BIOMARKERS OF TEA AND COFFEE-DERIVED POLYPHENOL EXPOSURE IN HUMAN SUBJECTS

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ABSTRACT

Tea and coffee are rich in polyphenols with a variety of biological activities. Polyphenols found in tea are predominantly flavonoids, of which up to 15% are present as free or esterified gallic acid. Coffee polyphenols are almost wholly comprised of chlorogenic acids. Many of the demonstrated activities of polyphenols are consistent with favourable effects on the risk of chronic diseases.

In investigating the relationships between intake and exposure to such compounds and chronic disease-related endpoints, it is important to be able to identify biomarkers that are specific to the compounds of interest. 4-O-methyl gallic acid (4OMGA) and isoferulic acid have been identified as potential biomarkers of intake and exposure to polyphenols derived from tea and coffee, respectively. 4OMGA is derived from gallic acid in tea, and isoferulic acid from chlorogenic acid in coffee.

The major objectives of the research which is the subject of this thesis were (1) to establish a dose-response relationship of 24h urinary excretions of 4OMGA and isoferulic acid following ingestions of black tea and coffee of different strengths, and (2) to explore relationships of tea and coffee intake with 24h urinary excretion of 4OMGA and isoferulic acid in human populations.

It was found that there was rapid excretion of both 4OMGA and isoferulic acid in the first 6h after tea and coffee ingestion, respectively. Approximately 60 – 80% of the ingested dose was excreted during the first 6h after ingestion. Urinary excretion of 4OMGA and isoferulic acid was directly related to the dose of tea and coffee, respectively. That is, higher intake resulted in increased urinary excretion of the metabolites.

The relationships of 24h urinary excretion of 4OMGA and isoferulic acid with long-term usual (111 participants) and contemporary recorded current (344 participants) tea and coffee intake were assessed. 4OMGA was strongly related to usual ($r = 0.50$, $P < 0.001$) and current ($r = 0.57$, $P < 0.001$) tea intake. Isoferulic acid was less strongly, but significantly associated with usual ($r = 0.26$, $P = 0.008$) and current ($r = 0.18$, $P < 0.001$) coffee intake.

Overall, the results are consistent with the proposal that 4OMGA is a good biomarker for black tea derived polyphenol intake and exposure, but isoferulic acid may have only limited use as a biomarker for coffee-derived polyphenol exposure.

TABLE OF CONTENTS

ABBREVIATIONS

4OMGA	4-O-methyl gallic acid
AUC	Area under curve
BSTFA	2, 2, 2,-Trifluro-N-O-Bis(Trimethylsilyl) Acetamide
CH_3CO_2Na	Sodium Acetate
CVD	Cardiovascular disease
DDI	Double deionised water
ECD	Electro chemical detector
GCMS	Gas chromatography mass spectrometry
HCl	Hydrochloric acid
Me-4OMGA	Methylated 4-O-methyl gallic acid
$NaHCO_3$	Sodium Bicarbonate

LIST OF FIGURES

Chapter 2

Chapter 4

Chapter 5

LIST OF TABLES

Chapter 2

Chapter 4

Chapter 5

PUBLICATIONS

Hodgson, J. M., Chan, S. Y., Puddey, I. B., Devine, A., Wattanapenpaiboon, N., Wahlqvist, M. L., Lukito, W., Burke, V., Ward, N. C., Prince, R. L., Croft, K. D. (2004). Phenolic Acid Metabolites as Biomarkers for Tea- and Coffee-Derived Polyphenol Exposure in Human Subjects. *British Journal of Nutrition* **in press**.

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CHAPTER 1

BACKGROUND AND HYPOTHESES

1.1 Background

Apart from water, tea and coffee are the most widely consumed beverages (Astill, C. *et al*., 2001; Balentine, D. A. *et al*., 1997; Dalluge, J. J. and Nelson, B. C., 2000; Das, S. K. and Tewari, V. K., 2002; Harbowy, M. E. and Balentine, D. A., 1997; Lakenbrink, C. L. *et al*., 2000; Nehlig, A., 1999; Olthof, M. R. *et al*., 2001b). Both contain chemical substances that may impact on human health. These substances include caffeine and polyphenols (Nehlig, A., 1999; Rechner, A. R. *et al*., 2001).

Polyphenols are of intense research interest because they exhibit potent antioxidant activity (Olthof, M. R., Hollman, P. C. H. and Katan, M. B., 2001a; Olthof, M. F. *et al*., 2001b; Rechner, A. R. *et al*., 2001). Further, polyphenols found in tea have several other demonstrated *in vitro* activities, which may translate into benefits relating to lowered risk of cardiovascular disease (CVD) and cancer (Ahmad, N., Gupta, S. and Mukhtar, H., 2000; Cao, J. *et al*., 1996; Chou, T. M. and Benowitz, N. L., 1994; Duffy, S. J. *et al*., 2001; Folts, J. D., 1998; Folts, J. D., 2002; Gao, Y. T. *et al*., 1994; Hakim, I. A. *et al*., 2003; Hertog, M. G. L. *et al*., 1993a; Hertog, M. G. L. *et al*., 1993b; Hertog, M. G. L. *et al*., 1995; Zhan, H. *et al*., 1999; Zhao, W. and Chen, J., 2001). A standard cup of tea contains approximately 200 to 400mg of polyphenols (Balentine, D. A., Wiseman, S. A. and Bouwens, L. C. M., 1997; Rice-Evans, C. A. *et al*., 1996). Coffee also contains high concentrations of a specific polyphenol, chlorogenic acid. Chlorogenic acid and its derivative, caffeic acid, have activities *in vitro* consistent with benefits to health (Olthof, M. R., Hollman, P. C. H. and Katan, M. B., 2001a; Rechner, A. R. *et al*., 2001).

Population studies investigating the relationships of tea and coffee intake with various chronic disease-related endpoints have used consumption levels to quantify tea and coffee intake. The identification of biomarkers of tea and coffee-derived polyphenol intake and exposure would provide an additional tool to investigate such

relationships. The biomarkers may have several advantages over the assessment of intake:

1. Tea and coffee intake may be poorly quantified using estimation of cups consumed.
2. The strength of tea and coffee, and therefore polyphenol content, can vary considerably.
3. There are individual differences in absorption of polyphenols, and therefore exposure.

1.2 Hypotheses

4-O-methyl gallic acid (4OMGA) and isoferulic acid have previously been identified as possible biomarkers of intake and exposure to polyphenols derived from tea and coffee, respectively. Therefore, the hypotheses being addressed are:

1. The measurement of 4OMGA in 24h urine samples provides a sensitive and specific marker of black tea intake and exposure to black tea-derived polyphenols.
2. The measurement of isoferulic acid in 24h urine samples provides a sensitive and specific marker of coffee intake and exposure to coffee-derived polyphenols.

Two studies were performed to address these hypotheses. In the first study, dose-response relationships of 24h urinary concentrations of 4OMGA and isoferulic acid following ingestion of black tea and coffee of different strengths were established. In the second study, the relationships of tea and coffee intake with 24h urinary excretion of 4OMGA and isoferulic acid in human populations were investigated.

CHAPTER 2

A REVIEW OF LITERATURE

2.1 Introduction

2.1.1 Tea: A Short Story

The tea beverage is made from processed leaves of the plant *Camellia sinensis*. Based on the Ch'a Ching (Classic of Tea), tea was discovered in 2723BC by Emperor Shen Nung of China and was used exclusively for medicinal purposes before the Tang Dynasty (618 – 906AD). Tea was consumed as soup with onions, ginger or orange peel and salt. In the Song Dynasty (906 – 1279AD), traditional tea leaves were powdered to produce a bright green and low astringency frothy drink, which is known as *Mattcha* in Japan today. It was not until the Ming Dynasty (1369 – 1644AD) that tea leaves were brewed in hot water. This coincided with the arrival of westerners in China. Today, various tea brewing techniques are used across cultures (Harbowy, M. E. and Balentine, D. A., 1997).

The earliest tea plantations were in China, and spread to India between 1818 and 1834. Tea cultivation then spread to the rest of the world; to tropical areas of Africa, South American and Russia, where localised practices and tea products developed. Today, tea is one of the most important agricultural products throughout the world, especially in the equatorial region where the conditions are most favourable for tea growth. The larger tea plantations are predominantly found in China, Sri Lanka, India, and parts of Europe and South America.

2.1.2 Coffee: A Short Story

Coffee is made from the beans of ripe berries of a tropical evergreen shrub, *Coffea*. There are three varieties of beans – *Arabica*, *Liberica* and *Robusta*. Coffee was introduced to Europe from the Arab world in the sixteenth century. Its consumption increased and spread rapidly throughout Europe in the seventeenth century. Before this, coffee was limited to the Arab world (Berthaud, J. and Charrier, A., 1988).

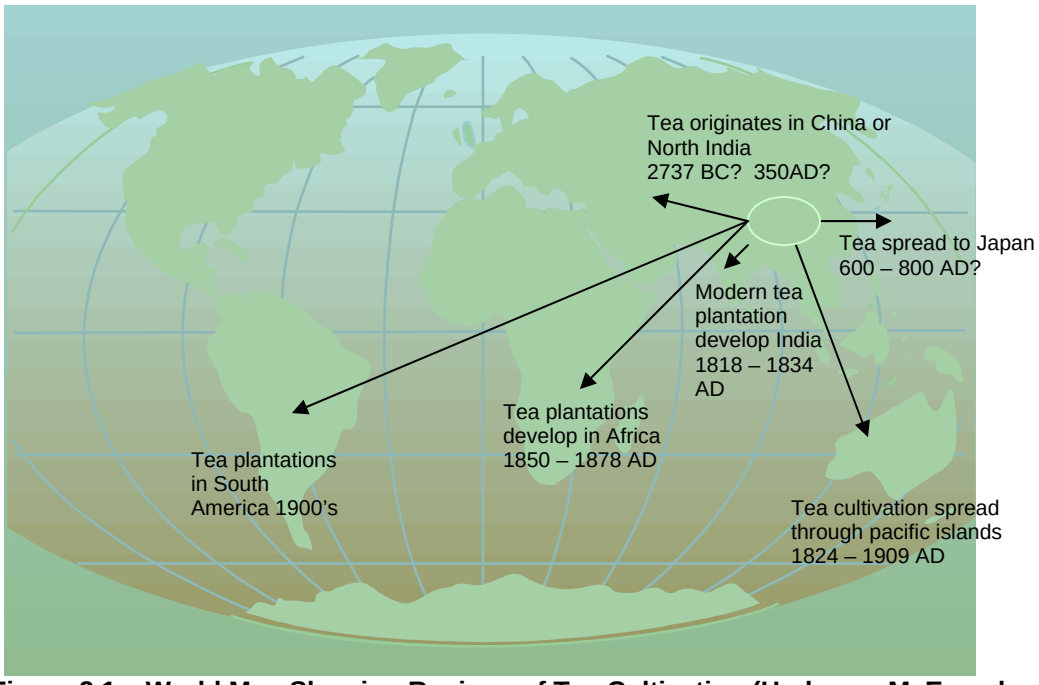


Figure 2.1 – World Map Showing Regions of Tea Cultivation (Harbowy, M. E. and Balentine, D. A., 1997)

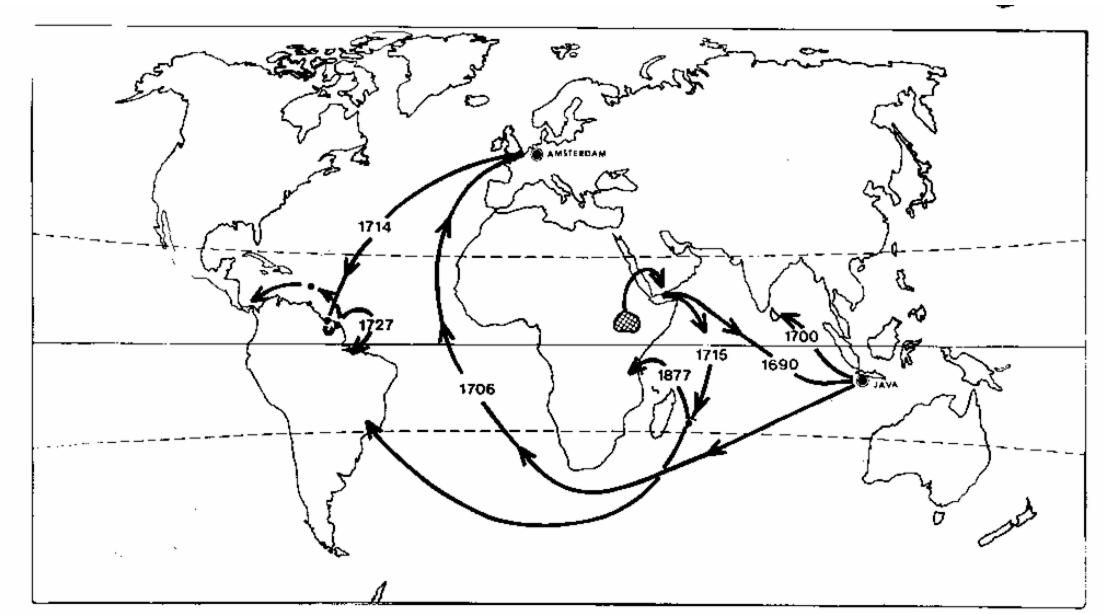


Figure 2.2 – History of Dissemination and Early Cultivation of Coffee (NB: Cross-hatched area = centre of origin) (Berthaud, J. and Charrier, A., 1998)

Coffee marketed throughout the world is made from the *beans* of ripe coffee berries. Today, most consumed coffee is either of the *Arabica* or *Robusta* variety. However, it is the *Arabica* that predominates, representing more than 70% of coffee consumed in most countries (100% in Finland and Sweden) (Berthaud, J. and Charrier, A., 1988; Nehlig, A., 1999; Spiller, M. A., 1984).

2.2 Types of Tea and Tea Manufacturing

In general, there are three types of manufactured tea: black tea, oolong tea and green tea. The type of tea manufactured depends on the degree of oxidation of leaves. Oxidation is an exogenous process: i.e. the natural browning of leaves catalysed by enzymes within the tea leaf (Harbowy, M. E. and Balentine, D. A., 1997; Unno, T. and Takeo, K., 1995; Unno, T. *et al*., 1996). Black teas are produced by promoting the enzymatic oxidation of polyphenols present in the tea leaf. Enzymes involved in polyphenol oxidation are inactivated to produce green tea.

Rapid steaming or pan firing of fresh leaves inactivates both endogenous and exogenous oxidative enzymes, stopping the oxidation of leaves and producing a dry, stable product. This is known as green tea. Green tea is further classified as either white or yellow green tea. Yellow green tea derives from naturally withered leaves, with a small degree of oxidation. White green tea derives from the unwithered but machine-dried leaves and is not subjected to any oxidation processes.

Oolong and black teas, on the other hand, are fresh leaves withered until their weight decreases to 55 – 72% of the fresh leaf weight (Harbowy, M. E. and Balentine, D. A., 1997). The withering process is an important element of the aromatic quality of the tea product. Withered leaves are rolled and crushed (releasing oxidative enzymes), initiating the oxidation process of the tea polyphenols. The final grade of tea is determined by the maceration of the tea leaves. Firing the tea leaves shortly after rolling to terminate the oxidation and drying processes produces oolong teas (Balentine, D. A. *et al*., 1997; Hampton, M. G., 1992; Harbowy, M. E. and Balentine, D. A., 1997). Cool air is circulated through the rolled and crushed leaves to moderate the oxidation reactions. The onset of the oxidation is directly coupled to the temperature from the exogenous reaction. Therefore, the oxidation starts from simple tea polyphenols to give more complex and condensed polyphenols, resulting in the bright red colour and brisk astringency of black teas. The oxidised leaves are fired and dried to stop further oxidation by the enzymes (Balentine, D. A. *et al*., 1997; Harbowy, M. E. and Balentine, D. A., 1997).

The final step in tea manufacturing is the sizing, grading and evaluation of the tea by professional tea tasters. The tea leaves are then packaged in sacks or wooden

chests, which are auctioned at warehouses throughout the world. Steps in tea manufacturing are outlined in Fig. 2.3.

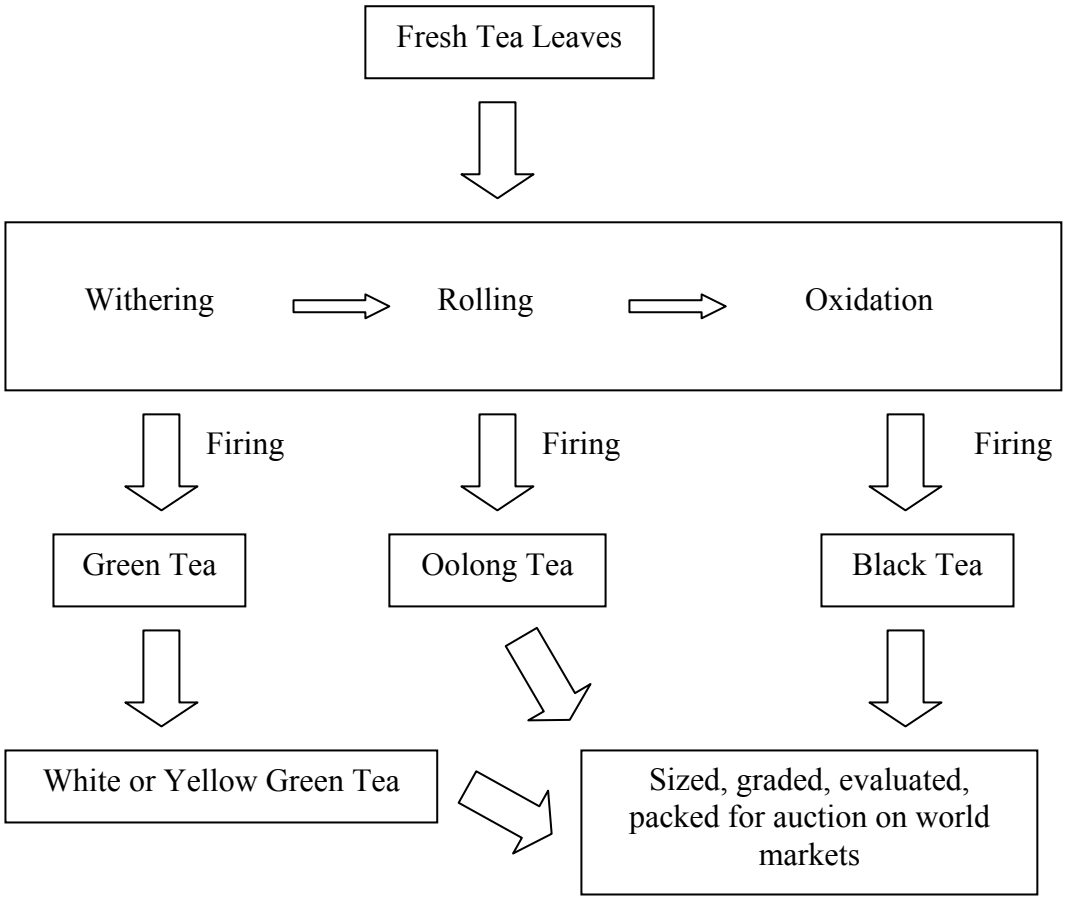


Figure 2.3 – The Tea Manufacturing Process

2.3 Types of Coffee and Coffee Manufacturing

After green coffee beans are harvested, they are prepared for market by removing the fruit, inner parchment, and outer hull of the bean, using either a wet or dry method.

The wet method involves the beans being mechanically de-pulped and soaked in fermentation tanks for up to three days. This method produces *washed coffee*, which is characterised by higher acidity and sharper flavour compared to the dry method (Mitchell, H. W., 1998). The dry method treatment produces coffees that are

lower in acidity, but fuller-bodied and more complex in flavour. This method involves natural drying of the whole green coffee beans in the sun, or machine drying. Beans are then de-hulled mechanically (Mitchell, H. W., 1998).

