PLANT POLYPHENOLS: CHARACTERISATION, METABOLISM AND BENEFICIAL ACTIVITY IN MODEL SYSTEMS

Reyna Gutiérrez Rivera

Submitted in accordance with the requirements for the degree of
Doctor of Philosophy

The University of Leeds
School of Food Science and Nutrition

May, 2015

The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

LIST OF ACCEPTED CONFERENCE ABSTRACTS

Polyphenol profile of German chamomile (*Matricaria recutita*) and its inhibition of COX-2 activity *in vitro* by R. Gutiérrez-Rivera, A. Kerimi and G. Williamson (2014)

8th World Congress on Polyphenols applications, Lisbon, Portugal

International PlantLIBRA conference, Vienna, Austria

How much do we know about polyphenols in traditional medicine? A plausible approach to understand the activity of polyphenols phase II metabolites on human health by R. Gutiérrez-Rivera, T.P. Dew and G. Williamson (2013)

XI Symposium of Mexican Studies and Students, Sheffield, UK

Polyphenol phase II metabolites: biological synthesis and characterisation by LC-ESI/MS by R. Gutiérrez-Rivera, T.P. Dew and G. Williamson (2013 -2012)

Industry Day at the School of Food Science and Nutrition, Leeds, UK

Warwick Mass Spectrometry 80/60 Conference, Warwick, UK

ACKNOWLEDGEMENTS

This journey has been an exciting adventure, full of learning and life-challenges. Firstly, I would like to thank the National Council of Science and Technology (CONACyT) for the award of my PhD scholarship. Next, I would like to thank my supervisor Professor Gary Williamson for giving me the opportunity to join his research group and guiding me through out this project. I would also thank Dr Tristan P. Dew for being my mentor during the beginning of my PhD and for sharing with me his knowledge and passion about mass spectrometry. Tristan, thank you for your patient and comments, you have always encouraged me to believe in myself.

I would like to thank Dr Asimina Kerimi for her support and work on the first screening of German chamomile. Also, I would like to thank all the staff in the teaching laboratories (Mr Kajda, Mr Ratcliffe, Dr Maycock and Mr. Hardy) and administrative staff of the School of Food Science and Nutrition who always gave me a hand when I needed it.

A special thank you goes to Dr Nicolai U. Kraut with whom I spent long hours in the laboratory dealing with problems about mass spectrometry and for his comments about this thesis. My dear friend, thank you so much for being there and share with me this wonderful stage of our lives. I am really grateful to Dr Kerstin Ziegler, Dr Joana Encarnaçao and Mrs Hilda Nyambe-Silavwe for all their honest comments, encouragement and company during those long experimental days.

This experience would have not been the same without the support of my dear friends Mr Marijan-Benedict Hubert, Mrs Kate Smith (née Timmins), Ms Michelle Morris, Ms Danielle Graham, Mrs Liliana Valles and Mr Gabriel Mata, and all those who have been part of this since the beginning.

My words are not enough to express my gratitude to my parents and my dear friend Ms Dina Arrieta back in Mexico. They coped with long calls, ups and downs, and they were a big part of this journey. Thank you so much for showing me the light when things looked pretty dark.

My deepest thank you goes to Mr Stephen Davies who joined this journey in the toughest part. Stephen, thank you for your encouragement, empathy and unconditional love; this achievement is also yours.

Finally, I would like to dedicate this thesis to the first person who thought that this dream could come true, Dr Francisco J. Gallardo Escamilla (R.I.P); you encouraged me and believed that it could be possible. Thank you for being not only my mentor, but also my friend. I will be always grateful for that.

ABSTRACT

The pursuit of efficient and safe alternatives to improve health and treat physical/mental illness has found in plant extracts a potential source of bioactive compounds such as polyphenols. The work presented within this thesis aimed to investigate the changes in the plant extract after ingestion, identify the remaining polyphenols and phase II conjugates, and to finally test their possible biological activity in *in vitro* assays.

German chamomile (GC) was used as experimental case which was treated with a multiple-enzyme hydrolysis to mimic the process most likely to occur in the gastrointestinal tract. This methodology was previously used in five different fruit matrices and it was successfully applied to plant-based materials. It was possible to identify and quantify a series of compounds before and after treatment by LC-ESI/MS, and these results were compared with data found in the literature. In addition, it was found that differences in sample preparation and a suitable organic solvent for liquid-liquid extraction had an important impact in the quantification of these compounds.

The transformation of polyphenols after the phase II metabolism was also investigated. For this purpose, hesperetin aglycone was used in the laboratory to produce the main monosulfate commonly found in urine samples. For the first time, *p*-nitropheyl sulfate (PNS) was used as co-cofactor in the biological sulfonation of polyphenols which enhanced the transformation to hesperetin-3'-*O*-sulfate (Hp3'SO_4). Indeed, it was the first time that Hp3'SO_4 was quantified in urine samples after orange juice consumption using a true standard.

Finally, the anti-inflammatory activity of tested plant extracts and their main polyphenols was assessed by measuring their inhibitory activity against recombinant human COX-2. An enzyme immunoassay (EIA) and *in vitro*-LC-MS methodologies were used for this purpose. Consistent COX-2 inhibition was found for green tea (GT) and its two major polyphenols, epigallocatechin gallate (EGCG) and epigallocatechin (EGC) which showed inhibition in a concentration dependence manner. On the other hand, various polyphenols and plant extracts also presented COX-2 inhibition, but did not show concentration dependence.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS & UNITS

<	Less than
>	More than
°C	Centigrade
µL	Microlitres
µm	Micrometres
ANOVA	Analysis of variance
ATP	Adenoside 5'-triphosphate
BW	Body weight
$CaCl_2$	Calcium chloride
COX-2	Recombinant human cyclooxygenase 2
cucurbita	Mexican chilacayote extract
DTT	DL-dithiothreitol
dw	Dry weight
EC	Epicatechin
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked immunosorbent assay
Ellman's reagent	5,5'-dithiobis-(2-nitrobenzoic acid)
g	Also known as relative centrifugal force
GC-MS	Gas chromatography mass spectrometry
ginseng	Ginseng Korean root powder
GT	Green tea
HCl	Hydrochloric acid
Hp3'Glu	Hesperetin-3'-*O*-glucuronide
Hp3'SO_4	Hesperetin-3'-*O*-sulfate
Hp7Glu	Hesperetin-7-*O*-glucuronide
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography diode-array

	detection
hr; hrs	Hour;hours
HUVEC	Human umbilical vein endothelial cells
IC_{50}	Half maximal inhibitory concentration
K3glu	Kaempferol-3-*O*-β-glucuronide
kg	Kilogram
L	Liter(s)
LC	Liquid chromatography
LC-ESI/MS	Liquid chromatography-electrospray ionisation mass spectrometry single quadrupole
LC-ESI/MS^2	Liquid chromatography-electrospray ionisation tandem mass spectrometry triple quadrupole
LC-MS	Liquid chromatography-mass spectrometry
LC-MS^2	Liquid chromatography tandem mass spectrometry triple quadrupole
LC-MSD-TOF-ESI/MS	Liquid chromatography time-off-flight electro spray ionisation mass spectrometry
LC-PDA-ESI/MS	Liquid chromatography-photodiode-array electro spray ionisation mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
M	Molar
m/z	Mass-to-charge ratio
mg	Miligram(s)
min	Minutes
mL	Mililitres
mm	Milimetres
mM	Milimolar
nm	Nanometres
NMR	Nuclear magnetic resonance spectroscopy
NSAIDs	Non-steroidal inflammatory drugs
PAP	Adenosine 3’,5’-diphosphate
PAPS	Adenoside 3’-phosphate-5’-phosphosulfate lithium
PG	Prostaglandins

PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2
pH	Negative common logarithm of the concentrartion of hydrogen ions [H^+] in moles/litre. Commonly known as *potential hydrogen*.
PNS	Potassium 4-nitrophenyl sulfate
PPX	20(S)-protopanaxatriol
rpm	Revolutions per minute
sec	Seconds
SIM	Single ion monitoring
SULTs	Sulfotransferases
TIC	Total ion counting
TPC	Total phenolic content
U	Unit (s)
UK	United Kingdom
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight
WHO	World Health Organization
x	Times

CHAPTER 1 Literature review

1.1 Plant extracts and their bioactive compounds

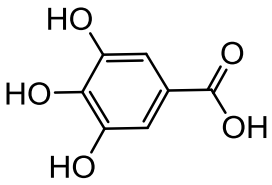
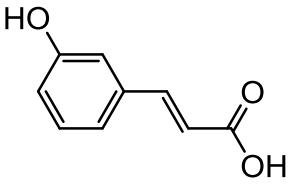
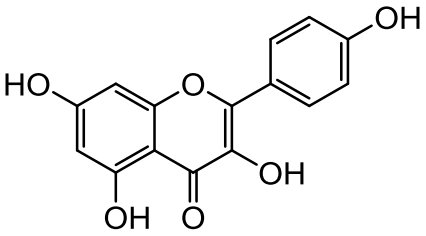
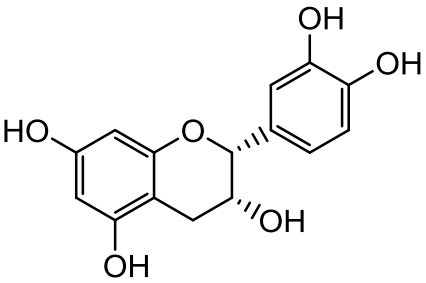
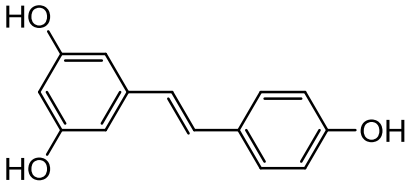
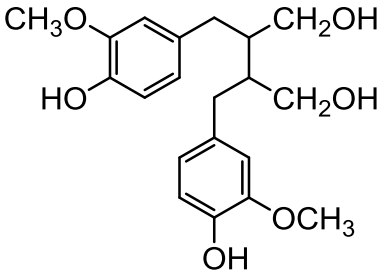
Plant extracts have been used as remedies to ease common afflictions for centuries and their popularity in Europe is increasing (Garcia-Alvarez *et al.*, 2014). Plants are used in different types of products such as food supplements, ointments and herbal medicines amongst others. The World Health Organisation (WHO) has listed them as part of the traditional/complementary/alternative medicine and included them as herbal medicine (WHO, 2002). Their importance has been addressed in the latest report by the World Health Organization (WHO, 2014), as their economic impact is growing in developing and industrialised countries. Information about their health benefits has been reported and it has been mostly attributed to different phytochemicals, in particular polyphenols (Crozier *et al.*, 2009; Chow and Hakim, 2011; Williamson *et al.*, 2011)

Polyphenols are plant secondary metabolites, widely found in fruits, vegetables, cereals and different beverages (Pandey and Rizvi, 2009). They contribute to general maintenance of the plant and are allocated in different cellular and subcellular levels *in situ*. It has been reported that the polyphenol content in plants can be affected by several factors, such as variety, growing environment, temperature, interaction with other polyphenols and many others (D'Archivio *et al.*, 2010). For instance, Alfaro *et al.* (2013) conducted a five year study to determine the impact of growing season on murtilla fruits (*Ugni molinae* Turcz) and found that the plant is affected by high rainfall and number of frosts during the season, increasing its polyphenol content. As well, Eid *et al.* (2013) tested the effect of cultivar and ripening on polyphenol content of dates. They found that the concentration of polyphenols was higher in unripe dates (kimri stage) than mature stages (e.g. rutab or tamr) and the cultivar Ajwa presented higher polyphenol content at the khalal stage than Barni and Khalas cultivars.

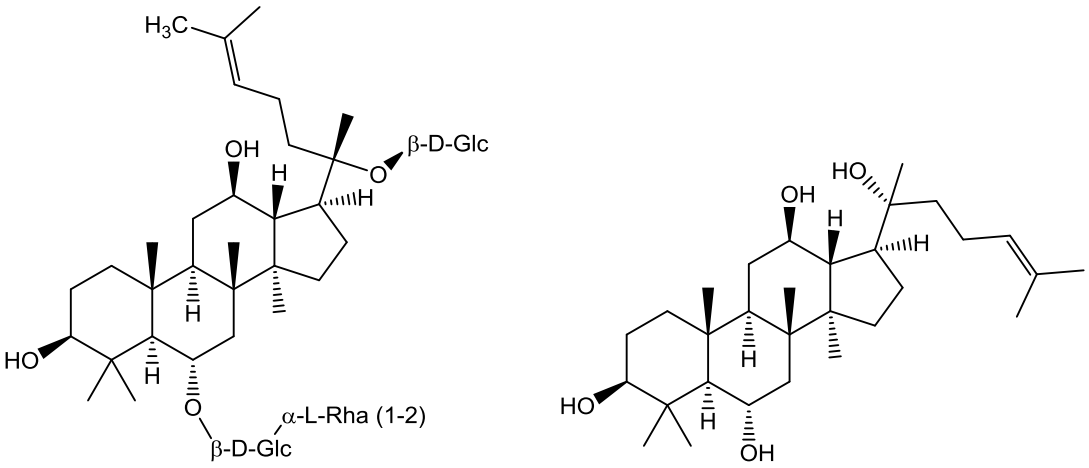
Different groups of polyphenols will be present in the plant and they are classified according to their number of phenol rings and the structure

that it binds them. They are: *phenolic acids, flavonoids, stilbenes* and *lignans* (see Table 1.1) (Manach *et al.*, 2004).

Table 1.1 (Poly)phenol classification and example(s) for each category.

Classification	Chemical structure
Phenolic acids	
Gallic acid (benzoic acid derivative)	
Coumaric acid (cinnamic acid derivative)	
Flavonoids	
Kaempferol (flavonol)	
Epicatechin (flavanol)	
Stilbenes	
Resveratrol	
Lignans	
Secoisolariciresinol	

Flavonoids are the most abundant polyphenols in the plant kingdom and their main subclasses are flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones and isoflavones. There are other minor subclasses which are dihydroflavonols, flavan-3,4-diols, coumarins, chalcones, dihydrochalcones and aurones (Crozier *et al.*, 2009). A combination of these subclasses can be found in a single plant. For instance, **German chamomile** (*Matricaria recutita*) is used to ease skin affections and it contains flavones (e.g. apigenin-7-*O*-glucoside), flavonols (e.g. quercetin), flavanones (e.g. naringenin) and coumarins (e.g. umbelliferone) (Singh *et al.*, 2011). **Green tea** (*Camellia sinensis*) is highly sought for its potential health benefits against cardiovascular diseases, cancer, osteoporosis and others, and it is a rich source of flavan-3-ols such as epicatechin and its esterified form epigallocatechin gallate. It also contains flavonols such as quercetin, kaempferol and myricetin (Crozier *et al.*, 2009). Indeed, other plant extracts, like ginseng and ginkgo, contain different compounds of notable interest. **Ginseng** (the roots of *Panax ginseng*) is commonly used in Traditional Chinese Medicine and it contains different compounds such as fatty acids, flavonoids, polysaccharide, saponins and others. It is believed that a class of saponins, known as ginsenosides (see Figure 1.1), are the major bioactive compounds of the plant and these are of major scientific relevance (Wang *et al.*, 2012a).



Ginsenoside Re

Protopanaxatriol

Figure 1.1 Ginsenosides examples. Ginsenoside Re is a natural glycoside and protopanaxatriol is a cyclic alcohol. Glc= glucose, Rha= rhamnose.

Ginkgo (*Ginkgo biloba*) is also widely used in Traditional Chinese Medicine and its commercially-available extracts contain different flavonoids (e.g. quercetin, isorhamnetin, kaempferol and their glycoside derivatives) and various terpene lactones (Dew *et al.*, 2014). The terpene lactones are divided into diterpenes (gingkgolides) and sesquiterpenes (bilobalide) (see Figure 1.2), and are reported to help the treatment against mental diseases like dementia (Solfrizzi and Panza, 2015).

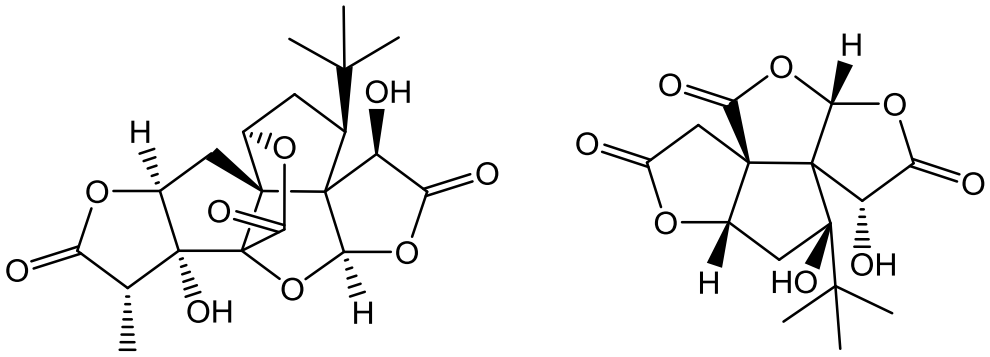


Figure 1.2 Ginkgolide examples. Ginkgolide A and bilobalide are terpene lactones.

Identification of the main compounds in plants is the first step to provide a profile of the possible active analytes. The composition is the key to understand the plant and it provides evidence to support further analyses. For example, German chamomile has been analysed by different research groups focusing on its content of apigenin aglycone and its derivatives (Švehlíková *et al.*, 2004; Srivastava and Gupta, 2009; Avula *et al.*, 2013), but few other papers have searched for other compounds such as umbelliferone, luteolin, ferulic acid hexoside and others (Lin and Harnly, 2012; Guimaraes *et al.*, 2013; Matic *et al.*, 2013). The quest for a simple methodology that provides sensitivity and selectivity to identify and quantify a wider range of plant polyphenols is still ongoing.

1.2 Metabolism of plant polyphenols

Flavonoids occur in plants in the form of esters, glycosides or polymers that cannot be absorbed in their native form. After plant extracts

have been consumed, flavonoids pass through the digestive system and undergo a series of reactions to be absorbed, metabolised and further excreted (O'Leary *et al.*, 2001; Manach *et al.*, 2004). The first step of metabolism is deglycosylation of flavonoid glycosides to release their aglycone form (see Figure 2.1) (Aherne and O'Brien, 2002). This process can occur in the plant itself, in the gastrointestinal mucosa, or in the colonic microflora which release simple aromatic acids during this process. Non-enzymatic deglycosylation does not happen in humans (Scalbert and Williamson, 2000).

Phase II metabolism is characterised by the conjugation of aglycones to yield glucuronidated, sulfated and methylated forms. These processes take place in the cells of the small intestine and liver, but sulfation mainly takes place in the liver (Dueñas *et al.*, 2011). A concise diagram of these pathways can be seen in Figure 1.3.

It has been suggested that conjugation will vary according to the nature of food and dose ingested. Indeed, the balance between sulfation and glucuronidation may be affected by type, gender and lack/abundance of food, as well as the composition and competence of each individual's gut microflora (Manach *et al.*, 2004; Crozier *et al.*, 2009). Conjugation reactions increase the solubility of the flavonoids which lead them to further excretion. Large conjugated forms are more likely to be eliminated in the bile while smaller conjugated forms are excreted in urine (O'Leary *et al.*, 2001; Manach *et al.*, 2004).

1.2.1 How polyphenol conjugates are identified and quantified?

The conjugated forms are chemically distinct from their aglycones and are more likely to have different physiological activities (Kroon *et al.*, 2004). Human studies investigating the pharmacokinetic parameters and biological effects of conjugate forms normally collect plasma and urine samples to perform the analysis. However, if plasma is not available, urine can be used to determine the degree of absorption of the target compounds.

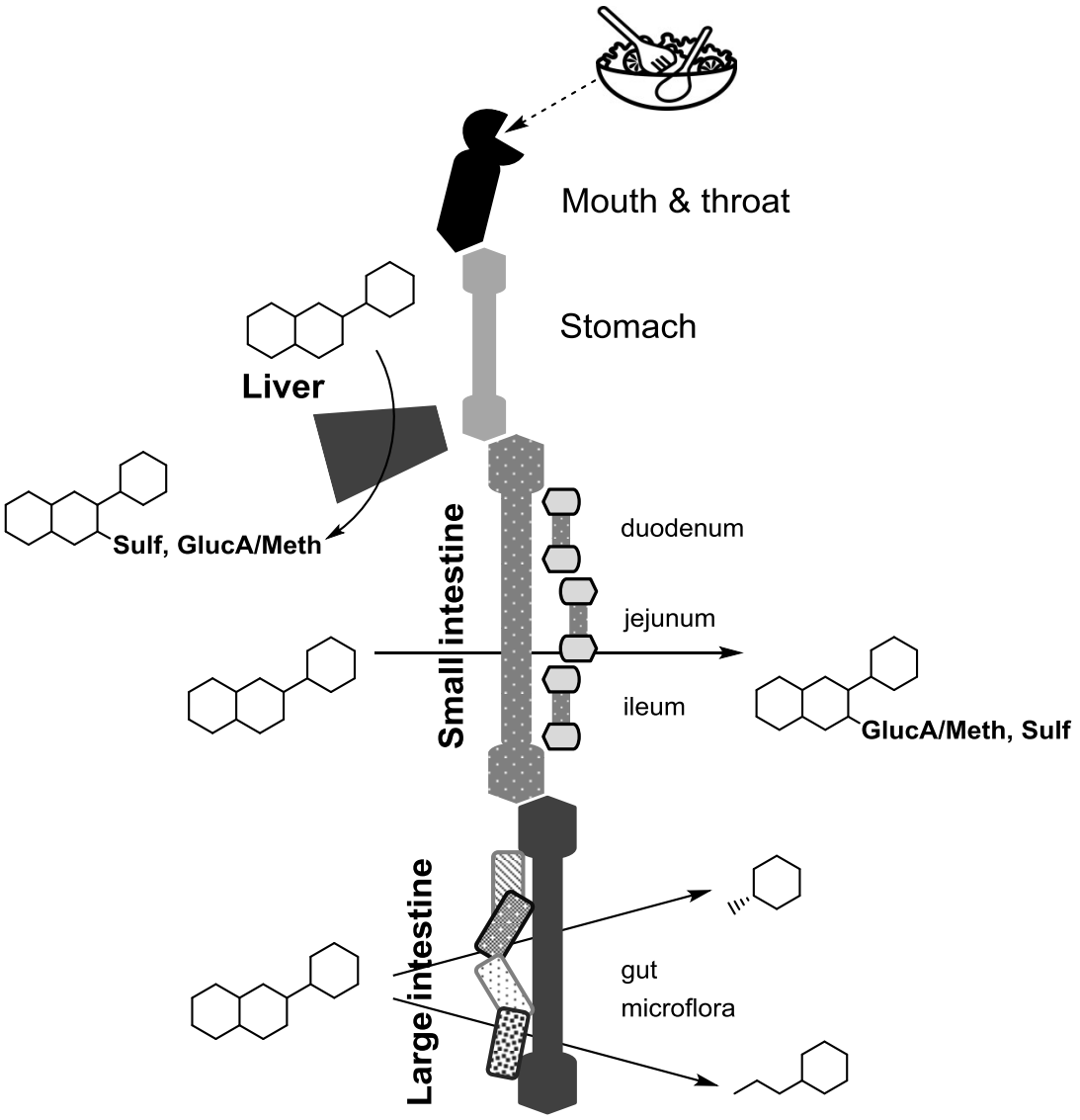


Figure 1.3 Diagram of phase II metabolism routes in humans. Conjugated forms: GlucA=glucuronidated, Sulf=sulfated and Meth=methylated.

Research on polyphenol conjugates has focused on the identification of the main conjugated forms that appear *in vivo* for which various analytical techniques have been developed. The most common techniques are liquid chromatography (LC), gas chromatography (GC), liquid chromatography-mass spectrometry (LC-MS) and immunoassays (e.g. ELISA) (Wilkinson *et al.*, 2002). The main advantages and disadvantages for each technique, e.g. sensitivity, multi-analyte detection, sample preparation, cost of preparation/reagents/equipment among others, are critical depending on the analyte and its chemical specifications. For instance, analysis of soy foods does not require high sensitivity techniques as the isoflavonoid analytes are

present at significant concentrations. Thus, HPLC-DAD is a suitable technique to identify and quantify the major soy metabolites (Wilkinson *et al.*, 2002).

Currently, the most used technique to determine the appearance of conjugated flavonoids after food ingestion is enzymatic hydrolysis. The quantification is performed measuring the aglycone forms for each metabolite by LC or LC-MS. However, it has been shown that not all conjugates are good substrates for these commercial enzymes which could likely underestimate the conjugates quantification (Farrell *et al.*, 2011; Saha *et al.*, 2012). Furthermore, the synthesis of true standards of the conjugate forms has become an important research field which will help to determine the appropriate concentration of flavonoid conjugates to use in *in vitro* assays. For example, hesperidin is a flavanone found in citrus that after deglycosylation (first metabolism) is transformed into hesperetin (aglycone form). Hesperetin conjugates (e.g. hesperetin-3'-*O*-glucuronide) have been identified in urine samples after orange juice consumption (Brett *et al.*, 2009; Bredsdorff *et al.*, 2010; Brand *et al.*, 2010; Pereira-Caro *et al.*, 2014), but the positive quantification of hesperetin sulfate has relied on LC-MS^2 and NMR identification. So far, these publications have agreed that hesperetin-3'-*O*-sulfate is the main monosulfate found in urine after orange juice consumption, but none has used a true standard for its identification or quantification.

1.2.2 Sulfate polyphenols and their occurrence in humans.

Sulfation is an important pathway in the transformation of polyphenols, hormones, drugs, chemical carcinogens and others. The enzymes in charge of this reaction are known as sulfotransferases (SULTs) and are located in the Golgi apparatus and in the cytosol of cells (Gamage *et al.*, 2006). The cytosolic SULTs catalyse the sulfation reaction where the activate sulfate, adenoside 3'-phosphate 5'-phosphosulfate (PAPS) donates a sulfite group (SO_3^{2-}) to a compound with a hydroxyl or amino group to form a sulfate or sulfamate ester (see Figure 1.4).

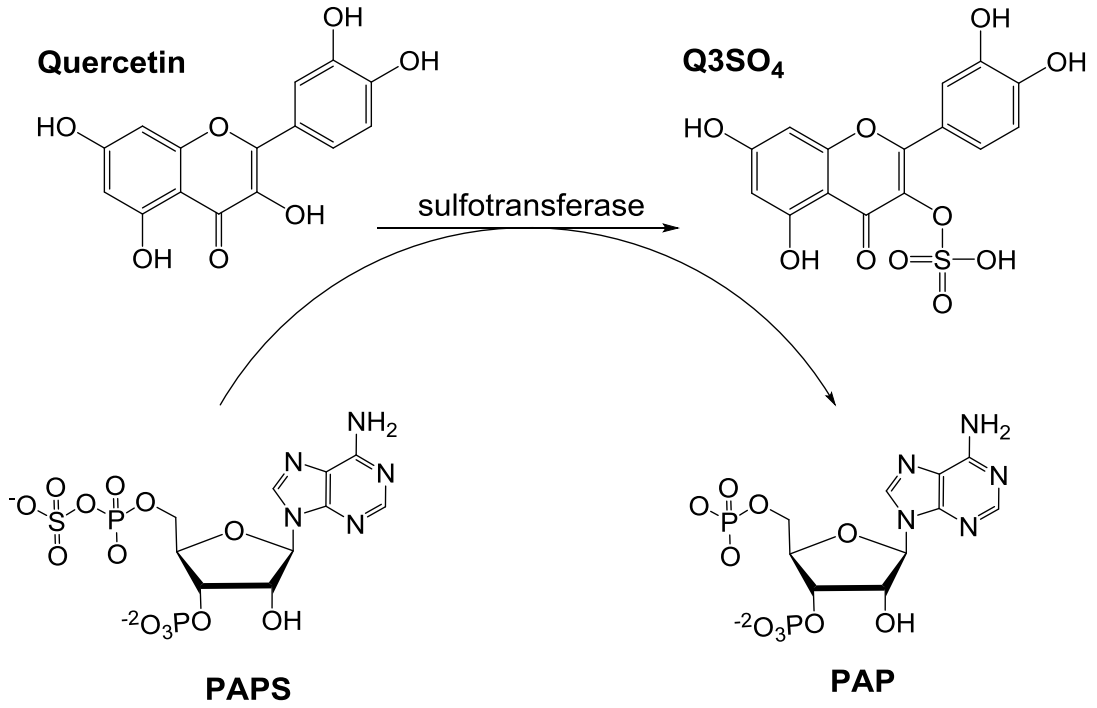


Figure 1.4 Sulfation reaction catalised by SULTs (sulfotransferase) using the universal donor PAPS with the example of quercetin to quercetin-3-*O*-sulfate ($Q3SO_4$).

Five distinct SULT families have been identified in mammals, but only three families and thirteen distinct members have been characterised in humans. These are: SULT1 – A1,A2,A3,A4,B1,C2,C4,E1; SULT2 – A1 and B1 and SULT4 – A1. Gamage *et al.* (2006) have published detailed information for each family and their function in the metabolism of xenobiotics (e.g. polyphenols).

Miksits *et al.* (2005) investigated the effect of different sources of SULTs (human liver cytosol and recombinant sulfotransferase isoforms) on the formation of resveratrol sulfate and they found similar synthesis rates between SULT1A1 and liver cytosol and suggested the conjugation of resveratrol-3-*O*-sulfate, resveratrol-3,4'-*O*-disulfate and resveratrol-4'-*O*-sulfate. The conjugation of hesperetin was reported by Brand *et al.* (2010) who compared individual sulfotransferases and rat and human tissue samples. They found that hesperetin was mainly sulfonated in the positions 7 and 3' and SULT1A1 was inhibited by substrate at concentrations > 0.15 µM. They also found that conjugation by the human cytosolic fractions (small intestine and liver) showed affinity to sulfonation of position 3'. Hesperetin was not conjugated by rat small intestinal cytosol, but hesperetin-7-*O*-sulfate

was formed by rat liver cytosol which contains SULT1C4, a form not detected in human liver.

In the sulfation reaction PAPS is commonly used as sulfate donor, but there are other compounds that have been reported to be donors such as *p*-nitrophenyl sulfate. Koizumi *et al.* (1990); (1991) reported the successful sulfonation of quercetin, phenolic acids (e.g. gallic acid) and other polyphenols using *p*-nitrophenyl sulfate (PNS) as sulfate donor instead of PAPS. They discovered that a novel type of sulfotransferase from the human intestinal bacterium (*Eubacterium* A-44) catalysed the transfer of the sulfate group using a phenol sulfate esters and not PAPS. In fact, it was until Tyapochkin *et al.* (2009) proposed a bypass mechanism between PAPS and PNS that PNS was considered not only a sulfate donor, but a co-cofactor of the sulfonation reaction. So far, there are not published articles that report experimental data of the combined mechanism PAPS-PNS for the biological sulfonation of polyphenols.

1.2.3 Polyphenol conjugates and their biological activity

It is known that polyphenols are present in foods as glycosides. Following ingestion, glycosides are deconjugated to their aglycone form and are further metabolised to phase II metabolites (see Figure 1.3) which can be found in biological fluids (e.g. blood and urine). Scientific publications have proposed that flavonoid conjugation and deconjugation play an important role in their biological activity and affect the absorption and bioavailability of the conjugates (Williamson *et al.*, 2005; Beekmann *et al.*, 2012).

Williamson *et al.* (2005) published an early review which compiled information about the activity of flavonoid aglycones and their conjugates, and hypothesised that flavonoids conjugates retain some biologically active properties from their aglycones. Firstly, they reported that quercetin glucuronides inhibited xanthine oxidase according to the position of substitution (e.g. 4' > 3') and suggested that the inhibition depended on the position of the substitution and less in the type of substitution. In the case of EGCG, EGC and their glucuronides they described that aglycones and conjugates inhibited the release of arachidonic acid in HT-29 human colon

cancer cells at different degrees. Some glucuronides were less effective than aglycones (e.g. EGC > EGC-3'-*O*-β-glucuronide, EGCG > EGCG-7-*O*-β-D-glucuronide) and EGCG-3'-, 3''- and 4''-*O*-β-D-glucuronides acted the same as EGCG. They reported that hesperetin glucuronides protected (25%) against UV-A-induced necrotic cells death in human foreskin derived fibroblast (FEK4), but hesperetin aglycone did not have effect. On the other hand, using *in vitro* mouse brain endothelial cell models (bEND5 and RBE4), hesperetin and naringenin aglycones passed more freely the transverse blood brain barrier than their conjugates. They presented information about the activity of other flavonoids such as isoflavones and baicalin.

A recent review, published by Beekmann *et al.* (2012) presented the effects of various flavonoids (e.g. EC, kaempferol, luteolin, quercetin and others) on different biological endpoints such as oxidative stress, cell adhesion, COX-2 transcription/activity and angiogenesis. They pointed that several general factors influence the effects of flavonoids conjugates on *in vitro* and *in vivo* assays. For instance, they suggested that conjugation can affect polarity and size of the compounds which will affect protein binding, cellular uptake and further affect the availability of the flavonoids to cells and tissues. Additionally, they reported that flavonoid conjugates can be taken up and then metabolised by various cell types during incubation of *in vitro* assays, but it is not usually specified as most reports do not characterise samples at the end of the incubation period. They also reported that flavonoids, due to their size and polarity, need to rely on active transport or deconjugation to pass cell membranes and act within cells. However, this phenomenon depends on the type of model system, as it has been reported that quercetin glucuronidated needs to be deconjugated before uptake into HUVEC cells, but it can be taken up without deconjugation by HepG2 cells. In addition, they also supported that the position of conjugation plays a more important role in the activity of the conjugate than the type of conjugation, and advised to take into account relevant concentrations, incubation times and to monitor the metabolic fate of the flavonoids in the chosen system to avoid misleading results. Finally, Del Rio *et al.* (2012) have published a comprehensive overview of flavonoids which provides information about their classification, bioavailability, *in vitro* biological activity, outcomes from *in*

vivo studies and their possible mechanism of action on chronic diseases such as cancer and neurodegenerative diseases.

Overall, these publications have made clear that information available for the biological activity of flavonoids conjugates is still insufficient, and it is necessary to perform improved *in vitro* assays and design better *in vivo* studies to obtain relevant data about the interactions of flavonoid conjugates with the human body and pathological disorders.

1.3 Polyphenols and their effect on inflammation

1.3.1 Inflammation: chronic or acute?

Inflammation is a complex reaction that it is often a response resulting from bacterial infection, injury, trauma, or ultraviolet light irradiation. This can be beneficial (restoration of homeostasis) or harmful (damage to cells and tissues) (Huang *et al.*, 2004). Inflammation is generally divided into **acute** and chronic. The first is believed to be a defence mechanism and facilitate wound repair. In contrast, **chronic** inflammation is the result of overproduction of pro-inflammatory enzymes and is mostly detrimental. Chronic inflammation is associated with immunopathological changes that play an important role in the development of degenerative diseases such as cancer, arthritis, atherosclerosis, diabetes, Alzheimer's, asthma and others (Huang *et al.*, 2004; Evans *et al.*, 2006).

1.3.2 Cyclooxygenase 2 and its role in health disorders

Cyclooxygenases are formally known as prostaglandin endoperoxide H synthases and they catalyse the oxidative reaction of essential fatty acids such as arachidonic acid and eicosapentaenoic acid for the biosynthesis of prostaglandins (PG). There are three isoforms: COX-1 is usually expressed in cells and tissues, COX-2 leads to the formation of PG and the less studied COX-3, which is expressed in specific areas including brain and spinal cord (Smith, 2007; Cerella *et al.*, 2010).

COX-1 and COX-2 have similar tertiary structures and perform essentially the same catalytic reaction; both convert arachidonic acid to

PGH_2 which it is further transformed into a variety of prostaglandins or thromboxane A_2 (see Figure 1.5).

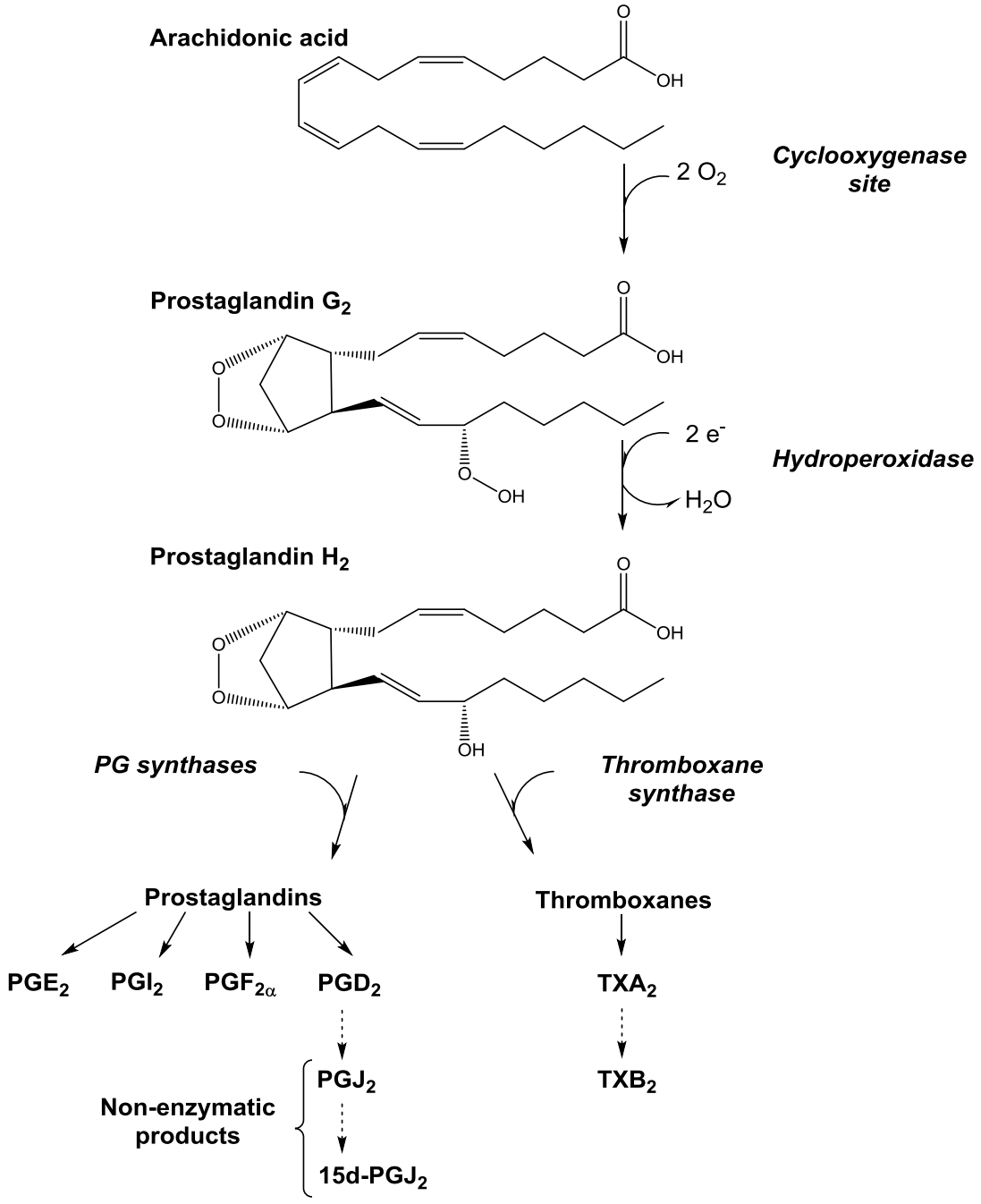


Figure 1.5 The action of COX on the arachidonic acid (AA) pathway. AA is released and metabolised by COX-1 or COX-2 into prostaglandin G_2 and further into prostaglandin H_2. Furthermore, prostaglandin H_2 is converted to prostaglandins and thromboxanes by specific terminal synthases. The cyclopentenones (PGJ_2 and 15d-PGJ_2) are non-enzymatic degradation products of PGD_2. PG = prostaglandin.

Despite the fact that COX isoforms produce the same PGH_2 precursor, COX-1 and COX-2 control very distinctive biological processes. **COX-1** is a

regulator of homeostatic functions, it is expressed at low levels in various tissues and it provides maintenance of physiological functions such as platelet aggregation, renal water balance and gastric mucosa protection. On the other hand, **COX-2** is not usually expressed and it appears in response to pathogens. Once its production is stimulated, its expression is high and it will cause the sudden production of PG (Cerella *et al.*, 2010).

Inflammation is critically regulated by COX-2 and its derived products (PG) are important in the early stages of the inflammatory response and the end of it (Greene *et al.*, 2011). The overproduction of PG has been mainly related to cancer, playing an essential role in oncogenesis and it is related to other diseases such as rheumatoid arthritis and diabetes (Cerella *et al.*, 2010; Schneider and Pozzi, 2011).

1.3.3 Inhibition of COX-2 by polyphenols

Clinically, COX-2 is targeted by non-steroidal inflammatory drugs (NSAIDs) like aspirin, ibuprofen and naproxen and other selective inhibitors (e.g. celecoxib). Its long-term use has been banned due to its severe side effects such as damage to the gastric mucosa and relationship with the increase of cardiovascular diseases (Greene *et al.*, 2011; Salvado *et al.*, 2012; Raman *et al.*, 2008). As a result, the search for safer and more effective substitutes is still in progress.

Natural occurring compounds (e.g. flavonoids) and plant extracts represent a major source of bioactive compounds with potential anti-inflammatory activity. For example, Cerella *et al.* (2010) have compiled information about the effects of curcumin, epigallocatechin gallate, resveratrol, apigenin, genistein, kaempferol, fisetin and chrysin on COX-2 inhibition. In this case, they reported that curcumin supresses PG production and prevents COX-2 gene expression where the NF-κB pathway is strongly inhibited. Indeed, epigallocatechin gallate (EGCG), resveratrol, apigenin, genistein and kaempferol inhibit COX-2 expression (e.g. transcription) and EGCG has been linked to modify the activities of NF-κB and AP-1. Chrysin, a flavonoid from honey, inhibits expression of COX-2 at mRNA and protein levels. Investigation of plant extracts (e.g. green tea, German chamomile) has focused in the anti-inflammatory activity of specific compounds and it

has revealed effective inhibition of COX-2 expression and suppression of the NF-κB pathway (Peng *et al.*, 2006; Srivastava *et al.*, 2009).

Despite the suggested anti-inflammatory activity for various plant extracts and their major compounds, experimental information is still insufficient and the use of a broader range of techniques to support current results is needed. Animal models are commonly used to test plant extracts, but they do not produce enough information about the mechanism of action against inflammation (Webb, 2014). Indeed, cell culture studies explain specific pathways were flavonoids or plant extracts work against COX-2, but the differences in cell lines and the variation in inflammatory responses makes it more difficult to draw decisive conclusions (Lotito *et al.*, 2011).

A different way to tackle this task is using analyses to determine COX inhibition at specific targets of the AA pathway. Some methods detect/measure reaction intermediates, meanwhile others detect final products (see Figure 1.6) (Petrovic and Murray, 2010). For example, O'Leary *et al.* (2004) used Caco-2 cells to measure the inhibition of COX-2 by quercetin and its metabolites. As well, they performed an *in vitro* assay measuring the peroxidase activity of COX-2 (TMPD oxidation) testing quercetin, its metabolites, and DuP-697 which is a selective COX-2 and a feeble COX-1 inhibitor. The combined techniques gave more information and a better approach, but increased the cost and time of analysis. As a consequence, new techniques such as liquid chromatography and mass spectrometry have been more used by researchers.

Mass spectrometry has emerged as a powerful tool to detect specific analytes (e.g. PG) which can be identified in a single run after they have been isolated using liquid-liquid extraction. Cao *et al.* (2010); (2011), have reported a faster, more convenient and less expensive cell-free assay to determine the inhibition of COX-2 by bioactive compounds. The *in vitro* assay is carried out at small scale (Eppendorf tubes) using purified COX-2 enzyme (human/ovine) and it includes a deuterium labelled standard (surrogate standard = d_4-PGE_2) to account for any losses during sample handling. Next, a liquid-liquid extraction is performed to obtain the PGE_2 and surrogate, and the concentration obtained is measured using LC-MS^2.

