glucose are contributing factors (Hanhineva *et al.*, 2010).

The World Health Organisation has reported that about 422 million people suffer from diabetes worldwide and that by the year 2030, diabetes will be the 7th leading cause of death. The WHO also reports that in 2012, about 1.5 million people died directly due to diabetes and 2.2 million deaths were due to high blood glucose related complications (WHO, 2016). Diabetes can be classified into type 1 (formerly known as insulin-dependent) and type 2 (formerly known as non-insulin dependent) diabetes. Type 1 diabetes is due to the absence of insulin production and thus requires daily injection of insulin to control it. Type 2 diabetes is largely due to diet, overweight and lack of physical activity characterised by high blood glucose levels (hyperglycaemia) followed by failure

of the pancreas to release enough insulin to overcome high blood glucose levels (fig 1-4).

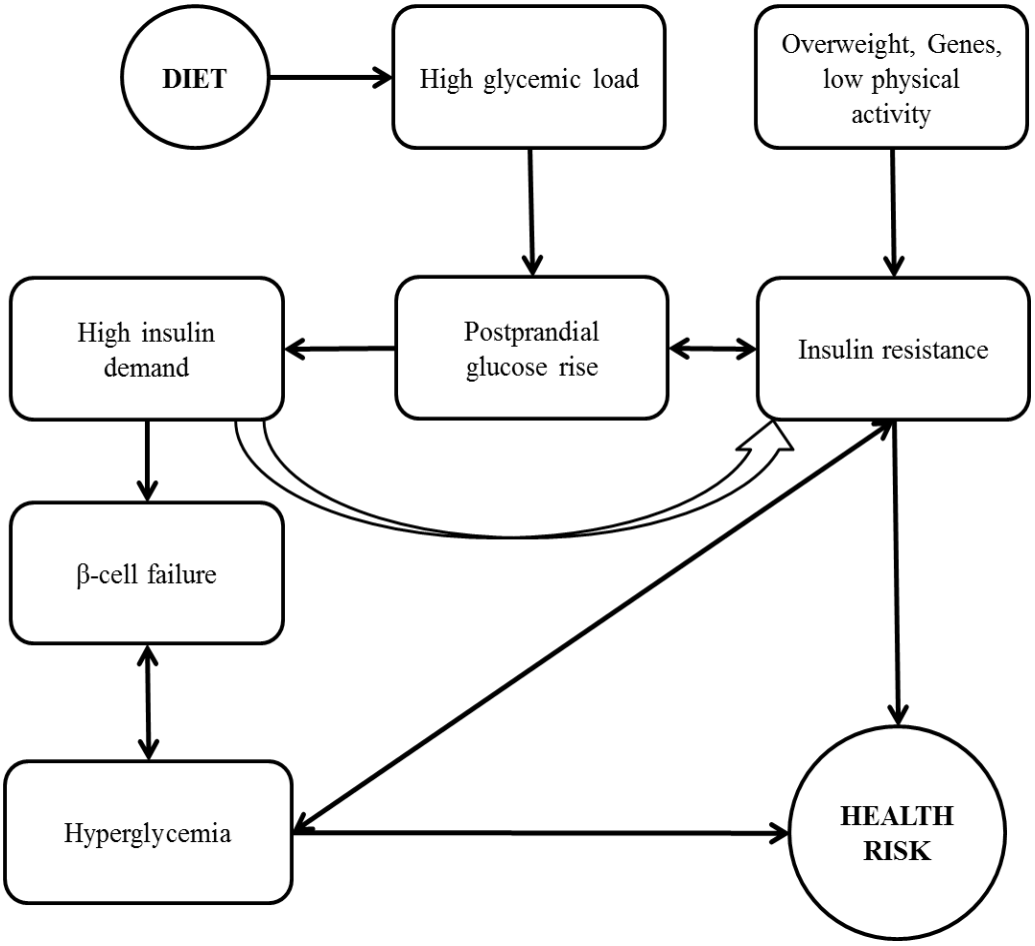


Figure 1-4: Proposed mechanism showing how high glycaemic load in the diet may lead to hyperglycaemia and consequently health risk adapted from (Aston, 2006).

Therefore, as much as carbohydrates are required in the human body as a major source of energy, too much in the diet can have adverse health effects especially high compared to low glycaemic index foods. Therefore, having anything in the diet that can slow down the digestion or absorption of carbohydrates can help reduce health risks (Barclay *et al.*, 2008). Two potential solutions are that of consumption of low glycaemic index foods or

having ingredients in the diet that can reduce the glycaemic index of foods as well as postprandial blood glucose levels. The presence of inhibiting components in the diet that can reduce postprandial glucose can also be a solution to reducing the risk. Polyphenols have the potential to inhibit the rise in blood glucose by hindering carbohydrate digestion and rapid absorption of glucose (Hanhineva *et al*., 2010, Williamson, 2013).

1.3.3 Polyphenols and carbohydrate digestion

The study of polyphenols has become very popular in the recent years due to the health benefits that are attributed to them. Epidemiological studies have attributed their presence in food to health effects which include protection against cardiovascular diseases and diabetes and have shown that regular consumption of fruits and vegetables give a positive effect to human health (Clifford, 2004, Scalbert *et al*., 2005, Crozier *et al*., 2009, Slavin and Lloyd, 2012). Scientific studies *in vivo* and *in vitro* have also shown that polyphenols have potential health benefits. Specific to this research, there is growing evidence that polyphenols have the potential to influence glucose metabolism (Hanhineva *et al*., 2010, Williamson, 2013, Morand and Sies, 2016) and hence can help in the prevention and management of type 2 diabetes. This research focuses on the potential of polyphenols in the prevention and management of type 2 diabetes through the inhibition of carbohydrate digesting enzymes and glucose transporters which may reduce postprandial blood glucose after a carbohydrate-rich meal (Williamson, 2013). More is discussed in chapters 5 and 6 on the health effects of polyphenols.

A number of *in vitro* studies have shown that polyphenols inhibit carbohydrate digestive enzymes (α-amylase and α-glucosidase) (Hanhineva *et al*., 2010). They also inhibit glucose transporters SGLT1 and GLUT2 *in vitro* using the intestinal brush border membrane vesicles and caco-2 cells. (Welsch *et al*., 1989, Johnston *et al*., 2005). This

property of polyphenols is important as it can affect carbohydrate digestion and absorption leading to a reduced postprandial blood glucose which is a risk factor for type 2 diabetes (fig 1-4). If each stage of the biochemical pathway of carbohydrate digestion and absorption is inhibited (fig 1-5), reduced postprandial glucose would be expected as this is the same mechanism used by acarbose, a drug in the treatment of type 2 diabetes. Polyphenols have a very high potential to inhibit carbohydrate digestion and absorption in humans. *In vitro* studies (table 1-1) have shown that polyphenols are able to inhibit all the steps in carbohydrate digestion and absorption. If this is the case in humans, polyphenols can thus help reduce the rises of blood glucose levels after a meal which is particularly important to diabetic people as well as those with metabolic syndrome and the general public as a whole in reducing the risk of cardiovascular diseases and type 2 diabetes. The glycaemic index of foods will thus be reduced when consumed together with polyphenols which may bring about desirable conditions that may help prevent the risk of developing cardiovascular diseases and type 2 diabetes (Ludwig, 2002).

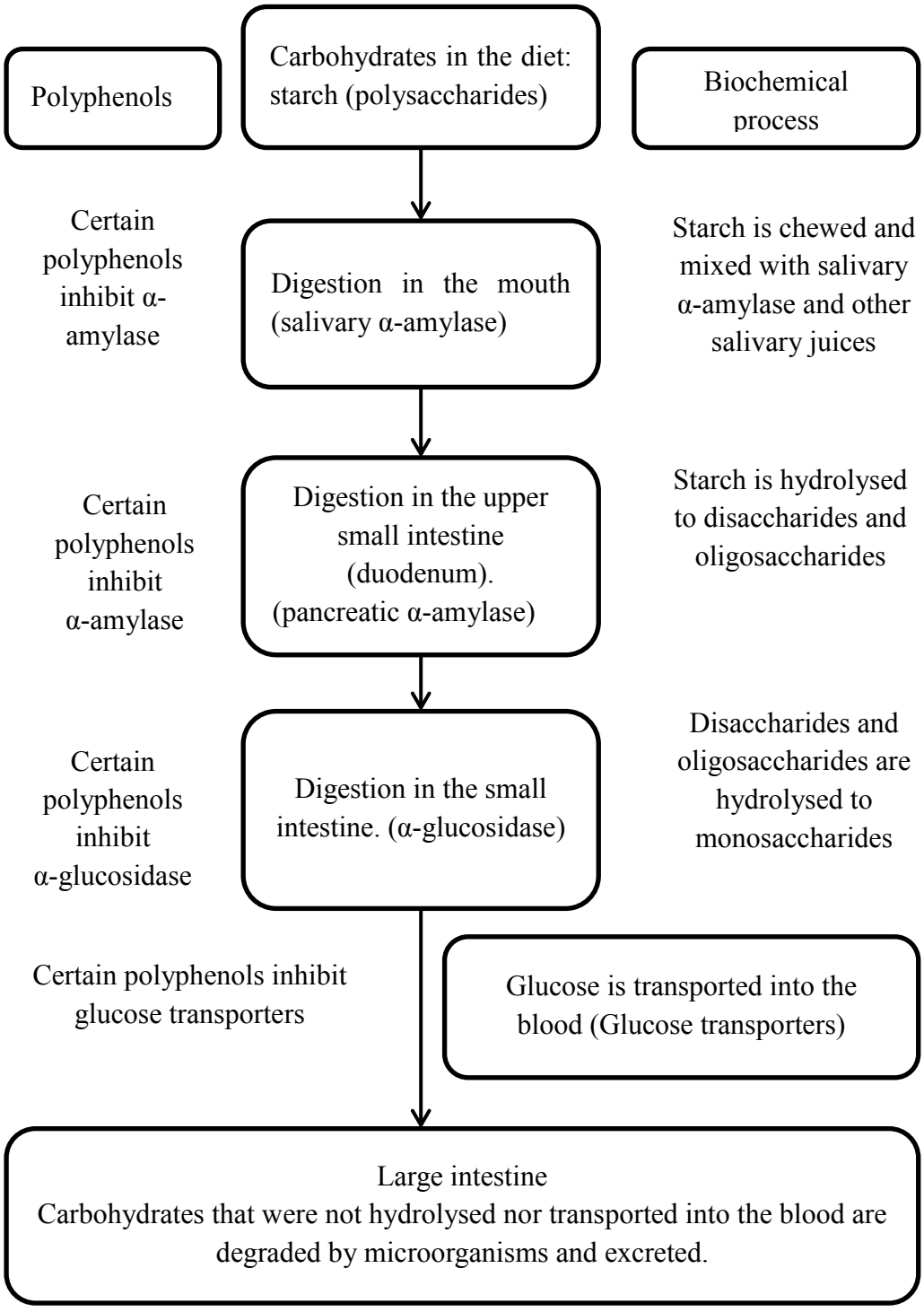


Figure 1-5: A summary showing the stages of carbohydrate digestion and glucose absorption with polysaccharides used as an example to show how polyphenols have the potential to affect postprandial plasma glucose by having the potential to inhibit both digestive enzymes and glucose transporters.

1.4 Research rationale

This research utilised available data from the literature on *in vitro* studies on the inhibition potential of different polyphenols on carbohydrate digesting enzymes (α-amylase and α-glucosidase) and glucose transporters sodium-dependant glucose transporter 1 (SGLT1) and glucose transporter 2 (GLUT2). From the data obtained, the half-maximal inhibitory concentration (IC_{50}) of the polyphenols for the enzymes and transporters were tabulated and converted to micromolar (µM) units (table 1-1). The criterion used was to select the polyphenols with the lowest IC_{50} value as well as only consider the ones that are mostly found in fruits and non-alcoholic beverages to help with design of the meal. Fruits and beverages were preferred because when selecting foods which have high amounts of polyphenols using the Phenol Explorer (web-based database on the polyphenol content of foods), it is important to estimate the amount of polyphenols in a serving rather than the total amount of polyphenols by Perez-Jimenez *et al.*, 2010. For example herbs and spices, even though they rank first in the total polyphenol contents, usually the amount consumed is very low. Using the ranking based on total polyphenols per serving by Perez-Jimenez *et al.*, 2010, changes the ranking from spices being first to fruits (berries) and non-alcoholic beverages (coffee and tea) ranking high. After identifying the polyphenols with the lowest IC_{50} values, Phenol Explorer was used to come up with the fruits and non-alcoholic beverages that contained the highest amounts of the named polyphenols (table 1-2).

Therefore, polyphenols with the lowest IC_{50} value mostly found in fruits and non-alcoholic beverages were selected as they would provide more polyphenols in a serving (cup/glass of beverage and/or portion of fruit).

Polyphenol	**Mammalian α-amylase inhibition (IC_{50})**	**Mammalian α-glucosidase inhibition (IC_{50})**	**Inhibition of glucose absorption from intestine**
Anthocyanins			
Cyanidin-3-O-galactoside	>1000 μM (Adisakwattana *et al*., 2009) >1000 μM (Akkarachiyasit *et* al., 2010)	Sucrase 500 μM, Maltase >3000 μM (Adisakwattana *et al*., 2009)	
Cyanidin-3-O-rutinoside	24.4 μM (Akkarachiyasit *et al*., 2011)	Sucrase 250.2 μM, Maltase 2323 μM (Adisakwattana *et al*., 2011)	
Cyanidin-3-O-sambubioside	960 μM (Iwai *et al*., 2006)	Sucrase 3260 μM, Maltase 3850 μM (Iwai *et al*., 2006)	
Cyanidin-3-O-glucoside	300 μM (Iwai *et al*., 2006)	Sucrase 970 μM, Maltase >3000 μM (Adisakwattana *et al*., 2009)	
Cyanidin-3-O-rhamnoside			Inhibited moderately(Hanamura *et al*., 2006)
Pelargonidin-3-O-glucoside			802 μM (Manzano and Williamson, 2010)
Pelargonidin-3-O-rhamnoside			Inhibited highly (Hanamura *et al*., 2006)
Isoflavones			
Daidzein		>500μM (Tadera *et al*., 2006)	
Flavanones			
Naringenin			8 -50 μM (Park, 1999)

Table 1-1: Summary of polyphenols that are good inhibitors of α-amylase, α-glucosidase and glucose transport. All IC_{50} values have been converted to μM which may be different units from the source.

Polyphenol	**Mammalian α-amylase inhibition (IC_{50})**	**Mammalian α-glucosidase inhibition (IC_{50})**	**Inhibition of glucose absorption from intestine**
Catechins			
Catechin		Sucrase 2490 μM, Maltase 1350 μM (Ishikawa *et al*., 2007)	
Epicatechin		Sucrase 2030 μM, Maltase 1750 μM (Ishikawa *et al*., 2007) Sucrase 1080 μM, Maltase 770 μM (Matsui *et al*., 2007) Sucrase 1000 μM, Maltase 290 μM (Kamiyama *et al*., 2010)	
Catechin gallate		Sucrase 86 μM, Maltase 62 μM (Kamiyama *et al*., 2010) Sucrase 610 μM, Maltase 150 μM (Ishikawa *et al*., 2007)	
Epicatechin gallate		Sucrase 172 μM, Maltase 53 μM (Matsui *et al*., 2007) Sucrase 140 μM, Maltase 40 μM (Kamiyama *et al*., 2010)	100 μM (35 %) (Johnston *et al*., 2005) 1000 μM SGLT1, μM GLUTs (1&3)(Kobayashi *et al*., 2000) 1000 μM (Kobayashi *et al*., 2000) Strongly 0.45 mM inhibition constant (Hossain *et al.*, 2002)

Table 1-1 (continued): Summary of polyphenols that are good inhibitors of α-amylase, α-glucosidase and glucose transport. All IC_{50} values have been converted to μM which may be different units from the source.

Polyphenol	**Mammalian α-amylase inhibition (IC_{50})**	**Mammalian α-glucosidase inhibition (IC_{50})**	**Inhibition of glucose absorption from intestine**
Catechins continued			
Epigallocatechin		Sucrase 921 μM, Maltase 1260 μM (Matsui *et al*., 2007) Sucrase 480 μM, Maltase 120 μM (Kamiyama *et al*., 2010)	100 μM (40 %) (Johnston *et al*., 2005)
Epigallocatechin gallate	400 μM (Tadera *et al*., 2006) 220 μM (Koh *et al*., 2010) 20 μM (34%) (Forester *et al*., 2012)	Sucrase 169 μM, Maltase 40 μM (Matsui *et al*., 2007) Sucrase 130 μM, Maltase 16 μM (Kamiyama *et al*., 2010) Sucrase 100 μM (Honda and Hara, 1993)	100 μM (63 %) (Johnston *et al*., 2005) 1000 μM (Kobayashi *et al*., 2000). Strongly 0.45 mM inhibition constant (Hossain *et al*., 2002)
Phenolic acids			
Caffeic acid	380 μM (Narita and Inouye, 2009)	Sucrase 2180 μM, Maltase 8280 μM (Ishikawa *et al*., 2007)	
Chlorogenic acid (5-Caffeoyl quinic acid)	70 μM (Narita and Inouye, 2009)	Sucrase 2180 μM, Maltase 2990 μM (Ishikawa *et al*., 2007) sucrase 1400 μM (Iwai *et al*., 2006)	2570 μM (Manzano and Williamson, 2010)
Quinic acid	25300 μM (Narita and Inouye, 2009)		

Table 1-1 (continued): Summary of polyphenols that are good inhibitors of α-amylase, α-glucosidase and glucose transport. All IC_{50} values have been converted to μM which may be different units from the source.

Polyphenol	**Mammalian α-amylase inhibition (IC50)**	**Mammalian α-glucosidase inhibition (IC50)**	**Inhibition of glucose absorption from intestine**
Flavonols			
Quercetin	500 μM (Tadera *et al.*, 2006)	Sucrase 1460 μM (Ishikawa *et al*., 2007). Maltase 710 μM (Ishikawa *et al*., 2007)	13 μM (Kwon *et al.*, 2007)
Quercetin-3-O-glucoside			64 μM (Kwon *et al*., 2007) 800 μM (Cermak *et al*., 2004) 640 μM (Kottra and Daniel, 2007)
Quercetin-4'-O-glucoside			37 μM (Kottra and Daniel, 2007) 103 μM (Kwon *et al*., 2007)
Quercetin-3-O-rhamnoside			31 μM (Manzano and Williamson, 2010)
Phloridzin			146 μM (Manzano and Williamson, 2010)
Myricetin	380 μM (Tadera *et al*., 2006)		8 -50 μM (Park, 1999) 17 μM (Kwon *et al*., 2007)
Flavones			
Apigenin			8 – 50 μM (Park, 1999)
Luteolin	360 μM (Tadera *et al*., 2006)		

Table 1-1 (continued): Summary of polyphenols that are good inhibitors of α-amylase, α-glucosidase and glucose transport. All IC_{50} values have been converted to μM which may be different units from the source.

Name of polyphenol	Good fruit/ non-alcoholic beverage source	Average total polyphenols in source (mg/100g FW) (Phenol Explorer)	Content in source (mg/100g FW or mg/100ml) (Phenol Explorer)
Cyanidin-3-O-rutinoside	Blackcurrant	821	160.8
	Sweet Cherry	173	143.3
	Fresh plums	410	33.9
	Blackberry	569	8.9
Cyanidin-3-O-glucoside	Black elderberry	1950	794.1
	Blackberry	569	138.7
	Sweet cherry	173	18.7
	Red raspberry	148	13.3
Epicatechin gallate	Green tea infusion	62	7.5
	Black tea infusion	1045	7.3
	Oolong tea infusion	-	4.9
Epigallocatechin gallate	Green tea infusion	62	27.2
	Oolong tea infusion	-	17.9
	Black tea infusion	105	9.1
Quercetin-3-O-glucoside	Black chokenberry	1752	41.9
	Red raspberry	148	3.6
	Blackcurrant	821	2.6
	Highbush-blueberry	223	1.5
Quercetin-3-O-rhamnoside	Lingonberry	652	12.2
	American-cranberry	315	6.2
	Apple dessert - whole	202	1.3
	Apple dessert pure juice	34	0.7
	Apple cider pure juice	143	0.2
Phloridzin	Apple puree	-	5.6
	Apple dessert-whole	202	2.8
	Apple cider-peeled	251	2.5
	Apple dessert pure juice	34	2.4
	Apple cider pure juice	143	1.5

Table 1-2: Identification of good sources of polyphenols that have good inhibitory effects (lowest IC_{50} value) towards α-amylase, α-glucosidase and glucose transporter.

From table 1-1, the best inhibitors for α-amylase are cyanidin-3-O-rutinosode, EGCG and cyanidin-3-O-glucoside which are present in highest amounts in blackcurrant, green tea and blackberry respectively (table 1-2). For α-glucosidase, EGCG was identified as the best inhibitor with green tea as the best source. Phloridzin, quercetin-3-O-glucoside, quercetin-3-O-rhamnoside and pelargonidin-3-O-glucoside were identified as the good inhibitors of glucose transport and are found in highest amounts in apple, blackcurrant, and strawberry respectively (tables 1-2 and 1-3). EGCG and EGC are also good inhibitors of glucose transport (table 1-1) and hence green tea would also contribute to the overall glucose transport inhibition. The information was used to propose the composition of a polyphenol and fibre-rich food (PFRF) mixture containing polyphenols that showed the highest inhibition towards carbohydrate digestion (carbohydrase inhibition) and absorption (glucose transport). Moreover, the consideration of just using one beverage in the test meal meant that even if coffee has good α-amylase inhibitory potential due to the presence 5-caffeoyl quinic acid, green tea was preferred because although it has the highest inhibition towards α-glucosidase, it also has inhibitory potential to both α-amylase and glucose transport (table 1-1 and table 1-2). Hence the composition of the PFRF mixture comprised of green tea, blackberry, blackcurrant, strawberry and apple (table 1-3). From the ranking table based on total polyphenols per serving, the PFRF mixture ranking was (3, 9, 10, 17, 18 and 19) for blackcurrant strawberry, blackberry, green tea, pure apple juice and apple fruit respectively (Perez-Jimenez *et al*., 2010).

Inhibition target	Good polyphenol inhibitor *in vitro*	IC_{50} value (reference)	Best food source (http://www.phenol-explorer.eu/)
α-amylase	Cyanidin-3-O-rutinoside	24.4 µM (Akkarachiyasit *et al*., 2011)	Blackcurrant
	Epigallocatechin gallate	220 µM (Koh *et al*., 2010)	Green tea
	Cyanidin-3-O-glucoside	300 µM (Iwai *et al*., 2006)	Blackberry
α-Glucosidase	EGCG	Sucrase 130 µM, Maltase 16 µM (Kamiyama *et al*., 2010)	Green tea
		Sucrase 169 µM, Maltase 40 µM (Matsui *et al*., 2007)	
		Sucrase 100 µM (Honda and Hara, 1993)	
Glucose transport	Phloridzin	146 µM (Manzano and Williamson, 2010)	Apple
	Quercetin-3-O-glucoside	64 µM (Kwon *et al*., 2007)	Blackcurrant
	Quercetin-3-O-rhamnoside	31 µM (Manzano and Williamson, 2010)	Apple
	Pelargonidin-3-O-glucoside	802 µM (Manzano and Williamson, 2010)	Strawberry

Table 1-3: Summary of best polyphenol inhibitors of carbohydrate digestion and glucose absorption selected from literature by comparing their IC_{50} values and their good food sources as obtained from Phenol Explorer database (Neveu *et al*., 2010).

1.4.1 Polyphenols and type 2 diabetes

The potential of polyphenols in the prevention and management of type 2 diabetes through the control of postprandial blood glucose in the PFRF mixture was reassessed *in vitro* using enzyme inhibition assays (chapter 4) before using the PFRF mixture in an intervention study (chapter 5) to test its effects on glycaemic response *in vivo*. The PFRF mixture was also analysed for the presence of polyphenols using the Folin assay and characterised for specific polyphenols using high pressure liquid chromatography (HPLC) as discussed in detail in chapters 2 and 3. This was necessary to ensure that the polyphenols of interest were in concentrations at least 3 times higher than the IC_{50} values to account for intestinal dilutions (Williamson, 2013). The PFRF mixture was also analysed for the presence of sugars which was an important control aspect for both the *in vitro* assays and the human intervention study as they could interfere with the assay and add to the amount of sugars present in the test meals respectively. This is the first research that intentionally utilised a combination of different polyphenol sources that are able to inhibit different stages of carbohydrate metabolism to determine the combined effect *in vivo*.

Thereafter, a single source of polyphenols in different food matrixes (juice and capsules) was used to determine the effects of polyphenols on glycaemic response *in vivo* as a step forward in the development of functional foods (chapter 6). Similarly, the source of polyphenols was reassessed *in vitro* for enzyme inhibition and was also characterised for the presence and amounts of specific polyphenols. Sugars were also determined to be taken into account for the design of the assays and human intervention study. This is also the first study to compare the effects of polyphenols on glycaemic response *in vivo* when using different food matrices of the same polyphenol source. Both studies used white bread as the source of starch and control meal to which the glycaemic response was

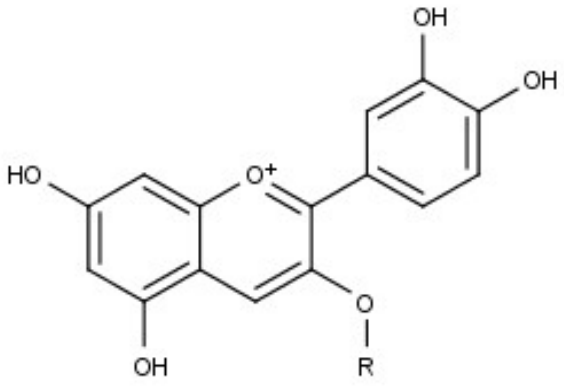
compared to when consumed on its own compared to consuming the bread with the polyphenols in addition to water and balancing sugars if needed. This is described in detail in chapters 5 and 6.

1.4.1.1 Blackcurrant and blackberry polyphenols

The major polyphenols found in blackcurrant and blackberries are the flavonoids called anthocyanidins. The most common anthocyanidin aglycones are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin. The named aglycones form conjugates with sugars and organic acids forming anthocyanins. Anthocyanins are water-soluble and they impart blue, purple or red colours to plant tissues making berries the richest sources of anthocyanins. Consumption of anthocyanin-enriched foods is associated with a reduced risk of chronic diseases such as diabetes (Akkarachiyasit *et al*., 2010). The berries are ranked highly in the list of the richest sources of polyphenols with blackcurrant being ranked number 3 and blackberry number 10 (Perez-Jimenez *et al*., 2010). For people who consume berries routinely, anthocyanins are major polyphenol sources in excess of 1 g per serving. Anthocyanins are said to be among the least absorbed polyphenols and would therefore make the small intestine as their site of action including the inhibition of α-amylase and α-glucosidase as they are found in high concentrations in the intestine (Manach *et al*., 2004, Manach *et al*., 2005). However, microbial metabolites of anthocyanins might also have an effect.

Cyanidin and its glycosides are reported to have promising potential health benefits to humans especially in reducing the risk of obesity, metabolic syndrome and type 2 diabetes (Akkarachiyasit *et al*., 2010). *In vitro* studies (Akkarachiyasit *et al*., 2011, Iwai *et al*., 2006) have shown that cyanidin-3-O-rutinoside and cyanidin-3-O-glucoside are good inhibitors of α-amylase. Cyanidin-3-O-sambubioside was also reported to have inhibitory properties though with a slightly higher IC_{50} value (table 1). Cyanidin-3-O-

rhamnoside inhibited glucose transport moderately whereas pelargonidin-3-O-glucoside highly inhibited glucose transport (Hanamura *et al*., 2006, Manzano and Williamson, 2010).



Cyanidin, R = H
Cyanidin-3-galactoside, R = galactose
Cyanidin-3-glucoside, R = glucose
Cyanidin-3-rutinoside, R = rutin

Figure 1-6: Chemical structure of cyanidin and its glycosides

Inhibition of carbohydrate digesting enzymes as well as glucose transporters has the potential to suppress postprandial hyperglycaemia and hence may play a very important role in reducing the risk of metabolic syndrome, insulin resistance, and subsequently diabetes type 2. Therefore the inclusion of blackberry, black currant and strawberry in the PFRF mixture will provide polyphenols to inhibit carbohydrate digestion and absorption.

1.4.1.2 Green tea polyphenols

Tea is one of the most consumed beverages in the world ranked second to water in popularity globally (Yang *et al*., 2009). Tea is produced from the leaves of *Camellia sinensis* and depending on the level of fermentation, four types are usually produced. White and green teas are both unfermented whereas oolong tea is semi-fermented and black tea is fermented. When tea is fermented, it induces enzymes to oxidise catechins

giving the characteristic black or orange-red colour of tea due to the formation of two major pigments known as theaflavins and thearubigins. (Horzic *et al*., 2009). Epidermiological studies have shown that consumption of tea is linked to health benefits and polyphenols found in them may play a role. The presence of the flavonoids catechins and theaflavins have been attributed to the great potential that tea has in reducing the risk of preventing various chronic diseases such as cardiovascular disease, obesity and diabetes (Higdon and Frei, 2003). In green tea, the beneficial effects observed are attributed to the presence of flavonoids. The five main flavonols found in green tea are catechin derivatives and include (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate (fig 1-7).

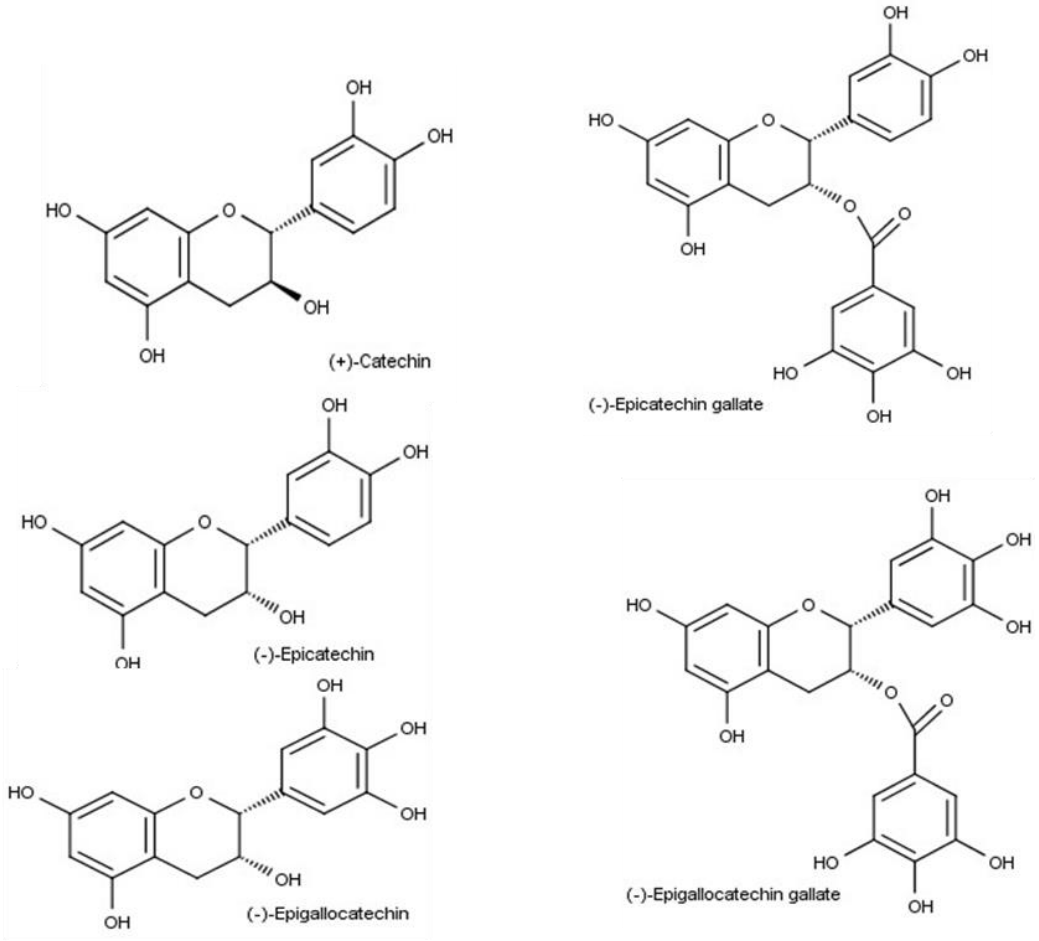


Figure 1-7: Chemical structures of green tea flavanols

Oral administration of 1.5, 3.0 and 4.5 g decaffeinated green tea solids to human volunteers gave maximal plasma concentrations (C_{max}) of 326, 550, and 190 ng/L for ECGC, EGC and EC at 1.4 - 2.4 h after ingestion of the tea preparations. About 14 and 31 % of EGC and EC was detected in plasma whereas <1 % EGCG was bioavailable in rats. It is presumed that EGCG converts to EGC in the small intestine hence the highest concentration of EGCG is found in the small intestine before it converts to EGC which was excreted in the urine together with EC (Yang and Landau, 2000). From the average data obtained from seven studies on green tea consumption, EC was highly absorbed and EGCG was the least absorbed (Williamson *et al*., 2011). Catechins are however potent enough to act in the gut and concentrations have been reported to be high and reach millimolar levels (Williamson, 2013). The consumption of green tea has been linked to the reduction of the risk of diabetes (Vinson and Zhang, 2005) and studies have shown the potential of green tea in reducing blood glucose levels. An *in vitro* study showed that different types of tea have different inhibitory potential on the two main carbohydrate digestion enzymes α-amylase and α-glucosidase (Koh *et al*., 2010). Among the catechins, the galloylated catechins EGCG and ECG have stronger inhibitory effects against mammalian α-glucosidase and moderate inhibition on human salivary α-amylase *in vitro* (Koh *et al*., 2010). A relatively low effective dose of EGCG acutely reduced postprandial blood glucose levels in mice when co-administered with common corn starch which gives a compelling case for human studies (Forester *et al*., 2012). This shows that green tea has the potential to inhibit the breakdown of starch *in vivo* and hence reduce the glycaemic index/load of the starch. Tea catechins inhibited glucose transport using caco-2 cells by 63, 40 and 35 % using 100 µM, EGCG, EGC and ECG as inhibitors respectively (Johnston *et al*., 2005).

Therefore, it is evident that green tea has the potential to affect postprandial blood glucose by acting as an inhibitor of carbohydrate digestion and absorption due to the inhibitory properties of its catechins on the digestive enzymes α-amylase and α-glucosidase as well as glucose transporters. This justifies its inclusion in the polyphenol-rich mixture for the human intervention study, specifically to act as the inhibitor for α-glucosidase for its inhibition (IC_{50} value <50 for maltase and <200 μM for sucrase) towards α-glucosidase *in vitro* (table 1-1).

1.4.1.3 Apple polyphenols

Apples are widely consumed worldwide and historically it has always being associated with health benefits as can be seen from the famous saying which says ‘an apple a day keeps the doctor away’ (Boyer and Liu, 2004). Research has since attributed the presence of polyphenols as a contributing factor to the health benefits of fruits and vegetables in addition to vitamins and fibre. A number of epidemiological studies have linked the consumption of apples to a reduced risk of cancer, cardiovascular disease, asthma, obesity and diabetes (Boyer and Liu, 2004). Apples are a good source of polyphenols and were ranked 18 (pure apple juice) and 19 (apple fruit) in the richest dietary sources of polyphenols per serving by Perez-Jimenez *et al*., 2010.

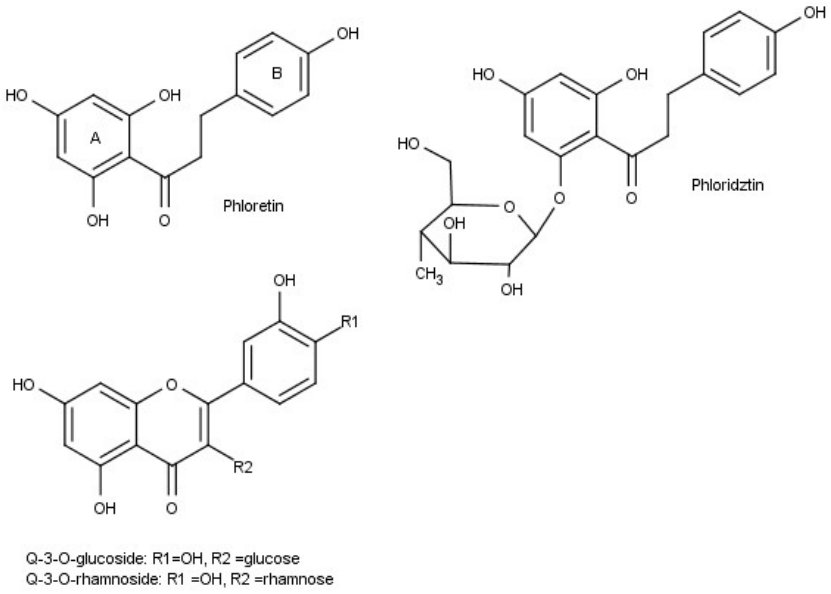


Figure 1-8: Chemical structures of apple polyphenols which have inhibitory effects on glucose absorption.

A review by Boyer and Liu (2004), reviews a number of studies that looked at the associations of consumption of apples to a number of chronic diseases including type 2 diabetes and results showed favourable effects in humans. Among the polyphenols found in apples, quercetin and its conjugates (Boyer and Liu, 2004) are reported to offer more protection against the chronic diseases including type 2 diabetes, with quercetin-3-O-rhamnoside and quercetin-3-O-glucoside having inhibitory properties against glucose transporters GLUT2 and SGLT1 (Ader *et al*., 2001, Cermak *et al*., 2004). In addition, phloretin and its glucosidic form phloridzin (phloretin-2'-O-glucoside) (fig-1-5) also inhibit glucose transporters GLUT2 and SGLT1 respectively (Minami *et al*., 1993, Gromova, 2006). Phloridzin is said to be a stronger inhibitor of glucose transport and may thus be beneficial for reducing the risk of obesity, type 2 diabetes and cardiovascular disease (Johnston *et al*., 2002, Manzano and Williamson, 2010).

1.4.1.4 Pomegranate polyphenols

Pomegranate is one of the oldest edible fruits and it is consumed fresh or processed into fresh juice, jams flavours and colourants. The different parts of the pomegranate tree have been used since ancient times in folk medicine for various diseases (Vidal *et al*., 2003). The presence of polyphenols is said to play a major role in the health properties and they include anthocyanins (which impart the red colour), phenolic acids (mainly ellagic and gallic acids), tannins (mainly punicalin and punicalagin) as well as flavonoids such as quercetin, phloridzin and catechins. The anti-diabetic activity (property to control blood glucose levels) of pomegranate is generally attributed to the presence of the tannins (punicalin, pendunculagin and punicalagin) (Medjakovic and Jungbauer, 2013). The tannins have high molecular weights and their chemical structures (fig 1-9) indicate the presence of hydroxyl groups which may play a major role in its anti-diabetic mechanisms which will be discussed in chapter 7. Other pomegranate polyphenols that also show anti-diabetic properties include: ellagic acid found in the flower, which increases insulin sensitivity (Huang *et al*., 2005), phenolic acids in seeds which decreased weight gain and improved insulin sensitivity and hence reduced the risk of type 2 diabetes in rats (McFarlin *et al*., 2009, Banihani *et al*., 2013). Details of their anti-diabetic properties are discussed in chapter 6.

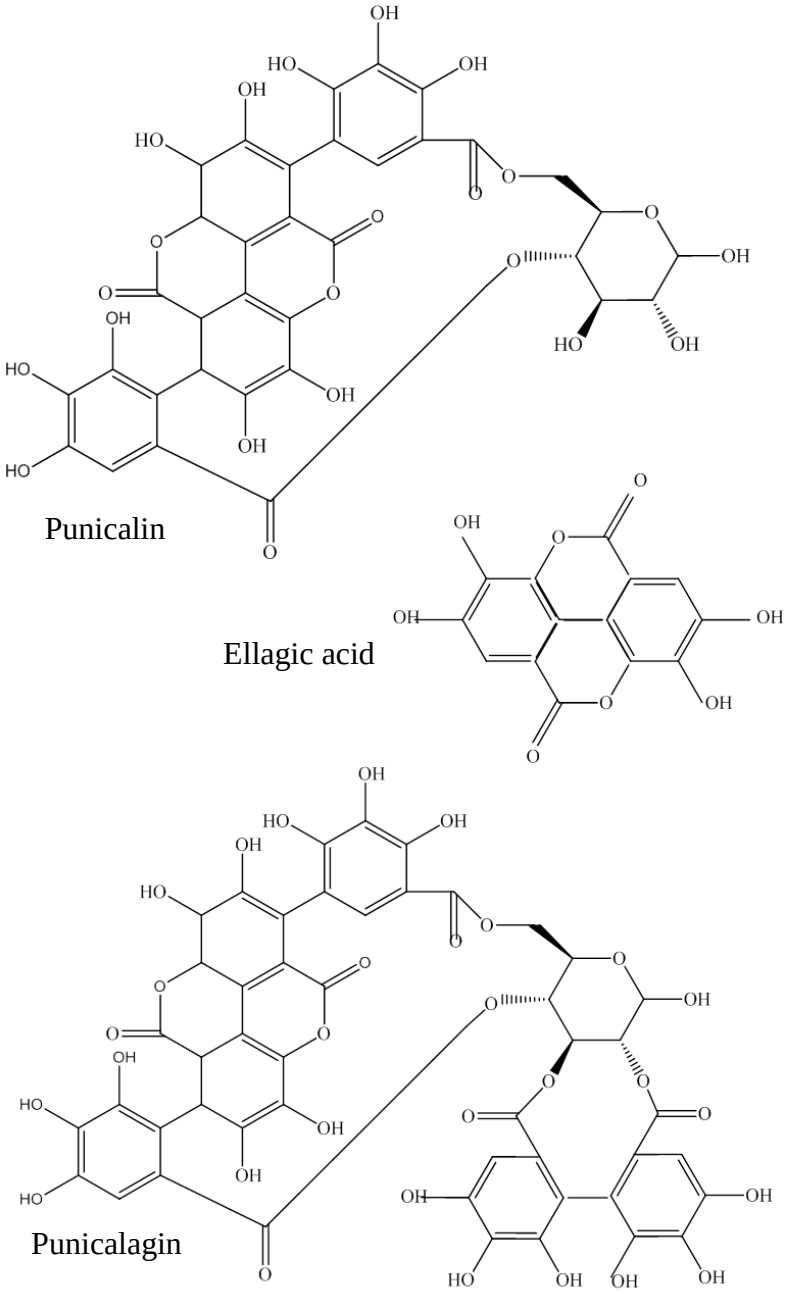


Figure 1-9: Chemical structures of pomegranate tannins punicalin and punicalagin as well as ellagic acid which will be investigated for inhibitory effects on carbohydrate digestive enzymes.

1.5 Hypothesis

By having a mixture of polyphenols in a meal that inhibit all the different stages of carbohydrate digestion and absorption (fig 1-5), a maximum effect of polyphenols on glycaemic response would be expected as all inhibitions would give a combined effect. Intervention studies will be carried out to determine the effects of polyphenols on glycaemic response *in vivo*.

1.6 Main Objective

To determine the effect of polyphenols on glycaemic response in healthy volunteers when consumed together with white bread (high glycaemic index food).

1.6.1 Specific objectives

- To determine the effects of polyphenols in the polyphenol and fibre-rich food (PFRF) mixture on glucose and insulin response in healthy volunteers.
- To determine the effects of different doses of polyphenols in the PFRF mixture on postprandial plasma glucose and insulin responses of healthy volunteers.
- To determine the effects of polyphenols from a single food source and compare the effects on postprandial glucose when different food matrices are used.

Chapter 2 . Materials and methods optimisation

2.1 Materials

Below is a list of equipment and materials used in this research.

2.1.1 Equipment

1. PHERAstar FS micro-plate reader (BMGlabtech, Ortenberg, Germany).
2. 96-well plate compatible for ultraviolet (UV) (Greiner Bio-one, Stonehouse, UK).
3. Accuchek Glucometer, Aviva Lancets and Strips (Roche Diabetes care Limited, Burgess Hill, West Sussex, UK).
4. 96-well plate for non UV range (Nunc A/S., Roskilde, Denmark).
5. Micro-plate sealing film (Z369659-100EA, Sigma-Aldrich, St Louis, MO, USA).
6. An Agilent 1200 SL system (Agilent Technologies, Dorset, UK).
7. Zorbax Eclipse plus C18 (1.8 µm, 100 x 2.1 mm) and Agilent-Zorbax eclipse XDB-C18 (1.8 µm, 50 x 4.6 mm) columns (Agilent Technologies, Dorset, UK).
8. Millipore water purifying system (Millipore, Hertfordshire, UK).
9. Genevac (EZ-2 plus model) (Fisher Scientific Ltd, Leicestershire, UK).
10. Eppendorf centrifuge (5810R) (Fisher Scientific Ltd, Leicestershire, UK).
11. Water bath (GLS Aqua 12 plus, Grant Instruments, Cambridge, UK).
12. Prevail Carbohydrate ES 5 µm column (GRACE, Lokeren, Belgium).
13. The Oasis Max SPE Cartridges (Waters Corporation Ltd, Milford, MA, USA).
14. UFLC$_{XR}$ Shimadzu system (Shimadzu, Kyoto 604-8511, Japan).

15. VISIPREP Manifold for solid phase extraction (Supelco Analytical, Sigma-Aldrich, St Louis, MO, USA).
16. Oasis MAX cartridge 1 mL (30 mg) and 3 mL (60 mg) (Waters Co-operation Ltd., Mildford, Mass, U.S.A).

2.1.2 Reagents

1. Glucose Hexokinase (G 3293) reagent (Sigma-Aldrich, St Louis, MO, USA).
2. Mercodia human insulin ELISA kit and Mercodia Diabetes Antigen Control (10-1164-01) (Mercodia AB, Uppsala,Sweden).
3. Formic acid, 3,5-Dinitrosalicylic acid, acetic acid, benzoic acid and trifluoroacetic acid (Sigma-Aldrich, St Louis, MO, USA).
4. Human salivary α-amylase type IX-A, powdered acetone protein extract from rat intestine, pancreatin, amyloglucosidase and invertase (Sigma-Aldrich, St Louis, MO, USA).
5. Folin-Ciocalteu Reagent (Merck, Steinheim, Germany).
6. Sodium carbonate, sodium phosphate dibasic, sodium phosphate monobasic sodium potassium tartrate, 3,5-dinitrosalicylic acid, disodium hydrogen phosphate (Na_2HPO_4 anhydrous), sodium dihydrogen phosphate (NaH_2PO_4 anhydrous), sodium chloride (NaCl), sodium acetate trihydrate, calcium chloride, acetonitrile and sodium hydroxide (NaOH) (Sigma-Aldrich, St Louis, MO, USA).
7. Methanol, DMSO and ethanol, (Sigma-Aldrich, St Louis, MO, USA).
8. Glucose, sucrose, fructose, maltose, iso-maltose, amylose, amylopectin and guar (Sigma-Aldrich, St Louis, MO, USA).

2.1.3 Polyphenols

1. Gallic acid (Alfa Aesar, Lancashire, UK)
2. Epigallocatechin, epigallocatechin, epicatechin gallate, epicatechin, cyanidin-3-O-rutinoside, cyanidin-3-O-glucoside, phloridzin, pelargonidin-3-O-glucoside, hesperetin, taxifolin, myricetin and quercetin-3-O-rhamnoside (Extra-synthase, Genay, France).
3. Ellagic acid, punicalagin, an isomeric mixture of punicalagin A and B, and punicalin, a mixture of A and B (Phytolab, Vestenbergsgreuth, Germany).

2.1.4 Food/supplements samples

1. Glucose, and fructose for human consumption (Holland and Barrett, Leeds, UK).
2. Warburton bread and sucrose (Morrison's and Tesco, Leeds, UK)
3. Green tea powder (Nestle, Research Centre, Lausanne, Switzerland).
4. Pomegranate juice (Healthy Supplies, Lancing, UK)
5. Freeze dried blackcurrant, blackberry and strawberry (Healthy Supplies, Lancing, UK)
6. Freeze dried apple peel (AppleBoost, USA)
7. Pomegranate supplements (CSIC-CEBAS, Spain)
8. Pomegranate extract (CSIC-CEBAS, Spain)

2.2 Hexokinase assay for the determination of blood glucose

2.2.1 Introduction

There are different methods that can be used to measure blood glucose. The common methods include glucose oxidase, automatic analysers, glucometers and hexokinase (HK). Due to the nature of the analysis (comparison of reference and test glucose response), any recognized and well standardised method of measuring glucose can be used. However it should have a coefficient of variation (CV) of analytical variation of <3 % and should give at least 98 % recovery from a glucose spike as is the recommendation for clinical trials (Widjaja *et al*., 1999) . The hexokinase assay was modified and optimised (Encarnacao, 2015) and will be used as it meets the requirements of CV of analytical variation of <3 % and at least 98 % recovery from a glucose spike.

2.2.2 Principle of the method

In enzyme kinetics, the rate of an enzyme reaction changes with substrate concentration. Determination of amount of product formed can thus be used to measure the substrate initially present. The glucose HK assay from Sigma (product code GAHK-20) utilises a second enzyme which converts the product into a measurable substance as shown below.

1) Glucose + ATP $\xrightarrow{\text{Hexokinase}}$ Glucose-6-phosphate + ADP
2) G6P + NAD^+ $\xrightarrow{\text{G-6-PDH}}$ 6-phosphogluconate + NADH

In the first reaction, glucose reacts with ATP in the presence of hexokinase enzyme and is phosphorylated to form glucose-6-phosphate (G-6-P). In the second reaction, G-6-P oxidation is catalysed by the enzyme glucose-6-phosphate dehydrogenase (G-6-PDH) in the presence of NAD^+ to give the oxidised form 6-phosphogluconate and an equimolar amount of NAD^+, is reduced to NADH. NADH is measured by a spectrophotometer at

340 nm and is used to obtain the concentration of glucose in the original sample, since the increase in absorbance at 340 nm is directly proportional to the glucose concentration in the original sample.

2.2.3 Equipment and reagents

The PHERAstar FS micro-plate reader and 96-well plate compatible for ultraviolet (UV) reading were used for UV absorbance readings. The glucose hexokinase reagent contained 1.5 mM of NAD^+, 1.0 mM ATP and 1.0 unit m/L hexokinase. A unit phosphorylates 1.0 µmol of D-glucose per min at 25 °C and pH 7.6 with phosphorylation rate constant of 0.12 mM at 30 °C and pH 7.5. It also contains 1.0 unit m/L of glucose-6-phosphate dehydrogenase with 1 unit defined to convert 1.0 µmol of glucose-6-phosphate to 6-phosphogluconate per min at 25 °C and pH 7.4 in the presence of NADH. D-glucose was used for glucose standard curves.

2.2.4 Procedure

The method was modified and optimised to use a 96 well plate to reduce analysis time and plasma volume from 1 mL to 10 µL (Encarnacao, 2015). A stock solution of 1.5 mg/mL D-glucose solution was prepared and different concentrations required for the standard curve were obtained by diluting the stock with millipore water accordingly to obtain concentrations of 0.025, 0.25, 0.5, 1.0 and 1.5 mg/mL (0.14, 1.39, 2.7, 5.55 and 8.33 mM). The sample blank, reagent blank, standard blank, standard and plasma were then mixed accordingly with either millipore water or reagent in 96 well plates and incubated for 15 min at 0 °C and then placed in the plate reader to read the absorption at 340 nm. After correcting for all the blanks (sample/standard and reagent blank), a standard curve for D-glucose was obtained and the equation used to calculate amount of glucose in the plasma**.**

2.2.4 Method optimisation

2.2.4.1 Glucose standard curves

To set up the basis to accept or disregard a standard curve for each experiment, three experiments were carried out to obtain the equation of D-glucose standard curves as shown in figure 2-1. The aim was to come up with a range of Slopes that should always be checked for each experiment to ensure that the standard curve equation is within the acceptable range.

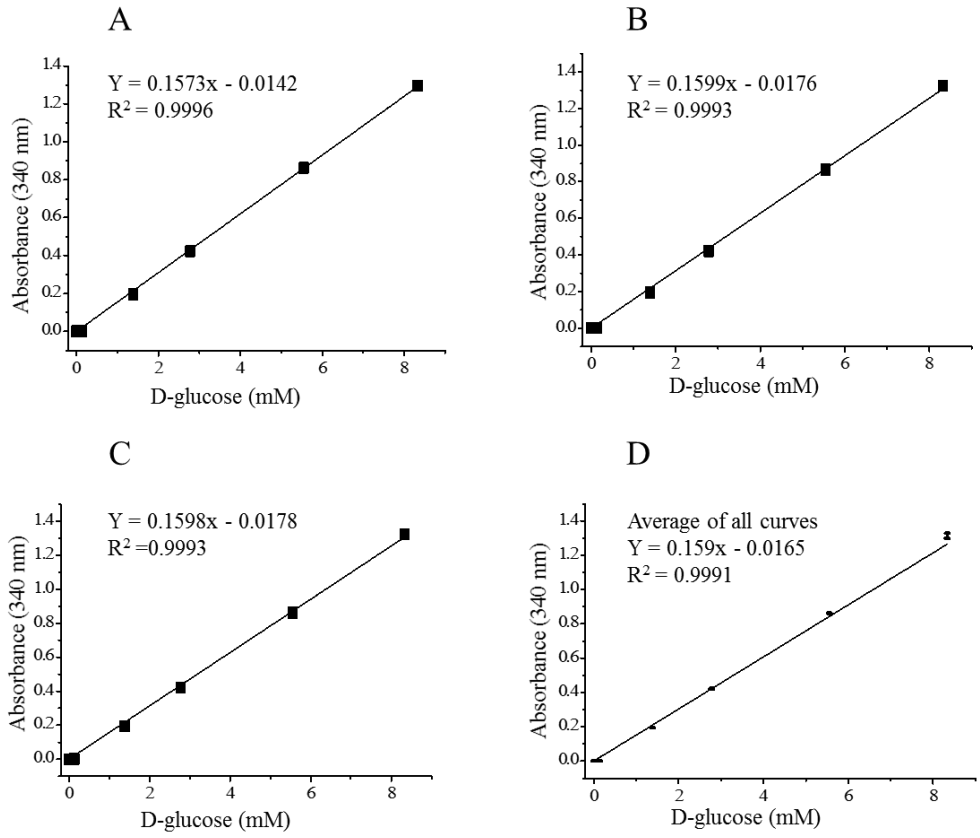


Figure 2-1: D-glucose standard curves for three biological triplicates (A, B and C) each data point being mean±SD, n=3, to determine an acceptable range of the slopes to be used as a guide for each experiment's glucose standard curve. The average of the D-glucose standard curves (D) with standard deviation error bars for standard curves (A, B and C), n=3.

The glucose standard curve slopes from the equations of the three biological triplicates gave a mean ±SD of 0.159±0.017 which was used as a reference for each glucose analysis experiment of which the slope had to be within this range to be acceptable. All experiments carried out had their slopes falling in the acceptable range. Linearity shows the proportionality of the response to the measured analyte and is determined by how well a standard curve follows a straight line. A common measure of linearity is the square of the correlation coefficient (R^2). The coefficient of determination R^2 was good for all the D-glucose standard curves that were carried out and was above 0.999.