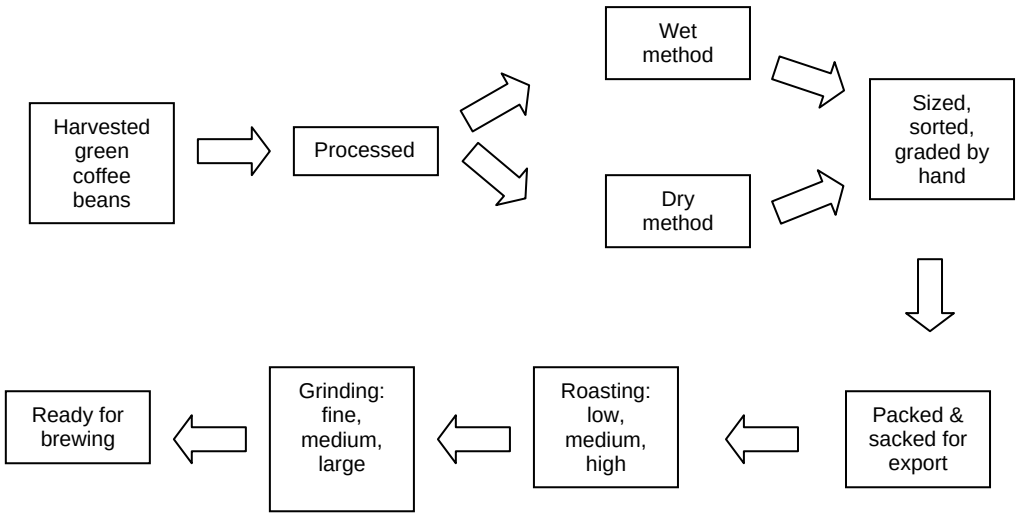


Figure 2.4 – Coffee Manufacturing

After either the wet or the dry method of bean extraction is complete, the beans are sized, sorted and graded by hand. Coffees are exported to countries all over the world, and each country has their own classification system for the hundreds of different types of coffee. However, there are three basic groups and classifications of coffee: i.e. *Milds*, *Brazils*, and *Robustas*.

Milds are all Arabicas that are grown outside of Brazil. These coffees are of premium or higher quality and used by the gourmet coffee industry. It is noteworthy that the term "mild" does not necessarily refer to the taste of the coffee, since some of these coffees taste quite bitter or acidic. *Brazils* refers to all coffees grown in Brazil, which are almost exclusively Arabicas. Brazils are the less expensive type of coffee used for tinned and instant coffee. Lastly, the *Robustas* which are African-grown coffees that are of low quality and are used almost exclusively for tinned and instant coffees (Berthaud, J. and Charrier, A., 1998; Carvalho, A., 1988; Snoeck, J., 1998; Sondahl, M. R., 1998).

There are three major steps in preparing green beans for consumption. Firstly, they must be precisely roasted to highlight any outstanding characteristics of the coffee. The beans are then ground according to brewing requirements. Lastly, the

freshly roasted and ground coffee must be brewed at the right temperature for the correct amount of time to bring out its best quality and flavour. The extent of the roasting determines the chemical composition of the resulting coffee. The higher the degree of roasting, the lower the content of polyphenols, specifically chlorogenic acid (Richelle, M. *et al*., 2001).

2.4 Chemistry of Tea

Polyphenolic compounds are secondary plant metabolites. They are aromatic molecules substituted with multiple hydroxyl (OH) groups (Figure 2.5) and have potent antioxidant properties. Whilst hydroxycinnamic acids are the main polyphenolic compounds in coffee, they also exist in tea, albeit at considerably lower concentrations. The dominating polyphenolic compounds found in tea are the flavonoids and the flavonols or flavones. They are commonly divided into five major groups: flavonoids, flavonols or flavones, flavanones, flavan-3-ols, and hydroxycinnamic acids (Balentine, D. A., Wiseman, S. A., and Bouwens, L. C. M., 1997; Beecher, G. R., Warden, B. A., and Merken, H., 1999; Dalluge, J. J. and Nelson, B. C., 2000; Finger, A., Kuhr, S. and Engelhardt, U. H., 1992; Harborne, J. B. and Williams, C. A., 2001; Harbowy, M. E. and Balentine, D. A., 1997; Knaggs, A. R., 2001; Lakenbrink, C. L. *et al*., 2000; Rechner, A. R. *et al*., 2001; Spiller, M. A., 1984; Yu, J., Vasanthan, T. and Temelli, F., 2001).

Polyphenol composition in tea is of primary interest to research on health benefits. The interest in these compounds is largely due to their antioxidant properties (Hanasaki, Y., Ogawa, S. and Fukui, S., 1994; Harborne, J. G., 2001; Hu, J. P. *et al*., 1995; Husain, S. R., Cillard, J. and Collard, P, 1987; Jovanovic, S. V. *et al*., 1994; Kanner, J. *et al*., 1997; Keli, S. O. *et al*., 1996; Kimura, M. *et al*., 2002; Kondo, K. *et al*., 1999; Torel, J., Cillard, J. and Collard, P., 1986; Valcic, S. *et al*., 2000; Vinson, J. A., *et al*., 1995; Waterhouse, A. L., Shirley, J. R., Donovan, J. L., 1996; Wiseman, S. A., Balentine, D. A. and Frie, B., 1997; Xie, B. *et al*., 1993). Antioxidants have the ability to scavenge reactive oxygen species (ROS), which are thought to play a causative role in diseases involving oxidative damage. Examples of such diseases are atherosclerosis and some cancers. While there are a large number of natural and synthetic polyphenols found in many dietary sources, tea can provide a major contribution to total polyphenol intake in the diet.

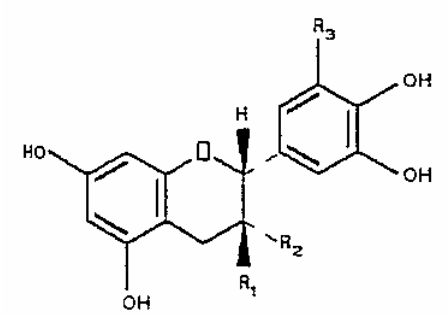


Fig. 2.5 – Structure of a Typical Polyphenol (Finger, A., Kuhr, S. and Engelhardt, U. H., 1992)

R_1 = H or OH; R_2 = OH or gallate ester; R_3 = H or OH

The main difference between black and green tea is in the polyphenol composition. The major classes of polyphenols identified in tea are the catechins, flavanols, flavonol glycosides and theaflavins (Table 2.1). Polyphenols are classified by the degree of oxidation to which they are subjected. Catechins are the raw or original polyphenols found in fresh leaves and theaflavins are the oxidised and more complex form of the catechins found in black teas.

Green teas contain both simple and complex polyphenols. The majority of the green tea polyphenols are flavanoid monomers (i.e. catechins) (Table 2.1). These compounds are synthesised from simple polyphenols and have 15 or more carbon atoms in their basic chemical structure. The catechins are a general class of flavanoid. The sub-groupings of these compounds differ in the degree of B-ring hydroxylation (Fig. 2.5). Catechins and gallocatechins are the dominant forms; their *epi*-isomers make up to 20 – 30% wt/wt of dissolved solids in tea (Rice-Evans, C. A. *et al*., 1996; Stagg, G. V. and Millin, D. J., 1975).

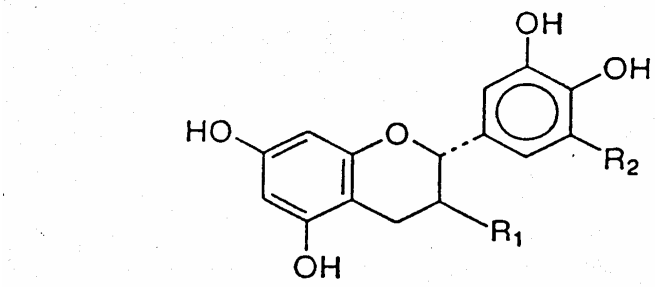
The majority of green tea catechins are gallic acid esters, with the gallation found principally at the 3-position. Other gallated species such as epigallocatechin digallate and epicatechin digallates are also found. The four most common gallic acid esters are the epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC) (Fig. 2.6). Catechin (C) and gallocatechins (GC) exist in small quantities, whereas gallocatechins gallate (GCG) and catechin gallate (CG) are racemization products that are not native to the tea plant (Balentine, D. A. *et al*., 1997; Harbowy, M. E. and Balentine, D. A., 1997; Rice-Evans, C. A. *et al*., 1996;

Stagg, G. V. and Millin, D. J., 1975). The differences in polyphenol content in green and black tea are presented in Table 2.1.

Table 2.1 – Principal Components of Green and Black Tea (% wt/wt Solids)

Chemical Component	Percentage in Green Tea Extract	Percentage in Black Tea Extract
Flavanols (= Catechins + Gallocatechins)	30 – 42	3 – 10
Theaflavins	2	2 – 6
Simple Polyphenols	2	3
Flavonols + Flavonol Glycosides	3 – 4	1
Other Polyphenols	6	23
Theanine	3	3
Amino Acids	3	3
Peptides/Proteins	6	6
Organic Acids	2	2
Sugars	7	7
Other Carbohydrates	4	4
Caffeine	3 – 6	3 – 6
Potassium	5	5
Other Minerals/Ash	5 – 8	5 – 8

(Balentine, D. A., *et al.*, 1997; Harbowy, M.E. and Balentine, D. A., 1997; Stagg, G. V. and Millin, D. J., 1975)



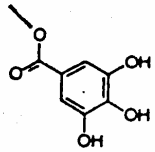
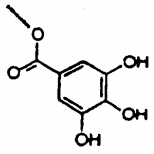
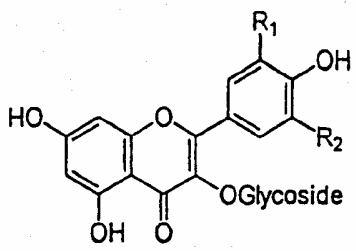
Compound		R_1	R_2	M.W.
Epicatechin	EC	OH	H	290.3
Epigallocatechin	EGC	OH	OH	306.4
Epicatechin gallate	ECg		H	442.4
Epigallocatechin gallate	EGCg		OH	458.4
Catechin	C	OH	H	290.3

Figure 2.6 – Structures of Principle Tea Catechins (Peitta, P. G. *et al.*, 1998a)

Flavonols and their glycosides (Fig 2.7) are also a significant component of green tea (Table 2.1). Chemical analyses have shown that in leaves, these compounds exist as flavonol-glycosides, flavonol-diglycosides and flavonol-triglycosides. The overall flavonol content of tea can be determined by following the hydrolysis of these compounds. The products of the hydrolysis reactions are flavonol aglycones, a compound that can be detected easily and used to represent the overall concentrations of flavonol in the beverage (approximately 0.5 – 2.5% wt/wt extract).

Green tea also contains simple and other polyphenols. High Pressure Liquid Chromatography (HPLC) analysis of green tea has indicated the presence of simple polyphenols such as gallic acid and its quinic acid esters, theogallin and other flavonol-glycosides such as apigenin.



		R_1	R_2
Kaempferol Glycoside	KaG	H	H
Quercitin Glycoside	QuG	OH	H
Myricitin Glycoside	MyG	OH	OH

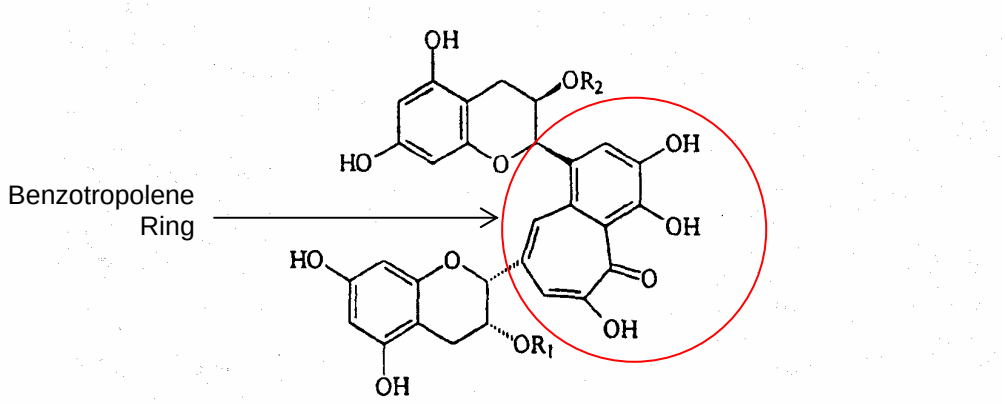
Figure 2.7 – Structures of Flavonol Glycosides (Harbowy, M. E. and Balentine, D. A., 1997)

Black tea polyphenols are produced from controlled enzymatic oxidation of polyphenols present in tea leaves. This produces some polyphenols that are unique to black tea. These compounds are more complex and difficult to characterise chemically. The majority of black tea polyphenols are unidentified. However, those that have been identified are classified as thearubigens (Table 2.1)

Catechins are the main "building blocks" of black tea polyphenols. As a result of the oxidation and thermal conditions during black tea production, some catechins are epimerized and degallated, which accounts for the presence of free gallic acid (FGA) and an increase in non-*epi*-isomers of catechins.

The majority of flavonols (kaempferol, quercetin and myricitine – see Fig. 2.7) including their glycosides in fresh leaves, are unoxidised during oolong tea and black tea production. Therefore, these are detectable in both green and black teas (Table 2.1) at similar concentrations. This is also the case for oolong teas. Unconverted polyphenols remain as the catechins and flavanols (Balentine, D. A. *et al*., 1997; Harbowy, M. E. and Balentine, D. A., 1997).

Theaflavins (Fig. 2.8) are polyphenols unique to black teas and typically make up 3 – 5% wt/wt of total extracted solids. The increase in oxidation time during manufacturing results in an increase in the concentration of theaflavins, which are responsible for the bright red-orange colour of black tea, and also decrease the astringency of black tea. Thus, theaflavins have a positive effect on the market value of tea.



		R1	R2
Theaflavin	TF	H	H
Theaflavin 3-Gallate	TF3G	Gallate	H
Theaflavin 3'-Gallate	TF3'G	H	Gallate
Theaflavin 3,3'-Digallate	TFDG	Gallate	Gallate

Figure 2.8 – Structures of Theaflavins (Harbowy, M. E. and Balentine, D. A., 1997)

The benzotropolene ring is unique to theaflavins. This part of the structure is accountable for the molecule's red colour, and thus makes theaflavins easily distinguishable amongst other polyphenols.

Thearubigens are a group of high molecular weight compounds that exhibit a bright orange-red colour, resulting from the oxidation of green tea polyphenols. They are a diverse range of compounds that are yet to be characterised.

The majority of polyphenols contained in tea are present as gallic acid esters. Gallic acid (3, 4, 5-trihydroxybenzoic acid, Fig. 2.11) is present at trace levels in fresh green leaf. It is accumulated during oxidation, most likely through the breakdown of 3-galloyl substituted catechins and gallocatechins, such as EGCG and ECG. (Harbowy, M. E. and Balentine, D. A., 1997; Hodgson, J. M. *et al*., 2000a; Hollman, P. C. H. *et al*., 1997). Therefore, levels of free gallic acid present in black tea will depend on the extent to which oxidation occurs. 4-O-methyl gallic acid (Fig. 2.9) is the 4-O-methylation product of gallic acid.

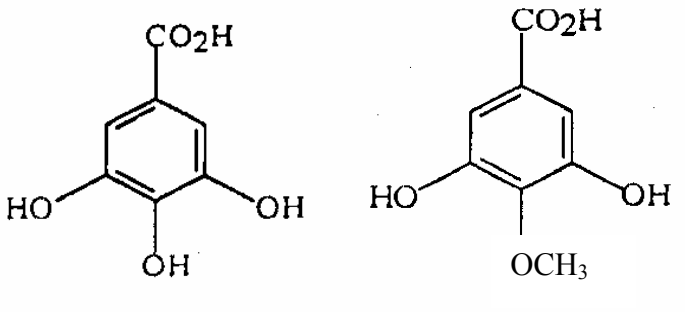
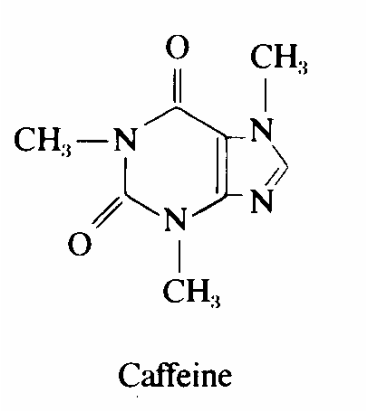


Figure 2.9 – The Gallic Acid and 4-O-Methyl Gallic Acid Molecules (Hodgson, J. M. *et al*., 2000a)

Caffeine (Fig. 2.10) is another important compound present in tea (Hindmarch, I. *et al*., 1998). The tea leaf contains 3.6% (Table 2.1) of caffeine on a dry weight basis. A consumer of a typical cup of tea (180ml), will ingest approximately 40 – 50mg of caffeine. There is little difference in caffeine concentration between green, oolong and black teas. The caffeine content of a tea beverage is determined by the brewing conditions (i.e. time, temperature, leaf size, and the amount of tea used to prepare the drink). Decaffeinated teas, on the other hand, yielde approximately 5mg of caffeine per 180ml serving (Balentine, D. A. *et al*., 1997; Drewnowski, A., 2001; Finger, A. *et al*., 1992; Groisser, D. S., 1978; Harbowy, M. E. and Balentine, D. A., 1997; Lakenbrink, C. *et al*., 2000; Unno, T. and Takeo, T., 1995).

Caffeine is synthesised from the adenosine molecule. Adenosine is converted into hypoxanthine, then xanthine or xanthosine. These xanthines are then converted to the final intermediate theobromine before caffeine is produced.



Caffeine

Figure 2.10 – The Caffeine Molecule (Taylor, D. A., 1994)

2.5 Chemistry of Coffee

There are five groups of compounds in coffee chemistry. These are the carbohydrates, nitrogenous components (caffeine), chlorogenic acid (polyphenols), volatile components and carboxylic acid (Ky, C. L. *et al*., 2001; McCamey, D. A., Thorpe, T. M., and McCarthy, J.P., 1990). Coffee represents one of the richest sources of dietary caffeine. Concentrations of caffeine vary with the variety of coffee. Polyphenols are also significant components of coffee, and contribute to aroma, an importantly quality of coffee as a beverage. To date, more than 800 aromatic compounds, including polyphenols, have been identified in coffee (Table 2.2) (Vitzthum, O. G., 1999). The majority of polyphenols found in coffee are present as chlorogenic acids, which are hydroxycinnamic acids (Fig. 2.11). The most common hydroxycinnamic aid derivatives are the esters of caffeic acid with quinic acid (i.e. 5-caffeoyl quinic acid). Chlorogenic acid makes up approximately 7 – 15% of the total dry weight of coffee (i.e. approximately 100 – 300mg/cup) (Cambrony, H. R., 1998; Poisson, J., 1998; del Castillo, M. D., Ames, J. M. and Gordon, M. H., 2002). These compounds are 100% soluble in water and contribute to the acidity of coffees, imparting the slightly sour and sharp taste characteristic.

Hydroxycinnamates are central compounds in polyphenol synthesis through the shikimate pathways, involving the metabolism of phenylalanine. The conversion of phenylalanine to *trans*-cinnamic acid is followed by a hydroxylation of the aromatic ring at the 4-position, which gives 4-hydroxycinnamic acid or p-coumaric acid. The aromatic ring is further hydrolysed at the 3-position giving caffeic acid, and subsequent O-methylation gives ferulic acid. Isoferulic acid (Fig. 2.12) is the 4-O-methylation product of caffeic acid (Rechner, A. R. *et al*., 2001).

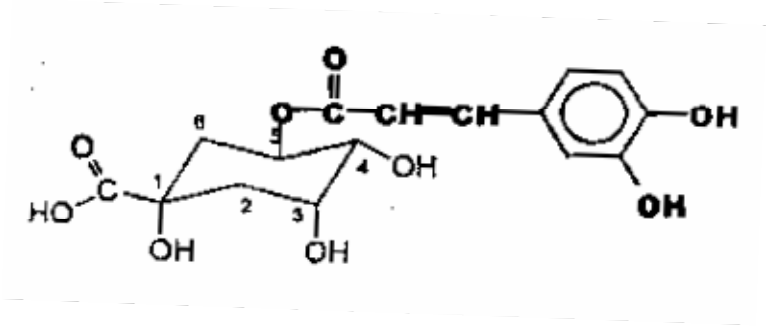


Figure 2.11 – The Chlorogenic Acid Molecule (Olthof, M. R., Hollman, P. C. H. and Katan, M. B., 2001a)

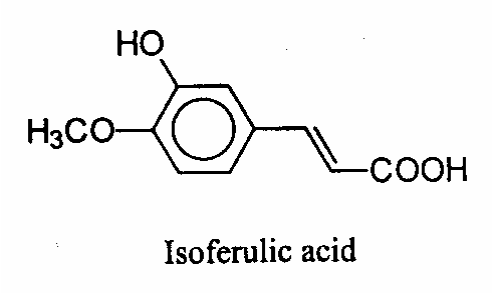


Figure 2.12 – Structure of Isoferulic Acid (Rechner, A. R. *et al*., 2001)

The concentration of caffeine in coffee varies with coffee variety. The average content of caffeine in *Robusta* coffee is approximately twice that of *Arabica* coffee (i.e. a standard cup of *Arabica* will contain 71 – 120mg of caffeine, whilst the *Robusta* will contain 131 – 220mg of caffeine) (Nehlig, A., 1999). Caffeine (Fig. 2.10) has a distinctly bitter taste, but it only accounts for approximately 10% of the perceived bitterness in coffee (Drewnowski, A., 2001). It is documented that the bitterness of caffeine is weakened when polyphenols are introduced, and the astringent taste of polyphenols is diminished by caffeine.

