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**Cocoa flavanol interactions with intestinal sugar
metabolism**

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The candidate confirms that the work submitted is her 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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Further details of the work from jointly authored publications/submitted manuscripts and the contributions of the candidate and the other authors to the work are included below.

Chapter 1

Ellam, S. and Williamson, G. (2013) 'Cocoa and Human Health', *Annual Review of Nutrition*, 33, in press.

Details of authorship contributions:

Ellam: All discussion and illustrations presented here is the authors own work.

Williamson: Additional discussion, guidance and editing of the final manuscript submitted for publication.

Chapter 2, 3 and 4

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Farrell: Experimental design and method development, except Caco-2 glucose transport protocol, data analysis, cell culture and practical work, data interpretation and primary authorship.

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Abstract

The interaction between cocoa polyphenols and sugars was investigated in the Caco-2 cell model. Previous investigations have suggested a link between sugar content of a food/meal and epicatechin bioavailability. To investigate this hypothesis further, the effect of sucrose, glucose and fructose on epicatechin absorption across the Caco-2 monolayer was studied, and is presented here. Apical to basolateral transport of epicatechin was enhanced by co-incubation with sucrose, but not glucose or fructose. It is proposed that sucrose-induced cell signalling stimulated activity of the sodium-dependent glucose transporter SGLT1, which lead to widening of tight junction pore size. Consequently paracellular permeability of epicatechin was enhanced. The presence of glucose or fructose, but not sucrose, reduced the total concentration of methylated epicatechin produced by Caco-2 cells. Decreased formation of methylated epicatechin is hypothesised to be a consequence of catechol-O-methyl transferase inhibition (COMT). COMT requires a magnesium cation cofactor, which is also required by some glycolytic enzymes. It is suggested that competition for the magnesium (II) cofactor leads to reduced epicatechin methylation.

Flavanol-rich dark chocolate extract also reduced total methylation of epicatechin. It is proposed that flavanols with a degree of polymerisation greater than monomer compete with epicatechin for methylation.

Dark chocolate is reported to have a low glycaemic index, which was hypothesised to be attributable to flavanol inhibition of sucrose hydrolysis and/or glucose uptake. In Caco-2 cells the rate of sucrose hydrolysis and the rate of glucose transport were attenuated by a flavanol-rich dark chocolate extract. Reduced rate of sucrose hydrolysis was partly attributed to the

epicatechin content of the dark chocolate extract, with a synergistic contribution from other flavanol components. Similarly, synergy between the flavanol components of dark chocolate extract, or those components with a degree of polymerisation greater than dimer, is postulated to be responsible for reducing the rate of glucose absorption. Moderate concentrations of flavanol monomers and B2 dimer enhanced the rate of sucrose hydrolysis; proposed to be a consequence of flavanol-enzyme binding, at a site other than the active site, resulting in a conformational change that lead to more exposed catalytic residues at the active site. Caffeine and theobromine increased the rate of cellular glucose uptake. This is suggested to be a consequence of stimulated SGLT1 trafficking to the apical membrane induced by elevated cyclic adenosine monophosphate concentration within the cells as a result of cyclic 3', 5'-nucleotide phosphodiesterase inhibition.

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Chapter 1 Literature review

1.1 Cocoa and chocolate

1.1.1 Production

Cocoa is a dry powdered product manufactured from seeds of the *Theobroma cacao* L. tree, and in its unsweetened form is the result of fat extraction from cocoa liquor. Cocoa liquor is the material used in chocolate confectionery manufacture along with other ingredients such as sugar, emulsifier, milk protein, etc. depending on the desired product.

The production of cocoa liquor begins with cleaning the seeds followed by a fermentation stage during which the chemical composition of the bean begins to alter and once dried the beans are ready to be roasted. Roasting may be performed before or after shelling (winnowing), and is a fundamental part of the process that affects the flavour characteristics and nutrient profile of the final product. The shelled bean, known as the nib, is then ground to a paste, which causes the fat to melt and form cocoa liquor. The liquor may then be treated with an alkali solution, 'Dutching', to increase the pH and improve palatability. Similar to the roasting process, the alkalising step affects the chemical composition of the cocoa liquor such that both stages in processing may be refined and strictly controlled to develop a product with a specific chemical profile.

1.1.2 Composition and biologically active components

The nutrient composition of cocoa and chocolate is distinct; dark chocolate containing 70% cocoa solids contributes more energy, lipid and sugars per 100 g edible weight than unsweetened cocoa powder. However comparing

percentage content is less relevant from a physiological perspective as the actual amount of dark chocolate and cocoa powder consumed is very different. A typical portion size of dark chocolate is approximately 28 g whereas a cocoa beverage may only contain around 5.5 g of unsweetened cocoa powder. Relative to the serving size, contributions to energy, lipid and sugar intake from unsweetened cocoa powder are much lower than from dark chocolate and quite insignificant compared with the United Kingdom (UK) reference nutrient intake (RNI) values. Similarly, micronutrient contribution from cocoa powder is slight with the exception of copper. A typical serving of unsweetened cocoa powder contributes approximately 16% of an adults daily UK RNI for copper (1.2 mg/d). A typical portion of 70% cocoa solids dark chocolate contributes quite considerably to total fat and non-milk extrinsic sugar (NMES) recommended intakes – 16% and 13% respectively. Additionally micronutrient contribution towards an adults intake are substantial – copper, 41%; iron and magnesium, each 23% (females); phosphorus 16% and zinc 13% (females). Deficiency of copper, magnesium, phosphorus and zinc is generally not a problem for the UK adult population however iron is a common deficiency in females. Although non-haem iron is less well absorbed than haem sources, when the body's iron reserves are low absorption becomes more efficient (Ministry of Agriculture 1995).

Of the non-essential nutrient components of dark chocolate and cocoa the most relevant, considering serving size, are caffeine, theobromine and flavonoids. Caffeine in cocoa powder is negligible; however a 28 g bar of dark chocolate may contain approximately 22 mg (U.S. Department of Agriculture 2011). For comparison a teaspoon of regular instant coffee contains around 31 mg and a cup of tea 47 mg (U.S. Department of

Agriculture 2011). Theobromine, a structurally similar precursor of caffeine in plant cells, is abundant in dark chocolate and cocoa. A typical serving of 70% dark chocolate would provide more than 200 mg whilst a typical serving of cocoa powder supplies >100 mg (U.S. Department of Agriculture 2011). Flavonoids present in dark chocolate and cocoa have attracted much attention in the past decade and are believed to stimulate biological activities that may be beneficial to human health. The main flavonoids identified in dark chocolate are flavanol monomers (-)-epicatechin and (+)-catechin, and polymers of these compounds up to decamer, and ferulic acid. Resveratrol and its 3-O-glucoside are also present but in >100-fold lesser amounts. The main flavanol present in dark chocolate is (-)-epicatechin, at approximately 70 mg 100 g⁻¹ fresh weight (Neveu *et al.* 2010). The (-)-epicatechin B2 dimer and C1 trimer constitute ~36 and 26 mg 100g⁻¹ fresh weight respectively. A 28 g typical portion of dark chocolate would supply almost 20 mg of (-)-epicatechin, 10 mg of the B2 dimer and 7 mg of C1 trimer.

1.1.2.1 Bioavailability of caffeine and theobromine

Caffeine is a 1,3,7-trimethylated xanthine that is metabolised *in vivo* to paraxanthine (1,7-dimethyl xanthine), theophylline (1,3 -dimethyl xanthine), theobromine (3,7-dimethyl xanthine) and 1,3,7-trimethyluric acid in pathways similar to those described for theobromine ([Figure 1.1](#)) (Thorn *et al.* 2012).

Caffeine is efficiently absorbed from the small intestine, such that the entire dose administered is absorbed with a reported peak in plasma concentration at approximately 30 min post-ingestion. The elimination half-life is on average 4.5 h but dependent on high individual variations (Blanchard and Sawers 1983).

There have been a limited number of bioavailability studies conducted in humans and other animals since the late 1950's; although the analytical methods have changed the metabolic outcomes remain relatively similar. In the first of such studies the authors identified unchanged theobromine and 3 metabolites in 48 h urine following consumption of the pure compound (Cornish and Christman 1957). The main metabolite was 7-methylxanthine which accounted for up to 30% of the dose, 3-methylxanthine accounted for approximately 20% and 7-methyluric acid accounted for around 4% of the dose. Later, 3,7-dimethyluric acid and a uracil compound was identified in urine following consumption of cocoa powder (Arnaud and Welsch 1979). These additional compounds have since been confirmed in human trials and the uracil compound identified as 6-amino-5-(N-methylformylamino)-1-methyluracil (Tarka *et al.* 1983). Metabolite recovery of around 80% of the ingested dose plus 18% unchanged theobromine have been reported (Tarka *et al.* 1983). Shively *et al.* (1985) also documented a small percentage of unchanged theobromine in the faeces of subjects which accounted for less than 1.5% of the ingested dose. Pharmacokinetic investigations have demonstrated an average half-life of theobromine in plasma of 7.2 h when consumed in the form of a gelatin capsule (Lelo *et al.* 1986) and 10 h when ingested from an aqueous solution (Shively *et al.* 1985) with total clearance from plasma measured at $1.2 \text{ mL min}^{-1} \text{ kg}^{-1}$ and $0.9 \text{ mL min}^{-1} \text{ kg}^{-1}$, respectively. The maximum plasma concentration of theobromine from chocolate is reached approximately 2 h post-ingestion suggesting that it is entirely absorbed in the small intestine (Shively *et al.* 1985). And although the mechanism for absorption through the intestinal epithelium has not been described, based on *in vitro* absorption of caffeine using colon

adenocarcinoma cells (Caco-2) (Smetanova *et al.* 2009), it can be surmised that theobromine similarly diffuses passively through the enterocytes into the hepatic circulation.

Most recent investigations have focused on the mechanisms by which theobromine is demethylated and oxidised to methyluric acid. Work conducted in the early 1990's suggested the hepatic cytochrome P450 pathway with specific involvement of the monooxygenase enzyme (EC 1.14.14.1) isoforms CYP1A2 and CYP2E1 (Gu *et al.* 1992, Tassaneeyakul *et al.* 1994, Rodopoulos *et al.* 1996). The main metabolite present in urine accounting for up to 40% of the ingested dose is 7-methyl xanthine which is a product of both enzyme isoforms. CYP2E1 appears to be the least specific as it also catalyses the formation of 3-methylxanthine and 3,7-dimethyluric acid (Gates and Miners 1999). Whilst the 3-methylxanthine isomer may undergo slight further metabolism to 3-methyluric acid (~1% of dose) (Rodopoulos *et al.* 1996), 7-methylxanthine is the main substrate, produced from theobromine catabolism, for xanthine oxidase (EC 1.17.3.2). Xanthine oxidase catalyses oxidation of carbon-8 to form 7-methyluric acid. The mechanism of uracil formation is yet to be established.

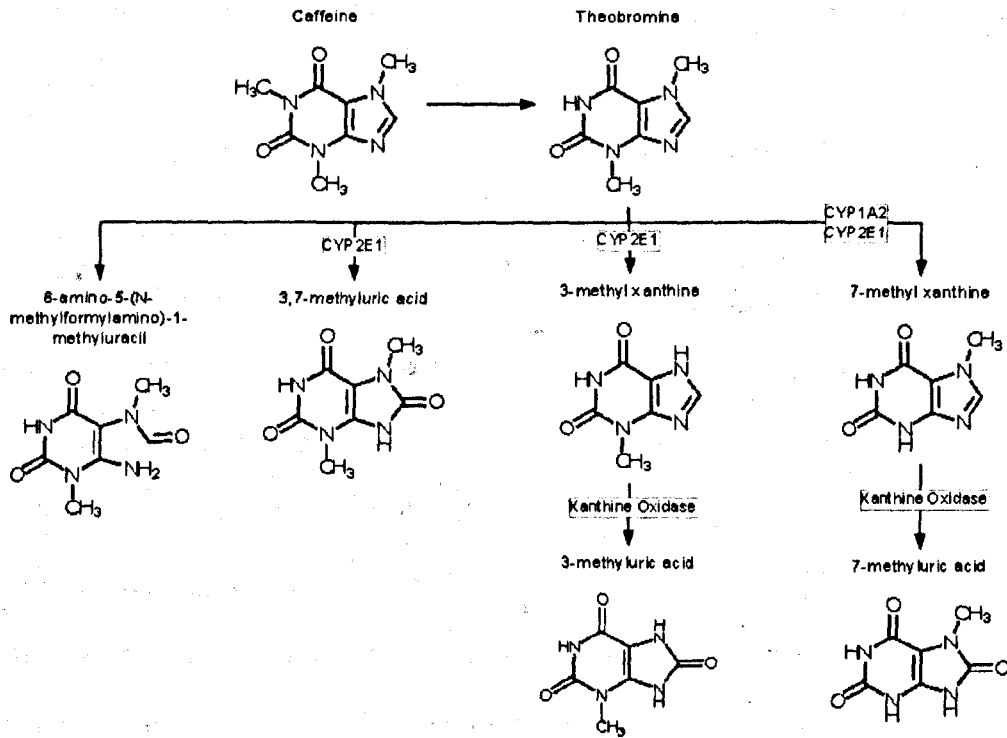


Figure 1.1 Metabolic pathway of theobromine

1.1.2.2 Bioavailability of flavanols

Flavanols are a type of phytochemical within the subgroup of flavonoids, other types within this subgroup include flavonols, flavones, flavanones, anthocyanidins and isoflavones. Phenolic acids and tannins are also a subclass of phytochemicals categorised as phenolics and are often incorporated in the general discussion of flavonoids. Human intervention studies investigating the bioavailability and effects of flavonoids have typically focused on phenolics in tea, coffee, red wine, fruit juice, onion and soy bean.

Epicatechin is the principal flavanol that has been investigated and to which many health benefits arising from consumption of tea and chocolate have been attributed.

A number of bioavailability studies in rats and humans have investigated the pharmacokinetics of epicatechin either administered as the individual compound or in a food matrix such as cocoa and tea. The specific mechanism of epicatechin uptake from the small intestine has not been elucidated although a transcellular route *in vivo* has been established through detection of phase II metabolites in plasma. *In vitro* studies demonstrate the absence of saturation kinetic properties, and similar apparent permeability coefficients (P_{app}) for apical to basolateral and basolateral to apical transport which together suggest a paracellular route (Deprez *et al.* 2001, Kosinska and Andlauer 2012).

Epicatechin absorption *in vivo* predominantly results in the production of phase II metabolites; very little, if any, free epicatechin is detected in humans. The peak plasma concentration of metabolites is reached 2-4 h post-ingestion indicating that absorption occurs mainly in the small intestine. There are some discrepancies in the pattern of metabolism between human and rat models. In both, epicatechin is predominantly conjugated with D-glucuronic acid in either the 3' or 4' position, a reaction catalysed by the membrane bound enzyme UDP-glucuronosyltransferase (EC 2.4.1.17) (Kuhnle *et al.* 2000, Baba *et al.* 2001b, Actis-Goretta *et al.* 2012). Methylation of epicatechin and epicatechin-O-D-glucuronides, catalysed by catechol-O-methyltransferase (EC 2.1.1.6), have also been reported in rat plasma but not human (Baba *et al.* 2001b). In humans methylated and non-methylated epicatechin is also conjugated with a sulphate group by sulfotransferase enzymes (EC 2.8.2.1) and some studies have reported the detection of sulfated glucuronide conjugates of epicatechin in both humans and rats (Baba *et al.* 2000b, Baba *et al.* 2001b).

Polymeric flavanols with a degree of polymerisation up to decamer have been identified in dark chocolate and cocoa. Monomeric units are joined by a carbon-carbon bond at various locations on the molecule. Currently commercial availability of these compounds is limited so many studies have focused attention on the B2 dimer and C1 trimer of (-)-epicatechin (Figure 1.2), or non-specific polymers extracted from cocoa or grape seed extract. *In vitro* studies have demonstrated that trimer to hexamer flavanols from cocoa degrade up to 100% to form monomers and dimers when incubated at pH 2 for up to 3.5 h at 37°C. The dimer degraded by ~20% to monomer. This was refuted by the results of a human study in which a cocoa beverage was consumed then gastric fluid extracted at ten minute intervals until the stomach was empty. The flavanol profile did not alter during the course of gastric fluid extraction indicating that there was no degradation of polymeric flavanols (Rios *et al.* 2002). The evidence for absorption of oligomeric flavanols is similarly contrasting. Perfusion of rat jejunum with dimeric flavanols demonstrated degradation of dimer to monomer with the detection of unmetabolised monomer at the serosal side. Some methylated dimer was also identified (Spencer *et al.* 2001). Procyanidin B2 administered to rats orally in water following a 12 h fast was detected in plasma along with epicatechin and 3'-O-methyl epicatechin; maximum concentration was achieved at 30-60 min. Up to 18 h, all three compounds were identified in urine along with methylated and non-methylated conjugates (Baba *et al.* 2002). Contradictory to this, plasma collected from rats 3 h following consumption of a meal prepared with grapeseed extract or the B3 dimer did not contain any B3 dimer and catechin and epicatechin were only detected in the plasma of rats fed the grapeseed extract. The concentration of

catechin in the plasma following consumption of grapeseed extract was comparable with the concentration following consumption of the same amount of pure catechin indicating that the dimer was not degraded to its monomer constituents (Donovan *et al.* 2002). A study published more recently reported similar results following human consumption of beverages containing either (-)-epicatechin only, monomer to decamer flavanols, or dimer to decamer. Monomeric flavanols were primarily detected in plasma following consumption of the (-)-epicatechin only beverage and the monomer to decamer-containing beverage. A small concentration of (-)-epicatechin was detected in plasma following ingestion of the dimer to decamer beverage but this was commensurate with low levels present in the beverage rather than a consequence of polymer degradation (Ottaviani *et al.* 2012). It appears that whilst rats may have the capacity to hydrolyse flavanol dimers to monomers and absorb some intact dimers, humans do not possess the same ability.

Flavanols that are not absorbed in the small intestine pass through to the colon where they are substrates for microflora metabolism. Metabolites detected in urine approximately 15 h after ingestion of tea included 5-(3',4',5'-trihydroxyphenyl)- γ -valerolactone attributed to epigallocatechin metabolism and 5-(3',4'-dihydroxyphenyl)- γ -valerolactone attributed to epicatechin metabolism (Li *et al.* 2000). *In vitro* assays of human colonic microflora with polymeric flavanols have revealed the formation of various phenolic acids including 2-(3-hydroxyphenyl)-acetic acid, 2-(4-hydroxyphenyl)-acetic acid, 2-(3,4-dihydroxyphenyl)-acetic acid, 3-phenylpropionic acid, 3-(3-hydroxyphenyl)-propionic acid and 3-(4-hydroxyphenyl)-propionic acid (Deprez *et al.* 2000, Appeldoorn *et al.* 2009a).

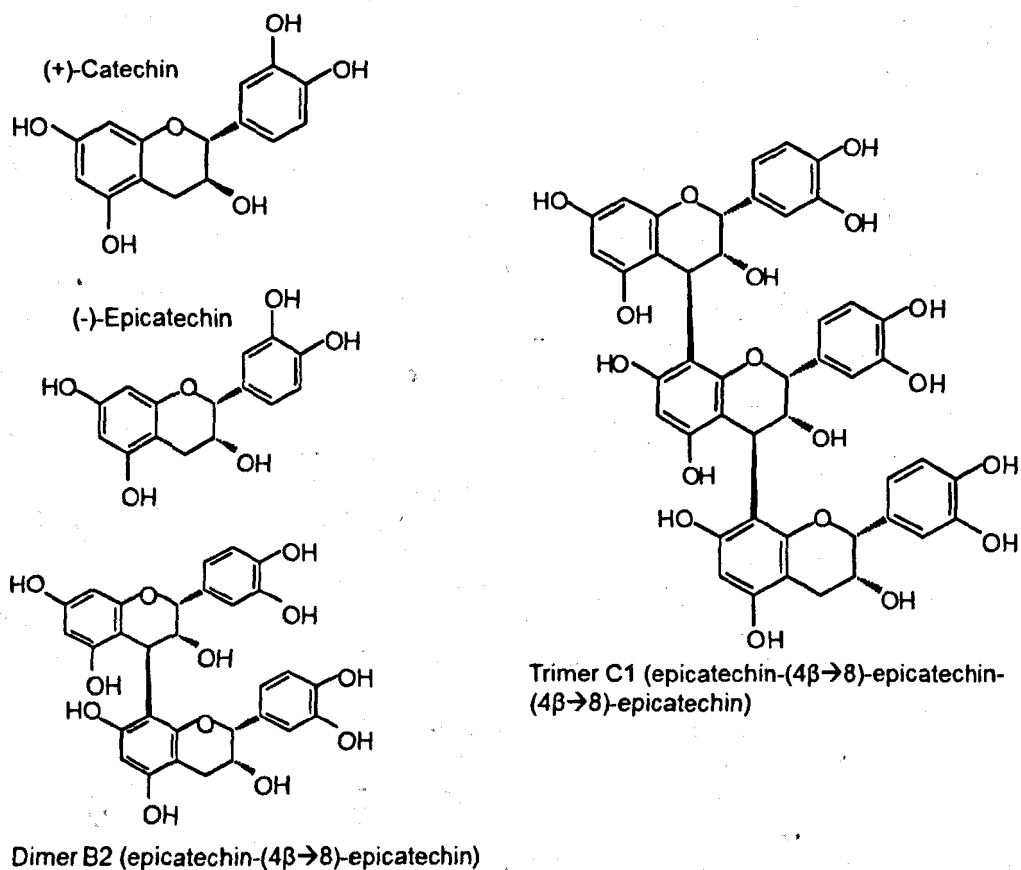


Figure 1.2 Cocoa flavanol structures

1.2 Cocoa and health

1.2.1 Consumption

A recent report by KPMG LLP (KPMG LLP 2012) demonstrated that whilst globally economies have been in recession, the chocolate market has remained stable, in fact its retail market value has risen marginally each year since 2007 and is predicted to continue its growth at a rate of 2% per year over the next 5 years. The majority of the global market is held by Western Europe (32%) followed by North America (20%), Asia (17%), Latin America (13%), Eastern Europe (12%), Middle East and Africa (4%) and Australasia

(2%), with large growth predicted in the smaller markets over the coming years.

The global population is increasing and with improvements in nutrition and health care so too is the age of populations. Concomitant to an ageing population is an increase in the number of non-communicable diseases which is the cause of more than 36 million deaths around the world each year, more than 80% of these deaths are due to cardiovascular diseases, cancers, diabetes and respiratory diseases (United Nations 2012). The economic impact of such chronic diseases has been estimated by the World Health Organisation (World Health Organization 2006) which predicts the greatest burden will be on the low and middle income countries. The estimated accumulated loss of national income between 2005 and 2015 is \$3.4 billion in the United Kingdom, whilst China, India and Russia have estimated national losses of \$53.3 billion, \$23 billion and \$29.8 billion, respectively. One factor associated with chronic non-communicable disease is an unhealthy diet; some governments have already introduced taxes on unhealthy foods whilst others are considering this possibility (KPMG LLP 2012).

The 2008/2009 UK National Dietary Nutrition Survey (NDNS) (Food Standards Agency 2010) reported that adults were consuming on average 21 g of chocolate confectionery per day and the 2010 Family Food survey (Department for Environment 2011) reported an average contribution to energy intake from confectionery (including chocolate) of 4% per person per day. In 2010 confectionery as a whole contributed 15% of the total non-milk extrinsic sugars (NMES) consumed per person per day from household purchases and 13% from foods purchased outside the home (Department

for Environment 2011); the current UK reference nutrient intake (RNI) recommends that NMES should not provide more than 11% of the daily energy intake (Food Standards Agency 2006). Considering potential taxes on unhealthy foods and the increasing global popularity of chocolate there appears to be scope for manufacturers to invest in research and development to manufacture products with functional health benefits.

1.2.2 Epidemiological evidence for beneficial effects

During the last 5 years there have been numerous reviews considering the role of cocoa in specific health issues in addition to a small number of epidemiological studies. The majority of published work during this time is human and non-human intervention trials particularly focusing on the effect of cocoa products or chemical components of cocoa individually. The majority of work relates to cardiovascular disease (CVD) with hypotheses typically that consumption of cocoa or intake of specific components of cocoa are able to lower risk or improve biomarkers of disease. Discussion in this section predominantly considers the literature pertaining to CVD but is inclusive of other areas of study with all human intervention trials summarised in table format ([Table 1.1](#)).

The risk of death from stroke, coronary heart disease (CHD) and CVD amongst participants of the Iowa Women's Health Study (IWHS) was lower in women who consumed chocolate relative to those who consumed no chocolate (Mink *et al.* 2007). Similarly data taken from the United States National Heart, Lung and Blood Institute Family Heart Study (Djousse *et al.* 2011) suggests that compared with no intake, participants with a greater frequency of chocolate intake have a reduced odds of CHD prevalence, the

lowest in consumers of 5 or more servings per week. However by contrast, a study of women participating in the Swedish Mammography Cohort (Mostofsky *et al.* 2010) demonstrated that the beneficial effect of chocolate consumption on risk of heart failure was reversed as intake exceeded 2 servings per week. It is relevant to note at this point that serving size is not stated in the aforementioned studies, although the authors of the Swedish investigation do suggest an average portion size somewhere between 19 and 30 g. An inverse association between chocolate consumption up to 7.5 g per day and risk of CVD among German adults participating in the European Prospective Investigation into Cancer was reported (Buijsse *et al.* 2010). In the latter study and that of the IWHS, the association appeared most compelling for stroke. Evaluation of data taken from The Stockholm Heart Epidemiology Program (Janszky *et al.* 2009) of individuals who have experienced a first acute myocardial infarction, show that the risk of cardiac mortality is lowest in those who consume a 50 g portion of chocolate at least twice per week and the risk of a non-fatal stroke was shown to be lowest in those consuming up to one portion per week.

From this small collection of epidemiological studies it can be surmised that the consumption of 50-100 g per week of chocolate may reduce the risk of cardiovascular disease, in particular stroke. What is not established in any of these investigations is the type of chocolate consumed. The type of chocolate, i.e. dark, milk or white, and the addition of other ingredients such as fruit or nuts, is an important factor in the overall analysis of evidence. Dark chocolate typically contains more non-fat cocoa solids (NFCS) than milk chocolate, and white chocolate contains no NFCS. As the percentage of NFCS content increases, the percentage content of sugar decreases

(U.S. Department of Agriculture 2011). Furthermore milk and white chocolate also contain milk powder that is not present in dark chocolate. One study investigating the effects of additional ingredients demonstrated that the lipid profile and inflammation biomarker concentration was improved following consumption of a cocoa product with the addition of hazelnuts, phytosterols and soluble fibre. The cocoa product without added ingredients had no significant effect (Sola *et al.* 2012).

Some epidemiological studies have investigated more specific interactions by focusing on particular components of cocoa. The primary focus has been flavonoids and in relation to cocoa, particularly the flavan-3-ol and proanthocyanidin sub-classes of polyphenols. An evaluation of incident hypertension, biomarkers of inflammation and endothelial dysfunction in relation to flavonoid intake of participants in the Nurses' Health Study (NHS) I and II, and the Health Professionals Follow-Up Study (HPFS) (Cassidy *et al.* 2011, Landberg *et al.* 2011) did not find any relationship with flavan-3-ol or proanthocyanidin intake. However, at the highest daily intake of catechin and epicatechin, the primary flavan-3-ol constituents of cocoa, there was a significantly lower risk of incident hypertension. In contrast, a significant reduction in the risk of CVD has been reported with increased intake of flavanols and proanthocyanidins (McCullough *et al.* 2012). The reduction was not linear and, similar to other epidemiological evidence, reversed in the highest intake groups.

1.2.3 Mechanisms of biological effects

1.2.3.1 Endothelial cell dysfunction

Endothelial cell dysfunction is an indicator of cardiovascular disease that has been widely studied *in vivo* and *in vitro*. Many of the human intervention trials described in this chapter involve quantification of products secreted by the endothelium such as nitric oxide, or expression of inflammatory response proteins such as interleukin-6 (IL-6). One study recently reported that nitric oxide (NO) production by platelets isolated from healthy subjects increased following 3 weeks daily consumption of dark chocolate (Nanetti *et al.* 2012). Systolic blood pressure (SBP) in spontaneously hypertensive rats (SHR) was reduced and considered to be a consequence of increased nitric oxide production following an acute dose of CocoanOX (a polyphenol-rich cocoa powder); the effect was prevented in the presence of an endothelial nitric oxide synthase (eNOS) inhibitor (M. Quinones *et al.* 2011). The same authors investigated the long-term effect of CocoanOX supplementation and observed attenuated development of hypertension in SHR with the effect being greatest in animals given the lowest dose (Quinones *et al.* 2010). Another study of SHR whose diets were supplemented with soluble cocoa fibre exhibited lower SBP and DBP during the treatment period. Throughout a 4 week post-treatment period when the test treatment had been discontinued SBP increased equivalent to control and DBP increased beyond that of control animals (Sanchez *et al.* 2010).

SHR administered 200 and 400 mg kg⁻¹ doses of CocoanOX exhibited higher plasma angiotensin converting enzyme (ACE) activity at the end of the intervention period, plasma concentration of angiotensin II was also raised.

The latter remaining elevated after treatment ended. Although this may be suggestive of a negative consequence, the authors discussed the possibility that inhibition of ACE leads to an increase in its plasma concentration without reducing efficacy of the inhibitor. The same study measured relaxation of pre-contracted intact aorta rings extracted from control animals induced by incubation with CocoonOX. Relaxation was reduced in the presence of N^ω-nitro-L-arginine methyl ester (L-NAME) but not indomethacin implying that dilation was a result of eNOS activity rather than prostaglandin-1 synthase (Mar Quinones *et al.* 2011). Similar effects have been reported using pre-contracted renal arteries isolated from wild-type (WT) and atherosclerotic mice (ATX) (Gendron *et al.* 2010). Catechin treatment in WT mice during months 9 to 12 improved dilation at 12 months compared with the untreated group, however this effect was not replicated in ATX mice. Incubation with N^G-nitro L-arginine (L-NNA) attenuated dilation in untreated WT mice but animals exposed to 3 months catechin treatment were unaffected, suggesting that eNOS was not inhibited in catechin treated cells or that addition of indomethacin to the incubation did not further affect this result.

In vitro studies using human umbilical vein endothelial cells (HUVEC) are widely used as a model to study endothelial function. HUVEC cells incubated with (-)-epicatechin for 2 hours had significantly increased nitrite levels up to 1 μM of (-)-epicatechin however beyond this, up to 10 μM, the nitrite concentration did not differ from control (Brossette *et al.* 2011). Similarly the greatest augmentation of nitric oxide produced by human coronary artery endothelial cells (HCAES) was reported following a 10 minute incubation with 1 μM (-)-epicatechin (Ramirez-Sanchez *et al.* 2010).

Following a 24 hour incubation with (-)-epicatechin Brossette *et al* (2011) observed no significant change in endothelial nitric oxide synthase (eNOS) mRNA expression nor any variation in its stability. It may be that increased production of nitric oxide is attributable to an interaction with the phosphatidylinositol 3-kinase (PI3K) pathway which is involved in controlling phosphorylation of the activation residues Ser1177 and Ser633. Treatment of HCAES with epicatechin increased phosphorylation of these serine residues and reduced phosphorylation of Thr495. In addition epicatechin treatment appeared to induce activation of eNOS through uncoupling from caveolin-1 (Cav-1) which it is bound to at the cytosolic side of the cell membrane in its inactive form, and binding with calmodulin (CaM) to stimulate solubilisation of the active enzyme. Further analysis suggested that this effect was mediated via interaction with phospholipase C. Whilst these conditions were dependent on the presence of calcium, the same authors have also demonstrated that epicatechin is able to stimulate NO production induced by phosphorylation of serine residues and activation of eNOS without uncoupling from Cav-1 in calcium-free conditions (Ramirez-Sanchez *et al.* 2011).

Similarly enhanced phosphorylation of non-specific serine/threonine protein kinase (Akt), an enzyme involved in the PI3K pathway, was measured in mice subjected to ischemia-reperfusion injury following 10 days of supplementation with 1 mg kg⁻¹ body weight (-)-epicatechin; infarct size was reduced. Co-supplementation with the opioid antagonist's naloxone or naltrindole eliminated the effect on Akt phosphorylation and infarct size. The results suggest that the protection conveyed by epicatechin was dependent on interaction with opioid receptors in the heart (Panneerselvam *et al.* 2010).

Endothelium secretion of endothelin-1 (ET-1) is associated with vascular constriction and therefore negative cardiovascular consequences. Bovine aortic endothelial cells (BAEC) treated with a procyanidin-rich extract of cocoa produced less ET-1 than control cells; the response was dose-dependent (Caton *et al.* 2010). Procyanidin-rich extracts of other fruits elicited similar results and the trimer to pentamer fractions of cranberry extract were shown to downregulate expression of ET-1 mRNA and upregulate Kruppel-like factor 2 (KLF2) mRNA, a transcription factor that mediates the synthesis of ET-1. Garcia-Conesa *et al.* (2009) demonstrated a similar reduction in ET-1 synthesis in HUVEC treated with a procyanidin-rich fraction of apple (Garcia-Conesa *et al.* 2009).

Increasing nitric oxide concentration *in vivo* is generally considered a positive consequence due to its functions of vasodilation (Figure 1.3) and inhibition of platelet aggregation. It is possible that reported increases in flow-mediated dilation (FMD) and reduction of blood pressure following cocoa consumption (Table 1.1) is due to an increase in the production of nitric oxide stimulated by epicatechin (Figure 1.4). Chronic intake may have a long-term protective effect in the event of CVD, although *in vitro* and non-human *in vivo* results are not necessarily transferrable to humans and consumption of cocoa products will contribute to energy and macronutrient intake that may be considered unhealthy.

1.2.3.2 Inflammation

Serum concentration of C-reactive protein (CRP) in a cohort of healthy Italian adults participating in the Moli-sani Project was lower amongst consumers of less than 3 x 20 g servings per week of dark chocolate (di

Giuseppe *et al.* 2008); this effect was not observed in consumers of 3 or more servings per week. A higher CRP concentration is associated with a higher risk of CHD therefore it can be inferred from this study that moderate consumers of dark chocolate have a lower risk of CHD than non- and high-consumers. Of the human intervention trials published since 2007 in which the serum CRP concentration is quantified following a chronic cocoa supplementation, the overall effect is inconclusive. In healthy subjects significant reductions in CRP following intervention have been observed (Hamed *et al.* 2008, Tzounis *et al.* 2011), whereas in subjects suffering from hypertension, hypercholesterolemia or type-2 diabetes there have been no significant changes observed (Grassi *et al.* 2008, Mellor *et al.* 2010, Sarria *et al.* 2012). By contrast, in a study of diabetic mice whose diets were supplemented with epicatechin significantly lower CRP levels were reported compared with diabetic mice whose diets were not supplemented (Si *et al.* 2011). Although serum CRP concentration is indicative of CVD it is yet to be determined whether high serum concentration contributes to the development of disease or whether it is a consequence of the disease. The level of intake of cocoa products or individual components, and whether any ability to lower CRP levels improves prognosis in patients or reduces risk in a healthy population, is yet to be determined.

1.2.3.3 Oxidative stress and dyslipidemia

Daily oral supplementation of 1 mg kg⁻¹ epicatechin for 10 days prior to ischemia-reperfusion injury, in rats, reduced infarct size and oxidized glutathione/reduced glutathione ratio (GSSH/GSH) at 48 hours post-injury (Yamazaki *et al.* 2008). Infarct size remained significantly smaller at 3

weeks post-injury. A further study by the same authors performed on animals exposed to permanent coronary occlusion (POC) yielded very similar results of infarct size, they also hypothesized that exposure to epicatechin prior to POC may elicit an increase of Akt phosphorylation however no significant changes were observed (Yamazaki *et al.* 2010).

Dyslipidemia is a major risk factor of CVD that has prompted an abundance of investigations, not least in relation to cocoa consumption. Many of the intervention trials described in Table 1.1 have reported increased HDL cholesterol levels and improved total cholesterol/HDL cholesterol ratio following cocoa intervention. A soluble cocoa fibre product fed to rats consuming a high cholesterol diet attenuated the negative consequences of the diet. The cholesterol-rich diet raised total- and LDL cholesterol concentration, and malondialdehyde (MDA) concentration of serum but these levels were significantly reduced in the group supplemented with the cocoa fibre product. In contrast the cholesterol-rich diet caused a reduction in HDL cholesterol concentration that was also improved by supplementation with the cocoa fibre product (Ramos *et al.* 2008, Bravo *et al.* 2008). A similar study of rats fed a high cholesterol diet with and without the addition of cocoa procyanidins reported that rats fed the cholesterol-rich diet experienced significantly higher plasma total cholesterol than rats fed a normal diet. This increase was significantly attenuated by the addition of 0.5 or 1.0% cocoa procyanidins to the high cholesterol diet. Furthermore rats consuming the high cholesterol diet all exhibited significantly more cholesterol and triglycerides in the liver than rats fed the normal diet. At the highest supplementation of cocoa procyanidins this increase was significantly less (Osakabe and Yamagishi 2009).

Obese-diabetic rats fed a diet supplemented with cocoa extract for 4 weeks demonstrated improved levels of plasma total cholesterol that was similar to that of non-diabetic rats consuming a normal diet. An improvement of similar magnitude was also noted for plasma triglycerides and LDL cholesterol. There were no significant changes in plasma HDL cholesterol concentration (Jalil *et al.* 2009). Diabetic mice treated with epicatechin for 15 weeks exhibited similar total- and LDL cholesterol levels to non-diabetic mice at the end of the study period that were significantly lower than untreated diabetic mice. Longevity of the epicatechin-supplemented mice was also improved; the number of mice surviving until the end of the study period was significantly greater than diabetic mice without supplementation, and not different from control mice. Markers of inflammation in the serum of epicatechin-supplemented diabetic mice were also affected by the treatment. Compared with non-treated diabetic mice, insulin-like growth factor-1 (IGF-1), CRP-1, interleukin-1 β (iL-1 β) and glutathione were either returned to control levels or reduced significantly. Interestingly, although there were no significant differences of superoxide dismutase (SOD) activity between control and diabetic mice, activity was significantly greater in the treated diabetic group (Si *et al.* 2011).

Investigations using the Zucker fatty rat model of obesity and metabolic syndrome demonstrated that supplementation of the diet with 5% soluble cocoa fibre resulted in a significant reduction of plasma MDA concentration such that levels were no longer significantly different from lean control rats. Zucker fatty rats fed a standard diet exhibited significantly higher levels of tumour necrosis factor- α (TNF- α) compared with the lean control animals but this was reduced in animals that consumed the 5% soluble cocoa fibre diet.

It is important to note that this reduction was not statistically significant however levels in the supplemented group were not significantly different from either fatty or lean rats. Fatty animals fed the 5% soluble cocoa fibre supplemented diet had significantly higher levels of plasma adiponectin compared with fatty rats and lean rats, which had similar levels (Sanchez *et al.* 2011). The same authors investigated the effect of a soluble cocoa fibre on development of hypertension in SHR and reported reduced MDA plasma concentration (Sanchez *et al.* 2010). A similar reduction in plasma MDA concentration was observed when SHR were treated with 100, 200 and 400 mg kg⁻¹ doses of CocoanOX; the effect was reversed by the end of a 4 week post-treatment period (Mar Quinones *et al.* 2011).

1.2.3.4 Conclusion

Cardiovascular diseases are responsible for more deaths globally than any other cause (World Health Organization 2011) and are the focus of much research, not least in relation to cocoa. The main focus of such research has been to identify associations between intake and disease, and to elicit mechanisms by which onset of disease can be delayed or prevented, or where treatment of cardiovascular events can be facilitated. Many of the human intervention studies described in Table 1.1 have involved intervention treatments that have demonstrated improvements in markers of disease risk factors such as total/HDL cholesterol ratio and FMD. Nitric oxide synthesis is possibly the most investigated endothelial function in relation to cocoa over the last 5 years with many authors reporting increases in NO concentration of plasma. At this time the predominant mechanistic hypothesis is that cocoa, or a component such as epicatechin, stimulates

eNOS activity. Although this doesn't appear to be the only mechanism that is affected, certainly increasing NO synthesis may be one reason why FMD and blood pressure are improved following intervention treatments.

	Intervention	Control	Study design	Outcomes	Ref.
1	Cu deficient patients supplemented with 10-40 g cocoa powder per day until improvement observed	None	Serum trace metal concentration monitored \leq 24 months post-enteral tube insertion in 23 patients (78.8 ± 10.4 years)	\uparrow serum [Cu] and neutrophil count	(Nishiwaki <i>et al.</i> 2011)
2	One cup of espresso coffee and 30 g DC (separate occasions)	None	50 term pregnant women without complicated gestation (31.8 ± 5.05 years)	\uparrow fetal HR accelerations and variability	(Buscicchio <i>et al.</i> 2012)
3	15 g pp-rich DC per day for 8 weeks	15 g macronutrient matched low-pp chocolate ($3.9 \text{ mg ECE g}^{-1}$)	Double-blind, randomised cross-over; 10 subjects diagnosed with CFS (6 females, 4 males) (52 ± 8 years)	Improved symptoms of CFS	(Sathyapalan <i>et al.</i> 2010)
4	HF beverage	Macronutrient-matched LF beverage	Cross-over; 10 healthy females (18-65 years)	\uparrow cutaneous blood flow	(Neukam <i>et al.</i> 2007)
5	20 g HF chocolate per day for 12 weeks	20 g LF chocolate ($<30 \text{ mg flavanols}$)	Double-blind, randomised; 30 healthy subjects (22 females, 8 males) (42.7 ± 10 years)	\uparrow minimal erythema dose	(Williams <i>et al.</i> 2009)
6	37 g DC and 237 mL cocoa beverage per day for 6 weeks	Low-pp bar and beverage containing 0.20 mg g^{-1} and 40.87 mg g^{-1} total proanthocyanidins, respectively	Double-blind, randomised; 101 healthy subjects (60 females, 41 males) (≥ 60 years)	\uparrow pulse rate at midpoint and end-of-treatment	(Crews <i>et al.</i> 2008)

	Intervention	Control	Study design	Outcomes	Ref.
7	Flavanol-rich cocoa beverage per day for 1 week	Flavanol-poor cocoa beverage (36 mg flavanols per day)	Double-blind, randomised; 21 healthy subjects (10 females, 11 males) (72.2 ± 6 years)	↑ cerebral blood flow in response to acute dose of cocoa beverage	(Sorond <i>et al.</i> 2008)
8	Cocoa beverage containing either 520 mg or 994 mg cocoa flavanols	Macronutrient-matched cocoa beverage containing 46 mg cocoa flavanols	Double-blind, cross-over; 30 healthy subjects (17 females, 13 males) (21.9 ± SE 0.61 years)	Improved cognitive performance and reduced mental fatigue	(Scholey <i>et al.</i> 2010)
9	35 g DC	35 g white chocolate	Cross-over; 30 subjects (22 females, 8 males) (18-25 years)	Improved contrast sensitivity, motion integration threshold, visual spatial working memory, and reaction time	(Field <i>et al.</i> 2011)
10	20 g chocolate beverage containing MF or HF content per day for 30 days	20 g chocolate beverage LF content	Double-blind, randomised; 63 subjects (52.30 ± 7.49 years)	↑ posterior parietal activity, synaptic excitation and neural information processing speed	(Camfield <i>et al.</i> 2012)
11	Mashed potato powder (1 g kg ⁻¹ BW) supplemented with cocoa butter (1 g kg ⁻¹ BW)	Mashed potato powder (1 g kg ⁻¹ BW) supplemented with olive oil (1 g kg ⁻¹ BW)	Randomised, cross-over; 10 healthy females (38.2 ± 10.7 years)	Plasma [TAG] and [IL-6] were altered (no significant difference between the meals)	(Tholstrup <i>et al.</i> 2011)

	Intervention	Control	Study design	Outcomes	Ref.
12	Cocoa beverage twice per day for 12 weeks	Cocoa-free beverage	Randomised; 25 healthy males (38 ± SE 1 years)	↓ LDL susceptibility to oxidation and urinary [dityrosine]; ↑ HDL cholesterol	(Baba <i>et al.</i> 2007b)
13	Low-, medium-, or high-pp cocoa beverage twice per day for 4 weeks	Nutrient matched beverage (trace amounts of cocoa pp)	Double-blind; 160 normo- and mildly hypercholesterolemic subjects (91 females, 69 males) (20-70 years)	↓ [Apo B] in MCP and HCP groups; ↓ oxidised LDL (kU L ⁻¹ plasma) in all groups	(Baba <i>et al.</i> 2007a)
14	40 g DC	Macronutrient matched, flavonoid-free chocolate	Double-blind, randomised; 22 heart transplant recipients (4 females, 18 males)	↑ Coronary artery diameter and % change of endothelial-dependent vasomotion; ↓ [8-iso-PGF2α] and platelet adherence	(Flammer <i>et al.</i> 2007)
15	22 g DC supplemented with 1.1 g canola sterol esters twice per day for 4 weeks	Macro- and micronutrient matched DC without the addition of plant sterols	Double-blind, cross-over; 49 subjects (32 females, 17 males) (24-70 years) with elevated cholesterol (5.20-7.28 mmol L ⁻¹)	↓ Serum total- and LDL cholesterol; ↓ SBP and DBP (combined intervention and control results)	(Allen <i>et al.</i> 2008)
16	MF or HF beverage	Macro- and micronutrient matched LF beverage	Double-blind, randomised, cross-over; 10 type-2 diabetic subjects (2 females, 8 males) (64.7 ± 9.9 years)	↑ FMD	(Balzer <i>et al.</i> 2008)

	Intervention	Control	Study design	Outcomes	Ref.
17	Flavanol containing beverage 3 times per day for 30 days	Macro- and micronutrient matched LF beverage	Double-blind, randomised; 41 type-2 diabetic subjects (29 females, 12 males) (intervention, 63.1 ± 8.3 years; control, 64.4 ± 8.6 years)	↑ FMD	(Balzer <i>et al.</i> 2008)
18	HF beverage twice per day for 12 weeks with and without 45 min physical activity 3 days per week	Macro- and micronutrient matched LF beverage with and without physical activity	Double-blind, randomised; 49 overweight and obese subjects (32 females, 17 males) (40-50 years)	↑ FMD (combined exercise and non-exercise results); ↓ insulin resistance, DBP and MAP (flavanol treatment nested in time)	(Davison <i>et al.</i> 2008)
19	Phase 1: Acute consumption of 74 g DC Phase 2: 2 cups of either sugar-containing cocoa beverage or sugar-free cocoa beverage	Phase 1: 74 g cocoa-free chocolate Phase 2: 2 cups of sugar-containing cocoa-free beverage	Single-blind, randomised, cross-over; 45 healthy subjects (35 females, 10 males) (52.8 ± 11.0 years)	↑ FMD; ↓ SBP and DBP; greatest FMD improvement following sugar-free cocoa beverage.	(Faridi <i>et al.</i> 2008)
20	100 g flavanol-rich DC per day for 15 days	100 g flavanol-free white chocolate per day	Double-blind, cross-over; 19 hypertensive, prediabetic subjects (8 females, 11 males) (44.8 ± 8.0 years)	↓ Insulin resistance and ↑ insulin sensitivity; ↓ clinical SBP and DBP, and ambulatory BP; ↑ FMD and ↓ serum total- and LDL cholesterol	(Grassi <i>et al.</i> 2009)

	Intervention	Control	Study design	Outcomes	Ref.
21	100 g DC per day for 1 week	None	28 healthy subjects (19 females, 9 males) (42 ± 12 years)	↓ platelet activated GP IIb/IIIa expression; ↓ LDL cholesterol; ↑ HDL cholesterol. ↓ C-reactive protein (females only)	(Hamed <i>et al.</i> 2008)
22	Cocoa beverage twice per day for 2 weeks	Macro- and micronutrient matched LF beverage	Double-blind, randomised, cross-over; 20 hypertensive subjects (12 females, 8 males) (51 ± SE 1.5 years)	↑ insulin-stimulated brachial artery diameter	(Muniyappa <i>et al.</i> 2008)
23	20 g cocoa powder in 250 mL skimmed milk twice per day for 4 weeks	250 mL skimmed milk without cocoa powder	Randomised, cross-over; 42 high-risk of CVD subjects (23 females, 19 males) (69.7 ± 11.5 years)	↑ HDL cholesterol; ↓ expression of adhesion molecules on the surface of monocytes and concentration of circulating soluble adhesion molecules	(Monagas <i>et al.</i> 2009, Khan <i>et al.</i> 2010)
24	HF cocoa beverage followed by 10 min cycling	Macronutrient matched LF cocoa beverage followed by cycling	Double-blind, randomised, cross-over; 21 healthy overweight/obese subjects (8 females, 13 males) (54.9 ± SE 2.2 years)	↑ FMD; ↓ AUC for DBP and MAP in response to exercise	(Berry <i>et al.</i> 2010)

	Intervention	Control	Study design	Outcomes	Ref.
25	Cocoa beverage containing 33, 372, 712, or 1052 mg total flavanols per day for 6 weeks	None	Double-blind, randomised; 52 mildly hypertensive subjects (20 females, 32 males) (42-74 years)	↓ 24 h ambulatory MAP, SBP and DBP (1052 mg); ↓ Overnight ambulatory SBP, DBP and HR	(Davison <i>et al.</i> 2010)
26	6 g or 25 g DC per day for 3 months	None	Single-blind, randomised; 91 cardiovascular high-risk patients (20 females, 71 males) (57-74 years)	↓ 24 h, day- and night-time MAP, SBP and DBP (6 g); ↓ 24 h and daytime MAP and SBP (25 g)	(Desch <i>et al.</i> 2010)
27	HF cocoa beverage twice per day for 30 days	Nutrient matched LF cocoa beverage	Double-blind, randomised, cross-over; 16 coronary artery disease (CAD) patients (3 females, 13 males) (64 ± 3 years)	↑ FMD (both conditions), FMD post-intervention was higher than post-control; ↑ % of CACs; ↓ plasma [nitrite]; ↑ SBP	(Heiss <i>et al.</i> 2010)
28	15 g DC 3 times per day for 8 weeks	Macronutrient matched cocoa solids-free chocolate	Double-blind, randomised, cross-over; 12 type-2 diabetic subjects (5 females, 7 males) (42-71 years)	↑ Serum [HDL cholesterol]; ↓ total cholesterol:HDL cholesterol ratio	(Mellor <i>et al.</i> 2010)
29	Dairy based cocoa beverage containing either natural-dose (NTC) or high-dose (TEC) theobromine once per day for 3 weeks	Unspecified dairy based placebo beverage	Double-blind, randomised, cross-over; 42 pre-/stage 1 hypertensive, healthy subjects (10 females, 32 males) (62 ± 4.5 years)	↑ 24 h DBP (NTC); ↑ 24 h SBP, daytime DBP, 24 h, day- and night-time HR (TEC); ↓ central SBP, HR and stroke volume (TEC)	(van den Bogaard <i>et al.</i> 2010)

	Intervention	Control	Study design	Outcomes	Ref.
30	Dairy-based high-fat liquid meal plus HF cocoa powder	Macronutrient matched dairy-based high-fat liquid meal containing LF cocoa powder	Double-blind, randomised, cross-over; 18 healthy subjects (16 females, 2 males) (25.2 ± 2.5 years)	↑ serum [triglycerides] and [free fatty acids] (both conditions); ↓ FMD (both conditions, higher following intervention)	(Westphal and Luley 2011)
31	40 g DC twice per day for 2 weeks followed by 1.5 h cycling	30.4 g bar of sugar- and fat-matched cocoa liquor-free chocolate followed by cycling	Single-blind, randomised; 20 healthy males (22 ± 4 years)	↑ Plasma [F ₂ -isoprostane] post-exercise (both conditions, lower following intervention); ↓ oxidised LDL pre- and post-exercise	(Allgrove et al. 2011)
32	40 g DC	40 g milk chocolate	Single-blind, randomised, cross-over; 20 healthy subjects (13 females, 7 males) (33 ± 11 years) and 20 smokers (13 females, 7 males) (33 ± 11 years)	↓ Serum sNOx ₂ -dp, urinary isoprostane excretion and platelet ROS, sNOx ₂ -dp and 8-iso-PGF ₂ α (healthy subjects); ↑ FMD, serum [NOx] and platelet NOx production (smokers); ↓ serum sNOx ₂ -dp, urinary isoprostane excretion and platelet ROS, sNOx ₂ -dp and 8-iso-PGF ₂ α (smokers)	(Loffredo et al. 2011, Carnevale et al. 2012)

	Intervention	Control	Study design	Outcomes	Ref.
33	Cocoa beverage containing either 2, 5, 13 or 26 g of cocoa	Macronutrient matched beverage without cocoa powder	Double-blind, randomised; 23 healthy subjects (14 females, 9 males) (63 ± SE 2 years)	Dose-dependent greater change in FMD (5, 13 and 26 g); ↑ SBP (2 and 26 g); ↑ DBP (2, 13 and 26 g); ↑ MAP (2, 13 and 26 g); ↓ [glucose] (0, 2 and 5 g)	(Monahan <i>et al.</i> 2011)
34	Sugar-free cocoa beverage or a sugar-sweetened cocoa beverage twice per day for 6 weeks	Cocoa-free sugar-sweetened beverage	Double-blind, randomised, cross-over; 39 overweight, healthy subjects (33 females, 6 males) (41-63 years)	↑ FMD (both conditions)	(Njike <i>et al.</i> 2011)
35	75 g DC	None	16 healthy subjects (6 females, 10 males) (20-45 years)	↓ Angiotensin-converting enzyme activity	(Persson <i>et al.</i> 2011)
36	13.5 g flavonoid-enriched chocolate twice per day for 1 year	Macronutrient matched placebo chocolate	Double-blind, randomised; 93 postmenopausal, type-2 diabetic patients (51-74 years)	↓ Plasma [insulin] and insulin resistance; ↑ insulin sensitivity; ↓ [LDL cholesterol]; ↑ CHD risk (both conditions, less following intervention)	(Curtis <i>et al.</i> 2012)

	Intervention	Control	Study design	Outcomes	Ref.
37	100 g DC followed by 2.5 h of cycling	Macronutrient matched cocoa solids-free bar (71 g) followed by cycling	Single-blind, randomised, cross-over; 14 healthy male subjects (22 ± 1 years)	↑ Plasma total antioxidant status (both conditions); ↑ [insulin] pre-exercise and 1 h post-exercise; ↓ plasma [glucose] post-exercise (both conditions)	(Davison <i>et al.</i> 2012)
38	100 g high-antioxidant dark chocolate (HADC) or 100 g DC	None	Double-blind, randomised, cross-over; 15 healthy subjects (9 females, 6 males) (30 ± 5 years)	↑ Plasma FRAP (both interventions); HADC FRAP remained higher at 4 and 5 h; ↑ urinary HADC FRAP up to 12 h; ↑ [triacylglycerol] (both interventions); ↑ [thiol] at 2- and 4 h (both interventions)	(Lettieri-Barbato <i>et al.</i> 2012)
39	50 g DC per day for 3 weeks	None	50 healthy subjects (25 females, 25 males) (28-45 years)	↑ [HDL cholesterol] and [triglyceride] and ↓ LDL cholesterol (females only); ↓ lipid peroxidation, conjugated diene and hydroperoxide content of HDL and LDL; ↑ platelet production of NO; ↓ peroxynitrite	(Nanetti <i>et al.</i> 2012)

	Intervention	Control	Study design	Outcomes	Ref.
40	Cocoa products: (B) cocoa + hazelnuts, (C) cocoa + hazelnuts + phytosterols, (D) cocoa + hazelnuts + phytosterols + soluble fibre per day for 4 weeks	(A) Cocoa	Double-blind, randomised; 113 pre/stage-1 hypertensive and hypercholesterolemic subjects (67 females, 46 males) (43-65 years)	↓ [Total-] and [LDL cholesterol] and [Apo B] and Apo B:Apo A ratio (C) and (D); ↓ hsCRP and oxidised LDL (D)	(Sola <i>et al.</i> 2012)
41	DC and cocoa beverage once per day for 3 months	None	5 type-2 diabetic patients with stage II and III heart failure (47-71 years)	↑ [HDL cholesterol]; enhanced expression of markers of mitochondrial structure in skeletal muscle	(Taub <i>et al.</i> 2012)

Table 1.1 Summary of intervention studies using cocoa published between 2007 and 2012. Only outcomes with a statistical significance of a maximum $p < 0.05$ are reported. Mean \pm standard deviation unless otherwise stated. Abbreviations used in the table: \uparrow , increase; \downarrow decrease; DC, dark chocolate; HR, heart rate; pp, Polyphenol; CFS, chronic fatigue syndrome; HF, high flavanol; LF, low flavanol; MF, medium flavanol; BW, body weight; TAG, triacylglycerol; IL-6, interleukin 6; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Apo B, apolipoprotein B; 8-iso-PGF 2α , 8-iso-prostaglandin F 2α ; SBP, systolic blood pressure; DBP, diastolic blood pressure; FMD, flow mediated dilation; MAP, mean arterial pressure; GP, glycoprotein; CVD, cardiovascular disease; AUC, area under curve; CACs, circulating angiogenic cells; sNO x 2-dp, soluble NO x 2 derived peptide; ROS, reactive oxygen species; FRAP, ferric reducing ability of plasma; NO, nitric oxide.

