Characterisation of polyphenolic compounds in herbal tea and berries: *in vivo* and *in vitro* studies on the bioavailability of anthocyanins

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This thesis is dedicated to my family:

A special feeling of gratitude to my loving parents, whose endless love, care, encouragement fills me with joy, strength and tenacity each day of my life. I'm so blessed to have you both.

To my true love, my husband, best friend and soul mate. Your love, support constant patience and sacrificing inspired me to complete this work as you made our future look brighter.

To my brothers and sisters, who have prayed for me and were by my side throughout all the process. I will always appreciate all what they have done.

At the end, I also dedicate this dissertation to my coming little angel, my baby, where another journey begins.

Abstract

Flavonoids and polyphenolic compounds are widely distributed in commonly consumed foods, beverages and herbs. Their consumption has been associated with improving the health and reducing the risk of several chronic diseases. In Saudi Arabia, recent interest in the health-promoting properties of traditional herbs has been increased. To-date researchers have focused on testing these therapeutic properties. However, information about their chemical profile is largely unexplored. Thus, the beginning of this project aimed to use HPLC–PDA–MS^2 to identify polyphenolic compounds in a medicinal herbal tea made from seeds of *Anastatica hirerochuntica*. Twenty compounds comprising a series of flavone *C*- and *O*-linked glycosides, phenolic acids, chlorogenic acids and flavonols were identified or partially identified on the basis of co chromatography with reference compounds and MS^2 and MS^3 fragmentation patterns. The flavones were the principal components, occurring as luteolin, apigenin and diosmetin conjugates. The antioxidant potential of individual compounds in *Anastatica* was assessed using HPLC with an on-line $ABTS^{\bullet+}$ detection system. Under the experimental conditions, 14 compounds exhibited antioxidant activity. The highest contribution to the antioxidant capacity of the tea, 56%, came from 3,4-dihydroxybenzoic acid and caffeoyl- and dicaffeoylquinic acids while 6-C-glucosides of luteolin and apigenin contributed 41%. These findings together with tests in the FRAP antioxidant assay and the Folin–Ciocalteu total phenolics assay revealed that aqueous infusion of *A. hirerochuntica* seed is rich in polyphenolic compounds.

Another perspective of investigating these polyphenolic compounds is not just knowing their chemical constituents but also studying their bioavailability upon ingestion. Therefore, blackberries and strawberries were chosen for intervention studies with human subjects to examine the bioavailability of anthocyanins, a group of flavonoids, due to their importance as natural antioxidants that have been linked with beneficial effects on health to humans. In the first *in vivo* study, plasma and urine were collected from eight healthy subjects after ingestion of 180 g of blackberries and analysed by HPLC-PDA-MS^2. Cyanidin-3-*O*-glucoside was metabolised principally to peonidin-*O*-glucuronide. Cyanidin-3-*O*-

glucoside and its metabolites appeared rapidly in the plasma in low concentrations ranging from 5 to 20 nmol/L with low urinary excretion of ~0.08% of the total intake. In the second *in vivo* study, both blackberry and strawberry were used to examine the small intestinal absorption and metabolism of both cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside by feeding five ileostomy volunteers with 180 g of berries. Samples of urine and ileal fluid were collected over a 0-24 h period after ingestion and analysed by HPLC-PDA-MS^2 Unmetabolized anthocyanins of blackberries and strawberries were recovered in ileal fluids at a level of 5.1% and 13.6%. This indicates either low level of absorption or lack of stability at the non-acid pH in the small intestine. The overall urinary excretion of anthocyanins was 0.06% and 0.87% of intake of blackberries and strawberries respectively. The urine data shows that both cyanidin and pelargonidin glucosides were metabolised primarily to peonidin-*O*-glucuronide and pelargonidin-*O*-glucuronide respectively. Although low recoveries of the two compounds are reported in this investigation in both ileal fluid and urine, pelargonidin-3-*O*-glucoside showed a slightly higher recovery compared to cyanidin-3-*O*-glucoside. The data from both *in vivo* studies reflect the influence of the aglycone structure on the bioavailability of these anthocyanins.

The stability of anthocyanins in the gastrointestinal tract was also assessed using *in vitro* models (Chapter 5). The results indicated that at neutral pH the salivary enzymes and oral microbiota had no impact on cyanidin-3-*O*-glucoside but degraded pelargonidin-3-*O*-glucoside by ~20%. With simulated and human gastric juice, both anthocyanins were unstable and the breakdown continued dramatically under pancreatic digestion where a 5.1% recovery was obtained. Their low recovery levels were comparable to those obtained *in vivo* with ileal fluid. These findings imply that the chemical structure of both anthocyanins influence their stability similarly. However, the data provide no explanation about the higher bioavailability of pelargonidin-3-*O*-glucoside compared to cyanidin-3-*O*-glucoside *in vivo*. This presumably is a consequence of the more ready access of pelargonidin-3-*O*-glucoside to the enterocytes of the small intestine.

In conclusion, the work here has shown the diversity of compounds with potential health benefits that are present in a natural product from Saudi Arabia. Experiments were carried out, using local products from the UK, to investigate factors that would affect their potential to provide health benefits. The *in vivo* and *in vitro* experiments show the complexity of investigations of potential bioactive compounds in human nutritional research. Polyphenols which differ by only a single hydroxyl group show very different absorption, metabolism and stability properties.

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Abbreviations

ABTS	2, 2'-Azino-bis-3-ethylbenzothiazoline- 6-sulfonic acid
AGly	Aglycon
Amu	atomic mass unit
AUC	plasma concentration-time area under curve
CBG	cytosolic ß-glucosidase
C_{max}	peak plasma maximum concentration
COMC	catachol-*O*-methyltransferase
DPPH	1,1-diphenyl-2-picrylhydrazyl
FRAP	ferric reducing antioxidant potential
GAE	gallic acid equivalent
GC-MS	gas chromatography tandem mass spectrometry
HDL	high density lipoprotein
HPLC	high performance liquid chromatography
λ_{max}	wavelength of maximum absorbance
LDL	low density lipoprotein
LC-MS	liquid chromatography-mass spectrometer
LPH	lactate phlorizin hydrolase
$[M-H]^-$	negatively charged molecular ion
$[M-H]^+$	positively charged molecular ion
MS^2	the second scan in MS-MS
MW	molecular weight
m/z	mass to charge ratio
n.d.	not detected
n.q.	not quantified
N.A.	not analysed
NMR	nuclear magnetic resonance
PDA	photodiode array
R_t	retention time
SGLT	sodium-glucose-co-trasporter
SULT	sulfotransferase
SRM	selective reaction monitoring
$T_{1/2}$	half-life elimination time
TEAC	trolox equivalent antioxidant capacity

TPTZ	ferric-2,4,6-tri-2-pyridyl-s-triazine
T_{max}	time reached at C_{max}
UV	ultra-violet
UDP-GT	uridine-5´-diphosphate glucuronosyltransferase
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

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Above all, I owe it all to Almighty God for granting me the strength to undertake this research task and enabling its completion. Thanks God for all the blessings that make the impossible possible.

Author's declaration

The composition of this thesis and the work described within it was carried out entirely by myself unless otherwise cited or acknowledged. The research for this thesis was carried out between October 2008 and October 2012.

Signed .....

Noura AlGamdi

April 2013

1 Introduction

Plants produce a large number of organic compounds which are classified into primary and secondary metabolites. Primary metabolites are defined as biochemicals that play an essential role in growth, photosynthesis, respiration, transport and differentiation. These biochemicals include phytosterols, acyl lipids, nucleotides, amino acids and organic acids. The secondary metabolites include less essential compounds and these products give plants their colour, flavour, and smell (Crozier *et al.,* 2006). Plant pigments, alkaloids, isoprenoids, terpenes and flavonoids are examples of secondary products. Secondary products are more complex than primary metabolites. The main roles played by secondary compounds are for defence, protection, and cell-to-cell signalling (Seigler 1977; Swain 1977).

Plant secondary metabolites include a wide variety of phytochemicals found in the diets of humans and other animals and recent studies have shown that they may have beneficial effects on health when ingested. The effects depend on the chemistry of the compounds, their concentration in the diet and on the amount consumed (Acamovic and Brooker 2005). Many epidemiological studies, which have been reviewed recently by (Del Rio *et al.*, 2012), have highlighted the link between the consumption of a diet rich in fruits and vegetables and the reduced incidences of chronic diseases. Therefore, recent attention has focused on trying to identify these components within the diet that contribute to good heath (Tomás-Barberán 1997; Crozier *et al.,* 2006). The uses of HPLC, HPLC-MS, GC, GC-MS and NMR have been employed to facilitate the identification and quantification of these compounds. However, it is likely that in the future many more compounds will be isolated and identified (Acamovic and Brooker 2005).

1.1 Phytochemicals in plants

Phytochemicals are natural bioactive, non-nutrient compounds in plants with potential therapeutic effect. There are more than 30,000 known compounds (Bidlack 2000) and they are structurally diverse and classified into four main groups: terpenoids, phenolics and polyphenolics, nitrogen containing alkaloids

and sulphur compounds (Crozier *et al.,* 2006). The focus of this investigation will be on the phenolic and polyphenolic compounds.

1.1.1 Phenolic and polyphenolic compounds

Phenolic compounds are wide spread throughout the plant kingdom. In recent years, over 8,000 phenolic compounds have been reported. Phenolics are characterised as having at least one aromatic ring attached to one or more hydroxyl groups (Crozier *et al.,* 2006). They range from simple compounds such as gallic acid, to large and complex tannins such as proanthocyanidins. In addition, phenolic substances tend to be water-soluble as they are normally conjugated with sugar and organic acids (Harborne 1998; Vermerris and Nicholson 2006). They can be classified into flavonoids and non-flavonoids (Crozier *et al.,* 2006). Previously, dietary phenolic compounds have been considered as non-nutrients and their possible benefit to human health has only recently been considered.

The term polyphenolic compounds will be used in the thesis to refer to flavonoids and related compounds, which also known as polyphenol. However, a number of compounds, including hydroxycinnamates and phenolic acids, have only one phenolic ring.

1.1.1.1 Flavonoids

Flavonoids are a large group of polyphenolics and include more than 4,000 separate compounds with different functions. They are the most numerous of the phenolics in the plant kingdom and they are concentrated mainly in the epidermis of leaves and in the skin of fruits. These compounds have been extensively investigated as they possess a wide range of biological and physiological activities (Hollman and Katan 1999).

Their chemical structure is based on a fifteen carbon skeleton, with two aromatic rings linked by a group of three-carbon bridge, known as a C_6-C_3-C_6 structure (Figure 1-1) (Vermerris and Nicholson 2006). Flavonoids are found as mixtures in almost all plant-based foods and beverages and it is very rare to find a single flavonoid component in plant tissues. Flavonoids are typically found as

glycoside conjugates. Hydroxyl groups are usually present at the 3´, 4´, 3, 5 and 7 positions. The presence of sugar and hydroxyl groups increases the water solubility of flavonoids. Other groups, such as the methyl group, give flavonoids lipopihlic properties (Harborne 1998).

The main dietary flavonoid subgroups are: flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins (Figure 1-1). However, minor flavonoids such as dihydroflavonols, flavan-3,4-diols (leucoanthocyanidins), coumarins, chalcones, dihydrchalcones and aurones are also found in low concentrations in the diet (Crozier *et al.*, 2006; Jaganath *et al.*, 2007).

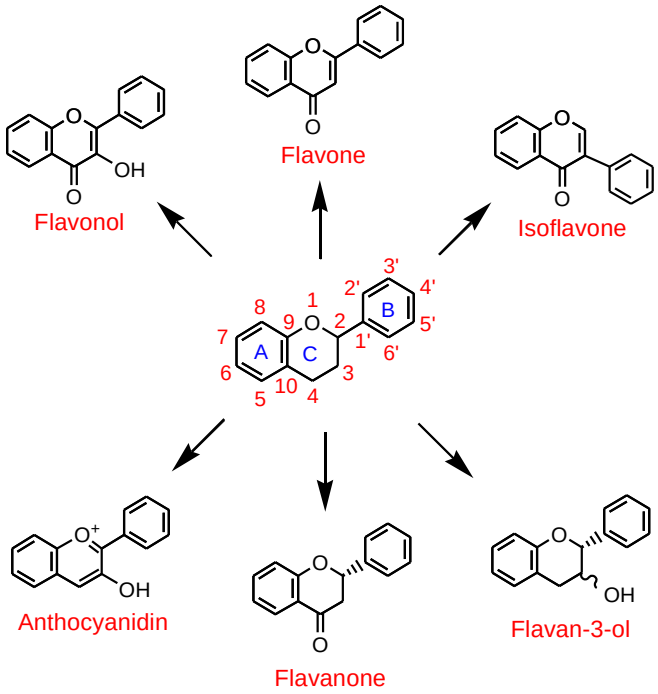


Figure 1-1: Structures of the major flavonoids (Crozier *et al.*, 2006).

1.1.1.2 Flavonols

Flavonols are the most widespread of the flavonoids throughout the plant kingdom with the exception of fungi and algae. They are widely distributed in plants as co-pigments to anthocyanins in petals and also in the leaves of higher plants. The common flavonols are: kaempferol, quercetin, myricetin and isorhamnetin (Figure 1-2) (Harborne 1998). They occur most frequently as

glycoside conjugates at position 3 of the C-ring but substitutions can also be present at the 5, 7, 4´, 3´and 5´ positions at the carbon ring allowing the production of numerous flavonol conjugates regardless of the limited number of aglycones. For example, there are more than 200 different sugar conjugates of kaempferol alone (Crozier *et al.*, 2006).

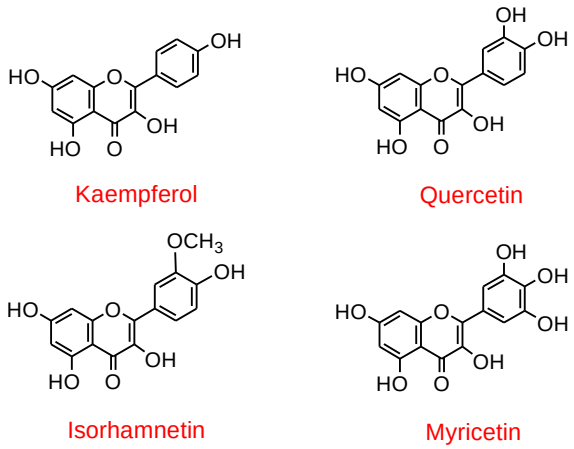


Figure 1-2: The flavonols kaempferol, quercetin, isorhamnetin and myricetin.

Numerous studies have reported on the different conjugates of flavonols found in commonly consumed fruit and vegetables. Crozier *et al.* (1997) found that tomatoes and onions are a rich source of flavonols, primarily present as sugar conjugates of quercetin. Conjugated quercetin and kaempferol with lower levels of myricetin are found in tea (Del Rio *et al.*, 2004). Kaempferol is also present in many fruits and leafy vegetables; isorhamnetin, the 3´-methoxy derivative of quercetin is found in onions and pears and myricetin occurs in berries, maize and tea (Peterson *et al.*, 2003).

1.1.1.3 Flavones

Flavones differ from flavonols as they lack the 3-hydroxyl substitution on the *C*-ring. Most flavones are present as 7-*O*-glucosides; however, hydroxylation, methylation, *O*- and *C*-alkylation can also possible to occur with flavones (Jaganath *et al.*, 2007). There are only two common flavones, apigenin and luteolin (Figure 1-3). They have limited distribution occurring in grains, leafy vegetables and some herbs (Peterson *et al.*, 2003).

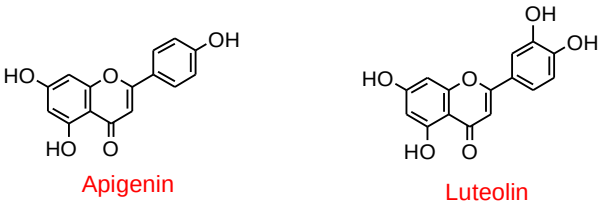


Figure 1-3: Structures of some flavonones.

1.1.1.4 Flavan-3-ols

Flavan-3-ols are a complex subclass of flavonoids that begin with the simple monomer (+)-catechin and its isomer (-)-epicatechin, and extend to oligomeric and polymeric proanthocyanidins (Figure 1-4) which are also known as condensed tannins. In addition to forming complexes with other flavan-3-ols, they are hydroxylated to form the gallocatechins, and also undergo esterification with gallic acid (Crozier *et al.*, 2006). Flavan-3-ols were found in high concentrations in purple grape juice (Mullen *et al.*, 2007). (-)-Epicatechin and its gallate derivates have been found in high concentrations in green tea relative to black tea (Del Rio *et al.*, 2004). Wine, berries, apples and chocolate are also good sources of flavan-3-ols (Sanoner *et al.*, 1999; Marks *et al.*, 2007).

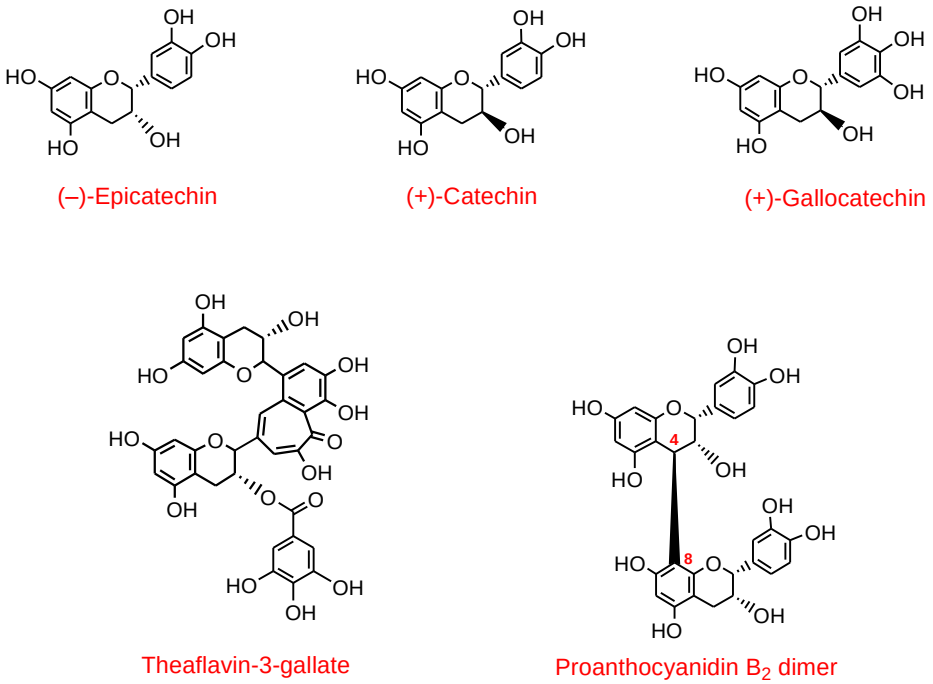
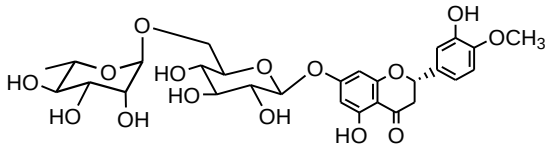


Figure 1-4: Chemical structure of the main flavan-3-ols.

1.1.1.5 Flavanones

Flavanones are characterised by the absence of the $\Delta^{2,3}$ double bond and the presence of a chiral centre at C2. They occur as glycosides, usually rutinosides and neoheperidosides attached at position 7 (Crozier *et al.*, 2006). The most common flavanone glycoside is hesperetin-7-*O*-rutinoside (Figure 1-5). Flavanones also have been reported to undergo hydroxylation, glycosylation and *O*-methylation reactions because of their highly reactive structure. Flavanones are present in high concentrations in citrus fruits such as grapefruit, lemons and limes (Peterson *et al.*, 2006). The bitter taste of grapefruits is due to the presence of flavanones 7-neohepesridoside but in oranges, rutinoside is responsible for the sweet taste (Tomas-Barberen and Clifford 2000).

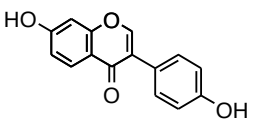


Hesperetin-7-*O*-rutinoside
(hesperidin)

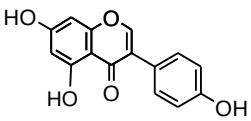
Figure 1-5: Structure of hesperidin.

1.1.1.6 Isoflavones

Isoflavones have a limited distribution in the plant kingdom. They are found only in leguminous species and are characterised by having a B-ring attached at the C3 rather than the C2 position. Soybeans are the most common source of isoflavones, with major compounds, genistein and daidzein (Figure 1-6)(Andersen and Markham 2006; Crozier *et al.*, 2006).



Daidzein



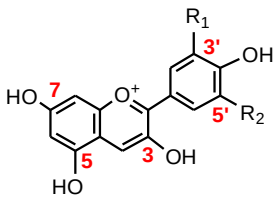
Genistein

Figure 1-6: Structures of isoflavonones.

1.1.1.7 Anthocyanidins

Anthocyanidins are the most important and widespread group of pigments in plants. They are responsible for nearly all the pink, scarlet, red-violet and blue colours in the petals, leaves and fruits of higher plants (Harborne 1998). They have an important role to play in attracting pollinating insects and also protect plants against excessive light by shading leaf mesophyll cells (Crozier *et al.*, 2006).

Anthocyanidins are chemically based on the single aromatic structure of cyanidin, and all are derived from this pigment by the addition or subtraction of a hydroxyl group, methylation or glycosylation (Harborne 1998). The most common coloured anthocyanidins are: pelargonidin (orange-red), cyanidin (red), peonidin (rose-red), delphinidin (blue-violet), petunidin (blue-purple) and malvidin (purple) (Vermerris and Nicholson 2006) (Figure 1-7). The colour varies among anthocyanidins based on the B-ring having more hydroxyl groups being more blue/violet and more methoxyl being more red. In plant tissues, anthocyanidins are found as sugar conjugates that are known as “anthocyanins”. Anthocyanidins could also form conjugation with hydroxycinnamates and organic acids such as malic and acetic acids. Conjugation mostly occurs on C3, however, it could also take place on carbons 5, 7, 3´, 4' and 5´ (Crozier *et al.*, 2006).



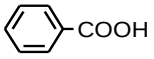
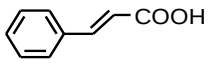
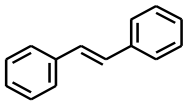
Anthocyanidin	R_1	R_2	MW	Colour
Pelargonidin	H	H	271	orange-red
Cyanidin	OH	H	287	red
Delphinidin	OH	OH	303	blue-violet
Peonidin	OCH_3	H	301	rose-red
Petunidin	OCH_3	OH	317	blue-purple
Malvidin	OCH_3	OCH_3	331	purple

Figure 1-7: Structures of the major anthocyanidins.

1.1.2 Non-flavonoids

The main non-flavonoid compounds are shown in (Table 1-1). They are the C_6-C_1 phenolic acids, the C_6-C_3 hydroxycinammates and the polyphenolic C_6-C_2-C_6 stilbenes.

Table 1-1: Structural skeletons of non-flavonid compounds (hydroxyl groups not shown).

Number of Carbons	Skeleton	Classification	Example	Basic structure
7	C_6-C_1	Phenolic acids	Gallic acid	
9	C_6-C_3	Hydroxycinnamic acids	*p*-Coumaric acid	
14	C_6-C_2-C_6	Stilbenes	Resveratrol	

1.1.2.1 Phenolic acids

The major component of phenolic acids, also known as hydroxybenzoates, is gallic acid. Gallic acid is the base unit of gallotannins while gallic acid and ellagic acid are both subunits of the ellagitaninins. Gallic acid and quninic esters of gallic have been reported in wine, grapes and green and black tea (Stewart *et al.*, 2005; Crozier *et al.*, 2006).

1.1.2.2 Hydroxycinnamates

Cinnamic acid is converted to a range of hydroxycinnamates. There are six common hydroxycinnamic acids and all plants will probably contain at least three of them. Common hydroxycinnamates are cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid, 5'-hydroxyferulic acid and sinapic acid. Apples are a rich source of hydroxycinnamates. Coumaric and caffeic acid can be found in tea and coffee (Del Rio *et al.*, 2004; Stewart *et al.*, 2005; Marks *et al.*, 2007).

1.1.2.3 Stilbenes

Stilbenes are phytoalexins produced by plants when they are attacked by fungal, bacterial and viral pathogens. The most common is resveratrol which occurs as *cis* and *trans* isomers. The main dietary sources of stilbenes are grapes, red wine, berries, soya and peanuts (Crozier *et al.*, 2006).

1.2 Dietary flavonoids and health

"Your food is your medicine" (Hippocrates cited in Tolonen,(1990).

It has been recognised for a long time that eating a diet rich in fruits and vegetables as part of a healthy, active lifestyle can protect humans against the risk of several serious diseases such as coronary heart disease, stroke, high blood pressure, diabetes and some types of cancer (Boyer and Liu 2004; Geissler and Powers 2005). The protective effects of fruits and vegetables have been attributed to the bioactive dietary phytochemicals such as flavonoids and phenolic acids, which display a wide range of biological activities (krzyzanowska *et al.,* 2010). For example, flavonoids, as one of the most widespread of the potentially bioactive secondary metabolites in the plant kingdom, were suggested extensively as an important dietary group that could be involved in the role of improving health and preventing diseases (Renaud *et al.,* 1992; Hertog *et al.,* 1993; Margetts *et al.,* 2003; Karlsen *et al.,* 2010). Flavonoids can play a number of roles as anticancer and antimicrobials and have been implicated in the prevention of a number of chronic diseases, initially due to their antioxidant activity and more recently for their anti-inflammatory properties (Rice-Evans *et al.,* 1996; Imai *et al.,* 2009). Arguably the targeting of flavonoids as compounds with potential health benefits came through the work, as previously cited, by Renaud *et al.,* (1992) and also by Hertog *et al.*, (1993). The work by Renaud *et al* showed an association between intake of red wine and a reduction in recorded death rates due to coronary heart disease, there was no direct link to flavonoids. The work by Hertog *et al* showed that those in the highest tertial of intake of apples, onions and tea again had a significant reduction in incidence and deaths from coronary heart disease. In the case of Renaud *et al* the stillbene resveratrol has attracted the most interest as the active ingredient whereas the work by Hertog *et al* the flavonoid quercetin has received the most attention. However, no direct evidence of the efficacy of these compounds has been seen in dietary supplementation (Princen *et al.,* 1998; Auclair *et al.,* 2010; van Mierlo *et al.,* 2010). This is one of the major difficulties of nutritional research as the potential benefits may only be seen after lifelong exposure.

Flavonoids can play a role as antioxidants against oxidation due to their free radical scavenging properties. However, there are differences in the antioxidative potential of different flavonoid subgroups, depending on their chemical structures (Knekt *et al.,* 2002). Flavonoids might offer protection from cancer by inhibiting oxidative damage, given their structure which is compatible with one-electron donor activity. They have been demonstrated to function as antioxidants by scavenging superoxide anion, singlet oxygen, lipid peroxy-radicals, and/or stabilising free radicals involved in the oxidative process through hydrogenation or complexing with oxidising species (Galluzzo *et al.,* 2009). Many of the flavonoid compounds have been identified as antioxidants such as quercetin, ellagic acid, (–)-epicatechin and (–)-epicatechin gallate (Terao *et al.,* 1994; Mullen *et al.,* 2002a; Mullen *et al.,* 2002b; Maatta-Riihinen *et al.,* 2004; Stewart *et al.,* 2005; Galluzzo *et al.,* 2009).

In vivo and *in vitro* studies showed an association between flavonoids and cardiovascular disease and cancer (Hollman and Katan 1999; Birt *et al.,* 2001; Benavente-Garcia and Castillo 2008). A significant inverse relationship between flavone intake and breast cancer was reported in a case-controlled study carried out by Peterson *et al.* (2003). In addition, Wu and his co-workers (1998) demonstrated that a daily intake of 10-20 mg of isoflavones, a consumption level found among the Asian population, is associated with a 10-30% reduction in the risk of breast cancer. The flavonol quercetin is also a well-known anti-cancer agent which can inhibit the growth of human gastric cancer cells (Yoshida *et al.,* 1990). Men with a high myricetin intake had a lower risk of prostate cancer (Knekt *et al.,* 2002).

In addition, many antimicrobial properties have been attributed to flavonoids, as they have the ability to inhibit human fungal, bacterial and viral pathogens. For example, quercetin, kaempferol, (+)-catechin and 5-*O*-caffeoylquinic acid, from quince fruit extract were found as a strong inhibitors of the bacterial growth (Fattouch *et al.,* 2007). Phenolic components in honey, naringenin, *p*-hydroxybenzioc acid, cinnamic acid, pinocembrin and chrysin, were the most effective compounds with high microbiological inhibition properties on a wide range of Gram-positive and Gram-negative bacteria (Estevinho et al., 2008). Pathogenic intestinal bacteria were also inhibited by berry ellagitannins

(Puupponen-Pimia *et al.,* 2005; Heinonen 2007). These *in vitro* studies showed that plant extracts inhibited bacterial growth but addressing how the natural bioactive molecules inhibit the growth of microorganisms is also an important area so that better, safer and cost effective drugs for treating bacterial infections can be developed. Flavonoids were also reported to inhibits HIV-1 infection (Li *et al.,* 2000) and act also as antifungal agents (Carlton *et al.,* 1991; Mbaveng *et al.,* 2008; Sisti *et al.,* 2008). Determining *in vivo* whether flavonoids after being absorbed by animals and humans will possess these antimicrobial properties is essential for drug discovery and improving health.

Although the protective effect of flavonoids against many serious diseases has been studied for about 50 years, the cellular mechanisms involved in their biological action are still not completely known (Benavente-Garcia and Castillo 2008). It is important to mention that each compound has different functions and the combination of these functions reduces the risk of chronic disease. For example, isoflavones reduce blood cholesterol, blood pressure and oxidative stress. This combination of bioactivity leads to a reduction in the risk of coronary artery disease. Moreover, other bioactive compounds may act in the same way to reduce the risk of these diseases which adds considerable complexity to the ability to understand their effect on health (Kris-Etherton *et al.,* 2004; Jaganath *et al.,* 2007).

1.3 Medicinal herbal plants

Herbal medicine is the oldest form of healthcare benefiting from a long and respected history. Medicinal herb defined as any herbs that had a therapeutic or prophylactic purposes or other health benefits due to the presence of active substances, such as alkaloids, flavonoids, glycosides, vitamins, tannins, and coumarin compounds, which physiologically affect the bodies of humans and animals or which are biologically active in relation to the causative agents of various diseases (Zoellner and Schwarz 2013).

For thousands of years, herbs have been used for many purposes including medicine, nutrition, flavourings, beverages, dyeing, repellents, fragrance and smoking (Zheng and Wang 2001; Wojdylo *et al.,* 2007). Many well-known

medications used in the 20th century were developed from ancient healing traditions that treated health problems with specific plants. About a quarter of the drugs used in medicine contain at least one active plant compound. For example, aspirin contains methyl salicylic acid derived from salicylic acid which occurs in white willow bark and acid meadow sweet plants (Magee 2005). Recently, scientists have extracted and isolated the medicinal properties of a large number of healing components from plants and they have synthesised several plant components in laboratories for use in pharmaceutical preparations (Zheng and Wang 2001).

Nowadays, traditional herbal medicines are receiving considerable attention from the public, researchers and health care agencies. In China and other Asian countries, traditional herbal medicine plays a major role in health care. In countries such as Germany and France, medicinal herbalism co-exists with modern medicine (Ernst 2005). However, medicinal herbs are not always free from the potential for adverse reactions which include toxicity and allergic reactions (Magee 2005; Parekh and Chanda 2007; Sekine-Osajima *et al.*, 2009).

The biological and pharmacological properties of medicinal herbs, with their antioxidant, anti-microbial, anti-inflammatory, anti-mutagenic and anti-carcinogenic potential, are attributed to the significant presence of phytochemicals. Medicinal herbs, as a source of phenolic compounds that can have a positive impact on health, have been extensively studied. However, many of these studies have not included a qualitative and quantitative analysis of phenolic compounds (Wojdylo *et al.*, 2007; Yu *et al.*, 2008). On the other hand, medicinal plants contain numerous phytochemicals in addition to phenolic compounds such as nitrogen compounds, terpenoids, carotenoids and ascorbic acid which may cause these biological activities. It is worth mentioning that the observed biological activities may not be due to a single compound in a plant but to a combination of several compounds (Harborne 1998; Zheng and Wang 2001).

Herbs and spices which contain phytochemicals can be rich sources of natural antioxidants (Wojdylo *et al.*, 2007; Coruh *et al.*, 2008). Estimation of the total antioxidant capacity and phenolic contents of 32 spice extracts from 21 botanical families in Poland showed a positive and significant correlation

between antioxidant activity and total phenolic compounds. The major phenolic acids identified in the analysed species were caffeic, *p*-coumaric, ferulic and neochlorogenic. The main flavonoids were quercetin, luteolin, apigenin, kaempferol and isorhamnetin (Wojdylo *et al.,* 2007). Chinese and Indian medicinal herbs, which contain high antioxidant components such as Vitamin E, flavonoids, phenolic acids, carotenoids and ascorbic acid, have been studied extensively. In (2008), Chan *et al.*, screened the antioxidant activity of 40 common Chinese medicinal herbs using both a DPPH and FRAP assay. The results indicated a strong positive antioxidant activity associated with total phenolic content. Ali and his co-worker (2008) reviewed 24 Indian medicinal herbs which were reported to have antioxidant properties. They concluded that the most common antioxidants among these herbs were flavanoids and tannins, followed by phenolics, ascorbic acids and alkaloids.

The majority of antimicrobial investigation studies have focused on the antimicrobial activity of traditional medicinal plants given pathogen resistance to common therapeutic agents. By screening plants used in folk medicine for new agents, various publications have documented the antimicrobial activities of plant extracts including tea, myrrh, basil and fennel (Hammer *et al.,* 1999; Oktay *et al.,* 2003; Bonjar *et al.,* 2004; Yigit *et al.,* 2009). Deans and Ritchie (1987) tested 50 different plant essential oils against 25 genera of bacteria. More than 30 oils were found to be inhibitory towards 10 or more of the test organisms. Moreover, the methanolic extract of *Rumex crispus*, a wild herb used extensively in traditional medicine in Turkey, showed strong antibacterial activity against *Agrobacterium tumefaciens, Bacillus cereus, Bacillus subtilis, Pseudomonas corrugate*, *Pseudomonas syringae* pv. *tomato, Salmonella typhimurium, Serratia liquefaciens, Vibrio cholerae, Yersinia frederiksenii* and *Yersinia pseudotuberculosis* (Coruh *et al.,* 2008). Another interesting study determined the antimicrobial activity of ethanol extracts from eight traditional medicinal plants in South Africa where they are used as a chewing stick against oral microorganisms that cause dental cavities. Six of the eight plants showed a positive inhibitory effect against five microorganisms (More *et al.,* 2008).

Yigit *et al.*, (2009) reported that the methanolic extract from eight Turkish plants used in folk medicine exhibited good anti-candidal activity against 99 *Candida* spp and they suggested that the presence of flavonoids and terpenes

might be responsible for this activity. Meanwhile, Bonjar *et al.,* (2004) tested the methanolic plant extracts of 221 species from 98 families in Iran for their antibacterial and antifungal activities. They found nine extracts that strongly inhibited the growth of *Candida albicans* and *C. Utilis*. Astragaloside IV, which is a triterpeniod saponin, was isolated from *Radix Astragali* and possesses anti-hepatitis B virus activity (Wang *et al.,* 2009). Further studies have shown the same inhibitory effect of two flavonoids (isoliquiritigenin and glycycoumarin) extracted from *Glycyrrhizae Radix* on hepatitis C virus replication (Sekine-Osajima *et al.,* 2009).

Herbal compounds have been reported to reduce the risk of cancer and to modify the tumor behaviour (Kaefer and Milner 2008). Several studies have reported that natural products from medicinal plant extracts have a positive effect against cancer when compared with chemotherapy or hormonal treatments (Wu *et al.,* 2002a; Helyer *et al.,* 2006; Liao *et al.,* 2013). The bioactive components in herbs may reduce cancer risks through their antimicrobial, antioxidant, and anti-inflammatory properties, as well as their direct suppressive effect on carcinogen bioactivation. For example, cinnamon bark oil inhibits *Helibacter pylori* and prevents its ability to invade and progress stomach cancer (Bergonzelli *et al.,* 2003; Kaefer and Milner 2008). In addition, the phenolic extract from pine bark was investigated in human breast cancer cells. The results show inhibition of the breast cancer cells in the range of 32.5-125 µg/mL (Yu *et al.,* 2008). Furthermore, the petroleum ether extracts from the root of the medicinal Asian plant *Platycodon grandiflorum* contains a strong polyacetylenic anti-cancer compound, which exhibited cytotoxicity on human cancer cells (Lee et al., 2004).

Flavonoids from herbal plants were reported to interfere with cancer processes such as proliferation, inflammation, angiogenesis, invasion, and metastasis (Prasad *et al.,* 2010). Although epidemiological studies suggest that flavonoids may reduce the risk of tumors of the breast, colon, lung, prostate, and pancreas (Sun *et al.,* 2006; Tang *et al.,* 2009; sing *et al.,* 2011). However, the generalizability of these anticancer effects need to be address since some of the benefits are specific to certain flavonoid subclasses (Messina *et al.,* 2006; Dong

and Qin, 2011), and some studies have reported inconclusive (Boehm *et al.*, 2009).

In vitro studies have indicated that herbs and their bioactive compounds can inhibit or induce pathways that regulate cell division, cell proliferation and detoxification, in addition to several enzyme systems involved in pathways that regulate the inflammatory and immune response (Kaefer and Milner 2008). For instance, Saffron offers different anti-carcinogenic and anti-tumour actions including having an inhibitory effect on cellular DNA and RNA synthesis and on free radical chain reactions (Abdullaev 2002). These *in vitro* experiments indicate the potential of these extracts for further investigation into identifying the potentially active ingredient, and then further evaluation of the compound or compounds to find out if their effects could be translated to a therapeutic level. It may be that the active ingredient does not reach the target source in sufficient concentration or it may be that the compound is metabolised during or after absorption to an inactive form. In many ways herbal medicine suffers from the same problems as nutrition and health, there is can be an association between two events, however, there may be no direct cause and effect.

1.4 Anthocyanins as natural pigments in plants: structure and occurrence

The study of natural colorants is a widespread and active area due to the increasing interest of substituting synthetic colorants which have toxic effects in humans (Castaneda-Ovando *et al.*, 2009; He *et al.*, 2009). Anthocyanins and carotenoids are amongst the most important natural colorants in the food industry (Delgado-Vargas *et al.*, 2000).

Anthocyanins are the largest group of water-soluble pigment in the plant kingdom (See Section 1.1.1.7). The word anthocyanin is derived from two Greek words: *anthos* meaning flower and *kianos* meaning blue (He *et al.*, 2009). Chemically anthocyanins naturally occur as glycosylated, polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium and contain two benzoyl rings (A and B) separated by heterocyclic (C) ring (Figure 1-8) (Jackman *et al.*, 1987).

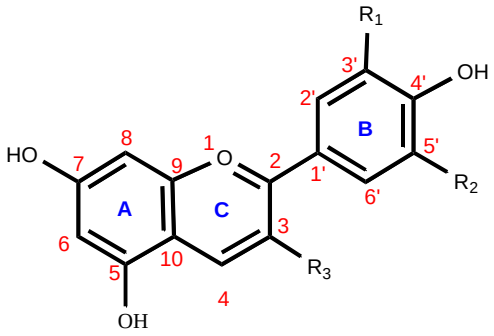


Figure 1-8: The flavylium cation. R_1, R_2 = H, OH or OCH_3; R_3= OH or Glycosyl.

The number of hydroxyl groups in the anthocyanins molecule, the degree of methylation of these hydroxyl groups, as well as the nature, number and position of sugar moiety attached to anthocyanidin (aglycone) and the number of aliphatic or aromatic acids attached to the sugar has contributed to more than 500 different anthocyanin pigments as reported in the literature (McGhie and Walton 2007).

With respect to molecular structure, some anthocyanins are more stable than others. Generally, the increased number of hydroxyl groups in the aglycone decreases anthocyanin stability, while methylation of one or two of these

hydroxyl groups tends to enhance the stability (Jackman *et al.,* 1987; Bakowska-Barczak 2005). Thus, under acidic conditions, glucosides of pelargonidin are more stable than those of cyanidin, which themselves are more stable than those of delphinidin (Fleschhut *et al.,* 2006). In alkaline pH, petunidin-3-*O*-glucoside, with two hydroxyl groups in the B-ring, was less stable than peonidin 3-*O*-glucoside, which has one hydroxyl group in the same ring (Cabrita *et al.,* 2000). Moreover, malvidin was more stable than pelargonidin, but less stable than cyanidin (Mulinacci *et al.,* 2001), and peonidin-3-*O*-glucoside was more stable than pelargonidin-3-*O*-glucoside in a buffered solution (Cabrita *et al.,* 2000).

1.5 Stability of anthocyanins

Anthocyanins are reactive compounds and are highly unstable in a food matrix compared to other flavonoids. The chemical structure of anthocyanins, concentration, glycosylation, acylation and co-pigmentation are factors that influence anthocyanins stability. Anthocyanins can also be affected and degraded under several factors such as pH, the presence of oxygen, enzymes, temperature and light. It is important to assess the influence of all these factors on the chemical stability of anthocyanins especially under physiological and experimental conditions to verify the contribution of degradation to anthocyanins bioavailability.

1.5.1 Glycosylation and acylation

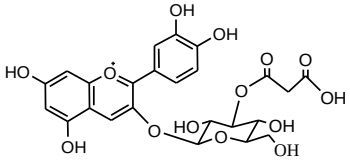
The hydroxyl group on the aglycone may be substituted by sugar moieties which may be further linked to other sugars through a glycosidic bond or acylated with aromatic or aliphatic acids through an ester bond. Both glycosylation and acylation can affect the stability of anthocyanins (Garzon and Wrolstad 2001).

Glycosylation has a significant effect on anthocyanin stability by forming an intramolecular H-bonding network within the anthocyanin molecule (Borkowski *et al.,* 2005). Glucose and rhamnose are common sugar moieties attached to the aglycone, but galactose, arabinose, xylose, rutinose, sambubiose and other sugars are also often found forming mono-glycosides, di-glycosides and tri-glycosides (Miguel 2011). As glycosylation of anthocyanindin enhances water

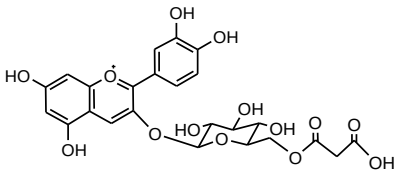
solubility, anthocyanins are more stable *in vitro* and more bioavailable *in vivo* than the aglycone (Kahle *et al.*, 2006; Woodward *et al.*, 2009; Gonzalez-Barrio *et al.*, 2010; Kraus *et al.*, 2010). The place of the glycosyl attachment influences the stability of the anthocyanin. Glycosylation at C3 position produced higher stability for anthocyanin compared to C5, C7, and C4´-glucosides (Rein 2005). Also the type of glycosyl units influences the anthocyanin stability; anthocyanins containing galactose are more stable than those with arabinose (Trošt *et al.*, 2008). The higher colour stability of red raspberry when compared to strawberry wine has been reported to be due to the diglycosidic nature of anthocyanins sophorose present in raspberry (Garzon and Wrolstad 2001).

Acylation was also found to be increasing the stability of anthocyanins colour and structure even at higher pH ranges (Giusti and Wrolstad 2003; Borkowski *et al.*, 2005). The stacking acyl groups with the pyrylium ring of the flavylium cation may reduce the susceptibility of nucleophile attack of water (hydration) and prevent the formation of pseudobase or chalcone (Brouillard *et al.*, 2003). The acyl group occurs mainly at the C3 and C6 of the monosaccharide of the monoacylated anthocyanins (Harborne 1998). Polyacylated anthocyanins are more stable than monoacylated anthocyanins and they posses high colour stability throughout the entire pH range from acidic to alkali. More stabilization was recorded when anthocyanins bond with aromatic acids (e.g. *p*-coumaric, caffeic, ferulic acids) than aliphatic acids (e.g. malonic, acetic, oxalic acids) (Figure 1-9). Within the aromatic acid group, the stability of acylated anthocyanins was also affected by the type of acids. Stabilisation occur more in acylated anthocyanins with caffeic acid compared with *p*-coumaric acid (Giusti and Wrolstad 2003). However, acylation decreases water solubility probably due to increased molecular size. Therefore, acylated anthocyanins are much less efficiently absorbed than nonacylated compounds (Kurilich *et al.*, 2005; Charron *et al.*, 2007).

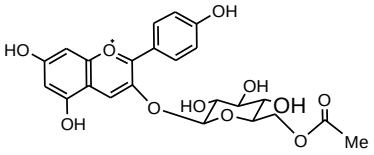
(Monoacylated anthocyanins-aliphatic acids)



Cyanidin-3-*O*-(3"-*O*-malonoyl) -glucoside

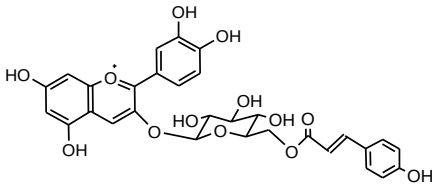


Cyanidin-3-*O*-(6"-*O*-malonoyl) -glucoside

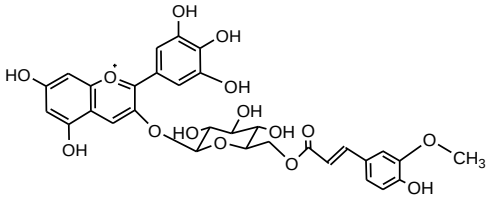


Pelargonidin-3-*O*-acetylglucoside

(Monoacylated anthocyanins-aromatic acids)



Cyanidin-3-*O*-(6"-*O*-*p*-coumaroyl) -glucoside



Delphinidin-3-*O*-(6"-*O*-feruloyl) -glucoside

Figure 1-9: Structure of some acyalted anthocyanins.

1.5.2 pH effect

The pH has a marked effect on anthocyanins stability. The ionic nature of anthocyanins enables them to change their molecule structure according to the pH, resulting in differences in colour variation, colour intensity and structure characterization (Cabrita *et al.*, 2000; Rein 2005; McGhie and Walton 2007). Within a different pH range, anthocyanins can exist in four forms: flavylium cation (red), anhydrous quinoidal base (purple to violet colour), colourless carbinol base and the pale yellow chalcone. At a very acidic condition, anthocyanins are more stable and the red flavylium cation is the predominant form of anthocyanins. Increasing the pH cause a reduction in both colour intensity and concentration of the flavylium cation. With high pH value the flavylium cation loses a proton to generate the blue quinonoidal bases. At the same time, slower hydration of the flavylium cation occurs by water to yield the colourless form, carbinol pseudobase. The carbinol form loses its double bond at the C-2 position of the pyranic ring C and thus dose not absorb visible light. The carbinol pseudobase further tautomerises through an opening of the C-ring to generate the chalcone (*cis* and *trans*) forms. With pH values higher than 7, the anthocyanins are degraded depending on their substituent groups (Figure 1-10).

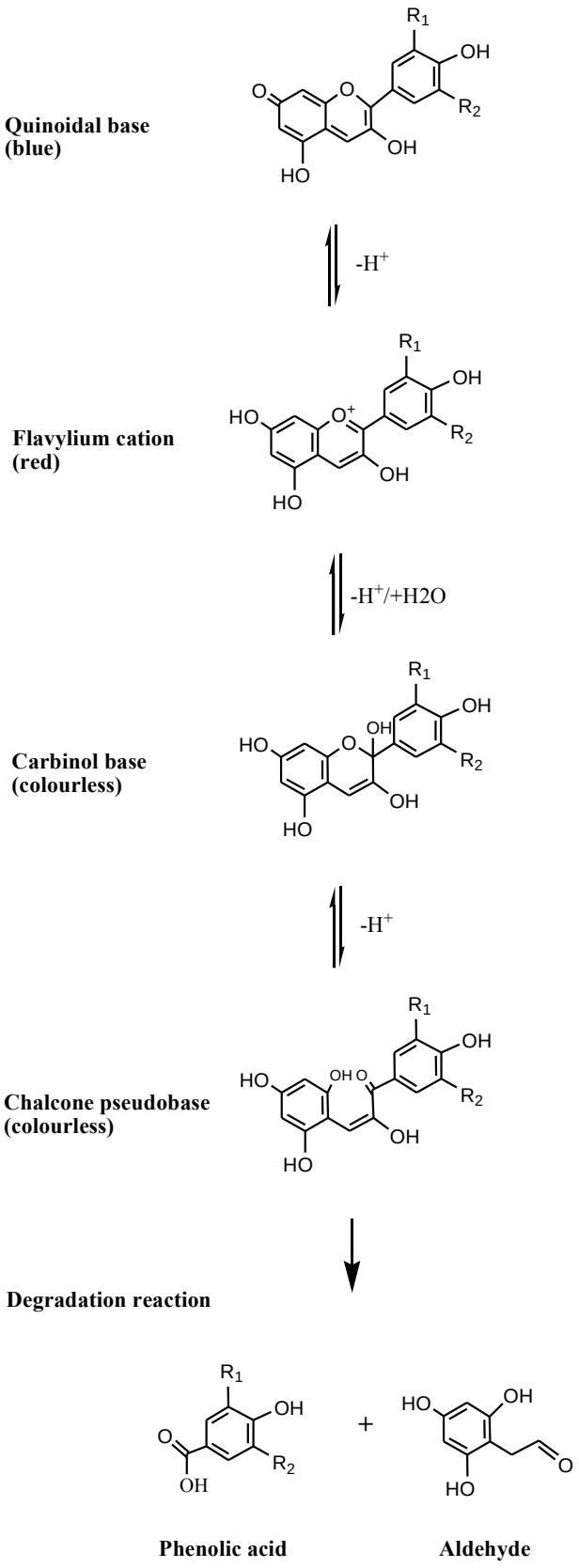


Figure 1-10: Structural transformations of anthocyanins and degradation reaction under different pH range; R1, R2= H or OH or OCH_3 (modified based on Castaneda-Ovando *et al.*, 2009).

As shown in Figure 1-10, acidic pH is favourable for the coloured form and as the pH increases the colour fades. Some anthocyanins are red in acid solutions, violet or purple in neutral solutions, and blue in alkaline pH.