2.2.4.2 Precision

Precision shows how well replicate measurements agree with one another and are usually expressed as a standard deviation. Instrument precision is the reproducibility obtained when the same quantity of one sample is repeatedly introduced in an instrument. To test this, three fasting plasma samples were aliquoted and analysed on five different days and results were calculated using the appropriate standard curve equations and the following results were obtained.

Biological triplicates	Glucose concentration (mM/L)					
	Week 1	Week 2	Week 3	Week 4	Week 5	Average ±SD
1	4.6	4.5	4.5	4.5	4.5	4.52±0.04
2	4.5	4.3	4.4	4.4	4.4	4.4±0.07
3	4.6	4.4	4.5	4.3	4.5	4.5±0.11

Table 2-1: Plasma glucose results of biological triplicates of fasting blood samples which were each aliquoted in five tubes and stored in the -80 °C freezer. Each week, one aliquot of each of the biological triplicates was analysed for the determination of glucose concentration using the hexokinase assay.

2.2.5 Conclusion

The method is reliable and reproducible as the aliquots of the same sample analysed on different days gave results that were very close with standard deviations of 0.04, 0.07 and 0.11 giving CV of 0.9, 1.6 and 2.4 % respectively which are all <3 %. In addition, a glucose spike result was 100±2 % which is also in the acceptable range of >98 %. This proved that the method and techniques were good and will be used for the analysis of blood glucose in the intervention study.

2.3 Insulin measurement by ELISA

2.3.1 Introduction

There are three types of methods that are used for the determination of insulin content in blood. They are *in vivo, in vitro* and immunological methods. In the *in vivo* methods, serum is injected into animals in which an insulin-like effect is produced. This effect is then compared with the response of known standard insulin solutions. The *in vitro* method involves incubating an isolated tissue in serum and one metabolic process produced by the tissue is compared to that produced by known insulin standard solutions Immunological methods determine the concentration of insulin in the serum by its binding to insulin antibody. The latter is the most commonly used method for insulin analysis especially the enzyme-linked-immuno-sorbent-assay (ELISA) (Federlin, 2012).

2.3.2 Principle of the method

The Mercodia insulin ELISA is based on the direct sandwich technique (solid phase two-site enzyme immunoassay). It is based on the affinity of an antibody (ABI) coated well which is specific to the antigen or molecule of interest on the insulin molecule. The sample to be analysed is added into the well and there is a specific binding reaction between antibody and molecule of interest. A washing buffer step then follows and it removes any unbound molecule. A second antibody linked to an enzyme (peroxidase) is then added into the antibody-antigen complex formed in the first step. A substrate solution, 3,3',5,5'-tetramethylbenzidine (TMB), is then added and reacts with the enzyme. The reaction is stopped by adding acid and a colour develops, proportional to the amount of molecule of interest present in the well. The colour intensity is measured spectrophotometrically using a micro-plate reader at 450 nm. The molecule of interest

can be quantified by using the standard curve equation obtained using the standard solutions enclosed in the kit.

2.3.3 Equipment and reagents

The PHERAstar FS micro-plate reader with 450 nm filter was used for absorbance reading. The plate shaker was used for incubation step with shaking. The kit contained ready-to-use coated plate, calibrators 0, 1, 2, 3, 4 and 5, enzyme conjugate buffer, substrate TMB and stop solution. It also contained 11 times concentrated enzyme conjugate buffer (11X) and 21 times concentrated wash buffer (21X).

The plate is coated with mouse monoclonal anti-insulin and calibrators 0, 1, 2, 3, 4 and 5 are recombinant human insulin with concentrations of 0, 3.2, 10.5, 31.1, 103 and 214 mU/L respectively. Substrate contains 3,3’,5,5’-tetramethylbenzidine (TMB) which is light sensitive and the stop solution contains 0.5 M sulphuric acid. Mercodia Diabetes Antigen Control was purchased separately.

2.3.4 Procedure

As recommended by the supplier, the ELISA kit was always stored at 4 °C and one batch was used for all the plasma samples to avoid batch related differences. The wash buffer (21X) was diluted 21 times with millipore water to 1X for use and once diluted could be stored for 8 weeks at 2-8 °C. Enzyme conjugate solution (11X) was also diluted to 1X fresh each time to use in the assay by diluting it 11 times with enzyme conjugate buffer.

For each analysis, insulin calibrators 0, 18, 60,180, 600 and 1200 pM were analysed to obtain a standard curve for insulin calculations in the plasma. They were taken out of the fridge and allowed to equilibrate to room temperature (20 °C). Plasma stored at -80 °C was thawed each time before analysis, hand mixed and centrifuged in a 5810R Eppendorf centrifuge at a speed of 4000 rpm at 4 °C for 10 min.

Using an appropriate pipette, 25 µl each of plasma and calibrators were added to clearly labelled wells of the coated plate. To each well 100 µL of enzyme conjugate 1X solution was added and the plate was incubated for 1 hr on a plate shaker at room temperature (20 °C). Incubation allows for the insulin molecules in the plasma to bind to the anti-insulin antibody on the plate. After incubation, the contents of the wells were discarded by inverting the micro-plate over the sink. The wells were then washed 6 times by adding 350 µL wash buffer 1X solution to each well and making sure excess liquid is removed between washes by tapping firmly several times against adsorbent paper. This washing step removes any unbound molecules. A second substrate TMB (200 µL) was added to each well and allowed to react with the bound complex by incubating at room temperature for 15 min. The reaction was stopped by adding 50 µL stop solution to each well. The plate was then placed on the shaker for 5 sec to mix and absorbance was read at 450 nm in the plate reader. Results were calculated by using the insulin standard curve equation to determine the amount of insulin in the plasma.

2.3.5 Method optimisation

The commercially available kit from Mercodia for insulin analysis is already optimised by the supplier. The assay kit guarantees high accuracy and avoids cross reactions with similar molecules such as pro-insulin because it uses monoclonal antibodies for both the primary and secondary antibodies. To validate this, insulin standard curve analysis with biological triplicates was carried out as shown in figure 2-2 to determine reliability of the results.

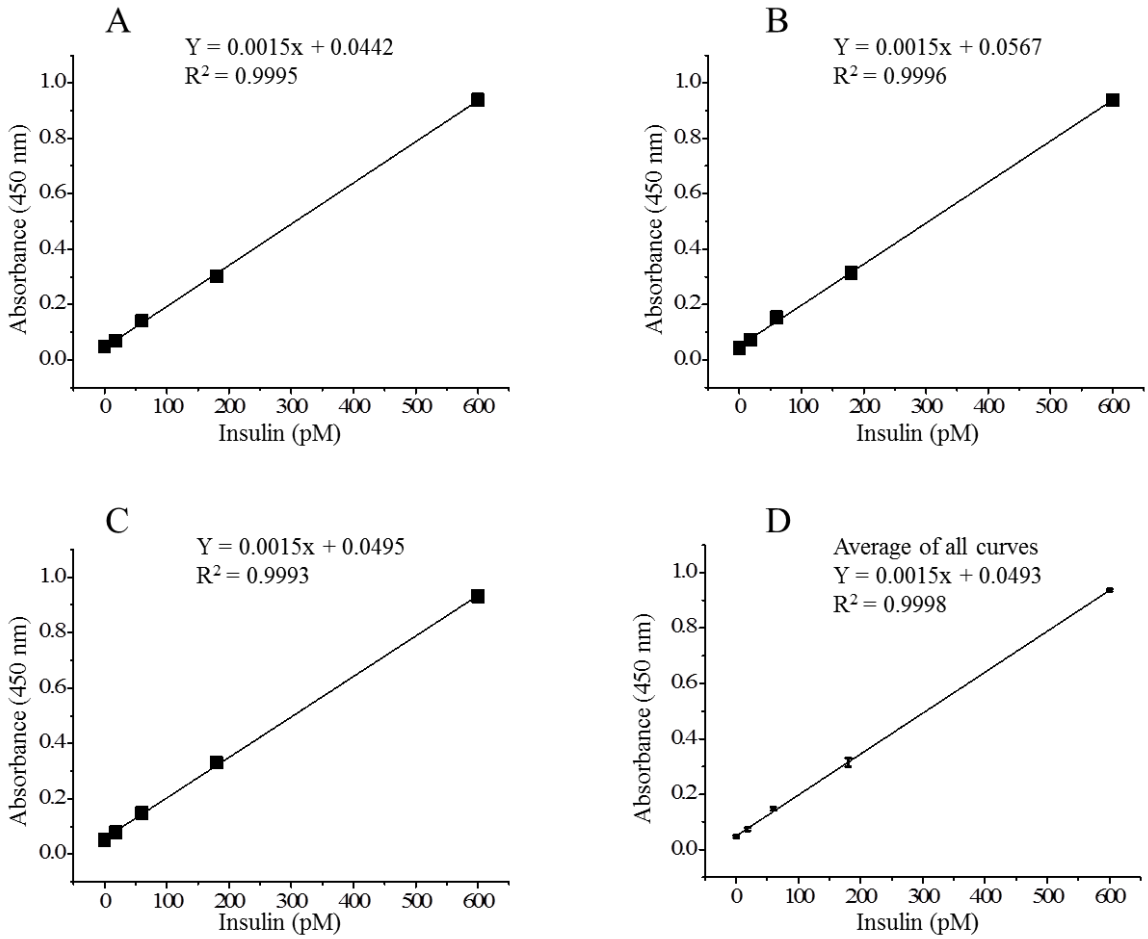


Figure 2-2: Insulin standard curves for three biological triplicates (A, B and C), for the determination of insulin concentration in plasma (data points are expressed as mean±SD n=3). (D) Is the average of the insulin standard curves (A, B and C) with standard deviation error bars (n=3).

The coefficient of determination R^2 was good for all the insulin standard curves and was all above 0.999. The average of the curves gave very small standard deviations as shown by the error bars in figure 2-1 (D). Mercodia antigen controls (high and low insulin concentrations) were always analysed together with samples and calibrators to ensure that the sensitivity of the assay was maintained. The coefficients of variation between the assays were 7.3 and 8.2 % for the low and high controls respectively.

2.3.6 Conclusion

The Mercodia kit for insulin was shown to be a good method for insulin determination as demonstrated by the standard curves and the R^2 values confirming good linearity. Inter assay repeatability was also confirmed as shown by the coefficients of variation values between the assays for the low and high controls. Hence this assay is precise and has good linearity and suitable to be used for determining insulin concentration in plasma.

2.4 Folin assay for the determination of total polyphenols

2.4.1 Introduction

There are different methods used to measure the total phenol contents of food with the Folin-Ciocalteu method being the most acceptable (Margraf *et al*., 2015). The Folin assay method determines the total polyphenol content in food samples using Folin-Ciocalteu reagent and the spectrophotometer. It is preferred as it is inexpensive, soluble in water, recrystallized easily from water, readily dried and stable in dry form Singleton *et al*., 1999). The Folin-Ciocalteu assay was first used by Singleton and Rossi in 1965 when they improved the method from using Folin-Denis reagent which gave precipitation problems. The Folin-Ciocalteu formulation gave greater colour, less variation and better recovery (Singleton, 1985). The method used in this research is adapted directly from Singleton *et al.* (1999) with modifications by including a control for each sample to account for any interference from other reducing agents in the food sample (Yahya, 2012).

2.4.2 Principle of the method

The assay involves the reduction of Folin-Ciocalteu reagent by phenols to form a blue coloured complex which gives maximum absorbance in the spectrophotometer at 765 nm. The Folin-Ciocalteu reagent measures total phenols and any reducing agents and will thus measure the total reducing capacity of a sample. Therefore, to measure the amount of total polyphenols, most commonly gallic acid is used as a standard so that all phenols are reported in gallic acid equivalents (GAE).

2.4.3 Reagent and standard preparation

Folin-Ciocalteu Reagent was prepared by diluting it 10 times with millipore water. Sodium carbonate solution was prepared by dissolving 75 g (Mw=75) in 1 L using a 1 L volumetric flask and 80 % methanol was prepared by mixing 200 mL of millipore water with 800 mL of pure methanol in a 1 L volumetric flask. Gallic acid stock standard solution (1 mg/mL) was prepared and used to obtain various concentrations (25, 50, 75, 100, 125, 150, 175, 200 µg/mL) for the standard curve, by diluting with millipore water.

2.4.4 Procedure

The assay was conducted by adding 1 mL of every solution (sample, standard and control) into separate 15 mL Falcon tubes followed by the addition of 5 mL of the Folin-Ciocalteu reagent to each tube. The tubes were capped, mixed on a vortex mixer and 4 mL of sodium carbonate solution was added within 3-8 min from the addition of Folin-Ciocalteu reagent. The tubes were capped and mixed quickly and then placed in the water-bath at 26 °C and incubated for 2 hr. The control sample had 4 mL sodium carbonate replaced with 4 mL millipore water. Absorbance was read using the plate reader spectrophotometer at 765 nm wavelength. A gallic acid standard curve was

obtained and using its equation, the amount of GAE of the sample and control were determined. The total polyphenol content was calculated as:

GAE of sample - GAE of the control.

2.4.5 Method optimisation

The method was optimised by including a control sample to account for other interferences from other reducing agents such as ascorbic acid (Yahya, 2012). The coefficient of determination (R^2) was good in all the experiments (>0.99). Moreover, the value of the Slope was also very similar in all the experiments as shown by the error bars in the average standard curve (figure 2-3D).

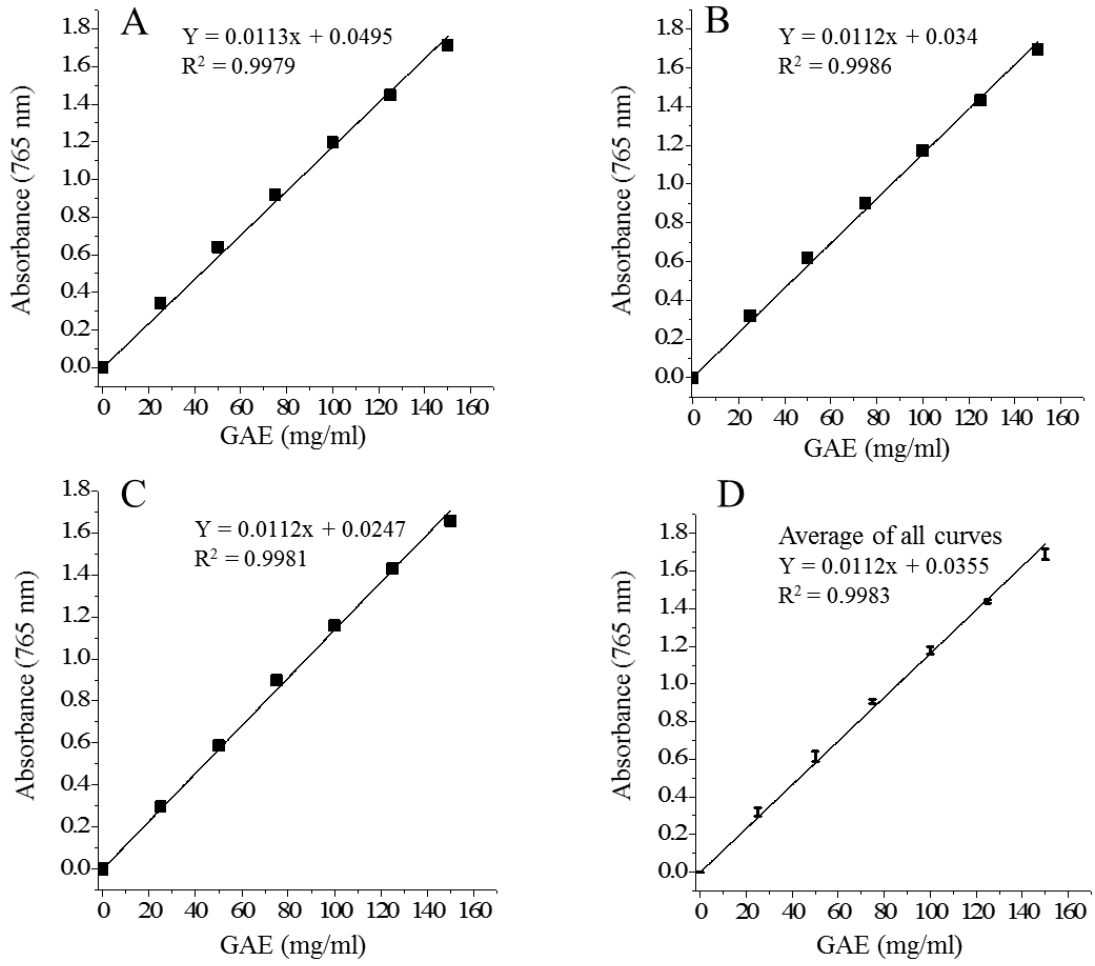


Figure 2-3: Gallic acid standard curves for three biological triplicates (A, B and C) for the determination of insulin concentration in plasma (each data point being mean±SD n=3). (D) is the average of the gallic acid standard curves (A, B and C) with standard deviation error bars (n=3).

2.4.6 Conclusion

The coefficient of determination for the gallic acid standard curve was good for all experiments. Hence the method is reproducible and reliable to use.

2.5 High Performance Liquid Chromatography (HPLC) for polyphenol identification and quantification

2.5.1 Introduction

HPLC is a chromatographic method that separates, identifies and quantifies components from a mixture. Unlike the Folin-Ciocalteu assay that gives the total amount of polyphenols in a food sample, HPLC is more specific as it will identify as well as quantify individual components. Reverse phase HPLC was used.

2.5.2 Materials and equipment

An Agilent 1200 SL system equipped with a diode array detector (DAD) was used for all HPLC analysis. It comprised of a binary pump, degasser, column oven (35 ˚C) and well plate auto-sampler (5 ˚C). Two columns were used depending on sample; a Zorbax Eclipse plus C18 column (1.8 µm, 100 x 2.1 mm) and Agilent- Zorbax eclipse XDB-C18 (1.8 µm, 50 x 4.6 mm). They are 600 bar, rapid resolution and high throughput columns. Other parameters include 5 µL injection volume, 0.5 mL/min flow rate with needle wash in flush point for 3 sec.

For all analysis, ultrapure, nuclease free water (≥18.2 MΩ cm at 25 ˚C) from a Millipore water purifying system was used. For sample preparation, a Genevac (EZ-2 plus model) was used for evaporation.

2.5.3 Reagents and standards preparation

1 mg/mL standard solutions were prepared in 50 % ethanol and aliquots of 100 µL (100 µg) were dried using the Genevac evaporator and stored dry at -20 °C until further use. Addition of 1 mL of 50 % ethanol to the tube containing 100 µg of the standard, gave a

concentration of 100 µg /mL which was used to obtain concentrations of 10, 30, 50, 70 and 90 µg /mL. The standards were analysed using reversed phase HPLC and using an internal standard each time. Internal standards are important for improving the precision and accuracy of results obtained by correcting analytical variability. Solvent A (mobile phase) consisted of (millipore water with 0.1 % formic acid) and solvent B (stationery phase) consisted of (Acetonitrile with 0.1 % formic acid).

2.5.4 Procedure

The separation started with 5 % solvent and chromatographic conditions of elution continued as follows: 0-5 min, increase of solvent B from 5 to 10 %, 5-20 min, increase solvent B to 40 %, 20-25 min, increase of solvent B to 90 %, 25-29 min, isocratic for 4 min, 29-30 min, decrease solvent B to 5 % and re-equilibration for 3 min at 5 %.

Standard curves were obtained using different concentrations of standards and were used to determine the amounts of specific polyphenols in a food mixture. The peaks were identified by use of the retention time, spectra as well as spiking the food samples with polyphenols of interest. The retention time of the particular polyphenol obtained in the standard analysis gave an estimate of what retention time the same polyphenol is likely to have in the food mixture. By comparing the spectra of the particular polyphenol standard with that obtained in the food mixture gave an indication of which peak represented the particular polyphenol. Finally, spiking was also used to confirm the identification of a compound of interest by adding a particular standard to the mixture and the peak that gave a corresponding increase was identified as the compound of interest.

2.5.5 Method optimisation

Reproducibility of the method was tested by using taxifolin as the internal standard for 5 different experiments on green tea extract. It was prepared on the first day of experiments and aliquots containing equal amounts were dried and stored at -20 °C in the same way as the standards. Taxifolin was used as internal standard for 5 experiments and peak areas were compared (table 2-2).

Experiment	**Mean peak areas ±SD (mAU)**	**COV (%)**
1	464.3±39.7	8.5
2	469.3±23.9	5.1
3	473.5±16.8	3.4
4	474.1±7.7	1.6
5	466.1±4.8	1.0
Average of All Experiments	468.7±26.2	5.6

Table 2-2: Mean±SD (n=3) peak areas and COV of taxifolin used as an internal standard in the characterisation of green tea and green tea polyphenols in 5 different experiments using reverse phase HPLC.

The coefficient of variation (COV) obtained from five different experiments was good (<10 %) in all the results. Blank and positive samples gave expected results giving an indication that the experiments were accurate. From the results shown, the COV reduced from the first experiment having the highest 8.5 % to the last experiment having 1.0 % showing an improvement in the skills of the analyst, but overall all were below 10 %.

2.5.6 Conclusion

The square of correlation coefficient R^2 was good in all the experiments that were carried out and was above 0.999 as will be shown in chapter 3. There was a good relationship of the standard solutions being measured which were used to determine the amounts of polyphenols in the food samples. The method has good specificity and selectivity as it was possible to distinguish a particular polyphenol from food extracts that contained a mixture of polyphenols. The method was also reproducible hence will be used for the quantification of polyphenols in the food extracts to be used in this research.

2.6 Englyst method for the determination of available carbohydrates in bread

2.6.1 Introduction

The Englyst method determines the different types of starch by controlled enzymic hydrolysis of starch and determines the glucose released using the glucose oxidase method. One modification to the method was that the glucose released was determined using hexokinase as it has been optimized and validated as discussed in section 2.2. The aim of this analysis was to determine the amount of bread to give a certain amount of glucose after digestion.

2.6.2 Materials and reagents

Sodium acetate buffer (0.1 M, pH 5.2) was prepared by dissolving 13.6 g sodium acetate trihydrate in 250 mL saturated benzoic acid solution and 4 mL of 1 M calcium chloride added to act as a stabiliser and activator of enzymes. The pH was adjusted to 5.2 with 0.1 M acetic acid. Using appropriate calculations, a concentration of 140 U/mL

amyloglucosidase was prepared from 300 U/mL stock. A stock of 150 mg/mL pancreatin was prepared by adding 20 mL millipore water to 3 g pancreatin which was mixed and stirred for 10 min. The mixture was placed in centrifuge tubes and centrifuged for 10 min at 4000 rpm. Supernatant was placed in another tube and was ready for use. Invertase (3000 U/mL) was prepared by dissolving 60 mg of the powder (1 mg = 300 U) in 6 mL sodium acetate buffer. The enzyme mixture was prepared by mixing pancreatin (150 mg/mL), invertase (3000 U/mL) and amyloglucosidase (140 U/mL) in the ratio 1: 13.5: 9

2.6.3 Procedure

Centrifuge tubes (50 mL) were correctly labelled for test samples and glucose standard curves. For the test samples, bread samples (1 g) were placed in labelled 50 mL centrifuge tubes. Then approximately 50 mg guar and 5 glass balls were added to each tube to help with the mixing upon addition of 20 mL acetate buffer. The same was done for the standards tubes except that 20 mL of known glucose solution in acetate buffer was added in place of the bread sample. To correct for any glucose present in amyloglucosidase, a blank tube was prepared containing glass balls, guar gum and 20 mL acetate buffer. All tubes (sample, blank and standards) were treated the same in the following steps.

The tubes were equilibrated to 37 °C in a water-bath. Thereafter, 5 mL of the enzyme solution was added to each tube and time set accordingly from this point allowing at least 2 min between tubes. The tubes were then placed horizontally in a shaking water-bath at 37 °C with the glass balls providing the crushing action.

After 120 min, 0.5 mL of the hydrolysate was removed (without stopping the shaking action) and placed in a labelled tube containing 20 mL 66 % ethanol, and mixed well to denature the enzymes.

The standards, samples and controls were all analysed for amount of glucose using the hexokinase assay as described in section 2.2. After correcting for blanks, the standard curve equation was used to calculate the amount of glucose in the samples. Knowing the amount of bread added in each tube, calculations were done to determine how much glucose was released from a gram of bread for use in subsequent calculations.

2.6.4 Method optimisation

Linearity was achieved for the glucose standard curve obtained by adding known concentrations of glucose in the enzymatic digestion mixture. The repeated curves gave similar slope and the coefficient of determination was above 0.99 in all (figure 2-4).

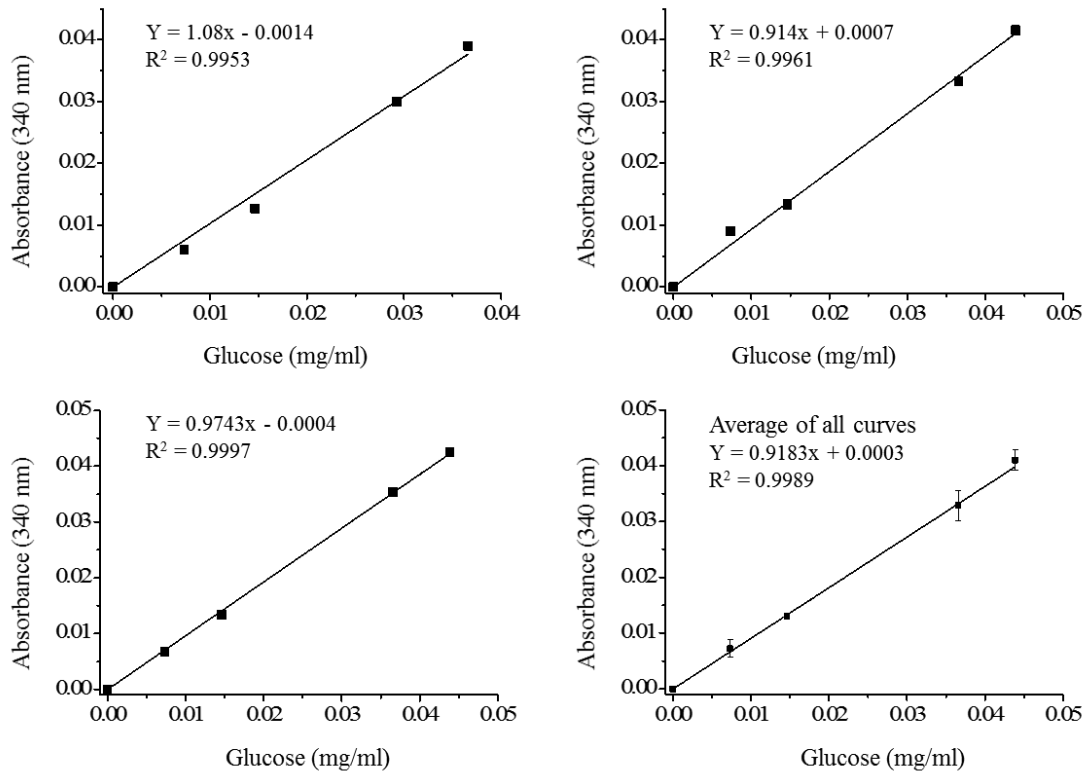


Figure 2-4: Glucose standard curves for three biological triplicates (A, B and C) used for the determination of glucose produced after enzymatic hydrolysis of bread (each data point being mean±SD n=3). The average of the standard curves (A, B and C) is shown in (D) with standard deviation error bars (n=3).

Bread samples from four batches were aliquoted and stored in the -20 °C freezer and were analysed for the amount of glucose produced after 2 hrs on different days. The aim was to determine the reproducibility of the method and to compare the variability between different batches of bread as shown in table 2-3. The method was reproducible and different batches of bread gave similar results of the amount of glucose released after the hydrolysis with low standard deviations.

Sample/batch number	**Glucose (g/100g bread)**				
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Mean ±S.D within batch
1	42.2	43.7	45.1	45.1	44.0±1.4
2	46	44	44.1	45.7	45±1
3	44.6	44.1	44.6	45.4	44.7±0.5
4	45.6	44.6	45.1	45.1	45.1±0.4
Mean ±S.D between batches	44.6±1.7	44.1±0.4	44.7±0.5	45.3±0.3	

Table 2-3: Mean ± S.D (n=4) results of amount of glucose produced (g) from 100 g bread for 4 batches of bread. Each batch was analysed 4 times (experiment 1-4) to determine variability within each batch and between batches.

2.6.5 Conclusion

The method by (Englyst *et al*., 1992) was validated and gave both good linearity and was reproducible. Warburton's brand of bread did not vary within batches and hence will be used in the intervention study meals.

2.7 α-Glucosidase assay for the determination of α-glucosidase inhibition by polyphenols

2.7.1 Introduction

Inhibition of α-glucosidase by polyphenols is determined by comparing the enzyme activity in the test (with inhibitor) to the control (without inhibitor). Glucose production is used as the end point as inhibiting α-glucosidase affects the amount of glucose produced from the hydrolysis of disaccharides. Maltose, sucrose or iso-maltose can be used as substrates for which the activity can be related to maltase, sucrase and iso-maltase respectively obtained from powdered acetone protein extract from rat intestine. The method was adapted from Adisakwattana *et al*. (2009) with some modifications.

2.7.2 Principle of the method

The assay involves enzymatic hydrolysis of disaccharides to release glucose and the enzymes activities are determined by analysing the amount of glucose produced. By comparing the production of glucose in the presence and absence of inhibitors, the inhibition of α-glucosidase by plant extracts or pure compounds is determined.

2.7.3 Chemicals and equipment

The PHERAstar FS micro-plate reader and 96-well plate compatible for UV reading were used for UV absorbance reading at 340 nm. Intestinal acetone powder extract from rat intestine, glucose, sucrose, maltose, iso-maltose, sodium phosphate dibasic, sodium phosphate monobasic and sodium hydroxide were used for the assay.

2.7.4 Preparation of reagents

Sodium phosphate buffer (10 mM, pH 7.0). 10 mM solutions of sodium phosphate monobasic and sodium phosphate dibasic were prepared separately. The buffer was prepared by mixing 256.85 mL sodium phosphate monobasic with 243.15 mL sodium phosphate dibasic both 10 mM. The pH was adjusted to 7 with sodium hydroxide or phosphoric acid.

Acetone extract of rat intestine. Stock (50 mg/mL) was prepared by mixing with the vortex mixer for 30 sec. The supernatant was obtained and placed in a new tube after micro-centrifuging the mixture at 4000 rpm for 10 min.

2.7.5 Determination of assay conditions

2.7.5.1 Sucrose apparent km determination for assay substrate concentration

The aim of this experiment was to determine the substrate concentrations (sucrose, maltose and iso-maltose) to use in the α-glucosidase inhibition assays.

Different concentrations of sucrose were prepared ranging from 1 to 100 mM. The assay was prepared by adding 200 µL substrate (for each sucrose concentration), 100 µL PBS, mixed well and allowed to equilibrate to 37 °C in the water-bath for 5 min. To start the reaction, 200 µL (20 mg/mL) RIP (previously warmed to 37 °C) was added to the assay mixture, mixed gently and placed in the water-bath for 20 min. After 20 min, the reaction mixture was placed in the hot water-bath at 100 °C for 10 min to stop the reaction by denaturing the enzyme. The mixture was cooled to room temperature on ice and samples analysed for the amount of glucose produced using the hexokinase assay described in section 2.2. The Lineweaver-Burk and Hanes-Woolf transformations were used to obtain the apparent km value (Lineweaver and Burk,1934).

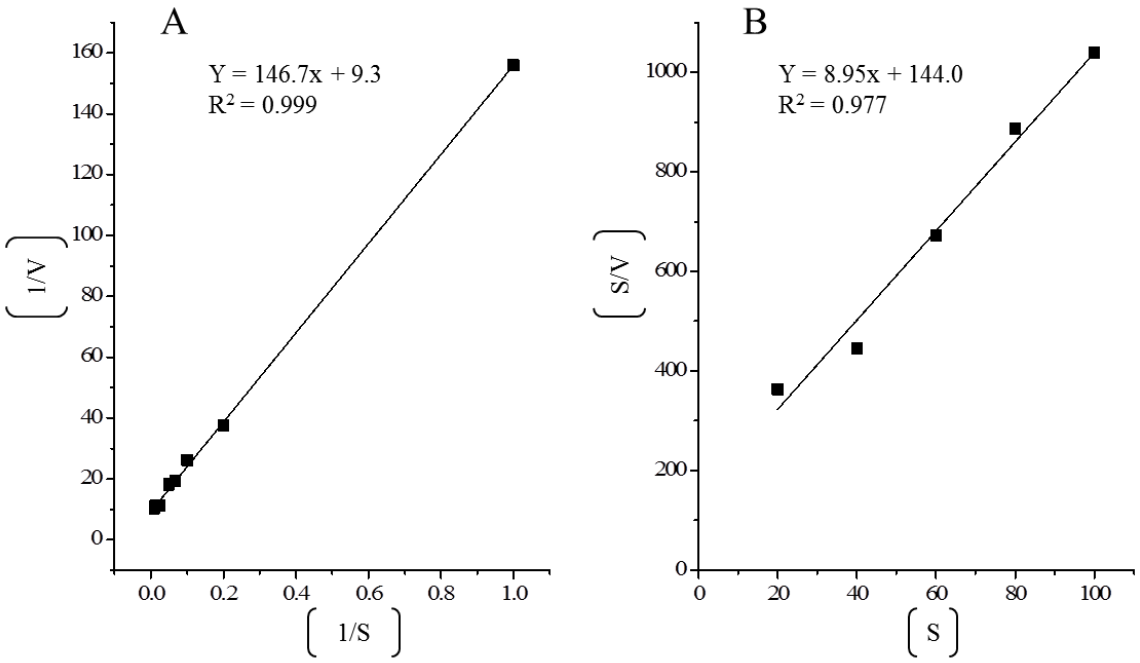


Figure 2-5: The Lineweaver-Burk (A) and Hanes-Woolf (B) plots showing the relationship between the substrate concentration [S] and the enzyme rate [V] used to determine the apparent kinetic parameter km with sucrose as substrate. Each data point being mean±SD, n=3.

The km values obtained for sucrose were 15.8 and 16.1 mM and hence 16 mM will be used as the substrate concentration because it is recommended to use the km value as it is optimal for all types of inhibitors. Higher than km favours uncompetitive whilst lower than km favours competitive inhibitors (Acker and Auld, 2014). This is because for competitive inhibition, the inhibitor binds to the active site where the substrate usually occupies the enzyme thereby competing with the substrate ability to bind. So as the substrate concentration increases above the km value, there is a higher probability of the substrate occupying the active site over the inhibitor at a fixed inhibitor concentration. For uncompetitive inhibition, the inhibitor binds to the enzyme only when the enzyme is already bound to the substrate molecule. Therefore at concentrations lower than the km value,there are fewer substrate-enzyme complexes existing which results in a lower probability of uncompetitive inhibitors to inhibit the enzyme (Acker and Auld, 2014).

2.7.5.2 Iso-maltose apparent km determination

The same procedure used for sucrose was used and used different iso-maltose concentrations ranging from 1 to 10 mM to obtain the Lineweaver-Burk and Hanes-Woolf plots.

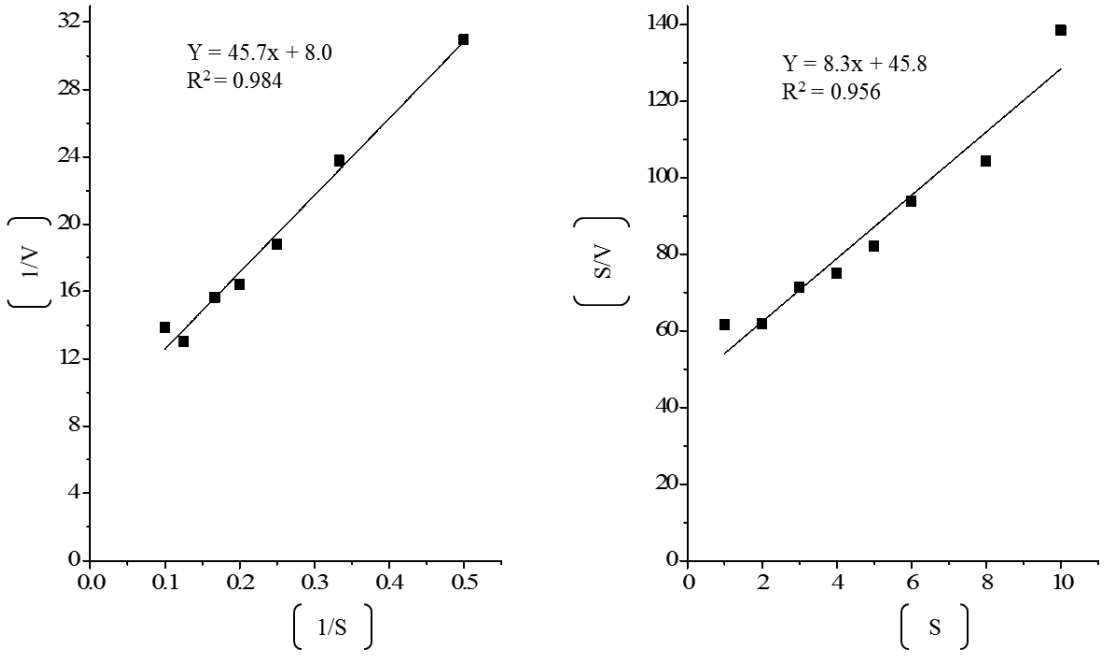


Figure 2-6: The Lineweaver-Burk (A) and Hanes-Woolf (B) transformations showing the relationship between the substrate concentration [S] and the enzyme rate [V] used to determine the apparent kinetic parameter km with iso-maltose as substrate. Each data point being mean±SD, n=3.

The apparent km values obtained for iso-maltose were 5.7 and 5.6 mM for the Lineweaver-Burk and Hanes-Woolf transformations respectively. A concentration of 6 mM substrate concentration for iso-maltose will be used as recommended to be optimal (Acker and Auld, 2014).

2.7.5.3 Maltose apparent km determination

The same procedure as for sucrose km determination was performed except that the different sucrose concentrations were replaced with different maltose concentrations ranging from 1 to 20 mM to obtain the Lineweaver-Burk and Hanes-Woolf transformations.

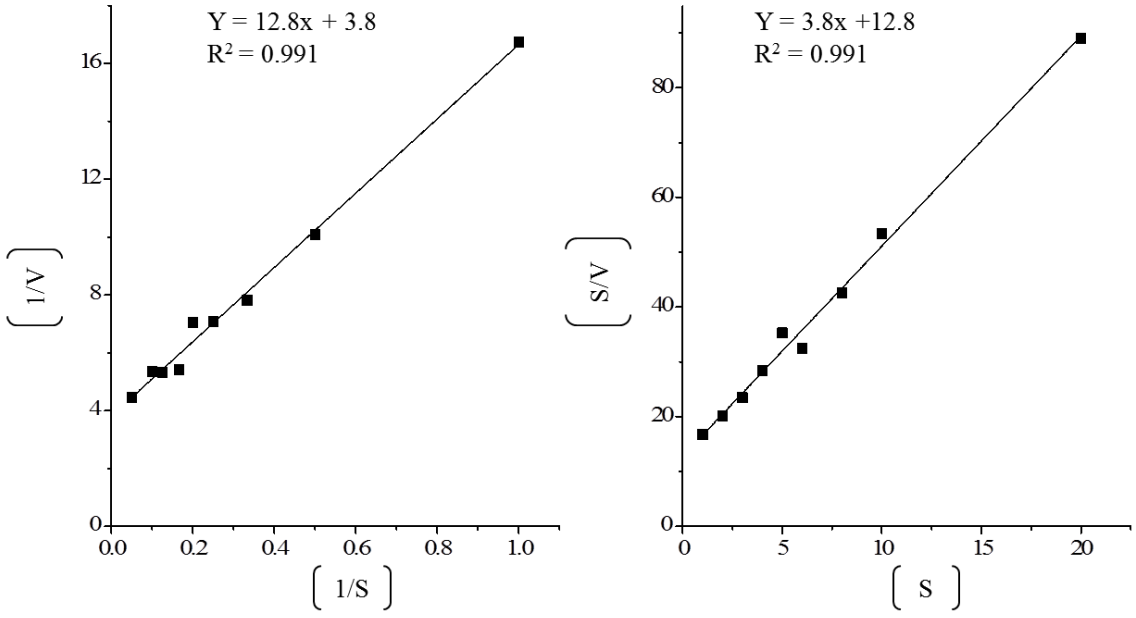


Figure 2-7: The Lineweaver-Burk (A) and Hanes-Woolf (B) transformations showing the relationship between the substrate concentration [S] and the enzyme rate [V] used to determine the apparent kinetic parameter km with maltose as substrate. Each data point being mean±SD, n=3.

The apparent km values obtained for maltose were 3 and 3.3 mM for the Lineweaver-Burk and Hanes-Woolf transformations respectively. A concentration of 3 mM substrate concentration for maltose will be used as recommended to be optimal (Acker and Auld, 2014).

2.7.5.4 Sucrase reaction time and enzyme concentration

The aim of this experiment was to determine the optimum reaction time and enzyme concentration when using sucrose, iso-maltose and maltose as substrates and rat intestinal acetone powder (RIP) as the source of the enzymes. This combination needs to be in the linear range of the enzyme activity versus incubation time. This is important to avoid misinterpretation of results as reduced glucose production may be due to other factors like depleted substrate or enzyme when not in the linear range.

Using 16 mM sucrose concentration (apparent km value) as substrate concentration in the assay, 3 enzyme concentrations were used for 3 different sets of samples treated exactly the same and only differing in enzyme concentration used. The three enzyme concentrations were 10, 20 and 30 mg/mL RIP in assay. All the three sets were then incubated for different times from 5 min to 60 min. This was achieved by removing the tubes for each set time out of the water-bath and immediately placing them in the hot water-bath at 100 °C to denature the enzymes. The 0 min incubation reaction tubes had their enzyme denatured immediately by placing the tubes in the water-bath immediately after adding the enzyme to the reaction tube. The amount of glucose produced for each sets of tubes were determined using the hexokinase as described in section 2.2. The amount of glucose produced versus incubation time was then plotted and is shown in figure 2-8.

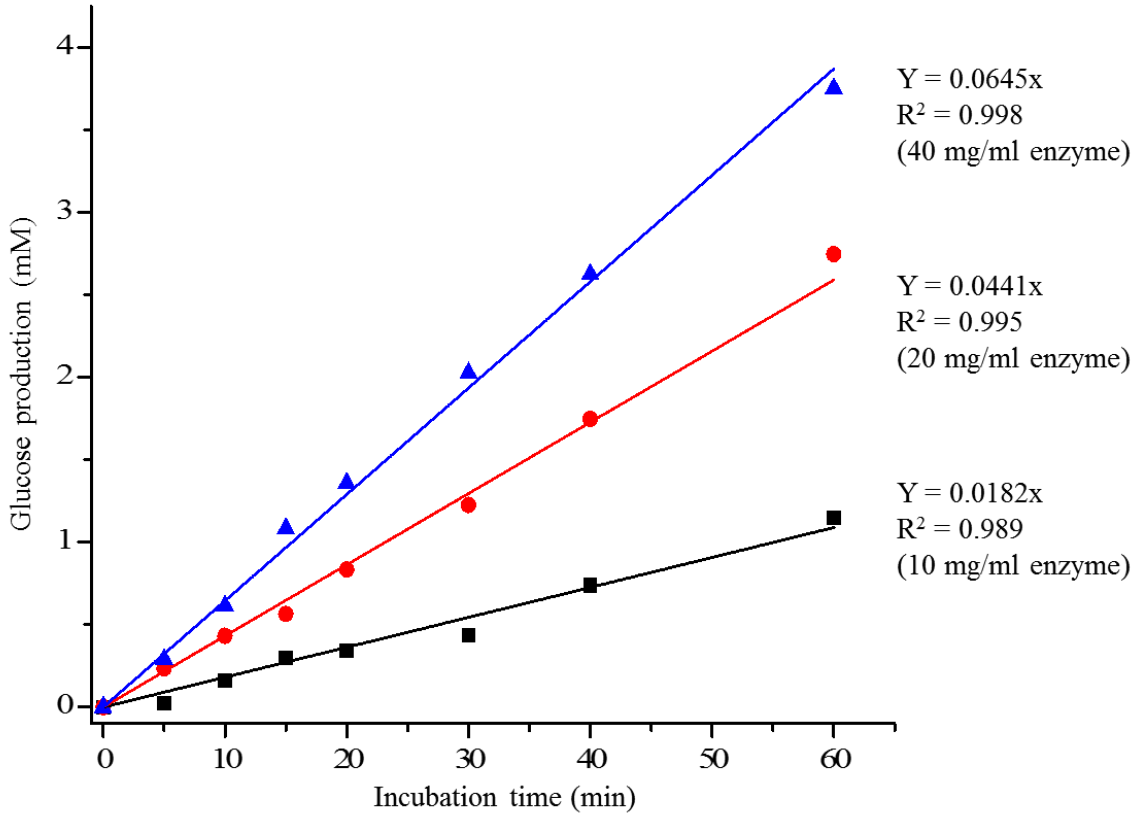


Figure 2-8: Different enzyme concentrations (10, 20 and 30 mg/mL) and different incubation times (0 – 60 min) were used to obtain the amount of glucose produced from sucrose. Glucose produced versus incubation time was plotted to show the pattern of enzyme activity at different concentrations and incubation times. Each data point being mean±SD, n=3.

All the 3 different enzyme concentrations used gave a linear relationship for the 60 min of incubation time tested. It was therefore decided to maintain the 20 mg/mL for 20 min as was in the adapted method (Adisakwattana *et al*., 2009).

2.7.5.5 Iso-maltase reaction time and concentration

The same procedure was used with iso-maltose and the amount of glucose produced versus incubation time was plotted.

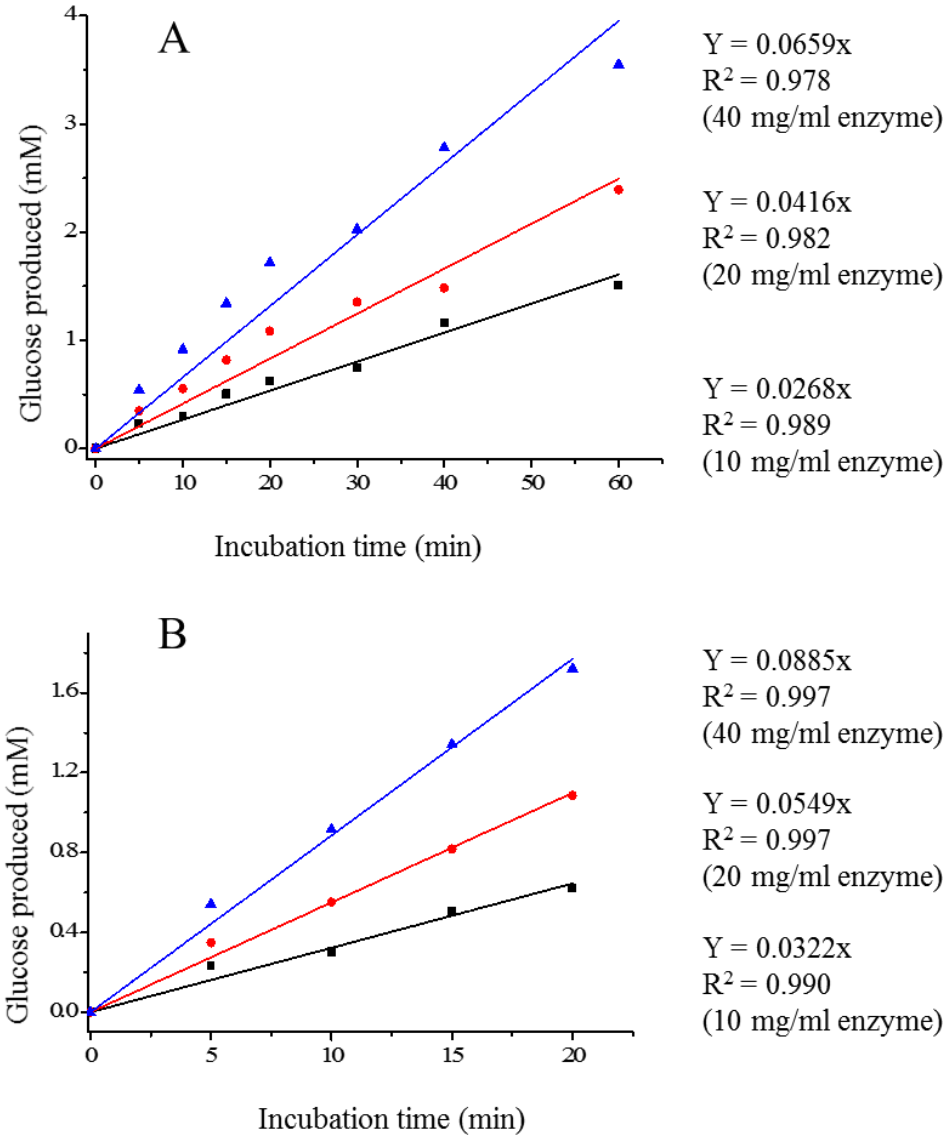


Figure 2-9: Different enzyme concentrations (10, 20 and 30 mg/mL) and different incubation times (0 – 60 min) were used to obtain the amount of glucose produced from iso-maltose. Glucose produced versus incubation time was plotted to show the pattern of enzyme activity at different concentrations and incubation times (A). B shows that linearity was better observed in 20 min. Each data point being mean±SD, n=3.

Similar to sucrase, all 3 different enzyme concentrations showed linearity for the incubation times tested from 5 to 60 min although had not so good coefficient of determination $R^2 \geq 0.98$. Linearity clearly shown with $R^2 \geq 0.99$ when only plotted up to 20 min (figure 2-7B). Hence 20 mg/mL for 20 min will also be used for iso-maltase.

2.7.5.6 Maltase reaction time and concentration

The same procedure was used with maltose and the amount of glucose produced versus incubation time was plotted.

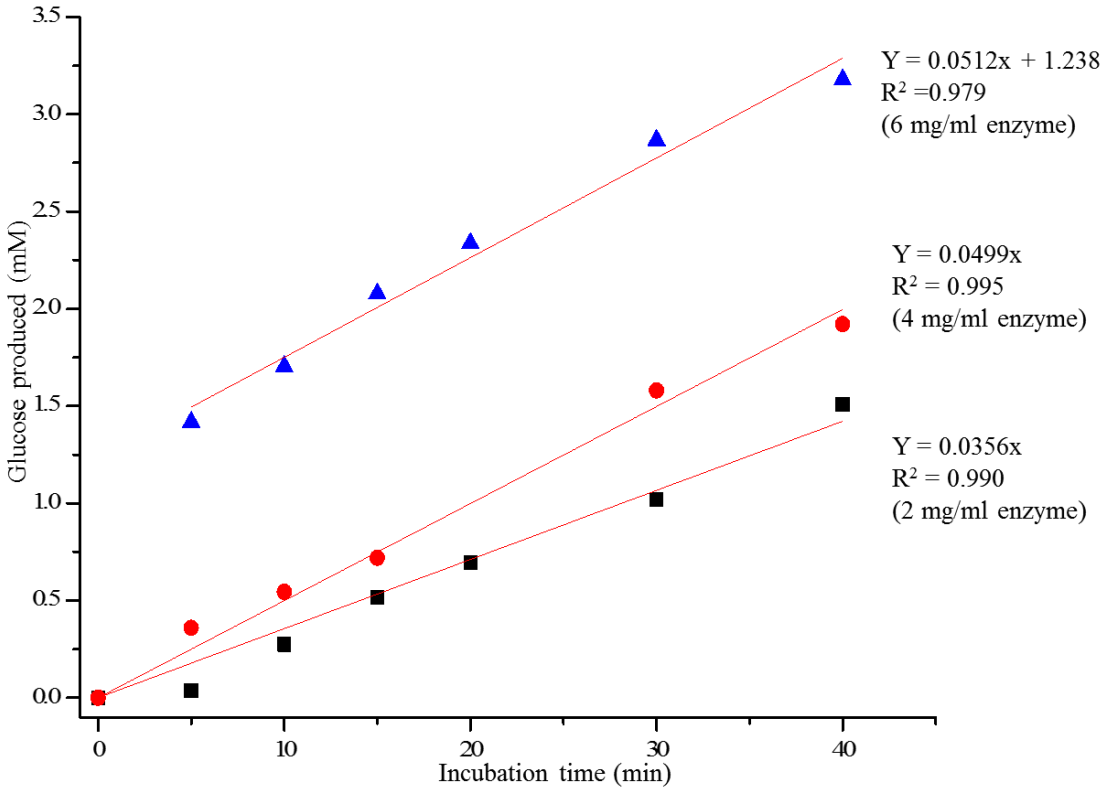


Figure 2-10: Different enzyme concentrations (10, 20 and 30 mg/mL) and different incubation times (0 – 40 min) were used to obtain the amount of glucose produced from maltose. Glucose produced versus incubation time was plotted to show the pattern of enzyme activity at different concentrations and incubation times. Each data point being mean±SD, n=3.

2.7.5.7 Conclusion

The α-glucosidase inhibition assay was optimised and conditions to be used are; substrate concentrations of 16, 6 and 3 mM for sucrose, iso-maltose and maltose. For sucrase and iso-maltase, enzyme concentration of 20 mg/mL RIP in assay and 4 mg/mL for maltase. All assays were to have a reaction time of 20 min as the activity of all enzymes was in the linear range.

2.7.6 Procedure for α-glucosidase inhibition by polyphenols

A total of 200 µL substrate, 50 µL sodium phosphate buffer (PBS) and 50 µL of inhibitor were mixed and pre warmed to 37 °C in a water-bath for 5 min. For the control assay, the inhibitor was replaced by 50 µL PBS. To start the assay, 200 µL of the enzyme preparation from powdered acetone protein extract from rat intestine also pre-warmed to 37 °C was added to the mixture (substrate, PBS and inhibitor) and mixed. The resulting mixture was incubated for 20 min at 37 °C in the water-bath. After 20 min, the tubes were placed in the water-bath at 100 °C to denature the enzymes. After cooling the assay mixtures to room temperature, SPE was carried out if the polyphenol/s reacted with hexokinase reagent and lastly hexokinase assay was performed as described in section 2.2. If the polyphenol used as the inhibitor does not react with hexokinase reagent, the SPE step was omitted. The rate of enzyme inhibition is calculated as a percentage of the control (without inhibitor) using the formula:

$$\% = \frac{\text{Abs Control} - \text{Abs sample}}{\text{Abs for control}} \times 100$$

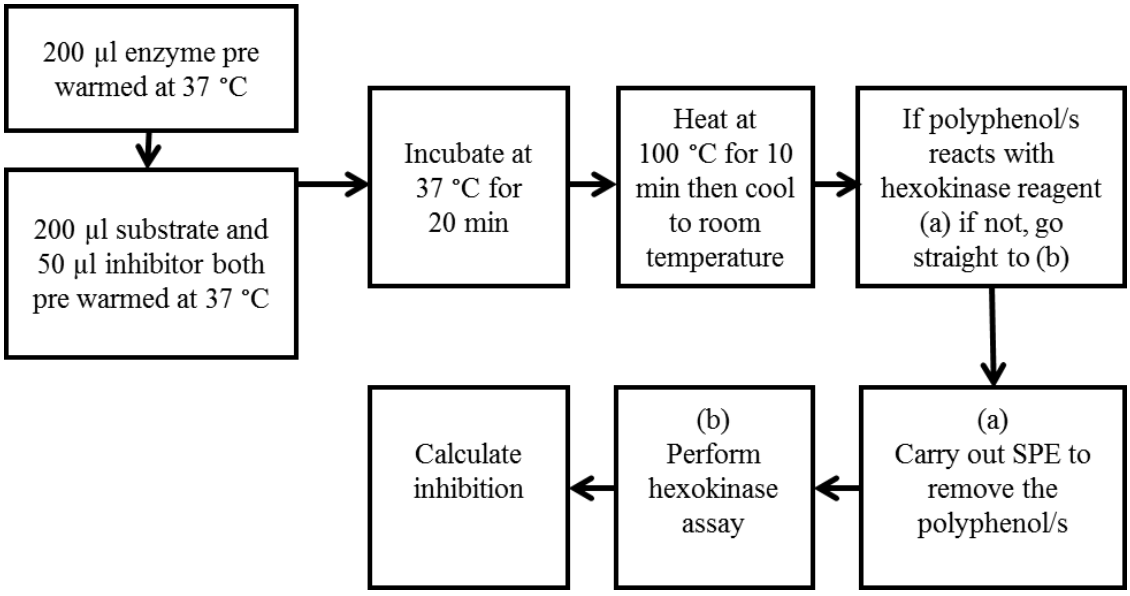


Figure 2-11: The flow diagram of α-glucosidase inhibition by polyphenols. The potential inhibitor was tested to determine if it reacted with hexokinase reagent. If reactive, then a solid phase extraction step (a) to remove the polyphenols was incorporated before performing the hexokinase assay.