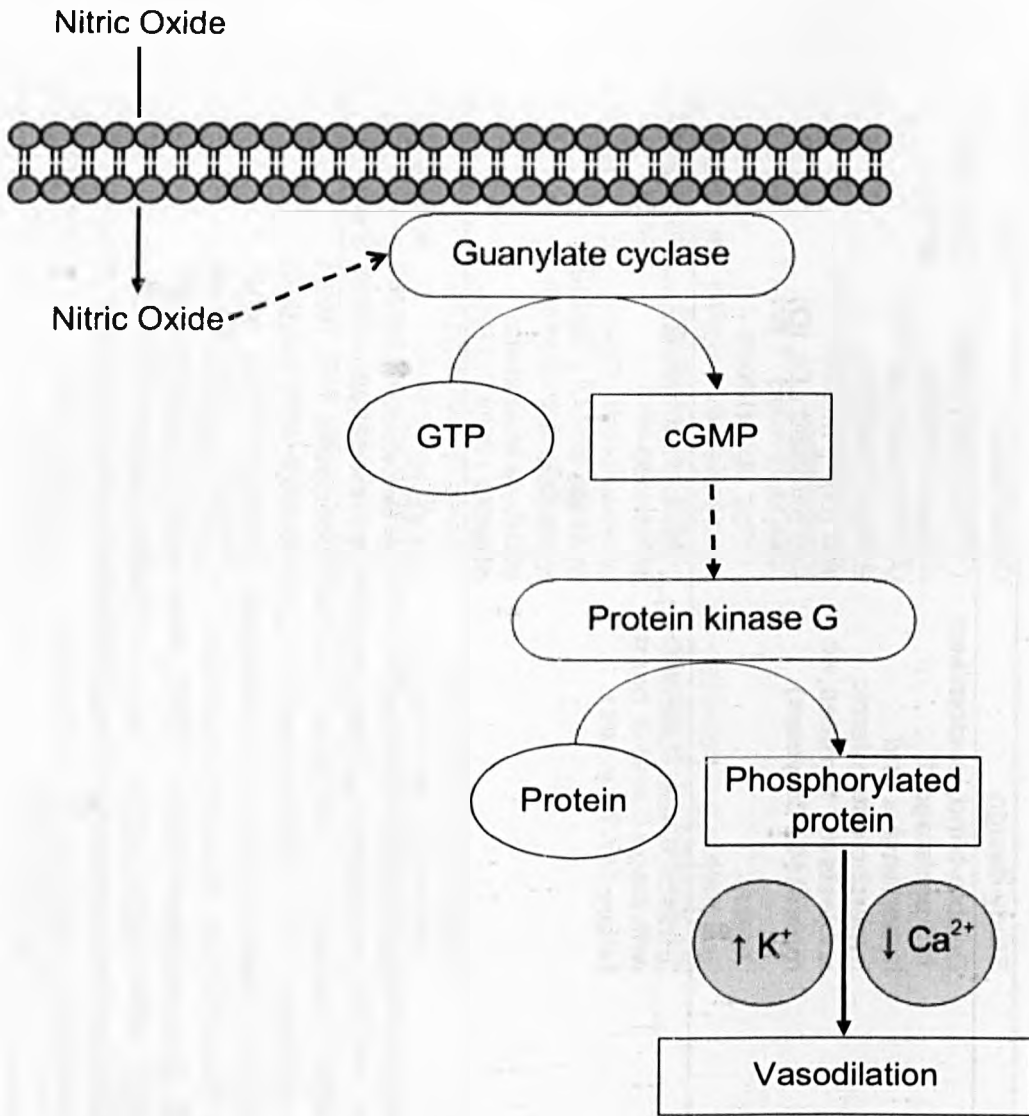


Figure 1.3 Nitric oxide stimulated vasodilation

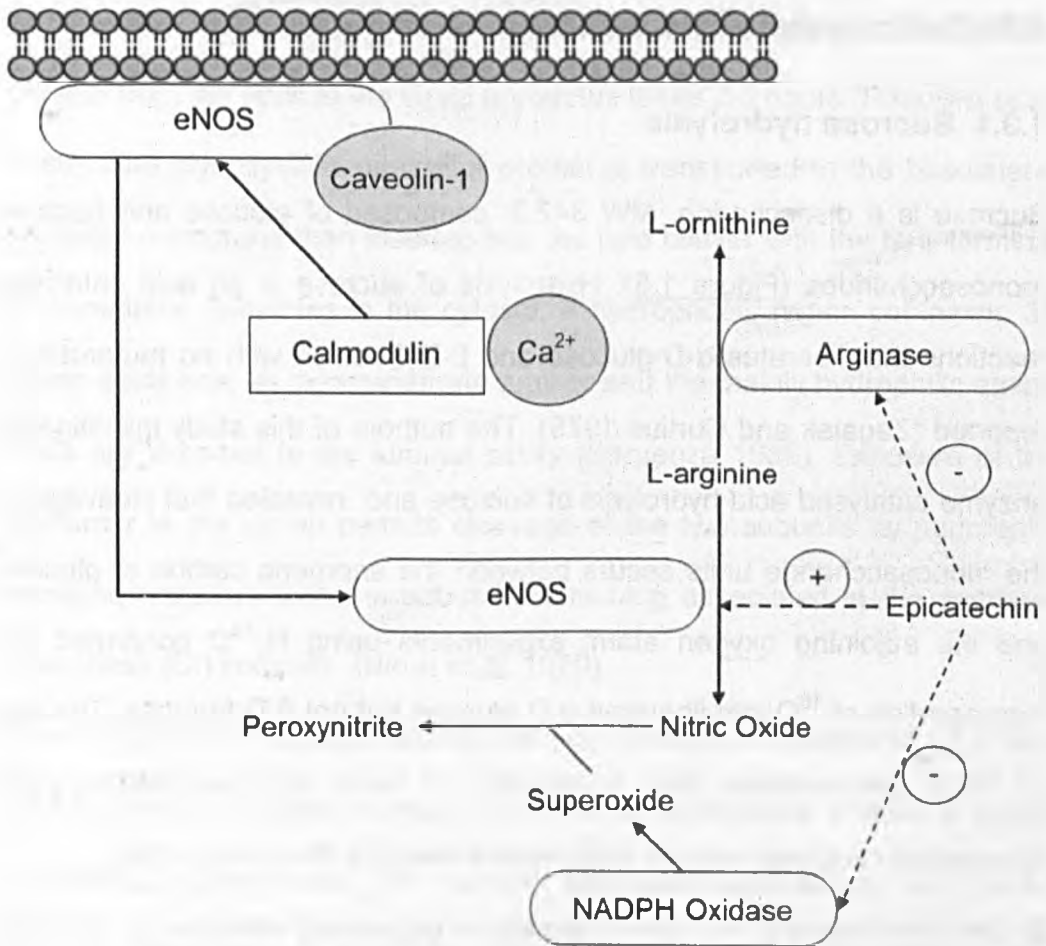


Figure 1.4 Potential mechanisms by which epicatechin increases nitric oxide concentration

1.3 Carbohydrate metabolism

1.3.1 Sucrose hydrolysis

Sucrose is a disaccharide, MW 342.3, composed of glucose and fructose monosaccharides (Figure 1.5). Hydrolysis of sucrose is an acid catalysed reactions that liberates α -D-glucose and β -D fructose with no mutarotation reported (Zagalak and Curtius 1975). The authors of this study investigated enzyme catalysed acid hydrolysis of sucrose and revealed that cleavage of the monosaccharide units occurs between the anomeric carbon of glucose and the adjoining oxygen atom; experiments using H_2^{18}O confirmed the incorporation of ^{18}O into liberated α -D-glucose but not β -D-fructose. The use of $^2\text{H}_2\text{O}$ demonstrated that protonation of each monosaccharide upon release from sucrose was not from water present in the reaction mix.

Enzyme hydrolysis of sucrose in humans is exclusively catalysed by sucrase (EC 3.2.1.48) although it also exhibits activity for maltose, a disaccharide comprised of two glucose units. Sucrase is located at the luminal surface of enterocytes, both crypt cells and microvillus cells express the enzyme in its mature and precursor form (Beaulieu *et al.* 1989). Sucrase is anchored to the epithelial cell membrane via another α -glucosidase enzyme, isomaltase (EC 3.2.1.10), that is responsible for the hydrolysis of maltose and isomaltose. The two subunits of sucrase and isomaltase are initially synthesised in the rough endoplasmic reticulum (RER) as a single polypeptide chain of 1827 amino acid residues with a MW in the region of 220,000-280,000 Daltons (Da) (Conklin *et al.* 1975). The exact molecular weight measured varies depending on the method of solubilisation, either papain or Triton-X-100 (Brunner *et al.* 1979). The protein is then transported

to the Golgi apparatus where *N*- and *O*-linked glycosylation takes place; transfer from the RER to the Golgi apparatus takes 2-3 hours (Beaulieu *et al.* 1989). The glycosylated precursor protein is transported to the basolateral epithelial membrane then inserted into the lipid bilayer with the NH₂-terminal of isomaltase remaining in the cytosol. A hydrophobic region containing 31 amino acids acts as the membrane anchor and the mainly hydrophilic amino acids are exposed to the luminal cavity (Semenza 1986). Exposure of the precursor to the lumen permits cleavage of the two subunits by pancreatic protease enzymes with the subunits remaining associated in the sucrase-isomaltase (SI) complex (Hauri *et al.* 1979).

The active site of sucrase displays activity towards hydrolysis of α 1-2 and α 1-4 glycosidic bonds. Human lysosomal α -glucosidase shares a highly conserved catalytic site with human and rabbit isomaltase, and rabbit sucrase (Hermans *et al.* 1991, Chantret *et al.* 1992). A study of the interaction between human lysosomal or yeast α -glucosidase and the inhibitor conduritol B epoxide (CBE) revealed that the β -carboxyl group of aspartic acid at the active site was essential for hydrogen bond formation with CBE and that proton donation was likely to be from an aspartic acid residue (Hermans *et al.* 1991, Okuyama *et al.* 2001). In support of these findings, structural studies using the inhibitors kotalanol and Man₂GlcNAc₂, an asparagine-linked N-acetylated oligosaccharide, have revealed hydrogen bond formation between aspartic acid residues and saccharide hydroxyl groups, with aspartic acid residues also identified as catalytic nucleophiles. Stabilisation of the enzyme-inhibitor complex was proposed to involve additional hydrophobic interactions with leucine, tryptophan, phenylalanine, valine and tyrosine (Sim *et al.* 2010).

1.3.1.1 Modification of sucrase activity

1.3.1.1.1 Diet and disease

The activity of SI may be enhanced or diminished in different disease states or through diet. A diet high in sucrose or medium-chain triacylglycerols resulted in significantly increased SI mRNA levels and increased activity in rats compared with animals fed a low-starch, high long-chain triacylglycerol diet (Yasutake *et al.* 1995). Intrajejunal administration of fructose or sucrose solution, but not glucose, compared with sugar-free solution, similarly enhanced SI activity and mRNA levels in rats (Kishi *et al.* 1999). A high-carbohydrate, low-fat diet fed to mice similarly enhanced the activity and mRNA levels of SI compared with animals fed a low-carbohydrate, high-fat diet (Honma *et al.* 2007). The authors reported the increase to be associated with enhanced acetylation of histone H3 and H4 on the promoter and transcription regions of the SI gene. Increased SI activity has also been reported in streptozotocin-induced diabetic rats due to a reduced rate of enzyme degradation (Olsen and Korsmo 1977). A more recent study demonstrated that increased SI mRNA levels, protein expression and enzyme activity were reduced to levels equivalent to those in non-diabetic rats, with insulin treatment (Liu *et al.* 2011). The authors treated Caco-2 cells with insulin and observed reduced SI mRNA levels and activity that was associated with a reduction in the level of caudal type homeobox 2 (CDX2) mRNA. Inhibition of the mitogen-activated protein kinase (MAPK)-dependent pathway, via which CDX2 is expressed, returned CDX2 mRNA levels, and SI mRNA levels and activity to a level equivalent to control cells not treated with insulin. Hepatocyte nuclear factor-1 α (HNF-1 α), another transcriptional

protein, is also implicated in the regulation of SI expression. Decreased expression and activity of SI in Caco-2 cells cultured with high concentration glucose media, was reversed when glucose concentration was lowered; HNF-1 α mRNA was similarly affected by glucose concentration (Gu *et al.* 2007).

Congenital sucrase-isomaltase deficiency (CSID) is an autosomal recessive disease of the human small intestine that results in abdominal pain and diarrhoea following ingestion of sugar. The pathogenesis of CSID has been mostly attributed to a mutation of the *SI* gene that encodes the precursor enzyme protein (Uhrich *et al.* 2012). Subsequent synthesis of the protein in the RER leads to the substitution of specific amino acids in the single polypeptide chain. Various amino acid alterations have been noted in biopsies obtained from patients suffering with CSID a small number of which appear more frequently. Consequently substituted amino acid residues lead to incorrect folding of the protein, insufficient transportation from either the RER or the Golgi apparatus, and cleavage of the hydrophobic membrane domain from the precursor protein within the RER ultimately preventing anchorage in the apical membrane of the epithelial cell (Jacob *et al.* 2000, Ritz *et al.* 2003, Keiser *et al.* 2006).

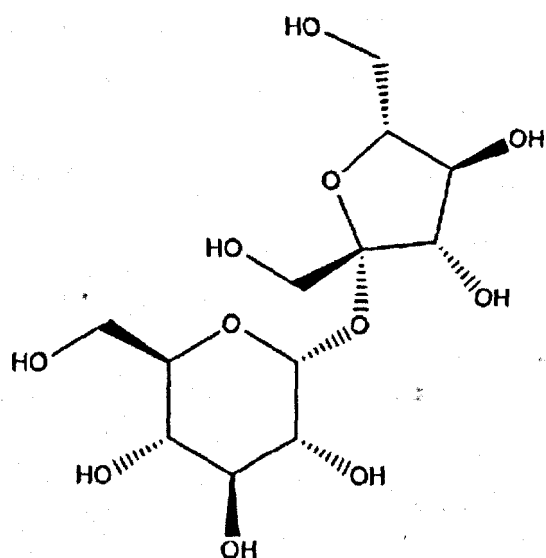


Figure 1.5 Structure of the disaccharide sucrose composed of α -D-glucose and β -D-fructose

1.3.2 Glucose transport

Glucose absorption from the lumen of the small intestine is controlled by two distinct protein carriers located at the apical membrane, SGLT1 and GLUT2, and one at the basolateral membrane, GLUT2. The primary glucose carrier, SGLT1, is a protein composed of 664 amino acid residues of which 14 helical regions span the apical membrane (Hediger *et al.* 1989, Turk *et al.* 1994, Turk *et al.* 1996). Activity of the carrier is dependent on the binding of sodium ions and maintenance of a negative electrochemical gradient within the cell. This gradient is a result of basolateral efflux of 3 sodium ions by the sodium-potassium pump and influx of fewer sodium ions via the glucose carrier protein. Affinity of this secondary active cotransporter for glucose is high as demonstrated by a low Michaelis-constant, ~ 5 mM (Scow *et al.* 2011), which represents the ratio of dissociation to formation of the transporter-substrate complex. Even at low luminal concentrations SGLT1 continues to transport glucose against a concentration gradient. Structural

evaluations have demonstrated that the number of sodium ions bound to the transporter affects the capacity for glucose binding and that cation- π interactions are involved in binding two sodium ions to the transporter which induces a conformational change that permits glucose binding (Jiang *et al.* 2012). Investigations using the SGLT1 inhibitor phloridzin and glucose stereoisomers implicate the C-terminal loop 13 and tryptophan residues in formation of the transporter-substrate complex (Raja *et al.* 2003, Raja *et al.* 2004, Kumar *et al.* 2007, Tyagi *et al.* 2007, Wimmer *et al.* 2009). In order to release sodium and glucose into the cytosol the transporter must undergo a reorientation from outward to inward facing (Sala-Rabanal *et al.* 2012). This conformational change is a result of a sequence of altered electrical charges (Longpre *et al.* 2012). Reorientation weakens the bond between the transporter and one of the sodium ions which confers a disruption to the hydrogen bonds that maintain the transporter-glucose complex thereby releasing glucose into the cell. It has been proposed that only one sodium ion is released into the cell and that the second remains bound to the transporter and returns to the extracellular facing orientation.

Sodium-independent passive diffusion of glucose is facilitated by the GLUT2 protein carrier. Located in the basolateral membrane GLUT2 allows transport of glucose and fructose out of the cell into the hepatic portal vein. Relatively recently this transporter has also been detected in the apical membrane where it is believed to assist SGLT1 uptake of glucose in the presence of a high glucose concentration in the intestinal lumen (Zheng *et al.* 2009, Chaudhry *et al.* 2012). The secondary structure of GLUT2 is similar to that of SGLT1. It is a protein comprised of 554 amino acids that span the cell membrane across 12 regions with both the C- and N-terminal cytosol

facing (Mueckler 1994, Olson and Pessin 1996). Passive diffusion of glucose is proposed to involve hydrogen bond formation of glucose at carbon atoms 1, 3 and 4 with carbon-6 involved in a hydrophobic association with the transporter (Colville *et al.* 1993); transmembrane regions 9 to 12 have been considered essential for transporter-glucose affinity (Wu *et al.* 1998). In contrast to SGLT1, GLUT2 is a low affinity-high capacity carrier of glucose, $K_M = \sim 20$ mM, that does not show saturated kinetics with increasing concentration of substrate (Scow *et al.* 2011, Zheng *et al.* 2012). Translocation of GLUT2 to the apical membrane occurs rapidly within minutes of cells sensing the presence of a high luminal concentration of glucose. The mechanism for apical GLUT2 translocation is proposed to involve two separate pathways that cooperate to enhance activity of protein kinase C isoform β II (PKC β II) (EC 2.7.11.13). Cotransport of sodium ions into the cell leads to depolarisation of the cell membrane which activates the calcium channel Ca_v1.3 permitting a flux of calcium ions into the cell; this is maximal at glucose concentrations that saturate SGLT1. The increased influx of calcium alters the cytoskeletal structure and increases PKC β II affinity for phosphatidylserine. Similarly at SGLT1 saturating concentrations of glucose, apical sweet taste receptors are activated and internalised by the cell; the alpha subunits of these receptors is cleaved and released into the cytosol. The remaining beta and gamma subunits activate phospholipase C isoform β II (PLC β II) (EC 3.1.4.11) which catalyses the removal of a diacylglycerol from phosphatidyl inositol-bisphosphate. The presence of diacylglycerol fully activates PKC β II which leads to translocation of GLUT2 to the apical membrane (Scow *et al.* 2011). Caco-2 cells co-incubated with

PKC inhibitors or activator demonstrate reduced and elevated glucose uptake, respectively (Zheng *et al.* 2012).

1.3.2.1 Modification of transporter activity

1.3.2.1.1 Diet and disease

In healthy subjects the plasma increase in glucose concentration following a meal stimulates the release of insulin from pancreatic beta cells. Insulin receptors on muscle cells and adipocytes bind to the plasma insulin subsequently resulting in the insertion of glucose transporter GLUT4 into the cell membrane to facilitate glucose uptake, thereby lowering plasma glucose concentration. Insulin receptors at the apical membrane of epithelial cells similarly bind to insulin in the lumen and induce a reduction of GLUT2 translocation to the apical membrane that lowers glucose absorption from the small intestine (Tobin *et al.* 2008). In the diabetic disease state, glucose uptake into adipocytes and muscle cells is impaired due to resistance to the action of insulin or through lack of insulin release from pancreatic beta cells. In response to both situations, enhanced insertion of GLUT4 into the plasma membrane of adipocytes and muscle cells is not stimulated and plasma glucose concentration remains elevated. At the intestinal level, GLUT2 internalisation is not promoted therefore apical GLUT2 levels remain high and glucose uptake continues to be facilitated (Tobin *et al.* 2008). Streptozotocin-induced diabetic rats exhibit enhanced expression of GLUT2 in enterocytes that is reversed with insulin treatment (Burant *et al.* 1994, Corpe *et al.* 1996). Increased levels of GLUT2 in the small intestine of human subjects with type-2 diabetes has also been reported (Dyer *et al.* 2002).

1.4 Objectives

The objective of this project was to investigate the interaction between dark chocolate flavanols and sugars, including the metabolic fate of each, and to identify potential mechanisms by which interaction may occur.

Chapter 2 Materials and methods

2.1 Chemicals

All chemical reagents were purchased from Sigma-Aldrich, Dorset UK, and all water is ultrapure with a resistivity of 18.2 M Ω ·cm at 25°C supplied using the Millipore Milli-Q Integral system, unless otherwise stated.

2.1.1 Reagent preparation

Transport buffer solution was prepared according to the Hanks' Balanced Salt solution (H6648) formulation without the addition of D-glucose. Briefly, 0.4 g L⁻¹ potassium chloride (P5405), 0.06 g L⁻¹ potassium phosphate (P0662), 0.05 g L⁻¹ sodium phosphate dibasic (S9763), 0.35 g L⁻¹ sodium bicarbonate (S5761), 8 g L⁻¹ sodium chloride (S5886), supplemented with 0.27 g L⁻¹ calcium chloride dihydrate (C7902) and 0.018 g L⁻¹ ascorbic acid (A4544) were dissolved in water and the solution adjusted to pH 7.4 at 37°C before being sterile filtered (Corning® 430049).

2.1.1.1 Dark chocolate extract preparation

A flavanol-rich dark chocolate extract was prepared as recently described (Robbins *et al* 2012). Nestlé Noir Intense 70% cocoa solids was ground to a powder and the fat removed by treatment with hexane. A volume (45 mL) of hexane (Fisher Scientific, H/0406/PB17) was added to 5 g of powder and the suspension sonicated at 50°C for 5 min before being centrifuged at 3,000 rpm for 5 min. The hexane was decanted to waste and the process repeated twice more; residual hexane was evaporated overnight in a fume cupboard. A flavanol-rich extract was prepared from the fat-free powder; 10 mL of extraction solution containing acetone (Fisher Scientific, A/0606/17), water

and acetic acid (Fisher Scientific, A/0360/PB15) (70:29.5:0.5) was added to the required quantity of powder and the suspension sonicated at 50°C for 5 min followed by centrifugation at 3,000 rpm for 5 min. The supernatant was collected and passed through a strong-cation exchange solid phase extraction cartridge (52686) and filtered through a polytetrafluoroethylene (PTFE) membrane syringe filter, 17 mm, 0.2 µm (Chromacol, 17-SF-02(T)). The filtrate was evaporated to dry under vacuum then reconstituted in 20 mL of test or control solution and corrected to pH 7.4 at 37°C.

2.1.1.1.1 HPLC-FLD protocol

The flavanol profile, monomer to decamer, of dark chocolate extract was analysed using an Agilent 1200 series HPLC with fluorescence detection, as recently described (Robbins *et al.* 2012). A Phenomenex Develosil Diol 100 Å 5 µm column, 250 x 4.6 mm, plus Phenomenex cyano guard column, 4 x 3 mm, was maintained at 35°C in a thermostatted column oven. The mobile phase consisted of solvent A) 2% acetic acid in acetonitrile (Fisher Scientific, A/0626/17) (98:2 v/v), and B) 2% acetic acid in aqueous methanol (Fisher Scientific, M/4058/17) (95:3:2 v/v/v). The flow rate was maintained at 1.0 mL min⁻¹ throughout the duration of the analytical run. The mobile phase gradient of solvent B in solvent A was as follows: 0 min, 7% B; 3 min, 7% B; 60 min, 37.6% B; 63 min, 100%; 70 min 100% B; 76 min, 7% B; 81 min, 7% B. Fluorescence detection was performed with excitation at 230 nm and emission at 321 nm. The photomultiplier level was set to 10. Standard epicatechin was used to prepare a calibration curve of fluorescence as a function of epicatechin concentration. Linear regression analysis, with the y-intercept fixed at zero, was conducted to obtain the gradient and adjusted R-

square value of the curve. The concentration of flavanols dimer to decamer was calculated using the epicatechin calibration curve with a relative response factor applied to each fraction. The flavanol content ($\text{mg } 100\text{g}^{-1}$) of fresh weight dark chocolate was calculated firstly by converting the concentration (mg mL^{-1}) to the content (mg g^{-1}) of defatted dark chocolate. A correction factor was applied to account for the dilution factor and fat content of the dark chocolate.

2.1.1.2 *In vitro* epicatechin transport and methylation

Sugar solutions containing 100 mM sucrose (S9378), glucose (Fisher Scientific, G/0450/60) or fructose (47740) were prepared in transport buffer solution and corrected to pH 7.4 at 37°C. Each sugar solution was subsequently used to prepare test solutions containing 250 μM epicatechin. Briefly, 2 mg epicatechin was dissolved in a mixture containing 10 μL dimethyl sulfoxide plus 990 μL sugar solution, or transport buffer for the experimental control. The suspension was sonicated at 37°C for 30 min then vortexed to ensure complete dissolution. A volume (725 μL) of epicatechin solution was diluted to 20 mL using the corresponding solution then corrected to pH 7.4 at 37°C.

2.1.1.3 Dark chocolate extract inhibits sucrose hydrolysis in the Caco-2 cell model

A 20 mM sucrose solution was prepared. Briefly, 684 mg of sucrose (S9378) was dissolved in 100 mL of transport buffer solution and then corrected to pH 7.4 at 37°C.

2.1.1.3.1 Pure compounds

Pure compounds were weighed and initially dissolved in 1 mL of a 1% solution of DMSO (60153) in transport buffer solution or 20 mM sucrose solution (v/v). Aliquots were diluted to 20 mL using transport buffer solution or 20 mM sucrose solution to obtain final concentrations as described below.

Compound	Weight (mg)	Aliquot (μ L)	Final Concentration (μ M)
Flavanol monomers	3	970	500
	2	581	200
	1	580	100
	1	290	50
	1	116	20
Procyanidin B2	3	385	100
(Extrasynthese, 0984)	3	193	50
Acarbose (A8980)	3	860	200
	2	645	100
	1	645	50

^a(-)-Epicatechin (Extrasynthese, 0977 S), (+)-Epicatechin (Nacalia Tesque, 02573-34), (-)-Catechin (C0567), (+)-Catechin (Extrasynthese, 0976 S).

2.1.1.3.2 Ethyl acetate extract of dark chocolate extract

A volume (0.75 mL) of ethyl acetate (Fisher Scientific, E/0906/17) was added to the dry extract; the suspension was vortexed to mix and centrifuged at 17,000 x g for 5 minutes. The supernatant was removed and evaporated to dry. To the remaining pellet the ethyl acetate extraction procedure was repeated and the supernatant evaporated to dry. Each ethyl acetate fraction was reconstituted in 20 mL of sucrose solution and then correct to pH 7.4 at 37°C.

2.1.1.4 Glucose transport inhibition by a flavanol-rich dark chocolate extract

A stock solution of 10 mM glucose was prepared. Briefly, 180 mg of glucose (Fisher Scientific, G/0450/60) was dissolved in 100 mL of transport buffer solution and 50 mL serial dilutions ranging from 0.25-7 mM prepared in transport buffer solution. A 20 mL aliquot of each concentration was taken to which 9 μL of 0.1 $\mu\text{Ci } \mu\text{L}^{-1}$ [^{14}C]-glucose was added to a final radioactivity concentration of 0.045 $\mu\text{Ci mL}^{-1}$ and then corrected to pH 7.4 at 37°C.

2.1.1.4.1 Pure compound preparation

Pure compounds were weighed and initially dissolved in 1 mL of a 1% solution of DMSO (60153) in transport buffer solution (v/v). Aliquots were diluted to 20 mL to obtain final concentrations as described below. A volume (9 μL) of 0.1 $\mu\text{Ci } \mu\text{L}^{-1}$ [^{14}C]-glucose was added to each 20 mL solution to obtain a final radioactivity concentration of 0.045 $\mu\text{Ci mL}^{-1}$, and then corrected to pH 7.4 at 37°C.

Compound	Weight (mg)	Aliquot (μ L)	Final Concentration (mM)
(-)-Epicatechin (Extrasynthese, 0977 S)	2	581	0.2
(-)-Epicatechin	6	969	1
(+)-Epicatechin (Nacalai Tesque, 02573-34)	2	581	0.2
(-)-Catechin (C0567)	2	581	0.2
(+)-Catechin (Extrasynthese, 0976 S)	2	581	0.2
Procyanidin B2 (Extrasynthese, 0984)	3	780	0.2
Caffeine (C0750)	1	971	0.25
Theobromine (T4500)	10	605	1.68

2.1.1.4.2 Solid phase extraction of flavanol fractions

There are no commercially available standards of flavanols with a degree of polymerisation greater than trimer. In order to obtain and investigate the effect of flavanol polymers larger than dimer, a solid phase extraction (SPE) procedure was developed based on the HPLC-FLD method used to quantify the compounds. Using a vacuum manifold, vacuum 0.2 bar, Supelclean™ LC-Diol tubes (57016) were pre-conditioned with 2 mL of acetonitrile. Dried dark chocolate extract was reconstituted in water and 2 mL of the aqueous extract was loaded into the SPE tube. To prevent dilution of the collected sample, the initial 2 mL of aqueous extract was eluted to waste. A further 1 mL of aqueous extract was loaded into the SPE tube and collected in a centrifuge tube. The SPE material was washed twice with 1 mL per wash of water; each of these washes was collected separately in a centrifuge tube.

Analytes that remained adsorbed to the SPE material were eluted with two washes, 1 mL each wash, of a predominantly organic solvent. The elution solvent contained 85% acetonitrile/acetic acid (98:2 v/v) and 15% methanol/water/acetic acid (95:3:2 v/v/v). The elution samples were collected separately in centrifuge tubes. Each sample was evaporated to dry then reconstituted in 0.5 mL acetone/water/acetic acid (70:29.5:0.5 v/v/v) for HPLC-FLD analysis.

2.2 Cell culture

The human colon adenocarcinoma cell line, Caco-2 (HTB-37™), was obtained from the American Type Culture Collection (ATCC®) at passage 18 (LGC Standards, Middlesex UK) and propagated in 25 cm² (Corning® 430639) and 75 cm² (Corning® 430641) polystyrene culture flasks using Eagle's Minimum Essential Medium (EMEM). Media containing 1g L⁻¹ D-glucose (30-2003, LGC Standards, Middlesex UK) supplemented with 15% (v/v) fetal bovine serum (F7524), 19.6 mL L⁻¹ L-glutamine (200 mM; G7513), 10 mL L⁻¹ penicillin-streptomycin (10,000 U mL⁻¹-10 mg mL⁻¹; P0781), 10 mL L⁻¹ MEM non-essential amino acid solution (100x; M7145) and 1 mL L⁻¹ amphotericin B (250 µg mL⁻¹; A2942). The incubation temperature was maintained at 37°C with an atmosphere of 95% air and 5% carbon dioxide (CO₂). At 2 or 3 day intervals growth media was replenished and at 80% confluence cells were detached from the flask surface using 0.25% trypsin-EDTA solution (T4049). For experiments cells were cultured in 24 mm Transwell® plates on a 4.67 cm², 0.4 µm pore polycarbonate membrane insert (Corning® 3412) at a density of 6.43 x 10⁴ cells cm⁻². Growth media contained 10% fetal bovine serum (v/v) in EMEM supplemented as

described previously, incubation conditions remained unchanged. Experiments were performed between 21 and 23 days after seeding on Transwell® membrane inserts.

2.2.1 Validation of cell membrane integrity

The integrity of the Caco-2 monolayer in each Transwell® insert was confirmed by measuring the trans-epithelial electrical resistance (TEER) of the cells using a Millicell ERS-2 Electrical Resistance System (Millipore, Watford UK). TEER value is indicative of the monolayer condition, a low value implying less well formed tight junctions between the cells which may increase the paracellular transport of a compound across the membrane.

Readings were taken at three positions per insert with the mean \pm SEM being calculated for each well. The mean resistance of a blank insert, i.e. with no cells, was subtracted from the mean of each Transwell® insert containing cells to provide the resistance of the cell monolayer.

2.2.2 Assay protocol

Initially cells were washed to remove traces of cell culture media in a process that involved aspiration of media from apical and basolateral compartments and replacement with 2 mL of pre-warmed transport buffer. The solution was immediately aspirated and replaced with a further 2 mL of transport buffer before being returned to the incubator for 30 min at 37°C, 5% CO₂ to facilitate tight junction formation between cells. Following incubation trans-epithelial electrical resistance (TEER) was measured. The transport buffer was aspirated and replaced with a further 2 mL of transport buffer in the basolateral compartment and 2 mL of control or test solution in the apical compartment. Transwell® plates were returned to the incubator,

for up to 55 minutes (refer to section 2.3.2, 2.3.3 and 2.3.4 for the specific duration of each assay) at 37°C, 5% CO₂.

Upon removal from the incubator the trans-epithelial electrical resistance was measured in each insert before collection of apical and basolateral samples. Cells were washed with the addition of 2 mL of transport buffer in each compartment to ensure collection of all analytes. Cells were then detached from the membrane using 1 mL of 1 M sodium hydroxide (71686) solution per insert, and placing the Transwell® plate on a rotating shaker for 30-40 min. Upon collection of the detached cells the solution was neutralised by the addition of 1 mL of 1 M hydrochloric acid (Fisher Scientific, H/1200/PB17) solution. All samples were stored at -20°C until required for analysis.

2.3 Analytical methods

2.3.1 Validation

Protocol validation was based upon the International Union of Pure and Applied Chemistry (IUPAC) harmonised guidelines for single-laboratory validation of methods of analysis (Thompson *et al.* 2002).

2.3.2 *In vitro* epicatechin transport and methylation

All pure compound epicatechin is the 2R, 3R enantiomer (Extrasynthese, 0977 S). One Transwell® plate was allocated per experimental condition to provide six replicates. Two passages of cells, passage 43 and 50, were utilised in the investigation of epicatechin transport and methylation. The cell culture assay duration was 60 minutes.

2.3.2.1 HPLC-DAD-MS² Protocol

2.3.2.1.1 Parameters

An Agilent 1200 series Rapid Resolution System equipped with a Phenomenex Kinetex® C18 2.1 x 150 mm column was employed for the liquid chromatographic separation of analytes. The stationary phase was fitted in a thermostatted column compartment maintained at $35 \pm 0.15^\circ\text{C}$. The mobile phase consisted of solvent A: 0.2% formic acid in water (v/v) and solvent B: 0.2% formic acid in LCMS grade acetonitrile (v/v) (Fisher Scientific, A/0638/17). The solvent flow was maintained at 0.3 mL min^{-1} throughout the following gradient schedule of solvent B, briefly: 0 min, 5%; 5.8 min, 5%; 35 min, 30%; 37.4 min, 95%; 41 min, 95%; 44.6 min, 5%; and 53.1 min, 5%. The diode array detector was set to measure signal intensity at 220 nm and 280 nm each with a bandwidth of 8 nm. Mass spectrometric detection of analytes was performed using the Agilent 6410 Triple Quadrupole LC/MS system. In negative electrospray ionisation mode the instrument parameters were as follows: gas temperature and flow 350°C , 11 L min^{-1} ; nebuliser gas pressure 30 psi; capillary voltage, -4000 V ; and delta EMV 400 V . The analytical method run time was divided into 4 segments; the first being a window of 0 to 2 min whereby flow to the mass spectrometer was diverted to waste so as to reduce the transfer of salts which may otherwise interfere with analyte detection and quantification by causing increased background noise and suppression of ionisation. The remaining time segments, along with analytical parameters for compound quantification and qualification are shown in Table 2.1.

Multiple reaction monitoring extraction from total ion counts and peak area integration for each compound was performed using Agilent MassHunter Workstation Software version B.03.01.

2.3.2.1.2 Linearity, precision and limit of quantification

Initially six calibration standard solutions were prepared in the range of 0-50 μM and analysed in triplicate. Briefly, 1 mg of epicatechin was dissolved in a 1 % solution of dimethyl sulfoxide (DMSO) (60153) in water (v/v). Serial dilutions were prepared using water to closely match the sample matrix. For each concentration the mean peak area \pm standard error of the mean (SEM) ($n=3$) was plotted against concentration; linear regression analysis was performed to calculate the adjusted R-square value and gradient of the calibration curve. The relative standard deviation (RSD) of replicate peak areas was calculated for each concentration to assess precision of the analysis at each concentration. Limit of detection and limit of quantification was defined as 6x and 10x respectively, the mean of replicate zero concentration solutions.

2.3.2.1.3 Sample preparation for HPLC-DAD-MS² analysis

Samples were vortexed to ensure homogeneity then centrifuged at 17,000 $\times g$ for 5 min at room temperature to remove cellular matter. Supernatant was collected into a 1.8 mL cryogenic vial for storage at -20°C , and 1 mL passed through a polytetrafluoroethylene (PTFE) membrane syringe filter, 17 mm, 0.2 μm (Chromacol, 17-SF-02(T)) pre-conditioned with 1 mL methanol (Fisher Scientific, M/4058/17) followed by 1 mL water. A volume of filtered sample (180 μL) was added to an amber micro-vial along with 20 μL of 16.4

μM taxifolin (Extrasynthese, 1036), final concentration 1.6 μM , chosen as a suitable internal standard for the relative identification of analytes.

Time segment	Retention time (min)	Compound	Precursor ion (m/z)	Quantification			Qualification		
				Product ion (m/z)	Fragmentor voltage (V)	Collision energy (V)	Product ion (m/z)	Fragmentor voltage (V)	Collision energy (V)
2	13.0	Catechin	289.0	244.8	120	8	202.9	120	15
3	17.4	Epicatechin	289.0	245.0	120	8	203.0	120	15
4	21.6	3'-O-methyl-epicatechin	303.1	136.8	110	12	165.0	110	10
4	23.4	4'-O-methyl-epicatechin	303.1	136.8	110	12	165.0	110	12
4	22.5	Taxifolin	303.1	284.9	110	15	124.9	110	2

Table 2.1: Mass spectrometry parameters for quantification and qualification of epicatechin, methylated epicatechin metabolites and taxifolin

2.3.3 Dark chocolate extract inhibits sucrose hydrolysis in the Caco-2 cell model

Cells between passage 25 and 49 were utilised in the investigation of sucrose hydrolysis. A minimum of three replicates was allocated per experimental condition. The cell culture assay duration was 20 minutes

2.3.3.1 Glucose oxidase/peroxidase assay

The rate of sucrose hydrolysis was initially quantified by determination of glucose concentration in the sample and dividing this by the incubation time. The initial method for measuring glucose concentration was the glucose oxidase/peroxidase assay in which glucose oxidation to gluconic acid and hydrogen peroxide is catalysed by glucose oxidase/peroxidase from *Aspergillus niger* (EC 1.1.3.4) (49180). In the presence of hydrogen peroxide, horseradish peroxidase (EC 1.11.1.7) (P8125) catalyses the oxidation of o-dianisidine (D9154) to form a brown colour, addition of sulphuric acid (Fisher Scientific, S/9160/PB17) to the reaction mix changes the wavelength of absorbance and thus a colour change to pink is observed. The absorbance of the solution was measured at 540 nm. The Caco-2 cell assay protocol was developed based on Michaelis-Menten kinetic analysis of sucrose hydrolysis using this method. Initial inhibition assays using a cocoa powder extract provided by Nestlé revealed an inhibitory effect on enzymes of the glucose oxidase/peroxidase assay. To overcome this problem a solid phase extraction procedure using hydrophilic-lipophilic balanced reversed-phase cartridges (Waters, 186003849) was investigated, however this proved unsuccessful. An alternative method using universally radiolabelled [^{14}C]-sucrose and liquid scintillation counting was proposed as

a more suitable method. This also had the benefit of allowing distinction between glucose produced from sucrose hydrolysis and residual glucose from the cell culture media which may cause inaccurate determinations of glucose concentration.

2.3.3.1.1 Linearity, precision, limit of detection and limit of quantification

Six standard calibration solutions were prepared in the range of 0-80 $\mu\text{g mL}^{-1}$ and analysed in triplicate. The concentration of each calibration solution was plotted against the mean absorbance at 540 nm \pm standard error of the mean (SEM) (n=3). Linear regression analysis was performed, with the y-intercept fixed at zero, to calculate the gradient and adjusted R-square value of the calibration curve. The relative standard deviation (RSD) of replicate absorbance values was calculated for each concentration to assess precision of the analytical method. Limit of detection and limit of quantification, as defined by The Food and Drug Administration Validation of Analytical Procedures, were calculated as shown in Equation 2.1 and Equation 2.2.

$$\text{Limit of detection} = \frac{3.3\sigma}{m} \quad \text{Equation 2.1}$$

$$\text{Limit of quantification} = \frac{10\sigma}{m} \quad \text{Equation 2.2}$$

Where σ = standard deviation of blank samples and m = gradient of the calibration curve.

2.3.3.1.2 Sample preparation for glucose oxidase/peroxidase assay

Samples were centrifuged at 17,000 $\times g$ for 5 minutes to remove cell debris and 0.06 mL of the supernatant added to a microplate well. To commence

the assay, 0.12 mL of assay reagent containing 12.5 units mL⁻¹ of glucose oxidase/peroxidase, 2.5 purpurogallin units mL⁻¹ of peroxidase and 2.5 µg mL⁻¹ o-dianisidine, was added to the microplate well containing cell culture sample and incubated for 30 min at 37°C. The reaction was stopped by addition of 0.12 mL of 6 M sulphuric acid. The absorbance of each well was measured at 540 nm using a microplate reader.

2.3.3.1.3 Calculations

The absorbance of each sample was initially corrected against a blank and the concentration of glucose calculated from the linear regression equation determined for the absorbance calibration curve. The molar concentration was converted to rate of product formed per minute by division of the total molar concentration of glucose by the incubation time. The rate of product formation was equated to the rate of sucrose hydrolysis. The kinetic properties of sucrose hydrolysis were measured using the Michaelis-Menten model. A plot of rate of sucrose hydrolysis as a function of initial sucrose concentration was prepared and nonlinear regression analysis based on the Michaelis-Menten equation performed (Equation 2.3). Rearrangement of the equation permitted the maximum velocity (V_{max}) and Michaelis constant (K_M) to be determined (Equation 2.4 and Equation 2.5).

$$V_0 = \frac{V_{max}[S]}{K_M + [S]} \quad \text{Equation 2.3}$$

$$V_{max} = \frac{V_0(K_M + [S])}{[S]} \quad \text{Equation 2.4}$$

$$K_M = \frac{V_{max}[S]}{V_0} - [S] \quad \text{Equation 2.5}$$

2.3.3.2 Liquid scintillation counting

Liquid scintillation counting was performed using a Packard 1600 TR Liquid Scintillation Analyser. The carbon-14 isotope emits high energy beta radiation and has a half-life of 5730 years which promotes sample stability allowing repeated sample analysis. Use of a liquid scintillation cocktail (National Diagnostics/Fisher Scientific, SCN-220-110F) containing an energy-collecting solvent and a phosphor facilitates the measurement of radiation emissions, referred to as disintegrations. One disintegration per second (DPS) equates to 1 Becquerel (Bq), the standard international unit of radioactivity, and can be converted to the commonly used unit of Curie (Ci). The radioactive component emits a beta particle, the energy from which is ultimately absorbed by the phosphor. The phosphor becomes excited from the absorption of energy causing it to emit light energy that is detected by the scintillation counting equipment. Each phosphor emits one photon but multiple phosphors may be excited by the emission of one beta particle creating a distinct intensity of light that is measured as a pulse. The pulses are expressed as the number of counts per minute (CPM) which can be converted to the number of disintegrations per minute (DPM) based on the efficiency of energy transfer of the liquid scintillation cocktail and subsequently converted to units of radioactivity. In the current investigation the protocol for liquid scintillation counting used a minimum ratio of scintillant to sample volume of 1: 10 to ensure sufficient energy transfer and therefore permit the equation of one count per minute to one disintegration per minute. Counts were converted to units of Curie based on the relation of 1 Curie being equal to 2.2×10^{12} disintegrations per minute. Counting efficiency of the equipment and background radiation was corrected for. The counts per

minute determined for a carbon-14 calibration solution, relative to its specified value was calculated, and the correction factor applied to the counts per min for each calibration solution. The counts per minute of a background calibration solution were then subtracted from the counts per minute measured for each sample.

Because the [^{14}C]-sucrose (Perkin-Elmer, NEC100X050UC) was universally labelled the basolateral radioactivity measured was a combination of glucose and fructose liberated during sucrose hydrolysis; apical radioactivity was the sum of sucrose, glucose and fructose. Because of this glucose and fructose could not be quantified in the apical compartment. Initial analysis using the glucose oxidase/peroxidase method had demonstrated that relatively only a small amount of glucose was detected in the basolateral compartment, the majority was present in the apical compartment. To overcome this issue a high pressure liquid chromatography (HPLC) separation with electrochemical detection was developed to quantify mono- and disaccharides. After development of the HPLC-IPAD method the presence of sucrose in the basolateral compartment of Transwell® plates was observed rendering the use of [^{14}C]-sucrose ineffective for quantification of sucrose hydrolysis.

Concentration of glucose and fructose was not quantified using this method therefore a calibration curve was not produced.

2.3.3.2.1 Sample preparation for liquid scintillation counting

Samples were vortexed to ensure homogeneity then a volume, 0.25 mL for apical samples and sample blanks, and 0.5 mL for basolateral and cell samples, was added to liquid scintillation vials containing 5 mL of scintillant.

Light emission was counted for 10 min per vial and the number of counts per minute calculated by the equipment software.

2.3.3.2.2 Calculations

Radioactivity in the basolateral compartment of Transwell® plates was converted from counts per minute to Curie and expressed as a percentage of the total (apical + basolateral + cellular) radioactivity in the well.

2.3.3.3 HPLC-Integrated Pulsed Amperometric Detection (IPAD)

Carbohydrate separation is based on the principle of anion exchange chromatography. In water at 25°C the dissociation constants of glucose and fructose are >12 ; a strong sodium hydroxide gradient, $\text{pH}>12$, causes ionisation of the sugars and permits separation of glucose, fructose and sucrose. Electrochemical detection employs the use of three electrodes, a gold working electrode, a combined pH-silver/silver chloride reference electrode and a titanium counter electrode. Application of a repeating sequence of potentials across the working and reference electrodes causes oxidation of the gold electrode. The titanium counter electrode receives the flow of electrons to ensure the potential applied across the working and reference electrodes remains unaffected by current flowing from the gold electrode. Anionic sugar molecules are attracted to the oxidised gold electrode and a transfer of electrons results in the detection of current. The current is integrated over time which gives a measure of charge that is expressed in units of Coulombs (C).

Separation of mono- and disaccharides was achieved using a Dionex CarboPac PA20 column (3 x 150 mm), with Dionex CarboPac PA20 guard column (3 x 30 mm), fitted into a thermostatted column compartment on a

Dionex LC system with autosampler (AS50), gradient pump (GS50), and electrochemical detector (ED50), controlled by Chromeleon 6 software. Sonicated solvents A) water, and B) 200 mM sodium hydroxide (71686) were stored under nitrogen gas for the duration of use and replaced with fresh solutions at least once per week. Solvent flow rate was maintained at 0.4 mL min^{-1} throughout the following gradient schedule of solvent B in solvent A: 0 min, 30%; 10 min, 50%; 17 min, 30%; 27 min, 30%. The repeating sequence of potentials was as follows: 0.00 s, 0.05 V; 0.20 s, 0.05 V; 0.40 s, 0.05 V; 0.41 s, 0.75 V; 0.60 s, 0.75 V; 0.61 s, -0.15 V; 1.00 s, -0.15V; integration of current over time began at 0.20 s and ended at 0.40 s. The electrode cleaning step from 0.41 s to 1.00 s firstly ensured full oxidation of analytes at the gold electrode with the application of a high positive potential, then repulsion of all analytes upon application of a negative potential. The autosampler temperature was maintained at 10°C and the column oven at 30°C .

2.3.3.3.1 Linearity, precision, limit of detection and limit of quantification

Ten standard calibration solutions were prepared in the range of 0-500 μM and analysed in triplicate. The molar concentration of each calibration solution was plotted against the mean peak area, expressed in units of $\text{nC}\cdot\text{min}$, \pm standard error of the mean (SEM) ($n=3$). Linear regression analysis was performed, with the y-intercept fixed at zero, to calculate the gradient and adjusted R-square value of the calibration curve. The relative standard deviation (RSD) of replicate calibration solutions was calculated for each concentration to assess precision of the analytical method. Limit of

detection and quantification were calculated as shown in [Equation 2.1](#) and [Equation 2.2](#).

2.3.3.3.2 Sample preparation for HPLC-IPAD

Samples were centrifuged at 17,000 x g for 5 minutes to remove cell debris. Supernatant was passed through a 1.7 mm, 0.2 µm PTFE filter pre-conditioned with 1 mL methanol (Fisher Scientific, M/4056/17) followed by 1 mL water. A volume of filtered sample (180 µL) was added to a polypropylene HPLC vial along with 20 µL of 1 mM fucose (F2252) used as an internal standard.

2.3.3.3.3 Calculations

The concentration of each glucose and fructose was calculated from the linear regression equation determined from the peak area calibration curve for each monosaccharide. The sum of apical and basolateral molar concentration for each monosaccharide was calculated and corrected against the appropriate sample blank. To account for any potential loss of glucose and fructose through cellular glycolysis the mean monosaccharide concentration was derived from the individual total concentrations of glucose and fructose. The rate of product formation was equated to the rate of sucrose hydrolysis and was calculated by dividing the total molar concentration by incubation time.

2.3.4 Glucose transport inhibition by a flavanol-rich dark chocolate extract

All glucose used in the investigation was the D- isomer. Cells between passage 33 and 50 were utilised in the investigation of [¹⁴C]-glucose

transport. One Transwell® plate was allocated per experimental condition to provide six replicates. The cell culture assay duration was 30 minutes.

2.3.4.1 Liquid Scintillation Counting

Liquid scintillation counting was performed using a Packard 1600 TR Liquid Scintillation Analyser. A universally labelled [¹⁴C]-glucose isotope (Perkin-Elmer, NEC042V250UC) was used in the measurement of apical to basolateral glucose transport and cellular uptake. To prevent residual glucose from the cell culture media interfering with determination of basolateral and cellular glucose concentration, each solution was supplemented with a fixed volume of radiolabelled glucose containing a specific radioactivity. The benefits of this were two-fold: firstly glucose analysis was precise and sensitive, and secondly minimal pre-analytical sample preparation was required due to lack of interference from other compounds in the sample matrix.

The carbon-14 isotope emits high energy beta radiation and has a half-life of 5730 years which promotes sample stability allowing repeated sample analysis. Use of a liquid scintillation cocktail containing an energy-collecting solvent and a phosphor facilitates the measurement of radiation emissions, referred to as disintegrations. One disintegration per second (DPS) equates to 1 Becquerel (Bq), the standard international unit of radioactivity, and can be converted to the commonly used unit of Curie (Ci).

