Liquid Chromatography Tandem Mass Spectrometry Identification of Apple Polyphenol Metabolites in Human Urine and Plasma

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Abstract

Apples are a major dietary source of polyphenols in the Western diet and contain procyanidins, hydroxycinnamic acids, flavanols, dihydrochalcones and flavonols. Despite their abundance and familiarity very little research into their metabolism has been performed; research is required to elucidate the metabolic products of these polyphenols and characterise their absorption and excretion pathways. A human intervention study was designed specifically to investigate the absorption, metabolism, excretion and biokinetcs of apple polyphenols. Male volunteers (n = 9) consumed a supermarket apple juice substituted with water as the control phase, and the same apple juice substituted with a high polyphenol cider apple extract as the test phase. Blood samples were taken over 0-24 h and urine samples were collected at 0-4 h, 4-8 h and 8-24 h. A rapid, validated and novel single LC/ESI/MS/MS method was developed and validated for the analysis of a wide range of polyphenols and their metabolites in these urine and plasma samples (after sample preparation). Apple polyphenol-related metabolites were identified using LC/MS/MS and MS^2; nine urinary metabolites and seven plasma metabolites were identified, mostly for the first time after apple consumption. Data on the excretion, bioavailability and biokinetics of these metabolites, including products of the colonic microflora, were obtained. In urine, the major apple-related polyphenolic metabolites identified were dihydroxyphenyl valerolactone sulfate and 5- (3′, 4′-dihydroxyphenyl) -γ- valerolactone glucuronide; both colonic bacterial metabolites which appear at their maximum concentrations 4-8 h post apple ingestion. Minor metabolites included (-) epicatechin sulfate and glucuronide conjugates. In plasma, 3, 4-dihydroxyphenylacetic acid, 5- (3′, 4′-dihydroxyphenyl) -γ- valerolactone glucuronide and dihydroxyphenyl valerolactone sulfate predominate; T_{max} values of 5-6 h were observed. Minor plasma metabolites included phloretin (C_{max} 291 ± 175 nM) and *p*-coumaric acid (C_{max} 634 ± 225 nM). In conclusion, the project has identified apple-related polyphenol metabolites in human urine and plasma; many for the first time after apple consumption. Important biokinetic parameters have also been reported for these metabolites.

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Table of Contents

Chapter Three – Identification of Apple Polyphenol Metabolites

Chapter Four – Biokinetics of Apple Polyphenol Metabolites

Chapter Five – Conclusions

Chapter Six - References

Alphabetical List of Abbreviations

APCI	Atmospheric Pressure Chemical Ionisation
BBSRC	Biotechnology and Biological Sciences Research Council
BMI	Body Mass Index
BUPA	British United Provident Association
DAD	Diode Array Detector
DMHA	Dimethylhexylamine
EPI	Enhanced Product Ion
ESI	Electrospray Ionisation
FAB	Fast Atom Bombardment
H_2O	Distilled water
HCl	Hydrochloric acid
HNU	Human Nutrition Unit
HRGC	Human Research Governance Committee
IFR	Institute of Food Research, Norwich
IS	Internal Standard
kg	kilograms
LC	Liquid Chromatography
LC/MS/MS	Liquid Chromatography tandem Mass Spectrometry
Lit. Hep.	Lithium Heparin
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
MeCN	Acetonitrile
MeOH	Methanol
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
m/z	Mass to charge ratio
nc	Not calculable
nd	Not detected
NH_4OH	Ammonium Hydroxide
NL	Neutral Loss
NREC	Norfolk Research Ethics Committee
RP-HPLC	Reversed Phase High Performance Liquid Chromatography
rt	Room temperature
T_r	Retention Time
SIM	Single Reaction Monitoring
SPE	Solid Phase Extraction
TFA	Trifluoroacetic acid
TIS	TurboIon Spray
UV	Ultra Violet

Chapter One

Introduction to Polyphenols, their Metabolism in Humans and Existing Analytical Methodology for their Determination

Summary

This chapter introduces polyphenols and flavonoids, and their metabolism in humans. A literature review of analytical methodology for the determination of polyphenols in food stuffs, and for the determination of polyphenols and their metabolites in human biological fluids, has been compiled. Justification for the proposed research has been given, in addition to the aims and objectives of the research.

1.1 Introduction to Polyphenols

Polyphenols are a diverse class of plant secondary metabolites, characterized structurally by the presence of one or more six-carbon aromatic rings and two or more phenolic hydroxyl groups [1]. Polyphenol nomenclature is illustrated in Figure 1.1 [2, 3].

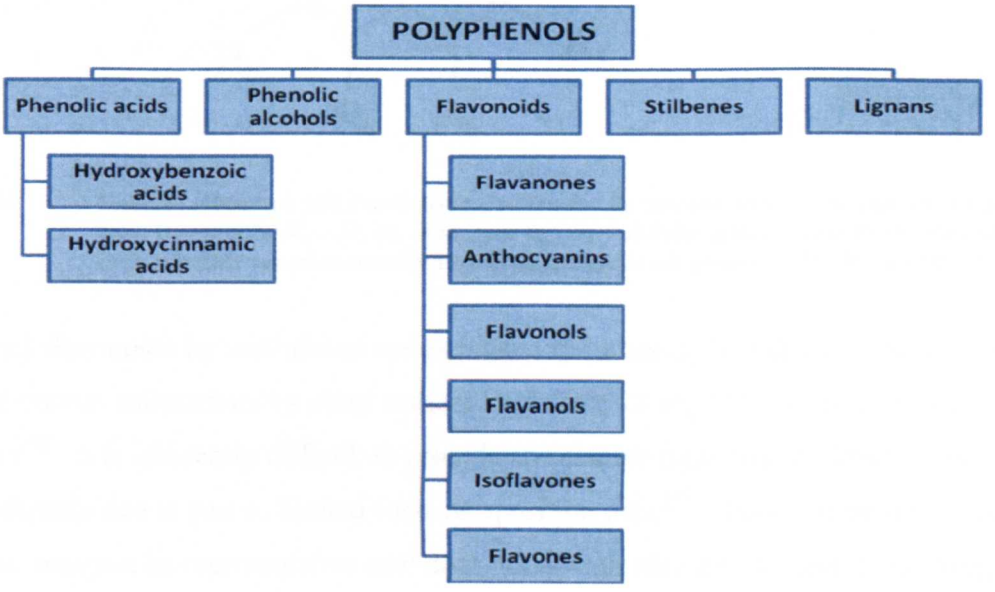

Figure 1.1 Classes of polyphenols

These ubiquitous compounds have various functions in the plants themselves, not all of which are fully understood. However, one undisputed function of polyphenols in plants, is their role in protection against microbial invasion [4]; they act as antibacterial and antifungal agents [5]. Mechanisms for this action include the high affinity of the hydroxyl groups on the aromatic rings for proteins, which bind to fungal enzymes and prevent their attack [5]. Polyphenols also contribute to the colour of flowers and fruit, for example the blue colour seen in many species of flowering plants is achieved by the presence of the anthocyanidin, delphinidin [4]; the same compound gives blueberries their name. They are also responsible for many of the organoleptic properties of fruit and vegetables [6].

1.2 Introduction to Flavonoids

The largest group of polyphenols are the flavonoids of which more than four thousand individual flavonoids have been characterised [3, 7]. The general structure of a flavonoid is shown in Figure 1.2.

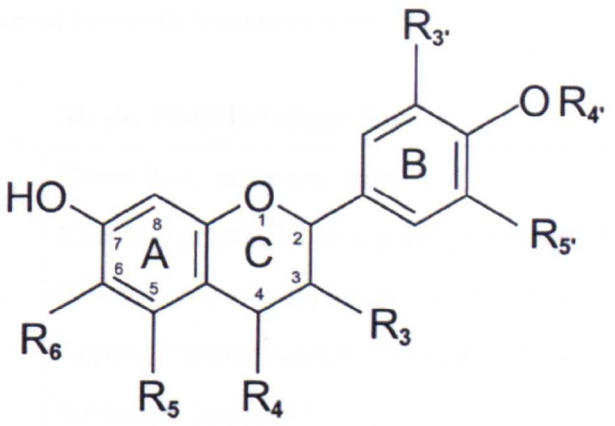

Figure 1.2 **General structure and numbering pattern for flavonoids. Few exceptions exist but for most flavonoids, $R_{4'}$—H, R_5—OH and R_6—H. Additional individual flavonoids within each subclass are characterized by unique functional groups at R_3, $R_{3'}$, and $R_{5'}$.[2]**

Intakes of flavonoids by individuals are estimated to be about 1g a day according to Kahle *et al.* [8], however estimations by other authors vary from 28 mg [9] to between 30 mg and 2300 mg a day [6]. It is inherently difficult to provide an accurate figure for the level of consumption of polyphenols due in part to limited food composition data [10]. Food composition data that is available, may not be representative as it does not always take into account the variety, season, stage of ripening, agroclimatic conditions, storage, processing and the cooking of the food [6, 11]. Each of these factors can also influence the class of flavonoid differently, for example phenolic acid concentrations in foods decrease during ripening, whereas concentrations of anthocyanins appear to increase [12]. The simple action of peeling a fruit or vegetable can eliminate a significant proportion of its polyphenol content. Other factors involved in the difficulty of assessing the level of consumption of polyphenols, lie in the vast variations of food preferences which can be due to nationality, socio-economic group and gender. For example, Nijveldt *et al.* [13] report that the lowest intakes of flavonoids (~2.6 mg/day) are found among the population of Finland and the highest intakes (~68.2 mg/day) are found among the Japanese. Overall, flavonoids account for two thirds of the daily intake of polyphenols, and phenolic acids make up the final third [8]. Some flavonoids are found in a wide variety of foods, for example quercetin which is present in onions, apples, tea and broccoli, whereas others are limited to only a few foods, such as apigenin found only in parsley and celery. The flavonol quercetin represents one of the most abundant flavonoids in food [14, 15], in addition to chlorogenic acid [16].

Table 1.1 Occurrence of flavonoids in common foods [3, 7]

Class of Flavonoid	Major Food/Beverage Sources
Flavanones	Citrus fruit, tomatoes, mint
Anthocyanins	Cherries, grapes, blackcurrants, blackberries, cabbage, beans
Flavonols	Onions, broccoli, apples, berries, tea, red wine
Flavanols	Apples, blueberries, tea, chocolate, apricots, red wine
Isoflavones	Soybeans, legumes
Flavones	Parsley, thyme, celery

Evidence for the health benefits of polyphenols is constantly accumulating. Epidemiological studies indicate that fruit and vegetable consumption is inversely related to cancer [17-19] and coronary heart disease mortality [8, 17, 20, 21], and researchers have suggested that this reduction is not solely due to increased levels of vitamins and fibre [20], but to the additional presence of polyphenols [17]. Activities observed *in vitro* with doses of dietary polyphenols higher than predicted physiological levels are not likely to represent the *in vivo* status because of the limited colonic and systemic availability of polyphenols [8, 17, 22]. There are many difficulties in extrapolating data from cell culture experiments to *in vivo* cancer prevention, due to lack of knowledge of the possible metabolites and mechanisms of metabolism [17], which is why human intervention studies are important. Knowledge of the bioavailability and metabolism of dietary polyphenolics *in vivo* is essential to understanding their role in disease prevention [23].

1.2.1 Flavonoid Functions in Humans

One of the most studied attributes of flavonoids is their ability to act as antioxidants. According to literature [1, 24], the 'antioxidant hypothesis' was first proposed by Gey in 1987 [25]. Summarised, this states that 'small molecules' in the diet act as antioxidants *in vivo,* preventing the oxidation of plasma and consequently lowering the incidence of some diseases induced by oxidative stress. However, metabolic modifications of flavonoids will alter their 'classical' antioxidant nature [26] and polyphenols do not appear to be present in the plasma at high enough concentrations to contribute to the total antioxidant capacity [1]. This suggests that radical scavenging by flavonoids is unlikely to be the mechanism by which diets rich in fruits and vegetables have shown to protect against chronic diseases [6].

Gey also reports that synergistic actions between dietary antioxidants could be more important than the value of individual ones, a suggestion still relevant today. Researchers have been looking for the 'magic bullet' of phytochemicals – a key compound or combination of several compounds that can be produced as a dietary supplement, which would have beneficial effects on human health [27]. However, isolated phytochemicals do not have the same effects as when they are present in a whole food. The additive and synergistic effects of phytochemicals in fruit and vegetables appears to be responsible for their potent antioxidant and anticancer activities, and that the benefit of a diet rich in fruit and vegetables is attributed to the complex mixture of phytochemicals present in whole foods [27, 28].

Recent increasing interest in flavonoids is due to the elucidation of their broad range of pharmacological activities, such as antimicrobial, antiallergic, anti-inflammatory and antiviral [29]. Flavonoids and their metabolites have been shown to exert modulatory actions in cells through actions at protein kinase and lipid kinase signalling pathways [26]. However, much of this work has been carried out *in vitro*. A small number of studies undertaken in rodents provide limited *in vivo* data but the situation in humans is still largely unknown.

Another recent emerging role of polyphenolic compounds is concerned with the treatment of neurodegenerative diseases, such as Alzheimer's and Parkinson's [30, 31]. In these diseases, polyphenolic compounds have been shown to decrease or block the death of neurons. In addition, they seem to exert beneficial effects through different pathways such as signalling cascades, anti-apoptotic processes or the synthesis/degredation of the amyloid β peptide [32]; this peptide is toxic to nerve cells when it deposits in the brain and forms fibrils. The main polyphenolics responsible for these effects are the flavanols found predominately in green and black teas [33, 34]. In the literature, epigallocatechin gallate (EGCG) is the most potent green tea-derived constituent against neurodegeneration [33-35]. EGCG has several possible mechanisms of action, such as the inhibition of lipid peroxidation and the inhibition of xanthine oxidase which reduces oxygen free radical production in the body [34]. The latter is fundamental as reactive oxygen species are known to be instigators in many physiological diseases. These polyphenolic compounds may not only influence the mechanisms of the disease, but may also delay its progression [35].

1.2.2 Flavonoid Metabolism in Humans

Most of the flavonoid glycosides present in food resist acid hydrolysis in the stomach and arrive intact in the duodenum [11]. Five to ten percent of polyphenols in the diet are absorbed from the duodenum [6], such as aglycones [11], certain cinnamate conjugates [36] and some flavonoid glycosides (the sugar moiety affects the absorption, for example glucosides can be absorbed this way but rutinosides cannot; they must reach the colon where hydrolysis by bacterial rhamnosidases occurs [11, 37, 38]). Glycosylation with rhamnosides results in less rapid and less efficient absorption than glycosylation with glucosides, due to the smaller surface area of the site of absorption (the colon compared to the small intestine) and lower density of transport systems [11].

A number of mechanisms are involved in the absorption from the duodenum, not all of which have been characterised [1]. One suggested mechanism for uptake of some flavonoid glycosides utilises the β-glycosidase enzyme lactase phloridzin hydrolase, present in the brush border membrane of the small intestine [38, 39]. Hydroxycinnamate absorption from the small intestine is rapid, and the same process of glucuronidation and conjugation occurs that is observed with flavonoids. However, most of the hydroxycinnamic acids in the plant materials we consume are esterified, and this affects their absorption. They may be esterified to simple acids or polysaccharides in plant cell walls. Human tissues and biological fluids do not possess the esterases capable of hydrolysing the ester bonds in the hydroxycinnamates; however, the colonic microflora may have such capability. Just as observed for some of the glycosylated flavonoids, the extent of absorption of these esterified hydroxycinnamic acids is greatly reduced [11]. One key example of the esterification of hydroxycinnamates to polysaccharides is the binding of ferulic acid to arabinoxylans in cereal husks where it plays an integral role in cell wall structure, shape and defence against microbial attack [40]. Of the five to ten percent of polyphenols absorbed from the upper gastrointestinal tract, five to ten percent is excreted as unchanged plant phenols and the remainder as conjugates [6].

Flavonoid metabolism occurs primarily in the liver and the colon, where transformations may be extensive. As flavonoids are absorbed by the upper digestive system, they enter the hepatic portal system and are carried though the portal vein to the liver; if absorption occurs further down the digestive tract, then the products of metabolism can be absorbed directly into the systemic circulation. Polyphenols are said to undergo extensive first pass metabolism, where

their concentrations are greatly reduced before they reach the systemic circulation. The metabolism that takes place in the liver and colon, accounts for ninety to ninety-five percent of polyphenols ingested in the diet. There is evidence for *O*-methylation, sulphation and glucuronidation of hydroxyl groups in the liver [7] and removal of sugars, phenolic hydroxyl groups and bacterial ring fission of flavonoids in the colon [6]. A study conducted by Sesink *et al.* [39] concluded that when subjects consumed quercetin-3-glucoside or quercetin-4′-glucoside in separate oral administrations, neither these intact glucosides or much of the aglycone were found circulating in the plasma; they had all been converted into glucuronides. Contradictory evidence suggesting phloridzin and quercetin rutinoside are present as these glycosides in human plasma is provided by Paganga and Rice-Evans [41]. Further evidence by Aziz *et al.* [42] indicates the flavonol glycosides isorhamnetin-4′-*O*-beta-glucoside and quercetin-4′-*O*-beta-glucoside accumulated in plasma as intact glycosides and Mauri *et al.* [43] also observe intact plasma flavonoid glycosides after ingestion of tomato extract.

Degradation products of bacterial ring fission in the colon, such as phenolic acids, can be reabsorbed by active transport mechanisms and are found in urine of animals [6, 7]. It is the phenolic metabolites formed in the small intestine and hepatic cells and products of the colonic microflora that travel in the circulatory system and reach the tissues beyond the gut. Bacterial ring fission by the colonic microflora breaks the heterocyclic structure of the aglycone at different points depending on the class of flavonoid involved (see Figure 1.3). The human colon consists of more than five hundred bacterial species, of which the majority are anaerobic (> 99 %) [44]. Habitual bacteria are both commensal and detrimental to health; the balance between the species is fundamental to the overall health of the individual. Polyphenols have been shown to modify gut bacteria, in a way beneficial to health [44]. The major metabolites produced by the population of microorganisms in the human gut resulting from polyphenol intake, are 3, 4-dihydroxyphenylacetic acid, 3-(3-hydroxyphenyl) propionic acid and 3-(4-hydroxyphenyl) propionic acid. These acids can be conjugated with glycine, glucuronic acid or sulfate and/or further metabolised into derivatives of benzoic acid [11]. The products of colonic metabolism also depend on the bacterial population, which varies among individuals.

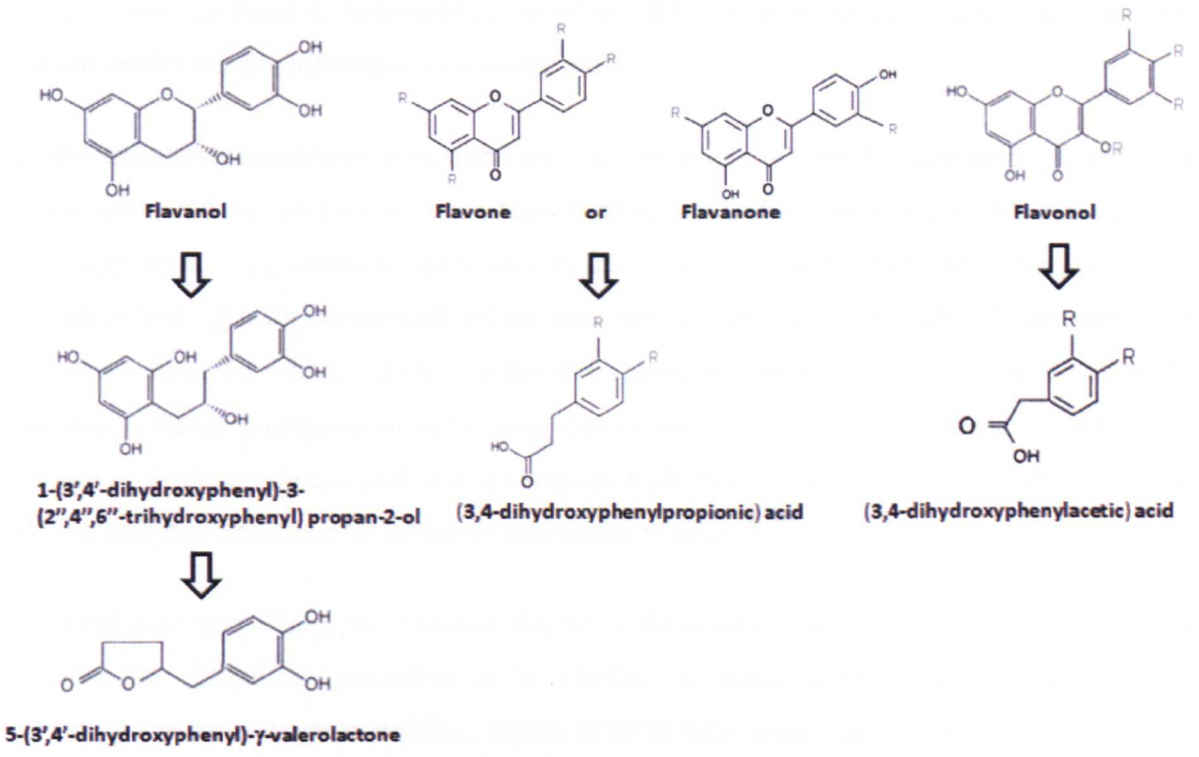

Figure 1.3 **Some of the major metabolites produced by the colonic microflora; the differences are due to the variations in structure across the flavonoid classes** [11, 45, 46]

Plasma metabolite concentrations are highly variable, depending on the subclass of polyphenol involved and the consumption matrix [6], and also on the individual [47]. For example, following an intervention study where subjects consumed 0.37 to 0.67 mg/kg (1300 to 2300 nmol/kg) of (+) catechin (via a reconstituted extract of red wine), Bell *et al.* [47] observed maximum plasma concentration of between 30 to 130 nmol/L, 1 h after ingestion. Individual subjects showed a variation of between 15 and 65 fold increases in total plasma (+) catechin concentrations; after 8 h, the levels had decreased to approximately 20 nmol/L. Studies undertaken with small numbers of volunteers such as the study by Hollman *et al.* [48] may not be so valuable due to this large inter-subject variation. In this study, two volunteers were given 64.2 mg of quercetin (as the aglycone) in a meal of fried onions. The mean peak plasma level of quercetin was observed 2.9 h after ingestion (196 ng/ml or 0.6 μM), and had a half life of between 0.71 and 1.02 h. Quercetin was still detectable in the plasma 48 h after ingestion, when the level had decreased to 10 ng/ml. McAnlis *et al.* [49] report that after a dose of 50.4 mg of quercetin from fried onions, peak plasma concentrations were obtained after 2 h (248 ng/ml). The greater peak concentration observed in this study, compared to the one executed by Hollman *et al.* [48], after a smaller initial dose of quercetin from the same type of dietary

source, could be down to inter-subject variation, differences in onion variety, place of onion harvest and/or method of preparation of the meal.

In the literature, maximum concentrations of polyphenol derived compounds observed in human plasma were not greater than 10 μM in total, and no more than approximately 1 μM for total aglycones. T_{max} values range from 1 to 2.5 h for compounds absorbed in the duodenum, and up to 5 to 12 h for compounds where microbial action is a prerequisite. Elimination half lives vary from 1 to 20 h. Owing to the difficulties in detecting dietary polyphenols in the plasma, as either conjugates or aglycones, due to low levels, lack of synthetic standards and the use of β-glucuronidase and/or sulphatase to hydrolyse the conjugates in plasma or urine, data on the pharmacokinetics of these compounds is limited [6].

As mentioned previously, the different classes of flavonoids undergo different mechanisms of metabolism. There is speculation as to whether the metabolism of larger more complex flavonoids, such as the procyanidins, results in them remaining intact or being depolymerised before absorption. Data in the literature support the concept that the dimers can be absorbed *in vivo*, although to a lesser extent than the monomer subunits of the depolymerized dimers [50-52]. In a study by Holt *et al.* [50], five subjects were given a drink containing 26.4 g cocoa, which provided 323 mg of monomers and 256 mg of dimers. Two hours after consumption, a peak mean plasma dimer B2 concentration of 41 nmol/L was observed. This dimer represented less than one percent of the circulating nonmethylated flavanols compared with an average plasma (-) epicatechin (monomer) concentration of 5.92 μmol/L at the same time point. In the cocoa drink, the ratio of (-) epicatechin to (+) catechin was 1:1, however in the plasma (-) epicatechin was the predominant flavanol, with plasma (+) catechin concentrations only accounting for three percent of the total plasma (-) epicatechin concentration. Rein *et al.* [51] demonstrate after a meal of chocolate supplying 557 mg of procyanidins, it is the (-) epicatechin levels which show a marked increase after 2 h. Plasma levels range between 100 and 800 nmol/L among volunteers, which even for the lower value is still a ten fold increase in the observed baseline levels. Once again, high inter-subject variability is observed. From further studies investigating the metabolism of flavanol polymers and monomers, it seems that if ingested in dimer form, significant proportions of unmethylated and unconjugated monomeric flavanols will be produced (in addition to small amounts of *O*-methylated dimer), but if ingested in the monomeric form, *O*-methylated and conjugated forms of flavanols predominate in the plasma [52]. This could be explained by high concentrations of the dimer

inhibiting phase I and phase II enzymes and so preventing the biotransformation (such as *O*-methylation) of any free (-) epicatechin produced.

Baseline levels of flavonoids in the plasma of individuals are also highly variable. For example, in a study involving 13 volunteers [51], eleven of the subjects had baseline (-) epicatechin levels that were measurable, with values between 10.3 and 80.3 nmol/L. The other two volunteers had levels below the limit of detection in this method. These participants were only instructed to abstain from vitamin supplements, alcoholic beverages and caffeine- or theobromine-containing foods for 24 h before the test day, so this may be representative of baseline levels on consumption of a relatively normal diet. In a study by McAnlis *et al.* [49] mean baseline quercetin levels of 28.4 ng/ml were observed in five volunteers after an overnight fast, with no dietary restrictions pre intervention.

Data on the metabolism of polyphenols in humans is limited, which is why it is important for further intervention studies to be undertaken, and also for them to include a larger number of test subjects. Researchers investigating the pharmacokinetics of phytochemicals in humans have observed substantial variation, as demonstrated by some of the articles cited in this chapter. One source of variation could be due to gut physiology; physiological parameters such as pH, intestinal fermentations and transit times can all vary [11]. These can vary due to the heath status of the individual, environmental exposures and the diet consumed. Diet also influences the population and activity of microorganisms in the gastrointestinal tract, as well as the genes expressed by an individual. In addition, it is likely that genetic variation in the pathways which affect absorption, metabolism and distribution of phytochemicals in terms of biotransformation enzyme expression, stability and activity, also affects the exposure of tissues to phytochemicals and their metabolites [53]. Variations in the receptors and signal transducers that interact with phytochemicals also have the potential to affect responses to these plant constituents at the tissue level. Figure 1.4 gives a diagrammatical representation of some of the factors that can yield inter-individual variation.

1.2.3 Bioavailability of Flavonoids in Humans

It is very difficult to give an exact definition of bioavailability as the area of research can influence the terms meaning. For example, some nutritionists, will assess bioavailability by how much of an orally administered phytochemical appears in urine [54]. The rationale behind this thought is that the compound has to enter the blood before it can be transported to the

kidney for excretion. If the compound is present in the blood it must have had access to all the tissues of the body, and hence the greater the proportion of the dose found in the urine, the more bioavailable the compound must be. This theory appears to have two major flaws. Firstly, if the compound appears in the urine rapidly after dosing then it has not be retained by the tissues or had much chance to provide beneficial effects. Secondly, the amount of compound available to the body depends on the rate of reabsorption by the proximal tubules of the kidney after initial filtration by the renal glomeruli. A compound which is completely reabsorbed will not appear in urine, so by this definition will be poorly bioavailable.

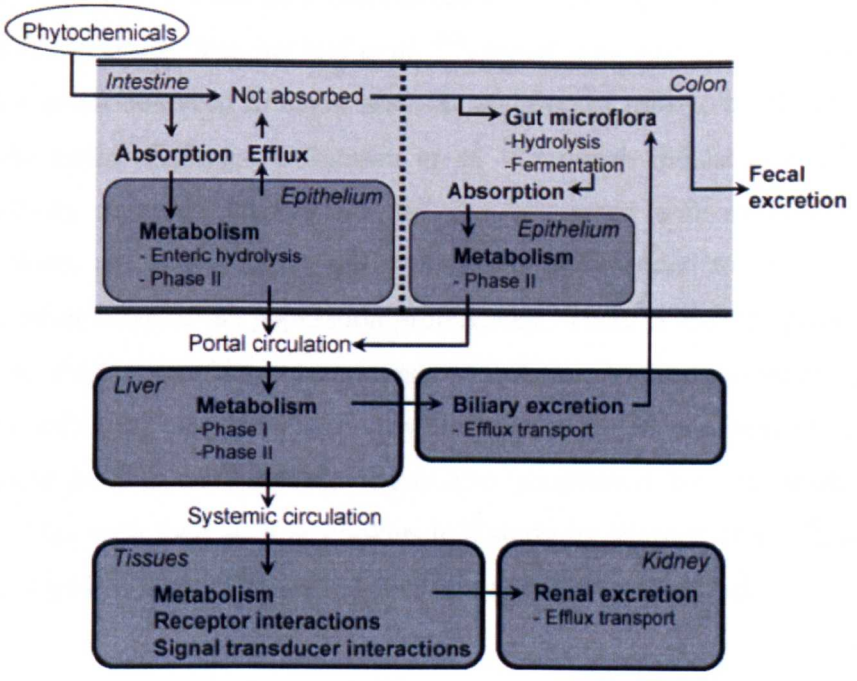

Figure 1.4 **Sources of variation in phytochemical metabolism and disposition** [53]

A further definition of bioavailability, more relevant to pharmacologists, compares the changes in area under the plasma concentration-time curve (AUC) when the compound under investigation is administered orally and intravenously. After the latter form of administration, the compound has direct access to the blood system and so can distribute directly to all the tissues of the body. The area under the curve for the time-dependent change in blood concentration gives a measure of oral bioavailability. However, in certain circumstances this may an underestimate of true bioavailability, as if a compound is taken up from the small intestine and excreted into bile, the proportion of unmetabolised parent compound in the blood is low, but the metabolites may have been utilised site-specifically in local tissues.

The most abundant flavonoids in the diet are not necessarily the most active; it depends on the processes of absorption, metabolism and excretion. Indeed, the process of metabolism greatly alters the native forms of flavonoids, and their resulting metabolites may differ in biological activity compared to the parent compounds [11]. It is generally accepted that the bioavailability of phenolics is rather low [22, 55, 56].

The food matrix itself has an effect on the bioavailability of polyphenols [11] and consumption of polyphenols with other food and drink items in the diet affects their bioavailabilty. There is conflicting information in the literature that suggests the consumption of tea with milk reduces the absorption of catechins. Van het Hof *et al.* [57], report that there is no significant effect on the (+) catechin levels observed in blood after the addition of milk to black tea. The authors suggest that the earlier findings of Serafini *et al.* [58], which indicated a reduction in the antioxidant activity observed when black tea was consumed with milk, compared to its consumption alone, are due to the larger amounts of milk added to the tea, which were generally unrepresentative of the population preferences. There is also the possibility that the milk reduced the absorption of other compounds contributing to the antioxidant activity of tea, and just did not affect the catechins. Work by Hollman *et al.* [48], confirmed that the increase in plasma concentrations of quercetin were also unaffected by the addition of milk. Consumption of tea with food such as cookies in a study by Warden *et al.*[59], suggested that the absorption of gallated catechins could have been affected by this food.

1.2.4 Polyphenol Metabolism Studies in Humans detailed in the Literature

Both *in vitro* and *in vivo* polyphenol metabolism studies are detailed in the literature. *In vitro* studies involve the application of varying concentrations of polyphenol standards to certain cell cultures to see the resulting physiological effects and metabolites produced [60-65]. *In vivo* animal feeding studies are also reported, mostly with rats, some using the individual polyphenol compound [66-69], some using whole foods [70] or mixed preparations and some involving perfusion [71]. A small number of studies have also been performed on people after surgical procedures, such as ileostomy patients [8, 36, 72], to determine the way in which polyphenols from food stuffs are metabolised when there are significant alterations to gut morphology.

Some of the relatively few studies of metabolism and bioavailability of polyphenols in healthy humans have been carried out after consumption of food and drink items such as tea [45, 59, 70, 73-77], coffee [78, 79], fruit juices [80] cocoa [50, 81-83], onions [15, 23, 42, 48, 84-86] and cider [72, 87]; none detail the effects when apples are consumed. A few studies have also been carried out using other food substances, such as artichoke [88, 89], oregano [90], endive [91], blackberries [92], soybean [93] herbal plants [94, 95] and specific flavonoid-rich preparations [96, 97]. One study by Rechner *et al.* [98], also investigates the metabolic fate of dietary polyphenols when a polyphenol rich meal is ingested, i.e. the meal contained a mixture of polyphenols from different sources. Due to the presence of quercetin and flavanols in the majority of food items investigated, any information on plasma and urine conjugates and ring fission metabolites is generally limited to these flavonoid classes. Table 1.2 details some of the most useful metabolism studies carried out in humans.

Summarised, it is easy to observe a lack of information in the literature on the ring fission metabolites of polyphenols and their conjugates, biokinetic and bioavailability data and quantification of metabolites. The same sources of polyphenols are investigated repeatedly; tea, cocoa and onions being the most studied. Thus, the same classes of polyphenol are researched; predominately the flavanols (catechin, epicatechin and EGCG) and flavonols (quercetin). Only two intervention studies using alcoholic cider could be sourced for information on the metabolism of apple polyphenols in healthy human subjects [72, 87]. However, the presence of alcohol in the cider, as well as the production method will have affected the metabolism compared to consumption of apples. No conjugated or ring fission metabolites were detected by DuPont *et al.* [87], except for hippuric acid, which was quantified. In addition, concentrations of aglycones were too low for pharmacokinetic parameters to be calculated. Marks *et al.* [72], provide more comprehensive information on one conjugated metabolite, reporting rare biokinetic data, but lack evaluation of ring fission metabolites. The only other studies reporting usage of apples or apple products as their source of polyphenols, involve the consumption of cloudy apple juice by ileostomy subjects [8, 99], which provide either none or limited details of conjugated or ring fission metabolites.

There is a requirement for a well designed human intervention study to be carried out, to detail the metabolism of apple polyphenols. Particular focus is needed with regards to the metabolism of the dihydrochalcones and the phenolic acids, in addition to quantitative

information on conjugated metabolites. A greater emphasis on identification of the ring fission metabolites of apple polyphenols and their conjugates would also contribute greatly to the knowledge.

1.3 Polyphenols in Apples

Apples (*Malus domestica Borkh.*) are a major source of flavonoids in the Western diet [65, 100], in addition to onions, berries, tea, coffee, wine and cocoa [101]. "An apple a day keeps the doctor away" is the old adage which has prompted many researchers to look for the 'magic' ingredient which lends truth to this saying [18, 21, 102]. Consumption of apples has been linked with a reduction in the risk of lung cancer, asthma, type-2 diabetes and heart disease [18, 103]. The most important commercial varieties of apple are Red Delicious, Golden Delicious, Granny Smith and Fuji [101].

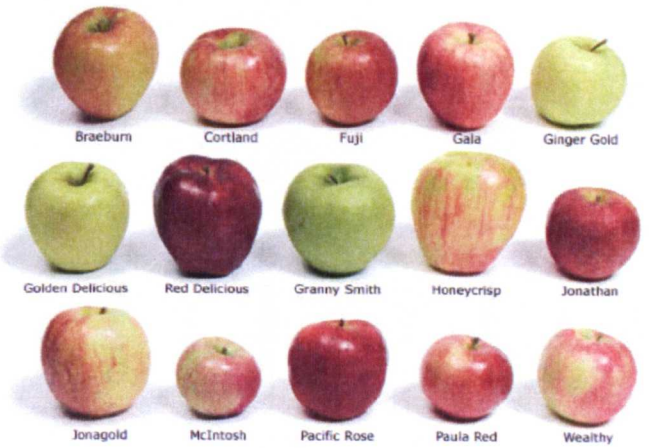

Figure 1.5 Some of the many varieties of apple *

The main classes of polyphenols in apple flesh are procyanidins, hydroxycinnamic acids, monomeric flavan-3-ols and dihydrochalcones, whereas flavonols and anthocyanins are found almost exclusively in the peels [104]. The phenolic profile is further complicated by the polymerisation of flavan-3-ols and glycosylation of other polyphenolics [102]. The predominant sugar involved in glycosylation in apple is galactose, followed by glucose, rhamnose, xylose, and arabinose, with the disaccharide rutinose also being associated with the phenolics [21]. Cider apples contain the same polyphenols as table varieties, but their concentrations are generally much higher [104], and unripe apples have a significantly higher proportion of procyanidins than ripe apples [9]. Despite the range of varieties of apples, the polyphenol profiles are practically identical; it is the concentrations that differ [11].

Table 1.2 **Summary of metabolism studies carried out in humans detailed in the literature**

Food Source	Polyphenol(s)	Biological Fluid	Conjugate /Ring Fission Information	PK/Bioavailability Data	Quantitative for aglycones	Quantitative for metabolites	Reference
Tea	Catechin	Blood	No	No	Yes	No	[57]
Tea	EGCG	Urine & Plasma	Yes	Yes	Yes	Yes	[74]
Green tea	Quercetin	Urine & plasma	No	No	Yes	No	[14]
Green tea	Catechins	Urine	Conjugates	No	No	No	[73]
Green tea	Catechins	Urine	Yes	No	No	No	[77]
Coffee	Hydroxycinnamates	Urine & plasma	Conjugates	Yes	Yes	Yes	[78]
Cloudy apple juice	Various	Ileostomy fluid	No	No	Yes	No	[8]
Cider	Flavonols	Urine & plasma	No	No	Yes	No	[87]
Cider	Dihydrochalcones	Urine, plasma & ileostomy fluid	Conjugates	Yes	Yes	Yes	[72]
Polyphenol-rich beverages	Various	Urine	No	No	Yes	No	[80]
Cocoa	Various	Urine & plasma	Yes	No	No	Yes	[83]
Cocoa	Various	Urine	Yes	No	Yes	Yes	[105]
Cocoa	Flavanols	Urine & plasma	No	Yes	No	Yes	[106]

Table 1.2 (cont.) **Summary of metabolism studies carried out in humans detailed in the literature**

Food Source	Polyphenol(s)	Biological Fluid	Conjugate /Ring Fission Information	PK/Bioavailability Data	Quantitative for aglycones	Quantitative for metabolites	Reference
Chocolate	Phenolic acids	Urine	Ring fission	No	Yes	Yes	[82]
Chocolate	Epicatechin	Plasma	Conjugates	No	Yes	No	[81]
Onions	Flavonols	Urine & plasma	Conjugates	No	No	No	[84]
Onions	Quercetin, Kaempferol	Urine	Conjugate	No	No	No	[86]
Oregano	Phenolic acids	Urine	Ring fission	No	Yes	Yes	[90]
Endive	Kaempferol	Urine & plasma	Conjugate	No	Yes	No	[91]
Danshen	Phenolic acids	Serum	No	No	Yes	No	[95]
Preparation	Epicatechin	Urine & Plasma	Conjugate	No	Yes	Yes	[97]
Capsule	Quercetin	Urine & Plasma	No	Yes	Yes	No	[96]
Artichoke	Various	Urine & plasma	Conjugates	Yes	Yes	Yes	[89]
Blackberry	Anthocyanins	Urine	Conjugate	No	Yes	Yes	[92]
Polyphenol-rich meal	Various	Urine & plasma	Yes	No	Yes	Yes	[107]

According to Barth *et al.* [17] the most promising candidates among bioactive apple constituents are the procyanidins which were shown to modulate cancer related processes *in vitro* and *in vivo*. These compounds were also shown to effectively inhibit colonic cancer cell growth mediated by activated MAPK pathways and activation of apoptosis-related caspase-3. The effects may be derived from absorption of active metabolites in the small intestine [17], or these metabolites reaching the colon [17] and there is also some evidence to suggest polyphenols modulate the bacterial contents of the colon [108].

In a study by Hollman *et al.* [37], after a single supplement of apple sauce and apple peel containing 325 μM of quercetin, peak concentrations of the aglycone in the plasma were observed at 2.5 h (0.30 μM or 92 ng/ml) post supplementation. Levels of quercetin remained detectable for 36 h post dose at approximately 0.03 μM. In this study, they compared the absorption of quercetin from onions (T_{max} 0.7 h), quercetin from apple and quercetin rutinoside (T_{max} 9 h) and concluded that the bioavailability of apple quercetin and quercetin rutinoside were both thirty percent that of onion quercetin. The variation in the time taken to reach the maximum plasma concentration indicates the site of absorption. For example, the short time taken for the quercetin from onions to peak suggests absorption from the small intestine as intact glucosides, whereas the longer time taken for the quercetin rutinoside suggests absorption from the colon, after hydrolysis of the rutinoside. Urinary excretion of quercetin was 1.39 %, 0.44 % and 0.35 % after ingestion of onions, apples and quercetin rutinoside, respectively. The observed differences in the bioavailability of quercetin from the food sources, is related to the variation in glycosidic moieties; the glycosides in onions being mostly glucosides which are rapidly absorbed. Factors such as differences in cell wall structure, location of glycosides in cells or binding to other cell components, could also influence the absorption and bioavailability.

The world produces a large quantity of apple residues (apple pomace) from juice processing [20], which could be exploited by the food industry and incorporated into dietary supplements [18], in turn improving the health of the population. Apple pomace is already utilised as a commercial source of pectin, used in the production of jams and fruit jellies [109].

1.4 Analysis of Polyphenols in Plant and Food Related Products

Historically, polyphenol analysis methods have evolved from the paper chromatography techniques of the 1950s and 1960s to the HPLC techniques of the 1970s and 1980s (Fisher and Wheaton were the first to use the technique of HPLC for the determination of polyphenols in 1976 [110]) to the current trend in the use of mass spectrometry. Recent advances in MS have enabled further progress of the elucidation of polyphenols in plants and food related products in this current decade, just as the development of paper chromatography did several decades ago [111].

The literature contains many articles detailing analysis of polyphenol standards [112-114], in addition to the polyphenolic profiles of a wide variety of fruits [115-119], vegetables [117, 120-125] and plants [126-131], as well as their products such as tea [132, 133], coffee [134], chocolate [135, 136], fruit juices [101, 137, 138], wine [133, 139] and honey [140]. Apples (both table and cider varieties) and their products have been subjected to polyphenol profiling [20, 101, 141, 142], but in general their composition has not been studied in as much detail as that of tea and cocoa. Table 1.3 details conditions of some of the analysis methods used for the determination of polyphenols in plants, food stuffs and beverages. The aqueous mobile phases utilised are almost always acidified; common acids used include formic acid and acetic acid. Organic solvents include MeCN and MeOH, or combinations of the two and are sometimes acidified. For determination of the polyphenols in apples, MeCN is preferred. Although the types of solvents remain fairly constant, there is variation in the percentage composition of solvent systems [143]; gradient elution is more commonly employed than isocratic. LC columns are nearly always composed of a C_{18} stationary phase and analysis times are usually in excess of twenty min. Some reported methods include time for the re-equilibration of the system prior to the next analysis, which is important as it is part of the time taken for the complete analysis, however other authors do not mention it.

Those studies which utilise LC/MS for the determination of polyphenols in plants, food stuffs and beverages generally use the negative ionisation mode, with capillary voltages of between 2 and 3.5 kV and temperatures of between 200 and 400 °C. It has been reported that positive ionisation mode results in rich fragmentation patterns, compared to limited fragmentation with negative mode, but negative mode provides the highest sensitivity for flavonoids [130, 140].

According to Abad-Garcia *et al.* [137], the reluctance of polyphenolic compounds to fragment in negative mode requires the use of higher cone voltages, particularly for flavonoid glycosides as the glycosidic linkages require more energy to break. Recent LC/MS/MS studies have been detailing specific members of a flavonoid class and attempting to identify members which differ structurally. For example, the determination of the fragmentation patterns for chlorogenic acids where esterification occurs on different carbons [134], or the analysis and separation of chiral flavonoids [144].

In general, approaches for the analysis of polyphenols in plants, food stuffs and beverages are quite comprehensive. More recent papers also profile the complex glycosylated polyphenols, previously neglected due to limitations in the instrumentation used. However, weak points of these methodologies include long analysis times and poor validation data. There is also a shortage of fully comprehensive methods for the determination of all classes of polyphenols in any one food item or beverage; research groups tend to focus on a particular class or individual.

Table 1.3 **Summary of information in the literature on the analysis of polyphenols in plants, fruit, vegetables and other food stuffs. NB most of the analysis times stated do not take into account time for re-equilibration**

Source	Polyphenol(s)	Analytical Method	Column	Mobile Phases	Run Time (min)	Validation	Reference
Cocoa	Flavanols Procyanidins Luteolin, Apigenin Naringenin	ESI LC/MS/MS	Luna C_{18}	A = 0.1 % formic acid in H_2O B = 0.1 % formic acid in MeCN	48	No	[135]
Cocoa	Flavanols Procyanidins	HPLC-F	Develosil Diol 100A	A = 2 % acetic acid in MeCN B = 2 % acetic acid in H_2O: MeOH 5:95	83	Yes	[136]
Orange juice	Flavanones Flavones Flavonols	LC/MS/MS	C_{18}	Formic acid in H_2O:MeCN 80:20	22	Yes	[138]
Apple juice	Phenolic acids Flavanols, Flavonols Dihydrochalcones Procyanidins	ESI LC/MS/MS (-ve)	Hypersil Gold C_{18}	A = 0.1 % formic acid in H_2O B = MeCN	40	No	[101]
Apples	Catechins, Flavonols Dihydrochalcones Hydroxycinnamic acids	HPLC	Nova-Pak C_{18}	A = 10 % acetic acid: H_2O B = MeOH	60	No	[145]
Apples	Catechins, Flavonols Dihydrochalcones Hydroxycinnamic acids	HPLC & ESI LC/MS/MS (-ve)	Purospher RP18	A = 2.5 % acetic acid in H_2O B = MeCN	45	NO	[146]
Fruit Juices	55 flavonoids, all classes	HPLC-UV	Luna C_{18}	A = 0.5% acetic acid in H_2O B = MeOH	150	Yes	[137]
Red wine Orange juice Tea	Selection of flavonoids, all classes	LC/MS QTOF	Waters Symmetry Shield RP-8	A = 0.1 % formic acid in H_2O B = MeOH	23	No	[133]
Sweet orange	Selection of flavonoids, all classes	ESI & APCI LC/MS/MS	Genesis C_{18}	H_2O:MeOH 50:50 or 60:40	60	No	[117]

Table 1.3 (cont.) Summary of information in the literature on the analysis of polyphenols in plants, fruit, vegetables and other food stuffs. NB most of the analysis times stated do not take into account time for re-equilibration

Source	Polyphenol(s)	Analytical Method	Column	Mobile Phases	Run Time (min)	Validation	Reference
Coffee beans	Chlorogenic acids	LC/MS/MS	Luna C_{18}	A = H_2O:MeCN:acetic acid 980:20:5 B = MeCN:acetic acid 1000:5	90	No	[78]
Grape stems	Flavanols Flavonols Proanthocyanidins	ESI LC/MS/MS	Nucleosil C_{18}	A = 2 % formic acid in H_2O B = 2 % formic acid in H_2O:MeCN 20:80	25	No	[119]
Herbal extract of *Crataegus*	Flavonoid glycosides	ESI LC/MS/MS (- ve) ESI LC/MS/MS (+ ve)	Xterra RP-18 MS	A = 0.1 % formic acid in H_2O B = 0.1 % formic acid in MeCN A = 0.5 % formic acid in H_2O B = 0.5 % formic acid in MeCN	30	No	[128]
Tu-Si-Zi (herbal drug)	Phenolic acids Flavonols and glycosides	LC/MS/MS	Zorbax Eclipse XDB C_{18}	A = MeCN B = 0.5 % acetic acid in H_2O	45	No	[126]
Broccoli Orange & apple peel Onion Tea	Flavones Flavonols Flavanones	APCI LC/MS/MS	Phenomenex RP C_{18}	A = 1 % formic acid in MeOH:H_2O 30:70 B = MeOH	50	Yes	[116]
Broccoli	Hydroxycinnamoyl derivatives Flavonol glycosides	LC/MS/MS	RP-18 LiChroCART	A = 5 % formic acid in H_2O B = MeOH	40	No	[123]
Compositae plants	Flavonoids, all classes Caffeoylquinic acids	LC-DAD-APCI/MS	Zorbax SB-C_{18}	A = MeCN B = 0.1 % formic acid in H_2O (pH 4 with NH_4OH aq.) solution)	100	No	[129]

Table 1.3 (cont.) Summary of information in the literature on the analysis of polyphenols in plants, fruit, vegetables and other food stuffs. NB most of the analysis times stated do not take into account time for re-equilibration

Source	Polyphenol(s)	Analytical Method	Column	Mobile Phases	Run Time (min)	Validation	Reference
Cabbage	Glycosylated kaempferol derivatives	ESI LC/MS/MS	RP-18 LiChroCART	A = 0.1 % formic acid in H_20 B = MeOH	37 & 52	No	[122]
Black carrots	Caffeoylquinic, Coumaroyl & feruloylquinic acids Hydroxycinnamates Acylchlorogenic acids Hydroxybenzoic acids & flavonols	ESI LC/MS/MS	Hydro-Synergi C_{18}	A = 2 % acetic acid in H_2O B = 0.5 % acetic acid in H_2O:MeCN 50:50	90	No	[125]
White Asparagus	Ferulic acid derivatives	ESI LC/MS/MS	Synergi HydroRP80A	A = 2 mM acetic acid in H_2O:MeCN 90:10 B = 2 mM acetic acid in MeOH:MeCN:H_2O 40:40:20	50	No	[124]
Sorrel leaf	Catechin Epicatechin	ESI LC/MS/MS	Luna C_{18}	10 mM H_3PO_4:MeCN 88:12	22	No	[130]
Dandelion	Caffeoylquinic acids Tartaric acid derivatives Hydroxycinnamates Luteolin, Quercetin	ESI LC/MS/MS	Hydro-Synergi C_{18}	A = 2 % acetic acid in H_2O B = 0.5 % acetic acid in H_2O:MeCN 50:50	90	No	[127]
Acacia catechu	Catechins	ESI LC/MS/MS	Not given	A = 0.1 % formic acid in H_20 B = 0.1 % formic acid in MeCN	50	No	[131]
Honey	Hydroxybenzoic & hydroxycinnamic acids Flavonols Flavanones	ESI & APCI LC/MS/MS	Atlantis C_{18}	A = 2 mM formic acid in H_2O B = MeOH	16	No	[140]
Chemical standards	Isoflavones Flavones, Flavanones Flavonols	ESI LC/MS/MS	YMC-Hydrosphere C_{18}	A = 0.1 % formic acid in H_2O B = 0.1 % formic acid in MeCN	50	Partial	[113]

1.5 Justification for the Proposed Research

As mentioned previously, there is a requirement for a well designed intervention study to investigate the metabolism of apple polyphenols in healthy human volunteers. Only four studies could be found in the literature detailing the metabolism of apple polyphenols in humans; polyphenol sources were cider and apple juice. Three of the studies involved the use of subjects with an ileostomy which will produce different findings to studies with healthy humans; in effect these studies only report half the story. Data is mainly provided for caffeoylquinic acid derivatives, one phloretin conjugate or aglycones; no information is given on conjugation of other apple components or metabolites of microbial origin produced in the gastrointestinal tract. Some of this information is provided by studies using alternative polyphenol sources, for example human intervention studies using tea and cocoa can be used to extrapolate some information; but differences will exist.

In addition to the intervention study, there is also a requirement for a fast, sensitive and validated method for the analysis of polyphenols, their conjugated metabolites and their ring fission products in biological matrices, all within one analytical run. Existing methodologies are generally not validated and involve lengthy run times of over forty min. A new hyphenated LC/MS/MS method will combine sensitivity with selectivity, in a time conscious and reproducible manner.

Discussion of available literature in this chapter highlights the lack of knowledge and understanding that exists for the metabolism of apple polyphenols in humans. Absence of fast and validated methods for biological sample preparation and instrumental analysis, a tendency for enzyme deconjuagtion experiments through which metabolite detail is lost, and an inclination to concentrate on one particular class of polyphenol or individual member, all contribute to the void in our comprehension. Apples are a familiar and popular food item and yet they have not been studied to the same extent as tea and cocoa.

This study will be the first to investigate the metabolism of apple polyphenols from a high polyphenol cider apple extract, in healthy human volunteers. Immediate and frequent blood sampling will enable as many metabolites to be detected and identified as possible and help to provide good biokinetic data for those that are identified. Attention to detail in all aspects of sample collection, sample processing, sample analysis and data processing will provide strong

evidence for the metabolic fate of the polyphenols present in apples. Comprehensive metabolite profiling by LC/MS/MS will encompass all classes of polyphenols in apples and their resulting metabolites, so a complete picture can be built.

1.6 Aims and Objectives of the Research

The aim of this project is to identify and quantify flavonoids and their metabolites in the plasma and urine of healthy humans after consumption of a high polyphenol cider apple extract. Information on the biokinetics of these metabolites will hopefully be obtained.

Specific objectives required to fulfil this aim are as follows:

1. To plan and execute a human dietary intervention study, including compiling appropriate documentation to gain suitable ethical approval for such an experiment and recruitment of suitable volunteers.

2. Development of suitable biological preparation methods for human urine and plasma which enable the metabolites of interest to be removed from the matrix unchanged for analysis.

3. Development of a new hyphenated liquid chromatography-mass spectrometry method for the quantitative analysis of the biological samples collected from volunteers participating in the afore mentioned intervention study.

4. Analysis of urine and plasma samples from the human intervention study to determine biokinetic information for absorbed polyphenols.

5. Identification of polyphenol metabolites, in particular bacterial ring fission metabolites and conjugates, to increase our understanding of the metabolism of apple polyphenols in humans.

Chapter Two

Development of an LC/MS/MS Method for the Quantitative Analysis of Polyphenols in Human Plasma and Urine

Summary

This chapter details the development of a novel LC/MS/MS method for the analysis of polyphenols and their metabolites in human urine and plasma samples. Initially, an LC-UV method was developed for the separation of a range of polyphenol standards; MS parameters were investigated for each of these polyphenol standards. Finally, LC/MS/MS experiments were performed with the polyphenol standards and some conjugates, to optimise the method. Extraction methods for human urine and plasma were then developed, optimised and validated and the method was applied to pooled study urine samples.

2.1 Introduction

Analytical methods documented in the literature for the determination of polyphenols from plants, plant products and biological fluids include HPLC with UV detection, HPLC with fluorescence detection, HPLC with electrochemical detection, LC/MS and LC/MS/MS. The literature also contains information on the way polyphenol standards behave when analysed by MS, for example fragmentation pathways and common fragments produced across the classes [114, 147-149]. LC in tandem with MS is a popular method of analysis combining the advantages of high speed separation with high selectivity and sensitivity [150, 151]. The interface between LC and MS is predominately reported to be electrospray ionisation (ESI) with a small number of studies using atmospheric pressure chemical ionisation (APCI).

When dealing with biological samples, the majority of the studies reported in the literature, involve deconjugating the metabolites using sulfatase and β-glucuronidase enzymes prior to analysis [14, 74, 76, 80, 85, 87, 89-91, 152] to remove the sulfate or glucuronide conjugates; this gives information on the total aglycone constituents of the metabolites, but it means a loss of the detail on the degree and ring position(s) of conjugation. Biological samples such as plasma cannot be assayed directly, but require a pretreatment to remove endogenous proteins, carbohydrates, salts, and lipids [153]. HPLC columns are packed with small-sized particles which are not designed to handle large volumes of biological fluids, therefore it is essential to remove matrix components that might contaminate or block the system [154] or go on to cause ion suppression in the MS reducing sensitivity. The sample preparation step of the analysis is fundamental to the performance of the method [54]. Acidified plasma extracted with solvent is a common approach to polyphenol extraction; acetonitrile (MeCN) [23, 72, 78, 84] is the most common solvent used. Solid phase extraction (SPE) has also been performed [81].

The urine matrix can also cause ion suppression in the MS, due to its high salt content; adduct formation during the electrospray process causing production of non volatile components, is another problem frequently encountered with urine, which leads to rapid deterioration of instrument performance [154]. However, urine extraction procedures are time consuming and some sample information may be lost due to the process. Common methods tend to be simple to reduce the negative effects of processing the samples; methods include filtering or centrifugation [72, 78, 82], addition of solvents [73, 77, 84, 86] and SPE [97].

2.1.1 Introduction to the Principles of High Performance Liquid Chromatography and Mass Spectrometry (HPLC/MS/MS)

Reversed Phase HPLC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The retention time of the analyte will vary depending on the interactions between the stationary phase, the molecules being analysed, and the solvent(s) used. In addition, the structural properties of the analyte molecule are fundamental to its retention characteristics. In general, the less water soluble the analyte (i.e. the more non-polar it is), the better the retention on the column, and the greater the retention time.

Mass spectrometry is an analytical technique that identifies the chemical composition of a compound or sample on the basis of the mass to charge ratio (*m/z*) of charged particles (ions). In order for this to occur, the analyte is ionised and the charge and mass of the ion are measured. The ratio of charge to mass of these resulting particles is deduced by passing the particles through electric and magnetic fields in the mass analyser of the MS.

There are various possible interfaces between the HPLC and MS. The technique of electrospray ionisation (ESI) involves the analyte solution passing through a narrow capillary, at a flow rate ranging from a few nanolitres per min to several hundred microlitres per min. The capillary has a high voltage applied to it, generating an electric field gradient between the capillary and the entrance to the MS. As a result, a spray of charged droplets (of the same polarity as the applied voltage), is forced from the capillary in the shape of a cone (the Taylor cone) [66]. As the droplets are exposed to high temperatures in the space between the capillary and the entrance to the MS, solvent evaporation occurs, shown circled in Figure 2.1.

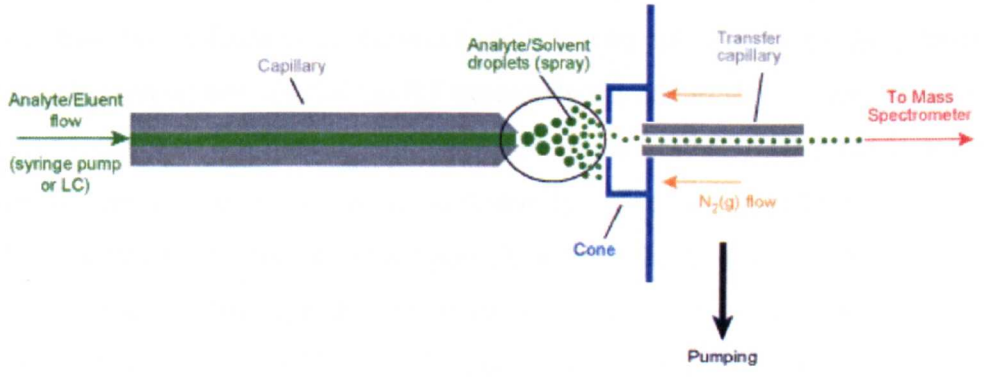

Figure 2.1 **Schematic representation of an ESI source. A cone shaped spray of charged droplets is forced from the capillary; high temperatures causes solvent evaporation [155].**

The droplets reduce in size until they reach a critical point where their surface-charge density reaches a maximum, known as the Rayleigh limit (Figure 2.2). Coulombic explosion occurs and the droplet is split apart to produce smaller particles, as illustrated in Figure 2.1. The mass to charge ratio of a charged droplet determines the fraction of its solute molecules that can become free ions [156].

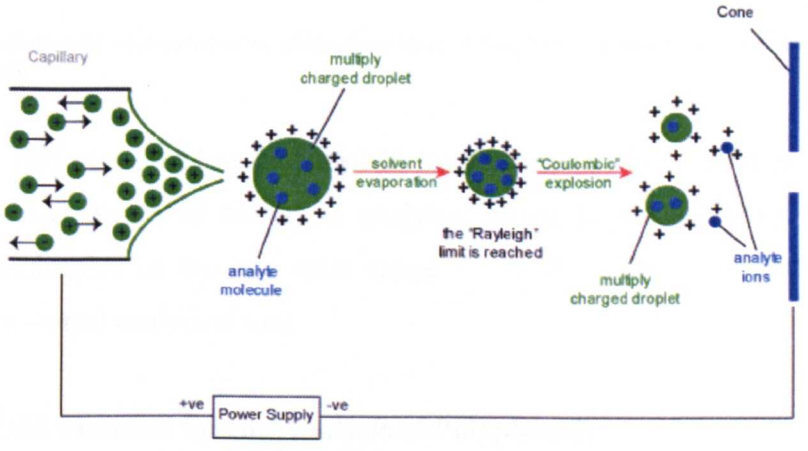

Figure 2.2 **Schematic representation of the mechanism of ion formation in ESI [155]. Free ions are formed from solvent droplets reducing in size; these enter the MS.**

Considerable variation in the electrospray ionisation response is observed among small polar molecules and often a great deal of time is required to optimise the specific analytical conditions for a particular analyte [74]. Many researchers have sought to study the factors that dictate the responsiveness of analytes to certain conditions. The ability of the ion to become charged is critically important in the process, and the response is also dependent upon the other molecules present in the sample matrix. This adds complexity to the detection of analytes in biological samples in particular, where matrix effects can be substantial.

There are many types of MS in existence. A quadrupole mass analyser consists of four parallel rods that have a fixed direct current (which can be positive or negative, depending on the analytes of interest) and alternating RF potentials applied to them. Ions produced in the source of the instrument are focused and passed along the middle of the quadrupoles. Their motion will depend on the electric fields so that only ions of a particular *m/z* that have been selected for will be in resonance and thus pass through to the detector. In a triple quadrupole mass analyser, three of these quadrupoles exist; the first acts as a filter in detecting ions of interest; the second acts as a collision cell where collision induced dissociation (CID) of the precursor ion occurs; the third quadrupole detects the product ions for the precursor ion of

interest. Many precursor ions can be selected for at any one time. Figure 2.3 illustrates the process.

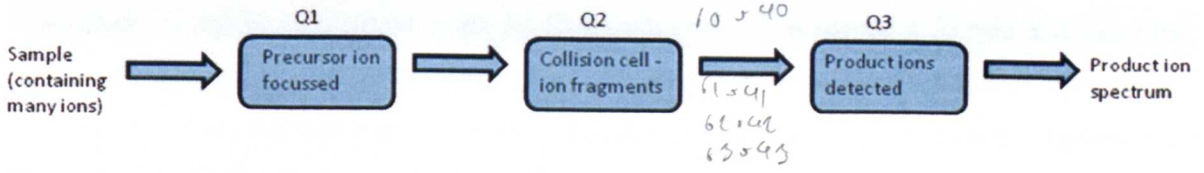

Figure 2.3 **Schematic representation of the function of the three quadrupoles in a triple quadrupole MS**

In recent years, HPLC coupled to ESI/MS/MS has become a widely used technique for the analysis of pharmaceutical and biological analytes, owing to its specificity, reliability and selectivity for molecules in the low mass range [66, 75, 76]. The combination of the two techniques is a powerful analytical tool.

2.1.2 LC/MS/MS Methods for the Analysis of Polyphenols

As mentioned previously, HPLC was first used for the determination of polyphenols in 1976 by Fisher and Wheaton [157]. However, the gold standard for the analysis of synthetic or plant derived products in both the pharmaceutical product and in body fluids and tissues today, involves the use of highly specific and quantitative, hyphenated chromatography-mass spectrometry techniques [54, 154]. Although gas chromatography-mass spectrometry was the first hyphenated chromatography-mass spectrometry technique to be implicated (developed during the 1950s), the introduction of suitable ionisation interfaces has led the majority of analysis of polyphenols in recent years to be carried out using LC/MS [158]. Polyphenols have been studied using the technique of MS since the 1970s [43, 159, 160]. Despite the high selectivity of tandem mass spectrometric detection, there are only a few examples of flow injection analysis (FIA) in bioanalysis and chromatographic separation still plays a key role [154].