2.8 Method set-up and optimization of the α-amylase inhibition assay by polyphenols

2.8.1 Introduction

As in the case of α-glucosidase inhibition assay, inhibition of α-amylase by polyphenols is determined by comparing the enzyme activity in the test (with inhibitor) to the control (without inhibitor). The measurement of the reducing ends in the products of starch hydrolysis (maltose, maltotriose and α-limit dextrins) is used as the end point as inhibiting α-amylase affects the amount of the products produced from the hydrolysis of starch (amylose and amylopectin), amylose or amylopectin. The method involving

dinitrosalicylic (DNS) acid was adapted from Adisakwattana *et al*. (2009) and was optimised and modified by Nyambe-Silavwe *et al*. (2015).

2.8.2 Principle of the method

The assay determines the inhibition of human salivary α-amylase by polyphenols by comparing the amount of product produced from enzyme hydrolysis of starch in the presence and absence of polyphenols by measuring their reducing ends. The enzyme α-amylase hydrolyses starch to produce maltose, maltotriose and α-limit dextrins. The presence of a free carbonyl group (C=O) in reducing sugars, enables them to participate in an oxidation-reduction reaction with DNS. The aldehyde functional group of the reducing sugar gets oxidised into a carboxyl group and the 3, 5-dinitrosalicylic acid gets reduced to 3-amino, 5-nitrosalicylic acid.

Aldehyde group $\xrightarrow{\text{oxidation}}$ carboxyl group

3,5-dinitrosalicylic acid $\xrightarrow{\text{reduction}}$ 3-amino, 5-nitrosalicylic acid

This reaction cause a colour change from yellow to orange or red, depending on the concentration of the reducing sugars produced. By reading absorbance of the coloured product, the amount of maltose equivalents/ reducing sugar ends produced can be calculated using the standard curve equation for maltose.

2.8.3 Chemicals and equipment

Micro-plate reader, 96-well plate for 540 nm and micro-plate sealing film were used for absorbance reading. Human salivary α-amylase type IX-A (1 unit will liberate 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20 °C), maltose, amylose, amylopectin, sodium potassium tartrate, 3,5-dinitrosalicylic acid, disodium hydrogen phosphate (Na_2HPO_4 anhydrous), sodium dihydrogen phosphate (NaH_2PO_4 anhydrous), sodium chloride (NaCl), sodium hydroxide (NaOH) and epigallocatechin gallate (EGCG) were used in

the assay. Oasis MAX cartridge 1 mL (30 mg) and 3 mL (60 mg) and the polyphenols phloridzin and gallic acid were also used in the assay. All the reagents were of the highest purity and standards were ≥98 %

2.8.4 Preparation of reagents

Amylopectin (0.93 mg/mL) The stock solution for amylopectin was prepared by dissolving 9.3 mg amylopectin in 10 mL boiling millipore water and was stirred on a hot plate for not more than 10 min until dissolved and clear.

Amylose (2.5 mg/mL) Amylose stock solution was prepared by adding 100 µL absolute ethanol to 25 mg amylose followed by addition of 1 mL millipore water and 200 µL 0.1M NaOH. The mixture was stirred on a hot plate at 100 °C until all amylose had dissolved (maximum 15 min). It was cooled to room temperature and a reasonable amount of millipore water just below 10 mL mark was added. The pH was adjusted to 6.9 with HCl or NaOH and volume adjusted to 10 mL with millipore water.

Phosphate-buffered saline (PBS) 0.1M, pH 6.9 Disodium hydrogen phosphate (Na_2HPO_4 anhydrous, 10.9 g), sodium dihydrogen phosphate (NaH_2PO_4 anhydrous, 3.2 g) and 90 g sodium chloride (NaCl) were all dissolved in 1 L millipore water and pH was adjusted to 6.9 with either 1 M NaOH or phosphoric acid.

Human salivary amylase (117.5 U/mL) According to supplier's recommendation, 1 mg/mL α-amylase gives 117.5 U/mL and was dissolved in 0.1 M PBS and stored in aliquots at -20 °C. The required concentration for the assay (1.25 U/mL) to give 0.5 U/mL in total assay volume was prepared by appropriate dilutions from the 117.5 U/mL stock.

Sodium potassium tartrate solution, 5.3 M (Reagent A) This was prepared by dissolving 12 g sodium potassium tartrate in previously heated 8 mL of 2 M NaOH at

50-70 °C. It was then heated directly on a heating/stir plate with constant stirring to dissolve taking the precaution not to boil the solution.

3,5- Dinitrosalicylic acid solution (DNS), 96 mM (Reagent B) This was prepared by dissolving 438 mg of DNS in a reasonable amount of millipore water and made up to 20 mL with millipore water. It was heated directly on a heating/stir plate with constant stirring to dissolve at 100 °C and taking the precaution not to boil the solution.

Colour reagent Solution The colour reagent containing DNS for the final step of the assay was prepared by slowly adding reagent A to reagent B with continuous stirring. It was diluted to 40 mL with millipore water. The quantities of reagent A, B and millipore water were adjusted 10 times and the solution was stored in an amber bottle at room temperature with expiry after 6 months.

2.8.5 Determination of assay conditions

2.8.5.1 Amylose and amylopectin km determination for assay substrate concentration

The aim of this experiment was to determine the substrate concentrations (amylose and amylopectin) to use in the inhibition assay. Different amylose and amylopectin concentrations ranging from 0 to 1 mg/mL were used in the determination of km. An assay volume of 500 µL was used containing 200 µL amylopectin/amylose, 100 µL PBS (0.1 M, pH 6.9) and the reaction was started with the addition of 200 µL human salivary α-amylase. The assay mixture was incubated for 10 min at 37 °C and the reaction was stopped by adding 1 mL of DNS to each tube and placing them in the 100 °C water-bath (GLS Aqua 12 plus) for another 10 min. After assay tubes were cooled to room temperature, 250 µL from each tube was put in a 96 well plate and absorbance read at 540 nm in a PHERAstar FS micro-plate reader. By using a maltose standard curve, the

rate of the enzyme reaction was determined and the Lineweaver-Burk and Hanes-Woolf plots were obtained.

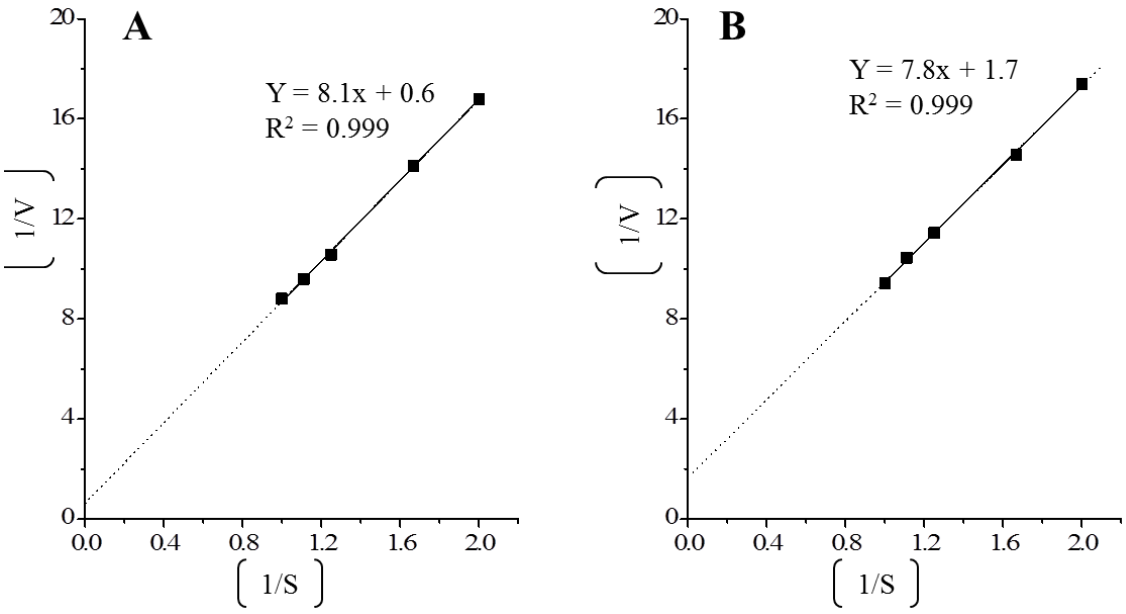


Figure 2-12: The Lineweaver-Burk plot showing the relationship between the substrate concentration [S] and the enzyme rate [V] used to determine the kinetic parameter km with amylose (A) and amylopectin (B) as substrates. Each data point being mean±SD, n=3.

The km values obtained for amylose and amylopectin were 12.9 and 4.8 mg/mL. The supplier of amylose and amylopectin recommends a maximum concentration of 2.5 mg/mL. Hence for amylose this concentration was to be used in all experiments to give an assay concentration of 1 mg/mL. Ratios of amylase and amylopectin km values were used to calculate the amount for amylopectin (0.93 mg/mL to give 0.38 mg/mL in assay).

2.8.5.2 Determination of reaction time and enzyme concentration

A series of experiments were done to obtain enzyme activities using different enzyme concentration and reaction time combinations. The enzyme activity was plotted against incubation time to identify linear range of the enzyme activity for amylose (figure 2-13) and amylopectin (figure 2-14) substrates.

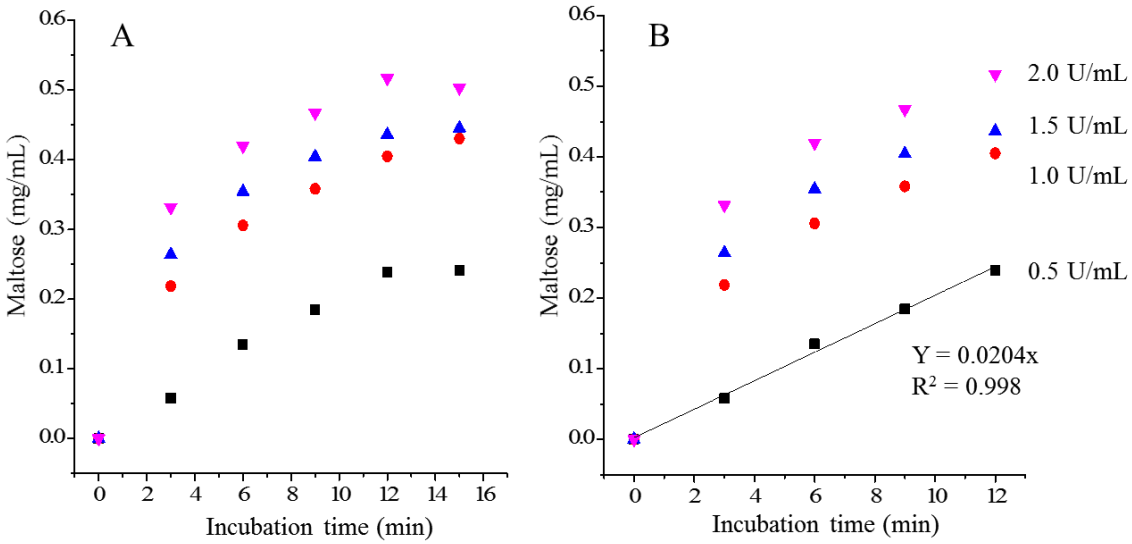


Figure 2-13: Different α-amylase concentrations (1, 1.5 and 2 U/mL) and different incubation times (0 – 16 min) were used to obtain the amount of maltose produced from amylose. Maltose produced versus incubation time was plotted to show the pattern of enzyme activity at different concentrations and incubation times (A) with each data point being mean±SD, n=3. B shows that linearity was observed in 12 min and 0.5 U/mL enzyme concentration. Error bars were smaller than data points.

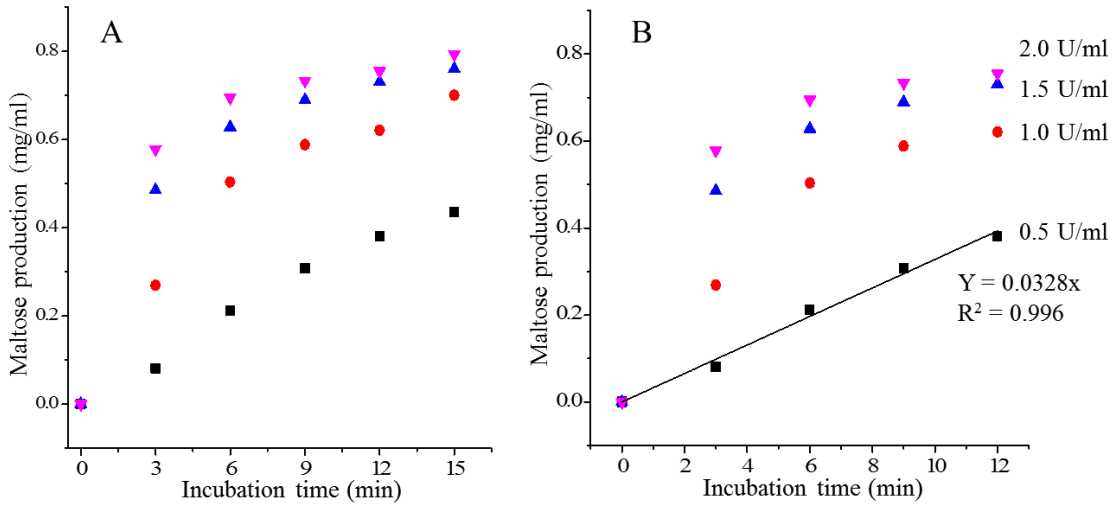
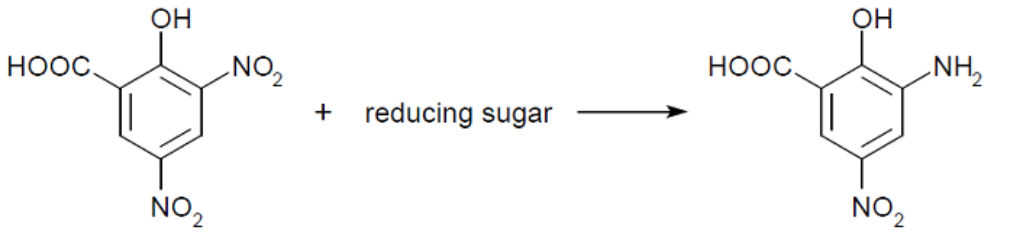


Figure 2-14: Different α-amylase concentrations (1, 1.5 and 2 U/mL) and different incubation times (0 – 16 min) were used to obtain the amount of maltose produced from amylopectin. Maltose produced versus incubation time was plotted to show the pattern of enzyme activity at different enzyme concentrations and incubation times (A) with each data point being mean±SD, n=3. B shows that linearity was observed in 12 min and 0.5 U/mL enzyme concentration.

In conclusion, amylose (1 mg/mL) or amylopectin (0.38 mg/mL) substrate concentrations, 0.5 U/mL enzyme concentrations in the assay of 500 µl total volume and 10 min incubation time gives a linear range of the enzyme activity. These conditions will be used in all experiments as they represent the optimised conditions.

2.8.6 Effect of polyphenols on colour reagent

Salivary α-amylase hydrolyses starch producing the disaccharide maltose and trisaccharide maltotriose and α-limit dextrins. DNS detects the presence of free carbonyl group (C=O) in the reducing sugar which gets oxidized to a carboxyl group and 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitro salicylic acid. The 3-amino-5-nitro salicylic acid produced forms an orange-brown colour and absorbs light at 540 nm.



3,5-dinitrosalicylic acid(DNS)

(Yellow)

3-amino-5-nitro salicylic acid

Orange-brown

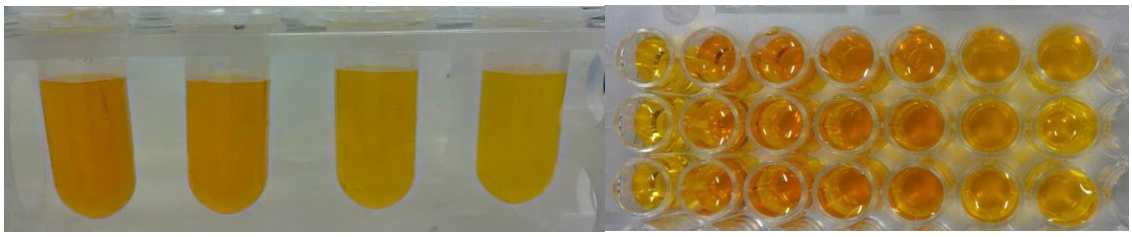


Figure 2-15: The colour change observed when 3, 5-dinitrosalicylic acid is reduced to 3-amino-5-nitro salicylic acid. The intensity of the colour is darker when more reducing sugar is present (positive control or less inhibition) and lighter if less (negative control or high inhibition).

The presence of hydroxyl groups in polyphenols makes them good reducing agents and hence also able to participate in a redox reaction with DNS, producing an orange brown color. This fact was examined by carrying out an experiment where different concentrations of polyphenols (500 µL) were mixed with 1 mL DNS, heated in the water-bath at 100 °C for 10 min and then cooled to room temperature. Absorbance was read at 540 nm and results were plotted against polyphenol concentrations (figure 2-16).

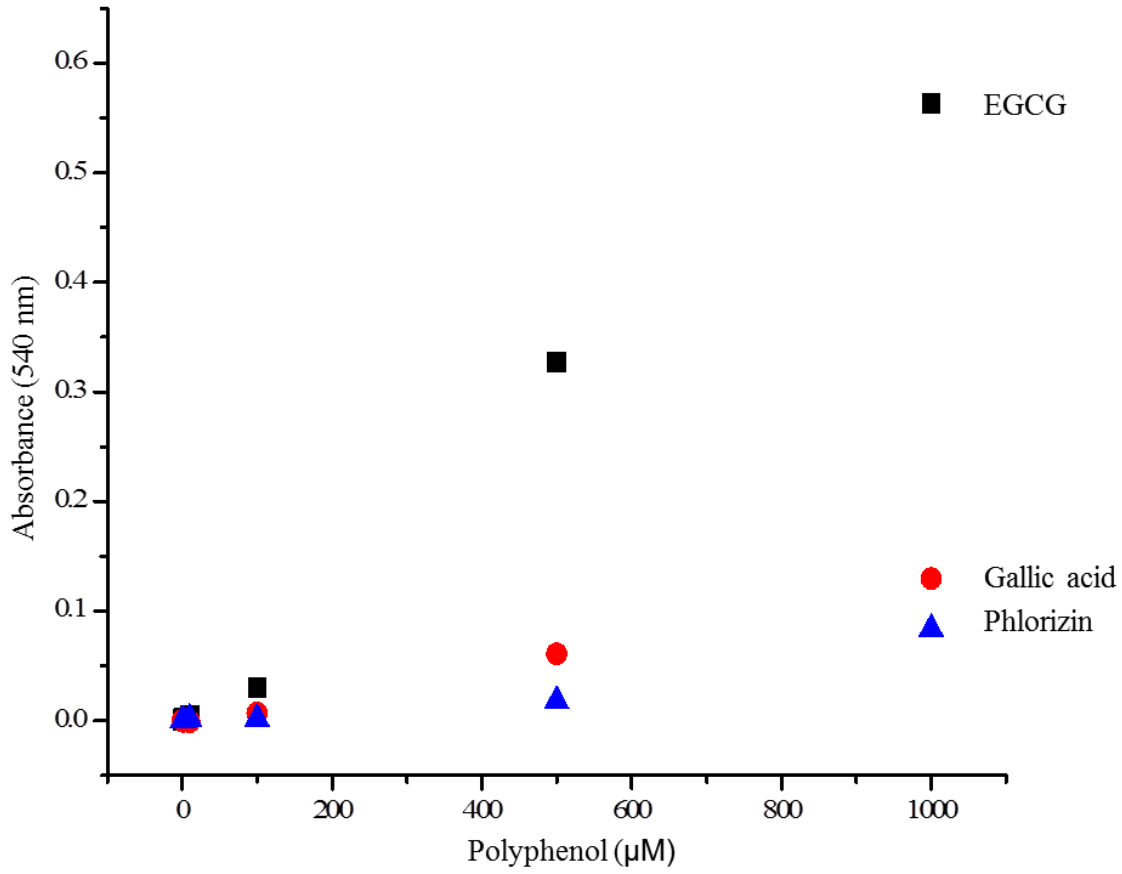


Figure 2-16: The reaction between EGCG, gallic acid and phloridzin with DNS at different polyphenol concentrations. Data points are expressed as mean ± SEM (n=3). EGCG has the highest reactivity and it also has the highest number of OH groups which make them good reducing agents (Nyambe-Silavwe *et al*., 2015).

Phloridzin, gallic acid and EGCG reacted with DNS in the absence of reducing sugars giving an orange brown colour confirming their participation in the redox reaction with DNS. Different polyphenols have different reducing capacity and react differently with DNS mostly due to the number of OH groups they have. For the three polyphenols above, EGCG reacted more, followed by gallic acid and finally phloridzin. Therefore a polyphenol removal step using solid phase extraction (SPE) was incorporated into the enzyme inhibition assay to ensure that a reaction mixture sample is free from polyphenols, which would react with DNS and give an incorrect result. However if a particular polyphenol does not react with DNS, the polyphenol removal step is not necessary.

2.8.7 Efficiency of SPE cartridges

The Oasis Max 1 cc cartridges containing 30 mg of sorbent were conditioned with 1 mL of methanol and cartridges were allowed to dry under vacuum (10 Hg) for 10 min. Millipore water (1 mL) was used to equilibrate the cartridges before adding 500 mL of assay mixture after the inhibition reaction with 100 µM EGCG used as inhibitor. After passing the sample through the cartridge, the filtrate collected was analysed to test the efficiency of removal of polyphenols using Oasis MAX SPE cartridge. The concentration of 100 µM EGCG was used in the analysis because it was going to be the highest concentration to be used in the inhibition experiments.

HPLC analysis of EGCG was carried out using a $UFLC_{XR}$ Shimadzu system consisting of binary pump, a photodiode array with multiple wavelength SPD-20A and a LC-20AD Solvent Delivery Module coupled with an online unit degasser DGU-20A3/A5 and a thermostat auto-sampler/injector unit SIL-20A (C). The detection was at 280 nm. A two phase gradient system consisting of millipore water with 0.1 % triflouroacetic acid

(HPLC grade) as mobile phase A and triflouroacetic acid, acetonitrile, and millipore water (50:49.9:0.1) was employed as mobile phase B. The gradient conditions were as follows: The initial conditions started with 92 % A and increasing to 18 % solvent B at 3.50 min, 32 % B at 18 min, 60 % B at 28 min reaching to 100 % B at 32 min for 4 min, returning to the initial conditions for 3.5 min. The column used for the analysis was a 5 µL Gemini C_{18} (250 x 4.6 mm, i.d.) with a flow rate of 1 mL/min, column temperature set at 35 °C with an injection volume of 10 µL.

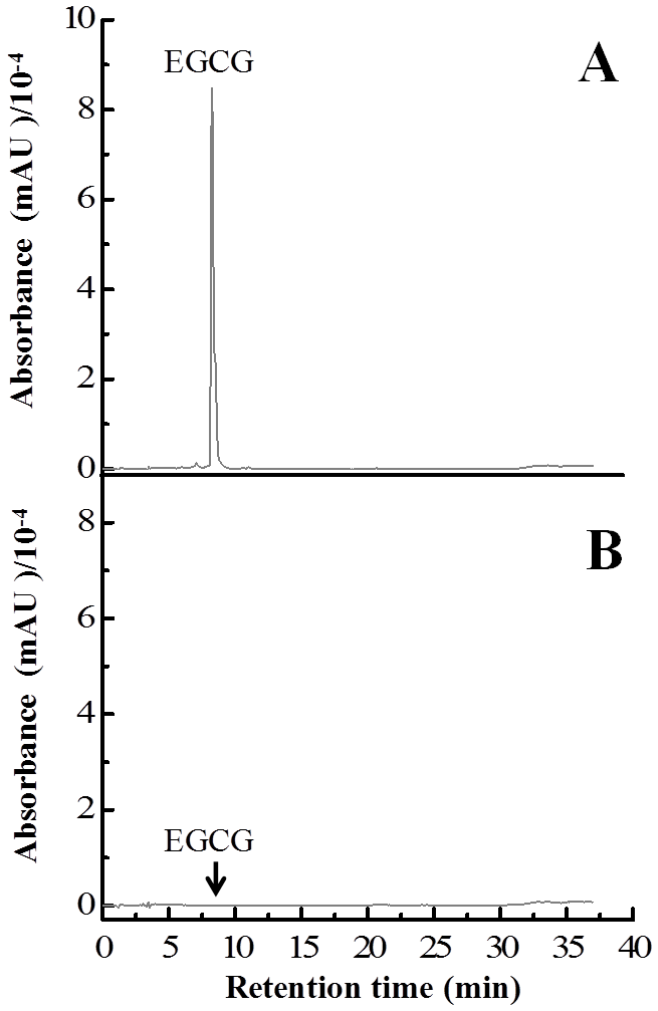


Figure 2-17: HPLC chromatogram of 100 µM EGCG (A) before SPE and (B) after SPE. The Oasis MAX SPE cartridge gave a removal efficiency of greater than 99 % for 100 µM EGCG.

The removal efficiency of the Oasis MAX SPE cartridge of greater than 99 % will ensure that polyphenols are removed before the addition of DNS for the final stage of the inhibition assay. 100 µM EGCG being the highest concentration to be used in the assay, indicates that lower concentrations would equally be removed efficiently.

2.8.8 Effect of enzyme and substrate concentration on inhibition (IC_{50}) value

As shown in section 2.8.5.2, it is important to use an optimised enzyme concentration which gives a linear relationship with time. Using an enzyme concentration that is not optimised can give different results as was observed in the literature where the same compounds were reported to have different IC_{50} values (Nyambe-Silavwe *et al*., 2015). Comparison between results obtained using the optimised 0.5 U/mL and 3 U/mL enzyme concentrations gave different results. There are different definitions of IC_{50} value. One defines it as the concentration of an inhibitor that reduces the activity of an enzyme by 50 %, with 100% being the activity of the enzyme without an inhibitor and 0 % being the control not containing either substrate or enzyme and hence no activity. The second definition is the concentration of the inhibitor required to bring the enzyme activity half way between the top and bottom plateaus of the curve (Neubig *et al*., 2003). The first definition is adopted for this research, and is the most commonly used because it gives a value that can be compared to other inhibitors. The second definition would for example show that a poor inhibitor has a low IC_{50} value compared to a good inhibitor only because its maximum inhibition was low and may give a lower value compared to a good inhibitor whose half inhibition concentration may be higher because it had a higher maximum inhibition.

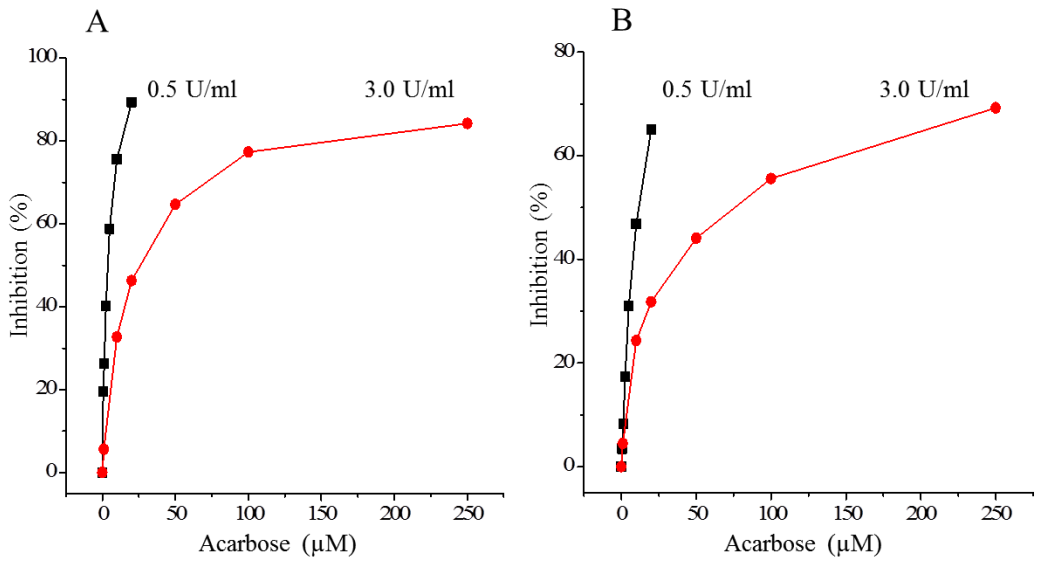


Figure 2-18: Acarbose inhibition of α-amylase using 0.5 U/mL and 3 U/mL enzyme concentrations and amylose (A) and amylopectin (B) as substrate. Data points are expressed as mean ± SD (n=3). The inhibition curves are different resulting in different IC_{50} values.

The use of different enzyme concentrations gave very different IC_{50} values for acarbose (table 2-4).

Substrate	**Enzyme concentration**	
	0.5 (U/mL)	3.0 (U/mL)
	IC_{50} (µM)	
Amylose	3.5	25
Amylopectin	7.5	70

Table 2-4: Acarbose IC_{50} values when different enzyme concentrations were used for amylase inhibition. There is a 7 and 9 fold difference in IC_{50} for amylose and amylopectin respectively.

The use of different enzyme concentrations (optimised 0.5 U/mL and sub-optimal 3 U/mL), gave a 7 and 9 fold difference in IC_{50} values for amylose and amylopectin. This partly explains the differences observed in the literature for reported IC_{50} values even for the same compounds (Nyambe-Silavwe *et al*., 2015)

2.8.9 Optimised assay procedure for amylase inhibition by polyphenols

The assay contained 200 µL each of substrate (amylose 2.5 mg/mL or amylopectin 0.93 mg/ml) and enzyme (0.5 U/mL), 50 µL phosphate saline buffer (PBS) and 50 µL of inhibitor of different concentrations. For the control assay, the inhibitor was replaced by an equal volume of PBS. The assay mixture containing the inhibitor, PBS and substrate were pre-incubated at 37 °C was added in a water-bath for 5 min and the enzyme also pre-incubated at 37 °C to start the assay. The reaction tubes were mixed and incubated at 37 °C in the water-bath for 10 min. The reaction was stopped by placing the samples in a water-bath (GLS Aqua 12 plus) at 100 °C for 10 min, then cooled down to room temperature and centrifuged for 5 min. The sample obtained was used for SPE which was carried out using Oasis MAX cartridges to remove polyphenols before adding colour reagent solution. To the resulting sample (500 µL), 1 mL of the colour reagent was added and heated at 100 °C for 10 min. After cooling to room temperature, 250 µL from each tube was place in a 96 well plate and the absorbance was read at 540 nm. The rate of enzyme inhibition was calculated as a percentage of the control (without inhibitor) using the formula:

$$\% = \frac{\text{Abs Control} - \text{Abs sample}}{\text{Abs for control}} \times 100$$

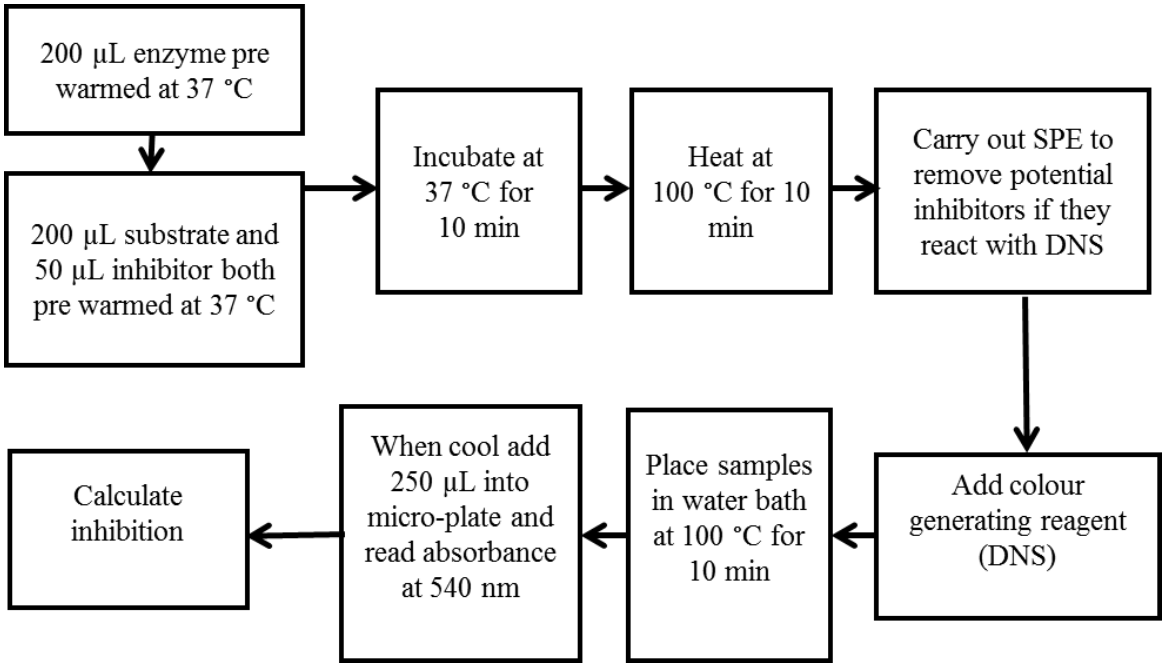


Figure 2-19: The flow diagram of α-amylase inhibition by polyphenols. The potential inhibitor has to be tested if it reacts with hexokinase reagent or not of which the former needs to have a solid phase extraction step to remove the polyphenols before performing the hexokinase assay (Nyambe-Silavwe *et al*., 2015).

2.8.10 Conclusion

For any enzymatic assay, it is important to determine the assay conditions and ensure that the optimum conditions are used. The km value needs to be determined so that the concentration of the substrate can be approximately equal to the km value. If results are to be compared between different substrates, ratios of their km values need to be taken into account for the substrate concentration used. Enzyme concentration and incubation time need to be in the initial rate of the enzyme activity of which the relationship is linear. There is a need to ensure that the detection method does not interfere with the inhibitor, and if it is the case, SPE must be employed to remove the polyphenol. The effectiveness of an inhibitor is usually determined by IC_{50} or Ki values with the IC_{50} being more

commonly used and usually compared with that of acarbose (digestive enzyme inhibitor). The optimisation of the amylase assay has shown that IC_{50} is very dependent on assay conditions which make it very difficult to compare results in the literature. The different assay conditions of importance are substrate type and concentration, enzyme concentration and origin, reaction duration, incubation temperature and pH. It is thus recommended to use Ki to report the effectiveness of pure compounds as it gives comparable results independent of inhibition mode, substrate and detection method.

2.9 Human Study design

Before commencing any human intervention study, it is important to choose the design of the study and ensure that it is correct for subsequent interpretation of results. Below are the different aspects that need to be considered for glycaemic index (GI) studies.

2.9.1 Subject number, gender and status

It is generally accepted that a minimum of 10 subjects provides a reasonable degree of power and precision (Brouns *et al*., 2005). However if greater precision and power is required, sample size can be increased upwards although this is more costly. When recruiting, a 20 % allowance for any drop-outs is recommended for GI studies. Male or female can be used as each person is a control of themselves and no differences in glycaemic response have been observed between males and females (Wolever and Mehling, 2002). Variations of the mean GI values are reported to occur in various groups (healthy, pre-diabetic, diabetic type 1 and diabetic type 2). The highest variations have been observed in individuals suffering from type 1 diabetes. It is therefore recommended that different groups of people as regards to their status should never be mixed in one study (Brouns *et al*., 2005). The subject status also depends on the hypothesis of the study

whether testing it in healthy, pre-diabetes or diabetes subjects. In cases where the hypothesis is tested in more than one group, data analysis is never to be mixed but treated separately.

2.9.2 Test meal number

In calculating glycaemic response, it is important to repeat the control meal at least once because the response of the test meal is always compared to that of the reference meal hence the importance of the reference area under the curve (AUC) being correct. Increasing the number of reference food measurement from one to two significantly reduced the margin of error whereas taking three or more measurements does not reduce it further (Wolever and Mehling, 2003).

2.9.3 Reference food

More than 90 % of studies on determination of GI use glucose or white bread as the reference food. Glucose was the original reference food and its GI is set to 100 (Jenkins *et al*., 1981). However, the use of glucose as a reference food has been reported to induce nausea in individuals when they drink it in the morning after a night fast (Brouns *et al*., 2005). White bread is preferred as it is a common food and can be used even in diabetic patients. However differences in the composition of white bread may entail that comparing results from different studies that used a different loaf of bread may be difficult. White bread can thus be used as long as the preparation of the bread is standardised. For determining polyphenol effects on GI, the reference meal depends on the main digestion step being inhibited. If the main inhibitor is targeting α-amylase, in order to test for inhibition, bread should be used as a reference because α-amylase hydrolyses amylose and amylopectin in starch (bread) into maltose and other disaccharides. Using glucose or sucrose as a reference meal in this situation is not

appropriate as the inhibition of α-amylase will have no effect on the amount of glucose going into the blood. If the main inhibitors being studied are α-glucosidases, sucrose or bread would be ideal reference food because the inhibition of the enzymes can be determined by the amount of glucose hydrolysed from either maltose (product of starch digestion) or sucrose. Finally if the stage of digestion to be inhibited is glucose transport, either glucose, sucrose or starch can be used as reference as the main target is the amount of glucose being transported.

2.9.4 Meal volume, composition and consumption time

A standardized amount of a drink such as 200 or 250 mL (one glass) should be used because increasing the amount of drink increases the area under the glucose response curve (Sievenpiper *et al*., 1998). Water is usually used for this purpose and any other water present in the food is not included in the standardised amount. In the case of reference food, for glucose, 50 g of glucose should be diluted in 200 or 250 mL water, whereas in the case of white bread, a portion containing 50 g of available carbohydrates should be given with 200 or 250 mL drink (usually water). The time for fluid ingestion should be between 5-10 min and 10-20 min for solids/semi solids. As regards the blood sample, it should be taken exactly 15 min after the first sip of the drink or bite of the food.

2.9.5 Amount of carbohydrate and dose

Available carbohydrate should be determined using validated methods to calculate how much bread gives 50 g. Another method called relative glycaemic response (RGE) can be used which compares the response from total carbohydrates in 50 g of test food in relation to 50 g glucose. Therefore, GI is based on available carbohydrate (CHO) whereas RGE is based on total CHO (Brouns *et al*., 2005). The amount of carbohydrate to be used

is dependent on the amount of carbohydrates present in the food. The recommended value is 50 g available CHO. In cases where there is a low amount of CHO in the test food, 25 g available CHO should be used and consequently compared to 25 g of glucose. This is done to avoid using a large meal to make up the 50 g available CHO which would be unrealistic.

2.9.6 Analysis of carbohydrates

The amount of available carbohydrates can easily be determined using the "Available Carbohydrates and Dietary Fibre Assay kit" (by Megazyme) or by the *in vitro* digestion method of Englyst (Englyst *et al*., 1992) . The assays utilises enzymes which convert the product into a measurable substance.

2.9.7 Time and duration of study and subject preparation

The test must be done in the morning before 10 am after an overnight fast of between 10-14 hours. A higher difference in glycaemic response between cereals was observed when tested at lunch time compared to the test done at breakfast (Wolever and Bolognesi, 1996). The recommended duration of the study (all visits) is no more than 4 months. When testing more than one food, a reference test should be done at the beginning of the test and again after every 6-8 weeks. For purposes of measuring glycaemic responses, it is not necessary to control exercise on the day before the test although unusual vigorous activity should be avoided. However, the subject should eat a meal of their choice the evening before the test but that they should eat the same meal the day before each test (Wolever *et al*., 1991).

2.9.8 Blood sampling

There are different methods and ways in which blood can be sampled with different sites giving different concentrations of blood glucose. Fingertip capillary sampling is recommended as the blood glucose concentrations are higher in the fingertip capillary than in the veins when blood glucose concentrations rise. In the same way when blood glucose concentrations fall, the concentration in the fingertip capillary is higher than in the vein (Ellison *et al*., 2002, van der Valk *et al*., 2002, Yang et al., 2012). However, according to the World Health Organisation (WHO) and Food and Agriculture Organisation (FAO), despite the criteria for diagnosis of diabetes referring to venous plasma glucose, finger prick and venous blood sampling can both be used for GI determination (FAO/WHO, 1998). On the other hand, insulin measurements have to be done from venous blood due to the higher volume required. With regards to sampling times, in healthy subjects blood should be sampled at the fasting stage (0) and then 15, 30, 45, 60, 90 and 120 min after starting to eat the test meal. Diabetic subjects should be sampled at (0) fasting state, then 30, 60, 90, 120, 150 and 180 min after starting to eat the test food (Brouns *et al*., 2005).

Blood can be processed into serum or plasma depending on the treatment after its collection. The difference between the two is their clotting factors. Plasma is the liquid part of blood that contains fibrinogen which is essential in blood clotting whereas serum is the liquid part obtained after coagulation and does not contain fibrinogen but contains proteins like globulins and albumin. Plasma is prepared by centrifugation to isolate blood cells which settle at the bottom of the tube leaving plasma above it, containing water and clotting factors and tubes with anticoagulants are usually used. Serum is prepared by allowing blood to clot after collection and the liquid squeezed out of the clot is serum. A study which compared serum and plasma glucose values from the same volunteers,

reported that serum gave lower glucose values than plasma values and it is therefore recommended to use plasma (Frank *et al*., 2012).

2.9.9 Calculating postprandial glucose/insulin response

Glucose concentrations at different time points are plotted against time to obtain the glucose curve. The method commonly used to determine glucose response is that of incremental area under the curve (IAUC) which calculates only the area over the baseline (fasting blood glucose) and does not take into account the area beneath the curve. The general formula for calculating AUC is given by:

Times are given by t=0, t=1…. t=n (equalling 0, 15….120 min)

Blood glucose concentrations are G=0, G=1….G=n respectively. The detailed calculations are obtained from (Brouns *et al*., 2005) as follows:

AUC = $\sum_{n}^{x=1}$ Ax

Where Ax = the IAUC for the xth time interval

For the first time interval (x=1),

If G1 > G0, A1 = (G1 – G0) x (t1 – t0)/2

Otherwise, A1 = 0

For the other time intervals (i.e. x > 1)

If Gx ≥ G0 and Gx-1 ≥ G0, Ax = [(Gx – G0)/2] + [(Gx-1 – G0)/2] x (tx – tx-1)

If Gx > G0 and Gx-1< G0, Ax = $[(Gx – G0)^2/ (Gx – Gx-1)]$ x (tx – tx-1)/2

If Gx < G0 and Gx-1 > G0, Ax = $[(Gx-1 – G0)^2/(Gx-1 – Gx)]$ x (tx –tx-1)/2

If Gx ≤ G0 and Gx-1 ≤ G0, Ax = 0

Chapter 3 . Characterization of polyphenol and fibre-rich food (PFRF) mixture and bread for intervention study test meals.

Abstract

In preparation for the human intervention study meals, the PFRF mixture was analysed for total polyphenol content, specific major polyphenols and sugar content. Bread analysis was also carried out to determine how much bread gave 50 g available carbohydrate. The total polyphenol contents were 541±25, 315±2, 303±1, 295±3 and 217±3 µg/mL GAE ± SD fresh weight for green tea, strawberry, blackcurrant, blackberry and apple peel respectively. Specific major polyphenols were 104±0.3, 70.1±0.1, 45±0.1, 19.9±0.7, 18.2±0.03 and 11.3±0.02 mg/100g of cyanidin-3-O-rutinoside, cyanidin-3-O-glucoside, EGCG, pelargonidin-3-O-glucoside and phloridzin and quercetin-3-O-rhamnoside in green tea, blackcurrant, blackberry, strawberry and apple respectively. All the constituents of the PFRF mixture had polyphenols in amounts within the Phenol Explorer range and specific polyphenols had concentrations higher than the reported IC_{50} values for carbohydrase inhibition. The PFRF mixture constituents were used in *in vitro* assays to determine enzyme inhibition potential. In a combination of 10 g each of the dried fruits (40 g total), there was a total of 4.3, 2.7 and 0.44 g of fructose, glucose and sucrose which were used to standardize the sugar concentrations in the intervention study meals. The amount of bread to give 50 g available carbohydrates was determined to be 109±1.2 g also to be used in the intervention study meals.

3.1 Introduction

From the literature, good inhibitors of α-amylase were identified as cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside (Akkarachiyasit *et al*., 2011) and epigallocatechin gallate (EGCG) (Koh *et al*., 2010). The α-glucosidase inhibitors are mostly the catechins (Honda and Hara, 1993, Ishikawa *et al*., 2007, Matsui *et al*., 2007, Kamiyama *et al*., 2010) and glucose transport inhibitors were quercetin-3-O-rhamnoside (quercetrin), phloridzin and pelargonidin-3-glucoside (Manzano and Williamson, 2010). The best sources of the named polyphenols are blackberry, blackcurrant, green tea, apple and strawberry (Neveu *et al*., 2010) hence constituting the polyphenol and fibre-rich food (PFRF) mixture. Green tea is the best source of catechins which are the best α-glucosidase inhibitors especially EGCG (Kamiyama *et al*., 2010) which also inhibits α-amylase and glucose transporters (Koh *et al*., 2010). It was added to the polyphenol-rich mixture to be the main α-glucosidase inhibitor but should however also contribute to the inhibition of α-amylase and glucose transport. The major polyphenols found in soft fruits (blackberry, blackcurrant and strawberry) are the anthocyanidins. The most common anthocyanidin aglycones are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin. The named aglycones form conjugates with sugars and organic acids forming anthocyanins. Anthocyanins are water-soluble and they impart blue, purple or red colours to plant tissues making berries the richest sources of anthocyanins. Consumption of anthocyanin-enriched foods is associated with a reduced risk of chronic diseases such as diabetes (Akkarachiyasit *et al*., 2010). The major polyphenol found in apple is phloridzin and together with quercetin-3-O-rhanmoside (quercetrin) have inhibitory properties against glucose transporters GLUT2 and SGLT1 (Ader *et al*., 2001, Cermak *et al*., 2004, Manzano and Williamson, 2010).

In order to be used in the human intervention study, the PFRF mixture constituents were analysed for total polyphenol contents and specific major polyphenols in order to determine the presence of polyphenols and especially the major polyphenols identified to have inhibitory properties. This was done to ensure that the PFRF mixture contained polyphenols in amounts above the reported IC_{50} values in order to have an effect *in vivo*. Sugar contents of the PFRF mixture were determined in order to standardize the sugar concentrations in the intervention study meals. Bread analysis was also carried out to determine how much bread gives 50 g available carbohydrate.

3.2 Materials and methods

3.2.1 Determination of total polyphenols in green tea

The Folin assay described in chapter 2 (2.4) was used for the determination of total polyphenols in green tea. It was prepared fresh for each experiment by dissolving the powder in millipore water, centrifuged at 4000 rpm for 10 min and supernatant collected in a new tube. Appropriate dilutions were made from the 1 mg/mL stock solution. Gallic acid standards were prepared as described in chapter 2 (2.4.3).

3.2.2 Determination of specific polyphenols in green tea

The HPLC method described in chapter 2 (2.5) was used to identify and quantify the main polyphenols in green tea (EGCG, EGC, ECG and EC). The standards EGCG, EGC, ECG and EC were analysed separately to obtain retention times, spectra and standard curves used for the identification and quantification of polyphenols in green tea.

The standards were dissolved in 50 % ethanol to a concentration of 1 mg/mL and placed in 100 μL aliquots. They were evaporated to dryness in a Genevac (EZ-2 plus model)

and stored in the -20 °C freezer until needed. Adding 1 mL 50 % ethanol gave 100 µg/mL and then dilutions were made to give different concentrations for the standard curves. Green tea (1 mg/mL) was prepared fresh for each experiment by dissolving the powder in millipore water. After centrifugation at 4000 rpm for 10 min, supernatant was collected in a new tube and appropriate dilutions were made from the 1 mg/mL stock solution.

3.2.3 Determination of total polyphenols in blackberry, blackcurrant, strawberry and apple

A stock solution of 10 mg/mL was prepared by water extracting polyphenols from 100 mg freeze-dried fruit in a total volume of 10 ml millipore water. This was done in 3 stages: the freeze-dried fruit was first vortexed vigorously for 3 min in 5 mL, centrifuged at 4000 rpm for 10 min and supernatant placed in a clean tube. This was repeated twice with 2.5 mL millipore water each time, giving a total volume of 10 mL. Appropriate dilutions were made from the 10 mg/mL stock solution. Gallic acid standards were prepared as described in chapter 2 (2.4.3). The Folin assay described in chapter 2 (2.4) was used for the determination of total polyphenols in all the fruits which were obtained as freeze-dried powders which were processed from the respective fruits. Freeze-dried fruits are produced by drying the previously frozen fruits under vacuum at a low temperature under reduced pressure. This is an excellent way of preserving heat sensitive materials/compounds in the fruits whilst improving their storage life without addition of artificial ingredients, flavouring or colours hence maintaining the natural components of the fruits except water.

3.2.4 Determination of specific polyphenols in blackberry, blackcurrant, strawberry and apple

The HPLC method described in chapter 2 (2.5) was used to identify and quantify the main polyphenols in the fruits. Cyanidin-3-O-rutinoside, cyanidin-3-O-glucoside, phloridzin and quercetin-3-O-rhamnoside standards were analysed separately to obtain retention times, spectra and standard curves used for the identification and quantification of polyphenols in the fruits. The standards were prepared in the same way as the green tea polyphenol standards (3.2.2).

3.2.5 Determination of sugar content in fruits by HPLC

Analysis was carried out to determine type and contents of sugars naturally present in the fruits in order to use the information to standardize the amount of sugars in the reference and test meals. The identification and quantification of sugars in the fruits was conducted using the method of Tembo, (2017) on a Shimadzu HPLC instrument equipped with a model DGU-20 A5 degasser, a LC-20 AD XR pump system, a SIL-20 AC XR auto sampler (Shimadzu), column oven and a Shimadzu ELSD-LTII low temperature evaporative light scattering detector. A sample volume of 10 µL was injected, and separations were achieved on a Prevail Carbohydrate ES 5 µm column (250 mm x 4.6 mm). The column was held at 20 ^{0}C, and individual sugars were eluted isocratically using a 1 mL/min flow of 75 % acetonitrile. Solutions of standard sugars prepared in millipore water with concentrations between 0 and 10 mg/mL were used for the calibration curve. The sugars were identified by their retention time characteristics. Quantification was achieved using standard calibration curves obtained by plotting area versus concentration ($r^2 > 0.98$).

3.2.6 Determination of available carbohydrate content in bread

Bread analysis was carried out as described in chapter 2.6. The aim was to determine the amount of bread required to give 50 g of available carbohydrate after 2 hr digestion.

3.3 Results

3.3.1 Total polyphenols

Total polyphenol contents of the PFRF mixture are shown in table 3-1 with the highest content being in green tea > strawberry > blackcurrant > blackberry > apple. They all had polyphenol contents that fall within the range reported in Phenol Explorer (Neveu *et al*., 2010) except for blackcurrant.

Extract	**Green tea**	**Strawberry**	**Blackcurrant**	**Blackberry**	**Apple peel**
Total polyphenols (µg/mL GAE) ± SD	54.1± 2.5	31.5 ± 2.0	30.3 ± 10	29.5 ± 3.0	21.7± 3.0
Phenol Explorer range (Neveu *et al*., 2010) (µg/mL GAE) Fresh weight basis	287-1029	72.5 - 443.4	498 - 1410	192.8 - 1056	66.2 – 430 (apple dessert whole raw)

Table 3-1: Total polyphenol contents of the PFRF mixture and the corresponding range of amounts as reported in Phenol Explorer. The amounts are expressed in mean±SD (n=3). Fresh weight basis is 10 times more of the dry weight according to the freeze dried fruits supplier (Healthy supplies UK). Hence to compare with Phenol explorer values, the Phenol Explorer values are divided by 10.

3.3.2 Specific polyphenols

3.3.2.1 Green tea

Green tea was characterized to determine the presence and amounts of the different catechins. Standards were used for the identification and quantification of the catechins. Standard curves for the catechins were identified separately and standard curves were used to quantify them in the green tea.

