CHARACTERISATION OF PHENOLIC ANTIOXIDANTS IN FRUITS AND VEGETABLES: BIOAVAILABILITY OF RASPBERRY PHENOLICS IN HUMANS AND RATS

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Dedicated to my children (my team mates). Finishing what we started together is a small present for them.

In memory of my son Diego,
a special and incredible brave boy that sadly passed away.
To my teenager son Tomás a charming and
lovely young man.

Shivatsa, symbol of infinite love.

Abstract

Epidemiological studies have suggested associations between the consumption of plant foods and beverages, especially those rich in phenolic compounds, and the prevention of chronic diseases, and there is a growing evidence indicating that these dietary phytochemicals are involved in enhancing long-term health. The antioxidant capacity of these compounds appears to be, at least in part, responsible the reduction of certain oxidative stress pathologies. Common fruits, vegetables and nuts available in supermarkets in Scotland were screened to identify products that are rich in phenolic antioxidants. The selection of the products analyzed was based on their availability in local supermarkets. Two different assays were used to assess antioxidant activity, electron-spin resonance spectroscopy (ESR) and Ferric Reducing Antioxidant Potential (FRAP). Total Phenolics content (TPC) was also quantified using the Folin-Ciocalteau assay. Due to the interest in the Zutphen study in the early 1990's that showed that dietary intake of flavonols was inversely correlated with the incidence of coronary heart disease, the analysis of quercetin, isorhamnetin and kaempferol was done. There were differences of up to 758-fold in the total FRAP antioxidant capacity (AOC) of the individual products. Those especially rich in antioxidants included peanuts, almonds, broad beans, blueberries, raspberries, strawberries and purple broccoli. The vitamin C and flavonol concentrations were not correlated to the AOC. The results obtained by FRAP and ESR are significantly high correlated. There was no correlation between FRAP and flavonols. The data obtained in this study were used in a separate collaborative investigation that is not included in this thesis to evaluate the overall intake of antioxidants in the UK.

Berries were selected for further analysis because they constitute a group of fruits with a very high AOC. Raspberries (*Rubus idaeus*), blueberries (*Vaccinium corymbosum*), blackcurrants (*Ribes nigrum*), redcurrants (*Ribes rubrum*) and cranberries (*Vaccinium oxycoccus*) were included in the study. A detailed analysis revealed not only the nature and concentration of individual phenolic compounds but also their contribution to the overall antioxidant activity of the berries. The importance of such a detailed quantitative and qualitative analysis of phenolics from

any specific source is considerable because their chemical structure has an impact on the absorption and bioavailability although the mechanisms involved are still unclear. Changes in the chemical structure of dietary phenolics following ingestion by humans and animal models can in part help unravel this puzzle. The phenolic compounds present in major quantities and are principally responsible for the antioxidant capacity in blackcurrants and blueberries were the anthocyanins. Blackcurrants contained large amounts of anthocyanins (5446 nmol/g) and vitamin C (2328 nmol/g) and had the highest AOC of the five berries. Blueberries were the second highest with anthocyanins levels of 4908 nmol/g but the sample studied contained no vitamin C. Raspberries, redcurrant and cranberries contained anthocyanins but in lower amounts. Ellagitannins such as sanguiin H-6 were responsible for 58% of the AOC of raspberries. Flavonols (16%) and vitamin C (23%) were important antioxidants in cranberries while in redcurrants a number of unidentified peaks were the major contributors for the AOC (33%) along with the 28% from anthocyanins.