Table 2.2 – Principle Chemical Compounds Found in Coffee

Compound	Concentration in Roasted Coffee (mg L^{-1})
Carbohydrates	
Sucrose + Cellulose	4000 – 6000
Volatile Compounds	
5-hydroxymethylfurfural	10 – 35
2-Methyl Furan	0.05
Furfuryl Alcohol	300
Chlorogenic Acid	20 – 100
Carboxylic Acids	
Caffeic Acid + Citric Acid	1800 – 8700
Malic Acid	1900 – 3900
Lactic Acid	0 – 3200
Pyruvic Acid	400 – 1700
Acetic Acid	900 – 4000
Nitrogenous Compounds	
Trigonelline	3000 – 10000
Pyrazine, Thiazole, Quinoline, and Phenyl Pyridine	17 – 40
Caffeine	10000 – 20000
Peptides + Proteins + Alicyclic Ketones Aromatic Ketones	5 – 80
Inorganic Compound	
Quinic	3200 – 8700

(McCamey, D. A., Thorpe, T. M., and McCarthy, J.P., 1990)

2.6 Biochemistry of Tea and Coffee

In a standard cup of tea, approximately 80% of caffeine will be extracted. Based on consumption of 5 – 6 cups/day, caffeine intake will be about 0.24g (~0.04g/cup) (Stagg, G. V. and Millin, D. J., 1975). However, in coffees, the caffeine content varies between coffee types and is dependent on the brewing procedure.

Table 2.5 – Caffeine Content in a Standard Cup of Coffee Based on Preparation Mode

Mode of Preparation	Volume of Serving (ml)	Caffeine Content (mg/cup)
Boiled	150 – 190	111 – 177
Filter	50 – 190	28 – 161
Espresso	50 – 150	74 – 99
Percolated	150 – 190	55 – 88
Instant	50 – 190	19 – 34

(Nehlig, A., 1999)

Once ingested, caffeine absorption from the gastrointestinal tract is complete and rapid, and 99% of total absorption can be achieved after 45min. The peak plasma concentration occurs between 15 – 120 minutes after oral ingestion (Fig. 2.13), with concentrations ranging from 41 – 52mM in adults, for doses of 5 – 8mg/kg.

Polyphenols in tea and coffee have been shown to exhibited antioxidant properties, free radical scavenging, and chelation abilities (del Castillo, M. D., Ames, J. M. and Gordon, M. H., 2002; Facino, R. M. *et al*., 1994; Hanasaki, Y., Ogawa, S. and Fukui, S., 1994; Harborne, J. G., 2001; Hu, J. P. *et al*., 1995; Husain, S. R., Cillard, J. and Collard, P, 1987; Jovanovic, S. V. *et al*., 1994; Kanner, J. *et al*., 1997; Keli, S. O. *et al*., 1996; Kimura, M. *et al*., 2002; Kondo, K. *et al*., 1999; Manzocco, L., Anese, M. and Nicoli, M. C., 1998; Morel, I. *et al*., 1994; Rice-Evans, C. A. *et al*., 1995; Young, J. F. *et al*., 2002). These compounds have also been reported to exert anti-inflammatory actions and modulate immune functions. Flavonoids may exert a cholesterol-lowering effect by enhancing reverse cholesterol transport and bile acid excretion, and decreasing the intestinal absorption of dietary cholesterol (Abe, I. *et al*., 2000; Abe, I., Seki, T. and Noguchi, H., 2000; Chugh, A., Ray, A. and Gupta, J. B., 2003; Fukuyo, M., Hara, Y. and Muramatsu, K., 1986; Kuhnau, J, 1976; Maron, D. J. *et al*., 2003; Stensvold, I. *et al*., 1992; Tebib, K., Besancon, P. and Rounat, J. M., 1994; Tokunaga, S. *et al*., 2002; Watkins, T. R. and Bierenbaum, M. L., 1998; Weisburger, J. H. and Chung, F. L., 2002; Yang, T. T. C. and Koo, M. W. L., 1997; Yee, W. L. *et al*., 2002; Younes, M. and Siegers, C. P., 1982). Anti-carcinogenic properties of flavonoids associated with cytotoxicity to cancer cells have also been suggested (Ahmad, N. Gupta, S. and Mukhtar, H., 2000; Bagchi, D. *et al*., 1997; Banks, B. A. *et al*., 1999; Cao, J. *et al*., 1996; Chatterjee, M. L., Agarwal, R. and Mukhtar, H., 1998; Gao, Y. *et al*., 1994; Imai, K. *et al*., 1997; Islam, S. *et al*., 2000; Jankun, J. *et al*, 1997; Katiyar, S. K., Ahmad, N. and Mukhtar, H., 2000; Krul, C. *et al*., 2001; Kono, S. *et al*., 1998; Kubo, I., Xiao, P. and Fujita, K., 2002; Mukhtar, H. and Ahmad, N., 2000; Naasani, I., Seimiya, H. and Tsuruo, T., 1998; Takasaki, M. *et al*., 2001; Vaidya, S. G. *et al*., 1997; Yang, C. S. and Wang, Z. Y., 1993). In addition, various epidemiological studies have observed an inverse relationship between flavonoid consumption and coronary heart disease and stroke (Beretz, A., Cazenave, J. P. and Anton, R., 1982; Chou, T. M. and Benowitz, N. L., 1994; de Lorgeril, M. S. P., 1999; Demrow, H. S., Slane, P. R. and Folts, J. D., 1995; Duffy, s. J. *et al*., 2001; Folts, J. D., 1998; Hakim, I. A. *et al*., 2003; Hertog, M. G. L., *et al*., 1993a; Hertog, M. G. *et al*., 1995; Iijima, K. *et al*., 2000; Katan, M. B., 1997; Knekt, P. *et al*., 1996; Kris-Etherton, P. M. *et al*., 2002a; Kris-Etherton, P. M. 2002b; Luc, G. and Fruchart, J. C., 1991; Moline, J. *et al*., 2000; Paquay, J. B. G. *et al*., 2000; Princen, J. M. G. *et al*., 1998; Riemersma, R. A. *et al*., 2001; Sato, M. *et al*., 1999; Sesso, H. D. *et al*.,

2003; Tijburg, L. B. M. *et al*., 1997; Whitehead, T. P. *et al*., 1995; Zhan, A. *et al*., 1997; Zhao, W. and Chen, J., 2001; Zhang, A. *et al*., 1997).

Studies have shown that upon oral administration of radioactively labelled catechins in humans, ~50% of the radioactivity was recovered in urine. Once ingested, catechins are biotransformed in the liver, through glucuronidation, sulphation and O-methylation. After oral administration to human subjects of EGCG and EC from green tea, major sulphated conjugates of these compounds were found in plasma, whilst EGC circulated as the glucuronide conjugate. Approximately 20% of EGCG, however, remained unconjugated. O-methylation of catechins was observed in *in vitro* incubation with liver homogenate, producing a 3-methoxy-catechin (Hollman, P. C. H., Tijburg, L. B. M., and Yang, C. S., 1997; Kuhnle, G. *et al*., 2000; Olthof, M. R. *et al*., 2000b; Scalbert, A. and Williamson, G., 2000; Spenser, J. P. E. *et al*., 2001; Wiseman, H., 1999).

After biotransformation in the liver, the sulphated, glucuronidated, or O-methylated derivatives have their catechin ring cleaved by microorganisms in the colon. This is followed by the hydrolysis of these compounds, resulting in free catechins and phenolic acid and lactone metabolites reabsorbed in the first enterohepatic circulation, whilst the methylated ester derivatives are excreted into the urine (Erlund, I. *et al*., 2001; Hollman, P. C. H., Tijburg, L. B. M., and Yang, C. S., 1997; Pietta, P. G. *et al*., 1998a; Spenser, J. P. E. *et al*., 2000; Spenser, J. P. E. *et al*., 2001).

Gallic acid, free or esterified, makes up approximately 5% wt/wt of the total solid extract of green and black tea. On the other hand, free gallic acid makes up about 1% of the total solid extract of black tea, and is lower in green tea (Bors, W., Michel, C., and Stettmaier, K., 2000; Finger, A. Kuhr, S., Engelhardt, U. H., 1992; Harbowy, M. E. and Balentine, D. A., 1997; Hodgson, J. M. *et al*., 2000a; Shahrzad, S. *et al*., 2001). The typical concentration of free gallic acid in a 200ml cup of black tea is approximately 10 – 50mg (Hodgson, J. M. *et al*., 2000a). Free gallic acid is rapidly absorbed, and *in vivo* metabolic pathways are likely to be similar to that of other absorbed polyphenols. Gallic acid has potent antioxidant activity *in vitro* and may contribute to any health benefits of drinking tea.

Chlorogenic acid and its conjugates are found in almost all fruits and vegetables. However, coffee is the richest dietary source of these compounds, contributing to more than 70% of total dietary intake (Rechner, A. R. *et al*., 2001). Isoferulic acid is derived from the 4-methoxylation of caffeic acid or a conjugate of caffeic acid. Chlorogenic acid concentration in coffees ranges from 20 – 100mg/L (McCamey, D. A., Thorpe, T. M. and McCarthy, J. P., 1990), depending on the coffee type and method of preparation of the beverage (del Castillo, M. D., Ames, J. M. and Gordon, M. H., 2002). Being a polyphenolic compound, isoferulic acid also has antioxidant properties similar to those of gallic acid and gallic acid esters (gallates) (Frankel, E. N. *et al*., 1993; Frankel, E. N. *et al*, 1995; Hanasaki, Y., Ogawa, S. and Fukui, S., 1994; Halder, J. and Bhaduri, A. N., 1998; Olthof, M. F., Hollman, P. C. H. and Katan, M. B., 2001a; Olthof, M. F. *et al*., 2001b; Rechner, A. R. *et al*., 2001). The antioxidant activity of chlorogenic acid is weaker than that of gallic acid and its esters because of the methylation of an OH group (Facino, R. M. *et al*., 1994; McCamey, D. A., Thorpe, T. M. and McCarthy, J. P., 1990; Olthof, M. F., Hollman, P. C. H. and Katan, M. B., 2001a; Olthof, M. F. *et al*., 2001b; Rechner, A. R. *et al*., 2001; Spiller, M. A., 1984).

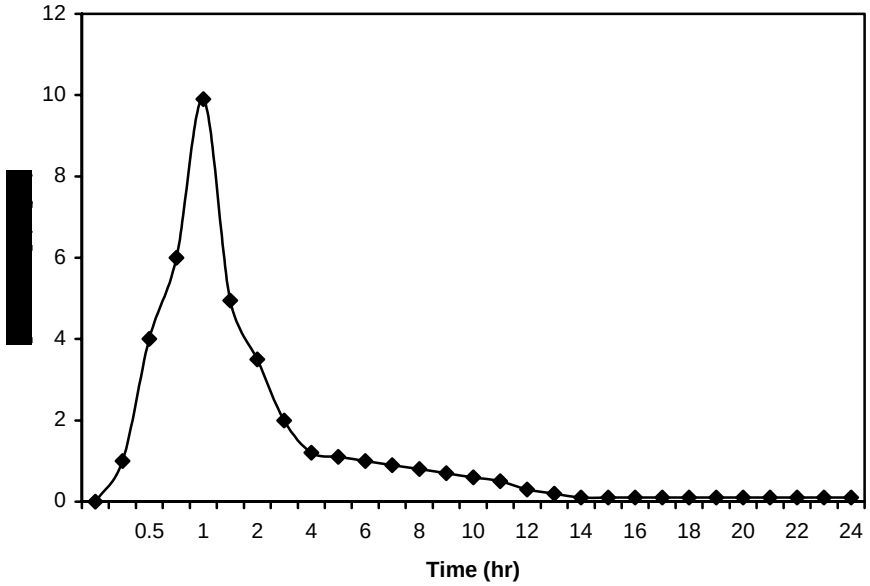


Figure 2.13 – Graph Showing Plasma Caffeine Concentration Over 24h After Oral Ingestion of 1 Standard Cup of Coffee (Derived from Nehlig, A., 1999)

2.7 Pharmacokinetics and Bioavailability of Polyphenols

To understand the mechanisms of actions of dietary polyphenols *in vivo*, we must first have an understanding of the pharmacokinetics and bioavailabilities of these compounds. Pharmacokinetics is the mathematical description of the rate and extent of uptake, distribution, and elimination of drugs or compounds of interest in the body (Gwilt, P. R., 1990). Bioavailability refers to the rate and extent of absorption for a given dose (Gwilt, P. R., 1990). Therefore, the definition of bioavailability evolves into the fraction of an oral dose (parent compound or active metabolite) from a particular preparation that reaches the systemic circulation (Stahl, W. *et al*., 2002).

In order to obtain the pharmacokinetics of tea and coffee derived polyphenols, various dose-response studies have been conducted by various research groups. These studies have identified the effective dose, time of peak concentration after ingestion, half-lives and elimination timelines. The bioavailabilities of polyphenols were traced through the administration of radiolabelled compounds using human and rat models, monitoring their possible routes of metabolism and excretion.

Figure 2.14, which derives from the work of Yang *et al*. (1998), depicts the typical plasma pharmacokinetics of polyphenolic compounds. The three polyphenols shown are EGCG, EGC and EC. These polyphenols are abundant in tea, with EGCG making up the majority of the flavonoids. Figure 2.14 shows a steep increase in initial plasma concentration from t = 0h to t ≈ 2.5h after ingestion. This is followed by a rapid decrease from t = 3h until t = 8h, by which time approximately 60% of the compounds are excreted. The remaining compound is excreted over the next 15 – 16h, with total elimination occurring 24h after ingestion. In other studies (Peitta *et al*., 1998a and 1998b, and Unno *et al*. 1996), a typical peak plasma concentration of 2μM was attained approximately 2h after administration of a single dose of EGCG equivalent to that in 2g of green tea brewed in 200ml of hot water. The compound was eliminated rapidly after the peak plasma concentration was reached, almost totally eliminated by 8h after ingestion and totally eliminated by 24h.

Nakagawa *et al*. (1997) looked at the dose-dependent incorporation of tea catechins into human plasma via the administration of pure EGCG and EGC capsules that corresponded to 2, 4 and 6 cups of green tea. They found that the two standards

(i.e. EGCG and EGC) reached peak plasma concentration 90min after administration, and the total amount of EGCG in blood mass was calculated to be 0.245 – 4.090μM/subject. This number accounted for 0.2 – 2.0% of the ingested EGCG when the whole blood mass was estimated to be 4L/subject. For EGC, the degree of incorporation into human plasma was slightly lower (i.e. 0.2 – 1.3%). Table 2.6 gives a summary of conclusions of similar research from various research groups.

Other research groups had found that as the dose of tea administered increased, the plasma concentration of the flavonoids also increase in an almost linear pattern. Van Het Hof *et al*. (1999) found that catechins levels increased 2.5-fold as a result of continuous consumption of green tea at 2h intervals for three days. Yang *et al*. (1998) also observed similar patterns after increasing the dose of EGCG, EGC and EC administered from 1.5 – 3g. The plasma concentration of EGCG increased 2.7-fold after the dose increase, whilst EGC and EC increased 3.4-fold.

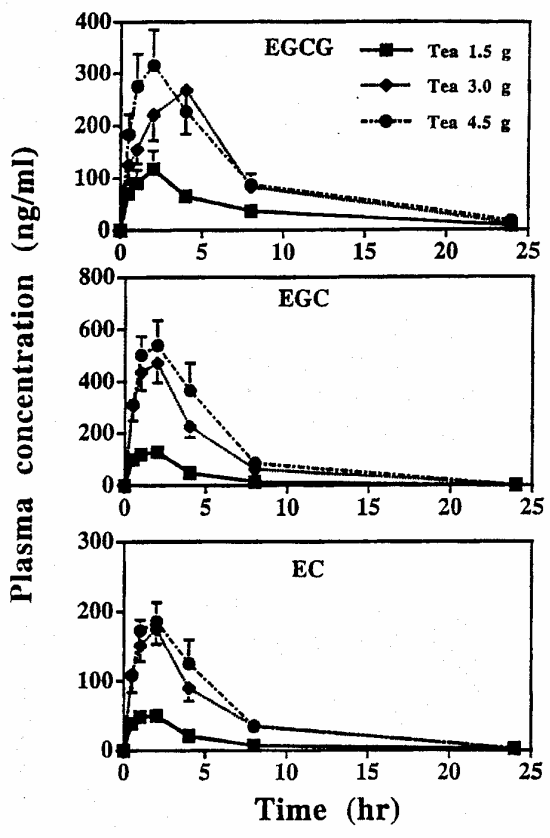


Fig. 2.14 – Graph Showing Plasma Level of Tea Catechins after Ingestion of Decaffeinated Green Tea (Yang, C. S. *et al*., 1998)

Table 2.6 – Summaries of Conclusions on the Pharmacokinetics of Tea Polyphenols by Various Research Groups

Research Group	Time of Peak Plasma Concentration after Treatment	Peak Plasma Concentration (μM)
Shahrzad, S. *et al*. 2001	1.5h (50mg of gallic acid from Assam black tea & 50mg of gallic acid from 2 acidum gallicum tablets)	2.4
Unno, T. *et al*. 1996	2.0h – green tea	2
Van Het Hof, K. H. *et al*. 1998	2.3h – green tea 2.2h – black tea	0.55 – green tea 0.17 – black tea

Based on documented studies, the amount of tea administered to subjects to achieve plasma concentration of ~2μM ranged from 1 – 4cups, or by administering pure standard compounds at an average of 400mg. These administrations should give a peak plasma concentration at approximately 2h after ingestion with a concentration that varies depending on the dose given. Additionally, previous studies had shown that when the dose of tea ingested was increased, the plasma concentrations of flavonoids also increased almost proportionally. Studies on the pharmacokinetics of chlorogenic acid are not available to date. However, it can be assumed that chlorogenic acid (being a polyphenol) should follow a pharmacokinetic pattern similar to other polyphenols.

Once the pharmacokinetics of polyphenols have been obtained, the next step is to study their bioavailability in biological systems. The bioavailability of flavanoids in animals was looked at by using a rat model (Hollman, P. C. H., Tijburg, L. B. M. and Yang, C. S., 1997). 47 – 58% of the radiolabelled (+)-catechin was excreted in urine after oral administration. This indicated that catechin and its microbial degradation products are well absorbed, at least in rats (Hollman, P. C. H., Tijburg, L. B. M. and Yang, C. S., 1997). However, 20% of the radioactivity was unaccounted for. Thus, it is thought that this 20% was incorporated into tissues. Unchanged catechin excreted into urine was around 0.1 – 2% of the administered dose (Hollman, P. C. H., Tijburg, L. B. M. and Yang, C. S., 1997). This model also showed that catechins are found in the portal vein and are able to cross intestinal membranes. The area under the curve (AUC) of EGC of the rat intestine was more than four times that of the kidney. The AUCs of liver EGCG, EGC and EC were less than that of both

intestine and kidney. Therefore, it was concluded that EGCG is mainly excreted through bile, and EGC and EC are excreted through bile and urine.

Human volunteers were recruited by Hollman, Tijburg and Yang (1997), and Van Het Hof *et al*. (1998) for the examination of flavanoid bioavailability after the administration of pure flavanoid standards (i.e. EGCG, EC and EGC, and green tea). It was found that 90% of the total ECG and EC administered was excreted between 0 – 8h, and their levels were below detection limit at 24h after ingestion. Although the concentration of EGCG is greater than EGC in green tea, plasma EGCG was found to be lower than plasma EGC. Therefore, the bioavailability of EGCG is lower than that of EGC in pure form, since EGCG may be converted into other metabolites (Hollman, P. C. *et al*., 1997; Shahrzad, S. *et al*., 2001; Van Het Hof, K. H. *et al*., 1998; Wiseman, H., 1999). Figure 2.15 shows the possible metabolic reactions of flavanoids in body tissues and colon. The flavanoid used as an example in this figure is (+)-catechin, showing that once ingested, part of the dose can undergo tissue incorporation, whilst a fraction will be metabolised in the colon before excretion through urinary or other portals.