This explained why most colorants containing anthocyanins can only be used at pH values below four (Delgado-Vargas *et al.,* 2000; Rein 2005; McGhie and Walton 2007; Castaneda-Ovando *et al.,* 2009; He *et al.,* 2009; Del Rio *et al.,* 2010). Understanding anthocyanins transformation is important during processing foods, product shelf-life, *in vivo* and *in vitro* studies including sample extraction and storage, sample analysis and anthocyanins changes as they are exposed to different pH when they pass through the gastrointestinal tract.

1.5.3 Other factors affecting anthocyanins stability

There are other factors affecting anthocyanins stability and responsible for their deterioration such as temperature, light, enzymes, oxygen and ascorbic acid.

Temperature is an important factor that causes degradation of anthocyanins during processing and storage as the temperature changes. This degradation can occur at higher temperatures which causes transition of anthocyanins to the unstable chalcone form which may be further degraded to a phenolic acid and aldehyde, respectively (Laleh *et al.,* 2006; Sadilova *et al.,* 2006; Sadilova *et al.,* 2007; Roobha *et al.,* 2011; Zhang *et al.,* 2012b). Adams (1973) proposed also hydrolysis of the glycosidic moiety and aglycon formation as the initial reaction. Therefore, storing anthocyanins at low temperature was recommended as an efficient means of preserving anthocyanins. Mullen *et al.,* (2002b) found that raspberry anthocyanins were stable when stored at 4°C for 3 days. 92% of grape anthocyanin was retained when stored at 3.5°C for 135 days (Palamidis and Markakis 1975). Kirca *et al.,* (2006) also reported that storage at 37°C resulted in much faster degradation of the black carrot anthocyanins than refrigerated storage at 4°C. Anthocyanins were also stable when stored at -80°C and pH≤2 while anthocyanidins were less stable (Woodward *et al.,* 2009).

Light is the most important environmental factor influencing anthocyanin biosynthesis in plants (Zhou and Singh 2004). However, light is also considered as a deteriorating factor affecting anthocyanin stability (Roobha *et al.,* 2011). Palamidis and Markakis, (1975) showed that after placing grape juice samples containing anthocyanins in the dark for 135 days at 20^{o}C, almost 30% of the pigments were destroyed while placing the same samples in the same condition with the presence of light destroyed more than 50% of total pigments. Therefore, it is recommended that anthocyanin colored foods and beverages are stored in the dark (Laleh *et al.,* 2006).

Inactivation of certain enzymes enhances anthocyanins stability (Garcia-Palazon *et al.,* 2004). The most known enzymes that cause anthocyanins degradation are glycosidase which are capable of hydrolyzing the glycosidic linkage between anthocyanidins and sugar moieties (Wightman and Wrolstad 1995). As noted above, anthocyanidins without the sugar moiety are extremely unstable and easily degraded. Therefore, caution must be taken when choosing an enzyme preparation for use in the food industry (Buchert *et al.,* 2005). ß-glycosidases in the animal digestive tract may also have an impact on anthocyanin stability and subsequently affect the availability of anthocyanins *in vivo*. Peroxidases and phenolases like phenol oxidase and polyphenol oxidase, which are native plant enzymes usually released from plant cells during anthocyanin extraction, are also common anthocyanin degradation enzymes (Garcia-Palazon *et al.,* 2004).

Atmospheric oxygen also has been well known to cause tissue browning for fruits rich in anthocyanins as it increases anthocyanin breakdown due to oxidation. The anthocyanins degradation level of acerola and acai fruits under a nitrogen atmosphere was less compared to under an oxygen atmosphere in an ascorbic acid fortified system (De Rosso and Mercadante 2007). In contrast, superatmospheric oxygen treatment has been demonstrated as an alternative to traditional low O_2 and a high CO_2 controlled atmosphere technique to keep the quality, colour and safety of fresh produce rich in anthocyanins (Wszelaki and Mitcham 2000), inhibiting microbial growth and enzymatic discoloration and preventing anaerobic fermentation reactions (Kader and Ben-Yehoshua 2000; Wszelaki and Mitcham 2000; Zheng *et al.,* 2003; Zheng *et al.,* 2007).

In addition, the effect of ascorbic acid on anthocyanins stability was reported intensively, however, without conclusive results. Anthocyanins degradation in the presence of ascorbic acid has been reported in several studies (Marti *et al.*, 2002; De Rosso and Mercadante 2007); however, the action mechanism still remains unclear. The cleavage of the pyrylium ring due to the formation of H_2O_2 through oxidation of ascorbic acid was proposed to cause anthocyanins degradation as well as a direct reaction between ascorbic acid and anthocyanins causing condensation (Talcott *et al.*, 2003; De Rosso and Mercadante 2007; He *et al.*, 2009). In contrast, adding ascorbic acid to fruits and berry juices is a common method to protect anthocyanins against oxidation and to enhance the nutritional value of a food product (Ozkan 2002; Rein 2005). Ascorbic acid has been reported to have protective effects against anthocyanins degradation (Kaack and Austed 1998; Sadilova *et al.*, 2009). In the presence of H_2O_2, ascorbic acid at both 60 and 80 mg/L concentrations protected anthocyanins in pomegranate juice (Ozkan 2002). Acylated anthocyanins stability was also increased in the presence of ascorbic acid (Del Pozo-Insfran *et al.*, 2004).

1.6 Anthocyanins in berries

Berries are an important source of anthocyanins and rich in a wide range of phenolic compounds including, flavonols, flavan-3-ols, ellagitannins, hydroxybenzoate derivatives as well as a source of vitamin C, folic acid, minerals and fibre (Del Rio *et al.*, 2010). In berries, phytochemicals concentrations will be influenced by many factors including environmental conditions, degree of ripeness, cultivar, cultivation site, processing and storage of the fruit (Hakkinen *et al.*, 2000; Hakkinen and Torronen 2000).

Anthocyanin content among these berries had a diversity of anthocyanin structures. For example, blackcurrants (*Ribes nigrum*) are characterised by the presence of the rutinosides and glucosides of delphinidin and cyanidin, with the rutinosides being the most abundant and with other minor anthocyanins. Redcurrants (*Ribes rubrum*), which are closely related to blackcurrants, contain mainly cyanidin diglycosides with minor cyanidin monoglucosides (Slimestad and Solheim 2002). Strawberries (*Fragaria x ananassa*) and blackberries (*Rubus* spp.) have a simple anthocyanin profile as cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside are the most predominant compounds with other minor derivatives

of cyanidin and pelargonidin respectively. On the other hand, red raspberries (*Rubus idaeus*) had more complex profile of cyanidin and pelargonidin derivatives. Moreover, in blueberries (*Vaccinium corymbosum*), the major components are 3-*O*-glucosides, 3-*O*-galactosides, and 3-*O*-arabinosides of malvidin, petunidin, cyanidin, and delphinidin, with many minor anthocyanins also being present (Wu and Prior 2005). Cranberries (*Vaccinium oxycoccos*) have only cyanidin- and peonidin-based compounds as their major anthocyanins (Brown and Shipley 2011). Elderberry (*Sambucus nigra*), is characterised by the presence of cyanidin derivatives (Wu *et al.*, 2002b) (Figure 1-11).

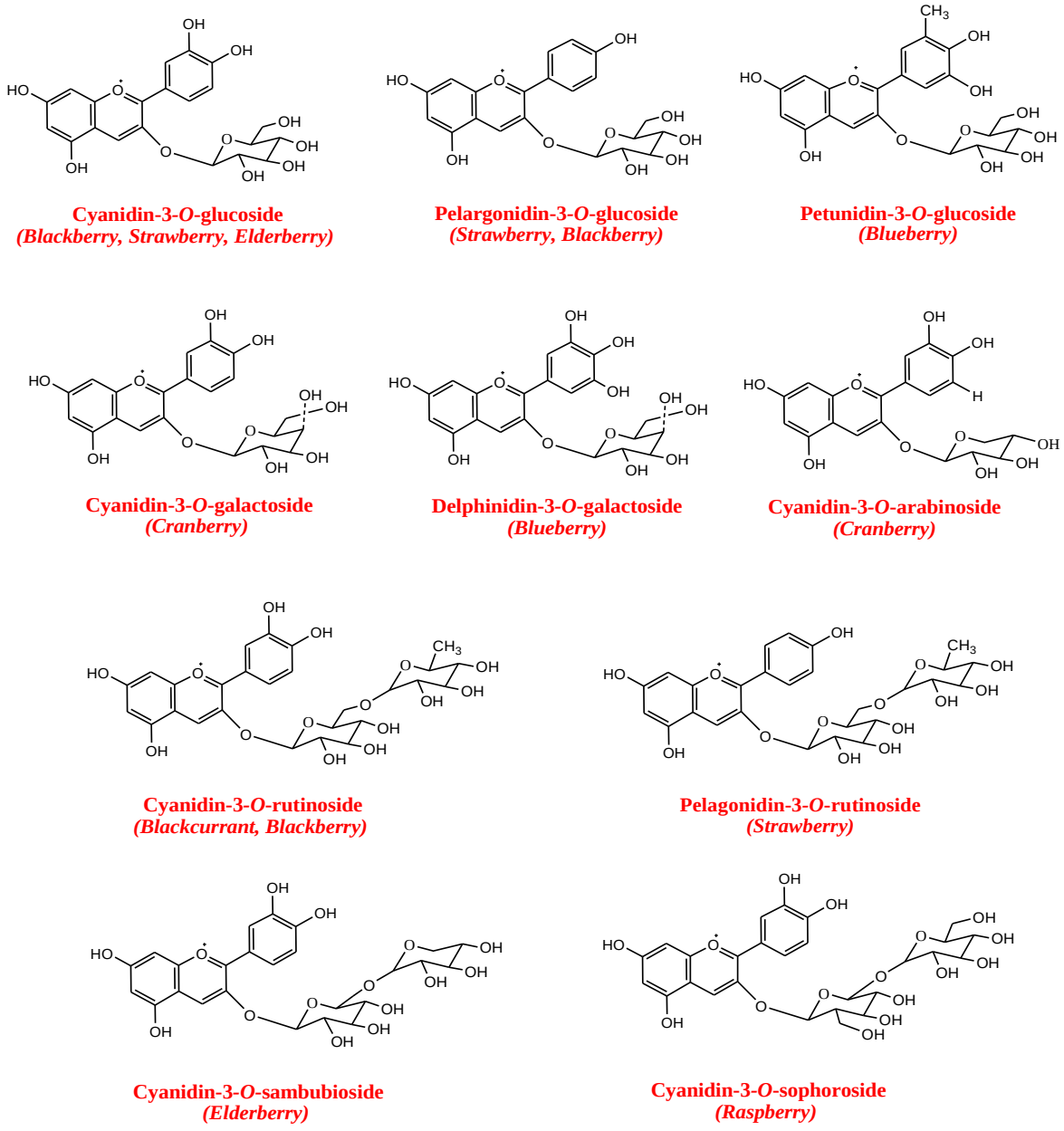


Figure 1-11: Structures of the major anthocyanins in berries.

The average intake of anthocyanins in the USA was estimated to be as high as 180-215 mg/day/person by Kuhnau, (1976) and this value is quoted extensively. However, Wu *et al.*, (2006a) recalculated the average intake of anthocyanins using a different food intake data to be 12.5 mg/day/person on the basis of the quantification and intake data from the National Health and Nutrition Examination Survey (NHANES 2001-2002) (Table 1-2). There are many factors that contribute to this great difference of total anthocyanins daily intake estimation in these reports. Kuhnau (1976) used the Folin Ciocalteau method to estimate the total intake of anthocyanins based on the reduction of the Folin reagent causing a colour change. However, reducing agents like vitamin C are present in many food especially berries and this may cause an overestimation. Other limitations must be considered in the Kuhnan estimation due to the lack of complete data on anthocyanin content and daily intake and information on individual anthocyanins. Knowing the concentrations and daily intake of specific anthocyanins is essential to get an accurate estimation of anthocyanins intake especially in food science, nutrition, and other related research fields. In addition, the differences between these reports also must result from different food intake data. The methods used in these estimations and the nature of the food analysis such as seasonal variation can contribute to variation in estimates of dietary intake of anthocyanins (Crozier *et al.,* 2000).

The daily intake of anthocyanins may also vary from these estimations. The consumers can more readily increase their consumption of anthocyanins by a regular daily consumption of berries as a single serving of some berries can contain >100 mg (McGhie *et al.,* 2003), while 200 mL of Austrian elderberry juice contains 2 g of anthocyanins, principally as cyanidin-3-*O*-glucoside and cyanidin-3-*O*-sambubioside (Murkovic *et al.,* 2000). Consumption of supplements or natural food colorants rich in anthocyanins is also another way to increase intake.

Epidemiological studies and human intervention trials have suggested an association between the consumption of anthocyanins-rich products, like berries, and the reduction in the risk of developing some chronic diseases such as cardiovascular disease, diabetes, arthritis and cancer, due to their antioxidant and anti-inflammatory properties (Rimm *et al.,* 1991; Wang *et al.,* 1999; Casto *et al.,* 2002; Sesso *et al.,* 2007). There are also several reports focused on the

Table 1-2 Estimation of daily consumption of anthocyanins from fruits, vegetable and beverages[a] of Wu *et al.*, 2006.

Food	Daily intake (g)	ACN (mg/100 g)	daily consumption (mg)
fruits			
apple, raw	11.77	0.6[b]	0.70
blackberry, raw	0.01	245	0.03
blueberry, raw	0.93	365	3.39
cherry, sweet, raw	0.46	122	0.56
cranberry, raw	0.12	140	0.17
grape, raw	4.83	36.7[c]	1.77
nectarine, raw	0.33	6.8	0.02
peach, raw	2.53	4.8	0.12
plum, raw	0.89	71.8[d]	0.64
raspberry, raw	0.24	390[e]	0.93
strawberry, raw	1.95	21.1[f]	0.41
subtotal			8.75
vegetables			
eggplant, raw	0.15	85.7	0.13
cabbage, red, raw	0.25	322	0.82
lettuce, red leaf, raw	0.27[g]	2.2	0.01
red radish, raw	0.14	100	0.14
onion, raw	7.92	12.1[h]	0.96
bean, black, raw	0.30	44.5[i]	0.13
subtotal			2.19
nuts			
pistachio nut	0.05	7.5	0.004
subtotal			0.004
beverages			
grape juice	6.61	14.0[j]	0.93
wine	12.13	10.7	0.66[k]
subtotal			1.68
total			12.53

[a] Food intake data from the National Health and Nutrition Examination Survey (NHANES 2001−2002). [b] Of total apple being consumed, 45% of apples consumed were 'Red Delicious', 10% were 'Fuji', and 13% were 'Gala' based upon market consumption data. [c] Fifty percent of total intake of grapes was estimated as red grapes. [d] Plum and black plum were estimated as 50% each of the total plum consumption. [e] Raspberry and black raspberry were estimated as 50% each of the total raspberry consumption. [f] Because strawberry from OSC is grown exclusively in Oregon, concentration data of other strawberries were used. [g] No intake data for red leaf lettuce are available; however, intake of red leaf lettuce was estimated to be similar to that of green leaf lettuce. [h] Twenty-five percent of total onion was estimated as red onion. [i] Black beans are not normally consumed in a raw state, but usually following domestic cooking or commercial canning. Because ACNs readily leach into cooking water or canning brine, only 50−70% of the ACN would likely be retained in the cooked beans. Hence, this value may be overestimated. [j] Mean of 23 different brands of grape juice (data, based upon determination by pH differential, were provided by Dr. JoLynne Wightman from Welch's). [k] Fifty percent of wine consumed was estimated as red wine on the basis of U.S. supermarket data.

effect of anthocyanins in decreasing LDL cholesterol, increasing HDL cholesterol (Qin *et al.*, 2009) and reducing blood pressure (Erlund *et al.*, 2008; Qin *et al.*, 2009). Therefore, due to the wide potential of natural anthocyanins as healthy pigments, researchers were interested in investigating anthocyanins in different fields such as developing analytical techniques for identification, quantification, purification and separation of anthocyanins (Giusti *et al.*, 1999; Chandra *et al.*, 2001), applications in food (Giusti and Wrolstad 2003), colour and pigment stability (Cabrita *et al.*, 2000; Garzon and Wrolstad 2001; Garzon and Wrolstad 2002; Kirca *et al.*, 2006), and more interestingly studying their bioavailability *in vivo* and *in vitro*.

1.7 Bioavailability and metabolism of anthocyanins

Anthocyanins have been reported to be strong antioxidants and have shown a wide range of health benefits. However, without clear knowledge on the rate and extent of their absorption, metabolism and tissue or cell distribution, the mechanism of anthocyanins in disease prevention remains unknown. This raises the importance of studying the bioavailability of these compounds upon ingestion.

1.7.1 Bioavailability definition

Generally, Holst and Williamson (2008) have defined the processes associated with bioavailability as the acronym LADME, as follows:

L = Liberation, processes involved in the release of a compound from its matrix.

A = Absorption, the diffusion or transport of a compound from the site of administration into the systemic circulation.

D = Distribution, the diffusion or transportation of a compound from the intravascular (systemic circulation) to the extra-vascular space (body tissues).

M = Metabolism, the biochemical conversion or biotransformation of a compound.

E = Excretion, the elimination of a compound, or its metabolite, from the body via renal-, biliary- or pulmonary processes.

Therefore, the bioavailability of a compound can be defined as the amount of ingested nutrient that is absorbed and may be metabolised to different forms from the parent compound ingested throughout the body and distributed to target tissue and which is eventually excreted out of the body, although storage may occur. Figure 1-12 shows the fate of dietary polyphenolic compounds after oral ingestion.

The first stage following consumption of anthocyanins, as a group of flavonoids of interest in this case is interaction with saliva, which contains α-amylase for starch digestion, in the mouth. Reports on the effects of saliva on phenolics in general are rare; saliva has been reported to be able to hydrolyze flavonoid glycosides (Walle *et al.,* 2005), but because of the limited period in the mouth this has not been considered to be an important factor in their bioavailability. Dietary anthocyanins then pass to the stomach were some anthocyanins might be absorbed (Passamonti *et al.,* 2003; Talavera *et al.,* 2003; He *et al.,* 2009). After anthocyanins enter the small intestine, they are absorbed, either as an intact form or metabolised to methylated, glucuronidated and sulphated forms. On entering the circulatory system, they are transported to the liver via the portal vein. Bile acts to emulsify fats, allowing greater absorption from the small intestine. From the liver, anthocyanins can be changed to form methylated, sulphated and glucuronide metabolites throughout phase I and phase II metabolism and then transported and distributed around the body tissues and/or to the kidneys, where they are excreted in urine. Unabsorbed compounds from the small intestine enter the colon, where they are catabolised by the action of colonic bacteria. Catabolic products will be excreted in faeces or absorbed into the blood stream and ultimately excreted in urine (Donovan *et al.,* 2006).

In most nutrition studies, the bioavailability of any dietary phenolic compounds can be assessed following a single-dose of pure compound, plant extract or whole food/beverage to experimental animals or humans, and then measuring their plasma pharmacokinetic parameters and their urinary excretion regarding the initial dose ingested. These pharmacokinetic parameters include the maximum plasma concentration (C_{max}), the time to reach the peak plasma concentration (T_{max}), the area under the curve of plasma concentration versus time (*AUC*) and elimination half-life ($T_{1/2}$) (Manach and Donovan 2004). Although

this assessment is simple for studying phenolic bioavailability, it does not reflect any information on the tissue distribution, storage or the site of metabolism.

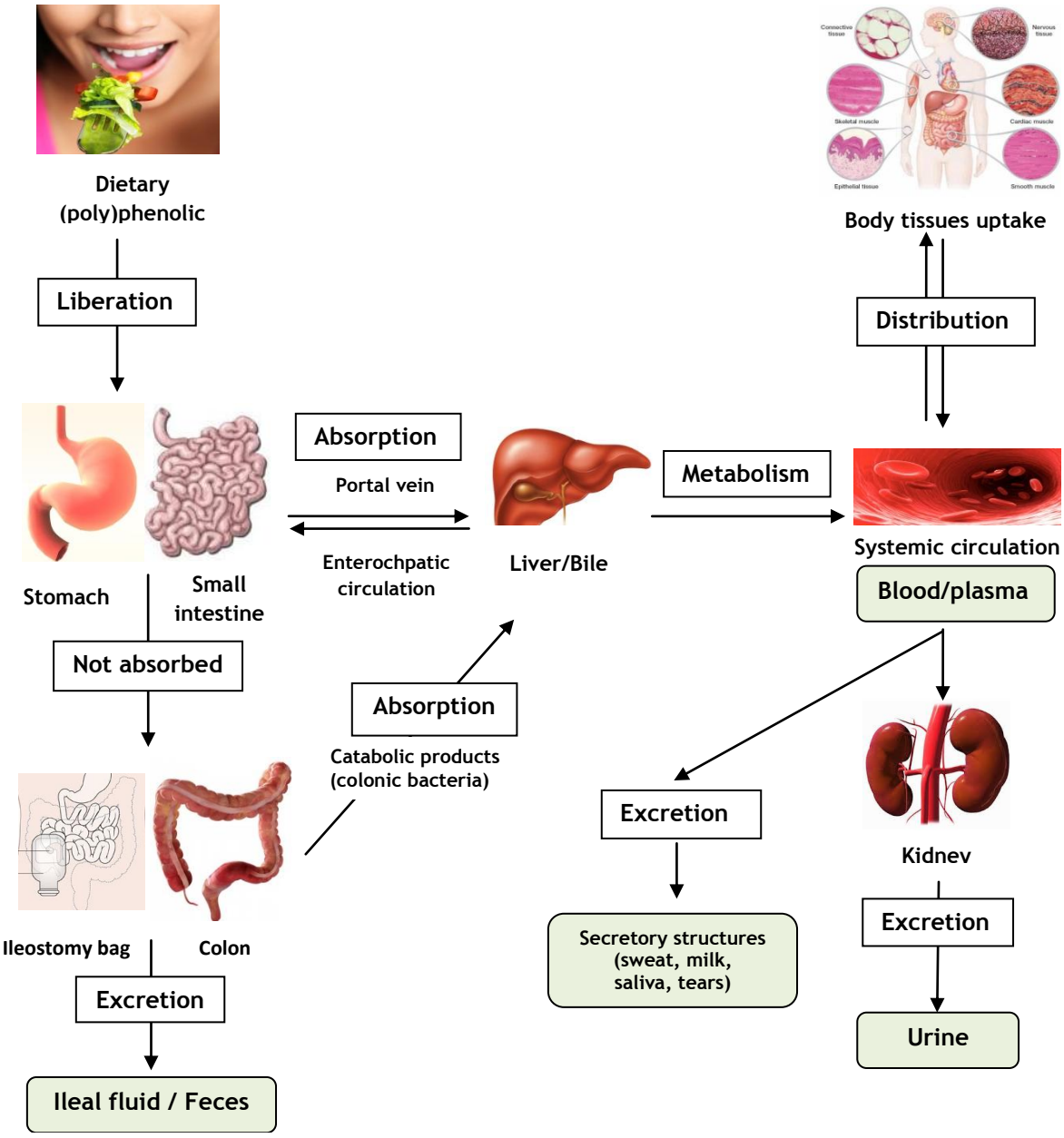


Figure 1-12: The fate of polyphenols after oral ingestion. Gray boxes represent the body fluid where samples can be collected and analysed for assessing the bioavailability of a compound of interest (Adapted from Holst and Williamson, 2008).

1.7.2 Bioavailability studies of anthocyanins

1.7.2.1 Animal studies

Animal studies have been used to evaluate anthocyanin bioavailability using mainly rats, pigs and rabbits. Most of these animal studies have been summarized by McGhie and Walton (2007) and Novotny (2012). Serving doses in most of these studies were high, in addition to using multi-component anthocyanin sources such as bilberry, elderberry and blackcurrant (Morazzoni *et al.,* 1991; Miyazawa *et al.,* 1999; Matsumoto *et al.,* 2001).

These studies indicated that anthocyanins are absorbed rapidly and reaching the circulatory system within the first two hours with a low peak plasma concentration mostly less than 1 µmol/L (McGhie and Walton 2007). Cyanidin-3-*O*-glucoside appeared in rat plasma after only 15 mins with C_{max} 0.18 µmol/L after oral administration of 100 mg of cyanindin-3-*O*-glucoside (Ichiyanagi *et al.,* 2005b). Similarly, malvidin-3-*O*-glucoside was detected in both portal and systemic plasma after only 6 mins after rats had been fed with a grape anthocyanin mixture. This finding shows that anthocyanin may permeate the gastric mucosa (Passamonti *et al.,* 2003). However, earlier animal studies indicated that anthocyanins appeared in plasma as intact forms ((Morazzoni *et al.,* 1991; Miyazawa *et al.,* 1999; Tsuda *et al.,* 1999; Matsumoto *et al.,* 2001). Neither aglycones nor metabolites were detected in these reports and this led to the suggestion that the flavylium cation structure of anthocyanins is resistant to enzymatic hydrolysis, preventing conversion to the aglycone and subsequent sulphation, methylation and/or glucuronidation. Recently published reports reported the occurrence of methylated and glucuronidated anthocyanins in plasma (Ichiyanagi *et al.,* 2004; Ichiyanagi *et al.,* 2005a; Ichiyanagi *et al.,* 2005b).

Urinary excretion of anthocyanins in animal studies was low with about 0.1% of the total ingested amounts being detected in urine as intact and metabolized forms (McGhie and Walton 2007). For example, methylated forms and glucuronidated derivatives of cyanidin-3-*O*-glucoside were detected in rat urine (Talavera *et al.,* 2004). More recently, Wu and colleagues (2006b) have shown a total of six parent anthocyanins and eight metabolites of anthocyanins excreted

in pig urine with a total recovery of 0.07% after feeding freeze-dried black raspberries. However, higher urine recoveries have been reported after acute intake of raspberries by rats (1·22%) (Borges *et al.,* 2007) and a bilberry extract by mice (0·62–2·45%) (Sakakibara *et al.,* 2009).

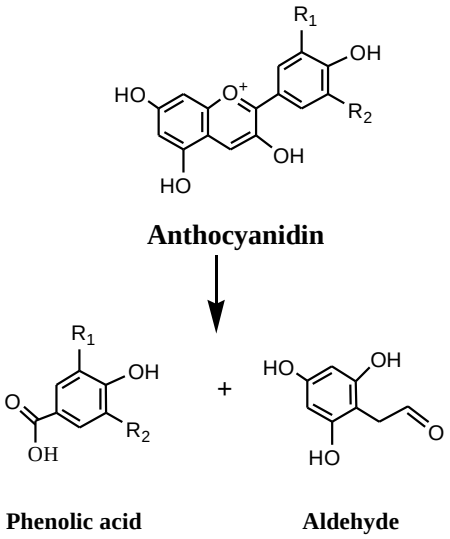
1.7.2.2 Human studies

An increasing number of human bioavailability studies with anthocyanins have confirmed the finding seen with animals studies. In human studies, large but more nutritional relevant doses of several hundred milligrams were used although in some cases more than one gram was ingested (Murkovic *et al.,* 2000; Cao *et al.,* 2001; Matsumoto *et al.,* 2001; Milbury *et al.,* 2002; Mulleder *et al.,* 2002; Wu *et al.,* 2002b; Felgines *et al.,* 2003; McGhie *et al.,* 2003; Felgines *et al.,* 2005; Mullen *et al.,* 2008b; Charron *et al.,* 2009; Stalmach *et al.,* 2012). From these reports, like animal studies, oral administration of berries and berry extracts showed that glycosylated anthocyanins have been detected in the blood stream rapidly with T_{max} values ranging from 0.5 to less than 2h. Maximum levels in human plasma are in the range of 1-100 nmol/L. Levels of absorption and excretion in urine are low, typically <0.1% of quantities ingested, with the highest accumulation occurring between 2 and 4 h within 24 h of consumption. Methylated, glucuronidated and sulphated metabolites have been detected in urine in addition to unmodified glycosidic anthocyanins. For instance, after human consumption of a chokeberry extract, cyanidin-3-*O*-galactoside and cyanidin-3-*O*-arabinoside, together with more substantial quantities of methylated and glucuronidated metabolites were detected in the plasma and urine (Kay *et al.,* 2004). Sulphate conjugates have been detected in a few investigations but in very low amounts (Felgines *et al.,* 2003; Mullen *et al.,* 2008b). These authors also reported higher urinary excretion of pelargonidin-3-*O*-glucoside intake corresponding to 1.80% and 0.75% respectively. These high urinary recoveries suggest that pelargonidin-3-*O*-glucoside may be absorbed more readily than other anthocyanins. In another human study by Felgines *et al.,* (2005) with cyanidin-3-*O*-glucoside only 0.16% of unmetabolised cyanidin-3-*O*-glucoside, cyanidin-*O*-glucuronide and a peonidin-*O*-glucuronide were excreted in urine. These findings concluded the importance of the aglycone structure on anthocyanin absorption, metabolism and stability throughout the gastrointestinal tract.

1.7.3 Factors affecting anthocyanin bioavailability

As shown from animal and human bioavailability studies, absorption and excretion of anthocyanins are different from other flavonoids in several ways. Their bioavailability is very low and they do not appear to undergo extensive metabolism to glucuronide and sulfate derivatives. Their peak absorption time (T_{max}) is occurring faster after consumption with low plasma peak concentration (C_{max}). In addition, urinary accumulation also occurs quickly within 2-4 h (McGhie and Walton 2007; Del Rio *et al.*, 2010). However, addressing the reasons behind the low bioavailability of anthocyanins is difficult with too many factors affecting their absorption.

Chemical structure is one of the main factors determining anthocyanins bioavailability. To some extent, low recoveries of anthocyanins could be a result of structural rearrangements of anthocyanins in response to a range of pH values as discussed previously (Section 1.5.2). In the stomach where the pH is low the stable form of anthocyanin, the flavylium cation, predominates and in more-basic conditions of the pH such as in the small intestine, the colourless carbinol pseudobase is possibly the major component along with smaller amounts of the colourless chalcone pseudobase and the blue quinoidal base (Del Rio *et al.*, 2012). The colourless carbinol pseudobase might undergo limited absorption, possibly being metabolised to conjugates that are unobserved because they cannot be converted back to the red flavylium forms before the final analysis. However, bioavailability and absorption studies of anthocyanins used detection methods (HPLC) that measure anthocyanins as the red flavylium cation. Although, from the previous discussion it is clear that the flavylium cation is not the only form that exists *in vivo*, which may have resulted in underestimating anthocyanins bioavailability (McGhie and Walton 2007). A further complication occurs when the parent anthocyanin and the carbinol pseudobase pass into the large intestine where they can be degraded to phenolic acid by the action of colonic bacteria, in addition to the possibility of breakdown to phenolic acid and aldehyde under physiological conditions and during sample processing before analysis (Figure 1-13) (Keppler and Humpf 2005; Fleschhut *et al.*, 2006).



Anthocyanidin	R_1	R_2	Phenolic acids
Pelargonidin	H	H	4-Hydroxybenzoic acid
Cyanidin	OH	H	Protocatechuic acid
Delphinidin	OH	OH	Gallic acid

Figure 1-13: Breakdown of anthoyanidin to phenolic acids (Woodward *et al.*, 2009)

Chemical structure of anthocyanidin (aglycone) and the sugar moiety were reported to limit absorption and excretion of anthocyanins (McGhie *et al.*, 2003; Wu *et al.*, 2005b; Prior and Wu 2006). Such an effect can be observed clearly in some sources of berries like raspberries and blueberries which contain a diversity of anthocyanin structures. These diverse structures complicate the interpretation of data on the anthocyanin content in biological fluids especially when these structures change throughout phase I and phase II metabolism. Like converting cyanidin to peonidin and delphinidin to petunidin through 3´-*O*-methylation, and like 5´-*O*-methylation converting petunidin to malvidin. However, assessing the influence of the aglycone on the anthocyanin bioavailability can be achieved using simpler anthocyanin profiles which can be found in strawberries and blackberries, where both of them contain one predominant anthocyanin, pelargonidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside respectively. This greatly simplifies monitoring post-ingestion absorption and metabolism as the anthocyanins pass through the body (Crozier *et al.*, 2009).

The chemical structure effect of the aglycone on the bioavailability of anthocyanins is most notably with pelargonidin-3-*O*-glucoside. The highest urinary recovery was recorded with pelargonidin-3-*O*-glucoside compared to other glucoside anthocyanins. In addition, pelargonidin-based anthocyanins metabolised mainly as glucuronide while methylation with glucuronidation were detected with other glucosides of anthocyanin such as cyanidin-3-*O*-glucoside (Felgines *et al.,* 2003; Wu *et al.,* 2004a; Felgines *et al.,* 2005; Carkeet *et al.,* 2008; Mullen *et al.,* 2008b).

The sugar moiety was also reported to have an effect on the bioavailability of anthocyanins. Recoveries of delphinidin and cyanidin rutinoside in the urine of weanling pigs after a meal of blackcurrants were higher than that of glucoside (Wu *et al.,* 2005b). Higher recoveries of cyanidin-3-*O*-sambubioside and cyanidin-3-*O*-sambubioside-5-*O*-glucoside than cyanidin monoglycoside were also documented (Mulleder *et al.,* 2002; Wu *et al.,* 2005a). A more recent study with rats indicated that the type of sugar moiety and aglycone largely affected phase II metabolism of anthocyanins. The type of sugar moiety affected glucuronyl conjugation in both the liver and small intestine and did not affect the *O*-methylation metabolism (Ichiyanagi *et al.,* 2008).

Anthocyanins and any other phytochemical compounds are typically consumed as part of a meal. However, little information is available on how their bioavailability is affected by the food matrix, especially food matrices of a normal diet. The available evidence suggests that the bioavailability of anthocyanins varies clearly depending on food matrices which consequently affect the absorption of anthocyanins (Yang *et al.,* 2011). Azzini *et al.,* (2010) reported lower bioavailability of strawberry anthocyanins after the consumption of fresh and stored strawberries alone by human subjects compared to other human studies done by Felgines *et al.,* (2003), Carkeet *et al.,* (2008) and Hollands *et al.,* (2008). Their data suggested that consumption of sweetened strawberries alone or by including a complete meal (typical breakfast) may raise the bioavailability of pelargonidin-3-*O*-glucoside. The bioavailability of pelargonidin-3-*O*-glucoside from strawberries was slightly affected by co-ingestion with cream by slowing its transit through the gastrointestinal tract resulting in delaying the urinary excretion of the compounds, without a

significant modification in the total amount excreted over 24 h or changing the plasma pharmacokinetics (Mullen *et al.,* 2008b). In keeping with this view, Murkovic *et al.,* (2000) showed that the maximum serum level is delayed significantly when anthocyanins are eaten together with a high fat meal. Peak plasma concentration for purple carrot juice was double the concentration of the whole carrots though the AUCs were similar. These results suggest that the total amount of anthocyanins absorbed in both cases were similar, but the time required for digestive processes to release anthocyanins from the plant matrix may result in a slower rate of anthocyanin absorption from whole purple carrots (Kurilich *et al.,* 2005; Charron *et al.,* 2009). Ingestion of sugar with an elderberry concentrate was also reported to reduce the excretion of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-sambubioside (Mulleder *et al.,* 2002). In general, the extent of the food matrix effect on anthocyanins bioavailability of anthocyanins is unclear and further investigation in this area is required.

1.7.4 Anthocyanins absorption and metabolism

Although there have been numerous reports examining the absorption and metabolism of anthocyanins in human, the absorption and metabolism of anthocyanins is still not sufficiently characterized due to the large variation in data in the literature. One of the main issues that cause this variation is the dosages used in clinical studies. Studies performed with large doses of anthocyanins will probably result in the saturation of metabolic pathways and consequently large amounts of unmetabolitezed compounds will enter the circulation system. However, lower doses or more similar doses to the levels found in the diet, would not be expected to saturate metabolic pathways and the parent compounds would most probably be largely conjugated. Therefore, feeding studies with single dose of anthocyanins will not establish a complete picture of anthocyanins absorption and metabolism. More studies using multiple-dosing are required to establish a true understanding of the metabolism of these compounds (Kay 2006; Prior and Wu 2006; McGhie and Walton 2007). More details about the absorption and metabolism will be highlighted in the coming section.

1.7.4.1 Absorption of anthocyanins

As discussed in Section 1.7.3, absorption of anthocyanins is different from other flavonoids due to many factors which influence their absorption and metabolism. However, from the current knowledge, anthocyanins seem to have the same absorption mechanisms as flavonoids. Generally, anthocyanidins are hydrophobic and can be absorbed through passive diffusion. Glycosylation of anthocyanidins increases their water solubility and limits their absorption with this mechanism as they become too hydrophilic. However, as anthocyanidins are present in the food diet as sugar conjugates other absorption mechanisms are required. Active transport mechanism or/and hydrolysis of the ß-glycoside before absorption are possible ways for anthocyanins to be absorbed (Crozier *et al.,* 2006; Del Rio *et al.,* 2012).

Absorption of anthocyanin glycoside can occur by two possible pathways. The two routes exist as many bioavailability studies reported the presence of the intact form of anthocyanin glucoside and also the metabolic form of anthocyanin. First, anthocyanins may be hydrolysed to the aglycone by lactate phlorizin hydrolase before being absorbed at the mucosal brush-border membrane in the small intestine (Day *et al.,* 2000). Then the aglycone can enter the intestinal epithelial cell passively where it could be conjugated or cross the intestinal membrane as an aglycone to the portal circulation heading to the liver (Day *et al.,* 2000; Scalbert and Williamson 2000; Nemeth *et al.,* 2003; Del Rio *et al.,* 2012). However, aglycones were not detected in the plasma and/or urine as aglycones are generally considered as unstable at physiological pH (Felgines *et al.,* 2003; Ichiyanagi *et al.,* 2005b; Galvano *et al.,* 2007).

The second route of anthocyanins absorption is anthocyanin glucoside absorbed directly by the sodium-glucose-co-trasporter (SGLT). Once they enter the intestinal epithelial cell, the intact glucoside could cross the membrane directly into the portal circulation or be hydrolysed by cytosolic ß-glucosidase (CBG) to form the aglycone which can be conjugated or cross the intestinal membrane to the portal circulation (Figure 1-14). Unabsorbed anthocyanins at the upper small intestine will pass to the colon where they will undergo bacterial metabolism by deglycosylation to form the aglycone which might be subjected to absorption or degraded to phenolic acid.

In contrast, Walton *et al.*, (2006) suggested that anthocyanins had different transport mechanisms to quercetin-3-*O*-glucoside. They found that the presence of glucose and phloridzin compounds, that involved inhibiting the active transport mechanism by (SGLT) and the hydrolysis of anthocyanins by lactate phlorizin hydrolase activity in the small intestine, did not affect the absorption of cyanidin-3-*O*-glucoside by jejunum tissue.

Other studies with rats indicate that anthocyanin absorption might occur in the stomach as well due to the quick appearance of anthocyanins in the blood stream. The gastric absorption was proposed to occur by the organic anion carrier bilitranslocase (Passamonti *et al.*, 2003; Talavera *et al.*, 2003; Talavera *et al.*, 2004; Fernandes *et al.*, 2012).

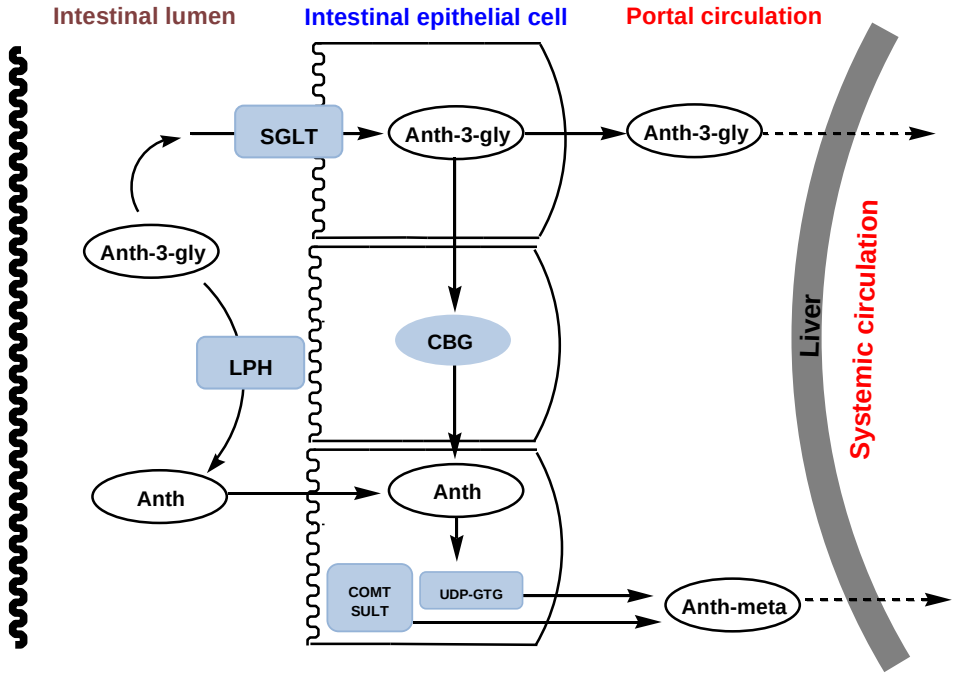


Figure 1-14: Proposed mechanisms for anthocyanin absorption. SGLT, sodium-glucose-co-transporter; LPH, lactate phlorizin hydrolase; anth-3-glu, anthocyanin-3-glucoside; anth, anthocyanidin; CBG, cytosolic ß-glucosidase; UDP-GT, uridine-5´-diphosphate glucuronosyltransferase; COMT, catechol-O-methyltransferase; SULT, sulfotransferase Anth-meta; anthocyanins metabolite (Adapted from Kay, 2006)

1.7.4.2 Metabolism of anthocyanins

Earlier bioavailability investigation suggested that anthocyanins are not metabolised and only the unmetabolised anthocyanin glucoside was detected in the blood and urine of both human and animal models (Miyazawa *et al.,* 1999; Cao *et al.,* 2001; Matsumoto *et al.,* 2001; Felgines *et al.,* 2002; Mulleder *et al.,* 2002).

The detection of anthocyanins metabolites plus the intact glucoside form were reported later in numerous studies. The major anthocyanins metabolites reported in the literature were glucuronidated and/or methylated conjugates (Wu *et al.,* 2002b; Wu *et al.,* 2002c; Kay *et al.,* 2004; Wu *et al.,* 2004a; Felgines *et al.,* 2005; Ichiyanagi and Konishi 2005; Ichiyanagi *et al.,* 2005a; Tian *et al.,* 2006; Wu *et al.,* 2006b; Borges *et al.,* 2007; Azzini *et al.,* 2010; Cai *et al.,* 2011; Stalmach *et al.,* 2012).

In addition, sulfation conjugates are also recovered in urine in low amounts (Felgines *et al.,* 2003; Hollands *et al.,* 2008; Mullen *et al.,* 2008b). The formation of these metabolites requires biotrasformation enzymes including UDP-glucuronosyl transferase, UDP-glucose dehydrogenase and catechol-*O*-methyltransferase (COMT) and these enzymes have been found to exist in the small intestine, liver and kidney. However, the possible pathways that led to the formation of anthocyanidin metabolites are still unclear. In the coming chapters, the formation about anthocyanin metabolites will be discussed.

1.8 Aims and objectives of the study

1.8.1 Background

The reported therapeutic effects of polyphenolics, and in particular those found in fruits, vegetables and herbs highlighted the need to increase our knowledge about these compounds further. One of the primary issues require to be investigate is their bioavailability. Therefore, the current research in this thesis will involve two parts. The first part will focus on studying polyphenolic content in a traditional Saudi herbal plant, whereas the second part will focus on how to

investigate the bioavailability of anthocyanin, found in local fruit, and how to obtain more details about these results through *in vivo* and *in vitro* experiments.

Regarding the polyphenolic content in medicinal herbal plants, the chemical constituents of many these plants are still unclear. Such knowledge is required, not only for drug discovery but also for finding new sources of phytochemicals, and for producing materials of economic value such as tannins and oils. In addition, the therapeutic effects of medicinal plants rich in polyphenolic compounds was reported to be related to their antioxidant capacity which appears to be, at least in part, responsible for the reduction of certain oxidative stress pathologies. Inflammatory disease, cardiovascular disease, cancer, and other chronic diseases are associated with increased oxidative stress (Rice-Evans *et al.,* 1996).

The antioxidant properties of plant polyphenolics are due to the hydrogen of the phenoxyl groups that is prone to be donated to stabilized the free radicals. Bors *et al.,* (1997) attributed the structure criteria for phenolic to had the best free radical scavenging activity as follow:
i) the presence of hydroxyl groups in the 3′ and 4′ positions and a B-ring structure resulting in stability of the radical formed mainly in the 3′ position; ii) a double bond in the 2,3 position providing higher conjugation with other double bonds; and iii) 3- and 5-hydroxyl groups with a 4-oxo function.

Due to these structural characteristic, polyphenolic compounds were studied for their scavenging properties, using various in vitro assays (Chan *et al.,* 2008). However, most polyphenolic compounds are modified during absorption, through conjugation and metabolism, and by the large intestine through the action of colonic bacteria. Thus, flavonoid metabolites that reach the cells and tissues are chemically, biologically and probably functionally different from the ingested dietary form (Kroon *et al.,* 2004). In terms of antioxidant activity glucuronidation or sulphation of quercetin, for example, reduces its free radical scavenging activity (Morand *et al.,* 1998). Although the mode of action of polyphenolic compounds following absorption is still unclear, their antioxidant protective effect could occur within the gastrointestinal tract itself.

On the topic of bioavailability, the available data about anthocyanin bioavailability in human models are limited, and sometimes confusing (see section (1.7.4). One of the reasons for the complicated picture that has emerged about anthocyanins bioavailability is that many feeds have involved berries or berry extracts containing several structurally diverse anthocyanins. For example, raspberries contain ten or more anthocyanins in the form of cyanidin- and pelargonidin-3-*O*-sugar conjugates ranging from mono-to trisaccharides, while blueberries contain more than fourteen anthocyanins, principally 3-*O*-glucosides, galactosides and arabinosides of cyanidin, delphinidin, petunidin and malvidin (Mazza *et al.,* 2002; Borges *et al.,* 2004; Kahle *et al.,* 2006). This complicates interpretation of data on anthocyanin content in biological fluids and complicates assessing of anthocyanin absorption and metabolism especially when 3´-*O*-methylation can convert cyanidin to peonidin, and delphinidin to petunidin, and 5´-*O*-methylation converts petunidin to malvidin. However, simple anthocyanin profiles are found in strawberries and blackberries, as they contain one predominant anthocyanin, pelargonidin-3-*O*-glucoside, in the former and cyanidin-3-*O*-glucoside in the latter. As a consequence, data on anthocyanin bioavailability after ingestion of these berries by human subjects are potentially more straightforward to interpret.

In addition, many studies claim to show heath effects of anthocyanins (Stoner *et al.,* 2007; Wang *et al.,* 2009). For anthocyanins to exhibit biological effects, they must reach the systematic circulation in meaningful quantities. However several studies have reported the very low bioavailability of anthocyanins, some less than 0.1% (Cao *et al.,* 2001; Mulleder *et al.,* 2002; Kay *et al.,* 2004; McGhie and Walton 2007; Gonzalez-Barrio *et al.,* 2010). Thus a clear understanding of anthocyanin has been elusive. Many details are still missing about anthocyanin absorption, metabolism and distribution and how the small differences in the chemical structures of anthocyanins can have a major influence on them. These details can have a major impact on their bioavailability and therefore their potential to provide health benefits.

1.8.2 Aims

1. To examine the total phenolic content and related total antioxidant potential of secondary metabolites in infusions of a medicinal herbal tea.

2. To evaluate the bioavailability of anthocyanins, especially cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside, occurring in blackberry and strawberry by an intervention study *in vivo* with human subjects.

3. To assess the effect of the aglycone structure of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside as both anthocyanins have the same sugar moiety but a different aglycone structure. Thus, such a comparison can reflect the effect of 3'-hydroxylation and 3',4'-dihydroxylation on anthocyanin bioavailability *in vivo* and on their stability *in vitro*.

1.8.3 Objectives

The objectives of this thesis are detailed as follows:

For the first aim

1. Analysing the content of polyphenolic in *Anastatica hirerochuntica* extract, a medicinal herbal tea, by HPLC-PDA-MS^n and measuring the total phenolic content with the Folin– Ciocalteu assay.

2. Assessing the antioxidant activity of the tea using the FRAP assay and identifying the major antioxidant compounds involved using an on-line HPLC antioxidant detection system.

For the second and third aims

1. Analysing the content of anthocyanins in blackberry and strawberry by HPLC-PDA-MS^2.

2. Bioavailability and metabolism studies:

    I. A single intake of blackberry by a group of healthy volunteers.

II. A single intake of blackberry and strawberry by a group of ileostomy volunteers.

3. *In vitro* digestion model with saliva and simulated stomach and duodenum digestion of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside in blackberries and strawberries.

4. Analysing anthocyanins and their metabolites in plasma, urine, ileal fluid and incubated anthocyanins with saliva, gastric and pancreatic juices by HPLC-PDA-MS^2.

2. Profiling of flavonoids and phenolic compounds in *Anastatica hirerochuntica* seeds by HPLC-PDA-MS^2

2.1 Introduction

The use of medicinal plant extracts to cure various diseases has been practiced since ancient times. Nowadays, traditional herbal medicines still receive a considerable amount of attention. As discussed in Chapter 1, these benefits may arise from their phenolic and polyphenolic content which may afford protective affects against many major chronic diseases through a diversity of mechanisms and not necessarily their antioxidant properties (Del Rio *et al.*, 2012).

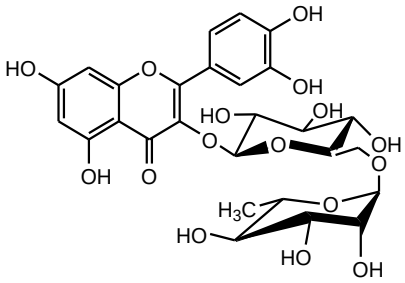
In Saudi Arabia, it is hard to find a city or village in the country where traditional medicines are not sold. They are also commonly used in home remedies for many ailments. Studies on medicinal plants in Saudi Arabia are of interest for researchers to explain the prominence and potential of Arabian medicinal plants on health (WHO 2001). Knowledge of the chemical constituents of these herbal plants is required, not only for drug discovery but also because such information may be of value in finding new sources of phytocemicals, and for producing materials of economic value such as tannins and oils. Therefore, the aim of the research in this chapter was to examine the total phenolic content and related total antioxidant potential of secondary metabolites in infusions of a medicinal plant that is widely used as a tea in Saudi Arabia.

