Bioavailability of Citrus Flavanones and Their Effect on Cardiovascular Health Biomarkers

Abdurrahman M Ali Sweidan

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

The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Abstract

Consumption of flavanones has been associated with a reduction in risk of developing CVD, however flavanone bioavailability varies greatly in the population. The thesis aim was to investigate variations in absorption, as assessed by urinary excretion and, for the first time, the effect of commercially-available citrus supplements on CVD risk biomarkers.

In study 1, 15 participants consumed orange juice (OJ; 500 ml: 8 mg naringenin, 42 mg hesperidin/100 ml) and/or soya-nuts (25g): 30 mg daidzein, 57 mg genistein/100 g). Urine was collected at 0, 0-4, 4-8, 8-12, 12-24 and 24-36 hr on each of three separate occasions; saliva was collected hourly (0-8 and 24 hr) after co-ingestion of OJ and soya-nuts. Daidzein was excreted to a greater extent than genistein (27-28% of dose ingested compared to 16-19%), whilst naringenin was excreted to a greater extent than hesperetin (9% vs 2.5-3%). There was a strong positive correlation between excretion of daidzein and genistein ($r=0.72$, $p<0.01$), and for hesperetin and naringenin ($r=0.72$, $p< 0.01$). Moreover, the amount of naringenin, and daidzein or genistein were correlated when co-ingested ($r=0.73$, $p<0.01$; $r=0.49$, $p=0.05$). No flavonoid was detected in saliva at any time point.

In study 2, 23 overweight participants ingested citrus supplements (114 mg hesperidin) or a placebo daily for 28 days in a randomised, cross-over intervention. Urinary excretion (24 hr) was analysed by LC-MS to determine flavanone metabolite concentration at day-1 or day-28. Hesperetin-3′-*O*-glucuronide, hesperetin-3′-*O*-sulfate and hesperetin-7-*O*-glucuronide were quantified. Supplement flavanones had similar bioavailability to OJ, and there was no cumulative increase in excretion over 28 days. There was a non-significant decrease in blood pressure and fasting blood glucose after supplementation compared to placebo, however pulse amplitude tonometry was not affected. Augmentation index was reduced after supplementation compared to baseline.

Although results from this thesis showed no significant change in CVD risk biomarkers, more work should verify the protective mechanism of hesperetin metabolites at different doses on vascular health.

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Abbreviations

ABC	ATP-binding cassette
ADMA	Asymmetric dimethylarginine
ACE	Angiotensin converting enzyme
ACN	Acetonitrile
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
AUC	Area under the curve
BHS	British Hypertension Society
BCRP	Brest Cancer Resistance Protein
BL	Baseline
BMI	Body mass index (kg/m2)
BOJ	Blood orange Juice
BP	Blood pressure
CaM	Calmodulin
CBG	Cytosolic β-glucosidase

CDP	Control drink placebo
cGMP	Cyclic guanosine 3,5,mono-phosphate
CHD	Chronic heart disease
C_{max}	Maximum plasma concentration
COMT	Catechol –O- methyltransferase
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
d	Day(s)
DAD	Diode array detector
DBP	Diastolic blood pressure
DDAH	Dimethylaminohydrolase
DM	Diabetes mellitus
DMD	Desmethylargolensin
DMSO	Dimethylsulphoxide
EDRF	Endothelium-derived relaxing factor
EGCG	Epigallocatechin gallate
ESH	European Society of Hypertension

ESI	Electrospray ionisation
EPIC	European Prospective Invesigation into Cancer and Nutrition
ET-1	Endothelin-1
ETS	Enzyme treated soymilk
FAD	Flavan adenine dinucleotide
FAO	Food Agriculature Organization
FBS	Food blance sheet
FBS	Fasting blood sugar
FFQ	Food frequency questioniare
FMD	Flow-mediated dilation
FMN	Flavan mono nucleotide
Fsoy	Fermented soy
FSM	Fermented soymilk
GlcA	Glucuronic acid
H_2O_2	Hydrogen peroxide
H4B	Tetrahydrobiopterin
HCT	Haematocite

HDL	High density lipoprotein
HPH	High-pressure homogenized
HPLC	High pressure liquid chromatography
hr	Hour (s)
HSA	Human serum albumin
ICC	Intra-class correlation coeffecient
IFCC	Inter Federation of Clinical Chemistry
INTERMAP	Epidomoligical investigation to examine the correlation between multiple nutrient and blood pressure
IS	Internal standard
ISO	International Organization for Standardzation
LC	Liquid chromatography
LDL	Low density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
LPH	Lactase phloridzin hydrolase
m/z	Mass to charge ratio

mAU	Milli absorbance unit.
MED	Mediterranean
min	Minute(s)
mM	Millimolar (mmol/l)
MRP	Multi-drug resistance-associated protein
MS	Mass spectrometry
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-B
NO	Nitric oxide
NOS	Nitric oxide synthase
OJ	Orange Juice
$ONOO^-$	Peroxynitrite
PAT	Pulsatile, sometimes peripheral, arterial tone
PTFE	Polytetrafluoroethylene
PVA	Pulse volume amplitude

PWV	Puse wave volume
RHI	Reactive hyperaemia index
ROS	Reactive oxygen species
Rt	Retention time
RCT	Randomized controlled trials
SBP	Systolic blood pressure
SD	Standard deviation
SE	Standard error
SGLT1	Sodium-dependent glucose transporter 1
SHR	Spontaneously hypertensive rats
SIM	Selective ion monitoring
SMBG	Self-monitoring of Blood Glucose
SULTs	Sulfotransferase
T2DM	Type-2-diabetes mellituse
TC	Total chlesterol
TG	Triglyceride
TIC	Total ion count
T_{max}	Time to reach maximum plasma

	concentration
TVP	Textured vegetable protein
UGTs	UDP-glucuronosyltransferases
USDA	United State Departement of Agriculture
UV	Ultraviolet
V	Visit
VCAM-1	Vascular cell adhesion molecule 1
v/v	Volume/volume
WHO	World health organization
$[M-H]^-$	Negatively charge molecular ion
µl	Microlitre
µm	Micrometre
µM	Micromolar
µmole	Micromole

Publications

Abstracts

Abdurrahman. M. Sweidan., Day, A. J. & Williamson G. Poster presentation: Food Science & Nutrition 1st Annual PhD Conference. University of Leeds. United Kingdom 2014.

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Sweidan, A.M., Day A.J. & Williamson, G. Effect of co-consumption of flavanones and isoflavones on urinary and salivary excretion in humans (to be submitted to BJN), based on chapter 3).

Sweidan, A.M., Day A.J. & Williamson, G. Effects of citrus bioflavonoid supplements on endothelial function and blood pressure among overweight adult in a placebo-controled cross-over intervention study. (to be submitted to EJCN) (Based on chapter 6).

Sweidan, A.M., Day A.J. & Williamson, G. Bioavailability and metabolism of hesperetin from citrus bioflavonoids in overweight adults. (to be submitted to Xenobiotica), (based on chapter 7).

Chapter 1: Introduction and Background

1.1 Introduction

Flavonoids are naturally occurring phytochemicals or secondary metabolites from plants. They are absorbed from food, metabolised, circulate in the bloodstream, and are excreted in the urine. Epidemiological studies have suggested that high intake of fruit; vegetables and particularly soy food reduce the risk of chronic diseases such as cardiovascular diseases and cancers. The gut microflora plays a significant role in the metabolism and absorption of some flavonoids. This role leads to a large inter-individual variation in the amount absorbed and excreted in urine.

Cardiovascular disease (CVD) is the leading cause of deaths in the Western world. Established risk factors include high LDL cholesterol, high blood pressure and diabetes. Poor blood vessel health is considered a predictor of future CVD risk, but can be reversed. Several different measurements can be used to determine blood vessel health; such as blood pressure (BP), and newer techniques which measure blood flow through the arteries after a blood pressure cuff restricts blood flow for a few minutes in one arm.

Literature review aimed to focus on the importance of bioavailability of the flavonoids flavanone and isoflavone and the effect of diet rich in flavanone in cardiovascular disease (CVD) risk. The research strategy focused on using the University of Leeds library system, Web of Science, Medline and EMBASE (both on Ovid.com), google scholar and PubMed database were searched between July 2010 and December 2014. The following search words were used: studies that investigated the bioavailability of flavanone and isoflavone their absorption and excretion OR the effect of food matrix and dose ingested. Biological samples were used to investigate the bioavailability absorption and excretions in (plasma, urine and saliva) were included.

Moreover, an advance search was carried out in the Phenol-Explorer database (http://phenol-explorer.eu/content) to retrieve mean content values for flavanone and isoflavone contained in orange juice and soya nut. The flavonoid content in the orange juice, soya nut and citrus supplement was considered as the mean after the experimental food samples were analyzed using HPLC.

Studies investigated the impact of orange juice and citrus fruit on CVD risk factors were included. The studies were included only if they were randomized (with either a parallel or crossover design); had flavonoid intervention, recruited healthy and overweight adults participants. We focused our primary outcomes on risk factors that have strong relationship with CVD eg. systolic and diastolic blood pressure. As endothelial function is measured as flow-mediated dilation (FMD), and Endo-PAT , which are the predictor of CVD events and which correlate with the CVD risk factors such as blood glucose as secondary outcome. All languages were included in the search, however only papers with English abstracts were used for the review. Non-English language articles were translated when possible.

The chapter will begin by introducing flavonoids classification, structural characteristics, dietary intake of flavonoids and their major food sources. Then, the absorption and metabolism of flavonoids and factors affecting their bioavailability was highlighted. One aim of the thesis was to investigate the the impact of citrus flavanones on cardiovascular risk factors, therefore search about endothelial cell function and blood flow related to dietary flavanones was conducted.

1.2 Overview of flavonoids

Polyphenols are the products of secondary metabolism of plants. They are compounds that can be widely found in high amounts in foods such as; fruits, vegetables, tea, cocoa, soya, wine (Nielsen *et al.,* 2002, Ito *et al.,* 2005) and nuts (Loke *et al.,* 2009), dry legumes and cereals (Han *et al.,* 2007). Polyphenols are available in

dietary sources predominantly as two main classes, phenolic acids and flavonoids (Crozier *et al.,* 2009, Manach *et al.,* 2004). The focus of this thesis is on flavonoids, which can be further sub-divided into different classes based on the structure groups around the phenolic rings (Crozier *et al.,* 2009). Flavonoids have a structure that consists of two phenolic rings A and B bound together with an oxygenated heterocycle ring C, with several hydroxyl groups attached to any/all of the rings. The basic structure is the flavonoid skeleton figure 1.1.

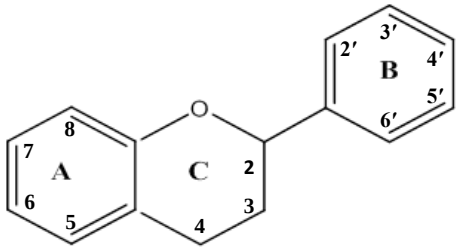


Figure 1.1: Chemical structure of the basic flavonoid structure. The rings A, B and C may have hydroxyl groups substitutions at any of the numbered positions, see table 1.1 for examples.

The division is dependent on what their molecular structure and substitution to functional groups in ring A and B. These can provide different sub-classes, such as flavonols, flavones, isoflavones, flavanones, anthocyanidins, flavanols and proanthocyanidins.

The majority of flavonoids occur naturally as glycosides rather than aglycones (Del Rio *et al.,* 2013). There are over 9000 different flavonoids (Thilakarathna and Rupasinghe 2013) as a result of the combination between the flavonoid aglycones and monosaccharides. The most common monosaccharides are D-glucose and L-rhamnose which bind the different hydroxyl groups of the flavonoid aglycones. The glycosides are

usually *O*-glycosides, which bind to the sugar moiety at the C-3 or C-7 position (Erlund, 2004).

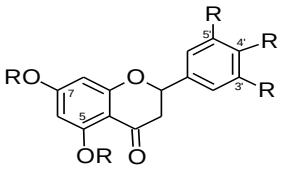
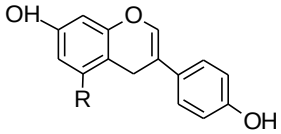
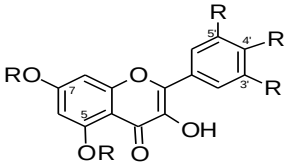
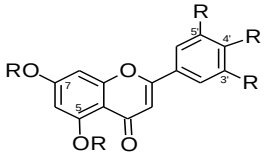
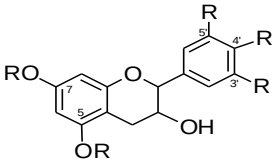
Table 1.1 shows the structure of the major classes of flavonoids and respective common dietary sources. Flavones, such as apigenin occur at high levels in parsley and celery (Linseisen and Rohrmann 2007). The richest source of flavonols in the diet is onion, which contains quercetin up to 1.2 g/kg fresh weight. Flavonols are also found in tea, which is an excellent source of flavanols/catechins found to be as high as 45 mg/L (Manach *et al.,* 2004). Moreover, high amounts of catechins are found in many kinds of plant-based foods, such as dark chocolate: 41.5 mg of (-) epicatechin and 11.9 mg of (+) catechin per 100g and red wine: up to 300 mg/L (Manach *et al.,* 2004). Anthocyanins are the coloured pigments rich in berry fruits and red wine.

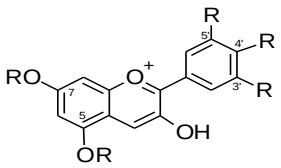
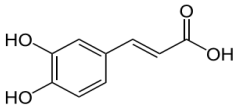
The sub-class of flavanones, which lack a double bond from position C2-3 in the C ring, are found almost exclusively in citrus fruits. The concentration of flavanones in the juice of these fruits can reach several hundred milligrams on a per-litre basis (Tomás-Barberán and Clifford, 2000). Orange juice is reported to contain between 200 and 600 mg hesperidin/L and 15–85 mg narirutin/L. The main flavonoids in orange and mandarins come from hesperidin (hesperetin-7-rutinoside) and narirutin (naringenin-7-rutinoside). On the other hand, the main flavanone in grapefruit is naringin (naringenin-7-neohesperoside) with lower amounts of narirutin (Kawaii *et al.,* 1999). Naringenin is also found in low levels in other fruits such as tomatoes and other tomato-based products. With regards to tomatoes as a specific example, naringenin chalcone can also be found particularly within the skin of the tomato, but during the processing from fruit to tomato ketchup this is converted into naringenin (Krause and Galensa, 1991).

Legumes and specifically soy foods are the main source of the isoflavone sub-class. Isoflavones have the B-ring attached at position C3 on the C-ring, instead of C2 as with all the other flavonoid classes. Daidzein and genistein are the main isoflavones in soya products. The amounts of isoflavones contained in soybeans and their products is

estimated between 580 – 3800 mg/kg fresh weight, with lower amounts, between 30 – 175 mg/L, found in soymilk (Manach *et al.,* 2004).

Table 1.1: Classification and sources of some dietary flavonoids

Flavonoids subclasses	**Dietary flavonoids**	**Chemical structure**	**Source (example)**
Flavanones	naringenin eriodictyol hesperetin		citrus fruits and juices (e.g. orange, grapefruit, lemon), peppermint
Isoflavones (Flavans)	genistein daidzein		soyabean and soya products
Flavonols	myricetin, querecetin, kaempferol, isorhamnetin		onions, tomatoes, tea (green, black), broccoli, apple
Flavones	apigenin luteolin		celery, peppers, parsley, herbal tea
Flavanols (Flavan-3-ols)	catechin, epicatechin,		red wine, tea (green), chocolate (dark, milk), cocoa, apples

Anthocyanidins	cyanidin, malvidin		cherry, red grapes, blueberries, red wine, strawberries
Phenolic acids	caffeic acid chlorogenic acid		blueberry, cranberry, orange, apple, lettuce, spinach, coffee, tea, coffee

Adapted from (Pérez-Jiménez *et a.,* 2010a) and (Crozier *et al.,* 2009).

1.3 Flavonoid intake

It has been estimated that in developed countries an average of 1-2 g/day of polyphenols may be consumed, largely provided from fruit and beverages such as tea, wine, coffee and fruit juices (Rothwell *et al.*, 2012). This level of dietary intake is much higher than the intake of vitamin C, E and other antioxidant compounds (Scalbert and Williamson, 2000, Jan *et al.,* 2010). The average intake of flavonoids appears to be between 65 – 250 mg/d (Manach *et al.*, 2004). In the US, the daily intake of flavonoids between 1999-2002 was calculated at 189 mg/d, and flavan-3-ols constituted the majority of this amount (Forester and Waterhouse, 2009). The intake of flavonols ranged from 13 – 64 mg/d in Germany; the flavone intakes were lower at 1-2 mg/d (Erdman *et al.,* 2007). A study in France reported that a total of 337 polyphenols were consumed including 258 polyphenols consumed by at least one half of the subjects and 98 polyphenols consumed in an amount > 1mg/d. Intake of individual polyphenols consumed were determined and the mean total of polyphenol intake was 1193 ± 510 mg/d or 820 ± 335 mg/d expressed as aglycone equivalents. Flavanone intake was 26 ± 29 mg/d mainly from oranges and orange juice (Pérez-Jiménez *et al.*, 2011).

Food frequency questionnaire (FFQ) is one of the common methods used to estimate flavonoid intake of the population (Tresserra-Rimbau *et al.*, 2013). For example, an FFQ was used in a large cohort study carried out in Greece to estimate the polyphenol content of foods consumed by adults. It was suggested that the intake of polyphenol came mainly from the common diet such as olive oil, vegetables and wine that constitute the main diet in the Mediterranean basin (Dilis and Trichopoulou, 2010).

It is very difficult to accurately measure the amount of flavonoid intake. This is due to the huge structural diversity in flavonoid classification and to the variation in content within foods. Moreover, the differences in content within similar food items results from the differences in growing environment, growing stage, storage, processing condition and the lack of standardised controlled analytical methods (Scalbert and Williamson, 2000).

The dietary intake of flavonoids is often estimated using the USDA flavonoids database or Phenol-Explorer database. Phenol-Explorer database holds data on 502 polyphenols contained in 452 foods and it has been developed and updated to be the most complete database currently available (Pérez-Jiménez *et al.*, 2011, Tresserra-Rimbau *et al.*, 2013) .

A large cohort study was conducted in 23 centres throughout 10 European countries. The aim of the project was to compare the total flavonoid dietary intakes between Mediterranean (MED) and non-MED countries participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. The results showed no significant differences between MED countries (370.2 mg/d) and non-MED countries (373.7 mg/d) in the total mean flavonoid intake. However, the main differences were in flavonoid class intakes and food sources. The main contributors in the non-MED countries were proanthocyanidins (48%) and flavan-3-ol (24.9%), and the main food sources were tea (25.7%) and fruits (32.8%); in the MED countries the most abundant

contributor was proanthocyanidins (59%) and fruits, wine, and tea were the main food sources (55%), (16.7%) and (6.8%), respectively (Zamora-Ros *et al.,* 2013).

Beking and Vieira (2011) relied on data from the international FAO Food Balance Sheets (FBS) to report the flavonoid intake among the populations of the UK and Republic of Ireland. Twenty-three flavonoids from five groups (anthocyanidins, flavonols, flavanols, flavanones, and flavones) were analysed. The results showed that the daily flavonoid intake (all five groups) was 182 mg and 177 mg for the UK and Ireland, respectively. In both cases, anthocyanidins and flavanols were the highest contributor for about 65% of total consumption. Combined intake of flavones, flavanones, and flavonols was 60 mg/day in the UK and 69 mg/day in Ireland (Beking and Vieira, 2011).

A study in China evaluated the intake of soya food and soya isoflavones. FFQ was the main tool in this study which recruited 1188 subjects. Results of the study revealed that the intake of soya isoflavones by Chinese rural adult women was much higher compared to women in western countries (Liu *et al.,* 2004). The dietary intake of soya foods and isoflavones in the UK diet was <1 mg for both men and women (Mulligan *et al.,* 2006). However, in soya consumers daily intake was higher at 8.6 mg, 7.5 mg in women and men, respectively. Bread and bread rolls were the main source for the intake (62.5% and 53%, respectively).

Data in table 1.2 shows the variation in the consumption and intake of flavonoids among different countries. This variation was explained by Scalbert and Williamson (2000) as the result of the dietary habits and preferences of the population involved in the study, as well as the differences in estimation from FFQ and database information.

1.4 Bioavailability of dietary flavonoids

1.4.1 Absorption and metabolism of flavonoids

Bioavailability has numerous definitions depending on the field of study. The most suitable one used in nutritional studies is “the fraction of the consumed ingredient that appears in the blood circulation” (Erdman *et al.,* 2007). The process of bioavailability is complicated as it includes ingestion, absorption, metabolism and excretion (Thilakarathna and Rupasinghe, 2013). The capability of flavonoids to be absorbed from the digestive tract depends on certain factors including the food matrix, the chemical structure of the flavonoid and the type of the conjugated sugar, which affect the rate and extent of small intestinal absorption (Terao *et al.,* 2008), large intestinal fermentation and transit time, and dietary habits (Bao and Fenwick 2004, Larkin *et al.,* 2008, Thilakarathna and Rupasinghe, 2013).

Table 1.2: Estimated daily dietary intake of flavonoids by adults in different countries.

Population	**Estimated dietary intake (mg/d)**	**Major food source of flavonoids and PP**	**Number of subjects and age**	**References**
Spanish	Total flavonoids, 443 ± 218 Total phenolic acids, 304 ± 156	Coffee, fruits, olives & olive oil, oranges	N= 7200 55 – 80 y	(Tresserra-Rimbau *et al.*, 2013)
French	820 ± 335 aglycone equivalents	Coffee, fruits, tea, wine, oranges	N=4942 45 – 60 y	(Pérez-Jiménez *et al.*, 2011)
European countries	Total flavonoids, Mediterranean, 370.2 Non-Mediterranean, 373.7	Tea , fruits, wine	N=521448 21 – 83 y	(Zamora-Ros *et al.*, 2013)
Spanish	Total flavonoids, 313	Apples, red wine, fruits, oranges	N=40683 35 – 64 y	(Zamora-Ros *et al.*, 2010)
Greek	Total flavonoids 92 Flavanones, 27, isoflavones, < 1	Onion, citrus fruits, apples, tea, wine	N=28572 51y for men, 54y for women	(Dilis and Trichopoulou, 2010)
Australian	Total flavonoids, 454 Flavanones, 7	Tea, wine, onion, apples, oranges	N=13858 >18 y	(Johannot and Somerset, 2006)
American	Total flavonoids, 189.7 Flavanone, 7.6%, Isoflavones 0.6%	Tea, wine, legumes, citrus fruits	8809 >19 y	(Chun *et al.*, 2007)
British and Irish	Total flavonoids, 182 for UK 177 for Ireland	NA	NA	(Beking and Vieira, 2011)

Hydrolysis and metabolism of flavonoids in the mouth will be limited. Some *in vitro* studies have suggested that some flavonoid glycosides can be hydrolysed by the β-glucosidase and esterase activity from oral bacteria after incubation with saliva (Yang *et al.*,. 1999, Walle *et al.*, 2005). However the short time they remain in the mouth suggests that early absorption is limited. The stomach may contribute to the absorption of some flavonoids that could be partly present in diet as aglycone, but not in their relative glycoside form (Crespy *et al.*, 2002). Flavonols, isoflavones, flavones and anthocyanins are usually found in the glycoside form linked with different sugars such as glucose, galactose, arabinose, xylose, rhamnose and glucuronic acid (Scalbert and Williamson, 2000).

Flavonoid glycosides will arrive to the small intestine and here hydrolysis, absorption and metabolism are more important. The small intestine has a large surface and a longer residential time, and there are an abundance of enzymes. Some dietary flavonoid glycosides are deglycosylated by lactase phlorizin hydrolyase (LPH) present in the brush-border of the small intestine lumen (Day *et al*., 2000). For example, Day *et al*. (2003) incubated quercetin-3-*O*-glucoside and quercetin-4′-O-glucoside with an inhibitor of SGLT1 and an inhibitor of the lactase domain of LPH using a rat everted-jejunum model. The results provided evidence that deglycosylation by LPH and subsequent diffusion of the aglycone is the major route of absorption for quercetin metabolism *in vivo (*Day *et al.*, 2000). Only glucoside conjugates appear to be hydrolysed by LPH (Németh *et al.*, 2003), and these flavonoids will be absorbed in the small intestine.

If the flavonoid is not a substrate of LPH, it will not be absorbed in the small intestine, but will continue to the large intestine where it will be deglycosylated by the action of microbial enzymes (Hollman, 2004; Thilakarathna and Rupasinghe, 2013). Both ways release the aglycone which is hydrophobic enough to pass through the

membrane of enterocyte by passive diffusion. The more efficient bioavailability of a flavonoid glucoside form was shown compared to the rhamnoglucoside form; Bredsdorff *et al.,* (2010) reported that hesperetin and naringenin absorption were increased in humans by conversion from rutinoside (the natural form in orange juice) to the glucoside after commercial orange juice was treated with an α-rhamnosidase enzyme.

The resulting aglycone will transport across the enterocyte of the small intestine or the colon and subsequently reach the liver. Flavonoids will undergo phase II metabolism in both the enterocyte and the liver. Aglycones will be metabolized by UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs) and catechol-O-methyltransferase (COMT; if an available catechol group is present) into, glucuronidated, sulfated and methylated metabolites, respectively (Manach *et al*., 2003, Matsumoto *et al*. 2004, Mullen *et al*., 2008, Brett *et al.*, 2009, Brand *et al*., 2010). These metabolites will then be distributed throughout the plasma, and will be excreted either through the urine or the bile. Significant amounts can be found in biological fluids such as plasma and urine (Matsumoto *et al*., 2004, Bredsdorff *et al*., 2010).

The metabolites that are excreted from the bile can also be hydrolysed again to their corresponding aglycone by the action of colon microflora enzymes, setting up enterohepatic circulation (Yuan *et al.*, 2007). The aglycone may also undergo further hydrolysis by the microflora to phenolic acids (Mullen *et al.*, 2008) which may also be absorbed and further conjugated. The absorption and metabolism processes are summarised in figure 1.2.

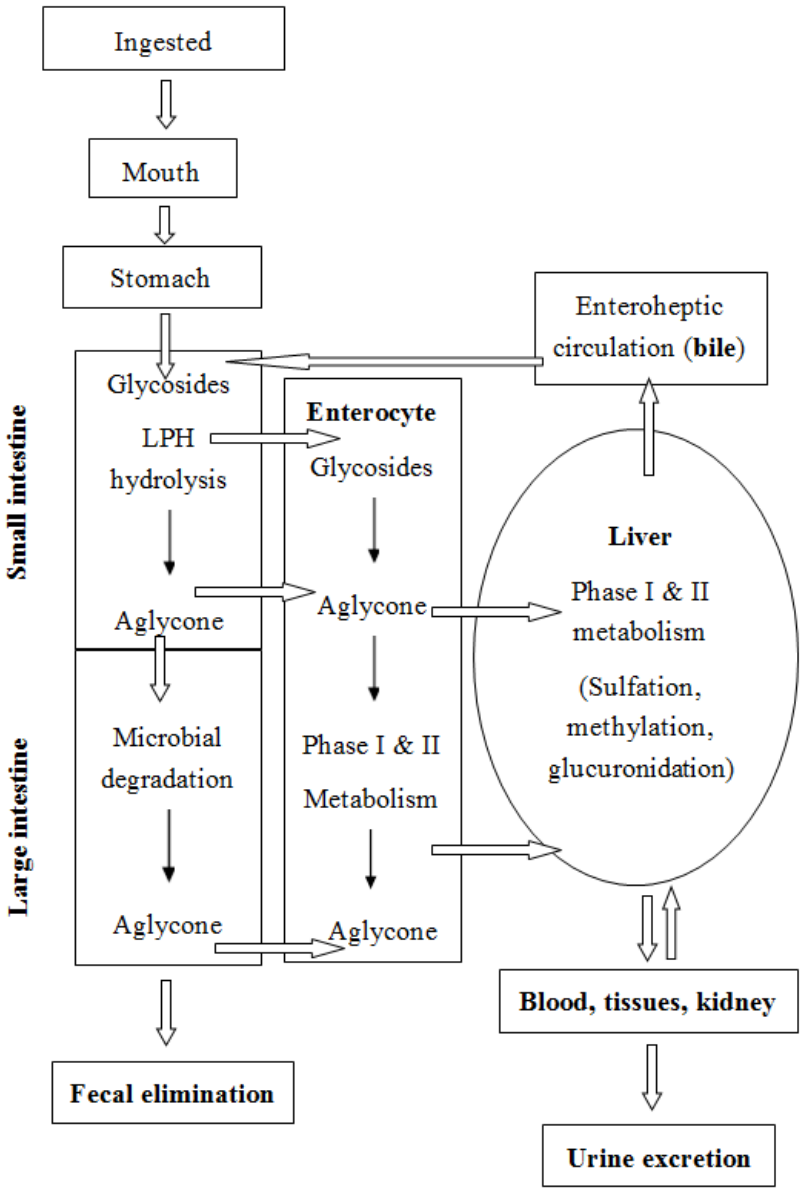


Figure 1.2: Metabolism of flavonoid glycosides in vivo. Flavonoids glycosides may be hydrolysed by lactase phlorizin hydrolyse (LPH) and the aglycone diffuses into enterocyte. Flavonoids which are not absorbed in the small intestinal will be hydrolysed by the microflora in the colon and released as aglycone form.

1.4.2 Bioavailability studies of flavanones and isoflavones

Human intervention studies on the flavanones hesperetin and naringenin, and the isoflavones daidzein and genistein are the most relevant flavonoids to be discussed in this thesis. The level of variability in the bioavailability of different sub-class of flavonoids is reported to vary greatly in vivo. The review by Manach *et al.* (2005) summarized the bioavailability of flavonoids on different sub-classes based on the data of 97 human studies conducted by that time. The review used several bioavailability measures, including the maximal plasma concentration (C_{max}), area under the plasma concentration time curve (AUC), time to reach C_{max} (T_{max}), elimination half-life, and relative urinary excretion and the data is presented in Table 1.3. Overall the plasma concentrations of total metabolites ranged between 0.1-1.5 µmol/L for flavanones, and 0.4-4.5 µmol/L for isoflavones, after an intake of 50 mg of aglycone equivalent. The T_{max} was similar for all these compounds, averaging at 5.4 hr. An enormous inter-individual variation in relative urinary excretion was shown, ranging between 1-30% and 7-62% of the ingested dose for flavanones and isoflavones respectively. In a review by Perez-Jimenez *et al*. (2010a) the recovery range for flavanones naringenin and hesperetin was 1.4 – 33% and 0.7 – 14%, respectively.

After orange and grapefruit juice were ingested (8 ml/kg of body weight), the concentration of both compounds as aglycones in plasma and urine was measured. The relative concentration of these compounds as excreted in the urine was 30 and 1.1% for naringenin from grapefruit and orange juice, respectively, and 5.3% for hesperetin from orange juice (Erlund *et al*., 2001). This study shows the food matrix and source of the compound is also of importance. Another study was on six healthy participants and they ingested 135 mg of hesperetin and naringenin (aglycone) orally. The result showed that the concentration in plasma reached a peak 4 and 3.5 hr for hesperetin and naringenin respectively. The mean values of relative cumulative urinary excretion as % of ingested

dose, were 3.2 ± 0.4 and 5.81 ± 0.8% for hesperetin and naringenin, respectively (Kanaze *et al*., 2006). Therefore there appears to be similarity in absorption and excretion behaviour from a non-food source.

In a cross-over study, 150 and 300 ml of blood orange juice (BOJ) were consumed by seven healthy females. The T_{max} in plasma was reached in 5.1 hr after BOJ was ingested. The C_{max} of hesperetin was 43.4 and 79.8 ng/ml after 150 and 300 ml consumed, respectively, and for naringenin the C_{max} was 16.4 and 34.0 ng/ml respectively (Gardana *et al*., 2007). This shows dose response in the same individuals.

Data were obtained on hesperidin and narirutin metabolites after ingestion of 0.5 and 1 L orange juice contain 444 mg/L hesperidin and 96.4 mg/L narirutin. The relative urinary excretion was 4.1 and 6.4% for hesperetin after 0.5 and 1L OJ consumed, and 7.1 and 7.8 % for naringenin as well. The circulating form of hesperetin was 13% sulfoglucuronides and 87% glucuronides (Manach *et al*., 2003).

The urinary excretion after five different beverages consumed was investigated. Commercial orange juice (29.2 – 70.3 mg flavanones/100 ml) was compared to experimental orange beverages enhanced by 110.2 mg/100 ml flavanones. The study reported that the solubility of the flavanones, particularly hesperidin is the key factor for the bioavailability of flavanones. Relative urinary excretion ranged from 0.5-5.4% for naringenin and 1.0-8.9% for hesperetin. There was a large inter-individual variation observed in the excretion of flavanones compounds. However, this variation was consistent for all participants after the consumption of different drinks. This could be interpreted by the fact that bioavailability is dependent on the occurrence of microflora in the colon of each individual (Vallejo *et al.*, 2010).

Table 1.3: Pharmacokinetic data of flavanones and isoflavones from 97 bioavailability studies *

	T_{max}		C_{max}		AUC		Urinary excretion		Elimination half-life	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
	hr		µ*mol/L*		µ*mol hr/L*		*% of intake*		*hr*	
Daidzin	6.3 ± 0.6	4.0–9.0	1.92 ± 0.25	0.36–3.14	21.4 ±6.5	2.7–38.6	42.3 ± 3.0	21.4–62.0	5.3 ± 0.8	3.4–8.0
Daidzein	4.9 ± 1.0	3.0–6.6	1.57 ± 0.52	0.76–3.00	12.2 ± 2.9	7.5–17.4	27.5		8.5 ± 0.8	7.7–9.3
Genistin	6.5 ± 0.6	4.4–9.3	1.84 ± 0.27	0.46–4.04	23.7 ± 6.7	6.2–45.1	15.6 ± 1.8	6.8–29.7	7.8 ± 0.7	5.7–10.1
Genistein	4.1 ± 0.6	3.0–5.2	2.56 ± 1.00	1.26–4.50	19.8 ± 6.5	10.4–32	8.6		7.1 ± 0.3	6.8–7.5
Hesperidin	5.5 ± 0.1	5.4–5.8	0.46 ±0.21	0.21–0.87	2.7 ± 0.7	1.9–4.1	8.6 ± 4.0	3–24.4	2.2	
Naringin	5.0 ± 0.2	4.6–5.5	0.50 ± 0.33	0.13–1.50	3.7 ± 1.5	0.9–7.0	8.8 ± 3.17	1.1–30.2	2.1 ± 0.4	1.3–2.7

*All data were converted to correspond to a supply of 50 mg a glycone equivalent. T_{max}, time to reach C_{max} AUC, area under the plasma concentration-time curve reproduced from (Manach *et al*., 2005).

The effect of food matrix on bioavailability and metabolism of flavanones was studied after orange juice (250 ml) was given with or without full fat yoghurt (150 ml). Pharmacokinetic of orange juice hesperetin–7–*O*-glucuronide and an unidentified hesperetin-*o*-glucuronide was detected after orange juice was consumed. The concentration of the metabolites reached a peak (C_{max}) of 924 nmol/L, 4.4 hr (T_{max}) after orange juice was ingested and has reached a peak (C_{max}) 661 nmol/L, and 5.5 hr (T_{max}) after orange juice with yogurt have been consumed. The level of flavanone metabolites excreted 0-5 hr after OJ consumed were significantly decreased by yogurt, however this was not true over the full 0-24 hr urine collection period. Excretion of hesperetin as percentage of intake was 6.3% without and 6.4% with yogurt. Naringenin excretion was 17.7% without yogurt combination and 15.7% with yogurt combination (Mullen *et al*., 2008).

The excretion level of phenolic acids was 62 µmol when OJ was ingested, but only 6.7 µmol and 9.3 µmol after ingestion of water and yoghurt, respectively. However, yogurt has a noticeable impact to slowing of the bulk of the meal reaching the large intestine and therefore effecting the excretion of phenolic acids resulting from the degradation of flavanones by the microflora in the colon (Roowi *et al.*, 2009). The resulting phenolic acids such as 3-hydroxyphenylacetic acid, 3-hydroxyphenyl hydracrylic acid, 3-methoxy-4-hydroxy phenylhydracrylic acid, dihydroferulic acid and 3-hydroxyl-hippuric acid, corresponded to 37% of the hesperetin-7-*O*-rutinoside ingested when the OJ was ingested individually (Roowi *et al*., 2009).

In an attempt to improve the bioavailability of hesperidin, a double-blind, randomised, cross-over study was conducted. Sixteen participants consumed three different orange juices (1) with natural hesperetin (low-dose), (2) with orange juice (low dose) treated with hesperidinase enzyme, and (3) orange juice (high dose) fortified to provide 3 times more hesperidin than naturally present. The C_{max} of hesperetin after OJ (2) was 4-fold higher (2.6 mmol/L) compound with the low dose OJ(1) and 1.5-fold

compared to high dose OJ(3). The T_{max} was earlier (0.6 hr) after consuming hesperetin-7-glucoside OJ(2) compared with 7.0 hr for the low-dose OJ (1) and 7.4 hr for the high-dose OJ(3). The study concluded that bioavailability was improved by enzymatic conversion of hesperidin to hesperetin-7-glucoside which changed the absorption site of the compound (Nielsen *et al.,* 2006). A study by Hubauzit *et al.* (2009) also reported that hesperetin-7-glucoside was more bioavailable than hesperidin, and the relative urinary excretion was increased from 2.4 to 3.6% by increase the dose between 0.25 and 0.5% hesperidin. Some human intervention studies after dietary intake of orange juice are shown in table 1.4.

The effect of single and repeated intakes of orange on flavanone absorption in humans was investigated. The level of hesperetin in the plasma increased after the consumption of 400 g of processed orange juice. The maximum level in plasma for hesperetin was 148 nmol/L, while 15 nmol/L for naringenin. After multiple-doses daily, there was significant increase compared to the baseline with 7 and 14 days after ingestion of 200 g orange. The concentration was undetectable in day one. After 7 and 14 days plasma levels of hesperetin were increased to 94 and 91 nmol/l respectively, and the concentration of naringenin increased to 3 and 2 nmol/l. This is an indication that levels of hesperetin and naringenin in plasma had an accumulative effect. However hesperetin and naringenin plasma levels showed no change from day 7 to 14 after orange fruits were consumed daily (De Pascual-Teresa *et al*., 2007).

Table 1.4: Human intervention studies, recovery of hesperetin and naringenin after dietary intake of orange juice

Study design	Source of flavanones	Dose ingested (mg)	Duration (hr)	Urinary excretion recovery (%)	C_{max} (nM)	T_{max} (hr)	References
Single dose N= 8 (M/F) 26 ± 5y	OJ	8 ml/kg	24	H=5.3 N=1.1	H= 2.2µmol/l N=0.6 µmol/l	-	(Erlund *et al.*, 2001)
Single dose N=6 (M/F) 25 ± 3.9 y	Oral capsule	(H)135 mg (N) 135	24	H=3.26 N=5.81	-	H=4.0 N=3.5	(Kanaze *et al.*, 2006)
Single dose N=7 (F) 25.7 ± 1y	Blood OJ	150 ml and 300ml (H) 51 -102 mg (N) 6-12 mg	24	-	H= 43 – 79 ng/ml N= 16.4 – 34 ng/ml	H= 5.3 – 5.3 N = 5.0	(Gardana *et al.*, 2007)
Cross-over	OJ	0.5 L	24	H=4.1 – 6.4	H=0.46 – 1.28	H=5.4 – 5.8	(Manach *et*

N =5 25 ± 1y		1L H = 444 mg/L N = 96.4 mg/L		N=7.1 – 7.87	µmol/L N=0.06 – 0.2 µmol/L	N=4.6-5.0	*al., 2003*)
Single dose N=10 (M)	Mixture juice	28 mg/L Each compound	48	H=14.2 N=22.6	H=0.25µmol/L N=0.18µmol	H=4.9 N=3.6	(Krogholm *et al.*, 2010)
Cross over N=10 (M/F)	OJ	29.2 – 70.3 mg/100ml	12	H=5.4 N=2.6	H=324 (nM) N=37(nM)	H=4.6 N=4.7	(Vallejo *et al.*, 2010)
Cross over N=8 21 – 50y	OJ	250 ml H=168 µmol N=12 µmol	24	7	OJ =922 nmol/L OJ with yogurt = 661 nmol/L	OJ=4.4 OJ W=5.1	(Mullen *et al.*, 2008)
Single dose N=10 (M/F) 19 – 51y	Polyphenol rich beverage	350 ml H=45 µmol	24	12	168 nmol/L	3.7	(Borges *et al.*, 2010)
Cross over	OJ low dose H	-	24	4.06	0.48	7.0	(Nielsen *et*

N=16 (M/F) 36 ±7y	OJ h-7-gly OJ high dose H			14.40 8.90	2.6 1.0	0.6 7.4	*al.*, 2006)
Cross over N=20 (F/M) 49y	Orange fruit OJ	150 g fresh fruit (79.7 H, 11.8 N) 300 g OJ (71.8H, 9.4 N)	48	H=4.5 N=12.5 H=4.6 N=10.2	28 µg/L 23 µg/L 30 µg/L 14.3 µg/L	7.0 5.8 6.2 4.46	(Brett *et al.*, 2009b)

OJ: Orange juice, (H) Hesperetin, (N) Naringenin

Isoflavones are the most well-absorbed dietary flavonoids with urinary excretion of metabolites typically 20 - 50% of the intake. The mean relative urinary excretion of isoflavones is 42% and 15.6% for daidzein and genistein, respectively (Manach et al., 2005). The bioavailability of isoflavones was reported by Watanabe *et al.* (1998). In their study seven male participants were given a 60 g of Kinoko (baked soybeans powder, containing 103 µmol/L daidzein and 112 µmol genistein. It reported that the plasma concentration of daidzein and genistein peaked at 1.5 ± 0.34 µmol/L and 2.4 µmol/L, respectively. The mean recovery rate of daidzein in urine was 35.8% and 17.6% for genistein. A randomized, double blind crossover study in seven men aged between 22-30 y was conducted to measure the pharmacokinetics of the isoflavones after ingestion of pure daidzein in both aglycone and glucoside form. After consumption of 1 mg (aglycone equivalent)/kg body weight of pure daidzein or pure daidzein-7-*O*-glucoside, blood and urine samples were collected. The cumulative recovery over 24 hr averaged 11.6% for daidzein and 38.9% after ingestion of daidzein glucoside (Rüfer *et al.*, 2008).

In another similar study, 19 healthy women were recruited and divided to 4 groups and asked to ingest standard 50 mg doses of daidzein, genistein, daidzin and genistin. The peak plasma concentration T_{max} of pure daidzein and genistein were reached in an average time of 5.2 and 6.6 hr, respectively, earlier than their corresponding glycosides where the T_{max} was delayed to 9.3 and 9.0 hr, respectively. The C_{max} was 0.76 and 1.5 µmol/L for daidzein and daidzin, respectively, and 1.26 and 1.22 µmol/L for genistein and genistin (Setchell *et al*., 2001).

The bioavailability of isoflavones from different soy-based foods was investigated with Isogen, soymilk, and fermented soybeans. The urinary recovery rates of daidzein and genistein were 42% and 17% after isogen, 46% and 23% after fermented soybeans, and 33% and 22% for the soymilk group. This study concluded that soy based food contain high amount of isoflavones aglycone such as fermented soy food are more

effective for maintaining high plasma isoflavone concentration (Chang and Choue, 2013).

A similar study investigated the effects of soymilk-based beverages containing different types of isoflavones on their bioavailability. Three different kind of soymilk (untreated soymilk, β-glucosidase-treated soymilk (ETS), and fermented soymilk (FSM) were ingested. The C_{max} reached 0.94 μmol/L at 6.0 hr for soymilk. However, the concentration was reached more quickly for ETS and FSM at 1.0 hr of (1.75 and 2.05 μmol//L), respectively. Moreover, the urinary excretion of isoflavones was significantly higher after ingestion of ETS and FSM compared with untreated soymilk up to 8 hr after consumption. The percentage of daidzein and genistein recovered in the urine after ingested was 37.3 and 20.2% for soymilk and 46 and 30% for ETS and 46.3 and 28.0 % for FSM. The aglycones isoflavone were reported to be absorbed faster and in greater amounts than their glucosides (Kano *et al.*, 2006).

The bioavailability of isoflavones after single consumption of fermented (Fsoy) and non-fermented soybeans (soy) products was investigated. The urinary recovery of total isoflavones was higher in the Fsoy group after 2 hr compared with that in the soy group with recovery rates 48% and 37% in 48 hr, respectively. The results demonstrated that the aglycone-rich Fsoy group were absorbed faster and in greater amounts compared with those consume glucoside-rich soy (Okabe *et al.,* 2011).

The pharmacokinetics of isoflavones in 10 healthy women was measured among participants who were asked to ingest 10, 20 and 40 g of soy nuts containing (9.8, 19.6 and 39.2 mg genistein and 6.6, 13.2 and 26.4 mg daidzein). The mean fraction of the isoflavones excreted in the urine was decreased with the increase of the intake. The excretion was expressed (as %) of the dose ingested was 63.2%, 84.4% and 44.0% respectively for daidzein and 25.2%, 23.4% and 15.8% for genistein. The result showed that daidzein and genistein were absorbed at range from 2-8 hr after ingestion, with means for daidzein and genistein of 6.1 hr and 5.0 hr, respectively (Setchell *et al.*,

2003a). Table 1.5 shows human intervention studies, recovery of daidzein and genistein after dietary intake of soy-based food products.

The effect of the matrix (supplements or foods) on urinary recovery of isoflavones was reviewed by Perez-Jimerez *et al.* (2010a). There was no difference between acute and chronic isoflavone studies in urinary excretion, and the recovery range for isoflavones were 8.3-60% and 1.7-47% for daidzein and genistein, respectively. Food matrix and processing might be good factor which affect the absorption of isoflavones in human. Three different food products (fruit juice, chocolate bar and cookies) were prepared and 455 mg isoflavone rich extract was added to each product before processing. Different aglycone levels of isoflavone in the plasma were reported but they were not affected by the different food matrix. The urinary excretion of daidzein was similar for all three foods consumed with total urinary recovery of 33-34% of dose ingested. However, urinary recovery of genistein was lower following consumption of juice compared with the other two foods, peak genistein levels were reached in plasma earlier after ingestion of a liquid matrix compared to solid matrix (De Pascual-Teresa *et al*., 2006). In a randomized crossover study, consumption of soy-bread or soya-beverage for 3 weeks was investigated. These foods contained 2 g soy protein with 99 and 93 g isoflavone aglycone equivalents /day respectively. Women excreted more metabolites after the bread intervention than men. However, the excretion of isoflavones was the same and no differences was reported after the soy beverage was consumed by men and women. It was suggested that food matrix significantly affects soy isoflavone absorption, particularly in women (Ahn-Jarvis *et al.*, 2012).

Table 1.5: Human intervention studies, recovery of daidzein and genistein after dietary intake of soy-based food products

Study design	Source of flavanones	Dose ingested (mg)	Duration (hr)	Urinary excretion recovery (%)	C_{max} (µmol/L)	T_{max} (hr)	References
Single dose N=7 (M) 33.3 y	Backed soybean powder	60 g of kinako 103 µmol (D) 112 µmol (G)	72	D = 35.8 G= 17.6	D= 1.56 G = 2.44	6	(Watanabe *et al.*, 1998)
Crossover N=7 (M) 22-30 y	Pure daidzein (D) Pure daidzein glucoside (DG)	3.9 µmol/kg body weight for both	24	D= 11.6 DG = 38.9	D= 0.48 DG = 2.5	D= 8.3 DG = 9.1	(Rüfer *et al.*, 2008)
Single dose N=19 (F) ≥ 18 y	Pure daidzein, diadzin, genistein, genistin	50 mg each compound	24	-	(D= 0.76 , DG= 1.5 (G = 1.26, G=1.22)	(6.6 , 9.0) (5.2, 9.3)	(Setchell *et al.*, 2001)

Single dose N=6 (M) 37 y	Soybean flour-based	0.84 g flour/kg body weight 27 and 3.6 µmol/kg body weight	35	D = 62 G= 22	D= 3.14 G = 7.42	D = 7.4 G = 8.4	(King and Bursill 1998)
Single dose N = 26 (F) 25.4 y	Isogen Fermented soybeans (FS) Soymilk	64.8 isoflavones 81.8 isogen 43.8 FS 600ml soymilk	24	Isogen (D=42,G=17) FS (D=46, G=23) Soymilk(D=33, G=22)	Isogen (D=230,G=160 ng/ml) FS (D=244, G=195 ng/ml) Soymilk (D=211, G=231 ng/ml)	Isogen (D=3.7,G=4.) FS (D=2.8, G=3.5) Soymilk (D=3.7, G=4.8)	(Chang and Choue 2013)
Single dose N=12 (M/F) 33.9 y	3 soymilk Untreated Treated (ESM) Fermented (FSM)	Untreated (D=130 , G= 116) ESM (D= 7, G= 5) FSM (D = 5 , G =15)	48		Untreated (D=0.47 , G= 0.41 µmol/L) ESM (D= 0.82, G=0.87) FSM (D =0.95 , G= 1.02)	Untreated (D=6 , G=5.9) ESM (D= 1.0, G=1.1) FSM (D =1.0 , G =1.0)	(Kano *et al.*, 2006)

Crossover N=12 (F) 49 – 65 y	F soy Soy	Isoflavones 95 µmol per 23g, for both	48	48 37	F soy (D= 1.51, G= 1.04) Soy (D= 0.9, G= 0.73)	F soy (D=1.0, G= 2.09) Soy (D= 3.9, G= 5.0)	(Okabe *et al.*, 2011)
Single dose N=10 (F) ≥ 18 y	Soy nuts	10,20,40g contain D=(6.6,13.2, 26.4 mg) G=(9.8,19.6, 39.2 mg)	120	D= (63, 54, 44%) G= (25, 13, 15%)	D=(428, 843, 1691 nmol/L) G=(585, 1222, 2209 nmol/L)	D=(5.8,6.4, 6.0) G=(4.9,4.0, 6.0)	(Setchell *et al.*, 2003a)
N=12 (F) 64 y	Cereal bars and yoghurt	100 mg isoflavones D= 118 µmol G= 241 µmol	36	D = 66 % G= 48 %	D= (1.68 day 1 to 2.27/ day 30) G= (3.8 day 1 to 5.3/ day 30)	2 peaks for both 2 and 6	(Vedrine *et al.*, 2006)

(D) Daidzein, (G) genistein

1.4.3 Gut microflora and absorption of flavonoids

The gut microflora plays an important role in metabolism, degradation and bioavailability of many flavonoids (Aura *et al.*, 2008). Flavonoids that reach the colon are subject to breakdown to lower molecular weight compounds. These microbial metabolites may contribute to the biological effects of dietary flavonoids (Manach *et al.*, 2005; Williamson and Clifford 2010). It has been estimated that 5-10% of the total polyphenol intake is absorbed in the small intestine, and between 90-95% of the total polyphenols consumed will reach the large intestine. Conjugated compounds excreted back through the bile into the small intestine are subjected to breakdown by the enzymatic activity of gut microflora to low-molecular weight compounds (Cardona *et al*., 2013). Figure 1.3 shows a proposed pathway for the breakdown of hesperetin-7-*O*-rutinoside in humans.

Effects of the intestinal microflora on absorption of hesperidin were assessed after intestinal microflora was treated with antibiotics. Hesperetin concentrations in non-antibiotic-exposed and pseudo-germ-free rats were 0.58 and 0.20 μg/ml, respectively, and areas under the curve (AUC) values were 6.3 and 2.8 μg-h/ml, respectively. AUC and C_{max} was significantly higher in normal compared to pseudo-germ-free rats. These results have concluded that pharmacokinetic differences between non-antibiotic-exposed and pseudo-germ-free rats may be attributed to differing hesperidin uptake, as well as changing in metabolic activities of intestinal microflora (Jin *et al*., 2010).

Metabolism of isoflavones and flavanones is mediated by both gut microflora and considerable inter-individual variations in the excretion of isoflavones and their metabolites among different individuals have been reported. This variation is recognized to be due to the gut microflora differences that are responsible for the hydrolysis of isoflavones (Rafii *et al*., 2003, Setchell *et al*., 2003a, Tamura *et al*., 2007). Daidzein, an isoflavone found in soya, may be metabolized and converted by gut microflora to equol (7-hydroxy-3-[4-hydroxyphenyl]-chroman), dihydroxydaidzein, *O*-desmethylangolensin (*O*-DMD), or 4-hydroxy equol (Yuan *et al*., 2007), and likewise dihydrogenistein may be converted from genistein (Pérez-Jiménez *et al*., 2010a). Only individuals that have the appropriate microflora are able to produce equol, estimated to be approximately 30% of the Western population.

Equol has several biological activities relating to human health, and therefore the ability to produce this may be advantageous (Cardona *et al.*, 2013). The production of equol might be influenced by particular dietary habits that stimulate/suppress individual microflora composition (Yuan *et al.*, 2007).

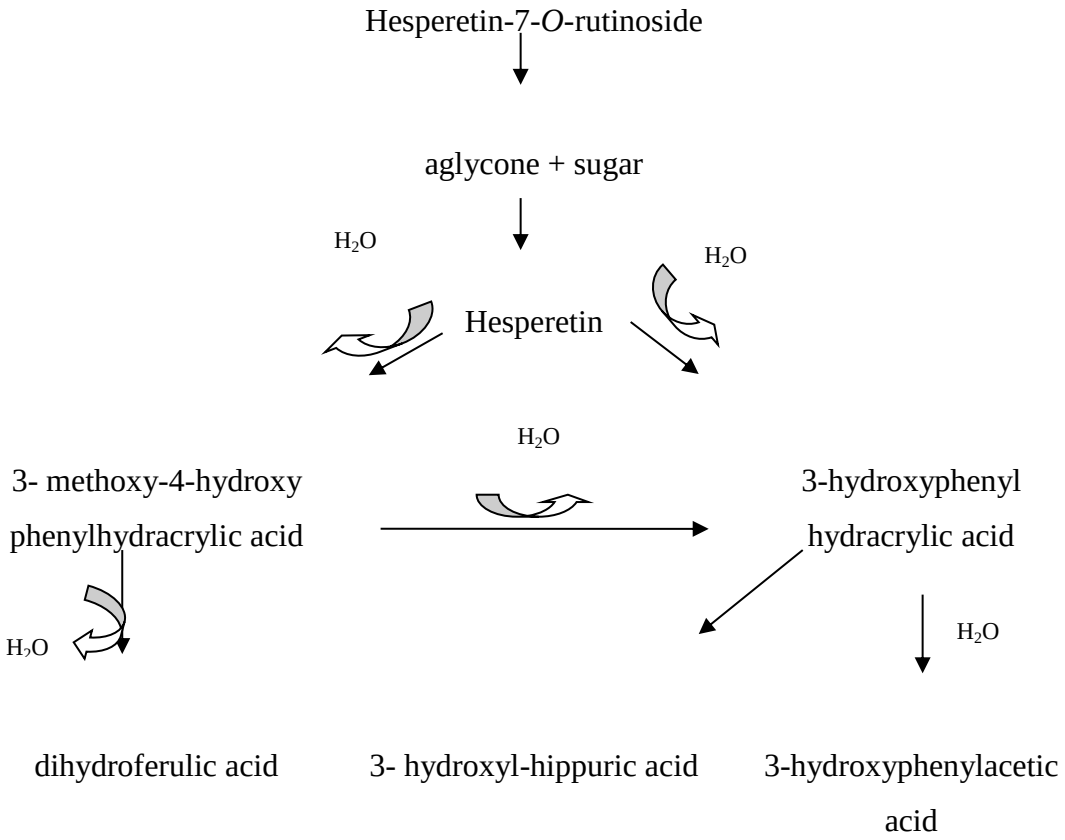


Figure 1.3: Proposed pathway for the breakdown of hesperetin-7-*O*-rutinoside in humans. Most conversions are the probably mediated by colonic microflora. Adapted from (Del Rio *et al.*, 2010).

1.5 Cardiovascular disease (CVD)

Cardiovascular disease (CVD) is a common term that is employed to describe diseases of the heart and blood vessels. In Europe, CVD is categorized as the main cause of mortality accounting for about four million deaths per year, nearly half of all deaths (48%) each year in Europe (Allender *et al.*, 2008; Chong *et al.*, 2010). Many environmental and genetic factors can cause this chronic and multi-factorial disease.

Well-known environmental factors which cause and/or enhance the CVD risk are high saturated fat consumption, hypertension, diabetes mellitus (DM), hyperlipidemia, physical inactivity and smoking (Huang, 2009, Vauzour *et al.,* 2010). Some of these risk factors can be controlled or modified, for example lifestyle modification; changing dietary habit and physical activity behaviour both led to decreasing CVD risk (Chong *et al.,* 2010), or if appropriate by pharmacological treatment (Kahn *et al.,* 2005, Huang 2009). Many studies have studied the relationship between diets rich in fruit and vegetables and decreasing CVD incidence. Fruits and vegetables contain many compounds that may have cardio-protective effects such as, folate, vitamin and non-nutrient phytochemicals, such as flavonoids and carotenoids (Chong *et al.,* 2010).

1.5.1 Endothelial cell function

Endothelial cells are arranged in a single layer known as the endothelium, separating the vascular lumen from the smooth muscle cells of the vessels. The endothelium is a semi-permeable barrier which helps in water and nutrient exchange between the blood and the vessel wall (Celermajer, 1997). Endothelial cells play a fundamental role in the regulation of vascular tone and homeostasis (Bonetti *et al.,* 2003). Responding to different hemodynamic and hormonal factors, the endothelium releases various vasoactive mediators to maintain vascular tone (Celermajer, 1997, Hinderliter and Caughey, 2003), namely nitric oxide (NO) as a vasodilator and endothelin-1 (ET-1) as a vasoconstrictor (Widlansky *et al.,* 2003, Shenouda and Vita 2007).

NO derived from endothelial cells is a strong vasodilator, and plays a crucial role in modulating both vascular tone and BP (Vallance and Chan, 2001, Moncada and Higgs, 2006). NO may play a crucial role in regulatory of atherosclerosis development, including restricting the activation of platelet aggregation, adhesion of leukocytes to the endothelial surface, synthesis of pro-inflammatory cytokines and vascular smooth muscle cell growth (Widlansky *et al.,* 2003, Rush *et al.,* 2005, Esper *et al.,* 2006).

NO is produced after the amino acid from L-arginine is oxidised (also producing L-citrulline), catalysed by endothelial nitric oxide synthase (eNOS) which can be found in caveolae (invaginations in cell membranes), in the presence of many cofactors including oxygen, tetrahydrobiopterin (BH4) and nicotinamide adenine dinucleotide phosphate (NADPH), flavan adenine dinucleotide (FAD), flavan mono nucleotide (FMN), and one heam group. The enzyme eNOS is activated to produce NO either by increase in blood flow (hyperaemia), which results in the increase of shear stress subsequently increasing intracellular Ca^{2+} (Davignon and Ganz, 2004), or by alteration in a number of chemical factors, such as histamine, bradykinin, acetylcholine (Moncada and Higgs, 1993, Kone *et al.* 2003, Fonseca *et al.*, 2004, Kawashima and Yokoyama, 2004, Stuehr and Griffith, 2006).

From the endothelial cells, NO will diffuse across the cell membrane into the smooth muscle cells in the vascular wall, where it activates guanylate cyclase resulting in an increase in cyclic guanosine 3,5-monophosphate (cGMP) level. This leads to smooth muscle cell relaxation (Vallance and Chan, 2001), as shown in figure 1.4.

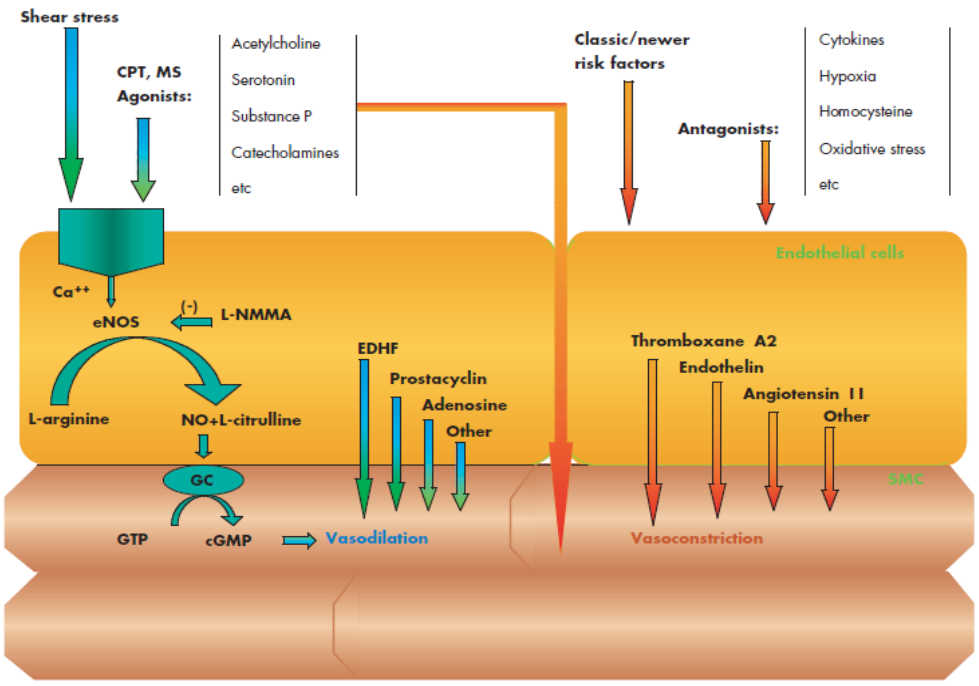


Figure 1.4: Vasoactive mediator effects on endothelial cells. Taken from Tousoulis *et al.*, (2005).

Low levels of NO bioavailability are recognised as an important factor in dysfunction of endothelial cells (Malek *et al.,* 1999, Vita and Keaney 2002). Alterations in normal function of endothelial cells play a fundamental role in pathogenesis of CVD. Thus atherosclerotic plaque formation, thrombosis, inflammation and vasoconstriction are all known as dysfunction of endothelial cells (Widlansky *et al.,* 2003, Bonetti *et al.,* 2003, Huang and Vita, 2006; Shenouda and Vita, 2007). Cardiovascular risk factors which raise vascular production of reactive oxygen species (ROS), and decrease the endothelial nitric oxide availability are shown in Figure 1.5. These include: reduced eNOS activity due to increased ADMA levels (an endogenous eNOS inhibitor), resulting from redox-sensitive inhibition of DDAH; eNOS "uncoupling" due to increased oxidation of the cofactor BH4; and inactivation of NO by reaction with superoxide resulting in peroxynitrite ($ONOO^-$) (Landmesser *et al.,* 2004). In fact NO bioavailability can be effected by many reasons such as decreased expression of eNOS, by altered activation of eNOS, or reduced level of eNOS cofactors (Venugopal *et al.,* 2002, Förstermann and Münzel, 2006), as well as increased synthesis of ROS from oxidative stress (Barua *et al.,* 2003, Mudau *et al.,* 2012).

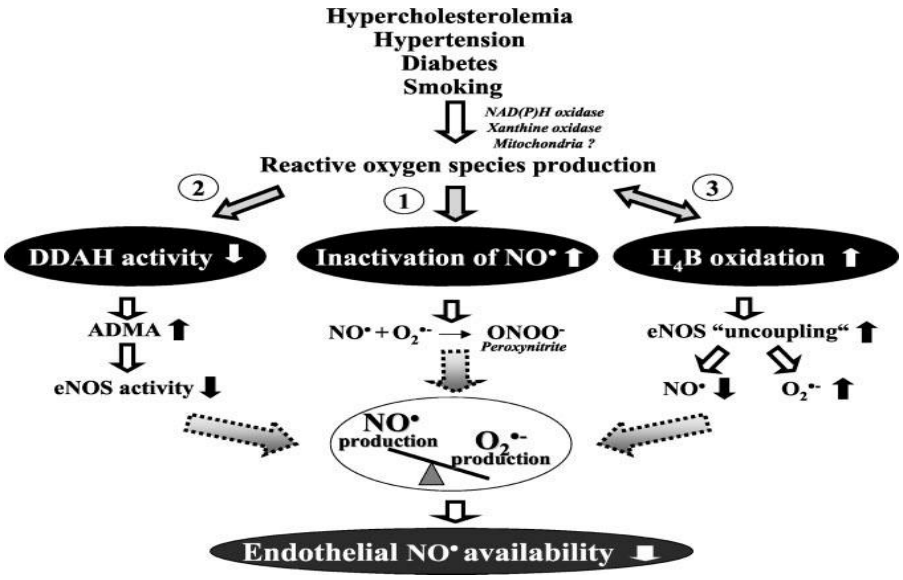


Figure 1.5: The major cardiovascular risk factors increase endothelial dysfunction through decreasing the endothelial NO availability, reproduced from (Landmesser *et al.,* 2004).

When there is deficiency in the enzyme cofactors or L-arginine, eNOS can raise the superoxide anion (O_2^-) and oxygen radical production. The O_2^- in the absence of antioxidant defence mechanisms will react with NO to form peroxynitrite ($ONOO^-$) or hydrogen peroxide (H_2O_2) decreasing NO bioavailability (Vanhoutte, 2003, Spieker *et al.,* 2006).

Dysfunction of endothelial cells is an early contributor to atherosclerosis (Bonetti *et al.,* 2003). It can therefore be an indicator for probable vascular complications (Anderson, 2004, Esper *et al.,* 2006,). Endothelial cell dysfunction is considered as an independent vascular risk factor that can be treated by medication, abstinent smoking, and increased physical activity. In addition to improving food habits and the healthy dietary food consumption rich in flavonoid compounds tends to reverse endothelial cell dysfunction, and thus reduce the CVD risk.