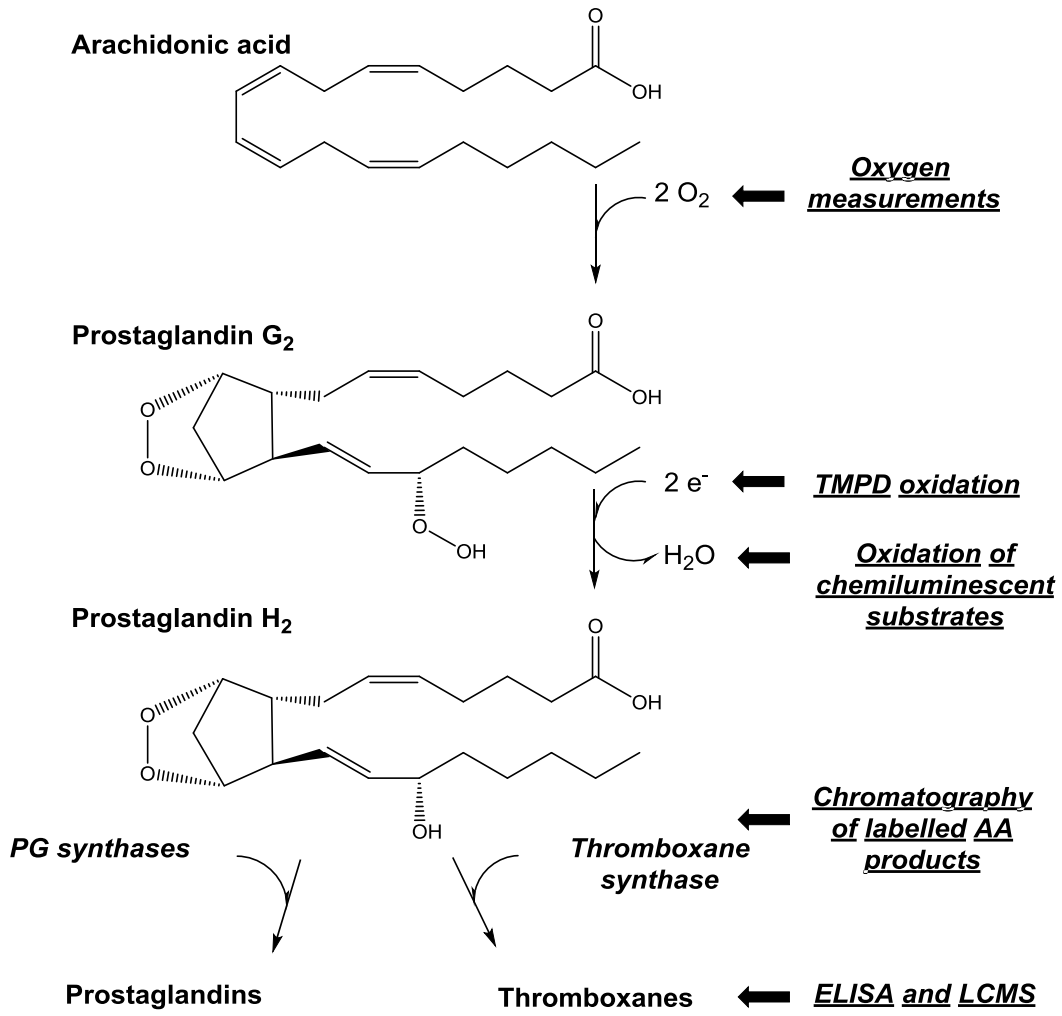


Figure 1.6 The AA pathway and the assays that can be used to measure activity/inhibition of COX-2. Modified from Petrovic and Murray (2010). TMPD = N,N,N',N'-tetramethyl-*p*-phenylenediamine.

The same proposal has been reported by Zhu *et al.* (2014) who compared the measurement of derivatized and underivatized PGE_2. They used (carboxymethyl)trimethylammonium chloride hydrazide, also known as Girard's reagent-T, to derivatize the PG. Derivatization is used to convert the carboxylic acid to a fixed-charge cationic derivative to improve detection sensitivity in LC-MS^2 and it has been previously reported by Bollinger *et al.* (2010) who developed a new derivatization reagent, *N*-(4-aminomethylphenyl)pyridinium (AMPP) for samples with limited amount of PGE_2. As well, Deng *et al.* (2014) used the same *in vitro* assay, but included a magnetic ligand fishing to characterise green tea and measure COX-2 inhibition at the same time. Both methodologies improve the measurement of COX-2 inhibition, but do not simplify the techniques already validated which it is the principal aim for the use of LC-MS. Indeed, measurements

using LC-ESI/MS are unusual, but they could provide an uncomplicated method to obtain equivalent results.

1.4 Aim of this thesis and main objectives

This thesis aimed to investigate the effect of plant extracts and their major compounds on inflammation processes after they have been ingested. Our targets were the transformation of the plant extract and its major compounds by the first step of metabolism; the synthesis of phase II metabolites and their accurate measurement by LC-ESI/MS, and their inhibition of recombinant human COX-2.

These objectives were achieved by:

1. Enzymatic hydrolysis of German chamomile and the identification of major compounds by LC-ESI/MS^2 and LC-ESI/MS. German chamomile was used as a case plant extract.
2. Biological synthesis of hesperetin sulfate using pig liver cytosol. The biological sulfation was accomplished by the synergic activity of PAPS and PNS. Indeed, biological samples (urine) were measured to compare the use of true standards and hydrolysis of metabolites in the quantification of these compounds.
3. The major compounds of various plant extracts with potential anti-inflammatory activity were used in an *in vitro* assay to screen their inhibitory activity against recombinant human COX-2. Next, selected compounds were used to perform a combined *in vitro*-LC-MS assay to determine their recombinant human COX-2 inhibition.

CHAPTER 2 Characterisation and enzymatic hydrolysis of German chamomile (*Matricaria recutita*)

2.1 Abstract

German chamomile (GC) contains a high number of compounds that are scarcely reported in the literature. Improvements in identification techniques, such as liquid chromatography and mass spectrometry, have provided the means to detect a broader range of chemical structures. However, the lack of standard compounds makes it challenging to provide an accurate quantification. The work here presented aimed to identify the major compounds of GC by the combined methodology of LC-ESI/MS^2 and LC-ESI/MS, and to mimic the transformation of these compounds after ingestion by using a multienzyme hydrolysis. Firstly, 25 compounds were identified and 8 compounds were accurately quantified in an aqueous extract of GC using true standards. Apigenin-7-*O*-glucoside was the most abundant flavonoid (5.7 g/100g dry weight) followed by luteolin-7-*O*-glucoside and apigenin (284 and 284 mg/100g dry weight respectively) which agreed with previous publications reporting that apigenin-7-*O*-glucoside and its derivative can be found in large quantities in GC. In addition, a two-enzyme hydrolysis procedure (hesperidinase and cellulase) was applied and samples before and after hydrolysis were analysed. A liquid-liquid extraction was performed after the hydrolysis was ended and it was found that acetonitrile was better remover than ethyl acetate. However, the concentration of aglycones (apigenin and luteolin) increased only up to 2.6% which might suggest that water-soluble compounds (glucosides) could have been lost during the extraction.

2.2 Introduction

German chamomile (GC), also known as Hungarian chamomile, mayweed, sweet false chamomile or wild chamomile, is cultivated in Germany, Hungary, Russia and other southern and eastern European countries. Traditionally, it has been used for minor infections in skin and mucosa. GC has been studied for its anti-inflammatory, analgesic and wound-healing properties, amongst other efficacies (Paulsen, 2002), and

has been listed by the European Food Safety Authority (EFSA) as a safe substance for human consumption (EFSA, 2012). Its use in the food and nutrition industry has increased during recent years and the awareness about its health benefits has drawn the attention of researchers. However, information about its specific activity and accurate composition is still scarce (Garcia-Alvarez *et al.*, 2014).

It has been reported that GC (flowers and plant) contains a vast number of components, including terpenoids, polyphenols (mainly flavonoids) and essential oils. Compounds such as monoterpenes, coumarins and flavonoids are considered to be bioactive ingredients (Srivastava *et al.*, 2010). For example, apigenin has been shown to decrease the release of inflammatory cytokines by inactivation of NF-κB and so defend against oxidative stress (Millington *et al.*, 2014). Indeed, Kato *et al*. (2008) found that GC tea decreased blood glucose concentrations in fasted mice after their treatment with sucrose and inhibited ALR2 (aldose reductase) which transforms glucose into sorbitol and in diabetic conditions also produces fructose.

Different techniques have been previously used to identify the composition of diverse plants and foodstuffs. For example, thin-layer chromatography, high performance liquid chromatography, supercritical fluid chromatography and others have been used to identify/quantify the polyphenol content of fruits and vegetables (Ignat *et al.*, 2011) and these techniques have been transferred to plant extracts. Mass spectrometry techniques (MS^2 and MS) have been previously used for the identification of the polyphenol content of GC and other plants (Raal *et al.*, 2012; Lin and Harnly, 2012), but a whole profile of GC polyphenols is still a work in progress. Plants are an intricate blend of compounds which are not easy to identify and it is laborious to quantify them.

Polyphenols in plants exist mainly as conjugates with one or two sugars attached (Bokkenheuser *et al.*, 1987). These are mostly glucose or rhamnose, but can also be galactose, arabinose, xylose, glucuronic acid or other sugars. After the polyphenols have been ingested, the first step of their metabolism is the removal of the sugar by enzymes. The process can occur

in the food itself or in the gastrointestinal tract (Scalbert and Williamson, 2000).

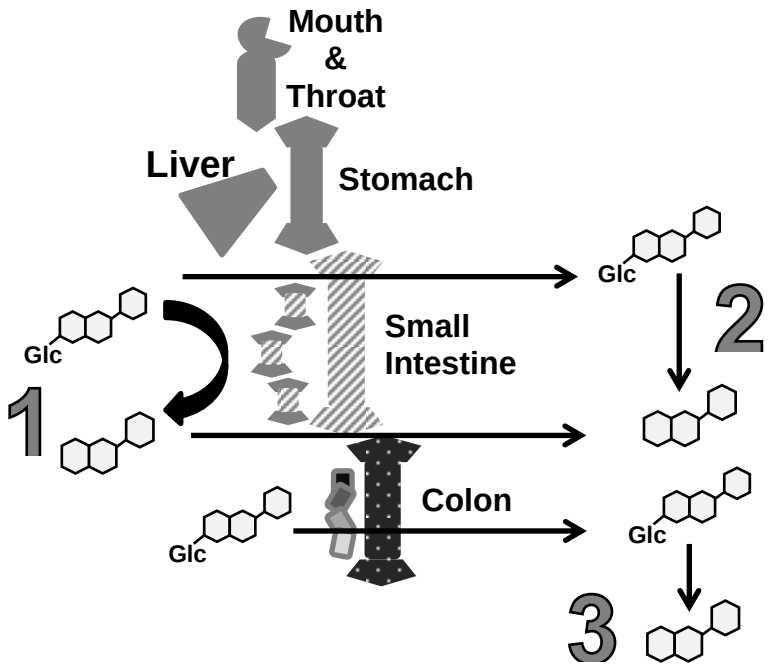


Figure 2.1 Possible pathway of deglycosylation of polyphenols. 1. Hydrolysis by lactase phlorizin hydrolase; 2. Deglycosylation by β-glucosidase and 3. Deglycosylation by gut microflora. Glc=glucose. Modified from Nielsen *et al*. (2006).

After a foodstuff or plant extract has been ingested, it is broken down and those compounds which survive the stomach acidity reach the small intestine and colon to be deglycosylated (see Figure 2.1) (Nielsen *et al.*, 2006). Specific enzymes (glycosidases and β-glucosidases) cut the sugars attached to liberate aglycones which will be further metabolised by other pathways. Hesperidinase is a mix of enzymes (glucosidases and rhamnosidases) which cuts rhamnose and glucose from the original compound (e.g. polyphenols) to liberate an aglycone (Wang *et al.*, 2012b). Cellulase catalyses the hydrolysis of β-glucosides to remove glucose and yieds free polyphenols to its aglycone form (Yan and Wu, 2013). Both enzymes are produce by the fungi *Aspergillus niger* and have been separately used to chemically mimic human digestion.

In this study, two mass spectrometry techniques were performed (MS and MS^2) to identify and quantify the major compounds of GC. The methodology was developed to be a straightforward approach to analyse

plant extracts and determine their polyphenol profile, both before and after hydrolysis.

2.3 Materials

German chamomile (*Matricaria recutita*), apigenin, luteolin, umbelliferone, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid were provided by PhytoLab (Vestenbergsgreuth, Germany) as part of the PlantLIBRA project. Ascorbic acid, sodium acetate, and hesperidinase and cellulase from *Aspergillus niger* were purchased from Sigma-Aldrich (Dorset, UK). Organic solvents were purchased from different suppliers: acetic acid and methanol from Sigma-Aldrich (Dorset, UK); ethyl acetate and formic acid from Fisher Scientific (Loughborough, UK); acetonitrile from VWR (Lutterworth, UK). All water refers to deionised Millipore water (Hertfordshire, UK) otherwise stated.

2.4 Methods

2.4.1 Screening of German chamomile by LC-ESI/MS^2.

Matricaria recutita, also known as German chamomile was provided by Phytolab as dry extract previously extracted with 60% ethanol, dried and mixed with maltodextrin to increase its solubility (50:50, w/w). German chamomile (GC) was dissolved in water and prepared every time prior to analysis. In collaboration with our project, this analysis was performed by Dr. Asimina Kerimi using an Agilent 1200 HPLC coupled with a 6410 LCMS triple quadrupole fitted with an electrospray ionisation used in negative mode (Workingham, UK). An analytical column, Kinetex C18 (2.10x150 mm, 2.6 µm) was used for the separation and was kept at 35°C. The injection volume was 5 µL at flow rate of 0.25 mL/min. Mobile phase A consisted of 95% water, 5% acetonitrile and 0.1% formic acid and mobile phase B consisted of 95% acetonitrile, 5% water and 0.1% formic acid. Elution started at 0% solvent B, reached 10% after 5 min and increased up to 25% after 5 min more. At 20 min solvent B was 35% and rose up to 50% at 25 min. This gradient was kept until 30.5 min when it was increased to 100%. Then, it was maintained for 5 min more and at 36 min decreased to 0%. The column

was equilibrated until 41 min. Each compound was compared with references from the literature (see Table 2.2) and only eight were matched with their respectively true standard (see 2.4.3) kindly provided by PhytoLab.

2.4.2 Enzymatic hydrolysis of German chamomile.

German chamomile was hydrolysed using a modified method from Pimpão *et al.* (2013). Firstly, GC was prepared at 20 mg/mL (w/v) with 0.1 mM ascorbic acid. However, due to its 50% maltodextrin content, the concentration was actually 10 mg extract/mL. The solution was adjusted to pH 3.8 with 0.2 M acetate buffer and 0.02 U/mL hesperidinase was added [1U=333 mg of protein and is defined as the amount required to liberate 1.0 µmol of glucose from hesperidin per minute at pH 3.8 at 40°C]; so, it was incubated for 16 hr at 40ºC/70 rpm. Then, the pH was increased to 5 using 0.2 M sodium acetate and incubated for 4 hr at 37ºC with 20 U/mL cellulase [1 U=0.885 mg of protein and is defined as the amount required to liberate 1.0 µmol of glucose from cellulose in 1 hr at pH 5 at 37°C] and the mix was shaken at 70 rpm. The reaction was stopped using 1:2 pure organic solvent (ethyl acetate or acetonitrile) and centrifuged at 4ºC/3 000 g for 10 min. Supernatant was separated and this procedure was followed twice more. The three different fractions were mixed, aliquoted and evaporated under vacuum (Genevac, HPLC fraction, 30-35°C) to obtain tubes with theoretical 1.11 mg/mL (w/w) concentrated sample. Then, samples were kept at -20ºC and reconstituted prior to LC-ESI/MS analysis with 30% methanol:water (v/v).

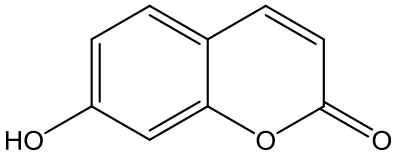
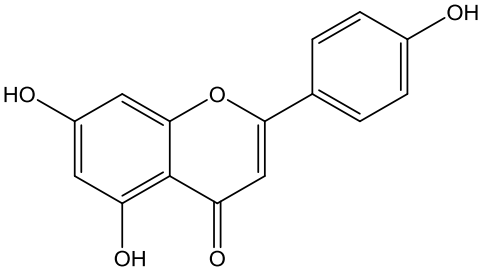
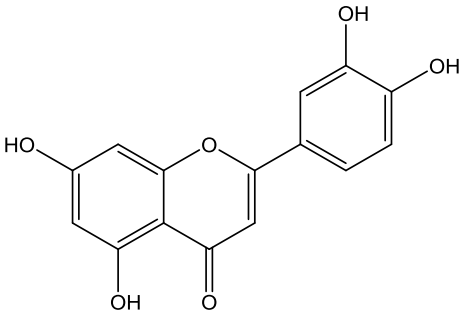
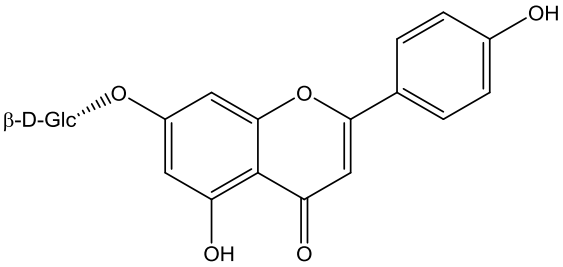
2.4.3 Identification and quantification of eight compounds of GC dissolved in water and after enzymatic hydrolysis by LC-ESI/MS.

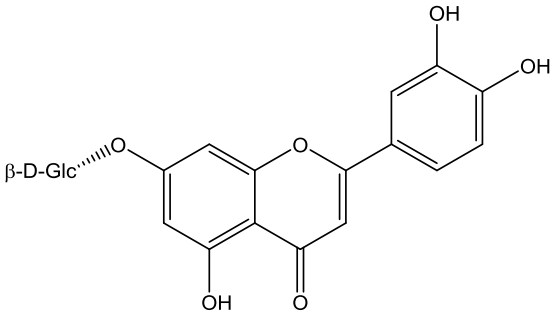
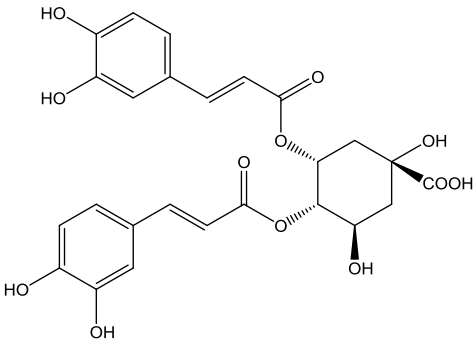
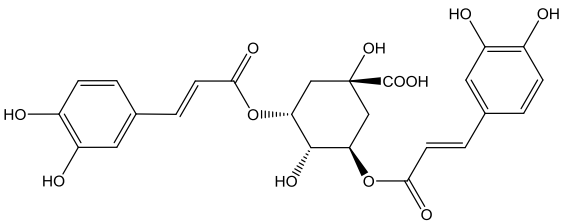
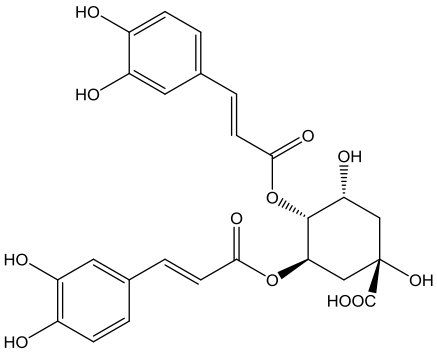
2.4.3.1 Mass spectrometry

Samples of GC (with/without hydrolysis) were characterised using a Shimadzu LC-2010 HT coupled with a LCMS-2020 quadrupole mass spectrometer fitted with an electro spray ionisation source (Milton Keys, UK). A Kinetex C18 analytical column (2.10x150 mm, 2.6 µm) was used for the separation and was maintained at 35ºC. The injection volume was 10 µL at flow rate of 0.25 mL/min. The running programme from screening (see 2.4.1) was followed and the same solvents composition was employed. Each

compound was identified according to its mass-to-charge ratio in negative mode ($m/z^{(-)}$) and compared to their respective true standard. Their general characteristics can be seen in Table 2.1.

Table 2.1 Chosen compounds identified and further quantified in GC and two treated samples. Commercially-available standards were used to ensure their appearance.

Compound	Molecular weight (g/mol)	RT (min)	Structure
Umbelliferone	162.15	11.7	
Apigenin	270.24	20.9	
Luteolin	286.24	18.1	
Apigenin-7-*O*-glucoside	432.38	15.1	

Luteolin-7-*O*-glucoside	448.38	13.9	
3,4-Dicaffeoylquinic acid	516.46	14.3	
3,5-Dicaffeoylquinic acid	516.46	14.7	
4,5-Dicaffeoylquinic acid	516.46	15.3	

Glc=glucose

2.4.3.2 Quantification using true standards

The concentration of eight compounds (see Table 2.3) in each sample was determined using external calibration. Stock solutions were prepared using 100% DMSO. Then, compounds were diluted in a mix methanol/water to reach < 1% DMSO. The curves were produced using a concentration range of 0.5 nM to 90 µM. Values from 5 to 30 µM were used to plot seven calibration curves and values from 5 to 70 µM were used for apigenin aglycone. The coefficient of determination was ≥ 0.97.

2.5 Results

2.5.1 Profile of German chamomile by LC-ESI/MS^2.

In collaboration with our project, Dr. Asimina Kerimi performed a screening of GC using a LC-ESI/MS^2 which followed various polyphenols already reported in the literature (see Table 2.2). Polyphenols were identified using their specific transitions in negative mode and published reports notifying their appearance in GC. The specific characteristics used for this purpose can be seen in Table 2.2. This screening provided general information about the composition of GC and further analysis were based on these results.

2.5.2 Identification of major compounds of German chamomile by LC-ESI/MS.

After the screening by LC-ESI/MS^2 was performed (see 2.5.1) GC was analysed by LC-ESI/MS and eight chosen polyphenols were identified (see Figure 2.2) in the sample dissolved in water (without hydrolysis).

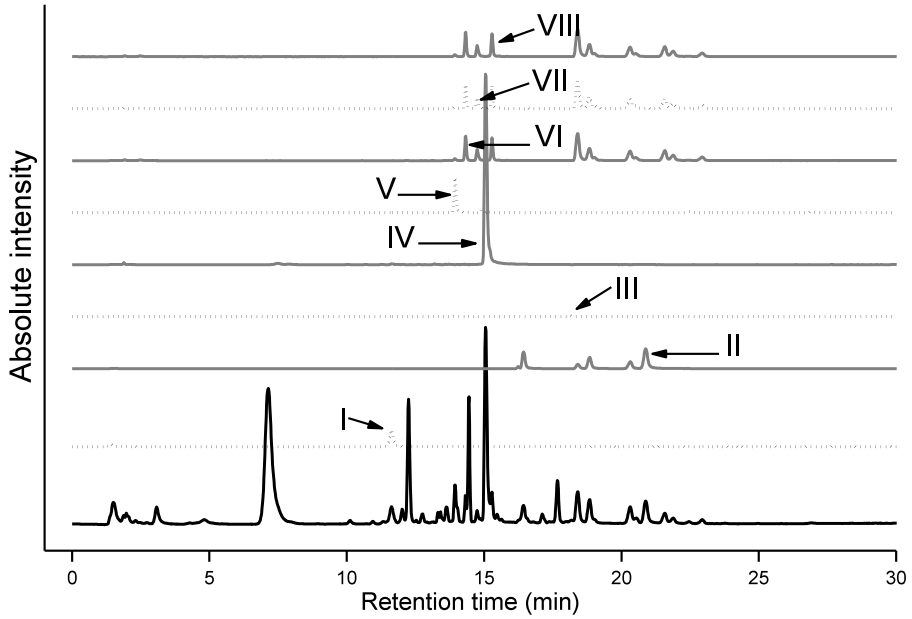


Figure 2.2 MS chromatography of GC dissolved in water (A: without hydrolysis). Total Ion Current (TIC) for GC and specific mass-to-charge ratio (*m/z*) in negative mode for identified polyphenols: umbelliferone (I) retention time (RT)= 11.7 min; apigenin (II) RT=20.9 min; luteolin (III) RT=18.1 min; apigenin-7-*O*-glucoside (IV) RT=15.1 min; luteolin-7-*O*-glucoside (V) RT=13.9 min; 3,4-dicaffeoylquinic acid (VI) RT=14.3 min; 3,5-dicaffeoylquinic acid (VII) RT=14.7 min and 4,5-dicaffeoylquinic acid (VIII) RT=15.3 min.

Table 2.2 Characteristics of compounds identified in German chamomile (GC) using LC-ESI/MS^2 detection and reference in the literature.

Compound name	Precursor ion	Product ion	Fragmentor	Collision energy	RT	RT	RT	RT	Reference
Protocatechuic acid aglycone	153	109	135	10	5.3				Guimaraes *et al.* (2013)
Umbelliferone aglycone	161.1	133	135	20	7.9	9.2	12.3		Novakova *et al.* (2010)
Ferulic acid (FA) aglycone	193	149	135	10	10.1	12.3			Guimaraes *et al.* (2013)
Apigenin aglycone	269.2	151.2	135	20	20.6				Novakova *et al.* (2010); Matic *et al.* (2013); Lin and Harnly (2012)
Luteolin aglycone	285.2	133.1	135	25	17.8				Novakova *et al.* (2010); Lin and Harnly (2012)
Kaempferol aglycone	285.2	151.1	135	20	17.7				Novakova *et al.* (2010)
Quercetin aglycone	301.1	151.2	135	20	17.5				Kato *et al.* (2008)
Isorhamnetin aglycone	315	300.3	135	20	21.5				Novakova *et al.* (2010)
Coumaroylquinic acid aglycone	337	191	135	30	11.2				Guimaraes *et al.* (2013)
4*O*- caffeolyquinic acid (CQA)	353	173	135	30	9.3				Guimaraes *et al.* (2013); Matic *et al.* (2013)
3*O*-CQA	353	179	135	30	6.6				Guimaraes *et al.* (2013); Matic *et al.* (2013); Lin and Harnly (2012)
5*O*-CQA	353	191	135	30	9.5				Guimaraes *et al.* (2013); Matic *et al.* (2013)
cis/trans FA hexoside	355	149	135	30	10.2				Guimaraes *et al.* (2013)
cis/trans FA hexoside	355	193	135	30	10.2				Guimaraes *et al.* (2013)
5*O*- feruloylquinic acid	367	194	135	30	11.8	13.7			Guimaraes *et al.* (2013); Matic *et al.* (2013); Lin and Harnly (2012)
Dimethoxy-cinnamic acid hexoside	369	189	135	30	14.7	16.9	19.5		Guimarães *et al.* (2013)
Luteolin *7 O*-glucoside	447	285	135	25	13.4	14.5			Guimaraes *et al.* (2013); Lin and Harnly (2012)

									Novakova *et al.* (2010); Lin and Harnly (2012)
Quercetin *3 O*-glucoside	463	301	135	25	12.5	13.1			Guimarães *et al.* (2013); Novakova *et al.* (2010); Lin and Harnly (2012)
Quercetin 3 *O*-glucuronide	477	301	135	35	13.3	14.5			Lin and Harnly (2012)
Myricetin *O*-hexoside	479	317	135	25	12.0				Guimaraes *et al.* (2013)
Luteolin *O*-acylhexoside	489	285	135	30	14.6	15.9			Guimaraes *et al.* (2013)
di-CQA	515	353	135	30	13.8	14.1	14.7		Guimaraes *et al.* (2013); Matic *et al.* (2013); Lin and Harnly (2012)
Kaempferol *3 O*-rutinoside	593	285	135	30	13.1				Lin and Harnly (2012)
Quercetin *3 O*-rutinoside	609	301	135	35	12.4				Lin and Harnly (2012)
FA hexoside dimer	711	193	135	35	10.2	12.3	12.9	13.9	Guimaraes *et al.* (2013)

Then, commercially-available standards were used to verify the nature of the eight compounds identified. This procedure provided validation of the retention time here reported. A typical chromatogram is shown in Figure 2.3.

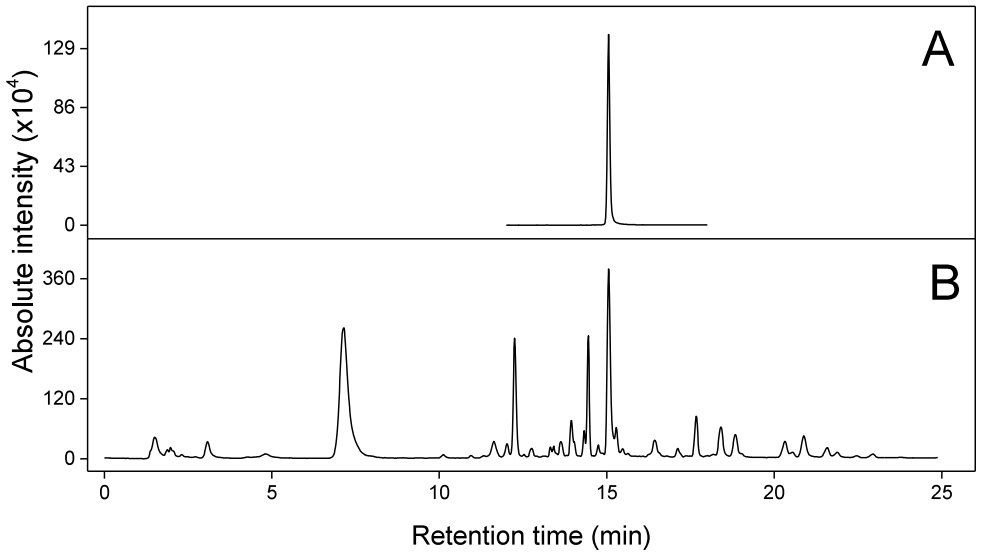


Figure 2.3 Typical MS chromatogram for validation of occurrence of a selected compound (apigenin-7-*O*-glucoside). TIC of a commercially available apigenin-7-*O*-glucoside (A) RT= 15.1 min dissolved in ethanol:water (10:90, v/v) and GC dissolved in water (B).

2.5.3 Effect of enzymatic hydrolysis on GC and identification of selected compounds.

German chamomile was enzymatically hydrolysed to determine the effect of gastrointestinal digestion, using hesperidinase and cellulase to mimic human digestion. Also, two different organic solvents (acetonitrile and ethyl acetate) were tested to choose the most efficient method for liquid-liquid extraction. Then, identification by LC-ESI/MS was performed following the protocol reported in 2.4.2. The chosen eight compounds (see 2.5.2) were also followed and their appearance can be seen in Figure 2.4-2.5. Each figure shows a different treatment from liquid-liquid extraction.

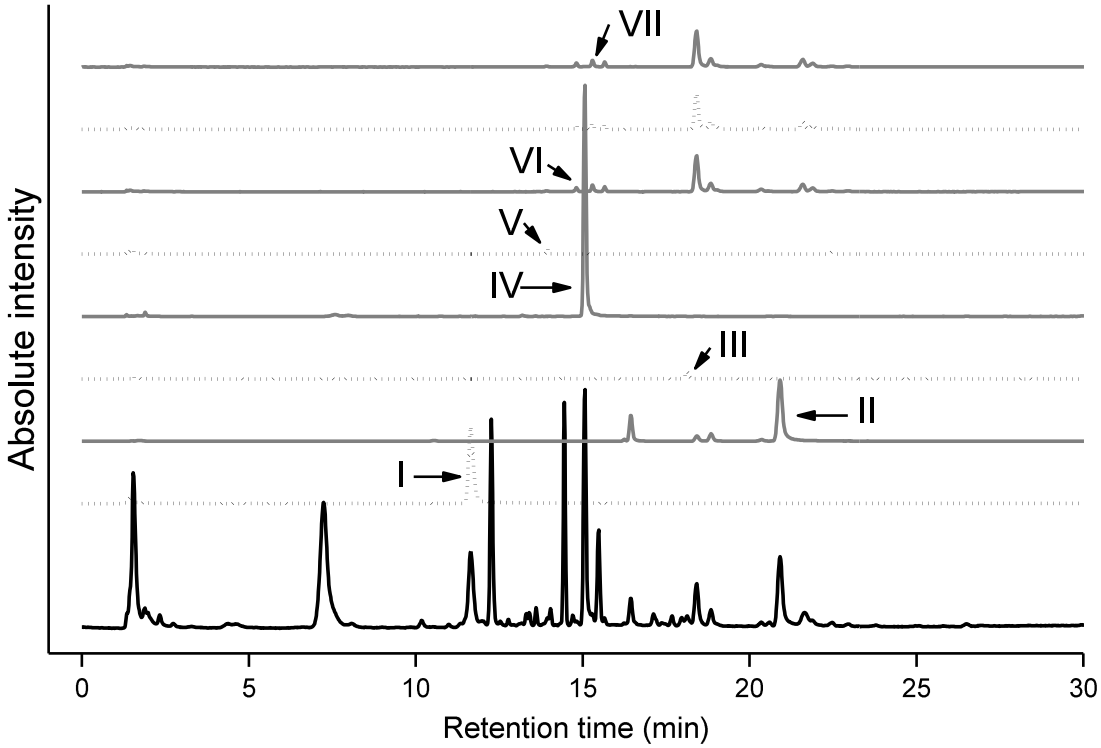


Figure 2.4 MS chromatography of GC fraction hydrolysed and extracted with acetonitrile. Total Ion Current (TIC) for each sample and specific mass-to-charge-ratio (*m/z*) in negative mode for identified polyphenols: umbelliferone (I) retention time (RT)=11.7 min; apigenin (II) RT=20.9 min; luteolin (III) RT=18.1 min; apigenin-7-*O*-glucoside (IV) RT=15.1 min; luteolin-7-*O*-glucoside (V) RT=13.9 min; 3,5-dicaffeoylquinic acid (VI) RT=14.7 min and 4,5-dicaffeoylquinic acid (VII) RT=15.3 min.

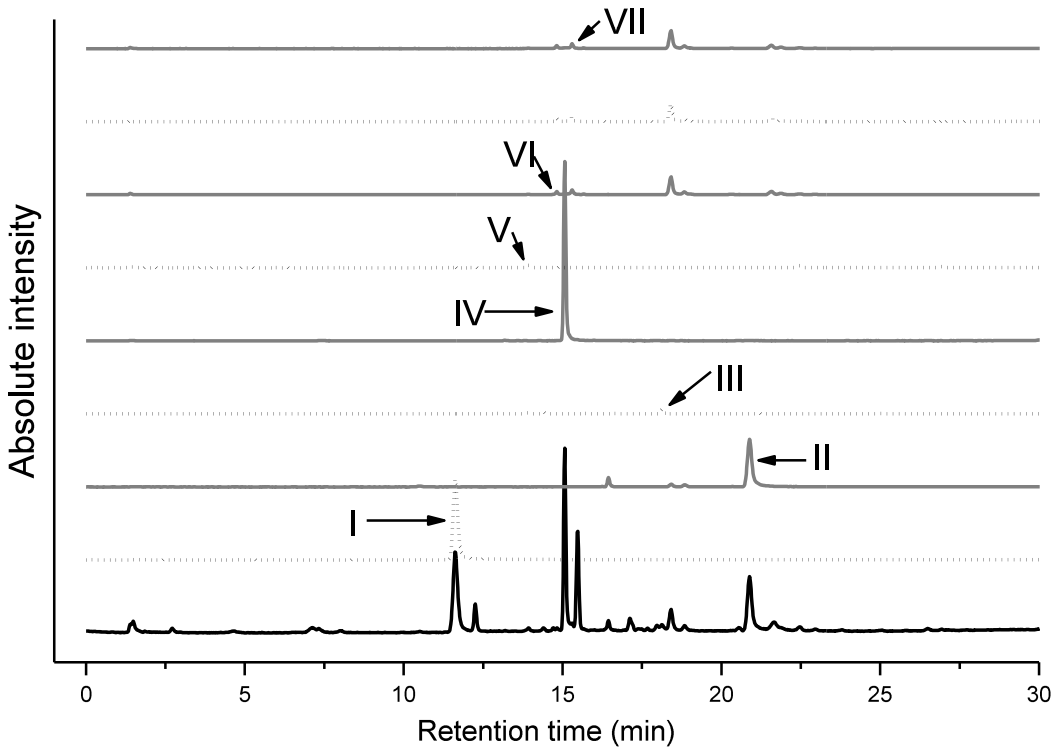


Figure 2.5 MS chromatography of GC fraction hydrolysed and extracted with ethyl acetate. Total Ion Current (TIC) for each sample and specific mass-to-charge-ratio (*m/z*) in negative mode for identified polyphenols. The compounds (I-VI) and retention time are stated in Figure 2.4.

It was found that seven of the eight compounds, identified in the sample dissolve in water, were still present after hydrolysis. Some (e.g. umbelliferone, apigenin) increased their MS signal after hydrolysis, suggesting the transformation from glucoside to aglycone. However, this phenomenon was not seen in the other compounds.

Next, calibration curves for the chosen polyphenols were produced and can be seen in Figures 2.6-2.9. Due to the variability in equations, the limit of detection (LOD) and limit of quantification (LOQ) were practically determined at 500 nM and 5 µM respectively for all compounds. The abundance of each identified polyphenols in every sample is reported in Table 2.3.

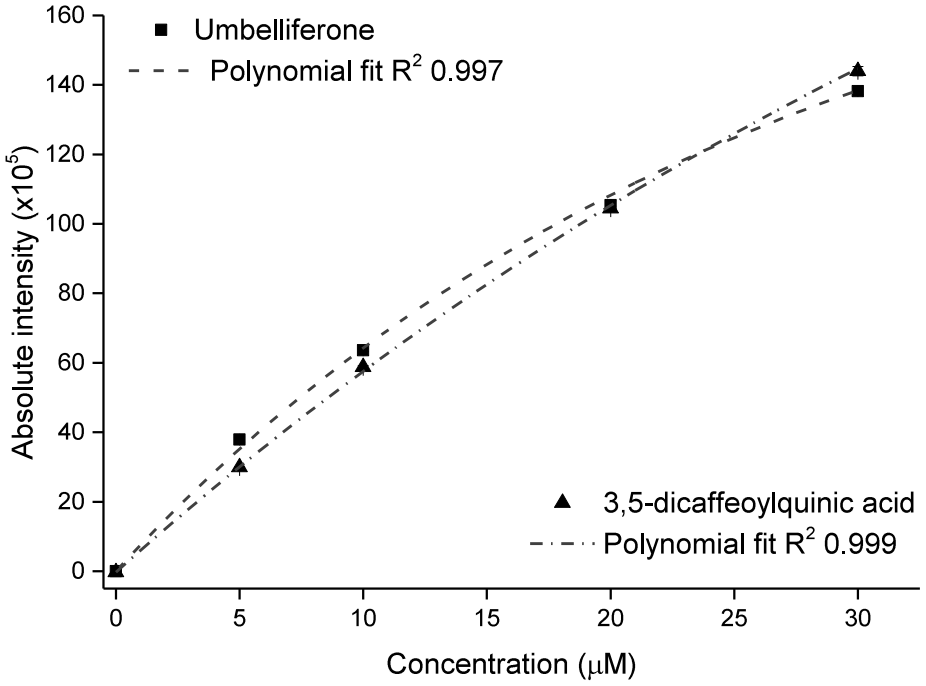


Figure 2.6 Calibration curve for umbelliferone and 3,5-dicaffoylquinic acid using commercially available standards. N=3 biological replicates and 1 technical measurement.

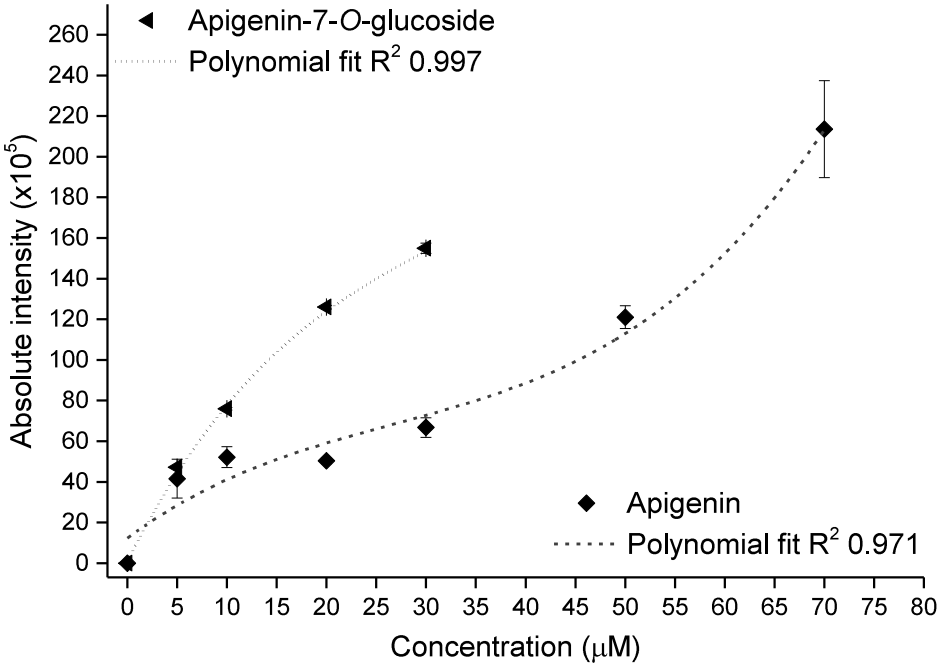


Figure 2.7 Calibration curve for apigenin and apigenin-7-*O*-glucoside using commercially available standards. N=3 biological replicates and 1 technical measurement.

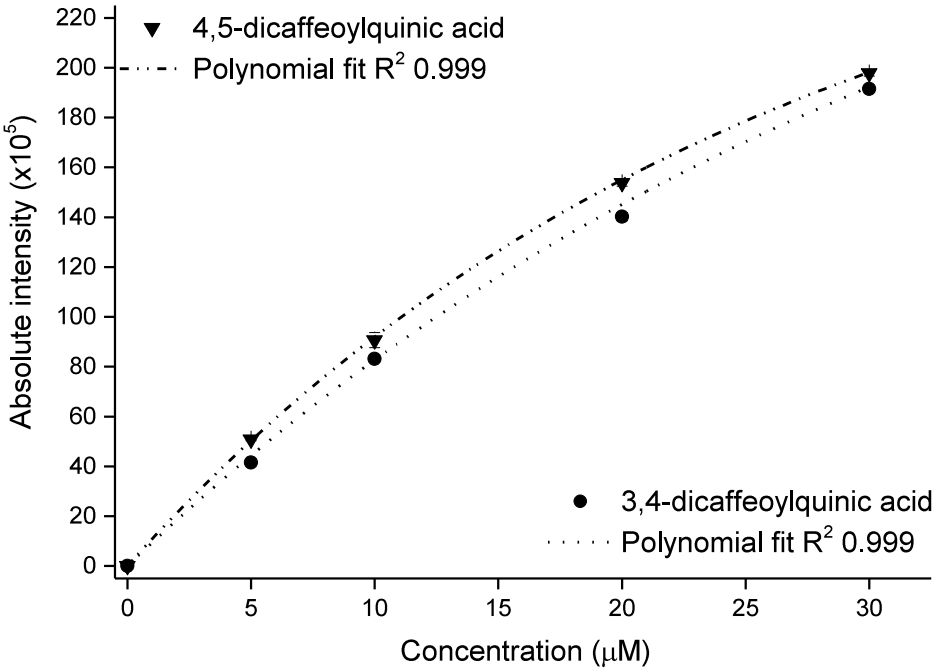


Figure 2.8 Calibration curve for 3,4 and 4,5-dicaffoylquinic acids using commercially available standards. N=3 biological replicates and 1 technical measurement.

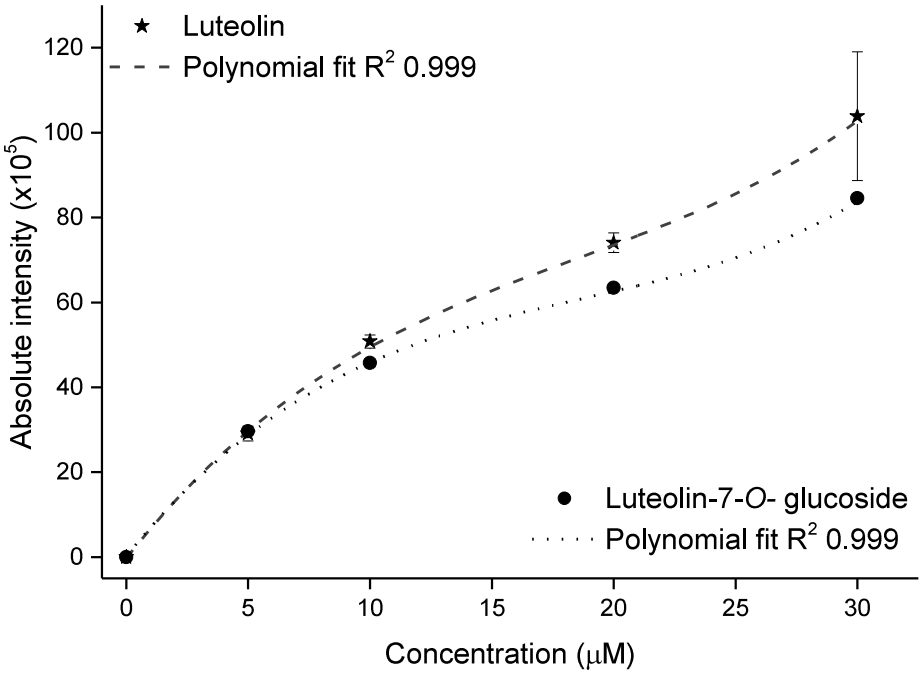


Figure 2.9 Calibration curve for luteolin and luteolin-7-*O*-glucoside using commercially available standards. N=3 biological replicates and 1 technical measurement.