Anastatica hirerochuntica, a local herbal plant grown in Saudi Arabia, was chosen for this study to investigate the phenolic compounds of its seeds and their antioxidant properties about which relatively little was known. *Anastatica hirerochuntica* is a small (5 cm high), grey, winter annual herb, found in the Sahara–Arabian deserts, it rolls inwards under dry conditions. Within the ball, the fruits remain attached and closed, protecting the seeds and preventing them from dispersing (Figure2-1). In Saudi Arabia, Jordan and Egypt it is known as Rose of Jericho, and is traditionally used as a medicine for difficult labours, uterine haemorrhage and to facilitate the expulsion of dead fetus' (Khalifa 1980). It is also used as a tea to treat asthma and respiratory diseases, dysentery, salmonella, colds, fevers and headaches, acting as a pain-killer, an

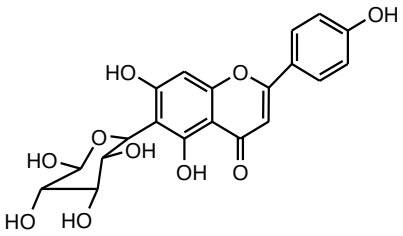
emmenagogue and for epilepsy (Mossa *et al.*, 1987). Drops from the sap of fresh leaves are prescribed for various ophthalmic problems such as conjunctivitis and an aqueous infusion of the herb is said to combat sterility. In addition, the dried plant crushed with sugar is taken as a strong purge for jaundice, followed by a milk diet (IUCN, 2005). Methanolic extracts of *A. hierochuntica* have antioxidant and antimicrobial properties (Mohamed *et al.*, 2009) and an aqueous extract had a hypoglycemic effect in both normoglycemic and diabetic rats, which was attributed to regeneration and repair of insulin-secreting β-cells (Rahmy and El-Ridi 2004).

Figure 2-1: *Anastatica hirerochuntica.*

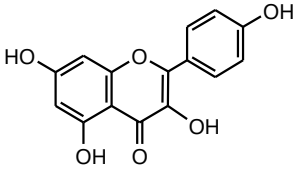
The reported information about the secondary metabolites occurring in *A. hirerochuntica* include phenolic compounds such as isovitexin, kaempferol, quercetin and rutin, β-sitosterol, campesterol, cholesterol, stigmasterol, coumarins, and alkaloids (Mossa *et al.*, 1987), (See Figure 2-2). Yoshikawa *et al.* (2003) isolated two novel skeletal flavanones, anastatins A and B, from *A. hierochuntica*, which were shown to have hepatoprotective effects on D-galactosamine-induced cytotoxicity in cultured mouse hepatocytes at concentrations as low as 3 μmol/L.



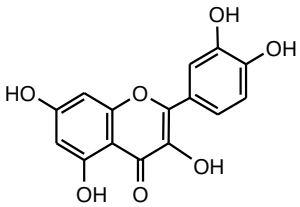
Rutin



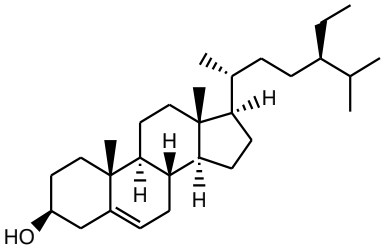
Isovitexin



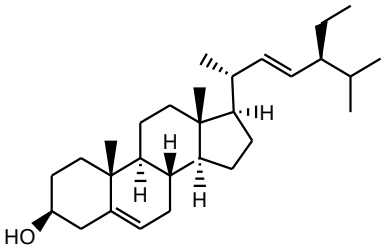
Kaempferol



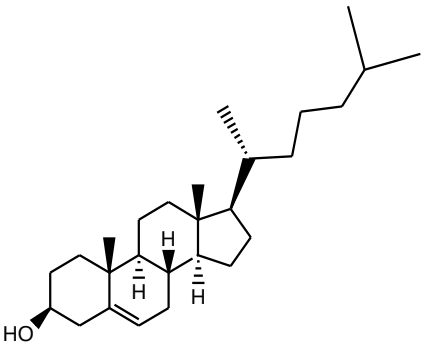
Quercetin



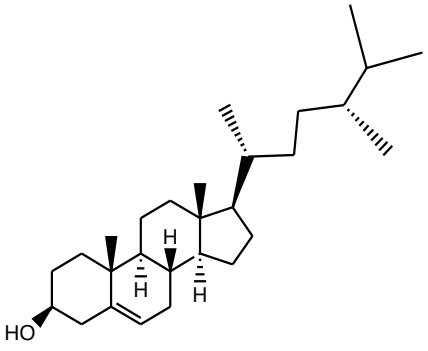
β–sitosterol



Stigmasterol



Cholesterol



Campesterol

Figure 2-2: Some of the reported compounds in *Anastatica hirerochuntica*.

In addition to anastatins A and B, after extensive sample purification this study also isolated and identified a number of other compounds from a methanolic extract of 3.5 kg of whole plants of *A. hirerochuntica*, including the flavonoids, naringenin, eriodictyol, aromadendrin, (+)-taxifolin (+)-epitaxifolin, 3`-methyl-taxifolin and quercetin. Unusually, Yoshikawa *et al.* found all these compounds occurred as aglycones. These compounds found normally as conjugated with sugar or organic acids in plants (Crozier *et al.,* 2006). However, despite its widespread use as a herbal medicine in parts of the Middle East, this is the sole study to use liquid chromatography (LC) coupled to mass spectrometry (MS) (LC/MS) to identity and quantify phenolic compounds occurring in *A. hierochuntica*. Flavonoids, which occur in plants as conjugated structures such as *O*- and *C*-mono and diglycosides (Ferreres *et al.,* 2007), were the main focus of interest in the study.

Phenolic compounds have a structure that affords them antioxidant activity. Generally, antioxidant activity depends on the number and positions of hydroxyl groups and other substituent, and glycosylation of flavonoid molecules. For example, the presence of hydroxyl groups on quercetin structure at the *C-3* position indicate the importance of the hydroxyl group for high antioxidant activity (Arora *et al.,* 1998). Also, glycosylation of flavonoids reduces their antioxidant activity *in vitro* when compared to the corresponding aglycones (Prochazkova *et al.,* 2011). Substitution patterns in the B-ring and A-ring as well as the 2,3-double bond (unsaturation) and the 4-oxo group in the *C*-ring also affect the antioxidant activity of flavonoids (Arora *et al.,* 1998; Cai *et al.,* 2004).

Flavonoids have been reported to exhibit antioxidant potential with different methodology such as the 1,1-diphenyl-2-picrylhydrazyl assay (DPPH$^{\bullet}$ assay), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS$^{\bullet+}$ assay) and the ferric reducing/antioxidant power assay (FRAP assay). These methods have been used to measure the antioxidant potential of pure chemicals, fruit juices, medicinal plant extracts, coffee and teas (Chan *et al.,* 2008). However, when used with complex matrixes, such as plant extracts, these procedures do not identify which particular compound(s) that are responsible for the antioxidant activity in the sample under investigation (Koleva *et al.,* 2000). To measure the antioxidant activity of the individual components in a complex mixture would require the

isolation and purification of each compound. This is a difficult and time consuming process with no guarantees that the results will be accurate. For this reason, a sensitive on-line HPLC method, which combines the advantages of rapid and sensitive antioxidant activity assay protocols with an HPLC separation for both, quantifying the contribution to the radical scavenging ability of each component in complex mixtures is useful (Koleva *et al.,* 2000; Stewart *et al.,* 2005).

In the current study, *Anastatica* tea was prepared by adding boiling water in the seeds, and the infusion analysed using HPLC with photodiode array (PDA) coupled to an electrospray interface tandem mass spectrometry (MS^2) operating in negative ion mode. The antioxidant activity of the tea was assessed using the FRAP assay and the total phenolic content was measured with the Folin–Ciocalteu assay. In addition, the infusion was analyzed using HPLC linked to a 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{\bullet+}$)-based on-line antioxidant detection system. This enabled the contribution of the individual components in the tea to the overall antioxidant capacity of the beverage to be assessed.

2.2 Materials and methods

2.2.1 Chemicals and reagents

HPLC grade methanol was obtained from Rathburn Chemicals Ltd., (Walkerburn, UK). Formic acid, Folin-Ciocalteu's phenol reagent and gallic acid were supplied by Sigma (Poole, Dorset, UK). Disodium carbonate was obtained from BDH Chemicals Ltd (Poole, Dorset, UK). Kaempferol-3-*O*-glucoside, luteolin, isovitexin, isoorentin, diosmetin were supplied by Extrasynthes (Genay, France) and caffeic acid from AASC Chemicals (Southampton, UK). All other chemicals and reagents were purchased from Sigma-Aldrich.

2.2.2 Preparation of *Anastatica hirerochuntica* extract

Seeds of *Anastatica hirerochuntica* were obtained from a local market in Jeddah, Saudi Arabia. Dry, powdered seeds were extracted by adding 10 mL boiling water to a 0.1 g sample. After 15 mins of continuous stirring, the extract

was centrifuged for 10 mins and filtered to remove particulate matter and then stored at -80^{o} C prior to analysis.

2.2.3 Colorimetric assays

2.2.3.1 Measurement of antioxidant activity using ferric reducing antioxidant potential (FRAP) assay

The total antioxidant potential of water extract was determined using the ferric reducing antioxidant potential (FRAP) assay. This method, as described by Benzie and Strain (1996), measures the ability of extracts to reduce a ferric-tripyridyl-triazine (Fe^{+3}-TPTZ) complex to the ferrous form (Fe^{+2}), producing an intense blue colour with an absorption at 593nm. In brief, 1.5 mL volume of freshly prepared FRAP reagent was added to 50 µL of the sample and 150 µL of water. The absorbance at 593 nm was measured after 4 min of reaction. This absorbance was compared to a standard curve prepared with 0 to 1 mM ferrous sulphate ($FeSO_4$) and results are expressed as the mean concentration of Fe^{2+} produced in mM.

2.2.3.2 Determination of total phenol content

The total phenolic content of *Anastatica hirerochuntica* was determined using the Folin-Ciocalteu method of Singleton and Rossi, (1965). This method determines phenols and other easily oxidized substances by producing a blue colour from reducing yellow phosphomolybdated-tungstate anions. In this method, 2.5 mL of (1:10) diluted Folin-Ciocalteu reagent was added to 50 µL of an appropriate diluted sample and 450 µL of distilled water. After 5 mins, 1.75 mL of sodium carbonate solution (Na_2CO_3) was added. The solutions were mixed and after 2 hours incubation at room temperature; the absorbance was read at 765 nm using a Unicam UV500 UV-visible spectrophotometer (Thermo Spectrenic, Cambridge, United Kingdom). Absorbance was calculated in gallic acid equivalents by reference to a standard calibration curve obtained with 50-800 µg/mL gallic acids. The plant sample was analysed in triplicate and the total phenolic content was expressed as µg of gallic acid equivalent (GAE)/mg of dry weight.

2.2.4 HPLC with diode array detection and tandem mass spectrometry

2.2.4.1 Analysis of phenolics compounds for *Anastatica hirerochuntica* extract

The aqueous extract of *A. hirerochuntica* was analysed using an HPLC system comprising of HPLC pump, photodiode array (PDA) detector scanning from 200 to 600 nm, and an autosampler set at 4^{o} C (Thermo Finnegan,San Jose, CA, USA) (Figure 2-3). The separation was carried out using a 250 x 4.6 mm i.d C12 4µm, Synergi RP-Max column (Phenomenex, Macclesfield, UK) maintained at 40^{o} C and eluted with a 60 min gradient of 10-70% of methanol in water containing 0.1% formic acid at flow rate of 1 mL/min. Separation of phenolics was monitored by the PDA detector at 365, 320 and 280 nm. After the mixture passed through the flow cell of the PDA detector, the column eluate was split and 20% was directed to a Finngan LCQ Advantage mass spectrometer with an electrospray interface, operating in full scan MS mode from 150 to 1000 *amu*. Samples were analysed in negative mode. ESI-MS parameters were as follows: potential of the ESI source, 4 kV; capillary temperature, 350°C. Data were analysed by Xcalibur software version 2.1.

Identifications were based on co-chromatography with authentic standards, when available. Absorbance spectra and mass spectra, using MS^2, were used to confirm the identification of these compounds which have been reported previously in the literature. The phenolic content of triplicate aqueous *Anastatica* extracts was quantified with calibration curves generated by the PDA detector using reference standards where available. When this was not possible, a closely related derivative was used instead. For example, all luteolin-derived compounds were quantified in luteolin equivalents; isovitexin was used to quantify apigenin-di-glucoside as it has a similar structure attached to a sugar unit and hydroxycinnamate derivatives were quantified by reference to the appropriate aglycone. In all instances, the standard curve of reference compounds ranged from 1 to 1500 ng.

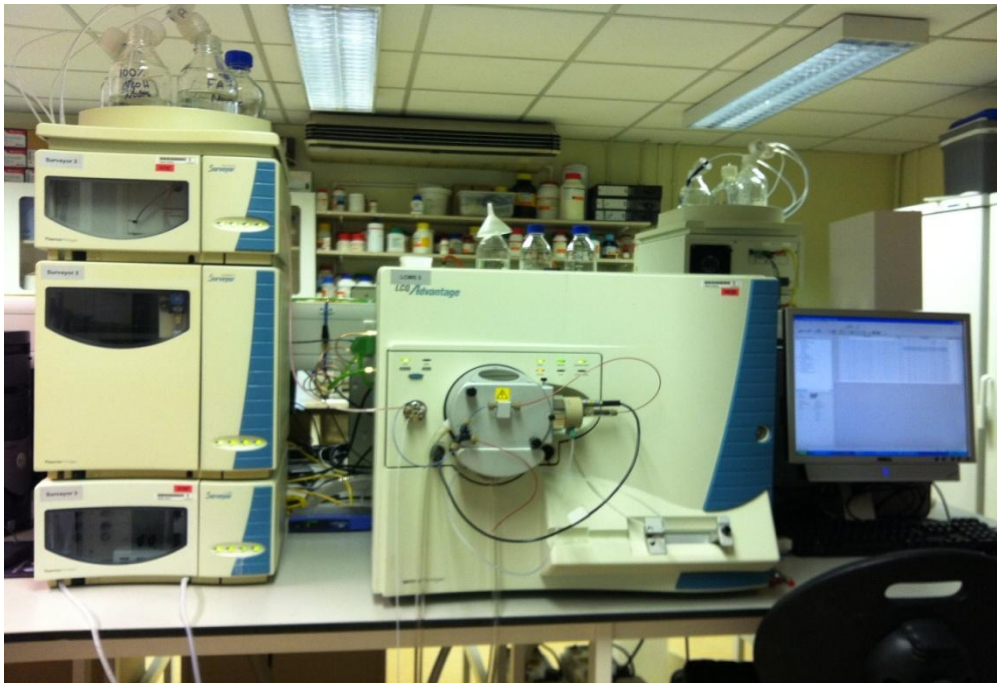


Figure 2-3: An HPLC system coupled to a mass spectrometrometer.

2.2.4.2 Trolox equivalent antioxidant capacity (TEAC) assay and on-line $ABTS^{\bullet+}$ assay systems

The antioxidant activity of *A. hirerochuntica* extract was determined using the radical cation ($ABTS^{\bullet+}$) on-line decolourisation assay based on the methods used by Dapkevicius *et al.,* (2001) and Koleva *et al.,* (2000) where the capacity of antioxidant molecules to reduces the blue-green radical cation 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) to ABTS which is colourless. The instrumental set-up is schematized in Figure 2-4. Briefly, a 2 mM $ABTS^{+}$ stock solution containing 3.5 mM potassium persulphate was prepared and incubated at room temperature in darkness overnight to allow the stabilization of the radical. $ABTS^{+}$ reagent was prepared by diluting the stock solution 8-fold in 0.1 M potassium phosphate buffer at pH 8. Triplicate of 50µL samples were injected into a Surveyor HPLC system comprising an LC pump, a PDA detector and a UV-VIS detector (Surveyor HPLC, Thermo Finnigan). HPLC separation was carried out as described in the previous section (2.2.4.1). HPLC eluate from the PDA was directed to a 'T' piece, where it was mixed with $ABTS^{+}$ delivered at a flow rate 0.5 mL/min by a Shimadzu LC-10 AP VP Liquid Chromatography pump. A Shimadzu GT-1543 vacuum degasser was used to remove oxygen in the reagent

prior to mixing. After passing through a 1.5 m x 0.4 mm loop maintained at 40° C, the absorbance was measured at 720 nm (Nemphlar Bioscience, Lanark, UK).

The total antioxidant potential was assessed using the methods described above. The antioxidant potential of the extract was calculated as the concentration of Trolox required to produce an equivalent antioxidant potential (μM) and expressed as Trolox equivalent antioxidant activity (TEAC), defined as the mM concentration of Trolox having equivalent activity to 1 mM concentration of the compound under investigation.

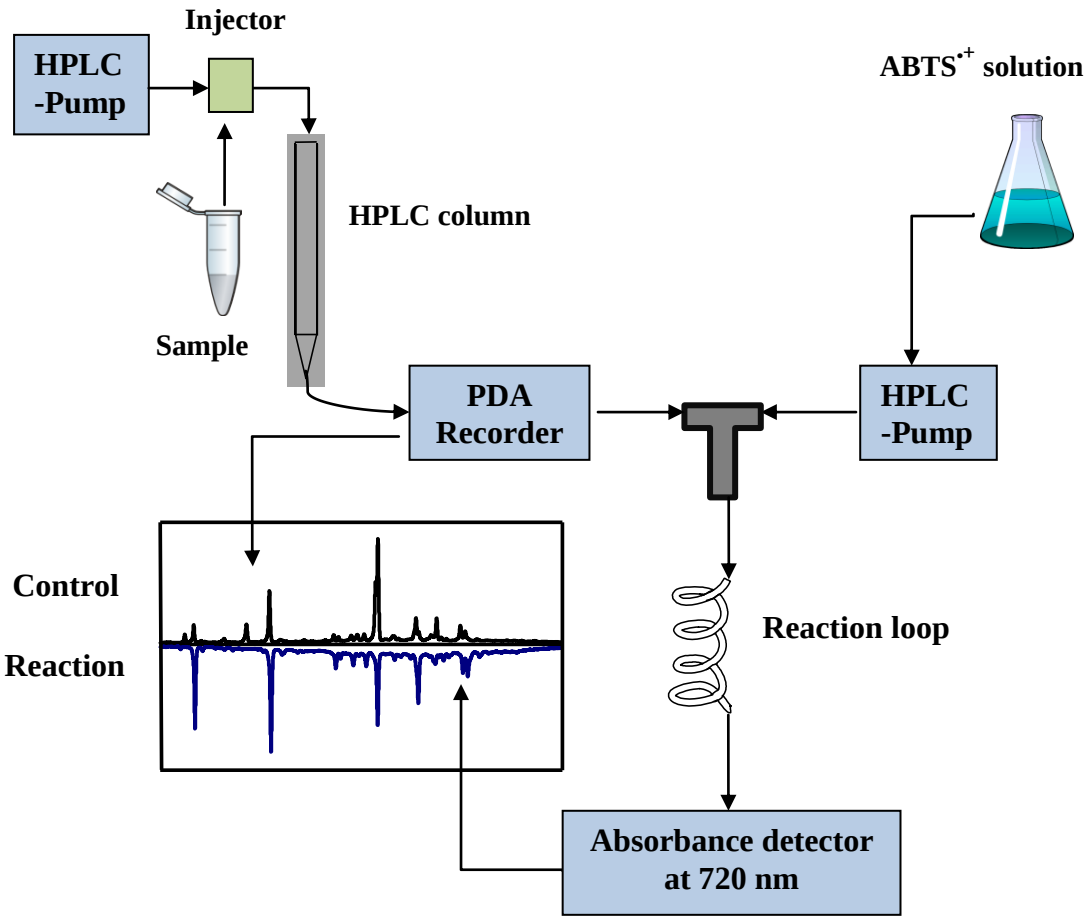


Figure 2-4: Schematics of ABTS-online HPLC system for the measurement of antioxidant activities.

2.2.5 Data analysis

Each sample was analysed in triplicate and data are presented as mean ± standard deviation. Data were analysed using Thermo Finnigan Chromquest™ software version 4.0.

2.3 Results and discussion

2.3.1 Qualitative analysis of *A. hirerochuntica* extract

The aqueous extract of *Anastatica hirerochuntica* was analyzed by reverse phase HPLC using a 60 min, 10 to 70% methanol gradient with the column eluate being directed first to a diode array absorbance monitor and then to a mass spectrometer with an electrospray interface operating in full scans MS^2 in negative ion mode. HPLC-MS^2 of the extract revealed the presence of numerous groups of *O*-glycosides, *C*-glycosides, *C*-di-glycosides and *O*-glycoside-*C*-glycosides. The 5-55 min absorbance traces obtained at 280 nm and 365 nm are illustrated in Figure 2-5 in which the 20 peaks are labeled. The identification of the numbered peaks based on MS^2 data and λ_{max}, are summarized below and outlined in Table 2-1.

Peak 1 (t_R, 9.2 min; λ_{max} 260 nm) has negatively charged molecular ion $[M-H]^-$ at *m/z* 299 and an MS^2 fragment at *m/z* 137. The loss of 162 *amu* indicates the presence of the glycoside unit. In accordance with molecular weight, fragmentation pattern and absorbance spectrum, this component was tentatively identified as a dihydroxybenzoic acid-*O*-hexoside as described by (Parejo *et al.*, 2004).

Peak 2 (t_R, 9.8 min; λ_{max} 295 nm) produced a $[M-H]^-$ at *m/z* 153 and an MS^2 ion at *m/z* 109, which is in keeping with the presence of 3,4 dihydroxybenzioic acid (**I** in Fig.2-6). The identity was confirmed by co-chromatography with a standard.

Peak 3 (t_R, 16.5 min; λ_{max} 325 nm) produced a $[M-H]^-$ at *m/z* 353 and MS^2 ions at *m/z* 191, the base peak, and a weak secondary ion at *m/z* 179, which indicates the presence of a chlorogenic acid, 5-*O*-caffeoylquinic acid (**II**), as described by Clifford *et al.*, (2003). This was confirmed by co-chromatography, and comparison with the MS^2 fragmentation patterns and absorbance spectrum of a standard.

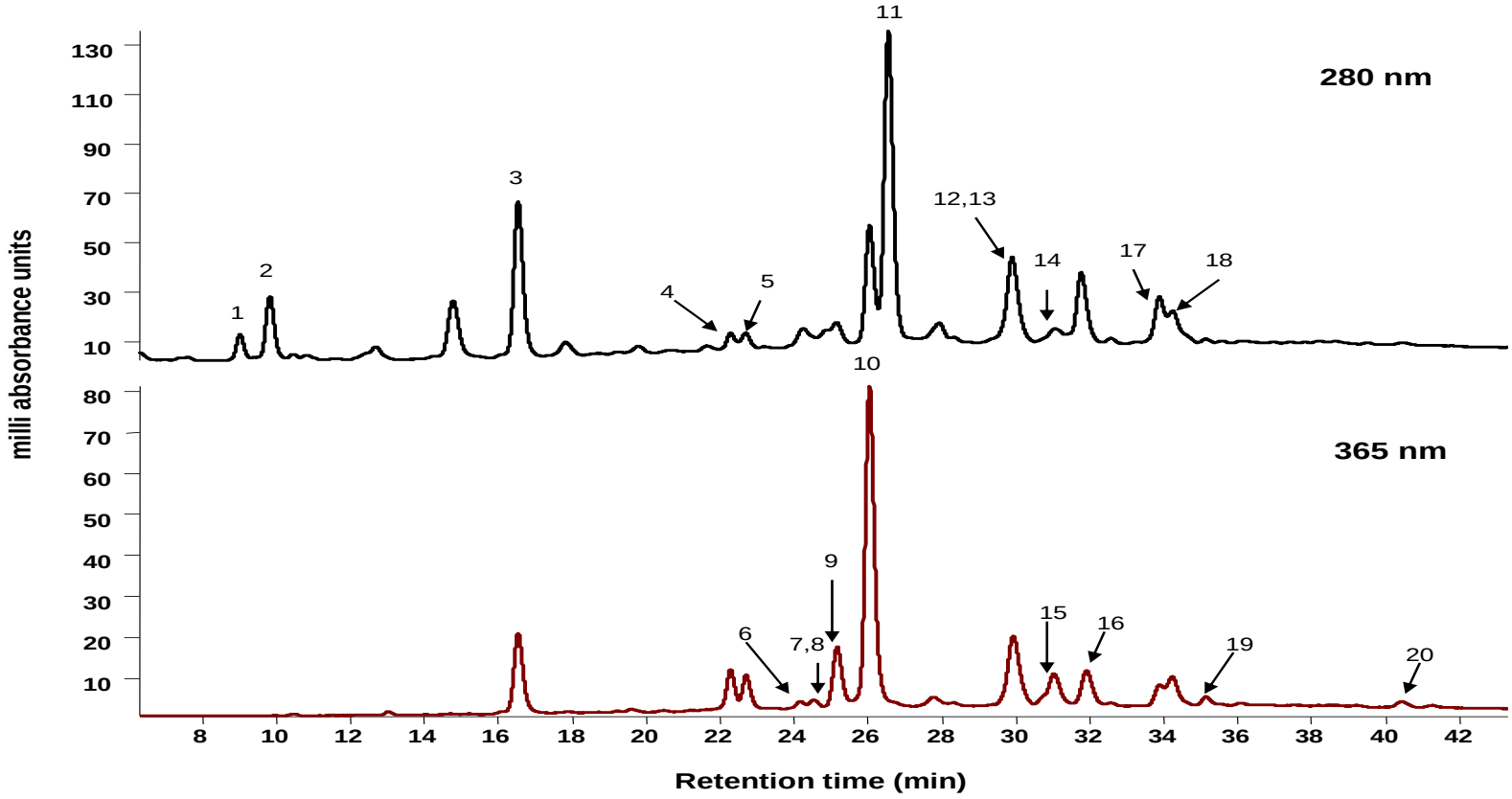


Figure 2-5: Gradient, reversed phase, HPLC analysis of a tea made from seeds of *A. hirerochuntica* with absorbance detection at 280 nm and 365 nm. For identification of numbered peaks see Table 2-1.

Table 2-1: HPLC retention times λ_{max} and mass spectral characteristics of polyphenolic compounds in tea made from seeds of *Anastatica hirerochuntica*

Peak	t_R (mins)	λ_{max}	[M-H]$^-$	MS^2 (*m/z*)	Compound
1	9.2	260	299	239,179, **137**	Dihydroxybenzioic acid-*O*-hexose
2	9.8	295	153	**109**	3,4-Dihydroxybenzioic acid (I)
3	16.5	325	353	**191**,179	5-*O*-Caffeoylquinic acid (II)
4	22.3	350	579	519, 561, 489, **459**, 399, 369	Luteolin-6-*C*-hexosyl-8-*C*-pentoside
5	22.7	350	579	519, 561, 489, **459**, 399, 369	Luteolin-6-*C*-pentosyl-8-*C*-hexoside
6	24.2	350	579	561, 519, **489**, 459, 369, 399	Luteolin-6-*C*-pentosyl-8-*C*-hexoside
7	24.5	330	593	473, 431, 341, **311**	Apigenin-6-*C*-7-*O*-diglucoside (isovitexin-7-*O*-glucoside)
8	24.5	330	563	545, 503, **473**, 443, 383, 353	Apigenin-6-*C*-arabinosyl-8-*C*-hexoside
9	25.2	350	447	369, 357, **327**	Luteolin-8-*C*-glucoside (orientin) (IV)
10	26	350	447	429, 357, **327**	Luteolin-6-*C*-glucoside (isoorientin) (V)
11	26.5	260	465	303, **285**	Taxifolin-*O*-hexoside
12	29.9	330	515	**353**,335,299,255,203,179,173	3,4-*O*-Dicaffeoylquinic acid (VI)
13	29.9	330	431	413, 341, **311**	Apigenin-6-*C*-glucoside (isovitexin)
14	30.8	350	447	**285**	Luteolin-*O*-glucoside
15	31	355	461	443, 371, **341**	Diosmetin-8-*C*-glucoside (VIII)
16	32	350	461	**285**	Luteolin-*O*-glucuronide
17	34.2	330	609	489, 447, **429**, 357, 339, 327, 309	Luteolin-6-*C*-glucosyl-2"-*O*-glucoside (isoorientin-2"-*O*-glucoside) (IX)
18	34.2	330	515	**353**, 317, 299, 255, 203, 179, 173	4,5-*O*-Dicaffeoylquinic acid (X)
19	35	380	447	**285**	Kaempferol-3-*O*-glucoside (XI)
20	40	350	461	**285**	Luteolin-*O*-glucuronide

[a] For peak numbers see Fig. 3.3. MS^2 ions in bold font indicate the base peak.

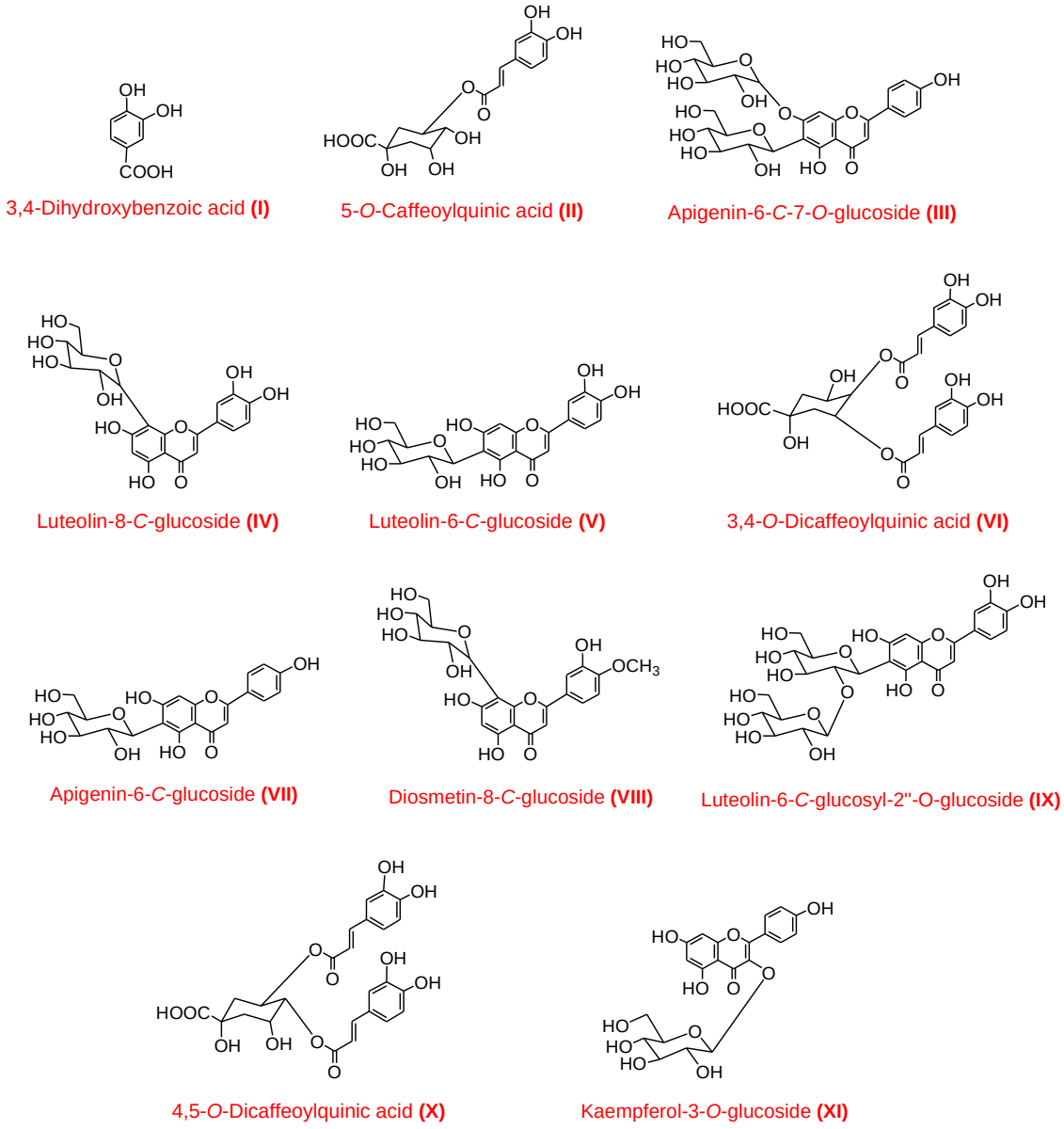


Figure 2-6: Structures of compounds identified in a tea made from seeds of *A. hirerochuntica*.

Peaks 4, 5 and 6 (t_R, 22.3, 22.7 and 24.2 min; λ_{max} 350 nm) appear to be flavonoid-*C*-diglycosides with a characteristic pattern of fragmentation ([(M-H)-18]$^-$, [(M-H)-90]$^-$, [(M-H)-120]$^-$, [AGly+83]$^-$ and [AGly+113]$^-$). This suggests the presence of luteolin *m/z* (285) + hexose *m/z* (162) + pentose *m/z* (132) giving the [M-H]$^-$ at *m/z* 579 as reported by (Ferreres *et al.*, 2003). In peak 4, the higher abundance of the [(M-H)-120]$^-$ ion as the base peak relative to [(M-H)-90]$^-$ indicates the presence of a 6-*C*-hexosyl-8-*C*-pentosyl substitution. In contrast, with peaks 5 and 6 [(M-H)-90]$^-$ was the base peak rather than [(M-H)-120]$^-$, which is characteristic of a 6-*C*-pentosyl-8-*C*-hexosyl substitution. Consequently, peak 4 was tentatively identified as a luteolin-6-*C*-hexosyl-8-*C*-pentoside and peaks 5 and 6 as luteolin-6-*C*-pentosyl-8-*C*-hexosides.

Peak 7 (t_R, 24.5 min; λ_{max} 330 nm) has [M-H]$^-$ at *m/z* 593 with an MS^2 fragmentation pattern characteristic of an *O*-glucosyl-*C*-glucosyl flavone, with losses of 162 and 120 amu, which were coincident with AGly+71 and AGly+41. This information allowed the characterization of apigenin-6-*C*-glucoside and the loss of 162 amu [Y^7_0, ([M-H]$^-$162)] suggesting an *O*-glucosylation at the 7 position. Therefore, this compound was identified as apigenin-6-*C*,7-*O*-diglucoside **(III)** (aka isovitexin-7-*O*-glucoside) as reported by Ferreres *et al.*,(2007).

Peak 8 (t_R, 24.5 min; λ_{max} 330 nm) has [M-H]$^-$ at *m/z* 563. The MS^2 fragmentation, like that of peak 6 is typical of a flavone-*C*-diglycoside with ([(M-H)-18]$^-$, [(M-H)-90]$^-$, [(M-H)-120]$^-$, [AGly+83]$^-$, and [AGly+113]$^-$). The higher intensity of the [(M−H)-90]$^-$ ion relative to [(M−H)-120]$^-$ is indicative of a 6-*C*-pentosyl-*8*-*C*-hexosyl-substitution. Therefore, this compound is apigenin-6-*C*-arabinosyl-8-*C*-hexoside (Ferreres *et al.*, 2008).

Peaks 9 and 10 (t_R, 25.2 min and t_R, 26 min λ_{max} 350 nm) show MS^2 fragmentation characteristic of a *C*-linked flavone-hexose conjugate, with a [M-H]$^-$ at m/z 447 and losses of 90 and 120 *amu* ($^{0,3}X^{-6}_0$ and $^{0,2}X^{-6}_0$) ,which were coincident with AGly+71 and AGly+41, respectively. This information allowed the characterisation of the aglycones. For peak 10, the loss of 18 *amu* indicates C6 substitution while with peak 9, the absence of the loss 18 *amu* is in keeping with a C8 substitution (Gattuso *et al.*, 2006). Therefore, compounds 9 and 10 were identified respectively as luteolin-8-*C*-

glucoside (**IV**, aka orientin) and luteolin-6-*C*-glucoside (**V**, aka isoorientin). This was confirmed by co-chromatography, and comparison of the MS^2 fragmentation patterns and absorbance spectra with standards of orientin and isoorientin.

Peak 11 (t_R, 26.5 min; λ_{max} 260 nm) had a $[M-H]^-$ at *m/z* 465 and yielded MS^2 ions at *m/z* 285 and 302. The MS^2 ion at *m/z* 285 is probably due to the presence of taxifolin (dihydroquercetin) [taxifolin-H_2O], which has been detected in *A. hirerochuntica* (Yoshikawa *et al.*, 2003) while the ion at *m/z* 302 indicates loss of a hexose unit, suggesting the presence of a taxifolin-*O*-hexoside as reported by Regos *et al.*, (2009). However, no standard of a taxifolin-*O*-glucoside was available to confirm this possibility.

Peak 12 (t_R, 29.9 min; λ_{max} 330 nm) produced a $[M-H]^-$ at *m/z* 515 and MS^2 ion at *m/z* 353 (base peak) with topical absorbance spectrum which indicate the presence of dicaffeoylquinic acid. MS^2 fragmentation showed a low intensity *m/z* 299 fragment (>15% of the base peak), along with less intense ions at *m/z* 255, 203, 179 and 173, which suggests the presence of either 3,4-*O*-dicaffeoylquinic acid or 4,5-*O*-dicaffeoylquinic acid. However, the presence of an *m/z* 335 ion allowed discrimination between these isomers and indicated the presence of 3,4-*O*-dicaffeoylquinic acid (**VI**). This was confirmed by the MS^3 and MS^4 fragmentation patterns. Both isomers produce an *m/z* 173 ion as the MS^3 base peak and an *m/z* 93 fragment in MS^4. They also yield an MS^3 ion at *m/z* 191 and a fragment at *m/z* 111 in MS^4 which in the case of the 3,4-isomer is double the intensity of the fragment in the spectrum of the 4,5-isomer as described by Clifford *et al.*, (2005) (also see peak 18).

Peak 13 (t_R, 29.9 min; λ_{max} 330 nm) was identified as apigenin-6-*C*-glucoside (isovitexin, **VII**) with a $[M-H]^-$ at *m/z* 431 and MS^2 fragments at *m/z* 311 and 341. The loss of 120 and 90 *amu* with the presence of AGly+71 and AGly+41 is a characteristic fragmentation of a flavone-*C*-hexoside. The identification of this peak as apigenin-6-*C*-glucoside (isovitexin) was confirmed by co-chromatography and the matching mass spectrum of a standard.

Peak 14 (t_R, 30.8 min; λ_{max} 350 nm) has $[M-H]^-$ at *m/z* 447 and MS^2 fragment at *m/z* 285. The loss of 162 *amu* indicates cleavage of a glucoside unit. In accordance with molecular weight, fragmentation pattern and absorbance spectrum, this compound was partially identified as luteolin-*O*-glucoside (Torras-Claveria *et al.*, 2007).

Peak 15 (t_R, 31 min; λ_{max} 350 nm) has an MS^2 fragmentation characteristic of a mono-*C*-hexosyl flavone as described previously for peaks 9 and 10. The $[M-H]^-$ at *m/z* 461 and MS^2 fragments at *m/z* 341 and 371 are similar to chrysoeriol-8-*C*-glycoside and diosmetin-8-*C*-glucoside. Gattuso *et al.*, (2006) reported that when bergamot juice was analysed by reversed phase HPLC, chrysoeriol- 8-*C*-glycoside eluted before diosmetin-8-*C*-glucoside. The two flavone glycosides have similar MS^2 fragmentation patterns, although the diosmetin glucoside has a more prominent *m/z* 371 ion. In the current analysis only one peak was present and no reference compounds were available. However, to distinguish between the relative abundance of both compounds, lemon juice, which contains both flavone conjugates, was analysed in the same manner as the *Anastatica* extract. The higher relative abundance of the *m/z* 371 ion in both lemon and *Anastatica* extract and a similar retention time indicated the presence of diosmetin-8-*C*-glucoside **(VIII)** rather than chrysoeriol-8-*C*-glycoside.

Peak 16 (t_R, 32 min; λ_{max} 350 nm) has $[M-H]^-$ at *m/z* 461 and MS^2 fragment at *m/z* 285. The loss of 176 *amu* indicates the presence of the glucuronide unit. In accordance with molecular weight, fragmentation pattern and absorbance spectrum, this component was identified as a luteolin-*O*-glucuronide (Torras-Claveria *et al.*, 2007).

Peak 17 (t_R, 34.2 min; λ_{max} 330 nm) has $[M-H]^-$ at *m/z* 609 that yielded MS^2 ions characteristic of a flavone-*O*-glucosyl-*C*-glucoside, as described for peak 7. Other abundant ions were $[(M-H)-180]^-$, the base beak, indicating the fragmentation of the sugar moiety from *O*-glycosylation. The characteristic AGly+71-18 and AGly+41-18 ions for this type of compound were also detected which confirms the *O*-glycosylation at 2″ position. Therefore, this compound

was identified as luteolin-6-*C*-glucosyl-2″-*O*-glucoside (**IX**, aka isoorientin-2″-*O*-glucoside) as reported by Ferreres *et al.*, (2007).

Peak 18 (t_R, 34.2 min; λ_{max} 330 nm) like peak 12 produced a $[M-H]^-$ at *m/z* 515 with the MS^2 base peak at *m/z* 353. The unique MS^2 fragmentation with low intensity of the *m/z* 299 fragment (>15% of the base peak), supported by less intense fragmentation at *m/z* 255, 203, 179, 317 and the absence of an *m/z* 335 ion is identical to the fragmentation of 4,5-*O*-dicaffeoylquinic acid (**X**) as described by (Clifford *et al.*, 2003; Clifford *et al.*, 2005). This was confirmed by MS^3 and MS^4 fragmentation as described in peak 12.

Peak 19 (t_R, 35 min; λ_{max} 380 nm) was identified as kaempferol-3-*O*-glucoside (**XI**) on the basis of co-chromatography with a standard and a mass spectrum with a $[M-H]^-$ at *m/z* 447 that yielded an MS^2 fragment at *m/z* 285 $[(M-H)-162]^-$; with loss of a glucosyl unit.

Peak 20 (t_R, 40 min; λ_{max} 350 nm) has $[M-H]^-$ at *m/z* 461 and MS^2 fragment at *m/z* 285. The loss of 176 amu indicates the presence of the glucuronide unit. The $[M-H]^-$ fragmentation pattern and absorbance spectrum are in keeping with the presence of a luteolin-*O*-glucuronide (Torras-Claveria *et al.*, 2007).

2.3.2 Quantitative analysis of *A. hirerochuntica* extract

The quantitative estimates of the concentrations of the identified polyphenolic compounds in *Anastatica hirerochuntica* based on HPLC-PDA data, are presented in Table 2-2. The predominant compounds were flavones, most notably luteolin conjugates. The level of flavones ranged from 0.5 ± 0.03 to 542 ± 35 μM with diosmentin-8-*C*-glucodside occurring in the highest concentration. Phenolic acids and hydroxycinnamates were also present in substantial amounts accounting for 36.5 % of the overall level of flavonoids and phenolic compounds with the level of the individual compounds ranging from 17 ± 0 to 210 ± 1 μM. Flavonols were present in relatively low concentrations.

2.3.3 On-line $ABTS^{\bullet+}$ analysis of antioxidants

The antioxidant potential of individual *Anastatica* phenolic compounds was assessed using HPLC with an on-line antioxidant detection system (Stewart et al., 2005). Following HPLC separation, HPLC eluate was mixed with a stabilised solution of the $ABTS^{\bullet+}$ radical and the solution directed to an absorbance detector operating at 720 nm. The $ABTS^{\bullet+}$ solution has a deep blue colour, and any quenching of the radical results in a loss of colour indicated by a negative peak on the HPLC trace as illustrated in A720 nm profiles in Figure 2-7.

The $ABTS^{\bullet+}$ activity profile of *Anastatica* showed that 13 compounds exhibited antioxidant activity (Table 2-2). The highest antioxidant contribution in the tea comes from phenolic acids and hydroxycinnamates with 56% of the antioxidant activity, most notably, 5-*O*-caffeoylqunic acid with a Trolox equivalents value of 60 µM (22% of the total antioxidant capacity). This result is in keeping with the finding of Stalmach *et al.,* (2006) who reported that the main antioxidant contributor in green coffee was 5-*O*-caffeoylqunic acid. He *et al.,* (2010) analysed antioxidant activity in a gardenia fruit extract with the on-line HPLC-$ABTS^{\bullet+}$ system, and showed that the main antioxidants were chlorogenic acids, principally 5-*O*-caffeoylqunic acid.

A range of flavone and flavonol glycosides, mainly luteolin derivatives were also present in the tea in low concentrations. Both luteolin-6-*C*-glucoside and apigenin-6-*C*-glucoside possess antioxidant potential similar to the phenolic acids and hydroxycinnamates. Zielinska and Zielinski (2011) also found that luteolin-6-*C*-glucoside and apigenin-6-*C*-glucoside had the highest antioxidant activity against the $ABTS^{\bullet+}$ radical compared to other flavones *C*-glucosides. The substitution of glucose at the *C*-6 position of the A ring increases antioxidant activity; whereas, the presence of glucose at the *C*-8 position results in a reduction as revealed when luteolin-6-*C*-glucoside and apigenin-6-*C*-glucoside were compared with luteolin-8-*C*-glucoside and apigenin-8-*C*-glucoside (Zielinska and Zielinski 2011).The low antioxidant activity associated with other flavone and flavonol glycosides, including diosmetin-8-*C*-glucoside and taxifolin-*O*-hexose, may be due to the conjugation of aglycone to sugars at loci which reduces their antioxidant potential (Prochazkova *et al.,* 2011).

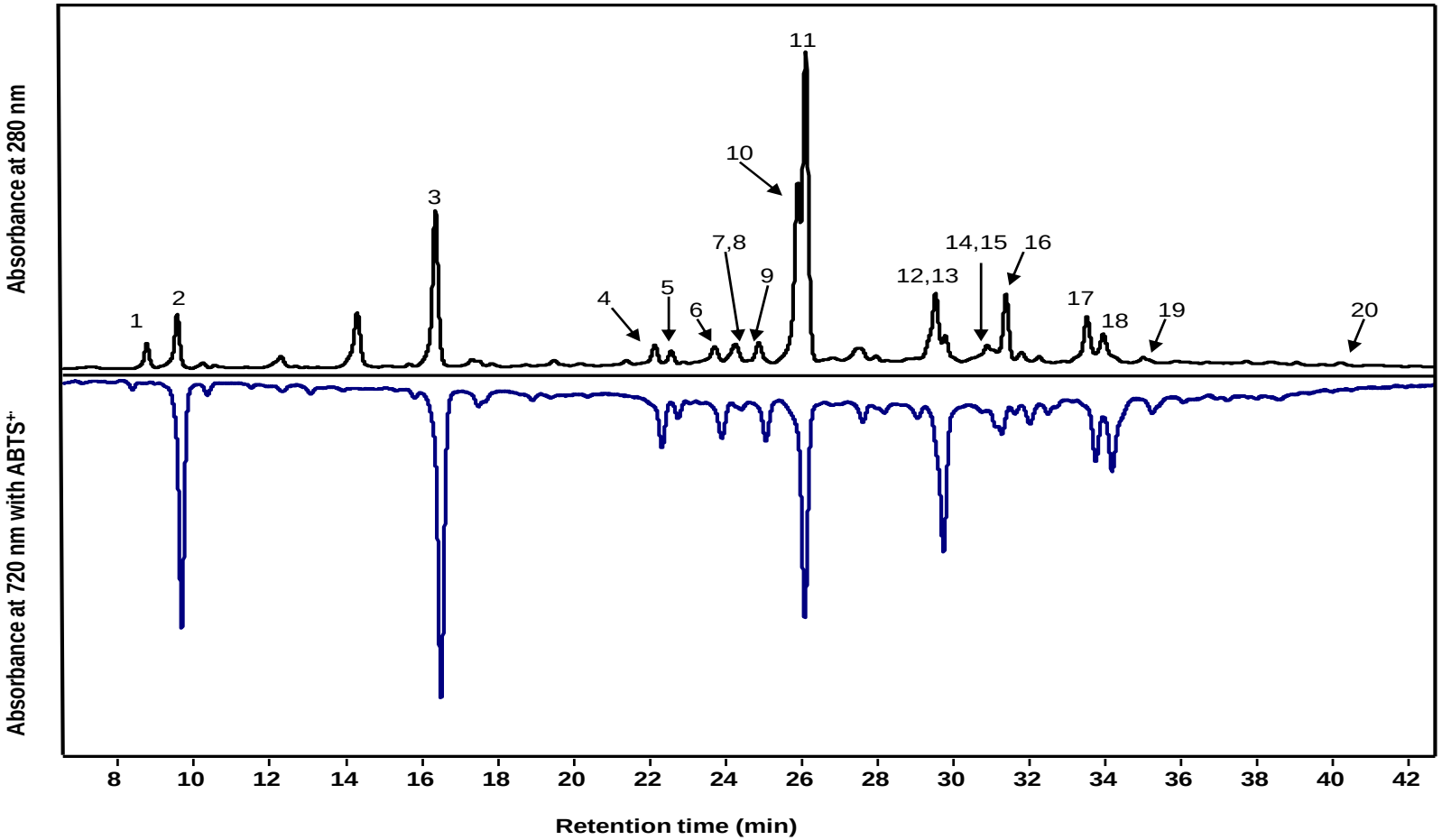


Figure 2-7: Gradient reversed phase HPLC analysis of a tea made from seeds of *A. hirerochuntica* with absorbance detection at 280 nm and on-line $ABTS^{\bullet+}$ antioxidant detection at 720 nm. For MS^2 identification of numbered peaks see Table 3.2.