1.5.2 Atherosclerosis

Atherosclerosis is a potentially serious condition whereby arteries become clogged up by fatty substances known as plaques or atheroma (Martini *et al.,* 2012). Atherosclerosis is a chronic vascular disease which entails thickening and toughening of arterial walls and narrowing of arterial lumen. As a result, blood flow and thus oxygen and nutrient supply to the body's tissues is diminished. Atherosclerosis is mainly observed in large and medium-sized arteries. Atherosclerosis pathogenesis requires several factors, such as vascular inflammation, endothelial dysfunction, and vascular cell and immune system interaction (Napoli *et al.,* 2006).

Atherosclerosis pathogenesis (theroma formation) comprises many steps as shown in figure 1.6. Firstly, low-density lipoprotein (LDL) accumulates within the intima layer where it is oxidized and modified. This gives rise to the release of leukocyte adhesion molecules by endothelial cells, which results in macrophages in the intima engulfing the modified LDL (De Winther and Hofker, 2000) eventually forming lipid-containing macrophages foam cells. It is the accumulation of foam cells which leads to fatty streaks. The fatty streak is covered by a cap of both smooth muscle cells and collagen matrix which forms the plaque. As the plaque grows, the blood vessel lumen narrows restricting the flow. The plaque might also rupture

which is of particular importance since it plays a crucial role in causing of ischaemic heart disease.

1.6 Dietary flavonoids and cardiovascular disease

1.6.1 Dietary flavonoids and CVD

As a diet high in fruit and vegetables has been linked to reducing the risk of chronic related diseases this has encouraged many researchers to investigate the possible protective effects of flavonoids. The association between flavonoid intake and health benefit can be measured through different approaches such as epidemiological studies, animal studies, cell culture models, and randomized cross-over intervention studies. Dietary intake of flavonoid-rich foods, or foods rich in specific flavonoid sub-classes such as anthocyanins and flavanones, were correlated with lower risk of death from CVD in large prospective cohort studies (e.g. Mink et al., 2007, McCullough *et al.,* 2012). Several studies have also found that even a small quantity of foods that are rich in flavonoids may reduce certain CVD risk factors, shown in a meta-analysis of studies (Hooper *et al.,* 2008). The role of flavonoid-rich foods on CVD has been reviewed by many (e.g. Manach *et al.,* 2005; Erdman *et al.,* 2007).

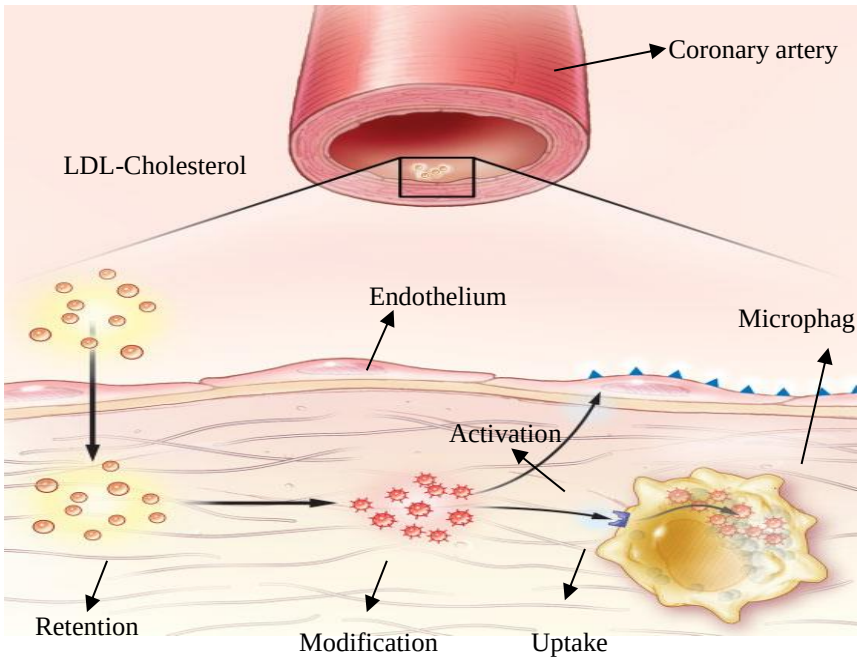


Figure 1.6: Foam cell formation in atherosclerosis. In hypercholesterolemia patients, excess LDL infiltrates the artery and is retained in the intima, particularly at sites of hemodynamic strain. Oxidative and enzymatic modifications lead to the release of in the modified LDL particles are taken up by scavenger receptors of macrophages, which evolve into foam cells, reproduced from (Hansson, 2005).

Flavonoids are found to possess both antioxidant and pro-oxidant effects. This gives them the ability to enhance the normal cell survival and suppress the risk of tumour cell proliferation (Jan *et al.*, 2004). A prior study reported that the level of antioxidant capacity in plasma increased after the consumption of various polyphenol-rich foods (Manach *et al.*, 2004). The study also showed that some of the polyphenol metabolites retain antioxidant activity. Thus it is considered that excessive consumption of flavonoids from food supplements should not be recommended (Halliwell, 2007). Polyphenols may affect the bioavailability of carcinogenic and toxic compounds by inhibiting/enhancing the enzymes implicated in their metabolic pathways, such as sulfotransferase, UDP- glucuronosyltransferase and cytochrome P450 isoforms (Manach *et al.*, 2005).

A randomized, placebo controlled double-blind crossover study to examine the effect of chocolate on lipid parameters, weight and glycaemic control was conducted among subjects with T2DM. The subjects (42-71 years) consumed 45 g chocolate with or without high flavonoid content for eight weeks crossover after four

weeks washout time. After the ingestion of chocolate-rich in flavonoids, a significant increase in HDL cholesterol and a reduction in total cholesterol to HDL-C level were reported (Mellor *et al.*, 2010).

1.6.2 Citrus flavanones and CVD

Some studies showed an inverse relationship between citrus consumption and the risk of CVD (Knekt *et al.* 2002, Chanet *et al.* 2012b). Although citrus fruits are the major dietary sources of flavanones, few epidemiological studies have studied the direct correlation between flavanone intake and CVD prevention. Flavanone intake, but not the total flavonoid intake, has been reported to be inversely related to the risk of stroke (Cassidy *et al.*, 2012). The main effects of flavanones/orange juice, and some other flavonoids, on CVD risk have been reported by Coelho *et al.* (2013), these effects include: decreasing blood pressure, improving lipid profiles, antioxidant and anti-inflammatory effects, and improving the function of endothelial cells (Egert *et al.*, 2009).

Interestingly, orange juice consumption is correlated with better diet quality and nutrient adequacy. The results of recent study have shown that people who consumed 210 ml/day had lower total cholesterol level, LDL cholesterol and body mass index (BMI) compared with <50 ml/day. Moreover, the risk of being obese and having metabolic syndrome amongst those people decreased by 21% and 36%, respectively (O'Neil *et al.*, 2012).

Hypercholesterolemia, which can lead to atherosclerosis, is one of the risk factors for CVD developing. A number of other potential impacts of citrus flavanone have been demonstrated in the case of lipid metabolism. It has been reported that flavanones can regulate apolipoprotein B secretion by HepG2 cells—potentially via cholesterol ester synthesis inhibition (Borradaile *et al.*, 1999, Kurowska *et al.*, 2004). One study showed that grapefruit intake decreased plasma TG levels in patients with CVD (Gorinstein *et al.*, 2006). Another study showed that the ingestion of 750 mL for 4 weeks, but not of 250 or 500 mL, increased HDL-cholesterol, and decreased the LDL:HDL cholesterol ratio (Kurowska *et al.*, 2000).

Other studies demonstrated that flavanones can lower blood cholesterol concentration (Jung *et al.,* 2003). Naringin supplements lowered the plasma total cholesterol (TC) and LDL-cholesterol, but not plasma triglyceride (TG) and high density lipoprotein (HDL)-cholesterol in hypercholesterolemic subjects (Jung *et al.,* 2003). However, when healthy overweight men aged between 50-65 years, consumed 500 ml orange juice containing hesperidin (292 mg) for one month, there was not any significant alteration in the level of TC, LDL-C, HDL-C and TG (Morand *et al.,* 2011). Moreover, pure flavanone compounds ingested did not show any lowering effect on plasma TC and LDL-C levels (Demonty *et al.,* 2010).

Worldwide, hypertension is categorised as one of the major public health problems (Medina- Remón *et al.,* 2011), with high BP shown to be one of the major risk factors of CVD. Differences in BP measurements by 20/10 mmHg above 115/75 mmHg were correlated with raised CVD risk by two-fold, for people aged between 40 and 70 years (Liu *et al.,* 2014b). Evidence from nutritional studies suggested that consumption of fruit and vegetables is negatively correlated with high BP (Alonso et al., 2004, Mancia *et al.,* 2013). Furthermore, intake of healthier foods such as the Mediterranean food pattern decreases the risk and the prevalence of high BP (Kokkinos *et al.,* 2005).

Fruit consumption may lower the risk of CVD through a BP reducing effect. Cross sectional data was used to quantify association of raw fruit and fruit juice intake with BP in different countries. BP was measured 8 times in 4 visits, four 24-h dietary recalls and two-24 hours urine samples were collected. The results concluded that diastolic BP was positively associated with consumption of fruit and fruit juice; moreover, there was positive association between citrus fruit consumption and diastolic BP in western regions (Savica *et al.,* 2010; Griep *et al.,* 2013). A meta-analysis on the effect of fruit juice on serum cholesterol and BP assessed a total of 618 participants from 19 studies. The study showed that fruit juice had a significant effect on decreasing DBP, but had no effect on blood lipid parameters such as, TC, HDL-C, LDL-C or SBP (Liu *et al.,* 2013a).

The effect of regular consumption of pasteurized orange juice on the nutritional status, biochemical profile, and arterial BP in healthy men and women was studied. Twenty one healthy women aged 20-35 years and 20 healthy men aged

21-44 years, were recruited to consume pasteurized orange juice (500 mL/d and 750 mL/d) for 8 weeks. Total cholesterol and LDL-C significantly decreased after the consumption of orange juice in both men and women, and a rise in HDL-C level was observed exclusively in women. FBS, DBP, and TG concentration were reduced in men after the consumption of orange juice. The study reported that regular consumption of pasteurized orange juice may decrease the risk of developing atherosclerosis, and raise the nutritional quality of their diets (Basile *et al.,* 2010).

To evaluate the antihypertensive effect of juice among patients with stage I hypertension, a double-blind, cross-over study was conducted in 12 patients and sweetie fruit juice, which is a hybrid between grapefruit and pummelo and contains a high level of flavonoids (mainly naringin and narirutin) (Reshef *et al.,* 2005). For a 5-week period, each participant consumed high-flavonoid sweetie juice and low-flavonoid sweetie juice. A tendency toward reduced DBP and SBP was seen with both low and high sweetie juice. However, a significant reduce in DBP was reported only in the group consuming high flavonoid sweetie juice (-3.7 mm Hg; p=0.04).

Consistent with this study, a randomized, controlled, crossover study investigated the effects of orange juice and hesperidin on microvascular reactivity, BP, and CVD risk biomarkers through both postprandial and chronic intervention in 24 healthy, overweight men (age 50–65 y) (Morand *et al.,* 2011). Participants consumed 500 mL orange juice, 500 mL control drink plus hesperidin (CDH), or 500 mL control drinks plus placebo (CDP) daily in three 4-wk periods. The study concluded that DBP was significantly decreased after four weeks consumption of orange juice or CDH compared to CDP. In addition, both orange juice and CDH consumption significantly enhanced postprandial microvascular endothelial reactivity compared with CDP when measured at the peak of plasma hesperetin concentration (Morand *et al.,* 2011).

Dysfunction of endothelial cells is a predictor for CVD events. Twenty two healthy participants were recruited in a single blind randomized crossover controlled study. The two groups consumed either commercial orange juice or fresh orange juice for the first four weeks and then switched to the second phase. The impacts of commercial and fresh orange juice on endothelial cell function and physiological characteristics in healthy humans was evaluated. Functionality of endothelial cells by

measuring flow-mediated dilation was evaluated alongside with serum lipids and inflammatory markers, such as adhesion molecule 1 (VCAM-1), E-selectin, high-sensitivity C-reactive protein, and interleukin-6. The results did not reveal any alteration in FMD (Asgary *et al.,* 2014).

Some studies have examined the effect of consumption of hesperidin on vascular function and BP among spontaneously hypertensive rats. Following a single oral dose of 50 mg/kg hesperidin, SBP was reduced in SHR; data have suggested that the hypertensive effect of hesperetin is associated with NO mediated vasodilatation, which may explain the mechanism of lowering BP (Yamamoto *et al.,* 2008b). Moreover hesperetin induced an increase in endothelium-dependent vasorelaxation of aortic ring (Yamamoto *et al.,* 2008a).

The estrogenic activities of naringenin and hesperetin were evaluated to verify whether they affect endothelial NO production. These were investigated via estrogen receptor activation. The results indicate that naringenin activated both estrogen receptor-α and estrogen receptor-β, whereas hesperetin activated estrogen receptor-α only (Liu *et al.,* 2008). Hesperetin has been shown to increase NO release from endothelial cells in a dose-dependent manner. The results showed that hesperetin exerts an anti-atherogenic activity via affecting estrogen receptor-mediated expression of eNOS and NO generation (Liu *et al.,* 2008).

Naringenin was reported to have a vasorelaxant effect via activation of Ca^{2+} channels in monocytes (Saponara *et al.,* 2006). A cell culture-based study was conducted to investigate the cellular and molecular mechanisms implicated in the role of flavanone in the decrease of CVD risk. The study reported that an inhibition effect of hesperetin and naringenin metabolites (hesperetin-3′-*O*-glucuronide, hesperetin-3′-*O*-sulphate and naringenin-4′-*O*-glucuronide) on the monocyte adhesion to tumour necrosis factor-α (TNFα) activated human endothelial cells by a gene expression effect (Chanet *et al.*, 2012a). Figure 1.7 shows the possible effects of citrus flavonoids on CVD risk factors.

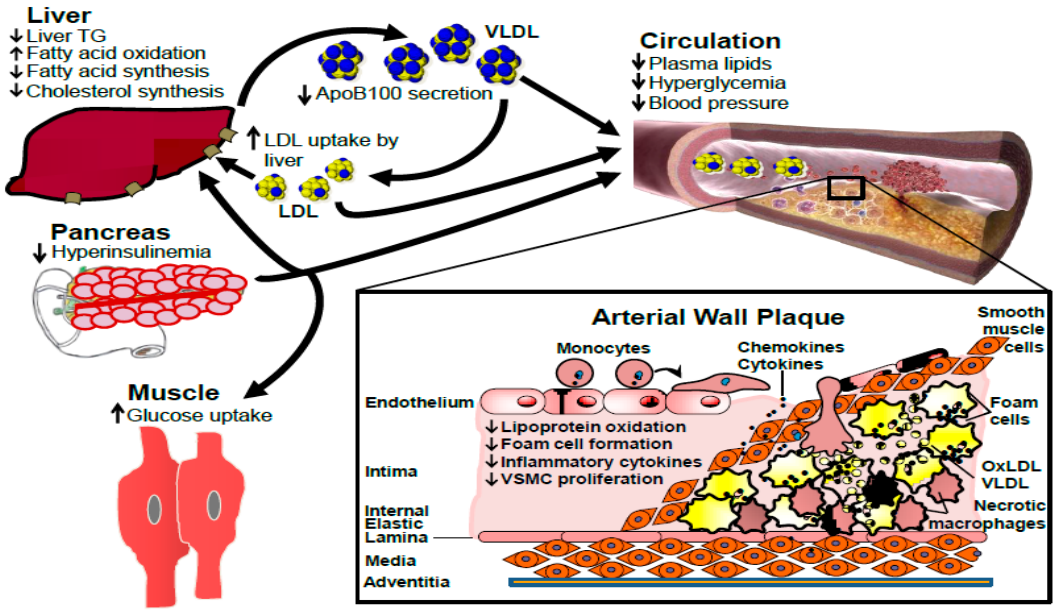


Figure 1.7: Possible effects of citrus flavonoids on CVD risk factors. Direction of arrows indicates effect. Reproduced from (Mulvihill *et al.*, 2012).

1.6.3 Dietary flavonoids and obesity

Worldwide, obesity is becoming an epidemic problem in both developed and developing states (Prentice, 2006; Velasquez and Bhathena, 2007). About one billion adults are considered overweight (Murthy *et al.*, 2009) and the rate at which it progressing is increasing rapidly across all aged groups (Velasquez and Bhathena, 2007). It has been reported that 24.5% of adults and 13.9% of children in the UK are obese (BMI $\geq$ 30 kg/m^2), and in the event that no action is taken, it is assessed that 47% of males and 36% of females will be categorised as obese by 2025 (Shepherd, 2010). Obesity is defined as the fat tissue aggregation to excess and to a degree that damages both physical and psycho-social wellbeing and health (James, 2004). Obesity has been shown to be associated with a number of diseases such as dyslipidemia, hypertension, stroke, metabolic syndrome and type 2 diabetes mellitus (T2DM), (Prentice, 2006; Velasquez and Bhathena, 2007; Meydani and Hasan, 2010; Ärnlöv *et al.*, 2010; Huang *et al.*, 1998; Nguyen *et al.*, 2008; Ding *et al.*, 2012).

Many studies have shown that whole plant foods, dairy products, dietary fibre and functional foods such as probiotics and prebiotics, and certain phytochemicals,

can play a protective role against obesity and their related diseases (Tuohy *et al.*, 2009). The regular consumption of fruit and vegetable was found to be correlated with body weight reduction (Vioque *et al.*, 2008). Furthermore, fruit and vegetables with high amounts of phytochemical and phenolic compounds have been shown to have anti-obesity and lipid-lowering effects and may help achieve body weight (Pal *et al.*, 2004, Nagasako-Akazome *et al.*, 2007, Vioque *et al.*, 2008, Hsu and Yen, 2008). Animal studies have also reported that consumption of foods/supplements rich in flavonoids can change lipid and energy metabolism and may help weight loss and prevent weight gain. The potential effect of these flavonoids may be explained through several mechanisms: inhibition of fat absorption from the gut, increased glucose absorption by skeletal muscles, inhibition of anabolic pathways, stimulation of catabolic pathways in adipose tissues, liver and other tissues, angiogenesis in adipose tissues, inhibition of differentiation of pre-adipocytes to adipocytes, stimulation of apoptosis of mature adipocytes, and reducing of chronic inflammation correlated with adiposity (Meydani and Hasan, 2010).

1.6.4 Dietary flavonoids and metabolic syndrome

Metabolic syndrome is characterized by a number of metabolic abnormalities including hyperglycaemia (impaired fasting glucose), abdominal obesity, hypertension and dyslipidaemia (Alberti *et al.*, 2009). Metabolic syndrome is correlated with developing T2DM, CVD, and all-cause mortality and is considered a highly prevalent health problem across the world (Yang *et al.*, 2012). The prevalence was estimated in the general population from 1.2% to 22.6% with rates of up to 60% observed in the overweight and obese (Tailor *et al.*, 2010). The unified diagnostic criterion for metabolic syndrome is having at least three of the following five abnormalities: serum TG level of 1.69 mmol/L or greater; HDL- cholesterol level lower than 1.03 mmol/L in men and lower than 1.29 mmol/L in women, waist circumference of 102 cm (40 inches) or greater in men and 88 cm (35 inches) or greater in women; BP of 135/85 mmHg or greater; or fasting blood glucose of 5.5 mmol/L or greater (Alberti *et al.*, 2009, Mitchell *et al.*, 2013).

Recently, there has been an increased interest in the various ways dietary polyphenols may influence carbohydrate metabolism. The beneficial effect of polyphenols consumption on glucose metabolism has been observed by number of human studies and the possible mechanism of action includes activation of insulin receptors and glucose uptake in the insulin-sensitive tissue, inhibition of carbohydrate digestion and absorption of glucose in the intestine, increased insulin secretion from the pancreatic β-cells, modulation of glucose release from the liver and intracellular signalling pathways and gene expression (Hanhineva *et al.,* 2010; Williamson, 2013).

The correlation to total fruits and vegetables intake and decreasing risk of developing DM remains unclear. Some epidemiological studies have supported the positive effect of flavonoid-rich foods against the development of T2DM (Williamson, 2013, Liu *et al.,* 2013b, Liu *et al.,* 2014a). A recent study evaluated whether dietary intakes of flavonoids i.e., flavanols, flavones, flavanones, flavan-3-ols and anthocyanins are associated with the risk of T2DM in US adults. A total of 70359 participants who were free of T2DM, CVD, and cancer at baseline were involved in this study. The results concluded that higher ingestion of anthocyanin-rich fruit was associated with a lower rate of T2DM (Wedick *et al.,* 2012). These results are also in agreement with a large prospective case cohort study a cross European countries. The results reported significant inverse correlation between flavan-3-ols, proanthocyanidins and myricetin and risk of T2DM (Zamora-Ros *et al.,* 2013). Another study investigated the association between dietary polyphenol intake including flavonoids and metabolic syndrome. The study was conducted in Iran on 2618 adults aged 19-84 years. Results of the study reported a relationship between high intake of flavonoids and lower incidence of WC, low HDL-cholesterol, hyperglycaemia, hypertriglyceridemia, BP and metabolic syndrome (Sohrab *et al.,* 2013).

Some flavonoid-rich foods have been shown to significantly decrease fasting blood glucose, SBP and DBP among human subjects with metabolic syndrome (Egert *et al.,* 2009, Almoosawi *et al.,* 2010, Khurana *et al.,* 2013) or with hypertension (Edwards *et al.,* 2007). However, fasting glucose, insulinemia, total cholesterol, LDL cholesterol, HDL-cholesterol, and triglycerides were not

significantly different after consumption of 500 ml of orange juice (Morand *et al.*, 2011). Also a meta-analysis study reported that fruit juice may have no overall effect on fasting blood glucose and insulin concentrations (Wang *et al.*, 2014). To confirm the positive implications of flavonoids on reducing the risk of metabolic syndrome and T2DM, more human studies with well-defined controlled diets and design are required.

The overall aims of this thesis were to investigate the bioavailability of citrus flavanones, specifically to consider intra- and inter-individual variation in excretion, and to consider the impact of citrus flavanones on cardiovascular health by assessing cardiovascular disease risk biomarkers after an intervention. The hypothesis is that flavanones improve cardiovascular disease risk biomarkers.

Many previous studies have investigated the absorption and excretion of flavonoids after a flavonoid-free period followed by an acute dose of a single food/beverage containing a specific flavonoid of interest. It is possible that co-ingestion of foods containing different flavonoids may affect the absorption and excretion, but this has not previously been investigated in humans. From cell culture studies there is some indication that absorption and metabolism of one flavonoid may be affected by another flavonoid using similar pathways of absorption. This study will monitor flavonoids excretion in 36 hr urine after single or co- ingestion of soya nuts and orange juice in 10-15 volunteers consuming a habitual diet.

Urine and blood are normally used to monitor the absorption of flavonoids from the diet, but saliva is a useful biological fluid that can represent exposure to some dietary compounds (e.g. caffeine). To the best of our knowledge, secretion of flavonoids into saliva after the ingestion of food has not previously been investigated. Due to improved analytical capability, it should now be possible to monitor salivary secretion to see if there is a significant pathway in humans. Saliva will therefore be collected concurrent with urine samples when orange juice and soya nuts are co-ingested by volunteers any salivary content correlated with the urinary content.

Flavonoids are compounds found in plant-based foods, and are associated with a reduced risk of CVD. From the previous studies, there is strong evidence that

orange juice and citrus foods which have higher amount of citrus flavonoids improved cardiovascular risk factors such as BP and blood vessel health. The absorption of citrus flavonoids occurs in the colon after bacteria breakdown of the precursor forms found in food. After flavonoids are absorbed into the blood they are modified by liver enzymes before they are excreted in the urine. A large range of citrus flavonoid have been found excreted in urine, ranging anywhere from 0-57% of the dose. Variation in the potential health effect may reflect the level of the citrus flavonoid absorbed. However, this is not often considered in human studies.

This study is a 4-week double-blinded, randomized, cross-over intervention trial using a commercially-available citrus supplement and a placebo control. It aims to determine whether citrus supplements reduce blood pressure and improve blood vessel health after 4 weeks. Furthermore, it aims to determine if there is a relationship between the absorption of flavonoids (as measured by urinary excretion) and changes in blood pressure or blood vessel health. For the first time, blood flow in fingertips is monitored before and after the consumption of citrus supplement using Endo-PAT technique. An improvement in blood pressure and/or blood flow should provide evidence that blood vessel health has improved through short-term (4 week) use of a citrus flavonoid supplement. Moreover, to our knowledge, this is the first time the excretion of hesperetin conjugates is measured in urine after the consumption of citrus supplement as tablets.

1.7 Thesis objectives

The overall aims of this thesis were to investigate the bioavailability of citrus flavanones, specifically to consider intra- and inter-individual variation in excretion, and to consider the impact of citrus flavanones on cardiovascular health by assessing cardiovascular disease risk biomarkers after an intervention. The hypothesis is that flavanones improve cardiovascular disease risk biomarkers

The specific objectives for this thesis were:

1- Determine the inter- and intra- variation in concentration of isoflavone and flavanones excreted in urine.

2- Evaluation of excretion of flavanones and isoflavones in saliva to assess whether saliva can be used as a non-invasive biological fluid for monitoring flavonoid intake.

3- Investigate the effect of co-ingestion of isoflavones and flavanones on excretion in urine.

4- Investigate the effect of acute or chronic exposure to flavanones on changes to metabolites excreted in urine.

5- Determine whether citrus bioflavonoid supplements reduce blood pressure after a 4 week placebo controlled cross-over intervention.

6- Investigate whether citrus bioflavonoid supplements affect endothelial function by measuring pulse amplitude tonometry (PAT) index after a 4 week placebo-controlled, cross-over intervention

7- Determine if there is a potential relationship between absorption of hesperidin (as measured by urinary excretion) and changes in blood pressure or blood vessel function (PAT index).

Chapter 2: Materials And Methods

2.1 Introduction

The general methods used throughout the thesis are described in detail in this chapter. More specific information and experimental details are described in the methodology section of the relevant chapter.

2.2 Material

2.2.1 Solvents

Several solvents and acids were used throughout the study and the following were purchased from Fisher Scientific, (Loughborough, UK) and from Sigma Aldrich (Gillingham, Dorset, UK): acetonitrile, methanol, ethanol and ethyl acetate (all HPLC grade); acetone, acetic acid glacial, formic acid, L-ascorbic acid (99%), and dimethyl sulfoxide (DMSO) (all analytical grade). Purified water (≥ 18.2 MΩ cm at 25 ºC) was obtained from a Millipore Direct-Q 5 purified system (Millipore UK Ltd, Hertfordshire, UK) and was used throughout the experiments in this project for both analytical purposes and the HPLC mobile phase. Water and acetonitrile (LC-MS grade) were purchased from VWR International (Leicestershire, UK).

2.2.2 Flavonoid standards

All flavonoid standards used in this study were purchased from Extrasynthase (Genay, France). These include the following list: daidzin, daidzein, genistin, genistein, narirutin, naringenin, hesperidin, hesperetin, apigenin, taxifolin, and rutin.

All standards were of HPLC purity (above 97 %). Hesperetin-3′-*O*-glucuronides and 7-*O*-glucuronides were kindly donated by Dr Denis Barron (Nestlé Research Centre, Switzerland). Hesperetin-3′-*O*-sulfate was kindly donated by Christine Morand (INRA, France).

2.2.3 Enzymes

All enzymes used were purchased from Sigma Aldrich (Dorset, UK) and stored at 4˚C or -20˚C until use. Hesperidinase (EC 3.2.1.40) from *Aspergillus niger*; the enzyme has both α-L-rhamnosidase and β–D–glucosidase activities and was stored at 4˚C; each unit of enzyme is able to liberate 1 µmol of glucose from hesperidin/min at pH 3.8 and 40˚C. Cellulase (EC 3.2.14) from *Aspergillus niger* was stored at 4˚C; one unit of enzyme will liberate 1 µmol glucose from cellulose/ hr at pH 5 and 37˚C. Sulfatase was from *Aerobacter aerogenes* Type II (EC B.1.61) and was stored at -20˚C; one unit will hydrolyse 1 µmole of *p*-nitrophenyl sulphate/min at pH 7.1 and 37˚C. β-Glucuronidase from *Escherichia coli* Type IX-A (EC 3.2.1.31) was stored at -20˚C; one unit will liberate 1 µg of phenolphthalein from phenolphthalein glucuronide/ hr at 37˚C.

2.2.4 Buffers

The following buffer was used throughout the study: sodium phosphate buffer (0.2 M, pH 7; monobasic sodium phosphate (0.2 M) and dibasic sodium phosphate (anhydrous). The buffer salts were purchased from Sigma (Gillingham, Dorset, UK).

2.3 HPLC Analysis

2.3.1 HPLC equipment

All high performance liquid chromatography (HPLC) analysis was performed on an Agilent 1200 SL system (Agilent Technologies, Dorset, UK) which comprised of a binary pump (model G1312B), that was optimised to cope with the repid resolution (up to 600 bars pressure), an in-line vacuum degasser (G1379B), and a photodiode array UV-VIS detector (model G1315C) with dual-lamp (deuterium and tungsten) design for high sensitivity with a wavelength range of 190–950 nm. The column temperature was held constant (35ºC) using model G1316 column heater. A high performance auto sampler SL (100-vial sampler tray) for high speed sample injection with lowest carryover was used. Data acquisition and analysis was done by ChemStation software under windows XP software. The mobile phases were

degassed in glass bottles using an ultrasonic waterbath (15 min; Clifton, Nickle-Electro, Oldmixon Crescent, UK) prior to HPLC analysis. A Zorbax Eclipse plus C18 micro-bore column (internal diameter 1.8 µm, 4.6 x 50 mm; Agilent Technologies, Dorset, UK) was used for all HPLC and LC-MS analysis. Prior to the analysis, any air bubbles from the solvent lines were flushed out by pumping both solvent lines individually at a flow rate of 5 ml/min for at least 5 min each. Solvent was then pumped through the system under the initial method conditions to allow the system and to come to the stable pressure and equilibration based of each method used.

2.3.2 HPLC consumables

The HPLC consumables used throughout this study were purchased from Chromos Express (Macclesfield, UK): 2 ml crimp top glass vials (12x32 mm), 11 mm aluminium crimp caps with butyl/PTFE septa, 17 mm PTFE syringe filter (0.2 µm pore size). Crimp top sample vials, 0.3 ml crimp top fixed insert amber glass vials (Fisher Scientific Ltd, Leicestershire, UK) were used when the sample volume was less than 1 ml to ensure accurate sampling by the auto sampler. Vials were capped by using a hand crimper until fully secure.

2.3.3 Chromatographic conditions

2.3.3.1 Method A for food samples (Glycoside Method)

This method was used to analyse flavanones in orange juice and isoflavones in roasted soy nuts. An Agilent 1200 series rapid resolution HPLC system was used with the diode array detector scanning from 190 to 450 nm. A gradient of water-formic acid (solvent A;100:0.1, v/v) and acetonitrile-formic acid (solvent B; 100:0.1, v/v) was applied using a flow rate of 0.5 ml/min. A 5 µl sample was injected on to the column and the gradient program was as follows: the initial composition consisted of 95% A and 5% B for 5 min, followed by a liner gradient to 10% B for 15 min, 30% B for 5 min, and 90% B for 4 min, 5% B for 1 min and 5% B for 3 min

(Figure 2.1). The absorbance of eluted flavonoids were monitored at 260 nm for isoflavones and at 280 nm for flavanones.

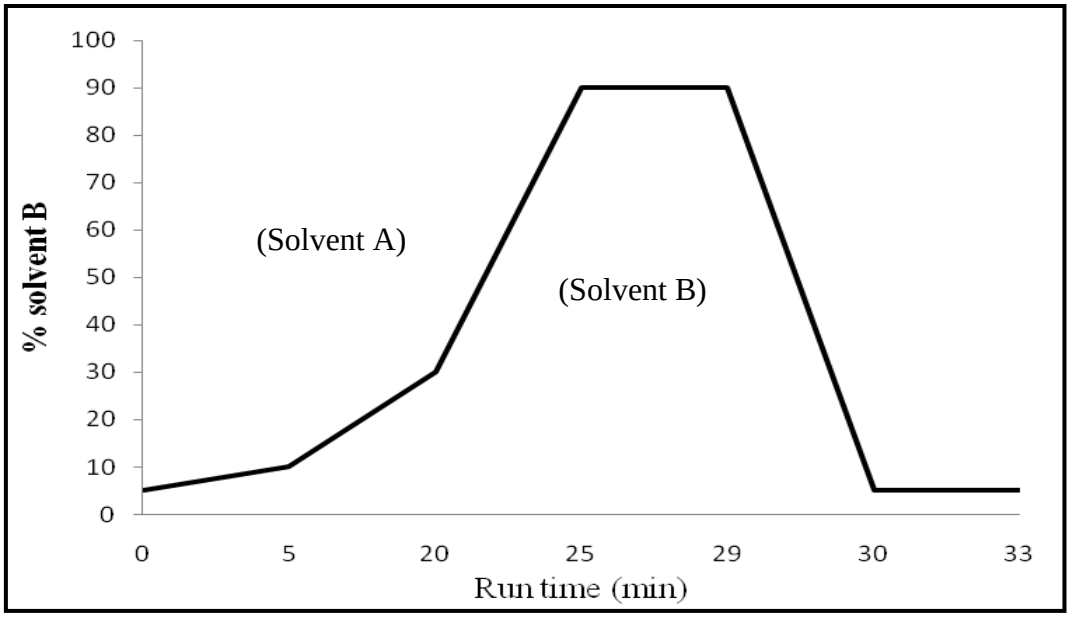


Figure 2.1: Solvent gradient throughout the HPLC run for method A. Solvent A is water + 0.1% formic acid, Solvent B is Acetonitrile + 0.1% formic acid).

2.3.3.2 Method B for biological fluid samples (Aglycone Method)

An Agilent 1200 series rapid resolution HPLC system was used to detect the flavonoids and analyse both saliva and urine samples. A gradient of water-formic acid (solvent A; 100:0.1, v:v) and methanol- formic acid (solvent B; 100:0.1, v:v) was applied using a flow rate and column temperature of 0.75 ml/min and 35ºC, respectively. The solvent gradient is shown in Fig. 2.2 and started at 30% solvent B, increasing to 40% (by 3 min), and to 70% by 15 min, and then to 95% (by 18 min). This was maintained for 5 min and then decreased back to 30% solvent B for 5 min post-run. A sample (10 µl) was injected on to the column and the DAD scanned from 220 to 450 nm. Specific detection was carried out at 220, 260, 280, 310 nm for optimal sensitivity to each compound. The needle was washed with methanol between injections to prevent sample run-over. Blank water samples and two different concentrations of mixture of standard were run at intervals of approximately every 20 unknown samples as a quality control measure.

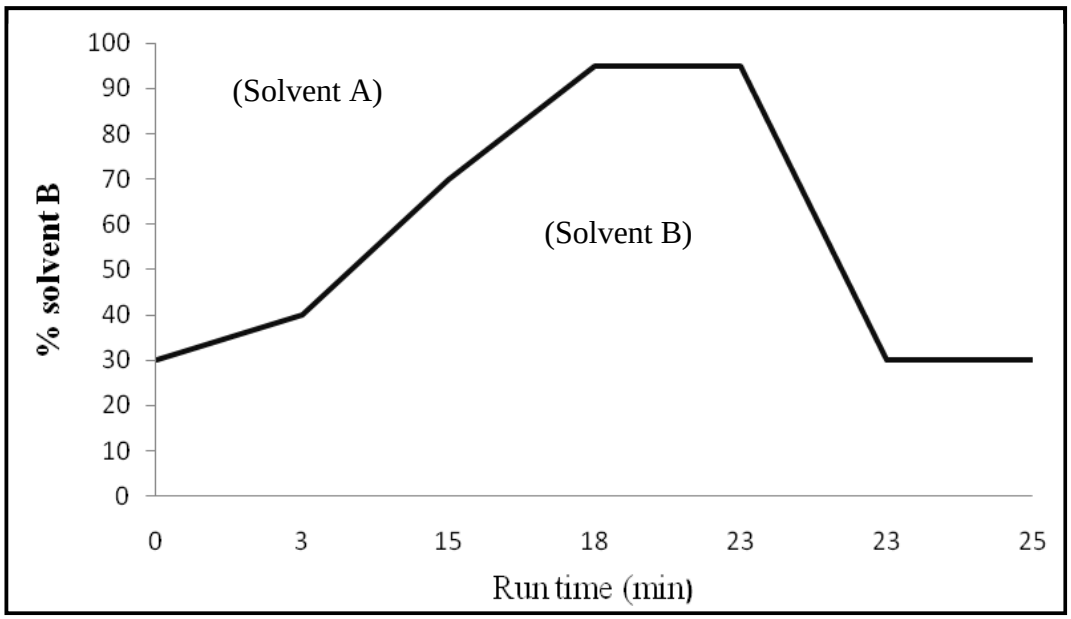


Figure 2.2: Solvent gradient throughout the HPLC run for method B. Solvent is 0.1% formic Acid, and solvent B is methanol containing 0.1% formic Acid.

2.3.4 LC-MS Analysis of conjugated flavanones in urine and saliva samples

The prepared samples were analyzed using LC-MS with the same C18 column used as described above. The analysis was carried out on a LC-MS Shimadzu LC-2010 HT HPLC coupled with a LC-MS-2020 quadrupole mass spectrometer fitted with an electro spray ionisation source used in negative mode (Milton Keys, UK). The column temperature was maintained at 30°C, mass detector was set at -1.80 kV, DL temperature 250°C, and the nebulizing gas flow and drying gas flow were 1.50 and 15 L/min respectively. The mass spectrophotometer was set to assay for ions with 301, 303, 381, 477 and 609 m/z ratios, corresponding to hesperetin, taxifolin, hesperetin sulfate conjugates, hesperetin mono-glucuronide conjugates and rutin respectively. The injection volume was 10 µL and a flow rate of 0.5 mL/min was used. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted in 0.1% formic acid in acetonitrile. Elution started at 5% solvent B and increased to10% after 5 min. From 5 to 20 min the gradient increased up to 40% and after 5 min reached 90%, which was kept for 4 min more. Then, after 1 min the gradient dropped down up to 5% and the run finished at 33 min (Figure

2.3). Pressure limits had a maximum of 370 bar and a minimum of 20 bars. The data files of the LC-MS were analyzed by Shimadzu Lab Solutions software.

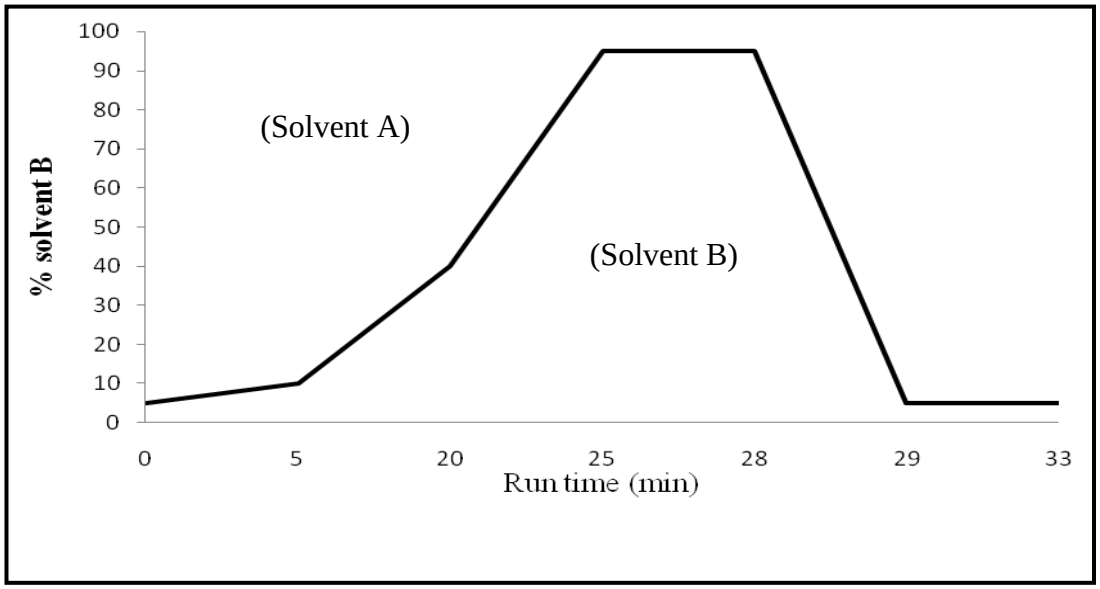


Figure 2.3 Solvent gradients throughout the LC-MS method. Solvent A is water + 0.1% formic acid, Solvent B is Acetonitrile + 0.1% formic acid).

2.4 Methods for extraction of flavonoids from food samples

2.4.1 Extraction of orange juice

A method by Mullen *et a.l,* (2008) was used with some modification. Orange juice (5 ml) in triplicate was mixed with 80% methanol (5 ml), vortexed (2 min) and then centrifuged (5 min at 3000 g, 20°C). The supernatant was removed and an additional 5 ml of 80% methanol was added to the remaining pellet, and the procedure was repeated. The pellet was extracted three times, and supernatants were combined and filtered (0.2 µm PTFE filter) and then analysed by HPLC using method A.

The flavonoids in the sample were also hydrolysed by enzymes. Sodium phosphate buffer (2 ml; pH 3.5) containing hesperidinase enzyme (0.02 U/ml) was added to 2 ml of orange juice. The mixture was incubated for 18 hr in a shaking water bath at 37°C and 100 rpm (Grant OLS 200,Grant Instruments, Ltd, UK). After incubation, methanol (80%, 8 ml) was added and the sample was centrifuged (5 min

at 3000 g, 20°C). The supernatant was taken, filtered through 0.2 µm PTFE filter and analysed by HPLC. Samples were analysed in triplicate with 5 µl injected, using the HPLC system, using method (A) (section 2.3.3.1).

2.4.2 Extraction of soya nuts

A method described by Liggins *et al.,* (1998) was used, with some modifications, to determine the daidzein and genistein in soya nuts. Triplicate samples of freeze dried soya nuts (2.5 g) were mixed with methanol (5 ml, 80%), the mixture was then vortexed for 2 min and left to incubate at 37°C for 1 hr in a waterbath. The mixture was vortexed again for 1 min and centrifuged for 5 min at 3000 g, 20°C. The same procedure was performed with the pellet, which was extracted another two times and the whole supernatant was combined and filtered through 0.2 µm PTFE filter before analysis by HPLC.

For enzyme hydrolysis, 0.5 g freeze dried soya nuts samples (in triplicate) were weighed and 10 ml of freshly boiled water was added and left for 5 min with occasional stirring. A volume of acetate buffer (10 ml; pH 5) containing cellulase (100 U/ml) was added. The mixture was incubated for 18 hr in a shaking waterbath at 37°C. After incubation, 30 ml of 80% methanol was added and centrifuged for 5 min at 3000 g, 20°C. The supernatant then was collected, and filtered through a 0.2 µm PTFE filter prior to HPLC analysis, (section 2.3.3.1).

2.4.3 Extraction of urine samples to assess flavonoid aglycones

Upon arrival in the lab, the volumes of urine sample were measured. Aliquots (2 x 50 ml) were centrifuged at (2000 g, 4°C for 10 min) to remove insoluble materials and cellular debris, and then the supernatant was taken and stored at -20°C until analysed (Appendix . 14).

Urine was defrosted and centrifuged for 5 min at (3000 g at 20 ˚C). A volume of apigenin solution (10 µl of 2 mg/ml) was added as internal standard to 1 ml portions of urine. Sodium phosphate buffer 150 µl (0.2 M at pH 7) enriched with 5

U/ml of β -glucuronidase and 0.03 U/ml of sulfatase was added to the urine sample. These samples were then incubated in a shaking water bath at 37°C and 100 rpm for 2 hr. A portion of 1 ml ethyl acetate was added to each hydrolysed urine samples and vortexed for 2 min. The samples were centrifuged for 5 min (3000 g, 20˚C) and the supernatant removed. The washing procedure was repeated twice more, so that there was 3 ml of ethyl acetate supernatant extract pooled together. The sample was then dried using the centrifugal evaporator (Genevac EZ-2 MK2, Ipswich, UK), using the low boiling point program (40^{o}C for 2 hr).

The evaporated samples then were reconstituted in 1 ml of aqueous methanol solution (50% methanol, 50% 200 μM ascorbic acid solution (v/v)). The samples were then vortexed for 2 min and sonicated for 2 min. until the samples were fully dissolved, and then centrifuged at 3000 g for 10 min. The supernatant was filtered (0.2 μm PTFE filters) prior to HPLC analysis.

2.4.4 Urine and saliva preparation to determine flavanone conjugates

The protocol for urine and saliva preparation was based on the method developed by Sang *et al.,* (2008) with some modification. The urine was defrosted and vortexed for 30 sec before sonication for 5 min. The urine was vortexed again for 30 sec and 200 μl was removed for analysis. This was mixed with 100 μl rutin as internal standard 1 (IS1, 4.5 μg/ml), 100 μl of ethanol and 800 μl of methanol. This solution was then placed in shaking waterbath at 50˚C, at 110 rpm for 10 min to achieve the solubilisation of the compounds. The mixture was then centrifuged for 10 min at 17000 g. The supernatant was removed as the first extract (extract A) and the process was repeated for the pellet (extract B) using water in the place of urine. The primary and secondary extracts were then dried down using centrifugal evaporation (5 hr, 46˚C; HPLC program with the lamp off) to achieve the evaporation of the methanol and ethanol layer. The dried samples were stored at -20˚C until analysis. Methanol (70 μl) was added to extract B. This solution was then vortexed for 30 sec, sonicated and placed in a shaking waterbath for 10 min. A 50 μl sample of extract B was then taken and placed with extract A, and vortexed for 30 sec, sonicated and placed in a shaking waterbath for 10 min.

For analysis, 50 µl of taxifolin as internal standard 2 (IS 2, 4 µg/ml in 0.2% ascorbic acid) was added to the combined solution, vortexed for 30 sec, and sonicated for 5 min. The sample was centrifuged for 10 min at 17,000 g and the supernatant was placed into amber HPLC vials. The vials were well capped and placed in the fridge (5˚C) until the LC-MS run. Triplicates were prepared and analyses using the LC-MS method (section 2.3.4).The same procedure was used to extract saliva samples with some modification in the volume where 100 µl instead of 200 µl, was taken.

2.5 Calibration curves and limit of detection

2.5.1 Methods

Stock solutions (2 mg/ml) of all standards were prepared in DMSO in methanol (0.1% DMSO): the flavanones - hesperidin, narirutin, hesperetin, naringenin and the isoflavones - daidzin, genistin, daidzein, genistein, and apigenin, taxifolin and rutin used as internal standards. Stock solutions were sonicated for 2 min until fully dissolved and stored at –20 °C until analysed. A serial dilution was prepared for each compound from 5 – 100 µg /ml. Samples were filtered (PTFE 0.2 µm pore size) prior to HPLC analysis.

Calibration curves were constructed for each standard at six concentrations in duplicate. Regression analysis was assessed to determine the linearity of calibration curves. The result showed that the peak area was linearly correlated with on-column amounts over the range of (20 – 393 µM), (18.5 – 370 µM), (16.5 – 331 µM), (18.3 – 367 µM) and (18.5 -370 µM) for daidzein, genistein, hesperetin, naringenin and apigenin, respectively.

There are several definitions which are used to define limit of detection (LOD) and limit of quantification (LOQ). In general, LOD is taken as the lowest concentration of an analyte in sample that can be detected, but not quantified. The LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated condition of the test (Shrivastava and Gupta, 2011).

The method used to estimate the detection and quantification limit is based on calculation from the calibration line at low concentration using the equation below:

LOD = F*SD/b

LOQ = F*SD/b

Where

F: factor of 3.3 and 10 for LOD and LOQ, respectively

SD: Standard deviation of intercept,

b: Slope of the regression line

The LOD and LOQ for each standard were established at their particular maximum UV wavelength when the signal to noise ratio of peak height was at three and ten respectively. Recovery, accuracy and selectivity of the standards were evaluated intra-day by performing triplicate injections for each concentration. The inter-day reproducibility test was carried out by analysing two injections on two different days. Each aliquot of these solutions were injected on to the HPLC system and calibration curves were done by plotting concentration against peak area of each compounds and then regression equation was calculated for each curve for each flavanone and isoflavone. Accuracy of HPLC methods was evaluated by analysing blank urine samples spiked and non-spiked by appropriate concentration of the target compounds. The recovery was calculated by comparing the detemined amounts of extracted urine with the known amounts added. Moreover, the areas of peaks and retention times were identified in human urine with and without enzymatic analysis to identify evidence of inference from the matrix or other analytes. Control samples were prepared for each urine sample in duplicate, replacing the enzyme-enriched buffer with non-enriched buffer.

2.5.2 Results

Calibration curves were performed for each standard at 6 concentrations in duplicate. The R^2 was greater than 0.99 for each standard using the chromatographic conditions mentioned in the methods previously (section

2.3.3.1) and (section 2.3.3.2) for glucoside and aglycone form, respectively. The slopes of peak area aginst the concentration of the standard (µg/ml) were as follows: daidzein 46.2, genistein 43.1, hesperetin 25.2, naringenin 26.1, daidzin 10.2, genistin 6.2, narirutin 13.3, hesperdin 13.3, apigenin 16.7, taxifolin 8.46 µg/ml. The limits of detection and quantification for these methods for isoflavones and flavanones standards are presented in Table 2.1. The precision of the methods were tested by both intra-day and inter-day and coefficient of variation was below 3% and 4.5%, respectively. The accuarcy of intra-day and inter-day accuracy were demonstrated across the concentration range with relative error of less than 6%. Standard curve of flavanones and isoflavones are shown in figure 2.4 and 2.5.

Table 2.1: Limit of detection and quantification of isoflavones and flavanones compounds.

	Limit of detection (LOD) *(µg/ml)*	*Limit of quantification (LOQ)* *(µg/ml)*
daidzein	0.19	0.66
genistein	0.34	1.12
naringenin	0.35	0.74
hesperetin	0.22	0.74
Daidzin	0.91	3.01
Genistin	1.04	3.4
narirutin	0.68	2.27
hesperidin	2.5	8.4

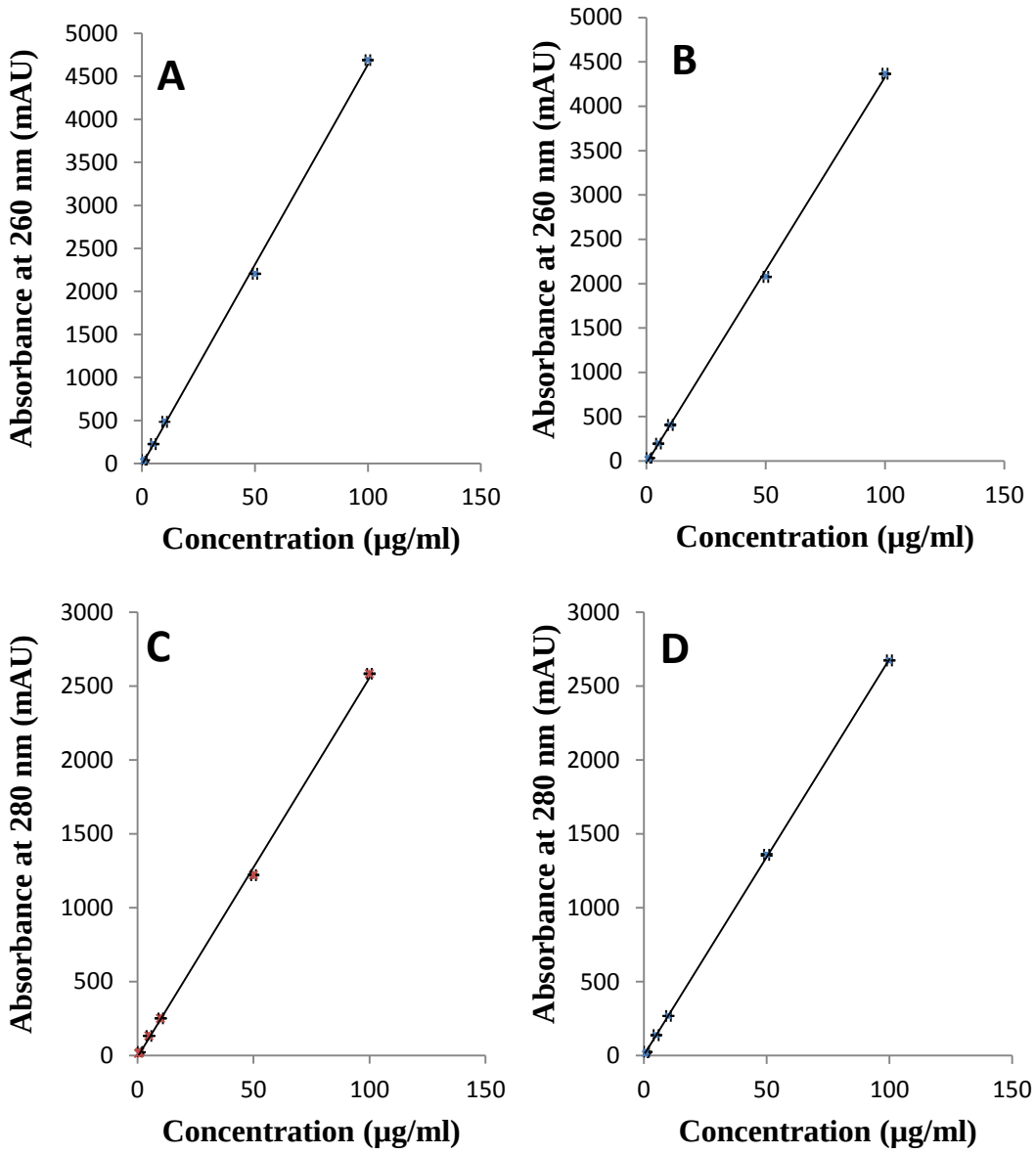


Figure 2.4 Standard curves: (A) daidzein, (B) genistein at 260 nm (C) hesperetin and (D) naringenin, 10 µl injected in the column at 280 nm, by using HPLC-DAD.

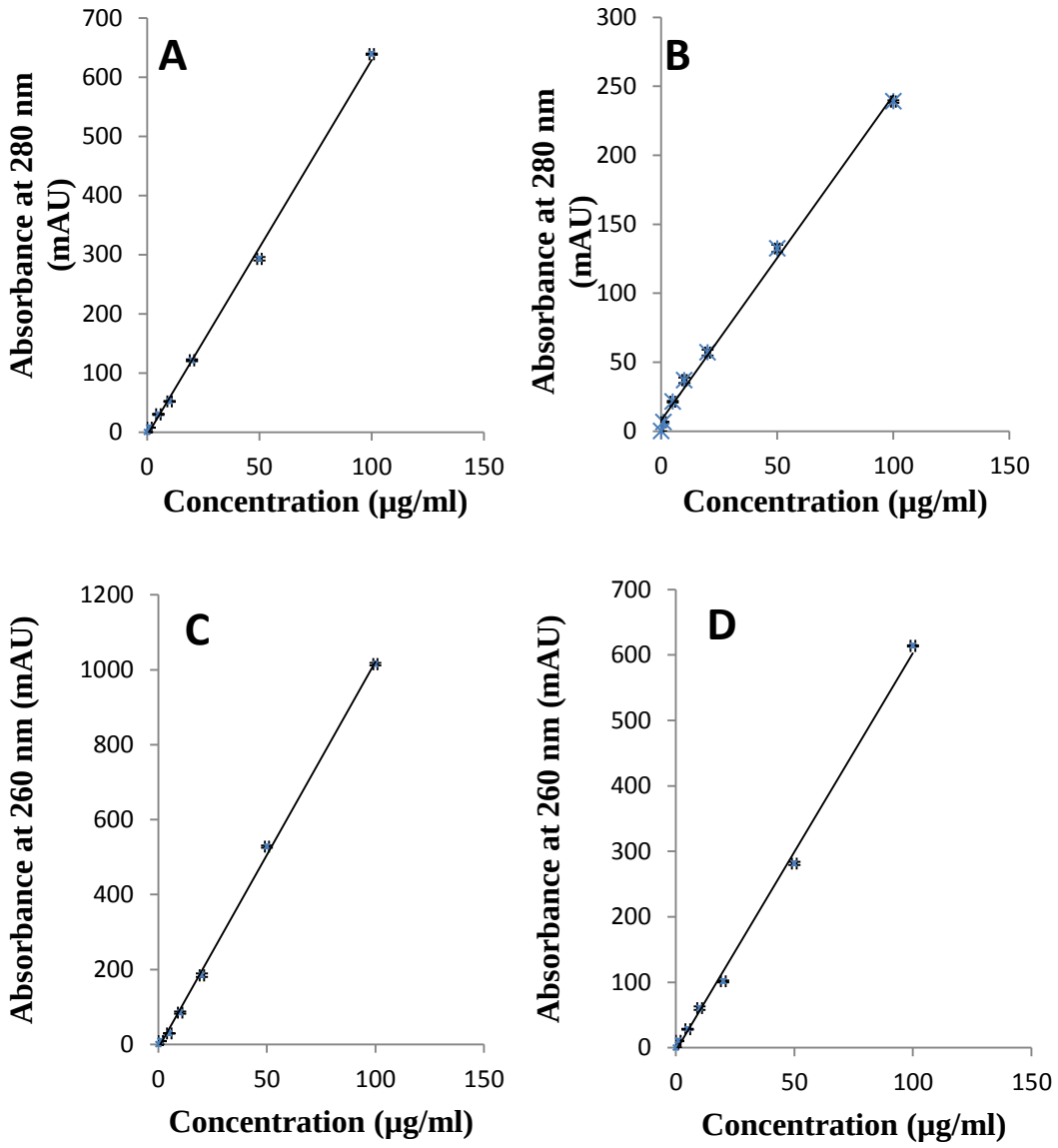


Figure 2.5 Standard curves of (A) Narirutin (B) Hesperidin at 280 nm and (C) daidzin (D) genistin, 5 µl injected in the column at 260 nm, by using HPLC-DAD.

The identification of flavanoids in the food and biological samples were based on comparison of retention times to authentic standards, and the UV absorption spectral characteristics, as shown in figure 2.6.

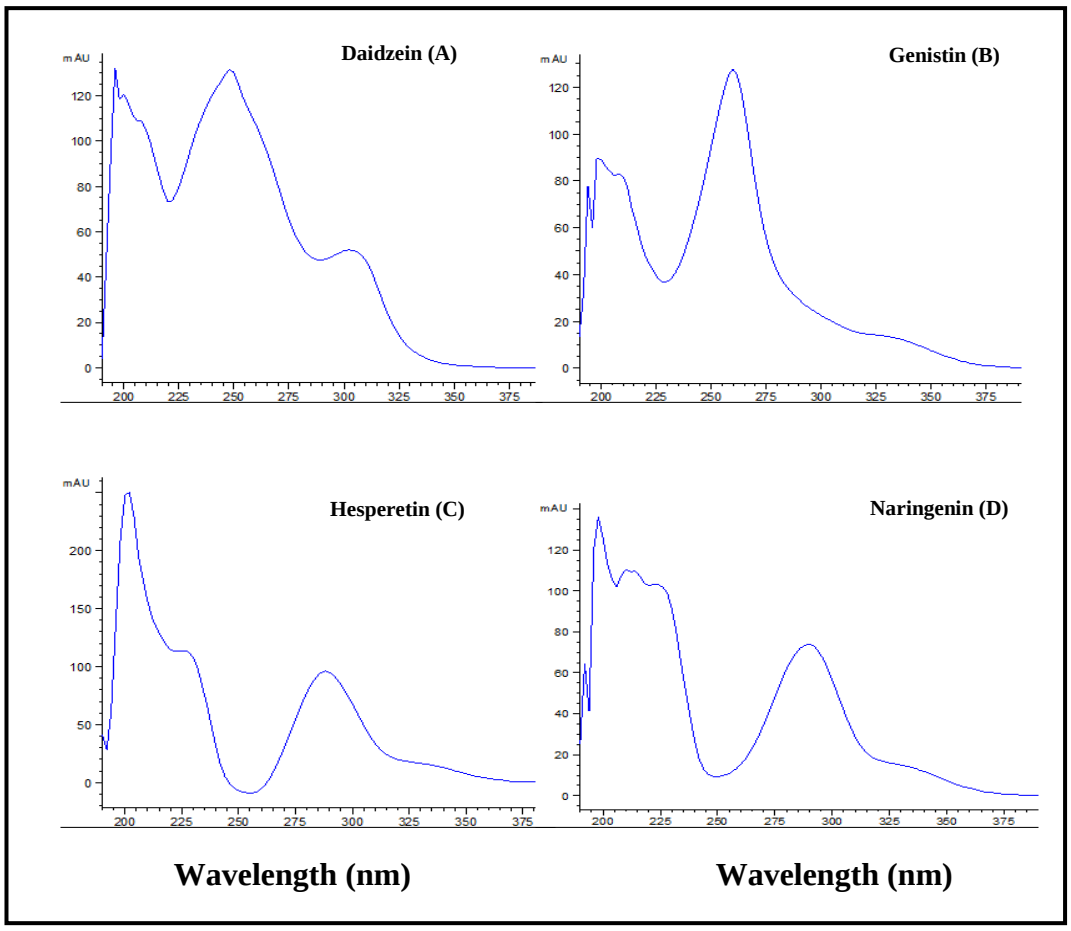


Figure 2.6: UV absorption spectra of flavanone and isoflavone standards daidzein (A), genistein (B) at 270 nm, hesperetin (C), naringenin (D) at 280 nm.

An HPLC chromatogram showing a mixture of flavanone and isoflavone aglycone standards is shown in Figure 2.7. The peaks of the flavanones and isoflavones were well separated at 6.8, 7.8, 8.4, 8.7 and 10 min for daidzein, naringenin, genistein, hesperetin and apigenin, respectively. An HPLC chromatogram of a mixture of flavanone and isoflavone glycosides standards are shown in Figure 2.8 The peaks are well separated at 11.1, 14.0, 15.4, 17.4 and 23.1 min for daidzin, genistin, narirutin, hesperidin and apigenin, respectively.

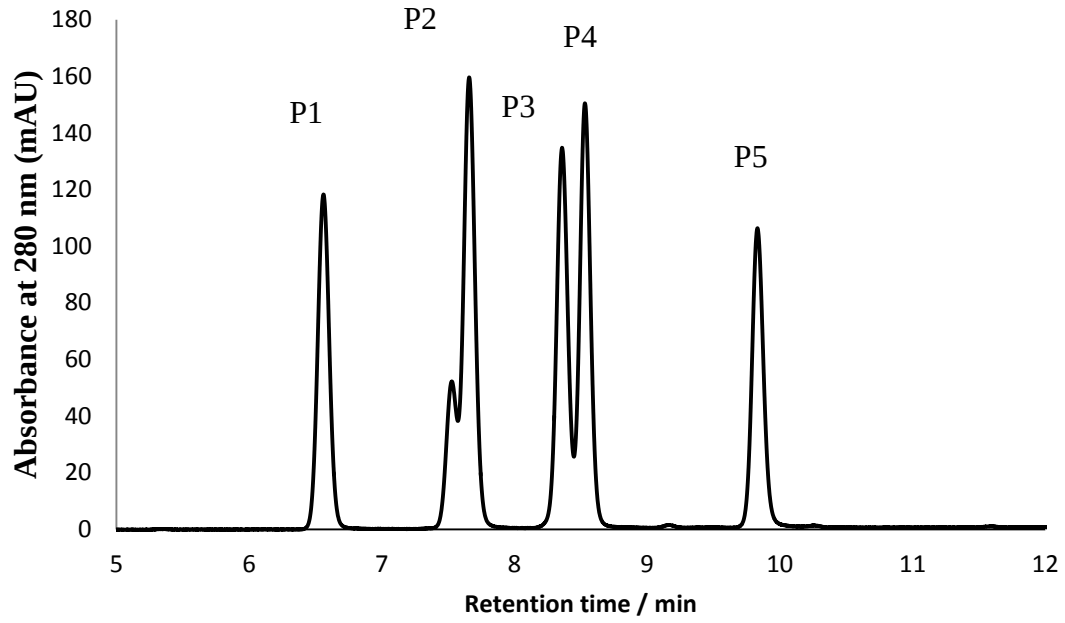


Figure 2.7: Reversed –phase HPLC chromatogram of a mixture of aglycone standards. Peak identity: P1- daidzein, P2- naringenin, P3- genistein, P4- hesperetin, P5- apigenin.

