

**The chronic and acute effects of (poly)phenols on sucrase  
isomaltase using the Caco-2/TC7 cell model**

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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## Abstract

Evidence from animal models and human intervention studies indicates that polyphenols can potentially attenuate the postprandial glycaemic response by inhibition of  $\alpha$ -glucosidases, critical for carbohydrate digestion, and by attenuation of monosaccharide transport across small intestinal enterocytes. Therefore, this study evaluated the effect of polyphenols, including an oleuropein-rich olive leaf extract, on sucrose hydrolysis and transport. Controlling the glycaemic response reduces the risk of development and progression of type 2 diabetes, much like the anti-diabetic drug acarbose. An *in vitro* inhibition assay for sucrase and maltase activity was optimised using Caco-2/TC7 cells as a human enzyme source and compared to a rat enzyme. Inhibitors were more effective on human compared to rat sucrase while the reverse was true for maltase. Chronic sucrose exposure led to altered N- and O-glycosylation and the sucrase  $K_m$  increased by 15% compared to cells cultured in glucose. A chronic 3-day treatment with 1.5 mg/mL olive leaf extract reduced the sucrase specific activity by 31% and 26% for cells cultured in glucose and sucrose, respectively. Sucrase N-glycosylation increased in cells cultured in sucrose and this could have led to the decrease in  $K_m$ . Oleuropein treatment decreased cell surface sucrase by 41% when cultured long-term in glucose but not sucrose. Transport studies for glucose-cultured cells showed that chronic treatment reduced sucrase hydrolysis and attenuated of fructose transport and GLUT2-mediated glucose transport. These results show for the first time that chronic treatment with olive polyphenols can reduce the sucrose hydrolysis and modulate glucose and fructose transport. Changes in post-translational modifications of sucrase with different treatments opens up new areas of research. Investigations are warranted regarding the use of olive leaf extract in humans for glycaemic control after sugar consumption with the suggestion that sugar intake might impact the effectiveness of the treatment.

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## List of Abbreviations

BCA	bicinchoninic acid
Caco-2	colonic adenocarcinoma cells
CSID	congenital sucrase isomaltase deficiency
CV	coefficient of variation
ddPCR	droplet digital polymerase chain reaction
DMEM	Dulbeccos's modified Eagle's Medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EFSA	European Food Safety Authority
EGCG	epigallocatechin gallate
ER	endoplasmic reticulum
F	fructose
FAM	6-carboxyfluorescein
FBS	fetal bovine serum
G	glucose
GI	glycaemic index
GIP	gastric inhibitory polypeptide
GL	glycaemic load
GLP	glucagon-like peptide
GLUT2	glucose transporter 2
GLUT5	glucose transporter 5
GR	glycaemic response
HDL	high density lipoprotein
HPAE	high performance anion exchange chromatography
HPLC	high performance liquid chromatography
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LOQ	limit of quantitation
MALDI-TOF	matrix-assisted laser desorption/ionization - time of flight
NCBI	National Centre for Biotechnology Information
ND	not detected
NDNS	National Diet and Nutrition Survey
NS	not significant
ORF	open reading frame
PAD	pulsed amperometric detection
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PET	polyethylene terephthalate
PTFE	polytetrafluoroethylene
Pro-SI	Immature sucrase isomaltase
RNA	ribonucleic acid
RT	reverse transcription
SACN	Scientific Advisory Committee on Nutrition
SD	standard deviation
SEM	standard error of the mean

SGLT1	sodium dependent glucose transporter 1
SI	sucrase isomaltase
SPE	solid phase extraction
T2DM	type 2 diabetes mellitus
TBP	TATA-box binding protein
TBS	Tris buffered saline
TEER	trans-epithelial electrical resistance
UDP	uridine diphosphate
UV/Vis	ultraviolet-visible spectrophotometry



# 1 Introduction

## 1.1 Diabetes

### 1.1.1 Pathogenesis

The prevalence of type 2 diabetes has doubled over the last thirty years and affects an estimated one in ten people worldwide, and non-communicable diseases including cardiovascular disease and type 2 diabetes account for more deaths than infectious diseases (WHO, 2012). The incidence of type 2 diabetes in 2013 was estimated to be 382 million people worldwide and is projected to reach approximately 600 million by 2035 (Guariguata et al., 2014). The pathogenesis of type 2 diabetes involves the development of insulin resistance as well as impaired  $\beta$ -cell function. During its development, patients pass through a phase of impaired glucose tolerance. Glucose is required as fuel source in the body and the levels of glucose in the blood need to be constantly maintained. To meet the requirement, glucose is produced during fasting by gluconeogenesis in the liver and kidney and from breakdown of glycogen in the liver. Following meal consumption, glucose is formed from the breakdown of carbohydrates and passes through the gut leading to a rise in blood glucose. This prompts the release of insulin from  $\beta$ -cells in the liver which signals for uptake in the insulin-responsive tissues, in turn reducing blood glucose levels (Macdonald, 2014). A sign of impaired glucose homeostasis is insulin resistance, in which the insulin is less effective at signalling glucose uptake and hyperinsulinemia occurs to keep blood glucose normal. This can progress to the development of type 2 diabetes in which insulin secretion is now impaired and there is not enough insulin released to compensate for the

insulin resistance (Merlotti et al., 2014). Individuals at risk of cardiovascular disease and type 2 diabetes are referred to as having metabolic syndrome which is defined by the International Diabetes Association as including a minimum of two of the following: low HDL cholesterol, high serum triglycerides, high systolic or diastolic blood pressure and increased fasting glucose (Gu and Lambert, 2013).

Interventions that improve insulin sensitivity or reduce the stress on  $\beta$ -cells by reducing hyperglycaemia should aid in preventing the development and progression of type 2 diabetes. Postprandial blood glucose levels range from 3.8 to 7.6 mmol/L in healthy individuals and the function of the  $\beta$ -cells drops by 60% at levels above 6.6 mmol/L (Augustin et al., 2015). Hyperglycaemia is also involved in pathways leading to endothelial function and further diabetic complications (Augustin et al., 2015).

### **1.1.2 Glycaemic load/glycaemic index and health**

The post-prandial glycaemic response (GR) is the change in concentration of blood glucose after a food or meal is ingested. The glycaemic index (GI) is a system for indexing foods based on their glycaemic response; it is defined as the GR for a portion of food containing 50 g of available carbohydrate expressed as a percentage of 50 g of the control, usually glucose or white bread (Wolever et al., 1991; Augustin et al., 2015). Foods that are digested and absorbed quickly with  $GI \geq 70$  are called high-GI foods, whilst those with  $GI \leq 55$  are called low-GI (Aston, 2006). Glycaemic load (GL) refers to the available carbohydrates present in a serving of food. The GI depends on many



properties of the food, firstly the carbohydrate content. For example, the GI of the monosaccharide glucose is greater than fructose (Atkinson et al., 2008). Amylose, a straight-chain starch, is more readily digested than amylopectin, a branched starch, therefore would exhibit a higher GI (Morris and Zemel, 1999). Other food components influence GI, including fat and fibre content, both which lower GI (Björck and Elmståhl, 2003). Standardised methods exist for the determination of GI (Brouns et al., 2005).

A meta-analysis of 37 observational studies found that high GI diets increased the risk of chronic type 2 diabetes and heart disease (Barclay et al., 2008) which supports the notion that postprandial hyperglycaemia contributes to chronic disease risk. A more recent meta-analysis of three large cohorts in which the primary endpoint was type 2 diabetes also suggests that high GI diets are unfavourable (Bhupathiraju et al., 2014). In addition to epidemiological studies that demonstrate a diet with low glycaemic response plays a role in the prevention of metabolic syndrome, an intervention study meta-analysis demonstrated that reducing the glycaemic impact of the diet has a positive effect on health markers (Livesey et al., 2008). Fasting blood glucose was reduced with consumption of lower GI/GL foods and this effect was greater in participants with metabolic disorder or type 2 diabetes. There was no effect on fasting insulin levels, however the insulin sensitivity was improved even in non-diabetics (Livesey et al., 2008).

There is some debate regarding the use of GI/GL as a dietary recommendation. The diets used to evaluate effects from high and low GI differ in composition other than just the carbohydrate amount making the results

difficult to interpret and distinguish the dietary factors involved. Furthermore, inter-individual variations occur so not everyone will benefit from low GI/GL. In light of these discussions, an international consensus summit was held in to review the current body of evidence related to glycaemic response. The panel agreed that reducing postprandial glycaemia has recognised beneficial physiological effects and reducing the GI/GL of the diet is a suitable approach, although more beneficial to those with metabolic syndrome or obesity (Augustin et al., 2015). Overall studies do provide strong evidence to suggest that a low-GI diet can reduce hyperglycaemia and body weight. These in turn are well established at reducing the risk of insulin resistance,  $\beta$ -cell dysfunction, inflammation, endothelial dysfunction and blood pressure to improve overall health status (Aston, 2006) (Figure 1-1).

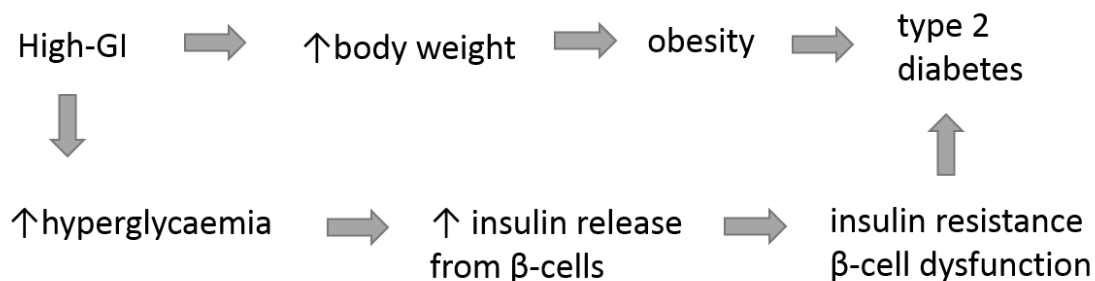


Figure 1-1. Relationship between GI and type 2 diabetes

### 1.1.3 Current diabetes treatment strategies

Pharmacological treatments for type 2 diabetes are based on the strategy of reducing the GI of the diet by slowing down carbohydrate digestion. These include the anti-hyperglycaemic drugs acarbose, volibose and miglitol, all of which are  $\alpha$ -glucosidase inhibitors (Breuer, 2003). Chronic 3-year treatment

with acarbose reduced the absolute risk of developing type 2 diabetes by 6% in patients with impaired glucose tolerance (Nijpels et al., 2008). In a similar double-blind randomised, placebo controlled trial, acarbose reduced the progression from impaired glucose tolerance to type 2 diabetes by 25% and improved glucose tolerance (Chiasson et al., 2002). Gastro-intestinal side effects are common with acarbose, and high study dropout rates have been reported in intervention trials (Nijpels et al., 2008).

In patients with metabolic syndrome, lifestyle modifications, diet and weight loss have been as effective as pharmacological treatments (Magkos et al., 2009). An intervention trial was performed on participants at high risk for diabetes to compare the anti-hyperglycaemic drug metformin to a group with intensive lifestyle modification. Participants with the modified lifestyle were guided to lose and maintain 7% of their initial body weight with a low calorie and low fat diet and perform moderate physical activity for 150 minutes per week. Lifestyle modification was able to reduce the incidence of diabetes by 58% compared to the placebo group while metformin led to a 31% reduction (Knowler et al., 2002). The large scale PREDIMED study investigate the relationship between the Mediterranean diet and health markers (Martínez-González et al., 2010). The intervention study group that followed a Mediterranean diet supplemented with olive oil was able to reduce relative risk of type 2 diabetes by 40% (Martínez-González et al., 2015). These results highlight that diet and lifestyle are important modifiable factors in both the prevention and treatment of type 2 diabetes.

## 1.2 Carbohydrate metabolism

### 1.2.1 Digestion and uptake

Carbohydrates play a key role in the diet as they yield the principle substrate of energy metabolism and influence the blood glucose response. The four main classes of readily available dietary carbohydrates are monosaccharides, disaccharides, oligosaccharides and polysaccharides. Monosaccharides and disaccharides are commonly referred to as sugars. The dietary monosaccharides are glucose, fructose and galactose whilst sucrose and lactose are disaccharides. A series of 3-7 or 10 monosaccharides linked together are defined as oligosaccharides and polysaccharides, respectively (Cummings and Stephen, 2007). Starch is a storage polysaccharides from plants such as cereals, root vegetables and legumes. It consists of two polymers: amylose and amylopectin, both containing only glucose molecules. Amylose contains glucose residues linked by  $\alpha$ -1,4 glycosidic bonds and amylopectin contains both  $\alpha$ -1,4 and  $\alpha$ -1,6 bonds and is highly branched (Morris and Zemel, 1999).

Carbohydrates must be digested into monosaccharides in order to be absorbed in the gut and this requires the action of several enzymes. Starch is digested by salivary and pancreatic  $\alpha$ -amylase (EC 3.2.1.1) which hydrolyses  $\alpha$ -1,4 bonds releasing maltose, maltotriose and  $\alpha$ -dextrans and some glucose (Cummings and Stephen, 2007). Further digestion to yield monosaccharides occurs by  $\alpha$ -glucosidases found at the brush border of the small intestine: sucrase isomaltase (EC 3.2.1.48, EC 3.2.1.10) (SI), maltase glucoamlyase (EC 3.2.1.20) (MGAM) and trehalase. The  $\beta$ -glucosidase lactase (EC 3.2.1.108) is

responsible for the hydrolysis of lactose from milk. The substrates for each of the enzymes are presented in Table 1-1. Sucrase isomaltase provides 80% of the total maltase activity as well as the majority of isomaltase activity (Van Beers et al., 1995) so plays a vital role in starch and sugar digestion and is further described in 1.2.4.

Table 1-1. Substrate specificities of intestinal brush border glucosidases. na= not applicable. (Adapted from Van Beers et al., 1995).

Enzyme	Subunit	Specificity	Substrates
<b>Trehalase</b>	na	$\alpha, \alpha'$ -trehalose	$\alpha, \alpha'$ -trehalose
<b>Maltase glucoamylase (MGAM)</b>	Maltase And Glucoamylase	$\alpha$ -1,4 glucosyl bonds	Maltose, amylopectin, glycogen, starch, amylose, isomaltose
<b>Sucrase isomaltase (SI)</b>	Sucrase	$\alpha$ -1,4 and $\alpha$ -1,2 glucosyl bonds	Sucrose, maltose
	Isomaltase	$\alpha$ -1,6 glucosyl bonds	Isomaltose, maltose
<b>Lactase</b>	na	$\beta$ -1,4 glucosyl bonds	Lactose, cellobiose, cellulose, glucosyl- and galactosyl-lactosylceramide

Glucose is primarily transported into small intestinal enterocytes by the sodium-dependent glucose co-transporter SGLT1 which is permanently present at the luminal (apical) side and has a  $K_m$  for glucose of 0.5 mM (Kellett et al., 2008; Röder et al., 2014). The facilitated transporter GLUT2 is also responsible for uptake of glucose at the apical side and it has been proposed that GLUT2 is translocated to the apical membrane to assist with glucose transport under conditions of high glucose (Kellett et al., 2008; Ait-Omar et al., 2011). Fructose is transported by GLUT2 and GLUT5, the latter being specific for fructose (Douard and Ferraris, 2008). GLUT2 is primarily responsible for transport of

sugars out of the enterocytes at the basolateral side and also for uptake into  $\beta$ -cells (Kellett et al., 2008).

### **1.2.2 Carbohydrate intake in the UK**

The Scientific Advisory Committee on Nutrition (SACN), who advise Public Health England, have recently recommended that daily intake of “free sugars” should be halved, down to no more than 5% of total daily calories. The newly adopted terminology “free sugars” is defined as “all monosaccharides and disaccharides added to foods by the manufacturer, cook or consumer, plus sugars naturally present in honey, syrup and unsweetened fruit juices. Under this definition lactose when naturally present in milk and milk products is excluded”. These were previously referred to as “non-extrinsic milk sugars” (SACN, 2015).

A recent report from SACN summarised data for the consumption of carbohydrates from The National Diet and Nutrition Survey with data from 3450 adults aged 19 years and over and 3378 children aged 1½-18 years collected over between 2008-2012 (PHE, 2014). For adults, 45% of total dietary intake was carbohydrates, in alignment with national recommendations. The mean intake of sugar was 95-103 g/day for adults, approximately 1/5<sup>th</sup> of total energy intake. All age groups on average had 11% of their total energy intake from sugar, higher than the original recommendation of 10%. The most commonly consumed sugar was sucrose, which accounted for half of the total sugar intake (40-50 g/day). Soft drinks and fruit were top contributors to total sugar, 13% each, followed by table sugar (sucrose) at 9%. The mean starch consumption was 110-135 g/day in adults (SACN, 2015). One of the key

factors behind the recommendation to decrease free sugars was the strong evidence that high sugar intake leads to increased energy intake, and therefore obesity, an established type 2 diabetes risk factor (Grundy et al., 2005).

### **1.2.3 Sugar and health**

Despite the fact that controlling glycaemic response has a positive effect on health markers, the commonly accepted perspective is that sugar is not a cause of diabetes, but is a cause of obesity which is a proven risk factor for diabetes (Zimmet et al., 2001; Grundy et al., 2005). However, a recent study found that sugar intake was an independent risk factor for diabetes and cardiovascular disease (Schmidt, 2014). In an analysis of population-level data from 174 countries, the relationship between obesity and diabetes was not directly associated. A statistical analysis of multi-variate data from numerous countries over time was conducted. It determined that sugar availability, based on food supply data, was found to be an independent and statistically significant determinant of diabetes (Basu et al., 2013). Similarly, the incidence of diabetes and obesity has been correlated to high availability of high fructose corn syrup, which consists of approximately 55% fructose and 45% glucose (Goran et al., 2013). The SACN report on carbohydrates highlights that there are a lack of studies to be able to draw conclusions on the health effect of individual sugars. Data from four randomised controlled trials with 1 to 6 months follow up provided evidence that higher consumption of sugars leads to increased energy consumption and is therefore detrimental to health (SACN, 2015). A meta-analysis of four cohort studies investigating sucrose, the most commonly consumed sweetener, found borderline association between high

sucrose and reduced incidence of type 2 diabetes (SACN, 2015). There was an association between sugar-sweetened beverages and type 2 diabetes and obesity (SACN, 2015). Dietary sugar intake can also indirectly lead to dysregulation of lipid and carbohydrate metabolism through increased body weight and fat. In agreement with the findings in the SACN report, a systematic review and meta-analysis of cohort studies and randomised controlled trials found that the intake of free sugars in *ad libitum* diets was associated to increased body weight due to an overall increase in energy intake (Te Morenga et al., 2013).

The metabolic consequences of glucose, sucrose and fructose intake differs. The GI of fructose and sucrose are lower than glucose (Atkinson et al., 2008) which prompted the food industry, particularly in the USA, to increase the use of fructose as a sweetener in the form of high-fructose corn syrup (Augustin et al., 2015; Stanhope, 2016). The correlation between fructose and fructose containing sugars with obesity and diabetes has prompted investigations into the potentially harmful effects of fructose, for which some controversy still remains. Glucose and fructose are utilised in glycolysis, gluconeogenesis, TCA cycle, lactate formation, pentose phosphate shunt and lipid synthesis however they enter these pathways differently (Sun and Empie, 2012). Glucose homeostasis requires insulin whereas fructose metabolism is insulin-independent (Laville and Nazare, 2009; Sun and Empie, 2012). Fructose stimulates *de novo* lipogenesis in the liver, which leads to increased triglycerides. This stimulation requires both glucose and insulin to be present; hence sucrose intake has lipogenic effects. The change in lipid composition of



cell membranes has an effect on the uptake of glucose, resulting in insulin sensitivity (Laville and Nazare, 2009; Lim et al., 2010; Douard, 2012). Furthermore, the elevated triglycerides from fructose or sucrose intake in turn causes obesity and leptin resistance, where satiety signals do not reach the brain and this leads to further consumption of food, to exacerbate the obesity problem (Lim et al., 2010). One of the harmful effects of fructose that is often reported is related to the involvement in the *de novo* lipogenesis pathway, however this pathway is minor in humans and requires excess energy intake, which could explain why some studies observed no deleterious effects compared to glucose (Laville and Nazare, 2009). Furthermore, a review of several studies suggests that fructose exerts harmful effects only with intake > 100 g/day and many studies reporting harmful effects of fructose use high concentrations that are not physiologically relevant and performed in a similar manner to toxicology studies (Augustin et al., 2015). The deleterious effects from fructose and fructose containing sugars on plasma lipids and *de novo* lipogenesis is still under debate (Sun and Empie, 2012; Stanhope, 2016). Further studies to investigate the effects of individual sugars on the development of diabetes will help provide valuable dietary recommendations to help tackle its rising incidence.

#### **1.2.4 Sucrase isomaltase**

Sucrase isomaltase (SI) is a highly N- and O-glycosylated brush border type-II membrane protein with various  $\alpha$ -glucosidase activities, and is trafficked to the cell surface through the association with lipid rafts (Naim et al., 2012). SI consists of two subunits, sucrase and isomaltase, which are cleaved by luminal

proteases but remain associated through non-covalent interactions (Quan and Gray, 1993). The sucrase subunit has both maltase ( $\alpha$ -1,4) and sucrase ( $\alpha$ -1,2) activities, while the isomaltase subunit has maltase ( $\alpha$ -1,4) and isomaltase ( $\alpha$ -1,6) activities (Jones et al., 2011) (Figure 1-2). SI is therefore involved in the digestion of both sugars and starch. Type 2 diabetic patients have increased levels of SI, which potentially increases the rate of sugar digestion and uptake and exacerbates the problem, making it an interesting target (Dyer et al., 2002). The sucrase subunit is the sole enzyme responsible for hydrolysis of sucrose. Maltase glucoamylase is another brush border which hydrolyses ( $\alpha$ -1,4) bonds from substrates including maltose and isomaltase as well as amylopectin, glycogen, starch and amylose, although its isomaltase activity is very minimal in comparison to SI (Van Beers et al., 1995). Sucrase isomaltase is more abundant in humans than maltase glucoamylase and accounts for 80% of maltase activity (Semenza, 1986). Of these two brush border  $\alpha$ -glucosidases, SI plays a greater role in starch and sugar digestion.

In the endoplasmic reticulum (ER), N-glycans are attached to SI which are essential for folding and movement out of the ER (Danielsen, 1992) and the size of 210 kDa represents this immature form of the enzyme, referred to as Pro-SI. At the Golgi complex, SI is further N- and O-glycosylated to yield mature SI, of size 240 kDa (Naim et al., 2012). In human intestinal biopsies, two subunits are detected due to the cleavage by luminal proteases, 120-140 kDa size is isomaltase and 140-150 size is sucrase (Conklin et al., 1975). The time required for processing of the Pro-SI to functional SI at the cell surface is between 1-3 hours (Danielsen, 1992). The first published sequence of the

human SI gene (Chantret et al., 1992) was used to predict that there are 18 N-glycosylation sites although the actual sites of glycosylation have not been confirmed. The active site of each subunit and the proteolytic cleavage site have been identified (Van Beers et al., 1995).

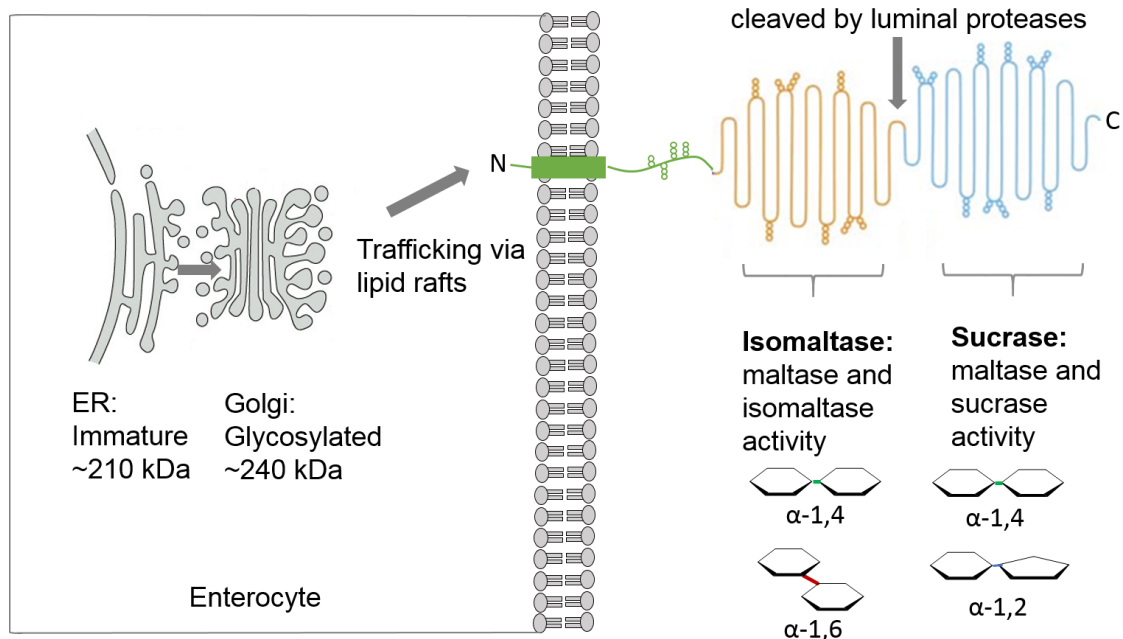


Figure 1-2. The sucrase isomaltase at the ER is N-glycosylated and this immature form is 210 kDa. Further N- and O-glycosylation occurs at the Golgi leading to the mature 240 kDa form which is then trafficked to the apical brush border via lipid rafts. The type-2 transmembrane protein is cleaved by luminal proteases into two active subunits which remain associated. The isomaltase subunit has maltase and isomaltase activity and sucrase subunit has maltase and sucrase activity.

The study of Congenital Sucrase Isomaltase Deficiency (CSID), in which digestion of sucrose is absent or reduced, has aided in uncovering information about SI trafficking and cell polarisation. The disorder is an autosomal recessive disorder that occurs in 0.2% of people of European descent and leads to abdominal pain and severe discomfort upon the ingestion of sucrose. The disorder has several phenotypes that have been identified and typically

only affects the sucrase subunit, with isomaltase activity remaining normal or sometimes absent. Point mutations in the isomaltase lead to phenotype I which has partial or absent sucrase and isomaltase activity due to alteration of trafficking. Only the immature 210 kDa form is present (Naim et al., 2012).

### **1.2.5 Regulation of sucrase isomaltase**

Regulation of SI has generally been reported as transcriptionally controlled (Van Beers et al., 1995). In Caco-2 cells, a common model of the small intestine, SI expression was related to glucose consumption; a low glucose consuming Caco-2 clonal cell line expressed higher levels of SI. In this clone, the amount of SI increased during differentiation and this corresponded to the mRNA expression levels (Chantret et al., 1994). The expression level of SI mRNA did not appear to change between cells cultured in 1 or 25 mM glucose (Mahraoui et al., 1994). Most previous reports on SI detected transcriptional regulation, for example forskolin and monensin both decreased SI activity in Caco-2 cells through reduced transcription (Chantret et al., 1993).

There have been some reports that SI can be regulated by its substrate and products. Studies in cells and rats have shown that monosaccharides have regulatory effects on sucrase at the mRNA level, but these compare the expression to that of a starved or low-carbohydrate state (Ferraris et al., 1992; Quan and Gray, 1993; Kishi et al., 1999) . In rats, a carbohydrate-free diet for 7-days decreased sucrase activity by 50%, there was no change in  $K_m$  but a decrease in  $V_{max}$ , indicating less available or active enzyme. The reduced activity was hypothesised to be due to changes in post-translational modifications, identified by altered migration on western blots (Quan and Gray,

1993). There have been several studies that investigated SI activity in response to dietary sucrose in rats and mice. Rats were fed a control sugar-free diet or either glucose, fructose or sucrose diets with three meals given in a 12 hour period. SI and SGLT1 mRNA levels were enhanced by sucrose in comparison to the no sugar diet (Kishi et al., 1999). Fructose and sucrose both increased mRNA to a greater extent than glucose. In another study in rats there was an increase in SI mRNA levels in as little as 3 hours after they were force-fed sucrose. Prior to sucrose loading, the rats had been fed a carbohydrate-free cellulose diet. The activity of sucrase also increased but an hour after the mRNA increase, suggesting approximately 1 hour for processing of SI to the functional form (Broyart et al., 1990). An increase in SI specific activity has also been observed in rats and mice fed a high sucrose diet in comparison to a low carbohydrate diet (Ferraris et al., 1992). A sucrose diet given to rats following a low starch diet induced sucrase and maltase activities within 6 hours and rats on high starch diet had increase level of SI activities compared to low-starch diet (Samulitis-dos Santos et al., 1992). These studies suggest that there is an increase in SI activity in response to sucrose after starvation or a low starch or no carbohydrate diet. The studies thus far do not provide evidence regarding whether sucrose, in comparison to glucose, can induce SI.

There is some evidence that the small intestine may be able to sense and respond to sugars (Le Gall et al., 2007). Human intestinal enterocytes in the duodenum express T1R2 and T1R3 taste receptors which are activated by sugar, upon which  $\alpha$ -gustucin within the cell activates adenylyl cyclase and cAMP increases (Alpers, 2010). The mRNA expression of the taste receptors

is negatively correlated to blood glucose levels in type 2 diabetic patients (Young et al., 2009). SGLT1 mRNA and protein are both upregulated by glucose, and in rats this was linked to the taste receptor cascade since mice without T1R3 or  $\alpha$ -gustducin no longer showed SGLT1 regulation by glucose (Margolskee et al., 2007). T1R2 and T1R3 have been identified on the basolateral membranes in Caco-2/TC7 cells (Le Gall et al., 2007). Glucose, fructose, sucrose and a mixture of glucose and fructose all stimulated the SI promoter activity in Caco-2/TC7 cells however the control was under starvation; it is expected that the addition of sugars will stimulate the promoter in comparison to the control which has no energy substrate provided (Le Gall et al., 2007).

Type 2 diabetic patients have increased levels of SI, GLUT2, SGLT1 and SGLT5 which potentially increases the rate of sugar digestion and uptake and exacerbates the problem (Dyer et al., 2002). The increase in SI was also observed in a diabetic rat model and was found to be due to a decrease in its degradation rate (Olsen and Korsmo, 1977). In obese patients with insulin resistance, an increase in GLUT2 was observed at the apical membrane of the gut, as compared to healthy lean individuals (Ait-Omar et al., 2011). An increase in intestinal digestion of carbohydrates was also observed in a diabetic mouse model (Adachi et al., 2003). Since the level of carbohydrate-digesting enzymes and transporters are altered during disease, a greater understanding of if and how their substrates are involved in their regulation is of interest.

## **1.3 Polyphenols and health**

### **1.3.1 Overview**

Historically, plant extracts have been used for therapeutic purposes, for example in Chinese traditional medicine and the observed health benefits are hypothesised to be in part due to their high phenolic content. It has been reported that up to 30% of patients with type 2 diabetes turn to alternative therapies such as plant supplements (Dragan et al., 2015), therefore an understanding of their mechanism of action is important. Polyphenols are plant secondary metabolites that are found in high abundance in plant based foods such as berries, fruits, soya, coffee, chocolate and beverages such as tea and coffee. Their basic structure includes an aromatic ring with one or more hydroxyl groups and they are classified into groups based on the number of phenol rings and how they are linked together. The estimated dietary intake of polyphenols is 1 g/day (de Bock et al., 2012). The effects of polyphenols on glucose homeostasis and their potential to aid in reducing the risk of diabetes has been extensively reviewed (Bahadoran et al., 2013; de Bock et al., 2012; Williamson, 2013; Hanhineva et al., 2010; Kim et al., 2016; Babu et al., 2013). Polyphenols have been associated with decreased incidence of disease including diabetes, cardiovascular disease and osteoporosis (Laville and Nazare, 2009; Hanhineva et al., 2010; Scalbert et al., 2005). For example, an epidemiological study suggests that polyphenol-rich tea and coffee can reduce the risk of type 2 diabetes (Huxley et al., 2009). Clinical intervention trials have been able to provide evidence for specific polyphenols; for example consumption of a dark chocolate rich in catechins and procyanidins for 15 days

was able to improve insulin sensitivity and reduce blood pressure in healthy patients and in those with hypertension (Grassi et al., 2005). A strong adherence to the Mediterranean diet was associated with a significant reduced risk of mortality from all causes, including a 9% reduction in mortality from cardiovascular disease (Sofi et al., 2008) and part of the protective effect is attributed to high consumption of polyphenol-rich foods (Martínez-González et al., 2015).

There are several mechanisms through which polyphenols could influence glucose homeostasis in the body and aid in prevention and development of type 2 diabetes and further complications (Thomas and Pfeiffer, 2012; Williamson, 2013; Hanhineva et al., 2010). Firstly, the small intestine is an important target for polyphenols to aid in glucose homeostasis by their ability to inhibit  $\alpha$ -glucosidases and subsequent transport of monosaccharides through the enterocytes (Hanhineva et al., 2010; Williamson, 2013). Postprandial polyphenol concentration is the highest in the gut lumen and can reach millimolar concentrations, making this a key site at which they may exert beneficial effects (Williamson, 2013). A common strategy for reducing the risk of metabolic syndrome is by inhibition of carbohydrate digestion to lead to reduced blood glucose response, much like the anti-hyperglycaemic drug acarbose (Van de Laar et al., 2005). Polyphenols have also exerted action at the liver with effects on  $\beta$ -cell function; ferulic acid and cinnamic acid derivatives promoted insulin secretion in a rat model (Adisakwattana et al., 2008). The uptake of glucose by tissues can also be influenced; for example, polyphenol polymers found in cinnamon exhibited insulin-like action (Anderson



et al., 2004). Finally, hepatic function can be affected; epigallocatechin gallate (EGCG) decreased endogenous glucose production in the liver (Wolfram et al., 2006) and the citrus polyphenols hesperetin and naringenin were able to reduce blood glucose through changed in hepatic glucose regulating enzymes (Jung et al., 2004). The observed health benefits of polyphenols in observational studies are a combination of these mechanisms exerted by complex mixtures of polyphenols in the whole diet. Further mechanistic studies and randomised controlled trials are required to identify particular polyphenols or combinations of polyphenols to make more detailed dietary recommendations and health claims.

### **1.3.2 Acute inhibition of $\alpha$ -glucosidases by polyphenols**

Polyphenols have the potential to act as  $\alpha$ -glucosidase inhibitors and therefore have the potential to lower the glycaemic response, much like the pharmacological treatments such as acarbose, described in Section 1.1.3. The *in vitro* inhibitory effect of polyphenols on  $\alpha$ -glucosidase activity has been previously reviewed and summarised (Hanhineva et al., 2010). Inhibition of  $\alpha$ -glucosidase and  $\alpha$ -amylase has been reported by flavonoids (anthocyanins, catechins, flavanones, flavanols, isoflavones), phenolic acids, and tannins. However, the published reports to date that evaluate the inhibition of  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes have substantial limitations. For example, it was recently reported that polyphenols can interfere with detection of assay products using the 3,5-dinitrosalicylic acid (DNS) assay which quantifies reducing sugars and is commonly used for  $\alpha$ -amylase assays (Nyambe-Silavwe et al., 2015). The aromatic DNS reagent reacts with reducing sugars

and the formation of 3-amino-5-nitrosalicylic acid is monitored by its absorbance at 540 nm. Inhibition assays for  $\alpha$ -glucosidase often use similar spectrophotometric techniques to quantify glucose, such as those based on glucose oxidase or hexokinase glucose assays. These assays are readily available and are commonly used because glucose does not have a chromophore so cannot be readily quantified directly without specialised analytical systems such as ion chromatography. It is hypothesised that polyphenols may also interfere with these enzymatic techniques and this is rarely accounted for in the literature.

Although human enzymes are always the intended target for therapeutic interventions, many studies use the more readily available rat intestinal preparations *in vitro*. However, there is only 74% homology between the rat and human sucrase isomaltase (Van Beers et al., 1995), which raises the question as to whether using the rat assay is physiologically relevant to humans. Some studies use yeast enzyme sources, but flavonoids exert different inhibitory effects towards yeast or rat  $\alpha$ -glucosidase; anthocyanidins, isoflavones and flavonols were strong inhibitors of yeast  $\alpha$ -glucosidase with  $IC_{50}$  values less than 15  $\mu$ M, whereas they only weakly inhibited rat  $\alpha$ -glucosidases with  $IC_{50}$  values at low mM concentrations (Tadera et al., 2006). There are limited studies investigating inhibition of sucrase from a human source. One report investigated inhibition of  $\alpha$ -glucosidases by an extract from mulberry leaves using the human Caco-2 cell model with cells cultured on porous filters, however this method involves the subsequent transport and metabolism of the enzymatic assay products (Hansawasdi and Kawabata,

2006). Further experiments to investigate the effects on transport independently from the digestion would be required to identify the effect on the digestive enzymes alone. Other inhibition assays have been developed using human Caco-2 cell lysates, however one failed to address the potential interference of the inhibitors on the detection method and limited assay kinetics were provided (Krog-Mikkelsen et al., 2011). Another assay provided details to satisfy assay kinetics, but samples required further processing including derivatisation before GC/MS analysis, which may not be as practical for large scale screening, and additionally the cells were not fully differentiated (Jockovic et al., 2013).

Additionally, some literature reports use *p*-nitrophenyl  $\alpha$ -D-glucoside as the substrate however this substrate is non-specific so the effects on specific enzyme activities are indistinguishable. The concentration of the inhibitors is another important consideration. Some polyphenols have poor solubility. For example, quercetin has been reported to be an inhibitor, with an  $IC_{50}$  value of 1450  $\mu$ M towards sucrase and 710  $\mu$ M towards maltase (Ishikawa et al., 2007). However, quercetin requires ethanol or DMSO for solubility (Srinivas et al., 2010) and would therefore have limited solubility in aqueous solutions so would not be soluble at these high concentrations in assay buffer. It is probable that quercetin had precipitated from the solution and interfered with the detection by spectrophotometry. Furthermore, flavonoids can absorb in UV/Vis so their presence in the sample will affect the glucose quantification.

In setting up an inhibition assay, the time course and enzyme activity need to fall within the linear range. Outside of the linear range, the reaction reaches the

steady-state does not apply so Michaelis-Menten kinetics are not valid (Bisswanger, 2014). Furthermore, if the substrate concentration is too high, the ability of competitive inhibitors to bind and inhibit an enzyme is reduced (Acker and Auld, 2014). The amount of enzyme units present in the assay can also affect the inhibition outcome (Nyambe-Silavwe et al., 2015). It is therefore essential that the assay is optimised prior to use; some literature reports fail to describe the assay conditions in full, making comparison between different reports difficult.

Although there are an abundance of reports on polyphenols and  $\alpha$ -glucosidase inhibition, experiments that take into account these issues are required to confirm and strengthen the evidence. There are very few results in the literature with strong evidence of sucrase inhibition by polyphenols using the human enzyme.

### **1.3.3 Acute inhibition of glucose transport**

Selected polyphenols can inhibit glucose transport and the first reported effect was by the flavonoid glucoside phloridzin and was evaluated in a hamster model using radiolabelled glucose (Alvarado, 1967). Phloridzin is now a well-established inhibitor of glucose transport that is specific to active transport via SGLT1. The first use of the human intestinal cell model Caco-2 to evaluate acute inhibition of glucose transport by polyphenols screened several classes of compounds (Johnston et al., 2005). Radiolabelled glucose (D-[6-<sup>3</sup>H]-glucose) was used as the substrate on the apical side and the radioactivity was determined after transport on apical and basolateral sides. Inhibition of glucose transport via SGLT1 occurred with 100  $\mu$ M neohesperidin dihydrochalcone by a

similar extent to the phloridzin control. The aglycones quercetin, phloretin, apigenin, and myricetin inhibited glucose transport via GLUT2. The catechins (-)-epigallocatechin gallate, (-)-epicatechin gallate and (-)-epigallocatechin were able to inhibit glucose transport by both facilitated transport by SGLT1 and active transport by GLUT2 (Johnston et al., 2005). A review of polyphenols and glucose homeostasis summarises other *in vitro* transport assays (Williamson, 2013). For example, strawberry and apple polyphenols inhibited transport via both GLUT2 and SGLT1, with stronger effects on GLUT2 (Manzano and Williamson, 2010).

#### **1.3.4 Chronic effects on $\alpha$ -glucosidases and transport**

Chronic intake of acarbose in patients with impaired glucose tolerance led to a 6% reduction in the risk of the progression to type 2 diabetes (Nijpels et al., 2008). The mechanism of action of acarbose is through its inhibition of starch and sugar digestion so it is possible that polyphenols, with their similar inhibitory effect, might also reduce the risk of diabetes. Other mechanisms by which acarbose could lead to chronic health benefits have not been established. There are other chronic mechanisms which could lead to reduction in starch or sugar digestion. For example, reduced expression or protein levels of an enzyme or decreased trafficking to the cell surface could lead to less active enzyme present at the cell surface. This in turn would lower the glycaemic response chronically without the requirement for direct inhibition. Acute effects require the inhibitor to be consumed with the meal, however a chronic change in the level of the enzyme would provide a more constant change. This approach would be particularly suited to diabetic patients who

have an increase in the level of  $\alpha$ -glucosidase enzymes and transporters (Dyer et al., 2002).

The *in vitro* studies that investigate the effects of polyphenols on  $\alpha$ -glucosidase enzymes and glucose transport have been predominantly acute studies (Section 1.3.3). There are limited reports where chronic treatments have been used (Section 1.3.4). One study found that an anthocyanin-rich berry extract reduced glucose transport by GLUT2 after a chronic 16 hour treatment in Caco-2/TC7 cells (Alzaid et al., 2013) but there are very few other studies investigating chronic effects of polyphenols in the small intestine. Polyphenol rich diets such as the Mediterranean diet are linked to reduction in chronic diseases, however, there is a gap in knowledge regarding mechanisms by which these chronic health benefits occur. More studies using chronic treatments are required to help understand the health benefits of regular consumption of polyphenol-rich foods.

#### **1.4 Caco2/TC7 cell model**

The Caco-2 cell line is a primary cell line that was derived from a colon carcinoma and is commonly used as a model for the small intestine. Upon differentiation, these cells form a polarised monolayer with an apical brush border, representing the gut lumen, and basolateral side, representing the interstitial fluid, and they express small intestinal hydrolases and transport proteins (Sambuy et al., 2005). Caco-2 cells have been used for the investigation of polyphenol transport (Teng et al., 2012; Kosińska and Andlauer, 2012) and to investigate the influence of polyphenols on glucose transport (Johnston et al., 2005). The model is also reported to be suitable for

screening compounds for their effect on glycaemic response. First, inhibition of sucrase was observed by the naturally occurring pentose *L*-arabinose using the Caco-2 cell model. Next, a cross-over design human intervention study determined the effect *in vivo* using sucrose beverages with and without *L*-arabinose. Attenuation of both the blood glucose and insulin responses occurred which validated the use of the *in vitro* assay in identifying inhibitors that will have *in vivo* effects (Krog-Mikkelsen et al., 2011).

Clones of the Caco-2 cell model were created for the purpose of investigating the transcriptional control of SI as it had been observed that glucose could play a role. The clones from a late passages had increased growth rate and cell density, were more homogeneous and had a 7-10 fold increase in sucrase activity whereas the clones from a lower passage demonstrated increased glucose consumption and lower SI activity (Chantret et al., 1994). The TC7 clone was described as a low glucose consuming clone and it is commonly used due to the fact that the cells are more stable and have more homogenous expression of proteins as compared to the parental line (Sambuy et al., 2005; Caro et al., 1995). Upon review of their characteristics, they have been recommended as a more suitable model for the small intestine as compared to the parental line (Sambuy et al., 2005). Further, they have also been characterised for the purpose of drug transport and transformation studies and are favourable to the parental line due to greater taurocholic acid transport, higher activity of UDP-glucuronosyltransferases and highly inducible cytochrome P450IA1 genes, all of which are important characteristics for drug metabolism studies (Caro et al., 1995). The experiments in this thesis involved

SI and the monosaccharide transporters SGLT1, GLUT2 and GLUT5, all of which are present in the small intestinal enterocytes and are concomitantly expressed in the Caco-2/TC7 cell line (Mahraoui et al., 1994). The glucose transporters GLUT1 and GLUT3 are also expressed in Caco-2/TC7 cells however they will not play a role in glucose transport in this model because their expression is high during the initial phase of growth and then very low after Day 20 (Mahraoui et al., 1994) and this is similar to the *in vivo* situation where glucose transport occurs through GLUT2 and SGLT1 (Kellett et al., 2008). The homogeneity of the Caco-2/TC7 cell line, expression of SI, GLUT2, and GLUT5 and the fact that SI mRNA and protein remain stable up until at least passage 40 (Chantret et al., 1994) make it a suitable model for studying sucrose hydrolysis and subsequent transport of the monosaccharides.

### **1.5 Project Aims and hypothesis**

This thesis investigates the hypothesis that in addition to acute inhibition of sucrase activity, polyphenols might exhibit chronic reduction in sucrase activity. The  $\alpha$ -glucosidase enzyme sucrase isomaltase is the main focus in this thesis since sucrose is the most commonly consumed sweetener in Europe and this enzyme is involved in both starch and sugar digestion. Furthermore, sucrose is specifically hydrolysed by sucrase whereas activities towards other starch and sugar substrates arise from several enzymes. The mechanisms that are evaluated include transcription, translation, processing and trafficking, hydrolysis and transport (Figure 1-3).



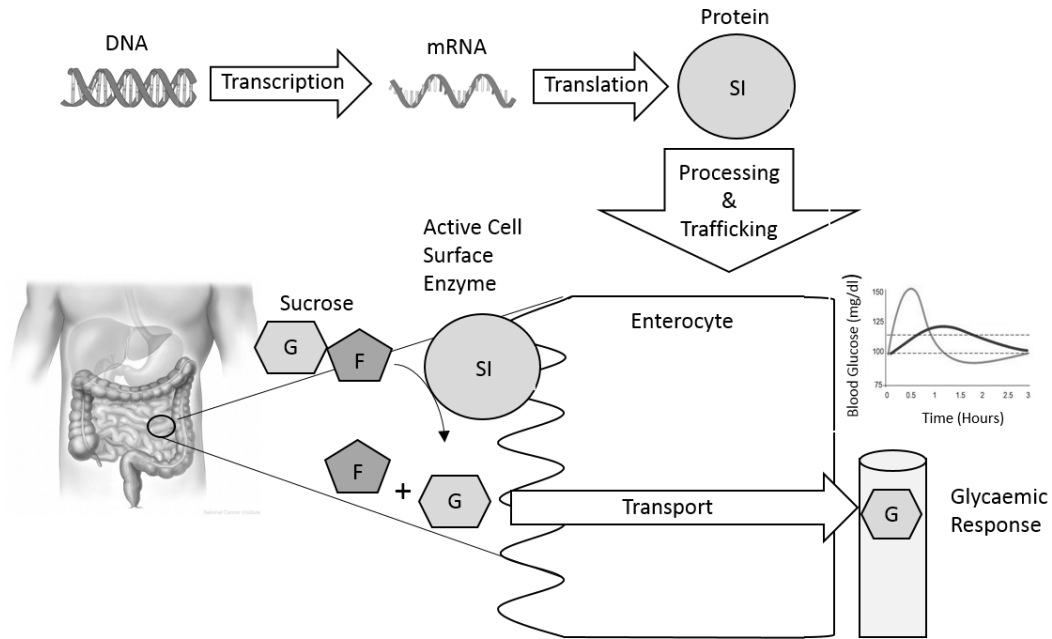


Figure 1-3. Potential mechanisms by which polyphenols could affect sucrase isomaltase and the subsequent glycaemic response. After a chronic treatment, the effects on transcription and translation can be determined by quantifying changes in mRNA and protein. The effects on post-translational modifications and trafficking can be evaluated by determining cell surface protein and analysis of the enzyme size. The activity of sucrase enzyme can be determined with *in vitro* enzyme assays and sugar transport studies can be performed.

Diabetic patients have increased levels of sucrase isomaltase therefore lowering its expression through chronic polyphenol intake would be beneficial in controlling the glycaemic impact in response to sucrose leading to health benefits. This hypothesis is investigated and addressed using the Caco-2/TC7 cell model with the following aims:

- 1) To optimise a method for the acute inhibition of sucrase and maltase by polyphenols which will address some of the limitations that were presented in Section 1.3.2. (Chapter 3).

- 2) To characterise the Caco-2/TC7 cell model and evaluate the effects of glucose and sucrose exposure on sucrase isomaltase (Chapter 4).
- 3) To evaluate if a polyphenol-rich extract from olive leaves that is known to be an acute sucrase and maltase inhibitor exhibits any chronic changes to sucrase isomaltase with the goal of reducing sucrase activity (Chapter 5).
- 4) To perform transport studies with glucose or sucrose as the substrate using Caco-2/TC7 cells that are cultured *in situ* on porous supports to further investigate the chronic and acute effects of the polyphenol-rich olive leaf extract (Chapter 6).

## **2 General methodology**

### **2.1 Caco-2/TC7 cell culture**

#### **2.1.1 Materials and equipment**

Caco-2/TC7 cells were a kind gift from Dr M. Rousset, U178 INSERM, Villejuif, France. Dulbeccos's modified Eagle's Medium, fetal bovine serum, glucose, non-essential amino acids, penicillin/streptomycin solution, sucrose, trypsin were from Sigma Aldrich, Dorset, UK. Dulbeccos's modified Eagle's Medium without glucose, Glutamax™ and SYBR® Safe stain were from Thermo Fisher, Loughborough, UK. Cell culture flasks and plates (75 cm<sup>2</sup> flasks, 6-well plates, 12-well plates, 6-well and 12-well transwell® plates with PET membrane at 0.4 µM) were from Corning, Amsterdam, The Netherlands. LDH cytotoxicity detection kit was from Roche, Burgess Hill, UK. Human intestine tissue total RNA reference sample was from DV biologics, California, USA. Zymoclean™ gel extraction PCR recovery kit was from Zymo Research, California, USA. Cloneamp HiFi PCR premix and In-fusion cloning kit were from Takara Bio Europe, Saint-Germain-en-Laye, France

#### **2.1.2 Medium preparation**

The standard Caco-2/TC7 cell culture medium contained high glucose (25 mM) Dulbeccos's modified Eagle's Medium (DMEM) supplemented with 20% (v/v) fetal bovine serum, 2% (v/v) Glutamax™, 2% (v/v) non-essential amino acids, 100 U/mL and 0.1 mg/mL streptomycin. Apical medium was prepared as above but with the absence of fetal bovine serum. For comparison of 25 mM glucose and 25 mM sucrose, DMEM medium with no glucose was supplemented with

either 25 mM glucose or 25 mM sucrose and with 20% (v/v) fetal bovine serum, 2% (v/v) non-essential amino acids, 100 U/mL penicillin and 0.1 mg/mL streptomycin.

### 2.1.3 Cell line maintenance and seeding

Caco-2/TC7 cells were routinely cultured at a density of  $1.2 \times 10^6$  cells per 75 cm<sup>2</sup> culture flask in standard medium and maintained in an incubator at 37°C with 10% (v/v) CO<sub>2</sub>. Medium was replaced every two days and at this seeding density, 80% confluence was reached in three days at which point the cells were sub-cultured by detaching with 0.25% (v/v) trypsin. All cell counting was carried out using a haemocytometer. Cell Seeding for experiments was carried out according to Table 2-1. A diagram of the transwell® filters is presented in Figure 2-1. Cell culture on transwell® filters allows the formation of a polarised monolayer and access to the apical side, representing the gut lumen, and the basolateral side. From day 1-7, regular medium containing FBS was added to both sides. From confluence at day 7, apical medium (no FBS) was added on the apical side for the remaining days of culture.

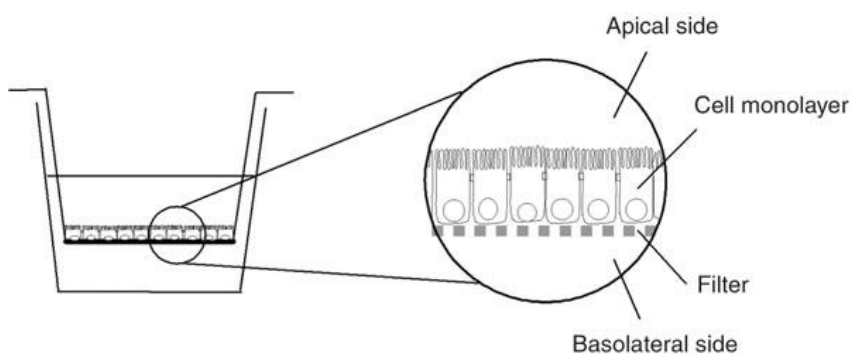


Figure 2-1. Transwell® filter plates for polarised cell culture

Table 2-1. Seeding Density for Caco-2/TC7 Cells

Plate Description	Seeding Density (cells/cm <sup>2</sup> )	Growth Area (cm <sup>2</sup> )	Medium Volume (mL)	Corning Product Number	Experiment Types
<b>6-well solid support</b>	64000	9.5	2 mL	3506	mRNA, protein
<b>12-well solid support</b>	64000	3.8	1 mL	3512	mRNA, protein, LDH Assay
<b>6-well transwell®</b>	64000	4.67	2 mL basolateral, 1 mL apical	3450	Cell surface biotinylation
<b>12-well Transwell®</b>	64000	1.12	1 mL basolateral, 0.5 mL apical	3460	Transport

#### 2.1.4 Chronic treatments

Intestinal enterocytes differentiate *in vivo* as they move from the crypt towards the villus tip where they are shed every 4-5 days as a self-renewal process (Umar, 2010). During cellular differentiation, the cells will be exposed to the nutrients within the intestinal lumen. In an attempt to mimic the *in vivo* exposure *in vitro*, Caco-2/TC7 cells were treated with polyphenol-rich extracts over the final three days of differentiation; days 18, 19, 20 with experiment on day 21. After chronic treatment, the effect on sucrase isomaltase mRNA, sucrase activity and sucrase isomaltase total and cell surface protein and the effect on glucose and sucrose transport across Caco-2/TC7 cells was evaluated. For treatment on solid supports, the treatment extract was dissolved directly into medium and then sterile filtered with 0.2 µM filter prior to adding to the cells. For treatment on transwell® filters, the treatment extract was dissolved into apical medium, sterile filtered and then added to the apical side only.