Raspberries were chosen for an intervention study with human subjects. The bioavailability of anthocyanins, ellagitannins and ellagic acid in raspberries was investigated. Plasma and urine were collected from six healthy human subjects after ingestion 300 g of raspberries. Three healthy volunteers with an ileostomy were included in the study, providing ileal fluid, plasma and urine at different time points for 24 h after consuming a 300 g raspberry portion. All samples were analyzed using HPLC-PDA-MS^2. No anthocyanins, ellagitannins or their metabolites or breakdown products were detected in the plasma of any of the volunteers. Eight of the anthocyanins identified in raspberries were detected and quantified in ileal fluid in their native form. They reached a maximum level of 36.5% of intake in samples collected 0-4 h after supplementation and after 24 h there was an overall anthocyanin recovery of 39.6%. With regard to ellagitannins, 16.3 μmoles of sanguiin H-6 was detected in ileal fluid after 0-4 h with 26.2% of intake being detected over the 0-24 h collection period. No lambertianin or sanguiin H-10 were found. The levels of ellagic acid in 0-24 h ileal fluid corresponded to 239.4% of intake 24 h with 162.9% being collected 0-4 h after ingestion. This coincided with the peak levels of ellagic acid in urine although the levels, 13.1 nmoles and 33.6 nmoles for non ileostomy and ileostomy volunteers respectively, were low and equivalent to no more that 0.4% of

intake. No ellagic acid or metabolites were detected in plasma at any time point. This study, therefore, found a low absorption and excretion of anthocyanins and ellagic acid in human subjects. The *ca*. 40% recovery of these compounds in ileal fluid indicates that in healthy subjects with a colon substantial quantities pass from the small to the large intestine where they will be catabolised by the gut microflora.

The present study also investigated the distribution of anthocyanins, ellagitannins and their metabolites in the gastrointestinal tract and their presence in other tissues of rats fed 2.77 mL of raspberry juice by gavage. One hour after feeding the ellagitannins, sanguiin H-6 and lambertianin C had disappeared with only traces of ellagic acid being detected in the stomach. Up to 2 h after supplementation there was a very high recovery of unmetabolised anthocyanins, principally cyanidin-3-sophoroside, cyanidin-3-(2^{G}-glucosylrutinoside) and cyanidin-3-glucoside, as they passed from the stomach to the duodenum/jejunum and ileum. After 3 h, less than 50% was recovered, after 4 h this declined to 11% of intake and after 6 h only 2% remained. Only trace quantities of anthocyanins were detected in the caecum, colon and faeces and they were absent in extracts of liver, kidneys and brain. These findings imply anthocyanins are poorly absorbed and that which does occur takes place before ileum, in keeping with evidence indicating that the stomach and the jejunum are sites of anthocyanin absorption in mice and rats. Because anthocyanins are poorly absorbed substantial amounts pass from the small to the large intestine where their rapid disappearance suggests they are degraded by faecal bacteria.

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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 July 2000 and February 2005.

Signed…….

Gina Borges

March 2008

ABBREVIATIONS

ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
$ABTS^{\cdot+}$	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation
amu	atomic mass unit
AOC	Antioxidant capacity
AUC	Area under plasma concentration/timre curve
CBG	Cytosolic-β-glucosidase
C_{max}	Peak plasma concentration
BMI	Body mass index
CHD	Coronary heart disease
DPPH	1,1-diphenyl-2-picrylhydrazyl
EDTA	Ethylene-dinitrilo-tetra-acetate
ESR	Electron-spin-resonance
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
HAEC	Human aortic endothelial cells
HPLC	High performance liquid chromatography
i.d.	Internal diameter
ISF	In source fragmentation
LC-MS	Liquid chromatography mass spectrometry
LDL	Low density lipoprotein
LPH	Lactase phloridzin hydrolase
MeOH	Methanol
MDR	Minimum daily requirement
$[M-H]^-$	negatively charged molecular ion
$[M+H]^+$	positively charged molecular ion
m/z	mass to charge ratio
ORAC	Oxygen radical absorbance capacity
PDA	Photodiode array
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SE	Standard error
TEAC	Trlox equivalent antioxidant capacity
TEAR	Trolox equivalent antioxidant ratio
T_{max}	Time of peak plasma concentration
TPC	Total phenol content
TPTZ	2,4,6-tripyridyl-s-triazine
t_R	retention time
UV	Ultraviolet
VSMC	Vascular smooth muscle cells
v/v	Volume to volume
λmax	**A**bsorbance maxima