The radioactive component emits a beta particle, the energy from which is ultimately absorbed by the phosphor. The absorption of energy excites the phosphor causing it to emit light energy that is detected by the scintillation counting equipment. Each phosphor emits one photon but multiple

phosphors may be excited by the emission of one beta particle creating a distinct intensity of light that is measured as a pulse. The pulses are expressed as the number of counts per minute (CPM) which can be converted to the number of disintegrations per minute (DPM) based on the efficiency of energy transfer of the liquid scintillation cocktail and subsequently converted to units of radioactivity. In the current investigation the protocol for liquid scintillation counting used a minimum ratio of scintillant to sample of 10:1 (v/v) to ensure sufficient energy transfer and therefore permit the equation of one count per minute to one disintegration per minute. Counts were converted to units of Curie based on the relation of 1 Curie being equal to 2.2×10^{12} disintegrations per minute. Counting efficiency of the equipment and background radiation was corrected for. The counts per minute determined for a carbon-14 calibration solution, relative to its specified value was calculated, and the correction factor applied to the counts per min for each calibration solution. The counts per minute of a background calibration solution were then subtracted from the counts per minute measured for each sample.

2.3.4.1.1 Linearity, precision and limit of quantification

Ten standard calibration solutions were prepared in the range of 0-0.014 μCi (0-30,000 CPM) and analysed in triplicate. Briefly, 2 μL of 0.1 $\mu\text{Ci } \mu\text{L}^{-1}$ [^{14}C]-glucose stock solution was diluted to 2 mL ($0.1 \mu\text{Ci mL}^{-1}$) and serial dilutions prepared using transport buffer solution. Based on the specific activity of the stock solution ($319 \mu\text{Ci } \mu\text{mol}^{-1}$) the molar content of each calibration solution was calculated and plotted against the mean number of counts per minute \pm standard error of the mean (SEM) (n=3). Linear regression analysis was

performed with the y-intercept fixed at zero to calculate the gradient and adjusted R-square value of the calibration curve. The relative standard deviation (RSD) of replicate counts per minute was calculated for each calibration solution to assess precision of the analysis at each molar amount. The limit of quantification was defined as the concentration equivalent to 10x the standard deviation of background corrected blank samples (Currie 1968).

2.3.4.1.2 Sample preparation for liquid scintillation counting

Refer to section 2.3.3.2.1, page 62.

2.3.4.1.3 Calculations

Each sample was corrected for background radiation and the content of [U-¹⁴C]-glucose calculated from the linear regression equation determined for the calibration curve. The content was converted to concentration per millilitre and, based on the ratio of radiolabelled glucose to non-radiolabelled glucose in the corresponding transport solution, the concentration per millilitre of non-radiolabelled glucose was calculated. Total glucose concentration per millilitre was calculated as the sum of radiolabelled and non-radiolabelled glucose, then converted to the concentration per litre. The rate of apical to basolateral transport and cellular uptake ($\mu\text{M min}^{-1}$) was calculated from the total glucose concentration in either the basolateral samples or cellular samples, respectively, divided by total incubation time.

The kinetic properties of apical to basolateral glucose transport and cellular uptake were measured using the Michaelis-Menten model. A plot of transport rate as a function of initial glucose concentration was prepared and nonlinear regression analysis based on the Michaelis-Menten equation was

performed (Equation 2.3). Rearrangement of the equation permitted the maximum velocity (V_{\max}) and Michaelis constant (K_M) to be determined (Equation 2.4 and Equation 2.5).

2.4 Statistical analysis

All statistical analysis of data was performed using IBM SPSS Statistics 19. Levene's homogeneity of variances was initially executed to calculate whether data variation within each group was significant. Where significant variations were measured a non-parametric test to determine significant differences between groups was applied. If data variations were not significant a parametric test was utilised. Data are expressed as mean values \pm standard error of the mean (SEM), and differences were considered statistically significant when $p < 0.05$, unless otherwise stated.

Chapter 3 *In vitro* epicatechin transport and methylation

3.1 Abstract

The investigation of epicatechin transport and methylation presented here was carried out to determine whether transport of pure epicatechin across the Caco-2 cell monolayer was significantly affected by co-incubation with sucrose, glucose or fructose; and whether transport of epicatechin from a flavanol-rich dark chocolate extract was significantly different from that of the individual compound. The results of this study reveal that whilst epicatechin transport is not significantly affected by the presence of higher molecular weight oligomers and polymers in a dark chocolate extract, *O*-methylation of epicatechin is significantly attenuated. The presence of sucrose significantly improved epicatechin transport but did not elicit a significant effect on the formation of 3'- and 4'-*O*-methylated metabolites. Conversely co-incubation with glucose and fructose did not significantly affect epicatechin absorption but did significantly reduce the synthesis of 3'-*O*-methylated metabolites. The role of paracellular permeability in epicatechin transport and the potential for competitive inhibition of catechol-*O*-methyltransferase by oligomeric flavanols and by glycolytic enzymes requiring the magnesium divalent cation is discussed.

3.2 Introduction

There are several reports in the literature of epicatechin bioavailability studies undertaken in humans and animals and absorption studies in cell culture models using flavanol-rich foods, beverages, plant extracts and the pure compound. Effects of the food matrix on gastrointestinal absorption and metabolism of epicatechin from cocoa in human and animal subjects has been investigated with particular attention to the carbohydrate and protein content, both of which are of significance to confectionary and beverages containing cocoa flavanols. The physical form in which the cocoa is ingested appears to significantly affect the pharmacokinetic properties of epicatechin with maximum concentration (C_{max}) of serum epicatechin greater in human subjects following ingestion of a cocoa beverage compared with a dark chocolate bar containing an equivalent amount of total flavanols (Neilson *et al.* 2009). Although this could be a consequence of the different cocoa butter content, and therefore fat content, of the bars and beverages. The ability of carbohydrates to enhance epicatechin absorption has been proposed but the evidence is inconclusive. Carbohydrate content of a meal and plasma flavanol area under the curve (AUC) was reported following an investigation of volunteers consuming either table sugar, bread or grapefruit juice immediately before consumption of a sugar-free flavanol-rich cocoa beverage (Schramm *et al.* 2003). Each test meal significantly increased plasma flavanol (epicatechin + catechin) 0-8 hour AUC relative to consumption of the cocoa beverage only. Table sugar and bread also increased the C_{max} . Augmented plasma epicatechin C_{max} and AUC following consumption of a dark chocolate bar containing sucrose compared with an

equivalent bar containing maltitol has also been reported (Rodriguez-Mateos *et al.* 2012). However the authors noted that the perceived enhancement induced by sucrose may be a consequence of reduced epicatechin absorption brought about by the presence of maltitol. A sugar-free flavanol equivalent control would be required to confirm the result.

Neilson *et al.* (2009) speculated that the presence of sucrose may enhance epicatechin bioavailability. This was based on the highest C_{max} following consumption of a high-sucrose confectionary bar compared with a low-sucrose equivalent, and a sucrose-containing beverage compared with sucrose-free beverage. However neither of these results was significant. Furthermore non-significant results were reported using a rat model to compare the effect of sucrose and milk-protein compared with a reference dark chocolate on epicatechin pharmacokinetics (Neilson *et al.* 2010).

These studies focus on the effect of sucrose on intestinal absorption of free epicatechin however it has been reported that following consumption of dark chocolate epicatechin in its free form is not detected in the plasma of human subjects (Actis-Goretta *et al.* 2012). The presence of free epicatechin in the plasma of human subjects following cocoa intake is controversial. In addition to the intervention studies discussed here others, including those based on animal models, have quantified free epicatechin along with methylated and non-methylated conjugates in the plasma of subjects (Baba *et al.* 2000b, Baba *et al.* 2000a, Baba *et al.* 2001a). Epicatechin and its metabolite concentrations in the plasma and urine of rats was examined following ingestion of increasing doses of pure epicatechin. The results were compared with concentrations following almost identical doses of epicatechin administered in a cocoa powder (Baba *et al.* 2001a). The

authors reported significantly lower plasma concentrations of methylated and non-methylated epicatechin conjugates post-ingestion of the cocoa powder compared with the pure compound. Conversely, free epicatechin concentration in plasma was significantly higher following cocoa powder treatment compared with the pure compound. Analysis of free epicatechin and its metabolite concentrations in urine revealed no significant differences between the two treatments suggesting an attenuated rate of epicatechin absorption from the gut lumen and/or metabolism within the intestinal epithelial cells.

The observation that free epicatechin concentration is higher and metabolite bioavailability is lower from cocoa than from the pure compound may be explained by the presence of higher molecular weight flavanols in the cocoa. Perfusion studies of rat small intestine with mixtures of flavanol oligomers have resulted in the detection of low levels of the B2 dimer when co-incubated with either a procyanidin tetramer or B5 dimer (Spencer *et al.* 2001, Appeldoorn *et al.* 2009b). Methylated dimer has also been detected along with free epicatechin following incubation of rat small intestine with a mix of B2 and B5 dimers (Spencer *et al.* 2001); although the detection of free epicatechin following incubation of flavanol oligomers has been contested (Donovan *et al.* 2002, Ottaviani *et al.* 2012).

Detection of methylated dimer but not methylated epicatechin suggested inhibition of the enzyme catechol-*O*-methyltransferase (COMT) within the cells. Dose-dependent reduction of COMT activity with epicatechin as the substrate, in the presence of increasing concentration of dimers was observed. Concurrently dose-dependent formation of *O*-methylated B2 and B5 dimers was noted at concentrations up to 300 μM of dimers. Above this

concentration methylation of both dimers decreased indicating that inhibition of COMT activity $\leq 300 \mu\text{M}$ is competitive whereas $>300 \mu\text{M}$ inhibition becomes non-competitive. Epicatechin and quercetin have been reported to inhibit the activity of human liver cytosolic COMT using 4-hydroxyestradiol as substrate, both flavonoids demonstrated a mixed mechanism of inhibition with non-competitive inhibition occurring at high concentrations of each (Nagai *et al.* 2004). The authors hypothesised that non-competitive inhibition is in part due to the presence of many COMT substrates that increases the formation of S-adenosyl-L-homocysteine (SAH) from S-adenosyl-L-methionine (SAM), SAH itself being a non-competitive inhibitor of COMT. In addition increased utilisation of SAM would reduce its availability as a methyl group donor.

Based on *in vivo* observations the objective of the investigation described in this chapter was to determine whether incubation of a flavanol-rich dark chocolate extract and the saccharides sucrose, glucose and fructose were able to modify *in vitro* epicatechin absorption and methylation using Caco-2 cells as a model of the human small intestine.

3.3 Results

3.3.1 HPLC-DAD-MS² Validation

3.3.1.1 Linearity, precision and limit of detection and quantification

Epicatechin calibration solutions in the range 0-50 μM were analysed in triplicate and the mean peak area \pm standard error of the mean (SEM) plot against concentration ([Figure 3.1](#)). Triplicate zero concentration epicatechin solutions generated small ion counts of which the peak areas were integrated. The signal to noise ratio of these peaks was calculated to be less than 10; subsequently the values were substituted for zero and for linear regression analysis the y-axis intercept was set to zero. An adjusted R-square value of 1.0 was calculated suggesting good proportionality of concentration with peak area.

The relative standard deviation was calculated for each of the five concentrations ranging from 1-50 μM ; because the zero concentration peak areas had been corrected to zero the mean and standard deviation was calculated as zero. For all other concentrations relative standard deviation ranged from 10-20 %, becoming smaller with increasing concentration; precision of the protocol was considered acceptable.

The limit of detection was defined as the smallest peak area that was significantly different from the mean peak area of replicate zero concentration solutions ($p < 0.05$). This equated to a concentration of 113 nM. The limit of quantification was calculated as the concentration at which the peak area was 10x that of the mean peak area of replicate zero concentration solutions. This equated to 188 nM.

Concentration of epicatechin and methylated metabolites were calculated by rearrangement (Equation 3.2) of the linear regression equation obtained from the epicatechin calibration curve (Equation 3.1). The concentrations of methylated metabolites were calculated as epicatechin equivalents assuming a relative response factor of 1.0.

$$y = mx + c \quad \text{Equation 3.1}$$

$$x = \frac{(y - c)}{m} \quad \text{Equation 3.2}$$

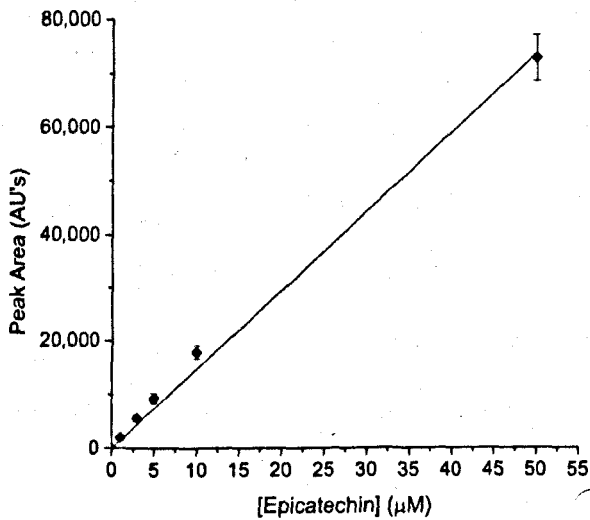


Figure 3.1 Epicatechin calibration curve. Mean \pm SEM (n=3); gradient = 1475, adjusted R-square = 1.0.

3.3.2 Epicatechin transport study

3.3.2.1 Caco-2 cell monolayer validation

Monolayer permeability is of importance when investigating the transport of a compound across the cell membrane. Blank corrected TEER values below 300 ohms may be demonstrative of less well formed tight junctions between the cells. Only cells with TEER values ≥ 300 ohms were used in the investigation presented here (Table 3.1).

For each cell culture assay performed the TEER values were compared to determine whether a significant difference existed between the conditions investigated. In the assay that investigated the effect of a flavanol-rich dark chocolate extract there was no significant difference in TEER values between the two groups consequently it was considered that any significant differences in basolateral epicatechin concentration could be reasonably considered a result of the condition being tested. In the experiment to test the effect of sucrose, glucose and fructose on epicatechin transport and methylation the mean TEER of control cells (250 μM epicatechin) was significantly lower ($p < 0.05$) than the mean TEER of cells used in the test conditions of sucrose, glucose and fructose. Partial correlation analysis controlling for condition showed no significant correlation between basolateral epicatechin concentration and TEER value (Figure 3.2). To avoid this problem replicates should have been distributed throughout the Transwell® plates rather than allocating one plate per condition.

Condition	TEER (Ohms)
250 μ M Epicatechin	416 \pm 12
Dark chocolate extract containing 241 μ M epicatechin	455 \pm 21
250 μ M Epicatechin	291 \pm 12 ^a
250 μ M Epicatechin + 100 mM sucrose	333 \pm 8 ^b
250 μ M Epicatechin + 100 mM glucose	342 \pm 10 ^b
250 μ M Epicatechin + 100 mM fructose	326 \pm 6 ^b

Table 3.1 Blank corrected trans-epithelial electrical resistance (TEER) measured before commencement of each assay. Mean \pm SEM (n=6) for each condition. Different superscript letters represent a significant difference, $p < 0.05$.

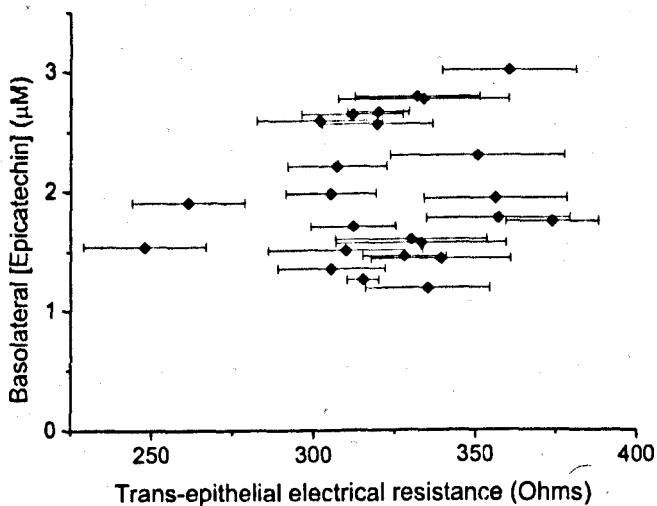


Figure 3.2 Basolateral concentration of epicatechin after the 60 minute incubation, in the absence and presence of 100 mM sucrose, glucose or fructose, as a function of mean \pm SEM (n=3) trans-epithelial electrical resistance per Transwell® insert measured before commencement of the assay.

3.3.2.2 Epicatechin transport and methylation

3.3.2.2.1 Effect of dark chocolate extract

Transport of epicatechin through a Caco-2 cell monolayer was assessed in the absence and presence of a flavanol-rich dark chocolate extract. [Table 3.2](#) shows the flavanol content of Nestlé NOIR Intense 70% cocoa solids and the extract used in the cell culture assay. A HPLC-FLD chromatogram representative of dark chocolate extract containing flavanol monomers to decamer is presented in [Figure 3.3](#). Caco-2 cells were incubated for 60 min with either a flavanol-rich cocoa extract containing 241 μM epicatechin or 250 μM epicatechin standard referred to as the control. TEER values measured after the 60 minute incubation revealed a significant reduction compared with the resistance measured before commencement of the assay ([Figure 3.4A](#)). The mean TEER of cells incubated with the flavanol-rich dark chocolate extract were also significantly lower than the mean control cell resistance post-incubation. The mean TEER did not fall below 300 Ohms in either condition investigated indicating that the monolayer maintained an acceptable level of integrity. The concentration of epicatechin in the basolateral compartment and 3'- and 4'-O-methylated epicatechin in the apical and basolateral compartments was calculated from the epicatechin calibration curve and corrected to account for the limit of quantification (LOQ). Concentrations below the LOQ were considered to be zero. Following incubation with the flavanol-rich dark chocolate extract epicatechin concentration in the basolateral compartment was not significantly different from the control ([Figure 3.4B](#)) however the total concentration of 3'-O-methylated epicatechin (sum of apical and basolateral concentrations) was

significantly lower than the control (Figure 3.5A). Basolateral concentration of 3'-*O*-methyl epicatechin was not significantly different from the control and neither was the total or basolateral concentration of 4'-*O*-methyl epicatechin (Figure 3.5B).

Degree of Polymerisation	Content	
	mg 100 g ⁻¹ FW Dark Chocolate	µg mL ⁻¹ Dark Chocolate Extract
Monomer	68.7	116.7
Dimer	43.2	73.5
Trimer	11.0	18.6
Tetramer	6.8	11.7
Pentamer	2.6	4.6
Hexamer	1.2	2.1
Heptamer	0.6	1.3
Octamer	0.3	0.6
Nonamer	0.2	0.4
Decamer	0.2	0.2

Table 3.2 Flavanol content of the dark chocolate extract used to investigate epicatechin transport and methylation by Caco-2 cells. Values represent the mean of two replicates and are expressed as the quantity (mg) per 100 g fresh weight (FW) of Nestlé NOIR Intense 70% cocoa solids dark chocolate and the amount present in the extract that was incubated with cells.

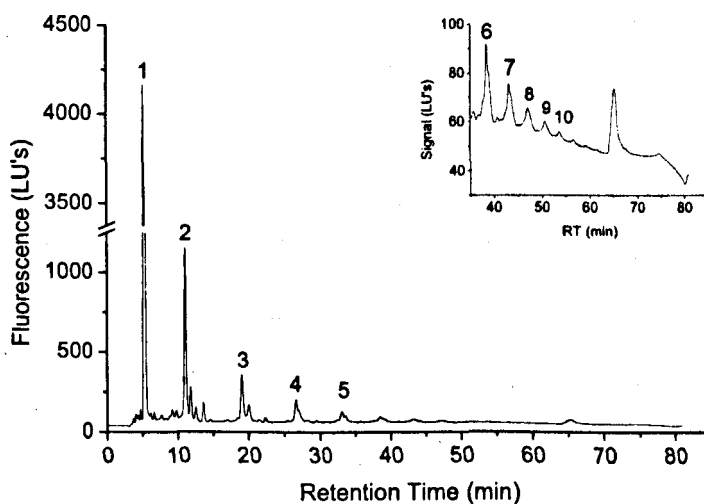


Figure 3.3 HPLC-FLD chromatogram of dark chocolate extract containing 64.3 mg of flavanol monomers 100 g⁻¹ fresh weight of dark chocolate. Peak numbering equates to the degree of polymerisation with 1 = monomer, 2 = dimer etc.

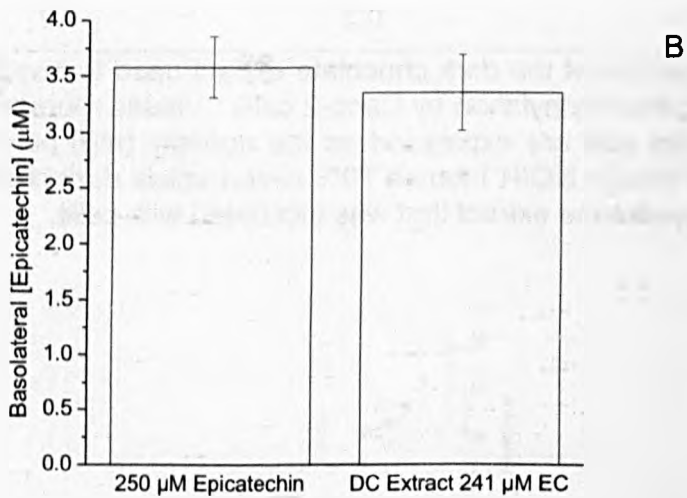
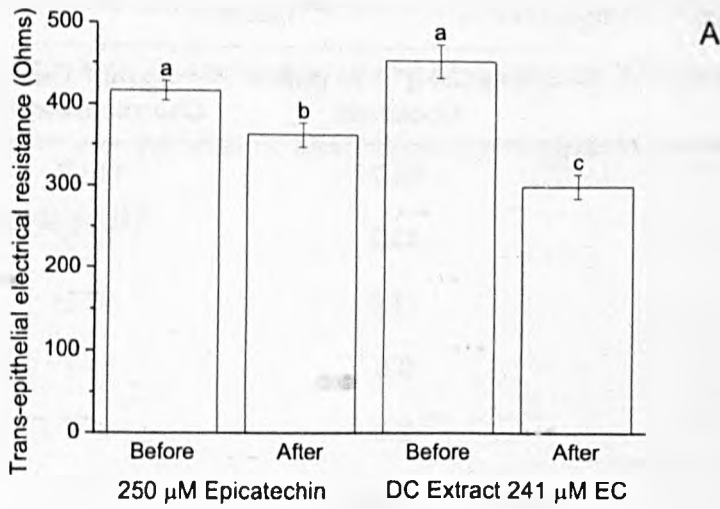


Figure 3.4 A) Blank corrected trans-epithelial electrical resistance (TEER) of Caco-2 monolayers measured before and after the 60 min incubation. Mean \pm SEM (n=6). Different letters represent significant differences, $p < 0.05$. B) Epicatechin concentration in the basolateral compartment of Transwell® plates post-incubation with 250 μ M standard epicatechin (EC) or a flavanol-rich dark chocolate (DC) extract containing 241 μ M EC. Mean \pm SEM (n=6).

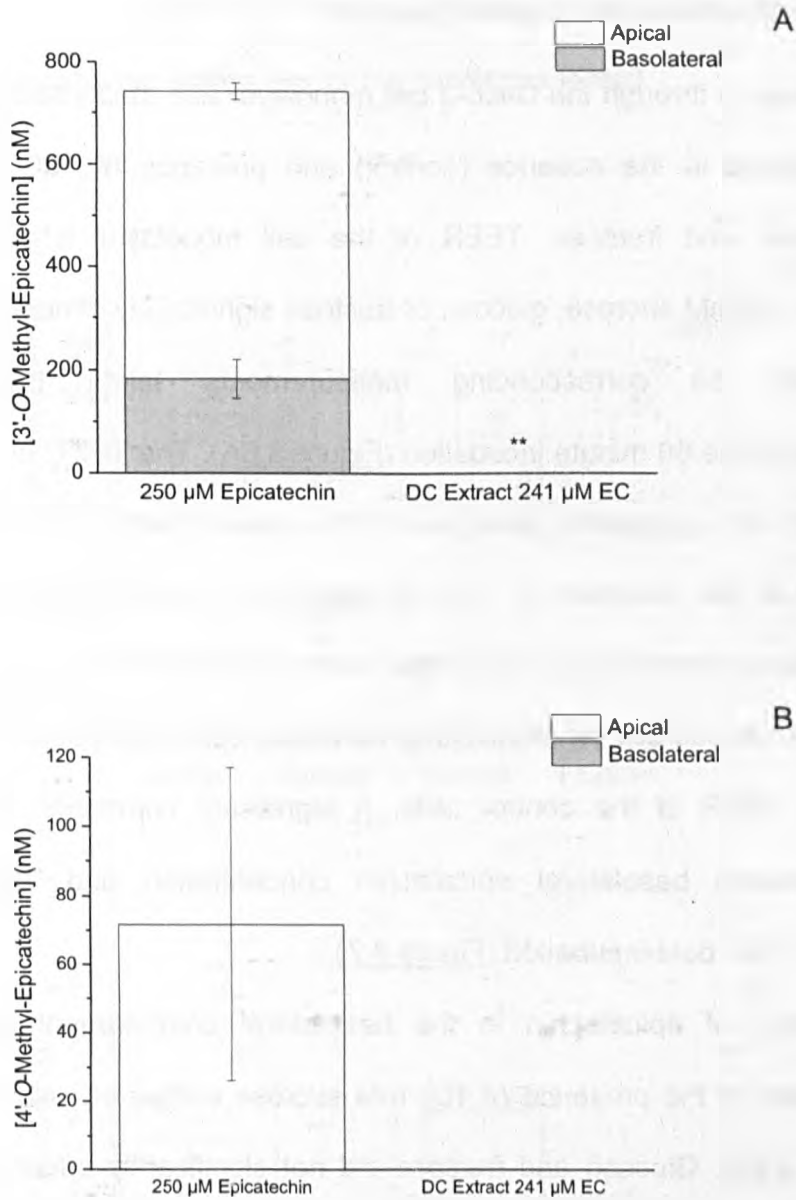


Figure 3.5 A) Concentration of 3'-*O*-methyl epicatechin, and B) 4'-*O*-methyl epicatechin in the apical and basolateral compartments of Transwell® plates after the 60 min incubation with 250 µM standard epicatechin (EC) or a flavanol-rich dark chocolate (DC) extract containing 241 µM EC. Mean ± SEM (n=6). Asterisk denotes a significant difference in the total concentration (sum of apical and basolateral concentrations) compared with 250 µM epicatechin, **p<0.01.

3.3.2.2.2 Effect of sucrose, glucose and fructose

Epicatechin transport through the Caco-2 cell monolayer and methylation by cells was assessed in the absence (control) and presence of 100 mM sucrose, glucose, and fructose. TEER of the cell monolayer following incubation with 100 mM sucrose, glucose or fructose significantly diminished compared with the corresponding measurements taken before commencement of the 60 minute incubation (Figure 3.6A). The TEER of the control cells did not significantly change and the post-incubation TEER of cells incubated in the presence of 100 mM glucose or fructose was not significantly different from the post-incubation control cells TEER. Incubation of cells with 100 mM sucrose did significantly lower the TEER compared with post-incubation TEER of the control cells. A significant correlation was established between basolateral epicatechin concentration and TEER measurements taken post-incubation (Figure 3.7).

The concentration of epicatechin in the basolateral compartment was significantly higher in the presence of 100 mM sucrose compared with the control (Figure 3.6B). Glucose and fructose did not significantly affect the basolateral epicatechin concentration.

Analysis of both apical and basolateral solutions revealed that only in control cells was 3'-*O*-methylated epicatechin metabolites effluxed across the basolateral membrane; 4'-*O*-methylated epicatechin metabolites were only effluxed across the apical membrane in control and sucrose-incubated cells (Figure 3.8A and B). Co-incubation with glucose or fructose significantly reduced the total and apical concentration of 3'-*O*-methyl epicatechin, but not the basolateral concentration, compared with the control; sucrose did not

elicit a significant effect. There was no significant change in 4'-*O*-methylation of epicatechin across any of the conditions tested.

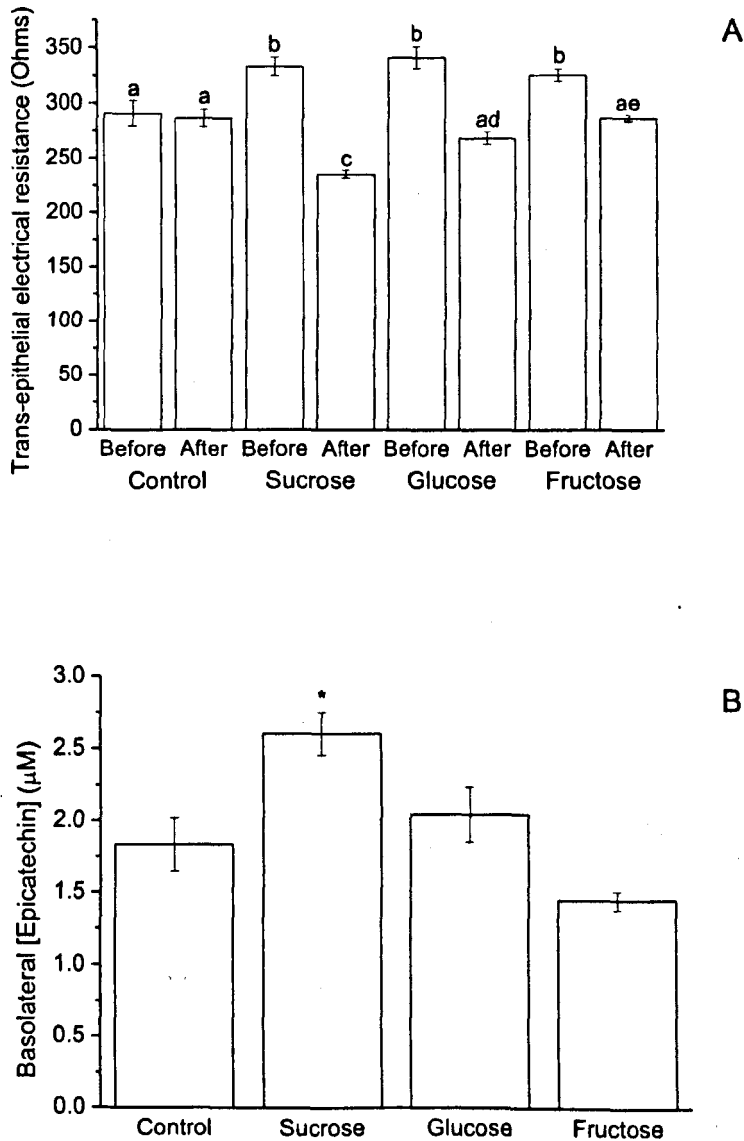


Figure 3.6 A) Blank corrected trans-epithelial electrical resistance of Caco-2 monolayers measured before and after the 60 min incubation in the absence (control) and presence of 100 mM sucrose, glucose or fructose. Mean \pm SEM (n=6). Different letters represent significant differences, $p < 0.05$. B) Epicatechin concentration in the basolateral compartment of Transwell® plates post-incubation. Mean \pm SEM (n=6). Asterisk denotes a significant difference from the control, * $p < 0.05$.

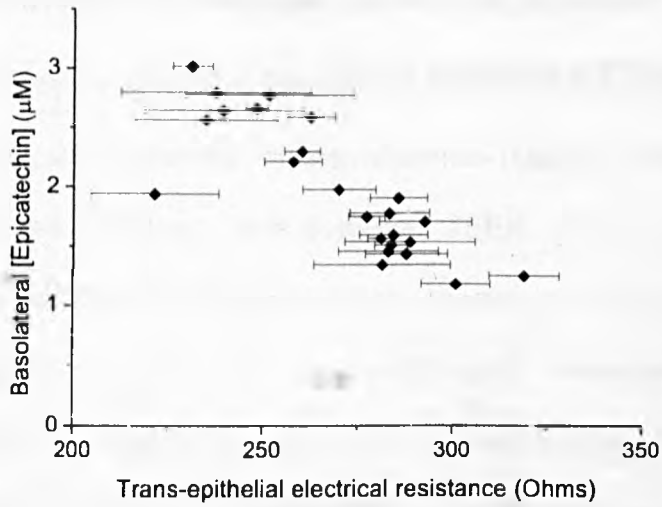


Figure 3.7 Basolateral concentration of epicatechin after the 60 minute incubation as a function of mean \pm SEM ($n=3$) trans-epithelial electrical resistance per Transwell® insert measured post-incubation. Partial correlation analysis controlling for condition revealed a significant correlation between basolateral epicatechin concentration and TEER, $p<0.001$.

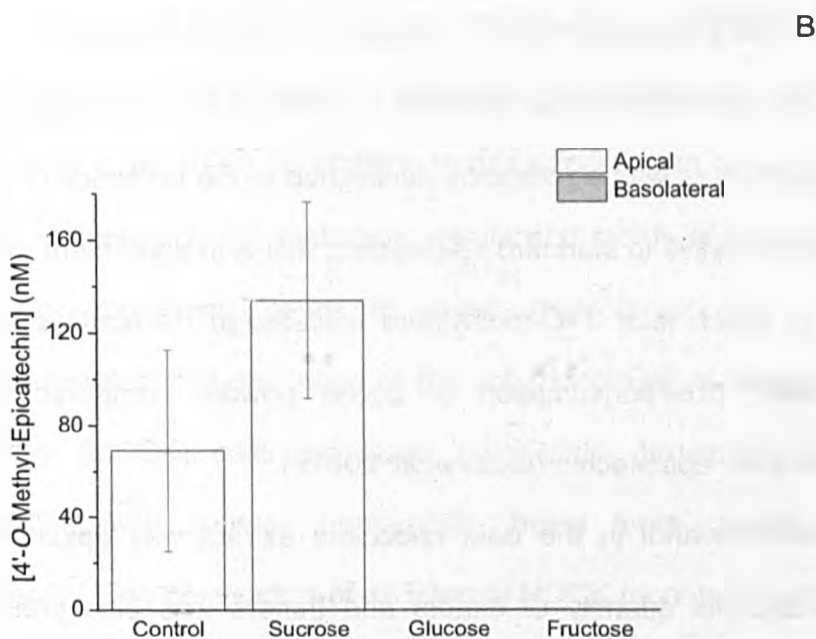
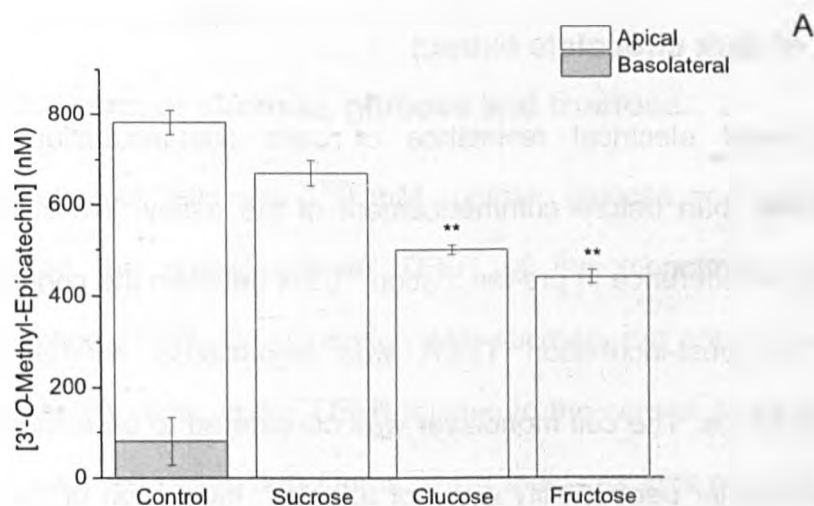


Figure 3.8 A) Concentration of 3'-*O*-methyl epicatechin, and B) 4'-*O*-methyl epicatechin in the apical and basolateral compartments of Transwell® plates after the 60 min incubation in the absence (control) and presence of 100 mM sucrose, glucose or fructose. Mean \pm SEM (n=6). Asterisk denotes a significant difference in the total concentration (sum of apical and basolateral concentrations) compared with the control, **p<0.01.

3.4 Discussion

3.4.1 Effect of dark chocolate extract

The trans-epithelial electrical resistance of cells post-incubation was significantly lower than before commencement of the assay. Whilst there was no significant difference in pre-incubation TEER between the conditions investigated, the post-incubation TEER was significantly different but remained ≥ 300 Ohms. The cell monolayer was considered to be sufficiently intact that paracellular permeability was not affected. Incubation of Caco-2 cells with the flavanol-rich dark chocolate extract containing 241 μM epicatechin did not significantly affect epicatechin transport compared with transport of 250 μM standard epicatechin. Formation of 3'-*O*-methylated epicatechin metabolites was significantly diminished in the presence of dark chocolate extract relative to standard epicatechin; this is in agreement with a study of rats in which total 3'-*O*-methylated epicatechin metabolites were significantly lower post-consumption of cocoa powder compared with ingestion of standard epicatechin (Baba *et al.* 2001a).

The predominant flavanol in the dark chocolate extract was epicatechin however a substantial quantity of dimers and trimers was also present. Tetrameric flavanols have been shown to enhance the cellular absorption of dimers (Appeldoorn *et al.* 2009b) which are reported to competitively inhibit the activity of catechol-*O*-methyltransferase reducing the formation of methylated epicatechin metabolites at concentrations relevant to this study (Spencer *et al.* 2001). It is postulated that the attenuation of 3'-*O*-methylated epicatechin observed in the present investigation may be a consequence of

cellular uptake of dimeric flavanols that compete with epicatechin for methylation by COMT.

3.4.2 Effect of sucrose, glucose and fructose

Incubation of cells with 100 mM sucrose, glucose or fructose significantly reduced the post-incubation TEER of the monolayer relative to pre-incubation TEER. Co-incubation with sucrose, but not glucose or fructose, significantly reduced the TEER relative to the control. In each test condition the value fell below 300 Ohms which indicates loss of monolayer integrity and potential for increased paracellular transport of solutes. The paracellular route has a net negative charge which results in a differential rate of solute permeability in the order from fastest to lowest cationic > neutral > anionic (Amidon *et al.* 1999). In addition to this principle the rate of permeability is also regulated by the molecular weight and radius of the solute, such that smaller molecules permeate faster than larger ones. The authors demonstrated that disruption of the cell monolayer of Madin-Darby canine-kidney (MDCK) cells increased paracellular transport of sucrose and mannitol with sucrose permeability being more greatly affected than mannitol. The pore radius of an integral MDCK monolayer was calculated as 6.1 Å; by comparison the radius of mannitol and sucrose is 4.1 and 5.6 Å, respectively. The disrupted monolayer, pore size 11.0 Å, was considered to become relatively less inhibiting to the larger molecule. The pore radius of Caco-2 cells is almost double that of MDCK cells however the permeability coefficient of mannitol is not significantly different between the two cell models. This is considered to be a function of other physical characteristics

of the cells including cell height and width, length of tight junctions and width of lateral space.

Incubation of Caco-2 cells with 100 mM sucrose, but not glucose or fructose, significantly increased the apical to basolateral transport of epicatechin. Concomitantly the TEER of cells incubated in the presence of sucrose significantly diminished compared with the control. It is probable that increased basolateral epicatechin concentration was a result of a significantly disrupted cell monolayer which allowed greater paracellular permeation. The absence of a significant change in basolateral epicatechin concentration following incubation with glucose or fructose, even though the monolayer resistance diminished below 300 Ohms, is believed to be a result of there being no significant difference between the post-incubation TEER of cells incubated with glucose and fructose compared with control cells.

In Caco-2 cells activation of the sodium-dependent glucose transporter, SGLT1, has been reported to increase tight junction permeability (Turner and Black 2000). The presence of sucrose in the diet of mice significantly increased SGLT1 activity compared with a carbohydrate-free diet or diet containing maltose (Weiss *et al.* 1998, Lam *et al.* 2002). Weiss *et al.* (1998) also demonstrated that sucrose α -glucosidase activity increased parallel with SGLT1 activity. Whilst it would be expected that the presence of glucose would induce activation of SGLT1, it has been reported that sucrose and fructose, but not glucose, enhances the activity of sucrose α -glucosidase (EC 3.2.1.48) in rats (Kishi *et al.* 1999). It could be postulated that incubation of Caco-2 cells with sucrose, but not glucose or fructose, induces SGLT1 activity that leads to increased tight junction pore size as determined by

reduced electrical resistance of the cell monolayer, and therefore increased paracellular permeability of epicatechin.

The presence of glucose or fructose, but not sucrose, elicited a significant reduction in 3'-*O*-methylation of epicatechin. The methylation of epicatechin is an enzyme catalysed reaction in which a methyl group is transferred from S-adenosyl-methionine to the substrate. It is a requirement of the enzyme activity for the presence of a divalent metal cation; this is usually magnesium (II). The metal cation ensures that the substrate binds to the enzyme-cofactor complex in the correct orientation to permit methyl group transfer; changing the metal ion affects the enzyme activity. In the present cell culture study magnesium is not present in the transport solution; the only divalent cation present is calcium (II). It has been reported that substituting calcium (II) for magnesium (II) produces a significant inhibition of the enzyme. Because the calcium (II) ion is larger than the magnesium (II) ion this prohibits the correct cofactor-substrate alignment within the enzymes active site (Sparta and Alexandrova 2012). The control transport solution is formulated using the same protocol therefore is it likely that magnesium (II) stored within the cells permits continued COMT activity. The inhibition of enzyme activity in the presence of glucose or fructose may be due to competition for magnesium (II) from other enzymes within the cell. Glucose is the primary substrate in the glycolytic pathway, it is initially phosphorylated by hexokinase (EC 2.7.1.1), an enzyme requiring magnesium (II) for its activity, to produce glucose-6-phosphate. The pathway continues with conversion to fructose-6-phosphate catalysed by glucose-6-phosphate isomerase (EC 5.3.1.9) and further phosphorylation to form fructose-1, 6-bisphosphate by 6-phosphofructokinase (EC 2.7.1.11), an enzyme that also

requires magnesium (II). It is probable that sucrose does not elicit a similar reduction of epicatechin methylation due to the relatively low levels of glucose and fructose liberated from enzymatic sucrose hydrolysis; the concentration of each monosaccharide produced may remain too low to exhibit an inhibitory effect of COMT.

Schramm *et al* (2003) demonstrated a positive association between carbohydrate content of a meal and plasma epicatechin area under the curve. Other studies have proposed similar associations but with less convincing results (Neilson *et al.* 2009, Rodriguez-Mateos *et al.* 2012). It may be that other components of the formulations are responsible for the effects observed or that lack of significant differences is due to the dose administered. The doses administered to subjects in the human studies described previously to determine the effect of food components on epicatechin bioavailability are shown in Table 3.3. In the Caco-2 study presented here the concentration of epicatechin and sucrose applied to cells was 250 μM and 100 mM, respectively. Assuming 500 mL of gastric fluid this would be the equivalent to a serving of approximately 53 g and 71 g of Nestlé NOIR Intense dark chocolate containing 70% cocoa solids to achieve the same epicatechin and sucrose concentrations.

Reference	Sucrose		Flavanol monomer	
	(g)	(mM)	(mg)	(μ M)
Schramm <i>et al</i> (2003)	35	204	100	689
Neilson <i>et al</i> (2009)	15	88	36	248
Rodriguez-Mateos <i>et al</i> (2012)	10	58	75	517

Table 3.3 Sucrose and flavanol monomer content per serving based on 65 kg body weight and equivalent concentration assuming 500 mL gastric fluid given to human subjects to investigate the effect of sucrose on epicatechin bioavailability.

3.5 Conclusion

In the Caco-2 cell model the apical to basolateral transport of epicatechin is not affected by the presence of higher molecular weight flavanols in a dark chocolate extract or monosaccharides glucose and fructose. Sucrose does significantly enhance epicatechin transport, possibly via its potential to increase paracellular permeability through stimulation of SGLT1 activity. The formation of 3'-O-methylated epicatechin is significantly attenuated in the presence of a dark chocolate extract and glucose or fructose. It is hypothesised that the effect of dark chocolate extract is attributable to the presence of dimeric flavanols in the dark chocolate extract which compete with epicatechin for methylation. The effect of glucose and fructose is proposed to be a consequence of glycolysis enzymes competing with COMT for magnesium (II) cations that are necessary for activity. Prospective studies in which the effect of sucrose on SGLT1 activity and TEER of Caco-2 cells are required to confirm the hypothesis presented here. Co-incubation of epicatechin with commercially available dimeric flavanol standards should be performed in Caco-2 cells to confirm whether epicatechin transport and COMT activity is significantly affected by the presence of dimeric flavanols.

Chapter 4 Dark chocolate extract inhibits sucrose hydrolysis In the Caco-2 cell model

4.1 Abstract

The rate of sucrose hydrolysis in Caco-2 cells was investigated in the presence of flavanol-rich dark chocolate and individual flavanol constituents of dark chocolate. Enantiomers of each catechin stereoisomer and acarbose, used as a positive control, were also assessed. Acarbose dose-dependently reduced the rate of hydrolysis. Dark chocolate extract containing 322 μM flavanol monomers and 500 μM (-)-epicatechin significantly decreased the rate of hydrolysis by 59% and 31% respectively. This suggests that the epicatechin content of dark chocolate is partly responsible with potential synergistic effects from other flavanol components contributing to the difference. All individual flavanol monomers and dimer investigated significantly enhanced the rate of sucrose hydrolysis at a concentration of 50 μM . With the exception of (+)-epicatechin, all other concentrations investigated did not affect the rate of sucrose hydrolysis. It is hypothesised that sucrase-flavanol binding may occur at two distinct locations; one that is not the active site, could alter the tertiary structure of the enzyme exposing more catalytic residues, thereby enhancing sucrose hydrolysis at moderate concentrations; and two at the more accessible active site, flavanol binding would elicit an inhibitory effect that becomes more prominent with increasing concentration of inhibitor.

4.2 Introduction

The effects of plant extracts and the individual phenolic components on carbohydrate metabolising enzymes present in the small intestine have been extensively investigated during the last decade (Kim *et al.* 2000, Hansawasdi *et al.* 2001, Matsui *et al.* 2001, Matsui *et al.* 2002, Ramachandra *et al.* 2005, McDougall *et al.* 2005, Barrenetxe *et al.* 2006, Iwai *et al.* 2006, He *et al.* 2007, Gupta *et al.* 2007, Ani and Naidu 2008, Kumarappan and Mandaol 2008, Adisakwattana and Chanathong 2011, Pereira *et al.* 2011, El-Beshbishy and Bahashwan 2012). Primarily these studies have focused on inhibition of enzymes belonging to the hydrolase family – α -amylase (EC 3.2.1.1), α -glucosidase (maltase) (EC 3.2.1.20), and sucrose α -glucosidase (sucrase) (EC 3.2.1.48). Common features of these enzymes are the catalytic residues of aspartic and glutamic acid that each contains an acidic side chain (Nichols *et al.* 2003, Lo Piparo *et al.* 2008). At physiological pH the ionised α -carboxyl group of each amino acid is able to participate in hydrogen bond formation with the hydroxyl groups of phenolic compounds. Many investigations have observed that more hydroxyl groups present in the molecule generally equates to stronger inhibition of the enzyme compared with similar structures containing fewer hydroxyl groups. This was demonstrated by a study of monomeric flavanols and condensed tannins typically present in tea. The most effective inhibition of maltase and sucrase was performed by those compounds esterified to gallic acid, such as theaflavin-3-*O*-gallate, epigallocatechin gallate and epicatechin gallate (Matsui *et al.* 2007, Kamiyama *et al.* 2010). A summary of the concentrations

required to reduce activity of the enzymes by 50% (IC_{50}) reported by Matsui *et al* (2007) is shown in Table 4.1.

The stereochemical configuration of hydroxyl groups was also found to be of importance to the efficacy of inhibition. The 3' hydroxyl group of theaflavin-3-O-gallate rotated in the '*R*' configuration inhibits maltase more potently than the '*S*' configuration. Similarly the '*R*' configuration of the C-ring hydroxyl group of both catechin and epicatechin inhibited maltase more than the '*S*' isomer (Matsui *et al.* 2007).

The number of hydroxyl groups present on the B-ring of flavonols affects the inhibitory capacity towards sucrase, maltase and α -amylase. The order of inhibition from strongest to weakest was reported to match the number of hydroxyl groups from most to fewest in a study of guava leaf constituents, with myricetin > quercetin > kaempferol (Wang *et al.* 2010). Apigenin, a flavone containing one B-ring hydroxyl group but no C-ring hydroxyl, exhibited only slight inhibition ($IC_{50} > 30$ mM), suggesting that hydroxylation of the C-ring is also important.

A double bond between carbon-2 and carbon-3 of the C-ring forms a conjugated system with delocalised electrons throughout the A- and C-rings, the presence of which has also been implicated in the ability of a flavonoid to inhibit glucohydrolase enzymes. Lo Piparo *et al* (2008) assessed the interaction between a variety of flavonoids and amino acid residues at the active site of human salivary α -amylase. They found the conjugated system was able to form π - π bonds with the aromatic side chains of tryptophan and tyrosine. Luteolin and quercetin, a flavone and flavonol containing the conjugated AC ring system and presenting identical structures except for the presence of a carbon-3 hydroxyl group in quercetin, exhibit similar IC_{50}

values, 18.4 μM and 21.4 μM respectively. Catechin and epicatechin, flavanols that do not possess the conjugated system were unable to achieve 50% inhibition of α -amylase at concentrations up to 100 μM .

The flavanols catechin and epicatechin are also only weak inhibitors of sucrase and maltase, refer to Table 4.1, and supported by the observations of Kamiyama *et al* (2010). Concentrations required to reduce enzyme activity to 50% are not physiologically easy to achieve. For example, one of the best sources of (-)-epicatechin is dark chocolate that contains approximately 70 mg 100 g⁻¹ fresh weight (Neveu *et al.* 2010). Assuming 500 mL of gastric liquid this equates to approximately 480 μM . Any benefits arising from inhibition of carbohydrate hydrolysis would likely be offset by the high sugar and fat intake from consuming 200 g of dark chocolate. As previously discussed the gallated flavanols seem to be more effective inhibitors of α -glucosidase enzymes at physiologically relevant concentrations, which may explain the medicinal use of green tea as an anti-diabetic treatment in some cultures.

	Maltase IC ₅₀ (μ M)	Sucrase IC ₅₀ (μ M)
(-)-Epicatechin (2 <i>R</i> , 3 <i>R</i>)	770	1080
(+)-Epicatechin (2 <i>S</i> , 3 <i>S</i>)	1320	Not measured
(-)-Catechin (2 <i>S</i> , 3 <i>R</i>)	1890	Not measured
(+)-Catechin (2 <i>R</i> , 3 <i>S</i>)	4320	Not measured
Epigallocatechin	1260	921
Epicatechin gallate	53	172
Epigallocatechin gallate	40	169
Theaflavin	500	>10,000
Theaflavin-3- <i>O</i> -gallate (3' <i>R</i>)	10	1024
Theaflavin-3- <i>O</i> -gallate (3' <i>S</i>)	83	Not measured

Table 4.1 Concentration of flavanols required to inhibit the activity of maltase (EC 3.2.1.20) and sucrase (EC 3.2.1.48) by 50% (IC₅₀) taken from Matsui *et al* (2007).

Plant extracts containing relatively high concentrations of polymerised flavanols such as grape seed extract have been assayed to determine the efficacy of α -glucosidase inhibition and protein binding. Sucrase activity in the Caco-2 cell model was reduced by 61% in the presence of 0.3 g L⁻¹ pre-digested grape seed extract containing approximately 54% flavanols with a degree of polymerisation greater than dimer (Laurent *et al.* 2007). The protein binding capacity of flavanols, ranging from monomer to hexamer extracted from grape seed, was assessed by fluorescence quenching of tryptophan residues in bovine serum albumin and α -amylase (Soares *et al.* 2007). Flavanols with a higher molecular weight (MW) exerted greater quenching capacity of α -amylase tryptophan residues than the smaller molecules, and flavanols with a gallate moiety were more effective than non-

gallated compounds. Tannic acid, a large phenolic compound with MW 1701.2, exerted the strongest fluorescence quenching capacity.

Tannic acid has been described to inhibit human salivary α -amylase by a mixed mode (Kandra *et al.* 2004) and similarly inhibits sucrase by mixed-type at physiological pH 7.2 (Gupta *et al.* 2010). The authors reported that binding of the phenolic compound altered the tertiary structure of the enzyme such that tryptophan residues in the active site became more exposed to the hydrophilic environment.

Synergistic effects between individual compounds and extracts have also been reported. Mulberry extract combined with either roselle, chrysanthemum or butterfly pea extract enhanced maltase inhibition compared with mulberry extract alone. Similarly, roselle extract combined with either chrysanthemum, mulberry, bael or butterfly pea extract increased α -amylase inhibition compared with roselle extract alone (Adisakwattana *et al.* 2012). The combined pure compounds quercetin and myricetin, hyperin and avicularin, kaempferol and quercetin increased sucrase and maltase inhibition compared with the compounds assayed individually; α -amylase was not affected (Wang *et al.* 2010).

The aim of this investigation was to determine whether flavanol-rich dark chocolate extract and individual flavanol components were able to inhibit the hydrolysis of sucrose in the Caco-2 cell model.

4.3 Results

4.3.1 Analytical protocol validation

4.3.1.1 Linearity, precision, limit of detection and limit of quantification

4.3.1.1.1 Glucose oxidase/peroxidase assay

Calibration solutions in the range 0-80 $\mu\text{g mL}^{-1}$ were assayed using the glucose oxidase/peroxidase method and a calibration curve of absorbance at 540 nm as a function of glucose concentration prepared. The linear regression equation was obtained, with the y-intercept fixed at zero the gradient = 0.014 and the adjusted R-square = 0.99, suggesting good proportionality of absorbance and concentration

Figure 4.1). The relative standard deviation of absorbance was calculated for each concentration of glucose, and ranged from 2.2 to 12.7% indicating that precision of the method was acceptable. The limit of detection was calculated as 1.9 $\mu\text{g mL}^{-1}$ and the limit of quantification as 5.7 $\mu\text{g mL}^{-1}$.