### **2.1.5 Cell viability by LDH**

Caco-2/TC7 cell viability was determined for experiments with chronic treatments. Cells were seeded on 12-well solid supports and maintained and treated as previously described (Section 2.1.3 and 2.1.4) and viability was determined using the LDH cytotoxicity detection kit according to the manufacturer's protocol. Lactate dehydrogenase (LDH) is released into cell culture medium in cells that are damaged and no longer viable, so the activity of LDH released into the medium can be used to determine the viability of the cells.

## **2.2 Gene expression**

### **2.2.1 Materials and equipment**

The QX200 Droplet Digital PCR system which includes the Biorad C1000 Touch™ Thermal Cycle with Deep Well Reaction Module, QX200™ droplet Reader, QX200™ Droplet generator and PX1™ PCR plate sealer, all consumables for the system and the ddPCR™ Supermix (1863024) were from Biorad Laboratories UK, Hemel, Hertfordshire, UK. The GoScript™ Reverse Transcription System was from Promega, Madison, WI, USA. The RNAqueous® kit was from Ambion, Life Technologies, Thermo Fisher Scientific, Loughborough, UK. The TaqMan® probes listed in Table 2-2 were from Life Technologies, Paisley, UK. The StepOne Real time PCR System was from Applied Biosystems, Life Technologies Ltd, Cheshire, UK.

Table 2-2. Taqman® Primers and probes used for digital PCR

Gene ID	Target	Life Technologies Primer/Probe ID
<b>CDX2</b>	CDX2	Hs01078080_m1
<b>SLC2A2</b>	GLUT2	Hs01096905_m1
<b>SLC2A5</b>	GLUT5	Hs01086390_m1
<b>SLC</b>	SGLT1	Hs01573790_m1
<b>SI</b>	sucrase isomaltase	Hs00356112_m1
<b>TAF1</b>	TBP	Hs00936234_m1

## 2.2.2 Digital PCR

### 2.2.2.1 Background

Droplet digital PCR (ddPCR) was used to quantitate changes in gene expression. The method of ddPCR involves partitioning a gene amplification PCR reaction into more than 15,000 nano-litre sized droplets prior to performing the PCR cycle, and then reading each individual droplet as either positive or negative for the gene of interest. The template for the reaction is spread over the droplets with the requirement that some of the droplets will be negative meaning they contain no template of the gene of interest and others will be positive for the template of the gene of interest. Quantitation is based on the proportion of positive droplets relative to the total number of droplets and their known volume, yielding a result in copies/ $\mu$ L of the gene of interest. This calculation is based on a Poisson distribution with Poisson correction to correct for the fact that some droplets may contain multiple copies of the template (Huggett et al., 2013). The PCR detection uses TaqMan® probes in which the probe for the gene of interest is fluorescently conjugated to 6-carboxyfluorescein (FAM) with absorption at 494 nm and emission at 518 nm and the probe for the reference gene is conjugated to reference dye VIC with

absorption at 538 nm and emission at 554 nm. The different fluorophores allow for simultaneous determination of the gene of interest with the reference gene. The tata-box binding protein (TBP) was used as the house-keeping/reference gene to correct for gene expression, allowing quantification and comparison of the relative expression of various genes analysed. A method summary is presented in Figure 2-1.

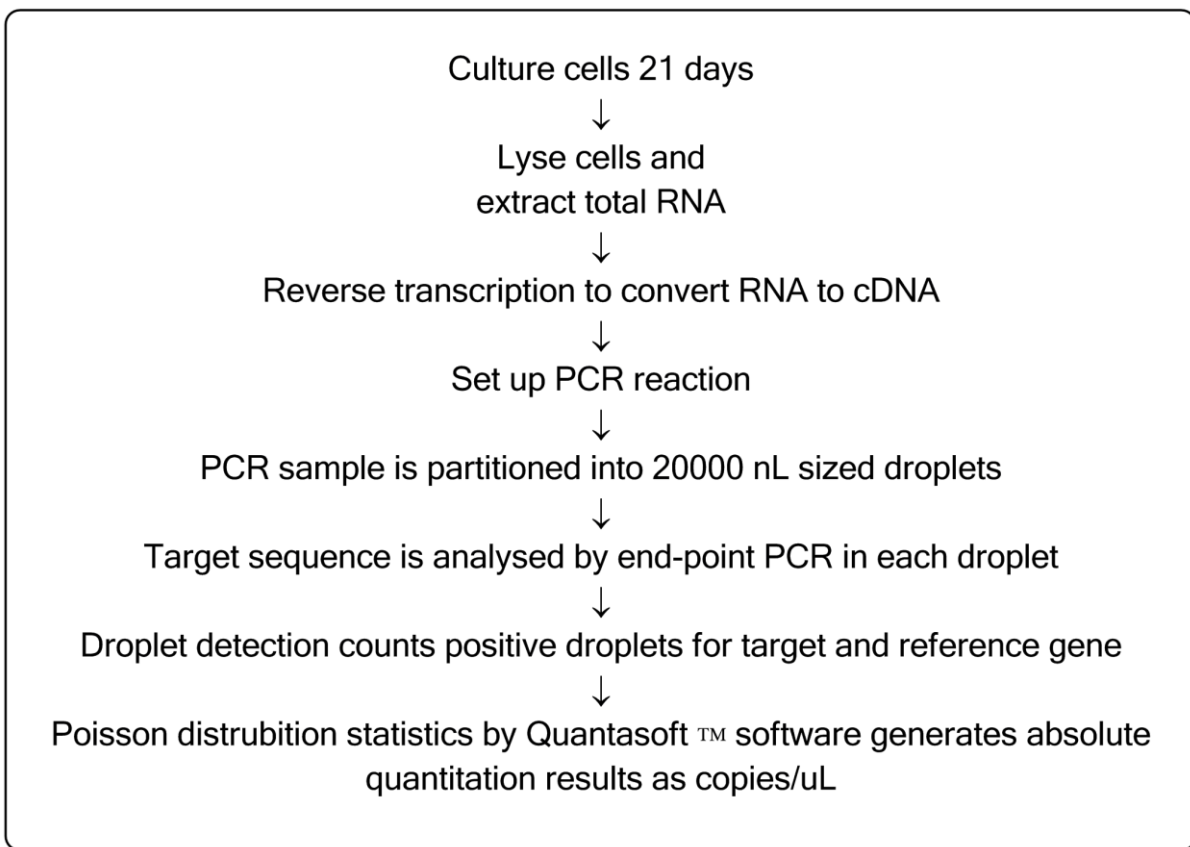


Figure 2-2. Droplet digital PCR method flow chart

#### **2.2.2.2 Procedure**

Caco-2/TC7 cells were seeded on 6-well culture plates and grown as described in 2.1.3. On day 21, total RNA was extracted using the RNAqueous Total RNA Isolation kit according to the manufacturer's protocol. The concentration of the



sample was determined using the Nanodrop®. 250 ng of total RNA was converted into cDNA by reverse transcription using the GoScript™ Reverse Transcription System, according to the manufacturer's protocol. Each 20 µL assay consisted of individual cDNA template (2.5 ng for SI, CDX2 or 5 ng for GLUT2, GLUT5, SGLT1), 1 µL of 20x FAM-labelled Taqman primer for gene of interest and 0.33 µL of 60x VIC-labelled Taqman primer for TBP (TATA-box binding protein) housekeeping control and 10 µL ddPCR Supermix. Droplets were prepared with QX100 droplet generator before cycling in C1000 touch thermal cycler: 95°C for 10 min followed by 40 cycles of 94°C for 30 sec, 57.8°C for 60 sec with a final incubation at 98°C for 10 min. The droplets were analysed on a QX100 droplet reader and analysed using QuantaSoft software which determines the concentration of target cDNA in copies/µL based on the Poisson distribution from the fraction of positive droplets (>10000 droplets per assay). Each individual cDNA sample was analysed with technical triplicates. Results are reported as the ratio between target and housekeeping gene copies/µL.

### **2.2.3 Sequencing the SI gene from Caco-2/TC7 cells**

#### **2.2.3.1 Background**

The method for the preparation of gene-specific DNA for sequencing analysis is presented in Figure 2-3. Briefly, total RNA was extracted from cells and converted to single stranded complementary cDNA using a gene-specific primer for the reverse transcription reaction. The primer was designed to be located outside of the open reading frame (ORF) for the gene of interest. The cDNA products were of varying lengths and then PCR was used to amplify the

cDNA containing the full length of the gene. For the PCR reaction, gene-specific PCR primers were used that anneal to the start and end of the gene of interest. The PCR product was run on an agarose gel next to a size ladder to identify the band at the size of the gene of interest. This band was extracted from the gel using a gel extraction kit and the purified gene-specific DNA was sent for Sanger sequencing analysis.

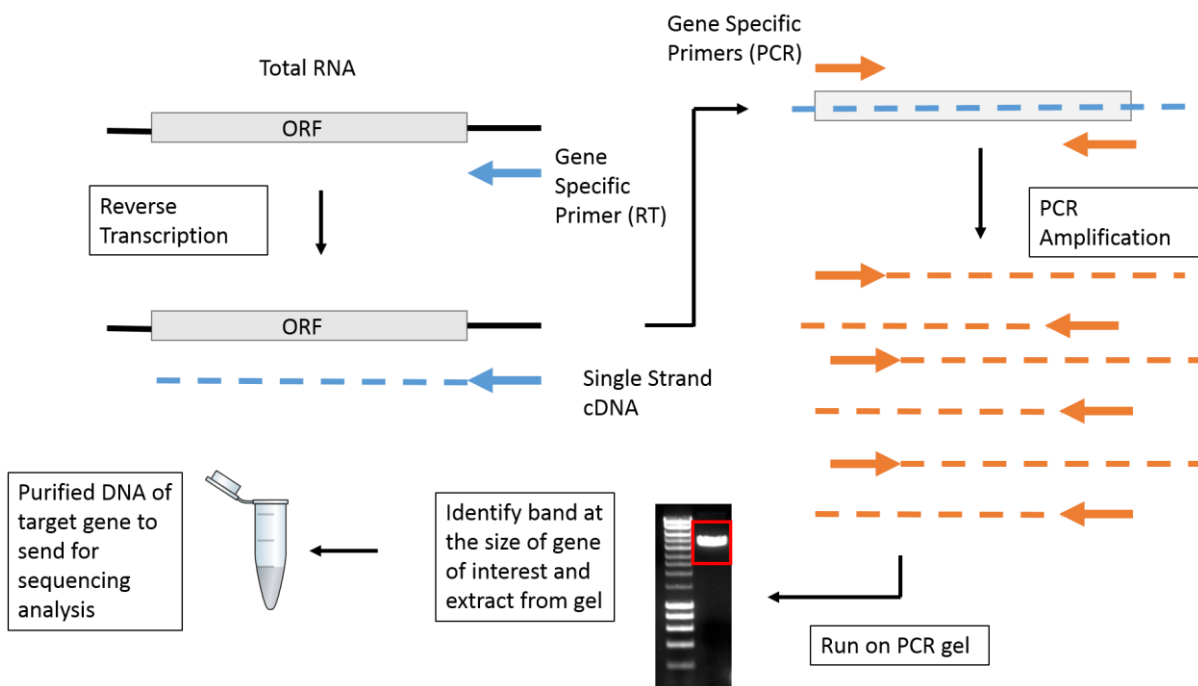


Figure 2-3. Method flow chart for preparation of gene-specific DNA for sequencing. Reverse transcription of total TNA is performed using a gene-specific primer and the resulting cDNA is amplified with gene-specific primers. The amplified product is run on a PCR gel and the band at the size of the target gene is extracted and sent for Sanger sequencing analysis.

### **2.2.3.2 Primer design and reverse transcription to generate cDNA**

The reference sequence for human sucrase isomaltase mRNA was obtained from the NCBI database (NM\_001041.3) and used to design primers. Sucrase isomaltase was reverse transcribed in two parts due to the length (~5.5 kb). Two sucrase isomaltase gene-specific primers were designed; the first was located beyond the stop codon of the open reading frame for sucrase isomaltase and the other was mid-way through the sequence at position 1811 of the reference sequence:

RT primer 1	5' tagagtacaagaaccaagtgaagaggggaaaattg
RT primer 2	5' cagcaaattgtgagcgggtaaga

Total RNA was extracted from Caco-2/TC7 as described in Section 2.2.2.2. The human intestine tissue total RNA sample was analysed as a control. Reverse transcription (RT) was performed on both total RNA samples using the GoScript™ RT System with the sucrase isomaltase gene-specific primers. One control sample was prepared without any RNA template and another control sample was prepared without any RT enzyme, to check for DNA contamination.

### **2.2.3.3 PCR amplification of sucrase isomaltase**

Two sets of primers were designed for the amplification of the two RT products:

1- forward	5' ccatggcgggcattgctgctgaccagttgatttctattg
1- reverse	5' cagcaaattgtgagcgggtaaga
2- forward	5' gcttcattcttscggctcaacatttgctg
2- reverse	5' cgctaccggtcgccaccatggcaagaagaatttag

The PCR reaction to amplify sucrase isomaltase from the cDNA sample was performed using the Cloneamp™ HiFi PCR Premix. The PCR sample contained 4% DMSO to enhance unwinding of the DNA. The PCR cycle started at 98°C for 1 min, followed by 40 cycles of 98°C for 30 sec and 72°C for 6 min. The samples were then held at 72°C for 12 min and finally kept at 4°C. The resulting PCR products were run on a 0.1% agarose gel containing SYBR® Safe stain for the visualisation of DNA. The two PCR product bands were extracted from the gel using the Zymoclean™ gel extraction PCR recovery kit. The two PCR products overlap and cover the entire coding region of SI so were cloned together using the In-fusion cloning kit which uses a recombinase enzyme to fuse the overlapping homologous sequences together. Lastly, the full SI product produced through the cloning kit was amplified by PCR using the original PCR primers 1-Forward and 2-Reverse and the product was run on a 0.1% agarose gel to confirm the size of the product. The band was extracted from the gel as described previously and sent for DNA Sanger sequencing by Beckman Coulter Genetics. Table 2-3 lists the primers that were used for the sequencing analysis.

Table 2-3. Primers used for Sanger sequencing of SI mRNA

Primer Name	Sequence
<b>Suc510-F</b>	5' actcaaaatcagacacccaatcgtttccgg
<b>Suc1174-F</b>	5' gtgtaaggagaaaccgggaagctggc
<b>Suc1811-F</b>	5' gcttcattctaccgctcaacattgctg
<b>Suc2461-F</b>	5' caagaaccagatgtaacaacaacagcaagc
<b>Suc3014-F</b>	5' cagtcaactcagctcgctattcatccatggg
<b>Suc3594-F</b>	5' ccagccaactcctgctctaactacc
<b>Suc4161-F</b>	5' caggactccacagcagagtgggtgggc

## **2.3 Protein analysis**

### **2.3.1 Materials and equipment**

Bradford reagent, octyl  $\beta$ -D-glucopyranoside, phosphate buffered saline with  $MgCl_2$  and  $CaCl_2$  (PBS+), protease inhibitor cocktail, sodium chloride, and trizma base were from Sigma-Aldrich, Dorset, UK. Rapid PNGase F was from New England Biolabs, Hitchin, UK. Cell surface protein isolation kit, NuPage LDS 4x sample buffer, and Pierce™ Microplate BCA protein assay kit were from Thermo Fisher Scientific, Loughborough, UK. The sucrase isomaltase antibody HBB2/614/88-s was from the Developmental Studies Hybridoma Bank, Iowa, USA. The  $\alpha$ -actinin antibody MAB8279 was from R&D Systems, Abingdon, UK. The Simple Western WES system and 66-440 kDa mouse master kit (PS-MK11 or MK09) were from ProteinSimple®, California. The Nanodrop ND-1000 spectrophotometer was from Thermo Scientific, Loughborough, UK. The PHERAstar FS plate reader was from BMG labtech, Ortenberg, Germany.

### **2.3.2 Determination of total protein**

Either the Bradford assay (Bradford, 1976) or the BCA assay (Smith et al., 1985) were used for total protein determination in cell lysates according to the manufacturer's protocol and protein standard curves were prepared using bovine serum albumin. The BCA assay was used when samples contained octyl  $\beta$ -D-glucopyranoside as the kit was reducing reagent compatible. All other samples use the Bradford assay. For the Bradford assay, the absorbance was measured at 595 and 450 nm and the ratio of 595/450 was used for the standard curve as the linearization is improved (Zor and Selinger, 1996).

### 2.3.3 Determination of target proteins using automated western blot technology: ProteinSimple® WES

#### 2.3.3.1 Background

ProteinSimple™ WES, was used as an alternative to traditional western blot as it is an automated system and can provide greater reproducibility. Furthermore, the system is highly sensitive and low sample volume is required for the analysis. The samples are loaded into capillaries containing a proprietary UV-activated chemical linked reagent and proteins are separated by size, immobilized and target proteins are identified using primary antibodies and immuno-probed using a horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate (Figure 2-4). The peak area of chemiluminescence is reported for each protein of interest, and results can be viewed in traditional western blot lane view or by pherogram peak view.

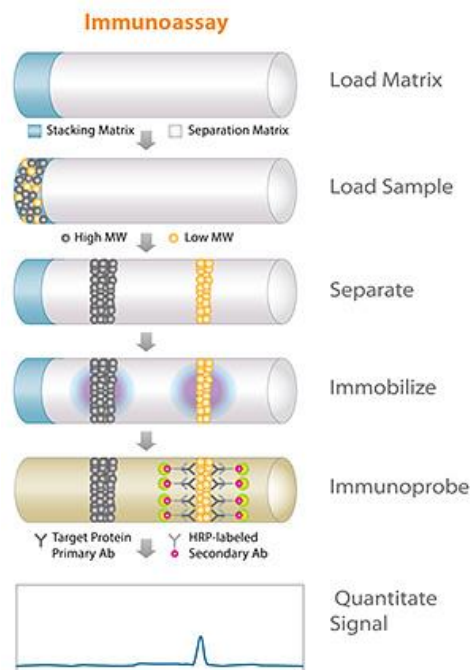


Figure 2-4. Schematic diagram for ProteinSimple® Simple Western WES system for quantification of target proteins.

### **2.3.3.2 Procedure**

Caco-2/TC7 cells were cultured as described in 2.1. Cells were washed with ice-cold PBS and scraped into lysis buffer containing 60 mM octyl  $\beta$ -D-glucopyranoside, 150 mM NaCl, 20 mM Tris pH 7.4 and 0.5% protease inhibitor cocktail. Samples were passed through a 21G needle 15 times then kept on ice for 10 min with vortexing every few minutes. The lysate was centrifuged at 14000 g for 10 min at 4°C. The total protein concentration of the supernatant was determined by the BCA assay (Section 2.3.2). The samples were then prepared for ProteinSimple WES™ analysis according to the manufacturer's recommendations. Samples were denatured at 95°C for 5 min prior to loading on the analysis plate. The sample concentration and antibody concentration were determined prior to any quantification runs, as detailed in section 2.3.3.3.

### **2.3.3.3 Antibody set-up**

For quantification by WES, the antibody for the protein of interest and a loading control were set up by determining the appropriate antibody concentration and linearity range. The two antibodies are analysed in the same sample capillary so the linearity must be checked when they are measured in the same capillary (multiplexed), rather than in independent capillaries, to ensure they do not interfere with each other. The manufacturer recommends that the antibody concentration used should be near saturation for the detection, providing that the chosen concentration gives linearity over the range of total protein concentrations and there is no interference or unusually high background. The SI antibody was evaluated from 0.48 to 4.8 mg/mL (Figure 2-5A) and saturation was achieved with 1.92 mg/mL which was a 1/25 dilution. The antibody

concentrations were also evaluated for  $\alpha$ -actinin and a 1/100 dilution was chosen. A representative pherogram is presented in Figure 2-5B for a Caco-2/TC7 lysate at 0.2 mg/mL using the chosen antibody concentrations. The assay had linear fit from 0.025 to 0.2 mg/mL total protein concentration for SI,  $\alpha$ -actinin and the ratio of SI/ $\alpha$ -actinin (Figure 2-5C and D).



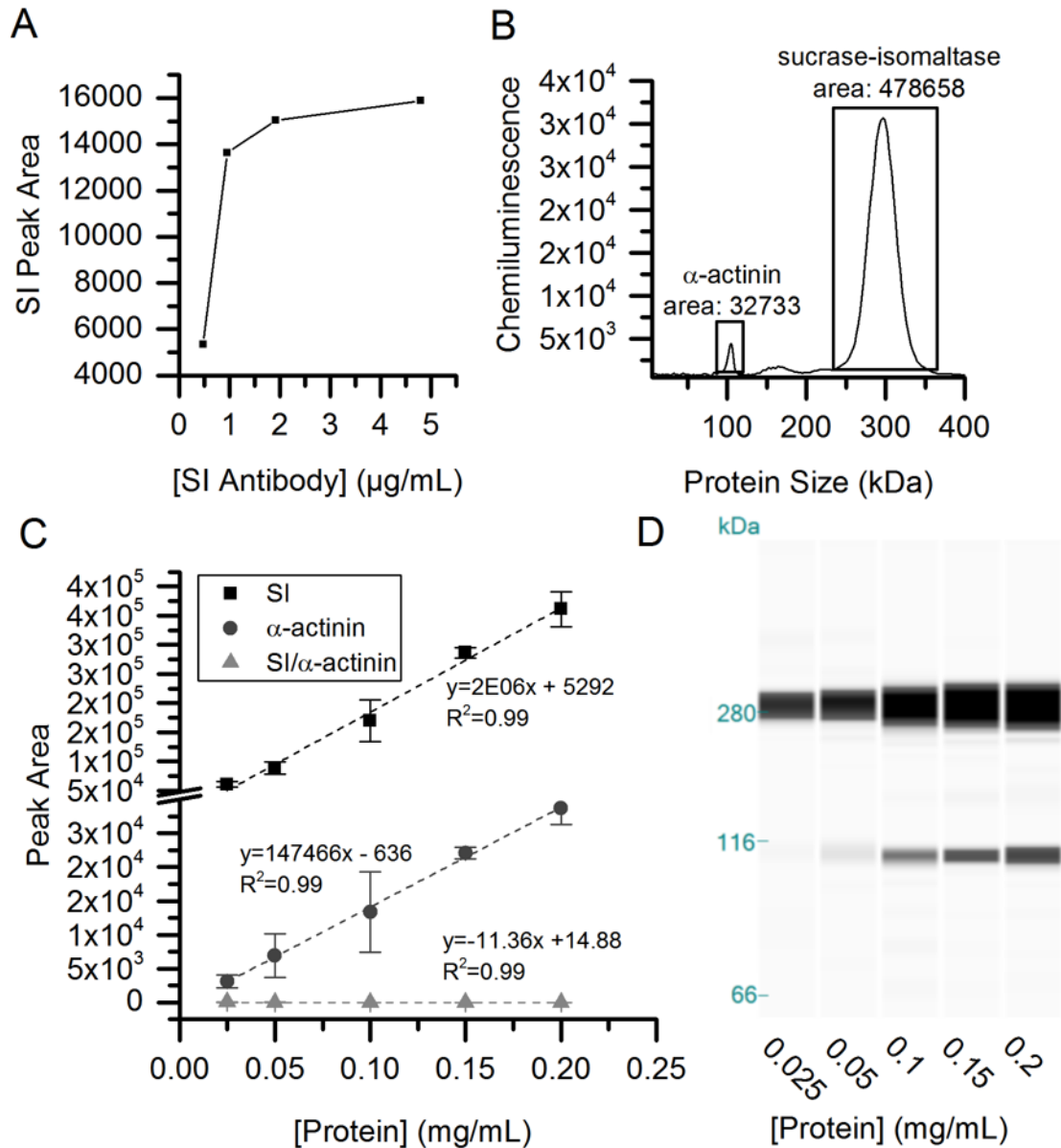


Figure 2-5. ProteinSimple™ WES optimisation for the quantification of sucrase isomaltase with  $\alpha$ -actinin as the loading control. A) The peak area of SI in Caco-2/TC7 cell lysate at 0.1mg/mL protein concentration use 4 different SI antibody concentrations. B) Representative pheroogram of SI/ $\alpha$ -actinin in Caco-2/TC7 lysates at 0.2 mg/mL total protein. C) Linearity of SI,  $\alpha$ -actinin and the ratio of SI/ $\alpha$ -actinin from 0.025 to 0.2 mg/mL total protein D) Lane view of multiplexed run with SI and  $\alpha$ -actinin in Caco-2/TC7 lysates from 0.025 to 0.2 mg/mL total protein.

#### **2.3.3.4 Data analysis**

The ProteinSimple® Compass software version 2.6.7 was used for analysis, which performs automatic peak integration to give the chemiluminescent peak areas for the proteins analysed. The ratio of SI/ $\alpha$ -actinin was used to perform quantification of sucrase isomaltase protein.

### **2.3.4 Cell surface protein – biotinylation method**

#### **2.3.4.1 Background**

The amount of sucrase isomaltase at the cell surface was determined to investigate effects on trafficking. A cell surface biotinylation method was used to purify apical cell surface proteins (Figure 2-6). The sulfo-NHS-SS- Biotin (sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate) reagent is commonly used for the biotinylation of cell surface proteins by reacting with primary amino groups on the side chains of lysine and the N-terminus of proteins to form a stable amine bond. The negative charge of the sulfonate group makes the reagent impermeable to the cell membrane. The biotinylated surface proteins are then extracted with NeutrAvidin agarose resin based on the strong non-covalent interaction between biotin and avidin. NeutrAvidin is used in this kit rather than streptavidin as it reduces non-specific binding (Daniels, 1998). Beads are added in excess to ensure the binding capacity is not fully saturated. The resin beads are washed to remove the non-biotinylated proteins then the surface proteins are eluted from with a reducing buffer. The protein released is only modified by a small sulfhydryl group. The method used here is based on the Thermo Fisher cell surface protein isolation kit (89881) with some modifications. The kit was scaled down for the labelling of 6-well

transwell® plates, as opposed to 75 cm<sup>2</sup> culture flasks as used in the provided protocol. To wash the beads, high and low ionic strength washes were used to help clear all non-biotinylated proteins, as recommended in published method in *Methods in Enzymology* (Daniels, 1998).

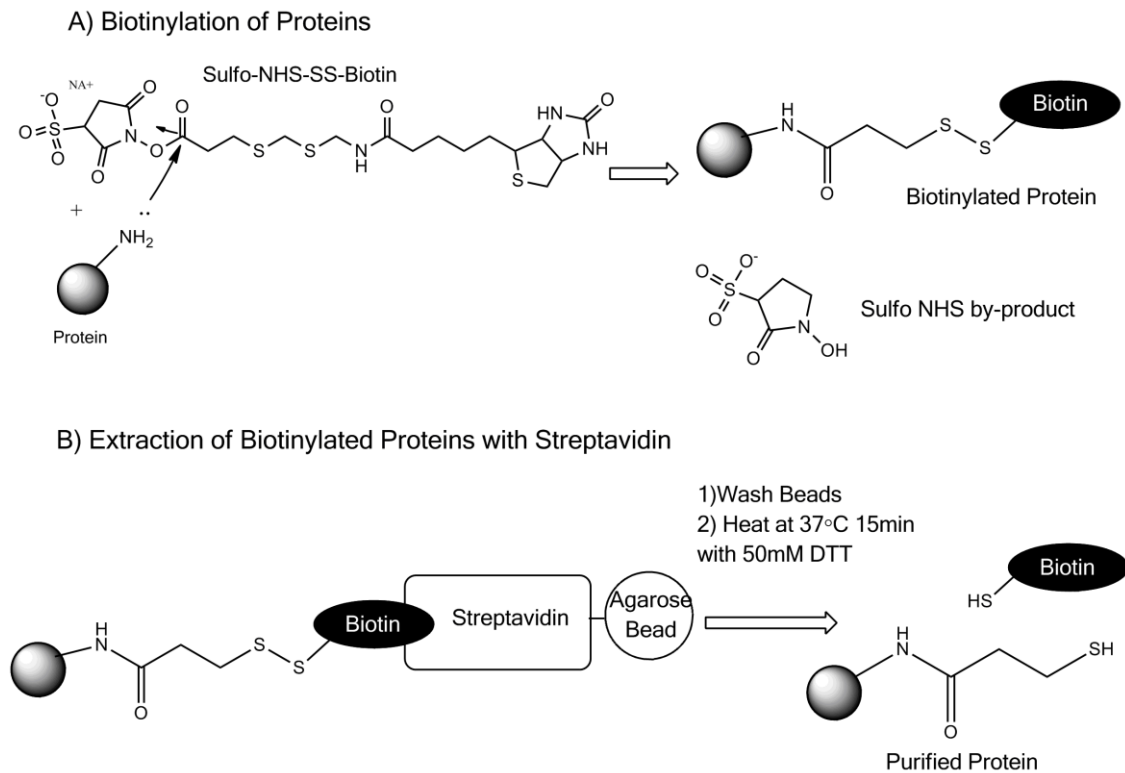


Figure 2-6. Biotinylation of cell surface proteins utilizing the strong nature of the biotin- streptavidin non-covalent bond. A) Protein amino groups at the N-terminus or on amino acid side chains are biotinylated using NHS-S-S-biotin reagent to yield the biotinylated protein and sulfo-NHS by-product B) The biotinylated proteins are added to streptavidin-agarose beads and incubated for 1 h at room temperature. The beads are washed to remove non-biotinylated proteins and then heated at 95°C in reducing sample buffer containing 50 mM DTT to cleave the disulfide bond, releasing the purified surface proteins from the biotin label.

#### **2.3.4.2 Preparation of reagents**

Stock solutions of 5 M sodium chloride and 1 M trizma base at pH 7.4, adjusted with hydrochloric acid, were prepared and stored at room temperature. A 1 mM dithiothreitol (DTT) stock solution was prepared by dissolving the pre-weighed tubes from the cell surface biotinylation kit in 50  $\mu$ L of deionised water. Cell lysis buffer was prepared containing 60 mM octyl  $\beta$ -D-glucopyranoside, 150 mM NaCl, 20 mM trizma base and 0.5% protease inhibitor. Wash solution 1 was prepared containing 10 mM octyl  $\beta$ -D-glucopyranoside, 500 mM sodium chloride and 20 mM trizma base. Wash 2 was prepared containing 20 mM trizma base pH 7.4. Tris buffered saline (TBS) was prepared as 150 mM sodium chloride and 20 mM trizma base pH 7.4. For the biotinylation reagent, 0.25 mg/mL of biotin EZ-Link™ Sulfo-NHS-SS-Biotin from the cell surface protein isolation kit was prepared in PBS+. The quenching solution was provided in the kit. The elution solution was prepared as 50  $\mu$ M DTT in 50% NuPage LDS sample buffer and 50% deionised water.

#### **2.3.4.3 Procedure**

Caco-2/TC-7 cells were cultured for 21 days on 6-well transwell® inserts as described in Section 2.1 to form a fully differentiated and polarised monolayer. The cells were washed three times with PBS+ and 1 mL of biotinylation reagent was added to the apical side of the filter and 1.5 mL of PBS+ to the basolateral side. The plate was rotated on ice at 4°C for 30 min. The reaction was stopped by adding 50  $\mu$ L of quenching buffer to the apical side and 100  $\mu$ L to the basolateral side then rotating for 5 min at 4°C. The wells were washed twice with TBS to bind any residual biotin and lysed with 0.5 mL of lysis buffer. The

cells were mixed vigorously with lysis buffer, transferred into a microcentrifuge tube and left on ice for 10 min with occasional vortexing. The lysate was centrifuged at 14000 g for 10 min at 4°C and the supernatant was removed and transferred to a clean microcentrifuge tube. The total protein concentration in the supernatant was measured using a Nanodrop™ and an aliquot of 0.3 mg of protein in 150 uL lysis buffer was prepared. 50 µL of streptavidin agarose resin slurry was pre-washed with PBS+ and then lysis buffer and sample was added and rotated for one hour at room temperature. The resin was washed with 0.5 mL of each of lysis buffer, wash 1 and wash 2. The purified surface proteins were eluted by the addition of 40 µL of elution solution followed by heating for 15 min at 37°C. The samples were stored at -80°C until analysis by ProteinSimple® WES, as described in section 2.3.3.

#### ***2.3.4.4 Data Analysis and Statistics***

Each sample well generates two samples: the total cell lysate containing all proteins within the cell, and the purified surface fraction from the streptavidin bead extraction. The peak area of SI and loading control  $\alpha$ -actinin were determined in both the total lysate and the purified surface fraction using WES. The absence of  $\alpha$ -actinin in the cell surface fraction indicates that the biotinylation specifically labels the cell surface and is not internalised, since  $\alpha$ -actinin is only located within the cell. The ratio of the peak area of SI to the peak area of  $\alpha$ -actinin in the total cell lysate was used to compare the amount of total sucrase between the control and treated cells. The absolute amount of SI in the cell surface purified fraction was reported.

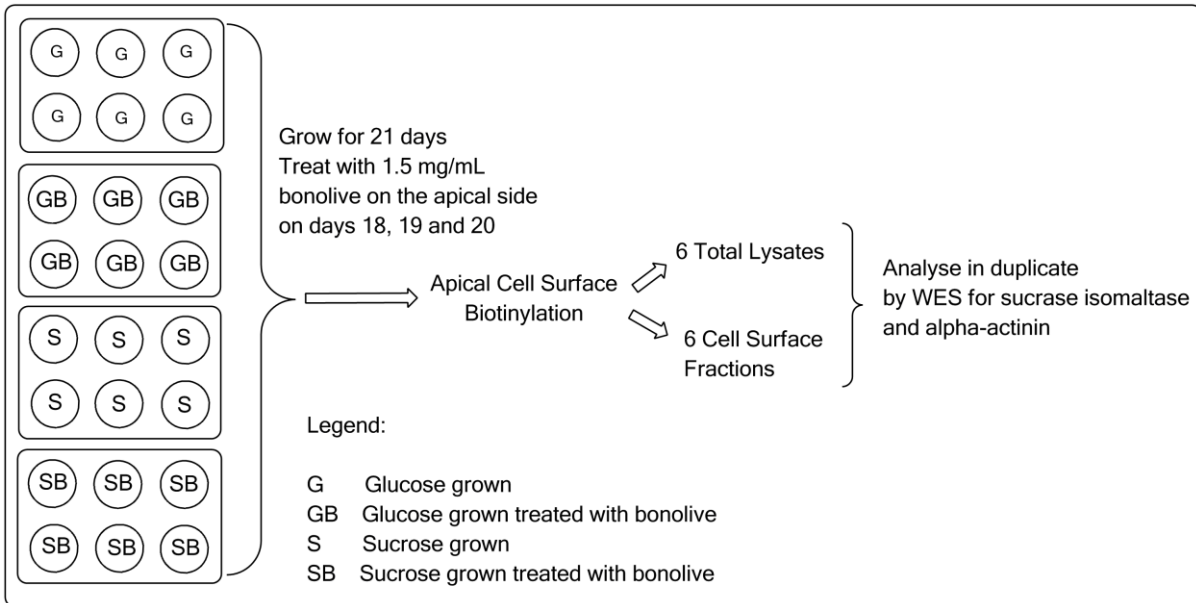


Figure 2-7. Cell surface biotinylation sample flow chart with example of bonolive treatment.

### 2.3.5 Protein sample digestion with PNGase F

PNGase F enzyme performs the de-glycosylation of all N-linked oligosaccharides from a protein so was therefore used to investigate the N-glycosylation of sucrase isomaltase. Protein lysate samples in lysis buffer were digested with rapid PNGase F. Following the manufacturer's instructions, 5  $\mu\text{g}$  of total protein from the cell lysate was digested with PNGase F alongside a mock treated sample in which water was added in the place of PNGase F enzyme. Samples were incubated for 10 min at 50°C. 5.6  $\mu\text{L}$  of the sample was added to 1.2  $\mu\text{L}$  of WES master mix and heated for 5 min at 95°C to deactivate the PNGase F enzyme and denatured for the analysis. The samples were then analysed using ProteinSimple® WES as detailed in section 2.3.3.

## 2.4 Quantification of glucose

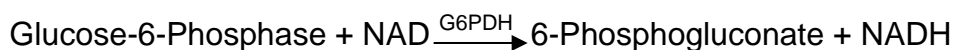
### 2.4.1 Materials and equipment

Glucose, Greiner 96-well flat bottom plates, fructose, hexokinase reagent, sodium phosphate mono- and di-basic and sucrose were from Sigma Aldrich, Dorset, UK. The PHERAstar FS plate reader was from BMG labtech, Ortenberg, Germany. The ICS4000 system for high-performance anion exchange with pulsed amperometric detection (HPAE-PAD), CarboPac PA20 column (0.4 x 150 mm), CarboPac PA20 Guard column (0.4 mm x 35 mm) 13 mm 0.2  $\mu$ M PTFE filters, 250  $\mu$ L polypropylene vials and pre-slit snap caps were from Thermo Fisher, Loughborough, UK.

### 2.4.2 Hexokinase assay

#### 2.4.2.1 Background

The hexokinase assay from Sigma was used for the quantitative determination of glucose in enzyme assay samples. The assay is based on the following reactions:



The absorbance of NADH is determined at 340 nm and is proportional to the concentration of glucose. The hexokinase reagent containing 1.5 mM NAD, 1.0 mM ATP, 1.0 unit/ml of hexokinase and 1.0 unit/ml of glucose-6-phosphate dehydrogenase (G6PDH) is used for the reaction.

### **2.4.2.2 Procedure**

Glucose standard solutions were prepared at 0.05, 0.1, 0.3, 0.5, 0.8, 1, 2, 5 and 10 mM glucose in 10 mM phosphate buffer. According to the manufacturer's protocol, 10  $\mu$ L of each standard or test sample was added to a 96-well plate in triplicate for  $A_{\text{test}}$  and triplicate for  $A_{\text{sample blank}}$ . 250  $\mu$ L of hexokinase reagent was added to  $A_{\text{test}}$  wells and 250  $\mu$ L of deionised water was added to  $A_{\text{sample blank}}$  wells. Reagent blank control and water blank control wells were also included in triplicate. The plate was shaken for 30 sec and incubated at 30°C for 15 min, followed by measurement of the absorbance at 340 nm.  $A_{\text{corr}}$  was determined by correction of the blank controls as follows:

$$A_{\text{corr}} = A_{\text{test}} - A_{\text{total blank}}$$

$$A_{\text{total blank}} = A_{\text{sample blank}} + A_{\text{reagent blank}} - A_{\text{water blank}}$$

The mean  $A_{\text{corr}}$  value for 0 mM glucose samples was subtracted from all glucose standards and plotted against glucose concentration in mM and the linear fit was determined.

### **2.4.2.3 Method accuracy, precision and linearity**

The method was validated by the independent preparation and analysis of four standard curves. The linearity was confirmed for glucose concentrations between 0.05 to 10 mM, and a representative standard curve is presented in Figure 2-8. At each concentration level, the variation between technical triplicates was less than 2% with the exception of the lowest concentration of 0.05 mM, for which the variation was less than 5%. To determine the accuracy,



the concentration at each standard level was calculated using the equation of the standard curve and the percent difference from the expected value was determined. For all concentration levels the percent difference was  $\leq 2\%$  and for the lowest concentration of 0.05 mM the percent difference was  $\leq 5\%$ . The slope from four independent standard curves had a variation of 0.4% indicating low inter-assay variation.

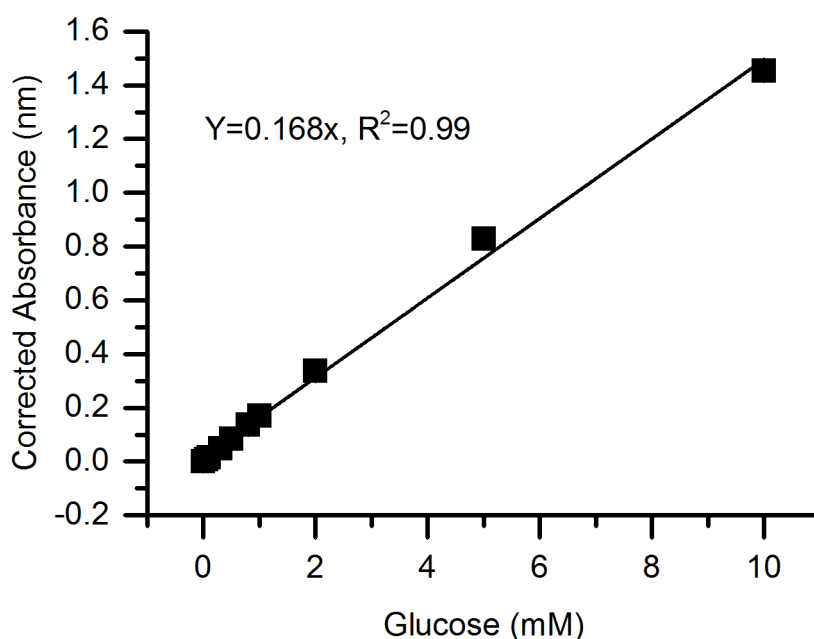


Figure 2-8. Calibration curve for the quantification of glucose standards by hexokinase assay. The values are presented as mean  $\pm$  SEM (linear fit  $Y=0.168x$ ;  $R^2=0.99$ ; average relative standard deviation 4.1%,  $n=4$ ). Error bars are smaller than the data points.

### 2.4.3 Method optimisation and validation for the quantification of glucose, sucrose and fructose by HPAE-PAD

#### 2.4.3.1 Background

The analysis of monosaccharides and disaccharides by conventional HPLC/UV method is challenging due to the highly polar nature of carbohydrates and their

lack of a suitable chromophore. Methods in the past have utilised derivatisation methods which introduce time and variability into the analysis. High performance anion exchange chromatography (HPAE) with pulsed amperometric detection (PAD) offers a solution to these challenges. Carbohydrates are weakly acidic and therefore ionised at high pH concentrations, and based on this HPAE separation utilises a basic NaOH eluent with a hydrophobic anion exchange resin to achieve separation. PAD detection utilises a gold working electrode upon which the analytes undergo an oxidation reaction and the resulting current is measured in coulombs. A method was set up using Thermo Dionex ICS-4000 HPAE-PAD system for the analysis of sugars in enzyme assay samples, cell transport assays and cell sugar uptake. The method was validated for the quantitation of glucose, sucrose and fructose.

#### ***2.4.3.2 Standard and sample preparation***

Glucose, sucrose and fructose standard solutions were prepared in deionised water at 50 mM. A mixed standard containing all three components was prepared at 1000  $\mu$ M and subsequently diluted to working standard concentration range from 0.5  $\mu$ M to 50  $\mu$ M. HPAE-PAD was used for the analysis of transport samples (Chapter 6) and inhibition assay samples (Chapter 3). Inhibition samples were diluted with deionised water to fall within the calibration range. Since assay samples contained biological material, the protein in the samples was precipitated prior to analysis by the addition of acetonitrile (1:1 v/v), vortexed and centrifuged at 17000 g for 10 min. The resulting supernatant was diluted with deionised water to fall within the

calibration range and the total sample dilution was 25-fold. All standards and samples were filtered with 13 mm 0.2  $\mu$ M PTFE filters into polypropylene vials and maintained at 4°C in the auto-sampler. Inhibition assay samples were in phosphate buffer and transport samples were in transport buffer, so standards were prepared in these buffers to confirm that they do not affect quantification. The assay samples contained 2% acetonitrile and this was also confirmed to have no impact on quantification.

#### ***2.4.3.3 Instrument and method parameters***

The HPAE-PAD system had an electrochemical detector, palladium reference electrode, gold working electrode and was operated with Chromeleon® software version 7.2 SR4. Capillary separation of carbohydrates was done using the capillary CarboPac PA20 column, 0.4 x 150 mm with CarboPac PA20 Guard, 0.4 x 35 mm with the column at 30°C and compartment at 15°C. The detection was performed using a gold working electrode and palladium reference electrode with a collection rate of 2.00 Hz using the “Gold, Carbo, Quad” waveform. Different concentrations of sodium hydroxide eluent were evaluated to achieve isocratic baseline separation of glucose, fructose and sucrose and 25 mM NaOH was chosen. The analysis method starts at 25 mM NaOH eluent for 10 min followed by a wash at 100 mM for 15 min to remove any salts or impurities and then re-equilibration at 25 mM for 13 min. The flow rate was 0.008 mL/min with 0.04  $\mu$ L injection volume and 20x capillary overfill.

#### 2.4.3.4 Specificity

Individual glucose, fructose and sucrose solutions were prepared at 10  $\mu$ M and analysed alongside a mixed standard to identify the elution order (Figure 2-9).

Glucose eluted first with a retention time of 7.8 min  $\pm$  0.13% followed by fructose at 9.3 min  $\pm$  0.17% and then sucrose at 10.9 min  $\pm$  0.20%.

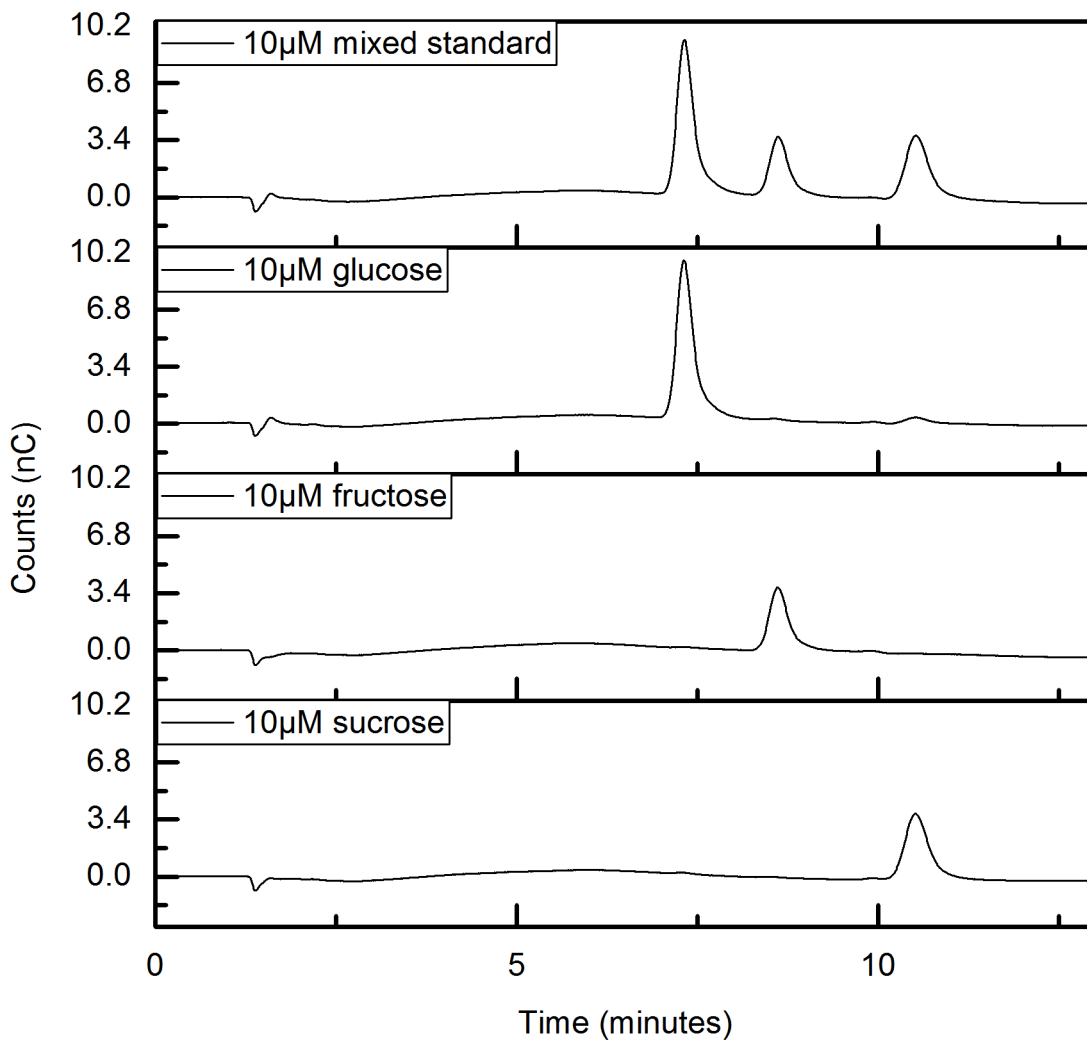


Figure 2-9. Identification of glucose, fructose and sucrose peaks from HPAE-PAD chromatograms by analysis of individual standards.

### 2.4.3.5 Precision

For the determination of method precision, 10 injections were performed from one vial containing 5  $\mu\text{M}$  standard mixture of glucose, fructose and sucrose. The actual concentration was determined for each injection and the mean was  $4.9 \pm 0.08$  (SD)  $\mu\text{M}$  with 1.7% CV for glucose,  $4.7 \pm 0.13$  (SD)  $\mu\text{M}$  with 2.9% CV for fructose and  $5.0 \pm 0.17$  (SD)  $\mu\text{M}$  with 3.3% CV for sucrose. The accuracy compared to the standard curve was -1.3%, -7.0% and -0.5% for glucose, fructose and sucrose respectively.

### 2.4.3.6 Linearity and Accuracy of Calibration Range

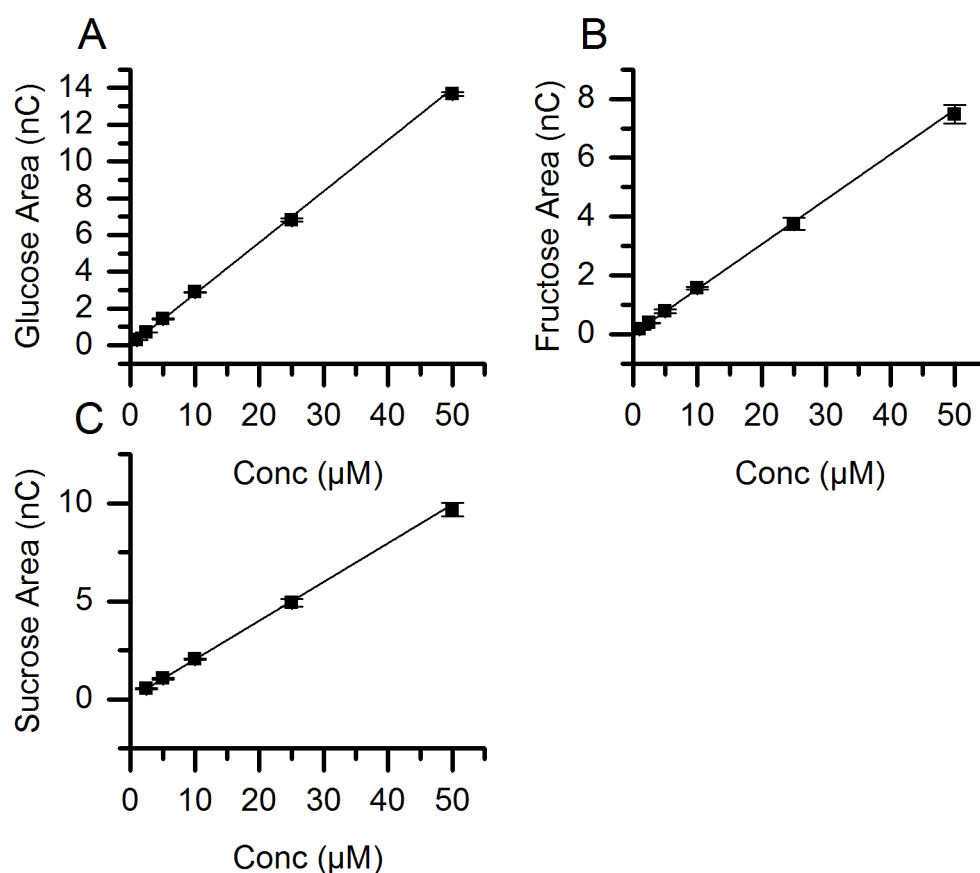


Figure 2-10. HPAE-PAD calibration standard curves for glucose (A) fructose (B) and sucrose (C) from 0.5  $\mu\text{M}$  to 50  $\mu\text{M}$ .