Table 2.3 Concentration (mg/100g dw) of each selected polyphenol in samples dissolved in water and after hydrolysis and extraction by different organic solvents (acetonitrile/ethyl acetate). Data from 3 biological samples, 1 technical measurement and ± SD.

Compound	*m/z*$^{(-)}$	RT (min)	Dissolved in water	Acetonitrile fraction	Ethyl acetate fraction
Umbelliferone	161	11.7	70.3 ± 1.1	154.9 ± 0.6	139.3 ± 0.9
Apigenin	269	20.9	245.3 ± 6.4	376 ± 23	204.2 ± 3.9
Luteolin	285	18.1	6.3 ± 0.3	18.1 ± 0.9	7.9 ± 0.1
Apigenin-7-*O*-glucoside	431	15.1	5683.7 ± 159.7	575.1 ± 6.6	315.7 ± 3.2
Luteolin-7-*O*-glucoside	447	13.9	284.1 ± 3.0	11.8 ± 0.1	5.2 ± 0.01
3,4-Dicaffeoylquinic acid	515	14.3	116.4 ± 0.5	ND	ND
3,5-Dicaffeoylquinic acid	515	14.7	82.1 ± 1.3	10.4 ± 0.2	7.7 ± 0.4
4,5-Dicaffeoylquinic acid	515	15.3	98.3 ± 1.2	10.7 ± 0.3	10.9 ± 3.2

ND. Not detected, dw= dry weight, SD= standard deviation.

This characterisation provided sufficient information to perform further analysis and the purification of those GC polyphenols which are not commercially-available (e.g. ferulic acid hexoside).

2.6 Discussion

Plant extracts and their polyphenol content have been a major research interest, as it has been suggested that they may provide health beneficial effects. For this reason, precise analytical techniques have been used to identify extract compositions and their application has been successful (Novakova *et al.*, 2010; Guimaraes *et al.*, 2013). In general, MS techniques are mainly used to identify and clarify chemical structures of specific compounds, such as polyphenols (Ignat *et al.*, 2011). Liquid mass spectrometry, particularly ESI/MS, has been used to identify GC polyphenols

(Lin and Harnly, 2012). Although it is a powerful technique, it does not identify isomers, losing the ability to specify the site of substitution in the polyphenol structure. However, its simplicity in comparison with LC-ESI/MS^2, GC-MS and NMR gives a substantial advantage over these other techniques.

Recently, GC polyphenols from dried chamomile flowers were identified by Lin and Harnly (2012) using LC-PDA-ESI/MS, but no quantification was reported. Also, Matic *et al.* (2013) analysed GC tea (infusion and decoction) by LC-MSD-TOF-ESI/MS and only reported the total phenolic content using the Folin-Ciocalteu method. It is a common practice to determine the polyphenol concentration by the Folin-Ciocalteu method which provides imprecise data about the polyphenol content in a sample. In fact, it has been showed that different substances, as vitamin C and tannins, interfere with the assay (Georgé *et al.*, 2005) and then could possibly lead to false results. Therefore, this technique must be used in combination with a more reliable methodology such as LC-MS.

Research has focused on identifying and quantifying apigenin-7-*O*-glucoside and its derivatives (Švehlíková *et al.*, 2004; Srivastava and Gupta, 2009; Avula *et al.*, 2013) but less attention has been paid to other compounds. Our methodology identified many compounds previously reported in different GC extracts (see Table 2.2). Indeed, it was possible to positively quantify eight compounds (see Table 2.3). The limit of detection and quantification (LOD and LOQ) here reported are lower than those already reported by Boiteux *et al*. (2014) who detected apigenin at 107 µM and quantified it at 355 µM. Raal *et al*. (2012) provided information about the polyphenol content of 13 commercial German-chamomile teas packaged in different countries and purchased in Estonia. These values can be seen in Table 2.4 in comparison with our sample dissolved in water (A).

Table 2.4 Content of major polyphenols (mg/100 g dw) in 13 commercial samples and in our sample dissolved in water (A=without hydrolysis).

	Samples													
Compound	**A**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Apigenin glu	**5684**	431	32	450	213	113	227	313	60	70	560	80	150	186
Luteolin glu	**284**	254	80	260	113	87	133	87	90	40	260	18	77	78
3,4-dcqa	**116**	354	32	260	93	207	193	153	150	110	250	58	147	68
3,5-dcqa	**82**	523	80	570	173	300	360	320	190	230	500	170	607	198
4,5-dcqa	**98**	392	24	350	93	153	227	187	110	80	300	102	260	78

Glu=glucoside, dcqa=dicaffeoylquinic acid; dw= dry weight. 1- Belin, 2- Wilken Tee, 3-Eko Natura, 4- Švenčionių vaistažoles, 5-Teekanne, 6-Rimi order, 7-Tea Trader, 8-Laplanta, 9-Bigelow, 10-Loodusravi, 11-Kubja Ürt, 12-Vadi Gild and 13-Elujõud.

They did not report their specific parameters of analysis (LOD and LOQ) for their quantification, but specified that each compound was identified comparing their fragmentation spectra (MS^2) to commercial standards or reports in the literature. They used calibration curves of chlorogenic acid, luteolin aglycone and apigenin aglycone for the quantification of apigenin glucoside, luteolin glucoside and the three dicaffeoylquinic acids (3,4-, 3,5-, 4,5-), and the quantification was done using the ratio of molecular weights of the related standard compounds. The concentration of apigenin glucoside in their samples varied from 32 (Wilken Tee; Germany) to 560 mg/100g dw (Loodusravi; Estonia) which was lower than our sample dissolved in water (5683.7 mg/100g dw). In the case of luteolin glucoside, samples varied from 18 (Kubja Ürt; Estonia) to 260 mg/100 g dw (Eko Natura- Poland; Loodusravi- Estonia). Our reported value for the sample dissolved in water (284.1 mg/100g dw) was higher than all the amounts reported. However, our comparison cannot be precise due to the lack of information about the specific glucoside measured by the publication. The concentration of 3,4-dicaffeoylquinic acid in our report (116.4 mg/100g dw) was similar to the concentration found in their sample Bigelow (USA) (110 mg/100 g dw). The sample Belin (Poland) showed the highest concentration (353.8 mg/100g dw) of 3,4-dicaffeoylquinic acid. Next, 3,5-dicaffeoylquinic acid concentration in the 13 samples varied from 80 (Wilken Tee) to 606.7 mg/100g dw (Vadi Gild). Only the lowest concentration matched our finding from the sample dissolved in water (82.1 mg/100g dw).

Finally, our measurement of 4,5-dicaffeoylquinic acid (98.3 mg/100g dw) was only comparable with the sample Švenčionių vaistažoles (Lithuania) (93.3 mg/100g dw). Discrepancy in our results could be attributed to our use of true standards instead of an indirect measurement for the quantification of each compound. The indirect measurement might have overestimated the concentration found in each sample, but other factors could have also influenced such as the preparation method, place of purchase and tea composition (tea leaves, flowers, etc).

Novakova *et al*. (2010) quantified methanolic extracts from loose tea and tea bags using a LC-ESI/MS^2 method. Their LOQ (5 - 50 nM) was up to 1000x lower than our method. This can be explained by the precision of the equipment used for their analysis. As it has been stated before, the inability of LC-ESI/MS to identify isomers will decrease the specificity of the identification which it might have a negative effect in the MS response, reducing the signal of each compound. They reported average values for apigenin-7-glucoside, apigenin, luteolin and umbelliferone in µmol/L (µM) in both types of samples (loose tea and tea bags). The comparison with our results cannot be 100% certain, since the change from µM to mg/100g dw was done using only the information provided in their article (see 2.3 in publication) and the results can be seen in the Table 2.5.

Table 2.5 Average content of 4 polyphenols (mg/100g dw) in 16 commercial samples from methanolic extracts of loose tea and tea bags, and content in our two liquid-liquid extractions.

	mg/100 g dw			
Compound	Loose tea	Tea bags	Fraction Acetonitrile	Fraction Ethyl acetate
Apigenin-7-glucoside	138	65	575	316
Apigenin	21	9	376	204
Luteolin	3	< 3	18	8
Umbelliferone	8	< 8	155	139

dw=dry weight

It was found that our two samples extracted with acetonitrile and ethyl acetate had higher concentrations of these 4 compounds in comparison with their methanolic fractions, where apigenin in tea bags presented the highest difference (up to 42x less than fraction acetonitrile). They reported that loose tea contained up to ½ higher concentration of apigenin and apigenin-7-*O*-glucoside than tea bags, suggesting that the manufacturing process has a negative effect on the content of major polyphenols.

Finally, one of the most recent reports on GC (Guimaraes *et al.*, 2013) presented a wider list of compounds using two different methods of sample preparation (infusion and decoction). According to their report, the major compound found in GC was luteolin-*O*-acylhexoside which we identified, but not quantified. The concentration of luteolin-7-*O*-glucoside in their sample extracted with methanol (60 mg/100g dw) was higher from our samples extracted with acetonitrile and ethyl acetate. However, our sample dissolved in water (284 mg/100g dw) was higher than their two methods of preparation (170 and 90 mg/100g dw from infusion and decoction respectively). We did not detect 3,4-dicaffeoylquinic acid in treated samples (liquid-liquid extraction), but we measured 116 mg/100 g dw in the sample dissolved in water. They quantified it in their three samples and at higher concentration (330-730 mg/100g dw) than our reported value. Low concentration (10-8 mg/100g dw) of 3,5-dicaffeoylquinic acid was found in our both treated samples in comparison with their value reported for their sample extracted with methanol (100 mg/100g dw). Their samples from infusion and decoction varied from 260 to 160 mg/100 g dw respectively. These values were higher than our sample dissolved in water (82 mg/100g dw). The compounds 3,4- and 3,5-dicaffeoylquinic acid were identified according to their fragmentation patterns and previous reports of their appearance, but it is not determine if a calibration curve was used to perform their quantification.

Overall, plant extracts are not only affected by external factors such as harvesting, growing season, processing, etc.; others might also influence the polyphenol content in the plant (Alfaro *et al.*, 2013). Polyphenols are transformed inside of the human body by the gastrointestinal tract and this phenomenon was mimicked by enzymatic hydrolysis. Two groups of

enzymes: hesperidinase and cellulase were used to mimic those enzymes found in the gut microflora (β-glycosidases and α-L-rhamnosidase) and intestinal cells (β-D-glucosidases) (Scalbert and Williamson, 2000). This approach has been reported since the late 1980's by Bokkenheuser *et al*. (1987) using intestinal bacteria from humans to hydrolyse quercetrin and robinin and obtain their aglycones, quercetin and kaempferol respectively. They also suggested that other intestinal species may perform the same hydrolysis. Then, the methodology followed in our experiments was previously tested in a complex mix of polyphenols in berries (Pimpão *et al.*, 2013) which showed that a combination of enzymes was more efficient to yield a higher concentration of aglycones than individual enzymes. Further, this method was tested in five different fruit matrices which revealed its versatility and potential use in other plant-based materials. We performed this method using the same combination of enzymes. In addition, GC polyphenols were extracted using two different organic solvents: acetonitrile and ethyl acetate.

Firstly, as it was expected, the concentration of the aglycone forms after hydrolysis and liquid-liquid extraction (umbelliferone, luteolin and apigenin) increased by up to 2.3x. Indeed, the concentration of conjugates (e.g. apigenin-7-*O*-glucoside) decreased more than 90% and some could not be detected (3,4-dicaffeoylquinic acid). It was assumed that once conjugates were hydrolysed, the concentration of its aglycones after extraction (apigenin and luteolin) would increase in a similar ratio. However, this did not happen as expected. For instance, luteolin-7-*O*-glucoside decreased more than 95%, but luteolin only increased up to 2.6%. The most drastic loss was seen in the concentration of apigenin-7-*O*-glucoside and its aglycone (apigenin) which did not increase more than 2%. One of the possible reasons for this phenomenon is due to solubility of the compounds which suggested that no hydrolysed glucosides were lost during the liquid-liquid extraction. Glucosides are more water-soluble than their aglycones and this might have affected their quantification. It is recommend that after enzymatic hydrolysis a liquid-liquid extraction should be carried out with other organic solvents miscible in water such as 2-propanol or ethanol. Methanol has been previously used by Guimaraes *et al.* (2013), but did not show effective

extraction of the most abundant compounds in GC, apigenin and its derivatives. Also, Ignat *et al.* (2011) reported that methanol in different aqueous mixes has been generally used for the extraction of these polyphenols, but it can promote spontaneous methylation and then deliver incorrect results (Xie *et al.*, 1993). Despite seeing a better extraction using acetonitrile, the concentration of each compound might have been underestimated.

In summary, the characterisation of a plant extract is an essential step towards a better understanding of the potential effects on human health. A methodology using LC-ESI/MS will provide the necessary information to select those compounds with more scientific interest, but it is important to take into account its limitations. Also, if two methodologies are combined (MS^2 and MS) the time of analysis will decrease and its accuracy will improve. Additionally, enzymatic hydrolysis will help to identify and quantify the polyphenol content of plant samples which by nature is a complex mixture. This approach can provide a complete profile of the plant which will improve the knowledge collected so far.

CHAPTER 3 Polyphenol phase II conjugation: sulfation of hesperetin using pig liver cytosol and identification by LC-ESI/MS

3.1 Abstract

Identification and quantification of polyphenol phase II metabolites has been an ongoing challenge for research, and is required to understand and determine the potential health benefit of polyphenols. The main methodology to avoid this challenge has been through enzymatic hydrolysis. However, due to the technique's limitations, it provides only general information of the phase II metabolites, but cannot report accurate quantification data. The objectives of this investigation were to develop a reproducible high-yielding method to sulfate polyphenols, to identify the main form biologically synthesised in humans and to analyse urinary samples to confirm their presence in biological fluids. The sulfation was achieved using pig liver cytosol and *p*-nitrophenyl sulfate (PNS) as co-cofactor, which produced up to 90% transformation. Hesperetin was used as the experimental polyphenol and the sulfated form was identified as hesperetin-3'-*O*-sulfate. Urine samples from a crossover human study were collected and analysed by Dr. Tristan P. Dew and Miss Isabella Procopiou using true standards for identification and quantification of hesperetin metabolites. The data was assessed, statistically analysed and interpreted by this researcher. Quantification by LC-ESI/MS showed an enhanced response of the sulfated form in comparison of its aglycone, which modified its concentration calculated in urine samples. This allowed the hesperetin excretion to be accurately quantified. The excretion data showed statistical difference between value brand and freshly squeezed orange juice (p=0.011). The individual excretion for each metabolite (hesperetin-7-*O*-glucuronide, hesperetin-3'-*O*-glucuronide and hesperetin-3'-*O*-sulfate) was also statistically different between value brand and freshly squeezed orange juice. The concentration of hesperidin ingested was significantly different in the three orange juices (p<0.001). The metabolite excretion was higher in freshly squeezed orange juice, even though the concentration of hesperidin ingested was the lowest.

3.2 Introduction

Polyphenols occurring naturally in plants can be classified into two groups, the flavonoids and the non-flavonoids (Crozier *et al.*, 2009). In the group of flavonoids, **flavanones** are present almost exclusively in citrus fruits. Hesperetin-7-*O*-rutinoside (hesperidin) is one of the most common in oranges and mandarin (see Figure 3.1). Its aglycone form, without sugar moiety, hesperetin is present in only very small quantities in the plant, and mostly appears after the first step of metabolism.

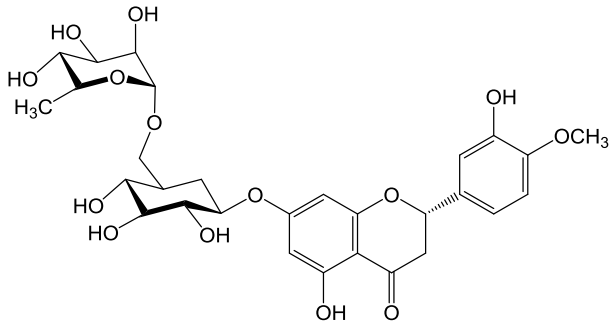


Figure 3.1 Hesperetin-7-*O*-rutinoside (hesperidin) structure.

Orange juice is one of the most widely consumed fruit juices in the UK, covering 54% of the fruit juices market (BSDA, 2014). Although its consumption has dropped during recent years, orange juice ingestion is still related to health benefits such as protection against oxidative-stress related to inflammation (Nakajima *et al.*, 2014) and improvement in cognitive function in healthy older adults (Kean *et al.*, 2015). After orange juice has been consumed, hesperidin undergoes cleavage and hesperetin is relased. Then, phase II reactions (sulfation, glucuronidation and methylation) occur as part of its absorption process (Nielsen *et al.*, 2006). Ultimately, these metabolites circulate through the bloodstream and significant quantities are excreted in the urine. Hesperetin and its metabolites have been recently quantified in urine samples using authentic standards commercially available (Bredsdorff *et al.*, 2010; Pereira-Caro *et al.*, 2014). However, most of the publications have used enzymatic hydrolysis to split glucuronide and sulfate group(s) and release its aglycone. Even though this method is widely used, some flavonoids have been shown to be poor substrates for commercial enzymes (Vallejo *et al.*, 2010; Farrell *et al.*, 2011; Saha *et al.*, 2012) which suggests an underestimation of their concentration. A series of

methodologies to chemically synthesise glucuronidated compounds have been reported (Khan *et al.*, 2014) and only a few research papers have performed analysis and biological synthesis of sulfated forms (Takumi *et al.*, 2012; Bredsdorff *et al.*, 2010; Brand *et al.*, 2010; Mullen *et al.*, 2010). Although these publications have positively identified hesperetin-3'-*O*-sulfate as the common monosulfate metabolite in urine after phase II metabolism, their methodology relies on differences in ion fragmentation in LC-MS^2 or specific resonance frequency in NMR which increases cost, time of analysis and could potentially report misleading information. Therefore, knowing these limitations and the need for authentic standards, we have developed an accessible method to biologically sulfate flavanones and we compared it with a chemically synthesised standard. We introduced a regenerator of adenoside 3'-phosphate 5'-phosphosulfate (PAPS) called *p*-nitrophenyl sulfate (PNS) which has been previously reported as a sulfate donor (Koizumi *et al*. 1990;1991), but in the presence of adenosine 3',5'-diphosphate (PAP) it donates its sulfate group and restores PAPS, thereby increasing the % transformed. The compound biologically synthesised was identified as hesperetin-3'-*O*-sulfate and it was used to quantify phase II metabolites in urine samples following orange juice consumption. To our knowledge, there is no reported methodology using our approach and it is the first data using an authentic standard to quantify hesperetin-3'-*O*-sulfate in urine samples.

3.3 Materials

Rutin, taxifolin and hesperetin were purchased from Extrasynthese (Genay Cedex, France). Potassium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Alfa Aesar (Ward Hill, USA). L-Ascorbic acid, adenosine 3'-phosphate-5'-phosphosulfate lithium (PAPS), DL-dithiothreitol (DTT), sodium sulphite, potassium 4-nitrophenyl sulfate (PNS) and formic acid were purchased from Sigma-Aldrich (Dorset, UK). Hesperetin-3'-*O*-sulfate (Hp3'SO_4) was kindly provided by Dr. C. Morand (INRA, France). Hesperetin-3'-*O*-glucuronide (Hp3'Glu) and hesperetin-7-*O*-glucuronide (Hp7Glu) were kindly donated by Professor Denis Barron (Nestle, Switzerland). Supermarket own-brand value range orange juice manufactured from concentrate, luxury range freshly squeezed orange juice

(Morrisons; Bradford, UK) and premium-brand (Tropicana; Boxford, UK) were purchased at a local supermarket (Morrisons; Leeds, UK). Pig liver cytosol suspension was prepared as described in 3.4.1. Ethyl acetate and acetonitrile were LCMS grade and purchased from VWR (Lutterworth, UK). Methanol HPLC grade was purchased from Fisher Scientific (Loughborough, UK) and ethanol absolute for HPLC use was purchased from Sigma-Aldrich (Dorset, UK). All water refers to deionised Milipore water (Hertfordshire, UK) unless otherwise stated.

3.4 Methods

3.4.1 Preparation of pig liver cytosol suspension

Preparation of liver cytosolic suspension was performed using a modified version of a method by Campbell *et al*. (1987). Fresh pig liver purchased locally was submerged in an ice-cold 250 mM sucrose solution to remove excess of blood. The liver was blotted dry and a sample (13 g) was finely chopped with scissors then homogenised with 50 mL of ice-cold Tris-HCl buffer (50 mM, pH 7.5) using a hand blender. The homogenate was separated in a refrigerated centrifuge (Beckman JA25.50; High Wycombe, UK) at 17,800 g for 10 min at 4°C. The resulting supernatant was decanted and further centrifuged for 90 min at 34,900 g at 4°C. The final supernatant was recovered, aliquoted and stored at -80°C for further use.

3.4.2 Synthesis of polyphenol sulfate

Biological sulfation was performed using a modified version of a method by Menozzi-Smarrito *et al*. (2011). The reaction was performed in potassium phosphate buffer solution (74 mM containing 100 µM ascorbic acid, pH 7.4) including adenosine 3'-phosphate 5'-phosphosulfate lithium (100 µM), DL-dithiothreitol (1mM) and sodium sulphite (10 mM) with or without potassium 4-nitrophenyl sulphate (various concentrations). The mixture was pre-warmed for 5 min at 37°C and a solution of hesperetin in ethanol (80 µM) was added. The reaction was initiated by the addition of the pig liver cytosol suspension and was incubated for 4 hrs at 37°C. The reaction was stopped by adding organic solvent (2:1, v/v) and vortexing for 60 sec. Two organic solvents, acetonitrile (method 1) and ethyl acetate (method 2), were used to remove unreacted compounds and other impurities.

After the incubation time was over, ice-cold acetonitrile (method 1) was added to the mixture (2:1, v/v), vortexed for 60 sec and centrifuged at 17,000 g for 15 min. The supernatant was collected and centrifuged again at 17,000 g for 15 min. The last supernatant was collected and was analysed by LC-ESI/MS.

In a similar way, after the incubation time had finished, ice-cold ethyl acetate (method 2) was added to the mixture, vortexed for 60 sec and the supernatant was discarded. This process was repeated 2x. Then, the remaining pellet was treated as described in method 1. Samples that were not analysed were stored at -80°C.

3.4.3 Semi-preparative chromatography and identification by LC-ESI/MS

Hesperetin sulfate was further purified via semi-preparative HPLC using a Shimadzu Prominence system (Milton Keys, UK) comprising a LC-20AD XR with parallel double micro plunger, a temperature-control autosampler and a SPD-M20A diode array detector. The HPLC system was coupled to a FRC-10A fraction collector. A 20 µL sample was injected and analytes were separated using an eclipse XDB-C18 analytical column (4.6x50 mm, 1.8 µM; Agilent, Cheadle, UK) maintained at 30°C. Mobile phase A consisted of 0.5% formic acid in water and mobile phase B consisted of 0.5% formic acid in acetonitrile. Elution started at 5% solvent B at a flow rate of 1 mL/min. Solvent B was increased to 10% after 5 min, then rose up to 40% until 20 min and after 25 min reached 90%; this percentage was held until 29 min. The gradient returned to 5% solvent B over 1 min, and the column re-equilibrated until 33 min. Hesperetin sulfate was collected between 7 and 8.5 min and was identified using a similar chromatographic separation with a flow rate of 0.5 mL/min, injecting a 10 µL sample using a 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. The retention time and sensitivity of the method for hesperetin-3'-*O*-sulfate (Hp3'SO_4) was evaluated using a chemically synthesised standard generously provided by Dr. C. Morand (INRA, France).

3.4.4 Human study to identify hesperetin metabolites from orange juice consumption

This crossover human study was carried out by Dr. Tristan P. Dew and Miss Isabella Procopiou in collaboration with our research project.

3.4.4.1 Subjects

Fifteen healthy subjects (eleven females and four males) were recruited and all completed this study. Inclusion criteria included: age 22-27 years, body mass index (BMI) 16.9-32.9 kg/m^2. Exclusion criteria included: regular medication, gastrointestinal surgery and intestinal or metabolic disease. The study was approved by the Faculty research ethics committee MEEC (Maths, Engineering and Physical Sciences) 09-019 which followed institutional guidelines.

3.4.4.2 Study design

The study was a 3-visit treatment with a randomised crossover design, including a wash out of 2 days prior to test day. Participants were provided with a food restriction list to avoid during the wash out and 24 hrs after orange juice consumption, including citrus and citrus derived food. Restricted foods included all types of juices, smoothies, jams, jellies, etc. After overnight fasting, baseline urine was collected prior to orange juice consumption. Doses of orange juice were administered on the basis of body weight (BW) (at the rate of 5 mL/kg BW). Next, a polyphenol-free breakfast and lunch were provided, and participants were asked to collect urine for 24 hrs recording the collection time and any irregularities.

3.4.4.3 Urine collection and processing

Urine was collected in sterilised and dried 3 L unisex collection vessels, each containing 3 g of ascorbic acid. The total volume of baseline and 24-hrs urine was recorded using the scale on the vessel and 10 mL aliquots were placed into 15 mL falcon tubes. To each 10 mL portion of urine, 0.1 mL of 10% sodium azide was added and sample was vortexed for 10 sec. If 24-hr urine was collected in two or more containers, all urine was mixed thoroughly before aliquots were taken. Samples were centrifuged at 2,000 g for 10 min at 4°C (Centrifuge 5810 R Eppendorf, UK) and supernatants were removed and stored at -20°C until analysis.

3.4.4.4 Extraction of hesperetin conjugates from urine

Baseline and post-supplementation urine samples were extracted in duplicate from all participants. For each replicate, 200 µL of urine was mixed with 100 µL of rutin internal standard (2.5 µg/mL), 100 µL of HPLC grade ethanol and 800 µL of HPLC grade methanol. This solution was then placed in a shaking water bath (GLS Aqua 12 Plus, Grant Instruments Ltd, UK) at 50˚C and 110 rpm for 10 min to solubilise the compounds. The mixtures were placed in a micro-centrifuge for 10 min at 17,000 g (IEC-MicroCL17, Thermo, Germany). The supernatant was removed as the first extract. The pellet was resuspended in 200 µL water, 100 µL of HPLC grade ethanol and 800 µL of HPLC methanol and the extraction process was repeated. The primary and secondary supernatants were stored in a freezer at -20°C. After all samples were processed, the primary and secondary supernatants were dried using a centrifugal evaporator (Genevac EZ2plus Evaporation System, USA), initially using the HPLC program with the lamp off to achieve the evaporation of methanol and ethanol layer, followed by the aqueous program at 65˚C to evaporate the remaining urine-water layer. Completely dried samples were placed back into the freezer at -20°C until their reconstitution.

3.4.4.5 Reconstitution of the dried samples

The dried samples were brought to room temperature and were reconstituted within 24 hrs of analysis. A 70 µL aliquot of HPLC grade methanol was added to secondary supernatant (see 3.4.4.4). This solution was vortexed, sonicated and placed in a shaking water bath for 10 min; then a 50 µL aliquot was combined with the corresponding primary extract. This mixture was vortexed, sonicated and placed in a shaking water bath. Subsequently, a 50 µL aliquot of 0.2% ascorbic acid (w/v) with 5 µg/mL taxifolin internal standard were added to the mix and vortexed until the samples were completely dissolved into the solution. This mixture was further centrifuged for 10 min at 17,000 g and the supernatant was transferred into amber HPLC vials. The determination of recovery was performed using blank urine spiked with hesperetin, hesperetin-7-*O*-glucuronide (Hp7Glu) and hesperetin-3'-*O*-glucuronide (Hp3'Glu) at concentrations of 41.6, 4.16 and 0.42 µM in triplicate.

3.4.4.6 Orange juice composition

Each orange juice (freshly squeezed, value-brand and premium-brand) was treated with aqueous methanol (80%, v/v). A 500 µL aliquot of orange juice was mixed with 10 µL of apigenin as internal standard (1 mg/mL) and 490 µL of aqueous methanol. The sample was centrifuged at 3,000 g for 5 min and the supernatant was collected. The pellet was washed three times using 500 µL of methanol each time and collecting all supernatants. Finally, a 0.2 µm PTFE filter was used to filter supernatants into HPLC vials. These were used in LC-ESI/MS quantification.

3.4.4.7 Analysis of urinary hesperetin-metabolites by LC-ESI/MS

Orange juice and urine samples were analysed using a Shimadzu LC-2010 HT HPLC coupled with a LCMS-2020 quadrupole mass spectrometer fitted with an electro spray ionisation source operated in negative mode, with a detector voltage of -1.80 kV, DL temperature 250°C, nebulizing gas flow and drying gas flow were set to 1.50 and 15 L/min respectively. The mass spectrometer was set to single ion monitoring (SIM) mode in negative mode, capturing data at mass-to-charge ratios (*m/z*) of 301, 303, 381, 477 and 609 corresponding to hesperetin, taxifolin, Hp3'SO_4, Hp3'Glu, Hp7Glu and rutin respectively. A 5 µL aliquot was injected and analytes were separated using a Kinetic C18 analytical column (2.1x150 mm, 2.6 µm; Phenomenex, Cheshire, UK) maintained at 35˚C. Mobile phase A consisted of 0.2% formic acid in a mix of water: acetonitrile (95:5, v/v) and mobile phase B consisted of 0.2% formic acid in a mix of acetonitrile:water (95:5, v/v). The flow rate was set at 0.26 mL/min. Elution started at 15% solvent B and remained until 13.6 min. From 16.4 to 22 min solvent B was increased up to 35% and at 23.4 min was increased to 90% and then held up to 27.2 min. At 30 min solvent B was decreased to 15% and the column was re-equilibrated until 37 min.

3.4.4.8 Statistical analysis of urine samples after orange juice consumption

IBM SPSS statistics 22 was used for all the statistical analysis (Portsmouth, UK) and orange juices were coded as 1, 2 and 3 corresponding to freshly squeezed, premium brand and value brand to facilitate the evaluation. Data was assessed by Shapiro-Wilk’s test to

determine normality. The non-parametric analysis, Friedman test, was used for data not normally distributed to determine any significant difference among the variables. A one-way repeated measurement ANOVA was conducted for data normally distributed. Significance level was determined as $p<0.05$.

3.5 Results

3.5.1 Optimisation of hesperetin sulfate biosynthesis

Biological synthesis of hesperetin sulfate was based on a modified method published by Menozzi-Smarrito *et al*. (2011). Even though previous work on hydroxycinnamic acids (Dr C.C. Wong, published) showed that human liver (diluted) sulfated these compounds, our early development work indicated that human pooled liver diluted was a poor driver of quercetin sulfation (< 1% transformation). Indeed, diluted cytosol from pig liver did not sulfate epicatechin. Then, cytosol from pig liver without dilution was used in further experiments for the transformation of hesperetin to hesperetin sulfate.

The biosynthesis of sulfate compounds is a reaction where PAPS is the universal donor of the sulfate group and it is transformed to PAP (adenosine 3',5'-diphosphate) after the reaction has occurred. It has been reported by Tyapochkin *et al.* (2009) that PAPS can be regenerated after being used in the reaction by PNS. Thus, the influence of PNS (20 mM) as co-cofactor of the reaction was investigated.

Firstly, to establish an optimum incubation time, the reaction was followed from 1 to 4 hrs in presence of a sulfatase inhibitor (sodium sulphite). Samples for each hr (PAPS and PAPS+PNS) were prepared following the methodology above described (see 3.4.2). Every hr, samples were taken out from the water bath and the reaction was stopped. Next, samples were assessed to determine their content of hesperetin sulfate. After 1 hr, samples with PAPS reached 32%, increasing to just 38% after 4 hrs incubation. On the other hand, samples with PAPS+PNS reached up to 90% transformation after the first hr and they did not present major changes after four hours (80% transformation). There was no significantly difference for the

time (hours), but there was a significant difference between treatments (p<0.001).

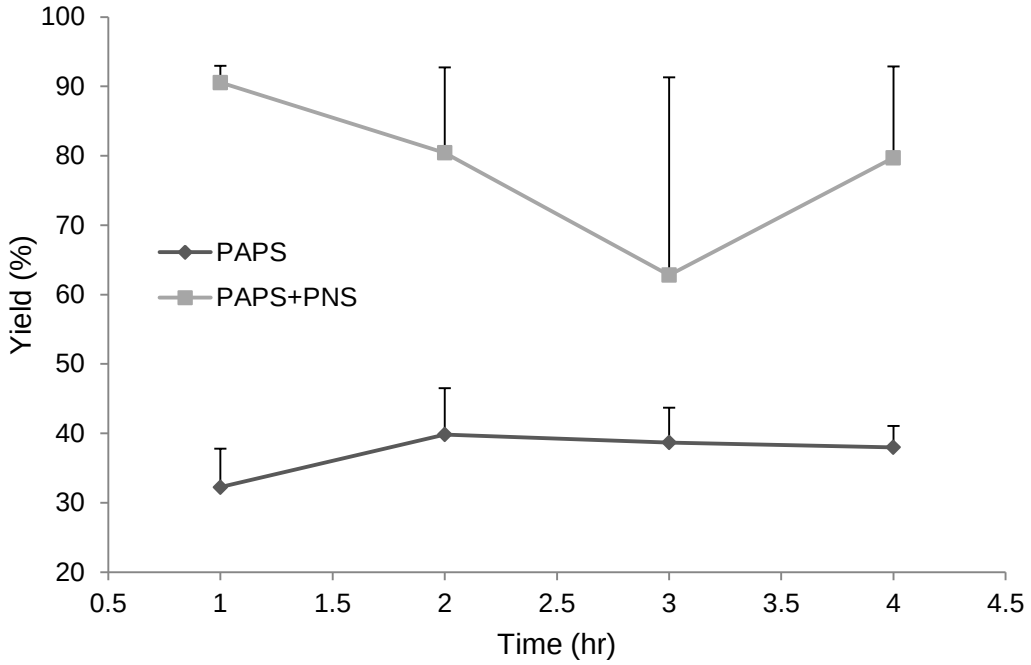


Figure 3.2 Hesperetin sulfate biosynthesis. Comparison between reaction with co-factor PAPS and reaction with mixture PAPS+PNS. N=3 biological replicates and 1 technical measurement, +SD.

The percentage of transformation was based on the appearance of sulfate versus loss of substrate after biosynthesis (see Figure 3.2). Further experiments were performed at 4 hrs and showed up to 90% transformation (see Figure 3.4).

3.5.2 Purification of hesperetin sulfate and its identification using a chemically synthesised standard and LC-ESI/MS

3.5.2.1 Method selectivity

All chromatograms were recorded following the mass-to-charge ratio of hesperetin aglycone (*m/z* 301) and its mono- and di-sulfates (*m/z* 381, 461) in negative mode. Hesperetin aglycone was identified in all samples and its mono-sulfate (*m/z* 381) was only detected following biosynthesis.

Additionally, it was found that the use of 0.5% (v/v) formic acid in LC-ESI/MS mobile phase increased the signal of sulfate metabolites in

comparison with 0.1% (v/v) formic acid, providing better separation and more stable signal (data not shown).

3.5.2.2 Hesperetin sulfate pre-treatment

A liquid-liquid extraction using ethyl acetate and acetonitrile was used as pre-treatment before purification. The methodology followed can be seen in 3.4.2. No major changes were observed after using two organic solvents (ethyl acetate and acetonitrile) instead of one (acetonitrile) to extract hesperetin sulfate, as both delivered more than 90% transformation (data not shown).

3.5.2.3 Identification of hesperetin sulfate

Hesperetin sulfate was collected following the methodology explained in 3.4.3. After several collections took place, the fraction was re-analysed in LC-ESI/MS and a chemically synthesised hesperetin sulfate was used to identify the monosulfate as hesperetin-3'-*O*-sulfate (Hp3'SO_4) (see Figure 3.3).

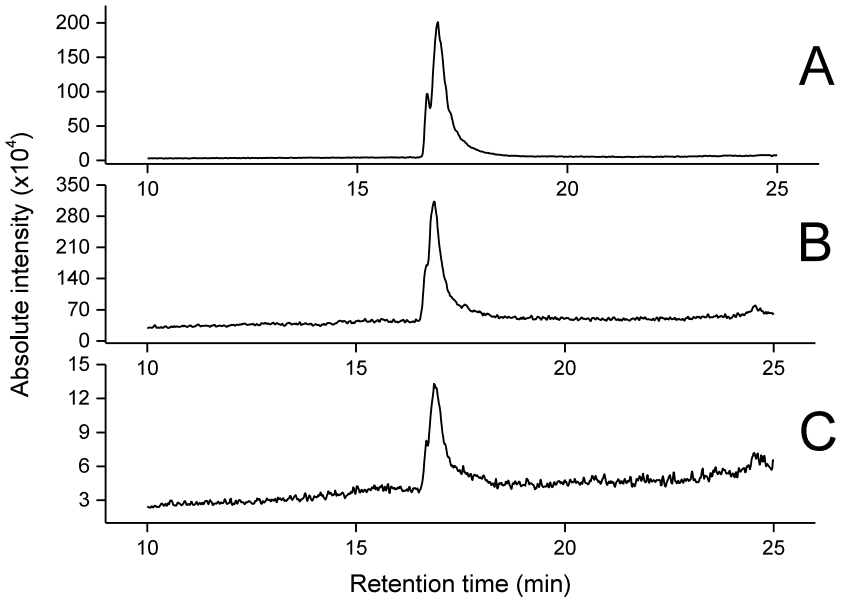


Figure 3.3 TIC chromatograms of hesperetin sulfate and its standard. A) Chemically synthesised hesperetin-3'-*O*-sulfate; B) Hp3'SO_4 using 1.5 mM PNS and C) Hp3'SO_4 using 20 mM PNS. Retention time (RT) = 16.7 min.

It was observed that after the sulfate sample was pre-treated (see 3.5.2.2), an amount of PNS still remained in the mix. For this reason, different concentrations of PNS (1 to 20 mM) were tested to estimate the

minimum amount to perform the reaction which will not remain in high quantities in the supernatant. The molar ratio between hesperetin and PAPS was kept at 1:1.25 and only the concentration of PNS was modified (see Figure 3.4).

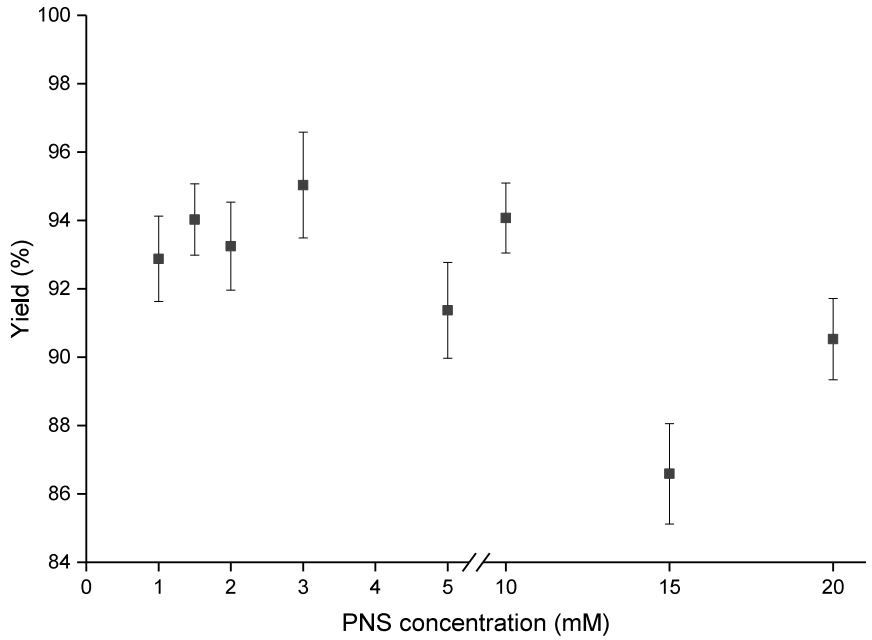


Figure 3.4 Percentage of conversion of hesperetin to its sulfate conjugate after 4 hrs using different concentrations of PNS. N=3 biological replicates and 1 technical measurement, ± SD.

From the lower concentrations, 1.5 and 3 mM yielded 94 and 95% transformation. The other concentrations yielded 90% or more, except for 15 mM (86%). Further experiments were performed using 10x less PNS from initial experimentation (20 mM). The chromatography was improved and the transformation was not compromised after the modification (see Figure 3.3).

3.5.2.4 Sensitivity of method for analysis of biosynthesis samples

The sensitivity of the analytical method was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) of the sulfate by LC-ESI/MS. Hesperetin aglycone, Hp3'SO_4 and a mix of both were analysed from 1 nM to 100 µM following the mass-to-charge ratio for each analyte (*m/*z 301 and 381) in negative mode. Compounds were dissolved in DMSO and diluted with ethanol/water at different ratios to provide a fully dissolved mix. The limits for hesperetin agycone were: LOD=1.1 µM and LOQ=3.7 µM. The limits for Hp3'SO_4 were LOD=2.9 µM and LOQ=9.7 µM. These values

were calculated using the equation LOD=(SD/slope)*3 and LOQ=(SD/slope)*10. Visually, hesperetin aglycone and hesperetin sulfate were detected at 0.1 µM and they could be confidently quantified at 0.5 µM. The calibration curves can be seen in Figure 3.5.

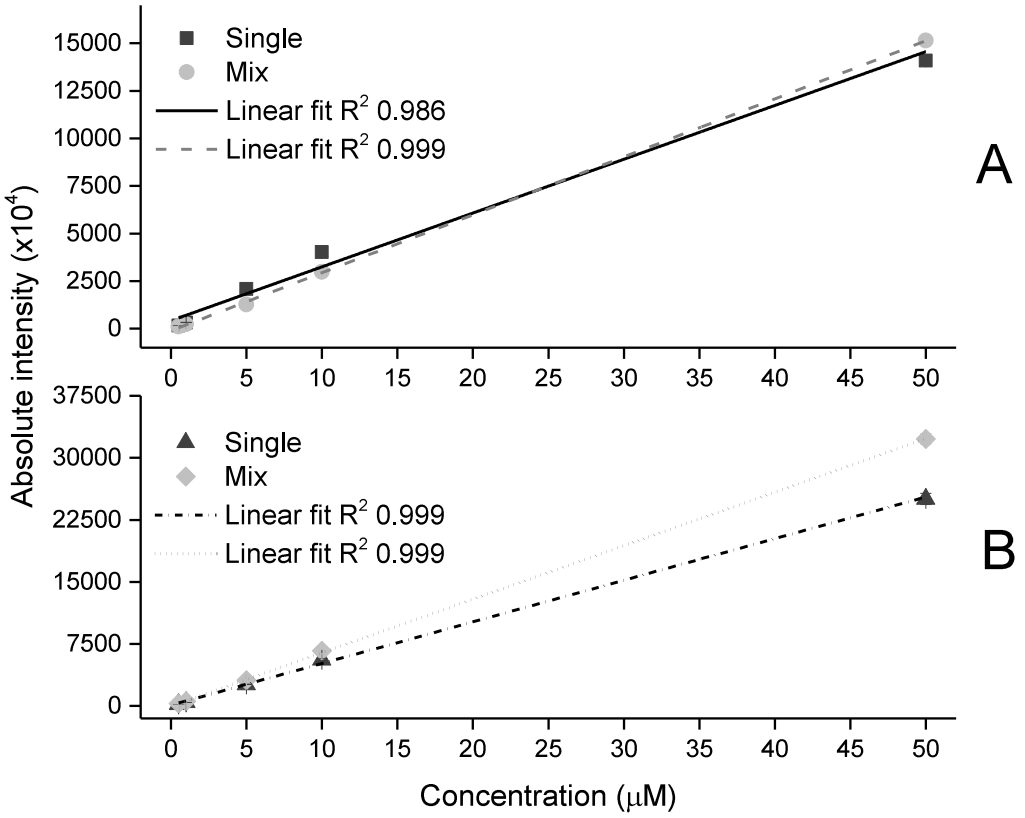


Figure 3.5 Calibration curves for hesperetin and Hp3'SO4 using single compounds and a mix of both. A) Hesperetin aglycone and B) Hp3'SO_4.