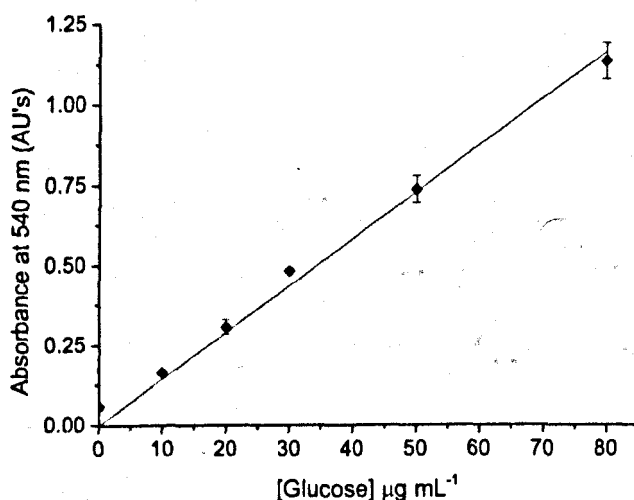


Figure 4.1 Glucose oxidase/peroxidase assay calibration curve; gradient = 0.014, adjusted R-square = 0.99. Mean \pm standard error of the mean (n=3).

The rate of sucrose hydrolysis in Caco-2 cells co-incubated with a cocoa powder extract provided by Nestlé, was investigated using the glucose oxidase/oxidase method to determine glucose concentration in the sample. At this point it was observed that the cocoa powder extract inhibited the activity of enzymes in the glucose/oxidase assay. To overcome interference a solid phase extraction procedure, using hydrophilic-lipophilic balanced cartridges, was trialled to assess whether the interfering compounds could be removed from the sample. Whilst the extraction improved glucose measurement in the presence of cocoa powder, some glucose was retained by the adsorbent (Figure 4.2). In addition this process was labour intensive and time-consuming such that alternative methods of glucose analysis were investigated.

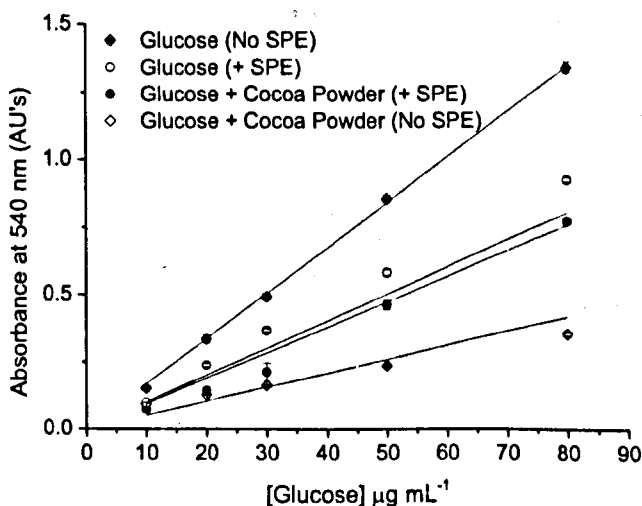


Figure 4.2 Absorbance of glucose calibration solutions, determined using the glucose oxidase/oxidase method, following incubation in the absence and presence of a cocoa powder extract, with (+ SPE) and without (No SPE) solid phase extraction. Mean \pm SEM (n=3).

4.3.1.1.2 HPLC-IPAD

Data analysis was performed over a 15 month period with a 3 month break between December 2010 and March 2011. At the beginning of each analytical period calibration solutions in the range 0-500 μM were analysed to ensure accurate data analysis and compare equipment function. Calibration curves of peak area as a function of glucose or fructose concentration were prepared (Figure 4.3A and B). The linear regression equation was obtained with the y-intercept fixed at zero the gradient for glucose and fructose were calculated as 0.19 and 0.16, respectively, and the adjusted R-square was 1.0 for each, suggesting good proportionality of peak area and concentration (Figure 4.3A and B). The relative standard deviation (RSD) of peak area was calculated for each concentration of glucose and fructose, and ranged from 0.7 to 11.2% and 0.8 to 8.7%, respectively. The limit of detection and limit of quantification were calculated as 0.02 μM and 0.06 μM for glucose and 0.01 μM and 0.04 μM for fructose.

Precision of the analytical method at the beginning of the second analytical period, determined by calculation of RSD, remained acceptable although there was a loss of sensitivity reflected in lower gradients and higher limits of detection (LOD) and quantification (LOQ). Glucose: gradient = 0.14, LOD = 0.44 μM , and LOQ = 1.32 μM ; fructose: gradient = 0.12, LOD = 0.05 μM , and LOQ = 0.14 μM .

Figure 4.4 represents a typical chromatogram of 200 μM calibration solution containing fucose, glucose, fructose and sucrose. Fucose was used as an internal standard. Figure 4.5 demonstrates the presence of sucrose in a basolateral cell culture sample.

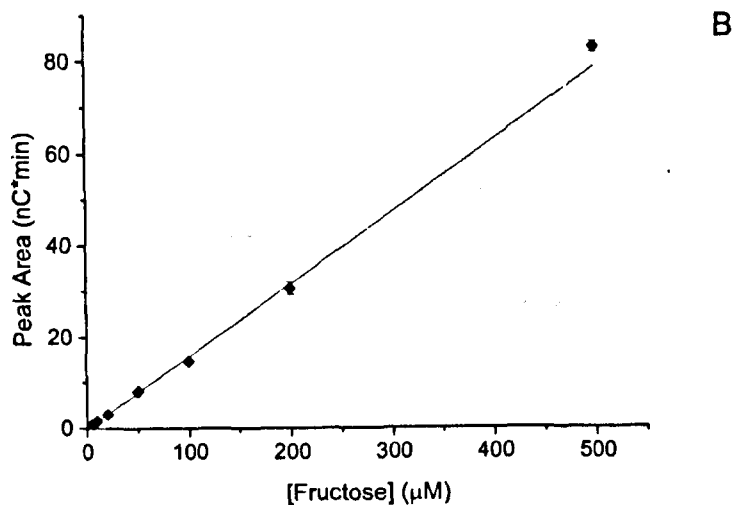
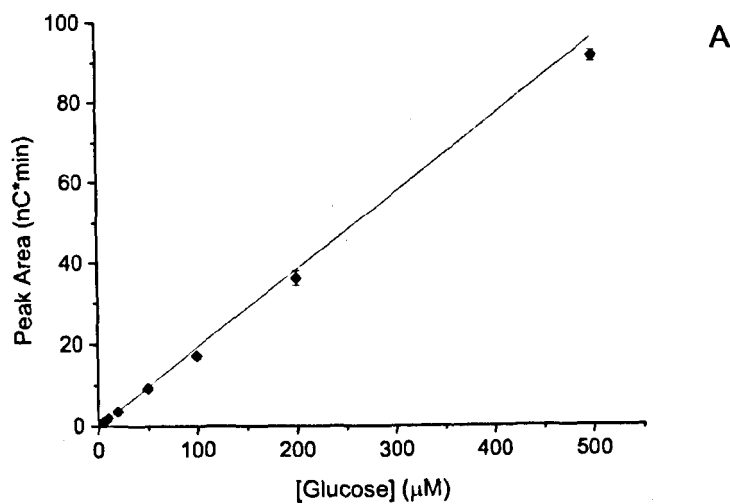


Figure 4.3 A) Glucose, and B) fructose HPLC-IPAD calibration curves. A) Gradient = 0.19, adjusted R-square = 1.0. B) Gradient = 0.16, adjusted R-square = 1.0. Mean \pm standard error of the mean ($n=3$).

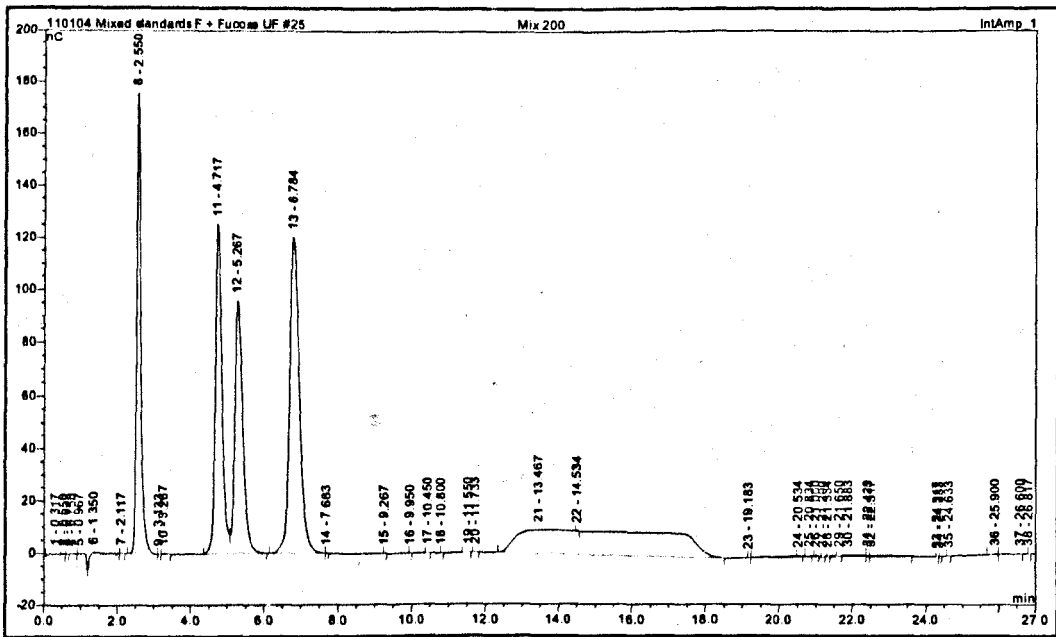


Figure 4.4 HPLC-IPAD chromatogram representing peaks of fucose, glucose, fructose and sucrose, from left to right. Concentration of each = 200 μ M.

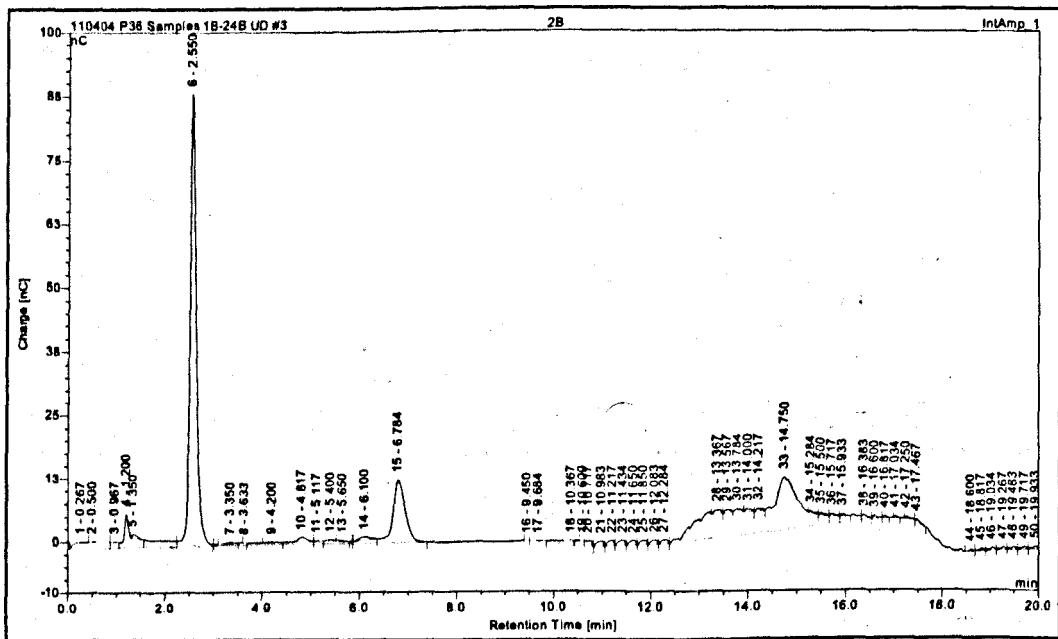


Figure 4.5 HPLC-IPAD chromatogram demonstrating the presence of sucrose in a basolateral cell culture sample. Fucose was added as the internal standard.

4.3.2 Cell culture

4.3.2.1 Validation of cell membrane integrity

Trans-epithelial electrical resistance (TEER) was measured before the 20 min incubation and corrected to account for resistance of the polycarbonate membrane to provide a measure of the cell monolayer resistance. Mean TEER varied from 130 to 464 (Table 4.2). The rate of sucrose hydrolysis significantly correlated with TEER even when the assay condition was controlled for, $p < 0.01$ (Figure 4.6). An association between TEER and cell passage number was considered but no significant correlation existed, whereas the rate of sucrose hydrolysis and passage number did correlate significantly, $p < 0.001$ (Figure 4.7). However, controlling for the condition and passage number did not alleviate the significant correlation between TEER and rate of sucrose hydrolysis, $p < 0.01$. During 2010, after the first period of cell culture assays whilst a new batch of cells was being prepared for the second period of cell culture assays, cell clumping was observed in culture flasks. It was proposed that high glucose concentration in the media may be a contributing factor to cells forming clumps in which rather than remaining as a monolayer they began to stack on top of one another. The concentration of glucose in the culture media was reduced from 25 mM to 5 mM which resulted in a general lowering of TEER of the cell monolayer. TEER and rate of sucrose hydrolysis significantly correlated with media glucose concentration, $p < 0.001$ (Figure 4.8A and B). Controlling for the assay condition, passage number and media glucose concentration removed any significant correlation between the rate of sucrose hydrolysis and TEER. In assays where the TEER of the test condition differed

significantly from the control condition partial correlation analysis, controlling for the condition, was performed and confirmed there was no association between TEER and the rate of hydrolysis, $p > 0.05$.

Condition	TEER (Ohms)	
	Control	Test
200 μ M Acarbose	195	236**
100 μ M Acarbose	209	310**
50 μ M Acarbose	319	385***
DC ^a extract 322 μ M epicatechin	213	254**
DC extract 141 μ M epicatechin	443	403
DC extract ethyl acetate fraction 65 μ M epicatechin	443	463
DC extract ethyl acetate fraction <1 μ M epicatechin	443	437
500 μ M (-)-Epicatechin	334	332
200 μ M (-)-Epicatechin	195	285**
200 μ M (-)-Epicatechin	130	177
200 μ M (-)-Epicatechin	392	423*
100 μ M (-)-Epicatechin	160	187
100 μ M (-)-Epicatechin	209	424***
50 μ M (-)-Epicatechin	319	415***
20 μ M (-)-Epicatechin	392	434**
200 μ M (+)-Epicatechin	195	289**
200 μ M (+)-Epicatechin	392	417
100 μ M (+)-Epicatechin	209	405***
50 μ M (+)-Epicatechin	319	402**
20 (+)-Epicatechin	392	424*
200 μ M (+)-Catechin	337	447***
100 μ M (+)-Catechin	209	363**
50 μ M (+)-Catechin	319	403***
20 μ M (+)-Catechin	337	464***
200 μ M (-)-Catechin	337	409*
100 μ M (-)-Catechin	209	367**

Condition	TEER (Ohms)	
	Control	Test
50 μ M (-)-Catechin	319	405**
20 μ M (-)-Catechin	337	447**
100 μ M Procyanidin B2	209	319**
50 μ M Procyanidin B2	319	401**

Table 4.2 Blank-corrected Caco-2 cell monolayer trans-epithelial electrical resistance (TEER) for each assay condition investigated. Mean \pm SEM. ^aAbbreviation: DC, dark chocolate. Asterisk denotes a significant difference from the control, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

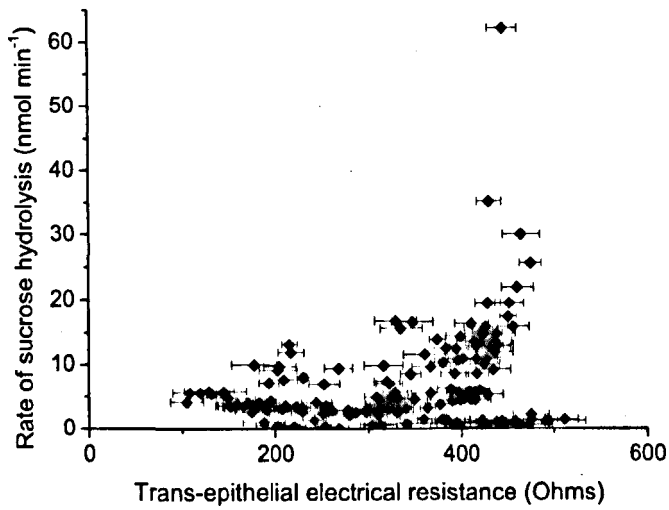


Figure 4.6 Rate of sucrose hydrolysis plotted against mean \pm SEM trans-epithelial electrical resistance (n=163).

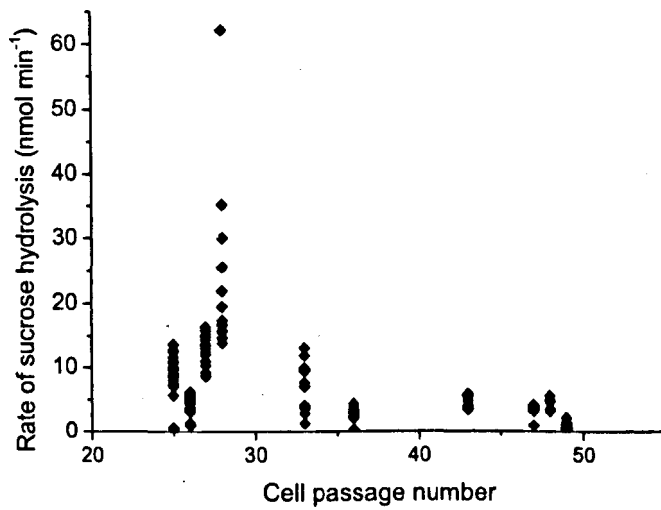


Figure 4.7 Rate of sucrose hydrolysis as a function of cell passage number.

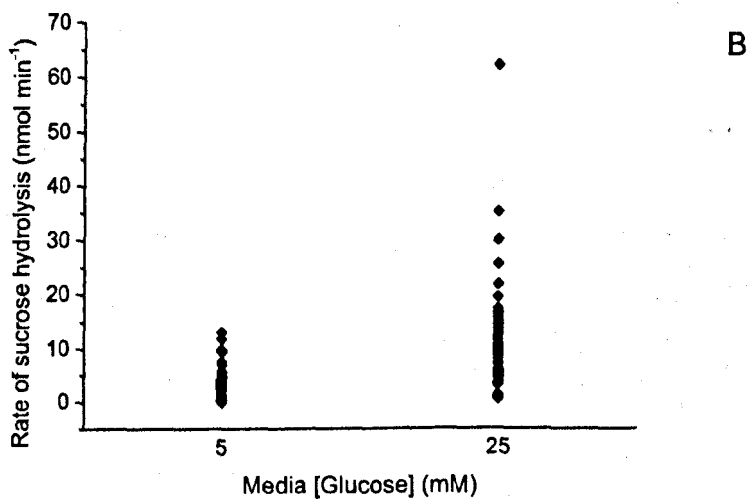
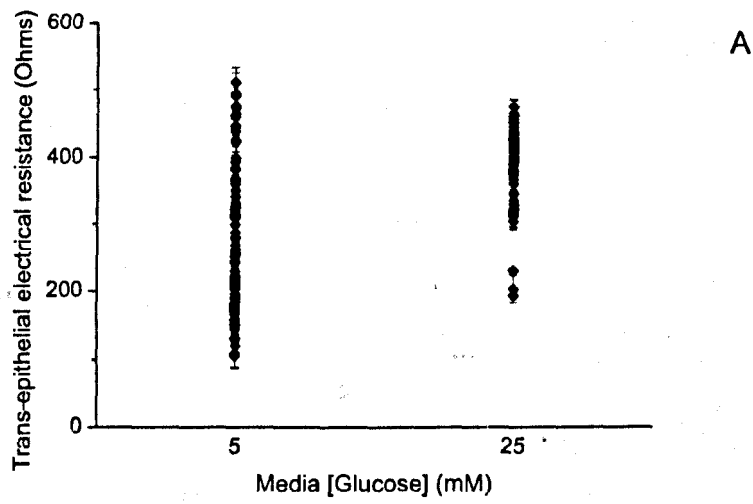


Figure 4.8 A) Trans-epithelial electrical resistance, and B) rate of sucrose hydrolysis as a function of glucose concentration in the cell culture media.

4.3.3 Sucrose hydrolysis study

4.3.3.1 Kinetic investigation

The kinetic properties of sucrose hydrolysis were determined using the glucose oxidase/peroxidase assay to measure glucose concentration. To establish the most appropriate sucrose concentration the kinetic properties were assessed using the Michaelis-Menten model. Assays of 1-120 mM sucrose revealed a typical hyperbolic growth curve when rate of hydrolysis was plotted against sucrose concentration (Figure 4.9). The maximum rate of hydrolysis (V_{\max}) = 20.5 nmol min⁻¹, and Michaelis constant (K_M) = 19.3 mM, was calculated from nonlinear regression analysis using the Michaelis-Menten function and rearrangement of the Michaelis-Menten equation. Subsequent assays were performed using 20 mM sucrose as this was within the linear range of the curve signifying that the enzyme was not saturated.

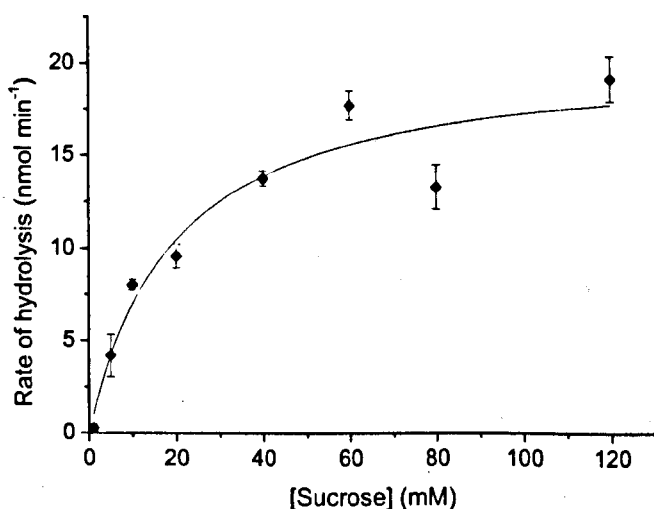


Figure 4.9 Plot of rate of hydrolysis against sucrose concentration with Michaelis-Menten nonlinear regression analysis. Mean \pm SEM (n=3).

4.3.3.2 Inhibition Investigation

The effect of dark chocolate extract and flavanol monomers and dimers on sucrose hydrolysis was investigated with the rate of sucrose hydrolysis calculated based on the concentration of mean glucose and fructose concentrations in the samples. Acarbose, an anti-diabetic prescription drug which inhibits the activity of carbohydrate hydrolysing enzymes, was used as a positive control. The total flavanol profile of monomers to decamers for the dark chocolate extracts and ethyl acetate fractions used in this investigation are displayed in Table 4.3. The mean concentration of glucose and fructose in each dark chocolate extract and ethyl acetate fraction is also presented.

Acarbose dose-dependently inhibited the rate of sucrose hydrolysis up to 93%, $p < 0.001$ (Table 4.4). Dark chocolate extract containing 322 μM flavanol monomers significantly reduced the rate of sucrose hydrolysis by 59% ($p < 0.01$). Dark chocolate extract containing 141 μM flavanol monomers and ethyl acetate fractions containing ≤ 65 μM flavanol monomers had no significant effect (Figure 4.10). Inhibition of sucrose hydrolysis in the presence of (-)-epicatechin was only significant at the highest concentration investigated (500 μM) = 31% inhibition, $p < 0.001$ (Figure 4.11). A 10-fold lower concentration of (-)-epicatechin (50 μM) significantly enhanced sucrose hydrolysis by 56%, $p < 0.01$. All other concentrations of (-)-epicatechin investigated did not elicit a significant effect. The (+)-epicatechin enantiomer also significantly enhanced sucrose hydrolysis, by 60%, at concentrations of 50 and 100 μM , $p < 0.05$ and $p < 0.01$ respectively (Figure 4.13A). Other concentrations of (+)-epicatechin investigated, 20 and 200 μM , did not significantly affect the rate of sucrose hydrolysis. The (+) and (-)-catechin enantiomers demonstrated significant enhancement of sucrose

hydrolysis at a concentration of 50 μM , increasing it by 51% ($p < 0.05$) and 64% ($p < 0.01$), respectively (Figure 4.12A and B). Similarly, procyanidin B2 significantly enhanced sucrose hydrolysis by 26% at a concentration of 50 μM , $p < 0.05$. All other concentration of (+)-catechin, (-)-catechin and procyanidin B2 did not produce a significant moderation of sucrose hydrolysis in Caco-2 cells.

Degree of polymerisation	Total flavanol content of dark chocolate extract ^a and ethyl acetate fractions ^b (mg) / Monosaccharide concentration (μM)			
	<i>Flavanol monomers (μM)</i>			
	322 ^a	141 ^a	65 ^b	<1 ^b
Monomer	3.09	1.36	0.63	0.01
Dimer	1.95	0.14	0.06	0.03
Trimer	0.49	0.04	0.02	0.01
Tetramer	0.31	0.02	0.01	0.0
Pentamer	0.12	0.01	0	0
Hexamer	0.05	0	0	0
Heptamer	0.03	0	0	0
Octamer	0.02	0	0	0
Nonamer	0.01	0	0	0
Decamer	0.01	0	0	0
Monosaccharide	377	120	4	0

Table 4.3 Total flavanol content (mg) and monosaccharide concentration (μM) of dark chocolate extract^a and ethyl acetate fractions^b used to investigate the effect on sucrose hydrolysis in Caco-2 cells.

Condition	Rate of hydrolysis (nmol min^{-1})		$\Delta\%$	Statistical analysis
	Control	Test		
Acarbose (μM)				
50	3.42	1.19	↓65	p<0.001
100	8.13	0.60	↓93	p<0.001
200	3.32	0.24	↓93	p<0.001

Table 4.4 Rate of hydrolysis for each concentration of acarbose investigated along with the corresponding control value, and percentage change relative to the control. Mean ($n \geq 3$).

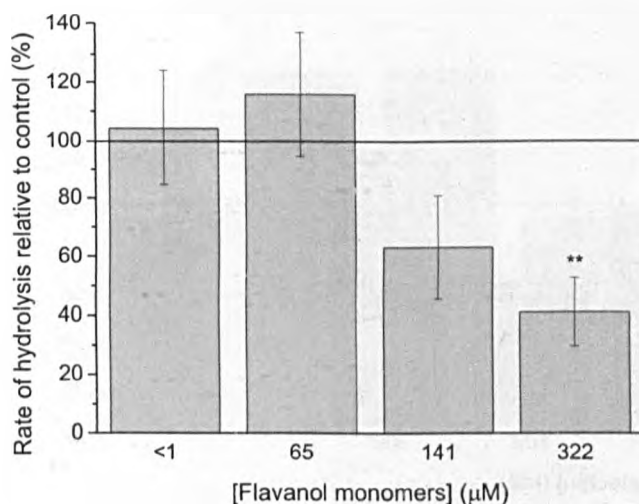


Figure 4.10 Rate of sucrose hydrolysis as a percentage of control following incubation of Caco-2 cells with dark chocolate extract and ethyl acetate fractions containing 322, 141, 65 and <1 μM flavanol monomers. Horizontal line at 100% represents the control. Mean \pm SEM (n=6). Asterisk denotes a significant difference from the control **p<0.01.

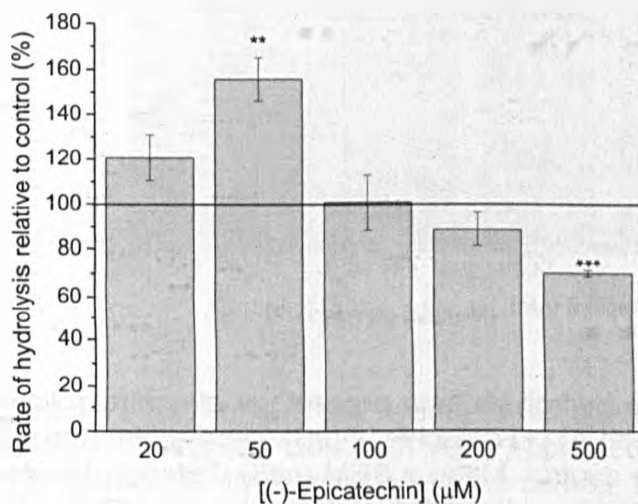


Figure 4.11 Rate of sucrose hydrolysis as a percentage of control following incubation of Caco-2 cells (-)-epicatechin ranging from 20-500 μM . Horizontal line at 100% represents the control. Mean \pm SEM (n \geq 3). Asterisk denotes a significant difference from the control; **p<0.01, ***p<0.001.

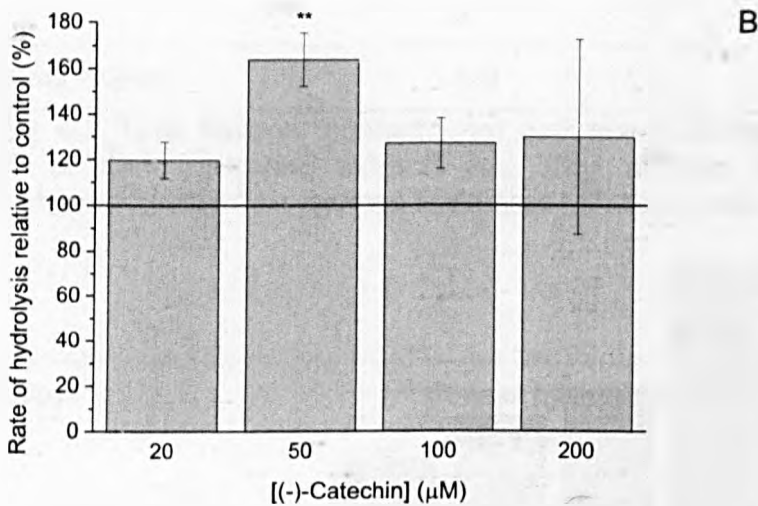
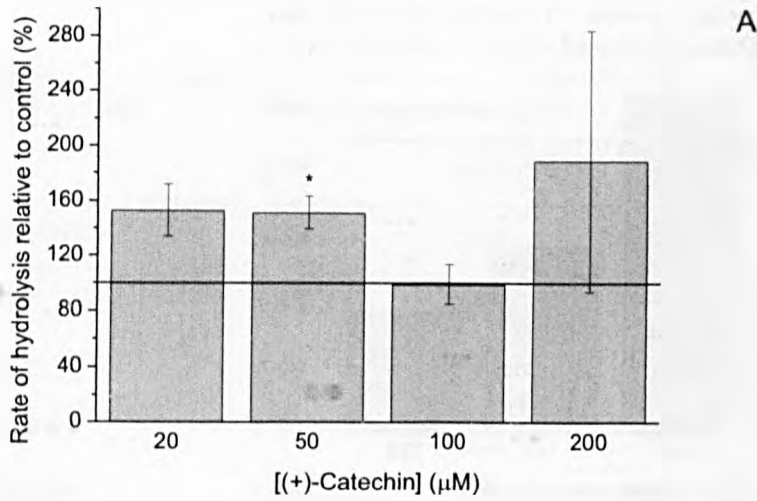


Figure 4.12 Rate of sucrose hydrolysis as a percentage of control following incubation of Caco-2 cells with A) (+)-catechin and B) (-)-catechin. Horizontal line at 100% represents the control. Mean \pm SEM ($n \geq 3$). Asterisk denotes a rate of hydrolysis that is significantly different from the control; * $p < 0.05$, ** $p < 0.01$.

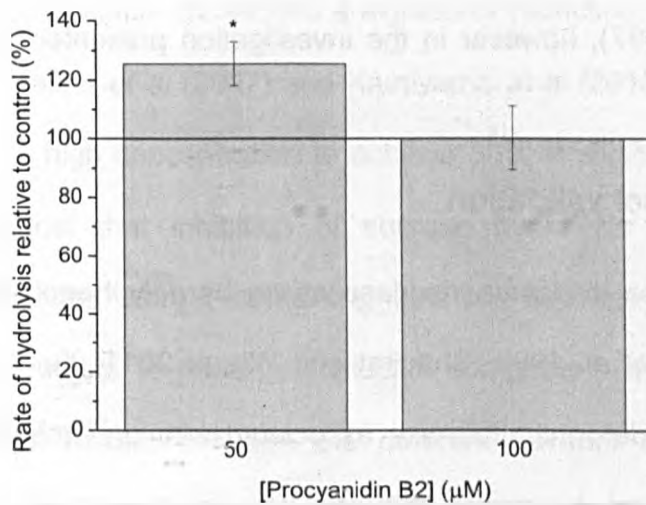
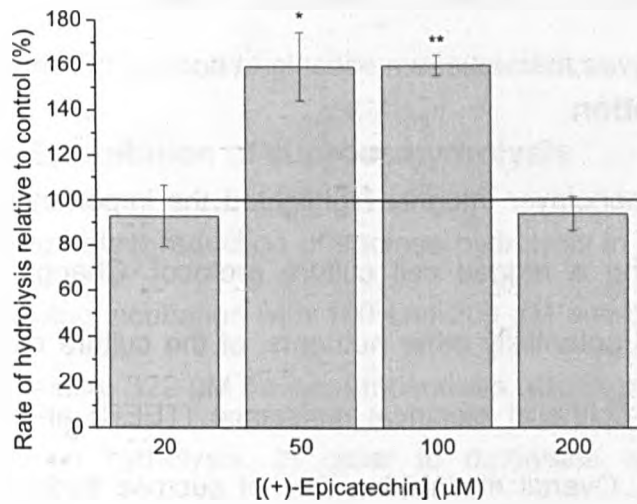


Figure 4.13 Rate of sucrose hydrolysis as a percentage of control following incubation of Caco-2 cells with A) (+)-epicatechin and B) procyanidin B2. Horizontal line at 100% represents the control. Mean \pm SEM ($n \geq 3$). Asterisk denotes a rate of hydrolysis that is significantly different from the control; * $p < 0.05$, ** $p < 0.01$.

4.4 Discussion

4.4.1 Cell culture validation

Validation of Caco-2 cell monolayer integrity highlighted the importance of establishing and maintaining a rugged cell culture protocol. Changes in glucose concentration, and potentially other nutrients, of the culture media significantly affected trans-epithelial electrical resistance (TEER) and the rate of sucrose hydrolysis. Overall diminishing rate of sucrose hydrolysis was associated with increasing cell passage number, which indicates that conducting experiments within a range that is as narrow as possible is essential for eliminating sources of error in the results. TEER has been reported to increase significantly with increasing cell passage number (BriskeAnderson *et al.* 1997), however in the investigation presented here this was not observed.

4.4.2 Analytical protocol validation

Interference of the glucose oxidase/oxidase assay by polyphenols has been reported (Nishioka *et al.* 1998, Shaukat and Waqar 2011, Xu *et al.* 2012). Reduced glucose determination was associated with prevention of chromophore formation and hydrogen peroxide scavenging resulting in oxidation of the active component, epigallocatechin gallate (Shaukat and Waqar 2011). Investigations whereby glucose concentration has been quantified using the glucose oxidase/oxidase assay in the presence of polyphenols require validation to confirm that the assay is not hindered by the presence of the polyphenolic component. In the event that this does

occur, a suitable extraction procedure should be incorporated or an alternative method of glucose measurement developed.

4.4.3 Inhibition of sucrose hydrolysis

The greatest reduction of sucrose hydrolysis in Caco-2 cells was measured following incubation with 100 and 200 μM acarbose. Dark chocolate extract containing 322 μM flavanol monomers also significantly reduced the rate of sucrose hydrolysis. In order to determine whether the reduction was attributable to one specific flavanol monomer or dimer constituent of the extract the rate of sucrose hydrolysis was measured in the presence of (-)-epicatechin, (+)-catechin and procyanidin B2. Inhibition of sucrose hydrolysis was observed in the presence of 500 μM (-)-epicatechin, neither (+)-catechin or procyanidin B2 elicited a significant reduction. This supports the findings of Matsui *et al* (2007) and Kamiyama *et al* (2010) that report the necessity for a high concentration to achieve 50% inhibition of activity. These results suggest that inhibition of sucrose hydrolysis in the presence of dark chocolate extract containing 322 μM flavanol monomers may be partly attributable to the (-)-epicatechin component. It may be that a synergistic effect occurs between the different flavanol monomers, oligomers and polymers similar to the synergistic inhibition of sucrase reported for flavonols and flavanol glycosides (Wang *et al.* 2010).

Each of the flavanol monomers and B2 dimer enhanced the rate of sucrose hydrolysis at concentrations of 50 μM . Gupta *et al* (2010) proposed that binding of a phenolic compound such as tannic acid to α -amylase altered the tertiary structure such that tryptophan residues in the active site became more exposed to the hydrophilic environment. It may be that flavanol

monomers and dimers are able to bind sucrose in two locations, one that induces a change in the structure of the enzyme exposing more of the catalytic residues subsequently enhancing activity, and the second at the active site causing inhibition of activity. This could explain how moderate concentrations appear to enhance enzyme activity and higher concentrations are inhibitory. At concentrations between, there is no apparent significant change due to the enhancement and inhibition counteracting one another. This supports previous evidence which shows that relatively high concentrations of non-gallated flavanol monomers are necessary to achieve 50% inhibition of enzyme activity (Matsui *et al.* 2007, Lo Piparo *et al.* 2008, Kamiyama *et al.* 2010). In order to determine whether stereochemical configuration of the flavanol monomer affects the inhibitory capacity, higher concentrations of each enantiomer must be investigated.

Enhancement of sucrose activity by each of the flavanol monomers and B2 dimer does not significantly differ between the groups indicating that stereochemical configuration of the B-ring and of the C-ring hydroxyl group is not a contributing factor. Superficially the degree of polymerisation also appears to be inconsequential although further investigations using more polymerised flavanols are required to confirm this proposal.

Sustained augmentation of sucrose activity measured following incubation with 100 μM (+)-epicatechin may suggest that the 2S, 3S configuration restricts effective binding at the active site, thereby preventing a relative reduction of sucrose hydrolysis, as observed with the other flavanol monomers at this concentration.

Previous studies that have investigated the effect of phenolic compounds on α -glucosidase activity have suggested that the number of hydroxyl groups,

stereochemical configuration of the C-ring hydroxyl and conjugation of the AC-ring system is of importance to the inhibition of sucrase, maltase and α -amylase (Matsui *et al*, 2007, Wang *et al*, 2010(Lo Piparo *et al*. 2008). Based on the results of the investigation presented here it would be necessary to compare the inhibitory profile of flavanols with compounds that contain the conjugated AC-ring system and the same number of hydroxyl groups, for example epicatechin and quercetin. It would also be beneficial to compare the inhibitory capacity of epicatechin with epigallocatechin and epicatechin-3-O-gallate to investigate whether the addition of hydroxyl groups affect sucrase inhibition in the Caco-2 model.

Analysis of the kinetic properties of sucrase in the absence and presence of flavanols would suggest a method by which inhibition occurs. Further analysis of the protein structure-activity association may be confirmed using fluorescence quenching techniques or computational ligand docking software.

4.5 Conclusion

Sucrose hydrolysis in the Caco-2 cell model was reduced as a consequence of incubation with a flavanol-rich dark chocolate extract. The (-)-epicatechin content of the dark chocolate extract is considered to be partly responsible and a synergistic effect with other flavanol components is proposed to account for the difference of inhibitory capacity between the dark chocolate extract and individual compound. Multiple binding sites on the enzyme are hypothesised to be the reason for elevated sucrose hydrolysis at moderate concentrations of each flavanol monomer and dimer investigated. Prospective studies should include investigation of stereochemical

configuration effects. Synergy between the flavanol components requires attention along with considering whether degree of polymerisation contributes to the overall effect. Kinetic analysis of sucrose hydrolysis in the Caco-2 cell model along with other structure-activity analytical methods may provide insight to the mechanism and binding properties of dark chocolate flavanols in relation to sucrase.

Chapter 5 Glucose transport inhibition by a flavanol-rich dark chocolate extract

5.1 Abstract

Glucose transport across the Caco-2 cell monolayer was significantly reduced in the presence of a flavanol-rich dark chocolate extract containing 35, 71, 142 and 322 μM epicatechin. Kinetic analysis revealed that the maximum rate of cellular uptake and apical to basolateral transport was significantly attenuated; this was dose-dependent for apical to basolateral transport. The concentration of glucose required to achieve half the maximum rate of apical to basolateral transport (K_M) was significantly increased, independent of the dose investigated. Overall inhibition was of the mixed-type, with non-competitive inhibition being tentatively attributed to the apical sodium-dependent glucose transporter SGLT1 and competitive inhibition attributed to the basolateral glucose transporter GLUT2. Inhibition was not shown to be the result of individual flavanol monomer or B2 dimer components of the dark chocolate extract but is hypothesised to be a consequence of higher molecular weight flavanols or a synergistic effect between monomers and dimers. Caffeine, theobromine, procyanidin B2 and (+)-epicatechin increased the rate of cellular uptake but not apical to basolateral transport. The role of caffeine and theobromine as inhibitors of cyclic 3', 5'-nucleotide phosphodiesterase and the potential for enhanced SGLT1 activity is considered and discussed in relation to the results presented here.

5.2 Introduction

Cellular uptake of glucose is primarily a secondary active process performed by the high-affinity sodium-dependent transporter SGLT1, $K_M = 0.8$ mM (Hediger and Rhoads 1994), that can transport glucose against its concentration gradient permitting uptake even when the concentration of glucose in the lumen is low. By contrast during post-prandial periods when the concentration of glucose in the lumen is high, glucose uptake is assisted by the sodium-independent hexose transport GLUT2 (Kellett and Brot-Laroche 2005). GLUT2 facilitates diffusion of glucose along its concentration gradient and although it has less affinity for glucose than its sodium-dependent counterpart, $K_M = 17$ mM (Thorens 1996), it possesses a high capacity for glucose transport.

A number of *in vitro* and *ex vivo* studies have investigated the interaction of flavonoids with intestinal glucose transporters. The majority of these focus on the effects of flavonols, in particular quercetin and its glycosides. It is now widely accepted that flavonoid glycosides are either deglycosylated by lactase phloridzin hydrolase (LPH) located at the epithelial brush border allowing the aglycone to diffuse into the cell, or alternatively the glycoside is transported into the cell via the sodium-dependent glucose transporter SGLT1, before being deglycosylated by cytosolic β -glucosidase. Entry of both the aglycone and glycoside has been established through detection of phase II metabolites of the aglycone.

Whilst quercetin-3-*O*-glucoside and 4'-*O*-glucoside have been observed to competitively inhibit the sodium-dependent uptake of glucose (Ader *et al.* 2001, Cermak *et al.* 2004), few studies report the ability of flavonoid

aglycones to reduce SGLT1 activity. Of three investigations to report significant reduction in sodium-dependent glucose uptake by non-glycosylated flavonoids, only one reported inhibition with physiologically realistic concentrations. Significant reduction of sodium-dependent glucose uptake was observed in Caco-2 cells with 100 μM (+)-catechin, (-)-epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate (Johnston *et al.* 2005). Studies prior to this also demonstrated inhibition of sodium-dependent glucose transport by epicatechin gallate and epigallocatechin gallate although the concentrations required to elicit a similar reduction were much greater, up to 1 mM (Kobayashi *et al.* 2000, Hossain *et al.* 2002). Hossain *et al.* (2002) also reported inhibition of sodium-dependent glucose transport by catechin, however the inhibition constant (K_i) was approximately 2.3 mM. The type of inhibition reported in these studies was contradictory; Kobayashi *et al.* (2000) suggested competitive inhibition whereas Hossain *et al.* (2002) proposed non-competitive.

Reports of sodium-independent glucose transport inhibition by non-glycosylated flavonoids are more prominent. In Caco-2 cells and *Xenopus* Oocytes, quercetin was reported to attenuate glucose uptake at physiologically relevant concentrations (Johnston *et al.* 2005, Kwon *et al.* 2007); the latter suggesting a non-competitive mode of inhibition. Epicatechin gallate and quercetin-3-*O*-glucoside were also reported to competitively inhibit sodium-independent glucose transport at a concentration of 100 μM each (Chen *et al.* 2007).

A more recent investigation of glucose uptake and transport across a Caco-2 cell monolayer reported the dose-dependent inhibition of both sodium-dependent and independent transport in the presence of strawberry or apple

extract; sodium independent inhibition was more potent as determined by the lower concentration required to achieve 50% inhibition (IC_{50}). Kinetic evaluation of the mechanism for transport inhibition indicated mixed-type for apical cellular uptake and non-competitive for transport across the basolateral membrane. Individual pure compounds were screened for inhibitory capacity, and quercetin-3-*O*-rhamnoside displayed the lowest IC_{50} for inhibition of apical and basolateral transport of those tested. (-)-Epicatechin elicited less than 50% inhibition at a concentration exceeding 500 μ M (Manzano and Williamson 2010).

The objective of the investigation presented here was to determine whether a flavanol-rich dark chocolate extract, and biologically active components of dark chocolate, were able to inhibit glucose uptake into Caco-2 cells and transport across the basolateral membrane.

5.3 Results

5.3.1 Liquid Scintillation Counting

5.3.1.1 Validation

5.3.1.1.1 Linearity, precision and limit of quantification

A calibration curve of radioactivity as a function of [U-¹⁴C]-glucose quantity was prepared and the linear regression equation obtained. With the y-intercept fixed at zero the gradient was calculated as 2441 and the adjusted R-square equal to 1.0, suggesting good proportionality of radioactivity and molar quantity (Figure 5.1). The relative standard deviation of radioactivity was calculated for each concentration of radiolabelled glucose, and ranged from 0.03 to 0.81% indicating that counting precision of the equipment was good. The limit of quantification was calculated as 0.02 pmol.

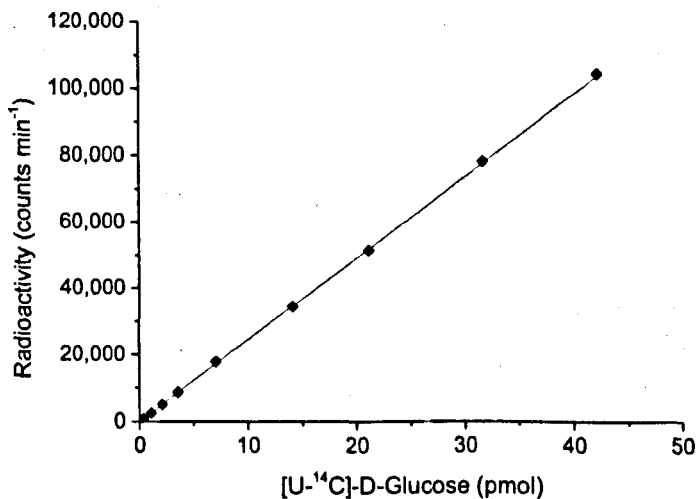


Figure 5.1 [U-¹⁴C]-Glucose calibration curve. Mean \pm SEM (n=3); gradient = 2441, adjusted R-square = 1.0.

5.3.2 Cell culture

5.3.2.1 Validation of cell membrane integrity

Trans-epithelial electrical resistance (TEER) was measured before and after the 30 min incubation and corrected to account for resistance of the polycarbonate membrane to provide a measure of the cell monolayer resistance (Table 5.1). Inter-assay mean TEER measurements taken before commencement of the assay showed a large variation ranging from 160 to 509 Ohms. Partial correlation analysis controlling for the assay condition confirmed that basolateral glucose concentration did not correlate with TEER measured pre- or post-incubation (Figure 5.2). Pre-incubation TEER value was significantly correlated with cell passage number ($p < 0.001$) (Figure 5.3).

Condition	TEER (Ohms)	
	Before	After
1 mM Glucose	423 ± 14	326 ± 9***
Dark chocolate extract 35 µM epicatechin	408 ± 7	345 ± 10***
0.25 mM Glucose	282 ± 11	259 ± 9
Dark chocolate extract 71 µM epicatechin	299 ± 9	289 ± 14
0.5 mM Glucose	322 ± 12	290 ± 12
Dark chocolate extract 71 µM epicatechin	303 ± 6	333 ± 10*
1 mM Glucose	310 ± 15	279 ± 10
Dark chocolate extract 71 µM epicatechin	347 ± 5	356 ± 7
2 mM Glucose	269 ± 6	254 ± 6
Dark chocolate extract 71 µM epicatechin	282 ± 5	295 ± 9
4 mM Glucose	326 ± 8	283 ± 7
Dark chocolate extract 71 µM epicatechin	321 ± 5	345 ± 11**

Condition	TEER (Ohms)	
	Before	After
7 mM Glucose	402 ± 12	353 ± 14*
Dark chocolate extract 71 µM epicatechin	423 ± 15	423 ± 8
0.25 mM Glucose	443 ± 22	268 ± 19***
Dark chocolate extract 142 µM epicatechin	462 ± 17	331 ± 13***
0.5 mM Glucose	451 ± 22	311 ± 17***
Dark chocolate extract 142 µM epicatechin	497 ± 16	416 ± 17**
1 mM Glucose	409 ± 3	305 ± 10**
Dark chocolate extract 142 µM epicatechin	398 ± 12	400 ± 5
2 mM Glucose	386 ± 14	384 ± 12
Dark chocolate extract 142 µM epicatechin	444 ± 10	404 ± 13*
4 mM Glucose	425 ± 15	393 ± 20
Dark chocolate extract 142 µM epicatechin	424 ± 6	450 ± 14
7 mM Glucose	509 ± 8	322 ± 14
Dark chocolate extract 142 µM epicatechin	475 ± 20	337 ± 12
1 mM Glucose	272 ± 11	259 ± 12
Dark chocolate extract 322 µM epicatechin	254 ± 11	272 ± 4
1 mM Glucose	160 ± 7	184 ± 8
200 µM (-)-Epicatechin	175 ± 15	187 ± 5
200 µM (+)-Epicatechin	252 ± 16	246 ± 11
200 µM (-)-Catechin	276 ± 8	265 ± 9
1 mM Glucose	236 ± 10	232 ± 11
200 µM (+)-Catechin	257 ± 7	237 ± 6
200 µM Procyanidin B2	313 ± 22	273 ± 4
1 mM (-)-Epicatechin	346 ± 5	278 ± 6***
1 mM Glucose	323 ± 7	275 ± 9**
250 µM Caffeine	366 ± 6	321 ± 8**

Condition	TEER (Ohms)	
	Before	After
1.68 mM Theobromine	355 ± 10	285 ± 8**

Table 5.1 Blank-corrected trans-epithelial electrical resistance (TEER) measured before and after each assay. Mean ± SEM (n=6) for each condition. Asterisk denotes significant difference from the corresponding pre-incubation measurement, *p<0.05, **p<0.01 and ***p<0.001.

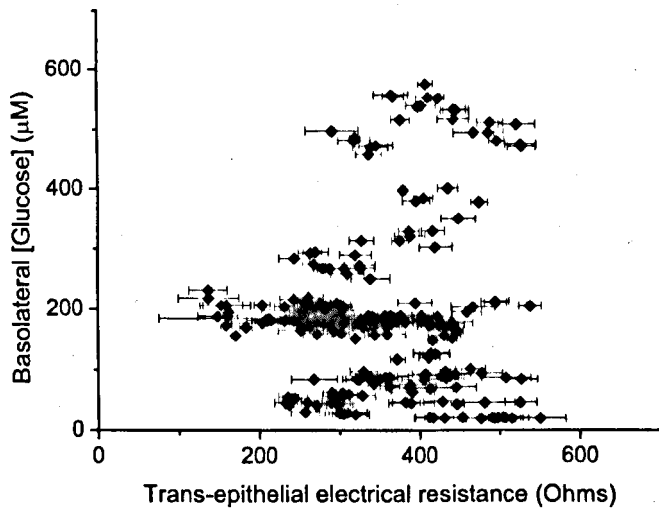


Figure 5.2 Basolateral concentration of glucose after the 30 min incubation in each assay replicate, as a function of mean \pm SEM ($n=3$) trans-epithelial electrical resistance per Transwell® insert cell monolayer measured before commencement of the assay.

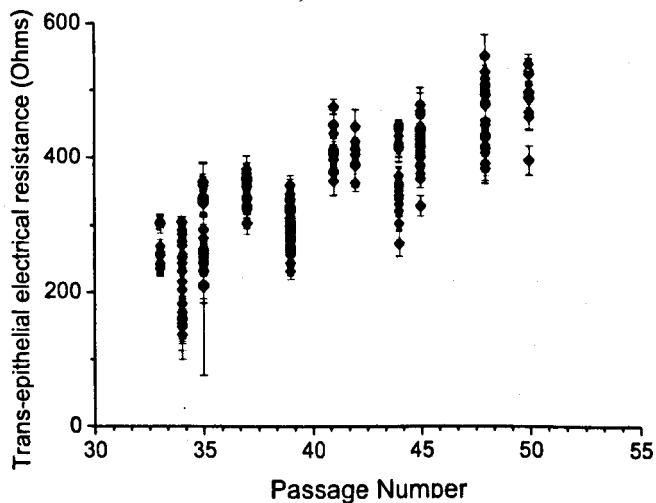


Figure 5.3 Pre-incubation trans-epithelial electrical resistance per Transwell® insert monolayer, mean \pm SEM ($n=3$), as a function of cell passage number.

5.3.2.2 [U-¹⁴C]-Glucose transport study

The effect of a flavanol-rich dark chocolate extract, containing between 35 to 322 μM flavanol monomers (Table 5.2), on apical to basolateral transport and cellular uptake of glucose was investigated. Biologically active components of dark chocolate were also individually assayed to explore the possibility that they were able to moderate glucose uptake and transport at concentrations relevant to the dark chocolate extract.

