The bioavailability and biological activity of sulphur-containing compounds from broccoli

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To my parents,

I dedicate this thesis to you, without whom I wouldn’t have achieved this.

You have guided me through life, encouraged and supported me at each stage of my academic career. You have been the most influential people in my life and this thesis represents the reward for all our hard work.

Abstract

The bioavailability of the broccoli-derived sulphur-containing compounds, glucoraphanin (GR), S-methyl-L-cysteine sulfoxide (SMCSO) and sulforaphane (SF), were quantified through a dietary intervention study in humans with the use of soups made with three broccoli genotypes that differed in their Myb28 alleles. One genotype was homozygous for the normal broccoli $Myb28^{B}$ allele while the other two were either heterozygous or homozygous for a $Myb28^{V}$ allele introgressed from the wild species *Brassica villosa*.

Phytochemical analysis of the soups confirmed that the presence of one or more $Myb28^{V}$ alleles led to higher levels of GR. A randomised, double-blinded, three-phase, crossover study was employed to quantify the pharmacokinetics of GR, SF and SMCSO following consumption of the soups. A significantly higher plasma concentration and urinary excretion of GR, SMCSO and SF and metabolites occurred following consumption of broccoli soup derived from the $Myb28^{B/V}$ and $Myb28^{V/V}$ genotypes compared to $Myb28^{B/B}$ genotype. There was considerable inter-individual variation in the excretion of these metabolites following consumption of the soups, with certain volunteers having consistently higher plasma levels of SF and higher levels of SF excretion for each of the different phases of the study. However, there was no correlation between plasma and urine levels of SF with SMCSO or GR.

The use of *in vitro* models indicated that intact GR and SMCSO may either require a transporter to diffuse across the enterocytes or diffuse paracellularly to reach the systemic circulation. As an initial investigation to explore the biological activity of SMCSO, experiments were undertaken to investigate whether SMCSO could induce nrf2-regulated genes within human and mouse liver cells, with the use of SF as a positive control. No gene induction was observed with SMCSO and it was concluded that the biological activity of SMCSO was likely to reside in its microbial degradation products.

Contents

List of Figures

List of Tables

Abbreviations

1-MIND	1-methoxyindole glucosinolate
3-MSP	3-methylsulfinylpropyl glucosinolate
4-MSB	4-methylsulfinylbutyl glucosinolate
4-MTB	4-methylthiobutyl glucosinolate
6PGDH	6-phosphogluconate dehydrogenase
AIF	Apoptosis-inducing factor
AITC	Allyl isothiocyanate
AKR1	Alpha-keto reductase
AKR1B1	Aldo-keto reductase family 1 member B
AP	Apical
ARE	Antioxidant response element
ATCC	American Type Culture Collection
B-ITC	*N*-butylthiocarbamoyl cysteine
BL	Basolateral
Caco-2	Human epithelial colorectal adenocarcinoma cells
CDK	Cyclin-dependent kinase
DPBS	Dulbecco's phosphate-buffered saline
DTCs	Dithiocarbamates
EMEM	Essential Minimum Eagle's Medium
ERN-NAC	Erucin-N-acetylcysteine
ESI	Electrospray Ionisation
ESP	Epithiospecifier protein
FBS	Fetal bovine serum
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCLC	Glutamate-cysteine ligase catalytic subunit
GPx	Glutathione peroxidase
GR	Glucoraphanin
GSH	Glutathione
GST	Glutathione-*S*-transferases
GTK	Glutamine transaminase K
HBSS	Hank's Balanced Salt Solution
HDK1	hexokinase domain containing 1

HepG2	Human hepatocellular carcinoma cell line
HK1	Hexokinase-1
HMOX1	Heme oxygenase-1
HNU	Human Nutrition Unit
HPLC	High-performance liquid chromatography
ICP-OES	Inductively coupled plasma optical emission spectrometry
IL	Interleukin
IND	Indoles
ITCs	Isothiocyanates
KAT	Kynurenine aminotransferases
Keap1	Kelch-like ECH-associated protein 1
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LPS	Lipopolysachcharide
LY	Lucifer yellow
MMTSI	S-methyl methanethiosulphinate (dimethyl disulphide sulphoxide)
MMTSO	S-methyl methanethiosulfonate (dimethyl disulphide sulphone)
MRM	Multiple reaction monitoring mode
MRP-1	Multidrug resistance associated protein-1
NADH/NADPH	Nicotinamide adenine dinucleotide/ Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NQO1	NAD(P)H; quinone oxidoreductase
Nrf2	Nuclear factor erythroid-derived 2-like
OH-IND	Hydroxy-indolyl methyl glucosinolate
PAMPA	Parallel artificial membrane permeability assay
P_{app}	Apparent permeability coefficient
PEITC	Phenethyl isothiocyanate
PGAM1	Phosphoglycerate mutase
PGD	Phosphogluconate dehydrogenase
Pgp-1	P-glycoprotein-1
PHITC	6-phenylhexyl isothiocyanate
QIB	Quadram Institute Biosciences
RT-PCR	Real-Time polymerase chain reaction
SF	Sulforaphane
SF-Cys	Sulforaphane-Cysteine
SF-Cys-Gly	Sulforaphane-Cysteine-Glycine

SF-GSH	Sulforaphane-Glutathione
SF-N	Sulforaphane-Nitrile
SF-NAC	Sulforaphane-*N*-acetyl-cysteine
SMCSO	S-methyl-L-cysteine sulfoxide
TALDO	Transaldolase
TCA	Trichloroacetic acid
TEER	Trans-epithelial electrical resistance
TKT	Transketolase
TLR4	Toll-Like receptor-4
TNF	Tumor necrosis factor
TXNRD1	Thioredoxin reductase
UGT1A1	UDP-glucuronosyltransferase family 1 member A1
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry

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CHAPTER 1

General Introduction

1.1. Summary

This Chapter introduces the primary focus of the thesis, sulphur-containing compounds from broccoli. Current knowledge regarding the synthesis and metabolism of these compounds is presented, followed by an overview of their bioavailability and biological activity *in vitro* and *in vivo*. Lack of knowledge in certain areas provides the rationale for the proposed aims of this thesis.

1.2. Cruciferous vegetables: sulphur compounds and their metabolism

Cruciferous is a lay term referring to all species in the family Brassicaceae belonging to the order Brassicales. Brassicaceae consist of more than 350 genera and 3000 species [1]. One of the genera within this family is *Brassica*, which includes vegetables consumed worldwide such as broccoli, cabbage, cauliflower, mustard and Brussel sprouts. Other more region-specific vegetables, mainly consumed in Eastern countries, include Bok choy, kai-lan and kohlrabi are also within the *Brassica* genus. A unique trait shared by the order Brassicales is to synthesise glucosinolates. In addition to glucosinolates, Brassicaceae also contains other sulphur compounds including S-methyl-L-cysteine sulfoxide (SMCSO) which is also found in other plant families [2]. These compounds account for the distinctive taste and smell associated with these vegetables.

1.2.1. Glucosinolates

To date, 120 glucosinolates have been identified in plants which can be classified into three main groups; aliphatic, aromatic, and indolic glucosinolates [1]. The synthesis of the glucosinolates from amino acids define which group they are categorised as. Aliphatic glucosinolates are mainly derived from methionine but also from isoleucine, leucine, or valine whereas aromatic glucosinolates are derived from phenylalanine or tyrosine, and indolic glucosinolates are derived from tryptophan [1]. The glucosinolates profile varies amongst the *Brassica* genus, for instance, broccoli predominantly accumulates aliphatic glucosinolates including 4-methylsulfinylbutyl glucosinolate (4-MSB, glucoraphanin, GR), 3-methylsulfinylpropyl glucosinolate (3-MSP, glucoiberin), and 4-methylthiobutyl glucosinolate (4-MTB, glucoerucin) [3]. On the other hand, 2-propenyl glucosinolate (PROP, sinigrin) is the major glucosinolate in Brussel sprouts and cabbage [4]. All glucosinolates are

structurally similar, consisting of a β-D-glucopyranose moiety, a sulfonated oxime groups, and a side chain **(Figure 1-1 and Table 1-1)** [3,5]. Due to the acidic nature of the sulphate groups, glucosinolates accumulate in plants as potassium salts [6]. The proportion of glucosinolates present in the plant varies amongst the *Brassica* species and within the same plant at various developmental stages and plant organs (heads vs. roots) [7]. The concentration of glucosinolates is also influenced by other factors including environmental conditions (temperature, pH, moisture) and genetic variations [7].

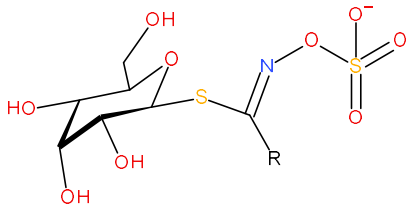
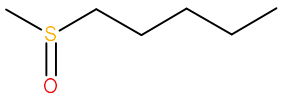
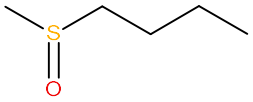
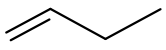
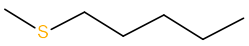
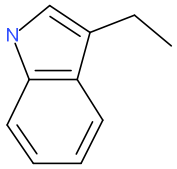
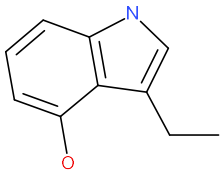
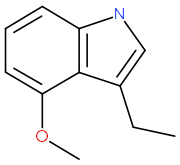
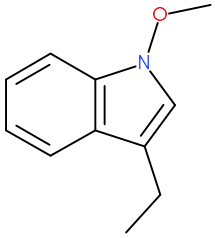
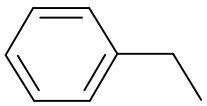
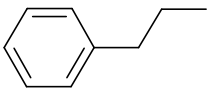


Figure 1-1: General structure of glucosinolates. The structure consists of a β-D-glucopyranose moiety, a sulfonated oxime groups and a variable side chain represented as R. Adapted from [1].

Table 1-1: Structures of the R chain of some of the glucosinolates found in Brassicaceae. This includes including aliphatic, indolic and aromatic glucosinolates. Adapted from [1,7].

Glucosinolate chemical name	Common name	R group structure
Aliphatic glucosinolates		
4-methylsulfinylbutyl	Glucoraphanin, GR	
3-methylsulfinylpropyl	Glucoiberin	
2-propenyl	Sinigrin	
4-methylthiobutyl	Glucoerucin	

Glucosinolate chemical name	Common name	R group structure
Indolic glucosinolates		
3-Indolylmethyl	Glucobrassicin	
4-hydroxy-3 indolylmethyl	4-hydroxyglucobrassicin	
4-methoxy-3-indolylmethyl	4-methoxyglucobrassicin	
1-methoxy-3-indolylmethyl	Neoglucobrassicin	
Aromatic glucosinolates		
Benzyl	Glucotropaeolin	
2-phenylethyl	Gluconasturtiin	

1.2.1.1. Glucosinolate breakdown products

Glucosinolates *per se* are not bioactive compounds but isothiocyanates (ITCs), their breakdown product, are biologically active. In comparison to its product, glucosinolates are more stable and found in higher levels in plants [1]. The breakdown of glucosinolates to ITCs is catalysed either by the plant β-thioglucosidase enzyme, myrosinase, or by our gut bacteria. Myrosinase, a glycoprotein, is spatially separated from its substrate, glucosinolates. Upon plant damage, the myrosinase comes into contact with glucosinolates thereby catalysing the breakdown. The activity of the myrosinase varies between *Brassica* species. Wilkinson *et al.* demonstrated the activity of myrosinase ranging from 0.3 μmol/min/mg protein to 10.5 μmol/min/mg protein from 12 cruciferous vegetables [8]. In addition, out of 18 cruciferous vegetable seedlings, the activity of myrosinase was highest in Daikon (280 μmol/min/mg of protein) [9].

Myrosinase appears to bind to the hydroxyl group at the C-2 position of the glucose moiety, cleaving the β-glucosyl moiety and releasing a β-D-glucose. In addition, an unstable intermediate, thiohydroxamate-*O*-sulfonate, is produced. Non-enzymatic re-arrangement of thiohydroxamate-*O*-sulfonate generates ITCs and releases other breakdown products including glucose and sulphate [1,9]. Hydrolysis of GR, a glucosinolate, results in the formation of the ITC, sulforaphane (SF) **(Figure 1-2)**.

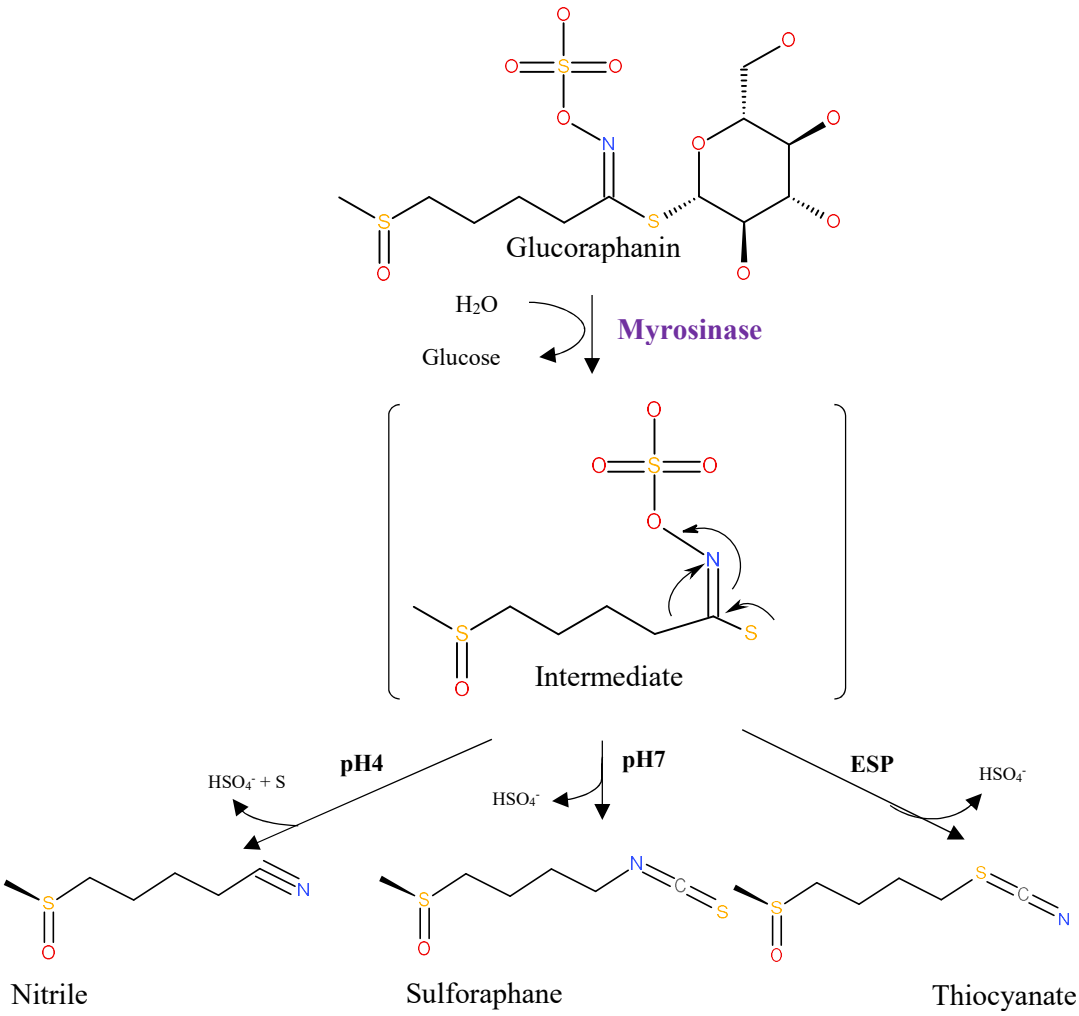


Figure 1-2: Hydrolysis of glucoraphanin by myrosinase to form SF, nitriles and thiocyanates. Re-drawn from [1,10] with permission of Elsevier and Taylor and Francis.

In addition, nitriles and thiocyanates can be produced from the breakdown of glucosinolates **(Figure 1-2)**. At pH 6-7, the main products are ITCs. However, under acidic or alkaline conditions, the production of nitriles are favoured over ITCs. Furthermore, epithiospecifier protein (ESP), a myrosinase co-factor, influences the production of nitriles over ITCs in fresh broccoli [11]. Mild cooking can denature the ESP-like protein but maintain an active myrosinase [12] as the ESP is not as stable as myrosinase and loses its activity at 60ºC [7]. However, further thermal damage such as cooking at high temperatures can denature the myrosinase as well as reduce glucosinolate levels [13]. Nevertheless, there is some indication that our gut microflora demonstrates thioglucosidase activity and thus is able to convert glucosinolates to ITCs. Although the mechanism remains unclear, there is evidence to support this notion including early work by Rabot *et al.* who demonstrated hydrolysis of glucosinolates

in germ-free rats when orally administered with human faecal flora from healthy adult male or a single strain of *Escherichia coli* or *Bacteroides* [14]. Further work by Shapiro *et al*. *in vivo* reported an 8.7-fold decrease in the conversion of glucosinolates to ITCs upon inhibition of gut microflora by a combination of cleansing and antibiotics [15]. The potential role of the gut microflora on the breakdown of glucosinolates to ITCs is an important breakthrough in understanding the bioavailability of ITCs from cruciferous vegetables.

At present, broccoli is purchased by the public either frozen or fresh. While the fresh broccoli contains active myrosinase, the frozen broccoli does not. Prior to freezing, broccoli undergoes blanching as a pre-treatment step at 80ºC for <3 minutes which inactivates myrosinase. It has been reported that the process of blanching and boiling affect the levels of glucosinolates [13]. Blanching resulted in a 2-37% reduction in the total levels of glucosinolates in a range of cruciferous vegetables whereas boiling resulted in a 35.3-72.4% loss of total glucosinolate levels [13].

1.2.1.2. Development of high-glucoraphanin broccoli varieties

Compared to a wild type species of *B. oleracea,* commercially available broccoli (*B. oleracea var. italica*) tends to have lower levels of glucosinolates [16,17]. Through a breeding programme at the John Innes Centre (Norwich, UK), a hybrid cross developed by Faulkner *et al*. between *B. oleracea var. italica* and wild type species *B. oleracea* resulted in a 10-fold enhanced levels of total aliphatic glucosinolates relative to standard edible broccoli, comparable to the levels found in the wild type [16]. With promising results, further work was undertaken by Mithen *et al.* in order to naturally enhance the levels of glucosinolates in edible *B. oleracea* [17]. Hybrids were produced by cross-breeding *B. oleracea var. italica* with *B. villosa*, a wild type species, to introduce three genome segments from *B. villosa* into the genetic background of standard broccoli [17]. Further backcrossing produced three hybrids with increased levels of 4-MSB and 3-MSP compared to the commercially available species, two of which were commercialised as Beneforté® [18]. Through genomic analysis of the hybrids, it was identified that one of the introgression segments from *B. villosa* found in the hybrids contained the Myb28 allele, which encodes for the Myb28 transcription factor. As described by others in *Arabidopsis thaliana* [19], the Myb28 transcription factor is implicated in the regulation of glucosinolate biosynthesis. It was demonstrated that these hybrids acquired one Myb28 allele from *B. villosa* and one from standard broccoli thus represented as $Myb28^{B/V}$. The $Myb28^{B/V}$ variety contained increased total sulphur and a 2.5-3-fold enhanced levels of GR compared to standard broccoli. However, lower levels of SMCSO were detected

in two of the hybrids, presumably due to increased assimilation of sulphate from the soil and channelling of sulphur into glucosinolate synthesis [18]. An additional variety possessing two Myb28^{V} alleles, accumulating even higher levels of glucosinolates, was developed.

1.2.2. Sulforaphane

Compared to the other ITCs including allyl ITC (AITC), benzyl ITC, phenethyl ITC (PEITC), SF is the most extensively studied ITC for its health-promoting effects. A considerable amount of *in vivo* and *in vitro* studies has been undertaken to explore its mechanisms. GR, the most abundant glucosinolate in broccoli is the precursor of SF **(Figure 1-2)**.

1.2.2.1. Sulforaphane metabolism

A majority of the studies have investigated the absorption of SF or other ITCs and its metabolism via the mercapturic acid pathway, with limited research undertaken to investigate the absorption of intact glucosinolates, presumably because possible absorption of intact glucosinolates was not considered. However, a small number of studies have measured intact GR indicating that GR is absorbed intact [20-22].

It was demonstrated that while glucosinolates are stable under acidic conditions of pH 2 in the stomach, only approximately 60% of intact glucosinolates reach the colon [7,23]. Following conversion of GR to SF in the intestinal lumen, SF is subject to absorption [7]. It is assumed that SF rapidly passively diffuses into the enterocytes due to its small molecular weight and lipophilicity [24]. Given that SF is an electrophile which can readily react with nucleophiles such as proteins, its central carbon reacts with the cysteine sulfhydryl group of glutathione (GSH) [25]. This conjugation reaction can occur non-enzymatically but is also stimulated by glutathione-*S*-transferases (GST) [25,26]. Through the use of an intestinal perfusion model, it was revealed that apart from the detection of conjugation to GSH, no other conjugates (cysteine or *N*-acetylcysteine) were observed in the enterocytes. This indicates that SF is conjugated to GSH in the enterocytes, but breakdown to other conjugates occurs elsewhere [27].

There are 3 families of GSTs; cytosolic GSTs, mitochondrial GSTs, and microsomal GSTs, the largest group being cytosolic GSTs which occur as a dimer comprising of 17 subunits grouped into 7 classes [28,29]. GSTs have been researched to a great extent due to their abilities to conjugate to a wide range of electrophiles maintaining cellular homeostasis [28],

including carcinogenic metabolites and several ITCs such as SF, benzyl-ITC, PEITC and AITC [25]. Although extensively researched, the conjugation of only 4 cytosolic GSTs including M1-1, A1-1, M4-4 and P1-1 were investigated with 14 ITCs [25]. Of the 4 enzymes, GST M1-1 presented itself as the most effective catalyst followed by GST P1-1 [25]. However, SF proved to be the slowest substrate to be conjugated out of all 14 ITCs tested [25]. Although the ITCs were conjugated to GSH non-enzymatically, it was demonstrated that the rate of conjugation was augmented in the presence of GSTs.

In the cells, reversible conjugation can occur rapidly within 30 minutes. Cells exposed to an extra-cellular SF concentration of 50-100 μM accumulated 95-98% as SF-Glutathione (SF-GSH) intracellularly with about 2-5% left as free SF. When the extracellular concentration was increased to 500 μM, the intracellular proportion shifted to 82% SF-GSH and 11% free SF [30].

SF can passively diffuse as free SF or can be transported out of cells as a GSH conjugate using the multidrug resistance associated protein-1 (MRP-1) and P-glycoprotein-1 (Pgp-1) [7,27,31], as revealed by the detection of SF-GSH in the lumen of the jejunum of 6 human volunteers following administration of SF from a broccoli extract [27]. In the plasma, due to its electrophilic nature, SF can reversibly bind to hydroxyl, sulfhydryl and amino groups of proteins such as albumin and glycoprotein enabling distribution around the body [32]. However, for SF to diffuse and accumulate in cells, they need to dissociate from proteins as only unbound SF compounds such as free SF can passively diffuse. Rapid distribution of ITCs around the body was demonstrated by administrating labelled ^{14}C benzyl-ITC to rats. Following oral administration, benzyl-ITC was rapidly absorbed in the plasma within 45 minutes followed by rapid excretion [Franklin, E. R., unpublished, cited in [33]]. In addition to the detection of high levels of ITCs in the sites of absorption and elimination such as GI tract, kidneys and liver, autoradiographic study in rats described high levels of ITCs in blood [Franklin, E. R., unpublished, cited in [33]]. Supporting this, another study in rats administrating two ITCs, ^{14}C PEITC and ^{14}C 6-phenylhexyl isothiocyanate (PHITC) demonstrated a rapid absorption of ^{14}C PEITC with a peak plasma concentration at 2.9 hours but slower absorption of ^{14}C PHITC peaking at 8.9 hours [34]. Following administration of ^{14}C PEITC, levels were detected in the stomach, small intestine and colon at high concentrations compared to lower concentrations in the pancreas and spleen and relatively lower in the heart and brain [34]. Peak concentrations are highly influenced by the rate of intestinal passage of the dose [34].

Following hydrolysis of GR to SF and conjugation in the enterocytes, SF undergoes metabolism in the liver. Similar to the conjugation process in the enterocytes, the first step of

the elimination process from the body, ITCs undergo conjugation in the liver. The liver, as the main site of xenobiotic metabolism, encompasses high levels of GSH and the highest GST activity [7]. Following conjugation to SF, SF-GSH undergoes a series of enzymatic reactions via the mercapturic acid pathway. Firstly, the γ-glutamyl residue is eliminated by cleavage catalysed by γ-glutamyltransferase to produce Sulforaphane-Cysteine-Glycine (SF-Cys-Gly). This is followed by cysteinylglycinase catalysing the release of a glycine residue to generate Sulforaphane-Cysteine (SF-Cys) **(Figure 1-3)**.

The final fate of SF is acetylation with *N*-acetylcysteine catalysed by *N*-acetyltransferases yielding Sulforaphane-*N*-acetyl-cysteine (SF-NAC) [10], primarily occurring in the kidneys, the major organ involved in excretion, due to high N-acetyltransferases activity [7]. In the urine, approximately 40-60% of dose consumption is excreted as SF-NAC, the most prevalent conjugate in urine excretion [32,35,36]. Dithiocarbamates (DTCs), referring to the mercapturic acids SF-GSH, SF-Cys, SF-Cys-Gly, SF-NAC, and SF **(Figure 1-3)**, are measured in the urine to assess the bioavailability of ITCs from cruciferous vegetable intake [10].

The predominate route of ITC excretion is via the urine, faeces, expiration and bile. Recovery of the labelled ^{14}C benzyl-ITC in rats was approximately 92% in the urine, 6% in the faeces, 0.4% expired in the air and 3.9 % in the bile three days following administration [33]. Similarly, another rat study reported 0.1% expired in the air, 89% in the urine, 10% in the faeces after 48 hours following administration [7]. Most of the urinary excretion of labelled compound occurred within 24 hours.

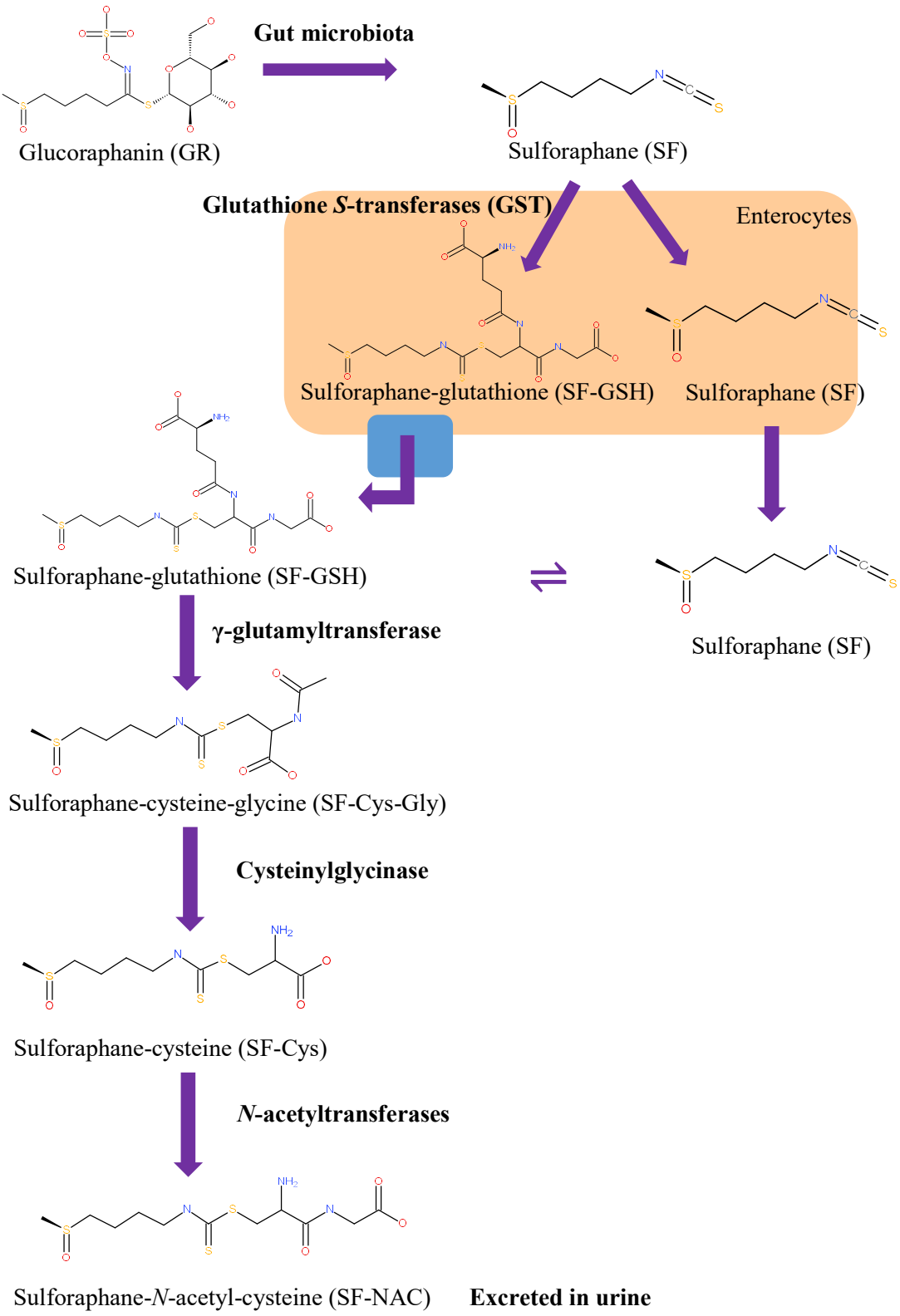


Figure 1-3: Metabolism of sulforaphane via the mercapturic acid pathway. Adapted from [37,38] with permission of Oxford University Press and under the terms of the Creative Commons Attribution License.

1.2.3. S-methyl-L-cysteine sulfoxide

Other than glucosinolates, many cruciferous vegetables have abundant levels of the amino acid derivative, 3-methylsulphinyl alanine, SMCSO, first identified to occur naturally in *Brassica* in 1956 [2].Compared to total glucosinolates, accounting for 0.1-0.6% dry weight in *Brassica* vegetables, SMCSO was quantified at higher levels, representing 1-2% of dry weight [2]. Other than *Brassica* vegetables, SMCSO is found in *Fabaceae* and *Alliaceae*. SMCSO, a secondary metabolite in plants, was implicated in the role of pathogenesis prevention by acting as a phytoalexin with anti-microbial activity. In ruminant animals, it was identified as a toxicant resulting in severe haemolytic anaemia, known as the 'kale anaemia factor' following consumption of *Brassica* crops, however, in humans, it has been associated to have potential health benefits but remains unclear due to limited research undertaken.

1.2.3.1. S-methyl-L-cysteine sulfoxide synthesis

Synthesis of SMCSO in plants is largely dependent on the assimilation of inorganic sulphate from soil. Inorganic sulphate is converted to 5-adenylsulphate then reduced to sulphite and sulphide. The sulphide reacts with *O*-acetylserine to yield cysteine and acetate. The proposed mechanism is the methylation of the cysteine producing S-methyl-L-cysteine followed by oxygenation of the sulphur yielding SMCSO [39]. In contrast, others have suggested a reaction between serine and methyl mercaptan followed by the oxygenation of sulphur from the thiomethyl moiety of methionine [2]. By feeding plants with labelled substrates either [^{14}C]-serine or [$^{35}SO_4$], it was demonstrated that SMCSO is synthesised via cysteine and via serine [2].

1.2.3.2. S-methyl-L-cysteine sulfoxide metabolism

Like glucosinolates and myrosinase, SMCSO and the enzyme that catalyses its breakdown, cysteine β-lyase, are separated in plants. Upon damage to the plant, cysteine β-lyase encounters SMCSO and hydrolyses it to yield ammonia, pyruvate and methanesulphenic acid [40]. Given that methanesulphenic acid is a highly reactive intermediate, it undergoes further reactions depending on various conditions to generate other products [41]. As such, disproportionation of methanesulphenic acid results in the formation of methanethiol and methane sulphinic acid whereas dimerisation produces S-methyl methanethiosulphinate (dimethyl disulphide

sulphoxide, MMTSI) as illustrated in **Figure 1-4** [40]. Using an enzymatic model comprising incubation of SMCSO and a purified cysteine β-lyase at 35ºC showed an increase in the formation of SMCSO-derived products, MMTSI, and pyruvate over 24 hours [41]. However, production of S-methyl methanethiosulfonate (dimethyl disulphide sulphone, MMTSO) was not detected until the 24-hour time-point at a relatively lower concentration compared to the formation of MMTSI (9.55 µM MMTSO vs. 1.72 mM MMTSI at 24 hours) [41]. Further, over 24 hours, SMCSO levels were significantly reduced by 52% at pH 4.4 and further reduced by 94% at pH 8.0 while the production of MMTSO increased. This indicates that both pH and time influence the degradation of SMCSO and formation of products as well as implicating the role of cysteine β-lyase in said breakdown [41]. Disproportionation of MMTSI generates MMTSO, and dimethyl disulphide, a volatile sulphur compound [2,40].

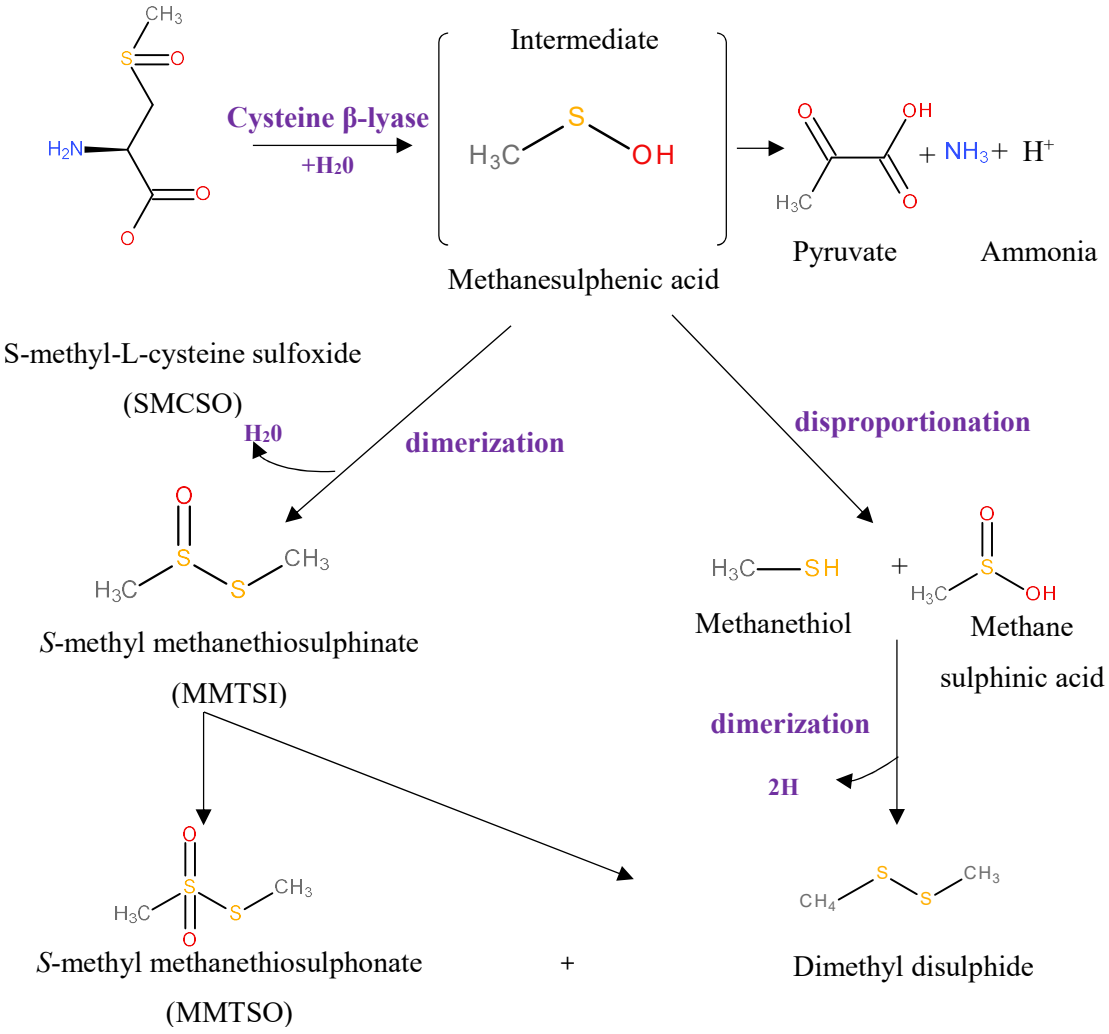


Figure 1-4: S-methyl-L-cysteine sulfoxide metabolism by cysteine β-lyase to form breakdown products. Re-drawn from [2] with permission of The Royal Society of Chemistry (RSC).

In addition to cysteine β-lyase being present in the plants, activity of cysteine β-lyase was established within the enteric bacterial population commonly found in the human intestines. Of 29 strains, 22 showed high substrate activity. However, the test compounds used in that investigation were S-methyl-L-cysteine and other cysteine conjugates, rather than SMCSO [42]. Recently, mammalian amino acid transferases including kynurenine aminotransferases (KAT I, II and IV) and glutamine transaminase K (GTK) have been identified as enzymes exhibiting cysteine β-lyase activity. Based on substrate specificity and subunit composition, it was demonstrated that KAT III is identical to the cysteine β-lyase isoform CCBL2, and KAT I is identical to the isoform CCBL1 [43] though the substrates used did not include SMCSO. Regardless, there are indications that there are enzymes/gut bacteria capable of hydrolysing SMCSO. Like myrosinase (the enzyme that catalyses glucosinolates), plant cysteine β-lyase could also be denatured when cooked thus relying on the gut bacteria to catalyse the breakdown [2].

1.3. Cruciferous vegetables: the bioavailability of sulphur compounds

The amount of the ingested dose that is absorbed by the body and reaches the systemic circulation is termed as bioavailability of compounds including drugs and dietary nutrients. Bioavailability of said compounds is influenced by the food matrix, absorption efficiency of individuals, distribution around the body and excretion. Doses that reach the systemic circulation after first-pass metabolism via the the liver circulate and reach target tissues [7]. A dose that reaches the target tissue is said to be physiologically relevant. However, many *in vitro* studies employ doses of SF that are not achievable *in vivo* after first-pass metabolism. The following sections discuss methods to assess the bioavailability of compounds using *in vitro* models and human intervention studies. While human intervention studies provide an insight into the absorption and metabolism of compounds, *in vitro* models address the transport mechanisms used by the compounds.

1.3.1. Transport and bioavailability of compounds using *in vitro* models

The permeability of compounds across the biological membrane is a fundamental factor considered when developing drugs and determining their pharmacokinetics [44]. The

composition of membranes defines the movement of compounds through them. Biological membranes consist of a phospholipid bilayer with cholesterol and transporters. *In vitro* models have been developed to mimic such membranes and can be used in the investigation of bioavailability of compounds.

1.3.1.1. Transport mechanisms across biological membranes

Three of the main transport mechanisms across membranes are passive transport (transcellular), active transport (transcellular) and the paracellular route via tight junctions. Passive transport follows the concentration gradient which either permeates across by simple diffusion or by non-specific carrier protein-mediated diffusion (facilitated diffusion) or filtration. Transport across membranes against the concentration gradient is via active transport using a specific transporter.

Parameters including lipophilicity, molecular weight, and polarity define diffusion of compounds [45]. Lipophilicity of compounds is expressed as the octanol/water partition coefficient [$\log_{10}$ pc]. A $\log_{10}$ pc <0 means that a compound is hydrophilic (lipophobic), whereas a $\log_{10}$ pc >0 means that a compound is hydrophobic (lipophilic). Lipophilic molecules tend to passively diffuse across membranes in and out of cells, whereas hydrophilic molecules cannot diffuse across the membrane without specific transporters. Polar surface area of compounds is another factor considered for intestinal permeability. The polar surface area is the total surface area of polar atoms such as nitrogen, oxygen and hydrogen. Compounds with a polar surface area of <60 A^2 are well absorbed in the gut with ≥90% absorbed whereas compounds with higher polar surface area (<140 A^2) correlates with poorer absorption of <40% in the gut [46,47]. A polar surface area of 140 A^2 is the highest boundary for compounds to be considered to be absorbed by gut cells [46].

It has generally been assumed that SF passively diffuses into the enterocytes due to its lipophilic nature and its small molecular weight of 177.29 g/mol [27]. Given that SF has a $\log_{10}$ pc of 0.72 classifying it as a lipophilic molecule implies that it can passively diffuse across a membrane [24]. On the other hand, GR is hydrophilic with a $\log_{10}$ pc of -3.8, thus potentially requiring a transporter to cross the membrane [24]. The $\log_{10}$ pc of SMCSO has not been calculated nor published elsewhere. In terms of the broccoli-derived compounds, SF, GR and SMSCO have a polar surface area of 80.7 A^2, 236 A^2 and 99.6 A^2, respectively. Taking into consideration their polar surface areas, it can be hypothesised that SMSCO, like SF, could be absorbed via passive diffusion (area <140 A^2).

1.3.1.2. *In vitro* models to study transport mechanisms

1.3.1.2.1 Human epithelial colorectal adenocarcinoma cells

The caco-2 cell-line is human epithelial colorectal adenocarcinoma cells. It originates from colon cancer and was first developed by Fogh *et al.* in the 1970s [48-50]. Over 21 days of culture, caco-2 cells can differentiate to columnar epithelial cells expressing microvilli, tight junctions, and alkaline phosphatase (brush border enzyme), resembling enterocytes [50-52]. In addition, caco-2 cells express transporters on both the apical and basolateral membrane [51]. While caco-2 cells are a useful model to investigate transport of compounds, there are limitations associated with the model. Some of these include (i) higher trans-epithelial electrical resistance (TEER) values in comparison to the human intestines; (ii) multilayer growth of cells, representing only one of the types cells expressed in human gut, and (iii) lack of mucus [47]. It has been observed that data obtained by using the caco-2 models are difficult to reproduce due to variation in the experimental conditions between different laboratories in terms of culture conditions, passage numbers and type of culturing plates (transwell plates with different membrane pore size and area) [50,53]. Furthermore, older passage number greatly increases the risk of cells growing in multilayers, as well as affecting TEER and markers expressed by enterocytes [50]. For this reason, comparison between data from caco-2 with older passage and younger passage has to be handled with caution [50].

1.3.1.2.2 Parallel artificial membrane permeability assay

Commonly used by pharmaceutical companies, the parallel artificial membrane permeability assay (PAMPA) was first established by Kansy *et al.* to investigate the permeability of compounds across membranes [54]. It was aimed to be suitable for high throughput and less laborious in comparison to culturing and performing transport experiments using caco-2 cells [54]. It was designed to correlate with oral permeability as well as provide a stable membrane mimicking the phospholipid layer.

Over the last few years (1998-2008), the PAMPA assay has been developed, most notably by modifications to the membrane. Historically, when first introduced, the original Kansy PAMPA model required pre-coating of the plates with phospholipids (1-20% lecithin in an organic solvent) by users causing significant inter-laboratory and intra-laboratory variations [54]. Concerns were also raised in terms of the lipid bilayer composition consisting of phosphatidylcholine and alkane or alkylidene that was found responsible of under-/over-

prediction of oral bioavailability. [55]. Modification to the phospholipids was the practical option proposed to overcome these issues [55]. Various modifications were made to the lipid composition including a lipid membrane comprising of 2% (w/v) dioleyoylphosphatidyl-choline (DOPC) in dodecane solution [56], 20% (w/v) dodecane solution in lecithin mixture developed by pION Inc [57], a mixture of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and cholesterol in an organic solvent to mimic the intestinal membrane by Sugano *et al.* [55]. However, poor predictability of permeability was still an issue due to the pre-coating requirement by users and the lipid compositions of membranes [58]. This was solved by developing a pre-coated membrane ready to use with a lipid-oil-lipid tri-layer, manufactured as the BD Gentest™ PAMPA assay developed by BD Bioscience [58], later acquired by Corning®.

The Gentest™ PAMPA assay is a validated assay comprising of pre-coated plates with a lipid-oil-lipid bilayer. The formation of the lipid-oil-lipid layer consists of introducing 1 µl hexadecane into the polyvinylidene fluoride (PVDF) membrane to form the oil layer coated with 40 µg of phospholipids mixture either side of the oil layer. This assay has been optimised and validated by Chen *et al.* [58]. The optimisation of this assay was undertaken by testing a wide range of compounds (n=12) that are not actively transported. Data from the Gentest™ PAMPA assay was compared with already known permeability coefficient with those obtained from the caco-2 model and two different lipid-solution based PAMPA methods (2% (w/v) DOPC in dodecane solution and 20% (w/v) dodecane solution in lecithin mixture). It was reported that the lipid-solution based PAMPA models have under predicted the permeability of some compounds possibly due to excess solution in the membrane. The predictability of the permeability of compounds was improved with the Gentest™ PAMPA assay due to its lipid-oil-lipid membrane. The permeability of compounds from the Gentest™ PAMPA assay correlated well with the permeability obtained from the caco-2 model and the human absorption data. Thus, the tri-layer mimics the biological membrane and generates reproducible results [58].

1.3.2. Bioavailability in humans

1.3.2.1. Glucoraphanin and sulforaphane bioavailability in humans

With a lot of interest regarding SF due to its possible health-promoting effects, it is unsurprising that clinical studies have been undertaken to identify the pharmacokinetics of SF from dietary sources. The extent of absorption and metabolism are key parameters in defining the bioavailability of compounds. Particularly, identifying concentrations of SF achievable *in vivo* is critical for exploring the mechanistic role of SF. A considerable amount of published research has been undertaken *in vivo* and *in vitro* using a range of SF or GR doses. A summary of 16 *in vivo* studies undertaken measuring mercapturic acids as DTCs or as SF and SF conjugates, in plasma and urine following consumption of SF or GR in the form of raw broccoli, cooked broccoli, capsules or soups is presented in **Table 1-2**. At present, in clinical studies, the urinary excretion of ITCs and conjugates is used directly as a measure of bioavailability of compounds derived from cruciferous vegetable intake [7]. This was confirmed as a good measure based on the positive correlation demonstrated between cruciferous vegetable intake and urinary excretion of ITCs [59]. However, to gain an insight into phase I metabolism of ITCs, a full metabolite analysis of blood, urine and faeces needs to be accomplished [7].

Initially, the total DTCs were measured using a method, known as cyclocondensation, first developed by Zhang *et al.* [60] and later modified within the same group, that enabled quantification via high-performance liquid chromatography (HPLC) or spectroscopy [61]. This method provides a quantitative measure of all ITCs present in plasma, urine and other samples. However, it does not allow identification of individual metabolites. Since then, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed by Vermeulen *et al.* [62] specific to measure *N*-acetylcysteine conjugates. Janobi *et al.* established a LC-MS/MS method to quantify individual mercapturic acids in plasma and urine samples following broccoli consumption [63].

The consensus observed from the *in vivo* studies was rapid absorption and excretion of SF and conjugates following direct consumption of SF compared to GR, the precursor. Results have consistently reflected that bioavailability of SF is 4-7 times higher upon direct oral consumption of SF versus consumption of GR (which must be converted to SF by the gut microbiota) [10,32,35,62,64-69]. As highlighted by others, the bioavailability of SF from GR is limited to the passage of GR through the gastrointestinal tract to the colon and is reliant on the gut microflora for the hydrolysation of GR to SF. Presumably, this takes time, and the gut microflora is not as efficient as myrosinase in converting glucosinolates to ITCs, resulting in

lower bioavailability. In contrast, bioavailability of SF from direct oral consumption of SF is dependent on the diffusion of SF across enterocytes and absorption by the jejunum without the dependence on the gut microflora [27]. Supporting this notion, several studies reported higher percentage of SF and conjugates recovered in urine (30-80%) following direct oral consumption of SF from raw broccoli, capsules, or extracts with intact myrosinase [10,32,35,36,38,65-71]. Rapid absorption of SF occurred with levels reaching 0.2-1.9 μM in the plasma [64,68,72] at 2-3 hours after oral SF consumption [32,38,68-71]. On the other hand, only ≤10% of ingested GR, in the form of cooked broccoli or extracts with inactivated myrosinase, was recovered as SF and conjugates [22,32,66-68,71], peaking much later around 4-6 hours [32,36,65,68,71] with low levels in the plasma of 0.02 μM [68]. In congruence with the plasma levels, urinary excretion peaks earlier following direct consumption of SF compared to GR [36,64,65,68,69,71].

The variation in urinary excretion reported within and amongst all the studies identified in **Table 1-2** is large irrespective of whether SF or GR was consumed. Fahey *et al.* reported an excretion of 48-96% of ingested SF [70] and excretion of 1-40% of ingested GR [66]. In accordance, Conway *et al.* reported an excretion of 14.2-61.8% and 3.8-24.6% following consumption of SF and GR respectively [32]. The variance in the urinary excretion was considerably lower following consumption of SF than following consumption of GR [22,70]. The variation in the recovery of SF following consumption of GR can be attributed to the reliance on the gut microflora, which is diverse and varies from individual to individual. In contrast, the variation observed in the recovery of SF following oral SF consumption cannot be justified by the gut microflora given that SF is directly absorbed without hydrolysation. Effective absorption of SF in an *in vivo* jejunum was demonstrated with 74% of SF passively diffusing [27].

Table 1-2: Summary of clinical studies investigating bioavailability of sulforaphane either from consumption of the precursor, glucoraphanin or sulforaphane in the form of various matrices

Reference	Intervention	Findings
Fahey *et al.* (2017) **[70]**	Prostaphane® tablets x 2: 100 µmol SF SF preparation in an α-cyclodextrin inclusion (SF-αCD): 200 µmol SF	**DTCs measured in 24h urine:** Prostaphane®:67.4 µmol (71% of dose) SF-αCD: 124.6 µmol (62.3% of dose)
Fahey *et al*. (2015) **[66]**	GR-rich BSE extract: 50 µmol, 69 µmol, 230 µmol GR Onco-PLEX™ capsules (broccoli seed extract):69 µmol, 230 µmol GR Freeze-dried broccoli sprouts (pre-hydrolysed with active myrosinase): 100 µmol GR + 49.6 µmol DTCs Freeze-dried broccoli sprouts (with active myrosinase, not pre-hydrolysed): 100 µmol GR + no DTCs Broccoli seed powder (active myrosinase): 100 µmol GR + No DTCs	**DTCs measured in 24h urine:** GR-rich BSE extract: 4.7 µmol (9.4% of 50 µmol dose) Onco-PLEX™ capsules: 11.2% of 69 µmol and 9.7% of 230 µmol Freeze-dried broccoli sprouts (pre-hydrolysed with active myrosinase): 41.8 µmol (41.8% of GR dose, 84% of DTC dose) Freeze-dried broccoli sprouts (with active myrosinase, not pre-hydrolysed): 42.8 µmol (35.1% of dose) Broccoli seed powder (active myrosinase): 32.6% of dose

Reference	Intervention	Findings
		SF and conjugates measured:
Atwell *et al.* (2015) **[72]**	Broccoli seed extract (BroccoMax™): 224 mg GR	Broccoli seed extract (BroccoMax™): Total SF and metabolites; 0.27 µM in plasma, 109.7 µmoles in 48h urine
	Placebo: no GR	Placebo: 0 µM SF and conjugates in urine and plasma
		SF and conjugates measured:
Atwell *et al.* (2015) **[64]**	Broccoli sprout extract treated with myrosinase: 200 µmol SF	Broccoli sprout extract treated with myrosinase: C_{max} 0.7 µM in plasma, AUC: 13.8, T_{max}: 3h, 109.7 µmoles in 48h urine
	Fresh broccoli sprout: 200 µmol SF	Fresh broccoli sprout: C_{max} 1.9 µM in plasma**,** AUC: 3.0, T_{max}: 3h, 345.7 µmoles in 48h urine
		SF and conjugates in 24h urine:
Oliviero *et al.* (2014) **[67]**	Broccoli powder with 100% myrosinase activity: 73 µmol GR + 34.0 µmol SF	Broccoli powder with 100% myrosinase activity: 40 µmol SF (58% of dose)
	Broccoli florets with 100% myrosinase activity: 73 µmol GR + 11.1 µmol SF	Broccoli florets with 100% myrosinase activity: 23 µmol SF (33% of dose)
	Broccoli florets with 80% myrosinase activity: 69 µmol GR + 6.7 µmol SF	Broccoli florets with 80% myrosinase activity: 15 µmol SF (22% of dose)

Reference	Intervention	Findings
	Broccoli florets with 2% myrosinase activity: 67 µmol GR + 2.8 µmol SF Broccoli florets with no myrosinase activity: 72 µmol GR + 0.3 µmol SF	Broccoli florets with 2% myrosinase activity: 11 µmol SF (17% of dose) Broccoli florets with no myrosinase activity: 7.1 µmol SF (10% of dose)
Saha *et al.* (2012) **[68]**	Fresh broccoli soup: no GR and 23.5 µmoles SF Frozen broccoli soup: 42.5 µmoles GR and no SF	**SF and conjugates measured:** Fresh broccoli soup: C_{max} 0.21 µM in plasma, 14 µmol in urine (58.5% of dose) Frozen broccoli soup: C_{max} 0.025 µM in plasma, 2 µmol in urine (9.6% of dose)
Fahey *et al*. (2012) **[73]**	Broccoli sprout extract (a): 200 µmol GR Broccoli sprout extract (b): 400 µmol GR	**DTCs measured:** Broccoli sprout extract (a): 23.5 µmol in 24h urine (11.8% of dose) Broccoli sprout extract (b): 42.1 µmol in 12h urine (10.4% of dose)
Cramer *et al.* (2011) **[36]**	Broccoli sprouts with active myrosinase: 74 µmol SF Broccoli sprout powder without myrosinase: no SF	**SF-NAC excreted in 24h urine:** Fresh broccoli sprouts with active myrosinase: 52 µmol (74% of dose)

Reference	Intervention	Findings
	Combination of both: 120 µmol SF	Broccoli sprout powder: 22.6 µmol (19% of dose) Combination: 93.8 µmol (49% of dose)
Clarke *et al.* (2011) **[69]**	Broccoli sprout with activated myrosinase: 150 µmol GR BroccoMax™ pills with inactivated myrosinase: 121 µmol GR	**SF and conjugates measured in 24h urine:** Broccoli sprouts: 74% of dose BroccoMax™ pills: 19% of dose
Clarke et al. (2011) **[65]**	Broccoli sprout with activated myrosinase: 218.4 µmol GR BroccoMax™ pills with inactivated myrosinase: 220.3 µmol GR	**SF and conjugates measured in 24h urine:** Broccoli sprout: 192 µmol (96% of dose) BroccoMax™ pills: 41.3 µmol (19% of dose)
Egner *et al.* (2011) **[22]**	GR-rich sprout beverage: 800 µmol GR SF-rich sprout beverage:150 µmol SF	**SF and conjugates measured in 12h urine:** GR-rich sprout beverage: 5% of dose SF-rich sprout beverage:70% of dose
Vermeulen *et al.* (2008) **[71]**	Cooked broccoli: 61.4 µmol GR Raw broccoli: 9.92 µmol SF	**SF and conjugates measured in 24h urine:** Cooked broccoli: C_{max}, 0.031, T_{max}: 6h, 3.4% of dose excreted in urine

Reference	Intervention	Findings
		Raw broccoli: C_{max}, 0.103, T_{max}: 1.6h, 37% of dose excreted in urine
		DTCs measured in 8h urine:
Shapiro *et al.* (2006) **[10]**	Broccoli sprout extract: 525 µmol glucosinolates over 7 days	Broccoli sprout extract: 17.8% of dose
	Broccoli sprout extract: 2,100 µmol glucosinolates over 7 days	Broccoli sprout extract: 19.6% of dose
	Broccoli sprout extract: 525 µmol ITCs over 7 days	Broccoli sprout extract: 70.6% of dose
		DTCs measured in 12h urine:
Kensler *et al.* (2005) **[74]**	Broccoli sprout infusions: 400 µmol GR	Broccoli sprout infusions: 49µmol (12% of dose)
		SF and conjugates excreted in 24h urine:
Gasper *et al.* (2005) **[38]**	Standard broccoli soup: 101.3 µmol SF	Standard broccoli: C_{max}: 2.3, T_{max}: 1.5h, 82% of dose in the urine as SF and conjugates
	Super broccoli soup: 344.25 µmol	Super-broccoli: C_{max}: 7.3, T_{max}: 2.0h, 55% of dose excreted in urine as SF and conjugates

Reference	Intervention	Findings
		Total ITCs excreted in 24h urine:
Conway *et al*. (2000) **[32]**	200g Fresh broccoli: 0.48 µmol GR/g	Fresh broccoli: 68.1 µmol (32.3% of dose)
	200g Steamed broccoli: 0.46 µmol GR/g	Steamed broccoli: 20.6 µmol (10.2% of dose)

This list is not exhaustive and it has been modified from the table from Houghton *et al.* [75] with permission of Oxford University Press.

Abbreviations used: DTC, dithiocarbamate; GR, glucoraphanin; SF, sulforaphane; ITC, isothiocyanate; SF-NAC, sulforaphane-N-acetylcysteine

The following search terms were used on PubMed: ‘sulforaphane’ and ‘bioavailability’ and ‘humans’. Results were manually curated for relevance.

1.3.2.1.1 Genetic polymorphisms in Glutathione-*S*-transferases

Considerable variations have been reported from clinical studies in the urinary excretion of SF and conjugates. This is highly influenced by the gut microflora, as well as genetic predispositions concerning genes involved in phase I and phase II metabolism resulting in interindividual variations [7]. Although it is clear that the gut microflora is required for the conversion of GR to SF and thus influences the variation in urinary excretion [15], Egner *et al*. observed variations regardless of whether the individuals consumed GR or SF, implying that another factor such as genetic polymorphisms could contribute to the interindividual variation observed [22].

Genetic polymorphisms have been identified in cytosolic GST, a phase I enzyme catalysing the conjugation of ITCs with GSH, resulting in the encoding of either an enzyme with reduced activity or no production of the enzyme [28]. Deletions in the *GSTM1* and *GSTT1* gene results in the null variants thus those homozygous for *GSTM1*-null, or *GSTT1*-null cannot produce the enzyme [28], with approximately 50% of the Caucasians homozygous for *GSTM1*-null [76]. Given the crucial role of GSTs in detoxification of electrophiles, it was presumed that individuals with polymorphisms in GST genes could be more susceptible to cancers [28]. Although this notion was supported by epidemiological studies [77-79], there are discrepancies between findings.

In theory, individuals with the null variants do not produce the GST enzyme. Thus, there is no conjugation of ITCs to GSH, and consequently, the excretion of ITCs is slower, exposing those individuals to ITCs for a longer period. Lin *et al*. first reported that subjects with *GSTM1*-null genotype had the lowest adenoma prevalence with high broccoli intake [80]. In contrast to the theory that *GSTM1*-null would retain ITCs and excrete slower, Gasper *et al*. observed a faster and greater urinary excretion of ITCs in individuals with *GSTM1*-null genotype compared to *GSTM1*-positive genotype [38]. Epidemiological studies from the US reported that with high cruciferous vegetable intake *GSTM1*-positive individuals gain greater protection against cancer risk [78,81]. In contrast, Vogtmann *et al*. reported no inverse associations between the risk of colorectal cancer and cruciferous vegetable intake in individuals with GST polymorphisms [82]. Similarly, Steck *et al*. observed no associations between breast cancer risk and consumption of cruciferous vegetables when stratified to the GST genotype [83]. In agreement, following consumption of GR or SF, no significant differences in the urinary excretion of ITCs were observed in two other studies when stratified to the GST genotype [22,68] contradicting the findings by Gasper *et al.* [38]. One limitation with regards to the studies discussed above is that most of the studies were retrospectively genotyped and therefore not powered for the genotype classification. With contradictory

findings from epidemiological and clinical studies, the effect of GST polymorphisms and cancer risk with higher cruciferous vegetable intake remains controversial.

1.3.2.2. S-methyl-L-cysteine sulfoxide bioavailability in humans

Inorganic sulphate has been shown to be poorly absorbed despite being needed to produce sulphate containing compounds essential for human functions. Thus, most of the sulphate required is acquired from protein, mainly via sulphur containing amino acids including methionine and cysteine. It is presumed that the degradation and oxidation of the sulphur moiety of these amino acids contributes to the sulphate pool, with cysteine being the major source. In addition to amino acids, S-substituted cysteine molecules namely SMCSO and S-carboxymethyl-L-cysteine have been indicated to contribute to the sulphate pool [84]. The metabolism of SMCSO has previously been investigated by employing labelled [^{35}S]-SMCSO in a human study and measuring radioactivity recovered in urine and faeces. Administration of 200 mg labelled [^{35}S]-SMCSO to 4 male volunteers resulted in a 96% recovery of [^{35}S] in 0-14 days [84]. Excretion of the [^{35}S] over 3 days appeared to occur predominantly via urine, with 77% recovered via this route and a significantly smaller amount (1.8%) excreted via faeces. Within the first 24 hours, approximately 60% of [^{35}S] was recovered in urine. Of the ingested [^{35}S]-SMCSO, 16% and 26% of radioactivity was recovered as inorganic sulphate in 24 hours and 3 days respectively, supporting the proposed mechanism of S-substituted cysteine molecules degrading to sulphate. Presumably, the acute dosing of SMCSO is not contributing to sulphate/sulphur containing molecules needed for human functions as a high percentage of the ingested [^{35}S]-SMCSO was excreted in urine as [^{35}S] and sulphate [84].

Despite the described studies, the metabolism and bioavailability of SMCSO in humans remains unclear due to limited research. Further work needs to be carried out to gain an understanding into the metabolism of SMCSO and whether products such as MMTSI and MMTSO are produced.

1.4. Cruciferous vegetables: health benefits through observational and experimental evidence

Regarding cruciferous vegetables, especially broccoli, as ‘superfood’ has originated from observational studies reporting associations between the intake of cruciferous vegetables and reduction of chronic disease risks. To elucidate the underlying mechanisms involved for the

protective effects associated with cruciferous vegetables, several animal and *in vitro* studies have been performed. The biological activity of two sulphur compounds; SF and SMCSO are discussed below.

1.4.1. Epidemiological evidence for cruciferous vegetable intake and chronic diseases

At present, chronic diseases including cancers, cardiovascular and type 2 diabetes are amongst the leading causes of death worldwide. Genetic predisposition highly influences the development of these diseases. However, it can still be reduced by lifestyle and dietary choices. Exploring the underpinning role of dietary intake with regards to the risks of developing chronic diseases is of high priority. It is widely known that the intake of fruit and vegetables is implicated with potential health benefits due to the presence of bioactive compounds, vitamins, fibre and other mineral elements. In particular, intake of cruciferous vegetables has been associated with a reduced risk of several types of cancers, cardiovascular diseases [85], and other chronic diseases, evidenced by considerable amount of epidemiological studies. Some of these cancers include lung cancer [86-88], breast cancer [89,90], prostate cancer [91,92], and colon cancer [93]. However, there are contradictions between epidemiological studies in regards to cruciferous vegetable intake and chronic disease risks. In the example of lung cancers, only three epidemiological studies demonstrated an inverse association between cruciferous vegetable intake and lung cancer risk [86-88], while other studies described insignificant associations [94-96]. There is a discrepancy between the studies that have reported associations. Feskanich *et al.* reported 26% lower risks of lung cancer (Relative risk= 0.74) in women with the highest intake of cruciferous vegetables (>4.8 servings/week) and no inverse associations observed in men [87]. Further, Lam *et al.* reported a significant inverse association between the highest intake of cruciferous vegetables and lung cancer risk in women (Odds ratio=0.57 Q4 vs. Q1) [88]. On the other hand, Mori *et al.* reported an association between the highest intake of vegetables and a decrease in the risk of lung cancers in non-smoking men (Hazard ratio=0.85) [86]. As emphasised by Feskanich *et al.*, confounding factors such as smoking have a huge impact on assessing relative risks of developing lung cancer [87]. This also supports the recommendation to treat evidence from epidemiological studies with caution as dietary intake and other influential factors such as smoking vary. Importantly, the intake of cruciferous vegetables differs between the western and eastern countries evidenced by the difference reported in literature. Eastern countries such as Japan consume on average 83.5 g/day of cruciferous vegetables, 3-fold higher compared to

an average consumption of 30.8 g/day in the US [97]. Regardless, the available evidence indicates that cruciferous vegetables have health-promoting effects which are associated with bioactive phytochemicals, ITCs.

1.4.2. Biological activity of SF

SF is an extensively researched ITC studied for its cancer-preventing and other health promoting properties based on the indicative data from epidemiological studies. While exploring the influence of SF from dietary sources and risks to chronic diseases *in vivo* using mice and humans is important, gaining an insight into the fundamental molecular mechanisms is just as significant. Pure commercially available SF has been applied to *in vitro* models to investigate how SF modulates metabolic pathways. SF is able to elicit its health-promoting effects notably through the induction of phase II enzymes, cell metabolism, inflammation and induction of apoptosis and cell cycle arrest [12]. In addition, there is emerging evidence implicating SF in histone modification, inflammation, angiogenesis and metastasis [24].

1.4.2.1. Induction of phase II enzymes and oxidative stress

Harmful xenobiotics such as carcinogens and electrophiles undergo detoxification, notably by conjugation catalysed by phase II enzymes, to generate inactive metabolites. These are excreted from the body preventing damage to DNA and proteins. Traditionally, the term "phase II enzymes" referred to those with conjugation abilities including GST, and UDP-glucuronosyltransferase. However, at present, it also includes other enzymes not directly involved in conjugation such as NAD(P)H: quinone oxidoreductase, heme oxygenase and glutamate cysteine ligase.

Regulation through the Nrf2-Keap1 pathway is a feature shared by many of the phase II enzymes. Nrf2 (nuclear factor erythroid-derived 2-like) is a transcription factor sequestered in the cytoplasm of cells by its repressor, Keap1 (Kelch-like ECH-associated protein 1) under basal conditions. In the bound form to Keap1, Nrf2 is targeted for ubiquitination and proteasomal degradation. Upon modifications of the Keap1 cysteine residues by Nrf2 agonists, Nrf2 dissociates enabling its translocation into the nucleus. Dissociated Nrf2 forms a heterodimer with Maf (small muscle aponeurotic fibrosarcoma protein) promoting its binding to a *cis*-acting antioxidant response element (ARE) located in the upstream region of many phase II genes [12,98,99]. Amongst several Nrf2 agonists, SF is known to be a potent

inducer. Due to its electrophilic feature, SF reversibly reacts with the thiol groups of the cysteine residues on Keap1 forming thionoacyl adducts [12,99-101]. Recently, *Hu et al.* confirmed that SF modifies several cysteine residues [100]. However, it is unclear whether modification of cysteine residues results in the dissociation of Nrf2 as work by *Eggler et al.* suggests that modification of Keap1 cysteine residues by SF does not alter its binding ability to Nrf2 [102].

The importance of Nrf2 for basal and inducible expression of phase II enzymes was presented in a transcriptional profile of small intestine from wild-type (Nrf2 +/+) and Nrf2-deficient (Nrf2 -/-) mice administered with 9 µmol/day of SF for 1 week [99]. Upregulation of a number of genes comprising *G6PD* (glucose-6-phosphate dehydrogenase), malic enzyme and *GPx* (glutathione peroxidase) were observed only in untreated wild-type mice, implying the dependence of Nrf2 for basal expression. Further, Nrf2-deficient mice expressed lower levels of basal enzyme activities and genetic expression of several genes including *NQO1* (NAD(P)H: quinone oxidoreductase) and *GST* compared to wild-type mice [99].

The upregulation of several phase II enzymes induced by SF has been established *in vitro* and *in vivo*, emphasising the fundamental role of SF on modulating phase II enzymes and thus protecting cells from damage. Through a microarray analysis, Agyeman *et al.* demonstrated significant upregulation of phase II genes including *AKR1* (alpha-keto reductase), *NQO1*, *TXNRD1* (thioredoxin reductase) following treatment of breast epithelial cells (MCF10A) with 15 µM SF and/or Keap1 knockdown [103]. *AKR1B10* was shown to be the most upregulated by a 302.9 and 69.4-fold increase by SF and Keap1 knockdown respectively [103]. In congruence, MacLeod *et al.* reported an upregulation by ≥2-fold of phase II enzymes including *ARK1*, *HMOX1* (heme oxygenase-1), *GCLC* (Glutamate-cysteine ligase catalytic subunit) and *NQO1* [104]. Further, *in vitro* work using prostate cancer cells [105], osteosarcoma cells [106], pancreatic β-cells [107] and microglia cells [108] demonstrated an upregulation of several phase II enzymes by SF.

Further, a study with wild-type mice (Nrf2 +/+) treated with SF reported upregulated expression of *NQO1*, *GST* and *6PGDH* (6-phosphogluconate dehydrogenase), amongst other genes, compared to the untreated wild-type and Nrf2-deficient mice (Nrf2 -/-) [99]. Interestingly, enzyme activity of *G6PD* and malic enzyme were upregulated in wild-type mice treated with SF even though the basal gene expression was not upregulated in wild-type mice treated with SF. Furthermore, enzyme activity of G6PD was upregulated in Nrf2-deficient mice treated with SF indicating that other transcription factors regulate its expression [99].

An imbalance in oxidative stress between ROS generation and elimination is linked to cancer development as well as other physiological conditions [12]. As many phase II enzymes are regulated by Nrf2, it is unsurprising that knockout Nrf2 mice develop lesions and a reduced survival rate when exposed to oxidative stress inducers [109-113]. Through the induction of phase II enzymes via Nrf2, SF ameliorates oxidative stress-induced physiological conditions. SF has the ability to increase the anti-oxidant capacity of cells by inducing anti-oxidant genes however it is not regarded as an anti-oxidant [12]. While SF is not classified as a pro-oxidant, it functions like a pro-oxidant to a certain extent by initially inducing oxidative stress followed by an upregulation of phase II enzymes. This was demonstrated in immortalised human hepatocytes exposed to SF for 3-6 hours resulting in a depletion of GSH, followed by an upregulation of GSH at 24 hours [114]. Oxidative stress-induced response was attenuated by SF in mice through the induction of Nrf2 thereby upregulating the expression of phase II genes including *NQO1*, *HMOX1* and *GPx* [115,116]. This further supports the protective effects of SF via the Nrf2-ARE pathway.

1.4.2.2. Cell metabolism

Evidence has emerged on the role of SF in modulating central metabolic pathways. Microarray analysis performed on breast cells treated with SF revealed upregulation of 6,378 transcripts, with those involved in carbohydrate metabolism and NADH/NADPH (Nicotinamide adenine dinucleotide/ Nicotinamide adenine dinucleotide phosphate) generation being the top pathways elevated [103]. Exposure to SF resulted in an increase in the expression of several genes including key enzymes involved in the pentose phosphate pathway (*PGD* (Phosphogluconate dehydrogenase), *G6PD*, *TALDO* (transaldolase), *TKT* (transketolase)), and enzymes implicated in the glycolysis pathway (*PGAM1* (Phosphoglycerate Mutase) ; *HK1* (Hexokinase-1) and *HDK1* (hexokinase domain containing 1)) [103]. These are also genes positively regulated by Nrf2-ARE pathway [117], possibly explaining the elevation observed when cells are subject to SF. Exposure to SF increases metabolism through glycolysis and the pentose phosphate pathway. The glycolysis pathway produces NADH, a cofactor that donates electrons to enzymes involved in xenobiotic metabolism. Likewise, NADPH, another cofactor involved in xenobiotic metabolism and anti-oxidant systems such as regeneration of GSH, is generated from the pentose phosphate pathway [103,117]. Congruently, SF increased the rate of the pentose phosphate pathway after 15 hours in astroglia cells [118]. Further, a recent three-phase study with a standard broccoli, high-glucoraphanin, and pea intervention reported an elimination of the differences observed pre-intervention in the TCA cycle intermediates in two genotypes following the broccoli intervention. Armah *et al.* suggested that a broccoli

intervention normalised the TCA cycle metabolism supporting the effect of SF on cellular metabolism [119]. There is supporting evidence to suggest SF can alter metabolism largely through the regulation of Nrf2 but further studies need to be focused on the implications of these metabolic changes.

1.4.2.3. Inflammation

In addition, SF modulates inflammatory responses by inhibiting lipopolysachcharide (LPS)-mediated cytokine secretion. A transcription factor, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and LPS, a glycolipid, are key mediators of inflammation. LPS induces the secretion of pro-inflammatory cytokines including interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-6 [12]. SF was shown to inhibit the translocation of the NF-κB to the nucleus as well as decreasing the pro-inflammatory cytokines (IL-1β and TNF-α) and increasing the anti-inflammatory cytokines (IL-4 and IL-10) in pancreatic β-cell lines [107]. Other *in vitro* studies have demonstrated a decrease in the elevated pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) induced by stimuli and increased anti-inflammatory cytokines (TGF-β) [115,120,121]. This is mainly occurring through the depletion in the translocation of NF-κB [115]. A considerable amount of evidence has shown the anti-inflammatory effects of SF attenuating inflammation induced responses **(Figure 1-5)**.

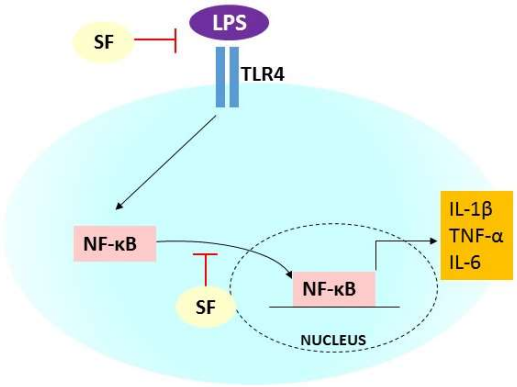


Figure 1-5: Suppression of pro-inflammatory cytokines by SF. SF inhibits inflammation by either modifying the cysteine residue of the Toll-Like receptor-4 (TLR4) and by inhibiting the translocation of NF-κB from the cytoplasm to the nucleus [120].

1.4.2.4. Induction of cell cycle arrest and apoptosis by SF

The cell cycle is regulated by cyclin-dependent kinase (CDK), CDK inhibitors, and cyclins. This occurs through five stages of the cycle, G_0, G_1, S, G_2 and M [12]. G_0 phase is the quiescence phase where cells leave the cycle to stop dividing, G_1 is the period of growth where cells increase their metabolic activity, S phase where DNA is duplicated, G_2 is the step where cells prepare to divide and finally M phase is where mitosis occurs. CDK and cyclins facilitate the progression of cells from one phase to the other. SF has the ability to induce cell cycle arrest thus inhibiting cell growth in various cell lines. SF induces a cell cycle arrest at G_2/M phase as demonstrated by *in vitro* studies. A decrease in the percentage of cells in the G_0/G_1 phase and an increase in the G_2/M phase was observed in lung cells exposed to 1 µg/ml (~5-6 µM) SF for 48 hours [122], prostate cells subject to 10 µM SF for 24 hours [123], bladder cell exposed to 20 µM SF for 48 hours [124], leukemia cells at 48-hour treatment with SF [125] and keloid fibroblasts exposed to 10-20 µM for 48 hours [126]. It is proposed that the accumulation of cells in the G_2/M phase is a result of a cell cycle arrest induced by SF [127]. This inhibits cells transitioning to the G_1 phase halting cell growth associated with reduced cell proliferation.

Apoptosis is a regulated programmed cell death fundamental in maintaining cellular homeostasis by eliminating dead cells. In certain scenarios, such as autoimmune diseases and some cancers, apoptosis is no longer properly regulated leading to aberrant death of cells [12]. There are two possible apoptosis mechanisms; caspase-dependent pathway and caspase-independent pathway. Caspase-dependent pathway is cell death mediated by a group of cysteine proteases known as caspases. They are synthesised as pro-caspases and become activated to caspases upon cleavage of specific aspartate residues [12]. Activated caspases lead to the cleavage of DNA characterised by apoptotic bodies and cell shrinkage. In contrast, cell death can also occur without the activation of caspases, via the release of the mitochondrial protein apoptosis-inducing factor (AIF).

Early reports of inhibition of proliferation and induction of apoptosis mediated by SF were evidenced by a decrease in viability of colon cancer cells subjected to 15 µM SF [128]. Thus far, a considerable number of *in vitro* studies have reported apoptosis induced by SF in various cell lines including lungs [122], cervical [127], liver [127], prostate [123] and bladder [124]. Even though SF inhibited proliferation of the lung adenocarcinoma cell line, no effect was observed in normal lung cells [122]. A growth inhibition of 82% and 73% was demonstrated in cervical and liver cells following exposure to 20 µM SF for 48 hours [127] and 75% growth inhibition of prostate cells treated for 48 hours with 10 µM SF [123].

SF was implicated in the activation of the intrinsic pathway, extrinsic pathway, and the caspase-independent pathway [12]. Activation of the intrinsic pathway involves release of cytochrome c from mitochondria induced by Bax and Bak resulting in the activation of caspase-9 and consequently activating caspase-3 [12]. Downregulation of anti-apoptotic proteins (Bcl-2 and $BclX_L$) and upregulation of pro-apoptotic proteins (Bax and Bak) involved in the intrinsic pathway is a key target in treating tumours. Overexpression of Bcl-2 is a classic feature of non-small cell lung cancer patients resulting in apoptotic resistance in cells [122]. SF was shown to downregulate Bcl-2 in lung cancer cells at a concentration of 1 μg/ml for 72 hours [122], in cervical cancer cells and in liver cells for 48 hours [127]. Whereas, upregulation of Bax and increased activation of caspase-3 was demonstrated in lung cells treated with 1 μg/ml SF for 72 hours [122]. In another study, upregulation of Bax in cervical cancer cells subject to SF for 48 hours was reported but not in liver cancer cells [127]. Upregulation of caspase-3 but no changes in caspase-9 was observed in cervical cancer cells and liver cancer cells [122,127]. Similarly, in bladder cells treated with SF, activity of caspase-3 was reported, but no activated caspase-9 was detected [124]. Activation of the extrinsic pathway comprises of activation of caspase-8 leading to the activation of caspase-3. SF significantly increased the activity of caspase-3 and caspase-8 in leukemia cells subject to 8 μM SF for 24-48 hours [125] and in prostate cells treated with 40 μM SF for 24 hours [129].

In many studies, the apoptotic effects of SF were demonstrated with ≥ 15 μM SF (IC_{50} of cell growth [122,127,129]). Given that these concentrations of SF does not reach the plasma following intake of commercially available broccoli by the general population, it is not physiologically relevant.

1.4.3. Biological activity of SMCSO

The combination of glucosinolates and SMCSO could contribute to the health benefits associated with cruciferous vegetables intake. In comparison to SF, SMCSO has not been extensively researched for its health benefits. However, it has been associated with anti-carcinogenic, anti-diabetic and cardiovascular effects using mice studies and *in vitro* work.

1.4.3.1. Anti-carcinogenic effects modulated by SMCSO

To establish whether SMCSO could be a chemopreventative agent, the effect of SMCSO and its metabolite MMTSI **(Figure 1-4)** was investigated by Marks *et al.* in a genotoxicity induced

mice model [41]. Genotoxicity was induced in mice by 8-fold with the treatment of benzo[*a*]pyrene, a tobacco carcinogen (0.75 mmol kg^{-1} body weight) resulting in the formation of micronucleated polychromatic erythrocytes. Mice that were pre-treated with either SMCSO (0.5 mmol kg^{-1} body weight) or MMTSI (0.05 mmol kg^{-1} body) presented lower levels of micronucleated polychromatic erythrocytes, diminished by 31% and by 33% respectively. These data indicated early signs that SMCSO and its metabolite, MMTSI could exhibit anti-carcinogenic effects. However, it is imperative to note that the metabolites derived from SMCSO such as MMTSI proved to be extremely toxic at high concentrations (1.0 mmol) evidenced by the death of 6 out of 7 mice [41]. Other *in vitro* studies have shown induction of apoptosis by thiosulfinates such as MMTSI leading to the inhibition of cell growth [130,131]. A crude extract of thiosulfinates largely made up of MMTSI demonstrated inhibitory effects on the growth of prostate cancer cells (RC-58T, DU-145, LNCap, PC-3), breast cancer cells (MCF-7) and liver cancer cells (HepG2) through the induction of apoptosis [130-132]. Mediation of apoptosis by thiosulfinates occurred via caspase-dependent and caspase-independent pathways. Thiosulfinates increased levels of caspase-3, caspase-8 and caspase-9 as well as increasing the levels of the pro-apoptotic protein Bax and decreasing the levels of the anti-apoptotic protein Bcl-2 in RC-58T cells [130] and in PC-3 cells [131]. Besides the activation of the caspase-dependent pathway, thiosulfinates induced the translocation of the AIF into the nucleus resulting in DNA fragmentation in RC-58T cells [130] and in PC-3 cells [131]. Furthermore, MMTSO, another SMCSO derived metabolite **(Figure 1-4)**, was reported to have chemopreventative effects in animal studies. Administration of MMTSO attenuated the development of aberrant crypt foci (lesions leading to colorectal cancer) induced by azoxymethane, a carcinogen [133]. Concurring with the work by Morishita *et al.* [133], co-administration of MMTSO and sulindac (an anti-inflammatory drug) in another study by Reddy *et al.* repressed the incidence of invasive adenocarcinomas [134]. SMCSO and its secondary metabolites, MMTSI and MMTSO have been demonstrated as promising chemopreventative agents. However further work is required in this area, especially the effect of these sulphur compounds *in vivo*.

1.4.3.2. Anti-diabetic and cardiovascular effects mediated by SMCSO

SMCSO has shown possible regulation of cholesterol in animal studies. Augmented cholesterol levels in the plasma and liver of rats induced by a hypercholesterolemic diet was suppressed following a diet with SMCSO [135,136]. In agreement, Kumari and Augusti.

reported that the treatment of SMCSO suppressed the elevated cholesterol levels in rats induced by a hypercholesterolemic diet [137]. In addition to the cholesterol levels, SMCSO treatment reduced the triglyceride, phospholipid and free fatty acid levels [137]. Another study by Kumari *et al*. found a depletion in the blood and urine glucose levels of diabetic rats following a two month SMCSO treatment [138]. Interestingly, increased bile acid excretion was observed following administration of SMCSO to rats [136,137]. The proposed mechanism for the lipid-lowering effects of SMCSO is through increased lipid catabolism and bile acid excretion [2]. Presumably, SMCSO, via its cysteine moiety, increases the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme responsible for bile acid synthesis from cholesterol [16,137]. This was supported by the increased activity of cholesterol 7α-hydroxylase detected in rats fed with SMCSO [136]. Possibilities for SMCSO to be used as anti-diabetic or anti-lipidemic agent have been demonstrated from the animal studies. Further work is necessary to confirm these effects using *in vitro* models and *in vivo* as well as to elucidate the mechanisms involved for said effects.

1.5. Thesis aims

A considerable amount of research has been undertaken to support the epidemiological evidence presenting an association between consumption of cruciferous vegetables and a reduction in the risk of chronic diseases. Much of this extensive research has employed human, animal and *in vitro* studies to investigate the effects of SF however many uncertainties remain. Further, SMCSO has been implicated to exhibit potential health benefits. However, the underlying mechanisms have not been extensively researched. As there are areas of SF and SMCSO research that remain unexplored, this thesis will address the bioavailability and bioactivity of sulphur-containing compounds with a series of aims as follows:

1. To date, a considerable number of *in vivo* studies have been conducted to assess the bioavailability of SF through the consumption of SF or its precursor, GR through a variety of matrices such as soups, florets, pills and extracts. The main limitation with most of these studies is the use of doses of SF/GR that may not be achievable in a day to day basis by the general population and are too high to encourage compliance during studies. Therefore, novel broccoli varieties with increasing levels of GR ($Myb28^{B/V}$ and $Myb28^{V/V}$) were previously developed at the John Innes Centre and manufactured into soups. Therefore, the aim of **Chapter 2** is to quantify the levels of sulphur compounds in the three types of soups to confirm that the increasing levels of GR observed in the broccoli florets is preserved in the soups.

2. The three types of soups produced by a leading food manufacturer as a portion size of 300g (comparable to commercially available soup portion sizes), enables compliance and is achievable *in vivo*. The aim of **Chapter 3** is therefore to quantify the bioavailability of sulphur-containing compounds including GR, SF and SMCSO in plasma and urine by means of undertaking a human intervention study.

3. The quantification of SF in biological samples has been reported, but limited studies have shown the quantification of GR and SMCSO in such samples. The presence of SMCSO and GR in plasma and urine poses an interesting question regarding their permeability. Previous reports assume that SF can passively diffuse through the enterocytes from the gut lumen into the blood circulation. However, the transport of GR and SMCSO remain unexplored. Therefore, **Chapter 4** aims to investigate the potential transport and the mechanism of these compounds using an artificial model (PAMPA) and a human colon model (caco-2).

4. Many of the health benefits reported with cruciferous vegetable intake has been primarily associated with SF. However, *Brassica* vegetables also contain high levels of SMCSO and early animal studies have indicated the possible anti-carcinogenic, anti-diabetic and anti-lipidemic effects of by SMCSO. Therefore, the aim of **Chapter 5** is to elucidate whether SMCSO, like SF, could exert changes to the expression of Nrf-2 regulated genes via the expression analysis of 10 genes following exposure of liver cells (HepG2) to SMCSO, SF, and a co-treatment of the two.

CHAPTER 2

Characterisation of bioactives in glucoraphanin enriched broccoli soups

2.1. Summary

Two high-glucoraphanin broccoli varieties were previously developed by cross-breeding standard commercially available broccoli, *Brassica oleracea var.italica*, with a wild broccoli species, *Brassica villosa*. This induced the introgression of several segments from *Brassica villosa* into the genome of standard broccoli, including a key transcription factor implicated in glucosinolate biosynthesis. Subsequently, broccoli soups were manufactured using these broccoli varieties to deliver high levels of sulphur-containing bioactives, GR and SMCSO *in vivo*. The soups were analysed to confirm whether the high levels of bioactives from the broccoli plant were retained during the soup production process. Analysis confirmed the soups accumulated increased levels of GR and SMCSO compared to standard broccoli soup. The development of these high-GR broccoli soups is of great importance to investigate the bioavailability and biological activity of sulphur-containing compounds from the novel broccoli varieties.

2.2. Introduction

Cruciferous vegetables including *Brassica oleracea* accumulate phytochemicals including glucosinolates and SMCSO. Glucosinolates uniquely accumulate in these plants at high levels as a defensive mechanism against pests and insects. Hence, glucosinolates and SMCSO have been associated with health-promoting effects because they may provide a defensive mechanism against carcinogens in the human body [2]. For this reason, developing cultivars with naturally enhanced levels of GR was a promising breakthrough. In the 1990s, pioneering research at the John Innes Centre (Norwich, UK) resulted in the development of cultivars by cross-breeding wild type broccoli *Brassica villosa* and commercially available broccoli. Due to the introgression of a novel Myb28^{V} allele from *Brassica villosa*, a transcription factor regulating glucosinolate biosynthesis, the two novel varieties (Myb28$^{B/V}$ and Myb28$^{V/V}$) accumulate enhanced GR levels compared to standard broccoli (Myb28$^{B/B}$) [16,18]. The consumption of GR from 1 portion of Myb28$^{B/V}$ florets is equivalent to ~ 3 portions of standard broccoli and 1 portion of Myb28$^{V/V}$ florets is equivalent to ~6 portions of standard broccoli (unpublished data). This provided a valuable dietary tool for clinical studies as many studies have employed doses of SF or GR not achievable in the general population.

Food matrix influences the palatability, compliance and absorption of dietary compounds. Dietary intervention studies summarised in **Chapter 1** used various matrices including pills, powder, raw or cooked florets, and soups. These matrices deliver problems concerned with

tolerance, diet compliance, and replication of cooking methods for long-term dietary studies and household cooking. Although pills made with pure SF seem to be an appealing idea to promote intake of SF especially for those who do not consume broccoli as their normal diet, it was not well tolerated by participants in a recent study [70]. In addition, synthesised SF is not stable at fridge/room temperatures and degrades easily. Although Fahey *et al.* developed a stabilised encapsulated SF [70], and drug company Evgen (Liverpool, UK) have developed Sulforadex®, a stabilised SF pill, it is a complex procedure. Homogenising florets to get an extract or powder may preserve the myrosinase and enable higher bioavailability of SF. However, it is not a food matrix that is palatable and it is laborious for long-term clinical studies/household cooking. Raw broccoli/lightly cooked broccoli is not appetising to the general population and it would be difficult to replicate cooking methods in households to ensure consistency. Soups used in previous studies represent a good food matrix, however previous studies have cooked broccoli in water and homogenised [38,68]. Again, this is not a palatable food source and compliance to diet may be low. Hence, there was scope to develop a food matrix that is palatable, ensures compliance, suitable for cooking in general households and clinical trials. Consequently, in collaboration with other companies including Monsanto®, A.P. East Anglia Ltd, Bakkavor Ltd, a leading food manufacturer, and Norbert Dentressangle, soups were developed according to a commercial broccoli and stilton recipe **(Figure 2-1)**. Three soups using the genotypes ($Myb28^{B/B}$, $Myb28^{B/V}$ and $Myb28^{V/V}$) were manufactured into freezable 300g portions. This provided a trouble-free dietary intervention suitable for long-term dietary interventions and cooking by volunteers in their households, unlike soups produced by other studies. With the potential to deliver enhanced levels of glucosinolates in human intervention studies, these palatable broccoli variety soups represent a feasible means of compliance in long-term dietary studies.

Figure 2-1: Collaborators involved in the process of developing broccoli soups using three genotypes. Broccoli cultivar seeds provided by Monsanto®, broccoli floreted and frozen by A.P. East Anglia Ltd, produced into broccoli and stilton soups by Bakkavor Ltd and stored in a food-grade long-term storage facility by Norbert Dentressangle.

This chapter explores whether the soup production, using the three broccoli varieties, $Myb28^{B/B}$ (standard broccoli), $Myb28^{B/V}$, and $Myb28^{V/V}$, preserved the high GR content and other sulphur containing bioactives. The main aim was to analyse the levels of glucosinolates, SMCSO, mineral elements and amino acids of the soups to establish whether the presence of one or more $Myb28^{V}$ alleles results in enhanced levels of glucosinolates and other compounds.

2.3. Materials and Methods

2.3.1. Materials

The following were purchased from Sigma® (UK); sinigrin hydrate, internal standard for GR (Cat. # 85440), DL-NorLeucine, internal standard for SMCSO (Cat # N1398), sulfate (TraceCERT®, 1000 mg/L) (Cat. # 90071), O-(carboxymethyl)hydroxylamine hemihydrochloride (OCMHA) (Cat. # C13408), sulfatase (Type H-1 from Helix pomatia) (Cat. # S9626), heptafluorobutyric (Cat. # 52411) and ammonium acetate (Cat. # 73594). Sulfatase was purified by passing through columns with DEAE Sephadex A25 (Cat. # 17-0170-01) and SP Sephadex C25 (Cat. # 17-0123-01) obtained from Amersham Biosciences (Sweden) as described previously [68]. The following was purchased from Cayman Chemical (Michigan, USA); S-methyl-L-cysteine sulfoxide (SMCSO) (CAS 6853-87-8) (purity ≥98%) (Cat # 17600) and GR (CAS 21414-41-5) (purity ≥95%) (Cat # 10009445). SF (CAS 4478-93-7) (purity > 98%) (Cat. # S8045) was purchased from LKT Laboratories (St. Paul, USA). Dulbecco's phosphate-buffered saline (DPBS) (Gibco®) 10X with no calcium and no magnesium was purchased from Thermo Fisher Scientific (Cat. # 14200059). Acetic acid was purchased from Biosolve (Cat. # 01074131).