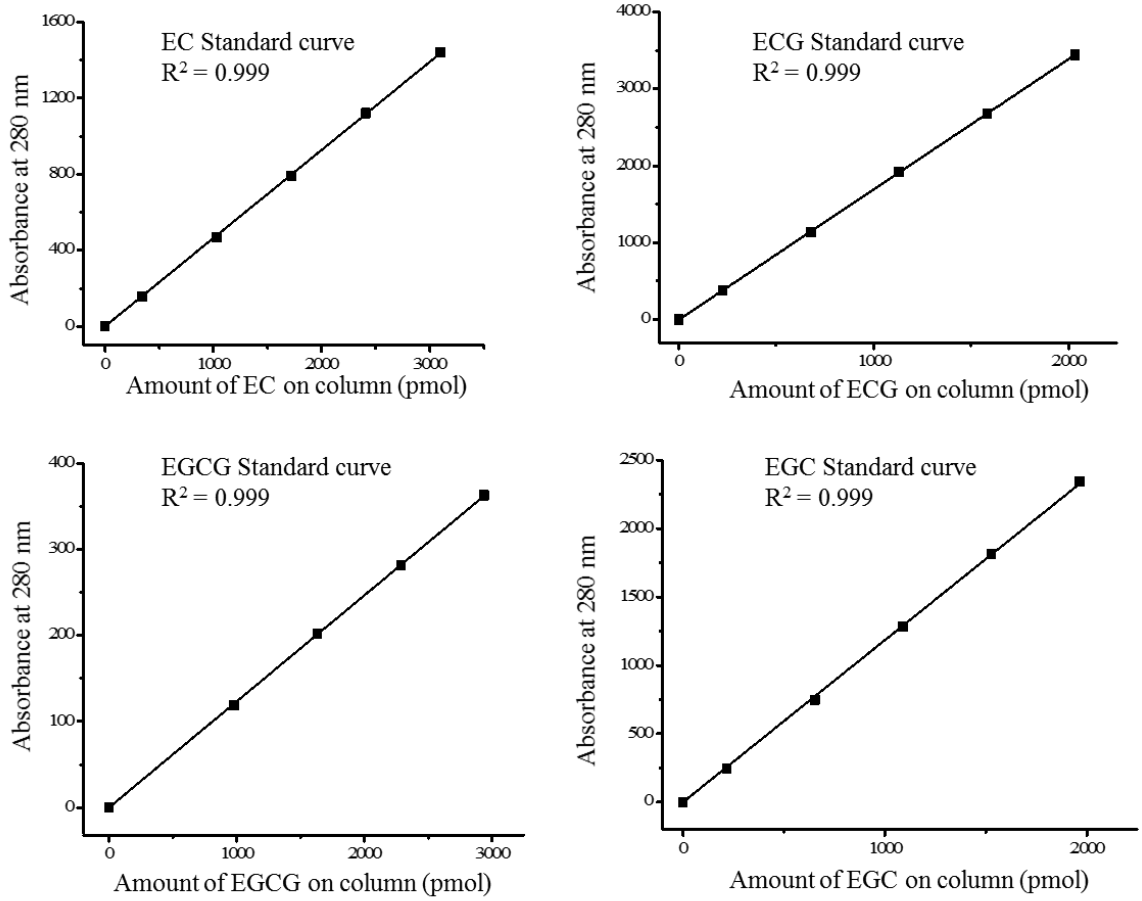


Figure 3-1: Standard curves for EC, ECG, EGCG and EGC used for the quantification of the catechins in green tea. All data points are mean±SD (n=3).

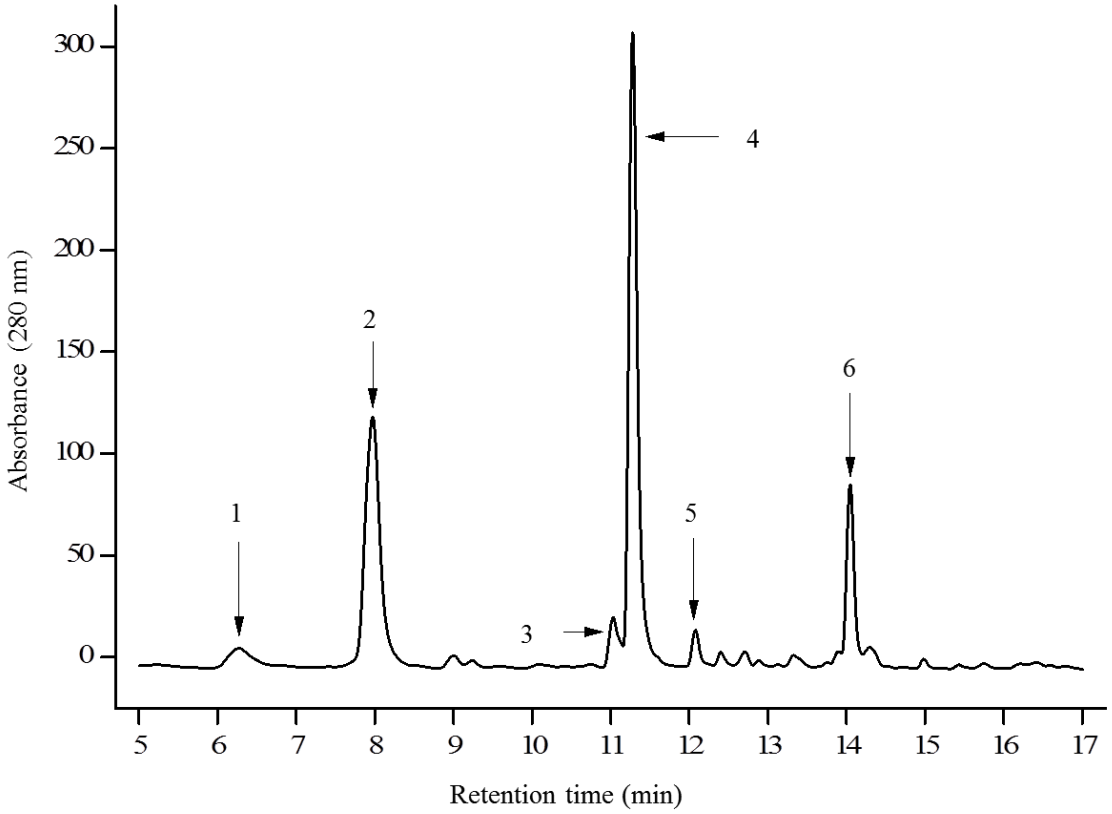


Figure 3-2: HPLC chromatogram of green tea: (1) EGC; (2) Caffeine; (3) EC; (4) EGCG; (5) Taxifolin (IS); (6) ECG.

The quantities of the catechins were in the order EGCG>EGC>ECG>EC and were 199.8± 6.7, 124.4 ± 9.3, 34.4 ± 1.9 and 23.3 ± 2.4 µg/mL giving 435.9, 406.1, 77.6 and 80.3 µM) respectively (in 1 mg green tea powder/mL).

3.3.2.2 Blackcurrant

Cyanidin-3-O-rutinoside standard curve was used to determine the amount of cyanidin-3-O-rutinoside and was 10.4±0.03 mg/100 g as determined by HPLC. The concentration lies within the range (9.3-24.1 mg/100 g DW) as reported in Phenol Explorer (Neveu *et al*., 2010). The polyphenol cyanidin-3-O-rutinoside was in the highest amount in blackcurrant powder as shown in Figure 3-3 B.

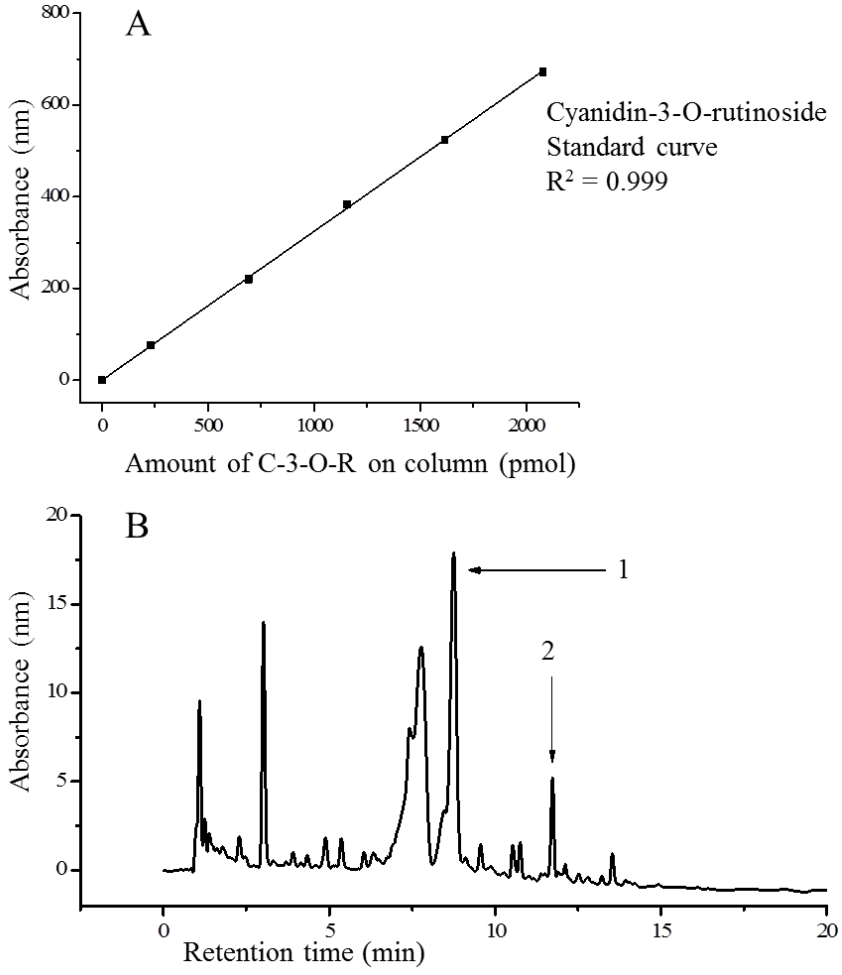


Figure 3-3: Standard curve (data points represents mean±SD, n=3) for cyanidin-3-O-rutinoside (A) used for its quantification in 10 mg/mL blackcurrant extract shown in chromatogram (B): (1) cyanidin-3-O-rutinoside; (2) myricetin (IS).

3.3.2.3 Blackberry

The polyphenol present in the highest amount in blackberry powder was cyanidin-3-O-glucoside (figure 3-4B) with a concentration of 7.01 ± 0.01 mg/100 g as determined by HPLC. It was slightly lower than the reported (8.52-19.06 DW) Phenol Explorer range (Neveu *et al*., 2010). Cyanidin-3-O-glucoside standard curve was used for the quantification.

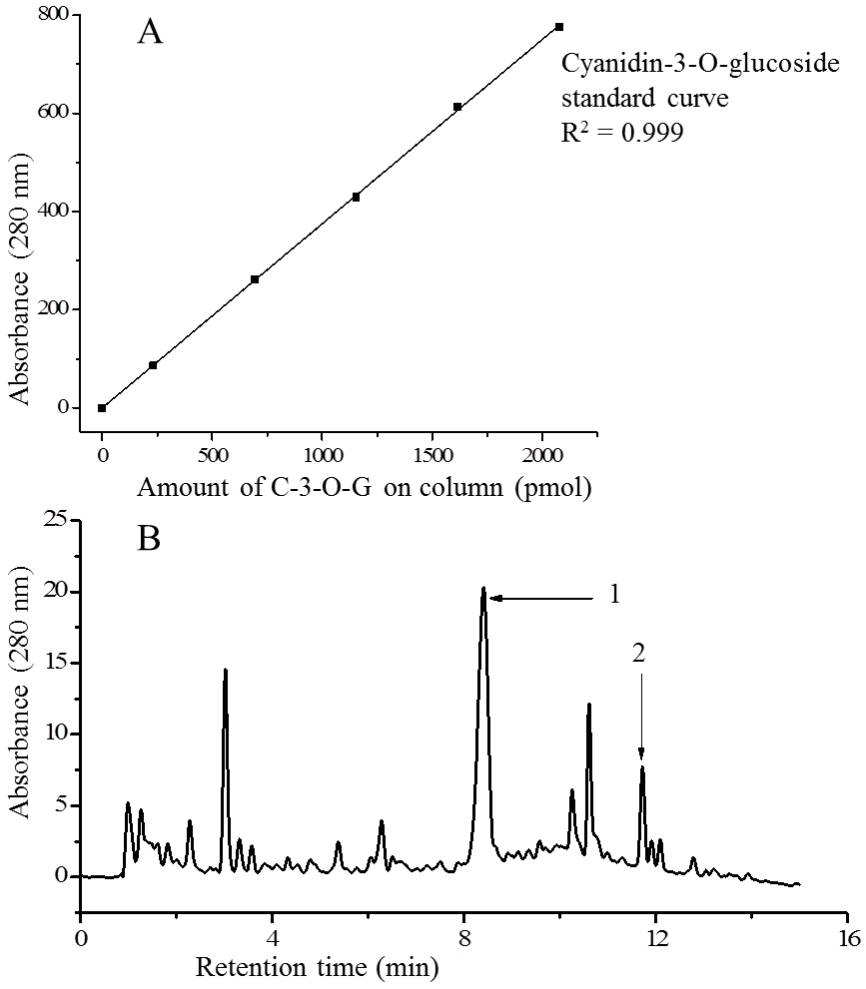


Figure 3-4: Standard curve (data points represents mean±SD, n=3) of cyanidin-3-O-glucoside (A) used for its quantification in 10 mg/mL blackberry extract shown in chromatogram (B): (1) cyanidin-3-O-glucoside; (2) myricetin (IS).

3.3.2.4 Strawberry

The polyphenol pelargonidin-3-O-glucoside was in the highest amount in strawberry powder and the amount was 4.5 ± 0.01 mg/100 g as determined by HPLC (figure 3-5B). The concentration lies within the range (2.03-6.83) reported in literature (Neveu *et al*., 2010).

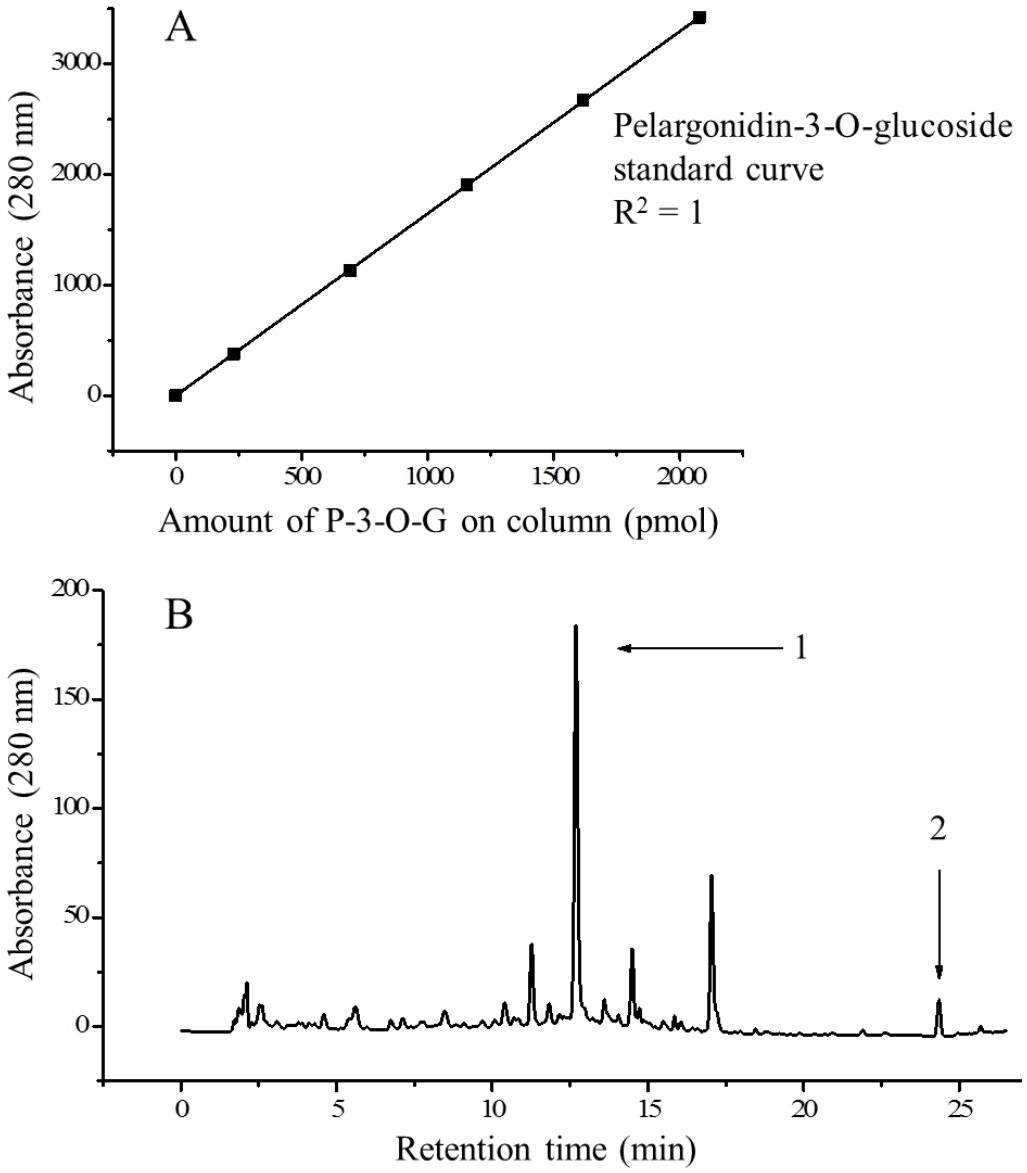


Figure 3-5: Standard curve (data points are mean±SD, n=3) of pelargonidin-3-O-glucoside used for its quantification in 1 mg/mL strawberry extract as shown in chromatogram (B): (1) pelargonidin-3-O-glucoside; (2) hesperetin (IS).

3.3.2.5 Apple peel

The two polyphenols of interest in apple peel powder (phloridzin and quercetin-3-O-rhamnoside) were identified and the concentrations were 18.2 ± 0.03 and 11.3 ± 0.02 mg/100 g fresh weight as determined by HPLC (figure3-6B) and quantified with the standard curves (figure 3-5A). The values lie above the Phenol Explorer range of 0.64-9.11and 0.52-5.30. This is because the apple in this study is just the peel which has higher concentrations of the polyphenols compared to the whole apple (apple dessert whole raw) reported in Phenol Explorer.

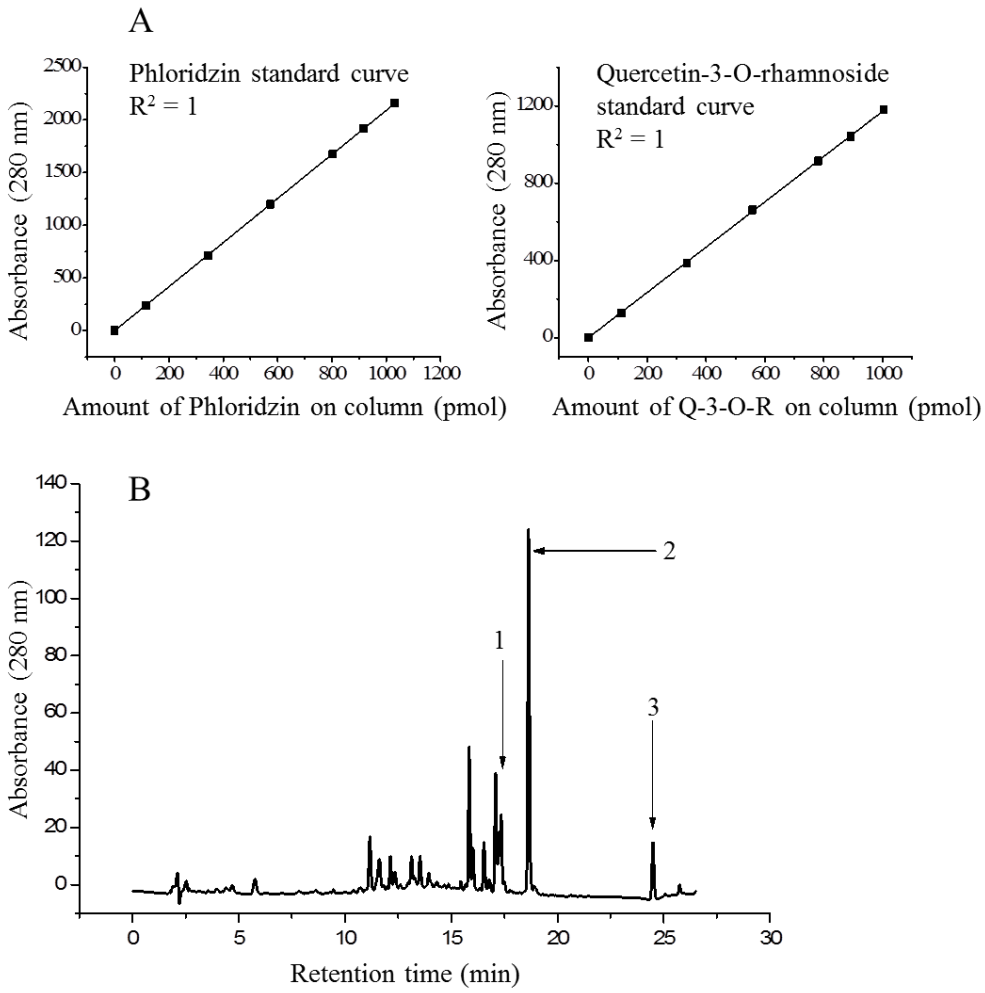


Figure 3-6: Standard curves (data points are mean±SD, n=3) for phloridzin and quercetrin (A) used for its quantification in apple peel extract as shown in chromatogram (B): (1) quercetrin; (2) phloridzin; (3) hesperetin (IS)

3.3.2.6 Sugar analysis in fruits

Fructose, glucose and sucrose were identified in the fruits with fructose being in highest amounts followed by glucose and lastly sucrose.

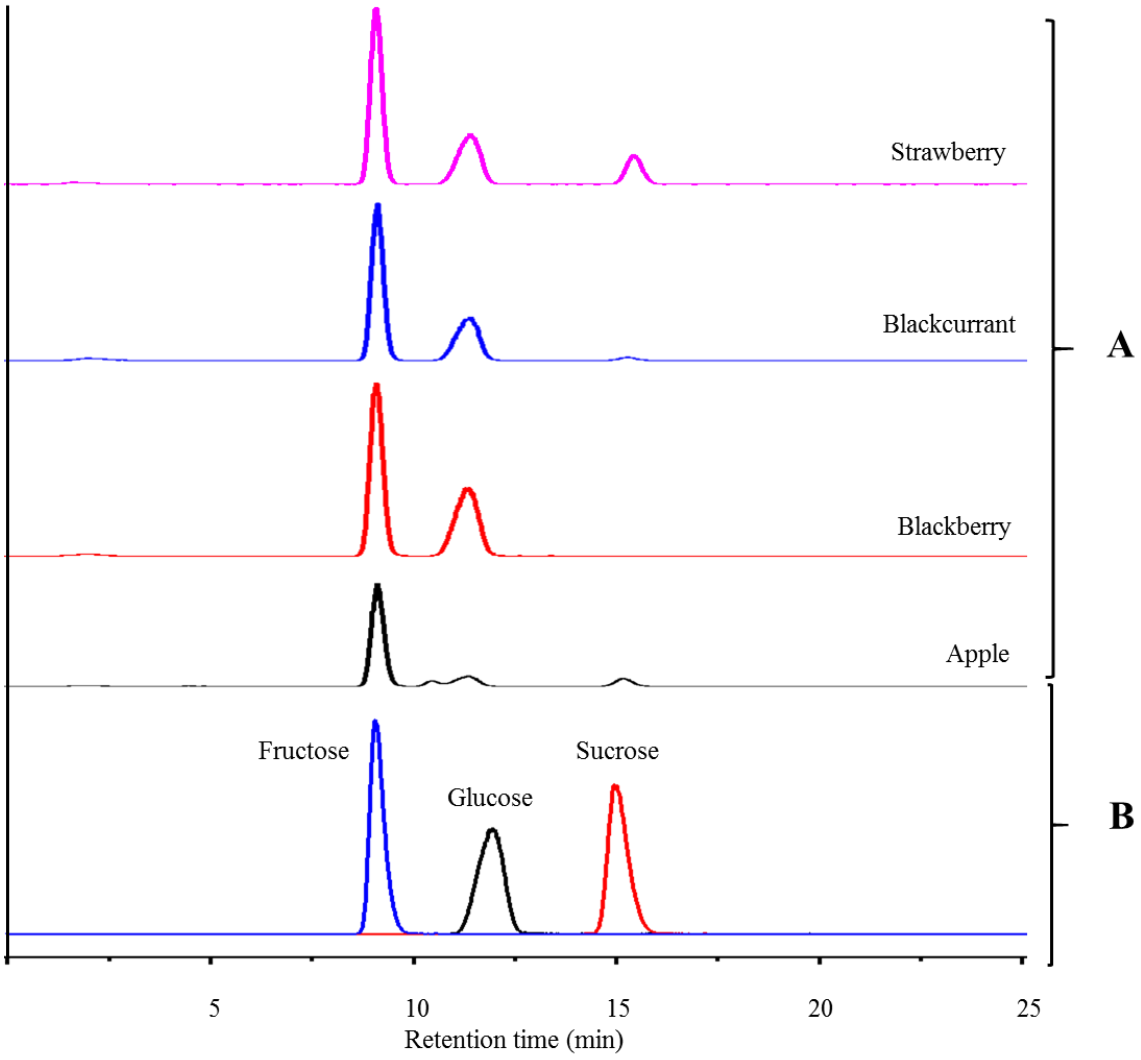


Figure 3-7: Chromatograms of blackcurrant, blackberry and apple (A) showing the presence of fructose, glucose and sucrose as identified from comparing with the chromatogram of standards (B) which are not representative of amounts but just qualitative to show the position of the peaks.

The sugars were quantified using fructose, glucose and sucrose standard curves. The quantities are as shown in table 3-2 and figure 3-8.

Fruit	**Concentration in fruit (g/10 g)**		
	Fructose	Glucose	Sucrose
Apple	0.64± 0.04	0.19± 0.05	0.07± 0.004
Blackberry	1.25± 0.06	0.93± 0.05	ND
Blackcurrant	1.10± 0.05	0.60± 0.03	0.04± 0.002
Strawberry	1.34± 0.04	0.77± 0.03	0.29±0.004
TOTAL in 40 g combination of all fruits.	4.3	2.7	0.44

Table 3-2: The amounts of fructose, glucose and sucrose in the fruits expressed as mean±SD, n=3. The total in 40 g is the sum of all the sugars in 10 g of each fruit (strawberry, blackcurrant, blackberry and apple).

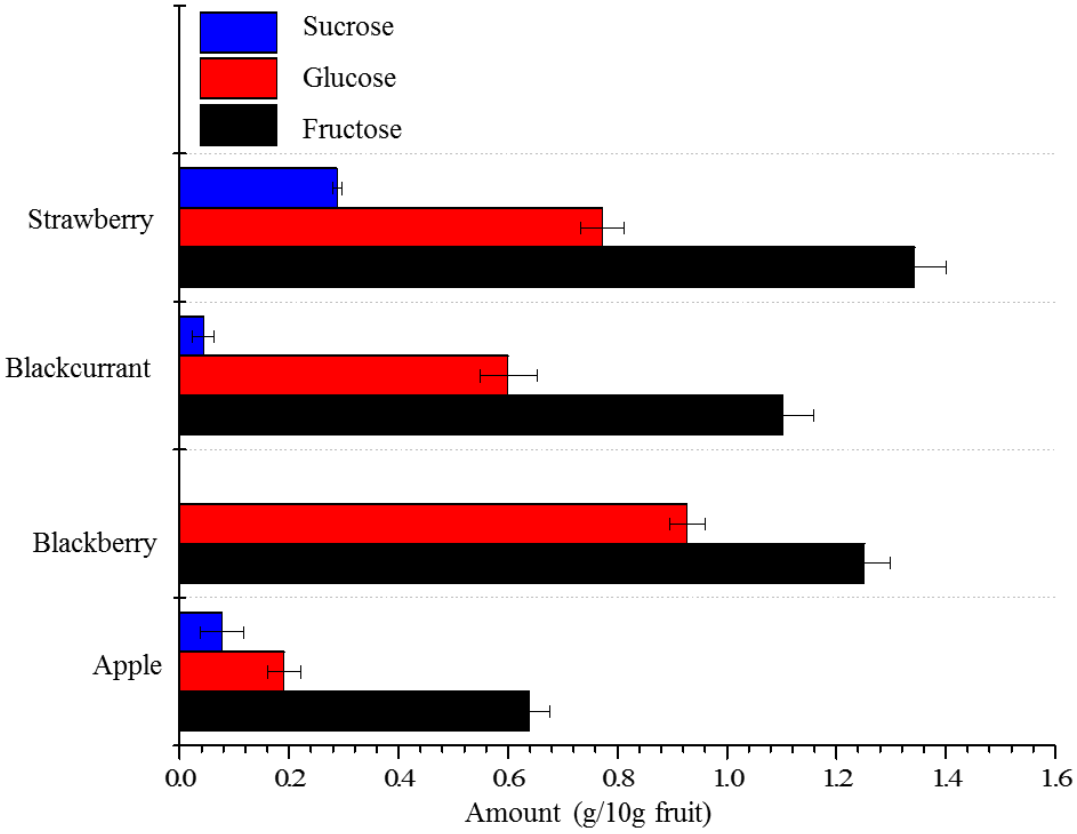


Figure 3-8: Amounts of fructose, glucose and sucrose in the dried fruits. Fructose was in highest amounts in all fruits, then glucose and lastly sucrose of which blackberry had no detectable sucrose.

3.3.2.7 Bread analysis for available carbohydrates

The amount of Warburton's white bread required to give 50 g available carbohydrate was determined to be 109±1.2 g bread as shown in table 3-3. Warburton white bread was selected because it was readily available at all local stores unlike supermarket branded bread which are not available in other stores.

Biological triplicates	Amount of glucose produced from 100g bread (g/100g)	Amount of bread to give 50 g available carbohydrate (g)
1	46.4	107.8
2	45.8	109.2
3	45.4	110.1
Average	45.9±0.5	109±1.2

Table 3-3: The amount of glucose produced from enzymatic hydrolysis of bread expressed as g/100 g of bread and was used to calculate the average amount (g) of bread required to give 50 g available carbohydrate in g (mean±SD, n=3).

3.4 Discussion

The aim of this chapter was to analyse the polyphenol and fibre-rich food (PFRF) mixture constituents for total and specific polyphenols to determine their suitability to be used in the human intervention study. Available carbohydrates in bread and sugar content of the fruits were also determined for the test and control meals study design. Polyphenol contents of different foods may differ depending on the variety and brand for processed foods. It was thus necessary to analyse the selected sources of polyphenols to ensure that they have polyphenols required for the inhibition of digestive enzymes, which is the primary aim of this research.

Typical percentage contents of the major tea polyphenols to the total catechin content are EGCG (59 %), EGC (19 %), ECG (13.6 %) and EC (6.4 %) (Hara *et al*., 2012). The green tea for this study had a similar trend of EGCG being in highest concentration (52.3 %) followed by EGC, ECG and EC with percentages of 32.6, 9.0, and 6.1 % respectively. Green tea was selected to be the main inhibitor of α-glucosidase because its major catechin (EGCG) gave low IC_{50} values *in vitro* (Sucrase 169 μM, Maltase 40 μM (Matsui

et al., 2007), Sucrase 100 μM (Honda and Hara, 1993) and Sucrase 130 μM, Maltase 16 μM (Kamiyama *et al*., 2010). In addition, EGCG also inhibited amylase as shown in table 1-1. In order for concentrations to have an effect *in vivo,* they should be at least 3 times more than the *in vitro* concentrations to allow for intestinal dilutions. This is an important aspect to look at when intending to determine the effects of polyphenols *in vivo* using *in vitro* data because for every 1 L of food, about 2 L of intestinal fluid is produced which entails that there will be a threefold dilution of the food in the stomach (Williamson, 2013). The results obtained for green tea quantification (435.9, 406.1, 77.6 and 80.3 μM for EGCG, EGC, ECG, EC indicate that EGCG concentration (435.9 μM) is more than 3 times the *in vitro* concentrations indicated above that gave 50 % inhibition as shown above.

All the freeze-dried fruits analysed except for apple peel, had polyphenol contents that fell within the range reported on Phenol Explorer (Neveu *et al*., 2010) as shown in table 3-1. Apple peel had higher (twice the amount) contents of phloridzin and quercetin-3-O-rhamnoside than the range reported in Phenol Explorer because the values in Phenol Explorer are those of apple juice whereas results in this research are for apple peel which confirms the fact that the apple peel contains polyphenols in highest amounts. There is currently no data for the contents in apple peel on Phenol Explorer. Blackcurrant was identified as the best source of cyanidin-3-O-rutinoside which was reported to have very good α-amylase inhibition with IC_{50} of 24.4 μM (Akkarachiyasit *et al*., 2011). Blackcurrant fruit also inhibited human salivary α-amylase due to the presence of polyphenols (McDougall *et al*., 2005). In the 10 g blackcurrant to be used in the human intervention study, there will be 10.4 mg cyanidin-3-O-rutinoside which is sufficient to give more than 3 times the IC_{50} concentration of 24.4 μM when made into a paste with 100 mL water (10.4 mg/100 mL = 165 μM). Cyanidin-3-O-glucoside inhibited amylase

with IC_{50} value of 300 µM (Iwai *et al*., 2006). Blackberry was added to the PFRF mixture to compliment blackcurrant as an amylase inhibitor because it has the highest amount of cyanidin-3-O-glucoside (Neveu *et al*., 2010). There will be 70.1 mg cyanidin-3-O-glucoside present in the 10 g blackberry for the PFRF mixture which is equally sufficient to provide a concentration 3 times more than the IC_{50} value (70.1 mg/100 mL =1.5 mM). Strawberry inhibited human salivary α-amylase with IC_{50} value of 120 µg phenols/assay (McDougall *et al*., 2005). Together with the other constituents of the PFRF mixture, strawberry will contribute to the inhibition of α-amylase although it was included to the PRFM as a glucose transport inhibitor. It inhibited glucose transport with its major polyphenol pelargonidin-3-O-glucoside contributing 26 % with an IC_{50} value of 802 µM (Manzano and Williamson, 2010). 10 g of strawberry will provide 45 mg pelargonidin-3-O-glucoside to the PFRF mixture. Pelargonidin-3-O-glucoside was the only polyphenol that did not reach the threefold concentration as it was 1 mM (45 mg/ 100mL) compared to the *in vitro* value of 802 µM. However the fact that the overall inhibition will be from a combination of all inhibitors, the amount of pelargonidin-3-O-glucoside is still a reasonable amount. Apple inhibited glucose transport with the major polyphenol phloridzin contributing 52 % with IC_{50} of 146 µM. Quercetin-3-rhamnoside also found in apple contributed 26 % with IC_{50} of 31 µM (Manzano and Williamson, 2010). Apple was added to the PFRF mixture to act as a glucose transport inhibitor in the PFRF mixture and will provide 18.2 mg phloridzin (18.2 mg/100 mL = 417 µM) and 11.3 mg quercetin-3-O-rhamnoside (252 µM) in 100 mL which are also enough to give at least threefold higher the *in vitro* IC_{50} concentration *in vivo*.

3.5 Conclusion

Green tea from Nestle will be used in the PFRF mixture as the main inhibitor of α-glucosidase as it contains polyphenols in quantities that will potentially have an effect *in vivo*. Blackberry and blackcurrant from Healthy Supplies will be used as the main amylase inhibitors leaving strawberry and apple as the main inhibitors of glucose transport. This will mean that all the three stages of carbohydrate digestion (α-amylase and α-glucosidase catalyzed hydrolysis and glucose transport) will be inhibited. However all the constituents of the PFRF mixture will be tested for potential inhibition of α-amylase and α-glucosidase. If the effects overlapped, it is possible that there would be a synergistic effect of the individual ingredients such that inhibition of different stages of carbohydrate digestion and absorption were affected. For example green tea was added to the PFRF mixture as the inhibitor of α-glucosidase but it is also known to inhibit α-amylase and glucose transport. This means that in the human intervention study test meal, green tea will be expected to contribute to the inhibition of α-amylase together with other α-amylase inhibitors (blackberry, blackcurrant and strawberry), inhibit α-glucosidase and contribute to the inhibition of glucose transport. The same will apply to the other constituents of the PFRF mixture if found to inhibit more than one stage of carbohydrate digestion.

Chapter 4 . Inhibition of α-amylase and α-glucosidase by a polyphenol and fibre-rich food (PFRF) mixture *in vitro*.

Abstract

Polyphenols have been shown to have inhibiting effects on the activities of carbohydrate digesting enzymes (Hanhineva *et al*., 2010). From a literature search, good inhibitors (with the lowest IC_{50} values) of α-amylase were identified as green tea, blackcurrant, blackberry and strawberry and green tea as the good inhibitor for α-glucosidase. The above will constitute the polyphenol and fibre-rich food (PFRF) mixture. The polyphenols from the PFRF mixture were assessed for inhibiting carbohydrate digesting enzymes *in vitro*. Green tea (EGCG 19.6, EGC 11.9, ECG 3.3 and EC 2.1 % by mass in the green tea) gave IC_{50} values of 0.035, 2.02 and 2.31 mg/mL for maltase, isomaltase and sucrase inhibition respectively. The IC_{50} values for α-amylase inhibition using amylose as substrate were 0.009, 1.22, 1.5 and 2.47 mg/mL for green tea, blackberry, blackcurrant and strawberry respectively. Using amylopectin as substrate gave IC_{50} values of 0.025, 1.57, 1.7 and 3.85 mg/mL for green tea, blackberry, blackcurrant and strawberry. The PFRF mixture inhibited the digestive enzymes with IC_{50} values achievable in the diet because the values are far less than one standard portion of fruit (100 g) or beverage powder (0.5 -1 g) and will thus be used in the test meal for the human intervention study to determine the effects of polyphenols from the PFRF mixture on glycaemic response *in vivo*.

4.1 Introduction

Postprandial hyperglycaemia is a risk factor for diabetes (Aston, 2006) which entails that strategies to control postprandial hyperglycaemia are considered important in the prevention and management of type 2 diabetes. A number of studies have shown that some polyphenols have inhibitory effects towards the carbohydrates digesting enzymes α-amylase and α-glucosidase (Hanhineva *et al*., 2010).

In the previous chapter, the PFRF mixture constituents (green tea, blackberry, blackcurrant, strawberry and apple) were analysed to determine the presence of polyphenols and were all found to have total and specific polyphenol contents that are comparable with those in the literature. The aim of this chapter was to investigate the inhibitory properties of the PFRF mixture towards the digestive enzymes α-amylase and α-glucosidase which are responsible for the breakdown of dietary carbohydrate into glucose. Salivary α-amylase starts the digestion of starch followed by pancreatic α-amylase and both hydrolyse starch into maltose, maltotriose and malto-oligosaccharides. Located on the membrane of the epithelium of the small intestine, membrane bound α-glucosidase hydrolyses the products of α-amylase hydrolysis producing glucose which is then transported into the blood by glucose transporters GLUT2 and SGLT1, resulting in a postprandial rise in glucose. Inhibiting α-amylase and α-glucosidase may thus reduce the amount of glucose produced and subsequently absorbed in the blood and lower the postprandial response *in vivo*.

The selection of the constituents of the PFRF mixture was based on their IC_{50} values from other studies (Hanhineva *et al*., 2010). The inhibitors with the lowest IC_{50} values for α-amylase, α-glucosidase and glucose transport were identified and their best sources identified accordingly as shown in table 4-1.

Inhibition target	Good polyphenol inhibitor *in vitro* (reference)	IC_{50} value (reference)	Best food source (http://www.phenol-explorer.eu/)
α-amylase	Cyanidin-3-O-rutinoside (Akkarachiyasit *et al*., 2011)	24.4 µM (Akkarachiyasit *et al*., 2011)	Blackcurrant
	Epigallocatechin gallate (Koh *et al*., 2010)	220 µM (Koh *et al*., 2010)	Green tea
	Cyanidin-3-O-glucoside (Iwai *et al*., 2006)	300 µM (Iwai *et al*., 2006)	Blackberry
α-Glucosidase	EGCG (Kamiyama *et al*., 2010) (Matsui *et al*., 2007) (Honda and Hara, 1993)	Sucrase 130 µM, Maltase 16 µM (Kamiyama *et al*., 2010)	Green tea
		Sucrase 169 µM, Maltase 40 µM (Matsui *et al*., 2007)	
		Sucrase 100 µM (Honda and Hara, 1993)	
Glucose transport	Phloridzin (Manzano and Williamson, 2010)	146 µm (Manzano and Williamson, 2010)	Apple
	Quercetin -3-O-glucoside (Kwon *et al*., 2007)	64 Mm (Kwon *et al*., 2007)	Blackcurrant
	Quercetin -3-O-rhamnoside (Manzano and Williamson, 2010)	31 µM (Manzano and Williamson, 2010)	Apple
	Pelargonidin-3-O-glucoside (Manzano and Williamson, 2010)	802 µM (Manzano and Williamson, 2010)	Strawberry

Table 4-1: Selection of PFRF mixture constituents from literature search for polyphenols with the lowest IC_{50} values and their best sources as reported in Phenol Explorer to inhibit the three stages of carbohydrate digestion and absorption (starch hydrolysis catalysed by α-amylase and α-glucosidase and glucose transport).

The PFRF mixture constituents were analysed for inhibitory activities *in vitro* to determine their suitability to be used *in vivo*. Different concentrations of polyphenols were tested as inhibitors in the assays to obtain dose-dependent inhibition curves from which IC_{50} values were calculated. If shown to be good inhibitors of α-amylase and α-glucosidase, it is hypothesized that the PFRF mixture have the potential to lower postprandial glucose *in vivo* and will thus be used in the human intervention study.

4.2 Inhibition of human salivary α-amylase by PFRF

4.2.1 Inhibition by Green tea and EGCG

Green tea was added to the PFRF mixture as the main α-glucosidase inhibitor. However since it also inhibits α-amylase, its inhibitory potential towards α-amylase was also determined as it would also contribute to the inhibition. Green tea as analysed by HPLC (discussed in chapter 2) contained: EGCG 19.6, EGC 11.9, ECG 3.3 and EC 2.1 % by mass in the green tea. The main catechin EGCG in green tea was also used as an inhibitor in the α-amylase inhibition assay.

4.2.1.1 Materials and methods

The materials and methods are as described in detail in chapter 2 (2.1 and 2.8). Green tea (1 mg/mL) was prepared fresh for each experiment by vortex mixing the powder in millipore water. It was centrifuged at 4000 rpm for 10 min and supernatant collected in a new tube. Appropriate dilutions were made from the 1 mg/mL stock solution to give a concentration range from 0 to 50 μg/mL green tea in the assay.

4.2.2 α-Amylase inhibition by fruits

The freeze-dried fruits (blackberry and blackcurrant) were included in the PFRF mixture as the main inhibitors of α-amylase. However although freeze-dried strawberry was added to the PFRF mixture as the main inhibitor of glucose transport together with apple, it is also reported to be a good inhibitor of α-amylase and its inhibitory potential was also determined as well as that of freeze-dried apple peel.

4.2.2.1 Materials and methods

The materials and methods are as described in detail in chapter 2 (2.1 and 2.8).

4.2.2.2 Sample preparation and Solid Phase Extraction (SPE) recovery

Stock solutions of 10 mg/mL of water extracts of the fruits were prepared by vortex mixing 100 mg freeze-dried fruit in a total volume of 10 ml millipore water. This was done in stages by first adding 5 mL water, vortexed vigorously for 3 min, centrifuged at 4000 rpm for 10 min and supernatant placed in a clean tube. This was repeated twice with 2.5 mL water each time, giving a total volume of 10 mL. Solid phase extraction was carried out to remove sugars as they would interfere with the assay because the final stage of the assay measures the reducing ends of the reducing sugars produced. Hence the presence of other reducing sugars not produced from the hydrolysis of starch would interfere with the results. The Oasis Max 3 cc cartridges containing 60 mg of sorbent were conditioned with 1 mL of methanol and cartridges were allowed to dry under vacuum (10 Hg) for 10 min. Millipore water (1 mL) was used to equilibrate the cartridges before adding 1 mL of the fruit extract sample (10 mg/mL). After passing the sample through the cartridge, the cartridges were washed with 1 mL of 50 mM sodium acetate pH 7 buffer containing 5 % methanol. Polyphenols were eluted with 1 mL methanol for the first wash, then with 2 % formic acid in methanol for three washes. Eluents were

dried using a Genevac and stored in the -20 ˚C freezer until needed. Recovery analysis was determined to determine the recovery percentage of polyphenols after SPE. This was carried out by determining the amount of the major polyphenols in each fruit before and after SPE. Percentage recovery was calculated as:

$$\frac{\text{Amount of polyphenol after SPE}}{\text{Amount of polyphenol before SPE}} \times 100$$

The sugar free samples were used in all inhibition assays by making appropriate concentrations and dilutions from the stock.

4.3 Inhibition of rat intestinal α-glucosidase

4.3.1 α-Glucosidase inhibition by green tea and EGCG

As the main inhibitor of α-glucosidase in the PFRF mixture, green tea and its main catechin EGCG were used in the inhibition assay to determine their potential as α-glucosidase inhibitors. Different concentrations were used to obtain dose-dependent inhibition curves to be used in the calculation of IC_{50}.

4.3.1.1 Materials and methods

The materials and methods are as described in detail in chapter 2 (2.1 and 2.7). Green tea (1 mg/mL) was prepared in the same way as in the α-amylase inhibition determination above (4.2.1.1).

4.3.2 α-Glucosidase inhibition by fruits

Although the freeze-dried fruits were included in the PFRF mixture as the main inhibitors of α-amylase (blackberry and blackcurrant) and glucose transport (strawberry and apple) from the literature search which identified them accordingly for having the lowest IC_{50} values, their potential as α-glucosidase inhibitors was also determined as they would contribute to the inhibition if found to be inhibitors.

4.3.2.1 Materials and methods

The materials and methods are as described in detail in chapter 2 (2.1 and 2.7) and sample preparation is the same as in the α-amylase inhibition (4.2.2.2). Similar to the α-amylase assay, sugar free water extracts of the fruits after SPE were used as the presence of glucose would affect the results because the final stage of the α-glucosidase assay utilizes the hexokinase assay to measure the amount of glucose produced and hence the concentration in the assay volume. The presence of glucose in the fruit water extracts (inhibitor) would add to the concentration of glucose and hence overestimate the amount being determined. The presence of extra glucose in the fruit water extracts (inhibitor) would result in lowered inhibition % as inhibition is calculated by comparing the amount of glucose produced in the test assay compared to the amount produced in the control assay as shown in chapter 2.7.6. The rate of enzyme inhibition is calculated as a percentage of the control (without inhibitor) using the formula:

$$\% = \frac{\text{Abs Control} - \text{Abs sample}}{\text{Abs for control}} \times 100$$

4.4 Results

4.4.1 α-Amylase inhibition by green tea and EGCG

Inhibition curves for green tea (fig 4-1) on α-amylase were obtained by using different inhibitor concentrations and different substrates (amylose and amylopectin). Green tea dose-dependently inhibited human salivary α-amylase and IC_{50} values of 0.009±0.001 and 0.025±0.001 mg/mL were obtained using amylose and amylopectin as substrate respectively (fig 4-1) and (table 4-1).

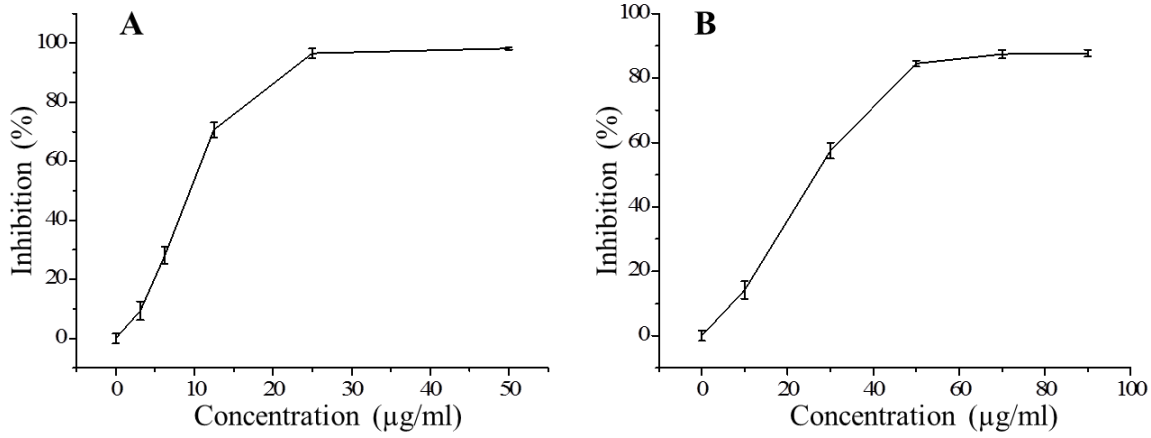


Figure 4-1: Inhibition curves for green tea on α-amylase using amylose (A) and amylopectin (B) as substrate. All data points are mean ±SD, n=3. The curves were used to obtain the IC_{50} values.

Enzyme substrate	Green tea IC_{50} values (µg/mL)	EGCG IC_{50} values (µM)	Acarbose IC_{50} values (µM)	EGCG Ki values (µM)
α-amylase amylose	8.9±0.1	5.3±0.6	3.5±0.3	0.3±0.6
α-amylase amylopectin	25±1	24±4	7.6±0.8	4.5±4.5

Table 4-2: Experimental IC_{50} values of green tea, EGCG and acarbose of α-amylase inhibition and Ki values of EGCG using amylose and amylopectin as substrates. Acarbose was used as a positive control. All values are expressed as mean±SD, n=3.

Similar to green tea, EGCG dose-dependently inhibited α-amylase with IC_{50} values of 5.3±0.6 and 24±4 µM (table 4-1) for amylose and amylopectin respectively. For EGCG, in addition to IC_{50} values, Ki values (the intersection point of all the lines) were also determined (fig 4-2) using amylose (A) and amylopectin (B) as substrate. Different substrate and inhibitor concentrations were used to obtain the Ki values of 0.3±0.6 and 4.5±4.5 µM (table 4-2).

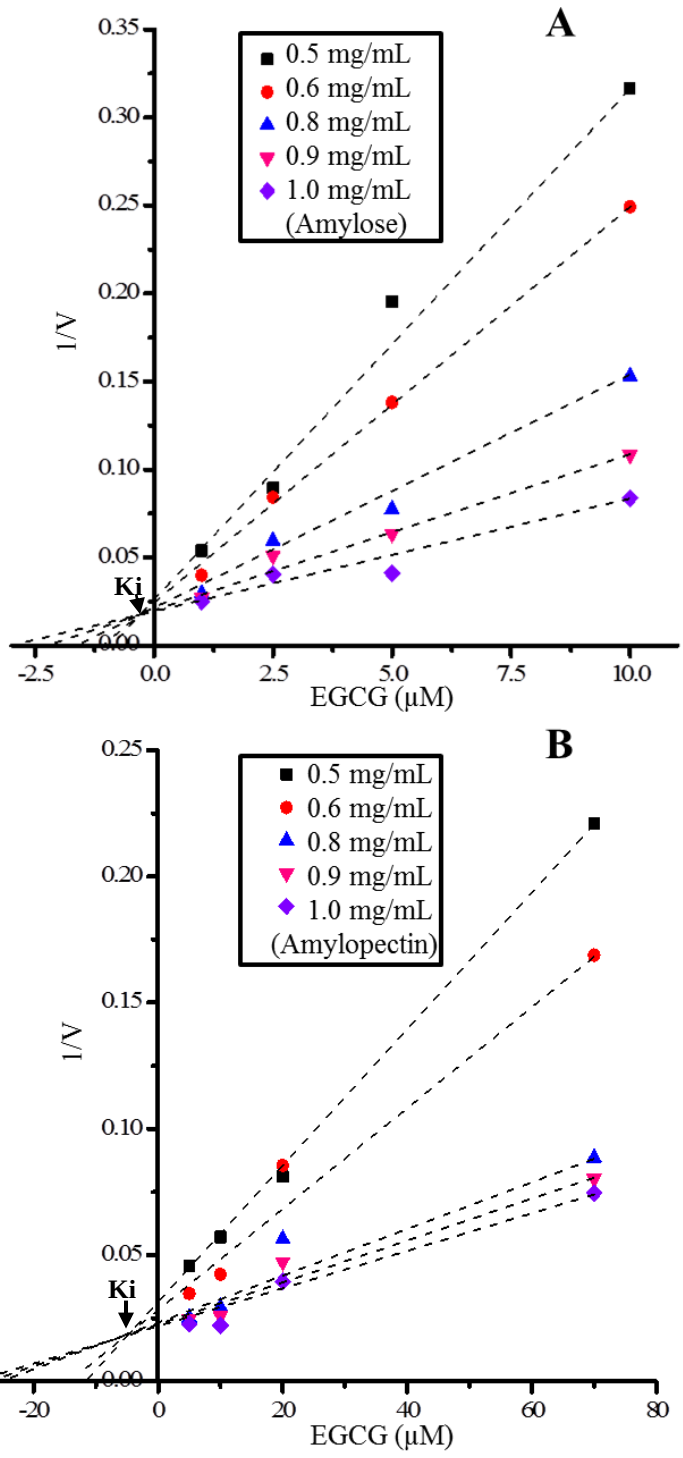


Figure 4-2: Determination of Ki values for EGCG inhibition of α-amylase using amylose (A) and amylopectin (B) as substrate. Different concentrations of the inhibitors EGCG up to 70 µM and substrate concentrations up to 1 mg/mL were used to determine the interaction between the enzyme and inhibitor for Ki determination (intersection point of all the lines). All data points are mean ±SD, n=3.

4.4.2 α-Amylase inhibition by fruits

Freeze-dried blackberry, blackcurrant and strawberry dose-dependently inhibited α-amylase with amylose and amylopectin as substrates (fig 4-3). Apple peel powder did not inhibit at the highest concentration tested (4 mg/mL). The IC_{50} values were 1.22±0.02, 1.5±0.1and 2.47±0.31 mg/mL for blackberry, blackcurrant and strawberry using amylose as substrate and 1.57±0.21, 1.7±0.1 and 3.85±0.05 mg/mL using amylopectin as substrate respectively.

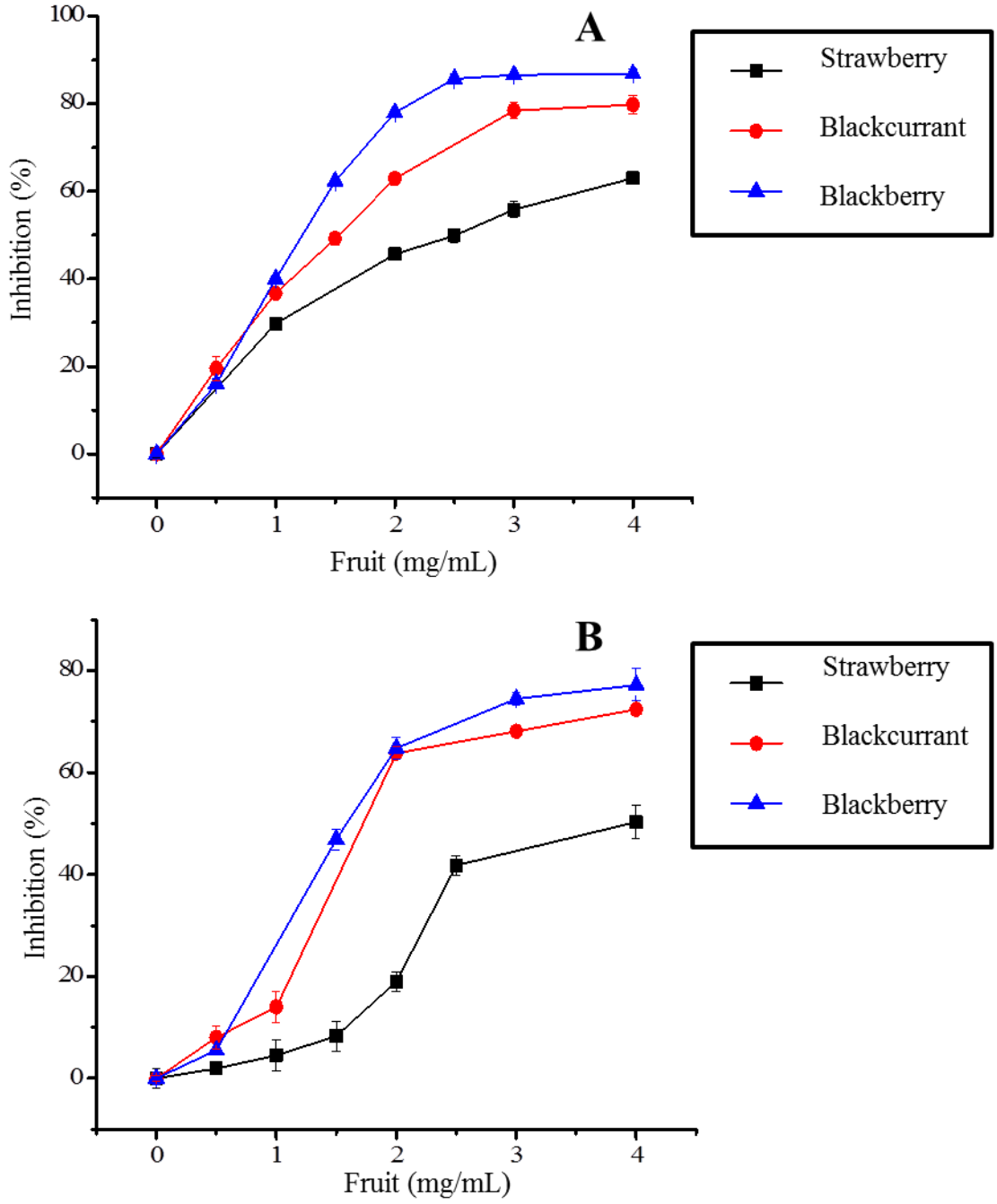


Figure 4-3: Inhibition curves for freeze-dried strawberry, blackcurrant and blackberry on α-amylase using amylose (A) and amylopectin (B) as substrate. All data points are mean ±SD, n=3. The curves were used to obtain the IC_{50} values.