Co-incubation of 1 mM glucose with dark chocolate extract dose-dependently attenuated the apical to basolateral transport and cellular uptake of glucose up to 75% and 68% respectively. Each preparation of dark chocolate extract significantly reduced the concentration of basolateral glucose, $p < 0.001$ (Figure 5.4A). Dark chocolate extract containing 142 and 322 μM epicatechin also significantly reduced the cellular concentration of glucose, $p < 0.001$ (Figure 5.4B). Of the flavanol monomers and B2 dimer tested, none significantly affected the concentration of glucose measured in the basolateral compartment (Figure 5.5A); 200 μM of each (+)-epicatechin or procyanidin B2 significantly increased cellular glucose concentration by 20% and 26% respectively ($p < 0.01$) (Figure 5.5B). Caffeine (250 μM) and theobromine (1.68 mM) did not significantly affect the basolateral concentration of glucose (Figure 5.6A). By contrast the cellular concentration of glucose was significantly increased by 25% and 9% in the presence of caffeine ($p < 0.05$) and theobromine ($p < 0.01$) (Figure 5.6B).

The kinetic properties of glucose transport in the absence and presence of dark chocolate extract containing 71 μM and 142 μM epicatechin were investigated. Basolateral and cellular concentration of glucose following incubation with initial glucose concentrations ranging from 0.25 to 7 mM are

displayed in Figure 5.7A and B. Both dark chocolate extract preparations significantly reduced the basolateral concentration of glucose at each substrate concentration investigated ($p < 0.01$ or $p < 0.001$). Cellular glucose concentration was significantly reduced at each substrate concentration investigated in the presence of dark chocolate extract containing $142 \mu\text{M}$ epicatechin ($p < 0.05$ and $p < 0.001$) and by dark chocolate extract containing $71 \mu\text{M}$ epicatechin at substrate concentrations of 0.25 , 4 and 7 mM ($p < 0.05$ and $p < 0.001$). Graphical representation of apical to basolateral rate of transport and cellular uptake as a function of substrate concentration demonstrates typical Michaelis-Menten hyperbolic saturation curves in the absence and presence of dark chocolate extract (Figure 5.8A and B). Nonlinear regression analysis using the Michaelis-Menten equation performed on each data set revealed the maximum rate (V_{max}) and Michaelis constant (K_{M}) of apical to basolateral transport and cellular uptake of cells incubated in the presence of substrate only, and when co-incubated with each preparation of dark chocolate extract (Table 5.3). The rate of apical to basolateral glucose transport was significantly attenuated dose-dependently in the presence of dark chocolate extract ($p < 0.01$), this is indicative of non-competitive inhibition. The Michaelis-constant was significantly augmented in the presence of dark chocolate extract ($p < 0.05$ and $p < 0.01$), independent of dose, indicating that transporter affinity for the substrate was reduced due to competitive-type inhibition. Together these kinetic parameters suggest that dark chocolate extract exerts a mixed-type inhibition of apical to basolateral glucose transport.

The rate of cellular glucose uptake was also significantly lower in the presence of dark chocolate extract ($p < 0.01$), dose-independent, suggesting

non-competitive inhibition of cellular glucose uptake. The Michaelis constant significantly diminished in the presence of dark chocolate extract containing 71 μM epicatechin implying that transporter affinity for the substrate was enhanced. Dark chocolate extract containing 142 μM epicatechin did not significantly affect the Michaelis constant relative to the control however in comparison to the dark chocolate extract containing 71 μM epicatechin it was significantly greater ($p < 0.01$). Cellular glucose uptake appeared only to be inhibited in a non-competitive manner.

	Defatted Dark Chocolate (g)			
	0.5	1	2	4.5
Total flavanol content (mg g ⁻¹ FW) ^a	0.7	1.4	2.7	6.1
Transport solution concentration				
Flavanol monomers (μM) ^a	35	71	142	322
Flavanol dimers (μM) ^a	11	22	45	102
Caffeine (μM) ^b	62	124	248	563
Theobromine (mM) ^b	0.7	1.3	2.7	6.1

Table 5.2 Total flavanol content (monomer to decamer) per gram of dark chocolate, fresh weight (FW), and concentration of flavanol monomers and dimers, caffeine and theobromine in the transport solution prepared using defatted dark chocolate. ^aBased on empirical measurement of flavanol content of an extract prepared using 1 g of defatted dark chocolate. ^bCalculated from values reported in the USDA Nutrient Database (U.S. Department of Agriculture 2011).

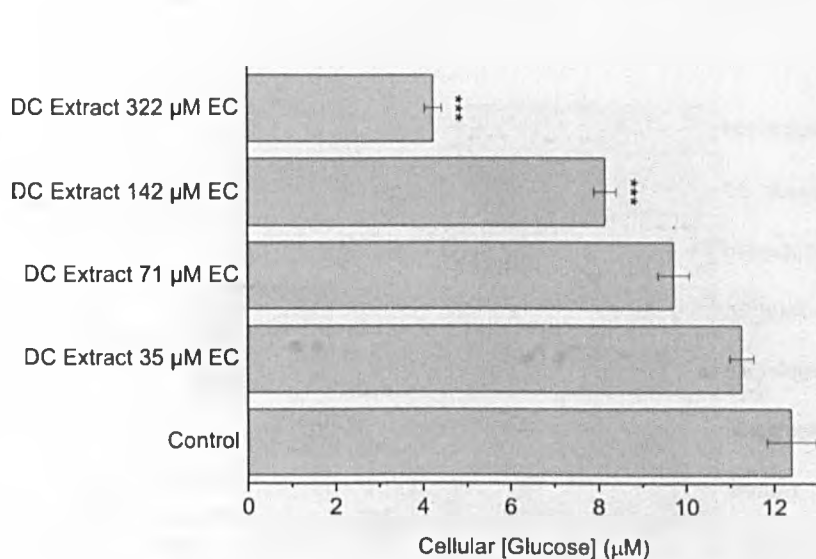
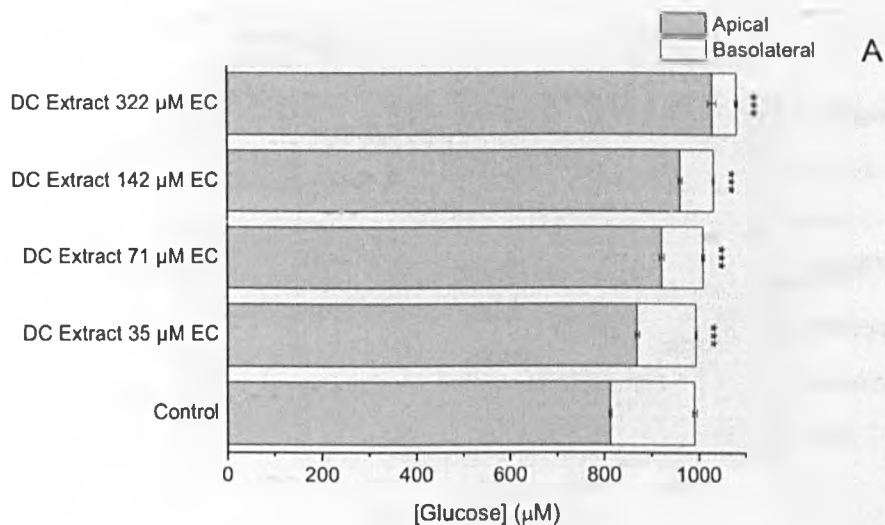


Figure 5.4 (A) Apical and basolateral, and (B) cellular concentration of glucose following a 30 min incubation of Caco-2 cells with 1 mM glucose in the absence (control) and presence of dark chocolate (DC) extract containing 35, 71, 142 or 322 μM epicatechin (EC). Control concentration equates to rate of apical to basolateral transport $5.9 \pm 0.2 \mu\text{M min}^{-1}$ and rate of cellular uptake $0.41 \pm 0.02 \mu\text{M min}^{-1}$. Mean \pm SEM ($n \geq 6$). Asterisk denotes a significantly different basolateral or cellular concentration relative to the control, $***p < 0.001$.

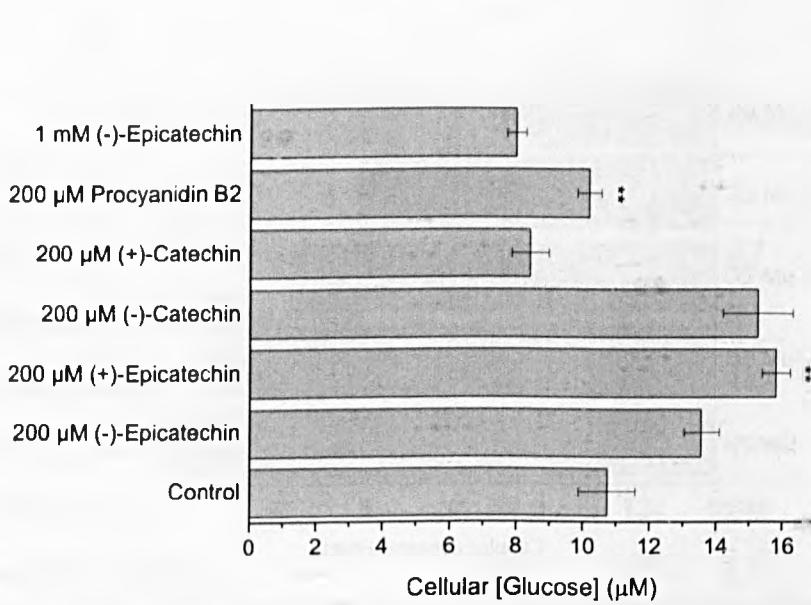
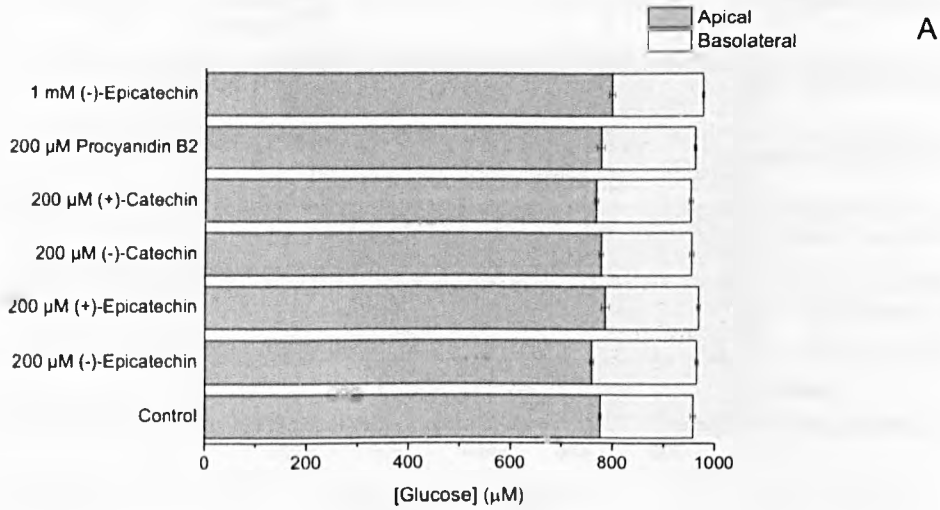


Figure 5.5 (A) Apical and basolateral, and (B) cellular concentration of glucose following a 30 min incubation of Caco-2 cells with 1 mM glucose in the absence (control) and presence of flavanol monomers and B2 dimer. Control concentration equates to rate of apical to basolateral transport $6.1 \pm 0.2 \mu\text{M min}^{-1}$ and rate of cellular uptake $0.36 \pm 0.03 \mu\text{M min}^{-1}$. Mean \pm SEM ($n \geq 6$). Asterisk denotes a significantly different cellular concentration relative to the control, $**p < 0.01$.

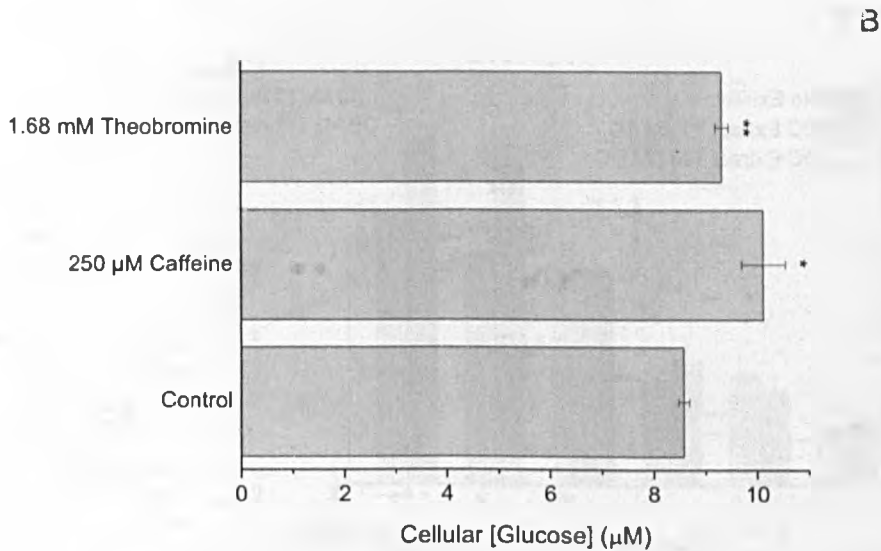
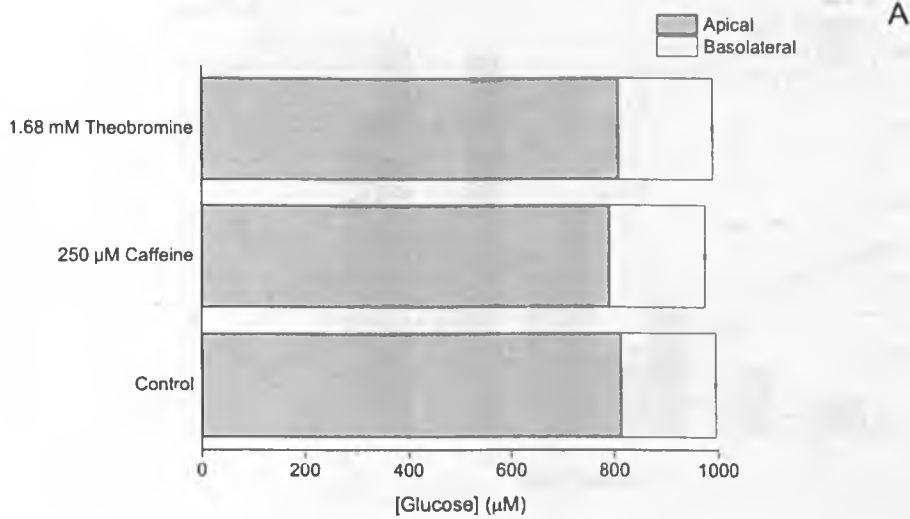


Figure 5.6 (A) Apical and basolateral, and (B) cellular concentration of glucose following a 30 min incubation of Caco-2 cells with 1 mM glucose in the absence (control) and presence of caffeine and theobromine. Control concentration equates to rate of apical to basolateral transport $6.0 \pm 0.1 \mu\text{M min}^{-1}$ and rate of cellular uptake $0.29 \pm 0.00 \mu\text{M min}^{-1}$. Mean \pm SEM ($n=6$). Asterisk denotes a significantly different cellular concentration relative to the control, * $p < 0.05$ and ** $p < 0.01$.

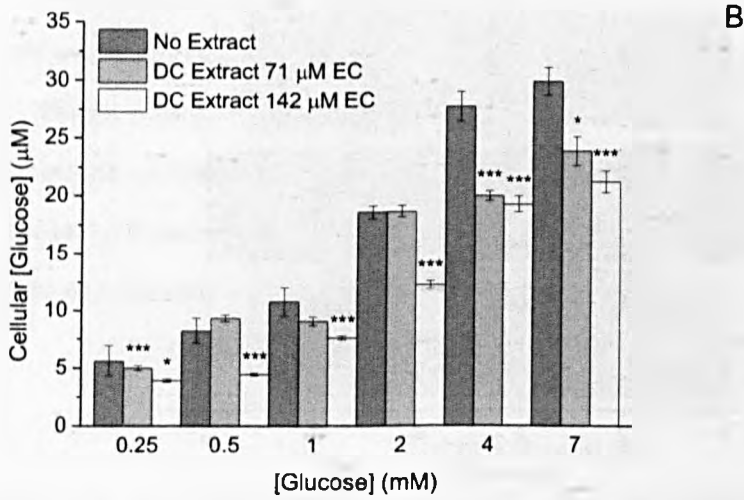
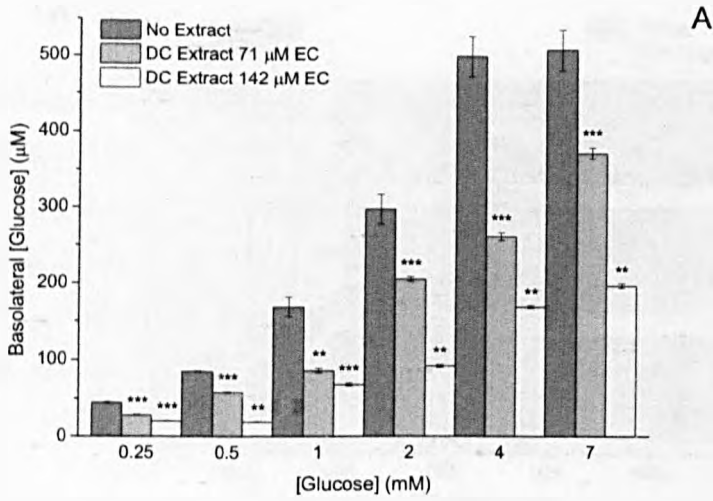


Figure 5.7 (A) Basolateral and (B) cellular concentration of glucose following a 30 min incubation of Caco-2 cells with 0.25-7 mM glucose in the absence (no extract) and presence of dark chocolate (DC) extract containing 71 or 142 μM epicatechin (EC). Values are mean \pm SEM ($n \geq 6$). Asterisk denotes significant difference from the corresponding no extract value, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

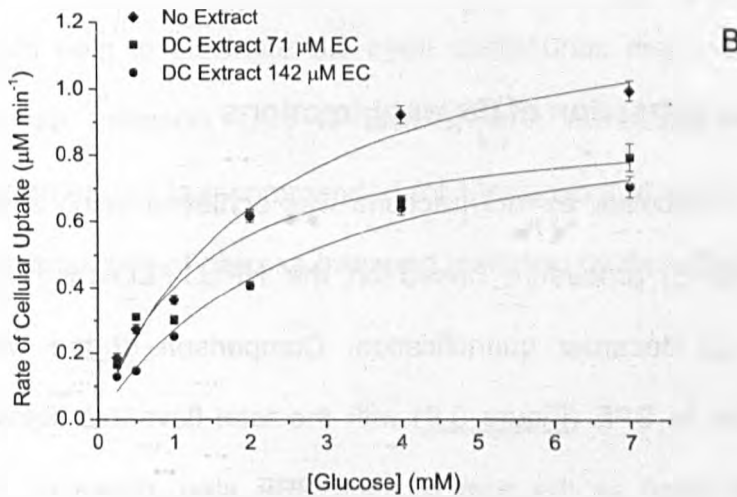
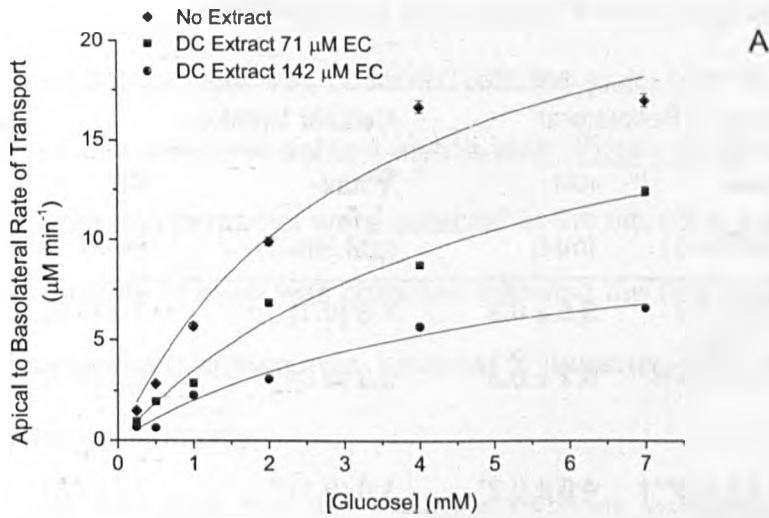


Figure 5.8 Rate of (A) apical to basolateral transport and (B) cellular uptake of glucose following a 30 min incubation of Caco-2 cells with 0.25-7 mM glucose in the absence (no extract) and presence of dark chocolate (DC) extract containing 71 or 142 μM epicatechin (EC). Mean \pm SEM ($n=26$).

	Apical to Basolateral		Cellular Uptake	
	Vmax	KM	Vmax	KM
	($\mu\text{M min}^{-1}$)	(mM)	($\mu\text{M min}^{-1}$)	(mM)
No Extract	27.2 \pm 0.9	3.5 \pm 0.3	1.3 (0.1)	2.2 (0.2)
DC Extract 71 μM EC	21.3 \pm 0.7**	5.2 \pm 0.3**	0.9 (0.04)**	1.3 (0.2)*
DC Extract 142 μM EC	11.2 \pm 0.3**†	4.6 \pm 0.2*	1.0 (0.1)**	2.7 (0.3)†

Table 5.3 Michaelis-Menten kinetic properties of cellular uptake and apical to basolateral transport of glucose in the absence (no extract) and presence of dark chocolate (DC) extract containing 71 μM or 142 μM epicatechin (EC). Values are mean \pm SEM ($n \geq 6$) per condition. Asterisk denotes significant difference from the corresponding no extract value, * $p < 0.05$ and ** $p < 0.01$ and † denotes significant difference from the corresponding DC extract containing 71 μM EC ($p < 0.01$).

5.3.3 Solid phase extraction of flavanol fractions

Separation of dark chocolate extract fractions was achieved using a solid phase extraction (SPE) procedure based on the HPLC-FLD method of flavanol monomer to decamer quantification. Comparison of the whole extract not subjected to SPE ([Figure 5.9](#)) with the total flavanols obtained following SPE, calculated as the sum of each SPE step, displayed very similar concentrations indicating that most of the flavanols monomer to decamer were collected ([Table 5.4](#)). Monomers mainly eluted with the 'initial' pass of sample ([Figure 5.10A](#)) and the water wash phases. Dimers eluted equally across each phase except the final organic solvent elution step demonstrating no preference for aqueous or organic solvent. The larger compounds with a greater degree of polymerisation appeared to be retained by the sorbent material more strongly. Trimeric flavanols, in total, eluted

equally between the aqueous and organic solvents although a preference for the organic solvent was observed with the majority of the compound eluting with the first organic solvent elution step (Figure 5.10B). A small amount of tetramer and pentamer were collected in the aqueous wash phases however the majority of each was collected following the first organic solvent elution. The remaining compounds, hexamer to decamer, were eluted in the organic solvent elution only.

It was intended that the aqueous fractions combined, rich in flavanols monomer and dimer, and the fractions eluted with organic solvent, containing relatively few flavanol monomers and dimers but rich in trimers to decamers, would be used in the assay of glucose transport to determine whether either fraction replicated the effect of the whole dark chocolate. This would help to elucidate the main compounds responsible for inhibition of glucose transport. Due to lack of time remaining this assay was not performed but is recommended for future work in order to contribute to the understanding of glucose transport inhibition by dark chocolate extract.

Degree of Polymerisation	Concentration ($\mu\text{g mL}^{-1}$)						
	A	B	C	D	E	F	G
Monomer	275.5	274.7	171.5	20.8	3.9	746.5	771.9
Dimer	35.1	42.5	30.9	39.9	4.0	152.6	145.5
Trimer	5.7	11.5	15.4	28.8	2.1	63.5	54.8
Tetramer	0.5	2.4	4.2	14.8	1.3	23.2	20.6
Pentamer	0.0	0.3	0.8	9.7	1.2	12.0	11.2
Hexamer	0.0	0.0	0.0	4.6	0.9	5.4	7.1
Heptamer	0.0	0.0	0.0	1.9	0.5	2.4	2.5
Octamer	0.0	0.0	0.0	0.7	0.2	1.0	1.1
Nonamer	0.0	0.0	0.0	0.3	0.1	0.4	0.5
Decamer	0.0	0.0	0.0	0.2	0.1	0.2	0.2

Table 5.4 Flavanols monomer to decamer determined by HPLC-FLD in fractions of dark chocolate extract separated by a solid phase extraction (SPE) procedure. A = initial collection of aqueous sample following SPE, B = first aqueous wash, C = second aqueous wash, D = first organic solvent elution, E = second organic solvent elution, F = total of all SPE steps A to E, and G = whole dark chocolate extract before SPE separation of fractions.

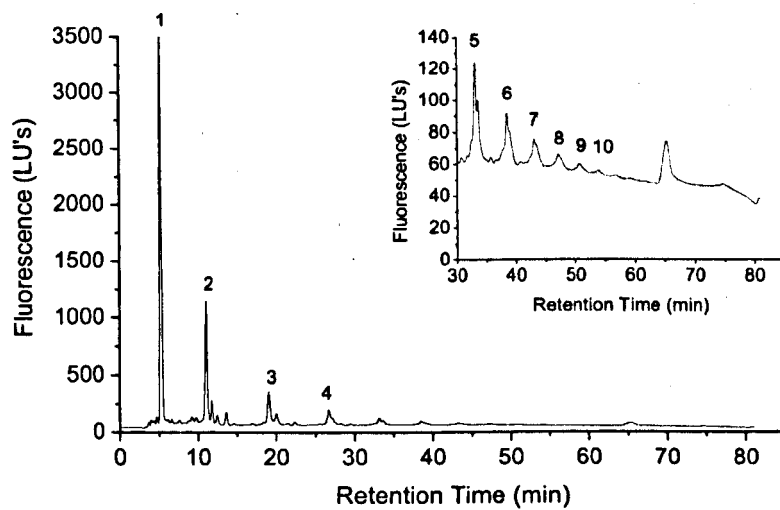


Figure 5.9 HPLC-FLD chromatogram of whole dark chocolate extract flavanols monomer to decamer before solid phase extraction separation of fractions. Peak numbers represent the degree of polymerisation; 1 = monomer, 2 = dimer, etc.

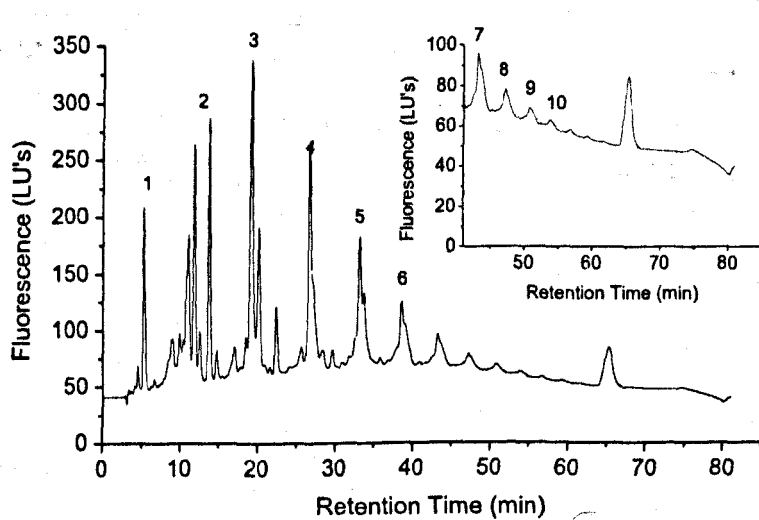
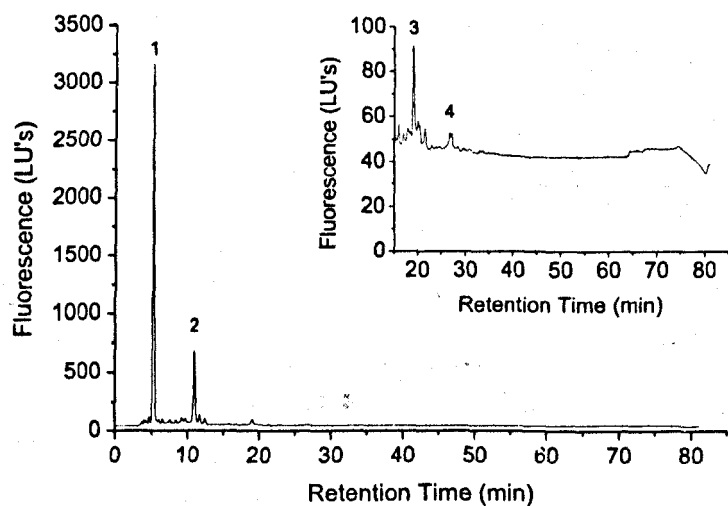


Figure 5.10 HPLC-FLD chromatogram of A) initial aqueous fraction collected following solid phase extraction, and B) the first organic solvent wash of sorbent retained compounds. Peak numbers represent the degree of polymerisation; 1 = monomer, 2 = dimer, etc.

5.4 Discussion

Preparations of flavanol-rich dark chocolate extract containing 35, 71, 142 and 322 μM epicatechin were shown to reduce basolateral glucose concentration in a dose-dependent manner. Cellular concentration of glucose was also dose-dependently reduced but only in the presence of dark chocolate extract containing 142 and 322 μM epicatechin. Glucose uptake into epithelial cells is predominantly governed by the sodium-dependent secondary active cotransporter SGLT1 and efflux from the basolateral membrane is maintained entirely by passive diffusion facilitated by the sodium-independent GLUT2. Kinetic analysis of apical to basolateral transport and rate of cellular uptake revealed that glucose transport and uptake reached saturation as substrate concentration increased; this is indicative of active transport. In the study presented here it is evident that the predominant mechanism of glucose transport is via the sodium-dependent cotransporter SGLT1.

The highest concentrations of epicatechin-containing dark chocolate extract, 142 and 322 μM , significantly reduced the concentration of cellular glucose which could be considered responsible for the observed reduction in basolateral glucose concentration, however at the lowest concentrations of epicatechin-containing dark chocolate extract, 35 and 71 μM , the basolateral glucose concentration was significantly reduced without a corresponding reduction of cellular glucose concentration. If the cellular uptake of glucose remained constant a reduction in basolateral glucose concentration would be expected to create an accumulation of glucose within the cell. The absence of increased cellular glucose concentration may be a consequence of

surplus glucose being either metabolised within the cell or effluxed from the apical membrane. Alternatively the results may indicate that apical glucose transport is also diminished but to a lesser extent than basolateral transport thereby negating any accumulation of glucose within the cell. Reduced cellular and basolateral concentration implies that apical uptake is affected still more to the point where a significant difference is observed. Inhibition of basolateral transport separate from that of apical transport demonstrates the ability of the inhibiting compound(s) to cross the apical cell membrane or permeate the tight junctions between cells to reach the basolateral membrane where the effect is elicited.

To assess whether inhibition of glucose transport was attributable to specific flavanol components of the dark chocolate extract, epicatechin was assayed at a concentration relevant to that in the dark chocolate extract. In this instance 200 μ M, and 1 mM, (-)-epicatechin did not significantly affect glucose concentration in the cells or basolateral compartment. For ease of comparison the same concentration of (+)-catechin and procyanidin B2, a flavanol monomer and dimer present in dark chocolate, were also investigated. (+)-Catechin did not elicit a significant effect on glucose transport. In contrast the cellular concentration of glucose was significantly greater in the presence of procyanidin B2 relative to the control; basolateral concentration was unaffected. The enantiomers (+)-epicatechin and (-)-catechin were also investigated to determine whether the structural configuration of the flavanol monomer was able to exhibit a significant effect. 200 μ M (-)-Catechin did not significantly moderate glucose transport however the same concentration of (+)-epicatechin significantly increased the cellular uptake of glucose without affecting basolateral efflux. From these results it

was apparent that individually flavanol monomers and B2 dimer were not responsible for the inhibitory effect induced by the dark chocolate extract, although a synergistic effect of flavanols in the dark chocolate extract could be responsible.

The methylated xanthine components of dark chocolate, caffeine and theobromine were also assayed at concentrations relevant to those expected in the dark chocolate extract. Both compounds significantly increased the cellular concentration of glucose without affecting the basolateral glucose concentration. Increased cellular uptake but not apical to basolateral transport of glucose following incubation in the presence of (+)-epicatechin, procyanidin B2, caffeine and theobromine is considered to be a consequence of enhanced apical glucose transporter activity without any significant change in the activity of basolateral glucose transport. It has been reported that the presence of caffeine significantly enhances intestinal glucose absorption in human subjects following ingestion of coffee (Johnston *et al.* 2003) and hypothesised that augmented carbohydrate oxidation during exercise is a consequence of a caffeine induced increase in glucose absorption (Yeo *et al.* 2005). Both caffeine and theobromine are inhibitors of the enzyme cyclic 3', 5'-nucleotide phosphodiesterase (EC 3.1.4.17) that catalyses the hydrolysis of a phosphate ester on a 3', 5'-cyclic nucleoside monophosphate (Butcher and Sutherland 1962). Inhibition of this enzyme results in the intra-cellular increase of a substrate such as cyclic adenosine monophosphate (cAMP), which has been implicated in the stimulation of SGLT1 activity (Sharp and Debnam 1994). cAMP-dependent protein kinase (EC 2.7.11.11) catalyses the phosphorylation of proteins, such as SGLT1, by transferring a phosphate group from adenosine triphosphate (ATP). cAMP-

activated protein kinase has been associated with enhancement of SGLT1 activity through additional trafficking of the transporter to the apical membrane. The maximum rate of glucose transport was increased without alteration of transporter affinity for the substrate (Wright *et al.* 1997). Previous studies have reported flavonoid inhibition of phosphodiesterase activity with cAMP as the substrate (Beretz *et al.* 1978, Ruckstuhl and Landry 1981) and stimulation of cAMP-dependent protein kinase (Eid *et al.* 2010, Zygmunt *et al.* 2010). This offers a hypothesis for the observed increase in cellular glucose concentration in the presence of caffeine, theobromine, procyanidin B2 and (+)-epicatechin.

Analysis of the kinetic effects of dark chocolate extract revealed that the maximum rate of apical to basolateral transport was dose-dependently reduced parallel with an increase in the Michaelis constant, not dose-dependent, that is overall indicative of a mixed-type inhibition. The maximum rate of reaction (V_{\max}) is representative of the rate at which an enzyme-substrate complex dissociates to form the product dependent on the concentration of total enzyme present. Attenuation of the rate of reaction, or in this instance the rate of transport, signifies non-competitive inhibition. The Michaelis constant (K_M) represents the ratio of enzyme-substrate complex dissociation, to enzyme and substrate or enzyme and product, relative to the formation of the enzyme-substrate complex. Increased K_M is associated with decreased affinity of the enzyme, or in this instance transporter, for the substrate which is indicative of competitive inhibition.

Kinetic analysis of cellular glucose uptake demonstrated that dark chocolate extract significantly attenuated the maximum rate of transport, independent of dose, implying non-competitive inhibition. Dark chocolate extract

containing 142 μM epicatechin did not significantly affect the Michaelis constant however the extract containing 71 μM epicatechin was responsible for a significant increase in the calculated K_M that suggests enhancement of transporter affinity for the substrate.

Reduced V_{max} of cellular uptake and apical to basolateral transport suggest that apical SGLT1 and basolateral GLUT2 are inhibited non-competitively. Increased K_M of apical to basolateral transport but not cellular uptake suggests that competitive inhibition affects GLUT2 but not SGLT1. The ability to competitively inhibit GLUT2 indicates that the inhibitor is able to enter the cell and is therefore limited to the less polymerised flavanols. Inhibition of SGLT1 could be a consequence of flavanol-transporter bond formation at one of the extracellular or intracellular loops which alters the structural conformation of the protein. The lack of inhibition by the flavanol monomers and B2 dimer investigated, imply that flavanols with a greater degree of polymerisation are involved or that a synergistic effect occurs. Protein-flavanol interactions are more prolific with increasing molecular weight (Soares *et al.* 2007) however the ability to enter the cell and interact with intracellular loops is expected to be limited to compounds with a degree of polymerisation no larger than dimer (Spencer *et al.* 2001, Ottaviani *et al.* 2012). The capacity to permeate the tight junctions and elicit an extracellular basolateral effect is probably limited to flavanols with a degree of polymerisation no greater than hexamer (Zumdick *et al.* 2012).

The observed increase in K_M of cellular uptake is hypothesised to be a consequence of enhanced SGLT1 trafficking to the apical membrane elicited by the methyl xanthine content of the dark chocolate extract. Whilst it would be expected to observe an increase in cellular glucose concentration and

therefore rate of transport, this effect may be negated by the presence of flavanols which non-competitively inhibited the activity of SGLT1. The proposed methyl xanthine induced effect may not be dose-dependent whereas dark chocolate extract induced reduction of cellular glucose concentration was dose-dependent. Dark chocolate extract containing 71 μM epicatechin may only inhibit cellular glucose uptake sufficiently to counteract the enhancement stimulated by caffeine and/or theobromine therefore there is no significant change in cellular glucose concentration. The addition of extra transporters that have the potential to increase glucose uptake are non-competitively inhibited, thus a reduced V_{max} and increased K_{M} is calculated. In the presence of dark chocolate containing 142 μM epicatechin SGLT1 is inhibited beyond the level of enhancement such that a significant reduction in cellular glucose concentration is observed. The V_{max} remains attenuated whilst the K_{M} is returned to a level similar to that of the control containing no dark chocolate extract.

5.5 Conclusion

Dark chocolate extract, but not individual flavanol monomers, B2 dimer and methyl xanthines, inhibit the cellular uptake and apical to basolateral transport of glucose. Non-competitive inhibition of both apical SGLT1 and basolateral GLUT2 along with competitive inhibition of GLUT2 is the proposed method of inhibition. Specific inhibitor/s remain unknown but are postulated to involve low molecular weight flavanols that are able to permeate the apical cell membrane where they interact with the intracellular active site of the protein carrier GLUT2. Inhibition of SGLT1 may involve low molecular weight flavanols that either enter the cell and form bonds with

intracellular regions of the protein or remain in the extracellular space where they similarly can interact with luminal facing regions of the protein. Larger molecular weight flavanols may also interact with the extracellular regions. Caffeine and theobromine are hypothesised to enhance cellular glucose concentration through inhibition of cyclic 3', 5'-nucleotide phosphodiesterase which may increase the cellular concentration of cyclic adenosine monophosphate ultimately leading to additional SGLT1 trafficking to the apical membrane. This effect potentially conveys an enhancement of the SGLT1 Michaelis dissociation constant (K_M) but is reversed by inhibition of the transporter.

Future studies should initially investigate inhibition of GLUT2 separately from SGLT1 as the kinetic properties of each transporter need to be determined separately. The potential for methyl xanthine inhibition of cyclic 3', 5'-nucleotide phosphodiesterase and whether this does lead to enhancement of SGLT1 membrane insertion and activity in the Caco-2 cell model requires confirmation. The inhibitory effect of flavanols with a degree of polymerisation greater than dimer should also be investigated. Synergistic effects between the flavanol components of dark chocolate and antagonism of methyl xanthine effects require further consideration. Where possible, the flavanol binding sites of SGLT1 and GLUT2 should be elucidated.

Chapter 6 Summary and future perspectives

6.1 Epicatechin transport

6.1.1 Effect of dark chocolate

Epicatechin present in dark chocolate extract was transported from the apical to basolateral membrane of the Caco-2 monolayer to the same extent as the individual compound. Transcellular transport of epicatechin was confirmed by the detection of *O*-methylated epicatechin in both apical and basolateral compartments following incubation of Caco-2 cells with standard epicatechin; the predominant form being 3'-*O*-methylated epicatechin. Incubation of cells with dark chocolate extract resulted in there being no *O*-methylated epicatechin detected in either apical or basolateral samples. Inhibition of epicatechin methylation by flavanol dimers has been reported using epicatechin as the substrate (Spencer *et al.* 2001). Reduced formation of 3'-*O*-methyl epicatechin was linear at concentrations of dimer up to 300 μM and increased dimer methylation was noted. The concentration of flavanol dimers in the dark chocolate extract used in the investigation presented here was 76 μM . The absorption of flavanol dimers remains controversial, *in vitro* and animal models appear to more readily transport these compounds whereas availability in humans is less likely. This highlights the difficulty of extrapolating results obtained from animal and cell culture studies to humans.

In the study of epicatechin transport across the Caco-2 cell monolayer presented here, only free and methylated epicatechin were analysed, detection of free flavanol dimers in the basolateral compartment and methylated dimers along with conjugated metabolites of epicatechin would

provide more insight as to the mechanisms that affected the metabolism of epicatechin present in the dark chocolate extract. In the absence of such analysis it is hypothesised that flavanol dimers present in the dark chocolate extract were taken up by the cell where they competitively inhibited methylation of epicatechin. Studies of dimer absorption report very low concentrations permeating the epithelial membrane, which may have little significance *in vivo*. Similarly attenuating the rate at which epicatechin is metabolised by intestinal, kidney or hepatic cells doesn't appear to affect total elimination in urine, thereby potentially restricting the impact this may have physiologically. Further investigations which address the bioactivity of metabolised forms of flavanol monomers and dimers may elucidate a physiological implication of these results.

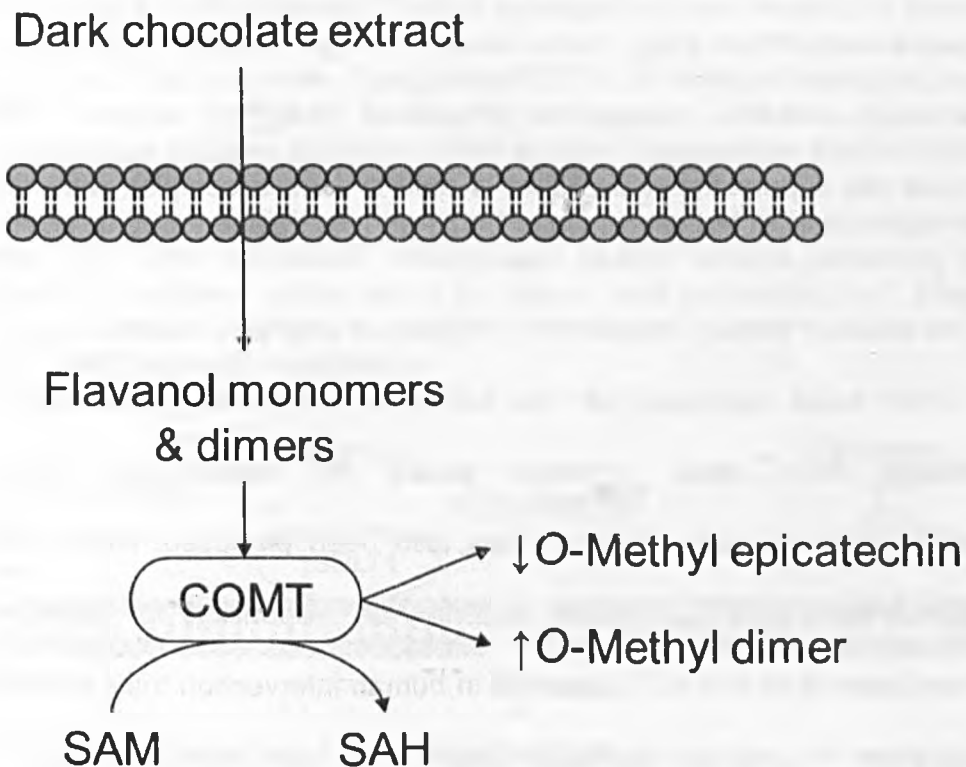


Figure 6.1 Illustration of proposed mechanism for dark chocolate extract inhibition of epicatechin methylation in Caco-2 cells.

6.1.2 Effect of sucrose, glucose and fructose

Sucrose enhanced the absorption of epicatechin in the Caco-2 cell model, which was attributed to stimulation of sodium-dependent glucose transporter activity at the apical membrane leading to increased tight junction pore size, determined by lower trans-epithelial electrical resistance; this resulted in greater paracellular permeability of epicatechin. A small number of human intervention studies have resulted in the speculation that sucrose may augment epicatechin bioavailability. Many investigations have focused on the bioactivity of epicatechin in its free form, with improvements in biomarkers of cardiovascular disease being its primary potential benefit. The mechanisms by which epicatechin may exert such influence include increased endothelial nitric oxide levels through inhibition of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which produces the superoxide radical that is able to form a complex with nitric oxide producing another radical, peroxynitrite. Endothelial nitric oxide may also be elevated through epicatechin inhibition of arginase, which competes with nitric oxide synthase for the substrate L-arginine. Stimulation of endothelial nitric oxide synthase activity by epicatechin induced phosphorylation of serine residues has also been proposed. Nitric oxide stimulates endothelial vasodilation and may be responsible for increased flow mediated dilation (FMD) observed in human intervention trials in which consumption of cocoa has resulted in greater FMD being measured.

Epicatechin has also been reported to improve biomarkers of CVD risk factors such as inflammation, oxidative stress and dyslipidemia. Reduction

of: C-reactive protein in diabetic mice supplemented with epicatechin, malondialdehyde concentration in rats supplemented with cocoa fibre, and total and LDL cholesterol levels of obese-diabetic rats supplemented with cocoa, have been reported.

Increasing bioavailability of epicatechin with formulations including sucrose may enhance such beneficial effects. However the impact of sucrose on biomarkers of disease must also be taken into consideration.

Glucose and fructose reduced the production of methylated epicatechin in the Caco-2 cell model although epicatechin transport was not affected. This suggested the inhibition of COMT activity. COMT inhibition in the presence of glucose or fructose is hypothesised to be a consequence of glycolytic enzymes competing with COMT for the magnesium cation cofactor required for activity in both pathways. Further investigations are required to determine whether, in the presence of magnesium (II) in the transport solution, glucose and fructose are able to inhibit COMT activity. It is possible that the inhibition observed in the epicatechin transport study presented here is unique to the specific conditions under which the assay was conducted, i.e. a lack of sufficient magnesium cations.

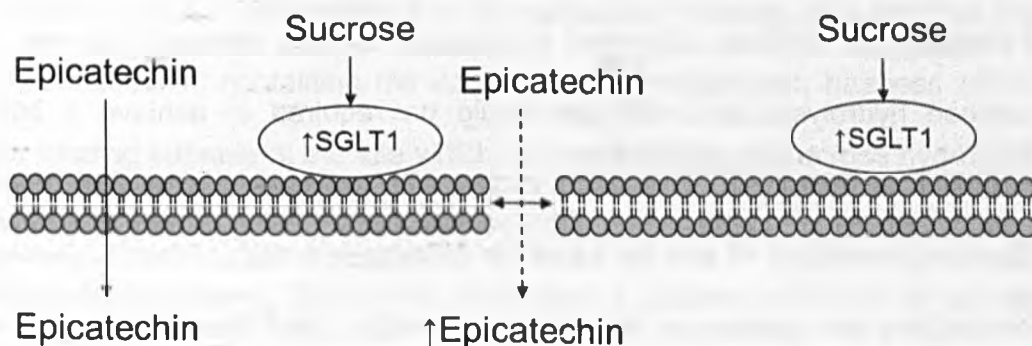


Figure 6.2 Illustration for the proposed mechanism by which sucrose may enhance epicatechin absorption in Caco-2 cells.

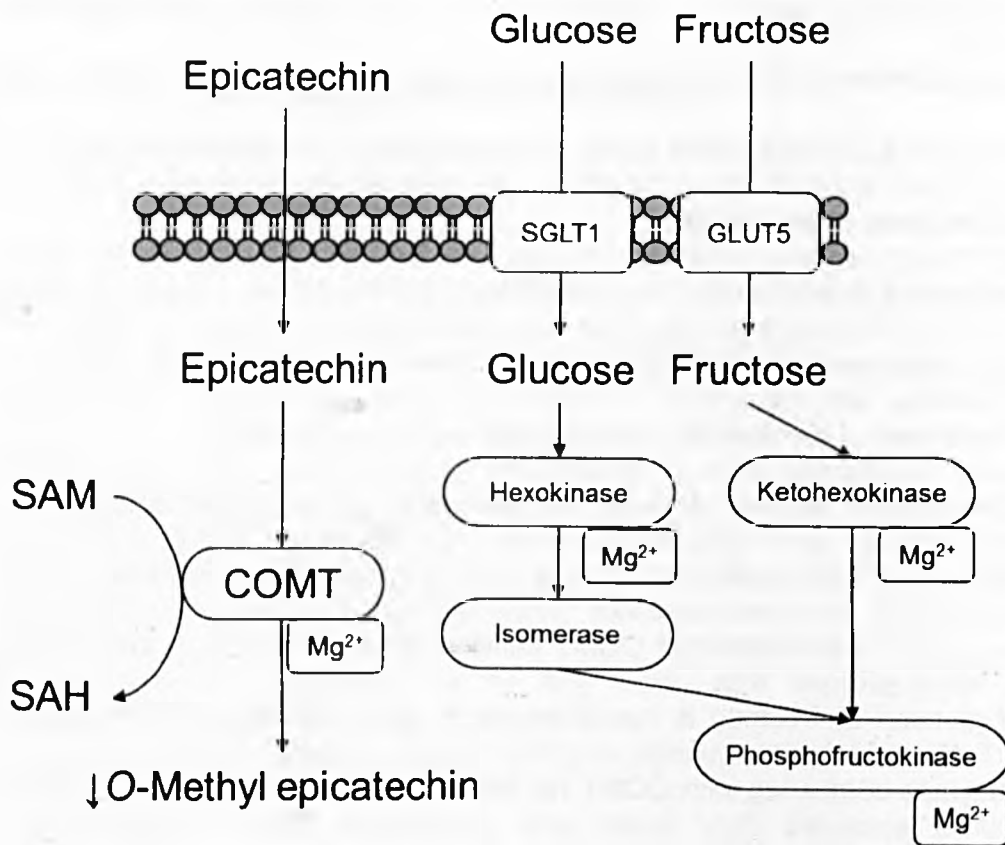


Figure 6.3 Illustration of the proposed mechanism by which glucose and fructose inhibit the production of methylated epicatechin in Caco-2 cells.

6.2 Sucrose hydrolysis

Dark chocolate extract containing 322 μM epicatechin reduced the rate of sucrose hydrolysis in Caco-2 cells by 59%, this was partly attributable to the (-)-epicatechin content. Standard (-)-epicatechin also reduced the rate of sucrose hydrolysis but >500 μM would be required to achieve a 50% reduction in hydrolysis. This is consistent with previous studies that report IC_{50} concentrations >1 mM for inhibition of sucrase activity. Flavanol-protein interactions are greater as the molecular weight, and thus the degree of polymerisation, increases. Combined with the results of the study presented here, it is hypothesised that synergistic effects between flavanols in dark

chocolate extract accounts for inhibition of sucrose hydrolysis in excess of that measured for (-)-epicatechin alone. Further investigation using commercially available standard compounds and/or those extracted by separation of flavanol fractions would confirm or refute this concept.

α -Hydrolase enzymes operate in a similar way; product formation is a function of acid hydrolysis of the substrate. The active site of sucrase shares some homology with α -amylase; it has been proposed that flavanol inhibition of the enzyme requires the presence of delocalised electrons in a conjugated AC-ring and is dependent on the number of hydroxyl groups across the molecule. Based on this evidence, it would not be expected that flavanols, containing just two hydroxyl groups located on the B-ring and a non-conjugated AC-ring system, would have a propensity for strong inhibition of sucrase. The stereoisomerisation of hydroxyl groups has also been implicated in the capacity to inhibit α -hydrolase enzymes. In order to examine this hypothesis, greater concentrations of each flavanol monomer enantiomer require investigation to allow comparison with the (-)-epicatechin isomer.

Based on the evidence presented here, it appears that enhancement of sucrase activity is independent of isomerisation. However, it is possible that (+)-epicatechin, containing the 2S, 3S bond configuration, has less affinity for binding sucrase at the site which causes inhibition of sucrose hydrolysis. Enhancement of activity was sustained at a concentration in which the other flavanol monomers, and dimer, displayed a relative reduction in activity, compared to the enhanced activity. Analysis of the kinetic properties of sucrose hydrolysis in the presence of dark chocolate extract and individual flavanol compounds, would confirm the type of inhibition. Computational

software could be used to predict the sites of interaction for ligands based on protein structure. Enhanced sucrose hydrolysis in the presence of moderate concentrations of flavanol monomers and dimer are proposed to be a consequence of flavanol-protein binding at a site other than the active site of sucrase. It has been suggested that such binding may stimulate a conformational change in the tertiary structure of the enzyme permitting more catalytic residues to be exposed to the hydrophilic environment (Gupta *et al.* 2010). In this instance the result may be enhanced hydrolysis of sucrose at the active site leading to an increase in the rate of product formation.

Impaired hydrolysis of sucrose in the small intestine is associated with congenital sucrase-isomaltase deficiency, a disease in which the patient experiences abdominal pain, bloating and diarrhoea. As there is no cure for the disease treatment involves elimination of sucrose, and other carbohydrates that are hydrolysed to maltose, from the diet or use of an enzyme replacement therapy. Inhibition of α -glucosidase enzymes by acarbose is an approved treatment for type-2 diabetes, although side-effects of flatulence and diarrhoea have been reported; the ability of dark chocolate extract and (-)-epicatechin to reduce sucrose hydrolysis could be considered to lead to similar side-effects. However this would depend upon whether sucrose hydrolysis was reduced sufficiently for sucrose to pass through to the colon where it would elicit negative side-effects, or whether hydrolysis was merely slowed down such that the peak in blood glucose concentration was lessened. This is a more probable outcome as consumption of dark chocolate is not associated with negative intestinal effects.

6.3 Glucose transport

Dark chocolate extract reduced cellular uptake and transport of glucose. This was not attributed to commercially available individual flavanol monomer or dimer components of dark chocolate or to caffeine and theobromine. It is hypothesised that a synergistic effect of the flavanol components is present. Individually caffeine and theobromine increased the rate of cellular glucose uptake without any change in the rate of transport. This supports previous reports that have observed greater glucose concentration in the plasma of subjects following ingestion of a caffeine containing beverage compared with a similar decaffeinated drink (Johnston *et al.* 2003). This is believed to be a result of increased sodium-dependent glucose transporter trafficking to the apical membrane due to elevated cellular concentrations of cAMP through inhibition of cyclic 3', 5'-nucleotide phosphodiesterase activity.