All solvents and other chemicals used were of high-performance liquid chromatography (HPLC) grade with a purity of ≥95%, Acetonitrile (Cat. # 10407440) and methanol (Cat. # 10674922) purchased from Fischer Scientific. Only pure water from Milli-Q® Integral Water Purification System (Millipore Advantage) was used to dissolve chemicals.

Broccoli cultivars were grown in Spain and made into broccoli and stilton soup by Bakkavor Group Limited. Soups produced from each cultivar were freeze-dried and powdered using a food mixer. The freeze-dried samples were stored at -80ºC for SMCSO analysis and at room temperature for GR and sulphate analysis. Dr Shikha Saha (QIB analytical chemist) freeze-dried soup samples, performed the extraction and quantification of glucosinolates, SMCSO and sulphate. Analysis of mineral elements and amino acids were undertaken by Eurofin.

2.3.2. Glucosinolate analysis by high-performance liquid chromatography

2.3.2.1. Extraction of glucosinolates from samples

Glucosinolates were extracted and quantified using a method described by Magrath *et al*. [139] and Shikha *et al.* [68] involving the conversion of glucosinolates to desulfoglucosinolates. Hot aqueous 70% methanol (10 ml) was added to 50 mg freeze-dried powder. Following the addition of known amounts of sinigrin (internal standard), samples were vortexed and then incubated at 70ºC for 20-30 mins. After samples were cooled and centrifuged at 4000 x g for 10 mins at room temperature. After centrifugation, the supernatant (3 ml) was passed through an ion exchange column. Once the samples had passed through, the columns were washed with water (2 x 0.5 ml) and 0.02 M sodium acetate (2 x 0.5 ml). Purified sulfatase (75 µl) was applied to the column and incubated overnight at room temperature for desulfonation reaction of glucosinolates. Following an overnight incubation at room temperature, the desulfoglucosinolates were eluted by sequential addition of 0.5, 0.5 and 0.25 ml water and collected into the autosampler vials and analysed by HPLC.

2.3.2.2. High-performance liquid chromatography set-up and quantification of glucosinolates

Desulfoglucosinolates were analysed using a Waters Spherisorb ODS2 (250 × 4.6 mm , 5 µM particle size) connected to an Agilent Technologies HPLC system model 1100 (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump, degasser, cool autosampler, column oven and diode array detector. Ultrapure water and acetonitrile were used as solvents with a flow rate of 1 ml/min, and a gradient of an increasing proportion of acetonitrile from 5% to 100% over 25 minutes was applied, prior to re-equilibration to 5% for 7 minutes. Desulphoglucosinolate quantification was achieved, using absorbance at 229 nm, by comparison with the peak area ratio of the internal standard (sinigrin), and the relevant desulphoglucosinolate ultraviolet (UV) light relative response factor.

2.3.3. Amino acid analysis by high-performance liquid chromatography

Freeze dried samples were sent to Eurofins UK (Eurofins Food Testing UK Ltd, i54 Business Park, Valiant Way, Wolverhampton, WV9 5 GB) for analysis of amino acids using an accredited HPLC method. All amino acids apart from cysteine, methionine and tyrosine, can be detected in their oxidised or unoxidized sample. However, cysteine and methionine must be oxidised to cysteic acid and methionine sulphone respectively, and tyrosine can only be analysed in the unoxidized sample. Oxidation of amino acids was carried out at 0ºC with a solution of performic acid/phenol mixture for 16 hours. Excess oxidation reagent was decomposed with sodium disulfite. Prior to analysis, oxidised or unoxidized samples were hydrolysed with hydrochloric acid for 23 hours and the hydrolysate was adjusted to pH 2.2 prior to running the samples on the HPLC. Internal standard and citrate buffer were added to the hydrolysate and filtered through a 0.2 µM filter. Amino acids were separated by ion exchange chromatography. The product from the reaction of the amino acids with ninhydrin was detected at 570 nm (440 nm for proline).

2.3.4. S-methyl-L-cysteine sulfoxide analysis by ultra-performance liquid chromatography-tandem mass spectrometry

2.3.4.1. Extraction of S-methyl-L-cysteine sulfoxide from samples

Extraction of SMCSO from freeze-dried soup samples was undertaken as described by Bernaert N *et al.* [140]. Freeze-dried powder (45-50 mg) was diluted with 1.1 mg/ml of OCMHA (O-(carboxymethyl)hydroxylamine hemihydrochloride). The addition of OCMHA inhibits the allinase activity to stop the breakdown of SMCSO. The solution was placed on a horizontal shaker for 10 minutes and centrifuged at 4000 x g for 10 minutes. The supernatant was further diluted by 10-fold with the addition of 0.1% formic acid in Milli-Q® water into autosampler vials and analysed by UPLC-MS/MS.

2.3.4.2. UPLC-MS/MS set-up and quantification of S-methyl-L-cysteine sulfoxide

A 1 mg/ml SMCSO stock was prepared in Milli-Q® water and a serial dilution was produced in the same matrix as the samples that were run. Serial dilutions were prepared prior to each run to generate a 4-6 point standard curve ranging from 0 µg/ml to 50 µg/ml. Analysis was performed using an Agilent 6490 Triple Quad LC-MS mass spectrometer equipped with a 4ºC autosampler, a degasser, binary pump, column oven, diode array detector and 6490 mass spectrometer. The column temperature and auto sampler were maintained at 20°C and 4°C respectively.

Samples were injected at 2 µl eluted at a flow rate of 0.3 ml/min on an Agilent SB-AQ 1.8 uM (100 x 2.1mm) C18 column. Separation was carried out using 10 mM ammonium acetate + 0.05% heptafluorobutyric acid in water (mobile phase A) and 10 mM ammonium acetate + 0.05% heptafluorobutyric acid in 90% methanol (mobile phase B). The gradient started at 2 % mobile phase B increasing over 2 mins to 5% mobile phase B and finally re-equilibrated to 2% mobile phase B for 2 mins. The LC eluent flow was sprayed into the mass spectrometer interface without splitting. SMCSO ion was monitored using mass spectrometry multiple reaction monitoring mode (MRM) in positive polarity with electrospray ionization (ESI) source. The source parameters were; a gas temperature at 200˚C with a gas flow of 16 l/minute, a sheath gas temperature at 300˚C with a sheath gas flow of 11 l/minute, a nebuliser pressure of 50 psi and capillary voltage at 3500V. The quantification was performed using matrix match calibration curve. Identification was achieved based on retention time and product ions monitored summarized in Table 2-1.

Table 2-1: Summary of the monitored product ions of SMCSO and the optimised MS operating parameters of the analyte.

Analyte	Retention time (mins)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy	Cell accelerator energy	Polarity
SMCSO	0.98	152.19	87.9	4	4	Positive
SMCSO	0.98	152.19	69.9	16	4	Positive
SMCSO	0.98	152.19	42.1	20	4	Positive

2.3.5. Sulphate analysis by liquid chromatography- mass spectrometry

2.3.5.1. Extraction of sulphate from samples

Analysis of sulphate was carried out as described elsewhere [141]. Milli-Q® water (1 ml) was added to 25g of washed and grounded PVPP (polyvinylpolypyrrolidone) and stored at 4ºC overnight. Powdered soup samples (20-30 mg) added to the solution of PVPP in water was vortexed briefly followed by shaking for 1 hour at 4ºC. Samples were then incubated at 95ºC for 15 mins and centrifuged at 4000 x g 4ºC for 15 mins. The supernatant was filtered using a PVDF 0.45 µm syringe filter into autosampler vials and analysed on an 1100 series Agilent Single Quad LC-MS mass spectrometer.

2.3.5.2. LC-MS set up and quantification of sulphate

Samples were eluted at 0.8 ml/min using an Acclaim™ Trinity™ P1 3 µm (3 x 50 mm) column and separated using 200 mM ammonium acetate with acetic acid, pH4 (mobile phase A) and 60% acetonitrile/40% ultrapure water (mobile phase B). The gradient started at 5 % mobile phase A increasing over 15 min to 90% solvent A and finally re-equilibrated to 5% solvent A for 5 min. The LC eluent flow was sprayed into the mass spectrometer interface without splitting. Sulfate was monitored using mass spectrometry in selective ion monitor mode (*m/z* = 97) in negative polarity with (ESI). The quantification was performed using matrix match calibration curve of the standard. Identification was achieved on the basis of retention time of the standard.

2.3.6. Data analysis from liquid chromatography-tandem mass spectrometry

Data files are exported and analysed on Agilent MassHunter Quantitative analysis B.06.00/Build 6.0.388.0 (Agilent Technologies©). The software manually integrates the peak area for the metabolites which is then exported as an Excel document. The concentration of the metabolites was calculated using the equation of the standard curve and the peak area of the metabolites.

2.3.7. Mineral elements analysis by inductively- coupled conductive plasma optical emission spectrometry

Freeze dried broccoli soup samples were sent to Eurofins UK (Eurofins Food Testing UK Ltd, i54 Business Park, Valiant Way, Wolverhampton, WV9 5 GB) for analysis of mineral elements including sodium, potassium, sulphur, calcium, magnesium, phosphorus, iron and zinc. Analysis of mineral elements was undertaken using inductively coupled plasma optical emission spectrometry (ICP-OES). Samples were first digested using microwave assisted accelerated digestion by adding concentrated nitric acid to 1 g of homogenised sample. Digested samples were then analysed on an Agilent 700 series ICP-OES. Limits of detection and quantification were provided by Eurofins UK **(Table 2-2)**.

Table 2-2: Limit of detection and quantification of mineral elements analysed by inductively coupled plasma optical emission spectrometry

List of Minerals	LOD	LOQ
Calcium	0.0001g/100g	0.0005g/100g
Phosphorus	0.00004g/100g	0.0002g/100g
Iron	0.2 mg/kg	1 mg/kg
Zinc	0.4 mg/kg	2 mg/kg
Sodium	0.0002g/100g	0.001g/100g
Potassium	0.001g/100g	0.005g/100g
Magnesium	0.0001g/100g	0.0005g/100g

2.4. Results

2.4.1. Increasing levels of glucoraphanin in soups with the presence of one or more Myb28^{V} alleles

To assess whether the presence of one or more Myb28^{V} alleles influenced the concentration of glucosinolates in the soups, freeze-dried samples were analysed using HPLC **(Figure 2-2)**. Amongst the glucosinolates derived from broccoli, the predominant glucosinolate is 4-MSB, GR as illustrated in **Figure 2-3**. The second major glucosinolate detected is 3-MSP, glucoiberin, followed by indoles (IND), 4-methoxyindole glucosinolate (4-MIND), hydroxy-indolyl methyl glucosinolate (OH-IND) and 1-methoxyindole glucosinolate (1-MIND). The level of 4-MSB is approximately 5-fold higher compared to MSP in the Myb28$^{B/B}$ broccoli soup. The presence of one or two Myb28^{V} alleles significantly increased the concentration of 4-MSB and 4-MIND ($p<0.0001$). Myb28$^{V/V}$ and Myb28$^{B/V}$ broccoli soups accumulate a statistically significant ~5-fold and ~3-fold elevated levels of 4-MSB respectively compared to Myb28$^{B/B}$ broccoli soup (Myb28$^{V/V}$, 452 µmoles; Myb28$^{B/V}$, 280 µmoles; vs. Myb28$^{B/B}$, 84 µmoles; $p<0.0001$). Similarly, the presence of one or more Myb28^{V} alleles resulted in a singificant ~2-fold and ~4-fold enhanced levels of 4-MIND respectively compared to Myb28$^{B/B}$ broccoli soup (Myb28$^{V/V}$, 49.1 µmoles; Myb28$^{B/V}$, 27.5 µmoles; vs. Myb28$^{B/B}$, 13.5 µmoles; $p<0.0001$). Significant increase in the levels of 3-MSP was observed in the Myb28$^{B/V}$ soups ($p<0.0001$) but not in Myb28$^{V/V}$ soups compared to Myb28$^{B/B}$. On the contrary, levels of IND were significantly lower in the Myb28$^{B/V}$ soups ($p<0.0001$) compared to Myb28$^{V/V}$ and Myb28$^{B/B}$. Whereas, the levels of OHIND were only significant in the Myb28$^{V/V}$ soups ($p<0.01$). No significant differences were detected in the levels of 1-MIND between the three varieties. No glucosinolate breakdown products was detected in the soups confirming that the soups only contain glucosinolates.

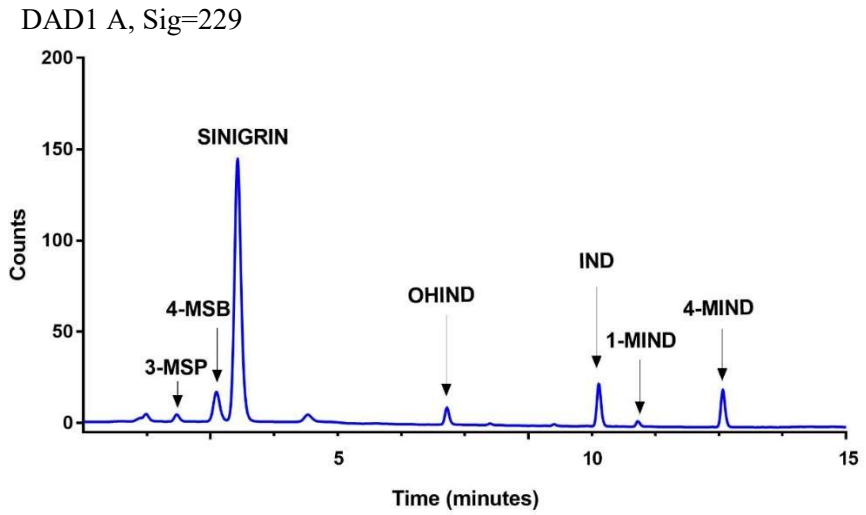


Figure 2-2: Chromatogram of glucosinolates profile in standard soup. An example of a chromatogram from the analysis of standard soup using HPLC system and absorbance measured at 229 nm. Quantification was relative to the internal standard peak sinigrin. Glucosinolates quantified include 3-MSP, 4-MSB, IND, 1-MIND and 4-MIND as shown in the chromatogram.

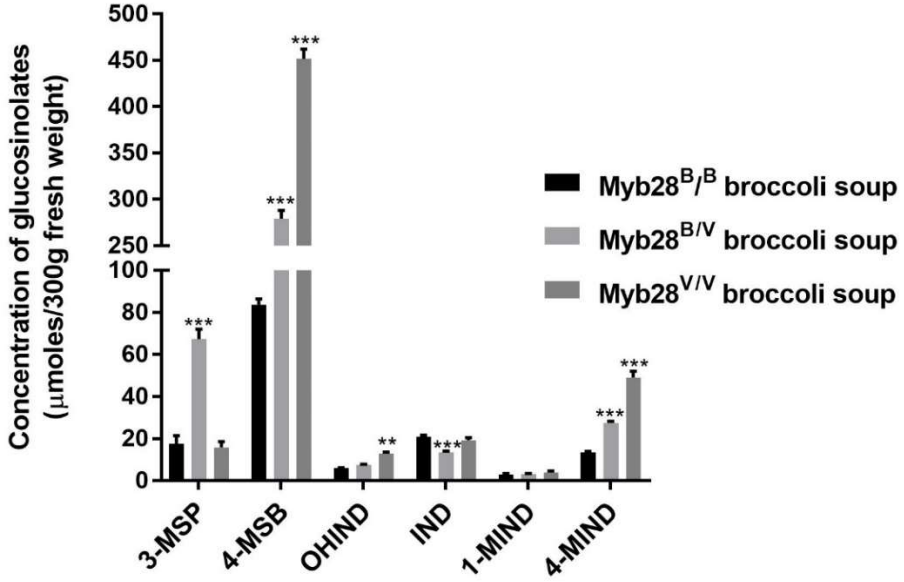
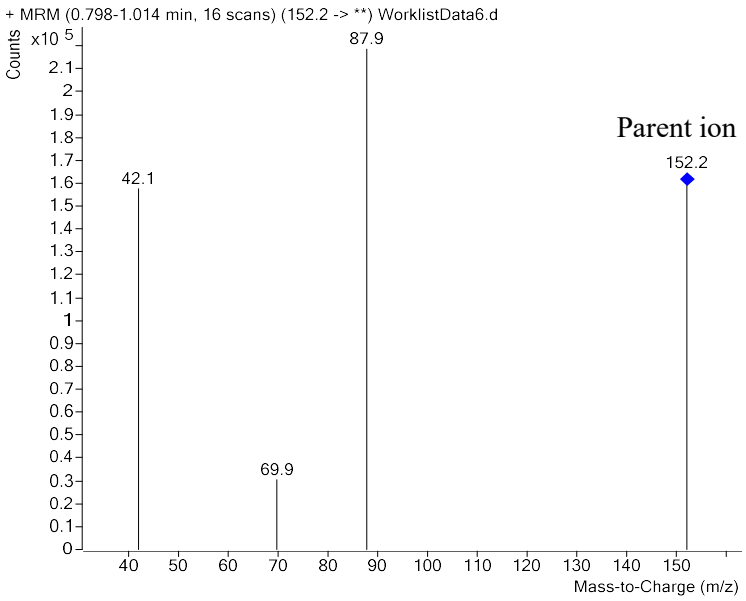


Figure 2-3: Concentration of glucosinolates in three varieties of broccoli soups. Freeze-dried soup samples were analysed and quantified for glucosinolates using HPLC. Glucosinolates measured includes 3-MSP, 4-MSB, IND, OHIND, 1-MIND and 4-MIND. Data is represented as mean ± SD (n=10, Myb28$^{B/B}$, Myb28$^{B/V}$ soups; n=4, Myb28$^{V/V}$ soups), 'n' varies due to differing stability of the broccoli lines. Statistical analysis of the data was undertaken by two-way ANOVA with Dunnett's multiple comparisons test (p<0.01 and *** p<0.0001 vs. Myb28$^{B/B}$ broccoli soup).**

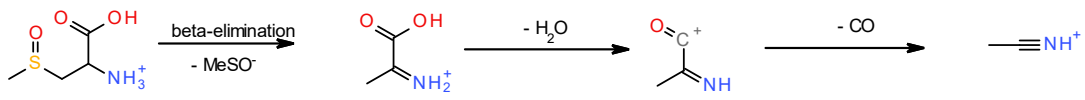
2.4.2. Increasing levels of S-methyl-L-cysteine sulfoxide in soups with the presence of one or more Myb28^{V} alleles

To evaluate whether the presence of one or more Myb28^{V} alleles impacts on the concentration of SMCSO in the soups, freeze-dried samples were analysed for SMCSO using UPLC-MS/MS. Spectrum from the UPLC-MS/MS shows the fragmentation of the parent ion to daughter ions **(Figure 2-4)**, of which the daughter ion *(m/z* = 87.9) was quantified in samples **(Figure 2-5).** As evidenced by **Figure 2-6**, the presence of one or more Myb28^{V} alleles resulted in significantly higher levels of SMCSO by ~1.4-1.5-fold compared to Myb28$^{B/B}$ broccoli soup (Myb28$^{V/V}$, 1454 µmoles; Myb28$^{B/V}$, 1514 µmoles vs. Myb28$^{B/B}$, 1030 µmoles; $p<0.001$). However, no significant differences were observed between the soups made with Myb28$^{B/V}$ and Myb28$^{V/V}$ broccoli.

A



B



m/z = 152.2 *m/z* = 87.9 *m/z* = 69.9 *m/z*= 42.1

Figure 2-4: Mass spectrum of the fragmentation of the SMCSO parent ion to produce daughter ions. An example of a chromatogram from the analysis of standard soup using UPLC-MS/MS system in the multiple reaction monitoring (A) and structures of the fragmentation ions produced from the parent ions (B).

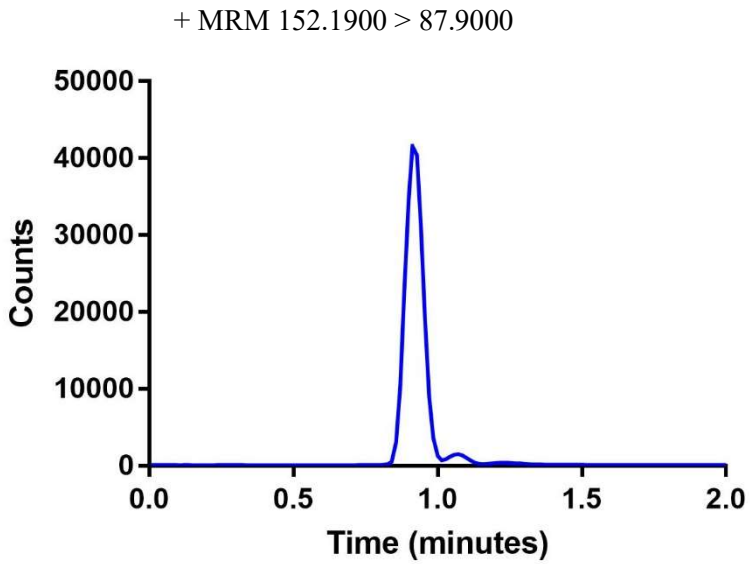


Figure 2-5: Chromatogram of the SMCSO daughter ion (*m/z* = 87.9) quantified in broccoli soup. An example of a chromatogram from the analysis of standard soup using UPLC-MS/MS system. Fragmentation of the daughter ion (*m/z* = 87.9) from parent ion (*m/z* = 152.2) was extracted using MRM mode from the ESI-MS positive mode.

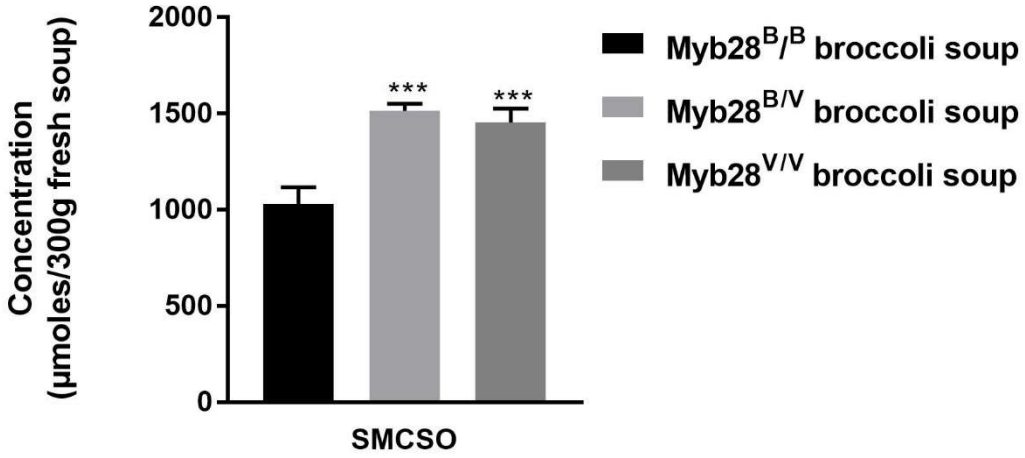
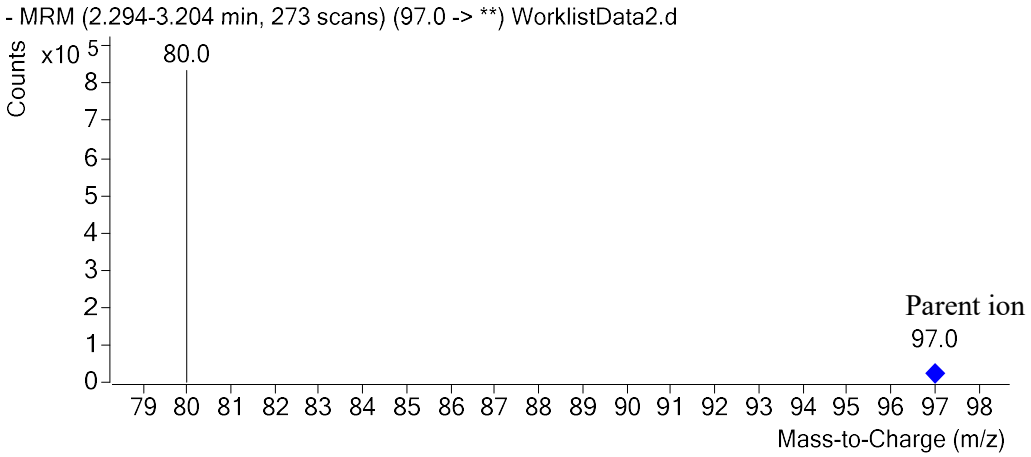


Figure 2-6: Concentration of SMCSO in three varieties of broccoli soups. Freeze-dried soup samples were analysed and quantified for SMCSO using UPLC-MS/MS. Data is represented as mean ± SD (n=3). Statistical analysis of the data was undertaken by one-way ANOVA with Dunnett's multiple comparisons test (* p<0.001 vs. Myb28$^{B/B}$ broccoli soup).**

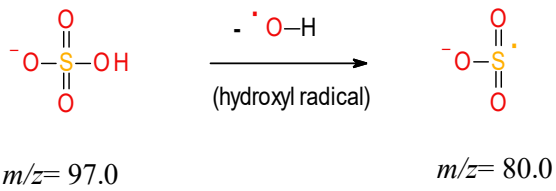
2.4.3. Sulphate levels in soups with the presence of one more Myb28^{V} alleles

Sulphate levels were measured in soups made with three varieties using LC-MS to determine whether the levels would vary with the three varieties. LC-MS spectrum illustrates the production of daughter ions from the parent ion **(Figure 2-7)**. The daughter ion (*m/z*= 80.0) was quantified in samples **(Figure 2-8)**. The three broccoli varieties accumulated high levels of sulphate. The levels in Myb28$^{B/V}$ were significantly reduced compared to Myb28$^{B/B}$ (Myb28$^{B/V}$, 445 µmoles vs. Myb28$^{B/B}$, 531 µmoles; $p<0.05$) whereas, the levels between Myb28$^{V/V}$ and Myb28$^{B/B}$ were not significantly different (Myb28$^{V/V}$, 555 µmoles vs. Myb28$^{B/B}$, 531 µmoles) **(Figure 2-9).**

A



B



m/z= 97.0 *m/z*= 80.0

Figure 2-7: Mass spectrum of the fragmentation of the sulphate parent ion to produce daughter ion. An example of a chromatogram from the analysis of standard soup using LC-MS system in the SIM mode (A) and structures of the daughter fragmentation ion produced from the parent ion (B).

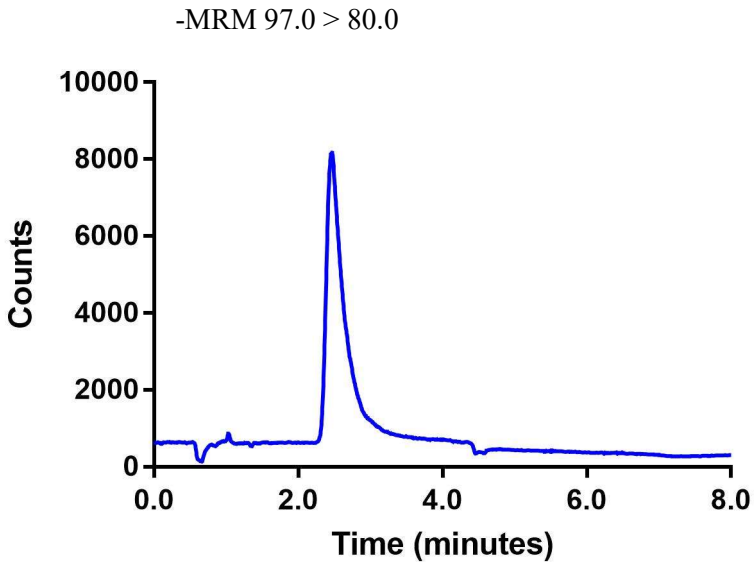


Figure 2-8: Chromatogram of the sulphate daughter ion (*m/z* = 80.0) quantified in broccoli soup. An example of a chromatogram from the analysis of standard soup using LC-MS system. Fragmentation of the daughter ion (*m/z* = 80.0) from parent ion (*m/z* = 97.0) was extracted using SIM mode from the ESI-MS negative mode.

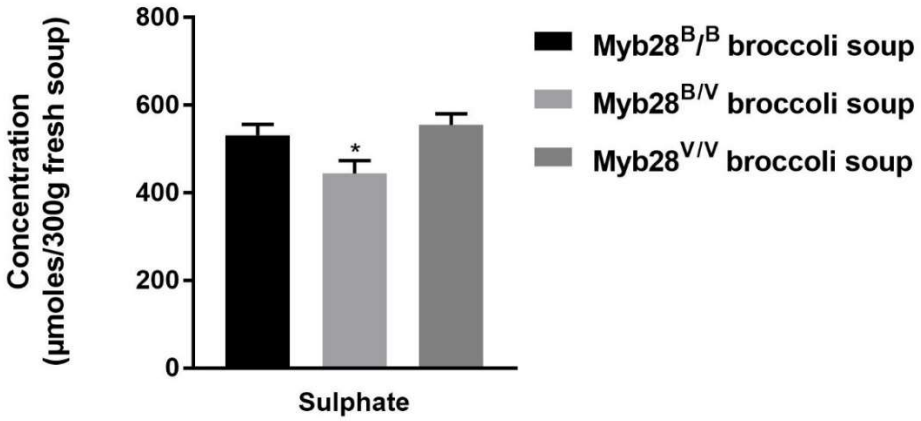
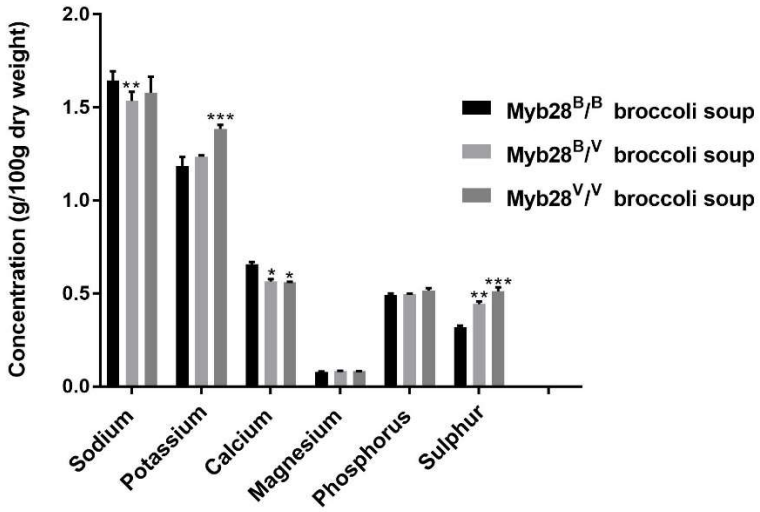


Figure 2-9: Concentration of sulphate in three varieties of broccoli soups. Freeze-dried soup samples were analysed and quantified for sulphate using LC-MS. Data is represented as mean ± SD (n=3, Myb28$^{B/B}$, Myb28$^{V/V}$ soups; n=2 Myb28$^{B/V}$ soup). Statitical analysis of the data was undertaken by one-way ANOVA with Dunnett's multiple comparisons test (* $p<0.05$ vs. Myb28$^{B/B}$ broccoli soup).

2.4.4. Increased levels of potassium, sulphur and iron in the presence of one or more Myb28^{V} alleles

In order to establish whether the presence of one or more Myb28^{V} alleles effects the concentration of mineral elements, soups made with three broccoli varieties were quantified using ICP-OES **(Figure 2-10)**. The soups had enhanced levels of sodium and potassium compared to other mineral elements. Potassium levels in Myb28$^{V/V}$ broccoli soup is significantly higher compared to Myb28$^{B/B}$ broccoli soup ($p<0.001$). No significant changes were observed in the levels of magnesium, phosphorus and zinc between the three broccoli varieties. Interestingly a significant decrease in calcium was detected in the presence of one or more Myb28^{V} alleles ($p<0.05$). In contrast, the levels of sulphur and iron significantly increased in the presence of one or more Myb28^{V} alleles ($p<0.001$). Sulphur levels increased by ~1-fold and ~2-fold in the Myb28$^{B/V}$ broccoli soup and Myb28$^{V/V}$ broccoli soup respectively, compared to Myb28$^{B/B}$ broccoli soup. Whereas the presence of one or two Myb28^{V} alleles enhanced the levels of iron by ~2-fold compared to Myb28$^{B/B}$ broccoli soup.

A



B

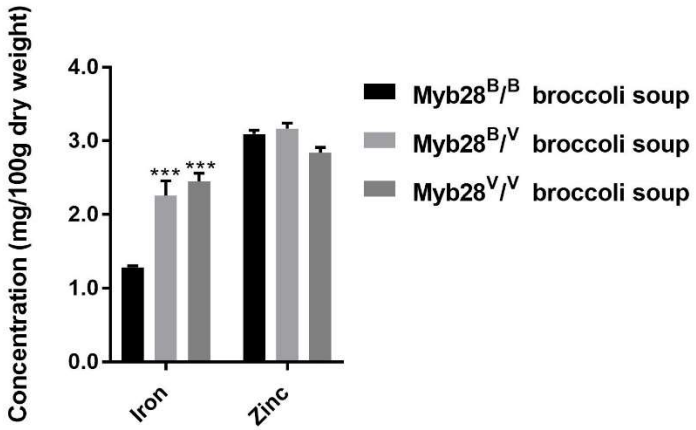


Figure 2-10: Concentration of mineral elements in three varieties of broccoli soups. Freeze-dried soup samples were analysed and quantified using ICP- OES. The following mineral elements were quantified; sodium, potassium, calcium, magnesium, phosphorus and sulphur in g/100g dry weight (A) and iron and zinc in mg/100g dry weight (B). Data is represented as mean ± SD (n=2). Statistical analysis of the data was undertaken by two-way ANOVA with Dunnett's multiple comparisons test (* p<0.05, ** p<0.01 and *** p<0.001 vs Myb28$^{B/B}$ broccoli soup).

2.4.5. Increased levels of amino acids in the presence of one or more Myb28^{V} alleles

Eighteen amino acids were analysed in the soups using HPLC to explore the levels of amino acids in the presence of one or more Myb28^{V} alleles **(Figure 2-11)**. Foremost is glutamic acid, found in high levels in all three broccoli varieties compared to all 18 amino acids. No significant differences were observed in the levels of phenylalanine, threonine, tyrosine, cysteine and cystine, methionine, or tryptophan. Interestingly, the levels of alanine, glycine, histidine, lysine, isoleucine, leucine, serine, and valine were only significantly increased in the presence of one Myb28^{V} allele ($p<0.05$). In contrast, the presence of one or more Myb28^{V} alleles resulted in significant increased levels of arginine, aspartic acid, glutamic acid, and proline relative to Myb28$^{B/B}$ broccoli soup ($p<0.0001$). There is approximately a 1.3-1.4-fold difference in the levels of glutamic acid in the presence of one or more Myb28^{V} alleles respectively compared to Myb28$^{B/B}$ broccoli soup.

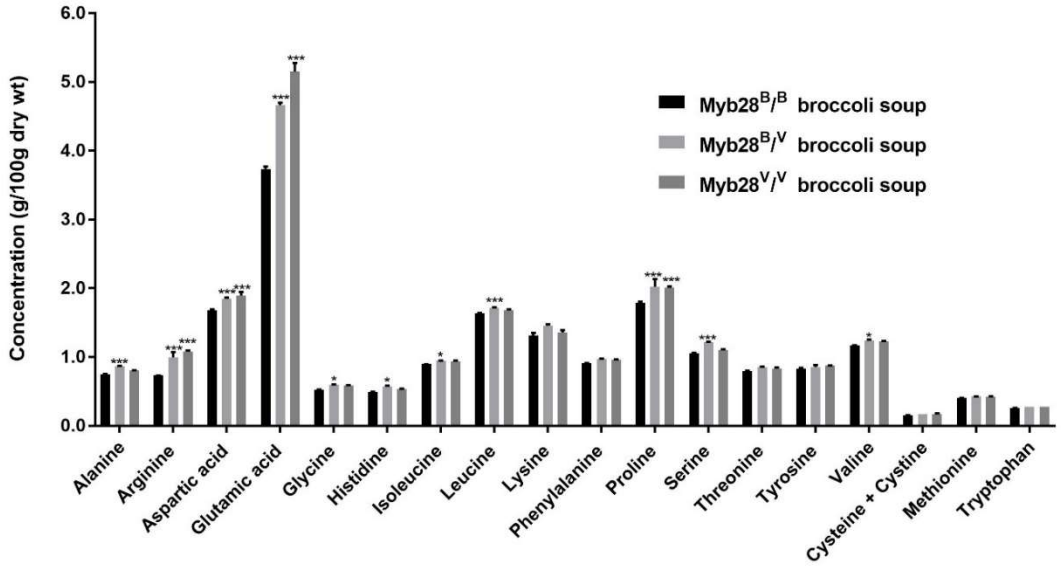


Figure 2-11: Concentration of amino acids in three varieties of broccoli soups. Freeze-dried soup samples were analysed and quantified using HPLC. Data is represented as mean ± SD (n=2). Statistical analysis of the data was undertaken by two-way ANOVA with Dunnett's multiple comparisons test (* $p<0.05$ and *** $p<0.001$ vs $Myb28^{B/B}$ broccoli soup).

2.5. Discussion

Broccoli varieties containing one or two $Myb28^{V}$ alleles were developed by conventional cross-breeding with enhanced levels of 4-MSB, the precursor of the bioactive ITC, SF. These broccoli varieties were made into soups for use in long-term human intervention studies. The three varieties of broccoli soups were analysed and quantified for glucosinolates, SMCSO, sulphate, mineral elements and amino acids. This chapter investigated the hypothesis that the presence of one or more $Myb28^{V}$ alleles would result in enhanced levels of GR and SMCSO being carried into the resultant soups.

The example of the chromatogram obtained from the analysis of glucosinolates from $Myb28^{B/B}$ soups, demonstrated that the highest peak observed is 2-propenylglucosinolate (sinigrin) followed by 4-MSB and 1-MIND. The high peak of sinigrin is a result of spiking samples with sinigrin, the internal standard for glucosinolates analysis. As hypothesised, the presence of one or two $Myb28^{V}$ alleles significantly increased the levels of 4-MSB in the broccoli soups compared to $Myb28^{B/B}$ broccoli soup by ~3-fold and ~5-fold, respectively ($Myb28^{B/V}$, 280 µmoles; $Myb28^{V/V}$, 452 µmoles; vs. $Myb28^{B/B}$, 84 µmoles; $p<0.0001$). The levels of 4-MSB in the broccoli plant were measured as a 6-fold and 3-fold increase with the $Myb28^{V/V}$ and

Myb28$^{B/V}$ broccoli genotypes respectively (unpublished data; Myb28$^{V/V}$, 31.30 µmoles/100 g fresh weight; Myb28$^{B/V}$; 17.60 µmoles/100 g fresh weight vs. Myb28$^{B/B}$, 5.40 µmoles/100 g fresh weight). The variation in the fold levels of GR between Myb28$^{B/B}$ and Myb28$^{V/V}$ in the soups and broccoli plant is due to variation in the 4-MSB levels in different Myb28$^{V/V}$ batches.

The increase in the GR levels with the presence of one or more Myb28^{V} alleles corroborates the implication of Myb28 transcription factor in glucosinolate biosynthesis. In addition, the levels of 4-MIND were also elevated in the broccoli soups containing one or two Myb28^{V} alleles compared to Myb28$^{B/B}$ broccoli soup. As the levels of 4-MSP is enhanced in the soups in the presence of one or two Myb28^{V} alleles and it is an aliphatic glucosinolate derived from mainly methionine, it was presumed that the methionine levels might be significantly different between the three varieties. However, as illustrated in **Figure 2-11**, methionine levels in the soups were not significantly different between the three varieties while the other amino acids used to synthesis aliphatic glucosinolates, valine and leucine, were significantly upregulated in Myb28$^{B/V}$ broccoli soups but not in Myb28$^{V/V}$ broccoli soups ($p<0.05$). Similarly, even though tryptophan-derived indolic glucosinolate, 4-MIND was elevated in Myb28$^{B/V}$ and Myb28$^{V/V}$ broccoli soups, tryptophan levels were not different between the three soup varieties as represented in **Figure 2-11**. Further, significant difference was observed in the levels of several amino acids in the presence one or more Myb28^{V} allele compared to Myb28$^{B/B}$ ($p<0.05$). This is probably due to the variation in growing conditions such as soil nutrient content. In agreement with Traka *et al.* [18], the presence of one or two Myb28^{V} alleles resulted in increased total sulphur levels **(Figure 2-10 A)** and no changes in sulphate **(Figure 2-9)** compared to Myb28$^{B/B}$. It is likely that the presence of Myb28^{V} allele increases sulphate assimilation, providing sulphur for glucosinolate biosynthesis resulting in enhanced levels of 4-MSB in Myb28$^{B/V}$ and Myb28$^{V/V}$ broccoli soups as proposed by Traka *et al.* [18]. Levels of 4-MSB were enhanced by ~3-fold in the Myb28$^{B/V}$ broccoli soup compared to Myb28$^{B/B}$ broccoli soup which is comparable to that reported previously in the florets [18]. In contrast to the lower SMCSO concentration reported in the florets of Myb28$^{B/V}$ compared to Myb28$^{B/B}$ previously [18], the current analysis of the soups demonstrated increased levels of SMCSO in the presence of one or more Myb28^{V} alleles compared to Myb28$^{B/B}$ broccoli soup (Myb28$^{V/V}$, 1454 µmoles; Myb28$^{B/V}$, 1514 µmoles vs. Myb28$^{B/B}$, 1030 µmoles; $p<0.001$). It is predicted that this could be a consequence of the soup production. The aim was to produce a food product that could deliver high levels of sulphur-containing bioactives *in vivo* and be used in future long-term intervention studies. These results confirm that increasing GR levels in the broccoli varieties is translated into increasing GR levels within the soups and that the processing does not affect the content. This is important for the development of a consistent dietary intervention.

Due to technical limitations with the internal standard D-NorLeucine for SMCSO, quantification was undertaken without the internal standard. An unknown interfering peak was appearing at the same retention time as D-NorLeucine (internal standard for SMCSO). The internal standard is crucial to allow monitoring and correction for signal fluctuation in the samples. However, the signal from the system suitability and calibration curve were monitored.

2.6. Conclusion

Through a breeding programme initiated at the John Innes Centre, two high-glucoraphanin trait broccoli varieties, heterozygous and homozygous for the $Myb28^{V}$ allele from *Brassica villosa,* were developed. These high-glucoraphanin varieties ($Myb28^{B/V}$ and $Myb28^{V/V}$) and standard commercially available broccoli ($Myb28^{B/B}$) were manufactured into soups. Analysis confirmed that the presence of one or two $Myb28^{V}$ alleles resulted in a ~3-fold and ~5-fold enhanced levels of 4-MSB (GR) in the soups ($Myb28^{V/V}$, 452 µmoles; $Myb28^{B/V}$, 280 µmoles; vs. $Myb28^{B/B}$, 84 µmoles). Further, the $Myb28^{B/V}$ and $Myb28^{V/V}$ soups accumulated increased levels of SMCSO compared to $Myb28^{B/B}$ broccoli soups ($Myb28^{V/V}$, 1454 µmoles; $Myb28^{B/V}$, 1514 µmoles vs. $Myb28^{B/B}$, 1030 µmoles). Consequently, a human intervention study was undertaken following on from this work to gain an insight into the bioavailability of GR and SMCSO from these soups containing increased levels of GR and SMCSO. In addition, the potential role of SMCSO will be explored based on the levels physiologically achievable from the soups.

CHAPTER 3

Bioavailability sulforaphane, glucoraphanin and S-methyl-L-cysteine sulfoxide in humans

3.1. Summary

Novel broccoli varieties that were heterozygous or homozygous for the wild-type broccoli, *Brassica villosa* allele Myb28^{V} were developed at the John Innes Centre. The levels of sulphur-containing bioactives such as GR and SMCSO were considerably higher compared to broccoli genotypes that were homozygous for the standard broccoli allele Myb28^{B}. As a practical means of delivering enhanced levels of bioactives, the three broccoli varieties (Myb28$^{B/B}$, Myb28$^{B/V}$ and Myb28$^{V/V}$) were manufactured into broccoli and stilton soups that retained the increased levels as described in **Chapter 2**. The aim of the work presented in this Chapter was to undertake a human intervention study to assess the pharmacokinetics of GR and other sulphur-containing bioactives (SF and SMCSO) from consumption of the soups. The presence of one or two Myb28^{V} alleles resulted in significantly higher plasma concentrations and a greater urinary excretion of GR, SF and SMCSO compared to standard broccoli. The results from this study have provided information on the levels of sulphur-containing compounds achievable *in vivo*.

3.2. Introduction

The previous Chapter demonstrated the enhanced concentration of GR and SMCSO in the novel varieties Myb28$^{B/V}$ and Myb28$^{V/V}$ compared to Myb28$^{B/B}$ (standard broccoli). The soups made from these varieties accumulated a ~5-fold (Myb28$^{V/V}$) and ~3-fold (Myb28$^{B/V}$) increase in GR content compared to those made with Myb28$^{B/B}$. Likewise, elevated levels of SMCSO, relative to Myb28$^{B/B}$ broccoli, were quantified in the Myb28$^{B/V}$ and Myb28$^{V/V}$ soups. In this project, a human study was designed and undertaken to investigate whether these enhanced levels are bioavailable. Bioavailability describes the amount of a nutrient from the consumption dose that is absorbed into the systemic circulation, distributed to target tissues, and is excreted.

A considerable amount of epidemiological evidence suggests that consumption of cruciferous vegetables such as broccoli is associated with a reduction in the risk of chronic diseases. This is mainly attributed to ITCs which exhibit health-promoting properties. To date, SF is the most extensively studied ITC in *in vivo* and *in vitro* models. SF is generated enzymatically by the hydrolysis of GR, the most abundant glucosinolate in broccoli. This reaction is catalysed by a plant enzyme called myrosinase that is denatured by thermal degradation through cooking at high temperatures. However microbial thioglucosidase, present in the gut, can also catalyse the reaction [12] **(Figure 1-2, Chapter 1)**. There is also evidence of the production of SF from

another isothiocyanate (4-methylthiobutyl isothiocyanate, erucin) and its precursor glucoerucin, commonly found in rocket salads. Like its analogue GR, glucoerucin is enzymatically converted by myrosinase to the ITC, erucin. Erucin and SF can undergo conversion by oxidation/reduction [142]. It is now well established that the health benefits associated with the consumption of cruciferous vegetables are the outcome of the biological activity of ITCs. SF is a potent inducer of Nrf2, a transcription factor causing the up-regulation of many phase II detoxification enzymes [65]. Recent work has also demonstrated the potential biological activity of erucin, including induction of phase 2 detoxification enzymes in rats [143] and inhibition of proliferating cells by p53 and p21 [144]. However, the potency of erucin is less than that of SF [145]. Following conversion of SF from GR by our gut microbial thioglucosidase, SF is absorbed by the enterocytes. Subsequently, SF is conjugated to glutathione, undergoes further metabolism via the mercapturic acid pathway and is excreted as a *N*-acetylcysteine conjugate [38] **(Figure 1-3, Chapter 1).** In addition to glucosinolates, *Brassica* vegetables such as broccoli contain high levels of another sulphur compound, SMCSO. Limited research has been conducted on SMCSO, but it has been reported that it may potentially have health benefits such as anti-carcinogenic, anti-diabetic and cardiovascular effects [2]. Therefore, this study aimed to assess the bioavailability of SMCSO as levels achievable *in vivo* will aid in investigating its biological activity at physiologically relevant concentrations.

Several studies have been undertaken to quantify the pharmacokinetics of SF using various doses of SF and GR. As previously reported, SF is more bioavailable from a direct intake of SF than from an intake of GR [22,38,66,69,71]. However, broccoli is often consumed cooked rather than raw, in which more SF would be present because the myrosinase will still be active. For this reason, novel broccoli varieties with enhanced levels of GR were developed to deliver increased concentrations of GR to the gut. This will be the first study to investigate the bioavailability of SMCSO and SF from these novel broccoli soups. Two other studies previously published by our group investigated the bioavailability of SF from different broccoli-rich "soups" [38,68]. However, the soups used in those studies were produced by the study scientists cooking broccoli in water and homogenising the cooked broccoli using a blender. To achieve palatable soups, a commercially available recipe was chosen and its production carried out by a leading international food manufacturer based in Lincolnshire (UK). The food manufacturer Bakkavor®, has produced broccoli and stilton soups for the purpose of this study using three broccoli varieties. This is a practical way to achieve increased GR levels in the diet as well as facilitating compliance from participants in long-term interventional studies. The bioavailability of SF from a high-glucosinolate variety, standard broccoli soup and water was previously assessed in a randomised, three-phase crossover study

(REC reference E/10/2003) [38]. Additional information on SF bioavailability following consumption of soups made from fresh and frozen plant material were reported by Saha *et al.* [68]. Both studies were very informative for the conception of the present study specifically designed to compare the bioavailability of SF from three broccoli varieties. While the other two studies have provided information about the bioavailability from simple soups, the current study is distinct due to the use of soups that closely approximate commercially available broccoli soups with increasing levels of GR. A double-blinded, randomised, cross-over design that is regarded as the gold standard trial for evaluating the bioavailability of interventions was chosen for the purpose of the research described in this Chapter with soups containing GR as in the study (LREC 2003/082) undertaken by Saha *et al*. [68].

This Chapter describes the design of the human study and the analysis of biological samples, i.e. urine and plasma, to determine the bioavailability of GR, SMCSO and SF using UPLC-MS/MS and LC-MS/MS. Based on the enhanced levels of bioactives measured in the broccoli soups ($Myb28^{B/V}$ and $Myb28^{V/V}$), it is hypothesised that higher levels of sulphur compounds (GR, SF, and SMCSO) will be delivered to the systemic circulation and excreted in the urine following consumption of these soups.

3.3. Materials and Methods

3.3.1. Materials

BD Vacutainer® EDTA (purple capped) (Cat # 367839) and trace-free (blue capped) (Cat # 368381) blood collection tubes were purchased from Becton, Dickinson and Company (New Jersey, USA). DNeasy® blood & tissue kit (Cat # 69504) and RNase (100 mg/ml) (Cat # 19101) were acquired from Qiagen. The following were purchased from Thermo Fischer Scientific; DPBS (Gibco®) 10X with no calcium and no magnesium (Cat. # 14200059), NanoDrop™ 1000 spectrophotometer, TaqMan®Drug Metabolism SNP Genotyping Assays (Cat # 4362691) and TaqMan® Universal Master Mix II without uracil-N-glycosylase (UNG) (Cat # 4440047) and Thermo Scientific™ insert vials (Cat # 10798345). Real-time Polymerase Chain Reaction (RT-PCR) was performed using the Applied Biosystems (AB) StepOnePlus™ machine.

3.3.2. Study design

The study was designed as a randomised, double-blinded, three-phase crossover trial carried out at the Human Nutrition Unit (HNU) at the Quadram Institute Bioscience (QIB), formerly known as the Institute of Food Research **(Figure 3-1)**. Each phase involved a 48-hour pre-intervention diet restriction, a study day involving a 9-hour stay at the HNU and a sample collection the following morning (24 hours post soup consumption) followed by a washout period of 2 weeks. Participants (n=10) were required to follow a glucosinolate-free diet as well as avoiding alcohol, spicy food and garlic (for SMCSO) for 48 hours prior to each study day until the collection of the 24-hour sample for each phase. This dietary restriction was required to ensure that glucosinolates from other food sources did not have an impact on the study results. During each study day, eleven blood samples (10 ml) were collected after the consumption of the soups at the following timepoints: 0, 30, 45, 60, 90, 120, 180, 240, 360, 480 minutes and 24-hour. Six urine samples were collected at the following timepoints: 0, 0-2, 2-4, 4-6, 6-8, 8-24 hours. Using an electronic randomization generator (www.randomization.com), a randomisation sequence was generated prior to the start of the intervention allocation. Blinding was undertaken by an independent person. The study lasted from September 2014-August 2015.

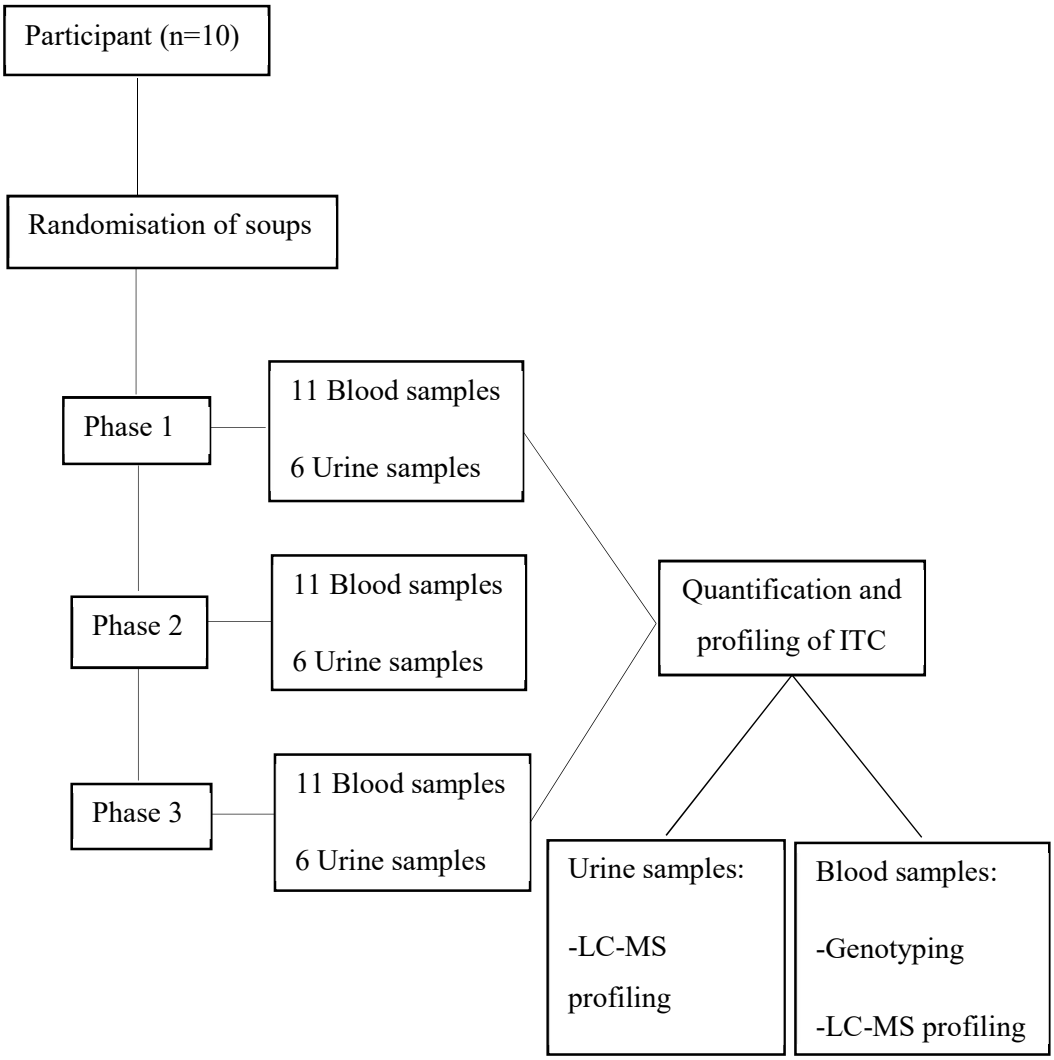


Figure 3-1: Study outline

3.3.3. Ethics approval

Prior to commencing the human study, approval was obtained from the institute's Human Research Governance Committee (HRGC) (reference IFR06/2014) and the NRES East of England Norfolk Ethics Committee (REC reference 14/EE/1121) **(Figure 3-2)**. The attendance to both meetings has been crucial for obtaining ethical approval in a time-effective manner. Following ethical approval, the study was registered on ClinicalTrials.gov (NCT02300324). All documents submitted to the REC can be found in **Appendix I** including the study protocol, study letters, ethical approval letters and subsequent amendments approvals.

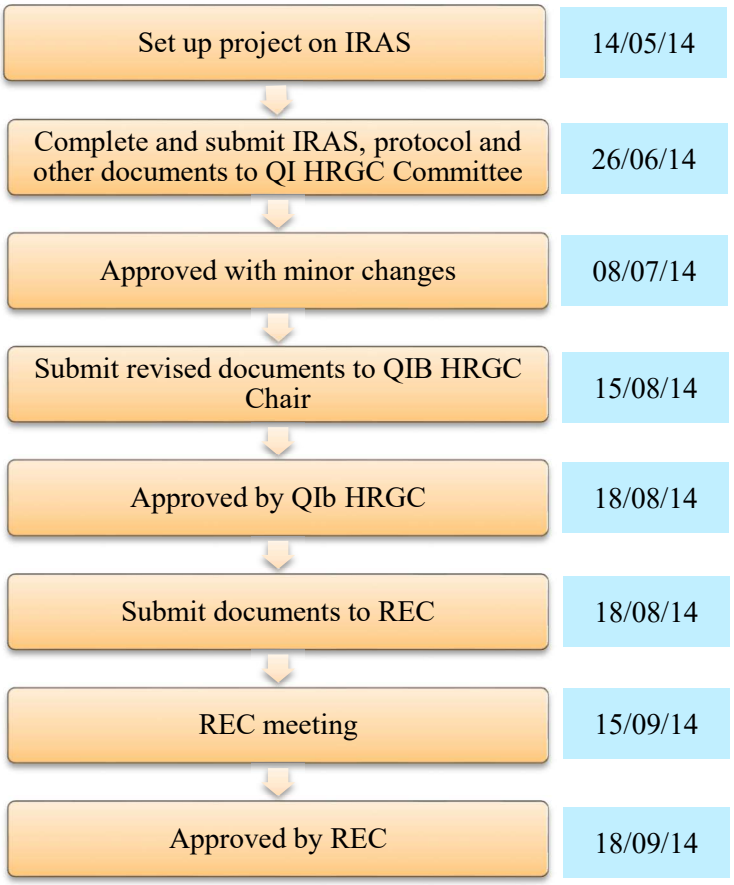


Figure 3-2: Flowchart of ethics approval process

3.3.4. Study recruitment

Ten apparently healthy men and women aged 18-65 years old were recruited onto the study. The recruitment strategy was mainly based on the use of the HNU volunteer database maintained by the HNU senior research nurse. The database consists of name and contact details of local people who have previously participated in studies or those who have registered interest in taking part in studies. Other recruitment resources included study advertisement around the Norwich Research Park and on the QIB website **(Annex 3, Appendix I)**. Interested participants who met the basic inclusion criteria were sent additional information about the study **(Annex 2, Appendix I)**. Further to this, interested participants were invited to the HNU to discuss the study and the inclusion/exclusion criteria.

3.3.5. Study inclusion/exclusion criteria

The basic eligibility criteria are listed in **Table 3-1**. Participants who were eligible to take part based on the basic inclusion/exclusion criteria underwent further eligibility assessment, prior to which they signed informed consent forms **(Annex 6, Appendix I)**. Eligibility assessments included anthropometric measurements (blood pressure, pulse, weight, height and Body Mass Index (BMI), a screening questionnaire assessing previous medical history and clinical laboratory analysis of urine and blood (screening for full blood count, urea and electrolytes and glycated haemoglobin, HbA1c). This assessment was carried out at the HNU by a senior nurse, and the screening results were reviewed by the HNU medical doctor. Participants' GP were informed of their patients' involvement into this study and they received copies of screening results to take further action in case of unexpected clinical findings.

Table 3-1: Eligibility criteria

Criteria	Included	Excluded
Aged between 18-65 years old	✓	
Non-smoker	✓	
Living within 40 miles of Norwich	✓	
BMI between 20 and 35 kg/m^2	✓	
Like broccoli and stilton soups.	✓	
Unwilling/unable to provide urine and blood samples,		✗
Clinical screening results were outside the standard range indicating a health problem		✗
Veins unsuitable for cannulation		✗
Known history of fainting when blood samples were taken/or during any clinical procedures		✗
Women who were or have been pregnant within the last 12 months/who were breastfeeding		✗
Diagnosed with any long-term medical condition (e.g. diabetes, haemophilia, cardiovascular disease, glaucoma, anaemia)		✗

Criteria	Included	Excluded
Requiring short or long term medication that may affect the study outcome, (including having used antibiotics within the previous one month)		✕
Taking dietary supplements/ herbal remedies which may affect the study outcome - unless the participant were willing to discontinue taking them for 1 month prior to starting study		✕
Allergic to members of the brassica family or the ingredients of the broccoli and stilton soups		✕
Participation in another dietary intervention/ blood sampling study		✕
Blood donors who have last donated in the last 16 weeks		✕
Depressed or elevated blood pressure measurements (<90/50 or 95/55 if symptomatic or >160/100)		✕
Unable to provide GP contact details/written informed consent and unable to complete the study diet.		✕

3.3.6. Study diet

Participants were randomly allocated to one of the three types of broccoli soups at each phase: (i) 300g Myb28$^{B/B}$ broccoli (standard broccoli) and stilton soup (providing 84 µmoles GR & 1031 µmoles SMCSO /300g soup), (ii) 300g Myb28$^{B/V}$ broccoli (Beneforte®) and stilton soup (providing 280 µmoles GR & 1514 µmoles /300g soup), (iii) 300g Myb28$^{V/V}$ broccoli and stilton soup (providing 452 µmoles GR & 1454 µmoles /300g soup), as reported in **Figure 2-3** and **Figure 2-6 (Chapter 2)**. The frozen soups were manufactured by Bakkavor, a leading international producer of freshly prepared foods. The study diet was heated in the microwave at the HNU for approximately six minutes on the morning of the study phases following a standard operating procedure strictly implemented by the study team for avoiding variability in the cooking process. All three types of soups appeared the same and had the same flavour enabling us to use them for a double-blinded study.

3.3.7. Sample preparation

Blood samples (10 ml) were collected via a cannula in EDTA tubes and trace free tubes at 0, 30, 45, 60, 90, 120, 180, 240, 360, 480 mins and 24-hour. Immediately after collection, blood samples were centrifuged at 1000 x g for 15 minutes at room temperature for plasma separation. Plasma samples were aliquoted and stored at -80°C. Six urine samples were collected at 0, 0-2, 2-4, 4-6, 6-8 and 8-24 hours. The total volume of urine was recorded and sub-samples aliquoted and stored at -80°C.

3.3.8. Extraction of DNA from whole blood samples

Genomic DNA was extracted from whole blood samples using DNeasy® blood & tissue kit from Qiagen per manufacturer's instructions. Samples were lysed with 20 µl proteinase K to 100 µl anticoagulated blood and made up to 220 µl with DPBS. To obtain RNA-free genomic DNA, 4 µl RNase was added to degrade RNA. Buffer AL (supplied with the kit) was added to the samples and incubated at 56°C for 10 minutes to provide an optimum buffer for lysis conditions. This was followed by the addition of 100% ethanol and vortexing to mix the samples. Samples were pipetted into DNeasy spin columns and centrifuged at 8000 rpm for 1 minute. Buffer AW1 was added to the DNeasy membrane inserted in a new column and centrifuged at 8000 rpm for 1 minute. Flow through was discarded followed by the addition of buffer AW2 and centrifuging at 8000 rpm for 3 minutes. DNA was eluted by adding buffer AE to the DNeasy membrane in a new column and centrifuging samples at 8000 rpm for 1 minute. To obtain maximum DNA yield, samples were eluted twice. Quantification was determined by using the NanoDrop™.

3.3.9. Quantification of the extracted DNA from whole blood samples

To determine the quantity and quality of DNA in the samples, a UV/VIS spectrophotometer (NanoDrop™) capable of measuring at 220 nm-750 nm was used. The spectrophotometer was normalised with 1 µl buffer AE as DNA was eluted in buffer AE. After which, 1 µl of the samples were pipetted on the pedestal of the spectrophotometer. Absorbance was measured at 260 nm, and 280 nm as nucleic acids including DNA absorb at 260 nm whereas proteins and phenols absorb at 280 nm. Using these absorbance values, the software calculates two

parameters that define purity of the DNA; the A260/280 and the A230/260. A 260/280 ratio of 1.8 indicates that the samples are 'pure' DNA, however, this is not a confirmation. A 230/260 ratio of 1.8-2.2 is a good indication of a low probability of contaminants present in the samples as contaminants absorb at 230 nm.

3.3.10. Real-time PCR to genotype blood samples

Real-Time polymerase chain reaction (RT-PCR) of *GSTM1* gene was undertaken using the AB StepOnePlus™ machine as reported by Cotton *et al.* [146]. The Corbett robot was provided with the master mix with the assay and DNA to prepare samples in a 96-well plate for the RT-PCR process. TaqMan® Drug Metabolism SNP Genotyping Assay for *GSTM1* (assay ID: C4420299720) was used. The assay includes the forward and reverse primers, two TaqMan® minor groove binder probes labelled at the 5' end of oligos with a reporter dye and a nonfluorescent quencher at the 3' end. The probes are labelled either with VIC® or FAM™ to distinguish between the two alleles. The master mix included a DNA polymerase enzyme (AmpliTaq Gold® DNA Polymerase), salts, buffer, and an internal reference (ROX™) to amplify the DNA. Each reaction consisting of 20 µl were run the AB StepOnePlus™. RT-PCR reactions comprised of the conversion of RNA to cDNA catalysed by the reverse transcriptase at 48°C for 30 minutes, activation of DNA polymerase enzyme at 95°C for 10 minutes followed by denaturation of the DNA at 95°C for 15 seconds and primer annealing and extension of DNA at 60°C for 1 minute.

3.3.11. Analysis of sulforaphane and conjugates, glucoraphanin and S-methyl-L-cysteine sulfoxide

Plasma (n=10) and urine samples (n=10) obtained from the study were analysed for SF and its conjugates; SF-GSH, SF-Cys-Gly, SF-Cys and SF-NAC using a validated LC-MS/MS method as described in Al Janobi *et al.* with minor modifications [63]. In addition, sulforaphane-nitrile (SF-N) and Erucin-*N*-acetylcysteine (ERN-NAC) were also measured by using the same method.

Dr Shikha Saha (QIB analytical chemist) developed a method to quantify GR (GR) and glucoerucin in urine and plasma samples. Quantification of SMCSO in the human study samples used the method described in **section 2.3.4.2**, **Chapter 2**.

3.3.11.1. Reagents and standards

SF (CAS 4478-93-7) (purity>98%) (Cat. # S8045) was purchased from LKT Laboratories (St. Paul, USA). SMCSO (CAS 853-87-8) (purity ≥98%) (Cat # 17600) and GR (CAS 21414-41-5) (purity ≥95%) (Cat # 10009445) were purchased from Cayman Chemical (Michigan, USA). Glucoerucin (CAS 21973-56-8) (purity≥97%) (Cat # 2280.1) was purchased from Carl Roth® (Karlsruhe, Germany). Dr Paul Needs (QIB chemist) synthesised the internal standard for SF *N*-butylthiocarbamoyl cysteine (B-ITC), SF conjugates including SF, SF-GSH, SF-Cys-Gly, SF-Cys, SF-NAC and ERN-NAC as published in [147] and SF-N as described by Zabala *et al.* [148]. The internal standard sinigrin for GR (Cat. # 85440) was purchased from Sigma-Aldrich®. Blank plasma from healthy participants with the same diet restriction described in **section 3.3.2** was ordered from Sera Laboratories International Ltd to make standard curves. Trichloroacetic acid (TCA) (Cat # 63399) and ammonium acetate (Cat. # 73594) was purchased from Sigma-Aldrich®. Hypergrade solvents were used for the LC-MS/MS. UV spectrometer was used to measure extinction coefficient. Agilent 6490 Triple Quad LC-MS/MS purchased from Agilent Technologies was used for analysis of SF and metabolites, GR and SMCSO in urine and plasma samples.

3.3.11.2. Plasma and urine extraction

Plasma samples were extracted on ice by adding 20 µl of pre-cooled 50% TCA and 10 µl of internal standard (B-ITC or Sinigrin) to 100 µl of plasma sample. The samples were vortexed for 30 seconds followed by centrifugation at 17,000 x g at 4°C for 10 minutes. The supernatant was transferred into Thermo Scientific™ insert vials and analysed on the LC-MS/MS.

Using 0.2 µM filters, urine samples were filtered and extracted by the addition of 890 µl of either ammonium acetate buffer for SF or 0.5% formic acid for GR or 5% TCA for SMCSO to 100 µl of the filtered urine samples. Samples were vortexed for 30 seconds then centrifuged for 10 minutes at 17,000 x g at 4°C. The supernatant was transferred into Agilent vials and analysed on the LC-MS/MS system.

3.3.11.3. Measuring the extinction coefficient of sulforaphane and its conjugates

SF and SF-GSH were diluted in water while the other conjugates were diluted in methanol to 1 mg/ml. All compounds were further diluted in methanol to measure the absorption using a UV spectrometer. The extinction coefficient ($M^{-1}cm^{-1}$) was calculated using the absorbance

values at 270 nm (and at 240 nm for SF) as follows; SF-Cys (8711), SF-Cys-Gly (4721), SF-GSH (5825), SF-NAC (7927) and SF (871), ERN-NAC (7854) comparable to published extinction coefficient [69,102,149].

3.3.11.4. LC-MS/MS analysis of sulforaphane and its metabolites

SF and metabolites were identified and quantified by LC-MS/MS. A serial dilution was conducted in either blank urine or plasma relevant to the sample run to minimise matrix effect. For plasma samples, the standards used were SF, SF-GSH and ITC-mix (SF-Cys, SF-Cys-Gly, SF-NAC, ERN-NAC) and the standards used for urine samples were SF, ITC-mix (SF-Cys, SF-Cys-Gly, SF-NAC, ERN-NAC, SF-GSH) diluted to generate a 10-point standard curve. The standard curve ranged from 0–10 µg/ml for SF and ITC-mix and 0–40 µg/ml for SF-GSH. Quality control consisting of pooled urine and blood samples and system suitability samples were run to check that the instrument operated without any issues.

The LC-MS/MS was set on a flow rate of 0.3 ml/min. The column used for the analysis was a HPLC Phenomenex® Luna 3u C18 (2) 100A 100 x 2.1 mm with a Phenomenex® C18 (2) 100A. The column temperature and autosampler were maintained at 40°C and 4°C respectively. Samples were injected at 5 µl, 'system suitability' was injected at 1 µl, and blank was injected at 20 µl. Separation of metabolites was carried out with 0.1% ammonium acetate in deionised water titrated with 0.1% acetic acid until pH 4 was reached (mobile phase A) and 0.1% acetic acid in acetonitrile (mobile phase B). The LC eluent flow was sprayed into the mass spectrometer interface without splitting. SF and SF conjugates ions were monitored using mass spectrometry in MRM mode in positive polarity with ESI source. The source parameters were; a gas temperature at 200˚C with a gas flow of 12 l/minute, a sheath gas temperature at 400˚C with a sheath gas flow of 12 l/minute, a nebuliser pressure of 60 psi and capillary voltage at 4000V.

The standard curve was used to calculate the concentration of the samples relative to the internal standard. For quantitative analysis in urine, the sample volume was considered to estimate the total amount of conjugates.

Table 3-2: Summary of the monitored product ions of SF and SF conjugates and the optimised MS operating parameteres of the analytes.

Analyte	Retention time (mins)	Precrusor ion (*m/z*)	Product ion (*m/z*)	Collision energy	Cell accelerator energy	Polarity
SF	7.1	178.01	136.0	10	5	Positive
SF	7.1	178.01	119.1	10	5	Positive
SF	7.1	178.01	114.1	10	5	Positive
SF-GSH	6.2	485.01	178.9	22	5	Positive
SF-GSH	6.2	485.01	113.9	38	5	Positive
SF-Cys-Gly	3.4	356.01	114.0	18	5	Positive
SF-Cys-Gly	3.4	356.01	75.9	24	5	Positive
SF-Cys	3.2	299.01	136.1	12	5	Positive
SF-Cys	3.2	299.01	114.0	10	5	Positive
SF-NAC	6.4	341.01	177.8	20	5	Positive
SF-NAC	6.4	341.01	114.0	18	5	Positive
ERN-NAC	7.9	325.01	164.0	10	5	Positive
ERN-NAC	7.9	325.01	122.0	14	5	Positive
ERN-NAC	7.9	325.01	102.9	18	5	Positive
SF-N	2.7	146.01	60.9	10	5	Positive
SF-N	2.7	146.01	55.0	22	5	Positive
B-ITC	9.2	279.01	122.0	10	5	Positive

3.3.11.5. UPLC-MS/MS analysis of glucoraphanin

GR and its reduced analog (glucoerucin) were analysed by UPLC-MS/MS in all study samples. GR stock (1 mg/ml) and glucoerucin (1 mg/ml) were diluted to a 9-point standard curve ranging from 0-2 µg/ml for urine and ranging from 0-0.25 µg/ml for plasma. Samples were injected at 5µl and eluted at a flow rate of 0.3 ml/min. GR was separated with 0.2% formic acid in water (mobile phase A) and 0.2% formic acid in acetonitrile (mobile phase B) using a Kinetex® 1.7 µm XB-C18 100 Å 100 x 2.1 mm UPLC column. The gradient started at 5% mobile phase B increasing over 7 mins to 80% mobile phase B and finally re-equilibrating to 5 % mobile phase B for 2 mins.

The LC eluent flow was sprayed into the mass spectrometer interface without splitting. GR ion and sinigrin ion was monitored using mass spectrometry MRM mode in negative polarity

with ES. The source parameters were the same as SMCSO analysis in **section 2.3.4.2**, **Chapter 2**. The quantification was performed using matrix match calibration curve. Identification and quantification of the GR peak was on the basis of retention time and peak area through the use of calibration standards relative to sinigrin.

Table 3-3: Summary of the monitored product ions of GR and glucoerucin and the optimised MS operating parameteres of the analytes.

Analyte	Retention time (mins)	Precrusor ion (*m/z*)	Product ion (*m/z*)	Collision energy	Cell accelerator energy	Polarity
GR	2.0	436.42	372	18	4	Negative
GR	2.0	436.42	96.9	22	4	Negative
Glucoerucin	5.5	419.99	96.8	22	4	Negative
Glucoerucin	5.5	419.99	74.8	34	4	Negative
Sinigrin	2.3	357.99	96.9	22	4	Negative
Sinigrin	2.3	357.99	74.8	34	4	Negative

3.3.11.6. UPLC-MS/MS analysis of S-methyl-L-cysteine sulfoxide

The UPLC-MS/MS set up is the same as the method in **section 2.3.4.2, Chapter 2**. Blood and urine samples were analysed for measuring SMCSO by using a novel UPLC-MS/MS method. A 10-point standard curve ranging from 0-20 µg/ml for urine and ranging from 0-10 µg/ml was generated by diluting 1 mg/ml SMCSO stock. Using an Agilent SB-AQ 1.8 uM (100 x 2.1mm) C18 column with an Agilent Zorbax guard column, SMCSO was separated in plasma with 10 mM ammonium acetate + 0.05% heptafluorobutyric acid in water (mobile phase A) and 10 mM ammonium acetate + 0.05% heptafluorobutyric acid in 90% methanol (mobile phase B). Analysis of urine samples was undertaken with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B).

3.3.12. Inductively coupled plasma-mass spectrometry to quantify trace elements in plasma samples

Plasma samples (0.5 ml) collected in BD Vacutainer® trace free blood collection tubes were sent to Dr Scott Young at the University of Nottingham to analyse 29 trace elements by

inductively coupled plasma-mass spectrometry (ICP-MS). Samples were run on a Thermo-Fisher Scientific iCAP-Q equipped with collision cell technology with kinetic energy discrimination. The analysis of the samples was undertaken as published in Hurst *et al*. [150]. The collision gas used for most elements was helium except for the use of hydrogen for selenium. A multi-element calibration standards including silver, aluminium, arsenic, barium, cadmium, cobalt, chromium, caesium, copper, iron, manganese, molybdenum, nickel, lead, rubidium, selenium, strontium, uranium, vanadium and zinc in the range 0 – 50 µg/L were purchased. Additional calibration standards were created comprising of calcium, magnesium, sodium and potassium ranging from 0-30 mg/L and phosphorus, sulphur and boron at 10 mg/L. Samples were diluted 1:20 with a solution consisting of 0.1% non-ionic surfactant Triton X-100, an anti-foam reagent, 1% HNO3, 2% methanol and an internal standard mix including scandium (10 µg/L), rhodium (5 µg/L) and iridium (2 µg/L).

Seronorm™ trace elements whole blood L-1 and L-2 were run as quality control to check instrument run without any issues. Quantification of samples from the instrument was also undertaken by Dr Scott Young using a Qtegra™ software by Thermo-Fisher Scientific. See **Appendix II** for results from ICP-MS.

3.4. Data Analysis

3.4.1. Sample size

Power calculation was performed by the statistician Dr Jack Dainty (UEA senior statistician) in order to determine the sample size for the study. To detect a significant ($p<0.05$) difference in mean total urinary thiol excretion of 18 µmoles between consumption of $Myb28^{B/B}$ (standard broccoli) and stilton soup and $Myb28^{B/V}$ (Beneforte®) broccoli and stilton soup, the study required ten participants giving a power of 80%. The SD of the differences between the two groups was estimated to be 17 µmoles. The basis for this calculation was preliminary unpublished data from 65 subjects from the ENGAGE study (REC 12/EE/0483) (data not published).

3.4.2. Statistical analysis

Statistical analysis was undertaken by Dr Jack Dainty (UEA senior statistician) and Dr Henri Tapp (QIB senior statistician). Response variables were modelled using sequential analysis of variance (ANOVA). These are linear models that account for the non-independence of the type

of data that were generated in this cross-over study (i.e. data that come from the same participants after different interventions). The aim of the analysis was to examine whether the intervention(s) had any effect on the various outcome measures (e.g. urinary excretion of total thiol conjugates, pharmacokinetic parameters generated from the time course of plasma samples). The four explanatory variables employed comprised the following: concentration prior to meal, volunteer identification code, sequence in which the soups were consumed, and either soup $Myb28^{B/B}$, $Myb28^{B/V}$ or $Myb28^{V/V}$. Standard model diagnostics were applied such as transformations of the response or removal of potential outliers. Significance levels for multiple comparisons are based on Tukey's honest significant difference.

3.4.3. Pharmacokinetic analysis

The plasma data from the study was imported into GraphPad Prism version 7.02 for Windows, (GraphPad Software, La Jolla California USA, www.graphpad.com). Prism was used to calculate area under the curve (AUC), T_{max} (time of maximum concentration), C_{max} (maximum concentration) as pharmacokinetic parameters. The percentage excreted from the initial dose was calculated for the urine samples as a biomarker of SF and metabolite, SMCSO and GR recovery.

3.5. Results

3.5.1. Participant recruitment

Eighty-seven potential participants expressed interest in taking part in the study. These potential participants were sent a participant information sheet to provide them more information about the study. Following this, 43 expressed an interest to come for a study talk at the HNU. Out of 24 participants that were subsequently screened, 11 participants were included into the study. The other 13 potential participants were excluded for various medical reasons as shown in **Figure 3-3**. One participant was excluded during the study thus of the 11 participants that were recruited in total, 10 completed all three phases.

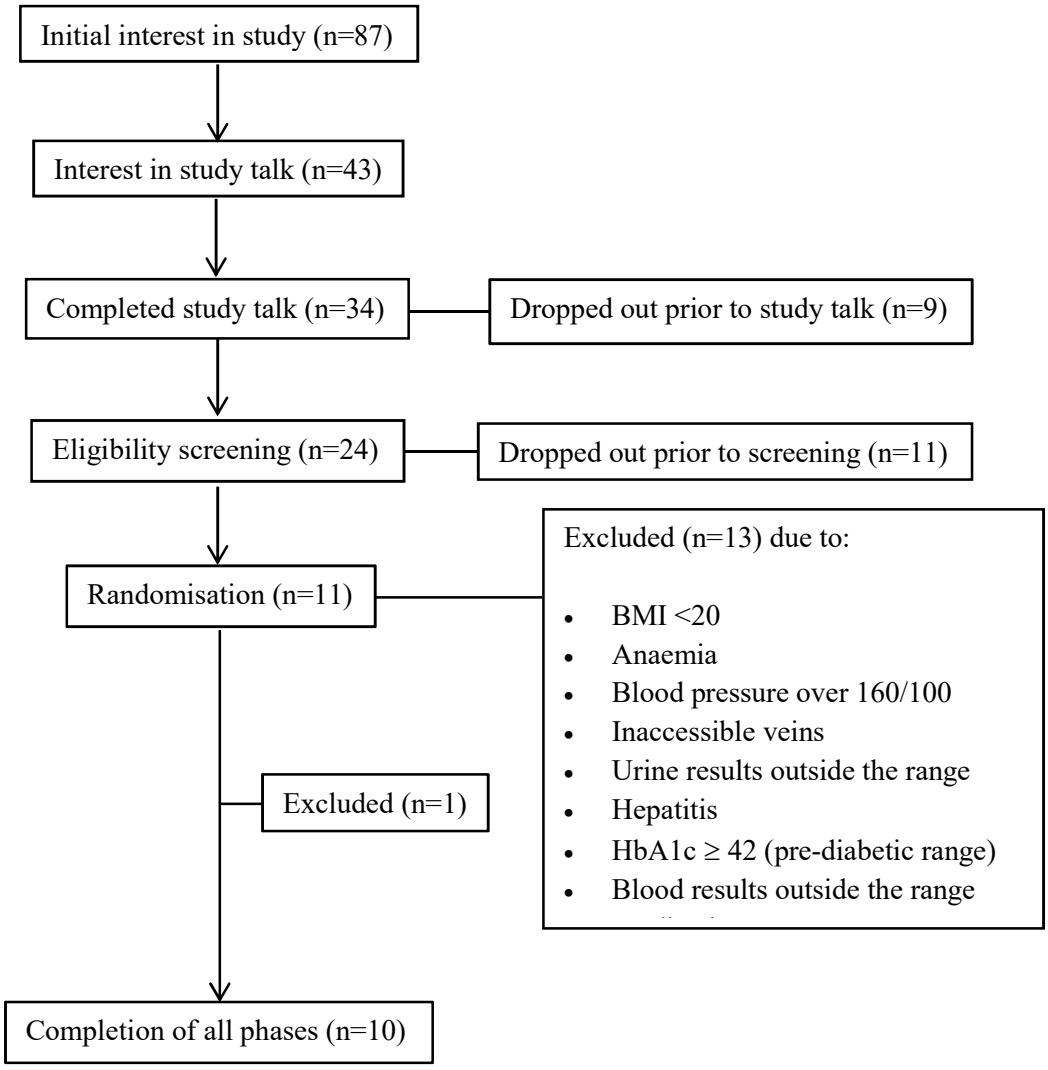


Figure 3-3: Flowchart presenting participant recruitment

3.5.2. Participant anthropometric measurements and their genotypes

Recruited participants had the following anthropometric characteristics as summarised in **Table 3-4**. Out of 10 participants, 6 were *GSTM1*-positive, and 4 were *GSTM1*-null. The occurrence of *GSTM1*-null genotype in this small study sample (n=10) is 40% which is within the range of 39-62% for Caucasians as published by Cotton *et al.* [146].

Table 3-4: Baseline characteristics of participants involved in the study.

Participant ID	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m²)
B02	24	161.8	67.2	25.7
B03	23	172.1	80.3	27.1
B07	61	158.9	61.3	24.3
B09	24	178.8	69.7	22
B15	59	173.5	66.3	22.0
B16	26	192.1	87.2	24.0
B20	45	179.7	97.6	30.2
B22	43	168.9	87.7	30.7
B23	62	162.0	61.0	23.2
B24	62	160.8	57.8	22.4
Mean ± SD	**42.9 ± 17.4**	**170.9 ± 10.6**	**73.6 ± 13.6**	**25.2 ± 3.2**

3.5.3. Bioavailability of sulforaphane and its conjugates following consumption of soups made with three broccoli genotypes

To determine the pharmacokinetics of SF and mercapturic acid metabolites from the broccoli soups, blood and urine samples collected from participants were analysed using LC-MS/MS. Prior to consumption of the soups, baseline plasma and urine samples were collected to check compliance to the glucosinolate diet restriction. Following consumption of the soups, SF and

metabolites were detected in both the plasma and urine samples. The concentration of SF and metabolites detected in the baseline samples was subtracted from subsequent time points to confirm that the concentration of SF and metabolites observed is a result of the broccoli genotypes. After consumption of all three types of soups, the concentration of SF and metabolites in the plasma was detectable within 30 minutes **(Figure 3-4)**. Following consumption of Myb28$^{B/B}$ broccoli soups, the levels of SF and metabolites peaked at 6 hours **(Figure 3-4)** subsequently declining to 0.02 ± 0.01 µM within 24 hours **(Table 3-5)**. On the contrary, after consumption of the soups with the Myb28$^{B/V}$ and Myb28$^{V/V}$ broccoli genotypes, the levels of SF and metabolites peaked later, at 8 hours, **(Figure 3-4)** subsequently decreasing to 0.07 ± 0.06 µM and 0.09 ± 0.05 µM respectively **(Table 3-5)**. There is a dose-dependent increase in the levels of SF and metabolites detected in the plasma at 8 hours following consumption of soups with Myb28$^{B/V}$ and Myb28$^{V/V}$ broccoli genotypes compared to Myb28$^{B/B}$ broccoli soup **(Figure 3-4)**.

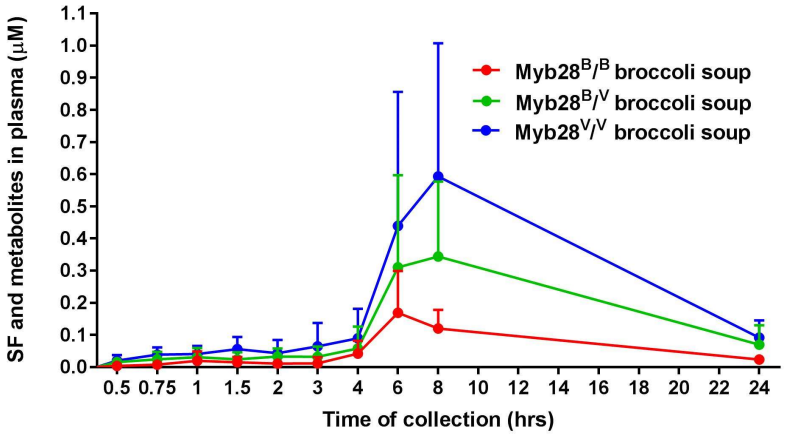


Figure 3-4: The plasma concentration of SF and metabolites (µM) following consumption of the three types of broccoli soups. Plasma samples were collected over 24 hours following consumption of Myb28$^{B/B}$ (84 µmoles GR /300g soup), Myb28$^{B/V}$ (280 µmoles GR /300g soup) and Myb28$^{V/V}$ (452 µmoles GR /300g soup). The samples were analysed for SF and metabolites including SF-GSH, SF-Cys, SF-Cys-Gly and SF-NAC using LC-MS/MS. Data (n=10) is represented as mean ± SD.

The pharmacokinetics of the broccoli soups are represented as the AUC, C_{max}, T_{max}, and percentage of excretion, as reported in **Table 3-5**. The levels of SF and metabolites reach a ~4 and ~2-fold significant increase in the plasma following consumption of the soups with the Myb28$^{V/V}$ ($p<0.0001$) and Myb28$^{B/V}$ ($p<0.01$) broccoli genotypes, respectively, compared to Myb28$^{B/B}$ broccoli expressed as the AUC and C_{max} **(Table 3-5)**.

Table 3-5: Summary table of the levels of SF and metabolites in plasma and urine after consumption of soups stratified by soups made with different broccoli genotypes

	Myb28$^{B/B}$ broccoli and stilton soup	**Myb28$^{B/V}$ broccoli and stilton soup**	**Myb28$^{V/V}$ broccoli and stilton soup**	**p-value for broccoli and stilton soup**			
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**ANOVA**	**Myb28$^{B/B}$ vs. Myb28$^{B/V}$**	**Myb28$^{B/B}$ vs. Myb28$^{V/V}$**	**Myb28$^{B/V}$ vs. Myb28$^{V/V}$**
SF and metabolites in plasma							
AUC (µmol.h/l)	1.99 ± 1.31	4.92 ± 3.77	8.08± 6.60	<0.0001	<0.01	<0.0001	0.0905
C_{max} (µmol/l)	0.17 ± 0.12	0.37 ± 0.26	0.61 ± 0.40	≤0.0001	<0.01	≤0.0001	0.0683
T_{max} (h)	6.10 ± 2.23	7.40 ± 0.97	9.20 ± 5.27	<0.05	0.5258	<0.05	0.2301
Concentration at 24 hours (µmol/l)	0.02 ± 0.01	0.07 ± 0.06	0.09 ± 0.05	<0.0001	<0.01	<0.0001	0.1797

	Myb28$^{B/B}$ broccoli and stilton soup	**Myb28$^{B/V}$ broccoli and stilton soup**	**Myb28$^{V/V}$ broccoli and stilton soup**	**p-value for broccoli and stilton soup**			
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**ANOVA**	**Myb28$^{B/B}$ vs. Myb28$^{B/V}$**	**Myb28$^{B/B}$ vs. Myb28$^{V/V}$**	**Myb28$^{B/V}$ vs. Myb28$^{V/V}$**
SF and metabolites in urine							
Total excreted in 24 hours (μmoles)	8.74 ± 4.95	23.14 ± 16.17	39.98 ± 26.22	<0.001	<0.05	<0.001	<0.01
Percentage excreted 24 hours after ingestion %	10.40 ± 5.90	8.26 ± 5.78	8.85 ± 5.80	0.5727			

Analysis modelled using sequential analysis of variance (ANOVA) by Dr Henri Tapp/Dr Jack Dainty. The variables underwent log transformation except for plasma concentration at 24 hours, which underwent a square root transformation. Significance level for multiple comparisons was analysed using Tukey's honest significance test. 'SF metabolites' refers to SF, SF-GSH, SF-Cys, SF-Cys-Gly and SF-NAC in plasma and SF, SF-NAC, SF-Cys, ERN-NAC and SF-Cys-Gly in urine.

The urinary excretion of SF and metabolites was detected within the first hour, peaked at 6-8 hours, and subsequently declined. SF and metabolites is slowly excreted in the first 0-6 hours followed by rapid excretion at 6-8 hours following consumption of the $Myb28^{B/B}$ broccoli soups, and rapid excretion 6-24 hours following consumption of the soups with $Myb28^{B/V}$ and $Myb28^{V/V}$ broccoli genotypes **(Figure 3-5 A)**. The amount of SF and metabolites excreted in the urine may not have reached a plateau following consumption of the soups with $Myb28^{B/B}$ broccoli, $Myb28^{B/V}$ and $Myb28^{V/V}$ broccoli genotypes **(Figure 3-5 A)**. Compared to the $Myb28^{B/B}$ broccoli, the cumulative amount of SF and metabolites excreted in the urine is significantly higher by ~5-fold 24 hours following consumption of the soups with $Myb28^{V/V}$ (39.98 ± 26.22 vs. 8.74 ± 4.95 µmoles, $p<0.001$) and significantly higher by ~3-fold following consumption of the soups with $Myb28^{B/V}$ (23.14 ± 16.17 vs. 8.74 ± 4.95 µmoles, $p<0.05$) **(Figure 3-5 A-B)**. Plasma and urine results have provided strong evidence that the presence of one or two $Myb28^{V}$ alleles enhances the pharmacokinetics of dietary compounds delivered by the broccoli soups.