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Chapter 1. INTRODUCTION

Plant secondary metabolites, which are also known as phytochemicals, are receiving increasing interest from consumers, food producers and researchers in the areas of nutrition, medicine and biology for several reasons. Epidemiological studies have suggested associations between the consumption of foods and beverages, especially those rich in phenolic compounds, and the prevention of chronic diseases, and there is a growing body of evidence indicating that these dietary phytochemicals are involved in enhancing long-term health (Scalbert and Williamson, 2000). Traditional medicines of ancient cultures like the Chinese, Egyptian, Asian, and Native Americans demonstrate an early knowledge of the use of plant constituents for preventing and curing diseases, and maintaining good health (Parkins, 2001; Higdon, 2007).

In this new era of phytochemicals and health research, past knowledge and experience in chemical, biochemical, physiology, nutrition, medicine and recently, molecular biology are allied to try to understand their health implications, efficacy and safety in order to produce dietary guidelines and new products to improve and promote well being. One of the greatest challenges today is to eliminate the misinformation and myth in the media regarding the health benefits of certain "super" foods and food supplements. The general public is constantly bombarded with propaganda and half-truths because of a desire to find the "magic-pill" that will make them thinner, stronger, healthier and free of disease. Recommendations on the beneficial effects of individual compounds or a specific "super food" are often made on anecdotal evidence and conclusions are often based on non-scientific observations. Although much of the early information regarding the significance of dietary compounds such as flavonoids was circumstantial and often contradictory, more mechanistic data are now being produced, contributing to the elucidation of their interaction with molecular pathways related to the development of diseases. Increasing state-of-the-art research is being directed toward isolating and identifying the active components in various foods and studying their molecular and bioavailability mechanism of action in order to produce scientific evidence to support dietary recommendations.

1.1. Phytochemicals or secondary metabolites in plants

All organisms need to transform and interconvert a vast number of organic compounds to enable them to live, grow, and reproduce. Plants are very efficient at synthesizing organic compounds via photosynthesis from carbon dioxide and inorganic materials found in the environment, whilst herbivores rely on obtaining their organic materials in their diet, i.e. by consuming plants. The chemical pathways for modifying and synthesizing the fundamental molecules (carbohydrates, proteins, fats and nucleic acids) are described as primary metabolism and the compounds involved as primary metabolites. Secondary metabolism in plants involves phytochemicals, which have a limited distribution in nature. In the majority of cases, their function is not known but there are examples where their role is being elucidated. The involvement of caffeine in allelopathy and as a chemical defence agent (Crozier and Ashihara, 2006) is one example. There are other instances where secondary metabolites function in plants as toxic materials for defence against fungal, viral, microbial or insect attack, while others provide protection against herbivores, act as volatile attractants, and as colouring agents to attract or warn off other species (Croteau *et al.,* 2000; Dewick, 2002). Most of the pharmacologically active natural products come from this area of secondary metabolism.

Recognition of the biological properties of natural products has fuelled the current interest on this field, namely, the search for new drugs, antibiotics, insecticides and herbicides and food supplements and dietary products.

Based on their biosynthetic origins secondary metabolites can be categorized into four groups: terpenoids, alkaloids, phenolics, and sulphur compounds (Crozier *et al*., 2003). Table 1.1 contains a list of various classes of phytochemicals.

1.1.1. Terpenoids

Terpenoids represent the most structurally varied class of phytochemicals with more than 30,000 compounds identified. The name terpenoid derives from the fact that the first members of the class were isolated from turpentine (“terpentin” in German) (Croteau *et al*., 2000).

Table 1.1. Classification of the main plant secondary metabolites.