The standard curve calibration samples at 0.5, 1.0, 2.5, 5.0, 10, 25 and 50 were injected 4 times. The linear equations were used to calculate the actual concentration of each injection and the accuracy was determined by comparing the calculated concentration to the expected concentration. For glucose and sucrose, accuracy at 0.5  $\mu\text{M}$  was 76% and 67% respectively and therefore quantitation at this level was not accurate as the values should be at least  $\pm 10\%$  of the expected value. For all other levels, accuracy was within  $\pm 5\%$  of the expected value. For sucrose, accuracy within  $\pm 5\%$  of the expected value occurred from 2.5 to 50  $\mu\text{M}$ . The accurate quantitation range for glucose and fructose was between 1.0 to 50  $\mu\text{M}$  and for sucrose was between 2.5 to 50  $\mu\text{M}$ . Samples below the lower limit for accuracy were reported as less than limit of quantitation (<LOQ). Samples for which no peak was observed are reported as not detected (ND).

## **2.5 Preparation of polyphenols and extracts and controls**

### **2.5.1 Materials**

Polyphenols: 5-caffeoyl-quinic acid, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, galangin, hesperitin, kaempferol, luteolin, naringenin and quercetin were from Sigma Aldrich, Dorset, UK. Apigenin was from Phytolab, Vestenbergsgreuth, Germany.

Extracts: Bonolive was kindly supplied by BioActor BV, The Netherlands as a powder containing 40% (w/w) of the bioactive oleuropein (OLE). Green tea water-soluble extract was from Nestlé, Choladi, India. The green tea extract was analysed by HPLC (Nyambe-Silavwe and Williamson, 2016) and 1 mg/mL

contained 199.8 mg/g (-)-epigallocatechin gallate, 124.4 mg/g (-)-epigallocatechin, 34.4 mg/g (-)-epicatechin gallate and 23.3 mg/g (-)-epicatechin. The German chamomile was from Phytolab, Vestenbergsgreuth, Germany and a maltodextrin-free sample was kindly prepared by Jose A. Villa-Rodriguez. Note that maltodextrin was removed as it can act as a substrate to sucrase isomaltase to produce glucose during the enzyme assays.

Positive Controls: acarbose and forskolin were from Sigma Aldrich, Dorset, UK.

### **2.5.2 Procedure**

Individual polyphenols were dissolved in DMSO and diluted for subsequent use into sample buffer. Control samples were spiked with an equivalent amount of DMSO and DMSO did not exceed 2% of the sample volume. Positive controls and extracts were water-soluble so were prepared in deionised water.

## **2.6 Statistics and sample replicate notation**

All values shown are the mean of  $n$  independent experiments  $\pm$  standard error of the mean, unless otherwise stated. Each experiment had between 3-6 technical replicates.  $N$  is the total number of technical replicates. For cell experiments, 3 different passages of cells were used. For analysis of statistical significance between multiple groups, ANOVA was used. If the ANOVA indicated a difference between the groups ( $p < 0.05$ ), Tukey post-hoc analysis was performed to determine the  $p$ -values. For the comparison of the means of two groups, unpaired Students  $t$ -test was performed, with confidence level at 95%. Levene's test was performed to confirm the equality of variances.

### **3 Inhibition of human and rat sucrase and maltase activities: optimisation of the assay using acarbose and polyphenols**

#### **3.1 Abstract**

The anti-diabetic drug acarbose reduces the post prandial blood glycaemic response by inhibiting  $\alpha$ -glucosidases (maltase and sucrase), required for the digestion of carbohydrates, and  $\alpha$ -amylase, required for the digestion of starch. Chronic intake of acarbose led to a 6% reduction of type 2 diabetes risk (Nijpels et al., 2008). Other compounds such as polyphenols have been reported to exhibit a comparable activity. However, for assessing this activity, many assays use a rat intestinal extract as the  $\alpha$ -glucosidase source, and then extrapolate to human tissues. Results in this chapter demonstrate that cell-free extracts from fully differentiated intestinal Caco-2/TC7 monolayers are a suitable source of the human enzymes sucrase and maltase, and the *in vitro* assay was optimised by comparing enzymatic and chromatographic methods to detect the products. Human sucrase was more susceptible than the rat enzyme to inhibition by acarbose, a polyphenol-rich green tea extract, and pure (-)-epigallocatechin gallate, whereas the reverse was observed when assessing rat maltase activity. 5-Caffeoyl-quinic acid up to 500  $\mu$ M did not inhibit any of the measured activities. We demonstrate that spurious results can be obtained if the amount of enzyme and assay conditions are not optimised, and if the tested compound inhibits or interferes with the product detection methodology. The data show that for sucrase and maltase activities, the inhibition of rat and human enzymes can be several-fold different.



### 3.2 Introduction

The prevalence of type 2 diabetes has doubled over the last thirty years and affects an estimated one in ten worldwide, and non-communicable diseases including cardiovascular disease and diabetes mellitus account for more deaths than infectious diseases (WHO, 2012). A meta-analysis of intervention studies suggests that reducing the glycaemic impact of the diet can favourably affect health markers for type 2 diabetes (Livesey et al., 2008). One current pharmaceutical therapy for diabetes is based on the strategy of reducing the glycaemic impact through inhibition of  $\alpha$ -glucosidase enzymes in the gut and these include the anti-hyperglycaemic drugs acarbose, voglibose and miglitol (Breuer, 2003). Type 2 diabetic patients have increased levels of SI which exacerbates the problem, making it an interesting target for inhibition (Dyer et al., 2002). Natural products have been sought as alternatives or adjuncts to these treatments, since the drugs exhibit gastro-intestinal side effects.

Consumption of polyphenols has been associated with decreased incidence of disease including type 2 diabetes, cardiovascular disease and osteoporosis (Scalbert et al., 2005; Laville and Nazare, 2009; Hanhineva et al., 2010). Part of this effect is the potential for polyphenols to influence sugar digestion and absorption (Williamson, 2013). The small intestine is an important target for polyphenols to aid in glucose homeostasis by the inhibition of  $\alpha$ -glucosidases and subsequent transport of monosaccharides through the enterocytes (Hanhineva et al., 2010). In an intervention study where acarbose was taken for three years, the risk for diabetes was reduced by 6% compared to the control

(Nijpels et al., 2008) so there is potential that chronic intake of a diet rich in polyphenols could improve diabetes risk through the same mechanism, i.e. the inhibition of  $\alpha$ -glucosidases.

Although polyphenols have the potential to act as  $\alpha$ -glucosidase inhibitors (Hanhineva et al., 2010) , there are substantial limitations to some of the methods used to date (Section 1.3.2). Although human enzymes are always the intended target for therapeutic interventions, many studies use the more readily available rat intestinal preparations *in vitro* with a variety of detection methods for the products, typically enzyme-linked spectrophotometric assays based on glucose oxidase or hexokinase (Jo et al., 2009; Gupta et al., 2010; Kim et al., 2011; Priscilla et al., 2014). However there has been no proper comparison between the inhibition of human and rat enzymes, and very few studies have used human enzyme. Furthermore, there is only 74% homology between the rat and human enzyme (Van Beers et al., 1995). Polyphenols inhibition assays are further complicated by their effects on the glucose detection methods, and this is not always taken into account and was addressed here.

The aim is to first optimise the assay for sucrase and maltase inhibition using a human enzyme source from Caco-2/TC7 cells. This includes evaluating methods for the preparation of the enzyme source and detection methods. Next, the optimised assay is used to determine inhibition of selected polyphenols and extracts; acarbose, green tea, (-)-epigallocatechin gallate since inhibition of rat sucrase and maltase has been observed in the Williamson lab (Nyambe Thesis, 2016). The results from the assay using the rat enzyme source are compared

here to use of a human enzyme source. Other polyphenols and extracts available in our laboratory are screened for sucrase inhibition for comparison with literature values.

### **3.3 Method optimisation**

#### **3.3.1 Background on human enzyme assay**

The human enzyme assay was set up using Caco-2/TC7 cell lysates as the enzyme source, based on same principle as the enzyme assay using rat intestinal powder (Nyambe-Silavwe and Williamson, 2016). Assay samples of 250  $\mu$ L contained sucrose or maltose substrate, Caco-2/TC7 cell lysate preparation in 0.1 M phosphate buffer (pH 7.0). Samples were incubated for the optimised time (determined in 3.3.7.1) at 37°C and the reaction was stopped by boiling for 10 min at 95°C which denatures the enzyme. Samples were centrifuged at 17000 g for 10 min. Finally, the concentration of glucose produced was quantified. For all assays, the enzyme was pre-warmed at 37°C and the substrate and inhibitor were pre-warmed in a separate tube. In the upcoming sections, the apparent  $K_m$  and  $V_{max}$  kinetic parameters were determined and the linear ranges for time and enzyme concentration were optimised. Two methods for the preparation of the cell lysate were evaluated. In addition, enzymatic and chromatographic detection of glucose, the effect of substrate concentration and the effect of enzyme amount on apparent inhibition were evaluated. The potential interference from inhibitors on the detection methods was addressed.

The assay flow chart for the final IC<sub>50</sub> determination is presented in Figure 3-1. First, the cell lysate was prepared from Caco-2/TC7. Next, using the optimised parameters, the specific activity of the cell lysate was determined. One enzyme unit (U) is equal the amount of enzyme that catalyses the hydrolysis of 1  $\mu$ mol of substrate per min. Finally, the assay was used to determine the inhibition of sucrase and maltase by selected polyphenols with the same amount of activity (based on number of units added per assay).

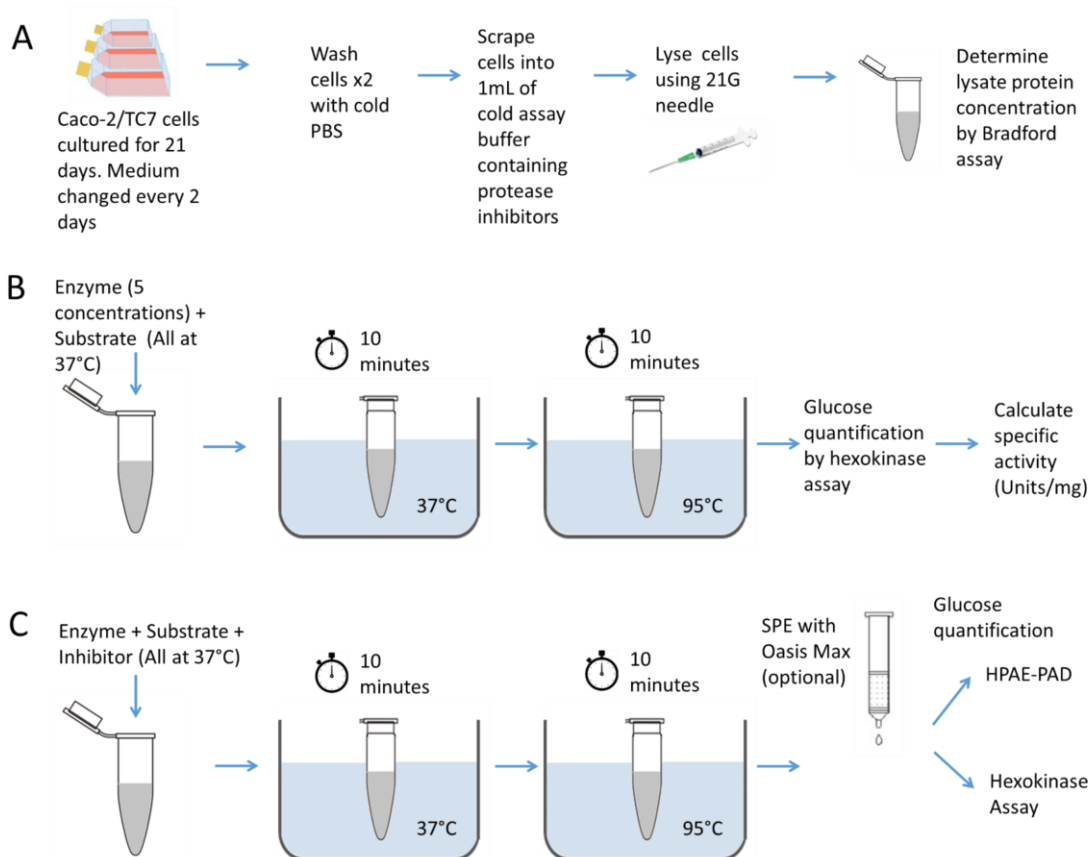


Figure 3-1. Method flow chart for the sucrase isomaltase enzyme assay. A) The cell lysate was prepared from Caco-2/TC7 cells. B) An enzyme activity assay was performed with varying enzyme concentrations to determine the Units/mg of total protein in the lysate. C) Enzyme inhibition assay with specified number of units. The inhibition assay includes the optional SPE step to remove the inhibitors from the assay if they were shown to interfere with the detection method. Two alternative detection methods were evaluated.

### 3.3.2 Materials and equipment

Sodium potassium monobasic, sodium potassium dibasic, glucose, sucrose, maltose, intestinal acetone powder from rat, protease inhibitors, L-cysteine, papain from papaya latex were from Sigma Aldrich, Dorset, UK. Oasis MAX cartridges were purchased from Waters Corporation *Ltd.*, Milford, MA, USA. Polyphenols and inhibitors are described in Section 2.5. Other methods are found in general methodology (Chapter 2).

### 3.3.3 Preparation of enzyme source

Caco-2/TC7 cells were cultured in 75 cm<sup>2</sup> flasks as described in Section 2.1 to full differentiation of 21 days. The cells were washed three times with cold phosphate buffered saline and cells were scraped with 1 mL of assay buffer (10 mM phosphate buffer, pH 7.0) containing protease inhibitors. The cell lysates were snap frozen in a dry ice and ethanol bath and stored at -80°C until required. On the day of assay, cell lysates were thawed, vortexed and then passed 10-15 times through a 21G needle syringe, and referred to as a cell-free extract since it contains no intact cells. Papain-digested lysates were also prepared to evaluate different lysate preparation methods. Fully differentiated cells were washed with warm PBS and then incubated at 37°C for 15 min with 2 mL papain solution containing 1 mg/mL papain and 0.5 mg/mL L-cysteine (Auricchio et al., 1963). After the incubation, cells were scraped and transferred into microcentrifuge tubes and centrifuged at 210 g for 10 min at 4°C. The supernatant was used for the assay. The protein content of the lysate, either crude or papain-digested, was determined by Bradford Assay (Section 2.3.2) and the lysate was diluted in assay buffer as required.

### **3.3.4 Assay controls**

A control was included where the enzyme was deactivated by 10 min boiling prior to the assay to confirm that the deactivation step was sufficient. A control sample with no substrate and no enzyme was also analysed with each experiment.

### **3.3.5 Glucose detection methods – hexokinase and HPAE-PAD**

The glucose produced in the enzyme reaction was quantitated enzymatically using hexokinase (Section 2.5), or chromatographically with high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Section 2.7). Before HPAE-PAD analysis, protein in the samples was precipitated by the addition of acetonitrile (1:1 v/v) and samples were vortexed and centrifuged at 17000 g for 10 min. The resulting supernatant was diluted with deionised water to fall within the calibration range, filtered through a 0.2 µm PTFE filter and maintained at 4°C in the autosampler.

### **3.3.6 Effect of polyphenols on hexokinase assay and removal by SPE**

To evaluate if polyphenols or polyphenol-rich extracts interfere with the hexokinase assay, 250 µL samples containing 5 mM glucose were prepared in assay buffer with and without polyphenols or extracts at the highest tested concentration. The hexokinase assay was performed to measure the amount of glucose and the samples were compared to the control. Independent sample T-test was performed to determine if the inhibitors had significant effect on the assay. Any inhibitors which interfered significantly were removed by SPE prior to analysis. Oasis MAX 3 cc cartridges were preconditioned with 1 mL of

deionised water and 1 mL of methanol then dried under vacuum for 5 min. The sample collection tube was placed under the cartridge and 200  $\mu$ L of sample was passed through and collected while polyphenols were retained.

### 3.3.7 Determination of assay conditions

#### 3.3.7.1 Determination of reaction time and enzyme concentration and kinetic parameters $K_m$ and $V_{max}$

A time course was determined for sucrase and maltase using 10 mM substrate for each. Sucrase exhibited a linear production of glucose up to 40 min and maltase was linear up to 15 min (Figure 3-2). An assay time of 10 min was selected for both as this falls well within the linear range and is convenient for handling assays with both sucrase and maltase simultaneously.

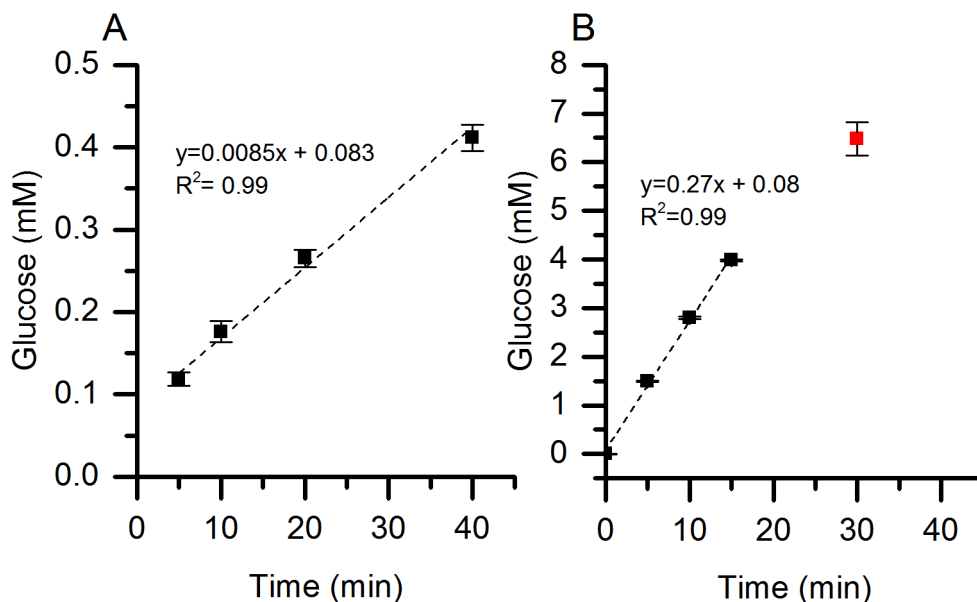


Figure 3-2. Time course for sucrase (A) and maltase (B) assay. Results are presented as mean  $\pm$  SEM (n=3). Error bars not visible are smaller than the data point.

A specific activity assay was performed for maltase and sucrase with different amounts of enzyme added based on the total protein concentration, up to 0.3 mg total protein with 10 mM substrate concentration and 10 min incubation. The specific activity of sucrase was  $130 \pm 4$  mU/mg and for maltase was  $725 \pm 36$  mU/mg (Figure 3-3). Sucrase specific activity was proportional to total protein up to 1.28 mg/mL whereas maltase was proportional up to 0.64 mg/mL. The amount of protein used for assays was selected to be within the linear range to satisfy the conditions of initial rate: 0.05 U per assay for maltase and 0.02 U per assay for sucrase.

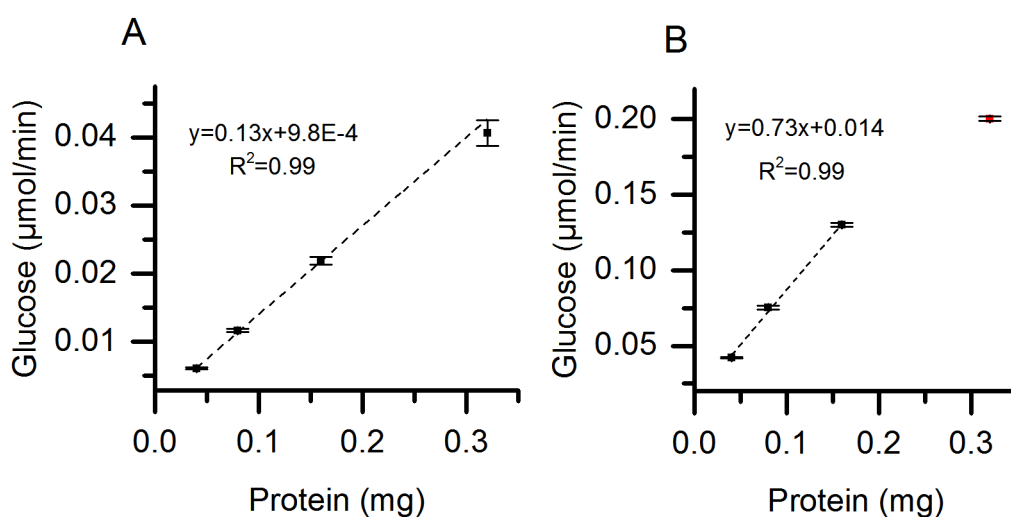


Figure 3-3. Specific activity in the Caco-2/TC7 cell lysate for sucrase (A) and maltase (B). The enzyme assay was performed with 10 mM substrate, 10 min incubation time and different amounts of enzyme up to 0.3 mg total protein. Results are presented as mean  $\pm$  SEM (n=3).

Using the conditions established, the apparent  $K_m$  and  $V_{max}$  for sucrase and maltase were determined using Lineweaver-Burk plots (Figure 3-4). For sucrose, apparent  $K_m = 9.5$  mM and  $V_{max} = 0.053$   $\mu$ mol/min; for maltose, apparent  $K_m = 7.5$  mM and  $V_{max} = 0.379$   $\mu$ mol/min.



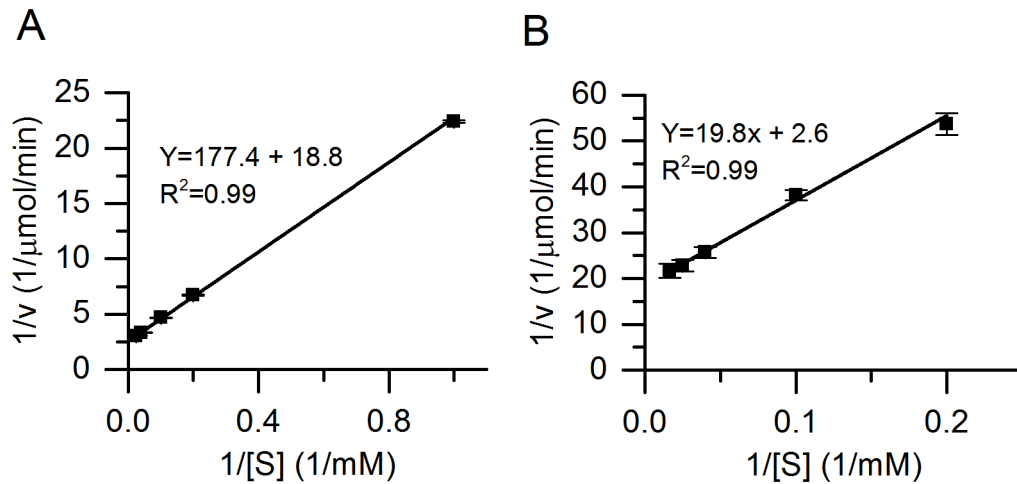


Figure 3-4. Lineweaver-Burk plot for the determination of apparent  $K_m$  and  $V_{max}$  for sucrose (A) and maltase (B). Results are presented as mean  $\pm$  SEM ( $n=3$ ). Error bars not visible are smaller than the data point.

### 3.3.7.2 Effect of substrate concentration on measured inhibition

The inhibition of maltase and sucrose by acarbose at 2.5  $\mu\text{M}$  was determined with different substrate concentrations. There was no significant difference on the sucrose inhibition using 1, 10 ( $\approx K_m$ ) or 100 mM substrate (Figure 3-5A). Maltase was evaluated with 1, 7 ( $\approx K_m$ ), 10 and 100 mM substrate. At 100 mM, which is  $\geq 10$ -fold the apparent  $K_m$ , the inhibition of maltase by acarbose was less than that determined with 1, 7 or 10 mM substrate ( $p < 0.001$ ) (Figure 3-5B). For convenience in handling multiple assay sample types, 10 mM was used for both sucrose and maltose.

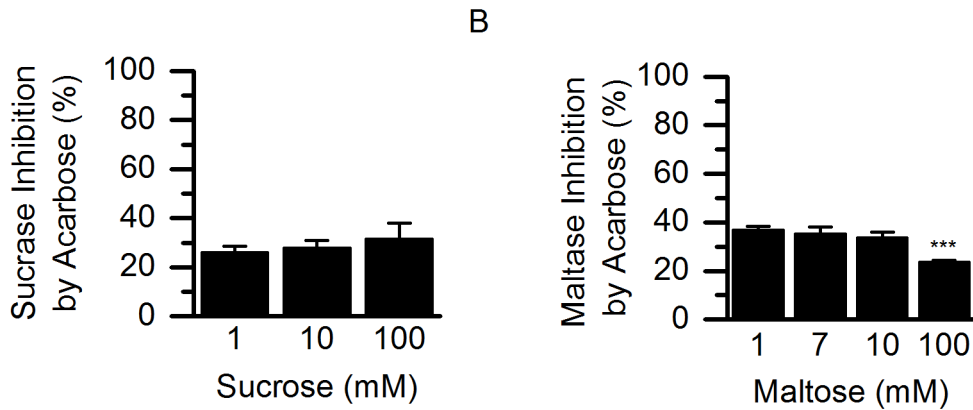


Figure 3-5. Effect of substrate concentration on apparent inhibition. Substrate concentrations of 1, 10 ( $\sim K_m$ ) and 100 mM were evaluated for sucrose (A) and 1, 7 ( $\sim K_m$ ), 10 and 100 mM for maltose (B) were used in the assay for inhibition by acarbose at 2.5  $\mu\text{M}$ . Results are presented as mean  $\pm$  SEM (n=3).

### 3.3.7.3 Effect on number of units on enzyme inhibition

The impact of the amount of enzyme in the assay was evaluated by determining the  $IC_{50}$  value for the inhibition of maltase by acarbose with two different enzyme concentrations: the optimised amount (0.05 units per assay) which falls within the linear range, and 0.2 units per assay which is above the linear range. As shown in Figure 3-6, the  $IC_{50}$  value was over-estimated when the number of units is above the linear range of the assay ( $p < 0.001$ ).

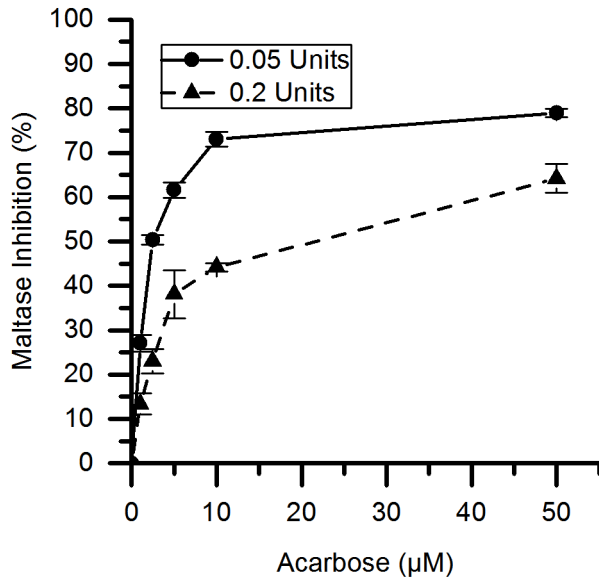


Figure 3-6. The effect of amount of enzyme in the assay for the inhibition of maltase by acarbose. Results are presented as mean  $\pm$  SEM (n=3).

#### 3.3.7.4 Effect of lysate preparation on activity and inhibition

Two different methods for the preparation of the cell lysate were evaluated to determine if release of sucrase from the brush border changes the inhibition observed. The papain-treatment gives an increase in enzyme specific activity when assessed using both sucrase and maltase activities (Figure 3-7). Next, the inhibition of sucrase and maltase by acarbose was evaluated with both preparations using equal amounts of activity per assay. As shown in Figure 3-8, the inhibition observed was the same regardless of the papain treatment. Therefore, both the crude lysate and papain-digested preparation are both suitable for inhibition assays.

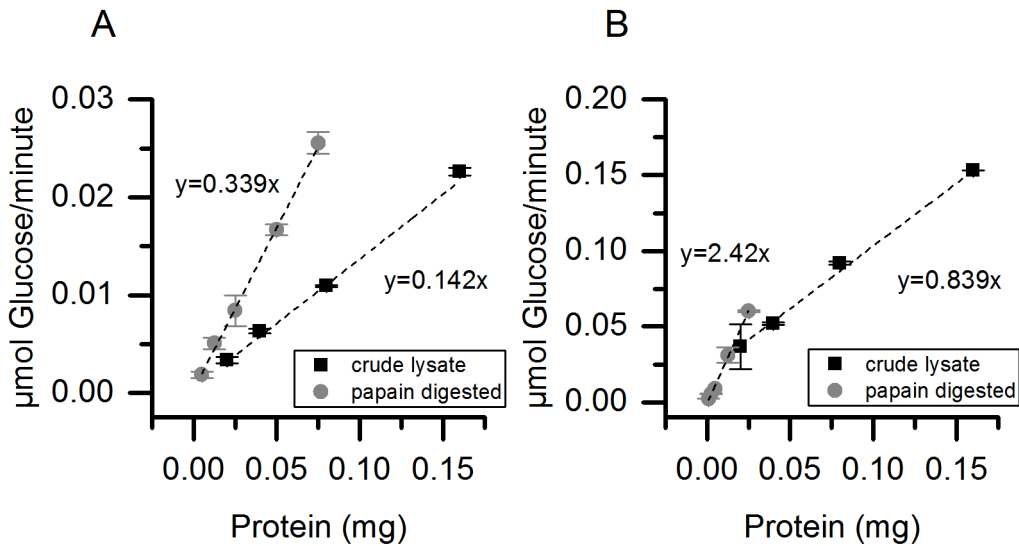


Figure 3-7. Effect of papain treatment on inhibition on the activity of sucrase (A) and maltase (B). Either a crude lysate of Caco-2/TC7 cells or the supernatant from papain-digested Caco-2/TC7 cells was used as the enzyme source for sucrase or maltase assay with 10mM substrate, 10 min incubation time and increasing amounts of total protein. Results are presented as mean  $\pm$  SEM (n=3).

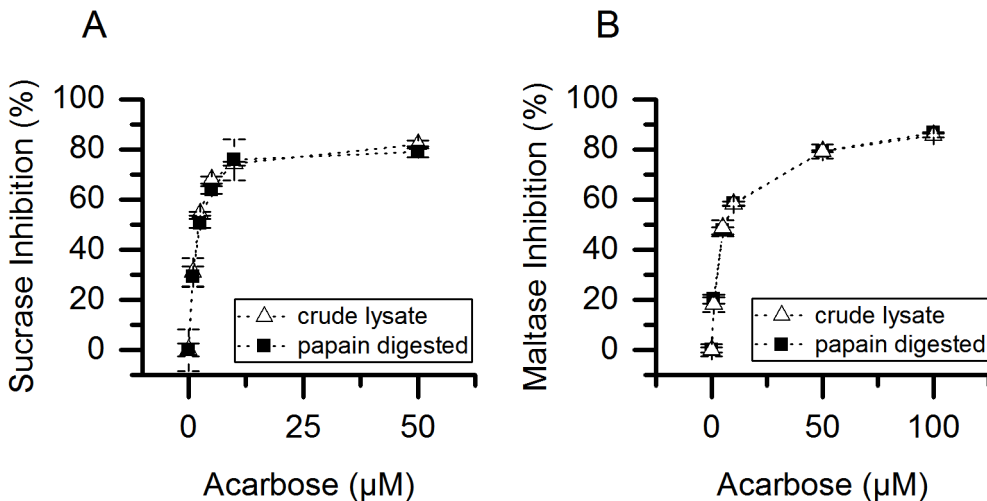


Figure 3-8. Effect of papain treatment on acarbose inhibition of sucrase (A) and maltase (B). Either a crude lysate of Caco-2/TC7 cells or the supernatant from papain-digested Caco-2/TC7 cells was used as the enzyme source with 10 mM substrate, 10 min incubation time and increasing concentrations of acarbose. Results are presented as mean  $\pm$  SEM (n=3).

### **3.3.7.5 Determination of possible interference in the hexokinase-linked estimation of glucose and removal by solid phase extraction**

All inhibitors were evaluated at their highest used concentration for interference with the hexokinase assay for the quantification of glucose according. Apigenin, (+)-catechin, (-)-epicatechin, galangin, hesperetin, kaempferol, luteolin, naringenin and quercetin were evaluated at their screening concentration (see Section 3.4.1) and there was no difference in glucose compared to the control (all  $p > 0.05$ ). EGCG at 1000  $\mu\text{M}$  led to an apparent 23% increase in glucose concentration ( $p < 0.001$ ,  $n=3$ ), green tea at 5.0 mg/mL caused an apparent 13% decrease in glucose concentration ( $p=0.002$ ,  $n=3$ ) and 5-caffeoyl-quinic acid at 500  $\mu\text{M}$  caused an apparent decrease of 10% ( $p < 0.001$ ,  $n=3$ ). Bonolive at 5 mg/mL led to an 11% decrease in glucose concentration ( $p < 0.001$ ,  $n=3$ ). The positive control acarbose at 100  $\mu\text{M}$  had no significant effect on the assay compared to the control ( $p=1.0$ ,  $n=3$ ). Based on these results, EGCG, green tea, 5-caffeoyl-quinic acid and bonolive assay samples all required solid phase extraction to remove polyphenols prior to glucose quantification by the hexokinase assay.

### **3.3.7.6 Comparison of detection methods for glucose**

Since several of the test compounds were shown to interfere with the hexokinase assay, chromatographic quantitation of sugars using HPAE-PAD was evaluated as a potential alternative for estimation of product compared to the enzymatic detection. The chromatographic method should allow elimination of the solid phase extraction (SPE) step, which could introduce errors and additional cost.

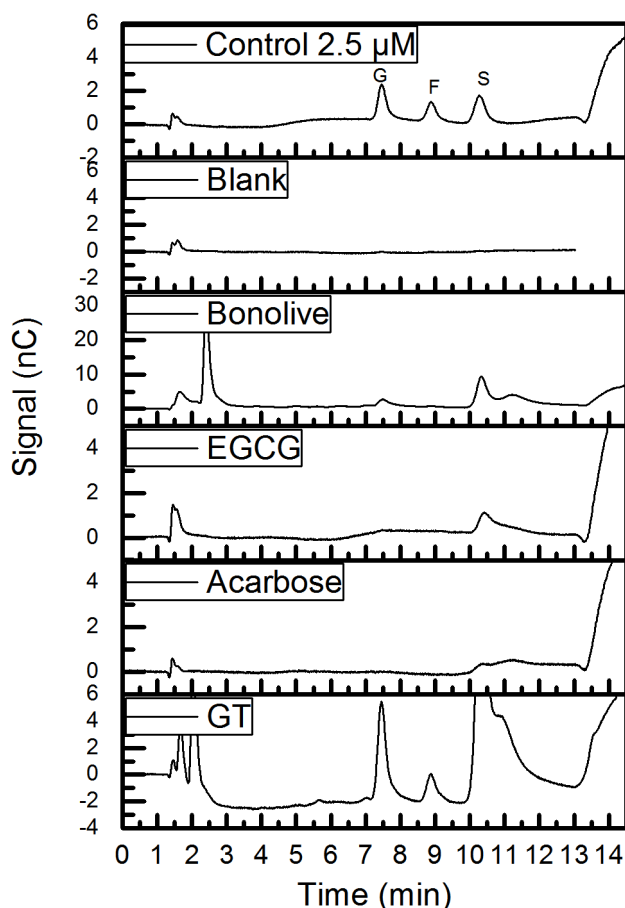


Figure 3-9. Evaluation of inhibitor interference in chromatographic analysis of sugars. Inhibitors were directly added at their highest evaluated concentration to assay samples containing no substrate. The “control” was spiked with 2.5  $\mu\text{M}$  glucose (G) fructose (F) and sucrose (S).

The selected inhibitors were first checked for interference with HPAE-PAD. Blank samples (no sugars) containing the inhibitors at their highest testing concentration were prepared and diluted for analysis by HPAE-PAD. Of the inhibitors tested, EGCG and acarbose did not interfere with glucose quantification, however bonolive and green tea did. After solid phase extraction, the bonolive interference was absent. For green tea, SPE did not completely remove the glucose interference; hence the extract contains some glucose (See 3.3.7.7). EGCG assay samples were selected to compare the HPAE-PAD

method with the hexokinase assay because they would normally require SPE prior to hexokinase analysis and EGCG does not interfere with HPAE-PAD.

Table 3-1. Comparison of glucose detection methods for the inhibition of maltase by EGCG. Glucose was quantified using HPAE-PAD or underwent SPE followed by the hexokinase assay. Results are presented as mean of three technical triplicates. SD= standard deviation, %CV= coefficient of variation.

EGCG ( $\mu\text{M}$ )	SPE/Hexokinase			No SPE/HPAE-PAD		
	Glucose (mM)	SD	% CV	Glucose (mM)	SD	% CV
<b>0</b>	1.7	0.2	8.6	2.7	0.1	2.6
<b>100</b>	1.1	0.0	2.4	2.1	0.1	3.6
<b>250</b>	1.1	0.1	7.0	1.8	0.0	2.6
<b>500</b>	0.7	0.1	17.4	1.3	0.1	9.7
<b>750</b>	0.8	0.1	13.7	1.4	0.1	5.2
<b>1000</b>	0.7	0.1	8.1	1.3	0.0	3.3
<b>Mean</b>			<b>9.5</b>			<b>4.5</b>

Glucose in the samples from the inhibition of maltase by EGCG was quantitated using both methods (Table 3-1). SPE-removal of EGCG was performed before analysis using hexokinase but not before analysis by HPAE-PAD. The variation between technical triplicates was from hexokinase method ranged from 2.4 to 17.4% (mean 9.5%) whilst variation was lower using HPAE-PAD, from 2.6 to 9.7% (mean 4.5%). Therefore, the enzyme-linked method showed ~2-fold greater variation than the chromatographic method. This variation is due to the SPE step; glucose standards analysed by hexokinase with and without SPE show greater variation after SPE. The concentrations of glucose determined enzymatically were  $43 \pm 4.3\%$  lower, but the loss was consistent and standards are also passed through SPE (Table 3-1). Importantly, the  $\text{IC}_{50}$  values were not different when determined enzymatically

and chromatographically ( $677 \pm 241$  and  $633 \pm 35$   $\mu\text{M}$  respectively;  $p=0.77$ ,  $n=3$ ) (Figure 3-10).

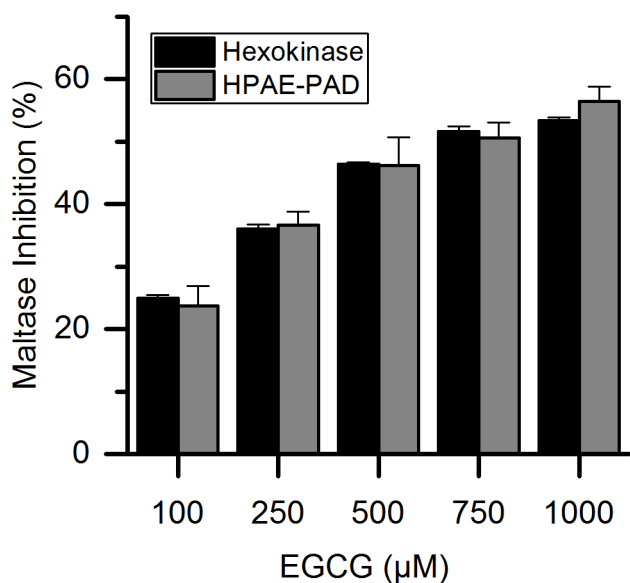


Figure 3-10. Comparison of glucose detection methods for the inhibition of maltase by EGCG. The amount of glucose produced was determined using HPAE-PAD or samples were passed through SPE and then glucose determined by the hexokinase assay. The percent inhibition was determined from  $n=3$  experiments and presented as mean  $\pm$  SEM.

Overall, the standard deviation was greater for the hexokinase detection due to the fact that the samples underwent SPE therefore the HPAE-PAD analysis offers a more accurate result. However, green tea and bonolive interfered with the glucose quantitation therefore the samples would still need to undergo SPE or blank correction on the HPAE-PAD system. Use of HPAE-PAD offers an alternative providing the inhibitors do not interfere with the analysis.



### 3.3.7.7 Green Tea analysis using HPAE-PAD

As mentioned in Section 3.3.7.5, the green tea extract contained glucose (Figure 3-9). Green tea samples were prepared between 1 and 5 mg/mL, the inhibition testing concentration range, and diluted as assay samples 25-fold for HPAE-PAD analysis. A standard curve is presented in Figure 3-11. Inhibition samples with green tea were analysed using HPAE-PAD and the results were corrected for the glucose content in the extract by subtraction using the standard curve.

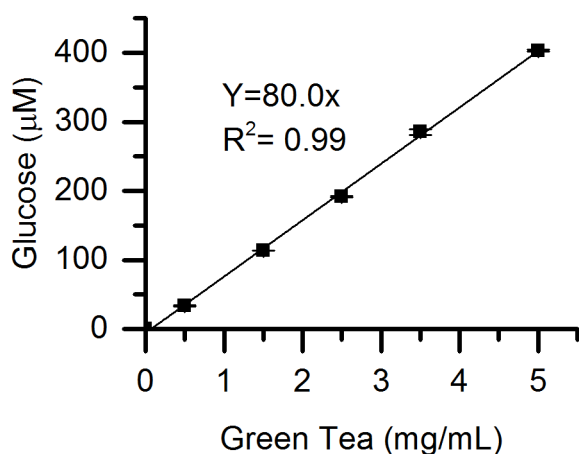


Figure 3-11. Green tea extract standard samples analysed using HPAE-PAD. Green tea samples from 1 to 5 mg/mL were diluted 25-fold for HPAE-PAD analysis to quantify the concentration of glucose present in the extract. Glucose results were multiplied by the dilution factor and presented as mean  $\pm$  SEM (linear fit,  $Y=80.0x$ ,  $R^2 = 0.99$ ,  $n=3$ ).

### 3.3.8 Human enzyme final inhibition assay

The final inhibition assay used 0.05 units per sample for maltase and 0.02 units per sample for sucrase, 10 mM substrate concentration for sucrose and maltose and 10 min incubation time. The hexokinase assay was primarily used as the detection method and inhibitors were evaluated for interference with the hexokinase and removed by SPE if necessary. Green tea inhibition samples

were analysed by HPAE-PAD and results were corrected with green tea standards because the green tea extract contained glucose, fructose and sucrose. The inhibitor was added to the reaction mixture at the reported concentrations. For inhibitors dissolved in DMSO, control samples were included to ensure no interference and a maximum of 2% of the total reaction volume was DMSO.

### **3.3.9 Rat enzyme assay method**

The rat enzyme assay procedure is the same as the human assay but with rat intestinal powder used as the enzyme source. The method was set-up in the same manner as the human assay here; time course, kinetics and specific activity were determined and the assay parameters were chosen so that glucose production is within the linear range and substrate concentration is near the  $K_m$  (Nyambe-Silavwe and Williamson, 2016). For maltase, 4 mg solid/mL was used with 3 mM maltose and 20 min incubation time. For sucrase, 20 mg solid/mL was used with 16 mM sucrose and 20 min incubation time. All samples from the rat assay were analysed by hexokinase and SPE was performed as required.

### **3.3.10 Data analysis and statistics**

The enzyme assay utilises cells as the enzyme source, which exhibit biological variation. Therefore,  $IC_{50}$  determinations and dose response experiments were performed in triplicate with three different passage of cells ( $n=3$ ) and the results are presented as mean  $\pm$  SEM. For screening experiments, one assay was done with three replicates and the results are presented as mean  $\pm$  SD.

The percent inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = 100 - \left( \frac{[\text{glucose}]_{\text{inhibitor}}}{[\text{glucose}]_{\text{control}}} \times 100 \right)$$

The IC<sub>50</sub> is defined as the concentration of inhibitor at which the amount of product produced in the assay is half of that in the control. The IC<sub>50</sub> values were determined graphically by plotting percent inhibition versus inhibitor concentration for each of the n=3 experiments and the mean value ± SD was reported. All statistics were performed using SPSS version 22.

### **3.4 Inhibition results**

#### **3.4.1 Human assay inhibition results – screening**

Selected polyphenol and polyphenol-rich extracts were screened for their inhibitory action towards sucrase (Table 3-2) and maltase (Table 3-3). The compounds chosen were based on inhibition reported in the literature as discussed in the literature review, or based on results from other lab members who used the rat inhibition assay. The screening concentration for some pure polyphenols was limited by their solubility, therefore, inhibition may occur at higher concentrations, although the concentrations tested are in the region of what is achievable in the gut lumen (Williamson, 2013).

Table 3-2. Screening pure polyphenols and polyphenol-rich extracts as inhibitors of sucrase. The results are presented as mean  $\pm$  SD (n=3). Significance was determined by independent sample T-test; \*\*\* p<0.001, \*\* p<0.01, \* p<0.05, ns= not significant.

Polyphenols/ Polyphenol-rich extracts	Screening Concentration	% Inhibition
<b>Apigenin</b>	30 $\mu$ M	NS
<b>Bonolive</b>	1 mg/mL	30 $\pm$ 3.0 ***
<b>(+)-Catechin</b>	100 $\mu$ M	NS
<b>5-Caffeoyl quinic acid</b>	500 $\mu$ M	24 $\pm$ 6.1 **
<b>(-)-Epicatechin</b>	100 $\mu$ M	NS
<b>(-)-Epigallocatechin Gallate</b>	500 $\mu$ M	48 $\pm$ 5.6 ***
<b>Galangin</b>	30 $\mu$ M	NS
<b>German Chamomile (maltodextrin free)</b>	2 mg/mL	NS
<b>Green Tea</b>	1 mg/mL	32 $\pm$ 5.9 **
<b>Hesperetin</b>	30 $\mu$ M	NS
<b>Kaempferol</b>	30 $\mu$ M	NS
<b>Luteolin</b>	30 $\mu$ M	NS
<b>Naringenin</b>	30 $\mu$ M	NS
<b>Quercetin</b>	30 $\mu$ M	11 $\pm$ 0.7 *

Table 3-3. Screening pure polyphenols as inhibitors of maltase. The results are presented as mean  $\pm$  SD (n=3). Significance was determined by independent sample T-test; \*\*\* p<0.001, ns= not significant.

Polyphenols/ Polyphenol-rich extracts	Screening Concentration	% Inhibition
<b>Bonolive</b>	2.0 mg/mL	78 $\pm$ 6.6 ***
<b>5-Caffeoyl quinic acid</b>	500 $\mu$ M	NS
<b>(-)-Epigallocatechin Gallate</b>	500 $\mu$ M	48 $\pm$ 5.6 ***
<b>Green Tea</b>	1.5 mg/mL	75 $\pm$ 1.5 ***

### 3.4.2 Human assay inhibition results – IC<sub>50</sub> determinations

The IC<sub>50</sub> values were determined for positive control acarbose as well as for green tea, EGCG and bonolive, all which exhibited statistically significant inhibition from the screening assays of both sucrase and maltase.

#### 3.4.2.1 Inhibition of sucrase and maltase by Acarbose

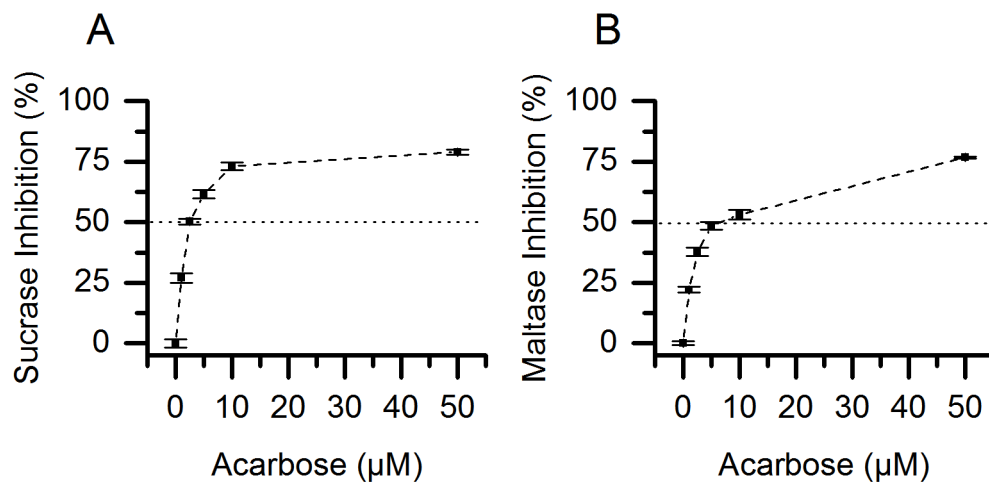


Figure 3-12. IC<sub>50</sub> determination for sucrase (A) and maltase (B) inhibition by acarbose. Results are presented as mean  $\pm$  SEM (n=3).

The IC<sub>50</sub> value for acarbose was  $2.5 \pm 0.5$   $\mu$ M for sucrase and  $5.7 \pm 1.4$   $\mu$ M for maltase.

### 3.4.2.2 Green tea inhibition of sucrase and maltase

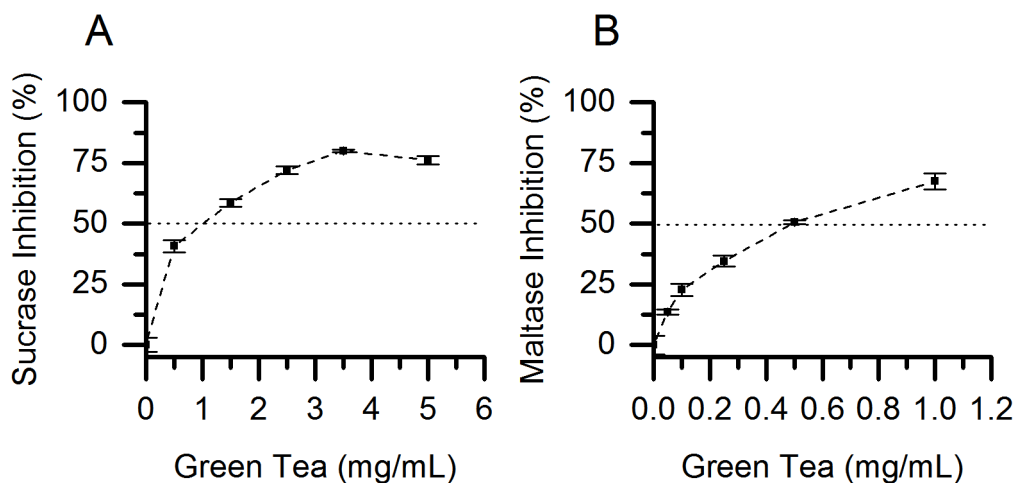


Figure 3-13. Green Tea IC<sub>50</sub> determination for sucrase (A) and maltase (B) inhibition. Results are presented as mean  $\pm$  SEM (n=3).

The IC<sub>50</sub> value for green tea was  $1.0 \pm 0.3$  mg/mL for sucrase and  $0.5 \pm 0.003$  mg/mL for maltase.

### 3.4.2.3 EGCG inhibition of sucrase and maltase

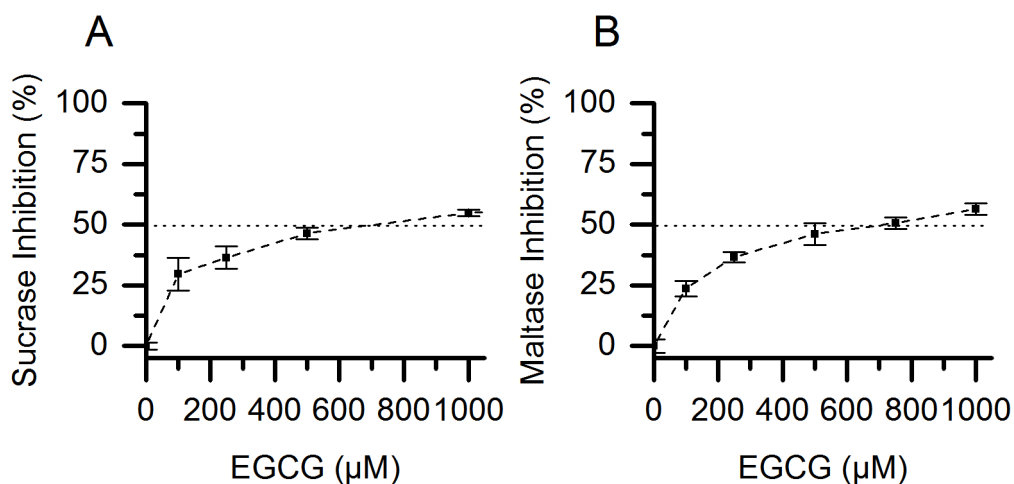


Figure 3-14. EGCG IC<sub>50</sub> determination for sucrase (A) and maltase (B) inhibition. Results are presented as mean  $\pm$  SEM (n=3).

The IC<sub>50</sub> value for EGCG was 657 ± 150 µM for sucrase and 677 ± 241 µM for maltase.