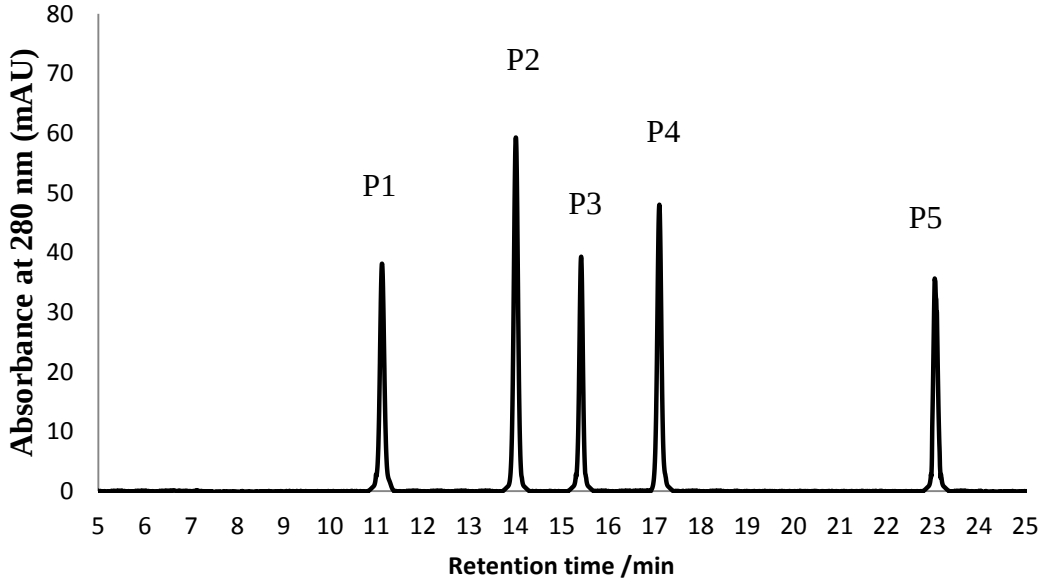


Figure 2.8: Reversed –phase HPLC chromatogram of a mixture of glycoside standards. Peak identity: P1- daidzin, P2- genistin, P3- narirutin, P4- hesperidin, P5- apigenin.

2.5.3 Verification and identification of flavanone and isoflavone peaks

The identification of eluted flavonoids was determined based on the following methods:

a) UV Absorption spectra. Most flavonoids have a distinct UV spectrum, which is very useful for determining compounds expected in samples. However it is difficult to identify the aglycone or conjugate, or very similar flavonoid subclasses using spectral characteristics.
b) Retention times. Hydrophobic aglycone flavonoids will elute later in reserved-phased HPLC, and hydrophilic glycosides or conjugate metabolites will elute earlier. Therefore comparing the retention time of the reference standard compound with the eluted unknown, especially in combination with the UV spectrum can help identify the flavonoid.
c) Spiking. The sample was spiked with a known amount of flavonoid standard to verify the change in peak area of the target flavonoids; the peak area was compared before and after spike.
d) Mass. For some samples (hesperetin conjugate metabolites), LC-MS was used to identify the mass of the parent compound. Along with the retention time and the UV spectrum this will add to the evidence to identify the right flavonoid. see chapter 7for more details

2.6 Data Analysis

Chemstation and Post-Run (LC-MS run) analysis took place using Chemstation and Lab-Solutions software. These data (peak areas and chromatograms) were further analyzed after combined with already known data (amount of juice ingested by each subject, hesperidin concentration in orange juice, urine volumes before and after each treatment). The calculations and the statistical analysis were performed using IBM SPSS Statistics 22. The comparison between and among the subjects or the treatments, was achieved using the paired t-test and ANOVA test, whereas the means and standard deviations were calculated using descriptive statistics.

Chapter 3: The Effect Of Flavanone And Isoflavone Co-Consumption On Salivary And Urinary Excretion In Humans

3.1 Abstract

Chapter 3 investigates the bioavailability of flavanones and isoflavones by measuring the urinary excretion after orange juice and soya were consumed separately or co-ingested. The hypothesis was that the intra-individual variation in excretion in these compounds would be similar despite the large inter-individual variation – i.e. a high isoflavone excretor will also be a high flavanone excretor. Furthermore, the excretion of isoflavones and flavanones in saliva was evaluated.

Fifteen subjects (eight women and seven men) with a mean age of 31±6.1 y (range 22 – 44 y) were recruited, with a mean BMI of 20±3.6 kg/m^2 (range 15 – 27 kg/m^2). After two days on a low flavonoid diet they consumed 25 g roasted soya nuts and/or 500 ml orange juice, on each of three separate days. Urine was collected in different fractions (0, 0-4, 4-8, 8-12, 12-24 and 24-36 hr) in each phase, and saliva was collected each 2 hr from 0-12 hr, then at 24 hr and 36 hr, but only in the co-ingestion phase. Urine and saliva samples were analysed by HPLC-DAD for flavanones and isoflavones after enzyme hydrolysis.

There was considerable inter-individual difference in urinary excretion of flavanones and isoflavones after single and co-ingestion of orange juice and soya nuts. Total urinary excretion (% dose ingested) of hesperetin, naringenin, daidzein and genistein was 2.5, 9.2, 27.1 and 19.2%, respectively, when the soya nuts and orange juice were ingested separately. When the compounds were co-ingested excretion (% dose ingested) was 3.0, 8.6, 28.1 and 16.8%. There was a strong positive correlation between urinary excretion of daidzein and genistein for both single and co-ingestion. Similar results were found with hesperetin and naringenin. Daidzein was excreted to a greater extent than genistein; naringenin was also excreted to a greater extent than hesperetin. The cumulative amount of naringenin, daidzein and genistein excreted in urine after a single or co-ingested dose was similar

for all compounds, but this was not the case for hesperetin which had a significantly higher average excretion between 8-12 hr. Flavanones and isoflavones were not detected in the saliva at any time point under the conditions used.

In conclusion a large inter-individual variation in urinary excretion of flavanones and isoflavones was observed between subjects. The largest variability based on the recovery in urine after 36 hr was observed for daidzein which ranged between 7% - 65% of dose ingested. High and low excretors of these flavonoids seem to have consistent excretion behaviour for different flavonoids only when co-ingested. Moreover, salivary flavonoid was not a reliable biomarker for citrus or soya intake.

3.2 Introduction:

Flavonoids are polyphenolic compounds that can be widely found in fruit and vegetables, whole grains and legumes. Consumption of flavonoid-rich foods is now generally accepted to be beneficial for health. Flavanones are a subclass of flavonoids found in considerable amounts in citrus fruits (Scalbert and Williamson, 2000). Hesperetin and naringenin are present in foods as their respective glycosides; hesperidin (hesperetin-7-*O*-glucose-(1-6)-rhamnoside) and narirutin (naringenin-7-*O*-glucose-(1-2)-rhamnoside (see Fig 1-5 chapter 1). The glycoside must be hydrolysed by the gut microflora before these compounds can be absorbed (Manach *et al.*, 2003). Intestinal microflora, which are responsible for breaking down naringenin and hesperidin, vary among individuals. As a result, varying degrees of absorption occur (Erlund *et al.,* 2002). There is a substantial inter-individual variation in bioavailability of flavanones (Brett *et al.,* 2009) with differences in bioavailability attributed to the differences in composition and metabolism of enteric microflora populations between individuals (Nielsen *et al.,* 2006, Spencer *et al.,* 2008). The consumption of flavanones has been associated with a reduction of the risk of cardiovascular disease as reviewed by Chanet *et al*., (2012b).

Isoflavones are dietary phytoestrogenic compounds found widely in legumes (Del Rio *et al.*, 2013). Soybeans are the main source of the isoflavones genistein and daidzein (Erlund, 2004). Considerable inter-individual variation in excretion of

daidzein and genistein has also been reported (King and Bursill, 1998). The main site of absorption is in the distal small intestine and colon after bacterial de-conjugation. This is despite the potential for early absorption of the mono-glucosides, daidzein-7-*O*-glucoside (daidzin) and genistein-7-*O*-glucoside (genistin) after hydrolysis by lactase phlorizin hydrolase (Day *et al.,* 2000b). This is probably a result of low affinity for the enzyme and the presence of malonyl and acetyl esters of daidzin and genistin in the food source (Wiseman *et al.,* 2002). Furthermore, daidzein may be metabolised by gut bacteria to equol, and only some people (approximately 30% of the population) have the right bacterial microbiome to produce this estrogenic compound (Chanet *et al.,* 2012, Del Rio *et al.,* 2013). Epidemiological evidence has shown that the high consumption of soy foods by many Asian populations reduced the incidence of chronic diseases such as breast and prostate cancer, and CVD (Cao *et al.,* 2010), with intervention trials indicating a reduction of blood pressure (Morand *et al*., 2011).

Although there is a large volume of literature on flavanones and isoflavones in plasma and urine, very few studies reported the level of flavonoids in saliva. Saliva as a biological fluid contains a large number of biochemical compounds some of which may indicate health status (Thomas *et al.,* 2009). To the best of our knowledge, only one study found isoflavones in saliva after food ingestion. In this study, 166 full term infants between birth and 1 year of age were recruited into soya formula, cow's milk formula or breast milk groups. The study investigated the correlation between urinary, salivary and blood concentration of the isoflavones. Daidzein and genistein were undetectable in most blood and saliva samples from children fed breast milk or cow's-milk formula. However, they were detected after soya milk formula. Both genistein and daidzein had the highest median concentration in the urine, followed by blood and then saliva (Cao *et al.,* 2009).

In another study (Tsuchiya *et al.,* 1997), catechins were determined in human saliva in the concentration range (0.05-25 µg/ml). However these salivary catechins were determined after a green tea extract solution was used to rinse the mouth. None of the catechins were detected in saliva before mouth-rinsing. However, significant amount were found after mouth-rinsing the oral cavity with green tea extract even after 1 hr. Catechins and theaflavins compounds, derived from tea, have been tested

for the delivery to the oral cavity after holding both green tea leaves (2 g) or brewed black tea (2 g) in the mouth for 2-5 min and thoroughly rinsing the mouth. Concentration of (2.2-131 μM) and (0.6-1.8 μM) of catechins and theaflavins, respectively, were observed in saliva in the first hr (Lee *et al.*, 2004).

Walle *et al.*, (2005) investigated the hydrolysis of flavonoid glucosides by saliva β-glucosidase. Un-stimulated saliva was collected from 17 participates. Flavonoid glycosides were hydrolysed to their aglycone in the oral cavity and high inter-individual variability was found; up to 20-fold for genistein-7-glucoside. Another cross-over study was conducted with 10 non-smoking volunteers between 19-61 years old. The result from a β-glucoside assay confirmed that whole and microflora-reduced saliva can hydrolyse black raspberry anthocyanins to their aglycone cyanidin (Mallery *et al.*, 2011). Similarly, beta-glucuronidase activity in saliva in relationship to some clinical parameters was investigated and it was concluded that high salivary β-glucuronidase activity existed (Lamster *et al.*, 2003). To the best of our knowledge, only one study to date conducted and compared the isoflavone concentrations between blood, saliva and urine (Cao *et al.*, 2009).

As saliva would make an excellent non-invasive biological fluid to study flavonoid exposure, it is important to assess if flavonoids can be detected easily. As one study has found evidence of isoflavones in saliva, it was useful to use isoflavones as a positive control when assessing potential flavanone secretion in the saliva. Furthermore, soya isoflavones and citrus flavanones have the following related characteristics which make them interesting to study:

(i) They are limited in the distribution across foods.

(ii) They are predominantly absorbed in the large intestine.

(iii) There is a wide variation in amounts excreted between individuals.

(iv) They both have a reasonable level of excreted compounds in urine (as % of dose), compared to other flavonoid sub-classes, which suggests they are more resistant to ring fission by gut bacteria,

(v) They have both been shown to reduce blood pressure in intervention trials.

3.3 Aims of the study

The purpose of this study is to assess the variation in excretion of flavanones and isoflavones in volunteers. The hypothesis is that the intra-individual variation in excretion in these compounds is similar despite the large inter-individual variation – i.e. a high isoflavone excretor will also be a high flavanone excretor.

The main aims are as follows:

- To investigate the pattern of isoflavone and flavanone excretion after single or co-ingestion of flavonoid-rich foods containing these compounds.
- To assess any correlation between excretion of isoflavones and flavanones.
- To determine the possibility of using saliva as a non-invasive biological fluid to monitor flavonoid intake from food.

3.4 Materials and Methods

For a detailed description of the materials, see chapter 2. Orange juice (Tropicana100% Orange Juice, "Not from Concentrate"), and sachets of dry-roasted soya nuts (25 g; Food Doctor, London, UK) were purchased from Morrison's supermarket between January 2011 and August 2011.

3.4.1 Human study design

The human study was designed as a cross-over, randomised (day 1 and 2), intervention using roasted soya nuts and fresh orange juice. The proposal was reviewed and approved by the MEEC Faculty Research Ethics Committee, University of Leeds, at its virtual meeting on 16th August 2010; Ethics reference number MEEC 10-001 (see Appendix 1). Healthy volunteers who responded to the posted flyers/circular email were invited to read the study information sheet (see Appendix 2) and the study protocol was fully explained to each subject prior to the start of the study. Having given their verbal and written informed consent (see

Appendix 3), participants were asked to fill in a basic health questionnaire recording age, gender, height, weight, medical and surgical history, medications taken to ensure the suitability of the volunteers for study inclusion (Appendix 4).

All subjects recruited were students (both male and female) from different Faculties (University of Leeds, UK) who fulfilled the following criteria: the volunteers were healthy, non-smokers, no use of vitamins or food supplements in the previous 3 months, no known history of allergy to soya or citrus fruit, no prescribed medication or antibiotics in the recent 3 months prior to study, no extreme diet (e.g vegan), no exercise regimes requiring significant dietary changes, they had normal body mass index (BMI) between 19 – 27 kg/m^2 , were not pregnant or breast feeding and aged between 20 – 65 y.

3.4.2 Study Protocol

The study consisted of 3 separate days. Each study day had a minimum of 24 hr between them. Participants were encouraged to consume their normal diets prior to the study. However, participants were asked not to consume citrus fruits and juices, and soya products (or supplements containing these) for 48 hr prior to, and for the duration of the study day. Participants were given a list of food to be avoided or allowed (table 3.1). Participants were randomized by dividing into groups A and B. Group A was assigned to start with ingestion of orange juice as the first phase, while group B in this period ingested roasted soya nuts. In the second phase, Group A switched to soya and Group B switched to orange juice. Finally in the third phase all participants consumed soya nuts and orange juice together. The three phases were separated by a washout period of 24 hr at least. The protocol is summarised in the follow diagram Figure 3.1.

Table 3.1: List of foods to avoid

Food items to avoid
Soya beans (fresh, dried roasted nuts, flakes, flour, etc) bread containing soya flour
Soya milk or drinks
Tofu, Tempeh, Miso, Natt , Aburaage
Soy-based desserts and yogurts containing phytoestrogens
Soy sauce
Soy protein Isolate
Cereal bars and breakfast cereals for vegetarians
Vegetarian Burgers and Nuggets and other vegetarian processed
Citrus fruits in any form (fresh, canned or dried)
Fruit juices, squashes and jams containing citrus fruits
Tomato and tomato-based products (juice, paste, sauce, etc.)
Red wine, Tea, coffee, onion (items try to reduce as much as possible)

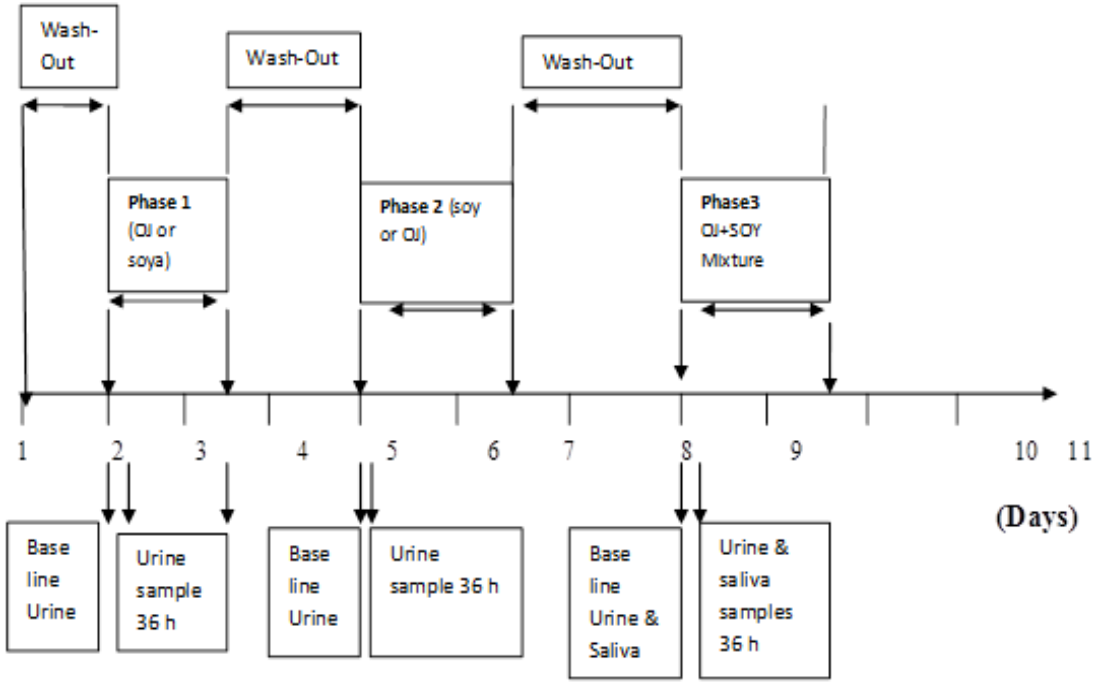


Figure 3.1: Intervention study 1 flowchart.

3.4.3 Phase 1 and 2: single food ingestion

Participants were provided with purpose-designed unisex urine collection containers and jug to be used for each study day. These were labelled by subject codes to maintain their anonymity. On the day of the study, participants were asked to void their bladder immediately on waking, and then have a glass of water (200-300 ml). Approximately an hour later (e.g. 8:00 am), the volunteers should again void their bladder and this time collect the urine sample in the baseline-labelled bottle. Soon after the baseline urine collection, the volunteers were asked to consume either the orange juice (500 ml), or the sachet of soya nuts (25 g) and water (both which had been provided), depending on whether they were in group A or B. They were also invited to consume a slice of bread or toast with butter. The time of consumption was recorded and no other foods were consumed for the first hour. All other foods consumed during the day were recorded using the questionnaire (see appendix 5). Urine was collected in separate labelled bottles containing 1g/L ascorbic acid was added in six different fractions (0 – baseline, 0-4, 4-8, 8-12, 12-24 and 24-36 hr), and the times recorded by the participant. All urine samples were returned to the School of Food Science and Nutrition. The

volume was measured and recorded for both baseline and all the 36 hr fractions. Urine samples were centrifuged (10 min, 2000 g, 4ºC) to remove insoluble materials and cellular debris (see appendix 6), and aliquots of the supernatant stored at -20°C until analysis.

3.4.4 Phase 3: Co-ingestion of foods

The volunteers were provided with 9 labelled salivette containers (Sarstedt, AG & Co., Germany) for the collection of saliva on the 3rd study day and given instructions on how to use it (Walsh *et al.*, 2006). After an overnight fast, the mouth was thoroughly rinsed with tap water, and then the un-stimulated whole saliva was collected on a cotton swab. Briefly the cotton swab was placed directly into the mouth by tipping the tube into the mouth and gently chewing and rolling the sponge around the mouth for 2 minutes before spitting it back into the tube (see Appendix 7). The salivette samples were then kept in the fridge until transported to the lab next day. The volume of the spot saliva was recorded and then all samples were centrifuged (3000 g, 5 min, 4ºC) to remove any solid debris from the saliva samples, before a portion (500 – 2000 µl) of the supernatant was transferred to a new eppendorf tube and stored at -20ºC.

Volunteers were asked to void their bladder immediately on waking, and then have a glass of water (200-300 ml). Approximately an hour later (e.g. 8:00 am), the volunteers should again void their bladder and this time collect the urine sample in the baseline-labelled bottle. A baseline saliva sample was collected at this time. Soon after the baseline urine collection, the volunteers asked to consume both the orange juice (500 ml) and a sachet of soya nuts (25 g) provided. The time of consumption was recorded and no food was consumed in the first hour after consumption of the foods. Urine was collected in separate labelled bottles for each time point, 0-4, 4-8, 8-12, 12-24, 24-36 hr. Saliva also was collected in salivettes as described above, after rinsing the mouth with tap water, at approximately 0, 2, 4, 6, 8, 10, 12, 24 and 36 hr (time was recorded). All other foods consumed during the day were recorded.

3.5 Sample analysis

Upon arrival in the lab, the volumes of urine sample were measured and 2X 50-ml aliquots were taken using ameasuring cylinder. Urine aliquots are then centrifuged at 2000 g, 4°C for 10 min to remove insoluble materials and cellular debris, then the supernatant was taken and samples were then stored at -20 °C until analysed (see Appendix 6). Urine samples were defrosted and centrfuged for 5 min at 3000 g at 20°C, and treated as follows prior the analysis by HPLC-DAD. The HPLC method used to measure the concentration of flavanones and isoflavones as aglycone in urine was previously described in chapter 2.

3.5.1 Extraction and hydrolysis of food flavonoids

Orange juice and soya nut samples (both in triplicate) were analysed with and without enzyme hydrolysis of the flavonoids, as described in sections 2.4.1 and 2.4.2, respectively. The extracted samples were analysed by HPLC using the methods described in 2.3.3.1 and 2.3.3.2 for glycosides and aglycones, respectively.

3.5.2 Extraction and hydrolysis of urine samples

Urine samples (in triplicate) were analysed with enzyme hydrolysis of the flavonoids, as described in 2.4.3, and analysed using the aglycone method as described in section 2.3.3.2).

3.5.3 Extraction and hydrolysis of saliva samples

Defrosted saliva samples were vortexed for 30 sec, and 100 µl of saliva was added to 5 µl of apigenin solution (2 mg/ml) and 20 µl of sodium phosphate buffer (0.2 M, pH 7) enriched with 5 U/ml of β-glucuronidase and 0.03 U/ml of sulfatase (details in section 2.2.3). These samples were then incubated in a shaking water bath at 37°C and 100 r.p.m for 2 hr.

Ethyl acetate (0.5 ml) was added to each hydrolysed saliva sample and vortexed for 2 min, followed by centrifugation (10 min, 17000 g, 20˚C). The supernatant was removed and the precipitate was extracted twice more, ending up

with 1.5 ml of ethyl acetate supernatant. This sample was then placed in the centrifugal evaporator, using the low boiling point program at 40°C for 1 hr.

The dried samples were reconstituted in 100 µl of aqueous methanol solution (50% methanol; 50% 200 µM ascorbic acid solution, v/v). The samples were vortexed for 2 min, and sonicated for 2 min until the samples were fully dissolved in the solution then 90 µl of supernatant was placed in HPLC vials for analysis. Samples were analysed by HPLC (see section 2.3.3.2) and by LCMS analysis (section 2.3.4).

3.5.4 Protein assay

The Bradford Coomassie-binding colorimetric method was used to determine total protein content in the saliva samples. Bovine serum albumin (BSA) and Bradford reagent were purchased from Sigma (Poole, UK). Stock solutions of BSA were made between 100-1500 µg/ml and saliva samples (in duplicate) were diluted 1:5 in buffer Aliquots (5 µl) of BSA standards and saliva samples were pipetted into a 96-well plate along with 250 µl Coomassie reagents. The plate was mixed on a plate shaker for 30 sec, incubated at room temperature for 10 min, and the absorbance read at 595 nm using a multi-well microplate reader (Multiskan FC, Thermo Scientific, China). The protein content of the saliva sample was calculated by subtracting the blank reagent value from the saliva sample reading. The protein content of extracted saliva samples was also monitored to confirm that all protein was precipitated by this process.

3.5.5 Recovery of flavonoids compounds from different matrices

Before analysing the biological samples (urine and saliva) from the human intervention study 1, the recovery of hesperetin, naringenin, genistein, daidzein and apigenin were confirmed by using blank baseline urine and saliva samples. The procedure could then be identified to allow for a good recovery of the compounds investigated. Two procedures have been used: free form of flavanones (ethyl acetate extraction) (see section 2.4.3) and one for hesperetin conjugates (present after precipitation of proteins using methanol and ethanol extraction). As the conjugated (sulfate or glucuronide) derivatives of isoflavones and flavanones were not available

commercially, the conjugates were not quantified by LC-MS in this study; however an assessment of the conjugate for was tentatively made by LCMS at m/z ratios relating to the conjugate form expected. Furthermore, to ensure that flavanones and isoflavones were recoverable from the saliva matrix various control experiments were carried out, as outlined in Table 3.2.

Table 3.2: Control experiments to determine potential recovery of flavonoids from saliva matrix.

Matrix	**Spiked compounds**	**Reaction**	**Rationale**
Water	daidzein + genistein	water	**Control** – standards without interference of matrix
Water	daidzein + genistein	hydrolysing enzymes	**Recovery control** – recovery of standards after enzyme hydrolysis without interference of matrix
Water	water	hydrolysing enzymes	**Control** - potential interfering compounds in enzyme mixture
Blank saliva	water	hydrolysing enzymes	**Control** - potential interfering compounds in enzyme mixture and saliva
Blank saliva	water	water	**Control** - potential interfering compounds in saliva
Blank saliva	daidzein + genistein	water	**Control** – identification of aglycones with interference of saliva matrix
Blank saliva	daidzein + genistein	hydrolysing enzymes	**Recovery control** – identification of aglycones with interference of saliva matrix and enzymes, and recovery after enzyme hydrolysis
Water (75%)	urine (subject 4, 12 hr) (25%)	water	**Control** – identification of metabolites without interference of saliva matrix
Blank saliva	urine (subject 4,	water	Evidence of conjugates after extraction

(75%)	12 hr) (25%)		from saliva matrix
Active saliva	water	water	Sample analysis for presence of conjugates
Active saliva	water	hydrolysing enzymes	Sample analysis for presence of aglycones after hydrolysis

3.6 Statistical analysis

The results were expressed as mean ± SE unless otherwise stated. For each flavonoids compounds detected and quantified, the maximum observed urine concentration (urinary C_{max} and time to reach urinary C_{max} (urinary T_{max}) were determined by visual inspectation of volunteers urine concentration time profile. The relative bioavaliblity of flavanones and isoflavones was calculated as precentage form the total a mount of these compounds excreted in urine over 36 hr divided by the amount consumed from the experimental food. Spearman's rank correction coefficients were calculated to evaluate the relation between the single and co-ingestion consumption patterns. A potential period effect was tested by a two-sample *t*-test to compare differences between different treatments. Moreover, the average of excretion was compared between phase 1,2 and 3, with the significance level set to 5%. A wide range of sample sizes was observed in the previous studies used for this analysis and the smallest sample used was 3 participants and the most common sample sizes were 5 – 20 participants to show important finding from this analysis. Therefore, sample size of 15 was the target for this study. All of the statstical analyses were preformed using the Statistical software (SPSS) for windows, version 22.

3.7 Results

3.7.1 Subjects

Fifteen healthy subjects gave their consent and completed all three phases of the study. Their characteristics are shown in Table 3.3. The study was reviewed and approved by the MEEC Faculty Research Ethics Committee, Ethics reference number is (MEEC 10-001) University of Leeds.

Table 3.3: Participant Characteristics

Characteristics	Mean ± SD, range
Number of subjects	15
Male	7
Female	8
Age	31 ± 6.1 (22 – 44)
Body mass index (kg/m2)	20 ± 3.6 (15-27)

3.7.2 Quantification of flavonoids in food samples

The identification of flavanones and isoflavones in food and biological fluid samples was based on the retention time of the eluted sample with that of the respective standard compound, the specific absorption spectra maxima for each reference compound, and confirmation on spiking of the sample with the authentic standard. LC-MS analysis was also carried out on selected saliva and urinary samples. Chapter 4 thoroughly explains the analysis of the flavonoid content for each food sample using enzyme hydrolysis.

Figure 3.2 shows a typical HPLC chromatogram of the orange juice used in the study. Narirutin and hesperidin were the main peaks identified (peaks 1 and 2), and the orange juice contained 80 mg/L and 420 mg/L of narirutin and hesperidin, respectively. Hesperetin and hesperetin-7-glucoside were not present in the sample above the limit of detection.

As daidzein and genistein are present as glucosides and also esterified to malonyl and acetyl groups in soya nuts, enzyme hydrolysis was used to hydrolyse the different conjugates in order to quantify the total amount of daidzein and genistein in the sample under the conditions applied. A typical HPLC chromatogram of hydrolysed soya nuts is shown in Figure 3.3. In this figure, the main peaks at 260 nm, with retention time of 6.95 and 8.67 min, were identified as peaks 1 and 2 and represent daidzein and genistein respectively. A third more minor peak eluted ~30 seconds after daidzein. This is likely to be glycetin, as it is normally present in soya foods at approximately 10% of the total isoflavones. Nevertheless, we did not

attempt to quantify this compound for the present study. The amount of daidzein and genistein found in the soya nuts were 30 mg/100 g and 57 mg/100 g, respectively.

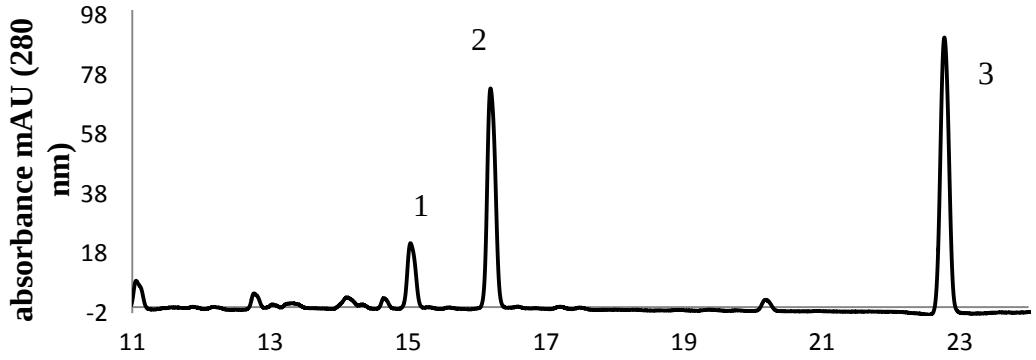


Figure 3.2: HPLC chromatogram of orange juice monitored at 280 nm. Peaks are identified as (1) narirutin (2) hesperidin and (3) apigenin used as internal standard. HPLC method is described in section 2.2.3.2.

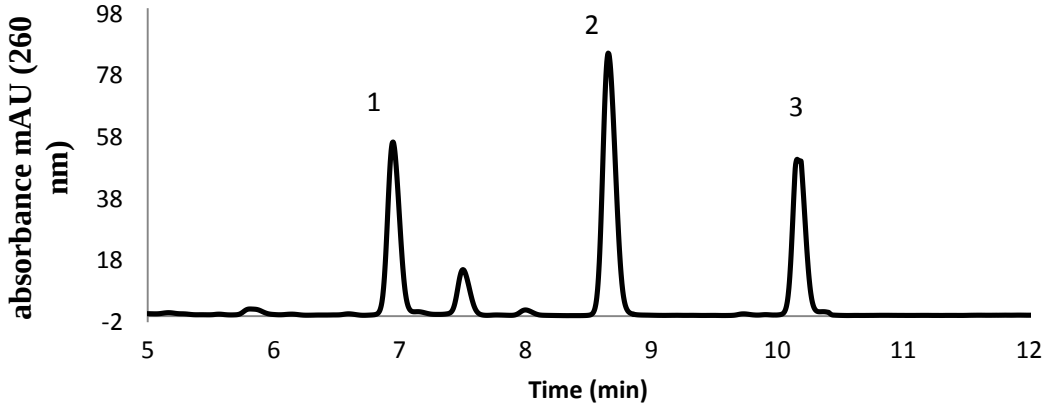


Figure 3.3: HPLC chromatogram of hydrolysed soya nuts monitored at 260 nm. Peaks identified in samples are: (1) daidzein, (2) genistein and (3) apigenin as internal standard. HPLC method is described in section 2.2.3.1.

3.7.3 Extraction and quantification of flavonoids in urine

On the basis of the authentic standards in Chapter 2, the identification of flavanones and isoflavones were achieved using HPLC-DAD. Flavanones were absent from all baseline samples, indicating compliance with the avoidance diet.

However, small amounts of isoflavones were detected in the baseline urine for three subjects who consumed food with hidden soya a day before the study day. Therefore, we have deducted the amount found in the baseline from the total of daidzein and genistein excreted. The total concentration of isoflavones and flavanones was quantified in the urine after de-conjugation by β-glucuronidase and sulfatase, and was corrected based on the recovery rate of internal standard with extraction efficiency of 87%. To ensure the quality of the data, quality control samples were analysed at the beginning and the end of each batch. An example HPLC chromatogram from baseline and an active urine sample (after enzyme hydrolysis) from one subject, after co-ingestion of orange juice and soya nuts is illustrated in figure 3.3. Peaks 1, 2, 3, 4 and 5 were detected in urine and in comparison with the retention time of authentic standards and relative spectra, these five peaks can be assigned as daidzein, naringenin, genistein, hesperetin and apigenin, respectively.

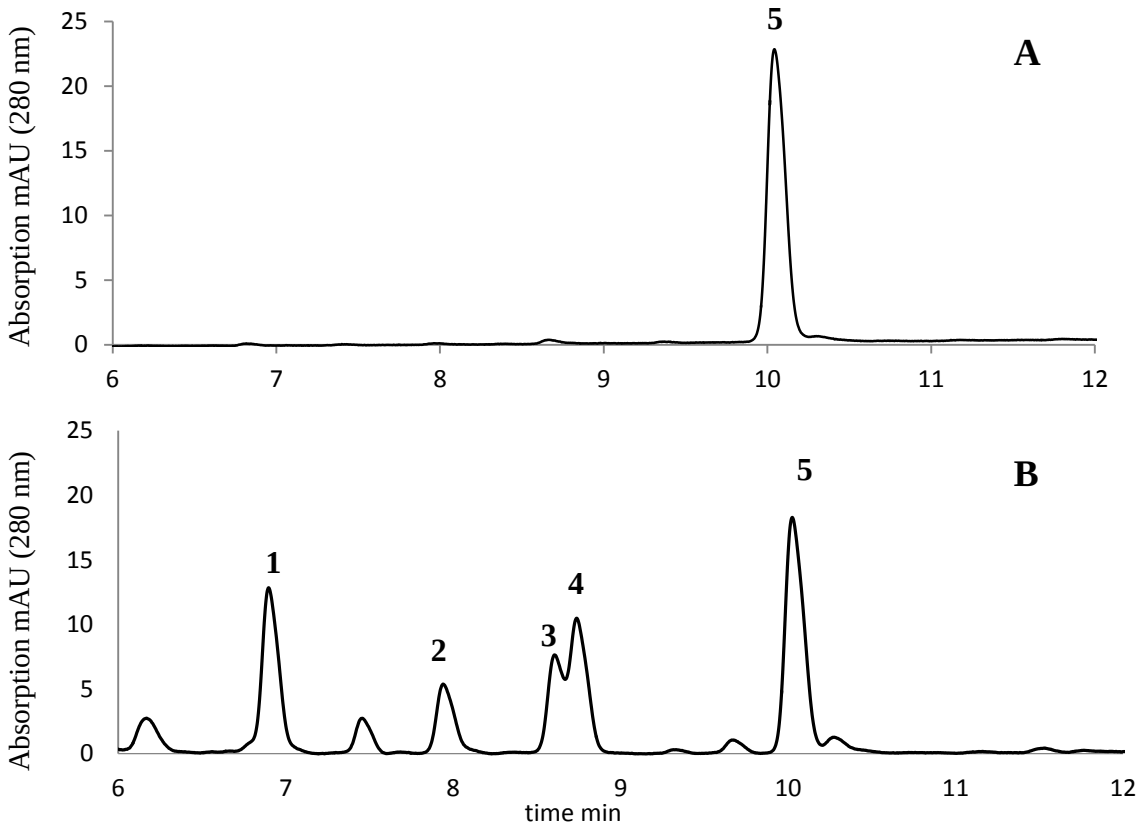


Figure 3.4: Typical HPLC chromatogram of a hydrolysed urine sample. Sample was from subject 16 at 12-24 hr after ingestion of 25 g soya nuts with 500 ml orange juice; (A) before enzyme hydrolysis and (B) after enzyme hydrolysis. The peaks were identified as (1) daidzein, (2) naringenin, (3) genistein, (4) hesperetin, and (5) apigenin (as internal standard).

Only flavanone and isoflavone aglycones were measured in the urine samples after de-conjugation by β-glucuronidase and sulfatase enzymes. The quantities of flavonoids excreted in each of the time fractions following single food or co-food ingestion are shown in Table 3.4. Figures 3.5 and 3.7 present this data for the flavanone and isoflavones, respectively, as bar charts for easy comparison. Figures 3.6 and 3.8 provide the cumulative excretion for the flavanones and isoflavones, respectively, over the 36 hr period of urine collection.

Very small amounts of flavanones were detected in the urine during the first 4 hr after ingestion. There was a rapid but fairly steady increase of naringenin from 4-8 hr and 8-12 hr, as shown by the linearity between these points in Figure 3.6A.

Similar amounts were excreted in the 12-24 hr fraction, but as this represented a 12 hour collection, the rate of excretion was slowing down. Excretion levels subsequently decreased and after 24 hr the baseline levels were likely to be reached before 36 hr. Naringenin excretion showed no difference between single and co-ingestion of orange juice with/without soya.

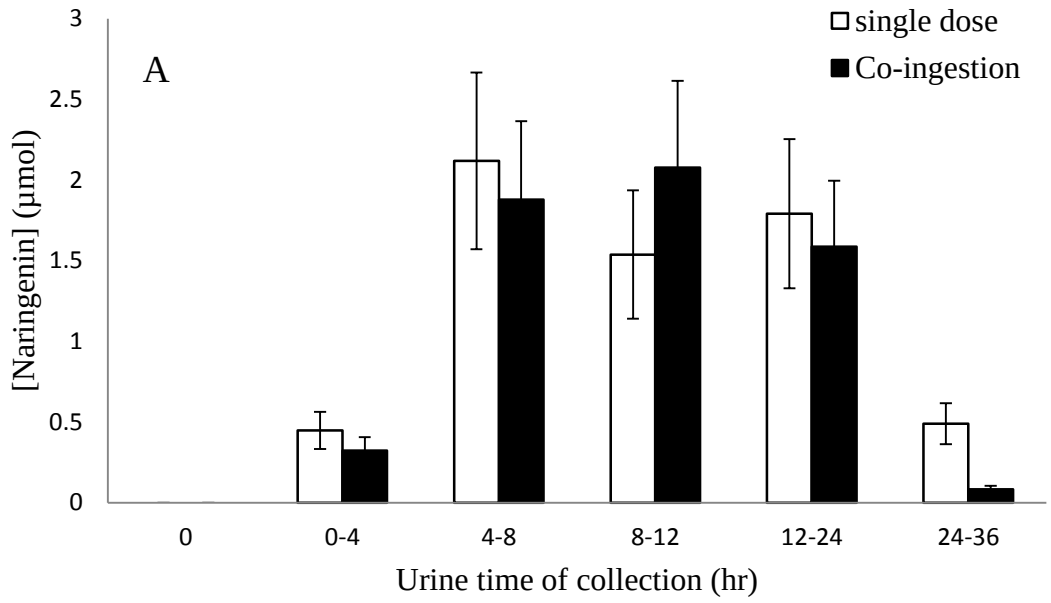
Hesperetin excretion followed a similar pattern, with little excretion up to 4 hr, and then an increase up to 12 hr. Although not significantly different, hesperetin excretion after co-ingestion did appear to be affected, with an increased excretion rate between 8 and 12 hr there is no significant difference ($p=0.3$), leading overall to a 20% increase in hesperetin excretion (Figure 3.6B).

Throughout the entire collection there was no significant difference in average daidzein or genistein excretion, after single or co-ingestion of soya with/without orange juice (Figure 3.8). Low levels of daidzein and genistein were excreted in urine for the first 4 hr, but the rate increased and was steady to 12 hr. Between 12-36 hr the excretion rate remained steady for single ingested soya, but for co-ingested soya the isoflavone excretion reached baseline by 36 hr (seen by the plateau in Figure 3.8).

Table 3.5 shows the fraction percent excreted in the urine over the 36 hr after single or co-ingestion of the foods. For the flavanones between 64 and 75% of total naringenin and hesperetin was excreted by 12 hr, with little difference after only orange juice or with soya nuts. On the other hand, only 52-54% of isoflavones were excreted by 12 hr when soya was ingested on its own, whereas 68% of both compounds were excreted by 12 hr when co-ingested with orange juice.

Table 3.4: Quantities of flavanones and isoflavones compounds (µmol) excreted in urine from 25 healthy volunteers 0 – 36 hr after ingestion of orange juice and soya nuts.

Flavonoids ingested	**Amount excreted in urine µmol (36 hr)**						**Total**
	0	0-4	4-8	8-12	12-24	24-36	
Naringenin (single dose)	0.0 ± 0.0	0.4 ± 0.1	2.1 ± 0.5	1.5 ± 0.4	1.8 ± 0.5	0.5 ± 0.1	6.4 ± 1.6
Naringenin (Co-ingestion)	0.0 ± 0.0	0.3 ± 0.1	1.9 ± 0.5	2.1 ± 0.5	1.6 ± 0.4	0.1 ± 0.0	5.9 ± 1.5
Hesperetin (single dose)	0.0 ± 0.0	0.6 ± 0.2	3.1 ± 0.8	2.5 ± 0.6	2.4 ± 0.6	0.3 ± 0.1	8.8 ± 2.3
Hesperetin (Co-ingestion)	0.0 ± 0.0	0.3 ± 0.1	2.7 ± 0.7	5.0 ± 1.3	2.4 ± 0.6	0.2 ± 0.0	10.6 ± 2.7
Daidzein (single dose)	0.0 ± 0.0	0.6 ± 0.2	4.2 ± 1.1	3.8 ± 1.0	4.6 ± 1.2	2.8 ± 0.7	16.0 ± 4.1
Daidzein (Co-ingestion)	0.0 ± 0.0	1.5 ± 0.4	3.7 ± 1.0	4.1 ± 1.1	3.6 ± 0.9	0.9 ± 0.2	13.8 ± 3.6
Genistein (single dose)	0.0 ± 0.0	0.6 ± 0.2	3.4 ± 0.9	2.5 ± 0.6	3.5 ± 0.9	2.7 ± 0.7	12.7 ± 3.3
Genistein (Co-ingestion)	0.0 ± 0.0	1.1 ± 0.3	2.5 ± 0.6	3.4 ± 0.9	2.5 ± 0.6	0.9 ± 0.2	10.4 ± 2.7



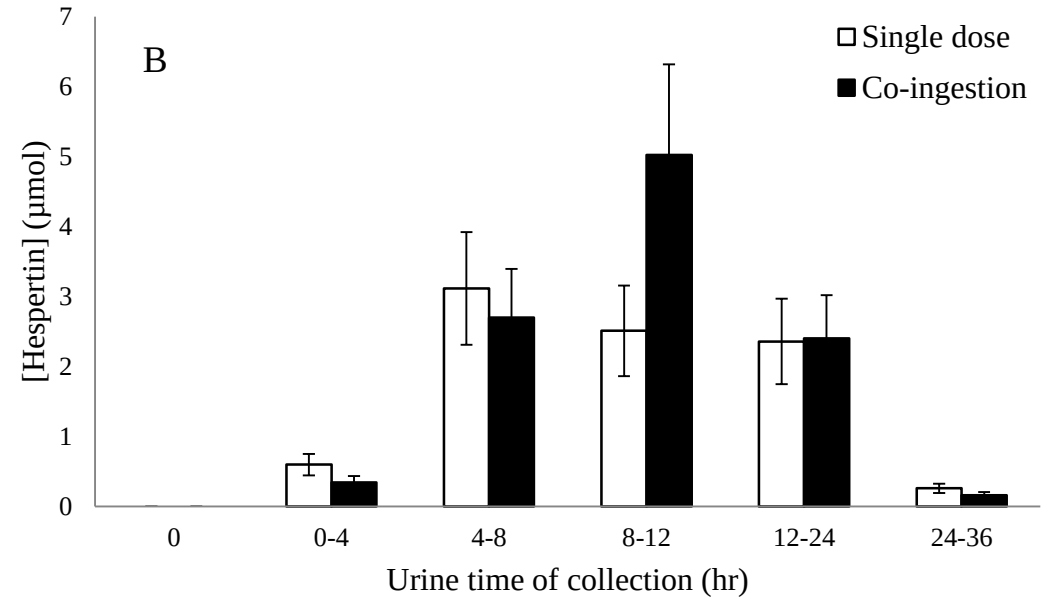


Figure 3.5: Averaged urinary excretion profile and concentration of (A) naringenin and (B) hesperetin in 15 volunteers after consumption of orange juice with and without soya nut.

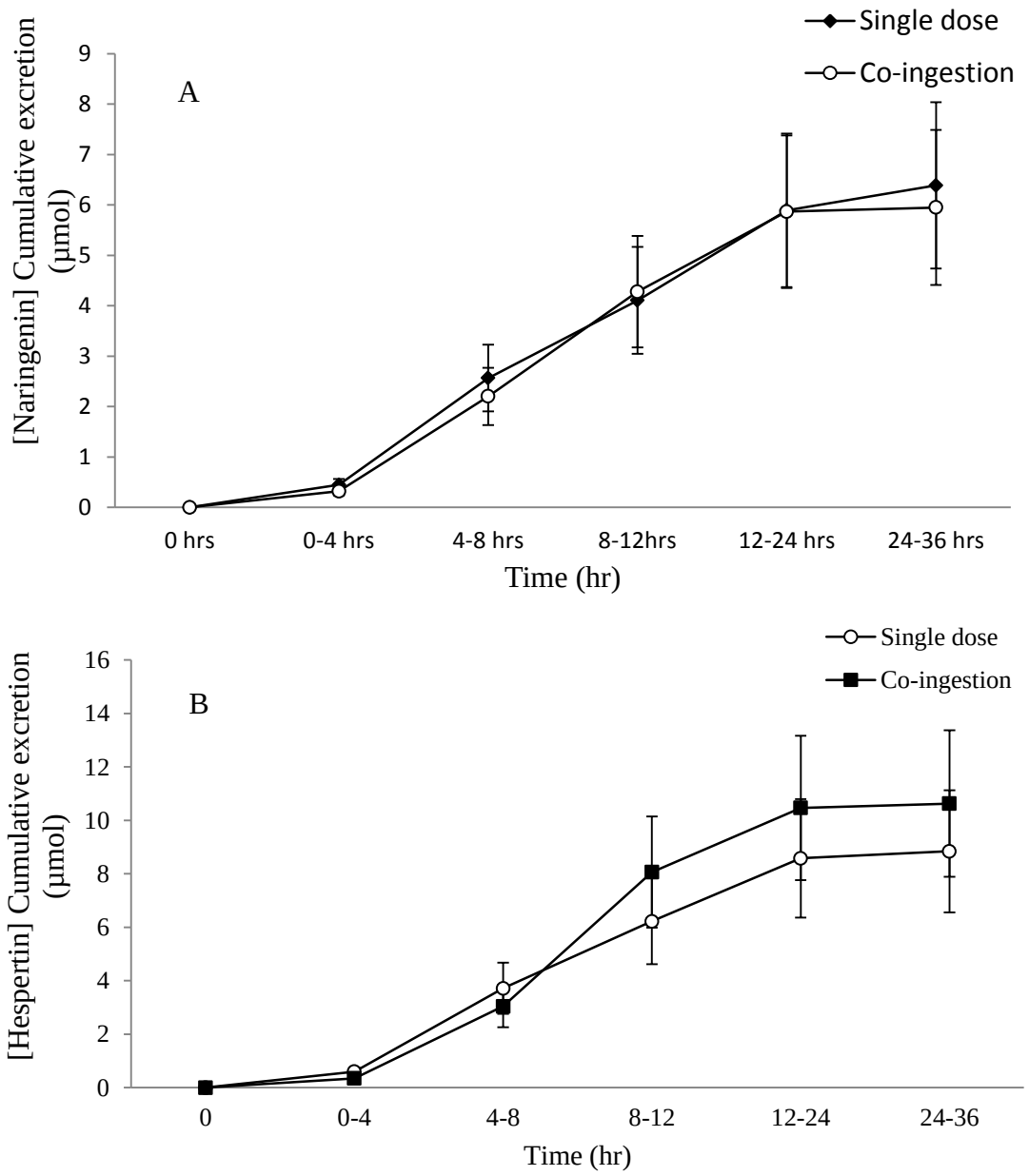


Figure 3.6: Cumulative urinary excretion of (A) naringenin and (B) hesperetin measured in urine collected up to 36 hr in healthy volunteers following consumption of orange juice with and without soya nut. Values are means ± SE (n=15).

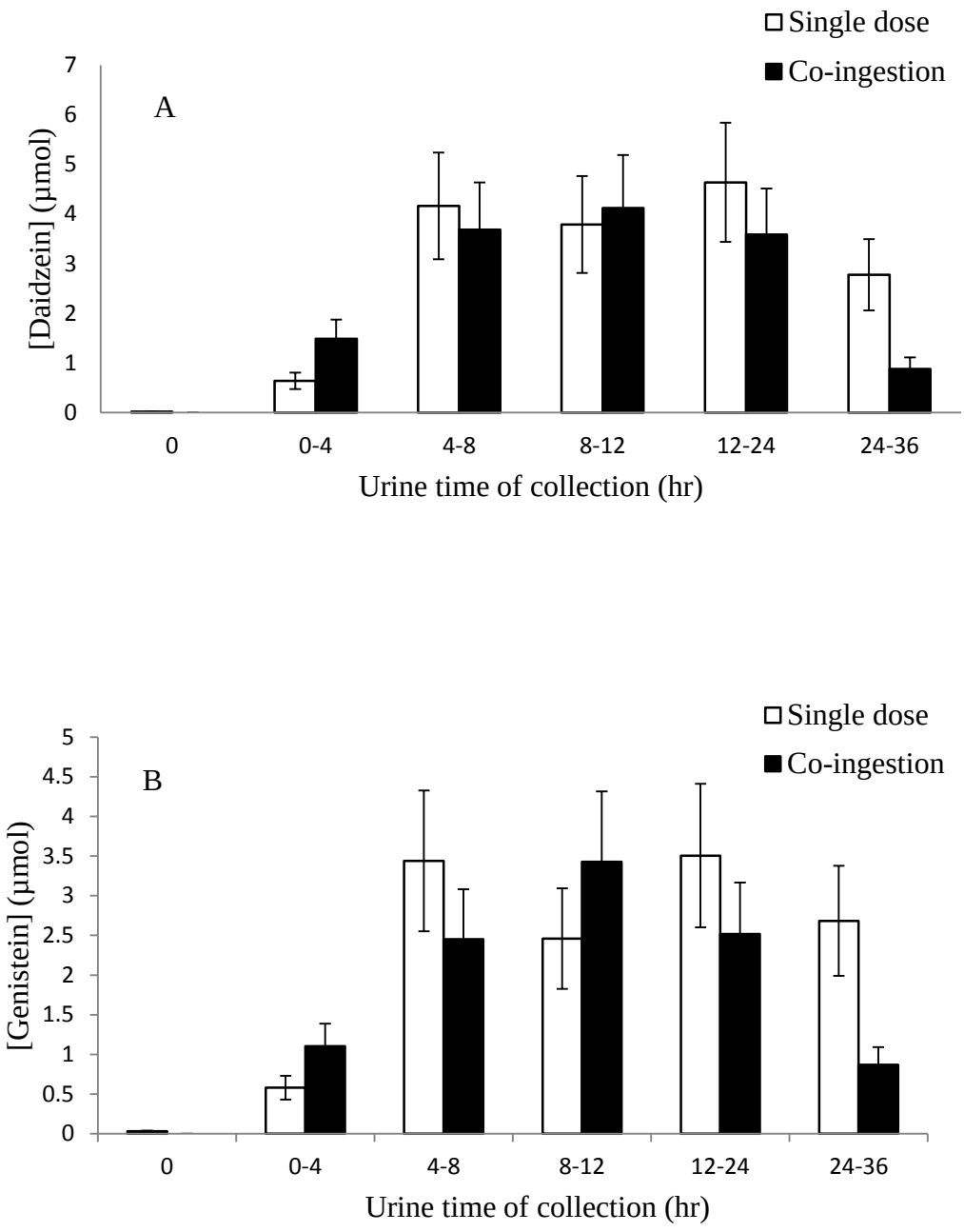


Figure 3.7: Average urinary excretion profile and concentration of (A) daidzein and (B) genistein in 15 participants after consumption of soya nut with and without orange juice.

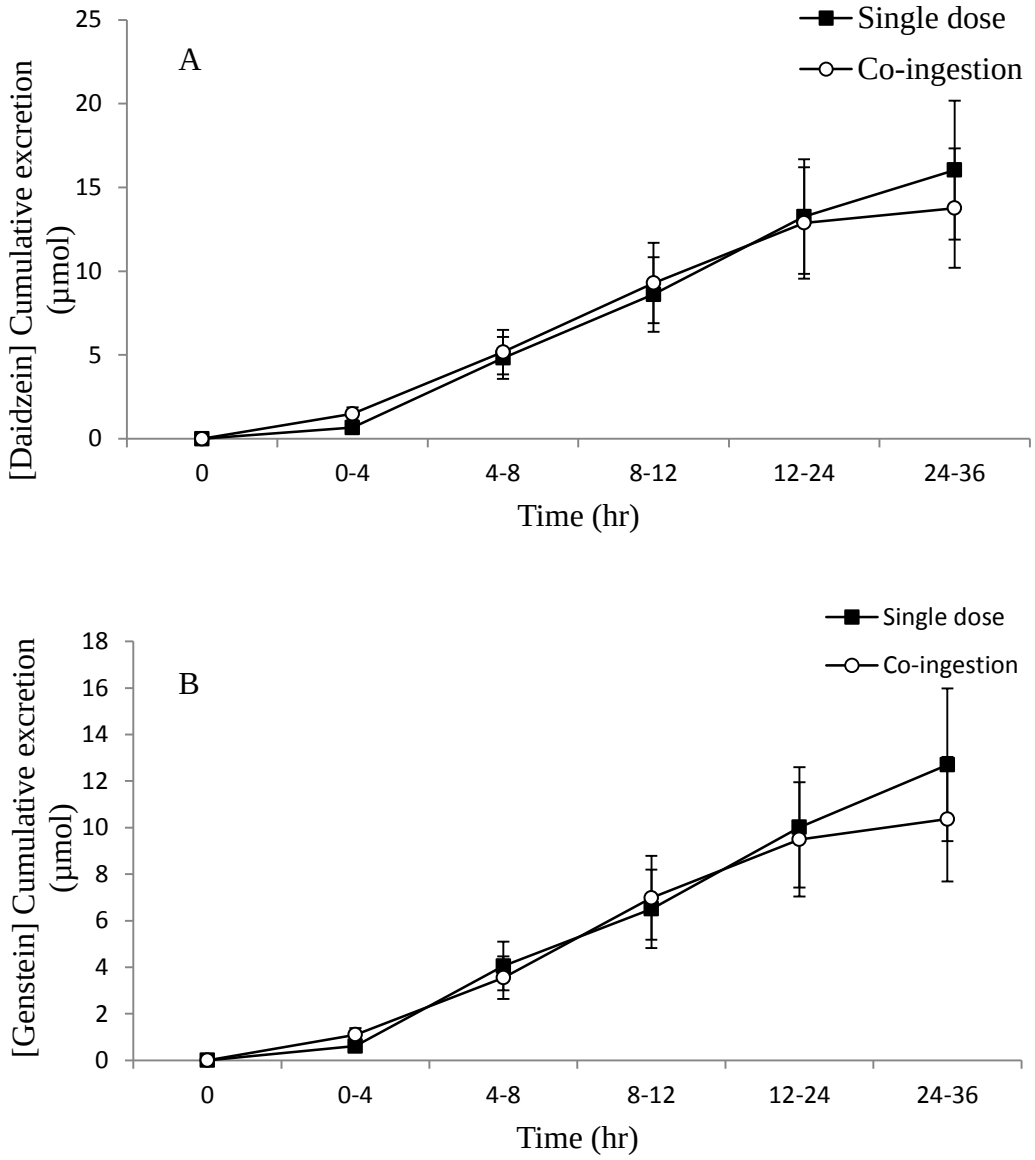


Figure 3.8: Cumulative urinary excretion of (A) daidzein and (B) genistein measured in urine collected up to 36 hr in healthy volunteers following consumption of soya nut with and without orange juice. Values are means ± SE (n=15).

Table 3.5: Fractional excretion of isoflavones and flavanones as (%) of total urinary excretion in healthy volunteers up to 36 hr after ingestion of orange juice and soya nut (n = 15).

Collection time (hr)	**Fractional excretion (% of total excretion)**							
	N 1	N 2	H 1	H 2	D1	D 2	G 1	G 2
0	0	0	0	0	0	0	0	0
0-4	7	5	7	3	4	11	5	11
4-8	33	32	35	25	26	27	27	24
8-12	24	35	29	47	24	30	20	33
12-24	28	27	27	23	29	26	28	24
24-36	8	1	3	2	17	6	21	8

N1 and N2 are values for naringenin after single and co-ingested foods respectively. Likewise H1 and H2 represent hesperetin; D1 and D2 represent daidzein; G1 and G2 represent genistein.

The total amount of isoflavones and flavanones detected in 36 hr urinary excretion from the 15 volunteers is presented in figure 3.9.

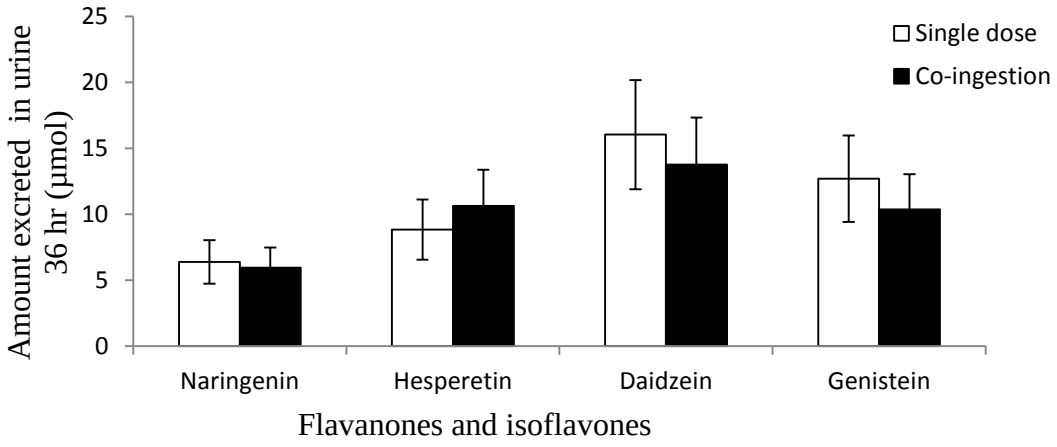


Figure 3.9: Mean urinary excretion of flavanones and isoflavones 36 hr after consumption of orange juice and/or soya nuts as single or co-ingested dose. Data presented as mean ± SE, n = 15 subjects.

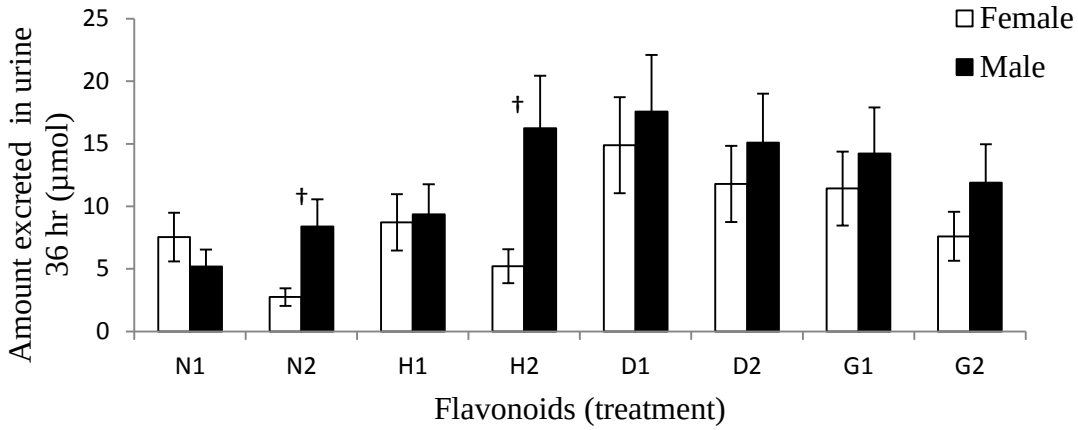


Figure 3.10: Mean urinary excretion of flavanones and isoflavones in male and females 36 hr after consumption of orange juice and/or soya nuts as single or co-ingested dose. Data presented as mean ± SE, n = 15 subjects. † indicates significant difference between gender, $p < 0.05$.

On the basis of gender, the mean urinary excretion of isoflavones and flavanones were compared between subjects. Figure 3.10 shows the excretion differences between males and females. It appears that while similar amounts where consumed, the urinary excretion of all compounds, except naringenin, tends to be higher among males compared to females. This higher excretion was statistically significant for naringenin and hesperetin when orange juice was co-ingested with soya nuts ($p= 0.01$; $p= 0.05$). Table 3.6 shows the individual total excretion of flavonoids based on gender.

Table 3.6: Total urinary amount excreted of flavanones and isoflavones in males and females, over 36 hr urinary collections, after consumption of orange juice and/or soya nuts.

Gender	code	N 1	N 2	H 1	H 2	D1	D 2	G 1	G 2
		(µmol excreted in 36 hr)							
Female	2	2.9	2.0	4.6	3.4	6.9	9.8	2.3	5.1
	4	8.5	3.0	12.6	12.7	16.8	14.4	2.6	5.1
	5	4.2	6.2	5.1	7.5	17.9	18.3	24.1	8.3
	8	2.3	3.9	2.6	3.2	11.8	8.4	7.8	6.0
	9	24.4	1.0	11.1	2.5	9.9	23.4	9.9	22.1
	14	2.8	0.9	2.2	4.5	28.8	4.3	28.0	3.7
	15	7.7	2.2	22.8	2.7	12.0	4.0	5.2	3.0
Female mean		7.5	2.7	8.7	5.2	14.9	11.8	11.4	7.6
Female SE		1.9	0.7	2.3	1.3	3.8	3.0	2.9	2.0
Male	1	6.6	11.2	6.1	9.2	13.3	18.2	10.9	19.0
	3	3.5	4.7	4.5	5.7	13.4	13.8	22.8	6.2
	6	0.4	5.5	0.3	22.2	5.1	5.2	3.2	1.6
	10	6.7	6.0	11.1	10.7	30.5	21.3	24.0	15.6
	11	6.3	19.8	11.6	44.7	25.2	23.3	19.4	30.1
	12	7.2	4.4	13.2	10.7	18.5	13.0	9.5	6.9
	13	0.8	8.3	0.5	13.5	21.1	19.9	11.2	13.8
	16	11.4	10.2	24.2	6.4	9.3	9.1	9.4	9.1
Male mean		5.4	8.4 †	9.4	16.3 †	17.6	15.1	14.2	11.9
Male SE		1.4	2.2	2.4	4.2	4.5	3.9	3.7	3.1
Overall Mean		6.4	5.9	8.8	10.6	16.0	13.8	12.7	10.4
Overall SE		1.6	1.5	2.3	2.7	4.1	3.6	3.3	2.7

N1 and N2 are values for naringenin after single and co-ingested foods respectively. Likewise H1 and H2 represent hesperetin; D1 and D2 represent daidzein; G1 and G2 represent genistein. Mean ± SE (N=15; †significantly different between male and female p= <0.05).

The mean urinary isoflavones and flavanones excreted as percentage of ingested dose was calculated and is presented in table 3.7. There was very little difference in the percent excreted for any compound between single or co-ingestion of the food. Daidzein was recovered in the urine to a greater extent than genistein; by

1.4- 1.7-fold increase for single dose compared to co-ingestion, respectively. Naringenin was recovered in urine to a greater extent than hesperetin; by 3.7-fold and 2.8-fold when orange juice was ingested separately or co-ingested with soya nuts.

Table 3.7: Percentage dose excreted in 36 hr for each flavonoid

Flavonoid	**Single/co-ingested**	**Average % of dose excreted (± SE, n=15)**
Daidzein	Soya nuts	27 ± 7.0
Daidzein	Soya nuts + OJ	28 ± 7.2
Genistein	Soya nuts	19 ± 4.9
Genistein	Soya nuts + OJ	16 ± 4.3
Naringenin	OJ	9.2 ± 2.3
Naringenin	OJ + soya nuts	8.6 ± 2.2
Hesperetin	OJ	2.5 ± 0.6
Hesperetin	OJ + soya nuts	3.0 ± 0.7

Figure 3.11 (A) and (B) demonstrate the inter-individual values corresponding to the percentage of total genistein and daidzein excreted in urine after soya nuts were consumed as single dose and co-ingested with orange juice. Figure 3.12 (A) and (B) illustrates the similar urinary recovery inter-individual variability of the flavanones. The percent dose excreted for naringenin ranged from 0.6 – 35%, and 1.3 – 28% for a single dose and co-ingestion, respectively. Hesperetin had a smaller range of 0.1 – 7.0%, and 0.7 – 13% respectively. The individual excretion profiles of naringenin and hesperetin after the consumption of orange juice separately and with soya are shown in figure 3.13 and figure 3.14, respectively. Likewise, figure 3.15 and figure 3.16 show daidzein and genistein after soya nuts alone or with orange juice, respectively. The large inter-individual variation in urinary excretion of isoflavones and flavanones is evident; range was between 1.6-30 µmol/36 hr for isoflavones and 0.8-45 µmol/36 hr for flavanones.