It was observed that the MS signal for Hp3'SO_4 in the mix was in average 2.3x more than hesperetin aglycone. Similarly, the MS signal of Hp3'SO_4 measured as single compound was 1.4x more than hesperetin aglycone. As a result, a 2.3 correction factor was used in further quantification of Hp3'SO_4, as hesperetin aglycone and Hp3'SO_4 would be measured mixed together.

3.5.3 Analysis of different orange juices and how their consumption impacts hesperidin urinary phase II metabolites on humans

3.5.3.1 Quantification of major polyphenols in orange juice

The concentration of hesperidin (which is the precursor of hesperetin and its metabolites) was assessed in each of the 3 orange juices used in the intervention study. Value brand orange juice contained the highest amount of hesperedin (276 mg/L) followed by the premium brand and freshly squeezed juice (189 and 177 mg/L respectively) (see Figure 3.6). Only traces of hesperetin and hesperetin-7-*O*-glucoside (< 0.01 mg/L) were detected.

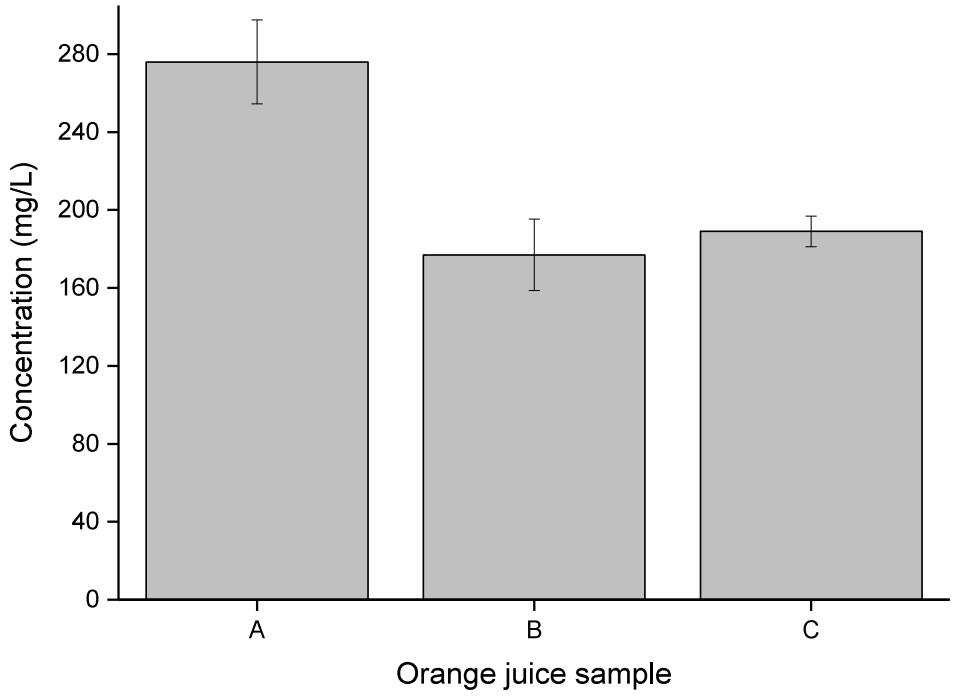


Figure 3.6 Hesperidin content of different orange juice samples. A) Value brand (276 ± 21.6 mg/L), B) freshly squeezed (177 ± 18.3 mg/L) and C) premium brand (189 ± 7.8 mg/L). N= 3 biological replicates and 1 technical measurement, ± SD.

3.5.3.2 Hesperetin and hesperetin conjugates evaluation

An external calibration curve for hesperetin aglycone, Hp3'Glu and Hp7Glu (prepared in 50% methanol, containing 0.2% ascorbic acid and 2.5 µg/mL taxifolin) were generated across a concentration range of 41.6 nM to 41.6 µM (see Figure 3.7). The LOQ was between 85-150 nM for Hp3'Glu and Hp7Glu, and below 40 nM for hesperetin. Each target-analyte area was

normalised to taxifolin as internal standard and results were transformed to log to provide linearity to all analytes.

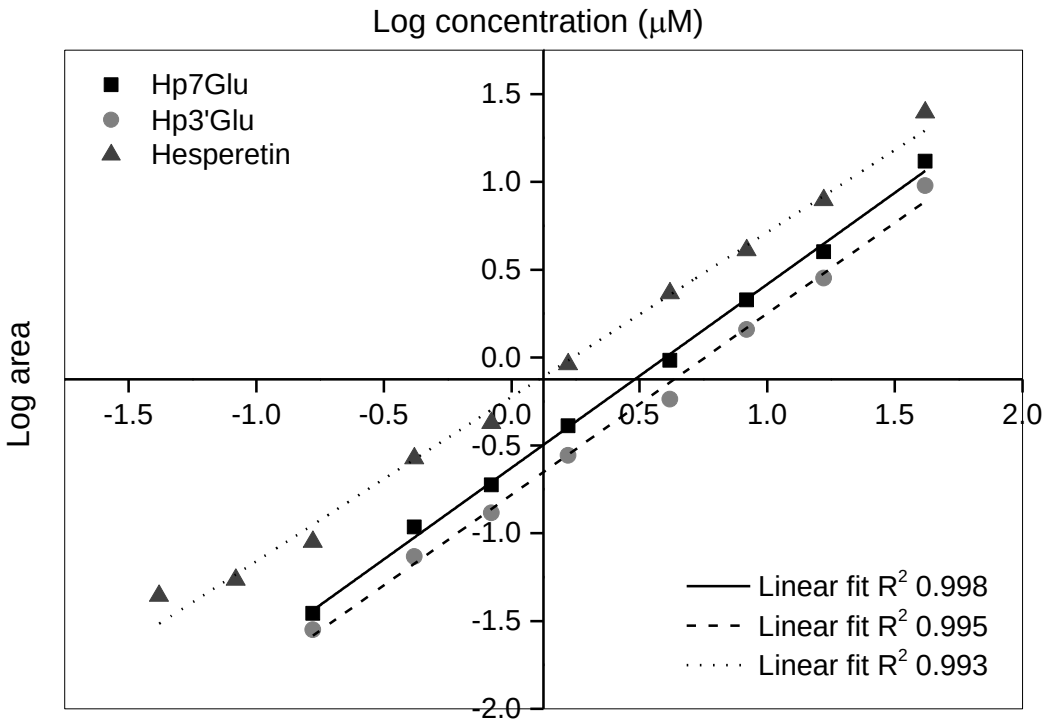


Figure 3.7 Logarithmic calibration curve for hesperetin, Hp7Glu and Hp3'Glu. The analysis was performed from 41.6 nM to 41.6 µM.

After these calibration curves were performed, it was found that Hp3'SO_4 provided more signal than hesperetin aglycone. From a previous calibration (see 3.5.2.4) it was calculated that the MS signal for Hp3'SO_4 was 2.3x more than hesperetin aglycone (see Figure 3.5). Thus, 2.3 was used as a correction factor to calculate Hp3'SO_4 excretion from urine samples.

3.5.3.3 Urine analysis

Initially, extraction efficiency of each compound was calculated following the protocol above stated (see 3.4.4.5). It was found that each analyte (hesperetin, Hp7Glu and Hp3'Glu) yielded 66.9%, 80.7% and 76.8% respectively. A limited amount of Hp3'SO_4 was available for accurate extraction test, so an estimated average extraction-efficiency of 74.8% was used after a one-time measurement. These values were used to adjust for analyte losses during sample handling. Samples were analysed in LC-ESI/MS (see 3.4.4.7) and each metabolite was quantified using the calibration curves previously performed (see 3.5.3.2). A typical

chromatography of urinary metabolites excretion for each orange juice can be seen in Figure 3.8.

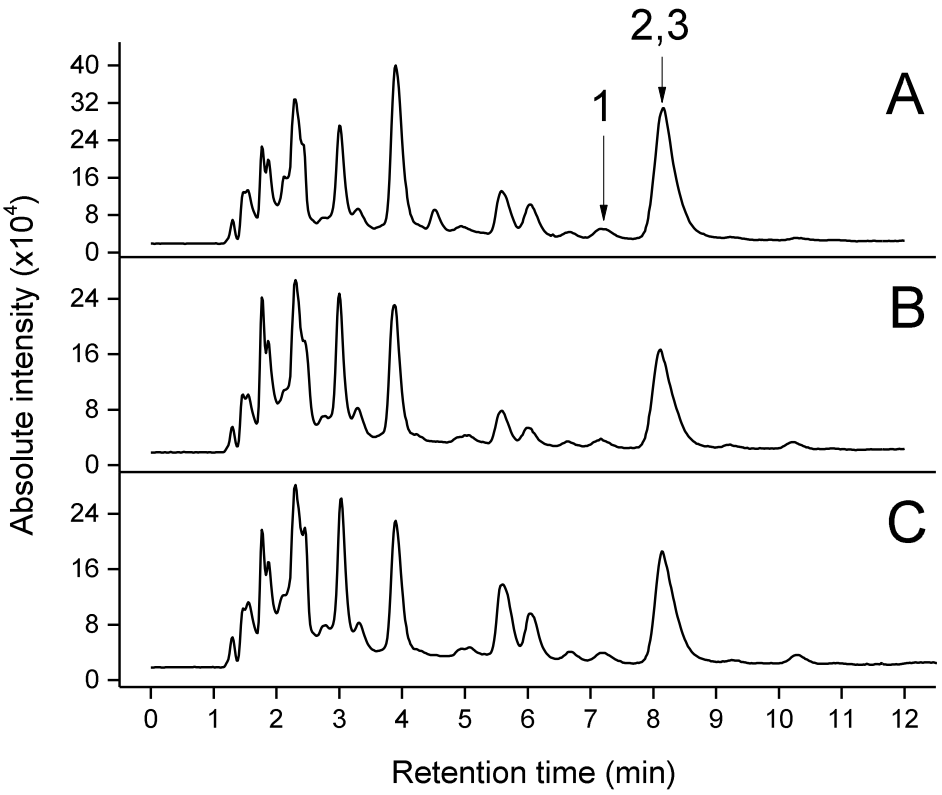


Figure 3.8 TIC chromatograms of urine samples from one participant after metabolite extraction for all three orange juices. A) Freshly squeezed, B) premium brand and C) value brand. Metabolites identified: 1- Hp7Glu, retention time (RT)=7 min; 2- Hp3'Glu, RT=7.9 min and 3- Hp3'SO_4, RT=8.1 min. Hp3'Glu and Hp3'SO_4 were confidently identified following their negative mass-to-charge ratio (*m/z* 477, 381) and comparing them to their authentic standards.

A total of 2 volunteers were excluded from any analytical measurement as they did not provide complete urine sets. The remaining data was revised and those samples which showed metabolites in baseline urine and low or no excretion were discarded from statistical analysis. Thus, data from 10 volunteers was statistically analysed to determine if the ingestion of different commercial orange juices had an impact on hesperidin metabolism. Firstly, concentrations of metabolites excreted were corrected by the volume of orange juice ingested. Then, results were analysed as percentage of dose excreted in urine (see Table 3.1).

Table 3.1 Urinary excretion data and hesperidin ingested (mean values and standard deviations).

	Freshly squeezed		Premium brand		Value brand	
N=10	Mean	± SD	Mean	± SD	Mean	± SD
Hesperidin ingested (µmol)	89.43	14.78	95.50	15.78	139.45	23.05
Urinary excretion (% dose)						
Total metabolites	13.00	14.11	10.26	13.84	5.55	6.45
Hp7Glu	1.57	2.01	1.36	1.86	0.56	0.41
Hp3'Glu	10.04	10.58	7.84	10.38	4.41	5.30
Hp3'SO_4	1.40	1.60	1.06	1.63	0.58	0.76

The data was tested for normal distribution. Data for Hp7Glu, Hp3'Glu and Hp3'SO_4 was not normally distributed, as determined by Shapiro-Wilk's test ($p < 0.05$) (see Figure 3.9, A-C). In contrast, hesperidin ingestion was normally distributed, determined by Shapiro-Wilk's test ($p > 0.05$) (see Figure 3.9, D).

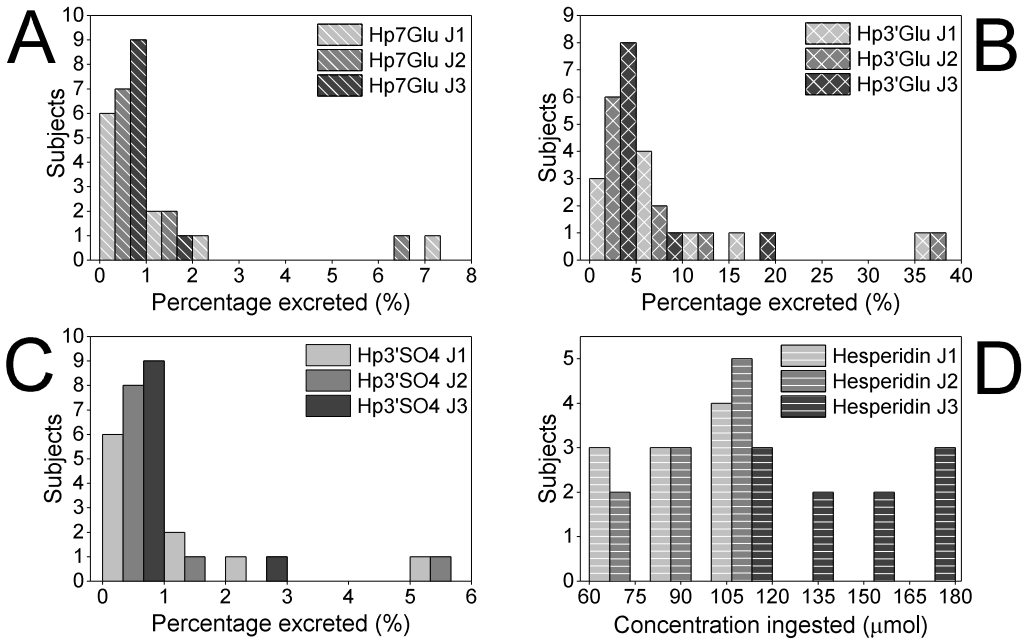


Figure 3.9 Histograms for data statistically analysed. Non-parametric data: A) Hp7Glu for three orange juices; B) Hp3'Glu for three orange juices; C) Hp3'SO_4 for three orange juices. Parametric data: D) hesperidin ingested for three orange juices. J1- Freshly squeezed, J2 – premium brand and J3 – value brand. N=10

A non-parametric analysis, the Friedman test, was used to determine if there was any significant difference in total metabolites excretion among the orange juices. The statistical results are showed in Table 3.2 and significant differences can be seen in Figure 3.10.

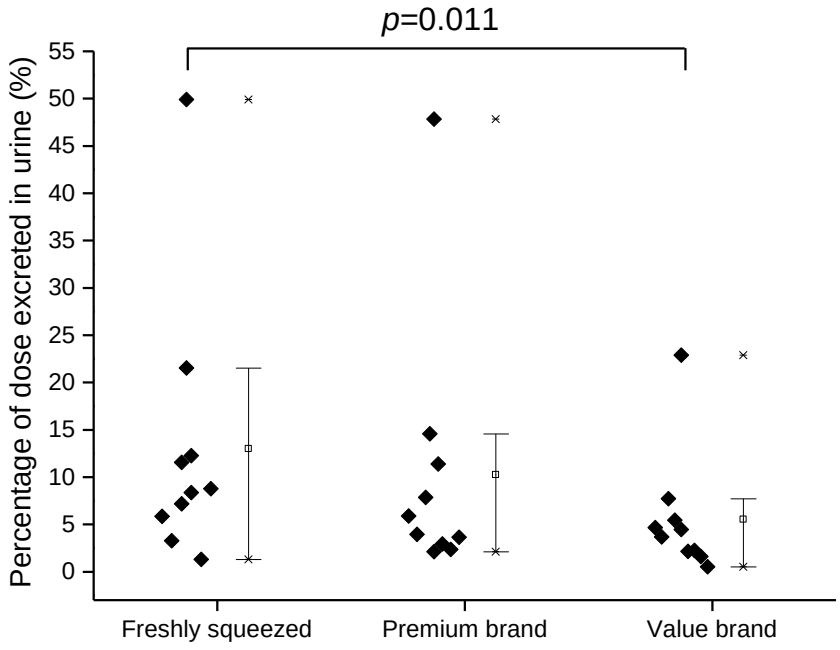


Figure 3.10 Total metabolites excretion of each subject in the three orange juices. N=10, significance level is 0.05.

A statistical significant difference was found between total metabolite excretion and the three orange juices (p=0.0014). The post hoc analysis showed only a significant difference between total metabolite excretion from value brand orange juice to total metabolite excretion from freshly squeezed orange juice (p=0.011) where total metabolite excretion increased from 5.55 ± 6.45 % with value brand orange juice to 13.00 ± 14.11 % with freshly squeezed orange juice.

In the same way, a Friedman test was used to determine if the significant difference between value brand and freshly squeezed orange juice was still valid in the samples without correction for Hp3'SO_4 excretion. The statistical significant difference was still found in the three orange juices (p=0.007) and the post hoc analysis corroborated that total metabolite excretion was significantly different (p=0.005) between value brand orange

juice and freshly squeezed orange juice, as the excretion increased from 6.30 ± 7.4% to 14.8 ± 16.2% respectively.

Table 3.2 Statistical results from the non-parametric test Friedman to analyse significant difference within metabolites excreted and the three orange juices ingested.

Parameter	Chi-squared distribution (X^2-distribution)	Degrees of freedom	*p* value
Hp7Glu excretion in three orange juices	7.2	2	0.027
Hp3'Glu excretion in three orange juices	9.8	2	0.007
Hp3'SO_4 in three orange juices	9.8	2	0.007
Total metabolite excretion in three orange juices	8.6	2	0.014

The analysis for each metabolite excretion in the three orange juices showed significant difference between value brand and freshly squeezed orange juice (see Figure 3.11), and the results can be seen in Table 3.2. Two outliers were identified and were not discarded from statistical analysis, as the concentration of metabolite excretion could have been affected by the nature of the study (crossover), the sample size (10 subjects) and the biological differences among the subjects (e.g. gut microflora) which should be taken into account.

It was found that percentage of Hp7Glu excretion was significantly different $X^2(2)=7.2$, $p=0.022$ comparing freshly squeezed and value brand orange juices where the percentage increased from 0.56 ± 0.41% with value brand to 1.57 ± 2.00 % with freshly squeezed orange juice. As well, the percentage of Hp3'Glu excretion was significantly different $X^2(2)=9.8$, $p=0.005$ between freshly squeezed and value brand orange where percentage increased from 4.41 ± 5.30% with value brand to 10.04 ± 10.56% with freshly squeezed orange juice. Finally, the percentage of Hp3'SO_4

showed statistically significant difference $X^2(2)=9.8$, $p=0.007$ between freshly squeezed and value brand orange juice where percentage increased from 0.58 ± 0.76% with value brand to 1.40 ± 1.60% with freshly squeezed orange juice. No other relationships were significantly different.

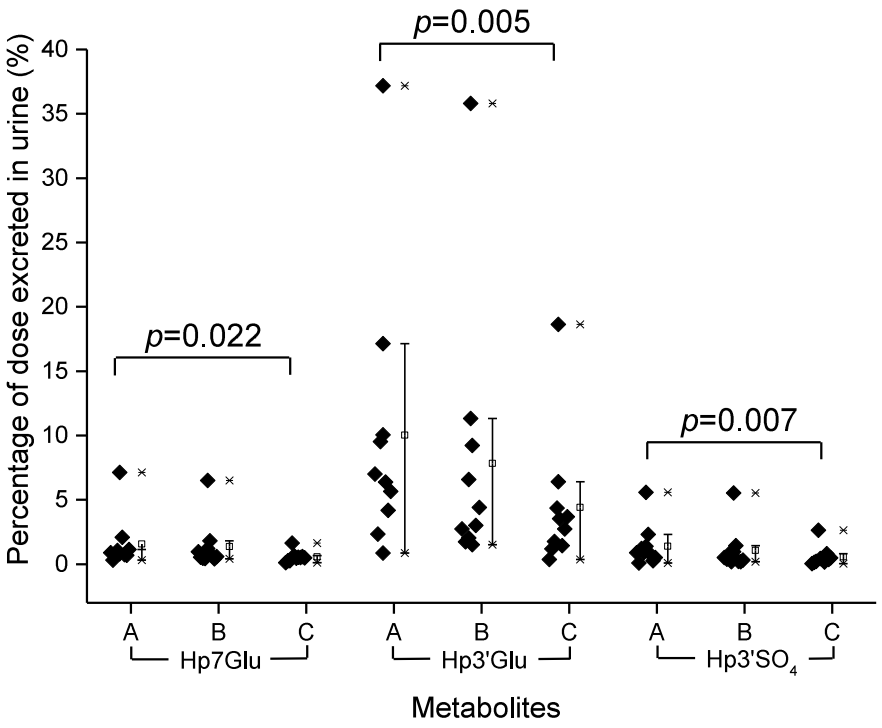


Figure 3.11 Percentage of dose excreted in urine by each subject in the three orange juices presented by urinary metabolite. A) Freshly squeezed, B) premium brand and C) value brand orange juice. N=10, significance level 0.05.

The data for hesperidin ingestion was normally distributed, as determined by Shapiro-Wilk's test ($p> 0.05$) and is graphically shown in Figure 3.9, D. No outliers were found by inspection of a boxplot (see Figure 3.12)

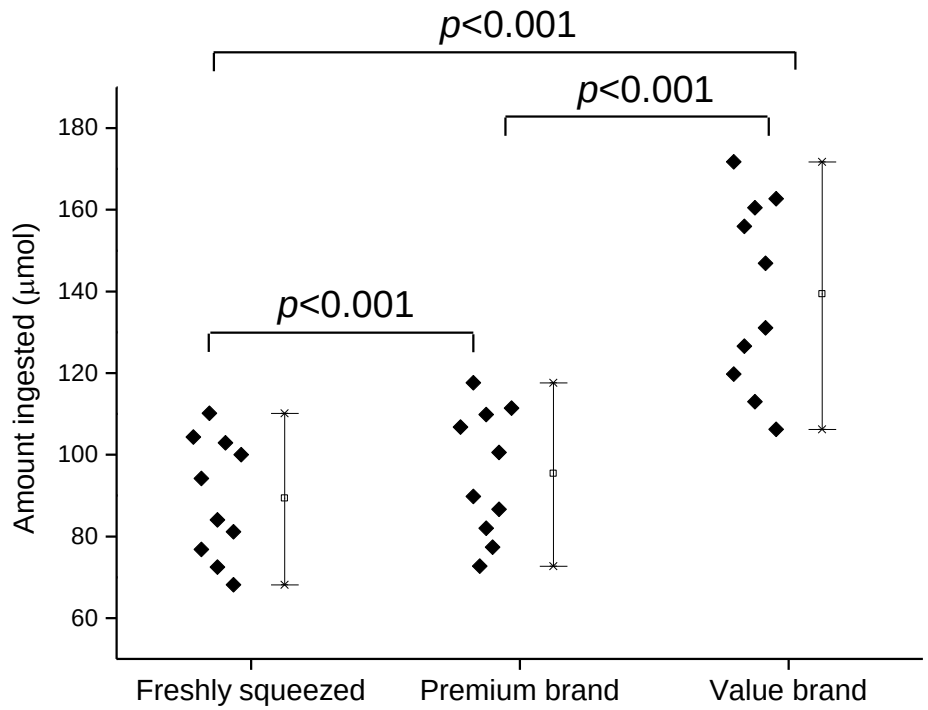


Figure 3.12 Hesperidin ingested by each subject in the three orange juices. N=10, significance level is 0.05.

As the data was normally distributed, a one-way repeated-measurement ANOVA was conducted to determine the statistically significant difference in hesperidin consumption in the three orange juices. Results from within samples showed a statistically significant change of hesperidin ingested depending of which orange juice was drunk, F (1,9)=366, p<0.001. The post hoc analysis with a Bonferroni adjustment indicated that the amount of hesperidin consumed increased from 89.4 ± 14.8 µmol with freshly squeezed to 95.5 ± 15.8 µmol with premium brand and to 139.5 ± 23.1 µmol value brand orange juice.

3.6 Discussion

Hesperetin urinary-metabolites have been reported in the literature using different methodologies including enzymatic hydrolysis, specific mass-to-charge ratios and NMR (Brett *et al.*, 2009; Bredsdorff *et al.*, 2010; Pereira-Caro *et al.*, 2014). Only a few publications have described the accurate identification and quantification of hesperetin urinary-metabolites using authentic standards. For example, Hp3'Glu and Hp7Glu have been

confidently identified by Brand *et al*. (2010) using true standards. They tested different glucuronosyltranferases and sulfotransferases from various sources (human and rat) as well as rat and human tissues to determine the kinetics for both biotransformations and explain the major hesperetin metabolites found *in vivo*. The identification of Hp3'SO_4 was done by NMR analysis. Its quantification was done indirectly using the calibration curve at 270 nm (UV range) for hesperetin and adjusting the results for the response factor 0.86. The sulfated form was collected, but not purified to obtain the authentic standard. Similarly, Pereira-Caro *et al*. (2014) identified Hp7Glu in urine samples after pulp-enriched orange juice consumption using a commercially-available standard (Extrasynthese, France). They also reported Hp3'Glu and Hp3'SO_4 metabolites, but true standards were not used for their identification which was based on HPLC-MS^2 fragmentation and previous information reported by Bredsdorff *et al*. (2010).

A common approach to avoid the use of authentic standards it is to assume that polyphenols (e.g. hesperetin) are glucuronidated and sulfated during phase II metabolism. Then, enzymatic hydrolysis using β-glucuronidase and sulfatase will liberate the aglycones, which are most likely to be commercially-available, and quantification can be done indirectly using the calibration curve of the aglycone. Nevertheless, Saha *et al*. (2012) observed a lack of hydrolysis for epicatechin sulfates and methylepicatechin sulfates in urine samples after consuming two small chocolate bars. They treated their urine samples using standard hydrolysis conditions reported by other publications. Various modifications were applied to their protocol: increasing the quantity of enzyme, different incubation periods, pH optimisation and different commercially-available sulfatases were tested. Finally, they found that epicatechin sulfates and methylepicatechin sulfates remained in high quantities in the urine samples, assessed by their chromatography. They suggested that other reports have underestimated epicatechin content in biological fluids, as these metabolites are not efficiently hydrolysed enzymatically. In a similar way, Vallejo *et al.* (2010) found that 10% of hesperetin sulfate was unhydrolysed in urine samples after being treated with β-glucuronidase and sulfatase. Indeed, Charoensuk (2014) (unpublished) also found that sulfatase from *Abalone entrails* type

VIII was inhibited by unknown compounds in urine and did not hydrolyse quercetin and epicatechin sulfated from urine samples collected after ingestion of onion soup and dark chocolate by healthy volunteers.

The need for authentic standards was one of the main objectives to this research project. We modified a published protocol (see 3.4.2) to sulfate polyphenols and we introduced two different components: the use of pig liver cytosol (see 3.4.1) as main source of sulfotransferases and a co-cofactor for the reaction (PNS). Firstly, adding pig liver cytosol instead of human recombinant SULTs decreases the cost for the *in vitro* assay and makes more accessible the protocol. Secondly, PNS helps to increase transformation to sulfated forms. PNS was earlier reported by Koizumi *et al.* (1990) as a sulfate donor in the sulfation of quercetin using arylsulfotransferases isolated from human faeces. A year later, Koizumi *et al.* (1991) tested tannins and alkyl gallates using the same protocol and they successfully sulfated these compounds, reporting up to 100% transformation for some of them. On the other hand, it was not until Tyapochkin *et al.* (2009) proposed a bypass mechanism where PAPS is regenerated by PNS after being used by the sulfotransferases (see Figure 3.13) that PNS was considered as a co-cofactor of the reaction and not only a sulfate donor. They tested their theory performing an activation assay using naphthol. They found that PNS intercepts PAP and converts it to PAPS immediately after it has been released, which later was used by naphthol to be converted to naphtyl sulfate. The result was explained as apparent ping-pong behaviour and supported their proposed mechanism. Our experiments showed that the addition of PNS into the mixture reaction increased the % transformation up to 40% changing from 38% without PNS to 80% with it (see 3.5.1). In addition, we showed that lower concentrations of PNS will work efficiently (see Figure 3.4) and will remain in less quantity in the mixture after the reaction has ended and a liquid-liquid extraction is performed.

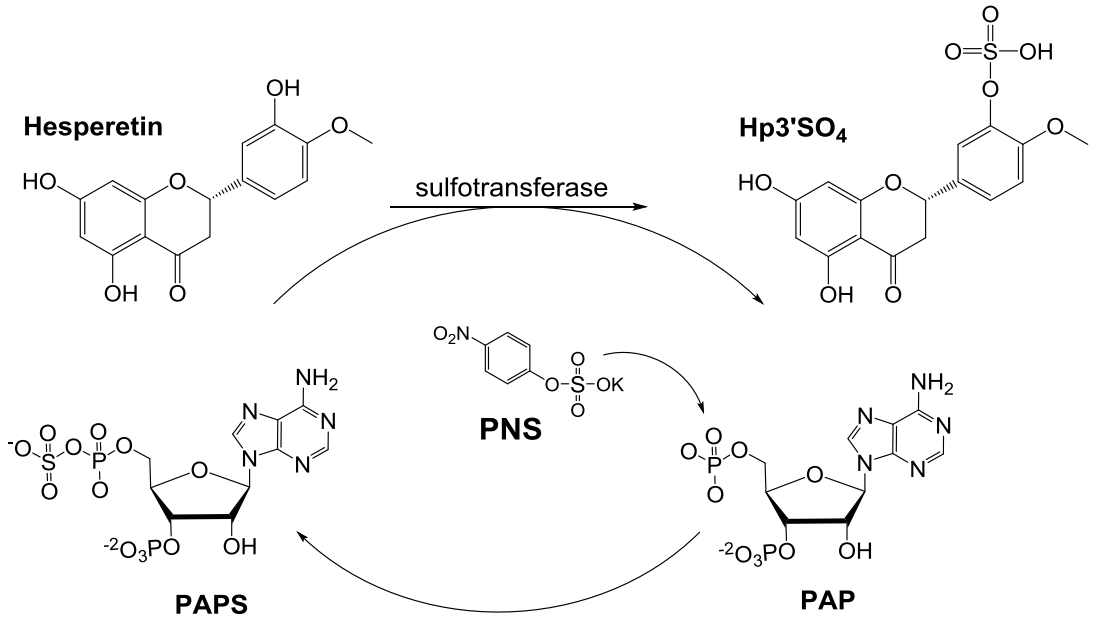


Figure 3.13 Bypass mechanism proposed by Tyapochkin *et al.* (2009). PAPS is regenerated by PNS after being used by sulfotransferase in a ping-pong behaviour.

The biological synthesis of hesperetin provided us with Hp3'SO_4, which has been reported to be one of the main urinary-hesperetin metabolites (Brett *et al.*, 2009) and allowed to quantify accurately Hp3'SO_4. Next, a cross-over human study was design with two major objectives: to quantify the main hesperetin urinary-metabolites and investigate the effect of type of orange juice consumed on the excretion of these metabolites. Urine samples were collected and analysed by Dr Tristan P. Dew and Miss Isabella Procopiou, and statistical analysis and interpretation was performed by this researcher.

First, the concentration of hesperidin (hesperetin glycoside) in two orange juices (freshly squeezed and premium brand, see Figure 3.6) was below what previous publications have reported (Manach *et al.*, 2003; Brett *et al.*, 2009). The variety of orange was not recorded, but the concentrations were similar to the amount found by Tomás-Barberán and Clifford (2000) for Navel orange juice (166-205 mg/L), and higher than the fresh and pasteurised fresh orange juice (47.2 ± 4.0 and 154.6 ± 47.2 mg/L respectively) by Silveira *et al.* (2014). The value-brand orange juice from concentrate showed similar hesperidin concentration (276 ± 21.6 mg/L) to those found by Erlund *et al.* (2001) and Brett *et al.* (2009), and it was higher

than those reported by Vallejo *et al.* (2010) for two commercial orange juices from concentrate (54-57 mg/L).

Most publications have reported the urinary excretion of hesperetin instead of its metabolites due to the lack of authentic standards. Our total metabolite excretion (Hp7Glu, Hp3'Glu and Hp3'SO_4) was compared to aglycone results from different publications, assuming that if enzymatic hydrolysis was performed, the conjugates will be fully 100% transformed to their aglycone form (hesperetin). The values for **freshly squeezed** (13 ± 14.1%) and **premium brand** (10.3 ± 13.8%) were higher from those reported by Manach *et al.* (2003) (4.1-6.4%), Nielsen *et al.* (2006) (low dose: 4.1%, high dose: 8.9%), Brett *et al.* (2009) (4.5%) and Silveira *et al.* (2014) (3.8-4.1%). On the other hand, the urinary excretion reported by Nielsen *et al.* (2006) for their treated orange juice (14.4%) was slightly higher than our results. They explained this as an effect of the enzymatic conversion of hesperidin to hesperetin-7-*O*-glucoside which increased the bioavailability of the compound. The concentration reported by Krogholm *et al.* (2010) (13.9%) was similar to our result, even though the origin of their 'juice mix' was not reported. The total metabolite excretion for the **value brand** juice (5.6 ± 6.5%) agreed with Erlund *et al.* (2001) (5.3%), Brett *et al.* (2009) (4.6%) and Vallejo *et al.* (2010) (orange juice A: 5.4%) values. The results reported by Pereira-Caro *et al.* (2014) (17.5%) cannot be confidently compared as the pulp-enriched orange juice was not identified as fresh or from concentrate orange juice. A summary of these results is shown in Table 3.3.

Table 3.3 Urinary excretion (%) of hesperetin after hesperidin ingestion (juice) and its comparison with previous reports.

Type of orange juice	Total metabolite excretion (%)	Reference
Freshly squeezed	13 ± 14.1	Our report
Premium brand	10.3 ± 13.8	Our report
100% pure juice	4.1 ± 1.2 - 6.4 ± 1.3	Manach *et al.* (2003)
Orange juice	4.1 ± 1.8	
Orange juice enriched	8.9 ± 3.8	Nielsen *et al.* (2006)
Orange juice treated	14.4 ± 6.8	

Orange fruit	4.5 ± 3.4	Brett *et al.* (2009)
Processed orange juice	4.1 ± 3.3	Silveira *et al.* (2014)
OJ freshly squeezed	3.8 ± 2.2	
Juice mix	13.9 ± 8.7	Krogholm *et al.* (2010)
Value brand	5.6 ± 6.5	Our report
OJ from concentrate	5.3 ± 3.1	Erlund *et al.* (2001)
OJ from concentrate	4.6 ± 3	Brett *et al.* (2009)
OJ from concentrate (A)	5.4 ± 1.2	Vallejo *et al.* (2010)
OJ from concentrate (B)	1.7 ± 0.4	
Pulp-enriched OJ	17.5	Pereira-Caro *et al.* (2014)

OJ-Orange juice.

Further, we found a significant difference in the total metabolite excretion and the type of orange juice consumed (see Figure 3.10) and our results did not agree the previous report by Brett *et al.* (2009) who did not find any significant difference in the bioavailability of flavanones between fresh fruit and orange juice from concentrate. Their concentrations were calculated using enzymatic hydrolysis which can underestimate the real concentration of total urinary metabolites. Indeed, they suggested the appearance of metabolites Hp7Glu, Hp3'Glu and Hp3'SO_4 relating their mass-to-charge ratio (LC-ESI/MS), UV spectra to each substitution site and confirming them by LC-MS^2. No quantification was performed.

Following the report by Nielsen *et al.* (2006), their urine samples were further analysed by Bredsdorff *et al.* (2010). The metabolites were first identified on the basis of their MS spectra; then, their sites of conjugation were determined by NMR and isolated fractions were compared to authentic standards or diluted urine samples. They found < 1% urinary excretion for Hp7Glu for natural and fortified juice, and their results were below our report (1.6 and 1.4%, freshly squeezed and premium brand respectively). The excretion for Hp7Glu from the treated juice was approximately 1.5% and matched the value found for our freshly squeezed value. Hp3'Glu was excreted < 1.5% for natural juice,> 3.5% for the fortified juice and above 5% with their treated juice. Our results for freshly squeezed and premium brand

were above their findings (see Table 3.1) and only the value brand juice showed a concentration below 5%. Hp3'SO_4 was < 1% for the natural and fortified juice, but above 1% for the treated juice. Our values for freshly squeezed and premium brand were < 1% and the excretion from value brand juice was around half the value (0.6 ± 0.8%). Our results are compared to their values graphically presented, as their precise concentrations were not reported in the text. These results and reported concentrations are shown in Table 3.4.

A recent publication for flavanone glycosides with a single dose of orange juice (fresh and pasteurised fresh orange juice) by Silveira *et al.* (2014) presented the quantification of Hp7Glu, Hp3'Glu and Hp3'SO_4, but their results (Table 5 in published article) are presented without any specification of units and their interpretation is based in the differences (x-fold) for each compound between both orange juices. Therefore, their concentrations and methodology are not discussed further. Finally, the report of orange juice polyphenols by Pereira-Caro *et al.* (2014) presented a quantification for hesperetin and naringenin metabolites. They used a pulp-enriched orange juice, but did not identify the juice as fresh or from concentrate. Their metabolite identification was done following the specific mass-to-charge ratio for each metabolite in negative mode in a MS^2 methodology. Once the metabolites were identified, Hp7Glu was confirmed by the chromatography of its authentic standard, but Hp3'Glu and Hp3'SO_4 were only related to previous information published by Bredsdorff *et al.* (2010). Their reported value for Hp7Glu (1.4%, 4.7 ± 1.6µmol) was similar to our finding for the premium brand juice (1.4 ± 1.9%). The Hp7Glu excretion for freshly squeezed was above their value and the value brand juice was below it (see Table 3.1). They reported 5.5% (19.0 ± 6.5µmol) Hp3'Glu excretion which was again above the value for our value brand juice (4.4 ± 5.3%), but below the data found for freshly squeezed and premium brand. In contrast, their concentration for Hp3'SO_4 (5.2%, 18.2 ± 7.3µmol) was up to 5x more than our report for all orange juices (see Table 3.4). This is due to the correction factor that we have applied to our measurements. We found that the signal in LC-ESI/MS of Hp3'SO_4 was in average 2.3x more than hesperetin aglycone (see 3.5.2.4).

Table 3.4 A comparison of the urinary excretion of hesperetin metabolites (% dose) for different type of orange juices and their values reported in the literature.

Metabolite	Type of orange juice	Urinary concentration (% dose)	Reference
Hesperetin-7-*O*-glucuronide	Freshly squeezed	1.57	Our study
	Premium brand	1.36	
	Value brand	0.56	
	Natural juice	< 1	Bredsdorff *et al.* (2010)
	Treated juice	approx. 1.5	
	Fortified juice	< 1	
	Pulp-enriched juice	1.35	Pereira-Caro *et al.* (2014)
Hesperetin-3'-*O*-glucuronide	Freshly squeezed	10.04	Our study
	Premium brand	7.84	
	Value brand	4.41	
	Natural juice	< 1.5	Bredsdorff *et al.* (2010)
	Treated juice	above 5	
	Fortified juice	> 3.5	
	Pulp-enriched juice	5.46	Pereira-Caro *et al.* (2014)
Hesperetin-3'-*O*-sulfate	Freshly squeezed	1.40	Our study
	Premium brand	1.63	
	Value brand	0.58	
	Natural juice	< 1	Bredsdorff *et al.* (2010)
	Treated juice	above 1	
	Fortified juice	< 1	
	Pulp-enriched juice	5.23	Pereira-Caro *et al.* (2014)

This phenomenon was also reported by Farrell *et al.* (2011) who found higher signal measuring hydroxycinnamic acids glucuronide and sulfate by LC-ESI/MS^2. They found a response of 3 to 10x higher from

metabolites compared to their aglycones. Also, Brand *et al.* (2010) used a correction factor of 0.86 to quantify Hp3'SO_4 produced after an *in vitro* assay using human and rat sulfotransferases. Their quantification was done using the calibration curve of hesperetin which was measured by HPLC-DAD. Therefore, as Pereira-Caro *et al.* (2014) are not using authentic standards for the quantification of Hp3'SO_4 and their measurements do not take into account the MS response of the sulfated form, this value might not reflect the real concentration of Hp3'SO_4 present in the urine samples.

In conclusion, to our knowledge, this is the first report showing the synergy between PAPS and PNS for the biological sulfation of polyphenols, obtaining a metabolite found in *in vivo* conditions. This methodology can lead to availability of Hp3'SO_4 as authentic standard which we have shown with the quantification of Hp7Glu, Hp3'Glu and Hp3'SO_4. It is important to address that enzymatic hydrolysis can provide general information about the metabolism of hesperetin or other polyphenols, but it will not help to identify and quantify accurately the metabolites produced *in vivo*.

CHAPTER 4 Selective inhibition of COX-2 by plant extracts and their unconjugated and conjugated polyphenols

4.1 Abstract

Cyclooxygenase 2 (COX-2) is an enzyme responsible for the conversion of arachidonic acid to prostanoids (e.g. PGH_2, PGE_2) which have been related to inflammatory diseases, and its inhibition has become the main goal for research. Plant extracts and their main polyphenols (unconjugated and conjugated) have become an alternative to drug therapies, but their behaviour is still not well understood. This project aimed to find the inhibition of recombinant human COX-2 by different plant extracts and a series of unconjugated and conjugated polyphenols. Two procedures were used to measure the % inhibition by the different analytes. Firstly, a COX inhibitor screening assay was used to quantify, via enzyme immunoassay (EIA), $PGF_{2\alpha}$ which is a reduced form of PGH_2. Green tea (0.1 mg/mL) and German chamomile (0.5 mg/mL) showed more than 75% inhibition of recombinant human COX-2, and following tests with unconjugated and conjugated polyphenols provided no consistent results. Next, a mass spectrometry method was used to quantify directly PGE_2. Unconjugated and conjugated polyphenols showed inhibition, but their results were not reproducible. Green tea (GT) was the only plant extract that showed coherent results and presented concentration dependence. It was found that GT IC_{50} for COX-2 was 16.1 µg/mL. Lastly, the two main GT polyphenols epigallocatechin gallate (EGCG) and epigallocatechin (EGC) were tested and both showed concentration dependence. Subsequently, their IC_{50} were calculated at 2.8 and 9.2 µM respectively. So far, no publications have shown the inhibition of recombinant human COX-2 by green tea, as other research has focused on effects on enzyme expression or other pro-inflammatory cytokines.

4.2 Introduction

Chronic inflammation appears to be associated with the malfunction of tissue, producing imbalance in the physiological systems that are not directly functionally related to host defence or tissue repair (Medzhitov, 2008). During chronic inflammation, pro-inflammatory enzymes are secreted in large amounts to eliminate foreign pathogens, but they also attack normal tissues which lead to cell damage (Pan *et al.*, 2009).

Pro-inflammatory enzymes, such as cyclooxygenase enzymes, convert polyunsaturated fatty acids (PUFA) into lipid mediators (eicosanoids). There are two main cyclooxygenase enzymes: COX-1 is normally present in most tissues and it is involved in essential maintenance necessary for normal physiological activity whereas COX-2 is the inducible form which is produced in response to inflammation and it is usually undetectable in healthy tissues (Stables and Gilroy, 2011; Cao *et al.*, 2011).

COX-2 has become an important target to battle against inflammatory conditions as atherosclerosis, arthritis and various types of cancer (Raman *et al.*, 2008). Selective inhibitors of COX-2, e.g. celecoxib, rofecoxib and others have been developed and historically used for the treatment of chronic inflammatory conditions. However, these drugs have been recently associated with an increase in blood pressure, myocardial infarction, or stroke (Raman *et al.*, 2008; Salvado *et al.*, 2012). Thus, researchers have started to seek for alternatives which can effectively inhibit COX-2 and provide less or no side effects.