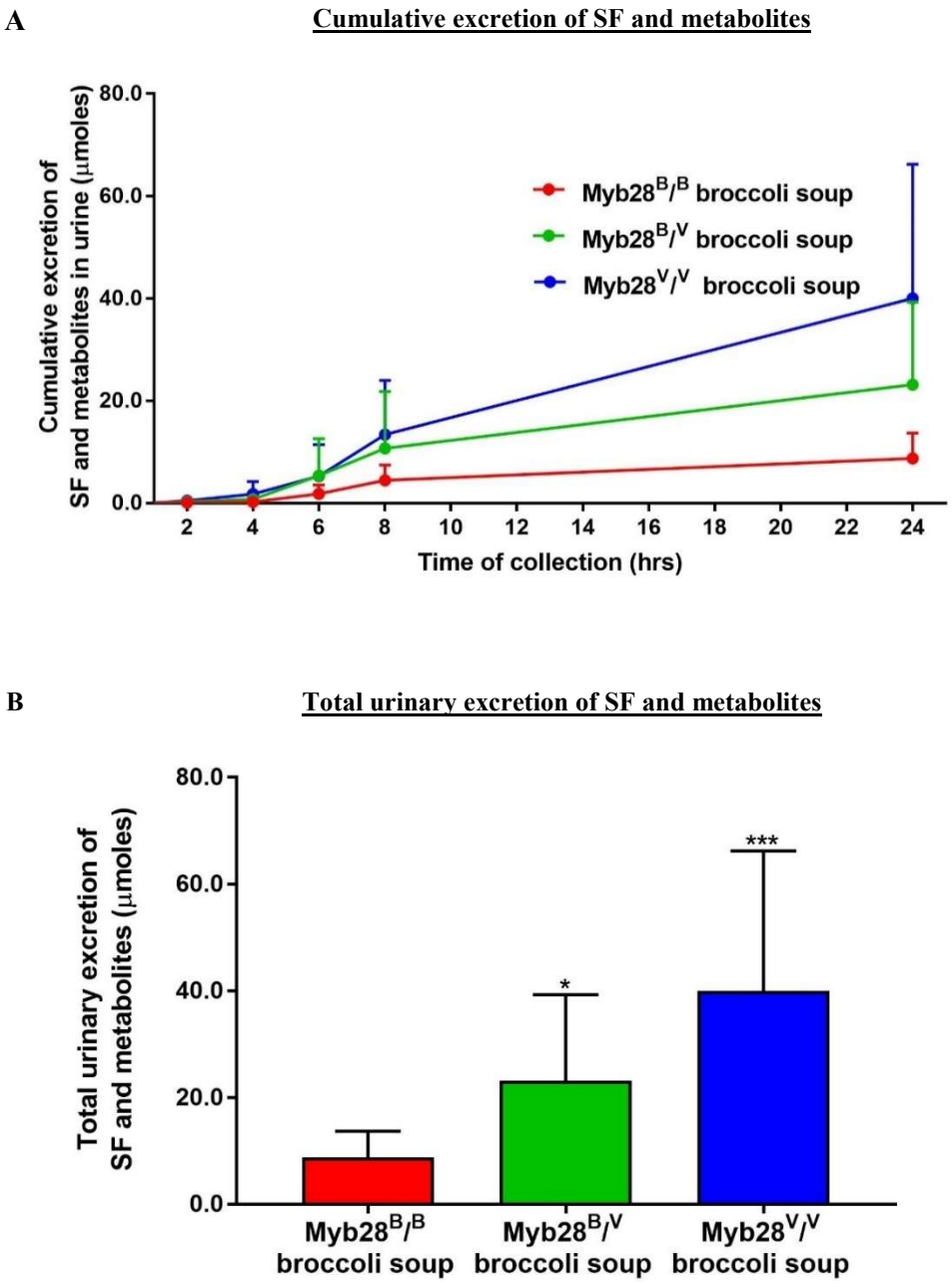


Figure 3-5: Urinary excretion of SF and metabolites (µmoles) in 24 hours following consumption of the three types of broccoli soups. The cumulative excretion of SF and metabolites (A) and the total urinary excretion of SF and metabolites (B) measured in urine samples collected over 24 hours after consumption of Myb28$^{B/B}$ (84 µmoles GR /300g soup), Myb28$^{B/V}$ (280 µmoles GR /300g soup) and Myb28$^{V/V}$ (452 µmoles GR /300g soup). Samples were analysed for SF and metabolites including ERN-NAC, SF-Cys, SF-Cys-Gly, and SF-NAC using LC-MS/MS. Data (n=10) is represented as mean ± SD. Total urinary excretion data underwent log root transformation followed by analysis by one-way ANOVA with Tukey's honest significance test (*p<0.05 and *** p<0.001 vs. My28$^{B/B}$ broccoli soup).

Plasma samples were analysed for the following SF metabolites; SF, SF-GSH, SF-Cys, SF-Cys-Gly, SF-NAC and ERN-NAC. The SF conjugates found in the plasma were, in order of the percentage of the total conjugates in 24 hours, SF = SF-GSH > SF-Cys-Gly > SF-Cys > SF-NAC, as represented in **(Table 3-6)**. ERN-NAC was not detected in the plasma samples. Whereas ERN-NAC was detected in the urine samples but SF-GSH was not detected. The percentage of urinary excretion of SF and conjugates in 24 hours were as follows, SF-NAC > ERN-NAC > SF-Cys > SF > SF-Cys-Gly **(Table 3-6)**.

Table 3-6: Summary table of the percentage of individual SF metabolite in plasma and urine after consumption of soups with increasing levels of GR

	$Myb28^{B/B}$ broccoli and stilton soup	**$Myb28^{B/V}$ broccoli and stilton soup**	**$Myb28^{V/V}$ broccoli and stilton soup**
	Mean ± SD (%)	**Mean ± SD (%)**	**Mean ± SD (%)**
Metabolites in plasma over 24-hours			
SF	32.20 ± 17.29	34.59 ± 14.67	36.58 ± 14.35
SF-GSH	34.57 ± 21.74	33.01 ± 23.80	30.45 ± 21.21
SF-CYS	8.93 ± 9.93	5.54 ± 3.53	8.77 ± 8.80
SF-CYS-GLY	21.60 ± 11.81	22.22 ± 10.27	20.23 ± 7.38
SF-NAC	2.70 ± 1.79	4.63 ± 4.29	3.97 ± 2.84
Metabolites in 24-hour urine excretion			
SF	8.53 ± 5.57	5.66 ± 3.04	5.41 ± 2.20
SF-NAC	57.78 ± 11.73	49.46 ± 14.08	51.26 ± 11.07
SF-CYS	18.29 ± 6.18	19.15 ± 9.20	21.08 ± 5.61
ERN-NAC	20.93 ± 9.04	25.30 ± 16.73	21.15 ± 13.51
SF-CYS-GLY	0.48 ± 0.90	0.44 ± 0.72	1.11 ± 2.74

Data (n=10) is represented as mean ± SD. The percentage was calculated as the concentration of each individual metabolite (µM) of the total concentration of SF and metabolites (µM).

In order to explore the role of the *GSTM1* genotype on the metabolism of SF, data collected from the urine and plasma samples were stratified according to the genotypes **(Table 3-7)**. There was no significance on the plasma pharmacokinetics (AUC, C_{max}, T_{max}) and the urinary excretion between *GSTM1*-null and *GSTM1*-positive participants as reported in **Table 3-7**. However, the study was not powered in such a way that one could definitively say that there is no effect.

Table 3-7: Summary table of the SF metabolites in plasma and urine after consumption of soups stratified by *GSTM1* genotypes

	***GSTM1*-positive (n=6)**	***GSTM1*-null (n=4)**	**p-value for genotype**
	Mean ± SD	**Mean ± SD**	
SF and metabolites in plasma			
AUC (µmol.h/L)			
$Myb28^{B/B}$ broccoli and stilton soup	1.45 ± 0.68	2.79 ± 1.72	0.2186
$Myb28^{B/V}$ broccoli and stilton soup	4.05 ± 2.75	6.22 ± 5.13	0.4815
$Myb28^{V/V}$ broccoli and stilton soup	7.39 ± 3.41	9.12 ± 10.43	0.7670
C_{max} (µmol/L)			
$Myb28^{B/B}$ broccoli and stilton soup	0.12 ± 0.06	0.25 ± 0.15	0.1882
$Myb28^{B/V}$ broccoli and stilton soup	0.32 ± 0.22	0.43 ± 0.33	0.5839
$Myb28^{V/V}$ broccoli and stilton soup	0.63 ± 0.29	0.58 ± 0.58	0.8866
T_{max} (h)			
$Myb28^{B/B}$ broccoli and stilton soup	5.50 ± 2.66	7.0 ± 1.15	0.2614
$Myb28^{B/V}$ broccoli and stilton soup	7.33 ± 1.03	7.50 ± 1.00	0.8065
$Myb28^{V/V}$ broccoli and stilton soup	10.33 ± 6.74	12.00 ± 8.00	0.7436
Total SF and metabolites in urine (µmoles)			

	GSTM1-positive (n=6)	*GSTM1*-null (n=4)	p-value for genotype
	Mean ± SD	**Mean ± SD**	
$Myb28^{B/B}$ broccoli and stilton soup	10.28 ± 4.86	6.42 ± 4.73	0.2541
$Myb28^{B/V}$ broccoli and stilton soup	25.47 ± 16.28	19.64 ± 17.77	0.6179
$Myb28^{V/V}$ broccoli and stilton soup	47.86 ± 23.92	28.17 ± 28.27	0.2972
Percentage excreted 24 hours after ingestion %			
$Myb28^{B/B}$ broccoli and stilton soup	12.23 ± 5.78	7.65 ± 5.64	0.2541
$Myb28^{B/V}$ broccoli and stilton soup	9.09 ± 5.81	7.01 ± 6.35	0.6180
$Myb28^{V/V}$ broccoli and stilton soup	10.59 ± 5.29	6.23 ± 6.26	0.2972

Data was analysed using unpaired t-test with Welch's correction. 'SF metabolites' refers to SF, SF-GSH, SF-Cys, SF-Cys-Gly and SF-NAC in plasma and SF, SF-NAC, SF-Cys, ERN-NAC and SF-Cys-Gly in urine.

3.5.4. Bioavailability of intact glucoraphanin following consumption of soups made with three broccoli genotypes

Plasma and urine samples collected from participants were analysed by UPLC-MS/MS to assess the bioavailability of intact GR. Following consumption of the soups, intact GR was detected in both plasma and urine samples. To our knowledge, this is the first human study to report the detection and quantification unmetabolized GR in plasma and urine. The concentration of GR detected in the baseline samples were subtracted from subsequent time points to confirm that the concentration of GR observed is because of the broccoli genotypes. In addition, glucoerucin was found in the urine samples but not detected in the plasma samples.

After consumption of all three types of soups, the concentration of GR in the plasma was detectable within 30 minutes **(Figure 3-6)**. The levels of GR peaked at 2 hours and then within 24 hours decreased to trace levels following consumption of all three types of soups **(Table 3-8)**. At 2 hours after the participants consumed the soups with the $Myb28^{V/V}$ and $Myb28^{B/V}$

broccoli genotypes, higher levels of GR were detected in the plasma in a dose-dependent manner compared to Myb28$^{B/B}$ broccoli soups.

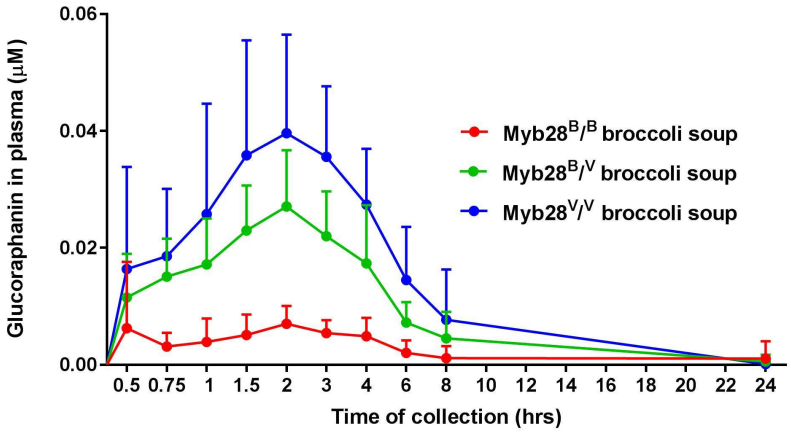


Figure 3-6: The plasma concentrations of unmetabolised GR (µM) following consumption of the three types of broccoli soups. Plasma samples were collected over 24 hours following consumption of Myb28$^{B/B}$ (84 µmoles GR /300g soup), Myb28$^{B/V}$ (280 µmoles GR /300g soup) and Myb28$^{V/V}$ (452 µmoles GR /300g soup). The samples were analysed for GR by UPLC-MS/MS. Data (n=10) is represented as mean ± SD.

The pharmacokinetics parameter including AUC, C_{max}, T_{max}, and percentage of excretion are all reported in **Table 3-8**. Significant ~5-fold and ~3-fold enhanced levels of GR were delivered to the plasma following consumption of soups enriched with GR (Myb28$^{V/V}$ and Myb28$^{B/V}$ broccoli soups respectively, $p<0.0001$) in contrast to Myb28$^{B/B}$ broccoli soup consumption as represented by the AUC.

Table 3-8: Summary table of the levels of unmetabolised GR in plasma and urine after consumption of soups stratified by soups made with different broccoli genotypes

	$Myb28^{B/B}$ broccoli and stilton soup	**$Myb28^{B/V}$ broccoli and stilton soup**	**$Myb28^{V/V}$ broccoli and stilton soup**	**p-value for broccoli genotypes**			
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**ANOVA**	**$Myb28^{B/B}$ vs. $Myb28^{B/V}$**	**$Myb28^{B/B}$ vs. $Myb28^{V/V}$**	**$Myb28^{B/V}$ vs. $Myb28^{V/V}$**
GR in plasma							
AUC (µmol.h/l)	0.05 ± 0.05	0.15 ± 0.08	0.24 ± 0.11	<0.0001	<0.0001	<0.0001	<0.05
C_{max} (µmol/l)	0.01 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	<0.0001	<0.0001	<0.0001	<0.01
T_{max} (h)	2.20 ± 1.16	2.23 ± 0.90	2.25 ± 0.95	0.8006			
Concentration at 24 hours (µmol/l)	<0.1	<0.1	<0.1	0.8389			

	Myb28$^{B/B}$ broccoli and stilton soup	**Myb28$^{B/V}$ broccoli and stilton soup**	**Myb28$^{V/V}$ broccoli and stilton soup**		**p-value for broccoli genotypes**		
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**ANOVA**	**Myb28$^{B/B}$ vs. Myb28$^{B/V}$**	**Myb28$^{B/B}$ vs. Myb28$^{V/V}$**	**Myb28$^{B/V}$ vs. Myb28$^{V/V}$**
GR and glucoerucin in urine							
Total excreted in 24 hours (μmoles)	0.54 ± 0.29	1.44 ± 0.66	2.12 ± 0.98	<0.0001	<0.0001	<0.0001	<0.05
Percentage excreted 24 hours after ingestion %	0.64 ± 0.34	0.51 ± 0.24	0.47 ± 0.22	0.6053			

Analysis modelled using sequential analysis of variance (ANOVA) by Dr Henri Tapp. The variables including AUC, total excreted and percentage excreted underwent a square root transformation, C_{max} and T_{max} underwent a log transformation. Concentration at 24 hours underwent a transformation of (y + 9.5463e-05)^-1. Significance level for mutliple comaprisons was analysed using Tukey's honest significance test.

GR and glucoerucin were excreted via urine within 2 hours after consumption of all three types of soups. The levels of GR and glucoerucin rapidly increased at 2-4 hours followed by a decline in the levels. The cumulative amount of GR and glucoerucin demonstrates a rapid excretion of these compounds from 0-8 hours, and then reaches a plateau following consumption of all three types of soups which is consistent with the trace levels measured at 8-24-hour urine samples **(Figure 3-7 A)**. A similar dose-dependent trend to that observed in the plasma is also demonstrated in the urine excretion **(Figure 3-7 A-B)**. Compared to $Myb28^{B/B}$ broccoli, ~4-fold and ~3-fold greater cumulative amount of GR and glucoerucin was significantly excreted in the urine following consumption of the soups with $Myb28^{V/V}$ broccoli genotype (2.12 ± 0.98 vs. 0.54 ± 0.29 μmoles, $p<0.0001$) and $Myb28^{B/V}$ broccoli genotype respectively (1.44 ± 0.66 vs. 0.54 ± 0.29 μmoles, $p<0.0001$) **(Figure 3-7 A-B)**. These data convincingly demonstrate that the presence of one or two $Myb28^{V}$ alleles delivers enhanced levels of GR and glucoerucin to the plasma as exhibited by the plasma and urine data.

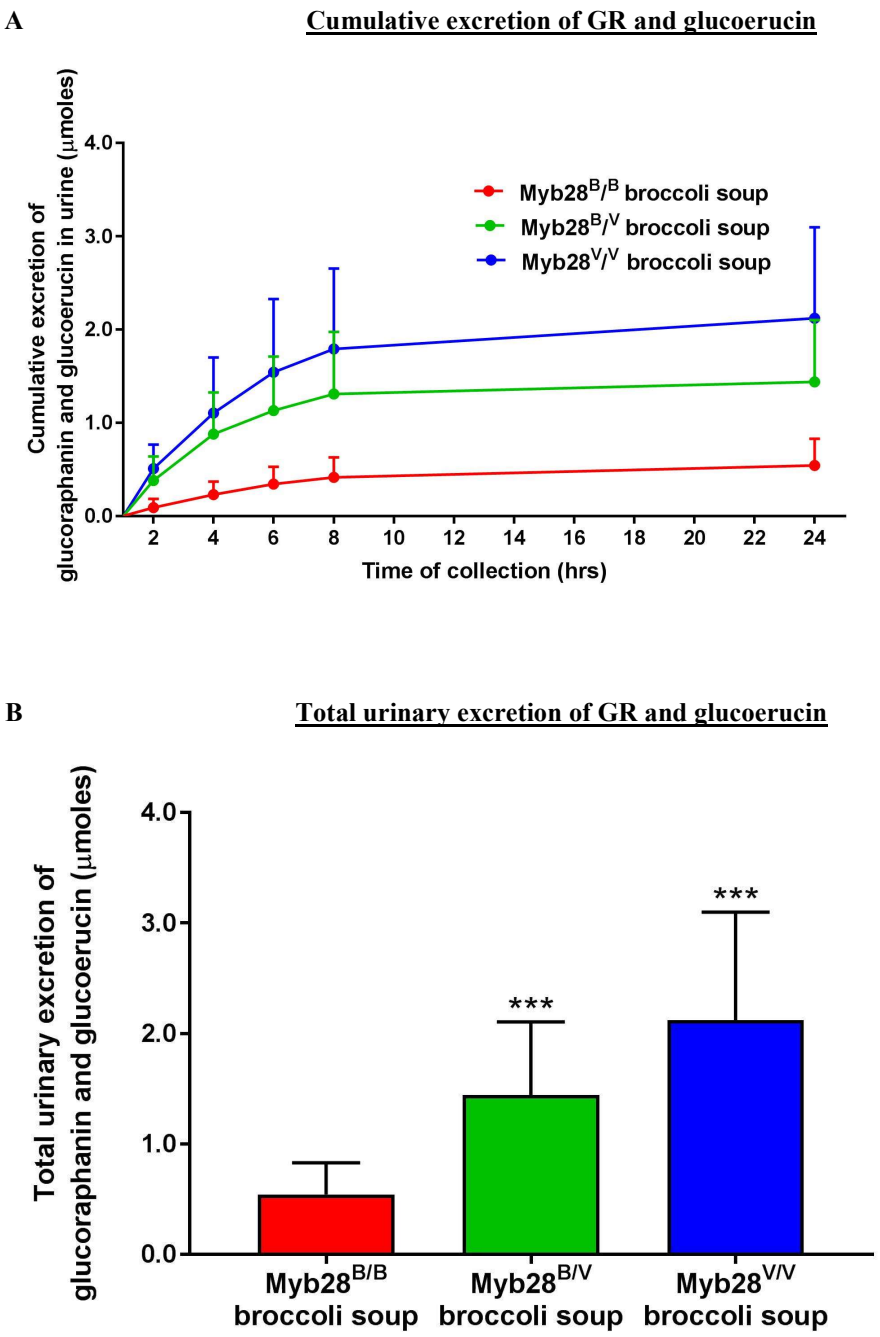


Figure 3-7: Urinary excretion of unmetabolised GR and glucoerucin (µmoles) in 24 hours following consumption of the three types of broccoli soups. The cumulative excretion of GR and glucoerucin (A) and the total urinary excretion of GR and glucoerucin (B) measured in urine samples were collected over 24 hours after consumption of Myb28$^{B/B}$ (84 µmoles GR/300g soup), Myb28$^{B/V}$ (280 µmoles GR /300g soup) and Myb28$^{V/V}$ (452 µmoles GR/300g soup). The samples were analysed for GR and glucoerucin by UPLC-MS/MS. Data (n=10) is represented as mean ± SD. Total urinary excretion data underwent square root transformation followed by analysis by one-way ANOVA with Tukey's honest significance test (* p<0.0001 vs. My28$^{B/B}$ broccoli soup).**

Glucoerucin, a product of the enzymatic conversion of GR by gut bacteria, was determined in the urine and plasma samples of participants who consumed the three soups. The levels of glucoerucin were undetectable in the plasma samples after consuming all three types of soups. In contrast, it was quantifiable in the urine samples collected from the participants. The percentage of GR and glucoerucin individually excreted in the urine is represented in **Table 3-9**. There was no significant difference in the percentage of GR or glucoerucin excreted following consumption of the three soups. However, excretion of GR was the major contributor to the total excretion in the urine. The percentage of GR excreted was twice the percentage of glucoerucin excreted following consumption of all three types of soups.

Table 3-9: Summary table of the individual percentage of GR and glucoerucin excreted in urine following consumption of soups made with different broccoli genotypes

	Myb28$^{B/B}$ broccoli and stilton soup	**Myb28$^{B/V}$ broccoli and stilton soup**	**Myb28$^{V/V}$ broccoli and stilton soup**
	Mean ± SD (%)	**Mean ± SD (%)**	**Mean ± SD (%)**
Urine metabolites			
GR	66.7 ± 11.02	60.7 ± 8.83	64.7 ± 13.24
Glucoerucin	33.3 ± 11.02	39.3 ± 8.83	38.6 ± 9.64

Data (n=10) is represented as mean ± SD. The percentage was calculated as the individual concentration of glucoraphanin and glucoerucin (µM) of the total concentration of glucoraphanin and glucoerucin (µM).

To assess whether the *GSTM1* genotype affects the bioavailability of GR, urine and plasma data were stratified per the participants' genotypes **(Table 3-10)**. There was no significant difference with the plasma pharmacokinetics (AUC, C_{max}, T_{max}) and the urinary excretion between *GSTM1*-null and *GSTM1*-positive participants.

Table 3-10: Summary table of unmetabolised GR in plasma and urine after consumption of soups made with different broccoli genotypes stratified by *GSTM1* genotypes

	***GSTM1*-positive (n=6)**	***GSTM1*-null (n=4)**	**p-value for genotype**
	Mean ± SD	**Mean ± SD**	
GR in plasma			
AUC (µmol.h/l)			
$Myb28^{B/B}$ broccoli and stilton soup	0.07 ± 0.06	0.03 ± 0.01	0.2390
$Myb28^{B/V}$ broccoli and stilton soup	0.18 ± 0.09	0.12 ± 0.03	0.1488
$Myb28^{V/V}$ broccoli and stilton soup	0.27 ± 0.12	0.20 ± 0.08	0.3559
C_{max} (µmol/l)			
$Myb28^{B/B}$ broccoli and stilton soup	0.01 ± 0.01	0.01 ± 0.00	0.4689
$Myb28^{B/V}$ broccoli and stilton soup	0.03 ± 0.01	0.03 ± 0.01	0.9463
$Myb28^{V/V}$ broccoli and stilton soup	0.04 ± 0.01	0.05 ± 0.03	0.3866
T_{max} (h)			
$Myb28^{B/B}$ broccoli and stilton soup	2.25 ± 1.48	2.13 ± 0.63	0.8591
$Myb28^{B/V}$ broccoli and stilton soup	1.88 ± 0.74	2.75 ± 0.96	0.1788
$Myb28^{V/V}$ broccoli and stilton soup	2.50 ± 1.18	1.88 ± 0.25	0.2597
Total GR and glucoerucin in urine (µmoles)			
$Myb28^{B/B}$ broccoli and stilton soup	0.53 ± 0.27	0.56 ± 0.36	0.9005
$Myb28^{B/V}$ broccoli and stilton soup	1.51 ± 0.46	1.34 ± 0.98	0.7583
$Myb28^{V/V}$ broccoli and stilton soup	2.11 ± 0.55	2.13 ± 1.53	0.9777

	GSTM1-positive (n=6)	*GSTM1*-null (n=4)	p-value for genotype
	Mean ± SD	**Mean ± SD**	
Percentage excreted 24 hours after ingestion %			
Myb28$^{B/B}$ broccoli and stilton soup	0.63 ± 0.32	0.66 ± 0.43	0.9004
Myb28$^{B/V}$ broccoli and stilton soup	0.16 ± 0.348	0.35 ± 0.164	0.7583
Myb28$^{V/V}$ broccoli and stilton soup	0.47 ± 0.12	0.47 ± 0.34	0.9777

Data (n=10) was analysed using unpaired t-test with Welch's correction.

3.5.5. Bioavailability of S-methyl-L-cysteine sulfoxide following consumption of soups made with three broccoli genotypes

To assess the bioavailability of SMCSO, plasma and urine samples collected from participants were analysed for SMCSO using a new validated UPLC-MS/MS method. All baseline samples collected prior to consumption of the soups detected SMCSO as SMCSO is present in some non-Brassica vegetables e.g. onions. Participants were only required to restrict glucosinolate-containing foods and garlic. This was considered and baseline levels were subtracted from all the subsequent samples. Following consumption of the soups, SMCSO was detected in both plasma and urine samples. To our knowledge, this is the first human study to report SMCSO detection and quantification in plasma and urine using UPLC-MS/MS.

SMCSO was detected in the plasma within 30 minutes of consumption of all three types of soups **(Figure 3-8)**. Subsequently, there is a steady increase in the levels of SMCSO in the plasma and peaks between 1.5-2 hours following consumption of Myb28$^{B/B}$ broccoli soups and soup with the Myb28$^{V/V}$ broccoli genotypes. After consumption of the soup with Myb28$^{B/V}$ broccoli genotype, the levels peak slightly earlier at 1 hour post-consumption. Consequently, the levels of SMCSO gradually decrease over the 24 hours after soup consumption but do not relapse to baseline levels. At 24 hours after consumption of the Myb28$^{B/B}$ broccoli soup, Myb28$^{B/V}$ broccoli soup, and Myb28$^{V/V}$ broccoli soup, SMCSO was quantified at 2.18 ± 1.53 µM, 3.57 ± 1.52 µM and 4.66 ± 1.52 µM respectively. There is an increase in the plasma levels of SMCSO following consumption of the soups with Myb28$^{B/V}$ broccoli genotype and Myb28$^{V/V}$ broccoli genotype compared to Myb28$^{B/B}$ broccoli soup **(Figure 3-8)**. In contrast to

GR and SF, the presence of one or two Myb28^{V} alleles results in similar levels of SMCSO in plasma but increased levels compared to Myb28$^{B/B}$.

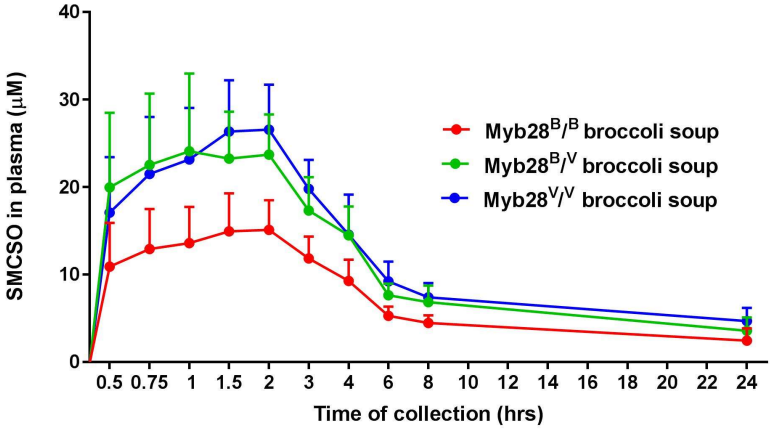


Figure 3-8: The plasma concentrations of SMCSO (µM) following consumption of the three types of broccoli soups. Plasma samples were collected over 24 hours following consumption Myb28$^{B/B}$ (84 µmoles GR & 1031 µmoles SMCSO /300g soup), Myb28$^{B/V}$ (280 µmoles GR & 1514 µmoles SMCSO /300g soup) and Myb28$^{V/V}$ (452 µmoles GR & 1454 µmoles SMCSO /300g soup). The samples were analysed for SMCSO by UPLC-MS/MS. Data (n=10) is represented as mean ± SD.

The pharmacokinetics of SMCSO from the broccoli soups are represented as the AUC, C_{max}, T_{max}, and percentage of excretion as reported in **Table 3-11**. There is a significant increase in the plasma levels of SMCSO by ~2-fold following consumption of the soups with the Myb28$^{B/V}$ and Myb28$^{V/V}$ broccoli genotypes ($p<0.0001$) compared to Myb28$^{B/B}$ broccoli as expressed by the AUC and C_{max} **(Table 3-11)**.

Table 3-11: Summary table of the levels of SMCSO in plasma and urine after consumption of soups stratified by soups made with different broccoli genotypes

	Myb28$^{B/B}$ broccoli and stilton soup	**Myb28$^{B/V}$ broccoli and stilton soup**	**Myb28$^{V/V}$ broccoli and stilton soup**	**p-value for broccoli genotypes**			
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**ANOVA**	**Myb28$^{B/B}$ vs. Myb28$^{B/V}$**	**Myb28$^{B/B}$ vs. Myb28$^{V/V}$**	**Myb28$^{B/V}$ vs. Myb28$^{V/V}$**
SMCSO in plasma							
AUC (μmol.h/l)	130.85 ± 51.49	195.34 ± 40.42	217.53 ± 43.22	<0.01	≤0.0001	<0.0001	0.2793
C_{max} (μmol/l)	16.93 ± 3.79	26.94 ± 7.70	28.03 ± 5.39	<0.01	<0.0001	<0.0001	0.6122
T_{max} (h)	1.78 ± 0.63	1.38 ± 0.54	1.70 ± 0.35	0.2548			
Concentration at 24 hours (μmol/l)	2.18 ± 1.53	3.57 ± 1.52	4.66 ± 1.52	<0.01	0.1360	<0.01	0.1273

	Myb28$^{B/B}$ broccoli and stilton soup	**Myb28$^{B/V}$ broccoli and stilton soup**	**Myb28$^{V/V}$ broccoli and stilton soup**	**p-value for broccoli genotypes**			
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**ANOVA**	**Myb28$^{B/B}$ vs. Myb28$^{B/V}$**	**Myb28$^{B/B}$ vs. Myb28$^{V/V}$**	**Myb28$^{B/V}$ vs. Myb28$^{V/V}$**
SMCSO in urine							
Total excreted in 24 hours (µmoles)	68.16 ± 23.98	104.71 ± 46.16	109.27 ± 34.50	<0.01	<0.05	<0.01	0.6820
Percentage excreted 24 hours after ingestion %	6.61 ± 2.33	6.92 ± 3.05	7.52 ± 2.37	0.4501			

Analysis modelled using sequential analysis of variance (ANOVA) by Dr Henri Tapp. The variables including C_{max}, total excreted and percentage excreted underwent a log transformation. Significance level for multipe comparisons was analysed using Tukey's honest significance test.

The excretion of SMCSO via urine commenced within 2 hours and peaked at 2-4 hours with consumption of all three types of soups. The cumulative excretion of SMCSO rapidly increases between 0-8 hours and then continues at a steady rate. Thus, excretion does not reach a plateau in any of the three types of soups **(Figure 3-9 A)**. The cumulative amount of SMCSO excreted in the urine 24 hours following soup consumption is significantly higher by ~1.6-fold with $Myb28^{V/V}$ (109.27 ± 34.50 vs. 68.16 ± 23.98 µmoles, $p<0.01$) and significantly higher by ~1.5-fold with $Myb28^{B/V}$ (104.71 ± 46.16 vs. 68.16 ± 23.98 µmoles, $p<0.05$) compared to $Myb28^{B/B}$ broccoli **(Figure 3-9 A-B)**. No significant difference was observed between the two enhanced broccoli types in terms of the excretion of SMCSO in the urine **(Figure 3-9 A-B)**.

The urine excretion **(Figure 3-9 A-B)** and plasma levels of SMCSO **(Figure 3-8)** depict that the occurrence of one or two $Myb28^{V}$ alleles in the broccoli genotypes results in increased pharmacokinetic parameters.

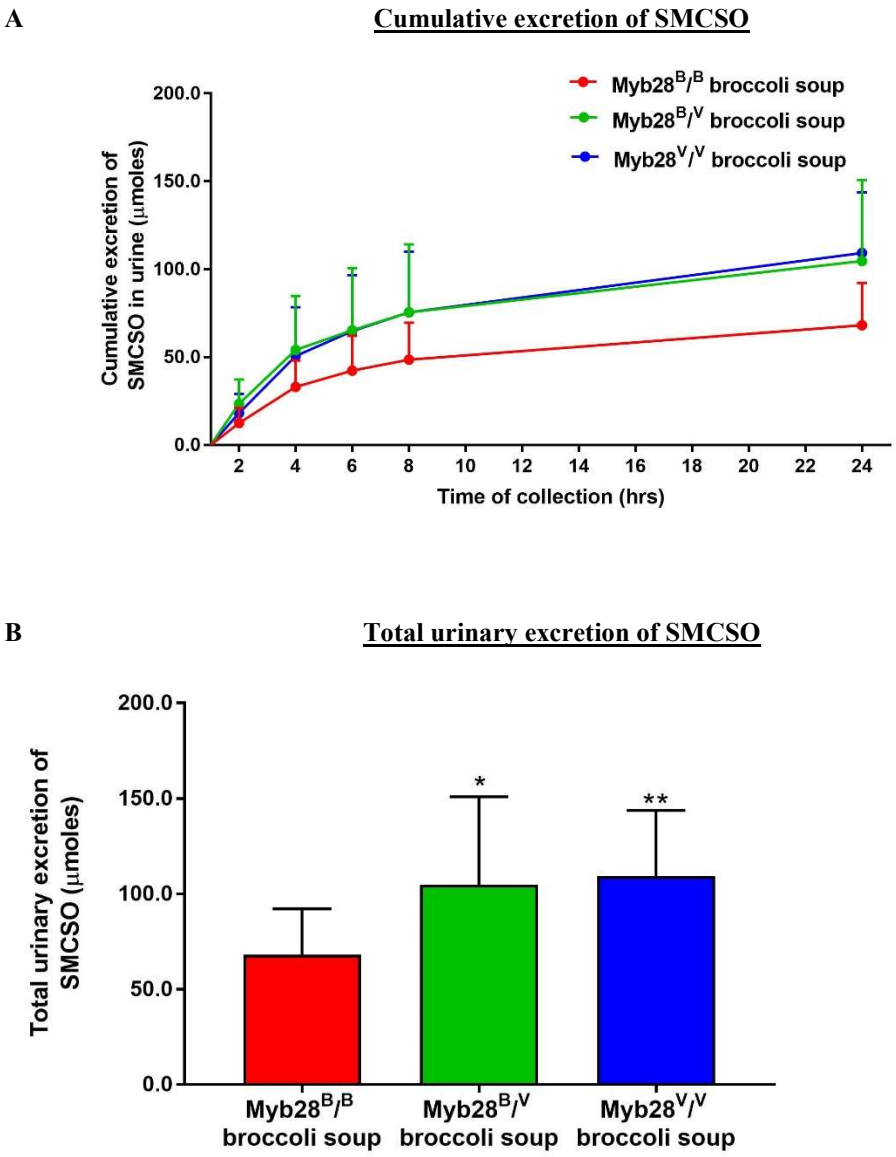


Figure 3-9: Urinary excretion of SMCSO following consumption of the three types of broccoli soups. The cumulative excretion of SMCSO (A) and the total urinary excretion of SMCSO (B) measured in urine samples collected over 24 hours after consumption of Myb28$^{B/B}$ (84 µmoles GR & 1031 µmoles SMCSO /300g soup), Myb28$^{B/V}$ (280 µmoles GR & 1514 µmoles SMCSO /300g soup) and Myb28$^{V/V}$ (452 µmoles GR & 1454 SMCSO µmoles /300g soup). The samples were analysed for SMCSO using UPLC-MS/MS. Data (n=10) is represented as mean ± SD. Data underwent log transformation followed by analysis by one-way ANOVA with Tukey's honest significance test (* p<0.05 and **p<0.01 vs. My28$^{B/B}$ broccoli soup).

Pharmacokinetic parameters of SMCSO were stratified by *GSTM1* genotype to determine its influence on the bioavailability of SMCSO. The analysis indicated no statistically significant difference between *GSTM1*-null and *GSTM1*-positive participants on the plasma pharmacokinetics (AUC, C_{max}, T_{max}) and the urinary excretion **(Table 3-12)**.

Table 3-12: Summary table of SMCSO in plasma and urine after consumption of soups made with different broccoli genotypes stratified by *GSTM1* genotypes

	***GSTM1*-positive (n=6)**	***GSTM1*-null (n=4)**	**p-value for genotype**
	Mean ± SD	**Mean ± SD**	
SMCSO in plasma			
AUC (µmol.h/l)			
$Myb28^{B/B}$ broccoli and stilton soup	152.72 ± 52.45	98.06 ± 31.26	0.0732
$Myb28^{B/V}$ broccoli and stilton soup	209.03 ± 44.68	174.80 ± 25.22	0.1617
$Myb28^{V/V}$ broccoli and stilton soup	231.03 ± 44.91	197.28 ± 36.49	0.2303
C_{max} (µmol/l)			
$Myb28^{B/B}$ broccoli and stilton soup	17.64 ± 2.96	15.88 ± 5.09	0.5627
$Myb28^{B/V}$ broccoli and stilton soup	29.83 ± 7.53	22.62 ± 6.45	0.1478
$Myb28^{V/V}$ broccoli and stilton soup	29.14 ± 5.28	26.36 ± 5.88	0.4747
T_{max} (h)			
$Myb28^{B/B}$ broccoli and stilton soup	1.75 ± 0.42	1.81 ± 0.94	0.9072
$Myb28^{B/V}$ broccoli and stilton soup	1.38 ± 0.54	1.38 ± 0.63	0.9999
$Myb28^{V/V}$ broccoli and stilton soup	1.75 ± 0.42	1.63 ± 0.25	0.5711
Total SMCSO in urine (µmoles)			
$Myb28^{B/B}$ broccoli and stilton soup	68.2 ± 25.52	68.2 ± 25.29	0.9995
$Myb28^{B/V}$ broccoli and stilton soup	110.8 ± 51.6	95.5 ± 42.11	0.6221

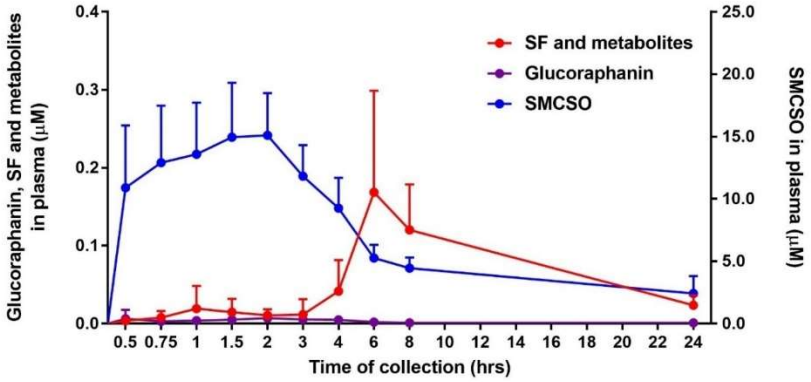
	GSTM1-positive (n=6)	*GSTM1*-null (n=4)	p-value for genotype
	Mean ± SD	**Mean ± SD**	
Myb28$^{V/V}$ broccoli and stilton soup	106.0 ± 17.04	114.2 ± 55.07	0.7885
Percentage excreted 24 hours after ingestion %			
Myb28$^{B/B}$ broccoli and stilton soup	6.61 ± 2.48	6.62 ± 2.45	0.9995
Myb28$^{B/V}$ broccoli and stilton soup	7.32 ± 3.41	6.31 ± 2.78	0.6221
Myb28$^{V/V}$ broccoli and stilton soup	7.29 ± 1.17	7.86 ± 3.79	0.7885

Data represented as mean ± SD and analysed using unpaired t-test with Welch's correction.

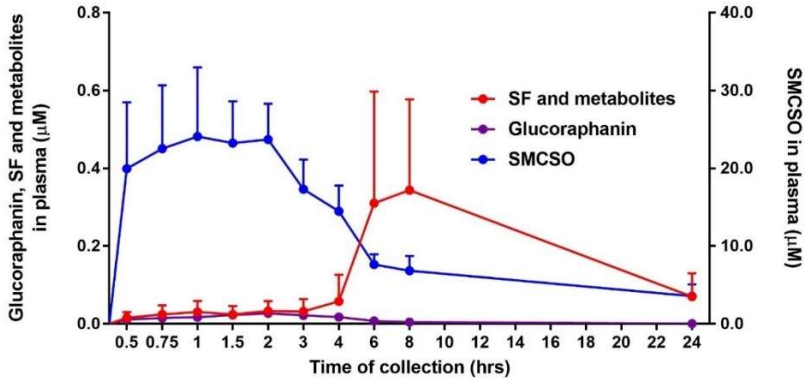
3.5.6. Comparison of the bioavailability of sulforaphane, glucoraphanin and S-methyl-L-cysteine sulfoxide in plasma following consumption of soups made with three broccoli genotypes

To compare the bioavailability of the three compounds; GR, SF and SMCSO, data were collated as represented in **Figure 3-10**. Following consumption of the soups, SMCSO and GR levels peaked much earlier at approximately 2 hours and started to decline, whereas SF peaked at 6-8 hours. Twenty-four hours after soup consumption, levels of SF and GR measured in the plasma were negligible. On the other hand, SMCSO levels were quantified at higher levels of 2.2-4.7 µM. A similar trend in GR, SMCSO and SF across 24 hours in plasma was observed following consumption of all three types of soups. The major distinction between the consumption of the three types of soups is the levels achieved in the plasma. Consumption of the soup with the Myb28$^{V/V}$ broccoli genotype accumulates enhanced levels of all three compounds compared to after consumption of the soup with Myb28$^{B/V}$ broccoli genotype and Myb28$^{B/B}$ broccoli. There was a dose-dependent increase in the levels of GR, SMCSO and SF and metabolites. A crucial finding is the augmented levels of SMCSO measured in the plasma, ranging from 15.1-26.6 µM, compared to SF (0.2-0.6 µM) following consumption of the soups. This is ~44-75-fold greater than the SF levels achieved in the plasma. Intact GR levels detected are much lower than those of SF in plasma.

A **Levels in the plasma after consumption of Myb28$^{B/B}$ broccoli soup**



B **Levels in the plasma after consumption of Myb28$^{B/V}$ broccoli soup**



C **Levels in the plasma after consumption of Myb28$^{V/V}$ broccoli soup**

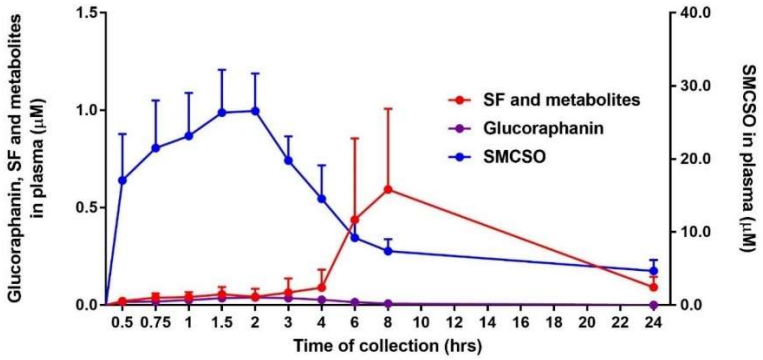
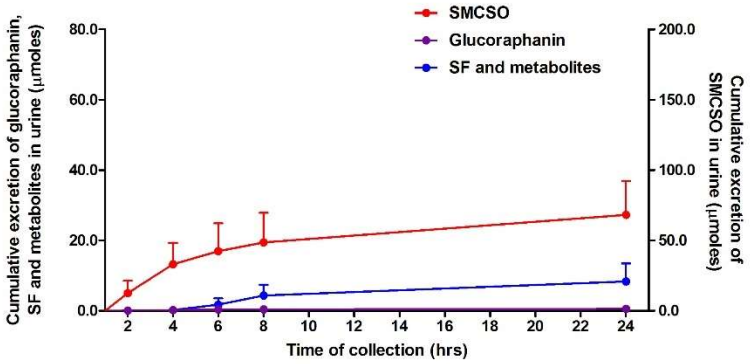


Figure 3-10: The plasma concentrations of GR, SF, and SMCSO (μM) following consumption of soups made with three broccoli genotypes. Blood samples collected from participants were analysed for metabolites. GR and SF concentration are plotted on the left y-axis and SMCSO is plotted on the right y-axis. SF metabolites measured in the plasma includes SF, SF-GSH, SF-Cys, SF-Cys-Gly and SF-NAC. The levels of metabolites in the plasma following consumption of standard broccoli soup, Myb28$^{B/B}$ (84 μmoles GR & 1031 μmoles SMCSO /300g soup) (A); Myb28$^{B/V}$ (280 μmoles GR & 1514 μmoles SMCSO /300g soup) (B) and Myb28$^{V/V}$ (452 μmoles GR & 1454 μmoles SMCSO /300g soup) (C). Data (n=10) is represented as mean ± SD.

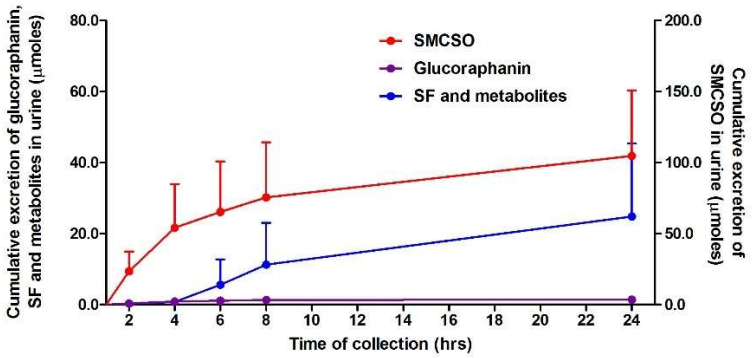
3.5.7. Comparison of the bioavailability of sulforaphane, glucoraphanin and S-methyl-L-cysteine sulfoxide in urine following consumption of soups made with three broccoli genotypes

Data from the bioavailability of the three compounds were compared as illustrated in **Figure 3-11.** Following consumption of the soups, the excretion of SMCSO and GR rapidly increases at 0-4 hours whereas SF does not increase until at 6-8 hours post consumption. While the cumulative excretion of GR reaches a plateau after 8 hours of excretion, SF and SMCSO do not plateau even after 24 hours, following consumption of all three types of soups. The levels of GR and SF excreted increase in a dose-dependent manner following consumption of soups made with $Myb28^{V/V}$ broccoli genotype versus those made with $Myb28^{B/V}$ broccoli and $Myb28^{B/B}$ broccoli. Comparable levels of SMCSO were excreted in the urine following consumption of the soups made with $Myb28^{V/V}$ broccoli genotype and $Myb28^{B/V}$ broccoli genotype, with both being greater than levels excreted following consumption of $Myb28^{B/B}$ broccoli. The fundamental difference in the levels of excreted metabolites in the urine following soup consumption is that the levels of GR excreted are considerably lower than those of SF and SMCSO. The levels of SMCSO excreted are notably higher than those of SF and GR. At 24 hours, SMCSO excretion ranges from 68.2-109.3 µmoles as opposed to 9.0-40.6 µmoles of SF excretion, which is a ~3-8-fold difference.

A **Levels in the urine after consumption of Myb28$^{B/B}$ broccoli soup**



B **Levels in the urine after consumption of Myb28$^{B/V}$ broccoli soup**



C **Levels in the urine after consumption of Myb28$^{V/V}$ broccoli soup**

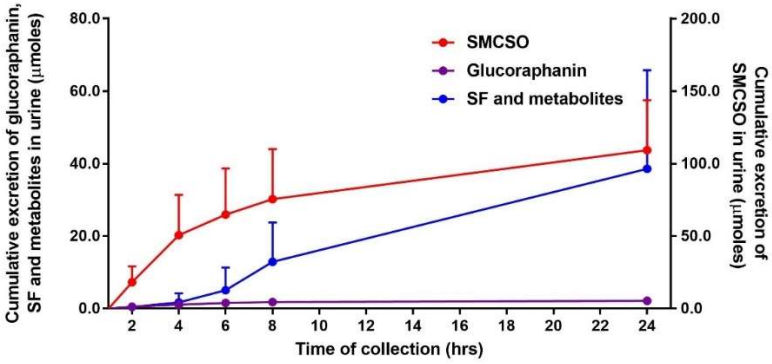
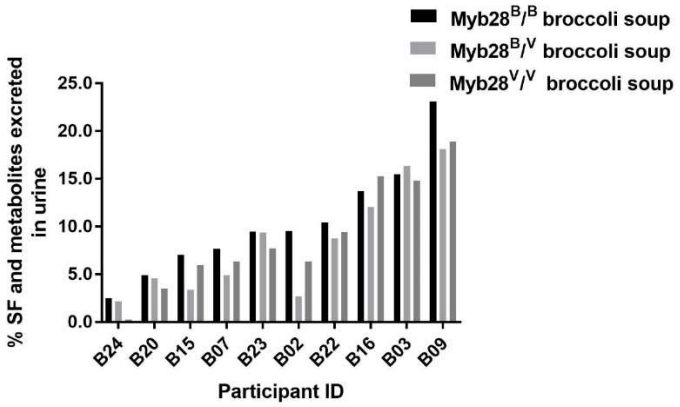


Figure 3-11: Cumulative urinary excretion of GR, SF, and SMCSO (µmoles) following consumption of soups made with three broccoli genotypes. Urine samples collected from participants were analysed for metabolites. GR and SF concentration are plotted on the left y-axis and SMCSO is plotted on the right y-axis. SF metabolites measured in the urine includes SF, ERN-NAC, SF-Cys, SF-Cys-Gly, and SF-NAC. GR levels shows also include glucoerucin levels. The levels of metabolites in the urine following consumption of standard broccoli soup, Myb28$^{B/B}$ (84 µmoles GR & 1031 µmoles SMCSO /300g soup) (A); Myb28$^{B/V}$ (280 µmoles GR & 1514 µmoles SMCSO /300g soup) (B) and Myb28$^{V/V}$ (452 µmoles GR & 1454 µmoles SMCSO/300g soup) (C). Data is represented as mean + SD (n=10).

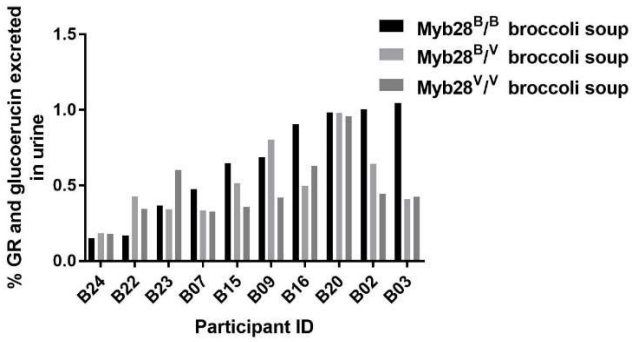
3.5.8. Comparison of the percentage of sulforaphane, glucoraphanin and S-methyl-L-cysteine sulfoxide excreted in the urine following consumption of soups made with three broccoli genotypes

The SF and GR data are represented here as the percentage of the ingested dose of GR that is excreted. However, SMCSO is expressed as the percentage of the ingested SMCSO. There is a considerable variation in the percentage of SF and metabolites excreted following consumption of the three types of soups compared to the percentage of GR and SMCSO excreted. The percentage of SF and metabolites excreted ranges from 2.5-23.1%, 2.2-18.1% and 0.2-18.9% following consumption of $Myb28^{B/B}$, $Myb28^{B/V}$ and $Myb28^{V/V}$ broccoli soups, respectively **(Figure 3-12 A)**. In contrast, the percentage of GR and metabolites excreted ranges from 0.2-1.0% following consumption of $Myb28^{B/B}$, $Myb28^{B/V}$ and $Myb28^{V/V}$ broccoli soups, respectively **(Figure 3-12 B)**. The percentage of SMCSO excreted ranges from 3.3-10.7%, 3.4-12.3%, 2.5-11.0% following consumption of $Myb28^{B/B}$, $Myb28^{B/V}$ and $Myb28^{V/V}$ broccoli soups, respectively **(Figure 3-12 C)**.

A **Percentage of SF and metabolites excreted**



B **Percentage of GR and glucoerucin excreted**



C **Percentage of SMCSO excreted**

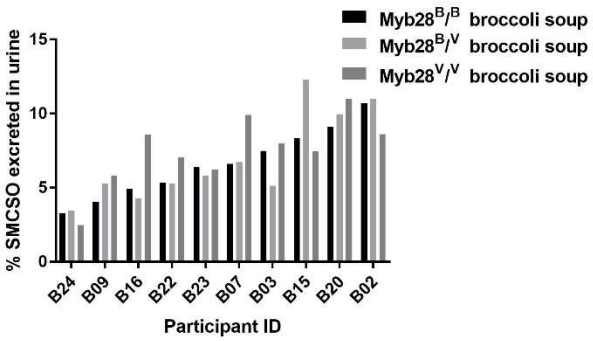
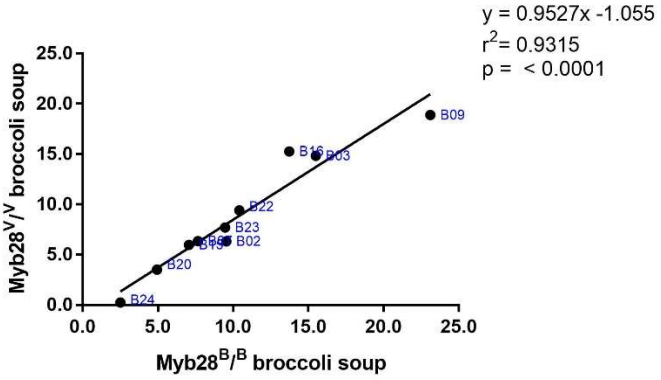


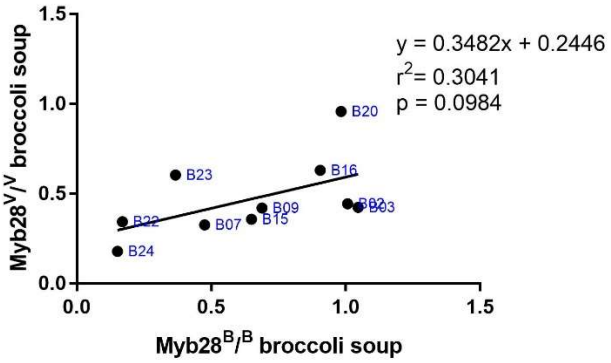
Figure 3-12: Percentage of SF, GR, and SMCSO excreted in the urine following consumption of soups made with three broccoli genotypes. Urine samples collected from participants were analysed for metabolites. SF metabolites measured in the urine includes SF, ERN-NAC, SF-Cys, SF-Cys-Gly, and SF-NAC. GR levels shown also include glucoerucin levels. The percentage of SF and metabolites (A), GR and glucoerucin (B) and SMCSO (C) excreted of the ingested dose of GR and SMCSO in the broccoli soups, Myb28$^{B/B}$ (84 µmoles GR & 1031 µmoles SMCSO /300g soup) (A); Myb28$^{B/V}$ (280 µmoles GR & 1514 µmoles SMCSO /300g soup) (B) and Myb28$^{V/V}$ (452 µmoles GR & 1454 µmoles SMCSO/300g soup).

In order to assess whether an individual would excrete similar percentages of metabolites following consumption of $Myb28^{B/B}$ and $Myb28^{V/V}$, correlation graphs were plotted for SF and its metabolites, GR and glucoerucin, and SMCSO. A strong statistically significant positive correlation was observed in the percentage of SF and metabolites excreted between consumption of $Myb28^{B/B}$ and $Myb28^{V/V}$ ($p<0.0001$) **(Figure 3-13 A)**. Additionally, a moderate statistically significant positive correlation was observed in the percentage of SMCSO excreted between consumption of $Myb28^{B/B}$ and $Myb28^{V/V}$ ($p<0.05$) **(Figure 3-13 C)**. In contrast, no significant correlation was observed in the percentage of GR and glucoerucin excreted between consumption of $Myb28^{B/B}$ and $Myb28^{V/V}$ **(Figure 3-13 B)**.

A **Urine excretion of SF and metabolites**



B **Urine excretion of GR and glucoerucin**



C **Urine excretion of SMCSO**

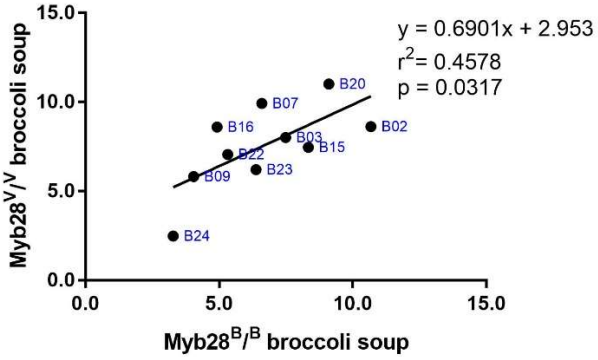
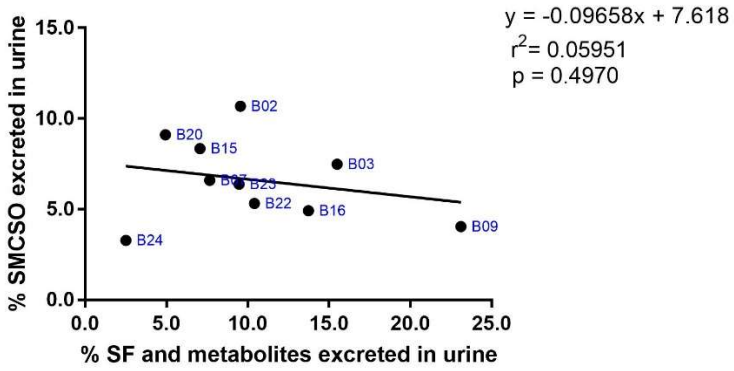


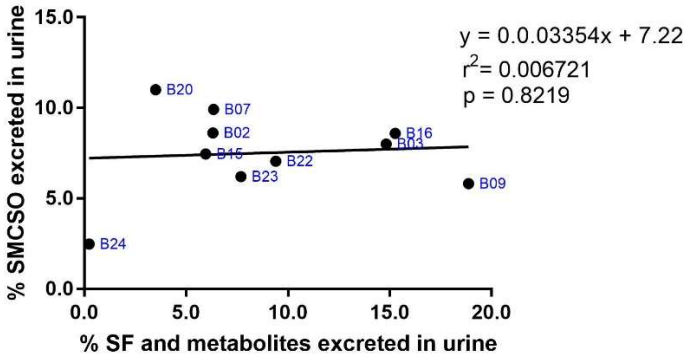
Figure 3-13: Correlation of the percentage of SF, GR, and SMCSO excreted in the urine following consumption of Myb28$^{B/B}$ and Myb28$^{V/V}$ soups. Urine samples collected from participants were analysed for metabolites. SF metabolites measured in the urine (n=10) includes SF, ERN-NAC, SF-Cys, SF-Cys-Gly, and SF-NAC. GR levels shown also include glucoerucin levels. The graphs represent the correlation of the percentage of SF and metabolites (A), GR and glucoerucin (B) and SMCSO (C) excreted of the ingested dose of GR and SMCSO in the broccoli soups between Myb28$^{B/B}$ (84 µmoles GR & 1031 µmoles SMCSO /300g soup) and Myb28$^{V/V}$ (452 µmoles GR & 1454µmoles SMCSO/300g soup).

To evaluate whether there is a correlation between the percentage excretion of the three metabolites following consumption of the three types of soups, correlation graphs were plotted for SF vs. SMCSO, SF vs. GR and GR vs. SMCSO. There was no statistically significant correlation between the percentage of SF and SMCSO excreted following consumption of the three types of soups **(Figure 3-14)**. Similarly, there was no statistically significant correlation between the percentage of GR and SF excreted following consumption of the three types of soups **(Figure 3-15)**. Although not statistically significant, there is a moderate relationship between the percentage of GR and SMCSO excreted following consumption of $Myb28^{B/B}$ and $Myb28^{V/V}$ soups **(Figure 3-16)**. Following consumption of $Myb28^{B/V}$ soup, a non-statistically significant weak correlation was observed between the percentage of GR and SMCSO excreted **(Figure 3-16 B)**.

A **Percentage urinary excretion after consumption of Myb28$^{B/B}$ broccoli soup**



B **Percentage urinary excretion after consumption of Myb28$^{B/V}$ broccoli soup**



C **Percentage urinary excretion after consumption of Myb28$^{V/V}$ broccoli soup**

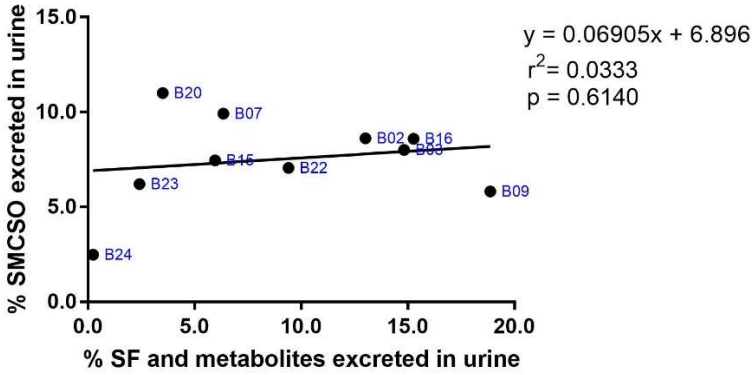
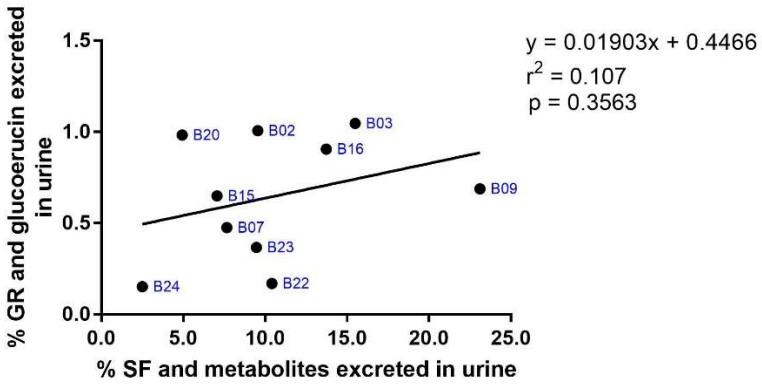
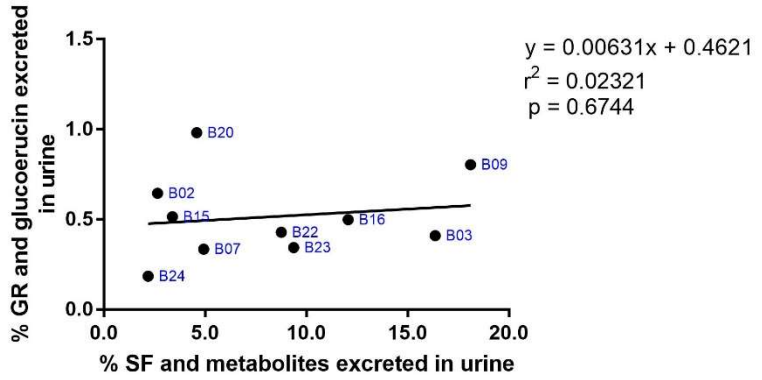


Figure 3-14: Correlation of the percentage of SF and SMCSO excreted in the urine following consumption of all three types of soups. Urine samples collected from participants were analysed for metabolites. SF metabolites measured in the urine (n=10) includes SF, ERN-NAC, SF-Cys, SF-Cys-Gly, and SF-NAC. The graphs represent the correlation between the percentage of SMCSO and SF excreted of the ingested dose following consumption of Myb28$^{B/B}$ (84 µmoles GR & 1031 µmoles SMCSO /300g soup) (A), Myb28$^{B/V}$ (280 µmoles GR & 1514 µmoles SMCSO /300g soup) (B) and Myb28$^{V/V}$ (452 µmoles GR & 1454 µmoles SMCSO /300g soup) (C).

A **Percentage urinary excretion after consumption of Myb28$^{B/B}$ broccoli soup**



B **Percentage urinary excretion after consumption of Myb28$^{B/V}$ broccoli soup**



C **Percentage urinary excretion after consumption of Myb28$^{V/V}$ broccoli soup**

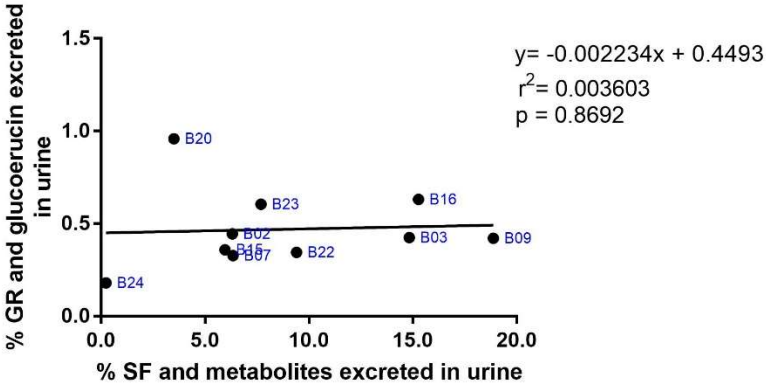
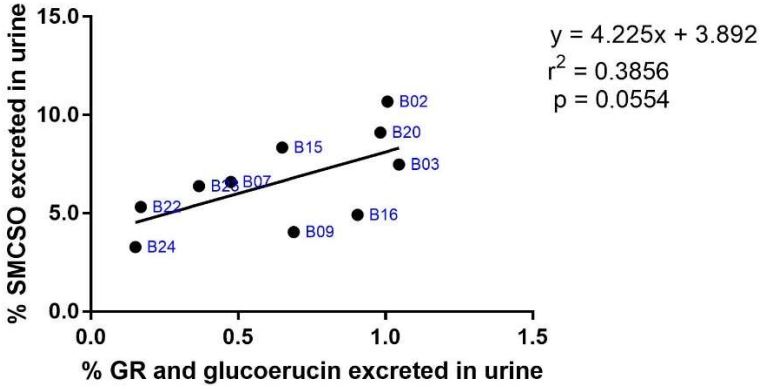
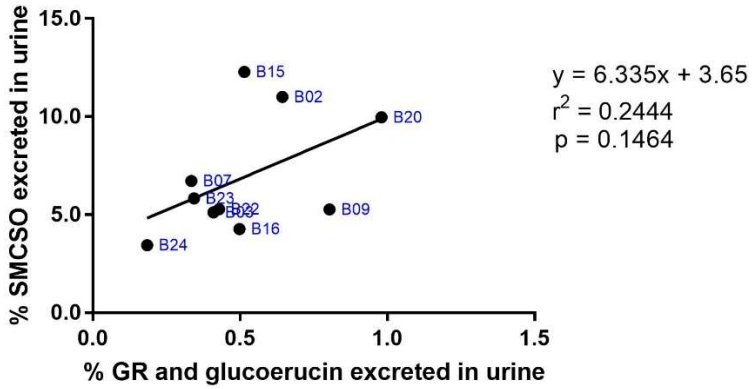


Figure 3-15: Correlation of the percentage of SF and GR excreted in the urine following consumption of all three types of soups. Urine samples collected from participants were analysed for metabolites. SF metabolites measured in the urine (n=10) includes SF, ERN-NAC, SF-Cys, SF-Cys-Gly, and SF-NAC. GR levels includes glucoerucin. The graphs represent the correlation between the percentage of GR and SF excreted of the ingested dose following consumption of Myb28$^{B/B}$ (84 µmoles GR & 1031 µmoles SMCSO /300g soup) (A), Myb28$^{B/V}$ (280 µmoles GR & 1514 µmoles SMCSO /300g soup) (B) and Myb28$^{V/V}$ (452 µmoles GR & 1454 µmoles SMCSO /300g soup) (C).

A **Percentage urinary excretion after consumption of Myb28$^{B/B}$ broccoli soup**



B **Percentage urinary excretion after consumption of Myb28$^{B/V}$ broccoli soup**



C **Percentage urinary excretion after consumption of Myb28$^{V/V}$ broccoli soup**

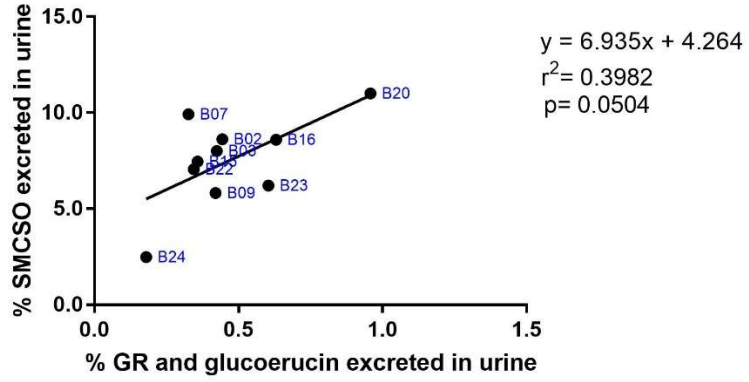


Figure 3-16: Correlation of the percentage of SMCSO and GR excreted in the urine following consumption of all three types of soups. Urine samples (n=10) collected from participants were analysed for metabolites. GR levels includes glucoerucin. The graphs represent the correlation between the percentage of GR and SMCSO excreted of the ingested dose following consumption of Myb28$^{B/B}$ (84 µmoles GR & 1031 µmoles SMCSO /300g soup) (A), Myb28$^{B/V}$ (280 µmoles GR & 1514 µmoles SMCSO /300g soup) (B) and Myb28$^{V/V}$ (452 µmoles GR & 1454 µmoles SMCSO /300g soup) (C).

3.6. Discussion

The aim of this work was to investigate the bioavailability of sulphur-containing compounds from enriched broccoli soups made with three broccoli genotypes. To accomplish this, 10 apparently healthy participants were recruited into a double-blind, three-phase, crossover, randomised intervention trial with three soups made with different broccoli genotypes. The three types of broccoli consist of standard broccoli ($Myb28^{B/B}$) and two novel varieties; $Myb28^{B/V}$ and $Myb28^{V/V}$. As reported in **Chapter 2** the presence of one or more $Myb28^{V}$ allele resulted in elevated levels of GR and SMCSO in the soups. The $Myb28^{V/V}$ soups accumulate 452 µmoles GR; 1454 µmoles SMCSO, $Myb28^{B/V}$ soups accumulate 280 µmoles GR; 1514 µmoles SMCSO compared to standard broccoli soup/$Myb28^{B/B}$ (84 µmoles GR;1031 µmoles SMCSO). The objective was to assess whether the enhanced levels of GR and SMCSO in the soups would be bioavailable resulting in enhanced levels absorbed and delivered to the plasma. Participants consumed each of the three types of soups in a random sequence, following which plasma and urine samples were collected at various time-points and quantitatively analysed for SF, GR and SMCSO using LC-MS/MS and UPLC-MS/MS.

Bioavailability of SF and metabolites from the intake of cruciferous vegetables including raw/cooked broccoli has been widely demonstrated by many human intervention studies. Further, Gasper *et al.* described increased absorption of SF following consumption of a high-glucosinolate soup, with the AUC and C_{max} in the plasma being 3-fold greater than that resulting from the consumption of standard broccoli [38]. The present study confirmed that the intake of increasing GR levels with the three broccoli genotypes delivered increasing concentrations of GR to the gut as demonstrated by a dose-dependent curve for SF **(Figure 3-4)** and GR **(Figure 3-6)**. Consumption of the $Myb28^{V/V}$ broccoli soup, containing 5 times more GR, led to an AUC for SF 4 times that of $Myb28^{B/B}$ broccoli. Similarly, $Myb28^{B/V}$ broccoli soup containing 3 times more GR resulted in an AUC for SF of 2 times that of $Myb28^{B/B}$ broccoli. In addition, both the $Myb28^{V/V}$ and $Myb28^{B/V}$ broccoli soups comprised levels of SMCSO 1.4 times that of $Myb28^{B/B}$ broccoli resulting in an AUC of 2 times that of $Myb28^{B/B}$, respectively. The enhanced levels of GR and SMCSO from the soups are bioavailable as indicated by the elevated levels of SF, GR and SMCSO in plasma.

As a result of the soup production process, the myrosinase enzyme was inactivated thus there was no presence of SF in the soups ingested. Following consumption of the three types of soups, intact SMCSO and GR was rapidly absorbed at 30 minutes and peaked approximately at 2 hours in the plasma **(Figure 3-6; Figure 3-8).** On the other hand, even though SF was detected at 30 minutes, it did not peak until 6 hours with $Myb28^{B/B}$ broccoli soup and 8 hours with $Myb28^{B/V}$ and $Myb28^{V/V}$ broccoli soups which is comparable to the T_{max}= 6 hours

reported by previous studies [68,69,71]. This is much later compared to GR and SMCSO presumably because time is required for the GR to reach the gut where it is hydrolysed to SF by the gut microbial thioglucosidase [15] followed by absorption through the enterocytes. The study by Gasper *et al*. demonstrated a T_{max} of 1.5-2 hours following ingestion of SF as the broccoli soups were produced without denaturing the myrosinase [38]. Evidently, SF was delivered to the gut and rapidly absorbed, peaking earlier at 2 hours without the pre-requirement of conversion by the microbial thioglucosidase. Given that this is the first human study intervention to report the detection of intact GR and SMCSO in plasma, the pharmacokinetics of these dietary compounds are unknown. However, it can be assumed that GR and SMCSO are absorbed in their native forms, without conversion by the gut bacteria, peaking in the plasma at 2 hours. Although this is the first study to report intact GR in human plasma, other studies have described measuring intact GR in the plasma of dogs and rats [21]. This suggests that GR is hydrolysed by our microbial thioglucosidase as well as absorbed intact through the enterocytes.

The present study has reported a plasma C_{max} of 0.2 µM for SF following consumption of soup containing 84 µmoles GR ($Myb28^{B/B}$ broccoli). Increasing the GR levels in the soups resulted in higher C_{max} of 0.4 µM and 0.6 µM following consumption of $Myb28^{B/V}$ and $Myb28^{V/V}$ broccoli soups, respectively. Despite providing participants soups with only ½ the amount of GR compared to the current study, Saha *et al.* reported that considerably less SF was detected with a C_{max} of 0.025 µM SF [68]. Similarly, Vermeulen and colleagues showed a C_{max} of 0.031 µM following consumption of 61.4 µmoles [71]. One of the potential reasons for the difference in the levels of measured SF across these interventions could be the use of a different column and LC-MS/MS system. The present study used a Luna 3u C18 presenting better separation than the Zorbax SB-Aq column used in the study by Saha *et al.* as well as the use of an Agilent 6490 Triple Quad LC-MS/MS [68]. Other explanations include the use of a different food matrix as the soups used in this study were rich in fat and other nutrients which could have aided absorption of GR. The C_{max} of intact GR was <0.1 µM following consumption of all three types of soups which is less in comparison to SF in plasma. On the other hand, elevated levels of SMCSO in the plasma were achieved. A C_{max} of 16.9 µM, 26.9 µM and 28.0 µM was measured following consumption of $Myb28^{B/B}$, $Myb28^{B/V}$ and $Myb28^{V/V}$ broccoli soups, respectively. In comparison to the levels of SF, this is considerably higher and could possibly be explained by the high levels of SMCSO present in the soups compared to the other dietary compound GR.

The prevalence of the SF conjugates in plasma in the current study is not in accordance with previous reports. SF and SF-GSH were the primary metabolites measured in the plasma

followed by SF-Cys-Gly. However, Saha *et al.* ranked the metabolites in the following order; SF, SF-Cys-Gly, SF-Cys, SF-GSH and SF-NAC [68] whereas Clarke *et al.* ordered them as SF-Cys-Gly, SF-NAC, SF-Cys, SF-GSH, SF following consumption of GR [69]. Gasper *et al.* reported a higher percentage of SF succeeded by SF-Cys, SF-Cys-Gly, SF-NAC and SF-GSH in that order following consumption of SF [38].

The discrepancy observed in the concentrations of SF, in addition to individual SF conjugates, could be justified by the intra-individual variations, potentially influenced by age, diet, gender, gut microbiota and other characteristics [22]. Gut microbiota variation amongst individuals, resulting in a deviation in the amount of SF hydrolysed from its precursor GR, is a plausible explanation [151], especially as greater variations have previously been reported in the excretion of SF following consumption of GR compared to direct consumption of SF [22,151,152].

This study has also reported that increasing levels of GR in soups resulted in increased levels of total SF **(Figure 3-5 B)** and GR excreted in the urine **(Figure 3-7 B)** in a dose-dependent manner. Likewise, the Myb28$^{B/V}$ and Myb28$^{V/V}$ broccoli soups with higher SMCSO levels led to greater urinary excretion concentration compared to Myb28$^{B/B}$ broccoli **(Figure 3-9 B)**. As the levels of SMCSO did not significantly differ between Myb28$^{B/V}$ and Myb28$^{V/V}$ broccoli soups, the urinary output also did not significantly differ. While another study has reported the detection of SMCSO in urine using NMR [153], this was not quantifiable and thus the present study is the first to report the bioavailability of SMCSO in humans. One human intervention study employing radiolabelled [^{35}S]-SMCSO was undertaken in 4 healthy males and quantified recovered [^{35}S] [84], however, this approach did not discriminate between [^{35}S] contained within SMCSO and that incorporated into metabolites, whereas this study specifically quantified SMCSO. Urinary excretion of intact GR has been discovered by others in human [14] and rats [20] although one study was not able to detect GR in human urine following consumption of broccoli sprouts [22].

GR and SMCSO are rapidly excreted at 0-4 hours **(Figure 3-7; Figure 3-9)** but SF is excreted much later **(Figure 3-5),** emphasising the role of gut microbiota in the hydrolysis of GR to SF. Consumption of the three types of soups resulted in a peak urinary excretion of SF at 6-8 hours, comparable to findings by others [65,69]. Consistent with other findings, the most abundant metabolite excreted in urine was SF-NAC [38,68,69]. Approximately 4-6% of ingested GR was excreted as SF-NAC which represented 49-58% of the total ITC recovered in urine **(Table 3-6)**, as demonstrated by Conaway *et al*. [32]. Erucin-NAC was the second major metabolite excreted with consumption of all three types of soups **(Table 3-6)**. Erucin enzymatically derived from glucoerucin by myrosinase was reported to be excreted in the urine of rats

[20,147] and humans [65,69] following consumptions of cruciferous vegetables [65,69]. Reduction of SF's sulfinyl group results in the interconversion between SF and erucin [68,69]. This was confirmed by the excretion of erucin in urine following consumption of broccoli without glucoerucin [68,154]. In the present study, glucoerucin was not detected in the broccoli soups confirming the interconversion between SF and erucin. Excretion of SF-NAC was ~2-fold greater than erucin-NAC, similar to the 3-fold difference reported by Saha *et al.* [68]. The current study has reported that 2% of the ingested GR was excreted as ERN-NAC in accordance with the findings described by Saha *et al*. [68].

The current study measured a high percentage of SF-NAC followed by ERN-NAC, SF-Cys, SF and SF-Cys-Gly excreted in the urine in congruence with the study by Saha *et al.* [68]. The present study recovered ~10%, ~8% and ~9% of the ingested dose as SF and its metabolites in the urine following consumption of $Myb28^{B/B}$, $Myb28^{B/V}$ and $Myb28^{V/V}$ broccoli soups respectively **(Table 3-5)**, consistent with published literature [32,66,67]. In contrast, Saha *et al*. [68] and Egner *et al.* [22] reported 5% excretion as SF of the ingested GR dose. Inclusion of the excretion of erucin-NAC in the total amount of SF excreted, which accounts for 2%, could be a plausible explanation as could the use of a more sensitive LC-MS/MS method than previously used by Saha *et al.* [68]. Approximately 7-8% of ingested SMCSO **(Table 3-11)** was excreted in urine following consumption of the soups which was inconsistent with the recovery of 60.2% of labelled [^{35}S] following consumption of [^{35}S]-SMCSO in the study by Waring *et al.* [84]. As noted earlier, however, that result should be viewed with caution as Waring *et al.* quantified the total [^{35}S], not SMCSO specifically, therefore also measuring metabolites derived from SMCSO [84]. Therefore, the discrepancy observed could be due to the metabolism of SMCSO to other metabolites which has not been quantified in this study [2]. The metabolism of SMCSO in humans remains unclear and is imperative in understanding the potential biological mechanisms of SMCSO. As glucoerucin is a reduced analogue of GR converted by gut microbiota [20,68,142], its excretion was also taken into account when calculating the excretion of GR following soup consumption. Of the ingested dose, <1% was excreted as GR and glucoerucin which is to a certain extent comparable to the 5% reported in rats [20] and 3% reported in humans by Egner *et al.* [22]. The difference can be justified by taking into consideration individual variations and sample size as Egner *et al*. recruited 50 participants as opposed to the 10 participants recruited for this study [22]. However, 17% of the ingested dose has been excreted as GR in mice [142], this being considerably more than the amount excreted reported by the other studies.

Despite the enhanced levels of SMCSO, relative to GR, in the soups, the bioavailability of these compounds was to a certain extent comparable. Although the SMCSO plasma C_{max} of

28 µM was higher compared to SF plasma C_{max} of 0.6 µM, the percentage recovery was similar with ~9% SF and ~8% SMCSO recovered in the urine. This can be rationalised by the fact that SF is represented by data comprising SF and its metabolites, whereas the metabolites of SMCSO have not been quantified and are therefore not represented in the bioavailability of SMCSO. Interestingly, there is only a ~3-fold difference in the levels of SMCSO and GR in $Myb28^{V/V}$ soup (1454 µmoles SMCSO and 452 µmoles of GR). However, the plasma concentration of SMCSO is ~47-fold higher than SF (28 µM SMCSO and 0.6 µM SF). Given that SMCSO is abundant in other species such as *allium*, participants were requested to exclude garlic 48 hours prior to the study day and during the study day but onions were not excluded. Consequently, baseline sample detection of SMCSO was subtracted from subsequent time points to ensure that the SMCSO quantified is a result of the soup intake as opposed to other dietary sources of SMCSO. Regardless, the levels of SMCSO is considerably higher than the SF plasma levels despite the differing soup levels.

Inter-individual variation in the urine excretion of SF and metabolites following consumption of GR and SF has been well documented. The current study presents a range of SF excretion between 2.5-23.1%, 2.2-18.1% and 0.2-18.9% following consumption of $Myb28^{B/B}$, $Myb28^{B/V}$ and $Myb28^{V/V}$ **(Figure 3-12)**. This is comparable to the SF recovery reported by others, e.g. 1-40% [70] and 3.8- 24.6% [32] following consumption of GR. The variation observed in the percentage of intact GR and SMCSO excreted is not unreasonably large compared to the percentage of SF recovered **(Figure 3-12)**. This further supports the notion that the inter-individual variation observed is influenced by the gut microflora as uptake of SF is dependent on the gut microbiota conversion from GR whereas the uptake of unmetabolised GR and SMCSO is not.

Interestingly, there is a strong, statistically significant, correlation in the urine excretion of SF following consumption of $Myb28^{B/B}$ and $Myb28^{V/V}$ ($p<0.0001$) suggesting that the variation observed is due to inter-individual variation, possibly influenced by the gut microflora **(Figure 3-13 A)**. A correlation was not observed between the GR and glucoerucin excreted following consumption of each soup, indicating that any variation is unlikely to be due to characteristics unique to the participants, e.g. gut microbiota **(Figure 3-13 B)**. However, it appears that there is inter-individual variation in the percentage of SMCSO excretion following consumption of $Myb28^{B/B}$ and $Myb28^{V/V}$ evidenced by the moderate significant correlation ($p<0.05$) **(Figure 3-13 C)**. Given that intact SMCSO was measured as requiring no metabolism by the gut microflora, the variation observed is presumably influenced by another participant-unique factor.

Inter-individual differences do not result in the variation observed with the percentage of SMCSO or GR excreted compared to the percentage of SF excreted in the urine **(Figure 3-14; Figure 3-15)**. On the other hand, there is a non-significant moderate correlation between the percentage of SMCSO and GR excreted **(Figure 3-16)**. This indicates that the urinary excretion of both compounds is potentially influenced by the same factor but not due to gut microbiota as seen with SF excretion.

Blood samples from participants were genotyped for *GSTM1* as it was previously suggested that the genotype influenced the metabolism, absorption, and excretion of SF [38]. Individuals with *GSTM1*-null genotypes excreted higher amounts of SF according to the study by Gasper *et al.* [38] and Steck *et al.* [155]. However, the current study revealed no significant difference in the metabolism of SF **(Table 3-7)** as shown by others [22,68]. It was previously assumed that upon consumption of SF as opposed to GR, the *GSTM1* genotype played a significant role in the metabolism of SF. However, the influence of the *GSTM1* genotype on SF metabolism remains controversial irrespective of whether SF or GR is consumed [22,68]. Further, the *GSTM1* genotype influence on the metabolism of GR **(Table 3-10)** and SMCSO **(Table 3-12)** also demonstrated no significant difference following consumption of three types of soups.

One of the principle strengths of this study was the fact that the subjects acted as their own control. This means that the inter-individual variability in response to the dietary intervention is accounted for. Lack of faecal collection was one of the major limitations of this study. Presumably, these compounds are excreted via faeces in humans as reported in mice [142]. To gain a complete understanding of the bioavailability of SF, GR and SMCSO, urine, blood as well as faecal samples would prove useful. Additionally, a longer period of urine collection of up to 48-72 hours would be essential in further work as the 24 hours samples in this study still showed excretion of SF and SMCSO. The cumulative excretion of SF and SMCSO illustrated that the excretion did not reach a plateau at 24 hours post-soup consumption **(Figure 3-5; Figure 3-9)**. The rationale to design the study with 24-hour urine collection was based on the findings that SF and conjugates were excreted by 24 hours in other studies [142]. No previous data about SMSCO pharmacokinetic in humans were available. This study has provided new and invaluable pharmacokinetic information on GR, SMCSO and SF and on the bioavailability of these compounds from the novel soups.

It is important to note that while SF and metabolites and GR were quantified relative to the internal standard, quantification of SMCSO was not relative to an internal standard as mentioned in **section 2.5, Chapter 2**.

3.7. Conclusion

For the first time, this study has demonstrated the use of novel soups with increasing levels of GR led to increased levels of SF and metabolites that reflected the ingested levels. Consumption of increased levels of GR and SMCSO led to elevated AUC, C_{max}, and total amount excreted for SF, GR and SMCSO. However, with only ~9% of SF and ~8% of SMCSO recovered in urine, it is reasonable to state that the bioavailability of SF and SMCSO is low. Though, it is important to note that the metabolism of SMCSO in humans remains unclear and this study only investigated the bioavailability on intact SMCSO, not its metabolites. Nevertheless, the present study has provided an insight into physiologically relevant concentrations of GR, SMCSO and SF to be used for cell treatments. Furthermore, this is the first human intervention study to investigate the bioavailability of intact GR in plasma and of SMCSO in plasma and urine. On the basis of these results, it can be hypothesised that intact GR and SMCSO are absorbed into the enterocytes either via passive diffusion or via transporters. This poses the question of how these compounds are absorbed in their native form given that GR is a large molecule, with a molecular weight of 437.50, and that SMCSO could potentially be charged in the physiological environment. With very limited evidence available on the transport of these compounds, *in vitro* models will be used to investigate the transport of GR and SMCSO. In conclusion, this study has provided conclusive evidence for the suitability of these novel soups with increasing GR levels for future human intervention studies that will be designed to assess clinical health benefits

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CHAPTER 4

Transport of sulforaphane, glucoraphanin, and S-methyl-L-cysteine sulfoxide using *in vitro* models

4.1. Summary

The human intervention study presented in **Chapter 3** is the first to indicate the transport of GR and SMCSO across the enterocytes into the systemic circulation. SF is widely accepted to passively diffuse across enterocytes however the transport mechanism of GR and SMCSO has not been previously reported. Given the limited literature on the transport of these compounds, the purpose of the experimental work presented in this Chapter was to investigate the mechanism of this transport across the enterocytes. Two *in vitro* models, caco-2 cells and the PAMPA assay, were used to determine whether GR and SMCSO permeate across the membrane via passive diffusion and/or active transport. SF was used as a positive control under the assumption that it can passively diffuse while glucose was used as negative control given that it is known to require a transporter. For the first time, evidence from the caco-2 model and the PAMPA assay indicates that GR and SMCSO may transfer across the membrane via active transporters. Further work is required to discover the transporters involved.

4.2. Introduction

Several human intervention studies have reported recovery of SF and its metabolites in plasma and urine following broccoli consumption [22,32,38,68]. The results from the BOBS human intervention study described in **Chapter 3** have shown for the first time that intact GR and SMCSO can transfer across across the gut, and be recovered in plasma and urine of human participants who have consumed broccoli-rich soups. It is has been reported by others that SF is likely to passively diffuse across the membrane of the enterocytes [27,156], but there is limited literature on the mechanism of transport of other broccoli-derived phytochemicals including GR and SMCSO. Transport of GR and SMCSO across the enterocytes could be either via passive diffusion, paracellularly via tight junctions, or by active transport.

To explore the transport mechanism of GR and SMCSO, two *in vitro* models were used to mimic the intestinal wall. This Chapter describes the use of a cell model (caco-2) and a cell-free system (PAMPA assay) to explore the potential mechanisms of transport that are responsible for the absorption of GR and SMSCO *in vivo*. At present, the caco-2 (human colon adenocarcinoma) cell line is the most well-established model to investigate intestinal permeability. While caco-2 cells give an insight into transport via passive diffusion, active transport, and paracellular diffusion, the PAMPA assay is an artificial membrane which only permits passive diffusion of compounds. It was envisaged that the combination of both models would give an insight into the transport mechanism of GR and SMCSO.

SF is reported to passively diffuse across the membrane, and it appeared to be well absorbed (absorption rate: 74 ± 29%) by the enterocytes as reported in an *in vivo* study using human jejunum [27]. Although the accumulation of SF in cells have been reported, implying that it can enter cells, to our knowledge the diffusion of SF has not been previously tested using *in vitro* models such as the PAMPA assay or caco-2 cells transport experiments. The hypothesis is that SF passively diffuses, given its lipophilicity (log_{10} pc of 0.72) [24] and small molecular weight (mw=177.29 g/mol) [27], and thus such experiments aim to confirm the concept. Furthermore, due to the assumption that SF can passively diffuse, it was used as a positive control for both *in vitro* models while glucose was used as a negative control since it is known to require a transporter [157].