4.4.3 α-Glucosidase inhibition by green tea and EGCG

The inhibitory potential of green tea and its main catechin EGCG on α-glucosidase was determined by using rat intestinal powder as the source of the enzymes. Maltose, sucrose and isomaltose were used as substrates in independent experiments. Green tea dose-dependently inhibited rat α-glucosidase with IC_{50} values of 0.035±0.005, 2.02±0.01 and 2.31±0.02 mg/mL for maltase, iso-maltase and sucrase respectively (fig 4-4).

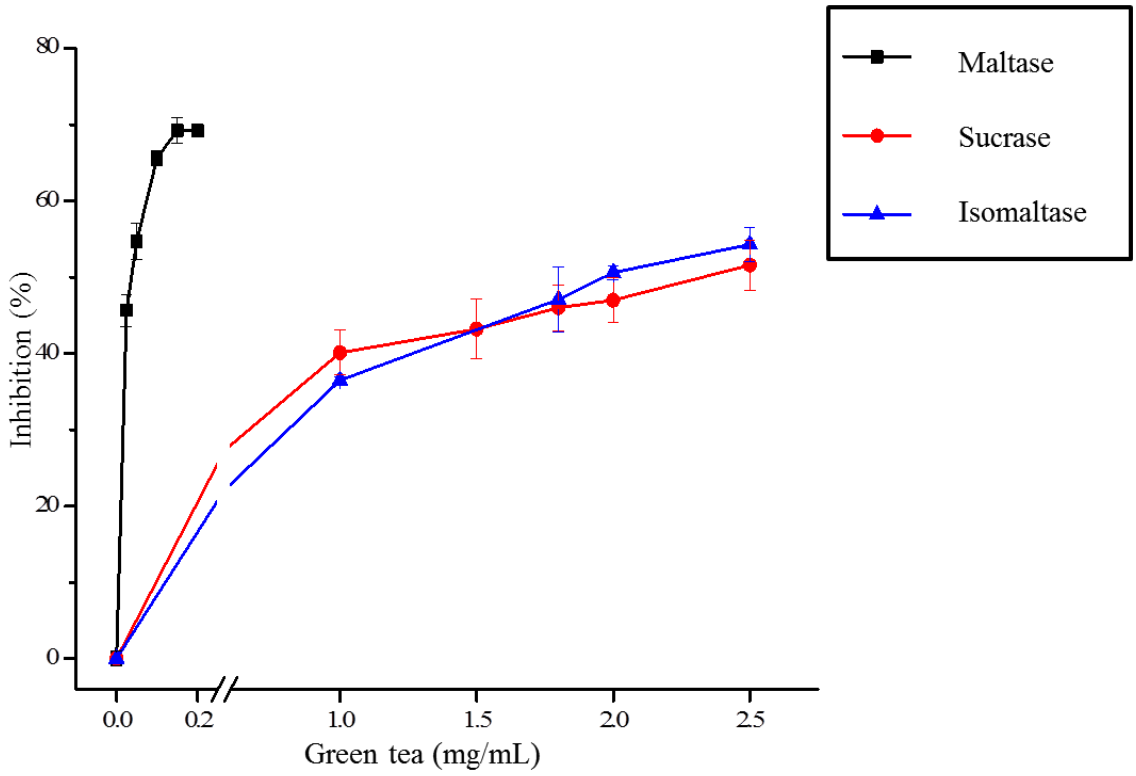


Figure 4-4: Inhibition curves for green tea on rat maltase, sucrase and iso-maltase using maltose, sucrose and iso-maltose as substrate. All data points are mean ±SD, n=3. The curves were used to obtain the IC_{50} values.

Similarly, EGCG inhibited rat maltase at the highest concentration tested (100 µM) with IC_{50} value of 14 µM. Different substrate and inhibitor concentrations were used to obtain

the Ki value of 21.7±1.8 µM (fig 4-5). However, rat sucrase and iso-maltase were inhibited weakly with percentages less than 20 % at 100 µM.

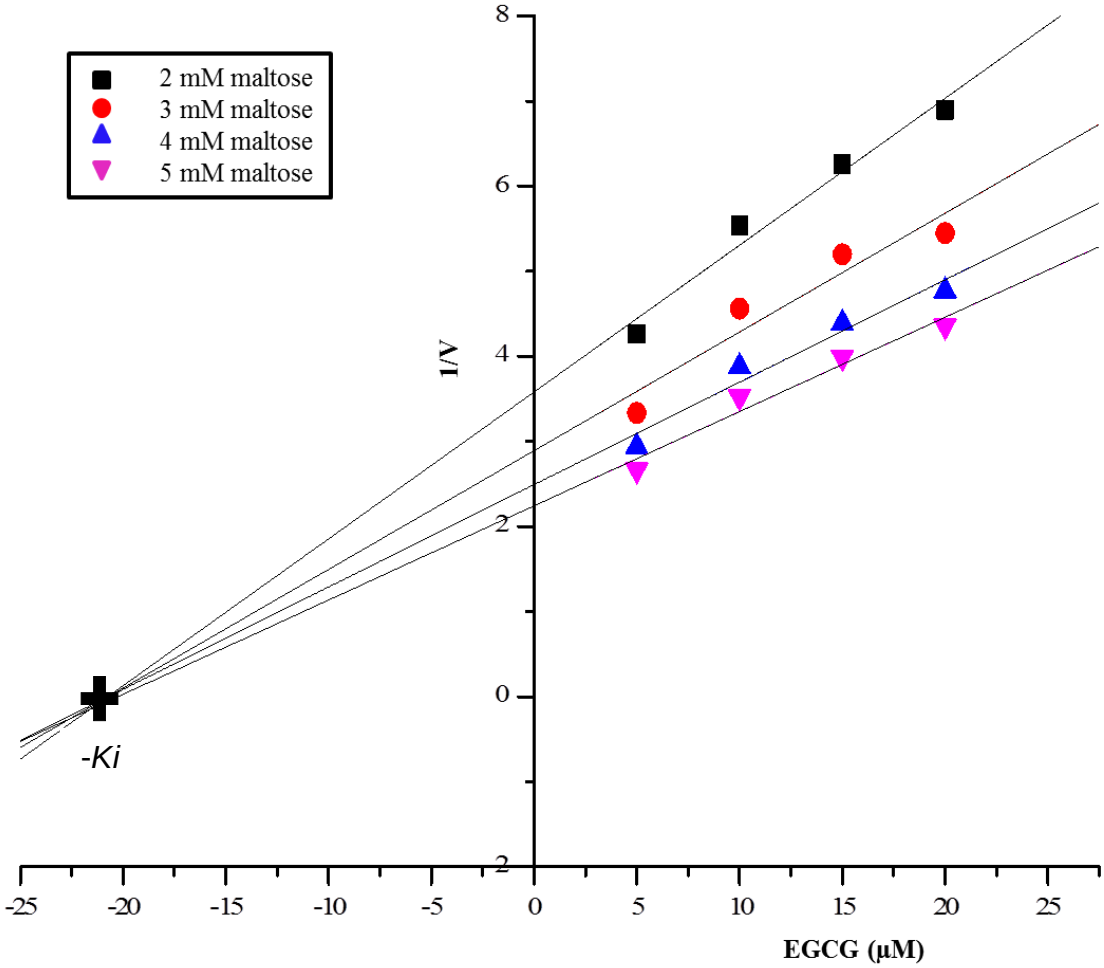


Figure 4-5: Determination of Ki values for EGCG inhibition of rat maltase using maltose as substrate. Different concentrations of EGCG up to 25 µM and maltose (2, 3, 4 and 5 mM) were used to determine the interaction between the enzyme and inhibitor for Ki determination. All data points are mean ±SD, n=3.

4.4.4 α-Glucosidase inhibition by fruits

The freeze-dried fruits blackberry, blackcurrant strawberry and apple peel did not inhibit α-glucosidase at the highest concentration (4 mg/L) tested.

4.4.5 SPE recovery

The major polyphenols in all the fruits gave good recovery (≥ 90 %) after solid phase extraction using Oasis Max cartridges (table 4-3).

Fruit	Polyphenol	Recovery after SPE (%)
Apple	Phloridzin	94±1
	Quercetin-3-O-rhamnoside	97±2
Blackberry	Cyanidin-3-O-glucoside	97±3
Blackcurrant	Cyanidin-3-O-rutinoside	90.0±0.7
Strawberry	Pelargonidin-3-O-glucoside	95.0±0.2

Table 4-3: Solid phase extraction recovery of the main polyphenols found in freeze-dried apple peel, blackberry, blackcurrant and strawberry expressed as percentages (4.2.2.2). All values are expressed as mean±SD, n=3.

4.5 Discussion

Salivary and pancreatic α-amylase catalyse the hydrolysis of the endo α-1,4-glycosidic bonds into maltose, maltotriose and α-limit dextrins from starch. Then α-glucosidases hydrolyse the terminal α-1,4-glycosidic bonds linking the glucose residues to release glucose which is transported into the blood by glucose transporters. The inhibition of α-amylase and α-glucosidase can thus reduce the amount of glucose produced and subsequently transported into the blood which can be considered to be an effective tool in the prevention and management of diabetes type 2.

The PFRF mixture components inhibited the digesting enzymes α-amylase and α-glucosidase *in vitro*. Green tea and EGCG showed inhibition towards both human salivary α-amylase and rat α-glucosidase. EGCG gave an IC_{50} value (5.3 µM) close to

that of the drug acarbose (3.5 µM) which is a known α-amylase and α-glucosidase inhibitor used in the management of type 2 diabetes. These results agree with previous studies (Tadera *et al*., 2006, Koh *et al*., 2010, Forester *et al*., 2012). Koh *et al*. (2010) reported an IC_{50} value of 1.4 mM for EGCG and 4.2 and 4.4 mg/mL of two different green tea plants. Although inhibition was reported, the IC_{50} values were higher than those obtained in the present study (5.3 and 24 µM) for EGCG using amylose and amylopectin as substrate. The IC_{50} value was also higher for green tea which was 8.9 and 25 µg/mL green tea powder for amylose and amylopectin as substrate. The differences observed could be due to a number of reasons including: the catechin content of the green tea used as their total polyphenols were 101 mg GAE/g green compared to 541 mg GAE/g green tea in this study, rice starch was used as substrate compared to amylose and amylopectin used in this study and finally the use of a different method and assay conditions which as discussed by Nyambe-Silavwe *et al*. (2015), can lead to varying results could add to the differences observed.

For α-glucosidase inhibition, results of the present study agree with the literature showing that green tea catechins are the best inhibitors of α-glucosidase (Honda and Hara, 1993, Matsui *et al*., 2007, Kamiyama *et al*., 2010). The $1C_{50}$ values obtained for EGCG were 16 and 40 µM for maltase and 130 and 169 µM for sucrase (Matsui *et al*., 2007, Kamiyama *et al*., 2010). The results are comparable to the present study results which gave IC_{50} of 14 µM for maltase and less than 50 % for sucrase at the highest concentration tested of 100 µM. Similarly, green tea inhibited α-glucosidase in this study and agrees with the literature where inhibition by green tea was reported with IC_{50} value of just below 200 µg/mL total phenolics (Kwon *et al*., 2008).

The freeze-dried fruits blackberry, blackcurrant and strawberry all inhibited human salivary α-amylase. A previous study (McDougall *et al*., 2005) showed that strawberry

and blackcurrant inhibited human salivary α-amylase with strawberry being a better inhibitor than blackcurrant whereas blackcurrant was better than strawberry in the present study. The difference could be attributed to different polyphenol contents due to different cultivars of fruits being used. At the highest concentrations tested, the freeze-dried fruits did not show inhibition towards α-glucosidase. Freeze-dried apple peel did not show inhibition towards neither α-amylase nor α-glucosidase, but will however still be used in the PFRF mixture for purposes of inhibiting glucose transport (Johnston *et al*., 2002, Manzano and Williamson, 2010).

4.6 Conclusion

The PFRF mixture components (green tea powder, freeze-dried blackberry, blackcurrant and strawberry) inhibited human salivary α-amylase *in vitro* with IC_{50} values that can be achieved in the diet after taking into account 3 fold stomach dilutions because for every 1 L of food consumed, about 2 L of intestinal fluid is produced which entails a threefold dilution of the food in the stomach (Williamson, 2013). Together with green tea that inhibited rat α-glucosidase and apple and strawberry that have been shown to inhibit glucose transport (Johnston *et al*., 2002, Manzano and Williamson, 2010), the PFRF mixture has the potential to reduce postprandial glucose *in vivo* by inhibiting the 3 stages of carbohydrate digestion (α-amylase and α-glucosidase hydrolysis of starch and glucose transport into the blood). Hence it will be used in the human intervention study to determine the effects of polyphenols on glycaemic response.

Chapter 5 . Human intervention study to determine the effects of polyphenol and fibre-rich food (PFRF) mixture on glycaemic response *in vivo*.

Abstract

There is increasing evidence that low glycaemic index diets reduce the risk of developing type 2 diabetes. Polyphenol and fibre-rich foods have the potential to affect postprandial glycaemic responses by reducing glucose absorption, and so decreasing the glycaemic response of foods when consumed together. This is the first study to examine the effects of a meal comprised of components designed to inhibit each stage of the biochemical pathway leading up to the appearance of glucose in the blood. The aim was to test whether polyphenol and fibre-rich foods analysed for *in vitro* inhibition in chapter 4, could attenuate post-prandial blood glucose in healthy volunteers when added to a source of carbohydrate (starch in bread). A randomized, single blind crossover study was conducted on 16 healthy volunteers. The volunteers were fasted and attended four visits: two control visits (bread, water, and balancing sugars) and two test visits where they consumed single and double dose of the PFRF mixture together with bread and water. Blood samples were collected at 0 (fasted), 15, 30, 45, 60, 90, 120, 150 and 180 min post consumption. Plasma glucose was lower after consumption of both doses compared to controls: Lower dose, change in incremental area under the curve (IAUC) =-27.4±7.5 % (mean ± SD) $p<0.001$; higher dose, IAUC=-49.0±15.3 %, $p<0.001$); insulin IAUC was

also attenuated by -46.9±13.4% (mean ± SD; $p<0.01$). The PFRF have a pronounced and significant effect on postprandial blood glucose and insulin response in humans.

5.1 Introduction

Diabetes is a global problem and scientific evidence suggests that postprandial hyperglycaemia in humans has a major role to play in the development of type 2 diabetes (Salmeron *et al*., 1997, Steven *et al*., 2002, Hodge *et al*., 2004, McKeown *et al*., 2004). Low glycaemic index (GI) diets show favourable changes in health markers which include fasting blood glucose (Livesey *et al*., 2008a), plasminogen activator inhibitor-1 (Järvi *et al*., 1999) and glycated proteins (Brand-Miller *et al*., 2003, Livesey *et al*., 2008a). Having low glycaemic index (GI) foods in the diet or components that reduce the GI of other foods is thus a good intervention in reducing the risk (Livesey *et al*., 2008b). Low carbohydrate and low GI diets have promising effects in diabetes management (Ajala *et al*., 2013). Strategies to reduce the glycaemic index of foods, even without altering the total carbohydrate content, are therefore of growing interest for reducing diabetes risk and maintaining a more "healthy diet". A number of related factors influence the glycaemic index and response and they include: the nature and amount of carbohydrate, the rate of carbohydrate digestion in the gastrointestinal tract, the rate of absorption of the resulting glucose, the insulin response to the absorbed sugar, and the intrinsic insulin sensitivity (Augustin *et al*., 2002). The presence of naturally-occurring polyphenols have been associated with low glycaemic index foods for many decades (Thomson and Yoon, 1984). Fibre can also play a role in reducing hyperglycaemia, by delaying glucose absorption, increasing insulin secretion and sensitivity, and binding of bile acids (Scazzina *et al*., 2013). In addition, soluble fibre attenuates postprandial glucose by increasing the viscosity in the gastrointestinal tract which disturbs

carbohydrate breakdown and glucose absorption (Dikeman and Fahey Jr, 2006). Possible mechanisms by which polyphenols may affect post-prandial glycaemia are the inhibition of carbohydrate-digesting enzymes and glucose transporters, stimulation of pancreatic β-cells to secrete insulin, activation of insulin receptors, modulation of the release of glucose from the liver, and effects on intracellular signalling pathways and gene expression (Hanhineva *et al*., 2010, Williamson, 2013). The potential action of polyphenols can be compared to that of acarbose, an α-glucosidase and α-amylase inhibitor, which reduces diabetes risk (Hanefeld *et al*., 2008). The Study To Prevent Non-Insulin dependent Diabetes Mellitus (STOP-NIDDM) trial in impaired glucose tolerant (IGT) subjects showed a 36% risk reduction in the progression to diabetes after treatment with acarbose (Chiasson, 2006). The use of a diet-related intervention either on its own or in combination with acarbose would be an alternative to the use of acarbose alone, which can lead to side effects such as flatulence, nausea and diarrhoea (Coniff *et al*., 1995, Chiasson *et al*., 2002).

Some polyphenols inhibit starch-digesting enzymes (α-amylase and α-glucosidase), in addition to glucose transporters SGLT1 (SLC5A1) and GLUT2 (SLC2A2) (Hanhineva *et al*., 2010). Most intervention studies so far have focused on the effect of polyphenols with the endogenous carbohydrates already present in the food, but addition of polyphenols and fibre to reduce the glycaemic index of that food has not been fully explored, but is the normal way in which most foods are consumed i.e. as a total meal in combination with other foods.

In this study, the hypothesis that a combination of components in the diet (polyphenols and fibre) capable of inhibiting the different stages of starch digestion would reduce postprandial blood glucose and insulin using a randomized, controlled, single blind, crossover intervention was thus tested. The test diet was designed to consist of inhibitors

for every stage of carbohydrate digestion. It consisted of α-amylase inhibitors (green tea, freeze-dried blackberry, blackcurrant and strawberry), α-glucosidase inhibitor (green tea) and glucose transport inhibitors (freeze-dried apple peel and strawberry). All freeze-dried fruits also contained fibre which is expected to contribute to the effects on glycaemic response.

5.2 Experimental methods

5.2.1 Subjects

The recruitment of subjects was carried out at the University of Leeds, School of Food Science and Nutrition, Leeds, UK. Poster advertisements around the University of Leeds notice boards were used to recruit interested potential volunteers, who were then screened for fasting plasma glucose (required to be between 3.9 and 5.9 mmol/L). They were then asked to assess themselves using criteria to ensure they could be classified as healthy and free of symptomatic disease. The eligibility criteria were: Aged 18-75, apparently healthy, not diabetic, not on long term prescribed medication, not pregnant nor lactating, and not on a special diet (e.g. for losing weight or fruit supplements). In total, 16 healthy volunteers aged 26 ± 4 y with BMI of 24 ± 3 kg gave their written informed consent and completed the 4 study visits as shown in Figure 5-1. Fasting plasma glucose and insulin concentrations were 4.8 ± 0.1 mmol/L and 24 ± 10 pmol/L respectively.

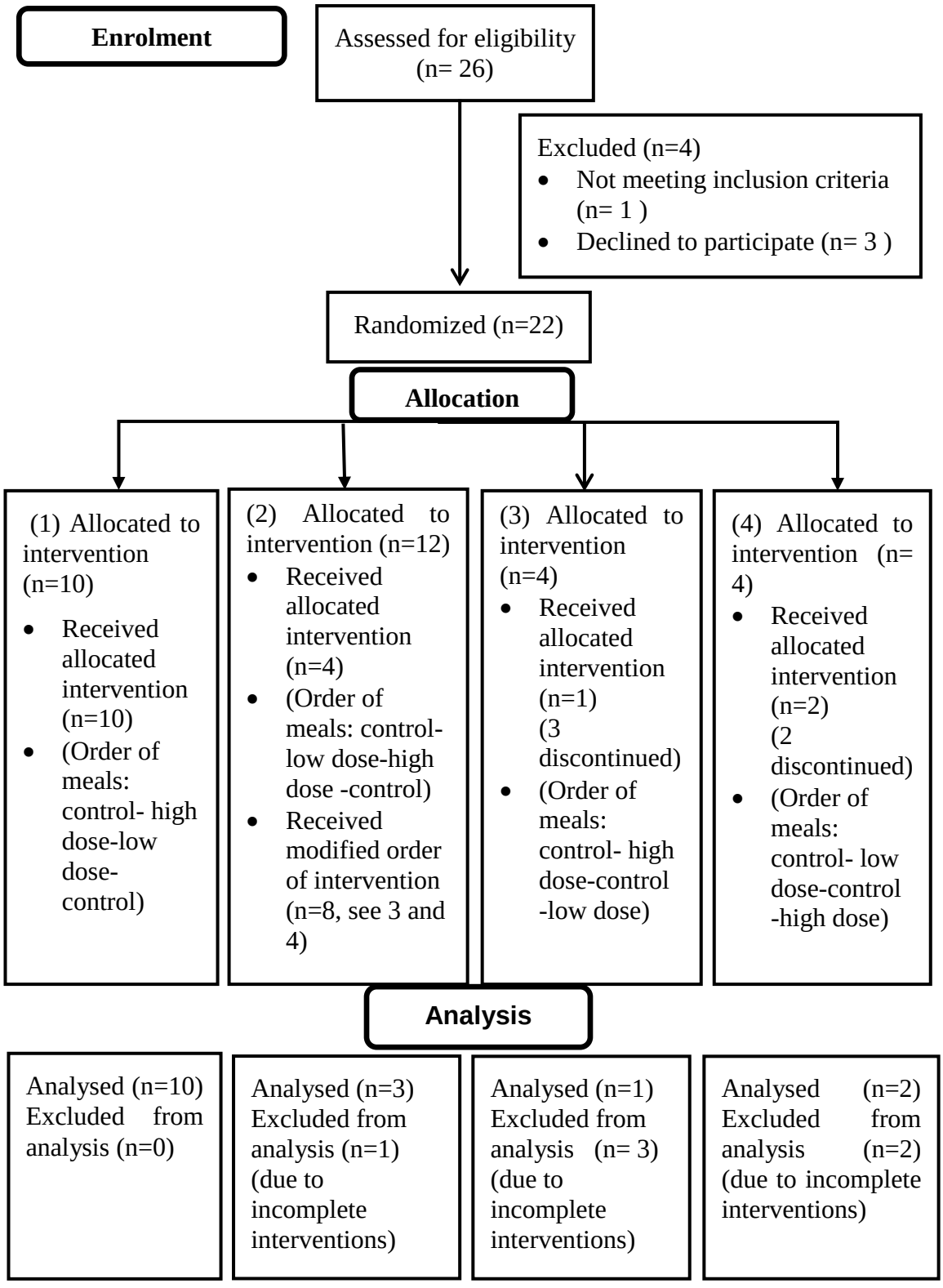


Figure 5-1: Participant enrolment flow diagram showing the randomization of participants in groups for the allocation of the order of meals. It also shows the number of participant's plasma analysed from each randomized intervention.

5.2.2 Study design

A randomized, controlled, single blind, crossover intervention was carried out on a total of 16 healthy volunteers with the primary outcome of post-prandial blood glucose area under the curve. Due to the nature of the test meals, it was impossible to blind participants. However analysis of the plasma samples was blinded and was only unblinded after data analysis. Subjects were cannulated (forearm vein) by a research nurse to ensure comfortable collection of blood samples. Each participant had four visits, two of which were reference meals (the first and last visits) and two visits were test meals (single and double dose of PFRF), administered in a randomized pattern.

All meals contained 109.0 ± 1.2 g white bread (50 g available carbohydrate as analysed by the method of Englyst (Englyst *et al*., 1992) explained in detail in chapter 2. The higher dose consisted of 1 g green tea powder in 200 ml drinking water, with 20 g each of apple peel, blackberry, blackcurrant and strawberry freeze-dried powders mixed with drinking water to make a paste and spread on the bread. The reference meal included 0.8, 5.4 and 8.6 g of sucrose, glucose and fructose respectively dissolved in 200 ml drinking water to standardize the amounts of sugars present in the extracts of the high dose. The lower dose of the test meal contained half the amount of fruits and green tea with half the amounts of balancing sugars dissolved in 200 ml drinking water to equalize the amount of sugars present in all doses. A polyphenol and fibre-rich food (PFRF) containing polyphenols that are effective inhibitors of different stages of starch digestion and absorption was used in this study. It comprised of an α-glucosidase inhibitor (green tea) (Honda and Hara, 1993, Matsui *et al*., 2007, Kamiyama *et al*., 2010), α-amylase inhibitors (green tea, blackberry, blackcurrant and strawberry) (McDougall *et al*., 2005, McDougall and Stewart, 2005, Forester *et al*., 2012) and glucose transport inhibitors (apple peel and strawberry) (Kobayashi *et al*., 2000, Hossain *et al.*, 2002, Manzano and

Williamson, 2010), with all fruits also providing fibre. The PFRF components were analysed for total polyphenol content, specific major polyphenols and for α-amylase and α-glucosidase inhibition *in vitro* (chapter 4). Figure 5-2 shows a summary of the study design from recruitment of volunteers to analysis of plasma.

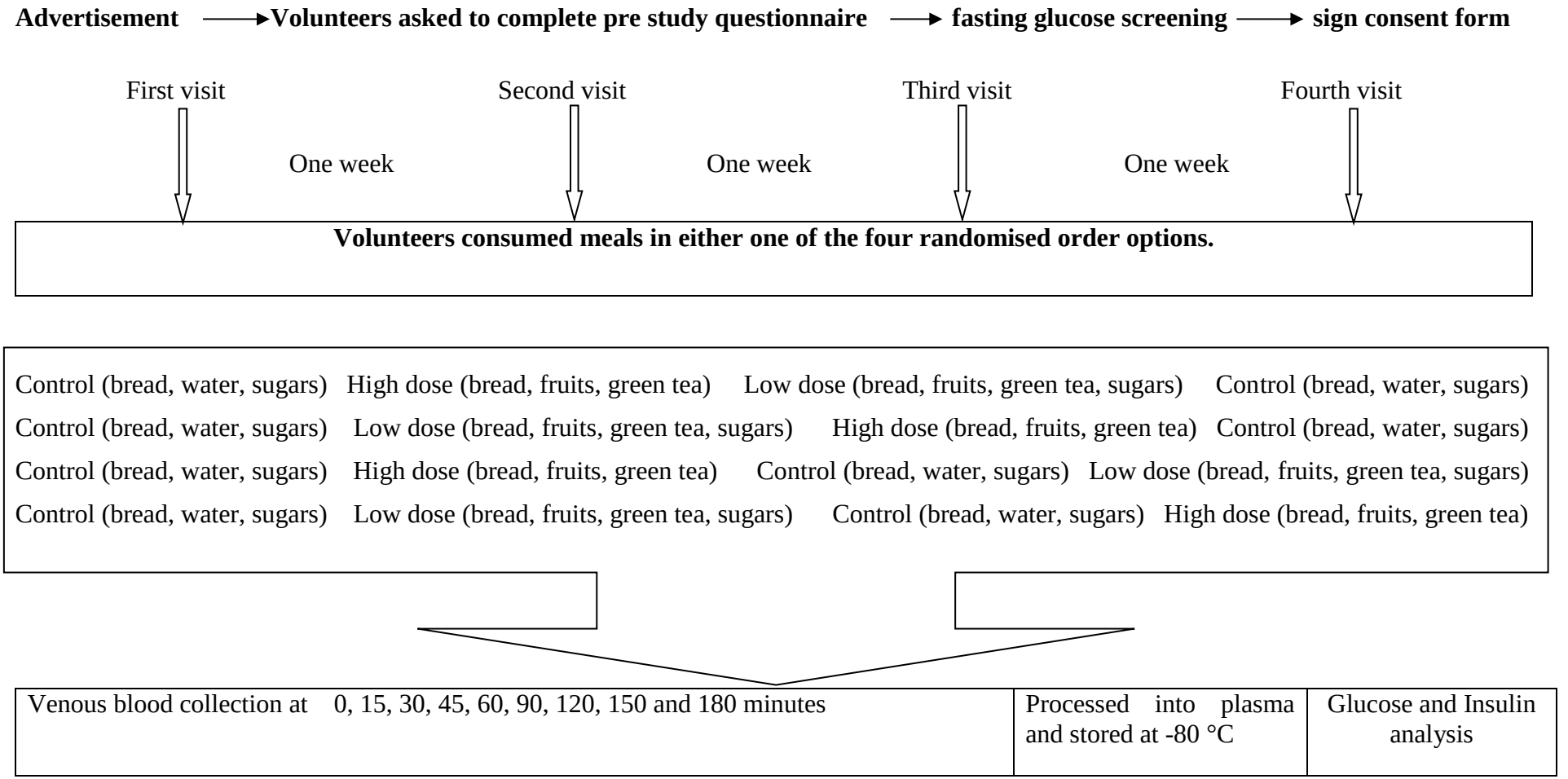


Figure 5-2: Flow diagram summarizing the design for the intervention study from recruitment of volunteers to analysis of the plasma samples for glucose and insulin.

5.2.3 Test meals

All meals contained 109.0 ± 1.2 g white bread (50 g available carbohydrate as analysed by the method of Englyst (Englyst *et al*., 1992) The higher dose consisted of 1 g green tea powder in 200 ml drinking water, with 20 g each of apple peel, blackberry, blackcurrant and strawberry freeze-dried powders mixed with drinking water to make a paste and spread on the bread. The reference meal included 0.8, 5.4 and 8.6 g of sucrose, glucose and fructose respectively dissolved in 200 ml drinking water to standardize the amounts of sugars present in the extracts of the high dose. The volunteers consumed the reference meal on two of the visits to determine any variability in the measurements (Brouns *et al*., 2005). The lower dose of the test meal contained half the amount of fruits and green tea with half the amounts of balancing sugars dissolved in 200 ml drinking water to equalize the amount of sugars present in all doses.

5.2.4 Study protocol

The University of Leeds, Faculty of MaPS and Engineering Ethics Committee (MEEC) approved the study protocol (MEEC 12-037) (appendix 1) and the protocol was registered with ClinicalTrials.gov, ID number NCT01994135. Each participant had one visit per week and hence did the study in 4 weeks with body weight and height measurements taken on the first visit. On each visit, a cannula was inserted in the forearm of the subject. A fasting blood sample was taken and after that the volunteer consumed the meal and the timer started upon first bite or sip. The volunteers consumed the whole meal and blood was collected after 15, 30, 45, 60, 90, 120, 150 and 180 min. Neither harm nor side effects were incurred during the consumption of the meals. Blood samples were collected in fluoride/oxalate and ethylenediaminetetraacetic acid (EDTA) tubes for glucose and insulin measurements respectively and immediately placed on ice. The tubes

were then centrifuged within 15 min at 4000 g at 4°C for 15 min. Thereafter, plasma was placed in storage tubes and stored at -80°C. Plasma glucose concentrations were determined using hexokinase linked to NADH oxidation and insulin concentrations by immunoassay. Both the methods are described in detail in chapter 2 (2.2 and 2.3).

5.2.5 Statistical analysis

The incremental areas under the glucose curves (IAUC) were calculated for each subject for each visit using the trapezoidal rule. Data were analysed using the two tailed paired T-test analysis and results were confirmed by using the one factor repeated measures analysis of variance (ANOVA) between the two references, reference and dose 1, reference and dose 2 and between lower and higher dose. Sample size was determined by designing the trial to have 90% power to detect a clinical difference of 15% IAUC between test and reference meal. For a power calculation, the following information is required:

1) A significance level at which the chance of wrongly concluding that a difference exists when in fact there is no real difference (type 1 error) one is willing to tolerate. Typically a 5% level of significance is chosen to reflect a 95% confidence $\alpha = 0.05$.

2) A desired power at which the chance of correctly detecting a difference when the difference truly exists one wishes to achieve. A conventional power is either 80 or 90% and will use the latter.

3) Specifying a clinically meaningful difference in terms of the primary endpoint denoted by Δ. For this study, clinically significant difference can either be the difference between healthy range and pre-diabetic range, pre-diabetic range and diabetes or from healthy to diabetic. For the purpose of getting a clinically meaningful difference in terms of glucose IAUC, we will consider the difference between healthy range and pre-diabetic range

although only healthy volunteers will be recruited. The difference in fasting plasma glucose from the highest concentration in normal range of 5.9 mmol/L to that of the pre-diabetic one of 6.9 mmol/L gives a 14.5 % change from the calculation:

(6.9 – 5.9) /6.9 X 100, hence will use a decrease of 15 %.

4) Knowledge regarding the standard deviation of the primary endpoint calculated as: Standard deviation X 100 = CV %.

Mean

An average of 11.9% was obtained from three references; (Josic *et al*., 2010) who had 13.9 %, (Johnston *et al*., 2003) with 12.2 % and (Wu *et al*., 2012) with 9.7 %.

By using statistics tables (Machin *et al*., 2009) with the above information assuming normal distribution and paired t-test,

Δ = 15 % / 11.9 % = 1.218 approximately 1.200

α = 2-sided 0.05

Power $1 - \beta = 0.90$, gives **15 participants**

The above calculation was also verified by SPSS statistics software (version 21) as follows:

To determine the estimated sample size for two-sample comparison of means when testing the hypothesis (Ho) that the mean in population 1 (m1) (consuming the control meal) is equal to the mean in population 2 (m2) (consuming the test meal).

Ho: m1 = m2

Assumptions:

alpha = 0.0500 (two-sided)

power = 0.9000

m1 = 200

m2 = 230

sd1 = 25

sd2 = 25

n2/n1 = 1.00

Estimated required sample sizes:

n1 = 15

n2 = 15

The study required 15 participants each for reference and test meal. Each participant being a control of themselves, a minimum of 15 participants were thus required.

5.3 Results

5.3.1 Post-prandial plasma glucose

The plasma glucose concentrations as analysed by hexokinase (chapter 2.2), were used to obtain the average postprandial glucose readings and the curves were obtained (fig 5-3) for each meal (control, low dose and high dose) for 16 volunteers.

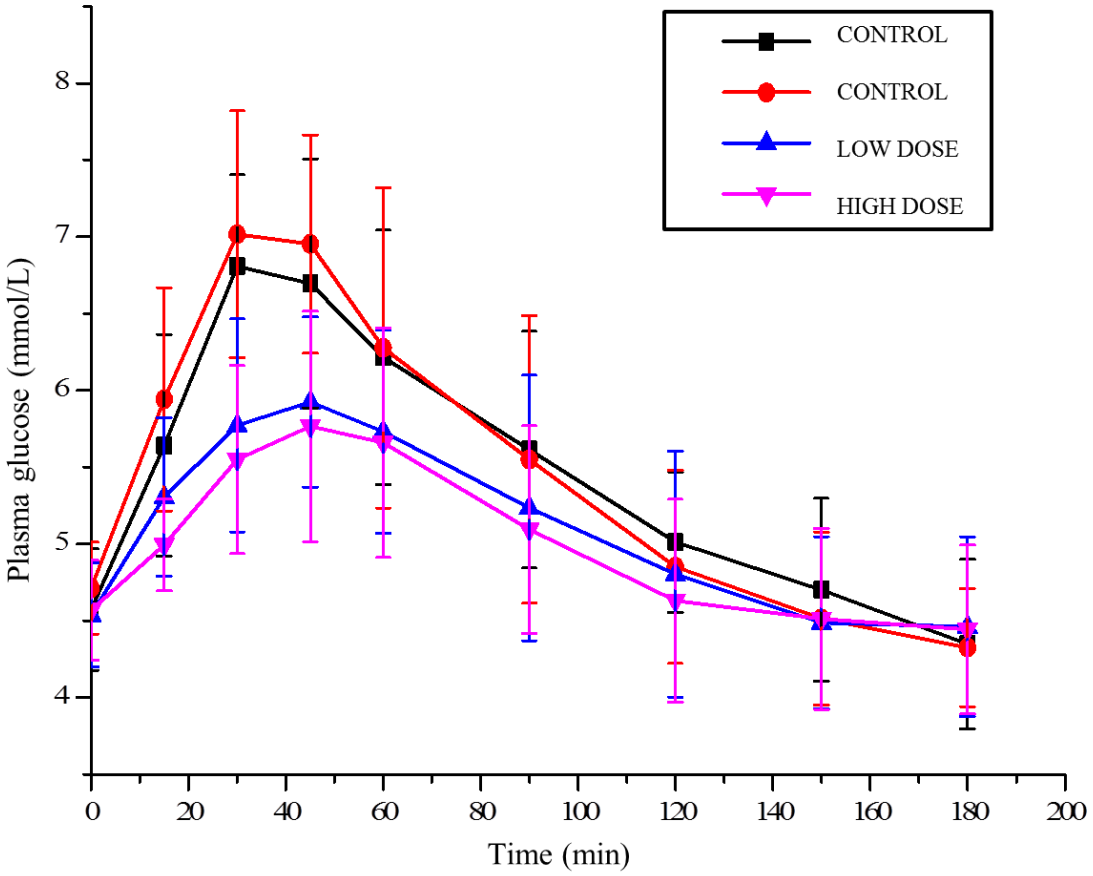


Figure 5-3: Average glucose curves after consumption of reference, test meal dose 1 and test meal dose 2 for 16 volunteers with 3 technical replicates each. There is a decrease in the area under the curve and the peak rise in glucose concentration between the control and test meals. All data points represent mean ± S.D, n=16.

Both the low and the high dose test meals containing PFRF showed a significant decrease in a dose-dependent manner in the glucose IAUC compared to the control meals (Figure 5-4), - 27.4±7.52 % (mean ± SD; $p<0.01$) and -49.0±15.3 % ($p<0.01$) respectively, with no significant difference between the two reference (control) meals. The peak glucose concentration was also significantly lower in both PFRF test meals compared to the reference meals.

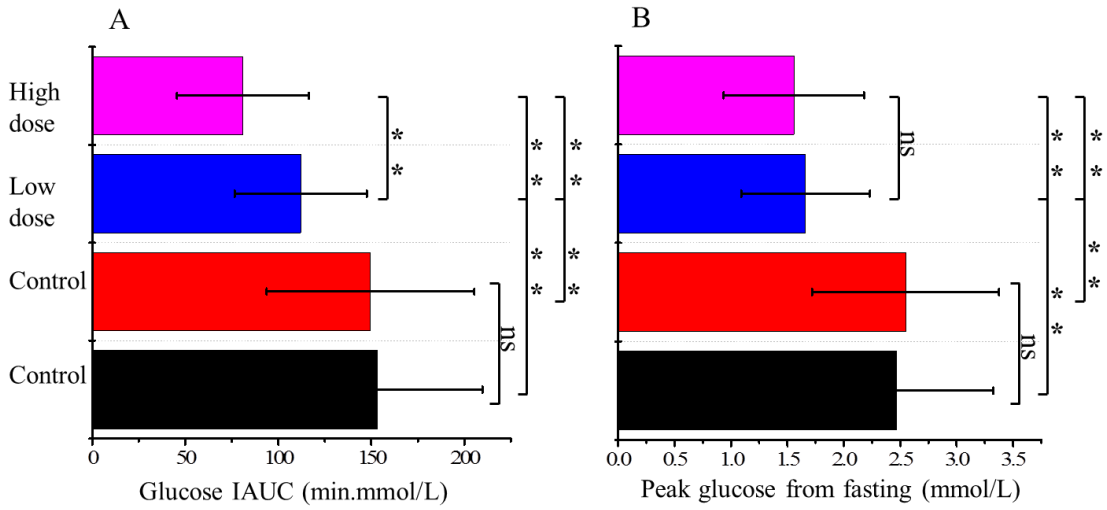


Figure 5-4: Average glucose IAUC (A) and peak glucose concentration from fasting after consumption of reference, test meal dose 1 and test meal dose 2 for 16 volunteers. There is a significance difference between IAUC of reference meals and test meals as well as between the peak rises in glucose concentration (B), $p<0.01$ (**), no significant difference (ns) ($p>0.05$).

5.3.2 Post-prandial plasma insulin

The Mercodia insulin ELISA method (described in chapter 2.3), was used to determine the plasma insulin concentrations which were used to obtain the average postprandial insulin readings and the curves were obtained (fig 5-5) for the control and high dose for 16 volunteers.

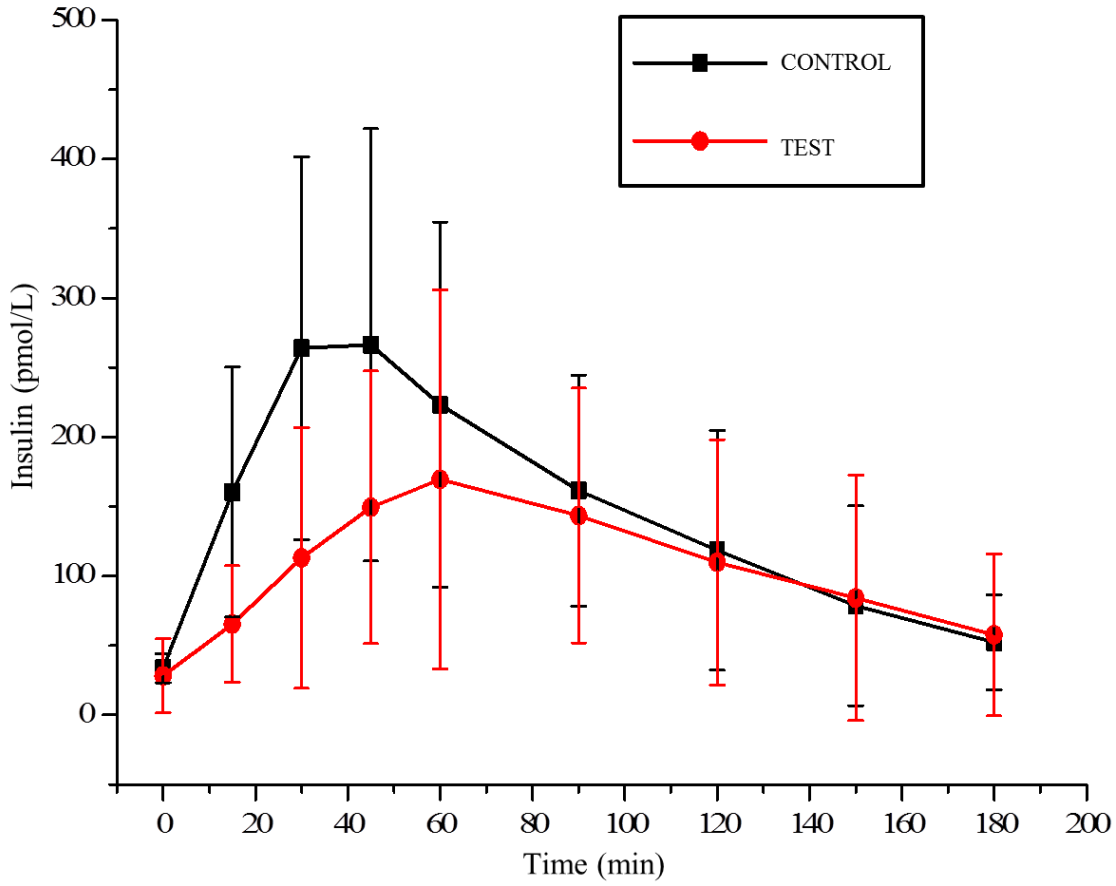


Figure 5-5: Average postprandial insulin curves after consumption of control and test meal for 16 volunteers with 3 technical replicates each. There is a decrease in the area under the curve and the peak rise in insulin concentration between the control and the test meal. All data points represent mean ± S.D, n=16.

There was a reduction in insulin IAUC for the PFRF meal compared to the reference meal of - 46.9±13.4 % (mean ± SD) ($p<0.01$) (Figure 5-5). The PFRF meal also attenuated the peak postprandial insulin concentration, and any differences had disappeared by 120 min.

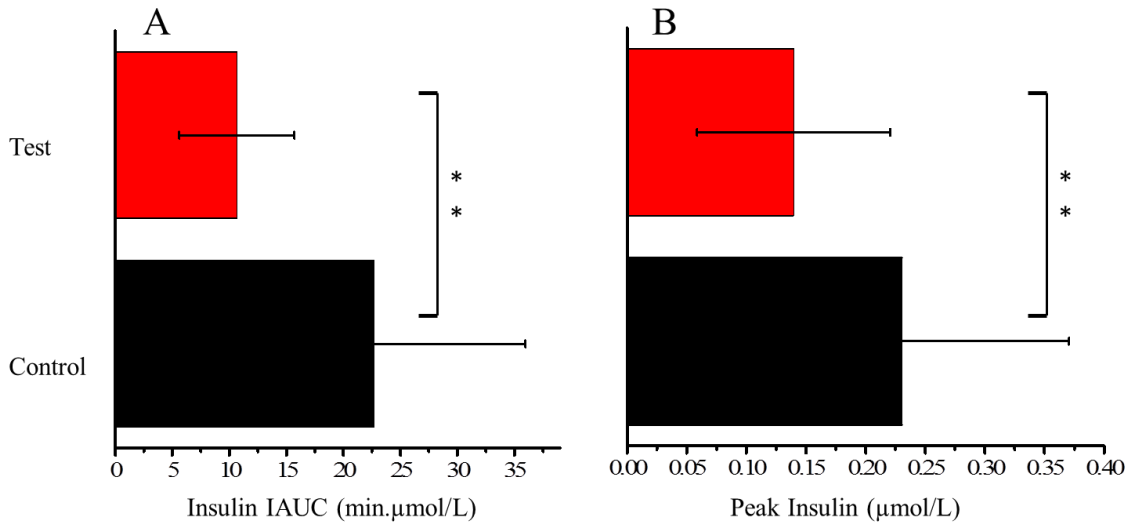


Figure 5-6: Average insulin IAUC (A) and peak insulin concentration from fasting after consumption of control and test meal for 16 volunteers. There is a significance difference between IAUC of control meal and test meal as well as between the peak rises in insulin concentration (B), $p<0.01$ (**).

5.3.3 Inter-individual differences

Inter-individual differences were observed in this study in both postprandial glucose and insulin IAUC (table 5-1). The decrease (%) in glucose IAUC for dose 2 test meal compared to control meal for 16 volunteers varied from 30.4 to 87.3 %. Similarly the decrease (%) in insulin IAUC for dose 2 test meal compared to control meal varied from 28.1 to 67.8 % There was no correlation (Pearson correlation coefficient of -0.219) between insulin and glucose IAUC % difference between control and dose 2 test meals (fig 5-7).

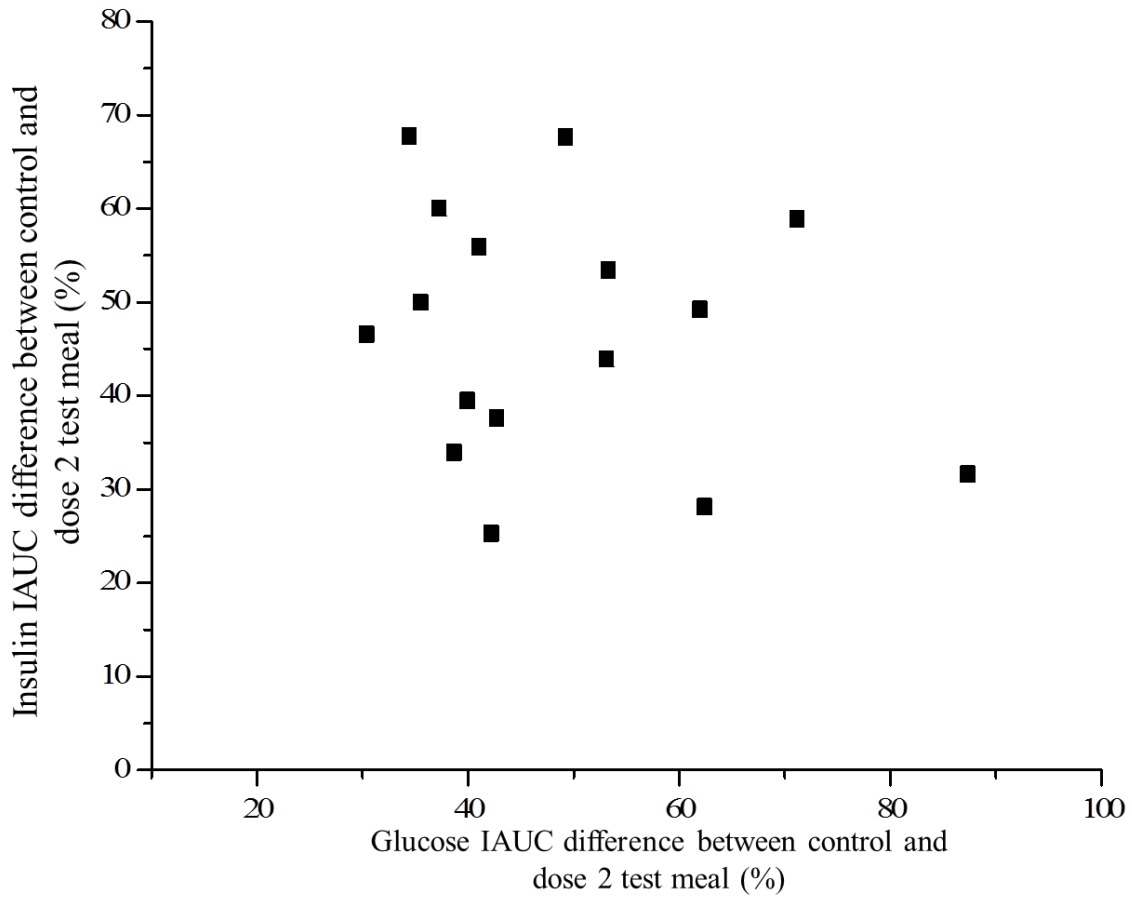


Figure 5-7: Correlation of insulin and glucose IAUC difference (%) between control and dose 2 test meals shows no correlation with Pearson correlation coefficient = -0.219 (n=16 each for glucose and insulin).

Participant number	Decrease (%) in IAUC between control meal and dose 2 test meal	
	Glucose	Insulin
1	38.7	33.9
2	62.4	28.1
3	87.3	31.6
4	71.2	58.9
5	42.7	37.6
6	49.2	67.6
7	42.2	25.3
8	30.4	46.6
9	39.9	39.5
10	53.1	43.9
11	61.9	49.3
12	35.5	50.0
13	34.4	67.8
14	41.0	55.9
15	53.3	53.4
16	37.2	60.0

Table 5-1: Percentage decrease in IAUC of insulin and glucose between control and test meal dose 2. There are inter-person differences in the decrease (%) for both the postprandial IAUC of glucose and insulin. There are also differences in the individual corresponding decrease in postprandial IAUC of insulin in comparison to glucose IAUC change.

5.3.4 Correlations between postprandial glucose and insulin IAUC.

There was positive correlation between insulin and glucose IAUC for both the reference meals (A) and dose 2 test meals (B) with Pearson correlation coefficient = 0.621 and 0.568 respectively.

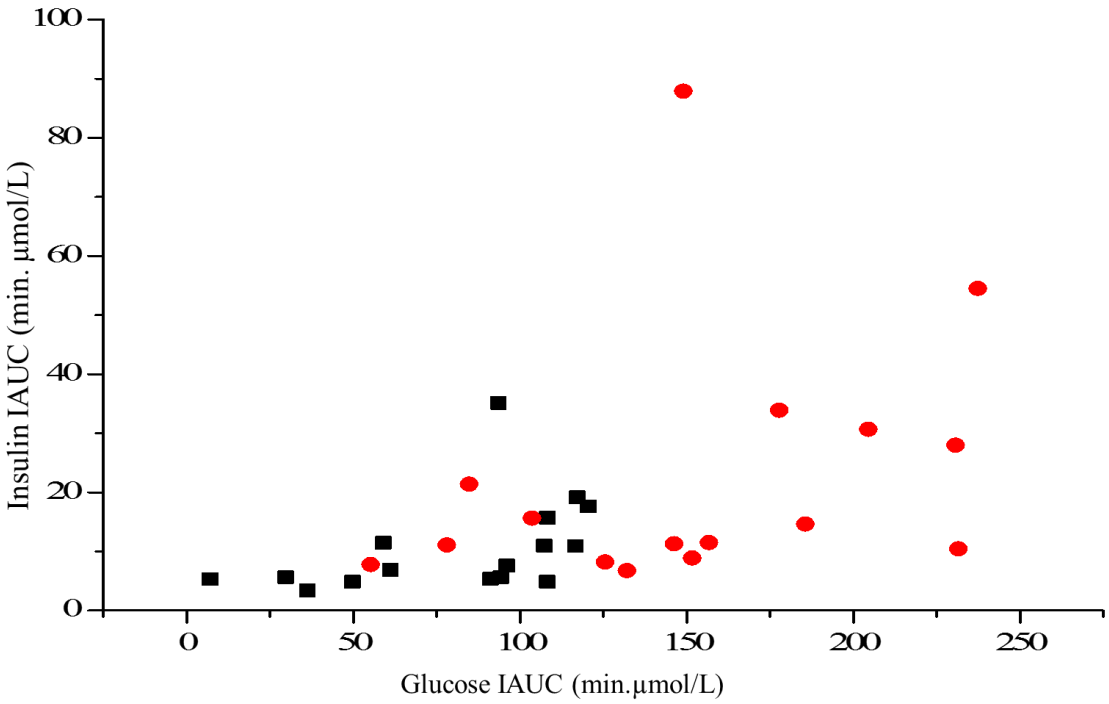


Figure 5-8: Correlation between insulin and glucose IAUC for both the reference (●) and test (●) (n = 16) dose 2 meals with Pearson correlation coefficient = 0.412.