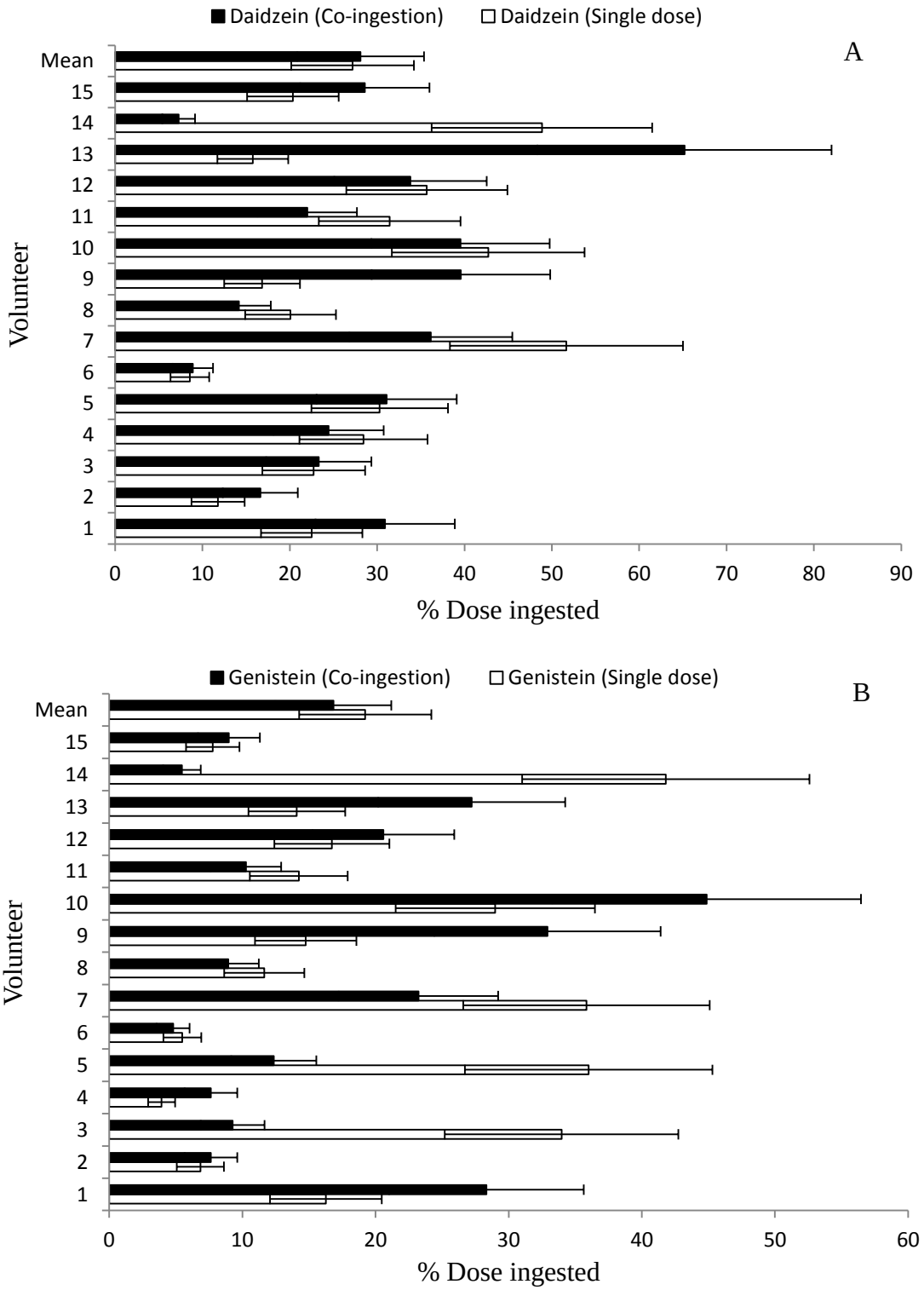


Figure 3.11: Inter-individual variation in recovery of daidzein (A) and genistein (B) as percentage of administrated dose. Data presented as mean ± SE (n = 15).

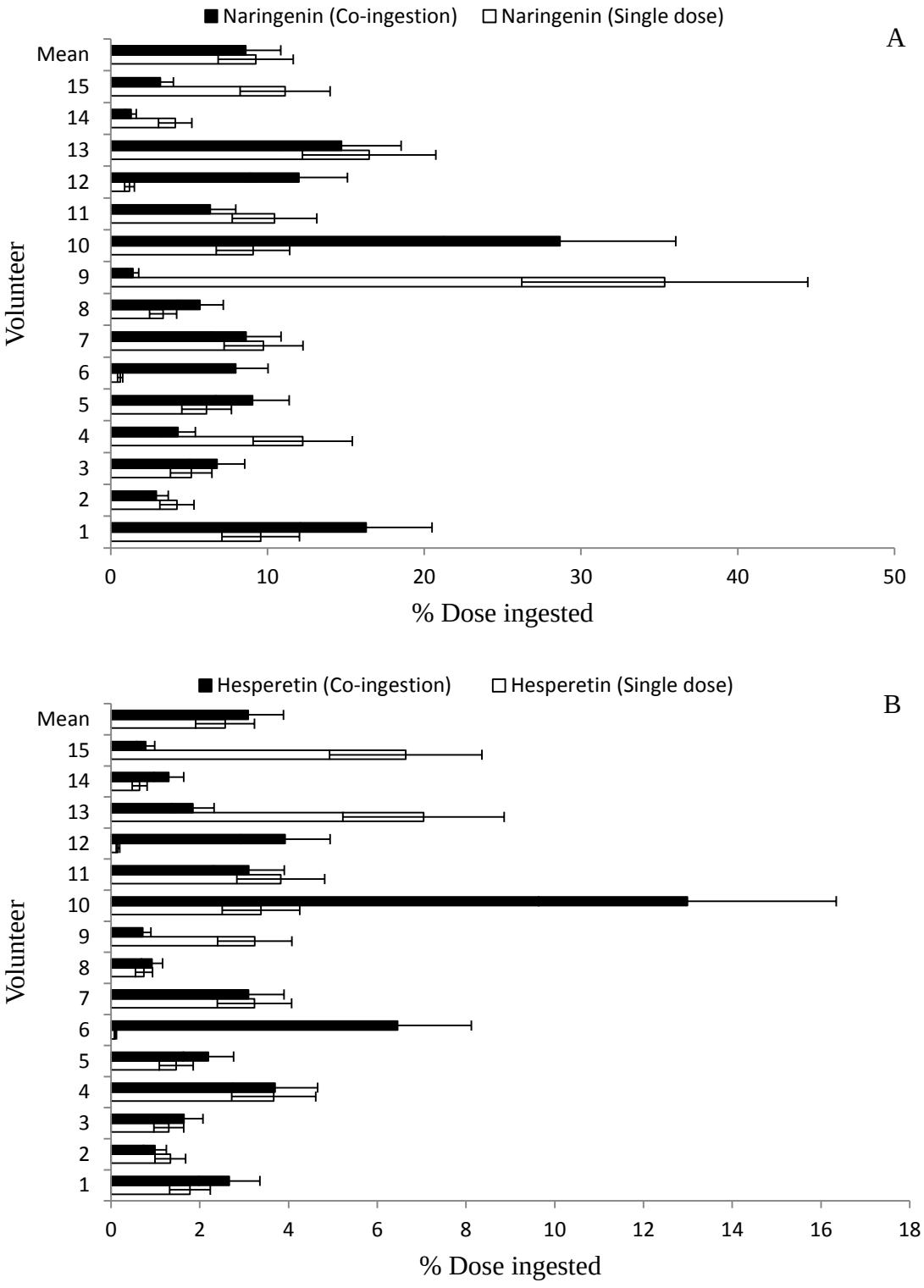


Figure 3.12: Inter-individual variation in recovery of naringenin (A) and hesperetin (B) and naringenin as percentage of administrated dose. Data presented as mean ± SE (n = 15).

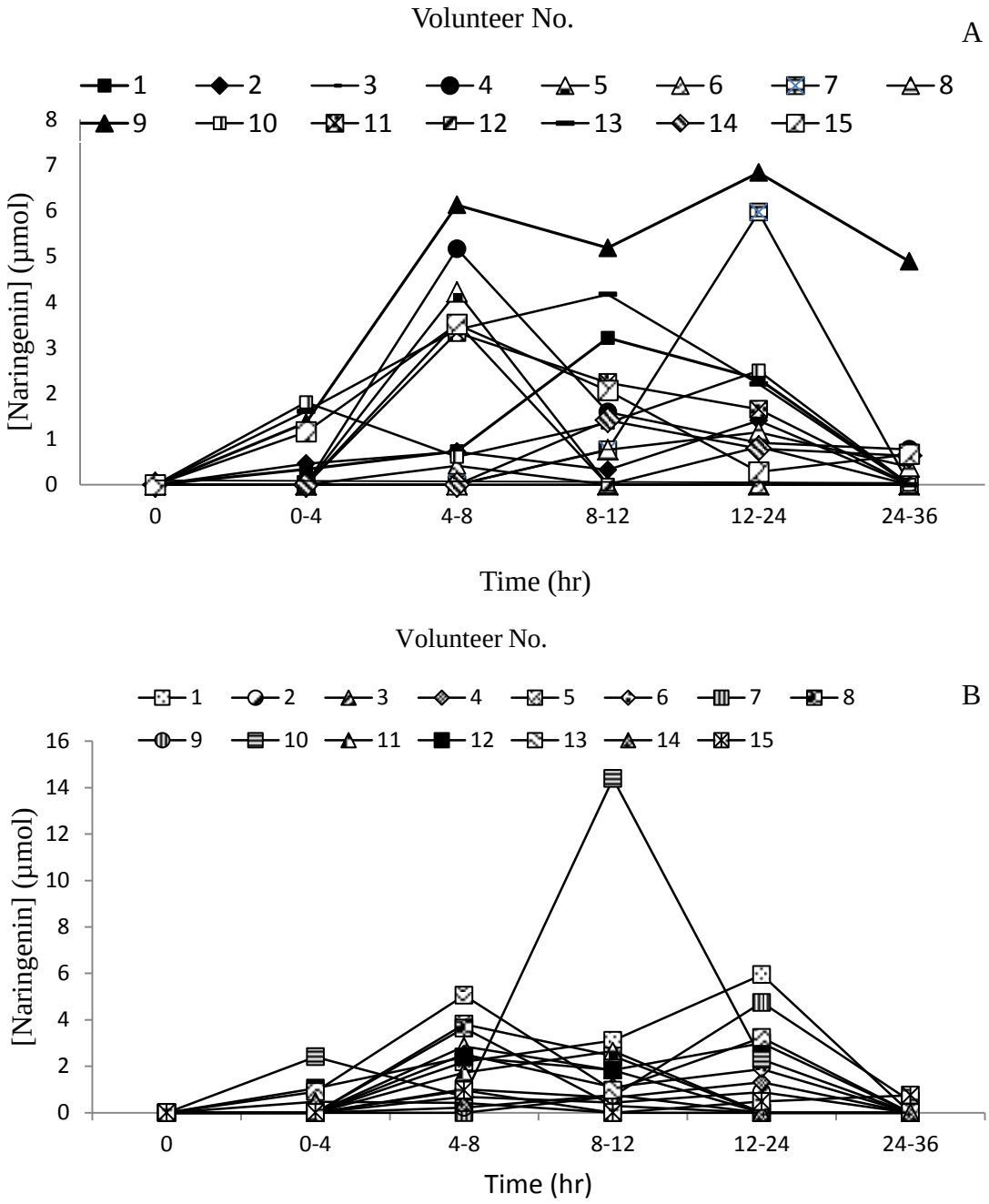


Figure 3.13: Naringenin excretion profiles of individual subjects. (A) Single dose of orange juice, (B) co-ingestion after consumption of orange juice with soya nuts.

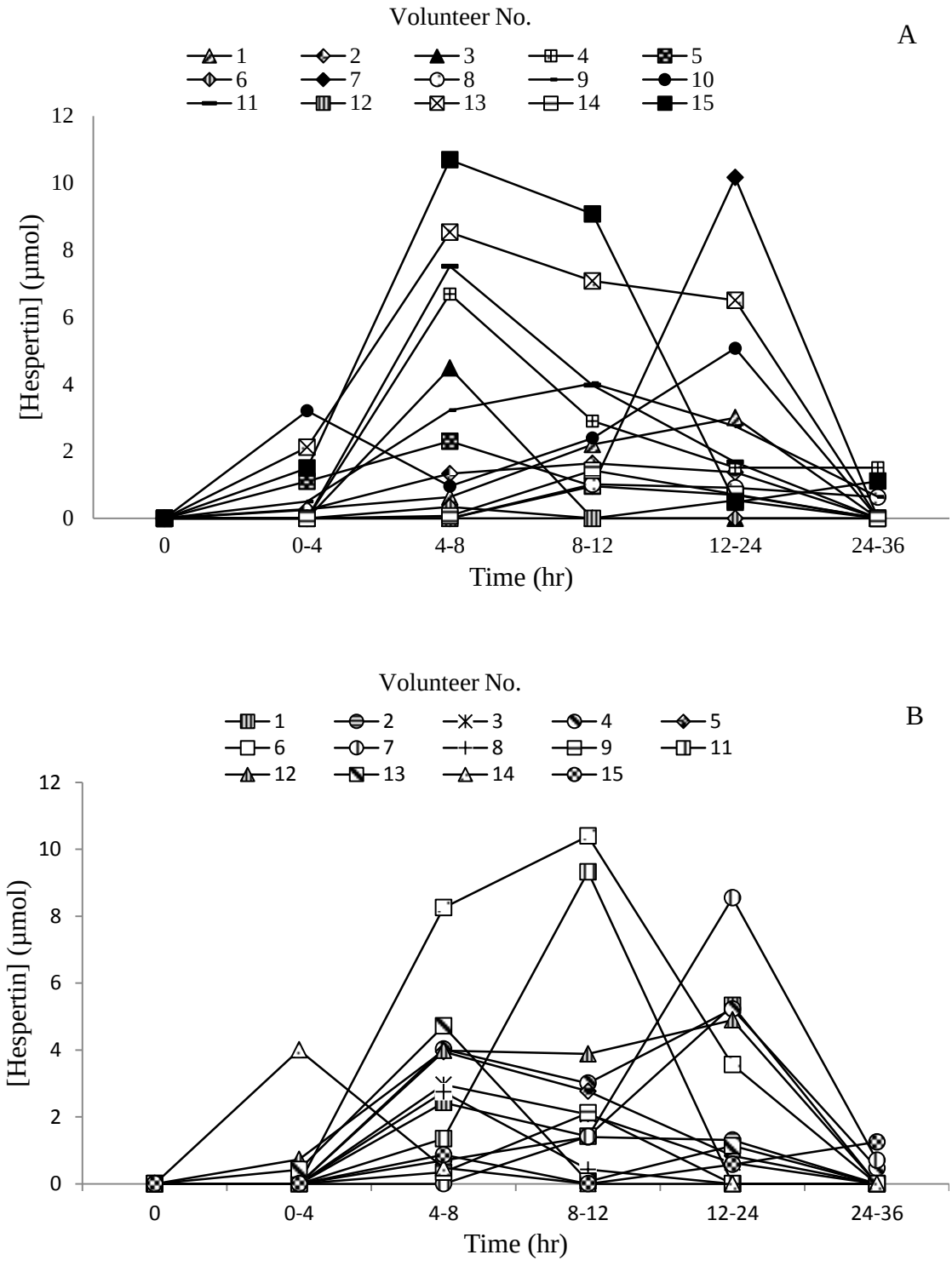


Figure 3.14: Hesperetin excretion profiles of individual subjects. (A) Single dose of orange juice, (B) co-ingestion after consumption of orange juice with soya nuts.

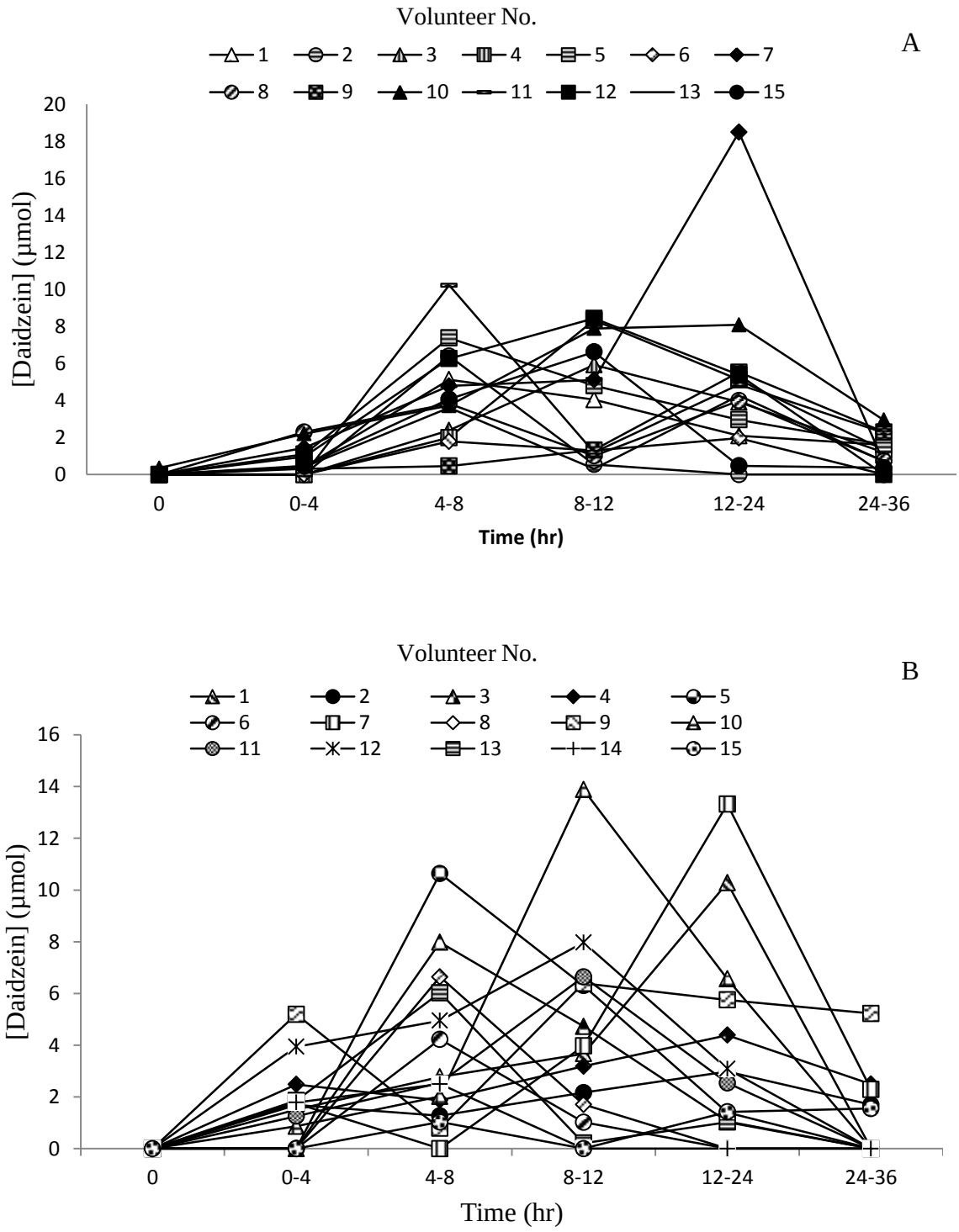


Figure 3.15: Daidzein excretion profiles of individual subjects. (A) Single dose of soya nuts, (B) co-ingestion after consumption of soya nuts with orange juice.

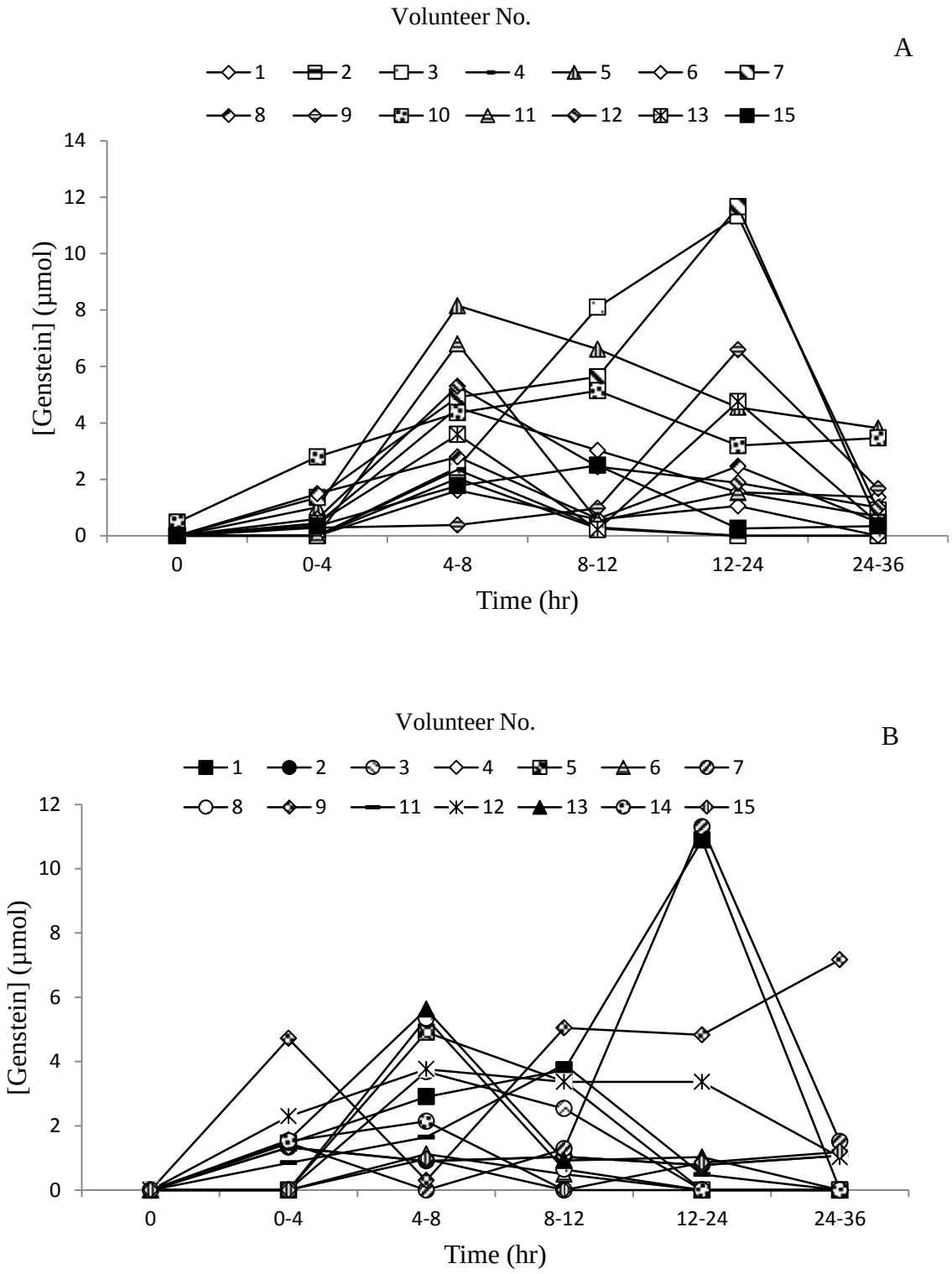


Figure 3.16: Genistein excretion profiles of individual subjects. (A) Single dose of soya nuts, (B) co-ingestion after consumption of soya nuts with orange juice.

Using the Spearman correlation coefficient, the correlation between the flavanones and isoflavones as percentage recovered in urine was investigated after orange juice and soya nuts were ingested separately. As can be seen from figure 3.17, the urinary excretion of hesperetin was correlated with the amount of naringenin recovered in urine samples ($r= 0.54$; $p= 0.03$), as was the amount recovered for daidzein and genistein ($r= 0.72$; $p = 0.002$). Conversely, neither hesperetin nor naringenin were correlated with the amount of daidzein and genistein excreted in urine samples when orange juice and soya nuts were consumed separately.

The results indicate that the urinary recoveries (% of intake) of isoflavones and flavanones when orange juice and soya nuts were co-ingested were correlated more significantly than after single ingestion (Figure 3.18). There was a strong positive correlation between urinary excretion of hesperetin and naringenin ($r= 0.72$; $p<0.0005$), and between genistein and daidzein ($r=0.72$; $p=0.002$). Moreover, urinary excretion of naringenin was strongly correlated with the excretion of genistein ($r=0.73$; $p=0.002$), and daidzein ($r=0.49$; $p=0.05$). Whereas, excretion of hesperetin was weakly correlated with the urinary excretion of genistein ($r=0.50$; $p=0.05$), and was not correlated at all with daidzein.

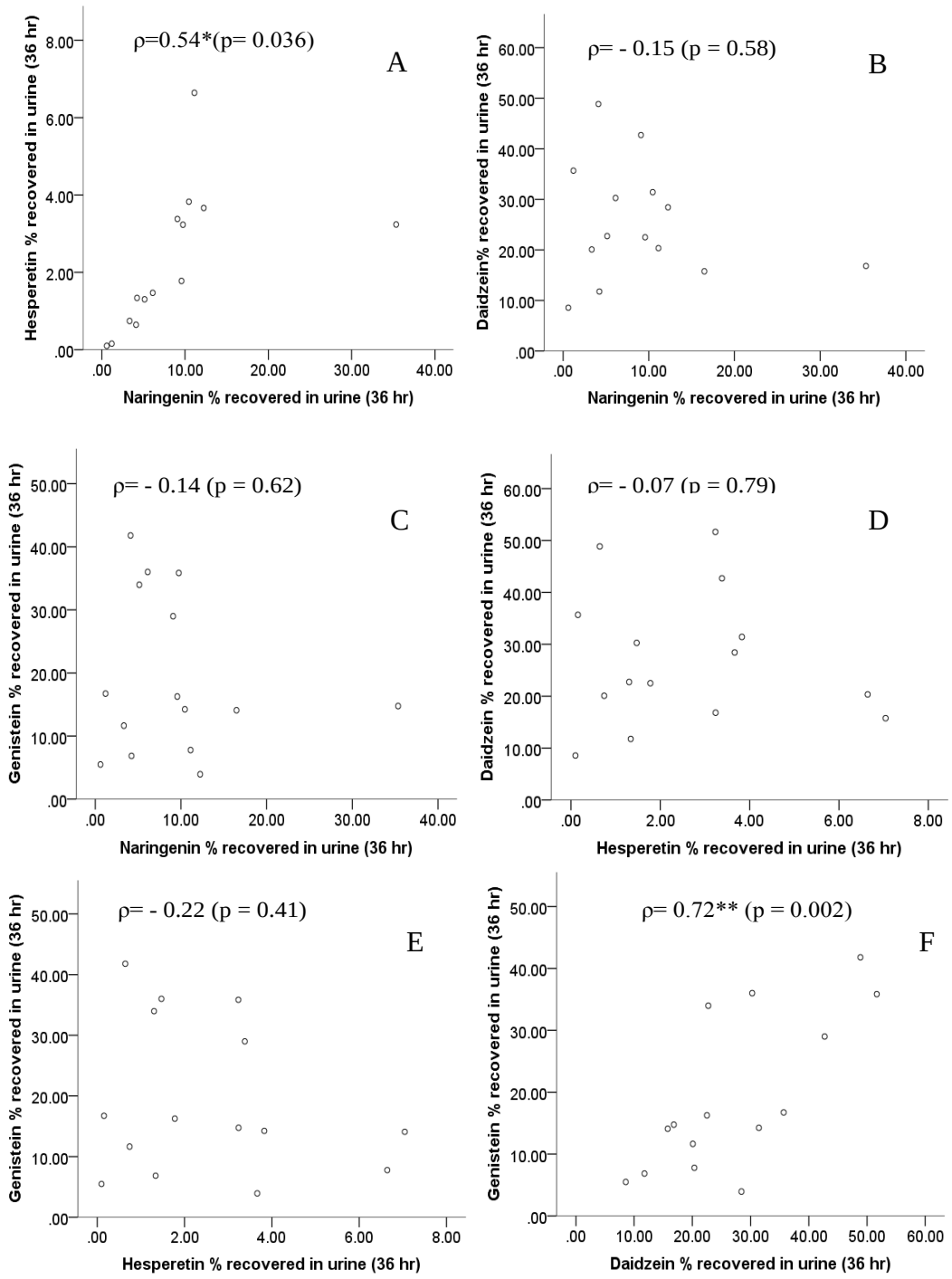


Figure 3.17: Correlation between (A) naringenin and hesperetin (B) naringenin and daidzein (C) naringenin and genistein (D) hesperetin and daidzein (E) hesperetin and genistein (F) daidzein and genistein on the percentage recovered in urine samples after experimental meals were separately ingested sing the Spearman correlation coefficient. Correlation is significant at the p =0.01 **, p = 0.05 *.

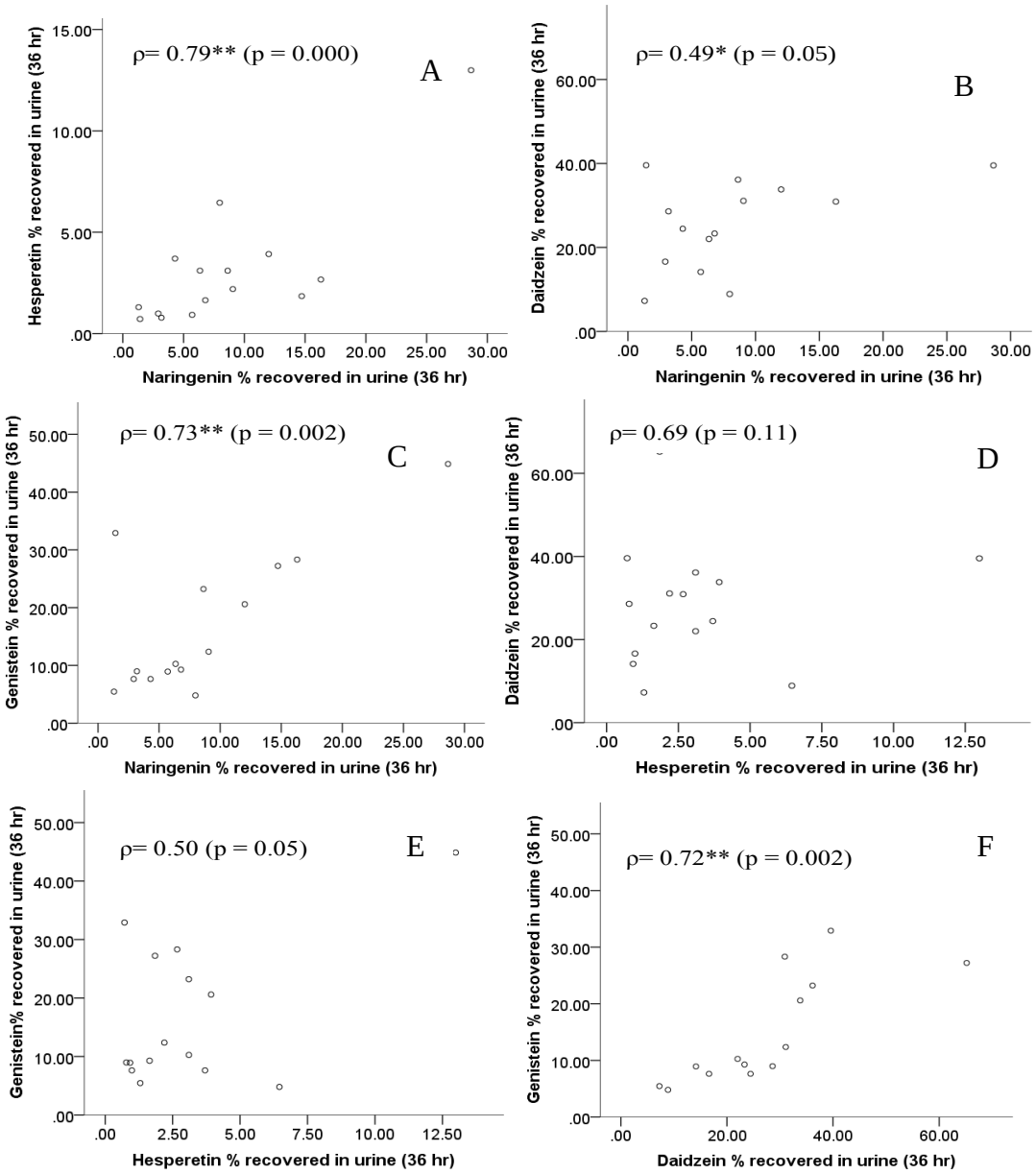


Figure 3.18: Correlation between (A) naringenin and hesperetin (B) naringenin and daidzein (C) naringenin and genistein (D) hesperetin and daidzein (E) hesperetin and genistein (F) daidzein and genistein on the percentage recovered in urine samples after experimental meals were co-ingested using the Spearman correlation coefficient. Correlation is significant at the p =0.01 **, p = 0.05 *.

3.8 Analysis of flavonoids in saliva

Saliva samples were collected form 15 volunteers during phase 3 of the study. The average total protein concentration in the saliva was 0.60 ± 0.03 mg/ml, which is similar to reported values (Lin and Chang, 1989). Three subjects who had excreted high levels of isoflavones and flavanones in their urine were chosen for further HPLC – DAD and LC-MS analysis. All saliva fractions between 0 – 36 hr were analysed by HPLC after enzyme hydrolysis for the presence of the hesperetin, naringenin, daidzein and genistein aglycones. Figure 3.19 shows the chromatograms for one set of the saliva samples. No flavonoid aglycones were detected in any of the samples analysed. In case the hydrolysis step resulted in lower concentrations of compounds in saliva, selected samples were also analysed prior to enzyme hydrolysis to monitor any potential peaks present. Figure 3.20 shows an 8 hr saliva sample with and without enzyme hydrolysis. Again, no peaks were detected in any of these samples.

As a result of the non-detectable values for all compounds in saliva by HPLC, more analysis using LC-MS was carried out. This analysis is more sensitive and selective for specific mass ratios expected, but even so the compounds were still not detected. Selected saliva samples were analysed, alongside urine samples from the same individual, and saliva samples spiked with 25% “active” urine (containing the flavanone metabolites) to confirm detection of conjugates was feasible under the extraction conditions used. Figure 3.21 shows the chromatograms from these analyses. Two hesperetin mono-glucuronides and one hesperetin sulfate peak were present in the urine sample diluted in water (25/75) and were also present in the saliva sample spiked with the urine sample. The key finding here is that there was no real difference between water and saliva spiked with the same amount of urine. Based on this observation, it is unlikely that saliva is irreversibly binding to the compounds and preventing detection in the samples collected.

Due to the non-detectable values of isoflavones and flavanones in the samples analysed, further analysis of all the saliva samples was not carried out. If there are hesperetin and isoflavone metabolites in saliva, they are not at levels suitable for use as a biomarker in future human studies.

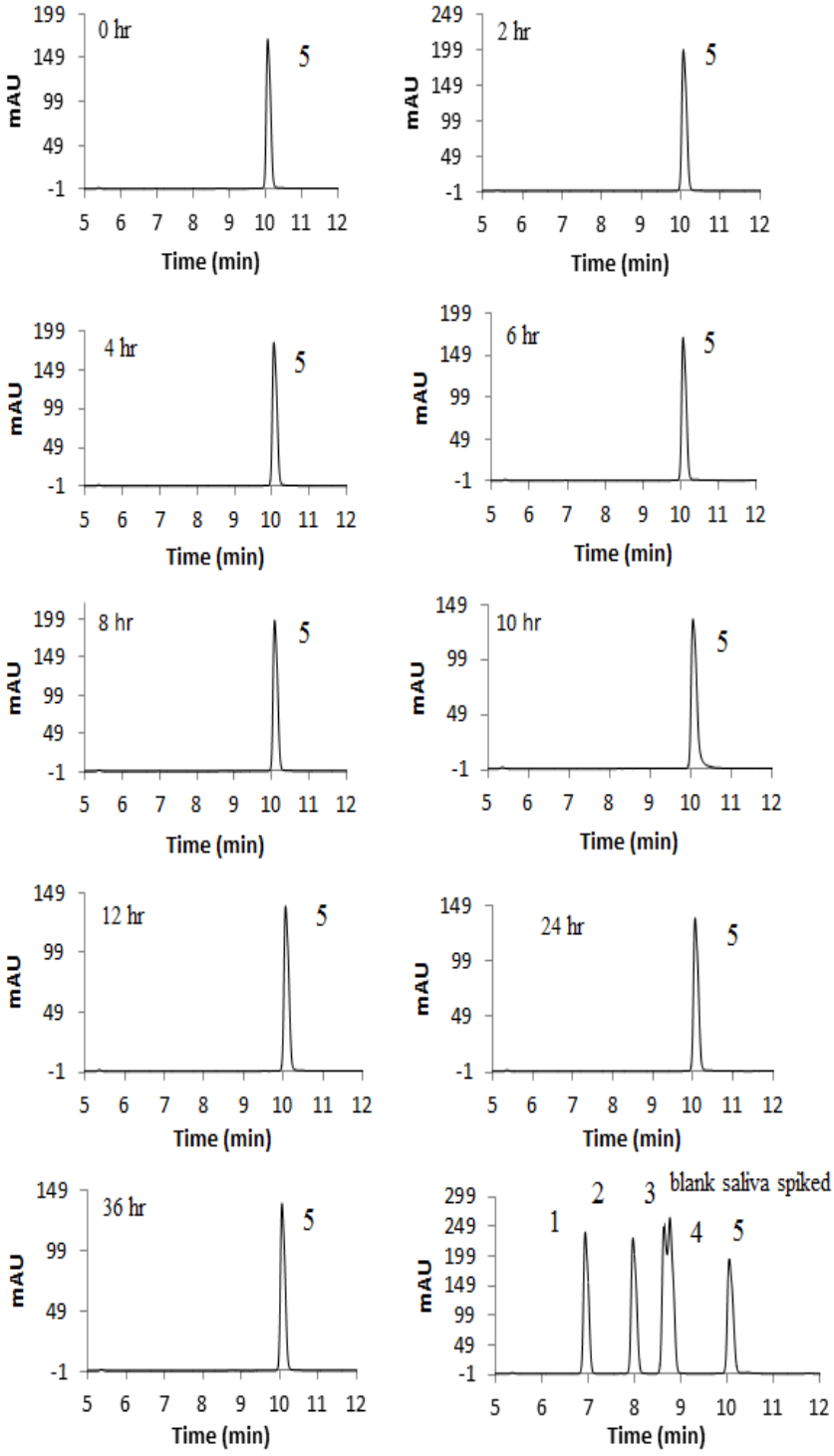


Figure 3.19: Example set of HPLC chromatograms representing analysis of active saliva (subject 4) 0-36 hr after the ingestion of 500 ml orange juice and 25 g soya nuts. Peaks identified as (1) daidzein, (2) naringenin, (3) genistein, (4) hesperetin, and (5) apigenin as internal standard. The peaks were monitored at 280 nm.

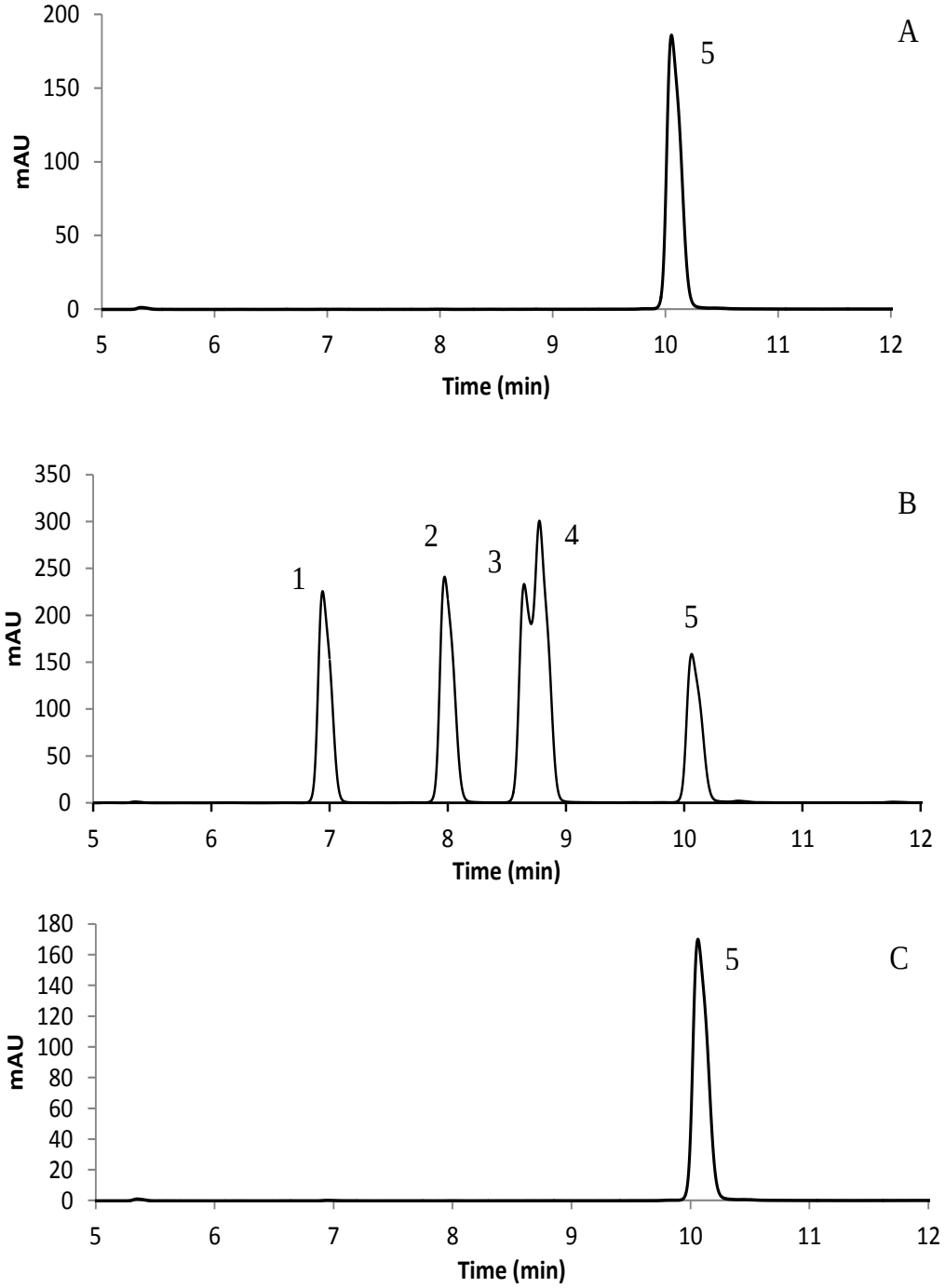


Figure 3.20: Example HPLC chromatogram representing analysis of saliva (subject 4) collected 8 hr after the ingestion of 25 g soya nuts and 500 ml orange juice. (A) Active (8 hr) saliva sample before enzyme hydrolysis (B) Active saliva spiked with aglycones (C) Active saliva sample after enzyme hydrolysis. The peaks are identified as (1) daidzein, (2) naringenin, (3) genistein, (4) hesperetin, and (5) apigenin as internal standard. The peaks were monitored at 280 nm.

3.9 Quantification of hesperetin from urine after enzyme hydrolysis.

To assess whether the enzyme hydrolysis was complete or not for the hesperetin metabolites, samples of urine were analyzed by LC-MS before and after enzyme hydrolysis. Different metabolites in urine were identified by their UV spectra (by diode array) and molecular mass (negatively charged precursor ions of interest $[M-H]^-$). The percentage of remaining un-hydrolyzed flavanone from the original amount was calculated.

Peak (1) in Figure 3.22 is the internal standard taxifolin (Rt = 13.0 min), detected at m/z 303. Peaks 2, 3, and 4 (Rt = 15.6, 15.8 and 16.8 min, respectively) were detected with an m/z of 477, 477 and 381, respectively, indicating that the presence of mono-glucuronide derivatives of hesperetin and the mono-sulfate. After hydrolysis (but not prior to enzyme action) another peak was observed, at Rt 20.3 min. This peak had an m/z at 301, and is assigned as hesperetin aglycone. Further confirmation of this was based on the Rt of the authentic standard of hesperetin and the UV spectrum. The percentage of hesperetin conjugate that was hydrolysed by β-glucuronidase and sulfatase enzymes was calculated and shown in table 3.7.

Table 3.8: Enzyme efficiency of beta-glucuronidase and sulfatase on hesperetin metabolites.

Metabolite	**RT (min)**	**Percent remaining after hydrolysis***	**Approximate percent hydrolysed**
Hesperetin-7-*O*-glucuronide	15.6	17.4 ± 1.4	82%
Hesperetin-3′-*O*-glucuroinde	15.8	12.3 ± 3.1	88%
Hesperetin-3′-*O*-sulfate	16.8	39.1 ± 7.8	61%

*Under conditions described in section 3.5.3.

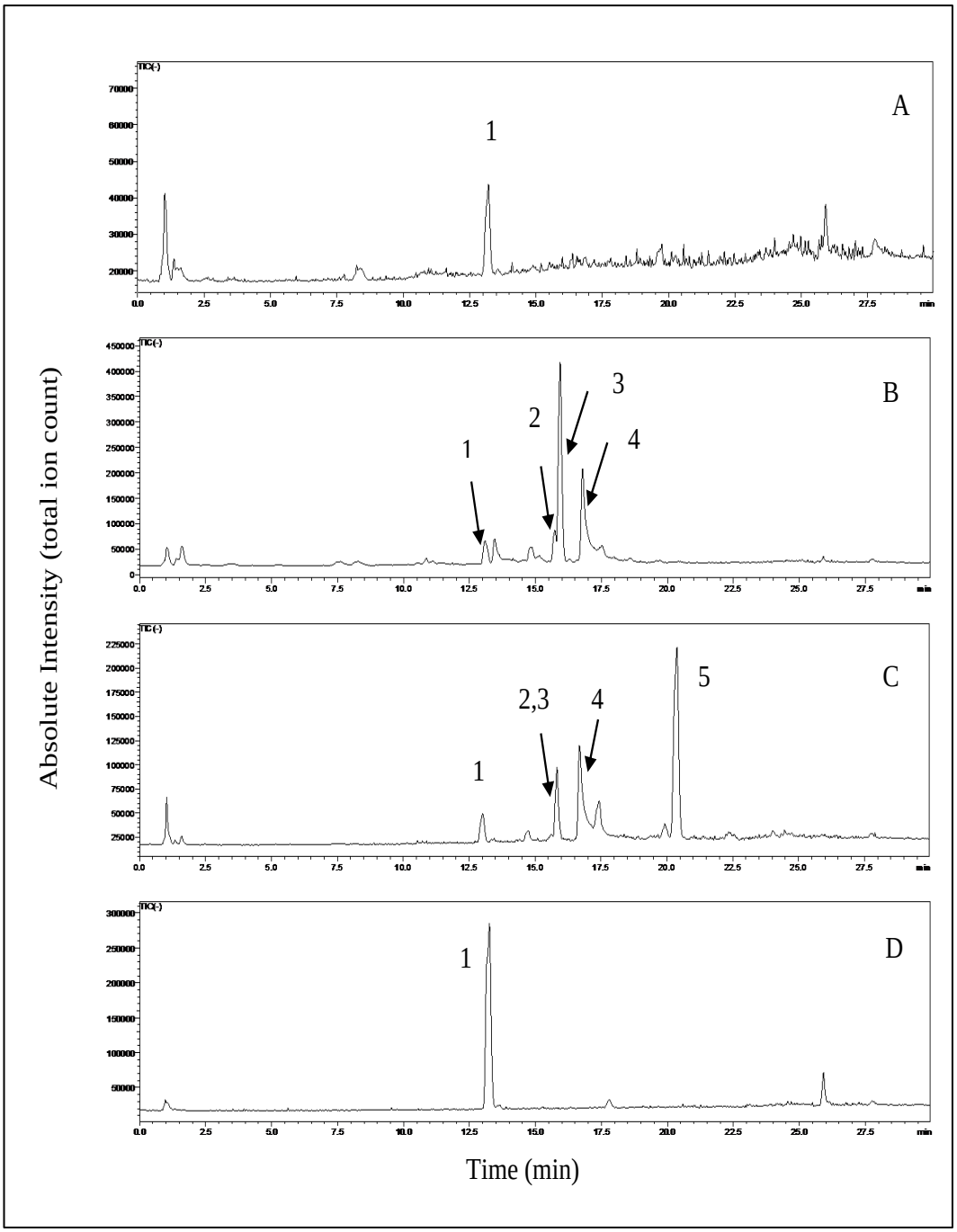


Figure 3.21: LC-MS Chromatogram of a saliva and a urine sample (from subject 4) 8 hr after the ingestion of 25 g soya nuts with 500 ml orange juice. (A) baseline (0 hr) urine sample, (B) active (8-12 hr) urine sample without enzyme hydrolysis (C) active (8-12 hr) urine sample after enzyme hydrolysis, (D) active (8 hr) saliva sample after hydrolysis. The peaks were identified as (1) taxifolin (internal standard) transition $(m/z)^-$ 303, (2) and (3) hesperetin–*O*-glucuronides form $(m/z)^-$ 477, (4) hesperetin-*O*-sulfate form $(m/z)^-$ 381, (5) hesperetin $(m/z)^-$ 301.

3.10 Discussion:

The purpose of this study was to investigate various aspects of bioavailability, as assessed by urinary excretion of flavanones and isoflavones in volunteers. Firstly, we wanted to assess if there was any influence of consuming the specific flavonoid-rich foods together or independently, and secondly to assess if there was any correlation in the amount excreted of each flavonoid. Lastly, saliva taken at various time points was analysed after both soya nuts and orange juice were consumed to see if this biological fluid would reflect flavonoid absorption. Overall the 36 hr urinary excretion of four dietary flavonoids (hesperetin, naringenin, genistein and daidzein) was investigated in 15 healthy volunteers.

Daidzein and genistein were absorbed quickly and appeared in urine within a few hours after ingestion of soya nuts. Moreover, daidzein was recovered to a larger extent in urine than genistein in all subjects; the average percent dose excreted was about 27-28% for daidzein compared to 16-19% for genistein. This finding is in accordance with previous studies (Watanabe *et al.,* 1998, King and Bursill, 1998, Richelle *et al.,* 2002, Faughnan *et al.,* 2004, Vedrine *et al.,* 2006), and the data for these is presented in Figure 3.23. The overall range reported by Manach *et al.,* (2005) is between 21.4 – 62% for daidzein and 6.8 – 29.7% for genistein. The lower urinary excretion of genistein compared to daidzein measured as % of dose intake is not due to lower systematic bioavailability and plasma levels (Setchell *et al.,* 2003a), but is more likely to reflect the more pronounced biliary elimination route for genistein. This has been shown as the major route for genistein-7-*O*-β-glucuronide (represents the major metabolite of genistein) in rats (Zheng *et al.,* 2004). The reason for this difference in pathways may be the lower molecular weight and the increased water solubility of daidzein compared to genistein which favours urinary excretion via the kidney (Tapiero *et al.,* 2002). Moreover, the 5-OH group in genistein is assumed to be more susceptible to microflora degradation leading to lower overall urinary recovery (Zheng *et al.,* 2004).

The variation in urinary recovery of genistein and daidzein is reasonable across different studies, likely due to different factors such as the design of the study, the form of isoflavones ingested (e.g. glycosides vs. aglycone), the food matrix and individual variation in microflora (Nielsen and Williamson, 2007). The

urinary recovery as % of dose after ingestion of tempeh (a fermented soya food high in aglycones) was higher than after soybean pieces (contain mainly glucosides) (Hutchins *et al.,* 1995). It has been reported that the absorption of isoflavones as cumulative uptake is higher in solid matrices, even though the absorption is faster from liquid matrix (Cassidy *et al.,* 2006, de Pascual-Teresa *et al.,* 2006). Processing soya foods such as steamed, fermented and roasted, has been reported to change the portion of the various isoflavones species and had the lowest quantities of malonyl-glycosides (Ahn-Jarvis *et al.,* 2013). Recent studies (e.g.) reported that under heat (100 ºC) moist and dry conditions, isoflavones (genistein and their glycoside forms) are converted from malonyl-genistein to genistein (Chien *et al.,* 2005). These changes have been also reported from malonyl-glycoside to acetyl-glycosides under dry and moist heating in soya flour and soya milk processing and this could explain the higher levels of aglycones in roasted soya foods products.

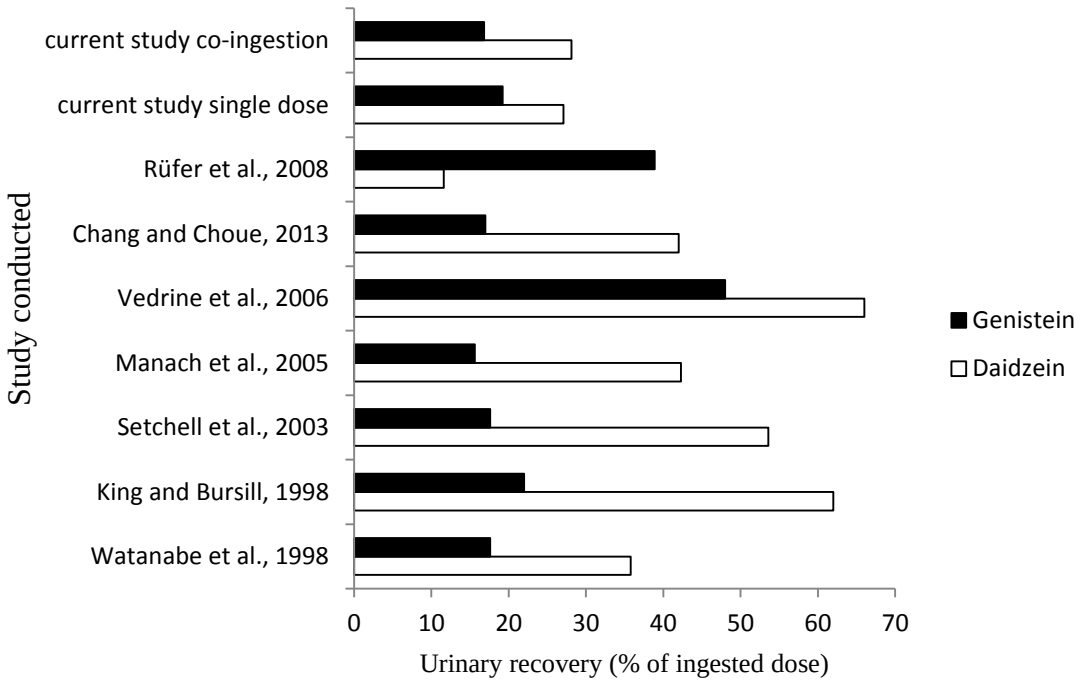


Figure 3.22: Data from various studies showing the urinary recovery of isoflavones in urine from original dose ingested from different type of supplements.

In the present study, the recovery of hesperetin in the urine was 2.5-3% based on the ingested dose of 500 ml orange juice; naringenin was recovered at a higher level of 9%. This finding is consistent with previous studies (Manach *et al.,*

2003, Kanaze *et al.*, 2006, Gardana *et al.*, 2007, Mullen *et al.*, 2008) as shown in figure 3.24. It has been suggested that this difference may be due to the structural differences where naringenin only has a 4′-OH group but hesperetin has a 3′-OH and 4′-methoxy group, which makes it less water soluble and also increases the molecular weight. Thus, like with genistein it may preferentially be excreted via the bile. The structural changes may also partly influence the absorption of the flavonoid (Manach *et al.*, 2003, Mullen *et al.*, 2008,), such as differences in colon microflora that mediated degradation of hesperetin and naringenin. However, there was no difference in the bioavailability of flavanones either after the consumption of orange juice or fruits (Brett *et al.*, 2009). In another study, the bioavailability of naringenin was reported to differ after ingestion of orange juice and grapefruit juice which suggest that this difference was determined by the type of juice (Erlund *et al.*, 2001). However, other factors that could also contribute include differences in the affinities of enzymes cleaving neohesperosides and rutinosides, or the dose-dependent renal clearance (Erlund *et al.*, 2001). Our result is within the range between (3 - 24.4%) for hesperidin and (1.1 – 30.2%) for naringenin that have been reported by Manach *et al.*, (2005).

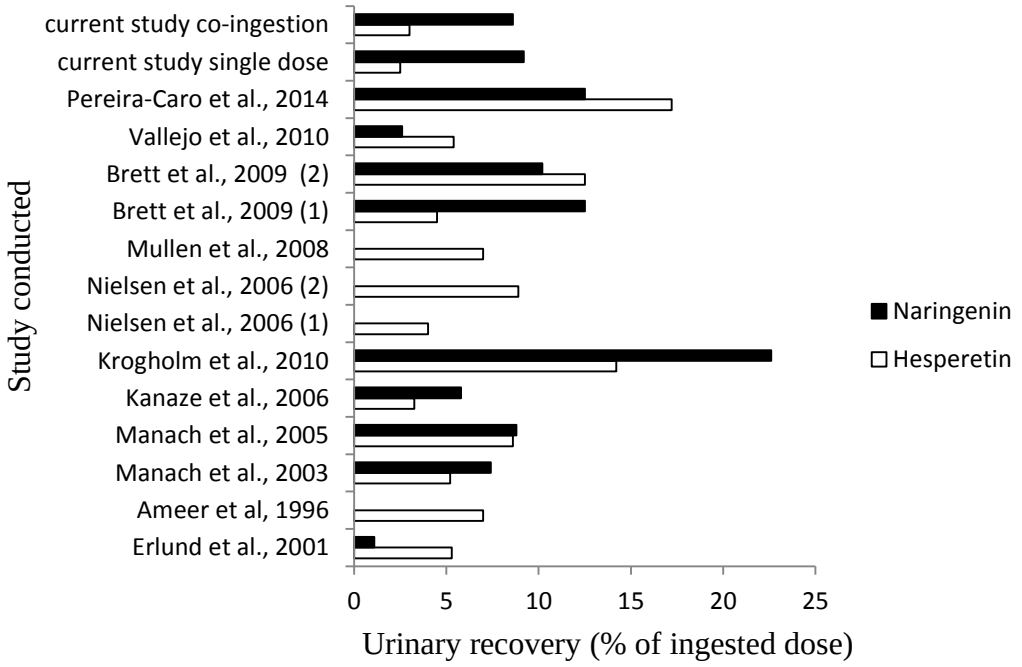


Figure 3.23: Data from various studies showing the urinary recovery of flavanones in urine from original dose ingested from different type of supplements.

Food matrix and solubility of flavanones was reported to affect the absorption. Recent studies by Tomas-Navarro and colleagues (2013) have investigated the effect of processing on the flavanone bioavailability based on the processing effect on flavanone solubility and particle size of the cloud. The result revealed that high-pressure homogenized (HPH) juice showed better absorption and excretion rate compared to conventional pasteurized juice among higher excretor volunteers. This high absorption among higher excretor volunteers could be explained by the gut microbiotas which were active in releasing hesperetin from hesperidin. As a result, they take the benefit of small particle size of hesperidin in HPH juice with more availability to interact with the gut microbiota than in pasteurized juice (Tomás-Navarro *et al.,* 2013).

In the present study, a gender related trend in urinary excretion of isoflavones and flavanones was observed. Specifically, the mean urinary excretion of isoflavones and flavanones were higher in males compared to females. Several studies had reported no differences between males and females in the recovery of isoflavones in urine and plasma sample (Faughnan *et al.,* 2004, Nielsen and Williamson, 2007). Similarly, the percentage of flavanones recovered in urine was not correlated with sex or BMI of the participants (Vallejo *et al.*, 2010). In contrast, in a study to determine if there were any change in urinary recovery of isoflavones after three different soya foods were ingested (soya milk, textured vegetable protein (TVP) or tempeh), suggests that the absorption and excretion of genistein was higher in females compared with males. The absorption was also is influenced by the food matrix and chemical composition. In addition, metabolism of daidzein suggested to be altered by the chemical composition of the isoflavones ingested. (Faughnan *et al.*, 2004). In addition, genetic variation could influence the degree of absorption, metabolism and excretion of flavonoids. Several studies showed that ethnic background (or cultural food background) may have an influence on the metabolism of glucoside forms of isoflavones (Lampe *et al.*, 1998, Zubik and Meydani, 2003).

In this study, large inter-individual variation was observed between volunteers in the urinary excretion of flavanones and isoflavones. The largest variability based on the recovery in urine after 36 hr was observed in daidzein which

ranged between 7% - 65% of dose ingested. High and low excretors of these flavonoids seems to have consistent excretion behaviour for different flavonoids only when co-ingested, and was more prominent with naringenin than with hesperetin, as shown by the spearman correlation coefficients. This is possibly due to the similarity in the intestinal bacteria composition which leads to changes in metabolic activity of the colonic microflora due to different levels of α-rhamanosidase and β-glucosidase activities. As a result of this there may be variation in the absorption of isoflavones and flavanones in the colon. This may also explain the delay to reach the urinary C_{max} at 8-12 hr in co-ingestion phase compared to 4-8 hr when these compounds were consumed separately.

In this study we hoped to investigate the concentration of flavonoids in saliva samples and to compare with urinary concentration to determine how reliable a saliva sample is as a biomarker for citrus and isoflavones consumption. No isoflavones or flavanones were detectable in the saliva. We expected the flavonoids might be available in saliva in conjugated form, therefore the hesperetin conjugate compound were tested using LC-MS, but this form was undetectable in saliva samples as well. These results are in contrast to the result obtained by Cao *et al.,* (2009), who investigated the level of isoflavones in infants (birth to 1 year). Isoflavones were not detected after cow or breast milk, but recordable levels were determined in saliva samples after soya formula was given, although levels were higher in urine. It could be that the continuous intake of soya milk formula led to higher and constant levels of isoflavones in the circulation and therefore a detectable level in the saliva. Our study was a low dose given on a single occasion, and so if isoflavones are able to reach the saliva the levels were too low to be detected.

Another study by Halm *et al.*, (2007) reported that isoflavones are excreted in urine at higher level among children than adults, with total excretion rate of +32% higher (Halm *et al.,* 2007). The higher urinary excretion level of flavonoids among children compared to adults also could be explained by the difference in microflora populated in the colon, which could be able to hydrolyse flavonoids more effectively to their aglycone form in children, however degrade the aglycones less efficiently (Halm *et al.,* 2007). Released aglycones normally undergo ring fission leading to the

production of phenolic acids and hydroxycinnamates (Crozier *et al.,* 2010, Del Rio *et al.,* 2010).

It should be noted that the salivary enzymes are capable of hydrolysing isoflavones and flavanones by the esterase and glucosidase activity from bacteria in the saliva. However, the activity may not be significant in physiological conditions. In practice, food will not normally stay in the mouth for more than 20-30 second. During the short stay of flavonoid glycoside in the mouth, the enzymes may continue to work through digestion, along the GI tract where PH changes greatly from 2-8. However, these enzymes in saliva were from a bacterial source as proposed by Walle *et al.,* (2005), and the activity of these enzymes were significantly decreased when the mouth was rinsed by anti-bacterial solution before the collection of saliva. Furthermore, as a result of high concentration of nitric oxide produced by the stomach, most of the bacteria would be killed after reaching the stomach (Gladwin *et al.,* 2005). For that reason, the activity of salivary enzymes will be limited, if their sources are from bacteria.

From our results, it can be concluded that saliva as a non-invasive biological fluid is not a good tool to monitor flavonoid intake and not reliable biomarker for citrus and isoflavones consumption.

Our result showed that the aglycone hesperetin yielded after enzyme hydrolysis of hesperetin conjugates from urine was incomplete. This is in agreement with Bredsdorff *et al.,* (2010). Thus, to have a detailed description of isoflavones and flavanones absorption and metabolism, the analytical method employed must be highly sensitive and be able to identify the forms of metabolites in the biological fluids without any enzyme treatment of conjugates. It is recommend that further analysis using more sophisticated methods such as the LC-MS can reveal more accurately the concentration of these compounds.

The current study was limited to only assessing the bioavailability of hesperetin and naringenin after consumption of orange juice and daidzein and genistein after consumption of soya nuts either separately or together. There is a need to investigate other foods which are commonly consumed together by the population for a better understanding of the flavonoid intake and bioavailability of these compounds, and if

interactions during absorption or metabolism by gut microflora occur. Ultimately a better understanding of inter-individual variability in bioavailability will be beneficial in assessing the ability of flavonoids in reducing the risk of chronic diseases.

Chapter 4: Quantification Of Flavonoid Contents In Orange Juice And Citrus Supplements

4.1 Abstract

The aim of this chapter was to assess the flavanone variability in commercially available orange juice, and orange drinks (being considered as a placebo-control), and citrus supplements, to determine the best options for carrying out a double-blind, placebo-controlled, cross-over intervention human study (chapter 6).

In this study the flavanone content of six selected brands of orange juice and orange juice drinks (< 3% from concentrate), and two citrus bioflavonoid tablets available in the UK market were extracted and analysed by HPLC. Hesperidin and narirutin were the major flavonoids detected. The orange drinks contained 2-5 mg/100 ml hesperidin and 0.1-0.3 mg/100 ml narirutin, which were considerably lower than the orange juices which contained 39-49 mg/100 ml hesperidin and 3-5 mg/100 ml narirutin. The supplements were analysed and were contained 55 mg/tablet hesperidin for Solgar citrus bioflavonoids, and 22 mg/tablet for the Health Aid: Citrus Bioflavonoid. No flavonoids were detected in a placebo tablet.

In conclusion, Solgar bioflavonoid supplements was analysed and had sufficient hesperidin levels of 55 mg/tablet, with 2 tablets equivalent to 200 ml orange juice, and so were selected as the experimental source to be used in study 2 (chapter 6). These could be used more appropriately in a blinded intervention with a placebo control.

4.2 Introduction

There is an increasing support to the view that many phenolic secondary metabolites (polyphenols) found in plant-derived foods have some beneficial effects in the prevention of degenerative diseases (Del Rio *et al.,* 2010, Khan and Dangles, 2014). Besides, citrus fruit and juices are vastly consumed around the world. This consumption has considerably increased during the decade. The global production of

citrus fruit was estimated to be 82 million tons in 2009–2010. Of this total, orange - the most important citrus fruit - accounts for about 50 million tons (Khan and Dangles, 2014).