Hollman *et al*. (1997) also looked at the bioavailability in animals of a flavonol, quercetin aglycone, by administering radioactive quercetin aglycone to rats. It was found that the compound was poorly absorbed. Only 4 – 13% of the dose, including the compound's conjugates, was recovered in urine. 40% of the dose was recovered in faeces, and this high level of radioactivity was associated with CO_2 that had resulted from the absorbed quercetin that was metabolised through the β-oxidation of phenylpropionic acid, where 12 – 14% of the dose originated.

Metabolism of quercetin was also traced in humans using its aglycone. After oral administration of a very high dose of quercetin aglycone (4000mg), no aglycone or conjugates of quercetin were detected in urine samples (Hollman, P. C. *et al*., 1997). Less than 1% of the administered quercetin was absorbed. It was hypothesised that the sugar moiety of the compound is important to the absorption of dietary quercetin (Hollman, P. C. *et al*., 1997; Olthof, M. R. *et al*., 2000), and that the elimination of quercetin from plasma was low. It is also possible that there may be an accumulation of quercetin in plasma throughout the day with repeated dietary intake. Figure 2.16 shows the possible metabolic pathways flavonols may undergo, using

quercetin as an example of the class of polyphenol. The schematic diagram shows how quercetin can undergo tissue incorporations once ingested, metabolised in the colon and then incorporated into the tissues. However, unlike flavanoids, the metabolites of flavonols can also undergo tissue incorporation. This may be the reason little or no flavonol and its metabolites were excreted in the urine.

Catechin condensation products (i.e. catechin dimers such as the B2 and B5 procyanidin dimers) (Fig. 2.17) are also relatively abundant in black tea. Therefore, gain further understanding concerning the metabolism of black tea polyphenols, the biological activities of these compounds should be scrutinised. It was found that procyanidin dimers are absorbed in mice (Spenser, J. P. E. *et al*., 2000; Spenser, J. P. E. *et al*., 2001), but absorption of these compounds intact in humans is less clear from the available evidence.

In humans, larger polyphenols may be absorbed. However, it is not known if or to what extent these larger polyphenolic compounds are absorbed intact. In addition, it is plausible that the large thearubigens may undergo gastrointestinal degradation or metabolism by colonic microflora, as is the case for simple catechins (Hollman, P. C. *et al*., 1997; Scalbert, A. and Williamson, G., 2000; Spenser, J. P. E. *et al*., 2000; Spenser, J. P. E. *et al*., 2001).

The compounds mentioned so far were polyphenols found in tea. The most abundant polyphenol in coffee is chlorogenic acid, which is a hydroxycinnamate. Therefore, it is possible to gain an insight to coffee metabolism by looking at the metabolic patterns of chlorogenic acid. Olthof *et al*. (2001a), using ileostomy subjects, found that 33% of ingested chlorogenic acid was absorbed from the small intestine of humans. In the urine collected from the volunteers, only traces of chlorogenic acid were found. This indicated that at least some of the ingested chlorogenic acid was absorbed as intact molecules, whilst most of it was metabolised extensively into other compounds after absorption.

In human subjects, urinary excretion of chlorogenic acid reached a peak at 1 – 3h after oral administration of a standard cup of coffee (149 ± 0.8mg of chlorogenic acid) (Olthof, M. R., Hollman, P. C. H. and Katan, M. B., 2001a; Rechner, A. R. *et al*., 2001). This indicated a relatively fast pathway of absorption, metabolism and elimination of caffeic acid metabolites (Fig. 2.18), which also indicated chlorogenic

acids are readily bioavailable. Figure 2.18 shows the metabolism pathway of chlorogenic acids, with caffeoyl quinic acid used as an example. Unlike tea polyphenols, chlorogenic acid undergoes metabolism in the colon before it is incorporated into tissues, and then excreted through urinary portals.

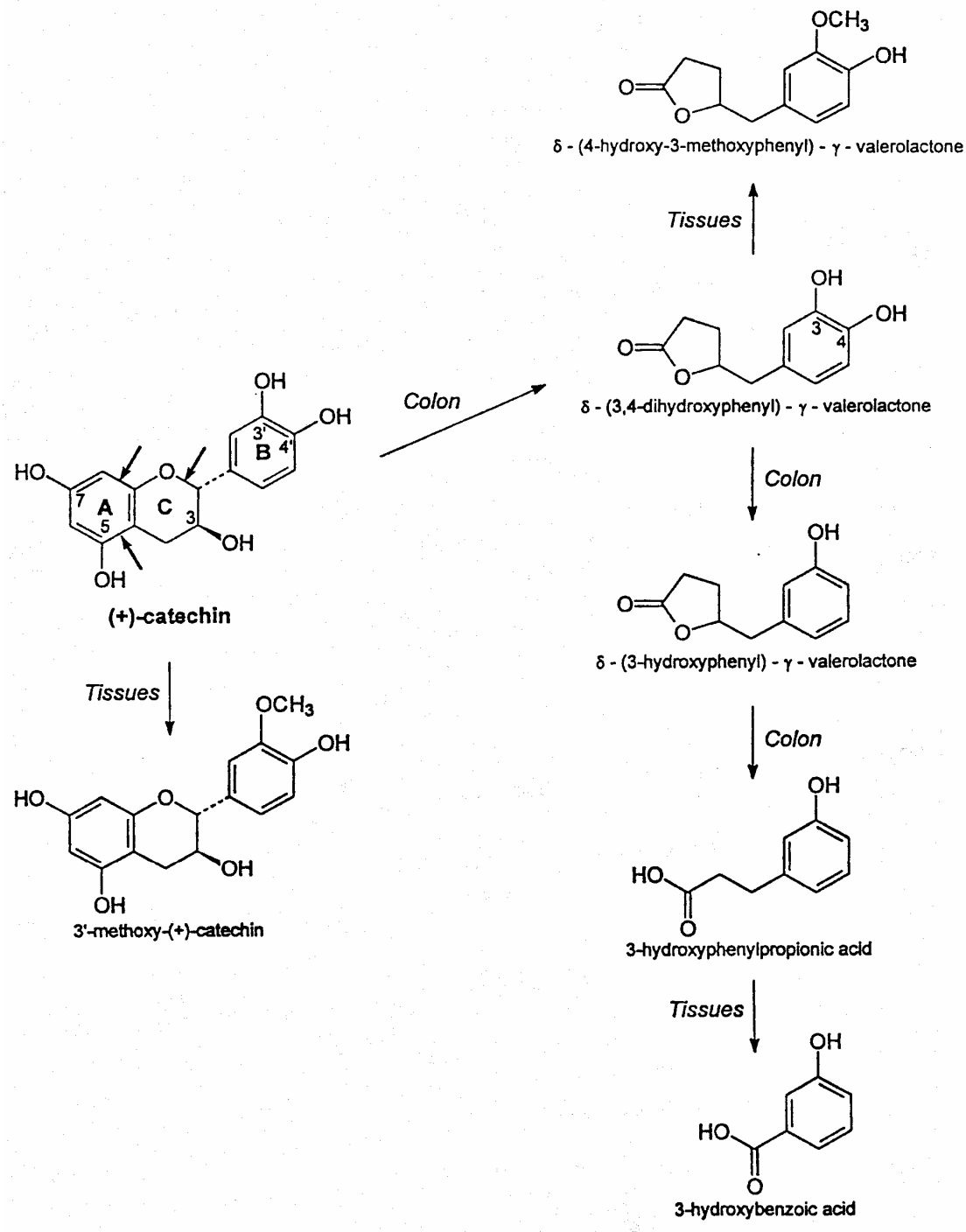


Figure 2.15 – Metabolic Reactions of Flavanoids in Body Tissues and Colon, with (+)-Catechin Shown as an Example (Hollman, P. C., Tijburg, L. B. M. and Yang, C. S., 1997)

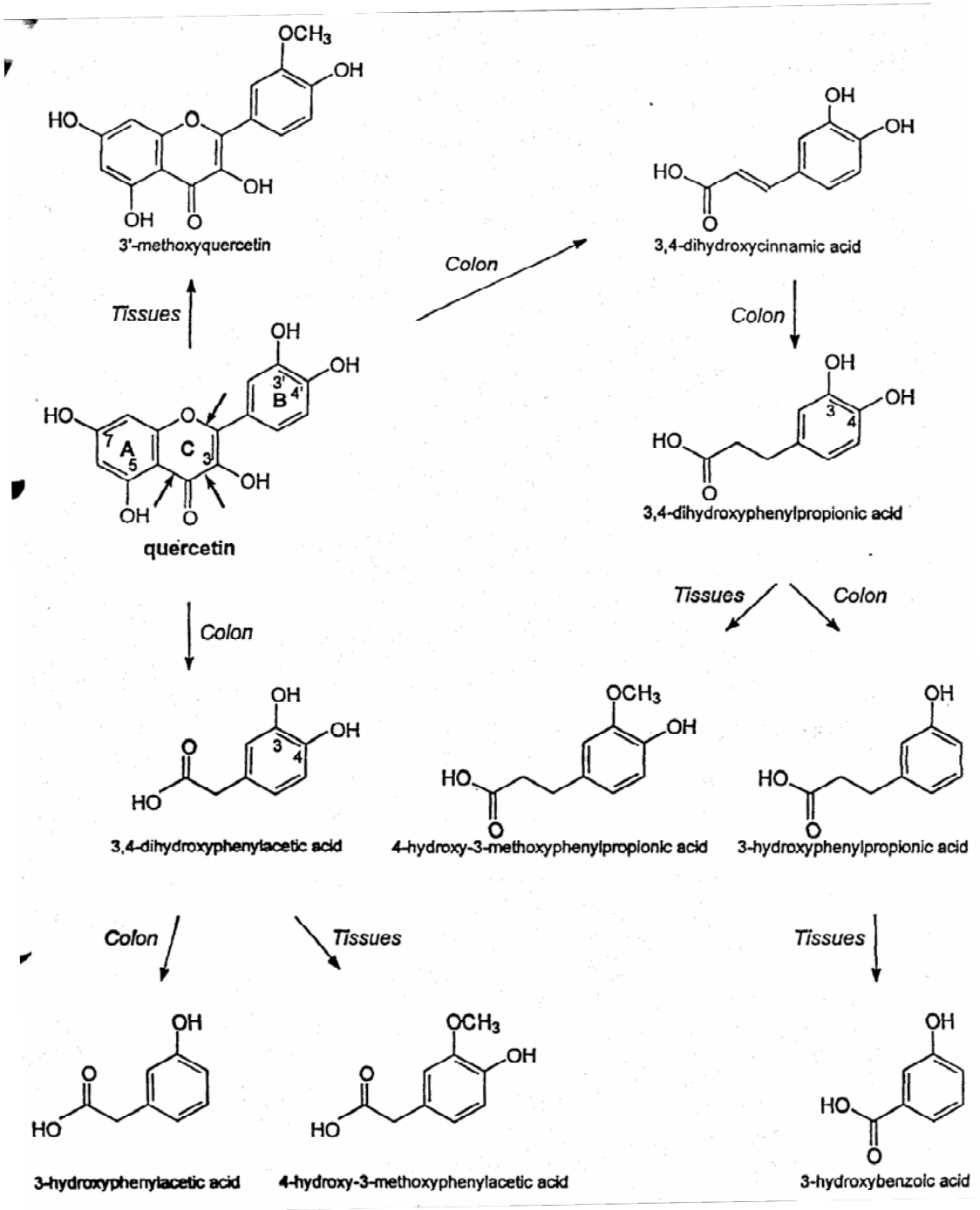


Figure 2.16 – Metabolic Reactions of Flavonols in Body Tissues and Colon, with Quercetin Shown as an Example (Hollman, P. C., Tijburg, L. B. M. and Yang, C. S., 1997)

It is a common practice to consume tea and coffee with milk. The question arises, therefore, as to whether the polyphenol concentrations of the beverages can be altered by the addition of milk. Figure 2.19 shows the effects of total catechin concentration in blood before and after the addition of milk to black tea. It can be seen that there is no significant effect on the total catechin levels in blood after the addition of milk.

It is thought that upon the addition of milk, there can be an inhibition of the antioxidant activities of polyphenols and bioavailability of flavanoids can be reduced (Richelle, M. *et al*., van Het Hof, K. H. *et al*., 1998). Figure 2.20 shows the effects of milk on the antioxidant activity of three beverages that are high in polyphenol content (i.e. coffee, cocoa and black tea). The antioxidant activity is determined by the lag time (i.e. the delay in LDL oxidation). There was no increase or decrease in the lag time of LDL oxidation after milk was added to the three solutions. Hence, it can be concluded that the addition of milk to beverages high in polyphenolic compounds appears to have little effect on the concentration or activities of these compounds *in vivo*.

However, a recent study has shown that the *in vivo* antioxidant activity of the dietary flavanoid from chocolates, (-)-EC was impaired upon the addition of milk (Serafini, M. *et al*., 2003). The experimental results using the ferric-reducing antioxidant potential (FRAP) assay showed that the antioxidant capacities were 147 ± 4.5 and 78.3 ± 3.4μmol reduced iron per 100g of dark and milk chocolate, respectively. In addition, when the volunteers consumed dark chocolate alone, their plasma antioxidant levels increased significantly from 100 ±3.5% to 118.4 ± 3.5% ($P < 0.001$) (Fig. 2.21).

As seen in the study by Serafini *et al*. (2003), the addition of milk during ingestion or the manufacturing processes can cause impairments to the *in vivo* antioxidant activity of chocolates and absorption of (-)-EC. It is hypothesised that this hindrance is due to the formation of secondary bonds between chocolate flavanoids and milk proteins, leading to a decrease in biological accessibility of the flavonoids, resulting in a decrease of the antioxidant potential of the compounds.

However, it is still debatable whether milk causes changes to the polyphenol contents of polyphenol rich foods, or impairments to their antioxidant potentials and bioavailabilities.

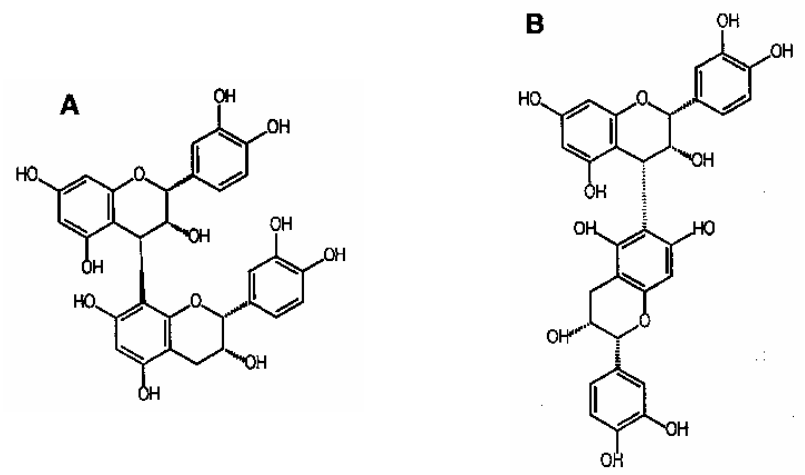


Figure 2.17 – Structure of B2 and B5 Procyanidin Dimers. (A) Epicatechin-(4b-8)-Epicatechin = B2 Dimer; (B) Epicatechin-(4b-6)-Epicatechin = B5 Dimer (Spencer, J. P. E. *et al.*, 2001)

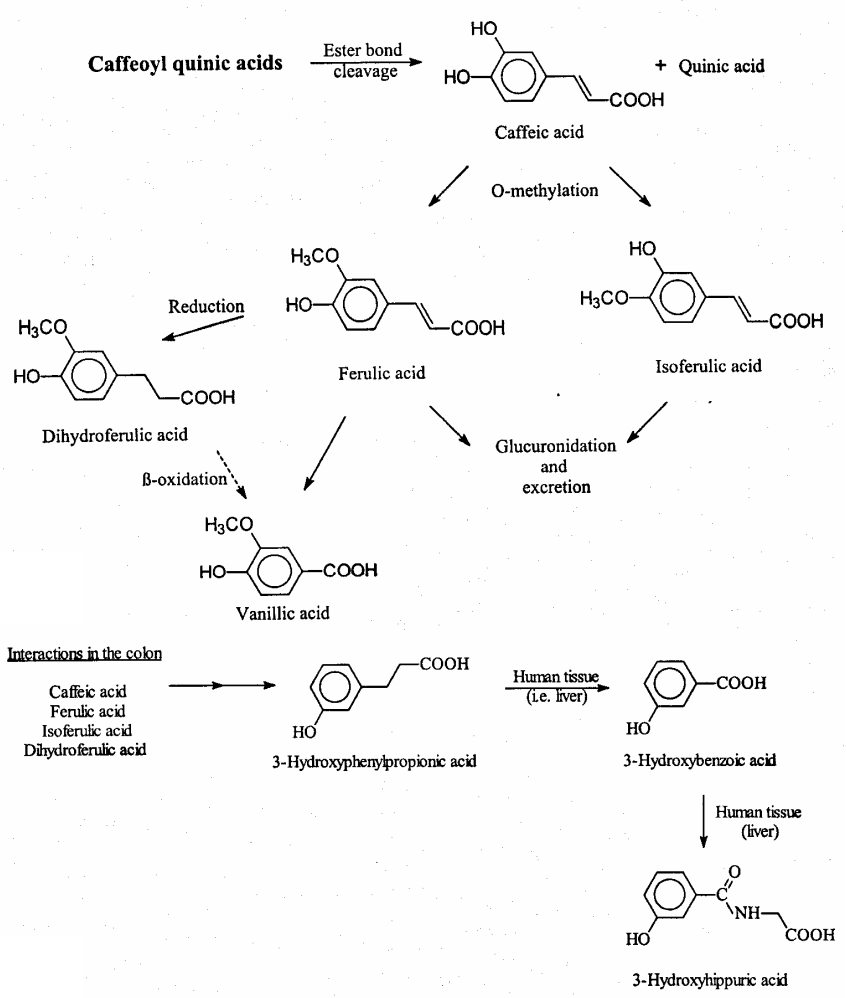


Figure 2.18 – Metabolism Pathway of Chlorogenic Acids in Body Tissue and Colon, with Caffeoyl Quinic Acid as an Example (Rechner, A. R. *et al.*, 2001)

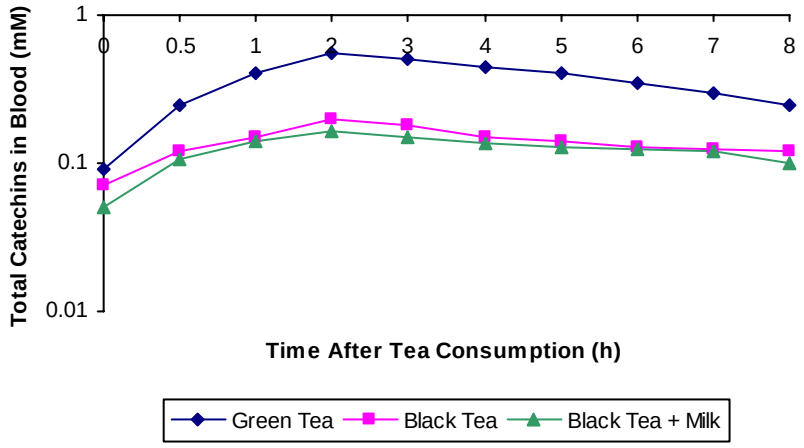


Figure 2.19 – Graph Showing Total Catechins in Blood after Consumption of Green Tea, Black Tea and Black Tea with Milk (adapted from van Het Hof, K. H. *et al*., 1998)

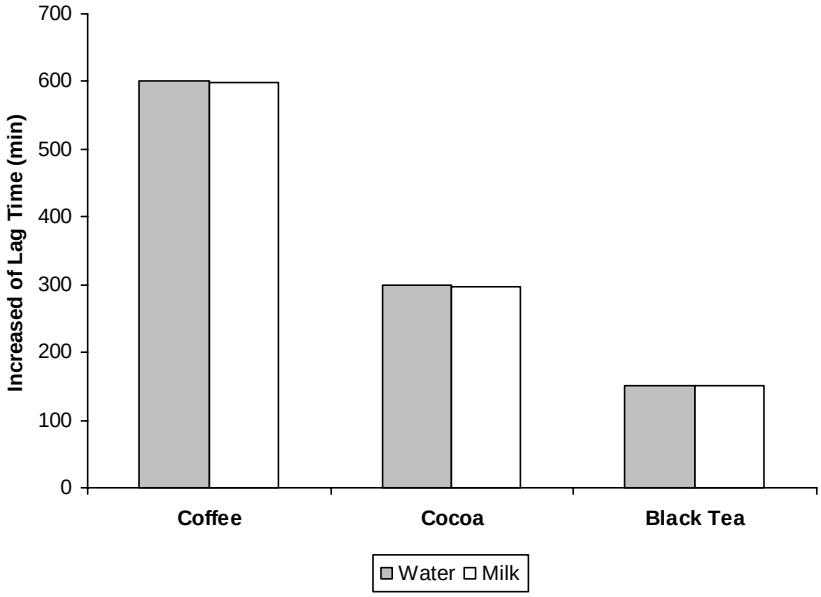


Figure 2.20 – Graph Showing the Effects of Milk on the Antioxidant Activity of Coffee, Cocoa and Black Tea (adapted from Richelle, M. *et al*., 2001)