#### 3.4.2.4 Bonolive Inhibition of Sucrase and Maltase

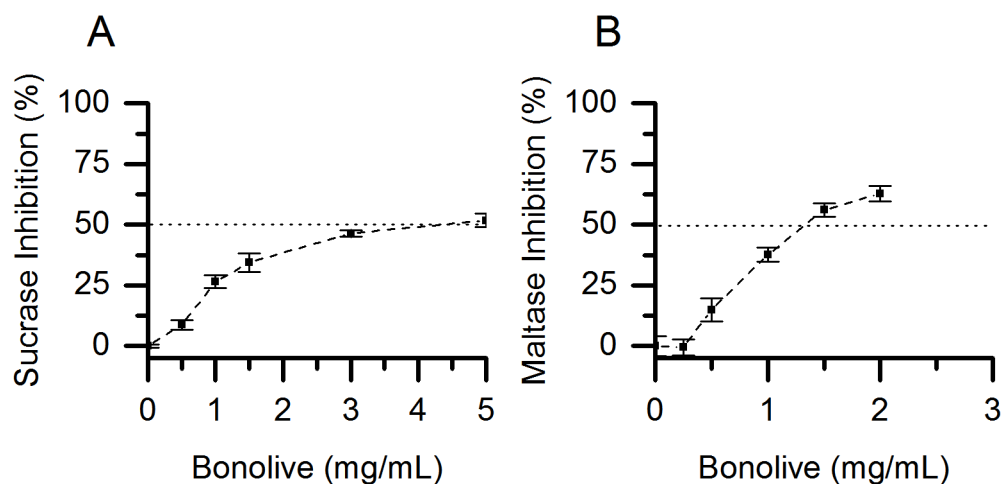


Figure 3-15. Bonolive IC<sub>50</sub> determination for sucrase (A) and maltase (B) inhibition. Results are presented as mean ± SEM (n=3).

The IC<sub>50</sub> value for bonolive was 3.2 ± 1.0 mg/mL for sucrase and 1.3 ± 0.2 mg/mL for maltase.

#### 3.4.3 Rat enzyme assay results

The rat enzyme assay was used to determine the IC<sub>50</sub> of EGCG on sucrase inhibition; 950 ± 86 µM (n=3). Bonolive inhibition of sucrase was evaluated up to 8 mg/mL and reached a maximum of 39.8 ± 2.9% by 5 mg/mL. Bonolive inhibition of maltase was evaluated up to 1.5 mg/mL and the IC<sub>50</sub> was 0.6 ± 0.2 mg/mL (n=3). All other rat assay results were completed by Hilda Nyambe (Nyambe-Silavwe, 2016). Rat enzyme assay results are presented in Table 3-4.

### 3.4.4 Summary of inhibition data from human and rat assays

Results from the human and rat inhibition assays are summarised in Table 3-4. Statistical significance for the difference between rat and human assays was determined by independent sample t-test. Of the polyphenols tested in the human assay, the strongest inhibitor of maltase was green tea, with IC<sub>50</sub> of 0.5 mg/mL, which contains 218 µM EGCG (Section 2.5.1). 677 µM of pure EGCG was required to achieve 50% inhibition. Green tea was also the strongest polyphenol inhibitor of sucrase, with IC<sub>50</sub> of 1.0 mg/mL, equivalent to 435 µM EGCG. The bonolive was 40% w/w oleuropein so 1.0 mg/mL was equivalent to 750 µM oleuropein. Maltase inhibition was always stronger against rat enzyme as compared to human enzyme whereas sucrase inhibition was always stronger against human enzyme, with the exception of bonolive.

Table 3-4. Inhibition of human or rat sucrase and maltase activities by selected compounds. Results are mean ± SD (n=3). Independent sample T-test was performed to compare results from rat with human; \*\*\* p<0.001, \*\*p<0.01, \* p<0.05, NA = not applicable. IC<sub>50</sub> was not reached for bonolive inhibition of sucrase when tested up to 8 mg/mL. # results are from Nyambe-Silavwe, 2016.

Inhibitor	Human		Rat	
	Sucrase IC <sub>50</sub>	Maltase IC <sub>50</sub>	Sucrase IC <sub>50</sub>	Maltase IC <sub>50</sub>
Acarbose (µM)	2.5 ± 0.5	5.7 ± 1.4	12.3 ± 0.6 ***#	0.42 ± 0.02 **#
Green Tea (mg/mL)	1.0 ± 0.3	0.5 ± 0.003	1.8 ± 0.3 *#	0.035 ± 0.005 ***#
EGCG (µM)	657 ± 150	677 ± 241	950 ± 86 *	14 ± 2.0 ***#
Bonolive (mg/mL)	3.2 ± 1.0	1.3 ± 0.2	> 5 mg/mL	0.6 ± 0.2 **

## 3.5 Discussion

In recent years, polyphenols have been investigated as potential α-glucosidase inhibitors for the treatment and prevention of type 2 diabetes, as an alternative



to pharmaceutical treatments such as acarbose. The methods used in the literature predominantly use enzyme preparations from rat intestine, rather than a human source (Matsui et al., 2007; Kamiyama et al., 2010; Honda and Hara, 1993). The homology between human and rat sucrase-isomaltase is only 74%, raising the question as to whether using non-human sources for enzyme assay is physiologically relevant to humans. The active site sequence is conserved, however there are differences in the N-glycosylation, which can affect inhibition due to steric hindrance. The human sequence has 18 predicted N-glycosylation sites while the rat has 16, only 9 of which are in homologous position to the human (Van Beers et al., 1995). Furthermore, some reports use a yeast  $\alpha$ -glucosidase with a non-specific substrate (*p*-nitrophenyl  $\alpha$ -D-glucoside), which provides even less relevant inhibition information. Another limitation of reports currently found in the literature is that the assay parameters and details are not always given in full, which makes comparison of inhibition values amongst different reports impossible. Here, an inhibition assay was optimised for sucrase and maltase using a human enzyme source and different assay parameters were evaluated to demonstrate their impact on inhibition values. The assay was used to evaluate inhibition by some selected polyphenols and polyphenol-rich extracts and the inhibition results from the human assay were compared to those determined using rat enzyme source.

During the assay optimisation, two different lysate preparations were compared. A crude cell lysate was compared to cells which had been digested with papain which proteolytically cleaves the isomaltase stalk from the membrane (Brunner et al., 1979). The papain digested enzyme had higher

specific activity for both sucrase and maltase and this is expected to be due to the fact that the cell debris was removed leading to a solution with higher purity of SI. Inhibition of maltase by acarbose was the same whether the enzyme was free, after proteolytic cleavage by papain or membrane-bound. The unprocessed crude lysate was used for the inhibition assays performed, however the papain method offers a suitable alternative with the benefit that more units are produced for a given amount of cells.

The quantitation of glucose by HPAE-PAD was evaluated in an attempt to eliminate the SPE step of the protocol, which provides cost savings and also reduced the variation between technical triplicates. Assay samples from EGCG inhibition were analysed using both glucose detection methods and both yield similar  $IC_{50}$  values, however the results from the HPAE-PAD analysis had a lower coefficient of variation, therefore providing a more precise result. However, green tea interfered with the glucose quantitation therefore the samples would still need to undergo SPE or blank correction on the HPAE-PAD system. Use of HPAE-PAD offers a suitable alternative providing the inhibitors do not interfere with the analysis.

In setting up the inhibition assays for sucrase and maltase, the time course and number of units were chosen to fall within the linear range. Outside of the linear range, steady-state reaction so Michaelis-Menten kinetics are not valid (Bisswanger, 2014). The importance of working within initial rates was shown by acarbose inhibition of maltase: when maltase activity in the assay was high and outside the linear range, the apparent  $IC_{50}$  value for acarbose was over-estimated. Further, an increased concentration of substrate decreases the

ability of competitive inhibitors to bind and inhibit an enzyme (Acker and Auld, 2014). This was demonstrated in the maltase assay, where maltose substrate at  $\sim 100\times$  the  $K_m$  led to a decrease in observed inhibition. A substrate concentration 10 mM was selected for both sucrase and maltase for consistency as it is near their determined  $K_m$  values, important for identifying all types of inhibitors (Acker and Auld, 2014). The sucrase apparent  $K_m$  measured here was 9.5 mM, within the reported range for human sucrase of 9 -11 mM (Krog-Mikkelsen et al., 2011; Jockovic et al., 2013; Trugnan et al., 1986) and maltase was 7.5 mM, also in agreement with values in the literature (Jockovic et al., 2013).

Results from the rat and human assay were performed under suitably determined assay conditions and both used SPE to remove polyphenols before analysis of glucose so the differences observed are due solely to the enzyme source. EGCG, green tea and acarbose exhibited 1.4 to 4.8-fold stronger inhibition of human, compared to rat, sucrase; for maltase, the reverse was true (13.5 to 48-fold weaker). Sucrase activity arises solely from the sucrase subunit of sucrase isomaltase whereas maltase is derived from both the isomaltase and sucrase subunits, as well as other  $\alpha$ -glucosidases such as maltase glucoamylase (MGAM) (Jones et al., 2012). The difference for the weaker inhibition of maltase could be due to the lack of MGAM in Caco-2/TC7 cells because there is a combined effect from each maltase catalytic sites. When evaluating literature inhibition reports, if a rat enzyme assay was used, the reported inhibition is much stronger than would be observed using a human

source for maltase. For sucrase, the opposite is true, and greater inhibition would be observed in the human assay.

The  $IC_{50}$  of EGCG for rat sucrase has been reported as 218  $\mu\text{M}$  (Honda and Hara, 1993) and 169  $\mu\text{M}$  (Matsui et al., 2007), both stronger than we observed here using rat; however both used detection methods for which EGCG interferes (Nyambe-Silavwe et al., 2015). The  $IC_{50}$  of EGCG for rat maltase has been reported as 40  $\mu\text{M}$ , somewhat weaker than we observed (14  $\mu\text{M}$ ). Reported results using the enzyme from rat source for acarbose, which does not interfere with detection methods, were more similar to ours ( $IC_{50}$  of 0.43  $\mu\text{M}$  for maltase and 1.2  $\mu\text{M}$  for sucrase) (Matsui et al., 2007). A  $K_i$  of 1.8  $\mu\text{M}$  has been reported for 5-caffeoyl-quinic acid for maltase activity using *Drosophila* expression system as the source of sucrase (Simsek et al., 2015), however, here it was not found to be a strong inhibitor of human sucrase or maltase at 500  $\mu\text{M}$ . Detection method interference or the use of sucrase mouse sequence for the expression could all explain the dramatic difference. Furthermore, glycosylation in the *Drosophila* expression system differs from mammalian: it lacks the ability to produce complex N-glycans due to absence of  $\beta$ -N-acetylglucosaminidase enzyme (Léonard et al., 2006).

The values from inhibition screening here differ from some literature reports. For example, quercetin, kaempferol, luteolin, naringenin and hesperetin  $IC_{50}$  values have been reported as 7, 12, 21, 75 and 150  $\mu\text{M}$ , respectively in an assay using yeast enzyme activity (Tadera et al., 2006). Here, these were screened at 30  $\mu\text{M}$  with human sucrase and only quercetin exhibited mild inhibition of 11%. The concentrations tested here were limited by solubility. For

example, limited solubility was observed for quercetin above 100  $\mu\text{M}$  when spiked into assay buffer. Quercetin was reported to inhibit rat sucrase with an  $\text{IC}_{50}$  of 363  $\mu\text{M}$  (Kim et al., 2011) which is weaker than we observed in the assay with human enzyme, as expected but to a greater extent possibly due to solubility issues. The  $\text{IC}_{50}$  for quercetin has even been reported as  $>1$  mM (Jo et al., 2009), where solubility is an issue and this concentration is not physiologically relevant. Another study reported an  $\text{IC}_{50}$  for naringenin as 384  $\mu\text{M}$  towards yeast  $\alpha$ -glucosidase using a generic substrate (Priscilla et al., 2014). Here, naringenin was only able to be screened up to 30  $\mu\text{M}$  since poor solubility was observed at greater concentrations, so it is unlikely that it would remain soluble up to the reported 384  $\mu\text{M}$ . Even more recent publications have limitations; the inhibition of  $\alpha$ -glucosidases by polyphenols from the plant *polygonum cuspidatum*, which is used in traditional Chinese medicine, was recently reported (Zhao et al., 2017). Although the assay does account for inference from the polyphenols by trapping them in an in-line SPE, the assay used a yeast enzyme with a *p*-nitrophenyl  $\alpha$ -d-glucopyranoside generic substrate which does not provide inhibition relevant to mammalian enzymes (Tadera et al., 2006).

The current pharmacological inhibitor acarbose often leads to gastrointestinal side-effects (Balfour and McTavish, 1993) due to undigested starch and sugar reaching the colon because of inhibition by acarbose towards maltase, sucrase,  $\alpha$ -amylase and isomaltase. Understanding the specific inhibition of  $\alpha$ -glucosidases by polyphenols or other inhibitors in humans can help to design therapeutic treatments with fewer side effects. For example, the naturally

occurring pentose *L*-arabinose, which is solely a sucrase inhibitor, did not cause gastrointestinal side-effects in humans (Krog-Mikkelsen et al., 2011). Digestion of sucrose by sucrase can be rate-limiting in the glycaemic response in rats; in an oral sucrose tolerance test with a formulation containing *L*-arabinose, the incremental area under the curve for blood glucose was significantly reduced while no change was observed in glucose or starch tests (Preuss et al., 2007b; Preuss et al., 2007a). The effect was also confirmed in humans; pure *L*-arabinose led to a delay in glucose absorption in response to sucrose intake along with a suppression of insulin (Krog-Mikkelsen et al., 2011). These studies demonstrate that the strategy of sucrase inhibition does translate in an *in vivo* effect.

### **3.6 Conclusion**

When performing assays or considering published inhibition results, the interference with any glucose detection method must be considered. Interfering compounds can be removed through solid phase extraction, although this introduces greater variation to the results, so HPAE-PAD offers an alternative detection method which can improve accuracy. The use of human or rat as the source of  $\alpha$ -glucosidase enzyme impacts the resulting inhibition and the optimised human assay used here is more physiologically relevant.

## **4 Chronic effects of sucrose exposure on sucrase isomaltase in the Caco-2/TC7 cell model**

### **4.1 Abstract**

Dietary guidelines in the UK now state that daily intake of sugar should be halved, down to no more than 5% of total energy intake. Consumption data from the National Diet and Nutrition Survey (NDNS) from 2008-2012 indicated that sucrose was the most commonly consumed sweetener and in adults its mean consumption was 1/5<sup>th</sup> of total energy intake (SACN, 2015). To date, there is no established mechanistic link between sugar and health and there is insufficient evidence to determine any direct effects of specific sugars on health. At the cellular level, there is evidence that small intestinal enterocytes can sense sugars (Le Gall et al., 2007) and Caco-2/TC7 cells have been reported to respond to maltose (Cheng et al., 2014). The aim here was to mimic nutritional sugar stress by evaluating the effects of long-term sucrose exposure on sucrase, the enzyme solely responsible for its hydrolysis. Compared to the glucose control, exposure to 25 mM sucrose did not change the SI mRNA levels, but increased SI protein levels while maintaining the same amount at the apical cell surface. In addition, both N- and O-glycosylation of SI, required for trafficking to the apical surface (Naim et al., 2012), were altered. This could explain the accumulation in the total lysate and could also be responsible for the apparent decrease in affinity for sucrose after sucrose exposure. Evidence here suggests that sugar exposure can influence post-translational modifications which could decrease starch or sugar digestion.

## 4.2 Introduction

Sucrose is hydrolysed solely by the sucrase subunit of SI at the small intestine brush border into fructose and glucose (Van Beers et al., 1995). Since SI plays a key role in digestion of sugar and starch, its activity will influence the postprandial blood glucose response. The focus of this chapter is specifically on SI because sucrase is the sole enzyme capable of sucrose hydrolysis and the also the main enzyme responsible for maltase and isomaltase activities, so has a key role in overall carbohydrate digestion. Furthermore, sucrose is the most commonly consumed sweetener in the UK (SACN, 2015). There is some evidence in the literature on the effect of dietary exposure to sucrose and its effect on sucrase. Studies in cells and rats have shown that monosaccharides have regulatory effects on sucrase at the mRNA level, but these compare the expression to that of a starved or low-carbohydrate state in which many genes can be activated (Ferraris et al., 1992; Quan and Gray, 1993; Kishi et al., 1999) (See Section 1.2.5). There is limited information in the literature as to whether sucrose is involved in the regulation of sucrase, in comparison to glucose, under normal rather than starved or low-carbohydrate conditions. Most reports of SI regulation are transcriptional, for example forskolin and monensin both decreased SI activity in Caco-2 cells through reduced transcription (Chantret et al., 1993); however in some phenotypes of congenital sucrase isomaltase deficiency (CSID) disorder, which affects 0.2% of people of European descent, lack of sucrase activity can result from mutations at the site of N- or O-glycosylation (Jones et al., 2012).



The cDNA sequence of SI in Caco-2 cells is identical in comparison to the human small intestine (Chantret et al., 1992) however the sequence in the Caco-2/TC7 clone has not been investigated. The first aim of this chapter is to characterise SI in the Caco-2/TC7 cell model, firstly by confirming if the sequence of SI in the Caco-2/TC7 clone is free from mutations since point mutations can lead to lack of function (Naim et al., 2012). The second aim is to evaluate sucrase in response to chronic exposure to sucrose with the hypothesis that sucrose will increase SI activity. The enterocyte life-span *in vivo* is between 3-5 days and they are exposed to the contents of the gut lumen throughout differentiation while they migrate from the crypt to villus tip, where they are shed (Ferraris, 2001). The Caco-2/TC7 cell model is exposed to sucrose throughout differentiation and the effects on SI mRNA, protein, activity and enzyme kinetics were evaluated in comparison to normal growth conditions in glucose.

### **4.3 Results**

#### **4.3.1 Sequencing results**

The sucrase isomaltase sequence from human cDNA in the NCBI database was used as the reference sequence (NM\_001041.3). Sequencing results (Appendix 1) show that the cDNA prepared from the Caco-2/TC7 cell line has one base different at position 4632 of the reference sequence. Position 4632 is an adenine rather than guanine, resulting in the codon ATA which codes for isoleucine, rather than ATG, which codes for methionine (Figure 4-1). The RNA from a human intestinal biopsy sample from DV Biologics was also sequenced for SI and the same single base difference from the reference was observed.

Furthermore, Chantret et al. reported the original sequence for SI from Caco-2 cells and is in agreement with the sequence found here in both the Caco-2/TC7 sample and the human biopsy sample (Chantret et al., 1992). Additionally, the National Institutes of Health Mammalian Gene Collection Program, whose aim was to identify and sequence a cDNA clone containing a complete ORF for each human and mouse gene, uploaded sequence BC132860.1 to the NCBI database and also report the same single base difference as compared to the reference (Mammalian Gene Collection Program, 2002). The agreement between the two samples tested here, the mammalian gene collection program and the sequence from Caco-2 cells suggests that there is no mutation present in the Caco-2 or Caco-2/TC7 cell line.

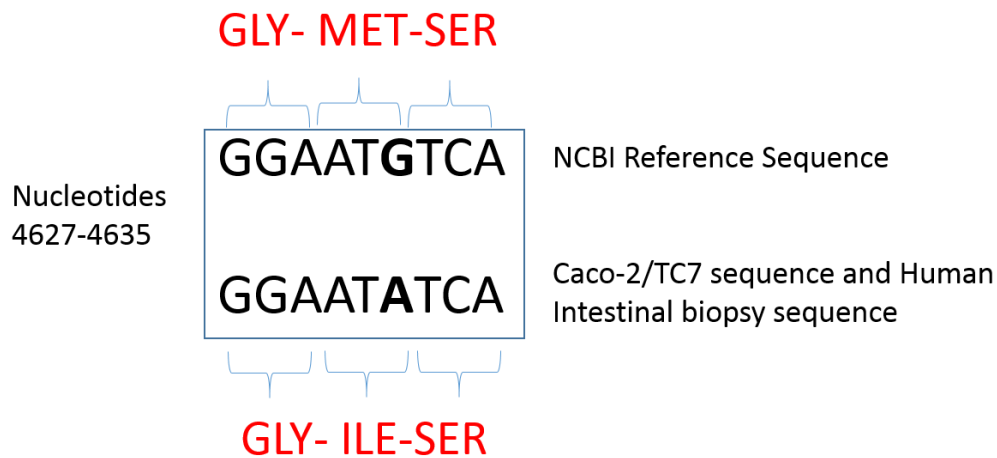


Figure 4-1. Sequence comparison of SI from Caco-2/TC7 cells and from human intestinal RNA to NCBI reference sequence.

#### 4.3.2 Comparison of Caco-2 with Caco-2/TC7 clone

The amount of SI relative to TBP (Tata-box binding protein) housekeeping gene was determined in Caco-2 cells and Caco-2/TC7 cells to confirm that the Caco-2/TC7 cell line has increased expression of SI and is therefore a more suitable model for this study. Results are presented in Figure 4-2. The Caco-2/TC7 cell line has 5-fold more SI mRNA as compared to the parental Caco-2 cell line ( $p < 0.001$ ) (Figure 4-2).

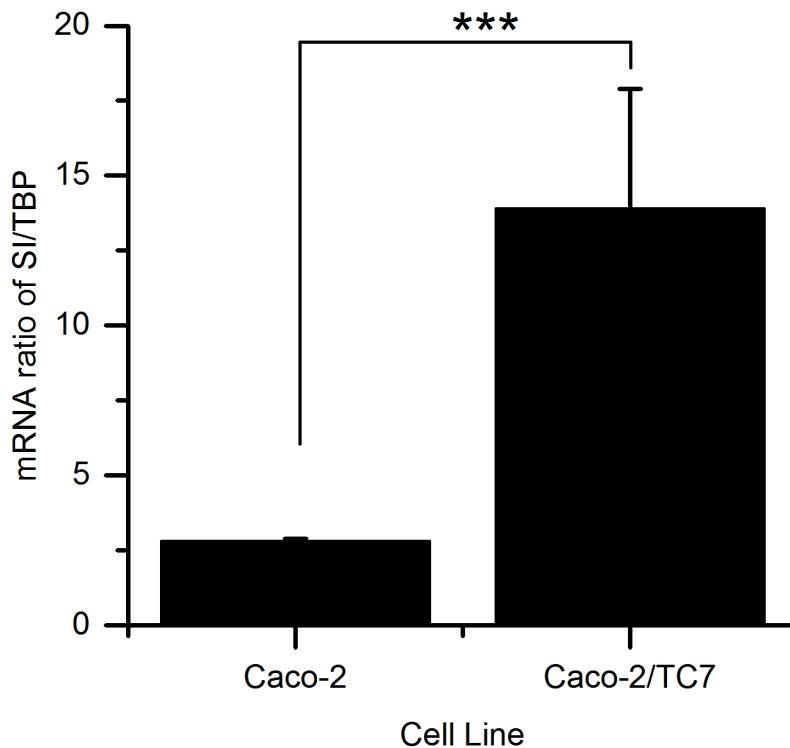


Figure 4-2. Comparison of the mRNA level of SI in Caco-2/TC7 clone in comparison to the parental Caco-2 cell line. Cells were cultured in 6-well plates for 21 days to reach full differentiation. Total RNA was extracted, reverse transcribed to cDNA and the mRNA copies/ $\mu$ L of SI and housekeeping gene relative to TBP were determined by digital PCR. Results are presented as mean  $\pm$  SD ( $n=3$ ) \*\*\*  $p < 0.001$

### 4.3.3 Results for the effects of high sucrose exposure in Caco-2/TC7 cells on sucrase isomaltase

#### 4.3.3.1 Sucrase isomaltase protein: total and cell surface

ProteinSimple WES was used to determine the amount of SI protein in the total cell lysate and in the cell surface fraction after the purification of cell surface proteins by biotinylation. Cells were cultured on 6-well transwell® filters in 25 mM glucose or 25 mM sucrose for 21 days, with four independent experiments with 3 wells per condition (n/N=4/12). Each sample was analysed in duplicate. There was a 71% increase in the absolute amount of total SI protein when cells were cultured in sucrose ( $p < 0.001$ ) (Figure 4-3A). The  $\alpha$ -actinin control had no significant change with the treatment (Figure 4-3B). The final result was corrected for the  $\alpha$ -actinin loading control; there was a 79% increase in total SI protein for cells that were cultured in sucrose ( $p < 0.001$ ) (Figure 4-3C).

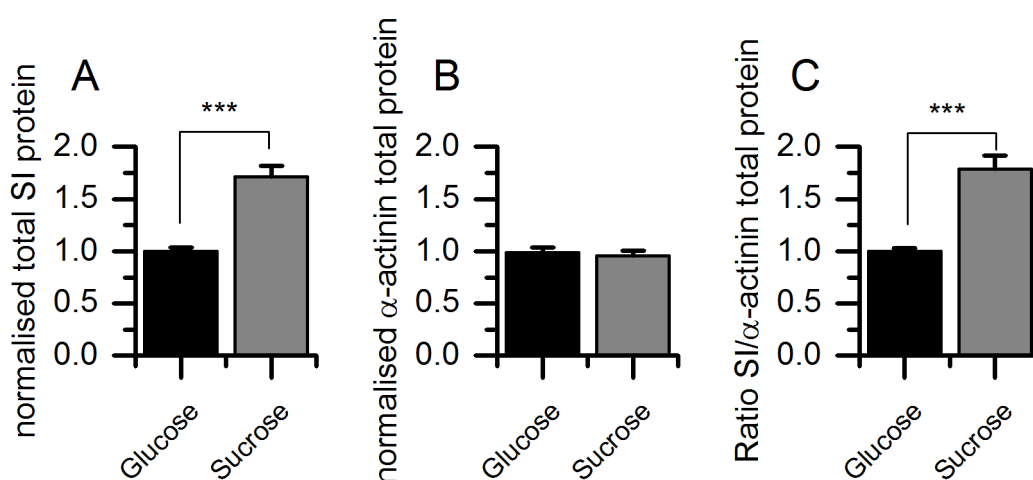


Figure 4-3. The effect of sucrose on SI protein in Caco-2/TC7 total cell lysate. Caco-2/TC7 cells were cultured in 25 mM glucose or 25 mM sucrose on transwell® plates for 21 days. ProteinSimple WES analysis was used to quantify sucrase (A) and the loading control  $\alpha$ -actinin (B) and the ratio of SI/ $\alpha$ -actinin was determined (C). Results were normalised to the glucose control and are presented as mean  $\pm$  SEM (n=4) \*\*\*  $p < 0.001$

In the surface fraction, there was no change in SI ( $p=0.1$ ) (Figure 4-4A). As a proportion of the total protein, there was a decrease at the surface by 30% ( $p=0.009$ ) (Figure 4-4B).

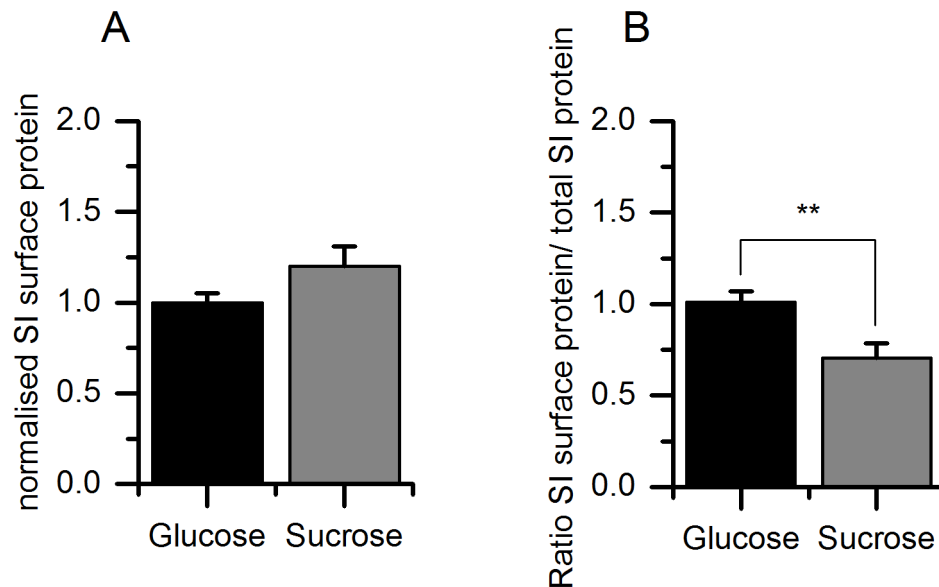


Figure 4-4. The effect of sucrose on surface SI protein in Caco-2/TC7 cells.

Caco-2/TC7 cells were cultured in 25 mM glucose or 25 mM sucrose on transwell® plates for 21 days. After purification of biotinylated cell surface proteins, samples were analysed by ProteinSimple WES analysis to quantify SI in the surface fraction (A). The ratio of SI at the cell surface to total SI (B), where the total SI is corrected for the  $\alpha$ -actinin loading control. Results were normalised to the glucose control and are presented as mean + SEM ( $n=4$ ), \*\*  $p<0.01$

#### 4.3.3.2 Protein size analysis

The ProteinSimple Compass® software assigns a size to the band based on the migration in the capillary by aligning the position of reference standards within each capillary to those in the ladder standard sample capillary. During the protein analysis on WES it was observed that the reported size of SI was higher for cells cultured in sucrose as compared to standard culture conditions in glucose. Three sample types were analysed; total cell lysate from cells cultured on Transwells®, purified cell surface protein for cells cultured on

Transwells® and total cell lysate from cells cultured on solid supports. In the cells cultured on transwells® the mean size of SI in the cell surface fraction and total lysate was determined from the 48 individual capillaries with samples from n=4 experiments. Note that at the cell surface there is no  $\alpha$ -actinin present. In the cells cultured on solid supports the mean size of SI was determined from n=3 independent experiments with results from 12 capillaries. In all three sample types, the size of SI for cells cultured in sucrose was greater than those cultured in glucose ( $p < 0.001$ ). The size of the loading control  $\alpha$ -actinin in the total lysate did not change ( $p > 0.05$ ) (Table 4-1).

Table 4-1. Protein size analysis of SI and the  $\alpha$ -actinin loading control on ProteinSimple WES. Caco-2/TC7 cells were cultured in 25 mM glucose (G; control) or 25 mM sucrose (S) for 21 days on solid supports or transwell® filters. The mean size of sucrose isomaltase was determined at the cell surface fraction after biotinylation (n=48). The mean size of SI and  $\alpha$ -actinin was determined in the total lysate from cells grown on transwell® filters (n=48) or solid supports (n=12). Results are presented as a percentage of the control (glucose), mean  $\pm$  SD, and statistical significance was determined with Student's T-test.

Sample	Condition	Sucrase Size (% of Control)	P-value	$\alpha$ -actinin (% of control)	P-value
<b>Cell Surface</b>	G	307.6 $\pm$ 1.5	<0.001	NA	NA
	S	310.3 $\pm$ 1.4		NA	
<b>Total Lysate (transwell)</b>	G	306.5 $\pm$ 4.0	<0.001	100.8 $\pm$ 0.8	0.1
	S	310.3 $\pm$ 4.1		101.2 $\pm$ 0.8	
<b>Total Lysate (solid support)</b>	G	294.9 $\pm$ 1.8	<0.001	100.0 $\pm$ 0.3	0.7
	S	297.1 $\pm$ 1.5		100.1 $\pm$ 0.6	

To further investigate the nature of this size increase for sucrose grown samples, the endoglycosidase PNGase F was used to remove N-glycosylation; resulting products of the same size would indicate that the size difference observed in untreated samples is due to N-glycosylation while products of a

different size would suggest that both N- and O-glycosylation were altered. Representative pherograms from mock treated (no PNGase F added) and PNGase F treated samples on ProteinSimple WES are presented in Figure 4-5. The PNGase F digestion product of sucrase isomaltase was significantly different between glucose and sucrose grown cells. No change in the  $\alpha$ -actinin loading control was observed (Table 4-2). These results suggest that the size difference observed in the untreated samples was not due to N-glycosylation alone, circumstantially suggesting a change in both N and O-glycosylation.

Table 4-2. Protein size analysis by WES of SI and  $\alpha$ -actinin after PNGase F deglycosylation. Caco-2/TC7 cells were cultured in 25 mM glucose or 25 mM sucrose on transwell® filters and the total lysate was subjected to PNGase F treatment or mock treatment (no PNGase F enzyme). Results for sucrase isomaltase and control  $\alpha$ -actinin are presented as mean  $\pm$  SD (n=7).

Sample	Sucrase Isomaltase Size (kDa)	p-value	$\alpha$ -actinin Size (kDa)	p-value
<b>Glucose Mock</b>	302.3 $\pm$ 0.9	<0.001	103.0 $\pm$ 0	0.57
<b>Sucrose Mock</b>	309.3 $\pm$ 0.4		103.5 $\pm$ 0.2	
<b>Glucose PNGase F</b>	278.3 $\pm$ 1.7	<0.001	103.4 $\pm$ 0.3	0.55
<b>Sucrose PNGase F</b>	286.9 $\pm$ 2.6		103.7 $\pm$ 0.4	

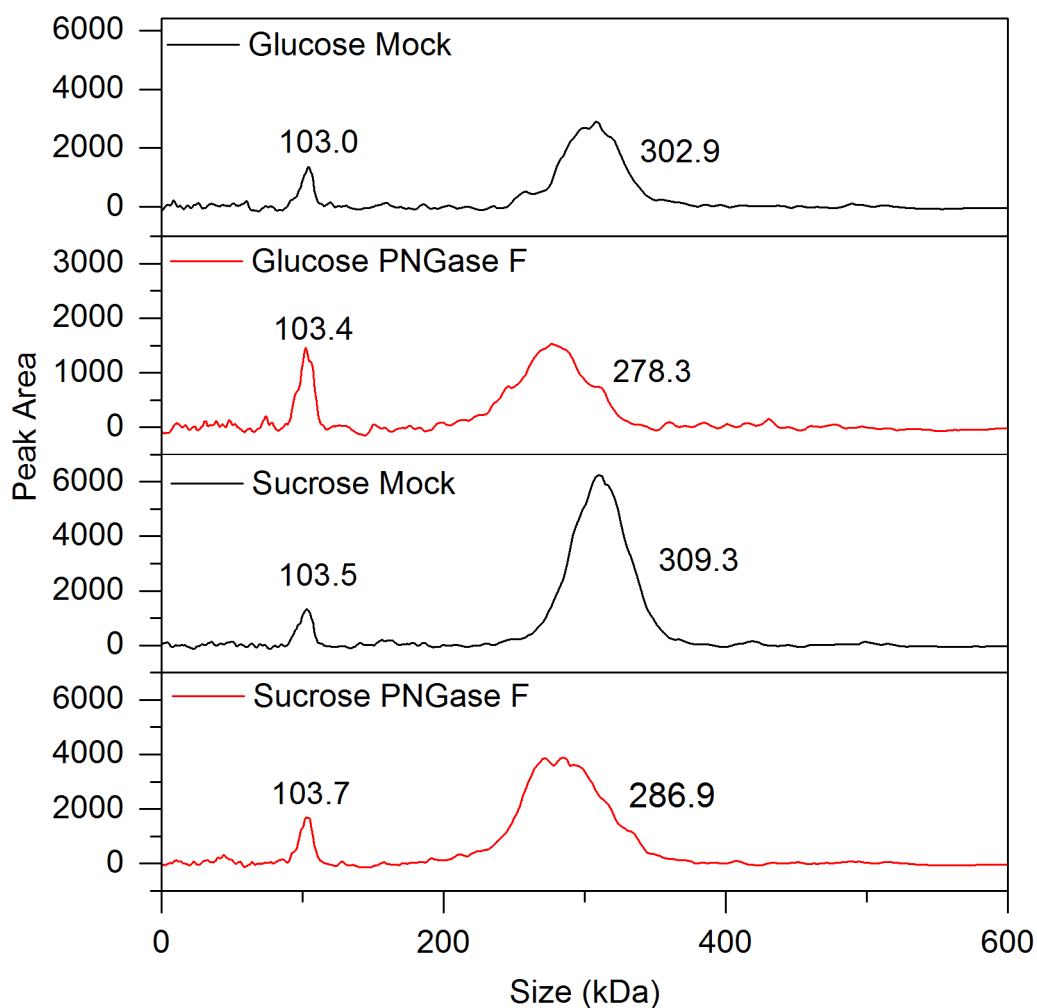


Figure 4-5. Representative pherograms from ProteinSimple WES of  $\alpha$ -actinin (first peak) and SI (second peak). Caco-2/TC7 total cell lysates from cells cultured in 25 mM glucose or 25 mM sucrose treated with and without PNGase F enzyme with the mean size from n=7 samples.

#### 4.3.3.3 Gene expression

The expression of SI mRNA was evaluated over differentiation in control cells and expression was at a maximal level at 14 days and decreased by 31% at day 21 when the cells were fully differentiated (Figure 4-6). The expression at each time point was significantly different than all the other points, as determined by ANOVA followed by Tukey's post-hoc test ( $p < 0.001$  for all). The



TBP housekeeping gene also changed with differentiation therefore the absolute copies/ $\mu\text{L}$  of SI mRNA was reported.

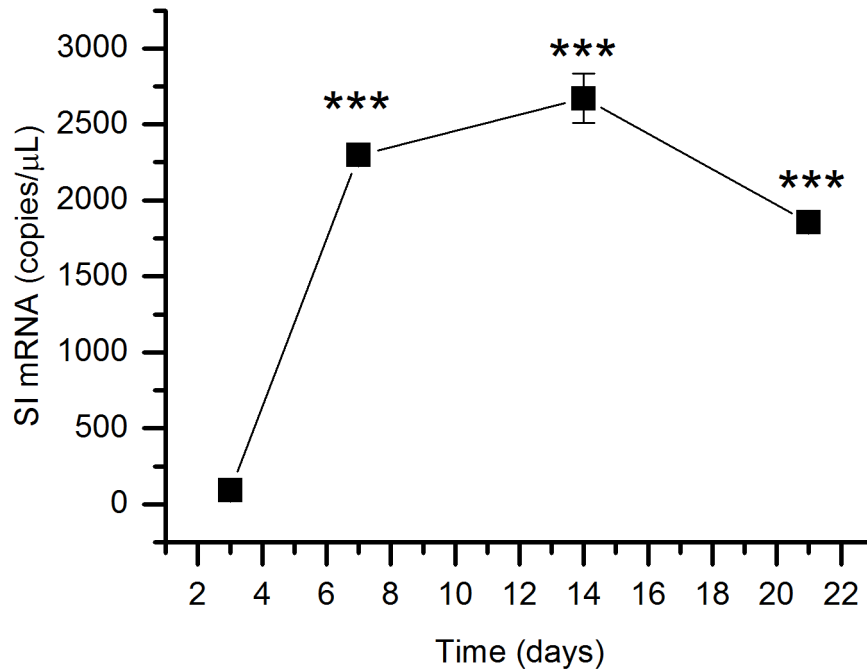


Figure 4-6. The expression of SI mRNA over differentiation in Caco-2/TC7 control cells cultured in 25 mM glucose. Presented as mean  $\pm$  SEM (n=3).

No change was observed in the mRNA levels of SI and GLUT2 when Caco-2/TC7 cells were cultured in 25 mM glucose or 25 mM sucrose. There was a significant 47% decrease in SGLT1 mRNA ( $p < 0.001$ ) and a 27% decrease in GLUT5 mRNA ( $p < 0.001$ ) when cultured in sucrose (Figure 4-7A). SI mRNA was the most abundant, followed by GLUT5, GLUT2 and SGLT1 (Figure 4-7B). There was 7.8-fold less SGLT1 as compared to GLUT2 ( $p < 0.001$ ). The TBP housekeeping gene was not significantly different between the two conditions, indicating it was a suitable control.

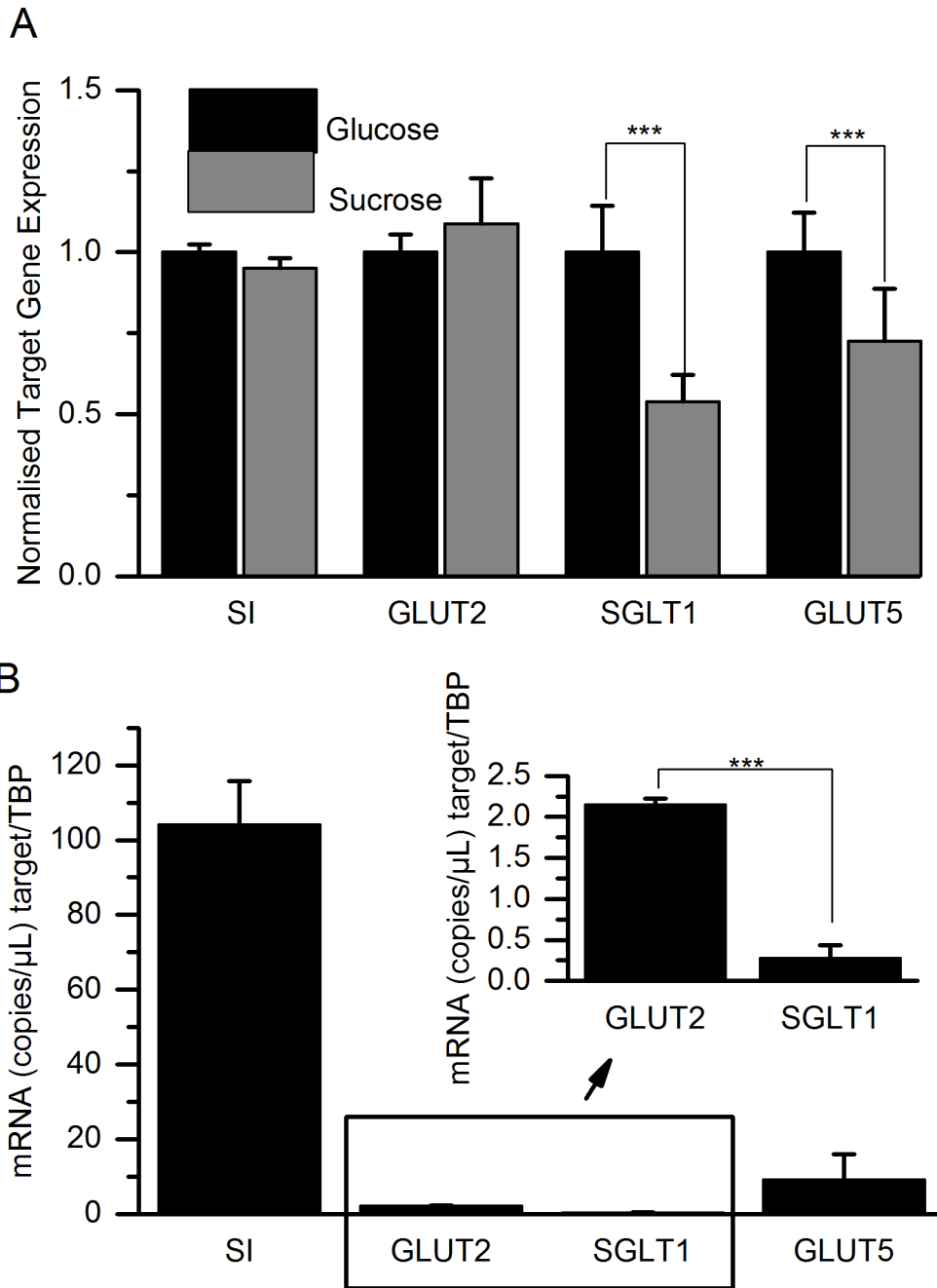


Figure 4-7. Effect of sucrose on the gene expression of SI, GLUT2, SGLT1 and GLUT5 in Caco-2/TC7 cells. Cells were cultured for 21 day in 25 mM glucose or 25 mM sucrose. Target gene in copies/ $\mu$ L was divided by the TBP housekeeping gene and then normalised to the glucose control. Results are presented as mean + SEM, \*\*\*  $p < 0.001$  from Student's T-test ( $n=3$ ). B) Relative gene expression of SI, GLUT2, SGLT1 and GLUT5 in Caco-2/TC7 control samples grown in 25 mM glucose. Results are presented as mean + SEM ( $n=3$ ), \*\*\*  $p < 0.001$  from ANOVA followed by Tukey's post-hoc test.

Since the protein and mRNA results did not correlate, The expression of SI mRNA was also determined from Caco-2/TC7 cells cultured on transwell® filters so that they are comparable to the cells used for protein analysis. Cells cultured on porous filters are polarised as they are exposed to FBS containing medium on the basolateral side only, from confluence day 7 until fully differentiated. As seen in Figure 4-8, there was no significant difference between SI mRNA from glucose or sucrose grown cells, however there was a difference in the absolute amount of SI mRNA between the two different cell supports; cells cultured on transwell® filters had a lower ratio of SI/TBP than those cultured on solid supports. Growth in 25 mM sucrose appears to have no impact on the mRNA of SI regardless of if they are cultured on solid or transwell® supports, however there was an increase in the total amount of protein when cultured in sucrose on transwell® supports.



Figure 4-8. Comparison of the SI mRNA from Caco-2/TC7 cells cultured in 25 mM glucose or 25 mM sucrose on either solid or transwell® supports. The amount of sucrase isomaltase in copies/ $\mu$ L relative to the TBP housekeeping gene is presented as mean + SEM (n=3). ns= not significant.

#### **4.3.3.4 Sucrase specific activity**

The specific activity of sucrase was determined for cells cultured in glucose and sucrose using the sucrase assay developed in Chapter 3. The amount of glucose produced in  $\mu\text{mol}/\text{min}$  was plotted against total milligrams of protein in the assay therefore the slope of the line is the specific activity in U/mg where 1 unit is defined as the amount glucose produced per min ( $\mu\text{mol}/\text{min}$ ). Significance was determined by independent sample t-test between the slope of the line from each replicate assay. After two weeks growth, at which the cells are not yet fully differentiated, the sucrase specific activity was 70.9 mU/mg for cells cultured in glucose and 55.3 mU/mg for cells cultured in sucrose and the 25% difference was significant ( $p < 0.001$ ) (Figure 4-9A). When the cells are fully differentiated at three weeks the specific activity was increased and there was no difference between cells cultured in glucose or sucrose ( $p = 0.6$ ) (Figure 4-9B).

The specific activity was then evaluated for cells cultured in 25 mM glucose and 25 mM fructose to see if the change in specific activity observed at two weeks was due to intact sucrose or to its hydrolysis products glucose and fructose. There was no significant difference between the specific activity of cells cultured in 25 mM sucrose or 25 mM glucose + 25 mM fructose (Figure 4-10A). The specific activity at two weeks was determined after treatment with forskolin as a negative control. Forskolin is reported to reduce SI mRNA through transcriptional regulation rather than changed in mRNA stability (Chantret et al., 1993). Caco-2/TC7 cells were cultured for two weeks and treated with 10  $\mu\text{M}$  forskolin for the last 5-days of growth and the SI specific activity was reduced

almost 2-fold from 95 mU/mg to 51 mU/mg ( $p < 0.001$ ) (Figure 4-10B). Finally, the specific activity of sucrase was also determined for cells cultured on transwell® filters for two weeks in comparison to solid supports. The same trend was observed on transwell® filters where the sucrase specific activity was decreased for cells cultured in sucrose, from 70 mU/mg in glucose to 41 mU/mg in sucrose ( $p < 0.001$ ) (Figure 4-10C).

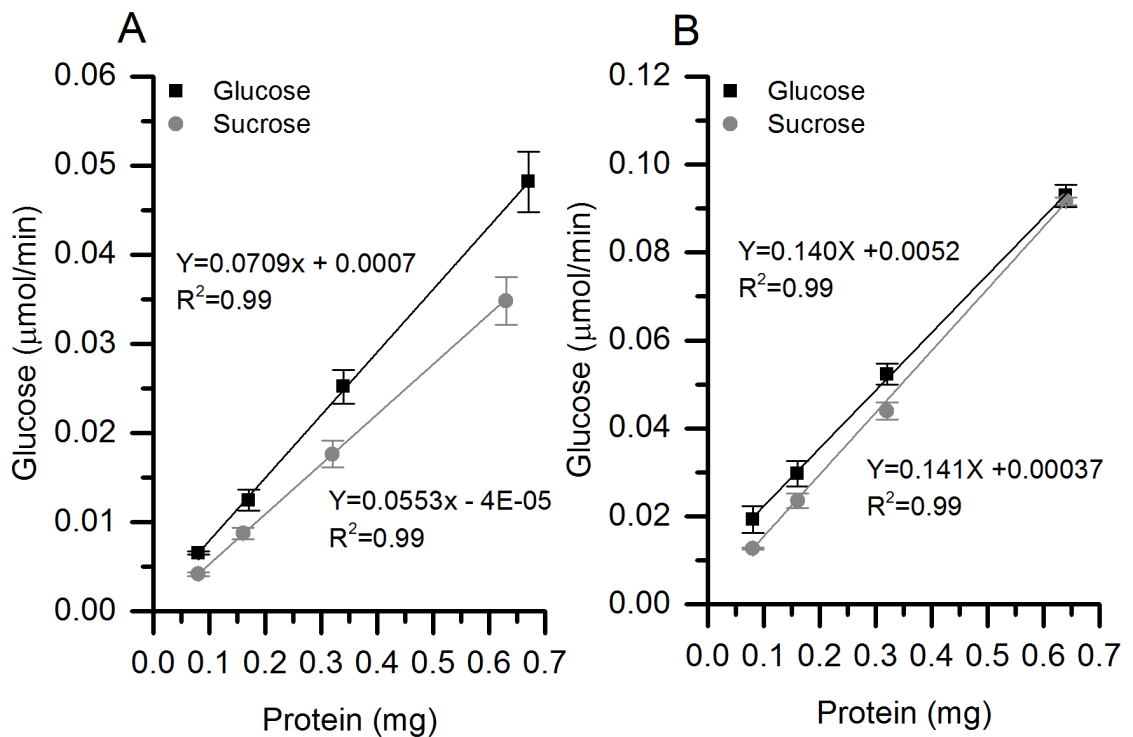


Figure 4-9. Sucrase specific activity for cells cultured in 25 mM glucose or 25 mM sucrose for two weeks (A) or three weeks (B). Presented as mean  $\pm$  SEM (n=3).

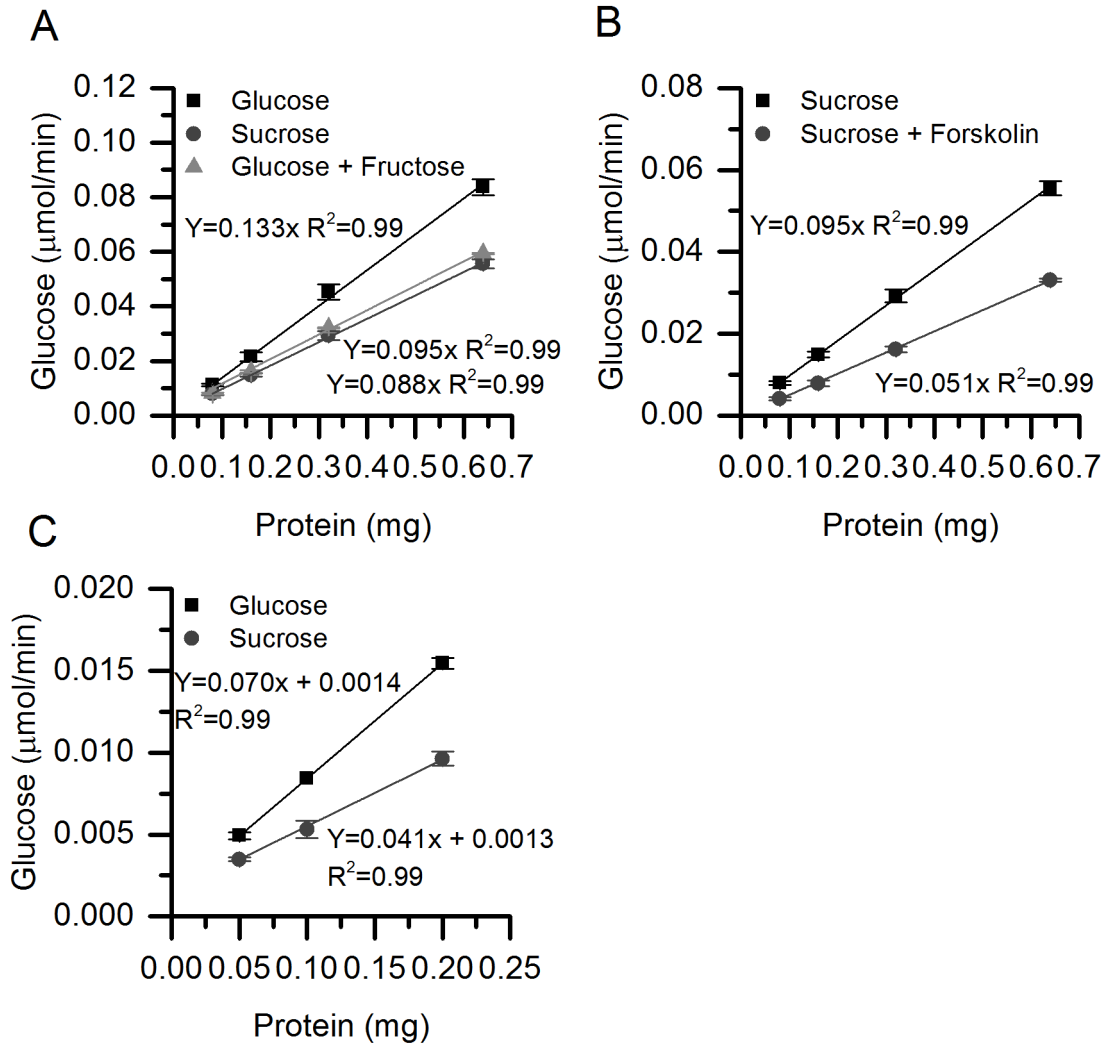


Figure 4-10. Sucrase specific activity determination in Caco-2/TC7 cells. A) 25 mM glucose, 25 mM sucrose or 25 mM glucose + fructose for 2 weeks B) 25 mM sucrose or 25 mM sucrose with 5-day treatment of forskolin at 10 μM as a negative control C) 25 mM glucose and 25 mM sucrose for 2 weeks on transwell® plates. Results are mean ± SD (n/N=1/3).