Transport of glucose by Caco-2 cells, presented here, was not linear with increasing concentration therefore it was considered that the primary route of cellular uptake was via the sodium-dependent glucose transporter SGLT1. Kinetic analysis of glucose transport in the presence of different concentrations of dark chocolate extract revealed a dose-dependent reduction in the rate of transport but not cellular uptake. The concentration of glucose required to achieve half the maximum rate of transport was increased for apical to basolateral transport without any change in cellular uptake, at the highest concentration of dark chocolate extract investigated. It is proposed that glucose uptake by SGLT1 was non-competitively inhibited whereas basolateral efflux of glucose by the hexose transporter GLUT2 was

inhibited by mixed-type. Non-competitive inhibition of GLUT2 is believed to be dose-dependent and responsible for the dose-dependent change in V_{\max} . Apparent enhancement of cellular glucose uptake in the presence of the lowest concentration of dark chocolate extract investigated, determined by a lower K_M , was believed to be a result of methyl xanthine induced stimulation of SGLT1 trafficking to the apical membrane concurrent with non-competitive inhibition of the transporter by the flavanol components.

Increased intestinal glucose concentration stimulates the insertion of GLUT2 into the apical membrane to assist with glucose absorption. In type-2 diabetic patients GLUT2 at the apical membrane remains elevated such that high blood glucose concentration, due to impaired uptake by muscle and adipose cells, is exacerbated. A capacity to reduce the activity of glucose transporters may be of benefit to patients with diabetes or pre-diabetes. In healthy subjects a general reduction in the glycaemic impact of a food/meal is considered beneficial for health. The presence of sugars in the colon may result in abdominal pain and diarrhoea, however similarly to the consideration for sucrase inhibition, a reduction in the rate of transport that lessens the post-prandial peak of blood glucose concentration without affecting the total concentration absorbed, is less likely to have negative side-effects.

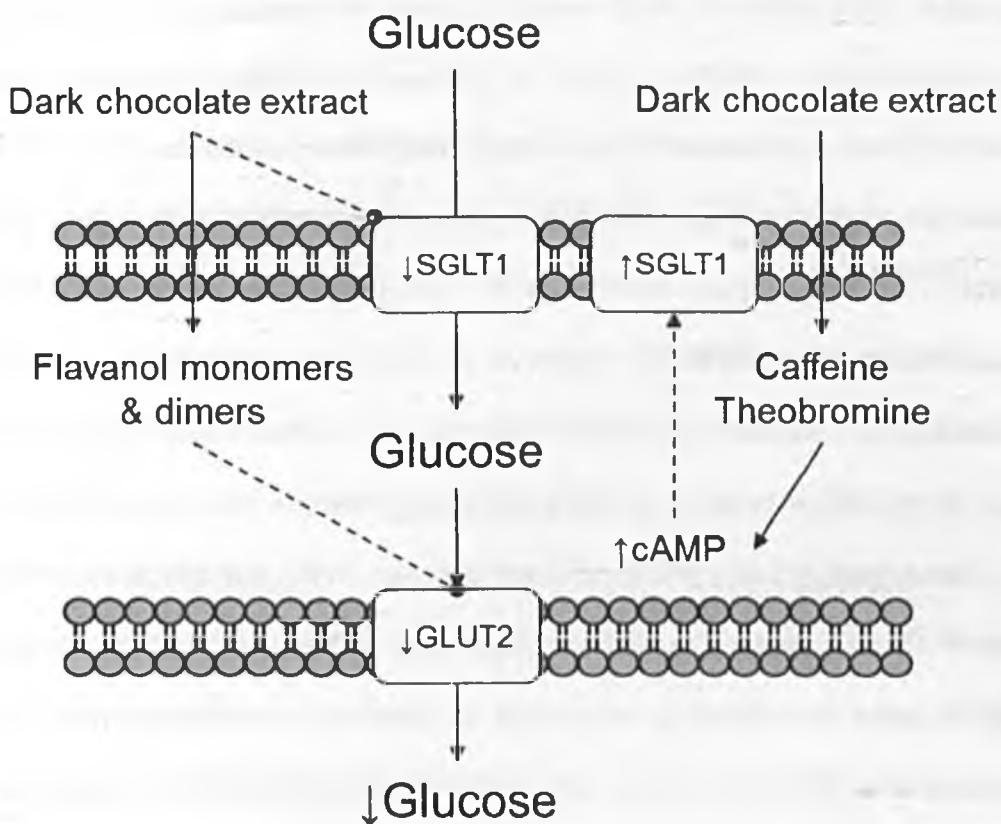


Figure 6.4 Illustration of the proposed mechanism for inhibition of glucose uptake and transport through the Caco-2 cell monolayer, and enhancement of glucose uptake by caffeine and/or theobromine.

6.4 Effect of dark chocolate on glucose uptake in healthy human subjects

To date there have been many studies conducted to investigate the effect of plant extracts and polyphenolic components on blood glucose levels. Several *in vivo* animal studies have investigated the chronic effects of dietary supplementation on biomarkers of metabolic disease and expression of genes related to glucose metabolism (Bose *et al.* 2008, Oliveira *et al.* 2008, Hininger-Favier *et al.* 2009, Kannappan and Anuradha 2009, Jia *et al.* 2009, Chen *et al.* 2011, Sae-tan *et al.* 2011, Bnouham *et al.* 2012, Kobori *et al.* 2012, Qin *et al.* 2012). Overall these studies reveal lowered blood

glucose concentration and insulin concentration/sensitivity following supplementation with the extract, in comparison to a non-supplemented control. Acute administration of various plant extracts has been shown to lower the rise in post-prandial blood glucose concentration in healthy and diabetic-induced animals (Motilva *et al.* 1983, Koga *et al.* 2006, Ishikawa *et al.* 2007, Ndong *et al.* 2007, Hogan *et al.* 2010, Abeywickrama *et al.* 2011, Ali *et al.* 2011, Ikarashi *et al.* 2011, Roy *et al.* 2011, Murase *et al.* 2012). The use of glucose, sucrose or maltose in the oral tolerance test of these studies has been useful to suggest a mechanism by which the hypoglycemic effects occur. For example 1 g kg⁻¹ polyphenolic extract of *Acacia mearnsii* administered to rats along with glucose, sucrose or maltose significantly reduced the 0-3 h area under the curve (AUC), 0.5 g kg⁻¹ of extract also reduced the 0-3 h AUC for maltose induced glycaemia. These results suggest that further to inhibition of glucose transport, the activity of sucrase and maltase enzymes may also be attenuated (Ikarashi *et al.* 2011). An extract of *Nerium indicum* leaves reduced the rise in blood glucose concentration in rats orally administered maltose or sucrose. Rise in blood glucose was suppressed at 30, 60 and 90 min following sucrose ingestion, and at 60 and 90 min following ingestion of maltose. Everted intestinal sacs were exposed to 1 mM chlorogenic acid, a component of the leaf extract, along with maltose or glucose. In both instances glucose absorption was significantly lower than the control (Ishikawa *et al.* 2007). Several *ex vivo* and *in vitro* assays have demonstrated the ability of plant extracts, and individual polyphenolic compounds to reduce glucose transport and sugar hydrolysis (Kobayashi *et al.* 2000, Song *et al.* 2002, Johnston *et al.* 2005, Hanamura *et al.* 2006, Kottra and Daniel 2007, Kwon *et al.* 2007, Wang *et*

al. 2008, Manzano and Williamson 2010, Adisakwattana and Chanathong 2011, Boath *et al.* 2012).

The results presented in Chapter 4 and Chapter 5 support the evidence that cocoa extracts, rich in polyphenols, are able to reduce sucrose hydrolysis and glucose transport *in vitro*.

A 50 g serving of dark chocolate, containing 31 g of carbohydrate, has a reported glycaemic index (GI) of around 42 compared with a 50 g portion of glucose with a GI of 100 (University of Sydney 1995-2007). The GI is a measure of the glycaemic response to a food or beverage during 120 min post-ingestion; it is compared with the GI of a standard reference food which is commonly glucose in water or white bread. The GI of the test food is calculated as a percentage of the reference; the higher the GI of a food, the greater its impact on glycaemia. The relatively low GI of dark chocolate may be partly attributed to it containing mainly sucrose; a 50 g portion of sucrose has a GI of ~60 compared with glucose (Foster-Powell *et al.* 2002). The glucose present in sucrose must first be liberated by the activity of intestinal sucrase before absorption can occur therefore attenuating the rise in blood glucose. Typically, post-prandial blood glucose concentration peaks at around 30 min; following ingestion of 50 g of glucose in water, the 30 min peak equates to a blood glucose concentration increase of approximately 2 mM above the pre-ingestion baseline concentration (Chlup *et al.* 2010). The change induced by ingestion of dark chocolate containing 50 g of carbohydrate was approximately 0.4 mM, at its maximum, however over the 120 min post-prandial period a distinct peak was not observed as the change remained relatively constant throughout (Figure 6.5) (Chlup *et al.* 2010).

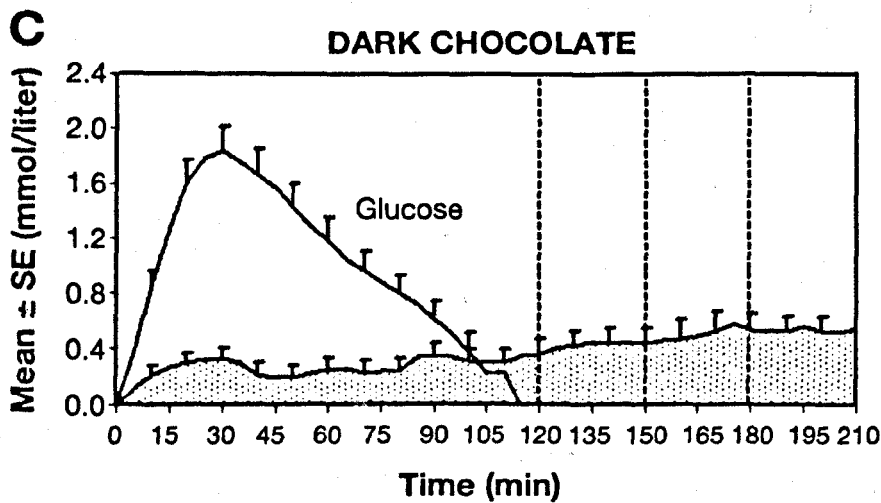


Figure 6.5 Mean glycaemic change from the pre-meal value following consumption of 50 g of carbohydrate in dark chocolate compared with glucose (Chlup *et al.* 2010).

The glycaemic index, determined in diabetic and non-diabetic subjects, of a variety of different foods containing 50 g of carbohydrate significantly correlated with polyphenol content (Thompson *et al.* 1984). Following ingestion of apple juice, compared with a control that was matched for sugar content, the rise in blood glucose concentration was significantly lower at 15 and 30 min in healthy subjects. Blood glucose concentration over 3 h did not differ significantly suggesting that absorption was delayed (Johnston *et al.* 2002). Ingestion of an instant tea beverage containing 75 g of glucose did not alter the 0-150 min AUC but did lower the blood glucose concentration measured at 120 min suggesting that the return to baseline was more rapid (Bryans *et al.* 2007). Similar results were observed in healthy subjects following consumption of a mixed berry purée, plus 250 mL water, containing 35 g sucrose, 4.5 g glucose and 5.1 g fructose, compared with 250 mL water containing the same sucrose, glucose and fructose content. There was no significant difference in the 0-3 h AUC, however blood glucose concentration

was significantly lower at 15 and 30 min. At 120 min post-ingestion, blood glucose was significantly higher than the control indicating that the rate of glucose absorption was reduced, not the overall concentration (Torronen *et al.* 2010).

Based on the results of *in vitro* studies presented in chapters 3 and 4 and the reported potential for plant extracts to attenuate the post-prandial peak of blood glucose concentration, a human intervention study is proposed to investigate whether consumption of dark chocolate in combination with a glucose-containing beverage could attenuate the post-prandial rise in blood glucose concentration over a 120 min post-ingestion period compared with a nutrient-matched control chocolate bar free from cocoa polyphenols.

Studies that investigate the post-prandial blood glucose concentration rely on a standard blood glucose monitor to measure the concentration. In a glycaemic index test this is adequate, however the human study proposed here is intended to investigate glucose absorption. As dark chocolate contains only sucrose, in a study such as this the use of a standard blood glucose monitor would measure blood glucose concentration resulting from the uptake of glucose from the beverage and of glucose liberated from the hydrolysis of sucrose. Thus the results would not be specific to glucose transport. The stable ^{13}C -D-glucose isotope has been routinely used as a metabolic tracer in human studies. Enrichment of the test food with this compound would enable specific measurement of ^{13}C -D-glucose and calculation of the ^{13}C to ^{12}C -D-glucose ratio; theoretically this should increase as glucose is absorbed from the test food. In order to measure ^{13}C -D-glucose in plasma a method for sensitive and precise measurement of plasma enriched with ^{13}C -D-glucose is described using HPLC separation

and mass spectrometry detection of glucose. Compared with a standard blood glucose monitoring method, use of the ^{13}C -D-glucose tracer provides a method to specifically investigate glucose absorption in the presence of a complex food matrix.

6.4.1 HPLC-MS Protocol

6.4.1.1 Parameters

A Shimadzu LC 2010 HPLC system equipped with a Phenomenex Rezex™ RNM-carbohydrate ion exclusion column, 8% cross-linked with sodium cations, 7.8 x 300 mm, was employed for the liquid chromatographic separation of glucose. The stationary phase was fitted in a thermostatted column compartment maintained at $60 \pm 0.1^\circ\text{C}$. The mobile phase consisted of 1 mM sodium formate (71539) in water. An isocratic gradient was maintained at a flow rate of 0.4 mL min^{-1} throughout the duration of the run. Maximum pressure was set to 65 bar and minimum pressure was 10 bar. Mass spectrometric detection of glucose was performed using the Shimadzu LCMS 2020 Single Quad Mass Spectrometer. Instrument parameters were as follows: gas temperature and flow 350°C , 15 L min^{-1} ; nebuliser gas flow 1.5 L min^{-1} ; desolvation line temperature 250°C ; heat block temperature 200°C . The analytical method run time was divided into 2 segments; the first being a window of 0 to 27.5 min during which analytes were detected in positive electrospray ionisation (+ESI) mode. During the second time segment, 27.5 to 28 min, negative electrospray ionisation (-ESI) was performed to eliminate analyte residue accumulation at the source which may suppress ionisation and reduce sensitivity of the mass spectrometer.

The autosampler was set to 8°C and a needle rinse step incorporated into the method before and after sample aspiration.

6.4.1.2 Sample preparation for LCMS analysis

Blood samples were centrifuged at 2,000 x *g* for 3 minutes to separate cellular matter from the plasma. The plasma supernatant was collected into centrifuge tubes and 50 µL diluted 10-fold with ice-cold ethanol. The ethanol-plasma mix was vortexed and stored at -20°C for 1 h before being centrifuged at 13,000 x *g* for 15 min and the supernatant syringe filtered through a 17 mm, 0.2 µm PTFE membrane (Chromacol, 17-SF-02(T)). The supernatant was evaporated to dry under vacuum and reconstituted in 50 µL water.

6.4.2 Pilot study

6.4.2.1 Ethical approval

Ethical approval was obtained from the University of Leeds Mathematics and Physical Sciences (MaPS) and Engineering joint Faculty Research Ethics Committee (MEEC FREC), reference MEEC 11-040.

6.4.2.2 Human study design

The design is a single-blind cross-over with participants required to attend two sessions, each session no longer than 3 h. Attendance at both sessions will qualify the volunteer to receive £5 to compensate for their time.

Volunteers will be asked to complete an informed consent form and pre-study questionnaire to determine suitability for the study. Volunteers will be excluded if they smoke, have been diagnosed with diabetes, pre-diabetes,

digestive disease, sugar intolerance, food allergy or hypertension, are taking prescribed medication, regular dietary supplements or pregnant/lactating.

Healthy, adult volunteers will be asked to maintain a normal, nutritionally balanced diet containing >150 g of carbohydrate per day and refrain from drinking alcohol and taking part in strenuous physical activity for 3 days prior to the study day. In addition the participants will be asked not to consume chocolate or cocoa during the 3 day period. On the evening prior to the study, subjects will be asked to consume a moderate meal then fast for 12 hours before their allocated start time on the study day.

On the day of the study participants will arrive at the designated time and rest for 10 minutes before providing the first sample of blood (baseline, T=0). The subject will then be asked to consume a 40 g bar of Nestlé NOIR Intense dark chocolate containing 70% cocoa solids, or a 28 g bar of macronutrient-matched chocolate free from cocoa solids. Immediately following ingestion of the bar, subjects will be asked to drink an aqueous beverage containing 25 g of D-glucose and 0.1 g of ^{13}C -D-glucose. Blood will be collected at 15, 30, 60, 90 and 120 min post-consumption.

Micro-volumes of blood will be drawn from the finger tip using BD Microtainer contact-activated lancets (MidMeds, 366594) designed specifically for the single puncture collection of blood volume up to 500 μL . Blood will be collected into BD Microtainer tubes containing sodium-ethylenediaminetetraacetic acid (EDTA) to prevent cell aggregation and sodium fluoride to prevent cellular glycolysis diminishing the glucose concentration (Fisher Scientific, SZV-110-130Y).

At the end of each session participants will be provided with a snack.

Subject confidentiality will be ensured by allocation of identification codes to each participant that will be kept securely in a restricted access area in a locked filing cabinet. Participants will be free to withdraw from the study at any point without providing a reason.

6.4.3 Qualification of D-glucose

^{12}C -D-Glucose has a molecular weight of 180.16, in positive electrospray ionisation mode the mass to charge ratio (m/z) for selective ion monitoring (SIM) is 181.16. The Shimadzu LCMS 2020 Single Quad Mass Spectrometer is not sensitive to detect intervals <1 , therefore all selective ion monitoring was set to integer m/z only. For example [^{12}C -D-glucose + H^+] = m/z 181. During a scan (+ESI) of standard ^{12}C -D-glucose m/z ratios of 203, 221, 383, 384 and 385 were detected; these were qualified as follows:

Mass/charge ratio	Compound + adduct
203	^{12}C -D-glucose + sodium
221	^{12}C -D-glucose + sodium + water
383	^{12}C -D-glucose + ^{12}C -D-glucose + sodium
384	^{12}C -D-glucose + ^{13}C -D-glucose + sodium
385	^{13}C -D-glucose + ^{13}C -D-glucose + sodium

Table 6.1 Mass/charge ratios detected during selective ion monitoring of ^{12}C -D-glucose.

During a scan (+ESI) of standard ^{13}C -D-glucose m/z ratios of 204 and 222 were detected and qualified as follows:

Mass/charge ratio	Compound + adduct
204	^{13}C -D-glucose + sodium
222	^{13}C -D-glucose + sodium + water

Table 6.2 Mass/charge ratios detected during selective ion monitoring of ^{13}C -D-glucose.

In addition to the ratios shown above, selective ion monitoring of m/z ratios 181 and 182 was included in the method to detect ionised ^{12}C -D-glucose (181) and ^{13}C -D-glucose (182). In the standards and plasma samples analysed during method development, m/z 181 and 182 were not detected. [Figure 6.6](#) and [Figure 6.7](#) show the absolute intensity of signals detected for ions of m/z 203, 221, 383, 384 and 385 from standard ^{12}C -D-glucose, and 204 and 222 from standard ^{13}C -D-glucose. Analysis of ^{12}C -D-glucose revealed the presence of ^{13}C -D-glucose, this would be expected due to the natural abundance of the carbon-13 isotope of glucose. The standard ^{13}C -D-glucose used in this study was enriched with D-glucose labelled with one carbon-13 atom. Natural abundance of carbon-13 isotopes of glucose decrease as the number of substituted carbon atoms increase.

The purity of the ^{12}C -D-glucose isotope can be confirmed by calculating the atom percent which takes into account the isotopic abundance of the sample relative to that of an international standard. The international standard used for carbon is the ratio of carbon-13 to carbon-12 in Vienna Pee Dee Belemnite (VPDB) that is equal to 0.0112372 ± 0.0000009 (R_{st}). Calculation of isotopic abundance and atom percent are shown in [Equation 6.1](#) and [Equation 6.2](#), respectively.

$$\delta^{13}C_{sample} (\text{‰}) = \left[\left(\frac{R_s}{R_{st}} \right) - 1 \right] \times 1000 \quad \text{Equation 6.1}$$

$$\text{Atom percent (AP)} = \left[\frac{100 \times R_{st} \times \left(\left(\frac{\delta^{13}C_{sample}}{1000} \right) + 1 \right)}{1 + R_{st} \times \left(\left(\frac{\delta^{13}C_{sample}}{1000} \right) + 1 \right)} \right] \quad \text{Equation 6.2}$$

Where:

R_s = ratio of carbon-13 to carbon-12 in the sample

To calculate the isotopic abundance and subsequently the atom percent of D-glucose the number of carbon-12 and carbon-13 atoms in each isotope must be accounted for. Integration of ion count peak area for each m/z ratio was performed then the sum of peak areas for each isotope in the sample calculated; from this, the ratio of ^{12}C -D-glucose to ^{13}C -D-glucose peak areas was determined. The ratio was then converted to the ratio of carbon-12 atoms to carbon-13 atoms by multiplying by 6 (6 carbon-12 atoms in one molecule of ^{12}C -D-glucose) plus 5 (5 carbon-12 atoms in one molecule of ^{13}C -D-glucose). Inversion of the figure obtained in this last calculation provided the ratio of carbon-13 atoms to carbon-12 atoms which is used to calculate isotopic abundance. The isotopic abundance of standard ^{12}C -D-glucose, used in the method development presented here, was equal to $65.5 \pm 17.8 \text{ ‰}$ and the atom percent calculated from this was 1.18 ± 0.02 (n=3). Isotopic abundance >1 indicates that the sample is enriched with carbon-13 atoms relative to the international standard (Godin *et al.* 2007). The plasma samples analysed in the development of this method contained an isotopic abundance of $63.9 \pm 2.3 \text{ ‰}$ and an atom percent of 1.18 ± 0.00 (n=3).

6.4.4 Plasma enrichment with ^{13}C -D-glucose

A trial was undertaken to confirm the method was sensitive to measure plasma enrichment following ingestion of 0.1 g ^{13}C -D-glucose and 25 g ^{12}C -D-glucose dissolved in 200 mL of tap water. A volunteer provided a sample of blood at baseline before drinking the beverage, then at 30 and 60 min post-ingestion. At baseline, isotopic abundance was 50.9 ‰ and the atom percent calculated as 1.17. Plasma collected at 30 and 60 min post-ingestion was calculated to have an isotopic abundance of 94.1 ‰ and 97.2 ‰, respectively. Atom percent at 30 and 60 min was 1.21 and 1.22, respectively. These results demonstrate the ability of the method to detect enrichment of plasma with ^{13}C -D-glucose following ingestion of a beverage containing a molar ratio of ^{13}C - to ^{12}C -D-glucose = 0.004.

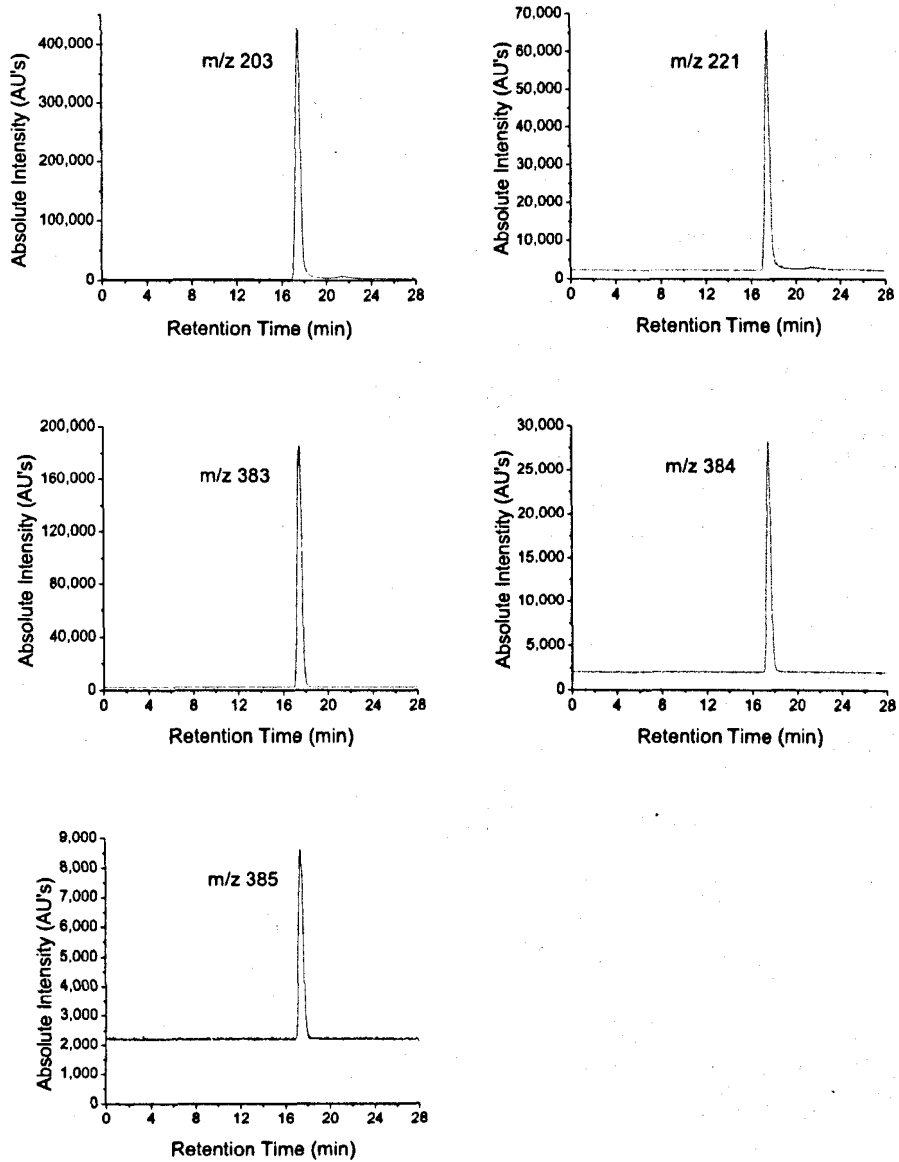


Figure 6.6 LCMS selective ion monitoring chromatograms extracted from the total ion count chromatogram of standard ^{12}C -D-glucose.

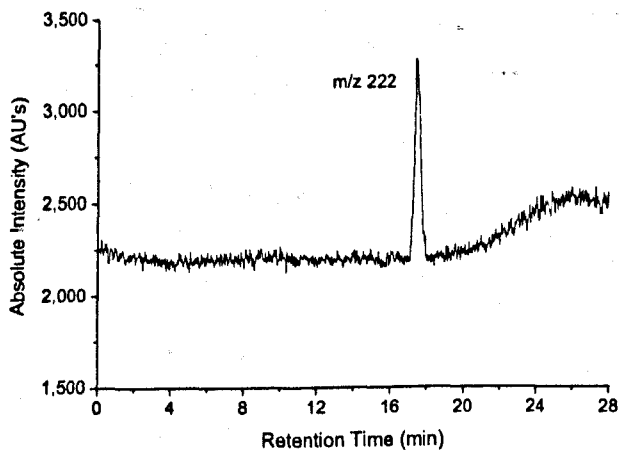
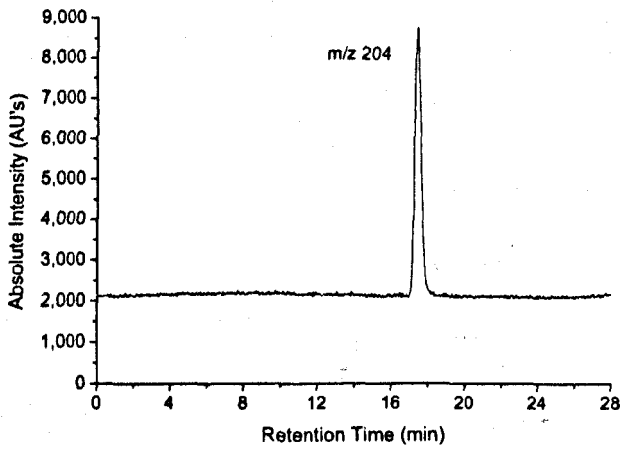


Figure 6.7 LCMS selective ion monitoring chromatograms extracted from the total ion count chromatogram of standard ^{13}C -D-glucose.

6.5 Conclusion

The results of the investigations presented here demonstrate the interaction between cocoa polyphenols and sugars. Sucrose has been shown to enhance epicatechin transport through the Caco-2 cell monolayer whilst dark chocolate extract, glucose and fructose attenuate epicatechin methylation. The apparent low glycaemic index of dark chocolate, compared with glucose

(and sucrose), may be attributable to its capacity to inhibit sucrose hydrolysis and glucose transport; the evidence presented here supports this hypothesis. The mechanism by which each of these outcomes occurs was not investigated but the hypotheses put forward provide a direction for future work to take. Namely, prospective investigations are recommended to include the following: determination of COMT activity in the presence of flavanols with a varying degree of polymerisation, and in the presence of glucose and fructose with sufficient magnesium (II) cofactor present in the transport solution; confirmation of enhanced sucrase activity at moderate concentrations, and reduced activity at high ($\geq 500 \mu\text{M}$) concentrations of flavanols; measurement of sucrase kinetic properties to determine the type of inhibition and structure-activity associations using different stereoisomers and computational software to reveal the requirements for interaction and the binding sites at which interactions may occur; sodium-dependent glucose transport should be more thoroughly investigated to confirm, or refute, the capacity for dark chocolate extract to inhibit GLUT2; the kinetic properties of methyl xanthine induced SGLT1 activity and the effect of varying concentrations of flavanols would further the understanding of this process; sucrase activity and glucose transport in the presence of separated flavanol fractions of dark chocolate would help to narrow the range of possible compounds responsible for the effects of dark chocolate extract; and, implementation of a human study in which ^{13}C -D-glucose plasma enrichment is measured following consumption of dark chocolate, compared with a nutrient-matched placebo control, may support the *in vitro* evidence for reduced glucose absorption induced by the flavanol components of dark chocolate.

Chapter 7 References

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Chapter 8 Appendix

8.1 Ethical application (MEEC 11-040) and supporting documents

UNIVERSITY OF LEEDS RESEARCH ETHICS COMMITTEE APPLICATION FORM¹

UNIVERSITY OF LEEDS

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

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

Ethics reference number:	
Grant reference and/ or student number:	200374840

PART A: Summary**A.1 Which Faculty Research Ethics Committee would you like to consider this application?²**

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

A.2 Title of the research³ The effect of cocoa powder on blood glucose concentration following an oral glucose tolerance test.

A.3 Principal Investigator's contact details⁴	
Name (Title, first name, surname)	Miss Samantha Eilam
Position	PhD Research Student
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	MAPS
Work address (including postcode)	School of Food Science and Nutrition, University of Leeds, LS2 9JT
Telephone number	07843 171548
University of Leeds email address	fs07sie@leeds.ac.uk

A.4 Purpose of the research:⁵ (Tick as appropriate) <input checked="" type="checkbox"/> Research <input checked="" type="checkbox"/> Educational qualification: <i>Please specify:</i> <u>Doctor of Philosophy</u> <input type="checkbox"/> Educational Research & Evaluation ⁶ <input type="checkbox"/> Medical Audit or Health Service Evaluation ⁷ <input type="checkbox"/> Other

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

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

- Research working with data of human participants
 - New data collected by questionnaires/interviews
 - New data collected by qualitative methods
 - New data collected from observing individuals or populations
 - Research working with aggregated or population data
 - Research using already published data or data in the public domain
- Research working with human tissue samples⁹

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

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

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

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

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

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

- Yes No

If yes, ethical approval must be sought from the University of Leeds. Please note that NHS R&D approval is needed in addition, and can be applied for concurrently www.myresearchproject.org.uk. Contact governance-ethics@leeds.ac.uk for advice.

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

- Children under 16 ¹⁸
- Adults with learning disabilities ¹²
- Adults with other forms of mental incapacity or mental illness
- Adults in emergency situations
- Prisoners or young offenders ¹⁴
- Those who could be considered to have a particularly dependent relationship with the investigator, e.g. members of staff, students ¹⁷
- Other vulnerable groups
- No participants from any of the above groups

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

There is likelihood that a staff member or student from the School of Food Science & Nutrition will be recruited to take part in this pilot study. Study participation is entirely voluntary and no person will be approached directly or coerced into taking part in the research. Volunteers will be free to withdraw from the study at any time without providing a reason.

A Criminal Record Bureau (CRB) check will be needed for researchers working with children or vulnerable adults (see www.crb.gov.uk)

A.9 Give a short summary of the research¹⁸

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

In vitro studies by the researcher demonstrated that an extract of dark chocolate reduced glucose transport through Caco-2 (colorectal adenocarcinoma) cells. These cells are commonly used as a model of the human small intestine.

The next step in this research is to assess whether consumption of cocoa by healthy human volunteers is able to reduce the absorption of glucose compared against a control. Volunteers will be required to take part in a three-phase pilot study which will implement the current World Health Organisation/United Kingdom National Health Service protocol for measuring blood glucose. Each phase will involve consumption of water containing 75 g of glucose followed by blood glucose monitoring over a two-hour period. 2 mL of blood will be collected from each subject at each time-point; plasma glucose concentration will be measured using mass spectrometry after the whole blood has been centrifuged and rendered acellular. Phases two and three will include cocoa powder dissolved in the beverage. Some subjects will only be required to provide up to 0.1 mL of blood per time-point.

Reduction of blood glucose concentration following consumption of a meal is desirable in healthy humans as well as those suffering from prediabetes and type-2 diabetes. Excessively high blood glucose concentration, particularly over a prolonged period, can damage arterial cells which increases the potential for narrowing of the arteries and consequently an increased risk of cardiovascular disease [1]. Additionally it is likely that slowing glucose uptake may help to reduce body weight [2]; being overweight is a risk factor for developing type-2 diabetes [3].

This research is funded by a BBSRC Industrial CASE award to Nestlé PTC, York. Recruitment will take place on the main University of Leeds campus.

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

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

Informed consent

The study co-ordinator will provide a clear, concise Participant Information Sheet (appendix 1) and Informed Consent Form (appendix 2) for the volunteers to read, complete and sign prior to commencement of the study. After the candidate has been determined as suitable to participate in the study, the background of the study (including purpose, duration, protocol, potential discomfort, associated risk, potential benefits, confidentiality, and disclosure of results, participation and withdrawal) will be explained to the participant. The study co-ordinator will fully answer all questions to the satisfaction of the individual. Written informed consent will be obtained by the study co-ordinator from each participant at least 7-days prior to enrolment in the study. The consent form will be signed and dated by both the participant and the study co-ordinator, and will be photocopied twice; one copy to be held with the study records (held by the study co-ordinator) and one copy for the individual. Written informed consent must be obtained before the individual can participate in the study and all participants will be free to withdraw from the study at any point without providing a reason.

Pre-study questionnaire

In order to assess candidate suitability for the study, each candidate will be required to complete a questionnaire (appendix 3) pertaining to lifestyle, including height and weight, gender, age, ethnicity and relevant medical history; this information may be considered a sensitive issue. Each volunteer will be assured that participation is entirely voluntary.

3-day restricted diet & overnight fasting

There may be some mild discomfort/inconvenience caused by the 3-day restricted diet and overnight fasting before the study. However the quantity and range of foods permitted is substantial and sufficient to provide a nutritionally balanced diet.

Subject confidentiality and data protection

Confidentiality of all participants will be maintained; identification will be coded using ID numbers that will be assigned on the day of the study. All data collected will be treated as confidential and stored securely in a locked filing cabinet according to current University regulations. It will not be possible to identify individual participants from the ID numbers; the linkage between individual identity and ID number will be kept in written form only and stored in a locked filing cabinet in a restricted access area. Data evaluation will only be performed using ID numbers. Anonymised data will be stored for no longer than 5 years in accordance with the University guidelines on the password protected M-drive of the University server.

Ethics committee approval

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

PART B: About the research team**B.1 To be completed by students only²⁰**

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

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

Name (Title, first name, surname)	
Position	
Department/ School/ Institute	
Faculty	
Work address (including postcode)	

Telephone number	
Email address	

Part C: The Research

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

The main objective of this pilot study is to determine whether consumption of cocoa affects blood glucose concentration over a 2-hour period post-ingestion.

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

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

Study design and programme

This is a placebo-controlled, crossover pilot study conducted over three-phases of no more than 3 hours per phase. All volunteers will be asked to complete a consent form and pre-study questionnaire prior to participation. It is anticipated that the pilot study will require no more than 12 subjects.

Days 1-3 prior to study day

Subjects will be required to maintain a normal nutritionally balanced diet containing >150 g of carbohydrate per day (appendix 4), and refrain from drinking alcohol and taking strenuous physical activity. Additionally the subjects will be asked not to consume chocolate or cocoa during this 3-day period. On the evening prior to the study, subjects will be asked to eat a moderate meal then fast for 12 hours before their allocated start time on the day of the study.

Day 4 – Study day

Subjects will be asked to arrive at a pre-designated time; they will then rest for 10 minutes before providing the first 2 mL sample of blood for glucose analysis (baseline). After this first sample has been taken, the subject will be asked to consume the beverage containing 75 g of ¹²C-D-glucose plus 0.15 g of ¹³C₄-D-glucose⁶ within 5 minutes. The subject will then provide a 2 mL blood sample at 15, 30, 60, 90 and 120 minutes post-consumption. Some subjects will be required to provide up to 0.1 mL of blood per time-point, drawn using a standard finger-prick device.

Sample analysis

Whole blood samples will be centrifuged within 30 minutes of collection and the acellular plasma fraction collected and stored at -80°C until required for analysis (appendix 5 and 6). The remaining cellular fraction will be treated with a

disinfectant to destroy the cells, as per manufacturer guidelines. Glucose concentration in the plasma samples will be analysed using liquid chromatography with mass spectrometry detection (LC-MS).

Statistical analysis

Blood glucose concentration in the test conditions will be assessed for statistically significant difference from the control condition at each time point using the Students T-test. Statistically significant difference will also be calculated between the two test conditions.

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

Participants will initially be asked to complete a consent form and pre-study questionnaire. The information provided on the questionnaire will be used to determine suitability for the study, and will include:

Height, weight, age, gender, ethnic background, smoking habit, pre-diagnosed gastrointestinal disease, pre-diabetes or diabetes, hypertension, pregnancy/lactation, use of prescribed medication or dietary supplements.

For 3 days prior to the study day, subjects will be asked to maintain a normal nutritionally balanced diet avoiding cocoa, chocolate and alcohol. Additionally each subject will be asked to refrain from strenuous physical activity during the 3-day period. The night before the study the participants will be required to fast for 12 hours (water to be permitted).

On the morning of the study, subjects will be asked to arrive at the study venue at an allocated time and then be asked to rest for 10 minutes before the first (baseline) blood sample is collected. The participants will then be asked to drink the beverage within 5 minutes and subsequent blood samples will be collected at 15, 30, 60, 90 and 120 minutes post-consumption. At the end of each study phase each subject will be provided with a snack.

Each participant will not be required to be present for longer than 3 hours during each phase of the study. There are three phases to the study so the total time required from each subject will be 9 hours (appendix 7 and 8).

The study will take place in the School of Food Science and Nutrition on weekdays during the University's normal opening hours. Subjects may have to travel to the building from elsewhere on campus or from outside the University campus, however it is envisaged that many of the volunteers will be staff or students at the University of Leeds and therefore travel will be minimal.

*There are two naturally occurring stable isotopes of carbon, carbon-12 and carbon-13 with an abundance of 98.9% and 1.1% respectively [4]. In this study the carbon atom in position 6 of the glucose molecule has an atomic mass of 13 (the remaining 5 carbon atoms have a mass of 12) therefore giving the glucose a molecular mass of 181.2 rather than 180.2 as in most cases. This difference in mass allows the specific analysis of $^{13}\text{C}_6\text{-D-glucose}$ concentration in the plasma samples, the presence of which will only originate from the beverage consumed at the beginning of the study. The use of $^{13}\text{C}_6\text{-D-glucose}$ in this study provides a sensitive and precise method to analyse the changes in blood glucose during the study period and compare the effects of cocoa consumption on the appearance of glucose in the blood. The use of carbon-13 in nutritional investigations is well documented and completely safe due to the stability of the carbon atom nucleus [5].

C.4 Does the research involve an international collaborator or research conducted overseas: ¹⁴

(Tick as appropriate)

Yes No

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

Describe the measures you have taken to comply with these:

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

C.5 Proposed study dates and duration

Research start date (DD/MM/YY): 30/04/12 Research end date (DD/MM/YY): 29/06/12

Fieldwork start date (DD/MM/YY): 30/04/12 Fieldwork end date (DD/MM/YY): 29/06/12

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

The study will be conducted in the School of Food Science and Nutrition at the University of Leeds.

RECRUITMENT & CONSENT PROCESSES

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

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

(i) identified?

Individual subject identification will be in the form of code and no personal details will be referred to in this study. Personal details are only required to determine subject suitability such that results will be generalised not specific to individual participants.

(ii) approached?

Volunteers will be sought by way of general invitation in the form of posters (appendix 9) displayed around the University of Leeds campus and by general email (appendix 10) to all staff and students within the School of Food Science and Nutrition.

(iii) recruited? ²⁶

Subjects will be recruited from the staff and student population. Participation in the study is entirely voluntary and participants will be free to withdraw from the study at any point without giving a reason.

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

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

The selection criteria have been chosen to minimise variable factors that can affect glucose absorption based on age, ethnicity, body mass index, pre-existing illnesses, pregnancy/lactation and use of prescribed medication.

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

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

This is a pilot study to determine whether a main study will be required and if so how many participants would be required based on power analysis. In this case, based on previous experience of the research team supervisor, it has been decided that 12 subjects will be sufficient to provide a reliable result.

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

C.10 Will the research involve any element of deception? ²⁹ If yes, please describe why this is necessary and whether participants will be informed at the end of the study.

No

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

Yes No

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

Written consent will be sought from each volunteer prior to commencement of the study. Volunteers will receive a participant information sheet and informed consent form after a positive reply to the recruitment advertisement. The main investigator will verbally explain the participant information sheet and informed consent form; all questions from the volunteer will be

answered at any time. The prospective participant will have approximately 7 days before the study commences to finally decide, sign and return the completed informed consent form (in duplicate – one copy for the participant and one for the research records, both to be co-signed by the main investigator).

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

Copies of any written consent form, written information and all other explanatory material should accompany this application. The information sheet should make explicit that participants can withdraw from the research at any time, if the research design permits.

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

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

Volunteers will be informed that withdrawal from the study is possible at any time without giving a reason and without questions being asked. No negative consequences or change of treatment of the participant will ensue.

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

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

The participant should decide whether or not to take part in the study in the 7 days prior to commencement of the study.

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

Volunteers who do not adequately understand the English language will not be recruited in the study. Email and mobile

phone contact details will be provided on the recruitment advert and participant information sheet.

C.15 Will individual or group interviews/ questionnaires discuss any topics or issues that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could take place during the study (e.g. during interviews/group discussions, or use of screening tests for drugs)?³³

Yes No

If Yes, give details of procedures in place to deal with these issues

The information sheet should explain under what circumstances action may be taken

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

Yes No

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

Participants will receive a snack at the end of each study phase to compensate for 12-hour fasting and study participation.

RISKS OF THE STUDY

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

There is very low potential risk involved in this pilot study. The participants may feel some discomfort from blood sampling, which will be conducted by a trained phlebotomist; and from the 12-hour fasting, however a snack will be provided at the end of each study phase to compensate for this. There are no direct benefits to the participant although following a 3-day nutritionally balanced diet may be considered a benefit to some individuals who would not normally follow such a diet.

C.18 Does the research involve any risks to the researchers themselves, or people not directly involved in the research? Eg lone working³⁶

Yes No

If yes, please describe: _____

Is a risk assessment necessary for this research?

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

Further information on fieldwork risk assessments is available at <http://www.leeds.ac.uk/safety/fieldwork/index.htm>.

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

- Examination of personal records by those who would not normally have access
- Access to research data on individuals by people from outside the research team
- Electronic transfer of data
- Sharing data with other organisations
- Exporting data outside the European Union

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

Publication of direct quotations from respondents

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

Use of audio/visual recording devices

FLASH memory or other portable storage devices

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

Manual files

Home or other personal computers

Private company computers

Laptop computers

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

See the answer explained in question A10.

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

___5___ years, ___ ___ months

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

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

CONFLICTS OF INTEREST

C.22 Will any of the researchers or their institutions receive any other benefits or incentives for taking part in this

research over and above normal salary or the costs of undertaking the research?³⁹

Yes No

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

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

Yes No *If yes, please explain* _____

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

Yes No *If yes, what is the source of this funding?* Nestlé PTC, York and Biotechnology and Biological Sciences Research Council (BBSRC)

PART D: Declarations

Declaration by Chief Investigators

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

Sharing information for training purposes

Optional – please tick as appropriate:

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

Principal Investigator

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

Print name:

Date: (dd/mm/yyyy):

Supervisor of student research

I have read, edited and agree with the form above.

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

Print name:

Date: (dd/mm/yyyy)

Please submit your form by email to I.M.Blaikie@leeds.ac.uk or if you are in the Faculty of Medicine and Health FMHUniEthics@leeds.ac.uk. Remember to include any supporting material such as your participant information sheet, consent form, interview questions and recruitment material with your application.

Checklist:

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

Ethical review**NOTICE OF AMENDMENT**

To be completed in typescript by the Principal Investigator in language comprehensible to a lay person and submitted to the FREC that gave the favourable opinion of the research.

Further guidance is available at

http://researchsupport.leeds.ac.uk/index.php/academic_staff/good_practice/managing_approved_projects-1/applying_for_an_amendment-1

Principal investigator's details:	
Name:	Samantha Ellam
Address:	School of Food Science and Nutrition
Telephone:	07843171548
Email:	fs07sle@leeds.ac.uk

Full title of study:	The effect of dark chocolate on blood glucose concentration following oral ingestion of a glucose beverage
Ethics reference number:	MEEC 11-040
Date study commenced:	Not yet started
Amendment number and date:	#1, 13.11.2012

Type of amendment (indicate all that apply in bold)

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

Yes **No**

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

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

Yes **No**

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

[Empty rectangular box]

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

Yes No

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

Yes No

If so has the amendment been submitted for R&D approval?

Yes No

Is sponsor sign off required for the amendment?

Yes No

Summary of changes

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

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

Initially the study was intended to provide the participants with a cocoa containing beverage however the sponsor prefers to use a dark chocolate bar matched with a white chocolate bar as the control. The glucose will still be consumed in beverage form following consumption of the chocolate bar, the glucose concentration has also been reduced. The change will make the results more comparable with the *in vitro* investigations previously conducted and more relevant for the sponsor who is providing the chocolate bars.

Blood collection has changed. Depending upon further method development it may be necessary to insert a cannula device in to the arm and collect up to 6 mL of blood per time point. A cannula allows blood to be drawn without a needle so is more suitable for multiple collections during a short period. However if the analytical method is suitable for a small volume of blood then only a finger prick will be required to draw the blood and the sample volume required will be a maximum of 0.6 mL per time point.

Any other relevant information

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

List of enclosed documents

<i>Document</i>	<i>Version</i>	<i>Date</i>
Participant Information Sheet	v3	23.11.12
Preparation of acellular human plasma	v2	09.11.12
Programme of Study	v2	09.11.12
Recruitment email	v2	09.11.12
Study flowchart	v2	16.11.12
Recruitment poster	v2	16.11.12

Declaration

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

Signature of Principal Investigator:

Print name:Samantha Ellam.....

Date of submission:16.11.12.....

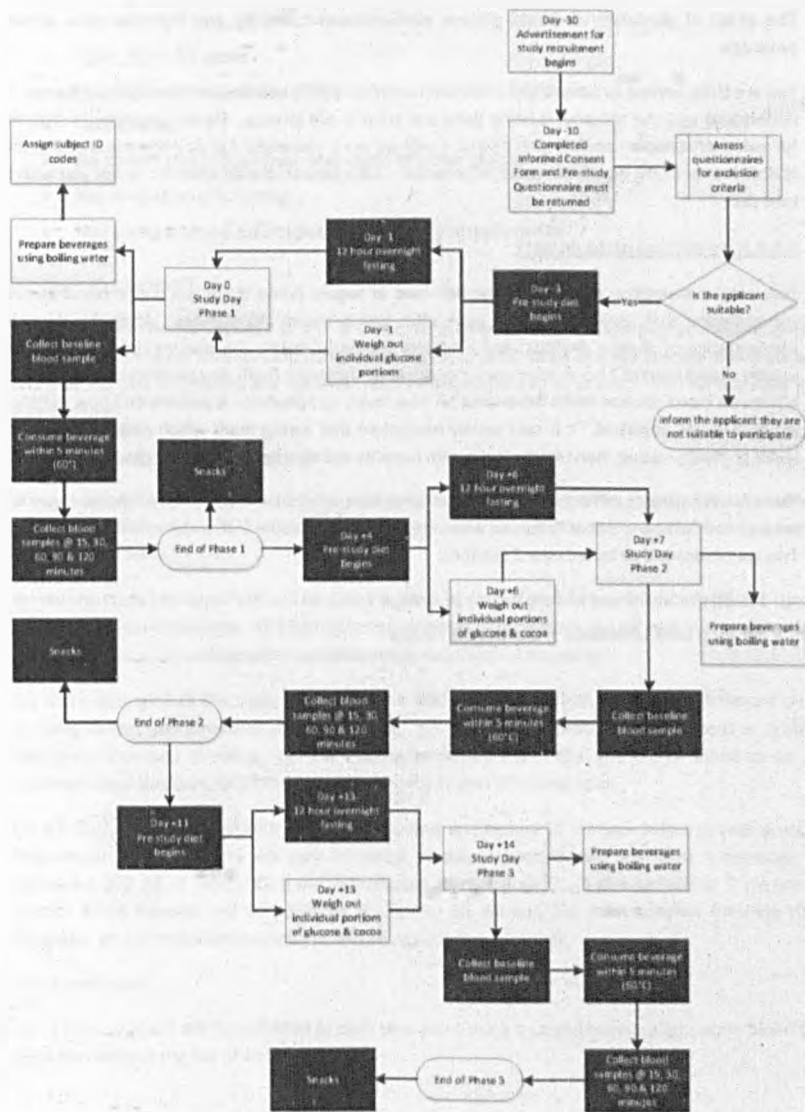
Signature of supervisor of student project:

Print name:Professor Gary Williamson.....

Date of submission:16.11.12.....

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

Study Flowchart



Participant Information Sheet

Research project title

The effect of chocolate on blood glucose concentration following oral ingestion of a glucose beverage.

You are being invited to take part in a research project. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the project?

Following consumption of carbohydrate-rich food or sugary drinks there is a rise in blood glucose concentration that usually reaches a peak after approximately 30 minutes. After this time the concentration of glucose declines until it returns to approximately the starting concentration, this usually occurs around 2 hours after eating or drinking. Different foods and beverages have different effects on blood glucose levels depending on how much carbohydrate is present and how quickly it is digested and absorbed. It is now widely recognised that eating foods which raise blood glucose levels gradually, rather than rapidly, has health benefits and may help control weight.

This research project will determine whether chocolate affects the absorption of glucose over a 2 hour period following consumption of a beverage containing 200 mL of water and 25 g of glucose. Two conditions will be tested over 2 sessions:

1. 28 g white chocolate + glucose beverage
2. 40 g dark chocolate + glucose beverage

Why have I been chosen?

The participant selection criteria are based upon the following:

- Healthy male or female
- Aged 18 to 60 years
- Normal body mass index (BMI) between 18.5 and 24.9 kg/m²
- Non-smoker
- No known gastrointestinal, metabolic or other chronic disease
- Not pregnant or lactating
- Not taking prescribed medication or dietary supplements

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep (and be asked to sign a consent form) and you can still withdraw at any time without it affecting any benefits that you are entitled to in anyway. You do not have to give a reason.

What will happen to me if I take part?

This is a two phase study that will take place over two weeks; you will be required to attend the School of Food Science and Nutrition twice, once per week on two separate weeks, for a maximum of 3 hours per visit.

Before the study can begin you will be asked to read, complete and sign an informed consent form and pre-study questionnaire. All information provided on the pre-study questionnaire is confidential and will only be used to determine suitability for participation in the study.

For three days prior to the study day you will be asked to eat a normal, nutritionally balanced diet avoiding alcohol and strenuous physical activity. You will also be asked not to eat foods or drinks containing chocolate or cocoa. On the evening before the study day, you will be asked to eat a moderate meal then fast for 12 hours before arriving at your allocated time.

On the study day you will arrive at your allocated time and rest for 10 minutes before providing your first sample of blood. You will then be asked to eat a chocolate bar and drink a beverage, containing 200 mL of water, 25 g of ¹²C-D-glucose and 0.1 g of ¹³C₁-D-glucose* within 5 minutes. Further blood samples will be collected at 15, 30, 60, 90 and 120 minutes after finishing the beverage. At the end of the session you will be provided with a snack.

To be confirmed:

Up to 6 mL of blood will be collected at each time point using a cannula device that allows blood to be drawn without the use of a needle.

Blood will be drawn from a fingertip using a single-use disposable lancet. The area where the finger-prick is performed will initially be sterilised using an alcohol wipe, and allowed to dry. A minimum 0.4 mL, maximum 0.6 mL, of blood will be collected into a microcollection tube at each time-point.

*This is a naturally occurring stable isotope-containing form of glucose. It is used in this study due to its low natural abundance which allows distinction between the glucose added to and consumed in the beverage, and any that may be naturally present in the chocolate.

What are the possible disadvantages and risks of taking part?

There are no additional risks involved as the procedures are according to standard guidelines. Due to the short duration of the study and the consumption of normal food, the risk of non-routine medication being required by a volunteer whilst the study is in progress is very low. This occurrence will be dealt with on a case-by-case basis at the discretion of the volunteer, and recorded by the study co-ordinator should the volunteer decide to participate, and be deemed suitable to continue. Any adverse effects observed will be treated in the same way, with emphasis on the choice of the volunteer whether or not to continue with the study. A decision by any volunteer to discontinue their involvement in the study shall not interfere in any way with the manner in which the volunteer is treated by the study co-ordinator. All data will be used even from subjects who withdraw (provided that the volunteer consents to this).