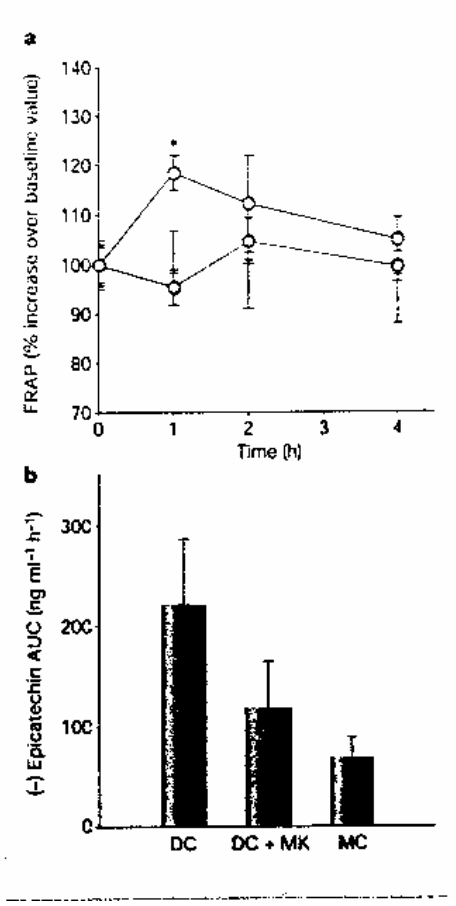


Figure 2.21 – Graphs Showing the Effects of Acute Dark Chocolate (100g), Dark Chocolate with Milk (100g), and Milk Chocolate (200g) Ingestions on the Total Antioxidant Capacity (A) and Human Plasma (-)-Epicatechin Concentration (B) (Serafini, M. *et al*., 2003)

2.8 Biomarkers of Exposure to Tea and Coffee-Derived Polyphenols

"Biomarker" is a word initially coined by free radical biologists to describe molecular changes in biological molecules due to attack by reactive oxygen, nitrogen or halide species (Griffiths, H. R. *et al*., 2002). However, with the recent increase in interests in food sciences, this term has evolved to accommodate a broader range of biological usage. Currently, study of biomarkers covers a range of biological interests, from antioxidant studies to disease end-points related studies. In the field of food science and nutrition, a biomarker can be any biological specimen that is an indicator of nutritional status with respect to intake or metabolism of dietary constituents

(Potischman, N. and Freudenheim, J. L., 2003). Biomarkers can be a biochemical, functional or clinical index of the status of an essential nutrient or other dietary constituent. Therefore, biomarkers can also be broadly defined as the biological consequences of dietary intakes or dietary patterns (i.e. they give an indication of a nutritional exposure) (Blanck, H. M. *et al*., 2003; Kohlmeier, L., 1991; Kok, F. J., 1991; Marshall, J. R., 2003; Mason, J. B., 2003; Potischman, N. and Freudenheim, J. L., 2003; Willett, W. C., 1991).

For a biological specimen or molecule to be used as a biomarker, it must satisfy two main criteria: (1) it is a compound that is unique to the item in question and primarily found within this item, and (2) it is traceable in the metabolic pathways of the item in question. By using biomarkers in scientific research, valuable information can be gathered about the item in question. Biomarkers can provide useful information on disease-related progressive outcomes (Griffiths, H. R. *et al*., 2002).

4-O-methyl gallic acid (4OMGA) is the major metabolite of gallic acid. It has been identified as a potential biomarker of tea intake and of tea-derived polyphenol exposure in humans (He, Y. H. and Kies, C., 1994; Hodgson, J. M. *et al*., 2000a; Shahrzad, S. *et al*., 2001). Gallic acid exists in free and bound forms and can be readily extracted into hot water infusions. It is a simple polyphenol that has potent antioxidant activity and anti-mutagenic and anti-carcinogenic properties. Despite the biological activities gallic acid possesses, there is limited data available about the extent of its absorption, elimination or relative bioavailability upon ingestion of dietary materials such as tea. However, dose-response studies have resolved the pharmacokinetics of gallic acid and its metabolite 4OMGA. Figure 2.22 shows that the peak plasma concentrations of both the original compound (gallic acid) and its metabolite (4OMGA) peaked at the same time (i.e. 2h after ingestion in the single dose administration of gallic acid and administration of tea (Assam black tea, 6.24g in 200ml hot water) (Shahrzad, S. *et al*., 2001). Therefore, since tea is a rich dietary source of gallic acid, these methylated ester derivatives of metabolised flavanoids may be used as biomarkers to monitor tea consumption, and as an indicator of tea-derived polyphenol exposure in humans.

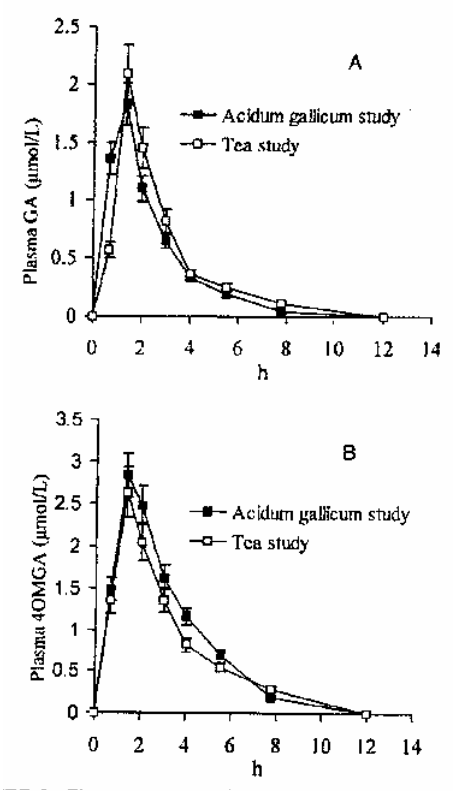


Figure 2.22 – Graphs Showing Time vs. Plasma Concentrations of (A) Gallic Acid, and (B) 4-O-Methyl Gallic Acid after a Single Dose Acidum Gallicum Tablets and Assam Black Tea, respectively (Shahrzad, S. *et al.*, 2001)

Isoferulic acid is a unique metabolite of caffeic acid, which is found in abundance in coffee. It has been identified as a potential biomarker of coffee intake and of coffee-derived polyphenol exposure in humans (Rechner, A. R. *et al.*, 2001). Isoferulic acid is derived from the 4-methoxylation of caffeic acid or a caffeic acid conjugate before or after its absorption (Fig. 2.18). In urine, isoferulic acid is present as a glucuronide conjugate, which is the most common caffeic acid derivative of chlorogenic acid.

Upon ingestion of coffee, the acidic environment of the gastric lumen does not cleave the caffeoyl quinic acid (chlorogenic acid) contained in the beverage. When chlorogenic acid reaches the colon, it undergoes extensive degradation by colon bacteria. Therefore, only a trace amount of chlorogenic acid is absorbed and/or excreted as an intact molecule, whilst the majority of the ingested compound is absorbed and/or excreted as degraded by-products (i.e. caffeic acid, isoferulic acid, ferulic acid, vanillic acid etc) (Olthof, M. R., Hollman, P. C. H., and Katan, M. B., 2001a; Rechner, A. R. *et al.*, 2001).

Gut microflora is responsible for the hydrolysis of chlorogenic acid into its corresponding derivatives, caffeic acid and quinic acid (Fig. 2.18). Caffeic acid then undergoes O-methylation in the liver, producing isoferulic acid and ferulic acid (Olthof, M. R., Hollman, P. C. H., and Katan, M. B., 2001a). After O-methylation, isoferulic acid is glucuronidated in the liver, then excreted into the urine. A notable feature of this series of reactions is that the gut microflora may be responsible for the rationing of the amounts of ferulic acid/isoferulic acid excreted into the urine, which mirrors individual difference in gut microflora (Rechner, A. R. *et al*., 2001).

Figure 2.23 shows the systemic concentrations of chlorogenic acid and caffeic acid after coffee ingestion. It can be seen that as chlorogenic acid builds up over time, so does caffeic acid. However, as is shown in Figure 2.23, the original compound and the metabolite do not reach peak concentration at the same time after coffee ingestion. Therefore, since coffee is a rich dietary source of chlorogenic acid, it is hypothesised that isoferulic acid, a metabolite of caffeic acid, may be used as a specific biomarker to monitor coffee consumption in humans, and to monitor the pharmacokinetics of coffee.

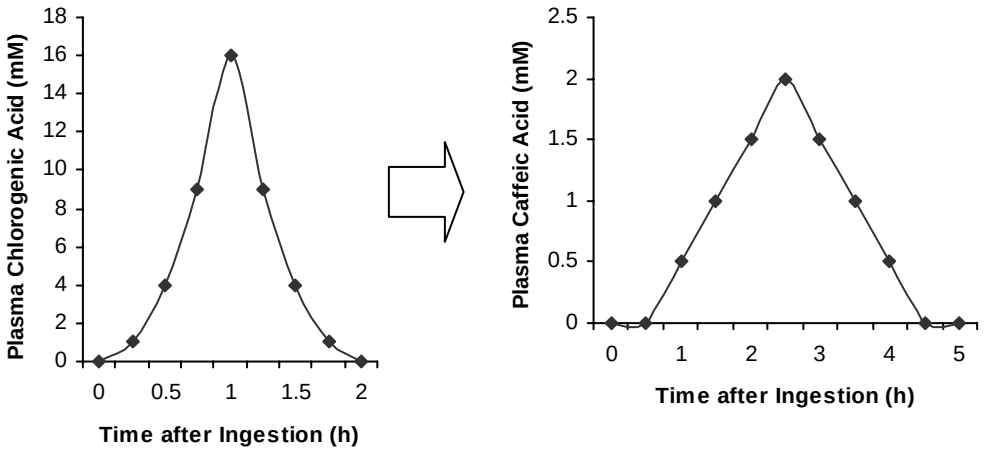


Fig. 2.23 – Schematic Representation of Chlorogenic Acid Metabolism into Caffeic Acid after Coffee Ingestion (Olthof, M. R., Hollman, P. C. H., and Katan, M. B., 2001a; Olthof, M. R. *et al*., 2001; Rechner, A. R. *et al*., 2001)

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Following is a list of analytical grade chemicals used and their sources:

1-hydroxy-2-naphthoic acid (Aldrich Chemical Company, Milwaukee, Wisconsin, USA)

Sodium Carbonate (Ajax Chemicals Pty. Ltd., Melbourne, Australia)

Ethyl Acetate, Hydrochloric Acid, Pyridine (AnalaR – Merck Pty. Ltd., Kilsyth, Victoria, Australia)

Sodium Acetate (BDH Chemicals, Australia Pty. Ltd., Kilsth, Victoria, Australia)

Air, Helium, Hydrogen, Nitrogen (BOC Gases, Perth, Australia)

Acetic Acid (Glacial), β-glucuronidase, 2,2,2-Trifluro-N-O-Bis(Trimethylsilyl) Acetamide (BSTFA), Isovanillin, Malonic Acid (Sigma Chemical Co., St. Louis, Missouri, USA)

3.1.2 Equipment

Glass vials, glass vial inserts, glass vial caps, and glass vial caps inserts were obtained from Alltech, Australia.

Derivatising tubes, 6ml glass tubes were obtained from InterPath Services Pty. Ltd., Australia.

Gas Chromatography Mass Spectroscopy (GCMS) system used was the HP ChemStation and HP 5890 gas chromatograph coupled to a HP 5970 mass-selective Mass Spectrometer obtained from Hewlett-Packard, Rockville, MD, USA. Results

were analysed using: HP G1034C MS ChemStation Software; HP-1 cross-linked methyl silicone column (12×0.20mm, 0.33mm film thickness); HP 1100 series HPLC system, which consisted of a G1311A QuatPump, G1322A Degasser, and a G1313A ALS; supercosil C_{18} reversed-phase column (150mm ×4.6mm, i.d.; particle size 5μm); HP 1049A programmable electrochemical detector (ECD); HP ChemStation software for LC, Rev. A.06.01 [043] (Copyright, HP 1990 – 1998); HP LaserJet 1100 printer.

3.2 Methods

3.2.1 Synthesis of Standard Isoferulic Acid

10g (0.066mol) isovanillin and 15g (0.144mol) malonic acid were refluxed in 30mls (i.e. 30g, 0.38mol) of pyridine and 0.5ml piperidine for 3h 20min. An equal amount of HCl as malonic acid was added into the reaction (i.e. 24ml of 6M HCl). 50ml of 32%HCl (10M) was diluted 1:1 in 50ml of DDI, and pre-chilled on ice. Excess pyridine used in the reflux reaction was flushed out with the ice-cold 5M HCl, and a white precipitate was formed. The precipitate was filtered and recrystallised from ethanol, containing a few drops of glacial acetic acid. The glacial acetic acid was added to maintain the acidic environment. The recrystallising solution was left to cool overnight at room temperature, as crystals precipitated out of the solution over the time span. Recrystallised isoferulic acid was filtered and tested for melting point. The literature melting point of isoferulic acid is 231°C, and the synthesised compound melted at 232°C. The synthesised compound was further confirmed to be isoferulic acid by Gas Chromatography coupled Mass Spectroscopy (GCMS).

3.2.2 Measurement of Polyphenol Metabolite 4-O-Methyl Gallic Acid and Isoferulic Acid in Urine Samples

1ml of urine was diluted in 4ml of 0.1M CH_3CO_2Na buffer, pH 4.6. An internal standard, 2-hydroxy-3-naphthoic acid (2.5μg/ml, 20μl) and 20μl of β-glucuronidase were added to the sample, which was incubated at 37ºC for 3h with half-hourly mixing. The final pH of the mixture was 4.8. After incubation, pH was adjusted to 1 – 2 before the addition of 4ml of ethyl acetate for 4OMGA extraction. The sample mixture was mixed vigorously for 1min then centrifuged at 3000×g for 5min. Ethyl acetate layer was back extracted into 3ml of 5% $NaHCO_3$, and the layers separated by spinning the mixture at 3000×g for 5min. $NaHCO_3$ was acidified with 5M HCl. Phenolic acids were finally extracted into 2ml of ethyl acetate, then dried under N_2 (g). Samples were then derivatised by the addition of 50μl of pyridine and 50μl of BSTFA are incubated at 40°C for 20min. The sample was analysed on the GCMS.

Samples were injected onto a HP-1 cross-linked methyl silicone column (12×0.20mm, 0.33mm film thickness, Hewlett-Packard) using He as carrier gas. An inlet pressure of 30kPa was used and injections were made in a splitless mode. The initial column temperature of 120ºC was held for 0.5min then programmed at 15ºC to 280ºC, where it was held for 5min. The mass-spectrometer was operated in the electron impact mode (70eV). SCAN monitoring was carried out to monitor the major characteristic ions for 4OMGA (M^+ = 370) and the molecular ion minus a methyl group for identification of internal standard (M^+-15 = 317). Isoferulic acid identification was by monitoring for its major characteristic ions at M^+-15 = 323 and M^+-15 = 338.

CHAPTER 4

DOSE-RESPONSE STUDY

4.1 Introduction

Tea and coffee are widely consumed beverages in contemporary society. Interest in the potential health effects of these beverages has developed in decades. Both beverages represent major dietary sources of polyphenols, including the phenolic acids and flavonoids. Polyphenols found in tea are predominantly flavonoids, of which up to 15% of the polyphenols are present as free or esterified gallic acid. Coffee polyphenols, on the other hand, are almost wholly comprised of chlorogenic acid. Since polyphenols are a class of compound that possess various biological activities, some of these may have positive effects on human health, such as the prevention or treatment of chronic diseases.

In order to investigate the relationships of tea and coffee polyphenol intake and exposure with chronic disease-related endpoints, it is crucial to be able to identify a biomarker that allows the measurement of dietary polyphenol exposure. For a compound to be classified as a biomarker, a clear dose-response relationship must be evident. That is, a linear relationship of the biomarker with exposure is important. A number of potential biomarkers have been identified in various studies, which included the specific O-methylated polyphenols derived from *in vivo* polyphenol metabolism (Rechner, A. R. *et al*., 2001; Shahrzad, S. *et al*., 2001). An example of these metabolites is 4-O-methylated gallate (4OMGA), the principal metabolite of gallic acid in humans, and isoferulic acid, a specific metabolite of dietary caffeic acid derivatives that are abundant in coffee (Olthof, M. F., Hollman, P. C. H. and Katan, M. B., 2001a; Olthof, M. F. *et al*., 2001b; Rechner, A. R. *et al*., 2001).

The objective of this study was to investigate the presence of a dose-response relationship between tea and coffee intake and urinary excretion of 4OMGA and isoferulic acid, respectively.

4.2 Methods

4.2.1 Study Participants

Eight healthy males and females were recruited to this study. Participants were non-smokers, had an alcohol intake <20g/day, tea and coffee intake less than 5 cups/d, had no major illness or history of heart disease, were not taking medications, had normal blood pressure and normocholesterolimia, with body mass index (BMI) <30kg/m^2. The experiment was carried out over ten weeks.

4.2.2 Study Design

In four participants, concentrations of 4OMGA were measured in urine following ingestion of 250ml of: (1) hot water, (2) 2g of black tea, and (3) 4g of black tea, in random order, 2 weeks apart, on the same day of the week and at the same time of day. In another four participants, concentration of isoferulic acid was measured in urine following ingestion of 250ml of: (1) hot water, (2) 2g of coffee, and (3) 4g coffee in random order, 2 weeks apart, on the same day of the week and at the same time of day. Prior to each visit, participants were asked to fast for at least 12 hours, and not to consume any foods or beverages containing high levels of polyphenols. Urine samples were collected over the same time intervals on each visit.

Foods and beverages avoided for 24h before the experiment were coffee and teas (including herbal teas), Coca Cola, Diet Coke, Pepsi, Schweppes Cola, Diet Pepsi, Jolt Cola, Bicardi or Bourbon and Coke, all chocolate products (cocoa, dark chocolate, milk chocolate, baker's or cooking chocolate, chocolate milk, chocolate flavoured syrup and Milo), and certain pharmaceutical products (Ergodry or Migral for migraines, Medislim for slimming, No-Doz for staying awake, Travacalm for anti-nausea, Naturale or Powder Active / Capsules or 4000 Energy Bar for Guarana).

4.2.3 Preparation of Tea and Coffee

Each tea bag containing 2g of tea leaves was brewed in 250ml of hot water with continuous movement for 5min. The tea or water was consumed over 5 – 10 minutes. Coffee samples of 2g and 4g were weighed out for participants. Coffee was

dissolved in 250ml of hot water and consumed over 5 – 10 minutes. All beverages (including water) were consumed hot and without the addition of milk or sugar.

4.2.4 Urine Sample Collection

Participants provided a spot urine sample on waking in the morning before each visit. A baseline urine sample was also collected in the department prior to the drinking of the tea, coffee or hot water. Following the drinking of tea, coffee or hot water, urine samples were collected from 0 – 6 hour and 6 – 24 hours. 10ml aliquots of each pool were collected into 5ml sample vials and frozen at –80ºC. 4OMGA and isoferulic acids excreted were analysed from each sample in relation to the dose of coffee or tea ingestion.

4.2.5 Recovery Assay

A recovery assay was performed to determine the loss of 4OMGA, if any, during the extraction described. To do this, standard 4OMGA was prepared by hydrolysing a sample of methylated-4OMGA. 1mg of Me-4OMGA ester was dissolved in 1ml of 1M methanolic potassium hydroxide, followed by the addition of a few drops of double deionised water, which is necessary for the hydrolysis reaction. The reaction mixture was flushed with nitrogen and incubated for 2 to 3 hours on a heating block with the temperature set at 45°C. Another 2 to 3ml of double deionised water was added to the reaction mixture after the incubation to dilute the sample. The pH of the sample was then adjusted to 3 or 4. 4OMGA was extracted into ethyl acetate (2ml), after centrifuging at 3,000×g for 5min. The extracted 4OMGA was dried under nitrogen and reconstituted in 1ml of redistilled ethanol. The standard 4OMGA was sealed and stored at 4°C for later use.