Plant extracts and natural compounds isolated from them are the main focus for potential bioactive molecules. For instance, catechins and flavonoids from **green tea** have been related to decrease COX-2 expression and NF-κB activation (Peng *et al.*, 2006; Aggarwal and Shishodia, 2006). NF-κB is a nuclear factor that activated induces cellular transformation, proliferation, cellular differentiation, chemo-resistance, and inflammation (Golan-Goldhirsh and Gopas, 2014). Apigenin and luteolin from **German chamomile** also inhibit NF-κB activation pathway and German chamomile extract reduced COX-2 mRNA in RAW 264.7 cells (Aggarwal and Shishodia, 2006; Srivastava *et al.*, 2009). Ginsenoside Rd from **ginseng** inhibited COX-

2 expression in RAW 264.7 cells and supressed PGE_2 production in stimulated RAW 264.7 cells (Kim *et al.*, 2013). ***Gingko biloba*** showed anti-inflammatory activity in ANA-1 cells inhibiting inflammatory markers as iNOS, TNF-α and COX-2 (Kotakadi *et al.*, 2008). Similarly, ***Cucurbita*** *ficifolia* has been tested in induced type-2-diabetes mice and the treatment with an aqueous fraction proved to decrease the pro-inflammatory cytokine TNF-α, but increased other cytokines such as IL-6 and IFN-γ (Roman-Ramos *et al.*, 2012). Despite the scientific research on plant extracts, experimental information is still scarce and more analyses are needed.

Diverse techniques to test the anti-inflammatory activity of plant extracts are used. The most common are animal and cell culture models which can be employed along with spectrophotometry or spectrometry methodologies. Recently, mass spectrometry has become a powerful alternative to identify and quantify compounds. One of its main advantages is the selectivity to target analytes, e.g. PGE_2 which contribute to faster analysis and validated results (Cao *et al.*, 2011; Zhu *et al.*, 2014). This research project examines the inhibitory activity of various plant extracts and their main polyphenols (unconjugated and conjugated) using two different techniques: spectrophotometric analysis by immunoassay test and mass spectrometry (LC-ESI/MS) by *in vitro* assay. According to our recent search, there is no report in the literature that compares these plant extracts and polyphenols for their recombinant human COX-2 inhibition.

4.3 Materials

Hesperetin, myricetin, galangin and naringenin were purchased from Extrasynthese (Genay Cedex, France); quercetin, baicalein, celecoxib, kaempferol-3-*O*-β-glucuronide (K3glu), 20(S)-protopanaxatriol (PPX), epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC) were purchased from Sigma-Aldrich (Dorset, UK). Hesperetin-3'-*O*-glucuronide (Hp3'glu) and hesperetin-7-*O*-glucuronide (Hp7glu) were kindly provided by Professor Denis Barron (Nestle, Switzerland). Apigenin, luteolin, umbelliferone, bilobalide, ginkgolides (A, B, C and J) and ginsenosides (C-K, Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, Rg2, Rg3, Rh1 and Rh2), as German chamomile (GC) (*Matricaria recutita*) and

green tea (GT) (*Camellia sinensis*) were bought or provided by PhytoLab as part of PlantLIBRA project. Gingko powder (*Gingko biloba*) was bought from Indigo Herbs (Glastonbury, UK). Ginseng Korean root powder (ginseng) (*Panax ginseng*) was bought from G Baldwin & Co. (London, UK). Quercetin-3'-*O*-sulfate (Q3'SO_4) was prepared enzymatically and provided by Dr. C.C. Wong. Hesperetin-3'-*O*-sulfate (Hp3'SO_4) was kindly provided by Dr. C. Monrad (INRA, France). Chilacayote extract (cucurbita) (*Cucurbita ficifolia*) was provided by Dr. F.J. Alarcon-Aguilar (UAM-I, Mexico). *Trans*-resveratrol (Resveratrol), COX inhibitor screening assay kit and human recombinant COX-2 were purchased from Cayman Chemical (Cambridge, UK). All water refers to deionised Millipore water (Hertfordshire, UK) unless otherwise stated.

4.4 Methods

4.4.1 COX inhibition assay testing different polyphenols and plant extracts

The effect of quercetin, hesperetin, cucurbita, GC, GT and other compounds on production of $PGF_{2\alpha}$ was tested using a COX inhibitor screening assay kit which measures directly $PGF_{2\alpha}$ via enzyme immunoassay (EIA). The pathway from arachidonic acid to $PGF_{2\alpha}$ can be seen in Figure 4.1. The assay is based on the competitive reaction between prostaglandins and an acetylcholinesterase conjugate (PG tracer) to bind a limited amount of prostaglandin antiserum. The amount of PG tracer is constant, so the concentration of $PGF_{2\alpha}$ produced will be inversely proportional to the absorbance measured. The quantification is achieved by measuring the activity of the acetylcholinesterase using acetylthiocholine and Ellman’s reagent. The product absorbs strongly at 412nm, but it can be detected from 405 to 420 nm. The schematic reaction can be seen in Figure 4.2.

Each test followed the manufacturer’s protocol and used reagents provided by it. Concisely, reaction buffer (0.1 M Tris-HCl, pH 8.0, containing 5 mM EDTA and 2 mM phenol), heme (Tris-HCl, pH 8.0), enzyme (COX-2) and inhibitor were mixed in an Eppendorf tube and incubated for 10 min at 37°C. Then, the reaction was initiated adding arachidonic acid, followed by 2

min incubation at the same temperature. HCl was added to stop the reaction. Samples with or without stannous chloride were performed.

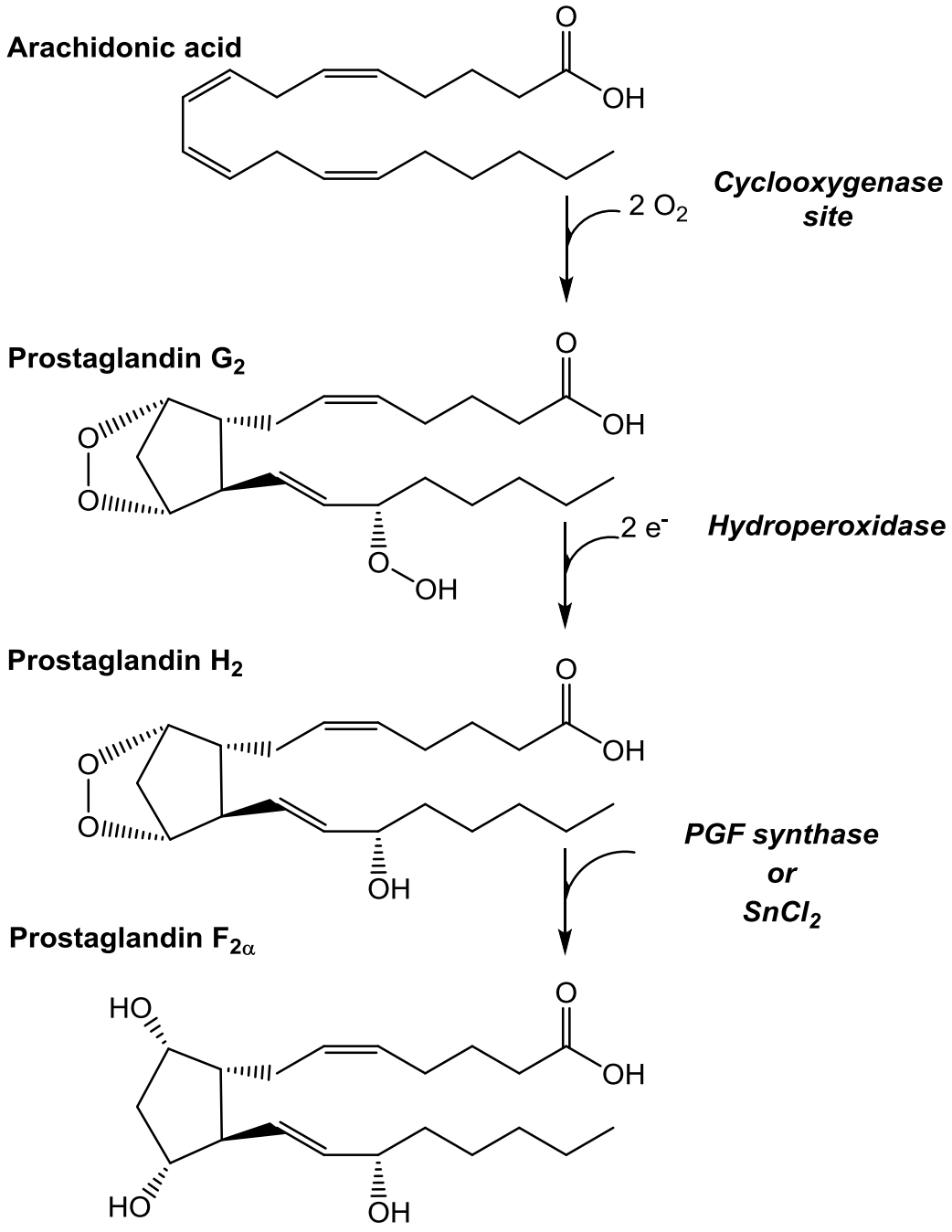


Figure 4.1 Schematic pathway of prostaglandin biosynthesis from arachidonic acid via COX-2 to $PGF_{2\alpha}$.

Afterwards, a 96-well plate was set and the binding reaction was developed up to 18 hrs in the dark, with orbital shaking (100 rpm) and at room temperature (25°C). Resveratrol (30 µM) and celecoxib (1 µM) were used as positive controls. After 18 hrs, the plate was developed using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB) and incubated

in the dark during 60 to 90 minutes with constant orbital shaking (100 rpm). All samples were read in a PHERAstar *FS* microplate reader set at 410 nm. Results were analysed using a 4-parameter logistic fit, calculated through myassays.com and interpreted using Microsoft Excel (2010).

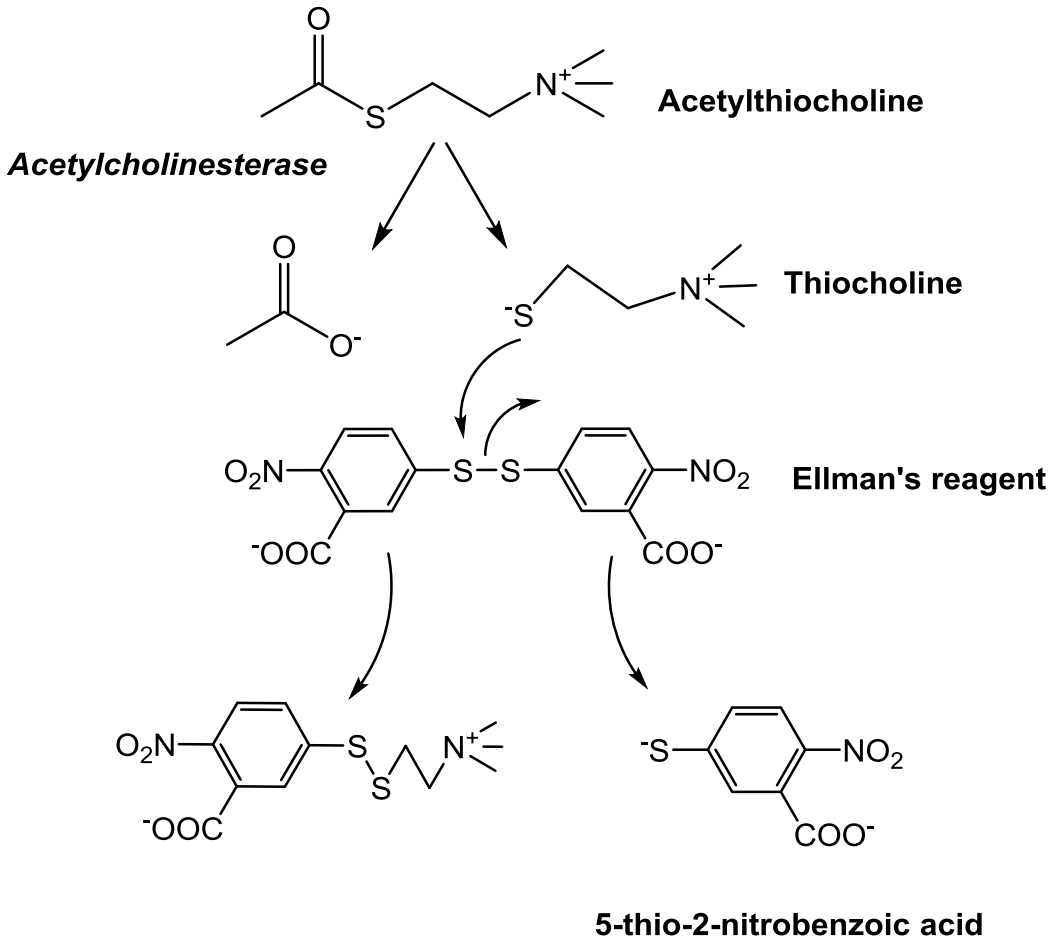


Figure 4.2 Schematic reaction between acetylthiocholine and Ellman's reagent to quantify the activity of acetylcholinesterase present after EIA. The product (5-thio-2-nitrobenzoic acid) is detected from 405 to 420 nm. Modified from COX inhibitor screening assay kit booklet (Cayman Chemical).

4.4.2 Cyclooxygenase inhibition measuring PGE_2 by LC-ESI/MS using solvent extraction.

Different plant extracts, conjugated and unconjugated polyphenols were tested for inhibition of COX-2 human recombinant, using a modified method by Cao *et al*. (2011). Reactions were performed in Eppendorf tubes using 146 µL of 100 mM Tris-HCl (pH 8.0) buffer, 2 µL of 100 µM hematin and 10 µL of 40 mM $_L$-epinephrine which were mixed at room temperature.

Then, 20 µL of recombinant human COX-2 (0.2-0.8 µg) diluted in Tris-HCl (pH 8.0) was added, and the mix was incubated at 25°C for 2-4 min. A 2 µL aliquot of inhibitor was added to the solution and pre-incubated for 10 min at 37°C in a water bath. Controls were carried out at the same time, substituting enzyme or inhibitor or both by Tris-HCl (pH 8.0) buffer.

The reaction was initiated by adding 20 µL of arachidonic acid in Tris-HCl (pH 8.0) to obtain a final concentration of 5 µM. After 2 or 4 min, the reaction was finished by adding 20 µL of 2.0 M HCl. Shortly, the surrogate standard d_4-PGE_2 was added (10 µL, 2.8 µM) to correct for any errors during liquid-liquid extraction. The solutions were incubated at 25°C for 30 min with constant orbital shaking (130 rpm). Next, PGE_2 and surrogate were extracted from each incubated mixture using 800 µL hexane/ethyl acetate (50:50,v/v). Samples were manually shaked for 1 min and vortex sideways for 30 sec to assure that both layers were mixed. Then, after 5 min ultracentrifuge (17,000 g, room temperature), the organic layer was removed and evaporated under vacuum (Genevac, EZ-2 plus model; Fisher Scientific Ltd., Leicestershire, UK) using a low BP mixture program without heat (lamp off). Samples were reconstituted with 100 µL methanol/water (50:50,v/v) and analysed using a Shimadzu LC-2010 HT coupled with a LCMS-2020 quadrupole mass spectrometer fitted with an electro spray ionisation source (Milton Keys, UK). A Waters XTerra MS C18 (2.1x50 mm, 3.5 µm) analytical column was used to perform the separation and was maintained at 35°C. The injection volume was 10 µL and the flow rate was set at 0.2 mL/min. The solvent A consisted of a mix of acetonitrile:water (35:65, v/v) with 0.1% formic acid and samples were run under an isocratic method. PGE_2 and its surrogate (d_4-PGE_2) were identified following their mass-to-charge ratio in negative mode (*m/z* 351,355 respectively). Those samples that were not immediately analysed were kept at -20°C until further analysis.

Statistical analyses were performed using IBM SPSS statistics 22 (Portsmouth, UK). Data was assessed to determine normality by Shapiro-Wilk's and a within-within-subjects ANOVA was conducted for data normally distributed. Significance level was determined as $p<0.05$.

4.5 Results

4.5.1 Measurement of prostaglandins by spectrophotometer using a screening assay.

Firstly, hesperetin and quercetin were measured (see Figure 4.3) at different concentrations, adding or not the reducing agent stannous chloride ($SnCl_2$). This test provided information about the capacity of the natural reductase to transform PGH_2 into $PGF_{2\alpha}$ and the efficacy of the positive controls. Hesperetin (5 µM) showed 24% inhibition and resveratrol and celecoxib (positive controls) showed 80% and 87% respectively. These values were calculated assuming 100% activity for no inhibitor samples. An independent t-test was used to determine the statistical difference between the control (no inhibitor) and samples with $SnCl_2$. It was found that hesperetin 5 µM was statistically different from the inhibitor (p=0.0292) as well as resveratrol (p=0.016) and celecoxib (p=0.0019).

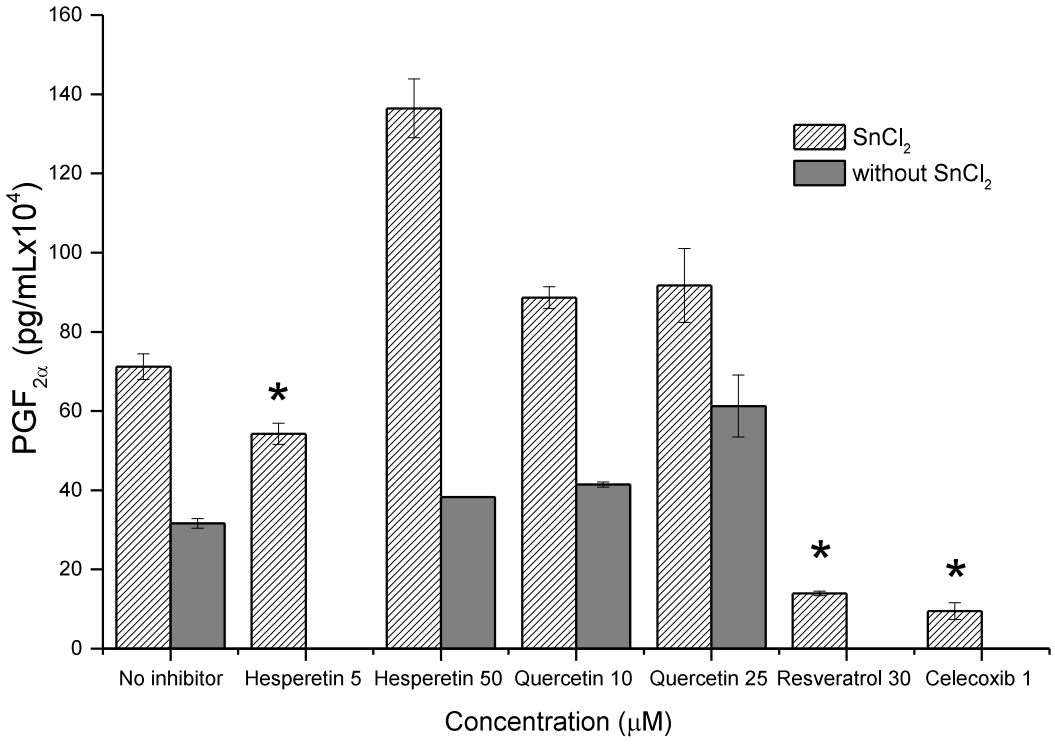


Figure 4.3 Measurement of $PGF_{2\alpha}$ to calculate COX-2 inhibition by unconjugated polyphenols. Resveratrol and celecoxib were used as positive controls. N=2 biological replicates, 1 technical measurement, ± SD. Asterix (*) represents statistical difference.

Then, various plant extracts were tested (see Figure 4.4) at 0.1 and 1 mg/mL adding stannous chloride. The final concentration of GC was

corrected for its 50% maltodextrin content. From these, GT (0.1 mg/mL) and GC (0.5 mg/mL) provided more than 75% inhibition. Indeed, an independent t-test showed that GT (0.1 mg/mL) and GC (0.5 mg/mL) were statistically different (p= 0.0056 and p=0.0074 respectively) from the control (no inhibitior), as well as resveratrol and celecoxib (see values above).

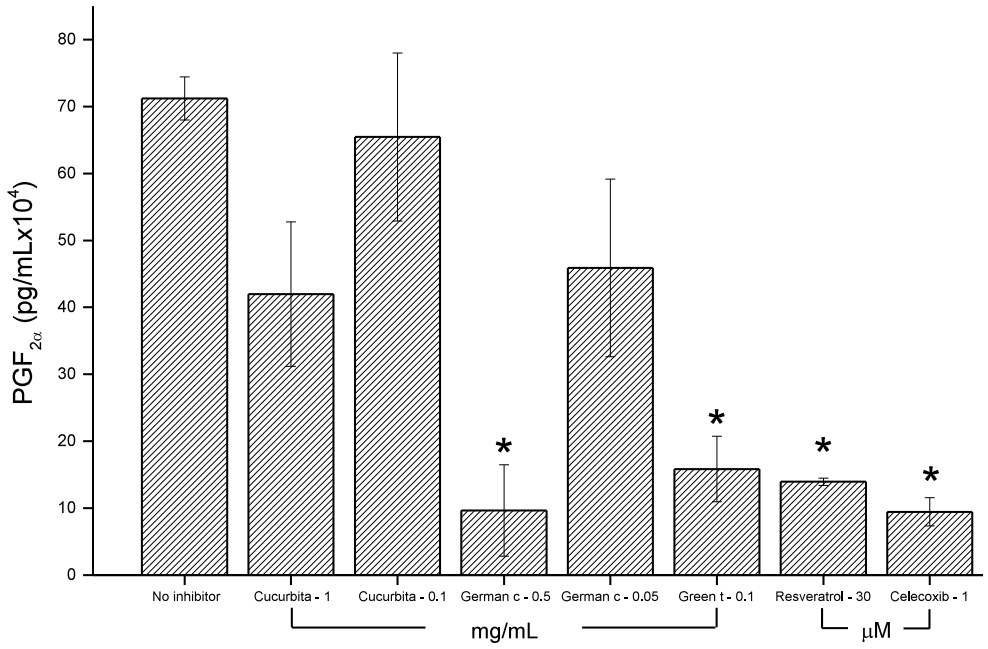


Figure 4.4 Measurement of $PGF_{2\alpha}$ to calculate COX-2 inhibition by different plant extracts. Resveratrol (30 µM) and celecoxib (1 µM) were used as positive controls. N=2 biological replicates, 1 technical measurement, ± SD. Asterix (*) represents statistical difference.

After the preliminary tests were done, several unconjugated and conjugated polyphenols were analysed (see Figure 4.5 and Table 4.1) to find those compounds which inhibit COX-2 activity. These compounds were chosen for their presence in the plant extracts and suggested health benefit reported by literature. All were tested at 5 µM concentration.

As a result, quercetin, apigenin, gingkolide J, ginsenoside Rg1, Hp3'SO_4, K3Glu, ginsenoside Re, Rf, Rd, kaempferol, ginsenoside Rg3 and Rh1 showed more than 25% inhibition. Then, these compounds were tested in further analysis. Statistical analysis (t-test) did not show any statistically significant difference between control (no inhibitor) and samples.

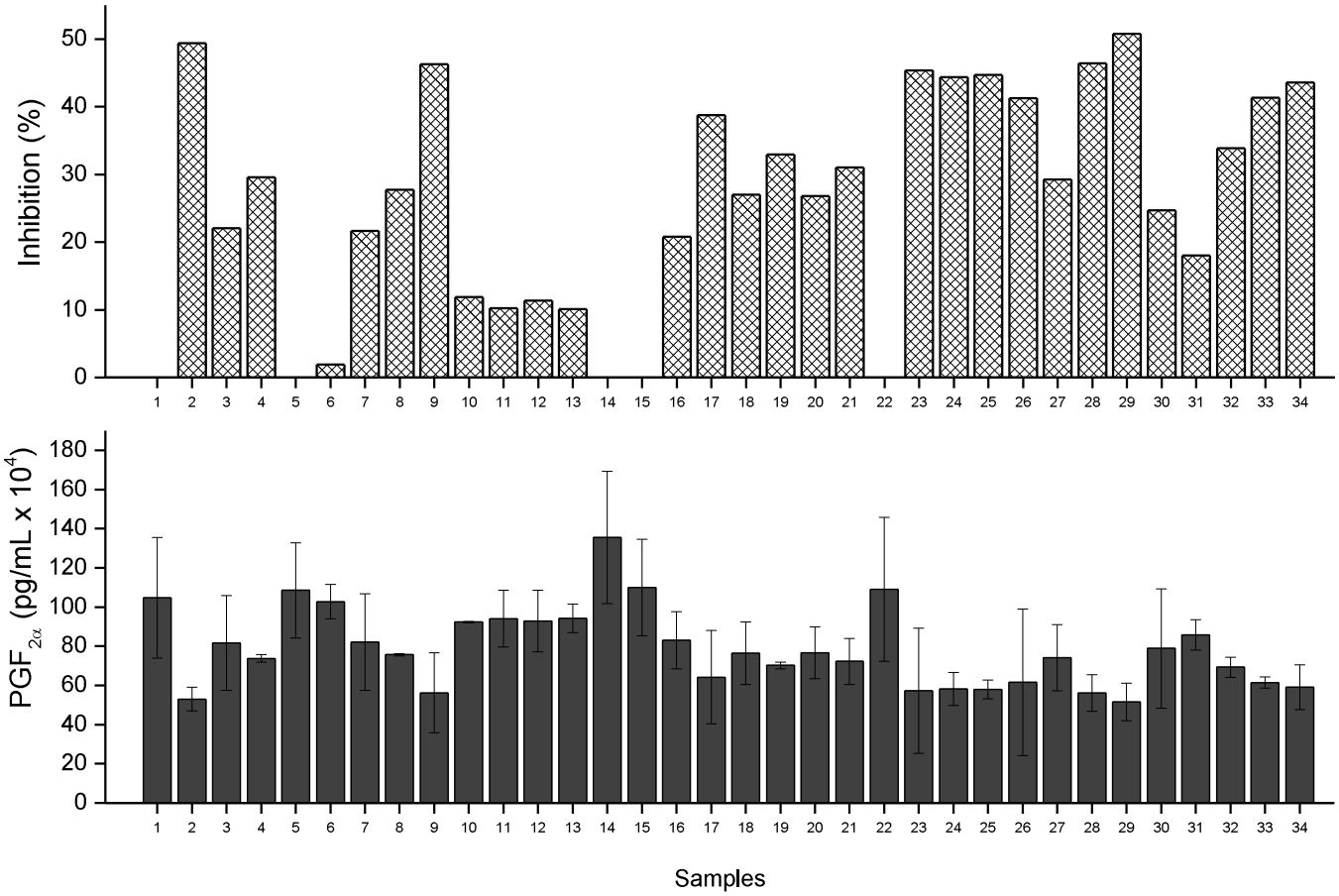


Figure 4.5 Measurement of $PGF_{2\alpha}$ to calculate COX-2 inhibition by various unconjugated and conjugated polyphenols. Compounds were tested at 5 μM except positive controls. Samples: 1- No inhibitor, 2-celecoxib (1μM), 3-resveratrol (30 μM), 4-apigenin, 5-luteolin, 6-baicalein, 7-myricetin, 8-quecetin, 9-kaempferol, 10-galangin, 11-hesperetin, 12-naringenin, 13-umbelliferone; gingkolides: 14-A, 15-B, 16-C and 17-J; ginsenosides: 18-C-K, 19-Rb1, 20-Rb2, 21-Rb3, 22-Rc, 23-Rd, 24-Re, 25-Rf, 26-Rg1, 27-Rg2, 28-Rg3, 29-Rh1 and 30-Rh2; 31-bilobalide, 32-Q3'SO_4, 33-Hp3'SO_4 and 34-K3glu. N=2 biological replicates, 1 technical measurement, ± SD.

Table 4.1 Measurement of $PGF_{2\alpha}$ and calculated COX-2 inhibition of various unconjugated and conjugated polyphenols. Compounds were tested at 5 µM. N=2 biological replicates, 1 technical measurement.

Compound	$PGF_{2\alpha}$ (pg/mL)	± SD	Inhibition (%)
Apigenin	737486	19499	30
Luteolin	1085403	243177	0
Baicalein	1027230	88122	2
Myricetin	820266	246552	22
Quercetin	756932	6011	28
Kaempferol	562220	203820	46
Galangin	922991	3698	12
Hesperetin	940270	145168	10
Naringenin	928185	156667	11
Umbelliferone	941181	72889	10
Gingkolide A	1355383	337331	0
Gingkolide B	1099976	246596	0
Gingkolide C	829517	145819	21
Gingkolide J	641232	238343	39
Bilobalide	788854	304371	25
Ginsenoside C-K	764144	158650	27
Ginsenoside Rb1	701859	17592	33
Ginsenoside Rb2	766448	132270	27
Ginsenoside Rb3	721991	117701	31
Ginsenoside Rc	1089860	367803	0
Ginsenoside Rd	572448	318552	45
Ginsenoside Re	582684	84359	44
Ginsenoside Rf	578948	48434	45
Ginsenoside Rg1	615319	373800	41
Ginsenoside Rg2	740595	168831	29
Ginsenoside Rg3	561421	92753	46
Ginsenoside Rh1	515738	95499	51
Q3'SO_4	858580	77600	18
Hp3'SO_4	692369	51985	34
K3Glu	614102	28188	41

Firstly, hesperetin and three conjugates (Hp3'glu, Hp7glu and Hp3'SO_4) were used to explore concentration dependence for COX-2 inhibition. Compounds were tested from 1 to 5 µM, using celecoxib (1 µM) as positive control (see Figure 4.6).

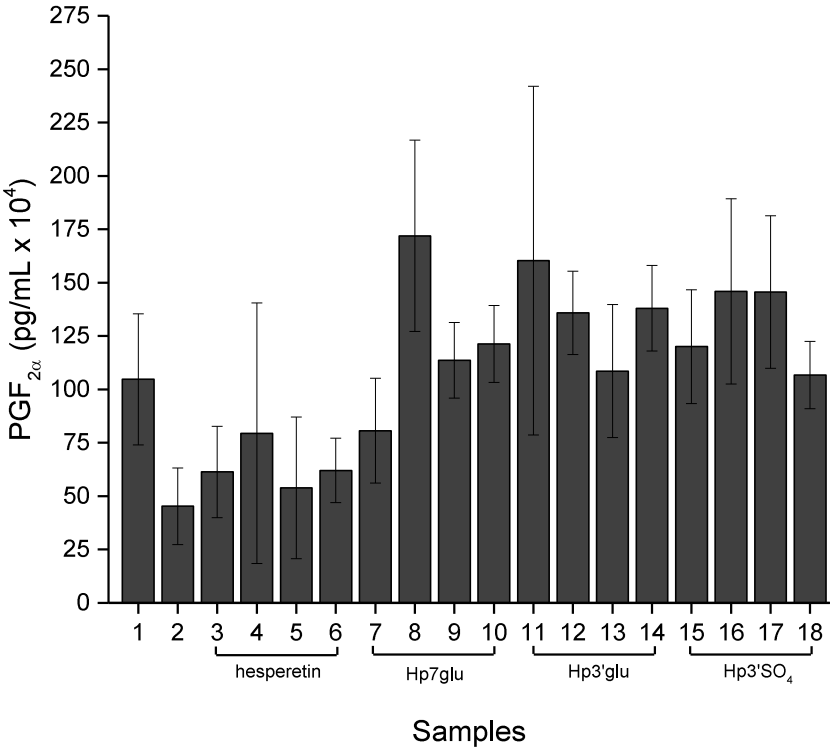


Figure 4.6 Measurement of $PGF_{2\alpha}$ to calculate COX-2 inhibition by hesperetin and hesperetin conjugates. Samples: 1-No inhibitor, 2-celecoxib (1 µM), hesperetin: 3- 1 µM, 4- 2 µM, 5- 3 µM, 6- 5 µM; Hp7glu: 7- 1 µM, 8- 2 µM, 9- 3 µM, 10- 5 µM; Hp3'glu: 11- 1 µM, 12- 2 µM, 13- 3 µM, 14- 5 µM and Hp3'SO_4: 15- 1 µM, 16- 2 µM, 17- 3 µM, 18- 5 µM. N=3 biological replicates, 1 technical measurement, ±SD.

Consequently, the inhibition was lower than previously observed for those compounds that were tested again. For instance, hesperetin (5 µM) inhibited 10% during the first screening, but showed 40% inhibition in the second test. On the other hand, the opposite effect was observed for Hp3'SO_4 (5 µM) which presented 41% in the first measurement and no inhibition in the second screening. An independent t-test showed that samples were not statistically different from the control (no inhibitor).

Thus, different modifications were made to the manufacturer's protocol. Initially, *in vitro* reactions were performed using kaempferol, K3glu and different ginsenosides (Re, Rg1, Rg2, Rh1 and PPX). The protocol

stated a 2000x dilution for each sample to be used in the EIA assay. So, samples following the protocol were used to develop the EIA assay and a 4000x dilution was also tested (data not shown). Next, only ginsenoside Rg1 and PPX (both 4000x dilution) showed 20% and 46% inhibition respectively. Celecoxib (1 µM) measurement was out of the standard values and could not be quantified.

Then, assays using different dilutions factors were performed using 250x, 500x, 1000x and 8000x dilution. For this effect, celecoxib (1 µM) and ginsenoside Re (5 µM) were used as testing compounds. Re (250x dilution) presented 45% inhibition, but other dilutions could not be quantified, as their measurements were out of the standard curve range.

Finally, two other dilutions were performed (100x and 200x) which represented samples 10x and 20x less diluted from the manufacturer's recommendation. Ginsenosides (Re, Rg1, Rg2, Rh1 and PPX) at 5 µM were tested, using celecoxib (5 µM) as positive control (see Figure 4.7). In both dilutions, celecoxib showed more than 80% inhibition.

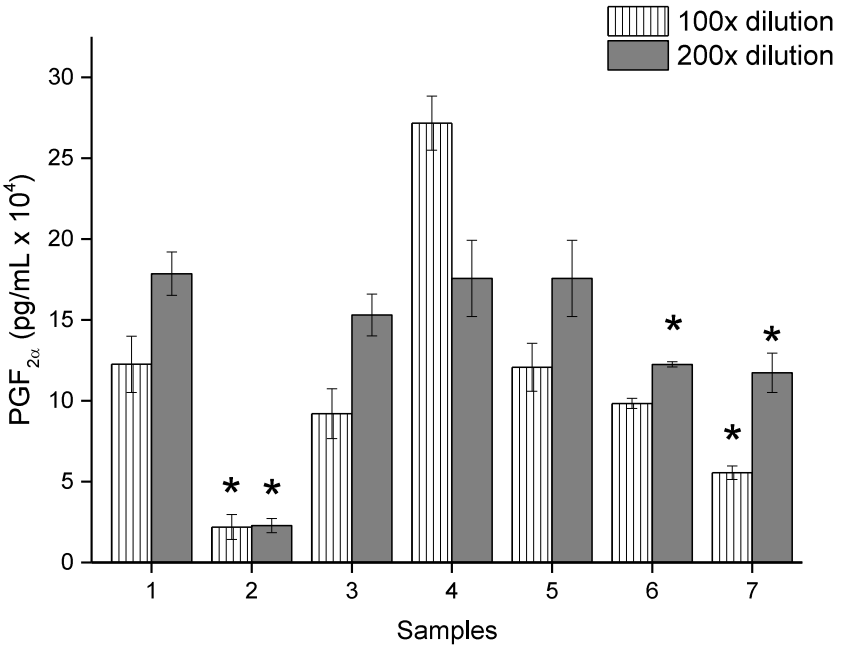


Figure 4.7 Measurement of $PGF_{2\alpha}$ to calculate COX-2 inhibition by ginsenosides. Samples: 1- No inhibitor, 2- celecoxib; ginsenosides: 3- Re, 4- Rg1, 5- Rg2, 6- Rh1 and 7- PPX. All compounds were tested at 5 µM. N=2 biological replicates, 1 technical measurement, ± SD. Asterix (*) represents statistical difference.

At 100x dilution, Re and PPX showed 25% and 55% inhibition respectively whereas celecoxib and Rh1 presented 82% and 20% each. On the other hand, at 200x dilution, the value for celecoxib and Rh1 increased up to 87% and 31%, but decreased for Re and PPX (14% and 34% respectively). An independent t-test showed that celecoxib at 100x and 200x was statistically different (p=0.0172 and p=0.0041 respectively) to the control (no inhibitior). Rh1 at 200x was statistically different from the control (p=0.0279) and PPX at both dilutions was also statistically different (p=0.0336 and p=0.0410 respectively) to the control.

Finally, it was decided to not further analyse any compounds using this methodology due to its unpredictable variation and the discrepancy of results. Therefore, an LC-MS assay was carried out to test those compounds with potential recombinant human COX-2 inhibition.

4.5.2 PGE_2 quantification by LC-ESI/MS using an in vitro assay and solvent extraction.

A series of plan extracts, conjugates and unconjugated polyphenols were tested and quantified using the methodology described above (see 4.4.2). This *in vitro* assay provided more selective measurement of PGE_2 and faster analysis than the immunoassay previously used.

The concentration of PGE_2 produced during the *in vitro* test was calculated using a PGE_2 calibration curve from 1.41 to 10.58 ng (40 nM to 3 µM respectively) (see Figure 4.8). The limit of detection (LOD) was 0.07 ng and the limit of quantification (LOQ) was 0.22 ng. These values were calculated using the equation LOD=(SD/slope)*3 and LOQ=(SD/slope)*10. The d_4-PGE_2 surrogate was used to correct any losses during solvent extraction; its LOD and LOQ were 0.09 ng and 0.29 ng respectively. A calibration curve was also performed for d_4-PGE_2, but it was found that after the liquid-liquid extraction the MS signal of the surrogate was enhanced. Then, the correction was done using the absolute intensity from a 0.43 ng sample which followed the same protocol, but without inhibitor. A typical chromatogram of both standards can be seen in Figure 4.9.

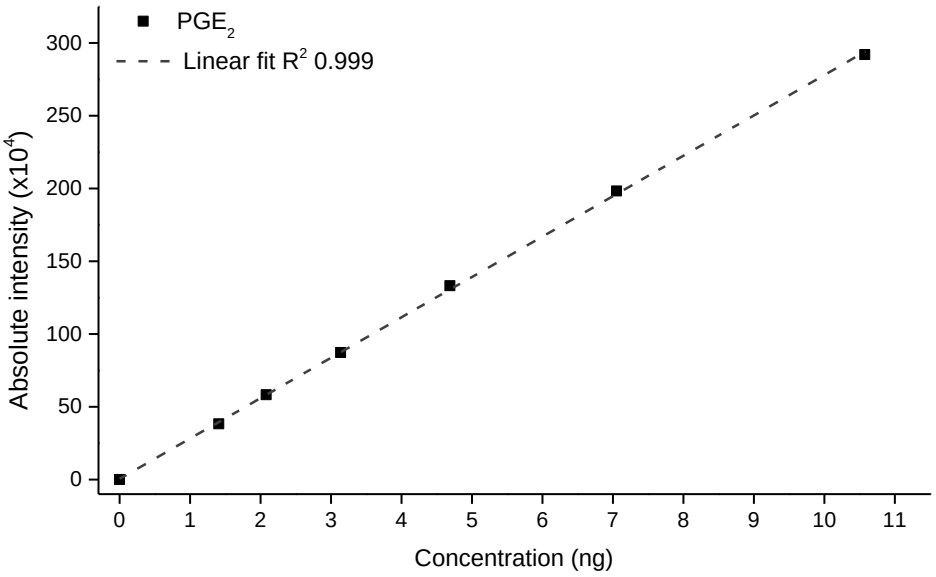


Figure 4.8 Calibration curve for PGE_2 using a commercially available standard diluted in methanol:water (50:50, v/v) at a high concentration and further diluted to various concentrations. N=3 biological replicates and 1 technical measurement.

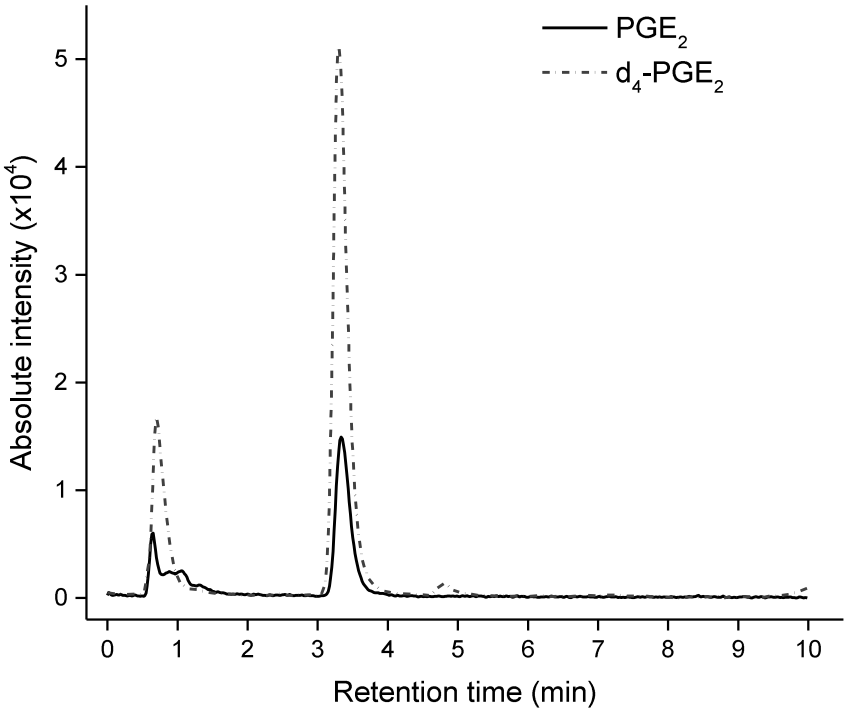


Figure 4.9 Example of selected ion monitoring (SIM) chromatography (LC-ESI/MS) showing the standards PGE_2 (*m/z* 351) and d_4-PGE_2 (*m/z* 351). Each was separately measured using a 10 µL aliquot of 0.7 µM solution (2.47 and 2.50 ng respectively).

4.5.2.1 *In vitro* assay method optimisation

The protocol published by Cao *et al.* (2011) suggested the use of 0.1-0.2 µg of protein (approximately 1 unit of recombinant human COX-2) and 2

min incubation for the *in vitro* assay. Consequently, the first test was to assess the impact of COX-2 concentration in the assay. This was done by performing the assay at two different COX-2 concentrations (0.2 and 2 µg). Firstly, samples (with/without inhibitor) at 0.2 µg did not provide with any PGE_2 signal in LC-ESI/MS. Samples at 2 µg did provide PGE_2 signal in LC-ESI/MS and their chromatograms can be seen in Figure 4.10.

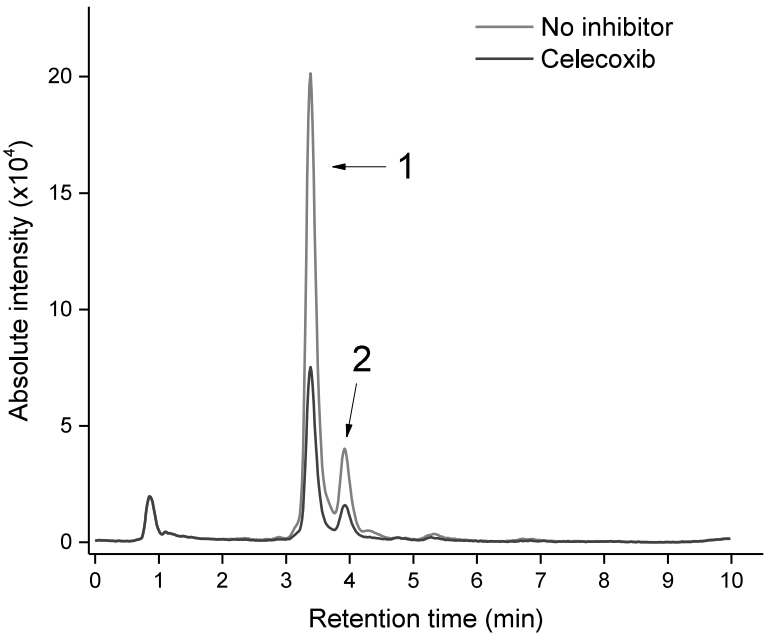


Figure 4.10 Typical MS chromatogram for the total ion counting (TIC) of sample without and with inhibitor (celecoxib 5 µM). The peak 1 is PGE_2 and 2 is PGD_2.

Next, lower concentrations (0.4 and 0.8 µg) and different incubation times were tested (seen Figure 4.11). The samples were developed without inhibitor and a statistical analysis (ANOVA) was performed. The data was normally distributed, as assessed by Shapiro-Wilk test (p>0.05) for all samples. It was found a statistically significant difference between treatments (p=0.001) where the amount of PGE_2 increased from 1.4 ± 0.2 ng with 0.4 µg COX-2 to 4.5 ± 0.6 ng with 0.8 µg COX-2. This difference was reflected in the signal registered during LC-ESI/MS analysis. The absolute intensity was higher which reduced the effect of background noise (see Figure 4.12).