#### 4.3.3.5 Sucrase $K_m/V_{max}$

The apparent  $K_m$  and  $V_{max}$  of sucrase were determined for cells cultured in glucose and sucrose using the sucrase enzyme assay developed in Chapter 3. Data from three independent experiments for each of 2 or 3 weeks growth were normalised to the glucose control and the average was used to plot the Lineweaver-Burk graphs presented in Figure 4-11A&B. The apparent  $K_m$  and  $V_{max}$  were calculated where the slope =  $K_m / V_{max}$  and the y-intercept =  $1 / V_{max}$ . Students' t-test was performed to determine the  $p$ -value for 95% significance. At two weeks, the  $K_m$  was 29% higher for cells cultured in sucrose, 17.2 mM compared to 12.3 mM for glucose ( $p < 0.001$ ) and the  $V_{max}$  was not significantly different, 0.036  $\mu\text{mol}/\text{min}$  in glucose to 0.032  $\mu\text{mol}/\text{min}$  in sucrose ( $p = 0.08$ ). The results from 3 weeks, at which cells are fully differentiated, are presented in Table 4-3. There was no change in the  $V_{max}$  but the  $K_m$  increased from 10.7 mM in glucose to 12.3 mM in sucrose ( $p = 0.01$ ).

Table 4-3. Kinetic parameters  $K_m$  and  $V_{max}$  determined from Caco-2/TC7 cells cultured in 25 mM glucose or 25 mM sucrose for 3 weeks.

Carbohydrate Source	Linear Fit	$R^2$	$K_m$ (mM)	P-value	$V_{max}$ ( $\mu\text{mol}/\text{min}$ )	P-value
<b>25 mM Glucose</b>	$y = 201.0x + 17.9$	0.99	$10.7 \pm 1.9$	<b>0.01</b>	0.06	0.3
<b>25 mM Sucrose</b>	$y = 225.5x + 19.5$	0.99	$12.3 \pm 3.5$		0.05	

The  $K_m$  and  $V_{max}$  of sucrase were also determined for cells cultured in 25 mM glucose + 25 mM fructose (Figure 4-12) after 2 weeks. As with the specific activity results, there was no difference between 25 mM glucose + 25 mM fructose and 25 mM sucrose ( $p < 0.001$ ), while both were different from 25 mM glucose ( $p < 0.001$ ).

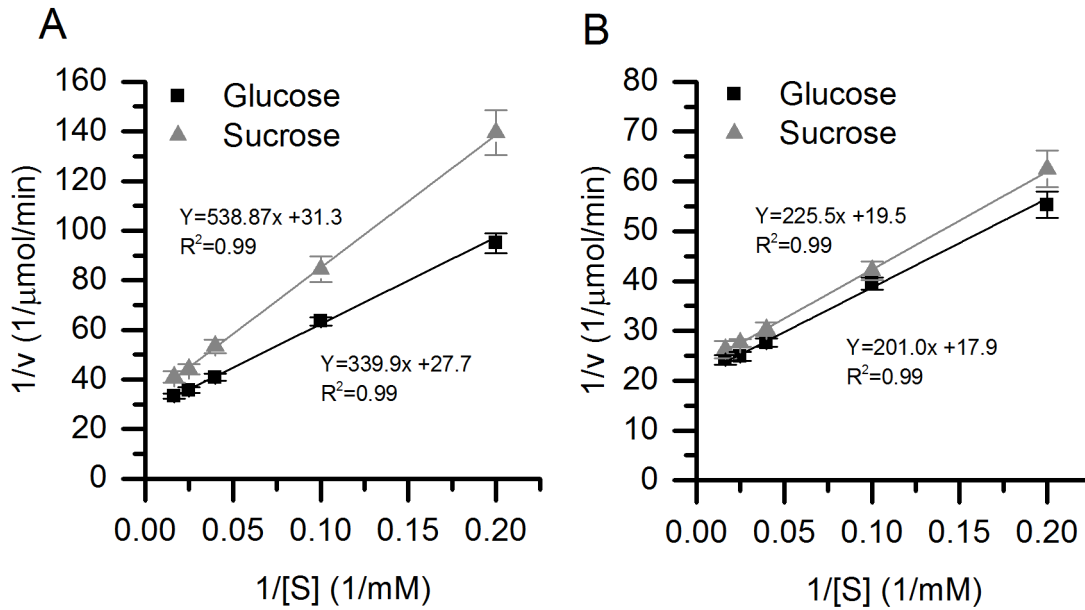


Figure 4-11. Sucrase Lineweaver-Burk plot for the determination of apparent  $K_m$  and  $V_{max}$  for cells cultured in 25 mM glucose or 25 mM sucrose for two weeks (A) or three weeks (B). Caco-2/TC7 cells were lysed and sucrase assay was performed with a range of substrate concentrations, fixed time and enzyme amount. Results from three independent experiments were normalised and plotted mean  $\pm$  SEM where  $[S]$  is the sucrose substrate concentration and  $v$  is  $\mu\text{mol}/\text{min}$  of glucose produced ( $n=3$ ).

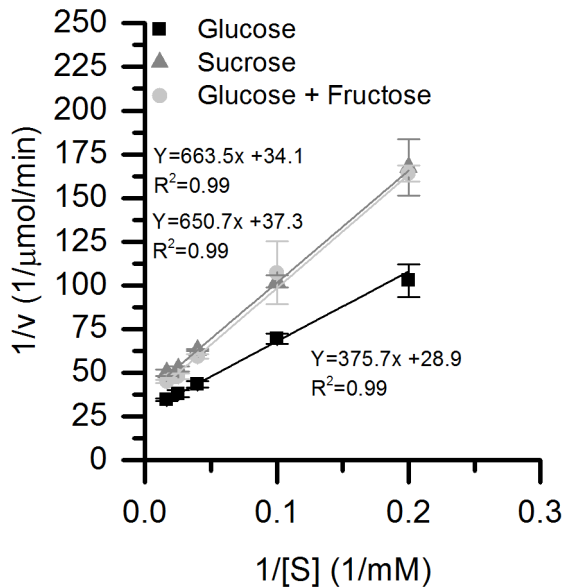


Figure 4-12. Sucrase Lineweaver-Burk plot for the determination of  $K_m$  and  $V_{max}$  for cells cultured in 25 mM glucose, 25 mM sucrose or 25 mM glucose + 25 mM fructose for two weeks. Caco-2/TC7 cells were lysed and sucrase assay was performed with a range of substrate concentrations, fixed time and enzyme amount. Results are presented as mean  $\pm$  SD where  $[S]$  is the



sucrose substrate concentration and  $v$  is  $\mu\text{mol}/\text{min}$  of glucose produced ( $n/N = 1/3$ ).

#### 4.3.4 Results summary

Table 4-4. Summary of the results for the effect of culturing Caco-2/TC7 cells for 21 days in 25 mM sucrose as compared to control cells grown in 25 mM glucose.

	Change in sucrose-grown compared to control
<b>K<sub>m</sub></b>	↑ 14% ( $p=0.01$ )
<b>V<sub>max</sub></b>	NS
<b>Total Specific Activity</b>	No change
<b>mRNA</b>	NS
<b>Total Sucrase Protein</b>	↑ 78% ( $p<0.001$ )
<b>Surface Sucrase Protein</b>	NS
<b>Sucrase Protein Size</b>	↑ 0.8% ( $p<0.001$ )
<b>Source of Size Change</b>	N- and O- glycosylation

#### 4.4 Discussion

In congenital sucrase isomaltase deficiency (CSID) disorder, which occurs in 0.2% of people of European descent, genetic differences as small as a point mutation can lead to lack of function (Naim et al., 2012). Therefore, it was important to confirm that the sequence of SI in the Caco-2/TC7 cell line used here is identical to the wild-type human SI. The Caco-2/TC7 clone was originally developed in order to investigate the transcriptional regulation of the expression of SI. It was created from a high Caco-2 passage number (198) which has increased growth rate, higher cell density and a 7-10 fold increase in sucrase activity (Chantret et al., 1994). To confirm that the provided Caco-2/TC7 cell line has increased SI expression, making it suitable for its study, the SI mRNA level in the cells was compared with the parental line and a 7-fold

increase in mRNA was observed. The sucrase specific activity from the standard culture conditions in 25 mM glucose was 140 mU/mg, comparable to that reported when the clone was prepared and the  $K_m$  was 10.7 which was again in agreement with the literature values reported in the region of 9-11 mM (Trugnan et al., 1986; Krog-Mikkelsen et al., 2011; Jockovic et al., 2013).

It could be hypothesized, that in the presence of high sucrose concentration, that sucrase could be up-regulated in order to allow sucrose hydrolysis for the subsequent uptake of glucose and fructose for energy and some studies support this hypothesis. For example, dietary sucrose upregulated SI at the mRNA level in an animal model. Rats were fed either no sugar, glucose, fructose or sucrose 3 times over 12 hours and mRNA levels of SI and SGLT1 were enhanced by sucrose and fructose but not glucose (Kishi et al., 1999). The mRNA amounts were determined from the band density on a Northern blot; however, technology has greatly advanced and digital PCR allows more accurate determination. Furthermore, the  $\beta$ -actinin housekeeping gene changed on the representative blot, suggesting it was not a suitable control. Other studies have reported that sucrose induces sucrase, however this was after a period of starvation and feeding after starvation is known to induce many genes (Section 1.2.5). Here, using ddPCR, there was no difference in the level of SI mRNA between Caco-2/TC7 cells cultured in sucrose or glucose. There was also no change in specific activity or amount of SI at the cell surface. There was however an increase in SI protein in the total cell lysate. It has been suggested that SI is primarily regulated through transcription whereby a change in SI mRNA will lead to a change in protein (Chantret et al., 1994), however

the level of protein depends on several factors including transcription rate, nuclear export, transcript stability, translational control and protein degradation therefore mRNA is sometimes a poor predictor of protein abundance (Pradet-Balade et al., 2001).

The results here suggest that sugar exposure affects post-translational modifications; sucrose led to an increase in the size of SI which was determined to be due to altered N- and O-glycosylation. Glycosylation refers to the carbohydrate oligosaccharide chains that are found attached to lipids and proteins at the brush border. There are two types of attachments; N-glycans have N-acetylglucosamine (GlcNAc) linked to the amide group of asparagine residues in the ASN-X-SER/THR sequence while O-glycans are attached to hydroxyl of certain amino acids, primarily serine and threonine. Nine monosaccharides are used in the glycosylation process in mammals: fucose, galactose, N-acetylgalactosamine, glucose, N-acetylglucosamine, mannose, sialic acid and xylose (Ohtsubo and Marth, 2006).

SI is highly glycosylated with threonine and serine rich regions that are O-glycosylated and there are 18 predicted N-glycosylation sites (Van Beers et al., 1995). These sites are only theoretical and the structure and branching of the modifications are unknown as well as how many are actually glycosylated *in vivo*. The fully functional size of SI is 240 kDa whereas the inactive precursor is 210 kDa so 12.5% of the total mass is due to post-translational modifications. Altered glycosylation could lead to improper protein folding and the conformational change could affect activity. The glycosylation of other brush border proteins is related to their activity, for example the activity of dipeptidyl

IV (DPP4) is related to its glycosylation state. DPP4 is another highly N-glycosylated brush border enzyme which exerts exoglycosidase activity and is involved in signal transduction and in type 2 diabetic patients, DPP4 inhibitors lower blood glucose levels. Inhibition of N-glycosylation by tunicamycin reduced the biological stability of DPP4 and decreased the activity (Aertgeerts et al., 2004). In the non-transformed intestinal cell model IEC-18, N $\omega$ -Nitro-L-arginine methyl ester (L-NAME) treatment resulted in decreased N-glycosylation on SGLT-1 corresponding to a decrease in  $K_m$  (Arthur et al., 2014). The increase in glycosylation observed here could be addition of further sugar residues or new additional glycosylation sites. The steric hindrance from the additional glycosylation could explain the change in  $K_m$  since the active site identified in the SI sequence is in close proximity to 4 of the 18 predicted glycosylation sites (Van Beers et al., 1995). The human SI crystal structure is not available so it is unknown how close the glycosylation sites are to the active site once in the active folded state. N- and O-glycosylation have wide ranging functions including protein quality control, trafficking and activity. Interestingly, the diabetes model rats Biobreed have altered glycosylation. This rat model for insulin-dependent diabetes displays hyperglycaemia, insulinitis, polydipsia, polyuria and requires insulin for survival. The size of the mature SI in the Biobreed rats is larger than in control rats (240 vs 230 kDa) but the size of the pro-SI precursor is identical, implying that the difference in the mature form arises from alterations in the complex glycosylation which was further confirmed to be changes in both N- and O-glycosylation. The SI was still appropriately trafficked to the cell surface but unfortunately the activity was not determined (Najjar et al., 2001). Human tissue biopsies show increased activity

of SI and increased levels of sugar transporters (Dyer et al., 2002), and since trafficking and sorting to the apical membrane is dependent on glycosylation, there is the potential that altered glycosylation in diabetes could play a role. The impact of altered glycosylation of brush border proteins can extend beyond their own activity; for example N-glycans at the brush border can bind to pancreatic  $\alpha$ -amylase therefore affecting starch digestion (Asanuma-Date et al., 2012). The intestinal brush border is rich in highly glycosylated proteins and the ability of luminal exposure to sugars to alter it could have widespread implications on overall starch and sugar digestion and overall enterocyte absorption.

The SI protein in the total lysate was increased by 78%, but there was no change in sucrase specific activity. The small increase in  $K_m$  is likely not the sole reason to explain the lack of change in activity. The antibody used here is specific for the sucrase subunit and would also detect inactive protein (Hauri et al., 1985). The amount of SI at the cell surface did not change with sucrose exposure so the post-translational changes observed could prevent trafficking of SI to the cell surface, leading to the accumulation within the cell. Both N- and O-glycosylation were modified here and both are involved in trafficking of SI to the cell surface (Naim et al., 2012). Alternatively, the trafficking process may be saturated, and therefore not capable of handling the increased internal SI. There are some phenotypes observed in the disaccharide malabsorption disease CSID in which mutations in SI lead to accumulation in the Golgi as 210 kDa form, or in the ER, as 240 kDa form (Naim et al., 2012). For example, COS-7 cells transfected with a mutation in SI that occurs in some chronic

lymphoma leukaemia cancer patients led to a lower sucrase activity and an increase in the immature 210 kD form of SI (Rodríguez et al., 2013). A heterozygous mutation of SI that occurs in a different CSID phenotype leads to accumulation of the complex glycosylated 240 kDa form in the ER in COS-7 transfected cells (Keiser et al., 2006; Alfalah et al., 2009). In the latter example, less SI was observed at the cell surface by confocal microscopy. Here, cell surface biotinylation results suggested no change at the cell surface, and this could be due to different protein half-life at the cell surface between the two cell lines, or the change observed here may still allow some trafficking to the surface. Since the protein change here is a post-translational modification, some of the SI produced could be processed as normal.

Preliminary work was done to purify SI from Caco-2/TC7 cells to send for N-glycan analysis at the University of York to determine the exact nature of the change in glycosylation. The PNGase F digestion used here was only able to identify the type of glycosylation difference, but not whether it is altered or additional sites. After immunoprecipitation with the SI antibody, the samples were run on a gel, stained with Coomassie and a strong band at the size of SI was observed (data not shown). Unfortunately, the University of York were unable to detect SI in the sample. The sample preparation they performed to enable MALDI-TOF analysis (Mariño et al., 2010) involved long incubations and glycoproteins are often prone to instability and aggregation. It is also possible that the amount of material provided was not enough for detection; however the intensity of the Coomassie stained protein band did meet their general detection limit requirements. This is an area worth future investigation; however

it is beyond the scope of the grant and the capability to undertake the analysis which requires outside expertise and equipment.

The expression of genes related to the transport of glucose and fructose were also evaluated. There was no change in GLUT2 expression, but SGLT1 and GLUT5 both decreased. A review of several animal studies found that GLUT5 expression was induced by the presence of fructose, whereas SGLT1 and GLUT2 expression is similar between presence of fructose, glucose and non-metabolizable glucose; however the induction of these genes was compared to starved state (Douard and Ferraris, 2008). Here, GLUT2 did not differ between cells cultured in glucose and sucrose, whereas SGLT1 and GLUT5 both decreased. A review of the literature on SGLT1 suggests that it may be primarily regulated by translational or post-translational mechanisms (Ferraris, 2001), similar to what we observed here for SI. There was approximately 8-fold less SGLT1 mRNA than GLUT2 mRNA, in agreement with the original characterisation of the Caco-2/TC7 clone (Mahraoui et al., 1994).

The chronic exposure to sucrose appeared to slow down the maturation of sucrase which occurs with differentiation. At two weeks when cells are not yet fully differentiated but have reached the peak SI mRNA level, the specific activity was decreased and the  $K_m$  was increased. Once the cells were fully differentiated, there was no difference in the activity between cells cultured in glucose or sucrose, but there was still a small but significant increase in  $K_m$ . Results indicated that it is the fructose part of sucrose which exerts the effect; there was no difference in sucrase activity or enzyme kinetics between cells cultured in sucrose or a mixture of glucose and fructose. Fructose, rather than

sucrose or glucose, has also been implicated in insulin resistance; glucose tolerance tests were performed in rats fed a starch diet for two weeks and then either starch (control), sucrose, glucose and fructose (G/F) or fructose and starch diets (F/starch). F/starch, sucrose and G/F diets all had the same changes and it was therefore proposed that fructose is mediating the effect, rather than glucose (Thresher et al., 2000).

Altered post-translational modifications in response to sugar has been previously reported; Caco-2/TC7 cells were reported to sense dietary maltose however the methods and results are questionable (Cheng et al., 2014). Caco-2/TC7 cells were grown to 10-days post confluence and then exposed to sucrose, glucose, maltose, isomaltose or fructose on the apical side for up to 48 hours, with no sugar on the basolateral side. A Western blot of SI was presented which suggested a potential size increase of SI for those exposed to maltose at 12 hours, but no difference at 3, 6, or 24 hours. Only one blot was shown, so it is possible that this altered band was just a case of poor loading on that particular lane. Despite this, strong conclusions were made stating that Caco-2/TC7 cells can detect and respond to dietary maltose. Furthermore, there was questionable cell viability, unclear methods and the cells were used at 10 days post confluence and reported to be fully differentiated. Typical culture conditions for the Caco-2/TC7 clone require 21 days to reach full differentiation, with confluence at 7 days (Chantret et al., 1994), so although they report that cells are fully differentiated, this may not be the case as the time to reach confluence was also not reported.



A limitation to this study is the use of a malignant cell lines and as with any cell study, it is possible the effects observed could be related to the malignant state in which cells are more glycolytic. Furthermore, regulation in cell models is sometimes different to that observed *in vivo*; for example, in Caco-2/TC7 glucose increases GLUT5 mRNA dose-dependently but this was not observed using an *in vivo* rat model (Ferraris, 2001), although this difference could be due to species rather than the experimental model. Another limitation to the experiment is that the cells were exposed to sucrose on the basolateral side, which would not be experienced *in vivo*. The cells were treated in this to ensure they utilised sucrose at the apical side rather than uptake glucose from the basolateral side. Furthermore, Caco-2 cells express the sweet taste receptors T1R1 and T1R2 only on the basolateral side, whereas *in vivo* they are found in the luminal gut mucosa, and it is possible they might play a role in the response to sugars, although it is not known if they respond to sucrose (Le Gall et al., 2007). These taste receptors are potentially involved in the regulation of glucose metabolism in cells have been implicated in the insertion of GLUT2 into the apical membrane in response to glucose (Alpers, 2010).

In summary, SI in the Caco-2/TC7 model was altered in response to chronic sucrose exposure in comparison to the control cells grown in glucose. Based on literature reports, it was expected that SI activity would increase, however no change was observed. Evidence here suggests that sugar exposure plays a role in post-translational modifications.

## **5 Chronic effects of olive leaf extract on sucrase expression and activity in Caco-2/TC7 cells**

### **5.1 Abstract**

There are many studies investigating the acute inhibition of SI, but limited studies investigating chronic effects. Bonolive, an olive leaf extract that is rich in oleuropein was used as a model compound to investigate its chronic impact on SI. Small intestinal enterocytes are exposed to the gut lumen as they differentiate and migrate up the villi and have a total life-span of 3-5 days. In order to mimic chronic nutritional exposure, the Caco-2/TC7 cell model was treated with 1.5 mg/mL of bonolive, a concentration achievable after consumption of bonolive supplement, for the last 3 days of differentiation. In Chapter 4, it was observed that sucrase from cells cultured in sucrose had altered glycosylation and  $K_m$ , and so the treatment here was evaluated on cells cultured in both glucose and sucrose. Chronic bonolive treatment reduced the sucrase specific activity by 31% and 26% for cells cultured in glucose and sucrose, respectively. SI mRNA expression decreased by 78% for cells cultured in glucose and by 74% for cells cultured in sucrose. An increase in N-glycosylation with bonolive treatment occurred for cells cultured in sucrose and this could be related to the decrease in  $K_m$  that was observed. Bonolive was reduced the amount of sucrase at the cell surface by 41% but only for cells cultured in glucose. These results warrant future investigation into the use of olive leaf extract in humans for glycaemic control after sugar consumption and the effect of the treatment may depend on sugar exposure.

## 5.2 Introduction

There have been many investigations into the health benefits of olive oil and other olive products in recent years due to the reported health benefits of the Mediterranean diet. The Mediterranean diet was first described in 1980 the Seven Countries study investigating cardiovascular health (Keys, 1980). It was further defined as a cultural model of healthy eating based on the observed long life expectancy and low rates of chronic diseases for people from Italy and Greece in the 1960s. The diet is tied closely to areas strongly involved in olive cultivation and generally is defined as consisting of fresh and local produce, rich in plant foods, low in processed foods, olive oil as the main fat source, low amounts of dairy consumed daily, fruit as desert daily with concentrated sugar or honey only consumed 1-2 times per week in low amounts, wine in moderate amounts consumed with food, low red meat intake and low to moderate fish and poultry intake (Willett et al., 1995). A meta-analysis of 12 prospective cohort studies totalling 1.5 million healthy individuals found that increased adherence to the Mediterranean diet was associated with a significant reduced risk of mortality from all causes, including a 9% reduction in mortality from cardiovascular disease (Sofi et al., 2008). Since the diet's main source of fat is olive oil and up to 25-35% of total intake, many studies have investigated the health benefit of olive oil and olive products. The large scale intervention study, PREDIMED, ran from 2003-2011 to monitor the long-term effects of the Mediterranean diet on cardiovascular health and follow-up is ongoing. A significant disease risk of cardiovascular disease and type 2 diabetes were reduced for Mediterranean diets supplemented with extra-virgin olive oil. The relative risk reduction for T2DM was 40% and the number of expected

prevented cases of T2DM was 26 for every 1000 hypothetical participants (Martínez-González et al., 2015; Martínez-González et al., 2010). These studies investigated the diet as a whole and took into account interactions between the components of a whole diet, however it is clear that olive oil poses a great health benefit, so the mechanisms are of interest.

The original health benefits of olive oil were originally attributed to its oleic acid content however more recently the effects of the polyphenolic components have been recognized (Martín-Peláez et al., 2013). The European Food Safety Authority (EFSA) accepted the first olive-based health claim 1924/2006 article 12 on 19<sup>th</sup> January 2007 stating that olive oil “contributes to the protection of blood lipids to oxidative damage”. This claim and other claims have been reviewed by the EFSA (EFSA, 2011) and they commented that the effect was due to its fatty acid composition rather than phenolic content. A claim has also been accepted to state that “Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress” and review by EFSA states that 5 mg of hydroxytyrosol and its derivatives, formed from oleuropein, must be consumed daily to meet the claim for the protection of LDL particles from oxidative damage and this is believed to be achievable in the diet (EFSA, 2011). Other claims are yet to be substantiated therefore there are still many ongoing studies to investigate other health benefits. A large amount of evidence from a range of *in vivo* and *in vitro* studies is required to support the claim and there must be a cause-and-effect relationship established along with mechanistic information.

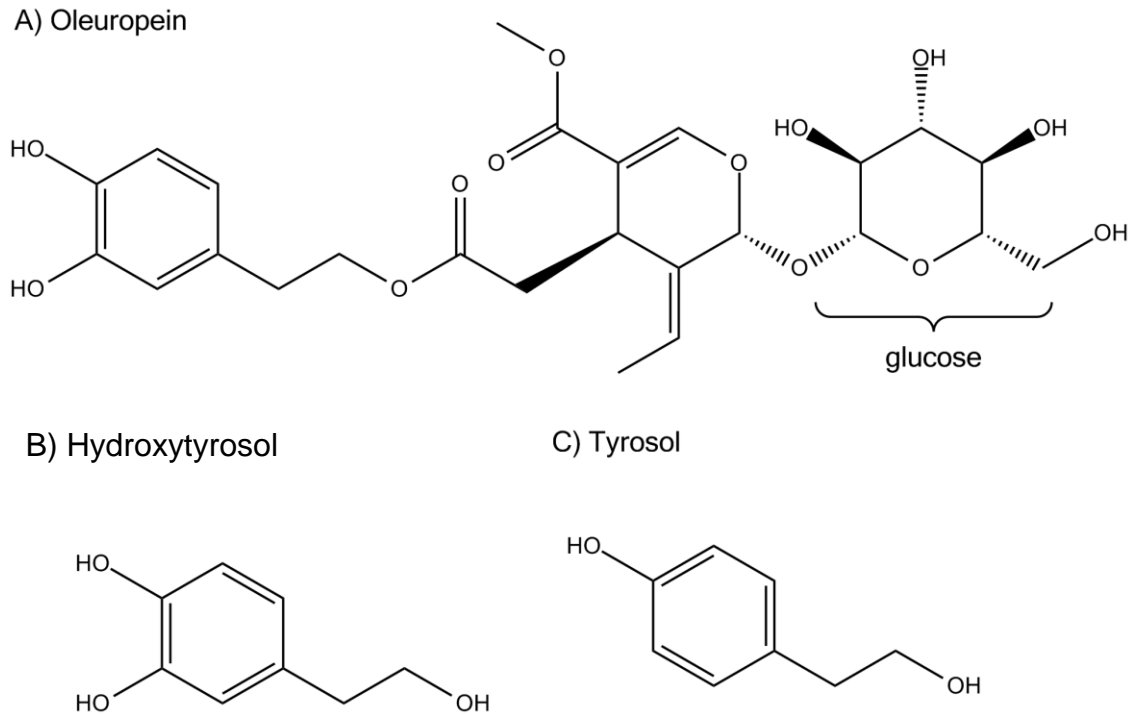


Figure 5-1. Structures of the key phenolic components found in olives.

The key phenolic compounds found in olives (*olea europaea*) include the parent compound oleuropein, in the class of secoiridoids, and its derivatives hydroxytyrosol and tyrosol (Figure 5-1). The concentration of polyphenols found in olives varies by cultivar and region (Garcia-Villalba et al., 2011) and further depends on the age and processing (Preedy and Watson, 2010). Olive leaves have a higher concentration of oleuropein as compared to the olive itself and the levels of oleuropein in olive leaf can reach up to 264 mg per g of dried leaf, expressed as tyrosol equivalents (Ryan et al., 2003). Oleuropein content is used a marker for olive quality (Preedy and Watson, 2010). During the processing of olives to produce olive oil, much of the oleuropein is converted to hydroxytyrosol and tyrosol (Vissers et al., 2002).

The bioavailability of olive leaf polyphenols has been studied in humans. Urinary excretion was evaluated in healthy subjects after consumption of a supplement containing oleuropein and found that 15% of the amount consumed was excreted as tyrosol and hydroxytyrosol metabolites. During the formation of the metabolites, the attached glucose is removed but this study was unable to determine if the glycoside is removed before or after intestinal absorption, however it was found to be stable *in vitro* in gastric and duodenal fluids (Vissers et al., 2002). After consumption of an olive leaf extract in capsule form, oleuropein concentration in the plasma peaked at 38 minutes and conjugated metabolites (glucuronidated and sulphated) of hydroxytyrosol peaked at 93 minutes. In both the plasma and urine, the phase II metabolites were the most predominant, however oleuropein was also present with the glucose group still attached. Males were more efficient at conjugation of oleuropein than females and overall inter-individual variation was observed (Bock et al., 2013). Caco-2 cells were unable to produce glucuronidated metabolites as these cells lack the enzymes required. The sulphated metabolites were formed and found in predominantly on the basolateral side (Rubió et al., 2014). In contrast, another study found that no uptake of oleuropein occurred in the Caco-2 cell model or across rat intestinal segments (Corona et al., 2006) however this was based on the fact that no oleuropein was detected. Their *in vivo* data demonstrate presence of conjugates so oleuropein is more likely taken up and the majority converted.

Recent investigations into the health benefits of olive polyphenols suggest that they may affect carbohydrate digestion and glucose transport. Inhibition studies

with a *Sacchariomyces cerevisiae* source of  $\alpha$ -glycosidases indicated that hydroxytyrosol was a strong inhibitor with  $IC_{50}$  of 150  $\mu$ M and oleuropein with  $IC_{50}$  of 400  $\mu$ M, while acarbose  $IC_{50}$  was 200  $\mu$ M (Hadrich et al., 2014). However, the limitations are the use of yeast enzyme source with a non-specific substrate *p*-nitrophenyl- $\alpha$ -D-glucopyranoside (pNPG) and the fact that the the interference of the polyphenols on the detection method was not taken into account (Nyambe-Silavwe et al., 2015). The potential for olive leaf polyphenols to act as acute  $\alpha$ -glucosidase inhibitors was confirmed using the optimised human sucrase assay in Chapter 3. The olive leaf extract bonolive exhibited inhibition towards sucrase and maltase with  $IC_{50}$  values of 3.2 and 1.3 mg/mL, respectively.

Chronic effects of an olive leaf extract containing 35% (w/w) oleuropein were observed in a type 2 diabetic mouse model. Mice that were fed 0.2% (w/w) olive leaf extract from 4-weeks of age showed a decrease in non-fasting blood glucose from 9-weeks onwards. Impaired glucose tolerance occurred at 8-weeks in this model and it was attenuated by the olive leaf extract at 11-weeks and 24-weeks, while no change in insulin levels or weight were observed. The results suggest that chronic intake of an oleuropein rich olive leaf extract can inhibit the progression of type 2 diabetes in mice (Murotomi et al., 2015). In humans, a double-blind crossover study was performed to evaluate the chronic consumption of an olive leaf extract on insulin sensitivity. Overweight middle-aged male participants, who were expected to be insulin resistant, consumed either placebo or olive leaf extract containing predominately oleuropein (51 mg per day) and hydroxytyrosol (9.7 mg per day) for 6-weeks, followed by a 6-

week washout and then the alternative treatment. A 75 g oral glucose tolerance test was performed at the end of each treatment period and a 15% improvement on insulin sensitivity occurred along with a 6% reduction in blood glucose (AUC) and insulin was decreased by 14% (de Bock et al., 2013). These studies suggest that oleuropein can influence glucose homeostasis.

A decrease in glucose transport is a potential mechanism by which the blood glucose and insulin levels were reduced in the aforementioned study. It is currently not known whether chronic intake had an effect on glucose transport or the expression level of transporters. The anti-diabetic drug acarbose, an  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibitor, reduces type 2 diabetes risk by 6% suggesting that acute inhibitors can exert chronic effects. Since it is reported that olive leaf polyphenols can inhibit  $\alpha$ -amylase and  $\alpha$ -glucosidase, a greater effect compared to glucose alone could be observed following consumption of starch or sucrose.

Bonolive extract was originally evaluated and is currently on the market as a treatment for osteoporosis. It has been standardised for its oleuropein content, which is >40% (w/w). A double-blind, randomised parallel group study was performed on 64 female subjects with osteoporosis in which the participants consumed 250 mg of extract and 1000 mg of calcium or 1000 mg of calcium (placebo) for one year. Pro-osteoblastic marker osteocalcin was found to increase with the treatment and bone mineral density remained stable in the treatment group but decreased in the control.



The aim here is to evaluate the chronic effects of the olive leaf extract bonolive on sucrase. Intestinal cells differentiate over 3-5 days and are constantly being regenerated and while they differentiate as they migrate from the base of the villi to the tip where they are exfoliated, continually being exposed to the contents of the gut lumen (Ferraris, 2001). The Caco-2/TC7 cell model was treated with bonolive for the final 3-days of differentiation and chronic effects are evaluated by determining mRNA and protein levels, activity, and kinetics of SI following treatment (Figure 5-2).

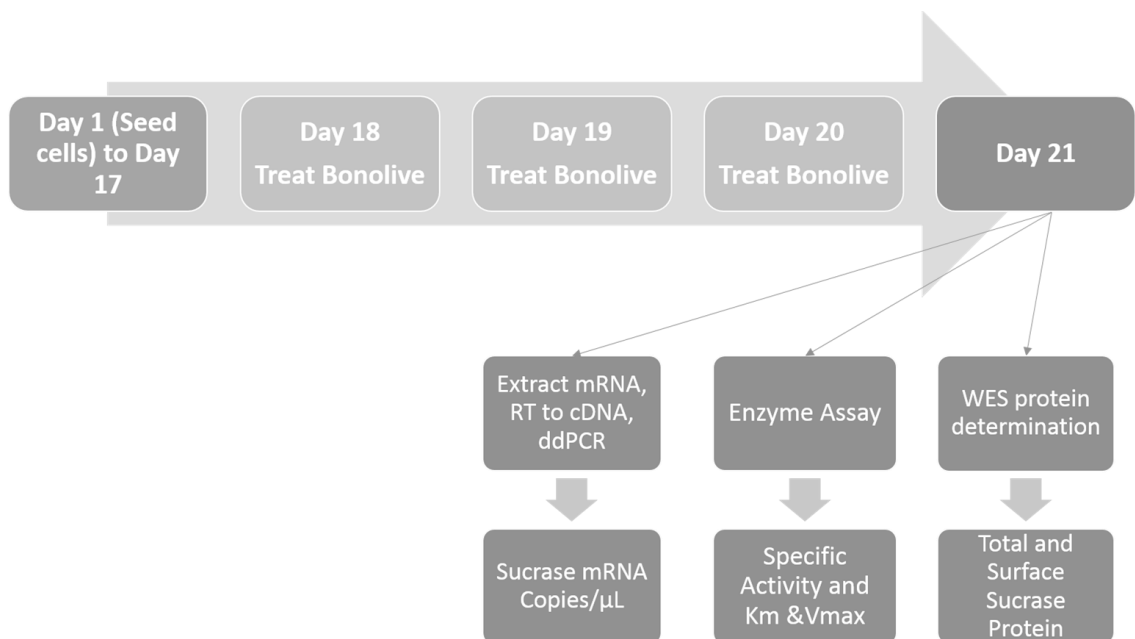


Figure 5-2. Determination of the effects from chronic 3-day bonolive treatment on sucrase isomaltase in the Caco-2/TC7 cell model. The medium was refreshed each day and the medium for the treatment wells contained bonolive.

## **5.3 Results**

### **5.3.1 Chronic effect of bonolive on Caco-2/TC7 viability**

Caco-2/TC7 cells were cultured on 12-well solid supports. The viability was determined after 3-day chronic bonolive treatment at 1.5 mg/mL using the LDH Assay (Section 2.1.5). There was no loss in viability as determined from n/N=3/9 replicates.

### **5.3.2 Chronic effect of bonolive on gene expression**

#### ***5.3.2.1 Chronic treatment: 3 days at 1.5 mg/mL***

The impact of chronic bonolive treatment on SI mRNA levels was investigated in the intestinal Caco-2/TC7 cell model. For all gene expression experiments, there was no significant change in the TBP housekeeping gene between the treatments. The ratio of the gene of interest to TBP was determined for each of n=3 biological replicates. There was variation in the SI/TBP ratio between the biological replicates so the results of each experiment were normalised to the control.

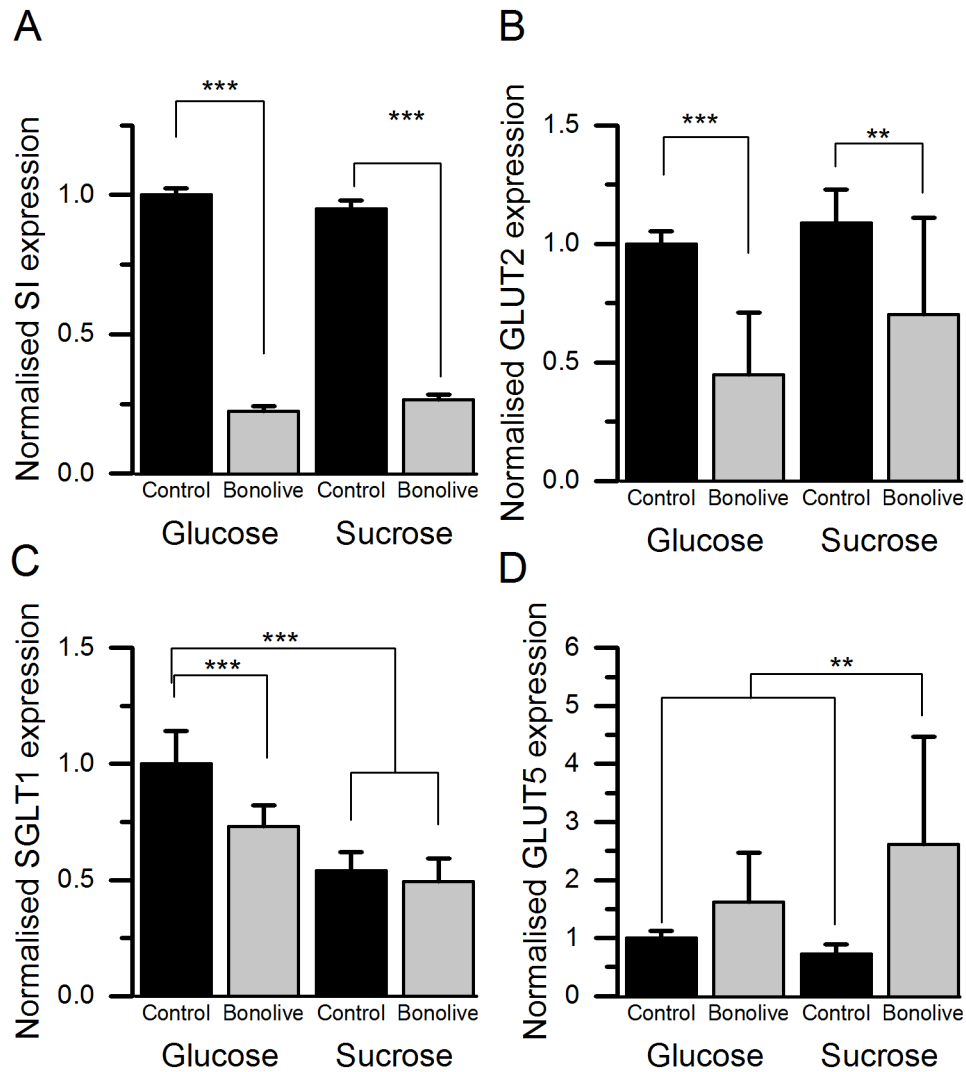


Figure 5-3. The effect of chronic bonolive treatment on gene expression for SI (A), GLUT2 (B), SGLT1 (C) and GLUT5 (D). Caco-2/TC7 cells were cultured in 25 mM glucose or 25 mM sucrose for 21 days and treated with 1.5 mg/mL bonolive for the final 3 days. Total mRNA was extracted, converted to cDNA and the copies/ $\mu$ L of target relative to TBP housekeeping gene were determined using ddPCR. Results were normalised to the glucose control and are presented as mean + SEM, \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ ,  $n/N = 3/3$ , analysed in triplicate.

A concentration of 1.5 mg/mL bonolive, equivalent to 1100  $\mu$ M oleuropein, was found to decrease sucrose-isomaltase mRNA by 78% ( $p < 0.001$ ) for cells cultured in glucose and 74% ( $p < 0.001$ ) for cells cultured in sucrose (Figure 5-3A). The effect of chronic bonolive treatment on the expression levels of the

related genes for GLUT2, SGLT1 and GLUT5 were also determined. GLUT2 was reduced by 55% ( $p < 0.001$ ) when cultured in glucose and by 30% in sucrose ( $p < 0.01$ ) (Figure 5-3B). There was a 27% ( $p < 0.001$ ) reduction in SGLT1 when cultured in glucose (Figure 5-3C). There was no statistically significant change in SGLT1 for cells cultured in sucrose. However, SI mRNA levels were already reduced by 54% from growth in sucrose, as discussed in Chapter 4. The GLUT5 gene expression showed the greatest variation, suggesting inconsistent expression, particularly when cultured in sucrose. Nevertheless, there was a statistically significant 360% ( $p < 0.01$ ) increase in GLUT5 with bonolive treatment for cells cultured in sucrose. When cultured in glucose, GLUT5 appeared to increase (62%), although the result was not statistically significant due to the high variation between samples ( $p = 0.6$ ) (Figure 5-3D). The analysis for GLUT5 was performed after storage of the cDNA samples for longer than 1 year so it is possible that the high variation is due to instability of the sample. The SI transcription factor CDX2 increased by 16% ( $p < 0.05$ ) with bonolive treatment when cultured in glucose but there was no change when cultured in sucrose (Figure 5-4) so the reduction of SI is not due to reduction in its transcription factor.

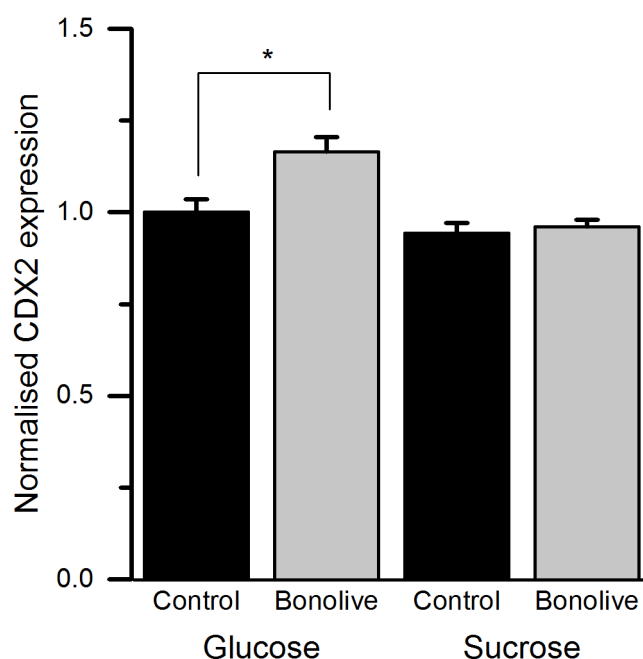


Figure 5-4. The effect of chronic bonolive treatment on the gene expression of transcription factor CDX2. Caco-2/TC7 cells were cultured in 25 mM glucose or 25 mM sucrose for 21 days and treated with 1.5 mg/mL bonolive for the final three days. Total mRNA was extracted, converted to cDNA and the copies/ $\mu$ L of CDX2 relative to TBP housekeeping gene was determined using ddPCR. Results were normalised to the glucose control and are presented as mean + SEM, \*  $p < 0.05$ ,  $n/N = 3/9$ , analysed in triplicate.

### ***5.3.2.2 Dose response and time Course for sucrase gene expression***

SI mRNA expression was determined after 12, 24, 48 and 72 hour treatment with 1.5 mg/mL bonolive. By 12 hours there was a significant ( $p < 0.001$ ) decrease in SI mRNA and the maximum reduction of ~80% occurred by 24 hours. There was no significant difference between 24 and 48 hours, however there was a slight increase between 48 to 72 hours, back up to 70% of the control ( $p < 0.01$ )(Figure 5-5A). The same pattern occurred at all four bonolive concentrations (Figure 5-5B).

Bonolive treatment at 0.5, 1.0, 1.5 and 2 mg/mL was applied for 72 hour and a linear concentration dependent reduction of SI mRNA expression was occurred ( $R^2 = 0.98$ ) (Figure 5-5C). The linear reduction also occurred for different treatment times (Figure 5-5D).

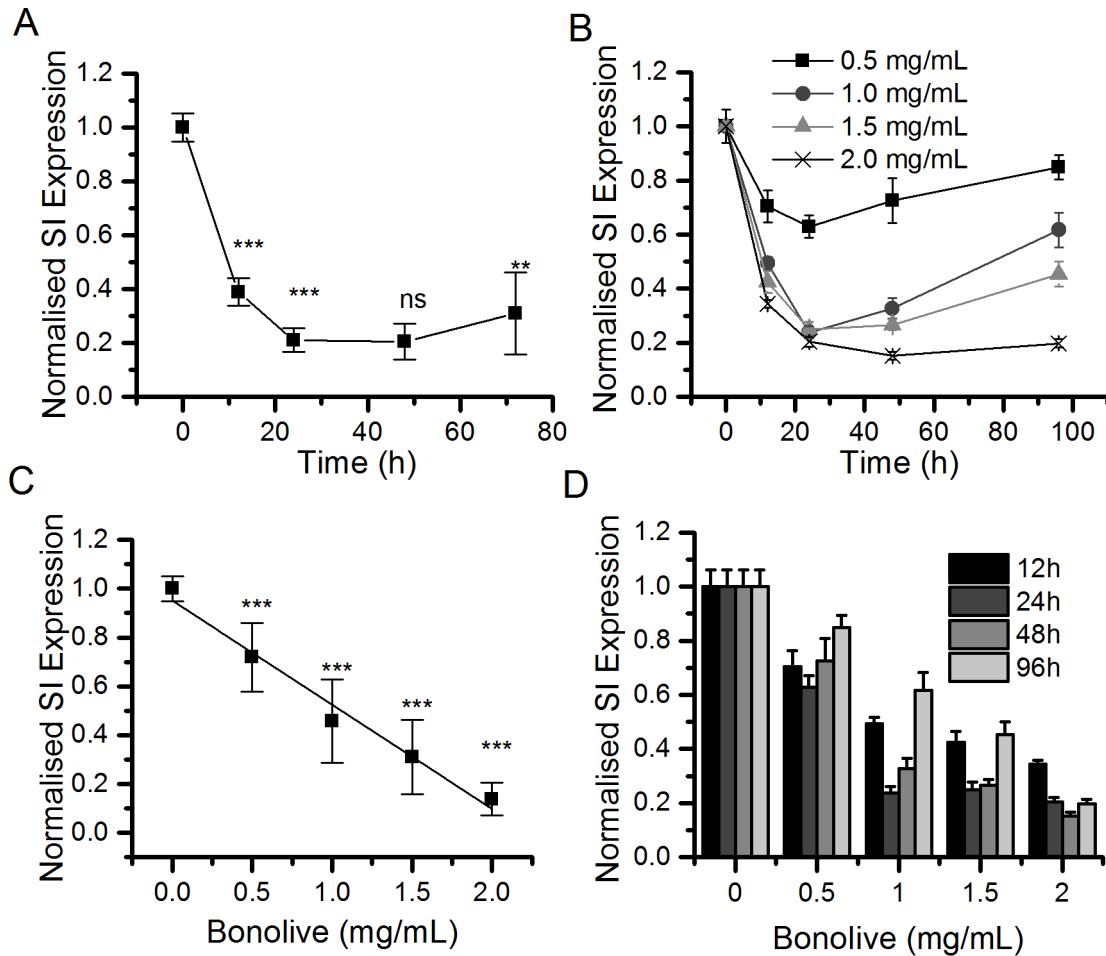


Figure 5-5. The time and concentration dependence for the effect of bonolive treatment on SI expression. Time course (A) with 1.5 mg/mL bonolive for 12, 24, 48 or 72 h and concentration response (C) with 72 h treatment, both with  $n/N=3/9$ , analysed in triplicate. Results presented as normalised mean + SEM, p-values determined from ANOVA followed by Tukey's post-hoc test, labels compare to previous point, \*\*\* $<0.001$ , \*\* $<0.01$ , ns= not significant. The time course was evaluated at four bonolive concentrations (B) and the concentration response (D) was evaluated at four time points for  $n/N=1/3$  replicates, presented as mean  $\pm$  SD.

### 5.3.2.3 Comparison of transwell® filters and solid support cell culture plates

Caco-2/TC7 cells are commonly cultured on transwell® filters which are porous supports and allow the polarised growth and access to basolateral and apical sides, with the apical side representing the gut lumen. The cells for mRNA experiments so far have been performed on solid supports due to high cost of the transwell® filters, therefore, a comparison was performed to evaluate if the decrease in mRNA with bonolive treatment still occurs when the cells are cultured on filters.

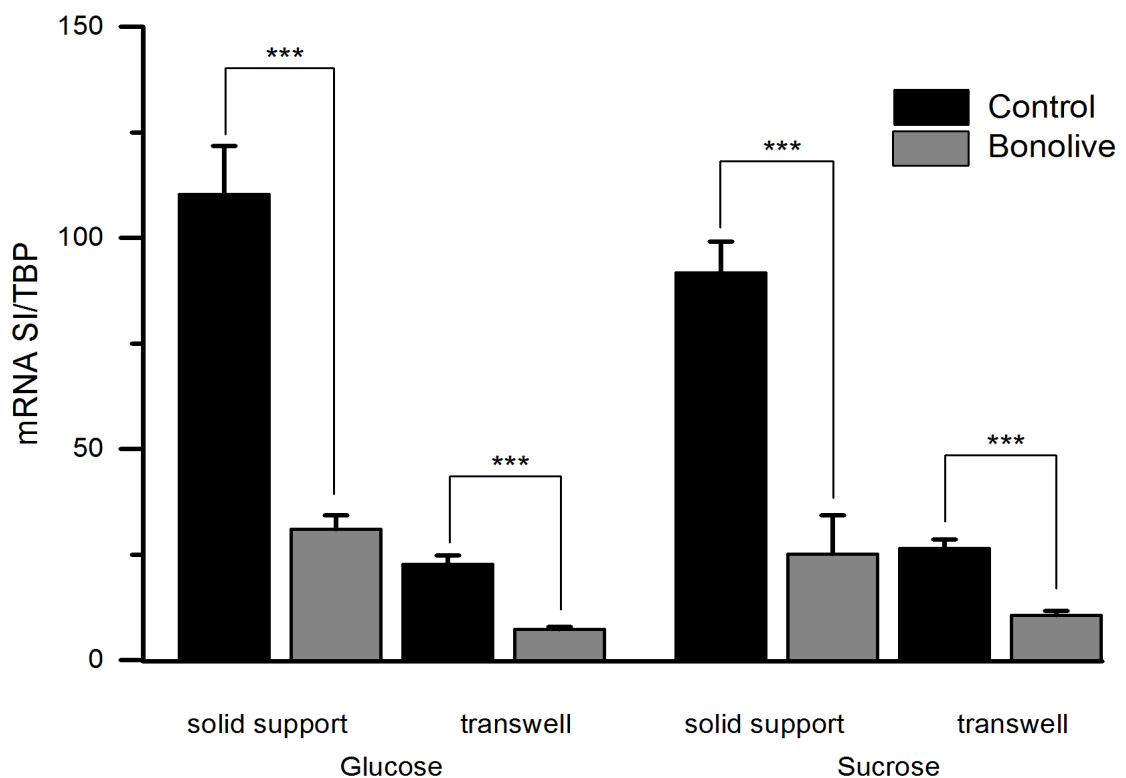


Figure 5-6. Comparison of mRNA from Caco-2/TC7 cells grown on solid or porous supports. Caco-2/TC7 cells were cultured in 25 mM glucose or 25 mM sucrose on either solid support or transwell® plates for 21 days and treated with 1.5 mg/mL with bonolive for the final 3 days of differentiation. Results are presented as mean + SD of the amount of SI in copies/ $\mu$ L relative to the TBP housekeeping gene. n/N = 1/3, analysed in triplicate. \*\*\*  $p < 0.001$

As seen in Figure 5-6, the decrease in SI mRNA still occurred when cultured on transwell® filters, for both sucrose and glucose grown cells, however the ratio of the absolute amount of SI relative to TBP is much lower from cells cultured on transwell® filters. The absolute concentrations of SI and TBP housekeeping gene, as determined by ddPCR are presented as copies/ $\mu$ L in Table 5-1. Cells cultured on solid supports had more SI mRNA than those cultured on transwell® filters, however they had a decrease in TBP. Nevertheless, a similar fold- decrease in SI relative to TBP was observed with chronic bonolive treatment on both solid supports and transwell® plates, with p-value of <0.001 (Table 5-1).

Table 5-1. Comparison of sucrase mRNA with chronic bonolive treatment for Caco2/TC7 cells cultured on transwell® or solid support plates. Caco-2/TC7 cells were cultured in 25 mM glucose (G) or 25 mM sucrose (S) for 21 days and treated with 1.5 mg/mL with bonolive for the final 3 days of differentiation. Results are presented as mean  $\pm$  SD, n/N=1/3.