CLASS	EXAMPLES
Terpenoids	
Monoterpenes	D-limonene, linalool, menthol
Saponins	soyasapogenol, quillaic acid
Limonoids	limonin, nomilin
Carotenoids	β-carotene, lycopene
Phytosterols	sitosterol, stigmasterol
Alkaloids	caffeine, atropine, solanine
Sulphur compound	
Glucosinolates	glucobrassicin, glucoerucin, glucoraphanin
Allium compounds	alliin, allicin, ajoene, cepaene
Phenolics	
Flavonoids	
Flavan-3-ols, procyanidins	(+)-catechins, (–)-epicatechin
Flavonols	quercetin, kaempferol
Flavones	nobiletin, tangeretin
Flavanones	hesperidin, naringin
Isoflavones	daidzein, genistein
Anthocyanins	malvidin, cyanindin
Non Flavonoids	
Stilbenes	Resveratrol
Phenolic acids	gallic acid, ellagic acid
Hydroxycinnamates	caffeic acid, ferulic acid

All terpenoids are derived by repetitive fusion of branched five-carbon units based on an isopentane skeleton. Carotenoids are an example of tetraterpenoids, a family of C_{40} compounds, which carry out essential functions in the life cycle of green plants and also play a role in human health by virtue of their metabolism to vitamin A (retinol) which is involved in sight, growth and development process and its deficiency in humans can have serious consequences for long-term health (Beyer *et al.,* 2002).

1.1.2. Alkaloids

These compounds have a 3000-year history of human use. They are nitrogen-containing compounds synthesized principally, but not exclusively, from amino acids. The latex of the opium poppy (*Papaver somniferum*) contains morphine and related alkaloids such as codeine. When the latex is dried, the hard brown substance called opium is formed. Opium has been the king of all medicines over the centuries. Analysis of the components of opium led to the isolation of morphine in 1806 by a German pharmacist Friedrich Sertuner. Because of their various pharmacological activities, alkaloids have influenced human history profoundly, for both good and ill. Atropine, cloroquine, heroin, cocaine, caffeine, nicotine are some of the alkaloids that have influenced medicine, societies and even geopolitics (Zulak *et al.,* 2006).

1.1.3. Sulphur-containing compounds

The main sulphur compounds in the human diet are the glucosinolates found in cruciferous crops like cabbages, broccoli, watercress, and the allium compounds in *Allium* crops including garlic, onions and leeks. Allylisothiocyanate and acrinylisothiocyanate are pungent isothiocyanates derived from glucosinolates. Glucosinolates become biologically active in the plant only in response to tissue damage releasing the enzyme myrosinase. Alliin is an odorless thiosulphinate present in garlic. It reacts with the enzyme alliinase when tissue is crushed or damaged producing allicin with its characteristic garlic flavour. It has a considerable antibacterial and antifungal properties (Dewick, 2002). Another well-known allium compound is the prophenyl sulphenic rearranged molecule, propanethial *S*-oxide, the lachrymatory factor of onions (Mithen, 2006).

1.1.4. Phenolic and polyphenolic compounds

Phenolic and polyphenolic compounds have played a role in the successful evolutionary adaptation of plants from an aquatic environment to the land. They are essential to the plant's physiology, being involved in diverse functions such as structure, pigmentation, ollination, allelopathy, pathogen and predator resistance,

growth and development (Croteau *et al.,* 2000; Dewick, 2002). Accounting for about 40% of organic carbon circulating in the biosphere, these phenolics are primarily derived from the phenylpropanoid and phenylpropanoid-acetate pathways in which *p*-coumaric acid is a key compound (Crozier *et al*., 2006). Phenolics range from simple, low molecular weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols. They can be classified into two main groups: flavonoids, a structurally diverse group of C_{15} compounds arranged in a C_6-C_3-C_6 configuration and non-flavonoids which include condensed and hydrolysable tannins, stilbenes, phenolic acids and hydroxycinnamates.