Table 2-2: Content and trolox equivalent antioxidant activity (TEAC) of individual polyphenolic compounds in a tea made from seeds of *Anastatica hirerochuntica*[a]

Peak	Compounds	Concentration (µM)	Trolox equivalents (µM)	Trolox equivalents % of total
	Phenolic acids & hydroxycinnamates			
1	Dihydroxybenzoic acid hexoside	89 ± 1	n.d.	-
2	3,4-Dihydroxybenzoic acid (**I**)	210 ± 1	40 ± 1	14
3	5-*O*-Caffeoylquinic acid (**II**)	58 ± 5	60 ± 0	22
12	3,4-*O*-Dicaffeoylquinic acid (**VI**)	44 ± 3	32 ± 0	12
18	4,5-*O*-Dicaffeoylquinic acid (**X**)	17 ± 0	23 ± 1	8
	Total	**418 (36.5%)**	**155 (56%)**	**56**
	Flavones			
4	Luteolin-6-*C*-hexosyl-8-*C*-pentoside	5.4 ± 0.2	5.5 ± 0	1.9
5	Luteolin-6-*C*-pentosyl-8-*C*-hexoside	4.3 ± 0.3	5.6 ± 0	2
6	Luteolin-6-*C*-pentosyl-8-*C*-hexoside	1.1 ± 0	5.2 ± 0	1.8
7	Apigenin-6,7-*C*-diglucoside (isovitexin-7-*O*-glucoside)	0.9 ± 0	n.d.	-
8	Apigenin-6-*C*-arabinosyl-8-*C*-hexoside	0.9 ± 0	n.d.	-
9	Luteolin-8-*C*-glucoside (orientin) (**IV**)	7.2 ± 1	7.6 ± 0.2	2.7
10	Luteolin-6-*C*-glucoside (isoorientin) (**V**)	37 ± 4	46 ± 0	16.5
13	Apigenin-6-*C*-glucoside (isovitexin)	13 ± 1	32 ± 0	11.5
14	Luteolin-*O*-glucoside	1.6 ± 0	n.d.	-
15	Diosmetin-8-*C*-glucoside (VIII)	542 ± 35	n.d.	-
16	Luteolin-*O*-glucuronide	0.5 ± 0	2.3 ± 0.1	0.8
17	Luteolin-6-*C*-glucosyl-2'-*O*-glucoside (isoorientin-2"-*O*-glucoside) (**IX**)	4.6 ± 0	11 ± 1	3.9
20	Luteolin-*O*-glucuronide	0.5 ± 0	n.d.	-
	Total	**619 (54%)**	**115 (41%)**	**41**
	Flavonols			
11	Taxifolin-*O*-hexose	87 ± 10	n.d.	-
19	Kaempferol-3-*O*-glucoside (**XI**)	4.9 ± 0	2 ± 0	1
	Total	**91.9 (8.1%)**	**2 (0.7%)**	**1**
	Total	**1129**	**272**	**98**

[a]Results represent concentration of polyphenolics and their antioxidant potential in an aqueous extract of A. *Hirerochuntica* seed (10 mg/mL). Data expressed as mean values ± standard deviation (n=3), n.d. not detected. For information on peak numbers see Table 2-1 and Fig. 2-6.

It is of note that the spectrum of compounds identified and quantified in the tea made from *A. hirerochuntica* seeds is significantly different to the flavonoids isolated from a methanolic extract of whole plants of *A. hirerochuntica* by Yoshikawa et al. (2003). This may be due to extracting different parts of this herb and using different solvents for extraction and different analysing methods.

The total antioxidant content of *Anastatica* was measured using the reaction of the aqueous of *Anastatica* with $ABTS^{\cdot+}$ radical solution using the on-line HPLC system but with the HPLC column removed (Stewart *et al.*, 2005). In this way there was no separation of *Anastatica* compounds and no material retained in the column. The total antioxidant activity obtained with this method can then be compared with the corresponding value obtained by adding the antioxidant capacity of the individual HPLC peaks in order to assess the contribution of compounds retained by the column. The data obtained by this method are presented in Table 2-3.

Table 2-3: Total antioxidant capacity of *Anastatica hirerochuntica* extract.

	Trolox Equivalent (uM)
Total antioxidant capacity [(a)]	1119 ± 6 (100%)
HPLC antioxidant capacity [(b)]	272 ± 3 (24%)
Difference	847 (76%)

(a) Trolox equivalent concentration of *A. hirerochuntica*.
(b) Addition of the Trolox equivalent concentration calculated for phenolic peak separated by HPLC.

The total antioxidant capacity of the extract when analysed without the HPLC column (Table 2-3) was increased from 272 ± 3 to 1119 ± 6 μM Trolox equivalents, an increase of 76% which is similar to the total antioxidant capacity of green coffee with 1125 ± 19 μM Trolox equivalents reported by (Stalmach *et al.*, 2006). However, the addition of the antioxidant potential of all individual *Anastatica* compounds separated by HPLC accounts only for 24% of the total antioxidant capacity. This indicates that other compounds may contribute to the antioxidant potential of this herb, possibly polymeric procyanidins which would be retained by the HPLC column.

2.3.4 Antioxidant capacity and total phenol content

The aqueous *A. hirerochuntica* infusions exhibited high antioxidant activity, 1262 ± 0.05 µmol/L of Fe^{+2}, in the FRAP assay (Benzie and Strain 1996) which is similar to the FRAP activity of coffees reported by Delgado-Andrade *et al.,* (2005) and Sanchez-Gonzalez *et al.,* (2005). The concentration of total phenolics was also high at 4.0 ± 0.01 mmol/L GAE, which is comparable to that of other herbs from the asteraceae family, namely *Echinainacea purpurea* and *Achillea millefolium* (Wojdylo *et al.,* 2007).

2.4 Conclusion

In this study, the use of HPLC-PDA-MS^2 has facilitated the identification and quantification of 20 polyphenolic compounds, in a tea prepared from dry seeds of *A. hirerochuntica*. The spectrum of polyphenolic compounds in the tea was very different to that detected in a purified methanolic extract of whole plants of *A. hirerochuntica* by Yoshikawa *et al.,* (2003) due to the use of different methods and different parts of the plant. Yoshikawa and his colleagues (2003) had isolated and identified two new flavonoids, anastantin A and B, by NMR and reported the presence of seven flavonoids, 11 aromatic compounds, three phenylpropanoids, 12 lignans and four flavonolignans. However, in this study the *Anastatica* tea contained 5 chlorogenic acids and hydroxybenzoic acids, 13 flavones conjugates and two flavonols glucosyl. The major components were flavone *C*-glycosides, *O*-glycosides, *C*-diglycosides and *O*-glycoside-*C*-glycosides occurring principally as luteolin conjugates.

This suggests that the technique described in this study seems to be quite useful for these compounds, when examined qualitatively and quantitatively even with the lack of reference standards. The mass spectrometer can be used in many ways to increase the sensitivity and selectivity of the analysis, depending on the circumstances of the analysis and the samples under study. For example, the mass spectrometer can be set up to detect only one ion, selected ion monitoring (SIM) (Mullen *et al.,* 2000c). Alternatively when limited availability of sample is not a problem, full scan analysis can provide detailed structural information at the expense of sensitivity. Selected reaction monitoring (SRM) is another mode

of analysis that offers high selectivity and sensitivity. With this type of analysis, in addition to the previous reported data in the literature, it was possible to identify or partial identify these compounds using HPLC-MS/MS (Mullen *et al.*, 2000b; Mullen *et al.*, 2007).

In addition, 14 of the 20 compounds in the *A. hirerochuntica* tea exhibited antioxidant activity when analysed by HPLC with an on-line antioxidant detection system. These findings together with tests in the FRAP antioxidant assay and the Folin–Ciocalteu total phenolics assay demonstrate that aqueous infusions of *A. hirerochuntica* seed, which is used as medicinal tea in Middle Eastern countries, is rich in polyphenolic compounds and is a good source of natural antioxidants which have given rise to a large number of publication on their potential impact on health (Hertog *et al.*, 1993; Crozier *et al.*, 2000; prior and Cao 2000; Tapiero *et al.*, 2002).

These finding raise the importance of studying the bioavailability of these compounds to understand their possible positive health effects in animal or human models. However, it would be extremely unlikely that ethical permission could be obtained for a human feeding study with *A. hirerochuntica* tea. The tea was purchased from a local market in Saudi and there was no documentation to show that the tea fit for human consumption. Even importation of the plant to the UK was not a simple matter. Therefore a human feeding with the herbal medicinal plant was not attempted. There were no facilities available to even carry out animal experiments using *A. hirerochuntic*. Therefore, on the advice of my supervisors, human bioavailability studies were carried out with anthocyanin-rich berries and the results obtained will be presented in the coming chapters.

3. Bioavailability of anthocyanins following consumption of blackberries by healthy subjects

3.1 Introduction

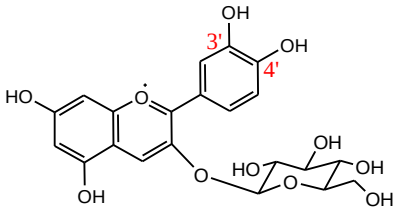
As it was not possible to do a feeding study with the herbal tea using humans or an animal model, the aim of the next phase of my research was to investigate fruits that are rich in polyphenolic compounds and test their bioavailability after consumption in humans in order to better understand their fate *in vivo* following ingestion. In the previous chapter the HPLC-PDA-MS^2 methodology that was used for the qualitative and quantitative analysis and antioxidant activity measurement of the polyphenolic content in a herbal tea is also used to measure the levels of these compounds both in the plant extracts, the *in vitro* experiments and in body fluids in the human bioavailabilty studies.

As discussed in Chapter 1, flavonoids are a large group of natural phenolic compounds, consisting mainly of flavones, flavonols, flavan-3-ols, isoflavones, flavanononones and anthocyanidins. Among these flavonoids, the water-soluble anthocyanin glycosides and acylglycosides are an important group of natural antioxidants and have been linked with beneficial effects on health including reducing cardiovascular disease risk, cancer-preventing, anti-obesity effects (Wang *et al.,* 1999; Crozier *et al.,* 2000; Casto *et al.,* 2002; Mulleder *et al.,* 2002; Acquaviva *et al.,* 2003). Given these biological effects, anthocyanins bioavailability and metabolism are considered to be important issues. *In vivo* studies with animal and humans have indicated that anthocyanins were absorbed rapidly through the small intestine and possibly through the stomach (Talavera *et al.,* 2003; Talavera *et al.,* 2004; Ichiyanagi *et al.,* 2005a; Borges *et al.,* 2007). However, the quantities appearing in plasma and urine indicate that anthocyanins have an extremely low bioavailability (Cao *et al.,* 2001; Mulleder *et al.,* 2002; Kay *et al.,* 2004; McGhie and Walton 2007; Gonzalez-Barrio *et al.,* 2010). They can be absorbed intact as the parent glycosides but can also appear as glucuronide, sulphated and/or methylated metabolites (Felgines *et al.,* 2003; Felgines *et al.,* 2005; He *et al.,* 2005; Talavera *et al.,* 2005).

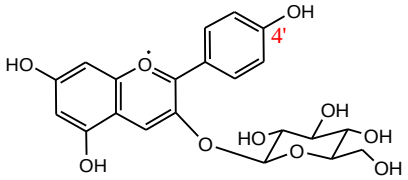
Berries in general are rich in anthocyanins and their intake was associated with beneficial health effects (see Chapter 1). Therefore, berries were of interest in this investigation and in the subsequent chapters.

Many bioavailability studies involved berries or berry extracts containing several structurally diverse anthocyanins. Raspberries for instance contain 3-*O*-glucoside, 3-*O*-sophoroside, 3-*O*-rutinoside, 3-*O*-2″-glucosylrutinoside, and 3-*O*-2″-xylosylrutinoside derivatives of cyanidin and 3-*O*-glucoside, 3-*O*-rutinoside, 3-*O*-sophoroside and 3-(2″-*O*-glucosyl) rutinoside derivatives of pelargonidin. The 3-*O*-glucosides, 3-*O*-galactosides, and 3-*O*-arabinosides of malvidin, petunidin, cyanidin, and delphinidin, a total of 12 anthocyanins, were found in blueberries (Mazza *et al.,* 2002; Borges *et al.,* 2004; Kahle *et al.,* 2006). As a result, the available data from these studies are confusing. The complexity of anthocyanins content in these berries complicates interpretation of data on anthocyanins content in biological fluids and complicates assessing anthocyanins absorption and metabolism (See chapter 1). However, simple anthocyanin profiles are found in strawberries and blackberries, as they contain one predominant anthocyanin, pelargonidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside respectively. Thus, the aim of this chapter was to evaluate the absorption, metabolism and urinary excretion of blackberry anthocyanins in human. As a consequence, the bioavailability data on anthocyanins after ingestion of blackberry by human subjects are potentially more straightforward to interpret.

In addition, some reports have also suggested the influence of the aglycone structure and sugar moieties on absorption and metabolism of anthocyanins, yet, very few studies have specifically investigated these effects (Wu *et al.,* 2004a; Bakowska-Barczak 2005; Felgines *et al.,* 2005; Wu *et al.,* 2005a; Borges *et al.,* 2007; Felgines *et al.,* 2008; Ichiyanagi *et al.,* 2008). In this study, to assess the influence of the aglycone on anthocyanin bioavailability, the current investigation with blackberry will be carried similarly to a previous feeding study with strawberries (Mullen *et al.,* 2008b). Both anthocyanins in blackberry and strawberry have the same sugar moiety but a different aglycone structure. Thus, taken together the data from both studies will highlight the effect of 3'-hydroxylation and 3',4'-dihydroxylation on the bioavailability of these two compounds (see Figure 3-1).



cyanidin-3-*O*-glucoside



pelargonidin-3-*O*-glucoside

Figure 3-1: Cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside.

3.2 Materials and methods

3.2.1 Chemicals and reagents

HPLC grade methanol was obtained from Rathburn Chemicals Ltd., (Walkerburn, UK). Formic acid and ascorbic acid were supplied by Sigma (Poole, Dorset, UK). Cyanidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside were supplied by Extrasynthese (Genay, France). Blackberries purchased frozen from a local Morrisons supermarket were defrosted prior to use.

3.2.2 Study design

The study was approved by the Glasgow Royal Infirmary Local Research Ethics Committee (REC reference number: 11/AL/0007). The participants criteria was to be in good health, non-smokers and not pregnant. Eight healthy human subjects (four males and four females) gave their written consent and participated in this study. From previous bioavailablity studies carried out in the laboratory in Glasgow, with a number of other food sources, (Jaganath *et al.*, 2006; Auger *et al.*, 2008; Mullen *et al.*, 2008a; Mullen *et al.*, 2008b; Marks *et al.*, 2009; Stalmach *et al.*, 2009a; Stalmach *et al.*, 2009b; Stalmach *et al.*, 2009c; Gonzalez-Barrio *et al.*, 2010; Mullen *et al.*, 2010; Stalmach *et al.*, 2010a; Stalmach *et al.*, 2010b) the proposed number of subjects recruited has been more than sufficient to give a good estimate of bioavailability, including a measure of the variability of response. In an investigation on quercetin bioavailability by Mullen *et al.*, (2006) the peak plasma concentration (C_{max}) of

the main metabolite was 665nmol/L with a standard error of 82 nmol/L, this was with six volunteers consuming a dose of 275 μmoL. In another study by Mullen *et al.,* (2008a) on the bioavailability of the polyphenols in orange juice the C_{max} was 924 nmol/L with a standard error of 224 nmol/L. This does have a higher variation however, the time to reach C_{max} is much later, indicating absorption from the large interstine, which would account for the greater variability. In addition, previous experiment with berries indicate that 180 g of the blackberries should contain levels of anthocyanins to facilitate detection and quantification of metabolites in plasma and urine by the HPLC-MS procedures in operation in our laboratory (Mullen *et al.,* 2008b; Gonzalez-Barrio *et al.,* 2010; Stalmach *et al.,* 2011).

The healthy volunteers were aged between 22 and 42 years with an average height of (1.70 m), average weight of (67 kg) and a mean body mass index of (24 kg/m). Subjects were required to follow a diet low in flavonoids and phenolic compounds for two days prior to the study, which excluded fruits and vegetables and beverages such as tea, coffee, fruit juices, and wine, whole grains, nuts, chocolate and dietary antioxidant supplements. On the morning of the study after an overnight fast, the volunteers consumed 180 g of blackberries.
Twelve mL of venous blood was collected in heparinized tubes from all healthy volunteers at 0, 0.5, 1, 2, 3, 4, 6, 8, and 24 h post-ingestion, and plasma was obtained by centrifugation at 4000*g* for 15 mins at 4 °C. The plasma was decanted and 1 mL aliquots were acidified to pH 3 with 15 μL of 50% formic acid, and 50 μL of 10 mmol/L ascorbic acid was also added to prevent oxidation. The samples were stored at -80°C prior to analysis. Urine was collected after feeding over 0-4, 4-7 and 7-24 h periods. The volumes of urine were measured and aliquots were acidified to pH 3 with 50% aqueous formic acid to keep the anthocyanins in the red flavylium cation form, resulting in increased peak sharpness with HPLC at 520 nm. Samples were then stored at -80ºC.

3.2.3 Preparation of blackberry extract

Ten grams of frozen blackberries were weighed and extracted with 10 mL of 50% aqueous methanol containing 1% of formic acid using an Ultra-Turrax T25 homogeniser (IKA Werke, Staufen, Germany). The resultant mixture was centrifuged for 10 mins at 4000g at 4ºC. The supernatant was removed and

stored and the pellet re-extracted with acidified 50% methanol. The two supernatants were combined and made up to an exact volume (50 mL) with acidified 50% methanol. The anthocyanin content of 10 µL aliquots was analyzed by HPLC-PDA-MS^2.

3.2.4 Analysis of anthocyanins in biological fluids

3.2.4.1 Extraction of anthocyanins from plasma

Plasma extraction for the analysis of anthocyanins and related metabolites was based on the method of (Stalmach *et al.*, 2011). Triplicate 500 µL volumes of plasma acidified with 50% aqueous formic acid, and containing 10 µL of 50ng/µL of delphinidin-3-*O*-glucoside added as an internal standard were loaded onto a Strata C18 (6 mL/500 mg) SPE cartridge (Phenomenex, Inc., Cheshire, U.K.), preconditioned with 5 mL of methanol acidified with 0.1% formic acid, followed by 5 mL of acidified water (0.1% formic acid). The cartridge was then washed with 5 mL of acidified water, after which anthocyanins were eluted with 5 mL of acidified methanol, which was dried for 2 h using a centrifugal vacuum concentrator at 37 ºC (SPSSpeedVac, Thermo Savant, Waltham, MA), prior to being dried under a stream of nitrogen at 35 ºC. Pellets were resuspended in 150 µL of 0.1% formic acid containing 10% methanol before being centrifuged for 15 mins at 4 ºC. Volumes of 100 µL were analyzed by HPLC-MS^n.

Extraction efficiency experiments were performed, and typical values of 68 ± 6.2 % were achieved for delphinidin-3-*O*-glucoside. Ratios of the individual recoveries relative to delphinidin-3-*O*-glucoside were computed and used as correction factors in the quantification of metabolites in the plasma samples.

3.2.4.2 Extraction of anthocyanins from urine

The urine samples were defrosted, vortexed and extracted using a solid-phase extraction procedure (Stalmach *et al.*, 2011). Five mL of urine, to which had been added 1 µg of delphinidin-3-*O*-glucoside as an internal standard, was loaded to a 500 mg/6 mL Strata C18E (Phenomonex, Macclesfield, U.K.) cartridge that had been conditioned with 5 mL of 5% formic acid in methanol and then 5 mL of 5% aqueous formic acid. The urine was applied to the cartridge and

washed with 5 mL of aqueous formic acid then anthocyanins were eluted with 5 mL of methanol containing 5% formic acid. The methanolic fraction was reduced to dryness with a rotary evaporator in vacuo at 30ºC. The dried samples were then re-suspended in 50 µL of 5% formic acid in methanol plus 450 µl of 5 % aqueous formic acid. Aliquots of 50 µL were analyzed by HPLC-PDA-MS^2 on the day of extraction. Recoveries of delphinidin-3-*O*-glucoside, the internal standard, in the urine were typically 70.5 ± 2 %.

3.2.5 Pharmacokinetic analysis of anthocyanins in plasma

Maximum plasma concentration of the metabolites from 0 to 8 h post-dose was defined as C_{max}. The time to maximum plasma concentration (t_{max}) was defined as the time in hours at which C_{max} was reached. The elimination half-life for the metabolites was computed by using the following formula: $t_{1/2} = 0.693/K_e$ where K_e is the slope of the linear regression of the plasma metabolite concentrations. Area-under-the-curve calculations were determined using a Kinetica software package (Thermo Electron Corp.).

3.2.6 HPLC with diode array detection and tandem mass spectrometry

Cyanidin-3-*O*-glucoside and their metabolites in blackberries, plasma and urine were analyzed on a Surveyor HPLC system comprising a HPLC pump, a diode array absorbance detector, scanning from 200 to 600 nm, and an autosamplercooled to 4°C (Thermo Electron Corp). Separation was carried out using a 250 x 4.6 mm i.d C12 4µm, Synergi RP-Max column (Phenomenex, Macclesfield, UK). Samples were eluted with a gradient 60 min of 5–50% with methanol in 1% formic acid at a flow rate of 1 mL/min and maintained at 40°C. After passing through the flow cell of the diode array detector, the column eluate was split, and 0.3 mL min was directed to an LCQ Advantage ion trap mass spectrometer fitted with an electrospray interface (Thermo Electron Corp). Analyses utilized the positive ion mode as this provided the best limits of detection for anthocyanins. Each sample was analyzed in the mass spectrometer in two separate ways: first, using full scan, data-dependent MS^2 scanning from *m*/*z* 200 to 600, then using a two segment selected reaction monitoring (SRM) method. The tuning of mass spectrometer was optimised by infusing a standard

of cyanidin-3-*O*-glucoside. Capillary temperature was 300°C, sheath gas and auxiliary gas were 40 and 20 units, respectively, and the source voltage was 3 kV. Anthocyanins and metabolites in blackberry extract and urine were quantified from their chromatographic peak and expressed as cyanidin-3-*O*-glucoside equivalents. The identifications were confirmed by full-scan MS^2 and/or SRM. For plasma, samples were quantified against standards of cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside based on the $[M-H]^+$ in the selected reaction monitoring (SRM) mode. Calibration curve for quantifying blackberry extract, ranged between 0.5-500 ng with linear coefficients of 0.99 and ranged between 0.1-50 ng with linear coefficients of 0.99 for quantifying plasma and urine.

3.2.7 Data analysis

HPLC-MS data were processed using Xcalibur QualBrowser version 1.0.1.03 software (Thermo Fisher Scientific Inc., 2009). Each sample was analysed in triplicate for each volunteer and data were presented as mean value ± standard error (n=8 for healthy volunteers).

3.3 Results

3.3.1 Analysis of blackberries

Several studies have identified individual anthocyanins of blackberries. Cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside have been reported as the respective major and minor anthocyanins in blackberries (Talavera *et al.,* 2003; Felgines *et al.,* 2005). Other studies reported the presence of trace quantities of cyanidin-3-*O*-xyloside, cyanidin-3-*O*-arabinoside and cyanidin-3-*O*-sambubioside (Wu and Prior 2005; Ogawa *et al.,* 2008). In addition, cyanidin-3-*O*-(6″-*O*-malonylglucoside) and an acylated cyanidin derivative have been identified in other investigations (Stintzing *et al.,* 2002; Ogawa *et al.,* 2008; Lopes-Lutz *et al.,* 2010). In the current study HPLC-PDA-MS analysis of a blackberry extract resulted in the detection of nine anthocyanins (Figure 3-2 and Figure 3-3). The main anthocyanin was cyanidin-3-*O*-glucoside. Other minor anthocyanins identified were cyanidin glycosides, with the exception of peak 4 which was identified as pelargonidin-3-*O*-glucoside. Peaks 8 and 9 co-eluted and their

identification were confirmed with SRM. Identification was based on published MS^2 fragmentation data and on co-chromatography with authentic standards, when available. The anthocyanins identified in blackberries are summarized below and outlined in Table 3-1.

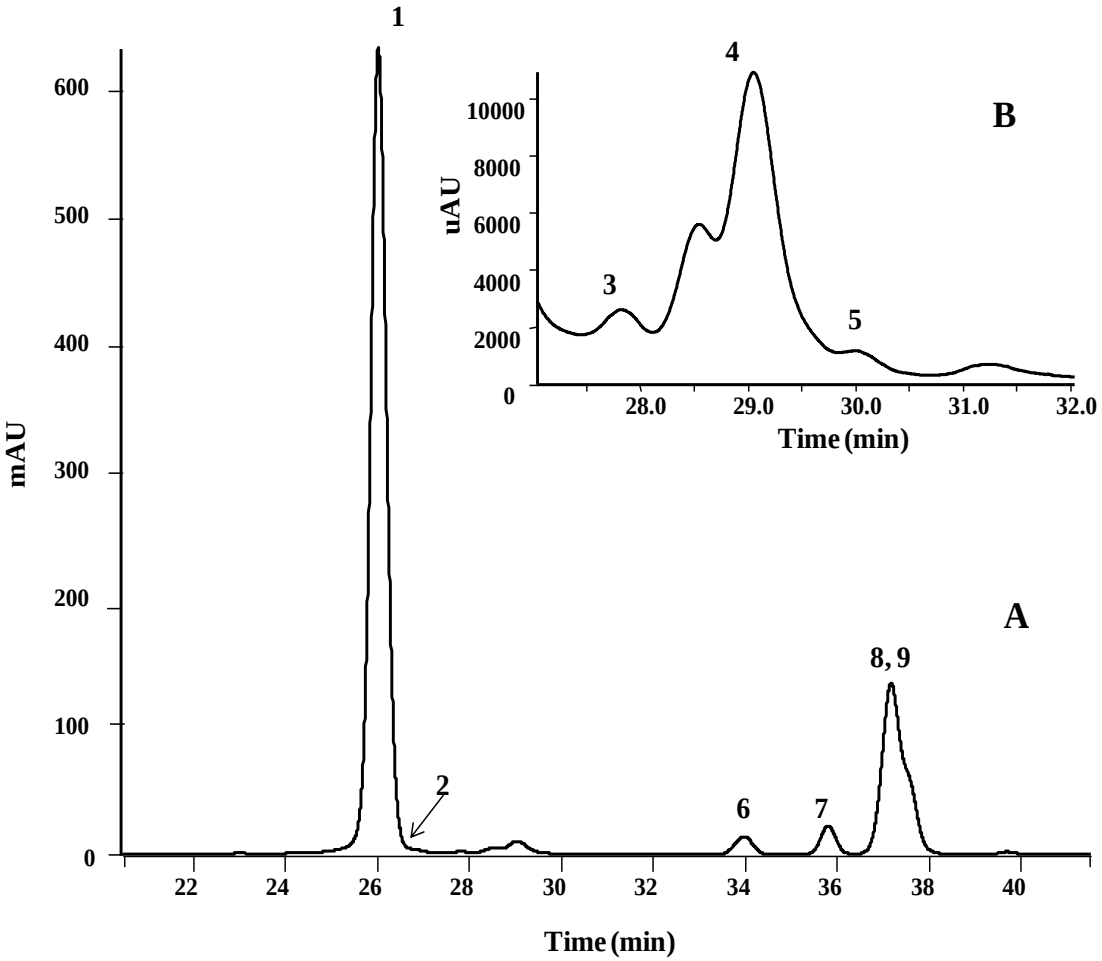
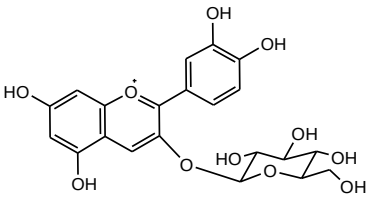
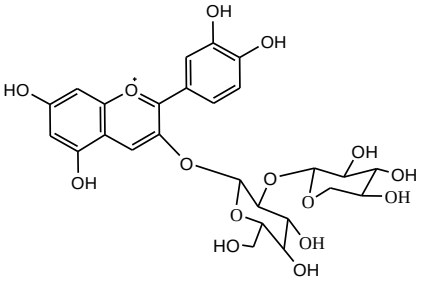


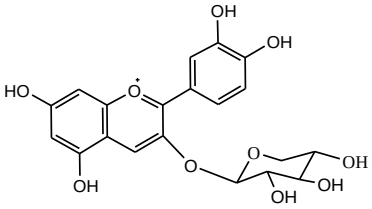
Figure 3-2: (A) Gradient reversed phase HPLC profiles at 520 nm showing the anthocyanin content of blackberries. For identification of numbered peak see Table 3-1. (B) Enlargement of minor peaks.



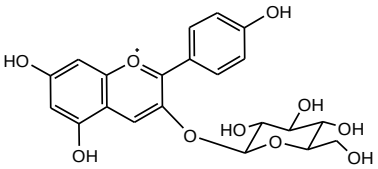
Peak 1. cyanidin-3-*O*-glucoside



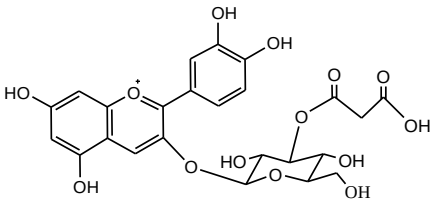
Peak 2. cyanidin-3-*O*-sambubioside



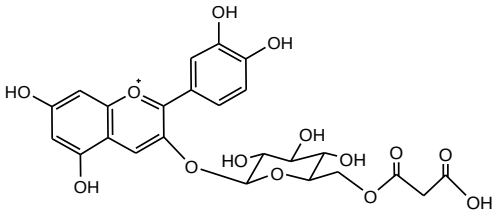
Peaks 3, 6. cyanidin-3-*O*-arabinoside



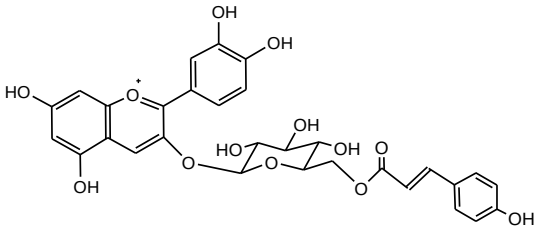
Peak 4. pelargonidin-3-*O*-glucoside



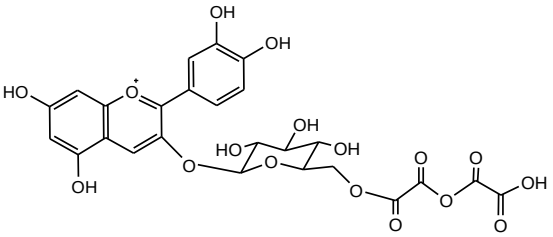
Peak 5. cyanidin-3-*O*-(3"-*O*-malonoyl) -glucoside



Peak 7. cyanidin-3-*O*-(6"-*O*-malonoyl) -glucoside



Peak 8. cyanidin-3-*O*-(6"-*O*-*p*-coumaroyl) -glucoside



Peak 9. cyanidin-3-*O*-(6"-*O*-dioxalylglucoside)

Figure 3-3: Structure of blackberry anthocyanins.

Table 3-1: Anthocyanins identified in blackberries by HPLC with diode array and MS^2 detection.

Peak	R_t	λ_{max}	$[M-H]^+$ *(m/z)*	MS^2 *(m/z)*	Anthocyanins
1	26.05	515	449	287	Cyanidin-3-*O*-glucoside
2	26.61	515	581	449,287	Cyanidin-3-*O*-sambubioside
3	27.83	515	419	287	Cyanidin-3-*O*-arabinoside
4	29.01	500	433	271	Pelargonidin-3-*O*-glucoside
5	30.1	515	535	287	Cyanidin-3-*O*-(3″-*O*-malonoyl)glucoside
6	33.99	515	419	287	Cyanidin-3-*O*-xyloside
7	35.82	515	535	449,287	Cyanidin-3-*O*-(6″-*O*-malonoyl)glucoside
8	37.68	515	595	287	Cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside
9	37.7	515	593	449,287	Cyanidin-3-*O*-(6″-*O*-dioxaylglucoside)

t_R, retention time; $[M+H]^+$, positively charged molecular ion; MS^2 daughter ion produced by fragmentation of $[M+H]^+$

Peak 1 (t_R, 26.05 min; λ_{max} 515 nm) was identified as cyanidin-3-*O*-glucoside based on cochromatography with a standard and a mass spectrum with a *m/z* 449 positively charged molecular ion ($[M-H]^+$) which fragmented with a loss of 162 *amu* (glucose) to produce a *m/z* 287 (cyanidin) daughter ion.

Peak 2 (t_R, 26.61 min; λ_{max} 515 nm) has a $[M-H]^+$ at *m/z* 581 and an MS^2 fragment at *m/z* 287. The loss of 294 *amu* indicates the presence of glucose and pentose units. In accordance with molecular weight, fragmentation pattern and absorbance spectrum, this component was identified as a cyanidin-3-*O*-sambubioside, previously detected by (Ogawa *et al.*, 2008).

Peak 3 and 6 (t_R, 27.83 min and 33.99; λ_{max} 515 nm) produced a $[M-H]^+$ at *m/z* 419 and an MS^2 ion at *m/z* 287. The loss of 132 *amu* is in keeping with the presence of pentose unit. The chromatographic profile for peak 3 and 6 and elution time indicate that they are cyanidin-3-*O*-arabinoside and cyanidin-3-*O*-xyloside, respectively, as previously reported in blackberry and chokeberry (Cho *et al.*, 2004).

Peak 4 (t_R, 29.01 min; λ_{max} 500 nm) was identified as pelargonidin-3-*O*-glucoside on the basis of co-chromatography with a standard and a mass spectrum with a $[M-H]^+$ at *m/z* 433 that yielded an MS^2 fragment at *m/z* 271 (M-162, loss of a

glucosyl unit). This minor anthocyanin has been previously reported to occur in blackberries (Dugo *et al.*, 2001; Sautebin *et al.*, 2004).

Peak 5 and 7 (t_R, 30.1 min and 35.82 min; λ_{max} 515 nm) had a $[M\text{-}H]^+$ at *m/z* 535 and MS^2 fragments. The loss of 248 *amu* indicates the presence of hexose and malonic acid (Giusti *et al.*, 1999). However, peak 7 has a lower intensity fragment at *m/z* 449 formed by the loss of 86 *amu* which can be ascribed to the release of the malonyl moiety. Based on the literature this fragmentation behaviour is in accordance with the presence of cyanidin-3-*O*-(6″-*O*-malonyl)glucoside (Wu and Prior 2005). Whereas, the more stable cyanidin- 3-*O*-(3″-*O*-malonoyl)glucoside (peak 5) yielded only one fragment ion at *m/z* 287, showing that the acyl linkage to the 6″-position of the sugar is more labile than the corresponding linkage to the 3″-position. The order of elution of compounds was found to be in accordance with that described previously under reversed-phase conditions (Schutz *et al.*, 2006; Ogawa *et al.*, 2008; Lopes-Lutz *et al.*, 2010). Therefore, these anthocyanins were tentatively identified as cyanidin-3-*O*-(3″-*O*-malonoyl)glucoside and cyanidin-3-*O*-(6″-*O*-malonoyl) glucoside, respectively.

Peak 8 (t_R, 37.68 min; λ_{max} 515 nm) had a $[M\text{-}H]^-$ at *m/z* 595 and yielded an MS^2 ion at m/z 287. This fragmentation with $[(M\text{-}H)\text{-}308]^+$ indicates the presence of *p*-coumarylglucoside moiety (Giusti *et al.*, 1999). Therefore, this peak was tentatively identified as cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside.

Peak 9 (t_R, 37.7 min; λ_{max} 515 nm) had a $[M\text{-}H]^+$ at *m/z* 593 and two MS^2 fragments at *m/z* 449 $[M\text{-}144]^+$ (cyanidin + glucose) and a main daughter ion at *m/z* 287 $[M\text{-}144\text{-}162]^+$ (cyanidin) corresponding to the loss of one hexose. The difference of *m/z* 449 [M^+- cyanidin – glucose] indicated two oxalic acid moieties. Thus, this peak is most probably cyanidin-3-*O*-(6″-*O*-dioxaylglucoside). This compound has already been tentatively identified on the basis of MS data by Stintzing *et al.*, (2002) in blackberries and Hillebrand *et al.*, (2004) by NMR techniques in blood orange juice.

The quantities of anthocyanins in the 180 g serving of blackberries consumed by the volunteers are presented in Table 3-2. Each serving contained a total of 295 µmoles of anthocyanins, 63% of which was cyanidin-3-*O*-glucoside as a main

component in the fruits. Other minor components (peaks 5, 7, 8 and 9) identified as acylated derivatives of cyanidin-3-*O*-glucoside accounted for 0.1% to 15% of total anthocyanins. Wu *et al.*, (2006b) reported that approximately 94% of blackberry anthocyanins occur in nonacylated form, and 90% of these exist as monoglycoside, whereas 10% are found as diglycosides.

Table 3-2: Anthocyanins quantified in blackberries by HPLC-PDA-MS^2.

Peak	Anthocyanins	µmol/180g
1	Cyanidin-3-*O*-glucoside	187 ± 1 ***(63%)***
2	Cyanidin-3-*O*-sambubioside	1.5 ± 0.3 ***(0.5%)***
3	Cyanidin-3-*O*-arabinoside	0.4 ± 0.0 ***(0.1%)***
4	Pelargonidin-3-*O*-glucoside	3.6 ± 0.4 ***(1.2%)***
5	Cyanidin-3-*O*-(3″-*O*-malonoyl)glucoside	0.3 ± 0.0 ***(0.1%)***
6	Cyanidin-3-*O*-xyloside	5.0 ± 0.2 ***(2%)***
7	Cyanidin-3-*O*-(6″-*O*-malonoyl)glucoside	6.7 ± 0.4 ***(2.3%)***
8	Cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	45 ± 0.3 ***(15%)***
9	Cyanidin-3-*O*-(6″-*O*-dioxaylglucoside)	45 ± 0.3 ***(15%)***
	Total	**295 ± 3**

Data expressed as mean values in µmol (n=3) ± standard error, % percentage of anthocyanins of the total.

3.3.2 Identification and quantification of anthocyanins in plasma

Data on the anthocyanins detected in plasma collected over 24 h following ingestion of 180 g of blackberries by eight healthy volunteers are shown in Table 3-3.

Excluding the delphinidin-3-*O*-glucoside used as an internal standard, no anthocyanins were observed in plasma collected before the consumption of the blackberries. Identifications were based on mass spectra data and cochromatography with authentic standard in the case of cyanindin-3-*O*-glucoside and peonidin-3-*O*-glucoside. Full-scan MS^2 and SRM were used to identify the methyl and glucuronide metabolites of cyanidin. Cyanidin and peonidin glucuronide metabolites had a $[M-H]^+$ at *m/z* 463, *m/z* 477, respectively, which fragmented with a 176 amu loss to yield respective to MS^2 fragments at *m/z* 287 and *m/z* 301. Peonidin-3-*O*-glucoside, which is a methylated form of cynaidin-3-*O*-glucoside (3´-*O*-methyl-cyanidin-glucoside),

had a $[M\text{-}H]^+$ at *m/z* 463, with a 162 amu cleavage of the sugar moiety gave rise to a m/z 301 peonidin daughter ion (Table 3-3). Although three glucuronide metabolites were detected, it was not possible to determine the point of attachment to the aglycone because reference standards were not available. It has been suggested that although cyanidin-3-*O*-glucoside has four free hydroxyl groups, all of which are potential sites for glucuronidation, glucuronidation is most likely to occur at the 3-position (Wu *et al.,* 2002b). However, earlier work in our lab by Mullen *et al.,* (2006; 2008b) with quercetin-3-*O*-glucuronide and pelargonidin-3-*O*-glucuronide using reversed phase HPLC, has shown that the 3-*O*-glucuronide of quercetin and pelargonidin elutes immediately after quercetin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside. Therefore, peak 2 and 4 may be cyanidin-3-*O*-glucuronide and peonidin-3-*O*-glucuronide respectively. Whereas, the later eluting peak 5 may be either a 3´- or 4´-*O*-glucuronide.

The concentration of peonidin-*O*-glucuronide (peak 4) in plasma reached 9 ± 1.9 nmol/L 30 mins after the intake of blackberries and attained a maximum concentration (C_{max}) of 17.4 ± 1.7 nmol/L after 2 h. It subsequently declined gradually and at 24 h was not detected. Cyanidin-3-*O*-glucoside (peak 1) was detected in all volunteers and reached C_{max} at 1.0 h with a value of 15 ± 2.2 nmol/L. However, peak 3 and 5, respectively peonidin-3-*O*-glucoside and a cyanidin-*O*-glucuronide, had a t_{max} of ~1.0 h and declined rapidly thereafter (Figure 3-4).

Pharmacokinetic analysis of the anthocyanin plasma data in Figure 3-4 is presented in (Table 3-4). C_{max} values ranged from 5-20 nmol/L, between 0.8-2.6 h (t_{max}) after ingestion. Peonidin-*O*-glucuronide which was the main metabolite in plasma had a C_{max} value of 20 ± 2.4 nmol/L and t_{max} value of 2.0 ± 0.4 h after the ingestion of blackberries. There was a slow rate of decline after C_{max} which is reflected in an elimination half-life of 15 ± 6 h. The C_{max} of the cyanidin-3-*O*-glucoside (peak 1) was 17 ± 2.4 nmol/L with a t_{max} at 0.9 ± 0.2 h and its elimination half-life at 4.6 ± 0.9 h was much shorter than the other metabolites (peaks 2, 3 and 4 with the exception of peak 5, a cyanidin-*O*-glucuronide).

Table 3-3: HPLC-PDA-MS^2 metabolites of anthocyanins in plasma collected after the ingestion of 180 g of blackberries by healthy human volunteers.

Peak	t_R	λ_{max}	$[M-H]^+$ *(m/z)*	MS^2 *(m/z)*	Anthocyanins
1	26.1	515	449	287	Cyanidin-3-*O*-glucoside
2	27.03	515	463	287	Cyanidin-*O*-glucuronide
3	31.13	515	463	301	Peonidin-*3*-O-glucoside
4	32.14	520	477	301	Peonidin-*O*-glucuronide
5	36.7	515	463	287	Cyanidin-*O*-glucuronide

t_R, retention time; $[M+H]^+$, positively charged molecular ion; MS^2 daughter ion produced by fragmentation of $[M+H]^+$.

Table 3-4: Pharmacokinetic analysis of anthocyanins detected in plasma of eight human subjects after the consumption of 180 g of blackberries.

peak	Anthocyanins	C_{max} (nmol/L)	t_{max} (h)	*t*1/2 (h)	AUC (nmol/L/h)	C_{max}/dose (nmol/L/µmol)	AUC/dose (nmol/L/µmol)
1	Cyanidin-3-*O*-glucoside	17 ± 2.4	0.9 ± 0.2	4.6 ± 0.9	45.04 ± 8.8	0.01 ± 0.001	0.03 ± 0.01
2	Cyanidin-*O*-glucuronide	5 ± 1	2.6 ± 0.3	18.2 ± 5.1	13.24 ± 3.8	0.003 ± 0.001	0.01 ± 0.002
3	Peonidin-3-*O*-glucoside	6 ± 2.3	1.3 ± 0.2	18 ± 8.2	1.9 ± 0.9	0.004 ± 0.001	0.001 ± 0.001
4	Peonidin-*O*-glucuronide	20 ± 2.4	2.0 ± 0.4	15 ± 6	93.6 ± 21.3	0.01 ± 0.001	0.1 ± 0.01
5	Cyanidin-*O*-glucuronide	5 ± 0.0	0.8 ± 0.2	1 ± 0.8	0.2 ± 0.0	0.003 ± 0.00	0.0001 ± 0.00

Data expressed as mean value ± standard error (n=8); C_{max}, maximum post-ingestion plasma concentration; t_{max}, time reach C_{max};*t*1/2, elimination half-life; AUC, area under the curve (0-8h).

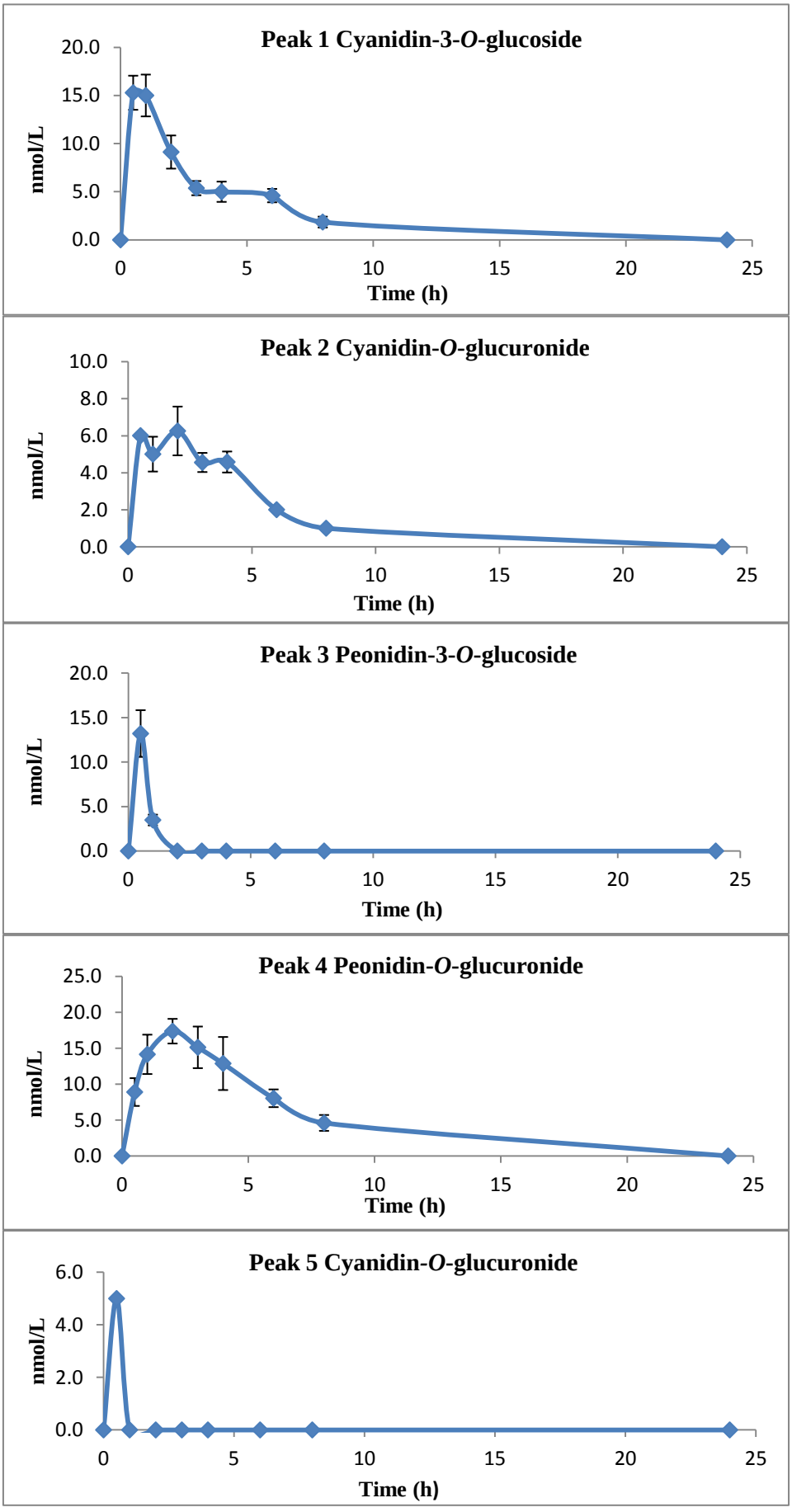


Figure 3-4: Pharmacokinetic profile of anthocyanins found in plasma of healthy volunteers (n=8), 0-24 h following acute intake of 180 g of blackberries.

3.3.3 Identification and quantification of anthocyanins in urine

Data on urinary excretion of anthocyanins after acute intake of 180 g of blackberries by healthy volunteers is presented in Table 3-5 while Figure 3-5 illustrates typical urinary HPLC profiles.

Excluding peak 1, the delphinidin-3-*O*-glucoside internal standard, no anthocyanins were observed in urine collected before the consumption of the blackberries. Seven anthocyanins were identified based on mass spectra data and cochromatography with authentic standards in the case of cyanindin-3-*O*-glucoside and peonidin-3-*O*-glucoside (3´-*O*-methyl-cyanidin-3-*O*-glucoside). Full-scan MS^2 and SRM were used to identify the cyanidin methyl and glucuronide metabolites as discussed in Section 3.3.2.

Unlike plasma, two methylated forms of cyanindin-3-*O*-glucoside were detected in urine at peak 3 and 4 which had a $[M\text{-}H]^+$ at *m/z* 463, which fragmented with a loss of 162 *amu* (glucose) to produce a *m/z* 301 (peonidin) daughter ion. To deduce the methylation site for these two peaks, they were cochromatographed with authentic peonidin-3-*O*-glucoside. The results clearly showed that peak 4 was peonidin-3-*O*-glucoside (3´-*O*-methyl-cyanidin-glucoside). Peak 3 had a shorter retention time and different absorbance spectrum (λ_{max}, 505 nm) from that of 3′-*O*-methyl-cyanidin-glucoside, indicating that the OH group at the 3′-position of the cyanidin B-ring was not the site of methylation. Although the methylation site of this metabolite remained unclear, it was possible to tentatively identify this peak based on the chromatographic behaviour of delphinidin-3-*O*-glucoside reported by Ichiyangi *et al*. (2004). The MS and NMR data showed clearly that the retention time of 4´-*O*-methyl-delphinidin-3-glucoside was shorter than that of 3´-*O*-methyl-delphinidin-3-glucoside (petunidin-3-*O*-glucoside). The same methylation pattern and chromatographic behaviour was observed with cyanidin-3-glucoside by (Ichiyanagi *et al.*, 2005b). Therefore, this peak 3 may be 4´-*O*-methyl-cyanidin-glucoside. In addition, based on the absorbance spectrum an unknown cyanidin derivative was detected (peak 7). This compound has $[M\text{-}H]^+$ at *m/z* 573 which fragmented with a loss of 286 *amu* yielding an m/z 287 daughter ion. However, no information was reported in the literature about this pigment.