Flavanones are a subgroup of flavonoids which are biologically active and have antioxidant activity (Brett *et al.,* 2009). In general, most flavonoids are commonly present in plants in a conjugated form along with sugars, forming glycosides. Hesperidin is a predominant flavanone presented in high levels in citrus fruits, especially in oranges (*Citrus sinensis*) as hesperetin-7-*O*-rutinoside (Nielsen *et al.*, 2006, Brett *et al.,* 2009). The intake of hesperetin in the diet can be substantial from citrus fruits and juices. Flavanones are found almost exclusively in citrus fruits. The concentration of flavanones in the juice of these fruits can reach several hundred mg on a per-litre basis (Tomás-Barberán and Clifford., 2000). An orange juice is reported to contain between 200 and 600 mg hesperidin/L and 15–85 mg narirutin/L. Additionally, a single glass of orange juice is expected to contain between 40 and 140 mg flavanone glycosides (Manach *et al.,* 2004). Mouly *et al.,* (1998) reported that orange juice from concentrate contains 475 mg/L hesperidin. The same study also reported that pure orange juice contains on average a lower concentration of hesperidin, compared to orange juice from concentrate. Gil-Izquierdo *et al.,* (2002) reported that the concentration of hesperidin may also depend on the quality of the oranges used for preparing the juice.

There are numerous factors that may influence the polyphenol content in orange juices. For instance, it has been reported in some studies that citrus flavanones exist in both juice serum and precipitated in the juice cloud (Silveira *et al.,* 2014). Another factor is that orange juice processing methods are also an important factor affecting the total concentration and solubility of the citrus flavanones. The processing method may likely influence the levels of bioavailable compounds following juice consumption. It has been reported by a number of studies (Gil-Izquierdo *et al.,* 2002, Bai *et al.,* 2013) that total concentrations of hesperidin and narirutin and their content in the juice cloud are higher in commercially processed orange juice (POJ) compared to fresh squeezed orange juice (FOJ). Processing such as pasteurization and clarification that is used for commersial juices, can have an impact on flavonoids content for example the

flavonoid content of fresh fruit juices is much higher than the commercial one and the possible reason for that might be because of their precipitation or binding to the proteins that get degraded in the process of pasteurisation (Mahdavi *et al.*, 2010).

It has also been documented that citrus fruits flavanones are present glycosides such as hesperetin-7-*O*-rutinoside (hesperidin) and naringenin-7-*O*-rutinoside (narirutin) which contain both glucose and rhamnose sugars (Kawaii *et al.*, 1999). The sugar moiety of flavonoids are proposed to be the major determinant of their absorption in human (Nielsen *et al.*, 2006). The presence of sugar is very important, because it can influence the bioavailability. It is of major interest in the current investigation whether these compounds (like hesperidin) are bioavailable in the body. Many studies have been trying to identify flavonoid bioavailability during the last two decades (Erlund *et al.*, 2002, Brett *et al.*, 2009, Bredsdorff *et al.*, 2010). Once a food is consumed, it starts to undergo the digestion process in the body. This is followed by absorption, a process in which the digested compounds enter the inner organs in order to become either modified or further metabolized or excreted. The site of absorption depends on the glycosylated or esterified form of the compound. It also depends on which specific sugars or other prosthetic groups are attached to on it (Scalbert and Williamson, 2000).

The increasing consumption of citrus around the world motivate researchers to investigate the most abundant citrus flavonoids, i.e. flavanones (Khan and Dangles, 2014). Many of these studies investigating the in vivo bioactivity of flavanones have used orange juice as intervention meal (Manach *et al.*, 2003, Bredsdorff *et al.*, 2010, Morand *et al.*, 2011, Buscemi *et al.*, 2012). Therefore, In order to assess any potential benefits, it is important to be aware of the composition of the biologically active compounds in commercially available orange juice and citrus tablets. In chapters 6 a human double-blind, placebo-controlled, cross-over intervention study on citrus flavanones and vascular health parameters has been designed and described. The aim of this study is to determine the concentration of flavanone in a commercially available orange juice and citrus supplements available in the UK market using HPLC technique.

The aims of this study are:

- To determine the total content of flavanones in a selected orange juice and orange drinks for possible use in a human study.
- To measure the concentration of hesperidin in citrus supplements for possible use in a human study.
- To decide on the best available source of hesperidin to be adopted the planned human study.

4.3 Materials

For details of the chemicals and solvents used in this analysis see chapter 2.2 for more information. All six orange juices were purchased from Morrison's, Leeds, UK and Sainsbury's, Leeds, UK between January 2011 and August 2011. Citrus supplements were purchased from online shops (Solgar vitamin and Herb, UK) between March 2013 and October 2013. Placebo tablets were purchased from (Fagron GmbH & Co. KG, Germany).

4.3.1 Orange juice

Six commercial brands of orange juice and orange juice drinks were analysed to determine their flavanone content. All orange juices are orange juice from concentrate except orange juice (# 3 and 4) which are diluted orange juice with added sweeteners, and orange juice (#1) which is orange juice not from concentrate, details in Table 4.1. For each brand, 2 bottles from 2 different batches were purchased. Samples were prepared as described in chapter 2 (see section 2.4.1) and analysed using HPLC with diode array.

4.3.2 Citrus and placebo supplements

Two citrus supplements were analysed. The label information of these supplements is provided in Table 4.2. A further placebo supplement was also assessed for flavonoid content. For sample preparation, the tablet was crushed using a mortar and pestle. The powder (200 mg) was extracted with 5 ml of 80% methanol for three times. The combined sample was centrifuged for 15 min, and the supernatant dried in the centrifugal evaporator overnight. All samples were extracted

in triplicate. The dried samples were reconstituted with 5 ml of 80% methanol, and an aliquot (250 µl) has spiked with standards of hesperetin and naringenin (20 µg/ml). The other two samples were spiked only with the internal standard apigenin (20 µg/ml). The samples were then analysed by HPLC using the method described in chapter 2 (section 2.3.31).

Table 4.1: Analysed commercial orange juice and drinks

Product code	Manufacturer and product name	Product labelled contents
1	Tropicana (purchased from Morrisons)	Orange juice not from concentrate (100%)
2	Princes (purchased from Morrisons)	Orange juice from concentrate (100%)
3	Sainsbury's orange juice drink	Water, orange juice from concentrate (3%), citric acid, sodium citrate, flavourings, sucralose, beta-carotene.
4	Disney "Cars" orange juice drink (purchased from Morrisons)	Natural mineral water, orange juice from concentrate (2%), citric acid, natural flavouring, pectin, sucralose, carotenes
5	Morrisons' orange juice from concentrate	Orange juice from concentrate (100%)
6	Sainsbury's smooth orange juice from concentrate	Orange juice from concentrate (100%)

Table 4.2: Information of the supplements

Supplement	A	B	C
Brand: name	Health Aid: Citrus Bioflavonoids	Solgar: Citrus Bioflavonoids Complex	Fagron: Placebo Tablet,Oval
Weight per tablet/capsule	1000 mg	1000 mg	770 mg
Eating suggestion	1 tablet/day with water	1 tablet/day with food	n/a
Flavonoid claim per tablet	Citrus Bioflavonoids (amounts not stated)	Flavanones: 110 mg, Hesperidin: 70 mg, Eriocitrin: 40 mg	none
Other ingredients	Bulking agent (di-calcium phosphate), acacia gum, anti- caking agent,stearic acid, magnesium stearate	Microcrystalline cellulose gum, vegetable stearic acid, hydroxypropylmethyl cellulose, vegetable glycerin	Lactose monohydrate, magnesium stearate, microcrystalline cellulose

4.3.3 Statistical analysis

Samples were prepared in duplicate for the validation analysis. All results from the HPLC analysis are presented as mean ± standard deviation and results are expressed as mg/ 100 ml or mg / tablets. Individual compounds were confirmed by comparison with literature values of their relative retention times, order of elution, and the UV spectra (compared to pure hesperidin and narirutin standards). Orange juice sample means were compared using the Tukey test at the 5% probability level. Pearson correlation was also used to calculate the total flavonoid content (mg) and unit price (UK sterling). All of the statistical analyses were conducted using SPSS for Windows, software version 22.

4.4 Results

4.4.1 Orange juice analysis

The flavanones are found mainly in the form of rutinoside conjugates in orange juice and citrus products. A small and large peak eluted after orange juice was analysed by HPLC (monitored at 280 nm) at retention times 14.9 and 16.1 min. This has a UV absorption spectra and retention times consistent with narirutin and hesperidin, respectively. Analysis of all various orange juices indicated the presence of these flavanones; typical chromatograms of flavanone content in six commercially orange juice form concentrate and orange drink drinks, using HPLC-DAD detector monitored at 280 nm are shown in Figure 4.1.

Hesperidin was the highest percentage found in the analysis. It is followed by narirutin, the concentrations of which are shown in table 4.3. The samples were analysed in duplicate and all four pure orange juices made from concentrate had a similar total concentration. The concentration of hesperidin and narirutin ranged from 39.1 ± 0.2 to 48.9 ± 0.6 mg/100 ml, and 3.5 ± 0.1 to 5.1 ± 0.1, respectively. The three orange juices made from concentrates had the highest values of hesperidin and narirutin compared to the Tropicana orange juice and orange juice drink. On the other hand, the lowest amount of hesperidin and narirutin in orange juice drinks ranged between 2.0 ± 0.1 to 5.3 ± 0.1 mg/100 ml. This result was not surprising, because these orange drinks were diluted orange juice from concentrate containing only 2-3% juice. Brands with higher hesperidin content were found to have higher concentration of narirutin level.

Flavanone hesperidin and narirutin contents were significantly ($P \leq 0.05$) higher in orange juice from concentrate 48.8 ± 5.02 mg/ 100 ml compared to orange juice drink 3.8 ± 1.8 mg/ 100 ml. The concentrations of flavanones were higher in orange juice from concentrate compared to orange juice drink as can be seen from Table 4.3. The concentration of flavanones in Morrisons' orange juice from concentrate were higher by 24 and 12-fold compared to the orange juice drink number 3 and 4, respectively.

However, no significant difference was found in the content of hesperidin and narirutin among orange juice 2 and 5 ($P = 0.28$). Moreover, there was low

variation of hesperidin content in this study. CV was 3.9% compared to CV 8.3% for narirutin. The concentration of hesperidin content in orange juice from concentrate was higher by 12-fold compared to the level found in the orange juice drink.

Table 4.3: Flavanones content in six commercial available brands of orange juices and drinks

Product code	**Manufacture; brand**	**Hesperidin mg /100 ml**	**Narirutin mg /100 ml**	**Total flavanone mg /100 ml**
1	Tropicana (Morrisons)	39.2 ± 0.2^{a}	3.57 ± 0.1^{a}	42.7 ± 0.3
2	Princes (Morrisons)	46.9 ± 2.2^{ab}	4.65 ± 0.1^{abc}	51.7 ± 2.1
3	Sainsbury's orange juice drink	5.3 ± 0.1^{b}	0.26 ± 0.4^{b}	5.5 ± 0.3
4	Disney "Cars" orange juice drink (Morrisons)	2.0 ± 0.1^{c}	0.14 ± 0.1^{ab}	2.1 ± 0.2
5	Morrisons' orange juice from concentrate	48.9 ± 0.7^{ab}	5.06 ± 0.1^{ab}	53.9 ± 0.4
6	Sainsbury's smooth orange juice from concentrate	42.7 ± 1.8^{d}	4.35 ± 0.6^{abc}	47.1 ± 1.6

Data are mean ± SD (n=4). Means with in the column without the same letter (a, b and c) are significantly different ($P \leq 0{:}05$; Tukey test).

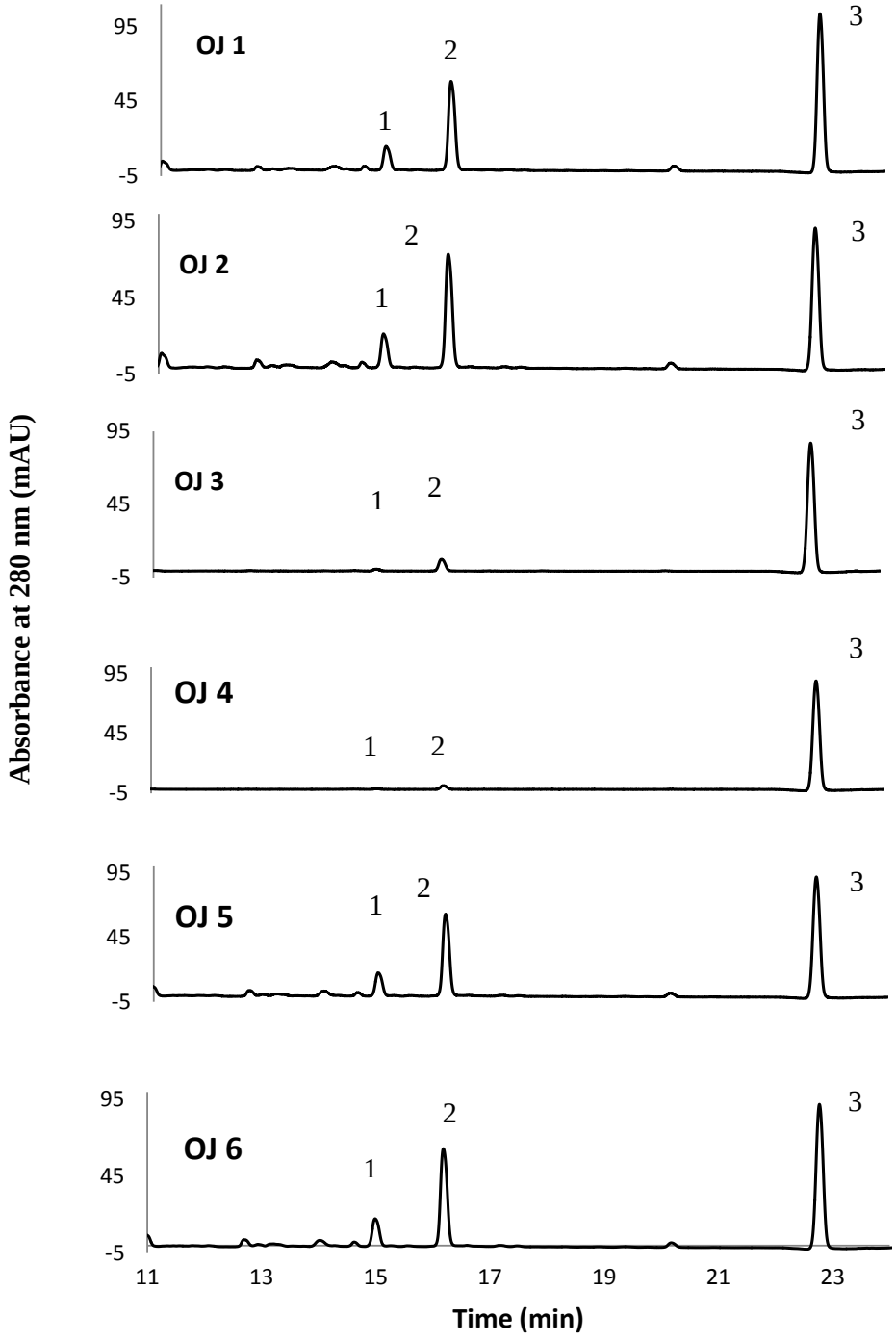


Figure 4.1: Chromatograms of flavanone content in six commercially orange juice and drinks. Narirutin peak (1), hesperidin peak (2) and apigenin as IS peak (3), using HPLC-DAD detector monitored at 280 nm. For details of HPLC conditions see chapter 2 (section 2.3.3).

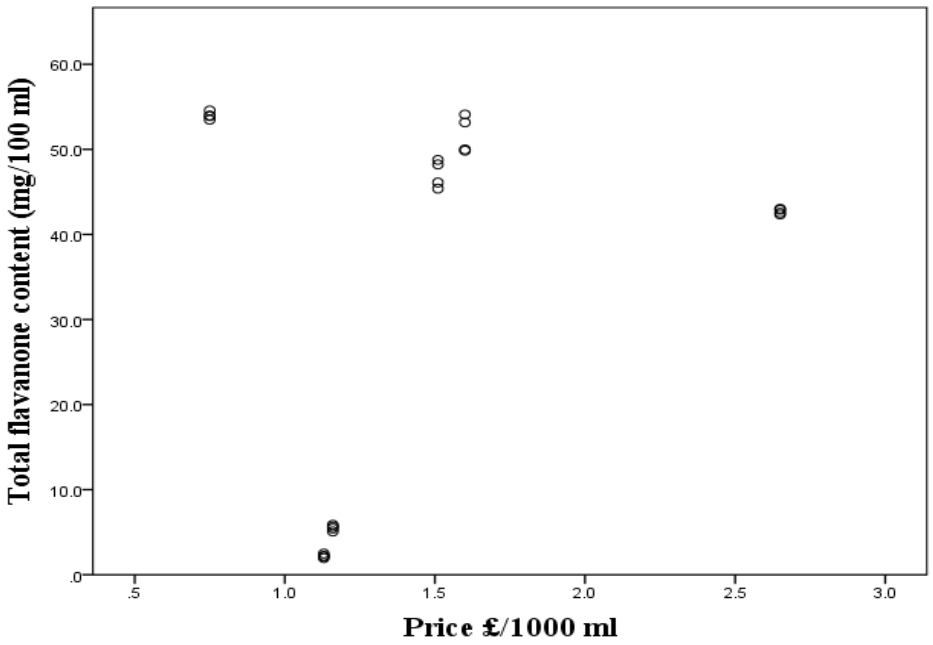


Figure 4.2: Pearson's correlation between price and total flavanone content for 6 commercial orange juice brand.

In this study, price did not correlate well with the total flavanones content in orange juice. Orange juices were shown to have weak correlation (r= 0.24; *P* = 0.26) between price and total flavanones content figure 4.2.

4.4.2 Supplement analysis

Analysis of compounds in supplements using HPLC was performed using the methods described in chapter 2 (section 2.3.3.1). The mean weight of the supplements was 1.48 ± 0.1 g for citrus bioflavonoids and 0.78 ± 0.1 g for placebo supplements. Table 4.5 shows the flavonoid content in the selected supplements. A comparison was made between the result achieved after the analysis and the amount of flavonoid claimed by the manufacturer. Supplement B has the closest amount of hesperidin as compared to the claim (55 mg/tablet). On the other hand, supplement A had a significantly lower hesperidin content (21.8 mg/ tablets) compared to supplement B (55 mg/ tablets), although no claim was indicated on the label. Citrus

supplement contain ≈ 78% of the claimed by manufacture (70 mg hesperidin/tablet) compared to the amount of hesperidin found in the tablet (55 mg/tablet). For the placebo supplement no flavanone compounds were found in the tablets, as expected.

Table 4.4: Flavanones content in the selected supplements.

	Hesperidin mg /tablet	**Narirutin mg /tablet**	**Total flavanone mg /tablet**
Health Aid: Citrus Bioflavonoids	21.8 ± 2.1^{a}	0.7 ± 0.55^{a}	22.5
Solgar: Citrus Bioflavonoids Complex	55.4 ± 5.4^{b}	1.8 ± 0.34^{b}	57.2
Fagron: Placebo Tablet,Oval	0.0 ± 0.0	0.0 ± 0.0	0.0

Average +/- S.D (n=4). Means with in the column without the same letter (a, b and c) are significantly different ($P \leq 0:05$; Tukey test).

4.5 Discussion:

The aim of this chapter is to investigate a potentially good dietary source of flavanones to be used in the human intervention study. A considerable amount of literature has been published on quantification of flavanones in orange juice. The results were firstly compared with the amount found in the Phenol-explorer database for orange juice from concentrate (www.phenolexplorer.eu) (Rothwell *et al.*, 2013). From this database the concentration of hesperidin ranged from 47.5 – 58.5 mg/100 ml (mean of 52.68 mg/100 ml, n=220) and narirutin ranged between 5.22 – 7.08 mg/100 ml (mean of 6.32 mg/100 ml, n=160). The amount of hesperidin and narirutin found by Manach *et al.*, (2003) was 44.4 and 9.6 mg/100 ml, respectively. A study in Turkey found lower amounts of hesperidin (17.1 mg /100 ml) and

narirutin (3.99 mg/ 100 ml) (Kelebek *et al.,* 2009). Mouly *et al,* (1998) reported that orange juice from concentrate contained 47.5 mg/100 ml hesperidin. Our results are in agreement with the results previously reported values by *Vanamala et al.,* (2006) where the total hesperidin and narirutin content in 13 made-from-concentrate orange juices were analysed and the concentration ranged from 32.9 ± 1.6 to 54.8 ± 3.4 mg/ 100 ml and 4.4 to 8.0 mg/100 ml, respectively. Vanamala *et al.,* (2006) also reported that the concentration of flavanone was significantly higher in orang juice made from concentrate than not-from-concentrate orange juice (Vanamala *et al.,* 2006).

The concentration of hesperidin and narirutin in the value-brand juice in this current study was higher compared to the Tropicana orange juice, which is considered a premium brand, and was used in study 1. This result supports the finding of research carried out by Mouly *et al.,* (1998), who reported that pure orange juice contained on average a lower concentration of hesperidin, compared to orange juice made from concentrate. The concentration of hesperidin may also depend on the quality of the oranges used for the juice. In this respect, the premium-brand juice had a comparatively high concentration of hesperidin (Gil-Izquierdo *et al.,* 2002), which is in disagreement with our finding. According to the label, orange juice drinks contain only 2-3% juice from concentrate. On the other hand, in juice made from concentrate the concentration of flavanones was 12 and 24-folds compared with orange juice drink analysed in this study.

Mahdavi *et al.* (2010) have shown that the amount of polyphenol content found in fresh fruit juices is significantly higher than that found in commercial ones. They also stated that, because of their precipitation, the availability of the phenolic compounds is reduced. It was also shown that processes like pasteurization and clarification which are adopted in manufacturing commercial juices, may influence the polyphenol content (Mahdavi *et al.,* 2010). However, in the juices used in the current study, the biggest hesperidin concentration was found in the value-brand orange juice, followed by the premium-brand and then by the orange juice drink. A possible reason for the lower apparent dose in different orange juices may be the variety of the oranges used in making the juice. It could also be due to the fact that hesperidin is binding to proteins that become degraded in pasteurisation (Mahdavi *et*

al., 2010). Hesperidin and narirutin are the major flavonoids in the orange juice. They occur as soluble in solution and precipitated in the juice cloud. A number of studies have reported that these distributions are influenced by commercial juice processing and storage techniques (Gil-Izquierdo *et al.*, 2002, Gil-Izquierdo *et al.*, 2003, Bai *et al.*, 2013). However, it is worth mentioning that the differences found in the flavanone content between different brands in this study may be explained by the variation in season and climate. Location may also influence the flavonoid content in orange juice. For example, naringin content of grapefruit juice from the same grove and trees fluctuated during the season and varied considerably between crop years (Albach *et al.*, 1981). In addition, fruit harvested in earlier in the season reported to had better quality in terms of higher juice content, ascorbic acid, and flavonoids compared with that fruit harvested later in the season (Bai *et al.*, 2009).

The aim of this study was to quantify the concentration flavanones in various orange juices and citrus supplements in order to better inform the choice for conducting a human intervention study. Only two citrus supplements were analysed in this study. The majority of citrus containing supplements available contain other added ingredients (e.g. vitamin C), which, if not controlled for, could affect the results in a human study. There was a large difference in the amount of flavanone between these supplements with "Solgar: Citrus Bioflavonoids Complex" containing the highest level. The placebo did not contain any flavonoid. In planning the second human intervention study (see chapters 6 and 7), we chose to use supplements instead of orange juice. Our choice was based on the following:

(i) Tablets are easy to distribute, and do not take up large areas of storage over the 4 week intervention (which would be required for 14 L of orange juice).

(ii) Tablets make the blinding of the study easier. Although the juice drink contained considerably less flavanone than the juice, the drink contained added ingredients and the taste was very different.

(iii) The juice-drink still contained flavanones which could have affected the study outcome for the control arm, but the placebo did not contain any flavonoids.

(iv) The tablets don't contain additional vitamin C, and more importantly, or sugar found in natural orange juice (40 g per day if 500 ml was used), which could influence both the CVD risk factors, and the energy consumed over the 4 weeks.

(v) The tablets contain approximately the same amount of flavanones that can be found in a 100 ml of orange juice.

(vi) It is thought that it is the flavanone component of orange juice that has the impact on some of the CVD risk factors which are the main focus of the intervention study.

(vii) To our knowledge, commercially-available health citrus supplements have not been used in previous studies. Therefore, it makes our study novel whilst adding to the evidence for citrus flavanone potential bioactivity.

In conclusion, "Solgar Citrus Bioflavonoids Complex" supplements were chosen to be used in a double-blinded cross-over intervention study, with placebo tablets acting as the control arm. Two flavanone tablets, approximately 200-250 ml of orange juice, were given per day to be consumed with breakfast. All tablets could be allocated in advance in coded sealed containers, blinded to the researcher, and given to the volunteer on the first day of the study. See chapter 6 for more information about the study design.

Chapter 5 Pilot Study Of Blood Pressure And Blood Glucose Measurements

5.1 Abstract

This study described in chapter 5 assessed the reproducibility of blood pressure measurements in healthy volunteers. Intra- and inter-operator reliability and repeatability were calculated at two different times, on three different days, in a sample of 7 subjects by the same researcher. Systolic and diastolic blood pressure (SBP, DBP) were measured in the morning vs afternoon. Data on visit-to-visit and within–visit BP variability were collected using a Panasonic (EW3152W) Blood Pressure Monitor and a Lloyds Blood Pressure Monitor (model LBPK1). Repeated measures analysis of variance (ANOVA) was used to assess the significance of the inter- and intra-operator variability. Reproducibility was assessed using the intra-class correlation coefficient (ICC) and a repeated measures analysis of variance.

The day-to-day variability was not significant and the operator showed excellent day-to-day reproducibility. The blood pressure measurements intra-session coefficient of variation at 2-hour intervals ranged between or between morning and afternoon session were (2.80 – 8.75%). The variability of the measurements measured on the same day (between morning sessions and afternoon session) was between 2.9 % and 11.8%. Reliability of blood pressure measurements made by the same operator was good to excellent (ICC = 0.6–0.90) for same day assessments morning and afternoon session, and good to excellent (ICC =0.24–0.95) when the measurements were taken in the morning of same day at 2-hour intervals.

In conclusion, the current study has shown that reliable measurements were obtained and inter- and intra-operator variability was not significant. The inter-operator variation in blood pressure measurements was higher in morning and afternoon measurements compared to that in the morning of same day at 2-hour intervals.

5.2 Introduction

In the intervention study 2, which will be presented in chapter 6, the results are based on repeatable measurements of blood glucose and blood pressure (BP) using standard equipment. For this reason, it was very important to validate the measurements for glucose and BP and to assess the operator variability in measuring blood pressure, in order to obtain accurate measurements and reliable results for a longer term intervention study.

Hypertension is among the most important preventable causes of death around the world. It is also one of the most commonly treated conditions in primary care in the UK. It affects more than a quarter of all adults and over half of those are over 65 years old (Krause *et al.*, 2011). A significant relationship between high blood pressure (BP) and cardiovascular risk has been long-established. Furthermore, the treatment of hypertension has been shown to reduce systolic and diastolic blood pressure, and consequently reduce the risk of CVD.

Reliable measurements of BP are important in monitoring hypertension accurately and in describing the nature of the risk (Altunkan *et al.*, 2007). However the accuracy and reliability of BP monitors should be investigated according to the standards of accepted validation protocols (Altunkan *et al.*, 2007). Blood pressure (BP) measurements based on repeated measurements readings are generally used to make clinical decisions and to investigate the prognoses (Shin *et al.*, 2012). In the UK, the National Institute for Health and Clinical Excellence (NICE) has recently produced guidelines and definitions of hypertension in adults (Krause *et al.*, 2011).

Previous studies have shown that there are a number of factors that need to be considered when measuring blood pressure. Factors that can cause error in measurement include: obtaining a measurement from an unsupported arm, lack of back support and crossed legs increase blood pressure, blood pressure measurement taken before bladder emptying, active listening or talking during blood pressure measurement. These factors can increase systolic blood pressure by 10 mm Hg or more (Handler, 2009). Previous studies have also shown that forearm BP measurements run 3.6/2.1 mmHg higher than upper arm generally. In addition, reading of BP decrease by $\geq$ 20 mmHg systolic pressure when BP is measured

standing (Handler, 2009, Domiano *et al.,* 2008). Finally, measurements taking with tight clothing pushed up on the arm or over clothing can result in a considerable effect (Handler, 2009).

In a research setting on healthy volunteers, only small changes in blood pressure may occur after an intervention, therefore accurate operator technique is essential. The aim of this study was to ensure reliability of the BP monitoring using repeat measurements on volunteers at different times of day and over a period of time, to ensure that the best operator protocol was in place and that variability in measurement was reduced prior to the intervention study.

Diabetes type II is an important healthcare problem. It was estimated that the prevalence of diabetes in 2010 was 4.3% (2.8 millions) in the UK with a dramatic of two-fold increase compared to 1996 (Clarke and Foster, 2012). Diabetes can be defined as a fasting blood glucose level of ≥ 7.0 mmol/L. Normal fasting blood glucose levels are in the range of 4-6 mmol/L, but as the body becomes more insulin resistant, the fasting blood glucose levels increase. A level of glucose between 6-7 mmol/L is classified as impaired glucose tolerance, and is one of the indicators for metabolic syndrome. Both diabetes and metabolic syndrome are significant risk factors for CVD.

In order to obtain reliable measurement results for clinical decisions, and nutrition research, the accuracy of the systems for self-monitoring of blood glucose is crucial. They play an important role in the management of diabetes, particularly in patients treated with insulin (Rheney and Kirk, 2000, Freckmann *et al.,* 2010). Blood glucose meters must have sufficient accuracy to allow patients and physicians to monitor glycaemic control and then modify treatment accordingly (Freckmann *et al.,* 2012). There is an internationally accepted standard defining the performance requirements for self-monitoring of blood glucose (SMBG) systems, e.g., concerning accuracy. The standard states that ≥95% of the measurement should fall within ±0.83 mmol/L of the results of the manufacturer's measurement procedure at glucose concentrations <4.2 mmol/L and within ±15% at glucose concentrations >4.2 mmol/L (Freckmann *et al.,* 2012).

A variety of factors can influence the performance and the accuracy of glucose meter result. Such factors as environmental factors, operator technique and patient factors (Tonyushkina and Nichols, 2009). The accuracy of blood glucose measurements can be altered and affected by substances that interfere with blood glucose measurements such as haematocrit (HCT), maltose, galactose, ascorbic acid and sodium (SKaron *et al.*, 2008, Dimeski *et al.*, 2010). As haematocrit has been shown that interfere glucose monitors are calibrated to provide accurate reading with a normal physiological range of values, typically 30 – 50% (Pfützner *et al.*, 2013).

In a research setting when only small changes in fasting blood glucose may be observed, accurate operator technique is vital. The aim of this study was to ensure reliability of the glucose monitor to be used over a period of time, and to ensure that the best operator protocol was in place to reduce errors in monitoring blood pressure and fasting blood glucose in volunteers.

5.3 Materials and methods

5.3.1 Subjects and study design:

Two studies were conducted as part of Ethics reference (MEEC 12-025) that was approved by the Ethics of University of Leeds Committee. These two small projects were carried out in the human study room at the School of Food Science and Nutrition, Parkinson building. Participants were mainly recruited from students of the University of Leeds to validate the use of blood pressure devices in adult healthy participants.

Seven healthy subjects (4 females and 3 males took part in the study I and 7 males took part in study II. The inclusion criteria were as follows: the volunteers were healthy, non-smokers, no use of vitamins or food supplements in the previous 2 months, no allergies, and no prescribed medication, no caffeine, high-fat foods, alcohol at least 2 hour before the measurements. All subjects had granted their informed consent form to participate in this pilot study. The subjects were instructed to refrain from exercise for at least 24 hours prior to taking the measurements. Subjects were allowed to consume their normal diet, but were asked not to ingest substances that might affect blood pressure such as caffeine, high-fat and nitrate- or flavonoid-rich foods for at least 24 hours prior to the measurements. Any individuals

with readings of systolic BP > 140 mm Hg or diastolic >90 mm Hg were omitted, because these were classified as mild hypertension (Krause *et al.*, 2011).

5.3.2 Method of the blood pressure measurements

In study I the measurements were taken using a Panasonic (EW3152W) Blood Pressure Monitor (Matsushita Electric Works, China), with automated blood pressure cuff. In study 2, the test was performed using Lloyds Pharmacy (model LBPK1) Automatic Blood Pressure Monitor (Kinetik Medical Devices Ltd, Elstree, Herts, UK). Two cuff sizes were available: the standard adult cuff which is appropriate for arm circumferences measuring 22 to 32 cm, and the large adult cuff which is appropriate for circumferences of 32 to 40 cm. The device has been designed and validated for blood pressure self-measurements by British Hypertension Society.

The protocol described by the manufacturers and the European Society of Hypertension (ESH) was followed with some modification based on O'Brien *et al.*, (2010). The tests were performed with the same conditions in a low light and noise room with temperature controlled between 19 – 23ºC. Prior to measurements anthropometric data, such as height, weight and gender were collected, to allow time for subjects to relax. Subjects were also asked to remove tight or restrictive clothing. All subjects were relaxed in a sitting position (study 1), and a supine position (study 2) with both arms resting on arm supports for at least 10 min, with the cuff placed on the upper non-dominant arm. They were asked not to do any activity that could affect the BP measurements such as exercise, activity, reading and talking.

To determine the BP once subject fully relaxed, the start button was pressed to begin automatic inflation and the LCD screen automatically displayed the systolic and diastolic pressure reading after each measurement. The BP was recorded and the cuff deflated. BP was measured in triplicate with five minute intervals between the three consecutive measurements. The average of the last two readings only was used in the calculation.

BP was monitored on three different days, separated by no more than 2 days, with two different sessions on each day, one in the morning between 9-11am and

one in the afternoon between 2-4 pm. The measurements were ideally taken at the same time, under the same conditions and in the same position each time and visit.

5.3.3 System accuracy evaluation of blood glucose monitoring systems

5.3.3.1 Materials and methods

The materials used were glucose standard Solution (product code G 3285). Accu-Chek Aviv meter (Roche Diagnostics Ltd, West Sussex, UK), Accu-Chek test strip, and multi-click lancet device. A stock solution of D-glucose standard (8.33 mmol/L) was prepared. This stock solution and the subsequent dilutions were prepared in Milli-Q water and vortexed before used. Different concentrations such as 1.39, 2.7, 5.5, 8.3 mmol/L were made. Testing was performed using the blood glucose monitoring system Accu-Chek Aviva. The measurement was taken in triplicate. Accu-Chek Aviva control solutions - low levels (ranged between 1.6 – 3.3 mmol/L) and high levels (ranged between 14.1 – 19.1 mmol/L). The purpose of this study was to determine the repeatability and precision of the Accu-Chek Aviva blood glucose system carried out in the same location by the same operator, using the same equipment. The test strips were dosed with control solution and the process was repeated over six days for each assigned level of control solution.

5.3.4 Protocol for measuring blood glucose using the Aviva Accu-Chek glucose meter

The blood samples were collected based on the method described by the manufacture and with some modification (Zueger *et al.*, 2012). The finger area where the sample would be taken from was first disinfected using a sterile wipe. The multi-click lancet was set with a new lancet drum and at a depth of 3 mm. The skin was pricked with pressing of the lancet. The first drop of blood was removed with a sterile tissue. All the measurements were carried out with one single subsequent drop from the same area. The drop was applied to the test strip inserted in the blood glucose monitor. The device was internally calibrated and directly reported the result as a plasma glucose value. The value was recorded and the test repeated on the same finger.

5.4 Statistical analysis

The parameters investigated were age, height, weight, gender, BMI, systolic (SBP) and diastolic (DBP) blood pressure. In order to compare the reproducibility of three measurements, the standard deviation was used to compare the means between measurements. The following statistical methods were used to assess the reproducibility of the measurements and the difference from days (1 vs 2 and 3), and between morning and afternoon time. A paired t-test compared the mean of two sessions, to identify the difference between the results of test and re-test obtained (Stergiou *et al.,* 1998). In addition, intraclass correlation coefficient (ICC) was calculated to measure the agreement between tests and re-tests results and to assess the variability that is caused by variation between the subjects and measurements error. The significant values of ICC have been defined as values of < 0.4, 0.5 – 0.75, and > 0.75, which correspond to poor, fair-to-good, and excellent agreement. The coefficient of variation (CV %) was calculated by dividing the SD by the mean values CV% = (SD/mean x 100). The alpha level was set at $p< 0.05$ for all statistical tests. The analysis of variance one-way ANOVA method allows the variability of the interaction between the sessions and days to be determined. Unless otherwise indicated, all the data reported are mean ± standard deviation. The measurements were done in triplicate in each session (three in the morning and three in the afternoon) and the results obtained were analysed based on the last two measurements. Calculations of blood glucose concentration were performed in mmol/L. All analyses were carried out using the Statistical Package for Social Science base 22 for windows (IBM SPSS Inc., Chicago, IL).

5.5 Results

5.5.1 Accuracy of blood pressure measurements study I, using Panasonic blood pressure monitor

Descriptive statistical for all variable characteristics and demographic data of the study participants in the first visit are shown in table 5.1. Age, BMI, height, weight and blood pressure measurements were measured. A total of 7 participants were included in the study, 4 females and 3 males with a mean age of 25 years, and

an average BMI of 20.2 kg/m^2 the group were all likely to be healthy and have normal BP values.

Table 5.1: Baseline characteristic of the seven participants study I (Panasonic BP monitor).

Age (y)	25±3.9
Height (cm)	1.7±0.10
Weight (kg)	96.4±13.5
BMI kg/m2	20.2±3.6
Systolic BP (mmHg)	112.7±8.4
Diastolic BP (mmHg)	71.9±4.9
Pulse ±	69.5±7.0

All values are expressed as mean ± SD

ANOVA analysis of variance was used to evaluate the reproducibility of measurements in the morning and afternoon. Tukey test was used to estimate the statistical differences. There were no significant differences among the measurements in the morning session or afternoon session for both SBP and DBP. The data shown in figures 5.1 and 5.2 show the results of SBP and DBP obtained on each session/day. The day-to-day variability was not significant and the operator showed excellent day-to-day reproducibility.

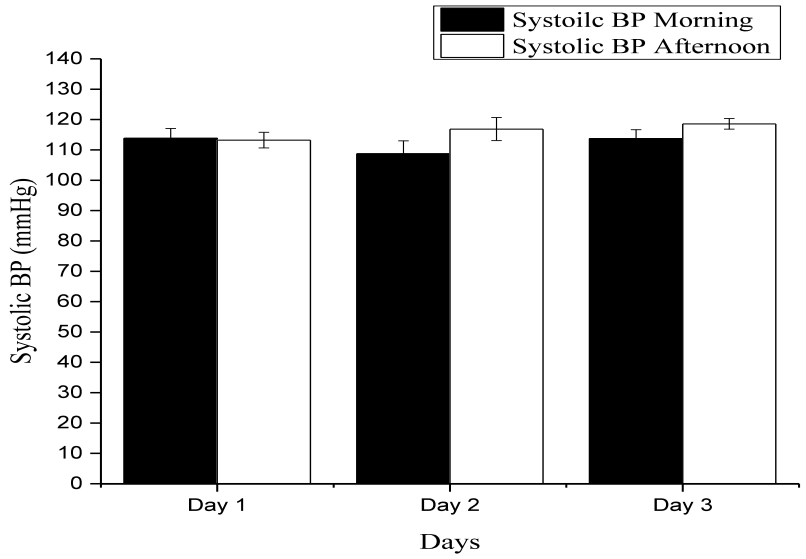


Figure 5.1: Systolic blood pressure reading (mm Hg), at the same time of day (morning and afternoon), and between different days. Result is expressed as mean ± SEM, significant within trials difference ($p<0.05$).

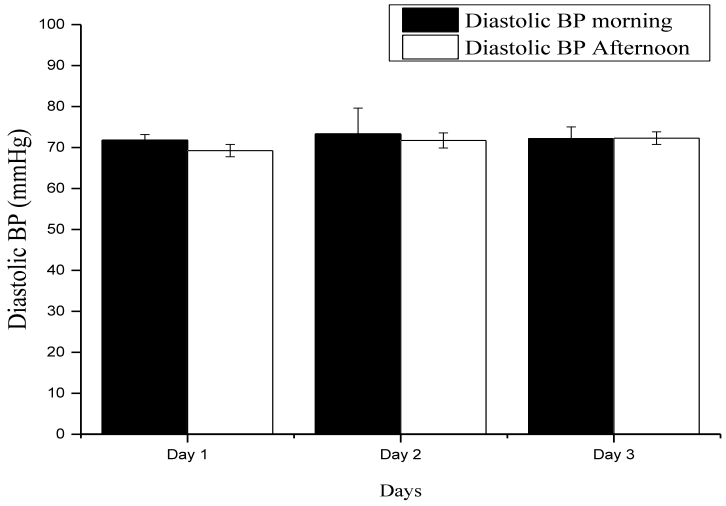


Figure 5.2: Diastolic blood pressure reading (mm Hg), at the same time of day (morning and afternoon), and between different days. Result is expressed as mean ± SEM, significant within trials difference ($p<0.05$).

Table 5.2 and 5.5 summarize the result of intraclass correlation coefficients (ICC), coefficient of variation and p-value from paired t-test. Reproducibility was defined as a non-significant paired t-test with an excellent intraclass correlation (>

0.7) within and between days. For SBP, the measurements were reproducible in day 1, 2 and 3 ranged between (0.59 – 0.87). For DBP all of the measurements were reproducible with t-test were not significant and ICC was excellent and ranged between (0.71 – 0.76). The same results were obtained for pulse rate where ICC was ranged between good to excellent correlation (0.62 – 0.90). The p-value was > 0.05 for all measurements of inter-day and intra-day and there was no statistical difference between measurements in the morning and afternoon in the same day and different days, and the probability of obtaining a different value within each day and between days was low. Therefore, the result confirms no difference within a single visit and between days and the result is reproducible. The variability of the measurements measured on the same day (between morning sessions and afternoon session) was between 2.9 % and 11.8%.

Table 5.2: Mean and standard deviation of blood pressure measurements across three days morning and afternoon in healthy subjects.

	Time point	***Day 1***	***Day 2***	***Day 3***	***One way ANOVA "p Value"***
Systolic BP	Morning session	113.8±8.6	113.0±6.1	112.6±8.6	0.95(NS)
	Afternoon session	111.4±7.4	115.4±9.7	115.10±2.2	0.54(NS)
Diastolic BP	Morning session	71.9±3.9	72.8±6.2	70.9±8.2	0.85(NS)
	Afternoon session	68.8±4.2	70.6±4.6	70.4±4.0	0.43(NS)
Pulse	Morning session	70.3±8.3	66.7±9.6	66.3±7.8	0.64(NS)
	Afternoon session	69.8±5.6	66.4±6.0	67.2±5.3	0.50(NS)

Values are means ± SD unless otherwise indicated. All measurements were taken between 8:00 am – 12:00 pm morning session and 1:00 – 4:00 pm afternoon session. $P \leq 0.05$ vs visit one using Tukey post hoc test, NS not significant.

Table 5.3: Reliability of blood pressure measurements on the same days (day1) in healthy subjects.

	Time point	*Mean ± SD*	*CV%*	*ICC*	*Paired t-test P-value*
Systolic BP	Morning session	113.9±8.6	7.5	0.78*	0.38
	Afternoon session	111.4±7.4	6.6		
Diastolic BP	Morning session	71.9±4.0	5.5	0.71*	0.05
	Afternoon session	69.0±4.2	6.1		
Pulse	Morning session	70.3±8.3	11.8	0.62	0.88
	Afternoon session	70.0±5.6	8.0		

Values are means± SD unless otherwise indicated. *p< 0.05, **p < 0.01.SD, standard deviation; CV, coefficient of variation; ICC, intraclass correlation coefficient, (0.5 – 0.6 moderate agreement,* 0.7-0.8 strong agreement, **> 0.8 perfect agreement).

Table 5.4: Reliability of blood pressure measurements on the same days (day 2) in healthy subjects.

	Time point	*Mean ± SD*	*CV%*	*ICC*	*Paired t-test P-value*
Systolic BP	Morning session	113.0±6.1	5.4	0.78*	0.38
	Afternoon session	115.4±9.7	8.4		
Diastolic BP	Morning session	73.0±6.2	8.5	0.73*	0.28
	Afternoon session	70.6±4.6	6.6		
Pulse	Morning session	66.7±9.6	14.4	0.79*	0.92
	Afternoon session	66.4±6.0	9.1		

Values are means± SD unless otherwise indicated. SD, standard deviation; CV, coefficient of variation; ICC, intraclass correlation coefficient (0.5 – 0.6 moderate agreement,* 0.7-0.8 strong agreement, **> 0.8 perfect agreement).

Table 5.5: Reliability of blood pressure measurements on the same days (day 3) in healthy subjects.

	Time point	*Mean ± SD*	*CV%*	*ICC*	*Paired t-test P-value*
Systolic BP	Morning session	112.6±7.6	7.6	0.59	0.49
	Afternoon session	115.1±2.2	2.9		
Diastolic BP	Morning session	71.0±8.2	11.6	0.76*	0.83
	Afternoon session	71.7±3.2	4.4		
Pulse	Morning session	66.3±7.8	11.8	0.90**	0.56
	Afternoon session	67.2±5.3	7.9		

Values are means± SD unless otherwise indicated. SD, standard deviation; CV, coefficient of variation; ICC, intraclass correlation coefficient (0.5 – 0.6 moderate agreement,* 0.7-0.8 strong agreement, **> 0.8 perfect agreement).

5.6 Accuracy of blood pressure measurements study II, using Lloyds blood pressure monitor

All 7 subjects completed the study. The baseline characteristics of the volunteers are presented in table 5.6. The mean age for all subjects was 39 years, with an average BMI 29.37 kg/m^2.

Table 5.6: Baseline characteristics of the 7 healthy participants study II (Lloyds BP monitor).

Age (y)	39 ± 4.27
Gender	Male
Room temp (ºC)	20.9 ± 0.8
Height (m)	1.7 ± 0.05
Weight (kg)	89.6 ± 13.5
BMI (kg/m^2)	29.4 ± 3.7
Systolic PB (mm Hg)	117.2 ± 10.3
Diastolic PB (mm Hg)	77.7 ± 4.5
Pulse	70.9 ± 4.0

All values are expressed as mean ± SD

An analysis of variance (ANOVA) for repeated measurements was used to estimate the reproducibility of measurements of all parameters in 3 days. There were no significant difference among the three recordings in test and re-test of blood pressure measurements (figures 5.3 and 5.4). Table 5.7 shows the results of the repeated-measures ANOVA. The day-to-day variability was not significant and the operator showed excellent day-to-day reproducibility. The results of the paired t-test showed that there was no significant difference between the pairs of measurements obtained on the same day. According to the results obtained to compare the values of the mean from two repeated measurements test and re-test the result of systolic, the diastolic and pulse rate in three days, the p-value was > 0.05 and ranged between (0.26 – 0.86). Therefore, the differences between the two measurements within the days were not statistically significantly different at the 5% level of significance.

Table 5.7: Mean and standard deviation of blood pressure measurements in three days test and re-test measurements in healthy subjects.

	Time point	***Day 1***	***Day 2***	***Day 3***	***One way ANOVA "p Value"***
Systolic BP	Measurements	113.8±6.7	115.2±4.6	113.3±6.6	0.87 (NS)
	Measurements	113.7±4.6	115.1±4.6	113.0±6.2	0.74 (NS)
Diastolic BP	Measurements	77.7±4.5	79.4±3.8	77.7±4.9	0.73 (NS)
	Measurements	78.9±5.3	80.3±4.7	78.9±4.5	0.87 (NS)
Pulse	Measurements	70.8±3.9	96.4±1.8	68.4±3.2	0.43 (NS)
	Measurements	68.8±4.8	67.7±2.1	66.4±4.2	0.57(NS)

Values are means ± SD unless otherwise indicated. All measures were collected between 8:00 am – 12:00 pm. P≤0.05 vs visit one using Tukey post hoc test, NS not significant.

The test and re-test of reliability and reproducibility was assessed with the intra-class correlation (ICC). The reliability of measurements for SBP, DBP, pulse

rate ranged between (0.87-0.95), (0.79-0.92) and (0.24-0.46), respectively. These values were within the acceptable ranges among 3 days according to Stanforth *et al*, (2000) which has consider CV of < 10% is good for most of the biological data (Stanforth *et al.,* 2000) . These results were obtained between and within-subject variance. A second measure of variability was the coefficient of variation (CV %), the results were within acceptable range. Tables 5.8- 5.10 show the result, where variability of measurements using CV was stated and coefficient of variation for SBP, DBP and pulse rate ranged between 2.80 – 8.75%.

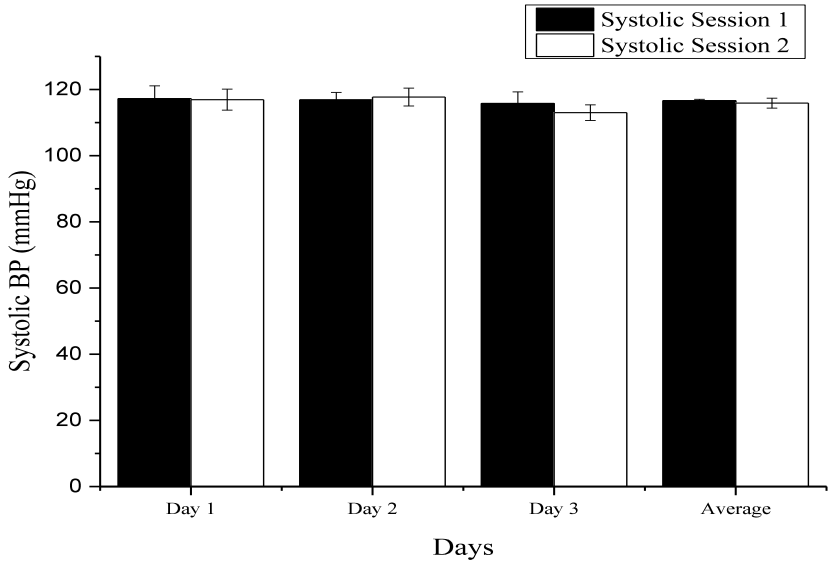


Figure 5.3: Systolic rate reading (mm Hg), at the same time of day (test and re-test), and between different days. The results are expressed as mean ± SEM, significant within trials difference ($p<0.05$).

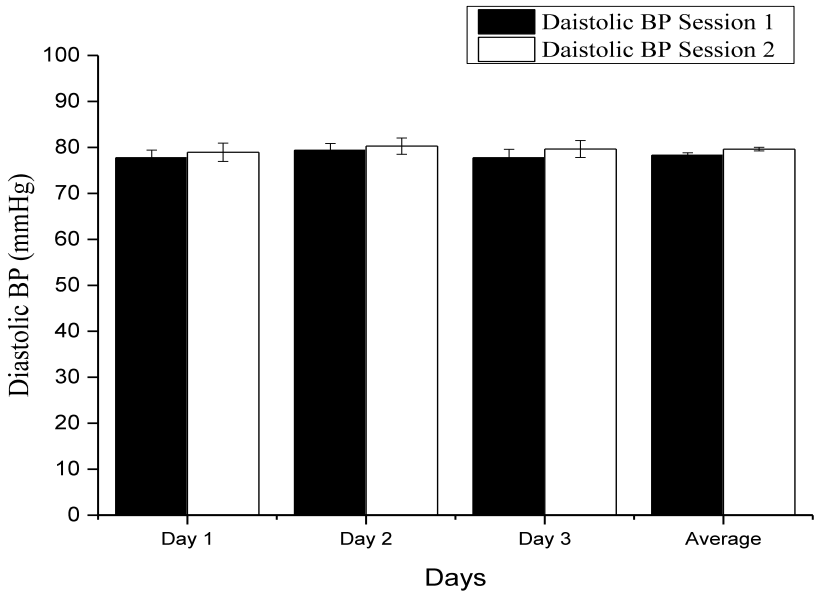


Figure 5.4: Diastolic reading (mm Hg), at the same time of day (test and re-test), and between different days. The results are expressed as mean ± SEM, significant within trials difference ($p<0.05$).

Table 5.8: Reliability of blood pressure measurements on the same days (day1) at 2 - hour intervals in healthy subjects.

	Time point	***Mean±SD***	***CV%***	***ICC***	***Paired t-test P-value***
Systolic BP	Measurements 1	113.8±6.7	8.75	0.95*	0.86
	Measurements 2	113.7±4.6	7.20		
Diastolic BP	Measurements 1	77.7±4.5	5.75	0.89*	0.33
	Measurements 2	78.9±5.3	6.66		
Pulse	Measurements 1	70.8±3.9	5.61	0.46	0.35
	Measurements 2	68.8±4.8	6.69		

Values are means± SD unless otherwise indicated, SD, standard deviation; CV, coefficient of variation; ICC, intraclass correlation coefficient (average measurements). (0.5 – 0.6 moderate agreement,* 0.7-0.8 strong agreement, **> 0.8 perfect agreement).

Table 5.9: Reliability of blood pressure measurements on the same days (day2) at 2 - hour intervals in healthy subjects.

	Time point	*Mean±SD*	*CV%*	*ICC*	*Paired t-test P-value*
Systolic BP	Measurements	115.2±4.6	3.96	0.87*	0.56
	Measurements	115.0±4.6	3.61		
Diastolic BP	Measurements	79.4±3.8	4.88	0.79*	0.42
	Measurements	80.3±4.6	5.84		
Pulse	Measurements	69.4±1.7	2.80	0.24	0.15
	Measurements	67.7±2.1	2.92		

Values are means± SD unless otherwise indicated, SD, standard deviation; CV, coefficient of variation; ICC, intraclass correlation coefficient, (0.5 – 0.6 moderate agreement,* 0.7-0.8 strong agreement, **> 0.8 perfect agreement).

The intra-operator variability in measurements of blood pressure measurement was assessed and figure 5.5 shows the measurements of SBP and DBP for each participant in two sessions on three different days. The results show that the CVs were between 0.5 – 7.4% for all measurements, which provides evidence of good operator variability.

Table 5.10: Reliability of blood pressure measurements on the same days (day3) at 2 - hour intervals in healthy subjects.

	Time point	***Mean±SD***	***CV%***	***ICC***	***Paired t-test P-value***
Systolic BP	Measurements	113.3±6.6	5.86	0.95*	0.73
	Measurements	113.0±6.2	5.50		
Diastolic BP	Measurements	77.7±4.9	6.37	0.92*	0.29
	Measurements	78.9±4.4	6.08		
Pulse	Measurements	68.4±3.2	4.62	0.37	0.26
	Measurements	66.4±4.2	6.35		

Values are means± SD unless otherwise indicated, SD, standard deviation; CV, coefficient of variation; ICC, intraclass correlation coefficient. (0.5 – 0.6 moderate agreement,* 0.7-0.8 strong agreement, **> 0.8 perfect agreement).

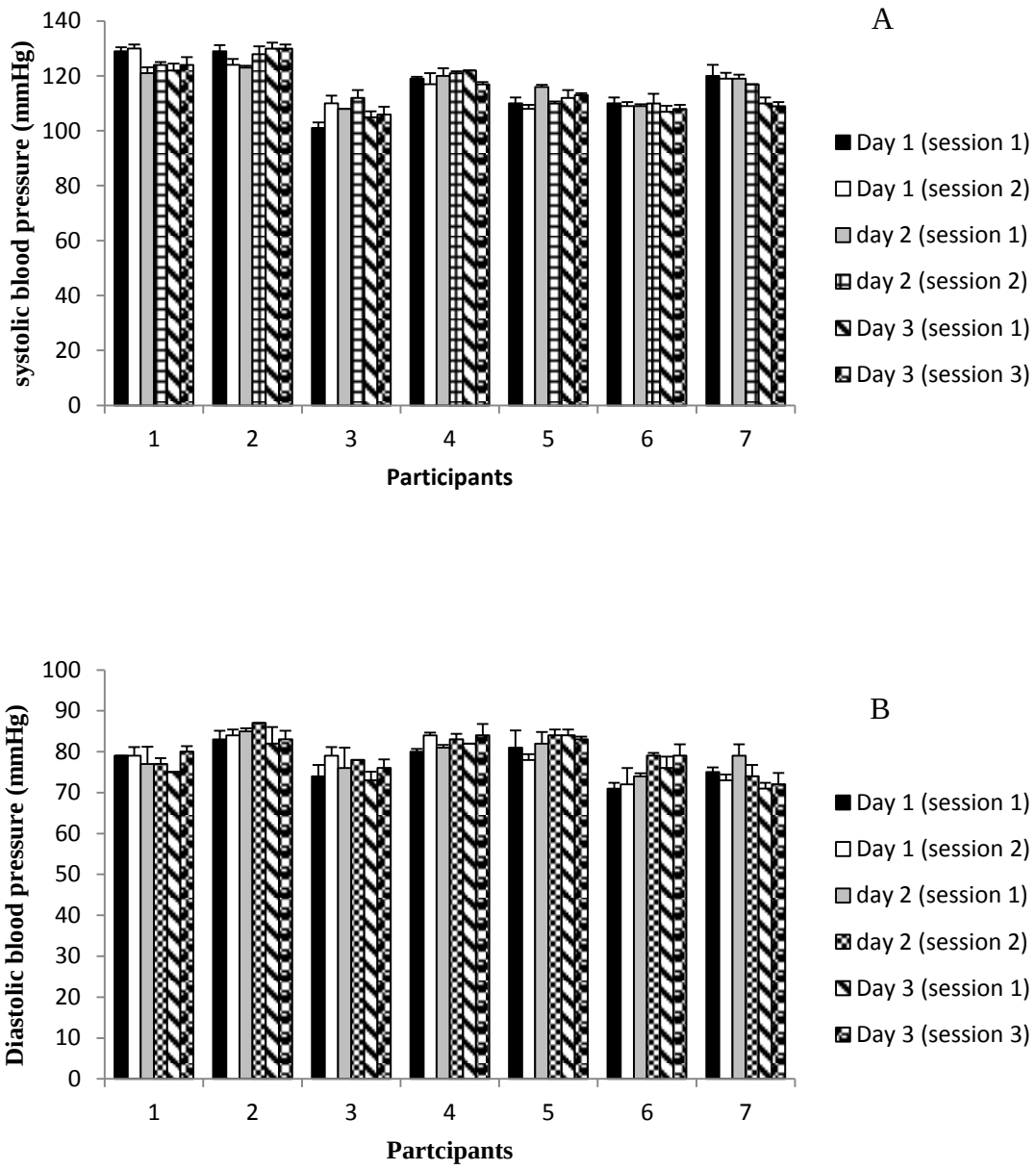


Figure 5.5: Blood pressure measurements among 7 participants in two sessions and three days. Systolic blood pressure (A). Diastolic blood pressure (B).

5.7 Accuracy and precision of the Accu-Chek Aviva blood glucose meter.

The determination of control prepared glucose solutions were made on Accu Chek Aviva meter. The results obtained were linear within the range from 1.4 – 8 4 mmol/L (Figure 5.5). The precision was good and the measurements are presented in Table 5.11.

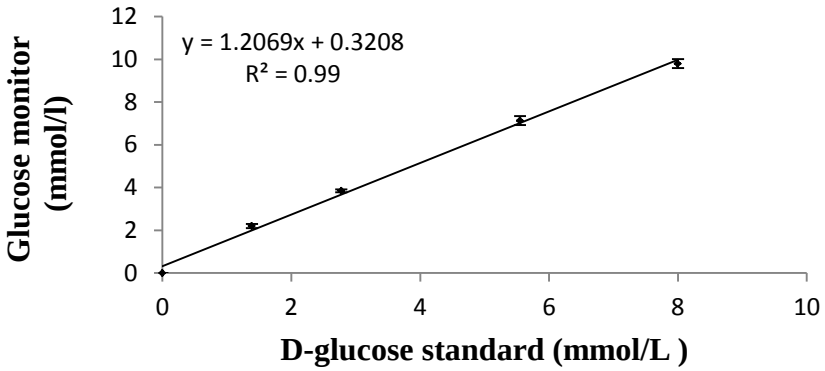


Figure 5.6: The concentration of standard glucose measured by Accu-Chek Aviva.

The variation of the test was calculated by the coefficient of variation (CV %). The performance of a method based on CVs of 5% or less is generally an indication that the method is reliable. From the result obtained, it can be seen that the performance and accuracy of the Accu check Aviva gave CVs values of less than ≤ 5%.

Table 5.11: The accuracy of and performance of Accu Chek blood glucose meter.

D-glucose standard conc. mmol/L	***Monitored glucose concentration (mmol/L)***		
	Mean	SD	CV%
1.4	2.2	0.1	4.5
2.8	3.9	0.1	1.8
5.6	7.1	0.2	2.9
8.0	9.8	0.2	2.2

Values are means ± SD (n=3)

The accuracy of Accu-Chek Aviva blood glucose meter was tested six times on each of six different days with the manufacturers two control solutions, data shown in tables 5.12 and 5.13. The CV expresses the variation as a percentage of the mean. The precision of device was less than < 5%. Moreover, the intra-class correlation ICC was excellent. It ranged between 0.76 and 0.93 for low levels and high levels measurements of Accu-Chek Aviva control solutions, respectively. The results of the repeatability results using Accu-Chek Aviva control solutions and Accu-Chek Aviva blood glucose test strips. Result of the paired t-test shows that the difference was not statistically significant ($p < 0.05$).

Table 5.12: Repeatability of Accu-Chek Aviva blood glucose meter at low concentration.

	Reading 1	*Reading 2*	*Mean ± SD*	*CV %*	*ICC*
day 1	2.6	2.6	2.6 ± 0.0	0.0	
day 2	2.6	2.6	2.6 ± 0.0	0.0	
day 3	2.7	2.6	2.6 ± 0.1	2.7	0.78*
day 4	2.6	2.5	2.5 ± 0.1	2.8	
day 5	2.5	2.4	2.4 ± 0.1	2.9	
day 6	2.6	2.6	2.6 ± 0.0	0.0	
Mean	2.6	2.6	2.6 ± 0.0	1.4	

Control solution 1 ranged 1.6-3.3 mmol/l; ICC *p< 0.05, **$p < 0.01$. †paired t-test $p < 0.05$.

Table 5.13: Repeatability of Accu-Chek Aviva blood glucose meter at high concentration.

	Reading 1	*Reading 2*	*Mean ± SD*	*CV %*	*ICC*
day 1	16.9	16.8	16.8 ± 0.1	0.4	
day 2	17.6	17.5	17.5 ± 0.1	0.4	
day 3	17.5	17.6	17.5 ± 0.1	0.4	0.93*
day 4	17.5	17.4	17.4 ± 0.1	0.4	
day 5	17.3	16.9	17.1 ± 0.1	1.7	
day 6	16.8	16.6	16.7 ± 0.1	0.8	
Mean	16.9	16.8	16.8 ± 0.1	0.7	

Control solution 2 ranged 14.1-19.1 mmol/l; *p< 0.05, **$p < 0.01$. †paired t-test $p < 0.05$.

5.8 Discussion

After examining 7 different subjects on two different blood pressure monitoring devices, both were found to be reliable in measuring systolic and diastolic blood pressure. In the present study, we determined the within day variability of BP by repeating measurements on the same day, and the between day variability was evaluated by repeating the measurements on different days. Analysis of variance using one-way analysis gives the variability of the interaction between the sessions and days to be determined. The accuracy of the instrument was not affected by the three days in-use assessment. The results were not significant when using both monitors. The standard deviations of the device–observer discrepancies were well within the Advancement of Medical Instrumentation (AAMI) requirement of a standard deviation of ≤ 8 mmHg (Bonso *et al.*, 2008). The results obtained showed that there was low intra-operator variability, providing confidence in accurate measurements, and both the Lloyds BP Monitor and Panasonic EW3152W Blood Pressure Monitor were reliable.

Several studies showed that accuracy of blood glucose measurements vary largely between different blood glucose devices (Baumstark *et al.*, 2012, Freckmann *et al.*, 2012), and different test strip lots of the same blood glucose system (Baumstark *et al.*, 2012). The Accu-Chek Aviva meter precision was acceptable and it met the accuracy requirement in comparison with the reference values recommended by the manufacture, which is in agreement with S.Karon *et al*., (2008). There was a difference between the standard glucose concentration and the glucose reading on the monitor, however the results were linear. This is likely to be as a result of internal calibration of the monitor which is calibrated to measure blood glucose, but to provide a plasma value reading that is also calibrated against interference from normal haematocrit values. Hence the absolute values obtained from the control glucose solutions were not relevant as long as linearity was observed. Ideally a hexokinase measurement would be made using a venous plasma sample, which would have low interference from collection of blood (which can occur from finger prick samples) and from matrix interference with maltose, galactose, haematocrit, ascorbic acid and sodium concentrations (S.Karon *et al.*, 2008, Dimeski *et al.*, 2010, Freckmann *et al.*, 2010, Pfützner *et al.*, 2013). However this was impractical for the intervention study described in chapter 6, and the repeatability of the Accu-chek blood glucose monitor was deemed sufficient to determine impaired glucose tolerance from fasting blood glucose measurements, but would be unlikely to be accurate in detecting changes in fasting blood glucose after an intervention of <0.6-0.8 mmol/L (15%).

In conclusion, the intra-operator variability in measuring blood pressure was good for both monitors, however the Lloyds upper-arm monitor was chosen as the results were dependable and repeatable. Furthermore, the blood pressure cuff was comfortable in a sitting and lying position, which was essential for the intervention study in chapter 6, whereas the Panasonic BP monitor was easier to use in a sitting position. The Accu-Chek Aviva blood glucose monitor gave reliable and repeatable results when tested against glucose standards and control solutions provided by the manufacture. Both devices were accepted for use in the human intervention study described in chapter 6 as the operator variability was low.