Baseline urine samples from one participant's water control were used to check whether the recovery of standard 4OMGA was 100%. Samples were prepared according to the following scheme (Table 4.1), and 4OMGA was extracted as described, followed by analysis on the GCMS.

Table 4.1 – Sample Preparations for Standard 4-O-Methyl Gallic Acid Recovery Assay

Sample	Preparations	Purpose of Sample
1	20μL of Internal Standard (50ng)	Standard control of AUC.
2	50μL 1mg/mL Standard 4OMGA (50μg)	Standard control of AUC.
3	Urine spiked with 50μL of Standard 4OMGA , i.e. 50μg	To assay for possible loss of standards after extraction procedure.
4	20μL of Internal Standard (50ng)+ 50μL of Standard 4OMGA (50μg)	For the determination of AUC ratios of compounds to ensure accuracies of calculations.

4.2.6 Analysis of Urinary 4-O-Methylated Gallic Acid and Isoferulic Acid

Urinary excretion of 4OMGA and isoferulic acid were measured in urine samples obtained from the participants after beverage administration. The compounds were extracted from the urine aliquots as described in Section 3.2.2.

4.3 Results

4.3.1 Identification and Measurement of 4-O-Methyl Gallic Acid and Isoferulic Acid by GCMS

4OMGA and isoferulic acid were measured against the same internal standard, 2-hydroxy-3-naphthoic acid. Chromatographic details of the ion extraction analysis of these compounds are given in Figures 4.1, 4.2 and 4.3. Isoferulic acid is a derivative of ferulic acid, one of the major metabolites of chlorogenic acid. Isoferulic acid and ferulic acid have the same mass spectrum and, therefore, characteristic ions of 323 and 338. However, isoferulic acid was eluted just before ferulic acid (Fig. 4.1). This was confirmed by injecting authentic standards. When scanning for the compounds chromatographically, ion extraction profiles used to identify isoferulic acid included ions of 323 and 338. The identification of 4OMGA was based on the elution of its major characteristic ions, which are 370 and 400 (Fig. 4.2). The internal standard was identified by its major characteristic ion of 317 (Fig. 4.3).

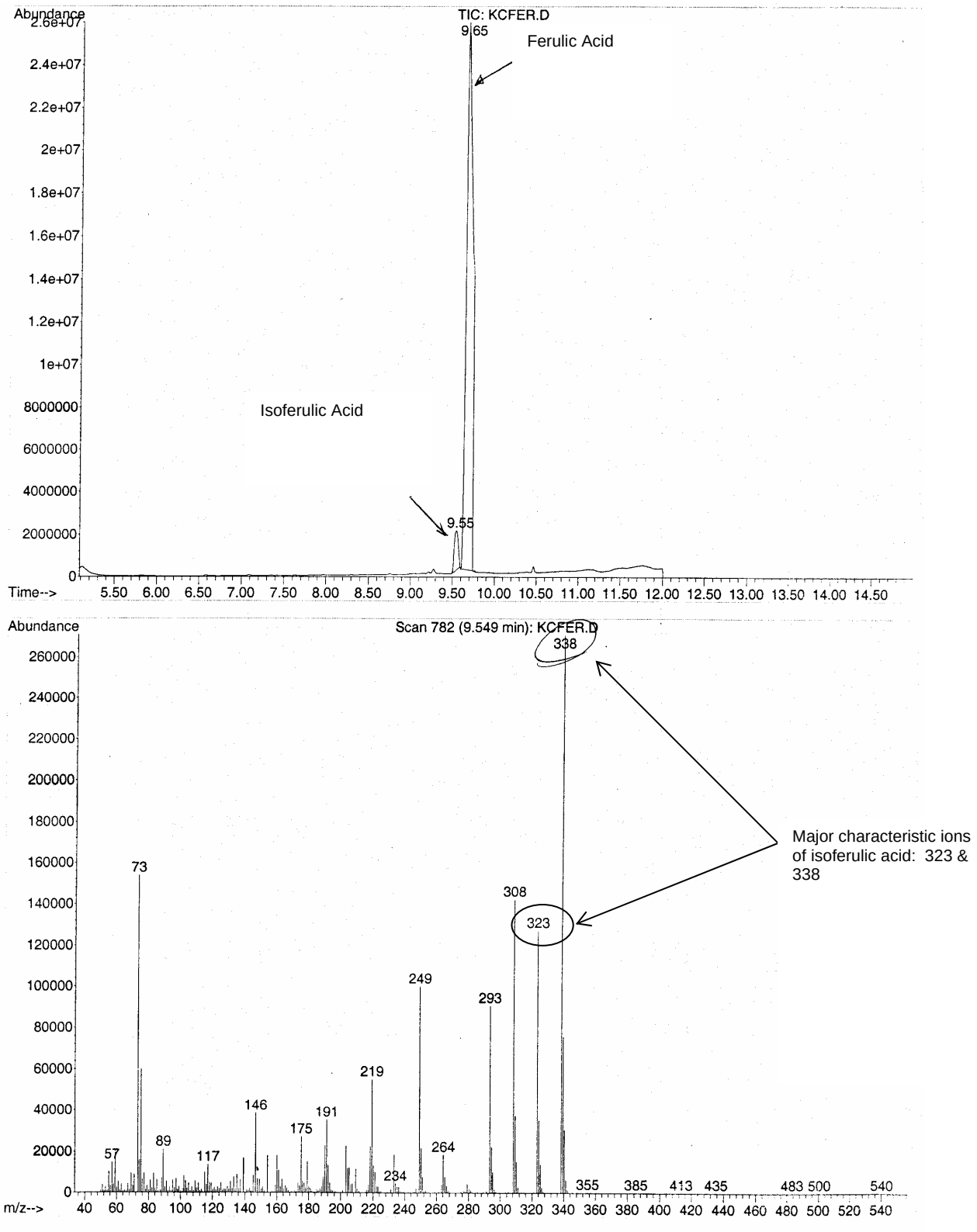
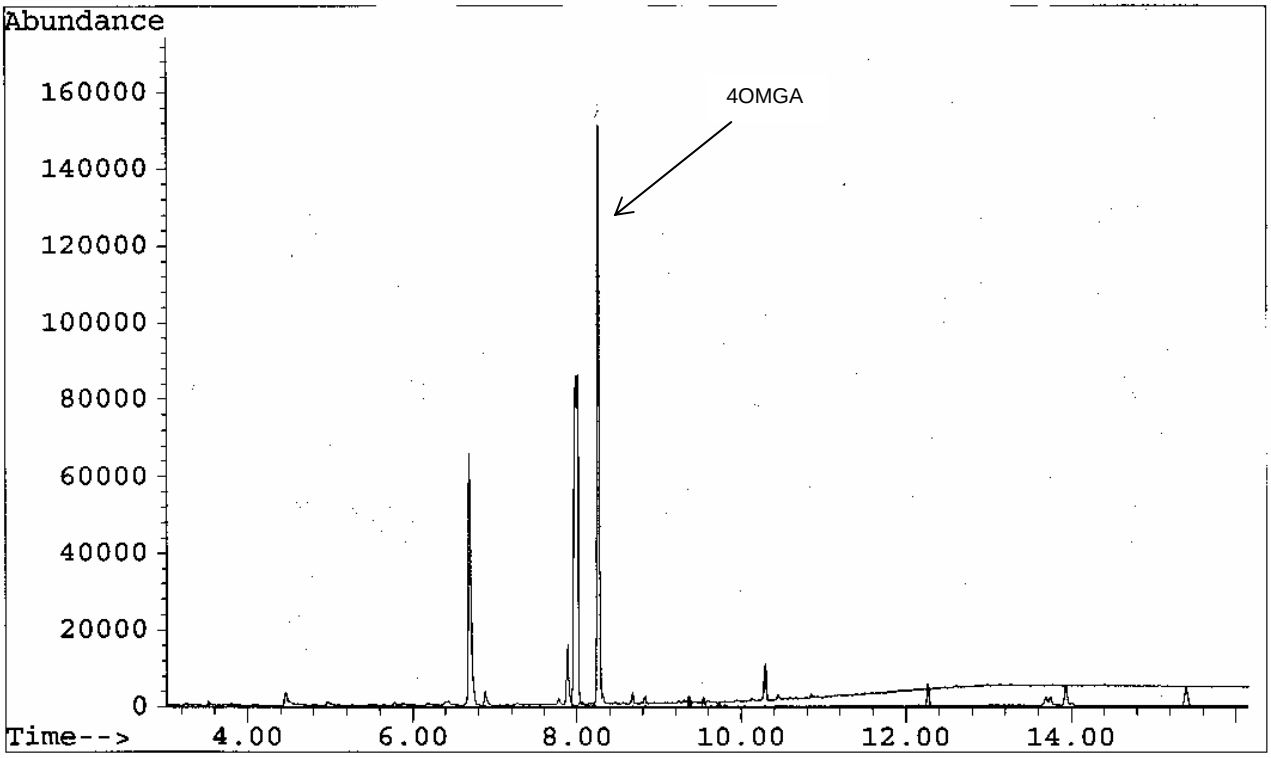


Figure 4.1 – Chromatographic Details of Isoferulic Acid



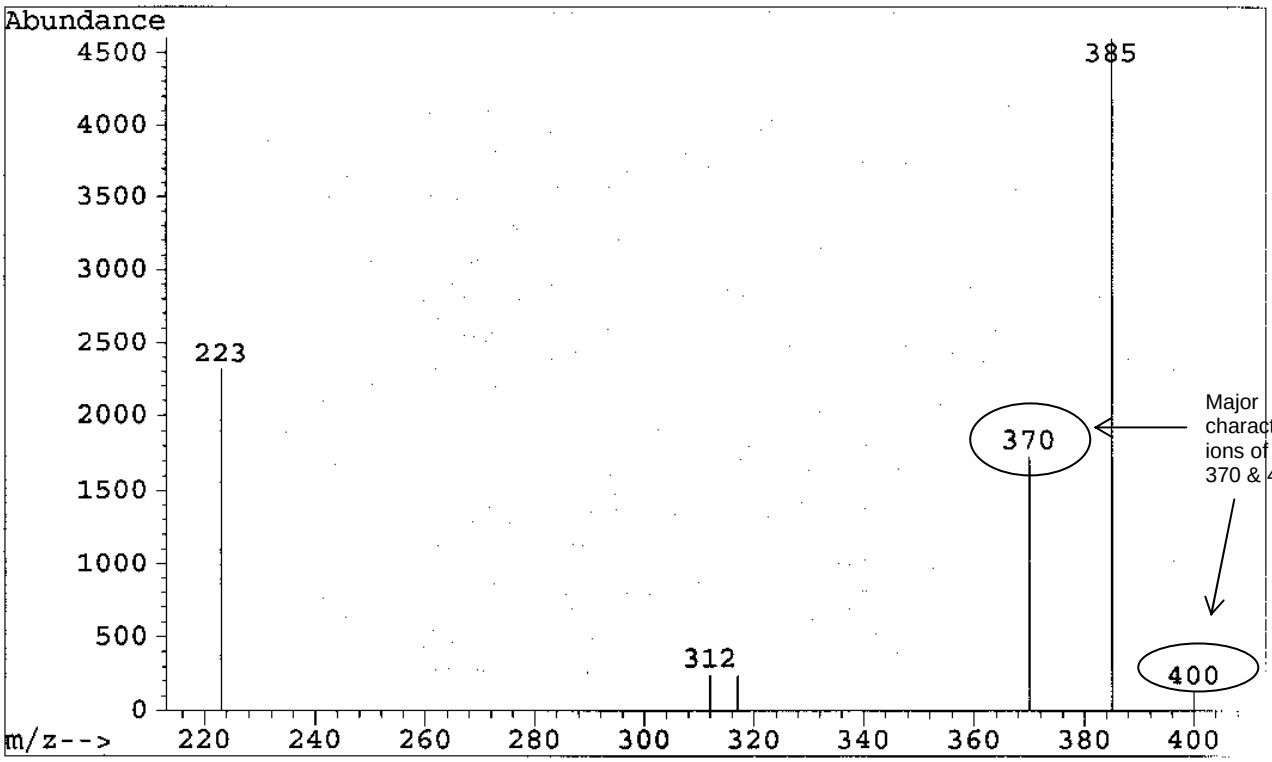


Figure 4.2 – Chromatographic Details of 4-O-Methyl Gallic Acid

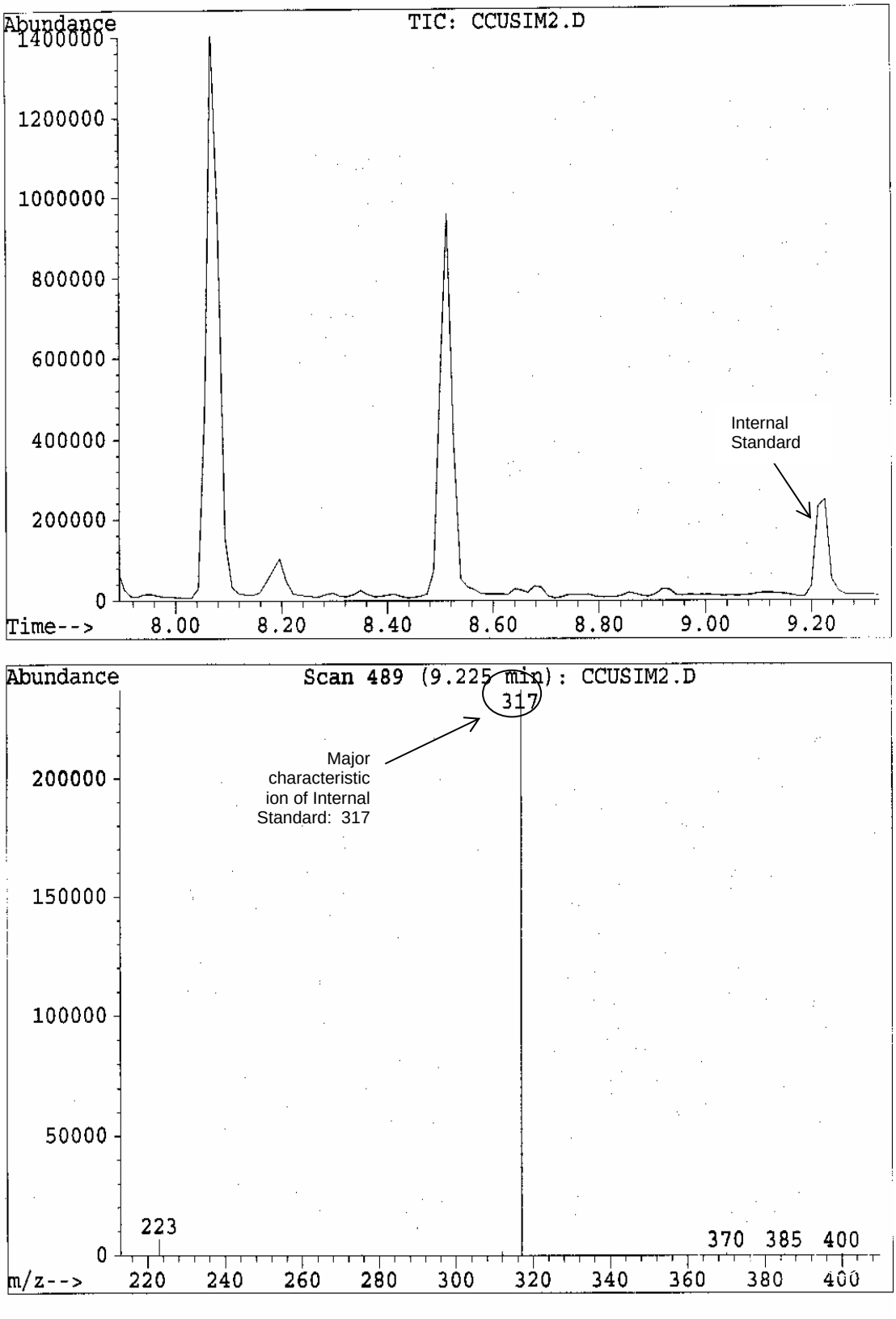


Figure 4.3 – Chromatographic Details of Internal Standard, 2-Hydroxy-3-Napthoic Acid

4.3.2 Results of GCMS Recovery Assay

Concentrations of 4OMGA from samples are calculated from the ratios of 4OMGA and the internal standard, and there is a chance that 4OMGA is lost during the extraction and or GCMS analysis procedures. Therefore, a recovery assay was carried out to verify this probability.

This recovery assay showed that the concentrations of standard 4OMGA added to the urine samples were lowered after the extraction procedure. The decrease in concentration was approximately 50% (i.e. decreased from 50ng to approximately 25ng after extraction). Although there is a loss of approximately 50% in the urine spikes, the ratios between the area under the curve (AUC) of 4OMGA and internal standard did not differ (see Table 4.1 and Table 4.2). Since the concentrations of 4OMGA were calculated from the AUC ratios of 4OMGA and internal standard, this loss will not influence accuracy of measured concentrations.

4.3.3 Urinary Excretion of 4-O-Methyl Gallic Acid and Isoferulic Acid

Urinary excretion of 4OMGA and isoferulic acid were calculated for urine samples obtained for two time periods: 0 to 6h and 6 to 24h after tea and coffee ingestion. Individual results of urinary excretion of 4OMGA and isoferulic acid during these time periods are shown in Figures 4.4 and 4.5. A clear dose-response was observed for 4OMGA in the 0 to 6h and 6 to 24h samples after black tea ingestion. As the dose of black tea ingested increased, the amount of 4OMGA excreted in urine also increased proportionally (Table 4.3). The dose-response observed for the coffee samples was only clear in the 0 to 6h samples and not the 6 to 24h samples. Figure 4.5A shows that as the dose of coffee ingested increased, the amount of isoferulic acid excreted into the urine also increased. This was also apparent over 24h (Table 4.3). However, there was considerable variability in response both between participants and in individual participants. In addition, isoferulic acid excretion after coffee consumption was >80% less than that of 4OMGA after black tea ingestion for both the 2g and 4g doses.

Table 4.2 – Comparisons of Areas under the Curves of Recovery Assay Samples

Sample	AUC		AUC Ratios of 4OMGA:Internal Standard
	4OMGA	Internal Standard	
1	-	1547263	-
2	25502577	-	-
3	15722002	710175	22.14
4	26209111	1170574	22.39

Table 4.3 – Mean 24h Urinary Excretion of 4-O-Methyl Gallic Acid and Isoferulic Acid Following Different Doses of Tea and Coffee, respectively.

Doses	0g		2g		4g	
Urine Collect at:	0 – 6h	6 – 24h	0 – 6h	6 – 24h	0 – 6h	6 – 24h
4OMGA (μg/mmol creatinine) from Black Tea	0	0	432.45 ± 287.41	252.869 ± 223.26	938.331 ± 592.65	500.60 ± 333.28
Isoferulic acid (μg/mmol creatinine) from Coffee	0	0	31.93 ± 53.23	34.53 ± 36.00	241.44 ± 277.73	51.75 ± 45.46

Data is presented as mean ± SD.

4.4 Discussion

Processing and differing methods of preparation of tea or coffee can lead to substantial differences in chemical composition. However, the final beverages consistently contain high concentrations of polyphenols, including gallic acid and chlorogenic acid, respectively. One cup of tea (2 g) will contain about 25 to 50 mg of free or esterified gallic acid (Hodgson, J. M. *et al*., 2000) and one cup of coffee (2 g) will contain 100 to 200 mg of chlorogenic acid as 5-caffeoyl quinic acid (Nardini, M. *et al*., 2002).

We proposed that 4OMGA and isoferulic acid might be useful as biomarkers of tea and coffee-derived polyphenol exposure. They may be used as markers of intake, compliance, polyphenol absorption and metabolism, and to investigate the

relationships with disease-related endpoints. They may have advantages over assessment of intake where an indication of polyphenol intake and absorption or metabolism is required. An important criterion for these potential biomarkers is that a dose-response relationship can be observed.