Given that there is no literature on the transport mechanism of GR and SMCSO, the work described in this Chapter sought to investigate for the first time their potential transport mechanism across the gut membrane. The combination of a large molecular weight of 437.49 g/mol and a partition coefficient (log_{10} pc) of -3.8 [24], making it hydrophilic, leads to the hypothesis that GR cannot passively diffuse across the membrane and thus requires a transporter. There is limited literature regarding the partition coefficient of SMCSO so it is not known whether it would be hydrophilic or hydrophobic. Based on its polar surface area which is <140 A^2, and its molecular weight of 151.2 g/mol, one might suggest that SMCSO may passively diffuse across the membrane. However, due to the structural similarity with the amino acid L-cysteine, it was hypothesised that SMCSO would require a transporter to permeate across the membrane.

In this Chapter, transport of broccoli-derived compounds (SF, GR and SMCSO) were investigated using two *in vitro* models; caco-2 cells and PAMPA assay. Exploring the transport of these compounds is of great importance facilitating the understanding of fundamental mechanisms involved in the bioavailability of bioactives from broccoli.

4.3. Materials and Methods

4.3.1. Materials

Human colon adenocarcinoma cell line (caco-2) (Cat # ATCC® HTB-37™ and Essential Minimum Eagle's Medium (EMEM) (Cat # ATCC-30-2003) containing Earle's balanced salt solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/l sodium bicarbonate was obtained from the ATCC. The following were purchased from Thermo Fischer Scientific; heat-inactivated fetal bovine serum (FBS) (Gibco™) (Cat # 10270-106), Penicillin-Streptomycin 10,000 U/ml (Cat # 15140122), DPBS (Gibco®) 10X with no calcium and no magnesium (Cat. # 14200059) and Hank's Balanced Salt Solution (HBSS) 10X with calcium, magnesium and no phenol red (Cat # 14065049). DPBS and HBSS were diluted to 1X in sterile water for use on cells. Amphotericin B (Cat # A2942), Corning® Transwell® polycarbonate membrane cell culture inserts (12 mm transwell with 0.4 μM pore) (Cat # CLS3401), Lucifer Yellow CH dipotassium salt (Cat # L0144), Corning ® 96 well black plate with clear bottoms, and glucose assay kit (Cat # GAGO20) were purchased from Sigma®. Corning® Gentest™ pre-coated PAMPA assay plates were purchased from Corning® (Cat # 353015). Only autoclaved glassware was used, and all items were sterilised with 70% ethanol prior to using in the laminar flow cabinet. T75 flasks were purchased from Starstedt (Cat # 83.3911.002). The epithelial Volt/Ohm meter ($EVOM^2$) to measure the transepithelial electrical resistance was acquired from the world precision instrument (WPI) (Florida, USA). FLUOstar® Omega 96-well plate reader was obtained from BMG labtech.

4.3.2. Cell culture

Caco-2 cells between passage 44 and 55 were cultured in 75cm² flasks in EMEM supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin and 0.1% amphotericin B. The cells were routinely cultured in a humidified atmosphere containing 5% CO_2 at 37 °C and were grown to 80% confluency on 75 cm² flasks.

4.3.2.1. Caco-2 culture conditions

Caco-2 cells were cultured on pore inserts in transwell wells (Corning® Transwell® polycarbonate membrane, 12 mm with 0.4 μM pore inserts) to enable them to grow in a monolayer with the formation of tight junctions and characterise the apical and basolateral

membranes **(Figure 4-1)**. The matrix above and below the pore membrane is the apical chamber (AP) and basolateral chamber (BL), respectively. Prior to seeding cells, the transwell plates were equilibrated with the addition of fresh medium to the AP and BL chamber and incubated for 1 hour. Cells were then seeded at a density of 2 x 10^4 cells/cm^2 and cultured for 21-25 days with media replaced every other day. One well without cells was used as a control for each plate. Media was first removed from the BL followed by removal from the AP chamber. Pre-warmed media was then added to the AP chamber (0.5 ml) and then to the BL chamber (1.5 ml). This process was followed carefully because the order of the medium replacement is critical in maintaining the hydrostatic pressure to reduce the risk of cells lifting off from the membrane.

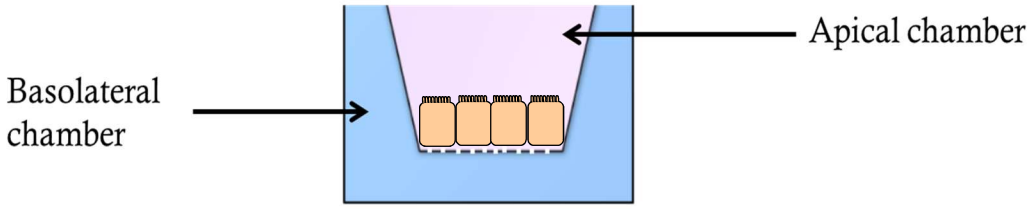


Figure 4-1: Illustration of the apical chamber and the basolateral chamber of the transwell plates.

4.3.2.2. Assessment of cell monolayer integrity

When performing transport experiments, it is crucial to assess the integrity of the monolayer to ensure the cells have formed tight junctions to characterise gut cells. This is to ensure that transport experiments provide reliable results on the movement of compounds via transporters, passive diffusion, and tight junctions as opposed to movement through 'gaps' between the cells. Two parameters were used to examine the integrity of the monolayers; TEER and Lucifer yellow leakage.

4.3.2.2.1 Trans-epithelial electrical resistance (TEER)

The Epithelial Volt/Ohm meter (EVOM^2) supplied with an electrode 'STX2' quantitatively measures the integrity of a monolayer. The transepithelial electrical resistance (TEER) is measured across a monolayer by placing the shorter end of the electrode in the transwell insert and the longer end of the electrode in the well. Current is passed through the transcellular (cells growing on the membrane) and paracellular routes (between the tight junctions). The

transcellular pathway presents a high resistance due to the AP and BL membrane of cells whereas the resistance through the tight junctions will vary and thus governs the TEER values [158,159]. A good monolayer is defined by formation of tight junctions which results in high TEER values.

TEER (Ohms) is calculated as below:

Equation 4-1: $R_{Cell\ Monolayer}\ (\Omega) = R_{Total}\ (\Omega) - R_{cell\text{-}free\ control}\ (\Omega)$

Equation 4-2: $R_{Corrected\ Cell\ Monolayer}\ (\Omega.cm^2) = R_{cell\ monolayer}\ (\Omega)\ x\ Area\ of\ membrane\ (cm^2)$

Although TEER is a widely used parameter to determine the monolayer integrity, it also poses limitations such as variations in the readings. This can be affected by the position of the electrode, temperature, media, pH, the transwell inserts used and seeding density of cells [160]. To ensure minimal variables such as temperature were introduced when TEER was measured, plates were taken out of the incubator and placed in the flow cabinet for 15 mins along with the pre-warmed media for temperature equilibrations, and TEER was measured after media was replaced [158]. TEER was measured after replacing media every two days for 21 days. As a safeguard, another procedure to assess the monolayer integrity was used due to the variations presented from the use of TEER.

4.3.2.2.2 Permeability of Lucifer yellow

The permeability of Lucifer yellow is a less commonly used method to establish the integrity of the monolayer [51,161]. Lucifer yellow is a fluorescent marker which diffuses through the paracellular route via tight junctions as its primary method of transport; it does not diffuse through the transcellular route due to its high molecular weight of 444.29 g/mol [162]. The permeability of Lucifer yellow is influenced by intact tight junctions thus low permeability of Lucifer yellow represents a good monolayer. In this case, it was used to verify the TEER values to eliminate erroneous TEER values due to limitations discussed above. The Lucifer yellow permeability assay was performed according to the Sigma® protocol. After the transport experiments were conducted, Lucifer yellow (1 mg/ml) was diluted to 0.1 mg/ml in HBSS buffer (transport experiment buffer) and added to the AP chambers (0.5 ml). HBSS was added to the BL chambers (1.5 ml). The transwell plates were incubated at 37°C for 60 minutes. Lucifer yellow permeability was measured by collecting 200 µl samples from the BL chamber of each transwell into a black plate and measuring excitation at 485 nm and emission at 520 nm in a fluorescent plate reader. Fluorescence was also measured for HBSS (blank) and 0.1

mg/ml Lucifer yellow in the same black plate. Permeability of Lucifer yellow is calculated using the following equation:

Equation 4-3: $\%\,permeability = \frac{sample - blank}{0.1\ mg/ml\ lucifer\ yellow - blank}\ x\ 100$

Less than 3% permeability of Lucifer yellow is considered a good monolayer as advised in the Sigma® protocol. Data were analysed from experiments performed in wells with a Lucifer yellow permeability of <3%.

4.3.2.3. Treatment with test compounds: sulforaphane, glucoraphanin and S-methy-L-cysteine

SF (CAS 4478-93-7) (purity > 98%) (Cat. # S8045) was purchased from LKT Laboratories (St. Paul, USA). SF stock solution (100 mM) in DMSO was used to obtain a final concentration in the media of 10 µM. SMCSO (CAS 6853-87-8) (purity ≥98%) (Cat # 17600) and GR (CAS 21414-41-5) (≥95%) (Cat # 10009445) were purchased from Cayman Chemical (Michigan, USA). Stock solutions (1 mg/ml) of GR and SMCSO were diluted in DPBS to obtain final concentrations of 100-200 µM. An aliquot (100 µl) of each final concentration of SF, GR and SMCSO was stored at -20°C for analysis to calculate % of transport from initial dose.

After 21 days of culture, transport experiments of SF, GR, SMCSO were performed at low temperature (4°C) and at physiological temperature (37°C).

For experiments at 4°C, media in the AP and BL chambers of the transwell plates were replaced with HBSS and transferred to the fridge (4°C) for 2 hours. This allowed the elimination of active transport processes and equilibration of cells. After 2 hours, HBSS was aspirated from the BL chambers followed by the AP chambers. Cells in the AP chambers were exposed to 0.5 ml of SF (10 µM), GR (100-200 µM) or SMCSO(100-200 µM). The BL chambers were replaced with 1.5 ml of HBSS. Transwell plates were incubated for 30, 60 and 120 minutes. After the given incubation period, samples were collected from the AP and BL chambers and transferred into eppendorf tubes for storage at -20°C until analysis by LC-MS/MS.

For the experiments at 37°C, the media from the AP and BL chambers were replaced with HBSS and incubated at 37°C for 15 mins. Cells were treated with the same concentrations of SF, GR and SMSCO used for the experiments at 4ºC. The BL chambers were loaded with 1.5

ml of HBSS. After the incubation time, samples from the AP and BL chambers were transferred into eppendorf tubes and stored at -20°C until analysis by LC-MS/MS.

4.3.3. Parallel artificial membrane permeability assay

PAMPA is a regularly used assay in the pharmaceutical industry. The assay investigates the permeability of compounds via passive diffusion and can be used as a high-throughput screening of many drug compounds. The assay is a 96-well plate consisting of one plate with filter membrane (acceptor plate) which sits on top of the other plate (donor plate) as illustrated in **Figure 4-2**. The artificial membrane has been designed to mimic the physiological lipid layer but as it is not biological, it has no active transporters thus only passive diffusion of compounds can be explored. This is an efficient way to screen drugs for their permeability coefficient as drugs with high permeability will be able to cross the blood-brain barrier and various lipid layers to reach their targets.

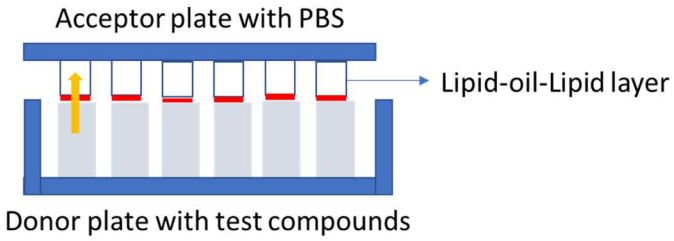


Figure 4-2. Diagram of the parallel artificial membrane permeability assay. Re-drawn from BD biosciences [58].

Various concentrations of glucose (200 µM, 1 mM and 10 mM) were added to the PAMPA assay plates as a negative control because it should not be able to pass through the lipid-oil-lipid membrane from the donor well to the acceptor well. Glucose is known to use active transporters to permeate across a membrane as it is large molecule [163]. Final concentrations of glucose were obtained by diluting an aqueous stock solution of 2 mg/ml solution in DPBS. Given that SF has a small molecular weight and is lipophilic, it was used as a positive control because it is likely to passively diffuse [156]. SF was tested at the final concentration of 1 µM, 5 µM and 10 µM.

Treatment with GR and SMSCO was carried out at the concentrations described below. GR stock solution of 1 mM was diluted in DPBS to 5 µM and 1 µM. Further dilution of 1 µM by 1:20 resulted in 0.05 µM. Final concentrations of SMCSO at 100 µM, 200 µM and 300 µM were prepared by diluting a stock solution of 1 mM.

The Corning Gentest™ PAMPA plate was stored at -20°C and thawed out at room temperature for 30 minutes before use. Once defrosted, plates must be used within 24 hours. The final concentrations of tested compounds (glucose, SF, GR, SMCSO) (300 µl) were dispersed into the receiver plate, donor plate and 200 µl of DPBS was added to the filter plate (acceptor plate). The filter plate is slowly positioned on top of the receiver plate and placed at room temperature for 5 hours. Post-incubation time, solutions from both plates were transferred into labelled eppendorf tubes and stored at -20°C until analysis by LC-MS/MS.

4.3.4. Analysis of sulforaphane, glucoraphanin and S-methyl-L-cysteine sulfoxide

4.3.4.1. Reagents and standards

HPLC columns (Luna 3u C18 (2) 100 A and Kinetex® XB-C18 100 Å) were purchased from Phenomenex®. Agilent SB-AQ 1.8 uM (100 x 2.1mm) C18 column was purchased from Agilent Technologies. All reagents used for LC-MS/MS were prepared following manufacture's advice. Water was obtained from a Milli-Q® Integral Water Purification System (Millipore Ltd). B-ITC used as the internal standard was synthesised by Dr Paul Needs (QIB chemist). The following were purchased from Sigma®; sinigrin (Cat. # 85440), ammonium acetate (Cat. # 73594) and TCA (Cat # 63399). TCA was diluted to 50% in deionised water and stored at 4°C. Agilent 6490 Triple Quad LC-MS mass spectrometer was used for the analysis.

4.3.4.2. Sample extraction

The extraction process is the same as the one describe in **section 3.3.11.2, Chapter 3**. Samples were thawed out, and extraction process was carried out on ice by adding 20 µl of pre-cooled 50% TCA, 10 µl of the internal standard (B-ITC for SF, sinigrin for GR) to 100 µl of samples, followed by vortexing for 10 minutes at 17,000 x g at 4°C. The supernatants were transferred into insert vials and run on the LC-MS/MS for SF and UPLC-MS/MS for GR and SMCSO.

4.3.4.3. LC-MS/MS and UPLC-MS/MS setup

The LC-MS/MS and UPLC-MS/MS set up and monitoring of ions is the same as **section 3.3.11.4, 3.3.11.5 and 3.3.11.6, Chapter 3**. Analysis was performed using an Agilent 6490 Triple Quad LC-MS mass spectrometer equipped with a degasser, binary pump, 4ºC autosampler, column oven, diode array detector and 6490 mass spectrometer. Samples were

eluted at a flow rate of 0.3 ml/min. Separation of SF, GR and SMCSO was carried out using HPLC columns Luna 3u C18 (2) 100 A, Kinetex® XB-C18 100 Å and Agilent SB-AQ 1.8 uM (100 x 2.1mm) C18 column respectively. The column temperature and auto sampler were maintained at 20°C and 4°C respectively. Samples were injected at 5 µl and separation carried out solvent A and solvent B.

Separation of SF was carried out with 0.1% ammonium acetate in deionised water titrated with 0.1% acetic acid until pH 4 was reached (mobile phase A) and 0.1% acetic acid in acetonitrile (mobile phase B). Separation of GR was using separated with 0.2% formic acid in water (mobile phase A) and 0.2% formic acid in acetonitrile (mobile phase B). Separation of SMCSO was using 10 mM ammonium acetate + 0.05% heptafluorobutyric acid in water (mobile phase A) and 10 mM ammonium acetate + 0.05% heptafluorobutyric acid in 90% methanol (mobile phase B).

The LC eluent flow was sprayed into the mass spectrometer interface without splitting. SF, GR and SMCSO ion were monitored using mass spectrometry in MRM mode in positive polarity (GR in negative polarity) with ESI. The quantification was performed using matrix match calibration curve. Identification and quantification of the SF, SMCSO and GR peak were based on retention time and peak area using calibration standards.

4.3.4.4. Identification and quantification of SF, GR and SMCSO

SF stock (1 mg/ml) was prepared in Milli-Q® water, and a serial dilution was produced in the same matrix as the samples that were run (e.g. HBSS for caco-2 transport experiments and DPBS for PAMPA assay). All serial dilutions were prepared fresh directly before each run. An 11-point standard curve ranging from 0-10 µM SF was generated as the highest concentration used for the treatments was 10 µM.

GR stock (1 mg/ml) was prepared in Milli-Q® prior to the run. For the caco-2 experiments, a 12-point standard curve from 0-200 µM was constructed by diluting the GR stock solution (1 mg/ml). For the PAMPA assay, lower concentrations were used so a 10-point standard curve ranging from 0-11 µM was prepared.

SMCSO stock solution (1mg/ml) was diluted to produce an 11-12-point standard curve ranging from 0-200 µM and 0-331 µM for analysis of caco-2 and PAMPA assay samples, respectively.

4.3.5. Analysis of glucose using a glucose detection assay kit

Glucose was quantified using a Sigma® glucose assay kit stored at 2-8°C. The purpose of the kit is to determine the quantity of glucose in various non-biological and biological samples. It comprises of a glucose oxidase/peroxidase reagent, o-Dianisidine reagent, and 1 mg/ml glucose solution in 0.1% benzoic acid. The glucose oxidase/peroxidase capsule contains 500 units of glucose oxidase (Aspergillus niger), 100 purpurogallin units of peroxidase (horseradish) and buffer salts. The oxidase/peroxidase reagent is made up with 39.2 ml of deionised water (Milli-Q®) in an amber bottle and is stable at 2-8°C for one month or at -20°C for 6 months. The vial containing 5 mg of o-Dianisidine dihydrochloride powder was dissolved with 1 ml of deionised water and inverted to mix contents. It can be stored for 3 months at 2-8°C and should be protected from light. To formulate the assay reagent, 0.8 ml of reconstituted o-Dianisidine dihydrochloride was pipetted to the amber bottle with glucose oxidase/peroxidase reagent and inverted. This should be kept away from light and stored at 2-8°C to keep it stable for 1 month. Sulfuric acid was prepared by Dr Paul Needs (QIB chemist). As sulfuric acid is a diprotic acid, a 12 N solution of sulfuric acid in water is equivalent to 6 M as explained in **Equation 4-4**. Stock solution of sulfuric acid will contain 18.38 M (36.76 N) in 1 L (**Equation 4-5**). To dilute 36.76 N to 12 N, 326.44 ml of 36.76 N sulphuric acid was slowly added to 1 L of deionised water (**Equation 4-6**). Sulfuric acid solution can be stored at room temperature.

Equation 4-4: *Molar concentration = Normality × Equivalence Factor*

Molar concentration = 12 N × 0.5 (equivalence factor for sulfuric acid)

Molar concentration = 6 M

Equation 4-5: $Molarity = \frac{weigh\ \ (\%) \times density}{molecular\ weight} \times 10$

$Molarity = \frac{98 \times 1.84}{98.08} \times 10$

Molarity= 18.38 M in 1 L

Equation 4-6: $\frac{12\ N}{36.76\ N}\ x\ 1000 = 326.44\ ml\ in\ 1\ L$

The principle of the assay kit is based on an enzymatic reaction producing a colour change in the presence of glucose which is then measured using a plate reader. Gluconic acid and hydrogen peroxide accumulate upon the addition of the enzyme glucose oxidase to the glucose samples. The reduced form of o-Dianisidine is colourless, oxidation by hydrogen peroxide

catalysed by peroxidase results in an oxidised o-Dianisidine, a brown coloured product. Incubation of the coloured product with sulfuric acid at 37°C in a water bath for 30 minutes develops a stable pink coloured product which is influenced by the glucose concentration **(Equation 4-9)**. The concentration of glucose in samples is determined from the standard curve generated using glucose samples of known concentrations.

Equation 4-7: *D-Glucose* + H_2O + O_2 → *D-Gluconic Acid* + H_20_2 catalysed by glucose oxidase

Equation 4-8: H_2O_2 + *Reduced o-Dianisidine (colourless)* → *Oxidised o-Dianisidine (brown)* catalysed by peroxidase

Equation 4-9: *Oxidised o-Dianisidine (brown)* → *Oxidised o-Dianisidine (pink)* in addition to sulfuric acid

Samples from PAMPA assay were defrosted and diluted with deionised water to contain 20-80 µg/ml. All samples and standard curve were pipetted into glass test tubes for the reactions. The concentrations of glucose added to the PAMPA donor plate were 200 µM (36.03 µg/ml), 1 mM (180.16 µg/ml), 10 mM (1801.60 µg/ml). Only the wells treated with 200 µM were within the range of glucose detection. The wells treated with 1 mM and 10 mM were diluted in deionised water by a factor of 1:5 and 1:50 respectively to give a glucose concentration of 36.03 µg/ml. Samples collected from both the donor plate and acceptor plate were diluted for consistency. The glucose detection assay was carried out as stated by Sigma®'s protocol with modifications. The main adjustment to the protocol was the change in the volume of samples and reagents added. This was a consequence of low volume of samples obtained from the PAMPA assay. A dilution of the 1 mg/ml glucose standard was undertaken to give a final concentration of 0-80 µg/ml in 200 µl in glass test tubes. The reaction commenced with the addition of 400 µl of assay reagent prepared previously followed by mixing of the test tubes. A 30-60 second interval was allowed between subsequent tubes prior to the addition of assay reagent. Tubes were placed in a water bath set at 37°C to react for 30 minutes. The reaction was terminated by pipetting 200 µl of sulfuric acid with an interval of 30-60 seconds between each tube. At this stage, a colour change from brown to pink was observed in the presence of glucose while samples without glucose remained colourless. To measure the absorbance, 200 µl of samples were transferred into a clear 96-well plate and measured at a wavelength of 540 nm against the blank sample (0 µg/ml) on the FLUOstar® Omega plate reader. Concentrations of the samples were interpolated from the standard curve.

4.3.6. Measurement of percentage of transport and apparent permeability coefficient

The percentage of transport (%) from the AP to the BL chamber was calculated from the initial concentration of compounds added to the AP chamber as shown in **Equation 4-10**. The apparent permeability coefficient (P_{app}) was calculated using **Equation 4-11** [164]:.

Equation 4-10: percentage of transport (%)= $\frac{\mathbf{Cf}}{\mathbf{C0}}\ x\ 100$

C_f= Final concentration in the BL chamber (µM)

C_0= Initial concentration added to the APchamber (µM)

Equation 4-11: $Papp\ (c\quad /s) = \frac{V}{A\ x\ Co}\ x\ \frac{dC}{dT}$

V= Volume in the BL chamber (1.5 ml)

A= area of the transwell (1.12 cm^2)

$\frac{dC}{dT}$ = change in the concentration of compound in the BL chamber over incubation time

4.4. Results

4.4.1. Assessment of membrane integrity in caco-2 cells by measuring the trans-epithelial electrical resistance

To assess the integrity of the caco-2 monolayer, caco-2 cells were grown on 12-well transwell plates, and TEER was measured using the Epithelial Volt/Ohm meter ($EVOM^2$) after media was replaced every two days as described in **section 4.3.2.2.1**. TEER from 3 repeated measurements from each well were recorded for 21 days and corrected from the cell-free control well **(Figure 4-1)**. This value was corrected for the area of the membrane **(Equation 4-2)**. The average TEER values of 24 wells (from two 12-well transwell plates) with standard deviation was plotted as illustrated in **Figure 4-3**. During the first 4 days of seeding the TEER values were low, almost the same as the TEER value from the blank well. After day 4, TEER values increased up to day 8. Thereafter, the TEER values stabilised until day 21, when the last measurement was obtained. Therefore, subsequent transport experiments were undertaken on day 21 of culturing cells on transwells. Wells with TEER values of <500 Ω.cm^2 were omitted from transport experiments.

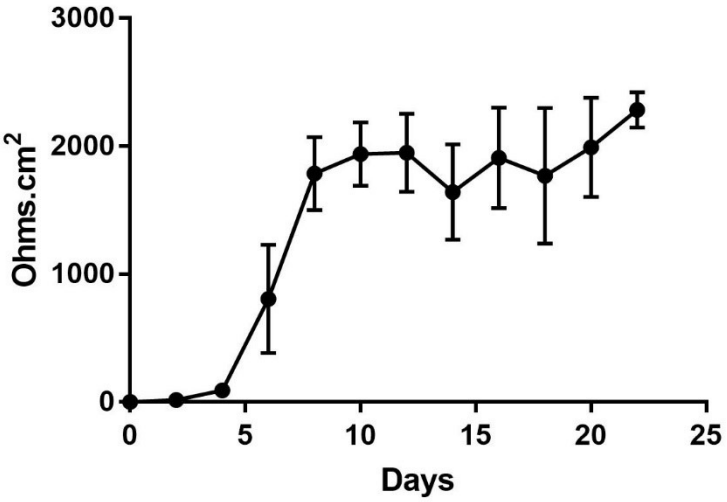


Figure 4-3: TEER values measured on caco-2 cells grown on 12-well transwell plates for 21 days. TEER values (Ohms) were measured after replacing media every two days. The values were subtracted from the cell-free control well and multiplied to correct for the membrane area of 1.12 cm². Data are represented as mean ± SD (n=22).

4.4.2. Assessment of membrane integrity in caco-2 cells by measuring Lucifer yellow fluorescence

To confirm membrane integrity independently, transwell plates were incubated with a fluorescent paracellular marker (Lucifer yellow), after treatment with tests compounds (SF. GR, SMCSO). Permeability of Lucifer yellow was calculated using **Equation 4-3**, and percentage values <3% were indicative of a good cell monolayer in accordance with manufacturer's instruction (Sigma®). As illustrated in **Figure 4-4**, Lucifer yellow permeability (%) (n=43 wells) was in the range of 0.02-2.8% with an average of 0.47 ± 0.60%. Each well containing cells had a Lucifer yellow permeability (%) <3% confirming a good membrane integrity as already demonstrated by TEER values. As expected, cell-free control wells showed much higher permeability values (n=4; 17.03 ± 2.98%).

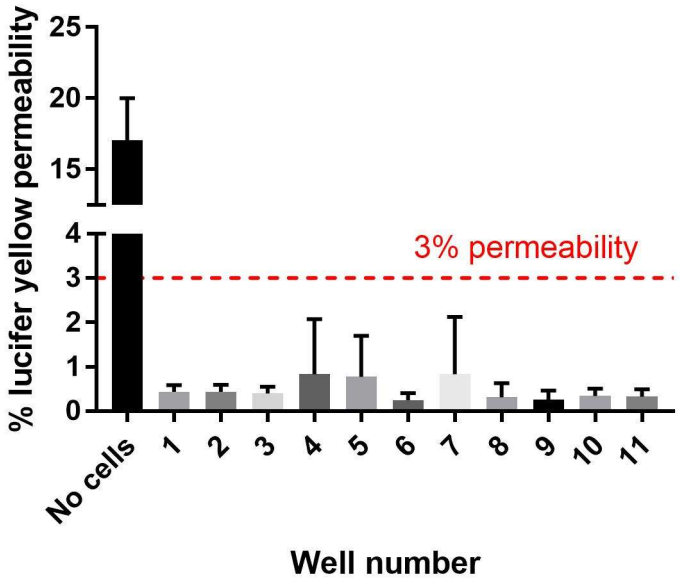


Figure 4-4: Percentage permeability of Lucifer yellow in transwell plates after treatment with test compounds. Fluorescence values were measured at excitation of 485 nm and emission of 520 nm on a FLUOstar® Omega plate reader. Lucifer yellow permeability (%) was calculated by correcting for the HBSS blank and 0.1 mg/ml Lucifer yellow. All values are represented as mean ± SD (n=47 wells).

4.4.3. Effect of temperature on sulforaphane transport through caco-2 monolayer

SF has been widely regarded as a small compound capable of passively diffusing through the monolayer. Caco-2 cells were exposed to SF (10 µM) at different temperature conditions (4°C and 37°C) to confirm that SF does use passive diffusion rather than active transport. It is well established that active transport is halted at 4°C, and thus by exposing SF-treated cells to this temperature its movement will only occur via passive diffusion. Results obtained by LC-MS/MS analysis of SF concentrations in AP and BL samples showed an increase of SF transport over time at both 4°C and 37°C **(Figure 4-5)**. Transport of SF (%) at physiological temperature (37°C) was 1.44 ± 0.05%, 1.84 ±0.51% and 2.92 ± 0.47% at 30, 60, and 120 minutes, respectively. SF transport (%) at 4°C was lower as only 0.61 ± 0.05%, 1.05 ± 0.13%, and 1.83 ± 0.15% at the same time intervals. Results obtained did show a significantly reduced transport of SF at 4ºC compared to the physiological temperature 37ºC at 30 minutes ($p<0.0001$) and at 120 minutes ($p<0.05$).

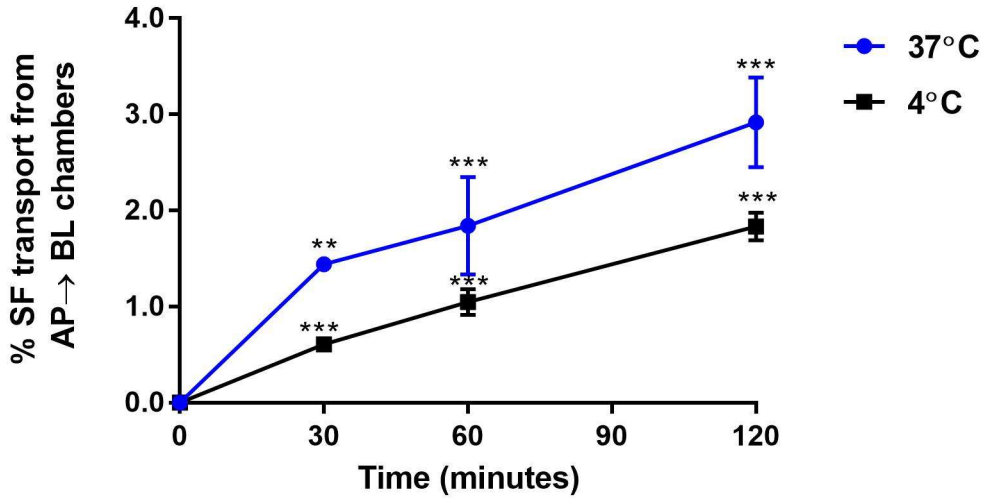


Figure 4-5: Percentage of SF transport from the AP to the BL chamber in caco-2 grown in transwells. Cells were treated with SF at the concentration of 10 µM and incubated at both 4°C, and 37°C. Diffusion of SF was quantified over time at 30, 60 and 120 mins. All values are represented as mean ± SD (n=3). Data points at 30 minutes have small errors hence the bars are not visible. Statistical analysis was undertaken by one-way ANOVA with Dunnett's multiple comparisons test (p<0.01 and *** p<0.001 vs. 0 minutes).**

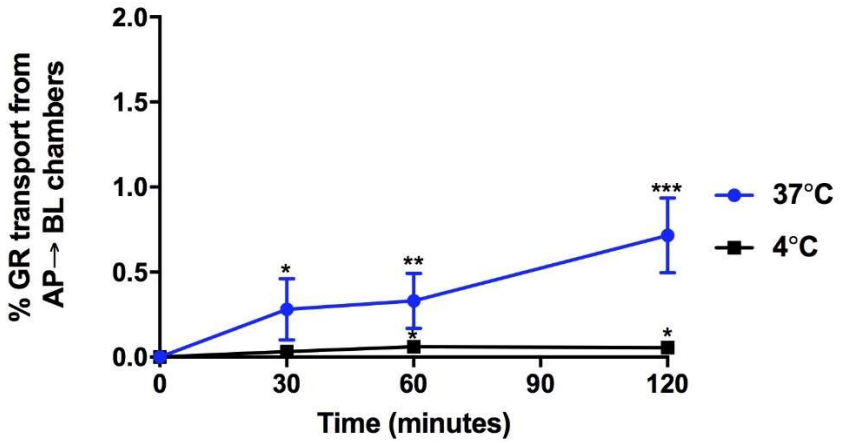
4.4.4. Effect of temperature on glucoraphanin transport through caco-2 monolayer

To date, there is no literature on the transport of GR; results obtained from the human intervention study (BOBS study) described in **Chapter 3**, revealed that unmetabolised GR can be detected in plasma and urine after consumption of GR-enriched food product. The transport of GR (100-200 µM) and the potential effect of different temperature conditions was evaluated by undertaking the transport experiments in caco-2 cells grown in transwells at 4°C and 37°C. Data obtained by UPLC-MS/MS analysis of samples showed that GR transport increases with time at both concentrations at 37°C **(Figure 4-6 A-B)**. Treatment of caco-2 cells with 100 µM GR at 37°C resulted in 0.28 ± 0.18%, 0.33 ±0.16 and 0.72 ± 0.22% at 30, 60, and 120 minutes respectively. In comparison, at 4°C only 0.03 ± 0.01%, 0.06 ± 0.03% and 0.06 ± 0.02% of the 100 µM GR diffused through the monolayer at 30, 60, and 120 minutes **(Figure 4-6 A)**. This difference in the percentage of 100µM GR transport induced by temperature change is significant at 60 and 120 minutes ($p<0.01$). The same trend was observed with caco-2 cells exposed to 200 µM GR where cells incubated at 37°C accumulated a significantly higher percentage of GR in the BL chamber compared to cells at 4°C ($p<0.01$

at 30 minutes, $p<0.0001$ at 60 and 120 minutes) **(Figure 4-6 B)**. Following exposure of cells to 100 μM and 200 μM GR treatment, a similar trend was observed with an increase in the permeation of GR from the AP to BL chamber with increasing time. However, there was a significant increase in the accumulation of GR in the BL when caco-2 cells were subjected to 200 μM versus 100 μM at 60 ($p<0.01$) and 120 minutes ($p<0.05$). The higher the concentration of GR dispersed in the AP chamber, the greater the recovery of GR in the BL chamber.

Caco-2 cells treated with 100 µM GR

A



Caco-2 cells treated with 200 µM GR

B

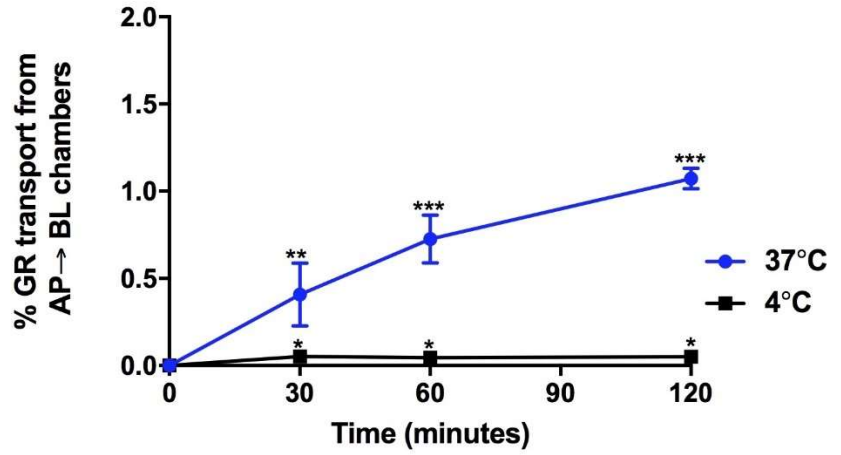


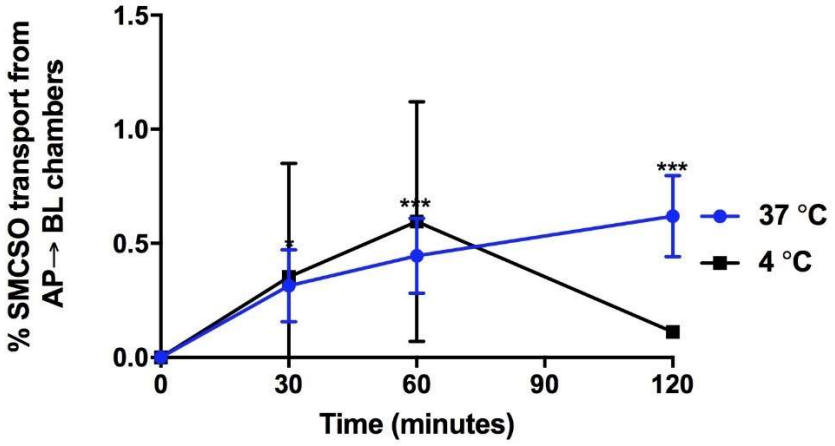
Figure 4-6: Percentage of GR transport from the AP to the BL chamber in caco-2 grown in transwells. Cells were treated with GR at the concentration of 100 µM (A) or 200 µM (B) and incubated at both 4°C and 37°C. The permeability of GR was measured over time at 30, 60, and 120 minutes. All values are represented as mean ± SD (n=6, 37°C experiments; n=3, 4°C experiments). Outliers were excluded when appropriate. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparisons test (*p<0.05, ** p<0.01 and *** p≤0.0001 vs. 0 minutes).

4.4.5. Effect of temperature on S-methyl-L-cysteine sulfoxide transport through caco-2 monolayer

Thus far, there are no reports on the transport of SMCSO. Unmetabolised SMCSO was detected in plasma and urine after consumption of GR and SMCSO enriched soups as described in **Chapter 3**. Consequently, the transport of SMCSO (100-200 μM) across the caco-2 monolayer was investigated at two temperatures. Transport experiments were undertaken in caco-2 cells grown in transwells at 4°C, and 37°C. Analysis by UPLC-MS/MS showed that SMCSO increases with time at both concentrations at 37°C **(Figure 4-7)**. Treatment of caco-2 cells with 100 μM SMCSO at 37°C resulted in 0.31 ± 0.16%, 0.45 ± 0.55%, 0.74 ± 0.39% at 30, 60, and 120 minutes respectively **(Figure 4-7 A)**. The temperature did not induce a significant change in the percentage of 100 μM SMCSO transport at 4°C at 30 and 60 minutes (0.35 ± 0.50% at 30 and 0.60 ± 0.53% at 60 minutes). The percentage of SMCSO transported across the monolayer at 30 and 60 minutes is comparable to 37ºC. However, at 120 minutes, the percentage of SMCSO transport is significantly reduced at 4ºC compared to 37ºC (0.11 ± 0.02%, 4ºC vs. 0.74 ± 0.39% at 37ºC**,** $p<0.0001$) **(Figure 4-7 A)**. On the other hand, caco-2 cells exposed to 200 μM SMCSO incubated at 37ºC accumulated a significantly higher percentage of SMCSO in the BL chamber compared to cells at 4°C ($p<0.05$ at 30 minutes, $p<0.01$ at 60 and 120 minutes) **(Figure 4-7 B)**. A similar trend to that observed with the percentage of 200 μM GR at 37º C and 4ºC **(Figure 4-6)**. Caco-2 cells exposed to 100 μM and 200 μM SMCSO treatment at 37ºC demonstrated a similar trend in the increase of SMCSO transport from the AP to BL chamber with increasing time. However, unlike the permeation of GR at 37ºC **(Figure 4-6)**, the accumulation of SMCSO in the BL chamber is not significantly different in cells subject to 100 μM or 200 μM SMCSO. A similar recovery of SMCSO in the BL chamber was observed.

Caco-2 cells treated with 100 µM SMCSO

A



Caco-2 cells treated with 200µM SMCSO

B

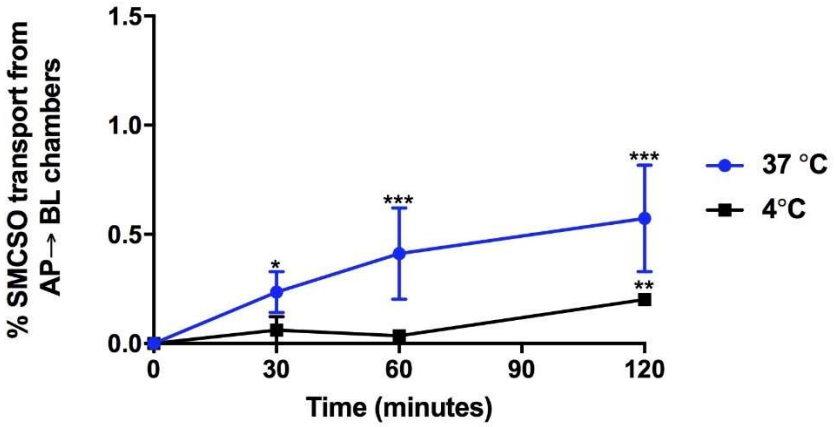


Figure 4-7: Percentage of SMCSO transport from the AP to the BL chamber in caco-2 grown in transwells. Cells were treated with SMCSO at the concentration of 100 µM (A) or 200 µM (B) and incubated at both 4°C and 37°C. The permeability of SMCSO was measured over time at 30, 60, and 120 minutes. All values are represented as mean ± SD (n=9, 37°C experiments; n=3, 4°C experiments). Outliers were excluded when appropriate. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparisons test (*p<0.05, ** p<0.01 and *** p<0.001 vs. 0 minutes).

4.4.6. Apparent permeability coefficient of sulforaphane, glucoraphanin and S-methyl-L-cysteine sulfoxide

The permeability coefficient of all three compounds in caco-2 cells incubated at 4ºC and 37ºC was calculated at 30 minutes to elucidate whether SF reported to passively diffuse, has a higher permeability coefficient compared to SMCSO and GR **(Table 4-1)**. SF (10 µM) displayed a higher permeability coefficient compared to 100-200 µM of GR and SMCSO by ~4-fold and ~5-fold respectively at 37ºC. Whereas, at 4ºC, the permeability coefficient of SF (10 µM), GR (100-200 µM) and SMCSO (200 µM) decreased compared to 37ºC, albeit with no difference in the permeability coefficient observed with 100 µM SMCSO. However, even at 4ºC the permeability coefficient of SF was higher than GR and SMCSO. Results obtained indicate that SF is more permeable than GR and SMCSO at both a physiological temperature (37ºC) and at 4ºC and that the temperature influences the permeability of compounds.

Table 4-1: Permeability coefficient (P_{app}) of SF, GR and SMCSO in caco-2 cells incubated at 4ºC and 37ºC.

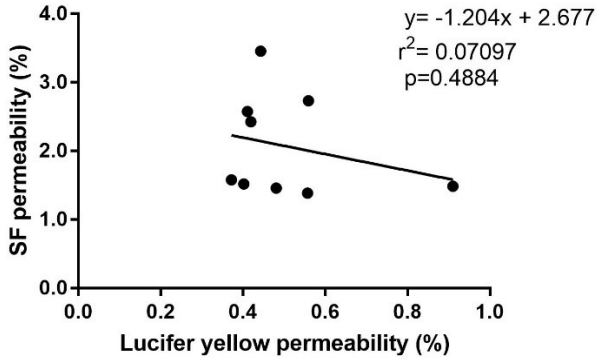
Treatment group	P_{app} AP→BL (x 10^{-6}cm/s)	n
Incubation at 37ºC,30 minutes		
10 µM SF	10.73 ± 0.38	3
100 µM GR	2.72 ± 1.83	5
200 µM GR	3.02 ± 1.34	5
100 µM SMCSO	2.33 ± 1.17	9
200 µM SMCSO	1.76 ± 0.71	8
Incubation at 4ºC, 30 minutes		
10 µM SF	4.53 ± 0.39	3
100 µM GR	0.25 ± 0.06	3
200 µM GR	0.39 ± 0.24	3
100 µM SMCSO	2.62 ± 3.71	2
200 µM SMCSO	0.46 ± 0.47	3

Data presented as mean ± SD, 'n' represents biological replicates, >n=3 indicates independent experiments. Outliers were excluded when appropriate.

4.4.7. Permeability of Lucifer yellow does not correlate with the permeability of sulforaphane, glucoraphanin and S-methyl-L-cysteine sulfoxide

The permeability of Lucifer yellow, a paracellular marker was assessed across the caco-2 monolayer to evaluate the monolayer integrity. A permeability of <3% was indicative of a satisfactory monolayer for transport experiments as reported in the protocol by Sigma®. However, the transport of the test compounds (SF, GR and SMCSO) was <3%. To elucidate whether the permeability of Lucifer yellow and the test compounds are related, correlation graphs were drawn as illustrated in **Figure 4-8**. There is no correlation between SF or SMCSO permeability and Lucifer yellow permeability. Although significant ($p<0.05$), there is a weak negative correlation between Lucifer yellow and GR permeability. The graphs show that there is no association in the permeability of Lucifer yellow and the test compounds.

A



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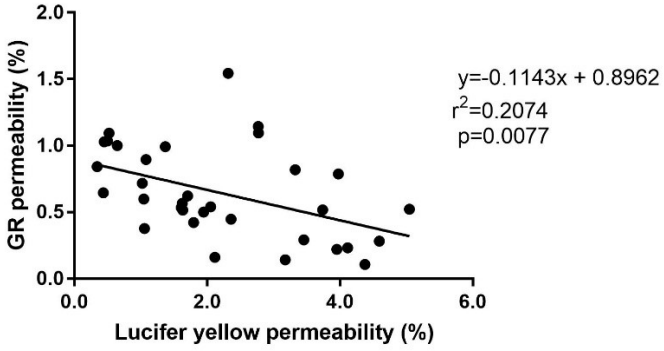

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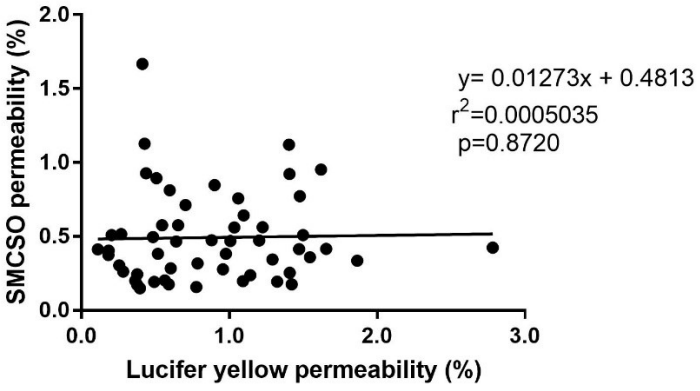


Figure 4-8: Correlation of Lucifer yellow permeability and SF, GR or S-methyl-L-cysteine permeability across caco-2 cells. Permeability (%) was calculated as the amount recovered in the BL chamber from the added concentration of Lucifer yellow/test compounds in the AP chamber. The relationship is illustrated between the permeability of Lucifer yellow and SF (A), permeability of Lucifer yellow and GR (B) and permeability of Lucifer yellow and SMCSO (C).

4.4.8. Sulforaphane transport occurs via passive diffusion through an artificial membrane

Several reports indicated that SF passively diffuses through biological membranes due to its chemical structure. To confirm that SF uses this mechanism of transport, an artificial membrane presenting no transporters (PAMPA assay) was exposed to a range of SF concentrations for 5 hours. The donor plate was treated with 1 µM (n=8 wells), 5 µM (n=8 wells), 10 µM SF (n=8 wells) and acceptor plate with DPBS. Chromatograms of samples analysed by LC-MS/MS are illustrated in **(Figure 4-9)**. As expected, SF peaks were detected in all donor plate samples loaded with various concentrations of SF. On the other hand, even though the acceptor plate was treated with DPBS only, peaks of SF were identified in all acceptor plate samples indicating transport via passive diffusion. SF peaks detected in the samples had the same retention time of 7.1 minutes such as the SF standard run at the same time.

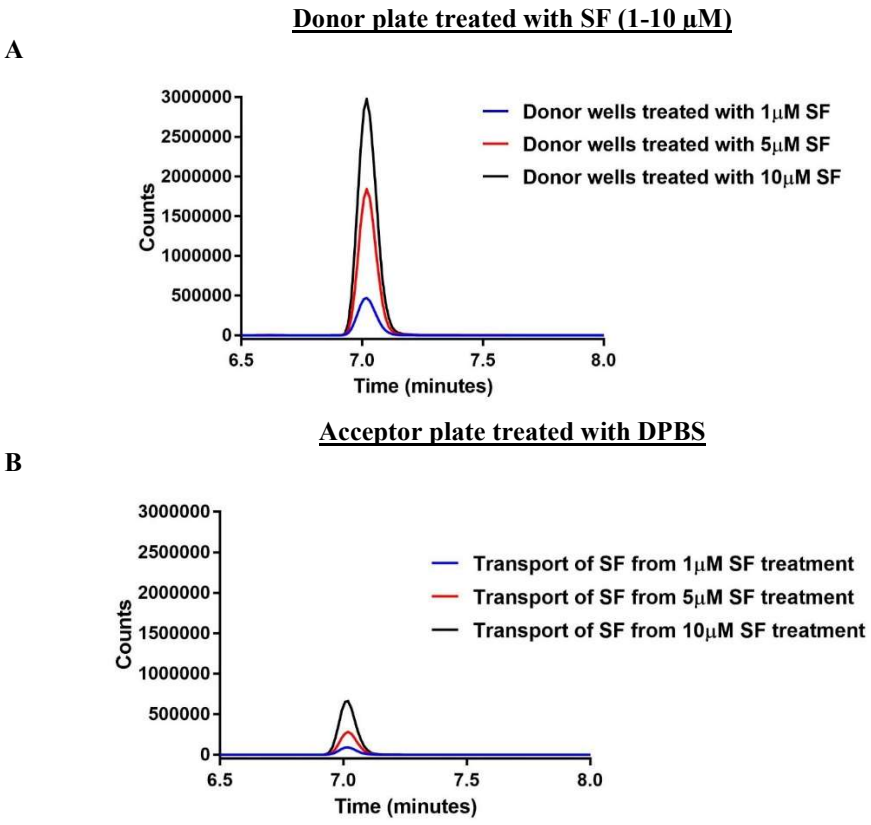
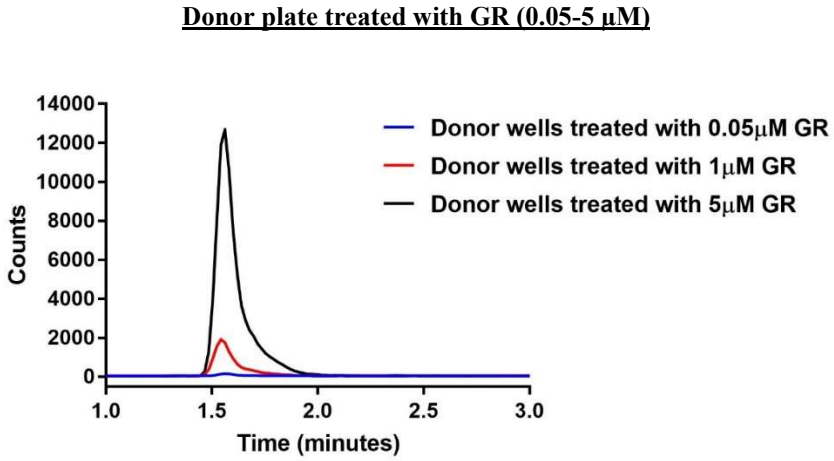


Figure 4-9: LC-MS/MS chromatograms of SF transport from the donor to the acceptor plate through an artificial membrane using PAMPA . Donor plates were treated with SF (1-10 µM) (A), and acceptor plates were treated with DPBS (B). Permeability of SF was measured using LC-MS/MS after 5 hours of incubation at room temperature.

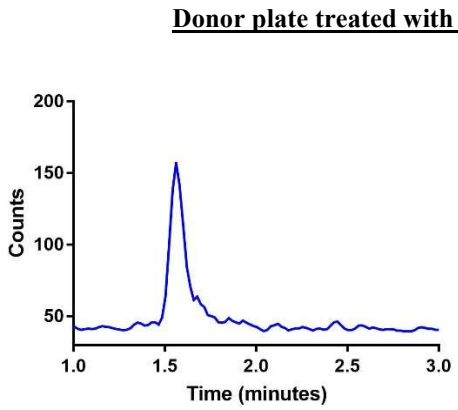
4.4.9. Glucoraphanin does not passively diffuse through an artificial membrane

Unlike SF, there is no literature on the transport of GR. To investigate whether GR can passively diffuse, the PAMPA assay was used in the same experimental conditions described in **section 4.4.3**. The donor plate was treated with 0.05 µM (n=8 wells), 1 µM (n=8 wells), 5 µM GR (n=8 wells) and the acceptor plate with DPBS. The incubation time was 5 hours as described for the experiments with SF. UPLC-MS/MS chromatograms are presented in **Figure 4-10**. GR peaks with a retention time of 2.0 minutes were identified in all the donor plate samples loaded with various GR concentrations **(Figure 4-10 A and B)**. Due to the difference in scale, the peak observed with 0.05 µM GR treatment is not visible in **Figure 4-10 A.** As such, this peak is redrawn in **Figure 4-10 B**. The retention time of GR detected in the assay samples was identical to the GR standard. On the other hand, samples from the acceptor plate revealed no GR peaks **(Figure 4-10 C)**. Unlike the assay with SF treatment, peaks were only detected in the donor plate which was treated with GR, and no peaks were detected in the acceptor plate, indicating that GR does not passively diffuse.

A



B



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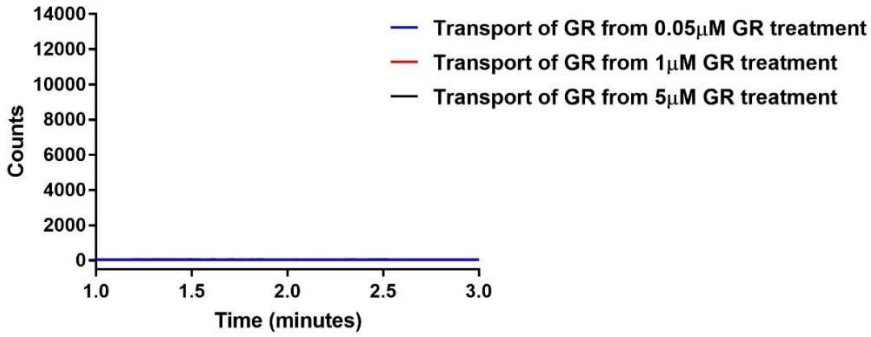
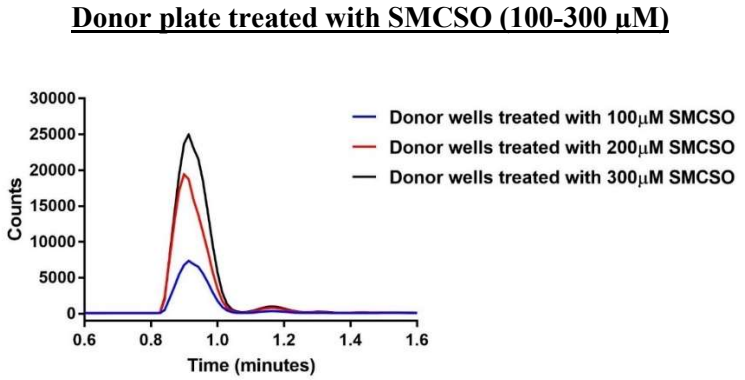


Figure 4-10: UPLC-MS/MS chromatograms of GR transport from the donor to the acceptor plate through an artificial membrane using PAMPA. Donor plates were treated with GR (0.05-1 µM) (A), and acceptor plates were treated with DPBS (C). GR peak detected in the donor wells treated with 0.05 µM GR (B). Permeability of GR was measured using UPLC-MS/MS after 5 hours of incubation at room temperature.

4.4.10. SMCSO does not passively diffuse through an artificial membrane

There is no previous report showing the mechanism of transport of SMSCO through biological membranes. The PAMPA assay was carried out to explore whether SMCSO can passively diffuse. As described for the experiments undertaken with SF and GR, donor plate was treated with a wide range SMCSO concentrations (100-300 µM) and the acceptor plate with DPBS for 5 hours. UPLC-MS/MS chromatograms of SMSCO are shown in **Figure 4-10**. Data obtained revealed that SMCSO, like GR, does not passively diffuse as confirmed from the absence of SMCSO peaks in acceptor plate samples **(Figure 4-10 B)**. However, SMCSO peaks with a retention time of 0.98 were detected in all samples from the donor plate loaded with SMCSO **(Figure 4-10 B)**; the same retention time was observed by using a synthetic standard.

A



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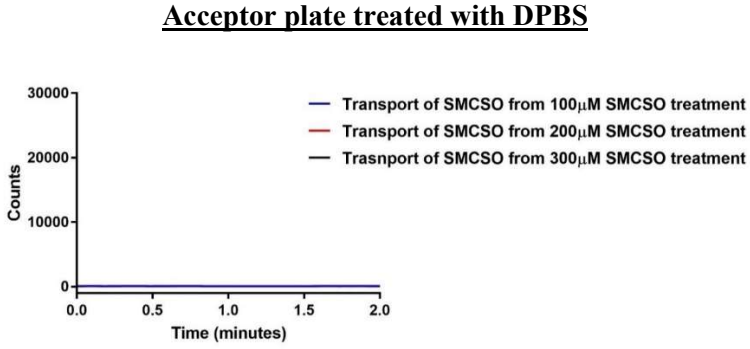


Figure 4-11: UPLC-MS/MS chromatograms of SMCSO transport from the donor to the acceptor plate through an articifical membrane using PAMPA. Donor plates were treated with SMCSO (100-300 µM) (A), and acceptor plates were treated with DPBS (C). Permeability of SMCSO was measured using UPLC-MS/MS after 5 hours of incubation at room temperature.

4.4.11. The use of glucose as a negative control for the parallel artificial membrane permeability assay

To ensure that the PAMPA assay was a suitable experimental tool for studying passive diffusion, glucose was used as a control because of its well-known transport mechanism based on specific transporters [157]. The PAMPA assay was carried out following the same experimental conditions used for the tests compounds (SF, GR, SMCSO). However, a different method based on absorbance readings was used for measuring glucose in assay samples. As **Table 4-2** shows, no samples from the acceptor plate detected any glucose. A glucose concentration of 201.79 µM, 0.85 mM and 9.18 mM was determined in donor plate samples treated with 200 µM, 1 mM and 10 mM. The concentration of glucose was underestimated (0.9 mM and 9.2 mM) in donor plate samples treated with 1 mM and 10 mM whereas the glucose concentration was overestimated in the donor plate samples treated with 200 µM **(Table 4-2)**. This may be due to technical problems related to the processing of many samples at the same time. Despite the technical problem, the assay confirmed that glucose does not passively diffuse corroborated with the absence of glucose in the acceptor samples.

Table 4-2: Glucose concentrations as measured by an absorbance-based method in samples from the donor and acceptor plates.

	Glucose concentration detected	
	Mean ± SD	**n**
Donor wells treated with 200 µM glucose	**201.79 ± 7.06 µM**	**8**
Acceptor plate treated with DPBS	**0**	**8**
Donor wells treated with 1 mM glucose	**0.85 ± 0.13 mM**	**8**
Acceptor plate treated with DPBS	**0**	**8**
Donor wells treated with 10 mM glucose	**9.18 ± 1.58 mM**	**15**
Acceptor plate treated with DPBS	**0**	**16**

Donor plates were treated with glucose (0.2-10 mM), and acceptor plates were treated with DPBS. Permeability of glucose was measured using an absorbance-based method after 5 hours of incubation at room temperature. Data represented as mean ± SD (n=8 wells, 200 µM and 1 mM glucose; n=16 wells, 10 mM glucose).

4.5. Discussion

This study aimed to investigate the transport mechanisms of the dietary compounds SF, GR and SMCSO using established *in vitro* models (caco-2 and the PAMPA assay). Previous reports have indicated that SF passively diffuses through membranes due to its low molecular weight and high lipophilicity [24]. For this reason, SF has been used as a positive control in both *in vitro* models described in this Chapter. To date, there has been no study published on the mechanisms of transport used by GR and SMCSO. Due to the high molecular weight and polar surface area of GR, it was predicted that GR would require a transporter to cross the membrane. Although SMCSO has a low molecular weight and a polar surface area comparable to that of SF, it was hypothesised that SMCSO would also require a transporter due to its structural similarity with L-cysteine.

Prior to the transport experiments, the monolayer integrity was assessed by measuring TEER and permeability of Lucifer yellow. Measurement of TEER is the leading method extensively used to assess the integrity of the caco-2 monolayer. Over 21 days of culture, the TEER values increased, eventually plateauing, indicating that tight junctions had developed to a satisfactory degree to follow with the transport experiments. TEER values reached >1000 $\Omega.cm^2$ on day 8 post-seeding, in congruence with previously published literature that reported that caco-2 monolayer density is established around day 6-7 [52]. While a lot of studies report lower TEER values, in the range of 450-500 $\Omega.cm^2$ [160,165,166], this is likely due to the low passage number cells used in those cases. TEER values are generally higher for cells at older passage numbers [50,53]. In this study, cells at a passage number of 44-55 were used hence the TEER was >1000 $\Omega.cm^2$ in accordance with the study by Ishida *et al*. who used cells at passage number 40 and recorded TEER values of 900 $\Omega.cm^2$ [167]. Wells with TEER values of <500 $\Omega.cm^2$ were omitted from transport experiments as it indicated that tight junctions were not fully developed. To further confirm the integrity of the cell monolayer, the fluorescence probe Lucifer yellow was used in all experiments described in this Chapter. Lucifer yellow permeability of <3% is indicative of a monolayer with intact tight junctions. A high permeability of Lucifer yellow, a paracellular marker, implies that the tight junctions are not intact in the monolayer and hence transport of compounds would be due to 'gaps' in the monolayer. While most wells used in the transport experiments exhibited a permeability of <3% **(Figure 4-8)**, some wells displayed a permeability of >3% such as those in the GR experiments **(Figure 4-8 B).** Although Lucifer yellow implied poorly formed tight junctions, TEER values, the more widely used method indicated othewise. Therefore, these wells were still included on the basis of the TEER values indicating that it was a good monolayer.

The effect of temperature on the transport of the test compounds across the cell monolayer was investigated at 37°C and at 4°C. This was undertaken to differentiate between passive diffusion (not energy-dependent) and active transport (energy-dependent). At 4°C active transport is halted due to less energy production, a key requirement for functional transporters [44,164,167-170]. The percentage of transport of 10 µM SF increases over time at 37°C and 4°C **(Figure 4-5)**. This implies that even when active transport is halted at 4°C, there is transport of SF occurring at a similar trend to the transport at 37°C but at a reduced rate. Two possible reasons for transport to occur at 4°C are either the transport of SF through unsealed tight junctions or via passive diffusion which is not energy-dependent. Given that the integrity of the monolayer was verified using TEER, the only plausible explanation is that SF passively diffuses across the membrane. Data obtained from this study represent the first experimental evidence of transport across caco-2 grown in transwells supporting the assumption that SF passively diffuses across membrane [27,156]. As observed with SF, the percentage of GR transport increases over time at 37°C. However, there is negligible transport of 100 µM and 200 µM GR at 4°C **(Figure 4-6)** in contrast to the percentage of SF transport at 4°C. In this case, the low percentage of GR transport at 4°C suggests that the diffusion of GR may require active transport to permeate across the membrane. Similarly, the percentage of SMCSO transport increases over time at 37°C. However, there is negligible transport of 200 µM SMCSO at 4°C. Transport of 100 µM SMCSO at 4ºC is erratic as it does not follow the trend observed with SF or GR at 4ºC or 37ºC. This may be due to technical issues as the lowest limit of detection of SMCSO by the UPLC-MS/MS is 0.19 µM. At 4ºC, the concentration of SMCSO might be <0.19 µM thus resulting in irregular detection of SMCSO reflected by the standard deviation bars. Taking into consideration the data from the transport of 200 µM SMCSO at 4ºC indicates that like GR, SMCSO may require an active transport. Incubating caco-2 cells at 4°C is not a commonly used procedure but has been applied previously by others to distinguish between passive diffusion and active transport [44,167-172]. The major concern with reducing the temperature is the consequent changes to the physical properties of the cells such as decreasing membrane fluidity [173].

Permeability of compounds can be determined by calculating the apparent permeability coefficient (P_{app}). In congruence with Song *et al*., incubation at 4ºC compared to 37ºC reduced the P_{app} for SF, GR (100-200 µM) and SMCSO (200 µM) bar SMCSO (100 µM) [169]. Artursson and Karlsson demonstrated that well absorbed drugs had a P_{app} of >1 x 10^{-6} cm/s [174] while, Yee, S. reported that a P_{app} <1 x 10^{-6} cm/s, 1-10 x 10^{-6} cm/s, and >10 x 10^{-6} cm/s correlates with a human oral absorption of 0-20%, 20-70%, and 70-100% respectively [175]. In this study, at 37ºC GR and SMCSO had a P_{app} >1 x 10^{-6} cm/s, suggesting that these compounds are moderately absorbed, while SF is well absorbed (70-100%) as a result of a P_{app}

>10 x 10^{-6} cm/s. In agreement with this, a SF absorption of 74 ± 29% was reported in human enterocytes using perfused jejunum segment by Petri *et al.* [27]. While the permeability coefficient does not distinguish whether a compound can passively diffuse or require active transport, it can aid classification of compounds according to their permeability. Given the similarity in the P_{app} between GR and SMCSO, it suggests that both compounds might use similar transport routes, e.g. active transport, whereas SF, which is more permeable based on the P_{app}, passively diffuses.

To further investigate the mechanism of transport of the tested dietary compounds, a cell-free model was selected to obtain additional information that would complement the data obtained from culturing caco-2 cells. LC-MS/MS analysis of samples obtained from the PAMPA assay showed that peaks of SF were detected in the donor plate and acceptor plate. The donor plate was treated with SF, thus it was expected to detect peaks. The presence of peaks in the acceptor plate implies that SF passively diffused across the membrane. This data corroborates the data from the caco-2 cells signifying that SF can passively diffuse as indicated in the literature [27,156]. On the other hand, GR and SMCSO were detected in the samples from the donor plate but not detected in the samples from the acceptor plate. This indicates that GR and SMCSO cannot passively diffuse which supports the data from the caco-2 model. Glucose, known to require active transport, was used as a control for the PAMPA assay and was added to the donor plate. As expected, glucose could not be detected in the samples from the acceptor plate but was detected in the samples from the donor plate where it was added and thus has validated the use of the PAMPA assay for the work presented in this Chapter.

It is important to note that the percentage movement of all three compounds (SF, SMCSO and GR) was lower than the percentage of Lucifer yellow permeating across the membrane, suggesting that the transport of the compounds may have been due to impaired tight junctions, allowing diffusion through the 'gaps' in the monolayer rather than via active transport. However, there is no correlation between the permeability of Lucifer yellow and that of the test compounds (SF, GR and SMCSO) **(Figure 4-8),** suggesting that the movement of the test compounds is due to a factor unrelated to that allowing the movement of Lucifer yellow. Thus transport of compounds may not be due to gaps. There are inconsistencies regarding the Lucifer yellow flux calculations as Debebe *et al.* excluded wells with a Lucifer yellow permeability of >2% [176]. Whereas, others [172,177-180] have calculated the apparent coefficient permeability (P_{app}) of Lucifer yellow as described by Lea, T. [50] and Hubatsch *et al.* [49]. The permeability of SF, GR and SMCSO is less than that of Lucifer yellow and therefore Lucifer yellow may not be a suitable assay. However, the TEER values alone are a suitable evaluation of the monolayer integrity thus we can still hypothesise. Given that

unmetabolized GR and SMCSO were detected in the plasma following consumption of soups, their transport by some means remains a plausible theory.

4.6. Conclusion

The findings from this study for the first time confirm experimentally that SF can passively diffuse using caco-2 and the PAMPA model. The data suggests that GR and SMCSO may require a transporter to permeate across the membrane, given the negligible transport of these compounds across the caco-2 monolayer at 4°C and lack of evidence for diffusion through the PAMPA membrane. The use of appropriate control (glucose and SF) and well-established analytical methods such as LC-MS/MS methods were used to investigate the hypothesis. For the first time, the work described in this Chapter indicates the potential involvement of a transporter in the influx of broccoli-derived compounds GR and SMCSO, however it is clear that further work is required to confirm the hypothesis.

CHAPTER 5

Exploring the biological activity of S-methyl-L-cysteine sulfoxide compared to sulforaphane through the expression of Nrf2-regulated genes in liver cells

5.1. Summary

Phytochemical analysis of the broccoli-rich food product described in **Chapter 2** has revealed that SMCSO is present at significantly higher levels compared to GR. Following consumption of these soups, high levels of SMCSO were measured in plasma and urine of healthy participants **(Chapter 3)**. SMCSO has recently drawn interest due to its potential protective effects including anti-carcinogenic, anti-diabetic and cardiovascular effects. As SF exhibits these biological effects by inducing a transcription factor Nrf2, and up-regulating anti-oxidant genes, it was postulated that the putative biological effects of SMCSO could also be similar. The purpose of this Chapter was to investigate whether SMCSO can up-regulate anti-oxidant genes through the induction of Nrf2 in human liver cells (HepG2). Interestingly, while SF up-regulated several Nrf2-regulated genes as predicted, SMCSO had opposing effects. However, this may not be biologically significant as fold changes induced by SMCSO are relatively low compared to the fold change elicited by SF. This data provides convincing evidence that Nrf2 induction is not a key molecular mechanism of SMCSO biological profile.

5.2. Introduction

Cruciferous vegetables have high levels of sulphur compounds such as glucosinolates [1,2,181]. These vegetables also accumulate another sulphur compound, SMCSO in significantly higher levels compared to glucosinolates [2] as reported in **Chapter 2** which are bioavailable as demonstrated from the *in vivo* work described in **Chapter 3**. Even though, the *in vitro* work using caco-2 cells **(Chapter 4)** suggested that <0.6% of SMCSO may diffuse through tight junctions or be actively transported, high levels of SMCSO were delivered to the plasma (17-28 μM) as reported in **Chapter 3**. In addition, the high levels of SMCSO in cruciferous vegetables poses the question of its influence on human health and whether this is comparable to the most studied phytochemical delivered from cruciferous vegetables, SF.

SF is a phytochemical extensively researched for its putative health benefits through the induction of phase II enzymes and its anti-carcinogenic activities [12]. Induction of transcription factor Nrf2 by SF facilitates its translocation from the cytoplasm into the nucleus [99]. Nrf2 binds to the ARE with a sequence of 5'-A/G TGA C/T NNN GC A/G-3'in the promoter region of several genes thereby inducing up-regulation of phase II enzymes [99]. It has been shown that SF stimulates the up-regulation of phase II genes such as *GCLC* and *NQO1* in several cell lines and mice models [99,103,108,117,125,182-184]. Through the up-

regulation of Nrf2-regulated genes, SF has been shown to modulate the central metabolic pathways and thus exert its chemo-preventative and anti-oxidant-like properties.

Previously published literature has regarded SMCSO as a bioactive phytochemical with positive health effects, including anti-diabetic, anti-carcinogenic, cardiovascular, and antioxidant-like effects [2]. The mechanism of action by which SMCSO achieves this is unknown. SMCSO's anti-oxidant effects have been mainly associated to its metabolite MMTSI and MMTSO [2]. MMTSI and MMTSO are formed from the dimerization of a highly reactive intermediate (methanesulphenic acid) produced by the enzymatic breakdown of SMCSO by cysteine β-lyase **(Figure 1-4, Chapter 1)**. It was proposed that the MMTSI induced apoptosis in PC3 [131] as well as in DU-145 cells [130] via induction of the caspase-dependent and caspase-independent cascade. Its anti-diabetic and cardiovascular effects were associated with the reduction of cholesterol levels in the plasma of rats that were fed a high cholesterol diet with the addition of SMCSO [135,137]. The proposed mechanism for the reduction of cholesterol was via the induction of the hepatic cholesterol 7-α hydroxylase activity [136,137]. This enzyme catalyses the conversion of cholesterol into bile acids for excretion. This theory was supported by an increased faecal excretion of bile acids in mice on the cholesterol and SMCSO diet compared to a cholesterol alone diet [136,137]. However, it is worth remembering that mice and human metabolism may differ in this regard. While research has been undertaken to investigate the potential health benefits, exploration into the underpinning mechanism such as transcriptional regulation by SMCSO remains unexplored [11, 12, 14-16, 18].

Given that SF, also derived from cruciferous vegetables, induces transcriptional changes via Nrf2, the rationale question was developed of whether SMCSO could induce Nrf2 and thereby up-regulate Nrf2-regulated genes like SF. On the basis of the putative health effects of SMCSO, it was hypothesised that SMCSO could induce Nrf2 and up-regulate phase II enzymes. To accomplish this objective, 10 genes positively regulated by SF and containing a putative ARE in their promoter region were selected. The following genes were selected; *NQO1*, *TXNRD1, UGT1A1* (UDP-glucuronosyltransferase family 1 member A1) *), HMOX1, GCLC, AKR1B1* (Aldo-keto reductase family 1 member B)*, PGD, G6PD, TALDO1 and TKT* **(Table 5-1).**

Table 5-1: Selection of 10 Nrf2-regulated genes, positively induced by SF, listed with their functions

Gene	Gene name	Function [117]
NQO1[a,b]	NAD(P)H quinone dehydrogenase 1	Detoxification of xenobiotics
TXNRD1[a,b]	Thioredoxin reductase 1	Part of thioredoxin system
UGT1A1[a,b]	UDP-glucuronosyltransferase family 1 member A1	Conjugation of compounds
HMOX1[a,b]	Heme oxygenase 1	Heme and Iron Metabolism
GCLC[a,b]	Glutamate-cysteine ligase catalytic subunit	Antioxidant enzymes
AKR1B1[b]	Aldo-keto reductase family 1 member B	Phase I enzyme
PGD[b]	6-phosphogluconate dehydrogenase	Carbohydrate metabolism enzymes
G6PD[b]	Glucose-6-phosphate 1 dehydrogenase	
TALDO1[a,b]	Transaldolase 1	
TKT[a]	Transketolase isoform 1	

[a] contains putative ARE [99,183], [b] induced by SF [103,106,108,125,182-184]

This Chapter explores this hypothesis by analysing the expression of an array of genes positively-regulated by Nrf2 in a human hepatocellular carcinoma (HepG2) exposed to physiologically relevant concentrations of SMCSO and SF. HepG2 is an extensively used model to investigate liver metabolism and primary site of detoxification of xenobiotics. However, potential regulation of these genes by SMCSO might not exclusively implicate Nrf2 as a key transcription factor as these genes can be regulated by other inducers/signalling pathways.

5.3. Materials and Methods

5.3.1. Materials

Human hepatocellular carcinoma cell line (HepG2) (ATCC® HB-8065™) and EMEM containing Earle's Balanced Salt Solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate (Cat # ATCC-30-2003) were purchased from ATCC. The following were obtained from Thermo Fischer Scientific; heat-inactivated FBS (Gibco™) (Cat # 10270-106), DPBS (Gibco®) 10X with no calcium and no magnesium (Cat. # 14200059) and penicillin-streptomycin (10,000 units/ml) (Cat # 15140122). T75 flasks were purchased from Starstedt (Cat # 83.3911.002). CellTiter-Blue® was purchased from Promega (Cat # G8080). RNeasy® mini kit (Cat # . 74014) supplied with RLT, RW1, RPE buffer, RNeasy® mini spin column, RNase-free water and QIAshredder™ (purchased additionally) (Cat # 79654) was acquired from Qiagen. NanoDrop™ 1000 spectrophotometer was purchased from Thermo Fisher Scientific. Primers were obtained from Integrated DNA Technologies® (IDT). Precision one step master mix with ROX (Cat # OneStepPLUS-R) was acquired from primer design. AB StepOnePlus™ was purchased from Applied Biosystems™.

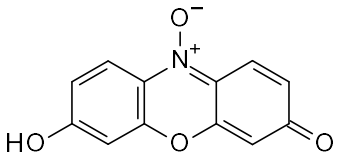
5.3.2. Culturing HepG2

HepG2 from passage 16-20 were routinely cultured in 75cm² flasks and maintained in a humidified atmosphere containing 5% CO_2 at 37°C. EMEM supplemented with 10% FBS, and 1% penicillin-streptomycin was replaced 3-4 times a week. Cells were grown to 80% confluency in a 75cm² flasks before subculturing as described in section **Chapter 4**.

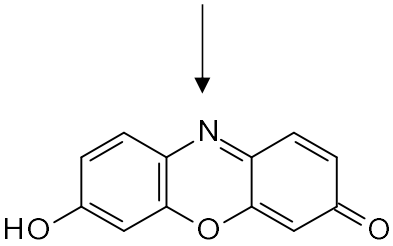
5.3.3. Cell viability of HepG2 cells treated with S-methyl-L-cysteine sulfoxide measured by CellTiter-Blue®

To measure cell viability, the CellTiter-Blue® viability assay was used, in which the end-product can be measured by fluorescence or absorbance. Firstly, HepG2 cells were seeded in a 96-well plate for 24 hours to allow cells to attach. SMCSO stock (CAS 6853-87-8) (purity ≥98%) (Cat # 17600) was purchased from Cayman Chemical (Michigan, USA) and reconstituted in water to prepare a 1 mg/ml stock solution that was stored at -20°C. Cells were exposed to 1-3000 µM of SMCSO in EMEM + 10% FBS for 24 hours. Water was used as vehicle control. As per the manufacturer's instruction, 40 µl of CellTiter-Blue® was pipetted

directly to the cells in the 96-well plate and incubated at 37°C for 30, 60, 120, 180 and 240 minutes. At each time point, plates were shaken for 10 seconds prior to measuring excitation at 560 nm, and emission at 590 nm on a fluorescent plate reader (FLUOstar® Omega plate reader). Background correction was performed by subtracting fluorescence values measured in cell-free wells containing culture medium. The principle of the assay is based on the ability of live cells to reduce resazurin (a blue indicator dye) to resorufin, a pink fluorescent product. Dead cells do not have the capacity to reduce resazurin due to loss of metabolic activity and thus fluorescent signal is not emitted.



Resazurin (dark blue and not highly fluorescent)



Resorufin (pink and highly fluorescent)

Figure 5-1: The principle of the cell viability assay. Viable cells are capable of reducing resazurin, a dark blue indicator dye to resorufin, a highly fluorescent product which excites at 560 nm and emits at 590 nm. Dead cells are not capable of reducing resazurin as a result of loss in metabolic activity.

5.3.4. Treatment of cells with sulforaphane and S-methyl-L-cysteine sulfoxide

HepG2 cells were cultured in 6-well plates until 80% confluency prior to treatment with SF and SMCSO. SF was diluted to a stock solution of 100 mM in DMSO stored at -20°C. Cells were subject to 10 µM, 100 µM and 300 µM SMCSO and 2 µM, 5 µM and 10 µM SF, based on the data from the human study which reported plasma concentrations of 30 µM SMCSO and 1 µM SF. Prior to the treatment of cells, 100 mM SF stock solution was diluted to 1 mM and further diluted to obtain a final concentration of 2 µM, 5 µM and 10 µM SF. SMCSO was diluted to 1 mM and further diluted to give final concentrations of 10 µM, 100 µM, 300 µM

and 1000 µM SMCSO. Cells were exposed to SF (0-10 µM) and SMCSO (0-1000 µM SMCSO) for 2, 6 and 24 hours at 37°C. In addition, cells were also subject to co-treatment of 10 µM SF and 300 µM SMCSO for 24 hours. DMSO was used as a vehicle control for SF and water was used as a vehicle control for SMCSO.

5.3.5. RNA extraction

Following treatment of cells with the dietary bioactives (SF, SMSCO), RNA was extracted using the RNeasy® mini kit, following the manufacturer's instructions. HepG2 cells were washed twice with cold DPBS. To lyse the cells, 350 µl of RLT buffer was added per well, and cells were harvested using cell scrapers. Subsequently, cell lysate was pipetted into QIAshredder™ spin columns and centrifuged for 2 minutes at 13,000 rpm. The QIAshredder spin columns consist of a biopolymer filter which homogenises cell lysate, disrupts high molecular weight DNA, filters out insoluble material, and reduces viscosity of the lysates. The addition of 350 µl of 70% ethanol to the lysate enables selective binding of RNA to the RNeasy membrane of the spin columns at the following step. The 700 µl sample was pipetted and transferred to the RNeasy spin column. Following centrifugation of the RNeasy spin column at 13,000 rpm for 15 seconds, the flow-through was discarded. Contaminants were washed away three times. The first wash comprised of adding 700 µl RW1 buffer to the spin column and centrifuging at 13,000 rpm for 15 seconds. This was repeated for the second wash. The third wash involved the addition of 500 µl of buffer RPE and centrifuging for 2 minutes at 13,000 rpm. The three-wash step efficiently washes contaminants. The RNA was eluted in 30 µl nuclease-free water and quantified using the NanoDrop™.

5.3.6. RNA quantification

To determine the quantity and quality of RNA in the samples, a UV/VIS spectrophotometer (NanoDrop™) capable of measuring at 220 nm-750 nm was used. The spectrophotometer readings were normalised by using 1 µl nuclease-free water. After which, 1 µl of the samples were pipetted on the pedestal of the spectrophotometer. Absorbance was measured at 260 nm, and 280 nm as in **section 3.3.9**.

5.3.7. Gene expression analysis by real-time PCR

RT-PCR was used to analyse the expression of genes in HepG2 cells treated with SF and SMCSO using the AB StepOnePlus™ machine. The Corbett robot was provided with probes, master mix and RNA to prepare samples in a 96-well plate for the RT-PCR process. The probes at a concentration of 2.5 nmoles were labelled at the 5' end of oligos with the fluorescent dye 6-Carboxyfluorescein (5'6-FAM™), at the 3'end with a quencher 3' Iowa Black® FQ (3'IBFQ) and with an internal quencher ZEN™. Double quenchers (ZEN/3'IBFQ) benefits by increasing signal and reducing background noise. The pre-designed primers purchased at a concentration of 5 nmoles are listed in **Table 5-2**. A master mix master mix consisting of a TAQ polymerase, buffer, $MgCl_2$, Moloney murine leukaemia virus reverse-transcriptase enzyme was acquired to enable a one-step RT-PCR reaction. RT-PCR reactions comprised of the conversion of RNA to cDNA catalysed by the reverse transcriptase at 48°C for 30 minutes, activation of DNA polymerase enzyme at 95°C for 10 minutes followed by denaturation of the DNA at 95°C for 15 seconds and primer annealing and extension of DNA at 60°C for 1 minutes.

Table 5-2: List of human genes purchased from IDT® for RT-PCR. PrimeTime® pre-designed standard qPCR assay was purchased consisting of a forward primer, reverse primer and a probe labelled with 5'6-FAM/ZEN/3'IBFQ.

Gene name	Ref seq number	IDT Assay ID	Primers
NQO1	NM_001025434	Hs.PT.58.2697277	Forward: 5'-GTGAGCCAGTACGATCAGTG- 3' Reverse: 5'-TCACCGAGAGCCTAGTTCC-3'
HMOX1	NM_002133	Hs.PT.58.45340055	Forward: 5'-TGCGCTCAATCTCCTCCT-3' Reverse: 5'-TCATGAGGAACTTTCAGAAGGG-3'
GCLC	NM_001197115	Hs.PT.58.2967824	Forward: 5'-TCTGTGCTACCTTCATGTTCTC-3' Reverse: 5'-ACTCTGCCTATGTGGTGTTTG-3'

Gene name	**Ref seq number**	**IDT Assay ID**	**Primers**
TXNRD1	NM_003330	Hs.PT.58.1 921030	Forward: 5'-CACTCCAAAGCGACATAGGAT-3' Reverse: 5'-CTGGTGACAAAGAATACTGCATC-3'
UGT1A1	NM_000463	Hs.PT.58.4 0269971	Forward: 5'-CTCTGGAATTTCTGAGACCATTG-3' Reverse: 5'-GGAATCAACTGCCTTCACCA-3'
AKR1B1	NM_001628	Hs.PT.56a. 41102241	Forward: 5'-AGGCAAGAAACACAGGTATAGG-3' Reverse: 5'-TACTCAGCTACAACAGGAACTG-3'
PGD	NM_002631	Hs.PT.58.3 368888	Forward: 5'-CCATACTCTATCCCGTTGTGC-3' Reverse: 5'-AGACCATCTTCCAAGGCATT-3'
G6PD	NM_000402	Hs.PT.58.2 5195058	Forward: 5'-GCTTCTCCACGATGATGCG-3' Reverse: 5'-CAACCGCCTCTTCTACCTG-3'
TALDO1	NM_006755	Hs.PT.58.2 2941498	Forward: 5'-TTCCCAGGTTGATGACAGC-3' Reverse: 5'-GTATCCACAGAAGTAGACGCAA-3'
TKT	NR_047580	Hs.PT.58.2 0474921	Forward: 5'-CATGCGAATCTGGTCAAAGG-3' Reverse: 5'-CCGCTTCATCGAGTGCTAC-3'
18S	NR_003286	Hs.PT.39a. 22214856.g	Forward: 5'-GGACATCTAAGGGCATCACAG-3' Reverse: 5'-GAGACTCTGGCATGCTAACTAG-3'
CCBL1	NM_001122 672	Hs.PT.56a. 2192206	Forward: 5'-GCTTCATCCTTCACAAAACAGA-3'

Gene name	Ref seq number	IDT Assay ID	Primers
			Reverse: 5'-GATGAGCCCTATGACAGACG-3'
CCBL2	NM_001008662	Hs.PT.58.14497240	Forward: 5'-GCTCATTATTCTTCATATCAGAGAG-3' Reverse: 5'GATCGGATGGTACGTTTACTTGA-3'
ACTB	NM_001101	Hs.PT.39a.22214847	Forward: 5'-CCTTGCACATGCCGGAG-3' Reverse: 5'-ACAGAGCCTCGCCTTTG-3'

5.3.8. Isolation of primary hepatocytes from wild-type mice

Primary hepatocytes isolated from healthy wild-type mice (BL6 C57) were provided by the research group led by Dr Naiara Beraza (QIB research leader). Briefly, mouse liver was perfused with a buffer containing collagenase I (Worthington, USA). Cells were washed and cultured in minimum essential media in 6-well plates pre-coated with rat collagen type I (BD Biosciences). Following 24-hour incubation, the hepatocytes were subject to 10 μM SF and 300 μM SMCSO for 2 hours. Subsequently, RNA was extracted and gene expression analysis was undertaken as described in **section 5.3.5, 5.3.6 and 5.3.7**. The expression of two genes; *HMOX1* and *G6PD*x (X-linked) was quantified relative to the *Polr2a*, mouse housekeeping gene **(Table 5-3)**.

Table 5-3: List of mouse genes purchased from IDT® for RT-PCR. PrimeTime® pre-designed standard qPCR assay was purchased consisting of a forward primer, reverse primer and a probe labelled with 5'6-FAM/ZEN/3'IBFQ.

Gene name	Ref seq number	IDT Assay ID	Primers
HMOX1	NM_010442	Mm.PT.58.8600055	Forward: 5'-TTGTGTTCCTCTGTCAGCATC- 3' Reverse: 5'-ACACTCTGGAGATGACACCT-3'
G6PDx	NM_008062	Mm.PT.58 13826440	Forward: 5'-TGGTTCGACAGTTGATTGGAG-3' Reverse: 5'-GAAGCAGTCACCAAGAACATTC-3'
Polr2a	NM_009089	Mm.PT.39a .22214849	Forward: 5'-CAGGGTCATATCTGTCAGCATG-3' Reverse: 5'-GGTCCTTCGAATCCGCATC-3'

5.4. Results

5.4.1. S-methyl-L-cysteine sulfoxide does not affect HepG2 cell viability

To determine the viability of HepG2 cells in response to various concentrations of SMCSO, cells were seeded on a 96 well-plate and exposed to SMCSO (0-3000 μM) for 24 hours. Examination under the microscope pre- and post-SMCSO treatment presented no apparent changes to cell morphology. Following the addition of CellTiter-blue® reagent, cells were incubated at 37°C, and fluorescence was measured at 30, 60, 120, 180 and 240 minutes. Over the time-course, metabolically active cells reduced resazurin (a blue dye) to resorufin (fluorescent product). Fluorescence values were blank corrected against EMEM + 10% FBS to minimise the signal from the media. There was no significant change in the viability of HepG2 with increasing SMCSO concentration **(Figure 5-2)**.

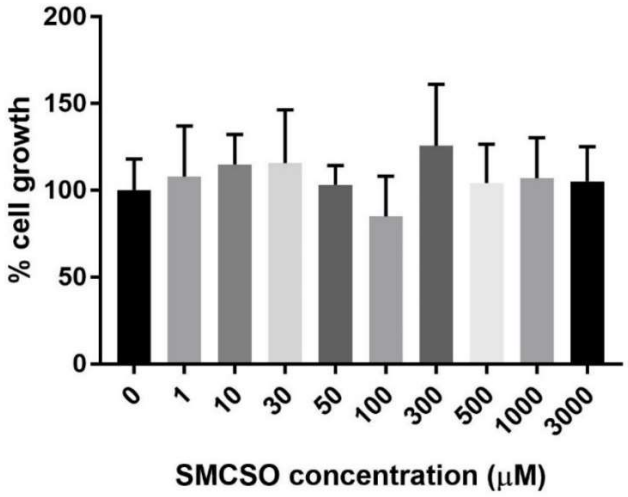
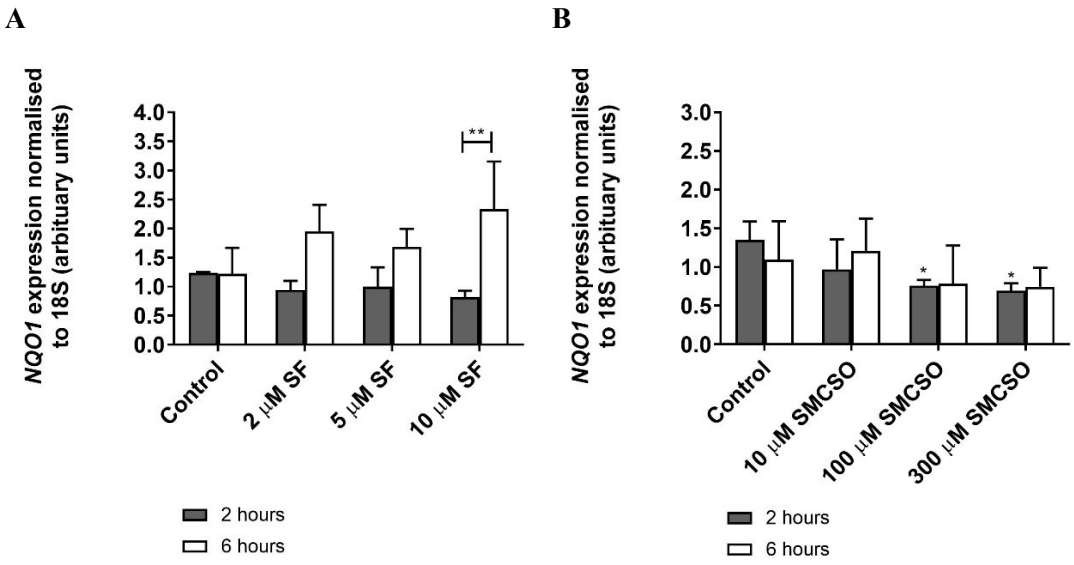


Figure 5-2: Viability of HepG2 cells treated with 0-3000 μM SMCSO for 24 hours. Cells were subject to vehicle control (water) and SMCSO for 24 hours. CellTiter-blue® reagent was added to the cells and emission at 560 nm, and excitation at 590 nm measured using a FLUOstar® Omega plate reader. Data (n=6) represented as mean ± SD for 240 minutes of incubation with CellTiter-blue® reagent.