Chapter 6: The Impact Of Oranges On Cardiovascular Health

6.1 Abstract

Cardiovascular disease (CVD) is the principal cause of death in the Western world. Established risk factors include high LDL cholesterol, high blood pressure (BP) and diabetes. Poor endothelial health is considered to be a predictor of future CVD risk. Previous studies have provided evidence that the consumption of orange juice improves cardiovascular risk factors such as BP and endothelial health. We tested the hypothesis that citrus bioflavonoid supplements improved BP and other CVD risk biomarkers in a double-blind, placebo-controlled, cross-over intervention study in overweight subjects.

Twenty three volunteers (males and females, age 29-44 y) were recruited and assigned to a treatment in random order for a 4 week period, with a 4 week washout between phases. On each study day, participants had weight, height, waist circumference, and blood pressure measured. A finger-prick blood sample monitored fasting blood glucose level using an Aviva Accu-chek glucometer. Pulse amplitude tonometry (PAT; blood flow through fingertips) was also monitored using an Endo-PAT, before and after forearm blood flow was restricted using a blood pressure cuff for 5 min (hyperaemia).

DBP, SBP and fasting blood glucose tended to be lower after 4 weeks consumption of citrus supplements compared to the placebo, however the data did not reach significance. Moreover, augmentation index (AI) significantly decreased when citrus supplement was ingested, compared to baseline, but no change was reported in the placebo intervention. There was no change in the reactive hyperaemia index (RHI) measured by the Endo-PAT.

For the first time commercially-available citrus bioflavonoid supplements were assessed for their ability to affect vascular parameters. It can be concluded that in healthy overweight subjects citrus supplements tended to decrease SBP, DBP and fasting blood sugar, however further studies are warranted at different doses and for

longer duration to determine if hesperidin, as the main compound in the citrus tablets, could be linked to the beneficial effects of orange juice in reducing the risk of CVD.

6.2 Introduction

Cardiovascular disease (CVD) is the main cause of morbidity and mortality around the world. Epidemiological studies showed that there is an inverse relationship between eating high quantities of vegetables and fruits and reducing the risk of CVD. The actual role played by these compounds is unclear and still under investigation (Chanet *et al.,* 2012b). Established risk factors of CVD include high LDL cholesterol, hypertension and diabetes, however, endothelial dysfunction has been defined as "the ultimate risk of the risk factors" as it can be considered a summation of the integrated effects of CV risk factors (Lerman and Zeiher, 2005). Endothelial dysfunction can be identified by an impaired endothelium-dependent vasodilation response to changes in blood flow or other stimuli (Allan *et al.*, 2013). Flow mediated dilation (FMD), pulse amplitude tonometry (PAT), and peripheral blood pressure (BP) can all be used to measure different aspects of underlying vascular dysfunction and are considered to be predictors of future CV risk (Tomiyama and Yamashina, 2010). Endothelial dysfunction is reversible (Inaba *et al.*, 2010, Green *et al.*, 2011).

The endothelium is the inner lining of blood vessels. It allows blood to flow smoothly within them and actively maintains the physiological condition. Normal endothelial function helps to protects blood vessels from formation of atherosclerosis. The endothelium has been recognized as the key regulator of vascular homeostasis. Healthy endothelial cells produces a wide range of factors that regulate vascular tone, thrombus formation, adhesion of circulating blood cells to the vessel wall, smooth muscle cell proliferation, and vessel wall inflammation, which are the key mechanism of the atherosclerotic process.

The effect on vascular tone is one of the most important functions of the endothelium, which is achieved by the production of several vasoactive compounds that relax the vessel (Poredos and Jezovnik, 2013). Nitric oxide (NO) is the most

important of these factors; it is an influential vasodilator, inhibits inflammatory activity, vascular smooth muscle cell proliferation and platelet adhesion and aggregation (Allan *et al.,* 2013). Endothelial dysfunction occurs when the balance between NO production and its consumption is disrupted (Poredos and Jezovnik, 2013).

Many non-invasive methods designed for measuring endothelial function have been investigated. Two of the most commonly used are flow mediated dilatation (FMD) and peripheral artery tonometry (PAT) (Arrebola-Moreno *et al.,* 2012). Both methods depend on increasing hemodynamic shear stress during reactive hyperaemia to stimulate NO release; changes in blood flow or vessel diameter explains the change in smooth muscle tone in resistance or conduit arteries (Ganz and Vita, 2003).

FMD is an ultrasound-based test and it is the most commonly used method to measure endothelial dysfunction. It measures alteration in conduit artery diameter stimulated by reactive hyperaemia (Corretti *et al.,* 2002, Lekakis *et al.,* 2011). The reactive hyperaemia phenomenon is a result of temporary arterial occlusion and then release which leads to increasing the blood flow (hyperaemia) and causes an increase in shear stress on the vessel wall. This activates endothelial nitric oxide synthase (eNOS) which oxidises L-arginine to produce NO. The NO enters the smooth muscle cell causing relaxation and vasodilation (Felmeden and Lip, 2005, Arrebola-Moreno *et al.,* 2012, Conraads, 2013, Allan *et al.,* 2013). FMD is measured as the percentage of change in brachial artery diameter between baseline (before occlusion) to the maximum diameter immediately after release (Al-Qaisi *et al.,* 2008). Figure 6.1 shows the setting and testing of FMD. Although the assessment of FMD is still one of the most widely used techniques to measure endothelial dysfunction, it requires good training and a high level of ultrasound experience in order to achieve replicable results (Conraads, 2013). Reports show that this technique has a considerable high intra- and inter- operator variability (Dhindsa *et al.,* 2008).

Digital peripheral arterial tonometry (PAT) is a relatively new and non-invasive technique. It measures the peripheral microvascular endothelial function by measuring pulse wave amplitude during reactive hyperaemia. It has been developed

to avoid the user-dependent measurement disadvantage of FMD (Conraads, 2013, Lumsden *et al.*, 2013). The Endo-PAT system (Itamar, Isreal), works by using pneumatic fingertip probes to automatically measure digital arterial pulse wave response when reactive hyperaemia is induced (Lekakis *et al.*, 2011), instead of the need for ultrasound.

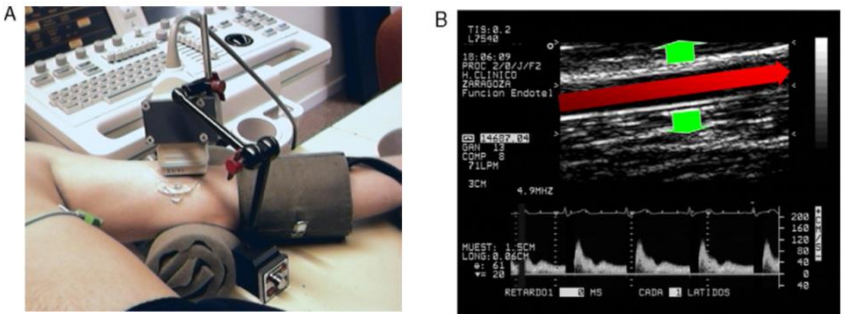


Figure 6.1: (A) Setting for flow-mediated dilation testing. The picture showing the ultrasound probe in a fixed position in the upper arm, and the blood pressure cuff on the lower forearm used to occlude blood flow. (B) Ultrasound image of brachial artery used to measure both the diameter changes and flow velocity. The red arrow shows the direction of blood flow through the vessel and the green arrows show the diameter which is the measurement taken before and after occlusion. Taken from Arrebola-Moreno *et al.*, (2012).

The Endo-PAT system is effectively an operator-independent device that is easy to use as it is computer controlled. The differences in technique are that Endo-PAT measures microvascular endothelial function, whereas FMD measures endothelial function at level of large conduit artery. Both techniques, however, are correlated well in healthy participants (Conraads, 2013).

The EndoPAT device has been approved by the Federal Drug Administration (FDA) and is commercially used to measure endothelial function in the USA (Hamburg and Benjamin, 2009). It consists of two probes placed on the index fingers of both hands to sense the change in blood volume in each arterial pulsation as shown in Figure 6.2. Using the pneumatic digital probes (figure 6.3) to sense the changes in pulsatile blood volume from the microvasculature of the fingertips, the peripheral arterial tone (PAT) can be measured.

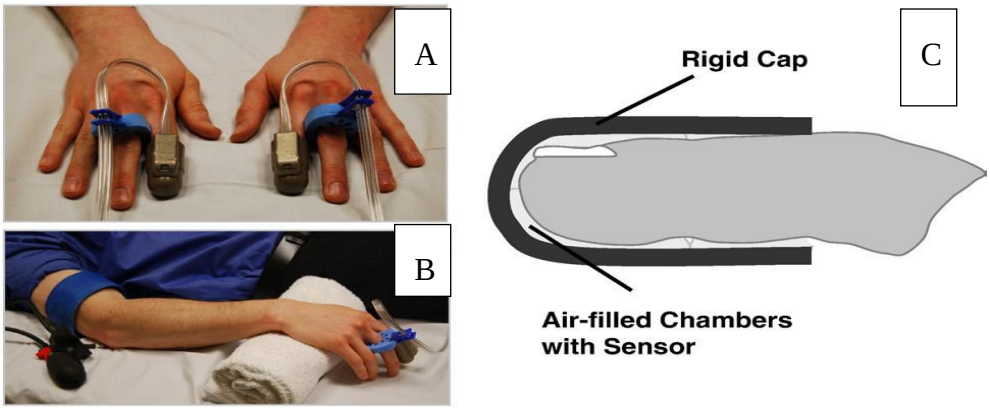


Figure 6.2: Diagram of Endo-PAT fingertip and inflatable cuff. (A) A thimble-like probe placed on the end of each index finger as shown in diagram. (B) A cuff is placed on one upper arm then inflated to supra systolic pressure to total occlusion, which on releases induce hyperaemia. (C) The air filled chamber senses changes in volume with each arterial pulsation. Reproduced from Hamburg and Benjamin., (2009).

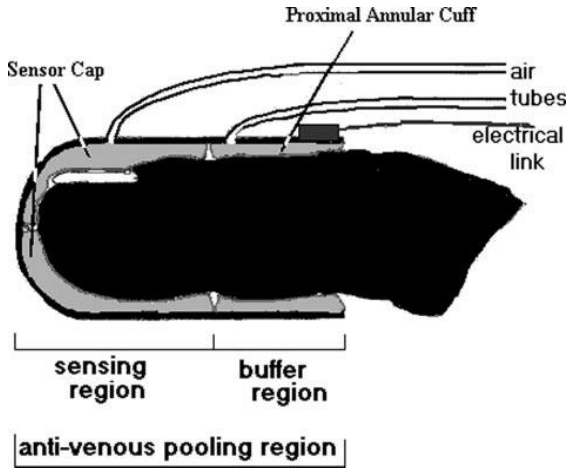


Figure 6.3: Cross-sectional view through the PAT finger probe. derived from Celermajer., (2008).

Each endothelial function test includes three phases; namely: baseline, occlusion and hyperaemia. A relative index (the reactive hyperaemia index, RHI) is then calculated by monitoring the change in pulsatile blood volume between baseline and hyperaemia, and compared to the control blood flow in the non-occluded arm. Endo-PAT score, or RHI, can be divided to 3 levels: RHI $\leq$ 1.67 indicates the need for further medical assessment for endothelial dysfunction; a score between 1.68–2 indicates the endothelium is fine, and >2.1 is considered a

good healthy endothelium. Figure 6.4 shows an example of the difference between low and high endothelial response measured by Endo-PAT 2000 device.

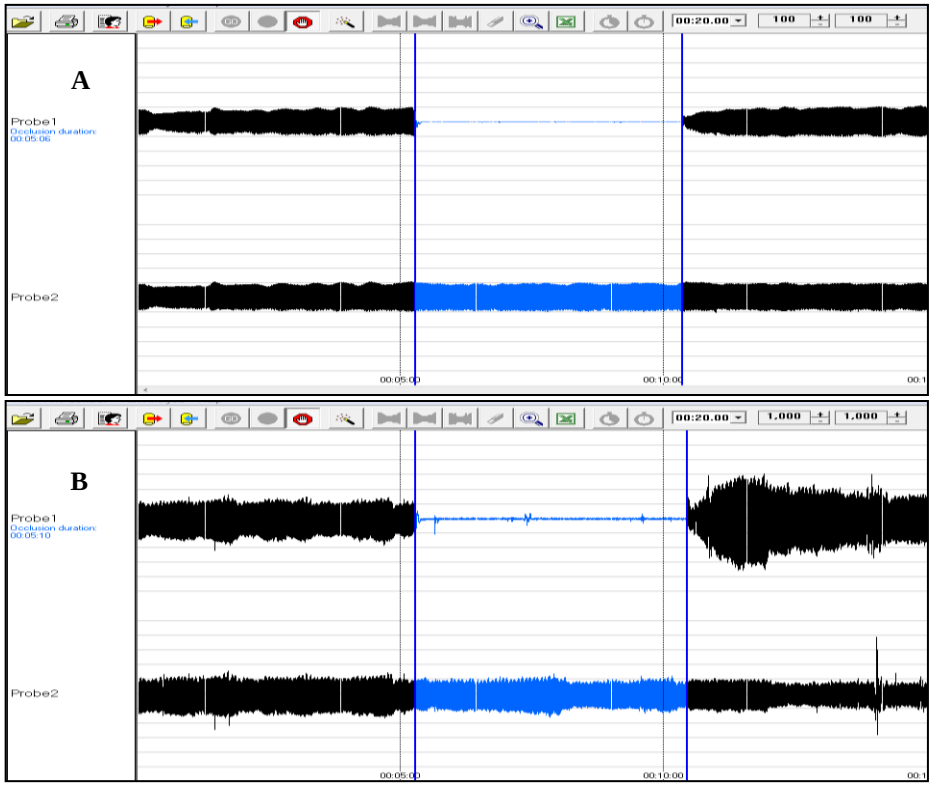


Figure 6.4: Low (A) and high (B) endothelial responses monitored by Endo-PAT 2000. The test has three phases: phase 1 (panel up to approx. 5 min) records the baseline, phase 2 (blue section) monitors occlusion during the cuff inflation, and phase 3 records the response after cuff release (hyperaemia). Probe 1 is test finger response, and probe 2 is the control finger response.

There have been several studies on citrus flavanones on CVD risk factors, such as BP and FMD. Buscemi *et al.*, (2012) showed that endothelial function, was significantly improved after was measured by flow-mediated dilation. Morand *et al.*, (2011) also showed in healthy, moderately overweight, men that orange juice decreased diastolic blood pressure when regularly consumed, and post–prandial increased endothelium–dependent microvascular reactivity. At the time of this study, to the best of our knowledge, no intervention had been published using commercially-available citrus supplements to improve CVD risk factors and,

furthermore, Endo-PAT had not been used to investigate chronic changes in endothelial response after flavanone intake.

6.3 Aims of the study

The purpose of this study is to assess changes in various CV risk factors after a four week placebo-controlled, cross-over intervention study after volunteers have consumed citrus supplements or a placebo supplement, on a daily basis with breakfast. The hypothesis is that chronic intake of flavanones improves endothelial health by decreasing blood pressure and increasing reactive hyperaemia.

The main aims were as follows:

- To investigate the change in systolic and diastolic blood pressure before and after citrus supplementation for four weeks, and compare to any changes after a placebo for the same time. This was the primary objective and was used as a basis for power calculations for determining volunteer numbers.
- To assess any changes in reactive hyperaemia as measured by Endo-PAT in the same intervention study.
- To assess any changes in fasting blood glucose, in the same intervention study.

6.4 Material and methods

This study was reviewed and granted permission by the MEEC Faculty Research Ethics Committee at its virtual meeting of 14th March 2013, under the Ethics reference number (MEEC 12-025) University of Leeds (see Appendix 2 for the Ethics forms). The study is registered with clinical Trial under the clinical Trials.gov identifier (NCT01935362).

6.4.1 Subjects

The study advertised for volunteers by (i) emails sent using the University of Leeds distribution lists, (ii) poster adverts displayed around the University and

across Leeds, (iii) an advert on the School of Food Science and Nutrition website, (iv) an advert in "The Reporter" the University monthly magazine. The Inclusion criteria specified in the advert were to have no known cardiovascular disease or diabetes, to be between 30-60 y, to be heavier than average (Body mass index, BMI $\geq$ 25 kg/m^2), to be a non-smoker, and willing to consume orange juice supplements for 2 months. The recruitment for this study was open to everyone; male and female were equally eligible to participate in the study.

The volunteers who responded positively to the adverts were invited to read the detailed study information sheet, and the study protocol in full was explained to each person. Having given their verbal and written informed consent, eligible participants were asked to fill in a basic health questionnaire recording age, gender, height, weight, medical and surgical history, medications taken and recent smoking status, to ensure the suitability for study inclusion.

Participants were recruited if they:

- had not taken any antibiotics in the 2 months prior to the study,
- did not consume dietary or herbal supplements,
- had not ever had any gastrointestinal tract surgery,
- were not currently taking medication,
- were happy to sign the informed consent form.

Figure 6.5 shows a flowchart of the recruitment period; in total 23 volunteers were excluded because they failed to meet the criteria required to participate in the study, a further volunteer did not complete the study as they started taking medication for blood pressure midway through the intervention, invalidating their data. Finally, 23 subjects complete in the entire study.

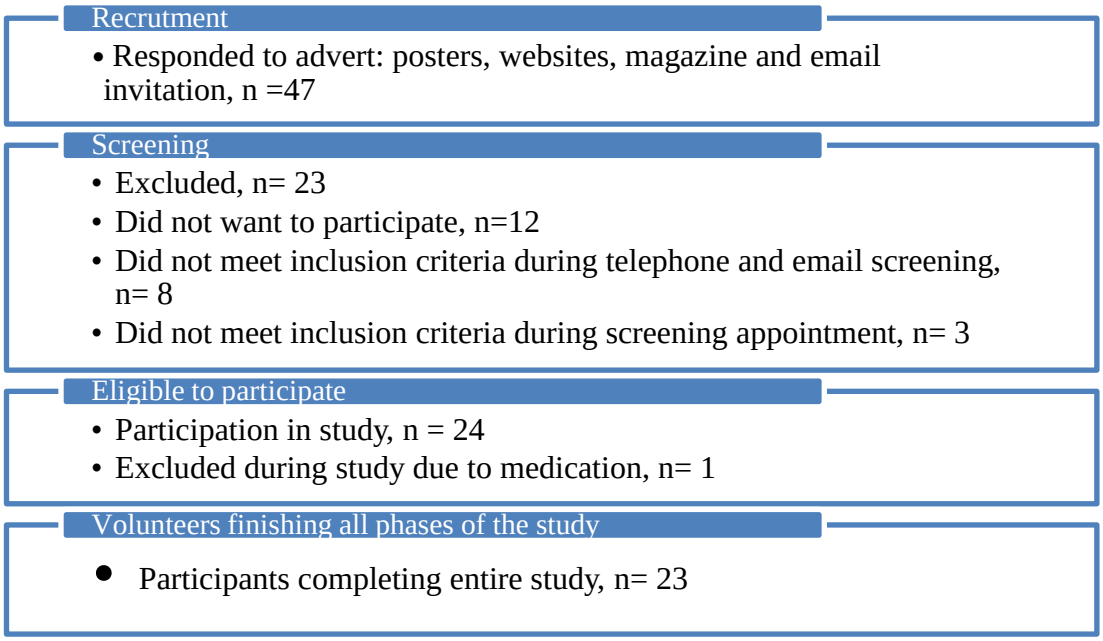


Figure 6.5: Flowchart of the study recruitment.

6.4.2 Design of the study

The study was designed as a 4-week double-blinded, randomized, cross-over intervention trial using a commercially-available orange juice supplement and a placebo control (Figure 6.6). The study lasted for 12 weeks and has 3 phases:

Phase 1 - a commercially-available orange juice supplement or a placebo control was given daily for the first 4 weeks

Phase 2 – a wash-out period of at least 4 weeks of no supplement

Phase 3 – the other supplement, either citrus supplement or a placebo control depending on first phase, was given for 4 more weeks.

The amount of flavanone absorbed and excreted by each individual was measured by collecting 24 hr urine samples at the beginning and end of each phase (analysed in Chapter 7). The vascular health of the individuals was measured by collecting height, weight, waist circumference, fasting blood glucose, BP and PAT, at the start and end of each phase.

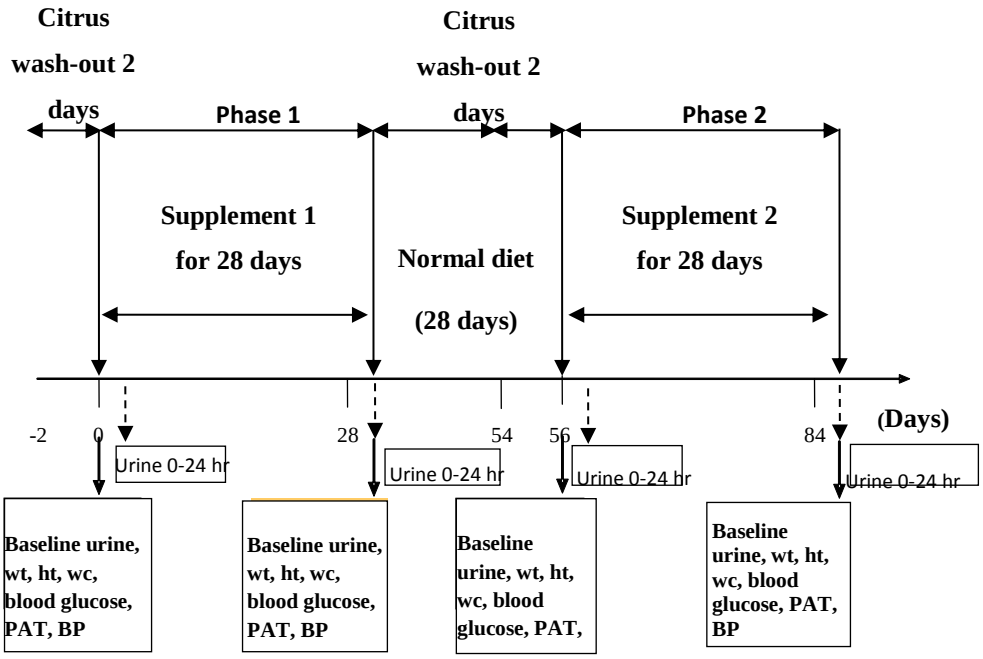


Figure 6.6: Experimental design of the study for 12 weeks. A 4-week double-blinded, randomized, cross-over intervention trial using a commercially-available orange juice supplement and a placebo control. Wt is weight, ht is height, wc is waist circumference, PAT is pulse amplitude tonometry, BP is blood pressure.

The 24 participants were randomised into the different supplement groups, coded A and N, and blinded to the study manager. At least one week after the consent form had been signed the participant attended the first study day. They were asked to refrain for consuming citrus foods/beverages (table 6.1) for 2 days prior to this. On each study day, the participant would arrive at the human study room at 9 am. They had been asked to fast from 9 pm the previous evening, drinking only water after this time. Height was measured using the Leicester height stand meter (Measure, Invicta plastics Ltd, UK); weight was recorded using a Marsden (MAR1047) weight scale, and waist circumference was determined with a Seca 200 measuring tape.

Table 6.1: Food items need to be avoided 48 hours prior to each experimental session.

Food items to avoid
Citrus fruit in any form (fresh, canned or dried): e.g. oranges, tangerines, clementines, satsumas, grapefruit, lemons, limes
Fruit juices, fruit squashes (cordials) and jam or marmalade containing citrus fruit

6.4.3 Evaluation of fasting blood glucose

Fasting blood glucose was monitored using a hand-held glucometer device, Accu-Chek Aviva (Roche Diagnostics; see chapter 5 for validation). Participants cleaned their fingers with an alcohol wipe prior to a finger-prick blood sample being taken using a single-use lancet. The Accu-Chek test strip would be placed adjacent to the blood droplet and allowed to soak into the strip. The blood glucose concentration was measured automatically and the value recorded. Triplicate measurements were taken on different fingers to improve reliability.

6.4.4 Evaluation of blood pressure

The participant was asked to remove tight or restrictive clothing and to relax in a comfortable chair, whilst they completed a citrus food habit questionnaire (see appendix 12). After sitting for 10 min, they were then ask to lie in a supine position on a bed with both arms resting on arm support pads to avoid muscular activities of the arms and fingers. A blood pressure cuff was placed on the upper left arm (see also 6.2.3 for set up of PAT) and then they were asked to continue to relax and refrain from talking. Measurement of blood pressure was calculated automatically using a portable blood pressure device (Lloyds Pharmacy Model (LBPK1) Automatic Blood Pressure Monitor measuring device, made by Kinetik medical devices Ltd, Elstree, Herts, UK). Blood pressure was measured in triplicate with at

least 5 min between each reading. The average of the last two readings were recorded for the study.

6.4.5 Evaluation of peripheral arterial tonometry (PAT)

The supine participant had a thimble-like sensor probe placed on the index finger of the hand undergoing hyperaemia testing (right hand), and a second PAT probe was placed on the control index finger (left hand). A blood pressure cuff was placed on the forearm, just below the elbow, of the right arm, but was left deflated. (See figure 6.2 for set up pictures, although in our study the blood pressure cuff was on the forearm). Once all was in position the sensor probes were inflated and the participant ask not to move their hands or fingers for the monitoring period. The Endo-PAT 2000 device was controlled by a laptop computer using Itamar EndoPAT software (version 2.0). The finger tip blood flow was continuously recorded from this point. After approximately 10 min in the relaxed state, the PAT baseline response was recorded for 5 min (i.e. PAT *pre*-occlusion). The radial artery was then occluded using the blood pressure cuff (220–240 mm Hg), for exactly 5 min. The pressure cuff was then released and the PAT response was monitored for a further 5 min (i.e. PAT *post-occlusion).* The finger probes are then deflated and removed, and the reactive hyperaemia index (RHI) automatically calculated by PAT device (Hamburg *et al* ., 2008, Hamburg & Benjamin., 2009).

The RHI is calculated as the ratio of the occluded arm's mean pulse wave amplitude at 90–150 s post-deflation to the mean amplitude of the same arm at baseline divided by the same ratio from the control arm, the quotient of which is multiplied by a proprietary baseline correction factor (Itamar Medical Ltd), As follows:

Equation 6.1:

$$\text{RHI} = \frac{\text{A/B}}{\text{C/D}} \text{ x baseline correction factor}$$

Where A is the mean digital pulse volume during the reactive hyperaemia, B is the baseline mean digital pulse volume and C and D are the respective values obtained in the control arm. Figure 6.7 shows the typical recording from the Endo-

PAT system and how estimation of peripheral arterial tonometry (PAT) ratio is calculated.

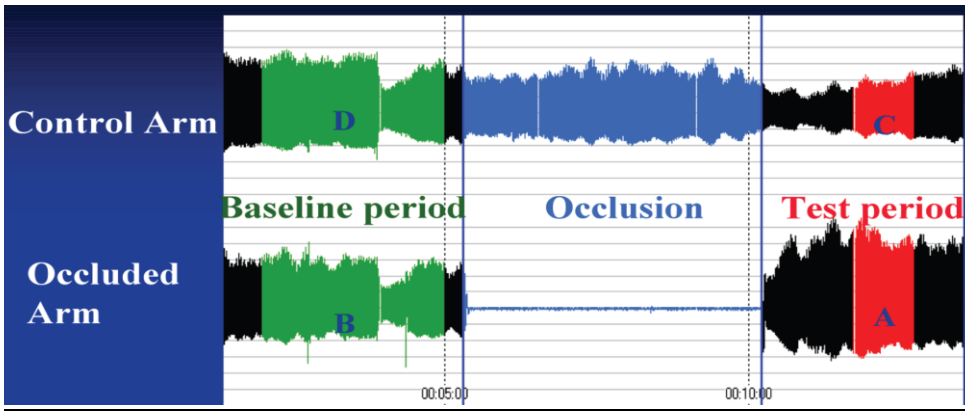


Figure 6.7: Typical recordings from the Endo-PAT. Estimation of peripheral arterial tonometry (PAT) ratio after correction of the reactive hyperaemia index for the control arm. Probe 1 corresponds to the occluded arm and probe 2 to the control arm. The blue-coloured interval corresponds to the duration of occlusion in the test arm (John Lekakis *et al.*, 2011). A, B, C, D refer to equation 6.1.

After finishing the experiment (approximately 30 min), the participant was provided with breakfast and asked to take the first supplements of the study. They were also asked to fill in a brief pain assessment questionnaire (appendix 18) regarding their impression on the intensity of pain from the Endo-PAT set up. The Wong-Baker Face Pain Rating Scale, ranged from (0–5) was used (Newman *et al.*, 2005, Tierney *et al.*, 2009).

6.4.6 Intervention

The supplements used in this study were (i) active phase - Solgar Citrus Bioflavonoid Complex 1000 mg (providing 70 mg hesperidin per tablet; equivalent to approximately 300 ml orange juice); (ii) control phase – a placebo (Fargon, placebo tablet, Germany) that was manufactured to European Pharmacy standards. Participants were asked to consume their normal diets for the duration of the study,

with the exception of 2 days prior to each study day when citrus fruits and juices should be avoided (see table 6.1). They were asked to take two of the provided supplement (in a seal opaque container, blinded to the study manager), every day at breakfast (and before 11 am), starting on day 1 of each phase after the vascular measurements had been taken. Any remaining tablets should be returned on day 28 of each phase. A supplementary questionnaire was provided so participants could write down any days missed, or deviations from the protocol. Urine was collected for 24 on day 1 and day 28. See appindex 11.

The participants were allowed at least 4 weeks without supplements before they were invited back for the third study day in phase 2. This was identical to the first study day, except the alternative supplement was given.

6.5 Statistical analysis

All statistical analyses were performed by using the statistical software package (IBM, SPSS statistics 21, Chicago, USA) and Origin Pro 8.6 software. Within-day reproducibility of the technique was determined by comparing the measurements during the three days. Between days reproducibility was evaluated by comparing the measurements obtained during the three days. A paired t-test was also used to check if there are any differences between the parameters at the beginning (baseline) and at the end of the 4-week period of treatment. Correlation coefficient was determined for PAT and blood pressure measurements with age, gender, and BMI, WC and blood glucose. in order to indicate the difference between these groups. A repeated measurement ANOVA has been used in order to determine any effect of time and treatments, and to trace any interaction between time and treatments on RHI and blood pressure measurements of the two supplements (citrus and placebo supplements) from baseline (day 1) to day 28. The alpha level was set at $P< 0.05$ for all statistical tests; all values are reported as mean +/- SD unless otherwise indicated. Based on a predicted reduction in diastolic blood pressure based on a previous study, and for a confidence level of <0.05, statistical power of >0.8, and to achieve an average reduction in diastolic blood pressure of 3.2 mmHg; 20 participants are required.

6.6 Results

The characteristics of the participants in the study are shown in Table 6.2. Subjects were predominantly male, older than the average student population, and had an average BMI of 30 kg/m^2 (classed as obese). The study was targeted at recruiting older, heavier volunteers, as although cardiovascular disease is not present, these characteristic may suggest greater potential for early metabolic syndrome.

Table 6.2: Baseline characteristics of study subjects

Characteristics	**Mean ± SD**	**Range**
Number of subjects	23 (19 male, 4 female)	
Age, y	37.0 ± 4.2	(29 – 44)
Weight, Kg	87.3 ± 15.1	(66 – 134)
Height, m	1.70 ± 0.07	(1.60 – 1.80)
BMI, kg/m^2	30.0 ± 4.4	(24.3 – 40.0)
Waist circumference, cm	98.0 ± 9.3	(80 – 122)

Values are means ± standard deviation (SD)

Results from the Wong-Baker faces pain rating questionnaire given to participants to evaluate the discomfort and pain of the Endo-PAT procedure are shown in Figure 6.8. In total 65% of participants described the discomfort as little or no pain, whereas 35% rated their discomfort as some pain. None of the participants rated the discomfort at the top two levels of more intense pain.

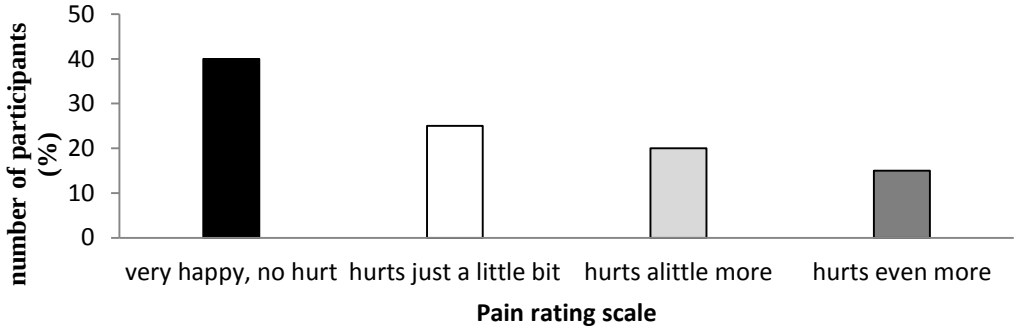


Figure 6.8: Assessment using Wong-Baker faces pain rating scale of the EndoPAT.

The cardiovascular risk factors monitored before and after the 28 day intervention are summarized in tables 6.3 and 6.4. Fasting blood glucose, systolic (SBP) and diastolic blood pressure (DBP) and Augmentation index (AI) all appeared to be reduced after 4 weeks of taking the citrus supplements, however only AI reached statistical significance in a paired t-test, with DBP not quite reaching significance. RHI, the index measuring the endothelial response to occlusion using the Endo-PAT was not affected at all. As expected, there was no difference between any of the parameters measured before and after the placebo intervention.

Table 6.3: Summary of paired t-test, within treatments, before and after consuming citrus supplements for 4 weeks.

Parameters	***citrus supplement baseline***	***citrus supplement 28 d***	***P value (0.05)***	***Paired difference***		
				Mean	**95% confidence interval**	
					Lower	**Upper**
SBP (mm Hg)	115.0 ± 7.9	113.8 ± 7.2	0.19	1.2	-0.7	3.1
DBP (mm Hg)	78.3 ± 6.7	76.9 ± 6.9	0.07	1.4	-0.5	3.2
AI	-3.1 ± 10.8	-4.7 ± 10.7	0.01*	1.7	0.3	3.0
RHI "ratio"	1.9 ± 0.4	1.9 ± 0.5	0.81	- 0.0	- 0.2	0.2
Fasting blood	5.5 ± 0.5	5.1 ± 1.3	0.17	0.4	-0.2	1.0
WC (cm)	98.6 ± 14.4	98.3 ± 14.5	0.14	0.1	-0.1	0.8
BMI (kg/m^2)	29.9 ± 4.4	29.9 ± 4.4	0.86	-0.0	-0.25	0.2

SBP systolic blood pressure; DBP diastolic blood pressure; RHI reactive hyperaemia index; AI Augmentation Index; WC Waist circumference; BMI body mass index. Data are expressed as mean ± SEM (n = 23); means are significantly different at, * significantly different at $P<0.05$.

For the anthropometric measurements, participants in the citrus and placebo group had an average BMI of 30.0 ± 4.4 kg/m^2 at the baseline, and there was no difference in weight loss for all participants, or between males and female for either the citrus or placebo group. Similar results were obtained for waist circumference. As expected, there was no significant change in any anthropometric measures observed in both groups from baseline to post intervention, or between groups after controlling for baseline values.

Table 6.4: Summary of paired t-test, within treatments, before and after consuming placebo supplements for 4 weeks.

Parameters	***Placebo supplement baseline***	***Placebo supplement 28 d***	***P value (0.05)***	***Paired difference***		
				Mean	**95% confidence interval**	
					Lower	**Upper**
Fasting blood glucose	5.4 ± 0.4	5.41 ± 0.5	0.8	0.0	-0.2	0.2
SBP (mm Hg)	114.7± 7.1	115.5 ± 8.4	0.4	-0.8	-2.7	-1.2
DBP (mm Hg)	77.5± 5.5	77.6 ± 6.3	0.9	-0.1	-1.9	1.7
RHI "ratio"	2.0 ± 0.6	1.9 ± 0.4	0.6	- 0.1	- 0.2	0.3
AI	-5.6± 9.5	-4.8 ± 9.9	0.8	-0.3	-3.3	2.6
WC (cm)	98.5± 8.8	98.2 ± 8.8	0.1	0.3	-0.1	0.7
BMI (kg/m^2)	29.9 ±4.3	29.9 ± 4.1	0.7	-0.0	-0.2	0.2

SBP systolic blood pressure; DBP diastolic blood pressure; RHI reactive hyperaemia index ; AI Augmentation Index; WC Waist circumference; BMI body mass index. Data are expressed as mean± SEM (n = 23)

Figure 6.9 shows the concentration of blood glucose before and after ingestion of supplements for 4 weeks. Although a decline in fasting blood glucose was observed, the large variation in response means this result was not significant with the number of participants in this study. The diastolic and systolic blood

pressure changes are shown in Figure 6.10 and 6.11, respectively. A non-significant (p =0.07) reduction in mean diastolic blood pressure pre- and post- citrus supplementation of -1.4 mmHg was observed, although for some individuals the decrease in DBP up to -11 mmHg. A trend in reduction of systolic blood pressure was also observed at -1.2 mmHg. No significant change in blood pressure was observed in the placebo group before and after the intervention with regard to systolic and diastolic blood pressure.

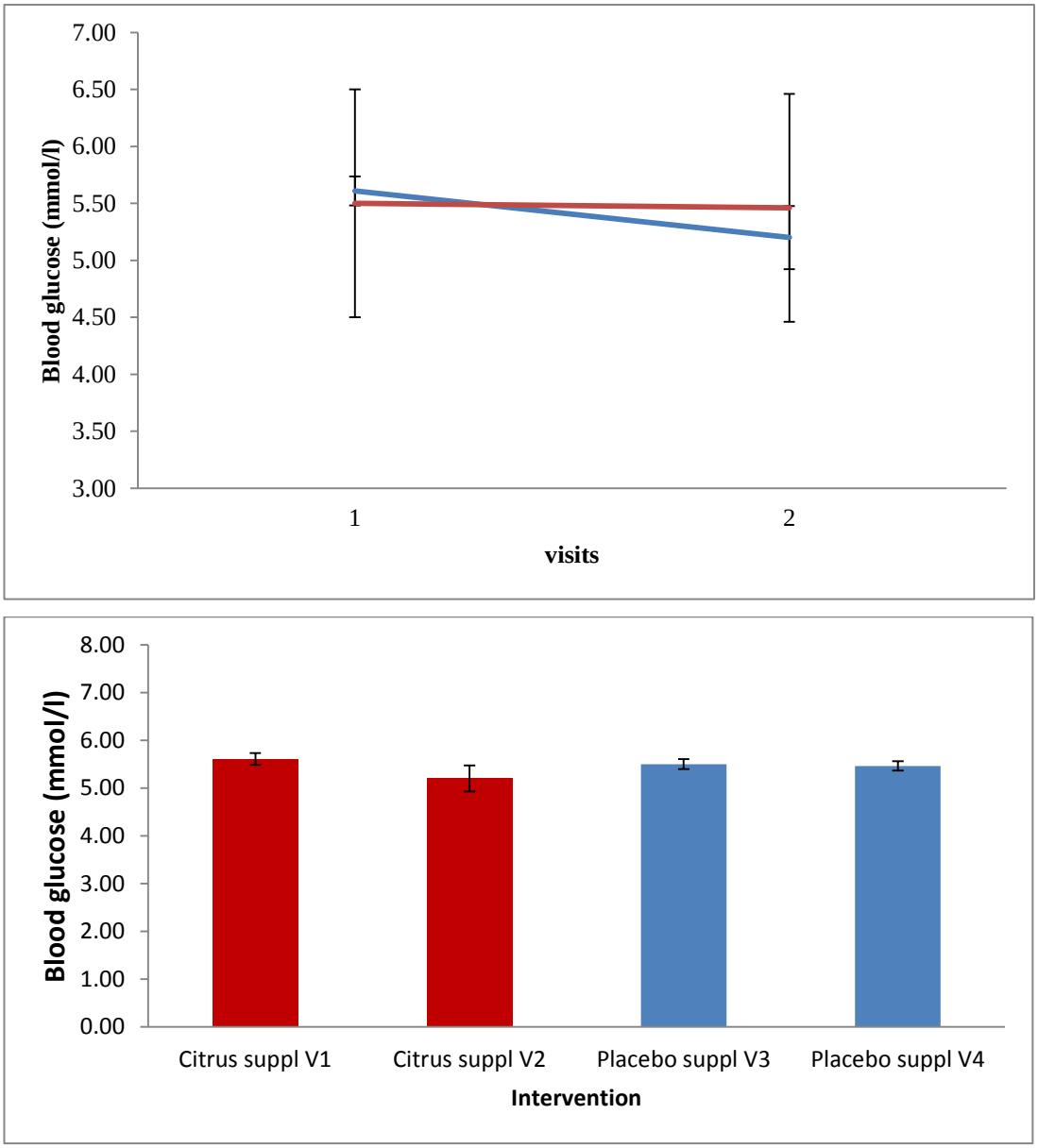


Figure 6.9: Change in fasting blood glucose level before and after consumption of citrus and placebo supplements (suppl). Data are expressed as mean ± SEM (n = 23), (Red) citrus treatment, (Blue) placebo treatment, * between group comparisons significant at (P < 0.05). (V: visit).

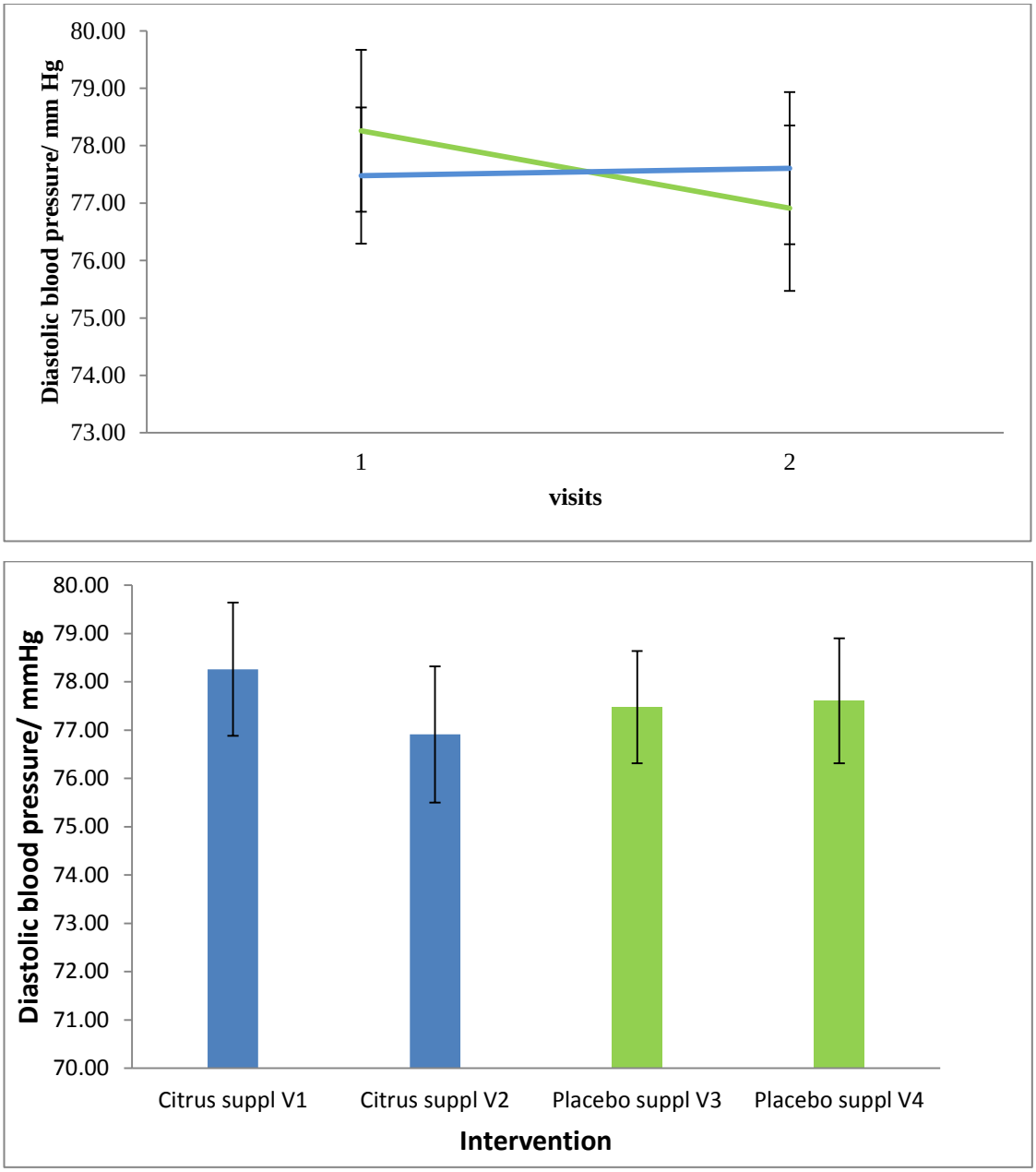


Figure 6.10: Change in diastolic blood pressure before and after citrus bioflavonoid and placebo supplements. Data are expressed as mean ± SEM (n = 23); (Blue) citrus treatment, (Green) placebo treatment. *between group comparisons significant at ($P < 0.05$). (V: visit).

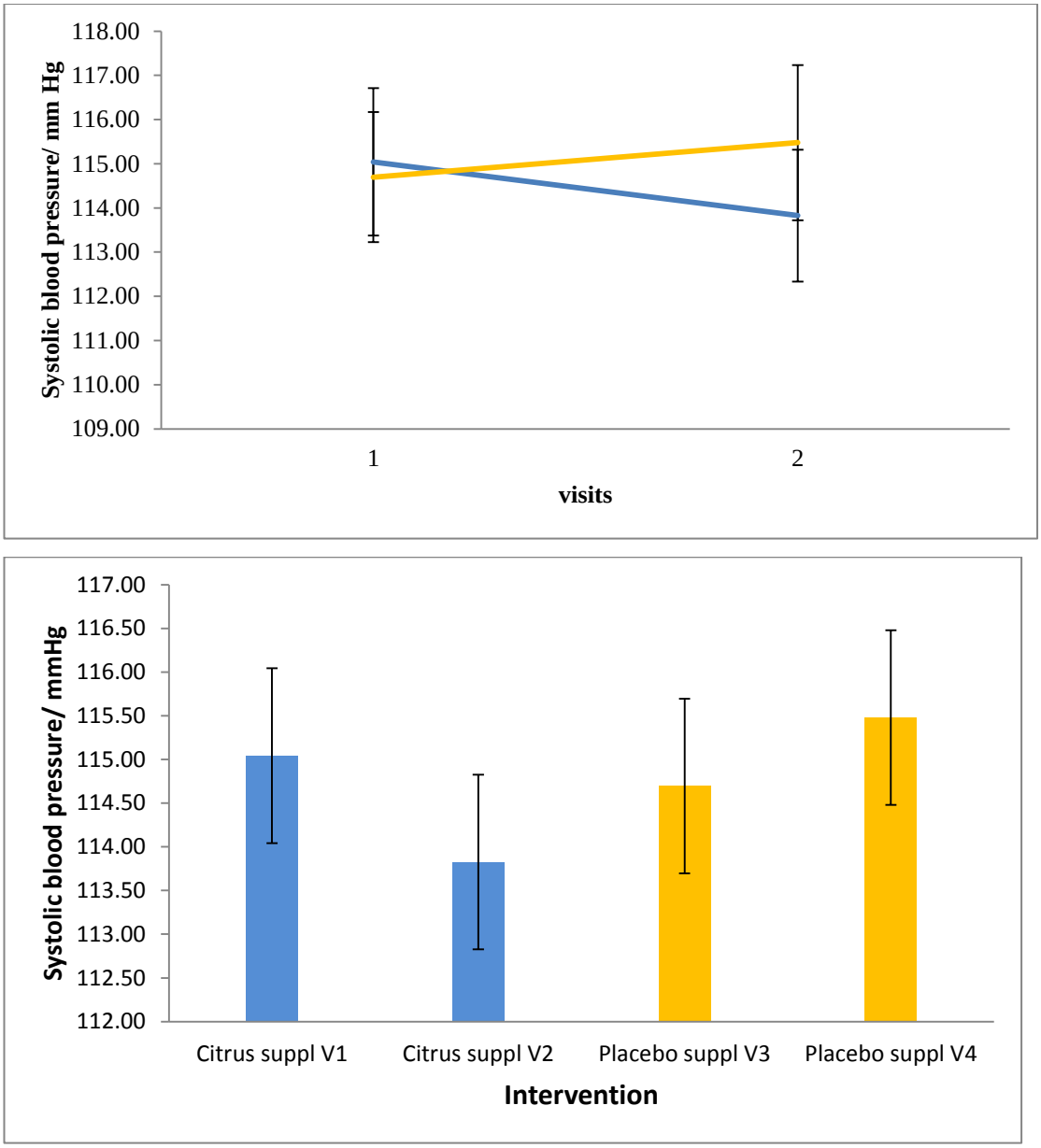
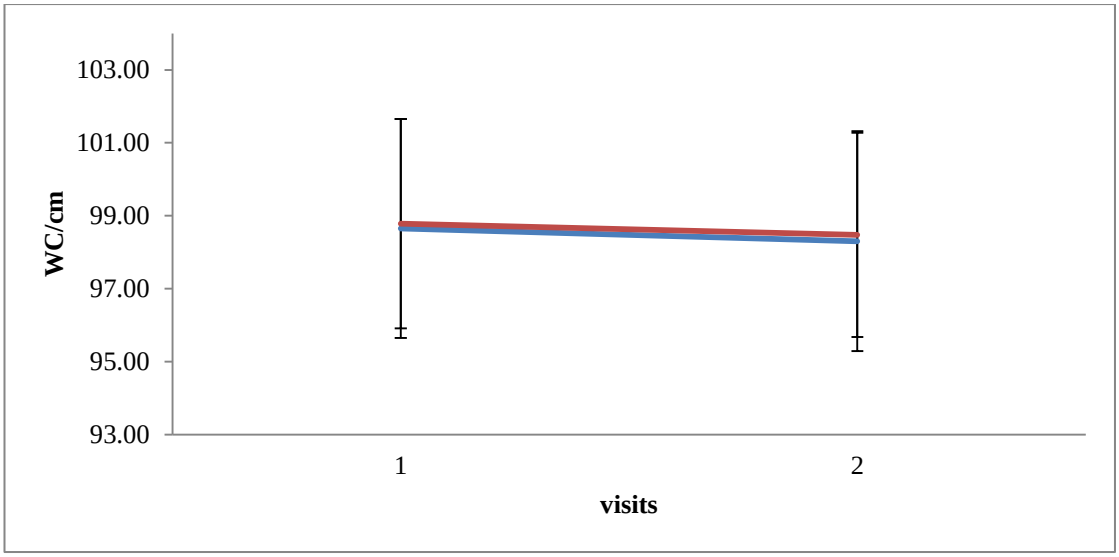


Figure 6.11: Change in systolic blood pressure before and after citrus bioflavonoid and placebo supplements (suppl). Data are expressed as mean ± SEM (n = 23); between group comparisons significant at *P < 0.05, (Blue) citrus treatment, (Orange) placebo treatment. (V: visit).



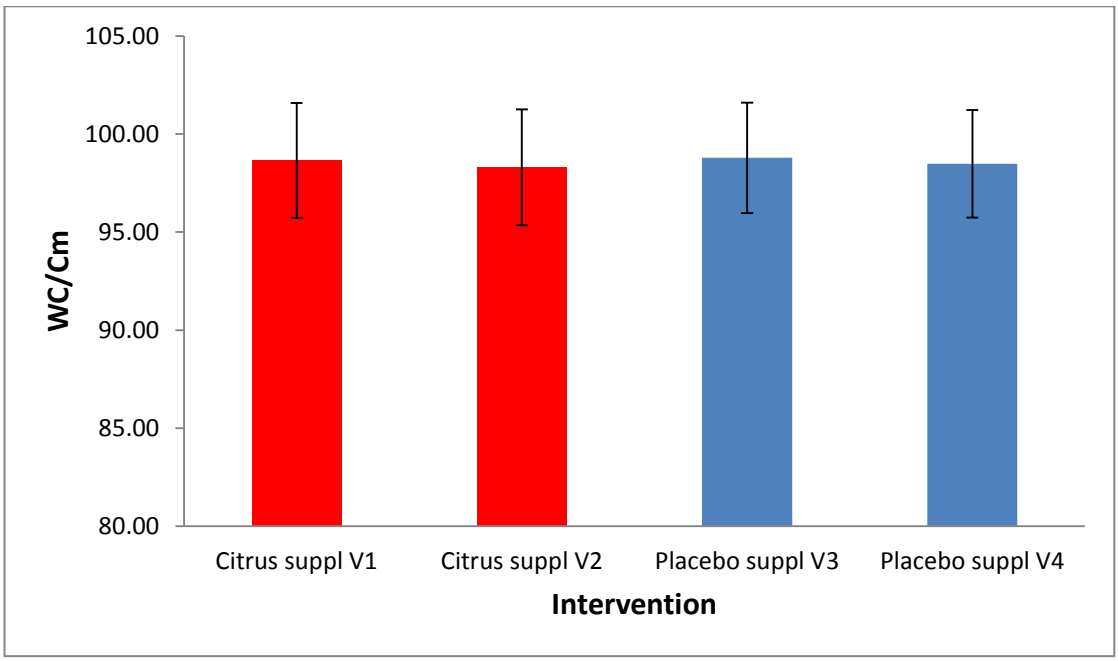


Figure 6.12: Change in waist circumference (WC) before and after citrus bioflavonoid and placebo supplements (suppl). Data are expressed as mean ± SEM (n = 23); (Red) citrus treatment, (Blue) placebo treatment, between group comparisons significant at *P < 0.05. (V: visit).

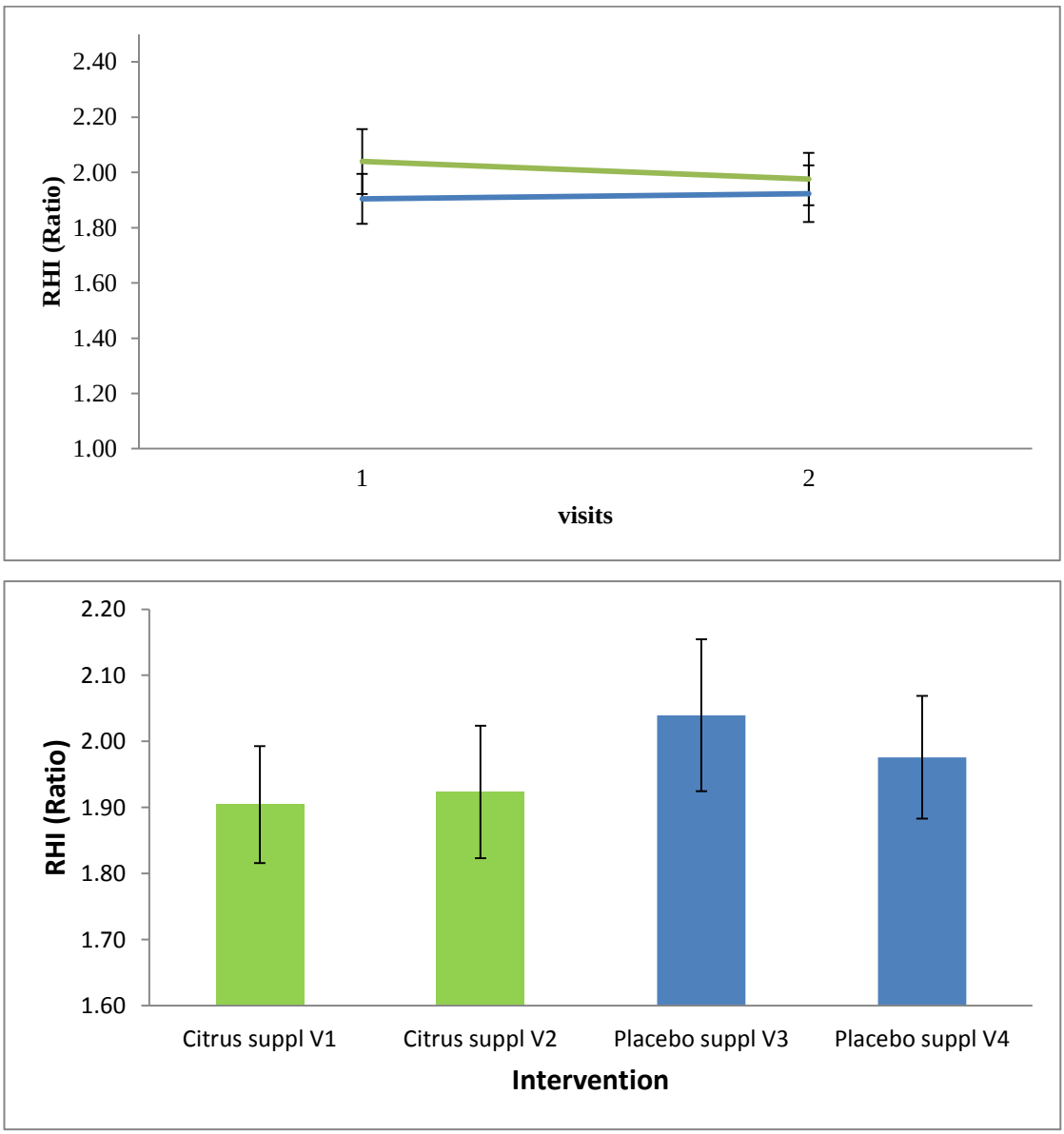


Figure 6.13: Mean change in reactive hyperaemia index (RHI) before and after citrus bioflavonoid and placebo supplements (suppl) intervention. Data are expressed as mean ± SEM (n = 23); (Green) citrus treatment, (Blue) placebo treatment, between group comparisons significant at *P < 0.05; between baseline measurements significant at α P < 0.05. (V: visit).

The result of the Endo-PAT assessment gives an automated reactive hyperaemia index (RHI), which is represented in Figure 6.13. The result represented the maximum response over the whole post-occlusion period (7-min). Notably, the

index of 46% of the subjects increased (improved condition) after 4 weeks of citrus supplement compared to the baseline, however, the increase overall was not statistically significant. On the other hand, the RHI after the placebo supplementation for weeks decreased, but again was not significantly different in comparison to the baseline values. Furthermore there was a statistically not significant difference in the baseline values between the citrus group and the placebo group ($p= 0.08$), with the placebo group displaying higher (more favourable) endothelial function. As the participants were all randomised at the start of the study to receive either the placebo or the citrus supplement first, there should not have been a difference in the baseline measurements.

The augmentation index (AIx) was derived from the digital pulse wave (automated output of the EndoPAT software). AIx expresses the (mathematically derived) augmentation of the central aortic pressure. Augmentation index (AI) as calculated from the pulse wave in the finger tips (Figure 6.14) displayed a clear marked decrease after citrus supplements intervention from -3.1 ± 10.8 to -4.7 ± 10.7, which was significant ($p=0.01$). As the decrease signifies improvement, it can be noted that chronic consumption of citrus consistently benefited digital augmentation index among 65% of the participants. The AI did not alter after placebo supplement intervention. However, just as with the RHI there was significant difference ($p = 0.07$) between the baseline values of citrus or placebo treatment, with the improved endothelial function represented in the placebo baseline.

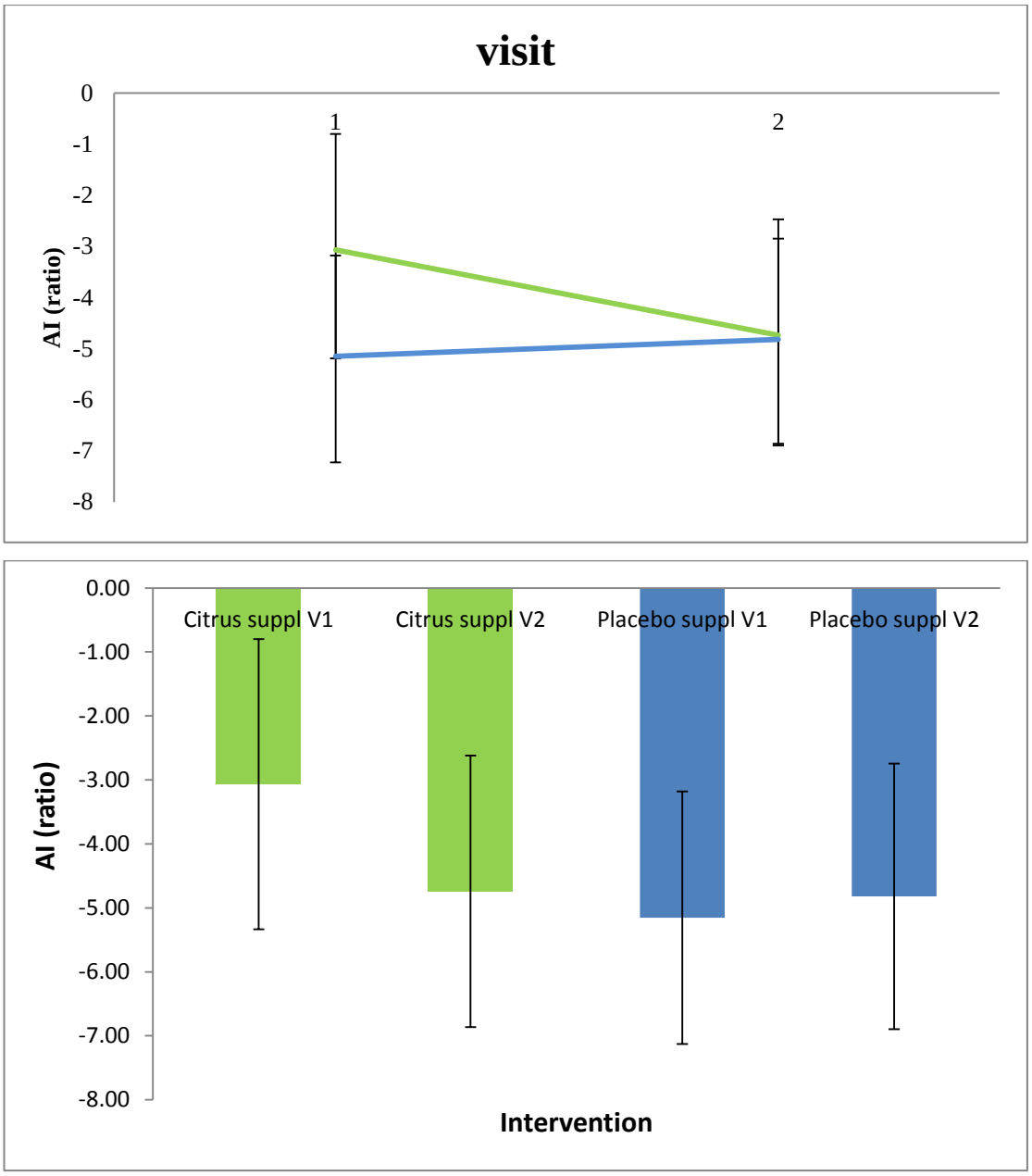


Figure 6.14: Mean change in augmentation index (AI) before and after citrus bioflavonoid and placebo supplements (suppl) intervention. Data are expressed as mean± SEM (n = 23); (Green) citrus treatment, (Blue) placebo treatment; between group comparisons significant at *P < 0.05; between baseline measurements significant at P < 0.05. (V: visit).

6.7 Repeated measures ANOVA design

This is a statistical test that is used when the same subjects take part in all the conditions of an experiment (placebo-controlled, cross-over design). We assume that the relationship between pairs of experimental condition is similar (known as the assumption of sphericity) and sphericity is met when these variances are equal. The hypothesis that the variances of the difference between conditions are equal is measured by Mauchly's test. Repeated measurement ANOVA used when there are repeated measurement on the same sampling units and the higher the F-value, the more likely the difference between samples are statistically different.

Table 6.5 summarises the descriptive and repeated-measures ANOVA results for all parameters assessed in four visits using 2 different supplements (citrus and placebo). According to the means ± SE, it can be concluded that some category means are different from each other. Mauchly's test for these data was checked and the significance value was less than 0.05 are <0.005, 0.03, for fasting blood glucose, AI, respectively. The hypothesis that the variances differences between levels were significantly different is accepted.

The next stage is which of the means are significantly different by using test of within-subjects effects, where we can determine the F and p value that indicate whether these means differ significantly or not. The result obtained from the test of sphericity, can be used to accept or reject the null hypothesis for this analysis. We should use sphericity assumed correction when sphericity test p value was > 0.05, and we should use Greenhouse-Geisser correction when the sphericity test p values were < 0.05 as shown in table 6.5. Using this correction, none of the F values were significant.

We have conducted Hotelling's Trace post-hoc tests to indicate significant differences between category means. These tests search for sources of the significant results by comparing two groups or two combinations of groups using t-tests; i.e. using paired-samples t-tests for a repeated-measures ANOVA's post hoc tests. It can be concluded that all p values were > 0.05 which mean that there were no differences in the means between groups and within the same group. Therefore, it can be stated that the effect of supplements were the same.

Table 6.5: A summary of repeated measures one way ANOVA between 4 visits.