In the literature, columns chosen for the determination of polyphenols in plant material and in biological fluids are almost exclusively composed of a C_{18} stationary phase [143]. An exception to this is the use of C_{12} columns with LC/MS/MS for the analysis of quercetin and its conjugated metabolites in human plasma and urine [14, 85]; the authors reported a reduction in peak broadening/tailing, and shorter run times with these coloumns compared with C_{18} columns.

Many of the polyphenol profiling methods reported in the literature have long analysis times, in excess of 50 min. For example, Hong and Mitchell [86] developed a method taking over 100 min for the separation of flavonol metabolites in human urine after consumption of onions. Separation of coffee chlorogenic acids by Stalmach *et al.* [78] involved a 70 min analysis time, or 40 min for plasma and urine. Sang *et al.* [73], also employ a 70 min analysis time for the separation of tea polyphenols in urine. Further details of these and other methods are summarized in Table 2.1. In contrast to these long analysis times, a few groups have reported success with shorter run times of less than 20 min [80-83]. Again, full details of the conditions are summarised in Table 2.1.

The majority of the LC/MS/MS methods detailed in the literature use common solvents for mobile phases, including methanol (MeOH), MeCN, formic acid, acetic acid and H_2O in varying combinations and concentrations. This is because solvents suitable for ESI/MS must permit the formation of ions in solution and allow for easy nebulisation and desolvation [128]. The choice of solvent can have marked effects on efficiency. For direct ESI/MS in the positive mode, MeOH gave the highest sensitivities for flavonoid glycosides and MeCN provided the highest sensitivities in the negative ion mode [128]. However, MeCN was preferred for LC/ESI/MS due to a lower background noise. Wang *et al.* [14], reported higher background noise using acetic acid, compared to formic acid, and higher ion suppression when using trifluoroacetic acid (TFA), compared to either of the other acids. The use of TFA is reported to provide more efficient reversed-phase separations of certain flavonoids than other acids [112, 161], but not for flavonoid glycosides [128]. It is universally agreed in the literature that the best separations for flavonoids are obtained by acidifying the mobile phase, because the hydroxyl groups are kept in their acidic form. This increases their retention on the column and decreases peak broadening, which can occur in the presence of the deprotonated form.

The literature reports the use of both positive and negative ionisation modes for the analysis of polyphenolic compounds and their metabolites, although the general consensus is that better sensitivity is achieved in negative ion mode. Wang *et al.* [14], report better sensitivity with negative ion for the analysis of the flavonol, quercetin. Mullen *et al.* [84], report that when quercetin-3-glucuronide is analysed as a standard, similar responses are observed with both negative and positive ionisation. However, when the standard was 'spiked' into urine, detection in negative mode was ten times more sensitive.

Table 2.1 **Summary of analytical methods reported in the literature, for the analysis of polyphenols in human biological fluids**

Food Source	Polyphenol(s)	Biological Fluid(s)	Analytical Method	Column	Mobile Phases	Run Time (min)	Reference
Chocolate	(-) Epicatechin	Plasma	LC/MS/MS (-ve)	Luna C_{18}	A = 0.1 % formic acid in H_2O B = 0.1 % formic acid in MeCN	14	[81]
Chocolate	Phenolic acids	Urine	LC/MS/MS & GC/MS	Hypersil BDS C_{18}	A = 0.1 % formic acid in H_2O:MeCN 95:5 B = 0.1 % formic acid in H_2O:MeCN 60:40	20	[82]
Cocoa	Various	Urine & plasma	LC/MS/MS (-ve)	Luna C_{18}	A = 0.1 % formic acid in H_2O B = 0.1 % formic acid in MeCN	14	[83]
Chocolate & Cocoa	(-) Epicatechin	Urine & plasma	LC/MS/MS (-ve)	Capcell Pack-UG 120	A = 0.03 % formic acid in H_2O B = MeCN	30	[162]
Tea (-/+ milk)	Catechins	Blood	HPLC-UV	C_{18}	H_2O:MeCN:Tetrahydrofuran: Acetic acid (975:25:3:5) to (535:400:70:5)	Not given	[57]
Green tea	Catechins	Urine	LC/MS/MS (-ve)	Gemini C_{18}	A = 0.2 % acetic acid in H_2O:MeOH 95:5 B = 0.2 % acetic acid in H_2O:MeOH 5:95	70	[73]
Green tea	Catechins	Urine & plasma	LC/MS/MS (-ve)	Supelco Discovery HS C_{18}	A = H_2O:MeOH 90:10 B = H_2O:MeOH 30:70	40	[70]
Green tea	Catechins	Urine	LC/MS/MS (-ve)	Supelcosil C_{18}	A = MeOH:H_2O:acetic acid 5:490:5 B = MeOH:H_2O:acetic acid 250:245:5	65	[77]
Green tea	Quercetin	Urine & plasma	LC/MS/MS (-ve)	C_{12}	0.2 % formic acid in MeCN:H_2O 40:60	Not given	[14]

Table 2.1 (cont.) **Summary of analytical methods reported in the literature, for the analysis of polyphenols in human biological fluids**

Food Source	Polyphenol(s)	Biological Fluid(s)	Analytical Method	Column	Mobile Phases	Run Time (min)	Reference
Coffee	Hydroxycinnamates	Urine & plasma	LC/MS/MS (-ve)	Synergi Polar RP	0.5 % acetic acid in MeCN, 5-16 %	40	[78]
Cider	Dihydrochalcones	Urine, plasma & ileostomy fluid	LC/MS/MS (-ve)	Synergi Max-RP	0.1 % formic acid in MeCN, 20-55 %	55	[72]
Cider	Flavonols	Urine & plasma	HPLC	Prodigy ODS-3 C_{18}	A = H_2O:Tetrahydrofuran:TFA 98:2:0.1 B = MeCN	70	[87]
Cloudy apple juice	Various	Ileostomy fluid	LC/MS/MS (-ve)	Hypersil Gold C_{18}	A = 0.1 % formic acid in H_2O B = 0.1 % formic acid in MeCN	45	[8]
Polyphenol-rich beverages	Various	Urine	LC/MS/MS (-ve)	Zorbax Eclipse XDB-C18	A = 0.1 % formic acid in H_2O B = 0.1 % formic acid in H_2O:MeCN 5:95	6	[80]
Blackberry	Anthocyanins	Urine	LC/MS/MS (+ve)	Hypersil BDS C_{18}	A = H_2O:H_3PO_4 99:1 B = MeCN	45	[92]
Oregano	Phenolic acids	Urine	HPLC	Intertsil ODS-3 C_{18}	A = 50 mM KH_2PO_4/H_3PO_4 buffer (pH 2.3) /MeOH 90:10 B = 50 mM KH_2PO_4/H_3PO_4 buffer (pH 2.3) /MeOH/ MeCN 40:40:20	Not given	[90]
Danshen	Phenolic acids	Serum	LC/MS/MS (-ve)	Capcell Pak C_{18}	0.5 % formic acid in H_2O:MeCN:MeOH 56:22:22	Not given	[95]
Endive	Kaempferol	Urine & plasma	LC/MS/MS (+ve & -ve)	Prodigy ODS-3 C_{18}	A = H_2O:Tetrahydrofuran:TFA 98:2:0.1 B = MeCN	55	[91]

Table 2.1 (cont.) **Summary of analytical methods reported in the literature, for the analysis of polyphenols in human biological fluids**

Food Source	Polyphenol(s)	Biological Fluid(s)	Analytical Method	Column	Mobile Phases	Run Time (min)	Reference
Onions	Quercetin	Plasma	LC/MS/MS (+ve)	Prodigy ODS-3 C_{18}	A = H_2O:Tetrahydrofuran:TFA 98:2:0.1 B = MeCN	45	[163]
Onions	Quercetin, Kaempferol	Urine	LC/MS/MS (+ve)	Prodigy ODS-3 C_{18}	A = 1 % formic acid in H_2O B = 1 % formic acid in MeCN	105	[86]
Onions	Flavonols	Urine & plasma	LC/MS/MS (-ve)	Synergi Max-RP	A = 1 % formic acid in H_2O:MECN 95:5 B = 1 % formic acid in H_2O:MeCN 60:40	60	[84]
Capsule	Quercetin	Urine & plasma	LC/MS/MS (-ve)	C_{12}	MeCN: H_2O with 0.2 % formic acid 40:60	Not given	[96]
Preparation	(-) Epicatechin	Urine & plasma	LC/MS/MS (-ve)	Capcell pack UG-5	A = 0.05 % acetic acid in H_2O B = 0.05 % acetic acid in MeCN	40	[97]
Polyphenol-rich meal	Various	Urine & plasma	HPLC & GC/MS	Novo-Pak C_{18}	A = MeOH:H_2O:5N HCl 5:94.9:0.1 B = MeCN:H_2O:5N HCl 50:49.9:0.1	80	[107]

2.1.3 Method Validation

Bioanalysis is one of the branches of analytical science that requires method validation [154] to generate confidence in the results. Analytical methods developed for the quantitative evaluation of compounds and their metabolites must be reliable and reproducible for their intended use. Table 2.2 illustrates the parameters to be considered during validation of a quantitative method.

Table 2.2 **Parameters to be considered during quantitative method validation in bioanalysis, adapted from Hartmann *et al.* [157]**

Parameters	Short description
Accuracy	How close the experimental value (determined from standards) is to the value determined by replicate analysis of 'spiked' samples
Precision	Determined by variation in the accuracy
Linearity	Ability of the method to obtain results proportional to the concentration in the 'spiked' sample
Selectivity	Ability to determine the analyte(s) in the presence of other compounds
Recovery	Percentage of the analyte(s) extracted from the 'spiked' samples
Ion Suppression	The effects of the sample matrix on the ionisation of components
Limit of detection	Lowest sample concentration that can be detected
Limit of quantitation	Lowest sample concentration that can be quantified

A minimum of three concentrations are required for determining the values for accuracy and precision, preferably at the low, medium and high ranges of the expected concentrations of the analytes in the samples.

2.1.4 Anticipated Problems in Development of an Analytical Method

Several problems can be foreseen in developing a rapid and validated method for the analysis of polyphenols in biological samples. Firstly, suitable extraction/sample preparation methods need to be developed for the anlaysis of the urine and plasma. This is fundamentally important to accurate analysis, and is a determinant in the success of the whole method. Urine contains many metabolites; the analytical approach has to be fairly targeted to avoid unwanted interference from substances not of polyphenol origin. As mentioned earlier, there are also the problems of high salt concentrations in the samples, which degrade the chromatography, cause

high system back pressure and deposit on the MS, reducing sensitivity. However, sample clean up procedures need to be as simple as possible to avoid initiating changes to the metabolite profiles and reduce associated errors and losses, and are also required to be rapid to increase usefulness. A compromise will need to be reached between these factors.

Compromises will also have to be made for the selection of solvents for the extraction procedures and for the solvents used for mobile phases. Although there is structural similarity across the different classes of polyphenols, they are analytically distinct. Many papers report the characterisation of members of the same class of flavonoid, but studies across the classes are rarer. Flavonoids possess phenolic hydroxyl groups which are weak acids and require conditions above their pKa of ~8-10 to become fully ionised. Phenolic acids possess carboxylic acids and are stronger acids than flavonoids; for ionisation pH must be above their pKa of ~3-5. This adds complexity to the conditions required for optimum ionisation, retention and extraction of both groups of polyphenols. Structural similarity among the flavonoids also causes problems in differentiating between individuals [114] if for example the molecular masses are identical; this effect can be overcome to some degree by the use of LC.

In addition to the compromises made between the choice of solvents for extraction and the choice of solvents for LC analysis of different polyphenols, one set of MS parameters is unlikely to suit all compounds. Currently there are no references to the specific identification and quantification of apple poylphenols and their conjugated and ring fission metabolites in healthy humans, using the technique of LC/MS/MS, so initial work was based on methods that had been applied to biological samples collected after ingestion of polyphenols from other sources, such as cocoa and tea.

The detection of multiple metabolites in any one sample is complex and problems are likely to arise. Firstly, these metabolites need to have been extracted from the biological matrix successfully; secondly the method has to be sensitive enough to detect the very low levels likely to be present; and thirdly the method is required to differentiate between likely metabolite isomers. If baseline resolution is not apparent between all peaks chromatographically or significant retention time differences do not exist between the isomers, MS fragmentation patterns are needed to distinguish between metabolites. The ring position of glycosylation or conjugation, impacts on the fragmentation pathways of the compounds and helps to identify the unknowns.

Further problems can be seen in developing a method to identify metabolites because of a lack of available polyphenol standards for the possible metabolites; it is impossible to unequivocally identify and quantify unknowns in biological samples without reference standards. Calibration curves can be constructed for those standards that are available, and subsequently quantification can be achieved for aglycones and some metabolites. Comparisons can be made for some metabolites, if they have a similar structure or have similar functional groups/positions, but definitive proof of identity and concentrations cannot be reported.

To eliminate some of the uncertainty associated with results from methods of this kind, due to all the problems discussed above, validation is performed. The process of validation imparts a way of defining whether a working method has been devised for the analysis of polyphenols and their metabolites in biological samples. The literature is predominately absent of fully validated LC/MS/MS methods for such analysis, as the task is complex and time consuming. Validation procedures also take into account the effect of the biological matrix (ion suppression/enhancement effects) on the analysis of the sample, which is an extremely difficult parameter to account for as each individual plasma or urine matrix will affect the analysis differently.

To summarise, an assessment of the anticipated problems in developing a validated LC/MS/MS method for the analysis of polyphenols and their metabolites in biological samples has been detailed. There are many difficulties associated with this type of research, but it is important to achieve the aims proposed for the progression of knowledge and the complete understanding of absorption, metabolism and excretion of apple polyphenols in humans.

2.1.5 Aim of the Chapter

To develop and validate a hyphenated liquid chromatography tandem mass spectrometry method for the measurement of polyphenols and their metabolites in human urine and plasma samples.

2.2 Materials

Chemicals for Chromatography

HPLC grade MeCN (far UV) and MeOH were purchased from Fisher Scientific UK Ltd (Loughborough, UK). Formic acid was obtained from Sigma Aldrich (Dorset, UK) and stored at ca. 4 °C. TFA and dimethylhexylamine (DMHA) were both purchased from Sigma-Aldrich (Dorset, UK) and stored at RT. All water used was purified using a MilliQ water purification system. Standards (listed in Table 2.3) were purchased mainly from Sigma Aldrich (Dorset, UK) with the exception of isorhamnetin-3-glucoside and kaempferol-3-glucoside purchased from Apin Chemicals (Oxon, UK) and taxifolin, (-) epicatechin gallate, (-) gallocatechin and (-) gallocatechin gallate, purchased from MP Biomedicals (London, UK). Polyphenol conjugates were supplied by Paul Needs (Phytochemicals and Health department, Institute of Food Research, Norwich).

Chemicals for Extraction and Experimental Procedures

Ethyl acetate and ammonium acetate were purchased from Fisher Scientific UK Ltd (Loughborough, UK). Ammonium hydroxide was purchased from Sigma-Aldrich (Dorset, UK). β-glucuronidase (Type 1X-A from *E. coli*) and sulfatase (from *Helix pomatia*) were purchased from Sigma-Aldrich (Dorset, UK) as solids and stored at -20 °C (NB the sulfatase also has glucuronidase activity).

HPLC/LC/MS Columns

Initial analysis used a HiChrom (Berkshire, UK) Hypersil C_{18} column of dimensions 150 x 4.6 mm (5 μm particle size). Further development was carried out with a Phenomenex (Macclesfield, UK) Luna C_{18} column of dimensions 150 x 4.6 mm (5 μm particle size). Optimisation of the method involved using Phenomenex (Maccelsfield, UK) Gemini C_{18} columns of dimensions 100 x 3.0 mm (3 μm particle size) and 100 x 2.0 mm (3 μm particle size). All columns were fitted with a Phenomenex guard column and cartridges of the same material as the column.

General Equipment

Compounds were weighed on a Mettler AE163 balance. Gilson pipettes and an Eppendorf multipipette were used for all liquids. A Cyclone vortex mixer and sonicator were used for sample preparation. A Jouan centrifugal evaporator was used for sample evaporation.

LC equipment consisted of:

- Hewlett Packard 1090 instrument, complete with autosampler, column oven, tertiary pumping system and diode array detector (DAD). Mobile phases were sparged with helium gas before and during use. Conditions are detailed in section 2.2.3.
- Agilent 1100 series HPLC instrument, complete with temperature controlled autosampler (G1367A), degasser (G1379A), column oven (G1316A) and quaternary pumping system (G1376A); this was coupled with a Waters Micromass Quattro Ultima (details below). Conditions are detailed in section 2.2.3.
- Shimadzu HPLC instrument, complete with temperature controlled autosampler (SIL-HTc), degasser (DGU-14A), column oven (CTO-10Avp) and binary pumping system (LC-10ADvp); this was coupled with an Applied Biosystems/MDS Sciex Model 4000 Q-TRAP (details below). Conditions are detailed in section 2.2.3.

Mass spectrometry instrumentation consisted of:

- Waters Micromass Quattro Ultima, a triple quadrupole with an electron spray ionisation (ESI) probe. Direct infusion experiments were performed with a syringe pump (Semat International) and 5 mL syringe (Fisher Scientific, Loughborough, UK) to optimise precursor and product ions of the polyphenol standards.
- Applied Biosystems/MDS Sciex Model 4000 Q-TRAP, a hybrid triple quadrupole/ linear ion trap instrument with an ESI probe. Direct MS infusion experiments were performed with a syringe pump (Semat International) and 100 μL syringe (Fisher Scientific, Loughborough, UK) to optimise precursor and product ions of the polyphenol standards.

Data Processing

Quattro - Results obtained from LC/MS/MS analysis were processed by MassLynx^{TM} Global Mass Informatics V4.0, Waters Micromass Ltd.

Q-TRAP - Results obtained from LC/MS/MS analysis were processed by Analyst Software 1.4.1, Applied Biosystems; the algorithm applied was IntelliQuan. Sample peaks were automatically integrated by the software. All results were checked manually, and manual integration was performed if necessary. The strategy applied to data processing was to ensure everything was analysed in the same way; for example, a standard with a characteristic shoulder would be integrated with or without that shoulder every time it was analysed. Manual integrations were necessary on occasion and care was taken to make sure this would

not introduce error. Quantitation methods were created for the analysis of standards in the mobile phases, urine and plasma, and analytes in the urine and plasma; these were applied to samples accordingly. Using this strategy it was hoped that data processing was consistent. Extracted ion chromatogram peak areas were taken from Analyst and transposed into Excel, where further data processing (means, standard deviations, etc) was undertaken. Graphs were also constructed in Excel.

2.3 Methods

2.3.1 Preparation of Polyphenol Standard Solutions

All standards were supplied as solids; appropriate amounts were weighed into Eppendorf tubes and dissolved in either MeOH: H_2O (50:50) or MeOH:MeCN (70:30) according to their solubility, to make 1 mg/ml stock solutions (Table 2.3). These solutions were stored at ca. -20 °C.

Stock solutions were diluted with mobile phase for HPLC and MS analysis (initially 0.01 % formic acid: MeOH: MeCN 80:8:12 v/v/v for early development and 0.01 % formic acid: MeCN 80:20 v/v for the final method and subsequent analysis). Taxifolin was used as an internal standard and was prepared in the same way as the other polyphenol standards. A 3.3 μM solution was prepared to 'spike' study samples. A 50 μM solution of all the polyphenol standards was prepared using the appropriate volumes of the 1 mg/ml stock solutions; this was further diluted and used in calibration and validation procedures.

2.3.2 Mobile Phase Preparation

Mobile phases were prepared in volumetric flasks and filtered using Whatman membrane filters (0.45 μm) before use. Details of the different mobile phases used throughout the method development process are given in the section RP-HPLC later in this chapter.

2.3.3 Optimisation of RP-HPLC

Initially, two mobile phases were utilised in a gradient system; 0.1 % formic acid in H_2O (A) and MeOH (B). A Hypersil C_{18} (HiChrom, Berkshire UK) column was maintained at a temperature of 25 °C with a mobile phase flow rate of 1.0 ml/min and wavelengths were monitored at 280 nm and 365 nm. The gradient was as follows: (0, 10), (15, 10), (30, 90), (31, 10) (time in min, % B); total analysis time was 35 min. Changes were made to the mobile phases, gradient, flow rate, and column temperature. A Luna C_{18} (Phenomenex, Macclesfield

UK) and Gemini C_{18} (Phenomenex, Macclesfield UK) column were also investigated. Further changes to the LC conditions were made when LC/MS/MS was performed (detailed in section 2.2.5).

Table 2. 3 **Concentrations and compositions of the stock solutions of the polyphenol standards. Compounds reported to be present in apples, or reported metabolites of apples are shown by an asterix (this data was taken from literature studies detailing the composition of apples and metabolism studies using foods containing the same polyphenols).**

Compound	MW	Concentration (mM)	Composition
Caffeic acid*	180	5.56	MeOH: H_2O
Chlorogenic acid*	354	2.82	MeOH: H_2O
Ferulic acid	184	5.43	MeOH: H_2O
Sinapic acid	224	4.46	MeOH: H_2O
p-Coumaric acid*	164	6.10	MeOH: H_2O
Gallic acid	170	5.88	MeOH: H_2O
Vanillic acid	168	5.95	MeOH: H_2O
p-Hydroxybenzoic acid	138	7.25	MeOH: H_2O
Syringic acid	198	5.05	MeOH: H_2O
Protocatechuic acid	154	6.49	MeOH: H_2O
Genistein	270	3.70	MeOH: H_2O
Daidzein	254	3.94	MeOH: H_2O
Procyanidin B1*	578	1.73	MeOH: H_2O
Procyanidin B2*	578	1.73	MeOH: H_2O
Phloridzin*	482	2.07	MeOH: H_2O
Phloretin*	274	3.65	MeOH: H_2O
(+) Catechin*	290	3.45	MeOH: H_2O
(-) Epicatechin*	290	3.45	MeOH: H_2O
(-) Epigallocatechin gallate	458	2.18	MeOH: H_2O
(-) Catechin gallate	442	2.26	MeOH: H_2O
(-) Epicatechin gallate	442	2.26	MeOH: H_2O
(-) Gallocatechin	306	3.27	MeOH:MeCN
Myricetin	318	3.14	MeOH:MeCN
Kaempferol	286	3.50	MeOH:MeCN
Kaempferol-3-glucoside	448	2.23	MeOH:MeCN
Quercetin*	302	3.31	MeOH:MeCN
Quercetin-3-glucoside*	464	2.16	MeOH:MeCN
Quercetin-3-galactoside*	464	2.16	MeOH:MeCN
Quercetin-3-rhamnoside*	448	2.23	MeOH:MeCN
Quercetin-3-rutinoside*	610	1.64	MeOH:MeCN
Quercetin-3-glucuronide*	478	2.09	MeOH: H_2O
Quercetin-3'-glucuronide*	478	2.09	MeOH: H_2O
Quercetin-3'-sulphate*	382	2.62	MeOH: H_2O
Isorhamnetin*	316	3.16	MeOH:MeCN
Isorhamnetin-3-glucoside*	478	2.09	MeOH:MeCN
Isorhamnetin-3-glucuronide*	492	2.03	MeOH:MeCN
Naringin	580	1.72	MeOH:MeCN
Naringenin	272	3.68	MeOH:MeCN
Hesperetin	302	3.31	MeOH:MeCN
Hesperidin	610	1.64	MeOH:MeCN
Luteolin	286	3.50	MeOH:MeCN
Apigenin	270	3.70	MeOH:MeCN
Taxifolin (IS)	304	3.29	MeOH: H_2O

2.3.4 Optimisation of MS Parameters

Initially, individual polyphenols standards were infused at a flow rate of 200 µl/min (400 ml/min on the Quattro) using both positive and negative ESI to establish optimum conditions. This involved varying ion spray voltage (termed capillary voltage in the software used with the Quattro), source temperature and gas flow rates/ temperatures. These parameters represent those which will have the greatest effects on ionisation, and were applied globally to all compounds. Collision energy and declustering potential (termed cone voltage in the software used with the Quattro) also have significant effects on the ionisation of compounds, but these were established individually for each compound. Minor parameters for investigation included resolutions, ion energies and entrance/exit potentials. The effect of the solvent present in the infusion solution was also investigated; TFA was compared with MeCN.

2.3.5 Optimisation of LC/MS/MS Method

Preliminary Development Work

The effect of different concentrations of TFA in the mobile phase was investigated. Concentrations of 0.001 % (pH 4.2), 0.002 % (pH 3.7), 0.005 % (pH 3.3) and 0.01 % (pH 3.1) were all investigated compared to the original mobile phase of 0.05 % TFA (pH 2.4). Investigations were also performed to establish which organic solvent gave the best results; MeOH and MeCN were compared. Experiments using the two solvents together in different proportions were also undertaken. In addition, the effect of formic acid on the signal intensities and chromatography was compared with TFA; concentrations of 0.05 %, 0.01 %, 0.005 % and 0 % formic acid in H_2O were investigated. This also required further gradient changes to be applied to the method; (0, 20), (4, 40), (5, 46), (8, 48), (8.1, 98), (9, 98), (9.1, 20) (time in min, % B), total analysis time was 12 min.

Further Development Work

Changes to the LC methodology were necessary after initial experiments with the polyphenol metabolite conjugates. A change to the organic mobile phase was incorporated, MeCN replaced MeCN:MeOH (60:40), and 20 % MeOH was added to the aqueous mobile phase of 0.01 % formic acid. Many alterations were also made to the gradient, flow rates and temperatures before the final optimised conditions were decided upon (Table 2.4).

Table 2.4 **Final optimised conditions for LC/MS/MS analysis of polyphenol standards and their metabolite conjugates. Phenomenex Gemini C_{18} column 100 x 3.0 mm (3 µm particle size); temperature was 65 °C. Mobile phase A: 0.01 % formic acid in H_2O, mobile phase B: MeCN**

Time (min)	Event	Parameter
0	% B	10
4	% B	40
5	% B	46
8	% B	48
8.1	% B	100
11	Total flow	0.225 µl/min
11.1	Total flow	0.850 µl/min
12	Total flow	0.850 µl/min
13	Total flow	0.225 µl/min
13.1	% B	100
17	% B	0
17.1	% B	10
20	Stop	-

2.3.6 Development of Sample Extraction Procedures for Human Plasma

Liquid-liquid extractions and SPE were evaluated as methods to prepare the plasma for LC/MS/MS analysis. Initially, different solvents and in varying volumes were investigated. The use of an internal standard was also considered. Extracts were subjected to filtering/centrifugation techniques also. Whatman VectaSpin Micro™ filters (manufactured from polypropylene with a membrane pore size of 0.45 µm) and Millipore YM3 Microcon filters were compared.

Liquid-Liquid Extractions

Plasma samples (0.5 ml) were 'spiked' with a mixture of polyphenol standards to give a concentration of 10 µM. The samples were extracted with solvent (Table 2.5), vortex mixed (1 min) and centrifuged (13,000 rpm for 3 min at 4 °C). All extractions used a 3:1 ratio of solvent to plasma. The supernatant was decanted into a clean Eppendorf tube and evaporated to dryness using nitrogen. A second extract (and third in the instance of methods J, K and L) were both performed in exactly the same way, with the addition of a sonication step (10 min) after vortex mixing. This/these extract(s) were combined with the first and evaporated to dryness. All extracts were reconstituted in MeOH: H_20; formic acid (10:89:1), however, 20 % (method H) and 50 % MeOH (method I) were also evaluated on some supernatants (produced from method F). Reconstitution involved vortex mixing (1 min) and centrifugation (13,000 rpm for 3 min at 4 °C), before decanting the supernatant into an HPLC vial for analysis.

Table 2.5 **Methods used for the optimisation of the extraction of polyphenol standards from plasma (n = 3)**

Method	Solvent
A	1 % acidified ethylacetate (formic acid)
B	MeOH
C	MeCN
D	50 % MeOH, 50 % MeCN
E	1 % acidified MeCN (formic acid)
F	0.1 mM NH_4OH in MeCN
G	30 % MeOH, 70 % MeCN
J	3 extractions with 2 % acidified MeCN (formic acid)
K	3 extractions with 0.1 mM NH_4OH in MeCN
L	2 extractions with 2 % acidified MeCN (formic acid) followed by 1 extraction with 0.1mM NH_4OH in MeCN

Method K was used for the preparation of all plasma samples collected from the human intervention study.

Solid Phase Extractions

Hydrophilic-liphophilic balance (HLB) cartridges (Oasis, Waters) were used; these had a particle size of 30 µm and a pore size of 80Å. The cartridge was preconditioned with 1 ml of MeOH, followed by 1 ml of H_2O. 1 ml of plasma 'spiked' with a mixture of polyphenols reported to be present in apples and some of the known metabolites of apples (see Table 2.1) was loaded onto the cartridge. Three methods were performed, each in triplicate. Extracts were combined for each method, evaporated to dryness using a centrifugal evaporator and reconstituted in 25 µl MeOH and 225 µl of 1 % formic acid in H_2O.

Method 1: Wash 1: 1 ml 5 % MeOH in H_20; wash 2: 1 ml 2 % formic acid in MeOH: H_20, (v/v 50:50); wash 3; 1 ml 2 % NH_4OH in MeOH: H_20, (v/v 50:50).

Method 2: Wash 1: 1 ml MeOH: H_20, (v/v 50:50); wash 2: 1 ml ethylacetate acidified with 2 % formic acid; wash 3: 1 ml MeOH.

Method 3: Wash 1: 1 ml 5 % MeOH in H_20; wash 2: 1 ml 25 % MeOH in H_20; wash 3: 1 ml 50 % MeOH in H_20; wash 4: 1 ml 2 % formic acid in MeOH: H_20, (v/v 50:50).

2.3.7 Urine Preparation

Blank urine (0.4 µl) was centrifuged (13,000 rpm for 3 min, at 4 °C) and compared to urine treated with 5 volumes of MeCN before centrifugation at the same speed and for the same length of time.

Enzyme Treatment of Non-Study Urine - Preparation of Solutions

Ammonium acetate buffer (100 mM) was prepared and adjusted to pH 5 by the dropwise addition of acetic acid (approximately 1.1 ml). Sulfatase was supplied at a concentration of 14,400 units/g; 0.20 g was added to 2.9 ml of ammonium acetate buffer to give the desired concentration for the experiments. β-glucuronidase was supplied at 1,134,600 units/g; 0.05 g were added to 455 µl of ammonium acetate buffer. Both enzyme solutions were vortex mixed to aid dissolution and used on the day of preparation.

Enzyme Treatment of Non-Study Urine – Method

Urine (200 µl) was incubated at 37 °C for 2 h with 100 µl of ammonium acetate buffer (100 mM, pH 5), 20 µl of enzyme solution (sulfatase or β-glucuronidase) and 10 µl of the internal standard taxifolin (3.3 µM). Concentrated formic acid (2 µl) was added to stop the reaction. The sample was vortex mixed, centrifuged (13,000 rpm for 3 min at 4 °C) and the supernatant was decanted into an HPLC vial before LC/MS/MS analysis. Suitable controls without enzymes present were incubated along with the test samples.

Human Intervention Study Urine – Pooling Procedure

Details of urine collection procedures are given in chapter 3. Samples were removed from -80 °C storage and allowed to defrost at room temperature. Vials were shaken occasionally to aid defrosting. Pools were made of each time point for all the volunteers, for each of the two phases (n = 8) and samples were stored at -80 °C. Pools of 0 – 24 h samples were also prepared for each of the two phases of the study. Aliquots of all pooled samples were centrifuged (13,000 rpm for 3 min, at 4 °C), and the supernatants decanted for injection and analysis by LC/MS/MS. These pooled urine samples were also enzyme treated, following the same procedure as detailed above and analysed by LC/MS/MS.

Further pools were made using equal quantities of urine from each time-point for each of the volunteers, hence 0 – 24 h pools for each volunteer were created (n = 9). These pools were enzyme treated as detailed above, and analysed by LC/MS/MS. These enzyme deconjugation experiments were undertaken to obtain values for the total aglycones excreted by each volunteer.

Human Intervention Study Urine – Individual Sample Analysis

Study urine collected which had not been defrosted before, was removed from storage at -80 °C and allowed to defrost at room temperature. An aliquot (400 µl) was removed and placed in an Eppendorf tube. The sample was centrifuged (13,000 rpm for 3 min, at 4 °C) and 200 µl of the supernatant was aliquoted into an HPLC vial. 30 µl of the internal standard Taxifolin was added, and the sample was inverted. Injections (20 µl) were made onto the LC/MS/MS. All results from these analyses are given in chapters 3 and 4.

2.3.8 Validation Procedures

Using a 50 µM solution of a mixture of polyphenol standards (prepared as detailed in section 2.3.1), a set of six calibration standards ranging in concentration from 25 nM to 1.0 µM were prepared in blank urine (pooled baseline samples) on the same days as the analysis of study urine samples. These samples were analysed at the start and at the end of the sample table. Peak area values for each of the polyphenol standards were plotted against concentration to give calibration graphs; a value for linearity could then be determined. In addition, blank urine was 'spiked' to give three different concentrations along these calibration graphs; low (0.09 µM), medium (0.277 µM) and high (0.83 µM). Samples were prepared in duplicate and centrifuged to represent the treatment of study samples. Accuracy values were calculated using actual peak areas produced from the analysis of these low, medium and high concentration 'spiked' urine samples, and calculated values from the calibration graphs. Mean (n = 7) accuracy values were calculated for each polyphenol standard, and a value for precision was determined by dividing the standard deviation by the mean.

Recovery was determined using data from two experiments: (1) blank urine (n = 6) was 'spiked' with a mixture of polyphenol standards to give the same low, medium and high concentrations as used previously, and centrifuged (same treatment as used for the study samples), before LC/MS/MS analysis. (2) Blank urine (n = 6) was centrifuged (same treatment as used for the study samples) and then 'spiked' with a mixture of polyphenol standards to give the same low, medium and high concentrations as previously, before LC/MS/MS analysis. Peak areas from the 'spiked' and then extracted samples were divided by peak areas from the extracted and then 'spiked' samples, and the value multiplied by a hundred to give a percentage recovery for each of the polyphenol standards in the mixture. Matrix effects were calculated at the medium and high concentrations by comparing the ratio between the low, medium and high concentrations with the ratio between the peak areas obtained for each of the

polyphenol standards. If the ratios were the same, the matrix was not affecting the MS response for the compounds, if the ratios differed then the MS response was either being suppressed or enhanced. Ratio values were used to calculate an expected peak area (which would be detected if the matrix was blank). The actual peak area detected was divided by the calculated peak area and multiplied by a hundred, to give the percentage of the response that was suppressed (negative value) or enhanced (positive value).

Procedures used for the validation of the method for the extraction of polyphenols from human plasma were the same as those used for the urine. The differences were that the set of six calibration standards prepared for linearity measurements ranged in concentration from 2.5 nM to 0.2 μM, and also the values for the low, medium and high concentrations of 'spiked' plasma were 0.05 μM, 0.10 μM and 0.20 μM, respectively.

2.4 Results and Discussion

The strategy undertaken for the development of a new LC/MS/MS method to quantify apple polyphenols and their metabolites in human urine and plasma was firstly to develop an HPLC-UV method which could separate the selected polyphenol standards. Following this, optimum MS parameters were investigated for each of these polyphenol standards. Further method development was performed via LC/MS/MS, using polyphenol standards and some conjugated polyphenol standards. Initial biological sample preparation was executed using blank urine and plasma, 'spiked' with polyphenol standards and qualitative assessments were made to highlight methods for further investigation; validation procedures were undertaken on the final methods. The optimised and validated method was then applied to pooled study urine samples to gather preliminary data to aid analysis of the individual study samples.

2.4.1 HPLC-UV and LC/MS/MS Method Development

Systematic changes to the mobile phases, gradients, flow rates, column, column temperatures and total analysis times were made in order to optimise the separation of the polyphenol standard mixture. Figure 2.4 (A) shows a chromatogram from the early development work with a HiChrom (Berkshire, UK) Hypersil C_{18} column (150 x 4.6 mm, 5 μm particle size). These columns have previously been used in the literature for the determination of polyphenols [8, 92]. The peaks observed at 8.7, 11.5, 12.5 and 18.5 min show characteristic tailing, probably caused by the ionised silanols and metal ions on the surface of the silica used

to pack the column. Hypersil columns are manufactured using an older style of silica, which has been superseded by a modern purer type of silica that lacks the impurities and therefore reduces these interactions, producing better chromatography.

A Phenomenex (Macclesfield, UK) Luna C_{18} column (150 x 4.6 mm, 5 μm particle size) was also evaluated (Figure 2.4, B) and the polyphenol peaks observed with this column were much sharper with no peak tailing. However, for a mixture of eight polyphenols, only seven peaks were observed, and these were not satisfactorily resolved, so additional gradient, flow rate and temperature (40 °C) changes were made. The best separation achieved from these early LC-UV experiments was suitable to begin work on the MS optimisation.

Further LC/MS/MS method development required the use of a column with smaller dimensions; a Phenomenex (Maccelsfield, UK) Gemini C_{18} column (100 x 3.0 mm, 3 μm particle size) was utilised. Preliminary ESI infusion experiments on the Micromass Quattro Ultima showed ion suppression effects when TFA was present in the standard mix infusion solution when compared to MeCN alone. Although TFA has proven to be more efficient in the separation of flavonoids in reversed-phase systems, compared to acetic or phosphoric acid [112, 161], TFA generally causes reduced signals for these compounds in the MS in negative mode [14]. To improve signal intensities, experiments to reduce the percentage of TFA in the infusion solution and in the mobile phase were undertaken; a compromise was made between signal intensities, resolution and peak shape. Concentrations of 0.001 %, 0.002 %, 0.005 %, 0.01 % and 0.05 % were investigated. The greatest ion signal intensities were observed at the lowest concentrations of TFA, but peak resolution was poor; e.g. a mixture of four polyphenols standards, which were resolved under the original TFA concentration of 0.05 %, co-eluted using 0.001 % TFA (Figure 2.5, A). The same concentration of 0.001 % TFA was added to mobile phase B to see if this could improve the resolution of the compounds, but only a slight change to the separation was observed. Experiments comparing MeOH and MeCN as the organic mobile phase resulted in MeOH providing resolution of these four polyphenolic standards at the same concentration of TFA (Figure 2.5, B).

However, analysis of some of the other polyphenolic standards using MeOH, such as gallic acid, resulted in broader peaks than observed with MeCN. Experiments were carried out with a combination of the two organic solvents in different ratios until resolution, peak shapes and

retention times were all optimised. This was achieved with a combination of MeCN and MeOH in the ratio of 60:40.

Despite significantly reducing the concentration of TFA in the mobile phase, a reduction in sensitivity was still observed in the MS. TFA was substituted with formic acid, which did not incur any losses in resolution or peak shape at the initial concentration of 0.1 %. However, retention times decreased, so a final change to the gradient was made. Presence of formic acid in the mobile phases utilised for LC/MS/MS analysis still caused a reduction in ionisation; 0.01 % formic acid gave the best signal intensities (Figure 2.6). Some previously unresolved peaks were also resolved at this lower concentration of formic acid, so a good compromise between resolution and ion signal intensities had been achieved.

2.4.2.1 Direct Infusion Experiments

Optimum MS conditions for individual compounds were established by direct infusion of the polyphenol standards in the mobile phases. Both negative and positive ionisation modes were investigated; with negative mode producing the best signal intensities for the greatest number of compounds. Table 2.6 details the optimised Q-TRAP parameters.

Table 2.6 Optimised global Q-TRAP MS parameters

Parameter	Value and units
Ion Spray Voltage	-4.5 kV
Temperature	225 °C
Curtain Gas	10.0 psi
Collision Gas	6.0 psi
Ion Source Gas 1	19.0 psi
Ion Soure Gas 2	10.0 psi

Preliminary experiments revealed that some of the standards had the same mass pairs, for example procyanidin B1 and B2, (+) catechin and (-) epicatechin, (-) catechin gallate and (-) epicatechin gallate and quercetin-3-glucoside and quercetin-3-galactoside. For the first three pairs, each compound in the pair had a different retention time (although the peaks for (-) catechin gallate and (-) epicatechin gallate are not baseline resolved) and so could be distinguished. However, the glycosides of quercetin had identical retention times, and no differences were observed in their fragmentation patterns so unfortunately these glycosides could not be distinguished by this method.

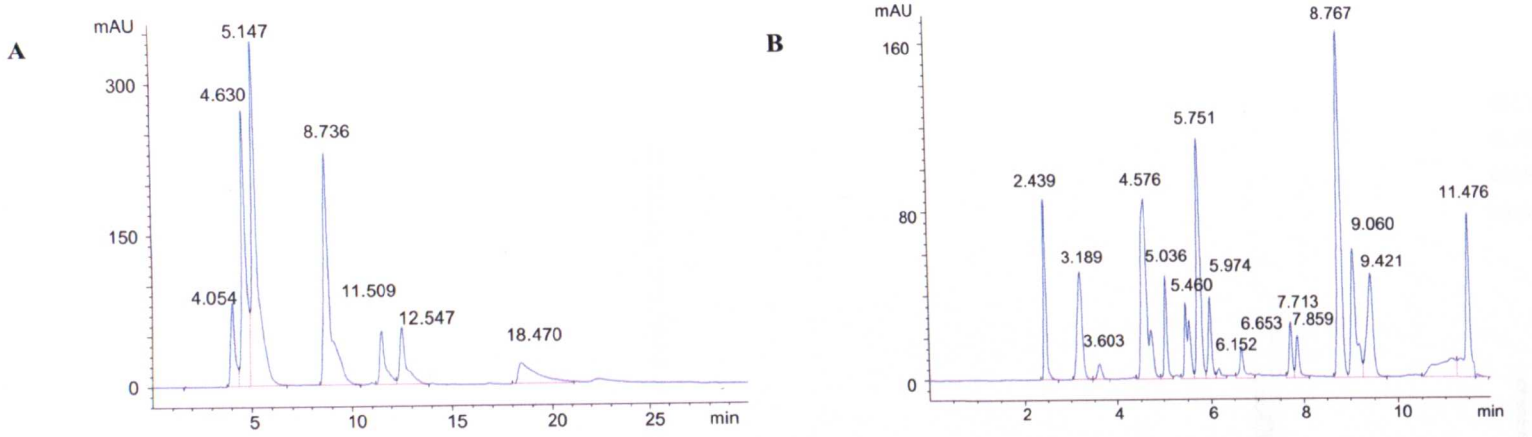

Figure 2.4 **(A) HPLC-UV chromatogram of polyphenol standard mixture (contains caffeic acid, chlorogenic acid, (+) catechin, vanillic acid, ferulic acid, quercetin, quercetin-3-rutinoside and hesperidin) using the following gradient (0, 10), (15, 50), (30, 90), (31, 10) (time in min, % B); total analysis time was 35 min and the analytical column used was a HiChrom Hypersil C_{18}. (B) HPLC chromatogram of same standard mixture, using a Phenomenex Luna column C_{18}.**

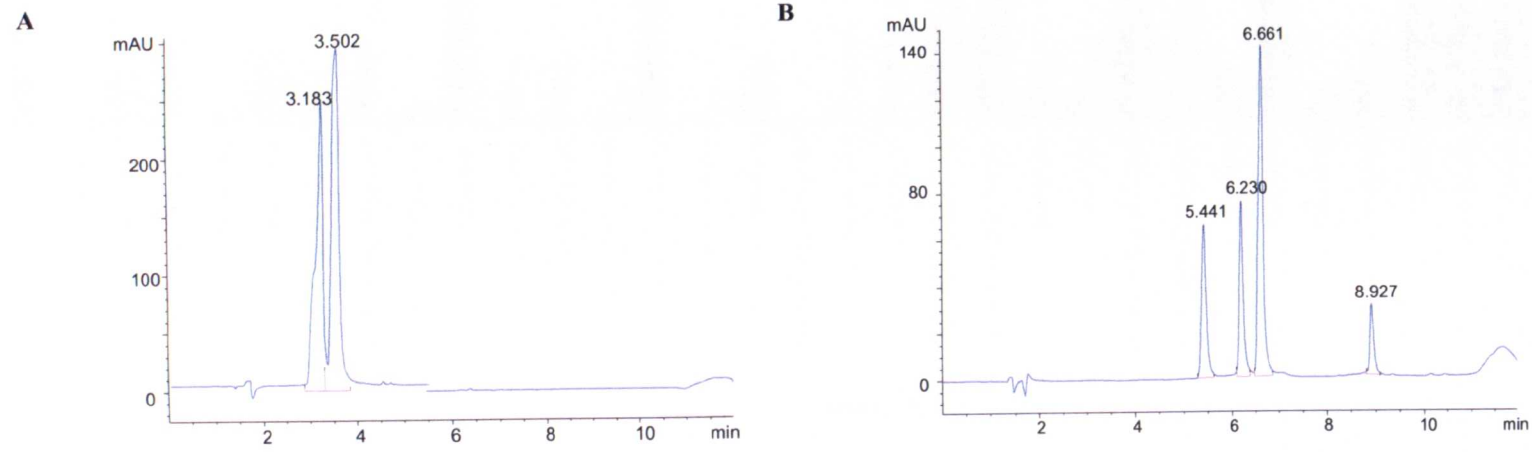

Figure 2.5 **(A) HPLC-UV chromatogram of caffeic acid, *p*-hydroxybenzoic acid, quercetin-3-rutinoside and syringic acid using a concentration of 0.001 % TFA in the aqueous mobile phase and MeCN as the organic mobile phase. (B) HPLC-UV chromatogram of caffeic acid, *p*-hydroxybenzoic acid, quercetin-3-rutinoside and syringic acid using a concentration of 0.001 % TFA in the aqueous mobile phase and MeOH as the organic phase. Gradient for both chromatograms: (0, 20), (4, 40), (5, 46), (8, 48), (8.1, 90), (9, 90), (9.1, 20) (time in min, % B); total analysis time was 12 min.**

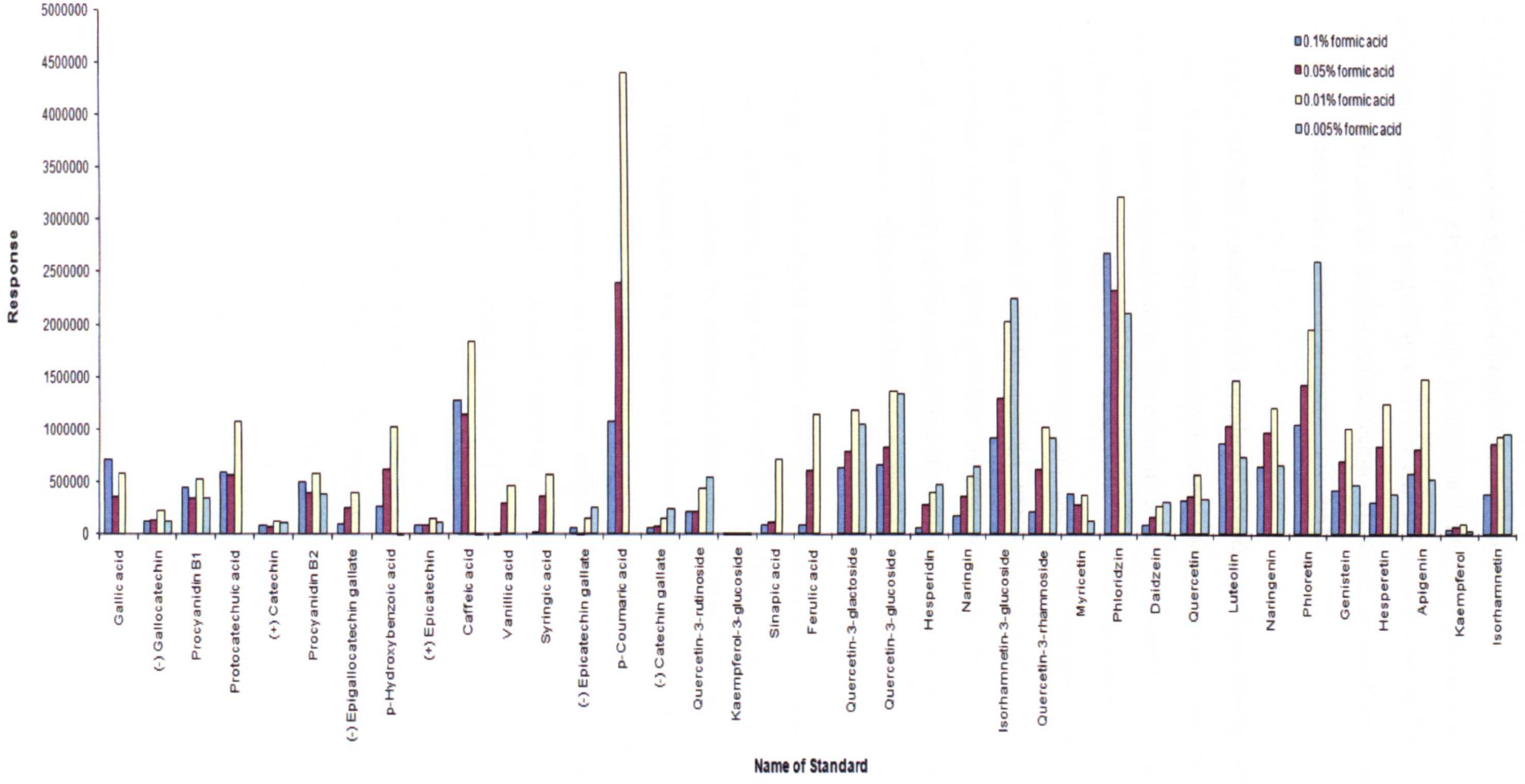

Figure 2.6 **Mass spectrometry responses obtained for polyphenol standards using concentrations of 0.1 %, 0.05 %, 0.01 % and 0.005 % formic acid in the mobile phase**

2.4.2.2 Atmospheric Pressure Chemical Ionisation (APCI)

The technique of APCI in both positive and negative modes was investigated for the polyphenol standards producing weak ion signal intensities using ESI, such as phloridzin, procyanidin B1 and B2 and (-) gallocatechin. However, no significant improvements to the signal intensities were achieved compared with ESI.

2.4.2.3 LC/MS/MS – Analysis of Polyphenol Standards (Non-Biological)

Figure 2.7 shows the extracted ion chromatogram for a mixture of the polyphenol standards, represented three dimensionally. Experiments using polyphenol metabolite conjugates in the polyphenol standard mix indicated some problems with the chromatography. Although the glucuronides of quercetin and isorhamnetin had earlier retention times compared to their aglycones, the quercetin-3′-sulfate standard appeared not to elute during the analysis time. Considerable time was spent problem solving, trying to ascertain whether the MS was detecting an impurity in the synthesised standard or whether poor compound ionisation or degradation was to blame for its lack of appearance.

After consulting the original method used for the identification of quercetin-3′-sulfate during synthesis, it was observed that elution resulted following a period of 100 % MeCN. MeCN is a much stronger solvent for decreasing retention of compounds on reversed phase columns. However, the method also had increased formic acid concentration and increased total analysis time, both undesirable conditions. Experiments using 100 % MeCN as mobile phase B and increasing the temperature and flow rate, instead of increasing formic acid concentration and analysis time, worked to reduce retention and elute quercetin-3′-sulfate within a 20 min analysis time; quercetin-3′-sulfate eluted at 17.3 min.

Table 2.7 details the retention times, precursor and product ions, optimised collision energies and declustering potentials for all of the polyphenolic standards evaluated. These parameters were used to construct the multiple reaction monitoring (MRM) method for the polyphenol standards, data from which was used for the construction of the calibration graphs. More polar polyphenolics such as the hydroxybenzoic acids, eluted at the beginning of the chromatogram followed in order by flavanols, hydroxycinnamic acids, coumarins, flavanones, dihydrochalcones, flavonols and flavones. This order is in agreement with published literature [136, 137, 164, 165]. Within the same class of polyphenol, the functional groups also play

an important role in retention. The greater the numbers of hydroxyl groups present on the molecule, the less the compound is retained, for example compare gallic acid with three hydroxyl groups (t_r 1.91 min) and protocatechuic acid with two hydroxyl groups (t_r 2.65 min). This is observed across all the classes in this study. The presence of apolar substituents, such as methoxy groups, also increase the retention time (compare quercetin t_r 6.68 min and isorhamnetin t_r 7.68 min, isorhamnetin differs in structure by the presence of a methyl group). This pattern is also seen with other polyphenols such as the hydroxybenzoic acids; gallic acid has three hydroxy groups present, when two of these are substituted for methoxy groups as in the case of syringic acid, the retention time increases from 1.91 min to 4.61 min. Retention times given by Abad-Garcia *et al.* [137] for a range of polyphenolic standards with and without methyl groups also supports this.

The retention times of the procyanidins B1 and B2 are an example of how the orientation of the functional groups also affects the retention time, as they differ only in the planar direction of a single hydroxyl group and this is the difference between t_r 2.12 min observed for B1 and 2.88 min recorded for B2. The later retention time for procyanidin B2 has also been reported by other authors [101, 137]. A further example of orientation affecting retention times is seen with the compounds (+) catechin (t_r 3.65 min) and (-) epicatechin (t_r of 4.32 min). These two flavanols are isomers, differing only in their orientation of the B ring about the C ring; retention time is critical for their identification as they are difficult to distinguish by other techniques. Supporting evidence for the elution order of flavanols is present in the literature [101].

If the compound has a sugar attached, the glycosylated form elutes before the aglycone (compare hesperidin t_r 5.07 min and hesperetin t_r 7.57 min; hesperidin contains glucose) under these conditions. This finding is supported by literature which also reports that if the sugars are acylated, this increases the retention time [126, 136]. Glycosylation affects retention times differently based on the nature of the sugar and the bond position; if compounds are glycosylated in the same bond position, the elution order is diglycoside, galactoside, glucoside, rutinoside, neohesperidoside and rhamnoside [137] (compare quercetin-3-glucoside t_r 5.19 min and quercetin-3-rutinoside t_r 5.76 min). Alterations to this order may be observed with different LC conditions; Kahle *et al.* [101] report the elution of quercetin-3-*O*-rutinoside before other *O*-glycosides. However, it is the position of the sugar moiety that is more important than the nature of the sugar [137]; glycosides in the 7 position elute before those on

the 4′ or 3 positions, and *C*-glycosides elute before *O*-glycosides [136]. Since the glycoside moieties on the flavonoids included in this method are all present on the 3 position, this aspect cannot be discussed.

The retention of conjugates is also influenced by the nature of the conjugate (methylated, sulfated or glucuronidated) and the position on the molecule. Isorhamnetin-3-glucuronide elutes after the glycoside (isorhamnetin-3-glucoside), but before the aglycone. This pattern is seen with quercetin-3-glucuronide also, however, the 3′-glucuronide elutes after both the glycosides and the aglycone. This elution pattern applies to the analysis of these compounds in biological matrices also; Mullen *et al.* [23], reported a t_r for quercetin-3-glucuronide as 28.4 min and a t_r for quercetin-3′-glucuronide as 36.3 min in human urine and plasma. However, elution of the aglycone in plasma was observed after all glycosides and glucuronides of quercetin. This may be due to differences in the LC conditions used for the analysis; a much higher concentration of formic acid is utilised in the mobile phases, or it may be due to other components in the plasma matrix.

When conjugated with a sulfate, the retention time of quercetin increased; t_r of aglycone was 6.68 min compared to t_r of quercetin-3′-sulfate 17.3 min. No other sulfated polyphenol standards were available for comparison in this study. Mullen *et al.* [23], also report the elution of quercetin sulfates after the aglycone, glucosides and glucuronides, in human urine and plasma. No methylated standards were purchased as standards for this present work, and so the effect of this type of conjugation cannot be considered. Literature indicates that methylation of polyphenols increases the retention time; Gonzalez-Manzano *et al.* [166], report t_r of (+) catechin to be 16 min, with 5-*O*-methylcatechin, 3′-*O*-methylcatechin, 7-*O*-methylcatechin and 4′-*O*-methylcatechin eluting after this time, in the order stated.

Table 2.7 **Retention times and optimised precursor and product ions, collision energies and declustering potentials for the polyphenolic standards. LC/MS/MS conditions were as described in Table 2.4.**

Standard	Retention Time (min)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)	Declustering Potential (V)
Caffeic acid	4.24	178.9	135.0	-22	-60
Chlorogenic acid	4.21	353.1	190.8	-24	-47
Ferulic acid	5.44	193.0	134.0	-22	-45
Sinapic acid	5.49	223.0	148.9	-28	-48
p-Coumaric acid	5.02	162.9	118.9	-20	-41
Gallic acid	1.91	169.0	124.9	-22	-56
Vanillic acid	4.39	167.0	107.9	-26	-40
p-Hydroxybenzoic acid	3.50	136.0	107.9	-27	-90
Syringic acid	4.61	197.0	181.9	-18	-50
Protocatechuic acid	2.65	152.9	109.0	-21	-41
Procyanidin B1	2.12	577.2	288.9	-34	-70
Procyanidin B2	2.88	577.2	407.1	-32	-90
(+) Catechin	3.65	289.0	109.1	-36	-62
(-) Epicatechin	4.32	289.0	109.1	-36	-70
(-) Epigallocatechin gallate	4.32	457.0	169.0	-26	-80
(-) Catechin gallate	5.09	441.1	169.0	-27	-62
(-) Epicatechin gallate	5.09	441.1	169.0	-26	-94
(-) Gallocatechin	2.16	305.0	124.9	-28	-61
Genistein	7.30	269.1	133.0	-42	-80
Daidzein	6.49	253.1	132.0	-56	-92
Phloridzin	5.85	481.1	273.0	-33	-32
Phloretin	7.16	273.1	167.0	-24	-68
Naringin	5.60	579.3	271.0	-44	-101
Naringenin	7.22	271.0	119.1	-34	-57
Hesperetin	7.57	300.9	151.0	-30	-111
Hesperidin	5.07	609.3	300.9	-35	-78
Luteolin	6.70	285.0	133.2	-49	-71
Apigenin	7.33	269.0	117.0	-50	-89
Myricetin	5.89	317.0	150.9	-32	-89
Kaempferol	7.42	285.0	116.9	-56	-94
Kaempferol-3-glucoside	4.90	447.1	222.9	-12	-24
Quercetin	6.68	301.0	106.9	-40	-71
Quercetin-3-glucoside	5.19	463.0	270.9	-63	-77
Quercetin-3-galactoside	5.19	463.1	300.2	-40	-81
Quercetin-3-rhamnoside	5.65	447.1	300.1	-37	-90
Quercetin-3-rutinoside (Rutin)	5.76	609.2	300.1	-54	-94
Quercetin-3-glucuronide	5.70	477.0	301.0	-32	-85
Quercetin-3′-glucuronide	6.90	477.1	300.9	-31	-66
Quercetin-3′-sulphate	17.3	381.0	300.9	-28	-45
Isorhamnetin	7.68	315.1	300.1	-31	-93
Isorhamnetin-3-glucoside	5.72	477.1	314.2	-37	-87
Isorhamnetin-3-glucuronide	6.22	491.0	315.0	-26	-123

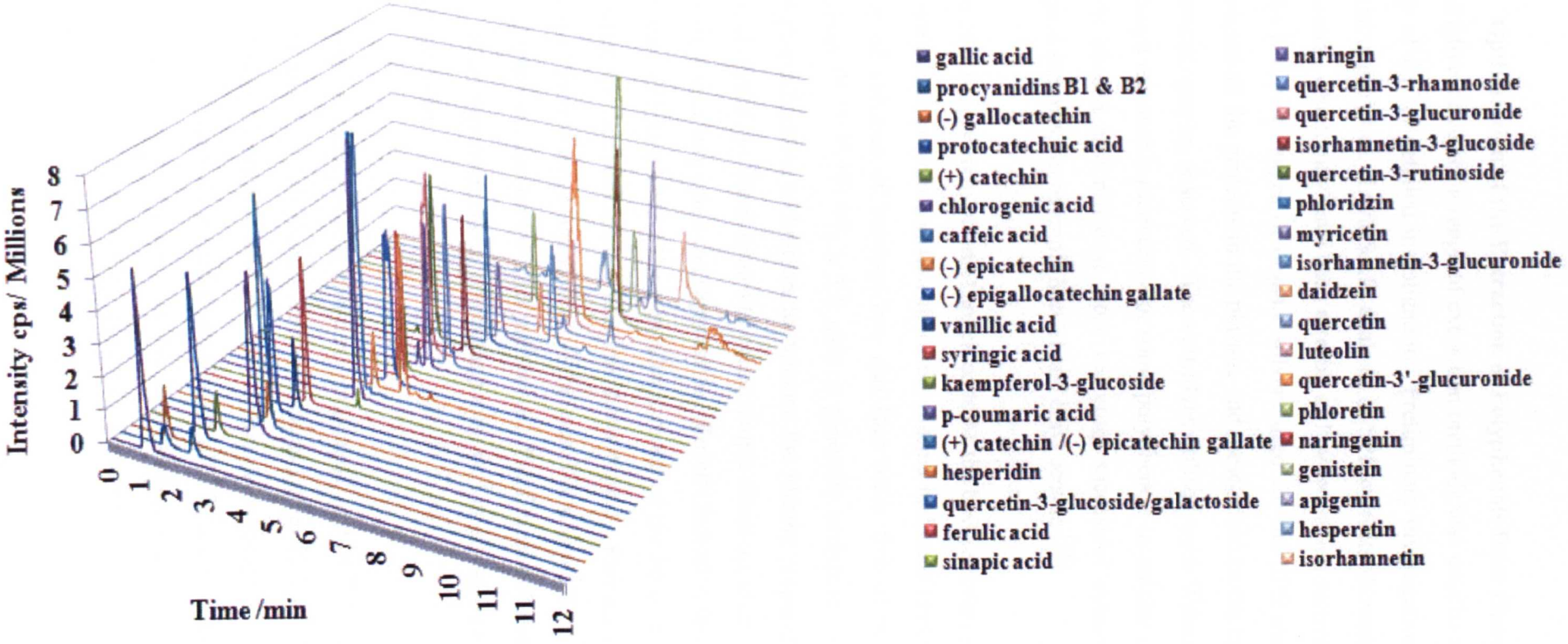

Figure 2.7 **3D extracted ion chromatogram of a mixture of some of the polyphenol standards. LC/MS/MS conditions were as described in Table 2.4.**

2.4.3 Optimisation of the Extraction of Polyphenols from Human Plasma

A qualitative survey of a range of extraction methods was used to assess which methods were worthy of further detailed investigation. Preliminary investigations eliminated a number of potential approaches. LC/MS/MS analysis of SPE extracts showed no peaks for the expected compounds, which may have been a result of low extraction recoveries. It was also observed that the low percentages of organic solvent used for the SPE methods may not have fully precipitated all the proteins in the plasma, and these could be the cause of the deterioration in the chromatography observed. The cartridges utilised were Waters HLB cartridges, as these had shown reasonable recoveries for some polyphenols in articles in the literature [156, 167] and solvents chosen also reflected those reporting successful extraction of polyphenols from biological samples, for example MeOH and ethyl acetate [70].