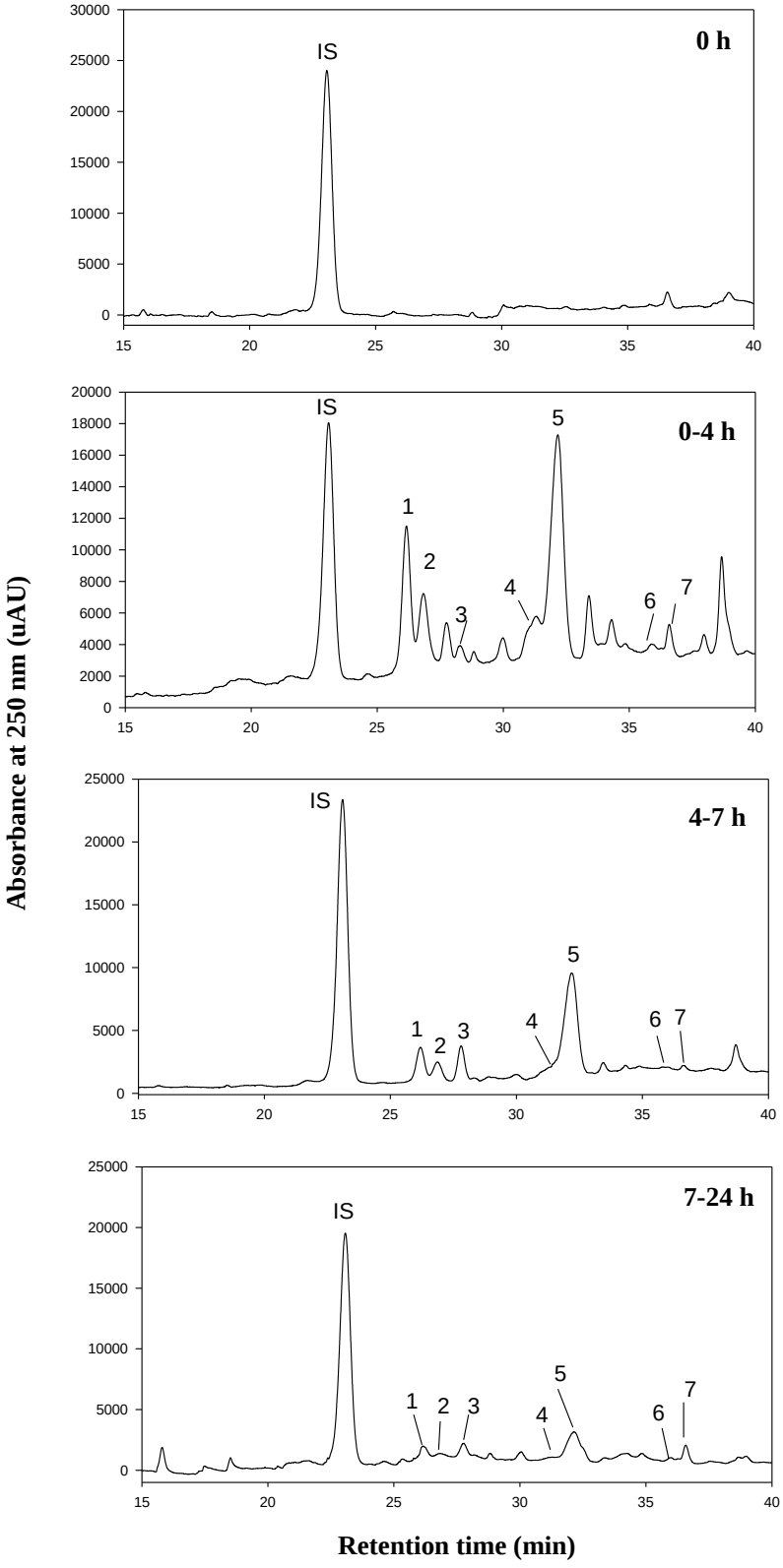


Figure 3-5: Changes on the anthocyanin profile of urine collected at 0h, 0-4h, 4-7h and 7-24h after ingestion of 180 g of blackberries by healthy volunteers. For numbered peak see Table 3-5. IS: internal standard delphinidin-3-*O*-glucoside.

Table 3-5: HPLC-PDA-MS^2 metabolites of anthocyanis in urine collected after the ingestion of 180 g of blackberries by healthy volunteers.

Peak	t_R	λ_{max}	$[M-H]^+$*(m/z)*	MS^2*(m/z)*	Anthocyanins
1	26.1	515	449	287	Cyanidin-3-*O*-glucoside
2	27.03	515	463	287	Cyanidin-*O*-glucuronide
3	27.7	505	463	301	4´-*O*-methyl-cyanidin-glucoside
4	31.13	515	463	301	3´-*O*-methyl-cyanidin-glucoside
5	32.1	520	477	301	Peonidin-*O*-glucuronide
6	36.7	515	463	287	Cyanidin-*O*-glucuronide
7	38.8	515	573	287	Unknown cyanidin

$t_{R,}$ retention time; $[M+H]^+$, positively charged molecular ion; MS^2 daughter ion produced by fragmentation of $[M+H]^+$.

Like the quantitative analysis of anthocyanins in plasma, the main anthocyanin which excreted in all urine samples was peak 5, peonidin-*O*-glucuronide, which was detected at all collection points for all the eight volunteers in quantities ranging from 19.5 to 144.5 nmoles. The total amount of anthocyanins excreted by the individual volunteers ranged from 102.8 to 289.8 nmoles which is equivalent to a 0.04% to 0.1% recovery of the total intake (Table 3-6).

The total mean urinary excretion of anthocyanins for healthy subjects over 24 h was low. The maximum excretion occurred between 0-4 h after the consumption of blackberries. Of the 294 µmol of anthocyanins ingested, only 206.5 nmole was detected in the 0-24 h urine which corresponds to 0.08% of the intake. The total excretion of the main metabolite, peonidin-*O*-glucuronide, was 84.7 ± 21.6 nmole, equivalent to a 0.03% recovery of the total anthocyanin intake (Table 3-7). The parent cyanidin-3-*O*-glucoside was detected in the urine of all volunteers' samples ranging from 16.4 to 63.3 nmoles with a total amount of 42 ± 8.6 nmole corresponding to 0.01% of the intake (Table 3-6 and Table 3-7).

Table 3-6: Quantities of anthocyanins and % excreted in urine collected from individual healthy volunteers 0-24 h after the consumption of 180 g of blackberries.

Subject	Anthocyanins	0-4 h	4-7 h	7-24 h	Total
1	Cyanidin-3-*O*-glucoside	22.6 ± 0.1	20.4 ± 0.4	2.12 ± 0.1	45.12 ± 0.6
	Cyanidin-*O*-glucuronide	13.8 ± 0.7	12 ± 0.7	n.q.	25.8 ± 1.4
	4´-*O*-methyl-cyanidin-glucoside	3.1 ± 0.2	12.2 ± 2	1.2 ± 0.1	16.5 ± 2.3
	3´-*O*-methyl-cyanidin-glucoside	5.9 ± 1	6.7 ± 0.2	5.4 ± 0.3	18 ± 1.5
	Peonidin-*O*-glucuronide	49.7 ± 0.2	90.7 ± 3.4	3.1 ± 0.4	143.5 ± 4
	Cyanidin-*O*-glucuronide	1.7 ± 0.1	2.5 ± 0.4	9.3 ± 0.3	13.5 ± 0.8
	Unknown cyanidin	2.8 ± 1.6	4.4 ± 0.0	20.2 ± 1	27.4 ± 2.6
	Total	99.6 ± 3.9	148.9 ± 7.1	41.3 ± 2.2	289.8 ± 13.2
	%	***(0.03 %)***	***(0.1 %)***	***(0.01 %)***	***(0.1 %)***
2	Cyanidin-3-*O*-glucoside	10.3 ± 4.1	4 ± 0.3	2.1 ± 0.01	16.4 ± 4.4
	Cyanidin-*O*-glucuronide	2.7 ± 0.6	0.8 ± 0.3	0.8 ± 0.2	4.3 ± 1.1
	4´-*O*-methyl-cyanidin-glucoside	6.8 ± 2.3	4.7 ± 0.8	5.5 ± 0.04	17 ± 3.1
	3´-*O*-methyl-cyanidin-glucoside	3.2 ± 0.7	0.9 ± 0.1	n.d.	4.1 ± 0.8
	Peonidin-*O*-glucuronide	13.01 ± 2.3	6.5 ± 0.4	n.d.	19.5 ± 2.7
	Cyanidin-*O*-glucuronide	1.2 ± 0.14	1.3 ± 0.4	8.8 ± 3.9	11.3 ± 4.4
	Unknown cyanidin	13.7 ± 4.3	10.7 ± 0.9	5.8 ± 2	30.2 ± 7.2
	Total	50.9 ± 14.4	28.9 ± 3.2	23 ± 6.2	102.8 ± 23.7
	%	***(0.02 %)***	***(0.01 %)***	***(0.01 %)***	***(0.04 %)***
3	Cyanidin-3-*O*-glucoside	26.3 ± 1.1	10.7 ± 3.4	5.2 ± 1.3	42.2 ± 5.8
	Cyanidin-*O*-glucuronide	14 ± 0.6	6.8 ± 0.4	n.q.	20.8 ± 1
	4´-*O*-methyl-cyanidin-glucoside	5.5 ± 1	9.7 ± 0.3	5.3 ± 2.4	20.5 ± 3.7
	3´-*O*-methyl-cyanidin-glucoside	5.03 ± 0.6	5.7 ± 1.2	n.q.	10.7 ± 1.8
	Peonidin-*O*-glucuronide	52.4 ± 6.2	44.9 ± 0.3	25.4 ± 6.0	122.7 ± 12.5
	Cyanidin-*O*-glucuronide	2.3 ± 0.2	2 ± 0.3	6.5 ± 0.3	10.8 ± 0.8
	Unknown cyanidin	13.4 ± 1.4	7.2 ± 1.1	4 ± 0.0	24.6 ± 2.5
	Total	118.9 ± 11.1	87 ± 7	46.4 ± 10	252.3 ± 28.1
	%	***(0.04 %)***	***(0.03 %)***	***(0.02 %)***	***(0.1 %)***
4	Cyanidin-3-*O*-glucoside	49.5 ± 6.1	9.1 ± 0.2	5.2 ± 0.7	43.8 ± 7
	Cyanidin-*O*-glucuronide	14 ± 5	2.4 ± 0.7	n.d.	16.4 ± 5.7
	4´-*O*-methyl-cyanidin-glucoside	10.6 ± 0.3	6.1 ± 0.4	7.1 ± 1.6	23.8 ± 2.3
	3´-*O*-methyl-cyanidin-glucoside	8.6 ± 2.5	1.7 ± 0.03	n.d.	20.6 ± 2.5
	Peonidin-*O*-glucuronide	50 ± 2.4	19.7 ± 2.3	14.9 ± 4.5	84.6 ± 9.2
	Cyanidin-*O*-glucuronide	n.q.	n.q	4.9 ± 1.6	4.9 ± 1.6
	Unknown cyanidin	18.8 ± 1.7	4.6 ± 0	n.q.	23.4 ± 1.7
	Total	151.5 ± 18	43.6 ± 3.6	32.1 ± 8.4	219.6 ± 30.1
	%	***(0.05%)***	***(0.02 %)***	***(0.01 %)***	***(0.1 %)***

Subject	Anthocyanins	0-4 h	4-7 h	7-24 h	Total
5	Cyanidin-3-*O*-glucoside	26.3 ± 28.4	8.4 ± 0.2	3.8 ± 0.0	38.5 ± 28.6
	Cyanidin-*O*-glucuronide	5.4 ± 0.03	n.q.	n.d.	5.4 ± 0.03
	4´-*O*-methyl-cyanidin-glucoside	2.7 ± 0.5	1.8 ± 0.4	n.q.	4.5 ± 0.9
	3´-*O*-methyl-cyanidin-glucoside	10.6 ± 0.1	2.6 ± 0.2	n.d.	13.2 ± 0.3
	Peonidin-*O*-glucuronide	15.8 ± 0.7	6.4 ± 1.4	n.q.	22.2 ± 2.1
	Cyanidin-*O*-glucuronide	1.6 ± 0.1	1.8 ± 0.2	8.6 ± 0.2	12 ± 0.5
	Unknown cyanidin	8.9 ± 2.6	6.3 ± 1.6	3.5 ± 0.5	18.7 ± 4.7
	Total	50.9 ± 24.94	27.9 ± 7.1	41.3 ± 2.2	114.5 ± 37.1
	%	***(0.02 %)***	***(0.01 %)***	***(0.01 %)***	***(0.1 %)***
6	Cyanidin-3-*O*-glucoside	20.8 ± 1.6	4.9 ± 0.01	n.q.	25.7 ± 1.6
	Cyanidin-*O*-glucuronide	16.3 ± 1.2	2.9 ± 0.02	n.q.	19.2 ± 1.2
	4´-*O*-methyl-cyanidin-glucoside	8.3 ± 0.5	5.8 ± 0.4	6.5 ± 0.5	20.6 ± 1.4
	3´-*O*-methyl-cyanidin-glucoside	5.8 ± 1.1	n.d.	n.d.	5.8 ± 1.1
	Peonidin-*O*-glucuronide	92 ± 5.2	30.7 ± 1.3	23.5 ± 1.6	146.2 ± 8.1
	Cyanidin-*O*-glucuronide	1.6 ± 0.1	1.7 ± 0.3	4.8 ± 0.3	8.1 ± 0.7
	Unknown cyanidin	19.5 ± 8.3	5.3 ± 1.1	n.q.	24.8 ± 9.4
	Total	164.3 ± 18	51.3 ± 3.1	34.8 ± 2.4	250.4 ± 23.5
	%	***(0.06%)***	***(0.02 %)***	***(0.01 %)***	***(0.1 %)***
7	Cyanidin-3-*O*-glucoside	32.3 ± 1.1	6.4 ± 1.4	2.4 ± 0.4	41.1 ± 2.9
	Cyanidin-*O*-glucuronide	19.5 ± 0.2	4.4 ± 1.4	2.7 ± 0.5	26.6 ± 2.1
	4´-*O*-methyl-cyanidin-glucoside	16.4 ± 0.9	11.5 ± 1.5	7.7 ± 0.4	35.6 ± 2.8
	3´-*O*-methyl-cyanidin-glucoside	4.3 ± 0.5	1.3 ± 0.0	3.7 ± 0.0	9.3 ± 0.5
	Peonidin-*O*-glucuronide	87.2 ± 0.1	40.5 ± 4.8	16.8 ± 0.6	144.5 ± 5.5
	Cyanidin-*O*-glucuronide	n.q.	n.q.	n.q.	n.q.
	Unknown cyanidin	20.1 ± 6.3	7.4 ± 0.0	2.4 ± 0.0	29.9 ± 6.3
	Total	179.8 ± 9.1	71.5 ± 9.1	34.7 ± 1.9	287 ± 20.1
	%	***(0.06 %)***	***(0.02 %)***	***(0.01 %)***	***(0.1 %)***
8	Cyanidin-3-*O*-glucoside	16.7 ± 0.13	39.5 ± 1.2	7.1 ± 0.3	63.3 ± 1.63
	Cyanidin-*O*-glucuronide	5.8 ± 0.3	11 ± 0.13	10.5 ± 1.5	27.3 ± 1.93
	4´-*O*-methyl-cyanidin-glucoside	n.q.	3.2 ± 0.0	n.q.	3.2 ± 0.0
	3´-*O*-methyl-cyanidin-glucoside	n.q.	5.4 ± 0.3	n.q.	5.4 ± 0.3
	Peonidin-*O*-glucuronide	10.2 ± 0.3	29 ± 0.3	n.q.	39.2 ± 0.6
	Cyanidin-*O*-glucuronide	1.2 ± 0.1	2.5 ± 0.1	8.6 ± 0.7	12.3 ± 0.9
	Unknown cyanidin	6.6 ± 0.5	13.5 ± 4.7	7.4 ± 1.1	27.5 ± 6.3
	Total	40.5 ± 1.3	104.1 ± 6.7	33.6 ± 3.6	178.2 ± 11.6
	%	***(0.01 %)***	***(0.04%)***	***(0.01 %)***	***(0.1 %)***

* Data for individual subjects are expressed as nmoles ± standard error (n=8). Figures in italics represent the percentage of the total amount of anthocyanins ingested. n.d.- not detected. n.q. trace levels not quantified.

Table 3-7: Quantities of anthocyanins and % excreted in urine collected from eight healthy volunteers 0-24 h after the consumption of 180 g of blackberries.

Peak	Anthocyanins	0-4 h	4-7 h	7-24 h	Total
1	Cyanidin-3-*O*-glucoside	25.6 ± 3.9	12.9 ± 4.0	3.5 ± 0.8	42.0 ± 8.6 ***(0.01%)***
2	Cyanidin-*O*-glucuronide	11.5 ± 2.0	5.0 ± 1.5	2.0 ± 1.3	18.5 ± 4.8 ***(0.01%)***
3	3´-*O*-methyl-cyanidin-glucoside	6.7 ± 1.7	6.9 ± 1.3	4.1 ± 1.1	17.7 ± 4.1***(0.01%)***
4	4´-*O*-methyl-cyanidin-glucoside	5.4 ± 1.1	3.0 ± 0.8	1.1 ± 0.7	9.6 ± 2.6 ***(0.01%)***
5	Peonidin-*O*-glucuronide	41.0 ± 8.8	33.2 ± 9.2	10.5 ± 3.6	84.7 ± 21.6 ***(0.03%)***
6	Cyanidin-*O*-glucuronide	1.2 ± 0.3	1.5 ± 0.3	6.4 ± 1.1	9.1 ± 1.7 ***(0.003%)***
7	Unknown cyanidin	12.9 ± 2.1	6.9 ± 1.4	5.0 ± 2.3	24.8 ± 5.8 ***(0.01%)***
	Total	104.4 ± 19.9	69.5 ± 18.4	23.7 ± 10.8	206.5 ± 49.2
	%	***(0.04%)***	***(0.02%)***	***(0.01%)***	***(0.08%)***

* Data presented as mean values in nmoles ± standard error (n=8 volunteers) and in italicised parentheses as percentage of the individual amounts of anthocyanins ingested.

3.4 Discussion

Although anthocyanins have been reported to promote health and prevent many chronic diseases, as yet there is a lack of information relating to their absorption, metabolism and the mechanisms involving absorption and transport of these pigments in the human body. To achieve a biological effect in a certain organ or tissue, these potentially active components in a daily diet must be bioavailable to be effectively absorbed, transported into the circulation system and delivered to target sites in a form with *in vivo* bioactivity.

Earlier bioavailability studies considered that anthocyanins were not absorbed into human body unless first hydrolyzed to an aglycone structure in the gastrointestinal tract (Wu *et al.*, 2002b). Horwitt, (1933), was perhaps the first to observe anthocyanins in the urine after feeding rats with Concord grapes. Morazzoni *et al.*, (1991) reported that anthocyanins were present in the plasma of rats 15 mins after ingestion of a mixture of 15 anthocyanins. Subsequently, several papers reported that parent anthocyanin glycosides, rather than their metabolites were detected in the plasma and urine of both human and animals models (Miyazawa *et al.*, 1999; Cao *et al.*, 2001; Matsumoto *et al.*, 2001; Felgines *et al.*, 2002; Milbury *et al.*, 2002; Mulleder *et al.*, 2002; Wu *et al.*, 2002b).

These findings led to the suggestion that the flavylium cation structure of anthocyanins is resistant to enzymatic hydrolysis, preventing conversion to the aglycone and subsequent sulphation, methylation and/or glucuronidation. In (1999) Miyazawa and his colleagues detected a trace amount of methylated cyanidin, peonidin-3-*O*-glucoside, in the liver of rats fed with cyanidin mono- and di-glucosides. Recent published reports about the bioavailability of anthocyanins in both humans and animals showed clearly that anthocyanins can be absorbed intact and can be excreted as glucuronide and/or methylated, sulphated conjugates (Matsumoto *et al.,* 2001; Felgines *et al.,* 2002; Felgines *et al.,* 2003; McGhie *et al.,* 2003; Borges *et al.,* 2004; Felgines *et al.,* 2005; Ichiyanagi *et al.,* 2005a; Kay *et al.,* 2005; Felgines *et al.,* 2006).

The purpose of this study was to evaluate only the bioavailability of cyanidin-3-*O*-glucoside by monitoring its post-ingestion absorption and metabolism in the human body and obtain insights into the effect of B-ring hydroxylation on anthocyanin bioavailability. A necessary step was to determine the anthocyanins content of blackberries. Cyanidin-3-*O*-glucoside was the major anthocyanin in blackberries contributing 63% of the 294 μmoles/180 g intake. However, several other glycoside and acylated anthocyanins were present in trace amounts. The presence of these minor anthocyanins can vary depending on species and cultivars (Kaume *et al.,* 2012).

In the present investigation, no anthocyanins were identified in the plasma or in the urine before the ingestion of blackberries by volunteers suggesting that the washout overnight and dietary exclusions were adequate. Glucuronidation and methylation were the major metabolic pathways for cyanidin-3-*O*-glucoside. The results showed clearly that cyanidin-3-*O*-glucoside was metabolised primarily to peonidin-*O*-glucuronide, a conversion that requires deglycosylation, glucuronidation and methylation steps. It is this metabolite rather than the parent cyanidin-3-*O*-glucoside that was the predominant anthocyanin in both plasma and urine samples. After ingestion of blackberries, this metabolite appeared in the bloodstream of all volunteers in low concentrations reaching a mean C_{max} of 20 ± 2.4 nmol/L after 2.0 ± 0.4 h. The total urinary excretion of this metabolite was 84.7 ± 22.6 nmoles which is 0.03% of the intake.

Monoglucuronides of cyanidin were also detected in both plasma and urine as minor metabolites. In plasma, two cyanidin-*O*-glucuronide (peaks 2 and 5) with similar low C_{max} but different t_{max} values were detected. Peak 5 reached the bloodstream faster within 30 mins of ingestion of the blackberry, with a t_{max} value of less than 1 h and $t_{1/2}$ of 1 h. The total urinary percentage of the excretion of the two cyanidin glucuronides was very low, equivalent to 0.01 and 0.003% of intake, respectively. As discussed, the exact sites of cyanidin-glucuronidation have not been clearly reported (Mullen *et al.,* 2006; Mullen *et al.,* 2008b). The conjugation with glucuronic acid is considered to be an important step in the detoxication pathway for many drugs and xenobiotic substances. This process is catalyzed by UDP-glucuronosyl transferase in the small intestine, liver and/or kidney (Wu *et al.,* 2002b; Kaume *et al.,* 2012).

Methylation at the 3'-OH moiety of cyanidin-3-*O*-glucoside has been largely reported in both animal and human studies (Wu *et al.,* 2002b; Wu *et al.,* 2004b; Felgines *et al.,* 2005; Ichiyanagi *et al.,* 2005a; Ichiyanagi *et al.,* 2005b). An earlier study by Miyazawa *et al.,* (1999) showed that the concentration of the methylated form of anthocyanin in the liver of rats was much higher than the parent anthocyanin, but they did not detect it in plasma and urine. A similar observation was obtained by Talavera *et al.*, (2003) in an animal feeding study with blackberries, where they found peonidin-3-glucoside as a major anthocyanin recovered in bile. However, they could not detect this metabolite in plasma from the abdominal aorta of the rats. This led them to assume that these methylated compounds were excreted directly from the liver into the bile rather than distributed into blood. However, in this current study, methylated forms were detected both in plasma and urine. Thus, anthocyanins were absorbed and enter the circulation system quickly and are then subjected to methylation in the liver prior to urinary excretion.

Tian and colleagues (2006) found that methylation can occur at both the 3´- and 4´- hydroxyl groups of the triglycoside, cyanidin-3-xylosylrutinoside, whereas mono- or diglycosides gave rise to only one methylated derivative due to their low concentration in black raspberries. The appearance of two methylated derivatives of cyanidin as 3´-*O*-methyl and 4´-*O*-methyl esters in the urine was also documented by Wu *et al.,* (2004b) and Ichiyanagi *et al.,* (2005b). The

analysis of urine samples in the present investigation indicates similar results with *O*-methylation of cyanidin probably occurring at 3´- and 4´-positions of the B-ring (peak 3 and 4, Table 3-5). The methylation site of these metabolites was confirmed by co-chromatography with authentic peonidin-3-*O*-glucoside (C-3´in the B-ring) for peak 4 and by retention time for peak 3 (C-4´) (Ichiyanagi *et al.*, 2005b). However, in plasma only the 3´-methylated derivative, peonidin-3-*O*-glucoside, was detected. This is in agreement with previous studies showing that only 3´-OH methylated cyanidin occurs in plasma (Wu *et al.*, 2002b; Kay *et al.*, 2005; Felgines *et al.*, 2008; Vanzo *et al.*, 2011). However, it is important to note that generally the detection of many of these metabolites, especially in the plasma, with the current instrumentation and methods were near the limits of detection and the concentration of some of these intermediates may be so low that they are undetectable.

The exact pathway that led to the formation of anthocyanidin monoglucuronide is still unknown. Two possible pathways could explain the formation of monoglucuronide of cyanidin and cyanidin-3-*O*-glucoside (See Figure 3-6). One possible pathway is that cyanidin-3-*O*-glucoside was absorbed intact and then partly methylated to form peonidin-3-*O*-glucoside in the liver probably by catechol-*O*-methyltransferase (COMT). Then, either cyanidin-3-*O*-glucoside or peonidin-3-*O*-glucoside could serve as substrates for UDP-glucose dehydrogenase to form the corresponding glucuronide from the glucose form.

Another possible route is that cyanidin-3-*O*-glucoside is cleaved by ß-glucosidase in the small intestine to release cyanidin. The released aglycone might be subjected to glucuronidation by UDP glucuronyltransferase in the intestinal tract to form a cyanidin-*O*-glucuronide which may be taken up in the plasma for further metabolism to form peonidin-*O*-glucuronide by COMT in the liver. However, no aglycone was detected in any urine or plasma samples in this study as anthocyanins aglycones are generally considered as unstable at physiological pH (Wu *et al.*, 2002b; Felgines *et al.*, 2003; Ichiyanagi *et al.*, 2005b; Galvano *et al.*, 2007).

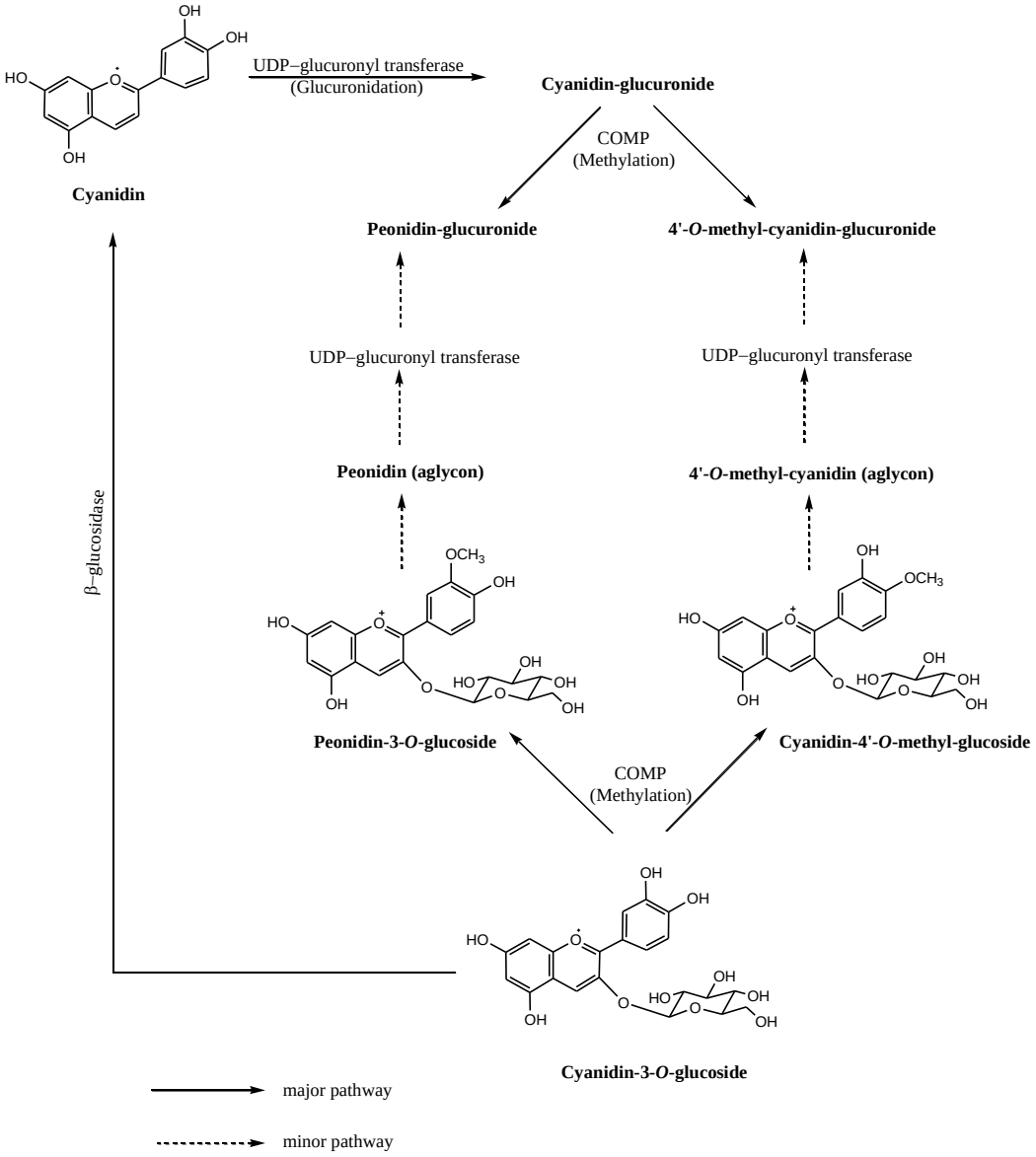


Figure 3-6. Possible pathway of cyanidin-3-*O*-glucoside metabolism (Adapted from Ichiyanagi *et al.,* 2005b).

Cyanidin-3-*O*-glucoside was also absorbed intact and appeared rapidly in the plasma reaching a C_{max} of 17 ± 2.4 nmol/L within less than one hour of the ingestion of blackberries (Figure 3-4, Table 3-4). Although some argue that anthocyanins cannot be absorbed into cells of animals and humans due to their hydrophilic nature (Hollman 2001), there are other studies that support this pathway for compounds such as cyanidin-3-*O*-glucoside (Passamonti *et al.,* 2002; Passamonti *et al.,* 2003; Ziberna *et al.,* 2012). The rapid appearance of cyanidin-3-*O*-glucoside in plasma could result in part from absorption through the gastric wall of the stomach. The acidity of the gastric contents would provide a favourable environment for the stability of anthocyanins. The evidence of anthocyanin absorption from the stomach has been demonstrated using in situ gastric exposure to anthocyanins (Passamonti *et al.,* 2003; Talavera *et al.,* 2003).However, the examination of plasma from the gastric veins of rats after *in situ* administration of blackberry anthocyanins by Talavera *et al.,* (2003) did not reveal the presence of anthocyanins metabolites, which suggests that unmetabolised anthocyanins are absorbed through the gastric wall.

In the current study, the excretion of unmetabolised cyanidin-3-*O*-glucoside in the urine was low, comprising only 20% of total anthocyanin excretion. Most anthocyanins were glucuronide (54%) and methylated (13%) metabolites. These findings were also highlighted by Borges *et al.,* (2007) and Felgines *et al.,* (2005). Other studies have detected proportionally higher excretion of cyanidin-3-*O*-glucoside but this could be a consequence of the higher dose consumed when compared to more normal dietary intakes (Matsumoto *et al.,* 2001; Felgines *et al.,* 2002; Kay *et al.,* 2004).

None of the other glucosides and acylated forms of cyanidin, identified in blackberries (peaks 2, 3, 5-9, Table 3-2), were detected in either plasma or urine samples in the present study. This differs from some of the mono/di-glucoside anthocyanins, such as cyanidin-3-*O*-arabinoside and cyanidin-3-*O*-sambubioside, which have been reported in low concentrations in plasma and/or urine in several bioavailability studies (Cao *et al.,* 2001; Milbury *et al.,* 2002; Mulleder *et al.,* 2002; Kay *et al.,* 2005; Lehtonen *et al.,* 2009). In these studies, cyanidin-3-*O*-arabinoside and cyanidin-3-*O*-sambubioside were the main components of the anthocyanins intake, whereas in the current study the low

intake of these compounds in blackberries could explain their absence in urine and plasma.

Acetylated anthocyanins are more stable than nonacylated anthocyanins (Giusti and Wrolstad 2003). However, studies have suggested that acylation of anthocyanins can significantly affect absorption. Recovery of acylated anthocyanins was 11 to 14-fold lower in urine and 8 to 10-fold lower in plasma compared to nonacylated anthocyanins in a human study with purple carrots (Kurilich *et al.,* 2005). Similarly, nonacylated anthocyanins from red cabbage were found to be four times more bioavailable than acylated anthocyanins (Charron *et al.,* 2007). In the current study acylated anthocyanins in blackberries were approximately 19% of the total intake and were not detected in either plasma or urine. This is similar to the findings of Mazza *et al.,* (2002) who were unable to quantify acylated anthocyanins in the serum of subjects after consumption of blueberries while many nonacylated anthocyanins were measurable. No clear explanation for the disappearance or lower recovery of acylated anthocyanins has been reported to date. It is possible those acylated anthocyanins are converted to their nonacylated counterparts or that the low concentration of these compounds in the fruits and food could affect their bioavailability. However, Kurilich *et al.,* (2005) in a feeding study with raw and cooked purple carrots could not prove any of these possibilities and concluded that large dose size significantly reduced recovery of both acylated and nonacylated anthocyanins, suggesting saturation of absorption mechanisms.

Generally, the total urinary excretion of cyanidin metabolites over 24 h was 206.5 ± 49.2 nmol/L accounting for 0.08 % of the initial dose. This observation supports the conclusion from other studies, which demonstrated urinary excretions ranging from 0.018 to 0.37 % (Galvano *et al.,* 2004; Galvano *et al.,* 2007). Felgines *et al.,* (2005) had also indicated the same low urinary excretion value of cyanidin-3-*O*-glucoside with 0.16% recovery after human subjects were fed 200 g of blackberries. They also highlighted that the metabolic fate of anthocyanins may differ according to anthocyanins aglycone structure based on a comparison of anthocyanins from blackberries and strawberries (Felgines *et al.,* 2003). They found that pelargonidin-3-*O*-glucoside from strawberries was more bioavailable than cyanidin-3-*O*-glucoside. They reported urinary recovery from pelargonidin-3-*O*-glucoside corresponding to 1.80% of the intake from 200 g of

strawberries. While still low it is >10-fold higher than the 0.16% recovery of cyanidin-3-*O*-glucoside observed in other studies. A similar conclusion can be drawn by comparing the urine recovery of cyanindin-3-*O*-glucoside in the current study with data obtained in a similar study design by our group with pelargonidin-3-*O*-glucoside in strawberries. Urinary recovery of cyanindin-3-*O*-glucoside was more than 9-fold lower (Mullen *et al.,* 2008b).

The different recoveries of these two anthocyanins in these reports clearly indicate that the chemical structure of cyanidin and pelargonidin have a marked influence on bioavailability because both compounds have the same conjugated glucose moiety. The low urinary recovery of cyanidin-3-*O*-glucoside compared to pelargonidin-3-*O*-glucoside could be due to the lower stability at physiological pH or from a lower rate of absorption from the gastrointestinal tract due to the limited ability of hydrolyzing enzymes to cleave cyanidin aglycone which will be subjected to glucuronidation in the small intestine. This observation supports the findings of McGhie *et al.,* (2003), which demonstrated that delphinidin-based anthocyanins were excreted in lower amounts than their malvidin counterparts. They concluded that this behaviour may result from either the greater number of hydroxyl groups in delphinidin and/or the more hydrophobic nature of malvidin which may facilitate more access into cells and tissues.

Moreover, the plasma data obtained in the current investigation also provided for the first time further comparative evidence of the bioavailability of these two different anthocyanins in humans. The C_{max} value for the main plasma metabolite in the blackberry feed was peonidin-*O*-glucuronide at 20 nmol/L. This value is an order of magnitude lower than the 274 nmol/L C_{max} of a pelargonidin-*O*-glucuronide obtained in the strawberry feeding study (Mullen *et al.,* 2008b).

Pelargonidin, which has only a 4` hydroxyl group in the B-ring, is not methylated and may be easily conjugated with glucuronic acid and sulfate. As previously reported after strawberry pelargonidin-3-*O*-glucoside consumption, glucuronidated conjugates were the main metabolites detected in both urine and plasma with minor quantities of a pelargonidin-*O*-sulfate as well as the parent pelargonidin glucoside and trace amounts of its aglycone pelargonidin (Felgines *et al.,* 2003; Mullen *et al.,* 2008b). In the current study, glucuronide and methylated conjugates of cyanidin were the major metabolites detected in

plasma and urine. Methylation and/or glucuronidation can occur with cyanidin as it has two B-ring hydroxyl groups. However, surprisingly no sulfate conjugates were produced from cyanidin-3-*O*-glucoside and likewise there was an absence of cyanidin.

Another important factor that may affect the bioavailability of these anthocyanins is the analytical issues which have been reported as possible contributor to the different level of anthocyanins recovery. Most bioavailability studies focused on only anthocyanins metabolites that have an anthocyanin skeleton and are detected at 524 nm. Any colorless forms or any degradation products, due to their chemical instability at physiological pH, would not be readily detected (see Chapter 1).

It has been shown that anthocyanins can be extensively transformed to phenolic acids though gut microflora-mediated C-ring fission in the colon (Aura *et al.*, 2005; Gonzalez-Barrio *et al.*, 2011). Support for this view was initially provided by Tsuda *et al.*, (1999) who reported the presence of protocatechuic acid (3,4-dihydroxybenzoic acid) in rats plasma in concentrations ~8-fold greater than that of cyanidin-3-*O*-glucoside. Protocatechuic acid was also found as a major metabolite of cyanidin-3-*O*-glucoside in humans, accounting for 44.4% of the ingested anthocyanin in plasma and 28% in faeces although it was not detected in urine (Vitaglione *et al.*, 2007). In the present investigation, no attempt was made to investigate the appearance of protocatechuic acid in plasma because of the lack of sensitivity of the HPLC-MS to protocatechuic acid and other phenolic compounds. There are, however, a large number of phenolic and aromatic acids that potentially could be formed from the degradation of anthocyanins in the gastrointestinal tract. Further studies involving anthocyanins labelled at several positions with a stable isotope such as ^{13}C are necessary for the complete understanding of anthocyanins bioavailability. The synthesis of such labelled anthocyanins has recently been reported (Zhang *et al.*, 2011).

3.5 Conclusion

Eight healthy volunteers were fed 180 g of blackberries containing 294 μmoles of anthocyanins. After the ingestion of blackberries, cyanidin-3-*O*-glucoside bioavailability was evaluated by urinary excretion and plasma concentrations of anthocyanins over 24 h when analysed by HPLC-PDA-MS^2. Nine anthocyanins were detected in blackberries, five in plasma and seven in urine. Plasma C_{max} values were low for all the metabolites ranging from 5-20 nmol/L. The mean t_{max} was ~1.3 h indicating that after ingestion cyanidin-3-*O*-glucoside was quickly absorbed and transported into the circulatory system. Urinary excretion was low with ~0.08% of the total intake being recovered within 24 h. Maximal urinary excretion of cyanidin was achieved in 0-4 h with a 0.04% recovery of intake.

Formation of monoglucuronides of cyanidin and its methylated derivative peonidin as well as intact form of cyanidin have been observed in both plasma and urine samples. However, cyanidin-3-*O*-glucoside was metabolised principally to peonidin-*O*-glucuronide, a conversion that required deglucosylation, glucuronidation and methylation steps. The absorption and excretion of the unmetabolised cyanidin-3-*O*-glucoside was also compared to its peonidin metabolite. Glucuronide and methylated conjugates were the major metabolites to be excreted in urine. Metabolites in urine had been subjected to methylation at either the 3´ or 4´position of the catechol unit of the B-ring of cyanidin while in plasma only the 3´methylation was detected.

The study also showed the effect of the 3'-hydroxylation on the absorption and metabolism. Although this study with blackberries used almost the same total of anthocyanins as our previous study with strawberries, the amount of anthocyanins absorbed/excreted in plasma and urine samples from volunteers who consumed the blackberries was much lower. Differences in the aglycone chemical structure influence the absorption and the metabolism of cyanidin and pelargonidin glucosides.

In this chapter time constraints prevented the investigation of the bioavailability of pelargonidin glucoside in the plasma and urine of healthy volunteers in parallel with the cyanidin glucoside study. However, in the following chapter the

same study design will be used to generate more information about cyanidin and pelargonidin glucoside bioavailability from urine and ileal fluid of ileostomy volunteers collected over a 24 h period post-ingestion. Absorption, metabolism and excretion of these anthocyanins will be discussed as well as the effect of 3'-hydroxylation on their bioavailability in a comparable study.

4. Bioavailability of anthocyanins following consumption of blackberries and strawberries by ileostomy subjects

4.1 Introduction

The aim of studying the bioavailability of a nutrient is to obtain information on the proportion of the prospective bioactive compound that is absorbed in the gastrointestinal tract including the metabolism, excretion and distribution in the circulation system (McGhie and Walton 2007). As was discussed in the previous chapter, cyanidin-3-*O*-glucoside is the predominant anthocyanin in blackberries. Monitoring the bioavailability of this compound in healthy subjects has shown its rapid appearance in the circulatory system and excretion in urine. However, there are further questions about the absorption and metabolism of this compound from the small intestine that require further investigation.

Bioavailability studies investigating the intestinal absorption and metabolism of various anthocyanins have been performed using an in situ intestinal perfusion model in rats (Talavera *et al.,* 2004; He *et al.,* 2009). However, animal models such as rats may metabolize anthocyanins differently from humans. For instance, the degradation product of cyanidin glucoside, protocatechuic acid, was reported to be stable in human plasma for up to 24h where it decomposed rapidly in mouse plasma (Chen *et al.,* 2012). Therefore, using a healthy subject with an ileostomy as well as subjects with an intact functioning colon, offers valuable information in studying the bioavailability and metabolism of dietary anthocyanins especially in the small intestine. However, published studies on the use of ileostomists in anthocyanin bioavailability research are limited (Kahle *et al.,* 2006; Gonzalez-Barrio *et al.,* 2010; Kraus *et al.,* 2010).

Comparing the intake of anthocyanins with the content in ileal fluid will help to assess metabolism and the absorption occurring in the small intestine from a different perspective to that gained from plasma pharmacokinetics and urine excretion by healthy subjects with an intact functioning colon. The

analysis of ileal fluid provides information on the compounds, which in healthy volunteers will pass from the small to large intestine where they have the potential to impact on colonic health and will also be subject to degradation to phenolic acids by the colonic microflora prior to absorption or excretion.

As noted in Chapter 3, pelargonidin-3-*O*-glucoside appears to be the most bioavailable of the anthocyanins with a 1.8% recovery in urine compared to ~0.1% for other anthocyanins (Felgines *et al.*, 2003). However, few human studies have dealt specifically with pelargonidin-3-*O*-glucoside bioavailability and there is still no investigation that has been designed to directly compare the absorption and excretion of this compound with other anthocyanins in human subjects at the same time.

Like cyanidin-3-*O*-glucoside in blackberries, strawberries represent an excellent food source to study the bioavailability of pelargonidin-3-*O*-glucoside as it is the single predominant anthocyanin in the fruit. Therefore, strawberries and blackberries were chosen to evaluate the absorption and metabolism of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside from the small intestine in ileostomy volunteers. The information that will be gained from this study and the previous study with healthy volunteers in chapter 3 will provide additional information about the bioavailability of these anthocyanins.

4.2 Materials and methods

4.2.1 Chemicals and reagents

HPLC grade methanol was obtained from Rathburn chemicals Ltd., (Walkerburn, UK). Formic acid and sodium diethyldiothiocarbamate was supplied by Sigma (Poole, Dorset, UK). Cyanidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside were supplied by Extrasynthes (Genay, France). Strawberries, purchased fresh from a local Marks and Spencer supermarket, were defrosted prior to use.

4.2.2 Preparation of blackberry and strawberry extract

Strawberries were homogenized using an Ultra-Turrax homogenizer at 24000 rpm then frozen at -20 ºC for the feeding study and for the extraction. Ten grams of frozen strawberries and blackberries were extracted as described in Chapter 3.

4.2.3 Study design

The study protocol was approved by the Glasgow Royal Infirmary Local Research Ethics Committee (REC reference number: 12/WS/0192). The participants' criteria were to be in good health, non-smokers, not pregnant and have had an ileostomy. Five ileostomy subjects (three males, two females) gave their written consent and participated in this study. They were aged between 45 to 74 years with an average height of (1.7 m), average weight of (75 kg) and a mean body mass index of (25 kg/m). Subjects were required to follow a diet low in flavonoid and phenolic compounds for two days prior to the study, which excluded fruits and vegetables and beverages such as tea, coffee, fruit juices, and wine, whole grains, nuts, chocolate and dietary antioxidants supplements. On the morning of the study after an overnight fast, the volunteers consumed 180 g of blackberries and strawberries on separate occasions with an interval of one month. All urine excreted for 24 h, as well as ileal fluid contained in the ileostomy bag, over the periods 0-4, 4-7 and 7-24 h were collected. The volumes of urine and weights of ileal fluid samples were measured and aliquots were acidified to pH 3 with 50% aqueous formic acid before being stored at -80ºC prior to analyses.

4.2.4 Analysis of anthocyanins in biological fluids

4.2.4.1 Extraction of anthocyanins from ileal fluids

Ileal fluid was defrosted and 2 µg of delphinidin-3-*O*-glucoside added as an internal standard to triplicate 0.5 g samples, which were homogenized in 3 mL of 1% formic acid in 50% aqueous methanol containing 20 mM sodium diethyldiothiocarbamate for 1 min at 24000 rpm using an Ultra-Turrax

homogenizer, after which they were centrifuged for 20 mins at 4600g at 4ºC. The supernatant was collected and the pellets were re-extracted using a solution of 1% formic acid in methanol, containing 20 mM sodium diethyldiothiocarbamate. The two supernatants were pooled and reduced to dryness in a centrifugal vacuum concentrator Speedvac Concentrator SPD131DDA (Thermo Electon Crop., San Jose, CA) at 45°C. The residues were resuspended in 50 µL of 1% acidified methanol and 450 µL of 1% formic acid, and aliquots of 100 µL were analyzed by HPLC-PDA-MS^2. Recoveries of delphinidin-3-*O*-glucoside, the internal standard, were typically 62.8 ± 5.5 % for the blackberries study and 55.2 ± 4.0 for the strawberries study.

4.2.4.2 Extraction of anthocyanins from urine

The urine samples from the blackberries feeding study were extracted using a solid-phase extraction procedure as described in chapter 3. Aliquots of 50 µL were analyzed by HPLC-PDA-MS^2 on the day of extraction. Recoveries of delphinidin-3-*O*-glucoside, the internal standard, were typically 65 ± 7 %; whereas, in the strawberry feeding study urine samples were thoroughly centrifuged at 4600g for 10 mins at 4ºC and 200 µL of samples were injected directly to HPLC-PDA-MS^2.

4.2.5 HPLC with diode array detection and tandem mass spectrometry

Anthocyanins and their metabolites in blackberries, strawberries, ileal fluid and urine were analyzed on a Surveyor HPLC system comprising a HPLC pump, a diode array absorbance detector, scanning from 200 to 600 nm, and an autosampler cooled to 4°C (Thermo Electron Corp). Separations were carried out using a 250 × 4.6 mm 5 µm C18 Gemini column (Phenomenex, Macclesfield, UK) for samples from ileostomists fed blackberries. A 250 x 4.6 mm i.d C12 4µm, Synergi RP-Max column (Phenomenex) was used for samples from ileostomists who ingested strawberries. Both columns were maintained at 40°C and eluted with a 60 min gradient of 5–50% with methanol in 1% formic acid at a flow rate of 1 mL/min. After passing through the flow cell of the diode array detector, the column eluate was split, and 0.3 mL min was

directed to an LCQ Advantage ion trap mass spectrometer fitted with an electrospray interface (Thermo Electron Corp). Analyses utilized the positive ion mode as this provided the best limit of detection for anthocyanins. Each sample was analyzed in the mass spectrometer in two separate ways: first, using full scan, data-dependent MS^2 scanning from *m/z* 200 to 600, then using a two segment selected reaction monitoring (SRM) method. The tuning of mass spectrometer was optimised by infusing a standard of cyanidin-3-*O*-glucoside. Capillary temperature was 300°C, sheath gas and auxiliary gas were 40 and 20 units, respectively, and the source voltage was 3 kV.

Anthocyanins and metabolites were quantified from their chromatographic peak areas and expressed as cyanidin-3-*O*-glucoside, peoinidin-3-*O*-gluciside or pelargonidin-3-*O*-glucoside equivalents. The identifications were confirmed by full-scan MS^2 and/or SRM. For urine collected from the strawberry study, samples were quantified against pelargonidin-3-*O*-glucoside based on the $[M+H]^+$ in selected reaction monitoring (SRM). Calibration curves ranged between 0.1-50 ng with linear coefficients of 0.999.

4.2.6 Data analysis

HPLC-MS data were processed using Xcalibur QualBrowser version 1.0.1.03 software (Thermo Fisher Scientific Inc., 2009). Each sample was analysed in triplicate for each of the five volunteers and data were presented as mean values ± standard error. When appropriate, data were subjected to statistical analysis using analysis of paired t test with Minitab software, version 13.3 (Minitab Ltd., Coventry, UK).

4.3 Results

4.3.1 Blackberry study

4.3.1.1 Analysis of blackberries

Eight anthocyanins were identified in the blackberry extract at 520 nm by HPLC-PDA-MS^2. The main anthocyanin was cyanidin-3-*O*-glucoside. Other

minor anthocyanins identified were cyanidin-3-*O*-sambubioside, pelargonidin-3-*O*-glucoside, cyanidin-3-*O*-(3″-*O*-malonoyl)glucoside, cyanidin-3-*O*-xyloside, cyanidin-3-*O*-(6″-*O*-malonoyl)glucoside, cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside and cyanidin-3-*O*-(6″-*O*-dioxaylglucoside) respectively. Peak identification was discussed in detail in Chapter 3, Section 3.3.1. The total quantities of anthocyanins in the 180 g serving of blackberries consumed by ileostomy volunteers was 199 µmoles of anthocyanins with 88% of the total being contributed by cyanidin-3-*O*-glucoside, the main component.

4.3.1.2 Identification and quantification of blackberry anthocyanins in ileal fluid

Extracts of ileal fluid collected after the ingestion of 180 g of blackberries were analyzed by HPLC-PDA-MS^2. Figure 4-1 illustrates typical HPLC profiles obtained at 520 nm. In addition to the delphinidin-3-*O*-glucoside internal standard, all the blackberry anthocyanins were detected in the ileal fluid (Table 4-1). No methyl or glucuronide anthocyanin metabolites were detected. Identification was based on mass spectral data and co-chromatography with an authentic standard in the case of cyanidin-3-*O*-glucoside as discussed in Section 3.3.1, in Chapter 3.

Table 4-1: Anthocyanins identified in ileal fluid after the ingestion of 180 g of blackberries.

Peak	R_t	λ_{max}	$[M-H]^+$ *(m/z)*	MS^2 *(m/z)*	Anthocyanins
1	26.05	515	449	287	Cyanidin-3-*O*-glucoside
2	26.61	515	581	287,449	Cyanidin-3-*O*-sambubioside
3	29.1	500	433	271	Pelargonidin-3-*O*-glucoside
4	29.9	515	535	287	Cyanidin-3-*O*-(3″-*O*-malonoyl)glucoside
5	33.99	515	416	287	Cyanidin-3-*O*-xyloside
6	35.82	515	535	287,449	Cyanidin-3-*O*-(6″-*O*-malonoyl)glucoside
7	37.68	515	595	287	Cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside
8	37.7	515	593	287,449	Cyanidin-3-*O*-(6″-*O*-dioxaylglucoside)

*t_R, retention time; $[M+H]^+$, positively charged molecular ion; MS^2 daughter ion produced by fragmentation of $[M+H]^+$.