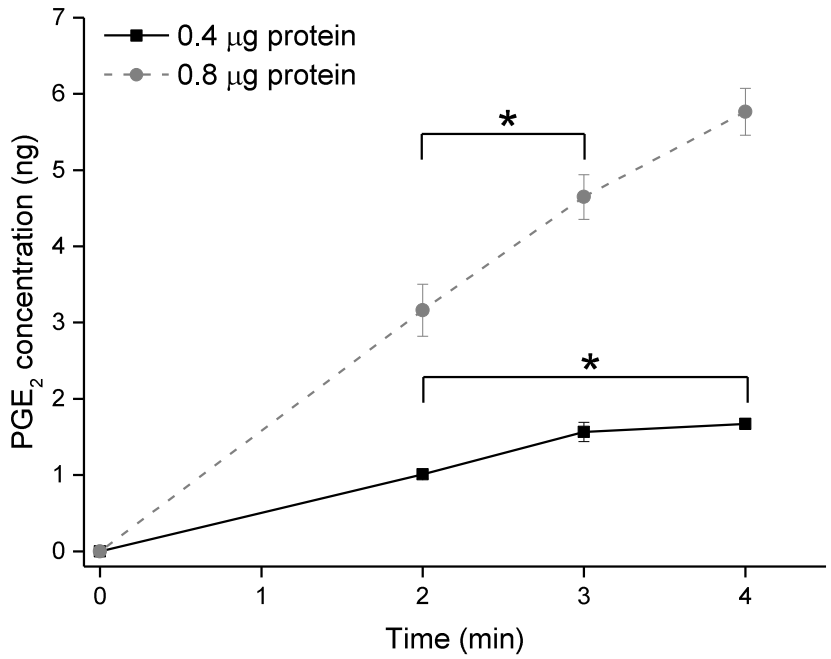


Figure 4.11 PGE_2 concentration of samples at two different enzyme concentrations (COX-2). Asterix (*) indicates those samples statistically significant different ($p<0.05$).

In addition, the three incubation times for each treatment were compared. Samples using 0.4 µg COX-2 were statistically significant different (p=0.003). Post hoc analysis with a Bonferroni adjustment revealed that the difference in PGE_2 concentration was from 1 ± 0.03 ng with 2 min to 1.7 ± 0.04 ng at 4 min (p=0.032). Equally, samples using 0.8 µg COX-2 were statistically significant different (p=0.002) and a post hoc analysis with a Bonferroni adjustment showed that PGE_2 concentration differed from 3.2 ± 0.20 ng at 2 min to 4.6 ± 0.17 ng at 3 min.

Eventually, 0.8 µg was chosen to perform the *in vitro* assay and two minutes remain as the incubation time. Even though statistically significant difference was found at times 2 and 3 min for 0.8 µg these reactions did not include inhibitor. Cao *et al.* (2011), concluded that the production of PGE_2 was linear up to 2 min (using different COX inhibitors); hence experiments at 2 and 4 min incubation were performed using 0.8 µg. Celecoxib (5 µM) was used as positive control to guarantee the activity of COX-2 and its inhibitory activity determined the time to use for further experiments. Samples at 4 min showed up to 52% inhibition (data not shown) and samples at 2 min

provided up to 85% inhibition (see 4.5.2.2). Further experiments were performed using 0.8 µg COX-2 human recombinant and 2 min incubation.

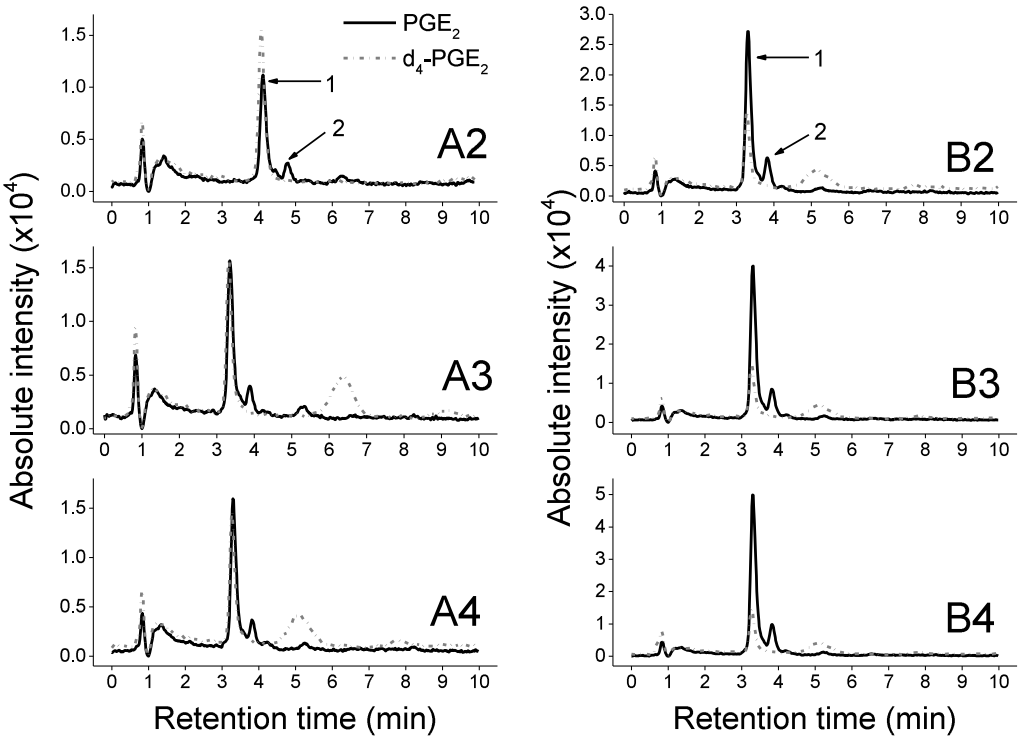


Figure 4.12 MS chromatograms for samples with different COX-2 concentration and at different incubation times. Samples A2, A3 and A4 show 0.4 µg samples and each number represent the min of incubation. Similarly, samples B2, B3 and B4 represent 0.8 µg samples. PGE_2 is arrow 1 and PGD_2 is arrow 2. The chromatograms are the mass-to-charge ratio for the specific compounds PGE_2 and d_4-PGE_2 (*m/z* 351 and 355).

4.5.2.2 COX-2 inhibition by plant extracts and their major polyphenols

Plant extracts were tested at 0.1 and 1 mg/mL concentrations (see Figure 4.13 – A). Conjugated and unconjugated (aglycones) polyphenols were tested at 5 µM (see Figure 4.13- B and C) and ginsenosides (see Figure 4.13- D) were also tested at 5 µM. For each experiment a control (no inhibitor, no enzyme), a 100% activity (no inhibitor) and a positive control (celexoxib, 5 µM) were tested alongside inhibitors.

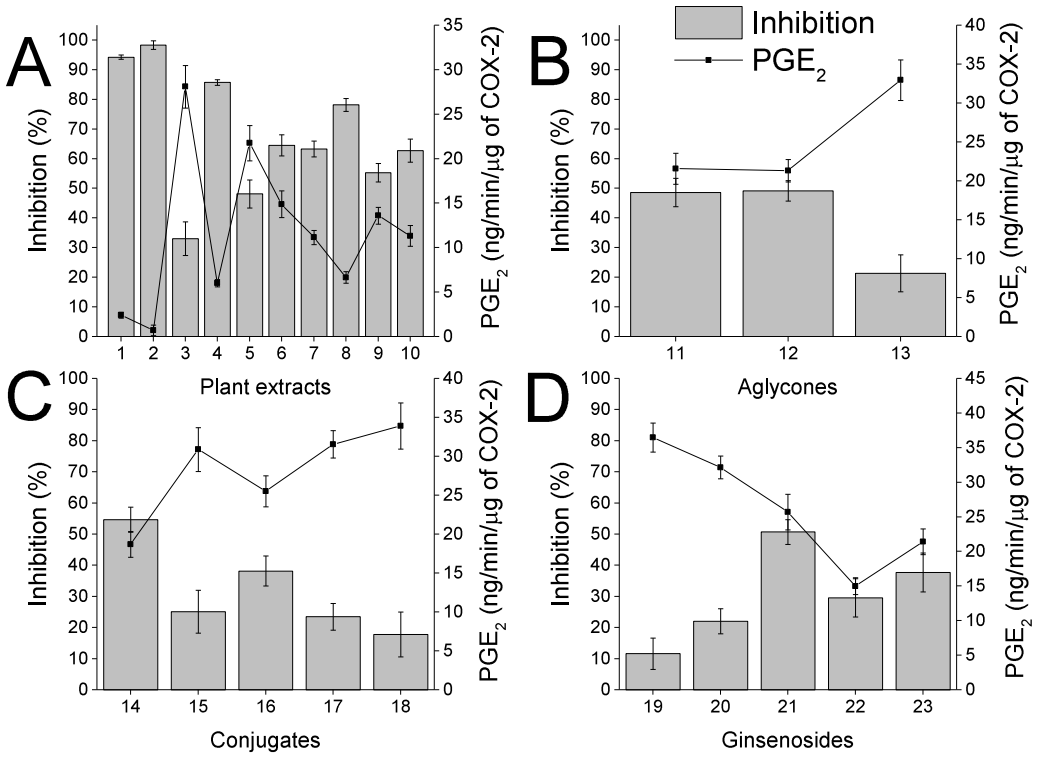


Figure 4.13 Percentage of inhibition and PGE_2 concentration for various compounds. <u>Plant extracts</u> (A): 1-GT 0.1 mg/mL, 2-GT 1 mg/mL, 3- GC 0.1 mg/mL, 4- GC 1 mg/mL, 5- cucurbita 0.1 mg/mL, 6- cucurbita 1 mg/mL, 7- gingko 0.1 mg/mL, 8- gingko 1 mg/mL, 9- ginseng 0.1 mg/mL and 10- ginseng 1 mg/mL. <u>Unconjugates</u> (B): 11- quercetin, 12- kaempferol and 13-hesperetin. <u>Conjugates</u> (C): 14- Hp3'SO_4, 15- Hp3'Glu, 16- Hp7Glu, 17- K3Glu and 18- Q3'SO_4. <u>Ginsenosides</u> (D): 19- Re, 20- Rh1, 21- Rg1, 22- Rg2 and 23- PPX. Compounds showed in graphs B, C and D were tested at 5 µM. Celecoxib (5 µM) was used as positive control and inhibited more than 75% (data not shown). N=3 biological replicates and 1 technical measurement, ± SD.

The first analysis showed that all plant extracts inhibit COX-2 more than 60% at 1 mg/mL and indicated that inhibition decreased at lower concentrations (0.1 mg/mL). The highest COX-2 inhibition was produced by GT (98%, 1 mg/mL), followed by GC (86%, 1 mg/mL). Quercetin and kaempferol were more efficient COX-2 inhibitors than their conjugates (Q3'SO_4 and K3Glu) with more than 45% inhibition. In contrast, hesperetin conjugates (Hp3'SO_4, Hp3'Glu and Hp7Glu) inhibited COX-2 from 25 to 55% meanwhile hesperetin aglycone inhibited only up to 21%. Ginsenosides inhibited from a range of 12 to 51%. The best inhibitor was Rg1 (50%) and the weakest was Re (12%).

Afterwards, conjugates, aglycones and ginsenosides were tested at three different concentrations (1, 5 and 10 µM) to seek for concentration dependence. First, quercetin, kaempferol and hesperetin were analysed (see Figure 4.14) at 1, 5 and 10 µM. Samples at 5 µM did not agree with previous results where quercetin at 5 µM inhibited 49% (see Figure 4.13) and after the second test decreased up to 18%. A similar phenomenon was observed among the others. For instance, kaempferol inhibited COX-2 in 49% during the first test and it did not show any inhibition after the second assay. On the contrary, the COX-2 inhibition increased for hesperetin (5 µM) up to 35% which was 10% difference from the first test.

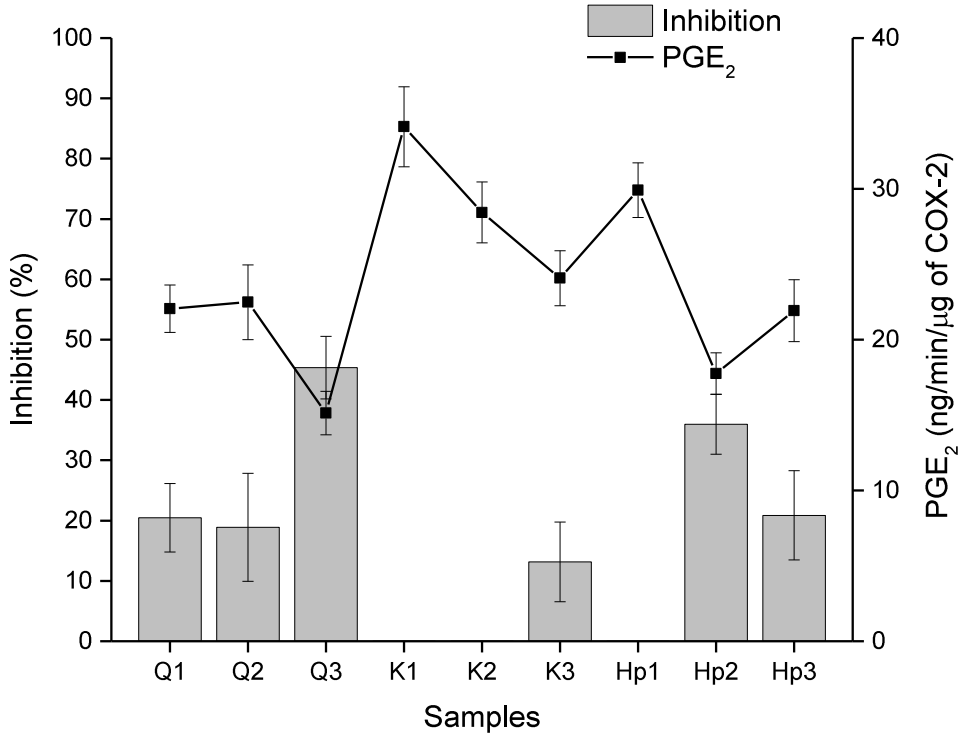


Figure 4.14 Percentage of inhibition and PGE_2 concentration for unconjugated polyphenols. Samples → <u>quercetin</u>: Q1- 1 µM, Q2- 5 µM and Q3- 10 µM; <u>kaempferol</u>: K1- 1 µM, K2- 5 µM and K3- 10 µM; <u>hesperetin</u>: Hp1- 1 µM, Hp2- 5 µM and Hp3- 10 µM. Celecoxib (5 µM) was used as positive control and inhibited more than 60% (data not shown). N=3 biological replicates and 1 technical measurement, ± SD.

Later, conjugates from quercetin, kaempferol and hesperetin were tested (see Figure 4.15). The conjugates used were Q3'SO_4, K3Glu, Hp3'SO_4, Hp3'Glu and Hp7Glu. The inhibitors were analysed at the same concentrations as their unconjugated forms were tested.

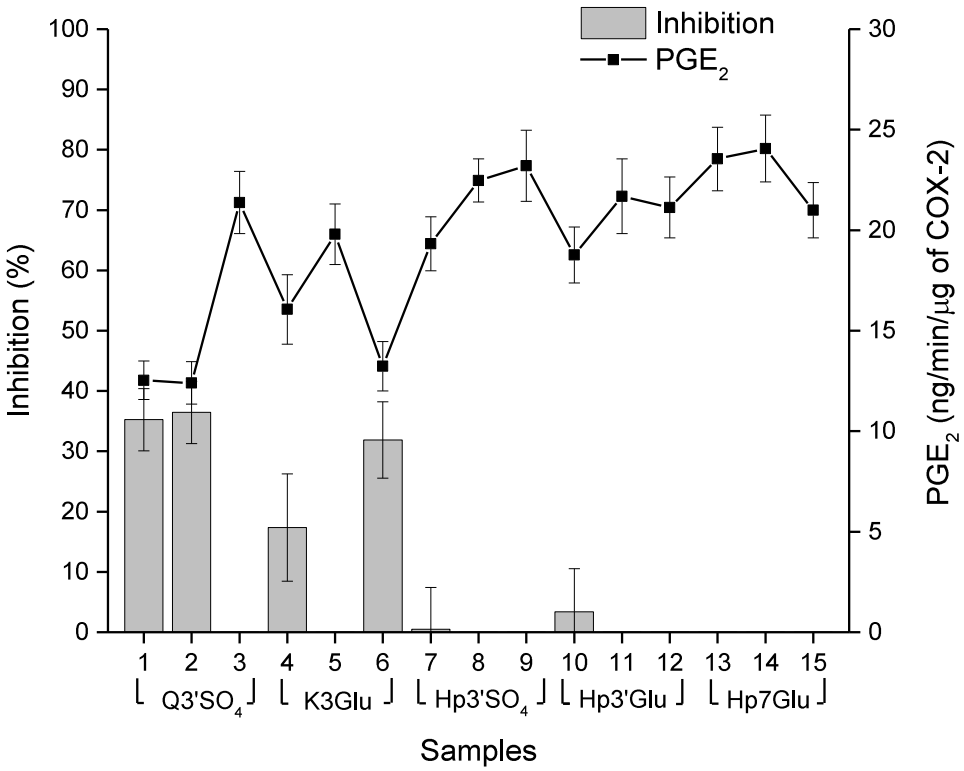


Figure 4.15 Percentage of inhibition and PGE_2 concentration for conjugated polyphenols. Samples → <u>Q3'SO$_4$</u>: 1- 1 µM, 2- 5 µM and 3- 10 µM; <u>K3Glu</u>: 4- 1 µM, 5- 5 µM and 6- 10 µM; <u>Hp3'SO$_4$</u>: 7- 1 µM, 8- 5 µM and 9- 10 µM; <u>Hp3'Glu</u>: 10- 1 µM, 11- 5 µM and 12- 10 µM; <u>Hp7Glu</u>: 13- 1 µM, 14- 5 µM and 15- 10 µM. Celecoxib (5 µM) was used as positive control and inhibited up to 77%. N=3 biological replicates and 1 technical measurement, ± SD.

Only 6 samples showed positive inhibition and results did not replicate previous findings. Q3'SO_4 produced around 36% inhibition for 1 and 5 µM, but provided only 18% during the first test. Indeed, K3Glu at 5 µM showed 23% inhibition after the first test, but no inhibition at 5 µM after replication. However, K3Glu samples at 1 µM and 10 µM inhibited 17% and 31% each. Hesperetin conjugates did not show more than 10% COX-2 inhibition after second assay. This occurrence was unexpected, since they provided up to 55% COX-2 inhibition in the first test (see Figure 4.13).

Next, ginsenosides (Re, Rh1, Rg1, Rg2 and PPX) were tested and only Rg2 (5 µM) and PPX (5 µM) presented around 20% COX-2 inhibition. Moreover, results were not consistent with earlier findings.

In the same way, plant extracts were tested to identify any concentration dependence and calculate the half maximal inhibitory concentration (IC_{50}) for COX-2.

Firstly, GT was tested from 10 to 100 µg/mL (see Figure 4.16), assuming that at 0.1 mg/mL inhibited 94%. The inhibition at the lowest concentration reached more than 35% and quickly increased. Finally, it was found that GT inhibited COX-2 in 50% (IC_{50}) at 16.1 µg/mL.

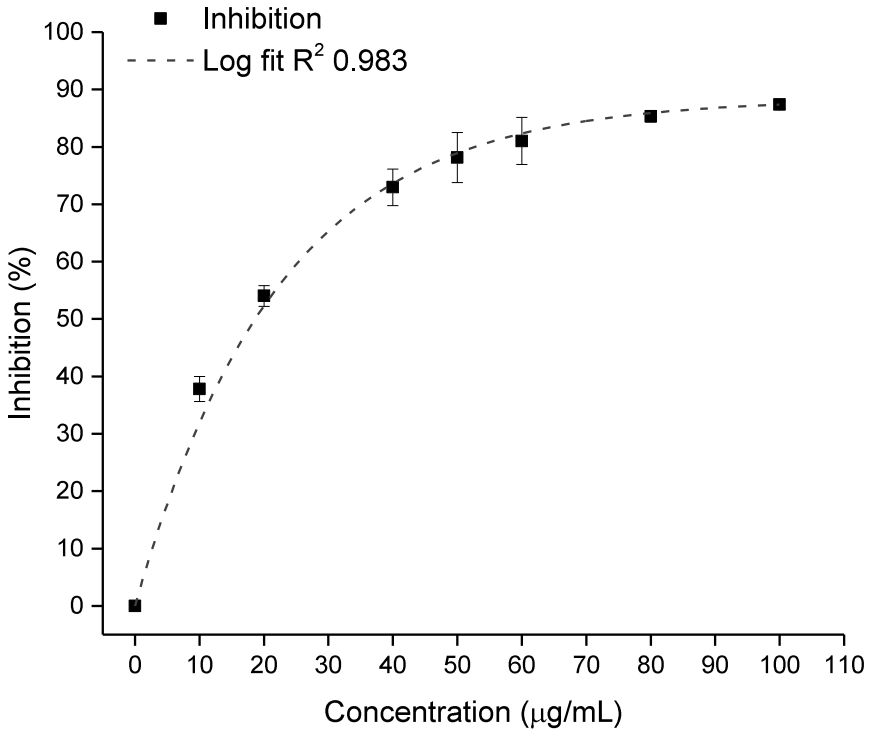


Figure 4.16 Percentage of inhibition by GT from 10 to 100 µg/mL. A logarithmic equation was obtained and it was calculated IC_{50} = 16.1 µg/mL. N=3 biological replicates and 1 technical measurement, ± SD.

Additionally, a 1 mg/mL of GT was characterised by Mrs. Hilda Nyambe as part of her research project. In brief, GT was characterised using a rapid resolution HPLC fitted with a diode array detector, binary pump, column and sample thermostat (1200 series Agilent Technologies; Dorset, UK). A Zorbax Eclipse Plus-C18 (2.1x100 mm, 1.8 µm) analytical column was used to perform the separation and was kept at 35°C. The injection volume was 10 µL and the flow rate was set at 0.25 mL/min. Solvent A was acidified water (0.1% formic acid, v/v) and solvent B was acetonitrile with 0.1% formic acid (v/v). The separation started with 5% solvent B and gradient

increase up to 10% B after 5 min. At 20 min the gradient was 40% B and reached 90% B at 25 min. This level was kept for 4 min and dropped to 5% B after 1 min. The column was re-equilibrated until 33 min.

Five major compounds (EGCG, EGC, ECG, EC and caffeine) were identified and quantified (see Figure 4.18). Their abundance was calculated as follows: EGCG 19.9%, EGC 11.9%, caffeine 5.4%, ECG 3.3% and EC 2.1%. These results were achieved using commercially-available standards and calibration curves for each compound.

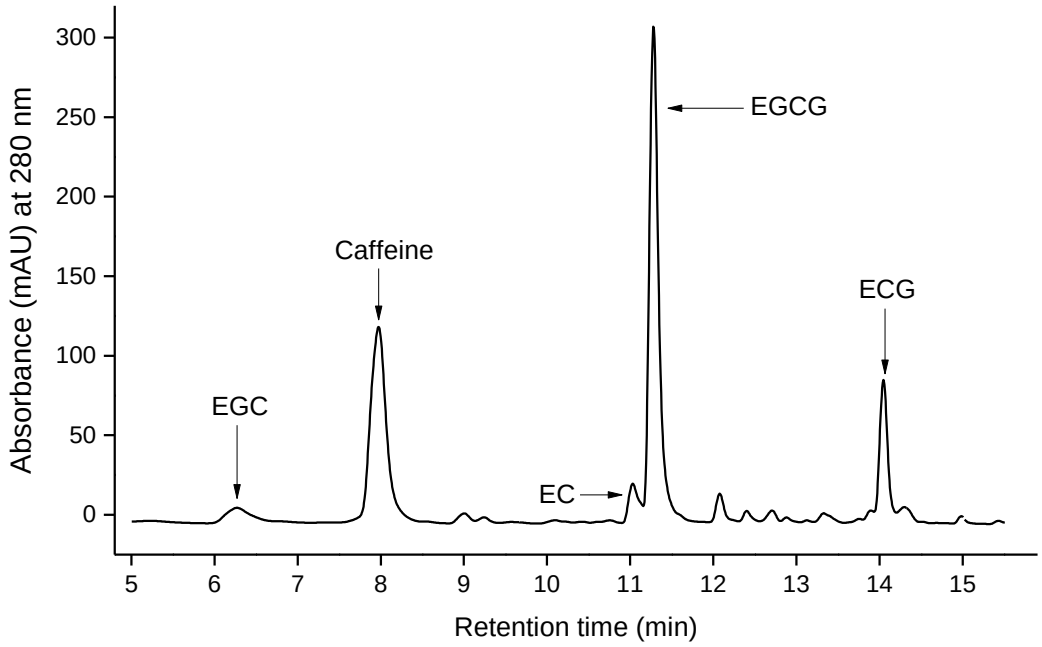


Figure 4.17 Reverse-phase HPLC analysis of 1 mg/mL of GT powder. In order of abundance: EGCG, EGC, caffeine, ECG and EC were identified and quantified using commercially available standards.

Next, based on GT IC_{50}, a theoretical IC_{50} for the two major compounds (EGCG 7 µM and EGC 6.5 µM) was calculated using their concentration in GT, and a test was carried out to verify each. At first, EGCG was tested using concentrations from 0.5 µM to 32 µM (see Figure 4.19) and its IC_{50} was calculated at 2.8 µM which was lower than the theoretical value (7 µM). Following, EGC was tested using the same concentration range from EGCG. However, no inhibition was found at 0.5 µM and similar inhibition was seen at 1 and 2 µM. Thus, values from 2 µM to 32 µM were used to calculate its IC_{50} which was 9.2 µM (see Figure 4.18). This value was higher than the theoretical previously estimated (6.5 µM).

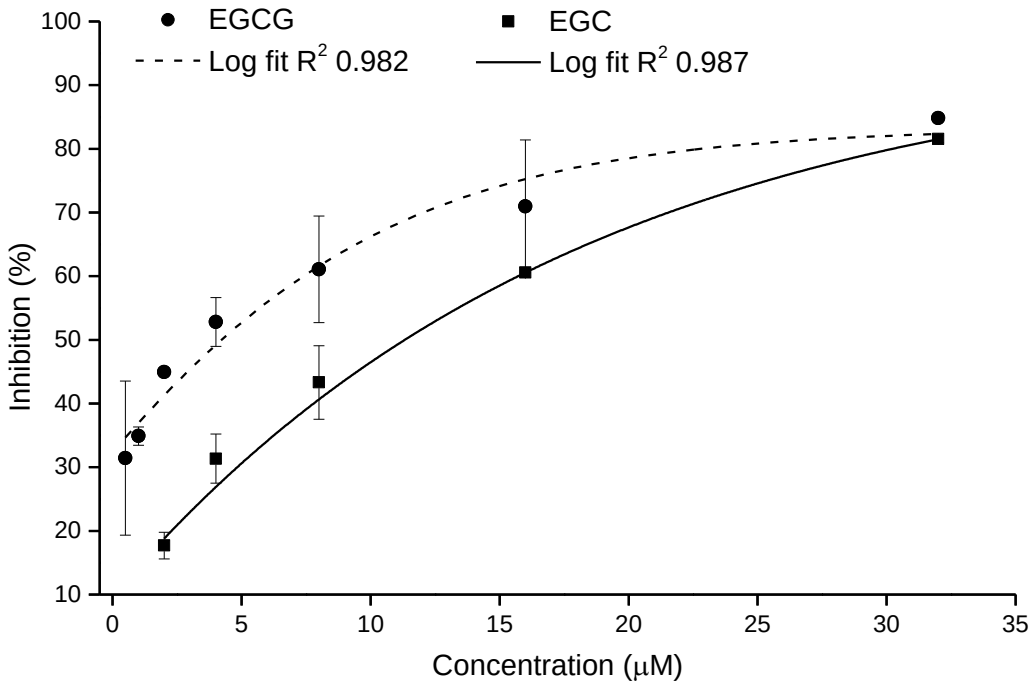


Figure 4.18 Percentage of inhibition by EGCG and EGC using different concentrations. IC_{50} was 2.8 μM and 9.2 μM respectively. N=2 biological replicates and 1 technical measurement, ± SD.

Other plant extracts, previously tested, were also analysed. The concentrations tested were calculated using as reference the % inhibition during first screening. GC was tested from 0.05 to 1 mg/mL and inhibited from 12% to 87%. However, the inhibition was not concentration- dependent and varied among all concentrations. Cucurbita was tested from 0.025 to 1.75 mg/mL, and only inhibited at 1.25 and 1.75 mg/mL (4 and 32% inhibition respectively). Gingko was tested from 0.01 to 1.50 mg/mL and inhibited from 1 to 21%, but it did not show any concentration dependence. Indeed, ginseng was tested from 0.01 to 1.75 mg/mL and inhibited from 1 to 48%, but samples did not show any concentration dependence.

Quercetin, hesperetin and $Q3'SO_4$ were selected to be analysed at different concentrations. Quercetin was performed from 0.2 to 25 μM and no inhibition was observed. Hesperetin was tested from 0.5 to 62.5 μM and it showed inhibition from 1 to 19%, but without concentration dependence. Finally, $Q3'SO_4$ was performed from 0.2 to 25 μM and inhibited from 3 to 21% without concentration dependence manner. A summary of all samples, concentration tested and % inhibition can be seen in Table 4.2.

Table 4.2 Plant extracts and compounds that inhibited recombinant human COX-2. The values for GT and its major compounds (EGCG and EGC) can be seen in Figure 4.16 and Figure 4.19.

Plant extract/ compound	Concentration (I = mg/mL, II = µM)	PGE_2 (ng/min/µg protein)	SD	Inhibition (%)	SD
German chamomile	0.05 (I)	46	8.57	12	16.40
	0.075 (I)	44	2.37	15	4.53
	0.1 (I)	47	5.96	11	11.41
	0.25 (I)	31	1.40	41	2.68
	0.5 (I)	25	0.03	52	0.06
	0.75 (I)	29	5.76	44	11.02
	1 (I)	7	2.05	87	3.92
Cucurbita	1.25 (I)	43	5.13	4	11.44
	1.75 (I)	30	3.18	32	7.10
Ginkgo	0.05 (I)	30	14.86	21	39.26
	0.1 (I)	37	0.79	1	2.09
	0.25 (I)	36	0.22	4	0.59
	1 (I)	33	4.08	12	10.78
	1.25 (I)	35	8.61	8	22.75
	1.5 (I)	36	0.07	5	0.17
Ginseng	0.01 (I)	34	0.26	1	0.74
	0.025 (I)	33	3.36	4	9.68
	0.05 (I)	33	2.86	4	8.24
	0.25 (I)	34	0.76	1	2.19
	0.5 (1)	20	4.26	41	12.25
	1 (I)	28	0.58	18	1.67
	1.25 (I)	20	1.68	41	4.83
	1.5 (I)	20	0.68	43	1.95
	1.75 (I)	18	4.25	48	12.25
Hesperetin	0.5 (II)	46	0.32	19	0.55
	1 (II)	57	5.17	1	9.04
	2 (II)	54	4.40	5	7.70
	4 (II)	48	2.57	16	4.49
	10 (II)	48	7.46	15	13.06
	25 (II)	48	6.61	16	11.57
	62.5 (II)	54	5.99	6	10.47

Q3'SO$_4$	0.2 (II)	46	3.37	21	5.86
	0.4 (II)	52	5.54	9	9.64
	0.8 (II)	49	1.64	14	2.85
	1.6 (II)	50	1.35	12	2.35
	4 (II)	50	3.03	14	5.28
	10 (II)	49	0.04	14	0.07
	25 (II)	54	0.99	3	1.48

4.6 Discussion

Cyclooxygenase 2 (COX-2) is an inducible isoform of COX enzymes that it is produced as response of inflammation. COX-2 produces inflammatory mediators such as prostaglandin E_2 (PGE_2) and it has become an important target for prevention and treatment of diseases like cancer (Srivastava *et al.*, 2009) .

Plant extracts are a complex mix of compounds which have attracted the attention of research during the last decade. Their use has been strongly criticised, as many of their beneficial effects are mainly based on experience (Calapai and Caputi, 2007). Therefore, scientific information has become fundamental to understand the mechanisms of action and elucidate their impact on human beings.

Polyphenols are the main bioactive compounds studied in the plant extracts and their effects are still under investigation (Cai *et al.*, 2004). The methodologies to test these compounds are diverse and provide different types of results which need to be analysed carefully to avoid discrepancy in the conclusions. Animal and cell culture studies are the most common assays used in research. For example, Maruyama *et al.* (2010) tested the anti-inflammatory activity of bee pollen from *Cistus sp*. (Spanish origin) using a carrageen-induced paw edema in rats. They described their results in % swelling and found that bee pollen and the fraction extracted with ethanol showed up to 73% less swelling (ethanol fraction). They characterised the ethanol fraction using NMR and found kaempferol glucoside, quercetin rhamnoside, quercetin, kaempferol and isoharmentin as main components, but quantification and experiments with individual compounds were not

performed. Gautam *et al.* (2011),tested anti-inflammatory properties of *Ajuga bracteosa* in mice using the TPA-induced mouse ear edema assay. They used a 70% ethanol-extracted fraction and compared two doses. As it was expected, the higher concentration inhibited up to 70% more compared with the control. They isolated five compounds, but none was used in this assay. Even though animal models are widely used to test drugs and plant extracts, they do not provide the complete information about how these compounds work (Webb, 2014). For example, carrageen-induced paw edema is tipically used as a model of inflammation, but it presents information about generalised inflammation and it is not specific of any mechanism (Whiteley and Dalrymple, 2001; Webb, 2014). Cell culture studies, using different sources and types of cells, have also contributed to understand how plant extracts/polyphenols function against pro-inflammatory enzymes (Peng *et al.*, 2006; Yang *et al.*, 2011). However, differences in cell lines and different inflammatory responses have an impact on results which can lead to inconsistent outcomes (Lotito *et al.*, 2011). Another way to undertake this challenge it is through *in vitro* assays. We compared two types of methodologies to measure the inhibition of COX-2 by plant extracts and their main components as conjugated or unconjugated polyphenols.

Firstly, a COX inhibition assay was used to test a wide range of polyphenols and various plant extracts. The assay was performed following the manufacturer's instructions, but the reproducibility was not consistent. To ensure that the recombinant human COX-2 worked and the procedure was followed correctly, celecoxib (5 µM) was used as positive control. Different dilutions were tested to examine if the binding response of PGE_2 during the immunoassay was affected by the dilution factor. No pattern was found and high variations in the results were seen. For example, ginsenoside Re (5 µM) was tested at different dilution factors (100x, 200x and 250x) and it showed 25%, 14% and 45% inhibition respectively, but celecoxib (5 µM) showed consistently more than 80% inhibition. In fact, distinct inhibition could be only seen for the plant extracts (see Figure 4.4) were GT and GC provided more than 75% inhibition. These results were used to perform further experiments. However, other researches have used this methodology to support their findings. Cheong *et al.* (2004), used the same *in vitro* assay to test different

concentrations of quercetin (1, 10, 50, 100 and 1000 µM) and compared them to resveratrol (30 µM) and NS-398 (10 µM). They did not provide any % inhibition and their graphic results are not comparable with our findings. Also, the procedure to analyse their results is not reported. Maruyama *et al.* (2010), performed the COX inhibitor screening assay testing bee pollen extracts at different concentrations. They did not report the use of a positive/negative control and their results were analysed by linear regression. No single compounds were tested. Finally, Gautam *et al.* (2011) also used the same *in vitro* assay for *Ajuga bracteosa* to test inhibition of COX-1 and COX-2. They analysed two different concentrations (25 and 50 µg/mL) and celecoxib at 30 µM, but none specific polyphenols were reported. The method to analyse their results was not reported. Overall, the COX inhibitor screening assay is a good method to determine if a plant extract has any inhibitory activity against COX-2, but it will not provide explicit information about the activity of its polyphenol content. Indeed, it is important to address that any data produced using this *in vitro* assay should be analysed using a 4-parameter analysis, as ligand binding assays are usually characterised by a sigmodial relationship between the mean response and the compound concentration (Findlay and Dillard, 2007).

A different strategy to establish if an analyte inhibits COX-2 it is through the combination of an *in vitro* assay and the measurement of PGE_2 by mass spectrometry. The methodology for the *in vitro* assay has been reported by Cao *et al*. (2010; 2011) and recently by Zhu *et al.* (2014) and Deng *et al.* (2014) who compared derivatization and magnetic ligand fishing of PGE_2 to the original protocol. The *in vitro* assay performed during our analysis used Tris-HCl buffer as negative control instead of DMSO. The quantification of PGE_2 produced after recombinant human COX-2 inhibition was carried out using the protocol published by Cao *et al.* (2011) using a LC-ESI/MS instead of a LC-ESI/MS^2. The LOD and LOQ calculated for PGE_2 during our quantification (see 4.5.2) were not as good as those previously reported by Cao *et al.* (2011) (LOD=0.2 pg, LOQ=1 pg) and Zhu *et al.* (2014) (LOD=0.006 pg, LOQ=0.0255 pg) who used a gradient protocol to perform the measurement of PGE_2. The variation could be due to the specificity of the LC-MS^2 which at lower concentrations provides higher signal in

comparison with LC-MS. None of the previous authors provided LOD and LOQ for the surrogate d_4-PGE_2.

Inhibition of recombinant human COX-2 by aglycones and conjugated polyphenols was not reproducible, as it showed inconsistent results after various repetitions, except for EGC and EGCG. The measurement of PGE_2 by mass spectrometry is one of the latest methodologies to detect prostaglandins in biological samples, and methodologies already published have only used LC-ESI/MS^2, but not LC-ESI/MS. During the LC-ESI/MS analysis, a standard curve for the surrogate was performed (data not shown) to determine the amount of d_4-PGE_2 remaining after liquid-liquid extraction. However, it was found that the absolute intensity (MS signal) was enhanced after the solvent extraction; then, the absolute intensity from a sample without inhibitor, but with a known amount of surrogate (0.43 ng) was used to correct for losses (see 4.5.2). This phenomenon might suggest that the sample mixture and the solvent extraction interfere with the evaluation of PGE_2 which could have led to instability of the measurement. Other organic solvents have been used to extract PGE_2 from biological samples (Brose *et al.*, 2011) which could improve the reproducibility of results. Even though results did not agree for most of the polyphenols and plant extracts tested, green tea and its two major polyphenols (EGCG and EGC) showed consistent inhibition. This could also suggest that the *in vitro* assay is affected by the type of polyphenol or plant extract to test, as different subclasses of polyphenols were analysed. For instance, EGCG and EGC are considered catechins and belong to the subclass flavanol, and quercetin and kaempferol are flavonols. On the other hand, the inhibition by plant extracts provided enough evidence to support previous reports suggesting that GT inhibits recombinant human COX-2. Lately, Deng *et al.* (2014) have reported the analysis of a variety of green tea (Yunwu, *Camellia sinensis*) obtained from the Tea Research Institute of Hunan province in China. They performed a modified protocol by Cao *et al.* (2011) and compared it to a new screening assay based on magnetic ligand fishing. Their IC_{50} for green tea was 437.3 µg/mL which is 27x more than our reported value (16.1 µg/mL) and it suggests that our green tea was richer in polyphenols (COX-2 inhibitors) than their sample. They performed the *in vitro* assay testing the

main polyphenols of green tea, but only reported IC_{50} for 3''-*O*-methyl-EGCG = 0.17 µM and 3''-*O*-methyl-ECG = 0.16 µM which were not tested by us. Publications about GT mostly report inhibition of COX-2 expression in cell lines models. For instance, Peng *et al.* (2006) cultured HT-27 and HCA-7 cells with various concentrations of EGCG (from 10 µM to 300 µM) and incubated them for 48 hrs. Next, they performed a series of different experiments which showed that EGCG inhibited the expression of COX-2 protein and mRNA in both cell lines. Western blot analysis showed that at 200 µM EGCG treatment, COX-2 protein expression was inhibited by 57% in HT-29 and 44% in HCA-7. Also, they found that COX-2 protein expression decreased due to reduction in COX-2 mRNA expression. Indeed, they showed that EGCG cut down COX-2 promoter activity in a dose-dependant manner and located the transcription factor NF-κB as the regulator of COX-2 expression. Recently, Singh and Katiyar (2011) also used cell lines to assess the inhibitory activity of EGCG in human melanoma cells. They used normal epidermal melanocytes and human melanoma cell lines (A375 and Hs294t) to perform their experiments. They found a reduction of endogenous expression of COX-2 in cell lysates (A375 and Hs294t lines) by Western blot analysis which was reduced in a concentration-dependant manner. They quantified the production of PGE_2 by the same immunoassay that we used to test COX-2 inhibition. However, their analysis involved melanoma cell homogenates and not single compounds. They treated A375 cells with various concentrations of EGCG (10,20 and 40 µg/mL) for 24 hrs and revealed that EGCG decreased the translocation of NF-κB/p65 into the nucleus in a dose-dependant pattern. Both publications agreed that EGCG modulates NF-κB and this information provides a key target to approach in anti-inflammatory and anti-cancer therapies.

Altogether, this is the first attempt to screen a series of different polyphenols and plant extracts for their recombinant human COX-2 inhibition activity using two different techniques (spectrophotometer and mass spectrometry). These analytes presented a wide range of characteristics and chemical structures which might have had impact on their response in both methodologies. Certainly, there is still a lack of information of COX-2 inhibition by GT and its two major compounds (EGCG and EGC), tested as

single compounds, in the literature. Although the mass spectrometry methodology is similar to other methods already reported, this assay provides a faster analysis than ELISA and it does not involve the use of derivatization or immobilization of COX-2 which could potentially minimise sample handling.

CHAPTER 5 Summary and Future Perspectives

5.1 Outcomes

Plant extracts and their constituent polyphenols have become an important research subject in recent years and their use in Western society is growing (Bodeker and Kronenberg, 2002). Different techniques have been used to identify and quantify the polyphenol content of plant extracts and determine their beneficial effect on human beings. The work here presented has focused in the impact of human metabolism on the polyphenols in the plant extracts. Mass spectrometry measurements and *in vitro* assays have been used to provide more information about the metabolism and activity of these compounds.

The first step of metabolism is characterised by the enzymatic cleavage of sugars, and it is most likely to occur in the gastrointestinal tract or in the food itself (Scalbert and Williamson, 2000). This step was mimicked using enzymatic hydrolysis on GC by a method using multiple-enzyme hydrolysis (hesperidinase and cellulase) (Chapter 2). This approach was previously tested in five different fruit matrices and demonstrated potential versatility for other plant based materials (Pimpão *et al.*, 2013). The methodology helped to simplify the identification of the main polyphenols in GC (see Figure 2.2, 2.4-2.5) which can be possibly applied to other plant extracts. The main polyphenol in GC is known to be apigenin-7-*O*-glucoside and publications have paid more attention to its quantification and identification of its by-product. Consequently, the identification and quantification of other polyphenols using true standards was our goal. Our results agreed with previous reports identifying various polyphenols in GC (see Table 2.2). The quantification of eight compounds (see Table 2.3) suggested that the variation in the origin of the plant extract and the use of different preparation methods cause high dissimilarity in the quantification of these compounds (Novakova *et al.*, 2010; Raal *et al.*, 2012; Guimarães *et al.*, 2013). Indeed, the specificity of the equipment (LC-MS^2, LC-MS or HPLC) had an impact in the identification and quantification of small quantities of polyphenol present in the plant, increasing with the complexity of the

analytical tool (Novakova *et al.*, 2010). Additionally, the deproteinization and extraction using acetonitrile showed higher recovery of aglycones after hydrolysis, but it was not as effective as expected. The concentration of the main GC aglycones (apigenin and luteolin) did not increase after hydrolysis which suggested that non-hydrolysed glucosides were lost during the solvent extraction. Glucosides are more water-soluble than their aglycones and their concentration might have been underestimated. Therefore, it is recommend that after enzymatic hydrolysis a liquid-liquid extraction should be carried out with other organic solvents miscible in water such as 2-propanol or ethanol. Methanol is not recommended as it can promote spontaneous methylation and deliver incorrect results (Xie *et al.*, 1993). Overall, the combined use of LC-ESI/MS^2 and LC-ESI/MS helped to identify with more accuracy those compounds with scientific interest and decreased the time of analysis.