What are the possible benefits of taking part?

You may not personally benefit from participating in the study, but results may be used for the advancement of knowledge and the future benefit of other individuals.

Will my taking part in this project be kept confidential?

The result of the data obtained will be reported in a collected manner with no reference to a specific individual. Hence, the data from each individual will remain confidential. As a subject only you have the right to know the results of the total analysis.

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

Data on your general health and blood plasma samples will be collected in the study.

What will happen to the results of the research project?

Once all participants have completed the study, the information obtained will need to be collected and analysed before any results are published. This is likely to take at least one year to be finalised. If you would also like to know the results of the study, the research team will be able to give this information to you when it becomes available. You will not be identified in any report or publication.

Who is organising and funding the research?

This study is organised by Professor Gary Williamson, Food Biochemistry Group, School of Food Science and Nutrition, University of Leeds, UK. The research is funded by a BBSRC industrial case award to Nestlé PTC, York, UK.

Who do I contact for further information?

Samantha Ellam, Study Co-ordinator
School of Food Science and Nutrition
Faculty of Mathematics and Physical Sciences
University of Leeds
Email: fs07sle@leeds.ac.uk
Mobile: 07843 171548

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

Informed Consent Form

Research project title The effect of cocoa powder on blood glucose concentration following an oral glucose tolerance test

Name of researcher Samantha Ellam

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

- | | | |
|---|---|--------------------------|
| 1 | I confirm that I have read and understand the Participant Information Sheet (dated TBC) explaining the above research project and I have had the opportunity to ask questions about the project. | <input type="checkbox"/> |
| 2 | I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without there being any negative consequences. In addition, should I not wish to answer any particular question or questions, I am free to decline. (You can contact the study co-ordinator by email: fs07sk@leeds.ac.uk or mobile: 07843 171548.) | <input type="checkbox"/> |
| 3 | I understand that my responses will be kept strictly confidential. I give permission for members of the research team to have access to my anonymised responses. I understand that my name will not be linked with the research materials, and I will not be identified or identifiable in the report or reports that result from the research. | <input type="checkbox"/> |
| 4 | I agree for the data collected from me to be used in future research. | <input type="checkbox"/> |
| 5 | I agree to take part in the above research project and will inform the principal investigator should my contact details change. | <input type="checkbox"/> |
| 6 | I agree to be re-contacted for future research projects related to this study. | <input type="checkbox"/> |

Name of participant	Date	Signature
---------------------	------	-----------

Name of person taking consent	Date	Signature
-------------------------------	------	-----------

Lead Researcher	Date	Signature
-----------------	------	-----------

Pre-study Questionnaire

Full name

Age: Height: Weight:

Contact phone no:

Email address:

Please circle or tick the appropriate answer.

Q1. What is your gender?

Male

Female

Q2. Do you smoke?

Yes

No

Q3. Have you been diagnosed with any of the following?

Diabetes

Yes

No

Pre-diabetes (also known as impaired fasting glucose, impaired glucose tolerance)

Yes

No

Digestive disease (e.g. Crohn's disease, celiac disease)

Yes

No

Sugar intolerance (sucrose, glucose, fructose)

Yes

No

Food allergy (trace amounts of nuts and gluten may be present in the cocoa powder)

Yes

No

Hypertension

Yes

No

Q4. Are you currently taking any prescribed medication?

Yes

No

Q5. Do you regularly take any dietary supplements

Yes

No

Q6. Are you pregnant or lactating?

Yes

No

Q7 What is your ethnic background?

<input type="checkbox"/> White – British	<input type="checkbox"/> Asian or Asian British – Bangladeshi
<input type="checkbox"/> White – Irish	<input type="checkbox"/> Chinese
<input type="checkbox"/> White – Scottish	<input type="checkbox"/> Other Asian background
<input type="checkbox"/> Irish traveller	<input type="checkbox"/> Mixed – White and Black Caribbean
<input type="checkbox"/> Other white background	<input type="checkbox"/> Mixed – White and Black African
<input type="checkbox"/> Black or Black British – Caribbean	<input type="checkbox"/> Mixed – White and Black Asian
<input type="checkbox"/> Black or Black British – African	<input type="checkbox"/> Other Mixed background
<input type="checkbox"/> Other Black background	<input type="checkbox"/> Other Ethnic background
<input type="checkbox"/> Asian or Asian British – Indian	<input type="checkbox"/> Prefer not to state
<input type="checkbox"/> Asian or Asian British – Pakistani	

Pre-study diet

For 3 days prior to the study day you will be required to maintain a nutritionally balanced diet containing at least 150 g of carbohydrate per day. Below is some information regarding what a nutritionally balanced diet is, followed by a list of carbohydrate-rich foods and some examples of how much to consume throughout the day to achieve the required intake.



This is the 'eatwell plate'. It shows the different types of food we need and the proportions in which they should be consumed.

For more information, visit:

<http://www.nhs.uk/livewell/goodfood>

As you can see carbohydrate-rich (starchy) foods should make up approximately one third of your daily diet. These foods include bread, rice, potatoes, pasta, oats and breakfast cereals; the table below shows average carbohydrate content based on medium portion sizes.

Food	Portion size	Carbohydrate
Bread	2 medium slices (72 g)	32 g
Rice, boiled	180 g	57 g
Potatoes		
Oven chips	165 g	30 g
Crisps	1 bag (30 g)	15 g
Baked	200 g	63 g
Boiled	175 g	30 g
Spaghetti, cooked	230 g	51 g
Breakfast cereals		
Porridge oats	160 g	106 g
Weetabix	2 biscuits (38 g)	29 g
Cornflakes	30 g	26 g

Food Standards Agency (1995) Manual of Nutrition Tenth Edition, UK: The Stationery Office

So, if you eat 2 slices of bread and a bowl of cornflakes for breakfast; a baked potato for lunch and spaghetti Bolognese for dinner you would consume approximately 170 g of carbohydrate.

Plasma collection form**To be completed by the study co-ordinator**

Subject ID code	
Test condition	
Time point	
Collection time	
Total sample volume (mL)	
Comments	

Preparation of acellular human plasma**Equipment**

0.6 mL Sodium fluoride tubes

1.5 mL centrifuge tubes

Tube labels

Protocol

1. Collect 0.4-0.6 mL of whole blood into a labelled medical grade PET tube containing sodium fluoride (required for inhibition of glycolysis). Immediately place on ice.
2. Within 15 minutes, centrifuge the whole blood sample for 10 minutes at 3000 x g.
3. Using a non-sterile tip, pipette 0.05 mL of supernatant and transfer to a labelled 1.5 mL centrifuge tube. Several aliquots may be prepared.
4. Seal and freeze upright at $\leq -20^{\circ}\text{C}$ until required for analysis.

Labelling

Blood collection tubes and centrifuge tubes will be labelled as follows:

- Subject ID code
- Study code, comprised of:
 - Test condition identification (C1, C2)
 - Timepoint (0, 15, 30, 60, 90, 120)
- Date
- Investigator initials

Recruitment email

Subject: Invitation to eat chocolate...

Main text:

As part of a PhD research project, healthy male and female adults aged 18 to 60 years are invited to take part in this pilot study. You will be required to attend 2 sessions over 2 weeks and provide blood samples over a 2 hour period at each session. There will be a 12 hour fasting period overnight before each session and a snack will be provided at the end of each session.

Volunteers will be asked to follow a nutritionally balanced diet for 3 days prior to each session and to avoid alcohol and strenuous physical activity during that time.

The aim of this pilot study is to determine whether absorption of glucose into the blood is affected by consumption of dark chocolate.

For more information please contact Samantha Ellam by email: fs07sle@leeds.ac.uk or mobile: 07843 171548.

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

Participation is entirely voluntary and you are free to withdraw at any time without providing a reason. All information that is collected during the study is anonymous and kept strictly confidential.

Thank you for your time.

Samantha Ellam
PhD Research Student
School of Food Science and Nutrition
University of Leeds

**8.2 International Conference on Polyphenols and Health
2009: Cocoa polyphenols inhibit sucrose hydrolysis in
Caco-2 cells**

Cocoa polyphenols inhibit sucrose hydrolysis in Caco-2 cells

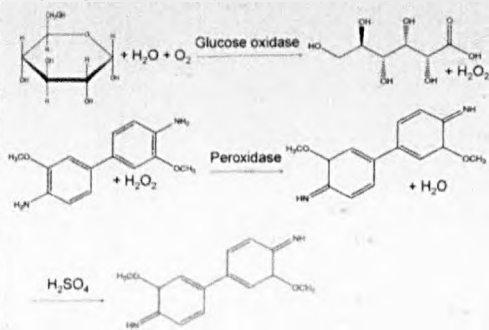
Samantha Ellam and Gary Williamson
School of Food Science and Nutrition, University of Leeds.

Introduction

- Dietary sucrose is hydrolysed in the small intestine by sucrose alpha-glucosidase (EC 3.2.1.48)
- Liberated glucose is primarily transported across the epithelium by a process of secondary active transport involving the sodium-dependent glucose transporter SGLT1
- During periods of elevated blood glucose, passive diffusion of glucose across the apical membrane is additionally facilitated by the sugar transporter GLUT2
- Rising blood glucose stimulates the release of insulin from pancreatic beta cells
- Insulin binds with receptors on the basolateral surface of skeletal muscle cells consequently promoting the uptake of glucose via the sugar transporter GLUT4
- Attenuation of glucose liberation and transport from the intestinal lumen may reduce post-prandial blood glucose concentration thereby reducing glycaemic load

Methodology

- Caco-2 cells were seeded into 6-well Transwell® plates at a density of 282,000 cells per well!
- 21 days post-seeding cells were ready for experiment:
 - ✓ Confluent
 - ✓ Polarised
 - ✓ Differentiated
 - ✓ Presence of tight junctions
- Cells were incubated with 20 mM sucrose plus test compound or extract (pH 7.4) for 20 minutes at 37°C, 5% CO₂
- Solid phase extraction of each sample was performed to remove polyphenols
- The concentration of glucose in each sample was measured using the glucose oxidase/peroxidase assay



Results and Discussion

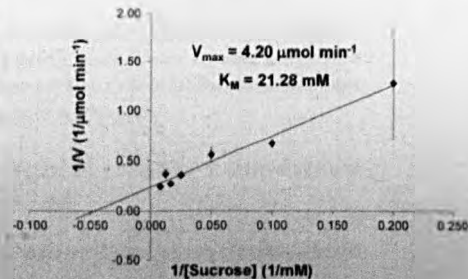


Figure 1. Lineweaver Burk plot of the mean rate of sucrose hydrolysis (n = 3) in the Caco-2 cell model. The maximum rate of reaction (V_{max}) and Michaelis-Menten constant (K_M) calculated from the linear regression equation is given.

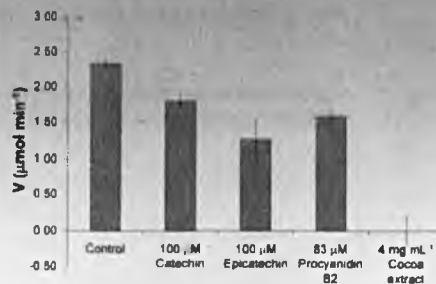


Figure 2. The effect of cocoa extract and cocoa polyphenols on the rate of sucrose hydrolysis in the Caco-2 cell model.

Conclusion and Future Work

- Preliminary results suggest that cocoa extract substantially inhibits the rate of sucrose hydrolysis in Caco-2 cells
- 100 μM (-)-Epicatechin demonstrated the greatest inhibition (45%) followed by 83 μM Procyanidin B2 (32%) and 100 μM (+)-Catechin (23%)
- Future work will include determining
 - The kinetic inhibition parameters of sucrose hydrolysis using the Caco-2 cell model
 - The effect of cocoa polyphenols on glucose transport across the Caco-2 cell monolayer

UNIVERSITY OF LEEDS

This project is funded by a BBSRC industrial case award to Nestlé, York, UK

- 8.3 Ellam, S. and Williamson, G. (2013) 'Cocoa and Human Health', *Annual Review of Nutrition*, 33, in press.

1 Cocoa and Human Health

2

3

4 Samantha Ellam and Gary Williamson

5 School of Food Science and Nutrition

6 University of Leeds

7

8 Email addresses: fs07sle@leeds.ac.uk g.williamson@leeds.ac.uk

9

10 Corresponding author: G Williamson, School of Food Science and Nutrition

11 University of Leeds, Leeds, LS2 9JT, UK

12

13

14

15 Keywords: Flavonoid; flavonol; polyphenol; theobromine; magnesium;

16 cardiovascular disease

17

18

19

20	Contents:
21	
22	Abstract
23	INTRODUCTION
24	BIOLOGICALLY ACTIVE COMPONENTS OF COCOA
25	<i>Flavonoids</i>
26	<i>Theobromine</i>
27	<i>Magnesium</i>
28	BIOAVAILABILITY OF INTACT FLAVONOIDS FROM COCOA
29	METABOLISM AND ABSORPTION OF PHENOLICS AFTER MICROBIAL
30	CATABOLISM
31	BIOAVAILABILITY OF THEOBROMINE
32	EFFECTS OF COCOA IN HUMAN INTERVENTION STUDIES
33	Epidemiological evidence
34	Mechanisms of biological effects
35	<i>Endothelial cell dysfunction</i>
36	<i>Inflammation</i>
37	<i>Oxidative stress and dyslipidemia</i>
38	<i>Cognitive function and exercise</i>
39	CONCLUSIONS
40	REFERENCES
41	
42	
43	

44 **Abstract:**

45 Cocoa is a dry powdered non-fat component product prepared from the
46 seeds of the *Theobroma cacao* L. tree and a common ingredient of many
47 food products, especially chocolate. Nutritionally cocoa contains biologically
48 active substances which may affect human health: flavonoids (epicatechin
49 and oligomeric procyanidins), theobromine and magnesium. Theobromine
50 and epicatechin are absorbed efficiently in the small intestine, and the nature
51 of the conjugates and metabolites are now known. Oligomeric procyanidins
52 are poorly absorbed in the small intestine, but catabolites are very efficiently
53 absorbed after microbial biotransformation in the colon. There have now
54 been a significant number of studies on the effects of cocoa and its
55 constituent flavonoids, using in vitro and in vivo approaches. Most human
56 intervention studies have been performed on cocoa as an ingredient,
57 whereas many in vitro studies have been performed on individual
58 components. ~70 human intervention studies have been carried out on
59 cocoa and cocoa-containing products over the last 12 years with a variety of
60 endpoints. These studies indicate that the most robust biomarkers affected
61 remain as endothelial function, blood pressure and cholesterol level.
62 Mechanistically, there is supporting evidence to show that epicatechin
63 affects, amongst other targets, nitric oxide synthesis (eNOS), breakdown
64 (via inhibition of NADPH oxidase) and the substrate arginine (via inhibition of
65 arginase). The evidence further supports cocoa as a biologically-active
66 ingredient with potential benefits on biomarkers related to cardiovascular
67 disease. However, when present in chocolate, consideration should always
68 be given to the calorie and sugar content in the total diet.

69

70 INTRODUCTION

71

72 Cocoa is a dry powdered non-fat component product manufactured from
73 seeds of the *Theobroma cacao* L. tree, and is the ingredient considered in
74 this review. Cocoa liquor also contains cocoa butter (~55%) and is the
75 material used in chocolate confectionery manufacture along with other
76 ingredients such as sugar, emulsifier, milk protein, etc. depending on the
77 desired product. The production of cocoa liquor begins with cleaning the
78 seeds followed by a fermentation stage during which the chemical
79 composition of the bean is altered. After drying, the beans are roasted either
80 before or after shelling (winnowing), a fundamental part of the process that
81 affects the flavour characteristics and nutrient profile of the final product (1).
82 The shelled bean, known as the nib, is then ground to a paste, which causes
83 melting of the fat and formation of the cocoa liquor. The liquor may then be
84 treated with an alkali solution, termed 'Dutching', to increase the pH and
85 improve palatability. Similar to the roasting process, the alkalisating step
86 affects the chemical composition of the cocoa liquor such that both stages in
87 processing may be refined and strictly controlled to develop a product with a
88 specific chemical profile (2). Being a roasted natural product, cocoa is a
89 complex material. The primary components impacting on health are currently
90 considered to be the naturally-occurring or process-derived flavonoids,
91 theobromine, and magnesium. The ingredients added to make chocolate,
92 sugar and cocoa butter, will not be discussed here.

93

94 In Europe, consumers prefer milk chocolate but dark chocolate is almost as
95 popular (3). In the US, milk chocolate is also the most popular, although the

96 majority of US confectionery consumption (~87 %) is not as pure chocolate
97 but rather enrobed with nuts, wafer, fruit, etc (4). Cocoa taken as a beverage
98 is also popular in some countries like Spain and this should also be taken
99 into account when surveying intake of chocolate and cocoa products.

100 Nutritionally, chocolate generates vehement debate in popular articles, and
101 opinions on the internet can be found that cover the whole range from
102 glorification of the benefits to emphasis on the evils of consumption! News
103 articles can be found claiming that "chocolate should be taxed to control
104 obesity" and at the other end of the scale that "chocolate may help keep you
105 slim". The 2008/2009 UK National Dietary Nutrition Survey (5) reported that
106 adults were consuming an average of 21 g of chocolate confectionery per
107 day and the 2010 Family Food survey (6) reported an average contribution
108 to energy intake from confectionery (including chocolate) of 4% per person
109 per day. In 2010 confectionery as a whole contributed 15% of the total non-
110 milk extrinsic sugars consumed per person per day from household
111 purchases and 13% from foods purchased outside the home (6); the current
112 UK reference nutrient intake recommends that non-milk extrinsic sugars
113 should not provide more than 11% of the daily energy intake (7). A recent
114 report (8) demonstrated that whilst economies globally have been in
115 recession, the chocolate market has remained stable; in fact its retail market
116 value has risen marginally each year since 2007 and is predicted to continue
117 growing at a rate of 2% per year over the next 5 years. The majority of the
118 global market is held by Western Europe (32%) followed by North America
119 (20%), Asia (17%), Latin America (13%), Eastern Europe (12%), Middle East
120 and Africa (4%) and Australasia (2%), with large growth predicted in the
121 smaller markets over the coming years.

122

123 There have now been several hundred human intervention studies on
124 flavonoids and flavonoid-rich foods, reviewed in many publications (3;9-12).
125 The role of flavonoid-rich foods on health has further been supported by
126 many epidemiological studies including meta-analyses comprising large total
127 numbers of volunteers (13). These studies infer a general and fairly
128 consistent protection by flavonoids against heart disease and biomarkers of
129 cardiovascular risk, while the evidence for protection against cancer is
130 weaker. This review will focus on recent work on the effect of cocoa,
131 including cocoa flavonoids, on biomarkers related to cardiovascular health.
132 Previously studies have shown that cocoa may improve cardiovascular
133 health by improving flow mediated dilation, a marker of endothelial function,
134 decreasing the susceptibility of LDL to oxidation, inhibiting platelet
135 aggregation and activation, and decreasing levels of F2-isoprostanes (3).
136 Recent meta-analyses on human intervention studies associated dark
137 chocolate with a reduction in systolic hypertension or diastolic pre-
138 hypertension (14) and both chocolate and cocoa with improvements in flow
139 mediated dilation (15).

140

141 **BIOLOGICALLY ACTIVE COMPONENTS OF COCOA**

142

143 ***Flavonoids***

144

145 Cocoa beans contain a high amount of flavonoids, a member of the broader
146 polyphenol class. The main constituents are flavan-3-ols (**Figure 1**), present
147 as monomeric (-)-epicatechin and (+)-catechin, together with type-B

148 proanthocyanidins, formed from monomeric flavanols by oxidative coupling
149 between the C-4 of the heterocyclic ring and the C-6 or C-8 positions of the
150 adjacent unit to create oligomers and polymers. Procyanidins in cocoa
151 include the B2 and B5 dimers and the C1 trimer, together with high levels of
152 longer chain polymers comprising four or more monomeric units (16;17).
153 Flavan-3-ols are lost during fermentation, treatment with alkali and roasting
154 (18) so that the total flavan-3-ol content of commercial cocoa varies by >10-
155 fold (19). Processing can also result in some epimerisation of (-)-epicatechin
156 to form (-)-catechin (20;21).

157

158 *Theobromine*

159

160 Theobromine (**Figure 2**) is a 3,7-dimethylated xanthine alkaloid that is also
161 formed during caffeine metabolism. It is most commonly consumed in the
162 human diet from chocolate and cocoa but is also present in tea. Especially
163 high levels are present in cocoa, about 2.5% of dry weight, whereas caffeine
164 is ~10-fold lower (~0.24%) (22). Theobromine is not degraded during cocoa
165 processing and can be used as a marker of cocoa content (21). The effect of
166 theobromine on arteriosclerotic pain was reported as far back as 1926 (23).
167 The more recent focus on cocoa flavonoids has meant that theobromine is
168 often not considered as a component responsible for an observed activity,
169 but its high bioavailability and potential biological activities mean that it
170 should not be ignored in cocoa intervention studies.

171

172 *Magnesium*

173

174 According to the USDA National Nutrient Database, magnesium is found at
175 significant levels in cocoa (2-4 mg/g dry powder). Although dependent on the
176 type of chocolate, this means that a 40 g portion of 70%-cocoa dark
177 chocolate would contain ≈40 mg of magnesium, enough to make a modest
178 ~10% contribution to the recommended daily allowance (300-400 mg
179 magnesium /day in adults) (24). Magnesium is an essential co-factor in
180 many hundreds of enzyme-catalysed reactions in vivo, and is essential for
181 maintenance of blood pressure, neuronal transmission and muscular
182 contraction. Deficiency has been linked to the metabolic syndrome, insulin
183 resistance and diabetes (24).

184

185 **BIOAVAILABILITY OF INTACT FLAVONOIDS FROM COCOA**

186

187 After consumption of food, any biologically active component must survive in
188 the digestive tract and be absorbed and metabolised in a form which
189 reaches and influences the target tissue. In the mouth, procyanidins bind to
190 salivary proteins, the degree of binding influenced strongly by the inter-
191 flavan-3-ol linkage and the chemical nature of the structural monomeric units
192 rather than size of the oligomers (25;26). In vivo, flavanols are stable in the
193 stomach (27), and reach the small intestine intact. The jejunal pH can reach
194 8.5, where flavanols are expected to be unstable, but epicatechin and
195 procyanidins appear to remain largely unaffected in the small intestinal
196 lumen, dependent on the food matrix, presumably due to the stabilising
197 influence of protein and other food constituents (28;29). Flavanols may also
198 be stabilised by the presence of fat in the intestinal lumen (30). Epicatechin
199 is absorbed in the small intestine and after consumption of dark chocolate,

200 conjugation produces (-)-epicatechin-3'-O- β -D-glucuronide, (-)-epicatechin
201 3'-O-sulfate, and 3'-O-methyl epicatechin sulfates (substituted in the 4', 5,
202 and 7 positions) in the plasma with a time of maximal concentration in the
203 blood of at 3-4 h (31). Epicatechin is subsequently excreted in urine with a
204 similar, but not identical, profile of conjugates and urinary data show that a
205 minimum of 20% of the epicatechin dose from cocoa is absorbed (31),
206 apparently higher than the dose of epicatechin absorbed from green tea
207 (32). The food matrix affects the rate and extent of absorption (33). Studies
208 on rats have indicated that flavan-3-ol monomer conjugates may be
209 transferred from the blood stream to the liver and subsequently returned to
210 the small intestine via bile (34;35). Regarding the oligomers, intact
211 procyanidin dimer B2 is poorly absorbed intact both in humans and animals
212 (36-38), and has only been detected at very low levels in human urine after
213 consumption of cocoa (39). Individual human intervention studies on
214 absorption and metabolism have been reviewed in detail (10;33).

215

216 **METABOLISM AND ABSORPTION OF PHENOLICS AFTER MICROBIAL** 217 **CATABOLISM**

218

219 There is now compelling evidence that the microbiota play a major role in the
220 metabolism of flavanols which reach the colon i.e. the proportion which are
221 not absorbed in the small intestine. After cocoa consumption by humans, 3-
222 (3'-hydroxyphenyl)propionic acid, 3'-hydroxy-phenylacetic acid, 3',4'-
223 dihydroxy-phenylacetic acid, 3-hydroxybenzoic acid, ferulic acid, 5-(3',4'-
224 dihydroxy-phenyl)- γ -valerolactone (5) and 5-(3'-methoxy-4'-hydroxyphenyl)-

225 γ -valerolactone were excreted in urine, peaking between 9 and 48 h,
226 indicative of microbial catabolism (39;40). These microbial metabolites
227 constitute about 80% of the dose of radiolabelled procyanidin B2 in rats, as
228 judged by urinary appearance and a late T_{max} (41), even though the intact
229 parent compound is almost absent from plasma and urine. The broad range
230 of metabolites produced by the microbiota has been shown using in vitro
231 incubations and analysis of the intermediate and final products, where
232 catabolism favoured removal of the 4' rather than the 3'-hydroxyl group,
233 along with both β -oxidation and α -oxidation and some scission of the
234 interflavan bond (42;43). Metabolism of radiolabelled procyanidin polymers
235 (average dp ~6) by human colonic microflora in vitro showed that they were
236 almost totally degraded after 48 h. The main metabolites detected were
237 similar to those described above found in urine (44). Some patterns in the
238 appearance of gut microbiota metabolites are apparent depending on the
239 class of parent polyphenol, and in general after consumption of
240 procyanidins, the flavan-3-ols are mainly converted to C_6C_2 and C_6C_3 -
241 dihydro forms partly via C_6-C_5 intermediates, the latter being unique to
242 flavan-3-ols (45).

243

244 **BIOAVAILABILITY OF THEOBROMINE**

245

246 Theobromine is absorbed extensively in the small intestine and the
247 metabolic pathway is quite well understood. The main metabolite is 7-
248 methylxanthine which accounts for up to 30% of the dose, 3-methylxanthine
249 accounting for ~20% and 7-methyluric acid for ~4% of the dose (46). 3,7-
250 dimethyluric acid and 6-amino-5-(N-methyl-formylamino)-1-methyluracil are

251 also present in urine following consumption of cocoa powder (47-49).
252 Unmodified theobromine is also found in urine and a small amount in feces
253 (<1.5% of the ingested dose) (49). The half-life of theobromine in plasma
254 was 7.2 h when consumed in the form of a gelatine capsule (50) and 10 h
255 when ingested from an aqueous solution (49). The maximum plasma
256 concentration of theobromine from chocolate is reached approximately 2 h
257 post-ingestion suggesting that it is entirely absorbed in the small intestine
258 (49). Although the mechanism for absorption through the intestinal
259 epithelium has not been described exactly, based on in vitro absorption of
260 caffeine using Caco-2 cells (51), it can be surmised that theobromine
261 similarly diffuses passively through the enterocytes into the hepatic
262 circulation.

263

264 The mechanisms by which theobromine is demethylated and oxidized to
265 methyluric acid involve hepatic cytochrome P450 enzymes with specific
266 involvement of the monooxygenase (EC 1.14.14.1) isoforms CYP1A2 and
267 CYP2E1 (52-54). The primary metabolite, 7-methylxanthine, is a product of
268 both enzyme isoforms. CYP2E1 appears to be the least specific as it also
269 catalyses the formation of 3-methylxanthine and 3,7-dimethyluric acid (55).
270 Whilst the 3-methylxanthine isomer may undergo limited further metabolism
271 to 3-methyluric acid (~1% of dose) (53), 7-methylxanthine is the main
272 substrate from theobromine catabolism for xanthine oxidase (EC 1.17.3.2)
273 which catalyses the oxidation of carbon-8 to form 7-methyluric acid. The
274 bioavailability of theobromine and high levels in cocoa mean that levels will
275 reach micromolar levels in plasma after consumption of modest amounts of
276 cocoa.

277

278 **EFFECTS OF COCOA IN HUMAN INTERVENTION STUDIES**

279

280 The 28 human intervention studies on the effect of cocoa between 2000 and
281 2007 have been reviewed (3). The main outcomes were improved
282 endothelial function, decreased susceptibility of LDL to oxidation, inhibition
283 of platelet aggregation and activation, and decreased levels of F2-
284 isoprostanes. Since then, there have been numerous reviews regarding the
285 role of cocoa in specific health issues, in addition to a small number of
286 epidemiological studies. Since 2007, the majority of published work in
287 human and non-human intervention trials focused on the effect of cocoa
288 products, or individual chemical components of cocoa, on risk of
289 cardiovascular disease as estimated using surrogate biomarkers. Discussion
290 in this section predominantly considers the literature pertaining to
291 cardiovascular disease but is inclusive of other areas of study, with all
292 human intervention trials conducted since 2007 summarised in **Table 1**. Of
293 these studies,, 15 have measured changes in parameters related to
294 endothelial function, either flow mediated dilation, angiotensin converting
295 enzyme activity or nitric oxide/nitrite levels. Blood pressure changed in 9 of
296 the studies and cholesterol levels were affected in 13 studies. Oxidative
297 parameters, such as F2-isoprostanes and susceptibility of LDL to oxidation,
298 were changed in 9 of the studies. Other parameters reported to be affected
299 by cocoa intervention were glucose/insulin levels (5 studies), platelet
300 function (5 studies), brain blood flow and cognitive function (4 studies),
301 inflammation (2 studies) and skin (2 studies). Six of the studies used
302 exercise in some form as a variable in at least one of the study arms. These

303 studies indicate that the largest changes measured remain as endothelial
304 function, blood pressure and cholesterol levels. However, variations in study
305 time, from a single dose up to 3 months intervention, and in the type of
306 cocoa given, make rigorous interpretation difficult. It is interesting to note
307 that positive changes in flow mediated dilation may be greatest when cocoa
308 is administered in the absence of sugar (56).

309

310 **Epidemiological evidence**

311

312 In the last few years, several epidemiological studies have reported on the
313 effects of cocoa intake in various population groups. Elderly male
314 participants in the Zutphen Elderly Study had lower cardiovascular mortality
315 when correlated with long-term cocoa intake (57). The risk of death from
316 stroke, coronary heart disease and cardiovascular disease amongst
317 participants of the Iowa Women's Health Study was lower in women who
318 consumed chocolate relative to those who consumed no chocolate (58).
319 Similarly data taken from the United States National Heart, Lung and Blood
320 Institute Family Heart Study (59) suggests that compared with no intake,
321 participants with a greater frequency of chocolate intake have reduced
322 coronary heart disease prevalence, the lowest found for consumers of 5 or
323 more servings per week. In contrast, a study of women participating in the
324 Swedish Mammography Cohort (60) demonstrated that the beneficial effect
325 of chocolate consumption on risk of heart failure was reversed as intake
326 exceeded 2 servings per week. It is relevant to note at this point that exact
327 serving size is not stated in the aforementioned studies, although the
328 authors of the Swedish investigation do suggest an average portion size

329 somewhere between 19 and 30 g. There was an inverse association
330 between chocolate consumption up to 7.5 g per day and risk of
331 cardiovascular disease among German adults participating in the European
332 Prospective Investigation into Cancer (61). In the latter study and that of the
333 Iowa Women's Health Study, the association appeared most compelling for
334 stroke. Evaluation of data taken from The Stockholm Heart Epidemiology
335 Program (62) of individuals who have experienced a first acute myocardial
336 infarction show that the risk of cardiac mortality was lowest in those who
337 consumed a 50 g portion of chocolate at least twice per week, and the risk of
338 a non-fatal stroke was shown to be lowest in those consuming up to one
339 portion per week.

340

341 From these epidemiological studies it can be inferred that the consumption
342 of 50-100 g per week of chocolate may reduce the risk of cardiovascular
343 disease, in particular stroke. What is not established in any of these
344 investigations is the type of chocolate consumed i.e. dark, milk or white, and
345 the possible addition of other ingredients such as fruit or nuts, important
346 factors in the overall analysis of the evidence, in addition to total diet and
347 numerous other cardiovascular disease risk factors. Dark chocolate typically
348 contains more non-fat cocoa solids than milk chocolate, while white
349 chocolate contains none. As the percentage of non-fat cocoa solids content
350 increases, the percentage content of sugar decreases. Further, milk and
351 white chocolate also contain milk powder that is not present in dark
352 chocolate. A case in point is demonstrated in a study (63) where the lipid
353 profile and inflammation biomarker concentration was improved following
354 consumption of a cocoa product with the addition of hazelnuts, phytosterols

355 and soluble fibre. However, the cocoa product without added ingredients had
356 no significant effect.

357

358 Some epidemiologists have investigated more specific interactions by
359 focusing on particular components of cocoa rather than the product *per se*.

360 As with the majority of all evidence discussed in this review, the
361 overwhelming theme is flavonoids and in particular the flavan-3-ol and
362 proanthocyanidin sub-classes of polyphenols, although other components
363 could conceivably have complementary, antagonistic or synergistic effects.

364 An evaluation of incident hypertension, biomarkers of inflammation and
365 endothelial dysfunction in relation to flavonoid intake of participants in the
366 Nurses' Health Study I and II, and the Health Professionals Follow-Up Study
367 (64;65), did not find any relationship with flavan-3-ol or proanthocyanidin
368 intake. However, at the highest daily intake of catechin and epicatechin, the
369 primary flavan-3-ol constituents of cocoa, a significantly lower risk of incident
370 hypertension was found. In contrast, a recent study (66) demonstrated a
371 significant reduction in the risk of cardiovascular disease with increased
372 intake of flavan-3-ols and proanthocyanidins although this attenuation was
373 not linear and, similar to other evidence from epidemiological studies,
374 reversed in the highest intake groups.

375

376

377

378 **Mechanisms of biological effects**

379

380 ***Endothelial cell dysfunction***

381

382 Endothelial cell dysfunction represents a risk factor for cardiovascular
383 disease and as such has been widely studied *in vivo* and *in vitro* (67). Many
384 of the human intervention trials discussed here involve quantification of
385 products secreted by the endothelium such as nitric oxide, or expression of
386 inflammatory response proteins such as interleukin-6 (IL-6). For example,
387 nitric oxide (NO) production by platelets isolated from healthy subjects was
388 increased following 3 weeks daily consumption of dark chocolate (68). At
389 least half of the studies listed in Table 1 reported improvements in
390 parameters related to endothelial function. Generally these are biomarkers
391 related to NO metabolism, including flow mediated dilation, angiotensin
392 converting enzyme activity or nitric oxide/nitrite levels. Mostly favourable
393 changes in blood pressure were reported in 9 of the studies. This marker is
394 especially important since average decrease in diastolic blood pressure in a
395 population of 1 mmHg is enough to reduce the incidence of coronary heart
396 disease events by 5% and of stroke by 7% in persons aged 50–69 y with a
397 systemic (high) blood pressure of 150 mm Hg and a diastolic blood pressure
398 of 90 mm Hg (69). Although an association between a sharp increase in
399 cardiovascular disease (CVD) mortality and blood pressure (BP) was made
400 very early, newer studies have introduced the notion of “background”
401 hypertension (pre-hypertension) and support the idea that pre-hypertension
402 increases the rate of cardiovascular disease only when accompanied with
403 other risk factors, the age having a major impact.

404

405 Systolic blood pressure in spontaneously hypertensive rats was reduced,
406 considered to be a consequence of increased nitric oxide production,

407 following an acute dose of *CocoanOX*; the effect was prevented in the
408 presence of an endothelial nitric oxide synthase (eNOS) inhibitor (70). The
409 same authors investigated the long-term effect of *CocoanOX*
410 supplementation and observed attenuated development of hypertension in
411 spontaneously hypertensive rats with the effect being greatest in animals
412 given the lowest dose (71). In another study, spontaneously hypertensive
413 rats whose diets were supplemented with soluble cocoa fibre exhibited lower
414 systolic and diastolic blood pressure during the treatment period. Throughout
415 a 4 week post-treatment period when the test diet had been discontinued,
416 systolic blood pressure increased equivalent to control and diastolic blood
417 pressure increased beyond that of control animals (72).

418
419 Spontaneously hypertensive rats administered 200 and 400 mg kg⁻¹ body
420 weight doses of *CocoanOX* exhibited higher plasma angiotensin converting
421 enzyme activity at the end of the intervention period, and plasma
422 concentration of angiotensin II was also raised. The latter remained elevated
423 after the treatment ended. The same study measured relaxation of pre-
424 contracted intact aorta rings extracted from control animals induced by
425 incubation with *CocoanOX*. Relaxation was reduced in the presence of *N^w*-
426 nitro-L-arginine methyl ester but not indomethacin implying that dilation was
427 a result of eNOS activity rather than prostaglandin-I synthase (73). Similar
428 effects were reported using pre-contracted renal arteries isolated from wild-
429 type and atherosclerotic mice (74). Catechin treatment in wild-type mice
430 during months 9 to 12 improved dilation, at 12 months compared with the
431 untreated group; however this effect was not replicated in atherosclerotic
432 mice. Incubation with NG-nitro L-arginine attenuated dilation in untreated

433 wild-type mice but animals exposed to 3 months catechin treatment were
434 unaffected, suggesting that eNOS was not inhibited in catechin treated cells
435 or that addition of indomethacin to the incubation did not further affect this
436 result.

437

438 *In vitro* studies using human umbilical vein endothelial cells (HUVEC) are
439 widely used as a model to study endothelial function. On incubation of
440 HUVEC cells with (-)-up to 1 μ M epicatechin for 2 h, nitrite, as an indirect
441 marker of the intracellular nitric oxide level, increased significantly (75).
442 Similarly the greatest augmentation of nitric oxide produced by human
443 coronary artery endothelial cells followed a 10 min incubation with 1 μ M (-)-
444 epicatechin (76). Following a 24 h incubation with (-)-epicatechin, there was
445 no significant change in endothelial nitric oxide synthase (eNOS) mRNA
446 expression nor any variation in its stability (75). It is hypothesized that
447 increased production of nitric oxide is attributable to an interaction with the
448 phosphatidylinositol 3-kinase pathway which is involved in controlling
449 phosphorylation of the activation residues Ser1177 and Ser633. Treatment
450 of human coronary artery endothelial cells with epicatechin increased
451 phosphorylation of these serine residues and reduced phosphorylation of
452 Thr495. In addition, epicatechin treatment appeared to induce activation of
453 eNOS through uncoupling from caveolin-1 (**Figure 3**). The latter binds to the
454 cytosolic side of the cell membrane in its inactive form, and binding with
455 calmodulin stimulates solubilisation of the active form, mediated via
456 interaction with phospholipase C. Whilst these conditions were dependent
457 on the presence of calcium, epicatechin is able to stimulate NO production
458 induced by phosphorylation of serine residues and activation of eNOS

459 without uncoupling from caveolin-1 in calcium-free conditions (77). Similarly
460 enhanced phosphorylation of non-specific serine/threonine protein kinase
461 (Akt), an enzyme involved in the phosphatidylinositol 3-kinase pathway, was
462 measured in mice subjected to ischemia-reperfusion injury following 10 d of
463 supplementation with 1 mg kg⁻¹ body weight (-)-epicatechin; infarct size was
464 reduced. Co-supplementation with the opioid antagonists naloxone or
465 naltrindole eliminated the effect on Akt phosphorylation and infarct size. The
466 results suggest that the protection conveyed by epicatechin was dependent
467 on interaction with opioid receptors in the heart (78). Endothelium secretion
468 of endothelin-1 is associated with vascular constriction and therefore
469 increased concentration has negative cardiovascular consequences. Bovine
470 aortic endothelial cells treated with a procyanidin-rich extract of cocoa
471 produced less endothelin-1 than control cells; the response was dose-
472 dependent (79). Procyanidin-rich extracts of other fruits elicited similar
473 results and the trimer to pentamer fractions of a cranberry extract were
474 shown to down-regulate expression of endothelin-1 mRNA and up-regulate
475 Kruppel-like factor 2 mRNA, a transcription factor that mediates the
476 synthesis of endothelin-1. There was a similar reduction in endothelin-1
477 synthesis in HUVEC treated with a procyanidin-rich fraction of apple (80).
478 Epicatechin also inhibits NADPH oxidase (81) and arginase activity (82),
479 leading to elevated intracellular NO levels (**Figure 4**).

480
481 An elevated nitric oxide concentration *in vivo* is generally regarded as
482 beneficial due to its vasodilating properties and inhibition of platelet
483 aggregation. It is possible that reported increases in flow-mediated dilation
484 and reduction of blood pressure following cocoa consumption (see **Table 1**)

485 are due to an increase in the production of nitric oxide stimulated by
486 epicatechin. Therefore, chronic intake may have a long-term protective
487 effect against cardiovascular disease via these mechanisms.

488

489 ***Inflammation***

490

491 Inflammation is a complex process and several biomarkers are used to
492 identify chronic inflammation in humans. Although multiple biomarkers are
493 now recommended to assess cardiovascular disease risk, a higher C-
494 reactive protein concentration is associated with a higher risk of coronary
495 heart disease (83-86). In a cohort of healthy Italian adult participants in the
496 Moli-sani Project, consumers of less than 3 x 20 g servings per week of dark
497 chocolate had lower plasma concentrations of C-reactive protein (87), but,
498 as observed in other studies, this effect was negated in those consuming 3
499 or more servings per week. The overall effect, as assessed from human
500 intervention trials published since 2007 in which serum C-reactive protein
501 concentration was quantified following chronic cocoa supplementation, is
502 inconclusive. In healthy subjects, significant reductions in C-reactive protein
503 following intervention were seen (88;89), whereas in subjects suffering from
504 hypertension, hypercholesterolemia or type-2 diabetes, no significant
505 changes were observed (90-92). In comparison, a study of diabetic mice,
506 where diets were supplemented with epicatechin, reported significantly lower
507 C-reactive protein levels compared with control diabetic mice (93). Although
508 serum C-reactive protein concentration is indicative of cardiovascular
509 disease, it is yet to be determined whether a high serum concentration
510 contributes to the development of disease or whether it is a consequence of

511 the disease. So at this point the level of intake of cocoa products or
512 individual components, and whether any ability to lower C-reactive protein
513 levels improves prognosis in patients or reduces risk in a healthy population,
514 is yet to be evaluated.

515

516 *Oxidative stress and dyslipidemia*

517

518 Oxidative stress is a broad term covering many aspects related to
519 generation of reactive oxygen species, with both chemical and biological
520 consequences. In a biological sense, the term must be carefully defined and
521 several biomarkers have been used to indicate a general oxidative stress in
522 vivo (94;95). Dyslipidemia constitutes a major risk factor for cardiovascular
523 disease that has prompted an abundance of investigations, not least in
524 relation to cocoa consumption. Many of the intervention trials listed in **Table**
525 **1** have reported increased HDL cholesterol levels and improved total
526 cholesterol/HDL cholesterol ratio following dietary cocoa intervention

527

528 Animal models have also supported the association. When fed to rats, a
529 cocoa fiber product protected against the effects of a high cholesterol diet,
530 namely total-, LDL and HDL-cholesterol and serum malondialdehyde (96;97)
531 and a similar effect was seen with 0.5 or 1.0% cocoa procyanidins (98). In
532 the Zucker rat model of obesity and metabolic syndrome, 5% soluble cocoa
533 fibre diet reduced plasma malondialdehyde and increased adiponectin (99).
534 The 5% soluble cocoa fibre diet also reduced malondialdehyde plasma
535 concentration in spontaneously hypertensive rats (72). A similar reduction in
536 plasma malondialdehyde concentration was observed when spontaneously

537 hypertensive rats were treated with 100, 200 and 400 mg kg⁻¹ body weight
538 doses of CoccoanOX, while the effect was reversed by the end of a 4 week
539 post-treatment period (73). Obese-diabetic rats fed a diet supplemented with
540 cocoa extract for 4 weeks demonstrated improved levels of plasma total
541 cholesterol, plasma triglycerides and LDL cholesterol. There were no
542 significant changes in plasma HDL cholesterol concentration (100). Similar
543 improvements were observed in diabetic mice treated with epicatechin for 15
544 weeks together with enhanced longevity. Compared with non-treated
545 diabetic mice, insulin-like growth factor-1, C-reactive protein, interleukin-1 β
546 and glutathione were either returned to control levels or reduced significantly
547 (93). Daily oral supplementation of rats with 1 mg kg⁻¹ body weight
548 epicatechin for 10 d prior to ischemia-reperfusion injury reduced infarct size
549 and oxidized glutathione/reduced glutathione ratio (GSSH/GSH) at 48 h
550 post-injury. Infarct size remained significantly smaller at 3 weeks post-injury
551 (101). A further study by the same authors performed on animals exposed to
552 permanent coronary occlusion (POC) yielded very similar results of infarct
553 size (102).

554

555 **Cognitive function**

556

557 Of the studies shown in **Table 1**, four have shown an improvement in some
558 biomarkers of cognitive function including cerebral blood flow, lowered
559 fatigue and increased processing of tasks (103-106). The changes are
560 modest but significant, and the mechanism may be related to vasodilation
561 and increased blood flow to the brain and nervous system.

562

563 **Conclusions**

564

565 Cardiovascular diseases are responsible for more deaths globally than any
566 other cause (107) and are the focus of much research, not least in relation to
567 cocoa. The main target of such research has been to identify associations
568 between intake and disease, and to discover mechanisms by which onset of
569 disease can be delayed or prevented, or where treatment of cardiovascular
570 events can be facilitated. Many of the human intervention studies described
571 in **Table 1** have involved intervention treatments that have demonstrated
572 improvements in markers of disease risk factors such as total/HDL
573 cholesterol ratio and flow-mediated dilation. Nitric oxide synthesis is
574 possibly the most investigated endothelial function in relation to cocoa over
575 the last 5 years with many authors reporting increases in NO concentration
576 of plasma. The predominant mechanistic hypothesis is that cocoa, especially
577 epicatechin, stimulates eNOS activity, inhibits arginase and inhibits NADPH
578 oxidase, leading to lower levels of superoxide and hence higher levels of
579 NO. Although this is not the only mechanism involved, a substantial increase
580 in NO synthesis may account for flow-mediated dilation and lower blood
581 pressure following intervention treatments.

582

583 **Future prospects**

584 Despite a substantial number of human studies which consolidated the
585 effects on endothelial function, blood pressure and cholesterol, the advances
586 in the last 5 years have been incremental and supportive, rather than
587 revolutionary. While important, there are other aspects that need addressing
588 in the future. One of the most important is the assessment of long term

589 nutritional doses as compared to high dose acute studies, and the relative
590 benefits of each. There are no easy ways to measure chronic effects, and
591 usually epidemiological studies have been used to fill this gap. Controlling
592 the diet of human in the long term is almost impossible, and novel strategies
593 need to be devised to test chronic effects. An important fact is that most
594 cocoa consumption worldwide is as chocolate, which also contains
595 additional calories, and these should be taken into account in any
596 intervention studies. Most of the recommendations made previously are still
597 relevant (3), especially the design of a relevant "edible" placebo (especially
598 important for chronic studies, and milk chocolate is not a real control for dark
599 chocolate owing to the milk content) and the need to publish the results of
600 studies where cocoa does not give a measured effect. (3)

601

602

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610

611

612

613 **Acronyms and definitions:**

614

615 **"Polyphenol(s)"** is used as a collective term for phenolic acids, flavonoids,
616 isoflavones and tannins. Hence polyphenols strictly should be written as
617 (poly)phenols, since phenolic acids are (mono)phenols, flavonoids and
618 isoflavones are (di)phenols (the C ring is not a true phenolic ring) and
619 tannins are (poly)phenols. Flavonoids consists of several classes, of which
620 the flavanols (also called flavan-3-ols, and including their oligomers, the
621 proanthocyanidins), flavonols, anthocyanins, flavanones and flavones are
622 the most abundant in the diet.

623 **Epicatechin** is the main flavanol in cocoa and there are many reports on
624 this flavonoid. It has anomeric carbons at positions 2 and 3, making 4
625 possible structures: (–)-epicatechin and (+)-catechin, which occur naturally
626 in plants, and (–)-catechin and (+)-epicatechin, which occur in foods owing
627 to isomerization during processing. Each form is absorbed to different
628 extents (108), supporting the concept of active processes involved in
629 absorption and metabolism. In this review, where the "(–)-epicatechin" form
630 is specified in the paper, then this notation is used. If the form is not
631 specified, then "epicatechin" is used.

632 **Cocoa** is a dry powdered non-fat component product manufactured from
633 cocoa beans. The full cocoa bean is used to make cocoa liquor, containing
634 the constituent cocoa butter (~55%), and is used to make **chocolate** after
635 addition of other ingredients such as sugar, emulsifier, milk protein, etc.

636 **CocoaOX** is a commercially available polyphenol-rich cocoa powder which
637 has been used in several experimental intervention studies.