5.3.5 Correlations between insulin sensitivity and insulin release

The homeostasis model assessment (HOMA) tool was used to estimate insulin resistance and β-cell function. It is a validated clinical and epidemiological tool which is a mathematical calculation that utilises fasting glucose and insulin concentrations (Wallace *et al*., 2004). HOMA-IR is HOMA of insulin resistance with the formula: fasting blood glucose (mmol/L) x fasting blood insulin (µLU/ml)/ 22.5 and HOMA-B is that of β-cell function/insulin release with the formula: 20 x fasting insulin concentration (µLU/ml)/

fasting glucose concentration (mmol/L) - 3.5 (Wallace *et al*., 2004). There was a positive correlation (Pearson correlation coefficient = 0.616) between HOMA-IR and HOMA-B showing that the more insulin sensitive a volunteer was, the less insulin was produced by the β-cells of the pancreas (fig 5-9).

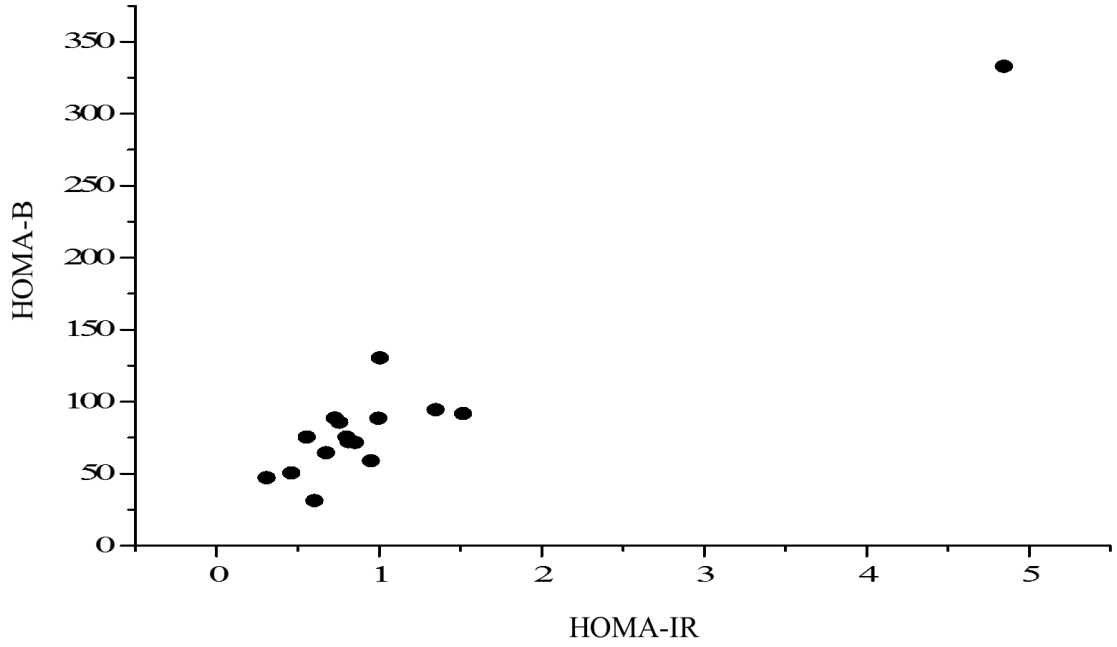


Figure 5-9: Correlation between insulin sensitivity (HOMA-IR) and insulin release (HOMA-B) using fasting insulin and glucose concentrations data from the volunteers (n=16). There is positive correlation with Pearson correlation coefficient = 0.962.

The healthy range for HOMA-IR is 0.5-1.4 with 1.0 as the average. HOMA-IR <1.0 indicates being very insulin sensitive, >1.9 early insulin resistance and >2.9 indicates significant insulin resistance. HOMA-IR and HOMA-B were calculated for all 16 volunteers (table 5-2).

Participant number	Fasting glucose mean±S.D (mmol/L)	Fasting insulin mean±S.D (pmol/L)	HOMA-IR	HOMA-B
1	4.2±0.12	9.9±3.2	0.3	47.2
2	5.2±0.28	15.7±5.6	0.6	31.3
3	4.5±0.34	23.7±10.4	0.8	75.4
4	4.5±0.42	20.0±14.2	0.7	64.5
5	4.3±0.09	31.5±0.6	1	130.4
6	5.0±0.34	41.0±9.7	1.5	91.7
7	4.8±0.13	37.7±10.7	1.3	94.4
8	5.0±0.19	25.8±6.4	1	59.0
9	4.7±0.06	24.7±1.8	0.9	71.7
10	4.4±0.14	23.1±4.5	0.8	85.8
11	4.6±0.14	23.8±2.8	0.8	72.2
12	4.4±0.40	22.6±3.3	0.7	88.6
13	4.4±0.35	14.0±4.0	0.5	50.5
14	4.6±0.16	29.2±10.2	1	88.5
15	4.3±0.22	17.5±4.3	0.6	75.4
16	4.9±0.24	134.9±15.3	4.8	333.0

Table 5-2: Fasting glucose and insulin plasma concentrations of volunteers used in the determination of HOMA-IR and HOMA-B.

5.4 Discussion

Consumption of foods rich in polyphenols and fibre (PFRF) together with bread resulted in a highly significant dose-dependent lowering of the glucose area under the curve and lower peak concentration, and an associated attenuation of insulin. The effect is most likely due to *in vitro* inhibition of human salivary α-amylase (mainly green tea, blackberry, blackcurrant and strawberry), α-glucosidase (green tea), and glucose transport (green tea, apple and strawberry Kobayashi *et al*., 2000, Manzano and Williamson, 2010), and additionally also the effect of fibre (Weickert and Pfeiffer, 2008, Anderson *et al*., 2009). Although it is not possible to define the exact contribution of inhibition of the different steps (inhibition of α-amylase, α-glucosidase or glucose transport) to the attenuation of blood glucose, we would speculate that partial inhibition of multiple steps is important for the observed effect on the glycaemic response. These

reductions in glycaemic response can play a major long term role in the management or risk reduction of diabetes type 2, comparable to the drug acarbose (Hanefeld *et al*., 2008), since high concentrations of postprandial glucose lead to insulin resistance, pancreatic exhaustion, glucose intolerance and an increased insulin demand (Willett *et al*., 2002).

Inter-individual differences were observed and this would be an important aspect to consider in the development of functional foods for the prevention and management of type 2 diabetes. However the PFRF mixture had a positive effect on all volunteers as they all responded by having a decreased glycaemic response compared to the control meal. Although there was an overall decrease for each volunteer in the postprandial glucose and insulin IAUC after a test meal (with the PFRF mixture) compared to the control meal, the differences varied from 30.4 to 87.3 % and from 25.3 to 67.8 % for glucose and insulin respectively (table 5-1). This was confirmed by the lack of correlation between insulin and glucose IAUC difference (%) between the controls and dose 2 test meals (figure 5-7) because volunteers had different glucose and insulin responses. This shows that the same dose of polyphenols from the PFRF mixture results in different percentage decrease in different individuals. For the differences observed in the glucose IAUC between individuals, this could be due to many reasons including the amount of digestive enzymes. The amount of intestinal fluids produced may also be different between individuals resulting in different dilution effects (Williamson, 2013). For the non-correspondence in the insulin IAUC with the glucose IAUC, differences could be due to differences in insulin sensitivity between individuals. To explain the inter-individual variation observed in the results of this study in terms of insulin sensitivity, the homeostasis model assessment (HOMA) tool was used to estimate insulin resistance and β-cell function or insulin release. Out of the 16 volunteers used in this study, one (volunteer 16) had significant insulin resistance with HOMA-IR of 4.8 (table 5-2) and

had a corresponding higher value of insulin release (HOMA-B) of 333. Volunteer 16 was 23 years old with BMI of 29.6, fasting plasma glucose of 4.9±0.2 mmol/L and fasting insulin concentration of 134.9 ±15.3 pmol/L. The BMI (29.6) places the volunteer in the overweight category (25-29.9) which is one of the risk factors for developing type 2 diabetes and partly explains the insulin resistance status. The fasting plasma glucose is however still in the normal healthy range (3.9 – 5.9 mmol/L) which indicates that the β-cells of the pancreas were still able to produce enough insulin to keep the plasma blood glucose in the healthy range although the high fasting insulin concentration shows that the β-cells were overproducing insulin to enable the removal of excess glucose from the blood. At the time of screening, only fasting glucose concentration was checked that's why the volunteer was considered healthy based on the fasting plasma glucose reading. On the other hand, although volunteers 6 and 7 had HOMA-IR values of 1.5 and 1.3, which are very close to the cut off value of 1.4 for the healthy range, their values are still lower than 1.9 which is the value indicating early insulin resistance. Moreover, their fasting insulin values were in the healthy range (41.0 and 37.7 pmol/L) which are less than 62.5 pM (Johnson *et al*., 2010) and are hence not insulin resistant. Volunteer 1 had the lowest fasting insulin concentration of 9.9 pmol/L and also had a corresponding lower HOMA-IR value of 0.3 (very insulin sensitive).

Therefore, differences in insulin sensitivities of the volunteers could be one of the reasons for the inter-individual variations observed. However despite all the inter-individual variations, the PFRF mixture affected all volunteers above the clinically significant difference of 15 % reduction on postprandial glucose and insulin IAUC even including volunteer 16 who had significant insulin resistance.

A limited number of studies have reported the effects of isolated polyphenols or polyphenol containing foods or extracts on post-prandial glycaemia (Johnston *et al*.,

2002, Johnston *et al*., 2003, Bryans *et al*., 2007, Hlebowicz *et al*., 2007, Tsujita *et al*., 2008, Tsujita and Takaku, 2008, Josic *et al*., 2010, Clegg *et al*., 2011, Linderborg *et al*., 2012, Törrönen *et al*., 2012, Chai *et al*., 2013, Coe *et al*., 2013, Schulze *et al*., 2014, Makarova *et al*., 2015) but the results are mixed with only some studies reporting significant differences between test meal and reference meal, and sometimes only at one or two time points, possibly owing to the use of different sugar sources, for example glucose (Johnston *et al*., 2002, Johnston *et al*., 2003, Bryans *et al*., 2007, Linderborg *et al*., 2012, Schulze *et al*., 2014, Makarova *et al*., 2015) or sucrose (Törrönen *et al*., 2012). A limited number of starch-based interventions, using rice, pancakes and white bread, have also shown mixed results (Hlebowicz *et al*., 2007, Tsujita and Takaku, 2008, Josic *et al*., 2010, Clegg *et al*., 2011, Coe *et al*., 2013). All of these studies above have not designed the study meal by considering the mechanism by including foods capable of attenuating the rate of each step of the digestive process. No significant difference was observed when a starch based meal (pancakes) was used together with 100 g berries as the source of polyphenols (Clegg *et al*., 2011). When used alone, green tea as the sole source of polyphenols also did not give a significant difference in the IAUC (Josic *et al*., 2010). There was a significant difference in the IAUC when apple juice was used as a polyphenol source and the decrease was clearly attributed to the inhibition of glucose transporters by polyphenols in apple especially phloridzin (Johnston *et al*., 2002). Polyphenols and fibre (14.7g) (Linderborg *et al*., 2012), present in lingonberries, nulled the glycaemic effect of the endogenous sugars present in the lingonberries. The PFRF mixture had fibre contents of 0.22, 0.53, 0.43 and 0.2 g/100g DW in apple peel, blackberry, blackcurrant and strawberry respectively giving the total amount of 1.1 g in the high dose and half the amount in the low dose. On its own fibre was not expected to have an effect on the postprandial glucose response because an average of >25 and > 38

g/day is recommended in order to have beneficial health benefits (Weickert and Pfeiffer, 2008). An average of 15 g/day fibre treatment for 5 weeks lowered the fasting plasma insulin values and increased insulin sensitivity (Anderson *et al*., 2009). Consumption of 6-20 g/day of fibre for ten weeks of treatment also reported significant reductions in both fasting plasma glucose and insulin (Anderson *et al*., 2009). Both studies indicate that at least more than 6 g/day of fibre was needed in order to have effects on glycemic response. Hence a conclusion can be made that the 1.1 g of fibre present in the PFRF mixture was not entirely responsible but contributed to the observed effects. *In vitro*, polyphenols, phenolic acids and tannins in strawberry and apple reduced glucose transport using caco-2 intestinal cell monolayers by inhibiting the glucose transporters SGLT1 and GLUT2. Phloridzin contributed 52% (IC_{50} = 146 μM) and pelargonidin-3-O-glucoside (IC_{50} = 802 μM) 26% to the total inhibition by apple and strawberry extracts respectively (Manzano and Williamson, 2010). These concentrations, together with those obtained for α-amylase and α-glucosidase inhibition (chapter 4), were theoretically obtained in the gut lumen (table 1) after taking into consideration calculated 3-fold dilution of consumed substances (Williamson, 2013). For example, the IC_{50} value for α-amylase inhibition by green tea was 0.009 mg/ml *in vitro*, 3-fold dilution *in vivo* would require 0.027 mg/ml in the original sample, and the test meals contained 2.5 and 5 mg/ml in the low and high dose respectively. It is therefore proposed that polyphenols and fibre present in the PFRF mixture act together by inhibiting α-amylase, α-glucosidase and glucose transporters and leading to the observed reduced glycaemic response *in vivo*. The observed reduction in postprandial blood glucose and insulin could play a major role in management and reducing the risk of type 2 diabetes, since hyperglycaemia is a risk factor for developing insulin resistance, impaired glucose tolerance and consequently type 2 diabetes.

5.5 Conclusion

Polyphenols and fibre present in fruits, together with a cup of green tea, have a pronounced lowering effect on postprandial glucose and insulin when consumed together with a starch food (bread), owing to inhibition of the different stages of starch digestion. For the current study, the inhibition of the different stages of digestion contributed to the overall inhibition because some studies that have used either one of the PFRF mixture components either did not report any significant difference between control and test meal (Josic *et al*., 2010) or reported a difference smaller (Johnston *et al*., 2005) than that found in this study. Hence to determine the importance and contribution of each step, this can be done either with well-designed *in vitro* assays and models or *in vivo* by carrying out separate human intervention studies using each component of the PFRF mixture separately as the test meal.

Chapter 6 . The effects of pomegranate polyphenols from different food matrices (food supplements and juice) on glycaemic response in healthy volunteers

Abstract

Results from chapter 5 have shown that together with lifestyle changes such as weight loss and physical activity, polyphenols have the potential to be used as functional foods in the prevention and management of type 2 diabetes, as the polyphenol-rich foods significantly reduced the postprandial glucose area under the curve as well as the glucose peak. The aim of this study was to determine the effects of polyphenols from a single source (pomegranate) on glycaemic response *in vivo* when consumed together with a source of carbohydrate (starch in bread) using pomegranate polyphenols in different food matrices (supplements and juice). Pomegranate extract dose-dependently inhibited human salivary α-amylase, maltase and sucrase *in vitro* with IC_{50} values of 0.06±0.01, 1.0±0.1 and 1.2±0.3 mg/mL respectively. One of its major polyphenols, punicalagin, also dose-dependently inhibited α-amylase with an IC_{50} value (9 µM) which is comparable to that of acarbose (3.5 µM), a well-known anti-diabetic drug. Two randomized, controlled crossover studies were conducted independently on 16 healthy volunteers for each study who were fasted and attended control (bread, water and balancing sugars if applicable) and test visits where a source of polyphenols (supplements or juice) were consumed together with bread. There was a significant difference between postprandial glucose IAUC (-33.1±18.1 %, $p < 0.01$) of control and test in the pomegranate juice study and no

significant difference when pomegranate capsules were used. The food matrix containing the polyphenols may play a role in the differences observed as it determines the speed of dissolution for availability of polyphenols to interact with digestive enzymes.

6.1 Introduction

There is growing evidence that certain polyphenols have the potential to lower postprandial blood glucose when consumed together with a starch meal due to inhibition of digestive enzymes α-amylase and α-glucosidase, as well as glucose transporters (Hanhineva *et al*., 2010, Williamson, 2013). A recent review recommended that a combination of polyphenols with the potential to inhibit the named different stages of carbohydrate digestion and metabolism would be the best strategy (Williamson, 2013). In the previous chapter (Chapter 5), a polyphenol and fibre-rich food (PFRF) mixture designed to contain polyphenols that had the potential to inhibit different stages of carbohydrate digestion was shown to have a significant effect on the postprandial glucose IAUC and peak rise in glucose. In order for polyphenols to be utilized in the prevention and management of diabetes type 2, functional foods or food supplements could be developed. The rise in diet related diseases such as type 2 diabetes (Shaw *et al*., 2010) requires a corresponding increase in research relating to the development of functional foods which should provide physiological benefits and play a role in disease prevention and management, in addition to their basic nutritional functions (Viuda-Martos *et al*., 2010).

Pomegranate qualifies to be a potential functional food as it has been used in folk medicine for years due to its reported therapeutic qualities which include anti-diabetic properties (Viuda-Martos *et al*., 2010). The beneficial effects of pomegranate are attributed to the presence of polyphenols, and research on its anti-diabetic and anti-

atherosclerotic effects have intensified in the past ten years (Medjakovic and Jungbauer, 2013). Different parts (peels, seed, leaves, fruit) of the pomegranate plant have bioactive properties attributed to its polyphenols, which include punicalagin, ellagic and gallic acids in addition to tannins and anthocyanins (Banihani *et al*., 2013).

A number of possible mechanisms by which pomegranate polyphenols confer the anti-diabetic properties have been proposed and may include; attenuation of glycaemic response by lowering fasting and postprandial glucose concentration (Jafri *et al*., 2000, Parmar and Kar, 2008), increasing insulin sensitivity and release (Medjakovic and Jungbauer, 2013, Parmar and Kar, 2008), reducing intestinal glucose absorption, promoting the synthesis of glycogen (Parmar and Kar, 2008) prevention of gluconeogenesis (Viuda-Martos *et al*., 2010) and up regulation of glucose transporter type 4 (GLUT4) mRNA expression which modulates glucose transporter expression and consequently glucose levels (Medjakovic and Jungbauer, 2013). Other mechanisms are through their anti-atherosclerotic and anti-inflammatory effects. Anti-atherosclerotic properties are through increasing the activities of paraoxinase 1 (PON1), the major anti atherosclerotic component of high density lipoprotein (HDL), and superoxide dismutase (SOD), which catalyses the conversion of the superoxide radical into oxygen (Banihani *et al*., 2013). Many of these effects are mediated through the activation of peroxisome proliferator-activated receptor (PPAR) pathways. This property is exhibited by drugs that are used to treat insulin resistance by sensitising the cells to the presence of insulin by activating PPAR-γ (Medjakovic and Jungbauer, 2013). Pomegranate and its polyphenols have been proven to act as modulators of PPAR-γ. Incubating either pomegranate juice, punicalagin or gallic acid with macrophages reduced oxidative stress in macrophages as a result of its stimulation by PPAR-γ release/action and the effects were cancelled when PPAR-γ was inhibited (Shiner *et al*., 2007, Mueller and Jungbauer, 2009). PPAR-γ also

plays a role in the anti-inflammatory effects of pomegranate. The loss of the PPAR-γ gene led to the loss of the anti-inflammatory properties of pomegranate polyphenol punicic acid as demonstrated in PPAR-γ null mice (Hontecillas *et al*., 2009).

A recent review reports that the mechanisms by which pomegranate polyphenols confer the anti-diabetic properties are not fully understood (Banihani *et al*., 2013). However, to establish the functions of pomegranate towards diabetes type 2 management and prevention, the mechanisms need to be identified *in vitro* and confirmed *in vivo* especially in humans in order to render scientific support. Among other mechanisms proposed, carbohydrase inhibition is not mentioned as one of the possible mechanisms, and hence the aim of this study was to determine whether pomegranate inhibits the digestive enzymes α-amylase and/or α-glucosidase and therefore affects glycaemic response *in vivo*. Pomegranate extract and its major polyphenols punicalagin, gallic acid and punicalin were tested for α-amylase and α-glucosidase inhibition *in vitro*. Thereafter two randomized crossover studies were conducted on 16 healthy volunteers using pomegranate supplements (double blind) and pomegranate juice as sources of polyphenols independently.

6.2 Experimental methods

6.2.1 Materials

Organic pure pomegranate juice, pomegranate extract which was produced from pressed pomegranate residue obtained during pomegranate juice processing. It included membranes and remaining seed from which juice had been removed.

Human salivary α-amylase, rat intestine powder, sucrose, maltose, D-glucose, glucose and fructose for human consumption, ellagic acid, punicalagin, an isomeric mixture of punicalagin A and B, and punicalin, a mixture of A and B, whose sources are listed in chapter 2.1 were used in this chapter.

For blood glucose readings, materials used were glucometer, test strips, gloves, cotton balls, pricking device and lancets.

6.2.2 Pomegranate analysis

6.2.2.1 Polyphenol characterization by HPLC

Polyphenol determination was carried out according to the method of (Kerimi Nyambe, unpublished data). Briefly juice samples were centrifuged and filtered and filtrates were directly injected on an Agilent 1200 HPLC system equipped with a photodiode array detector and an ion-trap mass spectrometer detector. Quantification of punicalin and punicalagin was performed at 360 nm with the calibration curve of punicalagin. Ellagic acid was quantified using the calibration curve of ellagic acid.

6.2.2.2 Sugar analysis by HPLC

The identification and quantification of sugars in the fruits was conducted according to the method by (Ifie *et al*., 2016) on a Shimadzu HPLC instrument equipped with a model DGU-20 A5 degasser, a LC-20 AD XR pump system, a SIL-20 AC XR auto sampler (Shimadzu), column oven, a diode array detector system (SPD-M20A) and a Shimadzu ELSD-LTII low temperature evaporative light scattering detector. A sample volume of 10 µL was injected, and separations were achieved on a Prevail Carbohydrate ES 5 µm column (250 mm x 4.6 mm). The column was held at 20 ^{0}C, and individual sugars were eluted isocratically using a 1 mL/min flow of 75 % acetonitrile. Solutions of standard sugars prepared in millipore water with concentrations between 0 and 10 mg/mL were used for the calibration curve. The sugars were identified by their retention time characteristics. Quantification was achieved using standard calibration curves obtained by plotting area versus concentration ($R^2 > 0.98$). Data from the sugar analysis allowed balancing of the control samples for glucose and fructose naturally present in the pomegranate juice.

6.2.3 Enzyme assays *in vitro*

The inhibition potential of pomegranate extract on starch-digesting enzymes α-amylase and α-glucosidase was tested *in vitro* as described in chapter 2.7 and 2.8 for α-glucosidase and α-amylase respectively. The major polyphenols in the pomegranate juice were also tested *in vitro* for human salivary α-amylase and rat α-glucosidase. Stocks of 50 mM of punicalagin, punicalin and ellagic acid were prepared in 100% DMSO and kept at -20 C. Fresh aliquots were diluted with 50 % ethanol to the required final concentration in the assay.

6.2.4 Subjects

Adverts were placed around the University of Leeds notice boards for the recruitment of volunteers and interested potential volunteers expressed their interest by means of email. An invitation email was then sent to them for the screening exercise for which they were asked to come in a fasted state. The screening and recruitment was done at the School of Food Science and Nutrition, University of Leeds. Pre-study questionnaires were used to enable the potential volunteers to assess themselves as being healthy and free of symptomatic diseases. They were also screened for their fasting blood glucose using a standard glucometer and the acceptable range was from 3.9 to 5.9 mmol/L. Other eligibility criteria included: Aged 18-75, apparently healthy, not diabetic, not on long term prescribed medication, not pregnant nor lactating, and not on a special diet (e.g. for losing weight or fruit supplements). For the pomegranate capsule intervention study, 16 healthy volunteers aged 26 ± 6 y with body mass index (BMI) of 23 ± 2 kg/m^2 gave their written informed consent and completed the 3 study visits (fig 6-1). Fasting plasma glucose concentrations were 4.7 ± 0.4 mmol/L. For the pomegranate juice study, 16 volunteers aged 31 ± 5 y with BMI of 23 ± 3 kg/m^2 and fasting blood glucose of 4.7 ± 0.5 mmol/L completed the 4 study visits (fig 6-2).

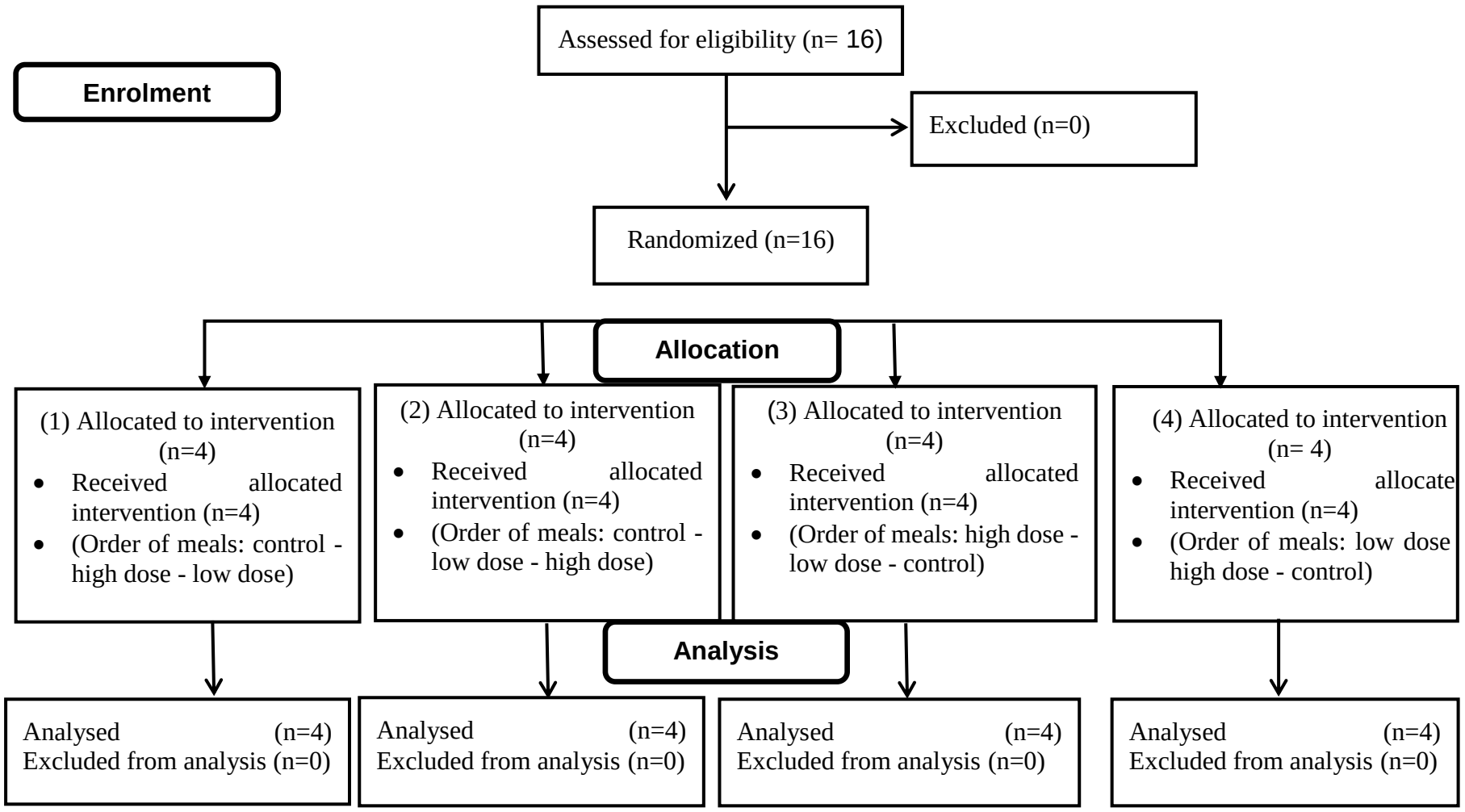


Figure 6-1: Participant enrolment flow diagram showing the randomization of participants in groups for the allocation of the order of meals.

It also shows the number of participants whose plasma glucose readings were used for data analysis.

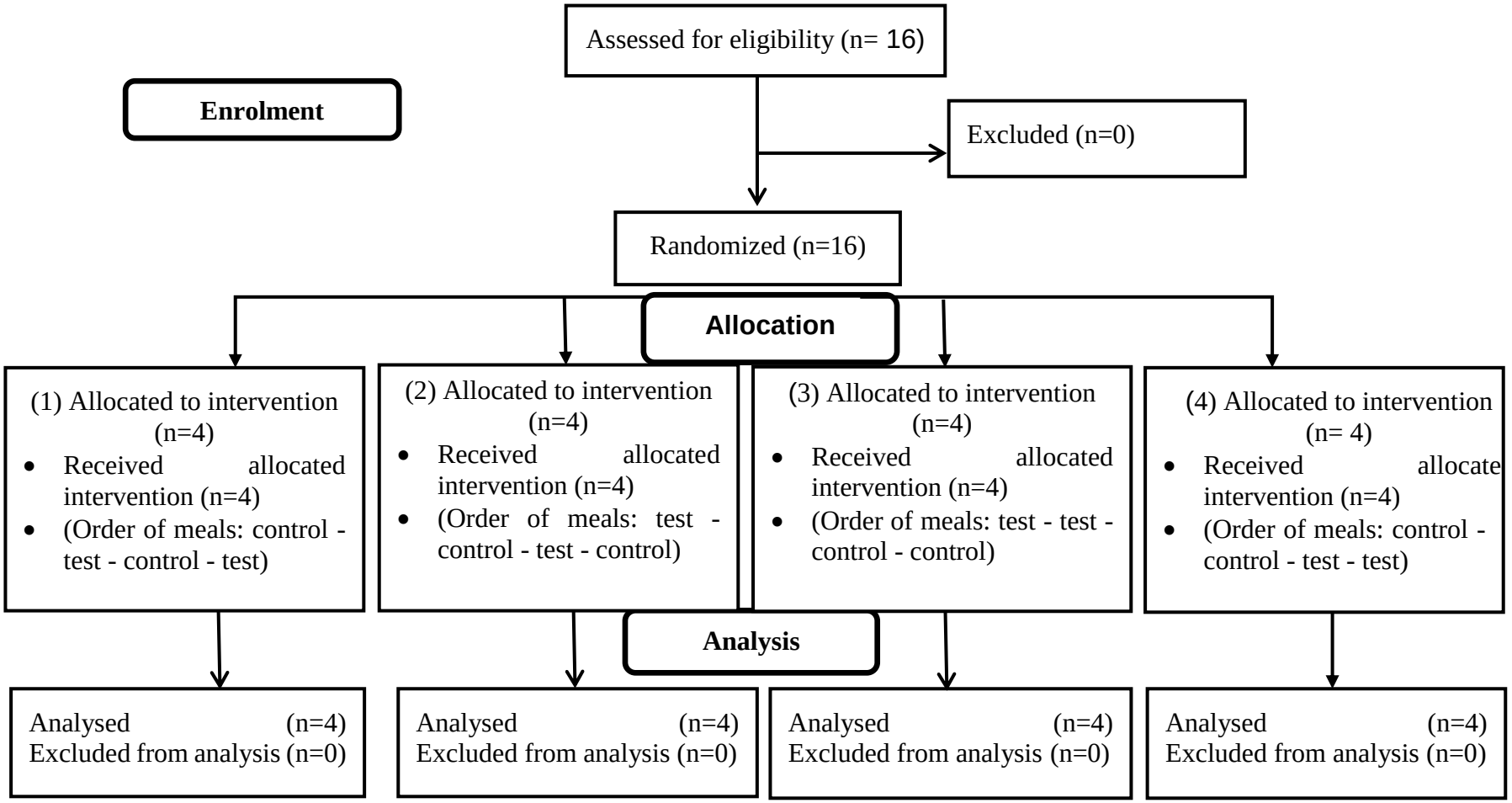


Figure 6-2: Participant enrolment flow diagram showing the randomization of participants in groups for the allocation of the order of meals.

It also shows the total number of participants whose plasma glucose readings were used for data analysis.

6.2.5 Study design

6.2.5.1 Intervention study using pomegranate capsules as source of polyphenols

A randomized, controlled, double blinded crossover intervention was carried out and each volunteer attended three visits; reference (400 mg placebo capsules), test dose 1 (200 mg placebo and 200 mg pomegranate supplement) and test dose 2 (400 mg pomegranate supplement). All meals contained 200 mL drinking water and 109.0 ± 1.2 g white bread (50 g available carbohydrate as analysed by the method of (Englyst *et al*., 1992) and explained in detail in chapter 2.6). Bread was consumed 5 min after taking the supplements to allow for dissolution of polyphenols from the capsules. The meals were administered in a randomized pattern as shown in figure 6-1 and a glucometer was used to instantly measure the blood glucose for each time point.

6.2.5.2 Intervention study using pomegranate juice as source of polyphenols

A randomized, controlled, crossover intervention was conducted and each volunteer had four visits, two of which were reference meals (200 ml drinking water with balancing sugars - 10.4 g glucose and 10.9 g fructose) and two were test meals of the same dose (200 ml pure pomegranate juice). Similarly all meals contained 109.0 ± 1.2 g white bread and were consumed in a randomized pattern as shown in figure 6-2. The bread and juice were consumed simultaneously and a glucometer was used to measure blood glucose concentrations at different time points.

6.2.6 Study protocol

The study protocols MEEC 12-037 (amended) (appendix 2) and MEEC 14-029 (appendix 3) were approved by the University of Leeds Faculty of Mathematics and Physical Sciences (MaPS) and Engineering Ethics Committee (MEEC) for the pomegranate supplements and juice studies respectively. They were registered with ClinicalTrials.gov with ID numbers NCT02486978 and NCT02624609. On each visit, fasting blood glucose was measured with a glucometer after finger prick and afterwards the volunteer consumed the meal and the timer started upon first bite or sip. The volunteers consumed the whole meal in less than 15 min and blood glucose was again measured at 15, 30, 45, 60, 90, 120, 150 and 180 min post meal consumption. Neither harm nor side effects were incurred during the consumption of the meals or during the finger prick and measurement of blood glucose.

6.2.7 Glucose reading by glucometer

6.2.7.1 Principle of the method

Glucose meters or glucometers are medical devices designed for individual use by diabetic's individuals for the determination of glucose concentrations in the blood. The glucometer uses electrochemical test strips to measure the concentration of glucose in the blood. A small drop of blood to be tested is placed on a disposable test strip that is used for the glucose measurement. Each strip contains an enzyme called glucose oxidase which reacts with the glucose in the blood sample to form gluconic acid. Also present in the testing strip is a chemical called ferricyanide which reacts with gluconic acid to form ferrocyanide. Ferrocyanide enables the device to run an electronic current through the blood sample on the strip. The current then reads the ferrocyanide and determines how

much glucose is in the blood sample on the testing strip and displays the concentration on the screen of the glucometer (Louie et al., 2000).

6.2.7.2 Procedure

After the volunteer is seated in a chair, with gloves on, the volunteer's hand was positioned with the palm-side up. The fingertip was cleaned with a disinfection swab by starting in the middle and working outward to prevent contaminating the area and the area was allowed to dry. Thereafter pressure was applied to the selected finger to help the blood to flow. The finger was held and a new sterile lancet/pricking device was placed on the centre of the fingertip and was firmly pressed to puncture the fingertip. The first drop of blood was wiped out with a sterile pad or cotton ball as it may contain excess tissue fluid. Thereafter a drop of blood was correctly placed onto the testing strip (inserted into the glucometer) and the glucose concentration was read and recorded. A cotton wool ball or pad was placed over the puncture site and the volunteer held it for a few minutes to stop any bleeding. If need be, a plaster could be placed afterwards.

6.2.7.3 Glucometer validation

During the intervention study using PFRF mixture (chapter 5), a glucometer was used for blood glucose readings from potential volunteers during the screening exercise. For each volunteer, 3 readings were obtained. Thereafter, each volunteer attended 4 visits and each time fasting blood was collected and later analysed for glucose concentration using the hexokinase assay. The hexokinase assay results and glucometer readings were tabulated and the average of all the fasting blood glucose readings obtained from both the glucometer and hexokinase assay was calculated and standard deviations for each volunteer was approximately ±0.1 which is an acceptable deviation (table 6-1).

Even if the use of a glucometer is a different method to the hexokinase assay for the determination of blood/plasma glucose, due to the fact that they are based on different enzymes (glucose oxidase and hexokinase) the fasting glucose results were comparable as shown in table 6-1 and hence it was decided to use the glucometer in this intervention study.

Volunteer code	**Glucose concentration (mmol/L)**							**Average of all (mmol/L)**	**SD**
	Hexokinase results				Glucometer readings				
1	4.1	4.1	4.3	4.3	4.4	4.3	4.4	4.3	0.058
2	5.5	5.2	5.2	4.8	5.3	5.1	5.2	5.2	0.100
3	4.4	4.2	4.6	5.0	5.3	5.1	5	4.8	0.153
4	4.7	4.0	4.5	5.0	4.9	5	5	4.7	0.058
5	4.3	4.4	4.3	4.2	4.5	4.6	4.6	4.4	0.058
6	5.5	4.8	4.9	4.8	5.4	5.5	5.4	5.2	0.058
7	4.7	4.9	4.7	5.0	5	5.3	5.1	5.0	0.153
8	5.2	4.9	4.9	4.8	5	5	4.9	5.0	0.058
9	4.6	4.7	4.7	4.6	4.7	4.7	4.5	4.6	0.115
10	4.5	4.2	4.5	4.4	4.3	4.5	4.3	4.4	0.115
11	4.7	4.4	4.6	4.7	4.7	4.9	4.9	4.7	0.115
12	4.4	4.0	4.1	4.9	4.3	4.4	4.3	4.3	0.058
13	4.0	4.3	4.8	4.6	4.8	4.8	5	4.6	0.115
14	4.4	4.6	4.6	4.8	4.6	4.8	4.6	4.6	0.115
15	4.2	4.6	4.1	4.2	4.3	4.4	4.2	4.3	0.100
16	4.7	4.8	5.2	4.7	5.1	5.0	5.3	5.0	0.153

Table 6-1: Comparison of fasting blood glucose readings obtained on the same volunteers using two different methods; hexokinase n=4 and glucometer n=3. The average of the combination of all the fasting glucose values obtained from both methods (n=7) was obtained for each volunteer and SD was approximately ±0.1.

6.2.8 Statistical analysis

The trial was designed to have 90 % power to detect a clinical difference of 15 % IAUC between test and reference meal. A power calculation was performed to determine the sample size required to achieve the above conditions. Estimated sample size for two-sample comparison of means: Test Ho: m1 = m2, where m1 is the mean in population 1 and m2 is the mean in population 2.

Assumptions: alpha = 0.05 (two-sided), power = 0.9000, m1 = 200, m2 = 230, sd1 = 25, sd2 = 25, n2/n1 = 1.0. Estimated required sample sizes: n1 = 15, n2 = 15 as explained in detail in 5.2.5.

Since a crossover design was used, a total of 15 participants in total were required since each volunteer consumed both diets (control and test). The trapezoidal rule was used to calculate the incremental area under the glucose curves (IAUC) for each volunteer as described in chapter 2.1.9. Data analysis was performed by the two tailed paired T-test and confirmed with the one factor repeated measures analysis of variance (ANOVA).

6.3 Results

6.3.1 Pomegranate analysis

Both the pomegranate extract and juice were analysed to determine the quantities of their main polyphenols punicalin, punicalagin and ellagic acid (table 6-2).

		Punicalagin	**Punicalin**	**Ellagic acid hexose**	**Ellagic acid**
Juice	Concentration (mg/L)	61.94±0.57	357.25±1.11	14.15±0.10	23.95±0.26
	Concentration in juice (µM)	57	460	30	79
	Per dose in 200 mL juice (g)	12.4	71.5	2.8	4.8
Capsules	Amount (mg/g)	121	6	5.9	101
	Concentration in 200 mL millipore water (µM)	221	15	64	669
	Per high dose of 400 mg capsule (mg)	48	2.4	5.9	40.4

Table 6-2: Major polyphenol content of pomegranate juice and extracts used in the intervention studies. Results are expressed as mean values of three replicates.

The extract and juice were also analysed for sugar contents in order to use the information in the design of the human intervention studies test meals for purposes of balancing the amount of sugars in the test and control meals. Moreover, the information was necessary in order to remove the sugars if present for the enzyme assays as the presence of sugars would interfere with the results (because the end point of the assay measures the amount of maltose or glucose produced for the amylase and glucosidase assay respectively

(chapter 2.7 and 2.8). Both the juice and extract contained glucose and fructose with the amounts in the extract being negligible (table 6-3).

		Glucose	**Fructose**
Juice	Concentration (mg/mL) mean±SD	51.8±0.1	54.7±0.1
	Amount per dose in 200 mL (g) mean±SD	10.357±0.098	10.929±0.091
Capsules	Concentration (mg/mL)	$8.3x10^{-6}$	$6.6x10^{-6}$
	Amount per high dose (400 mg) dissolved in 200 mL millipore water (mg)	$3.3x10^{-6}$	$2.6x10^{-6}$

Table 6-3: Sugar content of pomegranate juice and extracts used in the intervention studies. Pomegranate capsules had very little glucose and fructose and thus was not corrected for in the control meal. Results are expressed as mean values of three replicates.

6.3.2 Inhibition of α-amylase and α-glucosidase activities

Inhibition of human salivary α-amylase and rat α-glucosidase by pomegranate extract was determined and dose-dependently inhibited the digestive enzymes (fig 6-3) α-amylase, maltase and sucrase with IC_{50} values of 0.06±0.01, 1.0±0.1 and 1.2±0.3 mg/ml respectively (table 6-4).

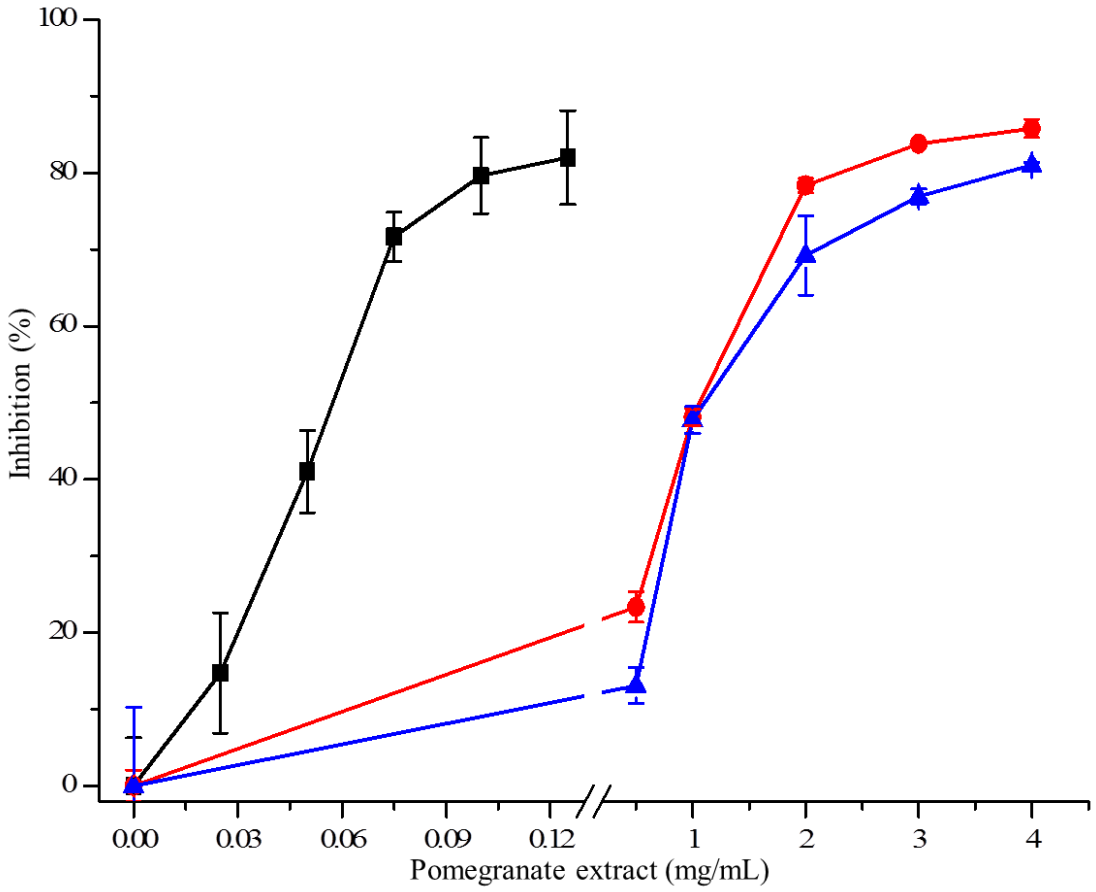


Figure 6-3: Inhibition of human salivary α-amylase (■) using amylose as substrate and rat intestinal α-glucosidase using maltose (●) and sucrose (▲) as substrates by pomegranate extract.

The inhibition potential of the major polyphenols in pomegranate: ellagic acid, punicalin and punicalagin were also determined. Punicalagin inhibited α-amylase with IC_{50} value of 9±1and Ki of 10.1±0.6 µM. Ellagic acid (26.5±0.5%) and punicalin (29.9±0.9 %) slightly inhibited α-amylase at 200 µM (table 6-4). Neither of the major polyphenols showed any inhibition towards α-glucosidase at 200 µM.

Enzyme	IC_{50} (mg/ml)	IC_{50} (µM)		Inhibition at: 50 and 200 µM	
	Pomegranate extract	**Acarbose**	**Punicalagin**	**Punicalin**	**Ellagic acid**
Amylase	0.06 ± 0.01	3.5 ± 0.2	9 ± 1	23.9±2.8 29.9±0.9	27.2±2.8 26.5±0.5
Maltase	1.0 ± 0.1	0.43 ± 0.1	NI	NI	NI
Sucrase	1.2 ± 0.3	12 ± 2	NI	NI	NI

Table 6-4: Experimental IC_{50} values for human salivary α-amylase using amylose as substrates and α-glucosidase using maltose and sucrose as substrates for acarbose and pomegranate extract and its major polyphenols (n=3), NI = no inhibition at 200 µM. Increasing the concentration for punicalin and ellagic acid 4 fold did not increase the inhibition for ellagic acid and only slightly for punicalin which could be indicative of maximum inhibition being attained.

6.3.3 Post-prandial blood glucose – pomegranate supplements

There was no significant difference ($p > 0.05$) between IAUC of the reference, low dose and high dose when pomegranate supplements were given (fig 6-4 and table 6-5) with values of 159 ± 57, 183 ± 87 and 184 ± 61 respectively. The peak glucose concentrations were also not significantly different ($p > 0.05$) with values of 6.8 ± 1, 6.8 ± 0.8 and 6.7 ± 0.9 mmol/L for reference, low dose and high dose respectively (Figure 6-4 and table 6-5).

Intervention study	Test meal	IAUC (mmol/L.min)	Peak glucose (mmol/L)
Pomegranate capsules	**Reference**	159 ± 57	6.8 ± 1.0
	200 mg pomegranate capsules	183 ± 87	6.8 ± 0.8
	400 mg pomegranate capsules	184 ± 61	6.7 ± 0.9

Table 6-5: Average IAUC and peak glucose concentration values for test and control meals for the pomegranate supplements study. All values are mean ± SD, n=3.

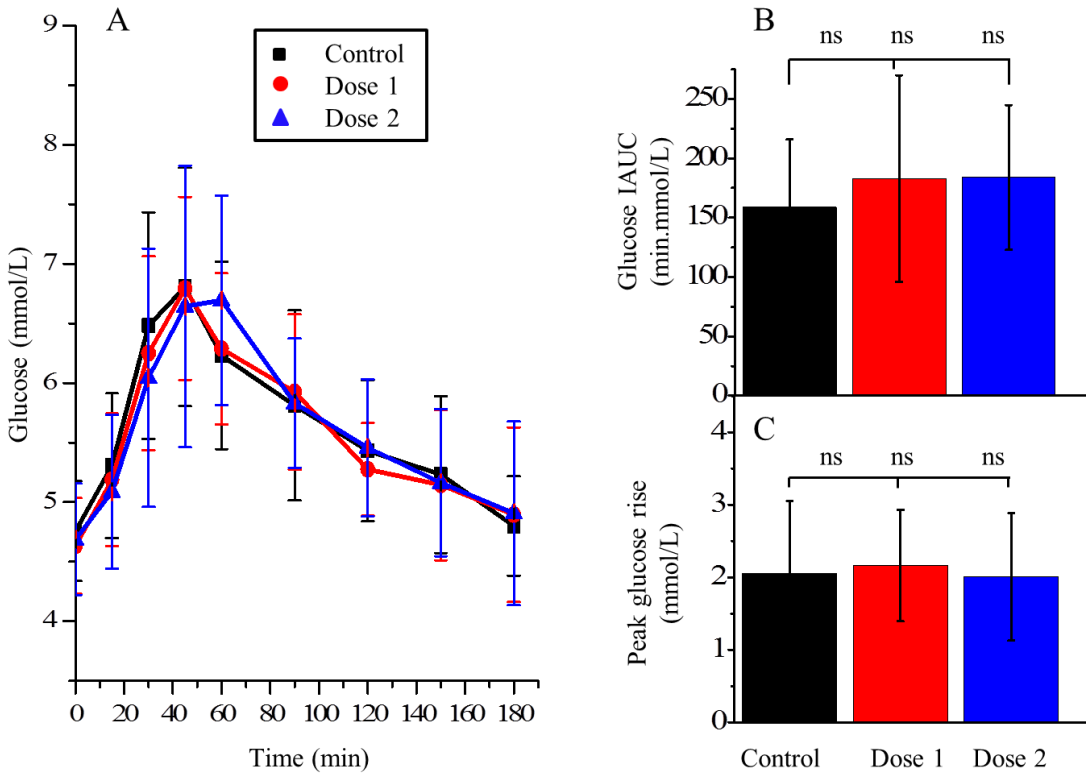


Figure 6-4: Average glucose curves after consumption of control, test meal dose 1 and test meal dose 2 for 16 volunteers (A). All data points represent mean ± S.D, n=16. There is no significance difference (ns) between IAUC of reference meals and test meals (B) as well as between the peak rises in glucose concentration (C).

6.3.4 Post-prandial blood glucose – pomegranate juice

Based on the *in vitro* data showing that punicalagin inhibited α-amylase, the hypothesis that this inhibition would be sufficient to affect the post-prandial response of bread as an added cooked starch source was tested. A randomized, controlled, crossover intervention was conducted on 16 healthy volunteers, and the control and treatment were both performed twice on the same volunteers, making a total of 4 visits for each volunteer. There was a significant difference ($p<0.01$) for both the IAUC and peak glucose concentration between the reference and test meal (fig 6-5 and table 6-6). Pomegranate juice brought about a decrease in the glucose IAUC of -33.1±18.1 % ($p = 0.000005$) n=16). No significant difference was observed between the two control meals, nor between the two test meals ($p>0.05$) (fig 6-5 and table 6-6).

Intervention study	Test meal	IAUC (mmol/L.min)	Peak glucose (mmol/L)
Pomegranate juice	**Reference**	202 ± 61	7.9 ± 1.0
	Reference	196 ± 69	7.8 ± 0.9
	Test (200 mL juice)	136 ± 57	7.2 ± 1.1
	Test (200 mL juice)	131 ± 69	7.2 ± 1.2

Table 6-6: Average IAUC and peak glucose concentration values for the test and control meals for the pomegranate juice study. All values are mean ± SD, n=3.

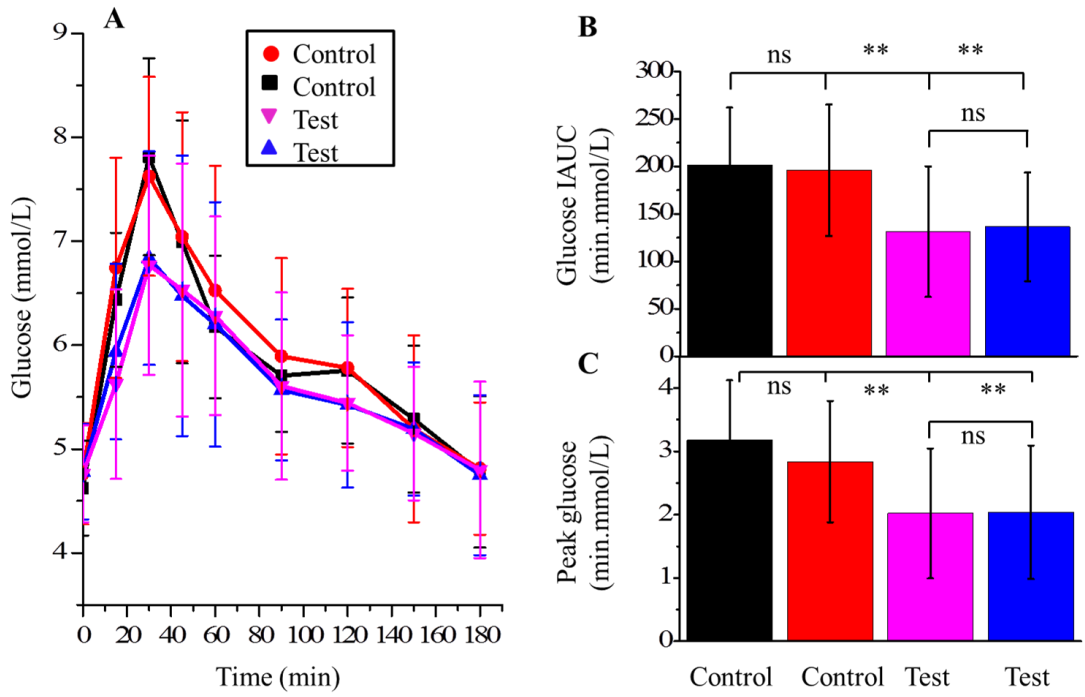


Figure 6-5: Average glucose curves after consumption of control and test meals both consumed on two visits for 16 volunteers (A). All data points represent mean ± S.D, n=16. There is significance difference (**) between IAUC of reference meals and test meals (B) as well as between the peak rises in glucose concentration (C). There is no significance difference (ns) between the two controls meals nor between the two test meals.

6.4 Discussion

Diabetes risk can be reduced by consumption of low glycaemic index foods and unavailable carbohydrates (Livesey *et al*., 2008a). Inhibition of carbohydrate digestive enzymes (α-amylase and α-glucosidase) is one of the potential mechanisms by which this can be achieved, and is the mechanism by which acarbose acts during the treatment of diabetes. Pomegranate juice reduced the glycaemic response in healthy volunteers after its consumption with bread. However there was no significant difference between the IAUC of the test meals (bread, water, low and high dose of pomegranate supplements) and the control meal (bread, water and placebo capsules).

In vitro studies showed that pomegranate polyphenol punicalagin inhibited human salivary α-amylase enzyme and not rat α-glucosidase. The IC_{50} of punicalagin was very low (9 µM) comparable to that of acarbose (3 µM) which showed that it was the major α-amylase inhibitor in pomegranate. Other polyphenols punicalin and ellagic acid also inhibited α-amylase by 29.9±0.9 and 26.5±0.5 % respectively at 200 µM concentration. The concentration of punicalagin in the juice (57 µM) was about six times the IC_{50} concentration and was therefore justified to use the juice in the human intervention study as it was more than the suggested concentrations of at least 3 times more than the IC_{50} value to allow for intestinal dilutions (Williamson, 2013). The total ellagic acid concentration in the juice was 109 µM and it only gave 28 % inhibition at 200 µM *in vitro*. Although punicalin was in the highest concentration in the juice of 460 µM, it only gave 23.9 % at 50 µM and 29.9 % inhibition at 200 µM which indicates a less inhibition despite a fourfold increase of punicalin concentration. Taking into consideration the intestinal dilutions of which the concentration of polyphenols should be at least 3 times more than the IC_{50} value (Williamson, 2013), it can be concluded that ellagic acid and

punicalin contributed little to the inhibition of amylase, hence making punicalagin the major inhibitor. However, although the three major polyphenols (punicalagin, punicalin and ellagic acid) did not inhibit α-glucosidase, the extract did (fig 6-3 and table 6-4). The inhibition could be from other polyphenols in pomegranate or contribution of each polyphenol combined together. Therefore the inhibition of α-glucosidase and α-amylase together contributed to the glucose lowering effects observed in the pomegranate juice intervention study.