1.1.4.1. Flavonoids

Plant flavonoids are the most numerous of the phenolics and are found throughout the plant kingdom (Harborne, 1993). They are of significance to mankind as well as to plants and have been strongly implicated as active contributors to the protective effect of fruits and vegetables, nuts, tea, wine, chocolate. The major dietary classes include flavan-3-ols, flavonols, flavones, flavanones, isoflavones and anthocyanins (Figure 1.1), (Crozier *et al.,* 2006).

1.1.4.1.1. Flavan-3-ols

Also known as flavanols, is a complex subclass of flavonoids ranging from the simple (+)-catechin and its isomer (–)-epicatechin, to the oligomeric and straight chain polymeric procyanidins, also known as condensed tannins (Figure 1.2). Proanthocyanidins can comprise as many as 50 monomer units. In addition to forming complexes with other flavan-3-ols, monomeric flavan-3-ols also undergo esterification with gallic acid to form catechin gallates, and hydroxylation reactions to form gallocatechins. Other polymer flavan-3-ols structures include the thearubigins, which form during fermentation of green tea and as a result are the dominant components in black tea (Balentine *et al.,* 1997).

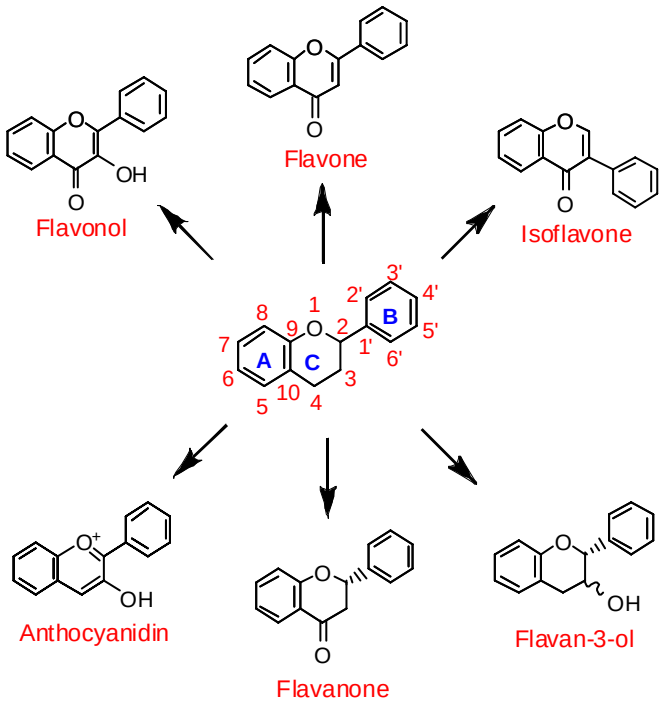


Figure 1.1. Chemical structures of the main flavonoids.

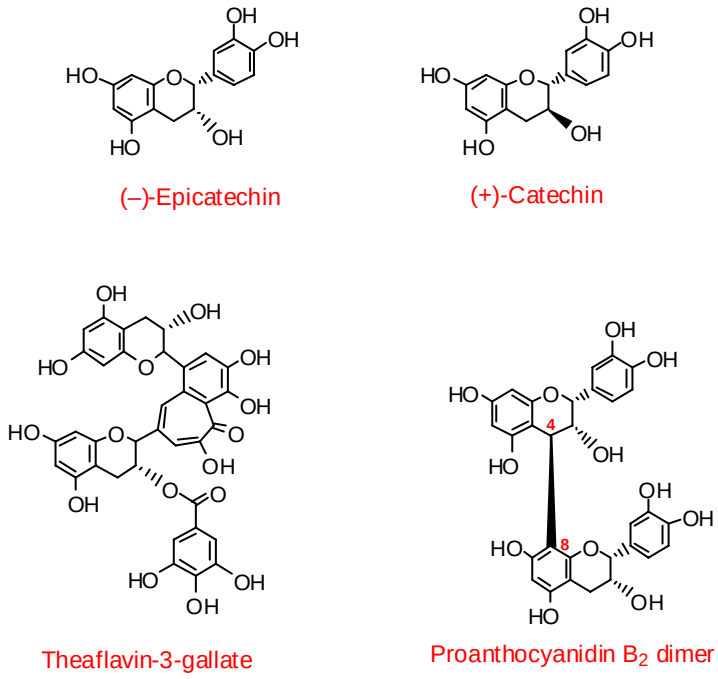


Figure 1.2. Chemical structures of the main flavan-3-ols.