	Citrus supplements (mean ± SE)		Placebo supplements (mean ± SE)		Mauchly's Test F-value	Tests of Within-Subjects Effects P-value		Hotelling's test p-value
	Visit 1	Visit 2	Visit 3	Visit 4		Sphericity Assumed	Greenhoue-Geisser	
Blood glucose mmol/L	5.5±0.5	5.1±1.2	5.4±0.4	5.4±0.4	<0.005	-	0.29	0.48
SBP (mm gh)	114.3±7.2	113.1±6.10	114.7±7.0	115.5±8.4	0.06	0.21	-	0.23
DBP (mm gh)	78.8±6.0	76.9±6.9	77.5±5.6	77.6±6.3	0.25	0.38	-	0.36
RHI "ratio"	1.90±0.4	1.92±0.4	2.03±0.5	1.97±0.4	0.34	0.51	-	0.40
AI	-3.06±10.8	-4.74±10.1	-5.15±9.4	-4.81±9.9	0.03	-	0.25	0.27

Bivariate correlations of PAT index with parameters measured in this study among 23 participants are described in table 6.6. Age and blood glucose level were significantly correlated, ($r = 0.41$, $p=0.04$). BMI and WC were strongly correlated, ($r = 0.80$, $p < 0.01$). Moreover, the correlation between diastolic blood pressure measurements and systolic blood pressure and augmentation index (AI) was significantly positively correlated ($r = 0.54$, $p < 0.01$) and ($r = 0.06$, $p < 0.01$), respectively.

Moreover, correlation between the total amount of hesperetin conjugate (glucuronides and sulfates) present in urine samples over 24 hr post-intervention (see chapter 7) with vascular reactivity and function measurements showed an inverse, or weak, correlation. Both RHI and SBP were inversely correlated ($r= -0.37$, $p=0.0.08$) and ($r = -0.12$, $p=0.57$). Similarly, other measures of vascular reactivity and function, such as DBP change ($r=0.12$, $p=0.55$), blood glucose change ($r=0.29$, $p=0.17$), AI change ($r=0.10$, $p=0.65$), all measurements displayed low or non-existent correlations with total urinary hesperetin metabolites after citrus bioflavonoids tablets were consumed for 4 wk. moreover, weak positive association was found when correlated with WC and BMI, ($r=0.05$, $p=0.79$), ($r=0.16$, $p=0.45$) respectively. as it can be seen in (figure 6.13).

Table 6.6: Adjusted Pearson's correlations of changes in CVD risk factors among participants consumed citrus bioflavonoids tablets (n=23).

	Age (y)	**BMI**	**WC Cm**	**Blood**	**DBP**	**SBP**	**Pulse rate**	**RHI "ratio"**	**AI**
Age (y)		-.043 (0.84)	.077(0.72)	**0.41(0.05)***	-0.21(0.33)	-0.38(0.06)	0.23(0.30)	0.17(0.39)	0.011(.962)
BMI (kg/m2)	-.043(0.84)		**0.80**(.00)**	.013(0.56)	.332(0.35)	.07(0.16)	.35(0.09)	.396(0.17)	0.24(0.26)
WC Cm	.077(0.72)	**0.80**(0.00)**		.18(0.40)	0.07(0.73)	0.05(0.80)	0.16(0.46)	0.21(0.33)	0.13(0.56)
Blood glucose	**0.41(0.05)***	.013(0.56)	.18(0.40)		0.15(0.47)	-0.14(0.51)	0.07(0.75)	-0.28(0.19)	0.32(0.12)
DBP(mm Hg)	-0.21(0.33)	.332(0.35)	0.07(0.73)	0.15(0.47)		**0.54** (<0.05)**	-0.15(0.47)	-0.3(0.89)	**0.63** (<0.05)**
SBP (mm Hg)	-0.38(0.06)	0.07(0.16)	0.05(0.80)	-0.14(0.51)	**0.54** (<0.05)**		0.22(0.30)	0.26(0.22)	0.28(0.18)
Pulse rate	0.23(0.30)	.35(0.09)	0.16(0.46)	0.07(0.75)	-0.15(0.47)	0.22(0.30)		0.07(0.72)	-0.02(0.93)
RHI "ratio"	0.17(0.39)	.396(0.17)	0.21(0.33)	-0.28(0.19)	-0.3(0.89)	0.26(0.22)	0.07(0.72)		0.03(0.87)
AI	0.01(.962)	0.24(0.26)	0.13(0.56)	0.32(0.12)	**0.63** (<0.05)**	0.28(0.18)	-0.02(0.93)	0.03(0.87)	

Data presented as r (p-value) are represented in bold value. Abbreviation: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; RHI, reactive hyperaemia index ; AI, Augmentation Index; WC, Waist circumference;

*Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed).

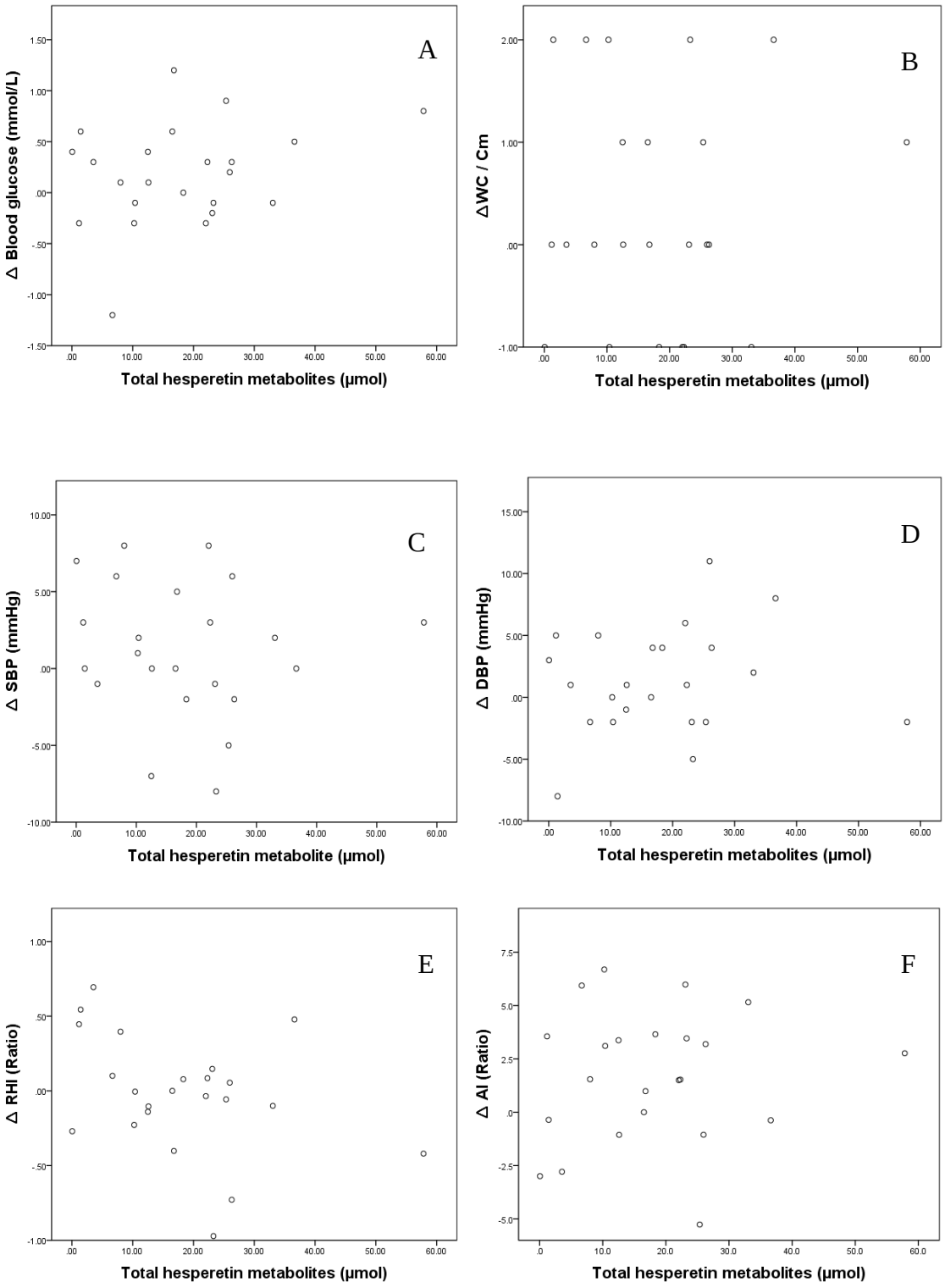


Figure 6.15: Correlation of change in endothelial function biomarkers with total hesperetin conjugates. Correlation of amount of metabolites (sum of hesperetin glucuronide and sulfate) excreted in urine in 24 hr after 4 weeks of citrus bioflavonoids tablets were ingested compared to the change observed in the parameters. Blood glucose (A), WC (B), SPB (C), DBP (D), RHI (E), AI (F).

6.8 Discussion

The overall aim of this study was to investigate, for the first time, the effect of commercially available citrus bioflavonoid supplements on endothelial function by measuring changes in some CVD risk markers. The specific focus was on changes in BP, as this parameter has been shown in previous studies to be reduced by orange juice consumption and the data from these studies had been used in the power calculations to determine number of volunteers required. The secondary aim was to monitor changes in PAT after supplementation, using an Endo-PAT. Changes in endothelial function after orange juice consumption have previously been shown using FMD measurements, but the Endo-PAT has rarely been used in any type of intervention studies (Hamburg and Benjamin, 2009, Moerland *et al.*, 2012, Allan *et al.*, 2013), and this is the first study, to the best of our knowledge, to measure Endo-PAT after flavavone intake.

Although the evidence was weak, and not statistically significant, there was a trend towards a reduction in both systolic and diastolic BP after 4 weeks of citrus supplementation compared to the placebo. The reduction in SBP and DBP were by 1.2 and 1.4 mmHg, respectively. Based on previous studies on citrus juices (Morand *et al.*, 2011, Buscemi *et al.*, 2012), DBP was significantly reduced by at least 3 mmHg. It has been estimated that even if the blood pressure lowering effect is moderate, in persons aged 50 – 69 y with an SBP of 150 mmHg and a DBP of 90 mmHg, a 3-4 mmHg decrease in DBP would reduce the incidence of coronary artery disease by 20% (Law *et al.*, 2009). Not all studies on flavanones have been consistent. For example, Rizza *et al.*, (2011) found no change in blood pressure after three weeks consumption with 500 mg of hesperidin (Rizza *et al.*, 2011). Orange and other juices also contain vitamin C, potassium, fibre, carotenoids, possibly anthocyanins, and also sugar. The supplements used in this study did not contain any of these additional compounds. These compounds might have contributed favourably (e.g. potassium) or negatively (e.g. sugar) to the blood pressure and endothelial effects. Larger trials, for a longer time periods, that monitor the influence of the non-flavonoid components as well, may be required to determine the effect of flavanones on BP.

DBP is a good indicator for peripheral vessel resistance (Franklin, 2007), however changes in overall endothelial function after 4 weeks of citrus supplementation was assessed by measuring changes in reactive hyperaemia index (RHI) and augmentation index (AI) using an EndoPAT. RHI was not affected by the citrus intervention. AI did show a significant change between baseline and 4 weeks after supplements were taken, however the baseline measurements between the supplement and the placebo were also significantly different which could indicate the variability in this measurement is less reliable. All the volunteers had an RHI in the normal healthy range.

A placebo-controlled crossover study involving participants with metabolic syndrome showed an improvement in FMD after three weeks consumption with 500 mg of hesperidin, despite no change on blood pressure (Rizza *et al.,* 2011). Morand *et al.,* (2011) also reported that daily intake of 500 ml OJ for 4 weeks improved endothelial function significantly. In another study (Buscemi *et al.,* 2012), FMD increased significantly (5.7% compared with 7.9%, $p < 0.005$) after one week of red orange juice (containing flavavones and anthocyanins) consumption in volunteers with increased CV risk. Recent studies on anthocyanin-rich juices have not find significant increases in FMD. A study conducted among 13 hypertensive men (39-68 y) investigated the acute effects of pomegranate juice on blood pressure and endothelial function measuring the change in FMD. A significant reduction in both SBP and DBP were observed, but brachial artery FMD did not change significantly (Asgary *et al.,* 2013). A study testing the effects of cranberry juice did show a decrease in pulse wave velocity, which is a measure of arterial stiffness, but the FMD, which measure the brachial artery, did not change (Dohadwala *et al.,* 2011). Acute changes (2 hr) in FMD were significantly improved in healthy adults after intake of grape polyphenols (Li *et al.,* 2013).

The possible mechanisms by which the flavonoids-rich foods are lowering BP and improving FMD may involve a chronic increase in the production of NO by vascular endothelium (Grassi *et al.,* 2009, Morand *et al.,* 2011, Asgary and Keshvari, 2013). In addition, inhibitory effect on angiotensin-converting enzyme could be another mechanism responsible for the blood reducing effects of flavanones (Actis-Goretta *et al.,* 2006, Morand *et al.,* 2011). Moreover, the study by

Rizza *et al.*, (2011) reported that hesperetin treatment increased FMD and reduced circulating markers of inflammation. This has the vascular-protective actions that may explain the beneficial effects of citrus intake on CVD. The same study reported that hesperetin acutely stimulated phosphorylation of AMP kinase, and eNOS which mediate NO production by endothelial cells.

The present study did not find a significant difference in fasting blood glucose levels after citrus supplement treatment (5.5 ± 0.5 mmol/L and 5.1 ± 1.2 mmol/L; p = 0.17). These findings are consistent with previous studies which showed that citrus flavonoids intake did not improve fasting blood glucose level after 500 ml orange juice daily for a 4 wk period (Morand *et al.*, 2011). In addition, our results were consistent with the results obtained from a recently published study which recruited 22 healthy volunteers and examined the effects of commercial fresh orange on endothelial function markers in healthy volunteers for 4 wk. The study concluded that fasting blood glucose did not change after treatments (Asgary *et al.*, 2014). No significant differences were observed in fasting plasma glucose, and fasting plasma insulin concentrations (Rizza *et al.*, 2011).

Hesperidin may have an important role in the modulation of gene expression and activities of hepatic glucose metabolic enzymes such as glucokinase and glucose-6-phosphatase. These enzymes are involved in the final step of catalysing the gluconeogenesis and glycolysis, and therefore can have a hypoglycaemic effect and play a role in regulating the homeostatic plasma glucose (Jung *et al.*, 2006, de Oliveira *et al.*, 2013). Moreover, isolated hesperidin in rats significantly increased the number of GLUT-2 and GLUT-4 carriers enhancing cellular signalling and glucose transport and consequently reducing insulin resistance (de Oliveira *et al.*, 2013). However, the mechanisms of how citrus flavanones may influence glucose control and insulin sensitivity in non-diabetic volunteers are unclear. One possible explanation is that participants have a fasting glucose concentration which fluctuates within the normal range, and hesperidin treatment does not affect the physiologic regulation of plasma glucose and insulin concentration under these parameters.

6.9 Limitations of the study

This study has certain limitations that need to be acknowledged. These are related to the size of the sample of participants, differences in health status of participants, food habits and physical activities of the participants, the length of the study and the dose of flavanones given, and the differences in the tablets and placebos given. This study did not recruit the same number of female and male (4 females and 19 males) and the influence of gender could not be taken into account. It assumed that the sample size of 23 participants would be enough to determine a change in BP of >3 mmHg, but the majority of participants were normotensive and therefore may be less responsive to changes. The same is true for RHI, in that normal RHI may not be influenced and only if an individual has endothelial dysfunction can an improvement be shown. The small sample size could account for the lack of detecting significant difference in blood pressure measurements, as a small decrease was observed and perhaps a larger cohort would have provided more evidence. Furthermore, a larger and/or longer treatment could influence the blood pressure measurements as many intervention studies on blood pressure use 3 months or longer.

Changes in RHI using Endo-PAT has not been used in many previous intervention studies, and although it has been shown to correlate well with endothelial dysfunction and overall CV risk, it is unclear whether short-term changes will be monitored in an intervention study. Few studies have investigated intra-individual variation in response to RHI measurement, and we were not able to do this in the current study (see Chapter 5 validation of BP), due to the prohibitive cost of the probes required. FMD measurements in previous studies have been affected by flavanones and other flavonoids in both chronic and acute studies (see chapter 1), and so this does appear to be a more robust measurement of endothelial function, however it can be significantly affected by end-user variability.

Although the study was a double-blind study, with neither the researcher nor the participants aware of which supplements they had been given, there were dramatic differences in the size, shape and colour of the tablets. This was because both supplements were commercially available and therefore had not been matched, as they would be in a larger scale RCT. The citrus supplements were larger and

brown in colour and the placebo were small and white. The researcher was blinded as far as possible by providing opaque pre-labelled bottles that were given to the participants, and the participant was not directly aware of which tablet was which. However it could be stated that these differences could have influenced the design of the study by making it not a fully double-blind study.

6.10 Summary

In summary, flavanones may reduce the risk of CVD, particularly by reducing the level of BP, vascular reactivity and fasting blood glucose, however in this study further supporting evidence for these affects were not found using commercially available citrus supplements in a 4 week intervention. We observed trends towards favourable changes in BP after the consumption of citrus supplements, but significance was not reached under the conditions of the study. The flavanone hesperidin could be responsible for the observed reduction, but it could be that other components of orange or citrus juices may contribute to the effects seen in other studies where significant reduction in blood pressure, or improvement in endothelial function were monitored.

More randomized controlled human studies are needed to evaluate the effects of hesperidin on blood glucose control, endothelial function and BP lowering effect. Before recommending that citrus bioflavonoid supplements are an option for people to promote CV health, long-term and high quality RCTs need to be conducted with more participants male and female from different age groups, especially those who are at high risk of CVD. Further studies are needed to identify the optimal dose of flavanones required to improve blood pressure, fasting glucose and endothelial health and additional parameters that could be influenced by flavanones.

Chapter 7: Bioavailability Of Hesperetin-Conjugate From Citrus Supplements

7.1 Abstract

The human study described in chapter 6 was also conducted to investigate the bioavailability, as measured by urinary excretion, of hesperidin from citrus supplements. The aim of this chapter was to identify and quantify metabolites of hesperetin excreted in urine after the acute 1 day and chronic 28 days ingestion of citrus bioflavonoids tablets by 23 participants. Urine was collected over 24 hr at the beginning and at the end of each phase after 4 weeks and hesperetin metabolites were quantified using LC-MS analysis.

Three hesperetin metabolites were detected in substantial amounts in urine samples taken 1 day and 28 days after the intake of citrus supplements. The main urinary metabolite present was hesperetin-3′-*O*-glucuronide, with 10.0 ± 6.4 µmol/24 hr and 8.5 ± 7.0 µmol/24 hr being excreted after 1 day and 28 days. Hesperetin-3′-*O*-sulfate was the second most abundant flavanone at 8.5 ± 5.2 and 8.3 ± 4.3 µmol/24 hr at 1 day and 28 days respectively. Hesperetin-7-*O*-glucuronide was also detected with an average of 4.1 ± 3.2 µmol and 3.1 ± 2.8 µmol/24 hr after 1 day and 28 days, respectively.

The urinary excretion of hesperetin-*O*-glucuronide as a proportion of the dose ingested was 7.5 ± 3.4% and 6.2 ± 3.7% after 1 day and 28 days, respectively. The excretion of hesperetin-3′-*O*-sulfate was 4.51 ± 2.71% and 3.36 ± 2.24% after 1 day and 28 days, respectively. Daily supplementation of hesperidin over 28 days did not significantly change the amount of glucuronide excreted, although hesperetin-3′-O-sulfate excretion significantly decreased after 28 days ingestion of citrus tablets ($p < 0.05$).

Hesperetin metabolites appear to be more bioavailable from citrus tablets compared to the orange juice used in this study based on the dose ingested, although further studies would need to confirm this.

7.2 Introduction

Citrus fruits are rich sources of flavanones and are widely consumed as part of human diets (Brett *et al.*, 2009, Turner and Burri, 2013). The regular consumption of orange juice has been associated with protective health benefits, such as reducing the incidence of cardiovascular disease (Morand *et al.*, 2011, Chanet *et al.*, 2012b), possibly improving endothelial function and reducing blood pressure (Morand *et al.*, 2011, Buscemi *et al.*, 2012).

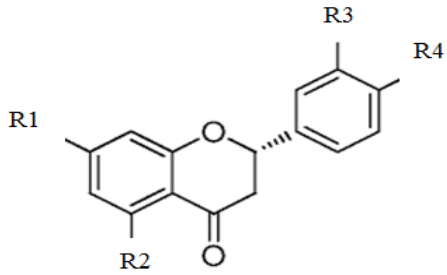
In general, flavonoids, mainly glycosides, are present in plants as conjugated forms. Hesperidin is the major flavanone in orange fruit and juice. It is found as the 7-*O*-rutinoside – hesperidin (Nielsen *et al.*, 2006, Bredsdorff *et al.*, 2010). The bioavailability of flavanones and the presence of their metabolites in biological fluids such as plasma and urine samples have been investigated using LC-MS (Brett *et al.*, 2009, Bredsdorff *et al.*, 2010, Yamamoto *et al.*, 2013). After oral ingestion, hesperidin is expected to be hydrolysed in the colon by β-glycosidases derived from gut microflora. The hesperetin aglycone is then absorbed and metabolised by UDP-glucuronosyltransferase (UGTSs) and sulfotransferase (SULTs) to glucuronides and sulfates metabolites in the intestinal cells (Brand *et al.*, 2010, Yamamoto *et al.*, 2013) or broken down by the colonic microbiota and produce phenolic acids catabolites (Roowi *et al.*, 2009). These potentially bioactive compounds are distributed through the plasma around the body and a significant amount is excreted in the urine (Matsumoto *et al.*, 2004). The main hesperetin conjugates are hesperetin-7-*O*-glucuronide, hesperetin-3′-*O*-glucuronide, hesperetin-di-glucuronides, hesperetin sulfa-glucuronides and hesperetin-3′-*O*-sulfate (Matsumoto *et al.*, 2004, Mullen *et al.*, 2008, Bredsdorff *et al.*, 2010). Hesperetin and its respective conjugates are shown in Figure 7.1.

Biological samples such as urine and plasma collected from human intervention studies after the consumption of OJ, have been used to measure

hesperetin compounds after de-conjugation with sulfatase and β-glucuronidase prior to LC-MS analysis (Nielsen *et al.*, 2006, Gardana *et al.*, 2007). However, due to some inconsistencies in hydrolysis by sulfatase (Bredsdorff *et al.*, 2010), and a growing interest in improving the knowledge of the form that OJ flavanones and metabolites are present and involve the analysis of conjugated forms of citrus flavanones metabolites mainly (glucuronide and sulfate) relevant to free-form standards (Pereira-Caro *et al.*, 2014). Free form standards usually generate standard curve for quantification of conjugates relative to their free forms.

Hesperetin metabolites have been assayed by LC-MS as this technique is very sensitive and selective, and has a high throughout it is particularly sensitive when dealing with complex matrices and mixtures as the peaks of the compounds of interest may overlap each other (Brett *et al.*, 2009, Bredsdorff *et al.*, 2010, Di Donna *et al.*, 2013), And cannot be separated by UV. MS improves identification by monitoring at selected mass for expected compounds.

The main aim of this study was to identify and quantify the metabolites of hesperidin from citrus supplement in humans using a 24 hr urinary collection, both in after an acute dose of 24 hr and after a 4 wk chronic intervention. Another aim was to compare the total urinary excretion from the OJ consumed in study 1 and the citrus supplement in study 2.



Compounds	R^1	R^2	R^3	R^4
Hesperidin	*O*-Glc-Rha	OH	OH	CH_3
Hesperetin	OH	OH	OH	CH_3
Hesperetin-7-*O*-glucuronide	O-GlcA	OH	OH	CH_3
Hesperetin-3′-*O*-glucuronide	OH	OH	O-GlcA	CH_3
Hesperetin-3′-*O*-sulfate	OH	OH	$O\text{-}SO_3^-$	CH_3

Table 7.1: Structure of hesperidin and hesperetin and their metabolites

7.3 Materials and methods

Urine from participants in study 2 (chapter 5) was analysed for levels of hesperetin conjugates.

7.3.1 Materials

For all common reagents and chemicals please see details in Chapter 2. Rutin (≥99%), hesperetin (>95%) and taxifolin (>90%) were purchased from Extrasynthèse (Genay, France). L-Ascorbic acid was purchased from Fisher Scientific (UK). Hesperetin-3’ and 7-glucuronides were kindly donated by Dr Denis Barron (Nestlé Research Centre, Switzerland). Hesperetin-3′-*O*-sulfate was donated by Christine Morand (INRA, France).

For details of the human intervention study, see method section in chapter 5. Urine was collected at several time points over 24 hr (baseline, 1 day and week 4) in designated bottles containing 1 g of ascorbic acid. The total volume of each urine sample was measured and recorded; samples were centrifuged at 3000 x g for 5 min at 4ºC to remove insoluble materials and cellular debris. Aliquots were stored at – 20 C until analysed. One out of the 24 volunteers was excluded due to health and medication issues see study information design in chapter 5 for more details.

7.3.2 Preparation of stock solutions

A stock solution of rutin (4.5 µg/ml in methanol), to be used as internal standard 1(IS1) was aliquot and stored at -20°Cuntil used for sample preparation. Taxifolin to be used as internal standard 2 (IS2) was prepared on the day of analysis, at a final concentration of 5 µg/ml, using 0.2% ascorbic acid as solvent.

Stock solutions of hesperetin, hesperetin-7-glucuronide and hesperetin-3′-glucuronide were used to spike blank urine (baseline) in order to calculate the extraction efficiency and prepare the standard curve. Hesperetin, hesperetin-7-glucuronide and hesperetin-3′-glucuronide were solubilised in methanol at a concentration of 1 mM. These solutions were then vortexed for 1 min, and sonicated for 5 min. The hesperetin-3′-*O*-sulfate stock solution was diluted with the 5% acetonitrile (ACN) as running solvent.

For the standard curve all stock solutions were diluted to produce solutions at concentrations of 0.5, 0.6, 1, 2, 5, 10, 50 µg/ml. For each sample, 50 µl of 0.005 mg/ml taxifolin (in 0.2% ascorbic acid) was included. The solutions were then filtered into vials for LC-MS analysis.

7.4 Extraction of hesperetin conjugates from urine

Urine samples were defrosted for 12 hr at 4˚C and 30 min at room temperature prior to extraction. Duplicate urine samples were extracted for each person at each time point. For each replicate of sample, 200 µl of urine was mixed with 100 µl of rutin-IS1 (4.5µg/ml), 100 µl of ethanol and 800 µl of methanol. This solution was then placed in a shaking water bath (GLS Aqua 12 Plus, Grant Instruments Ltd, UK;

50˚C, 110 rpm, 10 min) to achieve the solubilisation of the compounds. The mixtures were then centrifuged for 10 min at 17000 g (Eppendorf centrifuge, Thermo, IEC-Micro CL17, Germany). The supernatant was removed as the first extract (extract A) and the process was repeated for the pellet using methanol in the place of urine (extract B). The primary and secondary extracts were placed in the freezer at -20˚C until the day of the centrifugal evaporation

The samples were dried using a centrifugal evaporator (Genevac EZ2plus Evaporation System, US). To achieve a complete drying, the samples underwent a 5 hr HPLC program of drying with the lamp off to achieve the evaporation of methanol and ethanol layer. This was followed by an extra 2 hr and 15 min aqueous program at 65˚C to evaporate the remaining urine-water layer. If the samples were not dried after this session, an extra stage of 1.5 hr drying session was added in the aqueous program 65˚C. When the samples were completely dried, they were placed back in to freezer at -20˚C until their reconstitution.

The dried samples were reconstituted prior to analysis on the LC-MS. Methanol (100 µl) was added to extract B. This solution was then vortexed for 1 min, sonicated 2 min and placed in shaking waterbath for 10 min. Extract B was then added to the dried extract A and was vortexed, sonicated and placed in the shaking waterbath for 10 min. Then, 50 µl of 0.2% ascorbic acid containing 5 µg/ml taxifolin (IS2) was added to the combined solution and vortexed until the samples were fully dissolved in the solution. The sample was further centrifuged (10 min, 17,000g) and the supernatant was filtered and placed in labeled HPLC vials (brown vials). The vials were well capped and stored at 4˚C until LC-MS analysis.

7.5 LC-MS analysis

The LC-MS analysis was carried out on a single quad LC-MS Shimadzu LC-2010 HT HPLC coupled with a LCMS-2020 quadrupole mass spectrometer fitted with an electro spray ionisation source used in negative mode, detector -1.80 kV, DL temperature 250 °C, nebulizing gas flow and drying gas flow 1.50 and 15 L/min respectively (Milton Keys, UK). An eclipse XDB-C18 analytical column (4.6x50 mm, 1.8 µM; Berkshire, UK) was used for the chromatographic separation and semi-preparative purification protocol, and it was maintained at 30 °C. The mass

spectrophotometer was set in negative mode to assay for ions at m/z: 301, 303, 381, 477 and 609 m/z ratios, corresponding to hesperetin, taxifolin, hesperetin sulfate conjugates, hesperetin mono-glucuronides conjugates and rutin respectively. The injection volume was 10 µL at flow rate of 0.5 mL/min. A gradient of water-formic acid (Solvent A; 100:0.1, v:v) and acetonitrile-formic acid (Solvent B; 100:0.1, v:v) was applied. Elution started at 5% solvent B and increased to 10% after 5 min. From 5 to 20 min the gradient increased up to 40% and after 5 min reached 90%, which was kept for 4 min more. Then, after 1 min the gradient dropped down to 5% and the run finished at 33 min. Pressure limits had a maximum of 370 bar and a minimum of 20 bar. The data files of LC-MS were analyzed by Shimadzu Lab-Solutions software.

7.6 Data analysis

Post-Run (LC-MS run) analysis took place using Lab-Solutions software. These data (peak areas and chromatograms) were further analyzed after combined with already known data (amount of supplements ingested by each subject, hesperidin concentration in citrus and placebo supplements, urine volumes before and after each treatment). The calculations and the statistical analysis were performed using SPSS 21. The difference between 24 hr ingestion and 4wk ingestion was tested using paired t-test. The results were expressed as mean ± standard deviation (n=3) measurements obtained from urine samples from 23 participants. Differences were considered to be significant at $p < 0.05$.

7.7 Results

7.7.1 Extraction efficiency experiments

Recovery experiment was carried out in the urine samples during preparation. The samples are expecting to contain various compounds that could affect the chromatography performance and results. Urine samples were spiked with a known amount of available authentic standard and the percentage ratios between recovered after processing and expected concentration were calculated. Table 7.2 shows the mean recovery value obtained for each compounds at three concentrations (5, 10 and 20 µg/ml) spiked in two independent replicates of blank urine (collected

before experimental supplements consumption). The results showed good recovery values for all compounds, which is in the range of 81.5 ± 9.3 to 100.7 ± 1.5%. The average extraction efficiencies of the six compounds tested were acceptably good and reliable for every spiking concentration with coefficient variation value ranged from 3.2 - 16% across the concentration range tested. Moderate recovery values were obtained for the hesperetin conjugates, which were approximately in the range between 81.5 ± 9.3 and 90.7 ± 3.0%. From the results obtained can be concluded that the majority of flavanones tested had a high and moderate recovery percentage, therefore this method was suitable for the urine preparation step prior to LC-MS analysis. The data are summarised in Table 7.2.

Table 7.2: Recovery rate of the compound

Compounds	***Recovery (% of initial concentration)***	***Coefficient variation (CV %)***
Rutin	95 ± 3.1	3.1
Taxifolin	100 ± 1.5	1.6
H-7-*O*-glucuronide	90 ± 3.0	4.3
H-3′-*O*-glucuronide	89 ± 6.0	7.6
H-3′-*O*-sulfate	81 ± 9.0	16.8
Hesperetin	83 ± 1.9	3.2

Concentrations of the internal standards (IS) were the same for all spiking samples. Values are mean ± SD (n=3).

7.7.2 Identification of hesperetin metabolites in urinary excretion

The urine of 23 participants was collected over 24 hr at four separate time points, two pre-supplementation (baseline) and two post-supplements (day one and 28 days) for both citrus supplements (containing 188 µmol hesperetin equivalents to 300 ml OJ) and placebo. Urine was tested for compliance to the study. As the diet was restricted for 24 hr before urine collection a significant increase of metabolites excreted in urine after supplementation compared to baseline was indicative of compliance the citrus supplement.

Hesperetin conjugates were absent from all baseline urine samples except a small amount found in three volunteers (0.6, 0.7 and 1.0 µmol), indicating good compliance with the flavanone avoidance diet. Urine extracts before and after the consumption of the citrus supplements were analysed by LC-MS based on their mass (m/z ratio) and retention time. A comparison with the retention time and the peak of authentic standards, these three peaks appeared in the same mass window assigned as H-7-*O*-glcA, H-3′-*O*-glcA and H-3′-*O*-SO_4, respectively. Representative example of chromatograms for the standards and mixture of all standards are shown in Figure 7.1 and 7.2. Rutin and taxifolin were identified at 609 and 303 m/z at a retention time of 11.4 and 12.2 min, respectively**.** Also H-7-*O*-glcA, H-3′-*O*-glcA and sulfate H-3′-*O*-sulfate were identified at RT 14.5, 14.7 and 16.9 min, respectively. The mono-glucuronides eluted at similar retention times, without complete resolution of the peaks. Typical mass spectra used in identifying hesperetin glucuronides and sulfates are shown in Figure 7.3**.**

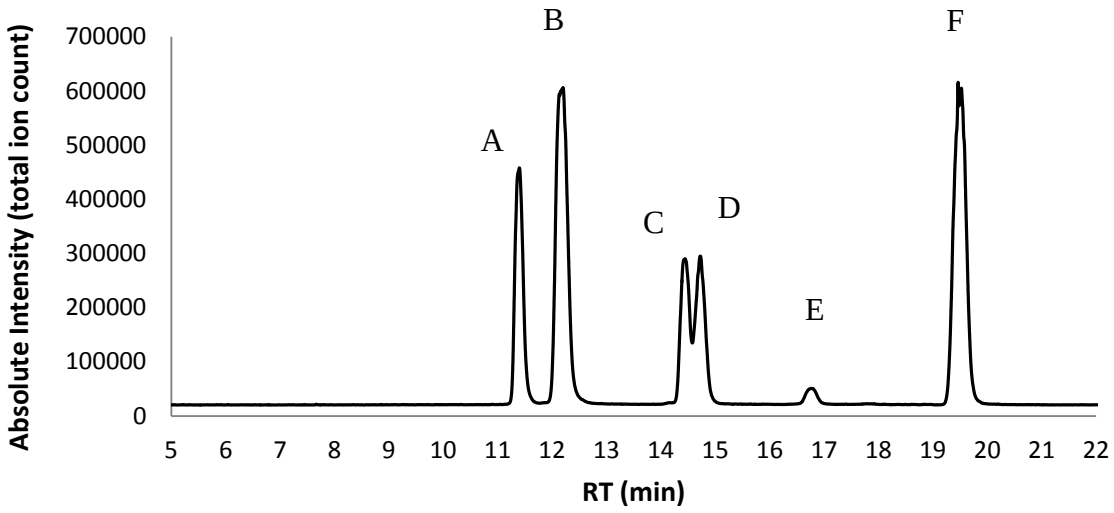


Figure 7.1: LC-MS chromatogram representing peaks of the mixed of standards. Peak (A) rutin; Peak (B) taxifolin; Peak (C) H-7-*O*-glcA; Peak (D) H-3′-*O*-glcA; Peak (E) H-3′-*O*-sulfate; Peak (F) hesperetin.

Urine extracts before and after the consumption of the two different supplements were analysed by LC-MS. A representative example of a chromatogram from subject 1 after the consumption of citrus supplements is shown in figure 7.4. Figure 7.5 shows the profile of peaks after the placebo intervention. A significant quantifiable amount of both hesperetin glucuronides and the sulfate were present in the urine samples of all volunteers except volunteer 20 and 21 where the amount was after both acute and chronic collections indicating that these volunteers were lower excretors.

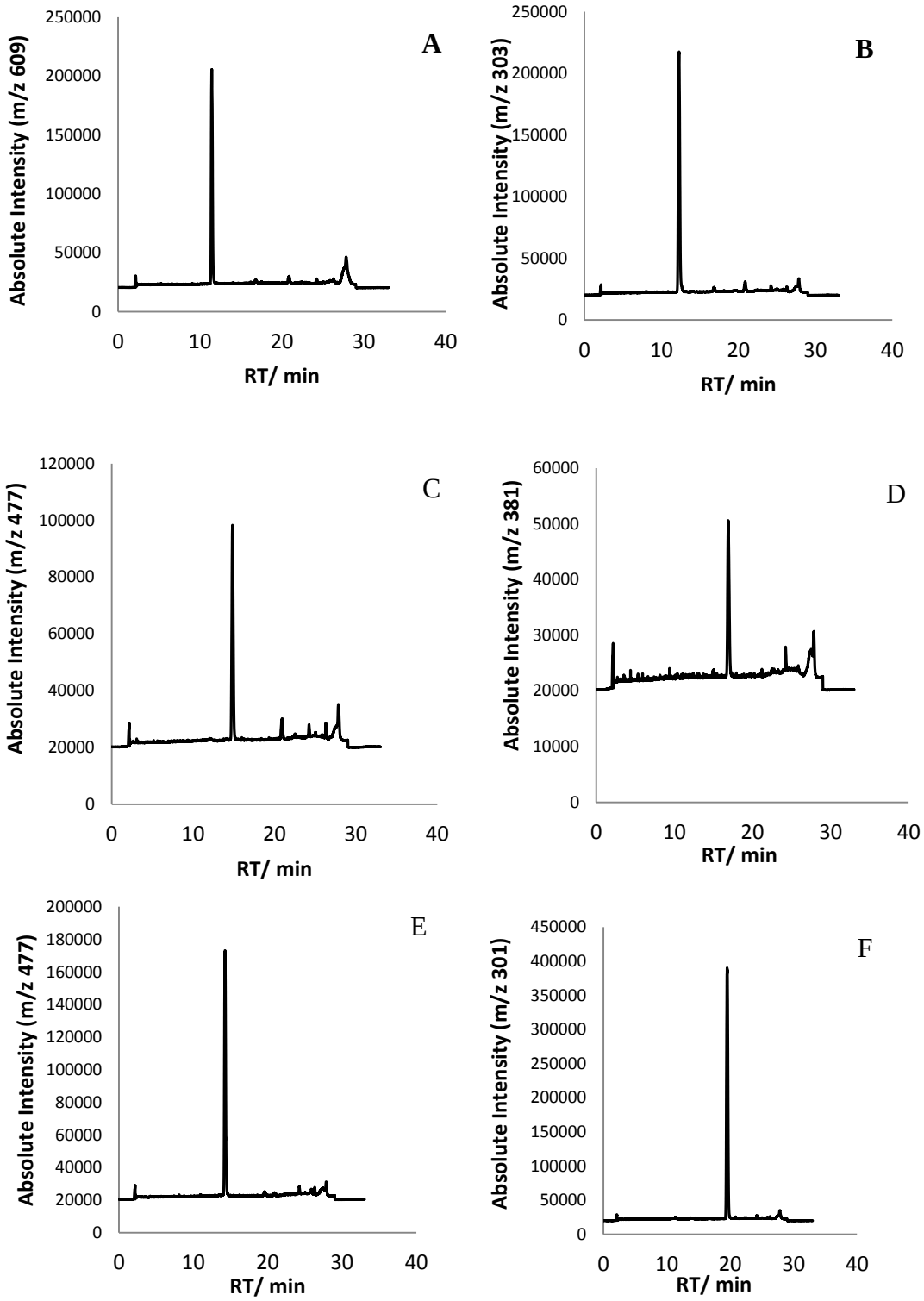


Figure 7.2: LC-MS chromatograms representing peaks of the standards. (A) rutin, 11.4 min; (B) taxifolin, 12.2 min; (C) Hesperetin-7-O-glcA, 14.4 min; (D) Hesperetin-3 ´ -O-glcA , 14.7 min; (E) Hesperetin-3 ´ -O-sulfate, 16.9 min ; (F) hesperetin, 19.0 min.

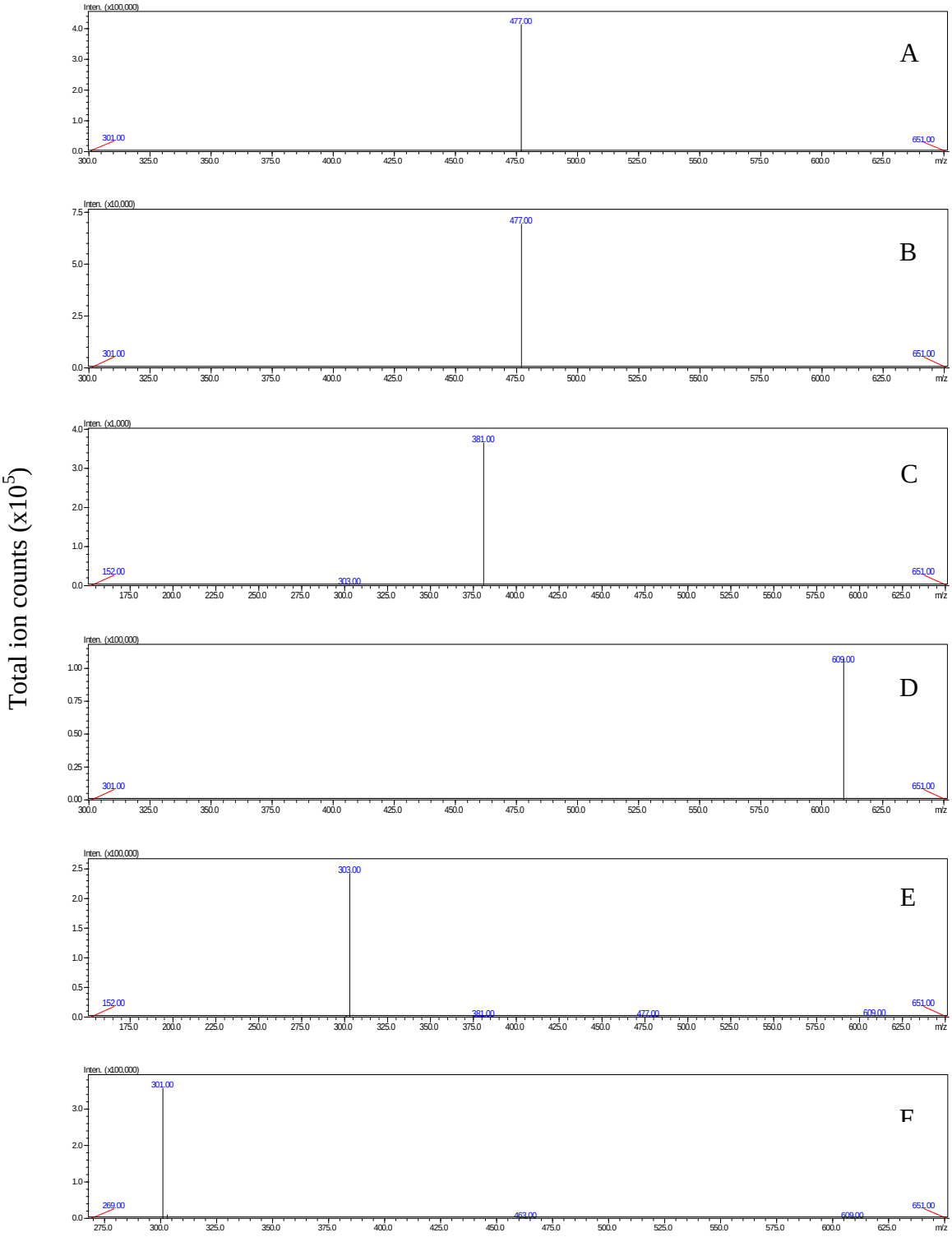


Figure 7.3: A typical MS spectrum of hesperetin conjugate standards and standard compounds obtained in negative ion mode [M-H]-. (A) rutin; (B) taxifolin; (C) hesperetin-7-*O*-glcA; (D) hesperetin-3′-*O*-glcA; (E) hesperetin-3′-*O*-SO4; (F) hesperetin.

As shown in Figure 7.4 three main hesperetin conjugates: hesperetin-7-*O*-glucuronide, hesperetin-3'-*O*-glucuronide and hesperetin-3′-*O*-sulfate. They were two mono-glucuronides peaks at 477 m/z. Hesperetin-7-*O*-glcA peak eluted first followed by a higher peak representing hesperetin-3'-*O*-glcA. Hesperetin-3′-*O*-sulfate was identified at an m/z ratio of 381 with a retention time of 16.9 min.

In some subjects (n= 5), very low amounts of hesperetin were present at 301 m/z at retention time of 19 min same as authentic standard aglycone. There were also two unknown compounds which had an m/z of 651 at RT 4.2 and 7.8 min, respectively. These compounds have an m/z for hesperetin di-glucuronide, therefore it is possible that these are hesperetin 3′, 7-*O*-diglucuronide and hesperetin 5, 7-*O*-diglucuronide based on the report by Bredsdorff *et al*., (2010).

Some participants (n=7), consuming the placebo excreted trace amounts of hesperetin metabolites < 0.2 µmol as the diet was not controlled. However, after citrus supplementation resulted in a > 50 fold higher excretion of hesperetin glucuronides in comparison to placebo supplementation.

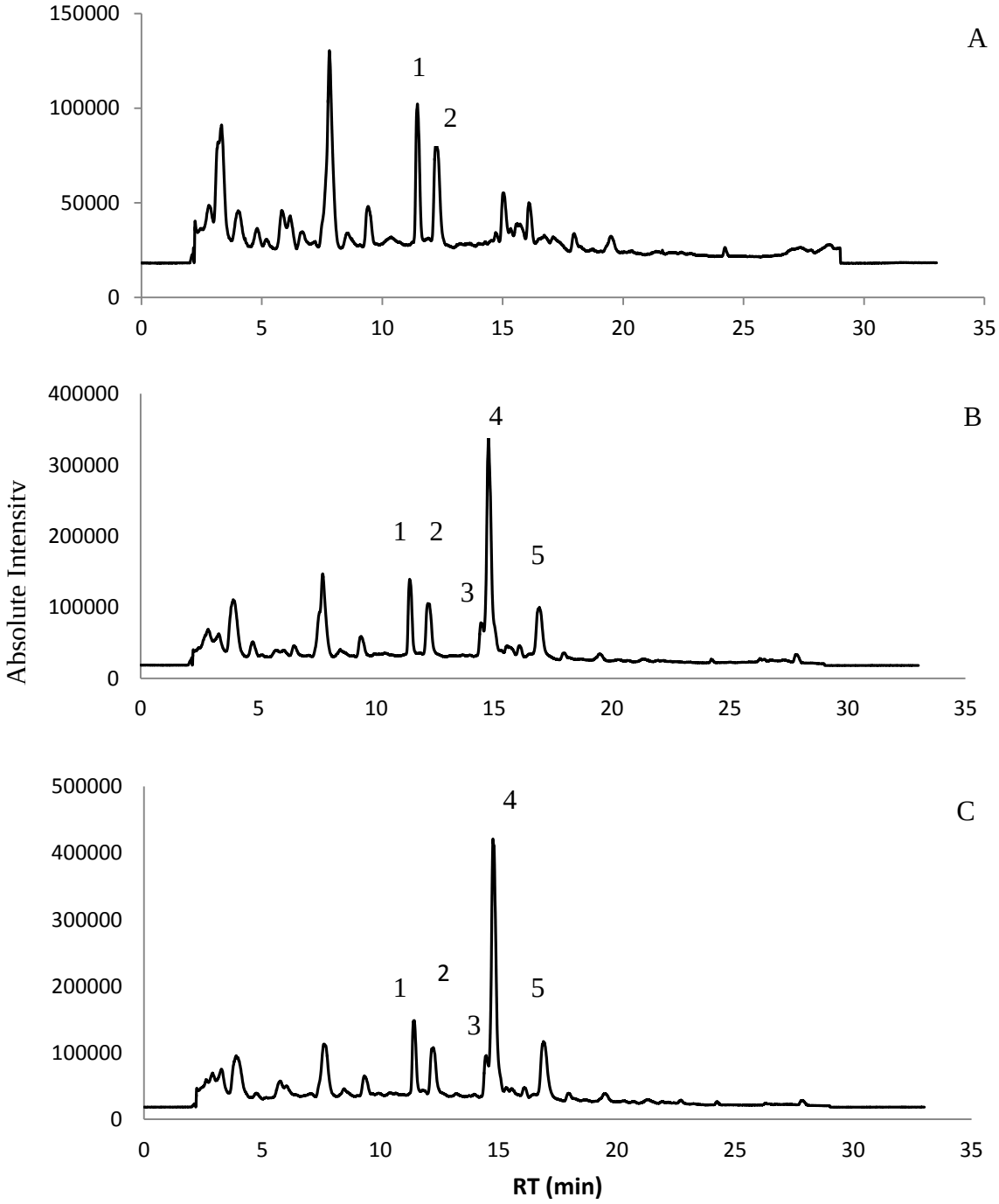


Figure 7.4: LC-MS chromatograms representing extracted urine samples from subject 1 after the consumption of citrus bioflavonoid supplements. (A) Baseline urine, (B) 1 day (C) urine after 28 days intervention. Internal standards are peaks 1, rutin; 2, taxifolin. Peaks 3 is hesperetin-7-*O*-glucuronide 4 is hesperetin-3'-*O*-glucuronide and 5 is hesperetin-3′-*O*-sulfate (5).

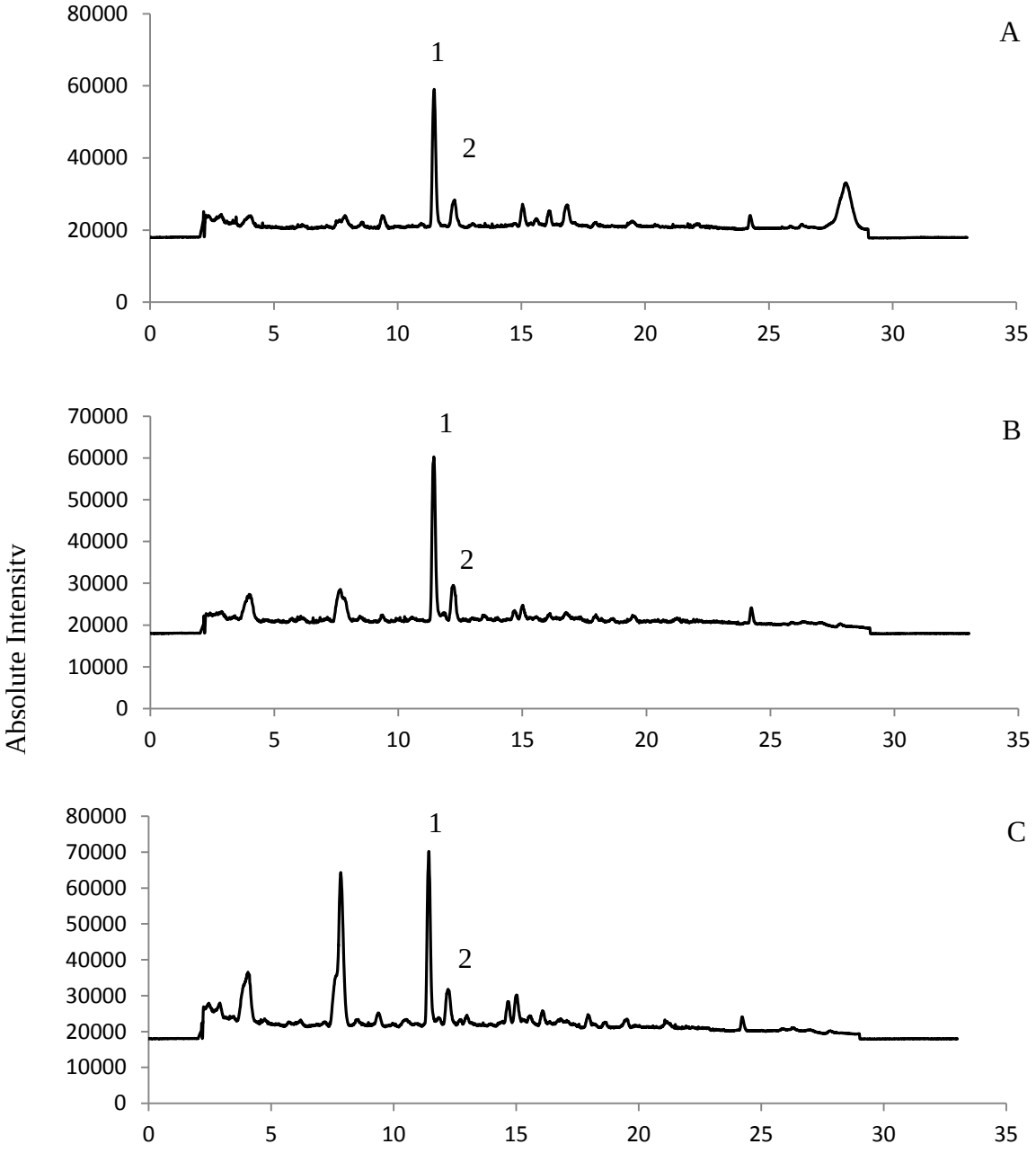


Figure 7.5: LC-MS chromatograms representing active urine sample from subject 1 after the consumption of placebo supplements. (A) Baseline urine, (B) 1 day (C) urine after 28 days intervention. Internal standards are peaks 1, rutin; 2, taxifolin. The three main metabolites of hesperidin: hesperetin-3′-*O*-sulfate, hesperetin-7-*O*-glucuronid and hesperetin-3'-*O*-glucuronide were not found.

7.7.3 Quantification of hesperetin metabolites in urine

The quantitative data of hesperetin metabolites excreted in urine over 24 hr at day 1 and day 28 is presented in Table 7.2 and Figure 7.6. Hesperetin-3′-*O*-glcA was the highest metabolite excreted followed by hesperetin-3′-*O*-sulfate and hesperetin-7-*O*-glcA was the lowest metabolite excreted. The proportion of sulphate to glucuronide metabolites was approximately 37.5:62.5. For all metabolites it appeared that the amount excreted in 24 hr was lower after 28 day than at 1day, however it was only significantly different ($p<0.05$) for hesperetin-3′-*O*-sulfate (see Table 7.3). Total hesperetin metabolites were also significantly different between day 1 and day 28 ($p= < 0.01$) (Fig. 7.6C).

The inter-individual variation of excretion of each metabolite is shown in Figure 7.7. It was apparent that some of the volunteers had a considerably higher excretion of hesperetin than other volunteers. In the case of hesperetin-3′-*O*-sulfate, hesperetin-7-*O*-glucuronide and hesperetin-3′-*O*-glucuronide, volunteer 8 was the highest excretor, with 2-3 x the average % dose excreted for both glucuronides. Volunteers 20 and 21 were the lowest excretors, with levels close to zero or the limit of detection in some cases. The highest amount of hesperetin metabolites excreted by volunteer 8 was 2-fold and 3-fold higher the average amount excreted 22 µmol and 18 µmol after citrus supplements were consumed for 1 day and 28 days, respectively. Interestingly, nothing was excreted by subject 15 after 1 day exposure; however, after 28 days the amount excreted was 1.8-fold lower than the average amount excreted.

Overall the total amount excreted ranged from 0-6.5% for hesperetin-7-*O*-glcA, 0-17% for hesperetin-3′-*O*-glcA and 0-10% for hesperetin-3′-*O*-sulfate. The total excretion as a percentage of doses is shown in Figure 7.7, and shows the range of amount excreted was varied from 0 – 32 µmol (average 22 and 18 µmol for day 1 and day 28 respectively). And the total excretion as percentage of dose ingested is shown in figuer 7.8.

Table 7.3: Quantification of hesperetin metabolites (µmol) excreted in urine from 23 healthy volunteers after consumption of citrus and placebo supplements.

Hesperetin metabolites (µmol)	***Citrus supplement***			***Placebo supplement***		
	Baseline	1 day	Day 28	Baseline	1 day	Day 28
H-7-*O*-glcA	0.02 ± 0.08	4.10 ± 3.15	3.13 ± 2.78	0.0 ± 0.0	0.00 ± 0.05	0.10 ± 0.26
H-3′-*O*-glcA	0.01 ± 0.02	10.04 ± 6.42	8.53 ± 7.03	0.01 ± 0.05	0.11 ± 0.18	0.23 ± 0.32
H-3′-*O*-sulfate	0.07 ± 0.12	8.49 ± 5.21	6.33 ± 4.26*	0.0 ± 0.0	0.06 ± 0.11	0.08 ± 0.23
Total excreted	0.1 ± 0.04	22.63 ± 4.90	17.99 ± 4.69	0.01 ± 0.05	0.17± 0.11	0.41 ± 0.27

All values are reported as mean ± SD (n=4). Asterisk (*) indicates that values are significantly different ($p < 0.05$) between day 1 and day 28. 188 µmol ingested.

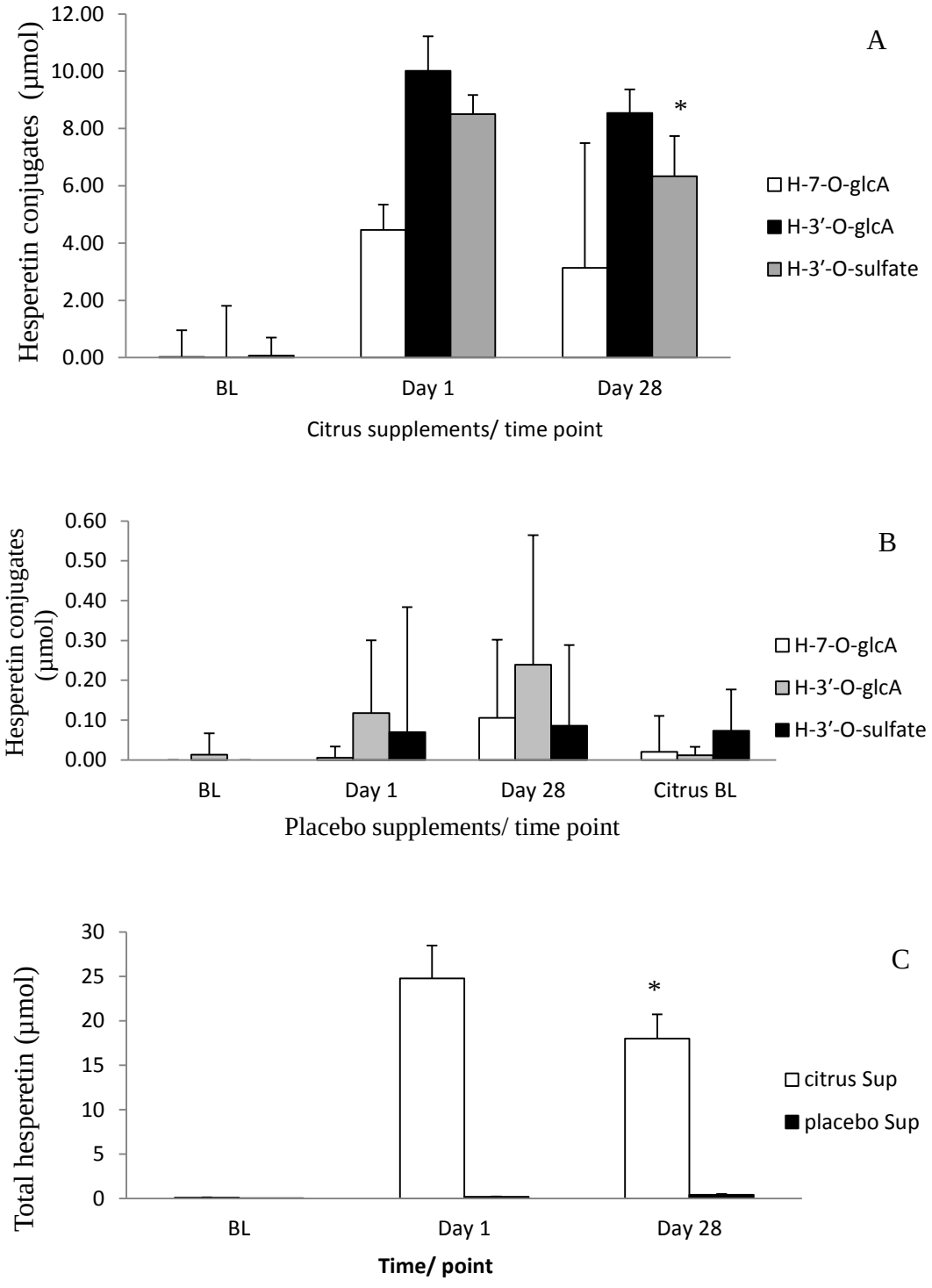


Figure 7.6: Excretion of hesperetin metabolites in urine after Day 1 and day 28 post consumption of (A) citrus bioflavonoid supplements, (B) placebo supplements, (C) comparison of total hesperetin metabolites excreted. Asterisk (*) indicates that values are significantly different ($p < 0.05$).

Table 7.4: P values for significance of hesperetin metabolites collected at different times.

Intervention	*Compounds*	*BL vs 1 day*	*Bl vs 28 days*	*1 day vs 28 days*	*BL (placebo) vs (Citrus supplement)*
Citrus supplement	H-7-*O*-	<0.01*	<0.01*	0.17	0.28
	H-3′-*O*-	<0.01*	<0.01*	0.27	0.91
	H-3′-*O*-	<0.01*	<0.01*	0.03*	0.1

BL = baseline. Samples were performed in technical duplicates, from 23 subjects. Asterisk (*) indicates that values are significantly different ($p < 0.05$).

As it can be seen from figure 7.9 (A and B), the correlation between the hesperetin glucuronide metabolites as an amount excreted in urine was investigated using the spearman correlation coefficient. The urine excretion of the hesperetin metabolites were highly correlated in the basis of the amount recovered with $p < 0.01$ (0.89 in 1 day and 0.93 in 28 days intervention. The data was assessed to identify whether there was a relationship between the excretion of hesperetin-7-*O*-glcA and hesperetin-3′-*O*-glcA. There was a high positive association ($R > 0.89$) between the excretion of the two hesperetin glucuronide conjugate after acute (day 1) and chronic supplementation over 28 days. As for the chronic dose, it appears that the association after the 28 day became stronger, although the concentration was less after 28 day intervention.

The correlation between urine maximum concentration of hesperetin glucuronide and urinary excretion of hesperetin sulfate metabolites was also investigated and shown in figure 7.10. The correlation was statistically significant between the concentration of hesperetin-*O*-glucuronides in urine and the total amount of hesperetin -3-*O*-sulfate excretion in urine ($r = 0.62$, $p = < 0.01$, Pearson coefficient) in 24 hr excretion. The correlation was observed to be stronger after were consumed for 28 days ($r = 0.80$, $p = < 0.01$).

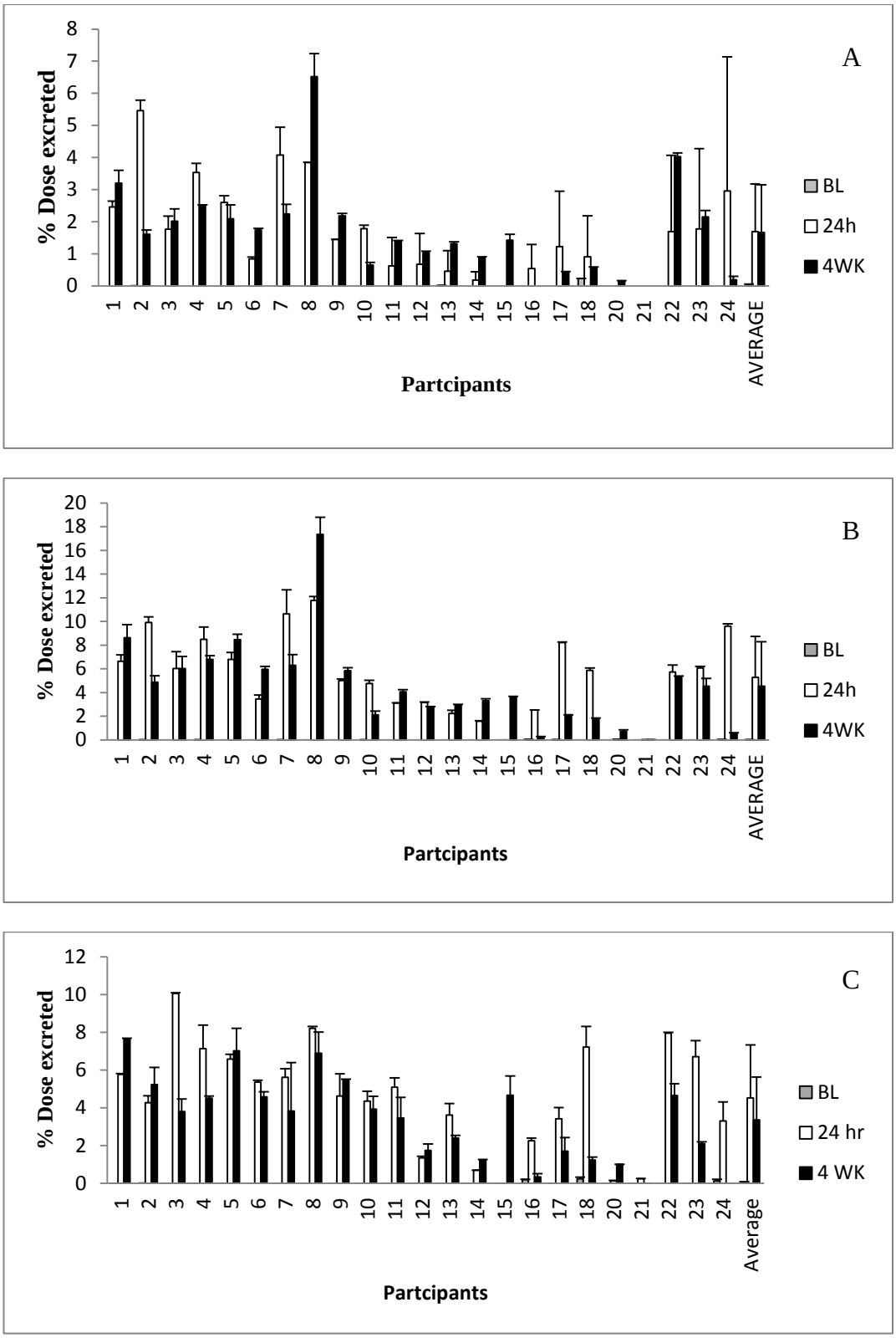


Figure 7.7: Presents the amount excreted as percentage from the dose ingested for hesperetin-7-*O*-glucuronide (A), and hesperetin-3′-*O*-glucuronide (B), hesperetin-3′-*O*-sulfate.