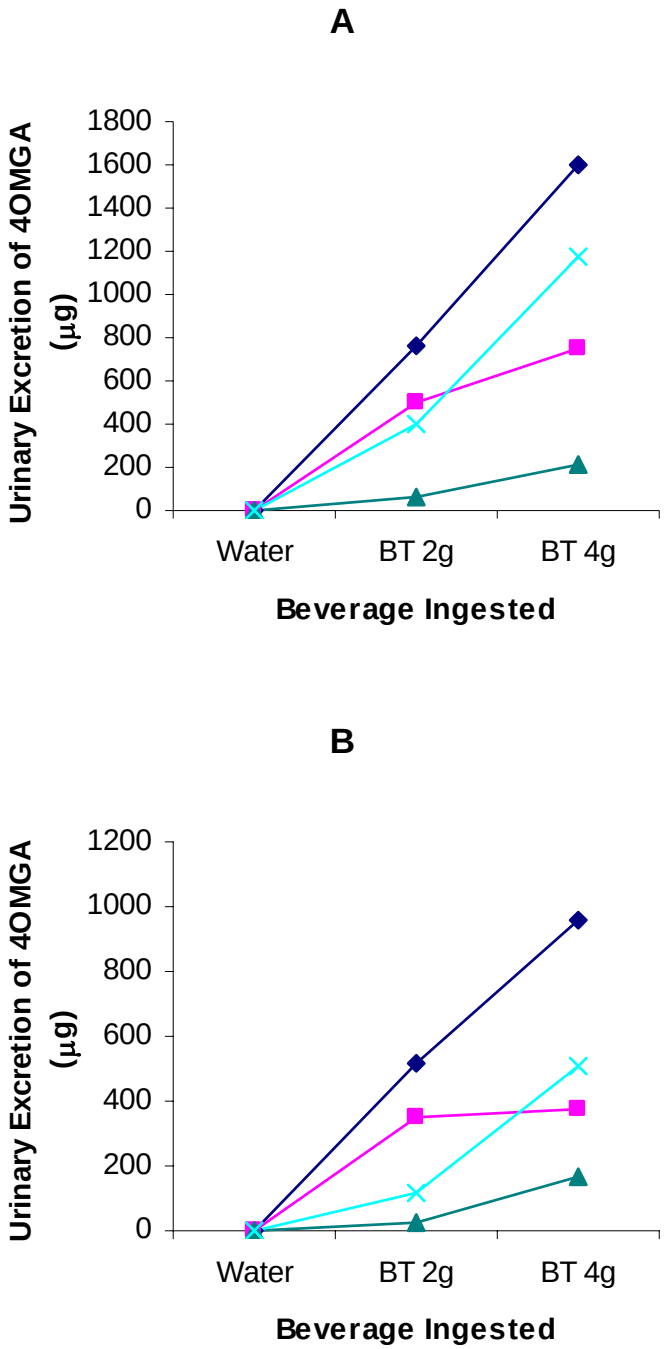


Figure 4.4 – Individual Urinary Excretion of 4-O-Methyl Gallic Acid from (A) 0 – 6h and (B) 6 – 24h after Ingesting 0g, 2g and 4g of Black Tea (♦ = participant 1; ■ = participant 2; ▲ = participant 3; × = participant 4)

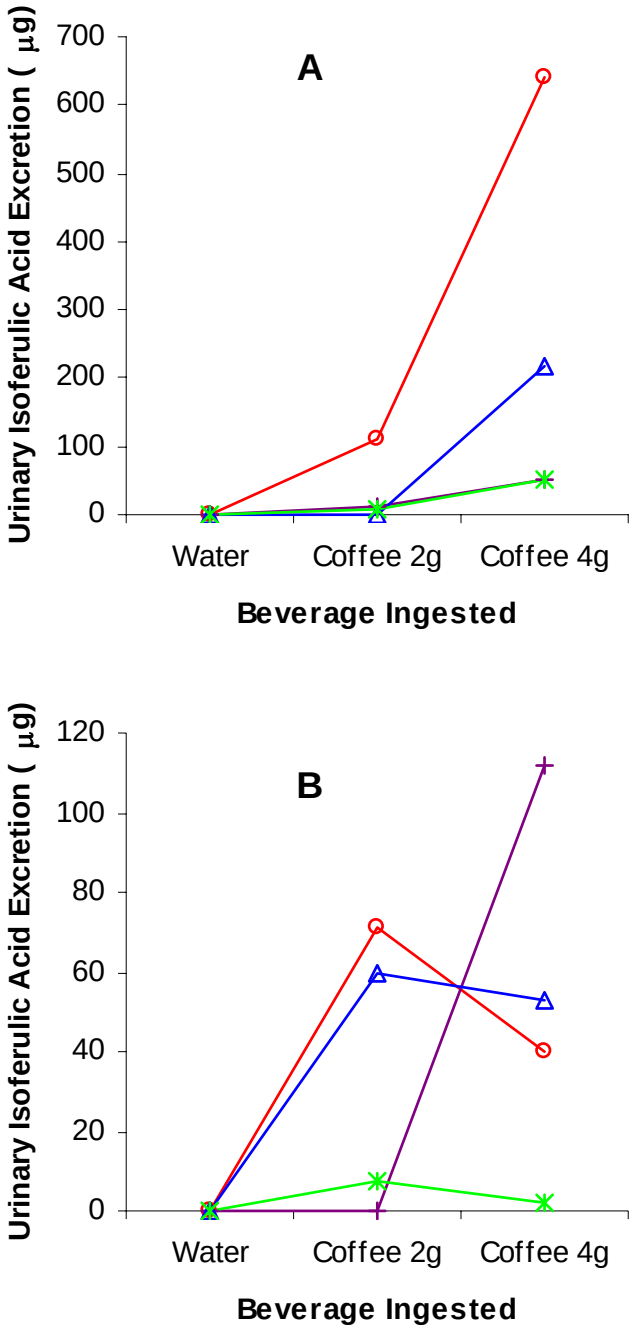


Figure 4.5 – Individual Urinary Excretion of Isoferulic Acid from 0 – 6h (A) and 6 – 24h (B) after Ingesting 0g, 2g and 4g of Coffee (○ = participant 1; + = participant 3; △ = participant 3; ✳ = participant 4)

In this study, a dose-related increase in urinary excretion of 4OMGA was found following ingestion of black tea. This supports the suggestion that 4OMGA is

a specific and major metabolite of gallic acid metabolism. There was considerable variability in the 4OMGA response between participants, but there was a consistent dose-related increase within individual participants. (A more detailed discussion of 4OMGA production and metabolism is presented in Chapter 5, Section 5.4). These results are an indication that 4OMGA may be a feasible biomarker of tea intake in humans.

A dose-related increase in urinary excretion of isoferulic acid was also found following ingestion of coffee. These results are consistent with the suggestion that isoferulic acid is a specific metabolite derived from chlorogenic acid (Rechner, A. R. *et al*., 2001). However, the absolute increase in isoferulic acid was less than that seen for 4OMGA, despite a larger dose of chlorogenic acid from coffee in comparison to gallic acid from tea. This suggests that less of the end metabolism of chlorogenic acid is in the form of isoferulic acid. Isoferulic acid, although a specific metabolite of chlorogenic acid metabolism, appears not to be a major metabolite. Alternatively, it may have undergone further metabolism before urinary excretion. In addition, there was considerable variability in the isoferulic acid response both between participants and within individual participants. This may be related to the metabolic pathways involved in isoferulic acid production and metabolism. (A more detailed discussion of isoferulic acid production and metabolism is presented in Chapter 5, Section 5.4)

CHAPTER 5

POPULATION STUDIES

5.1 Introduction

Tea and coffee are widely consumed beverages. They can be major dietary sources of poly-hydroxylated phenolic compounds (polyphenols), including phenolic acids and flavonoids. Tea contains primarily flavonoids, as well as up to 15% (25-50 mg/cup) of total polyphenols present as free or esterified gallic acid (Hodgson, J. M. *et al.*, 2000a; Shahrzad, S. *et al.*, 2001). Coffee polyphenols are almost entirely chlorogenic acid (100-200 mg/cup) (Clifford, M. N., 1999). Polyphenols have a range of activities (Morton, L. W. *et al.*, 2000) consistent with the potential to impact positively on human health. Results of population studies showing a higher intake of flavonoids to be associated with lower risk for chronic diseases would support this hypothesis (Hertog, M. L. *et al.*, 1995; Knekt, P. *et al.*, 2002).

The identification of biomarkers of dietary polyphenol exposure would provide an additional tool to investigate relationships with chronic disease-related endpoints. Several potential biomarkers of exposure to tea or coffee-derived polyphenols have been identified (Kivits, G. A. A. *et al.*, 1997; Hodgson, J. M. *et al.*, 2000a; Rechner, A., R. *et al.*, 2001; Shahrzad, S. *et al.*, 2001; Meng, X. F. *et al.*, 2002). These include specific O-methylated polyphenols derived from *in vivo* polyphenol metabolism, such as 4-O-methylgallic acid (4OMGA) and isoferulic acid (Hodgson, J. M. *et al.*, 2000a; Rechner, A. R. *et al.*, 2001; Shahrzad, S. *et al.*, 2001; Meng, X. R. *et al.*, 2002).

A major metabolite of gallic acid in humans is 4OMGA (Shahrzad, S. & Bitsch, I., 1998; Hodgson, J. M. *et al.*, 2000a; Shahrzad, S. *et al.*, 2001). 4OMGA has been used to assess compliance in intervention studies (Caccetta, R. A. A. *et al.*, 2001; Hodgson, J. M. *et al.*, 2000b; Hodgson, J. M. *et al.*, 2001; Hodgson, J. M. *et al.*, 2002a; Hodgson, J. M. *et al.*, 2002b), and as a marker of tea-derived polyphenol metabolism (Hodgson, J. M. *et al.*, 2003). It has been suggested that 4OMGA could be a biomarker of tea intake (Hodgson,

J. M. *et al.*, 2000a), and may be a good marker for tea-derived polyphenol exposure, which incorporates intake and absorption. The relationship between 4OMGA and tea intake has not been assessed within populations.

The chlorogenic acid in coffee is present primarily as 5-caffeoyl quinic acid (chlorogenic acid), which is an ester of caffeic acid with quinic acid (Herrmann, K., 1989; Clifford, M. N., 1999). Isoferulic acid has been identified as a specific metabolite of dietary caffeic acid derivatives, and was suggested as a useful biomarker for intake and metabolism of caffeic acid derivatives from dietary sources (Rechner, A. R. *et al.*, 2001). Since coffee is a rich dietary source of chlorogenic acid, we proposed that isoferulic acid might be useful as a biomarker for coffee intake and coffee-derived polyphenol exposure. The relationship between isoferulic acid and coffee intake has not been assessed within populations.

The ideal relationship between any exposure and its marker is linear. The major objective of this study was to explore relationships of 24 h urinary excretion of 4OMGA and isoferulic acid with usual and current tea and coffee intake in human populations.

5.2 Methods

5.2.1 Study Participants

The relationships of 4OMGA and isoferulic acid with usual (Population 1) and contemporaneously recorded current (Population 2) tea and coffee intake were investigated within two populations. For both populations, tea and coffee intake were assessed in terms of number of cups consumed. Almost all tea intake was black tea with added milk and most coffee intake was instant coffee. Tea and coffee intakes were not subcategorised due to small numbers within subcategories. The statistical prevalence within the population of gastrointestinal or renal disease, which may influence phenolic acid metabolism, is very low (<2%) and therefore it is considered unlikely that either disorder influenced the observed relationships.

5.2.1.1 Population 1 – Relationship of 4-O-Methyl Gallic Acid and Isoferulic Acid with Usual Tea and Coffee Intake

Population 1 included 111 participants, comprising 43 men and 68 women with a mean age of 55.8 (95%CI:53.1,58.5) years, recruited as a representative sample of Anglo-Celtic men and women residing in Melbourne, Australia (Dalais, F. S. *et al.*, 1998; Kouris-Blazos, A. *et al.*, 1999). Tea and coffee intake were assessed using a food frequency questionnaire (Kouris-Blazos, A. *et al.*, 1996). 4OMGA and isoferulic acid were measured in a 24 h urine sample collected at the time participants filled out the food frequency questionnaire. The relationships of usual tea and coffee intake with urinary excretion of 4OMGA and isoferulic acid, respectively, were assessed.

5.2.1.2 Population 2 – Relationship of 4-O-Methyl Gallic Acid and Isoferulic Acid with Current Tea and Coffee Intake

Population 2 included 344 participants, 50 men and 294 women with a mean age of 71.7 (95%CI: 70.8, 72.6) years, recruited to two studies. The first of these studies included 275 women with a mean age of 75.1 (95%CI:74.8,75.4) years, recruited from the Western Australian general population of women over 70 years of age (Dick, I. M. *et al.*, 2002). All participants were healthy and did not have any medical conditions likely to influence 5 year survival. Although the participants entering the study were weighted in favour of those in higher socio-economic categories, they did not differ from the whole population in health resource utilization (Bruce, D. G. *et al.*, 2002). Tea and coffee intake were assessed using a single interviewer administered 24 h diet recall. Informed consent was obtained and the Human Rights Committee of the University of Western Australia approved the study. The second study recruited 69 participants, comprising 50 men and 19 women with a mean age 58.0 (95%CI: 55.3, 60.7) y from the Western Australian general population to a cross-sectional and case-control study. Participants involved in this study were recruited on the basis of having normal or elevated blood pressure. Current tea and coffee intake were assessed using a 24 h beverage intake diary. Informed consent was obtained and the Royal Perth Hospital Ethics Committee approved the study.

For both studies, a 24 h urine sample was collected for the period corresponding to that for which tea and coffee intake were assessed. Relationships observed for tea and coffee intake with 4OMGA and isoferulic acid excretion, respectively, within the two studies were almost identical. Inclusion of participants from both these studies in Population 2 allowed analysis in both men and women.

5.2.2 Analysis of Urinary 4-O-Methyl Gallic Acid and Isoferulic Acid

Urinary excretion of 4OMGA and isoferulic acid were measured in urine samples obtained from the participants after beverage administration. The compounds were extracted from the urine aliquots as described in 3.2.2.

5.2.3 Statistics

All statistical analyses were performed using SPSS Version 11.5 (Chicago, IL, USA). Results are presented as means and 95 % confidence intervals (CI) and $P<0.05$ was used as the level of significance. The creatinine-adjusted urinary concentrations of 4OMGA and isoferulic acid were not normally distributed, and therefore log-transformed values were used. Univariate associations were assessed using Spearman's rank correlation. Linear regression was used to investigate the relationships of 4OMGA and isoferulic acid with tea and coffee intake after adjustment for potential confounding factors.

5.3 Results

The relationships of 4OMGA and isoferulic acid with usual tea and coffee intake were assessed in Population 1, and with current intake in Population 2. The tea and coffee intake, and the 24 h urinary excretion of 4OMGA and isoferulic acid for each population are presented in Table 5.1.

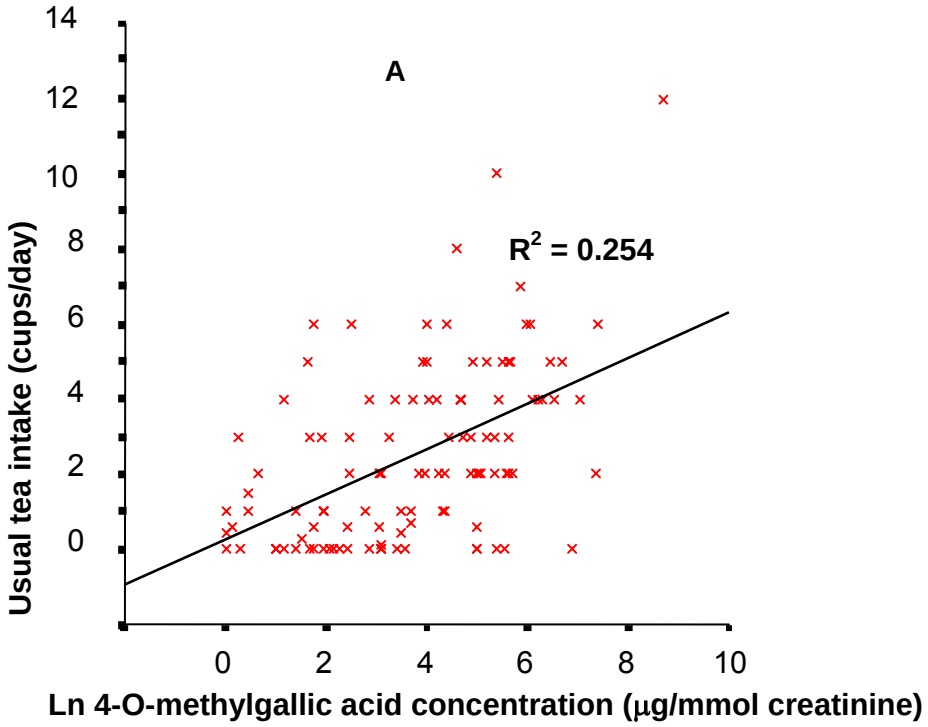
Table 5.1 – Tea and Coffee Intake, and Mean 24-hour Urinary Excretion of 4-O-Methyl Gallic Acid and Isoferulic Acid for Two Populations: Usual Tea and Coffee Intake was Assessed in Population One and Current Tea and Coffee Intake was Assessed in Population Two

	Population 1 (n=111): Usual intake		Population 2 (n=344): Current intake	
	Mean	95% CI	Mean	95% CI
Tea intake (cups/d)	2.49	(2.05,2.93)	1.99	(1.80,2.18)
Coffee intake (cups/d)	1.56	(1.23,1.90)	1.28	(0.98,1.52)
4-O-methylgallic acid (μg/mmol creatinine)[1]	42.1	(29.0,61.2)	38.9	(30.9,49.3)
Isoferulic acid (μg/mmol creatinine)[1]	11.1	(8.8,14.1)	7.8	(6.4,9.5)

[1] Results are geometric mean and 95% confidence intervals

4OMGA was strongly related to usual (r = 0.50, $P < 0.001$) and current (r = 0.57, $P < 0.001$) tea intake. Isoferulic acid, by comparison, was less strongly related to coffee intake, but the results were, nevertheless significant (usual intake: r = 0.26, $P = 0.008$ and current intake: r = 0.18, $P < 0.001$) coffee intake. Figures 5.1A and B show the relationship between 4OMGA and current and usual tea intake. Figures 5.2A and B show the relationship between isoferulic acid and current and usual coffee intake. The relationship observed between urinary isoferulic acid and usual and actual coffee intake is clear but weak. The results for both current and usual intake of tea and coffee were adjusted for age, sex and source of study participants. These adjustments did not alter the observed relationships.

The relationships of 4OMGA and isoferulic acid with current tea and coffee intake, respectively, are summarised in Figure 5.3. A cut-off concentration for 4OMGA excretion of 25μg/mmol creatinine had 82% sensitivity and 81% specificity for prediction of tea drinking status. A cut-off concentration for isoferulic acid excretion of 5 μg/mmol creatinine had 61% sensitivity and 57% specificity for prediction of coffee drinking status. The relationships of 4OMGA and isoferulic acid with usual tea and coffee intake, respectively, are summarised in Figure 5.4.