Procyanidins are astringent and are involved in the taste and texture of food and confer a bitter and astringent taste to red wine, chocolate and some fruits

(Frankel *et al*., 1993). This characteristic is due to the formation of complexes with salivary proteins rich in proline. The analysis of thearubigins and proanthocyanidins of greater size than tetramers is not straightforward because they are retained on most reverse-phase HPLC columns and therefore, their presence is often underestimated and even overlooked.

1.1.4.1.2. Flavonols

The variability of this flavonoid sub-group is extensive, with over 380 flavonol glycosides and more than 200 different quercetin and kaempferol glycosides currently described (Bravo, 1998). Quercetin is the most common flavonol and together with kaempferol, myricetin and isorhamnetin, is usually found as *O*-glycosides (Figure 1.3). Conjugation occurs most frequently at the 3 position of the C-ring, although substitutions at the 7, 3', 4' and 5' also occur (Hermann, 1976). Quercetin-3-*O*-rhamnosylglucoside (rutin) is present in tomatoes and asparagus (Peterson and Dwyer, 1998). Individual foods can differ in the number and nature of glycoside associated with a particular aglycone. For example, quercetin is found in onions in the form of glucose conjugates linked at the 3, 7 and 4' positions (Tsushida and Suzuki, 1995) whereas in apple peel it occurs as 3'-*O*-conjugates of glucose, arabinose, galactose, rhamnose and xylose (Dick *et al.,* 1985).

1.1.4.1.3. Flavones

They are structurally similar to flavonols differing only in the absence of hydroxylation at the 3-position in the C-ring (Figure 1.4). However, they have a much more restricted distribution occurring principally in celery (apigenin), sweet red pepper (luteolin), parsley and citrus fruits, which contain polymethoxyflavones such as tangeritin, nobiletin and sinensetin (Manach *et al.*, 2004).

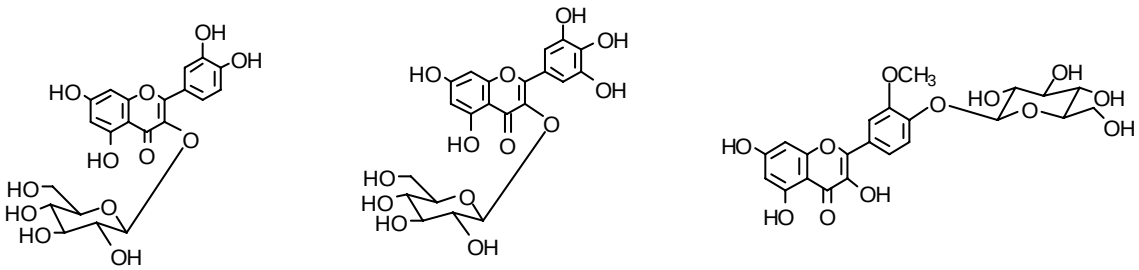


Figure 1.3. Chemical structures of the main flavonol glycosides.

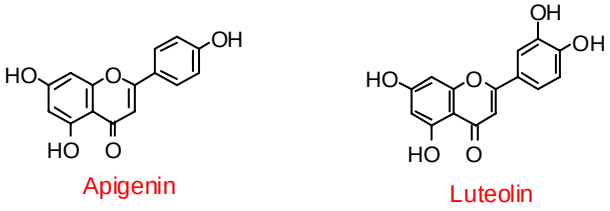


Figure 1.4. Chemical structures of the main flavone's aglycones.