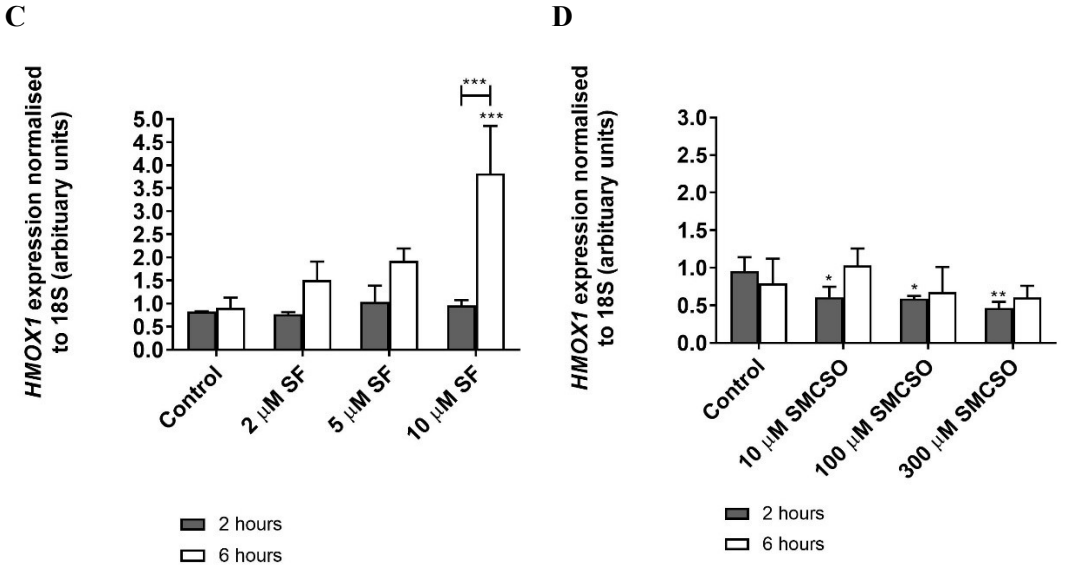
5.4.2. Modulation of Nrf2-regulated genes by a short-term treatment of HepG2 cells with sulforaphane and S-methyl-L-cysteine sulfoxide

To elucidate whether SMCSO could induce Nrf2 and up-regulate phase II enzymes like SF, as reported in the literature, HepG2 cells were exposed to SMCSO (0-300 μM) and SF (0-10 μM) for 2 and 6 hours. An array of 10 genes positively regulated by Nrf2 was analysed using RNA extracted from HepG2 cells. The NanoDrop™ spectrophotometer indicated that the quality of RNA was suitable for gene expression analysis using RT-PCR, based on the 260/280 and 230/260 ratios of 2.0 and 1.8-2.2, respectively. The expression of the following genes was quantified relative to the expression of 18S (housekeeping gene); *NQO1*, *HMOX1*, *GCLC*, *TXNRD1*, *UGT1A1*, *AKR1B1*, *PGD*, *G6PD*, *TALDO1*, and *TKT* **(Figure 5-3)**. Exposure of HepG2 cells to a range of concentrations of SF (0-10 μM) for 2 hours resulted in no change to the expression of the Nrf2-regulated genes except for *UGT1A1* and *AKR1B1*. A significant decrease was demonstrated in the expression of *UGT1A1* at 5 μM and 10 μM SF ($p<0.05$) **(Figure 5-3 I),** and in *AKR1B1* expression at 2 μM, 5 μM and 10 μM SF ($p<0.05$) **(Figure 5-3 K)**. Contrastingly, a significant up-regulation was observed following treatment for 6 hours with SF at 10 μM for *HMOX1* **(Figure 5-3 C)**, *GCLC* **(Figure 5-3E)**, *TXNRD1* **(Figure 5-3G)**, *G6PD* **(Figure 5-3 O)**, *TALDO1* **(Figure 5-3 Q)** and *TKT* **(Figure 5-3 S)** ($p<0.05$), while no change was observed in the expression of *NQO1* **(Figure 5-3 A)**, *UGT1A1*, *AKR1B1* or *PGD* **(Figure 5-3 M)**. Out of all the 10 genes analysed, the up-regulation of *HMOX1* and *TXNRD1* revealed the highest fold increase of ~4 vs. control after 6 hours at 10 μM SF while the other genes showed a ~1-3-fold increase. In contrast to SF, SMCSO showed a dose-dependent down-regulation of the Nrf2-regulated genes including *NQO1* **(Figure 5-3 B),** *HMOX1* **(Figure 5-3 D)**, *TXNRD1* **(Figure 5-3 H),** *AKR1B1* **(Figure 5-3 L)** and *G6PD* **(Figure 5-3 P)** at 2 hours. A significant decrease in the expression of *NQO1*, *HMOX1*, *TXNRD1*, *UGT1A1* **(Figure 5-3 J)**, *AKR1B1*, *G6PD* and *TKT* **(Figure 5-3 T)** was observed at 300 μM as early as 2 hours ($p<0.05$). This effect was abolished at 6 hours for all the genes except for *G6PD* which was significantly down-regulated by SMCSO at 100 μM and 300 μM ($p<0.05$). No change in the expression of *GCLC* **(Figure 5-3 F)**, *PGD* **(Figure 5-3 N)** or *TALDO1* **(Figure 5-3 R)** was detected in response to SMCSO. Results obtained from this study indicated that the expression of targeted genes was considerably lower following treatment with SMCSO compared to SF. Interestingly, most of the analysed genes were down-regulated by SMCSO. A down-regulation of ~0.4-fold in the expression of *AKR1B1* was the largest decrease observed at 2 hours (300 μM SMSCO). In summary, the gene expression analysis reported in this Chapter has demonstrated that SF increased the expression of Nrf2-regulated genes at 10 μM whereas SMCSO reduced their expression at 300 μM.

Expression of *NQO1* in HepG2 cells

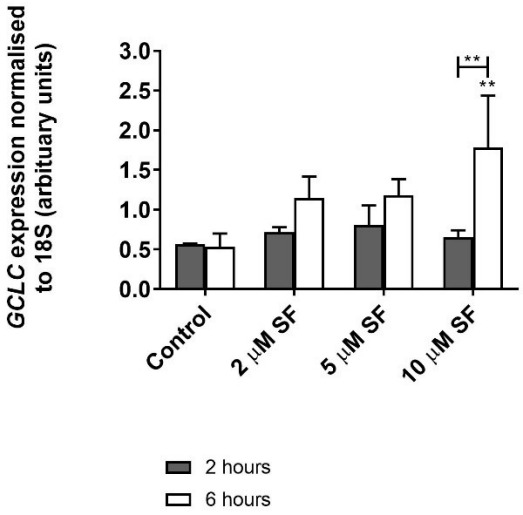


Expression of *HMOX1* in HepG2 cells

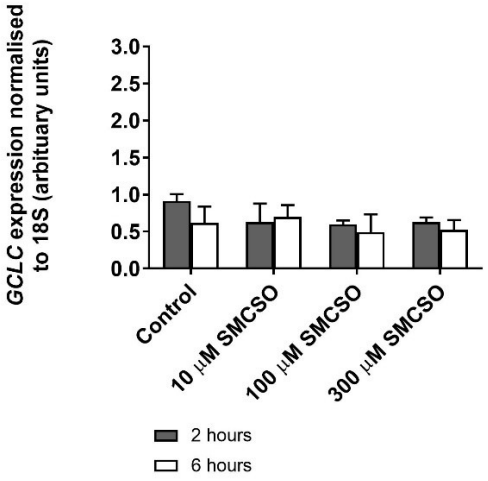


Expression of *GCLC* in HepG2 cells

E

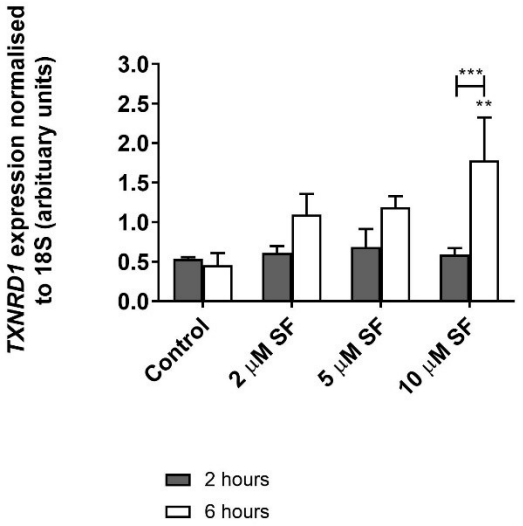


F



Expression of *TXNRD1* in HepG2 cells

G



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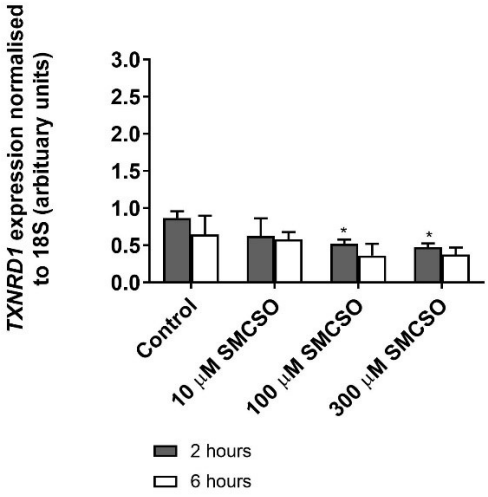
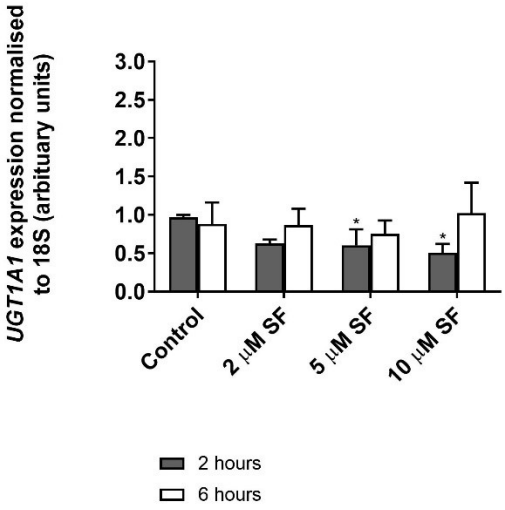


Expression of *UGT1A1* in HepG2 cells

I



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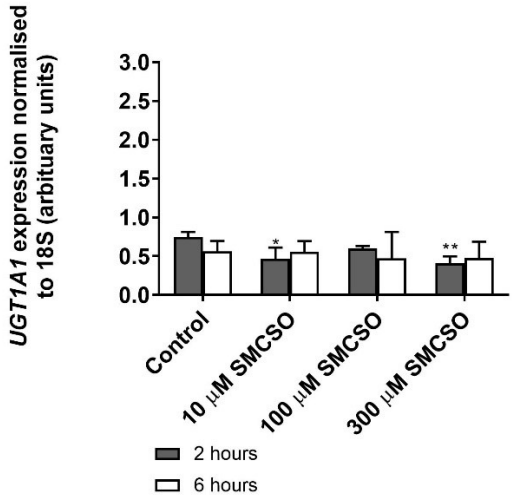
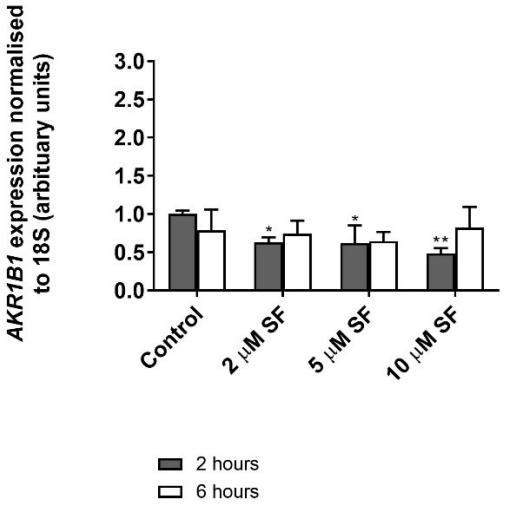


Expression of *AKR1B1* in HepG2 cells

K



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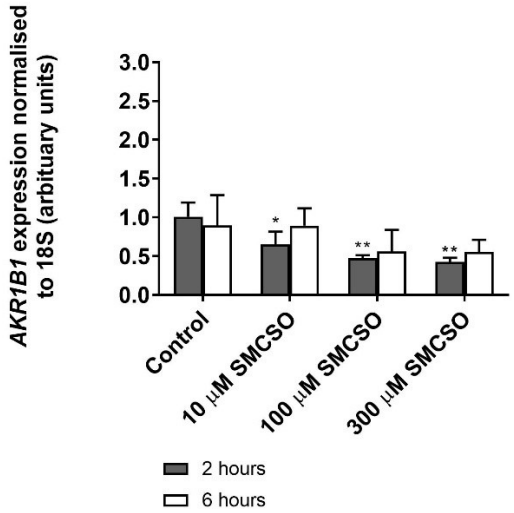
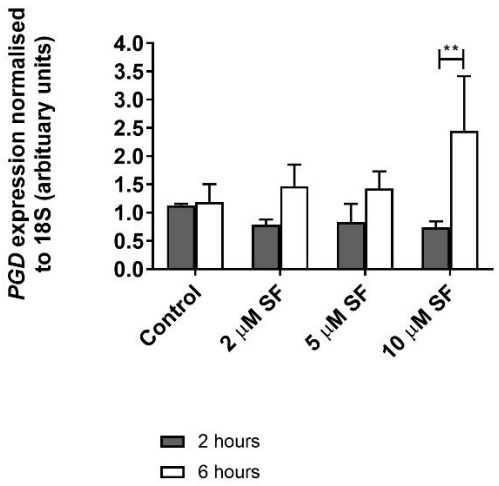
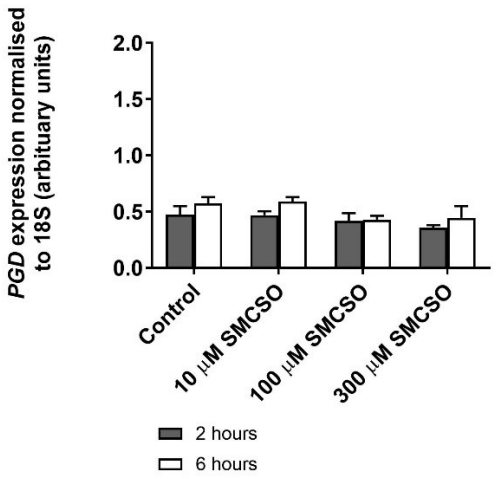


Expression of *PGD* in HepG2 cells

M

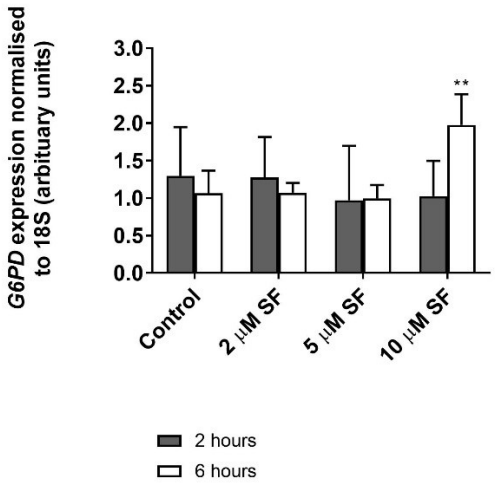


N

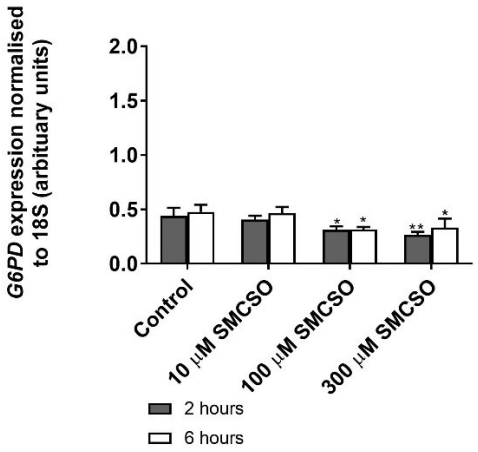


Expression of *G6PD* in HepG2 cells

O



P



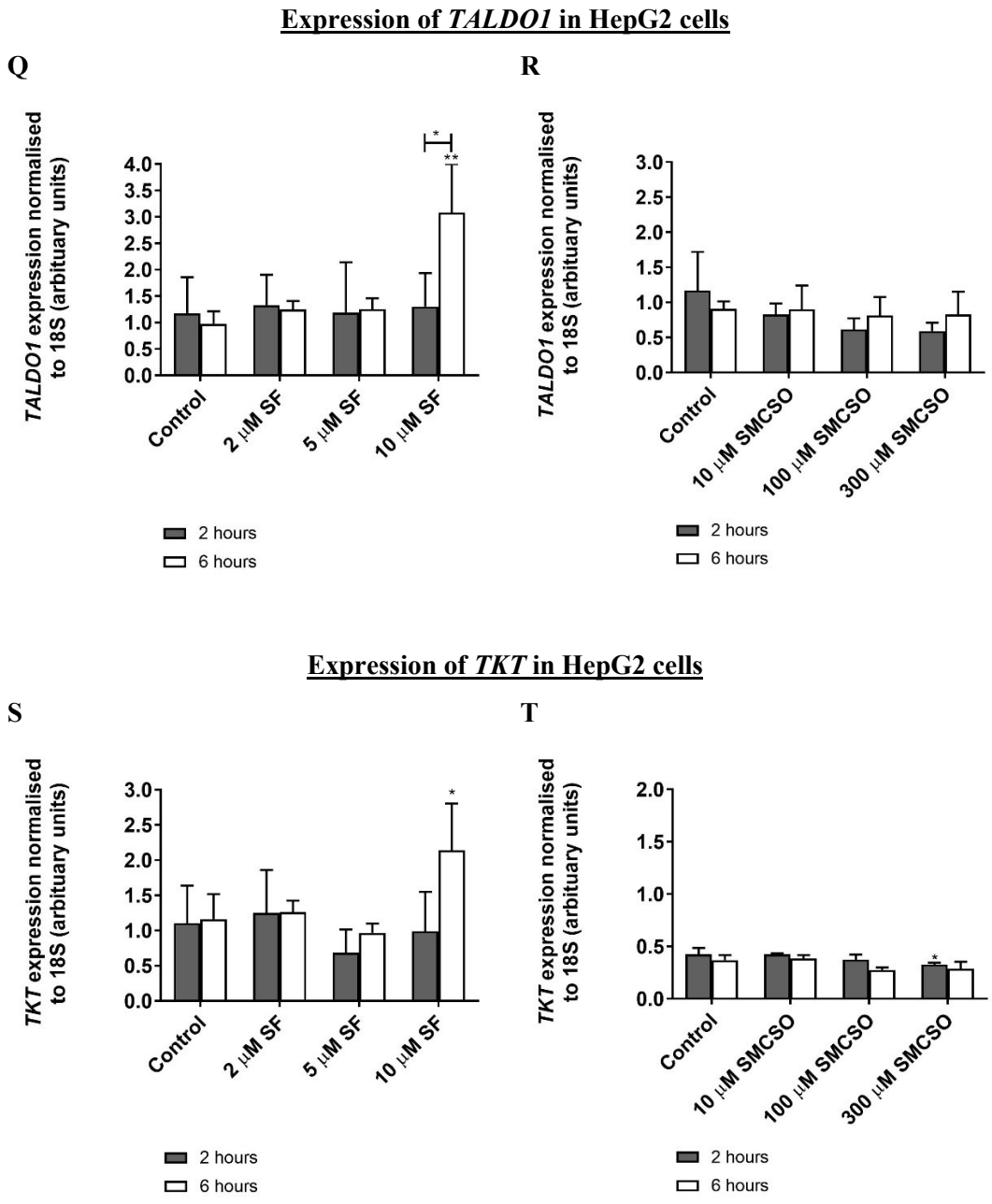
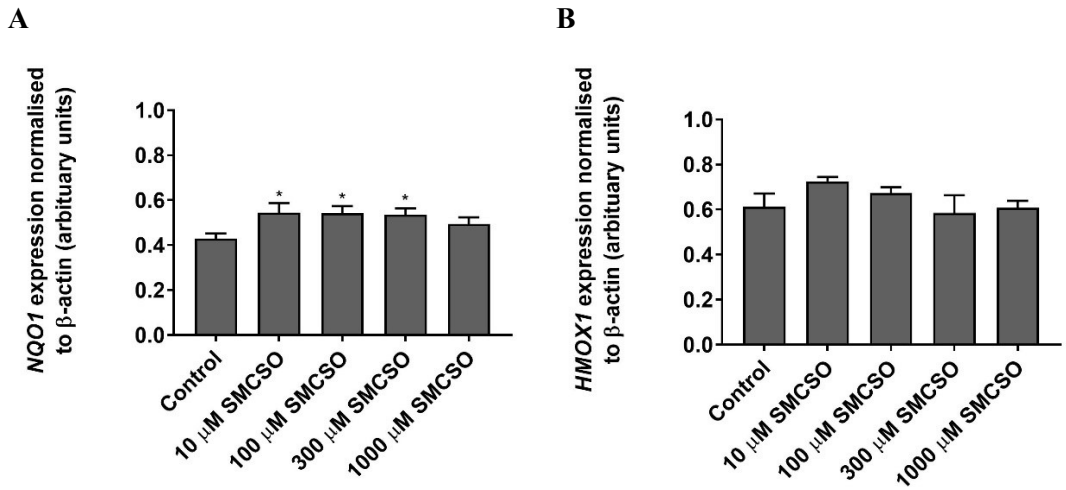


Figure 5-3: Expression of 10 Nrf2-regulated genes in HepG2 cells subject to SF or SMCSO. Cells were incubated with either vehicle control (DMSO) and 2-10 µM SF or vehicle control (water) and 10-300 µM SMCSO for 24 hours. Isolated RNA was used to perform RT-PCR to quantify 10 genes normalised to 18S including *NQO1* (A-B), *HMOX1* (C-D), *GCLC* (E-F), *TXNRD1* (G-H), *UGT1A1* (I-J), AKR1B1 (K-L), *PGD* (M-N), *G6PD* (O-P), *TALDO1* (Q-R), *TKT* (S-T). Data represented as average (n=3) ± SD. Statistical analysis was carried out by one-way ANOVA with Dunnett's multiple comparisons test (*p<0.05, ** p<0.01 and* p<0.001 vs. control).**

5.4.3. Modulation of Nrf2-regulated genes by a 24-hour treatment of HepG2 cells with S-methyl-L-cysteine sulfoxide

In order to examine the expression of Nrf2-regulated genes as a result of chronic exposure to SMCSO, HepG2 cells were treated with SMCSO (0-300 μM) for 24 hours. RNA was analysed for 10 Nrf2-regulated genes. The expression of Nrf2-regulated genes comprising of *NQO1*, *HMOX1*, *GCLC*, *TXNRD1*, *UGT1A1*, *AKR1B1*, *PGD*, *G6PD*, *TALDO1*, and *TKT* were normalised to β-actin (housekeeping gene) **(Figure 5-4)**. HepG2 treated with SMCSO (0-1000 μM) for 24 hours exhibited no changes in the expression of the following genes; *HMOX1* **(Figure 5-4 B)**, *UGT1A1* **(Figure 5-4 E)**, *AKR1B1* **(Figure 5-4 F)**, *PGD* **(Figure 5-4 G)**, *G6PD* **(Figure 5-4 H)** and *TALDO1* **(Figure 5-4 I)**. A significant up-regulation of *NQO1* **(Figure 5-4 A)**, *GCLC* **(Figure 5-4 C)**, *TXNRD1* **(Figure 5-4 D)** and *TKT* **(Figure 5-4 J)** genes was induced by 300 μM SMCSO ($p<0.05$). *NQO1* and *GCLC* genes were significantly induced by all SMCSO concentrations ($p<0.05$). The highest fold increase (~1.3-fold up-regulation) was observed for *GCLC* following treatment for 24 hours with SMCSO at the concentration of 300 μM.



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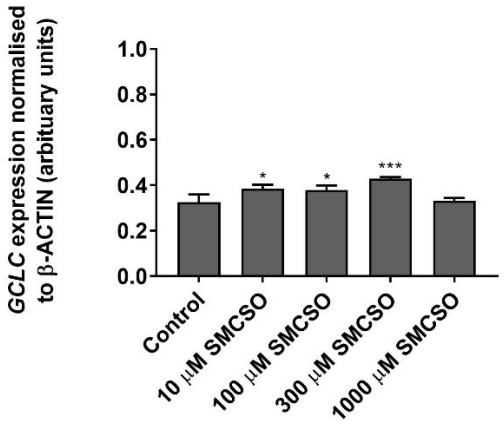
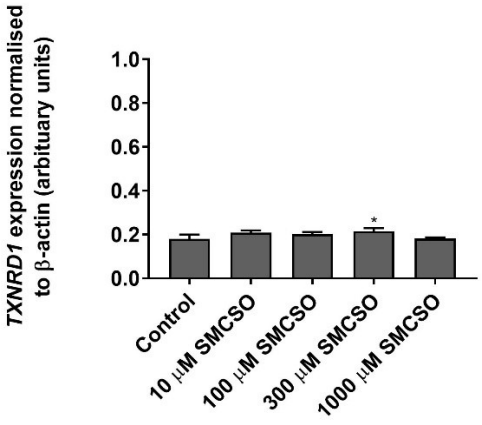
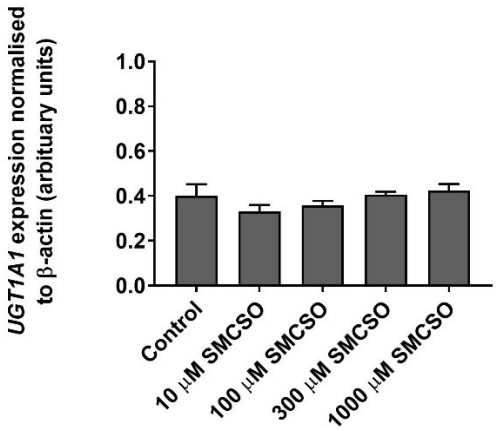

GCLC expression normalised to β-ACTIN (arbituary units)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
***
Control
10 μM SMCSO
100 μM SMCSO
300 μM SMCSO
1000 μM SMCSO

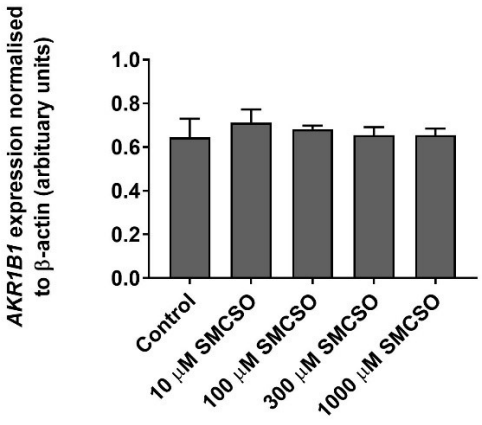
D


TXNRD1 expression normalised to β-actin (arbituary units)
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
10 μM SMCSO
100 μM SMCSO
300 μM SMCSO
1000 μM SMCSO

E


UGT1A1 expression normalised to β-actin (arbituary units)
1.0
0.8
0.6
0.4
0.2
0.0
Control
10 μM SMCSO
100 μM SMCSO
300 μM SMCSO
1000 μM SMCSO

F


AKR1B1 expression normalised to β-actin (arbituary units)
1.0
0.8
0.6
0.4
0.2
0.0
Control
10 μM SMCSO
100 μM SMCSO
300 μM SMCSO
1000 μM SMCSO

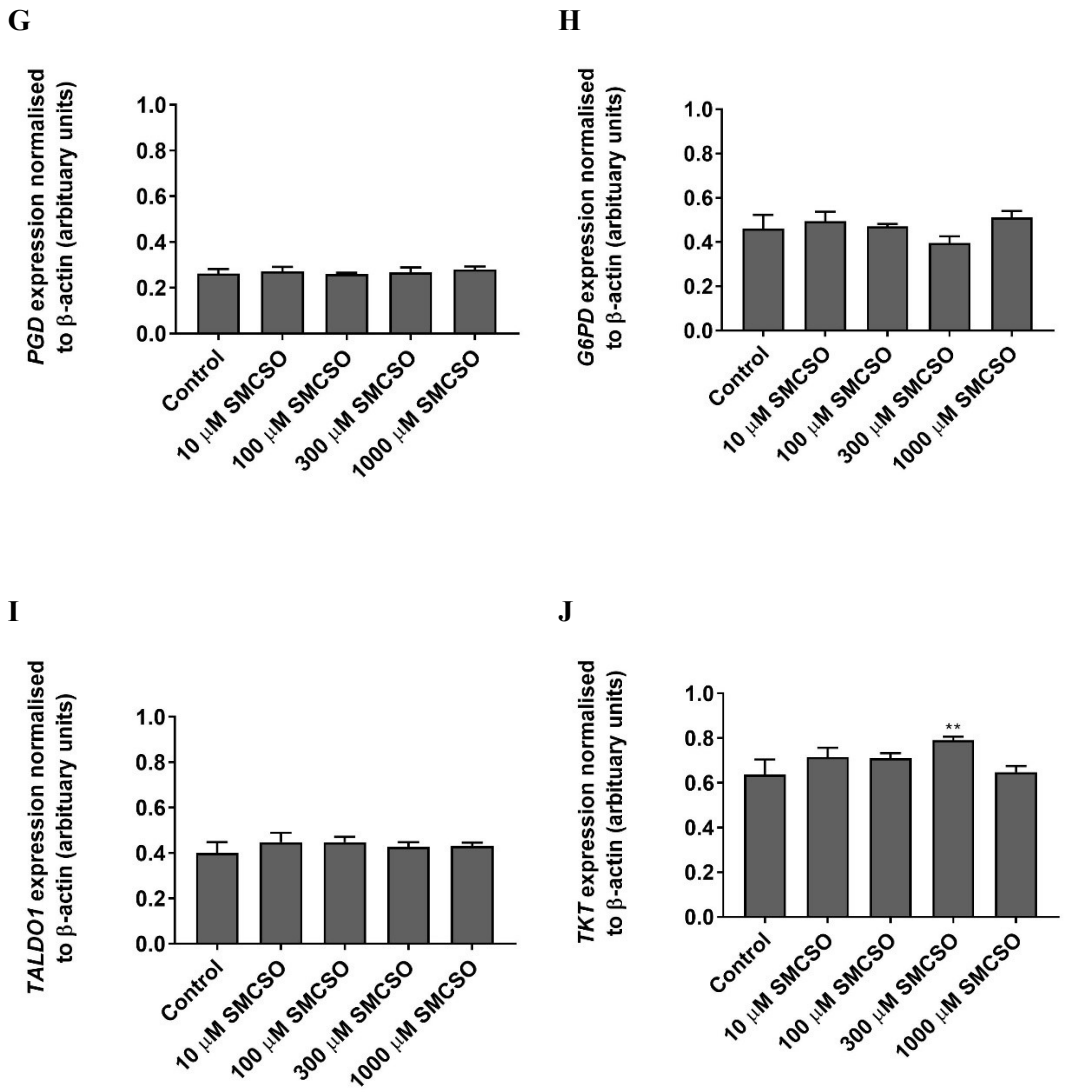
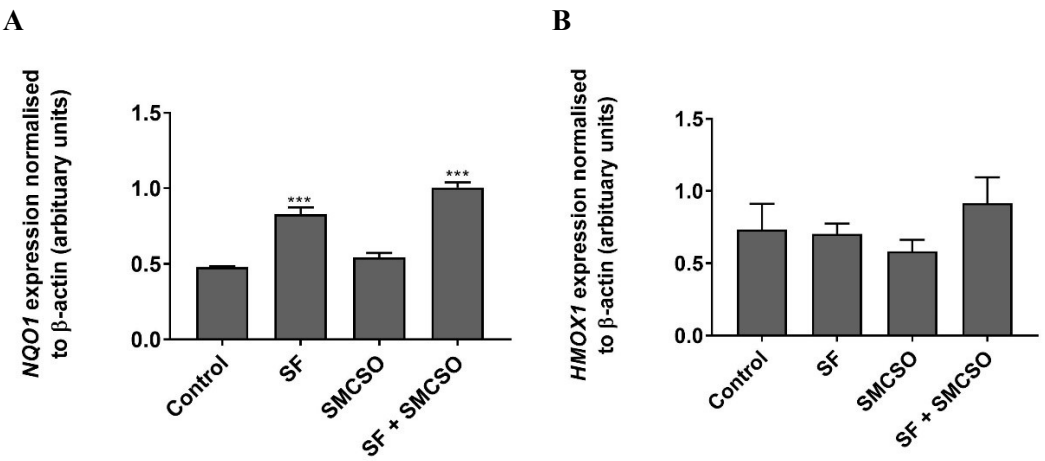


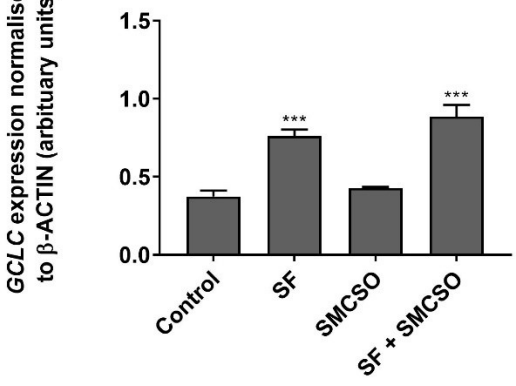
Figure 5-4: Expression of 10 Nrf2-regulated genes in HepG2 cells subject to SMCSO. Cells were incubated with vehicle control (water) and 10-1000 µM SMCSO for 24 hours. Isolated RNA was used to perform RT-PCR to quantify 10 genes normalised to β-actin including *NQO1* (A), *HMOX1* (B), *GCLC* (C), *TXNRD1* (D), *UGT1A1* (E), *AKR1B1* (F), *PGD* (G), *G6PD* (H), *TALDO1* (I), *TKT* (J). Data (n=3) represented as mean ± SD. Statistical analysis was carried out by one-way ANOVA with Dunnett's multiple comparisons test (*p<0.05, ** p<0.01 and *** p<0.001 vs. control).

5.4.4. Synergetic effects of sulforaphane and S-methyl-L-cysteine sulfoxide on Nrf2-regulated genes in HepG2 cells

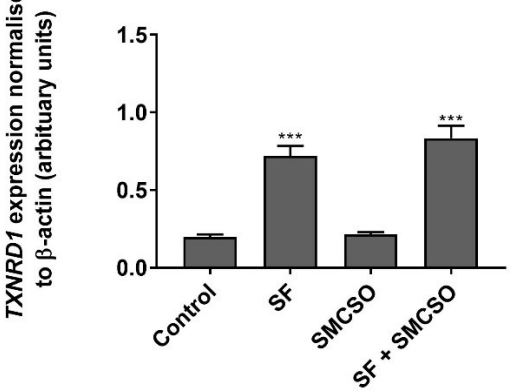
To assess whether both sulphur compounds had a synergetic effect on the induction of Nrf2-regulated genes, HepG2 cells were subject to co-treatment with 10 µM SF and 300 µM SMCSO for 24 hours. Using RT-PCR, 10 Nrf-2 regulated genes were quantified relative to β-actin (housekeeping gene) including *NQO1*, *HMOX1*, *GCLC*, *TXNRD1*, *UGT1A1*, *AKR1B1*, *PGD*, *G6PD*, *TALDO1*, and *TKT* **(Figure 5-5)**. SMCSO treatment for 24 hours did not significantly change the expression of any of the genes. Following stimulation with 10 µM SF and co-treatment with SF and SMCSO, 6 out 10 genes were significantly up-regulated in both cases, including *NQO1* **(Figure 5-5 A)**, *GCLC* **(Figure 5-5 C)**, *TXNRD1* **(Figure 5-5 D)**, *UGT1A1* **(Figure 5-5 E)**, *PGD* **(Figure 5-5 G)** and *TALDO1* **(Figure 5-5 I)** ($p<0.001$). Both *HMOX1* **(Figure 5-5 B)** and *AKR1B1* **(Figure 5-5 F)** showed no significant changes in response to 10 µM SF or co-treatment with SF and SMCSO. *TKT* **(Figure 5-5 J)** was significantly up-regulated only by the co-treatment ($p<0.01$), while *G6PD* was induced by SF alone **(Figure 5-5 H)**. In the case of *G6PD*, the co-treatment with SF and SMCSO did not induce the up-regulation observed following treatment with SF alone. A~4-fold up-regulation in the expression of *TXNRD1* represented the highest fold increase following SF treatment alone. Both *TXNRD1* and *PGD* exhibited a ~4-fold increase in response to co-treatment with SF and SMCSO.



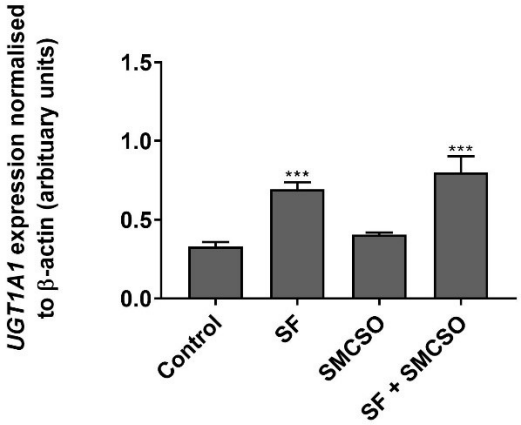
C


GCLC expression normalised to β-ACTIN (arbituary units)
1.5
1.0
0.5
0.0
***
***
Control
SF
SMCSO
SF + SMCSO

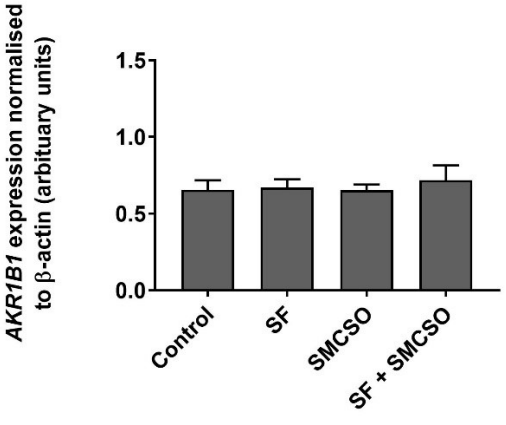
D


TXNRD1 expression normalised to β-actin (arbituary units)
1.5
1.0
0.5
0.0
***
***
Control
SF
SMCSO
SF + SMCSO

E


UGT1A1 expression normalised to β-actin (arbituary units)
1.5
1.0
0.5
0.0
***
***
Control
SF
SMCSO
SF + SMCSO

F


AKR1B1 expression normalised to β-actin (arbituary units)
1.5
1.0
0.5
0.0
Control
SF
SMCSO
SF + SMCSO

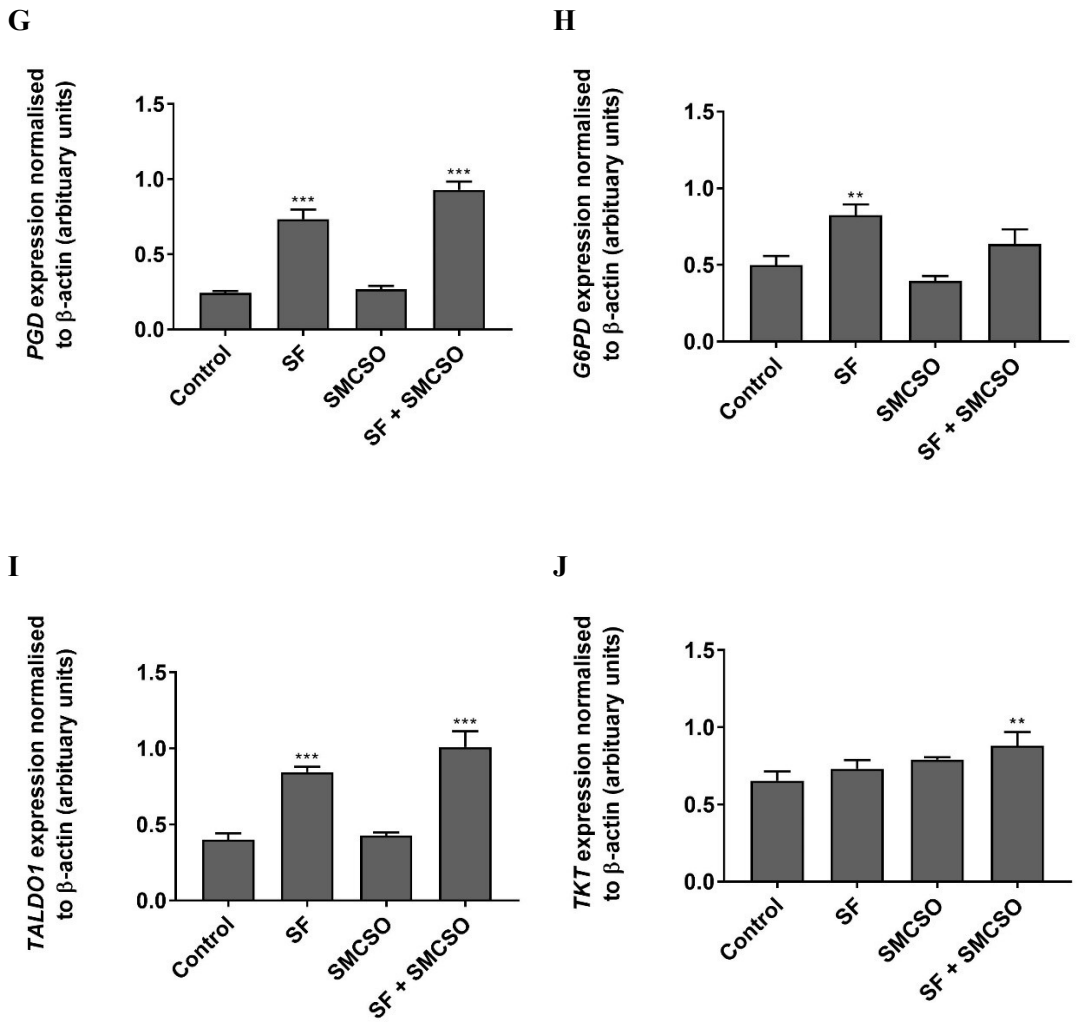
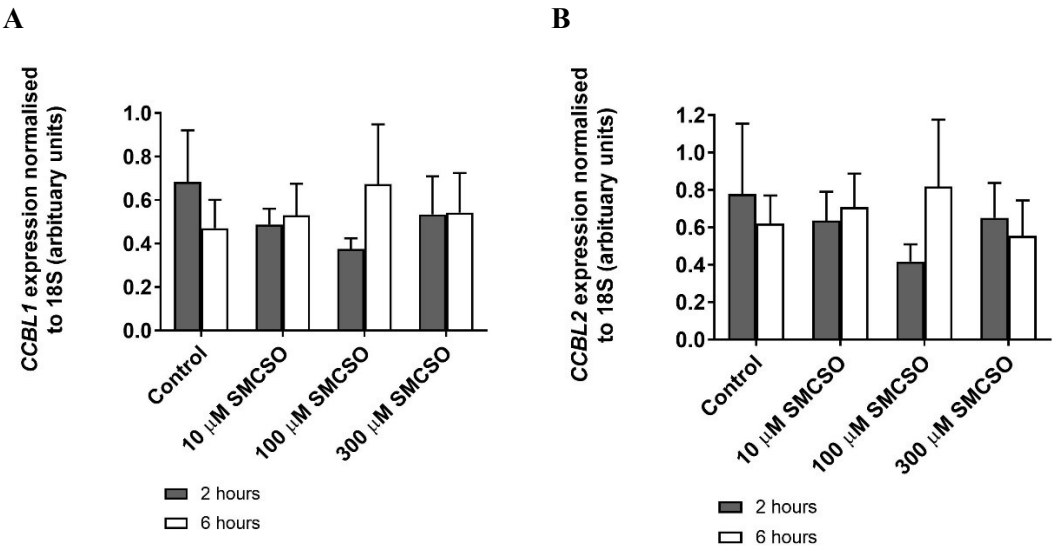


Figure 5-5: Expression of 10 Nrf2-regulated genes in HepG2 cells exposed to exposed to SMCSO, SF and co-treatment with SMCSO and SF for 24 hours. Cells were incubated with vehicle control (DMSO), SF (10 µM), SMCSO (300 µM) and co-treatment with SMCSO (300 µM) and SF (10 µM). RNA was used to perform RT-PCR to quantify 10 genes normalised to β-actin including *NQO1* (A), *HMOX1* (B), *GCLC* (C), *TXNRD1* (D), *UGT1A1* (E), *AKR1B1* (F), *PGD* (G), *G6PD* (H), *TALDO1* (I), *TKT* (J). Data (n=3) represented as mean ± SD. Statistical analysis was carried out by one-way ANOVA with Dunnett's multiple comparisons test (p<0.01 and *** p<0.001 vs. control).**

5.4.5. SMCSO did not affect cysteine β-lyase in HepG2

Gene expression analysis was carried out in order to identify whether SMCSO was able to induce the transcription of cysteine β-lyase (*CCBL1* and *CCBL2*), the enzyme that may catalyse the breakdown of SMCSO in humans. HepG2 cells were exposed to SMCSO (0-1000 μM) for 2, 6 and 24 hours before undertaking RT-PCR analysis **(Figure 5-6)**. The expression of two cysteine β-lyase isoforms, *CCBL1* and *CCBL2*, showed no significant changes following stimulation of SMCSO in doses ranging from 0-300 μM at any time points **(Figure 5-6 A-D)**. Additionally, HepG2 cells were exposed to higher concentration of SMCSO (1000 μM) for 24 hours, eliciting no changes in the expression of *CCBL1* or *CCBL2* **(Figure 5-6 C-D)**. Further, cells were incubated with SF (10 μM), SMCSO (300 μM), and a co-treatment with SF (10 μM) and SMCSO (300 μM) for 24 hours to establish whether SF alone or in combination with SMCSO could potentially regulate the expression of *CCBL1* and *CCBL2*. As reported by **Figure 5-6 E-F**, no changes in the expression of *CCBL1* and *CCBL2* genes were observed by SF alone or in combination with SMCSO.



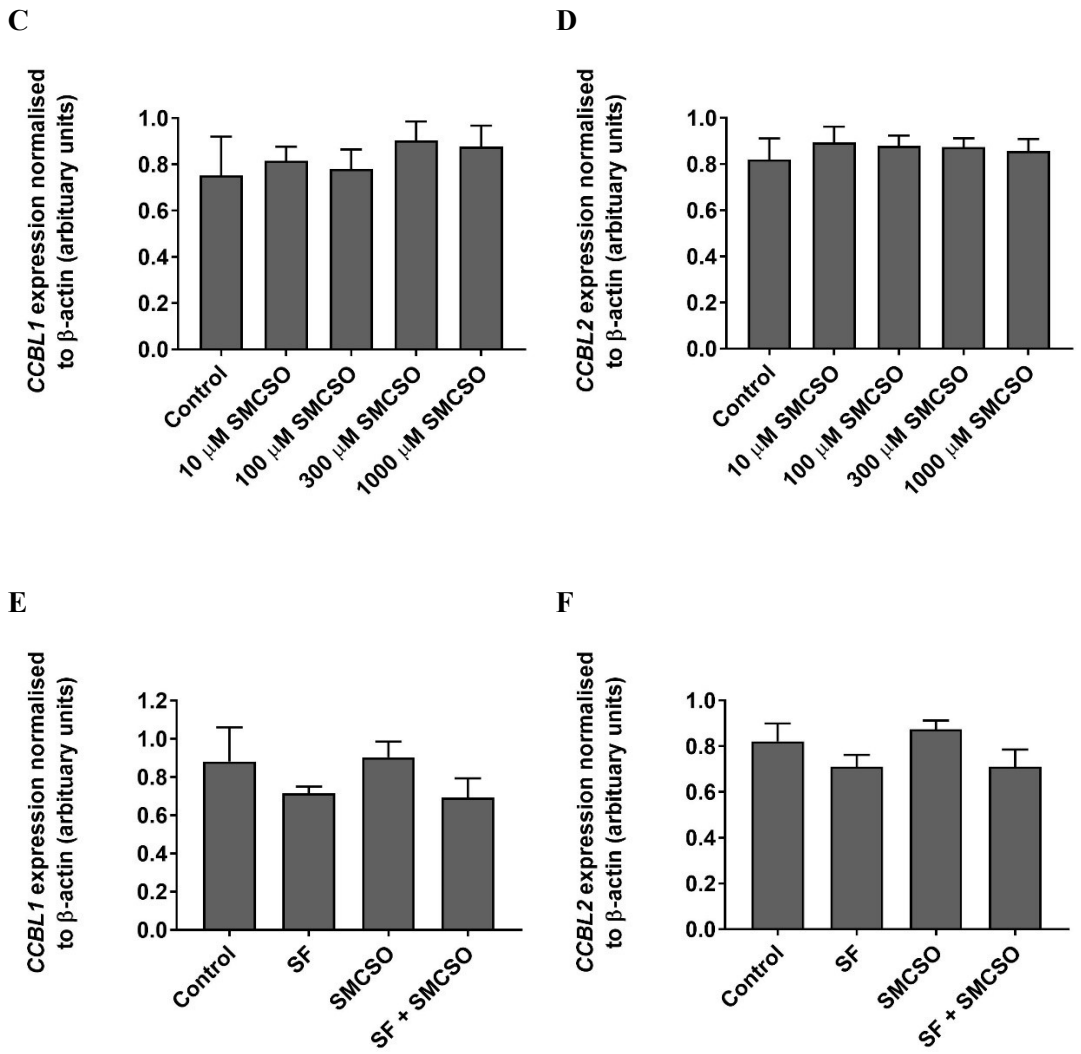


Figure 5-6: Expression of *CCBL1* and *CCBL2* in HepG2 cells exposed to SMCSO, SF and co-treatment with SMCSO and SF. RNA was used to perform RT-PCR to quantify *CCBL1* and *CCBL2* genes that were normalised to 18S or β-actin. HepG2 cells were incubated with 0-300 µM SMCSO for 2 and 6 hours (A-B), 0-1000 µM SMCSO for 24 hours (C-D), SF (10 µM), SMCSO (300 µM), co-treatment with SF (10 µM) and SMCSO (300 µM) for 24 hours (E-F). Data (n=3) represented as mean ± SD. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparisons test (treated cells vs. control).

5.4.6. SMCSO does not induce expression of Nrf2-regulated genes in primary hepatocytes isolated from wild-type mice

Isolated primary hepatocytes from wild-type mice were subject to 10 µM SF and 300 µM SMCSO for 2 hours to assess the effect of SMCSO on Nrf2-regulated gene expressions. Extracted RNA was analysed for the expression of two genes; *HMOX1* and *G6PD*x. A significant up-regulation of *HMOX1* gene expression by ~2-fold was observed in the hepatocytes treated with 10 µM SF ($p<0.0001$) **(Figure 5-7 A)**. However, no changes in the expression of *G6PD*x was observed following exposure to SF **(Figure 5-7 B)**. SMCSO (300 µM) did not affect the expression of both *HMOX1* and *G6PD*x.

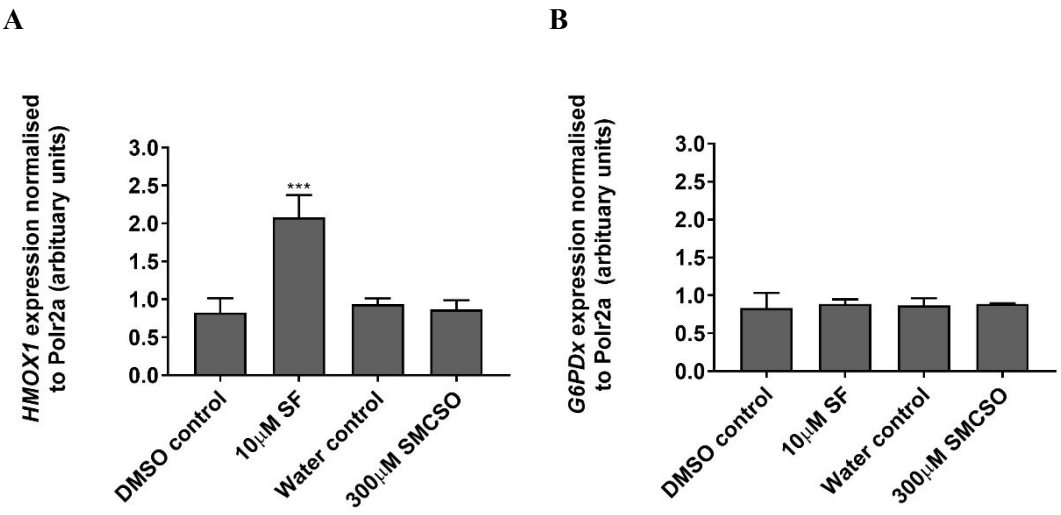


Figure 5-7: Expression of *HMOX1* and *G6PD*x in primary hepatocytes isolated from wild-type mice subject to SF and SMCSO. RNA was used to perform RT-PCR to quantify *HMOX1* (A) and *G6PD*x (B) normalised to *Polr2a*. Primary hepatocytes were incubated with vehicle controls (DMSO, water), SF (10 µM), and SMCSO (300 µM) for 2 hours. Data (n=3) represented as mean ± SD. Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparisons test (* p<0.0001 vs. control).**

5.5. Discussion

The experimental work described in this Chapter was designed to examine the potential biological effects of SMCSO at the level of gene expression in HepG2 cells. The human hepatocellular carcinoma cell line (HepG2) was selected to represent the liver, where fundamental detoxification of xenobiotics occurs. To address the question of whether SMCSO can induce Nrf2 and consequently up-regulate Nrf2-regulated genes like SF, an array of ten Nrf2-regulated genes were analysed in HepG2 cells exposed to SMCSO and SF. Due to its potential health benefits as reported in the literature, it was hypothesised that SMCSO could induce Nrf2 and up-regulate Nrf2-regulated genes.

This is the first study to report the viability of cells in response to SMCSO. However, as evidenced by **Figure 5-2**, no significant changes in the viability of HepG2 subject to 1-3000 μM SMCSO was observed, indicating that SMCSO does not have a potent toxic effect on cells. However, MMTSI, a secondary breakdown product of SMCSO, demonstrated a dose-dependent decrease in cell viability and cell proliferation in PC3 cells (IC_{50}:165.63 μM) [131], in DU-145 cells (IC_{50}:183.63 μM) [131], and in HepG2 cells (183.64 μM)[132].

The human intervention study undertaken as part of this PhD project **(Chapter 3)** revealed that approximately 30 μM SMCSO and 1 μM SF reach the systemic circulation after consuming $Myb28^{V/V}$ broccoli soup. Plasma levels of these dietary bioactives are achieved post first-pass metabolism in the liver. Therefore, the concentrations that the liver is exposed to must be considerably higher than those in the plasma. For this reason, HepG2 cells were exposed to >30 μM SMCSO (10 μM, 100 μM and 300 μM SMCSO) and >1 μM SF (2 μM, 5 μM and10 μM SF). In the case of SMCSO, the concentrations were shown not to affect viability of HepG2 cells **(Figure 5-2)**, with regards to SF, it has been reported by others that concentrations of ≥15-20 μM SF reduces cell viability [127,145]. In this work, HepG2 cells were treated with concentrations ≤10 μM SF.

Genes positively up-regulated by Nrf2; *NQO1*, *UGT1A1*, *AKR1B1*, *TXNRD1*, *GCLC*, *HMOX1*, *G6PD*, *TKT*, *TALDO1*, and *PGD*, were analysed in HepG2 cells stimulated with SF and SMCSO [117]. Two housekeeping genes, 18S and β-actin (*ACTB*), were used to normalise the quantification of the Nrf2 genes. The primary choice for the housekeeping gene was 18S due to its ubiquitous expression and the fact that its expression would be unchanged when stimulated by SMCSO and SF, given that it is a component of 40S ribosomal RNA [185,186]. Another housekeeping gene, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), was reported to be suitable for normalisation in HepG2. However, given that SF modulates central metabolic pathways including glycolysis, in which GAPDH is involved, it was deemed to be

unsuitable for this study. Gene expression data described in **Figure 5-3** were normalised to 18S, however, high variation between samples was observed in subsequent work when normalising to this gene. Chua *et al.* demonstrated that 18S had the least stable expression of 12 housekeeping genes, which could justify the variations seen in the data [186]. As a result, β-actin, a cytoskeleton component, was selected to normalise later work as represented by **Figure 5-4, Figure 5-5, Figure 5-6.** It should be noted that β-actin was ranked 7 out of 12 genes for its stability. Despite this, variation among samples was small.

The expression of 10 Nrf2-regulated genes and two non Nrf2-regulated genes (*CCBL1* and *CCBL2*) was compared following exposure to SF and SMCSO at different time points as summarised in **Table 5-4**. As previously published elsewhere in various cell lines including murine microglia cells, osteosarcoma cells, mice insulinoma cells, breast cancer cells, and colorectal adenocarcinoma cells [103,106-108,182-184], SF resulted in the induction of several Nrf2-regulated genes in the current study. In contrast, SMCSO did not result in a considerable up-regulation of Nrf2-regulated genes relative to SF.

Table 5-4: Summary of the changes in the expression of genes analysed in HepG2 cells exposed to SF (10 µM) or SMCSO (300 µM) for 2, 6 and 24 hours

Gene name	SF			SMCSO		
	2 hours	6 hours	24 hours	2 hours	6 hours	24 hours
NQO1	-	-	Up	Down	-	Up
HMOX1	-	Up	-	Down	-	-
GCLC	-	Up	Up	-	-	Up
TXNRD1	-	Up	Up	Down	-	Up
UGT1A1	Down	-	Up	Down	-	-
AKR1B1	Down	-	-	Down	-	-
PGD	-	-	Up	-	-	-
G6PD	-	Up	Up	Down	Down	-
TALDO	-	Up	Up	-	-	-
TKT	-	Up	-	Down	-	Up

Gene name	SF			SMCSO		
	2 hours	6 hours	24 hours	2 hours	6 hours	24 hours
CCBL1	-	-	-	-	-	-
CCBL2	-	-	-	-	-	-

Up denotes up-regulation of genes, Down denotes down-regulation of genes and – represents no significant changes.

Exposure of HepG2 cells to 300 µM SMCSO for 2 hours resulted in a down-regulation in several genes including *NQO1*, *HMOX1*, *TXNRD1*, *UGT1A1*, *AKR1B1*, *G6PD*, and *TKT*. However, these genes were no longer down-regulated after 6 hours, apart from *G6PD*. Following exposure to SMCSO (300 µM) for 24 hours, an induction was demonstrated in few genes including *NQO1*, *GCLC*, *TXNRD1* and *TKT*. On the contrary, although no up-regulation of Nrf2-regulated genes was observed with 2-hour treatment of SF, several genes including *HMOX1*, *GCLC*, *TXNRD1*, *G6PD*, *TALDO1* and *TKT* were significantly up-regulated at 6 hours ($p<0.05$), in accordance with published work [107,108,183,184]. Similarly, SF induced several genes at 24 hours, as previously reported by Agyeman *et al*. [103]. Some of these genes, such as *GCLC*, *TXNRD1*, *G6PD* and *TALDO1*, remained induced from the 6-hour incubation with SF. Interestingly, others including *NQO1*, *UGT1A1* and *PGD* were not up-regulated at 2 or 6 hours, in congruence with other studies [103,108,182]. *HMOX1* and *TKT* were up-regulated by treatment with SF for 6-hours but not with 24-hour stimulation. This concurs with Townsend and Johnson, as up-regulation of *HMOX1* in microglia cells was only observed between 3-9 hours but abolished at 24 hours exposure [108]. A similar time-dependent effect on *TKT* was also reported by Agyeman *et al*. [103] and Chorley *et al*. [183]. Despite published work reporting up-regulation of *AKR1B1* in response to 15 µM SF stimulation in breast cells for 24 hours [103], the current work presented in this Chapter showed no up-regulation of *AKR1B1* at 2, 6, or 24 hours.

The study described in this Chapter aimed to further understand the effects of both dietary bioactives alone (SF or SMCSO) and in combination (SF and SMCSO) on gene expression by using the same *in vitro* model. HepG2 cells exposed to SF and co-treatment with SF and SMCSO demonstrated a significant up-regulation in the gene expression of *NQO1*, *GCLC*, *TXNRD1*, *UGT1A1*, *PGD*, and *TALDO1* ($p<0.001$) **(Figure 5-5)**. Given that no up-regulation was observed following exposure to SMCSO alone, these data clearly suggest that the induction observed in the co-treatment is associated with the bioactivity of SF as opposed to SMCSO. It should be noted that 300 µM SMCSO showed statistically significant up-regulation of *NQO1*, *GCLC*, *TXNRD1*, and *TKT* ($p<0.05$) **(Figure 5-4)** when compared to its

control vehicle (water). However, when compared to the control vehicle for SF (DMSO) as illustrated in **Figure 5-5**, 300 µM SMCSO was not significant for any of those genes. Retrospectively a control vehicle of water and DMSO should have been used when comparing SF, SMCSO and the co-treatment. Regardless, the up-regulation observed with 300 µM SMCSO at 24 hours was relatively small. Further, SMCSO did not elicit any changes in the expression of *HMOX1* and *G6PD*x in primary hepatocytes, whereas an up-regulation of *HMOX1* was demonstrated with SF treatment.

It is important to highlight that the fold change in the up-regulation and down-regulation of genes observed with SMCSO treatment is relatively low compared to SF. For example, the highest up-regulation was a fold-change of ~4 for *HMOX1* in response to 10 µM SF for 6 hours. Whereas, the largest fold-change observed with SMCSO (300 µM) treatment was ~1.3-fold for *GCLC*, which is considerably lower in comparison to the induction of genes by SF. This suggests that even though the up-regulations observed with SMCSO were statistically significant ($p<0.05$); it might not necessarily be biologically relevant. A plausible explanation could be an initial metabolic stress on the cells resulting in the down-regulation of these genes. Subsequently, the cells recover by switching on a response to counteract the metabolic stress, evidenced by a lack of change in the expression of all the genes at 6 hours other than *G6PD*. This could support the role of SMCSO as a pro-oxidant like SF [114,187]. Taking into consideration the results obtained by using both an immortalised cell line (HepG2) and primary hepatocytes, this study provides the first *in vitro* evidence that SMSCO has the opposite effect on Nrf2-regulated genes in comparison to the well-studied ITC, SF.

Although SMCSO demonstrated no up-regulation of Nrf2-regulated genes; it does not necessarily imply that SMCSO does not exert any biological activities. Discussed below are three hypothetical scenarios that could support the role of SMCSO as a bioactive compound with health benefits.

Firstly, the work presented in this Chapter is specifically focused on Nrf2-regulated genes and SMCSO could be able to regulate other metabolic or signalling pathways. As such, Kumari and Augusti reported reduced enzyme activity of G6PD in the liver of cholesterol-fed mice receiving SMCSO treatment compared to mice fed only with cholesterol [137]. This supports the down-regulation of *G6PD* expression observed with SMCSO exposure at 2 hours in this Chapter **(Figure 5-3)** as well as the up-regulation observed with SF treatment alone at 24 hours, which was eliminated with the co-treatment of SF and SMCSO. However, no change in *G6PD*x was observed in primary hepatocytes from mice in the current study. Perhaps, SMCSO could influence protein expression and enzyme activity of G6PD metabolic enzymes but not their gene expression. Reducing G6PD could have biological relevance by decreasing

de novo fatty acid synthesis. NADPH, a product generated from the reaction catalysed by G6PD, is a co-factor for the fatty acid synthesis enzyme (FASN; fatty acid synthase) [188]. Therefore, SMSCO-induced reduction of G6PD enzyme activity could lead to a decrease of NADPH generation and *de novo* fatty acid synthesis that could play a key role for its potential use as an anti-diabetic agent possessing lipid-lowering effects. This is crucial for ameliorating cardiovascular diseases, as reported by Edmands *et al.* [2] and Kumari and Augusti [137].

Secondly, there is a distinct discrepancy between the recovery of SMCSO in urine reported in **Chapter 4** (~7-8% of administered SMCSO recovered) and that reported by Waring *et al.* (60% labelled [^{35}S] recovered in urine after consumption of [^{35}S]-SMCSO) [84]. Therefore, another explanation could be that the biological effects of SMCSO may be attributed to its secondary breakdown products, MMTSI and MMTSO **(Figure 1-4, Chapter 1)**. SMCSO undergoes hydrolysis, catalysed by either the plant cysteine β-lyase or our own gut microbiota presenting cysteine β-lyase activity or by human cysteine β-lyase, to produce a highly reactive intermediate, methanesulphenic acid [42]. This undergoes dimerization to generate MMTSI and MMTSO. It has been previously reported that MMTSI and MMTSO have chemopreventative effects such as inhibiting proliferation of cells and inducing apoptosis [130-134]. It has been proposed that cysteine β-lyase could be inactivated by these SMCSO products [189]. However, results reported in this Chapter demonstrated no changes in the expression of *CCBL1* or *CCBL2* in HepG2 cells subject to SMCSO alone at different time points. It could be hypothesised that SMCSO is an inert compound and further work needs to be undertaken to understand its metabolism in cells and humans. To date SMCSO metabolic pathway and the bioactivity of its metabolites remain unclear.

Finally, another factor to consider is the thermal degradation of SMCSO. Kubec *et al.* described SMCSO as 'thermoabile' compared to other amino acid containing compounds, given that its decomposition into sulphur and volatile compounds was detected at 80ºC [190]. Further, it was noted that water content as well as time also influenced the degradation of SMCSO [190]. Marks *et al.* demonstrated the formation of MMTSI and MMTSO over 24 hours at 35ºC in the presence of cysteine β-lyase [41]. Therefore, it is possible that the incubation of cells at 37ºC and the composition of culture medium may influence its degradation. As a result, the up-regulation observed at 24 hours may occur as a result of cells being exposed to the secondary metabolites of SMCSO rather than SMCSO itself. Nonetheless, if SMCSO undergoes thermal degradation at 37ºC, cells may be subject to lower concentrations than those intended.

5.6. Conclusion

The experimental work described in this Chapter aimed to elucidate the effect of SMCSO on hepatic gene expression *in vitro*. Results obtained clearly indicate that SMCSO does not affect Nrf2-regulated genes. The data in this Chapter are in accordance with published work showing the effects of SF in upregulating several Nrf2-regulated genes. On the other hand, SMCSO has the opposite effect, causing down-regulation of several genes at 2 hours followed by up-regulation at 24 hours, albeit with a small fold decrease that may not be biologically significant. To date there is very little published data on SMCSO biological profile, and this is the first *in vitro* study investigating the effect of SMCSO on gene expression. This warrants further work to investigate the metabolism of SMCSO in cells and humans as well as underpin the biological mechanism of SMCSO.

CHAPTER 6

General discussion

6.1. Summary of the thesis findings

The emphasis of the work described in this thesis was on assessing the bioavailability of sulphur-containing bioactives (SF, GR and SMCSO) through a multidisciplinary approach based on the use of *in vivo* and *in vitro* models. A broccoli-rich food formulation was specifically developed for this study by using novel broccoli varieties $Myb28^{B/V}$ and $Myb28^{V/V}$ **(Chapter 2)** were tested in an interventional trial in human participants. A three-phase, double-blinded, randomised crossover design, often considered the gold standard for clinical trials, was chosen as one of the experimental tool for investigating the bioavailability of broccoli-delivered phytochemicals. Bioavailability was assessed in plasma and urine samples of participants following consumption of the broccoli-rich soups **(Chapter 3)**. A higher plasma concentration and greater urinary excretion of SF, GR, and SMCSO was achieved following consumption of soups made with the $Myb28^{B/V}$ and $Myb28^{V/V}$ broccoli varieties. This lead to the investigation into transport mechanism of SF, GR, and SMCSO using two established *in vitro* models, caco-2 cells and the PAMPA assay **(Chapter 4)**. It was shown that SF can passively diffuse across the enterocytes whereas neither GR nor SMCSO can passively diffuse. However, the results were not indicative of a specific transport mechanisms, leaving open the possibility that GR and SMCSO may use an active transporter or diffuse paracellularly. Further studies are required to confirm either mechanism. Subsequently, the biological effects of SMCSO were investigated in an immortalised hepatic cell line of human origin (HepG2). No changes in the expression of Nrf2-regulated genes were observed, in contrast with SF which upregulated several genes upon exposure for 6 and 24 hours **(Chapter 5)**. Further exploration into other potential mechanisms will provide insight into the biological targets of SMCSO. Given the increased levels of bioactives measured *in vivo* following consumption of the novel broccoli soups, this food formulation represents a new tool for delivering enhanced levels of sulphur-containing bioactives in an effective and practical way. This is a promising tool to be used in large clinical trials to assess the health benefits of bioactives delivered by cruciferous vegetables, ensuring good compliance to the trial intervention.

6.2. Accomplishment of the thesis aims:

The overall aim of this thesis was to investigate the bioavailability and bioactivity of sulphur-containing compounds as described in **Chapter 1**.

The bioavailability of SF, an ITC derived from broccoli, has already been well established. Despite the use of various food matrices including pills, soups, florets, and extracts, there is still a substantial need for the development of a new food product easier to consume by the general population. Broccoli soups with increasing levels of GR and high levels of SMCSO, as described in **Chapter 2**, are a good example. Through the collaboration of several companies including Monsanto®, A.P. East Anglia Ltd, Bakkavor Ltd, and Norbert Dentressangle, broccoli and stilton soups were produced using three broccoli genotypes, $Myb28^{B/B}$, $Myb28^{B/V}$ and $Myb28^{V/V}$. Two of these, $Myb28^{B/V}$ and $Myb28^{V/V}$ are novel broccoli varieties developed by scientists at the John Innes Centre. The aim of the work presented in **Chapter 2** was to quantify the levels of sulphur-containing compounds to ensure that the soup production retained the increased levels of GR and SMCSO from the florets of the $Myb28^{B/V}$ and $Myb28^{V/V}$ broccoli varieties. The broccoli soups made with the broccoli varieties containing one or two $Myb28^{V}$ alleles indeed resulted in increased levels of GR (280 μmoles and 452 μmoles respectively) and SMCSO (1514 μmoles and 1454 μmoles respectively) compared to standard broccoli (84 μmoles GR and 1031 μmoles SMCSO). This has provided a dietary intervention that is both palatable and a practical means of delivering increased levels of bioactives for use in long-term clinical studies.

Prior to commencing a long-term study assessing biological and clinical endpoints, it is imperative to identify levels of dietary bioactives that are physiologically achievable *in vivo* to translate it into *in vitro* studies investigating their mechanisms of action. This warranted further exploration into the bioavailability of the bioactives from the broccoli-rich soups developed for the purpose of this study. Consequently, a human intervention study was undertaken to assess whether these increased levels of bioactives were 'bioavailable' in human subjects **(Chapter 3)**. Healthy participants (n=10) were recruited into a double-blind, three-phase, crossover study (ClinicalTrials.gov NCT02300324). At each phase, following consumption of one of the three types of broccoli and stilton soups, blood and urine samples were collected for 24 hours. A significantly higher plasma concentration of SF and metabolites, GR and SMCSO was observed following consumption of soups with the presence of one or two $Myb28^{V}$ alleles. Similarly, a greater amount of these metabolites was excreted in the urine following consumption of soups with the presence of one or two $Myb28^{V}$ alleles. This study for the first time not only demonstrated that the increased levels of GR and SMCSO were bioavailable from the new food product, but also showed that intact GR and SMCSO were quantifiable in plasma and urine. As well as confirming that the soups are a suitable dietary tool for long-term interventional studies, the results drew attention to the findings that intact GR and SMCSO were identified in plasma. This indicated that GR and SMCSO are able to permeate across the enterocytes to reach the systemic circulation.

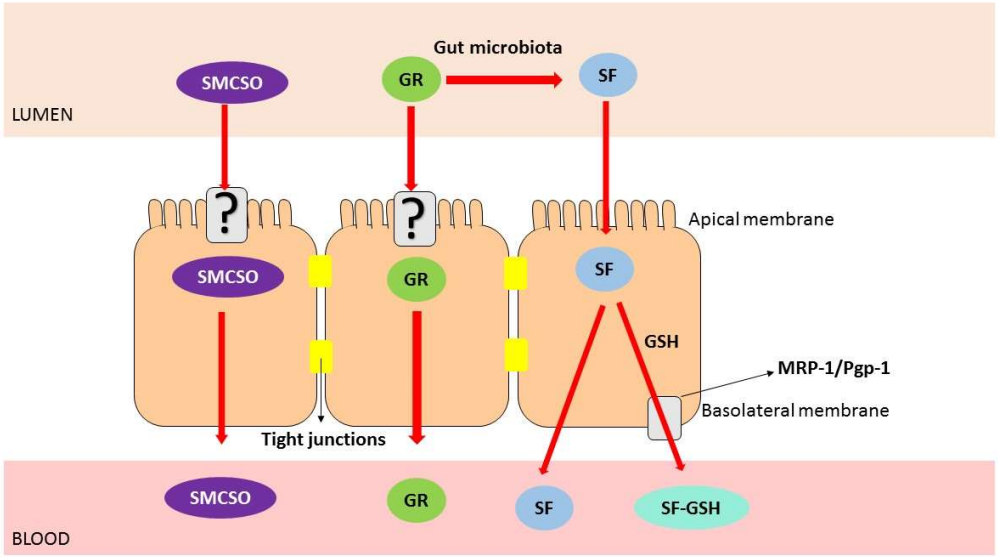


Figure 6-1: Schematic illustration of main findings of the bioavailability of sulphur-containing compounds from BOBS study and their potential transport mechanisms.

The above findings posed the question of how these bioactives were transported across the enterocytes. Therefore, the transport mechanisms of these compounds were investigated by using two *in vitro* models, caco-2 cells and the PAMPA assay **(Chapter 4)**. The purpose of both models was to differentiate between permeation of compounds via passive diffusion and via active transporters. Given that the caco-2 model differentiates into polarised enterocytes expressing active transporters, it enables permeation of compounds transcellularly via active transporters or via passive diffusion, as well as paracellularly through the tight junctions. On the other hand, the PAMPA assay is an artificial membrane (cell-free membrane) with no active transporters or tight junctions thus passive diffusion is the exclusive means of permeation across the membrane. The use of the PAMPA assay confirmed that while SF can passively diffuse through biological membranes, GR and SMCSO cannot. Caco-2 cells were incubated at different temperature conditions (4ºC and 37ºC) to discriminate between passive diffusion and active transport of compounds on the basis that transporters are deactivated at 4ºC, permitting transport of compounds either through tight junctions or via diffusion. Although the diffusion of SF was reduced at 4ºC, mainly due to reducing the rate of diffusion, the transport of SF at 4ºC and 37ºC indicated that SF can passively diffuse, supporting the results from the PAMPA assay. However, in the case of GR and SMCSO (apart from 100 µM SMCSO), the transport of said compounds at 4ºC was negligible compared to that at 37ºC.

This indicated that both compounds may require active transporters or can permeate paracellularly. Nonetheless, these results are not conclusive due to the high percentage of Lucifer yellow recovery (independent paracellular marker to assess monolayer integrity) compared to the low permeability of GR and SMCSO. Irrespective of the data from the caco-2 cells, the presence of GR and SMCSO in plasma and urine imply that these compounds permeate across the enterocytes, either via active transport or through tight junctions.

Interestingly, the levels of SMCSO measured in the plasma were considerably higher than the levels of GR and SF. Taking into account the high levels of this dietary bioactive in the systemic circulation and its potential association with human health benefits as demonstrated by previous studies, the hypothesis that SMCSO exhibits anti-oxidant properties was further investigated. In order to accomplish this, expression of anti-oxidant genes regulated by Nrf2 was explored in human HepG2 liver cells exposed to SF, a known Nrf2 inducer, and SMCSO **(Chapter 5)**. To date, there is no experimental evidence of the effects of SMCSO on the Nrf2 signalling pathway. The overall aim was to investigate whether SMCSO induced Nrf2-regulated genes in a similar way to the well-known Nrf2 inducer SF. HepG2 cells were exposed to SMCSO (0-300 μM) and SF (10 μM) for 2, 6 and 24 hours, and Nrf2-regulated gene expression was quantified. While SF upregulated the expression of several Nrf2-regulated genes at 6 and 24 hours, SMCSO did not show the same effect. It must be noted that while SMCSO was able to upregulate certain genes after treatment for 24 hours, this may not be biologically significant given the small fold-change. Further, SMCSO did not affect the expression of the two Nrf2-regulated genes measured in primary hepatocytes from wild-type mice. It is possible that SMCSO may exert its biological activity through a different mechanism of action rather than the induction of Nrf2-regulated gene expression. It could also be hypothesised that SMCSO activity require its metabolic transformation into active products.

The primary aims of the work described in this thesis were successfully completed, and interesting findings from the human intervention study have generated new research questions that go beyond the scope of the current study. Firstly, this study clearly supports the use of broccoli soups made with different broccoli genotypes as an effective dietary intervention for delivering higher doses of bioactives and minimising compliance issues in long-term interventional trials. In addition, it has shed some light into the bioavailability of other sulphur-containing bioactives, SMCSO and GR. Although the matter of whether SMCSO is a bioactive phytochemical has not been resolved in this thesis, preliminary work has demonstrated that SMCSO has a different bioactivity in terms of Nrf2-mediated pathways compared to the well-studied SF. Further work is essential to understand the molecular mechanisms by which SMCSO could exert health-promoting activities. This will definitely help our understanding

of the health benefits associated with the human consumption of broccoli and other plant material rich in sulphur-containing compounds.

6.3. Congruence of findings to published literature and implications of research

Although there are other broccoli-rich food matrices used as a SF/GR intervention tool, the use of a commercially available soup recipe and novel broccoli varieties with increased levels of bioactives represents a novel delivery formulation potentially useful in interventional trial. The development of this food product requires minimal effort by both the study scientists and the participants involved in the studies. However, a well-controlled platform, where scientists work closely with industrial partners, is crucial for the development of a high-quality product. As this is a new dietary tool with increasing levels of GR, its pharmacokinetic profile was investigated (BOBS study). When comparing human intervention studies that have either administered SF or GR, it very clear that the plasma levels and urinary excretion of SF are considerably higher following direct intake of SF rather than the precursor, GR **(Table 6-1)**. This is presumably because SF is readily absorbed by the enterocytes, whereas GR requires conversion to SF by the gut microbiota prior to absorption. This is reflected with a T_{max} of 1.5-2 hours observed in plasma following consumption of SF [38,64,68,71] compared to a T_{max} of 6-7 hours observed following consumption of GR [68,71]. In terms of the BOBS study, as the soups only contained GR, the T_{max} observed following consumption of $Myb28^{B/B}$ and $Myb28^{B/V}$ was 6-7 hours, comparable to those reported following consumption of GR. However, following consumption of $Myb28^{V/V}$ broccoli soup, a T_{max} of 9.20 hours was observed, slightly later than previously reported. This result is due to the T_{max} of 24 hours for one of the study participants. The C_{max} reported in previous studies following consumption of SF rather than GR is considerably higher, especially in the study by Gasper *et al.* possibly due the intake of super broccoli soup which contains 3-fold higher levels of SF than standard broccoli [38]. Nonetheless, they observed a C_{max} following consumption of standard broccoli soup more than ~10-fold higher than in a study undertaken by Vermeulen *et al.* [71], despite that the administered dose of SF levels was very similar. Likewise, despite participants in the BOBS study consuming twice the GR levels administered by Saha *et al.*, the C_{max} reported in this study is ~8-fold higher (0.2 µM vs. 0.025 µM) [68]. Similarly, the C_{max} following consumption of GR reported by Vermeulen *et al.* is considerably lower than the C_{max} reported in the BOBS study (0.031 µM vs. 0.2 µM respectively) [71]. Several factors could have influenced the discrepancies observed between the BOBS study and previous reports [68,71] including individual variability, analytical tools and food matrices. For examples, the use of HPLC column for analysing the study samples and the presence of fat in the intervention soups

may have contributed to the higher levels of bioactives measured in the BOBS study. In accordance with the data reported by Gasper *et al*. [38] increasing GR intake resulted in increased levels of SF in the plasma and the percentage of excretion excreted in the urine are to a certain extent similar between studies following consumption of GR or SF. The percentage of excretion following GR consumption is approximately 7.6-10.7% which is comparable to previous studies [22,32,67,68,70] **(Table 6-2)**.

Table 6-1: Comparison of the pharmacokinetics (C_{max}, T_{max}, AUC) following GR or SF consumption between the current study presented (BOBS) and other published intervention studies

	Intervention	Pharmacokinetics		
		C_{max} (µM)	**T_{max} (hours)**	**AUC (µmol.h/L)**
BOBS study (reported in this thesis)	Myb28$^{B/B}$ broccoli soup (standard broccoli) (84 µmoles GR)	0.2	6.1	2.0
	Myb28$^{B/V}$ broccoli soup (280 µmoles GR)	0.4	7.4	4.9
	Myb28$^{V/V}$ broccoli soup (452 µmoles GR)	0.6	9.20	8.0
Gasper *et al.* [38]	Standard broccoli soup (95.1-107.5 µmol/l SF)	2.3	1.5	10.7-13.0
	Super broccoli soup (342.7-342.8 µmol/l SF)	7.3	2.0	35.0-40.2
Saha *et al*. [68]	Frozen broccoli soup (42.5 µmoles GR)	0.025	6.0	0.144-0.414
	Fresh broccoli soup (23.5 µmoles SF)	0.21	2.0	1.26-1.27
Vermeulen *et al.* [71]	Cooked broccoli (61.4µmol GR)	0.031	6.0	0.286
	Raw broccoli (9.92 µmol SF)	0.103	1.6	0.495
Atwell *et al.* [64]	Sprout extract treated with myrosinase (200 µmol SF)	0.7	3.0	13.8
	Fresh broccoli sprout (200 µmol SF)	1.9	3.0	3.0

Table 6-2: Comparison of the percentage of SF and metabolites excreted following consumption of GR or SF current study presented (BOBS) and other published intervention studies

	Intervention	Percentage excretion (%)
BOBS study (reported in this thesis)	Myb28$^{B/B}$ broccoli soup (standard broccoli)	10.4
	Myb28$^{B/V}$ broccoli soup	8.3
	Myb28$^{V/V}$ broccoli soup	8.9
Gasper *et al.* [38]	Standard broccoli soup (95.1-107.5 µmol/L SF)	82
	Super broccoli soup (342.7-342.8 µmol/L SF)	55
Saha *et al*. [68]	Frozen broccoli soup (42.5 µmoles GR)	9.6
	Fresh broccoli soup (23.5 µmoles SF)	58.5
Vermeulen *et al.* [71]	Cooked broccoli (61.4µmol)	3.4
	Raw broccoli (9.92 µmol)	37
Fahey *et al.* [70]	Prostaphane® tablets (100 µmol SF)	71
	SF preparation in an α-cyclodextrin inclusion (200 µmol SF)	62.3
Egner *et al.*[22]	GR-rich beverage (800 µmol GR)	5
	SF-rich beverage (150 µmol SF)	70
Clarke *et al.* [69]	Broccoli sprout with myrosinase (150 µmol GR)	74
	BroccoMax™ pills (121 µmol GR)	19
Clarke *et al.* [65]	Broccoli sprout with myrosinase (218.4 µmol GR)	96
	BroccoMax™ pills (220.3 µmol GR)	19
Fahey *et al.* [73]	Broccoli sprout extract (200 µmol GR)	11.8

	Intervention	Percentage excretion (%)
	Broccoli sprout extract (400 µmol GR)	10.4
Oliviero *et al.* [67]	Broccoli powder with 100% myrosinase activity (73 µmol GR + 34.0 µmol SF)	58
	Broccoli florets with 100% myrosinase activity (73 µmol GR + 11.1 µmol SF)	33
	Broccoli florets with 80% myrosinase activity (69 µmol GR + 6.7 µmol SF)	22
	Broccoli florets with 2% myrosinase activity (67 µmol GR + 2.8 µmol SF)	17
	Broccoli florets with no myrosinase activity (72 µmol GR + 0.3 µmol SF)	10
Shapiro *et al.* [10]	Sprout extract (525 µmol glucosinolates over 7 days)	17.8
	Sprout extract (2,100 µmol glucosinolates over 7 days)	19.6
	Sprout extract (525 µmol ITCs over 7 days)	70.6
Kensler *et al.* [74]	Sprout infusion (400µmol GR)	12
Conway *et al.* [32]	Fresh broccoli (0.48µmol GR/g)	32.3
	Steamed broccoli (0.46 µmol GR/g)	10.2

It is well-established that SF is bioavailable from the intake of SF or GR; however, for the first-time, this study has reported the presence of intact GR and SMCSO in the systemic circulation. Due to limited literature, the pharmacokinetics of GR and SMCSO are unknown. The general observation is that increasing GR levels in the soups resulted in increasing levels of GR and SF in the plasma in a dose-dependent manner **(Table 6-3)**, as previously reported by Gasper *et al.* [38]. While $Myb28^{B/V}$ and $Myb28^{V/V}$ broccoli soups delivered higher levels of SMCSO in the plasma compared to $Myb28^{B/B}$ broccoli soup, there was no significant difference in the SMCSO levels between the two novel varieties. In comparison to SF and SMCSO, the plasma concentration and percentage excretion of GR was considerably lower.

Presumably this is because most of the intact GR is converted to SF and only a small amount is absorbed as intact GR. The amount of SF absorbed and excreted is dependent on the conversion efficiency of GR to SF. In accordance with published data, <1% was excreted as intact GR and approximately 9-11% as SF and metabolites, in congruence with other studies **(Table 6-2)**. However, this leaves ~88% unaccounted for. It is possible that this lost proportion may have been excreted, as intact GR and as SF and metabolites, in faeces, accumulated in tissues, or be excreted over the course of 48-72 hours (not measured in this study) **(Figure 6-2)**. Although, a previous study in rats detected no intact GR in faeces [20].

Table 6-3: Comparison of the plasma C_{max} and the percentage of sulphur-containing bioactives excreted in BOBS study following consumption of three types of broccoli soups

		Intervention diet		
		$Myb28^{B/B}$ broccoli soup	$Myb28^{B/V}$ broccoli soup	$Myb28^{V/V}$ broccoli soup
		Mean ± SD	**Mean ± SD**	**Mean ± SD**
Plasma C_{max}	**GR**	0.01 ± 0.01	0.03 ± 0.01	0.04 ± 0.02
	SF	0.17 ± 0.12	0.37 ± 0.26	0.61 ± 0.40
	SMCSO	16.93 ± 3.79	26.94 ± 7.70	28.03 ± 5.39
Percentage of excretion (%)	**GR**	0.64 ± 0.34	0.51 ± 0.24	0.47 ± 0.22
	SF	10.75 ± 7.48	7.68 ± 5.98	8.99 ± 6.27
	SMCSO	6.61 ± 2.33	6.92 ± 3.05	7.52 ± 2.37

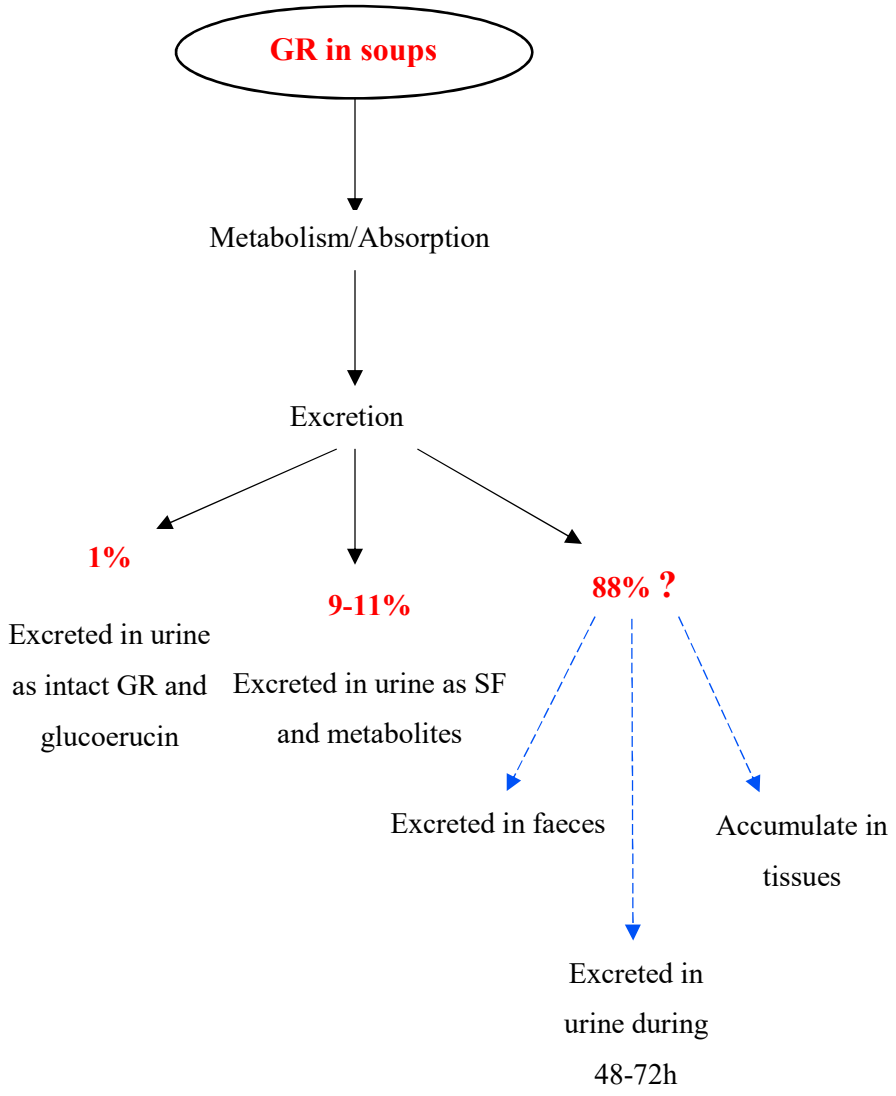


Figure 6-2: Schematic illustration of the percentage recovery from the intake of GR in the soups in BOBS study. While the black solid lines represent the findings from BOBS study, the blue dotted lines represent speculations about the unaccounted 88%.

The plasma concentration and urinary excretion of SMCSO was considerably higher than SF. Though the percentage of SMCSO excretion is to a certain extent similar to SF **(Table 6-3)**, it does not appear to align with evidence from the only published study regarding the bioavailability of SMCSO [84]. Of the administered SMCSO dose, 6-8% was recovered in the urine with 92% unaccounted for. Perhaps SMCSO accumulates in tissues, or is excreted in faeces or urine over 48-72 hours **(Figure 6-3)**. Data from another intervention study (SAP study) using the same soup intervention indicate accumulation of SMCSO in prostate and adipose tissues (Unpublished data from Mr. Jack Coode-Bate, MD research). Most importantly, these results lead to three main hypotheses, discussed below, including;

metabolism of SMCSO by cysteine β-lyase, metabolism of SMCSO by gut microbiota, and thermal degradation of SMCSO. These hypotheses arose from the large discrepancy between the 6-7% SMCSO excreted in this study and 60% ^{35}S of the administered ^{35}S-SMCSO excreted in the study by Waring *et al.* [84]. Although the study by Waring *et al.* recovered ^{35}S as opposed to SMCSO, a key distinction, the findings suggest that SMCSO undergoes further metabolism [84]. In addition, Waring *et al.* reported 16% of the ^{35}S-SMCSO excreted as sulphate, further supporting the concept of SMCSO undergoing further metabolism in humans, as the ^{35}S from the SMCSO has been incorporated into metabolites derived from SMCSO and excreted in the urine [84]. Thus, the low percentage of recovery reported by the BOBS study could be due to the fact that SMCSO metabolites were not measured.