After the first step of metabolism, a second series of reactions occurs and analytes are known as phase II metabolites. Polyphenols are conjugated into different metabolites to be further excreted through biological fluids (e.g. blood, urine). Urinary metabolites are usually investigated, as urine collection is not invasive and samples can be gathered by the subject. Moreover, for the second part of the project, the main targets were to sulfate hesperetin, identifty its main sulfated form and identify/quantify its main metabolites in human samples (urine) after orange juice consumption (Chapter 3). The first goal was established after various publications reported a lack of enzymatic hydrolysis for different polyphenols (e.g. epicatechin) using β-glucuronidase and sulfatase and which could potentially lead to flaw results (Vallejo *et al.*, 2010; Saha *et al.*, 2012; Charoensuk, 2014). Enzymatic hydrolysis is commonly used to identify and quantify urinary metabolites without the need of true standards. The methodology here reported introduced a new co-cofactor (*p*-nitrophenyl sulfate, PNS) which increased the transformation of hesperetin to hesperetin sulfate (Hp3'SO_4) up to 80% and its mechanism of action can be seen in Figure 3.13. The positive identification and quantification of Hp3'SO_4 by LC-ESI/MS provided enough information to quantify the main urinary hesperetin metabolites after orange juice ingestion. Samples from a cross-over human study were collected and analysed by Dr. Tristan P. Dew and Miss Isabella

Procopiou using true standards for identification and quantification of hesperetin metabolites. Then, the data was assessed, statistically analysed and interpreted by this researcher. Three commercial orange juices were used during the study and their main metabolites were quantified by LC-ESI/MS. It was found that the MS response (absolute intensity) from Hp3'SO_4 was 2.3x higher than its aglycone and this factor was used to correct the urine measurements for Hp3'SO_4 (see section 3.5.3.2). This phenomenon has been also detected in measurements by LC-ESI/MS^2 and HPLC-DAD using hydroxycinnamic acid conjugates and hesperetin sulfate (Brand *et al.*, 2010; Farrell *et al.*, 2011), but it was not reported in the latest measurement of hesperetin metabolites (Pereira-Caro *et al.*, 2014). The statistical analysis of the data showed a statistically significant difference between the freshly squeezed orange juice and value brand orange juice (see Figure 3.10) where the total metabolites excretion was 7% higher in freshly squeezed than the value brand. Statistically significant differences for each metabolite excretion were found between freshly squeezed and value brand orange juices. The concentration of Hp7Glu was 1% higher in freshly squeezed than the value brand. As well, Hp3'Glu was 5.6% higher in freshly squeezed than the value brand. Finally, Hp3'SO_4 was 0.8% higher in freshly squeezed than the value brand. A concise report of the measured values and those reported in the literature can be seen in Table 3.3 and 3.4. The considerable difference seen in Hp3'Glu could be explained due to the variability among the subjects, as two outliers were identified for this metabolite. It has been proposed that the excretion of these metabolites is affected by the gut microflora (Erlund *et al.*, 2001). Therefore, these inter-individual variations might suggest that the specific enzymatic activity of the colonic microflora in each participant plays an important role in the metabolism of flavonoids (Manach *et al.*, 2003; Pereira-Caro *et al.*, 2014). On the other hand, Bredsdorff *et al.* (2010) suggested that hesperetin and naringenin are glucuronidated/sulfated in the same pattern without being affected by the site of absorption. They concluded that the metabolism of these two polyphenols after absorption in the small intestine was not significantly different from the metabolism after absorption in the colon. Then, it is advised that further quantification of urinary-hesperetin metabolites are

designed studies using a large pool of participants which could potentially decrease the effects of the inter-individual variations. Indeed, it is recommended to pay attention to the MS response of polyphenol metabolites to avoid inaccurate measurements in future experiments.

After the plant extracts were characterised and their main polyphenols identified, the next step was to investigate their potential health benefit. Our target was the anti-inflammatory activity of these compounds and we focused in the inhibitory activity against cyclooxygenase 2 (COX-2). This pro-inflammatory enzyme has been related to arthritis and various types of cancer (Raman *et al.*, 2008). Selective inhibitors have been previously developed, but recent studies have found an association of their intake with an increase in blood pressure which leads to myocardial infarction or stroke (Raman *et al.*, 2008; Salvado *et al.*, 2012). Consequently, scientists are seeking for harmless alternatives to inhibit effectively COX-2 and avoid or provide fewer side effects. For that reason, the third experimental part focused on cell-free models to screen plant extracts and their main aglycones and conjugate polyphenols (Chapter 4). A COX inhibitor screening assay was used to quantify directly $PGF_{2\alpha}$ via enzyme immunoassay (EIA) (see Figures 4.1 and 4.2) and various plant extracts and their main aglycone/conjugated polyphenols were tested. It was found that the screening assay provided sufficient information to determine if the sample was or was not an inhibitor of COX-2, but it did not give reproducible results if concentration dependence was sought for. In this case, different dilutions were tested to investigate if the amount of $PGF_{2\alpha}$ binding in the EIA was affected by the dilution factor. The positive control (celecoxib) showed consistently more than 80% inhibition, but samples (e.g. ginsenoside Re) did not give any inhibition. Only plant extracts (GT and GC) provided more than 75% inhibition and they were further analysed by our second methodology. Recently, published reports using this *in vitro* assay testing natural materials (bee pollen, *Ajuga bracteosa*) and quercetin reported concentration dependence for their samples tested (Cheong *et al.*, 2004; Maruyama *et al.*, 2010; Gautam *et al.*, 2011). Even though their samples were compared with positive controls (resveratrol, NS-398 and celecoxib), they used these inhibitors at higher concentrations (resveratrol-30 μM, NS-398-10 μM and

celecoxib-30 µM) than our experiment (5 µM). In addition, only Maruyama *et al.* (2010) reported their method of data analysis (linear regression) which it is not the most recommended method to analyse ligand binding assays. The 4-parameter analysis was used to analyse our results from the *in vitro* assay by recommendation of the manufacturer and taking into account that data from ligand binding assays is usually characterised by a sigmodial relationship between the mean response and the compound concentration (Findlay and Dillard, 2007). We found that the COX inhibitor screening assay is a useful test to evaluate the inhibitory activity of plant extracts and polyphenols, but it is recommended to be used in combination with other techniques to assure their results. As a result, another approach was applied to calculate the inhibitory activity of COX-2. An integrated methodology using an *in vitro* assay and PGE_2 measurement by LC-ESI/MS was performed. This methodology is a novel approach to determine if plant extracts and their main polyphenols have inhibitory activity. It was firstly reported using various nonsteroidal anti-inflammatory drugs (celecoxib, indomethacin and diclofenac) and resveratrol, but it has been extended to other compounds (e.g. EGCG) and plant extracts (e.g. green tea, Chinese herbs) (Cao *et al.*, 2011; Zhu *et al.*, 2014; Deng *et al.*, 2014). Inhibition of recombinant human COX-2 by aglycones/conjugated polyphenols was not consistent and results could not been replicated throughout various repetitions. During the first stage of the optimisation, it was found that after liquid-liquid extraction the absolute intensity (MS signal) of the surrogate d_4-PGE_2 was enhanced. This anomaly might suggest that the sample composition and the solvent extraction interfere with the evaluation of PGE_2 which could affect the measurement. For further experimentation, it is advised to test different organic solvents to extract PGE_2 (e.g. acetone) and spike controls with sample mix after the reaction has ended to tackle any effect by sample composition. Our experiments using EGCG and EGC showed consistent inhibition that could indicate that the *in vitro* assay is affected by the type of polyphenol or plant extract to test, as different polyphenols were analysed. For example, flavonols (quercetin and kaempferol), flavanols (EGCG and EGC) and flavanones (hesperetin) and ginsenosides were used to perform the assay. In contrast, the inhibition by plant extracts provided enough

evidence to support previous reports suggesting that GT inhibits recombinant human COX-2. The last publication using mass spectrometry to test the inhibition of COX-2 by green tea and its major polyphenols (Deng *et al.*, 2014) reported a greater IC_{50} for green tea (437.3 µg/mL) than our reported value (16.1 µg/mL) and they did not report any IC_{50} for EGCG or EGC. Other publications testing GT and its main polyphenols have mainly looked into inhibition of COX-2 expression using different cell line models (Peng *et al.*, 2006; Singh and Katiyar, 2011) and they have agreed that EGCG modulates NF-κB and reduces COX-2 mRNA expression in a dose-dependant pattern. Overall, mass spectrometry could be used as a routinely methodology to quantify PGE_2, but different parameters such as solvent extraction, sample composition and chemical structure of the analyte should be further investigated. This methodology could provide faster analysis than ELISA and could also minimise sample handling procedures.

5.2 Future work

5.2.1 5-Lipoxygenase and the methods to quantify its inhibition.

Besides prostaglandin-endoperoxide synthase 2 that catalyses the first step of arachidonic acid pathway (see Figure 4.1), there is another family of enzymes in mammalian cells that are lipid mediators derived from oxidative modification of arachidonic acid. Lipoxygenase enzymes have been related to inflammatory and immunomodulatory functions in disease, as well as homeostatic effects in normal processes (Griesser *et al.*, 2011). 5-Lipooxygenase (5-LOX) is the main enzyme controlling the synthesis of leukotrienes (LTs) and hydroxyeicosatetroenoic acid (HETE) from arachidonic acid (see Figure 5.1) (Willey *et al.*, 2008). The products of 5-LOX have been related to certain disorders such as asthma, osteoporosis, cancer (e.g. prostate, breast) and cardiovascular diseases as artherosclerosis and stroke (Werz, 2007).

Numerous metabolites can be examined to determine the activity of 5-LOX. For example, 5-HETE is typically measured due to its stability and removes the possibility of interference due to additional enzymes (e.g. LTA_4

hydrolase) (Willey *et al.*, 2008). Researches have focused their efforts towards two types of systems: cell models and cell-free assays.

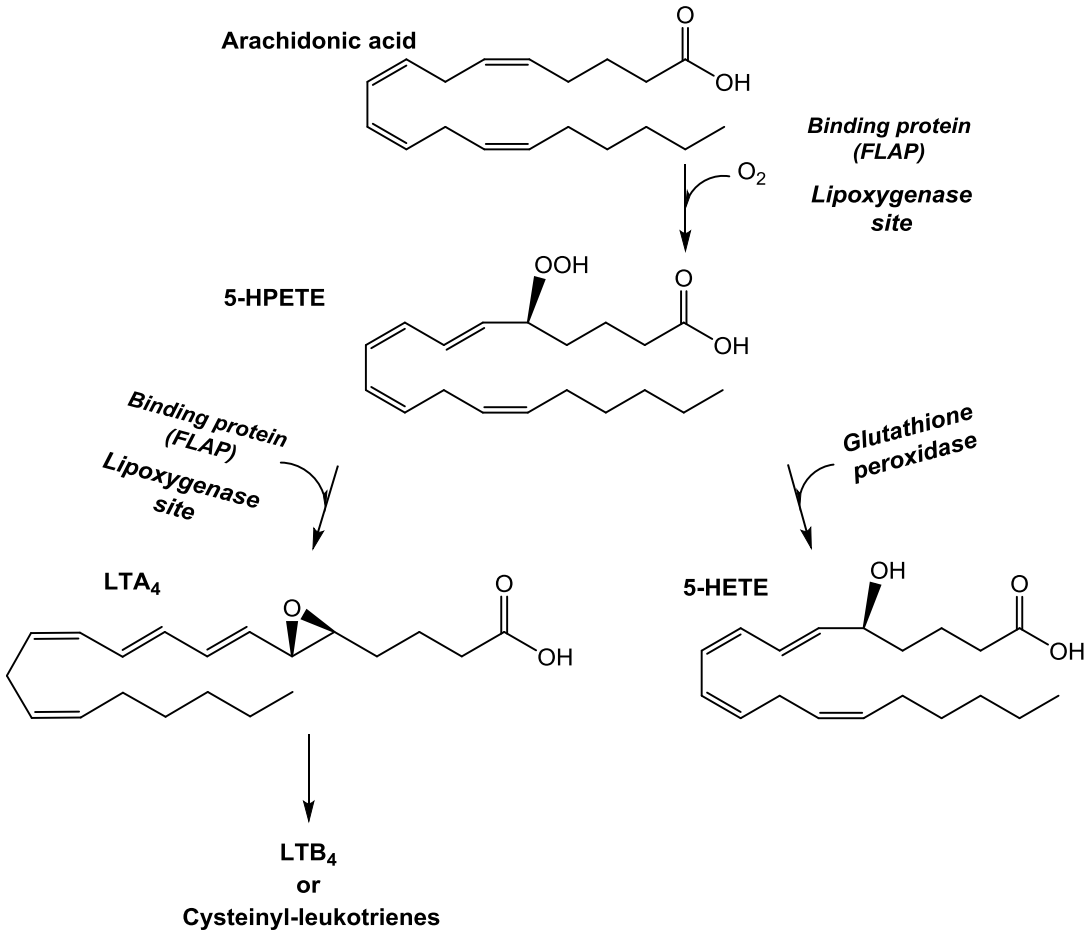


Figure 5.1 Conversion of arachidonic acid by 5-lipoxygenase. 5-HPETE: 5-hydroperoxyeicosatetraeinoic acid, 5-HETE: 5-hydroxyeicosatetraenoic acid, FLAP: 5-lipoxygenase activating protein, LTA_4: leukotriene A_4 and LTB_4: leukotriene B_4.

Cell-free models have been developed to be simpler and rapid assays which could decrease the time of analysis and contribute to the understanding of 5-LOX inhibitors. For instance, Lu *et al.* (2013) have proposed a colorimetric assay to indirectly measure 5-LOX activity using the ferric thiocyanate (FTC) method. Once the LOX-derived lipid peroxidases have been produced, they oxidise the ferrous ion (Fe^{2+}) to the ferric ion (Fe^{3+}) which binds with thiocyanate (SCN^-) and generates the red ferrithiocyanate (FTC) complex. In brief, the method is performed as follows: 95 µL of assay buffer (Tris-HCl, 50 mM, pH 7.5) is added to wells (96-well plate) in combination with recombinant human LOX. Then, 5 µL of arachidonic acid solution (2 mM) is added and the mixture is incubated for 6 min at room

temperature. The reagent FTC is added (100 µL) to terminate the reaction and after 5 min the absorbance is measured at 480 nm. If an inhibitor is used, the compound is incubated with the enzyme for 5 min before the addition of arachidonic acid. They found that the colour development was LOX and arachidonic dependant. Also, the absorbance of the background was consistently low if arachidonic acid and FTC reagent were prepared freshly. The limit of detection was determined at 0.5 nM.

We performed a preliminary test using recombinant human LOX and two known LOX inhibitors (fisetin and quercetin). The results can be seen in Figure 5.2.

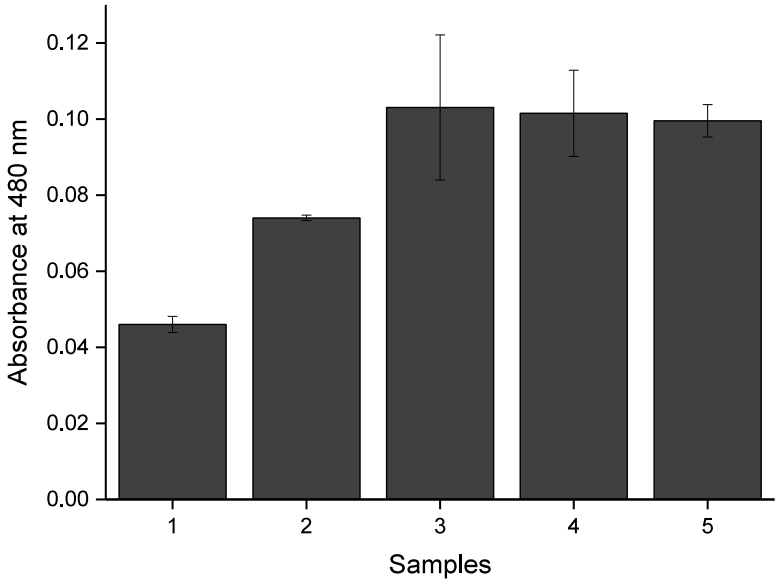


Figure 5.2 Absorbance values for different samples using the FTC complex method. 1- Blank1 (no LOX, no inhibitor), 2- Blank2 (no LOX), 3-100% activity, 4- Positive control (fisetin, 5 µM), 5- Quercetin, 5 µM. N= 2 biological replicates and 1 technical measurement.

The inhibition by fisetin or quercetin was not clear. Then, 5-LOX was compared to lipoxygenase from soybean which is used in other *in vitro* assays to determine the inhibitory activity of polyphenols (Chedea and Jisaka, 2011). We also measured the absorbance at three time points 5, 10 and 15 min. Results can be seen in Figure 5.3.

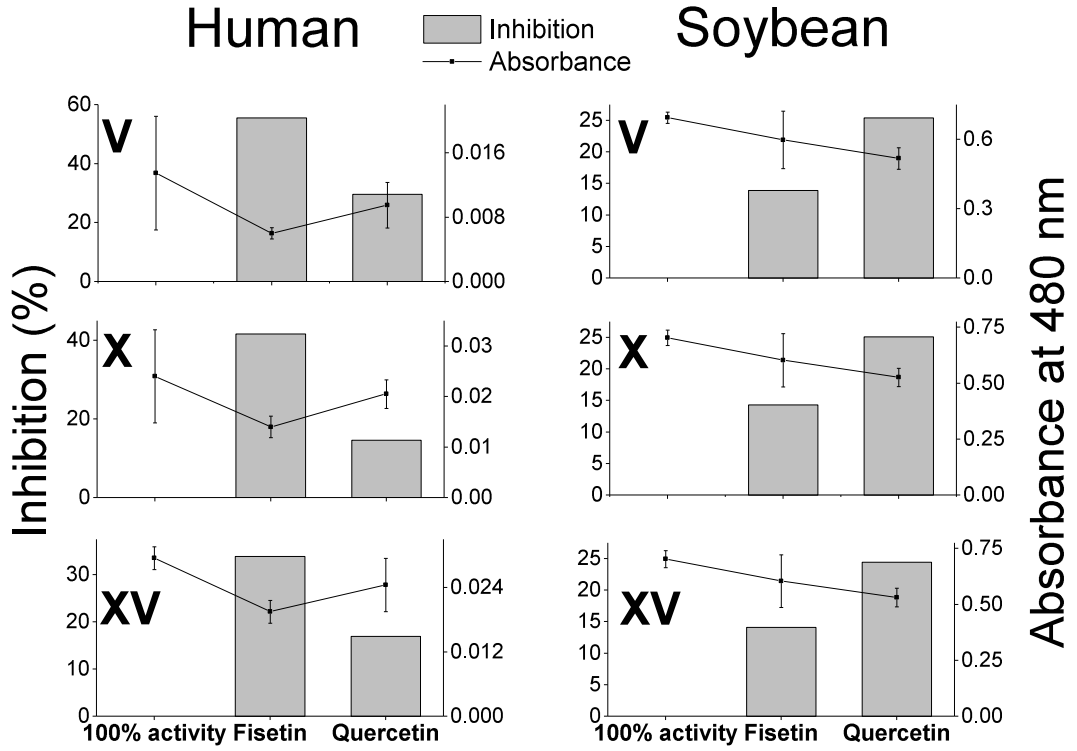


Figure 5.3 Comparison between human arachidonate-5-lipoxygenase (Human, 5-LOX) and lipoxygenase from soybean (Soybean). The Roman numberals represent the time point of measurement in min; e.g. V = 5 min. Fisetin and quercetin were tested at 5 µM. N=2 biological replicates and 1 technical measurement.

It was expected to obtain higher inhibition, as the IC_{50} for quercetin and fisetin reported by Lu *et al.* (2013) were 1.8 and 1.2 µM respectively. Further, it was noticed that absorbance values were too low for 5-LOX in comparison with lipoxygenase from soybean. This could suggest that the assay has a limited working range and it might represent an important restriction to take into account for further experiments. Even though the assay allows to process different compounds at the same time, it does not simplifies reagent preparation which could affect the replication of results.

Alternative approaches to identify and quantify the inhibition of 5-LOX have involved *in vitro* assays and mass spectrometry (Kanamoto *et al.*, 2009) which is similar to our rationale to measure the activity of recombinant human COX-2. Schewe *et al.* (2002), reported the inhibition of 5-LOX by the main polyphenols from cocoa. Their protocol uses aliquots of lysate supernatant mixed with Tris buffer (50 mM, pH 7.4) and pre-incubation for 10 min in the presence/absence of the polyphenol. Then, an assay mixture

containing ATP (1 mM), $CaCl_2$ (4 mM), EDTA (1 mM) and dipalmitoyl phosphatidylcholine (13 mg/L) is added. After 5 min more, the reaction is initiated by the addition of arachidonic acid (33 mM) in methanol and the reaction is stopped after 15 min by the addition of cold methanol. Their samples are treated with sodium borohydride and glacial acetic acid which later are centrifuged (5 min, 4 000 g) and directly used for HPLC analysis. In a similar way, Mohamed *et al.* (2014) reported the inhibition of 5-LOX by ascorbic acid 6-palmitate. They modified the method by Aharony and Stein (1986) that uses phosphate buffer (100 mM, pH 7.4), DTT (50 µM), ATP (200 µM), $CaCl_2$ (300 µM), arachidonic acid and enzyme (5 µg protein). Both protocols do not use any internal standard to correct losses by sample handling, so the surrogate d_8-5-HETE can be used for this purpose.

Furthermore, the appropriate method of detection is equally important. Various publications have reported the detection and measurement of different arachidonic acid metabolites, including 5-HETE. For instance, Kempen *et al.* (2001) measured various arachidonic metabolites (e.g. 5-HETE) in cultured tumour cells using LC-ESI/MS^2 applying a gradient protocol, ammonium acetate and methanol as mobile phases and a hydrophilic C18 column to perform the analysis. Zick *et al.* (2011), employed a LC-ESI/MS^2 to test ginger and its inhibitory effect in the production of eicosanoids in the colonic mucosa of healthy volunteers. The eicosanoid levels were measured by a linear methanol gradient and a phenyl-hexyl chromatographic column. Then, Yue *et al.* (2007) developed a method in LC-ESI/MS to measure simultaneously arachidonic acid and eicosanoids from cyclooxygenase and lipoxygenase pathways in rat brain tissue. They used a gradient protocol, acidified water and acetonitrile with formic acid as mobile phases and a C18 column for the separation. Finally, Blewett *et al.* (2008) developed a method utilising LC-ESI/MS to separate and quantify 23 different eicosanoids and their method was applied to rat kidney tissue. They employed a gradient protocol, acidified water and acetonitrile with formic acid as mobile phases and a C18 column for the separation. Once this information was gathered, we performed a preliminary identification of 5-HETE and its surrogate d_8-5-HETE to establish the suitable conditions for our LC-ESI/MS equipment. First, we modified the method by Blewett *et al.*

(2008). In brief, a Waters XTerra MS C18 column was used to perform the separation and it was kept at 40°C. The mobile phase A consisted of acidified water (0.1% formic acid, v/v) and mobile phase B of acetonitrile with 0.1% formic acid (v/v). The flow rate was set at 0.150 mL/min. The gradient elution started at 40% B and it was kept for 1.4 min. Then, solvent B increased up to 65% at 3.5 min and it was hold up to 5.6 min. Solvent B raised to 90% at 6.3 min and it was hold up to 7.7 min. Finally, the column re-equilibrated to 40% from 7.7 to 8.5 min. The injection volume was 2 µL. The chromatography obtained from this test can be seen in Figure 5.4

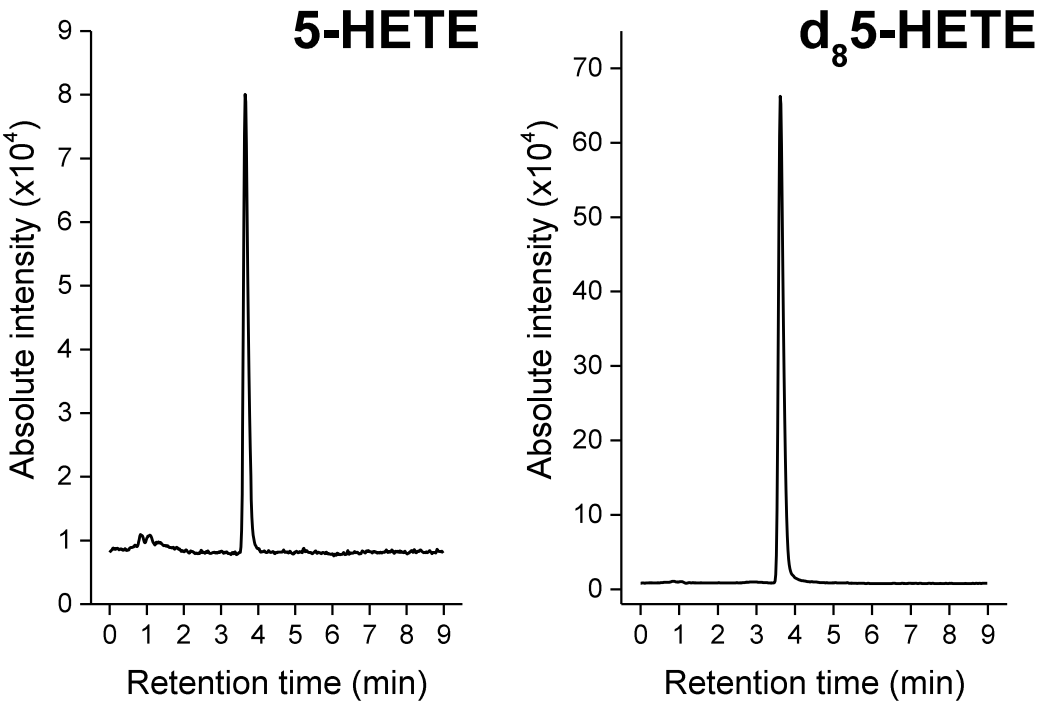


Figure 5.4 MS chromatography for the total ion counting (TIC) for 0.5 µM 5-HETE and 5 µM d_8-5-HETE.

The standards were measured at two different concentrations: 0.5 µM for 5-HETE and 5 µM for d_8-5-HETE, as we wanted to test if 10x higher concentration will show 10x more absolute intensity. The result agreed with our rationale. Next, we measured both standards at a lower concentration (100 nM). The MS chromatography can be seen in Figure 5.5.

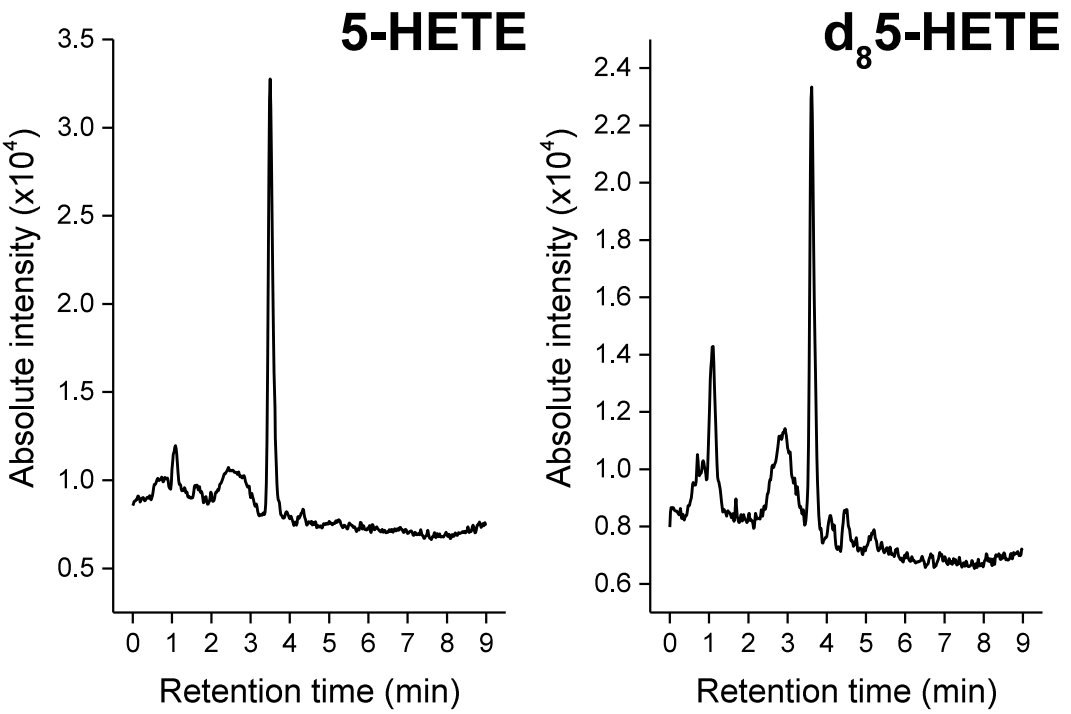


Figure 5.5 Total ion counting (TIC) for 5-HETE and d_8-5-HETE. Both standards were measured at 100 nM.

It was noticed that the chromatography was less clear than the first attempt which could suggest that background noise will interfere with the accurate measurement of each metabolite. At last, an *in vitro* assay was performed following a modified method by Mohamed *et al.* (2014). A mixture containing phosphate buffer (100 mM, pH 7.4), DTT (50 µM), ATP (200 µM) and $CaCl_2$ (300 µM) was pre-incubated with 5-LOX (0.1 µg) and the inhibitor zileuton (5 µM) for 2 min at room temperature. Then, arachidonic acid (150 µM) was added and 5 min later the reaction was stopped with HCl (6N). The surrogate, d_8-5-HETE was added (100 nM) and the mixture solution was extracted twice with diethyl ether:hexane (50:50, v/v). The combined supernatants were dried under a N_2 stream and reconstituted with methanol:water (50:50, v/v) prior to LC-MS analysis. Remaining samples were kept at -20°C. Even though the target metabolites, 5-HETE and d_8-5-HETE were visible, the chromatography was more instable than previous tests (see Figure 5.6).

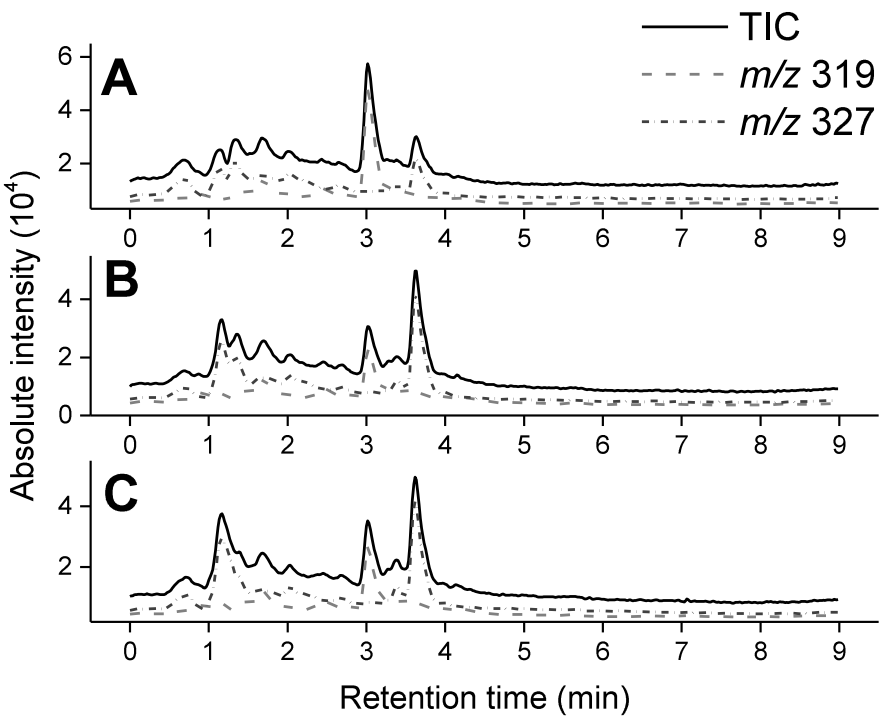


Figure 5.6 Total ion counting (TIC) and negative mass-to-charge ratio (*m/z*) for three different samples and each sought metabolite (319:5-HETE, 327:d_8-5-HETE) . A- blank, no enzyme (5-LOX) and no inhibitor, B- 100% activity, no inhibitor and C- zileuton (5µM).

Overall, the *in vitro* assay and mass spectrometry methodologies require a systematic-method development which could provide with a new validated method to identify and quantify the inhibitory activity of 5-LOX of plant based materials and their main polyphenols.

5.2.2 Plant extracts and future research

Plant extracts and plant-derived compounds are highly sought by the Western society as an alternative to prevent or treat diseases. Although some of the effects and doses have been suggested by the Traditional Medicine, there is still a lack of scientific background that validates this knowledge. The results reported in this thesis have shown that plant extracts, after ingestion, undergo a series of transformations which lead to a change in their biological activity. These effects would need to be deeply investigated and, in the future, tested in clinical trials.

Further research in plant extracts should show the composition of the plant first, then look for its changes after ingestion and finally establish a profile of the possible compounds to be metabolised. It is important to pay

attention of the type of compounds found (chemical structures) as their differences can determine their possible behaviour. Indeed, conjugated metabolites should be synthesised to confirm their occurrence after human ingestion which could be used in *in vitro* assays to test the possible interactions with target analytes, such as pro-inflammatory enzymes.

It is important to address that quantification of conjugated metabolites using enzymatic deconjugation should be used carefully or, if it is possible, to be avoided as it could lead to incorrect measurements. Analysis of these metabolites in biological samples (urine/blood) and concentration-dependence interventions should be targets in future work on plant extracts.

The information already documented about the possible effects of the plant extracts should be taken into account to evaluate the biological activity of the extract and its metabolites. For example, our research focused on pro-inflammatory enzymes as different publications suggested the positive effects of the plant extracts against inflammation. Certainly, these effects can be measured by different techniques such as animal models, cell models or cell-free assays which could be used independently or combined. In addition, equipment that is already used in pharmacokinetics as HPLC or LC-MS can help to minimise the time of analysis and reduce the cost of the investigation. It is also necessary to detect the toxic dose of the plant extracts to prevent any adverse effects. Overall, all this data could promote or dismiss the use of plant extracts in the future.

CHAPTER 6 References

Aggarwal, B. B. and Shishodia, S. 2006. Molecular targets of dietary agents for prevention and therapy of cancer. *Biochemical Pharmacology.* **71**(10), pp.1397-1421.

Aharony, D. and Stein, R. L. 1986. Kinetic mechanism of guinea pig neutrophil 5-lipoxygenase. *Journal of Biological Chemistry.* **261**(25), pp.11512-11519.

Aherne, S. A. and O'Brien, N. M. 2002. Dietary flavonols: chemistry, food content, and metabolism. *Nutrition.* **18**(1), pp.75-81.

Alfaro, S., Mutis, A., Palma, R., Quiroz, A., Seguel, I. and Scheuermann, E. 2013. Influence of genotype and harvest year on polyphenol content and antioxidant activity in murtilla (Ugni molinae Turcz) fruit. *Journal of Soil Science and Plant Nutrition.* **13**, pp.67-78.

Avula, B., Wang, Y. H., Wang, M., Avonto, C., Zhao, J., Smillie, T. J. and Khan, I. A. 2013. Determination and Characterization of Polyphenolic Constituents from Flowers of Chamomile Samples using UHPLC-QTOF-MS. *Planta Medica.* **79**(05), p128.

Beekmann, K., Actis-Goretta, L., van Bladeren, P. J., Dionisi, F., Destaillats, F. and Rietjens, I. M. C. M. 2012. A state-of-the-art overview of the effect of metabolic conjugation on the biological activity of flavonoids. *Food & Function.* **3**(10), pp.1008-1018.

Blewett, A. J., Varma, D., Gilles, T., Libonati, J. R. and Jansen, S. A. 2008. Development and validation of a high-performance liquid chromatography–electrospray mass spectrometry method for the simultaneous determination of 23 eicosanoids. *Journal of Pharmaceutical and Biomedical Analysis.* **46**(4), pp.653-662.

Bodeker, G. and Kronenberg, F. 2002. A Public Health Agenda for Traditional, Complementary, and Alternative Medicine. *American Journal of Public Health.* **92**(10), pp.1582-1591.

Boiteux, J., Vargas, C. S., Pizzuolo, P., Lucero, G. and Silva, M. F. 2014. Phenolic characterization and antimicrobial activity of folk medicinal plant extracts for their applications in olive production. *Electrophoresis.* **35**(11), pp.1709-1718.

Bokkenheuser, V. D., Shackleton, C. H. and Winter, J. 1987. Hydrolysis of dietary flavonoid glycosides by strains of intestinal Bacteroides from humans. *Biochemical Journal.* **248**(3), pp.953-956.

Bollinger, J. G., Thompson, W., Lai, Y., Oslund, R. C., Hallstrand, T. S., Sadilek, M., Turecek, F. and Gelb, M. H. 2010. Improved Sensitivity Mass Spectrometric Detection of Eicosanoids by Charge Reversal Derivatization. *Analytical Chemistry.* **82**(16), pp.6790-6796.

Brand, W., Boersma, M. G., Bik, H., Hoek-van den Hil, E. F., Vervoort, J., Barron, D., Meinl, W., Glatt, H., Williamson, G., van Bladeren, P. J. and Rietjens, I. M. C. M. 2010. Phase II Metabolism of Hesperetin by Individual UDP-Glucuronosyltransferases and Sulfotransferases and Rat and Human Tissue Samples. *Drug Metabolism and Disposition.* **38**(4), pp.617-625.

Bredsdorff, L., Nielsen, I. L. F., Rasmussen, S. E., Cornett, C., Barron, D., Bouisset, F., Offord, E. and Williamson, G. 2010. Absorption, conjugation and excretion of the flavanones, naringenin and hesperetin from α-rhamnosidase-treated orange juice in human subjects. *British Journal of Nutrition.* **103**(11), pp.1602-1609.

Brett, G. M., Hollands, W., Needs, P. W., Teucher, B., R. Dainty, J., Davis, B. D., Brodbelt, J. S. and Kroon, P. A. 2009. Absorption, metabolism and excretion of flavanones from single portions of orange fruit and juice and effects of anthropometric variables and contraceptive pill use on flavanone excretion. *British Journal of Nutrition.* **101**(05), pp.664-675.

Brose, S. A., Thuen, B. T. and Golovko, M. Y. 2011. LC/MS/MS method for analysis of E2 series prostaglandins and isoprostanes. *Journal of Lipid Research.* **52**(4), pp.850-859.

BSDA. 2014. *The 2014 UK Soft Drinks Report.* United Kingdom: British Soft Drinks Association.

Cai, Y., Luo, Q., Sun, M. and Corke, H. 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences.* **74**(17), pp.2157-2184.

Calapai, G. and Caputi, A. P. 2007. Herbal Medicines: Can We Do without Pharmacologist? *Evidence-Based Complementary and Alternative Medicine.* **4**(S1), pp.41-43.

Campbell, N. R. C., Van Loon, J. A. and Weinshilboum, R. M. 1987. Human liver phenol sulfotransferase: Assay conditions, biochemical properties and partial purification of isozymes of the thermostable form. *Biochemical Pharmacology.* **36**(9), pp.1435-1446.

Cao, H., Yu, R., Choi, Y., Ma, Z.-Z., Zhang, H., Xiang, W., Lee, D. Y.-W., Berman, B. M., Moudgil, K. D., Fong, H. H. S. and van Breemen, R. B. 2010. Discovery of cyclooxygenase inhibitors from medicinal plants used to treat inflammation. *Pharmacological research : the Official Journal of the Italian Pharmacological Society.* **61**(6), pp.519-524.

Cao, H., Yu, R., Tao, Y., Nikolic, D. and van Breemen, R. B. 2011. Measurement of cyclooxygenase inhibition using liquid chromatography–tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis.* **54**(1), pp.230-235.

Cerella, C., Sobolewski, C., Dicato, M. and Diederich, M. 2010. Targeting COX-2 expression by natural compounds: A promising alternative strategy to synthetic COX-2 inhibitors for cancer chemoprevention and therapy. *Biochemical Pharmacology.* **80**(12), pp.1801-1815.

Charoensuk, P. 2014. *The metabolism and excretion of quercetin and (-)-epicatechin after co-consumption of onion soup and dark chocolate by healthy volunteers*. Doctor of Philosophy thesis, University of Leeds.

Chedea, V. S. and Jisaka, M. 2011. Inhibition of Soybean Lipoxygenases - Structural and Activity Models for the Lipoxygenase Isoenzymes Family. In: Krezhova, D. D. ed. *Recent Trends for Enhancing the Diversity and Quality of Soybean Products.*

Cheong, E., Ivory, K., Doleman, J., Parker, M. L., Rhodes, M. and Johnson, I. T. 2004. Synthetic and naturally occurring COX-2 inhibitors suppress proliferation in a human oesophageal adenocarcinoma cell line (OE33) by inducing apoptosis and cell cycle arrest. *Carcinogenesis.* **25**(10), pp.1945-1952.

Chow, H. H. S. and Hakim, I. A. 2011. Pharmacokinetic and chemoprevention studies on tea in humans. *Pharmacological Research.* **64**(2), pp.105-112.

Crozier, A., Jaganath, I. B. and Clifford, M. N. 2009. Dietary phenolics: chemistry, bioavailability and effects on health. *Natural Product Reports.* **26**(8), pp.1001-1043.

D'Archivio, M., Filesi, C., Varì, R., Scazzocchio, B. and Masella, R. 2010. Bioavailability of the Polyphenols: Status and Controversies. *International Journal of Molecular Sciences.* **11**(4), pp.1321-1342.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M., Borges, G. and Crozier, A. 2012. Dietary (Poly)phenolics in Human Health: Structures, Bioavailability, and Evidence of Protective Effects Against Chronic Diseases. *Antioxidants & Redox Signaling.* **18**(14), pp.1818-1892.

Deng, X., Shi, S., Li, S. and Yang, T. 2014. Magnetic ligand fishing combination with high-performance liquid chromatography–diode array detector–mass spectrometry to screen and characterize cyclooxygenase-2 inhibitors from green tea. *Journal of Chromatography B.* **973**(0), pp.55-60.

Dew, T. P., Wang, G. and Williamson, G. 2014. Urinary excretion of ginkgolide terpene lactones following acute consumption of Ginkgo biloba extract. *BioFactors.* **40**(2), pp.268-274.

Dueñas, M., Surco-Laos, F., González-Manzano, S., González-Paramás, A. and Santos-Buelga, C. 2011. Antioxidant properties of major metabolites of quercetin. *European Food Research and Technology.* **232**(1), pp.103-111.

EFSA, E. F. S. A. 2012. Compendium of botanicals reported to contain naturally ocurring substances of possible concern for human health when used in food and food supplements. *EFSA Journal.* **10**(5), p60.

Eid, N. M. S., Al-Awadi, B., Vauzour, D., Oruna-Concha, M. J. and Spencer, J. P. E. 2013. Effect of Cultivar Type and Ripening on the Polyphenol Content of Date Palm Fruit. *Journal of Agricultural and Food Chemistry.* **61**(10), pp.2453-2460.

Erlund, I., Meririnne, E., Alfthan, G. and Aro, A. 2001. Plasma Kinetics and Urinary Excretion of the Flavanones Naringenin and Hesperetin in Humans after Ingestion of Orange Juice and Grapefruit Juice. *The Journal of Nutrition.* **131**(2), pp.235-241.

Evans, D. A., Hirsch, J. B. and Dushenkov, S. 2006. Phenolics, inflammation and nutrigenomics. *Journal of the Science of Food and Agriculture.* **86**(15), pp.2503-2509.

Farrell, T., Poquet, L., Dionisi, F., Barron, D. and Williamson, G. 2011. Characterization of hydroxycinnamic acid glucuronide and sulfate conjugates by HPLC–DAD–MS2: Enhancing chromatographic quantification and application in Caco-2 cell metabolism. *Journal of Pharmaceutical and Biomedical Analysis.* **55**(5), pp.1245-1254.

Findlay, J. A. and Dillard, R. 2007. Appropriate calibration curve fitting in ligand binding assays. *The AAPS Journal.* **9**(2), pp.E260-E267.

Gamage, N., Barnett, A., Hempel, N., Duggleby, R. G., Windmill, K. F., Martin, J. L. and McManus, M. E. 2006. Human Sulfotransferases and Their Role in Chemical Metabolism. *Toxicological Sciences.* **90**(1), pp.5-22.

Garcia-Alvarez, A., Egan, B., de Klein, S., Dima, L., Maggi, F. M., Isoniemi, M., Ribas-Barba, L., Raats, M. M., Meissner, E. M., Badea, M., Bruno, F., Salmenhaara, M., Mila-Villarroel, R., Knaze, V., Hodgkins, C., Marculescu, A., Uusitalo, L., Restani, P. and Serra-Majem, L. 2014. Usage of plant food supplements across six European countries: findings from the PlantLIBRA consumer survey. *PLoS One.* **9**(3), pe92265.