A ratio of 3:1 (solvent to plasma) appeared to be the best selection for liquid-liquid extractions to ensure all the protein constituents of the plasma had been removed. The literature details the use of volumes of between two and four times that of the plasma for liquid-liquid extractions [14, 69, 84, 163, 168]; a 3:1 ratio is generally preferred. Extracts were subjected to filtering/centrifugation techniques to enhance the plasma preparation. The rationale behind the use of centrifuge filters relates to better sample clean-up, eliminating other components in the matrix which may cause ion suppression. Results indicated that due to the very small pore size of the Millipore YM3 Microcon filters, much of the 50 µl extract was lost on the filter membrane; for this reason their use was discontinued. Perhaps use on larger sample sizes would have proved more successful. Whatman VectaSpin Micro™ filters also caused much of the sample to be lost on the filter membrane. The filtrate that did pass through did not show enough of an improvement in chromatography or ion signal intensities to warrant the high costs of the filters.

In the literature, mostly MeCN (see next paragraph), MeOH [8, 99] and formic acid [83] are used for the extraction of polyphenols from plasma and other biological fluids. To avoid the need for the addition of ascorbic acid which many research groups use to preserve the sample and stabilise the polyphenols at neutral or alkaline pH [46], sample handling times were kept to a minimum in these investigations. It was thought the presence of ascorbate in the sample may obscure analytes present in small quantities, in terms of the response of the MS, and may cause further interactions between metabolites.

After initial preliminary experiments, optimisation of the plasma extraction method was performed. The methods detailed in Table 2.5 were evaluated and percentage recoveries for all methods are shown in Tables 2.8 and 2.9. Comparing methods A – D, method C using MeCN produced the highest extraction recoveries for 40 % of the compounds analysed. Methods A, B and D contributed to the remaining 60 % equally. In the instances where method C did not produce the highest extraction recovery for a particular compound, the value was not usually much lower than the greatest value achieved. For these reasons, method C was chosen as the solvent to continue method development. The rationale behind the successful use of MeCN for extraction of polyphenols is due to their physio-chemical properties. Polyphenols are polar and water soluble and so they dissolve completely in MeCN. Protein in the sample is precipitated on contact with MeCN and some lipids are also extracted, so there is a small amount of selectivity. Many of the papers detailing the extraction of polyphenols from plasma use MeCN as the extraction solvent [23, 72, 78, 84, 106]. However, very few papers quote the extraction efficiencies for the polyphenols under investigation; the average recovery for phloridzin from plasma was quoted as 65 % by Marks *et al.* [72], and the average recovery for (-) epicatechin for plasma was typically 55 % reported by Mullen *et al.* [106], both of these values are close to those obtained here with MeCN. Mata-Bilbao *et al.* [70], report a much higher recovery for (-) epicatechin of between 80.6 % and 119.75 % using 70 % DMF containing 0.1 % formic acid.

Increasing and decreasing the pH of the MeCN, with a base and an acid respectively, and also MeCN in combination with MeOH were investigated (methods E – G) for plasma extraction. Although initially looking at the data, acidified MeCN (method E) provided the highest percentages of recovery for the majority of compounds (57.5 %), MeCN containing 0.1 mM NH_4OH (method F) appeared superior for most of the compounds specifically found in apples, for example the procyanidins, catechins and dihydrochalcones. The literature supports the acidification of the plasma before extraction, which would achieve a similar effect to acidifying the extraction solvent, but there are no references to the use of alkaline extraction solvents in the literature. Where the acidified MeCN offered the greater extraction efficiencies, the value for the extraction efficiency using MeCN containing 0.1 mM NH_4OH was not usually much lower. For these reasons, it was decided to proceed with method F - 0.1 mM NH_4OH in MeCN to continue method development.

It is possible that the solvents used for reconstitution could also have an effect on the values for the extraction efficiencies and so this was investigated (comparing methods H and I). The results show that there were no improvements to the recoveries by increasing the proportion of MeOH in the reconstitution step (20 % and 50 %); all the extraction efficiency values were lower than for the reconstitution using 10 % MeOH. The final experiment on the optimisation of the plasma extraction method combined the extraction with acidified MeCN (method E) and MeCN containing 0.1 mM NH_4OH (method F). Extracting the plasma a third time was also investigated (methods J – L). When both acidified MeCN and alkaline MeCN were used in combination, extraction efficiencies overall improved, but the extracts obtained were not as clean as those prepared when using each of the solutions on their own. It was also noted, that for the majority of the apple components, 0.1 mM NH_4OH in MeCN alone still gave the greatest recoveries. Extracting the plasma twice improved the recoveries, but no improvement in extraction efficiencies was noted with a third extract. This would unnecessarily increase the time taken for sample preparation, with no observed benefits.

The only paper in the literature to date, which deals with the extraction of procyanidins from biological fluids is by Tanaka *et al.* [68]; the authors measure the levels of procyanidins B2 and B3 in rat plasma after oral administration of the polyphenols. Recoveries were between 73.7 and 93.8 % for concentrations between 125 nM and 2000 nM; full method validation was not performed. The extraction methodology presented here did not produce the same high recoveries as reported in this paper (2:1 MeCN: plasma, reconstituted in 50 % MeOH); the best recoveries for the procyanidins B1 and B2 were 58.3 % and 56.5 %, respectively and were produced by extraction with 0.1 mM NH_4OH in MeCN.

In summary, the data shows that for the majority of compounds, method L, i.e. the combination of acidified MeCN and NH_4OH in MeCN, produced the greatest recoveries. However, for six of the compounds found in apples, the method using only NH_4OH in MeCN appeared more effective. Noting this, and acknowledging that an extraction with just one solvent is much easier to execute than with the combination of the two, and also considering that when the extraction efficiencies are improved with the treatment of acid and alkali together, the actual values for the recoveries are not significantly more than when NH_4OH is used alone; method K was used to extract all the plasma samples collected from the human intervention study.

The liquid-liquid extraction method using a 3:1 ratio of 0.1 mM NH_4OH in MeCN to plasma generally gave extraction recoveries greater than 50 % for the 40 polyphenols investigated. It is important to mention that recoveries for metabolites of apple polyphenols (the majority of which are not included in the standard solution) may be different to those of the standards. Also, where recoveries for some standards are low, quantitative data produced for the absorption and excretion of metabolites may be underestimated. The extraction efficiencies of certain polyphenols were compromised for others in these experiments, to attempt to find one method to suit all. The range of polyphenols under investigation adds a great deal of complexity to finding just one method that can be applied to all compounds. This method provides a simple, fairly rapid way of preparing plasma for LC/MS/MS analysis, which is not specific for polyphenols, although has proven to be suitable for members of the polyphenol family, regardless of class. A method more specific to the extraction of polyphenols, or some of the more difficult classes of polyphenol could also have been investigated.

2.4.4 Optimisation of the Extraction of Polyphenols from Human Urine

The resulting pellets formed at the bottom of the Eppendorf tubes from urine centrifuged compared to urine treated with 5 volumes of MeCN before centrifugation with the same conditions, indicated that the treatment with MeCN gave no benefit of increased removal of endogenous materials over centrifugation alone. Thus, all urine was centrifuged before analyses and this was the only sample preparation performed. This is in agreement with the majority of studies using polyphenols detailed in the literature [72, 74, 78, 82]. A few exceptions to this involved acidifying the urine with acetic acid before extraction with ethyl acetate [80] (no metabolite detection/identification performed), treating the urine with dichloromethane prior to centrifugation [77] and treating the urine with MeCN [73] both methods used for the identification of metabolites of tea.

Simple sample preparation such as used presently, also reduces the chances of altering the metabolite composition of the fluid, which may occur with extraction procedures. Small volumes of urine were analysed to avoid salt and contaminants which will not have been removed via this method, accumulating on the analytical column and reducing sensitivity in the mass spectrometer.

Table 2.8 **Mean percentage recoveries (n = 3) of compounds using plasma extraction methods A – I (% CV). Compounds known to be present in apples or known metabolites of apples are shown by an asterix. Greatest recoveries are shown in bold.**

Standard	Method								
	A	B	C	D	E	F	G	H	I
Caffeic acid*	51.9 (7.80)	71.2(8.9)	**76.7** (1.2)	70.1 (4.7)	**81.0** (5.0)	75.1 (11.4)	70.7 (6.4)	6.2 (18.0)	8.1 (3.6)
Chlorogenic acid*	40.8 (13.4)	**67.5** (10.5)	62.6 (8.7)	58.7 (2.7)	59.8 (3.0)	**63.9** (15.4)	54.6 (4.6)	5.7 (16.0)	6.2 (5.6)
Ferulic acid	60.2 (11.3)	**74.7** (5.7)	73.9 (6.0)	72.1 (6.8)	**71.1** (6.2)	64.6 (11.0)	61.8 (10.2)	7.1 (15.5)	8.9 (0.9)
Sinapic acid	57.5 (11.1)	70.8 (6.3)	**78.4** (6.7)	74.7 (0.6)	**60.3** (2.2)	55.6 (10.1)	58.0 (7.7)	5.7 (21.8)	6.3 (0.4)
p-Coumaric acid*	74.1 (6.0)	81.0 (9.3)	**83.3** (1.9)	79.5 (2.1)	**85.6** (3.1)	75.4 (6.2)	72.1 (4.3)	9.9 (16.4)	12.4 (4.4)
Gallic acid	14.3 (19.9)	13.7 (6.8)	9.8 (13.3)	**12.9** (10.9)	**43.3** (7.5)	22.0 (5.4)	9.9 (13.4)	0.3 (11.1)	0.2 (10.4)
Vanillic acid	34.4 (9.0)	**40.8** (13.6)	36.5 (6.0)	38.5 (3.6)	**37.2** (9.4)	29.0 (15.4)	30.8 (7.6)	2.9 (17.7)	3.6 (7.6)
p-Hydroxybenzoic acid	38.8 (16.4)	**69.9** (13.0)	68.3 (9.4)	66.1 (2.3)	70.5 (9.5)	**73.3** (15.9)	63.1 (5.4)	3.4 (20.4)	4.3 (22.0)
Syringic acid	**34.3** (13.8)	33.2 (16.8)	32.6 (7.0)	31.4 (9.4)	**33.7** (2.7)	30.0 (14.5)	28.4 (0.6)	2.8 (13.9)	4.1 (4.4)
Protocatechuic acid	34.7 (16.2)	45.8 (14.5)	**60.1** (1.3)	46.8 (2.6)	57.7 (11.0)	**63.3** (8.2)	52.0 (5.7)	5.0 (18.6)	6.6 (4.4)
Procyanidin B1*	3.4 (13.0)	1.7 (10.8)	**5.0** (9.6)	1.6 (6.7)	4.9 (12.5)	**10.5** (18.6)	2.6 (11.0)	0.3 (6.0)	0.2 (0.3)
Procyanidin B2*	4.5 (13.0)	6.7 (19.3)	**21.3** (3.1)	9.7 (2.9)	14.2 (12.5)	**25.6** (14.0)	8.4 (4.6)	0.5 (15.3)	0.5 (14.4)
(+) Catechin*	11.8 (9.7)	15.7 (17.7)	**31.7** (5.8)	17.7 (4.6)	33.5 (6.0)	**42.6** (19.3)	21.9 (14.6)	1.5 (17.1)	1.7 (4.0)
(-) Epicatechin*	23.5 (2.8)	32.1 (18.1)	**51.9** (2.0)	37.1 (6.2)	36.4 (6.2)	**54.3** (10.7)	29.0 (13.1)	2.0 (7.9)	2.3 (12.3)
(-) Epigallocatechin gallate	**15.9** (14.1)	0.7 (18.1)	2.7 (17.8)	3.5 (8.9)	**32.5** (16.7)	9.3 (9.3)	2.4 (19.1)	0.0 (16.6)	0.0 (0.2)
(-) Catechin gallate	25.3 (14.2)	26.1 (14.7)	28.4 (16.1)	**32.7** (6.0)	66.7 (10.6)	**67.1** (7.7)	40.4 (2.8)	5.5 (16.2)	7.5 (0.8)
(-) Epicatechin gallate	36.8 (7.9)	33.4 (18.4)	35.7 (14.5)	**43.2** (8.2)	**59.2** (19.5)	57.5 (1.9)	39.6 (12.8)	3.9 (20.1)	4.7 (0.1)
(-)-Gallocatechin	**6.8** (18.4)	0.1 (4.2)	1.1 (12.7)	0.4 (15.9)	**10.3** (4.4)	3.2 (11.9)	0.3 (15.4)	0.0 (8.4)	0.0 (1.7)
Genistein	63.5 (10.7)	**81.4** (13.5)	69.4 (11.2)	76.0 (6.7)	**70.0** (20.0)	69.4 (1.3)	44.6 (0.7)	10.8 (15.9)	12.2 (5.6)
Daidzein	60.7 (10.5)	76.5 (8.9)	**78.6** (10.4)	66.6 (10.1)	**74.7** (13.6)	60.6 (20.1)	51.8 (4.4)	7.5 (19.8)	8.5 (3.5)
Phloridzin*	**69.4** (10.6)	64.8 (7.4)	68.6 (9.3)	63.0 (2.9)	54.5 (6.5)	**60.3** (9.5)	51.2 (0.6)	6.1 (22.6)	6.5 (10.3)
Phloretin*	**44.1** (10.6)	15.0 (20.3)	37.8 (11.6)	37.3 (13.7)	67.7 (17.6)	**75.5** (5.9)	17.7 (10.2)	3.4 (25.5)	7.6 (5.7)
Naringin	57.5 (6.8)	66.0 (3.1)	**70.5** (1.3)	60.1 (1.6)	55.8 (2.0)	**59.2** (9.0)	54.1 (6.2)	8.2 (14.3)	9.0 (15.7)
Naringenin	62.7 (17.2)	62.9 (17.9)	61.1 (14.3)	**77.3** (9.1)	73.2 (14.4)	**78.4** (2.6)	45.0 (6.6)	8.8 (14.6)	12.5 (13.4)
Hesperetin	66.5 (20.6)	58.1 (18.7)	62.5 (4.2)	**67.3** (7.7)	**86.3** (16.8)	61.3 (5.3)	11.2 (21.0)	0.3 (18.0)	0.5 (17.2)
Hesperidin	44.6 (4.7)	74.1 (9.1)	**77.7** (3.5)	66.2 (12.6)	**68.0** (2.1)	64.7 (5.8)	58.8 (2.1)	10.7 (18.4)	12.7 (3.0)
Luteolin	**98.2** (7.7)	58.8 (11.4)	26.2 (20.2)	79.1 (4.2)	**76.3** (14.7)	66.1 (7.4)	49.8 (10.5)	5.4 (18.3)	8.8 (6.3)
Apigenin	61.4 (15.2)	**71.8** (13.8)	29.6 (16.8)	82.3 (7.2)	**84.3** (18.2)	45.3 (2.7)	43.6 (8.5)	5.1 (13.9)	6.9 (7.7)
Myricetin	**25.5** (15.5)	0.4 (17.4)	0.2 (12.7)	0.8 (17.6)	**42.1** (15.5)	1.2 (16.9)	0.7 (12.2)	0.0 (32.1)	0.0 (23.4)
Kaempferol	41.7 (17.8)	49.0 (13.6)	30.3 (18.1)	**55.2** (9.0)	**73.7** (15.9)	62.2 (12.9)	27.2 (9.3)	1.0 (24.9)	1.2 (3.7)
Kaempferol-3-glucoside	**46.0** (16.0)	31.4 (8.2)	36.9 (3.1)	34.5 (6.4)	15.5 (9.4)	14.2 (18.5)	**16.7** (5.2)	0.6 (12.2)	0.7 (10.1)
Quercetin*	55.5 (12.3)	63.6 (14.1)	60.2 (13.9)	**71.2** (2.0)	**54.3** (3.6)	48.7 (1.2)	41.4 (4.1)	7.1 (12.0)	3.8 (12.1)
Quercetin-3-glucoside/galactoside*	71.0 (5.9)	72.0 (8.0)	**75.2** (4.5)	66.3 (6.5)	67.3 (8.7)	**68.5** (3.4)	55.4 (9.1)	8.9 (18.0)	9.8 (3.8)
Quercetin-3-rhamnoside*	79.7 (6.4)	89.9 (6.3)	**94.2** (4.2)	82.5 (7.1)	79.8 (2.0)	**81.1** (3.5)	69.2 (7.5)	14.6 (16.3)	15.5 (7.9)
Quercetin-3-rutinoside* (Rutin)	53.6 (3.5)	69.7 (11.1)	**71.9** (4.6)	66.0 (4.4)	63.8 (6.7)	58.1 (7.9)	**67.0** (7.6)	8.8 (16.3)	9.2 (0.8)
Quercetin-3-glucuronide*	57.1 (7.5)	**84.8** (13.8)	81.1 (1.9)	77.5 (4.5)	**73.3** (4.6)	60.7 (6.6)	62.7 (1.5)	9.1 (17.3)	8.8 (2.1)
Quercetin-3'-glucuronide*	60.7 (15.1)	**87.3** (14.7)	83.3 (4.7)	77.4 (2.3)	**72.0** (7.1)	57.5 (0.4)	63.8 (2.)	10.4 (12.3)	10.6 (0.7)
Isorhamnetin*	93.2 (9.4)	30.2 (23.5)	27.1 (10.1)	**99.6** (19.5)	**73.9** (5.4)	59.9 (0.3)	56.0 (1.4)	2.1 (12.3)	1.6 (21.0)
Isorhamnetin-3-glucoside*	77.8 (3.7)	85.8 (9.0)	**89.7** (7.6)	78.6 (5.9)	77.7 (7.4)	**77.9** (8.6)	71.2 (7.2)	15.7 (20.3)	16.1 (6.0)
Isorhamnetin-3-glucuronide*	55.9 (3.9)	78.7 (16.0)	**79.1** (4.2)	74.5 (6.0)	**92.3** (14.2)	79.9 (4.0)	75.9 (8.6)	10.3 (16.3)	10.0 (0.6)

Table 2.9 **Mean percentage recoveries (n = 3) of compounds using plasma extraction methods J - L (% CV). Compounds known to be present in apples or known metabolites of apples are shown by an asterix. Greatest recoveries are shown in bold.**

Standard	Method		
	J	K	L
Caffeic acid*	87.6 (16.4)	82.5 (8.2)	**98.2** (8.0)
Chlorogenic acid*	110.4 (9.4)	131.1 (5.2)	**138.2** (1.9)
Ferulic acid	99.4 (13.6)	104.7 (3.7)	**122.7** (9.7)
Sinapic acid	88.7 (15.1)	88.7 (5.4)	**109.0** (10.4)
p-Coumaric acid*	112.1 (12.6)	101.6 (5.5)	**117.6** (7.8)
Gallic acid	53.4 (11.4)	7.8 (20.1)	**65.8** (7.7)
Vanillic acid	85.6 (20.4)	96.7 (2.7)	**113.5** (6.2)
p-Hydroxybenzoic acid	80.4 (9.3)	79.7 (9.6)	**90.8** (19.2)
Syringic acid	83.7 (18.9)	76.6 (2.3)	**97.7** (7.3)
Protocatechuic acid	77.7 (11.5)	88.8 (4.3)	**100.6** (5.8)
Procyanidin B1*	5.3 (17.2)	**58.3** (27.3)	29.6 (26.7)
Procyanidin B2*	13.1 (2.7)	**56.5** (18.9)	32.4 (17.2)
(+) Catechin*	26.9 (16.5)	**90.9** (6.6)	89.5 (7.0)
(-) Epicatechin*	16.7 (2.2)	**99.2** (20.3)	92.1 (16.9)
(-) Epigallocatechin gallate	15.6 (5.0)	32.5 (0.7)	**48.8** (9.9)
(-) Catechin gallate	27.7 (13.7)	**99.8** (15.0)	64.6 (6.2)
(-) Epicatechin gallate	25.4 (13.3)	**89.0** (13.5)	60.8 (3.6)
(-)-Gallocatechin	10.3 (11.4)	3.2 (2.1)	**39.3** (0.8)
Genistein	97.7 (10.4)	**149.2** (24.6)	107.5 (19.1)
Daidzein	86.2 (15.2)	109.3 (20.8)	**112.5** (16.3)
Phloridzin*	101.7 (10.4)	**123.2** (13.9)	114.8 (13.5)
Phloretin*	67.7 (21.2)	**121.7** (18.2)	80.9 (2.7)
Naringin	82.9 (16.0)	103.6 (1.6)	**113.4** (6.1)
Naringenin	98.9 (13.8)	**139.2** (24.4)	105.8 (24.8)
Hesperetin	**65.3** (14.4)	58.8 (22.2)	63.7 (11.6)
Hesperidin	96.3 (14.6)	123.3 (3.5)	**135.6** (2.6)
Luteolin	**86.0** (13.1)	68.7 (17.9)	77.5 (4.4)
Apigenin	89.6 (17.2)	82.6 (15.7)	**97.6** (27.8)
Myricetin	**34.1** (17.4)	0.4 (4.9)	30.5 (3.9)
Kaempferol	**68.1** (9.1)	55.6 (40.6)	67.4 (48.3)
Kaempferol-3-glucoside	49.7 (19.7)	45.3 (5.7)	**61.6** (17.5)
Quercetin*	**76.1** (13.3)	40.6 (10.7)	58.0 (2.2)
Quercetin-3-glucoside/galactoside*	122.1 (11.3)	119.1 (7.8)	**130.0** (4.4)
Quercetin-3-rhamnoside*	116.0 (9.2)	124.6 (8.9)	**128.7** (9.5)
Quercetin-3-rutinoside* (Rutin)	102.5 (11.4)	120.7 (2.9)	**131.1** (3.3)
Quercetin-3-glucuronide*	101.3 (23.3)	109.5 (10.6)	**125.8** (10.2)
Quercetin-3'-glucuronide*	96.5 (12.4)	99.3 (18.2)	**104.7** (3.1)
Isorhamnetin*	**81.2** (22.5)	48.5 (12.5)	67.0 (9.3)
Isorhamnetin-3-glucoside*	122.9 (12.0)	127.7 (7.5)	**130.4** (7.1)

2.4.5 Validation of the Methods for the Extraction of Polyphenols from Human Urine and Plasma

Values for linearity showed that for the concentrations under investigation, the response of the MS was on the whole proportional to the concentration of the analyte in the sample. Calibration graphs constructed from 'spiked' urine samples, for three of the polyphenols in the mixture are shown in Figure 2.8. Values for the linearity of the MS response to polyphenol standards in the urine were all above 0.99, with the exception of two polyphenols (Table 2.10). In plasma, the responses were not as linear as for the urine, but R^2 values were generally above 0.97 (Table 2.11). As 'blank' samples for both urine and plasma were baseline volunteer samples and not synthetically prepared matrices, they contained some endogenous levels of the compounds under investigation; this explains an intercept value of greater than zero.

Values for the recovery of polyphenols from the urine, was generally above 85 % and below 102 %, with a few exceptions. Recoveries from plasma were higher; generally between 92 and 120 %, with a few exceptions below and above these values. Values for recovery were generally better at the higher concentration compared with the low concentration. Evaluation of so many polyphenols using just one extraction method results in a loss of recovery of certain compounds to benefit the increased extraction of others. Mostly, literature presents methodology for the extraction of one class of polyphenol (for example catechins [57, 74, 77]) or even a specific member of one class, for example the reporting of the conjugates produced from (-) epicatechin [81, 97] or quercetin [23].

Matrix effects were investigated in both urine and plasma; the percentages calculated represent the proportion of the actual response observed compared to the expected response if the matrix was blank. For example, a value for the matrix effect in urine of -3.8 % as in the case of chlorogenic acid, indicates that only 96.2 % of the expected MS response for that particular polyphenol has been observed; there is 3.8 % suppression is due to other components in the matrix. Where a negative value is quoted, this represents a decreased MS response, and where a positive value is given it represents signal enhancement, where the matrix components have either been suppressed to allow the polyphenol to dominate or the matrix components have improved the degree of ionisation of the polyphenol, producing a better signal. In general, mostly ion suppression is observed. For those values which are not calculable, it is due to a high endogenous level of the polyphenol (or something with the same mass pair and retention

time), which obscures the results. In some cases, the endogenous level is greater than the lowest concentration used for the validation procedures.

Variation of the MS response for the same polyphenol in the same sample matrix on the same day should be minimal, if the method is precise. In urine, interday precision was generally less than 20 % for the lowest concentration (except for kaempferol, 22.1 %), and mostly below 15 % for the medium and high concentrations. Day to day variation of the MS response for the same polyphenols in the same sample matrix is greater, although intraday precision in urine was also generally less than 20 % for the lowest concentration, and below 15 % for the medium and high concentrations. However, there were a few more exceptions than noted for interday precision. Accuracy for interday experiments was generally between 85 and 100 %, with a few values falling below or above this range. Intraday accuracy was generally between 95 and 110 %. For plasma, interday and intraday values for precision were generally below 20 % for the lowest concentration and below 15 % for the high concentration, except for a few of the polyphenol standards. Interday and intraday accuracy values were generally between 80 and 110 %. Values for the effect of ion suppression show that the greatest effects were noticed at the lower concentrations, as expected.

The ability to report values within universally accepted ranges for some of the key parameters required for validation demonstrates that a working method has been developed; this method can be applied to both urine and plasma samples from humans. Although the purpose of the method was to identify some of the major metabolites of apples in humans, the method may also be successful for determining metabolites from consumption of other foods and beverages, indicating a much wider application.

Few studies documented in the literature contain any validation data, especially for those studies investigating polyphenols and their metabolites in biological fluids. Ito *et al.* [80], report validation of their method to quantify polyphenol levels in human urine after the intake of six different polyphenol-rich beverages. Sixteen polyphenol standards belonging to the hydroxycinnamic acid, hydroxybenzoic acid, flavonol, flavanone and dihydrochalcone classes were shown to have good linearity (r^2 0.986 - 0.999) in concentrations ranging from 0.5 to 50 μM (not all polyphenol standards were validated over the same concentration range). Accuracy values varied from -4.1 to 3.2 % and intraday and interday variabilities were lower than 10.1 and 10.3 %, respectively. Roura *et al.* [81], report partial validation of their method

to quantify (-) epicatechin metabolites in human plasma after ingestion of cocoa. Linearity was good (r^2 0.999) between concentrations of 15 and 300 μg/L 'spiked' into plasma, however precision and accuracy values were only obtained for variation in retention times. Recovery from 'spiking' into water was 93.5 %, whereas recovery from the blank human plasma was 71 %. The lower recovery in the matrix was hypothesized to be caused by the complexity of the matrix interfering with the ionisation of (-) epicatechin metabolites and/or interactions of phenols with plasma components such as protein, which can occur during the process of extraction.

One article which combines partial validation for both urine and plasma, shows the lower sensitivity achieved from 'spiking' polyphenol standards into plasma compared to urine; again this can be attributed to matrix components [76]. Intraday and interday variations are below 10.7 % for all three catechins measured, although only one concentration appears to have been investigated, and this is not stated. Recovery for (-) epicatechin from plasma was 79 – 91 % and from urine was 90 – 106 %. One disadvantage of the paper is the deconjugation of the urine and plasma samples using enzymes to cleave the conjugates, so information on the metabolites is not provided. Validation reported by Urpi-Sarda *et al.*, [105] for microbial metabolites of cocoa after intake in humans and rats uses synthetic urine (which will be consistent throughout analysis, unlike actual urine) and gives values for recovery similar to those established here; e.g. caffeic acid 95 – 109 %, ferulic acid 87.2 - 96.8 %, (-) epicatechin 84 – 90 %. Once again, only values for one concentration are given and the paper concentrates on the simple phenolic acids and breakdown products of the compounds present in cocoa; it does not deal with the more complex conjugates or wider range of polyphenolics in cocoa.

One limitation associated with LC/MS/MS methods such as this, developed for the quantitative analysis of biological samples, is that the biological matrices can affect the ionisation process resulting in either signal suppression or enhancement, as mentioned previously. The change in signal intensity observed is caused by the co-elution of endogenous compounds such as salts, amines, fatty acids, or triglycerides, as not all of these endogenous compounds will have been removed by the sample preparation methods. These effects are strongly compound-dependent, affecting each one differently and are also more pronounced with ESI than with APCI [154]. These effects also alter the efficiency and reproducibility of the analyte ionisation within the ion source and thus may be especially complex when analysing multiple analytes from a biological sample in a single analytical run [94], such as in this case.

Ion suppression effects are usually greater in urine than in plasma, as urine is a highly changeable matrix, varying between individuals and among one individual; the composition of plasma is much more constant.

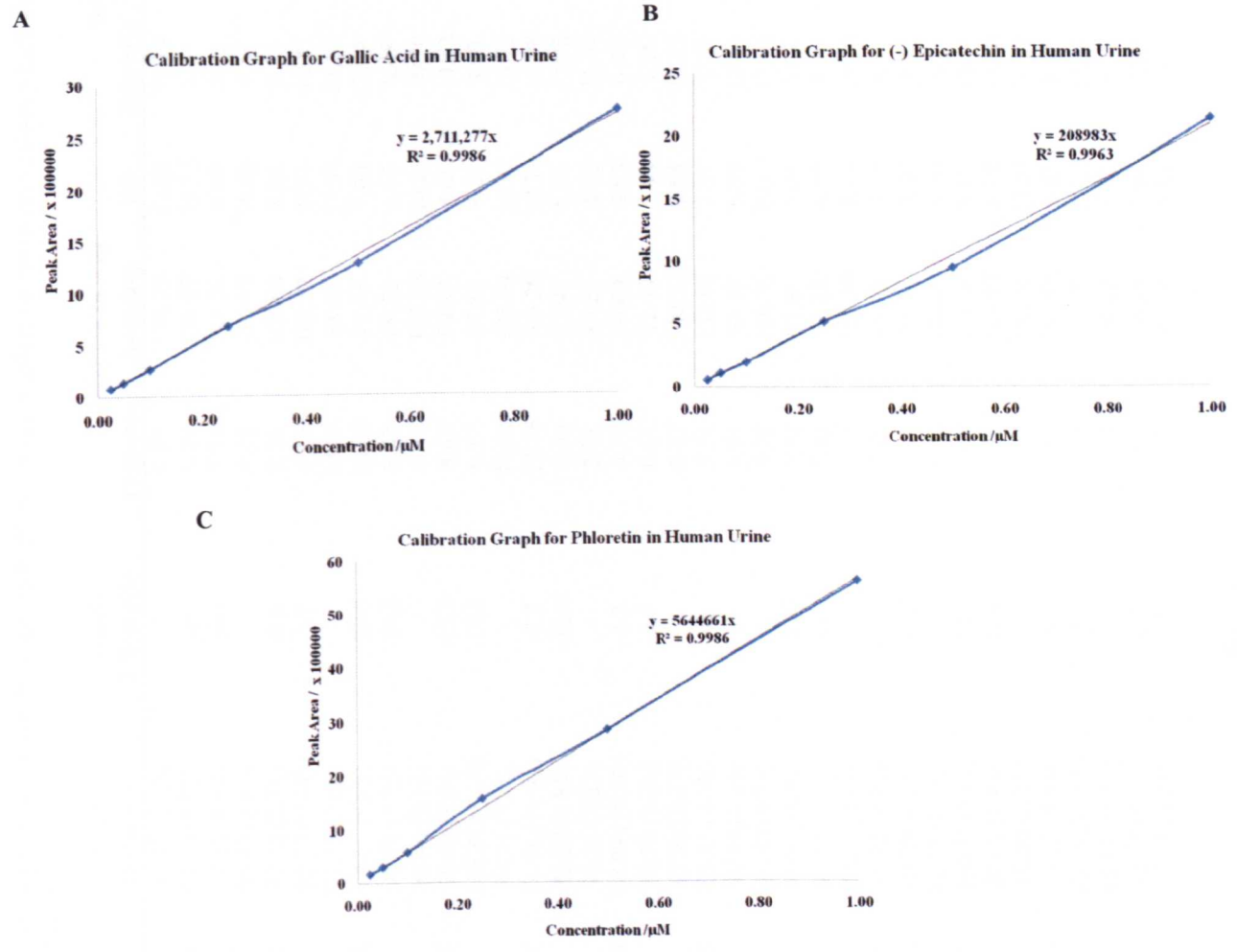

Figure 2.8 **Calibration graphs for the polyphenol standards gallic acid (A), (-) epicatechin (B) and phloretin (C) when 'spiked' into human urine at various concentrations. R^2 values are 0.9989, 0.9963 and 0.9986 for the three polyphenol standards, respectively.**

Table 2.10 **Validation parameters for the analysis of polyphenol standards when 'spiked' into human urine; nc represents values which were not calculable due to high endogenous levels of the polyphenol.**

Standard	Conc. (μM)	Linearity Equation	R^2	% Recovery (SD n = 6)	Matrix Effect (%)	Interday (n = 6) Precision	Accuracy	SD	Intraday (n = 7) Precision	Accuracy	SD
	0.09			48.42 (8.12)	-	9.59	130.35	12.50	39.14	97.36	38.10
Caffeic acid	0.277	y =3211693x + 76128	0.9945	91.87 (5.45)	nc	8.19	126.07	10.33	8.63	105.33	9.09
	0.83			87.11 (8.84)	nc	3.51	116.29	4.08	8.73	103.68	8.16
	0.09			93.53 (8.42)	-	9.98	109.17	10.90	9.35	116.57	10.90
Chlorogenic acid	0.277	y = 3459795x	0.9998	90.40 (2.76)	-3.8	8.26	106.96	8.84	8.81	111.33	9.80
	0.83			92.77 (9.14)	-5.7	6.35	103.52	6.57	15.57	100.98	14.17
	0.09			71.68 (7.00)	-	13.48	88.56	11.94	21.70	94.55	20.52
Ferulic acid	0.277	y =1158947x	0.9968	95.62 (8.93)	-28.0	10.59	71.75	7.60	7.19	105.76	7.61
	0.83			93.78 (3.76)	-24.5	8.82	69.53	6.13	8.48	108.19	8.27
	0.09			86.46 (9.10)	-	9.47	108.25	10.25	10.20	76.54	7.80
Sinapic acid	0.277	y = 448597x	0.9940	90.50 (8.64)	-11.3	8.67	99.89	8.66	14.43	84.01	12.12
	0.83			103.81 (6.79)	-10.8	6.17	97.84	6.04	8.40	95.65	7.24
	0.09			94.31 (5.41)	-	10.02	105.94	10.62	18.73	94.43	17.68
p-Coumaric acid	0.277	y = 3621413x	0.9974	86.78 (10.93)	-7.2	12.75	100.20	12.78	15.98	97.60	15.60
	0.83			85.28 (6.89)	-12.8	9.00	92.97	8.37	12.78	99.44	11.45
	0.09			93.96 (4.83)	-	5.33	89.22	4.75	11.08	108.03	11.97
Gallic acid	0.277	y = 1934571x	0.9965	88.99 (10.80)	-5.3	5.70	84.69	4.83	6.50	103.06	6.70
	0.83			88.10 (5.21)	-8.8	6.67	81.40	5.43	7.25	98.70	6.45
	0.09			95.93 (8.27)	-	6.82	140.52	9.58	11.18	106.95	11.96
Vanillic acid	0.277	y = 397017x	0.9983	85.81 (4.30)	nc	5.54	132.98	7.37	7.99	106.94	8.54
	0.83			90.15 (6.74)	nc	6.84	93.38	6.39	10.14	103.82	9.48
	0.09			82.80 (9.75)	-	8.98	116.56	10.47	12.63	109.32	13.80
Syringic acid	0.277	y = 793778x	0.9983	90.49 (10.34)	-27.8	5.92	91.81	5.44	7.62	103.33	7.87
	0.83			85.38 (6.72)	-23.9	5.41	91.22	4.93	9.70	103.59	9.05
	0.09			92.29 (4.42)	-	7.44	109.30	8.14	14.16	105.17	14.90
Protocatechuic acid	0.277	y = 8103771x	0.9991	85.80 (4.79)	-14.8	7.76	99.05	7.68	6.66	103.41	6.89
	0.83			89.29 (4.39)	-17.9	5.89	91.72	5.40	8.25	103.01	7.65
	0.09			92.73 (11.25)	-	6.58	122.42	8.06	16.26	99.39	16.16
(+) Catechin	0.277	y = 208915x	0.9751	100.49 (7.05)	-21.7	8.98	101.04	9.07	12.34	91.10	11.24
	0.83			92.48 (2.43)	-14.0	5.78	106.93	6.18	13.46	93.99	11.40
	0.09			85.00 (5.27)	-	11.65	108.46	12.63	11.23	101.60	11.41
(-) Epicatechin	0.277	y = 230599x	0.9954	84.14 (9.00)	-6.2	8.66	101.95	8.83	9.44	100.52	9.49
	0.83			89.75 (10.65)	-3.2	10.59	105.00	11.12	12.36	101.65	11.32
	0.09			96.14 (10.10)	-	5.87	94.87	5.57	9.75	91.99	8.97
(-) EGCG	0.277	y = 1182235x	0.9997	92.63 (6.26)	2.0	5.86	96.95	5.68	7.89	94.94	7.49
	0.83			95.21 (4.92)	3.5	5.63	98.18	5.53	7.63	101.44	6.97

Table 2.10 (cont.) **Validation parameters for the analysis of polyphenol standards when 'spiked' into human urine; nc represents values which were not calculable due to high endogenous levels of the polyphenol.**

Standard	Conc. (μM)	Linearity Equation	R^2	% Recovery (SD n = 6)	Matrix Effect (%)	Interday (n = 6) Precision	Interday (n = 6) Accuracy	Interday (n = 6) SD	Intraday (n = 7) Precision	Intraday (n = 7) Accuracy	Intraday (n = 7) SD
(-) ECG/CG	0.09	y = 3236963x	0.9979	80.68 (8.19)	-	7.52	102.45	7.70	10.44	116.01	12.12
	0.277			81.00 (11.93)	-5.8	12.96	96.78	12.54	10.84	112.11	12.15
	0.83			94.29 (5.16)	-10.3	5.18	91.99	4.77	11.73	115.00	12.15
(-) GC	0.09	y = 182106x	0.9951	113.62 (22.24)	-	17.36	90.20	15.66	21.38	91.96	19.66
	0.277			94.75 (4.45)	4.4	9.14	95.21	8.71	8.93	89.03	7.95
	0.83			92.95 (1.13)	4.7	3.63	94.77	3.44	9.79	91.40	8.06
Genistein	0.09	y =1523219x	0.9979	95.36 (6.03)	-	10.81	91.93	9.94	12.21	119.12	14.55
	0.277			94.61 (6.38)	-8.6	11.67	84.12	9.82	6.90	110.53	7.63
	0.83			110.09 (7.98)	-16.5	6.22	76.80	4.78	12.08	105.90	11.52
Daidzein	0.09	y = 526268x	0.9946	90.19 (13.21)	-	12.87	99.76	12.83	21.21	118.05	25.04
	0.277			83.34 (7.88)	-2.6	10.27	97.62	10.02	8.34	109.15	9.11
	0.83			84.54 (6.75)	-4.6	4.17	95.24	3.97	6.63	106.54	6.37
Phloretin	0.09	y = 6612507x	0.9979	90.88 (10.95)	-	15.77	89.71	14.15	3.00	108.49	3.25
	0.277			91.98 (7.49)	-6.6	9.14	83.84	7.67	6.51	106.83	6.96
	0.83			90.07 (6.77)	-8.7	9.75	81.89	7.98	6.21	102.87	5.76
Naringin	0.09	y = 1261742x	0.9907	94.68 (8.23)	-	8.55	85.86	7.34	11.27	113.83	12.83
	0.277			94.93 (5.71)	-6.9	10.14	80.09	8.12	11.62	112.28	13.04
	0.83			81.81 (9.60)	1.0	11.07	86.70	9.60	11.03	108.74	10.80
Naringenin	0.09	y = 3038045x	0.9975	101.43 (9.59)	-	8.05	80.11	6.45	8.87	114.47	10.15
	0.277			98.35 (6.20)	1.0	8.79	81.37	7.15	6.21	107.14	6.65
	0.83			101.47 (11.04)	-5.1	6.90	76.11	5.25	12.84	99.09	11.46
Hesperetin	0.09	y = 1452930x	0.9931	89.05 (3.45)	-	5.51	97.80	5.37	9.81	114.84	11.27
	0.277			78.59 (7.72)	-5.4	16.88	92.43	15.60	6.85	107.71	7.37
	0.83			88.88 (6.72)	-18.0	4.45	79.98	3.56	5.64	107.37	5.45
Hesperidin	0.09	y = 2731130x	0.9979	73.71 (7.49)	-	13.39	106.38	14.24	22.28	100.44	22.38
	0.277			78.95 (21.46)	-13.3	14.37	92.28	13.26	21.27	109.77	23.35
	0.83			93.50 (9.98)	-22.8	6.35	82.16	5.21	8.31	111.97	8.38
Luteolin	0.09	y = 1672709x	0.9976	91.24 (5.81)	-	3.57	98.45	3.52	9.05	112.39	10.17
	0.277			96.08 (6.28)	-11.0	17.99	87.69	15.78	5.53	107.13	5.92
	0.83			88.29 (4.09)	-12.0	5.90	96.61	5.11	3.72	102.23	3.43
Apigenin	0.09	y = 3772650x	0.9938	93.16 (11.67)	-	7.45	97.00	7.23	7.39	111.41	8.24
	0.277			100.86 (8.74)	-12.7	10.16	84.81	8.62	8.66	106.41	9.22
	0.83			102.31 (12.15)	-17.4	8.44	80.15	6.76	8.43	102.88	7.82
Myricetin	0.09	y = 437947x	0.9990	88.12 (4.09)	-	8.11	91.19	7.40	19.43	90.33	17.55
	0.277			91.26 (5.48)	-3.1	11.18	88.77	9.92	15.37	103.60	15.93
	0.83			85.36 (8.42)	10.8	7.86	101.09	7.94	14.41	115.63	15.01

Table 2.10 (cont.) **Validation parameters for the analysis of polyphenol standards when 'spiked' into human urine; nc represents values which were not calculable due to high endogenous levels of the polyphenol.**

Standard	Conc. (μM)	Linearity Equation	R^2	% Recovery (SD n = 6)	Matrix Effect (%)	Interday (n = 6) Precision	Interday (n = 6) Accuracy	Interday (n = 6) SD	Intraday (n = 7) Precision	Intraday (n = 7) Accuracy	Intraday (n = 7) SD
Quercetin	0.09	y = 496956x	0.9920	81.54 (12.37)	-	10.12	104.68	10.60	8.18	106.39	8.70
	0.277			87.84 (8.16)	-6.5	17.55	98.14	17.22	5.12	111.81	5.72
	0.83			92.08 (8.08)	-14.1	15.35	89.95	13.81	5.71	105.81	5.44
Quercetin-3-glu./gal.	0.09	y = 3832100x	0.9896	64.69 (9.21)	-	7.36	93.52	6.89	8.93	116.72	10.43
	0.277			86.61 (18.95)	-22.4	11.14	72.63	8.09	9.24	110.60	10.22
	0.83			99.20 (9.20)	-38.4	5.02	57.60	2.89	8.56	114.74	8.85
Quercetin-3-rham.	0.09	y = 1334560x	0.9951	92.98 (9.48)	-	9.49	85.76	8.14	19.66	120.19	23.63
	0.277			95.06 (3.33)	-6.0	3.67	80.65	2.96	20.95	116.65	24.44
	0.83			101.43 (12.32)	-8.6	6.93	78.37	5.43	21.93	113.55	22.43
Quercetin-3-rut.	0.09	y = 1951779x	0.9900	72.77 (4.85)	-	9.73	109.28	10.64	19.63	112.73	22.13
	0.277			76.42 (7.13)	-6.4	9.11	102.42	9.34	12.73	112.89	14.37
	0.83			85.35 (12.19)	-22.5	8.79	84.71	7.45	13.53	103.81	12.65
Isorhamnetin	0.09	y = 3783495x	0.9989	82.46 (5.16)	-	6.50	106.19	6.90	4.40	107.57	4.73
	0.277			86.33 (3.90)	-9.4	15.42	96.35	14.86	10.03	106.59	10.69
	0.83			83.47 (8.82)	-16.4	6.83	88.78	6.06	7.16	100.45	6.48
Isorhamnetin-3-glu.	0.09	y = 1585423x	0.9919	87.48 (10.26)	-	7.70	106.49	8.20	7.53	117.81	8.87
	0.277			87.72 (6.17)	-9.8	11.08	96.15	10.65	9.73	112.43	10.94
	0.83			81.67 (4.25)	-11.7	6.86	94.01	6.45	5.48	103.81	5.12
Phloridzin	0.09	y = 1328618x	0.994	93.43 (8.18)	-	8.57	89.00	7.62	5.40	119.01	6.42
	0.277			79.10 (11.20)	-0.4	6.75	88.75	5.99	10.76	113.18	12.18
	0.83			81.35 (4.12)	-2.3	9.18	86.98	7.98	13.80	110.32	13.72
Kaempferol	0.09	y = 224310x	0.9970	91.29 (7.07)	-	9.61	95.75	9.20	11.74	95.73	11.24
	0.277			90.55 (9.53)	-0.4	22.09	95.48	21.09	10.53	94.85	9.99
	0.83			100.16 (9.48)	-3.3	11.91	92.62	11.03	10.99	97.41	9.64

Table 2.11 **Validation parameters for the analysis of polyphenol standards when 'spiked' into human plasma; nc represents values which were not calculable due to high endogenous levels of the polyphenol.**

Standard	Conc. (μM)	Linearity Equation	R^2	% Recovery (SD n = 6)	Matrix Effects (%)	Interday (n = 6) Precision	Accuracy	SD	Intraday (n = 7) Precision	Accuracy	SD
	0.05			92.10 (7.06)	-	8.05	85.70	6.90	1.94	96.04	1.86
Caffeic acid	0.1	y = 1423622x	0.9799		nc	5.03	92.59	4.65	4.15	96.79	4.01
	0.2			114.67 (12.44)	nc	2.06	104.24	1.94	1.02	100.98	1.03
	0.05			108.63 (7.90)	-	9.12	80.91	7.38	9.85	89.76	8.85
Chlorogenic acid	0.1	y = 9167685x	0.9796		-8.0	10.12	88.60	8.97	2.86	96.82	2.77
	0.2			113.56 (8.26)	-6.6	2.52	106.30	2.41	1.03	102.32	1.05
	0.05			137.65 (16.54)	-	19.47	48.74	9.49	5.24	100.90	5.29
Ferulic acid	0.1	y = 550049x + 4782	0.9788		nc	3.05	96.73	2.95	1.94	100.90	1.96
	0.2			121.50 (14.81)	nc	2.59	111.83	2.61	0.79	99.82	0.79
	0.05			141.29 (36.01)	-	9.63	89.11	8.58	14.25	90.06	12.83
Sinapic acid	0.1	y = 263918x	0.9871		-15.3	5.01	96.08	4.81	20.14	93.37	18.80
	0.2			127.98 (11.73)	1.7	2.27	103.65	2.12	16.12	91.29	14.72
	0.05			129.23 (12.19)	-	7.27	99.85	7.26	9.13	105.49	9.63
p-Coumaric acid	0.1	y = 1502814x + 2965	0.9829		nc	4.93	97.64	4.81	6.21	96.66	6.00
	0.2			126.26 (6.50)	nc	1.74	101.91	1.60	2.73	99.77	2.72
	0.05			12.54 (4.96)	-	33.41	47.12	15.74	3.23	55.46	1.79
Gallic acid	0.1	y = 782472x	0.9347		-6.1	5.95	87.97	5.23	4.69	93.23	4.37
	0.2			49.74 (9.87)	-2.4	2.53	109.51	2.50	0.92	105.99	0.98
	0.05			119.22 (13.42)	-	17.82	99.27	17.69	3.30	110.24	3.64
Vanillic acid	0.1	y = 125208x + 6573	0.9797		nc	6.56	93.46	6.13	7.06	102.74	7.25
	0.2			113.44 (4.41)	nc	3.11	102.23	2.87	1.80	98.05	1.77
	0.05			141.54 (17.78)	-	12.35	94.20	11.64	12.06	105.84	12.76
Syringic acid	0.1	y = 401491x + 360	0.9953		nc	10.46	91.72	9.59	13.47	98.19	13.22
	0.2			135.51 (3.78)	nc	11.26	95.65	9.70	2.59	98.62	2.55
	0.05			114.37 (15.48)	-	7.22	87.22	6.30	6.39	92.67	5.92
Protocatechuic acid	0.1	y = 3968306x	0.9727		-17.0	4.80	93.14	4.47	7.28	98.50	7.18
	0.2			125.79 (10.04)	-27.1	2.07	104.07	1.94	1.40	101.10	1.41
	0.05			82.46 (13.85)	-	24.95	71.31	17.79	33.12	76.26	25.25
(+) Catechin	0.1	y = 59669x	0.9731		-37.9	9.40	93.90	8.83	34.44	92.80	31.96
	0.2			108.39 (16.92)	-40.9	3.76	105.01	3.55	16.36	96.47	15.78
	0.05			103.63 (8.59)	-	14.90	71.54	10.66	3.15	81.76	2.58
(-) Epicatechin	0.1	y = 118492x	0.9529		-47.6	6.74	88.09	5.94	3.08	93.33	2.88
	0.2			119.15 (6.91)	-58.0	2.98	107.09	2.87	0.85	103.29	0.88
	0.05			8.18 (1.16)	-	7.03	102.87	7.24	2.90	35.50	1.03
(-) EGCG	0.1	y = 106539x	0.8218		-4.6	5.92	103.65	6.14	1.95	87.45	1.71
	0.2			66.56 (8.08)	7.5	3.92	120.58	4.25	1.02	123.55	1.27

Table 2.11 (cont.) **Validation parameters for the analysis of polyphenol standards when 'spiked' into human plasma; nc represents values which were not calculable due to high endogenous levels of the polyphenol.**

Standard	Conc. (μM)	Linearity Equation	R^2	% Recovery (SD n = 6)	Matrix Effects (%)	Interday (n = 6) Precision	Accuracy	SD	Intraday (n = 7) Precision	Accuracy	SD
	0.05			82.57 (5.94)	-	10.48	78.48	8.22	18.23	52.64	9.60
(-) ECG/CG	0.1	y = 298798x	0.9054		-18.1	11.37	78.64	8.94	8.22	82.73	6.80
	0.2			144.16 (20.44)	-5.6	8.69	103.60	8.11	2.08	108.58	2.26
	0.05			53.00 (14.24)	-	60.67	24.12	14.63	42.20	21.43	9.05
(-) GC	0.1	y = 42372x	0.9267		nc	30.71	60.22	18.49	11.78	53.67	6.32
	0.2			98.96 (15.58)	nc	3.31	117.87	3.51	0.92	118.72	1.09
	0.05			174.88 (18.80)	-	41.61	45.63	18.99	2.33	78.21	1.82
Genistein	0.1	y = 492753x	0.9947		-9.3	9.09	99.98	9.09	8.33	95.03	7.92
	0.2			161.11 (11.55)	-13.0	2.11	111.73	2.13	2.75	102.57	2.82
	0.05			157.79 (21.26)	-	10.20	91.78	9.36	6.42	82.41	5.29
Daidzein	0.1	y = 221488x	0.9959		-9.2	9.76	100.12	9.77	2.18	92.32	2.01
	0.2			154.61 (38.98)	-18.7	0.99	103.76	0.93	0.88	104.09	0.92
	0.05			178.63 (19.09)	-	16.60	71.26	11.83	11.47	68.23	7.82
Phloretin	0.1	y = 1735744x	0.9723		-3.6	11.14	93.07	10.37	13.23	78.15	10.34
	0.2			163.77 (36.28)	-12.2	2.68	107.07	2.59	3.02	108.06	3.27
	0.05			193.16 (35.10)	-	7.78	83.16	6.47	3.91	97.49	3.81
Naringin	0.1	y = 533813x	0.9877		-2.0	8.54	102.45	8.75	16.01	103.33	16.54
	0.2			153.96 (25.11)	13.2	2.43	105.35	2.30	4.32	99.26	4.29
	0.05			225.60 (24.31)	-	15.60	93.49	14.59	13.70	82.57	11.32
Naringenin	0.1	y = 1035560x	0.9868		-30.4	8.75	99.10	8.67	12.49	100.64	12.57
	0.2			167.71 (21.23)	-21.6	2.18	104.65	2.06	3.92	101.06	3.96
	0.05			21.12 (5.10)	-	12.61	88.17	11.12	11.06	11.72	1.30
Hesperetin	0.1	y = 377567x	0.9100		-15.0	16.98	84.21	14.30	17.29	66.37	11.47
	0.2			90.47 (6.25)	-21.1	2.24	106.93	2.16	2.45	115.64	2.84
	0.05			66.24 (6.75)	-	11.29	79.84	9.01	6.26	74.22	4.65
Hesperidin	0.1	y = 1649165x	0.9543		0.2	5.41	98.16	5.31	5.69	99.24	5.64
	0.2			93.33 (14.48)	18.7	1.48	105.28	1.41	1.87	99.65	1.86
	0.05			71.15 (7.34)	-	18.02	99.42	17.92	8.01	77.73	6.23
Luteolin	0.1	y = 549124x	0.9759		-8.1	10.01	90.26	9.03	8.02	82.31	6.60
	0.2			77.00 (5.56)	-13.9	4.40	101.92	4.04	1.81	106.48	1.93
	0.05			57.08 (6.75)	-	55.64	32.01	17.81	12.04	84.00	10.11
Apigenin	0.1	y = 866525x	0.9744		-10.5	8.43	91.42	7.71	8.35	79.48	6.63
	0.2			53.01 (7.99)	-7.9	1.90	113.89	1.95	1.78	106.53	1.90
	0.05			25.70 (12.06)	-	54.06	53.50	28.92	67.83	75.85	28.69
Quercetin	0.1	y = 127661x	0.9023		17.9	14.08	89.21	12.56	20.18	92.92	18.75
	0.2			84.62 (12.11)	33.1	6.92	110.46	6.88	6.71	103.98	6.97

Table 2.11 (cont.) **Validation parameters for the analysis of polyphenol standards when 'spiked' into human plasma; values for the matrix effect represent the percentage of MS response observed compared to the MS response expected, negative values represent a decrease to the expected response and nc represents values which were not calculable due to high endogenous levels of the polyphenol.**

Standard	Conc. (μM)	Linearity Equation	R^2	% Recovery (SD n = 6)	Matrix Effects (%)	Interday (n = 6) Precision	Interday (n = 6) Accuracy	Interday (n = 6) SD	Intraday (n = 7) Precision	Intraday (n = 7) Accuracy	Intraday (n = 7) SD
Quercetin-3-glu./gal.	0.05	y = 2151808x	0.9761	113.30 (13.85)	-	7.98	100.55	8.02	9.50	88.95	8.45
	0.1				-10.9	8.27	92.96	7.69	13.75	92.07	12.65
	0.2			120.45 (12.98)	-12.0	2.02	102.00	1.85	3.40	101.92	3.46
Quercetin-3-rham.	0.05	y = 806083x	0.9866	125.52 (18.15)	-	5.48	99.36	5.44	17.65	88.05	15.54
	0.1				-15.5	7.53	97.41	7.33	7.84	102.79	8.06
	0.2			149.70 (14.94)	-25.0	1.96	103.33	1.82	2.91	100.2	2.91
Quercetin-3-rut.	0.05	y = 1401471x	0.9842	163.39 (40.19)	-	8.13	99.18	8.07	19.72	99.99	19.72
	0.1				-31.4	13.79	87.81	12.11	15.09	97.56	14.72
	0.2			139.39 (20.13)	-25.4	4.40	104.23	4.13	1.44	100.37	1.45
Isorhamnetin	0.05	y = 1218441x	0.9287	55.67 (11.51)	-	18.29	87.13	15.94	16.09	45.62	7.34
	0.1				-8.6	15.56	84.08	13.08	2.28	78.89	1.80
	0.2			115.11 (15.40)	-9.7	4.57	104.79	4.31	0.01	110.28	0.01
Isorhamnetin-3-glu.	0.05	y = 1179713x	0.9905	124.65 (19.88)	-	11.45	100.62	11.53	4.70	105.31	4.95
	0.1				-4.5	7.38	94.14	6.95	18.71	90.84	17.00
	0.2			139.59 (10.01)	-13.2	2.88	102.53	2.66	7.20	104.34	7.51
Phloridzin	0.05	y = 780806x	0.9905	164.87 (16.69)	-	8.47	93.72	7.94	1.28	82.73	1.06
	0.1				-11.5	4.96	94.37	4.68	3.57	90.40	3.23
	0.2			138.21 (14.32)	-5.8	2.35	103.44	2.19	0.80	103.77	0.83
Kaempferol	0.05	y = 56345x	0.9444	104.13 (26.48)	-	15.80	95.70	15.12	30.36	65.84	19.99
	0.1				-15.8	8.76	88.00	7.71	6.97	77.00	5.36
	0.2			126.91 (27.99)	-14.2	4.39	102.41	4.05	2.41	108.75	2.62

2.4.6 LC/MS/MS – Preliminary Analysis of Polyphenols in Biological Matrices

Enzyme Treatment of Non-Study Urine

Extracted ion chromatograms of enzyme treated and control urine were compared and tentative identities were assigned to the peaks in the chromatogram. Initial experiments were performed to see if a working method had been developed and to see if metabolites could be detected/identified. These results, a complete study of the structures of the flavonoid classes/likely fragmentation patterns and information on any apple, cocoa and tea metabolites reported in human urine and plasma in the literature, helped to construct a list of possible apple polyphenol metabolites; consisted of around 400 possibilities. These mass transitions were divided into three methods, to increase sensitivity for each mass transition.

Human Intervention Study Pooled Urine Samples

The enzyme treatment of non-study urine was repeated with pooled urine samples from the human intervention study. Figure 2.9 shows the extracted ion chromatograms from the analysis of control and test pooled urine, with and without enzyme treatment. Analysis of the pooled 0–24 h control and 0–24 h test urine samples from the human intervention study, gave preliminary information on the mass transitions that would be detected in the individual urine samples and extracted plasma samples. Comparison of the two sets of pooled samples also indicated which mass transitions were going to show the largest differences between the control and the test samples, i.e. those of greatest interest. Results from the pooled samples allowed the LC/MS/MS MRM method of over 400 mass transition possibilities to be refined to a smaller number of mass transitions (Tables 2.12, 2.13 and 2.14) for the analysis of the individual urine (and subsequent plasma) samples from the human intervention study. Table 2.12 details mass transitions from the polyphenol standard mix which showed significant increases in the test urine samples compared to the control urine samples. Table 2.13 details mass transitions from possible conjugated metabolites and Table 2.14 details mass transitions from possible ring fission metabolites. The method containing all these MRM transitions was applied to the urine and plasma samples from the human intervention study. Enhanced Product Ion (EPI) spectra were also acquired for the mass transitions of interest in the urine and plasma (this is explained in chapter 3). Comparison of retention times and the fragment ions produced for the standards, identities were proposed for the analytes (all detailed in chapter 3).