Although there was no significant difference in glycaemic response when the capsules were used in the intervention study, pomegranate extract also inhibited human salivary α-amylase with an IC_{50} value of 60 µg/ml, which is more than three times when compared to 2 mg/mL (400 mg in 200ml water) from the capsules, and the same applies to its inhibition of α-glucosidase (fig 6-3 and table 6-5). Moreover, the major human salivary α-amylase inhibitor, punicalagin, was at higher concentration (221 µM) compared to the concentration in the juice (57 µM). These results suggest that the food matrix in which the polyphenols are presented also has a role to play in the inhibition process. The observed differences in the results could be due to two possible reasons; absence of polyphenols in the supplements or unavailability of the polyphenols due to lack of stomach mixing and solubility. The former was ruled out because the amount of the extract and the concentration of punicalagin in the pomegranate capsules as analysed by HPLC were more than three times higher than the *in vitro* IC_{50} values of 60 µg/mL and 9 µM respectively, with the latter being comparable to that of acarbose (3.5 µM) which is a known drug used in diabetes treatment. The solubility or stomach mixing effects of the pomegranate supplements is therefore the most possible reason why no effect of postprandial glucose IAUC was observed because the polyphenols were not present in solution to inhibit the enzymes at the time of starch digestion.

The pomegranate juice intervention study results are in agreement with other studies on the anti-diabetic effects of pomegranate in which different parts (peels, seed, leaves, fruit) of the pomegranate plant have shown anti-diabetic properties with the effects being attributed to the presence of polyphenols (Banihani *et al*., 2013). Different doses of an extract from pomegranate flowers had glucose lowering effects on normal fasted rats and alloxan induced diabetic rats but the authors could not pinpoint the definitive mechanism of action (Jafri *et al*., 2000). They attributed the effects partly to the stimulation of insulin release because they compared the action observed in alloxan (which destroys β-cells) induced diabetic rats to the clinically used tolbutamide drug which lowers glucose levels by stimulating β-cells to release insulin, since the extract had a greater lowering effect compared to the drug tolbutamide. However due to the fact that the glucose lowering effect was also observed in normal rats, they suggested that the extract may also have effects on non-insulin dependent diabetes possibly by inducing increased peripheral utilization of glucose and inhibition of glucose reabsorption in the kidneys (Jafri *et al*., 2000). Pomegranate seeds also dose-dependently significantly reduced glucose levels in rats after 2 and 12 h by 6 and 40 % with a 150 mg/kg dose, and the reduction increased to 7 and 47 % with 300 mg/kg and further increased to 15 and 52 % with 600 mg/kg extract after 2 and 12 h respectively (Das *et al*., 2001). There are a limited number of human intervention studies that have examined the health effects of pomegranate (Basu and Penugonda, 2009, Banihani *et al*., 2013). However, it was shown that pomegranate reduced the level of lipid peroxidation (LPO) and cellular uptake of oxidized-LDL in type 2 diabetes patients which plays an important role in reducing the progression of atherosclerosis which may lead to a heart attack or stroke (Basu and Penugonda, 2009). Another study showed that consumption of 40 g/day of concentrated pomegranate juice for 8 weeks significantly reduced total cholesterol and low density lipoprotein-

cholesterol (Esmaillzadeh *et al*., 2004) which led to a conclusion that consumption of concentrated pomegranate juice could modify heart disease risk factors in diabetic patients with hyperlipidaemia (Esmaillzadeh *et al*., 2004).

Although there was no significant difference in the fasting blood glucose when capillary blood versus venous blood was used, this study however shows that the use of capillary blood as the sampling site for the determination of blood glucose compared to the vein (chapter 5) gives different readings at 180 minutes post meal consumption. This is because some of the glucose is consumed by tissue cells as it passes from the arteries, through the capillary bed and into the veins (Yang, 2012). This is shown in figure 5-3 (venous blood sampling) compared to figures 6-4 and 6-5 (capillary blood sampling). This confirms the earlier discussion in 2.9.8 that different sites for blood sampling may give different glucose concentrations. Readings from the venous blood were below the fasting blood glucose (fig 5-3) compared to readings from the capillary which were almost the same or slightly above the fasting blood glucose (fig 6-4 and 6-5). Moreover, a glucometer tests glucose of whole blood collected from a capillary through a finger prick whereas glucose was determined in plasma from blood collected from the vein which gives a difference in source (capillary versus vein) as well as nature of the samples analysed (whole blood versus plasma). For purposes of comparing results, it is therefore important to use one method of blood sampling site and nature for both the reference and test meals because the primary endpoint was to compare area under the glucose curve for the test meals compared with reference meals. These were maintained in all the intervention studies in this research. Moreover, the first intervention study (chapter 5) was independent to the second and third (chapter 6), hence it is alright that different methods were used. Each method has its own advantages and disadvantages with venous glucose measurement being more demanding as it requires pre-analytical conditions

compared to capillary blood (by use of glucometer) whereas on the other hand, venous blood allows for the determination of other biomarkers including hormones due to higher volumes. This agrees with a study by Yang, 2012 which showed that the fasting capillary and venous glucose concentrations did not differ but that the mean venous concentration was 35 % lower than that of capillary blood glucose postprandial.

6.5 Conclusion

Pomegranate polyphenols have potential anti-diabetic properties as they lowered the glycaemic response in healthy volunteers when the juice was consumed together with a source of starch (bread). The mechanism of action of pomegranate polyphenols is not fully understood (Banihani *et al*., 2013) and we hereby show for the first time that one way by which they infer glucose lowering properties is by the inhibition of the carbohydrate digestive enzymes α-amylase and α-glucosidase. On the other hand, pomegranate extract in the form of capsules did not alter the glycaemic response in healthy humans after consumption with bread. This could be due to delayed solubility and stomach mixing effects of the pomegranate supplements and hence lack of polyphenols at the time of starch digestion to inhibit α-amylase. Therefore the food matrix in which the polyphenols are presented is an important aspect to consider in the production of functional foods.

Chapter 7 . Summary discussion and conclusion

7.1 Research justification and novelty

The hypothesis that polyphenols have the potential to affect postprandial glycaemic response was tested. The potential of polyphenols to have a lowering effect on glucose and insulin after a carbohydrate-rich meal was investigated using a polyphenol and fibre-rich food mixture in the first instance and then with a single source of polyphenols in different food matrices. This property of polyphenols is important in relation to the prevention and management of type 2 diabetes of which high postprandial glucose is a risk factor (Aston, 2006, Livesey *et al*., 2008b, A.D.A, 2015). As type 2 diabetes is on the rise globally (Dunstan *et al*., 2002, Wild *et al*., 2004, Shaw *et al*., 2010), any intervention that can be used to reduce the risk of developing the disease as well as its management in diabetic patients would save governments money to overcome the burden. Drugs such as acarbose are already being used in some countries for the management of type 2 diabetes by acting as inhibitors of carbohydrate digesting enzymes. However, the use of acarbose has side effects such as nausea, flatulence and diarrhoea (Coniff *et al*., 1995, Chiasson *et al*., 2002). Therefore, food based carbohydrase inhibitors like polyphenols would provide a better alternative either on their own or in combination with acarbose to lessen the side effects.

The literature review (chapter 1, table 1-1) shows that different polyphenols inhibit the carbohydrate digesting enzymes α-amylase and α-glucosidase as well as glucose transporters with different inhibition potentials. A number of human intervention studies have been carried out with contradicting outcomes with some studies showing no

reducing effects of polyphenols on postprandial glycaemic response and a few showing an effect as discussed in chapter 5. This could be due to different reasons which mostly could be due to the study designs employed and may include: polyphenol dose, food matrix used and control sample used whether bread, glucose or sucrose as these may all affect the outcome of the study.

In this research, human intervention studies were designed to address the above issues. In the first study (chapter 5) a mixture of polyphenols that have shown inhibitory potential towards different stages of starch digestion (α-amylase and α-glucosidase catalysed hydrolysis and glucose transport) *in vitro* was used as a source of polyphenols. The justification was that if the potential inhibitors of the different stages of starch digestion as shown by *in vitro* studies can be present in the polyphenol-rich mixture at reasonable concentrations (3 times *in vitro* IC_{50} value), they can potentially provide one of the highest possible inhibitions. The most important objective was to establish whether polyphenols have an effect on glycaemic response *in vivo* or not by starting with one of the potential highest possible inhibition due to contradicting results (some studies show an effect whereas some don't show any effects) as discussed in chapter 5. If the highest possible combination confers no effect on glycaemic response, then a conclusion can be proposed that polyphenols have no effect on postprandial glucose *in vivo* when consumed together with a carbohydrate meal even if they inhibit carbohydrate digesting enzymes and glucose transporters *in vitro*. Two different doses of the polyphenol and fibre-rich food (PFRF) mixture were used to determine whether the inhibition was dose-dependent as is usually the case *in vitro*. This information is important for purposes of developing functional foods in order to have information on required dosages for different people. Future plans were to use single sources of polyphenols as well as different food matrices as a way forward in the development/identification of functional foods in the prevention

and treatment of type 2 diabetes. The second and third interventions were thus the use of pomegranate as a single source of polyphenols but in different food matrices (juice and capsules) to determine the effects of different food matrices on the glycaemic response as an important aspect to consider when developing functional foods. Pomegranate was used because it has been used for ages in folk medicine in the treatment of many diseases including type 2 diabetes. A recent review (Banihani *et al*., 2013) indicated that the mechanism of action was not clear and the present research attempted to determine whether inhibition of carbohydrate digesting enzymes was one of the mechanisms that pomegranate polyphenols act in the treatment of type 2 diabetes by carrying out *in vitro* and *in vivo* studies.

Healthy volunteers were used in this research to avoid any risks if the research gave opposite effects which could be risky for diabetes patients if the test meals lead to high glycaemic response. Bread was used in order to test the inhibition of both α-amylase and α-glucosidase because α-amylase catalyses the hydrolysis of starch into disaccharides, oligosaccharides and α-limit dextrin after which α-glucosidases catalyse the hydrolysis of the products of α-amylase catalysed hydrolysis into glucose. For the PFRF mixture study, a mixture of fruits and beverage was based on a portion of fruit (100 g) and a cup of tea (200 mL) as well as the pomegranate juice study (glass of juice, 200 mL). The dose for the pomegranate capsules study was based on the commonly used dose of 2 capsules of 500 mg each for food supplements. In all the studies, the concentrations of polyphenols of interest in the food source was also accounted for to ensure that they were at least 3 times higher than the *in vitro* IC_{50} value. This is an important aspect when *in vitro* studies results are to be used as a basis for *in vivo* studies because there is about 3 times dilution of food *in vivo* due to intestinal fluids produced (Williamson, 2013).

The novelty of this research is that this is the first study to use a mixture of polyphenols (PFRF) mixture which has the potential to inhibit different stages of carbohydrate digestion with the aim to obtain combined effects for maximum inhibition (Nyambe-Silavwe and Williamson, 2016). Comparison of the effect of pomegranate polyphenols on glycaemic response as a single polyphenol source in different food matrices (juice and capsules) is also novel.

7.2 Discussion of outcomes

The major results outcome for this research are *in vitro* carbohydrate digesting enzymes inhibition by polyphenols using the α-amylase (Nyambe-Silavwe *et al*., 2015) and α-glucosidase assays and the corresponding effects on glycaemic response *in vivo* (Nyambe-Silavwe and Williamson, 2016). In addition, the effect of using different food matrices to determine the effect of polyphenols on glycaemic response is also a major outcome of this research (Chapter 6 and publication in preparation).

7.2.1 *In vitro* carbohydrase inhibition

In vitro enzyme inhibition assays are in agreement with the results from the literature which show that different polyphenols have different enzyme inhibition potentials (chapter 1, table 1-1). However, results were very incomparable due to a number of reasons. As discussed by Nyambe-Silavwe *et al*. (2015), this could have been due to many reasons including differences in inhibition methods. For α-amylase using the dinitrosalicylic acid (DNS) detection method, the differences observed were due to type and concentration of substrate or enzyme, reaction time, and whether polyphenols react with DNS or not which results in false results. The α-amylase inhibition assay was optimized as discussed in chapter 2 and in Nyambe-Silavwe *et al*. (2015) in order to

highlight the important aspects to consider in enzyme inhibition assays for purposes of conducting the assays and reporting results in a manner that will enable comparison with the literature. The primary outcome of the optimization was that when reporting inhibition results, all the conditions which were used for the inhibition assays should be recorded and optimized conditions should be used in order to have correct interpretation of the results. Moreover, in cases where polyphenols react with the detection reagent DNS, an additional step should be included just before using DNS to remove polyphenols (solid phase extraction) from the reaction sample. Another important conclusion was that for pure compounds, results should be reported as Ki values instead of IC_{50} values as the Ki values do not depend on the assay conditions but it gives comparable results independent of inhibition mode, substrate and detection method.

From all the polyphenols analysed in this research, EGCG and punicalagin had the highest inhibition towards salivary amylase with IC_{50} values of 5.3±0.6 and 9±1 µM respectively, and are both very comparable to that of acarbose (3.5±0.3 µM). EGCG also gave the highest inhibition with IC_{50} of 14 µM towards α-glucosidase using maltose as substrate. Among the fruit extracts, blackberry had the highest inhibition against salivary α-amylase followed by blackcurrant and strawberry. Green tea was the only PFRF constituent that inhibited α-glucosidase with IC_{50} values of 0.035±0.005, 2.02±0.01 and 2.31±0.02 mg/mL for maltase, iso-maltase and sucrase respectively. Pomegranate extract also showed inhibition towards α-glucosidase with IC_{50} value of 1.0±0.1 and 1.2±0.3 mg/mL for maltase and sucrase respectively. A number of studies have reported the possible mechanisms of action of α-amylase inhibition by polyphenols with the most common being via hydrogen bonding between the hydroxyl groups of polyphenols and the catalytic residues of the binding sites of the enzyme (Tadera *et al*., 2006, Lo Piparo *et al*., 2008, Xiao *et al*., 2013). Computational ligand docking was used to determine the

structure-activity relationships of polyphenols in inhibiting α-amylase (Lo Piparo *et al*., 2008) and the inhibitory activity of flavonoids was attributed to; the presence of hydroxyl groups in particular positions in their structures which results in formation of hydrogen bonds with the enzymes binding site side chains of Asparagin[197] and Glutamine[233] as well as the formation of a conjugated π-system between the indole Tryptophan[59] and the heterocyclic ring (AC) or B-ring of the flavonoids (Lo Piparo *et al*., 2008). Higher inhibition was obtained with galloylated than non galloylated catechins (Xiao *et al*., 2013) as validated in this study with EGCG. Ellagitannins are also said to be good inhibitors if highly hydroxylated and the presesnce of β-galloyl groups at glucose C-1 positions (Xiao *et al*., 2013) as validated in this research by punicalagin. The glycosylation of flavonoids also plays a role in the inhibition potential against amylase depending on the conjugation site and the sugar moiety. Other structural properties that influence inhibition potential include methoxylation and methylation of flavonoids (Lo Piparo *et al*., 2008 and Xiao *et al*., 2013) as this reduces the number of hydrogen bonds that may be formed with the amino acids of the binding site. This agrees with others who also showed that the potency of inhibition correlated with the number of hydroxyl groups on the B ring of the flavonoids (Tadera *et al*., 2006).

7.2.2 Human intervention studies

This research has shown that polyphenols and possibly other components like fibre found in fruits have an effect on glycaemic response in healthy volunteers. The important outcomes that arose from this research include; dose dependence effects (PFRF), food matrix in which the polyphenols are presented (pomegranate polyphenols study) and inter-individual variations (both studies).

7.2.2.1 PFRF as source of polyphenols

The effect of dose on glycaemic response was observed in the intervention study with the higher dose giving a greater decrease in glycaemic response. This supports the proposal that the inhibition of digestive enzymes and glucose transporters by polyphenols contributed highly to the observed reduced glycaemic response when a PFRF was consumed by healthy volunteers together with bread. This is because *in vitro* results showed that different polyphenol concentrations have different inhibition effects on carbohydrate digestive enzymes. The pattern usually follows an inhibition curve where inhibition increases with increasing polyphenol concentration until maximum inhibition is reached, after which an increase in concentration gives no further increase in inhibition as shown in the inhibition curves in the previous chapters (4 and 6).

In the production of functional foods, this information is important in order to avoid the use of either too low a dose will not be enough to give any significant effects as well as too high a dose that does not offer more inhibition potential after maximum inhibition is achieved. Instead, inhibition can be increased by combining different polyphenol sources with different inhibition targets (α-amylase, α-glucosidase or glucose transporters) which was the aim of this study. However, even with the use of a combination of different polyphenols to potentially increase the inhibition outcome, a maximum inhibition is still expected as all individual polyphenols also reach their maximum inhibition. It is also important to ensure that polyphenol sources used show inhibition effects *in vitro* for them to have an effect *in vivo*. For this research all the polyphenol sources used, were in concentrations high enough in concentrations that were at least 3 times higher than the IC_{50} value (Williamson, 2013).

7.2.2.2 Pomegranate as source of polyphenols

This research has shown that the food matrix in which the polyphenols are presented also plays a role in the determination of inhibiting potential of polyphenols on carbohydrate digestion and glucose transport. Polyphenols need to be in a soluble state in order to interact with digestive enzymes and glucose transporters to inhibit their activities. The use of pomegranate polyphenols in the form of capsules did not show any inhibition properties to the digestive enzymes even though the pomegranate extract gave very high inhibition with IC_{50} value (0.06±0.01 mg/mL) and its main inhibitor punicalagin having an IC_{50} value (9 ± 1 µM) very close to that of acarbose (3.5 ± 0.2 µM) *in vitro*. However, the use of pomegranate juice as a source of polyphenols in the determination of their effect on glycaemic response showed a reduced glycaemic response when comparing the test (pomegranate juice and bread) and the control (bread and balancing sugars). The major difference between the two food matrices was that the polyphenols were already in solution in the pomegranate juice compared to polyphenols in the pomegranate capsules. This entails that polyphenols were readily available in the juice to interact with digestive enzymes which they inhibited. The capsules on the other hand did not provide polyphenols readily as the capsules needed to be dissolved first, followed by dissolution of the pomegranate powder inside the capsules which would then be available to inhibit the enzymes. Without the knowledge of how long this process takes place and in which part of the digestive tract, may indicate that the polyphenols where not available at the time they needed to be present in the small intestine.

The use of pomegranate juice also shows that polyphenols reduced the glycaemic response in healthy volunteers after a meal with white bread even in the absence of fibre compared to the PFRF mixture which had some fibre from the fruits which indicates that

polyphenols played a major role and they have the potential to be used as functional foods in the prevention and management of type 2 diabetes with or without fibre.

7.2.2.3 Interpersonal variations

In all the three intervention studies (PFRF mixture, pomegranate capsules and pomegranate juice), there was high variability between volunteers (chapters 5 and 6). This agrees with a recent study (Zeevi *et al*., 2015) which validates the accuracy of this data. Their study reported high variability in glycaemic response after consuming identical meals in an 800 person cohort of healthy and pre-diabetic volunteers in a total of 46,898 meals. However for the repeated tests, there was no intrapersonal variations observed which also agrees with the present study results as shown by lack of significant difference between the repeated meals (reference meals in the PFRF mixture study and both reference and test meals in the pomegranate juice studies) which further validates the accuracy and reproducibility of the present research data. This aspect is important in the formulation of functional foods as some individuals need higher doses compared to others who may get an effect without increasing the dose. The differences could be due to many reasons with individual insulin sensitivity and enzyme concentrations being some of the reasons as discussed in chapter 6. Other possible reasons as reported by (Zeevi *et al*., 2015) include; genetics, glucose transporters activity levels, lifestyle and possibly gut microbiota. Because depending on the baseline microbiota composition of an individual, certain effects are expected such as the Anaerostipes genus being associated with improved glucose tolerance when present in low levels (Everard *et al*., 2011) and both low levels of the Bacteroidetes phylum (Turnbaugh *et al*., 2009) and *Alistipes putredinis* species (Ridaura *et al*., 2013) are associated with obesity which is a risk factor for type 2 diabetes. Lifestyle can affect postprandial glycaemic response by

consistently changing the microbiota composition when consistently consuming bad diet (high sugar and fat) (Zeevi *et al*., 2015).

7.3 Overall conclusion and future perspectives

Polyphenols found in fruits (apple, blackberry, blackcurrant, strawberry) also containing fibre and green tea (PFRF) mixture significantly reduced the glycaemic response of healthy humans by acting in synergy by inhibiting carbohydrate digesting enzymes (α-amylase and α-glucosidase) and glucose transporters. Polyphenols played a major role compared to fibre because when pomegranate juice was used in the intervention study (chapter 6) as a source of polyphenols instead of the PFRF mixture (chapter 5), reduced postprandial blood glucose was also observed despite the absence of fibre.

The use of pomegranate as a single source of polyphenols showed that the food matrix in which the polyphenols are present plays a major role in the observed outcomes as the polyphenols need to be in a soluble state in order to interact with their inhibition targets. Polyphenols have the potential to be used in the prevention and management of type 2 diabetes and functional foods should be developed either on their own or in combination with drugs for this purpose. However lifestyle changes such as weight loss and physical activity also need to be present in order to achieve the benefits. Moreover, due to interpersonal variations observed, personalized doses should be utilized upon determination of individual glycaemic responses which may pose a challenge.

The objectives of this research have been achieved and they are:

- Overall the effects of polyphenols on glycaemic response in healthy volunteers when consumed together with bread (high glycaemic index food) have been determined and polyphenols have a reducing effect on the postprandial blood glucose and insulin.
- Specifically, the PFRF mixture containing sources of polyphenols with the potential to inhibit different stages of carbohydrate digestion produced a significant decrease on the postprandial blood glucose and insulin.
- Increasing the dose of polyphenol sources in the test meals gave lower postprandial blood glucose compared to the first dose in healthy volunteers.
- Food matrix in which the polyphenols are present in the polyphenol source play a role in the glycaemic response observed.

On the basis of the results of this research, more research should be carried out with different combinations of polyphenol sources as well as single sources to have a variety of combinations for the development of functional foods. Most importantly, future intervention studies should include pre-diabetic and diabetic volunteers to determine whether the observed beneficial effects of polyphenols on glycaemic response in healthy volunteers would be the same in the named groups that need the interventions the most. Although no side effects were observed in healthy volunteers, more studies on healthy volunteers need to be done to ensure their safety in pre-diabetic and type 2 diabetic volunteers.

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List of appendixes

APPENDIX 1

UNIVERSITY OF LEEDS

UNIVERSITY OF LEEDS RESEARCH ETHICS COMMITTEE APPLICATION FORM [1]

Please read each question carefully, taking note of instructions and completing all parts. If a question is not applicable please indicate so. The superscripted numbers refer to sections of the guidance notes, available at www.leeds.ac.uk/ethics. Where a question asks for information which you have previously provided in answer to another question, please just refer to your earlier answer rather than repeating information.

Research ethics training courses: http://www.sddu.leeds.ac.uk/sddu-research-ethics-courses.html

To help us process your application enter the following reference numbers, if known and if applicable:

Ethics reference number:	**MEEC 12-037**
Grant reference and/ or student number:	**200358739**

PART A: Summary

A.1 Which Faculty Research Ethics Committee would you like to consider this application?[2]

- ○ Arts and PVAC (PVAR)
- ○ Biological Sciences (BIOSCI)
- ○ ESSL/ Environment/ LUBS (AREA)
- ◉ MaPS and Engineering (MEEC)
- ○ Medicine and Health (Please specify a subcommittee):
  - ○ Leeds Dental Institute (DREC)
  - ○ Health Sciences/ LIGHT/ LIMM
  - ○ School of Healthcare (SHREC)
  - ○ Medical and Dental Educational Research (EdREC)
  - ○ Institute of Psychological Sciences (IPSREC)

A.2 Title of the research[3]

Effects of polyphenols on carbohydrase-inhibiting polyphenols on glycaemic response.

A.3 Principal investigator's contact details[4]	
Name *(Title, first name, surname)*	Ms Hilda Nyambe
Position	Provisional PhD Student
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	MAPS
Work address *(including postcode)*	School of Food Science and Nutrition, University of Leeds, LS2 9JT
Telephone number	07415780195

University of Leeds email address	fs07hs@leeds.ac.uk

A.4 Purpose of the research:[5] (Tick as appropriate)

- ☑ Research
- ☑ Educational qualification: ***Please specify: __Doctor of Philosophy_____***
- ☐ Educational Research & Evaluation[6]
- ☐ Medical Audit or Health Service Evaluation[7]
- ☐ Other

A.5 Select from the list below to describe your research: (You may select more than one)

- ☑ Research on or with human participants
- ☐ Research with has potential significant environmental impact.[8] If yes, please give details:

  _____

- ☐ Research working with data of human participants
  - ☐ New data collected by questionnaires/interviews
  - ☐ New data collected by qualitative methods
  - ☐ New data collected from observing individuals or populations
  - ☐ Research working with aggregated or population data
  - ☐ Research using already published data or data in the public domain
- ☐ Research working with human tissue samples[9]

A.6 Will the research involve any of the following:[10] (You may select more than one)

If your research involves any of the following an application must be made to the National Research Ethics Service (NRES) via IRAS www.myresearchproject.org.uk as NHS ethical approval will be required. There is no need to complete any more of this form. Contact governance-ethics@leeds.ac.uk for advice.

- ☐ Patients and users of the NHS (including NHS patients treated in the private sector)[11]
- ☐ Individuals identified as potential participants because of their status as relatives or carers of patients and users of the NHS
- ☐ Research involving adults in Scotland, Wales or England who lack the capacity to consent for themselves[12]
- ☐ A prison or a young offender institution in England and Wales (and is health related)[14]
- ☐ Clinical trial of a medicinal product or medical device[15]
- ☐ Access to data, organs or other bodily material of past and present NHS patients[9]
- ☐ Use of human tissue (including non-NHS sources) where the collection is not covered by a Human Tissue Authority licence[9]
- ☐ Foetal material and IVF involving NHS patients
- ☐ The recently deceased under NHS care
- ☑ None of the above

You must inform the Research Ethics Administrator of your NRES number and approval date once approval has been obtained.

If the University of Leeds is not the Lead Institution, or approval has been granted elsewhere (e.g. NHS) then you should contact the local Research Ethics Committee for guidance. The UoL Ethics Committee need to be assured that any relevant local ethical issues have been addressed.

A.7 Will the research involve NHS staff recruited as potential research participants (by virtue of their professional role) or NHS premises/ facilities?

☐ Yes ☑ No

If yes, ethical approval must be sought from the University of Leeds. Please note that NHS R&D approval is needed in addition, and can be applied for concurrently: www.myresearchproject.org.uk.

Contact governance-ethics@leeds.ac.uk for advice.

A.8 Will the participants be from any of the following groups? (Tick as appropriate)

- ☐ Children under 16[16]
- ☐ Adults with learning disabilities[12]
- ☐ Adults with other forms of mental incapacity or mental illness
- ☐ Adults in emergency situations
- ☐ Prisoners or young offenders[14]
- ☑ Those who could be considered to have a particularly dependent relationship with the investigator, eg members of staff, students[17]
- ☐ Other vulnerable groups
- ☐ No participants from any of the above groups

Please justify the inclusion of the above groups, explaining why the research cannot be conducted on non vulnerable groups.

Since adverts will be put around the University campus notice boards, it is very likely that students and members of staff may be recruited for the study as they are normally around the University campus. This will make it convenient to use students or members of staff as there will be no need to travel to get to the School of Food Science and Nutrition as they will already be on campus for work or for studies.

However, participation will be purely voluntary and the participants will not be approached directly or forced to take part. If they so wish, they can withdraw from participating at any time.

A Criminal Record Bureau (CRB) check will be needed for researchers working with children or vulnerable adults (see www.crb.gov.uk and http://store.leeds.ac.uk/browse/extra_info.asp?modid=1&prodid=2162&deptid=34&compid=1&prodvarid=0&catid=243)

A.9 Give a short summary of the research[18]

*This section must be completed in **language comprehensible to the lay person**. Do not simply reproduce or refer to the protocol, although the protocol can also be submitted to provide any technical information that you think the ethics committee may require. This section should cover the main parts of the proposal.*

Consumption of carbohydrate containing foods or sugary drinks brings about changes to the blood glucose levels. After a meal or drink, blood glucose levels rise until it reaches a peak concentration usually after 30 minutes. When the body senses the increase in blood glucose, a hormonal process involving insulin takes place to ensure that the glucose is taken up from the blood for storage and where it is needed for energy in the body. This process then brings about a decrease in the concentration of glucose until it reaches approximately the starting concentration. The original concentration of glucose is attained approximately 2 hours after eating or drinking a carbohydrate food or sugary drink respectively.

Different carbohydrates and sugary drinks have different effects on blood glucose response depending on the amount as well as the type of carbohydrate. Those that give rise to a high glucose response compared to a reference carbohydrate (usually glucose) are said to be high glycaemic index (GI) foods and those with a lower glucose response compared to a reference carbohydrate (usually glucose) are said to be low glycaemic index (GI) foods (Jenkins *et al*., 2002).

Research has shown that diets that give rise to a high glucose response are associated with a number of abnormalities like increased metabolic syndrome (Aston, 2006). Metabolic syndrome mostly comprises of insulin resistance and glucose intolerance which gives an increased risk of type 2 diabetes (McKeown *et al*., 2004) It also gives rise to other conditions like high blood pressure (arterial hypertension), elevated blood insulin levels (hyper-insulinemia), elevated amounts of fat in the liver (fatty hepatosis) and elevated amounts of lipids in the blood (dyslipidemia). After type 2 diabetes become clinically apparent, the risk of cardiovascular disease also rises (Beilby, 2004). Research has also shown that foods/drinks which raise blood glucose levels gradually (low GI) rather than rapidly (high GI) have health benefits which include reducing the risk of metabolic syndrome (Barclay *et al*., 2008). In vitro studies have shown that polyphenols found in fruits, vegetables and plant based foods have a positive effect on carbohydrate metabolism and can lower the blood glucose levels (Hanhineva et al., 2010).

This research will determine whether the presence of polyphenols in the diet has any lowering effect on the blood glucose levels and hence the glycaemic index of foods. This will be determined by asking volunteers to consume polyphenol rich drink/food together with white bread and determine the glycaemic response. The GI of bread will be determined initially as a reference.

Analysis will be done by measuring blood glucose response to white bread alone as reference and then to white bread with test sample containing polyphenols and then determine GI and see how the GI of bread will be affected. Other analyses to be done are plasma insulin, glucagon, gastric inhibitory polypeptide (GIP) and glucagon like peptides-1 (GLP-1) as they all relate to glycaemic response.

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A.10 What are the main ethical issues with the research and how will these be addressed?[19]

Indicate any issues on which you would welcome advice from the ethics committee.

Participation of human volunteers addressed by informed consent

Prior to commencement of the study, potential volunteers will be given a clear, concise Participant Information Sheet (appendix 1) and Informed Consent Form (appendix 2) by the investigator to read, complete and sign in their own time. The investigator will answer all questions to the satisfaction of the individual and will not influence the decision of taking part in the study. The participants will be free to withdraw from the study at any point without providing a reason. The written consent form will be signed and dated at least 2 days prior to enrolment in the study by the volunteers and the Investigator. It will be photocopied and one copy will be kept by the Investigator (for the study records) and one by the volunteer. Participation by the volunteers is conditional upon receiving the written consent by the volunteer.

Gathering of personal data addressed by confidentiality and data protection

Candidates will be asked to complete a questionnaire (appendix 3) in order to assess their suitability for the study. The information pertains to their lifestyle and may be considered a sensitive issue, however each volunteer will be assured of confidentiality and that participation is entirely voluntary. The information on the questionnaire will include height, weight, gender, age and relevant medical history.

Confidentiality of all participants' information will be maintained. Identification will be coded using random ID numbers that will be assigned on the day of study. All data collected will be treated as confidential and stored securely in a locked filling cabinet according to current University regulations. The linkage between individual identity and ID number will be kept in written form only and stored in a locked filling cabinet in a restricted access area, thus it will not be possible to identify individual participants from the ID numbers. Data evaluation will only be performed using ID numbers. In accordance with the University guidelines on the password protected M-drive of the University server, anonymised data will be stored for at least 5 years.

Blood sample collection addressed by presence of experienced nurse

To minimise any risk linked to venous blood collection, an experienced nurse will be present during the study to draw the venous blood (4 ml) samples from the elbow pit using a cannula. The use of a cannula will avoid the repeated insertion of a needle at every withdrawal. Capillary blood samples (200 µl) will be collected from participants by means of a finger prick using a sterile puncture device designed for this purpose for each time point. The finger prick protocol is the WHO/FAO 1998 glycaemic index approved protocol. Moreover research has shown that for blood glucose measurements, fingertip capillary blood is recommended as it is sensitive to changes in blood glucose concentrations compared to other sites. This is crucial due to the nature of the study design as blood will be collected at different times and hence we aim to get the real blood glucose concentration for each time point. It has been reported that the blood glucose concentrations are higher in the fingertip capillary than in other sites when blood glucose rise. In the same way when blood glucose concentrations fall, the concentrations in the fingertip capillary is lower than in other sites (Ellison, *et al*., 2002 and Van Der Valk, *et al*., 2002). Moreover, there is controversy on this issue as other researchers like Yang *et al*., 2012 have reported that the venous blood is better

for glucose analysis. We therefore aim to compare the results as well so that we can contribute to the knowledge about this issue.

For finger prick, since 7 points will be needed (0, 15, 30, 45, 60, 90, 120) thus we will aim to prick one finger once per session. An allowance of only two visits per week will also help in the healing of the pricks as well as one week rest after the 2 visits/ before the last 2 visits.

The analyses of the blood samples will be blood glucose, insulin, glucagon, GIP and GLP-1 concentrations. Blood collected from the participants will be rendered acellular within 1 hour of collection before storage. For the standard protocol being used for removing cellular debris, see Appendix 4.

Ethics Committee Approval

This human study protocol will be submitted to the Faculty of Mathematics and Physical Sciences Ethics committee at the University of Leeds, UK.

References

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PART B: About the research team	
B.1 To be completed by students only[20]	
Qualification working towards (eg Masters, PhD)	PhD
Supervisor's name (Title, first name, surname)	Professor Gary Williamson
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	MAPS
Work address (including postcode)	School of Food Science & Nutrition, University of Leeds, LS2 9JT
Supervisor's telephone number	0113 343 8380
Supervisor's email address	g.williamson@leeds.ac.uk
Module name and number (if applicable)	

B.2 Other members of the research team (eg co-investigators, co-supervisors) [21]	
Name *(Title, first name, surname)*	
Position	
Department/ School/ Institute	
Faculty	
Work address *(including postcode)*	
Telephone number Email address	

Part C: The research

C.1 What are the aims of the study?[22] (Must be in language comprehensible to a lay person.)

1. To determine the effects of polyphenol rich mixture/food on glycaemic index and glycaemic response.
2. To determine the effect of different doses of polyphenol rich mixture on postprandial plasma glucose, insulin, glucagon, GIP and GLP-1 responses of healthy humans and humans with metabolic syndrome.

C.2 Describe the design of the research. Qualitative methods as well as quantitative methods should be included. (Must be in language comprehensible to a lay person.)

It is important that the study can provide information about the aims that it intends to address. If a study cannot answer the questions/ add to the knowledge base that it intends to, due to the way that it is designed, then wasting participants' time could be an ethical issue.

Subject preparation

Unusual vigorous activity should be avoided and subjects should eat a meal of their choice the evening before the test and should eat the same meal the day before each test.

Study Design

A minimum of 10 subjects per group will be recruited most likely 12 participants will be recruited. The first group will be healthy individuals and the second will be those with metabolic syndrome. The study will involve 4 visits with each visit not lasting more than 4 hours.

Subjects will be expected to arrive at the school of food science and nutrition in the morning before 10 am after an overnight fast of between 10-14 hours. They will be asked to rest for 10 minutes during which they can report on what they had the previous evening prior to the fast. Body weight, height and blood pressure measurements will also be taken. Thereafter, a cannula will be inserted by a nurse in the participants elbow pit to avoid the use of a needle for every blood withdrawal. After the participant rests for another 5 minutes, the fasting capillary and venous blood samples will be collected.

The subject will then be asked to eat or drink the food test sample as shown in the appendix 5. Then about 200 µl capillary blood and 4 ml venous blood will be drawn at 15, 30, 45, 60, 90, 120, 150 and 180 minutes post consumption of the test food.

The Latin square design will be used to randomize which test food will be consumed by which volunteer on different days as shown in appendix 5. However the first and last visit will be the same for everyone as it will be the reference test (bread, natural yoghurt and water).

Sample analysis

For glucose analysis, blood samples will be collected in glucose preservation fluoride/oxalate tubes and placed on ice until they are centrifuged within 30 minutes. Centrifugation will be done at 3,500g at 4°C for 10 minutes. The acellular plasma fraction will be collected and stored in micro centrifuge tubes at -80°C until required for analysis. The hexokinase assay (commercially available kit and is commonly used) will be used to determine glucose concentration in the plasma samples. The cells in the cellular fraction will be destroyed by treating them with a disinfectant using the guidelines from the manufacturer.

For the analysis of insulin, glucagon, GIP, GLP-1, blood samples will be collected in heparinised tubes and immediately placed on ice until they are centrifuged within 30 minutes. Centrifugation will be done at 3,500g at 4°C for 10 minutes. The acellular plasma fraction will be collected and stored in micro centrifuge tubes at -80°C until required for analysis. Elisa assay kits (commercially available kits) will be used for plasma insulin, glucagon, GIP and GLP-1 measurements.

Statistical analysis

For each time point, blood glucose, insulin, glucagon, GIP and GLP-1 concentration will be analysed to show whether there is any significant difference with that of the reference by using the Students T-test. This will also be done for the different study groups.

Polyphenol analysis

The amount of polyphenols present in the test foods will be analysed by the Folin-Ciocalteu assay and by high performance liquid chromatography (HPLC)

C.3 What will participants be asked to do in the study?[23] *(e.g. number of visits, time, travel required, interviews)*

What will participants be asked to do in the study

Participants will be asked to complete a pre study questionnaire (appendix 3) to determine their suitability for the study as well as have their fasting glucose checked using a glucometer. Then if they qualify, they will be asked to complete the consent form. (Appendix 2)

The study is to be done over 4 visits with each visit not lasting more than 4 hours. Preferably twice a week according to which days are convenient for the volunteer but with at least one day in between the two visits. With one week washout period in between the two weeks, gives a total of three weeks.

Subjects will be expected to arrive at the school of food science and nutrition in the morning before 10 am after an overnight fast of between 10-14 hours. The participants will be asked to have a meal of their choice the night before the study day but should consume the same meal on all the days and a record of what they eat should be availed. They will be asked to rest for 10 minutes during which they can report on what they had the previous evening prior to the fast. Body weight, height and blood pressure measurements will also be taken. Thereafter, a cannula will be inserted by a nurse in the participants elbow pit to avoid the use of a needle for every blood withdrawal. After the participant rests for another 5 minutes, fasting venous and capillary blood samples will be collected.

The subject will then be asked to eat or drink the food test sample as shown in the appendix 6. Then about 200 µl capillary blood and 4 ml venous blood will be drawn at 15, 30, 45, 60, 90, 120, 150 and 180 minutes post consumption of the test.

C.4 Does the research involve an international collaborator or research conducted overseas:[24]

(Tick as appropriate)

☐ Yes ☑ No

If yes, describe any ethical review procedures that you will need to comply with in that country:

Describe the measures you have taken to comply with these:

Include copies of any ethical approval letters/ certificates with your application.

C.5 Proposed study dates and duration

Research start date (DD/MM/YY): As soon as ethical approval is obtained.

Research end date (DD/MM/YY): 31/09/2016

Fieldwork start date (DD/MM/YY):

Fieldwork end date (DD/MM/YY):

C.6. Where will the research be undertaken? (i.e. in the street, on UoL premises, in schools)[25]

On University of Leeds premises.

RECRUITMENT & CONSENT PROCESSES

How participants are recruited is important to ensure that they are not induced or coerced into participation. The way participants are identified may have a bearing on whether the results can be generalised. Explain each point and give details for subgroups separately if appropriate.

C.7 How will potential participants in the study be:

(i) identified?

Codes will be used to identify individual subjects and no personal details will be referred to in this study as reported in A10. Personal details will only be used to check suitability at the recruitment stage.

During the first contact with the potential participant, the main investigator will assign a code number to the participant which will be used to identify the participant throughout the study. An intermediate person who will never have any contact with the participants will be matching the names and give the corresponding codes to the Investigator.

(ii) approached?

Advertisements (appendix 6) in the form of posters and emails to the staff and students in the school of Food Science and Nutrition as well as other parts of the University of Leeds campus will be used.

(iii) recruited?[26]

Subjects will be recruited from the university of Leeds staff and Students. Written informed consent will be obtained from the volunteers and participation can be withdrawn at any time without giving a reason.

C.8 Will you be excluding any groups of people, and if so what is the rationale for that?[27]

Excluding certain groups of people, intentionally or unintentionally may be unethical in some circumstances. It may be wholly appropriate to exclude groups of people in other cases

Selection criteria is shown in **appendix**

Group 1 – Healthy subjects

Not diabetic
Fasting glucose (blood glucose level before breakfast) 3.9-5.9 mmol/L
Not on long term prescribed medication (except contraceptives)
Not pregnant or lactating
Not on special diet (for losing weight or fruit extracts supplements)
Aged 18-75

Group 2 – Metabolic syndrome risk group

Not diabetic
With fasting glucose (blood glucose level before breakfast) in the range of 6.0 – 8.0mmol/l
Not on long term prescribed medication (except contraceptives)
Not pregnant or lactating
Not on special diet (for losing weight or fruit extracts)
Aged 18-75

C.9 How many participants will be recruited and how was the number decided upon?[28]

It is important to ensure that enough participants are recruited to be able to answer the aims of the research.

According to a review on Glycaemic index methodology by Brouns et al., 2005, which looked at a number of papers on glycaemic index methodology, the conclusion was

that a minimum of 10 subjects is required for such type of study for each group and so will use 12-15 to allow for volunteers who may discontinue.

From experience in the school from previous human studies, the number suggested above is achievable and in the rare case that it's not achieved, recruitment will continue until the required number is obtained as the study is only 3 weeks in total per participant and so can be done at any time of the recruitment process.

If you have a formal power calculation please replicate it here.

Remember to include all advertising material (posters, emails etc) as part of your application

C10 Will the research involve any element of deception?[29]

If yes, please describe why this is necessary and whether participants will be informed at the end of the study.

No

C.11 Will informed consent be obtained from the research participants?[30]

☑ Yes ☐ No

If yes, <u>give details</u> of how it will be done. Give details of any particular steps to provide information (in addition to a written information sheet) e.g. videos, interactive material. If you are not going to be obtaining informed consent you will need to justify this.

Volunteers will receive a participant information sheet and informed consent form after a positive reply to the recruitment advertisement. Volunteers will consent in writing prior to commencement of the study. The main investigator will verbally explain the participant information sheet and informed consent form and questions from the volunteer will be answered at any time. The prospective participant will have 1-2 days prior to commencement of the study to decide, sign and return the completed informed consent form (two copies where one copy remains with the participant and the other copy for the research records) both to be co-signed by the main investigator.

If ***participants are to be recruited from any of potentially vulnerable groups, <u>give details of extra steps</u> taken to assure their protection. Describe any arrangements to be made for obtaining consent from a legal representative.***

Copies of any written consent form, written information and all other explanatory material should accompany this application*. The information sheet should make*

explicit that participants can withdraw from the research at any time, if the research design permits.

Sample information sheets and consent forms are available from the University ethical review webpage at http://researchsupport.leeds.ac.uk/index.php/academic_staff/good_practice/planning_your_research_project-1/approaching_and_recruiting_participants-1.

C.12 Describe whether participants will be able to withdraw from the study, and up to what point (eg if data is to be anonymised). If withdrawal is not possible, explain why not.

Volunteers will be informed that they can withdraw from the study at any time without giving any reason and without any negative consequences.

C.13 How long will the participant have to decide whether to take part in the research?[31]

It may be appropriate to recruit participants on the spot for low risk research; however consideration is usually necessary for riskier projects.

2 days prior to commencement of the study.

C.14 What arrangements have been made for participants who might not adequately understand verbal explanations or written information, or who have special communication needs?[32] *(e.g. translation, use of interpreters etc. It is important that groups of people are not excluded due to language barriers or disabilities, where assistance can be given.)*

Participants will be expected to understand the English language. Email and phone contact details will be provided on the recruitment advert and information sheet.

C.15 Will individual or group interviews/ questionnaires discuss any topics or issues that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could take place during the study (e.g. during interviews or group discussions)?[33] *The information sheet should explain under what circumstances action may be taken.*

☐ Yes ☑ No *If yes, give details of procedures in place to deal with these issues.*

C.16 Will individual research participants receive any payments, fees, reimbursement of expenses or any other incentives or benefits for taking part in this research?[34]

☑ Yes ☐ No

If Yes, please describe the amount, number and size of incentives and on what basis this was decided.

Participants will receive breakfast/snack after collection of blood (3 hours after consumption of test food) if they wish. A monetary reward of £15 for each visit will be given at the end of the study.

RISKS OF THE STUDY

C.17 What are the potential benefits and/ or risks for research participants?[35]

There is very low risk to the participants as the method used is a World Health Organisation (WHO) approved and very common method. Moreover, a nurse will be present to draw the blood. Breakfast will be provided after blood collection. There are no direct benefits to the participants although the consumption of polyphenol rich test samples may have beneficial effects to the participants who may not normally have them in their diets. Moreover the results of the study may help to add to the knowledge on how diet may affect health.

C.18 Does the research involve any risks to the researchers themselves, or people not directly involved in the research? *Eg lone working*[36]

☐ Yes ☑ No

If yes, please describe:

_____

Is a risk assessment necessary for this research?

NB: Risk assessments are a University requirement for all fieldwork taking place off campus. For guidance contact your Faculty Health and Safety Manager or visit http://www.leeds.ac.uk/safety/fieldwork/index.htm.

☐ Yes ☑ No If yes, please include a copy of your risk assessment form with your application.

DATA ISSUES

C.19 Will the research involve any of the following activities at any stage (including identification of potential research participants)? (Tick as appropriate)

☐ Examination of personal records by those who would not normally have access

☐ Access to research data on individuals by people from outside the research team

☐ Electronic transfer of data

☐ Sharing data with other organisations

☐ Exporting data outside the European Union

☑ Use of personal addresses, postcodes, faxes, e-mails or telephone numbers

☐ Publication of direct quotations from respondents

☐ Publication of data that might allow identification of individuals to be identified

☐ Use of audio/visual recording devices

☐ FLASH memory or other portable storage devices

Storage of personal data on or including any of the following:

☐ Manual files

☐ Home or other personal computers

☐ Private company computers

☐ Laptop computers

C.20. How will the research team ensure confidentiality and security of personal data? E.g. anonymisation procedures, secure storage and coding of data.[37] You may wish to refer to the data protection and research webpage.

See the answer explained in question A.10

C.21 For how long will data from the study be stored? Please explain why this length of time has been chosen.[38]

RCUK guidance states that data should normally be preserved and accessible for ten years, but for some projects it may be 20 years or longer.

***Students**: It would be reasonable to retain data for at least 2 years after publication or three years after the end of data collection, whichever is longer.*

5 years

CONFLICTS OF INTEREST

C.22 Will any of the researchers or their institutions receive any other benefits or incentives for taking part in this research over and above normal salary or the costs of undertaking the research?[39]

☐ **Yes** ☑ **No**

If yes, indicate how much and on what basis this has been decided

_____

C.23 Is there scope for any other conflict of interest?[40] *For example will the research funder have control of publication of research findings?*

☐ Yes ☑ No ***If yes, please explain*** _____

C.24 Does the research involve external funding? (Tick as appropriate)

☐ Yes ☑ No ***If yes, what is the source of this funding?*** _____

PART D: Declarations

Declaration by Chief Investigators

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.
2. I undertake to abide by the University's ethical and health & safety guidelines, and the ethical principles underlying good practice guidelines appropriate to my discipline.
3. If the research is approved I undertake to adhere to the study protocol, the terms of this application and any conditions set out by the Research Ethics Committee.
4. I undertake to seek an ethical opinion from the REC before implementing substantial amendments to the protocol.
5. I undertake to submit progress reports if required.
6. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer.
7. I understand that research records/ data may be subject to inspection for audit purposes if required in future.
8. I understand that personal data about me as a researcher in this application will be held by the relevant RECs and that this will be managed according to the principles established in the Data Protection Act.
9. I understand that the Ethics Committee may choose to audit this project at any point after approval.

Sharing information for training purposes: Optional – please tick as appropriate:

☐ I would be content for members of other Research Ethics Committees to have access to the information in the application in confidence for training purposes. All personal identifiers and references to researchers, funders and research units would be removed.

Principal Investigator

Signature of Principal Investigator: ..... (This needs to be an actual signature rather than just typed. Electronic signatures are acceptable)

Print name: HILDA NYAMBE Date: (dd/mm/yyyy): 15/08/2013

Supervisor of student research: I have read, edited and agree with the form above.

Supervisor's signature: ..... (This needs to be an actual signature rather than just typed. Electronic signatures are acceptable)

Print name: G. WILLIAMSON Date: (dd/mm/yyyy): 15-8-13

Please submit your form **by email** to J.M.Blaikie@leeds.ac.uk or if you are in the Faculty of Medicine and Health FMHUniEthics@leeds.ac.uk. **Remember to include any**

supporting material such as your participant information sheet, consent form, interview questions and recruitment material with your application.

Checklist:

- ❑ I have used layman's terms to describe my research (applications are reviewed by lay members of the committee as well).
- ❑ I have answered all the questions on the form, including those with several parts (refer to the guidance if you're not sure how to answer a question or how much detail is required)
- ❑ I have included any relevant supplementary materials such as
  - ❑ Recruitment material (posters, emails etc)
  - ❑ Sample participant information sheet
  - ❑ Sample consent form. Include different versions for different groups of participants eg for children and adults.
- ❑ If I am not going to be using participant information sheets or consent forms I have explained why not and how informed consent will be otherwise obtained.
- ❑ If you are a student have you discussed your application with your supervisor and are they satisfied that you have completed the form correctly? (This will speed up your application).
- ❑ I have submitted a signed copy of my application. (If you are a student your supervisor also needs to sign the form).

MEEC 12-037 Ethical application - Appendix 1

Participant's information sheets

Research Project Title

Effects of carbohydrase-inhibiting polyphenols on glycaemic response in vivo

You are being invited to take part in a research project. Kindly read carefully all the information given as it is important that you understand why the study is going to be done and what it will involve. Before making any decision, if there is anything that is not clear, feel free to ask us and take time to decide whether to take part or not. Thank you for taking time to read this.

What is the purpose of the project?

Carbohydrate rich foods can be said to be high glycaemic index (GI) foods or low glycaemic index foods depending on the blood glucose resulting from consumption. High GI foods give rise to high blood glucose levels due to being absorbed rapidly whereas low GI foods give a lower blood glucose response due to being absorbed slowly. Different foods and beverages have different effects on blood glucose levels depending on whether they are high GI foods or low GI foods. Observational studies as well as research have shown that eating low GI foods rather than high GI foods may have long term health benefits.

The research project will determine whether a polyphenol rich meal will have an effect on reducing the GI of foods and hence reduce absorption of glucose. Polyphenols are naturally occurring constituents found in fruits and vegetables and other plant derived foods like tea and coffee.

Am I a suitable candidate for this study?

Inclusion Criteria for group 1

- Male or female
- Aged 18 to 75 years
- Not diabetic
- Normal fasting glucose (blood glucose level before breakfast) of 3.9 - 5.9 mmol/L
- Non-smoker
- Not pregnant or lactating
- Not on long term prescribed medication

Inclusion Criteria for group 2

- Male or female
- Aged 18 to 75 years
- Not diabetic
- Fasting glucose (blood glucose level before breakfast) of 6.0 - 8.0 mmol/L
- Non- smoker
- Not pregnant or lactating
- Not on long term prescribed medication

Do I have to take part?

Taking part in this research study is entirely voluntary. You have the right to withdraw from it at any time without giving any reason. If you decide to take part, you will be given this information sheet to keep and you will be asked to sign a consent form two days before starting the study just to indicate that you have understood what it means to take part in this research. Even after signing the consent form, you can still withdraw at any time if you no longer feel comfortable.

What do I have to do? / What will happen to me if I take part?

The first phase will involve a screening process to come up with the two groups (with different fasting glucose levels). This will require you to fast overnight drinking only water from 9 pm the previous night and report to the school of Food Science and Nutrition before 10 am the following day. Then your fasting blood glucose will be measured using a standard glucometer. Afterwards you will be offered breakfast.

After the two groups have been set and if you qualify to take part in this study, you will then proceed to take part in the second phase.

The second phase is to be done over 4 visits with each visit not lasting more than 4 hours. Preferably twice a week according to which days are convenient for you but with at least two days in between the two visits.

You will be expected to arrive at the school of food science and nutrition in the morning before 10 am after an overnight fast of between 10-14 hours. You will be asked to have a meal of your choice the night before the study day but should consume the same meal on all the days prior to the visit and keep a record of what you eat. You will be asked to rest for 10 minutes during which you will be asked to report on what food you ate the previous evening prior to the fast. Body weight, height and blood pressure measurements will also be taken. Then a cannula will be inserted in your elbow pit by the nurse to avoid the use of a needle for every blood withdrawal. Thereafter the fasting (time 0 sample) glucose blood sample will be collected from the fingertip using the finger prick device and from your vein by the nurse.

You will then be asked to eat or drink the food test sample. Then about 200 µl blood will be drawn at 15, 30, 45, 60, 90 and 120 minutes after starting to eat the test food from the finger prick and venous blood will also be collected by a nurse.

Reference food will be white bread containing 50g available carbohydrate, yoghurt (natural, lactose free and fat free) and water. Test food will include a cup of green tea, yoghurt (natural, lactose free and fat free) mixed with commercially available fruit

extracts and white bread. All the procedures on the participants mentioned above will be carried out in an appropriate human study room.

What are the possible disadvantages and risks of taking part?

There is very low risk to the participants as the method used is a World Health Organisation (WHO) approved and very common method. If you decide to discontinue your involvement in the study, this will not interfere in any way with the manner in which you will be treated. All data collected up to the point of withdrawal will only be used if the participant consents. If the participant does not want any of their data to be used after withdrawal, it will not be included. There are no additional risks involved as the procedures are according to standard guidelines.

What are the possible benefits of taking part?

You may not personally benefit from the study but the results may be beneficial for advancement of knowledge on nutrition/diet as it relates to human health.

Will my taking part in this project be kept confidential? / What will happen to the results of the research project?