638

Intervention	Control	Study design	Outcomes	Ref.
Cu deficient patients supplemented with 10-40 g cocoa powder per day until improvement observed	None	Serum trace metal concentration monitored \leq 24 months post-enteral tube insertion in 23 patients (78.8 ± 10.4 years)	\uparrow serum [Cu] and neu trophil count	(109)
One cup of espresso coffee and 30 g DC (separate occasions)	None	50 term pregnant women without complicated gestation (31.8 ± 5.05 years)	\uparrow fetal HR accelerations and variability	(110)
15 g pp-rich DC per day for 8 weeks	15 g macronutrient matched low-pp chocolate ($3.9 \text{ mg ECE g}^{-1}$)	Double-blind, randomised cross-over; 10 subjects diagnosed with CFS (6 females, 4 males) (52 ± 8 years)	Improved symptoms of CFS	(111)
HF beverage	Macronutrient-matched LF beverage	Cross-over; 10 healthy females (18-65 years)	\uparrow cutaneous blood flow	(112)

20 g HF chocolate per day for 12 weeks	20 g LF chocolate (<30 mg flavanols)	Double-blind, randomised; 30 healthy subjects (22 females, 8 males) (42.7 ± 10 years)	↑ minimal erythema dose	(113)
37 g DC and 237 mL cocoa beverage per day for 6 weeks	Low-pp bar and beverage containing 0.20 mg g ⁻¹ and 40.87 mg g ⁻¹ total proanthocyanidins, respectively	Double-blind, randomised; 101 healthy subjects (60 females, 41 males) (≥60 years)	↑ pulse rate at midpoint and end-of-treatment	(114)
Flavanol-rich cocoa beverage per day for 1 week	Flavanol-poor cocoa beverage (36 mg flavanols per day)	Double-blind, randomised; 21 healthy subjects (10 females, 11 males) (72.2 ± 6 years)	↑ cerebral blood flow in response to acute dose of cocoa beverage	(106)
Cocoa beverage containing either 520 mg or 994 mg cocoa flavanols	Macronutrient-matched cocoa beverage/46 mg cocoa flavanols	Double-blind, cross-over; 30 healthy subjects (17 females, 13 males) (21.9 ± SE 0.61 years)	Improved cognitive performance and reduced mental fatigue	(105)
35 g DC	35 g white chocolate	Cross-over; 30 subjects (22 females, 8 males) (18-25 years)	Improved contrast sensitivity, motion integration threshold, visual spatial working memory, and reaction time	(104)

20 g chocolate beverage containing MF or HF content per day for 30 days	20 g chocolate beverage LF content	Double-blind, randomised; 63 subjects (52.30 ± 7.49 years)	↑ posterior parietal activity, synaptic excitation and neural information processing speed	(103)
Mashed potato powder (1 g kg^{-1} BW) with cocoa butter (1 g kg^{-1} BW)	Mashed potato powder (1 g kg^{-1} BW) supplemented with olive oil (1 g kg^{-1} BW)	Randomised, cross-over; 10 healthy females (38.2 ± 10.7 years)	Plasma [TAG] and [IL-6] were altered (no significant difference between the meals)	(115)
Cocoa beverage twice per day for 12 weeks	Cocoa-free beverage	Randomised; 25 healthy males ($38 \pm \text{SE } 1$ years)	↓ LDL susceptibility to oxidation and urinary [dityrosine]; ↑ HDL cholesterol	(116)
Low-, medium-, or high-pp cocoa beverage twice per day for 4 weeks	Nutrient matched beverage (trace amounts of cocoa pp)	Double-blind; 160 normo- and mildly hypercholesterolemic subjects (91 females, 69 males) (20-70 years)	↓ [Apo B] in MCP and HCP groups; ↓ oxidised LDL (kU L^{-1} plasma) in all groups	(117)
40 g DC	Macronutrient matched, flavonoid-free chocolate	Double-blind, randomised; 22 heart transplant recipients (4 females, 18 males)	↑ Coronary artery diameter and % change of endothelial-dependent vasomotion; ↓ [8-iso-PGF 2α] and platelet adherence	(118)

22 g DC supplemented with 1.1 g canola sterol esters twice per day for 4 weeks	Macro- and micronutrient matched DC without the addition of plant sterols	Double-blind, cross-over; 49 subjects (32 females, 17 males) (24-70 years) with elevated cholesterol (5.20-7.28 mmol L ⁻¹)	↓ Serum total- and LDL cholesterol; ↓ SBP and DBP (combined intervention and control results)	(119)
MF or HF beverage	Macro- and micronutrient matched LF beverage	Double-blind, randomised, cross-over; 10 type-2 diabetic subjects (2 females, 8 males) (64.7 ± 9.9 years)	↑ FMD	(120)
Flavanol containing beverage 3 times per day for 30 days	Macro- and micronutrient matched LF beverage	Double-blind, randomised; 41 type-2 diabetic subjects (29 females, 12 males) (intervention, 63.1 ± 8.3 years; control, 64.4 ± 8.6 years)	↑ FMD	(120)

HF beverage twice per day for 12 weeks with and without 45 min physical activity 3 days per week	Macro- and micronutrient matched LF beverage with and without physical activity	Double-blind, randomised; 49 overweight and obese subjects (32 females, 17 males) (40-50 years)	↑ FMD (combined exercise and non-exercise results); ↓ insulin resistance, DBP and MAP (flavanol treatment nested in time)	(121)
Phase 1: Acute consumption of 74 g DC Phase 2: 2 cups of either sugar-containing cocoa beverage or sugar-free cocoa beverage	Phase 1: 74 g cocoa-free chocolate Phase 2: 2 cups of sugar-containing cocoa-free beverage	Single-blind, randomised, cross-over; 45 healthy subjects (35 females, 10 males) (52.8 ± 11.0 years)	↑ FMD; ↓ SBP and DBP; greatest FMD improvement following sugar-free cocoa beverage.	(56)
100 g flavanol-rich DC per day for 15 days	100 g flavanol-free white chocolate per day	Double-blind, cross-over, 19 hypertensive, prediabetic subjects (8 females, 11 males) (44.8 ± 8.0 years)	↓ Insulin resistance and ↑ insulin sensitivity; ↓ clinical SBP and DBP, and ambulatory BP; ↑ FMD and ↓ serum total- and LDL cholesterol	(90)
100 g DC per day for 1 week	None	28 healthy subjects (19 females, 9 males) (42 ± 12 years)	↓ platelet activated GP IIb/IIIa expression; ↓ LDL cholesterol; ↑ HDL cholesterol. ↓ C-reactive protein (females only)	(89)

Cocoa beverage twice per day for 2 weeks	Macro- and micronutrient matched LF beverage	Double-blind, randomised, cross-over; 20 hypertensive subjects (12 females, 8 males) (51 ± SE 1.5 years)	↑ insulin-stimulated brachial artery diameter	(122)
20 g cocoa powder in 250 mL skimmed milk twice per day for 4 weeks	250 mL skimmed milk without cocoa powder	Randomised, cross-over; 42 high-risk of CVD subjects (23 females, 19 males) (69.7 ± 11.5 years)	↑ HDL cholesterol; ↓ expression of adhesion molecules on the surface of monocytes and concentration of circulating soluble adhesion molecules	(123; 124)
HF cocoa beverage followed by 10 min cycling	Macronutrient matched LF cocoa beverage followed by cycling	Double-blind, randomised, cross-over; 21 healthy overweight/obese subjects (8 females, 13 males) (54.9 ± SE 2.2 years)	↑ FMD; ↓ AUC for DBP and MAP in response to exercise	(125)
Cocoa beverage containing 33, 372, 712, or 1052 mg total flavanols per day for 6 weeks	None	Double-blind, randomised; 52 mildly hypertensive subjects (20 females, 32 males) (42-74 years)	↓ 24 h ambulatory MAP, SBP and DBP (1052 mg); ↓ Overnight ambulatory SBP, DBP and HR	(126)

6 g or 25 g DC per day for 3 months	None	Single-blind, randomised; 91 cardiovascular high-risk patients (20 females, 71 males) (57-74 years)	↓ 24 h, day- and night-time MAP, SBP and DBP (6 g); ↓ 24 h and daytime MAP and SBP (25 g)	(127)
HF cocoa beverage twice per day for 30 days	Nutrient matched LF cocoa beverage	Double-blind, randomised, cross-over; 16 coronary artery disease (CAD) patients (3 females, 13 males) (64 ± 3 years)	↑ FMD (both conditions), FMD post-intervention was higher than post-control; ↑ % of CACs; ↓ plasma [nitrite]; ↑ SBP	(128)
15 g DC 3 times per day for 8 weeks	Macronutrient matched cocoa solids-free chocolate	Double-blind, randomised, cross-over; 12 type-2 diabetic subjects (5 females, 7 males) (42-71 years)	↑ Serum [HDL cholesterol]; ↓ total cholesterol:HDL cholesterol ratio	(92)
Dairy based cocoa beverage containing either natural-dose (NTC) or high-dose (TEC) theobromine once per day for 3 weeks	Unspecified dairy based placebo beverage	Double-blind, randomised, cross-over; 42 pre-/stage 1 hypertensive, healthy subjects (10 females, 32 males) (62 ± 4.5 years)	↑ 24 h DBP (NTC); ↑ 24 h SBP, daytime DBP, 24 h, day- and night-time HR (TEC); ↓ central SBP, HR and stroke volume (TEC)	(129)

Dairy-based high-fat liquid meal plus HF cocoa powder	Macronutrient matched dairy-based high-fat liquid meal containing LF cocoa powder	Double-blind, randomised, cross-over; 18 healthy subjects (16 females, 2 males) (25.2 ± 2.5 years)	↑ serum [triglycerides] and [free fatty acids] (both conditions); ↓ FMD (both conditions, higher following intervention)	(130)
40 g DC twice per day for 2 weeks followed by 1.5 h cycling	30.4 g bar of sugar- and fat-matched cocoa liquor-free chocolate followed by cycling	Single-blind, randomised; 20 healthy males (22 ± 4 years)	↑ Plasma [F ₂ -isoprostane] post-exercise (both conditions, lower following intervention); ↓ oxidised LDL pre- and post-exercise	(131)
40 g DC	40 g milk chocolate	Single-blind, randomised, cross-over; 20 healthy subjects (13 females, 7 males) (33 ± 11 years) and 20 smokers (13 females, 7 males) (33 ± 11 years)	↓ Serum sNOx ₂ -dp, urinary isoprostane excretion and platelet ROS, sNOx ₂ -dp and 8-iso-PGF ₂ α (healthy subjects); ↑ FMD, serum [NOx] and platelet NOx production (smokers); ↓ serum sNOx ₂ -dp, urinary isoprostane excretion and platelet ROS, sNOx ₂ -dp and 8-iso-PGF ₂ α (smokers)	(132) (133)
Cocoa beverage containing either 2, 5, 13 or 26 g of cocoa	Macronutrient matched beverage without cocoa powder	Double-blind, randomised; 23 healthy subjects (14 females, 9 males) (63 ± SE 2 years)	Dose-dependent greater change in FMD (5, 13 and 26 g); ↑ SBP (2 and 26 g); ↑ DBP (2, 13 and 26 g); ↑ MAP (2, 13 and 26 g); ↓ [glucose] (0, 2 and 5 g)	(134)

Sugar-free cocoa beverage or a sugar-sweetened cocoa beverage twice per day for 6 weeks	Cocoa-free sugar-sweetened beverage	Double-blind, randomised, cross-over; 39 overweight, healthy subjects (33 females, 6 males) (41-63 years)	↑ FMD (both conditions)	(135)
75 g DC	None	16 healthy subjects (6 females, 10 males) (20-45 years)	↓ Angiotensin-converting enzyme activity	(136)
13.5 g flavonoid-enriched chocolate twice per day for 1 year	Macronutrient matched placebo chocolate	Double-blind, randomised; 93 postmenopausal, type-2 diabetic patients (51-74 years)	↓ Plasma [insulin] and insulin resistance; ↑ insulin sensitivity; ↓ [LDL cholesterol]; ↑ CHD risk (both conditions, less following intervention)	(137)
100 g DC followed by 2.5 h of cycling	Macronutrient matched cocoa solids-free bar (71 g) followed by cycling	Single-blind, randomised, cross-over; 14 healthy male subjects (22 ± 1 years)	↑ Plasma total antioxidant status (both conditions); ↑ [insulin] pre-exercise and 1 h post-exercise; ↓ plasma [glucose] post-exercise (both conditions)	(138)
100 g high-antioxidant dark chocolate (HADC) or 100 g DC	None	Double-blind, randomised, cross-over; 15 healthy subjects (9 females, 6 males) (30 ± 5 years)	↑ Plasma FRAP (both interventions); HADC FRAP remained higher at 4 and 5 h; ↑ urinary HADC FRAP up to 12 h; ↑ [triacylglycerol] (both interventions); ↑ [thiol] at 2- and 4 h (both interventions)	(139)

50 g DC per day for 3 weeks	None	50 healthy subjects (25 females, 25 males) (28-45 years)	↑ [HDL cholesterol] and [triglyceride] and ↓ LDL cholesterol (females only); ↓ lipid peroxidation, conjugated diene and hydroperoxide content of HDL and LDL; ↑ platelet production of NO; ↓ peroxynitrite	(68)
(B) cocoa+hazelnuts, (C) cocoa+hazelnuts+phytosterols, (D) cocoa+hazelnuts+phytosterols+soluble fibre daily, 4 weeks	(A) Cocoa	Double-blind, randomised; 113 pre/stage-1 hypertensive and hypercholesterolemic subjects (67 females, 46 males) (43-65 years)	↓ [Total-] and [LDL cholesterol] and [Apo B] and Apo B:Apo A ratio (C) and (D); ↓ hsCRP and oxidised LDL (D)	(63)
DC and cocoa beverage once per day for 3 months	None	5 type-2 diabetic patients with stage II and III heart failure (47-71 years)	↑ [HDL cholesterol]; enhanced expression of mitochondrial structure markers in skeletal muscle	(140)

Table 1. Summary of intervention studies on cocoa between 2007 and 2012.

Only outcomes with a statistical significance of a maximum $p < 0.05$ are reported. Mean \pm SD unless otherwise stated.

Abbreviations used in the table: \uparrow , increase; \downarrow decrease; DC, dark chocolate; HR, heart rate; pp, Polyphenol; CFS, chronic fatigue syndrome; HF, high flavanol; LF, low flavanol; MF, medium flavanol; BW, body weight; TAG, triacylglycerol; IL-6, interleukin 6; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Apo B, apolipoprotein B; 8-iso-PGF 2α , 8-iso-prostaglandin F 2α ; SBP, systolic blood pressure; DBP, diastolic blood pressure; FMD, flow mediated dilation; MAP, mean arterial pressure; GP, glycoprotein; CVD, cardiovascular disease; AUC, area under curve; CACs, circulating angiogenic cells; sNO x_2 -dp, soluble NO x_2 derived peptide; ROS, reactive oxygen species; FRAP, ferric reducing ability of plasma; NO, nitric oxide.

Figure legends

Figure 1: Chemical structures of cocoa flavanols

Figure 2: Metabolic pathways of theobromine in humans

Figure 3: General mechanism by which NO affect vasodilation

Figure 4: Possible mechanisms by which epicatechin affects nitric oxide levels.

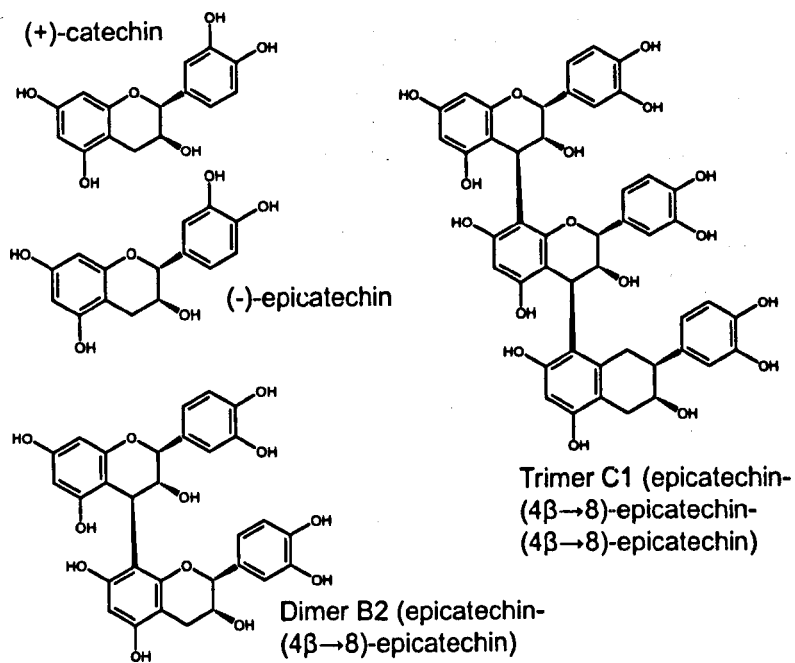


Figure 1

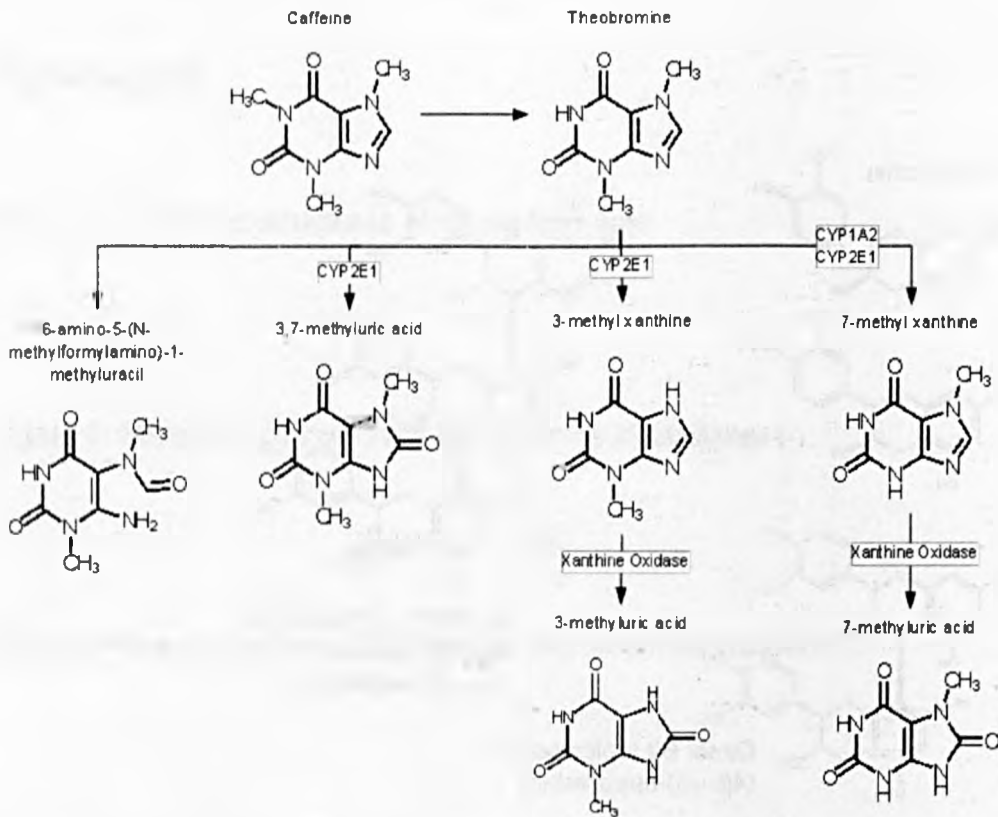


Figure 2.

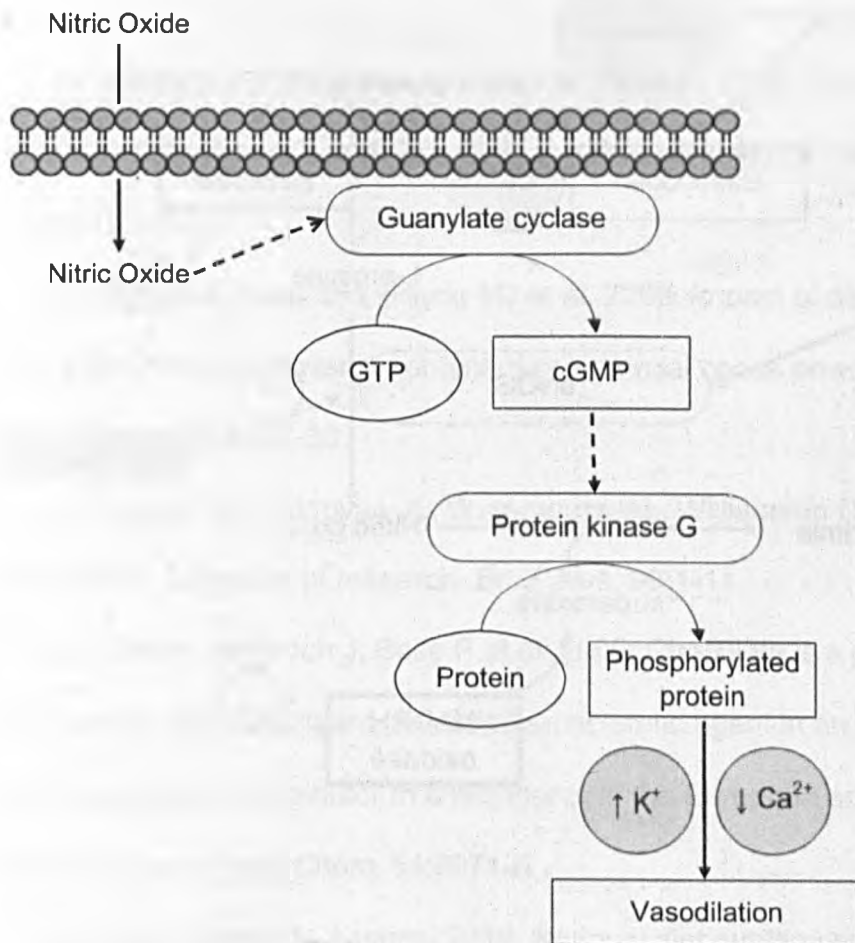


Figure 3.

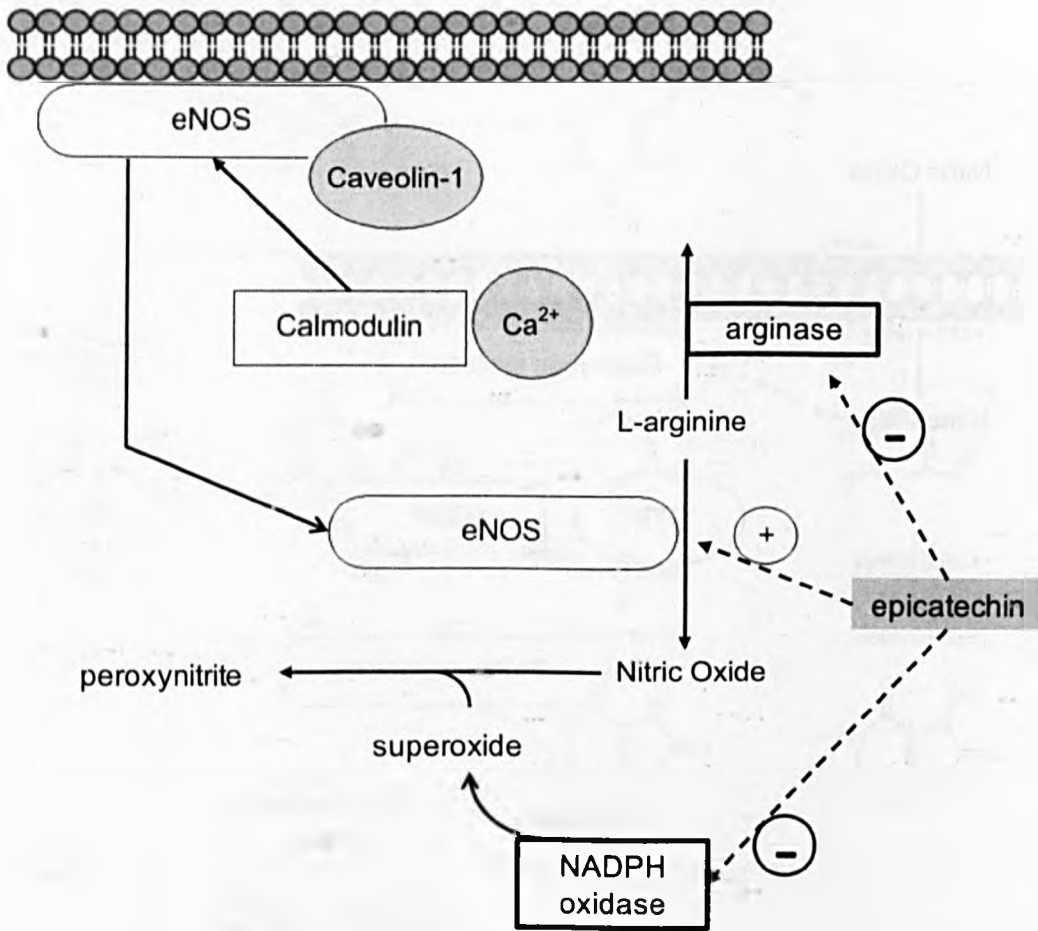


Figure 4.

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Title Page

1 **Polyphenol-rich herbal supplement attenuates glucose uptake and transport across**
2 **Caco-2 cell monolayers by interaction with SGLT1 and GLUT2 transporters**

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11 Key words: diabetes, glucose transporters, caco-2 cells, polyphenol, herbal.

12 Abbreviations:

13 EMEM: Eagle's minimum essential medium; FBS: Fetal bovine serum; GLUT2:
14 sodium-independent glucose transporter 2; LPH: lactase phlorizin hydrolase; MRM:
15 multiple reaction monitoring; MS: mass spectrometry; SGLT1: sodium-dependent glucose
16 transporter 1; TEER: transepithelial electrical resistance;

17

Abstract

18 We have investigated the inhibition of glucose transporters SGLT1 and GLUT 2 by
19 polyphenol-rich herbal supplement using the well-characterized Caco-2 intestinal model.
20 Glucose absorption under sodium dependent and sodium-free conditions was decreased by
21 54% and 35% respectively in the presence of the water soluble components from the herbal
22 supplement. Using sodium-dependent and sodium-free mechanistic studies, we demonstrate
23 that the inhibition of GLUT2 was greater than SGLT1. Glycosidase and esterase enzymatic
24 hydrolysis was used to assess the impact of human metabolism on the efficacy of activity.
25 Glucose transport across the membrane was reduced by 70% compared to the control and
26 the enhance inhibition was associated with significant increases in flavonoid aglycones,
27 caffeic acid and *p*-coumaric acid. These results suggest that intact and metabolized
28 polyphenols, likely to be found in the lumen after ingestion of the supplement, play an
29 important role in the attenuation of glucose absorption and may have potentially beneficial
30 anti-glycemic effects in the body.

31

Introduction

32 Over the past two decades the prevalence of diabetes has risen dramatically, and is
33 estimated to effect 347 million people globally [1]. Type 2 diabetes is the most common
34 form, which can develop following prolonged periods of elevated blood glucose
35 (hyperglycemia); and is a recognized risk factor for metabolic syndrome [2]. Lifestyle
36 modifications including increased fruit and vegetable consumption, weight loss and lowered
37 blood glucose may prevent or delay disease development [3,4].

38 Promising evidence is emerging concerning the use of certain herbs, botanicals and trace
39 elements for the control of blood glucose [5,6]. Currently there is insufficient evidence to
40 draw conclusions on the efficacy of herbal remedies, but beneficial effects on several risk
41 factors have been observed for chromium and *Gymnema sylvestre* (gymnema) [7],
42 cinnamon [8], green coffee [9] and grape seed [10] in controlled-trials. The mechanism of
43 action is currently unclear, but there is growing evidence that secondary plant metabolites,
44 known as polyphenols, can modulate carbohydrate metabolism [11]. The *in vitro* anti-
45 diabetic properties of a number of extracts have been attributed to polyphenols [12-15],
46 mainly cinnamates, glycosides, procyanidins and flavonol derivatives. Modulation of such
47 metabolic markers by natural botanicals may be beneficial to the treatment or prevention of
48 type 2 diabetes.

49 Previous cell culture studies using plant materials have demonstrated that some
50 polyphenols can attenuate intestinal glucose absorption and potentially "blunt" post-prandial
51 glucose spikes by inhibition of active uptake via sodium-dependent glucose transporter 1
52 (SGLT1) and facilitated transport by sodium-independent transporter 2 (GLUT2) [16,17].
53 However, very few have addressed the issue of identification and efficacy of the *in vivo* gut
54 lumen metabolites despite established knowledge that following ingestion, polyphenols
55 undergo metabolism by the intestinal β -glycosidase, LPH [18]. Growing evidence suggests
56 that the major forms present in the body are de-glycosylated and metabolized derivatives of

57 the parent aglycones [19,20], which have altered biological activities [21]. Attenuation of
58 glucose transport across the small intestine by polyphenols and their metabolites may be a
59 potential mechanism of glycemic control. Such activity may, in part, explain previous
60 observations of lowered blood glucose concentrations and delayed glucose absorption in
61 human studies [22,23] following consumption of polyphenol-rich foods.

62 The purpose of this study was to investigate the inhibitory potency of a botanical
63 supplement containing a broad spectrum of herbs and spices on glucose uptake by SGLT1
64 and GLUT2 transporters using a Caco-2 cell model of the small intestine. Consumption of
65 this botanical supplement can affect glucose absorption and may be useful for the
66 prevention or management of type 2 diabetes.

Materials and Methods

67 **Chemicals**

68 All chemicals were purchased from Sigma-Aldrich (Dorset, UK) unless stated otherwise.
69 *D*-glucose and HEPES (N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid) were
70 purchased from Fisher Scientific (Loughborough, UK). Magnesium sulfate was purchased
71 from VWR International Ltd (Leicestershire, UK). Transwell polycarbonate inserts (6 well,
72 0.4 µm pore size), tissue culture flasks and filter system (500 ml, 0.2 µm nylon membrane)
73 were manufactured by Corning® and supplied by Sigma-Aldrich (Dorset, UK). *D*-[U-¹⁴C]-
74 glucose was purchased from Perkin Elmer (Cambridge, UK) and Exoscint XR scintillation
75 cocktail from National Diagnostic (Yorkshire, UK). Eagle's Minimum Essential Medium
76 (EMEM, 1000 mg/ L glucose) was purchased from American Type Cell Culture (LGC
77 Promochem, Middlesex, UK). Herbal powders were supplied by NewChapter.com
78 (Vermont, USA).

79 **Preparation of cell culture test solutions**

80 The herbal supplement used in this study was composed of several herbs, spices and
81 botanicals and was prepared fresh on the day of use. Test solutions used in the sodium
82 dependent/ independent cell culture experiments were prepared as described. Herbal
83 powders equivalent to 1020 mg of extract were solubilized in DMSO and centrifuged at 17,
84 000 ×g for 5 min. Supernatants were combined and an aliquot (81 µl) of the mixture was
85 diluted to 20 ml using transport buffer (pH 7.4) of the appropriate sodium content modified
86 1 mM glucose and combined with *D*-[U-¹⁴C] glucose (0.05 µCi/ ml).

87 For the preparation of the polyphenolic extracts with and without enzymatic hydrolysis,
88 an aliquot (81 µl) of the solubilized herbal supplement was combined with ascorbic acid
89 (1mg/ ml) as a stabilizing agent and then diluted to 20 ml with warm (40 °C) buffer solution
90 (CH₃COOH, 0.2 M: CH₃COONa, 0.2 M, 90:10, v/v, pH 3.8) either with or without

91 hesperidinase from *Aspergillus niger* (40 U/ L). All samples were incubated at 40 °C for 5 h
92 in a shaking water bath (Grant Aqua 12 Plus, Grant Instruments Ltd, Cambridgeshire, UK).
93 Afterwards, a liquid-liquid extraction procedure was performed by adding 20 ml of warm
94 ethyl acetate. Phases were mixed by vortex (10 min), followed by centrifugation at 4000 ×g
95 (5 min) to allow separation and collection of upper ethyl acetate phase. These steps were
96 repeated twice more using 10 ml of ethyl acetate. The combined organic phases were dried
97 under nitrogen flow at 40 °C. The dried extracts were reconstituted with 50 µl DMSO,
98 sonicated (5 min) and vortexed to mix. Reconstituted solutions were then diluted to 20 ml
99 using transport buffer A (see below) modified with 1 mM glucose and *D*-[U-¹⁴C] glucose at
100 0.05 µCi/ ml.

101 In order to calculate the recovery efficacy of the extraction procedure, the above steps
102 were repeated in triplicate using samples that had been spiked with an internal standard,
103 dihydrocaffeic acid (0.01mg/ ml), immediately before enzyme hydrolysis. Samples were
104 reconstituted in transport buffer A without *D*-[U-¹⁴C] glucose.

105 Cell culture

106 The human colon adenocarcinoma cell line, Caco-2 (HTB-37) was obtained from the
107 American Type Culture Collection at passage 25 (LGC Promochem, Middlesex, UK).
108 Permeability studies utilized Caco-2 cells between passages 40 and 46. Caco-2 cells were
109 added to Transwell inserts (24 mm diameter, 4.67 cm² growth area) at a density 6 × 10⁴
110 cells/cm² and cultured for 21 d at 37°C under a humidified atmosphere of 95% air: 5% CO₂.
111 The culture medium, EMEM supplemented with 10% FBS, 100U/ml penicillin-
112 streptomycin and 0.25 µg/ml amphotericin B was replaced every other day.

113 Glucose transport measurements

114 On or after 22 d, permeability studies were initiated by careful aspiration of the culture
115 medium from apical and basal compartments and 2 ml of transport buffer A (HEPES, 20

116 mM; NaCl, 137 mM; KCl, 4.7 mM; CaCl₂, 1.8 mM; and MgSO₄, 1.2 mM; adjusted to pH
117 7.4 using NaOH, 10 mM) was added to each compartment to carefully wash cells. After
118 washing, the solutions were removed and fresh transport buffer (A) was added into each
119 compartment. Plates were incubated at 37 °C in a humidified 5% CO₂ atmosphere for 30
120 min to allow equilibration of tight junction integrity. Trans-epithelial electrical resistance
121 (TEER) measurements were recorded using a Millicell ERS volt-ohm meter fitted with a
122 chopstick probe (Millipore Ltd, Watford, UK). Wells with a resistance above 250 Ω, after
123 correction for membrane resistance, were used. Afterwards, the liquid was aspirated and
124 replaced with 2 ml of test solution (1 mM glucose and *D*-[U-¹⁴C] glucose at 0.05 μCi/ ml
125 dissolved in transport buffer A with or without the herbal supplement, 0.4% DMSO) at pH
126 7.4; all basal solutions were in transport buffer A (pH 7.4). Plates were incubated at 37 °C in
127 a humidified 5% CO₂ atmosphere for 25 min, the TEER measurements were repeated and
128 the solutions removed. Statistical analysis was performed on the final TEER for all test
129 conditions, and addition of herbal supplement had no effect on monolayer resistance
130 compared to respective controls (*p* > 0.22). Apical and basal compartments were washed
131 twice with 1 ml of transport buffer to remove any residual *D*-[U-¹⁴C] glucose from the cell
132 monolayer or compartment walls and the aliquots were collected. After this, 1 ml of NaOH
133 (1 mM) was added to the apical compartments and shaken for 30 min to lyse the cells. The
134 detached cells were then neutralized with 1 ml of HCl (1 mM), mixed and the aliquots were
135 collected. Radiochemical detection of *D*-[U-¹⁴C] glucose was performed by combining 5 ml
136 of scintillation cocktail with 0.25 ml of the apical solutions or 0.5 ml of the basal solutions,
137 the apical and basal wash solutions and lysed cell solutions. All samples were analyzed
138 using a Parkard Liquid Scintillation Analyzer 1600TR. A cold sample of transport buffer
139 solution was used to determine background noise and all samples were corrected for the
140 count efficiency of the analyzer.

141 To investigate the transport of glucose under sodium-free conditions, the experiments
142 above were repeated using transport buffer B; where the formulation of transport buffer A

143 was modified so that sodium chloride was replaced by potassium chloride (137 mM) and
144 adjusted to pH 7.4 using KCl (10 mM).

145 To assess the inhibitory potential of the herbal supplement in the native and digested
146 forms, the transport experiments described above were repeated using polyphenolic extracts
147 prepared with and without enzymatic hydrolysis.

148 HPLC-DAD and MS analysis

149 Analysis of the aqueous polyphenol extracts, prepared with and without enzyme
150 hydrolysis, was performed using the following HPLC-DAD-MS method. An aliquot (1 ml)
151 of each extract was removed to a 2 ml tube and centrifuged at 17,000 ×g for 5 min to
152 remove particulate matter. A small volume (100 µl) was removed to an amber vial, spiked
153 with internal standard and stored in the HPLC autosampler for analysis. A volume (5 µl)
154 was injected on to a Rapid Resolution HPLC (1200 series Agilent Technologies, Berkshire,
155 UK) with diode array (DAD). The methodology used was based on our previous work [24].
156 Chromatographic separation was achieved on an Eclipse plus C18 column (30 °C, 2.1 mm x
157 100 mm, 1.8 µm; Agilent Technologies) using a 75 min gradient of (A) premixed 5%
158 acetonitrile in water (5:95, v/v) and (B) premixed 5% water in acetonitrile (5:95, v/v) both
159 modified with 0.1% formic acid with a flow rate of 0.26 ml/min. Elution was initiated at 0%
160 of solvent B and maintained for 17 min; the percentage of solvent B was then increased to
161 35% over the next 43 min and immediately increased to 100% for 5 min before initial
162 starting conditions were resumed for a 10 min column re-equilibration. After separation the
163 analytes were quantified by DAD based on calibration curves for available standards
164 including caffeic acid, 5-*O*-caffeoylquinic acid, cinnamaldehyde, *p*-coumaric acid, coumarin
165 and dihydrocaffeic acid. Dicafeoylquinic acid and feruloylquinic acid were quantified
166 based on 5-caffeoylquinic acid, data are expressed as mean ±SD (n =3). Calibration curves
167 showed good linearity over the tested range; Pearson's coefficients were significant at the
168 1% level ($R^2 > 0.99$). Caffeic acid was used to determine DAD analytical performance

169 based on triplicate injections on the same day: the limit of quantification was 25 nM,
170 accuracy and precision were calculated at < 2% R.E. and R.S.D respectively.

171 Identification of analytes present in the extracts was confirmed by MS. In brief, after
172 separation the eluate was passed, without splitting, into an Agilent 6410 triple quadrupole
173 MS fitted with an electrospray ionization interface and the operation conditions are
174 described elsewhere [24]. Samples were analyzed in full scan (100-1000 *m/z*) mode under
175 both negative and positive ionization conditions to identify the relevant molecular ions.
176 Then the identity of the compounds was then confirmed using multiple reaction monitoring
177 (MRM) mode by comparison of molecular ion and associated fragmentation pattern to
178 available standards. Quercetin and kaempferol could not be determined by DAD due to co-
179 elution interference. Thus these compounds were quantified in MRM mode based on
180 quercetin and kaempferol reference standards using the response of the major product ion
181 achieved by fragmentation of the 301 and 285 negative molecular ions respectively.

182 **Statistical analysis**

183 Analysis of variance was used to confirm statistical difference in samples under different
184 experimental conditions and is a test of whether the means of two or more groups are equal.
185 Shilpro-Wilk and Levene's test were performed to confirm the normality of the data and the
186 equality of variances respectively. The mean difference was statistically significant at the
187 5% level (PASW statistics. 17)

188

Results

189 **Inhibition of glucose transport under sodium dependent/ sodium-free conditions**

190 The final concentration of the herbal supplement used in cell transport studies reflected
191 the estimated physiological concentration in the gut lumen following ingestion. In the
192 absence of the aqueous herbal supplement under sodium-dependent conditions, *D*-[U-¹⁴C]
193 glucose was taken up by the cells and transported into the basal chamber at a rate of 4.4
194 ±0.1 nmol/cm² • min. It is expected that the uptake of glucose in the presence of sodium is
195 mediated by both SGLT1 and GLUT2 located at the apical surface and exported to the
196 serosal side via GLUT2 in the basal membrane. Addition of the herbal supplement
197 significantly (*p*< 0.001) decreased glucose transport to 45.6 ±3.8% of the control value (Fig.
198 1A), ~55% inhibition. In the absence of aqueous herbal supplement under sodium free
199 conditions, the rate of *D*-[U-¹⁴C] glucose transport was reduced to 3.3 ±0.1 nmol/cm² • min
200 compared to sodium dependent conditions. In the absence of sodium, it is considered that
201 glucose transport is mediated solely by the GLUT2 transporters located at the apical and
202 basal membranes. Incubation of the Caco-2 cells with the herbal supplement under these
203 conditions lead to a significant (*p*< 0.001) decrease in glucose transport to the basal chamber
204 to 65 ±2.6% of the control value (Fig. 1A), ~35% inhibition.

205 Under sodium dependent conditions, the supplement had no effect on the accumulation
206 of *D*-[U-¹⁴C] glucose by Caco-2 cells compared to control (Fig. 1B). In contrast, the
207 inhibition of glucose transport by the extract of the herbal supplement under sodium-free
208 conditions was associated with a significantly (*p*< 0.001) lowered accumulation of glucose
209 in the Caco-2 cells compared to control conditions (Fig. 1B).

210 **Effect of de-conjugation on potency of glucose-transporter inhibition**

211 Cell transport studies were performed under sodium dependent conditions using
212 polyphenol extracts prepared from the aqueous herbal supplement following a liquid-liquid

213 extraction with or without an enzymatic de-conjugation step. Extraction efficiencies for
214 dihydrocaffeic acid show that $92 \pm 0.3\%$ and $93 \pm 1.4\%$ of the spiked amount was recovered
215 after extract with and without enzymatic de-conjugation respectively. The non-hydrolyzed
216 polyphenol extract efficiently reduced glucose transport to $50 \pm 1.5\%$ of the control value,
217 which was comparable with the un-extracted herbal supplement. Interestingly, when the
218 hydrolyzed polyphenol extract was used, there was a larger and significant ($p < 0.001$)
219 decrease in the rate of glucose uptake to $29.3 \pm 2.1\%$ of the control value (Fig. 2).

220 **Polyphenol composition of herbal supplement**

221 The herbal supplement used in the current study contained a combination of herbs, spices
222 and botanicals (Fig. 3). A liquid-liquid extraction was performed in the presence or absence
223 of glycosidase and esterase enzyme mixture and resulting polyphenol extracts were
224 analyzed by HPLC-DAD-MS. A typical chromatogram of the polyphenol profile is shown
225 in Fig. 4A, for details of peak numbers refer to Table 1. The major components of the non-
226 hydrolyzed polyphenol extract appear to be chlorogenic acids and cinnamaldehyde.
227 Following enzymatic de-conjugation, the polyphenol profile is distinctly altered due to
228 liberation of free-aglycone forms (Fig. 4B). A statistically significant increase in response
229 was observed for several of the compounds including caffeic acid, *p*-coumaric acid,
230 quercetin and kaempferol. The compounds were identified by comparison of the retention
231 time, UV spectra and the molecular ion produced by electrospray ionization relative to
232 available standards.

Discussion

233 There is growing evidence for the *in vitro* interaction of polyphenols with transporters
234 [16,17,25] and enzymes [15,26,27] of importance to glucose absorption and metabolism.
235 These observations indicate that these compounds may have beneficial influence on
236 glycemic control, which is a known risk factor for the development of type 2 diabetes. The
237 objective of this current study was to assess the capacity of a new herbal supplement to
238 reduce glucose transport across the small intestine, and demonstrate the mechanism of
239 action.

240 The data indicate that the combination of polyphenols from the herbs, spices and seeds in
241 the aqueous herbal supplement were able to decrease the transport of glucose by up to 54%
242 at concentrations predicted after ingestion of a normal dietary serving. Investigations with
243 and without sodium suggest that disruption of GLUT2 mediated uptake is the major apical
244 target for attenuation of glucose transport by Caco-2 cells. The predominant polyphenols
245 identified following HPLC-MS analysis were cinnamaldehyde and dicaffeoylquinic acids
246 derived from cinnamon and coffee beans respectively.

247 Evidence from clinical trials in humans have indicated that a daily high dose (> 3 g) of
248 cinnamon significantly lowered fasting blood glucose concentrations in type 2 diabetic
249 volunteers [28,29]. In other studies using a daily dose of 1- 1.5 g, these effects were not
250 observed [30-32]. The mechanism of action is unclear, but *in vitro* and animal studies, using
251 levels of cinnamon achievable in the diet, suggest these effects may be attributed to
252 enhanced glucose storage by adipocyte cells [33,34], increased glucose conversion to
253 glycogen in the liver [35] and reduced carbohydrate digestion in the small intestine via
254 inhibition of α -glucosidase [15]. Similarly, dicaffeoylquinic acids have been highlighted as
255 the principal compounds responsible for the inhibition of glucose transporters by herbal
256 beverages in the small intestine [16], leading to reduced glucose absorption. These

257 observations suggest that polyphenols found in cinnamon and coffee may improve insulin
258 sensitivity and reduce post-prandial glucose levels.

259 Enzymatic hydrolysis of the aqueous herbal supplement lead to significant increases in
260 the amounts of caffeic acid and *p*-coumaric acid. These compounds are likely to be released
261 from the caffeoylquinic acids present in the green coffee, resulting from esterase activity
262 within the hesperidinase used in this study. Analysis of the hydrolysed supplement by
263 HPLC-MS also revealed small, yet significant, increases in the amounts of quercetin and
264 kaempferol, which were present in small amounts in the unhydrolysed supplement. Overall,
265 glucose uptake across the epithelial cells was reduced by 70% compared to the control.
266 These results suggest that the anti-diabetic activity may be enhanced following metabolism
267 in the intestinal lumen and indicates that the free-aglycones have greater potency to inhibit
268 or delay glucose uptake than their glycosylated forms. However, since the herbal
269 supplement used in our current study was unpurified, it is not possible to assign these effects to
270 a specific component(s).

271 Currently, literature evidence for the influence of polyphenols on glucose absorption
272 across Caco-2 cells is limited. Our data are in agreement with those investigating the
273 inhibition of glucose transporters SGLT1 and GLUT2, by individual polyphenols derived
274 from fruit extracts [17]. The authors reported inhibitory activity of *p*-coumaric acid at
275 concentrations in the range of 10 to 500 μ M, which is comparable to the levels detected in
276 our current study. In a rodent model of type 2 diabetes, a moderate intake of caffeic acid
277 (approximately 40 mg/kg body weight) induced a reduction in blood glucose levels and
278 lowered glucose-6-phosphatase activity [36]. Interestingly glucose uptake was significantly
279 reduced by 10-100 μ M quercetin but not rutin, the glycosidic form, in several transport
280 models mainly via interaction with GLUT2 [25,37]. In contrast to our results, some studies
281 using individual hydroxycinnamic acids including caffeic acid, 5-caffeoylquinic acid and *p*-
282 coumaric acid, at a concentration of 100 μ M, reported no effect on glucose uptake under

283 either sodium-dependent or sodium-free conditions [25]. A likely explanation for these
284 differences may be the use of a mixture of polyphenols used in the current study, leading to
285 enhancement of the inhibitory activity. Co-incubation of polyphenols and their
286 physiological effects have received little attention *in vitro*, but human trials with
287 polyphenol-rich beverages offer promising benefits for the reduction of blood glucose in
288 healthy volunteers [22,38].

289 Our study demonstrates that water soluble components from a herbal supplement
290 composed of a variety of herbs, spices and seeds, efficiently reduced glucose transport
291 across Caco-2 intestinal cells mainly through interaction with the GLUT2 transporter
292 family. Since the aglycone metabolites released following enzymatic hydrolysis have
293 sustained biological activity, we propose that regular dietary consumption may improve
294 glucose control either by limiting or delaying glucose absorption in the intestine. These
295 encouraging findings require further evaluation in human controlled-trials to establish the
296 beneficial effects in the body.

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300

Conflict of Interests

301 The authors have declared no conflict of interests.

302

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Legends for Figures

Fig. 1. A: Effect of herbal supplement on glucose transport by Caco-2 cells under sodium dependent and sodium-free conditions. B: Effect of herbal supplement on the intracellular accumulation of glucose by Caco-2 cells under sodium dependent and sodium-free conditions. Values expressed as a percentage of the control value which was the glucose transport under each condition in the absence of herbal supplement. Each data point is the mean \pm SD (n = 3). **: p < 0.001.

Fig. 2. Effect of polyphenol extract prepared with or without enzymatic hydrolysis on glucose transport by Caco-2 cells under sodium dependent conditions. Values expressed as a percentage of the control value which was the glucose transport under sodium dependent conditions in the absence of polyphenol extract. Each data point is the mean \pm SD (n = 3). **: p < 0.001.

Figure 3. Percentage composition of the herbal supplement.

Fig. 4. HPLC-DAD trace (280 nm) showing the phenolic profile of the extracts prepared without (A) and with enzymatic hydrolysis (B). For peak numbers refer to Table 1. *: p < 0.01; **: p < 0.001.

Figure 1

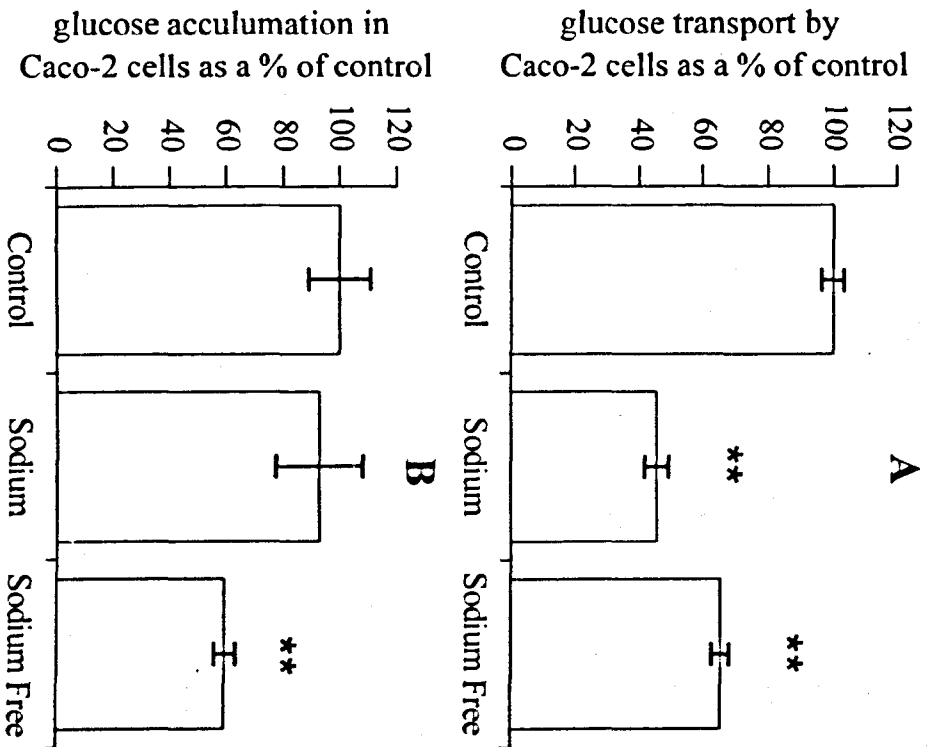


Figure 1

Figure 2

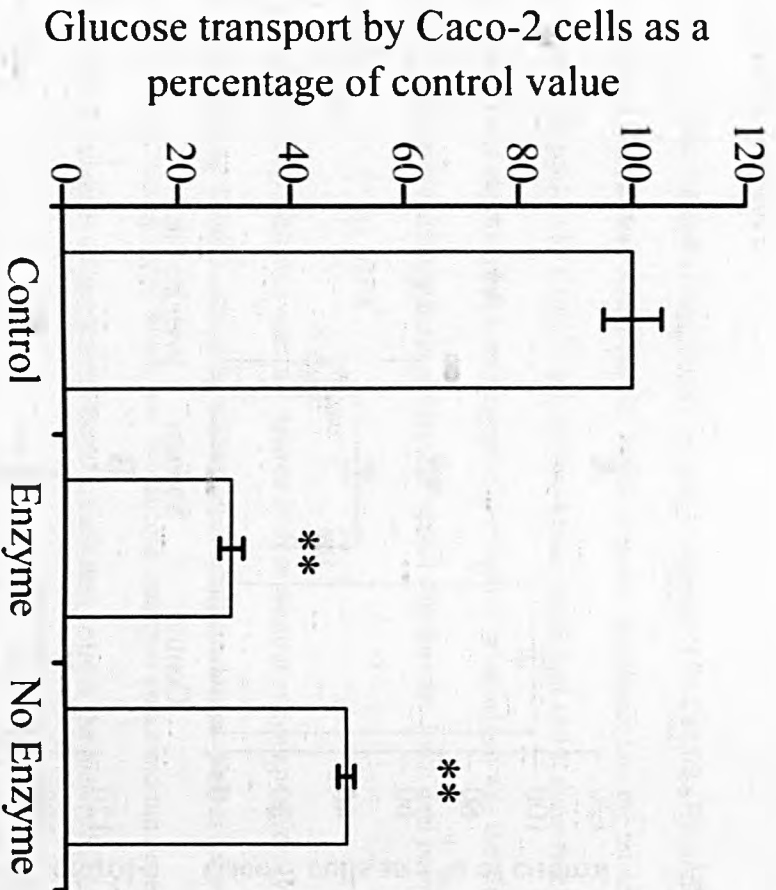


Figure 2

Figure 3

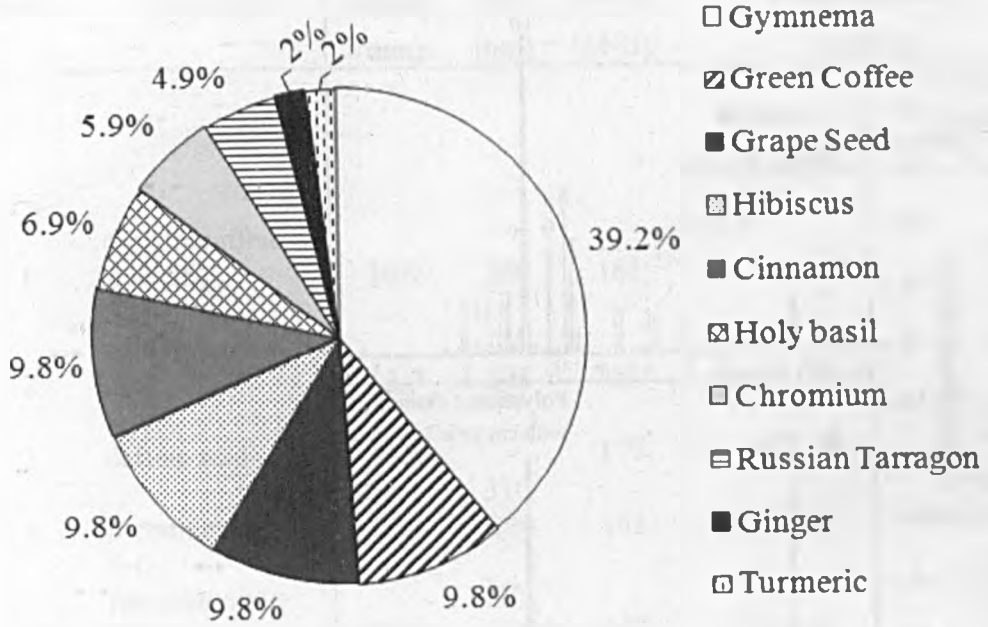


Figure 4

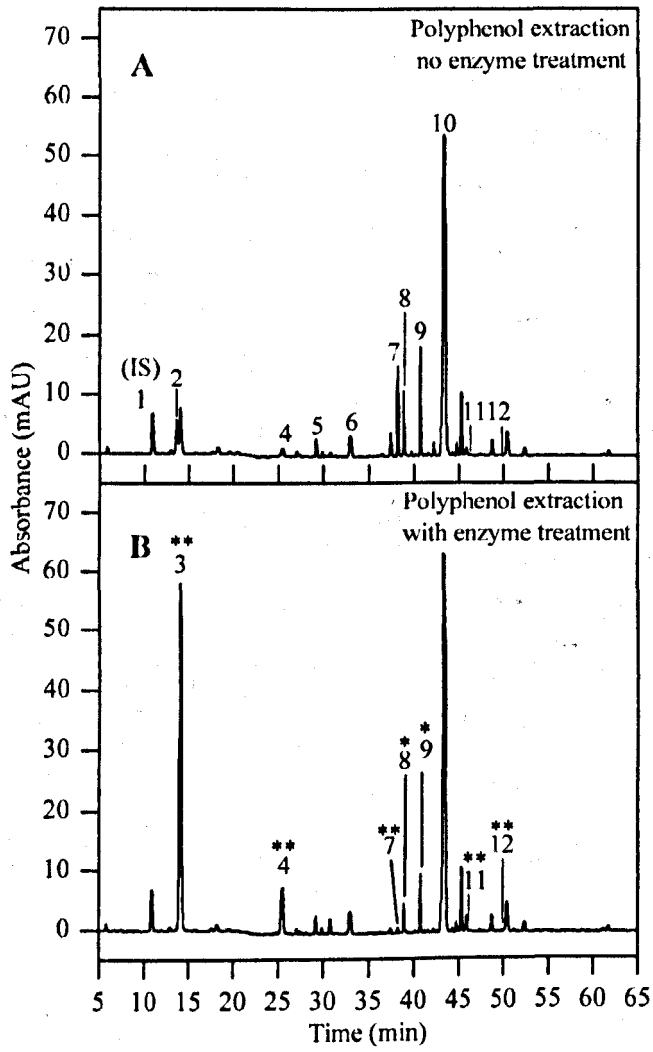


Figure 4

Table 1. Characterization of the polyphenols in the herbal supplement without and after enzyme hydrolysis by HPLC-DAD-MS

Peak number	Compound	Retention time (min)	λ max (nm)	Molecular ion [M-H] ⁻	Concentration (μ M)	
					Without Hydrolysis	With Hydrolysis
1	dihydrocaffeic acid (IS)	10.9	280	181	ND	
2	5- <i>O</i> -caffeoylquinic acid	13.7	325, 295	353	46 \pm 0.03	
3	caffeic acid	14.5	-	179	ND	77.1 \pm 0.2**
4	<i>p</i> -coumaric acid	25.6	310, 290	163	1.4 \pm 0.03	12.8 \pm 0.01**
5	5- <i>O</i> -feruloylquinic acid	29.4	325, 295	367	7.7 \pm 0.06	
6	coumarin	33.2	280, 310	[147] ⁺ *	8.0 \pm 0.09	
7	3,4-di- <i>O</i> -caffeoylquinic acid	38.4	325, 295	515	11.4 \pm 0.03	0.7 \pm 0.06**
8	3,5-di- <i>O</i> -caffeoylquinic acid	39.1	325, 295	515	8.3 \pm 0.09	4.0 \pm 0.03*
9	4,5-di- <i>O</i> -caffeoylquinic acid	40.9	325, 295	515	13.5 \pm 0.07	7.7 \pm 0.07*
10	cinnamaldehyde	43.5	290	[133] ⁺ **	98.2 \pm 0.05	
11	quercetin	46.0	370	301	0.1 \pm 0.004	0.8 \pm 0.03**
12	kaempferol		365	285	0.1 \pm 0.005	0.7 \pm 0.03**

A statistically significant change in concentration was observed *: $p > 0.01$; **: $p > 0.001$. [⁺]: positive molecular ion.