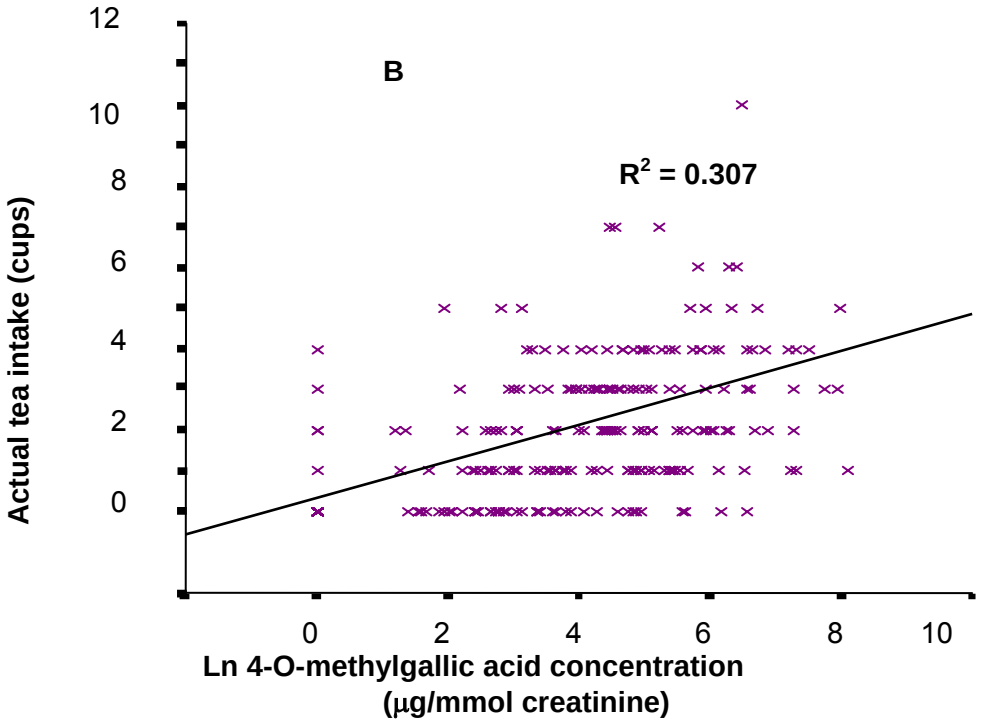


Figure 5.1 – Graphs Showing the Relationship of Urinary 4-O-Methyl Gallic Acid with (A) Usual Tea Intake and (B) Actual Tea Intake

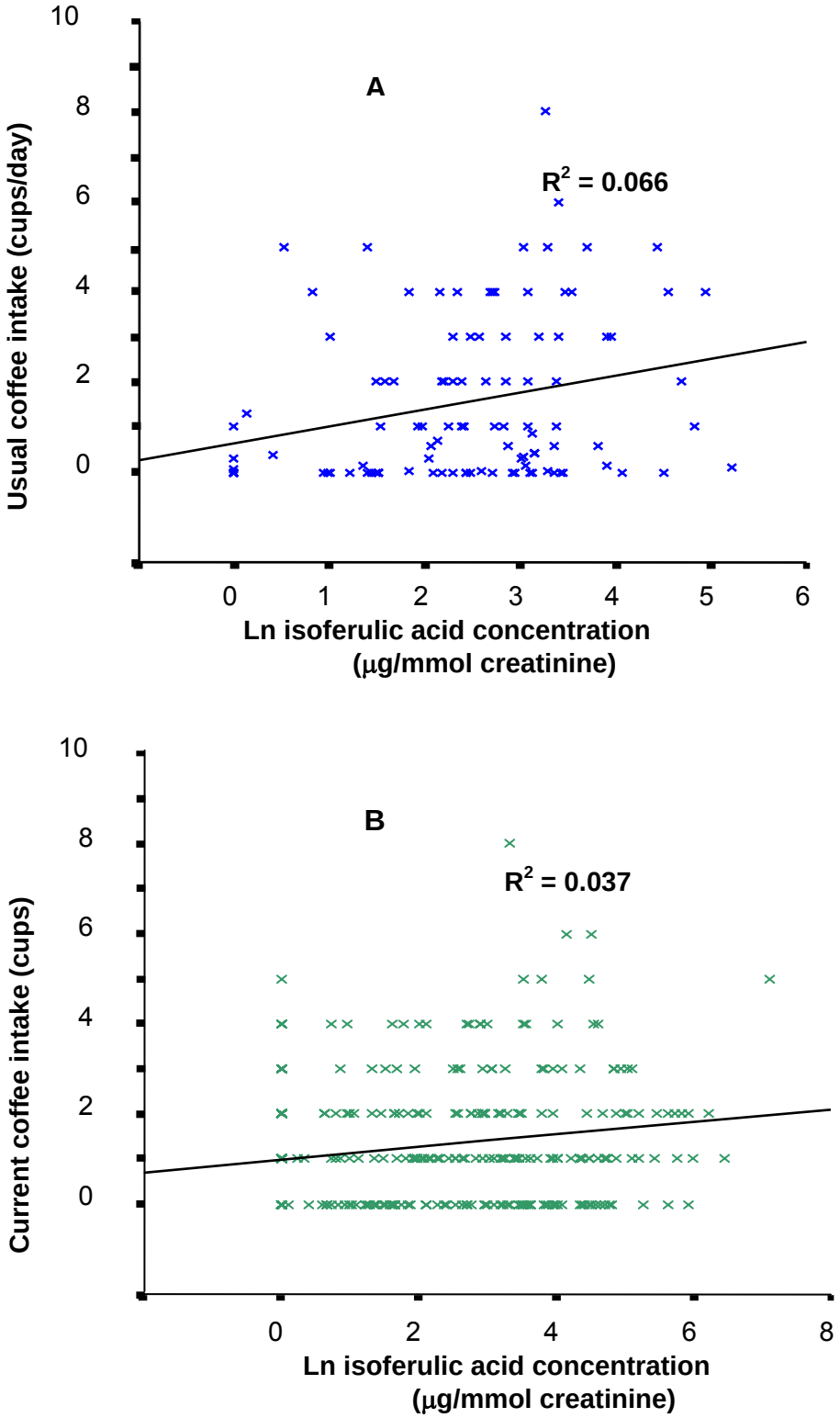


Figure 5.2 – Graphs Showing the Relationship of Urinary Isoferulic Acid with (A) Usual Coffee Intake and (B) Actual Coffee Intake

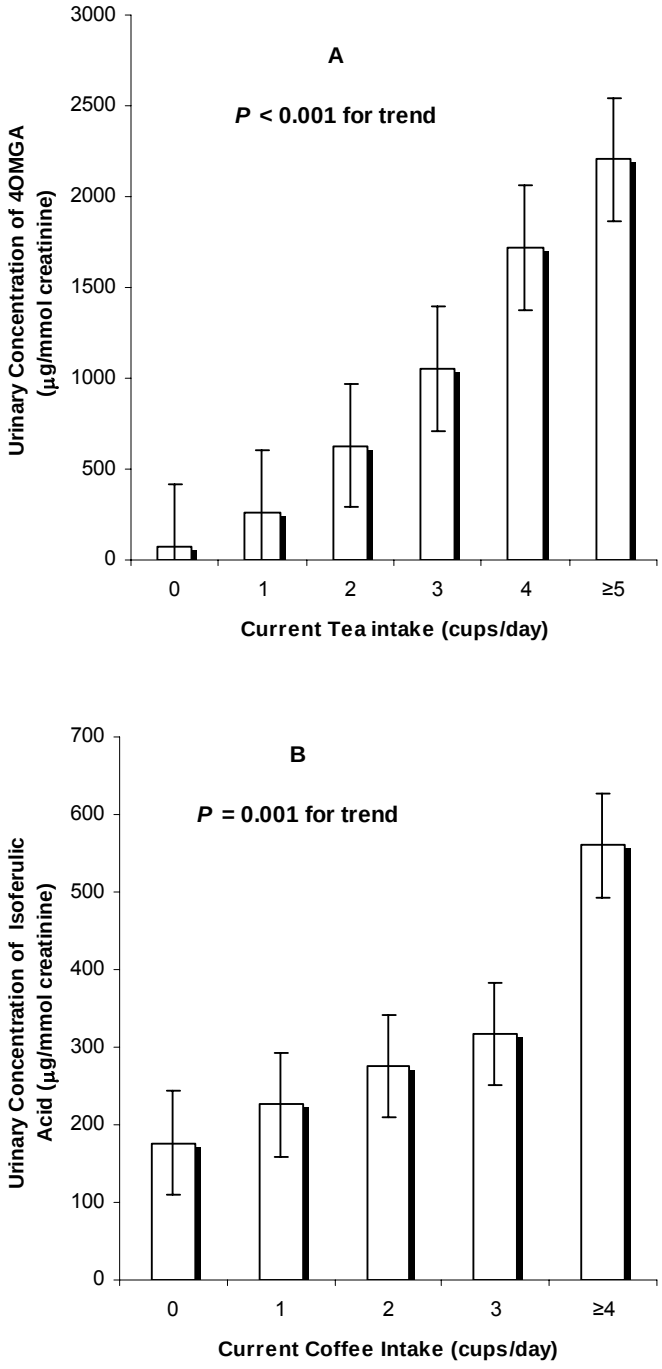


Figure 5.3 – Mean (A) 4-O-Methyl Gallic Acid Excreted by Cups of Current Tea Intake and (B) Isoferulic Acid Excretion by Cups of Current Coffee Intake in Population 2

Results are geometric means and 95% confidence intervals. P-Values are for linear trend analysed by linear regression.

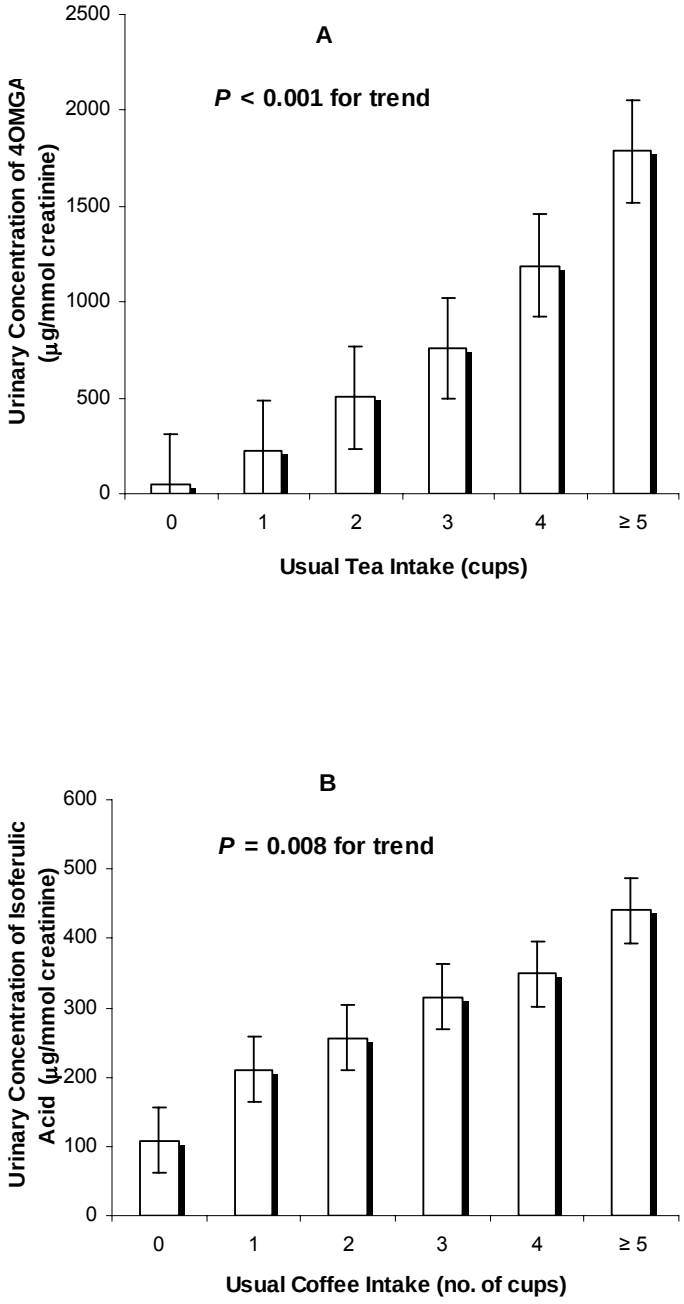


Figure 5.4 – Mean (A) 4-O-Methyl Gallic Acid Excreted by Cups of Usual Tea Intake and (B) Isoferulic Acid Excretion by Cups of Usual Coffee Intake in Population 1

Results are geometric means and 95% confidence intervals. P-Values are for linear trend analysed by linear regression.

5.4 Discussion

Tea and coffee are rich dietary sources of polyphenols. All tea derives from the plant *Camellia sinensis*. Coffee can be obtained from several *Coffea* species, with *C. Arabica* representing most of the world's coffee production. Tea can be processed into a variety of types, the two main types being black and green. Promoting the enzymatic oxidation of polyphenols produces black teas, whereas enzymes involved in polyphenol oxidation are inactivated in the production of green tea. Production of black tea results in changes to polyphenol structure and composition, including increased free gallic acid concentrations. Coffee can also be processed and prepared for drinking in a range of ways. Processing and differing methods of preparation of tea or coffee can lead to substantial differences in chemical composition. However, the final beverages consistently contain high concentrations of polyphenols, including gallic acid and chlorogenic acid, respectively.

Identification of biomarkers of intake of, and exposure to tea and coffee-derived polyphenols has not been straightforward. This has been largely due to the complex nature of many of the polyphenols present in tea, and the lack of knowledge regarding polyphenol metabolism. We proposed that 4OMGA and isoferulic acid might be useful as biomarkers of tea and coffee-derived polyphenol exposure, and may be used as markers of intake, compliance, polyphenol absorption and metabolism, and to investigate the relationships with disease-related endpoints. 4OMGA and isoferulic acid may have advantages over assessment of intake where an indication of polyphenol intake and absorption or metabolism is required.

Following ingestion of black tea, most urinary 4OMGA is likely to be derived from free, rather than esterified gallic acid. Much of the gallic acid present in black tea is free gallic acid, whereas green tea, which has similar total gallic acid present as gallate esters, contains little free gallic acid (Harbowy, M. E. & Ballentine, D. A., 1997, Hodgson, , J. M. *et al.*, 2000a). Hodgson *et al*. (2000b) have previously shown that following a set dose of black and green tea, the increase in urinary 4OMGA was 3-fold greater for black tea. Free gallic acid may be absorbed in the small intestine, transported to the liver where O-methylation and glucuronidation occurs, then excreted.

In contrast, most urinary isoferulic acid derives from cleavage of the caffeoyl quinic acid derivatives. This is likely to occur in the large intestine via esterase activity of gut microflora (Plumb, G. W. *et al.*, 1999, Rechner, A. R. *et al.*, 2001). Gallic acid may also be released from larger flavonoids containing gallate esters by the same means. The observed increase in urinary 4OMGA excretion following ingestion of green tea is consistent with this suggestion (Hodgson, J. M. *et al.*, 2000b). The free caffeic acid or gallic acid released may then be absorbed, transported to the liver where O-methylation to isoferulic acid and 4OMGA, respectively, and glucuronidation occurs, then excreted. Alternatively, the gut microflora could also be responsible for O-methylation, with the compounds absorbed in this form (Chesson, A. *et al.*, 1999). In addition, the phenolic acids may be further metabolised to smaller molecular weight compounds (Scalbert, A. and Williamson, G. 2000).

Peak plasma concentrations of phenolic acids occur about 1 to 2 h after ingestion (Shahrzad, S. *et al.*, 2001, Nardini, M. *et al*., 2002). The precise time may relate to the location of absorption: small or large intestine. Incorporation into tissues is possible (Youdim, K. A., Martin, A. and Joseph, J. A., 2000), but is likely to be small. The O-methylated phenolic acids are then rapidly excreted and fully eliminated from plasma within 12 to 24 h (Shahrzad, S. *et al.*, 2001). Therefore, 24 h urine samples will provide a better indication of short term consumption pattern and exposure than plasma measurements.

Urinary 4OMGA excretion was related to both usual and current tea intake. As might be expected, urinary 4OMGA predicted more of the variance in current tea intake than usual tea intake. However, the strength of the relationship was only slightly weaker for usual intake, suggesting that current tea consumption strongly predicts usual consumption. Although the mean 4OMGA concentrations were low in participants not drinking tea (Fig. 5.3A and 5.4A), the presence of 4OMGA in a substantial number of these participants is suggestive of other dietary sources. Therefore, some of the unexplained variance may be due to the presence of other dietary sources of gallic acid (Herrmann, K., 1989; Caccetta, A. *et al.*, 2001), but in comparison to tea, their contribution is likely to be minor. It was also seen that the specificity and sensitivity of 4OMGA prediction in usual tea intake dropped radically despite the same relationship being observed in current tea intake. Hodgson *et al*. (2003) have previously found that

regardless of background, diet urinary excretion of 4OMGA increases at least 3-fold and up to14-fold following an increase in regular ingestion of black tea by 5 cups/d. In addition, the 4OMGA response to a given dose of gallic acid is highly variable, and there are errors in estimation of intake, and variations in the strength of tea and coffee ingested. However, overall the results are consistent with the proposal that 24 h excretion of 4OMGA provides a good biomarker of exposure to tea-derived polyphenols, and a reasonable estimate of intake.

Isoferulic acid was significantly related to coffee intake, but the relationship was weak (Fig. 5.2A and B). The results are consistent with the suggestion that isoferulic acid is a specific metabolite derived from chlorogenic acid (Rechner, A. R. *et al.*, 2001). However, less than 7% of the variance in coffee intake was explained by isoferulic acid excretion, and the absolute increase in isoferulic acid was less than that seen for 4OMGA. This suggests that less of the end metabolism of chlorogenic acid is in the form of isoferulic acid. The weak relationship may be due to the presence of other dietary sources of caffeic acid derivatives as well as variability in isoferulic acid excretion in response to a given dose of chlorogenic acid. The presence of isoferulic acid in those not drinking coffee within the population studies is suggestive of other dietary sources. Therefore, isoferulic acid, although a specific metabolite of chlorogenic acid metabolism, appears not to be a major metabolite. Alternatively, it may have undergone further metabolism before urinary excretion. Differences in metabolism between free and esterified phenolic acids may help to further explain the weak association of isoferulic acid with coffee intake.

Overall, our results are consistent with the proposal that 4OMGA can be used as a biomarker for tea intake, and may provide a good indication of exposure to tea-derived polyphenols. However, isoferulic acid is quantitatively a minor metabolite in the metabolism of caffeic acid derivatives. Although isoferulic acid was significantly related to coffee intake in the population studies, the relationship was weak, limiting its usefulness as a potential biomarker of intake of coffee-derived polyphenols.

CHAPTER 6

FINAL DISCUSSION AND FUTURE DIRECTIONS

Tea and coffee are rich dietary sources of polyphenols. Dietary polyphenols, including those derived from tea and coffee, are thought to have a variety of health-related effects. Typically, one cup of tea brewed from 2g of tea will contain approximately 200 to 400mg of total polyphenols and 25 to 50mg of total gallic acid, whilst one cup of coffee brewed from 2g of coffee powder will contain 100 to 200mg of chlorogenic acid as 4-caffeoyl quinic acid. However, as a result of different processing and brewing methods applied in the manufacture and preparation of tea and coffee, the composition and concentration of polyphenols in the final brew can be influenced. The result is that exposure to tea and coffee-derived polyphenolic compounds may be poorly estimated using an assessment of number of cups ingested per day. Identification of biomarkers of exposure to tea and coffee-derived polyphenols may, therefore, provide a better indicator of exposure than estimated intake in cups.

It was proposed that 4OMGA and isoferulic acid may be useful biomarkers for tea and coffee-derived polyphenol exposure. Given that the ideal relationship between any exposure and its marker is linear, the major objectives were to establish a dose-response relationship of 24h urinary excretions of 4OMGA and isoferulic acid following ingestions of black tea and coffee of different strengths, and to explore relationships of tea and coffee intake with 24h urinary excretion of 4OMGA and isoferulic acid in human populations. Two studies were used to address these objectives.

In the first study, a dose-related increase in urinary 4OMGA and isoferulic acid excretion after the ingestion of black tea and coffee, respectively, was shown. These results support the suggestion that 4OMGA and isoferulic acid are specific metabolites of tea and coffee-derived polyphenols. It was also found that following a fixed dose of tea or coffee, most of the 4OMGA and isoferulic acid was excreted within the first 6 hours. These results are consistent with previous studies of polyphenol metabolism.

In the second study, it was found that urinary 4OMGA excretion was related to both usual and current tea intake. Up to 30% of the variance in tea intake was predicted by 4OMGA in the populations studied. The unexplained variance is likely to be due to the presence of other dietary sources of gallic acid, as well as inter-individual variation in 4OMGA response to a given dose of tea. However, the results of this study, as well as previous studies, suggest that tea-derived gallic acid is the primary contributor to urinary 4OMGA excretion within the populations.

Future intervention studies with black tea can now use 24 h urinary excretion of 4OMGA as an indicator of compliance. In addition, the measurement of 4OMGA in cross-sectional and prospective population studies could be used as a biomarker of tea-derived polyphenol exposure. If tea polyphenols are responsible for any health-related effects, measurements of 4OMGA may be more strongly associated with specific endpoints than estimation of tea intake in terms of cups consumed.

Isoferulic acid excretion was also associated with both usual and current coffee intake. However, despite the fact that the chlorogenic acid content of coffee is considerably higher than the content of gallic acid in tea, the increase in urinary isoferulic acid was lower than that of urinary 4OMGA. This suggests that isoferulic acid is not the major end product of chlorogenic acid metabolism, and although isoferulic acid is a specific metabolite of chlorogenic acid metabolism, it is not a major metabolite. Alternatively, it is possible that isoferulic acid may undergo further metabolism before urinary excretion.

Future work may look at alternative potential biomarkers of exposure to coffee-derived polyphenols. Although isoferulic acid is a specific metabolite of chlorogenic acid, its feasibility of acting as a biomarker for coffee-derived polyphenol exposure is questionable since it is also rich in other foodstuff such as plums. Isoferulic acid may be of use in intervention studies to monitor compliance, but measurement of 24 h excretion in cross-sectional and prospective population studies is unlikely to provide a better indicator of coffee-derived polyphenol exposure than assessment of intake in terms of cups consumed.

Overall, the studies are consistent with the proposal that 4OMGA is a good biomarker for black tea-derived polyphenol exposure, but isoferulic acid may be of limited value as a biomarker for coffee-derived polyphenol exposure.

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