Gautam, R., Jachak, S. M. and Saklani, A. 2011. Anti-inflammatory effect of Ajuga bracteosa Wall Ex Benth. mediated through cyclooxygenase (COX) inhibition. *Journal of Ethnopharmacology.* **133**(2), pp.928-930.

Georgé, S., Brat, P., Alter, P. and Amiot, M. J. 2005. Rapid Determination of Polyphenols and Vitamin C in Plant-Derived Products. *Journal of Agricultural and Food Chemistry.* **53**(5), pp.1370-1373.

Golan-Goldhirsh, A. and Gopas, J. 2014. Plant derived inhibitors of NF-κB. *Phytochemistry Reviews.* **13**(1), pp.107-121.

Greene, E. R., Huang, S., Serhan, C. N. and Panigrahy, D. 2011. Regulation of inflammation in cancer by eicosanoids. *Prostaglandins & Other Lipid Mediators.* **96**(1–4), pp.27-36.

Griesser, M., Suzuki, T., Tejera, N., Mont, S., Boeglin, W. E., Pozzi, A. and Schneider, C. 2011. Biosynthesis of hemiketal eicosanoids by cross-over of the 5-lipoxygenase and cyclooxygenase-2 pathways. *Proceedings of the National Academy of Sciences.* **108**(17), pp.6945-6950.

Guimaraes, R., Barros, L., Duenas, M., Calhelha, R. C., Carvalho, A. M., Santos-Buelga, C., Queiroz, M. and Ferreira, I. 2013. Infusion and decoction of wild German chamomile: Bioactivity and characterization of organic acids and phenolic compounds. *Food Chemistry.* **136**(2), pp.947-954.

Guimarães, R., Barros, L., Dueñas, M., Calhelha, R. C., Carvalho, A. M., Santos-Buelga, C., Queiroz, M. J. R. P. and Ferreira, I. C. F. R. 2013. Nutrients, phytochemicals and bioactivity of wild Roman chamomile: A comparison between the herb and its preparations. *Food Chemistry.* **136**(2), pp.718-725.

Huang, M. T., Ghai, G. and Ho, C. T. 2004. Inflammatory Process and Molecular Targets for Antiinflammatory Nutraceuticals. *Comprehensive Reviews in Food Science and Food Safety.* **3**(4), pp.127-139.

Ignat, I., Volf, I. and Popa, V. I. 2011. A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry.* **126**(4), pp.1821-1835.

Kanamoto, H., Takemura, M. and Ohyama, K. 2009. Detection of 5-Lipoxygenase Activity in the Liverwort *Marchantia polymorpha L. Bioscience, Biotechnology, and Biochemistry.* **73**(11), pp.2549-2551.

Kato, A., Minoshima, Y., Yamamoto, J., Adachi, I., Watson, A. A. and Nash, R. J. 2008. Protective Effects of Dietary Chamomile Tea on Diabetic Complications. *Journal of Agricultural and Food Chemistry.* **56**(17), pp.8206-8211.

Kean, R. J., Lamport, D. J., Dodd, G. F., Freeman, J. E., Williams, C. M., Ellis, J. A., Butler, L. T. and Spencer, J. P. 2015. Chronic

consumption of flavanone-rich orange juice is associated with cognitive benefits: an 8-wk, randomized, double-blind, placebo-controlled trial in healthy older adults. *The American Journal of Clinical Nutrition.* **101**(3), pp.506-514.

Kempen, E. C., Yang, P., Felix, E., Madden, T. and Newman, R. A. 2001. Simultaneous Quantification of Arachidonic Acid Metabolites in Cultured Tumor Cells Using High-Performance Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry. *Analytical Biochemistry.* **297**(2), pp.183-190.

Khan, M. K., Zill, E. H. and Dangles, O. 2014. A comprehensive review on flavanones, the major citrus polyphenols. *Journal of Food Composition and Analysis.* **33**(1), pp.85-104.

Kim, D. H., Chung, J. H., Yoon, J. S., Ha, Y. M., Bae, S., Lee, E. K., Jung, K. J., Kim, M. S., Kim, Y. J., Kim, M. K. and Chung, H. Y. 2013. Ginsenoside Rd inhibits the expressions of iNOS and COX-2 by suppressing NF-κB in LPS-stimulated RAW264.7 cells and mouse liver. *Journal of Ginseng Research.* **37**(1), pp.54-63.

Koizumi, M., Akao, T., Kadota, S., Kikuchi, T., Okuda, T. and Kobashi, K. 1991. Enzymatic Sulfation of Polyphenols Related to Tannins by Arylsulfotransferase. *Chemical & Pharmaceutical Bulletin.* **39**(10), pp.2638-2643.

Koizumi, M., Shimizu, M. and Kobashi, K. 1990. Enzymatic Sulfation of Quercetin by Arylsulfotransferase from a Human Intestinal Bacterium. *Chemical & Pharmaceutical Bulletin.* **38**(3), pp.794-796.

Kotakadi, V. S., Jin, Y., Hofseth, A. B., Ying, L., Cui, X., Volate, S., Chumanevich, A., Wood, P. A., Price, R. L., McNeal, A., Singh, U. P., Singh, N. P., Nagarkatti, M., Nagarkatti, P. S., Matesic, L. E., Auclair, K., Wargovich, M. J. and Hofseth, L. J. 2008. Ginkgo biloba extract EGb 761 has anti-inflammatory properties and ameliorates colitis in mice by driving effector T cell apoptosis. *Carcinogenesis.* **29**(9), pp.1799-1806.

Krogholm, K. S., Bredsdorff, L., Knuthsen, P., Haraldsdottir, J. and Rasmussen, S. E. 2010. Relative bioavailability of the flavonoids

quercetin, hesperetin and naringenin given simultaneously through diet. *European Journal of Clinical Nutrition.* **64**(4), pp.432-435.

Kroon, P. A., Clifford, M. N., Crozier, A., Day, A. J., Donovan, J. L., Manach, C. and Williamson, G. 2004. How should we assess the effects of exposure to dietary polyphenols in vitro? *The American Journal of Clinical Nutrition.* **80**(1), pp.15-21.

Lin, L. Z. and Harnly, J. M. 2012. LC-PDA-ESI/MS Identification of the Phenolic Components of Three Compositae Spices: Chamomile, Tarragon, and Mexican Arnica. *Natural Product Communications.* **7**(6), pp.749-752.

Lotito, S. B., Zhang, W.-J., Yang, C. S., Crozier, A. and Frei, B. 2011. Metabolic conversion of dietary flavonoids alters their anti-inflammatory and antioxidant properties. *Free radical biology & medicine.* **51**(2), pp.454-463.

Lu, W., Zhao, X., Xu, Z., Dong, N., Zou, S., Shen, X. and Huang, J. 2013. Development of a new colorimetric assay for lipoxygenase activity. *Analytical Biochemistry.* **441**(2), pp.162-168.

Manach, C., Morand, C., Gil-Izquierdo, A., Bouteloup-Demange, C. and Remesy, C. 2003. Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. *European Journal of Clinical Nutrition.* **57**(2), pp.235-242.

Manach, C., Scalbert, A., Morand, C., Rémésy, C. and Jiménez, L. 2004. Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition.* **79**(5), pp.727-747.

Maruyama, H., Sakamoto, T., Araki, Y. and Hara, H. 2010. Anti-inflammatory effect of bee pollen ethanol extract from Cistus sp. of Spanish on carrageenan-induced rat hind paw edema. *BMC Complementary and Alternative Medicine.* **10**(1), p30.

Matic, I. Z., Juranic, Z., Savikin, K., Zdunic, G., Nadvinski, N. and Godevac, D. 2013. Chamomile and marigold tea: chemical characterization and evaluation of anticancer activity. *Phytotherapy Research.* **27**(6), pp.852-8.

Medzhitov, R. 2008. Origin and physiological roles of inflammation. *Nature.* **454**(7203), pp.428-435.

Menozzi-Smarrito, C., Wong, C. C., Meinl, W., Glatt, H., Fumeaux, R., Munari, C., Robert, F., Williamson, G. and Barron, D. 2011. First Chemical Synthesis and in Vitro Characterization of the Potential Human Metabolites 5-O-Feruloylquinic Acid 4′ -Sulfate and 4′ -O-Glucuronide. *Journal of Agricultural and Food Chemistry.* **59**(10), pp.5671-5676.

Miksits, M., Maier-Salamon, A., Aust, S., Thalhammer, T., Reznicek, G., Kunert, O., Haslinger, E., Szekeres, T. and Jaeger, W. 2005. Sulfation of resveratrol in human liver: Evidence of a major role for the sulfotransferases SULT1A1 and SULT1E1. *Xenobiotica.* **35**(12), pp.1101-1119.

Millington, C., Sonego, S., Karunaweera, N., Rangel, A., Aldrich-Wright, J. R., Campbell, I. L., Gyengesi, E. and Munch, G. 2014. Chronic Neuroinflammation in Alzheimer's Disease: New Perspectives on Animal Models and Promising Candidate Drugs. *BioMed Research International.* **2014**, p10.

Mohamed, R., Tarannum, S., Yariswamy, M., Vivek, H. K., Siddesha, J. M., Angaswamy, N. and Vishwanath, B. S. 2014. Ascorbic acid 6-palmitate: a potent inhibitor of human and soybean lipoxygenase-dependent lipid peroxidation. *Journal of Pharmacy and Pharmacology.* **66**(6), pp.769-778.

Mullen, W., Borges, G., Lean, M. E. J., Roberts, S. A. and Crozier, A. 2010. Identification of Metabolites in Human Plasma and Urine after Consumption of a Polyphenol-Rich Juice Drink. *Journal of Agricultural and Food Chemistry.* **58**(4), pp.2586-2595.

Nakajima, V. M., Macedo, G. A. and Macedo, J. A. 2014. Citrus bioactive phenolics: Role in the obesity treatment. *LWT - Food Science and Technology.* **59**(2, Part 2), pp.1205-1212.

Nielsen, I. L. F., Chee, W. S. S., Poulsen, L., Offord-Cavin, E., Rasmussen, S. E., Frederiksen, H., Enslen, M., Barron, D., Horcajada, M.-N. and Williamson, G. 2006. Bioavailability Is Improved by Enzymatic Modification of the Citrus Flavonoid Hesperidin in Humans: A Randomized, Double-Blind, Crossover Trial. *The Journal of Nutrition.* **136**(2), pp.404-408.

Novakova, L., Vildova, A., Mateus, J. P., Goncalves, T. and Solich, P. 2010. Development and application of UHPLC-MS/MS method for the determination of phenolic compounds in Chamomile flowers and Chamomile tea extracts. *Talanta.* **82**(4), pp.1271-1280.

O'Leary, K. A., Day, A. J., Needs, P. W., Sly, W. S., O'Brien, N. M. and Williamson, G. 2001. Flavonoid glucuronides are substrates for human liver β-glucuronidase. *FEBS Letters.* **503**(1), pp.103-106.

O'Leary, K. A., Pascual-Tereasa, S. d., Needs, P. W., Bao, Y.-P., O'Brien, N. M. and Williamson, G. 2004. Effect of flavonoids and Vitamin E on cyclooxygenase-2 (COX-2) transcription. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis.* **551**(1–2), pp.245-254.

Pan, M.-H., Lai, C.-S., Dushenkov, S. and Ho, C.-T. 2009. Modulation of Inflammatory Genes by Natural Dietary Bioactive Compounds. *Journal of Agricultural and Food Chemistry.* **57**(11), pp.4467-4477.

Pandey, K. B. and Rizvi, S. I. 2009. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity.* **2**(5), pp.270-8.

Paulsen, E. 2002. Contact sensitization from Compositae-containing herbal remedies and cosmetics. *Contact Dermatitis.* **47**(4), pp.189-198.

Peng, G., Dixon, D. A., Muga, S. J., Smith, T. J. and Wargovich, M. J. 2006. Green tea polyphenol (−)-epigallocatechin-3-gallate inhibits cyclooxygenase-2 expression in colon carcinogenesis. *Molecular Carcinogenesis.* **45**(5), pp.309-319.

Pereira-Caro, G., Borges, G., van der Hooft, J., Clifford, M. N., Del Rio, D., Lean, M. E., Roberts, S. A., Kellerhals, M. B. and Crozier, A. 2014. Orange juice (poly)phenols are highly bioavailable in humans. *The American Journal of Clinical Nutrition.* **100**(5), pp.1378-1384.

Petrovic, N. and Murray, M. 2010. Using N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) to Assay Cyclooxygenase Activity In Vitro. In: Armstrong, D. ed. *Advanced Protocols in Oxidative Stress II.* Humana Press, pp.129-140.

Pimpão, R. C., Dew, T., Oliveira, P. B., Williamson, G., Ferreira, R. B. and Santos, C. N. 2013. Analysis of Phenolic Compounds in Portuguese

Wild and Commercial Berries after Multienzyme Hydrolysis. *Journal of Agricultural and Food Chemistry.* **61**(17), pp.4053-4062.

Raal, A., Orav, A., Püssa, T., Valner, C., Malmiste, B. and Arak, E. 2012. Content of essential oil, terpenoids and polyphenols in commercial chamomile (Chamomilla recutita L. Rauschert) teas from different countries. *Food Chemistry.* **131**(2), pp.632-638.

Raman, P., DeWitt, D. L. and Nair, M. G. 2008. Lipid peroxidation and cyclooxygenase enzyme inhibitory activities of acidic aqueous extracts of some dietary supplements. *Phytotherapy Research.* **22**(2), pp.204-212.

Roman-Ramos, R., Almanza-Perez, J. C., Fortis-Barrera, A., Angeles-Mejia, S., Banderas-Dorantes, T. R., Zamilpa-Alvarez, A., Diaz-Flores, M., Jasso, I., Blancas-Flores, G., Gomez, J. and Alarcon-Aguilar, F. J. 2012. Antioxidant and Anti-Inflammatory Effects of a Hypoglycemic Fraction from Cucurbita ficifolia Bouché in Streptozotocin-Induced Diabetes Mice. *The American Journal of Chinese Medicine.* **40**(01), pp.97-110.

Saha, S., Hollands, W., Needs, P. W., Ostertag, L. M., de Roos, B., Duthie, G. G. and Kroon, P. A. 2012. Human O-sulfated metabolites of (−)-epicatechin and methyl-(−)-epicatechin are poor substrates for commercial aryl-sulfatases: Implications for studies concerned with quantifying epicatechin bioavailability. *Pharmacological Research.* **65**(6), pp.592-602.

Salvado, M. D., Alfranca, A., Haeggström, J. Z. and Redondo, J. M. 2012. Prostanoids in tumor angiogenesis: therapeutic intervention beyond COX-2. *Trends in Molecular Medicine.* **18**(4), pp.233-243.

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

Schewe, T., Kühn, H. and Sies, H. 2002. Flavonoids of Cocoa Inhibit Recombinant Human 5-Lipoxygenase. *The Journal of Nutrition.* **132**(7), pp.1825-1829.

Schneider, C. and Pozzi, A. 2011. Cyclooxygenases and lipoxygenases in cancer. *Cancer and Metastasis Reviews.* **30**(3-4), pp.277-294.

Silveira, J. Q., Cesar, T. B., Manthey, J. A., Baldwin, E. A., Bai, J. and Raithore, S. 2014. Pharmacokinetics of Flavanone Glycosides after Ingestion of Single Doses of Fresh-Squeezed Orange Juice versus Commercially Processed Orange Juice in Healthy Humans. *Journal of Agricultural and Food Chemistry.* **62**(52), pp.12576-12584.

Singh, O., Khanam, Z., Misra, N. and Srivastava, M. K. 2011. Chamomile (Matricaria chamomilla L.): An overview. *Pharmacognosy Reviews.* **5**(9), pp.82-95.

Singh, T. and Katiyar, S. K. 2011. Green Tea Catechins Reduce Invasive Potential of Human Melanoma Cells by Targeting COX-2, PGE(2) Receptors and Epithelial-to-Mesenchymal Transition. *PLoS ONE.* **6**(10), pe25224.

Smith, W. L. 2007. Nutritionally essential fatty acids and biologically indispensable cyclooxygenases. *Trends in Biochemical Sciences.* **33**(1), pp.27-37.

Solfrizzi, V. and Panza, F. 2015. Plant-Based Nutraceutical Interventions against Cognitive Impairment and Dementia: Meta-Analytic Evidence of Efficacy of a Standardized Gingko biloba Extract. *Journal of Alzheimer's Disease.* **43**(2), pp.605-611.

Srivastava, J. K. and Gupta, S. 2009. Extraction, Characterization, Stability and Biological Activity of Flavonoids Isolated from Chamomile Flowers. *Molecular and cellular pharmacology.* **1**(3), pp.138-138.

Srivastava, J. K., Pandey, M. and Gupta, S. 2009. Chamomile, a novel and selective COX-2 inhibitor with anti-inflammatory activity. *Life Sciences.* **85**(19-20), pp.663-669.

Srivastava, J. K., Shankar, E. and Gupta, S. 2010. Chamomile: A herbal medicine of the past with a bright future (Review). *Molecular Medicine Reports.* **3**(6), pp.895-901.

Stables, M. J. and Gilroy, D. W. 2011. Old and new generation lipid mediators in acute inflammation and resolution. *Progress in Lipid Research.* **50**(1), pp.35-51.

Švehlíková, V., Bennett, R. N., Mellon, F. A., Needs, P. W., Piacente, S., Kroon, P. A. and Bao, Y. 2004. Isolation, identification and stability of acylated derivatives of apigenin 7-O-glucoside from chamomile

(Chamomilla recutita [L.] Rauschert). *Phytochemistry.* **65**(16), pp.2323-2332.

Takumi, H., Nakamura, H., Simizu, T., Harada, R., Kometani, T., Nadamoto, T., Mukai, R., Murota, K., Kawai, Y. and Terao, J. 2012. Bioavailability of orally administered water-dispersible hesperetin and its effect on peripheral vasodilatation in human subjects: implication of endothelial functions of plasma conjugated metabolites. *Food & Function.* **3**(4), pp.389-398.

Tomás-Barberán, F. A. and Clifford, M. N. 2000. Flavanones, chalcones and dihydrochalcones – nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture.* **80**(7), pp.1073-1080.

Tyapochkin, E., Cook, P. F. and Chen, G. 2009. para-Nitrophenyl Sulfate Activation of Human Sulfotransferase 1A1 Is Consistent with Intercepting the E·PAP Complex and Reformation of E·PAPS. *Journal of Biological Chemistry.* **284**(43), pp.29357-29364.

Vallejo, F., Larrosa, M., Escudero, E., Zafrilla, M. a. P., Cerdá, B. a., Boza, J., García-Conesa, M. a. T., Espín, J. C. and Tomás-Barberán, F. A. 2010. Concentration and Solubility of Flavanones in Orange Beverages Affect Their Bioavailability in Humans. *Journal of Agricultural and Food Chemistry.* **58**(10), pp.6516-6524.

Wang, J., Gao, W.-Y., Zhang, J., Zuo, B.-M., Zhang, L.-M. and Huang, L.-Q. 2012a. Advances in study of ginsenoside biosynthesis pathway in Panax ginseng C. A. Meyer. *Acta Physiologiae Plantarum.* **34**(2), pp.397-403.

Wang, J., Ma, Y.-L., Wu, X.-Y., Yu, L., Xia, R., Sun, G.-X. and Wu, F.-A. 2012b. Selective hydrolysis by commercially available hesperidinase for isoquercitrin production. *Journal of Molecular Catalysis B: Enzymatic.* **81**(0), pp.37-42.

Webb, D. R. 2014. Animal models of human disease: Inflammation. *Biochemical Pharmacology.* **87**(1), pp.121-130.

Werz, O. 2007. Inhibition of 5-Lipoxygenase Product Synthesis by Natural Compounds of Plant Origin. *Planta Medica.* **73**(13), pp.1331-1357.

Whiteley, P. E. and Dalrymple, S. A. 2001. Models of Inflammation: Carrageenan-Induced Paw Edema in the Rat. *Current Protocols in Pharmacology.* John Wiley & Sons, Inc.

WHO, W. H. O. 2002. *Traditional Medicine Strategy 2002-2005.* [Online]. Geneva, Switzerland. [Accessed 2014].

WHO, W. H. O. 2014. *WHO traditional medicine strategy 2014-2023.* [Online]. Geneva, Switzerland [Accessed 2014].

Wilkinson, A. P., Wähälä, K. and Williamson, G. 2002. Identification and quantification of polyphenol phytoestrogens in foods and human biological fluids. *Journal of Chromatography B.* **777**(1–2), pp.93-109.

Willey, M. B., Alborn, W. E., Lutzke, B. S., LeLacheur, R. M., White, R. J., Stavrakis, G., Konrad, R. J. and Ackermann, B. L. 2008. The development of methodology for clinical measurement of 5-lipoxygenase pathway intermediates from human peripheral blood mononuclear cells. *Journal of Pharmaceutical and Biomedical Analysis.* **48**(5), pp.1397-1403.

Williamson, G., Barron, D., Shimoi, K. and Terao, J. 2005. In vitro biological properties of flavonoid conjugates found in vivo. *Free Radical Research.* **39**(5), pp.457-469.

Williamson, G., Coppens, P., Serra-Majem, L. and Dew, T. 2011. Review of the efficacy of green tea, isoflavones and aloe vera supplements based on randomised controlled trials. *Food & Function.* **2**(12), pp.753-759.

Xie, Y. F., Reckhow, D. A. and Rajan, R. V. 1993. Spontaneous Methylation of Haloacetic Acids in Methanolic Stock Solutions. *Environmental Science & Technology.* **27**(6), pp.1232-1234.

Yan, S. and Wu, G. 2013. Secretory pathway of cellulase: a mini-review. *Biotechnology for Biofuels.* **6**(1), p177.

Yang, H.-L., Chen, S.-C., Senthil Kumar, K. J., Yu, K.-N., Lee Chao, P.-D., Tsai, S.-Y., Hou, Y.-C. and Hseu, Y.-C. 2011. Antioxidant and Anti-Inflammatory Potential of Hesperetin Metabolites Obtained from Hesperetin-Administered Rat Serum: An Ex Vivo Approach. *Journal of Agricultural and Food Chemistry.* **60**(1), pp.522-532.

Yue, H., Jansen, S. A., Strauss, K. I., Borenstein, M. R., Barbe, M. F., Rossi, L. J. and Murphy, E. 2007. A liquid chromatography/mass spectrometric method for simultaneous analysis of arachidonic acid and its endogenous eicosanoid metabolites prostaglandins, dihydroxyeicosatrienoic acids, hydroxyeicosatetraenoic acids, and epoxyeicosatrienoic acids in rat brain tissue. *Journal of Pharmaceutical and Biomedical Analysis.* **43**(3), pp.1122-1134.

Zhu, H., Zhuang, X., Liu, S., Pi, Z., Song, F., Lin, N. and Liu, Z. 2014. Ultrahigh-performance liquid chromatography/tandem mass spectrometry method for evaluating enzyme activity and screening inhibitors of cyclooxygenase-2. *Rapid Communications in Mass Spectrometry.* **28**(16), pp.1792-1800.

Zick, S. M., Turgeon, D. K., Vareed, S. K., Ruffin, M. T., Litzinger, A. J., Wright, B. D., Alrawi, S., Normolle, D. P., Djuric, Z. and Brenner, D. E. 2011. Phase II Study of the Effects of Ginger Root Extract on Eicosanoids in Colon Mucosa in People at Normal Risk for Colorectal Cancer. *Cancer Prevention Research.* **4**(11), pp.1929-1937.

APPENDIX A: Ethical application for human study

UNIVERSITY OF LEEDS RESEARCH ETHICS COMMITTEE APPLICATION FORM
TAUGHT STUDENT PROJECTS

The purpose of ethics review is to:

- protect the research subject from physical and mental harm including distress or offence
- protect the student, in terms of their personal safety and complaint if things go wrong
- protect the reputation of the University of Leeds

This form may be used for student research projects:

- that are 'low risk'; *and/or*
- that are essentially similar for all students within the group; *and/or*
- that are repeated from student cohorts for more than one year; *and/or*
- where the module is too short to allow time to seek individual consent.

As a condition of block approval for such projects, a member of University staff MUST approve each individual project conducted as part of this approval (i.e. a postgraduate student cannot do this role). The staff member will be responsible for ensuring that any legal and ethical standards are met and that the specific research is consistent with the type of research and procedures described in this application.

Consideration of ethics of research is also a vital element of research training. Thus, even if there is insufficient time for students to seek individual ethics approval for their research project, students should receive an training on ethics procedures, standards and legal requirements at some point within their course work or within module handbooks, as appropriate.

A1. Which Faculty Research Ethics Committee do you wish to consider this application? (Tick one box)

☐ Biological Sciences
xMathematics; Physical Sciences; Engineering
☐ Medicine and Health (please specify a subcommittee)
  ☐ Healthcare Studies
  ☐ Psychological Sciences
  ☐ Health Sciences; LIGHT
  ☐ Dentistry
  ☐ LIMM
  ☐ Medical and Dental Educational Research
☐ Social Sciences; Business; Arts; Performance, Visual Arts & Communications; Environment

A.2. For how long are you requesting block approval?

Approval may be given for up to five years before renewal is required, although audit reports may be required in the interim

Duration: 5 Years: ; 0 Months:

PART B: Description of module

B.1. Title of the module [4]

FOOD5070M: Food Science Research Project

B.2. Module or Course manager

Title:	Forename/Initials:	Surname:	
Prof	Gary	Williamson	
Department:	School of Food science and Nutrition		
Institution:	University of Leeds		
Work address:	Woodhouse Lane		
	Leeds		
Postcode:	LS2 9JT		Telephone: +441133438380
E-mail:	g.williamson@leeds.ac.uk		

B.3. Proposed start date for research within module and duration of project

Start date: 1st June each year

Duration: 0 Years: ; 3 Months:

B.4. What are the principal learning objectives of the module?

The application of fundamental scientific principles to solve problems in food research.

The research projects run for ~3 months, 2 months in the lab and 1 month writing the report. This application is for those projects related to research in nutrition, specifically bioavailability of nutrients.

PART C: Description of student research projects

C.1. Select from the list below to describe what the research within the module may involve:

(You may select more than one)

xExperiments or testing new equipment, products or procedures

- ☐ New data collected by administering questionnaires/interviews for quantitative analysis
- ☐ New data collected by qualitative methods
- ☐ New data collected from observing individuals or populations
- ☐ Working with aggregated or population data
- ☐ Using already published data or data in the public domain
- ☐ Working with human tissue samples
- ☐ Genetic modification
- ☐ Other research methodology

C.2. Will the module involve any of the following: (You may select more than one)

- ☐ Patients and users of the NHS (including NHS patients treated under contracts with private sector)
- ☐ Individuals identified as potential participants because of their status as relatives or carers of patients and users of the NHS
- ☐ Access to data, organs or other bodily material of past and present NHS patients
- ☐ Use of human tissue (including non-NHS sources) where the collection is not covered by a Human Tissue Authority licence
- ☐ Fetal material and IVF involving NHS patients
- ☐ The recently dead in NHS premises
- ☐ The use of, or potential access to, NHS premises or facilities
- ☐ NHS staff - recruited as potential research participants by virtue of their professional role
- ☐ Clinical trial of a medicinal product or medical device
- ☐ Research involving adults with incapacity in Scotland, Wales or England who lack the capacity to consent for themselves [10]
- ☐ A prison or a young offender institution in England and Wales (and is health related)

If student research involves any of the above, then an application must be made for individual projects to the National Research Ethics Service (NRES) via IRAS www.myresearchproject.org.uk

x None of the above

C.3. Will the participants be from any of the following groups? (Tick as appropriate)

☐ Children under 16

☐ Adults with learning disabilities

☐ Adults with other forms of mental incapacity or mental illness

☐ Adults in emergency situations

☐ Prisoners or young Offenders

x Those who could be considered to have a particularly dependent relationship with the investigator, e.g. members of staff, students

☐ Other vulnerable groups

Justify their inclusion.

There is the probability, but no certainty, that a staff member or student in the School would participate in the study. We will absolutely not induce anyone to agree to participate in the research and people will not be approached directly. Participation is completely voluntary. The students conducting the study will be allowed to participate in the study if they wish and will sign the consent form in the same way as any other volunteer. Relevant documents shown in **appendix 1**.

☐ No participants from any of the above groups

C.4. Who will be the member of University staff who will be responsible for ensuring that individual student projects comply with this block ethics approval?

Prof Gary Williamson

C.5. Who will be responsible for supervising individual student projects?

It is not necessary to name specific individuals please indicate whether they will be University members of staff, whether they are other students eg PhD students supervising undergraduate projects, or whether supervisors may be from outside the University.

Academic staff from within the School of Food Science and Nutrition only

C.6. Give a short summary of the type of research to be conducted by students as part of the module (maximum 1000 words)

This section must be completed in language comprehensible to the lay person.

Describe the methodologies that students may be using, any experiments/interventions involved, or the types of questions (especially on sensitive topics) to be asked of the participants

The research is to determine and quantify which components of foods are absorbed and appear in the urine within a period of up to 48 hours. This allows us to estimate the bioavailability of nutrients and other compounds in food, and to see how much and in what form the body takes up and excretes the compounds. Volunteers will be asked to consume food(s) or beverage(s), and urine will be collected, before and after consumption, for a period up to 48 hours (normally 24 hours). The urine will be rendered acellular according to **appendix 2**, and therefore does not qualify as human tissue according to the HTA. The cellular fraction will be destroyed and disposed of. Normally, volunteers will be requested to abstain from certain types of foods containing the compounds of interest for a day or two before the study. Subject will be coded and data will be stored in a form which cannot be traced back to the name of the volunteer. Amounts of the compound will be measured in the

urine samples, which will be stored in the freezer in small aliquots.

C.7. Where will students be conducting the research?

For example. at the University, in public areas, research participant homes

In the School of Food Science and Nutrition

C.8. Will the module be conducted overseas or involve overseas participants?

(Tick as appropriate)

☐ Yes xNo

If Yes, please describe any issues of cultural sensitivity that we need to be aware of, and explain how you students will tackle these issues?

C.9. What health and safety issues might arise within the research and how have these been addressed?

For example, laboratory protocols or safety of students conducting interviews off campus

Students will follow the SOPs (standard operating procedures) already running in the lab. COSSH forms will be completed as necessary according to local practice. Training will be provided by staff, postdocs or PhD students on equipment as needed.

C.10. How sort of techniques will students used to identify and recruit potential research subjects?

For example, e-mail or other form of advertisement asking for volunteers, stopping people in the street, or via membership of a particular organisation or group

The invitation sheet will be shown before the commencement of the study in the School of Food Science and Nutrition and in different places at University of Leeds. Anyone (including students or staff members) are free to participate in the research, provided they conform to the exclusion criteria (normally the inclusion criteria are (a) not allergic to the food to be given (b) healthy (c) normal weight (typically BMI of 19-27) (d) not taking prescription drugs and (e) not had gastrointestinal surgery. (**See appendix 3**).

C.11. Approximately how many participants will be recruited for each project?

An order of magnitude estimate will suffice e.g. will it be tens, hundreds of participants?

Tens of people (normally 6 to 25).

C.12. Will informed consent be obtained from the research participants?

xYes ☐ No

If yes, give an indication as to how consent will be sought, what sort of information will be given to participants (e.g. verbally or in writing, how long will potential participants have to consider the request, whether consent will be implied, in verbal or in writing. If consent is not to be obtained, please explain

why not.

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

Consent in writing will be given (**appendix 1**). Volunteers will have one week to decide. They will be free to withdraw at any time without giving a reason.

Copies of any standardised frameworks for written consent forms and information sheets that students will be expected to use should accompany this application.

C.13. What arrangements have been made for participants who might not adequately understand verbal explanations or written information given in English, or who have special communication needs? *(e.g. translation, use of interpreters etc.)*

Not anticipated to be necessary, participants will be competent in English.

C.14. Will individual or group interviews/questionnaires discuss any topics or issues that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could take place during the study (e.g. during interviews/group discussions, or use of screening tests for drugs)?

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

no

The Information Sheet should make it clear under what circumstances action may be taken

C.15. Will individual participants receive any payments, fees, reimbursement of expenses or any other incentives or benefits for taking part in this research?

xYes ☐ No

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

In some cases, a small reimbursement (typically £5 per person) would be made to compensate for time given.

C.16. What are the potential benefits, risks of harms for participants?

Only normal foods will be eaten prepared by standard kitchen methods. Urine will be collected. No unusual activities will be carried out so the risk will be minimal. People with food allergies to the tested foods will be excluded.

C.17. Will the module contain any issues of data protection, and how will these be addressed by the students?

Volunteers will be coded, and data will not be able to be traced back to volunteers. All data will be stored in such an anonymised form.

C.18. Is there scope for any other conflict of interest?

☐ Yes xNo

For example will the research funder have control of publication of research findings?

C.19. Will the module have any potential environmental impact?

☐ Yes xNo

If Yes, please describe?

C.20. What are the main ethical issues within research conducted as part of the module?

Summarise the main ethics issues, and say how you propose to address them.
Indicate any issues on which you would welcome advice from the ethics committee.

The main ethical issue is that the participation is completely voluntary and no student will be in any way coerced into participating. This will be explained verbally to the students and will also be part of the consent form (**appendix 1**), which will be signed by the student. Data will be anonymous as described above, and will be stored in coded form. Other data sheets and additional information are shown in **appendices 3-9**.

PART D: Declarations

Declaration by Chief Investigators

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.
2. I undertake to abide by the University's ethical and health & safety guidelines, and the ethical principles underlying good practice guidelines appropriate to my discipline (e.g. the Declaration of Helsinki for medical research)
3. If the research is approved I undertake to adhere to the terms of this application and any conditions set out by the Research Ethics Committee.
4. A member of University staff will approve each individual project conducted as part of this approval and will be responsible for ensuring that any legal and ethical standards are met and that the individual student research is consistent with the type of research and procedures described in this application.
5. Students should receive an training on ethics procedures, standards and legal requirements at some point within their course work or within module handbooks, as appropriate.
6. I undertake to seek an ethical opinion from the REC before implementing substantial amendments to the module.
7. I undertake to submit progress reports if required.
8. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer.
9. I understand that research records/data may be subject to inspection for audit purposes if required in future.
10. I understand that personal data about me as a researcher in this application will be held by the relevant RECs and that this will be managed according to the principles established in the Data Protection Act.

Optional – please tick as appropriate:

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

Signature of Module or Course Manager: .....  .....

Print Name: G Williamson

Date: (01/03/2010)

APPENDIX B: Information provided for advertisement and to subjects

Appendix 1a. Invitation sheet

*Do you like [*insert food*] ?*

***You are being invited to take part in this research project**. Before you decide it is important for you to understand why the research is being done and what it will involve.*
It is up to you to decide whether or not to take part. If you decide to take part you can still withdraw at any time without it affecting any benefits that you are entitled to in any way. You do not have to give a reason.
All the information that we collect about you during the course of the research will be kept strictly confidential and you will not able to be identified.

Thank you for reading this.

The main objective of this work is to observe and measure the urinary content of [*compound*], found in [*foods*], after ingestion of [*food*] in [x] people.

Volunteers will be asked to donate urine for [x] hours before and [x] hours after eating [food]. The urine collection after the consumption of [food] will be when each volunteers want, over the [x] hour period following eating the [food].

{*optional*: You will receive a 5 pounds gift voucher for the book shop WH Smith as a thank you for participating}.

[*foods*] must not be eaten for [x] days before the study and some food rich in [compounds] must not be eaten for [x] days before the study ([foods]) and for the dinner of the day of study.

If you need further informations about this study, you can contact **[name of student]** by an e-mail ([email address).

This study is part of the 5070M module (MSc student research project, module coordinator: Professor Gary Williamson), School of Food Science and Nutrition, University of Leeds, UK.

Many thanks for your participation.
[name of student], Study Coordinator

Appendix 1b. Information sheet

*"Human bioavailability of [*compound*], a [*class of compound*] rich in [*food*]"*

Responsible: [name of student], Study Coordinator, School of Food Science and Nutrition, The University of Leeds, UK.

Study Title: "Human bioavailability of [*compound*], a [*class of compound*] rich in [*food*]"

Introduction: This study is designed to measure the bioavailability of [*compound*], naturally rich in [*food*]. We aim to show if and how [*compound*] is absorbed in the human body after the consumption of a serving of [*food*].

Pre-requisites: Volunteers are required to complete a routine medical questionnaire one week prior to participation in the study. Written consent is required 1 week before should the volunteers be accepted on the study programme. Volunteers must refrain from eating any [*food*] for one week prior to commencement of the study. Some foods (see *"Foods to avoid"* sheet) must not be eaten for [x] days prior to commencement of the study and for the dinner of the day of study.
A pre-study compliance questionnaire will be completed by all volunteers on the day of the study to ensure compliance with such dietary restriction.

Study Programme: Volunteers will be required to donate urine immediately before the consumption of [food].
The urine collection after the consumption of [food] will be when each volunteers want, during [x] h after consumption by urinating in a purpose-designed unisex collection container. Subjects will provided with complete privacy during sample collection. The study will last for [x] h , with volunteers requested to arrive [x] minutes prior the start of the study at pre-arranged time, between [x] and [x].
A standard breakfast and lunch will be provided during the study. Water will also be available *ad libitum* and subject are encouraged to drink plenty of water during the study. The study will conclude after [x] hours from the commencement of the study ([x] h after eating [food]).

Any further questions will be answered in full by the study coordinator.

[student], Study Coordinator

Appendix 1c. Written consent form

Participant Consent Form

Research title: Human bioavailability of [compound], a [class of compound] rich in [food]

Name of Researcher: [student name]

	Y/N
1* I confirm that I have read and understand the information sheet explaining the above research project and I have had the opportunity to ask question about the project.	☐
2* I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without there being any negative consequences. In addition, should I not wish to answer any particular question or questions, I am free to decline.	☐
3* I understand that my responses will be kept strictly confidential. I give permission for members of the research team to have access to my anonymised responses. I understand that my name will not be linked with the research materials, and I will not be identified or identifiable in the report or reports that result from the research.	☐
4* I agree for the data collected from me to be used in future research.	☐
5* I agree to take part in the above research project.	☐

Name of participant	Date	Signature

Lead Researcher	Date	Signature

Appendix 2. Protocol for acellular 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 24hr 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 were then 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 bags.

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 3. Medical Questionnaire

To be completed by each potential volunteer:

Name:	
Age:	
Sex:	
Height:	
Weight:	
Contact (telephone number or e-mail):	

1* Do you consider yourself to be in good health?

Yes | No

2* Are you taking any routine medication?

Yes | No

If Yes, which kind of medication are you taking?_____

3* Have you ever received surgery on your stomach, small intestine or colon?

Yes | No

4* Do you have any history of metabolic disease?

Yes | No

5* Do you have any history of allergy?

Yes | No

If Yes, which kind of allergy do you have?_____

Is there any reason that you consider yourself to be unable to fully participate in the study?

_____
_____
_____

_____	_____	_____
Name of participant	Date	Signature

_____	_____	_____
Lead Researcher	Date	Signature

Appendix 4. Urine collection form

To be completed by the subject for each individual urine sample

Subject Code:	
Collection Time (please note hour and minute):	
Sample Collection Irregularities (eg. Loss of sample):	
Other Comments:	

To be completed by Study Coordinator:

Total Sample Volume (mL):	
Oral Comment:	
Study Coordinator comment:	

Appendix 5. Pre-study Compliance Questionnaire

To be completed by each individual subject:

Subject Code:	
Date & Time	

1* Did you ingest any [food] or meal containing [food] during the past week?

Yes	No

2* Did you ingest any [*food*] rich in [*compound class*] ([*list of foods*]) and other foods reported on the "to avoid" list during the last two days?

Yes	No

If Yes, please give details about which kind and how much of each you ate:

_____

_____

3* Have you taken any non-routine medication, or any medication not stated in the medical questionnaire in the last week?

Yes	No

If Yes, please give details of what and how much you took:

_____

_____

Appendix 6. Participant instruction sheet

Foods to avoid (2 days before study)

- [list of foods]

Appendix 7. Programme of the study

Days prior to Study	Actions

	- Study ends.

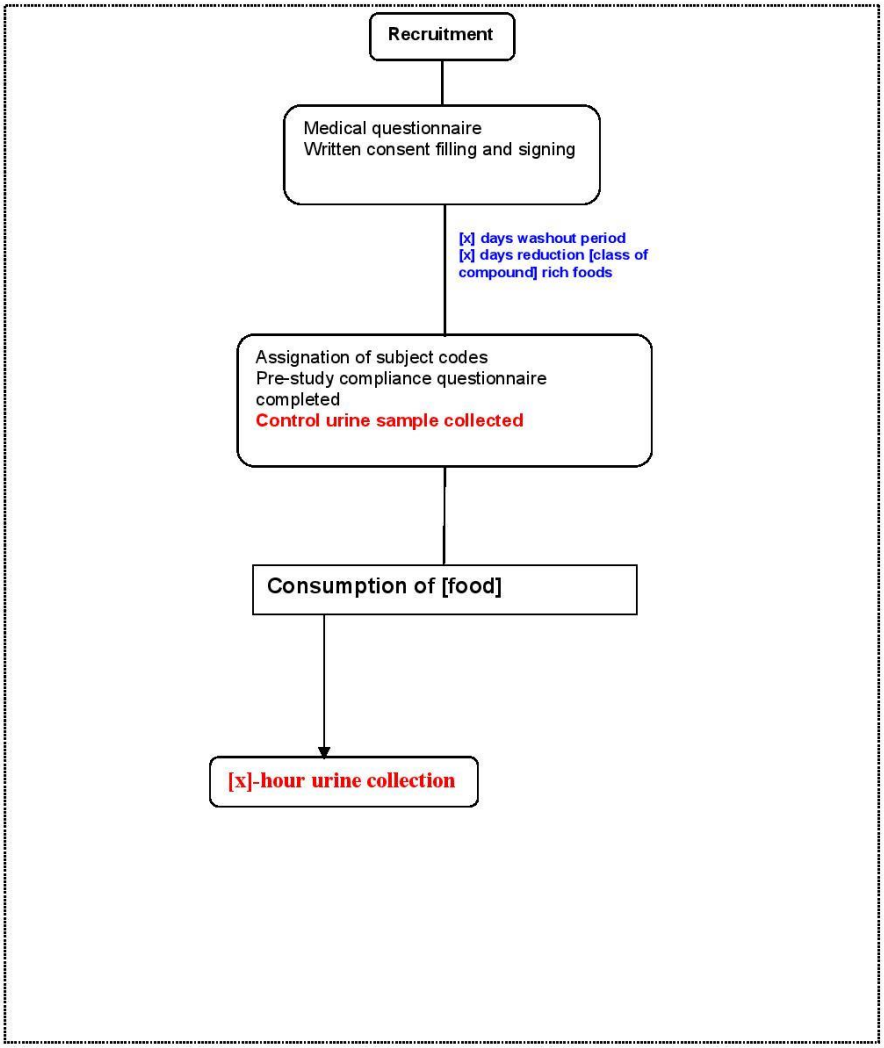
Volunteers	Arrival	Pre-study Questionnaire	Control urine	Eating time	Breakfast time					Lunch time
I										
II										
III										
IV										
V										
VI etc										
Urine samples will be collected when each volunteers wants (during 24 of study)										

Appendix 8. Meals of the day of the study

	Food
Breakfast	* [foods] * Water
Lunch	* [foods]

The volunteers should eat some of all foods reported above, in the amount that they want. If someone does not like or does not want to eat something, he/she can opt out of eating it, but the kind of food not eaten it will be recorded.

Appendix 9: Human study flowchart



APPENDIX C: Ethical approval

Research Support

3 Cavendish Road
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

18 August 2015

Prof Gary Williamson
Chair of Functional Food
Department of Food Science and Nutrition
University of Leeds

Dear Gary

Title of study: **FOOD5070M: Food Science Research Project**
Ethics reference number: **MEEC 09-019**

The above project was reviewed by the MEEC Faculty Research Ethics Committee at its virtual meeting on 17th March 2010.

The following documentation was considered:

Document	*Version*	*Date*
MEEC 09-019 Student_Block_Approval_Form 5070.pdf	1	01/03/10
MEEC 09-019 Appendix for ethical application 5070M.pdf	1	01/03/10

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

- The SOPs should outline procedures to be taken if an adverse incident occurs whilst eating the foods.

Yours sincerely

Jennifer Blaikie
Research Ethics Administrator
Research Support
On Behalf of
Professor Richard Hall, Chair, MEEC FREC.