The anthocyanins listed in Table 4-1 were quantified in the ileal fluid samples of the five volunteers (Table 4-2). Cyanidin-3-*O*-glucoside was detected at all collection points for all volunteers in quantities ranging from 916 nmoles to 7808 nmoles. Some acylated anthocyanins such as peaks 6, 7 and 8 were also presented in considerable amounts ranging from 96 nmoles to 5369 nmoles. The total amount of anthocyanins collected by the individual volunteers ranged from 2932 nmoles to 22768 nmoles which is equivalent to a 1.5% to 11.5% recovery of the total intake (Table 4-2).

Most anthocyanins appeared 0-4 h after ingestion. Of the 199 μmoles of anthocyanins after the ingestion of 180 g blackberries, a mean value of 10227 nmoles appeared in the 0-24 h ileal fluid, which represents a 5.1 % recovery (Table 4.3). Cyanidin-3-*O*-glucoside, as the main component that was detected in ileal fluid, accounted for 3136 nmoles which equates with to a 1.8% recovery of the amount ingested (Table 4-3). Peak 4, cyanidin-3-(3″-*O*-malamoyl)glucoside, a minor component in the blackberries had an 83.8% recovery and another minor anthocyanin, peak 5 cyanidin-3-O-xyloside had an 83.8% recovery. Recoveries of 25-32% were observed with cyanidin-3-*O*-(6″-*O*-malonoyl)glucoside, cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside and cyanidin-3-*O*-(6″-*O*-dioxaylglucoside) (Table 4.3)

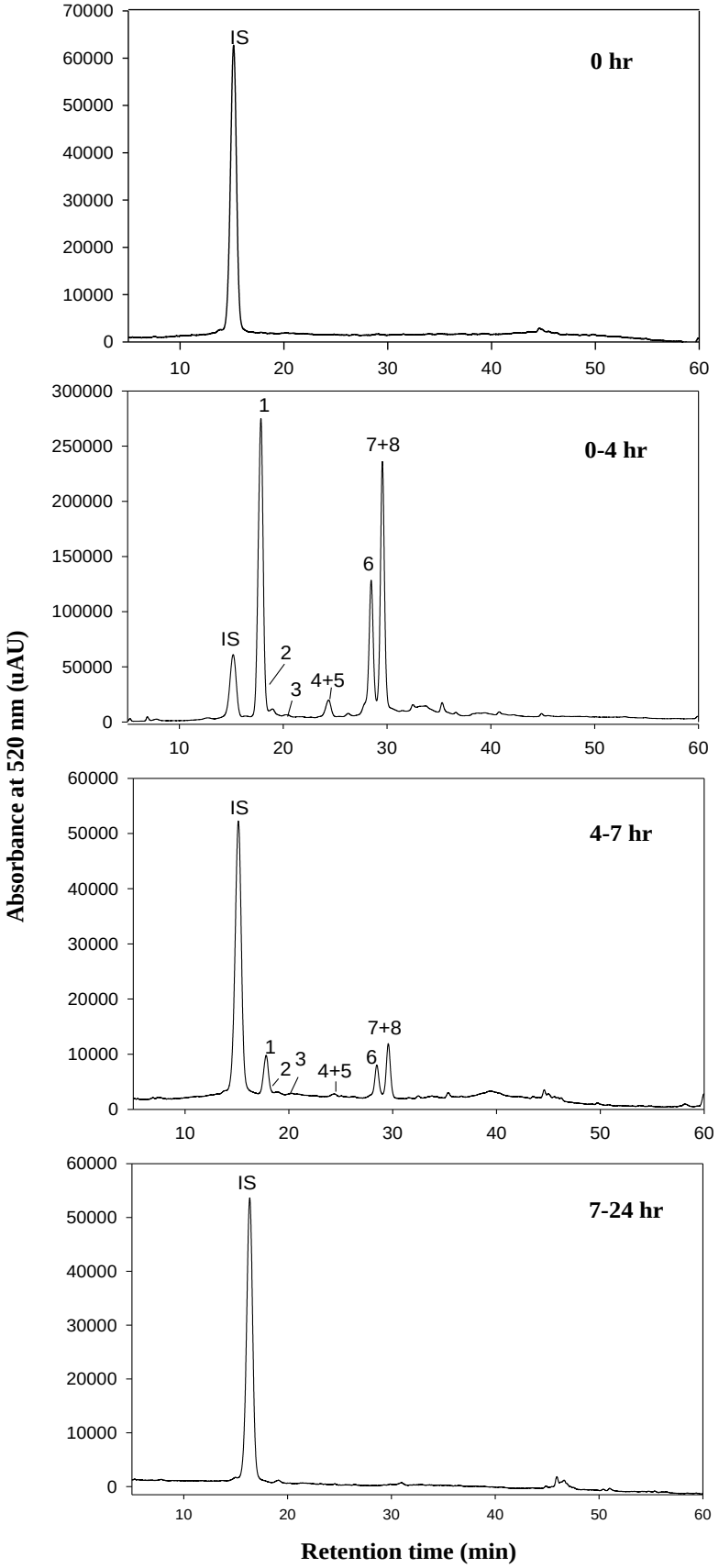


Figure 4-1: Changes on the anthocyanin profile of ileal fluid collected at 0 h, 0-4 h, 4-7 h and 7-24 h after ingestion of 180 g of blackberries by ileostomy volunteers. For numbered peak see Table.4.5. IS: internal standard delphinidin-3-*O*-glucoside.

Table 4-2: Quantities of anthocyanins collected in ileal fluid from individual volunteers with an ileostomy 0-24 h after the consumption of 180 g of blackberries.

Subject	Anthocyanins	0-4 h	4-7 h	7-24 h	Total
1	Cyn-3-*O*-glucoside	7716 ± 514	70 ± 0.7	22 ± 3	7808 ± 518
	Cyn-3-*O*-sambubioside	97 ± 28	8 ± 0.3	n.d.	105 ± 28.3
	Pelg-3-*O*-glucoside	83 ± 12	n.d.	n.d.	83 ± 12
	Cyn-3-O-(3″-*O*-malonoyl)glucoside	540 ± 27	10 ± 0.6	n.d.	550 ± 27.6
	Cyn-3-*O*-xyloside	540 ± 27	10 ± 0.6	n.d.	550 ± 27.6
	Cyn-3-*O*-(6″-*O*-malonoyl)glucoside	2870 ± 267	64 ± 1	n.d.	2934 ± 268
	Cyn-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	5274 ± 422	95 ± 2.4	n.d.	5369 ± 424.4
	Cyn-3-*O*-(6″-*O*-dioxaylglucoside)	5274 ± 422	95 ± 2.4	n.d.	5369 ± 424.4
	Total	22394 ± 1719	352 ± 8	22 ± 3	22768 ± 1730
	%	***(11.3%)***	***(0.2%)***	***(0.01%)***	***(11.51%)***
2	Cyn-3-*O*-glucoside	438 ± 33	263 ± 0.2	242± 27	943 ± 60.2
	Cyn-3-*O*-sambubioside	7 ± 0.6	7 ± 1.4	n.q.	14 ± 2
	Pelg-3-*O*-glucoside	9 ± 0.2	6 ± 0.8	n.q.	15 ± 1
	Cyn-3-*O*-(3″-*O*-malonoyl)glucoside	71 ± 4	13 ± 0.03	36 ± 3.4	120 ± 7.4
	Cyn-3-*O*-xyloside	71 ± 4	13 ± 0.03	36 ± 3.4	120 ± 7.4
	Cyn-3-*O*-(6″-*O*-malonoyl)glucoside	227 ± 21	103 ± 1	32 ± 5	362 ± 27
	Cyn-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	416 ± 27	162 ± 2	101 ± 11	679 ± 40
	Cyn-3-*O*-(6″-*O*-dioxaylglucoside)	416 ± 27	162 ± 2	101 ± 11	679 ± 40
	Total	1655 ± 117	729 ± 8	548 ± 61	2932 ± 185
	%	***(0.8%)***	***(0.4%)***	***(0.3%)***	***(1.5%)***
3	Cyn-3-*O*-glucoside	534 ± 64	336 ± 4	46 ± 2	916 ± 70
	Cyn-3-*O*-sambubioside	28 ± 0.1	12 ± 0.5	n.q.	40 ± 0.6
	Pelg-3-*O*-glucoside	15 ± 3	9 ± 1.4	n.q.	24 ± 4.4
	Cyn-3-*O*-(3″-*O*-malonoyl)glucoside	94 ± 2	26 ± 5	13 ± 1	133 ± 8
	Cyn-3-*O*-xyloside	94 ± 2	26 ± 5	13 ± 1	133 ± 8
	Cyn-3-*O*-(6″-*O*-malonoyl)glucoside	1065 ± 17	295 ± 27	52 ± 1	1412 ± 45
	Cyn-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	1431 ± 27	388 ± 30	65 ± 6	1884 ± 63
	Cyn-3-*O*-(6″-*O*-dioxaylglucoside)	1431 ± 27	388 ± 30	65 ± 6	1884 ± 63
	Total	4692 ± 142	1480 ± 103	254 ± 17	6426 ± 262
	%	***(2.4%)***	***(0.7%)***	***(0.1%)***	***(3.2%)***
4	Cyn-3-*O*-glucoside	1615 ± 97	322 ± 25	109 ± 14	2046 ± 136
	Cyn-3-*O*-sambubioside	32 ± 1	14 ± 1	n.d.	46 ± 2
	Pelg-3-*O*-glucoside	30 ± 7	8 ± 1	n.q.	38 ± 8
	Cyn-3-*O*-(3″-*O*-malonoyl)glucoside	156 ± 51	36 ± 5	30 ± 6	222 ± 62
	Cyn-3-*O*-xyloside	156 ± 51	36 ± 5	30 ± 6	222 ± 62
	Cyn-3-*O*-(6″-*O*-malonoyl)glucoside	39 ± 8	37 ± 6	20 ± 2	96 ± 16
	Cyn-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	366 ± 24	109 ± 11	56 ± 8	531 ± 43
	Cyn-3-*O*-(6″-*O*-dioxaylglucoside)	366 ± 24	109 ± 11	56 ± 8	531 ± 43
	Total	2760 ± 263	671 ± 65	301 ± 44	3732 ± 372
	%	***(1.4%)***	***(0.3%)***	***(0.2%)***	***(1.9%)***
5	Cyn-3-*O*-glucoside	2158 ± 2	1740 ± 90	70 ± 1.3	3968 ± 93.3
	Cyn-3-*O*-sambubioside	74 ± 6	63 ± 5	n.d.	137 ± 11
	Pelg-3-*O*-glucoside	37 ± 12	34 ± 0.5	n.d.	71 ± 13
	Cyn-3-*O*-(3″-*O*-malonoyl)glucoside	375 ± 1	237 ± 14	41 ± 0.1	653 ± 15.1
	Cyn-3-*O*-xyloside	375 ± 1	237 ± 14	41 ± 0.1	653 ± 15.1
	Cyn-3-*O*-(6″-*O*-malonoyl)glucoside	648 ± 57	780 ± 211	n.d.	1428 ± 268
	Cyn-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	2101 ± 90	2046 ± 209	41 ± 0.3	4188 ± 299.3
	Cyn-3-*O*-(6″-*O*-dioxaylglucoside)	2101 ± 90	2046 ± 209	41 ± 0.3	4188 ± 299.3
	Total	7869 ± 259	7183 ± 753	234 ± 2	15286 ± 1014
	%	***(4%)***	***(3.6%)***	***(0.1%)***	***(7.7%)***

* Data for individual subjects are expressed as nmoles ± standard error (n=3). Figures in italics represent the percentage of the total amount of anthocyanins ingested. n.d. not detected. n.q. trace levels not quantified.

Table 4-3: Recovery of anthocyanins in ileal fluid collected 0-4, 4-7 and 7-24 h after consumption of 180 g of blackberries by humans with an ileostomy

Peak	Anthocyanins	µmole/180g	0-4 h	4-7 h	7-24 h	Total
1	Cyanidin-3-*O*-glucoside	176	2492 ± 1368	546 ± 307	98 ± 39	3136 ± 1714 ***(1.8%)***
2	Cyanidin-3-*O*-sambubioside	0.7	48 ±17	21 ± 11	n.q.	69 ± 28 ***(9.9%)***
3	Pelargonidin-3-*O*-glucoside	0.6	35 ± 13	11 ± 6	n.q.	46 ±19 ***(7.7%)***
4	Cyanidin-3-*O*-(3″-*O*-malonoyl)glucoside	0.4	247 ± 92	64 ± 44	24 ± 8	335 ± 144 ***(83.8%)***
5	Cyanidin-3-*O*-xyloside	0.4	247 ± 92	64 ± 44	24 ± 8	335 ± 144 ***(83.8%)***
6	Cyanidin-3-*O*-(6″-*O*-malonoyl)glucoside	5	970 ± 515	252 ± 143	21 ± 10	1243 ± 668 ***(24.9%)***
7	Cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	8	1918 ± 915	560 ± 381	53 ± 17	2531 ± 1313 ***(31.6%)***
8	Cyanidin-3-*O*-(6″-*O*-dioxaylglucoside)	8	1918 ± 915	560 ± 381	53 ± 17	2531 ± 1313 ***(31.6%)***
	Total	**199**	**7874 ± 3927 *(3.9%)***	**2079 ± 1317 *(1.04%)***	**272 ± 99 *(0.2%)***	**10227 ± 5343 *(5.1%)***

*Data presented as mean values in nmol ± standard error (n=5). Italicized figures in parentheses represent the amount recovered as a percentage of the total quantity ingested. n.q. trace levels not quantified.

4.3.1.3 Identification and quantification of blackberry anthocyanins in urine

Figure 4-2 illustrates typical HPLC anthocyanin profiles of urine obtained from ileostomy volunteers 0-24 h after acute intake of 180 g of blackberries. Information on the identity of anthocyanins is summarised in Table 4-4. Five anthocyanins were identified based on mass spectra data and cochromatography with authentic standards in the case of cyanindin-3-*O*-glucoside and peonidin-3-*O*-glucoside (3´-*O*-methyl-cyanidin-glucoside). Full-scan MS^2 and SRM were used to identify the cyanidin methyl and glucuronide metabolites as discussed in Chapter 3, Section 3.3.2.

Table 4-4: HPLC-PDA-MS^2 metabolites of anthocyanis in urine collected after the ingestion of 180 g of blackberries by ileostomy volunteers.

Peak	t_R	λ_{max}	$[M-H]^+$ *(m/z)*	MS^2*(m/z)*	Anthocyanins
1	18.8	515	449	287	Cyanidin-3-*O*-glucoside
2	21.25	515	463	287	Cyanidin-*O*-glucuronide
3	24.01	515	463	301	Peonidin-3-*O*-glucoside
4	26.77	520	477	301	Peonidin-*O*-glucuronide
5	28.07	515	463	287	Cyanidin-*O*-glucuronide

t_R, retention time; $[M+H]^+$, positively charged molecular ion; MS^2 daughter ion produced by fragmentation of $[M+H]^+$

Like the healthy volunteers, the main anthocyanin which was excreted in all urine samples was peonidin-*O*-glucuronide (peak 4), which can be detected in all collection points for all the five ileostomy subjects in quantities ranging from 37.7 nmoles to 89.3 nmoles over 24 h. The total amount of anthocyanins excreted by the individual volunteers ranged from 99.6 nmoles to 135.5 nmoles which is equivalent to a 0.05% to 0.7% recovery of the total intake (Table 4-5). The total mean urinary anthocyanin recovery for subjects without a colon over 24 h was low. Of the 199 µmol anthocyanins ingested, only 117 nmol was detected in the 0-24 h urine which is 0.06% of the intake (Table 4-6). The maximum excretion occurred between 0-4 h after the consumption of blackberries. No anthocyanins were detected in urine collected 7-24 h after the ingestion of the blackberries with the exception of peonidin-*O*-glucuronide which had an overall 0-24 h excretion of 64 ± 13 nmole, equivalent to a 0.03% recovery of intake.

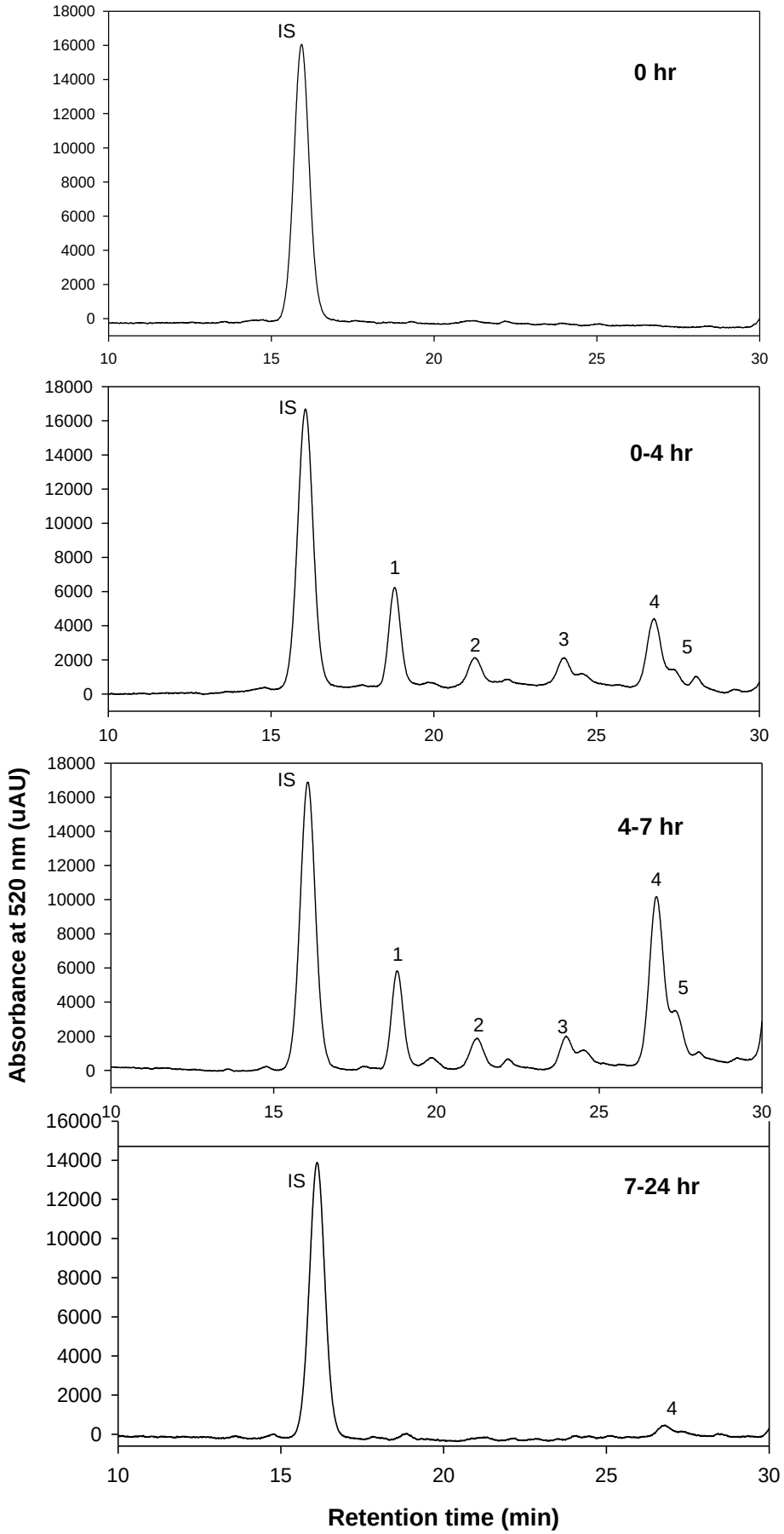


Figure 4-2: Changes on the anthocyanin profile of urine collected at 0 h, 0-4 h, 4-7 h and 7-24 h after ingestion of 180 g of blackberries by ileostomy volunteers. For numbered peak see Table 4.9. IS: internal standard delphinidin-3-*O*-glucoside.

Table 4-5: Quantities of anthocyanins and % excreted in urine collected from individual volunteers with an ileostomy 0-24 h after the consumption of 180 g of blackberries.

Subject	Anthocyanins	0-4 h	4-7 h	7-24 h	Total
1	Cyanidin-3-*O*-glucoside	15.5 ± 0.5	12.4 ± 0.9	n.d.	27.9 ± 1.4
	Cyanidin-*O*-glucuronide	5.5 ± 0.6	5.2 ± 0.6	n.d.	10.7 ± 1.2
	Peonidin-3-*O*-glucoside	7.2 ± 1.6	7.4 ± 0.4	n.d.	14.6 ± 2
	Peonidin-*O*-glucuronide	14.6 ± 1.7	29.7 ± 0.4	14.2 ± 0.8	44.3 ± 2.1
	Cyanidin-*O*-glucuronide	3.4 ± 0.7	2.1 ± 0.7	n.q.	5.5 ± 1.4
	Total	46.2 ± 5.1	56.8 ± 3	14.2 ± 0.8	103 ± 5.5
	%	***(0.02%)***	***(0.03%)***	***(0.01%)***	***(0.05%)***
2	Cyanidin-3-*O*-glucoside	10.1 ± 0.5	8.8 ± 0.6	n.d.	18.9 ± 1.1
	Cyanidin-*O*-glucuronide	6.8 ± 0.6	6 ± 0.5	n.d.	12.8 ± 1.1
	Peonidin-3-*O*-glucoside	4.5 ± 0.3	6.7 ± 0.3	n.d.	11.2 ± 0.6
	Peonidin-*O*-glucuronide	18.2 ± 3	37.6 ± 0.01	33.5 ± 7.9	89.3 ± 10.9
	Cyanidin-*O*-glucuronide	1.5 ± 0.1	1.8 ± 0.1	n.q.	3.3 ± 0.2
	Total	41.1 ± 4.5	60.9 ± 1.5	33.5 ± 7.9	135.5 ± 13.9
	%	***(0.02%)***	***(0.03%)***	***(0.02%)***	***(0.07%)***
3	Cyanidin-3-*O*-glucoside	8.1 ± 0.4	4.2 ± 0.5	n.d.	12.3 ± 0.9
	Cyanidin-*O*-glucuronide	9.3 ± 0.6	3.6 ± 0.3	n.d.	12.9 ± 0.9
	Peonidin-3-*O*-glucoside	5.8 ± 0.7	4.8 ± 0.5	n.d.	10.6 ± 1.2
	Peonidin-*O*-glucuronide	33 ± 1.1	23.8 ± 1.3	21 ± 0.5	77.8 ± 2.4
	Cyanidin-*O*-glucuronide	2.2 ± 0.2	1.8 ± 0.5	n.q.	4 ± 0.7
	Total	58.4 ± 3	38.2 ± 3.1	21 ± 0.5	117.6 ± 6.1
	%	***(0.03%)***	***(0.02%)***	***(0.01%)***	***(0.06%)***
4	Cyanidin-3-*O*-glucoside	35.5 ± 2.8	7.3 ± 2.1	n.d.	42.8 ± 4.9
	Cyanidin-*O*-glucuronide	6.5 ± 0.2	1.1 ± 0.04	n.d.	7.6 ± 0.24
	Peonidin-3-*O*-glucoside	15.2 ± 1.5	4.1 ± 2.2	n.d.	19.3 ± 3.7
	Peonidin-*O*-glucuronide	17.1 ± 1.7	5.5 ± 3.8	15.1 ± 2.2	37.7 ± 7.7
	Cyanidin-*O*-glucuronide	4.0 ± 0.5	0.8 ± 0.1	n.q.	4.8 ± 0.6
	Total	78.3 ± 6.7	18.8 ± 8.24	15.1 ± 2.2	112.2 ± 17.14
	%	***(0.04%)***	***(0.01%)***	***(0.01%)***	***(0.06%)***
5	Cyanidin-3-*O*-glucoside	10.2 ± 1	6.1 ± 0.7	n.d.	16.3 ± 1.7
	Cyanidin-*O*-glucuronide	9.1 ± 1.1	5 ± 0.8	n.d.	14.1 ± 1.9
	Peonidin-3-*O*-glucoside	5.5 ± 0.4	4 ± 0.1	n.d.	9.5 ± 0.5
	Peonidin-*O*-glucuronide	22 ± 1.9	18.1 ± 2	16 ± 6	56.1 ± 9.9
	Cyanidin-*O*-glucuronide	2.2 ± 0.2	1.4 ± 0.1	n.q.	3.6 ± 0.3
	Total	49 ± 4.6	34.6 ± 3.7	16 ± 6	99.6 ± 14
	%	***(0.02%)***	***(0.02%)***	***(0.01%)***	***(0.05%)***

* Data for individual subjects are expressed as nmoles ± standard error (n=3). Figures in italics represent the percentage of the total amount of anthocyanins ingested. n.d.- not detected. n.q. trace levels not quantified.

Table 4-6: Quantities of anthocyanins and % excreted in urine collected from five volunteers

Peak	Anthocyanins	0-4 h	4-7 h	7-24 h	Total
1	Cyanidin-3-*O*-glucoside	16 ± 5	8 ± 1	n.d.	24 ± 6 ***(0.01%)***
2	Cyanidin-*O*-glucuronide	7 ± 1	4 ± 1	n.d.	11 ± 2 ***(0.01%)***
3	Peonidin-3-*O*-glucoside	8 ± 2	5 ± 1	n.d.	13 ± 3 ***(0.01%)***
4	Peonidin-*O*-glucuronide	21 ± 3	23 ± 6	20 ± 4	64 ± 13 ***(0.03%)***
5	Cyanidin-*O*-glucuronide	3 ± 1	2 ± 0.2	n.q.	5 ± 1 ***(0.002%)***
	Total **%**	**55 ± 12** ***(0.03%)***	**42 ± 9** ***(0.02%)***	**20 ± 4** ***(0.01%)***	**117 ± 24** ***(0.06%)***

with an ileostomy 0-24 h after the consumption of 180 g of blackberries.
*Data presented as mean values in nmol ± standard error (n=5). Italicized figures in parentheses represent the amount excreted as a percentage of the amounts of anthocyanins ingested. nd, not detected. nq, trace levels not quantified.

4.3.1.4 Urinary excretion in healthy and ileostomist groups

In the previous chapter (Chapter 3), anthocyanins metabolism after blackberry consumption was investigated in a group of healthy volunteers (n=8) in identical condition to an ileostomy group (n=5). The total amount of 24 h urinary excretion in the healthy group accounted for 0.08 ± 0.02% (206.5 ± 49.2 nmol) of the dose ingested compared to 0.06 ± 0.01% (177 ± 24 nmol) in the ileostomy group (Table 4-7). The results show slight differences but not significant in the levels of anthocyanins excretion in the urine in both groups. However, two methylation forms of cyanidin glucoside were detected in healthy subjects and only the 3´-methylation was detected in the urine in the ileostomy study.

Table 4-7: Comparative levels of urinary excretion of anthocyanin metabolites in 24 h urine sample of healthy (n=8) and ileostomy (n=5) volunteers, following the ingestion of 180 g of blackberries[a]

Metabolites excreted	Healthy volunteers (n=8) 295 µmole ingested	Ileostomy volunteers (n=5) 199 µmole ingested
Cyanidin-3-*O*-glucoside	0.01 ± 0.003	0.01 ± 0.003
Cyanidin-*O*-glucuronide	0.01 ± 0.002	0.01 ± 0.001
3´-*O*-methyl-cyanidin-glucoside	0.01 ± 0.001	0.01 ± 0.001
4´-*O*-methyl-cyanidin-glucoside	0.01 ± 0.002	n.d.
Peonidin-*O*-glucuronide	0.03 ± 0.01	0.03 ± 0.01
Cyanidin-*O*-glucuronide	0.003 ± 0.001	0.003 ± 0.000
Unknown cyanidin	0.01 ± 0.002	n.d.
Total (% Dose ingested)	0.08 ± 0.02	0.06 ± 0.01

[a] Data represent mean values ± standard error as percentage of the ingested dose.

4.3.2 Strawberries study

4.3.2.1 Analysis of strawberries

HPLC-PDA-MS^2 analysis detected nine anthocyanins at 520 nm in strawberries (Figure 4-3 and Figure 4-4). Identification of individual compounds was based on the *m/z* of the positively charged molecular ion ($[M+H]^+$) and the MS^2 fragmentation, retention time of commercially available standards, absorbance spectra, and elution order, which depends upon the number of hydroxyl groups on the anthocyanidin aglycone and the attached sugar, as reported in the literature. The basis of the identification is outlined in Table 4-8 and summarized as follows:

Peak 1 (t_R, 13.6 min; λ_{max} 500nm) has a positively charged molecular ion ($[M+H]^+$) at *m/z* 595 and two MS^2 fragments. The most abundant fragment was at *m/z* 433, a loss of 162 amu which is indicative of the loss of a hexose sugar, indicating the presence of pelargonidin-*O*-glucoside. Another significant ion at *m/z* 271 was also present which corresponds to the pelargonidin aglycone, through the loss of another hexose sugar. In accordance with molecular weight, fragmentation pattern and absorbance spectrum, this component was identified as a pelargonidin-3,5-*O*-diglucoside which has previously been detected in strawberries (Lopes-da-Silva *et al.*, 2002).

Peak 2 (t_R, 22.9 min; λ_{max} 510 nm) has $[M+H]^+$ at *m/z* 681 and MS^2 fragments at *m/z* 519 $[(M+H)-162]^+$, *m/z* 433 $[(M+H)-248]^+$ and at *m/z* 271 $[AGly+248]^+$. This suggests the presence of pelargonidin diglucoside derivative acylated with malonic acid. The loss of 248 *amu* indicates the presence of hexose and malonic acid (Giusti *et al.*, 1999) while, *m/z* at 519 indicates the presence of another hexose unit. Therefore, according to a similar fragmentation pattern for cyanidin-3-malonylglucose-5-glucose reported previously by Lopes-de-Sliva *et al.*, (2002) this peak was tentatively identified as pelargonidin-3-*O*-(3"-*O*-malonoylglucoside)-5-*O*-glucoside.

Peak 3 (t_R, 25.6 min; λ_{max} 515 nm) was identified as cyanidin-3-*O*-glucoside based on cochromatography with a standard and a mass spectrum with a $[M+H]^+$ at *m/z* 449 which fragmented with a loss of 162 *amu* (glucose) to produce a *m/z* 287 (cyanidin) daughter ion.

Peak 4 (t_R, 28.3 min; λ_{max} 500 nm) was identified as pelargonidin-3-*O*-glucoside on the basis of co-chromatography with an authentic standard and a mass spectrum with a $[M+H]^+$ at *m/z* 433 that yielded an MS^2 fragment at *m/z* 271 (M-162, loss of a glucosyl unit).

Peak 5 (t_R, 31.4 min; λ_{max} 500 nm) produced a $[M+H]^+$ at *m/z* 579 and MS^2 ions at *m/z* 271 (pelargonidin) corresponding to the loss of 308 *amu* which indicates the presence of rutinoside moiety (hexose+rhamnose) and *m/z* 433 (loss of a rhamnose residue). This fragmentation pattern was reported by Lopes-de-Sliva *et al*. (2002) for pelargonidin-3-*O*-rutinoside.

Peak 6 and 8 (t_R, 32.6 min and 37.14 min; λ_{max} 500 nm) had $[M+H]^+$ at *m/z* 519 and MS^2 fragment *m/z* 271. The loss of 248 *amu* indicates the presence of hexose and malonic acid (Giusti *et al.,* 1999). However, peak 8 has a lower intensity fragment at *m/z* 433 formed by the loss of 86 *amu* which can be ascribed to the release of the malonyl moiety; whereas, peak 6 yielded only one ion at *m/z* 271. The identity confirmation of these two peaks was provided by comparing their fragmentation behaviour and order of elution with those in the literature for cyanidin- 3-*O*-(3″-*O*-malonoyl)glucoside and cyanidin-3-*O*-(6″-*O*-malonyl)glucoside, as discussed in chapter 3. Therefore, these anthocyanins were tentatively identified as pelargonidin-3-*O*-(3″-*O*-malonoyl)glucoside and pelargonidin-3-*O*-(6″-*O*-malonoyl)glucoside respectively (Lopes-da-Silva *et al.,* 2002; Lopes-da-Silva *et al.,* 2007).

Peak 7 (t_R, 35.4 min; λ_{max} 515 nm) has $[M+H]^+$ at *m/z* 595 and yielded an ion at m/z 287. Based on the retention time and fragmentation pattern that was described in chapter 3, this peak was identified as cyanidin-3-*O*-(6″-*O*-malonoyl)glucoside which had been identified in strawberries by (Lopes-da-Silva *et al.*, 2002; Lopes-da-Silva *et al.*, 2007).

Peak 9 (t_R, 43.02 min; λ_{max} 500 nm) had a $[M+H]^+$ at *m/z* 475 and an MS^2 fragment at *m/z* 271, a loss of 204 *amu* corresponding to cleavage of acetylglucosyl moiety. Thus, this peak was tentatively identified as pelargonidin-3-*O*-acetylglucoside (Kajdzanoska *et al.*, 2010).

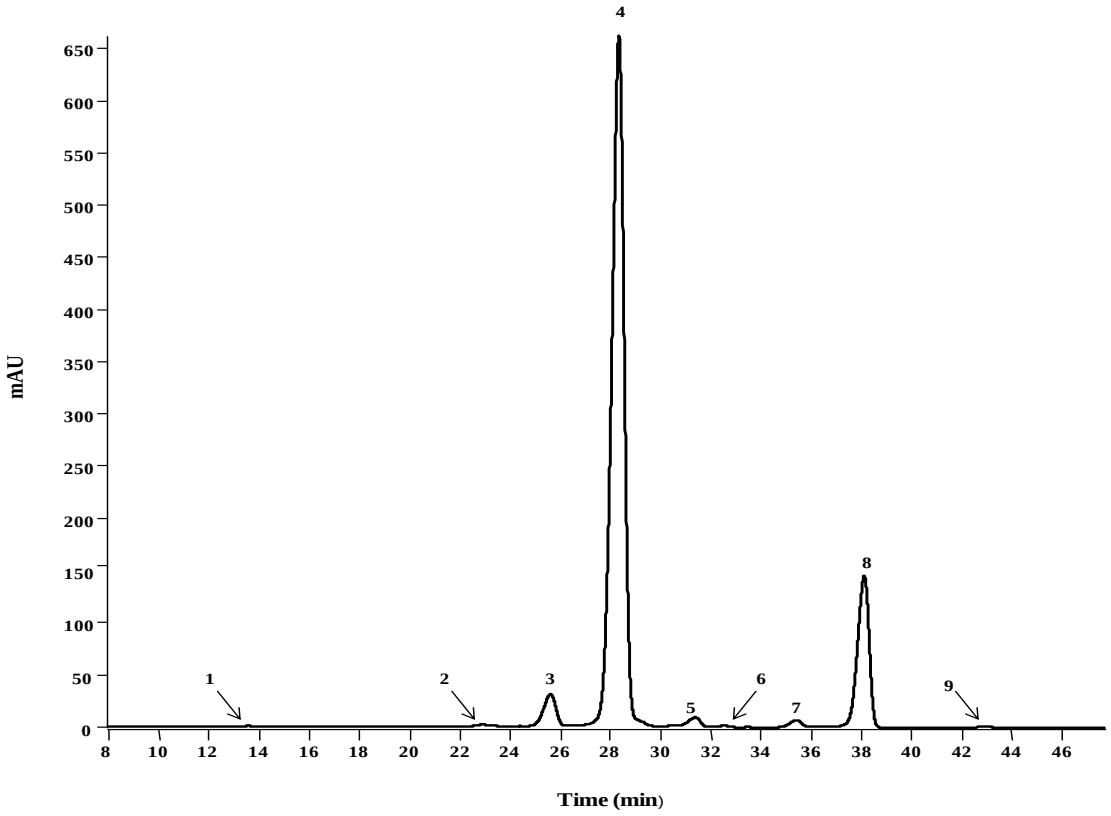
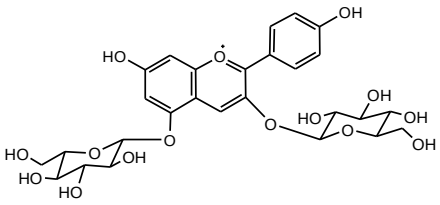
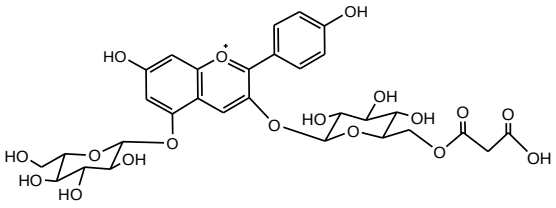


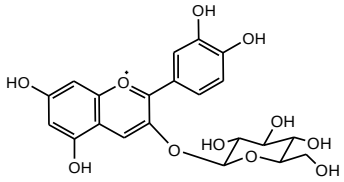
Figure 4-3: Gradient reversed phase HPLC profiles at 520 nm showing the anthocyanins content of strawberries. For identification of numbered peak see Table 4-1.



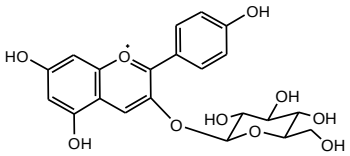
Peak 1. pelargonidin-3,5-diglucoside



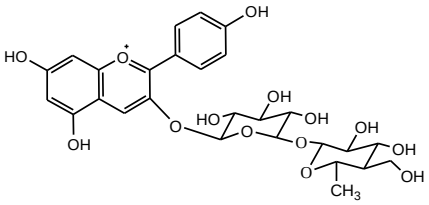
Peak 2. pelargonidin-3-*O*-(6"-*O*-malonoylglucoside)-5-glucoside



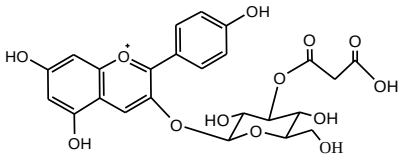
Peak 3. cyanidin-3-*O*-glucoside



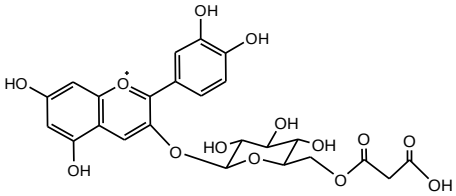
Peak 4. pelargonidin-3-*O*-glucoside



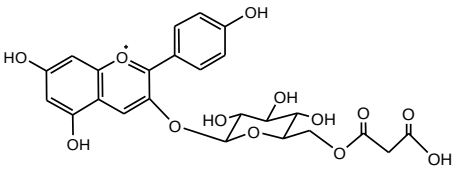
Peak 5. pelagonidin-3-*O*-rutinoside



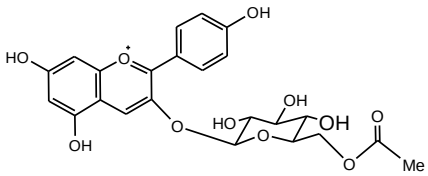
Peak 6. pelagonidin-3-*O*-(3"-*O*-malonoyl)glucoside



Peak 7. cyanidin-3-*O*-(6"-*O*-malonoyl) -glucoside



Peak 8. pelargonidin-3-*O*-(6"-*O*-malonoyl) -glucoside



Peak 9. pelargonidin-3-*O*-acetylglucoside

Figure 4-4: Structure of strawberry anthocyanins.

Table 4-8: Anthocyanins identified in strawberries by HPLC with diode array and MS^2 detection.

Peaks	R_t	$[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	Anthocyanins
1	13.6	595	433,271	Pelargonidin-3,5-*O*-diglucoside
2	22.9	681	519,271,433	Pelargonidin-3-*O*-(3"-*O*-malonoylglucoside)-5-*O*-glucoside
3	25.6	449	287	Cyanidin-3-*O*-glucoside
4	28.3	433	271	Pelargonidin-3-*O*-glucoside
5	31.4	579	271,433	Pelargonidin-3-*O*-rutinoside
6	32.6	519	271	Pelagonidin-3-*O*-(3"-*O*-malonoyl)glucoside
7	35.4	535	287	Cyanidin-3-*O*-(6"-*O*-malonoyl)glucoside
8	37.14	519	271,433	Pelargonidin-3-*O*-(6"-*O*-malonoyl)glucoside
9	43.02	475	271	Pelargonidin-3-*O*-acetylglucoside

t_R, retention time; $[M+H]^+$, positively charged molecular ion; MS^2 daughter ion produced by fragmentation of $[M+H]^+$

The quantities of anthocyanins in the 180 g serving of strawberries consumed by the ileostomy volunteers are presented in Table 4-9. Each serving contained a total of 146.4 µmoles of anthocyanins, 79% of which was pelargonidin-3-*O*-glucoside. Other minor acylated derivatives of pelargonidin and cyanidin were also detected ranging from 0.1% to 19% of the total anthocyanins. In addition, pelarogonidin-diglycosides (peaks 1 and 5) were detected in trace amounts while cyanidin-3-*O*-glucoside (peak 3) accounted for 0.6% of the anthocyanin content of the ingested strawberries.

Table 4-9: Anthocyanins quantified in strawberries by HPLC-PDA-MS^2.

Peak	Anthocyanins	µmol/180g
1	Pelargonidin-3,5-*O*-diglucoside	0.1 ± 0.01 ***(0.07%)***
2	Pelargonidin-3-*O*-(6"-*O*-malonoylglucoside)-5-*O*-glucoside	0.5 ± 0.02 ***(0.3%)***
3	Cyanidin-3-*O*-glucoside	0.9 ± 0.01 ***(0.6%)***
4	Pelargonidin-3-*O*-glucoside	116 ± 1.3 ***(79.2%)***
5	Pelargonidin-3-*O*-rutinoside	0.2 ± 0.02 ***(0.1%)***
6	Pelargonidin-3-*O*-(3"-*O*-malonoyl)glucoside	0.4 ± 0.01 ***(0.3%)***
7	Cyanidin-3-*O*-(6"-*O*-malonoyl)glucoside	0.2 ± 0.01 ***(0.1%)***
8	Pelargonidin-3-*O*-(6"-*O*-malonoyl)glucoside	28 ± 0.3 ***(19.1%)***
9	Pelargonidin-3-*O*-acetylglucoside	0.1 ± 0.01 ***(0.07%)***
	Total	**146.4 ± 1.6**

*Data expressed as mean values in µmol (n=3) ± standard error, % percentage of anthocyanins of the total.

4.3.2.2 Identification and quantification of anthocyanins in ileal fluid after ingestion of strawberries

Analysis of ileal fluid extract collected 0-24 h after supplementation of 180 g of strawberries revealed the presence of six anthocyanins of the total nine anthocyanins that were originally present in the fruit. No metabolites were detected in any of the samples (Table 4-10 and Figure 4-5).

Table 4-10: Anthocyanins identified in ileal fluid after the ingestion of 180 g of strawberries.

Peak	R_t	λ_{max}	$[M-H]^+$*(m/z)*	MS^2*(m/z)*	Anthocyanins
1	13.6	500	595	433,271	Pelargonidin-3,5-*O*-diglucoside
2	25.6	510	449	287	Cyanidin-3-*O*-glucoside
3	28.3	500	433	271	Pelargonidin-3-*O*-glucoside
4	31.4	500	579	271	Pelargonidin-3-*O*-rutinoside
5	32.6	500	519	271	Pelargonidin-3-*O*-(3"-*O*-malonoyl)glucoside
6	37.14	500	519	271	Pelargonidin-3-*O*-(6"-*O*-malonoyl)glucoside

*t_R, retention time; $[M+H]^+$, positively charged molecular ion; MS^2 daughter ion produced by fragmentation of $[M+H]^+$.

Among anthocyanins present in the ileal fluid pelargonidin-3-*O*-glucoside was quantified in samples from all five volunteers, at all collection points, in amounts ranging from 3744 nmoles to 21895 nmoles (Table 4-11). Pelargonidin-3-*O*-(3′′-*O*-malonoyl)-glucoside, peak 6, was also recovered in relatively high amounts ranging from 5921 to 17684 nmoles. The total amount of anthocyanins excreted by the individual volunteers ranged from 10194 nmoles to 33328 nmoles which is equivalent to a 7.0-22.8% recovery of the total intake (Table 4-11).

Unlike ileal fluid collected after blackberry ingestion, most of the strawberry anthocyanins appeared between 4-7h after ingestion. In total, a mean of 19995 nmoles were detected over 24 h after ingestion of 180 g of strawberries, which represent 13.6 % recovery of the anthocyanin intake (Table 4-12). This is higher than recovery of anthocyanins after the ingestion of blackberries (Table 4-3). Pelargonidin-3-*O*-(6"-*O*-malonoyl)glucoside and pelargonidin-3-*O*-glucoside were the main anthocyanins detected in ileal fluid after ingestion of strawberries at 11435 nmoles and 7902 nmoles,

respectively. However, the recovery as a percentage of the amount ingested was much higher for the malonoyl derivative (40.8%) than for the glucoside (6.8%). The recovery of the individual anthocyanins as a percentage of their intake ranged from 6.8 to 68.3%, indicating the different susceptibility of conjugated anthocyanins absorption and metabolism in the proximal gastric intestinal tract.

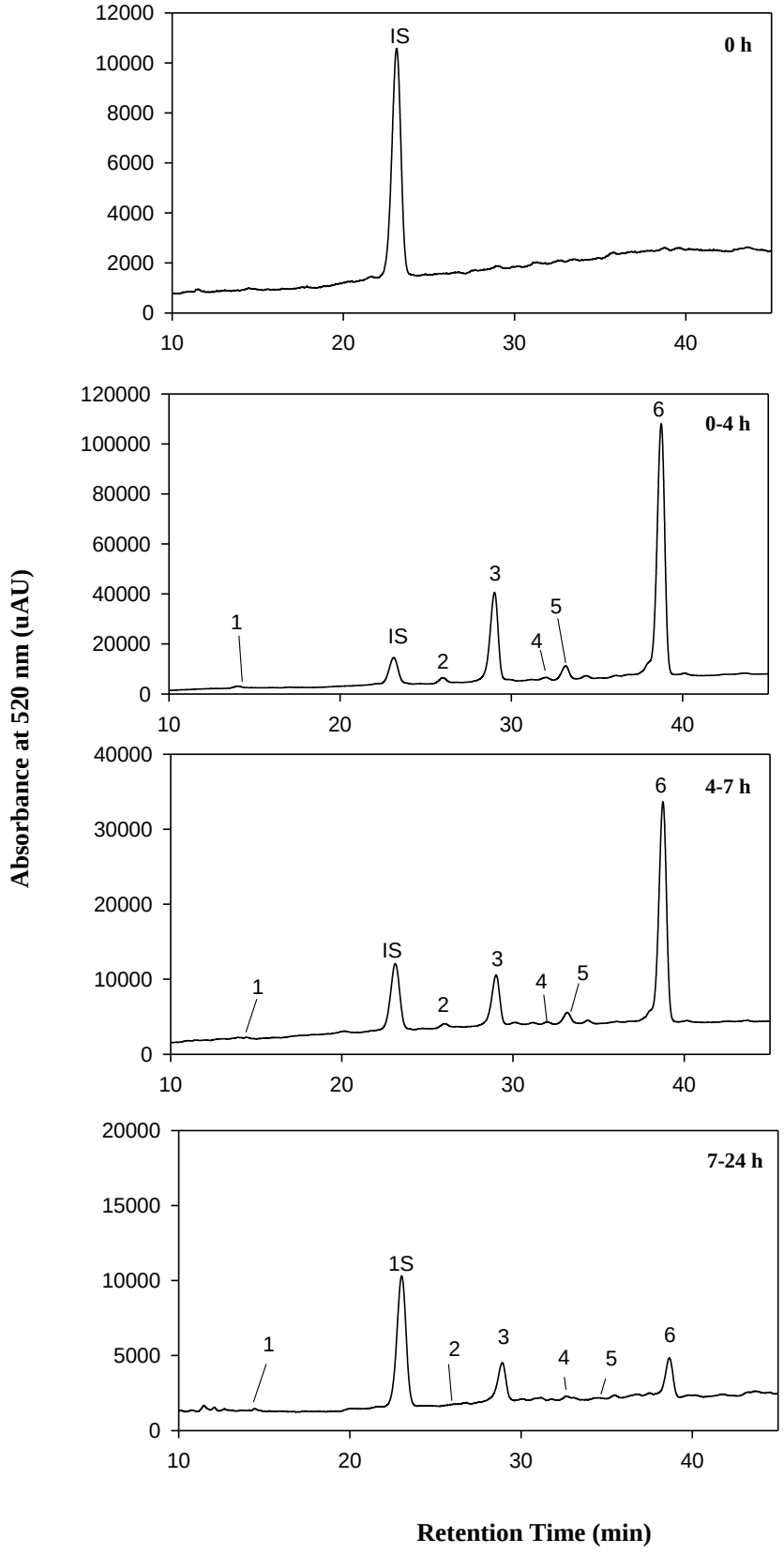


Figure 4-5: Changes on the anthocyanin profile of ileal fluid collected at 0 h, 0-4 h, 4-7 h and 7-24h after ingestion of 180 g of strawberries by ileostomy volunteers. For numbered peak see Table.4.5. IS: internal standard delphinidin-3-*O*-glucoside.

Table 4-11: Quantities of anthocyanins collected in ileal fluid from individual volunteers with an ileostomy 0-24 h after the consumption of 180 g of strawberries.