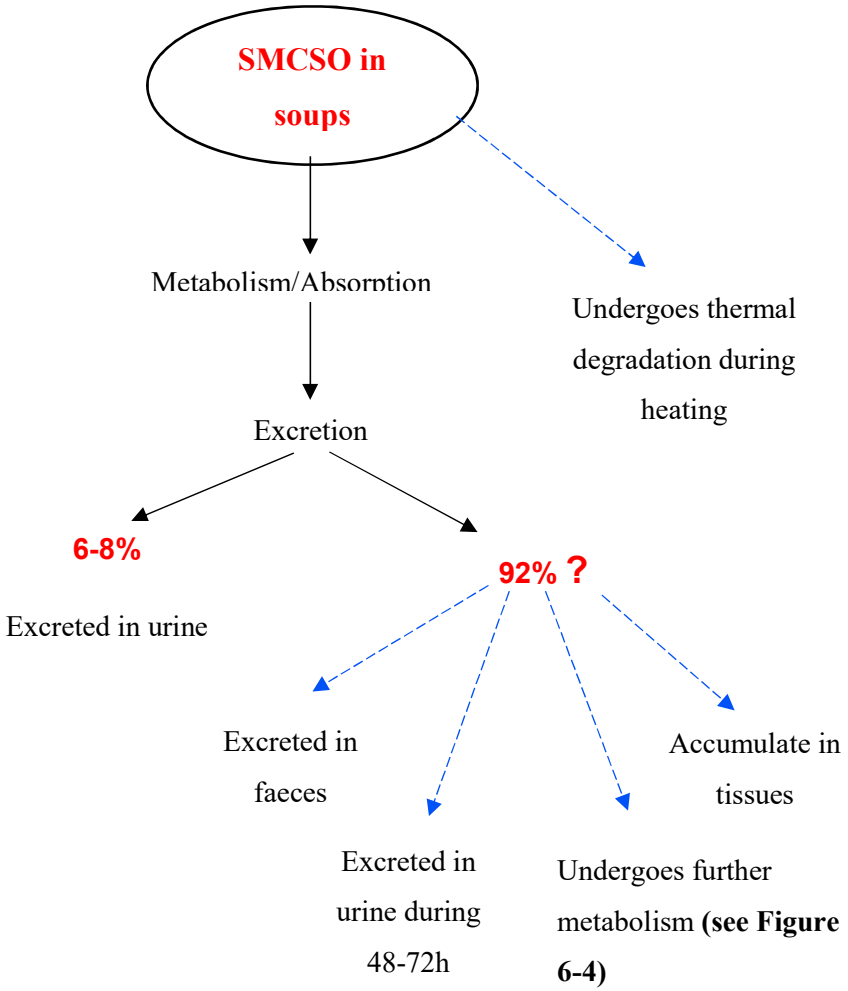


Figure 6-3: Schematic illustration of the percentage recovery of from the intake of SMCSO in the soups in BOBS study. While the black solid lines represent the findings from BOBS study, the blue dotted lines represent speculations about the unaccounted 92%.

As illustrated in **Figure 6-4**, SMCSO in the plant undergoes breakdown through the activity of plant cysteine β-lyase to generate an intermediate which undergoes further metabolism to generate MMTSI and MMTSO. This was confirmed using an enzymatic model consisting of SMCSO and purified cysteine β-lyase at 35ºC resulting in the generation of MMTSO, MMTSI, pyruvate and dimethyl trisulphide over 24-hours [41]. In addition, two human enzymes CCBL1 and CCBL2 were recently identified as presenting cysteine β-lyase activity, suggesting that SMCSO may undergo further metabolism in humans through the activity of cysteine β-lyase.

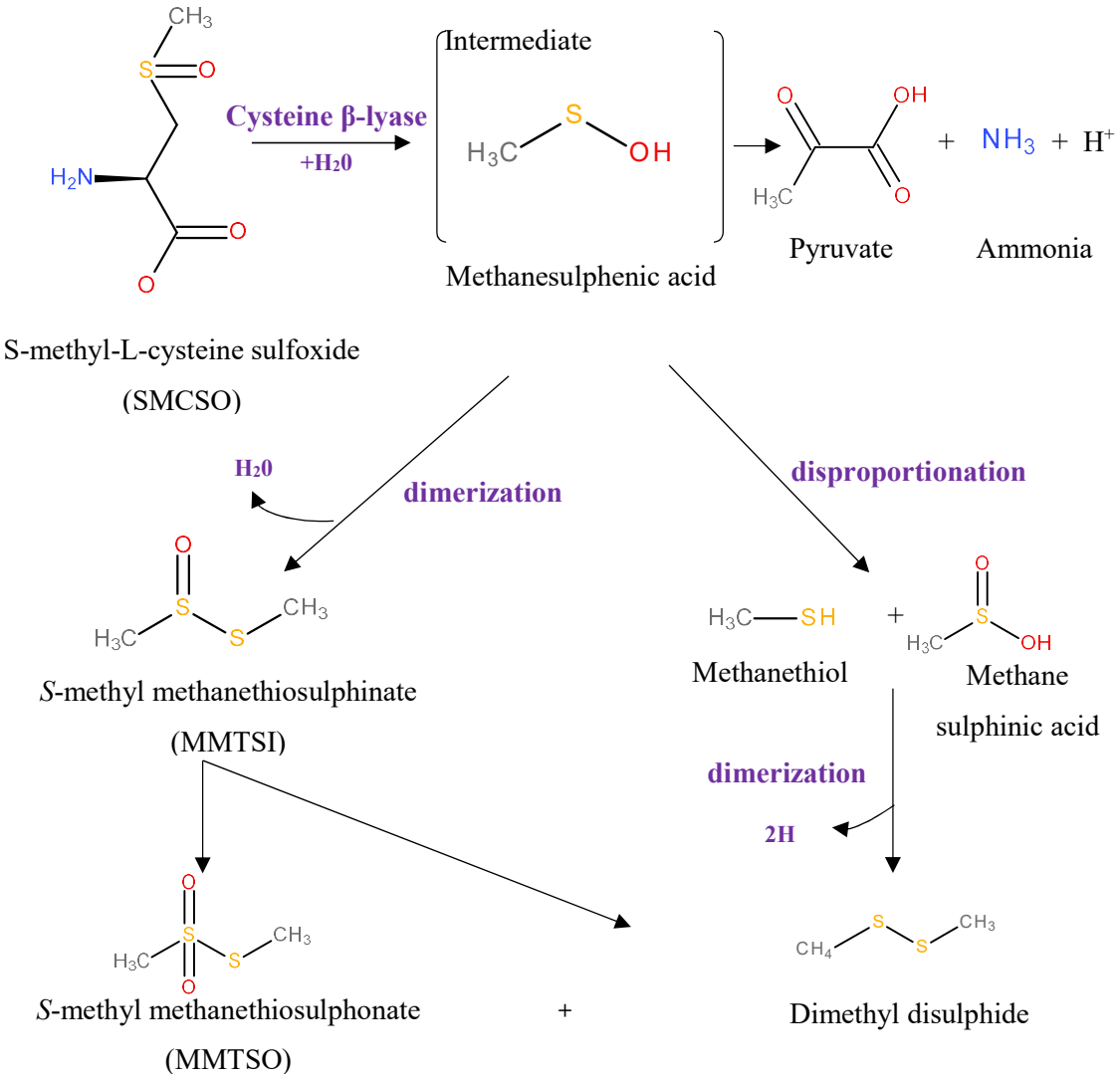


Figure 6-4: SMCSO metabolism by cysteine β-lyase to form breakdown products. Re-drawn from [2] with permission of The Royal Society of Chemistry (RSC) .

Nonetheless, metabolism of SMCSO in humans remains unclear due to limited research. An alternative metabolism route is via the gut microbiota. There are indications that SMCSO can undergo metabolism by the gut microbiota, as reported by Schwiertz *et al.* [42] and Dr Lee Kellingray (unpublished data from PhD thesis); however, the underpinning reactions and metabolite formation from this conversion remain unclear.

One final point to consider is that the process of cooking the soups in the microwave may result in thermal degradation of SMCSO and thus the actual amount administered may be much less than assumed. Further, physiological temperatures of 37ºC may also result in degradation of SMCSO, thus resulting in a lower amount reaching the gut. This has not been investigated as of yet but may be a significant factor.

There is very little evidence on the biological activity of SMCSO. In the current study, SMCSO presented no changes in Nrf2-regulated genes or viability of cells in HepG2. This could be due to the fact that SMCSO is an inactive phytochemical in its form like GR. It is possible that either MMTSI or MMTSO or other unknown metabolites from the breakdown of SMCSO in human exhibit biological activity as opposed to SMCSO. There is clear evidence to warrant further investigation to elucidate the metabolism of SMCSO by the gut microbiota and the biological activity of its metabolites.

Chapter 4 has provided novel data on the potential mechanisms of transport of GR and SMCSO through cell-free models and human intestinal cells. However, further work is required to elucidate their transport mechanism at the molecular level. The current work corroborates the widely-accepted hypothesis that SF passively diffuses across the enterocytes and confirms that neither GR or SMCSO are able to do likewise. However, as reported in **Table 6-4**, the transport of all three compounds were low in comparison to the independent paracellular marker, Lucifer yellow. This suggests that the transport was either through ‘gaps’ in the monolayer due to poorly formed tight junctions, via diffusion or active transport. It is unlikely that GR and SMSCO were passing through ‘gaps’ because all experiments were carried out using high quality cell monolayers as confirmed by the measured TEER values; however, it cannot be excluded because a 100% monolayer is not realistically achievable in laboratory conditions. On the other hand, the low permeability of GR and SMCSO could be justified by their inability to diffuse through the gap junctions that allow the diffusion only to certain compounds on the basis of on molecular weight, size, charge, structure and interactions of with the gap junction protein [191]. Lucifer yellow is known to permeate through the tight junctions [192], however, data obtained from the transport experiments of the tests compounds and Lucifer yellow permeability did not show any correlation suggesting that the transport of test compounds was not related to the transport of Lucifer yellow.

Furthermore, it was hypothesised that GR and SMCSO could permeate across membranes via active transport using membrane transporters. Taking into consideration the structural characteristics of GR, it could be hypothesised that GR might permeate across cell membranes using glucose transporters, either GLUT-2 or SGLT1, due to the presence of a glucose moiety in its structure. Similarly, given that SMCSO is derived from amino acids, it was hypothesised that it may permeate using amino acid transporters. This theory is further supported by Song *et al.* who investigated the transport of S-methyl-L-methionine, derived from *Brassicaceae* [164]. The authors have demonstrated that SGLT1 and Pgp-1 were not involved in the transport of S-methyl-L-methionine, but amino acid transporters were suggested as potentially responsible for its transport.

Once passively diffused across the enterocytes, given its electrophilic nature, SF binds to other proteins but most importantly conjugates to glutathione. Approximately 95-98% of SF was reported to accumulate as SF-GSH intracellularly and 2-5% as free SF of the initial dose used for the treatment of mouse liver cells [30]. This corroborates the findings from the study presented in **Chapter 4** as ~3% of the SF added to the apical chamber was measured in the basolateral. Free SF can passively diffuse out or it can be exported as SF-GSH conjugate using MRP-1 and Pgp-1 transporter [7,27,31]; however, SF-GSH was not measured in the study described in **Chapter 4** due to technical limitations. Interestingly, the low percentage of GR and SMCSO transport observed *in vitro* supports the data from obtained from the human intervention study (BOBS study).

Table 6-4: Transport of test compounds from the AP chamber to the BL chamber at 120 minutes, 37ºC.

	Test compounds		
	SF	**GR**	**SMCSO**
	Mean ± SD	**Mean ± SD**	**Mean ± SD**
Percentage of transport from AP→BL	2.92 ± 0.47	1.07 ± 0.06	0.57 ± 0.24

6.4. Limitations to the research

While specifically designed *in vivo* studies provide pharmacokinetic information such as the absorption, metabolism, distribution and excretion of dietary food/drugs, it is crucially important to carry out *in vitro* studies in order to elucidate their molecular mechanism of action. As *in vitro* studies are undertaken using isolated cells from their biological environment, they may behave differently under laboratory conditions. Consequently, it is difficult to translate data from *in vitro* studies into *in vivo* due to the complexity of human biology. Hence, the experimental work described in this thesis was carried out by using a combination of *in vivo* and *in vitro* models to provide information on the bioavailability of SMCSO, GR and SF in humans, and further mechanistic understanding in cultured cells. The main limitation of the research looking at the health benefits of broccoli-rich diets is the use of high concentrations of GR or SF that are not achievable in habitual diet by the general population. However, in this study, novel soups with higher levels of glucosinolates were successfully developed.

However, there are limitations to the research presented in this thesis. On reflection, given that there is a possibility that SMCSO could undergo possible thermal degradation, analysing sub-samples from soups after heating prior to participant consumption would have been valuable in determining the levels of SMCSO consumed by participants during the study. The percentage of excretion of SMCSO is based on the assumptions of the levels of SMCSO consumed prior to heating the soup; it does not take into account thermal degradation of SMCSO thus potentially under-estimating the percentage of excretion. Although a gold standard trial design was employed in this study, there were some limitations including absence of faecal collection and 48-72-hour urine sample collection. Even though Waring *et al.* reported only <2% of [^{35}S] excreted via faeces following intake of [^{35}S]-SMCSO ingestion in the form of a capsule [84], collection of faecal samples would have given an insight into the percentage of SMCSO excretion following soup consumption, a different matrix. The inclusion of faecal collection as part of the BOBS study protocol would have also provided useful information regarding excretion of GR and SF. Furthermore, one of the most challenging aspects of undertaking *in vitro* studies is choosing the concentration of the test compounds that are physiologically relevant. Whilst this study provides information on SF, GR, and SMCSO levels achievable *in vivo*, it does not reflect the concentrations that reach the liver prior to first-pass metabolism. A hepatic cell line (HepG2) was used for investigating the biological activity of SMCSO at the concentrations measured *in vivo* following consumption of the broccoli soups. However, the concentrations measured in the plasma reflect the levels distributed around the body following first-pass metabolism. The ideal sample collection to

gain such information would involve collection of blood samples from the hepatic portal vein which would give a representation of SMCSO concentrations achieved in the liver. However, such study has a high level of complexity and ethical issues would require serious consideration. Pharmacokinetic mathematical models to determine liver levels could be explored in future studies.

Additionally, there were drawbacks from the *in vitro* studies. The main limitation of the caco-2 studies in **Chapter 4** was the use of Lucifer yellow as an independent integrity marker. In this particular case, Lucifer yellow with higher permeability than the test compounds may have not been a suitable paracellular marker. However, this information was not available prior to the study. This has limited the comparison of the data obtained from the current study with published data. In contrast, the main issue in Chapter 5 was not further exploring the potential thermal degradation of SMCSO that would inform the experimental design in terms of the appropriate concentrations to be tested in HepG2 cells. Perhaps, the lack of activity of SMCSO in modulating Nrf2-regulated gene expression could be due to the SMSCO degradation during the incubation time.

6.5. Future research

The main outcome of this thesis is the discovery that SMCSO appears to be bioavailable in humans. This has led to further questions beyond the scope of this thesis, notably the question of how SMCSO is metabolised in humans and whether its biological activity could lead to significant health benefits. To date, there is very little experimental evidence which might contribute to a better understanding of the data reported in this thesis. A previous report has provided some information on the bioavailability of SMCSO by administrating radiolabelled SMCSO in humans, and measuring its recovery as ^{35}S and sulphate [84]; however the endpoint of radioactivity implies that SMCSO underwent metabolism. Although there is some speculation that either the gut microbiota or human cysteine β-lyase can catalyse SMCSO, neither has been previously demonstrated in humans. In addition, it may undergo thermal degradation either during the cooking process or at body temperature. At present, we know that (i) cruciferous vegetables have high levels of SMCSO, which is bioavailable in humans and (ii) it does permeate across the enterocytes to reach the systemic circulation.

The first question concerns whether SMCSO breaks down during the cooking process or at physiological temperatures. It has been demonstrated that SMCSO with the addition of a purified cysteine lyase undergoes degradation at 35ºC [41] or at 80ºC without the addition of the cysteine lyase [190]. However, further stability studies will need to be undertaken to

measure the levels of SMCSO at different time-points throughout the cooking process. In addition, it would be informative to investigate the effect of incubating synthetic SMCSO at various temperatures and monitor its stability.

The second question is whether the gut microbiota can metabolise SMCSO and what are the metabolic products. Dr Lee Kellingray demonstrated reduction of SMCSO to S-methylcysteine by an *E.coli* strain which also reduced GR (unpublished data). Further work will provide information on whether other gut bacteria can metabolise SMCSO. This could be achieved by adding a cocktail of gut microbiota, especially those known to have presented with cysteine β-lyase activity [42] to a sample of homogenised soup to the colon gut model described by Macfarlane *et al.* [193], which could be a suitable method in combination with analytical techniques for the analysis SMCSO end-products such as pyruvate and other known metabolites **(Figure 6-4)**. Target metabolites can be analysed using LC-MS/MS or GC-MS, whereas unknown metabolites can be measured using NMR. Furthermore, the target metabolites such as MMTSO and MMTSI and other metabolites indirectly derived from the metabolism of SMCSO in plants could be measured in the samples collected from the BOBS study. This will give an idea of whether MMTSO and MMTSI are formed from the metabolism of SMCSO in humans. However, the development of novel analytical methods will be required.

The third question is what metabolic pathways are modulated by SMCSO or its metabolites. While the current study demonstrated that SMCSO did not up-regulate Nrf-2 regulated genes, its potential biological activity cannot be dismissed. Perhaps SMCSO influences protein expression or the activity of enzymes rather than the expression of genes. Kumari and Augusti has previously reported a significant reduction in G6PD enzyme activity in rats on a SMCSO diet [137]. To follow on from the current work presented in this thesis, the protein expression of Nrf2-regulated genes can be investigated, or the enzyme activity of G6PD can be assessed using an assay kit following treatment with SMCSO. In addition, other researchers have shown that MMTSO and MMTSI are able to inhibit proliferation of cells, implying that biological activity associated with SMCSO may be due to its secondary metabolites [130,131]. The same experimental design as described in **Chapter 5** can be undertaken with MMTSO and MMTSI to investigate whether these metabolites can influence Nrf2-regulated genes in liver cells.

The experimental work on transport mechanisms **(Chapter 4)** has demonstrated interesting outcomes regarding the permeability of sulphur compounds in the gut. While the data is not conclusive, with GR and SMCSO potentially permeating via 'gaps' in the monolayer, there was a considerable difference in the transport of these compounds when incubated at 37ºC and 4ºC, indicating transport through cells via active transport. In addition, there is a possibility

that these compounds may diffuse through the tight junctions. To further elucidate this, inhibitors of active transporters and tight junctions may be used. In the case of GR, it was hypothesised that, due to the presence of a glucose moiety, it may permeate through either GLUT2 or SGLT1, both present on the apical side of the intestinal membrane [194]. Inhibition of either GLUT2 with phloretin **(Figure 6-5 A)** or SGLT1 with phlorizin **(Figure 6-5 B)** may provide further insight into whether GR permeated across these specific transporters [157,165]. With regards to SMCSO, given its structural similarity with amino acids, it was suggested that SMCSO may use an amino acid transporter. This is a potentially complex avenue of research because of the wide range of amino acid transporters [195,196]. It was reported that mercury bound to cysteine permeated across intestinal cells using amino acid transporters b0,+ and B0,+ [196]. Further investigation of whether SMCSO uses either amino acid transport systems or others could be investigated by knocking out the transporters using siRNA [196]. Finally it is important to highlight that the use of inhibitors such as quinidine and carbenoxolone or silencing the tight junctions [197] could be an effective experimental route in order to exclude the possibility that SMCSO and GR permeability reported in the current study is not due to their transport through gap junctions.

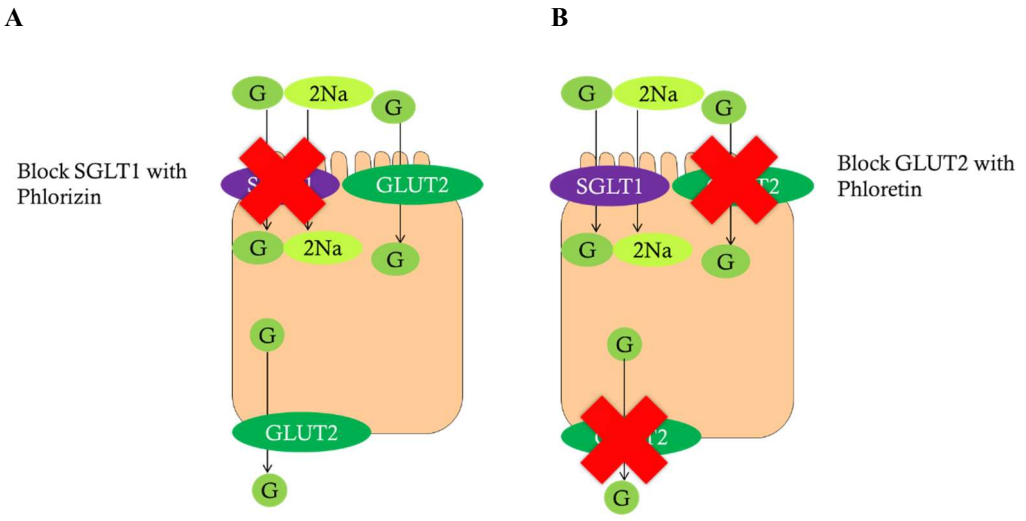


Figure 6-5: Inhibition of glucose transporters on the AP and BL membrane of enterocytes. SGLT1, a sodium-glucose transporter found on the apical membrane is inhibited by phlorizin (A) whereas GLUT2 found on the apical and basolateral membrane is inhibited by phloretin.

6.6. Future impact

Whilst the production of sulphur compounds in these novel broccoli varieties was already known in the field, their bioavailability in humans following consumption of soups made from the varieties was unknown. This study has provided the first evidence for dose-dependent bioavailability of beneficial sulphur compounds from novel broccoli soups.

There are still many unanswered questions regarding the epidemiological findings and the underpinning mechanisms of SF. The current study has provided valuable information on the physiological concentrations of SF that are achievable, following consumption of the novel broccoli varieties. Further work is being undertaken in the Mithen group using the concentrations of SF from the current study to understand the underlying mechanisms.

Currently the novel broccoli variety Myb28B/V, which is commercially available as 'Beneforte®', does not have any health claims associated with the product as there is not currently enough evidence from long-term studies to legally make such claims. Whilst this study is not sufficient for any health claims, it is an important step in the right direction. Companies currently selling 'Beneforte®' would see this research as progress. A product with associated health claims may be more attractive to consumers and by extension more attractive to retailers.

If the novel broccoli varieties were widely adopted, they could have wider positive implications on public health. The consumption of the enriched glucoraphanin soups would enable the population to get beneficial amounts of the bioactives investigated within this thesis without requiring the consumption of unreasonably large amounts of broccoli. A portion of the Myb28V/V soups (300g) is equivalent to five portions of broccoli florets. One can speculate that you would therefore get the same benefits from eating one portion of the soup per week as you would get from eating five portions of broccoli florets. In comparison to other similarly sized meals (in terms of calorific value), the broccoli soups would provide substantial quantities of the bioactives whilst not affecting the nutrient composition. A portion of the novel broccoli soups provides 9% energy, 16% fat, 22% saturated fats, 4% sugars, 16% salt based on the reference intake **(Section 6.6, Annex 2, p286)**, which is comparable to commercially available broccoli and stilton soups.

6.7. Conclusion

In conclusion, the data presented in this research emphasises the use of the novel broccoli-rich product as a suitable intervention diet. There is ongoing research showing the potential health-

benefit of consuming broccoli-rich diets against prostate cancer [92]. The broccoli soups tested in the current study could be an effective interventional strategy in a long-term trial recruiting men with organ confined prostate cancer to investigate whether this dietary approach may reduce cancer progression. Currently, two human intervention studies (ESCAPE study, clinical trials.gov: NCT01950143 and SAP study, clinical trials.gov: NCT02821728) recruiting prostate cancer patients are looking at the effect of broccoli-derived bioactives by using the soups well-characterised as part of this PhD project. Data obtained from this study will be very informative for the ongoing trials and will help the interpretation of their findings. Further, this study demonstrates another approach for delivering high levels of the bioactives, glucosinolates and SMCSO without unrealistic intake of cruciferous vegetables through our habitual diet. Further, this demonstrates that the soups with increasing GR levels results in increasing plasma levels and urinary excretion of SF and GR, and very importantly SMCSO is bioavailable. This is the first intervention study to report to bioavailability of intact GR and SMCSO. While it has been demonstrated that glucosinolates including GR do not exert biological activities in their form but only after conversion to ITCs, further research is needed for better understanding of the metabolism of SMCSO and its biological activity.

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Appendix I- Human study documents

IFR Institute of Food Research

An intervention study to assess the bioavailability of sulforaphane delivered by glucoraphanin-enriched broccoli soups in healthy subjects

Short title: The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

PROTOCOL

Version 3:

Chief Investigator:

Prof Richard Mithen

Investigators:

Miss Tharsini Sivapalan

Dr. Antonietta Melchini

Dr. Charlotte Armah

PROTOCOL SIGNATURE PAGE

Title: An intervention study to assess the bioavailability of sulforaphane delivered by glucoraphanin-enriched broccoli soups in healthy subjects

Version 3; 10th November 2014

Short title: The bioavailability of sulforaphane from broccoli soups (BOBS)

Sponsor's Approval:

This protocol has been approved by The Institute of Food Research's Human Research Governance Committee (HRGC)

Signature_____ Name_____

Role_____ Date_____

I have fully discussed the objectives of this trial and the contents of this protocol with the Sponsor's representative. I understand that the information in this protocol is confidential and should not be disclosed other than to those directly involved in the execution or ethical review of the trial.

I agree to conduct this trial according to this protocol and to comply with its requirements, subject to ethical and safety considerations and guidelines, and to conduct the trial in accordance with International Conference on Harmonisation (ICH) guidelines on Good Clinical Practices (GCP) and with the applicable regulatory requirements.

Chief Investigator name and address: Professor Richard Mithen,

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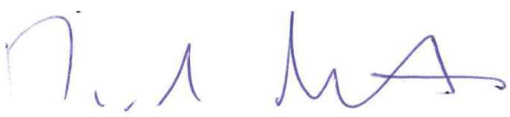

Signature:

Date: 10/11/14

Table of figures

List of annexes

Annex 1 Letter of invitation from HNU database

Annex 2 Participant Information Sheet (PIS)

Annex 3 Advertisement Poster

Annex 4 Letter of invitation from advertisement

Annex 5 Appointment cards

Annex 6 Consent Form

Annex 7 Medications declaration form

Annex 8 Eiligibility screening questionnaire

Annex 9 Case report form (CRF)

Annex 10 Clinical Results to GP

Annex 11 GP letter with results

Annex 12 List of ITC containing foods

Annex 13 Exclusion letter

Annex 14 Letter to GP informing of participants involvement in the study

Annex 15 GP letter with study description

Annex 16 Apology letter to participants

Annex 17 Appointment letter

Annex 18 Food choices for study day

Annex 19 GP letter informing participant exclusion due to blood pressure measurement

Annex 20 Consent form to be contacted for future studies

Annex 21 Soup information sheet

Contents

Executive Summary

ABSTRACT: There is a large body of evidence suggesting that the consumption of cruciferous vegetables such as broccoli is beneficial to our health and is associated with a reduced risk of different types of cancer. Cruciferous vegetables are able to deliver in our body a group of compounds called isothiocyanates (ITCs) that are thought to be responsible of their health-promoting effects. Sulforaphane (SF) from broccoli is one of the most studied ITCs and its anticancer properties have been extensively investigated in *in vitro* and *in vivo* models. We are currently undertaking a 12- month intervention to determine whether a diet rich in SF will result in changes in genes and metabolites within prostate tissue in a way that could reduce the progression of prostate cancer (ESCAPE, REC Ref Number 13/EE/0110). The ESCAPE intervention involves weekly consumption of broccoli + stilton soups made with standard broccoli and two high-glucoraphanin (SF precursor) broccoli varieties able to deliver different levels of SF. We now propose to undertake an intervention study to measure the bioavailability of SF from the soups used in the ESCAPE study in terms of rate and extent to which SF reaches the systemic circulation and is excreted in urine. We will measure SF and its metabolites in plasma and urine samples collected from apparently healthy subjects after consumption of the three types of broccoli + stilton soups.

PRIMARY AIM:

To measure the total excretion of SF in urine collected for 24 hours after consumption of one pot (300g) of three types of broccoli + stilton soup containing different concentrations of glucoraphanin, SF precursor.

SECONDARY AIMS:

To measure SF and its metabolites in plasma following consumption of one pot (300g) of three types of broccoli + stilton soup containing different concentrations of glucoraphanin.

To determine whether the glutathione S-transferase Mu 1 (*GSTM1*) genotype or other associated genotypes (e.g. GSST1) influence SF plasma pharmacokinetic parameters (C_{max}, T_{max}, area under the curve) and its total urinary excretion.

STUDY POPULATION: This study will recruit apparently healthy subjects aged 18-65 years with a BMI between 20 and 35 kg/m^2 (men and women). Recruitment will continue until ten participants have completed the study.

OUTLINE STUDY DESIGN:

The study outline is presented in ***Figure 1***. This study will be a randomized, double-blinded, three-phase crossover trial which will investigate the bioavailability of SF following consumption of broccoli + stilton soup containing different concentrations of glucoraphanin. The following soups will be tested in the three phases: i) 300g standard broccoli + stilton soup, ii) 300g Beneforte® broccoli + stilton soup, iii) 300g Beneforte Extra broccoli + stilton soup. Participants will undergo three phases separated by a minimum of two weeks (wash-out period). Each phase will consist of a 48 hour pre-intervention diet restriction, a study day involving an 8 hour cannulation at the Human Nutrition Unit (HNU) of the Institute of Food Research (IFR) and collection of a 24 hour urine and blood sample the following morning.

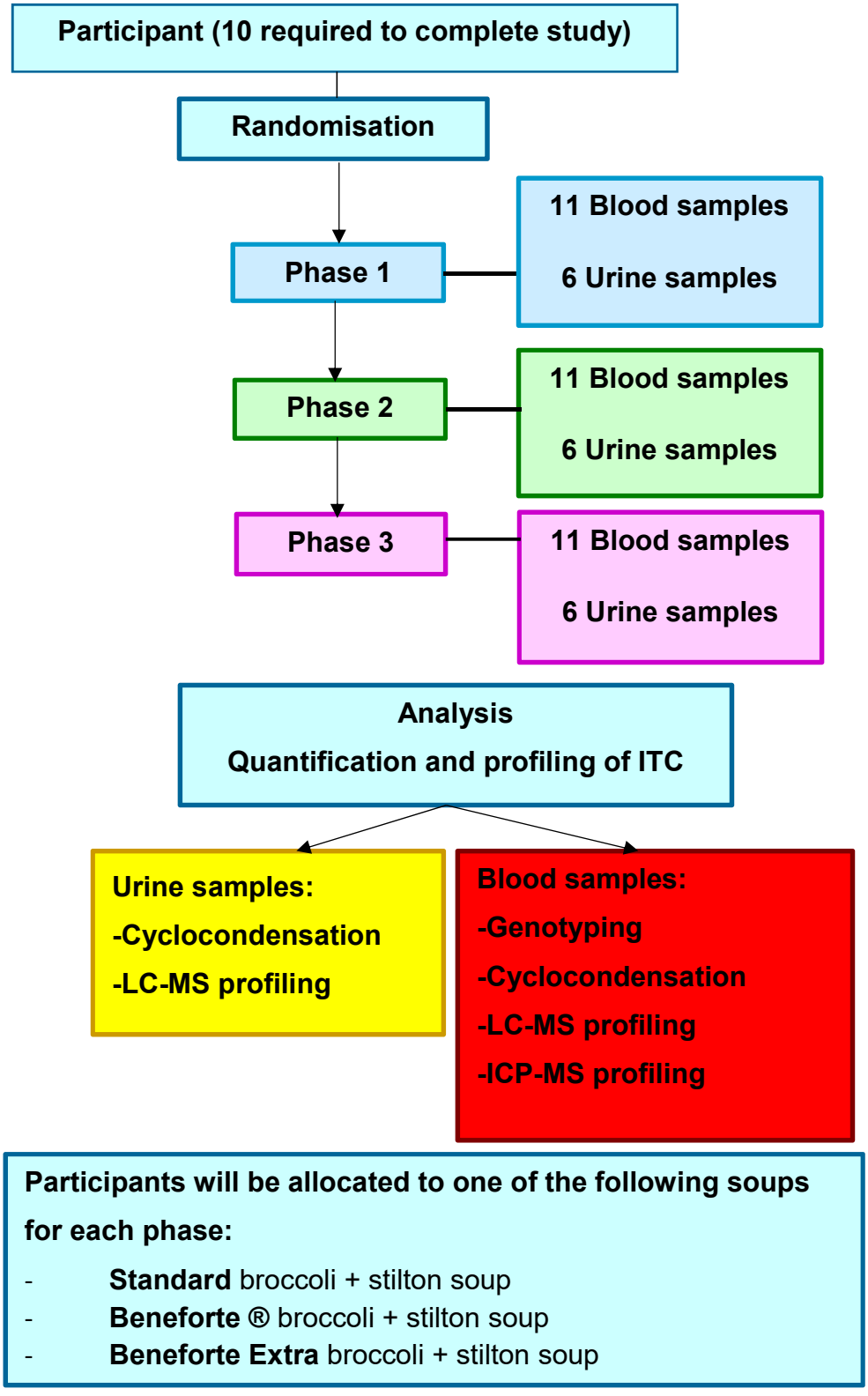

Figure 1: *Study outline*

1. Scientific background

There are many epidemiological studies that have reported reduction in the risks of cancers such as ovarian, pancreatic, breast and prostate with the consumption of cruciferous vegetables. Epidemiological studies from US, Singapore and China suggest that diets rich in cruciferous vegetables, e.g. broccoli, may reduce the risk of prostate cancer [91,92]. Studies have shown that an intake of one or more portions of broccoli per week can reduce the incidence of prostate cancer and also the progression from localised to aggressive forms of cancer.

1.1. Metabolism of glucosinolates

Cruciferous vegetables accumulate a group of compounds called glucosinolates which are sulphur containing phytochemicals. As well as containing glucosinolates, the broccoli florets also contain the thioglucosidase, myrosinase. The myrosinase is spatially separated from the glucosinolates, however when the plant structure is mechanically compromised, the plant thioglucosidase, myrosinase comes into contact with the glucosinolates [198] and catalyses the hydrolysis of the glucosinolates to isothiocyanates (ITCs) ***[Figure 2]***. In addition to the myrosinase, the glucosinolates can be hydrolysed by human gut microbiota with myrosinase-like activity ***[Figure 3]***.Glucoraphanin is the main glucosinolate naturally found in broccoli, which is catalysed to the ITC sulforaphane (SF).

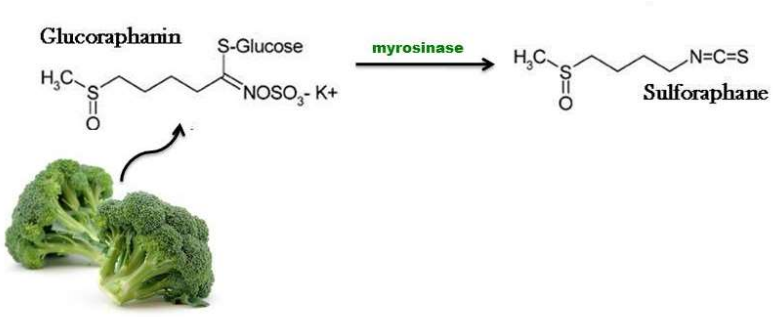

Figure 2: *Structure of the main glucosinolate in broccoli, Glucoraphanin and its isothiocyanate Sulforaphane*

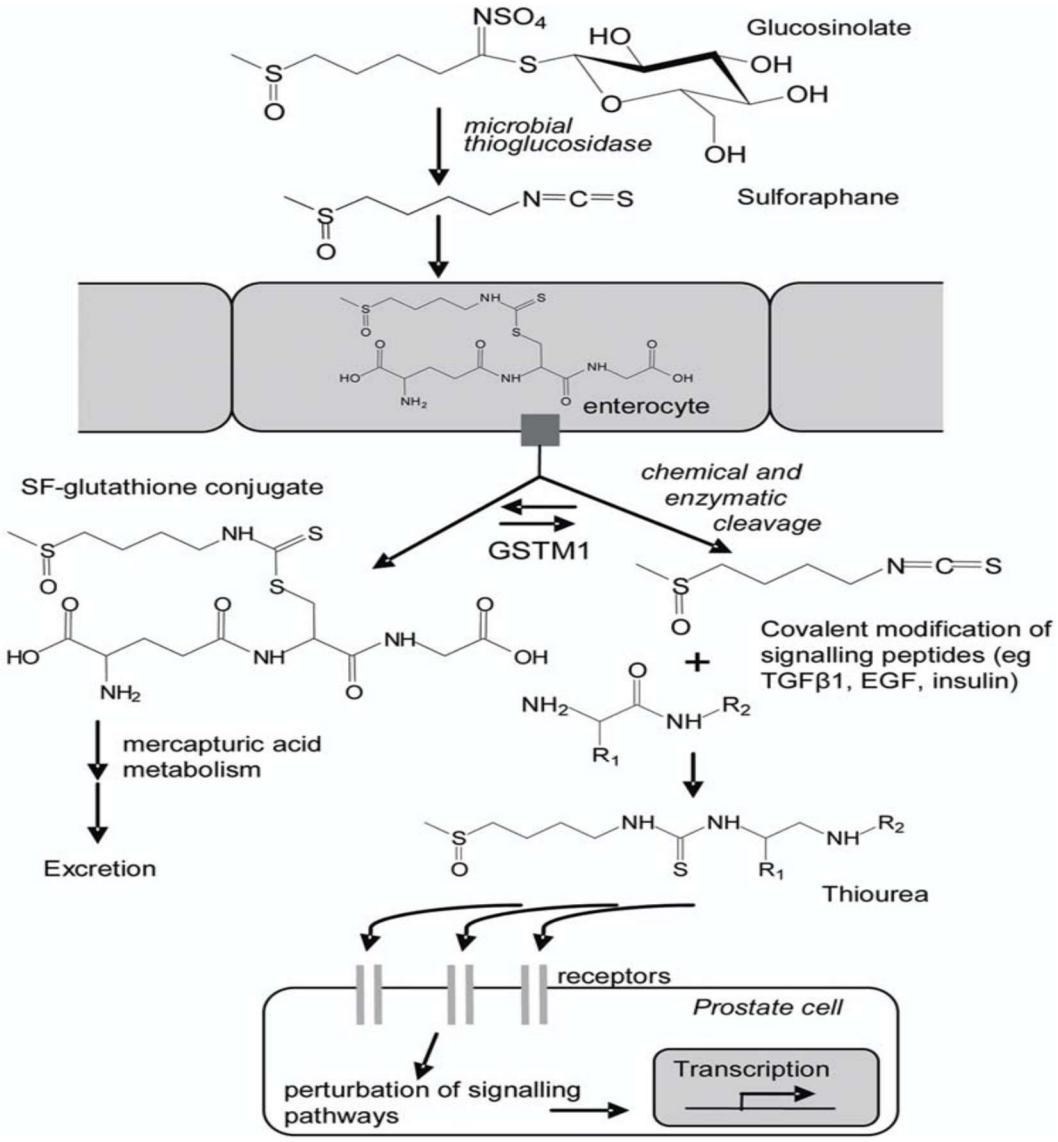

Figure 3: *Glucosinolate metabolism [37]*

1.2. Metabolism of sulforaphane

ITCs such as sulforaphane (SF) are bioactive compounds that exhibit anti-cancerous activity associated with cruciferous vegetable consumption. Overcooking of broccoli denatures the myrosinase enzyme so the glucoraphanin enters the gut where it is hydrolysed to SF by gut thioglucosidase. SF is then absorbed by the enterocytes, conjugated with glutathione via glutathione-S-transferases (GSTs) and transported into the systematic circulation to be metabolized by the mercapturic pathway ***[Figure 3]***. It is then excreted as N-acetylcysteine conjugates in the urine [37,38].Glutathione transferases (GSTs) are dimeric enzymes that catalyse the conjugation of glutathione

(GSH) to xenobiotics, e.g. ITCs or endogenous compounds, to facilitate their metabolism and excretion. There are three mammalian GSTs: cystolic, mitochondrial and microsomal. It is the *GSTM1*-1 and GSTP-1 class of GSTs that usually catalyse the conjugation between glutathione and SF [38].Approximately 45% of consumed SF occurs as free SF in the plasma with the rest occurring as thiol conjugates. The peak concentration of SF and its thiol is 2μm, which decreases within one hour [37].

1.3. *GSTM1* Genotype

The protective effects of SF appears to be linked with the *GSTM1* (glutathione S-transferase mu 1) genotype. One major *GSTM1* polymorphism refers to the presence or absence of the gene. Individuals with a homozygous deletion in the *GSTM1* gene (*GSTM1* -/-), termed as *GSTM1* null, do not have a functional *GSTM1* protein accounting for 39-63% of the population. On the other hand those with two *GSTM1* intact alleles or a heterozygous deletion in the *GSTM1* gene (*GSTM1* -/+ or *GSTM1* +/+) are classified as *GSTM1* positives [38]. Epidemiological studies reported that broccoli consumption provides greater protection for *GSTM1* positive individuals than *GSTM1* null individuals. It is thought that the *GSTM1* enzyme may catalyse the dissociation of the SF-thiol conjugates [38].

1.4. Beneforte® development

Broccoli contains approximately 6-9 μmol g^{-1} dry weight of glucosinolate in their florets [18]. A new variety of broccoli has been developed over the last twenty years by our research group delivering a threefold higher concentration of SF than standard broccoli because it accumulates 2.5-3 fold higher levels of glucosinolate in its florets [18]. This specific broccoli is now marketed under the trademark name Beneforte® broccoli which is also known as super broccoli. It is the F1 hybrid formed from the cross-breeding between a standard broccoli and a wild species broccoli known as *B.villosa* [17,18] that contains gene segments from *B. villosa* introgressed into the gene pool of the standard broccoli [18].

1.5. Bioavailability studies

Bioavailability is a term which describes the amount of a nutrient that is absorbed into the systemic circulation and becomes available at target tissues from the consumption dose.

In this project we propose to undertake a bioavailability study looking at the bioavailability of sulforaphane from three types of broccoli + stilton soups. This study design is based on two other similar bioavailability studies: a study by Gasper et al 2005 and a study by Saha et al 2012.

One of the studies was a randomised, two-phase crossover study undertaken to measure the bioavailability of SF from fresh versus frozen broccoli. The study consisted of two 5-day test phases with a washout period of 7 days (LREC 2003/082). Blood and urine samples were collected from 18 apparently healthy participants after they were fed 100g of fresh broccoli and 100g of frozen broccoli over the two phases. Participants were recruited based on their *GSTM1* genotype in order to achieved balanced groups [68].The other study was a randomised, three-phase crossover study with a washout period of 21 days, which recruited 16 participants. This study fed participants broccoli soup, super broccoli soup and water (reference E/10/2003). This study also recruited an equal number of participants with *GSTM1* positives and nulls [38].

In this proposed study, the design will be that of a three-phase crossover similar to Gasper et al (reference E/10/2003) with the blood and urine collection timepoints similar to that of Saha et al. One of the main differences between the proposed study and the two studies listed above is the study soup. Saha et al 2012 used 100g of fresh or frozen broccoli with 150g of water, microwaved for 75s and blended to create a soup. Gasper et al 2005 cooked 100g of florets with 150mL of water, microwaved for 90s then blended to create a soup. The light cooking of the broccoli in the Gasper study ensured that the activity of myrosinase enzyme was still preserved. In the study by Saha et al, the activity of the myrosinase enzyme was still preserved in the lightly cooked fresh broccoli. However, the myrosinase was inactivated in the lightly cooked frozen broccoli due to the process of blanching first before freezing the broccoli

florets. The soups used in this study are made and frozen by a food company so the myrosinase is inactive. Also the soups are in a form that can be made commercially available and thus more palatable than blended luke warm broccoli in water.

The studies also differ in the concentration of SF and glucaraphanin delivered by the soups. In the Gasper study 15 µmol and 52 µmol of SF; in the Saha study 23.5 µmol of SF and no glucoraphanin in the fresh broccoli soup and 42.5 µmoles of glucoraphanin in the frozen broccoli soup.

In this current study, the concentrations of glucoraphanin are:

- 43 µmoles in standard broccoli + stilton soup,
- 180 µmoles in Beneforte® broccoli + stilton soup
- 442 µmoles in the Beneforte Extra broccoli + stilton soup.

The proportion of glucoraphanin per frozen weight of broccoli soup in the Saha study is similar to that in this cannulation study (42.5 µmoles per 236g soup and 43 µmoles per 300g in the standard broccoli +stilton soup respectively). Following consumption of the soup made from frozen broccoli, SF conjugates were not detected in plasma until 1 hour after consumption and peaked at 6 hours and rate of excretion peaked during the 4-6 hours urine collection. Because the SF conjugates appeared later, participants will be cannulated in this study for 8 hours and blood will be collected at the following time points post soup consumption: 0, 30, 45, 60, 90, 120, 180, 240, 360, 480 mins and 24 hours. In addition, urine will be collected at the following timepoints: 0, 0-2, 2-4, 4-6, 6-8, 8-24 hours even though the rate of excretion peaked during the 4-6 hours. The early timepoints will be necessary for the additional sulphur analyses and for the other two soups that will deliver a higher concentration of glucoraphanin.

Due to the results from the study by Saha et al 2012, where there were no significant difference in the plasma levels or total urinary excretion between *GSTM1* nulls and *GSTM1* positives, participants will not be selected for this criteria. However, 5ml from the 15ml blood sample that will be collected from each participant at the eligibility screening will be used for genotyping purposes, which will be used to justify any outliers that may be seen in results from this study.

1.6. Use of broccoli soups in studies

In our research group, we are currently undertaking two intervention studies to investigate the beneficial effects of a diet rich in SF on prostate cancer (ESCAPE, REC Ref Number 13/EE/0110; ESCAPE-ING, IFR HRGC Ref Number IFR02/2014). The ESCAPE and ESCAPE-ING studies are double-blinded dietary interventions recruiting patients with early prostate cancer on active surveillance. Participants are asked to consume, for the duration of the study one portion per week of broccoli soups made with different broccoli varieties (standard, Beneforte® and Beneforte extra broccoli) delivering increasing levels of SF. Plant breeders at Seminis developed the Beneforte® broccoli cultivar, which is subject to very stringent quality control and is now widely commercialised in US and several European countries, including the UK. The breeders have developed another broccoli cultivar, Beneforte extra, which has almost double the amount of glucoraphanin compared to Beneforte® broccoli. Beneforte® and Beneforte extra have been developed by conventional breeding. Data obtained by analysing the study soups used in the ESCAPE and ESCAPE-ING studies have shown that one pot of the Beneforte extra broccoli soup (300g) contains approximately 442μmoles of glucoraphanin

In this study, we propose to undertake an intervention with standard broccoli + stilton, Beneforte® and Beneforte extra broccoli + stilton soups to measure the bioavailability of SF in apparently healthy subjects. Data obtained from this study will provide valuable information for our ongoing studies.

2. Hypothesis

Consumption of the Beneforte Extra broccoli + stilton soup will result in a higher excretion of SF and its metabolites in urine compared to Beneforte® and standard broccoli + stilton soup.

3. Aims

- To measure the total excretion of SF in urine collected for 24 hours after consumption of one pot (300g) of three types of broccoli + stilton soup containing different concentrations of glucoraphanin, SF precursor.

- To measure SF and its metabolites in plasma following consumption of one pot (300g) of three types of broccoli + stilton soup containing different concentrations of glucoraphanin.

- To determine whether the glutathione S-transferase Mu 1 (*GSTM1*) genotype or other associated genotypes (e.g. GSST1) influence SF plasma pharmacokinetic parameters (C_{max}, T_{max}, area under the curve) and its total urinary excretion.

4. Study design

This study will be a randomized, double-blinded, three-phase crossover trial **[Figure 4]** to assess the bioavailability of SF after consumption of (i) 300g standard broccoli + stilton soup (providing 43 µmoles/300g glucoraphanin), (ii) 300g Beneforte® broccoli + stilton soup (providing 180 µmoles/300g glucoraphanin), (iii) 300g Beneforte Extra broccoli + stilton soup (providing 442 µmoles/300g of glucoraphanin). The reported levels of glucoraphanin in the three types of soups come from recent analysis undertaken in February 2014.The study will be carried out at the Human Nutrition Unit (HNU) of the Institute of Food Research (IFR). Blood and urinary samples will be collected from participants following consumption of the broccoli + stilton soups with the aim of detecting plasma and urinary metabolites of SF, which are surrogate markers of bioavailability.

Each phase will consist of a 48 hour pre-intervention diet restriction, a study day (study day 1) comprising of approximately a nine hour stay at the HNU which will involve cannulation. The following morning participants will visit the HNU for up to 1 hour for collection of a single blood sample at 24 hour post-dose followed by breakfast. During study day 1, eleven 10ml blood samples will be collected over the course of the day from participants at the following timepoints: 0, 30, 45, 60, 90, 120, 180, 240, 360, 480 mins and a 24 hour blood sample. Six urine samples will be collected at the following timepoints: 0, 0-2, 2-4, 4-6, 6-8, 8-24 hours.

The three phases will be separated by a minimum of a two week washout period. This is to ensure that the veins recover from the 8 hour cannulation period for re-cannulation during the next phase. For the duration of the study, participants will be required to follow a glucosinolate-free diet for a total of nine days which will be three days per phase. The three days are split into 48 hours (2 days) prior to the study day, 24 hours (1 day) on the study day as well as the following morning until the 24 hour blood and urine sample have been collected. After the 24 hour urine and blood samples have been collected, participants can resume their normal diet for a minimum of two weeks until 48 hours prior to their next study day. This will reduce the contribution of glucosinolate from other foods having an impact on the results of the study. The involvement of the participants will last approximately 11-12 weeks.

The study will be led by Professor Richard Mithen. The study team members are Miss Tharsini Sivapalan, Dr Antonietta Melchini and Dr Charlotte Armah. Study talks, screening and study days will be carried out by the study team which includes by Miss Tharsini Sivapalan (a first year PhD student at the IFR) with the assistance of Dr Antonietta Melchini. Additional assistance with study talks, screening and study days will be provided by Dr Charlotte Armah in the absence of Dr Antonietta Melchini. All the biochemical analysis will be performed by Miss Tharsini Sivapalan with the assistance of Dr Antonietta Melchini under the supervision of Dr Shikha Saha, an IFR analytical expert. Screenings and cannulation will be performed by an appropriately trained and experienced HNU research nurse.

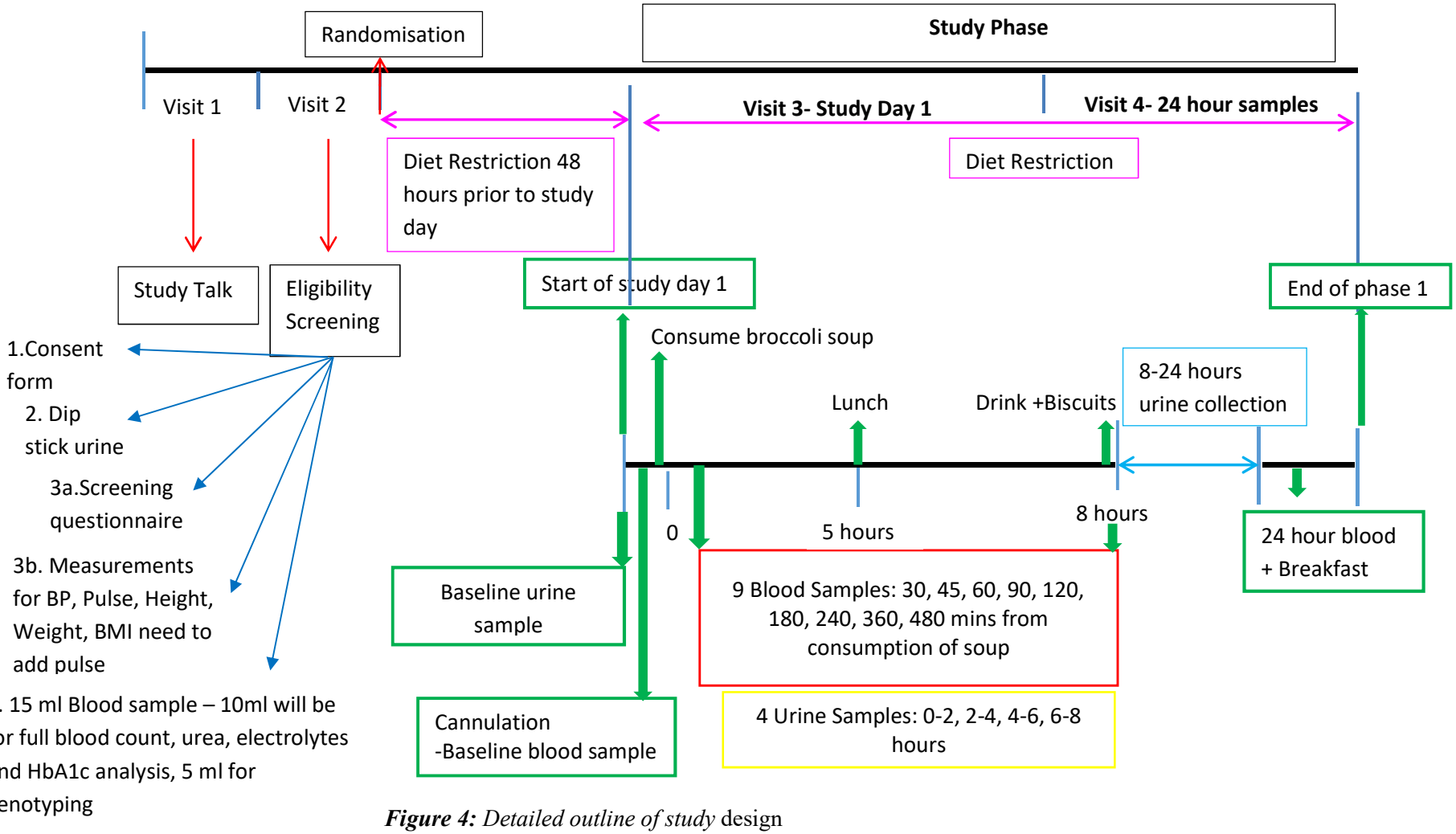

Figure 4: *Detailed outline of study* design

Each study phase will follow the same pattern as study phase after a minimum of 2 week washout

4.1. Recruitment

The aim will be to recruit apparently healthy participants aged 18-65 years old. Recruitment will continue until ten participants have completed the study. Potential participants will be recruited from the Human Nutrition Unit (HNU) volunteer database and the HNU senior research nurse will be responsible for this process. The database contains the names and contact details of local people who have previously registered an interest in participating in research studies at the IFR. Those individuals whose details are currently held on the database and who meet the basic inclusion criteria will be sent the study information pack. This will consist of a letter of invitation to take part in the study **(Annex 1)**, the Participant Information Sheet (PIS) **(Annex 2)** with a response slip and pre-paid envelope to return the response slip.

In addition to using the database for recruitment, advertisements **(Annex 3)** will be placed around the Norwich Research Park (University of East Anglia (UEA), John Innes Centre (JIC), The Genome Analysis Centre (TGAC) and IFR), and other appropriate locations for example, supermarkets and social clubs within an approx 40 miles radius of IFR.

If required, advertisements will be placed in the local newspapers; radio (and television) coverage will be obtained. Either the advertisement or a link to an IFR website containing the study advert will be placed on internet sites such as gumtree, social networking sites such as Facebook/ Twitter. The advertisement will contain contact details of members of the study team. It will be highlighted that in order to be eligible to take part in the study, participants need to live within a 40 mile radius of IFR. Those responding to the adverts will be sent an information pack consisting of a letter of invitation **(Annex 4)**, PIS, a response slip and pre-paid envelope.

Potential participants, who are interested in taking part in the study, after having read the PIS, will be asked to register their interest either by returning the PIS response slip or by contacting a member of the study team by telephone or email. When participants have registered their interest they will be contacted by a member of the study team to book an appointment to attend the HNU at IFR for a study talk.

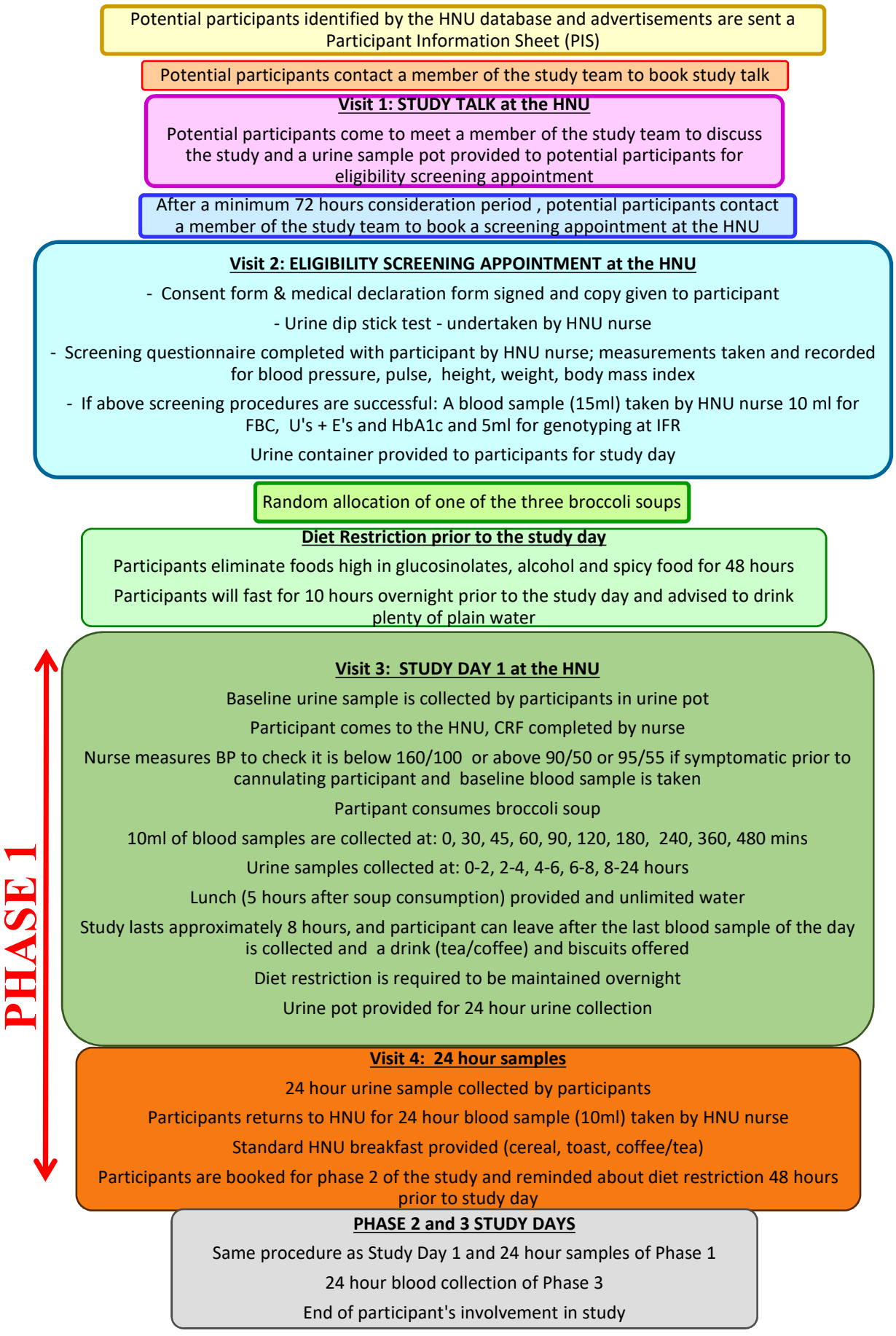

***Figure 5**: Study flowchart describing the involvement from the participants*

4.2. Study Talk (HNU-Visit 1)

During the study talk, a member of the study team will go through the PIS and explain the study fully including discussing all aspects of the participant's involvement **[Figure 5]**. The participant will be encouraged to ask any questions they have at the time. The participant will be able to speak to the HNU senior research nurse if they have any questions relating to cannulation, venepuncture or any of the clinical measurement prior to making any commitment. After the talk, potential participants will be given a minimum period of 72 hours (3 days) to consider whether they wish to participate in the study. During this period the potential participants will not be contacted. If after the 72 hour period, they still wish to participate in the study, they will be asked to contact a member of the study team to book an appointment at the HNU for an eligibility screening. At the study talk, potential participants will be provided with a container for their urine collection on the morning of the eligibility screening visit (**Visit 2**). If they do not wish to participate they will be told to discard the container.

If after the study talk the potential participant has decided to take part and request to book their eligibility screening appointment before leaving the HNU an appointment can be booked. However, this appointment will be scheduled for after a minimum of 3 days from the study talk. Any potential participants who book their eligibility screening appointment at the study talk will not be contacted during this time.

Potential participants who respond positively after the study talk will be invited to the HNU for an eligibility screening (**Visit 2**). They will be sent appointment cards with their appointment date and time and a reminder of what they need to do and bring with them for the eligibility screening **(Annex 5)**. Participants will also be reminded to bring with them details of any prescribed medication, herbal remedies or dietary supplements (i.e. name of medication, dose taken). For validity of the urine dipstick test the urine has to be tested within 2 hours of collection and therefore potential participants will be asked to collect a fresh midstream urine sample within 2 hours of their screening appointment. If the potential participant forgets to bring their urine sample or the urine sample provided is more than 2 hours old they will be provided with the opportunity to produce another sample at the HNU.

4.3. Eligibility Screening (HNU-Visit 2)

Potential participants are assessed for eligibility based on a set of specific inclusion and exclusion criteria, an eligibility questionnaire, anthropometric measurements (Blood pressure, pulse, weight, height and Body Mass Index (BMI)) and results of clinical laboratory tests (Combur 9 urine dipstick test and screening blood results (FBC,U/E and HbA1c)).

On the day of the eligibility screening at the HNU, potential participants will arrive at the HNU. They will be met by a member of the study team and taken into a confidential room where a member of the study team (Tharsini Sivapalan and Dr Antonietta Melchini) or the HNU senior research nurse will go through the consent form **(Annex 6).** Potential participants will be encouraged to ask any questions they have at this stage about the study and their involvement after which they will be asked to sign the consent form agreeing to participate in the study and to all their clinical results being sent to their GP. In addition they will be asked to sign a medications declaration form **(Annex 7)** agreeing to inform a member of the study team of any medication they may have to take, or if they become unwell or pregnant whilst on the study. A member of the study team (Tharsini Sivapalan or Dr Antonietta Melchini) or HNU senior research nurse will also sign the consent form and the participants will be given a copy to keep for their records. Each participant will be assigned a unique eligibility screening code number which will be used on their eligibility screening paperwork and samples.

Once the consent form has been signed by the participant, the HNU nurse will test the urine sample using Combur 9 urine dipstick test (Roche Diagnostics). The nurse will then complete an eligibility screening questionnaire with the participant **(Annex 8)**, a case report form (CRF) **(Annex 9)**, measure and record blood pressure (BP), pulse (P), height (cm) and weight (kg) and calculate Body Mass Index (BMI, kg/m^2).

If the BMI is $\leq$20 or $\geq$35 kg/m^2, the participant will be excluded from the study. If blood pressure is less than 90/50 or 95/55 mmHg if symptomatic, or greater than 160/100 mmHg they will be excluded from the study and advised to speak to their GP.

The urine dipstick test results will be known immediately. If any of the results for the urine dipstick test are flagged the nurses will refer to the HNU Protocol 'For the referral of abnormal urinalysis results at screening' which provides advice on exclusion or re-screening of the participant. This protocol has been authorised by the HNU medical advisor. In the event of a flagged urinalysis the HNU senior research nurse will speak

to the participant and they will be advised whether a re-screen or exclusion is appropriate.

In the event of a flagged urinalysis indicating re-screen is appropriate the HNU research nurse will speak to the participant regarding their results and they will be advised to visit their GP/practice nurse prior to returning for a re-screen. If the urinalysis results are flagged on the second occasion the participant may be excluded depending on the tests flagged. No blood sample will be taken until the re-screen appointment and then only if the urine test is satisfactory.

If blood is flagged in the urine sample of female participants they will be asked if they are menstruating or have just finished menstruating, if they answer yes to either they will be asked to provide a second urine sample for testing 5 days after finishing menstruation. In the event of a flagged urinalysis on the second occasion, which indicates they may be re-screened, the HNU research nurse will speak to the participant and they will be advised to speak to their GP regarding their results prior to coming back to the HNU for a re-screen. No blood sample will be taken until the re-screen appointment and then only if the urine test is satisfactory

In the event of a flagged urinalysis indicating exclusion is appropriate the HNU research nurse will speak to the participant regarding their results and they will be advised to visit their GP to discuss the results.

Although participants will be given a copy of their flagged urine results to take with them to their GP, a member of the study team will send a copy of all the eligibility screening results including urinalysis to the GP **(Annex 10)** within the timescale advised by the HNU senior research nurse, and this will be supported with a letter to their GP detailing their screening results (urine dipstick test, screening blood results (FBC,U/E and HbA1c), blood pressure, pulse, weight, height and BMI) **(Annex 11)**. The letter will also indicate whether the participant is included or excluded from the study.

As this study requires the participants to undergo an eight hour cannulation, plus a 24 hour blood sample over three phases, the vein status of the potential participants will be assessed by the HNU senior research nurse who is experienced at undertaking cannulation/phlebotomy procedures to ensure the veins are suitable and therefore minimising the risk of damage to the vein or to prevent putting participants through unnecessary attempted cannulation. Those who do not have suitable veins for

cannulation as assessed by the HNU senior research nurse will be excluded from the study.

If the result of the urine dipstick test is satisfactory and the participant is happy to continue, a 15ml blood sample (approximately 3 teaspoons) will be taken by the HNU nurse via venepuncture using a butterfly needle. 10ml (approximately 2 teaspoons) of the blood will be sent to the SPIRE Hospital for analysis of Full Blood Count (FBC), Urea and Electrolytes (Us + Es) and HbA1c (glycated haemoglobin). In addition, 5ml (approximately one teaspoon) of the blood sample will be used for genotyping which will be stored and subsequently analysed at IFR. After obtaining the blood sample the participants will be free to leave the HNU. Participants will be provided with a urine container for their urine collection the morning of study day 1 of phase 1 and a list of foods that will need to be eliminated from their diet **(Annex 12)** 48 hours prior to the study day, the study day itself and the following morning.

If any of the individual results from the screening blood tests analysed at the SPIRE Hospital falls outside the standard reference ranges, they will be assessed by the HNU medical advisor or in their absence a doctor from the UEA medical centre, who provides emergency cover for the HNU. They will consider whether the results could affect the study data or have implications for the health of the participant, and therefore, whether inclusion, re-screen or exclusion is appropriate. It may be that even though results are outside the standard reference ranges, either a re-screen or inclusion may be appropriate. Since some minor deviations will not warrant a re-screen and will neither affect the study data nor have health implications for the participant. If the participant may be included in the study the medical advisor will write ‘satisfactory for study’ on the blood results form indicating that inclusion onto the study is appropriate.

If a re-screen is considered appropriate, the participant will be given the option of returning for another blood test after an appropriate period of time, as advised by the HNU medical advisor. Participants who do not wish to be re-screened will be excluded from the study. Participants who display screening parameters outside the standard reference ranges for the study on the second occasion (rescreen) may be excluded from the study depending on the individual results flagged. This decision will be made by the HNU medical advisor.

By signing the consent form, the participant has agreed to clinical information being sent to their GP. Copies of all eligibility screening results from fresh samples (not

stored), which have been analysed by an accredited laboratory (SPIRE Hospital) will be sent to the participant's GP.

Participants will be informed of flagged blood results by a telephone call or an e-mail initially, followed by a letter **(Annex 13)** advising them to speak to their GP to discuss the results and/or to inform them that they will not be able to take part in the study. As the scientist in charge is not medically trained, they will not advise the participants which results are flagged or any other course of action. If the blood test results indicate a problem that requires immediate attention, the HNU medical advisor will advise the HNU senior research nurse to contact the participant by telephone to inform them of the results and advise them to make an appointment with their GP. In some cases the HNU medical advisor may ask the HNU senior research nurse to contact the participant's GP directly and results may be faxed to the GP. The participant will be advised by the HNU senior research nurse that their GP has been/will be contacted directly.

Participants that successfully pass the eligibility screening will be invited to commence the study within one month of the screening visit. They will be sent an appointment card with their study day 1 appointment. Participants are free to withdraw from the study at any time without giving any reason. Re-screen will be necessary if the period between screening and starting on the study is more than one month. A letter **(Annex 14)** will be sent to the GPs of the participants that are successfully recruited onto the study to inform them of their patient's involvement in the study. This letter will be accompanied with a summarised description of the study **(Annex 15)**. The GPs will also be sent a copy of all clinical results taken at the eligibility screening (blood pressure, pulse, weight, BMI, dipstick urine test, FBC, U+Es and HbA1c) and any clinical results collected during the study deemed significant by the HNU medical advisor.

If the participants that have passed the clinical screening experience any illness or change in their medication (between the period of passing the clinical screening and starting the study), they should immediately notify a member of the study team either by telephone, letter or email to ensure that they will still be eligible to participate in the study or whether they will need to be re-screened or any booked study days postponed/ rescheduled or excluded. This will be decided by the HNU senior research nurse and HNU medical advisor.

Once recruited onto the study, the participant's code number assigned to them at screening will be used on all their samples. This is to ensure the blood samples for

genotyping and the other samples collected during their involvement in their study all have the same code number and to ensure anonymity and confidentiality. Only the members of the study team named in the approved documentation will be able to link the codes to participant's names however, the code may be broken in the event of a medical emergency as deemed appropriate and necessary by the HNU senior research nurse, the HNU medical advisor or a UEA doctor who may provide cover in cases of emergencies. All personal information will be kept confidential and known only to the chief investigator, members of study team, HNU nurses, HNU medical advisor, UEA doctor and the participant's GP.

As recruitment approaches the required number of participants for this study, those who subsequently express an interest will be asked if they would be happy to be placed on a stand-by list in the event of participants dropping out or being withdrawn. At the study talk they will be reminded that they may not get the opportunity to take part in the study. If it turns out that they are not required then they will be notified by phone and letter **(Annex 16)**. If participants on the waiting list are required, they will be contacted to book an eligibility screening appointment. If participants from the waiting list no longer wish to participate in the study, they will be removed from the waiting list and will play no further part in the study. Further individuals from the waiting list will then be approached if required.

4.3.1. Basic inclusion criteria

- Men and women
- Aged 18-65 years
- Non-smokers
- Those that live within 40 miles of IFR
- Those who like broccoli + stilton soups

4.3.2. Basic exclusion criteria

- Those unwilling/unable to provide urine and blood samples
- Results of the clinical screening indicate, or are judged by the HNU medical advisor to be indicative of a health problem which could compromise the well-being of the participants if they participated, or which would affect the study outcome.

- Those whose vein status is assessed by HNU senior research nurse as unsuitable for cannulation
- Known history of fainting when blood samples are taken, feel unwell or faint during any clinical study day procedures at the HNU
- Women who are or have been pregnant within the last 12 months or who are breast feeding.
- Those diagnosed with any long-term medical condition (e.g. diabetes, haemophilia, cardiovascular disease, glaucoma, anaemia) or requiring medication that may affect the study outcome.
- Smokers (if smoked within 6 months prior to the study or during the study).
- Those taking dietary supplements or herbal remedies which may affect the study outcome - unless the participant is willing to discontinue taking them for 1 month prior to starting study. Please note that some supplements may not affect the study and this will be assessed on an individual basis
- Those allergic to any of the ingredients in broccoli + stilton soups
- Participants allergic to members of the Brassica family, e.g. mustard allergy (often combined with sensitivity to mugwort pollen, cabbage and peach) or to Brassica pollens (mustard or rapeseed).
- Those taking any prescribed or non-prescribed medication (short or long term), which may affect the study data or participant's wellbeing. This will be assessed by the HNU medical advisor on an individual basis.
- Those on an anti-coagulant therapy or have had anti-coagulant therapy in the past 3 months.
- Parallel participation in another research project that involves dietary intervention
- Any person related to or living with any member of the study team
- Participation in another research project, which involves blood sampling within the last four months unless total blood from both studies does not exceed 470mL (unless the participants are willing to wait 4 months and then be re-screened).
- Those unwilling to provide GP's contact details
- Those unable to provide written informed consent
- Those not suitable to take part in this study because of the screening results
- Those who have donated or intend to donate blood within 16 weeks prior to the study or during the study
- Those with a body mass index (BMI, kg/m^2) ≤20 or ≥35 kg/m^2

- Depressed or elevated blood pressure measurements (<90/50 or 95/55 if symptomatic or >160/100)
- Those that have used antibiotics within the previous one month or on long-term antibiotic therapy.
- Those who are unable to completely finish the 300g portion of broccoli + stilton soup on any of the study days as this will affect study data.

5. Study Procedures

5.1. Randomisation

The randomization will be undertaken by a third party with the use of an electronic randomization generator (www.randomization.com). This uses a method called 'second generator', that creates random permutations of treatments for human intervention studies where participants are to receive all of the treatments in random order.

5.2. Study Day Preparation (Diet Restriction):

Participants successfully recruited onto the study will be provided with a list of all the cruciferous vegetables to be eliminated from their diet at the eligibility screening **(visit 2)**. This will involve elimination of ITC-containing foods including all cruciferous vegetables, spicy foods and alcohol 48 hours (2 days) prior to the study day and for 24 hours (1 day) on the study day until the 24 hour blood and urine samples have been collected on the following morning. This will mean three days of diet restriction per phase and a total of nine days of diet restriction for the duration of the study. This diet restriction will continue until the participant's involvement on the study is complete. Based on previous studies, by avoiding food containing ITC precursors for at least 24 hours, we can ensure that ITCs will not be found in circulation [38].

Participants will also be provided with a urine container at the eligibility screening **(visit 2)** to collect their baseline urine sample for study day 1.

One week before study day 1, participants will be sent a letter or email to confirm the study day appointment **(Annex 17)** and reminding them that they will be in HNU for approximately nine hours, to wear comfortable loose clothing and to collect the first

urine sample on the morning of study day 1 in the container provided. In addition they will be reminded to maintain a diet restriction 48 hours before the study, and asked to fast for 10 hours overnight prior to the study day, but will be advised that it is important to drink as much plain water as they need during the period of fasting.

5.3. Study Day 1 (HNU-Visit 3)- Phase 1:

Participants will be asked to come to the HNU between 7:30 am and 8:00am having fasted for 10 hours overnight (except for plain water). This is to ensure the cannulation is completed while medical cover from the UEA medical service is available. During the study day, participants will be provided with the broccoli + stilton soup with two slices of bread and lunch. They will also be offered a drink (tea/coffee) and biscuits before they leave the HNU at the end of the day. All the meals will maintain the glucosinolate restriction. Participants will be given food choices for the lunch **(Annex 18)**.

Prior to cannulation, the HNU nurse will complete a CRF **(Annex 9)** with the participant to assess whether there have been any changes in health or medications since screening that would affect their ability to continue; and importantly to check if they are happy to continue with the study. If participants have consumed ITC containing foods, the study day will be postponed. The participant's blood pressure, pulse, weight, height and BMI will be measured and recorded on the CRF. If the participant's blood pressure is <=90/50 or 95/55 (if symptomatic) or 160/100 or above, three successive measurements will be taken at 15 minutes intervals as per the HNU 'Protocol for action to be taken for hypotensive/hypertensive participants at screening and study interventions'. If the blood pressure measurements remain outside the inclusion criteria and HNU's protocol then it will result in the participant being excluded from the study. Participants will also be advised to speak to their GP to discuss their blood pressure. The nurse will give the participant a copy of the blood pressure measurements to take to their GP. The participant's GP will be sent a letter **(Annex 19)** with the BP results notifying them of the exclusion and informing them that the participant has been asked to discuss their results with them.

The ITC metabolites will be measured in the blood and urine therefore if female participants are menstruating or have just finished menstruating, their study day

appointment will be rescheduled 7 days after their periods has ended, as blood in the urine will affect the study data.

➢ Baseline Urine Sample

A baseline urine sample will be collected by the participant at home on the morning of the study day in the urine container provided prior to the study day. If the participant has forgotten to bring the sample with them, they will be offered the opportunity to provide a urine sample at the HNU. This urine sample will be counted as the baseline urine sample (timepoint 0).

➢ Baseline Blood Sample

The baseline blood sample will be taken after the CRF has been completed and the participant is deemed eligible to proceed in the study. The HNU nurse will cannulate the participant using a sterile intravenous cannula of appropriate gauge for the vein (as assessed by HNU senior research nurse). A tube fitted with an integral 3-way tap will be flushed with approximately 0.2ml normal saline for injection to eliminate the air before attaching it to the cannula. The connection tube will allow blood samples to be taken using the 3-way tap, thus minimising risk of contamination to the iv site and risk of infection. Once the cannula has been checked, it will be fixed in place using a transparent sterile dressing to enable the nurse to observe the cannulation site for swelling/redness during the study day. Prior to the baseline sample being taken, the saline will be drawn back using a syringe and discarded. The baseline blood sample (time point 0) of 10ml will then be taken. The i.v.cannula will be flushed with a minimum of 5ml of normal saline to clear the line and maintain patency of the cannula. Occasionally, more than 5ml is required to clear the cannula therefore, up to 10ml of sterile normal saline may be used to clear the line. Flushing of the cannula will continue after each subsequent blood sampling and as required between sampling during the study day to maintain patency of the cannula. Occasionally, it is not possible to cannulate at the first attempt or the cannula may become blocked during the study day, and therefore the cannula may need to be re-sited. In the event that either of these situations arises one further attempt will be made to cannulate a vein. The cannula may need to be re-sited either initially or during the study day if patency is not maintained. However, if it is not possible to re-site the cannula and if there are only two blood samples left to take, the HNU senior research nurse will assess the

situation and if the participant is in agreement the last two samples may be taken as individual blood samples using a butterfly needle. If there are more than two samples that remains to be taken and the cannula cannot be resited then the study day will be stopped and the participant will be withdrawn from the study. The HNU senior research nurse is experienced at performing cannulation and the relevant techniques associated with maintaining patency and blood sampling via a cannula.

- **Consumption of Study Soup**

One pot (300g) of broccoli + stilton soup will be heated and two slices of bread (brown/white) with 20g or 2 portions of butter or margarine will be provided to participants to consume once the baseline blood sample has been taken. The participants will be permitted to consume up to 250 ml of water over the next 2 hours. After 2 hours, they will be allowed to drink as much water as they like, which will be measured and recorded.

A member of the study team will record the time after consumption of the soup. Throughout the study day, participants will be advised to drink plenty of plain water to stay hydrated. Ten 10ml blood samples will be collected at the following time points: 0, 30, 45, 60, 90, 120, 180, 240, 360, 480 mins.

Total blood volume collection per study day 1= 100ml [10ml × 10 (including baseline blood sample)]

Participants will be provided with 4 containers, with powdered ascorbic acid as a stabilising agent to collect their urine samples over the 24 hours labelled as: 0-2, 2-4, 4-6 and 6-8 hours.

Participants will be provided with a study specific glucosinolate-free lunch 5 hours after the consumption of the soup. The lunch will consist of approximately 130g of tuna, or 80 g of cheese made into sandwiches using 2 or 4 slices of brown/white bread, with or without butter/margarine and/or mayonnaise (2 tsp or 10 g each). In addition, an offer of a banana and natural yoghurt will be made **[Figure 6]**.

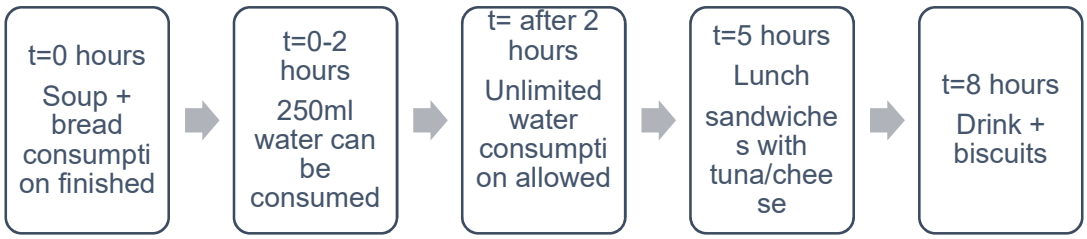

Figure 6: *Schematic diagram of timepoints for breakfast and lunch*

Following the last blood sample the cannula will be removed and the cannulation site dressed with a sterile gauze dressing. Participants will be offered a drink (tea/coffee) and biscuits before they leave the HNU. A container will be provided for the 24 hour urine collection (8-24 hours). Participants will be reminded to maintain their glucosinolate diet restriction until after their 24 hour urine and blood sample have been collected the following morning.

The participants will be required to attend the HNU the following morning **(visit 4)** to provide a 24 hour blood sample and hand in their 24 hour urine sample, which would have been collected up to the morning.

5.4. 24 hour samples (HNU-Visit 4)- Phase 1:

Having fasted again overnight for 10 hours (except for plain water), participants will be required to attend the HNU within 24 hours of consumption of the broccoli + stilton soup to have their 24 hour blood sample (10ml) taken. This appointment where the 24 hour samples will be collected will last approximately up to 1 hour at the HNU and will include breakfast. The 24hr blood sample will be taken by venepuncture using a butterfly needle from a suitable vein in the participant's arm. The procedure will be carried out by an appropriately trained HNU nurse. Their 24 hour urine sample will be handed over to a member of the study team. Following the blood sample, participants will be offered and encouraged to have a standard HNU breakfast. This will signify the end of study phase 1. Participants will be free to leave after they have finished their breakfast (cereal/ 2 slices of bread or toast with 20g (or 2 portions) of butter/margarine, coffee/tea) and will be booked in to attend study day 1 for phase 2 after a minimum of a two week washout period. Participants will be reminded to start a glucosinolate free diet 48 hours prior to phase 2 (study day 1). A list of all the foods that need to be excluded will be provided if necessary.

5.5. Study Days(HNU-Visit 5-8)-Phase 2/Phase 3:

Study day procedures for phase 2 and phase 3 will be the same as phase 1. The only exception is that participants will be given a different broccoli + stilton soup for each phase.

5.6. Completion of the study

After the 24 hour blood sample collection and urine collection of phase 3, participants will have completed the study. They will be informed that they no longer need to follow a glucosinolate-free diet. Participants who were recruited through advertisements will

be asked if they would consent to be contacted about future studies on broccoli (or similar) that may be undertaken at IFR **(Annex 20).**

6. Adverse Events (AE) and Serious Adverse Events/Suspected Unexpected Serious Adverse Reactions (SAEs/SUSARs)

The IFR complies with the National Research Ethics Service (NRES) and International Conference on Harmonisation –Good Clinical Practice (ICH-GCP) rules and the appropriate NRES form will be used to report the occurrence of serious adverse events and suspected unexpected serious adverse reactions. All adverse events/reaction and SAEs will be reported in the participant-specific case report form **(Annex 9)**. The report will include, the nature of the event, severity (mild, moderate or severe), and relationship to the study (definitely, probably, possibly, not related). Advice will be given by the HNU medical advisor as to the cause, relationship and any required further action (depending on the type of reaction); duration (dates and times), interventions and outcomes.

According to ICH GCP a serious adverse event (SAE) is defined as an untoward occurrence that:

- Results in death
- Is life threatening
- Requires hospitalisation or prolongation of existing hospitalisation
- Results in persistent or significant disability or incapability
- Is otherwise considered medically significant by the investigator

Any adverse event/reaction or a serious adverse event/reaction will only be recorded if they have occurred whilst the participant is on the study. The Chief Investigator will report all SAEs to the research ethics committee (REC) and human research governance committee (HRGC) as soon as they become aware, using the standard NRES form. Reports of SAEs will be provided to the REC within 15 days of the event being reported to a member of the study team. Once the incident has been resolved, both REC and HRGC will be informed of the outcome. The participants are asked questions relating to allergy at the eligibility screening to minimise the risk of any adverse events or reactions.

7. Study soup

The order in which the participants will consume their soups over the three phases will be decided randomly. All participants will consume all three soups containing:

- standard broccoli + stilton providing 43 µmoles/300g glucoraphanin,
- glucoraphanin-enriched broccoli + stilton- Beneforte® providing 180 µmoles/300g glucoraphanin)
- Beneforte extra broccoli + stilton providing 442 µmoles/300g of glucoraphanin

The reported levels of glucoraphanin in the three types of soups come from analysis undertaken in February 2014 **[Figure 7]**.

As mentioned in section **1.4** Beneforte® and Beneforte extra have been developed by conventional breeding. Standard broccoli, Beneforte® and Beneforte extra all have the same appearance and flavour which enables us to undertake a double-blinded human study.

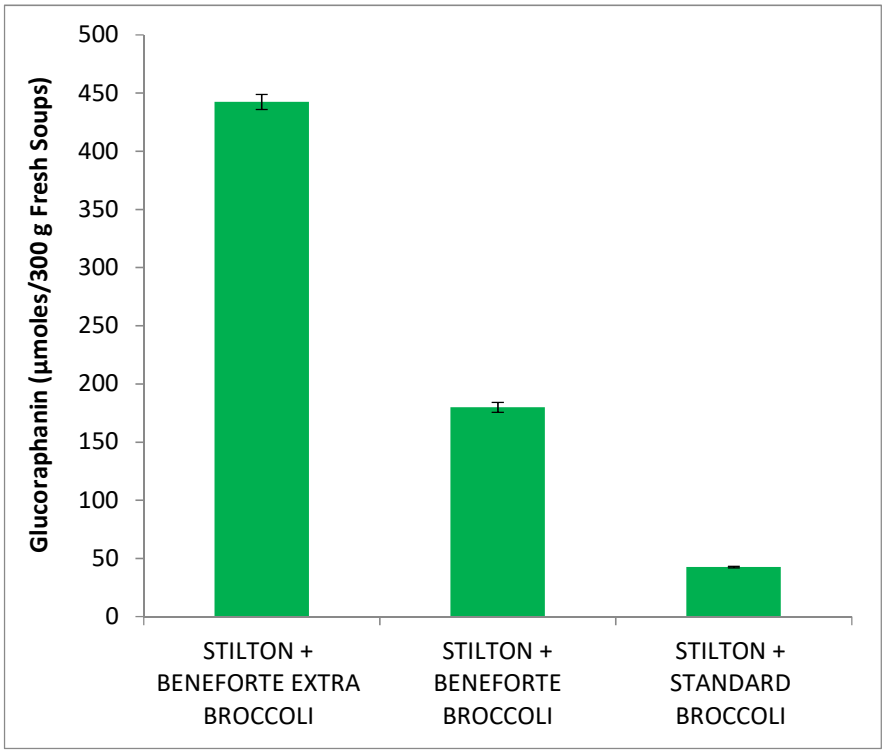

Figure 7: *Bar graph showing levels of Glucoraphanin in the three types of soups that will be used in this study from analysis undertaken in February 2014.*

7.1. Supply of study soup

Three types of broccoli soups: (i) standard, (ii) Beneforte® and (iii) Beneforte Extra will be prepared by Bakkavor, a leading international producer of fresh prepared foods **[Figure 8]**. The standard, Beneforte® and Beneforte extra broccoli soups we will have one recipe (+ stilton cheese). Ingredient declarations, nutritional information and allergy statements for the three broccoli + stilton soups have been provided from the producer (**Annex 21**). Bakkavor will make the soup, freeze them and then arrange for them to be delivered directly to the IFR. On arrival at IFR, the temperature of the soups will be checked and recorded by a member of the study team before they are unloaded and placed in the freezer. The soups will be stored in a dedicated food freezer at the HNU and member of the study team will monitor and record the temperature of the freezer on a daily basis to comply with HACCP (Hazard Analysis Critical Control Point).

Figure 8: Broccoli + stilton soups *provided by Bakkavor will be packed in a carton designed to freeze soups in individual portions.*

7.2. Preparation of study diet

The broccoli + stilton soups will be heated by the study scientist using a microwave in the HNU kitchen, using instructions provided by Bakkavor then given to participants for consumption. Participants are expected to finish the whole portion of soup. Two slices of bread with butter or margarine spread will be provided. The time taken by

each participant to consume the soup will be noted by a member of the study team. Once the soup is finished the time will be noted, which will commence the 24 hour period.

7.3. Preparation of food

The study soups, HNU standard breakfast and study specific lunch will be handled or prepared in the HNU diet kitchen by study team members who have completed formal *Level 2* training in food safety and the IFR in-house food hygiene training module included in the IFR Human Studies Training Program. All food preparation is carried out in the HNU diet kitchen in compliance with Environmental Health Guidelines. The HNU diet kitchen is registered with the local Environmental Health Office. All foods used in the breakfast and lunch are commercially available foods purchased in local supermarkets.

8. Experimental method

8.1. Blood sampling and processing

All venepuncture blood samples will be collected using a butterfly needle at the screening (15ml) and the 24 hour blood samples (10ml). Blood samples (10ml) collected on the study days will be collected via an intravenous cannula unless the last two samples are taken by venepuncture due to the cannula needing to be removed.

Total blood volume collection for whole study= (110ml × 3 phases) + 15ml from screening

= 345ml (unless participant is re-screened thus up to 355ml of blood could be collected)

Blood samples will be transferred to tubes containing EDTA anticoagulant, mixed well by inversion and the tubes will be centrifuged (1000 x g, 15 minutes, room temperature) for plasma separation. Aliquots of plasma will be stored at -80 °C and -20°C until analysis of SF and its metabolites are performed.

8.2. Urine sampling and processing

Participants will be given appropriately labelled specimen containers for their mid-stream morning urine samples. For their collection over 24 hours (0, 0-2, 2-4, 4-6, 6-8 and 8-24 hours), a number of pre-labelled containers containing 1g of powdered ascorbic acid as a stabilising agent will be provided. The participants will be advised to keep the containers containing urine samples they collect at home away from children and pets. If they accidentally get powder on their skin they will be told to wash it off as soon as possible.

The total volume of urine collected will be recorded, from which 10ml sub-samples will be taken and stored at –80 °C until analysis. The remaining urine will be discarded.

8.3. Genotype determination

From the 15ml of blood sample collected at the participant's eligibility screening visit, 5 ml will be used for genotyping. The genomic DNA will be extracted from the blood sample using the QIAamp DNA Mini kit protocol (Qiagen Inc.) prior to genotyping for *GSTM1* and other associated genotypes (e.g. GSST1) using real-time PCR according to the method of Cotton et al. [146], and others. These blood samples will be stored at IFR. All genotyping analysis will be carried out at IFR.

8.4. Quantification and profiling of ITC levels in blood and urine

8.4.1. Cyclocondensation Assay

Total SF and it conjugates (glutathione, cysteine-glycine, cysteine and N-acetyl cysteine) will be measured in plasma and urine samples using a method called cyclocondensation assay [61]. SF and its metabolites will be then identified by LC-MS analysis (as described below).

8.4.2. LC-MS

SF and its conjugates (glutathione, cysteine-glycine, cysteine and N-acetyl cysteine) in the urine and plasma sample will be measured by a validated liquid chromatography-mass spectrometry method in Gasper *et al [38]*.

8.4.3. ICP-MS

Plasma will be sent to the University of Nottingham for analysis of plasma sulphur and other elements using inductively coupled plasma-mass spectrometry (ICP-MS) *[199]*.

9. Confidentiality

All participants will be assigned a unique code number at screening. A lockable filing cabinet or cupboard will be used to keep paper documents and participants' personal data. The file that links the participant to the code and personal information will also be kept in a locked filing cabinet but separately to all the other documents. All electronic data will be stored on a password protected shared data file between the study team on IFR computers. The main computer storage will be on one main IFR computer, but as part of a password protected shared network. All IFR computers are individually password protected and the shared network access is limited to those working within the research area. Manual files/folders will consist of coded files for each participant. No data with the participants' name will be filed in the numbered file.