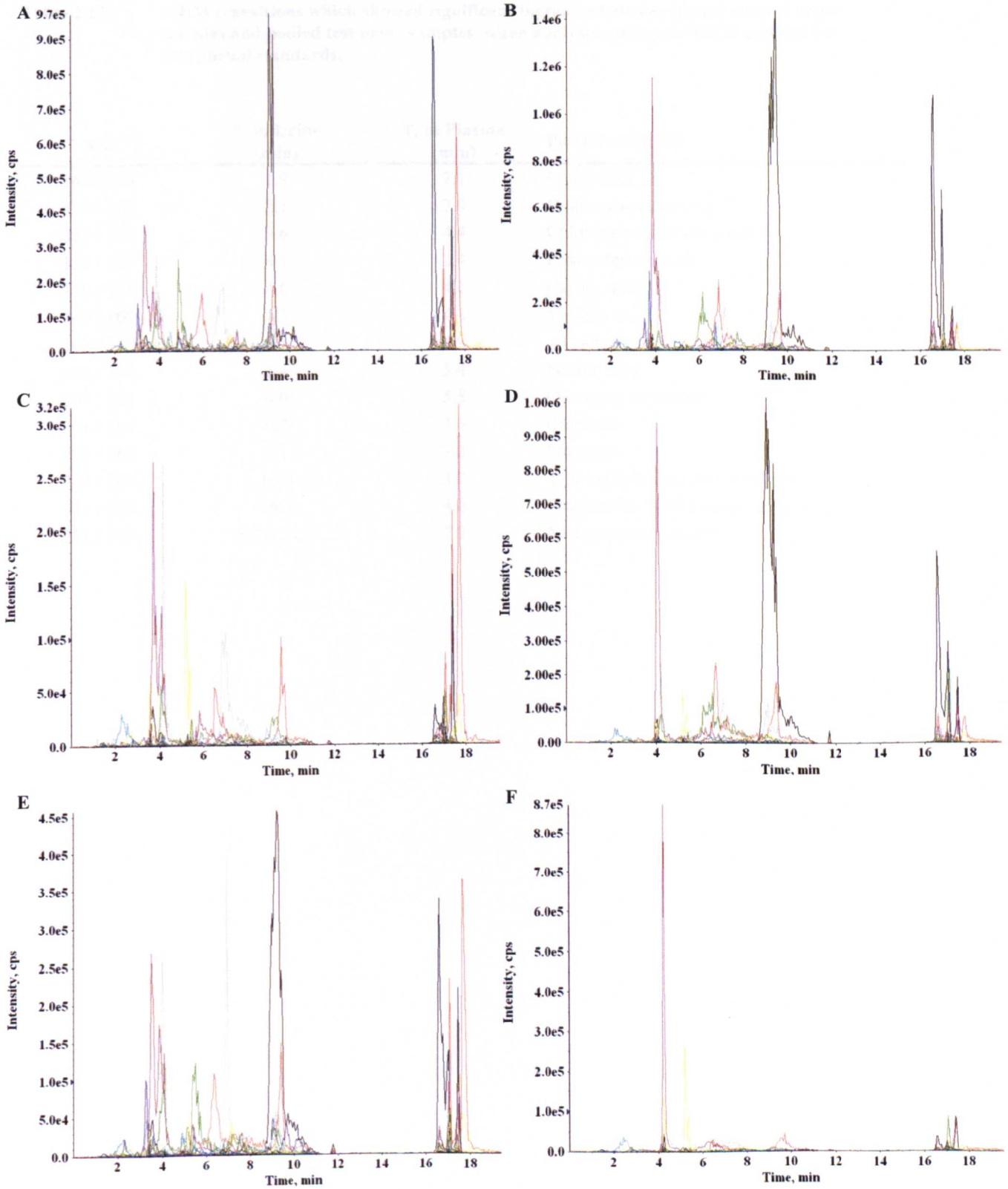

Figure 2.9 **Extracted ion chromatograms of pooled 0–24 h control urine, no enzyme treatment (A) and 0–24 h test urine, no enzyme treatment (B); 0–24 h control urine after treatment with sulfatase (C) and 0–24 h test urine after treatment with sulfatase (D); 0–24 h control urine after treatment with β-glucuronidase (E) and 0–24 h test urine after treatment with β-glucuronidase (F).**

Table 2.12 **MRM transitions which showed significant increases between pooled control urine samples and pooled test urine samples, when analysed using the MRM method for polyphenol standards.**

m/z	T_r in Urine (min)	T_r in Plasma (min)	Possible Identity
169 / 125	1.9	7.1	Gallic acid
137 / 108	2.3	2.0	Hydroxybenzoic acid
353 / 191	3.6	4.4	Chlorogenic acid conjugate
153 / 109	4.1	2.4	Protocatechuic acid
179 / 135	4.6	4.1	Caffeic acid
167 / 108	4.3	4.3	Vanillic acid
163 / 119	5.1	4.9	*p*-Coumaric acid
193 / 134	5.4	5.4	Ferulic acid
609 / 301	6.0	5.8	Quercetin rutinoside
301 / 107	6.7	7.6	Quercetin
273 / 167	7.1	7.2	Phloretin
289 / 109	16.0	3.7	(-)/(+) (Epi)/Catechin conjugate
577 / 289	16.1	4.0	Procyanidin B1/B2 sulfate conjugate
271 / 119	7.2 & 16.9	7.2	Naringenin conjuagte

Table 2.13 **MRM transitions and possible identities of conjugated metabolites which showed significant increase between pooled control urine samples and pooled test urine samples. – indicates no peak detected.**

m/z	T_r in Urine (min)	T_r in Plasma (min)	Possible Identity	Loss of...
194 / 179	8.4	5.6	Methylated caffeic acid	CH_3
259 / 179	8.6	17.0 – 17.8	Caffeic acid sulfate	SO_3
274 / 194	3.9	17.0	Methylated caffeic acid sulfate	SO_3
368 / 353	-	5.4	Methylated chlorogenic acid	CH_3
383 / 368	16.4	-	Dimethylated chlorogenic acid	CH_3
705 / 529	1.4	1.3	Chlorogenic acid diglucuronide	glucuronide
705 / 353	1.4	1.2	Chlorogenic acid diglucuronide	2 x glucuronide
544 / 368	5.4	19.5	Methylated chlorogenic acid glucuronide	glucuronide
609 / 433	1.4	2.5	Chlorogenic acid glucuronide sulfate	glucuronide
208 / 193	7.5	9.2	Methylated ferulic acid	CH_3
223 / 208	5.5	5.5	Dimethylated ferulic acid	CH_3
369 / 193	4.6	-	Ferulic acid glucuronide	glucuronide
545 / 369	10.5	1.3	Ferulic acid diglucuronide	glucuronide
273 / 208	16.0	6.1	Methylated ferulic acid sulfate	SO_3
384 / 369	16.5	18.8	Methylated ferulic acid glucuronide	CH_3
384 / 208	5.1	17.5	Methylated ferulic acid glucuronide	glucuronide
384 / 193	6.6	9.8	Methylated ferulic acid glucuronide	CH_3 & glucuronide
178 / 163	17.2	6.0	Methyl coumaric acid	CH_3
339 / 163	7.2	10.5	Coumaric acid glucuronide	glucuronide
515 / 339	1.5	1.3	Coumaric acid diglucuronide	glucuronide
264 / 169	16.0	1.5	Methylated gallic acid sulfate	CH_3 & SO_3
345 / 169	10.5	3.1	Gallic acid glucuronide	glucuronide
425 / 345	5.0	17.1	Gallic acid glucuronide sulfate	SO_3
182 / 167	1.7	-	Methylated vanillic acid	CH_3
197 / 182	4.6	4.6	Demethylated vanillic acid	CH_3
197 / 167	4.6	4.6	Dimethylated vanillic acid	2 x CH_3
247 / 167	6.2	16.9	Vanillic acid sulfate	SO_3
262 / 182	8.2	4.6	Methylated vanillic acid sulfate	SO_3
423 / 247	10.5	6.0	Vanillic acid glucuronide sulfate	glucuronide
233 / 153	5.6	17.0	Protocatechuic acid sulfate	SO_3
313 / 233	17.0	17.1	Protocatechuic acid disulfate	SO_3
168 / 153	4.3	7.7	Methylated protocatechuic acid	CH_3
248 / 168	4.9	3.8	Methylated protocatechuic acid sulfate	SO_3
248 / 153	4.9	19.8	Methylated protocatechuic acid sulfate	CH_3 & SO_3
409 / 233	1.3	17.4	Protocatechuic acid glucuronide sulfate	glucuronide

Table 2.13 (cont.) **MRM transitions and possible identities of conjugated metabolites which showed significant increase between pooled control urine samples and pooled test urine samples. – indicates no peak detected.**

m/z	T_r in Urine (min)	T_r in Plasma (min)	Possible Identity	Loss of...
369 / 289	8.5	17.0	(Epi)/Catechin sulfate	SO_3
465 / 289	4.0	5.2	(Epi)/Catechin glucuronide	glucuronide
449 / 369	9.7	17.5	(Epi)/Catechin disulfate	SO_3
449 / 289	8.4	3.7	(Epi)/Catechin disulfate	2 x SO_3
384 / 304	9.9	17.1	Methylated (epi)/catechin sulfate	SO_3
384 / 289	16.4	2.7	Methylated (epi)/catechin sulfate	CH_3 & SO_3
480 / 304	4.9	18.2	Methylated (epi)/catechin glucuronide	glucuronide
545 / 289	10.5	1.2	(Epi)/Catechin glucuronide sulfate	SO_3 & glucuronide
449 / 273	7.5	1.3	Phloretin glucuronide	glucuronide
433 / 353	16.4	17.4	Phloretin disulfate	SO_3
625 / 449	8.1	1.4	Phloretin diglucuronide	glucuronide
368 / 288	16.0	17.0	Methylated phloretin sulfate	SO_3
464 / 288	9.5	1.3	Methylated phloretin glucuronide	glucuronide
529 / 449	4.5	1.3	Phloretin sulfate glucuronide	SO_3
529 / 353	4.0	1.2	Phloretin sulfate glucuronide	glucuronide
529 / 273	4.2	1.2	Phloretin sulfate glucuronide	SO_3 & glucuronide
737 / 481	16.5	1.2	Phloridzin sulfate glucuronide	SO_3 & glucuronide
653 / 477	9.5	1.3	Quercetin glucuronide	glucuronide
653 / 301	18.3	17.4	Quercetin glucuronide	2 x glucuronide
667 / 653	1.3	1.3	Methyl quercetin diglucuronide	CH_3
667 / 477	1.27	0	Methyl quercetin diglucuronide	glucuronide
667 / 301	1.8	5.9	Methyl quercetin diglucuronide	CH_3 & glucuronide
557 / 477	19.1	1.3	Quercetin sulfate glucuronide	SO_3
557 / 381	19.3	1.3	Quercetin sulfate glucuronide	glucuronide
557 / 301	19.2	8.3	Quercetin sulfate glucuronide	SO_3 & glucuronide
639 / 463	1.2	1.2	Quercetin glucoside glucuronide	glucuronide
639 / 301	1.3	5.3	Quercetin glucoside glucuronide	glucoside
543 / 463	16.9	1.8	Quercetin glucoside sulfate	SO_3
543 / 381	16.9	1.4	Quercetin glucoside sulfate	glucoside
543 / 301	16.9	2.2	Quercetin glucoside sulfate	SO_3 & glucoside
557 / 477	19.1	1.3	Quercetin glucuronide sulfate	SO_3
557 / 381	19.2	1.3	Quercetin glucuronide sulfate	glucuronide
557 / 301	19.2	8.3	Quercetin glucuronide sulfate	SO_3 & glucuronide
491 / 315	10.9	1.4	Isorhamnetin glucuronide	glucuronide
491 / 301	2.4	4.9	Isorhamnetin glucuronide	CH_3 & glucuronide
381 / 301	17.2	17.0	Quercetin sulfate	SO_3

Table 2.14 **MRM transitions and possible identities of ring fission metabolites which showed significant increase between pooled control urine samples and pooled test urine samples. – indicates no peak detected.**

m/z	T_r in Urine (min)	T_r in Plasma (min)	Possible Identity	Possible Product
289 / 207	17.7	3.5	(-)/(+) (Epi)/Catechin	δ (3,4 dihydroxyphenyl) γ valerolactone
289 / 163	17.7	3.0	(-)/(+) (Epi)/Catechin	Coumaric acid
207 / 163	16.0	17.6	δ (3,4 dihydroxyphenyl) γ valerolactone	Coumaric acid
383 / 207	5.2	5.6	Dimethylated chlorogenic acid	δ (3,4 dihydroxyphenyl) γ valerolactone
383 / 163	5.2	10.7	Dimethylated chlorogenic acid	Coumaric acid
287 / 207	16.9	17.1	δ (3,4 dihydroxyphenyl) γ valerolactone sulfate	δ (3,4 dihydroxyphenyl) γ valerolactone
287 / 163	17.7	3.1	δ (3,4 dihydroxyphenyl) γ valerolactone sulfate	Coumaric acid
367 / 207	16.1	1.5	δ (3,4 dihydroxyphenyl) γ valerolactone disulfate	δ (3,4 dihydroxyphenyl) γ valerolactone
367 / 163	16.1	1.2	δ (3,4 dihydroxyphenyl) γ valerolactone disulfate	Coumaric acid
463 / 383	17.2	18.6	δ (3,4 dihydroxyphenyl) γ valerolactone glucuronide sulfate	δ (3,4 dihydroxyphenyl) γ valerolactone glucuronide
463 / 207	17.1	1.3	δ (3,4 dihydroxyphenyl) γ valerolactone glucuronide sulfate	δ (3,4 dihydroxyphenyl) γ valerolactone
463 / 287	17.0	9.4	δ (3,4 dihydroxyphenyl) γ valerolactone glucuronide sulfate	δ (3,4 dihydroxyphenyl) γ valerolactone sulfate
194 / 100	2.7	1.4	Hydroxyhippuric acid	?
289 / 165	15.9	3.0	(-)/(+) (Epi)/Catechin	Hydroxyphenylpropionic acid
166 / 90	18.1	17.3	Hydroxyphenylpropionic acid / Hydroxyphenylhydracrylic acid	?
166 / 107	17.2	8.6	Hydroxyphenylpropionic acid / Hydroxyphenylhydracrylic acid	?
223 / 109	19.0	5.5	Sinapic acid	Catechol
193 / 121	6.8	7.8	Ferulic acid	Ethylphenol
163 / 109	17.6	2.8	Coumaric acid	Catechol
353 / 179	5.2	4.3	Chlorogenic acid	Caffeic acid
289 / 125	17.5	4.3	(Epi)/Catechin	Phloroglucinol
289 / 109	17.4	4.2	(Epi)/Catechin	catechol
289 / 124	16.1	17.1	(Epi)/Catechin	A ring fragment
273 / 151	8.2	1.4	Phloretin	Hydroxyphenylacetic acid
273 / 125	8.2	7.2	Phloretin	Phloroglucinol
481 / 207	5.1	9.5	Phloridzin	δ (3,4 dihydroxyphenyl) γ valerolactone
218 / 138	7.4	17.1	A ring fission product sulfated	A ring
221 / 207	4.3	1.6	δ (4 hydroxy 3 methoxyphenyl) γ valerolactone	Valerolactone
224 / 207	-	8.5	Trihydroxyphenyl γ valerolactone	Valerolactone
400 / 224	5.4	3.3	Trihydroxyphenyl γ valerolactone glucuronide	Trihydroxyphenyl γ valerolactone

2.5 Conclusions

The LC/MS/MS and sample preparation methods developed herein represent a significant step forward in the timely analysis of biological samples for conjugated and ring fission metabolites of apple polyphenols. Although the literature contains articles on the analysis of different varieties of apples and apple products, such as juice and cider, there are no comprehensive methods to provide identification for the full range of metabolites produced in human biological fluids after consumption of these materials. Thus, there is an absence of quantitative information on the metabolism and bioavailability of polyphenols consumed from apples in healthy humans. To develop a complex method which can detect not only polyphenol conjugates but also products of bacterial action in the gut, and their conjugates is a significant achievement. The short analysis time required to perform this task is also a unique aspect of this novel method. This method may also be applied for the analysis of polyphenols in biological fluids after consumption of other foods and beverages, showing wider applications.

In summary, this chapter details the process of development of an LC/MS/MS method to detect polyphenols and their metabolites (conjugated, ring fission and ring fission conjugates) in the urine and plasma of humans. Preparation methods for human urine and plasma have also been reported, and more importantly validated. Validation supports the fact that this method is reproducible, reliable, sensitive and accurate for the purpose.

Chapter Three

Identification of Apple Polyphenol Metabolites in Human Urine and Plasma

Summary

The following chapter details the process of identification of some of the major polyphenolic metabolites, detected in human urine and plasma after the consumption of the control apple juice and high polyphenol cider apple extract (test phase). Identification involved comparing the retention times and EPI spectra of unknown metabolites with polyphenol standards and available information in the literature. Using the LC/MS/MS method detailed in chapter 2, conjugated, ring fission, conjugated ring fission and aglycone metabolites of apple polyphenols have been detected in urine and plasma; identities have been proposed for these metabolites. Time course data for these metabolites will be provided in chapter 4.

3.1 Introduction

Human intervention studies, using patients and healthy volunteers, have predominately focussed on the metabolism of tea, cocoa and onions; identifying polyphenolic aglycones (after deconjugation with enzymes) and conjugated metabolites. Recent research [83] has started to consider the identification of ring fission metabolites of polyphenols (and their conjugates), which are produced by the microflora present in the human gastrointestinal tract. Literature on the extent of microbial action on polyphenols is scarce, but there is a requirement for this information to enable a more comprehensive understanding of the metabolism of these reported 'health promoting compounds'.

There are four reports of apple metabolism in humans in the literature which use LC/MS/MS techniques for analysis; Marks *et al.* [72], DuPont *et al.* [87] and Kahle *et al.* [8, 99] None of these studies report any validation data. Using ileostomy patients, Kahle *et al.* [8] report data on the quantity of polyphenols reaching the colon after consumption of apple juice, in their paper published in 2005. They follow the time course of the excretion of hydroxycinnamic acid derivatives, dihydrochalcones, (-) epicatechin and quercetin-3-rhamnoside in ileostomy fluid, and also compare the quantities of hydroxycinnamic acids, flavanols and flavonols in the cloudy apple juice and in the ileostomy fluid. All compounds detected in biological fluids were already present in the cloudy apple juice ingested; only one new metabolite was reported; phloretin glucuronide. Biokinetic data reported is discussed in chapter 4. Work published by DuPont *et al.* [87], identified small amounts of methylquercetin glucuronide and caffeic acid in plasma and hippuric acid and phloretin in urine, after the consumption of alcoholic cider in healthy human volunteers. A limited amount of quantitative data is also reported for these metabolites and is discussed in chapter 4.

The paper published by Kahle *et al.* [99] in 2007, analysed the samples collected from their previous human study (using ileostomy patients) by LC/ESI/MS/MS. The aims of this paper were to report on new polyphenol metabolites present in the ileostomy fluid; six new metabolites were identified in addition to the detection of phloretin. Fragmentation patterns were provided for each of these new metabolites, as were time courses for their excretion in ileostomy fluid (details given in chapter 4). D-(-) quinic acid was identified as the most abundant metabolite, followed by 3-caffeoylquinic acid and other hydroxycinnamic acid derivatives. Trace amounts of phloretin and phloretin-2′-glucuronide were also detected.

Marks *et al.* [72], published a paper on the absorption, metabolism and excretion of dihydrochalcones in healthy human volunteers and ileostomy patients, after the consumption of cider. Plasma, ileal fluid and urine were all analysed by LC/MS/MS in negative ionisation mode. Six metabolites of phloretin were identified (two sulfates, three glucuronides and one sulfoglucuronide); retention times and fragmentation patterns were given. All metabolites identified were present in the ileal fluid, four were present in the urine (the three glucuronides and the sulfoglucuronide) and only one was found in the plasma; phloretin-2′-*O*-glucuronide. Phloretin-2′-glucuronide was identified as the major metabolite in urine also. In ileal fluid, the metabolites detected were phloretin glucuronides, phloretin sulfoglucuronide and phloretin sulfates. Two glycosides of phloretin present in cider were also detected in the ileal fluid; phloretin-2′-*O*-(2″-*O*-xylosyl) glucoside and phloretin-*O*-(*O*-xylosyl)hexoside. Another major phloretin glycoside found in cider, phloretin-2′-*O*-glucoside, was absent from ileal fluid suggesting it is more readily absorbed, than the previously mentioned glycosides.

Although literature reporting the identification of the metabolites of apple polyphenols found in human urine and plasma is limited, information on the metabolites of some of the apple polyphenols which are also found in tea, cocoa and onions can provide some details on possible metabolites. Studies concerning the metabolism and fate of polyphenols from tea, both black and green, dominate the literature. Tea contains a high proportion of flavanols, including (-) epicatechin, (-) epigallocatechin (EGC), (-) epigallocatechin gallate (EGCG) and (-) epicatechin-3-gallate (ECG). Of these compounds, (-) epicatechin is the only one found in apples and apple products, and so information on its metabolism established from tea studies can be evaluated to aid identification of (-) epicatechin metabolites produced post apple consumption.

Urinary metabolites have been reported more frequently than metabolites in plasma, presumably because a greater volume of urine is produced and the analytes are present in higher concentrations. (-) Epicatechin metabolites identified in human urine post tea ingestion, include (-) epicatechin glucuronide [73, 77], (-) epicatechin sulfate(s) [73, 76, 77, 169], methyl (-) epicatechin sulfate(s) [73, 77, 169] and methyl (-) epicatechin glucuronide(s) [73, 76, 169]. Research by Lee *et al.* [76], reports than (-) epicatechin from tea was found exclusively in the conjugated form in the urine; sulfate and glucuronide forms were present at a 2:1 ratio, but no structural conformations are proposed. In general, the absence of synthetic standards for all

possible metabolites has resulted in the proposal of tentative structural conformations, however, (-) epicatechin-3′-glucuronide has been reported to be the major urinary glucuronide of (-) epicatechin in humans [73]. Conjugation with a sulfate on the 3′ and 4′ positions has also been reported to be most likely conformation of (-) epicatechin sulfates detected [73]. The major methylated metabolites of (-) epicatechin in humans have been detailed as 4′-*O*-methyl-epicatechin and 3′-*O*-methyl-epicatechin, with the former being the most abundant one [73]. Methylated and glucuronidated epicatechin has also been described, with three possible conformations; 4′-*O*-methyl-epicatechin-3′-glucuronide, 4′-*O*-methyl-epicatechin-5-glucuronide and 4′-*O*-methyl-epicatechin-7-glucuronide [73]. Research carried out by Meng *et al.* [74] identified some methylated and ring fission products of tea catechins, in human urine. Several metabolites were identified, but only one related to the (-) epicatechin consumed from the tea; this was the ring fission metabolite 5- (3′, 4′-dihydroxyphenyl) -γ- valerolactone. Maximum levels of this metabolite were identified in the 3–6 h urine sample. Four volunteers were involved in this study, each consuming the tea solids as their initial phase and pure EGCG as their second phase, but only one volunteer was given a high dose of EGCG, EGC or (-) epicatechin.

In plasma, Lee *et al.* [76], found (-) epicatechin from tea present exclusively in the conjugated form in the plasma, with approximately two thirds sulfate and one third glucuronide. No details on structural conformations are given, and no details on possible ring fission metabolites are explained. Van Het Hof *et al.* [57], determined the bioavailability of tea catechins (including (-) epicatechin) in the blood of healthy human volunteers, after consumption of black and green tea, with and without milk (biokinetic data discussed in chapter 4). No conjugated catechin metabolites or ring fission metabolites were considered in this paper; only compounds measured in the original tea preparations were identified. Therefore, this work does not give a complete account of the bioavailability of these flavanols.

Similarly to tea, flavanols are found in high proportions in cocoa. Studies investigating the metabolism of (-) epicatechin from cocoa can also have relevance to the metabolism of (-) epicatechin from apples. Previously identified urinary metabolites post cocoa consumption include (-) epicatechin sulfate(s) [81, 83, 106], (-) epicatechin-3′-glucuronide [81, 106], epicatechin glucuronide(s) [83], methyl epicatechin sulfate(s) [81, 83, 106], methyl epicatechin glucuronide(s) [81, 83] and methyl epicatechin sulfoglucuronide [81]; the same metabolites detected after tea

consumption, with the addition of the methyl sulfoglucuronide. Non-methylated sulfate, glucuronide and sulfoglucuronide forms were present in the highest concentrations [81, 162]. Previously identified plasma metabolites post cocoa consumption include (-) epicatechin glucuronide [81], (-) epicatechin sulfate [106] and methyl epicatechin sulfate [106]; the latter methylated conjugate has not been reported in the plasma after consumption of tea. Mullen *et al.* [106], reported that additional metabolites seen after the consumption of green tea, were detected in samples after cocoa consumption, but since the (-) epicatechin intakes were higher in the green tea studies, the levels of these additional metabolites were too low to quantify. Structural elucidation work carried out by Urpi-Sarda *et al.* [83], indicated that (-) epicatechin glucuronidation occurred at the 7 and 3′ positions whereas sulfation occurred at the 7 or 5 positions.

Natsume *et al.* [97], performed structural elucidation work on the conjugates of (-) epicatechin, and reported that 3′-methyl-epicatechin, 4′-methyl-epicatechin-5 or 7-glucuronide and 4′-methyl-epicatechin-3′-glucuronide were present in human urine after intake of an (-) epicatechin preparation. The authors did not detect the presence of any sulfate conjugates, contrary to other literature. (-) Epicatechin-3′-glucuronide was also shown to be present in human plasma after ingestion of this flavanol preparation.

Three articles in the literature detail the identification of microbial metabolites of polyphenols, in urine, after the consumption of chocolate [82] and cocoa [83, 105] by healthy human volunteers. Compounds identified include: dihydroxyphenylvalerolactone*, methoxy hydroxyphenylvalerolactone, 3,4-dihydroxyphenylpropionic acid*, 3-hydroxyphenylpropionic acid*, 4-hydroxyphenylpropionic acid, *m*-coumaric acid, *p*-coumaric acid*, caffeic acid*, ferulic acid*, 3,4-dihydroxyphenylacetic acid*, 3-methoxy-4-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid*, protocatechuic acid*, phenylacetic acid*, vanillic acid*, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid*, 3-hydroxyhippuric acid*, 4-hydroxyhippuric acid* and hippuric acid. Compounds marked with an asterix were also reported to be present in plasma [83]. In the recent publication by Urpi-Sarda *et al.* [83], conjugates of some of these ring fission products were also identified, which is the first report of metabolites of this kind. In urine, glucuronide and sulfate conjugates of dihydroxyphenylvalerolactone and methoxy hydroxyphenylvalerolactone were identified; in plasma only the glucuronide conjugates were present.

Apples contain quercetin glycosides; human intervention studies performed with onions which also contain quercetin glycosides can be used to extrapolate some information on how these polyphenols are metabolised, although the precise glycoside profiles are different in the two food stuffs. Mullen *et al.* [23], reported the presence of the following quercetin metabolites in the plasma of volunteers post ingestion of fried onions; quercetin diglucuronide, quercetin sulfoglucuronide, quercetin-3-glucuronide, quercetin-3′-glucuronide, quercetin-3′-sulfate, quercetin sulfate, isorhamnetin-3-glucuronide and isorhamnetin-4′-glucuronide, in addition to the aglycone. Urinary metabolites included quercetin diglucuronides, quercetin-3-glucuronide, quercetin-3′-glucuronide, quercetin-4′-glucuronide, methyl quercetin diglucuronides, quercetin glucoside glucuronides, quercetin glucoside sulfates, quercetin-3′-sulfate, quercetin sulfoglucuronides, isohamnetin-3-glucuronide and isorhamnetin-4′-glucuronide. Some pharmacokinetic data are given in this paper, which is reported and discussed in chapter 4.

To summarise, literature available on the metabolism of apple polyphenols in healthy humans is very limited. However, information on the metabolism of the polyphenols in apples, from other food sources, can be used as a guide to the identification of novel apple metabolites. Absolute identification of metabolites was not possible, because of a lack of synthetic standards. Using the literature, information on available standards, retention times and fragmentation patterns of unknowns, tentative identities were proposed for the analytes detected in urine and plasma.

Aim of the Chapter

To identify apple polyphenol metabolites in human urine and plasma samples, using the LC/MS/MS method developed in Chapter 2.

3.2 Materials and Methods

3.2.1 The Human Dietary Intervention Study

A human dietary intervention study can provide valuable *in vivo* data on metabolism. This dietary intervention study was approved by the Human Research Governance Committee (HRGC) of the Institute of Food Research (IFR) and the Norfolk Research Ethics Committee (NREC). All documentation to support the approval for this research and the Protocol are supplied on CD with this thesis, and were been written and submitted by myself.

Volunteer Recruitment

Volunteers were recruited from the database held at the Human Nutrition Unit (HNU). In total 81 people were sent the Volunteer Information Sheet and letter of invitation. Interested individuals contacted study personnel for an interview. 20 people attended a one to one interview with myself, the Chief Investigator, to explain the study in person. In total, sixteen interested participants were given an appointment for a screening test. This comprised of a urine dipstick test, a health questionnaire (including height, weight, BMI, blood pressure and pulse rate) and a blood sample, tested for total blood count, fasted glucose level and kidney function. Volunteers whose screening test results were within standard ranges and the study inclusion criteria were given study day appointments. All screening results were sent with a letter to the volunteer's GP. Volunteers excluded on grounds of results outside of normal ranges were informed and advised to contact their GP for an appointment, if appropriate.

The Intervention

Coressence Ltd. cultivates high yielding apple varieties that have specific flavan-3-ol profiles. These Evesse^{TM} apples were freeze dried to produce apple granules which were then taken through an extraction procedure to produce the test material Evesse^{TM} Fructose (referred to as the high polyphenol cider apple extract). Simply, this extraction involved adding water (at 100 °C) to the granules and placing in a water bath for 15 min, shaking at timed intervals. Once cooled, the extract was filtered and the aqueous portion retained. This was subjected to various filtration procedures and a stage to reduce the extract to 70 % of its original volume. The extract was buffered to pH 3.5 with solid ascorbic acid, before being frozen for use.

Approximately 60 or 70 g (depending on the batch) of defrosted high polyphenol cider apple extract was dissolved into 250 mL of a supermarket apple juice (Tesco Pure Apple Juice, batch: 8048 1909) to provide one serving of the test drink. Water replaced the apple extract, for the control phase of the study. Exact weights of test material and test drinks were recorded. The drink was served in a coloured glass to disguise the red colour of the concentrate when added to the apple juice (Figure 3.1).

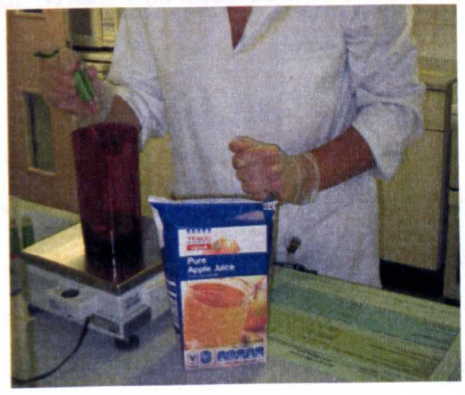

Figure 3.1 **Test drink preparation by an HNU Nurse, involving weighing 60 or 70 g of the high polyphenol cider apple extract and dissolving into 250 mL of 'Tesco' pure apple juice.**

Ten apparently healthy male volunteers aged between 39 and 60 (with BMI values of between 21 and 29) participated in this study. The volunteers followed a low polyphenol diet absent of certain fruits and vegetables, tea, coffee, chocolate, apples and alcoholic drinks for 48 h prior to the intervention and for 24 h after. Controlling polyphenol intake prior to the intervention will reduce the complexity of polyphenols from other dietary sources affecting the metabolism of the test material. Volunteers arrived fasted and baseline urine samples were collected before volunteers were cannulated. A baseline blood sample was taken (20 mL into Lithium Heparin tubes). 4 ml was prepared and sent to BUPA for cholesterol analysis and the remainder was immediately centrifuged at 3000 g for 10 min. 1 ml aliquots of the plasma were decanted into cryovials which were immediately frozen using liquid nitrogen and stored at -80 °C until analysis.

The volunteer was given breakfast (two slices of toast with butter) followed by one of the test drinks. Test drink preparation was carried out by the diet cooks at the HNU, as explained above. A new carton of 'Tesco' pure apple juice was opened for each volunteer and 6 vials (approx. 60 g weighted exactly) of the high polyphenol cider apple extract (batch 1, expiry date: 20.08.08) or 7 vials (approx. 70 g weighed exactly) of the high polyphenol cider apple extract (batch 2, expiry date: 09.10.08) were removed from -20 °C storage approximately 30 min before required. [Analysis of the second batch of the apple extract indicated that 7 vials would be necessary to provide the same concentration of (-) epicatechin as the first batch]. For control test drinks, 60 g or 70 g of water was used to replace the apple extract. 190 g of the 'Tesco' apple juice was used to make up the required volume of test drink. The drink was stirred to aid dissolution. Volunteers consumed the drink within 3 min; once the drink had been consumed by the volunteer, the empty glass weight was recorded to give the actual

weight of test drink consumed. Water was allowed ad libitum, two hours after consumption of the test drink.

Blood samples (10 ml) were collected at selected time-points after test drink consumption (20, 30, 40 and 60 min, then every half an hour until 4 h post dose (when lunch was provided), and then every hour until 8 h post dose). Lunch consisted of a sandwich, either tuna and mayonnaise or cheese, and natural yoghurt with honey. Blood samples were collected into Lith. Hep. vacutainers, and processed as for the baseline sample. All urine produced after consumption of the test drink was collected (0-4 h, 4-8 h and 8-24 h) into containers containing 1 ml of 4M HCl. After collection, an appropriate amount of HCl was added to the urine to give a pH lower than 4.0. The total volume of urine produced was recorded and three 20 ml portions were frozen at – 80 °C. Following a wash-out period of at least two weeks, the same volunteers were given the alternative test drink and the same blood and urine measurements were obtained.

3.2.2 Analysis of the 'Tesco' Pure Apple Juice and High Polyphenol Cider Apple Extract

Analyses of the 'Tesco' Pure Apple Juice, the high polyphenol cider apple extract and the test drinks were carried out by Christina Moyle, at IFR. Triplicate 300 µl aliquots of apple juice were diluted with 700 µl MeOH and 50 µl of internal standard. Galangin was used as an internal standard (1 mg/ml in MeOH). Samples were centrifuged at 1300 g for 10 min, and the supernatant filtered using a 0.22 µm Whatman filter. The filtrate was portioned into two vials. Each filtrate was analysed using two HPLC methods [170]: The first method involved RP-HPLC with UV DAD and the second NP-HPLC with Fluorescence Detection. Once the required weight of apple juice was removed from the carton on the morning of the study day, a 20 ml sample was taken and stored at 4 °C until analyis. This procedure was repeated three times.

Triplicate 100 µl aliquots of defrosted high polyphenol cider apple extract were diluted using 900 µl MeOH and 50 µl of galangin (0.1 mg/ml in MeOH). Samples were prepared as for the 'Tesco' Pure Apple Juice, and analysed with the same two methods. Two batches of the high polyphenol cider apple extract were required for the study, each was analysed on receipt for polyphenol content.

190 ul of 'Tesco' Pure Apple Juice was mixed with 60 ul of the high polyphenol cider apple extract, and triplicate 100 µl aliquots were diluted using 900 µl MeOH and 50 µl of galangin (0.1 mg/ml in MeOH). Samples were prepared as for the 'Tesco' Pure Apple Juice, and analysed with the same two methods.

3.2.3 LC/MS/MS Methodology

Development of the methodology for the analysis of the human urine and plasma samples is discussed in chapter 2. In summary the mobile phases used were 0.01 % formic acid in H_2O and MeCN, initial flow rate was 0.225 µl/min, increasing to 0.850 µl/min at 11.1 min for 2 min, and the column temperature was 65 °C. The gradient was as follows (0, 10), (4, 40), (5, 46), (8, 48), (8.1, 100), (13.1, 100), (17, 0) and (17.1, 10) (time in min, % B). Urine and plasma samples were analysed using the MRM method detailed in chapter 2 (Tables 2.12 – 2.14). In total, 115 mass transitions were monitored for in each and every sample. Analyst produced values for the peak areas for each of these transitions and those mass transitions showing the greatest increases, when test sample data were compared to control sample data, were investigated further.

The advantages of using a hybrid triple quadrupole linear ion trap MS for the identification of metabolites in plasma and urine, include MS^3 capabilities, multiple scan functions and more informative spectra. Triple quadrupole scan functions such as precursor ion scan, product ion and constant neutral loss are maintained, along with and in combination with the trap scan modes [171]. The ion trap can hold more ions than a conventional 3D ion trap, reducing space charge effects and leading to better spectra which can be regarded with more confidence in determining identification [172]. Not only do these hybrid instruments possess the capacity for greater ion storage, but they are also attributed with a higher trapping efficiency. The first quadrupole selects the precursor ion of interest; this is then accelerated into the pressurized collision cell (Q2), which induces fragmentation. Fragment ions are transmitted into the third quadrupole, acting as the linear ion trap, where they are mass selectively scanned out towards the detector, and at the same time ions are accumulated. This is known as an enhanced product ion (EPI) scan, and is unique to the Q TRAP instrument.

Enhanced Product Ion Spectra (EPI)

Functions regarded as 'enhanced' refer to the Q3 acting as a linear ion trap [171]. In a quadrupole collision cell (Q2), ions undergo multiple collisions and the fragment ions formed

become reactivated and undergo further fragmentation. In a 3D ion trap, fragmentation occurs from excitation of the precursor ion only; product ions are generally too cool to undergo further fragmentation. In a Q TRAP in the EPI mode, the precursor ion selected for in Q1 is fragmented in Q2 and subsequent fragmentation is isolated within the ion trap, giving the next generation of precursor ions. This produces a full spectrum for the fragmentation of the original precursor ion selected for in Q1.

Summary of Identification Strategy

The strategy employed for the identification of some of the major metabolites of apple polyphenols in human urine and plasma was derived from a series of experiments. Firstly, mass transitions showing the greatest increases between control samples and test samples were investigated in more detail; comparisons were made using peak area measurements. EPI spectra were generated and mass transitions with the same retention times were compared to eliminate the likelihood of more than one mass transition representing the same analyte. Tentative identifications of the metabolites were made from retention times and spectral fragments (comparisons were made with standards were appropriate), in both urine and plasma. Time course data for these metabolites is presented in chapter 4.

3.3 Results and Discussion

3.3.1 Analysis of the 'Tesco' Pure Apple Juice and High Polyphenol Cider Apple Extract

Table 3.1 details the average mg of phenolics per litre for the 'Tesco' Pure Apple Juice and the high polyphenol cider apple extract. Tables 3.2 and 3.3 show the quantity of phenolics ingested by the individual volunteers after consumption of the control drink and test drink, respectively. The test drink supplied over six times the amount of chlorogenic acid, forty-nine times the amount of phloretin, eleven times the amount of (-) epicatechin and forty-one times the amount of quercetin (as glycosides), compared to the control drink.

Table 3.1 **Comparison of polyphenol content between the average mg of phenolics per litre of the 'Tesco' Pure Apple Juice and the high polyphenol cider apple extract – nd idicates compound was not detected**

Compound	Mean (mg/phenolics)	
	Tesco Apple Juice	High Polyphenol Extract
Chlorogenic acid	72.13	1433.39
Phloretin	3.10	565.33
(-) Epicatechin	25.98	1120.79
Quercetin rutinoside	0.27	22.82
Quercetin-3-glucoside	0.53	120.27
Quercetin-3-galactoside	0.72	39.08
Quercetin-3-xyloside	nd	20.99
Quercetin-3-rhamnoside	nd	37.46
Total	**82.73**	**3360.13**

3.3.2 MS Fragmentation Pathways of Polyphenol Standards

MS allows identification of unknowns by observation of the fragment ions produced when a compound enters the MS. When collision induced dissociation (CID) was performed on polyphenol standards in the MS, common fragments were observed within the classes and sometimes across the classes. The presence of functional groups on the molecules, such as hydroxyl or methyl moieties, carboxylic acids or glycosides impact the characteristic way in which polyphenols fragment. The position and degree of hydroxylation, methylation and conjugation also affect the fragmentation. Figure 3.2 suggests five main fragmentation pathways for the CID of flavonoids.

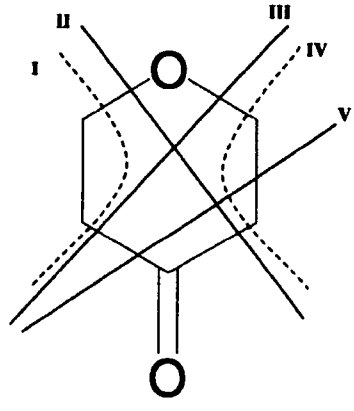

Figure 3.2 **Schematic representation of five common fragmentation pathways observed for the CID of flavonoids Hughes *et al.* [149]**

Table 3.2 **Quantity of phenolics ingested by individual volunteers after consuming the control drink (Tesco Pure Apple juice and 60 g or 70 f of water)**

Volunteer no.	Total weight of drink consumed (g)	Quantity of phenolics ingested (mg)							
		CHL	PHL	EPI	Rutin	Q-3-Gal	Q-3-Glu	Q-3-Xyl	Q-3-Rha
1	248.95	17.96	0.77	6.47	0.07	0.13	0.18	nd	nd
2	258.42	18.64	0.80	6.71	0.07	0.14	0.19	nd	nd
3	248.98	17.96	0.77	6.47	0.07	0.13	0.18	nd	nd
4	248.87	17.95	0.77	6.47	0.07	0.13	0.18	nd	nd
5	248.76	17.94	0.77	6.46	0.07	0.13	0.18	nd	nd
6	248.97	17.96	0.77	6.47	0.07	0.13	0.18	nd	nd
7	249.11	17.97	0.77	6.47	0.07	0.13	0.18	nd	nd
8	248.80	17.94	0.77	6.46	0.07	0.13	0.18	nd	nd
9	258.95	18.68	0.80	6.73	0.07	0.14	0.19	nd	nd
MEAN	**251.09**	**18.11**	**0.78**	**6.52**	**0.07**	**0.13**	**0.18**	-	-

Table 3.3 **Quantity of phenolics ingested by individual volunteers after consuming the test drink (190 g of Tesco Pure Apple juice and 60 g or 70 g of the high polyphenol cider apple extract)**

Volunteer no.	Total weight of drink consumed (g)	Quantity of phenolics ingested (mg)							
		CHL	PHL	EPI	Rutin	Q-3-Gal	Q-3-Glu	Q-3-Xyl	Q-3-Rha
1	246.52	111.26	35.31	71.56	0.60	7.96	2.93	1.48	1.94
2	254.47	135.66	47.75	82.22	0.79	9.56	3.87	1.77	2.31
3	244.77	110.87	35.06	71.06	0.59	7.90	2.90	1.47	1.93
4	246.94	111.32	35.37	71.69	0.60	7.97	2.93	1.48	1.94
5	246.48	111.29	35.30	71.55	0.60	7.96	2.92	1.48	1.94
6	246.00	111.23	35.23	71.41	0.60	7.94	2.92	1.47	1.94
7	247.15	111.21	35.40	71.75	0.60	7.98	2.93	1.48	1.95
8	246.59	111.28	35.32	71.58	0.60	7.96	2.93	1.48	1.94
9	256.24	135.66	48.08	82.80	0.80	9.63	3.89	1.78	2.33
MEAN	**251.43**	**116.64**	**38.09**	**73.96**	**0.64**	**8.32**	**3.14**	**1.54**	**2.02**

Flavonols

Direct infusion of quercetin and application of a collision energy of -24V, resulted in the formation of product ions of *m/z* 179, *m/z* 151 and *m/z* 121, in addition to the precursor ion at *m/z* 301 (Figure 3.3, A). The major product ions *m/z* 179 and *m/z* 151 resulted from fragmentation pathway IV or retro-Diels Alder reaction; the B ring gives the ion at *m/z* 151 and the A ring and partial C ring give the ion at *m/z* 179 [23, 140, 149, 173]. The product ion of *m/z* 121 is formed from the B ring (Figure 3.3, C). Another possible fragment of *m/z* 165 is not observed in this spectrum. The fragmentation pattern of myricetin, another member of the flavonol class of flavonoids, which has an extra hydroxyl group on the B ring compared to quercetin, reveals the same product ions (Figure 3.3, B). This time the minor spectral component of *m/z* 165 was observed. Kaempferol fragments in the same way, losing its methyl group and then behaving like quercetin. When there is a glycoside moiety present on the molecule, this is lost first, and then the standard behaves like the aglycone. The loss of HCO from the A ring and partial C ring can also give the ion at *m/z* 151.

Flavanols

The major product ion from the CID of (+) catechin and (-) epicatechin was *m/z* 245 (Figure 3.4, A); a loss of carbon dioxide from the molecule. The product ion of *m/z* 203, could indicate fragmentation at pathway V. Fragments of *m/z* 179 and *m/z* 151 were also observed as minor spectral components, as seen for the flavonols. Literature supports the presence of these product ions in the spectra of (+) catechin and (-) epicatechin [73, 77, 81, 149].

Procyanidins

The structures of procyanidin B1 and B2 are two units of (+) catechin and (-) epicatechin, respectively, attached by C4 (C ring). Their fragmentation patterns are therefore related to those of the flavanols; the presence of the flavonol monomer can be seen (*m/z* 289) [101] in addition to the product ion indicating a loss of CO_2 (*m/z* 245), and the characteristic ions of *m/z* 203, *m/z* 151 and *m/z* 179 caused by fragmentation at pathways V and IV, respectively (Figure 3.4, B). These observations are supported by evidence from Hellstrom *et al.* [174] Fragmentation pathway I also occurs after CID of many of the flavonoid standards, releasing the A ring part of the molecule and resulting in the minor spectral component of *m/z* 109, which appears in many EPI spectra (Figure 3.4, C).

Phenolic acids

Phenolic acids, both hydroxybenzoic and hydroxycinnamic, fragment to lose the carboxylic acid; for caffeic acid *m/z* 135 is the resulting product ion (Figure 3.5, A and C) [140]. Another hydroxycinnamic acid present in apples, *p*-coumaric acid, fragments to produce the major product ion *m/z* 119, which corresponds to the loss of COOH. Fragmentation of esters of hydroxycinnamic acids, such as chlorogenic acid, breaks the ester bond resulting in product ions of the same mass as the individual compounds; fragmentation then continues as if for a single acid [140].

Dihydrochalcones

Figure 3.5, B shows the fragmentation pattern produced by the dihydrochalcone, phloretin; product ions of *m/z* 167 and *m/z* 123 were observed [72, 99, 101, 175]. Phloridzin, a glycoside of phloretin, behaves in the same way as glycosides of flavonols; the glycoside is loss on fragmentation [99, 101, 175], and the compound behaves as the aglycone.

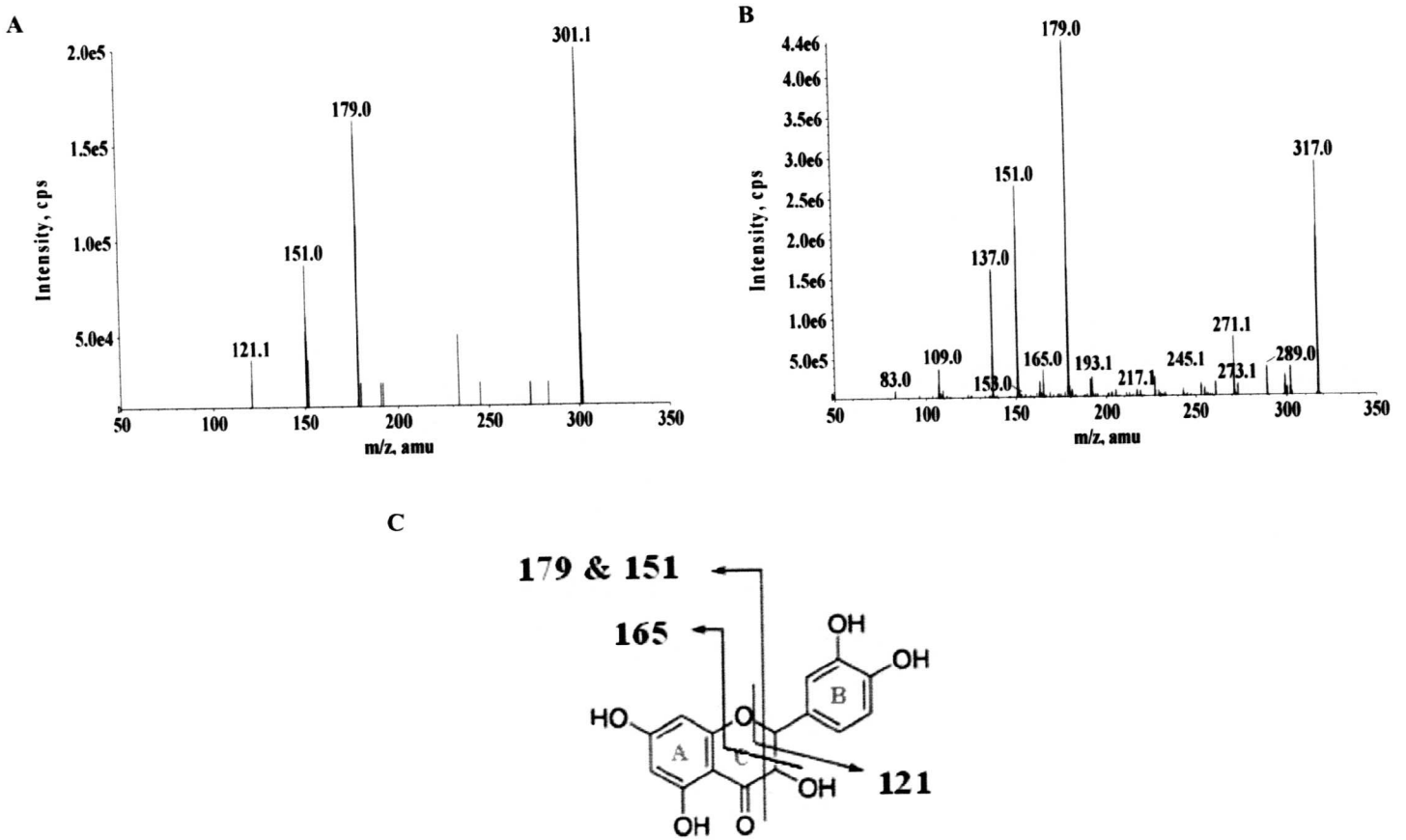

Figure 3.3 **Fragmentation pattern associated with the polyphenol standard quercetin (A), showing major product ions of *m/z* 151 and *m/z* 179 resulting from fragmentation pathway IV (Figure 3.2). Fragmentation pattern associated with the polyphenol standard myricetin (B), showing major product ions of *m/z* 151 and *m/z* 179 resulting from fragmentation pathway IV (Figure 3.2). Possible fragmentation pathways of quercetin and product ions (C).**

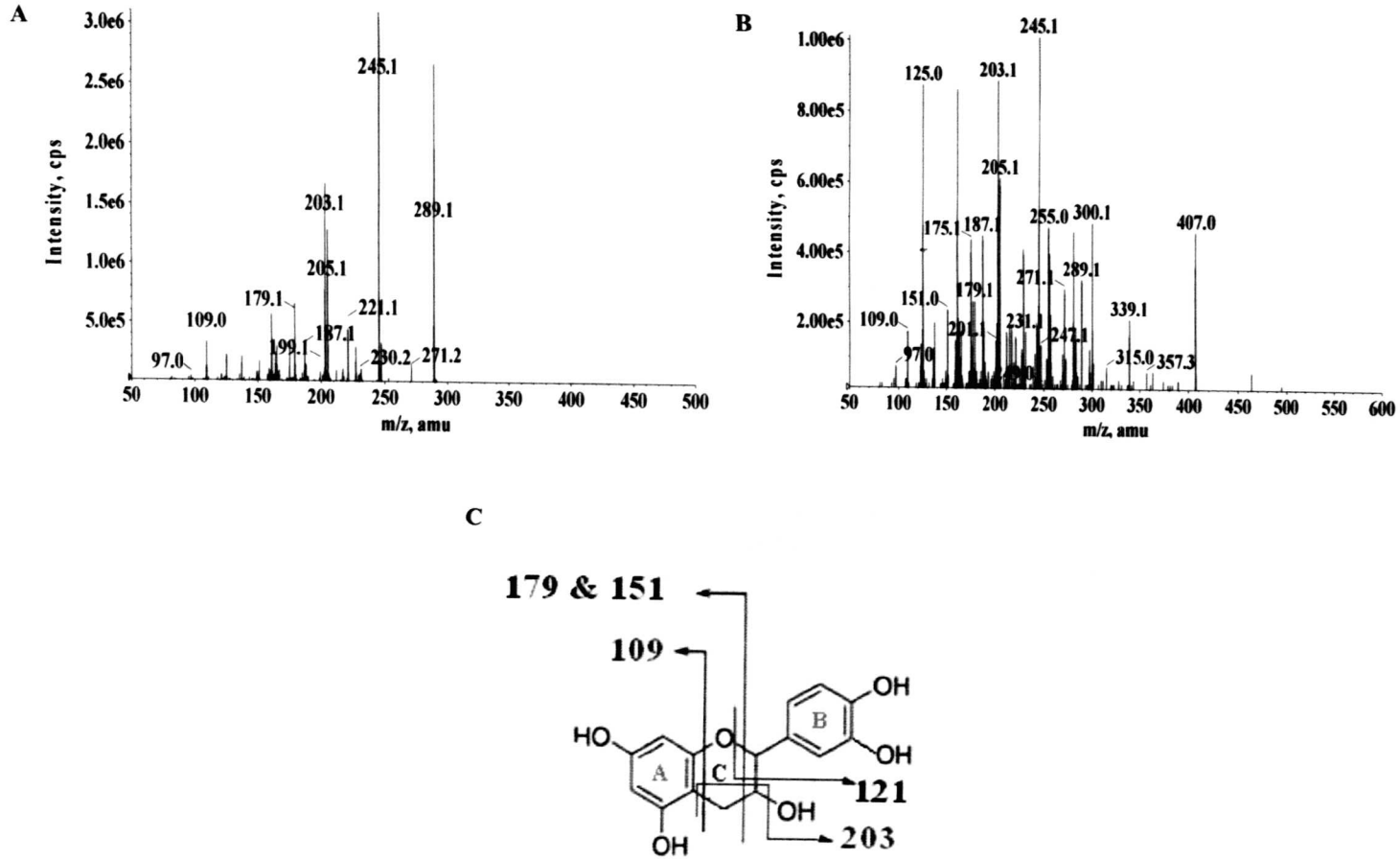

Figure 3.4 **Fragmentation pattern associated with the polyphenol standard (-) epicatechin (A), showing a major product ion of *m/z* 245 resulting from the loss of CO_2. Fragmentation pattern associated with the polyphenol standards procyanidins B1 and B2 (B). Much collision energy was applied to initiate fragmentation, causing a more complex spectrum. Product ions of *m/z* 289 and *m/z* 245 are observed, relating to the monomer and loss of CO_2, respectively. Possible fragmentation pathways and product ions of (-) epicatechin (C).**

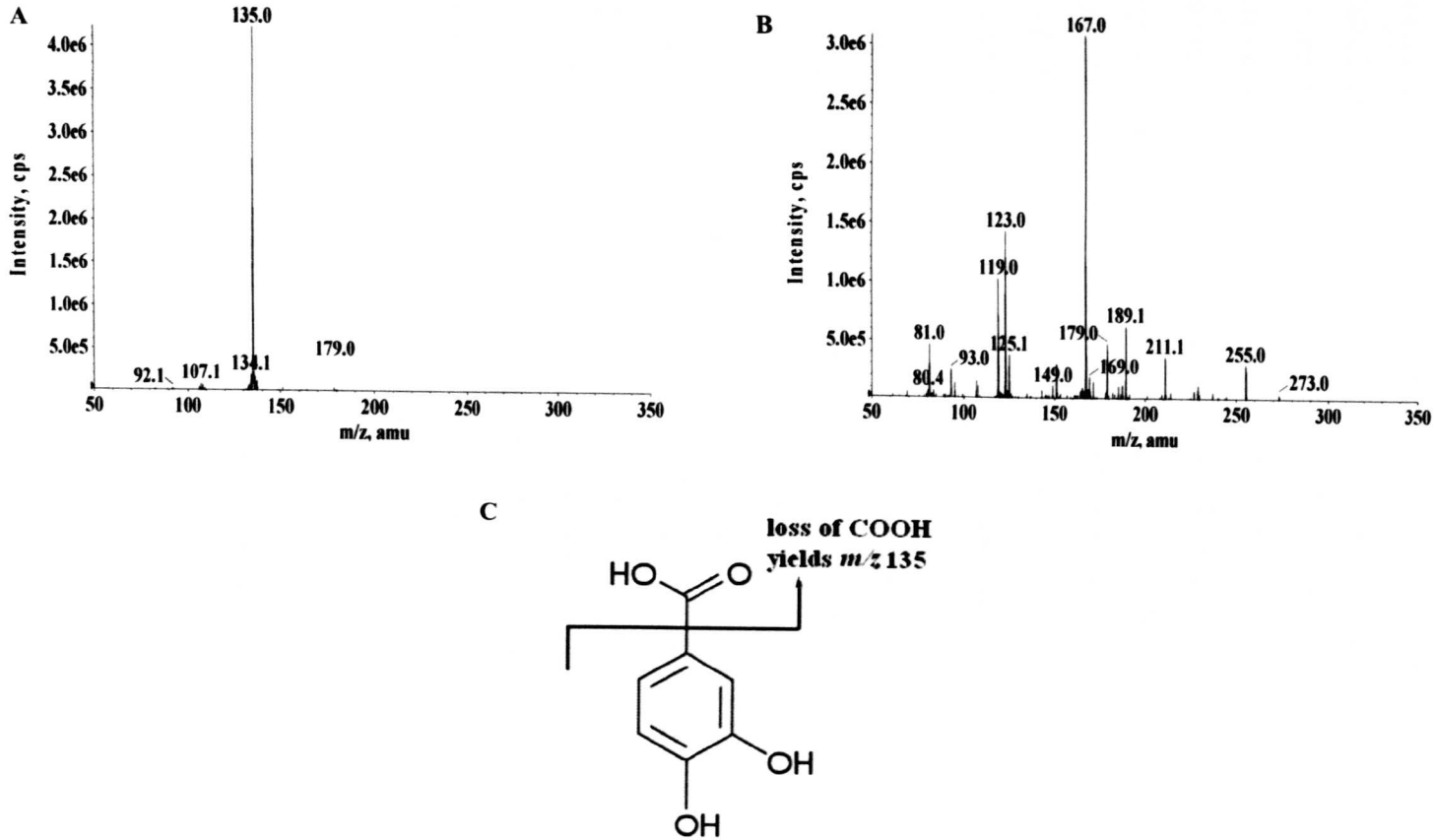

Figure 3.5 **Fragmentation pattern associated with the polyphenol standard caffeic acid (A), showing the major product ion of *m/z* 135, resulting from the loss of COOH. Fragmentation pattern associated with the polyphenol standard phloretin (B), showing the major product ion of *m/z* 167. Fragmentation pathway of caffeic acid (C).**

3.3.3 Identification of Metabolites in Human Urine

3.3.3.1 Screening of Urine for Polyphenol Metabolites Derived from the High Polyphenol Cider Apple Extract

As stated previously, 115 mass transitions were monitored for in the human urine samples. Of this number, 53 showed a significant increase when test urine samples were compared with control urine samples. Examples of mean data for two mass transitions are shown in Figures 3.6 and 3.7. The total peak area for all 53 mass transitions of interest in the test urine samples was calculated, and a percentage for each mass transition was determined. Four mass transitions accounted for over 10 % of the total area; the remainder were all below 5 %. However, closer inspection of each individual EPI spectra and retention times for these 53 mass transitions of interest indicated that they may not be 53 distinct compounds. Any one metabolite eluting from the column may enter the MS and dissociate to produce several product ions; different product ions for the same metabolite may have been detected here.

Figure 3.8 shows the extracted ion chromatograms for the mass transitions *m/z* 383 / 207 and *m/z* 383 / 163; two peaks were observed in each chromatogram, with similar retention times. The EPI spectra of both peaks in both chromatograms show product ions of *m/z* 207 and *m/z* 163. This suggests that both these transitions occur from fragmentation of the same metabolite. Experiments performed in the same way for all the 53 mass transitions concluded with 25 possible distinct metabolites. Three of the analytes accounted for over 10 % of the total area; the others accounted for less than 5 %. Tentative identities were assigned to each of these possible metabolites, using retention times and EPI spectra (compared to standards where appropriate). Metabolites were classified as conjugated metabolites, conjugated ring fission metabolites and unidentified apple-related metabolites. Table 3.4 details the mass transitions and their relative abundance in the urine. Evidence to support the identities of the mass transitions proposed in the table is given in the remainder of the chapter. The percentage areas given in Table 3.4 are not quantitative, as each metabolite may give a different response by MS analysis. However, the percentages do represent an approximation by which the most important metabolites may be identified.

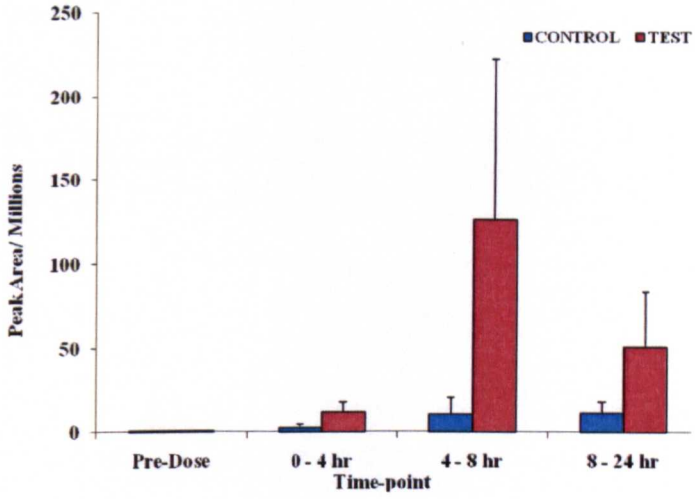

Figure 3.6 **Mean peak area (n = 9) for mass transition *m/z* 705 / 353 monitored in control and test urine. Error bars represent the standard deviation**

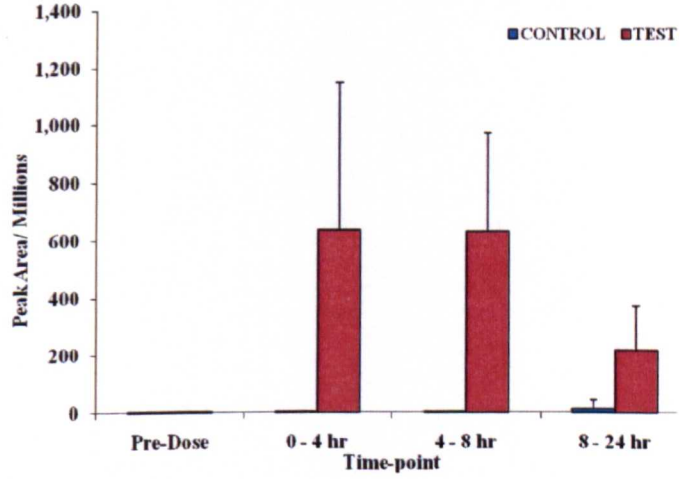

Figure 3.7 **Mean peak area (n = 9) for mass transition *m/z* 545 / 465 monitored in control and test urine. Error bars represent the standard deviation**

Table 3.4 **The following table details the mass transitions with the greatest increases in peak area, when test urine was compared to control urine, after the initial screening process. The percentage area represents the relative abundance of that particular mass transition compared to the others, but is not quantitative due to different metabolites giving different responses by MS analysis. Confidence level **** is assigned where there are good spectra and reference to the metabolite in the literature; confidence levels lower than this are given when spectra may not be as conclusive, retention times may fall in and around the solvent front and/or they may not have been reported in the literature.**

m/z	% Peak Area	Possible Identity	Confidence
287 / 207	39.0	Dihydroxyphenyl valerolactone sulfate	****
383 / 207	38.6	5- (3′,4′-Dihydroxyphenyl)-γ-valerolactone glucuronide	****
449 / 273	14.5	Phloretin-2′-glucuronide	****
367 / 207	3.1	Dihydroxyphenyl valerolactone disulfate	***
577 / 289	1.5	Unknown conjugate	
369 / 289	0.9	(-/+) Epicatechin-3′ or 4′-sulfate	****
653 / 477	0.6	Unknown diglucuronide conjugate	*
465 / 289	0.4	(-/+) Epicatechin-3′or 4′-glucuronide	****
264 / 169	0.3	Unknown methyl sulfate conjugate	*
545 / 465	0.2	(-/+) Epicatechin sulfoglucuronide	**
425 / 249	0.2	Unknown sulfoglucuronide conjugate	*
384 / 304	0.2	Unknown conjugate	
409 / 233	0.2	Unknown glucuronide conjugate	*
515 / 339	0.1	Dihydroxyphenylacetic acid glucuronide	**
289 / 163	0.1	Dihydroxyphenyl valerolactone sulfate	*
248 / 168	0.1	Unknown methyl sulfate conjugate	*
557 / 477	0.1	Unknown sulfate conjugate	*
737 / 481	0.1	Unknown conjugate	
705 / 353	< 0.1	Unknown conjugate	
271 / 191	< 0.1	Unknown sulfate conjugate	*
464 / 288	< 0.1	Unknown sulfoglucuronide conjugate	*
423 / 247	< 0.1	3, 4-Dihydroxyphenylacetic acid sulfoglucuronide	***
223 / 193	< 0.1	Sinapic acid	**
529 / 449	< 0.1	Unknown conjugate	
273 / 208	0	Unknown conjugate	

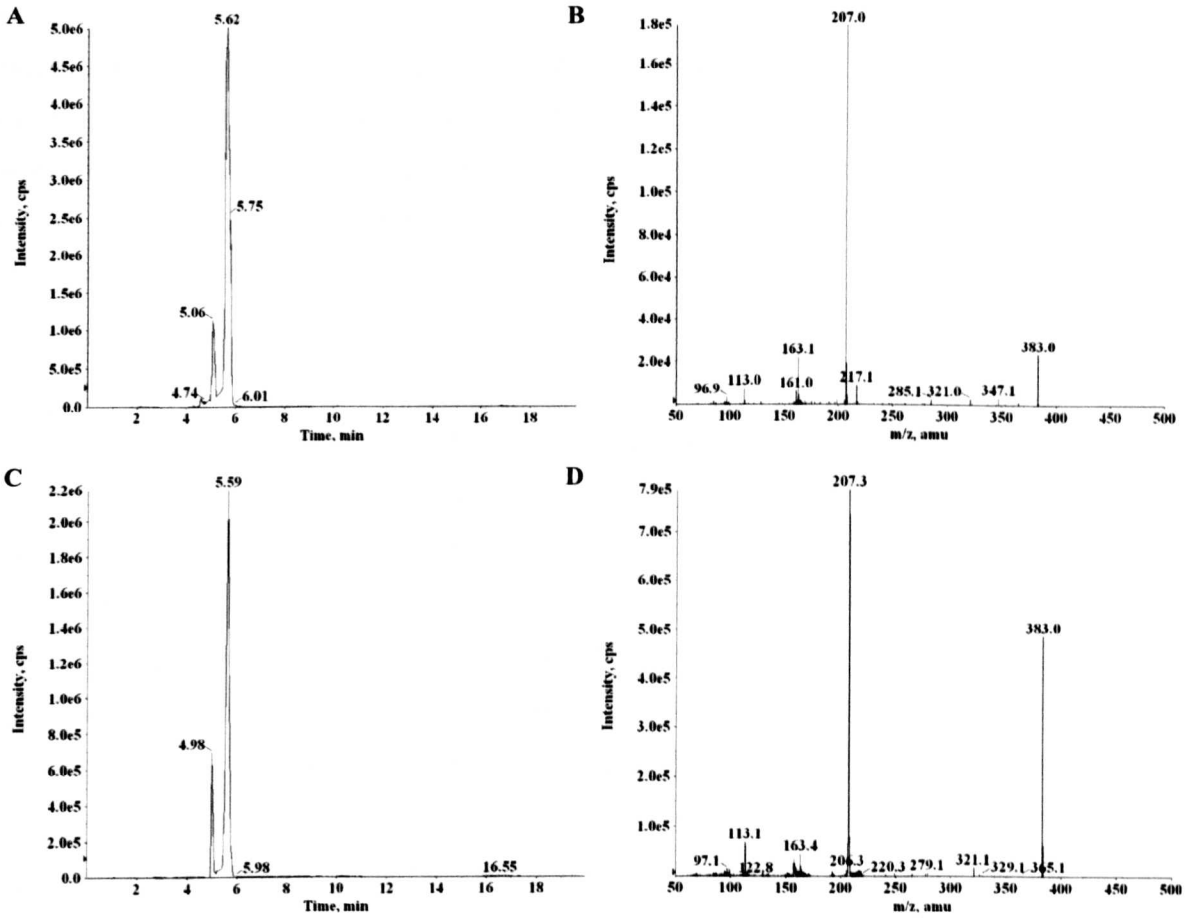

Figure 3.8 **Extracted ion chromatogram of *m/z* 383 / 207 (A) and EPI spectrum of the major peak at 5.62 min (B). Extracted ion chromatogram of *m/z* 383 / 163 (C) and EPI spectrum of the major peak at 5.59 min (D). LC/MS/MS conditions for all chromatograms include mobile phases consisting of 0.01 % formic acid in H_2O and MeCN; the initial flow rate was 0.225 µl/min increasing to 0.850 µl/min at 11.1 min for 2 min then returning to 0.225 µl/min for the remainder of the analysis time. Gradient was as follows: (0, 10), (4, 40), (5, 46), (8, 48), (8.1, 100), (13.1, 100), (17, 0) and (17.1, 10) (time in min, % B). Column temperature was 65 °C.**

3.3.3.2 Identification of Conjugated Metabolites in Human Urine

1. m/z 465 / 289 [(-/+) Epicatechin-3′ or 4′-glucuronide - 0.4 % of total analyte area]

The extracted ion chromatogram for the mass transition *m/z* 465 / 289 is shown in Figure 3.9; a major peak was observed at a retention time of 5.25 min with several smaller peaks also present. On MS^2, the analyte fragmented with a 176 amu loss, characteristic of the cleavage of a glucuronide unit. MS^2 experiments with conjugated standards of polyphenols indicated that the conjugate is readily lost on application of collision energy. Product ions of *m/z* 245 and *m/z* 179 were seen in this spectrum, in addition to *m/z* 289. These ions were also observed after the CID of the polyphenolic standards (+) catechin and (-) epicatechin (see Figure 3.4). This suggests that the identity of *m/z* 465 / 289 could be (+/-) catechin or (-/+) epicatechin

glucuronide. Research by Gonzalez-Manzano *et al.* [166] characterising catechin sulfates and glucuronides suggested fragmentation pathways and possible *m/z* values for fragments of some (+) catechin glucuronides, conjugated on the A and B ring (Figure 3.10). Comparing these fragments with those in the EPI spectrum for the largest peak detected with the mass transition *m/z* 465 / 289 (Figure 3.9), the same product ions of *m/z* 327 and *m/z* 381 were observed. This suggests that the position of conjugation could be on the B ring, hence 3′ or 4′ as these are the carbons with the hydroxyl groups attached.