Subject	Anthocyanins	0-4 h	4-7 h	7-24 h	Total
1	Pelargonidin-3,5-diglucoside	78 ± 6	n.d.	n.d.	78 ± 6
	Cyanidin-3-*O*-glucoside	240 ± 24	122 ± 4	n.d.	362 ± 28
	Pelargonidin-3-*O*-glucoside	3939 ± 431	1614 ± 61	171 ± 0.0	5724 ± 492
	Pelargonidin-3-*O*-rutinoside	96 ± 21	47 ± 9	n.d.	143 ± 30
	Pelg-3-*O*-(3"-*O*-malonoyl)glucoside	238 ± 13	163 ± 10	n.d.	374 ± 23
	Pelg-3-*O*-(6"-*O*-malonoyl)glucoside	10511 ± 1257	6795 ± 389	378 ± 0.0	17684 ± 1646
	Total	15102 ± 1752	8714 ± 473	549 ± 0.0	24365 ± 2225
	%	***(10.3%)***	***(5.9%)***	***(0.4%)***	***(16.6%)***
2	Pelargonidin-3,5-diglucoside	19 ± 3	35 ± 2	n.d.	54 ± 5
	Cyanidin-3-*O*-glucoside	9 ± 2	6 ± 0.4	n.d.	15 ± 2.4
	Pelargonidin-3-*O*-glucoside	1414 ± 44	1512 ± 203	1032 ± 341	3958 ± 580
	Pelargonidin-3-*O*-rutinoside	14 ± 0.01	15 ± 0.4	n.d.	29 ± 0.4
	Pelg-3-*O*-(3"-*O*-malonoyl)glucoside	117 ± 3	77 ± 5	n.d.	217 ± 8
	Pelg-3-*O*-(6"-*O*-malonoyl)glucoside	3197 ± 120	1880 ± 180	874 ± 323	5921 ± 623
	Total	4770 ± 172	3525 ± 391	1906 ± 664	10194 ± 1219
	%	***(3.3%)***	***(2.4%)***	***(1.3%)***	***(7.0%)***
3	Pelargonidin-3,5-diglucoside	n.d.	n.d.	n.d.	n.d.
	Cyanidin-3-*O*-glucoside	30 ± 1.4	237 ± 14	111 ± 38	378 ± 53.4
	Pelargonidin-3-*O*-glucoside	590 ± 23	2749 ± 196	853 ± 124	4192 ± 343
	Pelargonidin-3-*O*-rutinoside	11 ± 0.2	67 ± 2	37 ± 10	115 ± 12.2
	Pelg-3-*O*-(3"-*O*-malonoyl)glucoside	71 ± 1	211 ± 37	n.q.	282 ± 38
	Pelg-3-*O*-(6"-*O*-malonoyl)glucoside	1939 ± 24	11174 ± 290	2978 ± 320	16091 ± 634
	Total	2641 ± 50	14438 ± 539	3979 ± 492	21058 ± 1081
	%	***(1.8%)***	***(9.9%)***	***(2.7%)***	***(14.4%)***
4	Pelargonidin-3,5-diglucoside	n.d.	85 ± 20	n.d.	85 ± 20
	Cyanidin-3-*O*-glucoside	18 ± 2	65 ± 11	n.d.	83 ± 13
	Pelargonidin-3-*O*-glucoside	28 ± 4	19918± 4504	1949 ± 265	21895 ± 4773
	Pelargonidin-3-*O*-rutinoside	27 ± 2	156 ± 35	271 ± 23	454 ± 60
	Pelg-3-*O*-(3"-*O*-malonoyl)glucoside	27 ± 1	226 ± 19	n.q.	253 ± 18
	Pelg-3-*O*-(6"-*O*-malonoyl)glucoside	21 ± 2	9960 ± 2107	577 ± 66	10558 ± 2175
	Total	121 ± 11	30410 ± 6696	2797 ± 354	33328 ± 7039
	%	***(0.08%)***	***(20.8%)***	***(1.9%)***	***(22.8%)***
5	Pelargonidin-3,5-diglucoside	47 ± 1	40 ± 0.2	n.d.	87 ± 1.2
	Cyanidin-3-*O*-glucoside	n.d.	15 ± 1	n.d.	15 ± 1
	Pelargonidin-3-*O*-glucoside	184 ± 22	2074 ± 54	1486 ± 189	3744 ± 265
	Pelargonidin-3-*O*-rutinoside	n.d.	23 ± 3	n.d.	23 ± 3
	Pelg-3-*O*-(3"-*O*-malonoyl)glucoside	n.d.	261 ± 4	n.d.	261 ± 4
	Pelg-3-*O*-(6"-*O*-malonoyl)glucoside	n.d.	5756 ± 149	1132 ± 14	6888 ± 163
	Total	231 ± 23	8139 ± 211	2618 ± 203	11018 ± 437
	%	***(0.2%)***	***(5.6%)***	***(1.8%)***	***(7.6%)***

* Data for individual subjects are expressed as nmoles ± standard error (n=3). Figures in italics represent the percentage of the total amount of anthocyanins ingested. n.d. not detected. pelg. pelargonidin.

Table 4-12: Recovery of anthocyanins in ileal fluid collected 0-4, 4-7 and 7-24 h after consumption of 180 g of strawberries by humans with an ileostomy

Peak	Anthocyanins	0-4 h	4-7 h	7-24 h	Total
1	Pelargonidin-3,5-diglucoside	29 ± 15	32 ± 16	n.d.	61 ± 31 ***(61%)***
2	Cyanidin-3-*O*-glucoside	59 ± 46	89 ± 43	22 ± 0.0	170 ± 89 ***(19%)***
3	Pelargonidin-3-*O*-glucoside	1231 ± 730	5573 ± 3652	1098 ± 305	7902 ± 4687 ***(6.8%)***
4	Pelargonidin-3-*O*-rutinoside	30 ± 17	62 ± 26	62 ± 54	154 ± 97 ***(76.5%)***
5	Pelargonidin-3-*O*-(3"-*O*-malonoyl)glucoside	91 ± 43	182 ± 34	n.q.	273 ± 77 ***(68.3%)***
6	Pelargonidin-3-*O*-(6"-*O*-malonoyl)glucoside	3134 ± 1973	7113 ± 1669	1188 ± 473	11435 ± 4115 ***(40.8%)***
	Total	**4573 ± 2824** ***(3.1%)***	**13060 ± 5439** ***(8.9%)***	**2370 ± 832** ***(1.6%)***	**19995 ± 9096** ***(13.6%)***

*Data presented as mean values in nmol ± standard error (n=5). Italicized figures in parentheses represent the amount recovered as a percentage of the total quantity ingested. n.d.- not detected. n.q. trace levels not quantified.

4.3.2.3 Identification and quantification of anthocyanins in urine after ingestion of strawberries

Data on the excretion of anthocyanins in urine after the ingestion of 180 g of strawberries are presented in Table 4-13. Five metabolites were detected. Four were pelargonidin-*O*-glucuronides (peaks 1, 2, 3, and 5) plus pelargonidin-3-*O*-glucoside (peak 4) (Figure 4-6). No anthocyanins were observed in urine collected before the experimental meal. Identifications were based on mass spectral data and cochromatography with authentic standards in the case of pelargonidin-3-*O*-glucoside. Full-scan MS^2 and SRM were used to partially identify the pelargonidin glucuronide metabolites. Pelargonidin-3-*O*-glucoside has a $[M+H]^+$ at m/z 433 that yielded an MS^2 fragment at m/z 271 (M-162, loss of a glucosyl unit). The four glucuronide metabolites were characterized by a $[M+ H]^+$ at m/z 447, which fragmented with a 176 amu loss to yield a MS^2 fragment at m/z 271. Although four pelargonidin-*O*-glucuronides were detected, it was not possible to determine the point of attachment of the glucuronide units because reference compounds were not available. The fact that pelargonidin has hydroxyl groups at the 4´-, 3-, 5-, and 7-positions implies that the four pelargonidin isomers are glucuronidated at these positions. However, as explained in chapter 3 section 3.3.2, peak 5, which elutes immediately after pelargonidin-3-*O*-glucoside (peak 4), may be pelargonidin-3-*O*-glucuronide. No methyl or sulfation metabolites were detected.

Table 4-13: HPLC-PDA-MS^2 metabolites of anthocyanins in urine collected after the ingestion of 180 g of strawberries by ileostomy volunteers.

Peak	R_t	λ_{max}	$[M-H]^+$*(m/z)*	MS^2*(m/z)*	Anthocyanins
1	12.3	500	447	271	Pelargonidin-*O*-glucuronide
2	16.8	500	447	271	Pelargonidin-*O*-glucuronide
3	22.3	500	447	271	Pelargonidin-*O*-glucuronide
4	28.7	500	433	271	Pelargonidin-3-*O*-glucoside
5	29.9	500	447	271	Pelargonidin-*O*-glucuronide

t_R, retention time; $[M+H]^+$, positively charged molecular ion; MS^2 daughter ion produced by fragmentation of $[M+H]^+$.

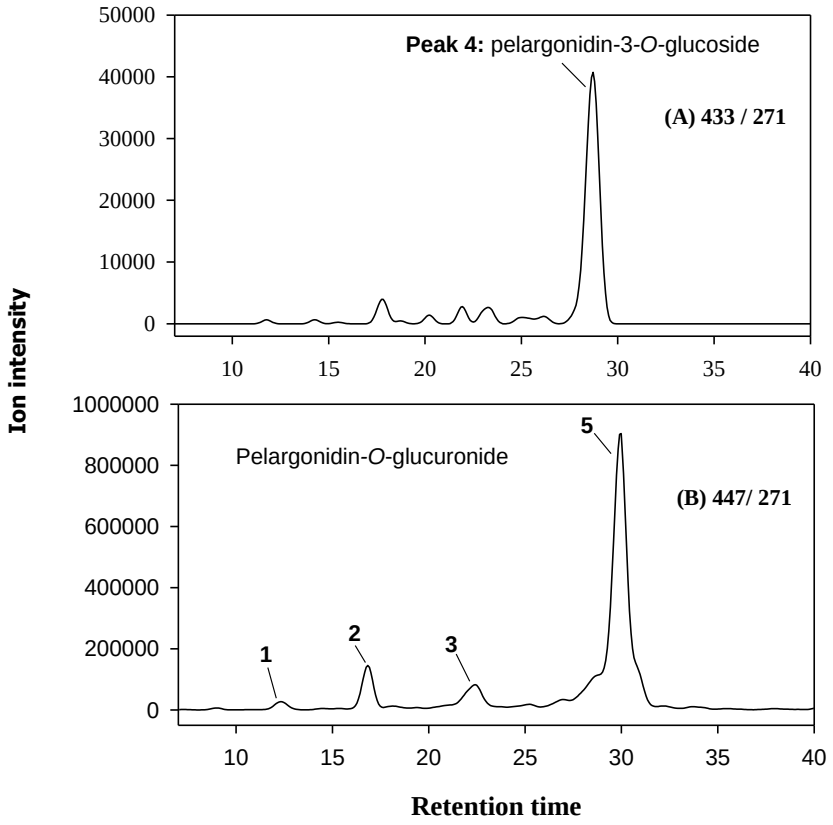


Figure 4-6: Gradient reversed phase HPLC of a urine sample collected 0-4 h after the ingestion of 180 g of strawberries by an ileostomy volunteer; **(A)** MS^2 with SRM (*m/z* 433/ 271): peak 4, pelargonidin-3-*O*-glucoside. **(B)** MS^2 with SRM (*m/z* 447/ 271): peaks 2, 3, 5 are pelargonidin-*O*-glucuronides (see Table 4-10).

Urinary excretion of the parent pelargonidin-3-*O*-glucoside was low, whereas the major portion of strawberry anthocyanins were excreted as pelargonidin-*O*-glucuronide (peak 5). The total urinary extraction of this pelargonidin glucuronide metabolite by the individual ileostomists ranged from 415 nmole to 1524 nmole which comprised of 65% and 85% of the total anthocyanins excreted over the 24 h post-ingestion period (Table 4-14).

The overall excretion of anthocyanins in urine over the 24 h period, with respect to the 146.4 µmol of pelargonidin-3-*O*-glucoside ingested, was 0.87% of the intake. This recovery still low but considered high compared to a 0.06% recovery of blackberry anthocyanins excreted in urine by the ileostomists. The pelargonidin-*O*-glucuronide, peak 5, comprised 79% of the anthocyanins excreted over the 24 h post-ingestion period (Table 4-15). Most pelargonindin-3-*O*-glucoside metabolites were excreted during the first 4 h after the consumption of the strawberries. The native strawberry anthocyanin,

pelargonidin-3-O-glucoside, was also present but in much smaller quantities than the glucuronides with 0.03% recovery of the total intake (Table 4-15).

Table 4-14: Quantities of anthocyanins and % excreted in urine collected from individual ileostomy volunteers 0-24 h after the consumption of 180 g of strawberries.

Subject	Anthocyanins	0-4 h	4-7 h	7-24 h	Total
1	Pelargonidin-*O*-glucuronide	16.5 ± 0.9	n.d.	n.d.	16.5 ± 0.9
	Pelargonidin-*O*-glucuronide	70.3 ± 4.6	28.3 ± 0.9	31.3 ± 1.4	129.9 ± 6.9
	Pelargonidin-*O*-glucuronide	27.13 ± 4.5	8.9 ± 0.4	n.d.	36.0 ± 4.9
	Pelargonidin-3-*O*-glucoside	27.1 ± 1.8	10.7 ± 0.3	6.4 ± 0.3	44.2 ± 2.4
	Pelargonidin-*O*-glucuronide	506.4 ± 2.6	229.9 ± 5.5	131 ± 5.3	867.3± 13.4
	Total	647.4 ± 14.4	278 ± 7.1	168.7 ± 7.0	1094 ± 28.5
	%	***(0.4%)***	***(0.2%)***	***(0.1%)***	***(0.7%)***
2	Pelargonidin-*O*-glucuronide	24.9 ± 2.5	8.5 ± 2.0	n.d.	33.4 ± 4.5
	Pelargonidin-*O*-glucuronide	100.8 ± 3.7	27.5 ± 0.7	n.d.	128.3 ± 4.4
	Pelargonidin-*O*-glucuronide	68.6 ± 9.4	11.5 ± 4.9	n.d.	80.1 ± 14.3
	Pelargonidin-3-*O*-glucoside	24.0 ± 2.4	6.8 ± 0.4	7.3 ± 0.0	38.1 ± 2.8
	Pelargonidin-*O*-glucuronide	1202.2 ± 56	188.5 ± 13.9	132.9 ± 5.1	1523.6 ± 75
	Total	1420.5 ± 74	242.8 ± 21.9	140 .2± 5.1	1803.5 ± 101
	%	***(1.0%)***	***(0.2%)***	***(0.1%)***	***(1.2%)***
3	Pelargonidin-*O*-glucuronide	19.7 ± 0.2	15.2 ± 2.5	n.d.	34.9 ± 2.7
	Pelargonidin-*O*-glucuronide	89.9 ± 1.9	86.3 ± 5.8	12.7 ± 4.8	188.9 ± 12.5
	Pelargonidin-*O*-glucuronide	67.2 ± 1.0	35.7 ± 1.8	n.d.	102.9 ± 2.8
	Pelargonidin-3-*O*-glucoside	32.9 ± 0.6	23.5 ± 2.9	0.12 ± 0.0	56.5 ± 3.5
	Pelargonidin-*O*-glucuronide	761.9 ± 15.6	708.8 ± 24.8	37.8 ± 2.0	1508.5 ± 42.4
	Total	971.6 ± 19.3	869.5 ± 37.8	50.6 ± 6.8	1891.7 ± 63.9
	%	***(0.7%)***	***(0.6%)***	***(0.03%)***	***(1.3%)***
4	Pelargonidin-*O*-glucuronide	20.8 ± 3.2	8.4 ± 0.9	23.3 ± 5.5	52.5 ± 9.6
	Pelargonidin-*O*-glucuronide	50.6 ± 0.8	19.6 ± 0.04	24.2 ± 3.4	94.4 ± 4.2
	Pelargonidin-*O*-glucuronide	24.8 ± 0.6	5.2 ± 0.5	11.1 ± 0.0	41.1 ± 1.1
	Pelargonidin-3-*O*-glucoside	21.0 ± 0.5	4.8 ± 1.1	6.0 ± 0.6	31.8 ± 2.2
	Pelargonidin-*O*-glucuronide	215.1 ± 1.5	98.5 ± 2.2	101± 10.3	414.6 ± 14
	Total	332.3 ± 6.6	136.5 ± 4.7	165.6±19.8	634.4 ± 31.1
	%	***(0.2%)***	***(0.1%)***	***(0.1%)***	***(0.4%)***
5	Pelargonidin-*O*-glucuronide	20.0 ± 1.7	n.d.	n.d.	20.0 ± 1.7
	Pelargonidin-*O*-glucuronide	54.3 ± 6.8	52.6 ± 4.9	20.7 ± 0.9	127.6 ± 12.6
	Pelargonidin-*O*-glucuronide	31.8 ± 1.5	13.1 ± 0.01	10.0 ± 0.0	54.9 ± 1.5
	Pelargonidin-3-*O*-glucoside	8.5 ± 3.3	7.9 ± 0.8	6.4 ± 1.1	22.8 ± 5.2
	Pelargonidin-*O*-glucuronide	286 ± 10.2	303 ± 9.4	92.6 ± 2.0	681.6 ± 21.6
	Total	401 ± 23.5	376.6 ± 15.1	129.7 ± 4	907 ± 42.6
	%	***(0.3%)***	***(0.3%)***	***(0.1%)***	***(0.6%)***

* Data for individual subjects are expressed as nmoles ± standard error (n=3). Figures in italics represent the percentage of the total amount of anthocyanins ingested. n.d.- not detected.

Table 4-15: Quantities of anthocyanins and % excreted in urine collected from five ileostomy volunteers 0-24 h after the consumption of 180 g of strawberries.

Peak	Anthocyanins	0-4 h	4-7 h	7-24 h	Total
1	Pelagonidin-*O*-glucuronide	20.4 ± 1.4	9.4 ± 4.6	1.7 ± 0.0	31.5 ± 6 ***(0.02%)***
2	Pelagonidin-*O*-glucuronide	73.2 ± 10	43.8 ± 12.0	16.9 ± 5.2	133.9 ± 27.2 ***(0.1%)***
3	Pelagonidin-*O*-glucuronide	43.9 ± 10	16.1 ± 5.0	3 ± 2	63 ± 17 ***(0.04%)***
4	Pelagonidin-3-*O*-glucoside	22.7 ± 4.1	11.0 ± 3.3	5.2 ± 1.3	38.9 ± 8.7 ***(0.03%)***
5	Pelagonidin-*O*-glucuronide	594.4 ± 182.4	306.1 ± 107.6	98.5 ± 17.5	999 ± 307.5 ***(0.68%)***
	Total	**754.6 ± 207.9**	**386.4 ± 132.5**	**125.3 ± 26**	**1266.3 ± 366.4**
	%	***(0.52%)***	***(0.26%)***	***(0.09%)***	***(0.87%)***

* Data presented as mean values in nmoles ± standard error (n=5 volunteers) and in italicised parentheses as percentage of the individual amounts of anthocyanins ingested.

4.3.2.4 Breakdown of anthocyanins in urine

Felgines *et al.*, (2003) reported in a human feeding study with strawberries, that urine metabolites of pelargonidin-3-*O*-glucoside disappeared or were substantially decreased if the samples were frozen prior to analysis. In this study, we also analyzed the urine sample immediately after collection. However, to investigate the potential breakdown we studied the stability of urinary anthocyanins from a healthy subject collected after strawberry intake, to assess the effect of freezing at −80°C for periods of one, two and five days. Triplicate urine samples of 200 µL were centrifuged at 4600g for 10 mins then injected directly to HPLC-PDA-MS^2. The data obtained in terms of total anthocyanins are presented in Table 4-16.

Table 4-16: Effect of freezing and storage on urine anthocyanin metabolites after the ingestion of 180 g of strawberries

Total anthocyanins metabolites	0-4 h	4-7 h	7-24 h	0-24 h
1st day*	3205 ± 34	640 ± 21	145 ± 11	3990 ± 66
2nd day	2810 ± 46	519 ± 6	146 ± 20	3475 ± 72
5th day	2710 ± 36	471 ± 3	91 ± 1	3272 ± 40

Data are expressed as nmoles ± standard error (n=1). 1st day* means samples were analyzed immediately after collecting.

The results show a reduction in the amount of anthocyanins in urine after freezing urine samples. The total amounts of anthocyanins excreted for 24h decreased from 3990 ± 66 nmoles to 3475 ± 66 nmoles in the second day of freezing accounting for 87% recovery on the first day (Figure 4-7). The reduction in the total amount of anthocyanins in the urine between the first and second days was statistically significant ($P<0.03$). At day 5, the total amount of anthocyanins continued to decrease significantly ($P<0.001$) where 82% was recovered compared to the first day (Figure 4-7). From this preliminary test, it seems that strawberry anthocyanins in urine reduced when the urine samples are stored for a longer time in -80 °C. However, to know the real effect of freezing on urine metabolites after the ingestion of strawberries, an appropriate investigation would give more details about this phenomenon but this was not in the scope of this study. In contrast, Mullen *et al.*, (2008b), observed that the pelargonidin glucuronide content of plasma samples collected after strawberry ingestion and stored at -80 °C for 1 month

prior to analysis was the same as that of samples analyzed immediately after collection. Therefore, this breakdown could be due to the presence of compounds in urine derived from the strawberries.

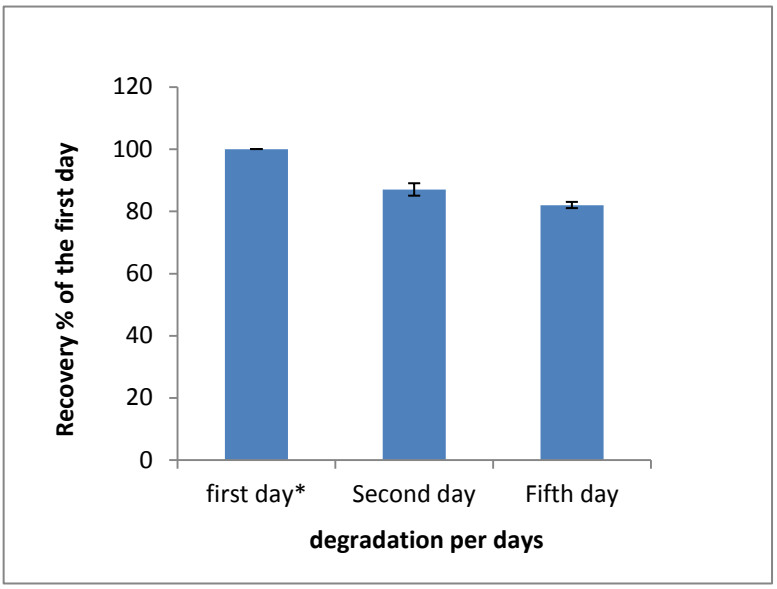


Figure 4-7: Breakdown of total anthocyanin metabolites in urine samples 0-24h stored in -80 ºC for one healthy subject after the consumption of 180 g of strawberries. Data are expressed as % ± standard error (n=1).1st day* means samples were analyzed immediately after collecting.

4.4 Discussion

The aim of this chapter was to evaluate the bioavailability of two anthocyanins, cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside with the use of ileostomy subjects. This is the first comparative investigation about the bioavailability of these two pigments in urine as well as ileal fluid after blackberry and strawberry ingestion by ileostomists. A preliminary step involved examining the anthocyanin content of blackberries and strawberries used in this study. Cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside represented 88% and 79% of the total anthocyanins in blackberries and strawberries, respectively. Other minor anthocyanins were present in much smaller amounts in both berries.

To date, little data have been available concerning the intestinal availability of anthocyanins in humans. Borges *et al.,* (2004) and Gonzalez-Barrio *et al.*, (2010) published data on raspberry bioavailability with an ileostomy and healthy subjects. Kahle *et al.,* (2006) and Kraus *et al.,* (2010) also investigated the intestinal availability of blueberry and bilberry anthocyanins in ileostomists.

The current study shows that after the ingestion of 180 g of blackberries and strawberries by ileostomy subjects on separated occasions, no metabolites were detected in the ileal fluid samples collected at any time point over the 24 h collection period. The anthocyanins detected in ileal fluid samples were the native, unmetabolised form of anthocyanins that were originally present in the berries. The analysis of ileal fluid collected 0-24 h after ingestion of blackberries and strawberries revealed the presence of low amounts of the native anthocyanins, with a 5.1% and 13.6% recovery of the total intake respectively (Table 4-3 and Table 4-12). The results also show that after ingestion of blackberries most anthocyanins appeared in ileal fluid in the 0-4 h collection period while with strawberries most anthocyanins were detected in the 4-7 h ileal fluid. This could be due to person to person variations which was clear in the strawberry study as two subjects had the highest anthocyanins concentration at 0-4h and the other three subjects at 4-7h. Aglycones of cyanidin and pelargonidin were not detected in either ileal fluid or urine and this is in agreement with Kahle *et al.,* (2006); Kraus *et al.,* (2010) and Gonzlez-Barrio *et al.*, (2010).

The limited information about anthocyanin bioavailability in ileal fluid reported their poor absorption from the small intestine. In total, 40% and 46% of unmetabolized anthocyanins were recovered in ileal fluid after the consumption of 300 g of raspberries and bilberries, respectively (Gonzalez-Barrio *et al.,* 2010; Kraus *et al.,* 2010). These findings were not in agreement with the current study results and are probably a consequence of the fruits containing different profiles of anthocyanins. Raspberries and bilberries contain a diversity of anthocyanin structures which complicates interpretation of data on the anthocyanin content of ileal fluids, whereas only one principal anthocyanin was present in the berries in the current study. It is of interest, however, to note that Borges *et al.,*(2004) and Gonzalez-Barrio *et al.,* (2010) both observed a low recovery of cyanidin-3-*O*-

glucoside in ileal fluids (5.9% and 6.2% respectively) compared to other cyanidin glycosides and to other anthocyanins. Kahle *et al.,* (2006) also reported a low recovery of cyanidin-3-*O*-glucoside in ileal fluid after blueberry intake.

This limited recovery of cyanidin-3-glucoside in ileal fluid is unlikely to be due to preferential absorption as plasma levels and urinary excretion of cyanidin-based anthocyanins are low (see Tables 3-4 and Table 4-6). The lower levels of cyanidin glucoside leaving the small intestine could be due to higher absorption from the stomach. The possibility of cyanidin glucoside absorption from the stomach has been demonstrated by (Passamonti *et al.,* 2003; Talavera *et al.,* 2003; He *et al.,* 2009). An *in vitro* study using mice intestinal segments has shown that cyanidin-3-glucoside absorption varied according to the intestinal segments, with the highest absorption occurring in the jejunum with a maximum absorption rate of 55.3%, minor absorption occurring in the duodenum and no absorption occurring in the ileum or colon (Matuschek *et al.,* 2006). This finding indicates that cyanidin absorption can occur in the small intestine. However, its failure to accumulate in quantity in plasma or urine remains a paradox. It could be a consequence of deposition in body tissues but there is limited evidence to suggest that this occurs in any quantity. The dilemma is likely to remain until radiolabelled cyanidin-3-*O*-glucoside is fed to an animal model and its fate within the body determined.

Differences in the absorption in the small intestine or conjugation via the human metabolism of the ingested anthocyanins have been observed. For instance, the recoveries of the malvidin, petunidin, and cyanidin glycosides in the ileostomy fluids after the ingestion of bilberries (Kraus *et al.,* 2010) and blueberries (Kahle *et al.,* 2006) revealed that arabinoside was recovered in ileal fluids in higher quantities than galactosides. In addition, glucosides of malvidin and petunidin, which have methyl groups on the B-ring, were absorbed and metabolized less than their cyanidin and delphinidin counterparts that have a hydroxylated B-ring. In the current investigation, cyanidin-3-*O*-glucoside showed lower recovery in ileal fluid at 1.8% of intake to a 6.8% recovery for pelargonidin-3-*O*-glucoside. Arguably, this may reflect a higher instability of cyanidin-3-*O*-glucoside at physiological pH as it is converted into a colourless form and/or degradation products.

Since only a small part of dietary anthocyanins are absorbed, large amounts of the ingested compounds are likely to enter the colon. *In vitro* studies with faecal slurries have shown deglycosylation and degradation of monoglucosides and diglucosides of anthocyanins due to the activity of colonic microflora and subsequently excreted in urine (Aura *et al.*, 2005; Gonzalez-Barrio *et al.*, 2011). Keppler and Humpf (2005), reported that cyanidin-3-*O*-rutinoside was first transformed by gut microflora into the corresponding glucoside and then converted to phenolic acids which can be further metabolized by the gut microflora or degraded due to pH condition in the colon (Vitaglione *et al.*, 2007). The anthocyanin nucleus is broken down and protocatechuic acid is detected as a product of the human colonic bacteria. Pelargonidin-3-O-glucoside was also reported to be breakdown to 4-hydroxybenzoic acid (Azzini *et al.*, 2010). Knowing which anthocyanins and in what forms and quantity they reach the colon remain questions of importance. Phenolic acids produced as a degradation products of anthocyanins by the action of colonic bacteria are acknowledged in the area of bioavailability as they might exert direct protective effects within the large intestine by mechanisms such as scavenging reactive nitrogen, antioxidant activity and inhibition of cyclooxygenases and lipoxygenases (Wang and Stoner 2008). While these mechanisms suggest that anthocyanins may prevent colon cancer, no direct link with a decrease in cancer risk were established yet (Del Rio *et al.*, 2012).

On the basis of the *in vitro* incubation data obtained compared with the urinary excretion of phenolic acid after anthocyanins ingestion, this would provide information on phenolic acids produced by colonic degradation that were absorbed and subjected to further phase II metabolism, most probably in the liver, before being excreted in urine. However, in the current study time constraints prevented the analysis of phenolic acids in urine as potential indicators of degradation of anthocyanins by colonic bacteria.

The current study also provided information on acylated anthocyanins in ileal fluid after the ingestion of berries. Relatively high amounts of acylated cyanidin and pelargonidin were detected, especially cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside, cyanidin-3-*O*-(6″-*O*-dioxaylglucoside) and pelargonidin-3-*O*-(6"-*O*-malonoyl)glucoside (Table 4-3 and Table 4-12). However, none of the

acylated anthocyanins were dectected in urine implying that they were not absorbed in the small intestine. Wu *et al.,* (2004a) reported that after pigs consumed blackberries acylated anthocyanins were less bioavailable than nonacylated forms. The relatively high recovery of acylated anthocyanins in ileal fluid indicates that acylation increases anthocyanin stability and/or reduces absorption from the small intestine.

Human studies on the metabolism of blackberry cyanidin and strawberry pelargonidin glucoside in ileostomiests and in healthy subjects are limited, particularly ileostomy data. To the best of our knowledge, only one study has reported the excretion of blackberry anthocyanins in human urine (Felgines *et al.,* 2005), while five studies have dealt with the bioavailability of strawberry anthocyanins. Three of them investigated only urinary anthocyanins (Felgines *et al.,* 2003; Carkeet *et al.,* 2008; Hollands *et al.,* 2008) and two reported on the anthocyanin content of both urine and plasma (Mullen *et al.,* 2008b; Azzini *et al.,* 2010). From these studies, the urinary excretion of pelargonidin-3-*O*-glucoside was high compared to other anthocyanins, with recoveries ranging from 0.75% to 2.4% of intake. In the present study, 0·87% of pelargonidin-3-*O*-glucoside were recovered in urine. This percentage is similar to those obtained by Azzini *et al.,* (2010) and Mullen *et al.,* (2008b) but lower than those reported by Felgines *et al.,* (2003); Carkeet *et al.,* (2008) and Hollands *et al.,* (2008). The higher recovery in these reports suggests that consumption of sweetened strawberries or strawberries as part of a typical breakfast may increase the bioavailability of pelargonidin-3-*O*-glucoside (Felgines *et al.,* 2003; Carkeet *et al.,* 2008; Hollands *et al.,* 2008).

The total urinary excretion of cyanidin and pelargonidin metabolites reported in this chapter accounted for 0.06 % vs. 0.87% of the respective intakes. The recovery of cyanidin glucoside in urine was >15-fold lower than that observed with pelargonidin glucoside. Because both anthocyanins were 3-*O*-glucosides this emphasizes the influence of the structure of the anthocyanidin moiety and the reduced absorption as a consequence of a 3′,4′-dihydroxy structure compared to that of 3′-hydroxylation.

Following deglucosylation of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside, glucuronidation was the major metabolic route while cyanidin was also methylated resulting in the formation of peonidin derivatives. The results showed that cyanidin-3-*O*-glucoside was metabolised primarily to peonidin-*O*-glucuronide as illustrated in Figure 3-6 in Chapter.3; whereas, pelargonidin-*O*-glucuronide was the main metabolite of pelargonidin-3-*O*-glucoside.

Among the urinary metabolites, monoglucuronides of cyanidin/peonidin in blackberries and pelargonidin in strawberries represented 68% and 79% of the total metabolites excreted in urine. Only a small amount, accounting for 21% and 3%, of the native glucoside form of cyanidin and pelargonidin, respectively, were detected in urine of ileostomists. These results are in accordance with the previous studies which reported monoglucuronides of cyanidin and pelargonidin as the most abundant anthocyanins excreted in human urine (Felgines *et al.,* 2003; Felgines *et al.,* 2005; Carkeet *et al.,* 2008; Mullen *et al.,* 2008b; Azzini *et al.,* 2010).

The current data with ileostomy subjects showed that the sulfated derivatives of pelargonidin were not detected in urine. This is in agreement with Carkeet *et al.,* (2008) and Azzaini *et al.,* (2010) with healthy subjects. Azzaini *et al.,* (2010) hypothesised that the absence of pelargonidin sulfate in their study and that of Carkeet *et al.,* (2008) reflected the low dose of pelargonidin glucoside ingested which were respectively 13 and 54 μmoles. In other studies where pelargonidin-sulfate was detected in urine, pelargonidin glucoside intake was higher than 100 μmoles (Felgines *et al.,* 2003; Hollands *et al.,* 2008; Mullen *et al.,* 2008b). Thus, it was hypothesised that at high doses, saturation of the pelargonidin glucuronidation pathway and initiation of pelargonidin-sulfation pathway may occur. However, this hypothesis is inconsistent with the absence of pelargonidin sulfate in the current study as we used a high dose of 146.4 μmoles. Mullen *et al.* (2008b), in a similar study with healthy subjects who consumed strawberries without cream, detected 1-2 nmoles of a pelargonidin sulfate in 5-8 h and 8-24 h urine. Therefore, the absence of pelargonidin sulfate might be due to very low metabolite concentrations (under the limit of detection) and/or late formation of this metabolite in the distal part of the small intestine. The absence of sulfate metabolites further implies that as far as cyanidin and pelargonidin-based anthocyanins are concerned sulfation does not occur in the liver.

The urinary excretion of anthocyanins by ileostomy volunteers after blackberry intake was slightly lower than with healthy volunteers, corresponding to 0.06% and 0.08% of the amount consumed, respectively (Table 4-6). This was due to the excretion of more peonidin metabolites in the urine of healthy subjects. Furthermore, this could well be a consequence of the small size of the sample groups and person to person variations rather than any inherent differences between the two groups of volunteers.

4.5 Conclusion

A summary of cyanidin-3-*O*-glucoside and pleargonidin-3-*O*-glucoside bioavailability data obtained from this chapter and chapter 3 are presented in Table 4-17.

Table 4-17: The main characteristics results found in blackberry and strawberry studies with ileostomists in the present chapter comparing to results found in blackberry and strawberry studies with healthy volunteers in chapter 3 and previously published data.

study	Dose ingested & Subject No.	Results		
		Plasma 24 h	Ileal fluid nmol in 24 h	Urine nmol in 24 h
Blackberry with healthy volunteers	295 ± 3 (µmole/180g) 8 volunteers	Pen-glu: C_{max} 20 ± 2.4nmol/L t_{max}: 2h. Cyn-glc: C_{max} 17 ± 2.4nmol/L t_{max}: 0.9h. Cyn: n.d.	NA	Total ANC 206.5 ± 49.2 ***(0.08%)*** Pen-glu: 0.03% Cyn-glc: 0.01% Cyn: n.d.
Blackberry with ileostomy volunteers	199 ± 7 (µmole/180g) 5 volunteers	NA	Total ANC 10227 ± 5343 ***(5.1%)*** Cyn-glc: 1.8% Cyn: n.d.	Total ANC 117 ± 24 ***(0.06%)*** Pen-glu: 0.03% Cyn-glc: 0.01% Cyn: n.d.
Strawberry with healthy volunteers*	222 ± 19 (µmole/200g) 8 volunteers	Plg-glu: C_{max} 274 ± 24 nmol/L t_{max}: 1.1h. Ple: n.d.	NA	Total ANC 1672 ± 551 ***(0.75%)*** Pel-glu: 0.67% Ple-glc: 0.01% Ple-sulph: 0.001% Pel: 0.01%
Strawberry with ileostomy volunteers	146.4 ± 1.6 (µmole/180g) 5 volunteers	NA	Total ANC 19995 ± 9096 ***(13.6%)*** Pel-glc: 6.8% Pel: n.d.	Total ANC 1266.3 ± 366.4 ***(0.87%)*** Pel-glu: 0.68% Ple-glc: 0.03% Ple-sulph: n.d. Pel: n.d.

Cyn-glc, cyanidin-3-*O*-glucoside; Cyn-glu, cyanidin-*O*-glucuronide; Pen-glu, peonidin-*O*-glucuronide; pel-glc, pelargonidin-3-*O*-glucoside; pel-glu, pelargonidin-*O*-glucuronide; Ple-sulph, pelargonidin-*O*-sulfate; Cyn, cyanidin; Pel, pelargonidin; ANC, anthocyanins; C_{max}, maximum post-ingestion plasma concentration; t_{max}, time reach C_{max}, NA, not applicable.

* Mullen, *et al.*, (2008b). Journal of Agricultural and Food Chemistry 56(3):713-719.

In this chapter, the absorption of cyanidin-3-*O*-glucoside and pleargonidin-3-*O*-glucoside into the human circulatory system and their excretion in urine was investigated. Five human subjects with an ileostomy consumed 180 g of blackberries and strawberries containing 199 and 146 μmoles of anthocyanins respectively. Samples of urine and ileal fluid were collected over a 0-24 h period after ingestion. All the anthocyanins identified in blackberries were detected and quantified in ileal fluid in their native form by HPLC-PDA-MS^2. Whereas, six of the nine native anthocyanins identified in strawberries were detected in ileal fluid.

The recovery of total anthocyanins in ileal fluids after the ingestion of blackberries and strawberries was 5.1% and 13.6% respectively. The low recovery of these compounds in ileal fluid indicates that in healthy subjects with a colon, small quantities of the intact anthocyanins pass from the small to the large intestine, where they will be catabolised by the gut microflora. Therefore, the low levels of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside emerging in ileal fluid do not appear to reflect substantial absorption of the anthocyanins in the small intestine and only trace levels were detected in plasma and urine. It could be a consequence of a lack of stability at the non-acid pH that prevails in the small intestine.

The overall urinary excretion of anthocyanins was 0.06% and 0.87% of intake of blackberries and strawberries respectively. Urinary excretion of both berries' anthocyanins occurred principally 0-4 h after consumption. These results are in keeping with those of Mullen *et al.*, (2008b) which demonstrated that pelargonidin and cyanidin glucoside were absorbed from the gastrointestinal tract and appear in the plasma and urine mainly as glucuronidated conjugates in humans. Following deglycosylation, glucuronidation was the major metabolic pathway for cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside in addition to methylation for cyanidin-3-*O*-glucoside. Cyanidin-3-*O*-glucoside was metabolised primarily to peonidin-*O*-glucuronide; whereas, pelargonidin-*O*-glucuronide was the main metabolite for pelargonidin-3-*O*-glucoside. However, the parent forms of these compounds were also detected in urine samples.

Although low recovery of the two compounds are reported in this investigation in both ileal fluid and urine, pelargonidin-3-*O*-glucoside showed slightly higher

recovery in human biological fluid compared to cyanidin-3-*O*-glucoside. This may reflect the influence of the aglycone structure on the bioavailability of these anthocyanins.

The results of this chapter and chapter 3 highlighted the bioavailability of cyanidin-3-*O*-glucoside in healthy subjects with intact functioning colon and ileostomy subjects. Cyanidin-3-*O*-glucoside was quickly absorbed and methylated mainly to peonidin-*O*-glucouronide and transported into the circulatory system. Urinary excretion by ileostomy volunteers was slightly lower than healthy volunteers corresponding to 0.06% to 0.08% of the amount consumed respectively within 24 h.

To have more information about the bioavailability of anthocyanins in general and for cyanidin and pelargonidin glucosides in particular, the present results suggest the importance of investigating the stability of anthocyanins in the gastrointestinal tract using *in vitro* models. Therefore, the coming chapter will aim to examine the stability of anthocyanins in the mouth and saliva, stomach and small intestine models.

5. Gastrointestinal stability of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside *in vitro*.

5.1 Introduction

The assessment of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside absorption, metabolism and excretion *in vivo* with human subjects (healthy and ileostomy volunteers) described in the previous chapters (Chapter 3 and 4) confirmed the low bioavailability of these compounds as has been documented in previous bioavailability studies with anthocyanins. Arguably, the seemingly low bioavailability of anthocyanins casts doubt on their ability to exert their proposed beneficial effects on health.

To understand anthocyanins bioavailability further, it is essential to determine their fate throughout the gastrointestinal tract and understand how the digestion process affects their stability as this, in turn, will affect their availability for uptake as well as their possible beneficial effects *in vivo*. As discussed in Chapter 1, there are many factors that may affect anthocyanin stability including the effect of pH, temperature, and gastrointestinal enzymes. Obtaining information on the relative stability of anthocyanins under gastrointestinal tract conditions is, therefore, a critical factor affecting their bioavailability.

The gastrointestinal tract is characterised by regions with different pH, enzymes and different microbial populations which effect and modify anthocyanins structure. In the upper gastrointestinal tract, digestion of food starts in the mouth by mixing with saliva which contains α-amylase for starch digestion at pHs ranging from 6.5–7 (Aura *et al.*, 2005). It has been suggested that saliva is able to hydrolyze flavonoid glycosides (Hirota *et al.*, 2001; Walle *et al.*, 2005), but because of the limited period in the mouth this has not been considered an important factor in flavonoids bioavailability. Insight regarding oral uptake and metabolism of anthocyanins remains limited. Recently, Kamonpatana and his co-researchers (2012), reported degradation of anthocyanins in human saliva which was dependant on the structure of anthocyanins and largely mediated by oral microflora.

In the stomach the pH of the gastric juice is low, ranging from 1–2, which should ensure that anthocyanins are maintained as the flavylium cation, which is the most stable anthocyanin structure (McGhie and Walton 2007). In contrast, the environment of the small and large intestines is largely at neutral pH, where anthocyanins are much less stable and converted to a combination of hemiketal, chalcone and quinonidal forms (McDougall *et al.,* 2005a; McGhie and Walton 2007; Tagliazucchi *et al.,* 2010). The information describing the effects of the digestion process on dietary anthocyanins in the stomach and small intestine *in vivo* and *in vitro* is limited. *In vivo* studies of the stability and metabolism of anthocyanins of rat intestine and stomach were reported by He *et al.,* (2005; 2009) and in the pig gastrointestinal tract by Wu *et al.,* (2006b). *In vitro* methods that simulate digestion conditions were used to investigate the effects of *in vitro* digestion on dietary anthocyanins in raspberry (McDougall *et al.,* 2005a), red wine and red cabbage (McDougall *et al.,* 2005b; McDougall *et al.,* 2007), chokeberry (Bermudez-Soto *et al.,* 2007), apple (Bouayed *et al.,* 2011), Mulberry (Liang *et al.,* 2012) and grape (Tagliazucchi *et al.,* 2010; Stalmach *et al.,* 2012). The results obtained from these studies have shown that *in vitro* methods are very useful for evaluating the effects of digestion conditions on the stability and properties of anthocyanins.

In this chapter we examined the effect of saliva on degradation of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside. In addition, the stability of blackberry and strawberry anthocyanins was investigated using an *in vitro* model that simulates some of the conditions (pH, temperature and enzymes) in the stomach and the duodenum. Monitoring the recovery of individual anthocyanins was achieved by using HPLC-PDA-MS^2. Furthermore, comparison of *in vitro* results with data obtained previously with the *in vivo* human studies will provide further details about anthocyanin bioavailability and stability during their passage through the gastrointestinal tract, especially in the mouth and stomach.

5.2 Materials and Methods

5.2.1 Chemicals and reagents

HPLC grade methanol was obtained from Rathburn chemicals Ltd., (Walkerburn, UK). Formic acid, Sodium chloride, hydrochloric acid, monobasic potassium phosphate, Sodium hydroxide, Sodium bicarbonate, penicillin/streptomycin antibiotic, pepsin, pancreatin and bile were supplied by Sigma (Poole, Dorset, UK). Cyanidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside were supplied by Extrasynthes (Genay, France). Blackberries and strawberries were purchased from a local supermarket as described in Chapters 3 and 4.

5.2.1 Preparation of blackberry and strawberry extracts

Ten grams of frozen strawberries and blackberries were extracted as described in Chapter 3.

5.2.2 *In vitro* digestion model

Collection of human saliva: after an overnight fast and prior to breakfast, saliva samples were collected in sterile centrifuge tubes from six healthy, non-smoking subjects (One man and five woman; 25-35 years old) without them having brushed their teeth since the previous evening (Walle *et al.*, 2005).

Collection of human gastric juice: human gastric juice with a pH of ~1.8 was collected from one volunteer in Gartnavel Hospital by Ms. Angela Wirz, a research nurse.

It should be noted here that an ethical approval for the use of biological samples such as saliva and human gastric juice must be obtained. However, as limitation of this work this has not been done.

Gastric juice was prepared by dissolving 2 g of sodium chloride and 3.2 g of pepsin in 7 mL of hydrochloric acid and made up to 1000 mL with distilled water and adjusted to pH 1.2.

Intestinal juice was prepared by dissolving 6.8 gram of monobasic potassium phosphate in 250 mL of water and adding to a mixture of 77 mL of 0.2 N of sodium hydroxide and 500 mL of distilled water. Ten g of pancreatin was added to the mixture and mixed well and adjusted to pH 6.8 with 0.2 N of sodium hydroxide. Finally the intestinal juice was diluted with water and made up to 1000 mL.

Bile extract was prepared by adding 10 g of bile into 100 mL of 0.5 N of sodium bicarbonate.

5.2.3 Incubation condition

5.2.3.1 Saliva incubation

The saliva incubation method was adapted from (Walle *et al.,* 2005). Collected saliva was mixed together and diluted 1:1 with distilled water and shaken to reduce viscosity. The diluted saliva was divided into two aliquots (A) diluted saliva and (B) diluted saliva containing 1% penicillin/streptomycin. For the two aliquots, 1 mL of diluted saliva was added to vials containing 0.1 g of homogenized blackberry and strawberry fruit, prepared using an Ultra-Turrax T25 homogeniser (IKA Werke, Staufen, Germany). The vials were then incubated in a shaking water bath in darkness for 0, 0.5, 1, 2 and 5 mins at 37ºC. At the end of the incubation period vials were placed immediately in liquid nitrogen to stop the enzymatic reaction and then stored at -80ºC prior to analyses. Control samples of 0.1 g of fruits in distilled water were subjected to the same procedure described above. Incubated samples were defrosted and centrifuged, after which 10 µL of aliquots were analysed by HPLC-PDA-MS^2.

5.2.3.2 Simulated gastric and pancreatic juices incubation

The *in vitro* gastric and pancreatic digestion of blackberries and strawberries was assessed following a previously published method (Bermudez-Soto *et al.,* 2007; Stalmach *et al.,* 2012). Three g of frozen blackberries and strawberries were homogenized using an Ultra-Turrax T25 homogeniser (IKA Werke, Staufen, Germany). Samples of the berries were placed in falcon tubes at

different times 0 h, 1 h, 2 h, 4 h and 6 h. For the gastric digestion, 1.7 mL of gastric juice containing 0.32 (w/v) of pepsin (USA Pharmacopoeia, 1990) was added to the sample tubes and adjusted to pH 2.0 with HCL. To avoid the effect of light and reduce the level of oxidation on the recovered anthocyanins, incubations were carried out in reduced light conditions and under N_2 (vials flushed for 10 mins) and sealed after the addition of the enzymes and incubated in a shaking bath in darkness at 37°C for 1 h. At the end of gastric digestion, aliquots were centrifuged and 10 µL samples of supernatant analysed by HPLC-MS. The remaining sample was neutralized with $NaHCO_3$ (pH ~7) and 3.3 mL of intestinal juice containing 1% (w/v) of pancreatin and 1.6 mL of bile juice (1% w/v) were added. Samples were further flushed with N_2 and incubated in darkness in a shaking water bath for for 2 h, 4 h and 6 h at 37°C. After the pancreatic digestion, aliquots of the digested samples were rapidly acidified to pH 2.0 with HCl prior to centrifugation and analysis by HPLC-MS.

5.2.4 Human gastric juice incubation

The same protocol that was used with simulated gastric juice was followed. Human gastric juice, 1.7 mL, was added to 3 g of the homogenised blackberries and strawberries in falcon tubes. The incubation tubes were covered with aluminium foil to avoid the effect of light and flushed for 10 mins with N_2 and sealed to reduce the level of oxidation on anthocyanins. The tubes were incubated in a shaking bath at 37°C for either 0 h or 1 h. At the end of gastric digestion, aliquots were centrifuged and the anthocyanin content 10 µL samples of supernatant were analysed by HPLC-MS.

5.2.5 HPLC with diode array detection and tandem mass spectrometry

Anthocyanins in blackberries, strawberries and their breakdown products in saliva and gastric and pancreatic juices were analyzed on a Surveyor HPLC system comprising a HPLC pump, a diode array absorbance detector, scanning from 200 to 600 nm, and an autosampler cooled to 4°C (Thermo Electron Corp). Separation was carried out using a 250 x 4.6 mm i.d C12 4 µm, Synergi RP-Max column (Phenomenex, Macclesfield, UK) with a gradient

60 min of 5–50% with methanol in 1% formic acid at a flow rate of 1 mL/min and maintained at 40°C. After passing through the flow cell of the diode array detector, the column eluate was split, and 0.3 mL min was directed to an LCQ Advantage ion trap mass spectrometer fitted with an electrospray interface (Thermo Electron Corp). Analyses utilized the positive ion mode as this provided the best limits of detection for anthocyanins. The sample was analyzed in the mass spectrometer in full scan, data-dependent MS^2 scanning from *m/z* 200 to 600. The tuning of mass spectrometer was optimised by infusing a standard of cyanidin-3-*O*-glucoside. Capillary temperature was 300°C, sheath gas and auxiliary gas were 40 and 20 units, respectively, and the source voltage was 3 kV.

Anthocyanins were quantified from their chromatographic peak areas and expressed as cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside equivalents. The identifications were confirmed by full-scan MS^2 and/or SRM.

5.2.6 Data analysis

HPLC-MS data were processed using Xcalibur QualBrowser version 1.0.1.03 software (Thermo Fisher Scientific Inc., 2009). Each sample was analysed in triplicate and the data presented as mean value ± standard error.