Confidential data will be accessed only by the members of the study team, HNU nurse, HNU medical advisor, UEA doctor providing emergency cover, participant's GP and CI. The participant's GP will have access to personal data. The samples sent to the SPIRE hospital for biochemical analyses (full blood count, U's + E's and other laboratory facilities) will be known only by their code number. All data collected will also be identified by code only.

Data will be stored for at least 15 years after completion or discontinuation of the study. The data will be stored in the IFR human studies archive. Access to archived data will be limited to the members of the study team and CI or the CI's successor. The quality assurance auditors may also be allowed access to data with the permission, and in the presence, of the CI however they will not have access to the participant's identifiable personal information.

10. Statistical Analysis

10.1. Sample size calculation

To detect a significant (P<0.05) difference in mean total urinary thiol excretion of 18 µmol between consumption of normal broccoli + stilton soup and Beneforte® broccoli + stilton soup, the study requires ten participants giving a power of 80%. The SD of the differences between the two groups was estimated to be 17 µmol. The basis for this calculation was preliminary unpublished data from 65 subjects on the ENGAGE study (12/EE/0483).

10.2. Data analysis

Data will be analysed using mixed effects models. These are linear models that account for the non-independence of the type of data that will be generated in this cross-over study (i.e. data that come from the same participants after different interventions). Some variation in the outcome measures will come from the genotype of the participants and allowances will be made for that by including it as an explanatory variable in the model. The aim of the analysis will be to examine whether the intervention(s) have any effect on the various outcome measures (e.g. urinary excretion of total thiol conjugates, pharmacokinetic parameters generated from the time course of plasma samples).

11. Ethical considerations

Food safety: IFR follows Environmental Health Guidelines for the storage and delivery of all food to IFR. These guidelines will be adhered to when processing and handling all food items.

Toxicity: There is no evidence from animal or human studies that broccoli is harmful.

The level of glucoraphanin that will be consumed in the broccoli + stilton soups with the highest concentration will be similar to that being consumed in the current intervention study (ESCAPE, REC Ref Number 13/EE/0110).

Cannulation/Phlebotomy: As with all procedures involving venepuncture the participants may feel a little discomfort on initial insertion of the intravenous cannula needle on the study day or the butterfly needle for single blood sampling, however, once the cannula/needle is in the vein there should be minimal or no pain. A small bruise may develop at the venepuncture/ cannula insertion site but, as with all bruises, this should fade after a few days. The HNU nurses are experienced in undertaking these procedures, therefore, the risks associated with these procedures are minimal. The well-being of the participants will be monitored by the HNU nurses throughout the study day and they are trained to deal with medical emergencies. A doctor from the UEA Medical Centre will also be contactable to provide medical support if the participant becomes unwell on the study day.

Allergies/sensitivity/dietary requirements: The participants are asked about any known allergies/dietary requirements they may have at the study talk and by the nurse at the eligibility screening and on each study day. This information is documented on their screening questionnaire and the CRFs. Anyone who has a known allergy/sensitivity to any of the contents of the broccoli + stilton soups will be unable to participate in the study. If any participant demonstrates any signs of possible allergy/sensitivity to the soup whilst on the study, they will be withdrawn from the study at that point. Information regarding the contents of the soup will be given to participants in the PIS.

12. Participants' expenses/payments

Each participant will receive a pro rota inconvenience payment for their participation in the study. The breakdown of the payment is outlined below:

Screening appointment:

Mid stream Urine sample	£2.00

Single venous blood sample	£5.00

Total per screening = £7.00 *(This could be £12.00 if a blood re-screen required and £9.00 if a urine re-screen is required)*

Each phase of the Study:

Three days of a restricted diet (no GSL and ITCs)	£15.00
Morning urine sample (Day 1)	£2.00
24 hour urine collection (Day 1-2)	£10.00
Insertion of cannula	£15.00
£3 per hour cannulated (x 8 hours)	£24.00
Single venous sample (1 sample at £5)	£5.00
Inconvenience payment	£94.00
Total per phase =	£165.00
The total expenses for each participant per 3 phase study will be =	£495.00

Total expense for whole study including screening (excluding re-screens) = £502.00

Participants will be reimbursed travel expenses to and from the HNU (at IFR). This will be reimbursed at the IFR's current mileage rate which is currently 45p per mile or by reimbursing public transport costs on production of a ticket or receipt. If participants require transport, this is provided by IFR as a taxi and is paid for by the study.

13. Study partners

The study will be funded by a Biotechnology and Biological Sciences Research Council (BBSRC) institute strategic programme grant (BB/J004545/1), BBSRC Doctoral Training Patnership grant (BB/J014524/1) and an award by the Prostate Cancer Foundation.

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Date

Dear_____,

Thank you for your interest in

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

at the Institute of Food Research

I have sent you details of this study which is in progress at present as your details currently held on the database indicate that you may fit the criteria. If you are interested in participating, please complete and return the reply slip on the enclosed participant information sheet (PIS). If you have any further questions about the study, please contact, Tharsini Sivapalan on 01603251425 (tharsini.sivapalan@ifr.ac.uk) or Dr Antonietta Melchini on 01603 255030 (antonietta.melchini@ifr.ac.uk) or as stated on the enclosed participant information sheet.

If, however, any of your details have changed or change in the future, or you would prefer to no longer remain on the database, please could you inform the Human Nutrition Unit on 01603 255305.

Thank you.

Yours sincerely,

Mrs Aliceon Blair

HNU senior research nurse

Norwich Research Park, Colney, Norwich NR4 7UA, UK

www.ifr.ac.uk Tel: +44(0) 1603 255000 GTN 6626 5000 Fax: +44 (0)1603 507723

PARTICIPANT INFORMATION SHEET

Bioavailability Of sulforaphane from Broccoli Soups (BOBS)

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen if you take part
- Part 2 gives you more detailed information about how the study will be conducted

Please 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.

This information sheet is yours to keep.

Thank you for reading this.

Study scientist contact details:

Miss Tharsini Sivapalan
(0)1603 251 425
tharsini.sivapalan@ifr.ac.uk

Dr Antonietta Melchini
(0)1603 255 030
antonietta.melchini@ifr.ac.uk

Chief investigator (CI)

Professor Richard Mithen

(0) 1603 255 259

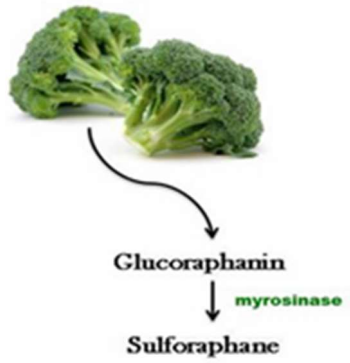
richard.mithen@ifr.ac.uk

INDEX PAGE

PART 1 of the information sheet

What is the purpose of this study?

There is current evidence that suggests eating cruciferous vegetables like broccoli, cauliflower, cabbage is beneficial to our health as they contain compounds which are thought to reduce the risk of diseases such as cancer and cardiovascular diseases. These compounds are called **glucosinolates** (GSLs) and more than 100 different GLSs are known to occur naturally in cruciferous vegetables.



Broccoli is a rich source of a GSL called **glucoraphanin**. When we eat broccoli, the bacteria in our gut break down the glucoraphanin to another natural compound called **sulforaphane (SF)**. It is thought that broccoli is good for our health because of the effect of SF in our body. We rely on our bacteria to do this even though the vegetable itself has proteins called **Myrosinase** which can convert GLS to SF. This myrosinase is however, destroyed when the broccoli is cooked.

What we aim to do?

We want to find out how SF is absorbed and metabolised by our bodies after eating three types of broccoli + stilton soups.

We would like participants to eat **one portion (300g)** of broccoli + stilton soup on three separate days. We will then collect blood and urine samples over the course of the three days to measure the levels of SF and its metabolites.

The three types of soups are:

- Standard broccoli + stilton
- Beneforte® broccoli +stilton - glucoraphanin-enriched broccoli
- Beneforte extra broccoli + stilton- higher levels of glucoraphanin than Beneforte®

The Beneforte® and Beneforte extra have been developed by conventional breeding so they are **not** genetically modified.

The Beneforte® broccoli contain 2-3 times higher levels of the glucoraphanin.

This study will be a **cross-over**, **double-blinded**, **randomised study** and will be carried out at the Human Nutrition Unit (HNU) of the Institute of Food Research (IFR).

Cross-over trial: means that each participant will have a different broccoli + stilton soup in each phase. There are a total of three phases (three study days) in this study which will be separated by a minimum of two weeks (washout period). By the end of the study you would have consumed all three soups.

Randomised trial: This means that the order in which you eat the soup will be randomly allocated. A computer program is used for the random allocation of the broccoli + stilton soup. Thus neither the scientist nor you can choose in which order you eat the three types of broccoli + stilton soups.

Double-blinded trial: This means that the scientist nor you will know which of the three broccoli +stilton soups you will be eating at each study day.

To investigate the aim of our study, we will need to collect:

- urine samples 
- blood samples 

From the blood and urine samples, we will **analyse the SF concentrations** to understand how it is metabolised over 24 hours after eating the three types of broccoli + stilton soups.

Genotyping

We all have about **25,000 genes**. Many of these genes have subtly different versions, which is why we all look slightly different to each other. The term genotype is used to describe which version individuals have. How beneficial certain vegetables may be for different people may depend upon what genotype they have.

This study will involve **genetic analysis** of your blood to see what genotype you have for two specific genes from the group of genes called GST (glutathione-S-transferases). This analysis may help us **understand** how the body absorbs and excretes SF in the plasma and urine **changes between different people.**

It is important to stress this genetic analysis has no clinical relevance to you or your family nor will we be able to tell you whether you have the genes.

Who can take part in the study?

We are aiming to recruit:

- Women and men aged 18-65 years
- Non-smokers
- Those who live within 40 miles of IFR
- Those who like broccoli + stilton soup

Who cannot take part in the study?

You **cannot** take part in this study if:

- You are unwilling/unable to provide urine and blood samples
- Results of the clinical screening indicate, or are judged by the HNU medical advisor to be indicative of a health problem which could compromise your well-being if you participated, or which would affect the study outcome.
- Your vein status is assessed by HNU senior research nurse as unsuitable for cannulation
- You have a known history of fainting when blood samples are taken, feel unwell or faint during any clinical study day procedures at the HNU
- You are or have been pregnant within the last 12 months or you are breast feeding.
- You have been diagnosed with any long-term medical condition (e.g. diabetes, haemophilia, cardiovascular disease, glaucoma, anaemia) or requiring medication that may affect the study outcome.
- You smoke (or have smoked within 6 months prior to the study or during the study)

- You are allergic to any vegetables of the Brassica family, e.g. mustard allergy (mugwort pollen, cabbage and peach) or to Brassica pollens (mustard or rapeseed).
- You are allergic to any of the ingredients of the broccoli +stilton soups
- You are taking dietary supplements or herbal remedies which may affect the study outcome - unless you are willing to discontinue taking them for 1 month prior to starting study. Please note that some supplements may not affect the study and this will be assessed on an individual basis.
- If you are taking any prescribed or non-prescribed medication (short or long term), which may affect the study data or your wellbeing. This will be assessed by the HNU medical advisor on an individual basis.
- You are on an anti-coagulant therapy or have had anti-coagulant therapy in the past 3 months.
- You are participating in another research project, that involves dietary intervention
- You are related to or living with any member of the study team
- You are participating in another research project, which involves blood sampling within the last four months unless total blood from both studies does not exceed 470mL (unless you are willing to wait 4 months and then be re-screened).
- You are unwilling to provide GP's contact details
- You are unable to provide written informed consent
- You are not suitable to take part in this study because of the screening results
- You have donated or intend to donate blood within 16 weeks prior to the study or during the study
- You have a body mass index (BMI, kg/m^2) $\leq$20 or $\geq$35 kg/m^2
- You have depressed or elevated blood pressure measurements (<90/50 or 95/55 if symptomatic or >160/100)
- You have used antibiotics within the previous one month or on long-term antibiotic therapy.

You are unable to completely finish the 300g portion of broccoli + stilton soup portion on any of the study days as this will affect study data

Why have I been invited?

You have been invited to take part either because:

- your details are currently registered on the IFR Human Nutrition Unit (HNU) database suggest that you may be able to take part in the study or,
- you responded to an advertisement for the study

Do I have to take part?

Taking part in this study is completely up to you. If after reading this information sheet, you would like to take part then you should send in the response slip (page 21) using the pre-paid envelope and if you wish you can also contact a member of the study team named on the front page. If you do not wish to take part in the study, then please ignore the letter sent to you and no one will contact you about joining the study. Expressing an interest in the study does not mean that you are obliged to take part. You are free to withdraw from the study at any time, without giving a reason. A decision to withdraw or not to take part will not affect your participation in future studies or being contacted to take part in other IFR studies if you are on the HNU volunteer database.

What next?

When you have sent in your response slip, a member of the study team will contact you to book an appointment for you to come to the HNU for the study talk. You will be asked to come to the HNU for 8 visits (see flowchart on p12):

VISIT 1- Study talk ~ approximately 1 hour

VISIT 2- Eligibility screening ~ approximately 1-1½ hours including breakfast

VISIT 3- Study Day 1- Phase 1 ~ approximately 9 hours at the HNU

VISIT 4– 24 hour samples- Phase 1 ~ approximately up to 1 hour including breakfast

VISIT 5-8- Study Days- Phase 2-3 will be the same as visit 3 and 4

We will try to book the screening and study day appointments at a time that is as convenient as possible for you but they will take place on weekdays. On the study days you will be required to come to the HNU between **7:30am and 8:00am** and you will have to stay at the HNU for approximately 9 hours.

- **VISIT 1-Study Talk**

A member of the study team will contact you to book an appointment to invite you to the HNU for a talk about the study. The study talk will last approximately 1 hour. At the talk a member of the study team will go through this information sheet, the procedures and your involvement in this study. You will be encouraged to ask any questions that you may have at the time. You will be able to speak to the HNU senior research nurse if you have any questions relating to cannulation, venepuncture or any of the clinical measurement prior to making any commitment.

After the talk you will be given a minimum of 72 hours (3 days) to decide whether you wish to take part in the study. During this time we will not contact you. If you wish, you can talk to your family, friends or GP about the study. After 3 days if you decide to take part then you will need to contact a member of the team to book a screening appointment. At the end of the study talk we will give you a container which will be used for the collection of the urine sample on the morning of the eligibility screening visit should you decide to take part in the study.

If you do not want to take part then you will not need to contact us, we will not contact you and you can dispose of the container given to you at the talk.

Not taking part will not affect you being approached for future studies at IFR if you are registered on the HNU database.

- **VISIT 2- Eligibility Screening**

This appointment will last approximately 1-1½ hours.

You will need to bring a fresh midstream urine sample with you in the container provided at the study talk. This sample has to be produced within 2 hours of your appointment as this is required for the urine test to work. It is important for the urine dipstick test results and study analysis that you do not use any containers other than the ones given to you during the study to collect your urine. If you lose the container or forget to bring your urine sample, you will have another opportunity to produce a sample at the HNU.

What will happen at the screening?

On arrival at the HNU you will be met by a member of the study team and taken to a confidential room where we will ask you to sign a consent form to say that you have understood what the study involves and that you agree to take part in the study. If you were recruited through adverts, then you will be asked to sign another consent form on completion of the study to ask if you would agree to be contacted about future studies on broccoli or similar. Completion of this consent to contact form is optional.

Please note: Participants already registered on the HNU volunteer database will be automatically contacted for future broccoli studies if your details fit the inclusion criteria.

A member of the study team or the HNU senior research nurse will also sign the consent form and you will be given a copy to keep. After signing the consent form, you are still free to withdraw at any time without giving a reason.

In addition, you will also be asked to sign a medications declaration form agreeing to inform a member of the study team of any medication you may have to take, or if you become unwell or pregnant whilst on the study. You will be given a copy to keep.

After consent is signed, the HNU nurse will carry out the urine dipstick test on your sample. The results will be known immediately and if any of the urine test results are 'flagged' the HNU nurse will explain this to you. Flagged means that the results do not match one or more of the standard test results and may mean you need to come back for a re-screen or may be excluded from the study. If any of the urine results are flagged the nurses will refer to the HNU Protocol 'For the referral of abnormal urinalysis results at screening' which provides advice on exclusion or re-screening of

the participant. This protocol has been authorised by the HNU medical advisor. You will also be advised to speak to either the nurse at your surgery or your GP to discuss the results and you will be given a copy of your urine results to take with you to the surgery.

The HNU nurse will complete an eligibility questionnaire and a case report form with you.

The HNU nurse will measure and record your:

- Blood pressure (BP)
- Pulse
- Height (cm)
- Weight (kg)
- Calculate BMI (kg/m^2) using your height and weight

Your BMI indicates whether you are a healthy weight for your height; if your BMI is below 20 or above 35, you will be excluded from the study.

As we need to insert a cannula into one of your veins in your arm for the study day and take a blood sample the day after is important that your veins are suitable for these procedures. Before taking the screening blood sample the HNU nurse will assess the veins in your arms to see whether they are suitable for cannulation. If the nurse does not find a suitable vein for cannulation, you may be excluded from the study.

If the eligibility screening results (dipstick urine, screening questionnaire, blood pressure, pulse and BMI) are within the range for the study and you are happy to continue, the nurse will take a 15ml (approximately 3 teaspoons) blood sample from a vein in your arms. 10ml (equivalent to 2 teaspoons) of the blood will be sent to the SPIRE Hospital for analysis of full blood count (FBC), urea and electrolytes (Us + Es) and HbA1c. The HbA1c test will provide us with a picture of your average blood glucose levels over a period of about 3 months.

In addition, 5ml (equivalent to 1 teaspoon) will be stored at IFR to be used for genetic analysis.

The HNU nurses are trained and experienced in carrying out the clinical procedures. Please ask them if you have any questions about the tests or procedures.

Your GP will be notified of your participation in the study and will be sent all copies of your clinical screening results (urine results, blood results, blood pressure, BMI and weight). You are agreeing to this when you sign the consent form.

If any of your clinical results are outside the standard reference ranges we may recommend that you speak to your GP about the results. All results outside the reference ranges are checked by the HNU medical advisor. The medical advisor will decide whether we may include you in the study, offer you the opportunity of a second screening (re-screen) or exclude you from taking part in the study.

If you do return for a re-screen and your results fall outside the standard reference ranges on the second occasion, you may be excluded from the study depending on the results flagged. We cannot tell you what your results may mean as we are not medically qualified to do so. You should not worry if your results are flagged as it may be a one off result or it may be perfectly normal for you, but your GP will be able to explain them to you. Please remember these tests are performed to determine if you are suitable for the study, not to find out if you are healthy.

You will be excluded from the study if the results of any of the screening tests are judged by the medical advisor to be unsuitable for your participation.

If the results of your screening indicate that you are eligible to take part in the study you will be contacted by a member of the study team to arrange a date for your first study day. You will be provided with a urine container at the screening to collect your urine sample for the study day and a list of foods that will need to be eliminated from your diet for 48 hours (2 days) before the study day and throughout the study intervention phase until the 24 hour blood sample has been collected the following morning.

Before study day:

- Eliminate all foods high in glucosinolates (list of food to avoid on page 19), spicy food and alcohol from your diet **48 hours** prior to the study day. We understand it is not easy to remember everything therefore, if you have accidentally consumed any food high in glucosinolates during the 48 hour restriction period please tell us as we will postpone the study day.

- Fast for **10 hours** prior to the study day and **drink plenty of plain water** during the fast.

- **VISIT 3- Study Day 1**

A study day will approximately last for 9 hours and you will be required to stay in the HNU for the duration of the study day.

-Morning of study day:

On the **morning of the study day**, collect a sample of urine in the container that was provided at the screening. This sample will be used as your baseline. If you forget to bring your sample, you will have an opportunity to collect a sample at the HNU.

Once you arrive at the HNU, you will hand your sample over to a member of the study team. The HNU nurse will then complete a case report form with you to check that you have not had any health problems or change of medication since your screening appointment. The nurse will also measure and record your blood pressure, height, weight and calculate your BMI. If your blood pressure measurement is outside the range for the study the nurse will take three further measurements at ten minute intervals. If your blood pressure measurement remains outside the range for the study you will be withdrawn from the study or if there are indications that your well-being maybe affected by any of the clinical procedure. If female participants are menstruating or have just finished menstruating, their study day appointment will be rescheduled 7 days after their period has ended.

If your blood pressure measurement is satisfactory for the study the HNU nurse will insert a cannula into a vein in one of your arms. A short clear tube will be attached to the cannula to allow the nurse to take the blood samples from the cannula throughout the day. Once in place the nurse will flush via the connecting tube with sterile normal saline to check it is in position and check that you are comfortable. The normal saline will then be withdrawn back through the tube attached to the cannula and discarded, the first study blood sample will be collected. This first blood sample is called the baseline sample (0 hours). After the first blood sample and all other blood samples taken through the cannula during the day, the HNU nurse will flush the tube and cannula with sterile normal saline to stop it becoming blocked.

If the cannula blocks during the study day the nurse may have to remove it and insert another cannula into a different vein in your arms. However, if there are only two blood samples left to take, the HNU nurse will assess the situation and if you agree the

nurse may be able to take them as individual blood samples using a butterfly needle the same as the way the blood was taken for your screening. The cannula site will be covered with a sterile dressing to protect it during the day. If there are more than two samples that remain to be taken and the cannula cannot be resited, then the study day will be stopped and you will be withdrawn from the study.

After the baseline blood sample has been taken you will be given one 300g pot of broccoli + stilton soup to eat with two slices bread (brown/white) with butter or margarine. You will also be able to drink up to 250ml of water over the next 2 hours. After 2 hours, you will be allowed to drink as much water as you like which will be recorded. After 5 hours, you will be provided with a study specific lunch consisting of tuna or cheese sandwiches made with 2-4 slices of brown/white bread, with or without butter/margarine and/or mayonnaise in addition to the offer of a banana and a natural yoghurt.

You will need to eat the study specific lunch on each of the study days.

During the whole study day, a total of **ten** blood samples will be collected at the following time points: 0, 1/2, 3/4, 1, 1.5, 2, 3, 4, 6, 8 hours.

You will also be asked to provide **five** individual urine samples during the study day at 0, 0-2, 2-4, 4-6, 6-8 hours. It is important that you drink enough water throughout the day so you can produce all **five** samples.

After the last blood sample has been collected the HNU nurse will remove the cannula from your arm and cover the puncture site with a sterile gauze pad. You will be offered a drink (tea/coffee) and biscuits. You will be provided with a urine container and asked to collect all of your urine (8-24 hours) until the following morning (before your appointment at the HNU). After which, you will be able to leave the HNU provided the HNU nurse has checked that you are well.

You will also need to eliminate food high in glucosinolates, spicy food and alcohol until you have had your 24 hour blood sample taken on the morning (list of foods to avoid at page 19).

- **VISIT 4– 24 hour samples**

The appointment that you attend at the HNU for sample collection 24 hours after consuming the broccoli soup, will last approximately up to one hour.

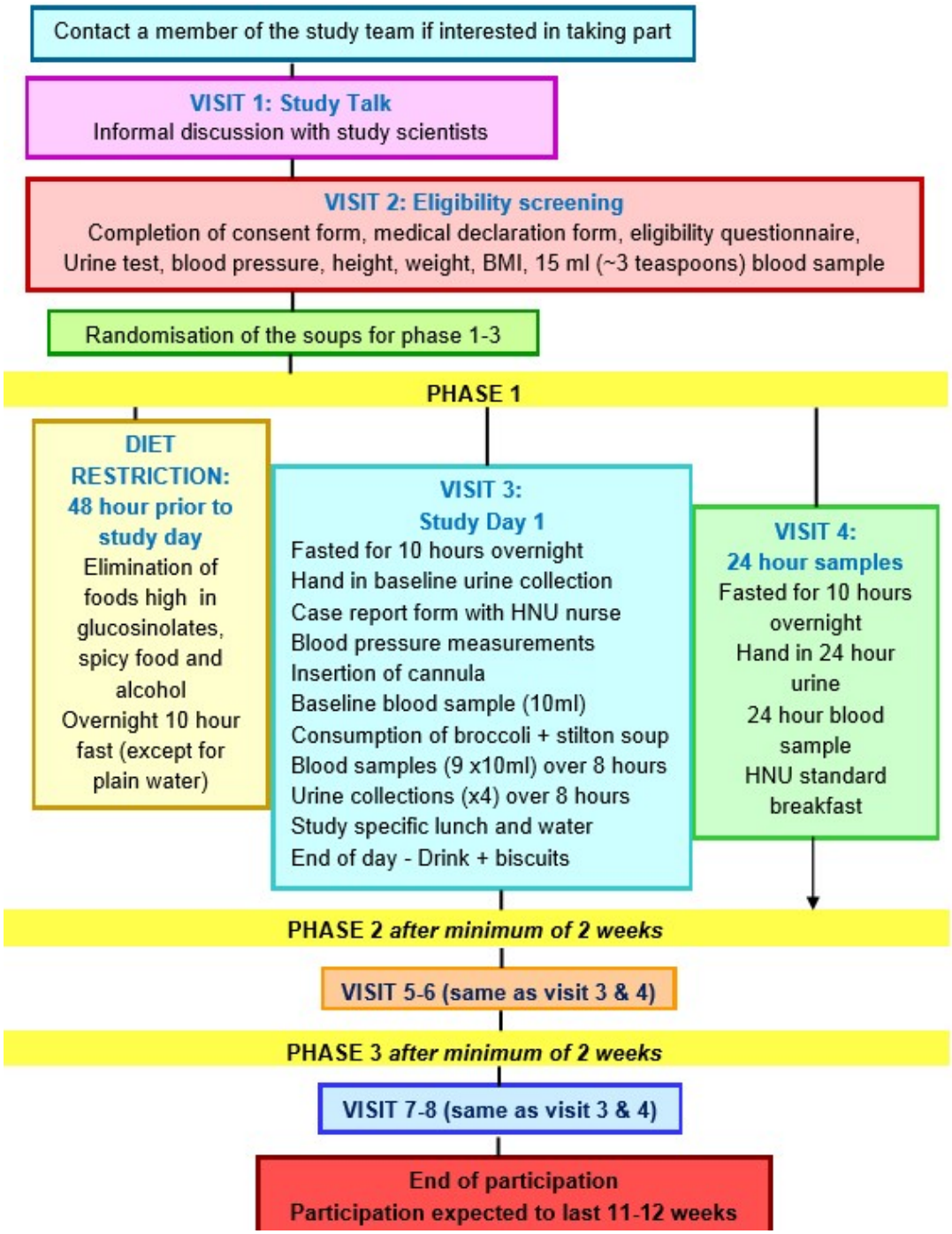
- You will have fasted overnight again for **10 hours** (except for plain water)
- You will need to bring the **8-24 hour** urine collection with you to the HNU
- You will need to arrive at the HNU within 24 hours of eating the soup to have your 24 hour blood sample taken. After this sample has been taken you will be offered and encouraged to have a standard HNU breakfast (cereal, toast, tea/coffee)

After breakfast, you will be able to leave the HNU. You will be able to follow a normal diet for a minimum of two weeks until your 48 hours prior to your phase 2 appointment.

- **VISIT 5-8- Study Days for phase 2-3**

The study procedure for phase 2-3 will be exactly the same as phase 1 consisting of a study day 1 and 24 hour sample. The only difference is that you will be given a different broccoli + stilton soup for each phase.

You will need to eliminate foods high in glucosinolates, spicy food and alcohol 48 hours prior to study day 1 until the 24 hour samples are collected the following morning in each phase.



Study flowchart outlining volunteer's involvement

Access to your personal information

During the screening visit, you will be given a code number. This code number is unique to you and will be used to protect your identity and make your samples anonymous. Only the study team, HNU nurses, the HNU medical advisor, your GP, UEA doctor providing medical cover in the event of an emergency and CI will have access to any information about you. There is more information about this in Part 2 of this information sheet.

Expenses and payments

Participating in these studies is on a voluntary basis. However, we do recognise that taking part can cause some inconvenience and there are associated travel costs. Thus, you will receive **£502** as an inconvenience payment on completion of the study; if you withdraw or are excluded from the study payment will be pro-rata. Travelling expenses to and from the HNU will be reimbursed on presentation of a receipt for buses or trains or at the current IFR mileage rate for private cars. If you require transport to and from the HNU, please let us know as we will organise and pay for a taxi.

All payments are liable to tax and you are responsible for declaring your own payments for tax purposes. If you are in receipt of benefits this payment may affect your benefits.

Members of staff at IFR are free to participate in this study; however we would like to point out that their inconvenience payment will be taxed at source in accordance with BBSRC and IFR rules and HM Revenue and Customs (HMRC).

What are the risks or side effects of participating in this study?

You may experience some discomfort on insertion of the needle for the screening blood test and the cannulation, which affects some people more than others. You should not experience pain during the procedure or afterwards. You may develop a small bruise at the site of the blood sample or cannula, but this will fade like any bruise. The nurses in the HNU are experienced at taking blood and cannulation.

As with any pressure measurement (like blood pressure) the inflation of the cuffs may cause slight discomfort.

The HNU nurses will be happy to answer any questions you may have about any of the procedures involved.

What are the potential benefits of taking part?

There will be no direct benefit for you by taking part in this study. However, the results that we get from this study will provide us with more information on how the natural compounds from broccoli are absorbed and metabolised by our bodies.

This completes Part 1 of the information sheet

If the information in **Part 1** has interested you and you are considering taking part, it is important that you read the additional information in **Part 2** before making any decision

PART 2 of the information sheet

What if relevant new information becomes available or changes to the study are made?

If this happens we will tell you. If changes to the study have to be made you may be asked to sign another consent form.

What happens if I don't want to carry on with the study?

You are free to withdraw from the study at any time without giving a reason. You will receive payment pro rata for samples given up to the point of withdrawal.

Samples collected will need to be kept until the study has been finished and any data collected until the point of withdrawal will still be used unless you decide otherwise.

What if there is a problem?

If you have a concern about any aspect of the study, you should ask to speak to the researchers who will do their best to answer your questions. You can telephone Miss Tharsini Sivapalan on (0)1603 251 425 or Dr Antonietta Melchini on (0)1603 255 030. If you have any questions about any of the clinical procedures then we can arrange for the HNU senior research nurse to talk to you.

If you remain unhappy and wish to complain formally, you can do this through the chairperson of the Human Research Governance Committee at IFR- Dr Linda Harvey (01603 255191). IFR accepts responsibility for carrying out trials, and as much will give consideration to claims from participants for any harm suffered by them as a result of participating in the study, with the exception of those claims arising out of negligence by the participant. Like all publically funded bodies, the Institute is unable to insure and thus cannot offer advance indemnity cover for participants.

Please note that the Institute will not fund any legal costs arising from any action unless awarded by a court.

Will my taking part in the study be kept confidential?

We will only collect personal information or samples from you that are needed for the study. Your personal information and samples will be stored in the strictest confidence with access only by members of the study team, the HNU nurses, HNU medical advisor, UEA doctor providing medical cover in the event of an emergency, your GP and CI. All personal information is kept in locked cupboards/filing cabinets at IFR.

During the eligibility screening you will be assigned a participant code number. This number is unique to you and it is this number that will be used to identify samples to protect your personal information. The document linking the code to your personal information is kept locked up. Study samples and data generated from this study will be stored at the Institute of Food Research for up to fifteen years after the end of the study.

All research is subject to inspection and audit and although your records may be accessed for this purpose any personal information remains confidential.

Please note that IFR has CCTV cameras in use for security purposes however, provision has been made so that volunteers attending the HNU will not be identified.

Will my GP be informed?

It is our policy to inform your GP that you are taking part in a study at IFR. All clinical screening results (blood tests-full blood count, urea and electrolytes and HbA1c, urine dipstick test, blood pressure, pulse, weight and BMI measurements) and any significant clinical results will be sent to the GP. This is one of the things you are agreeing to when you sign the consent form.

You will be advised to discuss your blood results with your GP if any of the blood results are outside the standard references ranges as the members of the study team are not medically qualified to discuss this with you. Sometimes the HNU medical advisor will discuss your results with your GP.

What happens to my samples?

Samples given at **screening**:

- the urine sample will be tested immediately using a urine dipstick test and then discarded.
- 10ml (2 teaspoons) of the blood sample will be sent to the SPIRE Hospital for full blood count, HbA1c, urea and electrolytes.
- 5ml (1 teaspoon) of blood sample will be stored at IFR for the genetic test described in Part 1 of this information sheet.

Samples given at **study days**:

- The blood and urine samples collected during the study days will be stored at IFR and analysed for SF concentrations.

This will be done using a liquid chromatography/mass spectrometry (LC/MS) and cyclocondensation method.

LC/MS uses a method that can separate components of a mixture. It will allow us to work out which compounds from the broccoli + stilton soup your body has absorbed, metabolised and/or excreted.

The cyclocondensation method is a specific chemical reaction and will help us to find out the amounts of total SF and related compounds in your blood and urine samples.

Some plasma samples from the blood samples will be sent to the University of Nottingham for analysis of sulphur and other elements. This will be done by a method called ICP-MS.

What will happen to the results of the study?

Unfortunately, we are unable to tell you any of your individual results. However, we will be able to report back the results of the study in the form of a basic summary. The data resulting from the study may be published in scientific journals or presented at meetings with our funders.

Any data presented is anonymous and your name will not appear in any results presented or published.

Who is funding this study?

This study is being funded by Biotechnology and Biological Sciences Research Council (BBSRC, UK). This study is part of an educational project and Miss Tharsini Sivapalan is a research student at the University of East Anglia (UEA) and IFR which is also funded by the BBSRC.

Who is performing this study?

This study will be run by Miss Tharsini Sivapalan, a research student at IFR and will form part of her research studies at UEA. The chief investigator and PhD supervisor is Professor Richard Mithen. Dr Antonietta Melchini and Dr Charlotte Armah who have experience of running trials will also assist where necessary. The analysis of plasma and urine samples will be performed by Miss Tharsini Sivapalan at IFR laboratories

under the supervision of an analytical expert at IFR. The clinical procedures and screening will be undertaken by the HNU research nurses.

Who has reviewed the study?

Human studies research carried out at IFR is reviewed by the Institute of Food Research Human Research Governance Committee (HRGC) and an independent group of people called a Research Ethics Committee (REC) to protect your safety, rights, wellbeing and dignity.

This study has been reviewed, and approved by HRGC and East of England Norfolk REC committee.

What you need to tell us?

We need you to tell us some things for your safety and for the success of the study. You need to tell us if you are taking **ANY** MEDICATION whilst on the study as some medication may affect the information we are collecting.

This includes anything you may have bought from the chemist. You will be asked to bring details of any medication i.e. name of medication and the dose you are taking when you come for your screening.

You will need also to tell us if you become unwell during the study or after a visit to the HNU. It is also important that you inform us if you think you may be pregnant or you become pregnant whilst on the study.

Thank you very much for reading this information sheet and considering whether or not to participate in this study.

Taking part in the research is entirely voluntary! You are free to withdraw from the study at any time without giving a reason.

For further information or to arrange a study appointment, please contact:

Miss Tharsini Sivapalan
(0)1603 251 425
tharsini.sivapalan@ifr.ac.uk

or

Dr Antonietta Melchini
(0)1603 255 030
antonietta.melchini@ifr.ac.uk

Alternatively you can complete the attached response slip on page 21 and return this to us in the prepaid envelope provided.

A list of foods that are high in glucosinolates and have to be avoided 48-hours before and during the study days:

Garlic

Mustard: all types including Ethiopian mustard, Indian mustard, Chinese mustard, red giant mustard, wrapped heart mustard cabbage, yellow mustard, black mustard, broad beak mustard, purple stem mustard, mustard spinach.

Broccoli: all types including rapini or broccoli raab, alboglabra kai-ian (Chinese broccoli), romanesco broccoli, broccoli, broccoflower.

Sprouting broccoli

Brussels sprouts

Cauliflower

Cabbage: all types including white cabbage (drum), head cabbage, Savoy cabbage, red cabbage, green cabbage.

Kale: all types including curley kale, Chinese kale, sea kale, pabularia siberian kale, acephala Kale.

Kohl rabi

Turnip and turnip tops

Spring greens and Collard greens

Chinese cabbage; Pak Choi; Bok choy and other Chinese brassica vegetables

Radish

Salad rocket

Horseradish: sauces and vegetables

Cress: all types, including watercress and garden cress

Papaya seeds, Wasabi

Soup ingredient information

Broccoli and Stilton Soup

Ingredients Declaration: Water, Broccoli (28%)*, Fresh Milk, Single Cream, Diced Onion, Potato, Stilton Cheese (4%), Cornflour, Rapeseed Oil, Salt, Black Pepper.

* Standard broccoli or Beneforte or Beneforte extra broccoli

Allergens: Milk, celery

Theoretical Nutrition:

		Energy Kcal	Fat	protein	CHO	Na	Sugar	Sat Fat	Salt	Fibre
Per:	300g portion	175	11.36	8.29	9.91	0.62	3.90	4.53	1.60	2.57
Per:	100g	58	3.79	2.76	3.30	0.21	1.30	1.51	0.53	0.86

✂ Cut along the dotted line

Bioavailability Of sulforaphane from Broccoli Soups

(BOBS)

I am interested in taking part and/or finding out more information about this study (please complete the personal details below).

Name: ……..

Address: ……

……..

Daytime telephone no.: ……...

Evening telephone no.:……...

Mobile no.:.....

I am happy for a message to be left via my daytime/evening number: YES/NO

please circle as applicable

Preferred time to call:.....

E-mail address: ……

Where did you hear about the study?

☐ **HNU database** ☐ **Study adverts**

☐ **Media (TV/newspapers)** ☐ **Social networks(facebook/twitter/gumtree)**

Other (please state):……...

Please return this form in the FREEPOST envelope provided to:

Miss Tharsini Sivapalan/Dr Antonietta Melchini/

Institute of Food Research

FREEPOST NC 252

Norwich Research Park

Colney

Norwich

NR4 7BR

Expressing an interest does not commit you to taking part in the study

We need your help!

Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

We want to find out how much of a naturally occurring compound called sulforaphane, that comes from cruciferous vegetables (e.g. broccoli, cauliflower, cabbage) is absorbed by our bodies by eating three types of broccoli + stilton soup.

- Are you interested in taking part in a study?
- Are you aged 18-65 (male and female)?
- Are you a non-smoker?
- Do you live within 40 miles of Norwich?
- Do you like broccoli and stilton soup?

If you answer yes to all the above questions then you may be eligible to take part in our study

You would need to:

- Follow a diet free of cruciferous vegetables for 3 days on 3 occasions separated by a minimum of 2 weeks
- Provide blood and urine samples
- Come to the Human Nutrition Unit for 8 visits

We will:

- Reimburse your travel costs
- Compensate you for taking part in the study

Contact us to find out further information:

Tharsini Sivapalan
01603251425
tharsini.sivapalan@ifr.ac.uk

IFR Institute of Food Research

Dr Antonietta Melchini
01603 255030
antonietta.melchini@ifr.ac.uk

Date

Dear_____,

Thank you for your interest in.

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

at the Institute of Food Research

I have sent you details of this study which is in progress at present as you have responded to the advert and you may fit the criteria. If you have any further questions about the study, please contact, Tharsini Sivapalan on 01603251425 (tharsini.sivapalan@ifr.ac.uk) or Dr Antonietta Melchini on 01603 255030 (antonietta.melchini@ifr.ac.uk) or as stated on the enclosed participant information sheet.

If you are interested in taking part or getting more information, fill out the reply slip of your participant information sheet, return it to the Institute of Food Research in the freepost envelope provided and a member of the study team will be in touch.

Thank you.

Yours sincerely,

Miss Tharsini Sivapalan

On behalf of the BOBS study team

Norwich Research Park, Colney, Norwich NR4 7UA, UK

www.ifr.ac.uk Tel: +44(0) 1603 255000 GTN 6626 5000 Fax: +44 (0)1603 507723

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

APPOINTMENT CARDS

Visit 2: Screening / Rescreening:

Day_____

For screening bring with you a mid stream urine sample produced within 2 hours of your appointment

Date_____

Time_____

Phase (1)

Day 1 ☐

Day 2 ☐

Preparation for Study days

Avoid eating foods high in glucosinolates,spicy food and alcohol for 48 hours (2 days) prior to study day

Visit 3: HNU Cannulation

Fast for at least 10 hours & drink as much plain water as you need during the fast period. Bring with you a mid-stream urine sample from that morning.

Day & Date _____

Start Time_____

Avoid eating foods high in glucosinolates, spicy food and alcohol throughout the day.

Visit 4: HNU 24 hour blood

Fast for at least 10 hours & drink as much plain water as you need during the fast period. Bring with you the 24 hour urine collection.

Day & Date _____

Start Time_____

No diet restrictions between the phases

Phase (2)

Day 1 ☐

Day 2 ☐

Preparation for Study days

Avoid eating foods high in glucosinolates,spicy food and alcohol for 48 hours (2 days) prior to study day

Visit 5: HNU Cannulation

Fast for at least 10 hours & drink as much plain water as you need during the fast period. Bring with you a mid-stream urine sample from that morning.

Day & Date _____

Start Time_____

Avoid eating foods high in glucosinolates, spicy food and alcohol throughout the day.

Visit 6: HNU 24 hour blood

Fast for at least 10 hours & drink as much plain water as you need during the fast period. Bring with you the 24 hour urine collection.

Day & Date _____

Start Time_____

No diet restrictions between the phases

Phase (3)

Day 1 ☐

Day 2 ☐

Preparation for Study days

Avoid eating foods high in glucosinolates,spicy food and alcohol for 48 hours (2 days) prior to study day

Visit 7: HNU Cannulation

Fast for at least 10 hours & drink as much plain water as you need during the fast period. Bring with you a mid-stream urine sample from that morning.

Day & Date _____

Start Time_____

Avoid eating foods high in glucosinolates, spicy food and alcohol throughout the day.

Visit 8: HNU 24 hour blood

Fast for at least 10 hours & drink as much plain water as you need during the fast period. Bring with you the 24 hour urine collection.

Day & Date _____

Start Time_____

If you are unable to make the appointment please contact Human Nutrition Unit on 01603 255305. Thank you.

IFR Institute of Food Research

Consent Form for Research Study

Study Title: The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

Chief Investigator: Prof Richard Mithen

Volunteer please initial each box

I confirm that I have read and understand the information sheet **dated** ……; **version no**. ……; for the above study, and I have had the opportunity to consider the information, discuss the study and ask questions. ☐

I confirm that I have received satisfactory answers to my questions. ☐

I have been informed about the purpose of the genetic test and that it has no clinical significance for me or my family and I will not receive the results of this test. ☐

I agree and consent to having a blood sample taken for this test. ☐

I understand that my participation is voluntary, and I am free to withdraw from the study (1) at any time without giving a reason and (2) without my withdrawal affecting future participation in other research studies at IFR ☐

With who have you discussed the information for this research study?

Name: ……..

Role: BOBS Study Scientist / Nurse

I understand that any of my personal information and data collected during the study may be looked at by individuals at IFR, where it is relevant to participation in this study. ☐

I give permission for these individuals to have access to my information and data. ☐

I understand that my personal information and data will be held confidentially at IFR and that it will be destroyed after 15 years. ☐

I agree to my GP being informed of my participation in the study, and for my clinical results to be sent to my GP

Name and address of your General Practitioner:

……
……..……
……
……

……
……

I understand that all research is subject to inspection and audit. ☐

NB: although your records may be accessed for this purpose your personal information remains confidential

I agree to take part in the above study.

Signed: ……..
(Name in BLOCK letters)……

Date: …….. **Date of Birth**: ……..……

I confirm that the participant above has been given a full verbal and written explanation of the study.

Signed: ……
(Name in BLOCK letters)……

Role: …… (BLOCK letters)
Date: ……..……

If you are unable to make the appointment please contact Human Nutrition Unit on 01603 255305. Thank you.

IFR Institute of Food Research

Study Title: The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

MEDICATION/ MEDICAL CONDITIONS

DECLARATION AGREEMENT

Certain illnesses and medication may affect the outcome of research studies.

Therefore, we would like you to inform the study organisers if you

start taking medication

suffer from any illness

Please sign below to confirm that you have agreed to this request.

I…….consent to inform the study organiser of the commencement of any medication/medical changes whilst participating in the study

Signature of volunteer…….

Date……..

Signature of scientist…….

If you are unable to make the appointment please contact Human Nutrition Unit on 01603 255305. Thank you.

IFR Institute of Food Research

Study Title: The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

CONFIDENTIAL Participant Eligibility Screening Questionnaire

Participant code number…… **Sex**: Male / Female

Date of birth: ……

Age: ……**years**

Height:……**Metres**

Weight: ……**Kg**

Body Mass Index (BMI, kg/m^2)……

Blood Pressure: **Right arm**…….. **Left arm**…… **Pulse**: ……

Urinalysis: see page 4

Have you ever had any of the following? If yes give details below each relevant section.

Angina/heart disease: Y N

……

……

Thrombosis: Y N

……

……

High Blood Pressure: Y N

……

……

High cholesterol: Y N

……

……

Chest problems: Y N **Diabetes:Y N**

…… ……

…… ……

Depression or anxiety: Y N **Gastrointestinal disorder/surgery: Y N**

…… ……

…… ……

Skin conditions: Y N **Liver problems: Y N**

…… ……

…… ……

Inflammatory disease: E.g. rheumatoid arthritis, psoriasis Y N

……

……

Other:

……

……

Are you currently on any of the following?

If yes, give details below each relevant section of brand, dosage, frequency, when started etc.

Prescribed medication: Y N

……

……

Dietary Supplements: Y N **Herbal remedies: Y N**

…… ……

…… ……

If taking broccoli and/or an ITC supplement, participant will have to discontinue use for at least 1 month before starting the intervention. If participant not happy to do so, please exclude from study.

WOMEN ONLY SECTION

Are you/could you be pregnant? Y N

Have you been pregnant within the last 12 months? Y N

Are you breast-feeding? Y N

End of women only section

Have you had a major physical injury/operation? If yes give details below: Y N

……

……

Are you currently suffering from any illness/injury? If yes give details below: Y N

……

……

SMOKING:

Are you currently a: **Non-smoker / Current smoker / Ex-smoker / Lifelong smoker (circle appropriate)**

What do/did you smoke? (E.g. cigarettes, roll ups, cigars, pipe etc.)……

If a non-smoker, have you ever smoked? Y N If yes, how long since you stopped smoking?.....

If an ex-smoker: How long since you **stopped smoking**:……How many did you smoker each day?.....

If currently a smoker/lifelong smoker: How many years have you been smoking?……..

DRINKING:

Do you drink alcohol: Y N How many units do you drink per week? ……

A unit of alcohol is approximately half a pint of beer or lager, a single pub measure of spirit e.g. gin/vodka or a small glass of wine (125mL).

DIETARY QUESTIONS:

Are you a vegan or vegetarian? Y N

Do you have any special dietary requirements: Y N

If yes state:……

……

Have you any known allergies: Y N

Food:……**Drugs**:……

……

Other:

……

……

BLOOD DONOR:

Are you a regular blood donor? Y N

If yes, when did you last donate blood?

……

If within 16 weeks months, suspend screening until 16 weeks has elapsed since last donor session

Are you happy not to donate blood while participating in this intervention study?

Y N

If no, exclude from study.

GENERAL PRACTITIONER:

Do you agree to us informing your General Practitioner of your participation in the study or of any results found?

Y N

If you have answered NO to this question then we are unable to accept you on this study.

Name and Address of your General Practitioner:

……

……

……Telephone number:…….

Form completed by (print):……Signature:……Date:……

As far as you know are you related to or living with any member of study team?

Y N

Are you currently participating in another research study?

Y N

If yes check with participant whether involves dietary intervention/blood sampling – refer to HNU Senior Research Nurse or scientist

Are you currently undergoing any GP/Hospital investigations?

Y N

(Add any other required information as per study exclusion criteria)

Form completed by (print):……Signature:……

Date: ……..

Designation:…….

URINE DIPSTICK TEST RESULTS

Attach to screening questionnaire to be kept at Study centre

Study Title:

Participant code number **(NOT NAME)**:……Date of Birth:……Male/Female (circle)

Date of sample:…… Time of sample:…….

URINE DIP STICK TEST RESULTS:

pH:…… Protein:……. Glucose:……

Ketones:……..Urobilinogen:…….

Bilirubin: ……. Blood:…….. Leucocytes:……..Nitrites:……Specific Gravity: ……..

Test performed by:…….

Signature:…….

Date:……Time:……

N.B. If positive for blood and participant is female please ask if they are menstruating, if answer is yes, repeat the test once volunteer has ceased menstruating.

Menstruating: Y N Date due to finish menstruating:……..

URINE DIP STICK TEST RESULTS REPEAT TEST RESULTS: Y N

pH:…….

Protein:……..Glucose:……..Ketones:……..Urobilinogen:……..

Bilirubin: ……Blood:……Leucocytes:……Nitrites:……Specific Gravity: ……

Date of repeat test:……Time:……

Repeat test performed by:…… Signature:……

Abnormal results referred to HNU protocol 'for the referral of abnormal urinalysis results at screening': Y N

Comments:……

……

……

……

……

……

Please note a copy of these results must be sent to the participant's G.P on the Clinical Test Results Form.

If you are unable to make the appointment please contact Human Nutrition Unit on 01603 255305. Thank you.

IFR Institute of Food Research

CONFIDENTIAL

CASE REPORT FORM

Study Eligibility Screening

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

PARTICIPANT STUDY ID:

Chief Investigator Prof Richard Mithen

Study Manager/Study Coordinator

INSTRUCTIONS FOR COMPLETION OF CRFS

The Case Report Forms (CRF) are to be completed in **black ballpoint pen** only. All entries must be printed and legible.

Ensure that the subject ID is consistent throughout the document.

Each part of the CRF is to be completed as indicated.

Dates are to be written as follows: 01 /01/2013

dd mm yyyy

Day Month Year

Times are to be written using the 24h clock configuration as follows:- 00 : 00 (Midnight)

Hours Minutes

When an answer to a question is required, mark the box corresponding to the response:

Example: "YES" YES ☑ NO ☐

Example: "NO" YES ☐ NO ☑

The following abbreviations are to be used:-

ND = Not Done, NA = Not Applicable, NK = Not Known

If comments are required, print them clearly and briefly in English using capital letters.

Corrections are to be made by striking out the erroneous data with a **single line** in such a way that the **original entry remains legible.** The correct datum is then written clearly alongside the erroneous datum. Do NOT use correction fluid.

All **corrections are to be dated**and **initialled**. A reason must be given for each correction or missing datum.

If a whole page is to be changed, the page is to be **struck out with a diagonal black line** and a copy inserted in the CRF, dated and initialled.

Ensure that all boxes are completed. If a subject discontinues early, ensure that the Header information for all of the remaining pages is completed and a single line drawn through each uncompleted page.

The Chief/Principal Investigator/Study Manager/Study Coordinator must sign and date the Study Completion page to certify completeness of the CRF.Only those members of staff trained and responsible for completing the specified duties may complete the CRF.

Participant ID	Date of Visit	Screening
☐☐☐	___/_____/____ dd mm yyyy	

INFORMED CONSENT		
Has the participant given signed Written Consent to be included in this study?	Yes ☐	No ☐ **If no, please do not continue**
Date consent obtained	____/_____/____ dd mm yyyy	
Has the participant kept a copy of the information sheet and the consent form?	Yes ☐	No ☐

INCLUSION CRITERIA **This should fit study inclusion criteria Examples below**

If the answer to any of the criteria below is **NO**, the participant does not qualify for the study

Is the participant *male/female?*	Yes ☐	No ☐
Is the participant18-65years of age?	Yes ☐	No ☐
Is the BMI within ≤20 or ≥35kg/m^2 range	Yes ☐	No ☐
Does the participant live within 40 miles of IFR?	Yes ☐	No ☐
Is the participant a non-smoker?	Yes ☐	No ☐
Does the participant like Broccoli and Stilton soup?	Yes ☐	No ☐
Is the participant able to provide written informed consent?	Yes ☐	No ☐

Participant ID	Date of Visit	Screening
☐☐☐	___/_____/____ dd mm yyyy	

EXCLUSION CRITERIA If the answer to any of the criteria below is **YES**, the volunteer does not qualify for the study		
Is the participant related to or living with any member of the study team?	Yes ☐	No ☐
Does the participant have an intolerance / allergy to any member of the Brassica family, Brassica pollens or any of the constituents of the test meal/study food?	Yes ☐	No ☐
Is the participant a smoker or have they smoked within the **last six months**?	Yes ☐	No ☐
Has the participant been diagnosed with any long term medical condition(e.g. diabetes, cancer, cardiovascular disease) requiring medication that may affect the study outcome?	Yes ☐	No ☐
Is the participant pregnant, have they been pregnant in the last 12 months or are they breast feeding?	Yes ☐	No ☐
Is the participant willing to provide blood and urine samples?	Yes ☐	No ☐
Does the participant have a known history of fainting when having blood samples taken?	Yes ☐	No ☐
Does the participant take any prescribed or over the counter (self-prescribed) medications which could affect the study data?	Yes ☐	No ☐
Is the participant receiving any anti-coagulant therapy or has received any anti-coagulant therapy in the last 3 months?	Yes ☐	No ☐
Is the participant taking part in another study which involves dietary intervention and/or blood sampling?	Yes ☐	No ☐

Participant ID	Date of Visit	Screening
☐☐☐	___/_____/____ dd mm yyyy	

Has the participant participated in another research study in the last 4 months which involves blood sampling? *If yes exclude unless total blood from both studies does not exceed 470 ml or participant is willing to wait 4 months and be re-screened.*	Yes ☐	No ☐
Is the participant's blood pressure greater than 160/100 or less than 90/50 or 95/55 if symptomatic? *Either systolic or diastolic or both*	Yes ☐	No ☐
Does the participant have any special dietary requirements which may affect the study data?	Yes ☐	No ☐
Does the participant agree to their GP being informed of their participation in study and to provide details of GP?	Yes ☐	No ☐
Is the participant on long term antibiotic therapy or used any antibiotics within the last month?	Yes ☐	No ☐
Is the participant a regular blood donor? If yes Date of last donation Date: ____/_____/_____ dd mm yyyy **If less than 16 weeks postpone screening until after 16 weeks**	Yes ☐	No ☐
Does the participant agree not to donate blood for the duration of their involvement on the study?	Yes ☐	No ☐
Print Name _____ Signed_____ Date: ____/_____/_____ (*dd mm yyyy)* Role:_____		

Participant ID	Date of Visit	Screening
	___/_____/____ dd mm yyyy	

URINE DIPSTICK TEST RESULTS			
Combur 9 urine dipstick test used		*Time urine voided:*	
pH		Protein	
Glucose		Ketones	
Urobilinogen		Biliruben	
Blood		Leucocytes	
Nitrites			

Urine should be tested within 2 hours of voiding for manufacturer's validity. If more than 2 hours has elapsed ask participant to collect another sample and discard first sample.

ANTHROPOMETRIC MEASUREMENTS			
Blood pressure		**Pulse**	
Right arm	Left arm	Right arm	Left arm
Height (cm)		**BP Cuff used** (circle as appropriate)	Small / Large
Weight (kg)		**BMI (kg/m^2)**	

Participant ID	Date of Visit	Screening
☐☐☐	___/_____/____ dd mm yyyy	

SUBJECT ELIGIBILITY	
Are the participant's veins suitable for cannulation?	Yes ☐ No ☐
Is the subject eligible for the study according to screening questionnaire and anthropometric measurements?	Yes ☐ No ☐
If No, what was the reason for decision?	
Print Name _____ **Signed** _____ **Date: ____/_____/_____** **HNU Senior Research Nurse** **dd mm yyyy**	

Medication	Prescribed Yes/No	Self- treated Yes/No	Nurse's Signature	Date
				____/_____/____ dd mm yyyy
				____/_____/____ dd mm yyyy

Participant ID	Date of Visit	Screening
	___/_____/____ dd mm yyyy	

				____/_____/____ dd mm yyyy
				____/_____/____ dd mm yyyy
				____/_____/____ dd mm yyyy
				____/_____/____ dd mm yyyy

Participant ID	Date of Visit	Screening
☐☐☐	___/_____/____ dd mm yyyy	

Dietary Supplements/Herbal remedies	Need to stop for study Yes/No	Checked with Scientist Yes/No	Nurse's Signature	Date
				___/_____/____ dd mm yyyy
				___/_____/____ dd mm yyyy
				___/_____/____ dd mm yyyy
				___/_____/____ dd mm yyyy

Participant ID	Date of Visit	Screening
☐☐☐	___/_____/____ dd mm yyyy	

SUBJECT ELIGIBILITY SCREENING BLOODS	
Has blood been taken for: Full Blood Count (FBC) Urea & Electrolytes (Us&Es) HbA1c	Yes ☐ No ☐ If No state reason:
Were there any problems when taking blood?	Yes ☐ No ☐
If Yes state what problems? If participant felt unwell/faint/fainted during or after blood sampling exclude from study	
Blood sample taken by: **Print Name**_____ **Signature:**_____ **Role:**_____ **Date:** ____/_____/_____ **dd mm yyyy**	
Were the screening blood results satisfactory for study inclusion? Yes ☐ No ☐ If NO which results were flagged? FBC Yes ☐ No ☐ Us&Es Yes ☐ No ☐ HbA1c Yes ☐ No ☐	

Participant ID	Date of Visit	Screening
☐☐☐	___/_____/____ dd mm yyyy	

Were flagged blood results referred to HNU Medical Advisor? Yes ☐ No ☐

If NO state why:_____

Referred by: Print Name:_____

Signature:_____

Date referred: **Role:**

Has the participant been referred for a re-screen? Yes ☐ No ☐

Re-screen blood? Yes ☐ No ☐ Re-screen urine? Yes ☐ No ☐

Date re-screened:_____

Did the re-screen results exclude the participant from the study? Yes ☐ No ☐

FEMALE PARTICIPANTS	
Do you menstruate regularly?	**Yes** ☐ **No** ☐ **If No state reason:**
How long is your typical menstrual cycle?	

Participant ID	Date of Visit	Screening
☐☐☐	___/_____/____ dd mm yyyy	

COMMENTS			
Any comments to record? Yes ☐ No ☐ **Please record any additional information relevant to the study. Date and initial.** **Each comment should be completed by the appropriate Study Staff member.**			
CRF Page Number	**Item**	**Comment**	**Date & Initials**

Participant ID	Date of Visit	Screening
☐☐☐	___/_____/____ dd mm yyyy	

PROTOCOL VIOLATIONS OR DEVIATIONS						
Were there any protocol violations or deviations during the Screening process? Yes ☐ No ☐ If Yes, please detail below:						
Description	**Reason**	**Deviation**	**Outcome**	**CRF Page**	**Personnel Initial**	**Date**
						___/_____/20__ dd mm yyyy

Participant ID	Date of Visit	Screening
☐☐☐	___/_____/____ dd mm yyyy	

						___/_____/20__ dd mm yyyy
						___/_____/20__ dd mm yyyy

STUDY STATUS

Did subject complete the screening process? **Yes** ☐ **No** ☐ If **No** complete box below.

Date of withdrawal: **____/_____/201**

dd mm yyyy

At whose request did subject withdraw?

☐ Subject

☐ Investigator

☐ Sponsor

☐ Subject's GP

☐ Other (specify) _____

Primary Reason for withdrawal due to (please tick one):

☐ Failure to meet inclusion criteria

☐ Failure to provide a screening blood sample

☐ Failure to comply with other requirements of the protocol (record on the comments page)

☐ Failure to meet Blood Pressure criteria

☐ Withdrawal of Consent

☐ Other (specify) _____

Was any Re-screen procedure necessary?	Yes ☐	No ☐
If yes, please provide details		

INVESTIGATOR STATEMENT

To the best of my knowledge, I confirm that I have made every reasonable effort to ensure that the data presented in this Case Record Form are a true, accurate and complete report and accurately reflect the study conduct during the screening process.

Print Name _____

Signed _____ **Date: ____/_____/201**

Investigator **dd mm yyyy**

IFR Institute of Food Research

CONFIDENTIAL

CASE REPORT FORM

Study Phase 1-3

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

PARTICIPANT STUDY ID:

Chief Investigator Prof Richard Mithen

Study Manager/Study Coordinator

INSTRUCTIONS FOR COMPLETION OF CRFS

The Case Report Forms (CRF) are to be completed in **black ballpoint pen** only. All entries must be printed and legible.

Ensure that the subject ID is consistent throughout the document.

Each part of the CRF is to be completed as indicated.

Dates are to be written as follows: 01 /01/2013

dd mm yyyy

Day Month Year

Times are to be written using the 24h clock configuration as follows:- 00 : 00 (Midnight)

Hours Minutes

When an answer to a question is required, mark the box corresponding to the response:

Example: "YES" YES ☑ NO ☐

Example: "NO" YES ☐ NO ☑

The following abbreviations are to be used:-

ND = Not Done, NA = Not Applicable, NK = Not Known

If comments are required, print them clearly and briefly in English using capital letters.

Corrections are to be made by striking out the erroneous data with a **single line** in such a way that the **original entry remains legible.** The correct datum is then written clearly alongside the erroneous datum. Do NOT use correction fluid.

All **corrections are to be dated** and **initialled**. A reason must be given for each correction or missing datum.If a whole page is to be changed, the page is to be **struck out with a diagonal black line** and a copy inserted in the CRF, dated and initialled.

Ensure that all boxes are completed. If a subject discontinues early, ensure that the Header information for all of the remaining pages is completed and a single line drawn through each uncompleted page.

The Chief/Principal Investigator/Study Manager/Study Coordinator must sign and date the Study Completion page to certify completeness of the CRF.Only those members of staff trained and responsible for completing the specified duties may complete the CRF.

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

STUDY DAY CRITERIA		
Has the participant given signed Written Consent to be included in this study?	Yes ☐ No ☐ **If no, do not continue**	
Is the participant happy to continue on study?	Yes ☐ No ☐ **If no, do not continue**	
Is the participant well today? *If **No** postpone study day and inform scientist*	Yes ☐	No ☐
Has the participant been well over previous five days? *If **No** postpone study day and inform scientist. List any illness on comments page with date and duration of illness.*	Yes ☐	No ☐
Does the participant have any known allergies? *If **Yes** state below and exclude if allergic to any content of the study foods/soups/Brassica family or pollens*	Yes ☐	No ☐
Allergies:		
Has the participant fasted for a minimum of 10 hours? *If **No** postpone study day and inform scientist*	Yes ☐	No ☐
When was the last time the participant consumed any of the foods below and which:	Date	Time
Foods in high glucosinolates Yes ☐ Alcohol Yes ☐ Spicy food Yes ☐		

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

Female participants only: **Are you menstruating or just finished menstruating?** *If menstruating or just finished then postpone study day 7 days after menstruating and inform scientist* **When did your last period end?** *If no menstrual cycle for 2 months or more ask 'could you be pregnant?'*	**Yes** ☐	**No** ☐
Did the participant have any problems when screening blood taken? *If participant felt unwell/faint/fainted during or after blood sampling exclude from study*	Yes ☐	No ☐
Has the participant take any prescribed or over the counter (self-prescribed) medications since their last visit? *If **Yes** list on medications sheet of CRF and postpone study day/exclude from study as advised by HNU medical advisor*	Yes ☐	No ☐
Has the participant received/taken any anti-coagulant therapy since last visit? *If **Yes** list on medications sheet of CRF and postpone study day/exclude from study as advised by HNU medical advisor*	Yes ☐	No ☐
Has the participant used/taken any antibiotics since last visit? *If **Yes** list on medications sheet of CRF and postpone study day/exclude from study as per study protocol*	Yes ☐	No ☐

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

Is the participant a blood donor?	Yes ☐	No ☐
If yes Date of last donation Date: ___/___/___ dd mm yyyy		No ☐
Has the participant donated blood since their screening?	Yes ☐	
If yes exclude from study		

Print Name _____ Signed_____

Date: ____/_____/_____ (*dd mm yyyy)*

Role:_____

URINE DIPSTICK TEST RESULTS			
Combur 9 urine dipstick test used		*Time urine voided:*	
pH		Protein	
Glucose		Ketones	
Urobilinogen		Biliruben	
Blood		Leucocytes	
Nitrites			

Urine should be tested within 2 hours of voiding for manufacturer's validity. If more than 2 hours has elapsed ask participant to collect another sample and discard first sample.

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

ANTHROPOMETRIC MEASUREMENTS			
Blood pressure		**Pulse**	
Right arm	Left arm	Right arm	Left arm
Height (cm)		**BP Cuff used** (circle as appropriate)	Small / Large
Weight (kg)		**BMI (kg/m^2)**	

SUBJECT ELIGIBILITY	
Are the participant's veins suitable for cannulation?	Yes ☐ No ☐
Is the subject eligible for the study according to screening questionnaire and anthropometric measurements?	Yes ☐ No ☐
If No, what was the reason for decision?	
Print Name _____ **Signed** _____ **Date:** ____/_____/_____ **HNU Senior Research Nurse** **dd mm yyyy**	

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

Medication	Prescribed Yes/No	Self- treated Yes/No	Nurse's Signature	Date
				___/_____/____ dd mm yyyy
				___/_____/____ dd mm yyyy
				___/_____/____ dd mm yyyy
				___/_____/____ dd mm yyyy
				___/_____/____ dd mm yyyy

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

COMMENTS			
Any comments to record? Yes ☐ No ☐ **Please record any additional information relevant to the study. Date and initial.** **Each comment should be completed by the appropriate Study Staff member.**			
CRF Page Number	**Item**	**Comment**	**Date & Initials**

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

STUDY DAY BLOOD PRESSURE MEASUREMENTS (Pre-cannulation)	
Measured by: Print Name_____Signature:_____ Role:_____	
Arm with highest reading at screening to be used **Left arm Right arm** (*circle as appropriate)*	**If BP >160/100 or <90/50 or <95/55 (if symptomatic) repeat x 3 at 15 minute intervals** *If BP remains above 160/100 or below 90/50 or 95/55 (if symptomatic) exclude participant from study*

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

	Initial Measurement	Repeat 1	Repeat 2	Repeat 3		
BP						
Pulse						
Time measured						
		Satisfactory for cannulation?	Yes ☐ No ☐	**Participant Excluded**	Yes ☐ No ☐	

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

CANNULATION	
Type of cannula used: Size (Gauge): Name of nurse cannulating:_____	
Cannulation site : 1)_____(2)_____ Time Cannulated: (1)_____(2)_____ Length of time cannulated:_____ Time cannula removed:_____	
Batch No:_____ Exp. Date:_____ Sterile normal saline for injection used to flush cannula: Total amount of saline used during study day:_____	

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

Any problems during cannulation: Yes ☐ No ☐	If **YES** give details:	
Was the cannulation site assessed by HNU nurse before leaving HNU? Yes ☐ No ☐	Was the participant well on leaving HNU? Yes ☐ No ☐ Time left HNU:_____ Name of nurse responsible for assessing the participant before they left HNU: Print name:_____Signature:_____ Role:_____	

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

24 HOUR SAMPLES (URINE AND BLOOD)	
Has the participant fasted for a minimum of 10 hours? Yes ☐ No ☐	Is the participant well? Yes ☐ No ☐
Venepuncture site used:_____	Has the 24hour blood sample been taken? Yes ☐ No ☐

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

	Any problems taking blood sample? Yes ☐ No ☐ If YES state:_____
URINE	Has the participant handed urine sample to member of study team? Yes ☐ No ☐
Did the participant comply with study diet restrictions during Phase 1? Yes ☐ No ☐	Was the participant able to eat the whole portion of soup? Yes ☐ No ☐ If No – exclude from study and give reason for not finishing the soup: _____ _____

Participant ID	Date of Visit	STUDY DAY 1-3 PHASE 1
☐☐☐	___/_____/____ dd mm yyyy	

PROTOCOL DEVIATIONS						
Were there any protocol violations or deviations during Phase 1? Yes☐ No ☐ If YES, please detail below:						
Description	**Reason**	**Deviation**	**Outcome**	**CRF Page**	**Personnel Initial**	**Date**
						____/_____/ dd mm yyyy
						____/_____/ dd mm yyyy

CONCOMITANT MEDICATIONS

Were there any medications taken during Phase 1?

Yes☐ No ☐ If YES, please detail below:

Medication (Trade name preferred)	**Date**	**Dose (Specify units)**	**Route**	**Frequency**	**Indication**	**Ongoing (√)**
	Start Date ____/____/____ dd mm yyyy					
	Stop Date ____/____/____ dd mm yyyy					
	Start Date ____/____/____ dd mm yyyy					
	Stop Date ____/____/____ dd mm yyyy					
	Start Date ____/____/____ dd mm yyyy					
	Stop Date ____/____/____ dd mm yyyy					
	Start Date ____/____/____ dd mm yyyy					

Participant ID	Date of Visit	STUDY DAY 1 PHASE 1-3
☐☐☐	___/_____/____ dd mm yyyy	

	Stop Date ____/____/____ dd mm yyyy					
	Start Date ____/____/____ dd mm yyyy					
	Stop Date ____/____/____ dd mm yyyy					
	Start Date ____/____/____ dd mm yyyy					
	Stop Date ____/____/____ dd mm yyyy					
	Start Date ____/____/____ dd mm yyyy					
	Stop Date ____/____/____ dd mm yyyy					
	Start Date ____/____/____ dd mm yyyy					

Participant ID	Date of Visit	STUDY DAY 1 PHASE 1-3
☐☐☐	___/_____/____ dd mm yyyy	

	Stop Date ____/____/____ dd mm yyyy					

NON-SERIOUS AND SERIOUS ADVERSE EVENTS

Categorisation of Severity

The grading of adverse events by the Investigator will take into consideration the following 3-point scale:

Mild:

For example, an adverse event that is easily tolerated by the volunteer, causing minimal discomfort and not interfering with everyday activities.

Moderate:

For example, an adverse event that is sufficiently discomforting to interfere with normal everyday activities.

Severe:

For example, an adverse event that prevents normal everyday activities.

Relationship to Study Procedures

The relationship of the clinical adverse events to the study procedure will be categorised as follows:

Not related:

This category includes such adverse events that, after careful medical consideration at the time of such evaluation, are judged to have clearly and incontrovertibly no relation with the study procedures. Adverse events must have a definite extraneous cause (disease, environmental reasons, etc.). Criteria listed under "remote", "possible", and "probable" must not apply.

Participant ID	Date of Visit	STUDY DAY 1 PHASE 1-3
☐☐☐	___/_____/____ dd mm yyyy	

Remote:

This category includes such adverse events for which, after careful medical consideration at the time of evaluation, a remote possibility for a relation with the study procedures is taken into consideration, but which are judged with a high degree of certainty to have an unlikely relation.

An adverse event is classified as "remote" if the following conditions apply:

1. It does not follow a reasonable temporal sequence from the performance of the study procedures.

2. It can very easily be explained by the subject's general condition, environmental conditions, toxic factors, or other kinds of therapy or treatment administered to the subject.

3. It does not follow a known response pattern to the study procedures.

Possible:

This category includes such adverse events for which, after careful medical consideration at the time of evaluation, a possible relation with the study procedures must be taken in consideration or cannot be ruled out with certainty, but which is judged to have more likely no relation. An adverse event is classified as "possible" if the following conditions apply:

1. It follows a reasonable temporal sequence from the performance of the study procedures.

2. It can also easily be explained by the subject's general condition, environmental conditions, toxic factors, or other kinds of therapy or treatment administered to the subject.

3. It follows a known response pattern to the suspected procedures.

Participant ID	Date of Visit	STUDY DAY 1 PHASE 1-3
☐☐☐	___/_____/____ dd mm yyyy	

Probable:

This category includes such adverse events which, after careful medical consideration at the time of evaluation, are related to the study procedures with a high degree of certainty. An adverse event is classified as "probable" if the following conditions apply:

It follows a reasonable temporal sequence from administration of the study procedures.

2. It cannot be reasonably explained by the subject's general condition, environmental conditions, toxic factors, or other kinds of therapy or treatment administered to the subject.

3. It follows a known response pattern to the suspected procedures.

ADVERSE EVENTS	
Did the subject experience any adverse events during the study? Yes ☐ No ☐ **If Yes, please complete <u>ALL</u> sections below:**	
Event Diagnosis	
Start Date	___/____/201 dd mm yyyy
Start Time	☐☐:☐☐
Stop Date	___/____/201 dd mm yyyy

Participant ID	Date of Visit	STUDY DAY 1 PHASE 1-3
☐☐☐	___/_____/____ dd mm yyyy	

Stop Time		☐☐:☐☐
Frequency	**1 = Constant** **2 = Intermittent**	
	If Intermittent (i.e. > 1 episode) please provide number of episodes	
Severity	**1 = Mild** **2 = Moderate** **3 = Severe**	
Relationship to Study Procedures	**1 = Not Related** **2 = Remote** **3 = Possible** **4 = Probable**	
Action Taken	**1 = None** **2 = Referred to Subject's GP** **3 = Other**	**If Other, please specify**
Outcome	**1 = Resolved** **2 = Ongoing** **3 = Lost to Follow Up** **4 = Death**	

Participant ID	Date of Visit	STUDY DAY 1 PHASE 1-3
☐☐☐	___/_____/____ dd mm yyyy	

Serious	**Yes** ☐ **No** ☐		
Principal Investigator's Signature	_____	**Date**	___/____/201 dd mm yyyy

Participant ID	Date of Visit	STUDY DAY 1 PHASE 1-3
☐☐☐	___/_____/____ dd mm yyyy	

STUDY STATUS
Did subject complete Phase 1? **Yes** ☐ **No** ☐ If **No** complete box below.
Date of withdrawal: **____/_____/201** **dd mm yyyy** **At whose request did subject withdraw?** ☐ Subject ☐ Investigator ☐ Nurse ☐ Subject's GP ☐ Other (specify) _____ **Primary Reason for withdrawal due to (please tick one):** ☐ Blood pressure (record on comments page) ☐ Failure to comply with other requirements of the protocol (record on the comments page) ☐ Serious Adverse Event/Reaction/ Adverse Event (record on the adverse event page)

Participant ID	Date of Visit	STUDY DAY 1 PHASE 1-3
☐☐☐	___/_____/____ dd mm yyyy	

☐ Withdrawal of Consent ☐ Other (specify) _____	
Was any Follow up procedure necessary?	Yes ☐ No ☐
If yes, please provide details	

INVESTIGATOR STATEMENT
To the best of my knowledge, I confirm that I have made every reasonable effort to ensure that the data presented in this Case Record Form are a true, accurate and complete report and accurately reflect the study conduct for the Phase. **Print Name** _____ **Signed** _____ **Date: ____/_____/201** **Investigator** **dd mm yyyy**

IFR Institute of Food Research

CLINICAL TEST RESULTS FORM- TO BE SENT TO THE

PARTICIPANT'S GP

Study Title: The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

Study Title ……

Participant's Name:……

Date of Birth:…….. Male/Female:……..

Dipstick urine test results: Combur[9] dipstick test strips used. Test areas compared after 60 seconds as per manufacturers guide.

pH…… Protein:…… Glucose:…… Ketones:…… Nitrites:…… Bilirubin:……

Urobilinogen:…… Blood: (ERY)……. (Hb):….. …. Leucocytes……

Menstruating: Y N N/A (circle as appropriate)

Test performed by (print name): …….. Signature:……

Date:…… Time:……

Repeat test required at surgery prior to re-screen: Y N N/A (circle as appropriate)

Repeat urine dipstick test results (HNU). Tested using urine dipstick test strips/guide times as above

pH……Protein:……Glucose:……Ketones:……Nitrites:……Bilirubin:…….

Urobilinogen:……. Blood: (ERY)…… (Hb):…..…… Leucocytes……

Test performed by (print name): …….. Signature:……

Date:…… Time:……

Observations:

Blood Pressure: Right arm:…… Left arm:……. Pulse: Rt…… Lt…….

Weight(kg): ……. BMI(kg/m^2):……Reference range for study: ≥ 20 - ≤35 kg/m^2

Copy of screening blood results attached: Yes No Not applicable (circle as appropriate)

The ……..(insert as appropriate) result(s) will / will not exclude your patient from this study.

Date:…….. Signature:……

Abnormal results are referred to the HNU Medical Advisor for comments regarding participation in the study.

If you are unable to make the appointment please contact Human Nutrition Unit on 01603 255305. Thank you.

Date

Dear Doctor (insert name of participant's GP)

RE: (insert name of participant and home address) I am writing to inform you that your patient has consented to take part in a research study at the Institute of Food Research (IFR) in Norwich.

The study is led by Professor Richard Mithen and is entitled:

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

The study has been approved by the IFR Human Research Governance Committee (HRGC) and (to add REC name after gaining approval).

I have enclosed a copy of the Patient Information Sheet for your reference.

Following consent, it is our standard practice to screen the volunteers to exclude any health factors that may affect the study data or indicate an issue which may require further investigation. We are looking for healthy people who have no chronic illness and are not taking any prescribed medication that may affect the study data.

Some/none of your patient's results fell outside the standard reference range on this occasion.

These results **will/will not** affect the study data.

Your patient **will/will not** be able to participate in the study.

If you require any further information about the study then please do not hesitate to contact the study scientists Miss Tharsini Sivapalan on (0)1603 251425 (email Tharsini.sivapalan@ifr.ac.uk), Dr Antonietta Melchini on (0)1603 255030 (email antonietta.melchini@ifr.ac.uk) or Dr Charlotte Armah on (0)1603 255000 .

Thank you for taking the time to read this letter.

Yours sincerely,

Miss Tharsini Sivapalan

On behalf of the BOBS study team

Encs: Participant Information Sheet, version (insert version number) dated (insert date)

Norwich Research Park, Colney, Norwich NR4 7UA, UK

www.ifr.ac.uk Tel: +44(0) 1603 255000 GTN 6626 5000 Fax: +44 (0)1603 507723

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

A list of foods that are high in glucosinolates and have to be avoided 24-hours before and during the study days:

Garlic

Mustard: all types including Ethiopian mustard, Indian mustard, Chinese mustard, red giant mustard, wrapped heart mustard cabbage, yellow mustard, black mustard, broad beak mustard, purple stem mustard, mustard spinach.

Broccoli: all types including rapini or broccoli raab, alboglabra kai-ian (Chinese broccoli), romanesco broccoli, broccoli, broccoflower.

Sprouting broccoli

Brussels sprouts

Cauliflower

Cabbage: all types including white cabbage (drum), head cabbage, Savoy cabbage, red cabbage, green cabbage.

Kale: all types including curley kale, Chinese kale, sea kale, pabularia siberian kale, acephala Kale.

Kohl rabi

Turnip and turnip tops

Spring greens and Collard greens

Chinese cabbage; Pak Choi; Bok choy and other Chinese brassica vegetables

Radish

Salad rocket

Horseradish: sauces and vegetables

Cress: all types, including watercress and garden cress

Papaya seeds

Wasabi

Please have a look at the photos that can help you in recognising all foods in the list:

Kohlrabi

Collard

Collard

Spring green

Kale

Wasabi

Cauliflower

Cabbage

Brussels sprout

Chinese cabbage

Pak Choi cabbage

Bok Choy cabbage

Savory cabbage

Broccoli

Salad rocket

Sprouting Broccoli

Turnip and turnip tops

Radish

Horseradish vegetable

Cress

Cress

Watercress

Watercress

Papaya seed

Mustard

Garlic

IFR Institute of Food Research

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

Date

Dear……..

I am writing to inform you that one or more of the results from your **blood tests** fell **outside** the standard reference range.

As you are aware from your interview it is our policy to refer such results to our medical doctor for a decision on whether you should participate in the study, as the results may affect the study data.

The doctor has advised that the results flagged **could affect** this particular study's data; therefore we are **unable to accept** you on this study. However, these results do not necessarily exclude you from taking part in future studies.

There may be no cause for concern about your health and copies of your results have been sent to your GP, therefore, we would suggest you contact your GP to discuss your results. Please find enclosed your expenses form for your screening samples. Please complete the form with your name, address, bank details and mileage (if appropriate) and enclosing any public transport receipts. Please make sure that you **sign the form** and return it to us in the freepost envelope provided.

We would like to thank you for your interest and willingness to participate.

Yours sincerely,

Miss Tharsini Sivapalan

On behalf of the BOBS study team

Norwich Research Park, Colney, Norwich NR4 7UA, UK

www.ifr.ac.uk Tel: +44(0) 1603 255000 GTN 6626 5000 Fax: +44 (0)1603 507723

IFR Institute of Food Research

Norwich Research Park
Colney
Norwich
NR4 7UA
UK

Date

Dear Doctor *(insert name of patient's GP)*

RE: (*insert name of patient and home address*)

I am writing to inform you that your patient has consented to take part in a research study organised by the Institute of Food Research in Norwich. The study is led by Professor Richard Mithen and is entitled '**The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)'**. The study has been approved by the Institute of Food Research (IFR) Human Research Governance Committee (HRGC) and the (insert committee name) NRES Research Ethics Committee (REC).

The study will investigate the isothiocyanate excretion following consumption of broccoli soups with different doses of glucoraphanin on three occasions. Twenty-four hour urine samples and blood samples, before and after consumption of one pot (300g) of broccoli soup, will be collected from participants. Following consent it is our standard practice to screen the volunteers to exclude any health factors which may affect study procedures or indicate an issue which may require further investigation. It is our policy to forward copies of all results (normal or abnormal) during the study to the volunteer's GP.

Thank you for taking the time to read this letter. If you require any further information about the study then please do not hesitate to contact Professor Richard Mithen at the Institute of Food Research **01603 255259** (email **richard.mithen@ifr.ac.uk** or the study scientists, Dr Antonietta Melchini on **01603 255030** (email **antonietta.melchini@ifr.ac.uk)** or Tharsini Sivapalan on **01603251425** (email **tharsini.sivapalan@ifr.ac.uk).**

Thank you for taking the time to read this letter.

Yours sincerely,

Miss Tharsini Sivapalan

On behalf of the BOBS study team

Norwich Research Park, Colney, Norwich NR4 7UA, UK

www.ifr.ac.uk Tel: +44(0) 1603 255000 GTN 6626 5000 Fax: +44 (0)1603 507723

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

Study description

This will be a randomized, three-phase, double-blinded crossover study that will investigate the bioavailability of sulforaphane (SF), following consumption of three broccoli + stilton soups with different doses of glucoraphanin. The study will take place in the Human Nutrition Unit (HNU) at the Institute of Food Research.

PRIMARY AIM:

To measure the total excretion of SF in urine collected for 24 hours after consumption of one pot (300g) of three types of broccoli + stilton soup containing different concentrations of glucoraphanin, SF precursor.

SECONDARY AIMS:

To measure SF and its metabolites in plasma following consumption of one pot (300g) of three types of broccoli + stilton soup containing different concentrations of glucoraphanin.

To determine whether the glutathione S-transferase Mu 1 (*GSTM1*) genotype or other associated genotypes (e.g. GSST1) influence SF plasma pharmacokinetic parameters (C_{max}, T_{max}, area under the curve) and its total urinary excretion.

STUDY DESIGN:

Participants will be asked to attend the HNU for a total of 8 visits during the study. Their involvement is expected to last 11-12 weeks. The following soups will be used as a test meal: i) 300g standard broccoli + stilton soup, ii) 300g Beneforte® broccoli + stilton soup and iii) 300g Beneforte Extra broccoli + stilton soup. The study will consist of three study phases separated by a minimum of two weeks washout period. Each phase will consist of a 48 hour pre-intervention diet restriction, a study

day (study day 1) consisting of approximately a 9 hour stay at the HNU and will involve the participant being cannulated for an 8 hour period to allow multiple blood samples to be taken. A 24 hour urine and blood sample will also be collected from the participants the following morning. During each study day 1, participants will also collect their urine in containers provided.

Participants will be asked to eliminate foods high in glucosinolates, spicy food and alcohol 48 hours prior to each study day 1 and during each study phase until the collection of the 24 hour blood and urine samples i.e. a total of 3 days per phase and a total of 9 nine days for the entire study (Phase 1-3). They will also be asked to fast for a minimum of 10 hours overnight, and advised to drink plenty of plain water prior to blood sampling days in each study phase.

During each phase, **six** urine samples will be collected after consumption of each of the soups at the following time-points: 0, 0-2, 2-4, 4-6, 6-8 and 8-24 hours. **Eleven** blood samples will also be collected at the following time-points: 0, 30, 45, 60, 90, 120, 180, 240, 360, 480 mins and 24 hours.

The total blood volume collection during the study is (110 ml × 3 phases) + 15 ml screening sample = 345ml or 355ml if participant is re-screened.

IFR Institute of Food Research

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

Date

Dear……..

I am writing to inform you that the recruitment for the Bioavailability of sulforaphane from broccoli soups (BOBS) study is now complete. I would like to thank you for your interest in the study and for your willingness to be on a stand-by list.

Unfortunately, on this occasion, you were not required and as such will not be able to participate in this study.

This will not affect your involvement in any future studies at the Institute.

Many thanks again for your interest.

Yours sincerely,

Miss Tharsini Sivapalan

On behalf of the BOBS study team

Norwich Research Park, Colney, Norwich NR4 7UA, UK

www.ifr.ac.uk Tel: +44(0) 1603 255000 GTN 6626 5000 Fax: +44 (0)1603 507723

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

Date

Dear……..

I am writing to remind you of your appointment on:

_____

You will be required to remain in the HNU for approximately 9 hours on the study days and you will have a cannula in your arm for 8 hours. The nurse has asked us to advise you to wear comfortable clothing, which you can easily remove for toilet purposes whilst cannulated.

Please also remember to collect the first urine sample on the morning of study day 1 in the container provided. You need to maintain a diet restriction 48 hours before the study and fast for 10 hours prior to the study day. It is advised that you drink as much plain water as you need during the period of fasting.

If you are unable to make the appointment, please ring the HNU on 01603 255305.

Yours sincerely,

Miss Tharsini Sivapalan

On behalf of the BOBS study team

Norwich Research Park, Colney, Norwich NR4 7UA, UK

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The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

Food Choices for study days

Lunch

2 - 4 medium slices of white or brown

20g or 2 portions Butter or Flora (optional)

130g Tuna Chunks in spring water or 80g grated mature cheddar cheese

10g Mayonnaise (optional)

Natural Yoghurt (optional)

Banana (optional)

Unlimited Water

IFR Institute of Food Research

Date

Dear Doctor ……..

Your patient, ……..,date of birth …….. has consented to take part in a Human Nutrition study at the Institute of Food Research entitled,

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

Prior to cannulation on the study day, it is our practice to measure and record the participant's blood pressure to ensure it falls within the HNU's reference range for cannulation, and the inclusion criteria for the study.

Your patient's blood pressure measurement was outside the range for cannulation and study inclusion criteria. Therefore, your patient will be excluded from the study.

Please find a copy of the blood pressure measurements attached with this letter. You will already have received a copy of the eligibility screening blood results and blood pressure measurements. Your patient has been advised to discuss these results with you.

Yours sincerely,

Miss Tharsini Sivapalan

On behalf of the BOBS study team

Norwich Research Park, Colney, Norwich NR4 7UA, UK

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Consent to contact form for future research studies

Study Title: The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

Chief Investigator: Prof Richard Mithen

For participants who were recruited through advertisements

Please sign below if you agree to be contacted about future studies on broccoli (or similar) that may be undertaken at IFR.

Signed: …… (Name in BLOCK letters)……

Date: …… **Date of Birth**: ……

Address:_____

_____

_____

_____

_____

Contact telephone number:_____

E-mail address:_____

Norwich Research Park, Colney, Norwich NR4 7UA, UK

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The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

Broccoli and Stilton Soup

Ingredients Declaration: Water, Broccoli (28%)*, Fresh Milk, Single Cream, Diced Onion, Potato, Stilton Cheese (4%), Cornflour, Rapeseed Oil, Salt, Black Pepper.

* Standard broccoli or Beneforte broccoli or Beneforte extra broccoli

Allergens: Milk, celery

Theoretical Nutrition:

		Energy Kcal	Fat	protein	CHO	Na	Sugar	Sat Fat	Salt	Fibre
Per:	300g portion	175	11.36	8.29	9.91	0.62	3.90	4.53	1.60	2.57
Per:	100g	58	3.78	2.76	3.30	0.21	1.30	1.51	0.53	0.86

Certificate
of Conformity

This is to certify that

B02729
Bakkavör Soup & Sauces
BRC Site Code: 1206376
West Marsh Road
Spalding
Lincolnshire
PE11 2BB

Has been evaluated by
Exova
Accredited Certification Body No: 185
and found to meet the requirements of
GLOBAL STANDARD FOR FOOD SAFETY
Issue 6: July 2011

Scope:

For the manufacture of chilled ready to eat / reheat soups and sauces hot filled into: plastic pots, elopak cartons and capkold bags. Also for the manufacture of sauce base, cold filled into pallecons for further processing.

Exclusions from Scope: Bakkavör Spalding Storage and Distribution Centre.

Product Categories: 10

Achieved Grade: A

Audit Programme: Announced

Authorised by:

Jon Revell
Certification Scheme Manager

Date of Audit:
4th & 5th November 2013

Certificate Issue Date:
12th December 2013

Re-Audit Due Date:
From 9th October 2014
to 6th November 2014

Certificate Expiry Date:
18th December 2014

Auditor Number:
221008

UKAS PRODUCT CERTIFICATION
0185

Exova (UK) Limited
121, Shady Lane, Great Barr, Birmingham B44 9ET UK
Tel. +44 (0) 121 251 4000 Email: auditadmin@exova.com

This certificate remains the property of Exova, a subsidiary of Exova Group Limited and must be returned on demand.

If you would like to feedback comments on the BRC Global Standard or the audit process directly to BRC, please contact enquiries@brcglobalstandards.com

BRC CERTIFICATION BODY

V6.03

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Norwich Research Park
Colney
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Ms Tharsini Sivapalan
Food and Health Programme
Institute of Food Research
Norwich Research Park
Norwich
NR4 7UA

8 July 2014

Dear Tharsini,

HRGC ref: IFR06/2014
Short title: Bioavailability of sulforaphane from broccoli soups (BOBS Study)

The above protocol was discussed at a meeting of the IFR Human Research Governance Committee (HRGC) held on Friday 4 July 2014. Following discussion, the committee's decision was that the proposal could be submitted to an NHS Research Ethics Committee, once minor changes have been made.

I list the main changes the committee requests and comments to consider. Please use the electronic version of the relevant text of this letter to detail your response to each of the points (eg. "Done", or any argument for not agreeing with any point) and highlight the changes in your revised documents. This will hasten our review.

General comments:

1. The committee felt that further justification for undertaking the cannulation aspect of the study is required. The study has been powered on urinary sulforaphane excretion, so further justification is needed to confirm that the glucoraphanin doses you plan to use will elicit a statistically significant plasma response. Please confirm the glucoraphanin doses that you will be using for the study. Please discuss with Jack Dainty (institute statistician).
2. Please justify the time points that you have chosen for blood sampling, there should be a scientific justification.
3. It appears that the study has been powered on a comparison of the low and high glucoraphanin broccoli rather than the low versus medium content. In order to obtain useful data from the latter group the committee felt that the study should be powered on this comparison. Please discuss with Jack Dainty (institute statistician).
4. The stated glucoraphanin content of the three types of broccoli used in this study is different from that stated in other submissions previously reviewed by the committee. Please clarify and amend.
5. Throughout the documentation you use the terms 'bioavailability' and 'plasma and urinary sulforaphane' interchangeably. The latter are surrogate markers of bioavailability and it is important to make the relevant distinction and use the appropriate terms when describing the study.