The retention times of (+) catechin and (-) epicatechin standards are 3.65 and 4.32 min, respectively. Only quercetin glucuronides were available for comparison, but if a comparison is made between how the position of conjugation affects retention, it appears that conjugation on the A ring results in earlier retention than the aglycone, and conjugation on the B ring results in later elution (retention time of quercetin is 6.68 min; quercetin-3-glucuronide is 5.70 min; and quercetin-3′-glucuronide is 6.90 min [Table 2.3, Chapter 2]). Flavanols (such as (+) catechin and (-) epicatechin) and flavonols (such as quercetin) are very similar in structure, only differing by the addition of C = O on the C ring of the latter. Therefore, the retention time phenomenon observed with flavonols could also apply to flavanols.

Thus, identification of the mass transition *m/z* 465 / 289 could be (+/-) catechin/ (-/+) epicatechin-3′ or -4′ -glucuronide. Since volunteers were given a test drink which contained a high level of (-) epicatechin (~74 mg), it is more likely that the identification of the mass transition is (-/+) epicatechin-3′ or -4′ glucuronide. It has been reported that in humans, (-) epicatechin-3′-glucuronide is the major urinary glucuronide metabolite of epicatechin from sources such as tea and cocoa [73, 83, 97].

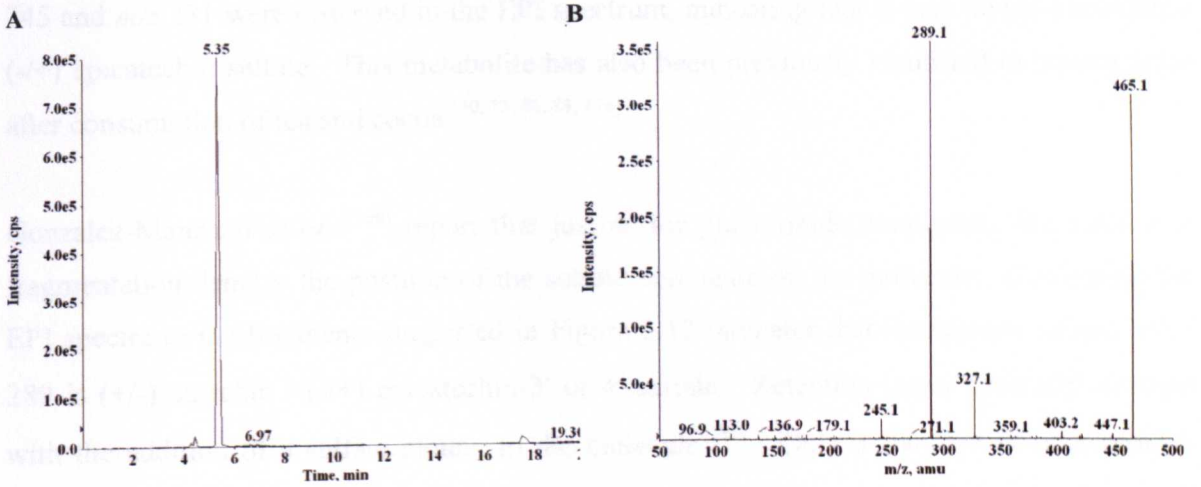

Figure 3.9 **Extracted ion chromatogram of *m/z* 465 / 289 (A) and EPI spectrum of the peak at 5.35 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

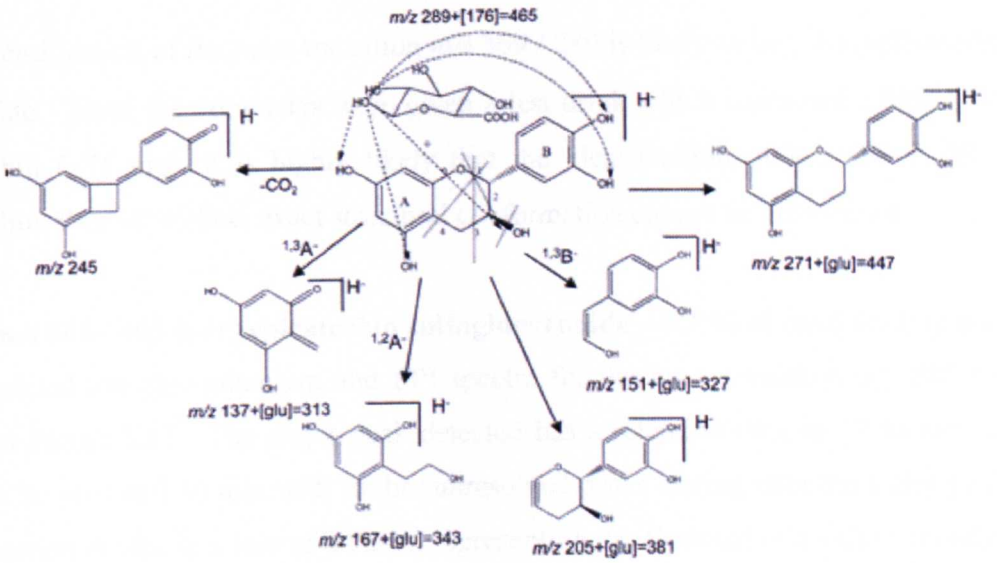

Figure 3.10 **MS^2 fragmentation pathways of (+) catechin glucuronides in negative ionisation mode** [166]

2. *m/z* 369 / 289 [(-/+) epicatechin-3′ or 4′-sulfate – 0.9 % of total analyte area]

Figure 3.11 shows the extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 369 / 289. A major peak was observed at a retention time of 10.71 min, with some minor peaks also present. MS^2 yields the loss of 80 amu, which indicates cleavage of a sulfate from a conjugate. Just as for glucuronide metabolites, sulfate moieties are readily fragmented from conjugates in the MS. As seen for the previous mass transition, product ions of *m/z* 289, *m/z*

245 and *m/z* 231 were observed in the EPI spectrum, indicating that it may be (+/-) catechin / (-/+) epicatechin sulfate. This metabolite has also been previously identified in human urine after consumption of tea and cocoa [70, 73, 81, 83, 176].

Gonzalez-Manzano *et al.* [166] report that just as for glucuronide conjugates, the pattern of fragmentation denotes the position of the sulfate conjugate on the molecule. Comparing the EPI spectra to the fragments suggested in Figure 3.12 indicates that the identity of *m/z* 369 / 289 is (+/-) catechin / (-/+) epicatechin-3′ or 4′-sulfate. Retention times generally increase with the addition of a sulfate moiety to the molecule [81]. Only a standard of quercetin-3′-sulfate was available for comparison in this study; its retention time was greatly increased compared to the aglyone and glucuronides (retention time of quercetin was 6.68 min; quercetin-3-glucuronide was 5.70 min; quercetin-3′-glucuronide was 6.90 min; and quercetin-3′-sulfate was 17.3 min [Table 2.3, Chapter 2]).

Thus, identification of the mass transition *m/z* 369 / 289 is likely to be (+/-) (epi)catechin-3′ or -4′ -sulfate. Since the volunteers were given a test drink which contained a high level of (-) epicatechin (~74 mg), it is highly likely that the identification of *m/z* 369 / 289 is (-/+) epicatechin-3′ or -4′ sulfate, exact structural conformation cannot be determined.

***3. m/z* 545 / 465 [(-/+) epicatechin sulfoglucuronide – 0.2 % of total analyte area]**

The extracted ion chromatogram and EPI spectra for the mass transition *m/z* 545 / 465 are shown in Figure 3.13. The major peak detected has a retention time of 17.53 min; a minor peak can be seen at 4.80 min with further unresolved peaks eluting after the major peak. MS^2 fragmentation results in a loss of 80 amu, representing the cleavage of a sulfate moiety from a conjugate. The late retention time of the mass transition also suggests the presence of a sulfate. Studying the EPI spectrum, ions of *m/z* 369 and *m/z* 289 are observed, in addition to *m/z* 465. These same product ions were seen in the EPI spectra of the previous two mass transitions, *m/z* 465 / 289 and *m/z* 369 / 289 and so the identity of this metabolite could be related to (-/+) epicatechin. Application of a greater collision energy (-60 to -90 eV), produced ions of *m/z* 245, 203, 179 and 271; all of which were observed in the spectra for (+) catechin and (-) epicatechin standards (Figure 3.4), providing further support for the suggestion of an (-/+) epicatechin conjugate.

The mass transition *m/z* 545 / 465 represents a loss of 80 amu (sulfate moiety) and *m/z* 545 / 369 represents a loss of 176 amu (glucuronide moiety). Alternatively, *m/z* 545 / 369 represents a loss of 176 amu and *m/z* 369 / 289 represents a loss of 80 amu. These different mass transitions suggest that the intact metabolite has both a sulfate and a glucuronide attached, which are fragmented in the MS in either order. Therefore, evidence indicates that the analyte could be an (-) epicatechin sulfoglucuronide. Roura *et al.* [81], also found evidence of this metabolite in human urine after consumption of cocoa, however no structural elucidation was performed. Using the data from Gonzalez-Manzano *et al.* [166], the presence of *m/z* 217 and *m/z* 151 in the EPI spectrum of *m/z* 545 / 465 could indicate that the sulfate is positioned on the A ring (Figure 3.12). However, presence of the ions of *m/z* 327 and 205 in the EPI spectrum of *m/z* 545 / 465 give conflicting evidence as to the position of the glucuronide (Figure 3.10); *m/z* 327 suggests B ring attachment and *m/z* 205 suggests A ring attachment. No ion of *m/z* 313 was observed, which is seen with A ring attachment; B ring attachment is more likely considering the previous identifications made.

The minor peak and unresolved peaks also observed in the extracted ion chromatogram are present in lower concentrations than the threshold value so an EPI spectrum is not triggered; product ions cannot be compared with those of the major peak.

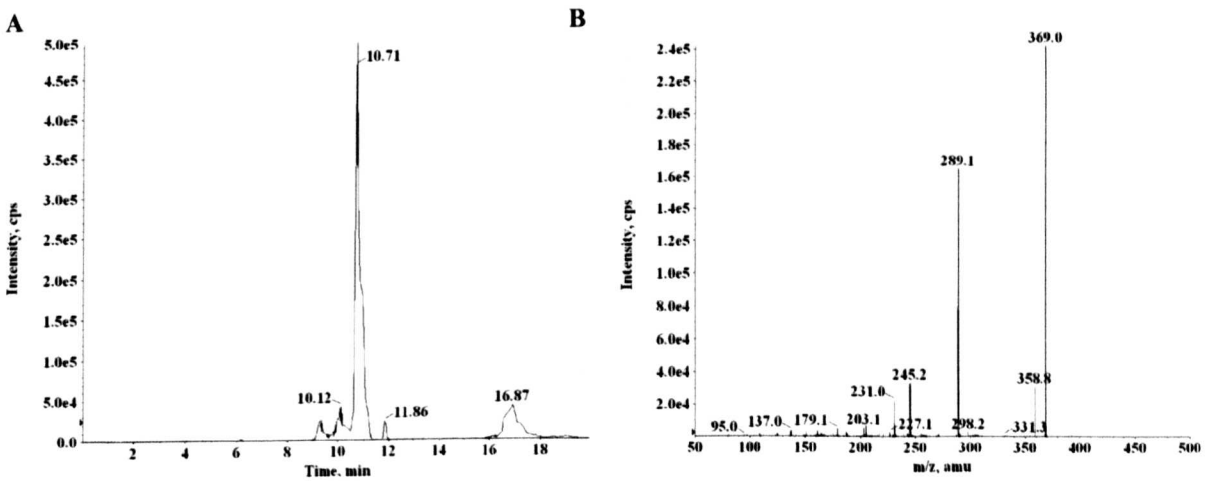

Figure 3.11 **Extracted ion chromatogram of *m/z* 369 / 289 (A) and EPI spectrum of the peak at 10.71 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

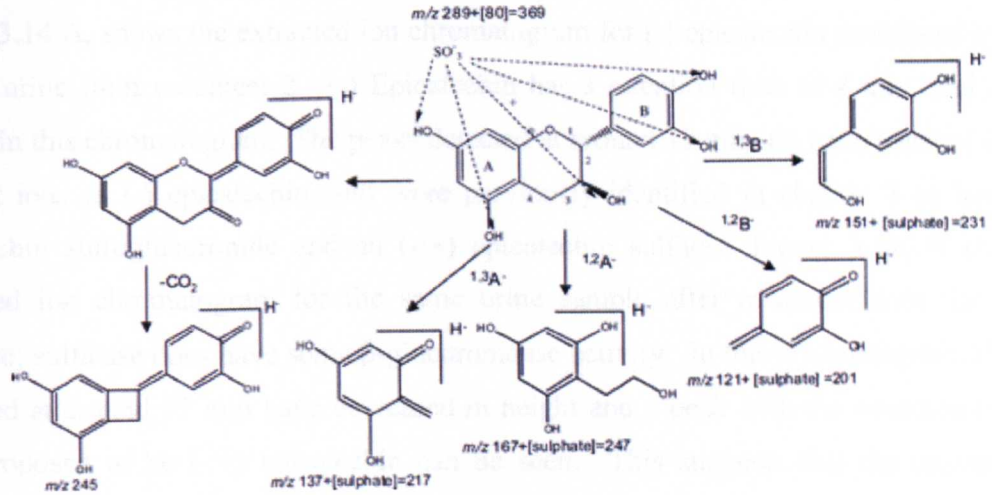

Figure 3.12 **MS^2 fragmentation pathways of (+) catechin monosulfates in negative ionisation mode [166]**

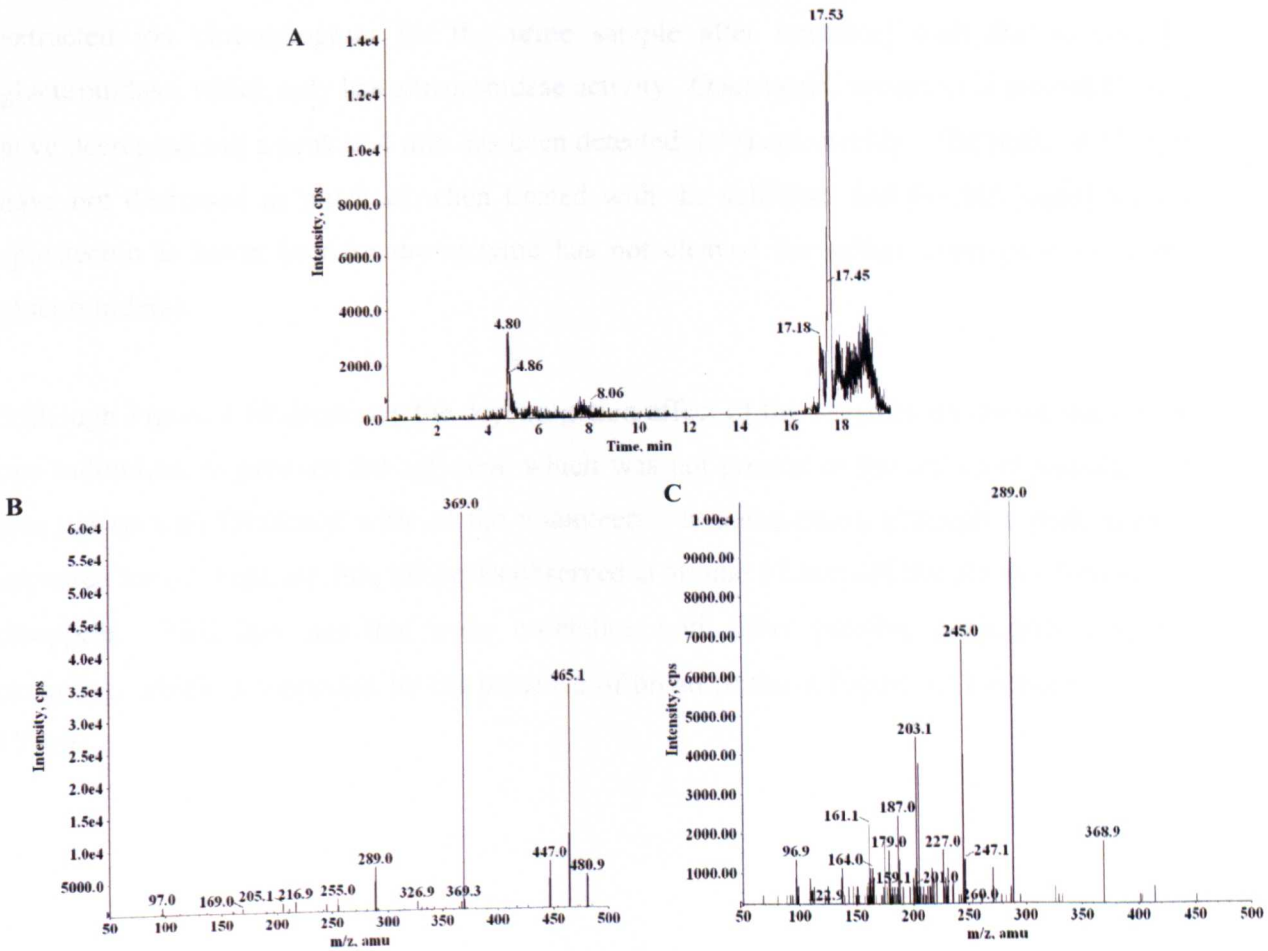

Figure 3.13 **Extracted ion chromatogram of *m/z* 545 / 465 (A), EPI spectrum of the peak at 17.53 min (B) and EPI spectrum of the peak at 17.53 min with application of greater collision energy. LC/MS/MS conditions were as described in Figure 3.8.**

Further Confirmation of Identity of (-/+) Epicatechin Conjugates in Human Urine

Figure 3.14 A, shows the extracted ion chromatogram for (-) epicatechin monitored in control 0-24 h urine from volunteer 2. (-) Epicatechin has a retention time of 4 min, and does not appear in this chromatogram. The peaks detected at around 17 min, do have some of the same product ions as (-) epicatechin, and were previously identified in chapter 3 as being (-/+) epicatechin sulfoglucuronide and an (-/+) epicatechin sulfate. Figure 3.14, B shows the extracted ion chromatogram for the same urine sample after treatment with the enzyme sulfatase; sulfatase does have some β-glucuronidase activity. In this chromatogram, the peaks observed at around 17 min have decreased in height and a peak with the retention time of 4 min, proposed to be (-/+) epicatechin can be seen. This suggests that the enzymes have cleaved the sulfate and glucuronide conjugates from the metabolites, leaving the aglycone (-) epicatechin. This confirms the identity of the peaks at 17 min as being possible sulfoglucuronide and sulfate conjugates of (-/+) epicatechin. Figure 3.14, C shows the extracted ion chromatogram for the urine sample after treatment with the enzyme β-glucuronidase, which only has glucuronidase activity. Once again, the peaks at around 17 min have decreased and a peak at 4 min has been detected; (-/+) epicatechin. The peaks at 17 min have not decreased as much as when treated with the sulfatase, and the MS signal for (-) epicatechin is lower because the enzyme has not cleaved the sulfate conjugates, only the glucuronide(s).

Although Figure 3.14 illustrates the deconjugation affect of the enzymes on the samples from one individual, to produce the aglycone which was not present in the untreated sample, this was not so well illustrated with all the volunteers. In some cases, although a peak always appeared for (-/+) epicatechin, the peaks observed at around 17 min did not always decrease or disappear. This indicates that some co-elution with other possible conjugates may be occurring, which is supported by the presence of broad peaks in Figure 3.11 between 16 and 17 min.

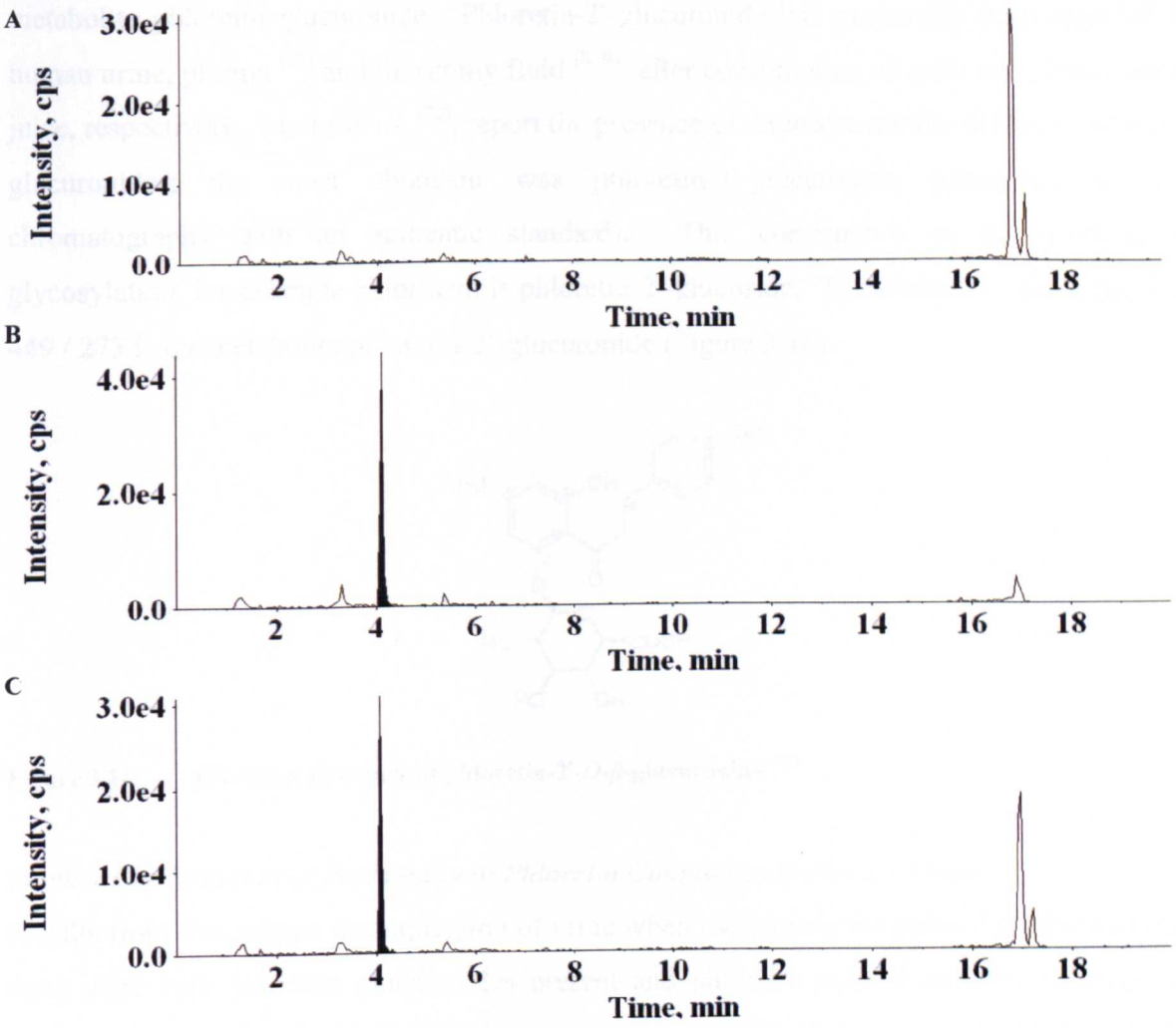

Figure 3.14 **Extracted ion chromatograms monitoring for (-) epicatechin. 0-24 h control urine contains no (-) epicatechin (A), 0-24 h urine after enzyme treatment with sulfatase (B) (which also contains some β-glucuronidase activity) shows (-) epicatechin with a retention time of 4 min and 0-24 h urine after enzyme treatment with β-glucuronidase (C), again showing (-) epicatechin with a retention time of 4 min.**

4. m/z 449 / 273 [phloretin-2'-glucuronide – 14.5 % of total analyte area]

The extracted ion chromatogram and EPI spectra for the mass transition *m/z* 449 / 273 are given in Figure 3.15. The major peak has a retention time of 7.93 min. On MS^2, this analyte fragmented with a 176 amu loss, characteristic of the cleavage of a glucuronide unit. Product ions of *m/z* 315, *m/z* 167 and *m/z* 345 were observed, in addition to *m/z* 273. These product ions also appeared after the fragmentation of the polyphenol standard phloretin (same collision energy applied).

These spectra provide compelling evidence that the mass transition *m/z* 449 / 273 is the metabolite phloretin glucuronide. Phloretin-2′-glucuronide has previously been reported in human urine, plasma [72] and ileostomy fluid [8, 99] after consumption of cider and cloudy apple juice, respectively. Marks *et al.* [72], report the presence of three structurally different phloretin glucuronides; the most abundant was phloretin-2′-glucuronide (identified by co-chromatography with an authentic standard). This corresponds to the position of glycosylation, for example phloridzin is phloretin-2′-glucoside. Therefore, it is likely that *m/z* 449 / 273 is the metabolite phloretin-2'-glucuronide (Figure 3.16).

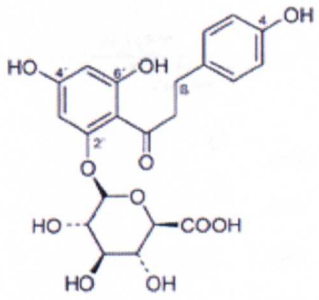

Figure 3.16 Chemical structure of phloretin-2′-*O*-*β*-glucuronide [99]

Further Confirmation of the Identity of Phloretin Conjugates in Human Urine

Results from the enzyme deconjuagtion of urine when monitoring for phloretin, indicated that there were both phloretin glucuronides present and phloretin sulfates present, although no sulfate conjugate has been identified as a major metabolite here. On treatment with the enzyme sulfatase, the amount of phloretin detected in the urine increased and on treatment with β-glucuronidase the amount of phloretin also increased, but to a lesser extent due to the enzymes only cleaving the glucuronides and not both conjugates as with the sulfatase enzymes.

Summary

The following conjugated metabolites have been identified in human urine;

1. (-/+) Epicatechin-3′ or 4′-sulfate,
2. (-/+) Epicatechin-3′ or 4′-glucuronide,
3. (-/+) Epicatechin sulfoglucuronide (sulfate on position 5 or 7, glucuronide on position 5, 7, 3′ or 4′)
4. Phloretin-2′-glucuronide

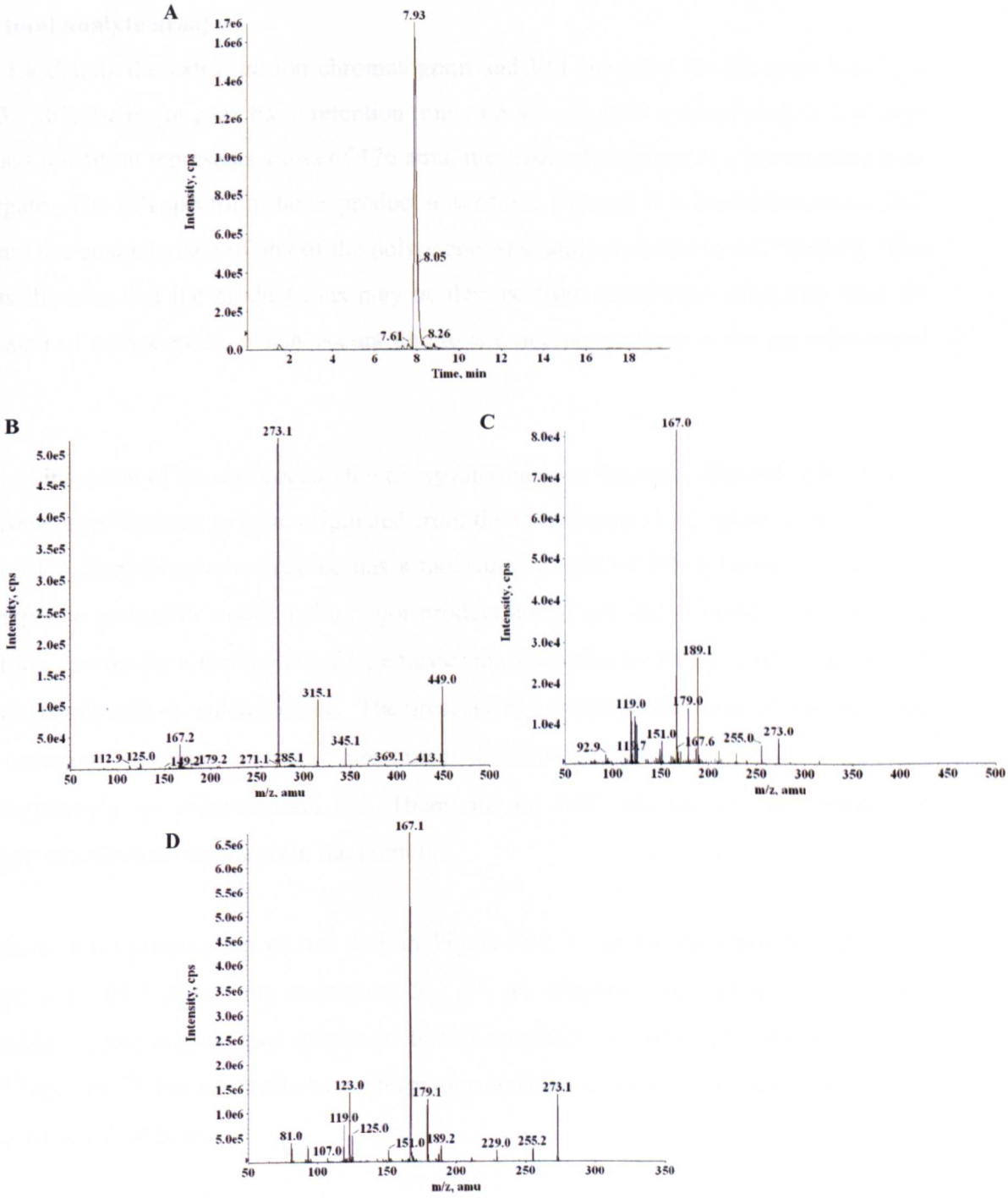

Figure 3.15 **Extracted ion chromatogram of *m/z* 449 / 273 (A), EPI spectra of the peak at 7.93 min (B and C) and EPI spectrum of the polyphenol standard phloretin (D). LC/MS/MS conditions were as described in Figure 3.8.**

3.3.3.3 Identification of Conjugated Ring Fission Metabolites in Human Urine

1. ***m/z* 383 / 207 [5- (3',4'-dihydroxyphenyl)-γ-valerolactone glucuronide – 38.6 % of total analyte area]**

Figure 3.8 details the extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 383 / 207; the major peak has a retention time of 5.62 min, with a minor peak at 5.06 min. This mass transition represents a loss of 176 amu, the result of cleavage of a glucuronide from a conjugate. The EPI spectrum shows product ions of *m/z* 163 and 113, in addition to *m/z* 207, which are not characteristic of any of the polyphenol standards analysed by LC/MS/MS. This supports the idea that the product ions may be derived from metabolites produced from the ring fission of polyphenols, which occurs due to the microorganisms in the gastrointestinal tract.

Following ingestion of tea and cocoa, dihydroxyvalerolactone has been detected in both urine and plasma, hypothesized to have originated from the breakdown of (-) epicatechin [77, 83, 177] (Figure 3.17). Dihydroxyvalerolactone has a molecular weight of 208 (observed as *m/z* 207 under negative ionisation mode) and a major product ion of *m/z* 163. Figure 3.18 shows the product ion spectra for a metabolite of (-) epicatechin, identified by Meng *et al.* [74] as 5 - (3', 4'- dihydroxyphenyl) -γ- valerolactone. The presence of a minor product ion of *m/z* 123 in the lower spectrum indicates a meta substituted dihydroxyphenyl structure; 5 - (3', 5'- dihydroxyphenyl) -γ- valerolactone [74]. There are no MS^2 spectra for the analysis of dihydroxyvalerolactone standards in the literature.

Since there is no product ion of *m/z* 123 in Figure 3.9, it can be proposed that the mass transition *m/z* 383 / 207 is the metabolite 5 - (3', 4'- dihydroxyphenyl) -γ- valerolactone glucuronide. There are just two references to this metabolite in urine after consumption of cocoa [83] and tea [73], but no structural conformation so either of the two structures proposed in Figure 3.19 could be likely.

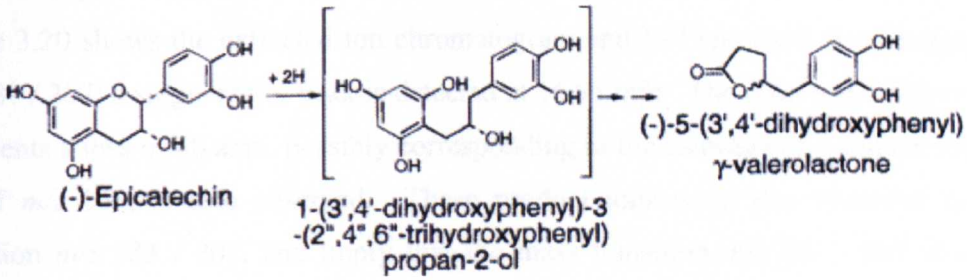

Figure 3.17 **Proposed pathway for conversion of (-) epicatechin to dihydroxyvalerolactone, adapted from Li *et al.* [177]**

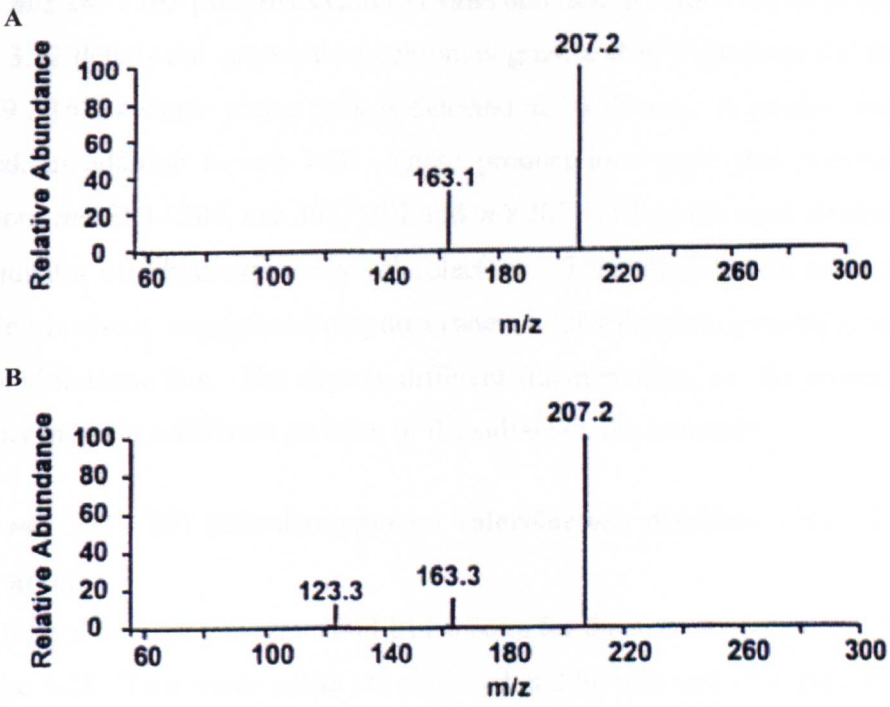

Figure 3.18 **Product ion spectrum of 5- (3′, 4′-dihydroxyphenyl)-γ-valerolactone (A) and product ion spectrum of 5- (3′, 5′-dihydroxyphenyl)-γ-valerolactone (B) adapted from Meng *et al.* [74]**

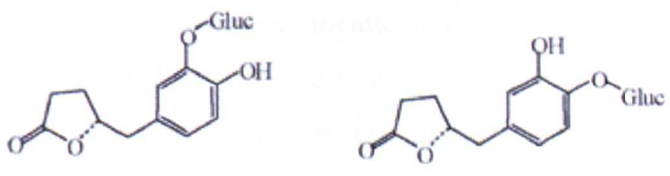

Figure 3.19 **Two possible structural conformations of dihydroxyphenyl glucuronide, adapted from Sang *et al.* [73]**

2. ***m/z* 287 / 207 [dihydroxyphenyl valerolactone sulfate – 39.0 % of total analyte area]**

Figure 3.20 shows the extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 287 / 207; a single major peak is detected at 16.91 min. The mass transition *m/z* 287 / 207 represents a loss of 80 amu, possibly corresponding to the cleavage of a sulfate; and a product ion of *m/z* 163, is also observed. These product ions were also observed for the mass transition *m/z* 383 / 207, and imply that the mass transition *m/z* 287 / 207 is a dihydroxy valerolactone sulfate. There is no evidence to assign whether the sulfate moiety is attached at the 3′ or 4′ position (Figure 3.21).

3. ***m/z* 289 / 163 [dihydroxyphenyl valerolactone sulfate – 0.1 % of total analyte area]**

Figure 3.22 details the extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 289 / 163; a single major peak is detected at 16.97 min. A product ion of *m/z* 207 was detected, in addition to *m/z* 163. These product ions were also detected with the mass transitions *m/z* 383 / 207, *m/z* 367 / 207 and *m/z* 287 / 207 which were all tentatively identified as conjugates of dihydroxyphenly valerolactone. This suggests that the mass transition *m/z* 289 / 163 is also a conjugate of dihydroxyphenyl valerolactone, possibly a sulfate as the mass loss could indicate this. The slightly different fragmentation, i.e. the presence of the ion *m/z* 289, may indicate a different position of the sulfate on the molecule.

4. ***m/z* 367 / 207 [dihydroxyphenyl valerolactone disulfate – 3.1 % of total analyte area]**

The extracted ion chromatogram and EPI spectra for the mass transition *m/z* 367 / 207 is given in Figure 3.23. Two major peaks are observed at 3.96 min and 17.4 min, in addition to some minor peaks. The mass transition *m/z* 367 / 207 represents a loss of 160 amu, or possibly the cleavage of two sulfate units. A product ion of *m/z* 163 is also seen in the EPI spectrum in addition to *m/z* 207, which is shown more clearly when greater collision energy is applied (Figure 3.23, C). These product ions appear identical to those detected for the mass transitions *m/z* 287 / 207 and *m/z* 383 / 207, suggesting that *m/z* 367 / 207 is dihydroxyphenyl valerolactone disulfate; this metabolite has not been detected in urine or plasma previously.

5. ***m/z* 423 / 247 [3, 4-dihydroxyphenylacetic acid sulfoglucuronide – <0.1 % of total analyte area]**

The extracted ion chromatogram and EPI spectra for the mass transition *m/z* 423 / 247 are given in Figure 3.24; many peaks were detected. This mass transition represents a loss of 176 amu, corresponding to the cleavage of a glucuronide unit. Product ions of *m/z* 343 and 167 were observed in addition to *m/z* 247. *m/z* 247 / 167 represents the loss of 80 amu; cleavage of a sulfate, therefore it is likely that this analyte has both a sulfate and a glucuronide present, which fragment in the MS in either order. Further fragmentation at a higher collision energy, gave a new product ion of *m/z* 123.

Comparison of these EPI spectra with those produced from the analysis of polyphenol standards did not result in any matches or similarities. Although vanillic acid has the precursor ion *m/z* 167, it fragments to *m/z* 108; a product ion not observed in Figure 3.24. Phloretin has the product ion *m/z* 167, but the precursor ion of *m/z* 273 is not observed in Figure 3.24. The literature reports the presence of 3, 4-dihydroxyphenylacetic acid in urine after consumption of cocoa [82, 83]; this has the mass transition *m/z* 167 / 123 which is seen in Figure 3.25. Evidence would therefore suggest that *m/z* 423 / 247 is the microbial derived metabolite 3, 4-dihydroxyphenylacetic acid sulfoglucuronide; no data exist to assign structural positions to the sulfate or glucuronide groups.

6. ***m/z* 515 / 339 [dihydroxyphenylacetic acid glucuronide – 0.1 % of total analyte area]**

Figure 3.26 shows the extracted ion chromatogram and EPI spectrum for this mass transition; a single peak is detected at a retention time of 1.47 min. This mass transition suggests a loss of a glucuronide, as there is a difference of 176 amu between the two masses. A product ion of *m/z* 167 can be seen in addition to *m/z* 339. No polyphenol standards analysed in this study produced these fragments in their EPI spectra. Dihydroxyphenylacetic acid has the precursor ion *m/z* 167, but it produces an ion of *m/z* 123 on fragmentation [82, 83]; not seen in Figure 3.26. No literature supports the presence of an analyte with *m/z* 515 / 339, and the only conclusion that can be made on its identity is that it probably has a glucuronide attached.

7. ***m/z* 223 / 193 [sinapic acid – < 0.1 % of total analyte area]**

Figure 3.27 shows the extracted ion chromatogram and EPI spectrum for the precursor product ion pair *m/z* 223 / 193. Several peaks can be seen; the sharpest and largest peak at 5.69 min

and a broader peak at 9.05 min; all peaks show the same product ions in their EPI spectra. Product ions of *m/z* 149 and *m/z* 121 are observed in the EPI spectrum in addition to *m/z* 193. The polyphenol standard sinapic acid has the precursor product ion pair of *m/z* 223 / 149, and fragments to give product ions of *m/z* 208, *m/z* 193 and *m/z* 121. These spectra show the same ions present, just at different intensities. The retention time for sinapic acid is 5.49 min, and the analyte is 5.69 min, so there is a possibility that the mass transition *m/z* 223 / 193 is sinapic acid. Sinapic acid has been found in the urine of humans after consumption of cereal bran [178], and is a major urinary metabolite of green coffee extract in humans [179].

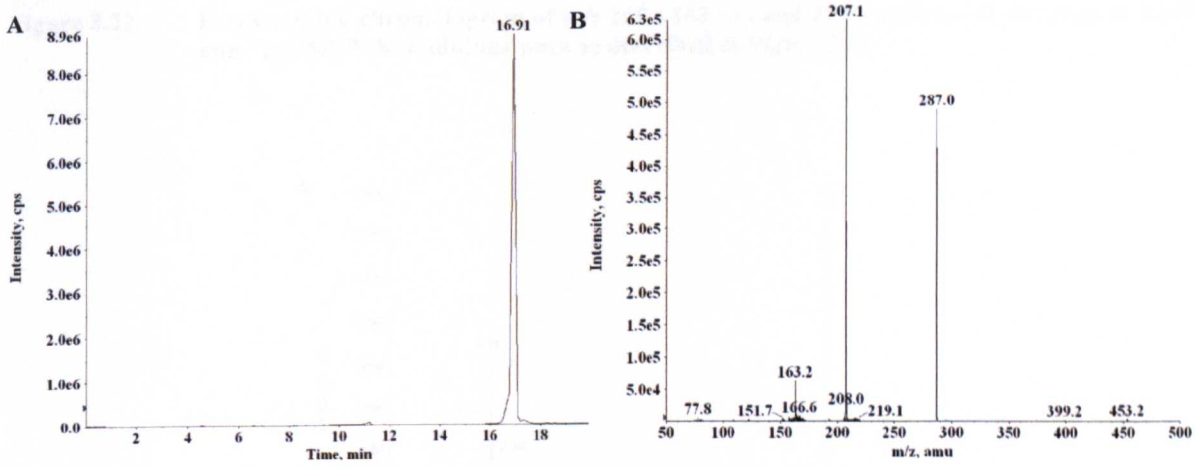

Figure 3.20 **Extracted ion chromatogram of *m/z* 287 / 207 (A) and EPI spectrum of the peak at 16.91 min. LC/MS/MS conditions were as described in Figure 3.8.**

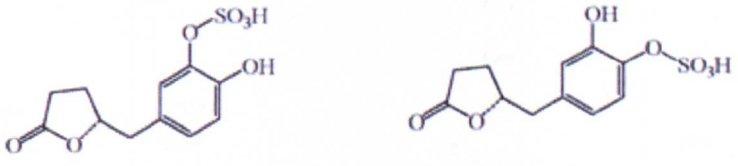

Figure 3.21 **Two possible structural conformations of dihydroxyphenyl valerolactone sulfate, taken from Sang *et al.* [73]**

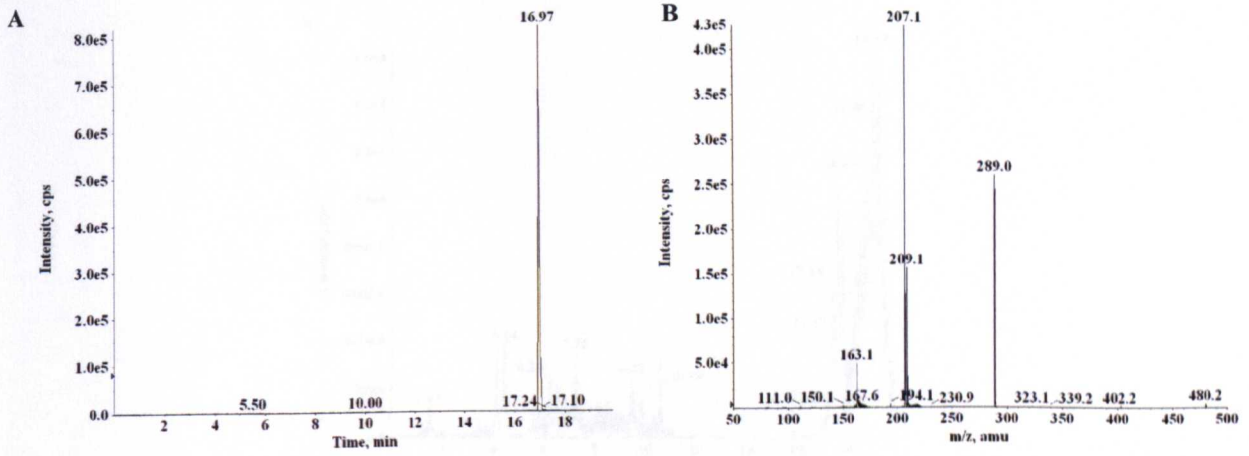

Figure 3.22 **Extracted ion chromatogram of *m/z* 289 / 163 (A) and EPI spectrum of the peak at 16.97 min. LC/MS/MS conditions were as described in Figure 3.8.**

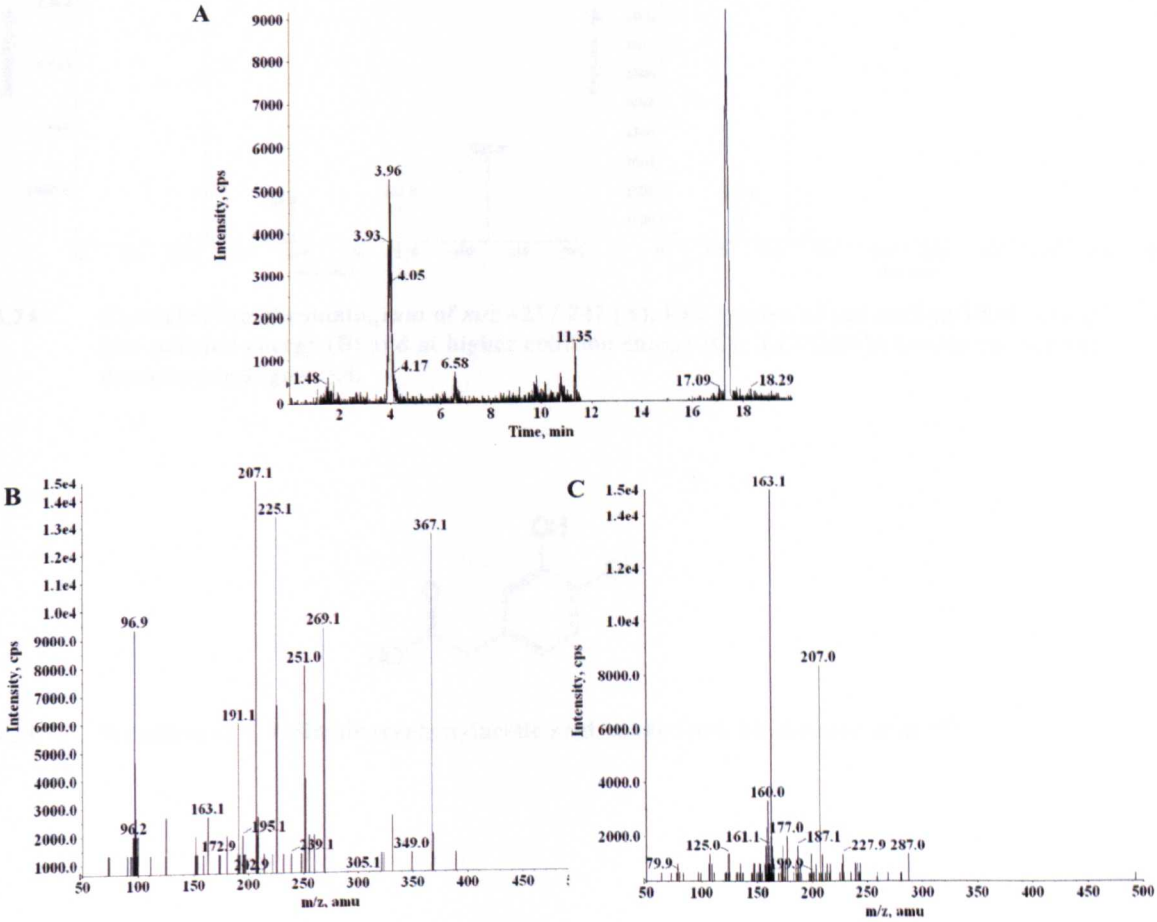

Figure 3.23 **Extracted ion chromatogram of *m/z* 367 / 207 (A), EPI spectra of the peak at 3.96 min at low collision energy (B) and at higher collision energy (C). LC/MS/MS conditions were as described in Figure 3.8.**

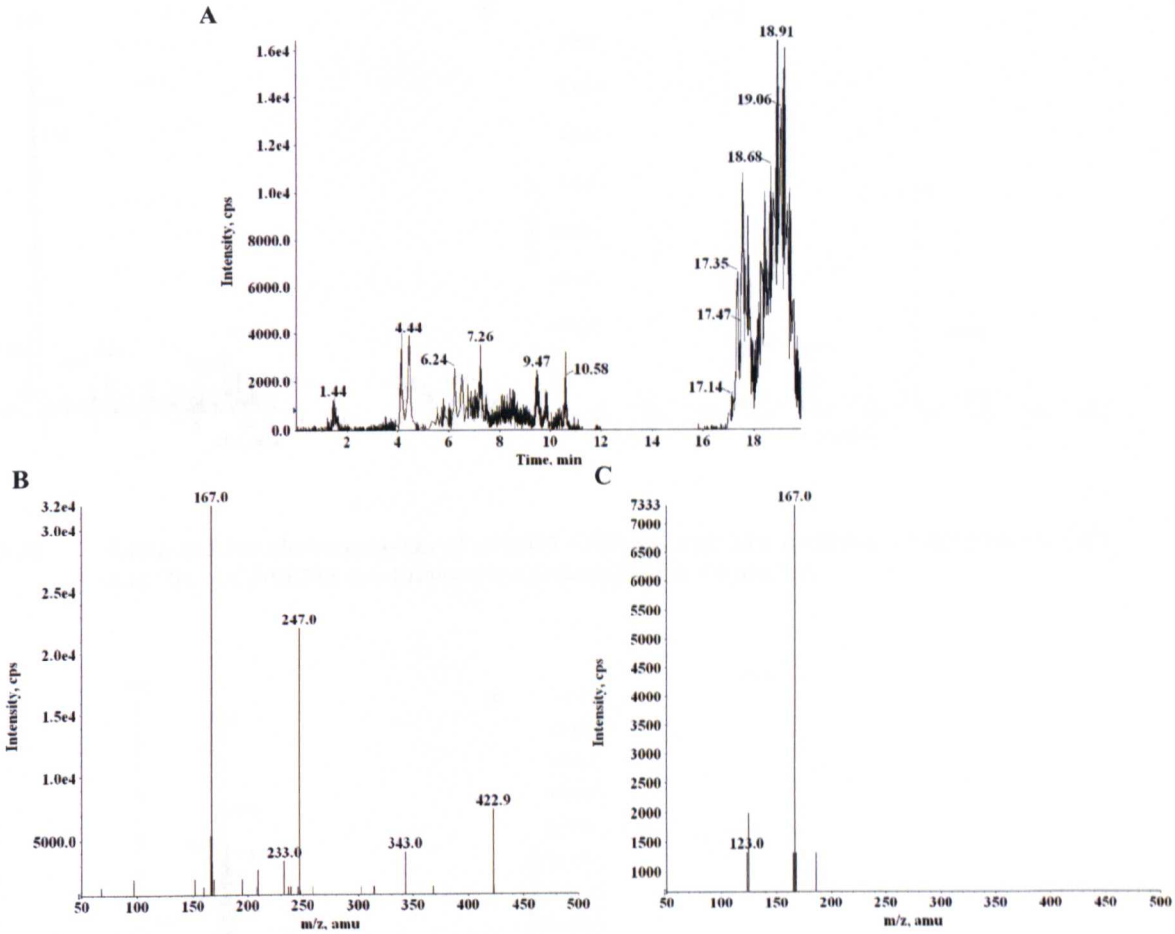

Figure 3.24 **Extracted ion chromatogram of *m/z* 423 / 247 (A), EPI spectra of the peak at 18.91 min at low collision energy (B) and at higher collision energy (C). LC/MS/MS conditions were as described in Figure 3.8.**

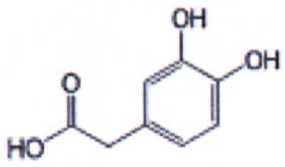

Figure 3.25 **Structure of 3, 4-dihydroxyphenylacetic acid, taken from Urpi-Sarda *et al.* [83]**

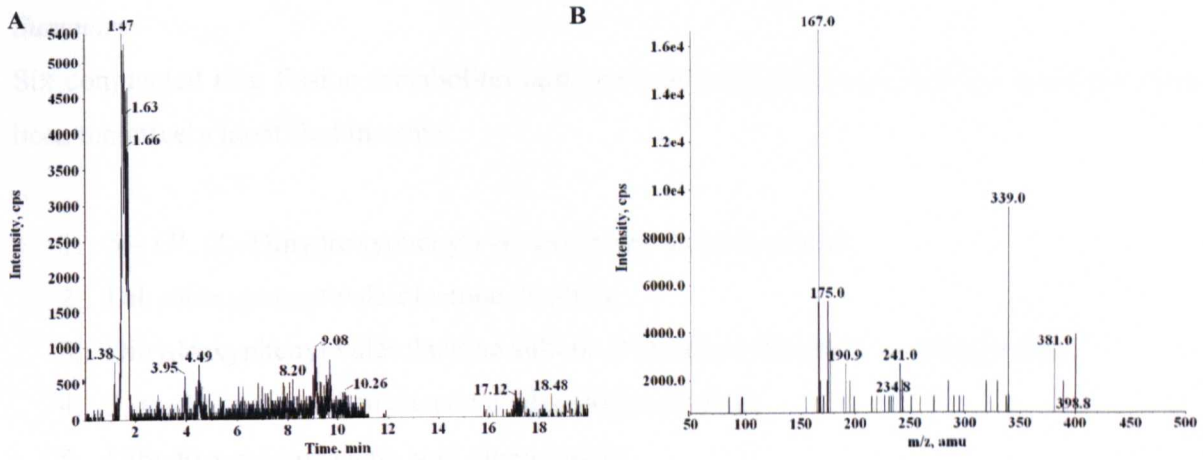

Figure 3.26 **Extracted ion chromatogram of *m/z* 515 / 339 (A) and EPI spectrum of the peak at 1.47 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

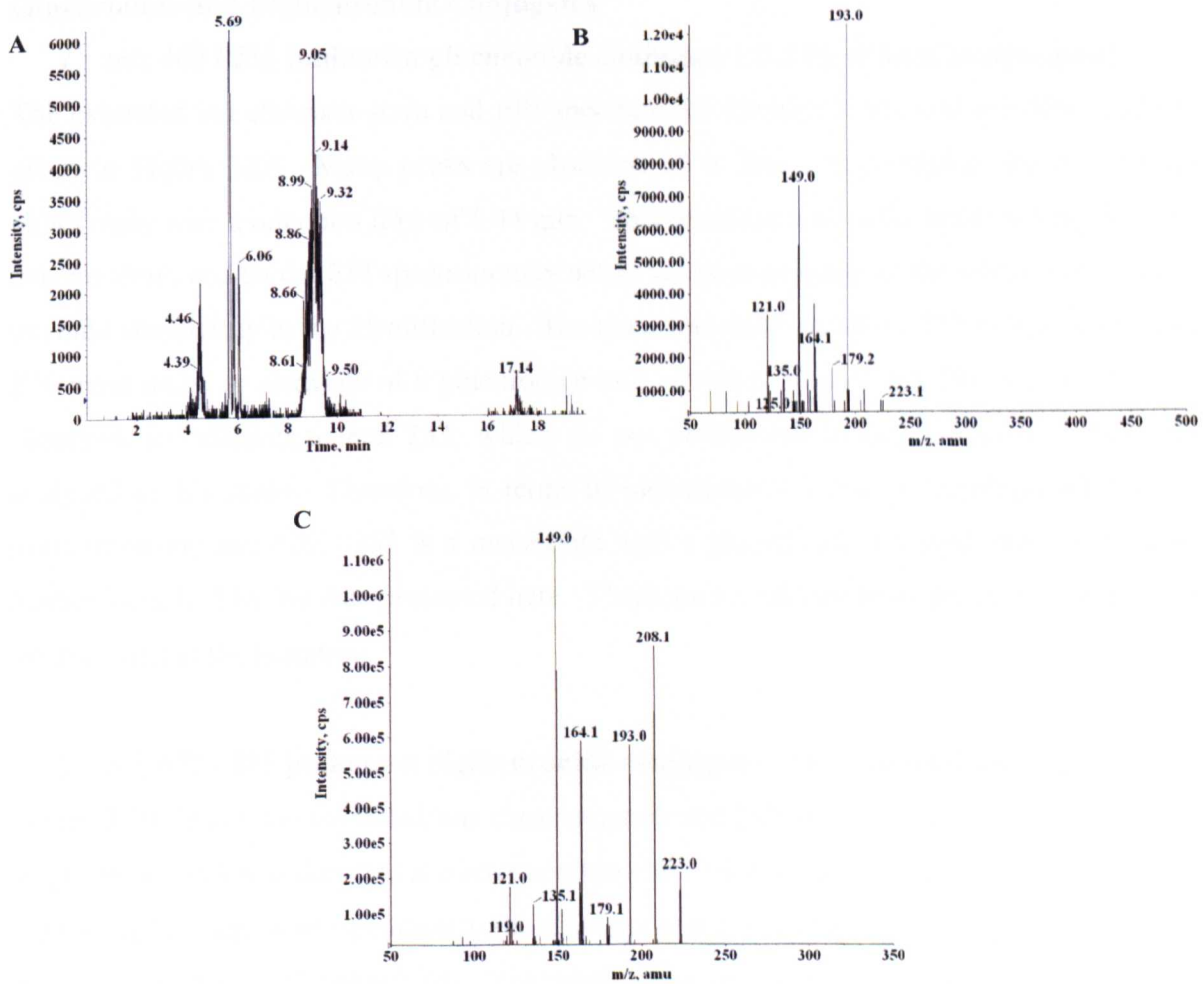

Figure 3.27 **Extracted ion chromatogram of *m/z* 223 / 193 (A), EPI spectrum of the peak at 5.69 min (B) and EPI spectrum for the polyphenol standard sinapic acid (C). LC/MS/MS conditions were as described in Figure 3.8.**

Summary

Six conjugated ring fission metabolites and one non-conjugated ring fission metabolite have been tentatively identified in urine;

1. 5 – (3′, 4′ –Dihydroxyphenyl) -γ- valerolactone glucuronide,
2. Dihydroxyphenyl valerolactone disulfate,
3. Dihydroxyphenyl valerolactone sulfate (2 different structural conformations),
4. 3, 4 – Dihydroxyphenylacetic acid sulfoglucuronide,
5. Dihydroxyphenylacetic acid glucuronide,
6. Sinapic acid

3.3.3.4 Unidentified Apple Related Metabolites in Human Urine

Glucuronide and Diglucuronide Conjugates

***1. m/z* 409 / 233 [unknown glucuronide conjugate – 0.2 % of total analyte area]**

The extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 409 / 233 are given in Figure 3.28. Many peaks are observed all in low concentrations; the major peak elutes early with a retention time of 1.44 min. This retention time is the same or very near the solvent front, and so the EPI spectrum may not be as clean as many of the others, which leads to some uncertainty in the identification. The mass transition *m/z* 409 / 233 indicates a loss of 176 mass units, or cleavage of a glucuronide unit. Product ions of *m/z* 191 and *m/z* 175 are observed, in addition to *m/z* 233, which do not correspond to any polyphenol standards analysed in this study. Therefore, in terms of identification it can be hypothesized that the mass transition *m/z* 409 / 233 is a metabolite with a glucuronide attached, but it cannot be further identified by the data presented here. There are no references to this mass transition or product ions in the literature.