Plate Type	Condition	Bonolive	SI (Copies/ $\mu$ L)	TBP (Copies/ $\mu$ L)	Ratio SI/TBP	Fold	P-value
<b>Transwell®</b>	G	-	532.6 $\pm$ 64.4	23.4 $\pm$ 2.3	22.8 $\pm$ 2.1	3.2	<0.001
	G	+	166.2 $\pm$ 25.6	23.5 $\pm$ 5.6	7.2 $\pm$ 0.7		
	S	-	591.4 $\pm$ 70.2	22.5 $\pm$ 3.2	26.4 $\pm$ 2.2	2.6	<0.001
	S	+	271.2 $\pm$ 96.9	25.0 $\pm$ 6.9	10.7 $\pm$ 1.1		
<b>Solid Support</b>	G	-	1215.6 $\pm$ 179.8	11.0 $\pm$ 1.1	110.3 $\pm$ 11.4	3.5	<0.001
	G	+	477.8 $\pm$ 36.5	15.6 $\pm$ 1.7	31.0 $\pm$ 3.3		
	S	-	991.4 $\pm$ 239.1	10.7 $\pm$ 2.1	91.7 $\pm$ 7.5	3.6	<0.001
	S	+	480.7 $\pm$ 99.1	18.1 $\pm$ 0.6	25.1 $\pm$ 9.3		



#### **5.3.2.4 Effect of green tea treatment on SI mRNA expression and cell viability**

Green tea was determined to be an acute sucrose and maltase inhibitor in Chapter 3. The effect of chronic green tea treatment was evaluated here to see if other acute sucrose inhibitors in addition to bonolive would also lead to changes in mRNA expression. Caco-2/TC7 cells were treated with 1.0 mg/mL green tea from day 3 to 21 and mRNA was extracted and analysed by ddPCR (n/N=1/6). Viability was confirmed for the treatment using the LDH assay. There was no significant change in SI mRNA expression with the 18 day green tea treatment.

### **5.3.3 Chronic effect of bonolive on sucrose isomaltase protein**

#### **5.3.3.1 Total and surface SI protein**

Caco-2/TC7 cells were cultured on 6-well transwell® filters and the total and surface SI protein were determined by ProteinSimple WES analysis. All protein results were normalised to the glucose control for each experiment due to variation in the amount of SI between the biological replicates. There was no change in SI protein in the total lysate after chronic bonolive treatment for cells cultured in glucose or sucrose grown cells when looking at total absolute amount of SI (i.e. not corrected for loading control) (Figure 5-7A). There was a difference between glucose and sucrose grown, as discussed in Chapter 4. The  $\alpha$ -actinin results were presented independently to demonstrate its suitability as a loading control (Figure 5-7B). Loading controls normalise the results for loading of sample into the capillaries. The same amount of total protein of each sample was loaded into each lane, however the loading control

can account for pipette error in sample loading and the actual separation into the capillary and the ideal loading control will remain consistent throughout different sample types. There was no significant difference between the absolute amount of  $\alpha$ -actinin between the bonolive treated samples and their respective controls, or between glucose and sucrose controls, indicating that it is a suitable loading control; only the difference between glucose + bonolive and sucrose + bonolive was significantly different. For the final result, the normalised amount of SI was divided by the normalised amount of  $\alpha$ -actinin (Figure 5-7C). There was no change in SI in the total lysate with bonolive treatment for cells cultured in glucose, however there was a 22% decrease ( $p=0.004$ ) for cells cultured in sucrose.

The amount of SI at the apical cell surface is presented (Figure 5-8A) and results were also determined as a fraction of the total lysate, to represent the proportion of SI at the cell surface (Figure 5-8B). The amount of SI at the cell surface decreased by 47% ( $p=0.01$ ) for cells cultured in glucose and there was no significant change for cells cultured in sucrose ( $p=0.4$ ) (Figure 5-8A). When the surface sucrose was evaluated relative to total sucrose corrected by the loading control  $\alpha$ -actinin, the proportion of SI at the surface decreased by 41% ( $p=0.004$ ) for glucose-grown and increased by 25% for sucrose-grown cells ( $p=0.018$ ) (Figure 5-8B).

Since there was no change in total SI protein for cells cultured in glucose with the 3-day bonolive treatment, a longer treatment time was also evaluated. Caco-2/TC7 cells grown on solid supports were treated with 1.5 mg/mL bonolive for the final 7 days of differentiation and the SI in the total lysate was

determined as previously described. There was no change in SI in the total lysate after 7-day bonolive treatment ( $p=0.1$ ,  $n/N=1/3$ ) (data not shown).

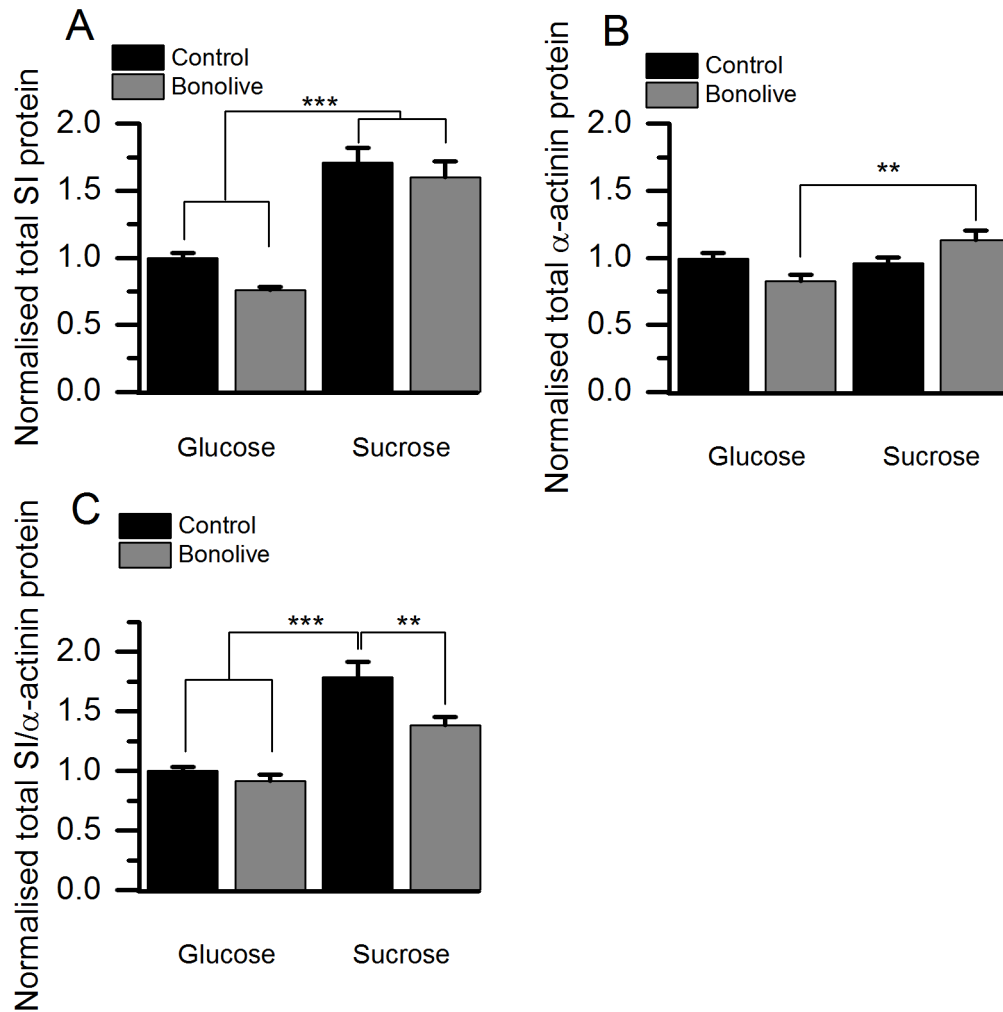


Figure 5-7. The effect of chronic bonolive treatment on sucrase-isomaltase protein in Caco-2/TC7 total cell lysate. Caco-2/TC7 cells were cultured in 25 mM glucose or 25 mM sucrose on transwell® plates for 21 days and treated with 1.5 mg/mL with bonolive for the final 3 days of differentiation. ProteinSimple WES analysis was used to quantify SI (A) and the loading control  $\alpha$ -actinin (B) and the ratio of SI/ $\alpha$ -actinin was determined (C). Results were normalised to the glucose control and are presented as mean + SEM,  $n/N = 3/12$ , analysed in duplicate.

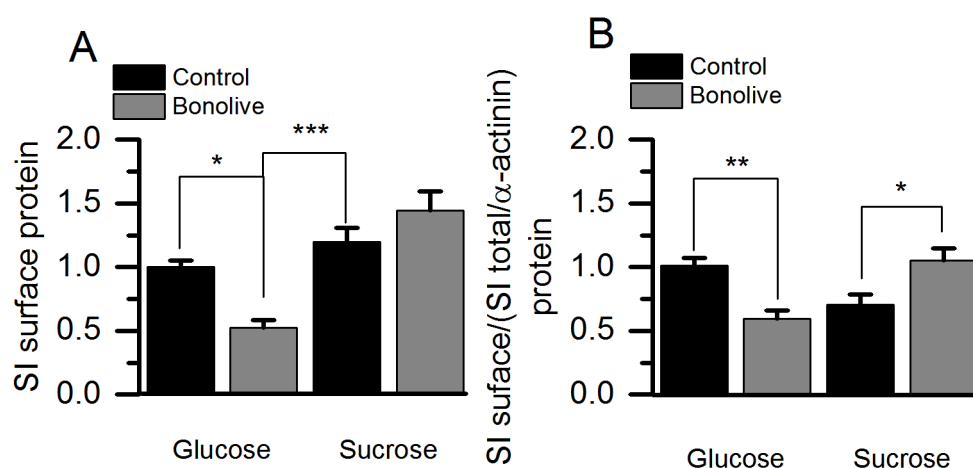


Figure 5-8. The effect of chronic bonolive treatment on surface sucrase-isomaltase protein in Caco-2/TC7 cells. Caco-2/TC7 cells were cultured in 25 mM glucose or 25 mM sucrose on transwell® plates for 21 days and treated with 1.5 mg/mL with bonolive for the final 3 days of differentiation. After purification of biotinylated cell surface proteins, samples were analysed by ProteinSimple WES analysis to quantify SI (A). The ratio of SI at the cell surface to total SI corrected to the  $\alpha$ -actinin loading control was determined (B). Results were normalised to the glucose control and are presented as mean + SEM, n/N = 3/12, analysed in duplicate.

### 5.3.3.2 Protein size analysis

In Chapter 4 Section 4.3.3.2, the size of SI and  $\alpha$ -actinin were analysed for differences between cells cultured in glucose or sucrose. Cells cultured in sucrose had an increase in the size of SI which was determined to be due to N- and O-glycosylation. In a similar fashion, protein lysate samples were evaluated for the effect of bonolive on the size of SI and  $\alpha$ -actinin loading control. For SI at the cell surface and in the total lysate from cells cultured on transwells® or solid supports, the size increased with chronic bonolive treatment when the cells were cultured in sucrose (Table 5-2). The increase was by 1.0% ( $p=0.001$ , n/N=4/12), 0.9% ( $p<0.001$ , n/N=4/12) and 1.0% ( $p=0.04$ , n/N=2/12), respectively. The increase is equivalent to ~2.3 kDa, a small but significant increase. There was no change for cells cultured in glucose and

there was no change for the  $\alpha$ -actinin loading control. Since the protein samples were analysed in duplicate, these results are from the data from 48 individual capillaries for samples cultured on transwells® and from 24 individual capillaries for cells cultured on solid supports. As explained in Section 4.3.3.2, the accurate size result reported on WES is determined by comparison with standard peaks.

Table 5-2. Protein size analysis of SI and the  $\alpha$ -actinin loading control on ProteinSimple WES. Caco-2/TC7 cells were cultured in 25 mM glucose (G;control) or 25 mM sucrose (S) for 21 days on solid supports or transwell® filters. The mean size of SI was determined at the cell surface fraction after biotinylation (n=48). The mean size of SI and  $\alpha$ -actinin was determined in the total lysate from cells grown on transwell® filters (n=48) or solid supports (n=12). Results are presented as a percentage of the control (glucose), mean  $\pm$  SD, and statistical significance was determined with ANOVA following by Tukey's post-hoc test.

Sample	Condition	Bonolive	Sucrose Size (% of Control)	P-value	$\alpha$ -actinin (% of control)	P-value
<b>Cell Surface</b>	G	-	307.6 $\pm$ 1.5	0.4	NA	NA
	G	+	307.6 $\pm$ 1.5		NA	
	S	-	310.3 $\pm$ 1.4	<0.001	NA	NA
	S	+	313.1 $\pm$ 2.3		NA	
<b>Total Lysate (transwell)</b>	G	-	306.5 $\pm$ 4.0	0.9	100.8 $\pm$ 0.3	0.7
	G	+	306.6 $\pm$ 4.4		100.1 $\pm$ 0.7	
	S	-	310.3 $\pm$ 4.1	<0.001	101.2 $\pm$ 0.5	0.8
	S	+	312.1 $\pm$ 3.4		101.8 $\pm$ 0.5	
<b>Total Lysate (solid support)</b>	G	-	294.9 $\pm$ 1.8	0.06	100.0 $\pm$ 0.0	0.6
	G	+	296.5 $\pm$ 1.1		100.2 $\pm$ 0.4	
	S	-	297.1 $\pm$ 1.5	<0.001	100.1 $\pm$ 0.6	0.9
	S	+	299.4 $\pm$ 1.0		100.2 $\pm$ 0.6	

To further investigate the nature of this size increase, samples cultured in sucrose with and without bonolive treatment were treated with PNGase F to remove N-glycosylation. Representative WES pherograms from the PNGase F digests are presented in Figure 5-9. A total of n=7 samples were treated with

PNGase and the size of the resulting products are presented in Table 5-3. The resulting SI products were the same size after PNGase F digestion ( $p=0.6$ ,  $n=7$ ) indicating that the size increase observed from bonolive treatment was due to N-glycosylation. There was no change in size for the  $\alpha$ -actinin control.

Table 5-3. Protein size analysis by WES of SI and  $\alpha$ -actinin after PNGase F deglycosylation. Caco-2/TC7 cells were cultured in 25 mM sucrose +/-chronic bonolive treatment. The total lysate was subjected to PNGase F treatment or mock treatment. Results for SI and control  $\alpha$ -actinin are presented as mean  $\pm$  SD ( $n=7$ ).

Sample	SI size (kDa)	P-value	$\alpha$ -actinin size (kDa)	P-value
<b>Sucrose Mock</b>	$308.8 \pm 1.1$	<0.001	$103.2 \pm 0.8$	0.8
<b>Sucrose + Bonolive Mock</b>	$311.3 \pm 0.8$		$103.3 \pm 0.8$	
<b>Sucrose PNGase F</b>	$286.9 \pm 6.6$	0.6	$103.7 \pm 1.0$	0.8
<b>Sucrose + Bonolive PNGase F</b>	$289.9 \pm 7.9$		$103.9 \pm 0.7$	

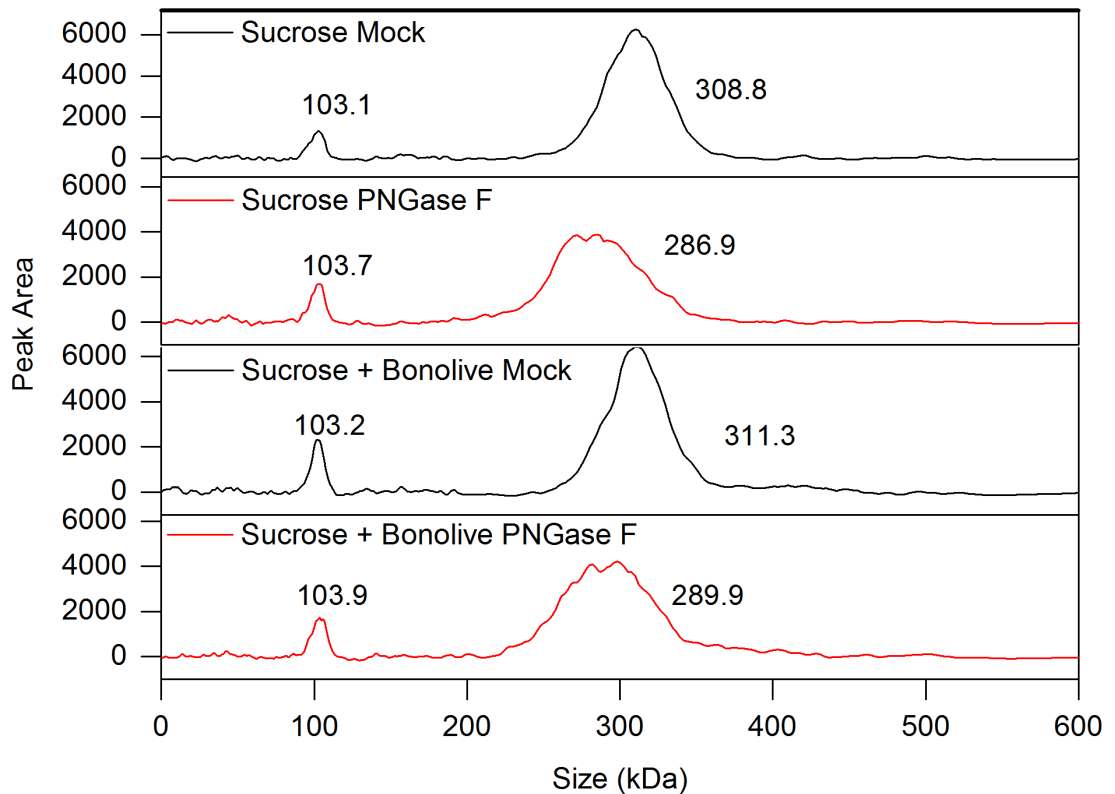


Figure 5-9. Representative pherograms from ProteinSimple WES analysis of  $\alpha$ -actinin (first peak) and SI (second peak) in samples digested with PNGase F. Caco-2/TC7 cells were cultured in 25 mM sucrose and treated +/- bonolive for the final three days of differentiation. Total cell lysate was digested with (red) and without (black) PNGase F enzyme with the mean size from n=7 samples.

### 5.3.4 Chronic effect of bonolive on sucrase-isomaltase enzyme activity

#### 5.3.4.1 Chronic treatment: 3 days at 1.5 mg/mL

Sucrase specific activity was determined after chronic bonolive treatment at 1.5 mg/mL for the final 3 days of differentiation (n/N=3/9) (Figure 5-10). Chronic bonolive treatment reduced the specific activity of sucrase by 31% for cells cultured in glucose ( $p < 0.001$ ), from  $140.1 \pm 0.6 \mu\text{mol}/\text{min}/\text{mg}$  to  $96.9 \pm 0.05 \mu\text{mol}/\text{min}/\text{mg}$ . The reduction in sucrase specific activity was 26% for cells cultured in sucrose ( $p < 0.001$ ), from  $141.6 \pm 0.01 \mu\text{mol}/\text{min}/\text{mg}$  to  $104.5 \pm 0.003 \mu\text{mol}/\text{min}/\text{mg}$ . Statistical significance was determined by ANOVA followed by

Tukey's post-hoc test for the slopes of each replicate. There was no difference in the specific activity between growth in glucose or sucrose, or bonolive treated cells grown in either glucose or sucrose.

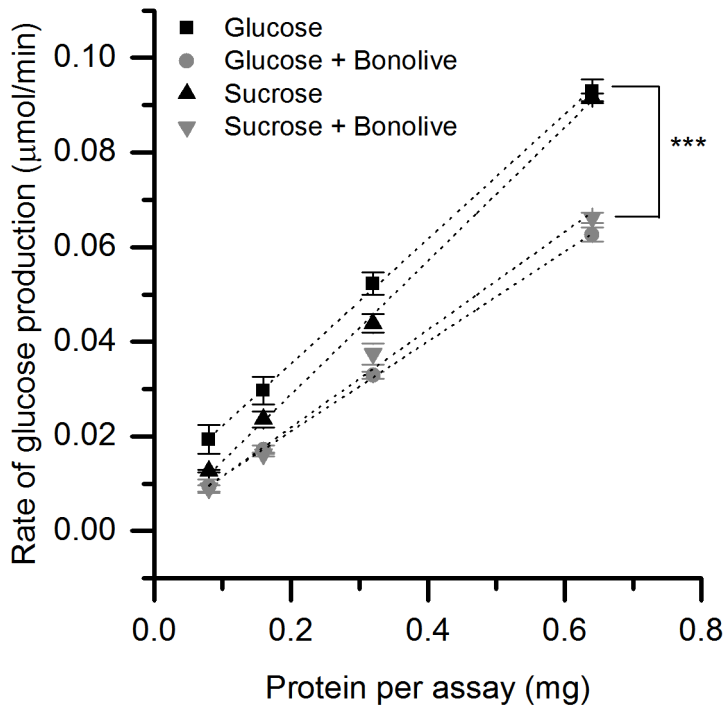


Figure 5-10. Effect of chronic bonolive treatment on sucrose specific activity. Caco-2/TC7 cells were cultured in 25 mM glucose or 25 mM sucrose for 21 days and treated for the final three days with 1.5 mg/mL bonolive. The cells were lysed and used for sucrose enzyme assay to determine the  $\mu\text{mol}/\text{min}$  of glucose produced per milligram of total protein. The slope of the line represents the enzyme activity in  $\mu\text{mol}/\text{min}/\text{mg}$  or  $\text{mU}/\text{mg}$  where 1 unit is equal to 1  $\text{mol}/\text{min}$ . Mean  $\pm$  SEM, \*\*\*  $p > 0.001$ ,  $n/N = 3/9$ .

#### 5.3.4.2 Time course and concentration response

Sucrose specific activity was determined after bonolive treatment for 0, 0.5, 3 and 7 days. After 0.5 days there was no significant change compared to the control. There was a 12% reduction between 12 and 24 hours ( $p=0.04$ ) to 26% of the control. The decrease plateaued after 24 hours and no significant difference was observed between 1, 3 and 7 day treatment time (Figure 5-11A).



A range of bonolive concentrations up to 2.5 mg/mL were evaluated with 3 day treatment and the specific activity decreased with all doses compared to control ( $p < 0.001$ ). There was no significant difference between 0.5 and 1.0 and between 1.5 and 2.5 mg/mL, but there was a decrease of 27% between 1.0 to 1.5 mg/mL. The maximal reduction of 39% of the control occurred at 1.5 mg/mL (Figure 5-11B).

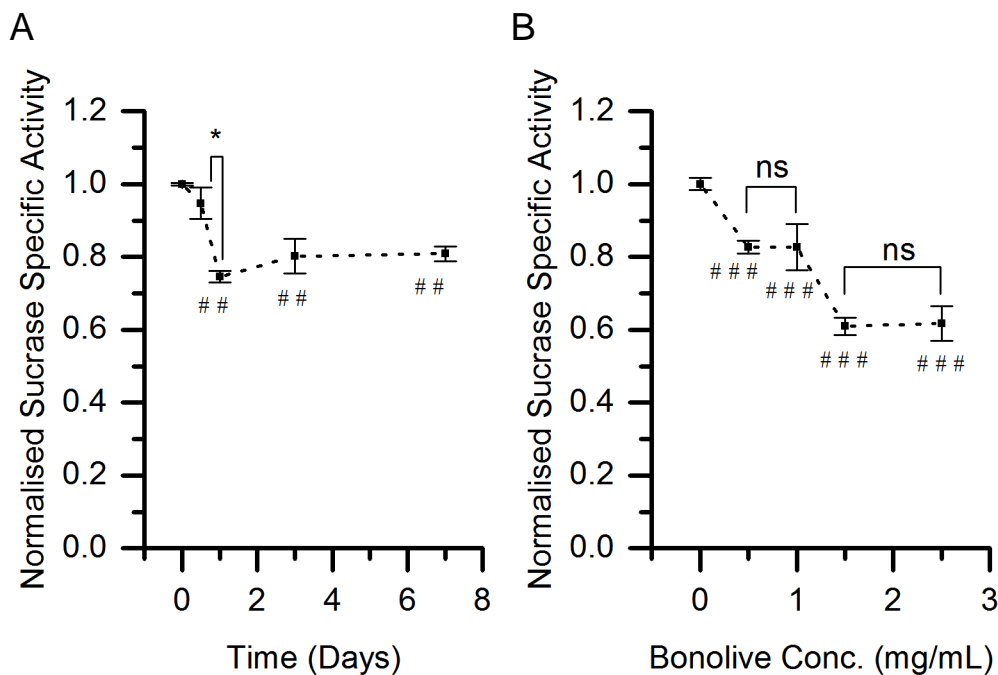


Figure 5-11. Effect of treatment time and bonolive concentration on sucrase specific activity. Caco-2/TC7 cells were cultured on solid support 75 cm<sup>2</sup> flasks in 25 mM glucose for 21 days and treated for the final 0.5, 1, 3 or 7 days with 1.5 mg/mL bonolive for the time course (A) or treated for 3 days with 0.5, 1, 1.5, 2.5 mg/mL bonolive for 3 days. The specific activity was determined for each treatment based on the  $\mu\text{mol}/\text{min}/\text{mg}$  of protein. Results were normalised to the control of each experiment and are presented as mean  $\pm$  SEM, ###  $p < 0.001$  compared to control, \*  $p < 0.05$ , ns = not significant,  $n/N=3/6$ .

### 5.3.4.3 Effect on $K_m$ and $V_{max}$

The chronic bonolive treatment was evaluated for its effect on the kinetic parameters  $K_m$  and  $V_{max}$  using the sucrase enzyme assay. For glucose and sucrose grown cells, the chronic bonolive treatment reduced the  $V_{max}$  by 34% ( $p < 0.001$ ) and 20% ( $p < 0.001$ ), respectively. The  $K_m$  decreased with chronic bonolive treatment only in sucrose grown cells from 12.3 to 9.0 ( $p < 0.001$ ) (Figure 4D&E). The  $K_m$  increased from 10.7 mM to 12.3 mM when cultured in sucrose compared to glucose ( $p = 0.01$ ) and there was no change in  $V_{max}$  (Table 5-4).

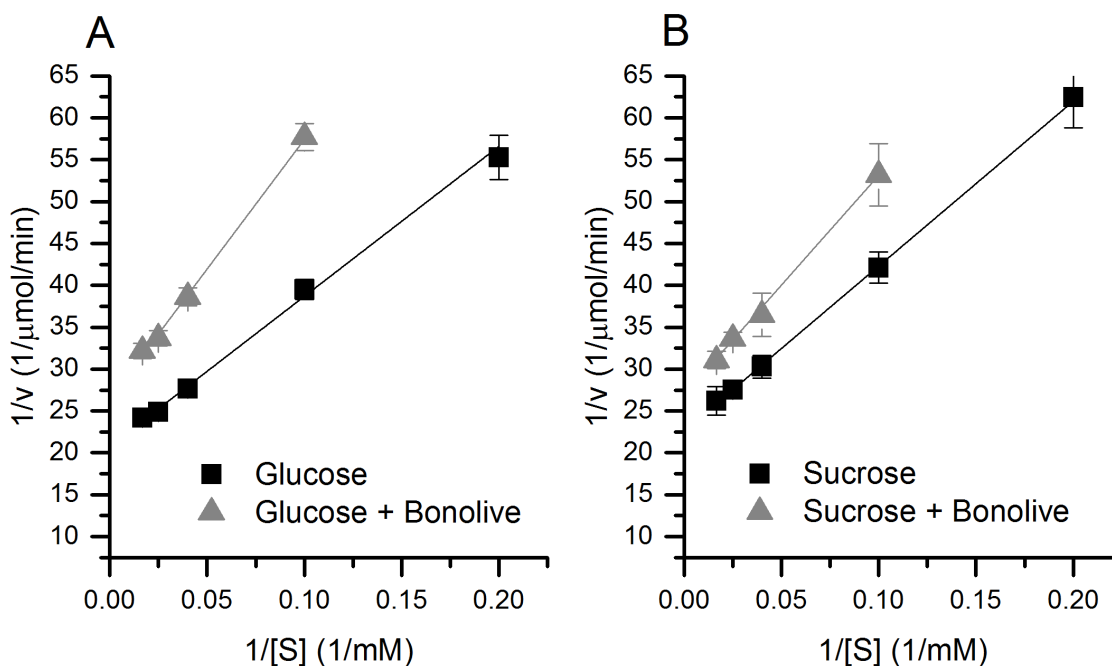


Figure 5-12. Sucrase Lineweaver-Burk plot for the determination of  $K_m$  and  $V_{max}$  for cells cultured in 25 mM glucose (A) or 25 mM sucrose (B) with and without chronic bonolive treatment. Caco-2/TC7 cells were for 21 days and for cells treated with bonolive, 1.5 mg/mL was added to the medium for the final three days. The cells were lysed and a sucrase assay was performed with substrate concentrations from 5-60 mM, fixed time and fixed enzyme amount. Results from three independent experiments were normalised and plotted mean  $\pm$  SEM where  $[S]$  is the sucrose substrate concentration and  $v$  is mmol/min of glucose produced ( $n/N = 3/9$ )

Table 5-4. Kinetic parameters  $K_m$  and  $V_{max}$  determined from Caco-2/TC7 cells cultured in 25 mM glucose or 25 mM sucrose for 21 days with chronic bonolive treatment.

Cell Medium	Treated	Linear Fit	$R^2$	$K_m$ (mM)	P-value	$V_{max}$ ( $\mu\text{mol}/\text{min}$ )	P-value
<b>Glucose</b>	-	$y = 201.0x + 17.9$	0.99	$10.7 \pm 1.9$	0.1	$0.06 \pm 0.01$	$p < 0.001$
	+	$y = 280.1x + 26.4$	0.99	$10.7 \pm 3.0$		$0.04 \pm 0.00$	
<b>Sucrose</b>	-	$y = 225.5x + 19.5$	0.99	$12.3 \pm 3.5$	0.05	$0.05 \pm 0.01$	$p < 0.001$
	+	$Y = 280.1x + 26.4$	0.99	$9.0 \pm 2.5$		$0.04 \pm 0.01$	

### 5.3.5 Results summary

The results for the effects on sucrase from chronic exposure to 25 mM sucrose rather than 25 mM glucose (Chapter 4) are summarised in Table 5-5, along with the effects from chronic bonolive treatment (3 days, 1.5 mg/mL).

Table 5-5. Summary of the effects of chronic bonolive treatment on sucrase in Caco-2/TC7 cells. Cells were cultured in 25 mM glucose or 25 mM sucrose for 21 days and treated for the final 3 days of differentiation with 1.5 mg/mL bonolive. NS = not significant, NA = not applicable.

	Glucose $\rightarrow$ Sucrose	Glucose $\rightarrow$ Glucose + Bolive	Sucrose $\rightarrow$ Sucrose + Bolive
<b><math>K_m</math></b>	$\uparrow 15\%$ ( $p=0.01$ )	NS	$\downarrow 27\%$ ( $p < 0.001$ )
<b><math>V_{max}</math></b>	NS	$\downarrow 22\%$ ( $p < 0.001$ )	$\downarrow 20\%$ ( $p < 0.001$ )
<b>Specific Activity</b>	No change	$\downarrow 31\%$ ( $p < 0.001$ )	$\downarrow 26\%$ ( $p < 0.001$ )
<b>mRNA</b>	NS	$\downarrow 78\%$ ( $p < 0.001$ )	$\downarrow 74\%$ ( $p < 0.001$ )
<b>Total Sucrase Protein</b>	$\uparrow 78\%$ ( $p < 0.001$ )	NS	$\downarrow 22\%$ ( $p = 0.004$ )
<b>Surface Sucrase Protein</b>	NS	$\downarrow 41\%$ ( $p = 0.01$ )	NS
<b>Sucrase Protein Size</b>	$\uparrow 0.8\%$ ( $p < 0.001$ )	NS	$\uparrow 0.9\%$ ( $p < 0.001$ )
<b>Source of Size Change</b>	N- and O-glycosylation	N/A	N-glycosylation

## 5.4 Discussion

Following meal ingestion, carbohydrate hydrolysis and sugar transport across the intestinal wall is one of the critical points affecting subsequent glucose levels in the blood and supply to the tissues, presenting a unique target for interventions to limit excessive levels of blood glucose. This process requires coordinated action of glucosidases and sugar transporters to deliver monosaccharides to the target organs. SI is the only enzyme capable of hydrolysing the  $\alpha$ -1,4 glycosidic bond of sucrose, one of the most commonly used sweeteners (Van Beers, 1995). It was established in Chapter 3 that bonolive exerts acute inhibitory action on both sucrase and maltase activities, similar to the anti-diabetic drug acarbose. Chronic intake of acarbose reduced the risk of type 2 diabetes (Nijpels et al., 2008) so it is hypothesised that other  $\alpha$ -glucosidase inhibitors such as bonolive may also exert chronic effects whereby long term consumption could lead to altered activity or expression of the SI enzyme. Caco-2 cells were exposed to a chronic 3-day treatment and the mRNA expression levels, cell surface and total lysate SI protein amounts, SI enzyme specific activity and kinetic parameters were determined to evaluate the potential chronic effects. A concentration of 1.5 mg/mL bonolive was used, equivalent to 1100  $\mu$ M oleuropein, which would be achievable in the gut after consumption of bonolive supplement. For example the consumption of two 500 mg capsules with 200 mL of water would yield a concentration of 5 mg/mL. Assuming a 3-fold dilution from digestion, the concentration reaching the gut would be greater than 1.5 mg/mL. The oleuropein-rich extract was applied directly into the cell culture medium on the apical side because it has previously

been established that oleuropein is stable in the stomach and to digestion in the proximal gut (Vissers et al., 2002; Markopoulos et al., 2009).

Chronic bonolive treatment dramatically decreased mRNA expression under growth in both glucose and sucrose which did not correspond to total SI protein levels. The SI mRNA half-life has been reported as 30 hours (Chantret et al., 1993) but that can sometimes be a poor predictor of protein levels which depend on transcription rate and additional control mechanisms including nuclear export and mRNA localisation, transcript stability, translational regulation and protein degradation (Pradet-Balade et al., 2001); there is low correlation between mRNA and protein if the protein half-life is longer than mRNA (Raj et al., 2006). The protein half-life for SI has not been reported but it is possible that it is longer than for mRNA because even 7-day treatment did not lead to a reduction of SI mRNA expression in glucose grown cells.

There was no reduction in SI mRNA with green tea treatment, which is also an acute sucrase-isomaltase inhibitor, even though the treatment time was longer than that of bonolive. This implies that not all acute inhibitors will lead to chronic effects. The reduction in SI mRNA expression by bonolive occurred whether the cells were cultured on solid or transwell® supports and was observed to decrease linearly with concentration. A reduction in CDX2, one of the transcription factors required for SI expression along with HNF-1 $\alpha$  and GATA-4 (Boudreau et al., 2002), has been shown to lead to a reduction in SI mRNA (Liu et al., 2011). Here, no change in CDX2 mRNA was observed implying that the bonolive effect on SI mRNA is achieved through an alternative mechanism. GLUT2 mRNA expression also decreased with chronic bonolive treatment,

although the decrease was not as great as for SI. SGLT1 mRNA expression was only reduced when cultured in glucose, however cells cultured in sucrose already had lowered expression of SGLT1. GLUT5 mRNA expression increased with bonolive treatment for cells cultured in sucrose. As mentioned in Section 5.3.2.1, there was high variation in GLUT5 mRNA expression and this was potentially due to sample degradation. Despite the variation, there was still a significant increase in GLUT5 mRNA with bonolive treatment for cells cultured in sucrose. For cells cultured in glucose, GLUT5 mRNA appeared to increase but due to the variation was not statistically significant. The reduction of SI mRNA is not just a total reduction of all genes; GLUT5 increased and the TBP housekeeping gene did not change with bonolive treatment.

Chronic bonolive treatment reduced the amount of SI protein in the total cell lysate only when the level was already elevated by exposure to sucrose. The amount of apical surface SI decreased with bonolive only in cells cultured in glucose, implying a decrease in trafficking. Thus it appears that bonolive pleiotropic effects on protein depend on the previous sugar exposure and may be interplay between altered protein expression and trafficking. A change in N-glycosylation and  $K_m$  with bonolive treatment only occurred in cells cultured in sucrose, however specific activity and  $V_{max}$  decreased in both growth conditions. Specific activity was expected to decrease for cells cultured in sucrose due to the fact that there was less SI in the total lysate, and therefore less available active sites. However, specific activity also decreased for cells cultured in glucose, which had no significant change in the SI protein in the total lysate. Furthermore, cells cultured in sucrose had an increase in total

protein compared to those cultured in glucose, and that was not related to a change in activity. Therefore, it appears that the specific activity here is dependent on the glycosylation which can impact active site availability through steric hindrance and can therefore reduce or increase enzyme affinity for its substrate. This is supported by the fact that changes in glycosylation occurred alongside changes in  $K_m$ .

In the glucose grown cells, a potential decrease in trafficking was observed alongside an increase in N-glycosylation. Although the sucrose grown cells also had an increase in N-glycosylation, there was no change in trafficking in this case. However, cells cultured in sucrose already have altered glycosylation compared to cells cultured in glucose, and as discussed in chapter 4, this change is hypothesised to be both N and O-glycosylation. Post-translational modifications of SI are essential for correct folding and subsequent movement from ER to Golgi (Danielsen, 1992) and for trafficking to the cell surface by association with lipid rafts (Jacob and Naim, 2001). The glycosylation of other brush border proteins is related to their activity, for example the activity of DPP4 is related to its glycosylation state (Aertgeerts et al., 2004) and treatment of IEC-18 cells with L-NAME resulted in decreased N-glycosylation on SGLT-1 corresponding to a decrease in  $K_m$  (Arthur et al., 2014). Altered glycosylation on SI has been reported in the diabetic Biobreed Wistar rats (Najjar et al., 2001). Also, the impact of altered glycosylation of brush border proteins can extend beyond their own activity; for example N-glycans at the brush border can bind to pancreatic  $\alpha$ -amylase therefore affecting starch digestion (Asanuma-Date et al., 2012). The ability to detect these small changes in SI

protein size demonstrates a benefit of using automated capillary western blotting and although the size change observed is small relative to the overall protein size, it is large in terms of the change in glycosylation; glycosylation increased by 2.3 kDa which is approximately 7% increase of glycosylation.

A limitation to the protein results is the lack of loading control in the cell surface fraction. Attempts were made to choose a suitable control for this purpose however it required many conditions; firstly, it must be greater than 100 kDa in size but not near SI size of 240 kDa. It must be highly abundant because sucrase is abundant in the samples and requires a low protein concentration to fall within the quantification range without saturating the detection. It should also not change with the treatment and needs to be an anti-mouse secondary to be compatible with the sucrase isomaltase antibody. MRP2 and DPP4 were evaluated and found to be unsuitable due to poor abundance and an antibody unsuitable for the WES system. Despite having no loading control, the results from the analysis of SI at the cell surface had low variation as compared to the analysis of the total lysate and this is likely due to the purification procedure. To account for the lack of loading control, samples were analysed in duplicate and then the average of the two results was used.

The mechanism for the chronic effect of the anti-hypoglycaemic compounds acarbose, the pharmacological inhibitor, and berberine, found in plants and used in traditional Chinese medicine, on sucrase has been investigated (Liu et al., 2010). Berberine was not an acute inhibitor of sucrase, however a 5-day chronic treatment led to a dose-dependent reduction in sucrase activity when tested from 10 to 50  $\mu$ M. Berberine reduced SI mRNA levels whereas acarbose



did not. The effects were also confirmed *in vivo* using rats, where berberine attenuated the disaccharidase activity in the diabetic and normal models. Berberine is poorly absorbed and therefore remains at high concentrations in the gut. Similarly, it has been reported that oleuropein is poorly absorbed in the Caco-2 model (Corona et al., 2006). The reduction of sucrase activity after chronic treatment is therefore independent to the acute inhibitory effect, as was seen with green tea, and the actions appear to be exerted primarily in the gut lumen. The effect by berberine was linked to a protein kinase A (PKA) – dependent pathway, therefore it was able to affect signalling pathway through unknown receptor at the cell surface. It is therefore possible that bonolive may exert effects through a similar mechanism, however further investigations into the pathways involved are required to elucidate by which signalling pathways these effects occur.

The *in vitro* evidence here suggests that chronic treatment with an olive leaf extract can reduce sucrose hydrolysis by sucrase. Reduction of sucrase activity *in vivo* can lead to reduced glycaemic response to sucrose. In rats, digestion of sucrose by sucrase was rate-limiting towards the glycaemic response; in an oral sucrose tolerance test with a formulation containing *L*-arabinose, the incremental area under the curve for blood glucose was significantly reduced while no change was observed in glucose or starch tests (Preuss et al., 2007b; Preuss et al., 2007a). In humans, pure *L*-arabinose led to a delay in glucose absorption in response to sucrose and there were significant positive changes in GLP, GIP and insulin (Krog-Mikkelsen et al., 2011). Any reduction in SI levels would be beneficial beyond sucrose since the enzyme complex is the

primary source of maltase and isomaltase activity (Van Beers et al., 1995), therefore there could be wider impact on total starch digestion. Experiments here indicate that chronic olive leaf extract affects the activity of SI with several mechanisms at play, primarily post-translational modification. Chronic treatment with olive leaf extract has the potential to decrease saccharide hydrolysis warranting future investigation of the functional impact. Transport experiments in cells would be able to confirm in the chronic effects on sucrase could play a role in decreasing the glycaemic impact to sucrose.

## 6 Acute and chronic effects of bonolive on sucrose and glucose transport in Caco-2/TC7 cells

### 6.1 Abstract

The olive leaf extract bonolive was shown to be an acute inhibitor of sucrase and maltase (Chapter 3). Chronic treatment reduced sucrase activity and the amount of SI at the cell surface for cells cultured in glucose (Chapter 5). The *in vitro* methods were performed on whole cell lysate, with the exception of cell surface protein, which was determined from cells cultured on transwell® filters. The aim here was to determine if the same the acute and chronic effects of bonolive will occur with cells *in situ* on transwell® filters to give further information on the functional impact of reduced sucrase. Method parameters for a transport assay were set-up and HPAE-PAD detection was utilised to quantify glucose and fructose on the apical and basolateral sides. Both chronic and acute effects were evaluated. For acute effect, cells were cultured for 21-days and then exposed to bonolive and sucrose during the transport incubation. To measure chronic effects, cells were treated with bonolive for the final 3 days of differentiation, bonolive was removed and transport experiments with sucrose or glucose were performed. The acute inhibition of sucrase by bonolive was greater for cells *in situ* than for inhibition on the whole cell lysate. Chronic bonolive treatment reduced glucose transport only under sodium-free conditions, indicating an effect on GLUT2. Chronic bonolive treatment reduced the hydrolysis of sucrase, as observed by less apical glucose and fructose. Basolateral fructose decreased, however no change in basolateral glucose occurred and this is hypothesised to be due to the glycolytic nature of the cells.

The results indicate that both chronic and acute bonolive treatments have the potential to reduce the glycaemic response to sucrose and warrant future investigations in humans.

## 6.2 Introduction

Previous studies suggest that the olive polyphenol oleuropein can influence glucose homeostasis. Chronic intake of an oleuropein-rich olive leaf extract attenuated hyperglycaemia in a rat model (Murotomi et al., 2015). In humans, a double-blind crossover study with 6-week consumption of an olive leaf extract led to a 15% improvement on insulin sensitivity, a 6% reduction in blood glucose and 14% reduction of insulin (de Bock et al., 2013). In Chapter 5, chronic treatment of Caco-2/TC7 cells with an olive leaf extract decreased the specific activity of sucrase in the whole cell lysate and cells cultured with normal growth conditions had decreased levels of SI at the cell surface. The olive leaf extract was also an acute sucrase and maltase inhibitor, as determined with an *in vitro* assay using Caco-2/TC7 whole cell lysates (Chapter 3). Reduction of sucrase hydrolysis would be expected to reduce the glycaemic response and additional effect on glucose and fructose transport would strengthen the effect. This will be evaluated with transport experiments using Caco-2/TC7 cells *in-situ* in order to evaluate the functional impact of both chronic and acute treatments. The transport method takes into account hydrolysis of sucrase and subsequent transport of glucose and fructose products. Henceforth, the term “sucrose transport assay” will be used for the sake of brevity when sucrose is used as the substrate although sucrose itself is not transported.

Reduction of sucrase activity *in vivo* can lead to reduced glycaemic response to sucrose. In rats, digestion of sucrose by sucrase was rate-limiting towards the glycaemic response; in an oral sucrose tolerance test with a formulation containing *L*-arabinose, the incremental area under the curve for blood glucose was significantly reduced while no change was observed in glucose or starch tests (Preuss et al., 2007b; Preuss et al., 2007a). In humans, pure *L*-arabinose led to a delay in glucose absorption after sucrose intake and there were significant improvements in GLP, GIP and insulin (Krog-Mikkelsen et al., 2011). These results imply inhibition of sucrase can be rate-limiting in the glycaemic response and combined with effects on glucose transport, the olive leaf extract has the potential to yield a significant impact on glycaemic response.

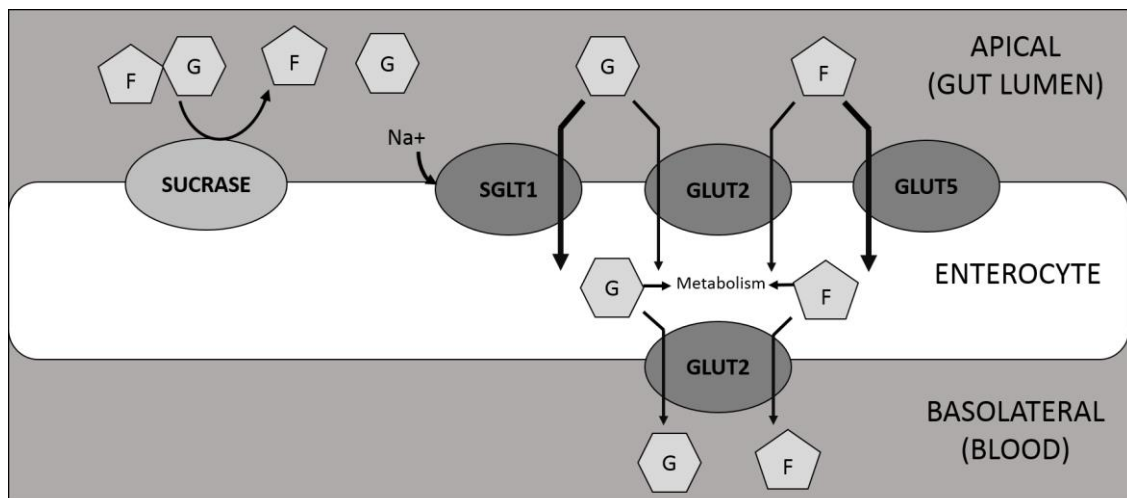


Figure 6-1. Sucrose is hydrolysed at the brush border of the small intestine into glucose (G) and fructose (F) which are subsequently transported. At the apical side, glucose is transported by SGLT1 and GLUT2 and fructose is transported by GLUT2 and GLUT5, a fructose specific transporter. GLUT2 is responsible for transport of both glucose and fructose at the basolateral side.

A schematic of the transporters involved is presented in Figure 6-1. Glucose transport at the apical membrane primarily occurs by active transport through

SGLT1, which is reported to reach saturation at 30 mM glucose. SGLT1 depends on sodium and the transport is driven by the gradient towards the lumen of  $\text{Na}^+$  which is maintained by the basolateral  $\text{Na}^+\text{K}^+$ /ATPase (Kellett et al., 2008). At concentrations greater than 30 mM, transport of glucose is reported to occur by diffusive transport by GLUT2, which is recruited and inserted into the apical membrane (Kellett et al., 2008). SGLT1 has a greater affinity ( $K_m = 0.5$  mM) (Röder et al., 2014) for glucose than GLUT2 ( $K_m = 17$  mM) (Manolescu et al., 2007). To determine the relative contribution of each of the glucose transporters in the Caco-2/TC7 cell model, experiments can be performed in the absence of sodium in the transport buffer. This suppresses SGLT1 activity and therefore can be used to evaluate effects on GLUT2. Apical transport of fructose occurs primarily through the fructose specific transporter GLUT5 ( $K_m = 11-15$  mM) (Douard and Ferraris, 2008) with some contribution of GLUT2 ( $K_m = 66$  mM) (Cura and Carruthers, 2012). Basolateral transport of glucose and fructose occurs through GLUT2 (Kellett et al., 2008).

Caco-2 or Caco-2/TC7 cells have been commonly used for transport experiments with acute polyphenol treatment. For example, grape phenolics acutely inhibited both glucose and fructose transport (Moser et al., 2016), polyphenols from peanuts inhibited glucose transport by up to 50% (Tamura et al., 2015) and strawberry and apple polyphenols were able to reduce glucose uptake and transport (Manzano and Williamson, 2010). There are limited transport studies evaluating chronic effects; one study found that an anthocyanin-rich berry extract reduced glucose transport by GLUT2 after a chronic 16 hour treatment in Caco-2/TC7 cells (Alzaid et al., 2013). There have

been no published *in vitro* studies found that evaluated transport in cells after chronic treatments using sucrose as the substrate. One *in vivo* study in rats investigated the chronic effects of berberine on sucrase and observed a decrease in postprandial glucose following a sucrose load (Liu et al., 2010). One study evaluated the acute effects of an extract from mulberry leaves on sucrose and maltose hydrolysis and transport and observed mild inhibition (Hansawasdi and Kawabata, 2006). The glucose oxidase assay was used to quantify glucose, however, as discussed in Chapter 3, polyphenols can interfere with the detection method. Other transport methods often utilise radiolabelled substrates and scintillation counting to quantify the counts present on the apical and basolateral sides after transport. The disadvantage is that only the total radioactivity counts are presented and the individual sugars present cannot be distinguished. Here, the HPAE-PAD system (Section 2.4.3) is used to quantify sugars present in the apical and basolateral transport solutions. This is particularly important for investigating sucrose transport so that both glucose and fructose could be detected. With the ability to detect individual sugars, the contribution of hydrolysis or transport mechanisms is investigated.

## **6.3 Method set-up for sucrose hydrolysis and glucose transport**

### **6.3.1 Background**

The method for transport studies in Caco-2 cells has been well established in this laboratory (Aydin, 2015). However, sucrose transport assay had not been performed in Caco-2/TC7 cell model or with HPAE-PAD detection so the aim here was to set up the method parameters. For chronic experiments, briefly,

cells were seeded on transwell® filters and treated with bonolive for the final 3 days of differentiation. The cells were washed to remove polyphenols and medium, pre-incubated in transport buffer and TEER values measured to ensure monolayer integrity. The transport solution with sucrose was added to the apical side, incubated for the specified time and apical and basolateral solutions were removed and glucose and fructose were quantified by HPAE-PAD (Figure 6-2). The acute experiments were performed as described but without the chronic treatment and bonolive was added with sucrose during the transport incubation.

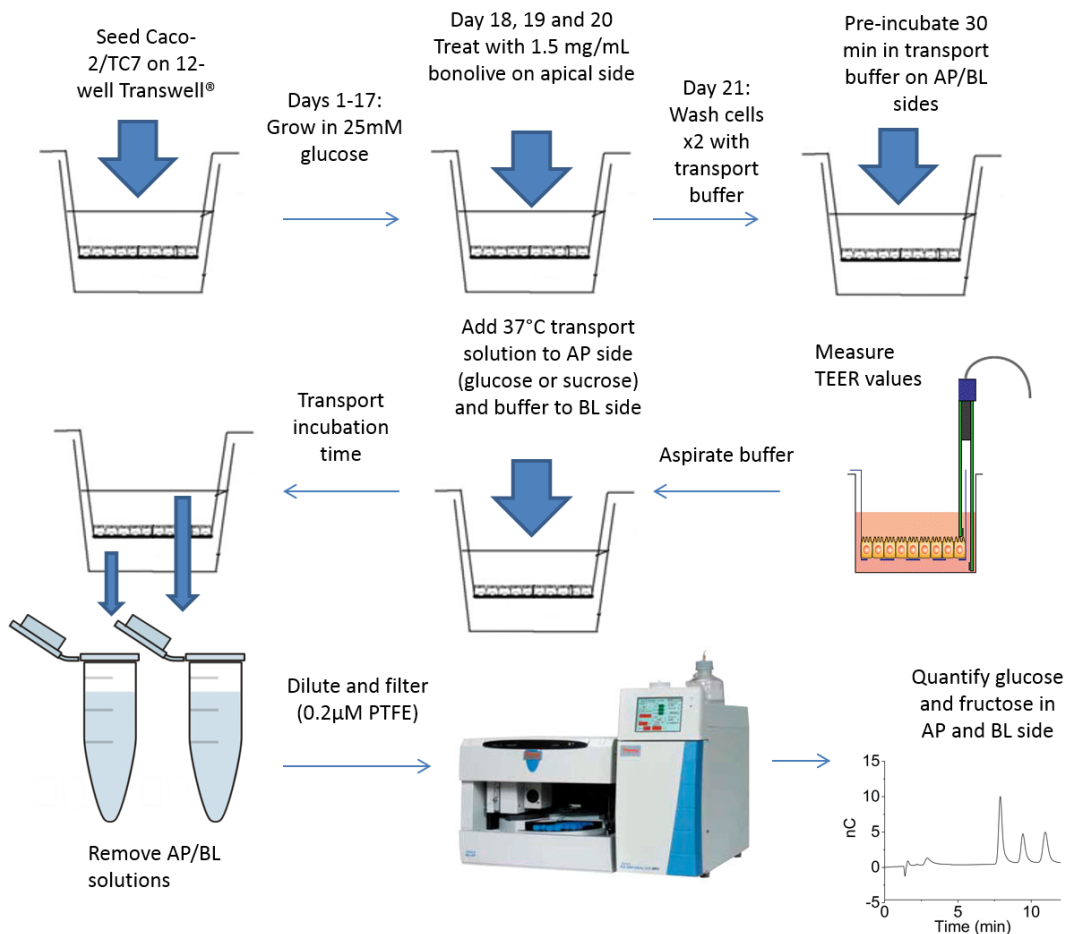


Figure 6-2. Method flow chart for sugar transport in Caco2/TC7 cells.