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IFR receives strategic funding from the Biotechnology and Biological Sciences Research Council

6. The committee felt that genotyping such a small number of participants would not provide any statistically significant data and this aspect should be removed from the study design. Please discuss with Jack Dainty (institute statistician).
7. In the eventuality that the majority of participants do not take up the opportunity to provide an optional faecal sample, the likelihood is that collection of such a small number of samples (maximum 8) will not provide any useful data. The collection of the samples also does not appear to be relevant to the aims of the study and it is unclear how they would be used. Please discuss with Jack Dainty (institute statistician).
8. Dr Charlotte Armah (HRGC committee member) has offered to provide further guidance and advice on the preparation of the general aspects of your submission. Please discuss with Charlotte.
9. Mrs Aliceon Blair (HNU senior research nurse) has offered to provide advice on the clinical and HNU related aspects of your study. She has prepared appropriate wording that will be helpful. Please discuss with Aliceon.
10. As this is the first study that you will be undertaking at IFR, the committee presumes that you will be fully supported by the study research team during the initial study talks and screening and study days?
11. The soups you plan to use in the study are variously referred to throughout the documentation as broccoli soup, broccoli and stilton soup, and broccoli and stilton flavoured soup. Please confirm which type of soup you plan to use and amend the documentation accordingly.
12. The title of the study is inconsistent in the submitted documentation. Please check and amend as appropriate.
13. Please note: the protocol is a standalone document and all the annexes are an annex to the protocol. The protocol is not an annex.
14. Please provide a copy of the letter confirming insurance details for the study from Ian Bartlett.
15. Please resubmit an HRGC registration form including the signature of the senior research nurse.
16. There are several typos and grammatical errors throughout the documentation. Please amend.
17. Please ensure that the information in the submission is consistent between documents.

Specific comments:

Protocol (version 1, dated 26 June 2014)

18. Why is the study single-blinded? Could it be a double-blinded? Please clarify.
19. As your eligibility screening does not include glucose measurements there is no need for the participants to be fasted as HBA1c analysis does not have this as a requirement. Therefore, this will allow slightly more flexibility in the timing of screening appointments (please note: all blood samples need to be at SPIRE Hospital by 3pm). Please speak to Aliceon for further clarification and amend documentation as appropriate.

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20. The washout period between study days should be stated as a minimum of 2 weeks throughout the documentation. Please amend.
21. **Pg 4, scientific background, 3rd line:** Gasper et al (2005) is not an epidemiological study.
Please amend.
22. **Pg 17, eligibility screening:** There is some repetition in this section. Please amend.
23. **Pg 17, eligibility screening:** Who is taking written informed consent form the study participants? Please clarify and include details.
24. **Pg 27/28:** Do participants really need a breakfast of two slices of toast two hours after they have had soup with two slices of toast? They may be quite full, especially as they are also having sandwiches etc. at the five hour time point. However, on page 32 it states that the participants will be having bread rather than toast. Please clarify and amend.
25. **Pg 28, 31 and 32:** The information relating the HNU kitchen etc. is repeated on these pages. Please amend.

26. **Pg 29, last para:** What do you mean by 'linked studies'? It is better to ask participants if they would consent to be 'contacted' (rather than recalled) about future studies on broccoli (or similar) that may be undertaken at IFR. You will also need to amend the wording on the consent from to reflect this, or you could consider a separate consent form for this aspect of the study. Please discuss with Aliceon the process for keeping a list of participants for future studies to make sure that they are not contacted too many times about studies, especially if they are also on the HNU volunteer database.

IRAS form
27. Clinical aspects of study details on this form need amending. Please discuss with Aliceon.
28. A6-2 suggests that you are going to pay participants for loss of wages if they take the day off work to participate in study days. The committee presumes that this is incorrect. Please clarify and amend.

Annex 3: PIS (version 1, dated 26th June 2014)
29. Whilst the committee was not against the booklet format for the PIS, it felt that it needed to be reformatted. Currently the font is quite large and text rather spaced out, which means that at almost 40 pages, the booklet is very long. This format would also benefit from an index because it is much harder to find your way around the information contained within, due to lots of pages of solid text. This booklet format will also be more difficult (although not impossible) to upload electronically to NRES for review. It is essential that it is submitted in the format that you will be presenting it to study participants i.e. two pages of text per A4 sheet. It will also be necessary to have a standard A4 version of the PIS for the purposes of
e-mailing it to potential participants. The booklet edition must also contain a version number and date.
30. Some of the clinical and HNU aspects of the study need rewording. Please discuss with Aliceon who will advise accordingly.
31. There are several sections of text in the PIS that need re-writing in lay

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language. Please amend as appropriate.
32. Pg 2, 2nd para: It is arguable that the health benefits of broccoli are purely due to sulforaphane. Please amend.
33. Pg 2, 3rd para: There is some text missing.
34. Pg 3: The aim of the study is not worded appropriately. Please amend.
35. Pg 3: The soup is described variously, see earlier comment. Please clarify and amend.
36. Pg 8: It would be helpful to state on which page the flow chart is located when it is referred to here.
37. Pg 16: It reads as though participants will receive £250 for the faecal samples alone. Please amend.
38. Pg 18: The flow chart needs simplifying, it is too wordy. Please also remove the references to the types of analysis as participants will not know what U's and E's are etc. Please also remove the reference to a CRF for the same reason.
39. Pg 25: Please remove the word 'ladies' in reference to pregnancy as it is not necessary.
40. Pg 28: The soup ingredients sheet should refer to three types of broccoli soup, not just two? Please clarify and amend.

Annex 4 Poster (version 1, 26 June 2014)
41. You should give an example and explain the 'natural compounds' you refer to in the first sentence of the advert.
42. Some of the text needs rewording e.g. potential participants will not necessarily understand what is meant by a 'glucosinolate free diet', and 'recompensate' is used incorrectly in this context. It would be better to ask if potential participants like broccoli and stilton soup, as they may like cheese and broccoli separately, but maybe not together in a soup. Please amend.

Annex 5, invitation letter (version 1, 26th June 2014)
43. This letter needs re-wording to reduce the over-use of the word 'study'.

Annex 7, consent form (version 1, 26th June 2014)
44. Please refer to earlier comment regarding the consent form and amend as appropriate. Please do not hesitate to contact me if you require clarification on any point.
Yours sincerely,

Yours sincerely,

Linda Harvey PhD, cert CRGCP
Vice chair, IFR Human Research Governance Committee cc. HRGC members

cc. HRGC members

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29th July 2014

Dear Linda,

HRGC ref: IFR06/2014
Short title: Bioavailability of sulforaphane from broccoli soups (BOBS Study)

Please find responses to all points discussed at the meeting of IFR Human Research Governance Committee (HRGC) held on Friday 4th of July 2014.

The changes in the revised documents have been highlighted.

General comments:

1. The committee felt that further justification for undertaking the cannulation aspect of the study is required. The study has been powered on urinary sulforaphane excretion, so further justification is needed to confirm that the glucoraphanin doses you plan to use will elicit a statistically significant plasma response. Please confirm the glucoraphanin doses that you will be using for the study. Please discuss with Jack Dainty (institute statistician

The documentation has been amended taking into account this comment.

2. Please justify the time points that you have chosen for blood sampling, there should be a scientific justification.

The documentation has been amended taking into account this comment. The time points selected for this study have been based on the same time points used in the study by Shikha et al 2012. Eventhough in the study by Shikha et al 2012 the SF peaks were seen after 1 hour post soup consumption and peaked at 6 hours, the earlier timepoints will be necessary for the additional sulphur analyses and for the other two soups that will deliver a higher concentration of glucoraphanin.

3. It appears that the study has been powered on a comparison of the low and high glucoraphanin broccoli rather than the low versus medium content. In order to obtain useful data from the latter group the committee felt that the study should be powered on this comparison. Please discuss with Jack Dainty (institute statistician).

The documentation has been amended taking into account this comment.

4. The stated glucoraphanin content of the three types of broccoli used in this study is different from that stated in other submissions previously reviewed by the committee. Please clarify and amend.

The stated glucoraphanin content of the three types of broccoli + stilton soups that will be used in this study are from a recent analysis on the broccoli and stilton soups carried out in February 2014. The stated Glucoraphanin content

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in previous submissions are from broccoli florets instead of broccoli + stilton soups which has resulted in the difference.

5. Throughout the documentation you use the terms 'bioavailability' and 'plasma and urinary sulforaphane' interchangeably. The latter are surrogate markers of bioavailability and it is important to make the relevant distinction and use the appropriate terms when describing the study.

The documents were checked to ensure that the relevant disctinction has been made. In addition, on p11 of the protocol the following phrase has been added 'Blood and urinary samples will be collected from participants following consumption of the broccoli + stilton soups with the hope of detecting plasma and urinary metabolites of SF which will be surrogate markers of bioavailability'.

6. The committee felt that genotyping such a small number of participants would not provide any statistically significant data and this aspect should be removed from the study design. Please discuss with Jack Dainty (institute statistician).

This aspect was discussed with Jack Dainty and was agreed that even though genotyping the small number of participants will not provide statistically significant data, it can be used to justify any outliers that could be seen from the results. Therefore, the study team have decided to genotype the participants in this study.

7. In the eventuality that the majority of participants do not take up the opportunity to provide an optional faecal sample, the likelihood is that collection of such a small number of samples (maximum 8) will not provide any useful data. The collection of the samples also does not appear to be relevant to the aims of the study and it is unclear how they would be used. Please discuss with Jack Dainty (institute statistician).

The collection of the faecal samples will no longer be part of this study. This aspect has been removed from the study.

8. Dr Charlotte Armah (HRGC committee member) has offered to provide further guidance and advice on the preparation of the general aspects of your submission. Please discuss with Charlotte.

The general aspects of the submission were discussed with Dr Charlotte Armah and feedback provided by Dr Charlotte Armah was taken into account in amending the documents.

9. Mrs Aliceon Blair (HNU senior research nurse) has offered to provide advice on the clinical and HNU related aspects of your study. She has prepared appropriate wording that will be helpful. Please discuss with Aliceon.

The clinical and HNU aspects of the study were discussed with Mrs Aliceon Blair and feedback provided by Mrs Aliceon Blair was taken into account in amending the documents.

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10. As this is the first study that you will be undertaking at IFR, the committee presumes that you will be fully supported by the study research team during the initial study talks and screening and study days?

The study research team which includes Dr Antonietta Melchini and Dr Charlotte Armah (in the absence of Dr Antonietta Melchini) will provide assistance in the initial study talk, screening and study days. A paragraph on the roles of the members of research team has been added to the protocol on p 11/12.

11. The soups you plan to use in the study are variously referred to throughout the documentation as broccoli soup, broccoli and stilton soup, and broccoli and stilton flavoured soup. Please confirm which type of soup you plan to use and amend the documentation accordingly.

The documentation has been amended taking into account this comment.

12. The title of the study is inconsistent in the submitted documentation. Please check and amend as appropriate.

Done

13. Please note: the protocol is a standalone document and all the annexes are an annex to the protocol. The protocol is not an annex.

Done

14. Please provide a copy of the letter confirming insurance details for the study from Ian Bartlett.

A copy of the letter confirming insurance details for the study from Ian Bartlett has been enclosed with the documents.

15. Please resubmit an HRGC registration form including the signature of the senior research nurse.

The HRGC registration form including the signature of the senior research nurse has been enclosed with the documents.

16. There are several typos and grammatical errors throughout the documentation. Please amend.

All documents were checked and have been amended taking into account this comment.

17. Please ensure that the information in the submission is consistent between documents.

All documents were checked and have been amended taking into account this comment.

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Specific comments:

Protocol (version 1, dated 26 June 2014)

18. Why is the study single-blinded? Could it be a double-blinded? Please clarify.

Members of the study have decided that the study will be a double-blinded study.

19. As your eligibility screening does not include glucose measurements there is no need for the participants to be fasted as HBA1c analysis does not have this as a requirement. Therefore, this will allow slightly more flexibility in the timing of screening appointments (please note: all blood samples need to be at SPIRE Hospital by 3pm). Please speak to Aliceon for further clarification and amend documentation as appropriate.

This clinical aspect was discussed with Mrs Aliceon Blair. Participants will not need to fast for the HbA1c analysis and all documents have been amended appropriately.

20. The washout period between study days should be stated as a minimum of 2 weeks throughout the documentation. Please amend.

Done

21. Pg 4, scientific background, 3rd line: Gasper et al (2005) is not an epidemiological study. Please amend.

Done

22. Pg 17, eligibility screening: There is some repetition in this section. Please amend.

Done

23. Pg 17, eligibility screening: Who is taking written informed consent form the study participants? Please clarify and include details.

The written consent form will be taken by the HNU senior research nurse or by members of the study team.

24. Pg 27/28: Do participants really need a breakfast of two slices of toast two hours after they have had soup with two slices of toast? They may be quite full, especially as they are also having sandwiches etc. at the five hour time point. However, on page 32 it states that the participants will be having bread rather than toast. Please clarify and amend.

Participants will no longer have breakfast. They will have the study soup with 2 slices of bread then lunch. All documents have been appropriately amended.

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25. Pg 28, 31 and 32: The information relating the HNU kitchen etc. is repeated on these pages. Please amend.

Done

26. Pg 29, last para: What do you mean by 'linked studies'? It is better to ask participants if they would consent to be 'contacted' (rather than recalled) about future studies on broccoli (or similar) that may be undertaken at IFR. You will also need to amend the wording on the consent from to reflect this, or you could consider a separate consent form for this aspect of the study. Please discuss with Aliceon the process for keeping a list of participants for future studies to make sure that they are not contacted too many times about studies, especially if they are also on the HNU volunteer database.

There are now two consent forms (Annex 6 and Annex 20) produced having taking this comment into account. The wording has been amended and a separate consent form (Annex 20) consent form will be given to participants who were recruited through adverts upon completion of the study to ask whether they would be happy to consent to be contacted for future studies by registering their details on the HNU volunteer database.

IRAS form

27. Clinical aspects of study details on this form need amending. Please discuss with Aliceon.

The clinical aspects of the submission were discussed with Mrs Aliceon Blair and feedback provided by Mrs Aliceon Blair was taken into account in amending the IRAS.

28. A6-2 suggests that you are going to pay participants for loss of wages if they take the day off work to participate in study days. The committee presumes that this is incorrect. Please clarify and amend.

The IRAS has been amended taking into account this comment.

Annex 3: PIS (version 1, dated 26th June 2014)

29. Whilst the committee was not against the booklet format for the PIS, it felt that it needed to be reformatted. Currently the font is quite large and text rather spaced out, which means that at almost 40 pages, the booklet is very long. This format would also benefit from an index because it is much harder to find your way around the information contained within, due to lots of pages of solid text. This booklet format will also be more difficult (although not impossible) to upload electronically to NRES for review. It is essential that it is submitted in the format that you will be presenting it to study participants i.e. two pages of text per A4 sheet. It will also be necessary to have a standard A4 version of the PIS for the purposes of e-mailing it to potential participants. The booklet edition must also contain a version number and date.

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The booklet format has been reformatted. The font and text space has been reduced. The booklet is now 25 pages. An index has also been included. For the online upload, the booklet created on publisher (two pages of text per A4) will be converted to a PDF file then uploaded which will be in the same format that will be presented to participants.

30. Some of the clinical and HNU aspects of the study need rewording. Please discuss with Aliceon who will advise accordingly.

The clinical and HNU aspects of the study were discussed with Mrs Aliceon Blair and feedback provided by Mrs Aliceon Blair was taken into account in amending the documents.

31. There are several sections of text in the PIS that need re-writing in lay language. Please amend as appropriate.

The documentation has been amended taking into account this comment.

32. Pg 2, 2nd para: It is arguable that the health benefits of broccoli are purely due to sulforaphane. Please amend.

The documentation has been amended taking into account this comment.

33. Pg 2, 3rd para: There is some text missing.

The documentation has been amended taking into account this comment.

34. Pg 3: The aim of the study is not worded appropriately. Please amend.

The documentation has been amended taking into account this comment.

35. Pg 3: The soup is described variously, see earlier comment. Please clarify and amend.

The documentation has been amended taking into account this comment.

36. Pg 8: It would be helpful to state on which page the flow chart is located when it is referred to here.

The page of the flowchart is added on p5 of the PIS in the section 'What next?' which introduces the HNU visits.

37. Pg 16: It reads as though participants will receive £250 for the faecal samples alone. Please amend.

This has been amended as faecal sample collection is no longer part of this study.

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38. Pg 18: The flow chart needs simplifying, it is too wordy. Please also remove the references to the types of analysis as participants will not know what U's and E's are etc. Please also remove the reference to a CRF for the same reason.

Flowchart has been amended into a simplified version p12 of the PIS.

39. Pg 25: Please remove the word 'ladies' in reference to pregnancy as it is not necessary.

Done

40. Pg 28: The soup ingredients sheet should refer to three types of broccoli soup, not just two? Please clarify and amend.

The documentation has been amended taking into account this comment.

Annex 4 Poster (version 1, 26 June 2014)

41. You should give an example and explain the 'natural compounds' you refer to in the first sentence of the advert.

Examples of the natural compounds have been included.

42. Some of the text needs rewording e.g. potential participants will not necessarily understand what is meant by a 'glucosinolate free diet', and 'recompensate' is used incorrectly in this context. It would be better to ask if potential participants like broccoli and stilton soup, as they may like cheese and broccoli separately, but maybe not together in a soup. Please amend.

The document has been amended taking into account this comment.

Annex 5, invitation letter (version 1, 26th June 2014)

43. This letter needs re-wording to reduce the over-use of the word 'study'.

The document has been amended taking into account this comment.

Annex 7, consent form (version 1, 26th June 2014)

44. Please refer to earlier comment regarding the consent form and amend as appropriate.

There are now two consent forms (Annex 6 and Annex 20) produced having taken the earlier comment regarding the consent form into account. Annex 20 consent form will be given to participants who were recruited through adverts upon completion of the study to ask whether they would be happy to consent to be contacted for future studies by registering their details on the HNU volunteer database.

Yours sincerely,

Miss Tharsini Sivapalan

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Miss Tharsini Sivapalan
Food and Health Programme
Institute of Food Research
Norwich Research Park
Norwich
NR4 7UA

15 August 2014

Dear Tharsini,

HRGC ref: IFROG/2014

Short title: Bioavailability of sulforaphane from broccoli soups (BOBS Study)

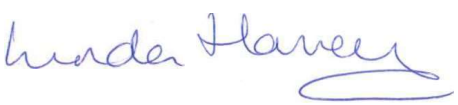
The revised documentation you have provided has been reviewed by Dr Sian Astley, Dr Jack Dainty, Mrs Aliceon Blair and myself and we are happy to take Chair's Action on behalf of the Human Research Governance Committee (HRGC) to give approval for this proposal *(Protocol version 2, dated 2910712014)* to be submitted to an East of England NHS Research Ethics Committee. This approval means that the HRGC is satisfied both with the originality and quality of the science proposed and considers that adequate expertise and resources are available to undertake the research.

Please enclose this letter, along with the previous correspondence from the HRGC *(dated 0810712014)* and your response *(dated 2910712014)* with your ethics submission.

May I take this opportunity to remind you that (i) all staff involved with the project must complete the relevant modules of the HRGC Human Studies training course prior to starting work on the study, and (ii) your study should be registered on a publicly-accessible database such as www.clinicaltrials.gov.

The HRGC general conditions of approval are appended.

Yours sincerely,



Linda Harvey. PhD, certCRGCP
Chair, IFR Human Research Governance Committee
cc: HRGC members

Institute of Food Research
Norwich Research Park
Colney
Norwich NR4 7UA
UK
www.ifr.ac.uk

29th July 2014

REC ref 14/EE/1121

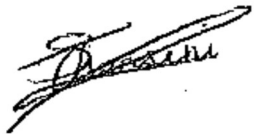
Short title: Bioavailability of sulforaphane from broccoli soups (BOBS Study)

Dear Tracy,

I would like to apply for SSA exemption for the above study.

Please see the exemption request from the sponsor of this study below.

Kind regards,



Miss Tharsini Sivapalan

SSA exemption request

Dear Tharsini,

Following our earlier conversation regarding the requirement for SSA for the above study, I can confirm that as sponsor's representative for this study, I consider that the Institute of Food Research would be SSA exempt in accordance with sections 4.4 - 4.6 (Requirement for site specific assessment) of the Standard Operating Procedures for Research Ethics Committees (version 5.1, March 2012). The SOPs can be found at the following link: **www.hra.nhs.uk/documents/2013/08/standard-operating-procedures-for-research-ethics-committees-sops.pdf**

If you require any further clarification on this matter then please do not hesitate to contact me.

Kind Regards,

Linda

NHS
Health Research Authority
NRES Committee East of England - Norfolk
The Old Chapel Royal Standard Place
Nottingham NG1 6FS

Telephone: 0115 883 9525:

18 September 2014

Prof Richard Mithen
Associate Director (Nutrition) and Head of Plant Natural Products and Health Programme and Chair of the Graduate Studies Committee
Institute of Food
Research Norwich
Research Park
Colney
NR4 7UA

Dear Prof Mithen

Study title:	**An intervention study to assess the bioavailability of sulforaphane delivered by glucoraphanin-enriched broccoli soups**
REC reference:	**14/EE/1121**
Protocol number:	**N/A**
IRAS project ID:	**157660**

The Research Ethics Committee reviewed the above application at the meeting held on 15 September 2014. Thank you to Miss Tharsini Sivapalan and Dr Antoinetta Melchini for attending to discuss the application.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager Ms Tracy Leavesley, NRESCommittee.EastofEngland-Norfolk@nhs.net

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below. .

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start

of the study.

1. Please ensure that it is clear on page 11 of the Participant Information Sheet that the 24 hour testing may take up to an hour.

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on question 2 of the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Summary of discussion at the meeting

Ethical issues raised by the Committee in private discussion, together with responses given by the applicant when invited into the meeting

Recruitment arrangements and access to health information, and fair participant selection

The Committee agreed the randomisation process would need to be clarified and asked the applicants to explain how this would be done. *The applicants advised randomisation will take place before the participants enter the study room by another member of the team and participants will be randomised into the study by the other member of the team to ensure that the applicants are unaware which group they are allocated to.*

The Committee queried why the applicants had opted to set the upper age limit at 65. *The applicants advised once a person passes a certain age, their metabolism and taste can change which may skew the results of the study. The applicants went on to advise that cannulation in older people can also carry a higher risk.*

The Committee asked the applicants why they had opted to exclude smokers from the study. *The applicants advised in smokers, the metabolism can be affected, which could skew the results of the study.*

Favourable risk benefit ratio; anticipated benefit/risks for research participants (present and future)

The Committee considered and discussed the length of time participants would be cannulated. The Committee asked the applicants whether this could be reduced as other reports have indicated that participants would only need to be cannulated 2-6 hours after ingesting the broccoli to detect the levels of glucoraphanin and sulforaphane. *The applicants advised that other studies had not sought the same levels of sulforaphane and glucoraphanin for analysis and by cannulating participants earlier in the study a wider reporting range would be achieved.*

Informed consent process and the adequacy and completeness of participant information

It was noted that on page 11 of the Participant Information Sheet (PIS), it states "the 24 hour sample will last up to 1 hour" which the Committee considered confusing. The Committee asked the applicants to clarify this sentence. *The applicants confirmed the sentence means when participants attend for their 24 hour screening, the appointment is expected to take an hour.*

The Committee asked the applicants what was meant by the "unexpected disclosures" detailed in the paperwork. *The applicants advised this means if a Research Nurse is made to feel uncomfortable by something the participant says during the study, they may ask another member of the team to remain present in the room.*

The Committee commented on the format of the PIS and noted that the index made it particularly easy to use.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Copies of advertisement materials for research	2	29 July 2014
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance letter]		24 July 2014
GP/consultant information sheets or letters [Annex 15- Study Description for GP]	2	29 July 2014
IRAS Checklist XML [Checklist_18082014]		18 August 2014
IRAS Checklist XML [Checklist_20082014]		20 August 2014
Letters of invitation to participant [Annex 1- Invitation letter from HNU database]	2	29 July 2014
Other [Annex 5-Appointment card]	2	29 July 2014
Other [Annex 12- List of high glucosinolates]	2	29 July 2014
Other [Annex 19- GP letter with study day	2	29 July 2014
Other [HRGC response letter]		29 July 2014
Other [Annex 7- Medical Declaration Form]	2	29 July 2014
Other [Annex 13- Exclusion Letter]	2	29 July 2014
Other [Annex 20- Consent form on completion of	2	29 July 2014
Other [HRGC approval letter]		15 August 2014
Other [Invitation letter from advertisements]	2	29 July 2014
Other [GP letter with clinical results]	2	29 July 2014
Other [Annex 18- Food choices for study day]	2	29 July 2014
Other [HRGC minor changes letter]		08 July 2014
Other [Annex 9- Case Report Form]	2	29 July 2014
Other [Annex 16- Apology letter for not	2	29 July 2014
Other [CV for study scientists]		24 April 2014
Other [Annex 10-Clinical Results]	2	29 July 2014
Other [Annex 17- Appointment Letter]	2	29 July 2014
Other [CV for study scientists]		24 April 2014
Other [Annex 8- Eligibility Screening	2	29 July 2014
Other [Annex 14- Letter to GP involvement of	2	29 July 2014
Other [Annex 21- Soups information sheet]	2	29 July 2014
Participant consent form [Annex 6-Consent form]	2	29 July 2014
Participant information sheet (PIS) [Annex 2-		29 July 2014
REC Application Form [REC_Form_18082014]		18 August 2014

REC Application Form [REC_Form_20082014]		20 August 2014
Research protocol or project proposal [Study	2	29 July 2014
Summary CV for Chief Investigator (CI) [CV for		24 April 2014
Summary CV for student [CV for student]		29 July 2014
Summary CV for supervisor (student research) [CV for supervisor]		24 April 2014

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Dr Linda Harvey is the Sponsor representative for this study. The Committee agreed that this was a conflict of interest and Dr Harvey was not present during the discussion and decision for this study.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

Notifying substantial amendments

- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the- hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

14/EE/1121	Please quote this number on all correspondence

With the Committee's best wishes for the success of this

project.

Yours sincerely

Dr Michael Sheldon (Chair)

E-mail: NRESCommittee.EastofEngland-Norfolk@nhs.net

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

"After ethical review – guidance for researchers"

Copy to: *Linda Harvey, Institute of Food Research*

NRES Committee East of England - Norfolk Attendance at Committee meeting on 15 September 2014

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mr Ron Driver	Retired Lecturer/Statistician	Yes	
Miss Sheila Ginty	Tissue Viability Specialist Nurse	No	
Ms Leanne Groves	Psychological Therapist/Practice	Yes	
Mrs Janette Guymer	NHS Administrator	Yes	
Dr Linda Harvey	Senior Research Scientist	No	
Dr Peter Langdon	Senior Lecturer in Clinical Psychology and Disability, Honorary Consultant Clinical	Yes	
Dr Elizabeth Lund	Independent Consultant, Nutrition and Gastrointestinal	Yes	
Mr George Mak-Pearce	Psychological Therapist	Yes	
Dr Michael Sheldon (Chair)	Retired Clinical Psychologist	Yes	
Dr Robert Stone	Retired General Practitioner	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Tracy Leavesley	REC Manager
Ms Helen Sutherland	Observer

Institute of Food Research
Norwich Research Park
Colney
Norwich NR4 7UA
UK
www.ifr.ac.uk

10 November 2014

Miss Tracy Leavesley
REC Manager NRES Committee East of England-Norfolk
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

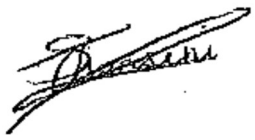
REC reference: 14/EE/1121
Short title: Bioavailability of sulforaphane from broccoli soups (BOBS Study)

I would like to submit a substantial amendment to the NRES Committee East of England - Norfolk. The amendment covers some changes to the BOBS study protocol.
Please find a revised copy of the following documents enclosed with changes highlighted and in bold:

- The substantial IRAS amendment form
- Study protocol version 3
- Annex 2- PIS version 4
- Annex 9- Case Report Form version 3

Should you require any further information please do not hesitate to contact us.

Yours sincerely,



Miss Tharsini Sivapalan

Institute of Food Research
Norwich Research Park
Colney
Norwich NR4 7UA
UK
www.ifr.ac.uk

10 November 2014

Dr Linda Harvey,
Chair, IFR Human Research Governance Committee

REC reference: 14/EE/1121
Short title: Bioavailability of sulforaphane from broccoli soups (BOBS Study)

I would like to submit a substantial amendment to the NRES Committee East of England - Norfolk. The amendment covers some changes to the BOBS study protocol.

Please find a revised copy of the following documents enclosed with changes highlighted and in bold:

- The substantial IRAS amendment form
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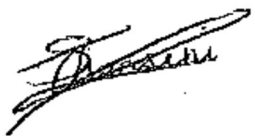
- Annex 9- Case Report Form version 3

Should you require any further information please do not hesitate to contact us.

Yours sincerely,



Miss Tharsini Sivapalan

We request an amendment to the BOBS study protocol. The amendment covers three parts of the protocol.

Exclusion criteria

We are requesting an amendment to one of the exclusion criterion (IRAS form: A6-2, A17-2, A32; Study protocol version 2: Exclusion criteria- p 21; Annex 2_PIS_version 3: p 3; Annex 9 case report form_version 3: Screening day-exclusion criteria-p 3).

In the current protocol version 2, the exclusion criteria include:

Smokers

The exclusion of smokers was decided because smoking affects metabolism which would affect the study data. However, if smokers have stopped smoking for the last 6 months prior to the start of the study then it would not affect our study data. Therefore we would like to change the previous exclusion criterion to the following exclusion criterion to increase the number of potential participants:

Smokers (if smoked within 6 months prior to the study or during the study).

Study day procedure

We are requesting an amendment to the study day procedures which applies to females only (IRAS form: A6-2, A13,A32; Study protocol version 2: Study day-p 24; Annex 2_PIS_version 3: p 10; Annex 9 case report form_version 3: Screening day- p 8, Phase 1- p 14, Phase 2- p 28, Phase 3- p42).

In the current protocol version 2, there is no consideration on the collection of urine from females during study days if they are menstruating. Therefore we would like to make an amendment to the study day procedures so that if females are menstruating or have just finished menstruating then the study day will be rescheduled to after 7 days of having finished menstruating. This is because blood in the urine sample will affect the study data as we are measuring the ITC metabolites in the urine samples. This will also minimise inconvenience caused to female participants to attend a cannulation day where blood samples and urine samples will be collected for 24 hours whilst menstruating.

Inconvenience payment (IRAS form: A46; Study protocol version 2: p 37; Annex 2_PIS_version 3: p 13)

The current financial compensation the volunteers receive for taking part in the study is £220. This sum was calculated on the basis of standard compensation paid for cannulation, obtaining blood and urine samples and dietary restriction. However, no compensation was included for the time requirement to be at the Human Nutrition Unit, equivalent to three whole days. Following a review of other studies of a similar nature, we would like to increase the total

compensation to £500. This has been discussed with Dr Linda Harvey, Chair of IFR HRGC who agrees this revised compensation sum is appropriate.

Note: Annex 9 case report phase 3 was missed out in the original ethics submission. The phase 3 case report is exactly the same as phase 1 and phase 2 that were approved. The only difference is the mention of phase 3.

Health Research Authority

NRES Committee East of England - Norfolk

The Old Chapel Royal Standard Place
Nottingham NG1 6FS
Tel: 0115883939

26 November 2014

Prof Richard Mithen
Associate Director (Nutrition) and Head of Plant Natural Products and Health Programme and Chair of the Graduate Studies Committee
Institute of Food Research Norwich Research Park Colney
NR4 7UA

Dear Prof Mithen

Study title: An intervention study to assess the bioavailability of sulforaphane delivered by glucoraphanin-enriched broccoli soups in healthy subjects
REC reference: 14/EE/1121
Protocol number: N/A
Amendment number: Substantial amendment 1 Amendment date: 12 November 2014
IRAS project ID: 157660

The above amendment was reviewed on 24 November 2014 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Notice of Substantial Amendment (non-CTIMP) [Signed by Richard Mithen]	Substantial amendment 1	12 November 2014
Other [Case Report Form - Annex 9]	3	10 November 2014
Participant information sheet (PIS)	4	10 November 2014
Research protocol or project proposal	3	10 November 2014

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

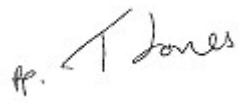
Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/EE/1121:	**Please quote this number on all correspondence**

Yours sincerely



Dr Michael Sheldon Chair
E-mail: NRESCommittee.EastofEngland-Norfolk@nhs.net

Enclosures: List of names and professions of members who took part in the review

Copy to:

Linda Harvey, Institute of Food Research

Institute of Food Research
Norwich Research Park
Colney
Norwich NR4 7UA
UK
www.ifr.ac.uk

NRES Committee East of England - Norfolk

Attendance at Sub-Committee of the REC meeting on 24 November 2014

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Michael Sheldon (Chair)	Retired Clinical Psychologist	Yes	
Dr Robert Stone	Retired General Practitioner	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Mr Tad Jones	REC Assistant

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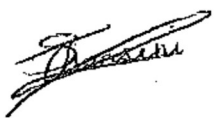
26th August 2016

Georgia Copeland
REC Manager NRES Committee East of England-Cambridgeshire and Hertfordshire
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

REC reference: 14/EE/1121
Short title: Bioavailability of sulforaphane from broccoli soups (BOBS Study)

I would like to submit a substantial amendment to the NRES Committee East of England - Cambridgeshire and Hertfordshire. The amendment covers changes in the sample storage after notification of the end of the study. We will notify the end of the study when the last participant has signed the consent form agreeing for samples to be stored at the Norwich biorepository or declined sample storage or we have been unable to contact the last participant 6 months after sending out the letter (Annex 22).

Please find the following documents enclosed:
Letter to participants- Annex 22 version number 2 (01/06/16)
Biorepository REC approval letter
Tissue bank participant information sheet and consent form- Annex 24 version number 15 (21/02/14)
Should you require any further information please do not hesitate to contact us.
Yours sincerely,



Miss Tharsini Sivapalan

We request an amendment to the BOBS study in regards to the storage of samples after notification of the end of the study. We would like to notify the end of the study after we have attempted to contact each of the study participants to ask for consent to store their remaining blood and urine samples in the Norwich Biorepository. We will declare the end of study when either the last participant has signed the consent form agreeing for samples to be stored in the Norwich Biorepository, or has declined sample storage in the Biorepository, or alternatively we have been unable to contact them within 6 months of sending the letter.

Use of biological samples after research

We would like to request an amendment to transfer the remaining of blood and urine samples to the local Human tissue bank (The Norwich Biorepository) of the Norfolk and Norwich University Hospital (Annex 23 Biorepository REC approval letter) at the end of the study to help future research studies. The proposed banking will be done exclusively after obtaining appropriate consent from study participants (Annex 24 Version 15, 21 February 2014, Biorepository information sheet/consent form).

In the current IRAS form it is stated that the urine and blood samples will be discarded according to the Human Tissue Authority's Code of Practise at the end of the study. However, the analysis of these samples has provided novel information about the absorption of bioactive compounds from broccoli. This was the first study that has fed volunteers with a special variety of broccoli (Beneforte extra) for measuring the bioavailability of sulphur-containing compounds. The use of these samples for the research purposes approved by the current ethics approval has provided valuable data about sulphur compounds that can now be further investigated using *in vitro* models. These findings have not been previously reported in literature and are extremely valuable to inform our research group, which is currently undertaking parallel human intervention studies at the Norfolk and Norwich University Hospital using the same broccoli-rich diet (ESCAPE, REC NRES Committee East of England-Cambridge South 13/EE/0110; ESCAPE-ing, REC NRES Committee East of England-Cambridge South 14/EE/1149; SAP, REC NRES Committee East of England-Cambridge East: 16-EE-0054). Furthermore, results obtained by analysing these samples were valuable for the recent submission of a grant proposal to the Prostate Cancer Foundation (PCF, USA) to seek additional financial support for future research at the Institute of Food Research.

We would like contact study participants and ask their written informed consent to bank their biological samples already collected as part of the study at the Norwich Biorepository according to the Norwich Biorepository procedures. Once the last participant has consented or declined sample storage at the Norwich Biorepository or we have been unable to contact the last participant 6 months after sending the letter we will notify the REC the end of the study. It is important to stress that we are not asking study participants to donate any additional samples but only to give permission for the storage and use of existing samples in the future in accordance with local policies of the Norwich Biorepository. In order to do this, we would like to contact the participants that took part in the study via a letter sent to their address and email address (in case either their postal or email has changed) (Annex 22 Version 2 1st June 2016, letter template) and follow this up with a telephone call if we don't receive a reply from them one month after sending out the letter. We will attempt to call no more than 3 times for a period of 6 months. Their contact details are up to date because we recruited subjects registered on the Human Nutrition Unit Volunteer Database at the Institute of Food Research. Participants will be provided with a copy the Biorepository information sheet for 'The donation, collection, storage and use of samples of tissue and/or fluids and/or other material from a healthy adult donor for research' (Annex 24 Version 15, 21 February 2014). If participants do not wish to give consent for the proposed banking or the study team is not able to contact them after 6 months of sending out the letter, their biological samples will be disposed of in accordance with the Human Tissue Authority guidance after notifying the end of the study. If participants do not want their samples stored at the Norwich Biorepository then we will discard them in accordance to the Human Tissue Authority guidance and this will not accept their participation in future studies. However, if they wish to give consent for their samples to be stored at the Norwich Biorepository we would request them to come to the Human Nutrition Unit for them to sign the appropriate consent form (Annex 24, The Norwich Biorepository consent form, Version 15, 21 February 2014) to enable us to bank their samples in accordance with the local policy for tissue banking. Consent will be obtained by appropriately members of the study team or by a senior research nurse at the Human Nutrition Unit. Participants will be reimbursed travel expenses to and from the Human Nutrition Unit. This will be reimbursed at the IFR's current mileage rate or by reimbursing public transport costs on production of a ticket or receipt. If volunteers require transport by taxi, this will be arranged and pre-paid by us. It is envisaged that the banking of the remaining of blood and urine samples at the end of the study at The Norwich Biorepository will be valuable for obtaining additional information for this study and for designing further studies.

However it is important to stress that any further analysis will be carried out in full compliance with ethical requirements.

IRAS form Part B: Section 5 (14)

In the IRAS form **Part B: Section 5 (14)**, it is stated that the samples will be discarded according to the Human Tissue Authority's Code of Practise at the end of the study. We would like to request an amendment to store at the tissue bank.

To attach as supporting documents:

Annex 22-Letter to participants to explain the reason of being contacted

Annex 23- Biorepository REC approval letter

Annex 24- Biorepository information sheet and consent for 'The donation, collection, storage and use of samples of tissue and/or fluids and/or other material from a healthy adult donor for research'

IFR Institute of Food Research

The Bioavailability Of sulforaphane from Broccoli Soups study (BOBS)

1st June 2016

Dear _____,

I would like to thank you again for taking part in this study. The study analysis has now come to an end and the data from the study has provided new information about the absorption of sulforaphane from the broccoli soups. This has provided new results that have not been previously reported in literature. We will be sending out a summary of the study results shortly.

We are contacting you by both letter and email (in case either your postal or email address has changed) to request your consent to store your urine and plasma samples at the Norwich Biorepository, which is based at the Norfolk and Norwich University Hospital. These samples may be used for any future analysis of compounds that we, or other researchers may do. We only want to store the samples that have already been collected from the study and you do not need to provide any more samples. Please see enclosed the information sheet/consent form about storage of samples at the Norwich Biorepository.

If after reading the enclosed information sheet you might be willing for us to store your samples then please send the reply below in the provided pre-paid envelope or contact me on tharsini.sivapalan@ifr.ac.uk. I will then contact you to organise a visit it to the Human Nutrition Unit to go through the participant information sheet and consent form and answer any of your questions. You will be reimbursed for your travel expenses to and from the Human Nutrition Unit. This will be reimbursed at the IFR's current mileage rate or by reimbursing public transport costs on production of

a ticket or receipt. If you require transport by taxi, this will be arranged and pre-paid by us. This will be at a convenient time for you, within normal working hours.

If you do not wish for your samples to be stored then please send the reply below and we will discard them according to the Human Tissue Act, 2004 as initially discussed when the study started. This will not affect your participation in any future studies at the Institute.

If we haven't heard back from you one month after sending out this letter then we will follow up with no more than 3 telephone call attempts for a period of 6 months. If after this we can't get in contact with you then we will discard them according to the Human Tissue Act, 2004 as initially discussed when the study started. This will not affect your participation in any future studies at the Institute.

I would like to thank you again for your participation in this study.

Yours sincerely,

Miss Tharsini Sivapalan

BOBS Study manager

✂ Cut along the dotted line

Name:

Contact number:

Email address:

☐ I will be willing to store my samples at the Norwich Biorepository for future research. I'm happy to be contacted to organise a visit to the Human Nutrition Unit.

☐ I don't want my samples to be stored at the Norwich Biorepository for future research. Please don't contact me regarding this.

NHS
Health Research Authority

East of England - Cambridgeshire and Hertfordshire Research Ethics Committee

The Old Chapel Royal Standard Place
Nottingham NG1 6FS

16 September 2016

Miss Tharsini Sivapalan
Institute of Food Research
Norwich Research Park
Colney
NR4 7UA

Dear Miss Tharsini Sivapalan

Study title:	**An intervention study to assess the bioavailability of**
REC reference:	**14/EE/1121**
Protocol number:	**N/A**
Amendment number:	**2**
Amendment date:	**01 June 2016**
IRAS project ID:	**157660**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Discussion

There were no ethical issues raised.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper		26 August 2016
Letters of invitation to participant	2	01 June 2016
Notice of Substantial Amendment (non-CTIMP)	2	01 June 2016
Other [Biorepository REC approval letter]		05 March 2014
Other [Biorepository Information Sheet and Consent Form]	15	21 February 2014
Other [e-mail]		31 August 2016

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/EE/1121:	**Please quote this number on all correspondence**

Yours sincerely

PP

Mr David Grayson Chair

E-mail: nrescommittee.eastofengland-cambsandherts@nhs.net

Enclosures: List of names and professions of members who took part in the review

Copy to:

Professor Richard Mithen, Institute of Food Research Linda Harvey, Institute of Food Research

East of England - Cambridgeshire and Hertfordshire Research Ethics Committee Attendance at Sub-Committee of the REC meeting on 13 September 2016

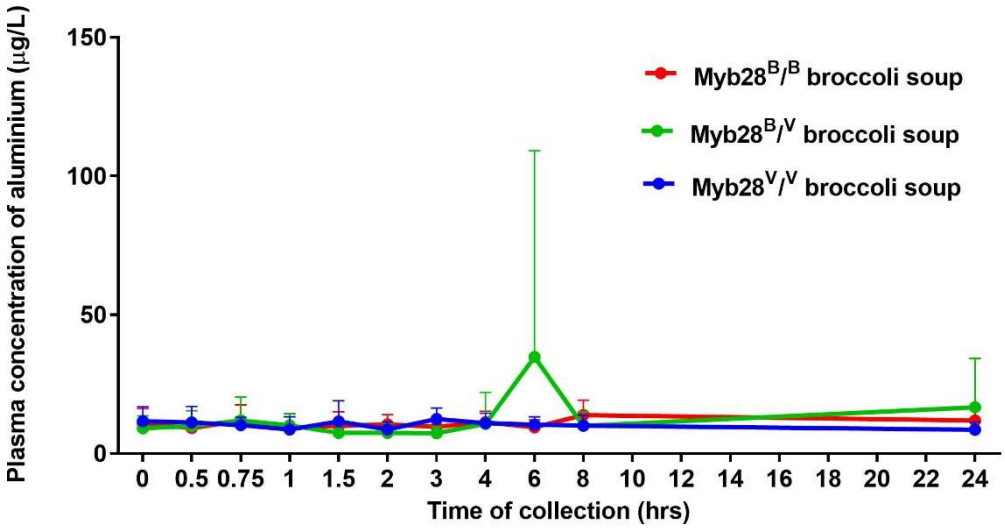
Committee Members:

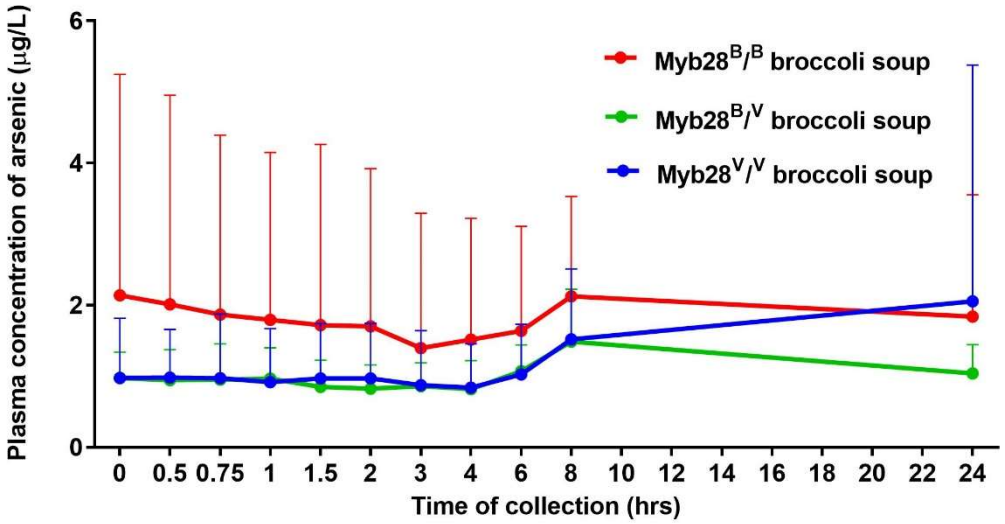
Name	*Profession*	*Present*	*Notes*
Mr David Grayson (Chair)	Retired Local Government Administrator	Yes	
Dr Wassim Matta	General Practitioner	Yes	

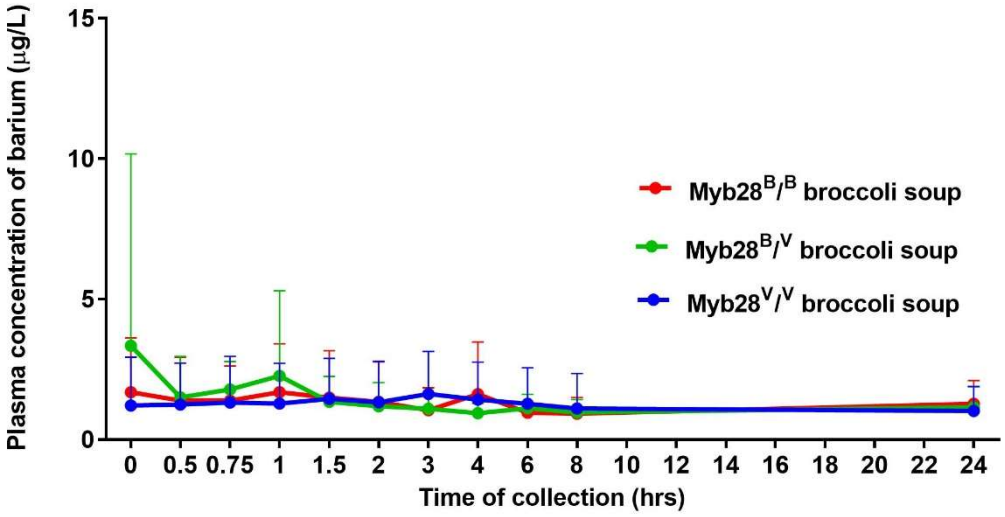
Also in attendance:

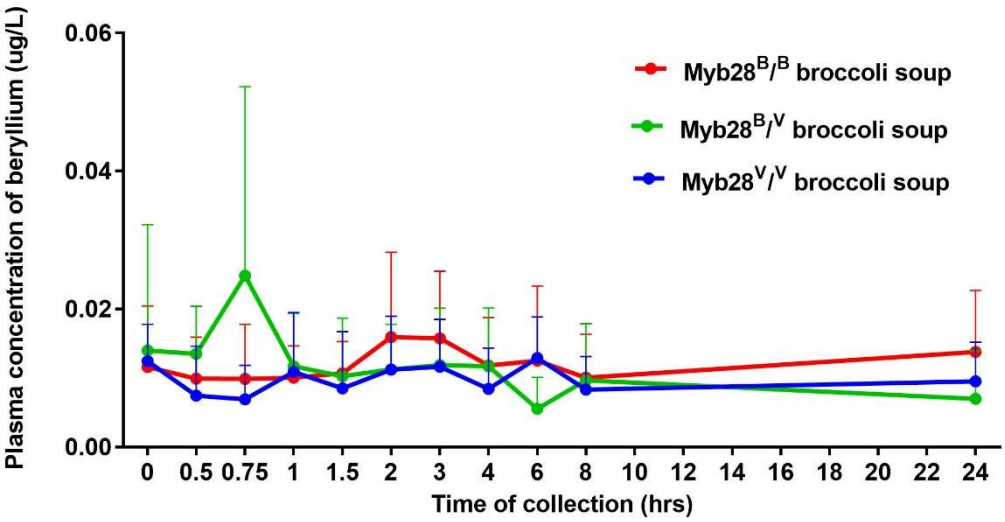
Name	*Position (or reason for attending)*
Miss Lindsey Wallace	REC Assistant

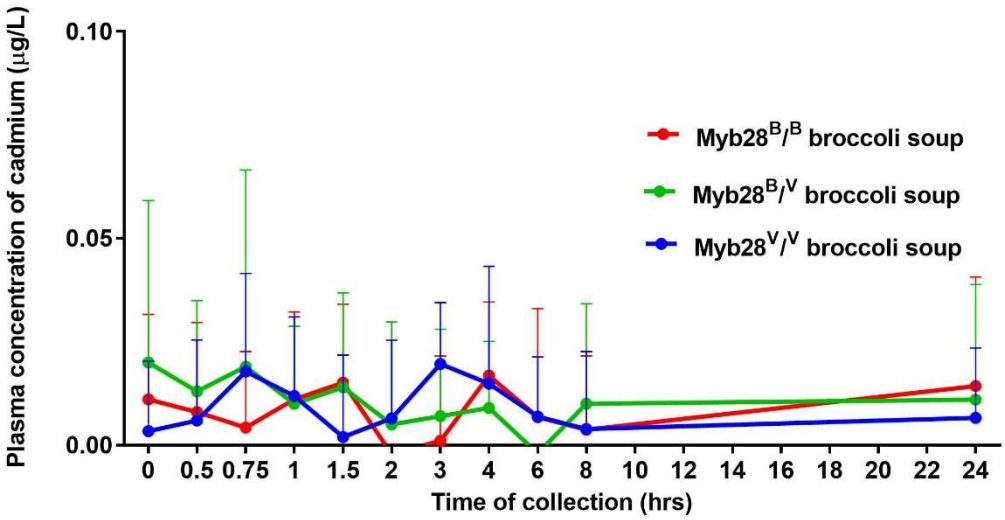
Appendix II-Mineral elements graphs

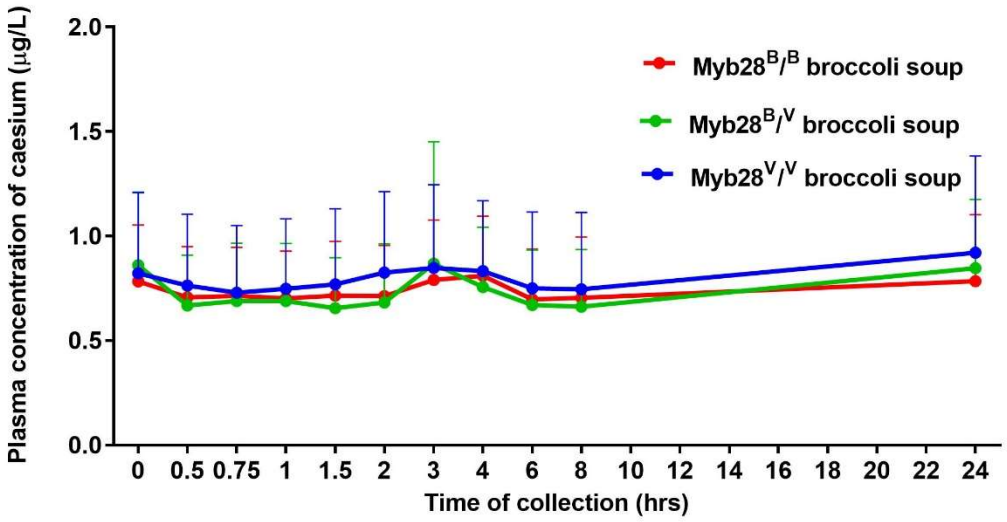


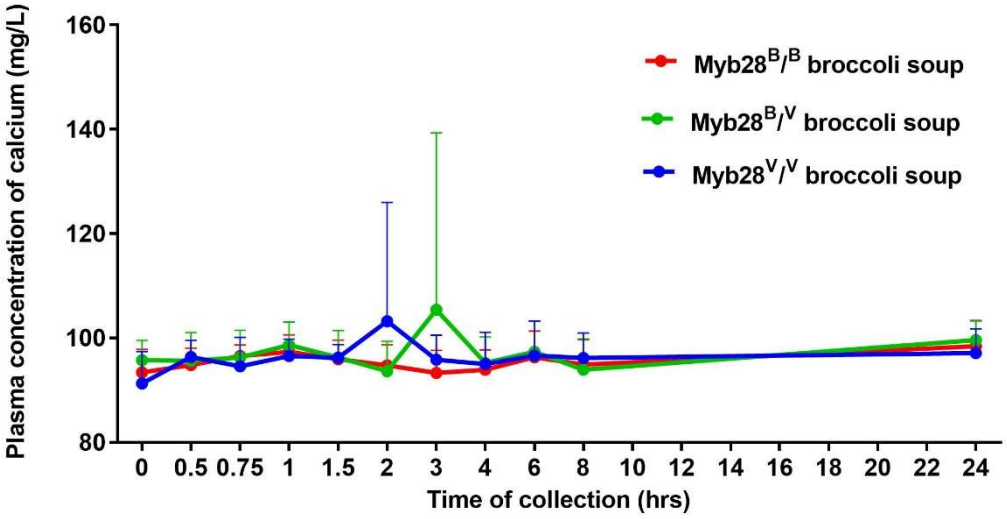


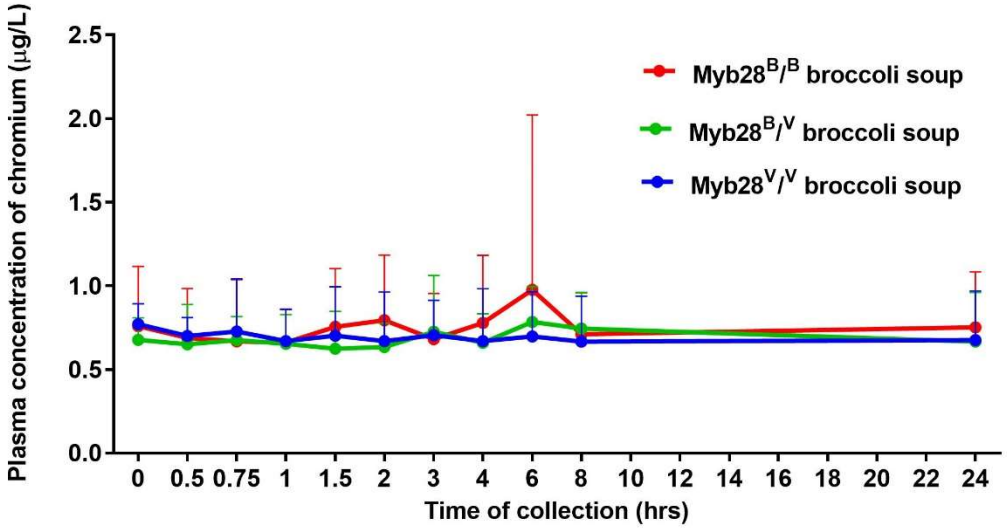

Plasma concentration of barium (µg/L)
15
10
5
0
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup

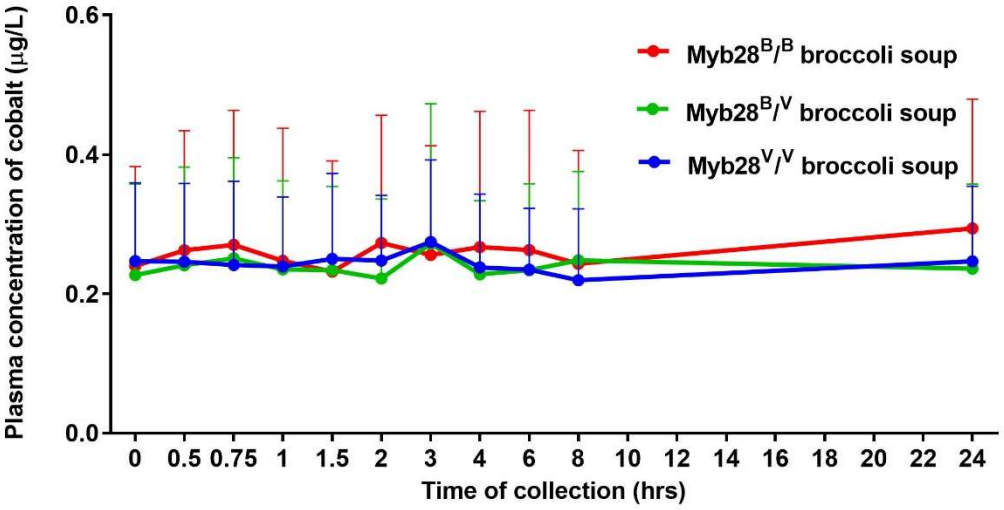

Plasma concentration of beryllium (ug/L)
0.06
0.04
0.02
0.00
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup

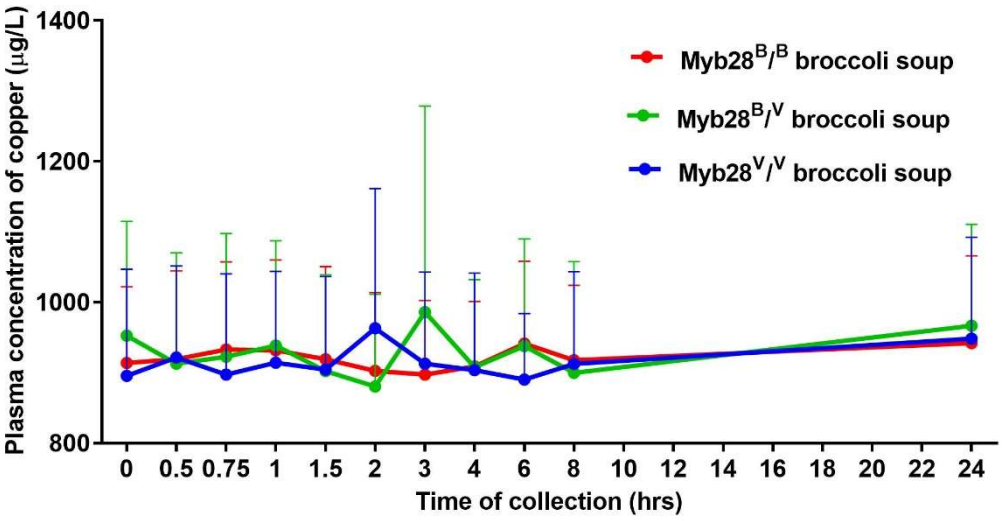

Plasma concentration of cadmium (µg/L)
0.10
0.05
0.00
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup

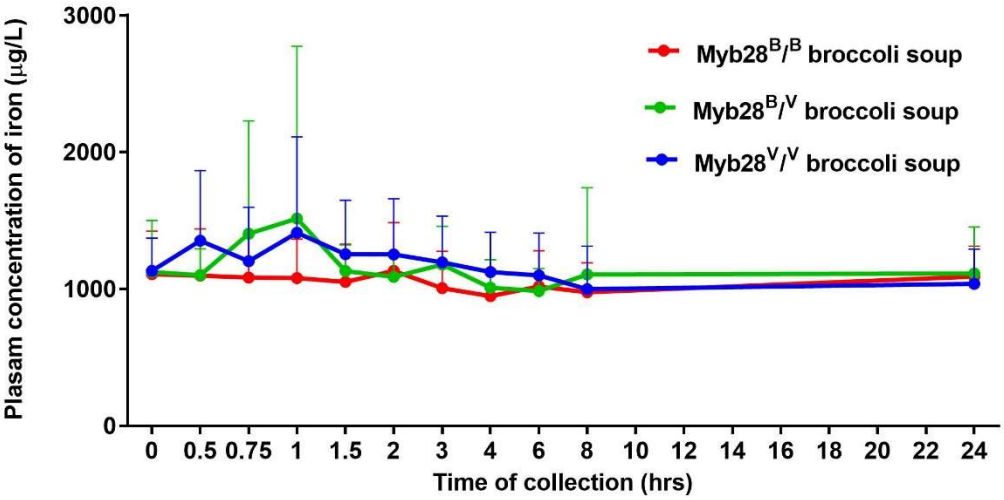

Plasma concentration of caesium (µg/L)
2.0
1.5
1.0
0.5
0.0
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

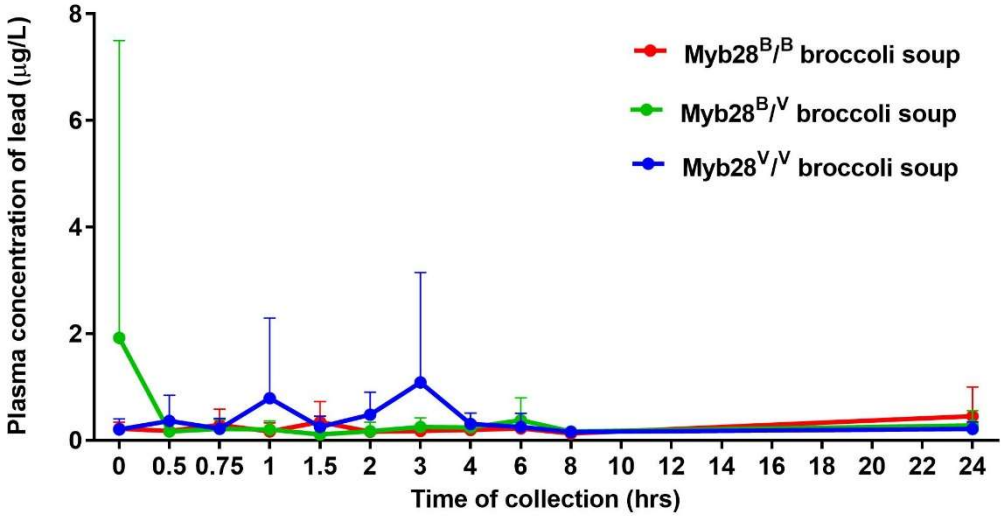

Plasma concentration of calcium (mg/L)
160
140
120
100
80
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

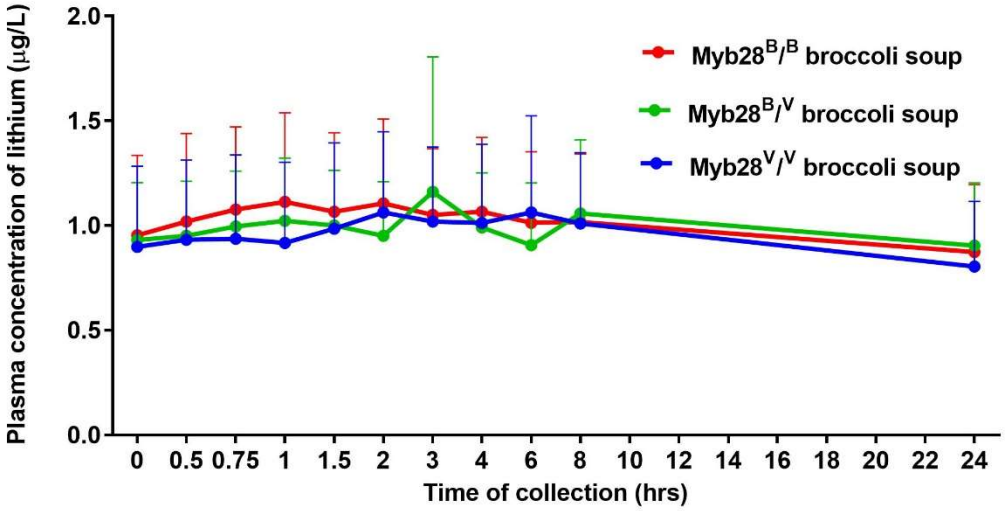

Plasma concentration of chromium (µg/L)
2.5
2.0
1.5
1.0
0.5
0.0
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

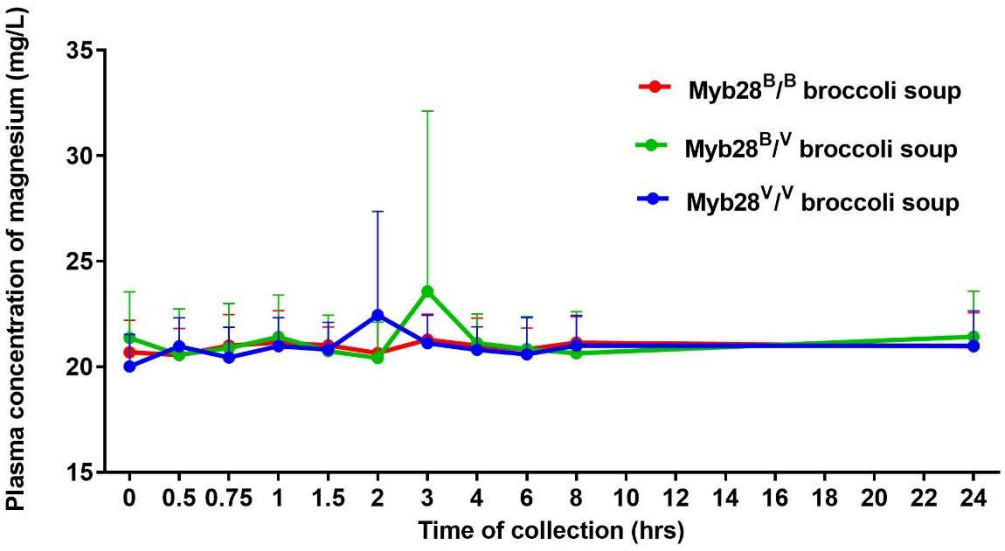

Plasma concentration of cobalt (µg/L)
0.6
0.4
0.2
0.0
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

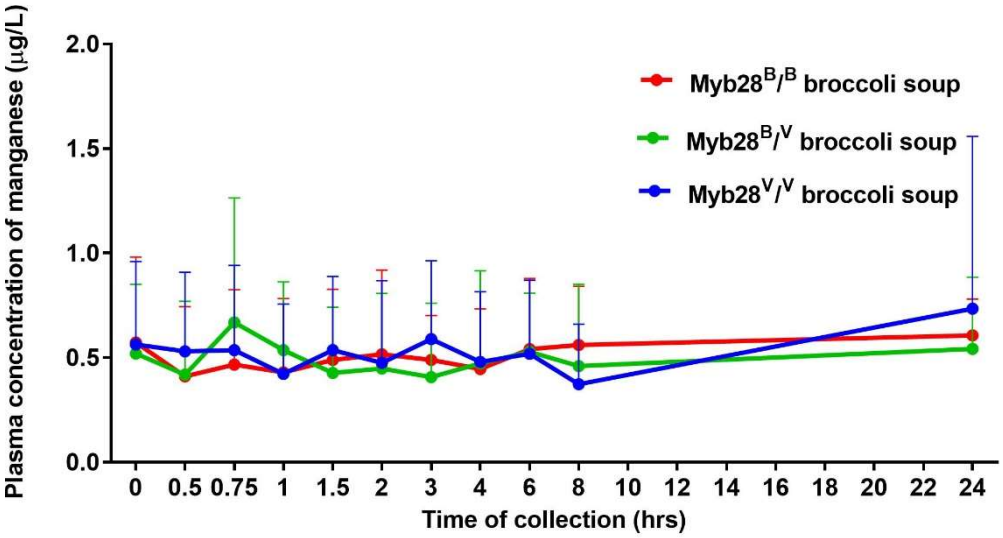

Plasma concentration of copper (µg/L)
1400
1200
1000
800
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

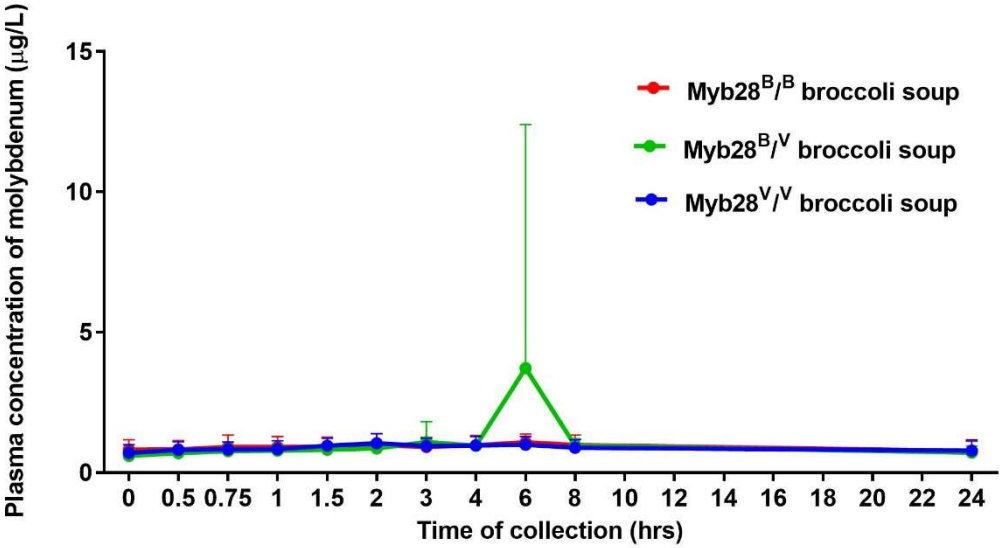

Plasam concentration of iron (µg/L)
3000
2000
1000
0
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

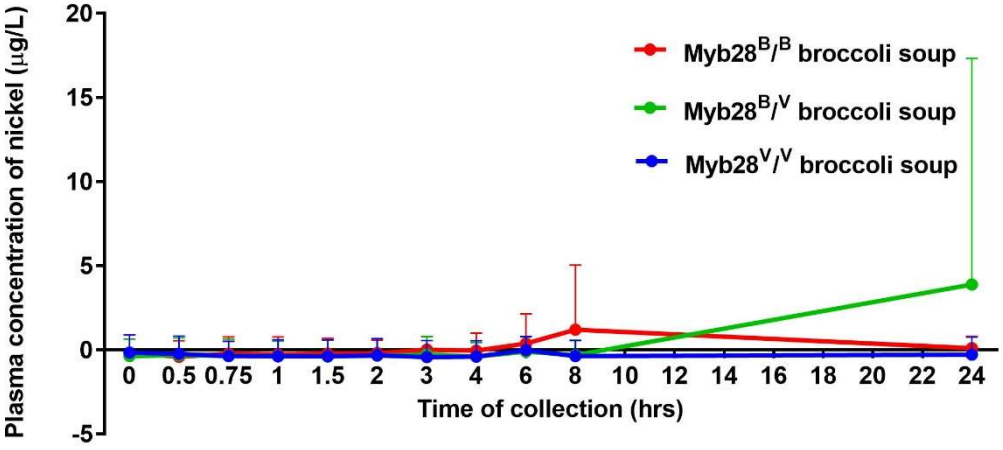

Plasma concentration of lead (µg/L)
8
6
4
2
0
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

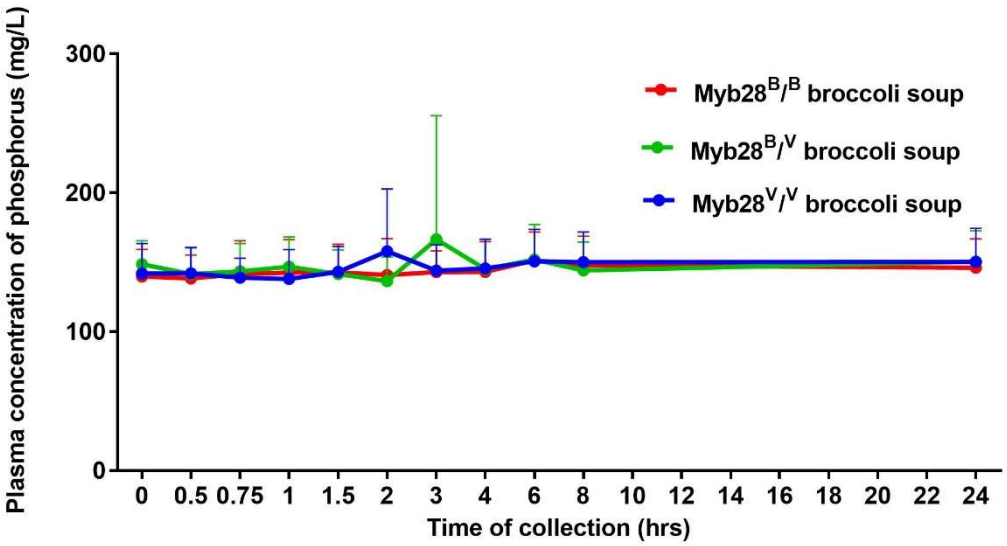

Plasma concentration of lithium (µg/L)
2.0
1.5
1.0
0.5
0.0
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

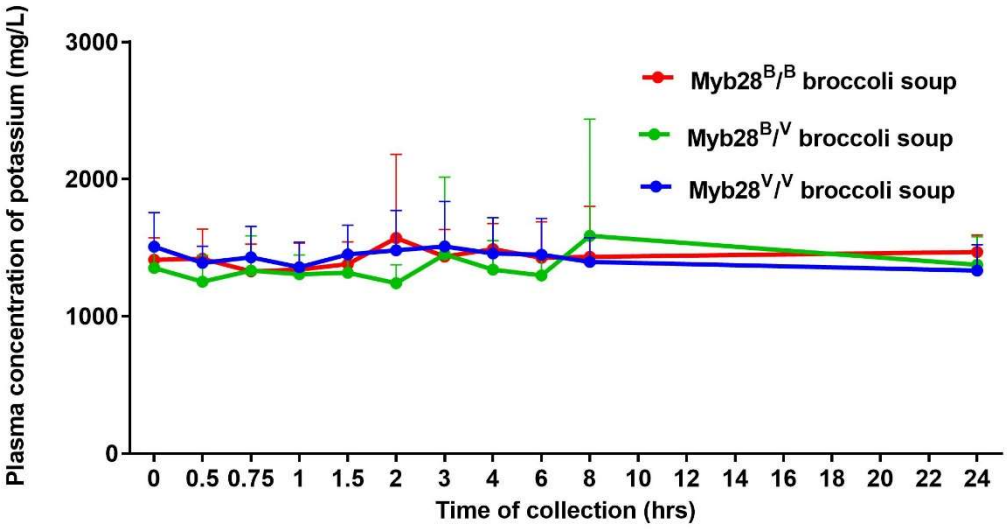

Plasma concentration of magnesium (mg/L)
35
30
25
20
15
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

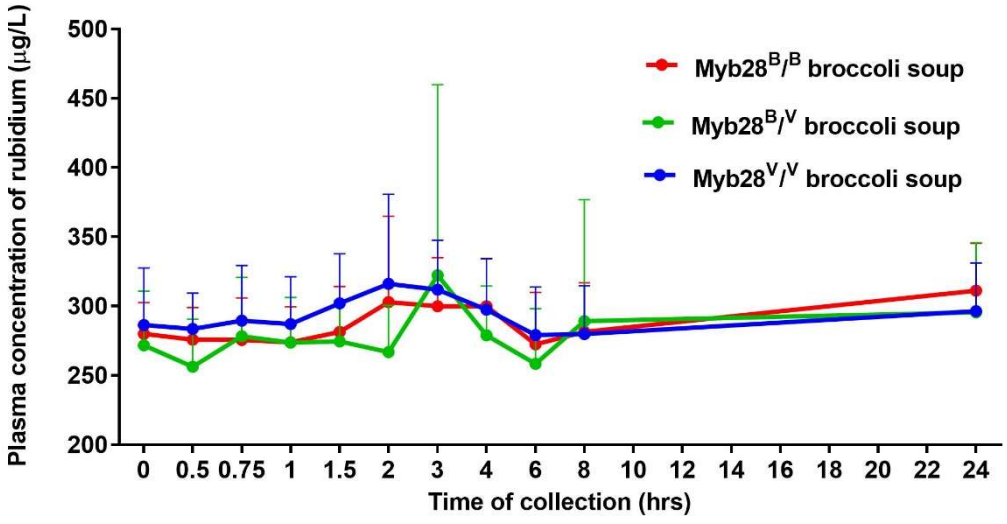

Plasma concentration of manganese (µg/L)
2.0
1.5
1.0
0.5
0.0
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

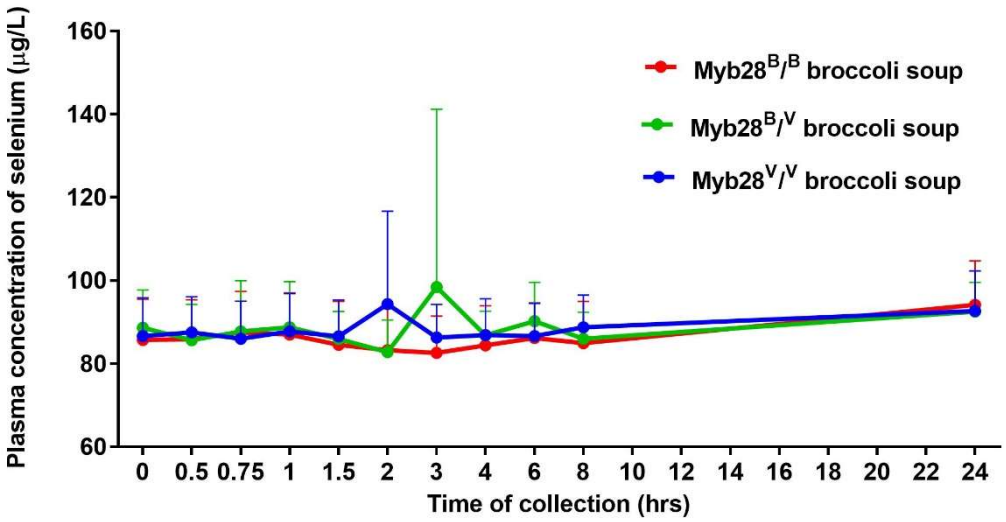

Plasma concentration of molybdenum (µg/L)
15
10
5
0
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

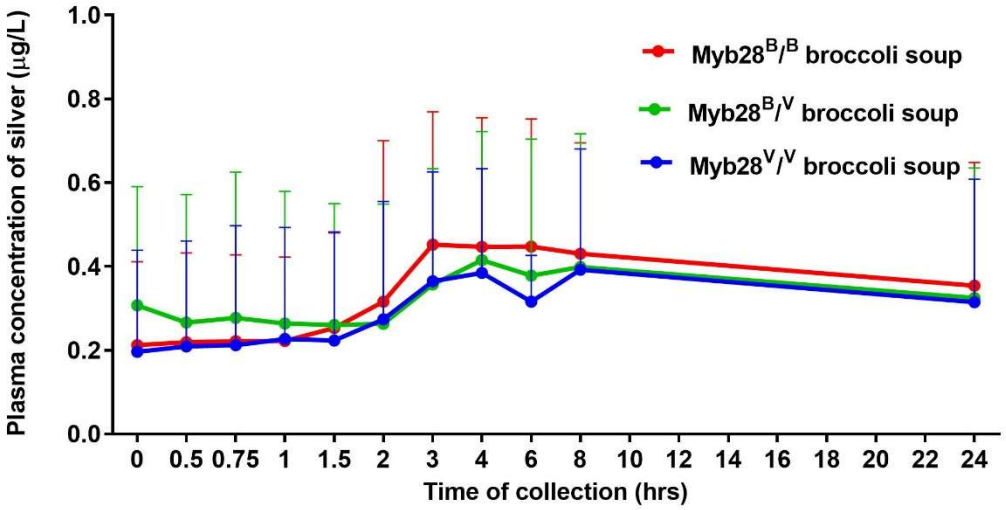

Plasma concentration of nickel (µg/L)
20
15
10
5
0
-5
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

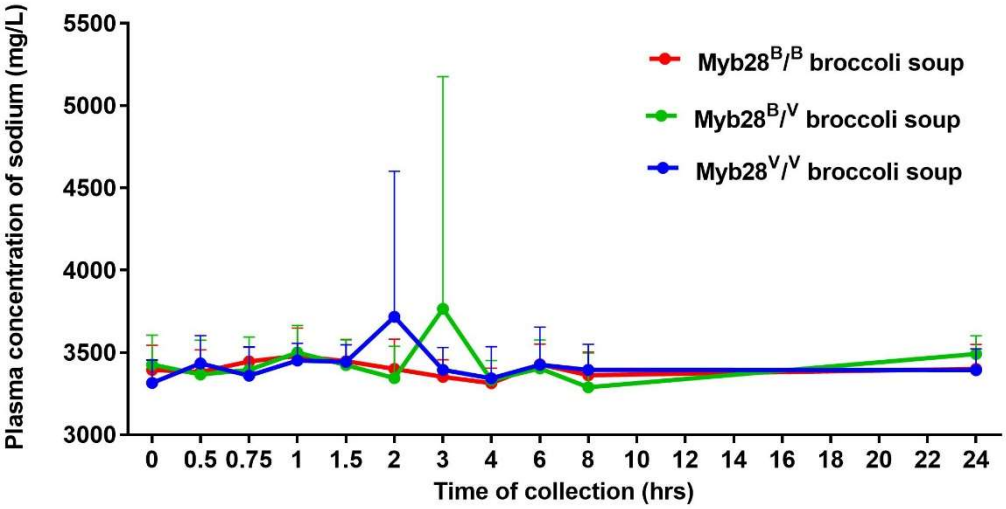

Plasma concentration of phosphorus (mg/L)
300
200
100
0
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

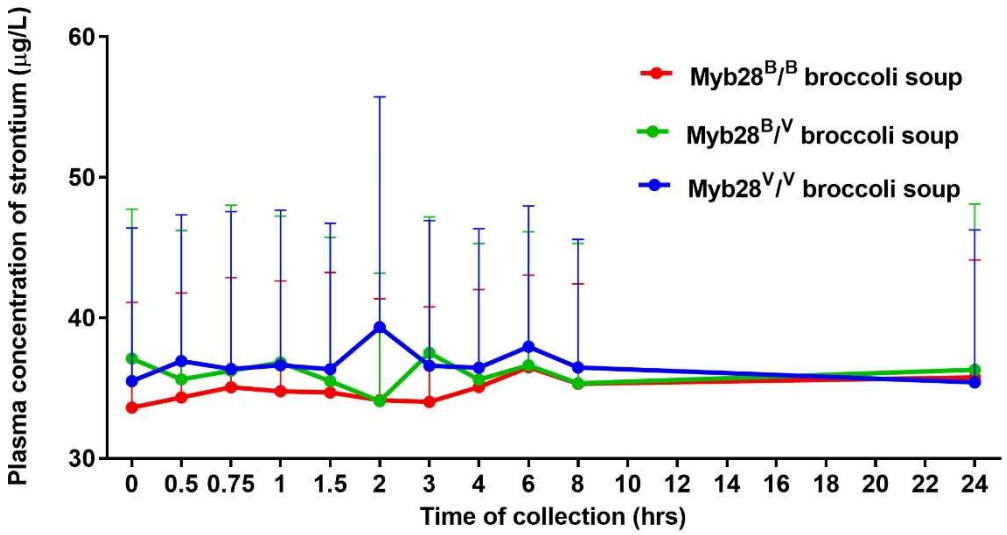

Plasma concentration of potassium (mg/L)
3000
2000
1000
0
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

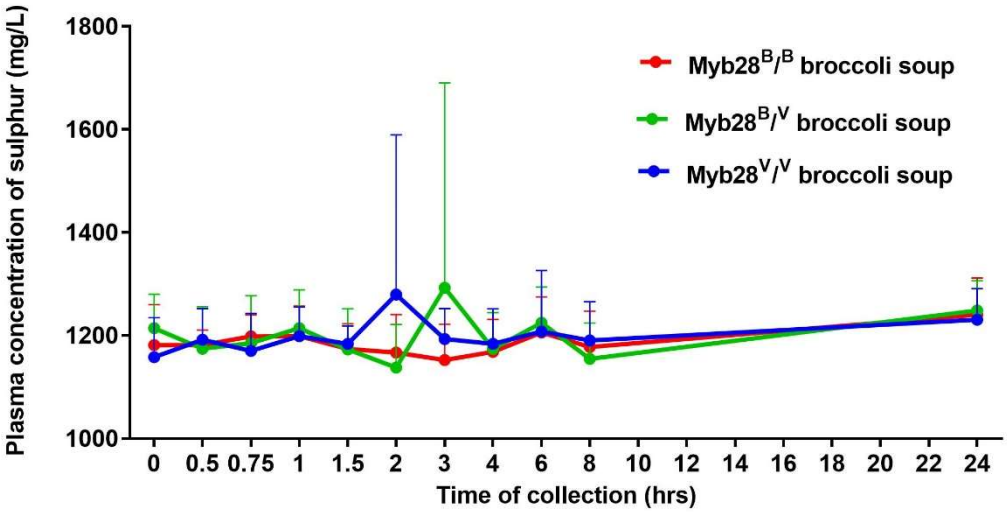

Plasma concentration of rubidium (µg/L)
500
450
400
350
300
250
200
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

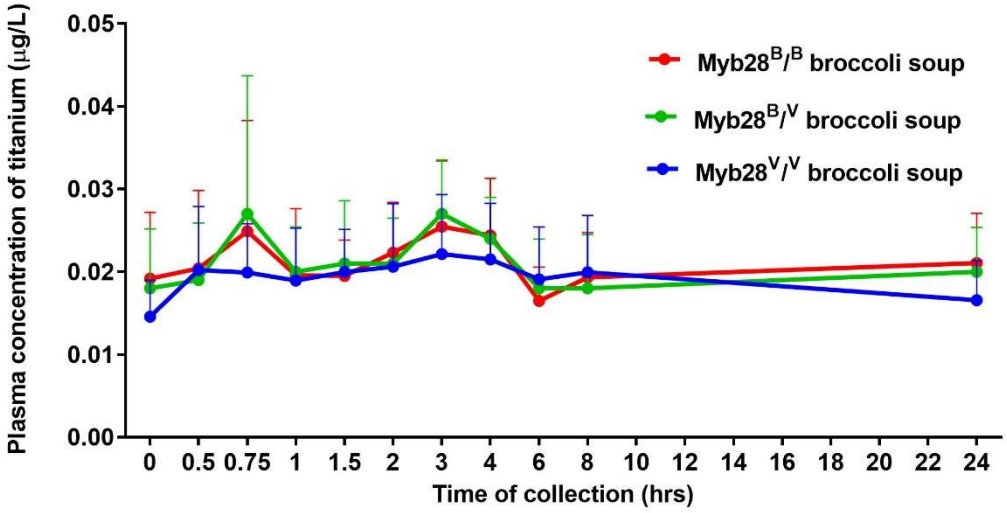

Plasma concentration of selenium (µg/L)
160
140
120
100
80
60
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup


Plasma concentration of silver (µg/L)
1.0
0.8
0.6
0.4
0.2
0.0
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup


Plasma concentration of sodium (mg/L)
5500
5000
4500
4000
3500
3000
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup


Plasma concentration of strontium (µg/L)
60
50
40
30
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)


Plasma concentration of sulphur (mg/L)
1800
1600
1400
1200
1000
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)


Plasma concentration of titanium (µg/L)
0.05
0.04
0.03
0.02
0.01
0.00
Myb28B/B broccoli soup
Myb28B/V broccoli soup
Myb28V/V broccoli soup
0 0.5 0.75 1 1.5 2 3 4 6 8 10 12 14 16 18 20 22 24
Time of collection (hrs)

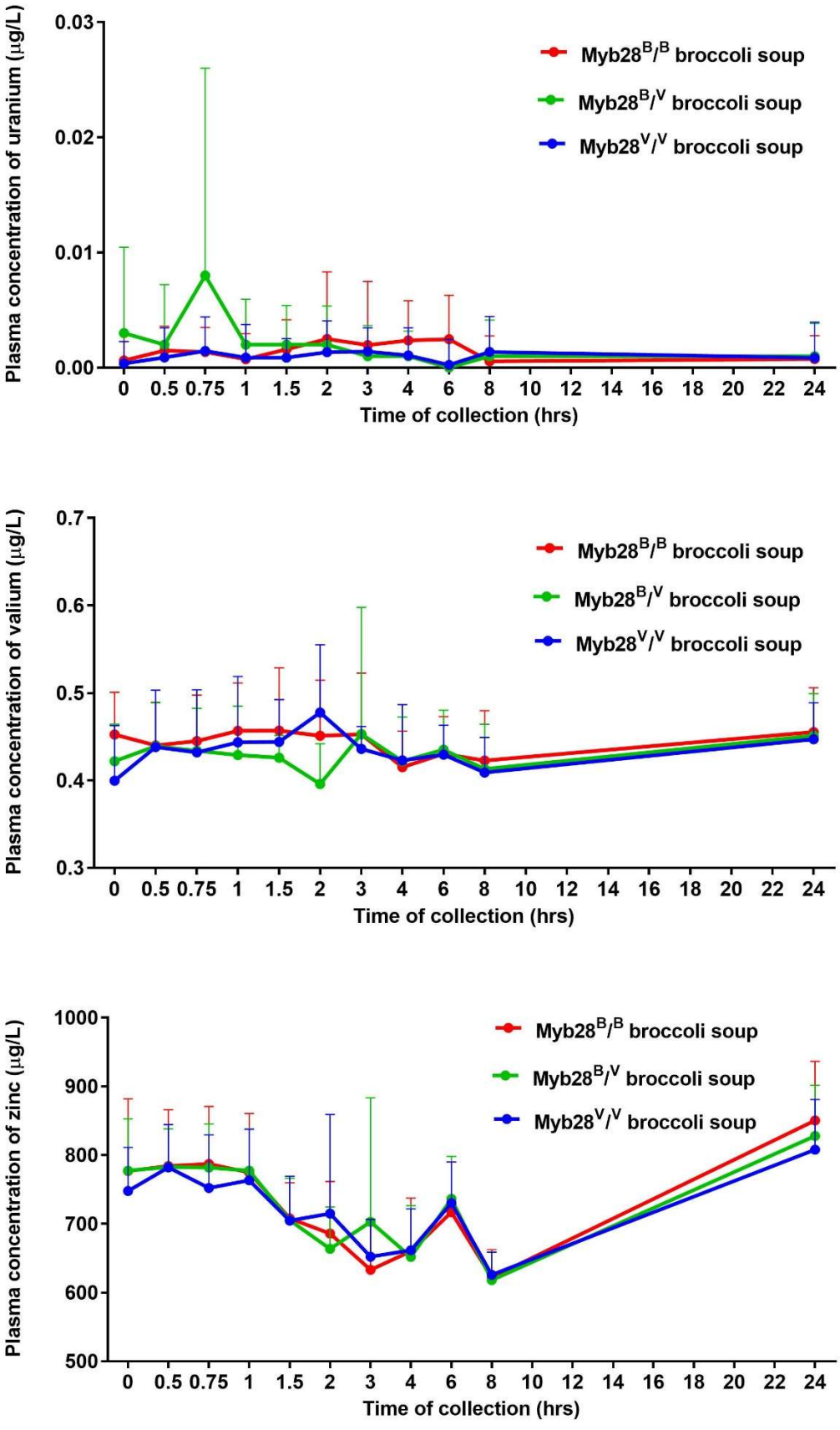


Figure: Concentration of mineral elements in plasma measured by ICP-OES.