5.3 Results

5.3.1 Saliva incubation with cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside

The influence of human saliva on cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside from blackberry and strawberry fruits was investigated in two groups of tests under aerobic conditions at 37ºC for 5 mins. Data on the recovery of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside after saliva incubation are presented in Figure 5-1. In group A, the blackberry and strawberry anthocyanins were incubated with the diluted saliva. Under this condition, cyanidin-3-*O*-glucoside was stable with a recovery of 101 ± 4% indicating a lack of effect of the salivary enzymes. Hydrolysis of the anthocyanins into the corresponding aglycone by the salivary enzymes was not observed within the 5 min incubation period. In group B, incubations

were performed with saliva in the presence of antibiotics (penicillin/streptomycin) to determine the importance of bacterial flora. Cyanidin-3-*O*-glucoside was stable and its recovery was 101 ± 6% and no hydrolysis was recorded. These results demonstrate that oral enzymes and oral microflora had no effect on cyanidin-3-*O*-glucoside. However, in both groups A and B, pelargonidin-3-*O*-glucoside was unstable, with 79 ± 9% and 78 ± 4%, respectively, being recovered. No pelargonidin aglycone was detected. This indicates that the pelargonidin glucoside is less stable in the mouth than its cyanidin counterpart.

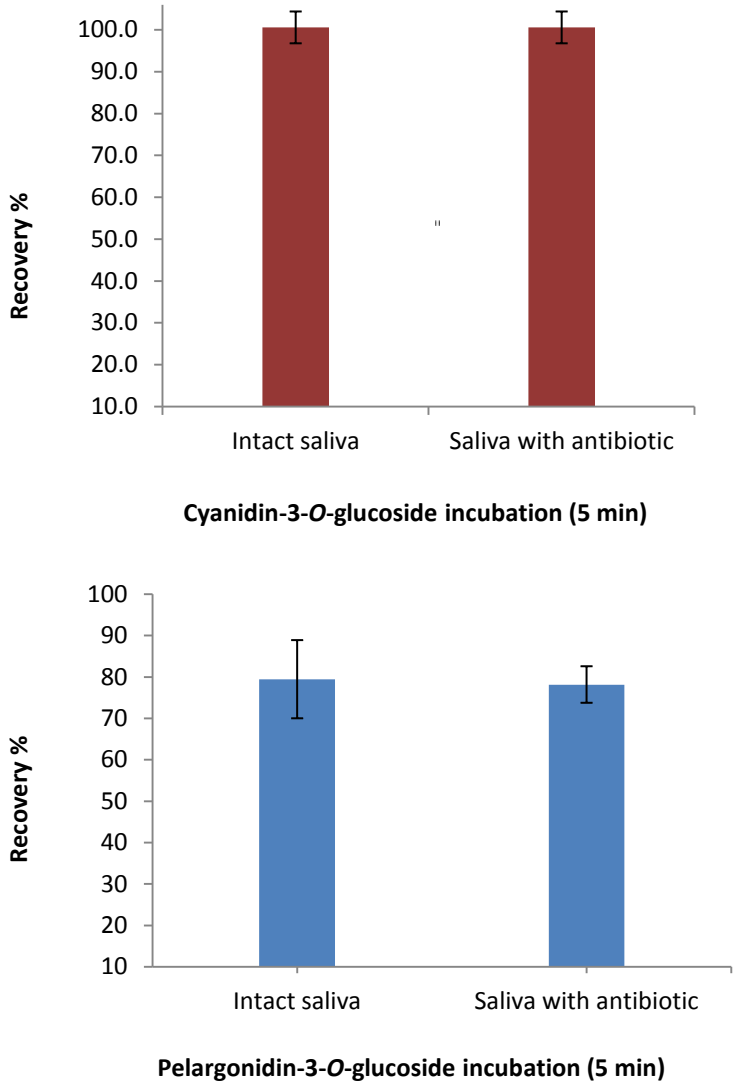


Figure 5-1: Total recovery of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside from blackberries and strawberries incubated with human saliva. Data represent mean values ± standard error (n = 3) as percentage of the incubated dose

5.3.2 Human gastric and simulated gastric juice incubation

The *in vitro* stability of anthocyanins from blackberries and strawberries in the stomach was assessed by incubation with human and synthetic gastric juices.

Changes during the *in vitro* digestion of blackberries are presented in Table 5-1. After one hour incubation with the gastric juices at pH ~2.0, anthocyanin levels decreased with 157.1 ± 1.9 and 130 ± 17.4 µmol/180 g being detected in the human and artificial gastric juices respectively, which corresponds to 53.5 ± 0.6% and 44.1 ± 5.9% recoveries of the incubated anthocyanin. A similar recovery of cyanidin-3-*O*-glucoside in both the gastric juices confirms the occurrence of degradation during gastric digestion. In addition, some minor acylated cyanidins in blackberries such as cyanidin-3-(3″-*O*-malonoyl)glucoside and cyanidin-3-*O*-(6″-*O*-malonoyl)glucoside were also detected in decreased amounts after gastric incubation with recoveries of 39.1 ± 0.4% and 78.8 ± 0.0 % with human gastric juice and 30.7 ± 7% and 51.6 ± 11% with synthetic juice respectively.

Table 5-2 shows the recovery of pelargonidin-3-*O*-glucoside after incubation of homogenised strawberries with human and artificial gastric juice. In the case of the human gastric incubation, only 59.6 ± 1.3 µmole/180 g of the 146.4 µmole/180 g of incubated anthocyanins were detected after 1 h. This represents about a 40.7 ± 0.9% recovery. The simulated gastric juice incubation used a different batch of strawberries with lower anthocyanin content and only the three anthocyanins were evaluated: cyanidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside and pelargonidin-3-*O*-rutinoside. The results showed that overall only 41.1 ± 3.8% of the anthocyanins were recovered after gastric ingestion of 30.4 ± 1.4 µmole/180 g. Pelargonidin-3-*O*-rutinoside recoveries were 40.9 ± 1.1 % and 29 ± 2.6% with the human and artificial gastric juices respectively, while 36 ± 0.9% of pelargonidin-3-*O*-(6"-*O*-malonoyl)glucoside remained after incubation with human gastric juice. Generally, the results indicate a similar low recovery of anthocyanins in both human gastric and simulated gastric juice incubation reflecting the instability of anthocyanins in the stomach.

Table 5-1: Concentration of anthocyanin compounds in blackberries obtained following *in vitro* incubation with real human gastric juice and simulated gastric juice (n=3) and percentages recovered in 1 h after incubation.

Anthocyanins	Initial concentration (µmol/180 g)	Human gastric digestion (1 h) (µmol/180 g)	Artificial gastric digestion (1 h) (µmol/180 g)	% recovery in human gastric digestion	% recovery in artificial gastric digestion
Cyanidin-3-*O*-glucoside	187 ± 1	134 ± 1.8	109.1 ± 14	72 ± 0.9	58.4 ± 7.5
Cyanidin-3-*O*-sambubioside	1.5 ± 0.3	0.37 ± 0.03	0.38 ± 0.05	24.9 ± 1.7	25.4 ± 3.5
Cyanidin-3-*O*-arabinoside	0.4 ± 0.0	0.18 ± 0.00	0.10 ± 0.03	44 ± 0.03	23.2 ± 6.4
Pelargonidin-3-*O*-glucoside	3.6 ± 0.4	0.11 ± 0.001	0.25 ± 0.02	3.1 ± 0.05	6.9 ± 0.5
Cyanidin-3-*O*-(3″-*O*-malonoyl)glucoside	0.3 ± 0.0	0.12 ± 0.001	0.10 ± 0.02	39.1 ± 0.4	30.7 ± 7.0
Cyanidin-3-*O*-xyloside	5.0 ± 0.2	0.86 ± 0.03	2.10 ± 0.4	17.1 ± 0.4	41.7 ± 7.6
Cyanidin-3-*O*-(6″-*O*-malonoyl)glucoside	6.7 ± 0.4	5.3 ± 0.00	3.4 ± 0.7	78.8 ± 0.00	51.6 ± 11
Cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	45 ± 0.3	8.1 ± 0.05	7.2 ± 1.1	17.9 ± 0.08	16 ± 2.5
Cyanidin-3-*O*-(6″-*O*-dioxaylglucoside)	45 ± 0.3	8.1 ± 0.05	7.2 ± 1.1	17.9 ± 0.08	16 ± 2.5
Total	**295 ± 3**	**157.1 ± 1.9**	**130 ± 17.4**	**53.3 ± 0.6**	**44.1± 5.9**

Data expressed as mean values in µmol ± standard error.

Table 5-2: Concentration of anthocyanins compounds in strawberries obtained following *in vitro* incubation with real human gastric juice and simulated gastric juice (n=3) and percentages recovered in 1 h after incubation.

Anthocyanins	Initial concentration (µmol/180 g)	Human gastric digestion (1 h) (µmol/180 g)	Artificial gastric digestion (1 h) (µmol/180 g)	% recovery in human gastric digestion	% recovery in artificial gastric digestion
Pelargonidin-3,5-diglucoside	0.1 ± 0.01	0.03 ± 0.00	N.A.	30 ± 0.5	N.A.
Pelargonidin-3-*O*-(3"-*O*-malonoylglucoside)-5-glucoside	0.5 ± 0.02	0.11 ± 0.01	N.A.	22 ± 1.2	N.A.
Cyanidin-3-*O*-glucoside	0.9 ± 0.01	0.44 ± 0.01	0.48 ± 0.04[a]	48.8 ± 0.6	25.3 ± 2.1[a]
Pelargonidin-3-*O*-glucoside	116 ± 1.3	48.7 ± 1.1	27 ± 2.5[b]	41.9 ± 0.9	43.5 ± 4.1[b]
Pelargonidin-3-*O*-rutinoside	0.2 ± 0.02	0.08 ± 0.001	2.9 ± 0.3[c]	40 ± 1.1	29 ± 2.6[c]
Pelagonidin-3-*O*-(3"-*O*-malonoyl)glucoside	0.4 ± 0.01	0.08 ± 0.001	N.A.	20 ± 1.8	N.A.
Cyanidin-3-*O*-(6"-*O*-malonoyl)glucoside	0.2 ± 0.01	0.06 ± 0.001	N.A.	30 ± 1.3	N.A.
Pelargonidin-3-*O*-(6"-*O*-malonoyl)glucoside	28 ± 0.3	10.10 ± 0.2	N.A.	36 ± 0.9	N.A.
Pelargonidin-3-*O*-acetylglucoside	0.1 ± 0.01	n.d.	N.A.	n.d.	N.A.
Total	**146.4 ± 1.6**	**59.6 ± 1.3**	**30.4 ± 1.4**	**40.7 ± 0.9**	**41.1 ± 3.8**

Data expressed as mean values in µmol ± standard error. n.d, not detected; N.A, not analysed.
For a, b and c the initial concentration was different as follows: a) 1.9 ± 0.1 ; b) 62 ± 3.5 ; c) 10.1 ± 0.4 µmol/180 g.

5.3.3 Simulated pancreatic juices incubation

Following the 1 h incubation under simulated gastric conditions, blackberry and strawberry samples were transferred to the *in vitro* pancreatic digestion model with samples being analysed after 1, 3 and 5 h corresponding to overall incubation periods of 2, 4 and 6 h. Results are presented in Table 5-3 and Table 5-4. Total anthocyanins in blackberries decreased gradually with time to reach a 5.1 ± 0.9 % recovery of the initial dose after 6 h. This recovery was in accordance with the ileal fluid recovery detected with ileostomy volunteers in chapter 4 (see Table 4-3). All acylated forms of cyanidin, especially cyanidin-3-(3″-*O*-malonoyl)glucoside and cyanidin-3-*O*-(6″-*O*-malonoyl)glucoside, declined after 2 h incubation with pancreatic juice but thereafter did not continue to degrade significantly up to the end of the 6 h incubation.

Strawberry anthocyanins were affected in a similar manner with a final recovery of 5.1 ± 0.1% of the digested dose after the 6 h incubation. Once again, the recovery is similar to the recovery in ileal fluid (7.0 ± 2.8 % of the total) obtained after ileostomists ingested strawberries. Note that a different batch of strawberries was used in the *in vitro* incubations and the lower anthocyanin content meant that only the three main anthocyanins were evaluated.

Comparing the digestive stability of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside using the *in vitro* model that simulates some of the conditions (pH, temperature and enzymes) in the stomach and the duodenum, it is clear that both anthocyanins were unstable (Figure 5-1). The recoveries from the *in vitro* models were similar to those obtained *in vivo* with ileostomists.

Table 5-3: Concentration of anthocyanin compounds in blackberries obtained following *in vitro* incubation with simulated pancreatic juice (n=3) and percentages recovered in 0-24h ileal fluid following ingestion of 180 g of blackberries by ileostomy volunteers (n=5).

Anthocyanins	**Initial concentration (µmol/180 g)**	**Pancreatic digestion (µmol/180g)**			**% recovery in ileal fluid (24 h)**
		(2 h)	**(4 h)**	**(6 h)**	
Cyanidin-3-*O*-glucoside	187 ± 1	17 ± 1.1 ***(9 ± 0.6)***	16 ± 1.3 ***(8.6 ± 0.7)***	12.5 ± 2.5 ***(6.7 ± 1.3)***	1.6 ± 1.0
Cyanidin-3-*O*-sambubioside	1.5 ± 0.3	0.13 ± 0.04 ***(8.7 ± 2.9)***	0.04 ± 0.002 ***(2.7 ± 0.1)***	0.04 ± 0.002 ***(2.7 ± 0.2)***	0.03 ± 0.0
Cyanidin-3-*O*-arabinoside	0.4 ± 0.0	0.02 ± 0.00 ***(5 ± 0.01)***	0.01 ± 0.003 ***(2.5 ± 0.7)***	0.01 ± 0.001 ***(2.5 ± 0.3)***	n.d.
Pelargonidin-3-*O*-glucoside	3.6 ± 0.4	0.04 ± 0.002 ***(1.1 ± 0.04)***	0.04 ± 0.002 ***(1.1 ± 0.04)***	0.03 ± 0.002 ***(0.8 ± 0.01)***	0.02 ± 0.1
Cyanidin-3-*O*-*(*3″-*O*-malonoyl)glucoside	0.3 ± 0.0	0.02 ± 0.002 ***(6.7 ± 0.5)***	0.02 ± 0.00 ***(6.7 ± 0.01)***	0.02 ± 0.003 ***(6.7 ± 0.6)***	0.02 ± 0.1
Cyanidin-3-*O*-xyloside	5.0 ± 0.2	0.31 ± 0.04 ***(6.2 ± 0.8)***	0.27 ± 0.04 ***(5.4 ± 0.7)***	0.19 ± 0.04 ***(3.8 ± 0.8)***	0.02 ± 0.1
Cyanidin-3-*O*-(6″-*O*-malonoyl)glucoside	6.7 ± 0.4	0.52 ± 0.05 ***(7.8 ± 0.7)***	0.52 ± 0.03 ***(7.8 ± 0.5)***	0.41 ± 0.05 ***(6.1 ± 0.7)***	0.6 ± 0.5
Cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside	45 ± 0.3	1.1 ± 0.07 ***(2.4 ± 0.1)***	1.01 ± 0.06 ***(2.2 ± 0.1)***	0.9 ± 0.07 ***(2 ± 0.1)***	1.3 ± 0.6
Cyanidin-3-*O*-(6″-*O*-dioxaylglucoside)	45 ± 0.3	1.1 ± 0.07 ***(2.4 ± 0.1)***	1.01 ± 0.06 ***(2.2 ± 0.1)***	0.9 ± 0.07 ***(2 ± 0.1)***	1.3 ± 0.6
Total	**295 ± 3**	**20.2 ± 1.4** ***(6.8 ± 0.4)***	**19 ± 1.5** ***(6.4 ± 0.5)***	**15 ± 2.8** ***(5.1± 0.9)***	**5.1 ± 2.8**

Data expressed as mean values in µmol ± standard error. n.d. not detected.

Table 5-4: Concentration of anthocyanins compounds in strawberries obtained following *in vitro* incubation with simulated pancreatic juice (n=3) and percentages recovered in 0-24h ileal fluid following ingestion of 180 g of strawberries by ileostomy volunteers (n=5).

Anthocyanins	**Initial concentration (µmol/180 g)**	**Pancreatic digestion (µmol/180 g)**			**% recovery in ileal fluid (24 h)**
		(2 h)	**(4 h)**	**(6 h)**	
Cyanidin-3-*O*-glucoside	1.9 ± 0.1	0.09 ± 0.003 ***(4.7 ± 0.2)***	0.09 ± 0.001 ***(4.7 ± 0.1)***	0.08 ± 0.003 ***(4.2 ± 0.2)***	0.15 ± 0.6
Pelargonidin-3-*O*-glucoside	62 ± 3.5	4.03 ± 0.3 ***(6.5 ± 0.4)***	3.9 ± 0.1 ***(6.3 ± 0.2)***	3.2 ± 0.08 ***(5.2 ± 0.1)***	6.7 ± 2.2
Pelargonidin-3-*O*-rutinoside	10.1 ± 0.4	0.6 ± 0.03 ***(5.9 ± 0.3)***	0.6 ± 0.02 ***(5.9 ± 0.2)***	0.5 ± 0.01 ***(4.9 ± 0.1)***	0.13 ± 0.1
Total	**74 ± 4**	**4.7 ± 0.3** ***(6.3 ± 0.4)***	**4.6 ± 0.1** ***(6.2 ± 0.2)***	**3.8 ± 0.1** ***(5.1 ± 0.1)***	**7 ± 2.8**

Data expressed as mean values in µmol ± standard error.

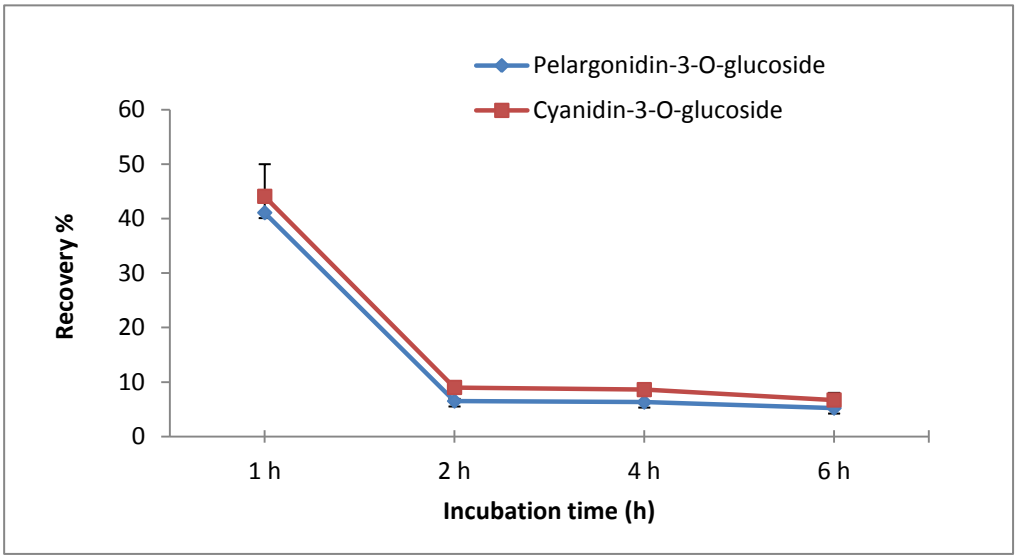


Figure 5-2: Recovery of cyanidin-3-*O*-glcoside and pelargonidin-3-*O*-glucoside expressed as a percentage of the initial amount incubated in simulated gastric juice at pH 2 (1 h) and simulated duodenal juice at pH 7 (2 h, 4 h, 6 h).

5.4 Discussion

Anthocyanins are ingested as a part of our daily diet. The chemical structure of anthocyanins is believed to be a major factor influencing their stability. They may be glycosylated and acylated by different sugars and acids. The increase in glycosidic substitution and the presence of aromatic acyl groups have been reported to improve anthocyanins stability (Cabrita *et al.*, 2000; Bakowska-Barczak 2005; Sari *et al.*, 2012; Zhang *et al.*, 2012a).

As mentioned in previous chapters, anthocyanins bioavailablity *in vivo* is low. The chemical structure and stability of these compounds affected their bioavailability throughout the gastrointestinal tract. Therefore, the chemical instability of anthocyanins must be taken into consideration when their biological properties are investigated using *in vitro* models because it can be assumed that the degradation and/or reaction products contribute to the observed biological effects. In this chapter, a number of experiments were carried out using *in vitro* digestion procedures that mimic the physiochemical and biochemical conditions that occur in the upper gastrointestinal tract to gain a greater understanding of the steps involved in the digestion of blackberry and strawberry anthocyanins in humans.

After consumption of food containing berries, the anthocyanins are released from the food matrix by chewing. Changes and probably absorption could start in the mouth as the anthocyanins come in contact with saliva. However, information about anthocyanins susceptibility to degradation under the effect of saliva is limited and unclear. Understanding the influence of anthocyanin structure on their stability and accessibility in the oral cavity is expected to facilite the development of products rich in anthocyanins that promote oral health. Several previous studies have demonstrated that saliva can hydrolyze certain flavonoid glycosides (Hirota *et al.*, 2001; Walle *et al.*, 2005; Kahle *et al.*, 2011). Walle *et al.,* (2005) have reported that hydrolysis of genistein conjugates is inhibited by antibacterial agents, suggesting a key role for oral microbiota in flavonoids metabolism in the oral cavity.

In the current study, the extent of degradation of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside in blackberries and strawberries in saliva collected from six healthy subjects was investigated. After incubation for 5 mins at 37°C with saliva and saliva containing antibiotics (penicillin/streptomycin), cyanidin-3-*O*-glucoside was found to be more stable than pelargonidin-3-*O*-glucoside, in both instances (Figure 5-1). In contrast, ~20% degradation of pelargonidin-3-*O*-glucoside was observed and as the antibiotics had no impact, this suggests that the breakdown was mediated principally by oral enzymes rather than bacteria. Recently, Kamonpatana *et al.,* (2012) examined the effect of anthocyanin structure on susceptibility to degradation by saliva and concluded that breakdown of anthocyanins during a 60 min incubation at 37°C was, in part, due to microbial activity. Degradation delphinidin and petunidin glycosides exceeded that of cyanidin, peonidin and malvidin conjugates. This contrasted with the data obtained in the current study presumably because the investigation of Kamonpatana and his co-researchers is a largely artificial situation as during the course of normal ingestion, foods are not retained in the oral cavity for 60 mins.

Another explaination for the degradation of pelargonidin-3-*O*-glucoside in the saliva is the pH impact. In the saliva, the pH was found to range from 6.5 to 7.0. Woodward *et al.,* (2009) investigated the stability of anthocyanins under physiological conditions where pelargonidin, cyanidin and delphinidin glucoside were incubated in water (pH 7 with the addition of dilute NaOH) at 37° C for 0,

2, 6, 12 and 24 h. They observed a low rate of degradation for the three anthocyanins over 24 h. Pelargonidin-3-*O*-glucoside showed the highest instability at 0 and 2 h. Anthocyanins have been also reported to degrade at neutral and alkaline pH in aqueous solution during a period of 0-60 days storage at 10 and 23° C (Cabrita *et al.,* 2000). They found that the stability of the 3-*O*-glucosides of pelargonidin, peonidin and malvidin (group A) decreased rapidly at pH 5-7 but were more stable at pH 8-9. On the other hand, delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside (group B) behaved differently as they exhibited higher stability in the pH 5-7 range and were unstable at more alkaline pH. In contrast, cyanidin-3-*O*-glucoside, which has a similar structure to group B anthocyanins, had stability behaviour closer to those in group B but more stable at pH 5-7 than pelargonidin glucoside.

Based on these findings by Woodward *et al.,* (2009) and Cabrita *et al.,* (2000), it seems that the presence of a single free hydroxyl group in the B-ring of anthocyanins, such as pelargonidin, peonidin and malvidin, decreases stability at neutral pH whereas it is improved under alkaline conditions; this is not the case for anthocyanins with a dihydroxy-B ring structure such as delphinidin-3-*O*-glucoside and petunedin-3-*O*-glucoside with the exception of cyanidin-3-glucoside as noted above.

The hydrolysis of flavonoids into the corresponding aglycone by salivary enzymes has been reported by (Hirota *et al.,* 2001; Walle *et al.,* 2005; Kahle *et al.,* 2011). However, this effect was not observed in the current study with blackberry and strawberry anthocyanins incubated with saliva for 5 mins. It is, however, in agreement with the findings of (Kamonpatana *et al.,* 2012) who reported that the degradation of cyanidin-3-*O*-glucoside after incubation with saliva for up to 60 min led to the presence of the cyanidin aglycone. However, assessing the ocurrence and the importance of oral hydrolysis on the stability of these compounds when they come into contact with the epithelium cells in the mouth is difficult due to the relatively short residence time of most food in the mouth.

Blackberries and strawberries were also incubated with *in vitro* models simulating gastric and pancreatic digestion. Under acidic conditions in the presence of pepsin, blackberry and strawberry anthocyanins were not stable in

both synthetic and human gastric juices with an average recovery of 53.3 ± 0.6% and 44.1 ± 5.9% for blackberries and 40.7 ± 0.9% and 41.1 ± 3.8% for strawberries (Table 5-1 and Table 5-2). However, these recoveries fell to 5.1± 0.9% and 5.1± 0.1% for blackberries and strawberries respectively, following simulated digestion with pancreatin and bile extract (Table 5-3 and Table 5-4). These recoveries where similar to the results obtained in 0-24 h ileal fluid collected from ileostomists after blackberry and strawberry intake (Table 5-3 and Table 5-4). These findings indicate that a small amount of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside will most likely pass from the small to the large intestine.

Anthocyanins have been reported to be stable with recoveries ranging from 88-100% under the acidic condition of simulated gastric juice *in vitro* due to the stability of the flavylium cation of anthocyanins in pH range of 1-2 (McDougall *et al.,* 2005a; McDougall *et al.,* 2005b; Bermudez-Soto *et al.,* 2007; McDougall *et al.,* 2007; Bouayed *et al.,* 2011; Stalmach *et al.,* 2012). This is in agreement with an *in vivo* study in which rats were fed blackberries for 15 days and cyanidin-3-*O*-glucoside recovery in the stomach tissues was 91% (Talavera *et al.,* 2005). This contrasts with the data obtained in the current study where only 44.1% and 41.1% of blackberry and strawberry anthocyanins were recovered after gastric juice incubation. It is difficult to isolate a single factor that explains the *in vitro* loss of anthocyanins in the synthetic gastric juice incubations. Therefore, we repeated the same study with gastric juice collected from humans and incubated the berries for 1 h under the same conditions. Similar recoveries were detected for both berries. The lower recovery of anthocyanins after gastric digestion in the current study of the blackberries and strawberries may be attributed to the food matrix factor as in this study homogenized berries were used while earlier investigations used juices or freeze-dried powders mixed with water. There is also the possibility that the fate of anthocyanins in the proximal gastrointestinal tract of rats and humans is very different.

Under simulated pancreatic conditions, blackberry and strawberry anthocyanins were unstable. This is in agreement with previous *in vitro* studies investigating the stability of anthocyanins after pancreatic digestion with strawberries, where only 29% was recovered after incubation for 2.5 h, (Gil-Izquierdo *et al.,* 2002)

and 25% and 5% for cyandin conjugates with red cabbage and mulberry respectively (McDougall *et al.*, 2007; Liang *et al.*, 2012). For *in vivo*, similar findings were reported in a study investigating the gastrointestinal stability of anthocyanins using animal models. Wu *et al.*, (2006b) showed that the recovery of anthocyanins from the gastrointestinal tract of pigs fed a raspberry meal ranged from ~40% to 80%, depending on the anthocyanin sugar moiety. Among the anthocyanins examined, cyanidin-3-*O*-glucoside exhibited the lowest recovery (~2%) and pelargonidin-3-rutinoside the highest at ~50%. However, this was not associated with parallel recoveries of these compounds or their metabolites in urine, indicating the possible degradation of these anthocyanins in the distal gastrointestinal tract of the pigs.

The reason for the high loss of anthocyanins in the small intestine remains unknown. McDougall *et al.*, (2005a) reported that the low stability of cyanidin-3-*O*-glucoside from raspberries after pancreatic digestion may have been caused by preferential oxidation due to its higher antioxidant activity. In addition, It is likely that anthocyanins converted partly into reversible pseudobases, quinoidal bases and chalcones under neutral pH and alkaline conditions (McDougall *et al.*, 2005a; Fleschhut *et al.*, 2006; McGhie and Walton 2007). This assumption was supported by the study of McGhie *et al.*, (2003) which did not detect anthocyanins in the small intestine after dosing rats with a boysenberry extract for 60 min. However, after acidifying the intestinal tissue, a red color appeared, representing the conversion of colorless forms of anthocyanins into the red flavylium cation. It is also possible that anthocyanins are broken down to various phenolic acids, such as 3,4-dihydroxybenzoic acid (aka protocatechuic acid) and *p*-hydroxybenzoic acid (Tsuda *et al.*, 1999; Perez-Vicente *et al.*, 2002; Hassimotto *et al.*, 2008; Azzini *et al.*, 2010), which would escape detection under the HPLC-MS conditions employed in the current study. However, no aglycones accumulated after pancreatin and bile digestion, in accordance with our previous findings with ileal fluid and other studies showing that the anthocyanidins are typically more unstable than their anthocyanin counterparts (Kahle *et al.*, 2006; Gonzalez-Barrio *et al.*, 2010; Kraus *et al.*, 2010).

As mentioned in previous chapters, the different structures of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside may influence their bioavalability and stability. Despite the 20% reduction of pelargondin in saliva, the *in vitro*

digestion showed little difference in their stability under both gastric and pancreatic conditions (Figure 5-2). These findings were in agreement with those of Cabrita *et al.,* (2000) who reported similar stabilities of cyanindin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside under a range of pH conditions in aqueous solution. However, *in vivo* studies with ileostomy volunteers indicated a slightly higher recovery of pelargonidin-3-*O*-glucoside compared to cyanindin-3-*O*-glucoside as discussed in Chapter 4. These differences could be due to the different matrix between ileal fluid and *in vitro* digestion juices and incubation condition and enhanced absorption of pelargonidin-3-*O*-glucoside compared to cyanindin-3-*O*-glucoside.

Furthermore, the current data provided information on the stability of minor acylated anthocyanins in the berries after the *in vitro* digestion in the stomach. Cyanidin-3-*O*-(3″-*O*-malonoyl)glucoside, cyanidin-3-*O*-(6″-*O*-malonoyl)glucoside and pelargonidin-3-O-(6″-*O*-malonoyl)glucoside (Table 5-1 and Table 5-2) were relatively stable in the stomach but degraded after incubation with pancreatic juice for 2 h while incubation for an additional 4 h did not result in further degradation (Table 5-3). Although these minor acylated anthocyanins were somewhat stable *in vitro* and *in vivo*, none of the acylated anthocyanins were detected in urine and plasma after berry ingestion as discussed in chapter 4.

5.5 Conclusion

The *in vitro* digestion model is straight forward and reproducible and can be used to screen the stability of large numbers of samples or to study a wide range of experimental conditions such as using an *in vitro* digestion procedure to test the effect of co-digestion of vitamin C and meat on red wine antioxidant capacity and total phenol content (Argyri *et al.,* 2006) or co-digestion of raspberry extracts with meat, bread and cereals (McDougall *et al.,* 2005a). This procedure can also help identify which compounds survive under gastrointestinal tract conditions and are likely to reach the colon where they can be bio-transformed into phenolic and aromatic acids that can be absorbed from the large intestine into the portal vein (Aura *et al.,* 2005; Gonzalez-Barrio *et al.,* 2010). However, it cannot mimic the active transport processes, which may interact with bilitranslocase in the stomach (Passamonti *et al.,* 2003) or the

active transport processes in the small intestine (Walton *et al.*, 2006). Therefore, feeding trials with humans or animals, though they are technically more complex and expensive, will provide more information about absorption, metabolism, distribution and excretion of certain compounds or mixtures of components. Consequently, comparing the results from both *in vivo* and *in vitro* is useful for the bioavailability studies.

In this chapter *in vitro* incubation with saliva and simulated stomach and duodenum digestion of blackberries and strawberries were performed to determine the stability of anthocyanins, especially cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside. The results showed the effects of different pH through the gastrointestinal tract. In the upper gastrointestinal tract, under neutral pH conditions, the salivary enzymes and oral microbiota had no impact on cyanidin-3-*O*-glucoside but degraded pelargonidin-3-*O*-glucoside by ~20%. In both simulated and human gastric juice, at pH ~2, both anthocyanins were unstable and the breakdown continued dramatically under pancreatic digestion where a 5.1% recovery was obtained. These findings were in agreement with the low recovery detected in ileal fluid *in vivo* with ileostomy volunteers. Generally, the chemical structure of cyanindin-3-*O*-glucosude and pelargonidin-3-*O*-glucoside influence their bioavaliblity and stability in a similar way with higher bioavailablity of pelargonidin-3-*O*-glucoside *in vivo*, which could be a consequence of it having more ready access to the enterocytes of the small intestine.

6 General discussion

Interests in the biochemistry and biological effects of polyphenolic compounds, particularly flavonoids, have become an important area for human nutrition research due to the evidence of their potential therapeutic effects (Renaud *et al.*, 1992; Hertog *et al.*, 1993; Margetts *et al.*, 2003; Karlsen *et al.*, 2010). Knowing the chemical constituents in potentially protective foods and beverages requires an understanding of their properties and the transformations they undergo in the body.

Therefore, interest in profiling chemical components in Arabian herbal plants has led to the first investigation of this study. The polyphenolic content and antioxidant capacity of *A. hirerochuntica,* an Arabian herbal tea, were screened. HPLC-PDA-MS^2 in addition to the use of an on-line HPLC antioxidant detection system facilitated the identification or partial identification and quantification of the antioxidant activity associated with several individual polyphenolic compounds in this tea. This analytical method revealed the presence of numerous groups of *O*-glycosides, *C*-glycosides, *C*-di-glycosides and *O*-glycoside-*C*-glycosides. Among these compounds, flavone glycosides have been shown to be predominant compound, most notably as luteolin conjugates. Furthermore, the highest antioxidant contribution in the tea comes from phenolic acids and hydroxycinnamates mostly from 5-*O*-caffeoylqunic acid.

The HPLC-MS used in this study is a common method to identify polyphenolic compounds, however, using other analytical methods such as gas chromatography (GC) would have expanded the range of compounds that were detected (Acamovic and Brooker 2005). Moreover, the antioxidant assessments of the herb were preformed with different antioxidant assays that had different mechanism of action. Scavenging of ABTS radicals in the ABTS assay and reduction of ferric ion in the FRAP assay. The results from these two assays confirmed the antioxidant potential of the herb.

Generally, this study has given information for a preliminary database to increase interest among researchers about the chemical profile and antioxidant potential of herbal plants. Knowing what happens to these compounds after

ingestion could make a valuable contribution in investigating the potential health effects of the tea. However, obtaining ethical permission for such a feed would have been difficult with evidence of the *A. hirerochuntica*, which was purchased at a local market in Saudi Arabia, being fit for human consumption. Furthermore, the tea, contain numerous (poly)phenolic compounds, including chlorogenic acids and flavones-*C*-glucoside which meant that a bioavailability study would almost certainly produce a very complex array of low level plasma and urinary metabolites that would not be especially informative.

The next phase of my research focused on investigating fruits that are rich in phenolic compounds and examine their bioavailability after consumption by humans in order to understand their fate *in vivo* following ingestion. Berries were chosen as a rich source of anthocyanins that been linked to several beneficial effects on health (see chapter 1). The same HPLC-PDA-MS^2 technique was used to examine their bioavailability.

To achieve any biological effects of anthocyanins, they must reach the circulation system in biological meaningful quantities. However, a clear understanding of anthocyanins bioavailability has been elusive as there is a consistent discrepancy between the bioactivity of anthocyanins and what have been observed to their bioavailability (Kay 2006; Prior and Wu 2006; McGhie and Walton 2007; Crozier *et al.,* 2010).

A number of studies were published in the last few years concerning the bioavailability of anthocyanins *in vivo* (Matsumoto *et al.,* 2001; Felgines *et al.,* 2002; Felgines *et al.,* 2003; McGhie *et al.,* 2003; Borges *et al.,* 2004; Felgines *et al.,* 2005; Ichiyanagi *et al.,* 2005a; Kay *et al.,* 2005; Felgines *et al.,* 2006). From these reports, it is clear that anthocyanins can be absorbed intact and can be excreted as glucuronide, methylated and/or sulfated conjugates. Although some studies suggested the influence of the aglycone structure and sugar moieties on absorption and metabolism of anthocyanins, very few studies have specifically investigated these effects (Wu *et al.,* 2004a; Bakowska-Barczak 2005; Felgines *et al.,* 2005; Wu *et al.,* 2005a; Borges *et al.,* 2007; Felgines *et al.,* 2008; Ichiyanagi *et al.,* 2008). Therefore, in the current investigation, the main aim was to perform *in vivo* studies focusing on cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-

glucoside bioavailability and their metabolism differences that could be associated with their respective 3', 4'-dihydroxyl and 3'-hydroxy structures (see Figure 3-1). Both anthocyanins have the same sugar moiety but a different aglycone structure. Thus, such a comparison can reflect the aglycone effect on anthocyanin bioavailability. In addition, *In vitro* stability of these anthocyanins was also investigated in model systems simulating different sections of the gastrointestinal tract. These models will help in understanding how the digestion process including the effect of pH, temperature and gastrointestinal enzymes, can affect the stability of these two compounds. The stability of anthocyanins under gastrointestinal tract condition is considered a critical factor affecting their bioavailability (McDougall *et al.,* 2005a; McGhie and Walton 2007; Tagliazucchi *et al.,* 2010).

In vivo studies have been designed with two groups, healthy and ileostomy groups. With healthy volunteers, the absorption, metabolism and urinary excretion of cyaniding-3-*O*-glucoside were evaluated. The current feeding study data with blackberry compared to the previous, similar design study with strawberry (Mullen *et al.,* 2008b) indicated clearly low bioavailability of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside, however, pelargonidin-based anthocyanins appeared to be absorbed and metabolized differently than cyanidin based anthocyanin. First, pelargonidin-3-*O*-glucoside was found in plasma and urine mainly as glucuronidated metabolite while more metabolites were detected with cyanidin-3-*O*-glucoside as methylated and glucuronidated metabolites. Second, plasma and urine recovery of pelargonidin-3-*O*-glucoside were higher than cyanidin-3-*O*-glucoside. In the plasma, pelargonidin-*O*-glucuronide had a peak plasma concentration C_{max} of 274 nmol/L whereas lower C_{max} ranging from 5-20 nmol/L were detected for cyanidin-3-*O*-glucoside and its metabolites. Urinary recoveries, corresponded to 0·75% of intake for pelargonidin-3-*O*-glucoside and 0.08% for cyanidin-3-*O*-glucoside. These findings confirm the effect of the B-ring hydroxylation on the absorption and metabolism of the two anthocyanins.

With ileostomy volunteers, absorption, metabolism of cyaniding-3-*O*-glucoside and pelargonidin-3-*O*-glucoside from the small intestine and urine excretion were investigated. Urine recovery after the ingestion of blackberries and

strawberries indicted similar results with a recovery of 0.06% of and 0.87% of cyanidin and pelargonidin glucoside respectively. The recovery of cyanidin glucoside was >15-fold lower than that observed with pelargonidin glucoside. Furthermore, only 5.1% and 13.6% of unmetabolized cyanidin and pelargonidin were recovered in the 0-24 h ileal fluid. This indicates i) poor recovery despite apparent low levels of absorption in the small intestine and ii) that in humans with an intact functioning colon only low amounts of both anthocyanins will pass from the small to the large intestine, where they will be catabolised by the gut microflora (Aura *et al.,* 2005; Gonzalez-Barrio *et al.,* 2010).

Generally, the low recovery for both anthocyanins in ileal fluid is unlikely to be due to preferential absorption as plasma levels and urinary excretion were also low. The failure of these compounds to accumulate in quantity in plasma or urine remains a paradox. Underestimating anthocyanin metabolites with the use of ultraviolet-visible light detection that only detects anthocyanins in the colored flavylium cation may explain the low bioavailability of anthocyanins (McGhie and Walton 2007). In terms of the notable biological effects of anthocyanins and the low bioavailability, the low amount of anthocyanins reported *in vivo* could be enough for such an effect or alternatively, degradation products and small metabolites may be responsible for the physiologic action of dietary anthocyanins (Tsuda *et al.,* 1999; Prior and Wu, 2006). Deglycosylation of intact anthocyanins will produce an unstable anthocyanidin aglycone, which around neutral pH will degrade to phloroglucinaldehyde and phenolic acids (see Figure 1-13). In addition, anthocyanins that reach the colon may be converted to phenolic acids under the effect of intestinal microflora (Keppler and Humpf 2005; Fleschhut *et al.,* 2006).

Another explanation of the low bioavailability of anthocyanins could be a consequence of accumulation in body tissues but there is limited evidence to suggest that this occurs in any quantity. For instance, Borges *et al.,* (2007) investigated the distribution of anthocyanins and their metabolites in the gastrointestinal tract and their presence in other tissues of rats fed 2.77 mL of raspberry juice by gavage. Only trace quantities of anthocyanins were detected in the caecum, colon and faeces and they were absent in extracts of liver, kidneys and brain. Rats fed with blackberries resulted in low concentration of

anthocyanins distribute in several organs (prostate, bladder, testes and heart) as methylated and glucurono-conjugated anthocyanins (Felgines *et al.,* 2009).

The dilemma about what happens to anthocyanins post-ingestion is likely to remain until radiolabelled cyanidin and pelargonidin glucosides are fed into an animal model and their fate within the body determined. Previous attempts to obtain data like this by using radiolabelled compound have been preformed with flavonoids. [2-^{14}C]quercetin-4´-glucoside has been used to provide a detailed insight of flavonol glucoside bioavailability in rats (Mullen *et al.,* 2003a; Graf *et al.,* 2005). A recent study by Felgines *et al.,* (2010) was carried using ^{14}C-cyanidin-3-*O*-glucoside to follow the distribution of anthocyanins in mice fed by gavage. Cyanidin-3-*O*-glucoside was labelled on its B ring. This study was limited in its success due to the positioning of the carbon label. ^{14}C will thus be present only in anthocyanins and metabolites derived from the B-ring. Therefore, studies involving anthocyanins labelled at several positions with a stable isotope such as ^{13}C, which been synthesis recently, are necessary for complete understanding of anthocyanins bioavailability (Zhang *et al.,* 2011).

Furthermore, the chemical structure of anthocyanins is believed to be a major factor influencing their stability (see chapter 1). Many factors may affect anthocynains stability including the effect of pH, temperature and gastrointestinal enzymes (Wightman and Wrolstad 1995; Cabrita *et al.,* 2000; Laleh *et al.,* 2006). Thus, the possibility that the low bioavailability of anthocyanins could be a consequence of a lack of stability at the different pH environments occurring in the gastro-intestinal tract, led to an investigation of the stability of the two anthocyanins in the upper gastrointestinal tract using *in vitro* models.

The *in vitro* digestion models that mimic the physiological and biochemical conditions occur in the upper gastrointestinal tract can be of use for a greater understanding about anthocyanins stability throughout the gastrointestinal tract. These models are straightforward, reproducible and can be used to screen many samples under a wide range of experimental condition (McDougall *et al.,* 2005a, 2005b; McDougall *et al.,* 2007).

The results of the *in vitro* digestion showed that at neutral pH the salivary enzymes and oral microbiota had no impact on cyanidin-3-*O*-glucoside but, in contrast, lowered the recovery of the incubated pelargonidin-3-*O*-glucoside by 20%. In the stomach, where the gastric juice is at pH~2, somewhat surprisingly both anthocyanins degraded and this reduction continued dramatically under neutral pH through the pancreatic digestion to reach low recovery levels comparable to those obtained *in vivo* with ileal fluid. The high loss of anthocyanins under simulated pancreatic condition and in the ileal could be due the partial conversion of anthocyanins into reversible pseudobases, quinoidal bases and chalcones under neutral pH and alkaline conditions (McDougall *et al.*, 2005a; Fleschhut *et al.*, 2006).

Although the data in the current *in vitro* study showed that the chemical structure of cyanidin-3-*O*-glucoside and of pelargonidin-3-*O*-glucoside influence their stability similarly, the data, however, provide no information regarding why pelargonidin-3-*O*-glucoside is more bioavailable than cyanidin-3-*O*-glucoside. This presumably is a consequence of the more ready access of pelargonidin-3-*O*-glucoside to the enterocytes of the small intestine. This hypothesis was supported by McGhie *et al*. (2003). Their data show lower excretion of delphinidin-based anthocyanins than their malvidin counterparts. They concluded that the greater number of hydroxyl groups in delphinidin and/or the more hydrophobic nature of malvidin may facilitate more access into cells and tissues.

The *in vitro* digestion model used in this study provided insight on the anthocyanins stability in the gastrointestinal tract. However, it cannot mimic the active transport processes of anthocynains in the stomach and in the small intestine. Therefore, the feeding studies with human, though they were technically more complex and expensive, provided us more information about absorption, metabolism and excretion of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside. As a result, the combination of both *in vivo* and *in vitro* studies was very useful for studying the bioavailability of these two compounds.

7 Conclusion and recommendations

The study of bioavailability of dietary compounds is essential in order to determine the form in which the absorbed compounds circulate and to determine their fate after absorption. Based on the current studies data and the reviewed literature data in this thesis about anthocyanins bioavailability, it is cleared that anthocyanin glucoside can be rapidly absorbed from the stomach by a process that may involve bilitransloc (Passamonti *et al.,* 2003; Talavera *et al.,* 2003; He *et al.,* 2009). Then, they enter the systemic circulation and a portion of anthocyanins will be metabolised by methylation and glucuronidation reactions after they pass into the liver (Wu *et al.,* 2002b; Wu *et al.,* 2002c; Kay *et al.,* 2004; Wu *et al.,* 2004a; Felgines *et al.,* 2005; Ichiyanagi and Konishi 2005; Ichiyanagi *et al.,* 2005a; Tian *et al.,* 2006; Wu *et al.,* 2006b; Borges *et al.,* 2007; Azzini *et al.,* 2010; Cai *et al.,* 2011; Stalmach *et al.,* 2012). Some of the metabolites will transport back to the small intestine as bile (Talavera *et al.,* 2003). Anthocyanin glucosides that are not absorbed from the stomach move into the small intestine where they may convert to hemiketal, chalcone and quinonoidal forms under the effect of higher pH (McDougall *et al.,* 2005a; McGhie and Walton 2007; Tagliazucchi *et al.,* 2010). Further absorption appears to take place in the jejunum (Matuschek *et al.,* 2006). Absorbed anthocyanins enter the systemic circulation after passage through the liver and may be metabolised. Unabsorbed anthocyanins reach the colon and exposed to gut microflora were they maybe degraded to phenolic acid and aldehydes (Aura *et al.,* 2005; Gonzalez-Barrio *et al.,* 2010). These products, derived from the ingested anthocyanins may contribute to the health effect of anthocyanins.

Furthermore, the current data raises the important points that need to be addressed with future research. First, most of the studies on anthocyanin bioavailability have used detection methods that are based on the measurement of anthocyanins as red flavylium cations either by absorbance detection or by mass spectrometric analysis with HPLC. This may lead to underestimating anthocyanin bioavailability because it is not known what forms predominate *in vivo*. *In vivo*, the red flavylium form will probably be found only in the stomach. When the anthocyanins encounter the higher pH of the small intestine, the carbinol pseudobase is likely to predominate. Therefore, developing analytical

techniques to identify the colourless forms of anthocyanins would be a major technical advance that would enhance our knowledge of the fate of dietary anthocyanins. Second, for assessing anthocyanins bioavailability it is important to identify and quantify the degradation products and small metabolites of anthocyanins as they may be responsible for the physiologic action of dietary anthocyanins.

Another area that should be further investigated is the gastric and intestinal absorption mechanism. Only a few studies have investigated gastric absorption of anthocyanins using *in situ* gastric administration (Passamonti *et al.,* 2003; Talavera *et al.,* 2003; He *et al.,* 2009) or more recently using human gastric epithelial MKN-28 cells (Fernandes *et al.,* 2012). On the basis of data obtained in these studies, it has been proposed that anthocyanins are absorbed at the gastric level possibly through bilitranslocase transport (Passamonti *et al.,* 2003). Moreover, the transport mechanism involved intestinal absorption of anthocyanins has not been identified, but if similar to flavonols, it is associated with cleavage and release of the aglycone as a result of the action of lactase phloridzin hydrolase (LPH) in the brush border of the small intestine epithelial cells. The released aglycone may then enter the epithelial cells by passive diffusion as a result of its increased lipophilicity (Day *et al.,* 2000). An alternative hydrolytic step is mediated by a cytosolic β-glucosidase (CBG) within the epithelial cells. For CBG-catalyzed hydrolysis to occur, the polar glucosides must be transported into the epithelial cells, possibly with the involvement of the active sodium-dependent glucose trans- porter 1 (SGLT1) (Gee *et al.,* 2000).

Furthermore, the use of ileostomists has proven to be a useful means of assessing the intestinal absorption of anthocyanins and metabolism of other polyphenols (Stalmach *et al.,* 2011; Stalmach *et al.,* 2012). However, there are a few limitations associated with this *in vivo* model. Ileostomists are healthy subjects whose colons have been surgically removed. These subjects had their surgery a few years ago, resulting in removal of colonic bacteria which catabolise anthocyanins and other polyphenols that reach the large intestine. The small intestine usually has fewer than 10^4 organisms per mL (Hao and Lee, 2004). Therefore, the small intestinal lumen is not totally free of bacterial activity especially if the terminal ileum has developed a bacterial population

over the years (Hao and Lee, 2004). However, to our knowledge, no studies have been carried out to evaluate the effect of the small intestine microflora on dietary compounds such as anthocyanins. In contract, the role of colon microflora in the metabolism of anthocyanins has been studied by *in vitro* incubations with faecal slurries. A range of phenolic acids and aldehydes were detected after the incubation as a result of the colonic degradation (Aura *et al.*, 2005; Keppler and Humpf 2005; Gonzalez-Barrio *et al.*, 2011). Therefore, in future, the analysis of possible phenolic compounds in the ileal fluid, plasma and urine by the GC-MS would help to complete the picture of the bioavailability of the blackberry and strawberry anthocyanins.

Assessing the bioavailability of anthocyanins compared to other flavonoids is not straightforward because of the impact of pH on their structure. More details are required on how anthocyanins are absorbed, how the potential variation in structures generated *in vivo* contribute to their health benefits. Therefore, it is important to understand the nature of their absorption and metabolism *in vivo*. Understanding these processes will enable the development more stable food products with more healthy benefits.

Appendices

1. Tea prepared from *Anastatica hirerochuntica* seeds contains a diversity of antioxidant flavonoids, chlorogenic acids and phenolic compounds.
   Noura AlGamdi, William Mullen and Alan Crozier; Phytochemistry 72 (2011): 248–254.

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