### **6.3.2 Materials and equipment**

Calcium chloride, glucose, HEPES buffer, sodium bicarbonate solution, sodium chloride and sucrose were from Sigma Aldrich, Dorset, UK. Sterile 0.2 µm filters were from Whatman, Maidstone, UK. All cell culture materials are listed in Section 2.1. HPAE-PAD materials and equipment are listed in Section 2.4.3.

### **6.3.3 Cell culture and treatments**

Caco-2/TC7 cells from passages 31 to 40 were seeded on 12-well transwell® plates according to section Section 2.1.3. Chronic treatments were performed according to Section 2.1.4.

### **6.3.4 Preparation of reagents**

The transport buffer contained 20 mM HEPES buffer, 5.4 mM potassium chloride, 137 mM sodium chloride, 2.4 mM calcium chloride and 26.8 mM sodium bicarbonate. The pH was adjusted to 7.4 and then the solution was sterile filtered. Sodium-free transport buffer was prepared similarly but without sodium chloride and sodium bicarbonate, and with potassium chloride increased to 142 mM. Glucose and sucrose solutions were prepared at 50 mM in transport buffer and then diluted to the working concentration as reported for each experiment, referred to as transport solution. For acute experiments, bonolive was weighed and dissolved into transport solution containing sucrose at the specified concentrations.

### 6.3.5 Procedure

Cells were washed three times with warmed transport buffer (37°) and pre-incubated in transport buffer at 37°C with 10% CO<sub>2</sub> for 30 min. The trans-epithelial electric resistance (TEER) was measured using Millipore Millicell® Voltohmmeter three times per well, with TEER values greater than 200 Ω deemed acceptable monolayer formation for Caco-2/TC7 cells grown on 12-well transwell®. For transport incubation, 1 mL of transport buffer was added to the basolateral side and 0.5 mL of transport solution containing glucose or sucrose was added to the apical side. Samples were incubated for the specified time in the incubator at 37°C with 10% CO<sub>2</sub>. Following incubation, the apical and basolateral solutions were transferred to micro-centrifuge tubes and stored at -80°C until further analysis.

### 6.3.6 Transport sample analysis by HPAE-PAD

Samples were analysed by HPAE-PAD according to the method in Section 2.4.3. Samples were diluted to fall within the calibration range and the final sample vial contained up to 20% transport buffer, representing a 5-fold sample dilution. A matrix matched blank and 5 µM standard were prepared in 20% transport buffer to compare with standards in water. There were no interfering peaks observed in the blank and the peak areas for glucose, fructose and sucrose were within 2% of the standard in water. Therefore, transport buffer in the samples does not impact the quantitation of glucose, sucrose or fructose so standard curves were prepared in water.

### **6.3.6.1 Interference from bonolive on HPAE-PAD**

The samples from the acute experiments contain bonolive and were diluted 5-fold for analysis, and therefore the final samples contained bonolive at 20% of the specified testing concentration. A matrix-matched blank and 5  $\mu\text{M}$  standard were prepared to evaluate potential interference from bonolive (Figure 6-3). There are interfering peaks present in the bonolive blank (Figure 6-3B) so to correct for the interference, standard curves were prepared with the same amount of bonolive present as the samples. Standards were prepared at 0, 1, 2.5, 5 and 10  $\mu\text{M}$  with 20% of either 0.5 mg/mL bonolive, 1.5 mg/mL bonolive or 3.0 mg/mL bonolive. Representative calibration curves for glucose and fructose with bonolive are shown in Figure 6-4. These standard curves were used to quantify glucose and fructose in samples from acute experiments.

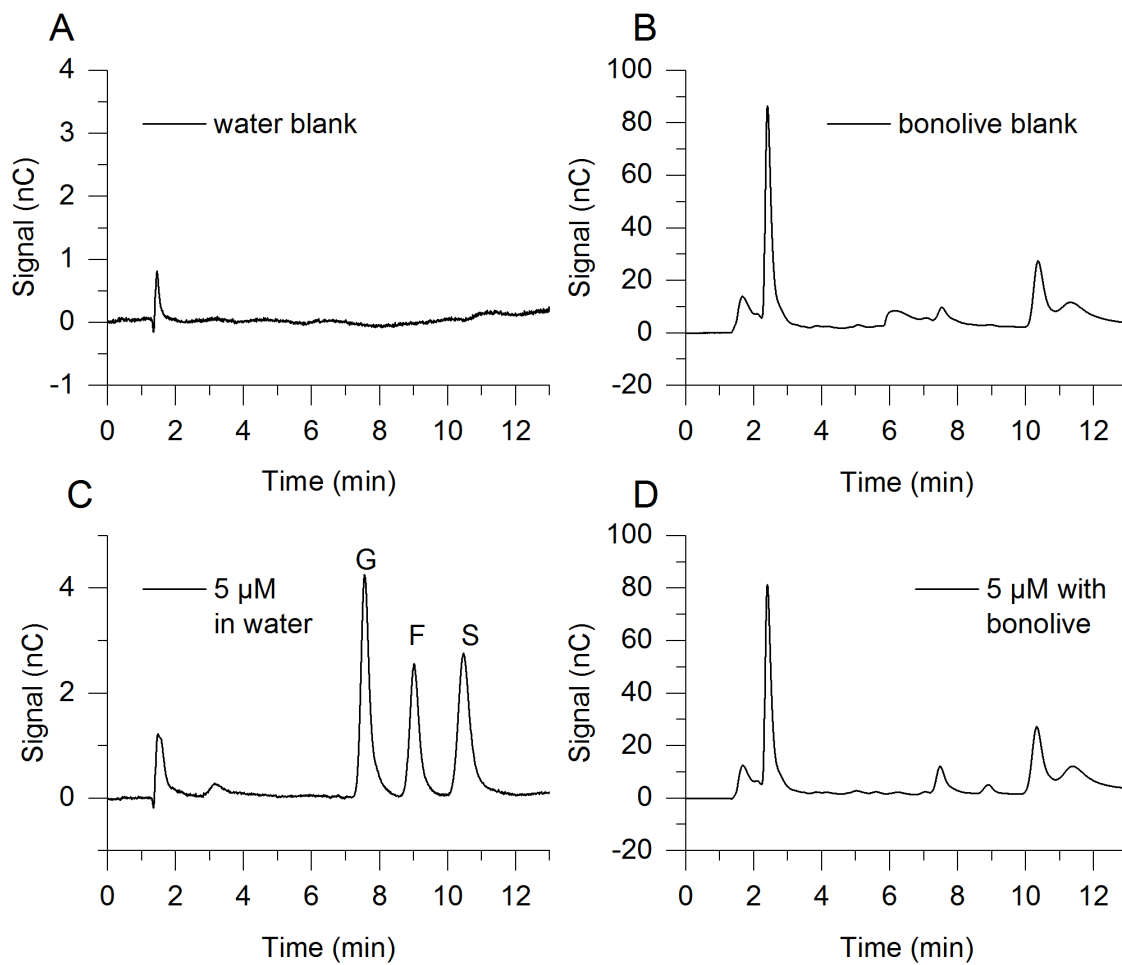


Figure 6-3. Representative chromatograms from HPAE-PAD analysis of glucose (G), fructose (F) and sucrose (S) for the evaluation of matrix effects. Water blank (A), blank containing 20% 1.5 mg/mL bonolive (B), 5  $\mu$ M standards in water (C) and 5  $\mu$ M standards containing 20% 1.5 mg/mL bonolive (D).

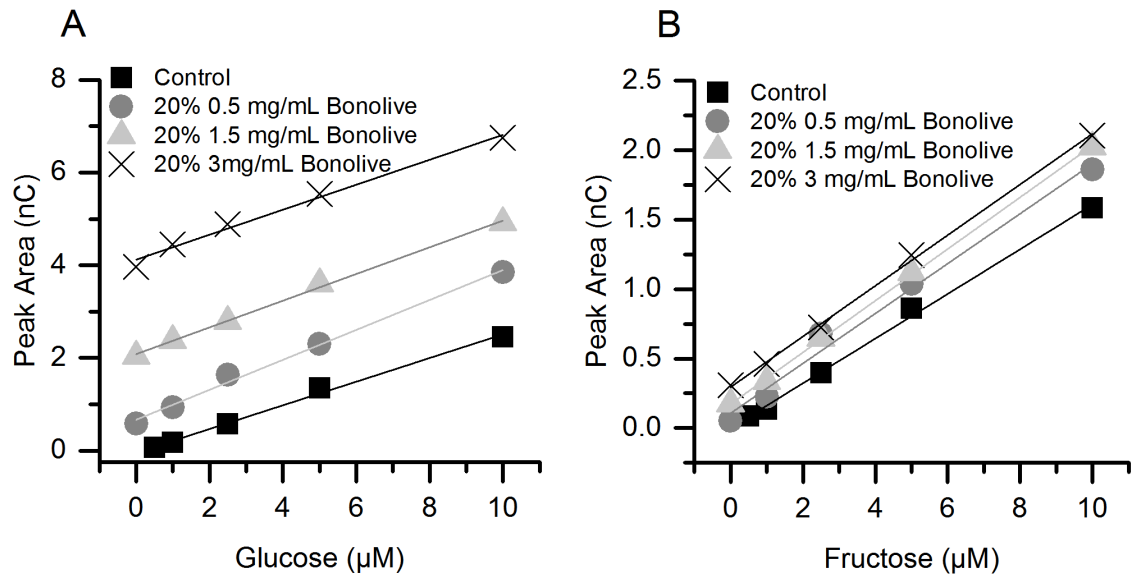


Figure 6-4. Glucose (A) and Fructose (B) standard curves prepared in 20% bonolive at 0.5 mg/mL, 1.5 mg/mL or 3 mg/mL.

### 6.3.7 Set-up of sucrose hydrolysis and transport

#### 6.3.7.1 Time-course

Different transport incubation times (30, 60 and 90 min) were evaluated with 5 mM sucrose as the substrate to select an incubation time for subsequent experiments. High biological variation was observed between the three experiments (Table 6-1).

Table 6-1. Comparison between apical glucose and apical fructose after 60 min incubation with 5 mM sucrose. Three independent experiments were performed on subsequent passages of Caco-2/TC7 cells and results are presented as mean  $\pm$  SD.

Experiment (n)	Apical Glucose (µM)	Apical Fructose (µM)	% Difference
1	16.9 $\pm$ 1.50	28.4 $\pm$ 1.77	50.8%
2	71.8 $\pm$ 4.81	84.6 $\pm$ 6.66	16.4%
3	99.9 $\pm$ 15.8	123 $\pm$ 20.1	20.5%
		<b>Mean</b>	<b>29.2%</b>
		<b>%CV</b>	<b>64.3%</b>

Different absolute amount of apical fructose and apical glucose were measured in the 3 independent experiments (Table 6-1) and the percentage difference between apical fructose and apical glucose also varied (%CV = 64.3%). This difference highlights the high biological variation, possibly from the long time in culture. Each experiment was done with independent reagents and transport solutions which could also contribute. Despite this, the same pattern was observed where there is more apical fructose than apical glucose. Hydrolysis of sucrose should lead to equivalent amounts of glucose and fructose, so this pattern suggests that glucose is transported at a faster rate than fructose.

Due to the detected biological variation between the three experiments, data were normalised by experiment to the 60 min time-point so that changes relative to the 60 min time-point could be evaluated. The normalised data from three experiments were combined and are presented in Figure 6-5. Statistical significance between time-points was determined for each of apical glucose, basolateral glucose, apical fructose and basolateral fructose using ANOVA followed by the Tukey post-hoc test.

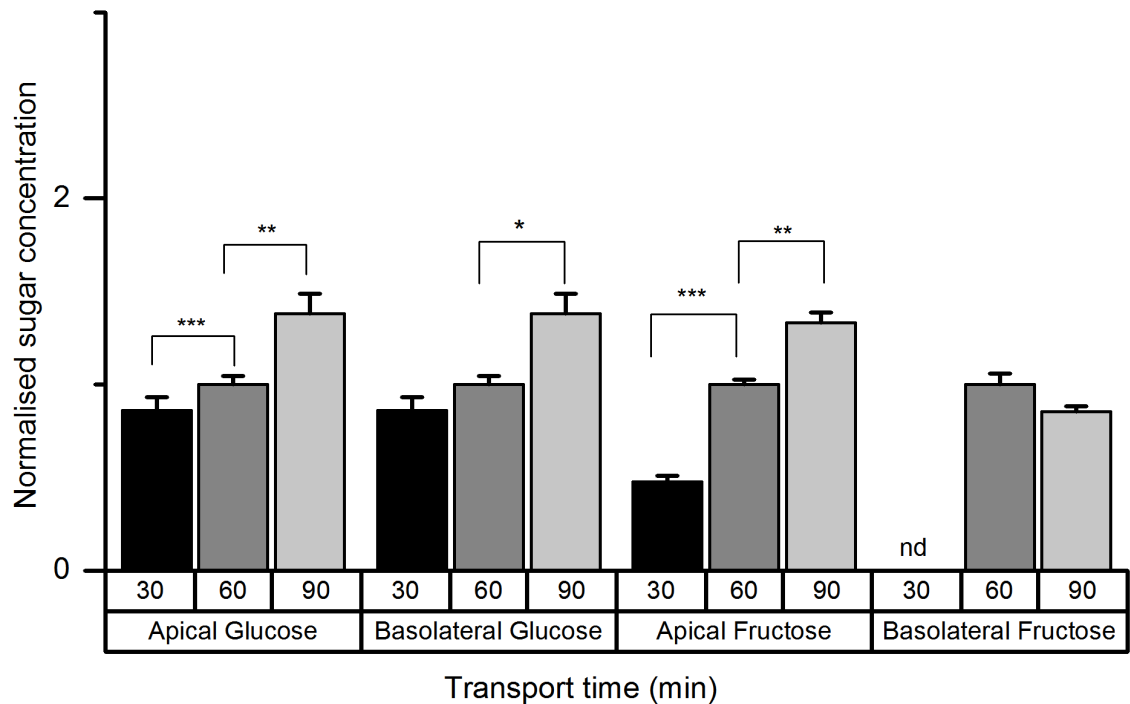


Figure 6-5. Evaluation of transport time for sucrose transport experiments.

Transport experiments were performed with 5 mM sucrose with 30, 60 or 90 min incubation and the glucose and fructose at the apical and basolateral sides were quantified by HPAE-PAD. The sugar concentrations in  $\mu\text{M}$  were normalised for each experiment to the mean of the 60 min time-point and presented as mean  $\pm$  SEM (n/N=3/18). \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$ , nd= not detected.

Both apical glucose and fructose levels increased between all transport times (Figure 6-5). For basolateral glucose, there was no change from 30 to 60 min ( $p=0.5$ ) and a significant increase between 60 to 90 min ( $p=0.004$ ). Basolateral fructose was not detected at 30 min and there was a slight decrease between 60 and 90 min ( $p=0.04$ ). This suggests that the transport of fructose to the basolateral side is saturated or has reached equilibrium. For further tests, 60 min was chosen as the transport time.

The concentration of sucrose on the apical side was only quantified for the first experiment and results are not presented. The sample required a second

dilution to quantify sucrose because the concentration of 5 mM required a 100-fold dilution to fall within the calibration range, which doubled the analysis time.

#### **6.3.7.2 Effect of different sucrose substrate concentration**

Different substrate concentrations were evaluated using 60 min incubation. Apical glucose, basolateral glucose and apical fructose all increased in response to increased sucrose (Figure 6-6). When plotted against substrate concentration, the amount of apical glucose and fructose produced was not linear at 25 mM. This implies that sucrose could be saturated when using 25 mM as the substrate concentration. Basolateral fructose was below the detection limit using 1 or 5 mM sucrose, and was 3.9  $\mu\text{M}$  with 25 mM sucrose. During the time course experiments, basolateral fructose was detected at an average of  $6.7 \pm 1.7 \mu\text{M}$  at 60 min, however here was not detected. This further highlights the biological variation that occurs with different passages of cells. Basolateral glucose did not increase in response to increased apical glucose; the transport of glucose could be rate-limiting at either the apical or basolateral side. Furthermore, the difference observed could be due to metabolism within the cell because cultured cells tend to be more glycolytic; Caco-2/TC7 cells require 25 mM glucose in the culture medium for normal growth. Apical glucose and fructose values were significantly different with 1 mM sucrose ( $p < 0.001$ ) but not with 5 mM or 25 mM sucrose ( $p = 0.08$  and  $0.9$ , respectively) (Figure 6-6).



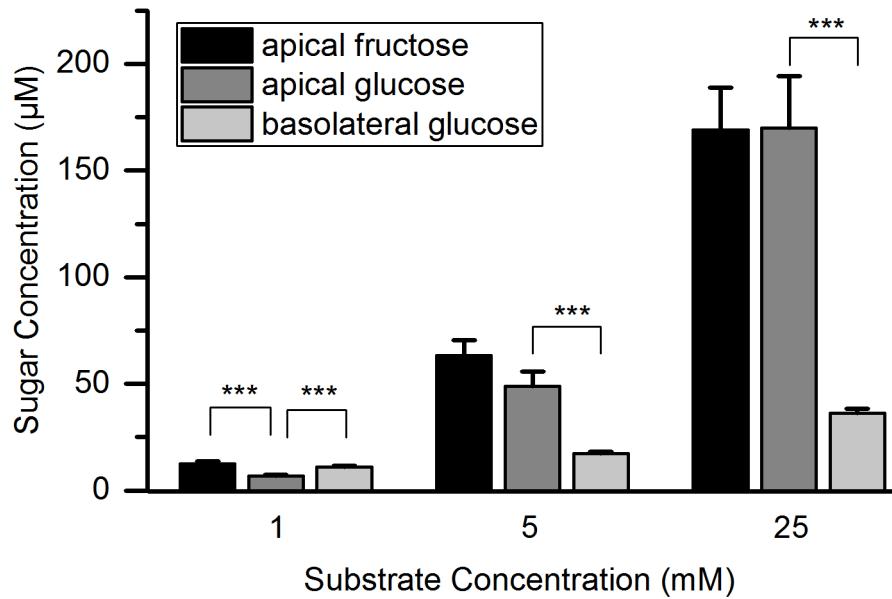


Figure 6-6. Sucrose transport with 1, 5 or 25 mM sucrose and 60 min incubation. Apical glucose, basolateral glucose and apical fructose were quantified by HPAE-PAD and the concentrations are reported as mean  $\pm$  SEM (n/N=3/18). \*\*\*  $p > 0.001$ .

### 6.3.8 Method summary

The final transport incubation times and substrate concentrations that were used for subsequent experiments are summarised in Table 6-2. Both chronic and acute effects of bonolive were evaluated with sucrose as the substrate. The acute effect of bonolive on glucose transport has already been evaluated in this laboratory (Kerimi, 2017), therefore only the chronic effects are determined here. The optimal substrate concentration and incubation time for glucose transport were previously determined in this laboratory and are used as standard lab protocols (Aydin, 2015).

Table 6-2. Transport Method Details

Substrate	Treatment	Substrate Concentration (mM)	Incubation Time (min)
Glucose	Chronic	1	25
Sucrose	Acute	1, 5 and 25	60
Sucrose	Chronic	5	60

## 6.4 Results

### 6.4.1 Glucose transport

#### *6.4.1.1 Chronic bonolive: regular and sodium-free conditions*

The effect of chronic bonolive treatment (3 days, 1.5 mg/mL) on glucose transport was determined under regular transport conditions using 1 mM glucose and 25 min incubation. The results from each of 3 independent experiments (n/N=3/18) are presented in Figure 6-7 A&B. Statistical significance was determined with Student's t-test. No significant change in either apical or basolateral glucose was observed. One experiment was done at higher glucose concentration of 5 mM (n/N=1/6) and again no change was observed (data not shown).

The glucose transport experiments were repeated under sodium-free conditions to suppress participation of SGLT-1 which requires a Na<sup>+</sup> gradient for its function. The results from each of 3 independent experiments (n/N=3/18) are presented in Figure 6-7 C&D. Under these conditions, GLUT2 is expected to be the dominant transporter. Bonolive inhibited glucose transport under sodium-free conditions with a 4.5% increase in glucose on the apical side (p<0.05) and a 24% reduction in glucose on the basolateral side (p<0.01).

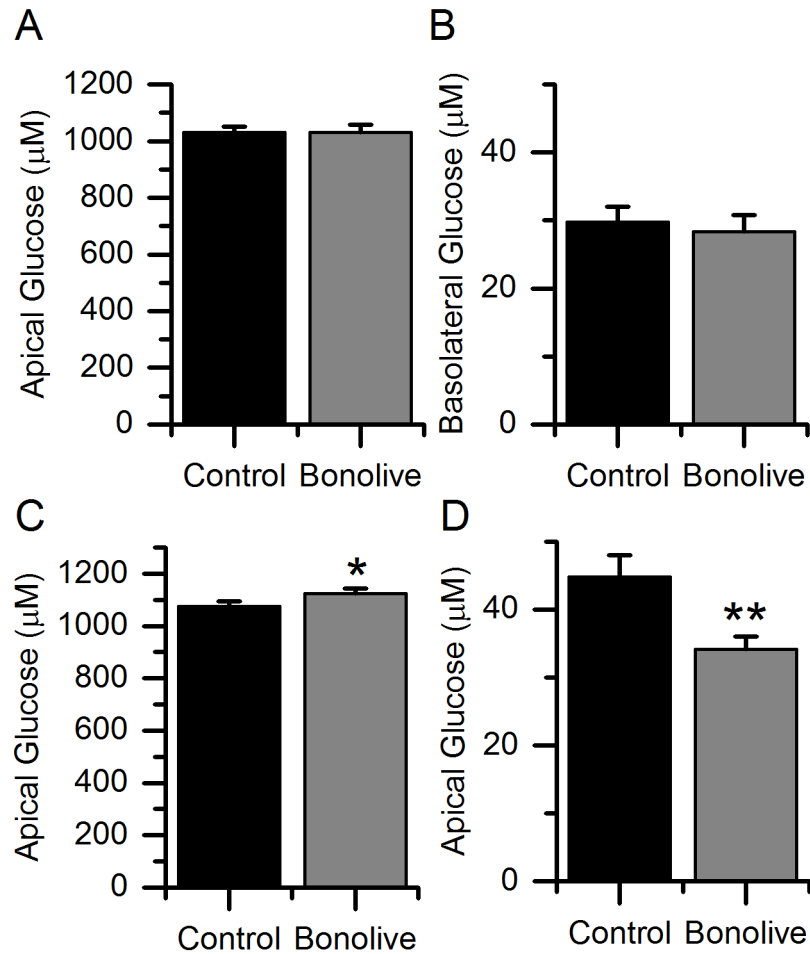


Figure 6-7. Glucose transport in regular (A&B) and sodium-free (C&D) conditions after chronic bonolive treatment. Caco-2/TC7 cells were cultured for 3 weeks and treated with bonolive for the final three days of differentiation. Transport experiments were done with 1 mM glucose for 25 min and the apical and basolateral glucose concentrations were determined by HPAE-PAD. Normalised results are presented as mean  $\pm$  SEM, n/N=3/18, \*  $p < 0.05$ , \*\*  $p < 0.01$

#### 6.4.1.2 Contribution of SGLT1 and GLUT2

The contribution to glucose transport by SGLT1 and GLUT2 was determined by comparing the percentage of glucose transported under regular and sodium-free conditions (n/N=1/6). Under regular conditions,  $4.8 \pm 2.3\%$  of the total glucose added reached the basolateral side whereas for sodium-free conditions,

significantly less glucose ( $2.6 \pm 0.4\%$ ,  $p=0.04$ ) was transported. Therefore, 54% of total glucose transport was attributed to SGLT1.

## 6.4.2 Sucrose hydrolysis and transport

### 6.4.2.1 *Chronic bonolive treatment*

The effect of chronic bonolive treatment (3 days, 1.5 mg/mL) on sucrose hydrolysis and transport was evaluated at 60 min using 5 mM sucrose. During the transport experiment, no bonolive would be present as it has been washed off the cells, and therefore any effects observed are due to changes from the chronic treatment. The results from three independent experiments ( $n/N=3/18$ ) are presented in Figure 6-8. On the apical side, bonolive treatment resulted in a 38% decrease in glucose ( $p<0.001$ ) and a 21% decrease in fructose ( $p<0.001$ ) (Figure 6-8). No change in basolateral glucose was observed, however the basolateral side is also impacted by cellular metabolism; as glucose passes to the basolateral side some is used by the cell. Since basolateral fructose levels are near the detection limit, basolateral fructose was only detected in the third experiment ( $n/N=1/6$ ) reflecting some biological variation.

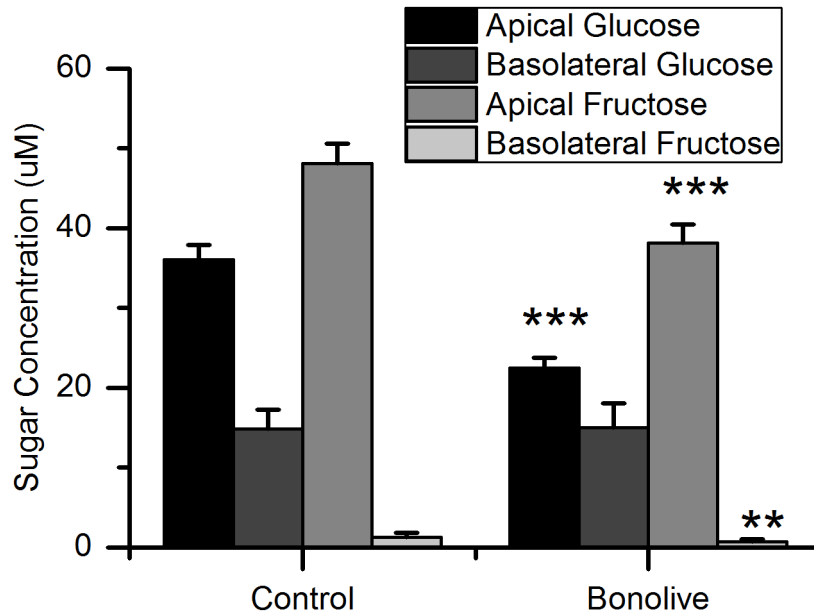


Figure 6-8. The effect of chronic bonolive treatment on sucrose hydrolysis and transport. Caco-2/TC7 cells were cultured for 21 days and treated with 1.5 mg/mL for the final 3 days of differentiation. Transport experiment was performed with 5 mM sucrose and 60 min incubation. Glucose and fructose from the apical and basolateral sides were quantitated by HPAE-PAD. Results are presented as mean  $\pm$  SEM with n/N = 3/18 for all except for basolateral fructose where n/N= 1/6, presented as mean  $\pm$  SD. \*\*\* p<0.001, \*\* p<0.01.

#### 6.4.2.2 Effect of chronic forskolin treatment

Forskolin was used as a positive control because chronic treatment with forskolin leads to reduced sucrase mRNA, biosynthesis and activity (Rousset et al., 1989). The biosynthesis of SI, determined with pulse-chase experiments, was only 15% of the control for a treatment of 25  $\mu$ M forskolin for 48 hours in Caco-2 cells at day 14. The decrease in biosynthesis corresponded to decreases in activity and mRNA levels and it has been reported that the forskolin leads to reversible decreased transcription of mRNA (Chantret et al., 1993). Here, the cells were treated with forskolin for the final 3-days of differentiation to be consistent with the treatments with bonolive. Under these

conditions, less hydrolysis of sucrose occurred as seen by the 17.8% ( $p=0.05$ ) and 10.1% ( $p=0.04$ ) reduction in apical glucose and fructose, respectively (Figure 6-9). There was no change in basolateral glucose and basolateral fructose was below the detection limit. The reduction by this positive control was not as great as observed in the literature, however a lower concentration was used and the treatment began at day 18 rather than day 14, during which time the mRNA levels for SI are still increasing.

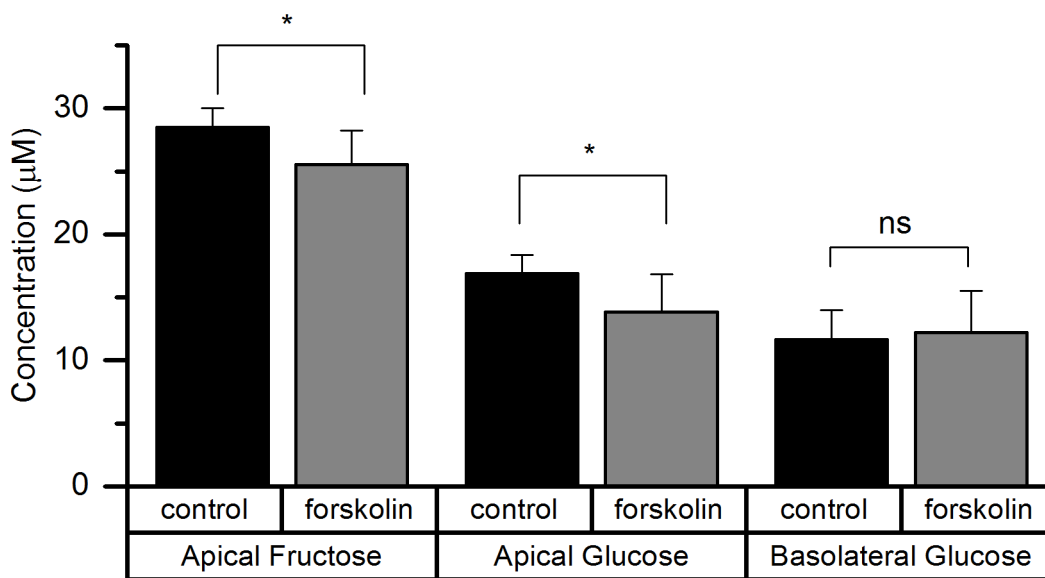


Figure 6-9. The chronic effects of forskolin on sucrose hydrolysis and transport. Caco-2 cells were treated with 10  $\mu\text{M}$  forskolin for the final 3 days of differentiation. Transport experiment was performed with 5 mM sucrose and 60 min incubation. Glucose and fructose from the apical side and glucose from the basolateral sides were quantified by HPAE-PAD. Results are presented as mean  $\pm$  SD. \*  $p<0.05$ , ns = not significant,  $n/N=1/6$ .

### **6.4.2.3 Acute bonolive treatment**

The acute effect of bonolive on sucrose hydrolysis and subsequent glucose and fructose transport was determined by including 0.5, 1.5 and 3.0 mg/mL bonolive in the transport solution with 5 mM sucrose with 60 min incubation. A reduction in apical glucose, apical fructose and basolateral glucose occurred as the concentration of bonolive increased (Figure 6-10). The results are presented as a percent reduction from the control samples which had no bonolive treatment. Statistical significance was determined by ANOVA followed by Tukey's post-hoc test. No basolateral fructose was detected in the control or treated samples.

Bonolive at 0.5 mg/mL decreased apical glucose to 52.9% of the control ( $p < 0.001$ ) and further decreased to 23.9% of the control at 1.5 mg/mL ( $p < 0.001$ ). There was no change in apical glucose between 1.5 and 3 mg/mL ( $p = 0.5$ ). There was no change in apical fructose with 0.5 mg/mL bonolive but it decreased to 54.8% and 30.7% of the control with 1.5 mg/mL and 3.0 mg/mL bonolive, respectively ( $p < 0.001$ ). Basolateral glucose decreased to 34.7% of the control with 0.5 mg/mL ( $p < 0.001$ ) and 9.9% of the control with 1.5 mg/mL ( $p = 0.03$ ) and no further decrease was observed at 3 mg/mL bonolive (Figure 6-10).

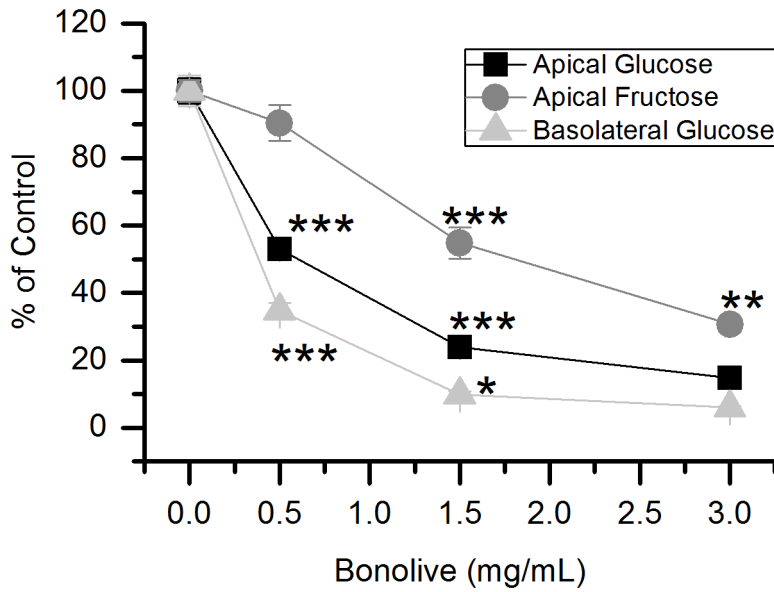


Figure 6-10. Acute effects of bonolive on sucrose hydrolysis and subsequent glucose and fructose transport with 60 min incubation. The 5 mM sucrose transport solution contained bonolive at 0.5, 1.5 or 3.0 mg/mL. Results for apical glucose and fructose and basolateral glucose are presented as a percentage of the apical glucose control, mean  $\pm$  SEM. Significance between time-points was determined by ANOVA followed by Tukey's post hoc test. \*\*\*  $p < 0.001$ , \*  $p < 0.05$ ,  $n/N = 3/18$ .



Glucose transport and sucrose hydrolysis were determined for each bonolive concentration using following equations:

$$\% \text{ Glucose Transport} = 100 \times (\mu\text{M Basolateral Glucose}) / (\mu\text{M Apical Glucose} + \mu\text{M Basolateral Glucose})$$

$$\% \text{ Sucrose Hydrolysis} = 100 \times (\mu\text{M Apical Glucose} + \mu\text{M Basolateral Glucose}) / (\mu\text{M Sucrose Substrate at time zero})$$

$$\% \text{ Sucrase Inhibition} = 100 - (\mu\text{M Total Glucose} / \mu\text{M Total Glucose in Control}) \times 100$$

$$\% \text{ Glucose Transport Inhibition} = 100 - (\mu\text{M Basolateral Glucose} / \mu\text{M Basolateral Glucose in Control}) \times 100$$

These calculations make the assumption that the results of glucose and fructose are independent of each other and that the cell metabolism is not affected by the treatment. The results are presented in Table 6-4. The  $IC_{50}$  for sucrase was approximately 0.5 mg/mL and 87.9% inhibition was achieved at the highest concentration. Slightly stronger inhibition was observed for glucose transport, with  $IC_{50}$  below 0.5 mg/mL. Up to 94% inhibition of glucose transport occurred with 3 mg/mL bonolive.

Table 6-3. Acute effects of bonolive on sucrose hydrolysis and subsequent glucose and fructose transport. Bonolive from 0.5 to 3.0 mg/mL was present in the transport solution with 5 mM sucrose and incubated for 60 min. Results from apical glucose and fructose and basolateral glucose are presented as mean  $\pm$  SD.

Bonolive Concentration (mg/mL)	0 (Control)	0.5	1.5	3.0
Percent of Glucose Transported	29.8 $\pm$ 4.3	21.7 $\pm$ 3.4	14.9 $\pm$ 2.3	12.1 $\pm$ 2.4
Percent of Sucrose Hydrolysed	0.2 $\pm$ 0.005	0.1 $\pm$ 0.003	0.05 $\pm$ 0.001	0.03 $\pm$ 0.002
Percent Inhibition of Sucrase	NA	52.5 $\pm$ 4.1	80.3 $\pm$ 3.8	87.9 $\pm$ 3.9
Percent Inhibition of Glucose Transport	NA	65.3 $\pm$ 3.7	90.1 $\pm$ 3.2	94.1 $\pm$ 3.1

#### 6.4.3 Effect of bonolive on TEER values

The TEER values were determined for each experiment and an increase of 42.7  $\pm$  2.7% occurred in wells with chronic bonolive treatment (3 days at 1.5 mg/mL) compared to the control (Figure 6-11).

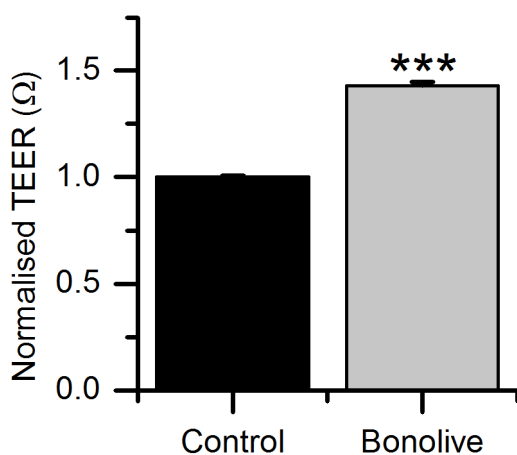


Figure 6-11. Trans-epithelial electrical resistance (TEER) from Caco-2/TC7 cells treated chronically with bonolive for the final 72 h of differentiation. Results are presented as mean  $\pm$  SEM, n/N = 11/66, \*\*\* p<0.001.

## 6.5 Discussion

The transport methodology that is traditionally used involves the use of a  $^{14}\text{C}$  radiolabelled substrate followed by liquid scintillation counting of the apical and basolateral sides. If sucrose were used as the substrate, both the glucose and fructose products are radiolabelled and therefore the counts observed in the final samples cannot distinguish between glucose and fructose. Here, HPAE-PAD quantification of carbohydrates was used to improve the method so that more detailed information regarding specific changes in glucose and fructose was obtained. For acute transport experiments, it was essential to consider the interference from bonolive on the quantification. Bonolive was found to interfere with the quantification of carbohydrates and therefore appropriate standard curves containing bonolive were prepared to correct for this. Overall, the use of HPAE-PAD as an alternative to the previously used radiochemistry method improved the analysis time and cost. It also provided more information which allowed better interpretation of the results as compared to traditional methods.

Oleuropein has been reported to acutely inhibit  $\alpha$ -glucosidase enzymes such as sucrase (Hadrich et al., 2014) and in Chapter 3, the oleuropein-rich bonolive was observed to inhibit human sucrase and maltase in the assay using a Caco-2/TC7 cell-free extract. Evidence from human studies suggest that oleuropein may inhibit glucose transport (de Bock et al., 2013) and this was confirmed in our lab where bonolive acutely inhibited glucose transport in Caco-2/TC7 cells in a concentration dependent manner. Using 5 mM glucose and a 25 min incubation time, 44% inhibition of glucose transport occurred with 1 mg/mL of bonolive under regular transport conditions (Kerimi, 2017). Here, the acute

effects of bonolive were evaluated using intact cells where both hydrolysis and product transport are involved.

In experiments with acute bonolive treatment where sucrose was the substrate, the reduction in basolateral glucose compared to the untreated cells was greater than the reduction in apical glucose. This is because the basolateral glucose includes the combined effects of the inhibition of sucrose hydrolysis and inhibition of glucose transport. As already mentioned, previous experiments demonstrated that bonolive can acutely inhibit glucose transport and sucrose hydrolysis, so the *in situ* experiments here were successful in reflecting the combined effect. If there was no effect on glucose transport, the ratio between the apical and basolateral sides would be expected to have remained constant. The fact that the ratio of apical to basolateral glucose increased supports the hypothesis that both sucrose hydrolysis and glucose transport were inhibited. In Chapter 3, there was 34.4% sucrose inhibition with 1.5 mg/mL bonolive using the cell-free assay developed. The inhibition of sucrose was stronger here using intact cells (87.9%, Section 6.2.4.3).

The effect of chronic 3-day treatment with 1.5 mg/mL bonolive was evaluated to further extend the results from Chapter 5 to cells *in situ*. Glucose transport was not affected in regular transport conditions confirming that the decrease in apical glucose observed with sucrose as the substrate was due to lower hydrolysis of sucrose and not because of altered transport. On the other hand, the fact that there was more apical fructose than glucose suggests a small inhibition on fructose transport. The chronic treatment did lead to a decrease in GLUT5 mRNA (Chapter 5). Chronic bonolive treatment only led to a change in

glucose transport under sodium free conditions, in which SGLT1 is not active (Kellett et al., 2008), therefore the treatment predominantly affects GLUT2 and not SGLT1. Under regular conditions, 54% of transport was attributed to SGLT1, in agreement with the literature (Kellett et al., 2008). SGLT1 and GLUT2 gene expression were reduced with bonolive treatment, with GLUT2 to a greater extent (Chapter 5). Based on the gene expression data, bonolive treatment would be expected to reduce glucose transport by both GLUT2 and SGLT1, however it is possible that for SGLT1, the effects observed for the mRNA were not translated on the protein level. A similar phenomenon was observed in a study by Alzaid et. al; glucose transport inhibition was only observed in sodium-free conditions for a 16 h treatment with anthocyanin-rich berry extract on Caco-2/TC7 cells. GLUT2 and SGLT1 mRNA levels were both significantly decreased but only GLUT2 protein level was decreased (Alzaid et al., 2013). When considering the impact that these results could have *in vivo*, the effect on GLUT2 observed here under sodium-free conditions is important because at high glucose concentrations, GLUT2 is the main glucose transporter (Kellett et al., 2008). Chronic bonolive treatment reduced sucrase hydrolysis, supported by results in Chapter 5 where less surface protein, decreased specific activity and mRNA occurred.

The trans-epithelial resistance increased with chronic bonolive treatment. This could be similar to previously reported effect of quercetin increasing the trans-epithelial resistance which was correlated to an increase in claudin-4, a tight junction protein (Amasheh et al., 2008). Tight junction proteins link cells together and help dictate paracellular permeability, and is not expected to

impact transport of sugars which occurs through the cells. However, one of the major roles of the small intestine is to provide a protective barrier against harmful substances. Inflammation can lead to dysregulation of tight junction proteins in patients with ulcerative colitis (Schmitz et al., 1999). There is clinical interest in improving tight junction permeability since a “leaky gut” occurs in cases of inflammatory bowel diseases such as Crohn’s and celiac disease (Hollander, 1999).

There are some limitations to the transport experiment methodology. Firstly, the amount of fructose on the basolateral side was not detected in the experiments so the effect of fructose transport can only be speculated by the amount on the apical side. The amount of sucrose that is hydrolysed in the 60 minute time point was low so longer times could be evaluated to try and increase the concentration of basolateral fructose. Since the experiments involve the effect of hydrolysis and transport, the interpretation of the results is challenging but the independent results from glucose transport and sucrose hydrolysis support the observed results. Finally, the calculations for the acute effects of bonolive made the assumption is made that the cellular metabolism and glucose and fructose is not affected. In order to verify this, cell metabolism studies could be performed with bonolive treatment.

In summary, the transport model used here in Caco-2/TC7 cells was able to detect changes in sucrose hydrolysis by sucrase for both acute and chronic bonolive treatments. Chronic treatment inhibited glucose transport via GLUT2, however this effect was masked by SGLT1 transport under normal conditions, when sucrose was used as a substrate. Chronic treatment with olive leaf

extract has the potential to decrease disaccharide hydrolysis and transport warranting future investigation in humans for glycaemic control after sugar consumption for the reduction of type 2 diabetes risk.

## 7 Summary, discussion and future prospective

### 7.1 Summary of results

#### 7.1.1 Acute inhibition assay

One strategy for reducing postprandial glycaemia is through inhibition of starch and sugar digesting enzymes. Type 2 diabetes treatment with the drug acarbose is based on this approach (Breuer, 2003). Due to side effects of pharmacological treatments, natural compounds such as polyphenols are often screened as potential inhibitors of  $\alpha$ -glucosidases (Hanhineva et al., 2010). Most inhibition assays use a readily available rat intestinal powder preparation as a source of  $\alpha$ -glucosidase enzymes and it was hypothesised that human or rat enzyme sources might yield different inhibition results due to their different genetic sequence. An assay using human Caco-2/TC7 cells was optimised in Chapter 3 and used to compare the inhibition by selected polyphenols and acarbose towards sucrase and maltase with results from an assay using the rat enzyme source. Caco-2/TC7 cells have high levels of SI and the sequence was confirmed for the first time to be identical to human SI in Chapter 4, making this a suitable source of enzyme.

Using this *in vitro* assay, inhibitors were more effective towards human compared to rat sucrase. Therefore, it is possible that *in vivo* studies the effect in humans could be greater than that observed in rat studies. The opposite situation occurred for maltase where inhibitors were stronger towards the rat enzyme source. This is important because the rat intestinal preparation is commonly used as the enzyme source and then the *in vivo* effect is determined



in rats. If the study is then progressed into human interventions, a lack of effect could occur. The enzyme assay based on the rat enzyme is useful for comparing the strength between different inhibitors, for example in the design of pharmacological treatments. Since the rat enzyme source is readily available, it could be used for screening purposes however the strength of inhibition observed would not be the same as towards a human enzyme source. If screening is performed using an assay with rat enzyme, the inhibitors evaluated will also inhibit the human enzyme but the enzyme assay with the human enzyme source should be used to quantify and confirm the result. The evidence here highlights the importance of confirming enzyme inhibition with the more physiologically relevant human source.

Experiments in Chapter 3 also demonstrated that inconsistent results can be obtained if the amount of enzyme and assay conditions are not optimised. Further, results were presented that demonstrate the importance of considering interference by the inhibitors on the detection methods. Taken together, this information established an improved method for inhibition of  $\alpha$ -glucosidases. Previously published literature results should be critically considered for these factors. In the literature there was limited strong evidence that polyphenols can specifically inhibit sucrase. Here, inhibition was observed with EGCG, green tea, an olive-leaf extract and quercetin and the inhibition values obtained would be achievable in the gut lumen. For example, one 200 mL portion of green tea extract prepared as a drink would contain approximately 5 mg/mL of green tea polyphenols. The  $IC_{50}$  value obtained for sucrase inhibition was 1 mg/mL, a concentration required in the gut lumen. Taking into account an approximate 3-

fold dilution due to intestinal dilution (Williamson, 2013), a 200 mL cup of green tea would lead to a concentration in the gut lumen which could inhibit sucrase by at least 50%.

Other studies have demonstrated that acute sucrase inhibition observed *in vitro* does lead to an effect *in vivo*, so this is a suitable strategy (Krog-Mikkelsen et al., 2011). Interestingly, a synergistic effect with acarbose has also been described for several polyphenols and polyphenol-rich extracts. For example, using an *in vitro* inhibition assay, an anthocyanin-rich blackcurrant and chlorogenic acid-rich rowanberry extracts were able to recover the inhibition that was lost when the acarbose dose was lowered (Boath et al., 2012). The anthocyanin cyanidin-3-rutinoside has also been reported to work synergistically towards  $\alpha$ -glucosidase inhibition (Adisakwattana et al., 2011). Polyphenols that are identified as strong  $\alpha$ -glucosidase inhibitors could potentially be used as a replacement for or in conjunction with pharmaceutical drugs for maintaining glycaemic control.

### **7.1.2 Effect of sucrose on sucrase in Caco-2 model**

Some reports in the literature suggest that sucrase expression is modulated by its substrate sucrose. As discussed in Chapter 1, many of these reports compare the expression of SI to a starved state. There is no evidence comparing the effect of glucose exposure with sucrose exposure. This is of interest due to the fact that sucrose is the most commonly consumed sugar in the UK (SACN, 2015). The effect of sucrose on sucrase was evaluated because it is the sole enzyme capable of its hydrolysis. Since SI has sucrase and maltase activities, a decrease in sucrase activity would have beneficial

effects on both starch and sugar hydrolysis and could affect the postprandial glycaemic response. The results from Chapter 4 indicated that chronic sucrose exposure in the Caco-2/TC7 cell model did not lead to a change in SI mRNA expression, surface protein or specific activity. There was a slight decrease in the affinity for sucrose and it is hypothesised that this is due to the change in N- and O-glycosylation. A limitation to this work is that regulation in cell culture models can be different than what occurs *in vivo* (Ferraris, 2001). The small intestine has enteroendocrine cells in addition to enterocytes and these have been suggested to be involved in sugar sensing in the gut (Le Gall et al., 2007; Miguel-Aliaga, 2012). The regulation of brush border enzymes and transporters *in vivo* most likely involves coordinated action of the different cell types therefore the regulation of SI *in vivo* could be different to what was observed in the cell model here. However, the use of this model was able to identify the fact that sugar exposure can affect glycosylation. Future work to identify the effect of sucrose, glucose and fructose on the steps of glycosylation would be of interest. As an aside, it would also be interesting to evaluate the effects from synthetic sweeteners since they are commonly used by the food industry as a "healthier" sugar replacement however limited work has been done to evaluate any chronic effects from long-term consumption. Overall, the evaluation of sucrose exposure as a nutritional sugar stress indicated that sucrose can affect the post-translational modification of the enzyme. Glycosylation plays a role in protein localisation, activity and degradation so therefore there several widespread implications. These results add to the body of evidence that cells can respond to sugars.

### 7.1.3 Acute and chronic effects of bonolive

There are many reports that investigate the acute inhibition of a range of polyphenols towards  $\alpha$ -glucosidase enzymes, with the aim of reducing sugar and starch digestion for the attenuation of the postprandial glycaemic response. Up to 5 mg of polyphenols are consumed per day (Scalbert and Williamson, 2000) so any beneficial effects from long-term exposure are of interest. An olive-leaf extract was used here as the model compound due to the fact that olive polyphenols have been reported to aid in glucose homeostasis (de Bock et al., 2013) and bonolive was found to be an acute sucrase and maltase inhibitor (Chapter 3). In the Caco-2/TC7 cell model, a chronic 3-day treatment with 1.5 mg/mL of bonolive led to a reduction in sucrase activity. The activity, mRNA, total SI protein, enzyme kinetics were first evaluated in whole cell lysates (Chapter 5) and then further investigated in more physiological state *in situ* with transport studies, where a greater effect occurred. In transport studies, both chronic and acute treatments with bonolive reduced the hydrolysis of sucrose by sucrase. The chronic bonolive treatment also reduced glucose transport by GLUT2 when SGLT1 was suppressed. The determination of glucose on the basolateral side depends on intra-cellular metabolism and efflux from the cell and the model assumes that these are not affected. Cells in culture are more glycolytic and utilise majority of the glucose they take up so it is possible that effects at the basolateral side cannot be detected. Nevertheless, changes were observed on the apical side that suggest sucrase hydrolysis was reduced after chronic treatment.

Reduction of sucrase activity *in vivo* can lead to reduced glycaemic response to sucrose. In rats, digestion of sucrose by sucrase was rate-limiting towards the glycaemic response; in an oral sucrose tolerance test with a formulation containing *L*-arabinose, the incremental area under the curve for blood glucose was significantly reduced while no change was observed in glucose or starch tests (Preuss et al., 2007b; Preuss et al., 2007a). In humans, pure *L*-arabinose led to a delay in glucose absorption in response to sucrose and there were significant positive changes in GLP, GIP and insulin (Krog-Mikkelsen et al., 2011). These results suggest that sucrase can be rate-limiting in the uptake of sugars *in vivo*. In contrast, an isotopic tracer study in humans demonstrated that the uptake rate of glucose after consumption of either sucrose or a mixture of glucose and fructose was the same, for which it was suggested that sucrose digestion is rapid and not rate-limiting *in vivo* (Sun and Empie, 2012). However, the fact that the same glucose uptake rate was observed does not provide alone enough evidence to claim which steps are limiting. Any reduction in SI levels would be beneficial beyond sucrose since the enzyme complex is the primary source of maltase and isomaltase activity (Van Beers et al., 1995), therefore there could be wider impact on total starch digestion. Reduction in the level of SI at the brush border of the small intestine would be of particular importance to diabetic patients who have elevated levels (Dyer et al., 2002). These findings enhance the understanding of chronic effects by which polyphenols can lead to health benefits. The results are the first to identify chronic changes in SI with chronic treatment with an olive leaf extract.

## 7.2 Future prospective

### 7.2.1 Human intervention study

One of the main questions raised from these results is whether the effects observed using the *in vitro* cell model would also occur *in vivo*. Intra- and inter-individual variation of the expression of transporters and SI *in vivo* makes comparison to the cell model challenging (Zeevi et al., 2015). Furthermore, level of glucose transporters *in vivo* is not constant due to the proposed recruitment of GLUT2 to the apical cell surface at high glucose concentrations (Kellett et al., 2008). In the transport studies in Chapter 6, there was no change detected on the basolateral side, which is representing the blood glucose response. This was one main limitation and is in part due to the fact that the cultured cells are highly glycolytic and most of the glucose taken up in the cell would have been metabolised and used within the cell. The next logical step to test the hypothesis that bonolive is able to reduce sucrase activity and therefore influence the glycaemic response to sucrose is to evaluate using human intervention trials.

Human studies to evaluate the acute effects of bonolive have already been undertaken in the Williamson laboratory. In the first study, after an overnight fast, participants consumed 100 g of white bread with or without 125 mg of bonolive and on the second visit, the opposite treatment (cross-over design). Blood glucose was measured up to 3 hours and there was no change between the bonolive and placebo (Li, 2016). The use of white bread involves the effect on starch digestion and glucose transport. The second study was performed in the same manner but with 25 g of sucrose instead of white bread and a dose of

290 mg bonolive. In this case, there was statistically significant reduction in blood glucose response (13%) (Shelley, 2017). The differences between these two studies highlight the fact that sucrase inhibition combined with inhibition of glucose transport is a good strategy to reduce the postprandial glycaemic response; bonolive was more effective against sucrose than starch digestion.