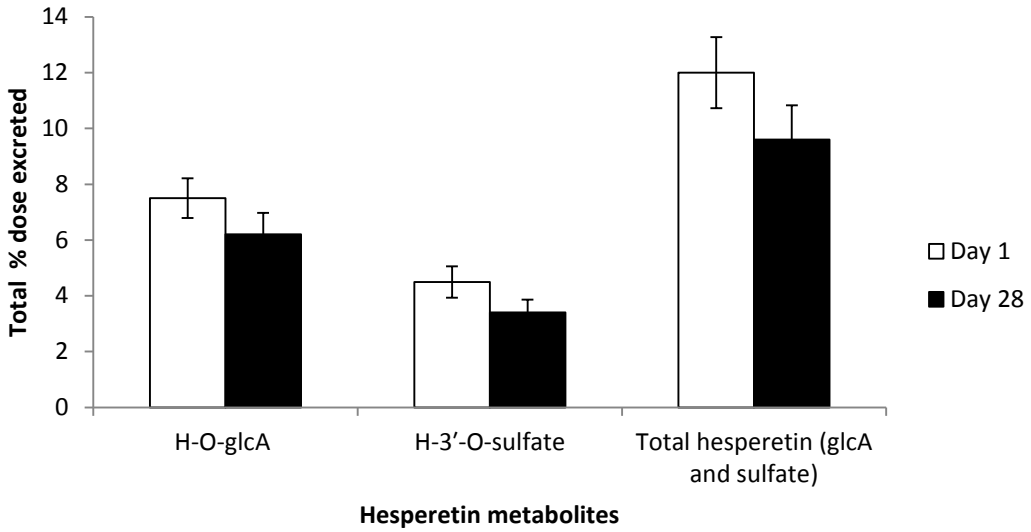


Figure 7.8: The total excretion as a percentage of doses ingested. Day 1 and 28 days after consumption of the citrus supplements.

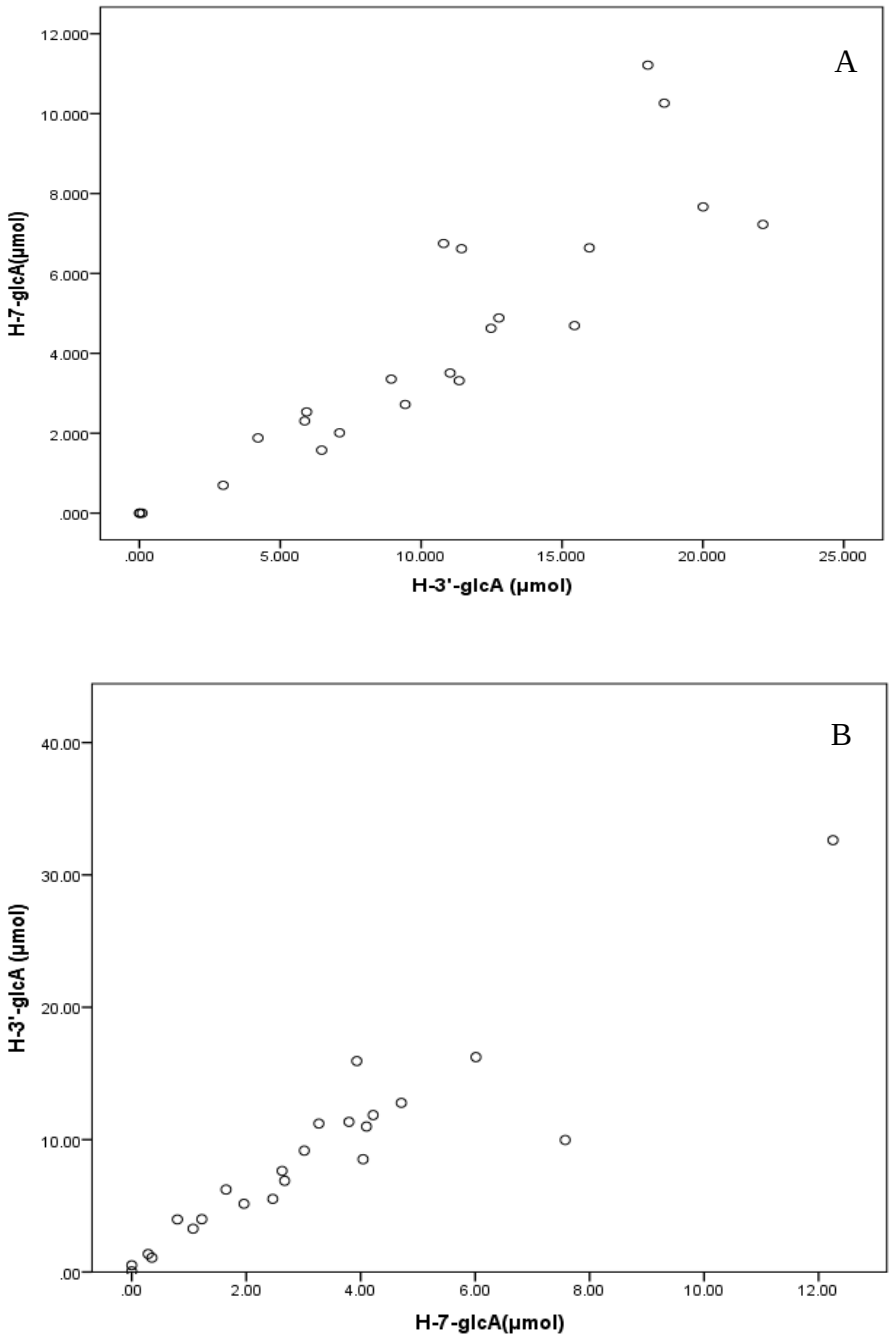


Figure 7.9: Correlation between excretion of H-7-*O*-glcA and H-3′-*O*-glcA in urine collected for 24 hr post-intervention of citrus supplements of 1 day (A), or after 28 days supplementation (B). Correlation is significant at the * 0.05 and 0.01** level (2-tailed).

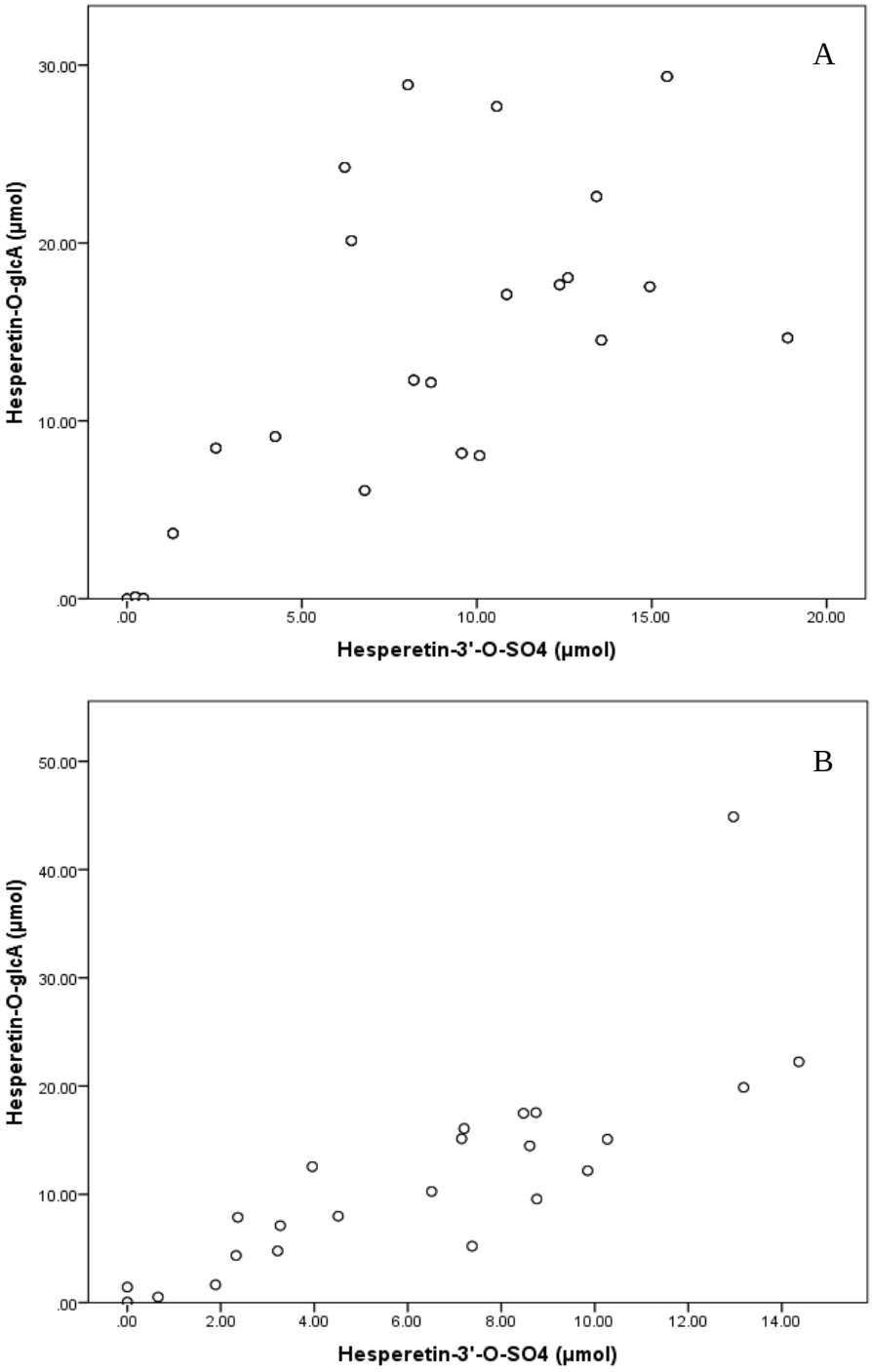


Figure 7.10: Correlation between a) hesperetin-*O*-glucuronide and hesperetin -3′-*O*-sulfate after 24 hr ingestion b) hesperetin-*O*-glucuronide and hesperetin -3′-*O*-sulfate after 28 days ingestion based on the amount recovered in urine samples using the Spearman correlation. Correlation is significant at the * 0.05 and 0.01** level (2-tailed).

7.8 Comparison between bioavailability of orange juice and citrus supplement

The total urinary excretion between the orange juice in study 1 (see chapter 3) and the citrus supplements in study 2 (described in this chapter 6) were compared. In study 1 the metabolites were hydrolysed by sulfatase and β-glucuronidase before analysis of the aglycone. As shown in chapter 4, these enzymes, and particularly the sulfatase, did not completely hydrolyse the metabolites. In this chapter the metabolites were therefore analysed individually and the total metabolites determined. However despite the differences in methodology and the different volunteers taking part, we wanted to compare the datasets to see if any comparisons could be made between the different sources of flavanones.

Table 7.4 summarises the data from the two studies. Despite nearly a 2-fold increase in dose provided from the orange juice, and a longer (36 h) and therefore complete collection of hesperetin metabolites, the % dose excreted was only 9.3%. The percent excreted from the supplements was at least twice this value. The percent excreted from the supplements was 2.5x this value. However we know that the hydrolysis enzymes did not fully hydrolyse the conjugates in study 1 (see chapter 3). So assuming a partial (50%) hydrolysis of hesperetin-3′-*O*-sulphate and only a partial (80%) hydrolysis of the glucuronides then based on the ratio of conjugates at 37.5:62.5 for sulphate:glucuronide respectively found in study 2 (this chapter), the following estimated calculation could be applied:

% recovery from study 2 (day 1) = 22.6%

37.5% of this is sulphate metabolites and 62.5% is glucuronide metabolites.

> Therefore 22.6 x 37.5% = 8.475% is from sulphates

> Therefore 22.6 x 62.5% = 14.125% is from glucuronides

Assumption: If enzymes were only 50% efficient for sulphate metabolites and 80% efficient for glucuronide metabolites then,

> 50% x 8.475 = 4.2%

> 80% x 14.125 = 11.3%

TOTAL expected recovery in study 2, day 1, if enzyme hydrolysis had been used is 15.5%.

Hence based on the assumption of enzyme efficiency, the crude estimation in recovery of hesperetin was 15.5% in study 2 if enzyme hydrolysis had been used, compared to 9.3% determined in study 1. This suggests that the bioavailability is potentially higher after supplements than after fresh orange juice, and is certainly not worse. However, in order to verify this hypothesis a human intervention cross-over study would be required to ensure that the variables of excretion time, volunteer differences and enzyme activity were all fully taken into account.

Table 7.5: Bioavailability of hesperidin between study1 ingested orange juice and study 2 ingested citrus tablets

	Study 1 (orange juice)	*Study2(citrus supplements)*
No. of participants (M/F)	15	23
Urine collection period (hr)	36	24
Form of hesperidin measured	Aglycone form after enzyme hydrolysis	Hesperetin conjugates "glucuronide and sulfate"
Dose ingested (µmol)	500 ml orange juice (344 µmol)	2 citrus bioflavonoid tablets (188 µmol)
Dose excreted in urine %	9.27 ± 8.09	$(22.6 \pm 4.9)^{\dagger}$ $(18 \pm 4.7)^{\ddagger}$

(†Day 1; ‡28 Days)

7.9 Discussion

A number of aims have been pursued in this study. The first aim was focussed on the bioavailability of hesperetin metabolites following consumption of citrus supplements. The second aim was to investigate whether or not there were differences between acute and chronic intake of supplements in the excretion of hesperetin metabolites. Finally, this study seeked to answer whether the hesperetin was more bioavailable from the supplement intake than from orange juice (compared to the results obtained in chapter 4 of this thesis). LC-MS analysis was chosen as the most appropriate method to identify and quantify the hesperetin metabolites in urine. The study examined the excretion profiles of the compounds the three main hesperetin metabolites in 23 subjects who followed a single 1 day and chronic dose (28 days)of citrus supplements starting from a washed out point. For the first time, this study provided evidence to show that hesperidin was bioavailable in reasonable quantities following the consumption of citrus bioflavonoids supplements and apperaed as bioavailble as orange juice as a source of flavanones.

We have identified and quantified three hesperetin metabolites hesperetin-7-*O*-glcA, hesperetin-3′-*O*-glcA and hesperetin-3′-*O*-sulfate. Our results are in agreement with recent studies that have identified these hesperetin metabolites in human urine after the consumption of orange juice (Matsumoto *et al.,* 2004, Mullen *et al.,* 2008, Brett *et al.,* 2009, Bredsdorff *et al.,* 2010, Pereira-Caro *et al.,* 2014).

The quantification of metabolites in the present study was based on the corresponding metabolites of respective authentic synthesised standards, monitored by LC-MS. In the present study, hesperetin glucuronides were the most abundant metabolites recovered in the urine after 1 day and 28 days of citrus supplementation. This was consistent with the other studies, however in these studies hesperetin-7-*O*-glucuronide was more abundant than hesperetin-3′-*O*-glucuronide, opposite to the finding in chapter 7. Hesperetin sulfate in other studies was also found to be the least excreted metabolite. Manach *et al*., (2003) found 87% of hesperetin as glucuronide, with 13% as sulfo-glucuronide, which was similar to the present study at 68% hesperetin glucuronide (di-glucuronides were observed but not quantified). The variation in positioning of the glucuronide may be as a result of variations in UDP-glucuronosyl transferase activity in different individuals (Brand *et al.,* 2010).

In all studies that have considered flavavone excretion a large variation in metabolite excretion has been observed, highlighted by Brett *et al.,* (2009) who showed 0-57% variation in excretion. The large inter-individual variation is likely to be a result of the difference in gut microflora which will alter the hydrolysis of the rutinoside sugar, allowing the aglycone hesperetin to be absorbed, and the extended breakdown of the flavavone into smaller phenolic acids (shown by Roowi *et al,* 2009). Table 7.5 summarises the previous studies along with the results from this thesis. The percent dose recovered in the current studies is higher than many of the other studies; all carried out with orange juice, but is similar to the results of previous studies; Pereria-Caro *et al.,* (2014), Borges *et al*., (2010) and Krogholm *et al*., (2010). Many of the studies monitored the aglycone after enzyme hydrolysis, which will have resulted in a lower recovery due to the poor ability of the sulfatase to deconjugate hesperetin-3′-*O*-sulfate (Bredsdorff *et al.,* 2010).

The quantification of metabolites in our study was based on the corresponding metabolites of their respective authentic synthetic standards. The concentration of hesperetin-7-*O*-glcA was slightly lower than that of H-3′-*O*-glcA. This result is in disagreement with the findings by Matsumoto *et al.,* (2004). In this study, there was a significantly greater amount of hesperetin metabolites excreted after the consumption of citrus supplments as a single dose compared with that after a chronic consumption for 4 weeks. previous results reported by Bredsdorff *et al.,* (2010), indicated that increasing the dose of hesperidin 3-fold affected the overall concentration, but not the profile of the conjugate formed after colonic absorption. A correlation test between the relative urine excretion % of intake and the dose ingesed in (µmol) was done with our results and the results obtained from previous studies (table 7.6) and low correlation was found ($r = 0.19$; $p < 0.51$).

This inter-individual difference in the ability of the enterocytes to hydrolyse glucosides, glucuronidate, sulfate or methylate flavonoids could lead to differences in metabolism of these compounds (Brett *et al.,* 2009). It can be stated that a possible reason for our observation is that the participants recruited in this study had sulfotransferase enzymes that worked more effectively than the UDP glucuronosyltransferase (UDP) which catalyse the conjugation of flavanones with sulfate group and glucuronic acid (Scalbert and Williamson, 2000, Brand *et al.,*

2010, Bredsdorff *et al.*, 2010). Another possible explanation is related to sulfate conjugates. Because of their excellent response in negative ESI compared to aglycone, sulfate are more readily ionised and give higher values. Moreover, hesperetin-3′-*O*-sulfate has been reported not to respond well to enzymatic hydrolysis with sulfatase (Bredsdorff *et al.*, 2010).

The excretions of flavanones between the two different treatments 1 day and 28 days were compared. The consumption chronic dose of supplements up to 28 days did not statistically significantly change the amount of hesperetin-7-*O*-gluA and hesperetin-3′-*O*-glcA recovered in urine. However, the excretion of hesperetin-3′-*O*-sulfate was significantly decreased after 28 days ($p < 0.05$). This lead to the conclusion that there is not a cumulative change in bioavailability however, the amount of hesperetin metabolites was statistically higher after 1 day ingestion compared with 28 days ingested.

In conclusion, flavavones from citrus supplements are bioavailable and the amount excreted is at least equivalent, if not more than from orange juice. There is no evidence of accumulation of flavanone metabolites over time, and possibly a reduction in excretion over time due to changes in sulfation.

Future work should focus on understanding the bioavailability of hesperidin by examining the influence of the environmental or genetic factors on the enzyme expression and activity. In this respect, a study of microflora composition would be a very promising approach. This could be achieved by taking faeces samples from individuals who underwent a hesperidin intervention treatment. Improving the method to determine the hesperidin metabolites will also help understand the fate and metabolism of these compounds more clearly.

Table 7.6: The relative urine excretion of hesperidin from different human studies

Reference	**Source**	**Ingested dose (µmol)**	**Relative urine excretion, % of intake**
(Manach *et al.,* 2003)**	OJ[1]	363 hesperetin	4.13 ± 1.18
		727 hesperetin	6.41 ± 1.32
(Nielsen *et al.,* 2006)**	OJ	98 hesperidin	4.06 ± 1.77
		314 hesperidin	8.9 ± 3.83
(Erlund *et al.,* 2001)**	OJ	206 hesperetin	5.3 ± 3.1
(Brett *et al.,* 2009)*	OJ	117 hesperetin	4.63 ± 3.05
(Pereira-Caro *et al.,* 2014)*	OJ	348 hesperetin	17.2
(Borges *et al.,* 2010)*	P-R juice[2]	45 hesperidin	12 ± 1.6
(Mullen *et al.,* 2008)*	OJ	168 hesperidin	7
(Roowi *et al.,* 2009)*	OJ +Yoghurt	168 hesperidin	37
(Krogholm *et al.,* 2010)**	Mix juice	52 hesperetin	14.2 ± 9.1
Study 1**(chapter 3)	OJ	344 hesperidin	9 ± 9.4
			10 ± 8.3
Current study* (chapter 7)	Citrus supplements	188 hesperidin	22.6 ± 4.9
			17.9 ± 4.7

[1]OJ, orange juice; [2]P-R, polyphenol-rich juice drink; Asterisk (*) indicates that sample was not hydrolysed; (**) indicates that samples were hydrolysed enzymatically and the aglycone measured.

Chapter 8: General Discussions And Future Perspectives

8.1 Overview of thesis

The overall aims of this thesis were to investigate the absorption of citrus flavanones, specifically to consider intra- and inter-individual variation in excretion, and the impact of citrus flavanones on cardiovascular health by assessing cardiovascular disease risk biomarkers during an intervention.

Cardiovascular disease (CVD) is the major cause of death worldwide (WHO, 2015). Diet plays a significant role in both causation (e.g. increasing blood LDL levels, and blood pressure), and in reducing risk of CVD. Reduction in risk has been linked to an improvement in endothelial health, and recently there has been a lot of interest in whether flavonoids are associated with the beneficial effects on endothelial function of fruit and vegetable intake. Only certain flavonoids may improve CVD risk, and citrus flavanones have been a subclass of flavonoids that show potential in lowering blood pressure and improving endothelial health. Moreover, flavonoid bioavailability varies widely across populations, based primarily on the gut microflora composition, and bioavailability is key to the impact on health.

The work in this thesis was based on two human intervention studies. The first study (chapter 3) investigated the inter-individual variation and absorption of both flavanones and isoflavones by measuring urinary excretion, after they were consumed individually or co-ingested. In the second study (chapter 6 and 7), the absorption of flavanones was assessed by means of the urinary excretion after a single dose or after 28 days of taking a citrus flavonoid supplement. Moreover, a comparison was done between the amount of flavanone excreted after orange juice or citrus supplements were consumed. Saliva, as a potential non-invasive biomarker fluid of exposure, was also analysed to assess the absorption of isoflavone and flavanone after the study. The second study (chapter 6) was designed as a randomised, double-blind, placebo-controlled, cross-over intervention to investigate the effects of citrus flavanone supplements on CVD risk biomarkers, such as blood pressure (BP), and pulse amplitude tonometry

(PAT), after 28 days of daily intake. Augumentation index (AI), fasting blood glucose, waist circumference and weight were also monitored.

The main findings of the thesis are summarised below:

Outcome of intervention studies:

- There was a trend towards decreasing systolic and diastolic blood pressure after 28 days of supplementation compared to placebo, but this was not statistically significant (p= 0.19) and (p= 0.07).
- Changes in augmentation index were decreased significantly after 28 days of supplementation compared to baseline (p= 0.01), but not to placebo.
- There was no difference in waist circumference, weight and reactive hyperaemia index (measurement of PAT) after 28 days of supplementation compared to placebo.

Outcome of bioavailability studies:

- The inter-individual variation in flavanone and isoflavone 36 hr urinary excretion which ranged from 0.6-35% and 5.4-65%, respectively.
- There was little difference between urinary excretion of flavanones and isoflavones after single or co-ingestion of foods.
- The percent dose excreted for daidzein and naringenin was higher than genistein and hesperetin, respectively.
- Excretion of genistein and daidzein, and hesperetin and naringenin, were correlated after single food or co-food ingestion.
- Naringenin excretion was correlated to genistein and daidzein excretion only after co-food ingestion.
- There was little difference in 24 hr urinary excretion of flavanones between 1 day and 28 days intake of citrus supplements.
- The major metabolites excreted in the urine were hesperetin-3′-*O*-glucuronide > hesperetin-3′-*O*-sulfate > hesperetin-7-*O*-glucuronide.
- The major metabolites were always excreted in the same relative order for all individuals.
- Genistein, daidzein, hesperetin and naringenin were not detected in saliva at any time point after foods were consumed. Which indicate that saliva was not suitable as biological fluid for assessment of exposure to

flavanones and isoflavones compounds.

- Citrus bioflavonoid supplements appeared to be just as bioavailable, as measured by urinary excretion, as orange juice.

8.2 General discussion and limitations of thesis

The first study compared excretion of flavonoids from orange juice and/or soya nuts when given seperately or co-ingested. Very few previous studies have investigated impact on absorption and excretion after different flavonoid-rich foods are co-ingestion. One study showed that after ingestion of three foods (grapefruit, soyamilk and onion), daidzein had a slower fecal disappearance rate but also an increased urinary recovery than genistein, naringenin, quercetin and hesperetin (Simons *et al.,* 2009). The authors suggested this was a direct result of the lack of 5-hydroxyl group on daidzein compound compared to the other flavonoid moieties. The rate of degradation of flavonoids by gut microflora are different and affected by structural differences. For example human microflora degraded flavonoids with 4,7- and 4′-OH group more quickly than those flavonoids lacking any one of these hydroxyl groups. These results may explain why rapidly degraded flavonoids such as genistein may not be as bioavailable in the colon, because they have less time to be absorbed before being metabolised by gut microflora (Simons *et al.,* 2009).

Pearson correlations were performed based on the relative urinary excretion as percentage of intake. There was a strong correlation between daidzein and genistein and between hesperetin and naringenin when they were ingested individually. When co-ingested urinary excretions of naringenin was strongly correlated with the excretion of genistein ($r=0.73$; $p=0.002$), and daidzein ($r=0.49$; $p=0.05$). This suggests that similar mechanisms may affect the gut hydrolysis and subsequent absorption for these two classes of compound. From the inter-individual differences observed, many of the results obtained from individuals with high levels of urinary excretion of hesperetin also show a correspondingly high level of urinary excretion of daidzein.

The hesperetin metabolites excreted in urine after the ingestion of citrus supplements (2 tablets contain 188 µmol/hesperidin), were similar to other studies

(Nielsen *et al.,* 2006, Bredsdorff *et al.,* 2010, Brett *et al.,* 2009). The observation that some of the volunteers may be better absorbers than others, may not only be a result of gut microflora differences, but also possibly due to particular polymorphisms for transporters or enzymes expression in the gastrointestinal tract (Manach *et al.,* 2005), and/or in the liver and kidney. In a recent study (Brand *et al.,* 2010) using an intestinal cell model, Caco-2 cells, showed that the co-administration of hesperetin with specific flavonoids such as quercetin improved the transport of hesperetin metabolites to the basolateral side; in addition there was a decrease in the amount of metabolites formed. Some dietary flavonoids have been shown to be potent BCRP-transporter inhibitors. This could lead to a decrease in the apical efflux of hesperetin metabolites and an increase in the transport to the basolateral sides (Brand *et al.,* 2010). Furthermore, competitition of the phase II conjugation enzymes upon co-exposure with quercetin and hesperetin may result in an increase in the transported aglycone.

Study 1 monitored excretion in different time periods over 36 hr, whereas study 2 collected the total 24 hr sample. Flavanones are mainly excreted within 24 hr, although isoflavones are circulating in the body for longer. This is also due to the enterohepatic circulation as a result of flavonoid metabolite excretion in the bile followed by β-glucuronidase activity of colon microflora releasing the aglycone allowing further absorption. Long collection times would also allow phenolic acid breakdown products of the flavonoids to be investigated which would give a broader view of absorption. Moreover, combining urinary excretion data with plasma measurements would provide complete understanding of bioavaliblity. Catabolite 3-hydroxyphenylacetic acid was identified in urine of some participants (n=8) by LCMS (data not shown). Further analysis of phenolic acids following the consumption of citrus food is required to fully understand the degradation by microflora and the complete bioavailability of flavanones. Further studies should investigate not only commonly co-consumed flavonoid-rich foods, and the impact of the food matrix, but also the competition between transporters, enzymes and bacterial metabolism in vitro. Bacterial breakdown products should also be followed in plasma and urine.

A limitation of the study in chapter 3, and for many other researchers on

bioavailability of flavonoids, is the incomplete hydrolysis achievable with the deconjugating enzymes available. Different flavonoid subclasses, and different positions of conjugation all have different affinities for the enzymes, and especially in the case of hesperetin-3′-*O*-sulfate, only 50-60% was hydrolysed. Other researchers have reported this with flavanones and other sub-classes (Bredsdroff *et al.,* 2010, Saha *et al.,* 2012). LC-MS can improve identification by providing m/z of the parent ion, and a daughter ion providing unequivocal identification of the metabolites in analysis. For this thesis, LC-MS was used to quantify the metabolites from study 2 (chapter 6&7), using synthesized hesperetin metabolites from Denis Baron (Nestle Research Center, Lausanne, Switzerland) and Christine Morand (INRA, Clemond Ferrand, France). Thus, ideally future studies should be conducted using LC-MS/MS with the full range of standards for flavonoid metabolites to allow complete quantification of all compounds, without the need for enzyme hydrolysis.

The total dietary intake of polyphenol is reported to be 1 g/d (Scalbert and Williamson, 2000) and polyphenol intake was negatively correlated with blood pressure in people who are at high risk of CVD (Medina-Remón *et al.,* 2011). The beneficial effects of flavanones on cardiovascular health outcomes have been reported in many studies (Giles *et al.,* 2005, Morand *et al.,* 2011, Buscemi *et al.,* 2012b, Asgary *et al.,* 2014). Hesperidin, which exists as a rich dietary source in citrus fruits, was suggested to contribute to reducing the incidence of CVD and particularly in reducing BP. The consumption of grapefruit was found to reduce SBP significantly compared with baseline (-3 ± 10.13 mm Hg, p=0.03; Dow *et al.,* 2012). Moreover, evidence from a cross-sectional international study (INTERMAP) reported that citrus fruit consumption is clearly correlated with diastolic BP in western consumers (Griep *et al.,* 2013). A meta-analysis study of 19 randomized controlled trials (RCT) has reported that a reduction of 2 mm Hg in DBP results in a reduction of incidence rates of coronary heart disease and hypertension by approximately 6% and 17%, respectively (Liu *et al.,* 2013a).

In chapter 6, the effects of hesperidin (from a commercially-available health supplement) on BP, on markers of endothelial function through measurements of PAT index, and on blood glucose were investigated. Our results were in accordance with those obtained by Morand *et al.,* (2011), who showed that consumption of 500

ml orange juice for 4 wk decreased DBP in healthy subjects when compared with the control drink plus placebo group. Moreover, in a similar study investigating the effect of the consumption of high naringenin sweetie fruit on blood pressure reported that DBP was reduced after 5 wk consumption in hypertensive volunteers when compared with low naringenin juice (Reshef *et al.,* 2005). The same observation that regular intake of OJ decreased SBP and DBP in young adult men was also reported (Bonifácio and César, 2009). On the other hand, a study conducted by BasiLe *et al.,* (2010) to investigate the effect of intake of pasteurized OJ (500 ml and 750 ml/d) for 8 wk, found SBP in both males and females did not change from baseline. However, DBP significantly reduced in male subjects (BasiLe *et al.,* 2010).

An animal study by Yamamoto *et al.,* (2008b) studied the mechanism by which hesperidin lowered blood pressure in spontaneously hypertensive rats (SHR). They concluded that the major metabolite, hesperetin glucuronide had a significant hypotensive effect at 9–12 hr after administration of hesperidin. This effect of hesperetin was associated with nitric oxide-mediated vasodilation. Increasing the production of NO by the vascular endothelium is one possible mechanism of action by which these flavanones may lower BP (Grassi *et al.,* 2009). Other mechanisms that could be responsible for lowering the BP include an inhibitory effect on angiotensin converting enzyme (ACE), which is a regulator of BP catalysing the conversion of angiotensin I to activated form angiotensin II. ACE can also degrade and inactivate the vasodilator bradykinin (Actis-Goretta *et al.,* 2006, Moore *et al.,* 2009).

It is important to examine the mechanism by which hesperetin metabolites and catabolites might exert a protective effect at physiological doses (Pereira-Caro *et al.,* 2014). In this context, a recent study by Chanet *et al.,* (2013), reported that hesperetin metabolites such as hesperetin-3′-*O*-sulfate, hesperetin-3′-*O*-glucuronide and naringenin-7-*O*-glucuronide significantly reduced monocyte adhesion to TNF-α-stimulated endothelial cells and modulated the expression of related genes involved in atherogenesis. Also reported were that flavanone metabolites conjugated on the B-ring were more efficient in decreasing cell adhesion compared with those conjugated on the A-ring, regardless of the nature of substituent glucuronide or sulfate.

Results from studies using orange juice suggested improvements in endothelial reactivity (Morand *et al.,* 2011, Rizza *et al.,* 2011). Hesperetin reported to play a crucial rule in BP regulation via activation and up-regulation of endothelium NO synthase (eNOS) which results in increased endothelial NO production (Liu *et al*., 2008). Endothelial function was assessed in the present study (chapter 6) by using Endo-PAT, which is an alternative method of assessment of endothelial function, which obtains radial artery pressure waveforms of smooth muscle tone (Wilkinson *et al.,* 2002). To the best of our knowledge, this is the first study to link ingestion of the effects of citrus tablets and a placebo to PAT index in a randomized, crossover, double-blind design. The results showed no significant effect on the PAT/ reactive hyperaemia index (RHI). Morand *et al*., (2011), demonstrated that the daily intake of 500 ml OJ for 4 wk improved endothelial function significantly, measured as the cutaneous acetylcholine micro-vascular reactivity by using Laser Doppler photometry. Moreover, hesperidin resulted in an improvement in the flow-mediated dilation of the brachial artery (Rizza *et al.,* 2011).

The findings of study 2 suggest there is no beneficial effect of short-term citrus hesperidin ingestion on markers of vascular health monitored by RHI. However, the augmentation index (AI, derived from the microvasculature and measured by the EndoPAT) improved significantly after citrus supplementation in 65% of the participants by 1.67% decrease from -3.0 ± 10.8 to -4.7 ± 10.7 ($p<0.01$). Additionally, the improvements in AI observed in this study were correlated with increases in diastolic blood pressure but not in RHI ($r= 0.63$; $p <0.005$). It has been reported that augmentation index showed no correlation with Endo-PAT RHI in 100 hypertensive patients (Yang *et al.,* 2011), suggesting improved AI to be independent of microvasculature dilation. Further, in another study a decreased augmentation index after chocolate intake was not accompanied by a drop in PWV (Vlachopoulos *et al.,* 2005).

A different study recruited 186 patients to assess the relationship between AI obtained from peripheral arterial tonometry (PAT) with cardiovascular risk factors. The results showed that AI measured by PAT was found to be correlated with central aortic blood pressures (Patvardhan *et al.,* 2011). Moreover, in the same study a correlation between age, heart rate, systolic blood pressure, mean arterial pressure,

pulse pressure, body weight and body mass index with AI were observed. Although, Yang *et al.* (2011) reported that age and sex were not correlated with AI in their study. The importance of the current study is that no previous assessments have been made of the relationship between citrus flavanone ingestion and arterial stiffness. However, these results should be considered with caution, due to the lower AI in the placebo group, which cannot be explained in the present study. Further investigations on this biomarker parameter may be worthwhile after a thorough validation of the measurement. This was a major limitation of the thesis as the probe costs were prohibitively high which meant a pre-study could not be conducted.

The fasting blood glucose levels in study 2 were not significantly different between the citrus and placebo treatments, although there was a trend towards a decrease at the end of the citrus phase. These results are in agreement with the results observed by Asgary *et al.,* (2014) where 22 healthy subjects consumed 500 mL OJ twice a day for 4 wk, and in accordance with the results by Morand *et al.,* (2011), where no changes in blood glucose level were reported. However, another study observed a significant decrease in fasting glucose levels after daily consumption of 750 ml OJ for 2 months in normo-lipidemic subjects (BasiLe *et al.,* 2010). It is feasible that the length of time for the intervention, the dose of flavanone given, or the well-controlled normal fasting blood glucose levels of the current subjects was not sufficient to provide a positive outcome. Moreover, the study was designed to primarily test for a decrease in BP, therefore more subjects may be required and a more sensitive analytical method for measuring blood glucose would be of benefit.

8.3 Future work

Orange juice is a popular drink in developing and developed countries. Hesperidin (hesperetin-7-*0*-rhamnoglucoside) is the main flavanone that is present in oranges and in their juice. The bioavailability of hesperidin from orange juice is highly bioavailable compared to other flavonoid compounds. In addition, hesperidin is potentially important to CV health and therefore could be recommended as a good dietary source to improve a healthy lifestyle. It is essential to note that many of the aspects attributed to these effects are not fully understood. This is specifically due to the contributory impact of flavonoids and polyphenols, in the diet, that also may have

similar biological effect. Furthermore there could be interaction between flavanones and other nutrients present in orange juice (e.g. potassium, folate, vitamin C), or elsewhere in the diet (other B-vitamins, vitamin D), that synergistically improve endothelial function vascular health. The following directions could be pursued to provide more evidence on the effect of flavonoids and specifically flavanones on CV health.

- Since glucuronidated and sulfated hesperetin were found in urine and plasma samples, well-designed studies are recommended to investigate the bioavailability of these compounds.

- Based on inter individual variation of flavanone absorption through the percentage excretion of the amount ingested, it would be very important to correlate the observed changes in CVD risk factors parameters with the plasma concentrations of these compounds not only with urine excretion. The information about the amount of metabolites available in plasma after the consumption of orange juice is very useful for future studies attempting to assess the impacts of flavanones on human health.
- Future studies should assess a dose response regarding absorption and in relation to CV health markers. Additionally, inflammation and endothelial dysfunction markers in plasma should be measured at the same time along with the concentration of hesperetin metabolites in plasma.
- Future large-scale human intervention studies in hypertensive and/or hyperglycaemic participants should be performed, in order to determine the effect of orange juice/hesperidin supplementation on endothelial function markers in humans.
- Ideally, participants should have a fully controlled and standardized environment and diet when conducting an intervention.
- More intervention studies in different populations using isolated flavanones compounds are recommended in order to explain the effect of these compounds in humans.
- Since the absorption mainly occurred in the large intestine, and the types of colonic microflora vary with individual, this will affect the absorption of

these compounds. Therefore, it will be worthwhile to assess the type of microflora populated in the colon to understand the bioavailability of hesperidin in humans and relate this to other flavonoid classes.

- Interactions between flavanones and other flavonoids, and indeed flavonoids and other nutrients should be investigated in both model systems and further well designed interventions.

8.4 Conclusion

The data provided from this thesis contributes to the growing knowledge on flavonoids found in orange and soya, with specific emphasis on the bioavailability of flavanones in an acute or chronic intervention, and from a matrix of commercially-available supplements and juice. The findings of this thesis provide good information about the forms of hesperetin metabolites using LCMS which verify the extensive modification in the human body after consumption of citrus-rich food before excreted in urine. Moreover, for the first time dietary health supplements were assessed for their ability to alter CVD risk factors. Although a significant decrease in blood pressure and fasting blood glucose were not found, there was a trend towards a reduction in these parameters in healthy overweight subjects, which does warrant further investigation both as a longer term study and in subjects who have higher blood pressure or metabolic syndrome. No changes in endothelial function were observed as measured by Endo-PAT, but this does not rule out potential changes in function, and should be further investigated by FMD or PWV in longer term studies. An extensive study on bioavailability of flavanones monitoring plasma concentrations as well as excretion levels of metabolites should determine the importance of absorption, metabolism and overall bioavailability on CVD risk. Although the results achieved in this thesis showed no significant change in CV risk biomarkers, more work is needed to verify the protective mechanism of hesperetin metabolites and the effects of different doses on the vascular protection in humans.

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Appendices

Appendix 1: Ethics approval study I

Research Support

University of Leeds

Leeds LS2 9JT Tel: 0113 343 4873

e-mail: j.m.blaikie@adm.leeds.ac.uk

MEEC Faculty Research Ethics Committee

University of Leeds

Abdurrahman Sweidan

PhD Research Student

School of Food Science and Nutrition

Leeds LS2 9JT

Dear Abdurrahman

Title of study: **Effect of acute and chronic co-ingestion of flavonoids on absorption**

Ethics Reference Number: **MEEC 10-001**

The above project was reviewed by the MEEC Faculty Research Ethics Committee at its virtual meeting of 16th August 2010.

The following documentation was considered:

Document	*Version*	*Date*
MEEC 10-001 ethical review form.docx	1	02/08/10
MEEC 10-001 study flyer FINAL.docx	1	02/08/10
MEEC 10-001 saliva protocol 6.docx	1	02/08/10
MEEC 10-001 Participant information sheet FINAL.docx	1	02/08/10
MEEC 10-001 consent form.docx	1	02/08/10
MEEC 10-001 Food record QUES.docx	1	02/08/10
MEEC 10-001 Appendix 5.doc	1	02/08/10
MEEC 10-001 Intervention study final chart.docx	1	02/08/10

On the basis of the information provided, the Committee is happy to approve the project, but would like to offer the following comments and advice:

1. You said that participants would be recruited by e-mail using "appropriate university distribution lists" – could you specify which distribution lists you mean?

2. You should have a protocol for dealing with any adverse events that might arise during the research.

3. Could you clarify whether only hard copies of personal data will be obtained and no personal data will be stored electronically, or if it is to be stored electronically will you take measures to ensure the data is encrypted?

4. On the flyer you mention that participants will collect "all of your urine", do you mean "samples of your urine"? The £10 gift voucher could be mentioned on the flyer.

5. On the consent form you do not really need to make reference to not answering particular questions as the participants are not going to be interviewed, but given that they need them to fill in the questions about their health/lifestyle etc accurately this could be confusing.

6. The saliva samples need to be labelled with their participant code rather than their own name. Alternatively you could pre-label the samples with the participant codes and then distribute them to the relevant participants.

Yours sincerely

Jennifer Blaikie

Research Ethics Administrator/ Research Support

On Behalf of

Professor Richard Hall

Chair, MEEC FREC.

Appendix 2: Participant information sheet study I

Dear participants,

There are many links between diet and health, a high intake of vegetables and fruits are associated with reduced risk of many cancers and coronary heart disease. In addition, a high intake of flavonoids or flavonoid rich foods has been inversely correlated with heart disease and cancer. Flavonoids (naturally-occurring antioxidants) are widespread in the human diet and samples such as urine and blood will allow us to measure the intake of fruit and vegetables. However, to the best of our knowledge, secretion of flavonoids into saliva after ingestion from food has rarely been investigated. Saliva will therefore be collected concurrent with urine samples when orange juice and soya nuts are co-ingested by volunteers.

I am writing to ask you to take part in this University of Leeds research project to assess the normal concentration of urinary flavonoids excreted after consumption of orange juice and soya nuts, ingested separately or together.

The decision to take part and participate in this study is yours and you can withdraw at any time without having to give a reason and without penalty. Please take your time to read and understand the study protocol before making your decision. Moreover, to help you make your decision, a short explanation of the study is given and you have one week to decide whether to participate or not.

What is the purpose of this study?

The purpose of this study is to investigate the effect of co-ingestion of flavonoids on their absorption, as most studies only look at a single food source when measuring bioavailability. Some studies have shown differences in absorption in model systems when flavonoids are given in combination and we would like to see if there is any change in absorption in people. The concentration of flavonoids in urine and saliva among subjects after eating soya and orange juice will be monitored.

Who is doing the study?

The study is being carried out by Mr Abdurrahman Sweidan, a research student at the School of Food Science and Nutrition, University of Leeds, towards the award of a PhD.

What will be involved if I take part in this study?

The study consists of 3 separate days; each will have a minimum of 24 hrs between them. Volunteers who have responded to the posted flyers/circular email will be invited to read the study information sheet, and the study protocol in full will be explained to each subject prior to the start of the study. Having given their verbal and written informed consent, participants will be asked to fill in:

- basic health questionnaire recording age, gender, height, weight, medical and surgical history, medications taken, life style (e.g. smoking and drinking habits and physical activity) to ensure the suitability of the volunteers for study.
- A urine and saliva samples collection record, a sample provision form will be asked to be completed after provision of each urine and saliva samples.

What are the advantages and disadvantages of taking part?

There are no direct benefits for taking part. But you will receive a £10 voucher as a thank you for taking part. The information obtained from this study and future studies will allow a better understanding of the role of flavonoids in the prevention of nutrition-related diseases, and in the inter and intra individual variation in the absorption and metabolism of these compounds.

Can I withdraw from the study at anytime?

Participants are free to withdraw at anytime they like. It is voluntary participation and you can stop at any stage of the study without giving any reasons.

Will the information I give be kept confidential?

Yes, all the information obtained will be kept confidential and handled in accordance with the data protection Act 1998. Participant's names will be number coded. In addition, all data will be kept in secure locked cabinet at the School of Food Science and Nutrition, University of Leeds.

What will happen to the results of study?

The result achieved will help to plan a large-scale dietary study on fruit and vegetables and health.

Who has reviewed this study?

This study has been reviewed by the University of Leeds Research Ethics Committee and ethical approval granted.

If you agree to take part, and would like more information and explanation please do not hesitate to contact:

Mr. Abdurrahman Sweidan

PhD Research student

School of Food Science and Nutrition

University of Leeds, Woodhouse Lane, Leeds, LS2 9JT

E-mail: *fsasw@leeds.ac.uk* **Tel: 07882019642**

You can contact the academic project supervisor Dr. Andrea Day or Prof Gary Williamson:

Tel: 01133432965

E-mail*:* ***a.j.day@leeds.ac.uk*** **,** ***G.Williamson@leeds.ac.uk***

If you have no more questions and you feel confident and agree to take part in this study, could you please complete the participant consent form and hand it to the researcher.

Thank you so much for your time to read this information and for your cooperation and help.

Appendix 3: Consent form

Name of researcher: Mr. Abdurrahman Sweidan **fsasw@leeds.ac.u**

AcademicSupervisors: Dr. Andrea Day **a.j.Day@leeds.ac.uk**

Prof. Gary Williamson **g.williamson@leeds.ac.uk**

		Yes/No
1	Have you read participant information sheet?	
2	Have you understood the information sheet which describes the above research?	
3	Have you had the opportunity to consider the information and ask questions about the study?	
4	Do you understand that participation is voluntary and you are free to withdraw from the study at anytime without giving any reasons?	
5	I understand that my responses will be kept strictly confidential. I give permission for members of the research team to have access to my anonymised responses. I understand that my name will not be linked with the research materials, and I will not be identified or identifiable in the report or reports that result from the research.	
6	Do you agree for the data collected from you to be used in future studies?	
7	Do you agree to take part in the above study?	

_____ _____ _____

Name of participant Date Signature

_____ _____ _____

Lead Researcher Date Signature

Appendix 4: Health Questionnaire

Please complete this form and answer all the questions.

Section 1: Background

Date		***ID***	
Name		**Marital status**	
Gender		**Educational level**	
Age		**Occupation**	

Section 2: anthropometry measurements

Parameter	**Measurement**
Height (cm)	
Weight (kg)	

Did you consume any dietary or health supplements? Yes No

If yes, which type .....

Did you have any antibiotic in the last two months? Yes No

Do you consider yourself as healthy person? Yes No

Are you allergic to orange juice or soya products? Yes No

Are you currently taking any medication? Yes No

Thank you for your time,

Abdurrahman Sweidan

Appendix 5: Food record (3 days)

Complete the table below for the food and drink that you eaten last 2 days and during the day of the study.

Date		Day	1	2	3		Subject code	

time	**Meal type**	**Where?**	**What kind of food eaten**	**Amount eaten**	**Brand name**
6 am to 8am					
8am to 12 noon					
12 noon to 3 pm					
3 pm to 5 pm					
5 pm to 8 pm					
8pm to 12 am					
12 am to 6 am					

- **Meal type**: snack, breakfast...etc.
- **Where?** Home, bar, restaurant...etc.
- **What kind of food eaten**? Bread, orange juice, tea ...etc.
- **Amount eaten**: description of portion size of item eaten, example (small slice, white bread, tea spoon, glass of water, bowl of soup...etc.

Appendix 6: Protocol for a cellular urine

Protocol for cellular urine

Urine preparation:

Urine samples of healthy volunteers will be transported to the lab within 12 hours of the collection where they will be processed within 24 hr before being frozen at -20°C.

- Upon arrival, the volume of sample will be measured and 2X 50-ml aliquots are taken.
- Urine aliquots are then centrifuged at 2000 g, 4°C for 10 min to remove insoluble materials and cellular debris.
- Being careful not to disturb the sediment and cell pellet, the supernatant is removed to within approximately 1-2 ml of the cell pellet.
- The supernatant is then filtered through a syringe filter (0.45 µm pore size).
- The samples will then be stored at -20°C until analyzed.

Waste disposal:

- The sediment will be decontaminated before disposal by adding Vikron (a disinfectant) (1% solution, 2X 5-g tablet /1 L) to the sediment and leaving over night.
- The sediment can then be discarded in the clinical waste.

General Safe operation:

- The urine samples are only processed on a designated bench area highlighted by biohazard labels.
- The centrifuge is disinfected before and after centrifugation by spraying 70% EtOH inside the chamber and wiping.

Reference:

Magistroni R. *et al*.; 2009; " Proteomic analysis of urine from proteinuric patients shows a proteolitic activity directed against albumin"; *Nephrol Dial Transplant*; **24**; pp. 1672-1681.

Appendix 7: Protocol for saliva collection using salivette collection container

Please follow the instruction and steps below before you collect your saliva sample:

- Do not drink or eat for 30 minutes prior to collection of saliva sample.
- Do not brush or floss your teeth before the collection. Also, remove lipstick, lip balms and any creams prior to collection.
- Do not collect the sample if your mouth is bleeding.
- Saliva sample should be collected at the time specified by the researcher.

Please collect saliva samples as follows:

1- Remove the top cap of the tube to expose the round sponge, taking care not to remove the holder that the sponge is sitting in.

2- Place the cotton tube into your mouth by tipping the tube so the sponge falls into your mouth. Do not touch the sponge with your fingers.

3- Keep the sponge in your mouth and place the cotton tube under your tongue. Very gently roll the cotton in the mouth and chew it for 10 minutes to start a flow of saliva until it is saturated.

4- Spit the saturated cotton tube back into the plastic salivette container. Do not touch the sponge with your fingers.

5- Close the cap firmly and make sure cap is on tightly.

6- Label the salivette collection container; write your name, date and time of collection.

7- Store the sample in the cool place and then take it to the laboratory next day.

Appendix 8: Ethical approval (Impact of oranges on cardiovascular health)

Performance, Governance and Operations
Research & Innovation Service
Charles Thackrah Building
101 Clarendon Road
Leeds LS2 9LJ Tel: 0113 343 4873
Email: ResearchEthics@leeds.ac.uk

UNIVERSITY OF LEEDS

Abdurrahman Sweidan
Food Science and Nutrition
University of Leeds
Leeds, LS2 9JT

MEEC Faculty Research Ethics Committee/ University of Leeds

Dear Abdurrahman

Title of study **Impact of oranges on cardiovascular health**

Ethics reference **MEEC 12-025**

The above project was reviewed by the MEEC Faculty Research Ethics Committee at its meeting of 14th March 2013. The following documentation was considered:

Document	*Version*	*Date*
MEEC 12-025 Orange juice and CVD - ethical review form.docx	1	28/02/13
MEEC 12-025 Orange juice and CVD - Study Appendices.docx	1	28/02/13
MEEC 12-025 signed page ethics.JPG	1	28/02/13

On the basis of the information provided, the Committee requested further information/ clarification on the following matters:

1. A4: This project is for a research qualification.
2. A8: The short summary of the research does use quite complicated concepts, technical terms and acronyms that may not be understandable. Later on there

is a succinct and plain description of the research aims. The summary should be similarly written.

3. A9: main ethical issues – reviewers felt this was too lengthy and some points not relevant to the question. Please review this. The information for participants is very good; perhaps some of the text could be used in the form to ensure simplified language.

4. C8: Why is the project restricted to recruiting from the University population, which is not very inclusive?

5. C24: Having read the Participant Information Sheet, this project appears to be externally funded and if so details of this should be included on the application form.

6. Have you considered telling the participants how they as a group responded to the tests?

A response, in summary form, should be sent to the Committee which addresses each of these points. Consideration will be given to your response and we will write to you with a further opinion.

Please note that the Committee is not able to approve your application at this stage so you are unable to begin the parts of your research requiring ethical approval.

Yours sincerely

Jennifer Blaikie

Senior Research Ethics Administrator, Research & Innovation Service
On behalf of Professor Gary Williamson, Chair, MEEC_FREC

CC: Student's supervisor(s)

Appendix 9: Participant information sheet study II

Dear Participants,

I am writing to ask you to take part in this University of Leeds research project to assess the effects of compounds (flavonoids) found in oranges on cardiovascular health. There is limited information on the effects of orange flavonoids, but so far the information published is very positive, indicating that these compounds are able to improve cardiovascular health in some individuals. With your help we would like to investigate this further.

The decision to take part and participate in this study is yours and you can withdraw at any time without having to give a reason and without penalty. Please take your time to read and understand the study protocol before making your decision and feel free to ask any questions to the study researchers.

What is the purpose of this study?

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the Western world. Measuring the health of blood vessels is now considered a predictor of future CVD risk; moreover blood vessel health can be improved and this will reduce the risk of CVD in the future. Flavonoids are found in all plant-based foods and beverages, and are associated with a reduced risk in CVD. There is accumulating evidence that flavonoids from oranges and orange juice can improve blood vessel health, but further studies are required to establish this relationship, especially given the fact that different people will absorb more or less of these flavonoids into their blood stream.

We will measure every volunteer's ability to absorb the flavonoids from an orange juice supplement by analyzing a 24 hour urine sample. We will also determine whether blood vessel health changes during two 4-week periods when different supplements are given to the volunteers.

Who is doing the study?

The study is being carried out by Mr. Abdurrahman Sweidan, a research student at the School of Food Science and Nutrition, University of Leeds; towards the award of

a PhD. Dr Andrea Day and Prof Gary Williamson are the PhD supervisors for this study.

What will be involved if I take part in this study?

Volunteers who have responded to the posted flyers/circular email will be invited to read the study information sheet, and the study protocol in full will be explained to each subject prior to the start of the study. Having given their verbal and written informed consent, participants will be asked to:

- Fill in basic health questionnaire recording age, gender, height, weight, medical and surgical history, medications taken, life style (e.g. smoking) to ensure the suitability of the volunteers for study.
- have a slightly restricted diet for 48 hours prior to each study session (see table below)
- Undergo a complete fast (water allowed) from 9 pm on the day before each study day.
- Take an orange juice supplement with water and collect urine for 24 hr on each of the study days.
- Take the provided supplements daily for two sets of 4 weeks.
- Attend 4 sessions on 4 separate study days, every 4 weeks for 12 weeks.

On each study day we will:

- Measure your weight, height and waist circumference.
- Measure your blood pressure
- Measure your fasting blood glucose level by taking a finger-prick blood sample using a single-use lancet (Accu-Chek Aviva),
- Measure the blood flow in your fingertips before and after reducing blood flow in your forearm using a blood pressure cuff (called an Endo-PAT – see later for more details)
    - Each study day will take approximately 1.5 hours
    - 4 study days in total, 1 every 4 weeks

- Total duration of study is 12 weeks

Restricted diet:

For 48 hours prior to each experimental session:

- All citrus foods and drinks need to be avoided.

ITEMS NOT ALLOWED

Food items to avoid
Citrus fruit in any form (fresh, canned or dried): e.g. oranges, tangerine, clementine, satsuma, grapefruit, lemon, lime
Fruit juices, fruit squashes (cordials) and jam or marmalade containing citrus fruit

Please eat your normal evening meal the day prior to each experimental session before 9 pm. Do not consume any food or drink, except water, after 9 pm.

Do not consume any food or drink, except water, on the morning of the experimental session.

Experimental session days:

We would like to measure the effects of orange juice compounds on the cardiovascular system, by using an Endo-PAT measurement which is non-invasive, completely safe and risk free. Firstly, however, we need to know how well the orange juice compounds are absorbed in you, as there is a large variation between individuals. Therefore, all participant need to have a restricted citrus diet for 48 hours prior to all experimental sessions, and also fast overnight starting the evening before all four experimental days from 9:00 pm.

When you arrive at the study room in the School of Food Science and Nutrition, we will:

(1) ask you to complete a short questionnaire on your normal citrus intake over the past month(s)

(2) take a finger prick blood sample, in which we can measure your blood glucose level

(3) ask you to provide us with a urine sample

(4) measure your height, weight and waist circumference

(5) take your blood pressure, using a standard inflatable cuff on your upper arm

(6) measure your finger-tip blood flow using a non-invasive technique called Endo-PAT

- You will be asked to lie down on a bed and relax for about 30-40 minutes total
- We will place a thimble-like probe on the end of each index finger as shown in diagram A below.

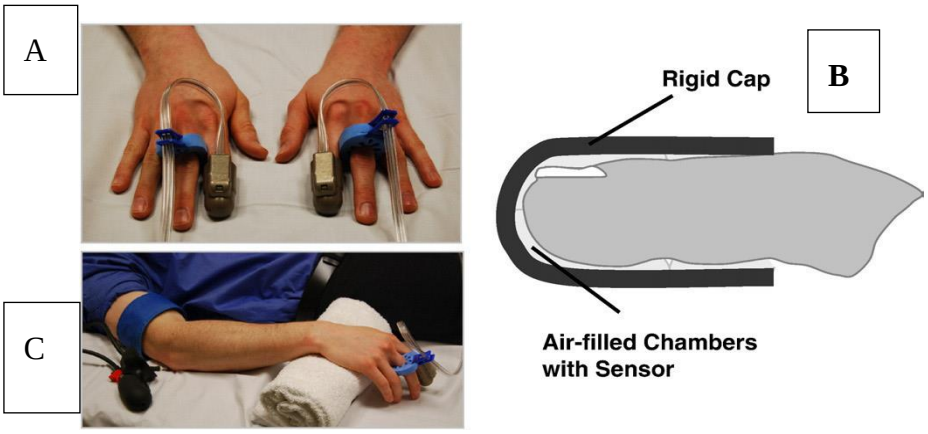

Diagram: Endo-PAT fingertip and inflatable cuff. *Reproduced from Hamburg, N., & Benjamin, E. (2009)*

- We will take a repeat blood pressure measurement after 5 and 10 minutes
- After 15 minutes we will inflate the finger tip probe with air (diagram B) and start to measure the blood flowing through your finger tip
- When you have a stable blood flow (about 20-25 minutes) we will inflatable a blood pressure cuff (diagram C) around your forearm to interrupt blood flow to your lower arm and fingers. This procedure is completely safe and without any risk or danger, but you may experience a discomforting, throbbing sensation in your arms and fingers as the cuff is slightly tighter than for a blood pressure measurement. The cuff will be released after exactly 5 minutes, and blood flow resumes. Any discomfort you felt will immediately cease.

- You will need to remain in a relaxed state for a further 5-10 minutes while the measurement reading is completed.

(7) We will then ask you to take an orange juice supplement with water, and provide you with a light breakfast of toast and/or cereal.

(8) We will provide you with a urine collection bottle in which you need to collect all your urine for the following 24 hours. You will need to return this to the University or arrange for it to be collected.

(9) We will provide you with supplements to take home to consume with water on a daily basis for the following 4 weeks. We would like you to bring back the supplement bottle at the end of the 4 week period and to note down any days you forgot to take the supplement on.

You are then free to leave. The experimental procedure should take about 1 hr (1.5 hr maximum), plus time for breakfast.

What are the advantages and disadvantages of taking part?

There are no direct benefits for taking part, however information obtained from the study will allow a better understanding of the role of flavonoids from citrus fruits in the prevention of cardiovascular diseases. You will receive £30 as a thank you for completing the study.

There may be a slight discomfort in the overnight fast from 9 pm prior to each study day (water is allowed), but you will be given breakfast as soon as all measurements have been taken at approx 10 am. The supplements are commercially available, and should not cause any discomfort. We recommend you consume them with your regular breakfast.

The experimental procedure involves using a blood pressure cuff on the forearm to restrict blood flow for 5 minutes. This may cause some slight discomfort and a tingling sensation in the fingers. The procedure is not associated with any risk, and feelings of discomfort cease immediately with the release of the pressure cuff after 5 minutes. There is the possibility of slight bruising of the forearm, but this is unlikely in most people.

Can I withdraw from the study at anytime?

Participants are free to withdraw at anytime they like. It is voluntary participation and you can stop at any stage of the study without giving any reasons.

Will the information I give be kept confidential?

Yes, all the information obtained will be kept confidential and handled in accordance with the data protection Act 1998. Participants will be number coded and all data obtained will not be identifiable to you. In addition, all data will be kept in secure locked cabinet at the School of Food Science and Nutrition, and will be destroyed after 5 years, in accordance with University of Leeds regulations.

What will happen to the results of study?

The results achieved may be published in academic journals or presented at research conferences or meetings. The results will also be used for Abdurrahman Sweidan's PhD thesis. Participant will not be identified in any publication/thesis.

Who has reviewed this study?

This study has been reviewed by the University of Leeds Research Ethics Committee and ethical approval granted.

Who is funding this study?

Mr Abdurrahman Sweidan is an international PhD student funded by Ministry of Higher Education in Libya.

Contact for further information:

If you agree to take part, and would like more information and explanation please do not hesitate to contact:

Mr. Abdurrahman Sweidan

PhD Research student

School of Food Science and Nutrition

University of Leeds, Leeds, LS2 9JT

E-mail: *cvd2013@leeds.ac.uk* **Tel: 0784 849 8746**

Alternatively you can contact the academic project supervisors:

Dr. Andrea Day

E-mail: a.j.day@leeds.ac.uk ,

If you have no more questions and agree to take part in this study, could you please complete the participant consent form and hand it to the researcher.

Appendix 10: 24-hour urine instruction form

Subject code: ☐ Date ☐ / /

- collect all urine into the labeled bottles provided
- write down the day, time and date you finish the collection.

  Day (.....), date (...../...../.....), time (.....: .....).
- keep the urine sample bottles in dark and cool place if possible, until you bring them back to the University.
- if you lose any specimens or have any problems during the whole period of the study please report that in the following table:

Day/Time:	
Sample Collection Irregularity (eg. loss of all sample/ some sample):	
Comments:	
Day/Time:	
Sample Collection Irregularity (eg. loss of all sample/ some sample):	
Comments:	

Appendix 11: Supplement instruction form

Subject code: [ ] Date [/ /]

- Take 2 tablets of the supplements provided every morning with breakfast, or before 11am.
- If you forget to take the tablets, then please take them as soon as you remember but not past 6 pm.
- If you do not take a tablet on a day, please make a note of this on the form below.
- Please return this form and any remaining supplements to the study investigators when you return after 4 weeks.
- Please make a note of the last day for taking the supplement:

  Day (.....), date (...../...../.....), time (.....:.).

Supplement Irregularities:

Date on which supplements taken later than 11 am	**Time taken (if after 11 am)**
Date on which supplements were completely missed:	
Number of supplements remaining	

Appendix 12: Food frequency questionnaire for citrus fruits and juices

Fresh fruit: portion size = 1 whole											
	Daily			**Weekly**			**Monthly**				
	1	2	3	1	2-3	4-5	<1	1	2	3	**Never**
Orange											
Grapefruit											
Lemon											
Lime											
Tangerine											
Clementine											
Satsuma											
Other citrus fruit; please specify:											
Fruit Juice: portion size = 100 ml (small) glass											
Orange juice (fresh or from concentrate)											
Grapefruit juice (fresh or from concentrate)											
Other (please specify):											
Dried citrus fruit: portion size = handful											
Type (please specify):											
Canned fruit: portion size = 1/3 regular sized can, or 1 small can											
Type (please specify):											
Marmalade: portion size = cafe packet (i.e. amount thinly spread on 1 piece of toast)											
Orange marmalade											
Grapefruit marmalade											
Other marmalade (please specify):											

Appendix 13: Pain assessment questionnaire

Participan code:

Date: / /

- Please tell us how do you feel? You may feel happy because you have no pain (hurt), or you may feel sad because you have some or a lot of pain.

Wong-Baker Faces Pain Rating Scale

- **Face 0** is very happy because he or she doesn't hurt at all.
- **Face 1** hurts just a little bit.
- **Face 2** hurts a little more.
- **Face 3** hurts even more.
- **Face 4** hurts a whole lot.
- **Face 5** hurts as much as you can imagine, although you don't have to be crying to feel this bad.

- You should point to each face using the words to describe the pain intensity. You should then choose the face that best describes how you feel.

Thank you for your participation,

Abdurrahman Sweidan

Main study investigator

Appendix 14: Protocol for adverse events during intervention

- Orange juice and soya nuts are normal foods; anyone with a suspected allergy to these foods will not be allowed to participate in study.
- Water will be offered during food consumption and participants will be advised to drink water during the study day (bottled water will be provided).
- Participants will be supervised during intervention and advised to eat and drink slowly.
- Participants will be asked to collect their urine in labelled containers. If a spillage occurs they must inform researcher investigator immediately who will take measures to clean and decontaminate the relevant area.
- People who are allergic to orange juice and soya will not be allowed to participate in this study. However, if allergic symptoms appear, food which causing the symptoms will be eliminated and then a doctor and dietician who is expert must be consulted.
- If there are any adverse events, actions will be taken if necessary:
    - First aid will be used by administered by a trained person within the school.
    - Call University security number (32222) to call 999 if ambulance is required.