***2. m/z* 653 / 477 [unknown diglucuronide conjugate – 0.6 % of total analyte area]**

Figure 3.29 details the extracted ion chromatogram and EPI spectrum for *m/z* 653 / 477. A single broad peak was detected at a retention time of 10.4 min. This mass transition represents the loss of 176 amu, corresponding to the cleavage of a glucuronide. The EPI spectrum shows product ions of *m/z* 477 and *m/z* 301. The mass transition *m/z* 301 is a further loss of 176 amu, indicating the possibility of a second glucuronide attached to the molecule. Greater collision energy was applied to fragment the ions further and a comparison was made with the EPI spectrum of quercetin, which also produces a precursor ion at *m/z* 301 in negative ionisation

mode. Product ions of *m/z* 179, *m/z* 151 and *m/z* 273 seen for quercetin, indicate that the two spectra are very different. Therefore, it can be suggested that the mass transition *m/z* 653 / 477 is a diglucuronidated metabolite, but there is not enough evidence to suggest any further identification. There is no supporting evidence in the literature either.

Sulfoglucuronide Conjugates

1. ***m/z* 464 / 288 [unknown sulfoglucuronide conjugate – 0.1 % of total analyte area]**

Figure 3.30 shows the extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 464 / 288; one major peak was detected at 17.1 min, and several other minor peaks were also observed. This mass transition represents the loss of 176 amu, or cleavage of a glucuronide. Product ions of *m/z* 384 and *m/z* 208 were seen in the EPI spectrum, in addition to *m/z* 288. The transition *m/z* 464 / 384 is a loss of 80 amu, representing the cleavage of a sulfate. Fragments indicate the sulfate and glucuronide units can be lost in either order. Further fragmentation yielded a product ion of *m/z* 163/164, which was also seen in the fragmentation of dihydroxyphenyl valerolactone conjugates. However, the spectra do not look the same when compared with those of the dihydroxyphenyl valerolactone conjugates, and therefore the mass transition *m/z* 464 / 288 is unlikely to be a dihydroxyvalerolactone conjugate. There is likely to be a sulfate and glucuronide present on the molecule, but further identification of this analyte is not possible.

2. ***m/z* 425 / 345 [unknown sulfoglucuronide conjugate – 0.2 % of total analyte area]**

Figure 3.31 shows the extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 425 / 345; a major peak was observed at a retention time of 4.82 min and several minor peaks were also seen. This mass transition represents a loss of 176 amu, or cleavage of a glucuronide. The EPI spectrum shows product ions of *m/z* 345 and *m/z* 234, in addition to *m/z* 249. The mass transition *m/z* 425 / 345 represents a loss of 80 amu, or cleavage of a sulfate unit. There is no mention of a metabolite with this fragmentation pattern in the literature, and therefore other than suggesting that there is a sulfate and a glucuronide unit attached, no further identification can be made.

3. ***m/z* 557 / 477 [unknown sulfoglucuronide conjugate – 0.1 % of total analyte area]**

Figure 3.32 shows the extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 557 / 477; the major peak has a retention time of 17 min, and several minor peaks were also detected. This mass transition represents a loss of 80 amu, indicating the cleavage of a

sulfate. Analysis of the EPI spectrum shows product ions of *m/z* 381 and *m/z* 187 which suggests that the mass transition *m/z* 557 / 477 has a sulfate attached, but there is not enough evidence to propose an identity. The EPI spectrum does not correspond with the previous unidentified metabolite either, despite both having the product ion of *m/z* 477. There is no evidence of this mass transition being detected or identified in the literature.

Sulfate Conjugates

1. *m/z* 271 / 191 [unknown sulfate conjugate < 0.1 % of total analyte area]

The extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 271 / 191 are shown in Figure 3.33; there are three major peaks at retention times 1.13, 1.73 and 16.99 min. The fist two peaks have retention times which are the same or very near to the solvent front, so the EPI spectrum of the peak at 16.99 min was used for identification. The mass transition *m/z* 271 / 191 represents the loss of 80 amu, or cleavage of a sulfate. There are no further characteristic product ions observed in the spectrum, so an identity for this analyte cannot be proposed. The literature does not report the presence of an analyte of this mass transition after consumption of polyphenol related food and drink items.

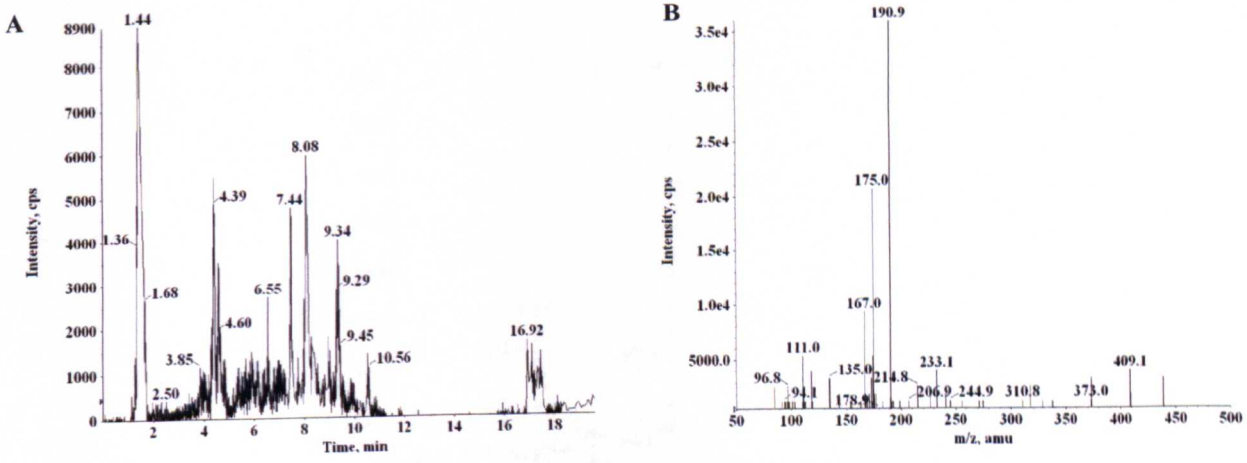

Figure 3.28 **Extracted ion chromatogram of *m/z* 409 / 233 (A) and EPI spectrum of the peak at 1.44 min. LC/MS/MS conditions were as described in Figure 3.8.**

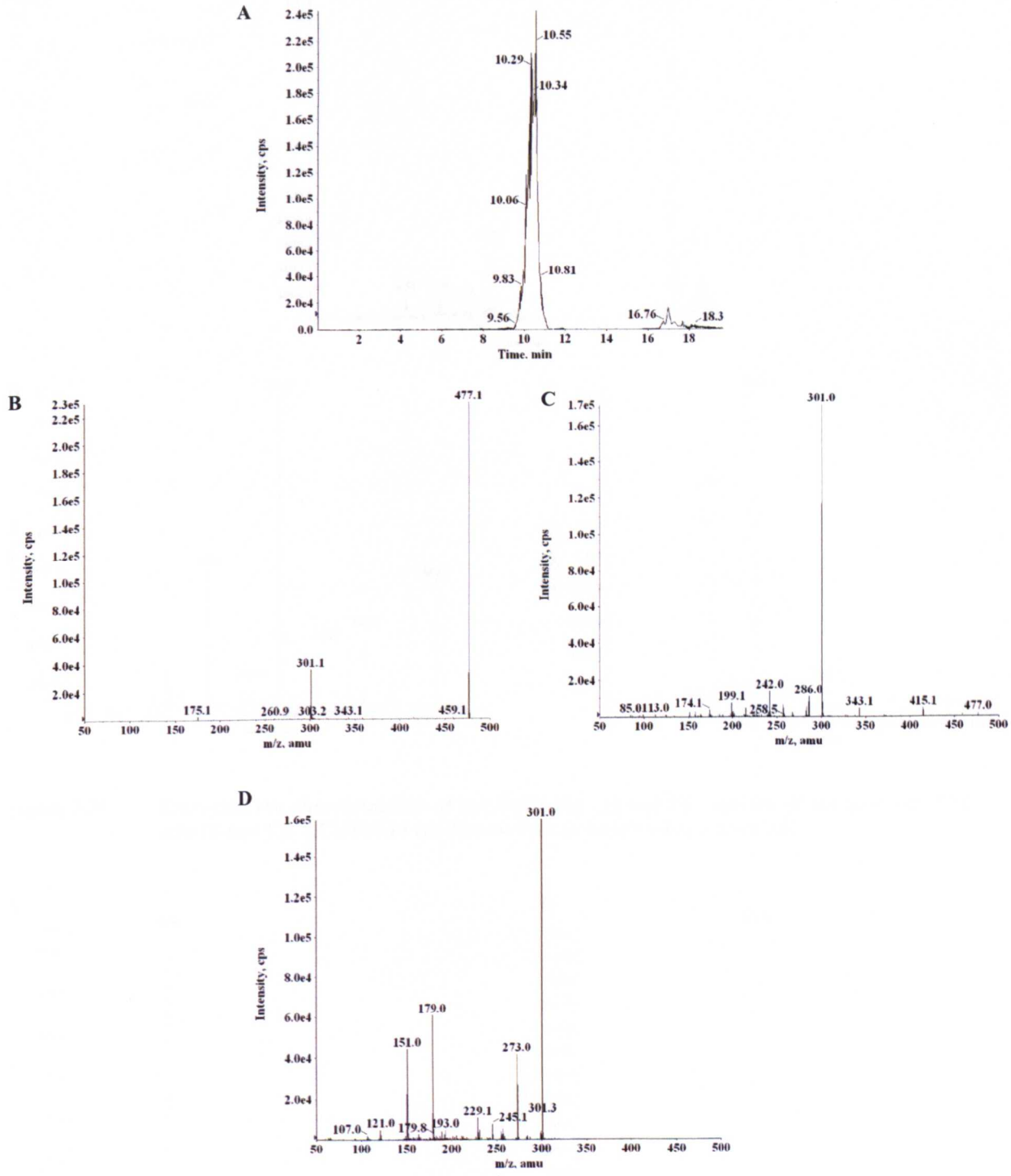

Figure 3.29 **Extracted ion chromatogram of *m/z* 653 / 477 (A), EPI spectra of the peak at 10.29 min (B and C) and EPI spectrum of the polyphenol standard quercetin (D). LC/MS/MS conditions were as described in Figure 3.8.**

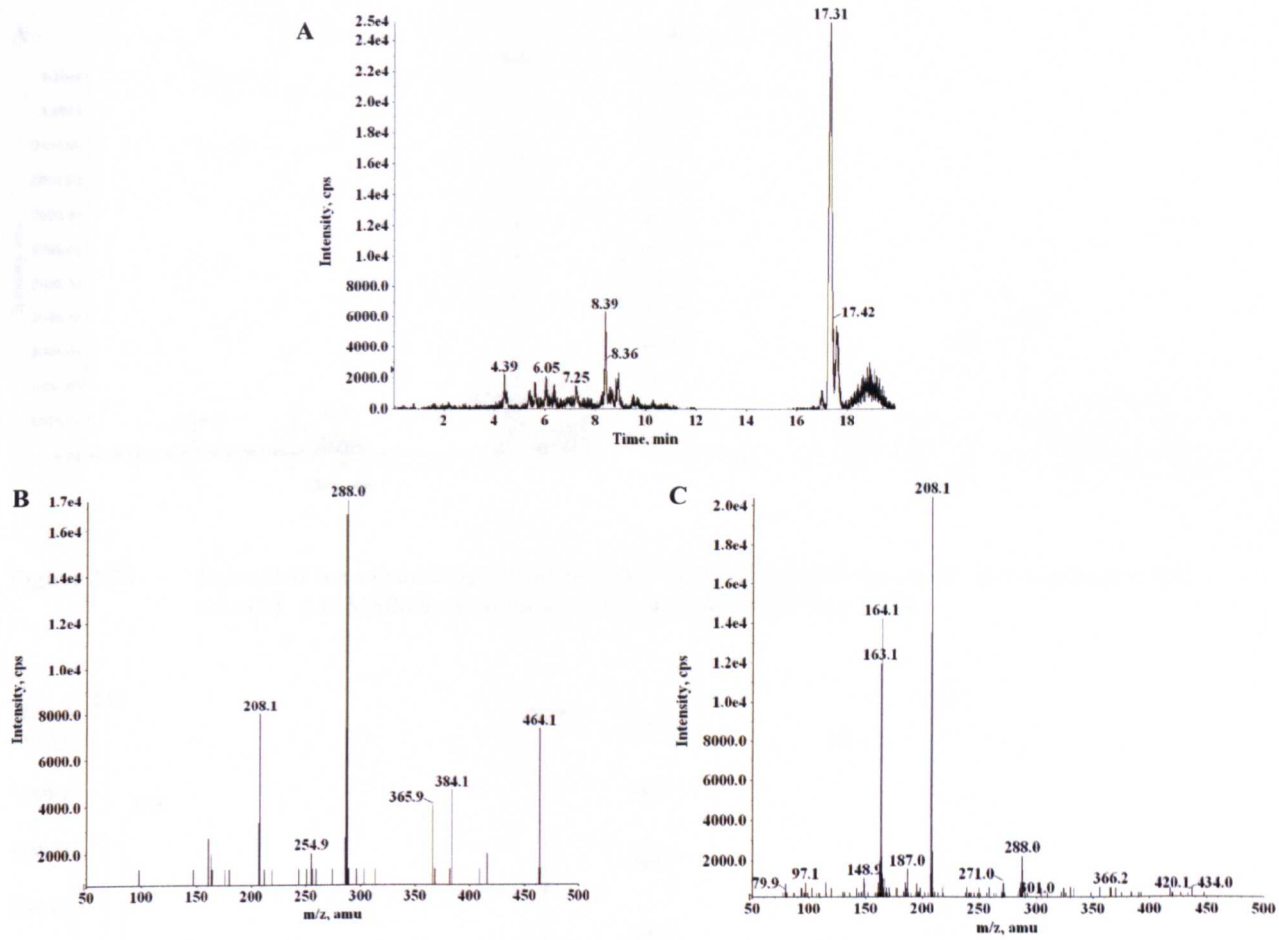

Figure 3.30 Extracted ion chromatogram of *m/z* 464 / 288 (A) and EPI spectra of the peak at 17.31 min (B and C). LC/MS/MS conditions were as described in Figure 3.8.

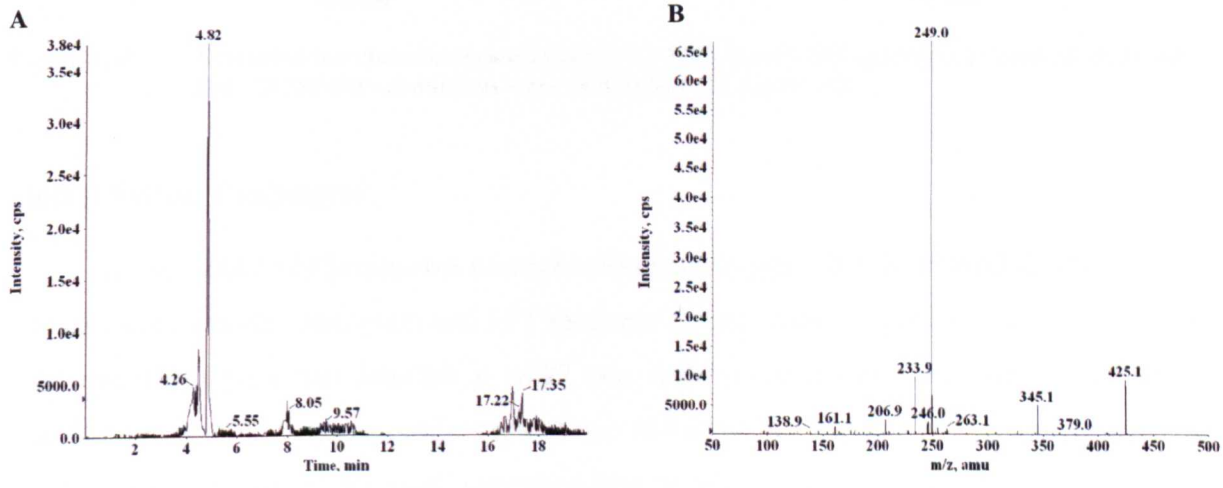

Figure 3.31 Extracted ion chromatogram of *m/z* 425 / 249 (A) and EPI spectrum of the peak at 4.82 min (B). LC/MS/MS conditions were as described for Figure 3.8.

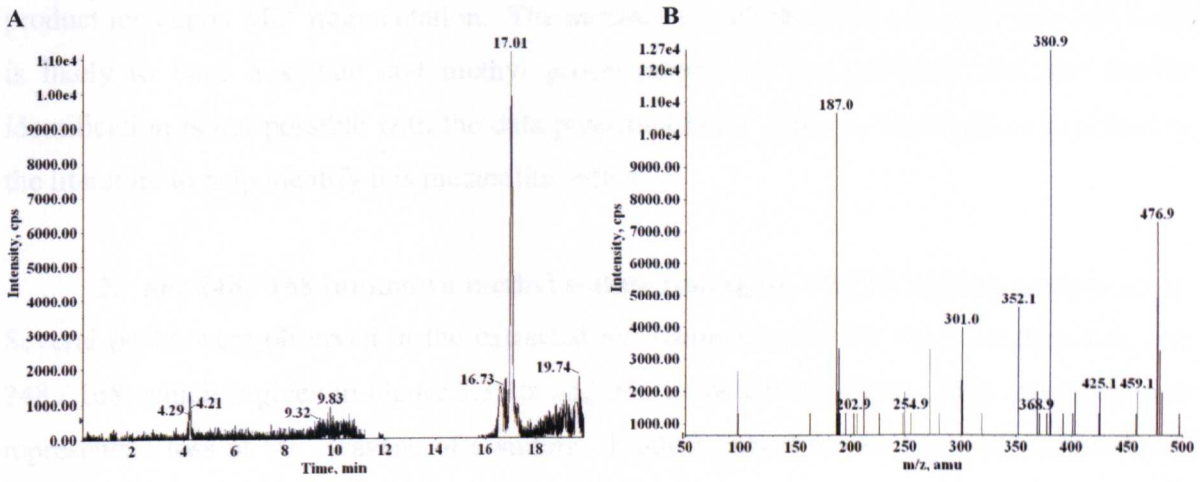

Figure 3.32 **Extracted ion chromatogram of *m/z* 557 / 477 (A) and EPI spectrum of the peak at 17.01 min (B). LC/MS/MS conditions were as described for Figure 3.8.**

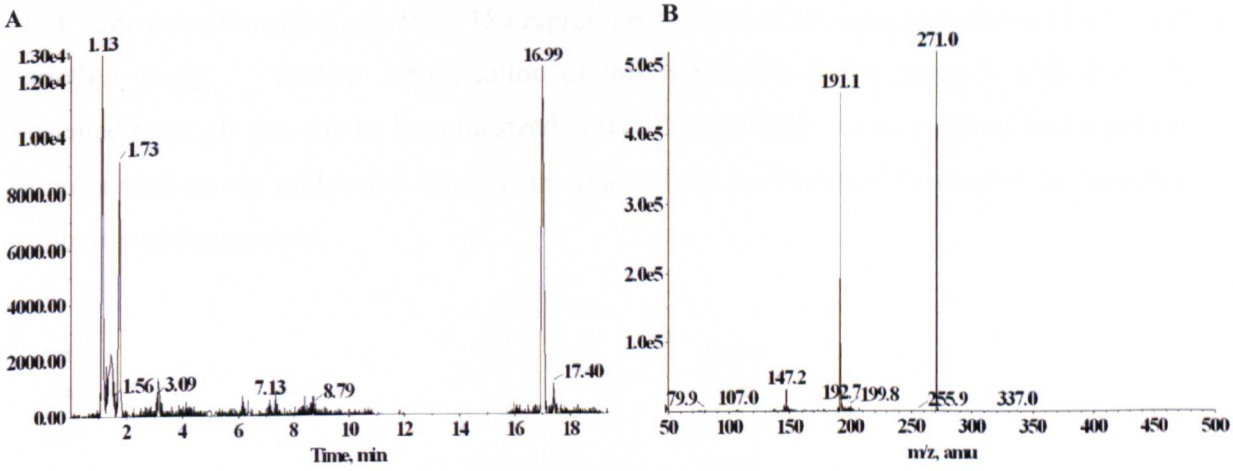

Figure 3.33 **Extracted ion chromatogram of *m/z* 271 / 191 (A) and EPI spectrum of peak at 16.99 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

Methyl Sulfate Conjuagtes

1. *m/z* 264 / 169 [unknown methyl sulfate conjugate – 0.3 % of total analyte area]

The extracted ion chromatogram and EPI spectrum for this mass transition is given in Figure 3.34; one major peak was detected at 16.97 min, and several minor peaks can also be seen. The EPI spectrum indicates a product ion of *m/z* 184 in addition to *m/z* 169; the latter product ion represents a loss of 80 amu, corresponding to the cleavage of a sulfate. The mass transition *m/z* 184 / 169 represents the loss of 15 amu, and could indicate cleavage of a methyl group. The precursor ion for the polyphenol standard gallic acid is *m/z* 169, but the characteristic product ion of *m/z* 125 seen with this standard is not observed in the EPI spectrum given in Figure 3.32. No polyphenol standards analysed in this study gave these

product ions upon MS^2 fragmentation. The metabolite with the mass transition *m/z* 264 / 169 is likely to have a sulfate and methyl group present on the molecule, but any further identification is not possible with the data presented here. There is no evidence provided in the literature to help identify this metabolite, either.

2. *m/z* 248 / 168 [unknown methyl sulfate conjugate – 0.1 % of total analyte area]

Several peaks were observed in the extracted ion chromatogram for the mass transition *m/z* 248 / 168, which is given in Figure 3.35 in addition to the EPI spectrum. This mass transition represents a loss of 80; cleavage of a sulfate. Product ions detected after MS^2 included *m/z* 124, *m/z* 153 and *m/z* 204 in addition to *m/z* 168. The same product ions were noted in the EPI spectra from all the peaks in the extracted ion chromatogram, however their intensities varied. The product ion of *m/z* 204 could represent the loss of a carboxylic acid (44), as could *m/z* 168 / 124. The mass transition *m/z* 168 / 153 represents the loss of 15 amu, or possibly cleavage of a methyl group. Further identification of this metabolite is not possible with the data presented here; all that can be hypothesized is that there is likely to be a sulfate and a methyl group present on the molecule. There is no evidence in the literature to support the presence or identity of this analyte.

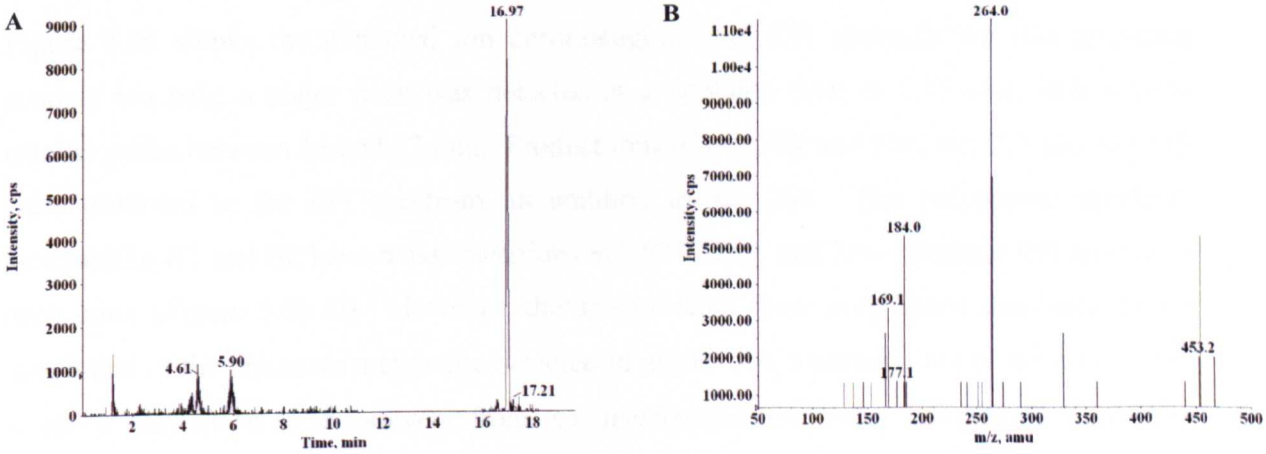

Figure 3.34 **Extracted ion chromatogram of *m/z* 264 / 169 (A) and EPI spectrum of the peak at 16.97 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

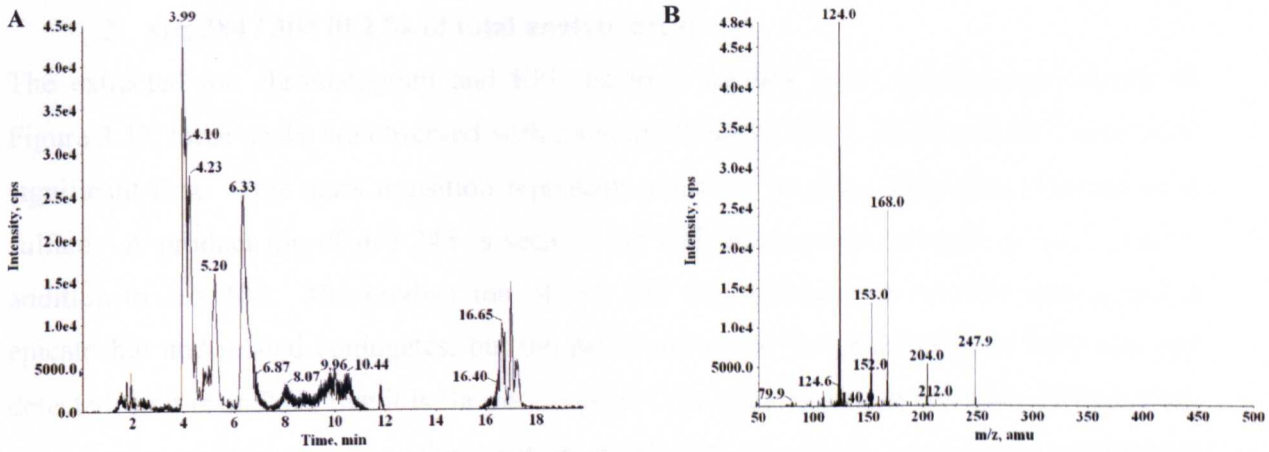

Figure 3.35 **Extracted ion chromatogram of *m/z* 248 / 168 (A) and EPI spectrum of the peak at 3.99 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

Unknown Conjuagtes

In some cases, after CID of the unknown metabolites, no fragments common to polyphenols were observed in the spectra. Thus, it was concluded that these metabolites may not be of polyphenolic origin, and are therefore beyond the scope of this project; they remain unidentified.

1. *m/z* 577 / 289 [1.5 % of total analyte area]

Figure 3.36 shows the extracted ion chromatogram and EPI spectrum for this precursor product ion pair; a major peak was detected at a retention time of 1.12 min, with several smaller peaks between 16 and 17 min. Product ions of *m/z* 305, *m/z* 175, *m/z* 273 and *m/z* 159 were observed in the EPI spectrum, in addition to *m/z* 289. The polyphenol standards procyanidin B1 and B2 have mass transitions *m/z* 577 / 289, and have identical EPI spectra to each other (Figure 3.36 C). However, the spectra from these polyphenol standards do not match that of the unknown metabolite detected in urine. The retention time of the major peak is on or very close to the solvent front, so interference from other compounds may have affected the EPI spectrum; the minor peaks are present in a low concentration so the EPI spectra of these is also unreliable. Therefore, it is not possible to propose an identity for this mass transition, based on the data supplied here. There are no characteristic losses of certain masses which have been observed with analytes and standards previously, and therefore this analyte may not be of polyphenol origin. The literature lacks any further information on such a metabolite.

2. *m/z* 384 / 304 [0.2 % of total analyte area]

The extracted ion chromatogram and EPI spectrum for this mass transition are shown in Figure 3.37; three peaks are observed with retention times of 16.59, 16.94 and 10.3 min, most significant first. This mass transition represents a loss of 80 amu, indicating cleavage of a sulfate. A product ion of *m/z* 245 is seen in the EPI spectrum of the peak at 10.3 min, in addition to *m/z* 304. The product ion of *m/z* 245 was observed in the EPI spectra of (-) epicatechin and related conjugates, but the precursor ion of this standard (*m/z* 289) was not detected, so it is unlikely that it is flavanol related. The mass transition *m/z* 384 / 304 contains masses of an even nature, something not observed with any of the polyphenolic metabolites which have been tentatively identified so far. This may indicate that the metabolite is not of polyphenol origin, but further identification is not possible. There is no data in the literature to support the presence or identity of this metabolite.

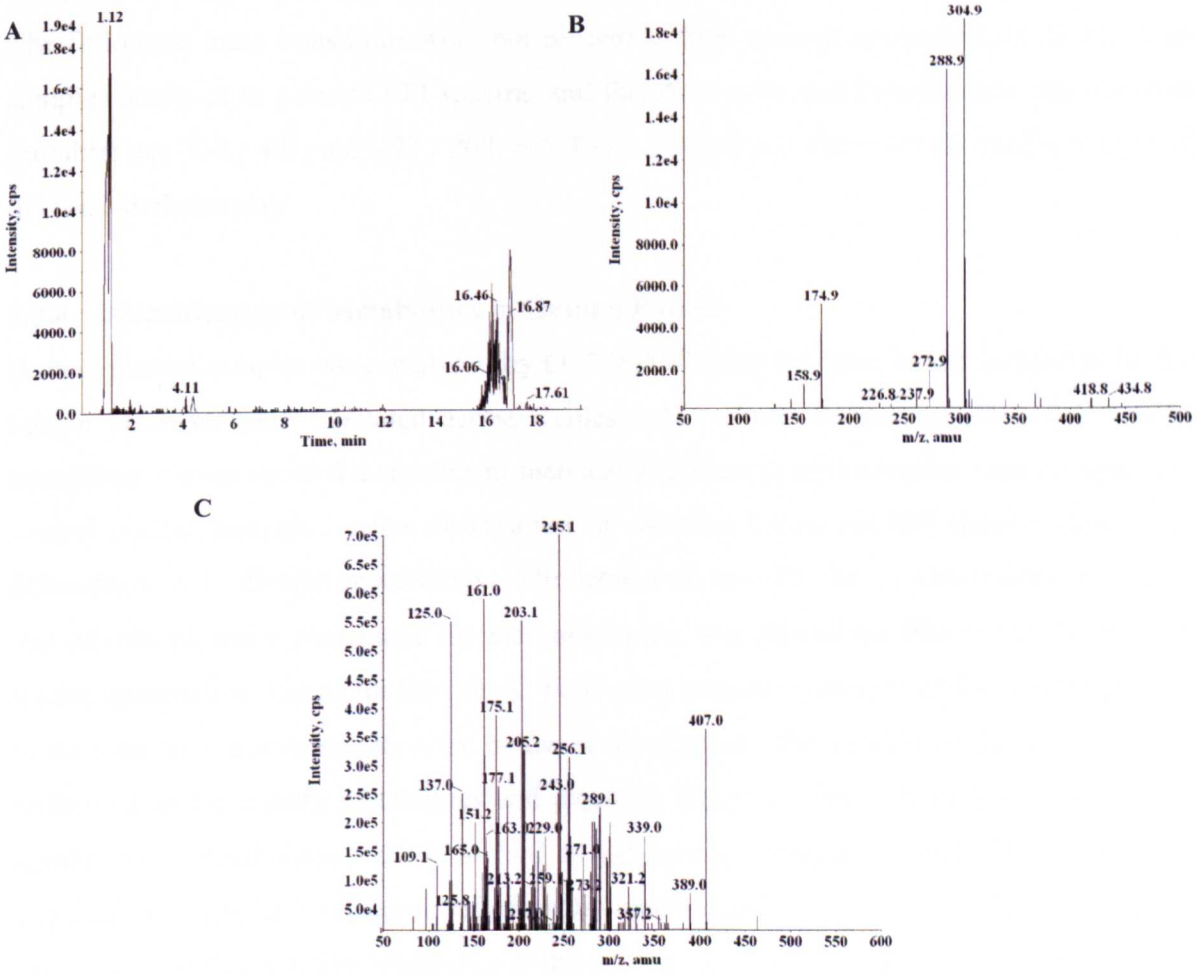

Figure 3.36 **Extracted ion chromatogram of *m/z* 577 / 289 (A), EPI spectrum of the peak at 1.12 min (B) and EPI spectrum of the polyphenol standard procyanidin B1 (C). LC/MS/MS conditions were as described in Figure 3.8.**

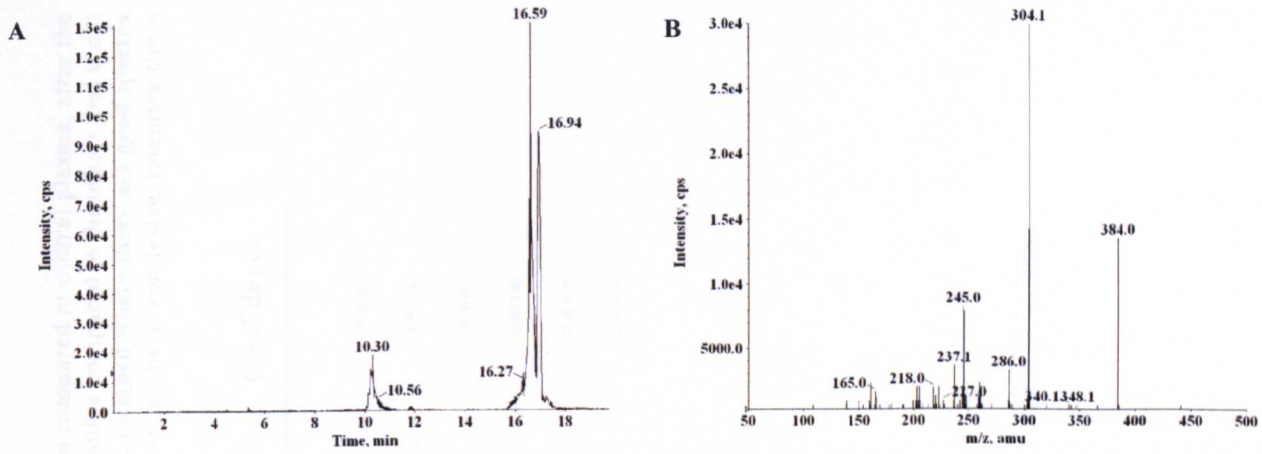

Figure 3.37 **Extracted ion chromatogram of *m/z* 384 / 304 (A) and EPI spectrum of the peak at 16.39 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

Summary

In summary, ten urinary metabolites have been partly identified, with elimination of possible identities in some cases, but further identification was not possible with the data presented. The following mass transitions were not present in high enough concentrations in the urine samples analysed to provide EPI spectra, and therefore even part identification has not been possible; *m/z* 737 / 481, *m/z* 273 / 208, *m/z* 705 / 353 and *m/z* 529 / 449 (in total < 0.15 % of the total analyte area).

3.3.4 Identification of Metabolites in Human Plasma

Human plasma samples were analysed by LC/MS/MS using the same MRM method as for the human urine samples, tabulated across Tables 2.12 – 2.14, chapter 2. Out of 115 mass transitions, eleven showed a significant increase when test plasma samples were compared to control plasma samples. After observation of retention times and EPI spectra, these were determined as 11 distinct metabolites. The total peak area for the 11 metabolites of interest was calculated, and a percentage for each metabolite was determined (the values for the area are not quantitative, just as for the urine). Four mass transitions accounted for over ten percent of the total area; the remainder were below seven percent. The process of identification was performed as for urinary metabolites and tentative identities were assigned to each of these metabolites. Metabolites were classified as aglycones, conjugated metabolites, conjugated ring fission metabolites and unidentified apple-related metabolites. Table 3.5 details the mass transitions and their relative abundance in the plasma. Evidence to support the identities of the mass transitions proposed in the table is given in the remainder of the chapter.

Table 3.5 **The following table details the mass transitions with the greatest increases in peak area, when test plasma was compared to control plasma, after the initial screening process. The percentage area represents the relative abundance of that particular mass transition compared to the others, but is not quantitative due to different metabolites giving different responses by MS analysis. Confidence level **** is assigned where there are good spectra and reference to the metabolite in the literature; confidence levels lower than this are given when spectra may not be as conclusive, retention times may fall in and around the solvent front and/or they may not have been reported in the literature.**

m/z	% Peak Area	Possible Identity	Confidence
381 / 301	32.7	Unknown conjugate	
247 / 167	21.1	3, 4-Dihydroxyphenylacetic acid sulfate	***
383 / 207	15.8	5- (3′,4′–Dihydroxyphenyl valerolactone) –γ- glucuronide	****
287 / 207	15.2	Dihydroxyphenyl valerolactone sulfate	***
465 / 289	7.2	(-/+) Epicatechin-3′-glucuronide	****
369 / 289	2.6	(-/+) Epicatechin-3′ or 4′-sulfate	****
163 / 119	1.9	*p*-Coumaric acid	****
289 / 124	1.3	Unknown conjugate	
273 / 167	0.5	Phloretin	**
353 / 191	0.6	Unknown conjugate	
289 / 109	0.1	Unknown conjugate	

3.3.4.1 Identification of Aglycone Metabolites in Human Plasma

1. *m/z* 273 / 167 [phloretin – 0.5 % of total analyte area]

Figure 3.38 shows the extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 273 / 167; one major peak was detected at 1.58 min, with two additional peaks at 7.23 and 8.48 min and several minor peaks. EPI spectra of all the peaks indicated product ions of *m/z* 179, *m/z* 123 and *m/z* 93 in addition to *m/z* 167; these ions were observed in the EPI spectrum of the polyphenol standard phloretin, (r_t of 7.2 min; same as observed for the metabolite). This suggests that phloretin may have been detected in the plasma; derived from phloretin-2'-glucuronide detected in the urine. Marks *et al.* [72], report the presence of phloretin in ileal fluid after consumption of cider, but not in plasma. Kahle *et al.* [8, 99], also report the presence of the glucuronide conjugate of phloretin in the ileal fluid of volunteers after consumption of cloudy apple juice; plasma was not analysed in either study.

2. *m/z* 163 / 119 [*p*-coumaric acid – 1.9 % of total analyte area]

The extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 163 / 119 are given in Figure 3.39; several peaks were detected with the major one eluting at 5.09 min. Product ions of *m/z* 93 and *m/z* 119 were observed in the EPI spectrum; both of which were previously detected with the polyphenol standard *p*-coumaric acid, which had a retention time of 5.1 min. These data suggest that the mass transition *m/z* 163 / 119 detected in plasma, is the metabolite *p*-coumaric acid, which was not found in urine. Urpi-Sarda *et al.* [83], report the presence of *p*-coumaric acid in the plasma of human volunteers after consumption of cocoa.

Summary

It has been possible to identify two aglycone metabolites in human plasma;

1. Phloretin
2. *p*-Coumaric acid

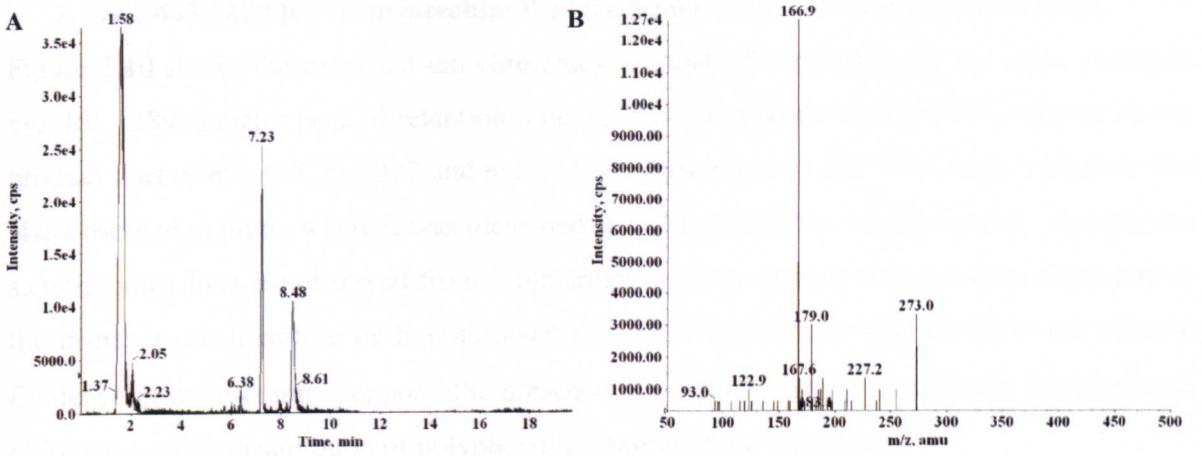

Figure 3.38 **Extracted ion chromatogram of *m/z* 273 / 167 (A) and EPI spectrum of the peak at 7.23 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

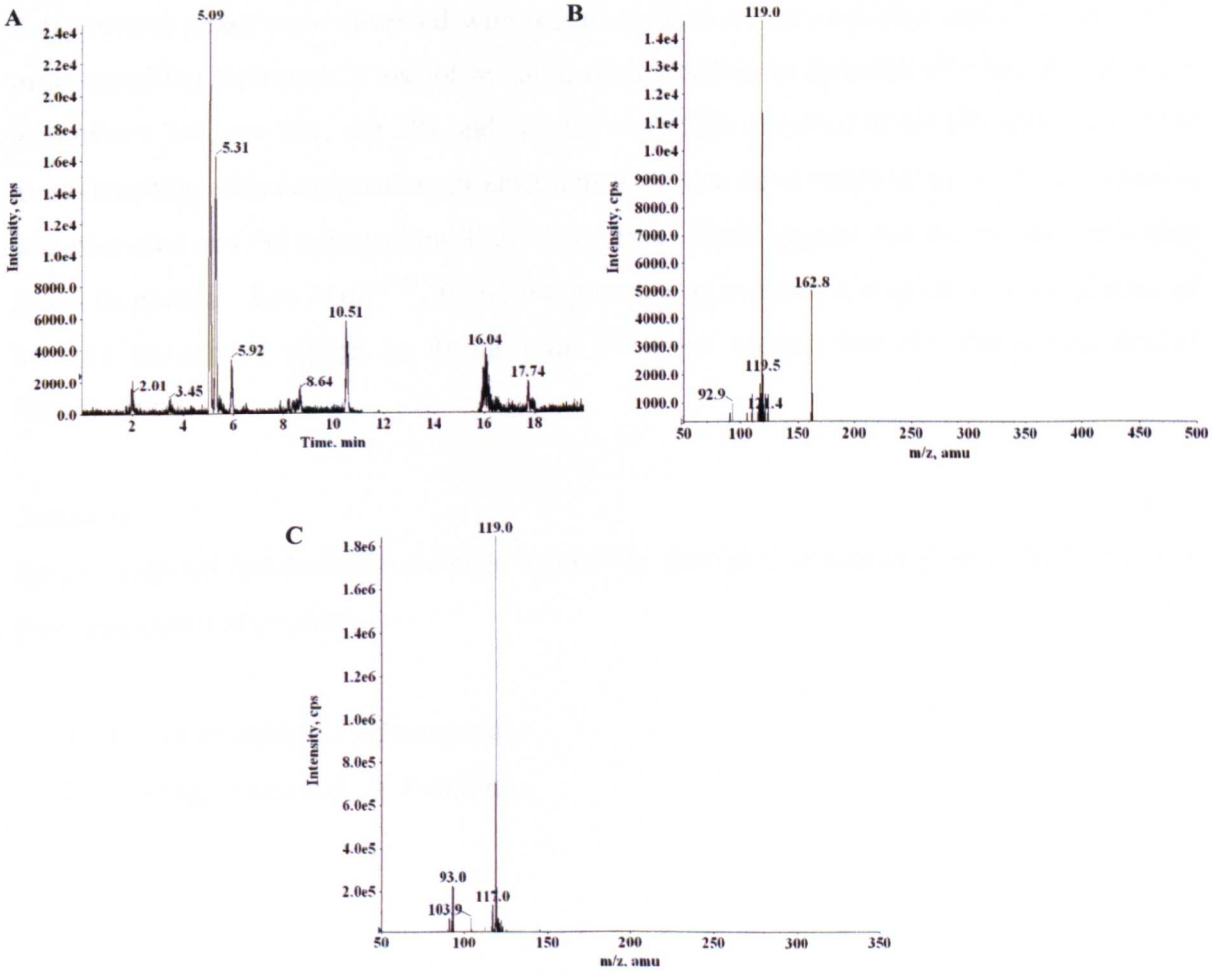

Figure 3.39 **Extracted ion chromatogram of *m/z* 163 / 119 (A), EPI spectrum of the peak at 5.09 min (B) and EPI spectrum of the polyphenol standard *p*-coumaric acid (C). LC/MS/MS conditions were as described in Figure 3.8.**

3.3.4.2 Identification of Conjugated Metabolites in Human Plasma

***1. m/z* 465 / 289 [(-/+) epicatechin-3′-glucuronide – 7.2 % of total analyte area]**

Figure 3.40 shows the extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 465 / 289; a major peak of retention time 5.27 min was observed. The EPI spectrum shows product ions of *m/z* 427, *m/z* 327 and *m/z* 245 in addition to *m/z* 289. This mass transition was also observed in urine, where it was identified as (-/+) epicatechin-3′-glucuronide. Exactly the same product ions are observed from fragmentation of the analyte in the plasma compared to the urine; it can therefore be hypothesised that this metabolite is also found in the plasma. Evidence in the literature supports the presence of (-) epictechin-3′-glucuronide in the plasma of humans after consumption of polyphenol containing food and drink [83, 97].

***2. m/z* 369 / 289 [(-/+) epicatechin- 3′ or 4′ – sulfate – 2.6 % of total analyte area]**

The extracted ion chromatogram and EPI spectrum for this mass transition is given in Figure 3.41; several peaks were observed with retention times of between 16.5 and 17.5 min. This mass transition represents a loss of 80 amu, corresponding to cleavage of a sulfate. Product ions of *m/z* 245, *m/z* 231, *m/z* 187 and *m/z* 277 were also observed in the EPI spectrum. This mass transition and corresponding product ions were also observed in urine, where the identity was proposed as (-/+) epicatechin- 3′ or 4′ – sulfate. Data suggest that the metabolite is also found in plasma. Lee *et al.* [76], found the presence of sulfated epicatechin in the plasma of humans, contrary to reports by Roura *et al.* [81] which suggest that only the glucuronidated form is found.

Summary

Two conjugated metabolites have been tentatively identified in human plasma; both of which were also detected in urine.

1. (-/+) Epicatechin-3′-glucuronide
2. (-/+) Epicatechin-3′ or 4′-sulfate

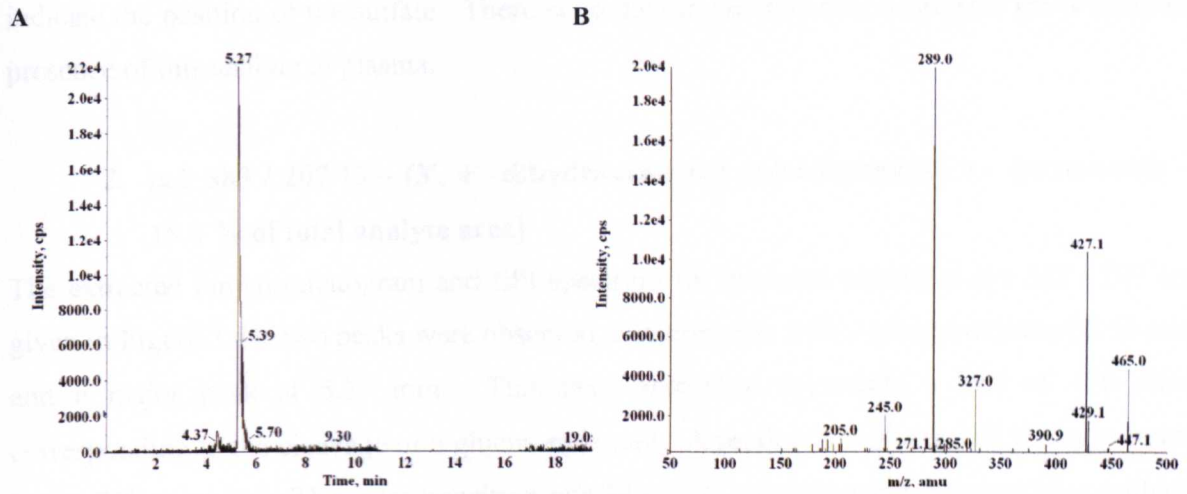

Figure 3.40 **Extracted ion chromatogram of *m/z* 465 / 289 (A) and EPI spectrum of the peak at 5.27 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

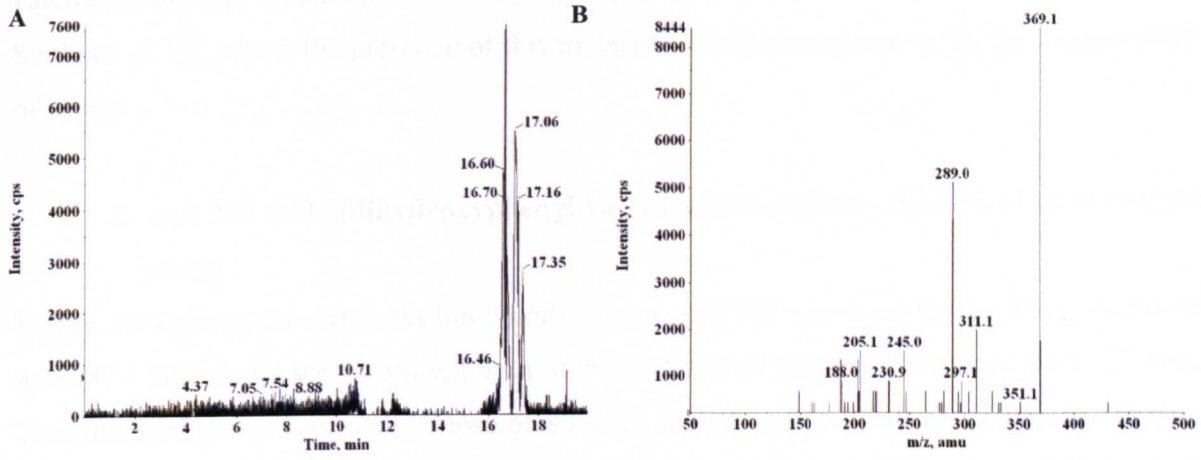

Figure 3.41 **Extracted ion chromatogram of *m/z* 369 / 289 (A) and EPI spectrum of the peak at 16.6 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

3.3.4.3 Identification of Conjugated Ring Fission Metabolites in Human Plasma

1. *m/z* 247 / 167 [3, 4-dihydroxyphenylacetic acid sulfate – 21.1 % of total analyte area]

Figure 3.42 shows the extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 247 / 167; several peaks were observed the majority of which have retention times of around 15 to 16 min. This mass transition represents the loss of 80 amu, corresponding to the cleavage of a sulfate unit. The EPI spectrum indicates product ions of *m/z* 123, *m/z* 149, *m/z* 184 and *m/z* 212, in addition to *m/z* 167, which are identical to the ones observed with the metabolite *m/z* 423 / 247 found in urine, which was identified as 3, 4-dihydroxyphenylacetic acid sulfoglucuronide. This would suggest that 3, 4-dihydroxyphenylacetic acid sulfate (without the glucuronide) is found circulating in the plasma; however there is no evidence to

indicate the position of the sulfate. There is no data in the literature to support the identity or presence of this analyte in plasma.

2. ***m/z* 383 / 207 [5 – (3′, 4′ -dihydroxyphenyl valerolactone) - γ - glucuronide – 15.8 % of total analyte area]**

The extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 383 / 207 are given in Figure 3.43; two peaks were observed, a minor peak with a retention time of 5.53 min and a major peak at 5.77 min. This mass transition represents a loss of 176 amu, corresponding to the cleavage of a glucuronide unit. A product ion of *m/z* 163 was also noted in the EPI spectrum. The mass transition *m/z* 383 / 207 with the same fragmentation pattern, was detected in urine where the metabolite was identified as 5 – (3′, 4′ -dihydroxyphenyl valerolactone) - γ - glucuronide. Evidence suggests this is also present in the plasma. Urpi-Sarda *et al.* [83], report the presence of this metabolite in human plasma after the consumption of cocoa.

3. ***m/z* 287 / 207 [dihydroxyphenyl valerolactone sulfate – 15.2 % of total analyte area]**

Figure 3.44 shows the extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 287 / 207; peaks were detected with retention times of around 13 min and 16 to 17 min. This mass transition represents a loss of 80 amu, indicating the cleavage of a sulfate from a conjugate. The EPI spectrum shows a product ion of *m/z* 163, in addition to *m/z* 207, which were also observed in urine, where the analyte was identified as dihydroxyphenyl valerolactone sulfate; evidence suggests the presence of this analyte in plasma, also. Urpi-Sarda *et al.* [83] did not find this metabolite in plasma, only in urine.

Summary

Three conjugated ring fission metabolites have been identified in human plasma;

1. 3, 4 – Dihydroxyphenylacetic acid sulfate
2. 5 – (3′, 4′ – Dihydroxyphenyl) - γ - valerolactone glucuronide
3. Dihydroxyphenyl valerolactone sulfate

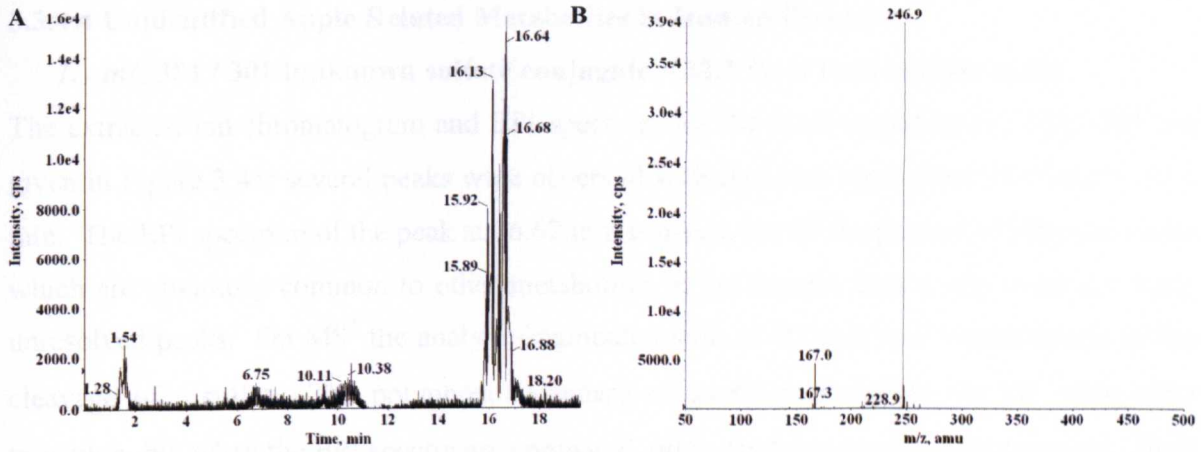

Figure 3.42 **Extracted ion chromatogram of *m/z* 247 / 167 (A) and EPI spectrum of the peak at 16.64 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

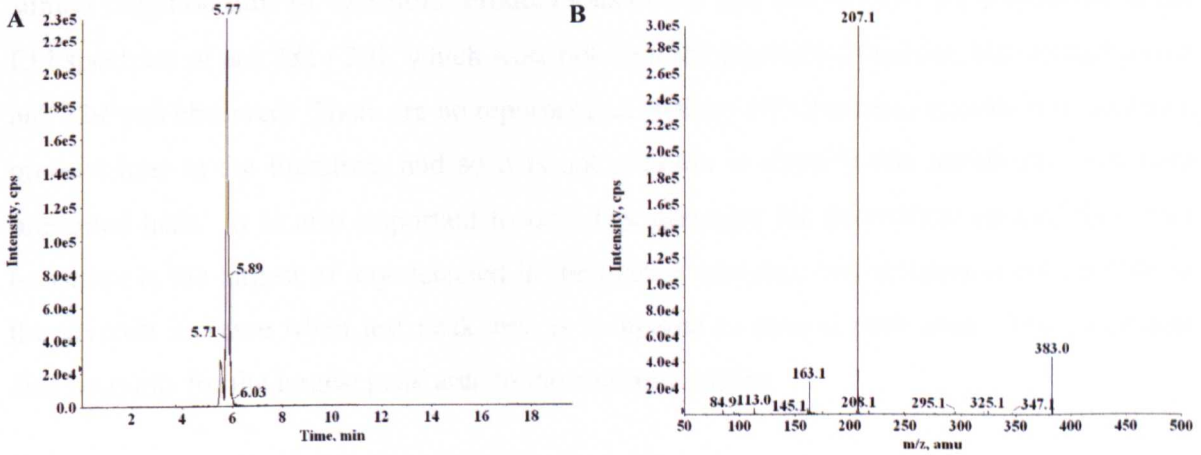

Figure 3.43 **Extracted ion chromatogram of *m/z* 383 / 207 (A) and EPI spectrum of the peak at 5.77 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

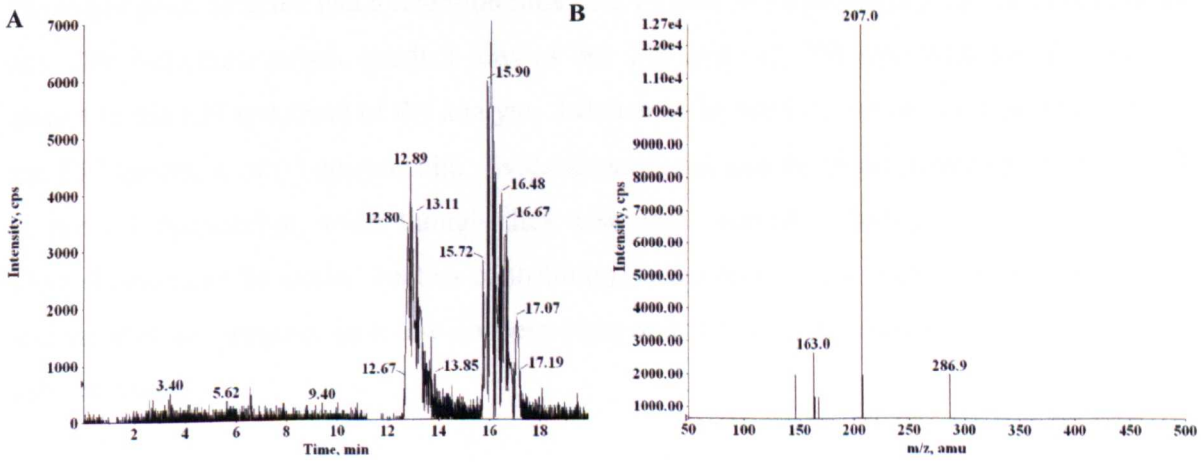

Figure 3.44 **Extracted ion chromatogram of *m/z* 287 / 207 (A) and EPI spectrum of the peak at 15.9 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

3.3.4.4 Unidentified Apple Related Metabolites in Human Plasma

1. m/z 381 / 301 [unknown sulfate conjugate – 32.7 % of total analyte area]

The extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 381 / 301 are given in Figure 3.45; several peaks were observed with retention times from 14.5 min to 17.5 min. The EPI spectrum of the peak at 16.62 min is given, but all the peaks yield the same ions which are obviously common to other metabolites in the sample, hence why there are many unresolved peaks. On MS^2 the analyte fragmented with an 80 amu loss, characteristic of the cleavage of a sulfate. The polyphenol standard of quercetin-3′-sulfate has the same mass transition, but when the EPI spectra are compared, other product ions do not correspond. Both quercetin and quercetin-3′-sulfate yield product ions of *m/z* 179 and *m/z* 151, which are not observed in the EPI spectra of the analyte. The standard of quercetin-3′-sulfate also has a similar retention time of 17.3 min. Product ions of *m/z* 255 and *m/z* 291 were observed in the EPI spectrum of *m/z* 381 / 301, which were not seen for quercetin-3′-sulfate, but another ion of *m/z* 229 was observed. There are no reports of an analyte with this mass transition or common product ions in the literature, and so it is not possible to identify this metabolite from data presented here. It is also important to note that although the percentage area of this mass transition is the largest of any detected in the plasma samples, this metabolite does not show the greatest increase when test peak area is compared to control peak area. The metabolite also accounts for the largest peak area in the control samples.

2. m/z 289 / 124 [unknown conjugate – 1.3 % of total analyte area]

Figure 3.46 details the extracted ion chromatogram and EPI spectrum for this mass transition; the major peak detected had a retention time of 2.13 min. (-) Epicatechin has the precursor ion *m/z* 289, but characteristic product ions of *m/z* 245 and *m/z* 203 seen with this flavanol are absent in the EPI spectrum of the analyte. Likewise, the product ion of *m/z* 124 is not seen in the EPI spectrum of (-) epicatechin. Evidence suggests that the mass transition *m/z* 289 / 124 is not (-) epicatechin, even though they also have similar retention times. No further identification can be made. Factors contributing to the uncertainty of the identification of this analyte include presence in a low concentration and the peak detected is on or very near the solvent front.

3. m/z 353 / 191 [unknown conjugate – 0.6 % of total analyte area]

The extracted ion chromatogram and EPI spectrum for the mass transition *m/z* 353 / 191 are given in Figure 3.47; the major peak had a retention time of 4.21 min. The EPI spectrum of the peak at 4.21 min shows product ions of *m/z* 295 and *m/z* 315 in addition to *m/z* 191. The polyphenol standard chlorogenic acid has the same precursor / product ion mass pairs as seen for this unknown metabolite, but observation of the fragmentation pattern of this standard revealed that they are very different. The mass transition *m/z* 353 / 191 detected in plasma cannot be chlorogenic acid. Further identification of this metabolite cannot be made from data presented here, or from the literature.