As we consume polyphenols regularly over the course of our lifetime, chronic effects that they could potentially exert are of interest. The results in Chapter 5 and Chapter 6 primarily focused on the chronic effect of bonolive. It is therefore recommended that a pilot study to evaluate the chronic consumption of bonolive capsules on the glycaemic response to sucrose should be undertaken. The proposed study is a randomised, double-blind, placebo controlled, cross-over intervention trial. The study would have two intervention periods of one week, during which the participant would consume either the placebo or treatment daily, with a one month minimum washout between interventions to allow any effects from treatment to be eliminated. The cross-over design in which participants undertake both interventions and therefore serve as their own control would help account for inter-individual variation.

Healthy participants with BMI 18-25 (healthy weight according to NHS) and fasting blood glucose within the normal range of 4.0 to 5.9 mmol/L (according to National Institute for Clinical Excellence Guidelines) would be recruited and then screened based on the inclusion criteria of the study. Providing they met the inclusion criteria, participants would be coded and then randomly assigned the order of interventions. During the intervention phase, participants would consume the placebo/treatment for a total of seven days. The dose of the

treatment would be two 500 mg per day with 200 mL of water. With digestive dilution, a concentration near 1.5 mg/mL, the concentration that was evaluated with the *in vitro* model, would be achieved in the gut lumen. At the end of each intervention, participants would undertake an oral sucrose tolerance test. The baseline blood glucose would be measured and then the participant would consume 50 g of sucrose dissolved in 250 mL of water (within 5 min) and the blood glucose would be determined every 15 minutes up to 3 hours. Some factors would be controlled in order to help with consistency of the blood glucose measurements (Brouns et al., 2005). It is recommended that participants consume the same meal before the 10-12 h overnight fast and testing, therefore a standardised meal could be provided to ensure compliance. Participants should be asked to consume 250 mL of water upon waking on the testing morning, to ensure that they are hydrated when the testing begins. A sleep diary could also be recorded because evidence suggests that sleep duration is associated with fasting blood glucose (Twedt et al., 2015). The effect on the incremental area under the curve (IAUC) from the blood glucose response would be determined and data analysed using previously described methodology (Brouns et al., 2005).

There is high inter-individual variation in the postprandial glycaemic response and individuals have different levels of sugar transporters (Zeevi et al., 2015). Furthermore, cell studies here saw different effects of bonolive depending on the whether cells were exposed chronically to glucose or sucrose therefore carbohydrate consumption habits could be taken into consideration. An ideal situation would be to provide a standardised diet to control carbohydrate



composition; however this type of study is not practical due to expense. Food frequency questionnaires would help in understanding the general eating patterns of participants.

### **7.2.2 Glycosylation analysis**

Long-term culture of Caco-2/TC7 cells in sucrose led to altered N- and O-glycosylation (Chapter 4) and the chronic bonolive treatment led to altered N-glycosylation for cells cultured in sucrose (Chapter 5). It has been previously discussed that both N- and O-glycosylation play a role in the processing and trafficking of SI and the investigation of the phenotypes of sucrase isomaltase deficiency disorder (CSID) has aided in understanding the role of glycosylation in these processes (Lee et al., 2010; Naim et al., 2012; Naim et al., 1999). The carbohydrates attached to proteins at the cell surface also play an important role in cell-cell interactions. Specific alterations in glycosylation has been used as a biomarker for disease, such as cancer (Ohtsubo and Marth, 2006). Altered glycosylation has been observed in the diabetic rat model so it is possible that it plays a role in diabetes (Najjar et al., 2001). Dietary sugar and its relation to glycosylation and human health have not been established so the results here add to the gap in knowledge.

Glycotransferases are the enzymes responsible for the formation of the glycans in the ER and Golgi during translation. Regulation of glycosylation is a complex interplay of processes involving the gene transcription of the glycosyltransferase enzymes, synthesis of sugar donors and transport to the ER and Golgi, protein turnover and other factors (Ohtsubo and Marth, 2006). Lectins are glycan binding proteins that are highly specific for glycan structures

and can be useful for studying altered glycosylation. Glycans can play a role in health and disease based on their interaction with lectins and effect on cellular mechanisms including cell adhesion, cell recognition, molecular trafficking and clearance, receptor activation and endocytosis, all of which have been reviewed (Ohtsubo and Marth, 2006).

Detailed glycosylation analysis would aid in understanding the changes observed. Here, an increase in glycosylation was observed and this could be due to additional sites or changes to the oligosaccharide chains. Two analyses would be required to distinguish if one or both of these changes occurred. To determine sites of N-glycosylation, the sample is first de-glycosylation with PNGase F. The removal of N-glycans converts asparagine into aspartic acid and this alters the mass of the peptide by +1 Da. MALDI-MS analysis would allow for the identification and exact mass determination of the peptides (Wilson et al., 2009). Identification of the O-glycans is complicated by the fact that a mixture of enzymes is required to remove all types of O-glycans. The most favoured method of removal is an ammonia-based non-reductive  $\beta$ -elimination followed by MS detection (Mariño et al., 2010). The detailed structure of the removed N- and O-glycans is complex and several methods exist. One method is based on hydrophilic interaction chromatography (HILIC) to allow good separation of glycans including separation of structural isomers. The glycans are first labelled with 2-aminobenzamide to allow fluorescent detection. A series of exoglycosidase digestions coupled with the analysis allows for the determination of detailed N-glycan structures through comparison to a database (Campbell et al., 2008; Mariño et al., 2010). MALDI-TOF-MS of

permethylated glycan mixtures offers another alternative. The determination of O-glycans is more challenging because they are more structurally diverse and have smaller mass. The methods for the analysis of glycans and their sites of occupancy has been improving with advances in technology but still proves very challenging and requires highly specialist instrumentation and use of several techniques. Once the post-translational changes have been identified, the functional impact and mechanisms could be investigated.

### **7.3 Conclusion**

The health burden and increasing incidence of diabetes is of great concern. Any lifestyle factors which can aid in glucose homeostasis are important and diet is an easily modifiable factor. Evidence here suggests that olive polyphenols can decrease the digestion of sucrose and could therefore aid in reduction of the glycaemic response to foods containing sucrose. Since SI also has maltase and isomaltase activities, the reduction of activity or levels at the cell surface could have a beneficial impact on starch digestion. Inter-individual variation occurs in the glycaemic response therefore the therapeutic use may not benefit everyone and the effect could potentially depend upon the amount of dietary sugars consumed. Evidence from the *in vitro* cell studies here demonstrated that bonolive treatment was only able to reduce the amount of SI at the cell surface when cells were cultured under their regular conditions in glucose. When exposed long-term to sucrose, there was no change in SI at the cell surface, where it exerts its function. From this it could be speculated that *in vivo*, those with high sucrose exposure may not see the benefit from bonolive treatment. The *in vitro* studies using the Caco-2/TC7 cell model indicated that

both acute and chronic olive treatment can inhibit sucrase activity. These results warrant investigation in humans to determine if the effects observed in the cell model will translate into a positive effect *in vivo* therefore future studies in humans, such as the pilot study described above, are recommended. The Mediterranean diet is well established as a pattern for healthy eating, and the olive-leaf polyphenols could play a role, in part through the mechanisms discovered here. Polyphenols have many modes of actions and health benefits are due to a combined effect. Every small individual change such as observed here could contribute to overall improved health status.

## List of References

- Acker, M.G. and Auld, D.S. 2014. Considerations for the design and reporting of enzyme assays in high-throughput screening applications. *Perspect. Sci.* 1(1–6), pp.56-73.
- Adachi, T., Mori, C., Sakurai, K., Shihara, N., Tsuda, K. and Yasuda, K. 2003. Morphological changes and increased sucrase and isomaltase activity in small intestines of insulin-deficient and type 2 diabetic rats. *Endocr. J.* 50(3), pp.271-279.
- Adisakwattana, S., Moonsan, P. and Yibchok-Anun, S. 2008. Insulin-releasing properties of a series of cinnamic acid derivatives in vitro and in vivo. *J. Agric. Food Chem.* 56(17), pp.7838-7844.
- Adisakwattana, S., Yibchok-Anun, S., Charoenlertkul, P. and Wongsasiripat, N. 2011. Cyanidin-3-rutinoside alleviates postprandial hyperglycemia and its synergism with acarbose by inhibition of intestinal alpha-glucosidase. *J. Clin. Biochem. Nutr.* 49(1), pp.36-41.
- Aertgeerts, K., Ye, S., Shi, L., Prasad, S.G., Witmer, D., Chi, E., Sang, B.C., Wijnands, R.A., Webb, D.R. and Swanson, R.V. 2004. N-linked glycosylation of dipeptidyl peptidase IV (CD26): Effects on enzyme activity, homodimer formation, and adenosine deaminase binding. *Protein Sci.* 13(1), pp.145-154.
- Ait-Omar, A., Monteiro-Sepulveda, M., Poitou, C., Le Gall, M., Cotillard, A., Gilet, J., Garbin, K., Houllier, A., Château, D. and Lacombe, A. 2011. GLUT2 accumulation in enterocyte apical and intracellular membranes. *Diabetes.* 60(10), pp.2598-2607.
- Alfalah, M., Keiser, M., Leeb, T., Zimmer, K.P. and Naim, H.Y. 2009. Compound heterozygous mutations affect protein folding and function in patients with congenital sucrase-isomaltase deficiency. *Gastroenterol.* 136(3), pp.883-892.
- Alpers, D.H. 2010. Nutrient sensing in the gastrointestinal tract. *Curr. Opin. Gastroenterol.* 26(2), pp.134-139.
- Alvarado, F. 1967. Hypothesis for the interaction of phlorizin and phloretin with membrane carriers for sugars. *Biochimica et Biophysica Acta (BBA)-Biomembranes.* 135(3), pp.483-495.
- Alzaid, F., Cheung, H.-M., Preedy, V.R. and Sharp, P.A. 2013. Regulation of glucose transporter expression in human intestinal Caco-2 cells following exposure to an anthocyanin-rich berry extract. *PLoS ONE.* 8(11), pe78932.
- Amasheh, M., Schlichter, S., Amasheh, S., Mankertz, J., Zeitz, M., Fromm, M. and Schulzke, J.D. 2008. Quercetin enhances epithelial barrier function and increases claudin-4 expression in Caco-2 cells. *J. Nutr.* 138(6), pp.1067-1073.
- Anderson, R.A., Broadhurst, C.L., Polansky, M.M., Schmidt, W.F., Khan, A., Flanagan, V.P., Schoene, N.W. and Graves, D.J. 2004. Isolation and characterization of polyphenol type-A polymers from cinnamon with insulin-like biological activity. *J. Agric. Food Chem.* 52(1), pp.65-70.
- Arthur, S., Coon, S., Kekuda, R. and Sundaram, U. 2014. Regulation of sodium glucose co-transporter SGLT1 through altered glycosylation in the intestinal epithelial cells. *Biochim. Biophys. Acta.* 1838(5), pp.1208-1214.
- Asanuma-Date, K., Hirano, Y., Le, N., Sano, K., Kawasaki, N., Hashii, N., Hiruta, Y., Nakayama, K.-i., Umemura, M., Ishikawa, K., Sakagami, H. and Ogawa, H. 2012. Functional regulation of sugar assimilation by N-Glycan-specific interaction of pancreatic  $\alpha$ -amylase with glycoproteins of duodenal brush border membrane. *J. Biol. Chem.* 287(27), pp.23104-23118.

- Aston, L.M. 2006. Glycaemic index and metabolic disease risk. *Proc. Nutr. Soc.* 65(01), pp.125-134.
- Atkinson, F.S., Foster-Powell, K. and Brand-Miller, J.C. 2008. International tables of glycemic index and glycemic load values: 2008. *Diabetes care.* 31(12), pp.2281-2283.
- Augustin, L.S.A., Kendall, C.W.C., Jenkins, D.J.A., Willett, W.C., Astrup, A., Barclay, A.W., Björck, I., Brand-Miller, J.C., Brighenti, F., Buyken, A.E., Ceriello, A., La Vecchia, C., Livesey, G., Liu, S., Riccardi, G., Rizkalla, S.W., Sievenpiper, J.L., Trichopoulou, A., Wolever, T.M.S., Baer-Sinnott, S. and Poli, A. 2015. Glycemic index, glycemic load and glycemic response: An International Scientific Consensus Summit from the International Carbohydrate Quality Consortium (ICQC). *Nutr. Metab. Cardiovasc. Dis.* 25(9), pp.795-815.
- Auricchio, S., Dahlqvist, A. and Semenza, G. 1963. Solubilization of the human intestinal disaccharidases. *Biochim. Biophys. Acta.* 73(4), pp.582-587.
- Aydin, E. 2015. *Effects of Natural Products on Sugar Metabolism and Digestive Enzymes.* PhD Thesis thesis, University of Leeds.
- Babu, P.V.A., Liu, D. and Gilbert, E.R. 2013. Recent advances in understanding the anti-diabetic actions of dietary flavonoids. *J. Nutr. Biochem.* 24(11), pp.1777-1789.
- Bahadoran, Z., Mirmiran, P. and Azizi, F. 2013. Dietary polyphenols as potential nutraceuticals in management of diabetes: a review. *J. Diabetes Metab. Disord.* 12(1), p43.
- Balfour, J.A. and McTavish, D. 1993. Acarbose. *Drugs.* 46(6), pp.1025-1054.
- Barclay, A.W., Petocz, P., McMillan-Price, J., Flood, V.M., Prvan, T., Mitchell, P. and Brand-Miller, J.C. 2008. Glycemic index, glycemic load, and chronic disease risk—a meta-analysis of observational studies. *Am. J. Clin. Nutr.* 87(3), pp.627-637.
- Basu, S., Yoffe, P., Hills, N. and Lustig, R.H. 2013. The relationship of sugar to population-level diabetes prevalence: an econometric analysis of repeated cross-sectional data. *PLoS ONE.* 8(2), pe57873.
- Bhupathiraju, S.N., Tobias, D.K., Malik, V.S., Pan, A., Hruby, A., Manson, J.E., Willett, W.C. and Hu, F.B. 2014. Glycemic index, glycemic load, and risk of type 2 diabetes: results from 3 large US cohorts and an updated meta-analysis. *Am. J. Clin. Nutr.*, pajcn. 079533.
- Bisswanger, H. 2014. Enzyme assays. *Perspect. Sci.* 1(1), pp.41-55.
- Björck, I. and Elmståhl, H.L. 2003. The glycaemic index: importance of dietary fibre and other food properties. *Proc. Nutr. Soc.* 62(01), pp.201-206.
- Boath, A.S., Stewart, D. and McDougall, G.J. 2012. Berry components inhibit  $\alpha$ -glucosidase in vitro: Synergies between acarbose and polyphenols from black currant and rowanberry. *Food Chem.* 135(3), pp.929-936.
- Bock, M., Thorstensen, E.B., Derraik, J.G., Henderson, H.V., Hofman, P.L. and Cutfield, W.S. 2013. Human absorption and metabolism of oleuropein and hydroxytyrosol ingested as olive (*Olea europaea* L.) leaf extract. *Mol. Nutr. Food Res.* 57(11), pp.2079-2085.
- Boudreau, F., Rings, E.H.H.M., van Wering, H.M., Kim, R.K., Swain, G.P., Krasinski, S.D., Moffett, J., Grand, R.J., Suh, E.R. and Traber, P.G. 2002. Hepatocyte nuclear factor-1 $\alpha$ , GATA-4, and caudal related homeodomain protein Cdx2 interact functionally to modulate intestinal gene transcription: implication for the developmental regulation of the sucrase-isomaltase gene. *J. Biol. Chem.* 277(35), pp.31909-31917.

- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72(1), pp.248-254.
- Breuer, H.W. 2003. Review of acarbose therapeutic strategies in the long-term treatment and in the prevention of type 2 diabetes. *Int. J. Clin. Pharmacol. Ther.* 41(10), pp.421-440.
- Brouns, F., Bjorck, I., Frayn, K., Gibbs, A., Lang, V., Slama, G. and Wolever, T. 2005. Glycaemic index methodology. *Nut. Res. Rev.* 18(1), p145.
- Broyart, J.P., Hugot, J.P., Perret, C. and Porteu, A. 1990. Molecular cloning and characterization of a rat intestinal sucrase-isomaltase cDNA. Regulation of sucrase-isomaltase gene expression by sucrose feeding. *Biochim. Biophys. Acta.* 1087(1), pp.61-67.
- Brunner, J., Hauser, H., Braun, H., Wilson, K.J., Wacker, H., O'Neill, B. and Semenza, G. 1979. The mode of association of the enzyme complex sucrase. isomaltase with the intestinal brush border membrane. *J. Biol. Chem.* 254(6), pp.1821-1828.
- Campbell, M.P., Royle, L., Radcliffe, C.M., Dwek, R.A. and Rudd, P.M. 2008. GlycoBase and autoGU: tools for HPLC-based glycan analysis. *Bioinformatics.* 24(9), pp.1214-1216.
- Caro, I., Boulenc, X., Rousset, M., Meunier, V., Bourrié, M., Julian, B., Joyeux, H., Roques, C., Berger, Y. and Zweibaum, A. 1995. Characterisation of a newly isolated Caco-2 clone (TC-7), as a model of transport processes and biotransformation of drugs. *Int. J. Pharm.* 116(2), pp.147-158.
- Chantret, I., Lacasa, M., Chevalier, G., Ruf, J., Islam, I., Mantei, N., Edwards, Y., Swallow, D. and Rousset, M. 1992. Sequence of the complete cDNA and the 5' structure of the human sucrase-isomaltase gene. Possible homology with a yeast glucoamylase. *Biochem. J.* 285(3), pp.915-923.
- Chantret, I., Lacasa, M., Chevalier, G., Swallow, D. and Rousset, M. 1993. Monensin and forskolin inhibit the transcription rate of sucrase-isomaltase but not the stability of its mRNA in Caco-2 cells. *FEBS Lett.* 328(1), pp.55-58.
- Chantret, I., Rodolosse, A., Barbat, A., Dussaulx, E., Brot-Laroche, E., Zweibaum, A. and Rousset, M. 1994. Differential expression of sucrase-isomaltase in clones isolated from early and late passages of the cell line Caco-2: evidence for glucose-dependent negative regulation. *J. Cell Sci.* 107(1), pp.213-225.
- Cheng, M.-W., Chegeni, M., Kim, K.-H., Zhang, G., Benmoussa, M., Quezada-Calvillo, R., Nichols, B.L. and Hamaker, B.R. 2014. Different sucrose-isomaltase response of Caco-2 cells to glucose and maltose suggests dietary maltose sensing. *J. Clin. Biochem. Nutr.* 54(1), p55.
- Chiasson, J.-L., Josse, R.G., Gomis, R., Hanefeld, M., Karasik, A., Laakso, M. and Group, S.-N.T.R. 2002. Acarbose for prevention of type 2 diabetes mellitus: the STOP-NIDDM randomised trial. *The Lancet.* 359(9323), pp.2072-2077.
- Conklin, K., Yamashiro, K. and Gray, G. 1975. Human intestinal sucrase-isomaltase. Identification of free sucrase and isomaltase and cleavage of the hybrid into active distinct subunits. *J. Biol. Chem.* 250(15), pp.5735-5741.
- Corona, G., Tzounis, X., Assunta Dessi, M., Deiana, M., Debnam, E.S., Visioli, F. and Spencer, J.P. 2006. The fate of olive oil polyphenols in the gastrointestinal tract: implications of gastric and colonic microflora-dependent biotransformation. *Free Radic. Res.* 40(6), pp.647-658.

- Cummings, J. and Stephen, A. 2007. Carbohydrate terminology and classification. *Eur. J. Clin. Nutr.* 61, pp.S5-S18.
- Cura, A.J. and Carruthers, A. 2012. Role of monosaccharide transport proteins in carbohydrate assimilation, distribution, metabolism, and homeostasis. *Compr. Physiol.*
- Daniels, G.W. 1998. Selective labeling of neurotransmitter transporters at the cell surface. *Methods Enzymol.* 296, pp.307-318.
- Danielsen, E.M. 1992. Folding of intestinal brush border enzymes. Evidence that high-mannose glycosylation is an essential early event. *Biochemistry.* 31(8), pp.2266-2272.
- de Bock, M., Derraik, J.G., Brennan, C.M., Biggs, J.B., Morgan, P.E., Hodgkinson, S.C., Hofman, P.L. and Cutfield, W.S. 2013. Olive (*Olea europaea* L.) leaf polyphenols improve insulin sensitivity in middle-aged overweight men: a randomized, placebo-controlled, crossover trial. *PLoS One.* 8(3), pe57622.
- de Bock, M., Derraik, J.G.B. and Cutfield, W.S. 2012. Polyphenols and glucose homeostasis in humans. *J. Acad. Nutr. Diet.* 112(6), pp.808-815.
- Douard. 2012. The role of fructose transporters in diseases linked to excessive fructose intake. *The Journal of Physiology.* 591, pp.401-414.
- Douard, V. and Ferraris, R.P. 2008. Regulation of the fructose transporter GLUT5 in health and disease. *Am. J. Physiol. Endocrinol. Metab.* 295(2), pp.E227-237.
- Dragan, S., Andrica, F., Serban, M.-C. and Timar, R. 2015. Polyphenols-rich natural products for treatment of diabetes. *Curr. Med. Chem.* 22(1), pp.14-22.
- Dyer, J., Wood, I.S., Palejwala, A., Ellis, A. and Shirazi-Beechey, S.P. 2002. Expression of monosaccharide transporters in intestine of diabetic humans. *Am. J. Physiol. Gastrointest. Liver Physiol.* 282(2), pp.G241-G248.
- EFSA. 2011. EFSA Panel on Dietetic Products - Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL cholesterol concentrations (ID 1639), maintenance of normal blood pressure (ID 3781), "anti-inflammatory properties" (ID 1882), "contributes to the upper respiratory tract health" (ID 3468), "can help to maintain a normal function of gastrointestinal tract" (3779), and "contributes to body defences against external agents" (ID 3467) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal.* 9(4).
- Ferraris. 2001. Dietary and developmental regulation of intestinal sugar transport. *Biochem. J.* 360(2), pp.265-276.
- Ferraris, R.P., Villenas, S.A. and Diamond, J. 1992. Regulation of brush-border enzyme activities and enterocyte migration rates in mouse small intestine. *Am J Physiol Gastrointest Liver Physiol.* 262(6), pp.G1047-G1059.
- Garcia-Villalba, R., Pacchiarotta, T., Carrasco-Pancorbo, A., Segura-Carretero, A., Fernandez-Gutierrez, A., Deelder, A.M. and Mayboroda, O.A. 2011. Gas chromatography-atmospheric pressure chemical ionization-time of flight mass spectrometry for profiling of phenolic compounds in extra virgin olive oil. *J. Chromatogr. A.* 1218(7), pp.959-971.
- Goran, M.I., Ulijaszek, S.J. and Ventura, E.E. 2013. High fructose corn syrup and diabetes prevalence: A global perspective. *Global Public Health.* 8(1), pp.55-64.
- Grassi, D., Lippi, C., Necozione, S., Desideri, G. and Ferri, C. 2005. Short-term administration of dark chocolate is followed by a significant increase in insulin



sensitivity and a decrease in blood pressure in healthy persons. *Am. J. Clin. Nutr.* 81(3), pp.611-614.

Grundy, S.M., Cleeman, J.I., Daniels, S.R., Donato, K.A., Eckel, R.H., Franklin, B.A., Gordon, D.J., Krauss, R.M., Savage, P.J., Smith, S.C., Spertus, J.A. and Costa, F. 2005. Diagnosis and management of the metabolic syndrome - An American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation.* 112(17), pp.2735-2752.

Gu, Y. and Lambert, J.D. 2013. Modulation of metabolic syndrome-related inflammation by cocoa. *Mol. Nutr. Food Res.* 57(6), pp.948-961.

Guariguata, L., Whiting, D., Hambleton, I., Beagley, J., Linnenkamp, U. and Shaw, J. 2014. Global estimates of diabetes prevalence for 2013 and projections for 2035. *Diabetes Res. Clin. Pract.* 103(2), pp.137-149.

Gupta, S., Mahmood, S., Khan, R.H. and Mahmood, A. 2010. Inhibition of brush border sucrase by polyphenols in mouse intestine. *Biosci. Rep.* 30(2), pp.111-117.

Hadrich, F., Bouallagui, Z., Junkyu, H., Isoda, H. and Sayadi, S. 2014. The  $\alpha$ -glucosidase and  $\alpha$ -amylase enzyme inhibitory of hydroxytyrosol and oleuropein. *J. Oleo Sci.* 64(8), pp.835-843.

Hanhineva, K., Torronen, R., Bondia-Pons, I., Pekkinen, J., Kolehmainen, M., Mykkanen, H. and Poutanen, K. 2010. Impact of dietary polyphenols on carbohydrate metabolism. *Int. J. Mol. Sci.* 11(4), pp.1365-1402.

Hansawasdi, C. and Kawabata, J. 2006.  $\alpha$ -Glucosidase inhibitory effect of mulberry (*Morus alba*) leaves on Caco-2. *Fitoterapia.* 77(7), pp.568-573.

Hauri, H.P., Sterchi, E.E., Bienz, D., Fransen, J.A. and Marxer, A. 1985. Expression and intracellular transport of microvillus membrane hydrolases in human intestinal epithelial cells. *J. Cell Biol.* 101(3), pp.838-851.

Hollander, D. 1999. Intestinal permeability, leaky gut, and intestinal disorders. *Curr. Gastroenterol. Rep.* 1(5), pp.410-416.

Honda, M. and Hara, Y. 1993. Inhibition of rat small intestinal sucrase and  $\alpha$ -glucosidase activities by tea polyphenols. *Biosci., Biotechnol., Biochem.* 57(1), pp.123-124.

Huggett, J.F., Foy, C.A., Benes, V., Emslie, K., Garson, J.A., Haynes, R., Hellemans, J., Kubista, M., Mueller, R.D., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T. and Bustin, S.A. 2013. The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. *Clin. Chem.* 59(6), pp.892-902.

Huxley, R., Lee, C., Barzi, F. and et al. 2009. Coffee, decaffeinated coffee, and tea consumption in relation to incident type 2 diabetes mellitus: A systematic review with meta-analysis. *Arch. Intern. Med.* 169(22), pp.2053-2063.

Ishikawa, A., Yamashita, H., Hiemori, M., Inagaki, E., Kimoto, M., Okamoto, M., Tsuji, H., Memon, A.N., Mohammadi, A. and Natori, Y. 2007. Characterization of inhibitors of postprandial hyperglycemia from the leaves of *Nerium indicum*. *J. Nutr. Sci. Vitaminol.* 53(2), pp.166-173.

Jacob, R. and Naim, H.Y. 2001. Apical membrane proteins are transported in distinct vesicular carriers. *Curr. Biol.* 11(18), pp.1444-1450.

Jo, S., Ka, E., Lee, H., Apostolidis, E., Jang, H. and Kwon, Y. 2009. Comparison of antioxidant potential and rat intestinal  $\alpha$ -glucosidases inhibitory activities of quercetin, rutin, and isoquercetin. *Int. J. App. Res. Nat. Prod.* 2(4), pp.52-60.

- Jockovic, N., Fischer, W., Brandsch, M., Brandt, W. and Drager, B. 2013. Inhibition of human intestinal alpha-glucosidases by calystegines. *J. Agric. Food Chem.* 61(23), pp.5550-5557.
- Johnston, K., Sharp, P., Clifford, M. and Morgan, L. 2005. Dietary polyphenols decrease glucose uptake by human intestinal Caco-2 cells. *FEBS Lett.* 579(7), pp.1653-1657.
- Jones, K., Eskandari, R., Naim, H.Y., Pinto, B.M. and Rose, D.R. 2012. Investigations of the structures and inhibitory properties of intestinal maltase glucoamylase and sucrase isomaltase. *J. Pediatr. Gastroenterol. Nutr.* 55, pp.S20-S24.
- Jones, K., Sim, L., Mohan, S., Kumarasamy, J., Liu, H., Avery, S., Naim, H.Y., Quezada-Calvillo, R., Nichols, B.L., Mario Pinto, B. and Rose, D.R. 2011. Mapping the intestinal alpha-glucogenic enzyme specificities of starch digesting maltase-glucoamylase and sucrase-isomaltase. *Bioorg. Med. Chem.* 19(13), pp.3929-3934.
- Jung, U.J., Lee, M.K., Jeong, K.S. and Choi, M.S. 2004. The hypoglycemic effects of hesperidin and naringin are partly mediated by hepatic glucose-regulating enzymes in C57BL/KsJ-db/db mice. *J. Nutr.* 134(10), pp.2499-2503.
- Kamiyama, O., Sanae, F., Ikeda, K., Higashi, Y., Minami, Y., Asano, N., Adachi, I. and Kato, A. 2010. In vitro inhibition of  $\alpha$ -glucosidases and glycogen phosphorylase by catechin gallates in green tea. *Food Chem.* 122(4), pp.1061-1066.
- Keiser, M., Alfalah, M., Pröpsting, M.J., Castelletti, D. and Naim, H.Y. 2006. Altered folding, turnover, and polarized sorting act in concert to define a novel pathomechanism of congenital sucrase-isomaltase deficiency. *J. Biol. Chem.* 281(20), pp.14393-14399.
- Kellett, G.L., Brot-Laroche, E., Mace, O.J. and Leturque, A. 2008. Sugar absorption in the intestine: the role of GLUT2. *Annu. Rev. Nutr.* 28, pp.35-54.
- Kerimi, A., Pyner, A., Nyambe-Silavwe, H., Oladele, E., Gauer, J., Stevens, Y., Williamson, G. 2017. Inhibition of sucrose hydrolysis and sugar transport by oleuropein can delay post-prandial glucose spikes. . *Manuscript for submission*.
- Keys, A. 1980. *Seven countries. A multivariate analysis of death and coronary heart disease*. Cambridge: Harvard University Press.
- Kim, S.-H., Jo, S.-H., Kwon, Y.-I. and Hwang, J.-K. 2011. Effects of onion (*Allium cepa* L.) extract administration on intestinal  $\alpha$ -glucosidases activities and spikes in postprandial blood glucose levels in SD rats model. *Int. J. Mol. Sci.* 12(6), pp.3757-3769.
- Kim, Y., Keogh, J.B. and Clifton, P.M. 2016. Polyphenols and glycemic control. *Nutrients.* 8(1), p17.
- Kishi, K., Tanaka, T., Igawa, M., Takase, S. and Goda, T. 1999. Sucrase-isomaltase and hexose transporter gene expressions are coordinately enhanced by dietary fructose in rat jejunum. *J. Nutr.* 129(5), pp.953-956.
- Knowler, W.C., Barrett-Connor, E., Fowler, S.E., Hamman, R.F., Lachin, J.M., Walker, E.A. and Nathan, D.M. 2002. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N. Engl. J. Med.* 2002(346), pp.393-403.
- Kosińska, A. and Andlauer, W. 2012. Cocoa polyphenols are absorbed in Caco-2 cell model of intestinal epithelium. *Food Chem.* 135(3), pp.999-1005.
- Krog-Mikkelsen, I., Hels, O., Tetens, I., Holst, J.J., Andersen, J.R. and Bukhave, K. 2011. The effects of L-arabinose on intestinal sucrase activity: dose-response studies in vitro and in humans. *Am. J. Clin. Nutr.* 94(2), pp.472-478.

- Laville, M. and Nazare, J.A. 2009. Diabetes, insulin resistance and sugars. *Obes. Rev.* 10, pp.24-33.
- Le Gall, M., Tobin, V., Stolarczyk, E., Dalet, V., Leturque, A. and Brot-Laroche, E. 2007. Sugar sensing by enterocytes combines polarity, membrane bound detectors and sugar metabolism. *J. Cell. Physiol.* 213(3), pp.834-843.
- Lee, S.H., Yu, S.Y., Nakayama, J., Khoo, K.H., Stone, E.L., Fukuda, M.N., Marth, J.D. and Fukuda, M. 2010. Core2 O-glycan structure is essential for the cell surface expression of sucrase isomaltase and dipeptidyl peptidase-IV during intestinal cell differentiation. *J. Biol. Chem.* 285(48), pp.37683-37692.
- Léonard, R., Rendić, D., Rabouille, C., Wilson, I.B., Préat, T. and Altmann, F. 2006. The *Drosophila* fused lobes gene encodes an N-acetylglucosaminidase involved in N-glycan processing. *J. Biol. Chem.* 281(8), pp.4867-4875.
- Li, S. 2016. Effect of Oleuropein on Post-Prandial Blood Glucose Respons to White Bread: A Randomized, Controlled, Cross-over trial. Master's thesis, University of Leeds.
- Lim, J.S., Mietus-Snyder, M., Valente, A., Schwarz, J.-M. and Lustig, R.H. 2010. The role of fructose in the pathogenesis of NAFLD and the metabolic syndrome. *Nat. Rev. Gastroenterol. Hepatol.* 7(5), pp.251-264.
- Liu, L., Yu, Y.-L., Yang, J.-S., Li, Y., Liu, Y.-W., Liang, Y., Liu, X.-D., Xie, L. and Wang, G.-J. 2010. Berberine suppresses intestinal disaccharidases with beneficial metabolic effects in diabetic states, evidences from in vivo and in vitro study. *Naunyn-Schmiedeberg Arch. Pharmacol.* 381(4), pp.371-381.
- Liu, L., Yu, Y.L., Liu, C., Wang, X.T., Liu, X.D. and Xie, L. 2011. Insulin deficiency induces abnormal increase in intestinal disaccharidase activities and expression under diabetic states, evidences from in vivo and in vitro study. *Biochem. Pharmacol.* 82(12), pp.1963-1970.
- Livesey, G., Taylor, R., Hulshof, T. and Howlett, J. 2008. Glycemic response and health--a systematic review and meta-analysis: relations between dietary glycemic properties and health outcomes. *Am. J. Clin. Nutr.* 87(1), pp.258S-268S.
- Macdonald, I.A. 2014. Dietary strategies for the management of cardiovascular risk: Role of dietary carbohydrates. *Proc. Nutr. Soc.* 73(02), pp.167-171.
- Magkos, F., Yannakoulia, M., Chan, J.L. and Mantzoros, C.S. 2009. Management of the metabolic syndrome and type 2 diabetes through lifestyle modification. *Annu. Rev. Nutr.* 29, pp.223-256.
- Mahraoui, L., Rodolosse, A., Barbat, A., Dussaulx, E., Zweibaum, A., Rousset, M. and Brot-Laroche, E. 1994. Presence and differential expression of SGLT1, GLUT1, GLUT2, GLUT3 and GLUT5 hexose-transporter mRNAs in Caco-2 cell clones in relation to cell growth and glucose consumption. *Biochem. J.* 298(3), pp.629-633.
- Mammalian Gene Collection Program, T. 2002. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proceedings of the National Academy of Sciences of the United States of America.* 99(26), pp.16899-16903.
- Manolescu, A.R., Augustin, R., Moley, K. and Cheeseman, C. 2007. A highly conserved hydrophobic motif in the exofacial vestibule of fructose transporting SLC2A proteins acts as a critical determinant of their substrate selectivity. *Mol. Membr. Biol.* 24(5-6), pp.455-463.

- Manzano, S. and Williamson, G. 2010. Polyphenols and phenolic acids from strawberry and apple decrease glucose uptake and transport by human intestinal Caco-2 cells. *Mol. Nutr. Food Res.* 54(12), pp.1773-1780.
- Margolskee, R.F., Dyer, J., Kokrashvili, Z., Salmon, K.S., Ilegems, E., Daly, K., Maillet, E.L., Ninomiya, Y., Mosinger, B. and Shirazi-Beechey, S.P. 2007. T1R3 and gustducin in gut sense sugars to regulate expression of Na<sup>+</sup>-glucose cotransporter 1. *Proc. Natl. Acad. Sci.* 104(38), pp.15075-15080.
- Mariño, K., Bones, J., Kattla, J.J. and Rudd, P.M. 2010. A systematic approach to protein glycosylation analysis: a path through the maze. *Nat. Chem. Biol.* 6(10), pp.713-723.
- Markopoulos, C., Vertzoni, M., Agalias, A., Magiatis, P. and Reppas, C. 2009. Stability of oleuropein in the human proximal gut. *J. Pharm. Pharmacol.* 61(2), pp.143-149.
- Martín-Peláez, S., Covas, M.I., Fitó, M., Kušar, A. and Pravst, I. 2013. Health effects of olive oil polyphenols: recent advances and possibilities for the use of health claims. *Mol. Nutr. Food Res.* 57(5), pp.760-771.
- Martínez-González, M.Á., Corella, D., Salas-Salvadó, J., Ros, E., Covas, M.I., Fiol, M., Wärnberg, J., Arós, F., Ruíz-Gutiérrez, V., Lamuela-Raventós, R.M., Lapetra, J., Muñoz, M.Á., Martínez, J.A., Sáez, G., Serra-Majem, L., Pintó, X., Mitjavila, M.T., Tur, J.A., Portillo, M.d.P. and Estruch, R. 2010. Cohort Profile: Design and methods of the PREDIMED study. *Int. J. Epidemiol.* 41(2), pp.377-385.
- Martínez-González, M.A., Salas-Salvadó, J., Estruch, R., Corella, D., Fitó, M., Ros, E. and INVESTIGATORS, P. 2015. Benefits of the Mediterranean diet: insights from the PREDIMED study. *Prog. Cardiovasc. Dis.* 58(1), pp.50-60.
- Matsui, T., Tanaka, T., Tamura, S., Toshima, A., Tamaya, K., Miyata, Y., Tanaka, K. and Matsumoto, K. 2007.  $\alpha$ -Glucosidase inhibitory profile of catechins and theaflavins. *J. Agric. Food Chem.* 55(1), pp.99-105.
- Merlotti, C., Morabito, A. and Pontiroli, A. 2014. Prevention of type 2 diabetes; a systematic review and meta-analysis of different intervention strategies. *Diabetes Obes. Metab.* 16(8), pp.719-727.
- Miguel-Aliaga, I. 2012. Nerveless and gutsy: intestinal nutrient sensing from invertebrates to humans. *Semin. Cell Dev. Biol.* 23(6), pp.614-620.
- Morris, K.L. and Zemel, M.B. 1999. Glycemic index, cardiovascular disease, and obesity. *Nutr. Rev.* 57(9), pp.273-276.
- Moser, S., Lim, J., Chegeni, M., Wightman, J., Hamaker, B. and Ferruzzi, M. 2016. Concord and Niagara grape juice and their phenolics modify intestinal glucose transport in a coupled in vitro digestion/Caco-2 human intestinal model. *Nutrients.* 8(7), p414.
- Murotomi, K., Umeno, A., Yasunaga, M., Shichiri, M., Ishida, N., Koike, T., Matsuo, T., Abe, H., Yoshida, Y. and Nakajima, Y. 2015. Oleuropein-rich diet attenuates hyperglycemia and impaired glucose tolerance in type 2 diabetes model mouse. *J. Agric. Food Chem.* 63(30), pp.6715-6722.
- Naim, H.Y., Heine, M. and Zimmer, K.-P. 2012. Congenital sucrase-isomaltase deficiency: heterogeneity of inheritance, trafficking, and function of an intestinal enzyme complex. *J. Pediatr. Gastroenterol. Nutr.* 55, pp.S13-S20.
- Naim, H.Y., Joberty, G., Alfalah, M. and Jacob, R. 1999. Temporal Association of the N- and O-Linked Glycosylation Events and Their Implication in the Polarized Sorting of Intestinal Brush Border Sucrase-Isomaltase, Aminopeptidase N, and Dipeptidyl Peptidase IV. *J. Biol. Chem.* 274(25), pp.17961-17967.

- Najjar, S.M., Broyart, J.P., Hampp, L.T. and Gray, G.M. 2001. Sucrase- $\alpha$ -dextrinase in the spontaneously diabetic BioBreed Wistar rat: Altered intracellular carbohydrate processing. *J. Cell. Biochem.* 81(2), pp.252-261.
- Nijpels, G., Boorsma, W., Dekker, J., Kostense, P., Bouter, L. and Heine, R. 2008. A study of the effects of acarbose on glucose metabolism in patients predisposed to developing diabetes: the Dutch acarbose intervention study in persons with impaired glucose tolerance (DAISI). *Diabetes Metab. Res. Rev.* 24(8), pp.611-616.
- Nyambe-Silavwe, H. 2016. *Effects of carbohydrase inhibiting polyphenols on glycaemic response in vivo*. PhD thesis, University of Leeds.
- Nyambe-Silavwe, H., Villa-Rodriguez, J.A., Ifie, I., Holmes, M., Aydin, E., Jensen, J.M. and Williamson, G. 2015. Inhibition of human  $\alpha$ -amylase by dietary polyphenols. *J. Funct. Foods.* 19, pp.723-732.
- Nyambe-Silavwe, H. and Williamson, G. 2016. Polyphenol-and fibre-rich dried fruits with green tea attenuate starch-derived postprandial blood glucose and insulin: a randomised, controlled, single-blind, cross-over intervention. *Br. J. Nutr.* 116(03), pp.443-450.
- Ohtsubo, K. and Marth, J.D. 2006. Glycosylation in cellular mechanisms of health and disease. *Cell.* 126(5), pp.855-867.
- Olsen, W.A. and Korsmo, H. 1977. The intestinal brush border membrane in diabetes: studies of sucrase-isomaltase metabolism in rats with streptozotocin diabetes. *J. Clin. Invest.* 60(1), pp.181-188.
- PHE. 2014. *Public Health England and Food Standards Agency - National Diet and Nutrition Survey Rolling Programme (NDNS RP). Results from years 1–4 (combined) for Scotland (2008/9-2011/12)*. [Online]. [Accessed 06 January 2017]. Available from: <https://www.gov.uk/government/statistics/>
- Pradet-Balade, B., Boulmé, F., Beug, H., Müllner, E.W. and Garcia-Sanz, J.A. 2001. Translation control: bridging the gap between genomics and proteomics? *Trends Biochem. Sci.* 26(4), pp.225-229.
- Preedy, V.R. and Watson, R.R. 2010. *Olives and olive oil in health and disease prevention*. Cambridge: Academic Press.
- Preuss, H.G., Echard, B., Bagchi, D. and Stohs, S. 2007a. Inhibition by natural dietary substances of gastrointestinal absorption of starch and sucrose in rats 2. Subchronic studies. *Int. J. Med. Sci.* 4(4), pp.209-215.
- Preuss, H.G., Echard, B., Bagchi, D. and Stohs, S. 2007b. Inhibition by natural dietary substances of gastrointestinal absorption of starch and sucrose in rats and pigs: 1. Acute studies. *Int. J. Med. Sci.* 4(4), pp.196-202.
- Priscilla, D.H., Roy, D., Suresh, A., Kumar, V. and Thirumurugan, K. 2014. Naringenin inhibits  $\alpha$ -glucosidase activity: A promising strategy for the regulation of postprandial hyperglycemia in high fat diet fed streptozotocin induced diabetic rats. *Chem.-Biol. Interact.* 210, pp.77-85.
- Quan, R. and Gray, G.M. 1993. Sucrase-alpha-dextrinase in the rat. Postinsertional conversion to inactive molecular species by a carbohydrate-free diet. *J. Clin. Invest.* 91(6), pp.2785-2790.
- Raj, A., Peskin, C.S., Tranchina, D., Vargas, D.Y. and Tyagi, S. 2006. Stochastic mRNA synthesis in mammalian cells. *PLoS Biol.* 4(10), pe309.

Röder, P.V., Geillinger, K.E., Zietek, T.S., Thorens, B., Koepsell, H. and Daniel, H. 2014. The Role of SGLT1 and GLUT2 in Intestinal Glucose Transport and Sensing. *PLoS ONE*. 9(2), pe89977.

Rodríguez, D., Ramsay, A.J., Quesada, V., Garabaya, C., Campo, E., Freije, J.M. and López-Otín, C. 2013. Functional analysis of sucrase–isomaltase mutations from chronic lymphocytic leukemia patients. *Hum. Mol. Genet.* 22(11), pp.2273-2282.

Rousset, M., Chantret, I., Darmoul, D., Trugnan, G., Sapin, C., Green, F., Swallow, D. and Zweibaum, A. 1989. Reversible forskolin-induced impairment of sucrase-isomaltase mRNA levels, biosynthesis, and transport to the brush border membrane in Caco-2 cells. *J. Cell. Physiol.* 141(3), pp.627-635.

Rubió, L., Macià, A., Castell-Auví, A., Pinent, M., Blay, M.T., Ardévol, A., Romero, M.-P. and Motilva, M.-J. 2014. Effect of the co-occurring olive oil and thyme extracts on the phenolic bioaccessibility and bioavailability assessed by in vitro digestion and cell models. *Food Chem.* 149, pp.277-284.

Ryan, D., Prenzler, P.D., Lavee, S., Antolovich, M. and Robards, K. 2003. Quantitative changes in phenolic content during physiological development of the olive (*Olea europaea*) cultivar Hardy's Mammoth. *J. Agric. Food Chem.* 51(9), pp.2532-2538.

SACN. 2015. *Scientific Advisory Committee on Nutrition - Carbohydrates and Health Report*. [Online]. [Accessed 20 March 2017]. Available from: <https://www.gov.uk/government/publications/sacn-carbohydrates-and-health-report>

Sambuy, Y., Angelis, I., Ranaldi, G., Scarino, M.L., Stamatii, A. and Zucco, F. 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol. Toxicol.* 21(1), pp.1-26.

Samulitis-dos Santos, B.K., Goda, T. and Koldovsky, O. 1992. Dietary-induced increases of disaccharidase activities in rat jejunum. *Br. J. Nutr.* 67(2), pp.267-278.

Scalbert, A., Manach, C., Morand, C., Rémésy, C. and Jiménez, L. 2005. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* 45(4), pp.287-306.

Scalbert, A. and Williamson, G. 2000. Dietary Intake and Bioavailability of Polyphenols. *J. Nutr.* 130(8), pp.2073S-2085S.

Schmidt, L.A. 2014. New Unsweetened Truths About Sugar. *JAMA Int. Med.*

Schmitz, H., Barmeyer, C., Fromm, M., Runkel, N., Foss, H.-D., Bentzel, C.J., Riecken, E.-O. and Schulzke, J.-D. 1999. Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. *Gastroenterol.* 116(2), pp.301-309.

Semenza, G. 1986. Anchoring and biosynthesis of stalked brush border membrane proteins: glycosidases and peptidases of enterocytes and renal tubuli. *Annu. Rev. Cell Biol.* 2(1), pp.255-307.

Shelley, M. 2017. A Crossover trial to determine the effect of Bonolive and the polyphenol oleuropein on reducing or modulating the GI of foods and hence reduce blood sugar "spikes". Bachelor of Science thesis, University of Leeds.

Simsek, M., Quezada-Calvillo, R., Ferruzzi, M.G., Nichols, B.L. and Hamaker, B.R. 2015. Dietary Phenolic Compounds Selectively Inhibit the Individual Subunits of Maltase-Glucoamylase and Sucrase-Isomaltase with the Potential of Modulating Glucose Release. *J. Agric. Food Chem.* 63(15), pp.3873-3879.

- Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150(1), pp.76-85.
- Sofi, F., Cesari, F., Abbate, R., Gensini, G.F. and Casini, A. 2008. Adherence to Mediterranean diet and health status: meta-analysis. *BMJ.* 337, pa1344.
- Srinivas, K., King, J.W., Howard, L.R. and Monrad, J.K. 2010. Solubility and solution thermodynamic properties of quercetin and quercetin dihydrate in subcritical water. *J. Food Eng.* 100(2), pp.208-218.
- Stanhope, K.L. 2016. Sugar consumption, metabolic disease and obesity: The state of the controversy. *Critical Reviews in Clinical Laboratory Sciences.* 53(1), pp.52-67.
- Sun, S. and Empie, M. 2012. Fructose metabolism in humans - what isotopic tracer studies tell us. *Nutrition & Metabolism.* 9(1), p89.
- Tadera, K., Minami, Y., Takamatsu, K. and Matsuoka, T. 2006. Inhibition of  $\alpha$ -Glucosidase and  $\alpha$ -Amylase by Flavonoids. *J. Nutr. Sci. Vitaminol.* 52(2), pp.149-153.
- Tamura, T., Ozawa, M., Kobayashi, S., Watanabe, H., Arai, S. and Mura, K. 2015. Inhibitory effect of oligomeric polyphenols from peanut-skin on sugar digestion enzymes and glucose transport. *Food Sci. Technol. Res.* 21(1), pp.111-115.
- Te Morenga, L., Mallard, S. and Mann, J. 2013. Dietary sugars and body weight: systematic review and meta-analyses of randomised controlled trials and cohort studies. *BMJ.* 346, pe7492.
- Teng, Z., Yuan, C., Zhang, F., Huan, M., Cao, W., Li, K., Yang, J., Cao, D., Zhou, S. and Mei, Q. 2012. Intestinal absorption and first-pass metabolism of polyphenol compounds in rat and their transport dynamics in Caco-2 cells. *PloS one.* 7(1), pe29647.
- Thomas, T. and Pfeiffer, A.F. 2012. Foods for the prevention of diabetes: how do they work? *Diabetes Metab. Res. Rev.* 28(1), pp.25-49.
- Thresher, J.S., Podolin, D.A., Wei, Y., Mazzeo, R.S. and Pagliassotti, M.J. 2000. Comparison of the effects of sucrose and fructose on insulin action and glucose tolerance. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 279(4), pp.R1334-R1340.
- Trugnan, G., Rousset, M. and Zweibaum, A. 1986. Castanospermine: a potent inhibitor of sucrase from the human enterocyte-like cell line Caco-2. *FEBS Lett.* 195(1-2), pp.28-32.
- Twedt, R., Bradley, M., Deiseroth, D., Althouse, A. and Facco, F. 2015. Sleep duration and blood glucose control in women with gestational diabetes mellitus. *Obstet. Gynecol.* 126(2), pp.326-331.
- Van Beers, E.H., Büller, H.A., Grand, R.J., Einerhand, A.W.C. and Dekker, J. 1995. Intestinal brush border glycohydrolases: structure, function, and development. *Crit. Rev. Biochem. Mol. Biol.* 30(3), pp.197-262.
- Van de Laar, F.A., Lucassen, P.L., Akkermans, R.P., Van de Lisdonk, E.H., Rutten, G.E. and Van Weel, C. 2005. Alpha-glucosidase inhibitors for type 2 diabetes mellitus. *The Cochrane Libr.*
- Vissers, M.N., Zock, P.L., Roodenburg, A.J., Leenen, R. and Katan, M.B. 2002. Olive oil phenols are absorbed in humans. *J. Nutr.* 132(3), pp.409-417.
- WHO. 2012. *World Health Organisation- World Health Statistics* [Online]. [Accessed 24 January 2017]. Available from: [http://www.who.int/gho/publications/world\\_health\\_statistics/2012/en/](http://www.who.int/gho/publications/world_health_statistics/2012/en/)

- Willett, W.C., Sacks, F., Trichopoulou, A., Drescher, G., Ferro-Luzzi, A., Helsing, E. and Trichopoulos, D. 1995. Mediterranean diet pyramid: a cultural model for healthy eating. *Am. J. Clin. Nutr.* 61(6), pp.1402S-1406S.
- Williamson, G. 2013. Possible effects of dietary polyphenols on sugar absorption and digestion. *Mol. Nutr. Food Res.* 57(1), pp.48-57.
- Wilson, N., Simpson, R. and Cooper-Liddell, C. 2009. Introductory glycosylation analysis using SDS-PAGE and peptide mass fingerprinting. *Glycomics: Methods and Protocols.* pp.205-212.
- Wolever, T.M., Jenkins, D.J., Jenkins, A.L. and Josse, R.G. 1991. The glycemic index: methodology and clinical implications. *Am. J. Clin. Nutr.* 54(5), pp.846-854.
- Wolfram, S., Raederstorff, D., Preller, M., Wang, Y., Teixeira, S.R., Riegger, C. and Weber, P. 2006. Epigallocatechin gallate supplementation alleviates diabetes in rodents. *J. Nutr.* 136(10), pp.2512-2518.
- Young, R.L., Sutherland, K., Pezos, N., Brierley, S.M., Horowitz, M., Rayner, C.K. and Blackshaw, L.A. 2009. Expression of taste molecules in the upper gastrointestinal tract in humans with and without type 2 diabetes. *Gut.* 58(3), pp.337-346.
- Zeevi, D., Korem, T., Zmora, N., Israeli, D., Rothschild, D., Weinberger, A., Ben-Yacov, O., Lador, D., Avnit-Sagi, T. and Lotan-Pompan, M. 2015. Personalized nutrition by prediction of glycemic responses. *Cell.* 163(5), pp.1079-1094.
- Zhao, Y., Chen, M.X., Kongstad, K.T., Jäger, A.K. and Staerk, D. 2017. Potential of *Polygonum cuspidatum* Root as an Antidiabetic Food: Dual High-Resolution  $\alpha$ -Glucosidase and PTP1B Inhibition Profiling Combined with HPLC-HRMS and NMR for Identification of Antidiabetic Constituents. *J. Agric. Food Chem.*
- Zimmet, P., Alberti, K. and Shaw, J. 2001. Global and societal implications of the diabetes epidemic. *Nature.* 414(6865), pp.782-787.
- Zor, T. and Selinger, Z. 1996. Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Anal. Biochem.* 236(2), pp.302-308.



## Appendix 1

Results from cDNA sequencing of SI from Caco-2/TC7 cells and DV Biologics human intestinal RNA sample. The bold capitalised letter represents a difference as compared to the NCBI reference sequence NM\_001041.3.

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