Confidentiality will be maintained at all stages of the study as allocation of codes will be done from the beginning of the study. Identification will be coded using ID numbers that will be assigned on the day of study. All data collected will be treated as confidential and stored securely in a locked filling cabinet according to current University regulations. The linkage between individual identity and ID number will be kept in written form only and stored in a locked filling cabinet in a restricted access area, thus it will not be possible to identify individual participants from the ID numbers. Data evaluation will only be performed using ID numbers. In accordance with the University guidelines on the password protected M-drive of the University server, anonymised data will be stored for at least 5 years. When any data needs to be published, the results will be reported in a manner without making reference to any individual. If you wish, we can inform you of the outcome of the total analysis although you will not be able to identify yourself in any results.

Confidentiality and data protection

Confidentiality of all participants' information will be maintained..

What type of information will be sought from me and why is the collection of this information relevant for achieving the research project's objectives?

A questionnaire on suitability to participate will only ask you questions on your general health just to ensure that you are suitable for this research.

Who is organising /funding the research?

This research will be conducted at the School of Food Science and Nutrition under the supervision of Professor Gary Williamson, Food Biochemistry Group, University of Leeds, UK.

Who do I contact for further information?

Hilda Nyambe (Study Co-ordinator)

School of Food Science and Nutrition

Faculty of Mathematics and physical Sciences

University of Leeds

Email: fs07hs@leeds.ac.uk

Mobile: 07415780195

Thank you for taking the time to read this information sheet.

MEEC 12-037- Appendix 2

Participant Consent Form

Research project title **Effects of carbohydrase- inhibiting polyphenols on glycaemic response in vivo**

Name of researcher Hilda Nyambe

Initial the box if you agree with the statement to the left

1	I confirm that I have read and understand the Participant Information Sheet dated (TBC) explaining the above research project and I have had the opportunity to ask questions about the project.	☐
2	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without there being any negative consequences. In addition, should I not wish to answer any particular question or questions, I am free to decline. In this case I can contact the study co-ordinator by email: fs07hs@leeds.ac.uk or mobile: 07415780195	☐
3	I understand that my responses will be kept strictly confidential. I give permission for members of the research team to have access to my anonymised responses. I understand that my name will not be linked with the research materials, and I will not be identified or identifiable in the report or reports that result from the research.	☐
4	I agree for the data collected from me to be used in future research.	☐
5	I agree to take part in the above research project and will inform the principal investigator should my contact details change.	☐
6	I agree to be re-contacted for future research projects related to this study.	☐

Name of participant	Date	Signature
Address of participant	Mobile phone number	Email address
Lead Researcher	Date	Signature

MEEC 12-037 - Appendix 3

Pre-Study Questionnaire

A. Personal Information

Full name:

Age: Gender: Male Female

Height: Weight:

Contact phone no:

Email address:

B. Health History

Q2. Are you pregnant?

Yes No

Q3. Are you breast feeding?

Yes No

Q4. Do you have any of the following diseases/conditions?

Diabetes	Yes	No	I don't know
Digestive disease (e.g. Crohn's disease, celiac disease)	Yes	No	I don't know
Food allergy If yes state to what	Yes	No	I don't know
High blood pressure	Yes	No	I don't know

Q5. Do you regularly take any dietary supplements?

Yes No

Q6. If yes to Q5 above, what supplements?

C. <u>Lifestyle</u>

Q7. Are you currently taking any long term prescribed medication?

Yes No

Q8. Do you smoke?

Yes No

Q9. Are you currently on a special diet (for losing weight or fruit extracts supplements)?

Yes (specify)	No

Q10. 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 per day?

Yes (specify) (frequency)	No

Q11. 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 10 minutes continuously per day?

Yes	No

(specify) (frequency)	

Q12. Is there any more additional information you wish to give about your health and lifestyle?

Yes	No

Q13. If yes to Q12, kindly write below

MEEC 12-037 - Appendix 4

Blood collection and acellular Plasma preparation

Equipment and Materials

0.6 ml fluoride/oxalate collection tubes

0.6 ml micro centrifuge tubes

Tube labels

-4°C centrifuge

BD Contact –activated Lancets

Ice box

Disinfection swabs

Gloves

Spray bottle with 70% ethanol

Plasters

100 µl pipettes

Cotton wool balls

Protocol

1. Label all appropriate tubes with all needed information.
2. After the volunteer is seated in a chair, with gloves on, position the volunteer s hand with the palm-side up.
3. To the selected finger, apply pressure to help the blood to flow.
4. Clean the fingertip with a disinfection swab by starting in the middle and working outward to prevent contaminating the area. Allow the area to dry.
5. Hold the finger and place a new sterile lancet on the centre of the fingertip and firmly press the lancet to puncture the fingertip.
6. Wipe out the first drop of blood with a sterile pad or cotton ball which may contain excess tissue fluid.
7. Collect approximately 200 µl blood by dropping the blood drops into a sterile 0.6 ml fluoride/oxalate collection tubes.
8. After collection, immediately place the tubes on ice.
9. Place a cotton wool ball or pad over the puncture site and let the volunteer hold it for a few minutes to stop the bleeding. If need be, place a plaster afterwards.
10. Centrifuge the whole blood sample within 15 minutes of collection for 10 minutes at 3500 X g.
11. By using a sterile tip, pipette at least 100 µl of supernatant and transfer to a labelled 0.5 ml micro centrifuge tube.
12. Seal and freeze upright at -80°C until required for glucose analysis.

Labelling

Labelling will include the following information:

1) Subject ID code
2) Study code, comprised of test (reference, test dose 1, test dose 2) identification and time point (0,15,30,45,60,90,120,150,180)
3) Date
4) Investigator initials

Example

1) S1R 30 HN 2/5/13

MEANS, Subject Id code 1, Reference sample, 30 minutes time point, Hilda Nyambe as Investigator, the date being 2/5/2013

2) S11T1 90 HN 13/6/13

MEANS, Subject Id code 11, test sample dose 1, 90 minutes time point, Hilda Nyambe as investigator, the date being 13/6/13

3) S9T2 15 HN 28/7/13

MEANS, Subject Id code 9, test sample dose 2, 15 minutes time point, Hilda Nyambe as Investigator, the date being 28/7/13

Blood collection form

To be completed by the study co-ordinator

Subject ID code	
Test Sample	
Time point	
Collection time	
Approximate sample volume (µl)	
Comments	

Waste disposal

Diluted bleach will be added to the cellular fractions of the blood samples in the collection tubes. These will be left overnight for destruction of cellular fractions and can then be discarded within 24 hours in the clinical waste bags.

All other wastes should also be discarded in suitable waste bags.

Responsibilities and emergency procedures

Procedure	Person responsible	Potential Hazards	Existing controls	Emergency procedures
Finger prick with puncture device	Investigator	Injury and infection	Appropriate human study room will be used. Use of disinfectant wipes to clean the fingertip area before pricking with the puncture device. Use of one finger per time to avoid pricking the same finger more than once per visit.	Wash thoroughly the injured area with soap and warm water and disinfect injured area Avoid cross contamination and infection by keeping own injury covered with waterproof dressing. Seek immediate medical advice
Blood collection from finger tip	Investigator	Spillage/contamination Over bleeding	Appropriate human study room will be used. Compulsory use of personal protective equipment (laboratory coat, gloves, goggles). Usage of plastic or metal tray to handle blood and plasma samples (ease of cleaning any spillage) Placing a cotton wool ball or pad over the puncture site after blood collection and plaster afterwards.	Use diluted bleach to wash thoroughly the contaminated area or spilled area. Disinfect contaminated area Appropriate waste disposal of all samples in closed bag that will then be discarded into a clinical waste bin bag. Place a cotton wool ball and plaster if still bleeding and seek immediate medical advice
Cannular insertion and blood collection	Nurse	Injury and infection Contamination	Appropriate human study room will be used. Certified nurse will insert the cannula into the participants and withdraw blood from the participants. Disinfectant wipes will be used to clean the skin area before inserting the cannular. Nurse will wash his/her hands before proceeding to blood withdrawal. Used needle will immediately be disposed into a bin.	Wash thoroughly the injured area with soap and warm water. Encourage bleeding by squeezing around the injured area and disinfect it. Avoid cross contamination and infection by keeping own injury covered with waterproof dressing. Seek immediate medical advice Report incident to safety officer

MEEC 12-037 - Appendix 5

Study design

Advertisement → Volunteers asked to complete pre study questionnaire → fasting glucose screening → sign consent form → Four visits

First visit — At least 2 days — **Second visit** — One week — **Third visit** — At least 2 days — **Fourth visit**

Volunteers will consume

First visit	Second visit	Third visit	Fourth visit
Bread, yoghurt (natural lactose and fat free) and water	Bread, yoghurt (natural, lactose and fat free) fruit extracts and green tea (dose 1)	Bread, yoghurt (natural, lactose and fat free), fruit extracts and green tea (dose 2)	Bread, yoghurt (natural, lactose and fat free) and water

Fingertip blood collection at 0, 15, 30, 45, 60, 90, 120, 150 and 180 minutes	Glucose analysis
Venous blood collection at 0, 15, 30, 45, 60, 90, 120, 150 and 180 minutes	Glucose, insulin, glucagon, GIP and GLP-1 analysis

HUMAN STUDY VISITS DESIGN

	1ST VISIT	2ND VISIT	3RD VISIT	4TH VISIT
Volunteer 1	A	B	C	A
Volunteer 2	A	C	B	A
Volunteer 3	A	B	C	A

	1ST VISIT	2ND VISIT	3RD VISIT	4TH VISIT
Volunteer 4	A	C	B	A
Volunteer 5	A	B	C	A
Volunteer 6	A	C	B	A

	1ST VISIT	2ND VISIT	3RD VISIT	4TH VISIT
Volunteer 7	A	C	B	A
Volunteer 8	A	B	C	A
Volunteer 9	A	C	B	A

	1ST VISIT	2ND VISIT	3RD VISIT	4TH VISIT
Volunteer 10	A	B	C	A
Volunteer 11	A	C	B	A
Volunteer 12	A	B	C	A

A= REFERENCE (White bread, natural yoghurt, water)

B= TEST SAMPLE DOSE 1 (White bread, natural yoghurt with fruit extracts, green tea)

C= TEST SAMPLE DOSE 2 (White bread, yoghurt with twice the amount of fruit extracts and green tea concentration in dose 1)

MEEC 12-037 Appendix 6 - Recruitment Poster

Volunteers needed to help investigate the effects of foods on Glycaemic response (blood glucose levels).

Are you

- **Healthy male or female aged 18-65**
- **Able to attend 4 sessions over three weeks not lasting more than 4 hours per session**
- **Willing to give small blood samples by finger prick and from vein.**

The study is looking at the effects of polyphenol rich food on blood glucose response.

Blood glucose will be measured after consumption of test food samples.

If you need further information about this study, you can contact Hilda Nyambe by an e-mail fs07hs@leeds.ac.uk.

This study is supervised by Professor Gary Williamson, Food Biochemistry Group, School of Food Science and Nutrition, University of Leeds, UK.

£15 as a thank you for participating

Hilda Nyambe fs07hs@leeds.ac.uk Mobile # 07415780195	Hilda Nyambe fs07hs@leeds.ac.uk Mobile # 07415780195	Hilda Nyambe fs07hs@leeds.ac.uk Mobile # 07415780195	Hilda Nyambe fs07hs@leeds.ac.uk Mobile # 07415780195	Hilda Nyambe fs07hs@leeds.ac.uk Mobile # 07415780195	Hilda Nyambe fs07hs@leeds.ac.uk Mobile # 07415780195	Hilda Nyambe fs07hs@leeds.ac.uk Mobile # 07415780195	Hilda Nyambe fs07hs@leeds.ac.uk Mobile # 07415780195	Hilda Nyambe fs07hs@leeds.ac.uk Mobile # 07415780195	Hilda Nyambe fs07hs@leeds.ac.uk Mobile # 07415780195

APPENDIX 2

Ethical review

NOTICE OF AMENDMENT – MEEC 12-037

To be completed in typescript by the Principal Investigator in language comprehensible to a lay person and submitted to the FREC that gave the favourable opinion of the research. *Further guidance is available at* http://ris.leeds.ac.uk/EthicsAmendment.

Principal Investigator's details:	
A.1 Name:	HILDA NYAMBE
Address:	SCHOOL OF FOOD SCIENCE AND NUTRITION WOODHOUSE LANE LS2 9JT
Telephone:	07415780195
Email:	fs07hs@leeds.ac.uk

Full title of study:	Effects of carbohydrase-inhibiting polyphenols on glycaemic response
Ethics reference number:	MEEC 12-037
Date study commenced:	4th AUGUST 2014
Amendment number and date:	08/09/2015

Type of amendment (indicate all that apply in bold)

(a) Amendment to information previously given on the University of Leeds ethical review application form

Yes No

If yes, please refer to relevant sections of the FREC application in the "summary of changes" section below.

(b) Amendment to the information sheet(s) and/or consent form(s) for participants, or to any other supporting documentation for the study

Yes No

If yes, please submit all revised documents with new version numbers and dates, highlighting new text using a different colour font or the track changes feature.

Is this a modified version of an amendment previously notified to the FREC/ School REC and given an unfavourable opinion?

Yes ***No***

Is this an amendment to a project which underwent NHS ethical review?

Yes ***No***

Is sponsor sign off required for the amendment?

Yes ***No***

If applicable, has the amendment been submitted for NHS management permission (R&D approval)?

Yes *No* ***N/A***

Summary of changes

Briefly summarise the main changes proposed in this amendment using language comprehensible to a lay person. Explain the purpose of the changes and their significance for the study. In the case of a modified amendment, highlight the modifications that have been made.

If the amendment significantly alters the research design or methodology, or could otherwise affect the scientific value of the study, supporting scientific information should be given (or enclosed separately). Indicate whether or not additional scientific critique has been obtained.

1) The food used in the test meal
   We are changing the food we used for the test meals. We are going to use pomegranate juice as well as olives. This is because they are also good sources of carbohydrase-inhibiting polyphenols.

2) Blood collection
   There will be no collection of blood as a glucometer will be used to measure blood glucose instantly from a finger prick.

Any other relevant information

Applicants may indicate any specific ethical issues relating to the amendment, on which the opinion of the REC is sought.

The ethical issue is that the foods that were used in the test meal in the original ethical approval were different to the ones we want to use now.

High risk ethical issues

(to be completed by applicants who applied for light touch ethical review only)

	Yes	**No**
Does the study involve participants who are particularly vulnerable or unable to give informed consent (eg children, people with learning disabilities, your own students)?		
Will the study require the cooperation of a gatekeeper for initial access to groups or individuals who are taking part in the study (eg students at school, members of self-help groups, residents of a nursing home)?		
Will participants be taking part in the research without their knowledge and consent (eg covert observation of people in non-public places)?		
Will the study involve discussion of sensitive topics (e.g sexual activity, drug use)?		
Are drugs, placebos or other substances (e.g food substances, vitamins) going to be administered to the participants or will the study involve invasive, intrusive or potentially harmful procedures of any kind?		
Will blood or tissue samples be obtained from the participants?		
Is pain or more than mild discomfort likely to result from the study?		
Could the study induce psychological stress or anxiety or cause harm or have negative consequences beyond the risks encountered in normal life?		
Will the study involve prolonged or repetitive testing?		

Will financial inducements (other than reasonable expenses and compensation for time) be offered to participants?		
Will the study involve an international collaborator or research conducted overseas?		
Will the study involve the transfer of data outside the European Economic Area?		
Are there any potential conflicts of interest?		
Does the research involve any risks to the researchers themselves, or individuals not directly involved in the research?		
Will the study require ethical review from the NHS? (Refer to http://ris.leeds.ac.uk/NHSethicalreview for guidance in identifying circumstances which require NHS review)		

If you answer yes to any of these questions please apply for full ethical review rather than an amendment to your light touch application:

http://ris.leeds.ac.uk/uolethicsapplication.

List of enclosed documents

Document	*Version*	*Date*
Amended ethical application form with appendices	2	08/09/15

Declaration

- I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.
- I consider that it would be reasonable for the proposed amendment to be implemented.

Signature of Principal Investigator: ..... be .....

Print name: Hilda Nyambe

Date of submission: 08/09/15

Signature of supervisor of student project: ..... G.Wm .....

Print name: G WILLIAMSON

Date of submission: 08/09/15

Please submit your form **by email** to ResearchEthics@leeds.ac.uk or if you are in the Faculty of Medicine and Health FMHUniEthics@leeds.ac.uk.

APPENDIX 3

UNIVERSITY OF LEEDS

UNIVERSITY OF LEEDS RESEARCH ETHICS COMMITTEE APPLICATION FORM [1]

Please read each question carefully, taking note of instructions and completing all parts. If a question is not applicable please indicate so. The superscripted numbers refer to sections of the guidance notes, available at www.leeds.ac.uk/ethics. Where a question asks for information which you have previously provided in answer to another question, please just refer to your earlier answer rather than repeating information.

Research ethics training courses: http://www.sddu.leeds.ac.uk/sddu-research-ethics-courses.html

To help us process your application enter the following reference numbers, if known and if applicable:

Ethics reference number:	**MEEC 14-029**
Grant reference and/ or student number:	**200358739**

PART A: Summary

A.1 Which Faculty Research Ethics Committee would you like to consider this application?[2]

- ○ Arts and PVAC (PVAR)
- ○ Biological Sciences (BIOSCI)
- ○ ESSL/ Environment/ LUBS (AREA)
- ◉ MaPS and Engineering (MEEC)
- ○ Medicine and Health (Please specify a subcommittee):
  - ○ Leeds Dental Institute (DREC)
  - ○ Health Sciences/ LIGHT/ LIMM
  - ○ School of Healthcare (SHREC)
  - ○ Medical and Dental Educational Research (EdREC)
  - ○ Institute of Psychological Sciences (IPSREC)

A.2 Title of the research[3]

Effects of carbohydrase-inhibiting polyphenols on glycaemic response

A.3 Principal investigator's contact details[4]	
Name *(Title, first name, surname)*	Ms Hilda Nyambe
Position	Provisional PhD Student
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	MAPS
Work address *(including postcode)*	School of Food Science and Nutrition, University of Leeds, LS2 9JT
Telephone number	07415780195

University of Leeds email address	fs07hs@leeds.ac.uk

A.4 Purpose of the research:[5] (Tick as appropriate)

- ☑ Research
- ☑ Educational qualification: ***Please specify: __Doctor of Philosophy_____***
- ☐ Educational Research & Evaluation[6]
- ☐ Medical Audit or Health Service Evaluation[7]
- ☐ Other

A.5 Select from the list below to describe your research: (You may select more than one)

- ☑ Research on or with human participants
- ☐ Research with has potential significant environmental impact.[8] If yes, please give details:

  _____

- ☐ Research working with data of human participants
  - ☐ New data collected by questionnaires/interviews
  - ☐ New data collected by qualitative methods
  - ☐ New data collected from observing individuals or populations
  - ☐ Research working with aggregated or population data
  - ☐ Research using already published data or data in the public domain
- ☐ Research working with human tissue samples[9]

A.6 Will the research involve any of the following:[10] (You may select more than one)

If your research involves any of the following an application must be made to the National Research Ethics Service (NRES) via IRAS www.myresearchproject.org.uk as NHS ethical approval will be required. There is no need to complete any more of this form. Contact governance-ethics@leeds.ac.uk for advice.

- ☐ Patients and users of the NHS (including NHS patients treated in the private sector)[11]
- ☐ Individuals identified as potential participants because of their status as relatives or carers of patients and users of the NHS
- ☐ Research involving adults in Scotland, Wales or England who lack the capacity to consent for themselves[12]
- ☐ A prison or a young offender institution in England and Wales (and is health related)[14]
- ☐ Clinical trial of a medicinal product or medical device[15]
- ☐ Access to data, organs or other bodily material of past and present NHS patients[9]
- ☐ Use of human tissue (including non-NHS sources) where the collection is not covered by a Human Tissue Authority licence[9]
- ☐ Foetal material and IVF involving NHS patients
- ☐ The recently deceased under NHS care
- ☑ None of the above

You must inform the Research Ethics Administrator of your NRES number and approval date once approval has been obtained.

If the University of Leeds is not the Lead Institution, or approval has been granted elsewhere (e.g. NHS) then you should contact the local Research Ethics Committee for guidance. The UoL Ethics Committee need to be assured that any relevant local ethical issues have been addressed.

A.7 Will the research involve NHS staff recruited as potential research participants (by virtue of their professional role) or NHS premises/ facilities?

☐ Yes ☑ No

If yes, ethical approval must be sought from the University of Leeds. Please note that NHS R&D approval is needed in addition, and can be applied for concurrently: www.myresearchproject.org.uk.

Contact governance-ethics@leeds.ac.uk for advice.

A.8 Will the participants be from any of the following groups? (Tick as appropriate)

- ☐ Children under 16[16]
- ☐ Adults with learning disabilities[12]
- ☐ Adults with other forms of mental incapacity or mental illness
- ☐ Adults in emergency situations
- ☐ Prisoners or young offenders[14]
- ☑ Those who could be considered to have a particularly dependent relationship with the investigator, eg members of staff, students[17]
- ☐ Other vulnerable groups
- ☐ No participants from any of the above groups

Please justify the inclusion of the above groups, explaining why the research cannot be conducted on non vulnerable groups.

Since adverts will be put around the University campus notice boards, it is very likely that students and members of staff may be recruited for the study as they are normally around the University campus. This will make it convenient to use students or members of staff as there will be no need to travel to get to the School of Food Science and Nutrition as they will already be on campus for work or for studies.

However, participation will be purely voluntary and the participants will not be approached directly or forced to take part. If they so wish, they can withdraw from participating at any time.

A Criminal Record Bureau (CRB) check will be needed for researchers working with children or vulnerable adults (see www.crb.gov.uk and http://store.leeds.ac.uk/browse/extra_info.asp?modid=1&prodid=2162&deptid=34&compid=1&prodvarid=0&catid=243)

A.9 Give a short summary of the research[18]

*This section must be completed in **language comprehensible to the lay person**. Do not simply reproduce or refer to the protocol, although the protocol can also be submitted to provide any technical information that you think the ethics committee may require. This section should cover the main parts of the proposal.*

Consumption of carbohydrate containing foods or sugary drinks brings about changes to the blood glucose levels. After a meal or drink, blood glucose rises until it reaches a peak concentration usually after 30 minutes. When the body senses the increase in blood glucose, a hormonal process involving insulin takes place to ensure that the glucose is taken up from the blood for storage and where it is needed for energy in the body. This process then brings about a decrease in the concentration of glucose until it reaches approximately the starting concentration. The original concentration of glucose is attained approximately 2 hours after eating or drinking a carbohydrate food or sugary drink respectively in healthy people.

Different carbohydrates and sugary drinks have different effects on blood glucose response depending on the amount as well as the type of carbohydrate. Those that give rise to a high glucose response compared to a reference carbohydrate (usually glucose) are said to be high glycaemic index (GI) foods and those with a lower glucose response compared to a reference carbohydrate (usually glucose) are said to be low glycaemic index (GI) foods (Jenkins *et al*., 2002)

Research has shown that diets that give rise to a high glucose response are associated with a number of abnormalities like increased risk of metabolic syndrome (Aston, 2006). Metabolic syndrome mostly comprises of insulin resistance and glucose intolerance which gives an increased risk of type 2 diabetes (Mckeown *et al*., 2004) It also gives rise to other conditions like high blood pressure (arterial hypertension), elevated blood insulin levels (hyper-insulinemia), elevated amounts of fat in the liver (fatty hepatosis) and elevated amounts of lipids in the blood (dyslipidemia). After type 2 diabetes become clinically apparent, the risk of cardiovascular disease also rises (Beilby, 2004) Research has also shown that foods/drinks which raise blood glucose levels gradually (low GI) rather than rapidly (high GI) have health benefits which include reducing the risk of metabolic syndrome (Barclay *et al*., 2008). Laboratory studies have shown that polyphenols found in fruits, vegetables and plant based foods have a positive effect on carbohydrate metabolism and can lower the blood glucose levels. (Hanhineva *et al*., 2010)

This research will determine whether the presence of polyphenols in the diet has any lowering effect on the blood glucose levels and hence the glycaemic index of foods. This will be determined by asking volunteers to consume commercially available food supplements together with white bread and then determining the glycaemic response. The blood glucose response of bread will be determined initially as a control reference. All will be consumed in random order.

Analysis will be done by measuring blood glucose response after consumption of the control reference meal and the test meal containing polyphenols and then determining the incremental area under the glucose curve.

References

Aston, L. M. (2006). Glycaemic index and metabolic disease risk. *Proceedings of the Nutrition Society,* **65,** 125-134.

Barclay, A. W., Petocz, P., Mcmillan-Price, J., Flood, V. M., Prvan, T., Mitchell, P. & Brand-miller, J. C. (2008). Glycemic index, glycemic load, and chronic disease risk - a metaanalysis of observational studies. *American Journal of Clinical Nutrition,* **87,** 627-637.

Beilby, J. (2004). Definition of metabolic syndrome: Report of the national heart, lung and blood institute/ American Heart Association Conference on scientific Issues Related to Definition. *Circulation* , **25**, 195-198.

Hanhineva, K., Torronen, R., Bondia-pons, I., Pekkinen, J., Kolehmainen, M., Mykkanan, H. & Poutanen, K. (2010). Impact of Dietary Polyphenols on Carbohydrate Metabolism. *International Journal of Molecular Sciences,* **11,** 1365-1402.

Jenkins, D. J. A., Kendall, C. W. C., Augustin, L. S. A., Franceschi, S., HamidI, M., Marchie, A., Jenkins, A. L. & Axelsen, M. (2002). Glycemic index: overview of implications in health and disease. *American Journal of Clinical Nutrition,* **76,** 266S-273S.

Mckeown, N. M., Meigs, J. B., Liu, S., Saltzman, E., Wilson, P. W. F. & Jacques, P. F. (2004). Carbohydrate nutrition, insulin resistance, and the prevalence of the metabolic syndrome in the Framingham Offspring Cohort. *Diabetes Care,* **27,** 538-546.

A.10 What are the main ethical issues with the research and how will these be addressed?[19]

Indicate any issues on which you would welcome advice from the ethics committee.

Participation of human volunteers addressed by informed consent

Prior to commencement of the study, potential volunteers will be given a clear, concise Participant Information Sheet (appendix 1) and Informed Consent Form (appendix 2) by the investigator to read, complete and sign in their own time. The investigator will answer all questions to the satisfaction of the individual and will not influence the decision of taking part in the study. The participants will be free to withdraw from the study at any point without providing a reason. The written consent form will be signed and dated at least 2 days prior to enrolment in the study by the volunteers and the Investigator. It will be photocopied and one copy will be kept by the Investigator (for the study records) and one by the volunteer. Participation by the volunteers is conditional upon receiving the written consent by the volunteer.

Gathering of personal data addressed by confidentiality and data protection

Candidates will be asked to complete a questionnaire (appendix 3) in order to assess their suitability for the study. The information pertains to their lifestyle and may be considered a sensitive issue, however each volunteer will be assured of confidentiality and that participation is entirely voluntary. The information on the questionnaire will include height, weight, gender, age and relevant medical history.

Confidentiality of all participants' information will be maintained. Identification will be coded using random ID numbers that will be assigned on the day of study. All data collected will be treated as confidential and stored securely in a locked filing cabinet according to current University regulations. The linkage between individual identity and ID number will be kept in written form only and stored in a locked filling cabinet in a restricted access area, thus it will not be possible to identify individual participants from the ID numbers. Data evaluation will only be performed using ID numbers. In accordance with the University guidelines on the password protected M-drive of the University server, anonymised data will be stored for at least 5 years.

Blood sample collection

Capillary blood glucose will be measured using a commercially available glucometer which is available to be bought by any member of the public for less than £50 and people already use them on their own. It measures glucose concentrations immediately and meets the accuracy requirement for the ISO 15197 standard. A drop of blood is enough and is obtained from participants by means of a mild finger prick using a sterile puncture device designed for this purpose for each time point. The finger prick protocol is the WHO/FAO 1998 glycaemic index approved protocol. Since 9 points will be needed (0, 15, 30, 45, 60, 90, 120, 150 and 180) thus we will aim to prick one finger once per session. An allowance of only one visit per week will also help in the healing of the pricks.

Ethics Committee Approval
This human study protocol will be submitted to the Faculty of Mathematics and Physical Sciences Ethics committee at the University of Leeds, UK.

PART B: About the research team

B.1 To be completed by students only[20]	
Qualification working towards (e.g. Masters, PhD)	PhD
Supervisor's name (Title, first name, surname)	Professor Gary Williamson
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	MAPS
Work address (including postcode)	School of Food Science & Nutrition, University of Leeds, LS2 9JT
Supervisor's telephone number	0113 343 8380
Supervisor's email address	g.williamson@leeds.ac.uk
Module name and number (if applicable)	

B.2 Other members of the research team (eg co-investigators, co-supervisors) [21]	
Name *(Title, first name, surname)*	Akshay Yashish, Clitheroe Richard and Huang Linlin
Position	Masters students
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	MAPS

Work address *(including postcode)*	School of Food Science & Nutrition, University of Leeds, LS2 9JT
Telephone number	
Email address	

Part C: The research

C.1 What are the aims of the study? [22] (Must be in language comprehensible to a lay person.)

3. To determine the effects of pomegranate and olive supplements on glycaemic response in comparison to a reference.
4. To determine the effect of different doses of the supplements on postprandial plasma glucose responses of healthy humans.

C.2 Describe the design of the research. Qualitative methods as well as quantitative methods should be included. (Must be in language comprehensible to a lay person.)

It is important that the study can provide information about the aims that it intends to address. If a study cannot answer the questions/ add to the knowledge base that it intends to, due to the way that it is designed, then wasting participants' time could be an ethical issue.

Subject preparation

Unusual vigorous activity should be avoided and subjects should eat a meal of their choice the evening before the test and we recommend that they eat the same meal the day before each test.

Study Design

In total, 40 healthy participants will be recruited of which some will consume pomegranate supplement as test sample and others will consume olive supplement as test sample. The study will involve 3 visits with each visit not lasting more than 4 hours.

Subjects will be expected to arrive at the school of food science and nutrition in the morning before 10 am after an overnight fast of between 10-14 hours. They will be asked to rest for 10 minutes during which they can report on what they had the previous evening prior to the fast. Body weight and height measurements will also be taken.

Thereafter, the fasting capillary blood glucose concentration will be measured with the glucometer.

The subject will then be asked to drink water with either test supplement dose 1 or dose 2 or placebo and eat white bread after 5 minutes. Then blood glucose will be measured at 15, 30, 45, 60, 90, 120, 150 and 180 minutes post consumption of the test food with a glucometer.

The Latin square design will be used to randomize which test food or reference will be consumed by which volunteer on different days as shown in appendix 5.

Sample analysis

Blood glucose will be measured using a glucometer at each time point which includes a mild finger prick.

Statistical analysis

The incremental area under each glucose curve will be calculated using the trapezoid rule. The area under the glucose curve for the three visits will be analysed to show whether there are any significant differences using the Students paired T-test.

C.3 What will participants be asked to do in the study?[23] *(e.g. number of visits, time, travel required, interviews)*

What will participants be asked to do in the study

Participants will be asked to complete a pre study questionnaire (appendix 3) to determine their suitability for the study as well as have their fasting glucose checked using a glucometer. The eligibility criteria will be explained to the potential volunteers and they will be informed that if their blood glucose falls outside the range, they will not be enrolled to take part in the study. If their fasting blood glucose is between 3.9 and 5.9 mmol/L, they will be enrolled in the study and thus asked to complete the consent form. (Appendix 2)

The study is to be done over 3 visits with each visit not lasting more than 4 hours. Each visit will be done once a week according to which days are convenient for the volunteer giving a total of three weeks.

Subjects will be expected to arrive at the school of food science and nutrition in the morning before 10 am after an overnight fast of between 10-14 hours. The participants will be asked to have a meal of their choice the night before the study day but should consume the same meal on all the days and a record of what they eat should be availed. They will be asked to rest for 10 minutes during which they can report on what they had the previous evening prior to the fast. Body weight and height measurements will also be taken. Thereafter, fasting capillary blood concentration will be measured with a glucometer.

The subject will then be asked to eat and drink the food test sample as shown in the appendix 6. Then blood glucose will be measured 15, 30, 45, 60, 90, 120, 150 and 180 minutes post consumption of the test/reference food using a glucometer.

C.4 Does the research involve an international collaborator or research conducted overseas:[24]

(Tick as appropriate)

☐ Yes ☑ No

If yes, describe any ethical review procedures that you will need to comply with in that country:

Describe the measures you have taken to comply with these:

Include copies of any ethical approval letters/ certificates with your application.

C.5 Proposed study dates and duration

Research start date (DD/MM/YY): As soon as ethical approval is obtained.

Research end date (DD/MM/YY): 31/09/2016

Fieldwork start date (DD/MM/YY):

Fieldwork end date (DD/MM/YY):

C.6. Where will the research be undertaken? (i.e. in the street, on UoL premises, in schools)[25]

On University of Leeds premises.

RECRUITMENT & CONSENT PROCESSES

How participants are recruited is important to ensure that they are not induced or coerced into participation. The way participants are identified may have a bearing on whether the results can be generalised. Explain each point and give details for subgroups separately if appropriate.

C.7 How will potential participants in the study be:

(i) identified?

Codes will be used to identify individual subjects and no personal details will be referred to in this study as reported in A10. Personal details will only be used to check suitability at the recruitment stage.

During the first contact with the potential participant, the main investigator will assign a code number to the participant which will be used to identify the participant throughout the study. An intermediate person who will never have any contact with the participants will be matching the names and give the corresponding codes to the Investigator.

(ii) approached?

Advertisements (appendix 6) in the form of posters and emails to the staff and students in the school of Food Science and Nutrition as well as other parts of the University of Leeds campus will be used.

(iii) recruited?[26]

Subjects will be recruited from the university of Leeds staff and Students. Written informed consent will be obtained from the volunteers and participation can be withdrawn at any time without giving a reason.

C.8 Will you be excluding any groups of people, and if so what is the rationale for that?[27]

Excluding certain groups of people, intentionally or unintentionally may be unethical in some circumstances. It may be wholly appropriate to exclude groups of people in other cases

Selection criteria is shown in **appendix**

Measured

Fasting glucose (blood glucose level before breakfast) 3.9 -5.9 mmol/L

Self-assessed
Apparently healthy
Not diabetic
Not on long term prescribed medication (except contraceptives)
Not pregnant or lactating
Not on special diet (for losing weight or fruit extracts supplements)
Aged 18-75

C.9 How many participants will be recruited and how was the number decided upon?[28]

It is important to ensure that enough participants are recruited to be able to answer the aims of the research.

According to a review on Glycaemic index methodology by Brouns *et al*., 2005 which looked at a number of papers on glycaemic index methodology, the conclusion was that a minimum of 10 subjects is required for such type of study for each group depending on desired power. In this study a total of 40 participants will be used with some to consume pomegranate supplement and others the olive supplement.

From experience in the school from previous human studies, the number suggested above is achievable and in the rare case that it's not achieved, recruitment will continue until the required number is obtained as the study is only 3 weeks in total per participant and so can be done at any time of the recruitment process.

If you have a formal power calculation please replicate it here.

Estimated sample size for two-sample comparison of means

Test Ho: m1 = m2, where m1 is the mean in population 1
and m2 is the mean in population 2
Assumptions:

alpha = 0.0500 (two-sided)
power = 0.9000
m1 = 200
m2 = 230
sd1 = 25
sd2 = 25
n2/n1 = 1.00

Estimated required sample sizes:

n1 = 15
n2 = 15

Since using crossover design this is 15 participants in total as they are testing both diets.

Remember to include all advertising material (posters, emails etc) as part of your application

C10 Will the research involve any element of deception?[29]

If yes, please describe why this is necessary and whether participants will be informed at the end of the study.

No

C.11 Will informed consent be obtained from the research participants?[30]

☑ Yes ☐ No

If yes, <u>give details</u> of how it will be done. Give details of any particular steps to provide information (in addition to a written information sheet) e.g. videos,

interactive material. If you are not going to be obtaining informed consent you will need to justify this.

Volunteers will receive a participant information sheet and informed consent form after a positive reply to the recruitment advertisement. Volunteers will consent in writing prior to commencement of the study. The main investigator will verbally explain the participant information sheet and informed consent form and questions from the volunteer will be answered at any time. The prospective participant will have 1-2 days prior to commencement of the study to decide, sign and return the completed informed consent form (two copies where one copy remains with the participant and the other copy for the research records) both to be co-signed by the main investigator.

If ***participants are to be recruited from any of potentially vulnerable groups, <u>give details of extra steps</u> taken to assure their protection. Describe any arrangements to be made for obtaining consent from a legal representative.***

Copies of any written consent form, written information and all other explanatory material should accompany this application. *The information sheet should make explicit that participants can withdrawn from the research at any time, if the research design permits.*

Sample information sheets and consent forms are available from the University ethical review webpage at http://researchsupport.leeds.ac.uk/index.php/academic_staff/good_practice/planning_your_research_project-1/approaching_and_recruiting_participants-1.

C.12 Describe whether participants will be able to withdraw from the study, and up to what point (eg if data is to be anonymised). If withdrawal is <u>not</u> possible, explain why not.

Volunteers will be informed that they can withdraw from the study at anytime without giving any reason and without any negative consequences.

C.13 How long will the participant have to decide whether to take part in the research?[31]

It may be appropriate to recruit participants on the spot for low risk research; however consideration is usually necessary for riskier projects.

2 days prior to commencement of the study.

C.14 What arrangements have been made for participants who might not adequately understand verbal explanations or written information, or who have special communication needs?[32] *(e.g. translation, use of interpreters etc. It is important that groups of people are not excluded due to language barriers or disabilities, where assistance can be given.)*

Participants will be expected to understand the English language. Email and phone contact details will be provided on the recruitment advert and information sheet.

C.15 Will individual or group interviews/ questionnaires discuss any topics or issues that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could take place during the study (e.g. during interviews or group discussions)?[33] *The information sheet should explain under what circumstances action may be taken.*

☐ Yes ☑ No *If yes, give details of procedures in place to deal with these issues.*

C.16 Will individual research participants receive any payments, fees, reimbursement of expenses or any other incentives or benefits for taking part in this research?[34]

☑ Yes ☐ No

If Yes, please describe the amount, number and size of incentives and on what basis this was decided.

Participants will receive snacks after collection of blood (3 hours after consumption of test food) if they wish. A monetary reward of £15 will be given at the end of the whole study (3 visits).

RISKS OF THE STUDY

C.17 What are the potential benefits and/ or risks for research participants?[35]

There is very low risk to the participants as the method used is a World Health Organisation (WHO) approved and very common method. There are no direct benefits to the participants although the consumption of polyphenol rich test samples may have beneficial effects to the participants who may not normally have them in their diets. Moreover the results of the study may help to add to the knowledge on how diet may affect health.

C.18 Does the research involve any risks to the researchers themselves, or people not directly involved in the research? *Eg lone working*[36]

☐ Yes ☑ No

If yes, please describe:

_____

Is a risk assessment necessary for this research?

NB: Risk assessments are a University requirement for all fieldwork taking place off campus. For guidance contact your Faculty Health and Safety Manager or visit http://www.leeds.ac.uk/safety/fieldwork/index.htm.

☐ Yes ☑ No If yes, please include a copy of your risk assessment form with your application.

DATA ISSUES

C.19 Will the research involve any of the following activities at any stage (including identification of potential research participants)? (Tick as appropriate)

☐ Examination of personal records by those who would not normally have access

☐ Access to research data on individuals by people from outside the research team

☐ Electronic transfer of data

☐ Sharing data with other organisations

☐ Exporting data outside the European Union

☑ Use of personal addresses, postcodes, faxes, e-mails or telephone numbers

☐ Publication of direct quotations from respondents

☐ Publication of data that might allow identification of individuals to be identified

☐ Use of audio/visual recording devices

☐ FLASH memory or other portable storage devices

Storage of personal data on or including any of the following:

☐ Manual files

☐ Home or other personal computers

☐ Private company computers

☐ Laptop computers

C.20. How will the research team ensure confidentiality and security of personal data? E.g. anonymisation procedures, secure storage and coding of data.[37] You may wish to refer to the data protection and research webpage.

See the answer explained in question A.10

C.21 For how long will data from the study be stored? Please explain why this length of time has been chosen.[38]

RCUK guidance states that data should normally be preserved and accessible for ten years, but for some projects it may be 20 years or longer.

***Students**: It would be reasonable to retain data for at least 2 years after publication or three years after the end of data collection, whichever is longer.*

5 years

CONFLICTS OF INTEREST

C.22 Will any of the researchers or their institutions receive any other benefits or incentives for taking part in this research over and above normal salary or the costs of undertaking the research?[39]

☐ **Yes** ☑ **No**

If yes, indicate how much and on what basis this has been decided _

_____

C.23 Is there scope for any other conflict of interest?[40] *For example will the research funder have control of publication of research findings?*

☐ Yes ☑ No ***If yes, please explain***

_____

C.24 Does the research involve external funding? (Tick as appropriate)

☐ Yes ☑ No ***If yes, what is the source of this funding? _***

_____

PART D: Declarations

Hilda Nyambe 200358739

PART D: Declarations

Declaration by Chief Investigators

1. The information in this form is accurate to the best of my knowledge and belief and I take fu responsibility for it.
2. I undertake to abide by the University's ethical and health & safety guidelines, and the ethic principles underlying good practice guidelines appropriate to my discipline.
3. If the research is approved I undertake to adhere to the study protocol, the terms of this app and any conditions set out by the Research Ethics Committee.
4. I undertake to seek an ethical opinion from the REC before implementing substantial amen the protocol.
5. I undertake to submit progress reports if required.
6. I am aware of my responsibility to be up to date and comply with the requirements of the lav relevant guidelines relating to security and confidentiality of patient or other personal data, i the need to register when necessary with the appropriate Data Protection Officer.
7. I understand that research records/ data may be subject to inspection for audit purposes if r future.
8. I understand that personal data about me as a researcher in this application will be held by relevant RECs and that this will be managed according to the principles established in the D Protection Act.
9. I understand that the Ethics Committee may choose to audit this project at any point after a

Sharing information for training purposes: Optional – please tick as appropriate:

☐ I would be content for members of other Research Ethics Committees to have access to the information in the application in confidence for training purposes. All personal identifiers and references to researchers, funders and research units would be removed.

Principal Investigator

Signature of Principal Investigator: ..... (This needs to be an ac signature rather than just typed. Electronic signatures are acceptable)

Print name: Hilda Nyambe Date: (dd/mm/yyyy): 22/05/2015

Supervisor of student research: I have read, edited and agree with the form above.

Supervisor's signature: ..... (This needs to be an actual signatu than just typed. Electronic signatures are acceptable)

Print name: G WILLIAMSON Date: (dd/mm/yyyy): 22-05-2015

Please submit your form **by email** to J.M.Blaikie@leeds.ac.uk or if you are in the Faculty of Medicine and Health FMHUniEthics@leeds.ac.uk. **Remember to include any supporting material** such as your participant information sheet, consent form, interview questions and recruitment material with your application.

Checklist:

- ❑ I have used layman's terms to describe my research (applications are reviewed by lay members of the committee as well).
- ❑ I have answered all the questions on the form, including those with several parts (refer to the guidance if you're not sure how to answer a question or how much detail is required)
- ❑ I have included any relevant supplementary materials such as
    - ❑ Recruitment material (posters, emails etc)
    - ❑ Sample participant information sheet
    - ❑ Sample consent form. Include different versions for different groups of participants eg for children and adults.
- ❑ If I am not going to be using participant information sheets or consent forms I have explained why not and how informed consent will be otherwise obtained.
- ❑ If you are a student have you discussed your application with your supervisor and are they satisfied that you have completed the form correctly? (This will speed up your application).
- ❑ I have submitted a signed copy of my application. (If you are a student your supervisor also needs to sign the form).

MEEC 14-029 - Appendix 1

Participant's information sheets

Research Project Title

Effects of pomegranate and olive supplements on glycaemic response (blood glucose) in vivo.

You are being invited to take part in a research project. Kindly read carefully all the information given as it is important that you understand why the study is going to be done and what it will involve. Before making any decision, if there is anything that is not clear, feel free to ask us and take time to decide whether to take part or not. Thank you for taking time to read this.

What is the purpose of the project?

Carbohydrate rich foods can be said to be high glycaemic index (GI) foods or low glycaemic index foods depending on the blood glucose resulting from consumption. High GI foods give rise to high blood glucose levels due to being absorbed rapidly whereas low GI foods give a lower blood glucose response due to being absorbed slowly. Different foods and beverages have different effects on blood glucose levels depending on whether they are high GI foods or low GI foods. Observational studies as well as research have shown that eating low GI foods rather than high GI foods may have long term health benefits.

The research project will determine whether pomegranate and olive supplements will have an effect on blood glucose concentrations after a meal.

Am I a suitable candidate for this study?

Inclusion Criteria

Measured on your first visit

Fasting glucose (blood glucose level before breakfast) 3.9 - 5.9 mmol/L

Self-assessed

Apparently healthy
Not diabetic
Not on long term prescribed medication (except contraceptives)
Not pregnant or lactating
Not on special diet (for losing weight or fruit extracts supplements)
Aged 18-75

Do I have to take part?

Taking part in this research study is entirely voluntary. You have the right to withdraw from it at any time without giving any reason. If you decide to take part, you will be given this information sheet to keep and you will be asked to sign a consent form two days before starting the study just to indicate that you have understood what it means to take part in this research. Even after signing the consent form, you can still withdraw at any time if you no longer feel comfortable.

What do I have to do? / What will happen to me if I take part?

Firstly, you will be required to come for a screening to ensure that you meet the inclusion criteria. This will require you to fast overnight drinking only water from 9 pm the previous night and report to the school of Food Science and Nutrition before 10 am the following day. Then your fasting blood glucose will be measured using a standard glucometer. Afterwards you will be offered breakfast.

If you qualify to take part in this study, you will then be asked to come for 3 visits with each visit not lasting more than 4 hours according to which days are convenient for you.

You will be expected to arrive at the school of food science and nutrition in the morning before 10 am after an overnight fast of between 10-14 hours. You will be asked to have a meal of your choice the night before the study day but should consume the same meal on all the days prior to the visit and keep a record of what you eat. You will be asked to rest for 10 minutes during which you will be asked to report on what food you ate the previous evening prior to the fast. Body weight (fully clothed), height and blood pressure measurements will also be taken. Thereafter the fasting (time 0 sample) glucose blood sample will be collected from the fingertip using the finger prick device and immediately measured using the glucometer.

You will then be asked to consume the supplements as 2 capsules with water and then eat white bread after 5 minutes with water within 10 minutes. Then glucose blood concentration will be measured using a glucometer after pricking your fingertip using a mild finger prick device at 15, 30, 45, 60, 90, 120, 150 and 180 minutes after starting to eat bread.

All the procedures on the participants mentioned above will be carried out in an appropriate human study room at the school of food science and nutrition located in parkinsons building.

What are the possible disadvantages and risks of taking part?

There is very low risk to the participants as the method used is a World Health Organisation (WHO) approved and very common method. If you decide to discontinue your involvement in the study, this will not interfere in any way with the manner in which you will be treated. All data collected up to the point of withdrawal will only be used if the participant consents. If the participant does not want any of their data to be used after withdrawal, it will not be included. There are no additional risks involved as the procedures are according to standard guidelines.

What are the possible benefits of taking part?

You may not personally benefit from the study but the results may be beneficial for advancement of knowledge on nutrition/diet as it relates to human health.

Will my taking part in this project be kept confidential? / What will happen to the results of the research project?

Confidentiality will be maintained at all stages of the study as allocation of codes will be done from the beginning of the study. Identification will be coded using ID numbers that will be assigned on the day of study. All data collected will be treated as confidential and

stored securely in a locked filling cabinet according to current University regulations. The linkage between individual identity and ID number will be kept in written form only and stored in a locked filling cabinet in a restricted access area, thus it will not be possible to identify individual participants from the ID numbers. Data evaluation will only be performed using ID numbers. In accordance with the University guidelines on the password protected university server, anonymised data will be stored for at least 5 years. When any data needs to be published, the results will be reported in a manner without making reference to any individual. If you wish, we can inform you of the outcome of the total analysis although you will not be able to identify yourself in any results.

Confidentiality and data protection

Confidentiality of all participants' information will be maintained..

What type of information will be sought from me and why is the collection of this information relevant for achieving the research project's objectives?

A questionnaire on suitability to participate will only ask you questions on your general health just to ensure that you are suitable for this research.

Who is organising /funding the research?

This research will be conducted at the School of Food Science and Nutrition under the supervision of Professor Gary Williamson, Food Biochemistry Group, University of Leeds, UK.

Who do I contact for further information?

Hilda Nyambe (Study Co-ordinator)

School of Food Science and Nutrition

Faculty of Mathematics and physical Sciences

University of Leeds

Email: fs07hs@leeds.ac.uk

Mobile: 07415780195

Thank you for taking the time to read this information sheet.

MEEC 14-029 - Appendix 2

Participant Consent Form

Research project title Effects of pomegranate and olive extracts on glycaemic response in vivo.

Name of researcher Hilda Nyambe

Initial the box if you agree with the statement to the left

1	I confirm that I have read and understand the Participant Information Sheet dated (TBC) explaining the above research project and I have had the opportunity to ask questions about the project.	☐
2	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without there being any negative consequences. In addition, should I not wish to answer any particular question or questions, I am free to decline. In this case I can contact the study co-ordinator by email: fs07hs@leeds.ac.uk or mobile: 07415780195	☐
3	I understand that my responses will be kept strictly confidential. I give permission for members of the research team to have access to my anonymised responses. I understand that my name will not be linked with the research materials, and I will not be identified or identifiable in the report or reports that result from the research.	☐
4	I agree for the data collected from me to be used in future research.	☐
5	I agree to take part in the above research project and will inform the principal investigator should my contact details change.	☐
6	I agree to be re-contacted for future research projects related to this study.	☐

Name of participant	Date	Signature
Address of participant	Mobile phone number	Email address
Lead Researcher	Date	Signature

MEEC 14-029 - Appendix 3

Pre-Study Questionnaire

D. Personal Information

Full name:

Age: Gender: Male Female

Height: Weight:

Contact phone no:

Email ddress:

E. Health History

Q2. Are you pregnant?

Yes No

Q3. Are you breast feeding?

Yes No

Q4. Do you have any of the following diseases/conditions?

Diabetes	Yes	No	I don't know
Digestive disease (e.g. Crohn's disease, celiac disease)	Yes	No	I don't know
Food allergy If yes state to what	Yes	No	I don't know
High blood pressure	Yes	No	I don't know

Q5. Do you regularly take any dietary supplements?

Yes No

Q6. If yes to Q5 above, what supplements?

F. <u>Lifestyle</u>

Q7. Are you currently taking any long term prescribed medication?

Yes No

Q8. Do you smoke?

Yes No

Q9. Are you currently on a special diet (for losing weight or fruit extracts supplements)?

Yes (specify)	No

Q10. 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 per day?

Yes (specify) (frequency)	No

Q11. 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 10 minutes continuously per day?

Yes (specify) (frequency)	No

Q12. Is there any more additional information you wish to give about your health and lifestyle?

Yes	No

Q13. If yes to Q12, kindly write below

_____

_____

MEEC 14-029 - Appendix 4

Blood glucose reading

Equipment and Materials

Glucometer with pricking device

Disinfection swabs

Gloves

Spray bottle with 70% ethanol

Plasters

Cotton wool balls

Protocol

13. After the volunteer is seated in a chair, with gloves on, position the volunteer s hand with the palm-side up.
14. To the selected finger, apply pressure to help the blood to flow.
15. Clean the fingertip with a disinfection swab by starting in the middle and working outward to prevent contaminating the area. Allow the area to dry.
16. Hold the finger and place a new sterile lancet on the centre of the fingertip and firmly press the lancet to puncture the fingertip.
17. Wipe out the first drop of blood with a sterile pad or cotton ball which may contain excess tissue fluid.
18. Place the strip and read the glucose concentration and record it according.
19. Afterwards, place a cotton wool ball or pad over the puncture site and let the volunteer hold it for a few minutes to stop the bleeding. If need be, place a plaster afterwards.

Waste disposal

During both the screening and the actual human study, all waste will be disposed in disposable bench top clinical waste bins. At the end of each session, the waste bins will be sealed, placed in orange bags and finally disposed in the clinical waste bins found in the laboratories to be treated as the rest of the waste according to the university regulations on disposing clinical waste.

Responsibilities and emergency procedures

Procedure	Person responsible	Potential Hazards	Existing controls	Emergency procedures
Finger prick with puncture device	Investigator	Injury and infection	Appropriate human study room will be used. Use of disinfectant wipes to clean the fingertip area before pricking with the puncture device. Use of one finger per time to avoid pricking the same finger more than once per visit.	Wash thoroughly the injured area with soap and warm water and disinfect injured area Avoid cross contamination and infection by keeping own injury covered with waterproof dressing. Seek immediate medical advice

MEEC 14-029 - Appendix 5

Study design

Advertisement → Volunteers asked to complete pre study questionnaire → Fasting glucose screening → Sign consent form → Three visits

First visit ⇓

One week

Second visit ⇓

One week

Third visit ⇓

Volunteers will consume

Either:

Reference (bread, water and 2 x 500 mg placebo capsules)

Dose 1 (bread, water, 500 mg placebo capsules and 500 mg pomegranate/olive capsules)

Dose 2 (bread, water, 2 x 500 mg pomegranate/olive capsules)

⇓

Blood glucose reading from finger prick using glucometer at 0, 15, 30, 45, 60, 90, 120, 150 and 180 minutes → Data analysis

MEEC 14-029 - Appendix 6

HUMAN STUDY VISITS DESIGN

	1ST VISIT	**2ND VISIT**	**3RD VISIT**
Volunteer 1	A	B	C
Volunteer 2	B	C	A
Volunteer 3	C	A	B

	1ST VISIT	**2ND VISIT**	**3RD VISIT**
Volunteer 4	B	A	C
Volunteer 5	A	C	B
Volunteer 6	C	B	A

	1ST VISIT	**2ND VISIT**	**3RD VISIT**
Volunteer 7	C	A	B
Volunteer 8	B	C	A
Volunteer 9	A	B	C

	1ST VISIT	**2ND VISIT**	**3RD VISIT**
Volunteer 10	A	C	B
Volunteer 11	C	B	A
Volunteer 12*	B	A	C

*Randomization will continue up to volunteer number 40

A = Reference (bread, water and 2 x 500 mg placebo capsules)

B = Dose 1 (bread, water, 500 mg placebo capsules and 500 mg pomegranate/olive capsules)

C = Dose 2 (bread, water, 2 x 500 mg pomegranate/olive capsules)

MEEC 14-029 Appendix 7 - Recruitment Poster

Volunteers needed to help investigate the effects of pomegranate and olive supplements on Glycaemic response (Blood glucose levels).

Are you

- **Healthy male or female aged 18-65**
- **Able to attend 3 sessions over three weeks not lasting more than 4 hours per session**
- **Willing to give small blood samples by finger prick.**

The study is looking at the effects of pomegranate and olive supplements on blood glucose response. Blood glucose will be measured after consumption of reference as well as after reference with 2 different doses of test capsules per visit.

If you need further information about this study, you can contact **Hilda Nyambe** by an e-mail fs07hs@leeds.ac.uk.

This study is supervised by Professor Gary Williamson, Food Biochemistry Group, School of Food Science and Nutrition, University of Leeds, UK.

You will receive £15 at the end of the study.

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