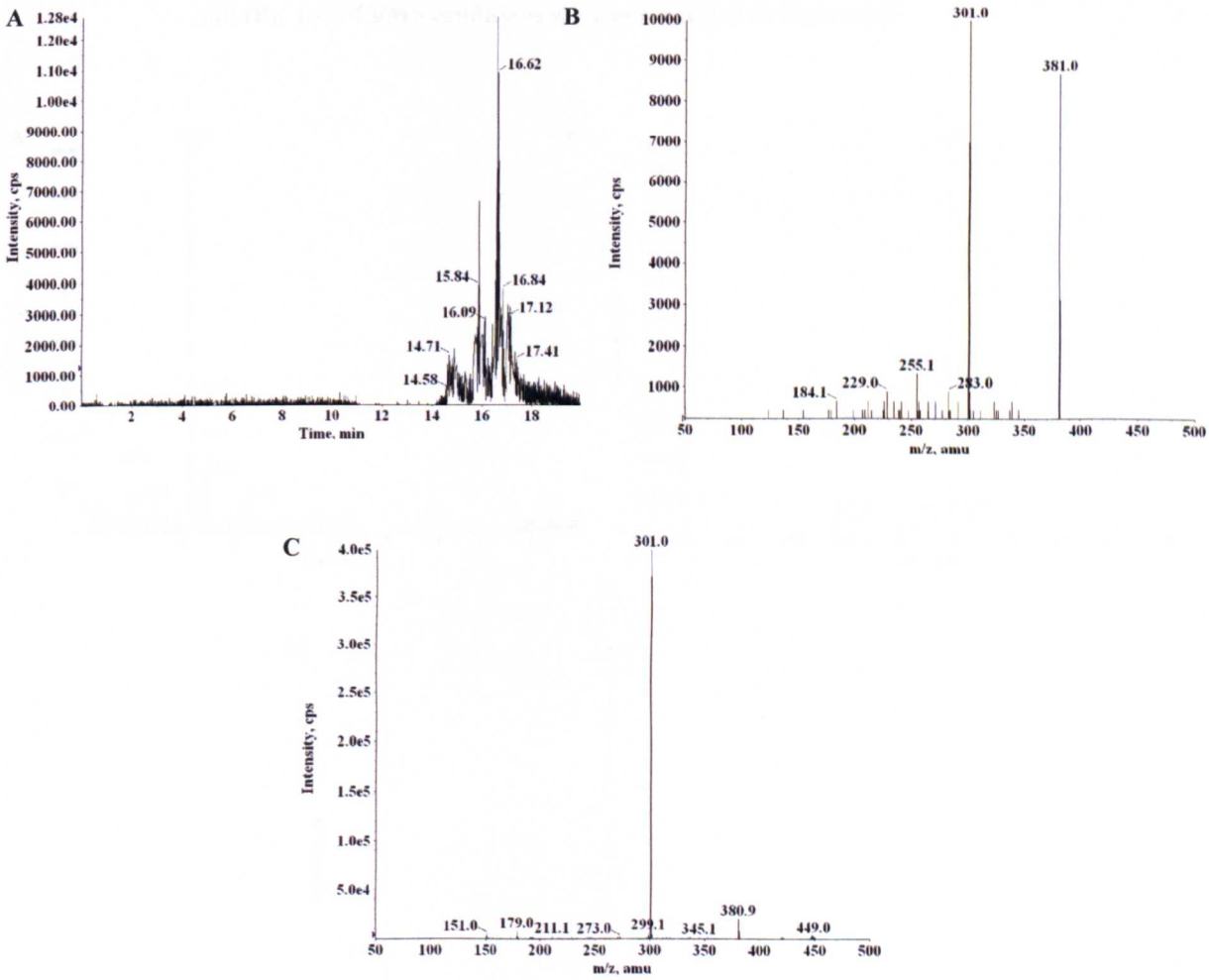

Figure 3.45 **Extracted ion chromatogram of *m/z* 381 / 301 (A), EPI spectrum of the peak at 16.62 min (B) and EPI spectrum of the polyphenol standard quercetin-3′-sulfate (C). LC/MS/MS conditions were as described in Figure 3.8.**

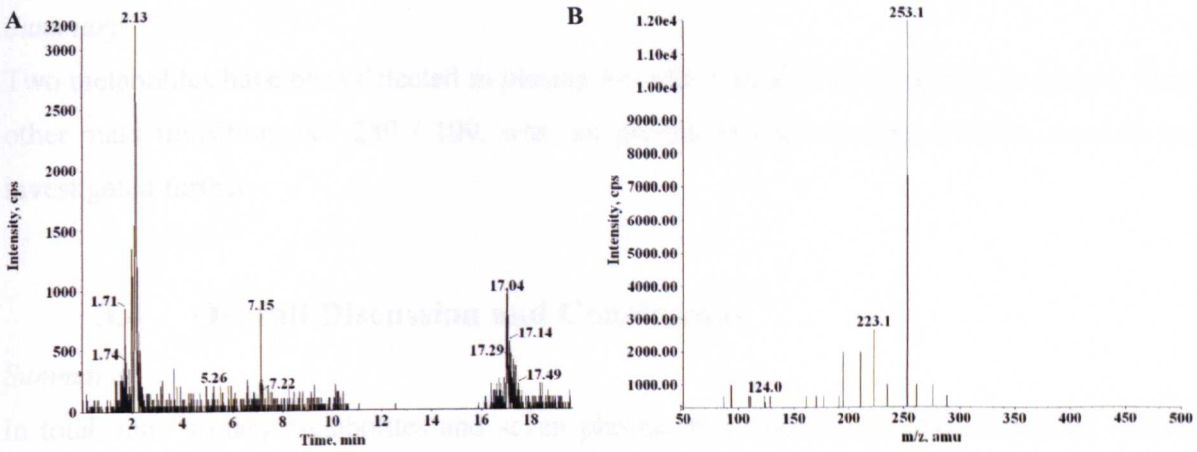

Figure 3.46 **Extracted ion chromatogram of *m/z* 289 / 124 (A) and EPI spectrum of the peak at 2.13 min (B). LC/MS/MS conditions were as described in Figure 3.8.**

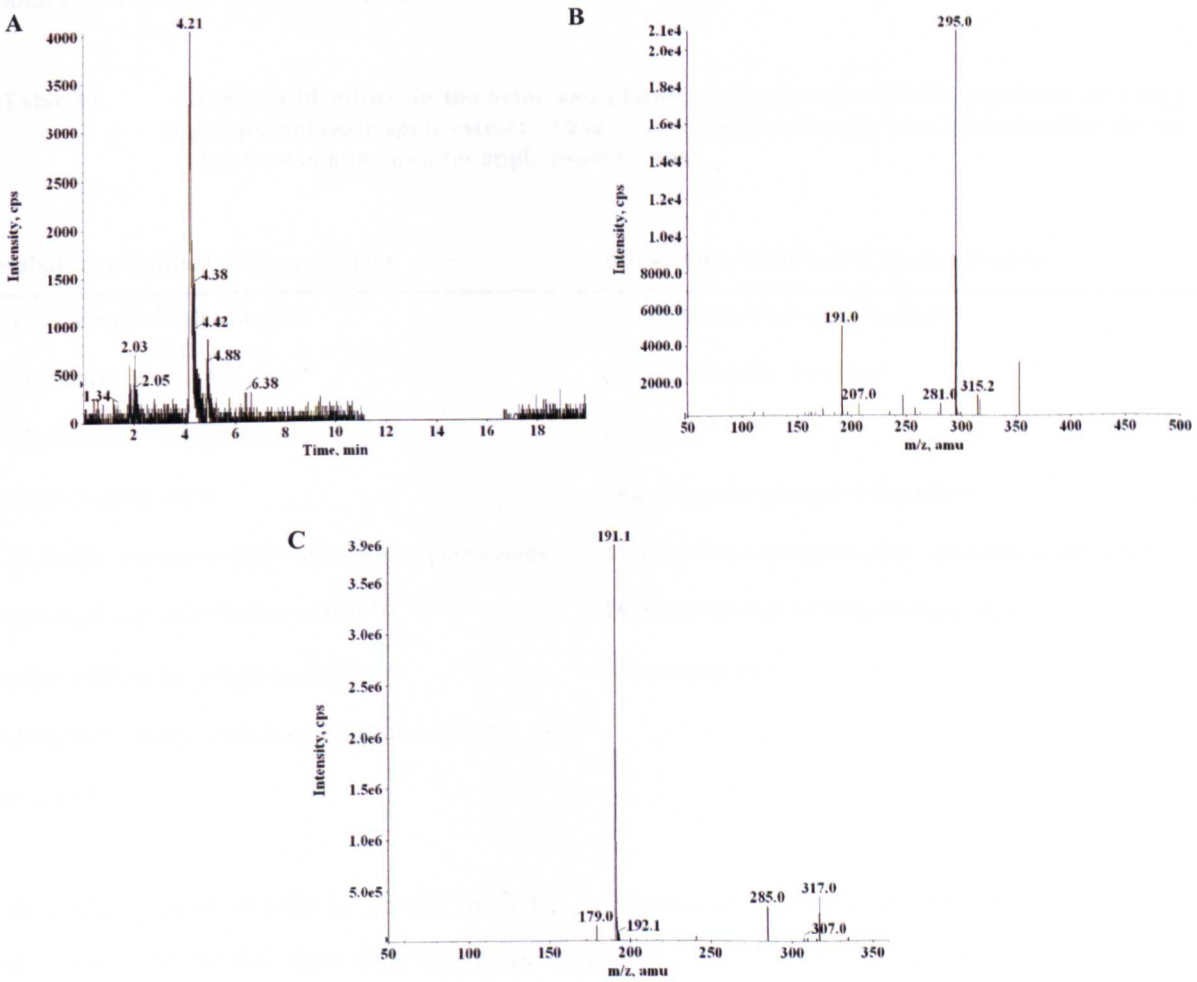

Figure 3.47 **Extracted ion chromatogram of *m/z* 353 / 191 (A), EPI spectrum of the peak at 4.21 min (B) and EPI spectrum of the polyphenol standard chlorogenic acid (C). LC/MS/MS conditions were as described in Figure 3.8.**

Summary

Two metabolites have been detected in plasma for which an identity cannot be proposed. One other mass transition, *m/z* 289 / 109, was not present in high enough concentrations to be investigated further.

3.4 Overall Discussion and Conclusions

Summary

In total, nine urinary metabolites and seven plasma metabolites have been identified (Table 3.6), with a further twelve urinary and two plasma metabolites partly identified. Definitive identification and structural elucidation was not always possible due to a lack of synthetic standards for comparison, but strong evidence for identity has been provided by LC/MS/MS analysis and MS^2 fragmentation.

Table 3.6 **Analytes identified in the urine and plasma of humans after the consumption of a high polyphenol cider apple extract. Those marked with an asterix have been identified for the first time in humans after apple consumption.**

Metabolites Identified in Human Urine	Metabolites Identified in Human Plasma
(-/+) Epicatechin-3'-glucuronide*	(-/+) Epicatechin-3'-glucuronide*
(-/+) Epicatechin-3' or 4'-sulfate*	(-/+) Epicatechin-3'-sulfate*
(-/+) Epicatechin sulfoglucuronide*	Phloretin
Phloretin-2'-glucuronide	Dihydroxyphenylacetic acid sulfate*
5 – (3',4'-Dihydroxyphenyl)-γ-valerolactone glucuronide*	5 – (3',4'-Dihydroxyphenyl)-γ-valerolactone glucuronide*
Dihydroxyphenyl valerolactone sulfate*	Dihydroxyphenyl valerolactone sulfate*
Dihydroxyphenyl valerolactone disulfate*	*p*-Coumaric acid*
3, 4-Dihydroxyphenylacetic acid sulfoglucuronide*	
Sinapic acid*	

Metabolites marked with an asterix in Table 3.6, have been identified in the urine and plasma of humans for the first time after apple consumption. The only previously reported metabolite of apples in humans is phloretin-2'-glucuronide [8, 72, 99].

Fate of (-/+) Epicatechin in Humans Post Ingestion of a High Polyphenol Cider Apple Extract

Glucuronide and sulfate metabolites of (-/+) epicatechin were identified in human urine and plasma, with the addition of a sulfoglucuronide conjugate present in the urine (Figure 3.48). All metabolites detected in urine are derived from the blood, so it is likely that the sulfoglucuronide conjugate of (-/+) epicatechin was present in the plasma also, but may have been broken down into the sinlge glucuronide or sulfate conjugates.

Glucuronide and sulfate conjugates of (-) epicatechin have been previously detected in human urine and plasma after ingestion of tea and cocoa; in this study, (-/+) epicatechin was found exclusively in the conjugated form in both urine and plasma, (in agreement with the literature [76]) and (-/+) epicatechin conjugates accounted for 1.5 % of the total analyte area in urine and 9.7 % of the total analyte area in plasma. The major urinary conjugates of (-/+) epicatechin were the sulfate conjugates, accounting for 0.9 % of the total analyte area, followed by the glucuronide conjugates (0.4 % of the total analyte area) and the sulfoglucuronide (0.2 % of the total analyte area). The major plasma conjugate of (-/+) epicatechin was (-/+) epicatechin-3′-glucuronide, which accounted for 7.2 % of the total analyte area. In the literature, (-) epicatechin-3′-glucuronide has been reported to be the major plasma glucuronide of (-) epicatechin in humans [73, 81, 97, 169], which agrees with the data presented here; there are a couple of reports of (-) epicatechin sulfoglucuronides in human biological samples, after consumption of cocoa and chocolate [81, 162].

Sulfate conjugates of (-/+) epicatechin are generally reported at higher concentrations than glucuronide conjugates in both urine and plasma, up to twice [76, 81]; in this research they account for approximately 2.5 times the proportion of glucuronide conjugates in urine. In plasma the opposite was observed; the glucuronide conjugates account for approximately three times the sulfate conjugates. Up to four different (-) epicatechin glucuronides [83] and three different (-) epicatechin sulfates have been reportedly detected in human urine after consumption of food and beverages containing (-) epicatechin [83, 169]. In this study, (-/+) epicatechin-3′ or 4′-sulfate was identified in urine, corroborated by other reports of (-) epicatechin metabolism [73, 76, 77, 83, 169]. In contrast to the findings presented here, Roura *et al.* [81] report that only the glucuronide form of (-) epicatechin was found in plasma and not the sulfate, after 40 g of cocoa powder was consumed by the volunteers. However, only one blood sample was taken, 2 h after cocoa consumption so the presence of the sulfate may have

been missed (time taken to maximum concentrations or excretion and biokinetic parameters are discussed in chapter 4). Mullen *et al.* [106] report the opposite; that sulfate conjugates of (-) epicatechin were present in plasma and not glucuronides, after 10 g of cocoa powder was consumed by the volunteers (6.7 mg of (-) epicatechin). This may be due to the low concentration of (-) epicatechin ingested; there is always competition between the phase II metabolism reactions of glucuronidation and sulfation, and at low concentrations sulfation predominates as it is a high affinity and low capacity pathway [11].

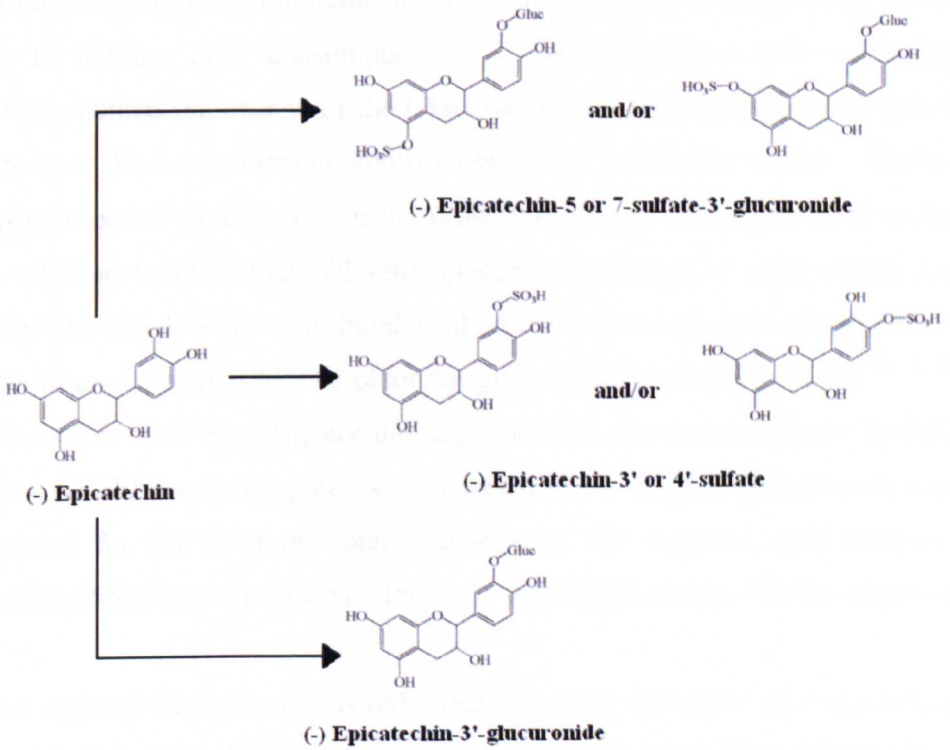

Figure 3.48 **Suggested metabolism pathway of (-/+) epicatechin in humans after consumption of a high polyphenol cider apple extract. (-/+) Epicatechin-5 or 7-sulfate-3′-glucuronide was the major urinary (-/+) epicatechin metabolite followed by (-/+) epicatechin-3′ or 4′-sulfate and (-/+) epicatechin-3′-glucuronide. In plasma, (-/+) epicatechin-3′-glucuronide was the major (-/+) epicatechin metabolite, followed by (-/+) epicatechin-3′ or 4′-sulfate. The sulfoglucuronide conjugate of (-/+) epicatechin was not found in plasma.**

A major difference between reports in the literature on epicatechin metabolism in humans and this study, is that here no methylated conjugates of (-/+) epicatechin were detected as major metabolites. This may be due to the difference in the source of the epicatechin, i.e. all the reports use epicatechin as a preparation or in tea/cocoa; (-/+) epicatechin metabolism will undoubtedly be affected by the matrix in which it is supplied. Another possibility is that the pathway for methylation may have become saturated by the high dose of the (-/+) epicatechin provided, or by the high dose of some of the other polyphenols in the cider apple extract.

Methylated conjugates may indeed have been formed but not in proportionally large enough concentrations to have warranted further investigation.

Some of the ring fission metabolites identified in this study may also have originated from the action of the gut microflora on (-/+) epicatechin; this is discussed at the end of this section.

Fate of Phloretin in Humans Post Ingestion of a High Polyphenol Cider Apple Extract

A glucuronide conjugate of phloretin, identified as phloretin-2′-glucuronide was detected in the urine of humans after consumption of a high polyphenol cider apple extract; this metabolite accounted for 14.5 % of the total analyte area making it the only non-ring fission metabolite to make a considerable contribution to the metabolite profile. Marks *et al.* [72], identified phloretin-2′-glucuronide in the urine and plasma of humans after consumption of cider, in addition to two other phloretin glucuronides found in urine which could not be structurally elucidated. They also found a sulfoglucuronide conjugate of phloretin, which was not detected here. The presence of phloretin glucuronide in ileostomy fluid [8, 72, 99] suggests that the liver and small intestine, not the large intestine, are responsible for formation of this metabolite. No phloretin conjugates were detected in plasma, but the aglycone was identified and accounted for 0.5 % of the total analyte area; this suggests rapid elimination of the glucuronidated form from the plasma. Phloretin has been found in ileal fluid previously [8].

It has been reported that phloretin is only found in apple and apple related products [67], and hence there is very limited information on its metabolism particularly in humans. However, Ito *et al.* [80], identified its presence in urine samples collected after consumption of grapefruit and orange juice, suggesting that its formation could be due to reductive opening of the C-ring of naringenin by the gut microflora. No naringenin was consumed in this study, so any phloretin detected must have been supplied directly by the high polyphenol cider apple extract.

Some of the ring fission metabolites identified in this study may also have originated from the action of the gut microflora on phloretin; this is discussed at the end of this section.

Fate of Chlorogenic Acid in Humans Post Ingestion of a High Polyphenol Cider Apple Extract

Various groups have reported that chlorogenic acid is stable during the passage through the small intestine and will be subsequently hydrolysed by the gut microflora [99]. In this study, although no conjugates of chlorogenic acid were identified, some may have been detected.

Increases in many of the peak areas for possible metabolites given in Tables 2.13 and 2.14, chapter 2, were noted in urine and plasma samples, in particular metabolites which have been tentatively identified as methylated and/or glucuronidated chlorogenic acid, methylated and/or sulfated caffeic acid, methylated and/or sulfated ferulic acid, methylated and/or glucuronidated ferulic acid and glucuronidated coumaric acid. The reason further investigation was not performed on these metabolites and identity was not confirmed, is that the metabolites did not show large enough differences between their peak areas when test samples were compared with control samples. Those metabolites which did show significant differences between test and control samples, still had peak areas which were much smaller than those for the metabolites which were investigated further (they accounted for <0.01 % of the total analyte area for all the MRM transitions monitored for).

In addition to these conjugates, some of the ring fission metabolites that have been identified here may also have originated from chlorogenic acid; sinapic acid was detected in urine (<0.1 % of the total analyte area) and *p*-coumaric acid (1.9 % of the total analyte area) was identified in plasma. Figure 3.49 suggests the metabolism pathway for the formation of sinapic acid and *p*-coumaric acid from chlorogenic acid. Observation of several peaks in the extracted ion chromatogram with the same *m/z* as the tentatively identified *p*-coumaric acid could indicate several conformations exist in the plasma. Urpi-Sarda *et al.* [83], report the presence of *p*-coumaric acid in urine and plasma, and *m*-coumaric acid in urine, after consumption of cocoa. In another study by the same research group [105], both *m*- and *p*-coumaric acids were found in human urine after cocoa consumption; *p*-coumaric acid was seen to increase by 467 percent compared to pre-dose levels. However, both these studies involved deconjugation of samples with β-glucuronidase and sulfatase enzymes so some of the detail of the metabolite profiles has been lost.

Chlorogenic acid is reported to be a constituent of coffee. In one study, after consumption of green coffee extract containing 170 mg of chlorogenic acid [179], the major urinary phenolic compounds detected were sinapic, gallic, *p*-hydroxybenzoic, and dihydrocaffeic acids; these represented ~85 % of the total amount of phenolic compounds in urine. In plasma, *p*-coumaric acid was detected and accounted for 1.9 % of the total phenolics. Other simple phenolic acids detected included caffeic, ferulic and isoferulic acids. The literature suggests that chlorogenic acids are subjected to extensive metabolism in humans, and are well absorbed [78].

Besides these two phenolic acids, some of the other ring fission metabolites identified in urine and plasma may also have originated from the action of the gut microflora on chlorogenic acid; this is discussed at the end of this section.

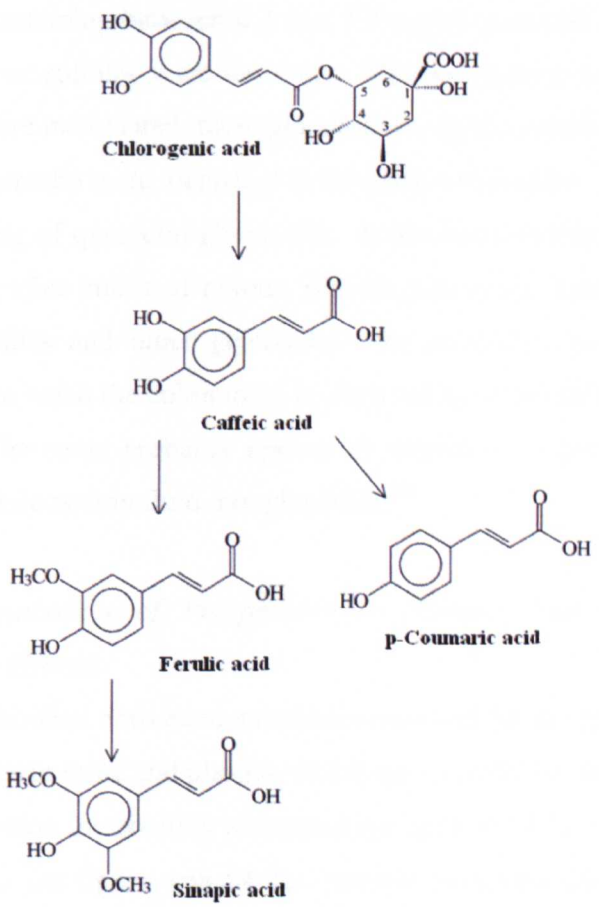

Figure 3.49 **Suggested metabolism pathway of chlorogenic acid in humans after consumption of a high polyphenol cider apple extract; *p*-coumaric acid was detected in plasma and sinapic acid was detected in the urine. Reactions involve the gut microflora.**

Fate of Quercetin in Humans Post Ingestion of a High Polyphenol Cider Apple Extract

The average dose of quercetin (in glycoside form) for each volunteer was 17.16 mg. This is much lower than for the other polyphenols such as (-) epicatechin and phloretin. No quercetin conjugates were detected in urine or plasma from this study, which suggests all the quercetin consumed in the test drink was converted to the ring fission metabolites by gut microflora (discussed later in this section), or that the levels were too low for detection. Urpi-Sarda *et al.* [83], do not report the presence of quercetin conjugates in urine or plasma after administration of 0.46 mg of quercetin (as glycosides) in cocoa. Dupont *et al.* [87], suggest extensive methylation of quercetin occurs in humans after low levels of quercetin intake; the cider

consumed by the volunteers in their study contained 1.57 mg of quercetin (a small amount was supplied as quercetin glycosides). At higher doses (such as those used in this study), they report that the pathway for methylation becomes saturated, and this maybe why no methyl conjugates are detected here. Volunteers for the study by Kahle *et al.* [8], consumed one litre of cloudy apple juice containing between 0.2 and 3.9 mg of quercetin (as glycosides), but no quercetin glucuronides or sulfates were detected. This is opposite to results following the ingestion of onions, where methylated, monoglucuronide, diglucuronide, sulfoglucuronide and sulfate conjugates of quercetin were identified in the urine and plasma [23, 84] after consumption of approximately 120 mg of quercetin glycosides. It has been established that absorption of quercetin is more rapid after intake of onions, rich in glucosides, than after intake of apples containing both glucosides and other glycosides. In particular, polyphenols linked to a rhamnose moiety have to reach the colon to be hydrolysed by rhamnosidases of the microflora prior to absorption. The same probably applies to arabinose – quercetin-3-rhamnose and quercetin-3-arabinose in ileostomy fluid, not glucoside [8].

Microbial Derived Metabolites of Polyphenols in Humans Post Ingestion of a High Polyphenol Cider Apple Extract

In this study, colonic microbial derived metabolites accounted for the largest proportion of the total phenolic metabolites in urine and plasma, which agrees with the findings of Urpi-Sarda *et al.* [83] In urine, ring fission metabolites accounted for at least 80 % of the total analyte area identified and in plasma the figure was 54 %. Several microbial-derived metabolites were identified in urine, such as glucuronide, sulfate and disulfate conjugates of dihydroxyphenyl valerolactone, and a sulfoglucuronide of dihydroxyphenylacetic acid. Figure 3.50 suggests some possible metabolism pathways for these species, from the polyphenols in apples.

Dihydroxyphenyl valerolactone glucuronide was identified in urine (38.6 % of the total analyte area) and plasma (15.8 % of the total analyte area); hypothesized to be 5-(3′, 4′-dihydroxyphenyl)-γ-valerolactone glucuronide, supported by Sang *et al.* [73] and Urpi-Sarda *et al.* [83] , after consumption of tea and cocoa, respectively. The glucuronide could be positioned on the 3′ or 4′ position, with the hydroxyl groups on the 3′, 4′ or 5′ positions; no structural conformation is given in either reference. Two dihydroxyvalerolactone sulfate conjugates were tentatively identified in urine (39.0 % of the total analyte area) and plasma (15.2 % of the total analyte area); conjugation could be on the 3′ or 4′ position, just as in the case of

glucuronidation. Sang *et al.* [73] found two dihydroxyvalerolactone sulfates present in urine (none in plasma) after the consumption of tea, one conjugated on the 3′ position and one conjugated on the 4′ position, but which was which was not possible to confirm. In agreement with these findings, Urpi-Sarda *et al.* [83], also found two dihydroxyvalerolactone sulfates present in urine (again, none in plasma) after cocoa consumption. However, the authors report that this particular conjugate did not show a significant change when control urine was compared with test urine, due to large inter-individual variations. This contradicts the findings in this study, where large increases in the presence of dihydroxyvalerolactone conjugates were detected. Due to enzyme deconjugation experiments performed by research groups, information on the conjugation possibilities of these ring fission metabolites is limited [74]. A dihydroxyphenyl valerolactone disulfate was also tentatively identified in urine in this study, accounting for 3.1 % of the total analyte area. However, no other research groups present information on a disulfate in urine or in plasma. The increased retention time of the major peak detected in the chromatogram would also support the presence of two sulfate units.

Dihydroxyphenylacetic acid sulfate was identified as the major metabolite in the plasma, accounting for 21.1 % of the total analyte area; the unconjugated form was observed in plasma after cocoa consumption[83]. To the best of my knowledge, no other reports of this metabolite in the literature are available for comparison. In addition to these conjugated ring fission metabolites, dihydroxyphenylacetic acid sulfoglucuronide was detected in urine. Urpi-Sarda *et al.* [83], report the presence of 3, 4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid and phenylacetic acid in human urine, but no conjugates. Rios *et al.* [82], also found 3, 4-dihydroxyphenylacetic acid in urine, but the authors only detailed phenolic acids rather than any conjugates. There is no evidence to support or contradict the presence of a sulfoglucuronide conjugate of dihydroxyphenylacetic acid in urine. It is important to highlight that any studies investigating the urinary and/or plasma metabolites in humans after consumption of apples or apple related products, do not report on bacterial ring fission metabolites or their possible conjugates [8, 72, 87, 99].

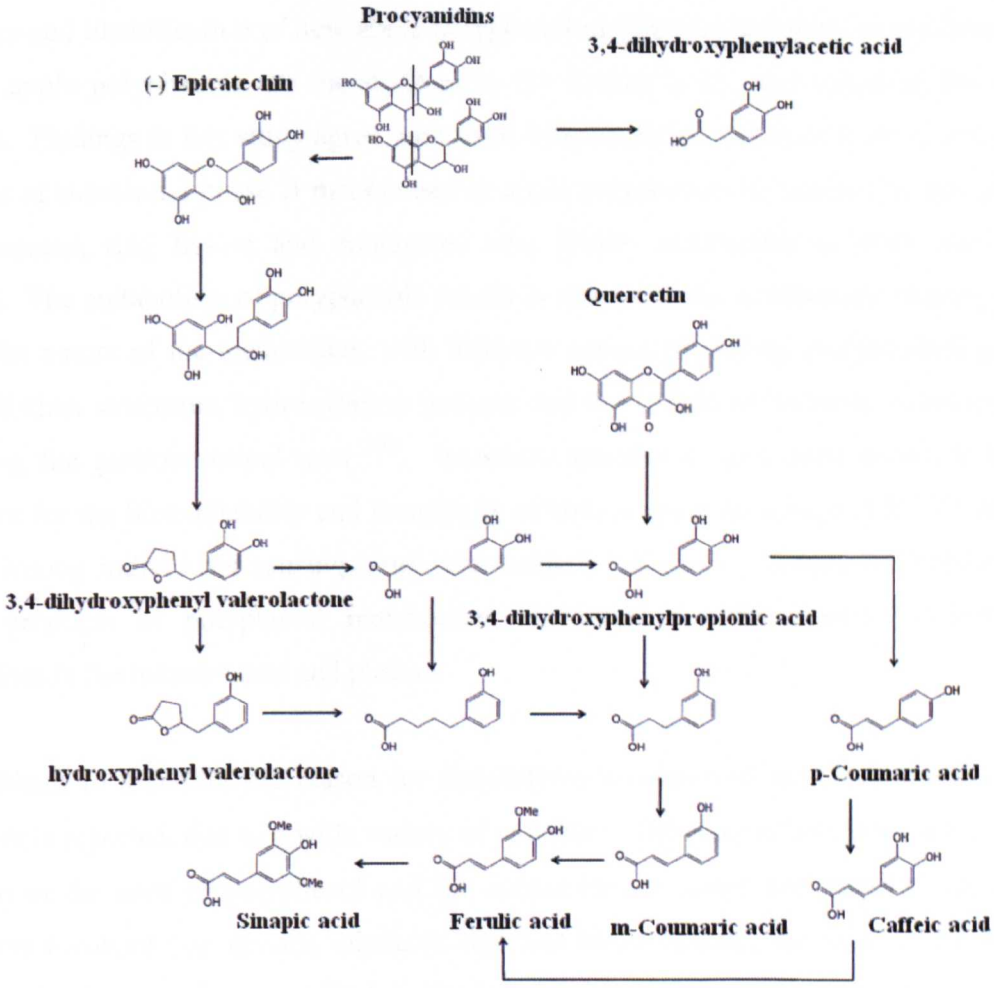

Figure 3.50 **Microbial metabolites of apple polyphenols. Sulfate and sulfoglucuronide conjugates of 3,4-dihydroxyphenylacetic acid were detected in plasma and urine, respectively. Glucuronide and sulfate conjugates of 3,4-dihydroxyphenylvalerolactone were detected in urine and plasma and a sulfoglucuronide conjugate was also identified in urine.**

Hippuric acid is a common metabolite present in urine after the consumption of polyphenols [87]. It is derived from either the microbial degradation of polyphenols in the colon followed by hepatic conjugation with glycine, or by metabolism of catecholamine neurotransmitters. Urpi-Sarda *et al.* [83], report the presence of 3-hydroxyhippuric acid and 4-hydroxyhippuric acid in urine and plasma after cocoa consumption. This analyte was detected in urine, but levels in test urine samples were not significantly higher than in control urine samples, so the metabolite was not investigated any further.

Conclusions

This study reports the first time identification of nine urinary metabolites and seven plasma metabolites in humans, after consumption of a high polyphenol cider apple extract. The

discovery and identification of new apple polyphenol metabolites enhances our understanding of how apple polyphenols are metabolised in the human body, and advances the area of research. Findings in this study agree with what little available literature there is and provide evidence of substantial phase II metabolism of apple polyphenols in humans by the detection of conjugated, ring fission and conjugated ring fission metabolites in urine and plasma samples. The metabolism of polyphenols results in many diverse metabolites; structure seems key to the nature of the metabolites, with different classes producing characteristic products due to distinct structures, hydroxylation patterns and the variety of bacterial microorganisms inhabiting the gastrointestinal tract [46]. Intestinal microbiota have been shown to be very important for the bioavailability and bioactivity of certain phenolic compounds [180], and vary greatly among individuals causing large inter-subject variations. Conjugated bacterial ring fission products of polyphenol metabolism, accounted for the greatest proportion of metabolites in the human urine and plasma.

It is difficult to ascertain the reason for the differences observed in metabolite profiles of polyphenols reported, due to a wide variety of variables. Differences between studies include variation in the dose of polyphenol and the source (tea or cocoa compared to apples), the volunteers involved (i.e. gender, ethnicity, age and health status), the time points at which plasma and urine samples are collected, the length of time the samples are stored for before use, the method used to process the samples before analysis and the sensitivity/accuracy of the LC/MS/MS method employed. This study was performed with great attention to detail, thereby reducing as many sources of error as possible and producing results which have a high level of confidence attached to them.

Chapter Four

Biokinetics of Apple Polyphenol Metabolites in Human Urine and Plasma

Summary

In chapter 3, some major metabolites of apple polyphenols detected in human urine and plasma samples were identified; this chapter evaluates some of the biokinetic parameters of these metabolites which provides evidence for their origin and gives information on their rates of absorption and excretion. The proportions of metabolites identified after the consumption of the control apple juice and the high polyphenol cider apple extract (test phase) have been compared and contrasted to reveal differences in the metabolic pathways at different concentrations of polyphenol intake. This is the first time biokinetic data has been reported for apple polyphenols in healthy humans.

4.1 Introduction

This section first reviews intervention studies performed by other researchers with apples or apple related material, and is followed by literature detailing information about polyphenols that are contained in apples, but where the intervention studies have been conducted with other food stuffs such as tea and cocoa.

4.1.1 Absorption/Excretion Profiles of Apple Polyphenols

As mentioned in the previous chapter, literature concerned with apple intervention studies is restricted to four papers [8, 72, 87, 99]; two of which only report data for metabolites found in ileostomy fluid [8, 99]. Maximum excretions of polyphenols in ileostomy fluid occurred 2 h after ingestion of the cloudy apple juice, and most of the polyphenols were already present in the juice; an exception was the presence of D-(-) quinic acid (major metabolite) and phloretin glucuronide (minor metabolite). No biokinetic data was provided in this study. Phloretin-2′-glucuronide was found as the major metabolite in plasma, urine and ileostomy fluid by Marks *et al.* [72] when comparing results from healthy volunteers and ileostomy patients after the consumption of cider. Biokinetic data reported from both subject groups revealed no statistical differences, which suggested that the principle site for the absorption of dihydrochalcones from cider was the small intestine. The presence of the glucuronide conjugate of phloretin reached a maximum 0.6 h after cider consumption in plasma, also indicating rapid absorption in the small intestine; maximum excretion occurred in the 0-2 h urine sample. After consumption of cider, Dupont *et al.* [87], report the presence of caffeic acid and 3′-methyl quercetin in plasma, both of which were present in the ingested cider. In urine, phloretin (present in the cider) and hippuric acid were detected, but enzyme deconjugation experiments were performed so any detail on the conjugation of these and other metabolites would have been lost. No biokinetic data was reported by these authors.

Using the data presented in these four papers it is only possible to form a limited understanding of the metabolism of apple polyphenols. Although the studies performed with ileostomy patients provided different data, no colonic metabolism can occur, hence data would be absent for the large proportion of the metabolites from apple polyphenols formed by colonic bacteria (as noted in chapter 3). Also, analysis methods utilised in previous studies were often not fully validated leading in some cases to a lack of certainty as to identification and to the accuracy of quantification. Many previous studies have used enzymes to

deconjugate polyphenol metabolites, and whilst this provides an overall estimate of conjugation, it results in a lack of detail on specific polyphenol metabolite conjugation, for example whether a glucuronide, sulfate or other conjugate has been formed.

4.1.2 Absorption/Excretion Profiles of Apple Polyphenols from Alternative Food Sources

Catechins and their metabolites present in plasma and urine of volunteers after consumption of tea, generally reach a maximum quickly; greatest excretion is between 0-4 h and highest plasma levels occur around 2 h [57, 75, 169]. Van Het Hof *et al.* [57], compared the consumption of black and green tea, with and without milk. Results indicated that green tea had three times the concentration of catechins when compared to black tea, which was reflected in the results of total catechins in the blood after consumption; milk showed no significant effects on these levels. In another study comparing the ingestion of three different amounts of decaffeinated green tea [75], a two fold increase in consumption levels produced a 3.4 fold increase in maximum circulating levels of (-) epicatechin; however, a further increase in quantity ingested did not produce a corresponding increase in plasma levels. The same effect was observed in the urine. The authors reported that most of the catechins were present in the sulfated and glucuronidated forms, but no biokinetic data was provided for these metabolites.

After consumption of cocoa in liquid form with milk or water, Mullen *et al.* [106], reported rare biokinetic data for two conjugates of (-/+) epicatechin identified in the plasma; epicatechin sulfate and methyl epicatechin sulfate. The maximum concentrations of these two metabolites were observed at 1.4 and 1.0 h, and were slightly lower when cocoa was ingested with milk compared to water, although not significantly. These metabolites were also identified in urine, in addition to another epicatechin sulfate and a glucuronide; values calculated for the proportion excreted as a percentage of what was ingested showed significant differences when cocoa prepared with water was compared to cocoa prepared with milk. A lower value was obtained after the cocoa-milk drink, suggesting components from the milk such as proteins, may have interfered with the transport of polyphenolic metabolites or bound to them, reducing their bioavailability. Absorption is affected by the matrix in which the polyphenols are present in, and therefore adds further complexity to the evaluation of bioavailability. Roura *et al.* [176], reported that although the amounts of epicatechin metabolites identified in urine did not significantly differ when milk was used to make the cocoa drink compared to water, the milk did affect the profile of metabolites; sulfation was found to increase compared to

glucuronidation in the first 6 h post ingestion. The ring position of these sulfate conjugates was also noted to be different.

There is agreement throughout the literature that T_{max} values for (-/+) epicatechin in plasma as the aglycone, are approximately 2 h, regardless of whether the epicatechin has been provided by tea or cocoa [81, 162]. One intervention study comparing the biokinetic parameters for (-) epicatechin after chocolate and cocoa intake [162], found maximum plasma concentrations were very similar, however, higher levels of non-methylated (-) epicatechin were observed in the plasma at 1 and 2 h post ingestion of cocoa compared to chocolate. After chocolate intake, concentrations of methylated (-) epicatechin were higher at 2 and 4 h compared to cocoa intake. This supports evidence provided by Roura *et al.* [176], that although maximum concentrations of polyphenols and their metabolites do not necessarily change with different food sources, the actual profile of the metabolites may change. In urine, these differences were not replicated.

One point to mention when comparing (-/+) epicatechin metabolism from tea and cocoa, is that cocoa also contains procyanidins which are in effect dimers, trimers, etc of (+/-) catechin and (-/+) epicatechin and therefore could be the origin of some of the flavanol metabolites observed in intervention studies carried out with cocoa.

After consumption of coffee, Stalmach *et al.* [78], provided some biokinetic data for chlorogenic acids and their metabolites in human urine and plasma. Twelve hydroxycinnamate derivatives were identified in plasma, with T_{max} values of between 0.6 and 5.2 h; the time is indicative of the site of absorption, as explained in the previous chapter. Early T_{max} values of between 0.6 and 1 h indicate absorption in the small intestine and are observed for metabolites which have been formed by simple phase II metabolism reactions, such as glucuronidation, sulfation and methylation. Longer values for the time taken to reach maximum concentrations indicate large intestine absorption, and are observed for metabolites produced as a result of greater transformations, for example those reactions of the colonic microflora such as ring fission. Major metabolites detected in the plasma, were those which had the longest T_{max} values of between 4.7 and 5.2 h; most metabolites were eliminated from the circulatory system rapidly, indicated by elimination half lives of between 0.3 and 1.9 h.

4.1.3 Absorption/Excretion Profiles of Ring Fission and Conjugated Ring Fission Metabolites

The identification of ring fission metabolites and their conjugates following consumption of polyphenols is scarce, but quantitative or biokinetic data is even more elusive, particularly for the conjugated ring fission metabolites. Urpi-Sarda *et al.*, reported identification of some metabolites of bacterial origin, after cocoa [83, 105] and almond [181] consumption (some quantitative but no biokinetic data provided), Meng *et al.* [74], provided some evidence of the formation of these metabolites after consumption of tea (quantitative and some biokinetic data provided) and Rios *et al.* [82] showed some urinary excretion profiles for six ring fission metabolites after chocolate consumption (quantitative data only).

After cocoa consumption [105], large increases in the excretion of many microbial-derived phenolic metabolites were reported, the largest increases were observed with ferulic acid, *p*-coumaric acid, 3-hydroxybenzoic acid and vanillic acid. Although percentage increases compared to baseline levels are reported, no actual biokinetic data is given. Some quantitative data for (-/+) epicatechin conjugates, ring fission and conjugated ring fission metabolites was provided by a more recent study [83], (but still no biokinetic data) however, this study was concerned with the regular consumption of cocoa throughout the experimental period and therefore results will be different to those generated by a single dose; the ability of phenolic compounds to modify the populations of microorganisms in the gut over time was previously discussed in chapter 3. Conjugates of hydroxyphenyl valerolactone significantly increased in the urine and plasma of volunteers post cocoa consumption; maximum excretion was observed 3-6 h post dose. The longer time taken to reach maximum excretion increases the likelihood that the metabolite is of bacterial origin.

Following green tea ingestion, 5- (3′, 4′-dihydroxyphenyl) –γ- valerolactone was identified in urine and plasma; maximum excretion occurred 3-6 h post dose and maximum plasma concentrations were observed 5 h post dose [74]. This supports the findings of Urpi-Sarda *et al.* [83] reported after cocoa consumption. Urine and plasma samples were both deconjugated with enzymes prior to the analysis in this green tea intervention, which could be the reason no conjugated ring fission metabolites were detected. Rios *et al.* [82], reported T_{max} values of between 9 and 48 h for ring fission metabolites in urine, which is later than values reported after tea or cocoa consumption. No conjugated ring fission products were identified, probably due to enzyme deconjugation prior to analysis.

Following the consumption of almonds, which contain (-) epicatechin and procyanidins similar to apples, two glucuronide and one sulfate conjugate of hydroxyphenyl valerolactone were identified in urine and plasma, in addition to hydroxymethoxyphenyl valerolactone glucuronides and sulfates [181]. Samples were analysed before and after deconjugation with enzymes for a more detailed account of metabolism. Levels of phenolic acids derived from the further breakdown of these hydroxyphenyl valerolactones, were also reported. Concentrations of all metabolites were given in the plasma and urine, where detected, but no further biokinetic data was supplied. Due to sample collection at limited time-points (plasma was taken at baseline, 2.5 h and 4.5 h after ingestion of the test food, and urine was collected from 0-24 h) time profiles could not be constructed, which limits the information on bioavailability which can be acquired from this study.

4.1.4 Summary

Limited biokinetic data for some polyphenol-related aglycones identified in human urine and plasma has been reported in the literature, in addition to some data for conjugated metabolites. However, quantitative and/or biokinetic data has not been detailed for ring fission and ring fission conjugates of polyphenols. This review of the literature highlights the lack of information available on the metabolism of polyphenols in humans; and the purpose of the research reported here is to contribute to this poorly investigated area. Many new metabolites have already been identified for the first time in humans, after the consumption of a high polyphenol cider apple extract, in chapter 3, and the aim of this chapter is to further our understanding of the metabolism of apple polyphenols by evaluating the biokinetics and bioavailability of these metabolites.

There are several reasons for the absence of detailed information in the literature, firstly human studies are rarely well designed and fail to take sufficient samples at a range of time-points to obtain good pharmacokinetic type data. Sample taking requires a great deal of care and attention to detail, in order to gain the most from the samples. Volunteer numbers are very often less than five, and high inter-subject variation is always observed, which has a greater impact in small-number studies. The focus of studies is often very narrow; researchers concentrate on one or two specific polyphenols or one class and fail to consider the broader outcomes. Analytical measurements are not always appropriate, for example the analysis of enzyme deconjugated urine and plasma samples may be carried out in conjunction with the analysis of the unhydrolysed samples, but often it is performed instead, and serves only to

conceal the detail. The literature is not comprehensive for any fruit, vegetable or food stuff, especially for apples. This chapter attempts to address these shortcomings by applying a validated analytical method to analyse urine and plasma samples from a human intervention study specifically designed to investigate apple polyphenol metabolism and biokinetics.

4.1.5 Aim of the Chapter

To evaluate the biokinetic data in human urine and plasma following administration of a high polyphenol cider apple extract to nine volunteers.

4.2 Methods

4.2.1 Summary of the Analytical Method used for the Analysis of Apple Polyphenol Metabolites in Human Urine and Plasma Samples

Full details are provided in chapter 2, section 2.3. In summary, mobile phase A constituted of 0.01 % formic acid in H_2O and mobile phase B was MeCN. A Gemini C_{18} column (Phenomenex, Macclesfield UK) of dimensions 100 x 3.0 mm and a 3 μm particle size was used at a temperature of 65 °C. Urine was centrifuged at 3,000 rpm and 4 °C for 3 minutes prior to analysis and plasma was extracted twice using a 3:1 solvent: plasma liquid-liquid extraction procedure with 0.1 mM NH_4OH in MeCN, prior to analysis.

4.2.2 Summary of the Human Intervention Study

Full details are provided in chapter 3, section 2.3. In summary, ten healthy male volunteers were recruited for a two-phase blinded intervention study; the control phase involved consumption of 'Tesco' Pure Apple Juice supplemented with water and the test phase involved consumption of the same apple juice supplemented with a high polyphenol cider apple extract (only nine volunteers completed both phases of the study; one volunteer dropped out due to medical reasons). Baseline urine and plasma samples were taken before consumption of the test material. Drinks were consumed within three min; urine samples were collected in the following time periods, 0-4 h, 4-8 h and 8-24 h and plasma samples were taken after 20, 30, 40 and 60 min, every half an hour until 4 h and then every hour until 8 h, with a final sample 24 h post consumption. At least two weeks passed between each phase.

4.2.3 Biokinetic Calculations for Apple Polyphenol Metabolites in Human Urine

Normally, in order to obtain biokinetic data, concentrations of metabolites in the biological fluid would be plotted against time, but here only the extracted ion peak areas were available for urine and plasma. Peak areas were used to represent concentration because of lack of available standards; although the polyphenol metabolites have closely related structures, there will still be differences in MS responses, so data for individual polyphenol metabolites will be broadly comparable, but nevertheless represent an approximation. Total LC/MS/MS extracted ion peak areas for each metabolite were calculated using the data for the total volume of urine collected at each time-point, and therefore are directly proportional to the total excreted amount of each polyphenol metabolite. Peak areas for individual metabolites are consistent between the control apple juice and the high polyphenol cider apple extract and are therefore directly comparable; responses vary between metabolites as mentioned previously, so a direct comparison cannot be made. Mean peak area data (n = 9) were calculated from all the volunteers and used to construct excretion profiles for the urinary metabolites. The time-point at which maximum excretion of a particular metabolite occurred was defined as T_{max} (h). Kinetics determines changes over time which applies to concentrations or peak areas similarly. The use of relative peak areas allows accurate measurement of the T_{max} values for individual metabolites as concentration data are not required in the calculation.

4.2.4 Biokinetic Calculations for Apple Polyphenol Metabolites in Human Plasma

As explained above, normally to determine biokinetic data, concentrations of metabolites in the plasma would be plotted against time, but here only extracted ion peak areas were available. Peak areas were used to represent concentration because of the lack of available standards. Peak area versus time profiles for the metabolites identified in the plasma were constructed from mean (n = 9) data. Peak areas for the metabolites identified in plasma represent the amount of polyphenol metabolite in 1 mL of plasma, and so are different from the peak areas of urinary metabolites. Although the polyphenol metabolites have similar structures, there will be differences in MS responses, as observed for urinary metabolites, and in addition there will also be differences in the recovery efficiencies of the individual polyphenol metabolites from the plasma matrix. This suggests that caution should be taken when attempting to make quantitative comparisons between the individual metabolite profiles. However, the values of T_{max} and apparent $T_{1/2}$ for individual metabolites are valid since concentration data are not required in their calculation.

T_{max} was defined as the time taken in hours to reach the maximum concentration (C_{max}) observed in the plasma. C_{max} was defined by the maximum peak area recorded for a particular metabolite during the sampling time; where polyphenol standards were available C_{max} values (nM) were calculated using the mean value for the slope of the calibration curve for the polyphenol standard in control plasma (matrix-matched). Normally, plasma $T_{½}$ or the elimination half-life is determined following an intravenous bolus injection and represents the time taken for the metabolite to be decrease to half of its maximum concentration in plasma. However, in this study an oral 'dose' was given to the volunteers, so it was only possible to estimate the elimination half-life. An estimate was given by examining the absorption profiles; T_{max} represents the time of maximum concentration of the metabolite in the plasma and indicates where the rate of absorption is equal to the rate of excretion, the point at which the rate of excretion is greater than the rate of absorption must be used to calculate the elimination half life. Therefore calculations were made from the slope of the graph at a significantly later time, for example 1-2 h after the T_{max} had been recorded.

Area under the curve (AUC) calculations, give a measure of bioavailability of the metabolites under investigation. In this study, values were based on measured peak areas derived from LC/MS/MS extracted ion peaks from the individual metabolites due to the lack of standards mentioned previously. Once again, quantitative comparisons between metabolites rely on the extraction efficiencies and the MS responses of the individual polyphenol metabolites being the same; they will be similar due to the similarities in structures, but there will be variations, which have been observed by examination of the calibration lines for each of the polyphenol standards in plasma. Therefore, the values of the AUC calculations in this study are an estimated measure of comparison between the major plasma metabolites identified. Both $T_{½}$ and AUC values were calculated using Microsoft Excel.

4.3 Results and Discussion

4.3.1 Biokinetics of Apple Polyphenol Metabolites in Human Urine

Table 4.1 details the percentages of each of the identified polyphenol metabolites in the urine samples collected at the various time-points; T_{max} is the time-point at which the greatest percentage of the analyte is recorded and is indicated by bold typeface. Table 4.2 gives the percentage of each urinary polyphenol metabolite out of the total analyte area, for control and test urine samples.

Table 4.2 **Summary of the mean (n = 9) values for the distribution of the metabolites identified in urine collected at the four time-points after the consumption of a high polyphenol cider apple extract by human volunteers; ± standard deviation. T_{max} is indicated by bold typeface.**

Metabolites Identified in Human Urine	Percentage of Analyte in Urine Samples			
	Baseline	0-4 h	4-8 h	8-24 h
(-/+) Epicatechin-3'-glucuronide	0.2 ± 0.3	**51.8** ± 35.2	27.5 ± 27.4	20.5 ± 23.2
(-/+) Epicatechin-3'-sulfate	0.1 ± 0.2	**50.9** ± 40.7	33.4 ± 35.6	15.5 ± 30.1
(-/+) Epicatechin sulfoglucuronide	< 0.1 ± < 0.1	35.8 ± 23.7	**50.4** ± 30.0	13.8 ±9.1
Phloretin-2'-glucuronide	< 0.1 ± < 0.1	**60.8** ± 28.0	33.4 ± 24.5	5.9 ± 9.0
5 – (3',4'-Dihydroxyphenyl)-γ-valerolactone glucuronide	< 0.1 ± < 0.1	7.6 ± 10.7	**56.5** ± 21.8	35.9 ± 23.7
Dihydroxyphenyl valerolactone sulfate	2.5 ± 6.2	12.5 ± 22.9	**54.9** ± 41.7	30.2 ± 45.2
Dihydroxyphenyl valerolactone disulfate	< 0.1 ± < 0.1	14.6 ± 26.2	**55.8** ± 30.1	30.0 ± 32.8
3, 4-Dihydroxyphenylacetic acid sulfoglucuronide	17.2 ± 23.0	**52.5** ± 31.4	7.1 ± 14.2	23.2 ± 35.6
Sinapic acid	Metabolite only detected in 3 volunteers, 2 had T_{max} at 8-24 h			

Table 4.3 **Summary of the mean (n = 9) percentage area for each of the metabolites identified in control and test human urine samples following ingestion of 'Tesco' pure apple juice (control) and a high polyphenol cider apple extract (test).**

Metabolites Identified in Human Urine	Percentage of Total Analyte Area	
	Control	Test
(-/+) Epicatechin-3'-glucuronide	0.2	0.4
(-/+) Epicatechin-3'-sulfate	5.4	0.9
(-/+) Epicatechin sulfoglucuronide	0.1	0.2
Phloretin-2'-glucuronide	12.2	14.5
5 – (3',4'-Dihydroxyphenyl)-γ-valerolactone glucuronide	11.4	38.6
Dihydroxyphenyl valerolactone sulfate	63.6	39.0
Dihydroxyphenyl valerolactone disulfate	0.5	3.1
3, 4-Dihydroxyphenylacetic acid sulfoglucuronide	0.1	< 0.1
Sinapic acid	0	< 0.1

4.3.1.1 Biokinetics of Conjugated Metabolites in Human Urine

Mean (n = 9) maximum excretions of (-/+) epicatechin-3'-glucuronide and (-/+) epicatechin-3' or 4'-sulfate were detected in the 0-4 h urine sample (Table 4.2). These metabolites continued to be excreted in the 4-8 h and 8-24 h samples, the amount decreasing each time, Figure 4.1. The literature reports that maximum excretion of conjugated metabolites of (-) epicatechin from cocoa [106] and tea [169] were in the 0-2 h and 0-4 h urine samples, respectively; this is the first time data has been reported for (-/+) epicatechin metabolites after the consumption of apples. Levels of (-) epicatechin glucuronide and (-) epicatechin sulfate have been reportedly detected in urine samples up to 24 h after tea consumption [73]. A decaffeinated green tea intervention study undertaken by Van Het Hof *et al.* [57], reported that over 90 % of the ingested (-) epicatechin was excreted within 8 h (mainly as sulfate and glucuronide conjugates) and after 24 h the levels were below those of detection. Hence, the results presented here are in agreement with previous published data. Maximum excretion for the sulfoglucuronide conjugate of (-/+) epicatechin (not previously identified after other intervention studies) was observed in the 4-8 h urine sample, later than for the other conjugates of (-/+) epicatechin. Individual volunteers showed variation in their excretion profiles for all these metabolites indicated by the large error bars which represent the standard deviation (Figure 4.1, A-D). High inter-subject variation has been reported in the literature with all studies of this kind [8, 83, 87, 99].

Mean (n = 9) maximum excretion of phloretin-2'-glucuronide was also detected in the 0-4 h urine sample (Table 4.2). Marks *et al.* [72], reported that the maximum excretion of phloretin-2'-glucuronide post cider consumption was in the 0-2 h urine sample; this is the only paper to my knowledge detailing the presence of this conjugate in the urine of healthy volunteers. More data exists for the analysis of ileostomy fluid after consumption of apple-related material; Kahle *et al.* [99], reported that maximum excretion of polyphenols such as hydroxycinnamic acid derivatives (chlorogenic acid and coumaroylquinic acids), dihydrochalcones (phloretin and phloretin-2'-xyloglucoside), (-) epicatechin and quercetin-3-rhamnoside (present in the ingested apple juice) occurred at 2 h in ileostomy fluid. The only new metabolite to be reported that was not present in the cloudy apple juice was phloretin-2'-glucuronide, and this was also reported at maximum concentrations in the 0-2 h urine sample; a further analytical study of the composition of ileostomy fluid collected from this study

showed that phloretin and the glucuronide conjugate only accounted for a minor proportion of the metabolites [8].

Phloretin-2′-glucuronide accounted for the largest proportion (14.5 %) of analyte area in urine (Table 4.3), of the non-ring fission metabolites identified after consumption of the high polyphenol cider apple extract. This phenomenon was also observed after the consumption of the control drink and was not much lower, (12.2 %); this suggests that the pathway of formation of this metabolite is saturated at a low level of phloretin intake; the high polyphenol cider apple extract provided around fifty times the quantity of phloretin than the supermarket apple juice, but no such increase is observed in conjugated phloretin metabolites excreted. The process of absorption is complex and there are a multitude of variables which could be affecting the fate of the phloretin.

Proportional amounts of (-/+) epicatechin glucuronide and sulfoglucuronide are very similar after the intake of both the control and the test drinks, but there is a greater proportion of (-/+) epicatechin sulfate produced after consumption of the control drink when compared to the test drink (Table 4.3). This supports the suggestion made previously in chapter 3, that the pathway for the formation of sulfate conjugates is readily saturated, by low doses of polyphenols [11]; however, for the effects of saturation to be obvious, the data must be presented as actual values rather than percentages of the metabolite pool, which is not possible here. The quantity of (-/+) epicatechin ingested is approximately eleven times higher after the consumption of the high polyphenol cider apple extract when compared to the supermarket apple juice. No significant increase in the production of the glucuronide conjugate is noted, which would be assumed if the sulfation pathway is saturated. One explanation for this could be that the low concentration of (-/+) epicatechin is enough to saturate the phase II conjugation pathways, so more passes through to the large intestine for bacterial colonic metabolism; a large increase in these ring fission metabolites was observed.

4.3.1.2 Enzyme Deconjugation Experiments in Human Urine

These experiments were described in chapter 2, section 2.3.7, and involved deconjugation of pooled test urine samples (0-24 h) with the enzymes sulfatase and β-glucuronidase, to obtain a figure for the total amount of (-/+) epicatechin and phloretin sulfate and glucuronide metabolites present in the urine (methyl conjugates will not be deconjugated). These two polyphenols are the only ones measured in the high polyphenol cider apple extract that

produced conjugates which were identified in the urine, and standards were also available for them. The mean (n = 9) amounts of these two polyphenols ingested were 73.96 mg for (-) epicatechin and 38.09 mg for phloretin.

Calculation of Total Proportion of (-/+) Epicatechin and Phloretin Metabolites in Human Urine

Since the values for the peak area after treatment of sulfatase would have included cleavage of all of the sulfate conjugates and some glucuronides (the source of the sulfatase contained some β-glucuronidase activity as well), this value was used as the total aglycone value for the 0-24 h urine samples. Using the sum of the peak areas after treatment of sulfatase and β-glucuronidase separately would have been an overestimate; calculated this way the value will be an underestimate as not all the glucuronides will have been deconjuagted. However, due to the use of pooled urine samples, the values have probably been overestimated as the same volume of urine was taken from each time-point sample to make the pool, regardless of the total volume, hence not accurately reflecting the relative contribution of each individual metabolite because of the volume differences at each time period. This was an error in the experimental design and therefore is only an approximation.

The mean (n = 9) excretion of sulfate, glucuronide and sulfoglucuronide conjugates of (-/+) epicatechin was 37.8 mg, which represented 51.2 % of the ingested (-/+) epicatechin and the mean (n = 9) excretion of phloretin sulfate, glucuronide and sulfoglucuronide conjugates was 19.2 mg, which corresponded to 50.4 % of the ingested phloretin (Table 4.4). These values were calculated by taking the sum peak area for the polyphenol after enzyme treatment, for each pooled urine sample from each volunteer (n = 9), and dividing by the slope of the calibration graph of the polyphenol standard in blank plasma (matrix-matched) and then multiplying by the molecular weight. These values do not consider any methylated derivatives of either polyphenol which may have been present; these would not have been deconjugated by sulfatase and β-glucuronidase enzymes. The values are highly variable among the volunteers for both polyphenols.

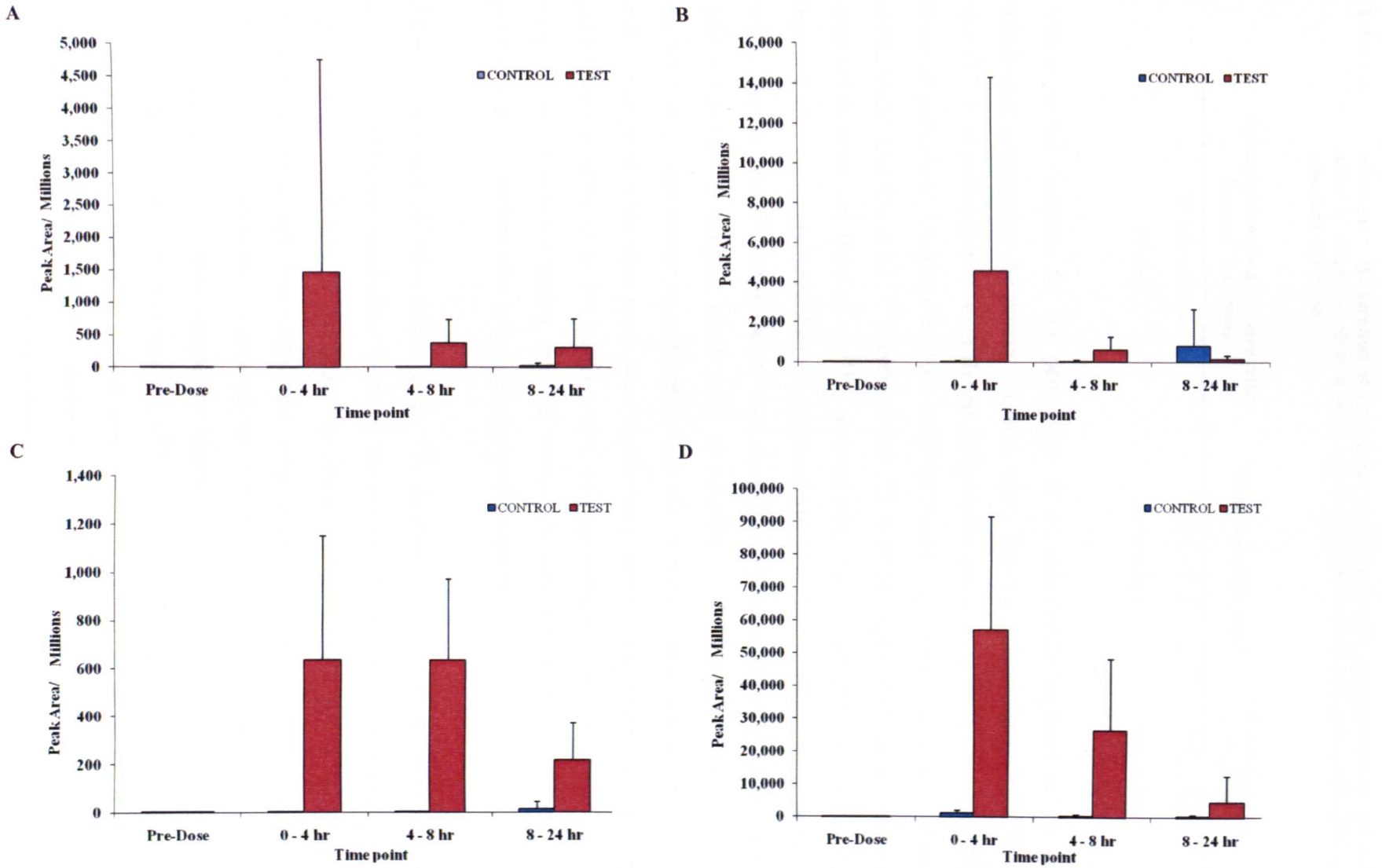

Figure 4.1 **Bar graphs representing the presence of (-/+) epicatechin-3′-glucuronide (A), (-/+) epicatechin-3′ or 4′-sulfate (B), (-/+) epicatechin sulfoglucuronide (C) and phloretin-2′-glucuronide (D) in control and test urine samples. Error bars represent the standard deviation (n = 9).**

Table 4.4 **Mean (n = 9) excretion of conjugates of (-) epicatechin and phloretin in the pooled test urine of volunteers (0-24 h) after treatment with sulfatase and β-glucuronidase ezymes, ± standard deviation.**

Aglycone Identified In Urine After Enzyme Treatment	Mean Excretion (mg)	% of Polyphenol Ingested
(-/+) Epicatechin	37.8 ± 17.8	51.2 ± 9.3
Phloretin	19.2 ± 24.1	50.4 ± 24.4

Values in the literature for the percentage of (-) epicatechin excreted in the urine as metabolites compared to the amount ingested, vary. For example, Mullen *et al.* [106], reported that 18.3 % of the ingested dose of flavon-3-ols from a cocoa-water drink were excreted in the urine of volunteers, compared to 10.5 % of the intake when milk was used to prepare the drink, whilst Del Rio *et al.* [169], reported that 7.2 % of the ingested flavanols from tea were excreted in urine and Baba *et al.* [162], reported that 29.8 % and 25.3 % of (-) epicatechin ingested after cocoa and chocolate consumption, respectively, was excreted in urine. These values are all derived from different food sources, different populations of volunteers and different method of analysis, which could all contribute to the variation. The values for the excretion of (-) epicatechin related metabolites after consumption of cocoa with and without milk investigated in the same study, were significantly different and indicate that absorption of polyphenols is affected by the matrix which they are consumed in, which may explain the variation noted between apples, tea and cocoa. Components in the matrix may bind to polyphenols, or interfere with their mechanisms of transport.

After consumption of cider, Marks *et al.* [72], reported that 5 % of the ingested dose was excreted as phloretin metabolites in urine; the amount of phloretin-2′-glucuronide excreted was equivalent to 4.2 % of the intake in healthy volunteers and 4.4 % of the intake in ileostomy patients, and represented the most abundant phloretin conjugate. In total, dihydrochalcone metabolites accounted for 2.0 % of the total quantity of metabolites determined in the 0-2 h urine samples for healthy volunteers in this study. In ileal fluid, the phloretin glucuronides detected totalled 18.7 % of the ingested dose, while the phloretin sulfoglucuronide and phloretin sulfates accounted for 3.6 and 3.0 % of the ingested dose, respectively. After consumption of alcoholic cider, DuPont *et al.* [87], identified phloretin and hippuric acid in the urine of healthy volunteers, but samples were subjected to deconjugation with enzymes prior to the analysis so any conjugates would have been cleaved. Between 16

and 26 % of the dose of phloretin (4.8 mg) was reportedly excreted in urine. Volunteers consumed 5.5 and 8 times the amount of phloretin by consumption of the high polyphenol cider apple extract in the present study, compared to the consumption of cider from the two studies, respectively. The differences observed between the results may be due to the alcohol present in the cider which would have affected the absorption of the polyphenols, the higher dose administered or the matrices.

4.3.1.3 Biokinetics of Conjugated Ring Fission Metabolites in Human Urine

Maximum excretion of 5 – (3′, 4′-dihydroxyphenyl) -γ– valerolactone glucuronide, sulfate and disulfate conjugates occurred in the 4-8 h urine sample (Table 4.2). T_{max} is consistently later for the ring fission metabolites and their conjugates, as they require time to travel the length of the gastrointestinal tract and reach the colon, where metabolism by the microflora occurs. This is the first time that kinetics have been established for these metabolites after apple consumption. Literature reports T_{max} values of between 3-6 h for dihydroxyvalerolactone metabolites of (-) epicatechin [74, 76, 83] from tea and cocoa, which aggress with the data presented here. One study concerned with an intervention where volunteers consumed tea and a supplement of the three main catechins in the tea separately, [74] provided a time concentration profile for a ring fission metabolite of (-) epicatechin, (5- (3′,4′-dihydroxyphenyl)-γ-valerolactone), showing a maximum concentration of 4.7 μM in the 3-6 h sample; the total excretion (0-24 h) of 5- (3′, 4′-dihydroxyphenyl)-γ-valerolactone ranged from 0.6 to 10 mg across the four volunteers. Enzyme deconjugation experiments were performed on the urine samples from this study and so if this metabolite was conjugated, the information has been lost. No other available literature reports a value for the total excretion of this ring fission metabolite. Some other studies identifying this ring fission metabolite do not deconjugate with enzymes prior to analysis, so identification of conjugates was possible, but no kinetics were evaluated.

Maximum excretion of the other conjugated ring fission metabolite to be identified in human urine, dihydroxyphenylacetic acid sulfoglucuronide, occurred in the 0-4 h urine sample (Table 4.2). Data for the identification and biokinetics of this metabolite have not previously been reported post consumption of apples, or other food sources. The earlier T_{max} observed with the sulfoglucuronide conjugate of dihydroxyphenylacetic acid, suggests that this metabolite may have been derived differently from the other metabolites. For example, after consumption of

chocolate, the presence of vanillic acid in the 0-3 h urine sample suggests that it may have originated from oxidation of the vanillin in the chocolate and not from microbes in the gastrointestinal tract [82]. There is evidence in the literature to support the finding that dihydroxyphenylacetic acid is a characteristic major metabolite of procyanidins, which were also present in the high polyphenol cider apple extract, but not from their monomers (i.e. (-) epicatechin) [180]. Figure 3.50 in chapter 3 shows a possible metabolism pathway for the formation of this metabolite, prior to conjugation. An excretion profile of 3,4-dihydroxyphenylacetic acid given by Rios *et al.* [82], showed maximum excretion occurred in the 9-24 h urine samples after chocolate consumption, which would indicate that the difference in the matrices between the chocolate and the high polyphenol cider apple extract could have altered the rate of formation of this metabolite.

After consumption of the control supermarket apple juice, dihydroxyphenyl valerolactone sulfate accounted for the greatest percentage of the total analyte area (63.6 %), as shown in Table 4.3. The glucuronide and disulfate conjugates accounted for 11.4 and 0.5 %, respectively. However, after the consumption of the high polyphenol cider apple extract (test phase), the proportions of metabolites were different. Glucuronide and sulfate conjugates of dihydroxyphenyl valerolactone accounted for almost equal proportions of the total analyte area, 38.6 and 39.0 %, respectively. This could be explained by the phenomenon mentioned previously, the metabolic route for the formation of sulfate conjugates becomes readily saturated, and so at higher levels of polyphenol ingestion, there is a shift towards the production of glucuronide conjugates; the pathway for sulfation is a high affinity, low capacity one [11]. Urpi-Sarda *et al.* [83], reported increases in conjugates of hydroxyphenyl valerolactones in urine after cocoa consumption, increases of 145 % were noted when compared to the control; large inter-subject variation was observed between the concentrations of dihydroxyvalerolactone sulfate in the urine of the volunteers and so this metabolite did not register as increasing significantly. Large inter-subject variation was observed in the profiles of the conjugated ring fission metabolites just as for the (-) epicatechin and phloretin conjugates (Figure 4.2), and was much greater for the sulfate and disulfate conjugates of dihydroxyphenyl valerolactone, than for the glucuronide. The excretion profile of 5 – (3′, 4′-dihydroxyphenyl) -γ– valerolactone glucuronide represents the lowest inter-individual variation of all the metabolites identified in urine; in particular, the 4-8 h sample shows that peak area values for the nine volunteers were very similar. This may suggest that the pathway

for sulfate formation is saturated by different concentrations of polyphenols, depending on the individual, but the pathway for the formation of glucuronides is more consistent.

4.3.1.4 Biokinetics of Non-Conjugated Ring Fission Metabolites in Human Urine

Maximum excretion of sinapic acid occurred in the 8-24 h urine sample (Figure 4.3), suggesting formation by colonic bacteria. Levels in the control urine samples from all volunteers were below the limit of detection. Sinapic acid was only found to be present in the urine of three volunteers; two showed maximum excretion in the 8-24 h sample and one in the 0-4 h sample; this metabolite shows large inter-individual variation maybe due to formation by populations of bacteria which may be present in some individuals, but not others. Sinapic acid has been detected in the urine of volunteers post coffee consumption, but T_{max} was not determined [179]; levels increased by an average of 12.6 fold, compared to control samples.

After cocoa consumption, Urpi-Sarda *et al.* [105], reported increases in the excretion of many microbial-derived phenolic metabolites; the largest percentage increases were noted for ferulic acid (619 %), *p*-coumaric acid (467 %), 3-hydroxybenzoic acid (413 %) and vanillic acid (265 %). From the schematic of the possible formation of these phenolic metabolites given in chapter 3 (Figure 3.50), it can be seen that sinapic acid may be a further metabolite of dihydroxyphenyl valerolactone, *p*-coumaric and ferulic acid. The cocoa intervention study involved regular consumption of cocoa, which as evidence exists to support the theory that polyphenols have the ability to modify the population of microorganisms in the gut, may have also affected the metabolites produced (previously discussed in chapter 3).

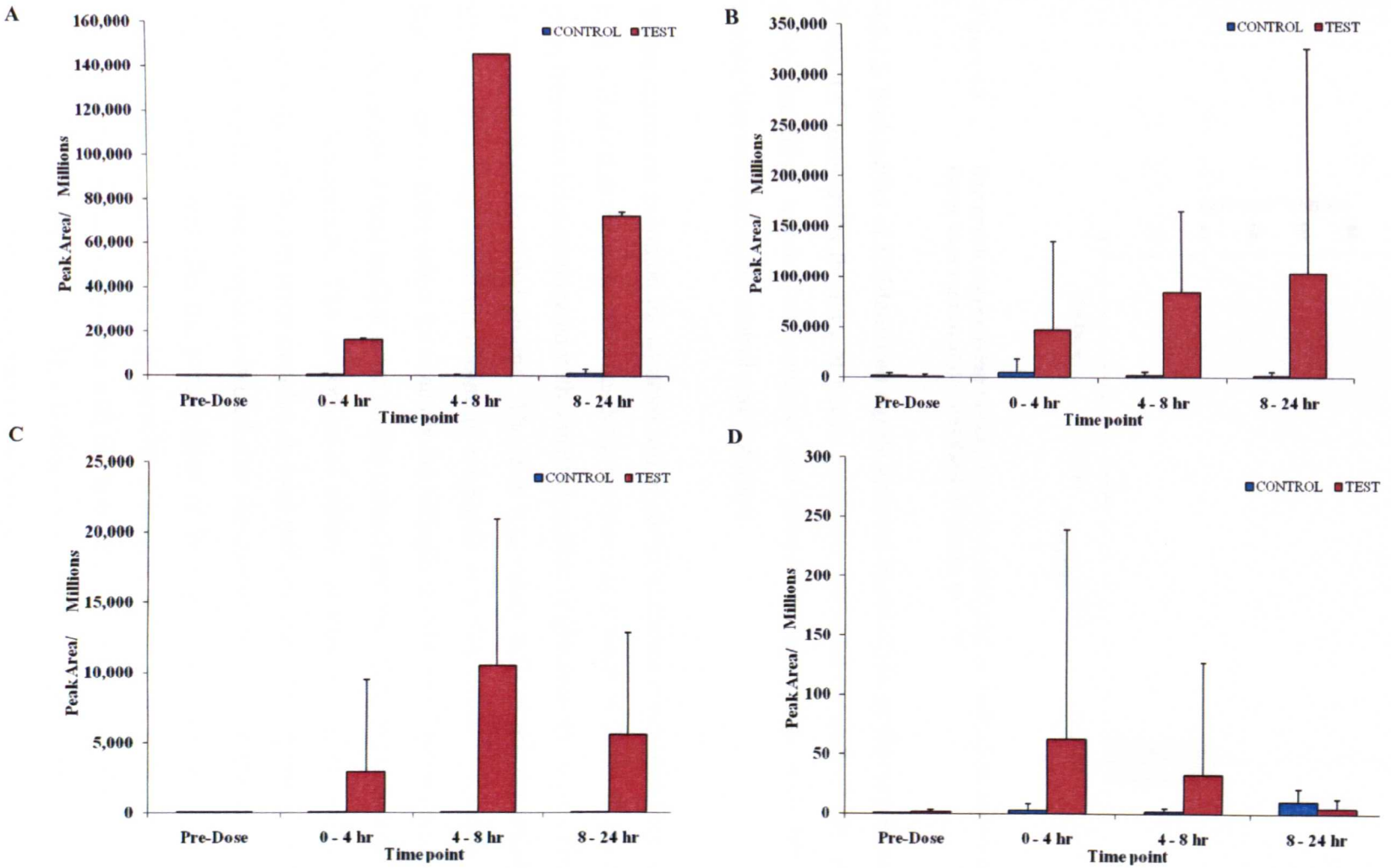

Figure 4.2 **Bar graphs representing the presence of 5 - (3′, 4′-dihydroxyphenyl) -γ- valerolactone glucuronide (A), 5 - (3′, 4′-dihydroxyphenyl) -γ- valerolactone sulfate (B), 5 - (3′, 4′-dihydroxyphenyl) -γ- valerolactone disulfate (C) and dihydroxyphenylacetic acid (D) in control and test urine samples. Error bars represent the standard deviation (n = 9)**

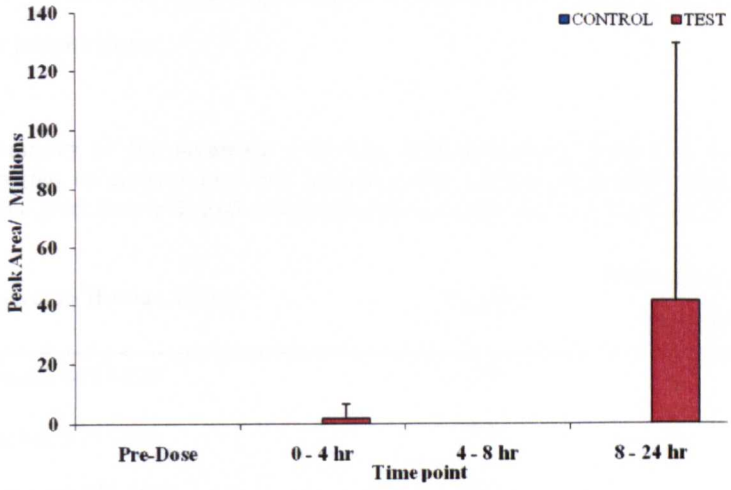

Figure 4.3 **Bar graph representing the presence of sinapic acid in control and test urine samples. Error bars represent the standard deviation (n = 9)**

4.3.1.5 Biokinetics of Unidentified Apple Related Metabolites in Human Urine

Table 4.5 summarises the time at which maximum excretion for each of the unidentified or partly identified metabolites occurred and details the percentage area for each of the metabolites detected in the control and test urine.

When maximum excretion for an unknown or partly identified metabolite occurs between 0-4 h, it is likely that the metabolite has been produced as a result of phase II reactions, and the polyphenol has been conjugated with a methyl, sulfate or glucuronide, or a combination of one or more of these functional groups. The later T_{max} values indicate that the metabolite is more likely to be a ring fission metabolite or conjugate of a ring fission metabolite; the result of bacterial action in the colon, for example the sulfoglucuronide conjugates detected. Values for the percentage of total analyte areas for the control and the test urine samples are very similar, with a few exceptions. The proportion of sulfate conjugates is higher in the control urine samples, than in the test urine samples; in total sulfate conjugates represent 4.9 % of the total analyte area for urine samples collected after the control phase of the study and only 1.1 % of the total analyte area after the consumption of the high polyphenol cider apple extract (test phase). In particular, the sulfoglucuronide conjugate with the mass transition *m/z* 425 / 345 and the methyl sulfate conjugates with the mass transitions *m/z* 264 / 169 and *m/z* 248 / 168 show the greatest increases. This further supports the evidence for the saturation of the pathway forming sulfate conjugates at low polyphenol concentrations, due to its high affinity

and low capacity. However, actual quantitative values are required for confirmation of this effect, rather than proportions.

Table 4.5 **Summary of the mean (n = 9) T_{max} and percentage area for each of the metabolites detected in control and test human urine samples following ingestion of 'Tesco' pure apple juice (control) and a high polyphenol cider apple extract (test).**

Unidentified Metabolites in Human Urine	T_{max} (h)	Percentage of Total Analyte Area	
		Control	Test
Glucuronide conjugate *m/z* 409 / 233	4-8	0.2	0.2
Glucuronide conjugate *m/z* 515 / 339	4-8	0.1	0.1
Diglucuronide conjugate *m/z* 653 /477	0-4	0.7	0.6
Sulfate conjugate *m/z* 557 / 477	8-24	0.4	0.1
Sulfate conjugate *m/z* 289 / 163	0-4	< 0.1	0.1
Sulfate conjugate *m/z* 384 / 304	0-4	0.1	0.2
Sulfoglucuronide conjugate *m/z* 464 / 288	8-24	0.2	0.1
Sulfoglucuronide conjugate *m/z* 425 / 345	8-24	1.9	0.2
Methyl sulfate conjugate *m/z* 264 / 169	8-24	1.5	0.3
Methyl sulfate conjugate *m/z* 248 / 168	4-8	0.8	0.1
m/z 577 / 289	4-8	0.2	1.5
m/z 271 / 119	4-8	0.1	< 0.1
m/z 737 / 481	0-4	< 0.1	< 0.1
m/z 705 / 353	4-8	0.1	< 0.1
m/z 529 / 449	0-4	0.1	< 0.1

4.4.1 Biokinetics of Apple Polyphenol Metabolites in Human Plasma

The pharmacokinetic data for the metabolites detected in human plasma are summarised and presented in Table 4.6. Data represent mean values (n = 9); plasma profiles for identified and unidentified metabolites are given in Figures 4.4, 4.5 and 4.6. The AUC values for each of the metabolites detected after consumption of the control apple juice and the high polyphenol cider apple extract have also been given. Table 4.7 details the percentage area for each of the metabolites detected in control and test plasma samples.

4.4.1.1 Biokinetics of Aglycone Metabolites in Human Plasma

Profiles for these metabolites are given in Figure 4.4, A and B. The two aglycones identified in plasma, phloretin and *p*-coumaric acid, showed mean maximum concentrations in the plasma within 1 h, and have elimination half lives of around 2 h (Table 4.6). The glucuronide conjugate of phloretin was identified in urine, which suggests that the glucuronides are rapidly eliminated from the body which leaves free phloretin circulating in the plasma. However, one study by Marks *et al.* [72], does report the presence of phloretin-2′-glucuronide in the plasma; T_{max} was 0.6 h, C_{max} was 73 nM and $T_{½}$ was 0.7 h.

The maximum concentration of *p*-coumaric acid in the plasma reported here before 1 h indicates that this phenolic compound has probably not originated from microbial action in the colon, as previously suggested. Since *p*-coumaroylquinic acids are present in large quantities in apples [101, 146], it can be assumed that they are also present in this high polyphenol cider apple extract (although not measured). The biokinetics reported suggest that the small intestine is the principle site for the absorption of the dihydrochalcones and hydroxycinnamic acids from this cider apple extract. One recently published study reported the presence of phloretin and *p*-coumaric acid in the plasma of healthy male volunteers after the consumption of organically and conventionally produced apples [182], however, samples were subjected to enzyme deconjugation prior to LC/MS analysis, so any possible conjugates would have been cleaved. The study reported a C_{max} of 13 ± 5 nM and a T_{max} of 1.7 h ± 1.2 h for phloretin, and a C_{max} of 35 ± 12 nM and a T_{max} of 3 h ± 0.8 h for *p*-coumaric acid. The maximum plasma concentrations reported for both these aglycones are considerably lower than values obtained in the present study, and times taken to reach the maximum concentrations are slightly earlier in the present study compared with those reported for the organically and conventionally grown apples. The differences are probably due to a different ingested level (the high polyphenol cider apple extract provided approximately three times the concentration of phloretin than the apples contained) and also suggest that the matrix in which the polyphenols are supplied in is affecting the bioavailability; the high polyphenol cider apple extract is in a liquid form and may be metabolised more rapidly than the apples, which require further breakdown of the food stuff. The time taken to consume 1 kg of apples will also impact the results; the drink in this present study was consumed within 3 min, but the consumption of the apples is assumed to have taken much longer than this although the data for this is not reported in the published paper. Apart from this paper, comparable studies on the biokinetics of *p*-coumaric acid after the consumption of apples or apple products have not yet been published.

Farah *et al.* [179], reported values for *p*-coumaric acid in the plasma of healthy volunteers after the consumption of 170 mg of chlorogenic acids in decaffeinated green coffee; T_{max} occurred at 2.5 h ± 1.8 and C_{max} was 0.4 μM ± 0.03. These values are similar to those obtained in the present study, although the amount of *p*-coumaric acid present in the green coffee and the cider apple extract were not quantified so the ingested values cannot be compared. Urpi-Sarda *et al.*, also reported the presence of *p*-coumaric acid in the plasma of volunteers, post cocoa [83] and almond [181] consumption; C_{max} values were 0.03 nM ± 0.01 and 19.14 nM ± 2.66, respectively. However, both these values are not significantly greater than the values obtained after analysis of the control samples; *p*-coumaric acid was not reported to be present in either food stuff ingested which could account for the differences between these studies and the data presented here.

When the presence of these aglycones are compared after consumption of the control supermarket apple juice and the high polyphenol cider apple extract, the proportion of phloretin remains very similar but the proportion of *p*-coumaric acid is higher after the control drink (Table 4.7). The AUC values given in Table 4.6 show that phloretin has increased approximately 3.4 fold after consumption of the test drink compared to the control and *p*-coumaric acid has increased approximately 2.1 fold after consumption of the test drink compared to the control. These are relatively minor increases compared to the difference in the polyphenol concentrations between the two drinks, and compared to the increases observed with other metabolites monitored.

Table 4.6 **Summary of the mean (n = 9) pharmacokinetic data for the metabolites detected in human plasma after the consumption of a high polyphenol cider apple extract, ± standard deviation; nc represents not calculable as no standard available. Not possible to calculate a standard deviation where there is an asterix as the value is taken from mean data.**

Metabolites Identified in Human Plasma	T_{max} (h)	C_{max} (nM)	$T_{1/2}$ (h)	AUC (µmol/h/L)	
				Control	Test
(-/+) Epicatechin-3′-glucuronide	1.4 ± 0.5	nc	1.7 ± 0.4	30277 ± 35611	17421418 ± 6244268
(-/+) Epicatechin-3′-sulfate	1.2 ± 1.0	nc	3.34 *	2964483 ± 2301194	6920378 ± 4147017
Phloretin	0.4 ± 0.1	291 ± 175	2.0 ± 0.4	239412 ± 99264	804120 ± 249841
Dihydroxyphenylacetic acid sulfate	1.3 ± 1.1	nc	4.2 ± 1.5	24784430 ± 12742312	68014125 ± 61717336
5 – (3′,4′-Dihydroxyphenyl)-γ-valerolactone glucuronide	6.3 ± 1.3	nc	2.5 ± 0.8	647911 ± 1178900	114693059 ± 98627689
Dihydroxyphenyl valerolactone sulfate	5.7 ± 1.9	nc	4.1 *	5075677 ± 4704869	132245253 ± 198609487
p-Coumaric acid	0.8 ± 0.8	634 ± 225	2.3 ± 0.9	2230697 ± 1281068	4611583 ± 2066763
Sulfate conjugate *m/z* 381 / 301	2.8 ± 2.5	nc	2.0 ± 0.7	28645210 ± 21497990	92646359 ± 131033469
Unknown conjugate *m/z* 353 / 191	1.5 ± 0.7	nc	1.5 ± 0.5	455676 ± 237427	1516830 ± 629622
Unknown conjugate *m/z* 289 / 109	0.8 ± 0.5	nc	1.9 ± 1.1	300846 ± 143800	432781 ± 264647

Table 4.7 **Summary of the mean (n = 9) percentage area for each of the metabolites identified in control and test human plasma samples following ingestion of 'Tesco' pure apple juice (control) and a high polyphenol cider apple extract (test).**

Metabolites Identified in Human Plasma	Percentage of Total Analyte Area	
	Control	Test
(-/+) Epicatechin-3'-glucuronide	< 0.1	7.2
(-/+) Epicatechin-3'-sulfate	3.0	2.6
Phloretin	0.3	0.5
Dihydroxyphenylacetic acid sulfate	31.1	21.1
5 – (3',4'-Dihydroxyphenyl)-γ-valerolactone glucuronide	0.5	15.8
Dihydroxyphenyl valerolactone sulfate	6.4	15.2
p-Coumaric acid	4.5	1.9
Sulfate conjugate *m/z* 381 / 301	48.8	32.7
Unknown conjugate *m/z* 353 / 191	0.6	0.6
Unknown conjugate *m/z* 289 / 109	0.3	0.1

4.4.1.2 Biokinetics of Conjugated Metabolites in Human Plasma

The profiles for these metabolites are given in Figure 4.4, C and D. The mean time taken to reach maximum concentration in the plasma for the conjugates of (-/+) epicatechin are very similar, 1.4 h ± 0.5 h for the glucuronide and 1.2 h ± 1.0 for the sulfate (Table 4.6); both conjugates are the result of phase II metabolism reactions and it would be expected that these values would be close. This is the first time biokinetic data for metabolites of (-/+) epicatechin have been reported after the consumption of apples. These values are consistent with the maximum concentration of the metabolites in the urine, 0-4 h. Values for the elimination half lives are difficult to compare, as much more variation between volunteers was observed for the sulfate conjugate, so only a single value could be obtained from the mean profile (Figure 4.4, D). It can be suggested that the time taken for the excretion of (-/+) epicatechin-3' or 4'-sulfate is slightly extended when compared to the glucuronide, but this value is possibly an overestimate.

There is agreement throughout the literature that T_{max} values for (-/+) epicatechin in plasma are around 2 h, regardless of whether the epicatechin has been provided by tea or cocoa [81, 162]; this is in regards to epicatechin produced as a result of enzyme deconjugation experiments,

and so represents the free aglycone. In an intervention study performed by Van Het Hof *et al.* [57], the bioavailability of tea catechins in the blood of healthy human volunteers, after consumption of black and green tea, with and without milk were investigated. Analysis of both types of tea indicated that the green tea had three times the concentration of total catechins when compared to the black tea, which was reflected in the results of the total catechins in the blood after consumption; C_{max} for total catechins was 0.55 μM after ingestion of green tea and 0.17 μM after black tea, milk showed no significant effects on these levels. T_{max} was 2.3 h ± 0.2 h for green tea and 2.2 h ± 0.2 h for black tea; values determined in the present study are earlier than these reported data, suggesting the matrices of tea and apples are affecting the absorption differently. In another study investigating the bioavailability of tea catechins [75], (-) epicatechin reached a maximum concentration in the plasma between 1.4 h ± 0.6 h post dose in volunteers. Most of the catechins were present in their sulfate and glucuronidated forms, although no pharmacokinetic data is provided for these metabolites. Some biokinetic data for (-/+) epicatechin sulfate and methyl epicatechin sulfate, identified in plasma after cocoa consumption, are provided by Mullen *et al.* [106]. The dose of epicatechin was 6.7 mg supplied in the form of a cocoa drink with either water or milk, T_{max} for (-) epicatechin sulfate was 1.4 h ± 0.2 h and 1.3 h ± 0.2 h with water and milk, respectively, and T_{max} for methyl epicatechin sulfate was 1.0 h ± 0.2 h and 1.3 h ± 0.2 h with water and milk, respectively. The values for the sulfate conjugate are consistent with the findings in the present study, despite the differences in the source of the epicatechin. C_{max} values were 83 nM and 77 nM for (-) epicatechin sulfate with water and milk, respectively, and 60 nM and 50 nM for methyl epicatechin sulfate with water and milk, respectively. The values obtained with water and with milk were not significantly different. T_{max} values for (-) epicatechin sulfate were 1.5 h ± 0.1 h with water and 2.0 h ± 0.2 h with milk; values were significantly different suggesting milk was causing the metabolite to remain in the circulatory system for longer. Although cocoa is a different matrix to apples, the longer elimination half life reported in the present study suggests a slight overestimate has been made.

Roura *et al.* [81], reported that after ingestion of 54.4 mg of (-) epicatechin, C_{max} was approximately 0.63 μM in five healthy volunteers, and Baba *et al.* [162], reported a concentration of 4.9 μM after a 220 mg dose of (-) epicatechin; the (-) epicatechin was consumed as a cocoa drink in both studies. In the study by Baba *et al.* [162], the bioavailability of (-) epicatechin from cocoa was investigated by feeding chocolate and cocoa (both containing 220 mg of (-) epicatechin) to five volunteers. Similar C_{max} values for (-)

epicatechin from both food sources were reported; 4.77 μM after chocolate intake and 4.92 μM after cocoa intake. One noteable difference were the levels of non methylated (-) epicatechin in the plasma; at 1 and 2 h post ingestion of the test materials the levels were higher after cocoa intake compared to chocolate intake, at 2 and 4 h after ingestion of the test materials the concentrations of methylated (-) epicatechin were much higher after chocolate intake compared to coca intake. Other metabolites did not show significant differences between the test foods.

Comparing the proportions of these conjugated metabolites of (-) epicatechin after control and test drink consumption (Table 4.7), indicates that significantly more of the glucuronide conjugate is detected after consumption of the high polyphenol cider apple extract compared to the supermarket apple juice, and that the reverse is true of the sulfate conjugate (Table 4.7). AUC values reported in Table 4.6 indicate that (-) epicatechin-3′-glucuronide has increased 575 fold (the largest increase of any metabolite detected in the present study), compared to an increase of 2.3 fold noted with (-) epicatechin-3′ or 4′-sulfate. This suggests once again the possibility of saturation of the sulfate forming pathway, resulting in a shift towards glucuronidation. The high polyphenol cider apple extract contained over eleven times the amount of (-) epicatechin than the control drink, however increases in conjugates of (-) epicatechin represent a much greater increase. Consumption of three different amounts of decaffeinated green tea, 1.5 g, 3.0 g and 4.5 g were investigated by Yang *et al.* [75]; after 3.0 g of tea solids were consumed, increases in the C_{max} and AUC for (-) epicatechin were 3.4 fold and 3.8 fold, respectively, compared to the dose of 1.5 g, but further increases were not observed after the dose of 4.5 g. The authors reported that it was unexpected that these values increased more than two fold after doubling the amount of catechins ingested, and surprising that a further increase was not observed after the higher dose. The authors suggested a possible saturation effect for the capacity of the plasma to take up or bind catechins; from data reported in the present study it appears that the capacity of the plasma to take up (-) epicatechin from apples is greater than observed with tea.

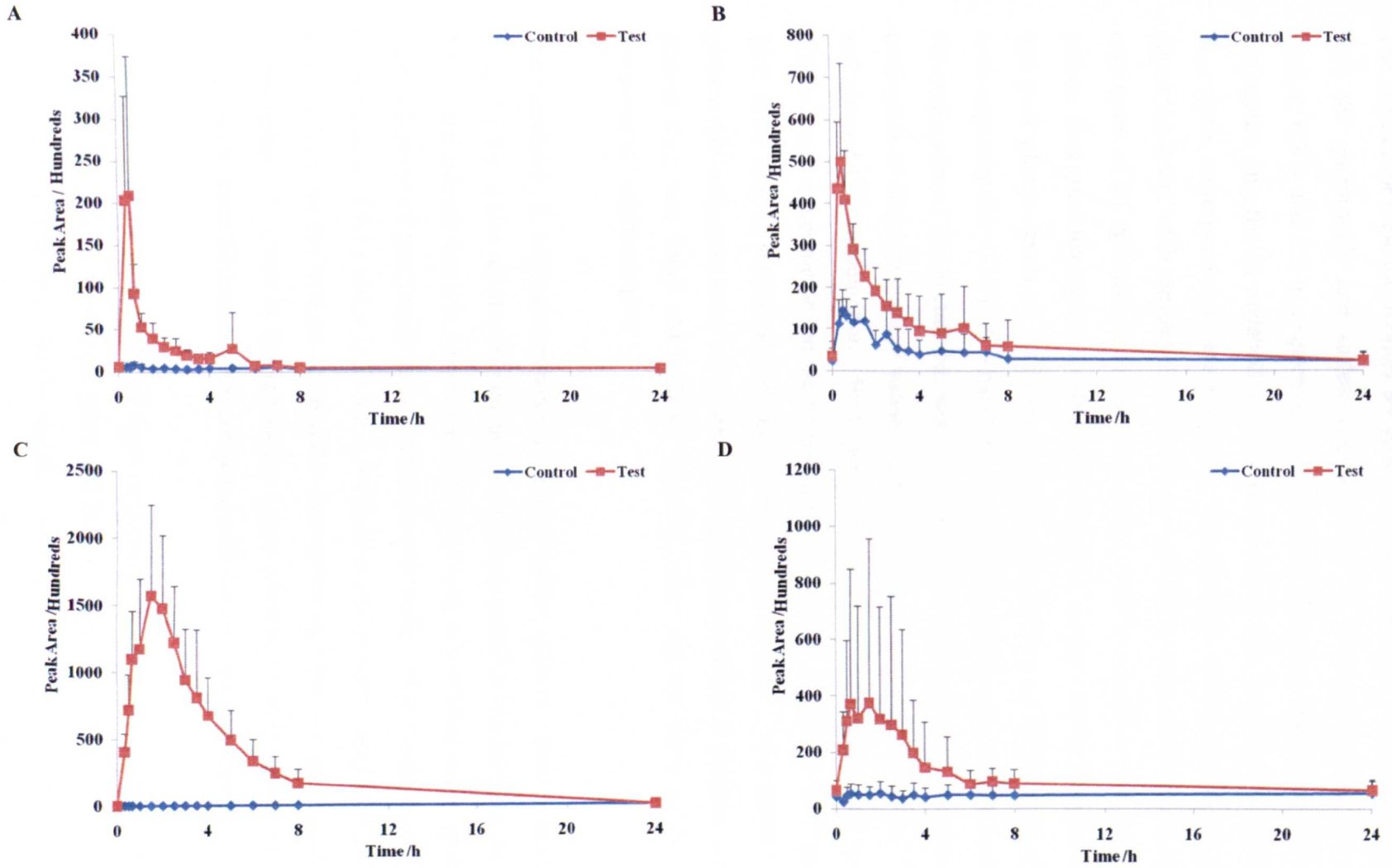

Figure 4.4 **Time profiles for phloretin (A), *p*-coumaric acid (B), (-) epicatechin-3′-glucuronide (C) and (-) epicatechin-3′ or 4′-sulfate (D) metabolites in control and test human plasma (A). Error bars represent the standard deviation (n = 9)**

4.4.1.3 Biokinetics of Conjugated Ring Fission Metabolites in Human Plasma

The profiles for these metabolites are given in Figure 4.5. Table 4.6 indicates that the T_{max} for both the glucuronide and sulfate conjugates of 3′, 4′-dihydroxyphenylvalerolactone are similar, 6.3 h and 5.7 h, respectively. This is the first time that biokinetic data for these conjugated ring fission metabolites has been provided after consumption of apples. These T_{max} times correspond with the maximum concentration of the metabolites in urine, both appearing in the 4-8 h sample. The later times recorded for these metabolites compared to the conjugates of (-) epicatechin, further demonstrate their formation in the colon by microbial action. Post green tea ingestion containing 200 mg of (-) epicatechin, Meng *et al.* [74], reported that peak plasma levels of 3′, 4′-dihydroxyphenylvalerolactone appeared at around 5 h and were approximately 0.36 μM. Enzyme deconjugation experiments were performed so no data for conjugates of this metabolite were provided. Some quantitative data was provided for conjugates of dihydroxyphenyl valerolactone in plasma, by Urpi-Sarda *et al.* [83] after cocoa and almond [181] consumption. Post cocoa consumption, two glucuronide conjugates were detected and concentrations in the plasma were reported as 39.1 nM ± 14.1 and 113.9 nM ± 34.5; these showed significant increases to the control samples. After almond consumption, glucuronide conjugates were only detected in the urine; a sulfate conjugate was quantified in plasma C_{max} was 368.2 nM ± 224.4, but this value did not show much increase when compared to control samples.

The metabolite, 3, 4-dihydroxyphenylacetic acid sulfate, appears earlier in the plasma, with a T_{max} of 1.3 h; it also reached maximum concentration earlier in the urine, in the 0-4 h sample. This could indicate that it is formed via a different route to microbial action, possibly from the direct cleavage of procyanidins, as mentioned previously. Urpi-Sarda *et al.* [83], reported a concentration of 0.11 nM ± 0.02 for 3, 4-dihydroxyphenylacetic acid in plasma after cocoa consumption, but the increase compared to the control samples was only small. Post almond consumption [181], levels of this metabolite waere reported as 385.8 nM ± 30.0, which again did not show much difference when compared with control plasma samples.

Comparisons of the proportions of these conjugated ring fission metabolites in plasma after consumption of the control apple juice and high polyphenol cider apple extract (Table 4.7), showed the same pattern as for the metabolites in urine. After the control drink, 5 – (3′, 4′-dihydroxyphenyl valerolactone) - γ - glucuronide accounted for 0.5 % of the total analyte area,

compared to 6.4 % for the sulfate; after test drink consumption, the glucuronide and sulfate conjugates accounted for approximately equal proportions of the total analyte area (15.8 and 15.2 %, respectively). The metabolite 3, 4-dihydroxyphenylacetic acid sulfate, also accounted for a greater percentage of the total analyte area after control drink consumption, compared to the test drink. The AUC values given in Table 4.6 indicate that 5 – (3′, 4′-dihydroxyphenyl valerolactone) - γ - glucuronide has increased by 177 fold and the sulfate conjuagte has increased by 26.1 fold; the metabolite 3, 4-dihydroxyphenylacetic acid sulfate showed a 2.7 fold increase. This further supports the theory for the saturation of the sulfate pathway at low concentrations of polyphenols, explained previously.

4.4.1.4 Biokinetics of Unidentified Apple Related Metabolites in Human Plasma

Profiles for these unidentified metabolites are given in Figure 4.6. The times taken for maximum concentration of these metabolites indicate that they are absorbed in the small intestine, and not as a result of microbial action in the gut. Their elimination half lives also indicate that they are rapidly eliminated from the body. The partly identified sulfate metabolite with the mass transition *m/z* 381 / 301 represents 48.8 % of the total analyte area for metabolites after the consumption of the control drink, but only 32.7 % of the area after test drink consumption (Table 4.7), which has been seen for all of the other metabolites containing a sulfate moiety. The AUC values reported in Table 4.6 indicate that the mass transition *m/z* 381 / 301 increased 3.2 fold after test drink consumption compared to after control drink consumption, which is a similar increase as that observed with (-/+) epicatechin-3′ or 4′-sulfate. The two unidentified metabolites with mass transitions *m/z* 353 / 191 and *m/z* 289 / 109 represent the same proportion of the total analyte area after the control drink as for the test drink (Table 4.7); AUC values (Table 4.6) indicate an increase of 1.4 and 3.4 fold, for the two mass transitions, respectively.

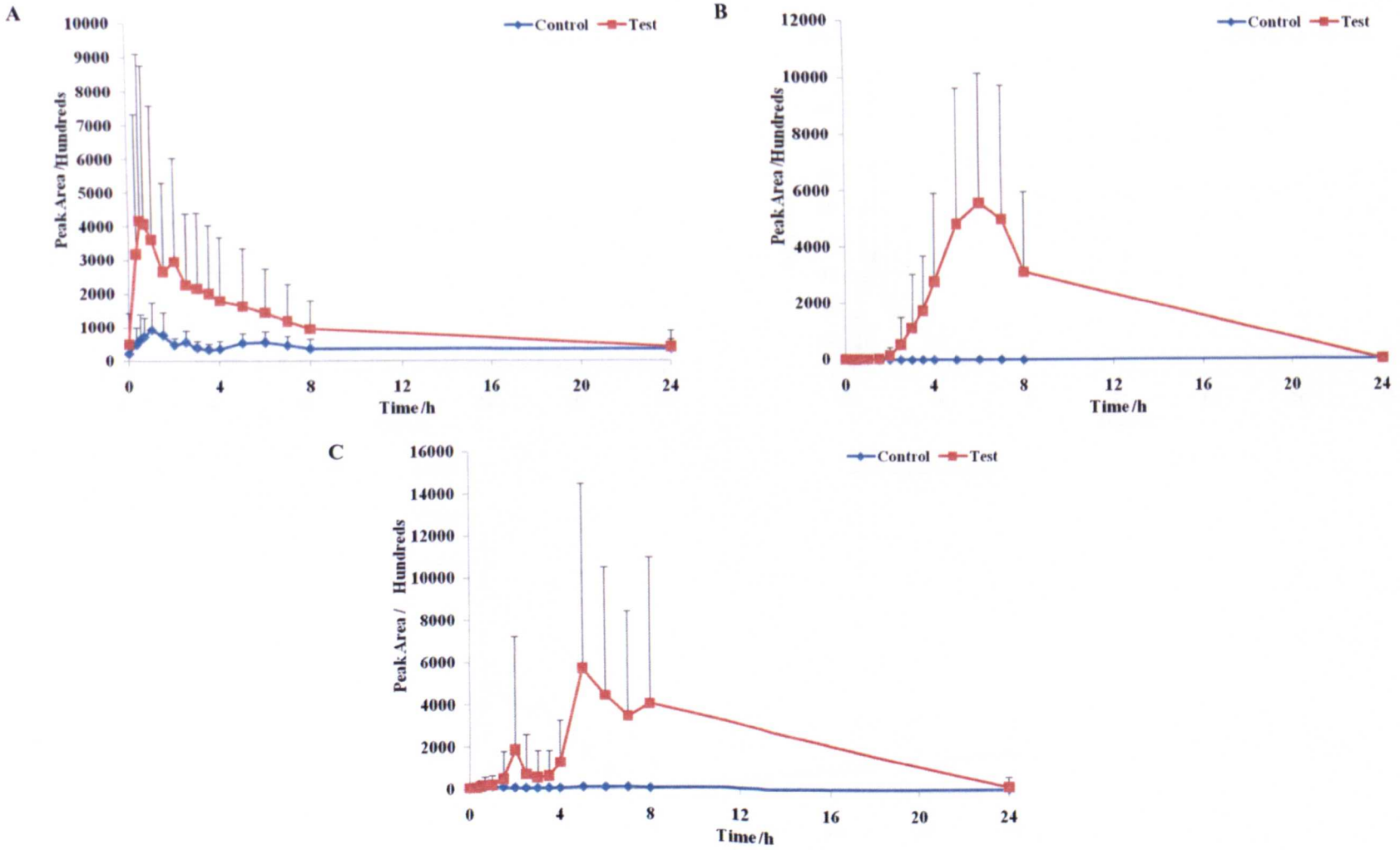

Figure 4.5 **Time profiles for the conjugated ring fission metabolites 3, 4-dihydroxyphenylacetic acid sulfate (A), 5 – (3′, 4′-dihydroxyphenyl valerolactone) - γ - glucuronide (B) and dihydroxyphenyl valerolactone sulfate (C) metabolites in control and test human plasma (A). Error bars represent the standard deviation (n = 9)**

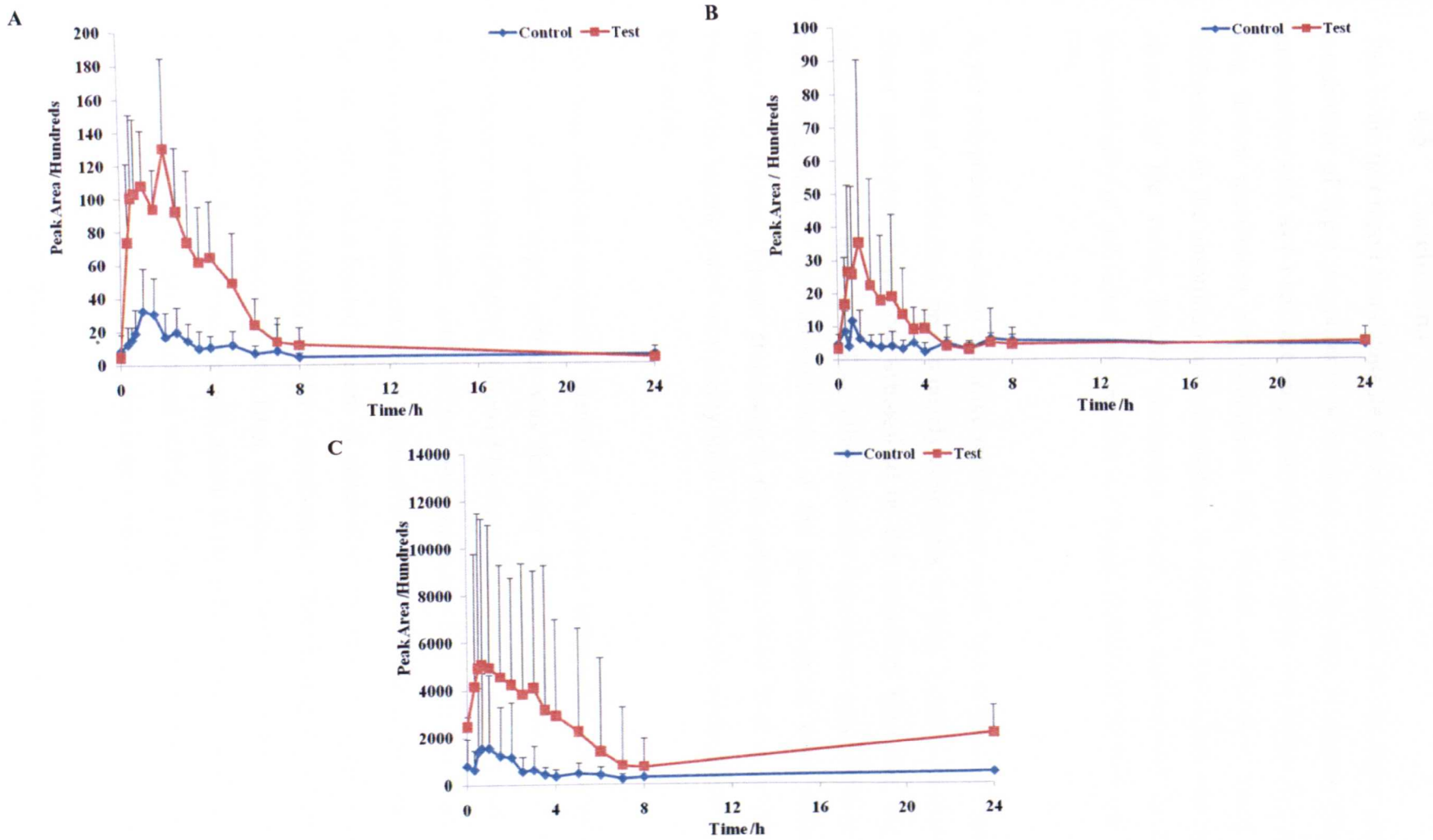

Figure 4.6 **Time profiles for the unknown metabolites *m/z* 353 / 191 (A), *m/z* 289 / 109 5 (B) and 381 / 301 (C) in control and test human plasma (A). Error bars represent the standard deviation (n = 9)**

4.5 Conclusions

This is the first report that details the biokinetic parameters for the major urinary and plasma metabolites of apple polyphenols in humans; not only does it include data on conjugated metabolites such as (-) epicatechin glucuronide and sulfate, but it also discusses data for the ring fission metabolites and conjugated ring fission metabolites. There are substantial differences in the absorption and elimination of different flavonoids and their metabolites, shown by the varied kinetic parameters which are summarised in Table 4.8. The bioavailability of individual polyphenols is strongly influenced by their chemical structures [181].

Apple polyphenols undergo extensive metabolism in the human digestive system to produce an array of metabolites, predominately conjugates of ring fission compounds. These ring fission metabolites have been synthesised by the microflora inhabiting the gastrointestinal tract, indicated by their later T_{max} values; current hypotheses suggest these compounds are then conjugated in the mucosal cells of the gastrointestinal tract, before entering the circulatory system. Further processing is also possible in the liver as these metabolites pass through the hepatic portal vein; methylation, deconjugation and conjugation reactions could all be feasible.

The most abundant metabolite identified in human urine after consumption of the high polyphenol cider apple extract was the ring fission metabolite 3′, 4′-dihydroxyphenyl valerolactone sulfate (39.0 %), followed by the glucuronide conjugate (38.6 %); in plasma, 3, 4-dihydroxyphenylacetic acid sulfate predominated (21.1 %), followed by 5- (3′, 4′-dihydroxyphenyl valerolactone) –γ- glucuronide (15.8 %) and sulfate conjugates (15.2 %). This indicates that a limited amount of metabolism occurs in the upper gastrointestinal tract and liver producing conjugates of (-) epicatechin, while the majority of the metabolism for apple polyphenols occurs in the large intestine; in urine, over 81 % of the metabolites identified were derived from bacterial action in the gut, in plasma the figure was over 54 %. Resulting metabolites are eliminated within 24 h, shown by their short elimination half lives and suggests that regular consumption is necessary for their accumulation in the human body.

Evidence of maximum plasma concentrations for metabolites such as phloretin, *p*-coumaric acid and 3, 4-dihydroxyphenylacetic acid sulfate occurs half an hour after test drink

consumption, implying that absorption is fairly rapid. Initial plasma concentration peaks and early values calculated for T_{max} confirm the early absorption of compounds in the small intestine. Later peaks in concentrations and longer values for T_{max} indicate absorption of the compound through the large intestine and/or recycling through digestive fluids. More than eight hundred bacterial species inhabit the human intestinal tract, impacting the health and nutritional status of the host [183]. The majority of the bacteria are present in the colon; concentrations of up to 10^{12} cells have been recorded per gram of faeces and 99 % of these bacteria are anaerobic [44]. A balanced composition of gut microflora confers benefits to the host, whereas microbial imbalances are associated with metabolic and immune related diseases [184, 185], such as inflammatory bowel disease and colorectal cancer.

As mentioned in previous chapters, the composition of microorganisms in the gastrointestinal tract can be influenced by diet among other factors, but in general the composition of an adults gut consists of mainly *Bacteroides*, followed by several genera belonging to the division *Firmicutes,* such as *Eubacterium*, *Ruminococcus* and *Clostridium*, and the genus *Bifidobacterium*. Transformations by these microbes via esterase, glucosidase, demethylation, dehydroxylation and decarboxylation activities, directly affect the bioavailability of polyphenols in the diet. The intestine is the preferred site of absorption of these bacterial products and enterohepatic circulation, discussed previously in this chapter, ensures their presence in plasma is extended compared to that of the parent compounds, before final urinary excretion.

Inter-Subject Variation

The length of time taken for food to be absorbed and eliminated depends on a huge number of factors, such as the type of food matrix, other dietary constituents consumed with the item under investigation, and many individual factors such as age, gender, rate of metabolism, health status, diet and gut inhabitants. In this study, it was observed that for most of the major metabolites of apple polyphenols, variation is mainly restricted to proportions, times taken to reach maximum levels or time taken for excretion; variation in the actual types of metabolites produced seems to occur only for the more minor metabolites. With such a small number of volunteers it is difficult to make any conclusions regarding the differences observed, and the data shows more similarities than differences.

Summary

The most important findings from the present study include the identification of predominately conjugated ring fission metabolites in the urine and plasma of volunteers; evaluation of their biokinetics indicates that their production is dependent on the colonic microflora. Also, much evidence has been provided to support the theory that at low concentrations of polyphenol intake, more sulfate conjugates are produced; the pathway is one of high affinity and low capacity. Following ingestion of higher intakes of polyphenols, this pathway becomes saturated, and glucuronide conjugates predominate. It has also been demonstrated that at the higher polyphenol intakes, the levels and proportions of metabolites do not necessarily increase; some increases are observed but they do not represent the same fold increases as the intakes, some are much greater and others are not.

Although the data presented here has been reported for the first time after the consumption of apples in humans, the data supports some of the evidence provided by researchers investigating different food sources which contain similar polyphenols; any variations identified can be attributed to the differences in the food sources of the polyphenols, the matrices and other foods consumed with the test items. This data extends the very limited research already published following apple consumption, particularly in healthy volunteers, and advances the area of understanding of the metabolism of apple polyphenols in humans.

Table 4.8 **Summary of the apple polyphenol derived metabolites identified by LC/MS/MS in the urine and plasma of human volunteers after consumption of a control apple juice and a high polyphenol cider apple extract, their relative abundance in these biological fluids and their biokinetic parameters. An asterix represents metabolites identified for the first time after the consumption of apples.**

Metabolites Identified in Human Urine	Relative % Analyte Area Control / Test	T_{max} (h)	Metabolites Identified in Human Plasma	Relative % Analyte Area Control / Test	T_{max} (h)	C_{max} (nM)	$T_{½}$ (h)	AUC (h/µmol/L) Control	AUC (h/µmol/L) Test
(-/+) Epicatechin-3'-glucuronide*	0.2 / 0.4	0-4	(-/+) Epicatechin-3'-glucuronide*	< 0.1 / 7.2	1.4 ± 0.5	nc	1.7 ± 0.4	30277 ± 35611	17421418 ± 6244268
(-/+) Epicatechin-3' or 4'-sulfate*	5.4 / 0.9	0-4	(-/+) Epicatechin-3'-sulfate*	3.0 / 2.6	1.2 ± 1.0	nc	3.34	2964483 ± 2301194	6920378 ± 4147017
(-/+) Epicatechin sulfoglucuronide*	0.1 / 0.2	4-8	Phloretin	0.3 / 0.5	0.4 ± 0.1	291 ± 175	2.0 ± 0.4	239412 ± 99264	804120 ± 249841
Phloretin-2'-glucuronide	12.2 / 14.5	0-4	Dihydroxyphenylacetic acid sulfate*	31.1 / 21.1	1.3 ± 1.1	nc	4.2 ± 1.5	24784430 ± 12742312	68014125 ± 61717336
5 – (3',4'-Dihydroxyphenyl)-γ-valerolactone glucuronide*	11.4 / 38.6	4-8	5 – (3',4'-Dihydroxyphenyl)-γ-valerolactone glucuronide*	0.5 / 15.8	6.3 ± 1.3	nc	2.5 ± 0.8	647911 ± 1178900	114693059 ± 98627689
Dihydroxyphenyl valerolactone sulfate*	63.6 / 39.0	4-8	Dihydroxyphenyl valerolactone sulfate*	6.4 / 15.2	5.7 ± 1.9	nc	4.1	5075677 ± 4704869	132245253 ± 198609487
Dihydroxyphenyl valerolactone disulfate*	0.5 / 3.1	4-8	*p*-Coumaric acid*	4.5 / 1.9	0.8 ± 0.8	634 ± 225	2.3 ± 0.9	2230697 ± 1281068	4611583 ± 2066763
3, 4-Dihydroxyphenylacetic acid sulfoglucuronide*	0.1 / < 0.1	0-4							
Sinapic acid*	0 / < 0.1	8-24							

Chapter Five

Conclusions

Summary

The development of the novel and validated LC/MS/MS method detailed in the present study for the identification of a whole range of metabolites of apple polyphenols, in human biological fluids, represents a significant contribution to this area of research. In addition to providing evidence for the identification of new major and minor urinary and plasma metabolites of apple polyphenols, some biokinetics of these metabolites have been reported and used to evaluate absorption and excretion of these compounds. This has not been previously reported in the literature. The methods utilised in this research have wider applications for the study of polyphenols from other dietary sources, including tea and cocoa derived polyphenols.

5.1 Analytical Methodology

The analytical method developed in the present study has made many improvements over existing methodologies; a full range of aglycones, conjugated metabolites, ring fission metabolites and conjugated ring fission metabolites of apple polyphenols have all been detected and the majority identified, in both human plasma and urine using the same single method. Validation was successfully performed, which lends credibility to the quality of the results reported. Great care and attention to detail were applied in the development of the method, which is reflected in the quality of the results obtained. Good quality data requires good experimental design and properly validated methodology. There is potential for wider applications of this method; it could be applied to any study involving polyphenols not just those using apples.

This is a complex and difficult area of research, indicated by the lack of studies detailing the metabolism of polyphenols, particularly from apples, in healthy humans. Analysis of this range of phenolic metabolites in biological samples in just a single analytical run makes this method unique; the only other comprehensive report of urine and plasma metabolites, in this case post cocoa consumption, uses one method for the determination of conjugated metabolites and one for the identification of ring fission metabolites [83]. One of the strengths of the method presented here is the broad range of polyphenolic compounds and their metabolites which can be detected and identified; many researchers focus on one group of flavonoids, in particular the catechins from either tea [57, 73-75, 77, 169] or cocoa [81, 106, 176]. However, use of a more specific method containing less MRM transitions, would have increased the sensitivity which may have enabled more minor metabolite constituents to be detected and identified, increasing its usefulness further. There are always compromises to be made when developing a method and this method demonstrates a balance between many of the important characteristics of a method which increase its usefulness and suitability. Another positive approach of the method is that analysis time is short; a further compromise as extension of the LC/MS/MS run time would increase overall analysis time, which has its draw backs, but it would mean that the sulfate conjugates, which appear to have much longer retention times under the current conditions, would have more time to elute, and better separation would be apparent. One paper reporting the analysis of conjugated and ring fission metabolites in human urine and plasma after almond consumption, utilises a method taking over 120 min [181].

One of the major limitations with the methods reported presently is the lack of commercially available standards to provide definitive identifications and quantitative data. Under the circumstances, the metabolite identifications proposed and the biokinetic data reported here is of the best possible standard and has significantly contributed to the understanding of the metabolism of apple polyphenols in humans. If time and funds were not placing constraints on the research, it may have been possible to organise some synthesis of apple polyphenol metabolites, such as some of the conjugates of (-/+) epicatechin and ring fission products which could have been used to finely tune the MS for the specific detection of these metabolites and further improve detection levels. Presence of these compounds in the 'spiking' solution would also have allowed validation parameters to be determined for these metabolites, currently it can only be assumed that they behave in a similar way to their aglycone or glycosylated forms due to similarities in structures. Standards would also have enabled quantitative values to be placed on biokinetic data, such as area under the curve which would have given a better estimate of bioavailability.

One particularly challenging area of the method development was performing the validation and calculating the effect of the urine and plasma matrices on the polyphenols and their metabolites. Most of the available papers reporting data on the identification and /or quantification of polyphenol metabolites in human urine and plasma do not undertake any validation procedures, because it is time consuming and complex, but this represents a major weakness in the literature [57, 72, 73, 75, 77, 83, 106, 169, 181]. An estimate of the effect of urine and plasma matrices on the ionisation of polyphenols in the MS has been given in the present study, although because each and every plasma and urine sample is different, it is impossible to put an absolute value on the suppression or enhancement effects of each polyphenol. The efficiency of ionisation was affected differently, depending on the class of polyphenol being analysed although similarities were apparent due to common structural features and similarities in response factors (slopes of the calibration curves). Thus, the metabolites of polyphenols such as the sulfate and glucuronide conjugates and the ring fission metabolites will probably be affected by the matrix differently to the aglycones and glycosides. This highlights another area where standards for all the possible metabolites would have contributed greatly to the research. The extraction of the polyphenols and their metabolites from plasma would also have been improved by the inclusion of more standards.

A further strength of the research presented here, is the well-designed human intervention study, purposely designed to produce good biokinetic data. This involved collection of many samples after control and test drink consumption, to provide detailed profiles of the changes in concentrations of the metabolites in urine and particularly those in plasma. The majority of intervention studies in the literature do not collect as many samples for plasma [57, 72, 74, 75, 87], sometimes only one or two samples [76, 81, 83, 97, 181] which limits the information which can be concluded from the studies; some reports are solely concerned with urinary analysis [73, 77, 169]. Accurate timings and rapid processing of the samples contributed to the high quality data produced. One major limitation with the intervention study was the small number of volunteers that participated; greater numbers would reduce the effects of inter-individual variations and provide better quality data. Numbers of volunteers used for intervention studies reported in the literature are generally smaller than the sample size used presently, commonly two to nine [72-74, 77, 81, 106, 162, 181], although some do use larger sample sizes of eighteen to forty-two [75, 83, 105, 169]. Additional urine collection time-points would also have improved the data on the excretion pattern of apple polyphenol metabolites, and defining an amount of water for consumption per time-point by each volunteer would have kept sample volumes more consistent.

5.2 Identification of Apple Polyphenol Metabolites in Human Urine and Plasma

The identification of polyphenol metabolites achieved in this study has contributed to the research area greatly; confirmation of the presence of known metabolites of apples such as conjugates of (-) epicatechin and phloretin has been provided, in addition to the identification of novel metabolites not previously reported after apple consumption. The use of LC, MS and LC/MS/MS for the identification of these metabolites provides a strong argument for their presence in biological fluids. The combination of enzyme deconjugation experiments to gain total aglycone concentrations excreted and analysis without enzyme treatment, enables the detail of conjugation for these metabolites to be investigated and structural conformations to be proposed; many other researchers only analyse samples post enzyme deconjugation which results in loss of these details [74-77, 87, 105, 177]. Many of the metabolites identified presently are also relevant to other dietary materials, and the methods used to obtain this information could have many wider applications. One limitation to the identification of the metabolites of apple polyphenols, relates to the lack of commercially available standards.

As mentioned previously, the identification of the metabolites of polyphenols in the literature is mainly concerned with the conjugates of flavanols, such as (-) epicatechin. Recently, there has been a move towards the identification of the ring fission products of microbial action in the gut, and their conjugates. Literature published in the last half of 2009 [83, 105, 181], shows the increasing trend for the analysis of these metabolites, as their importance to bioavailability and understanding the full metabolism of polyphenols has been realised. This supports the direction of the current research and it can be predicted that much more research in this area will follow over the coming years.

5.3 Biokinetics of Apple Polyphenol Metabolites in Human Urine and Plasma

The execution of the detailed and appropriate human intervention study reported presently, the attention to detail in the sample handling and the rigorous use of the LC/MS/MS method allowed the determination of high quality biokinetic data for the metabolites of apple polyphenols in human urine and plasma; this data has not previously been published. This data extends the information in the literature already available for apple polyphenols, which is scarce and only relates to the analysis of compounds already present in the ingested apple-related material, or to a single glucuronide conjugate of phloretin identified, or to volunteers with an ileostomy. This data also compares well with biokinetic data published for the same polyphenols present in different food sources, such as tea and cocoa, but does also indicate some differences partly due to the food matrices. Finally, additional information on novel metabolites of bacterial origin has been provided to give a better understanding of the absorption of apple polyphenols. The techniques and methods utilised for the determination of the biokinetic data, can be applied to other areas also, suggesting a much wider application. Again, the main limitation to the biokinetic data reported for apple polyphenol metabolites is the lack of commercially available standards, which has been discussed previously.

The trend towards the identification of the microbial metabolites of polyphenol metabolism in humans in the later part of last year, as discussed in the previous section, will lead to further studies in the area of the biokinetics of these metabolites, which is currently greatly under-researched. This will enhance the understanding of the absorption and excretion of polyphenols, and may also be applied to the metabolism of drug-related material.

5.4 Future Work

Over the course of this study, new literature concerning the identification of the ring fission and conjugated ring fission metabolites of polyphenol metabolism in humans has been published, using new LC/MS/MS methodologies and new food sources, such as the recent publication with almonds [181]. Two papers were published one concerning the fate of apple polyphenols in healthy humans [72] and one in subjects with an ileostomy [99] since commencing this research; prior to this very little was known. These papers concerned with apple polyphenol metabolism are far from comprehensive and indicate how much the research carried out presently was required. Recently, the focus has moved away from experiments with the aglycone and glycosylated forms of polyphenols found in food and beverages, as research indicated it was the metabolites of these compounds that are actually transported around the circulatory system, reach the tissues and contribute to beneficial heath effects hypothesised to result from diets high in fruit and vegetables. The trend towards microbial metabolite analysis supports the direction of this current work, and highlights the need for more research in this area to be undertaken; a viewpoint expressed by Del Rio *et al.* [186] in a publication last month.

Some additional work could be performed to enhance the results of this study and further investigate the metabolism of apple polyphenols. LC/MS^3 would have provided further detail on the fragmentation pathways and the inclusion of synthesised standards would have enabled more confirmed identifications and quantitative data to be reported. The accurate mass of the novel metabolites could have been determined by the use of time of flight mass spectrometry (TOF), which would also have contributed further to the validity of the identification. The use of NMR techniques coupled to LC and MS may also be of use in determining metabolite identities, particularly isomeric forms.

Chapter Six

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