1.1.4.1.4. Flavanones

Two main structural features characterise the flavanones, the absence of the C_2-C_3 double bond, and the presence of a chiral centre at C_2. The main flavanone aglycones are naringenin in grapefruit, hesperitin in oranges and erioctyol in lemons. Flavanones are generally glycosylated with a disaccharide at position 7, either typically neohesperidose, which imparts a bitter taste, or a rutinose, which is flavourless (Figure 1.5) (Kannes *et al.*, 1993; Manach *et al.*, 2004).

1.1.4.1.5. Isoflavones

Structurally isoflavones differ from other flavonoids in the orientation of the B ring (Figure 1.1, Figure 1.6). Isoflavonoids are best known for their oestrogenic activity as they seriously affect the reproduction of grazing animals. In humans, it has been

suggested that they may have a role in the prevention of breast cancer and osteoporosis (Scalbert and Williamson, 2000). The most studied isoflavonoids are daidzein and genistein. The principal sources are legumes such as soybeans, black beans, green peas and sprouts.

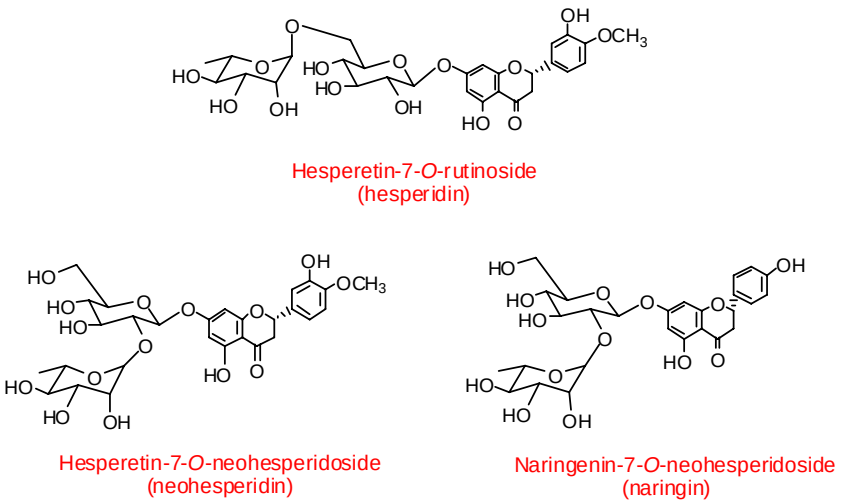


Figure 1.5. Chemical structures of the main flavanones.

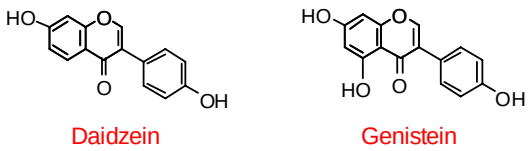
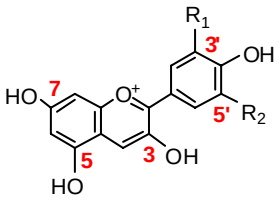


Figure 1.6. Chemical structures of the main isoflavones.

1.1.4.1.6. Anthocyanidins

Anthocyanins are pigments dissolved in the vacuolar sap of the epidermal tissues of flowers and fruit, to which they impart a pink, red, blue, or purple colour (Harborne, 1994). They have an important role for attracting insects to flowers to facilitate pollination and are involved in protecting the plant against UV light (Crozier, 2003). They exist in different chemical forms, both coloured and colourless, according to

pH. Although anthocyanins are highly unstable in the aglycone form (anthocyanidins), while they are in plants, they are resistant to light, and oxidation conditions that are likely to result in degradation. Degradation is prevented by glycosylation, generally with glucose at position 3. The most common anthocyanidins are malvidin, cyanidin, delphinidin, pelargonidin, petunidin and peonidin (Figure 1.7). Glycosylated anthocyanidins are referred as anthocyanins. Berries and red grapes are rich sources of anthocyanins.



Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 1.7. Chemical structure of the main anthocyanidins.

1.1.4.2. Non-flavonoids

Non-flavonoid compounds consist of one or two benzene rings and are derivates of benzoic acid, cinnamic acid and their respective aldehydes. They are subdivided into stilbenes, phenolic acids, hydroxycinnamates and other minor families.

1.1.4.2.1. Stilbenes

Stilbenes are produced in plants as a defence response to stress, microbial infection and UV irradiation (Fremont, 2000). They occur in grapes and consequently are

found in grape products. The most studied stilbene is resveratrol (3,5,4′-trihydroxystilbene) (Figure 1.8), a phytoalexin found in wine that has been one of the most widely phenolic studied for its potential effects on human health. Another important stilbene in wine is the δ-viniferin present as a result of oxidation of resveratrol by fungus (Vitrac *et al.,* 2005). Japanese knotweed (*Polygonum cuspidatum*) is an extremely rich source of resveratrol (Burns *et al*., 2002).

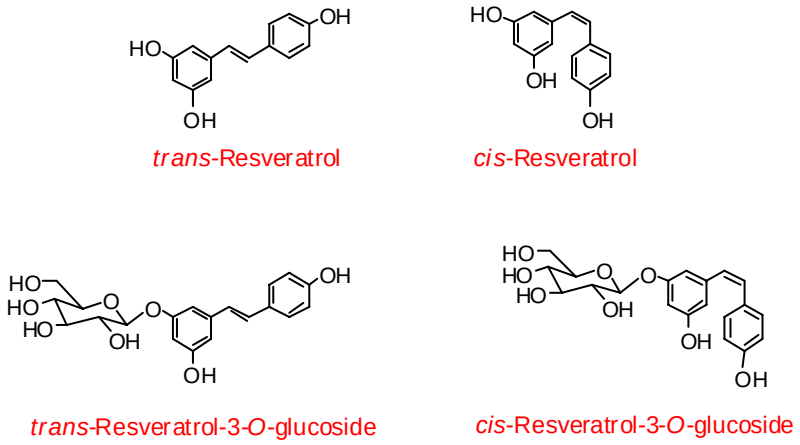


Figure 1.8. Chemical structures of resveratrol.

1.1.4.2.2. Phenolic acids and related compounds

Phenolic acids are derivatives of gallic acid and ellagic acid and are also known as hydroxybenzoates. The name gallic acid comes from the French word *galle*, which is a swelling in a plant tissue after an attack by insects. Such galls contain *ca*. 70% of the total phenolics as gallic acid esters (Gross, 1992). Gallic acid is the base unit of gallotannins whereas gallic acid and ellagic acid are both subunits of the ellagitannins (Figure 1.9). Ellagitannins are esters of hexahydroxydiphenic acid and a polyol, usually a glucose. When exposed to acids or bases, ester bonds are hydrolysed and the hexahydroxydiphenic acid spontaneously rearranges forming water-insoluble ellagic acid. Over 150 ellagitannins have been isolated and

identified from Chinese medicinal plants (Okuda *et al.*, 1993). They have been traditionally used as astringent drugs to cure diarrhoea, gastric ulcers and burns (Okuda *et al.*, 1981). Ellagitannins often accumulate in a few specific tissues such as the skin of walnut, the seeds of raspberries and the husk in pomegranate fruit. Raspberries and blackberries contain sanguiin H-6 as the main ellagitannin (Haslam, 1998) while the tetramer lambertianin C also occurs in raspberries (Mullen *et al.,* 2003b). Pomegranate juice contains the ellagitannin punicagallin at concentrations >2 g/L and it is responsible for the high antioxidant activity of the juice (Gil *et al.*, 2000).

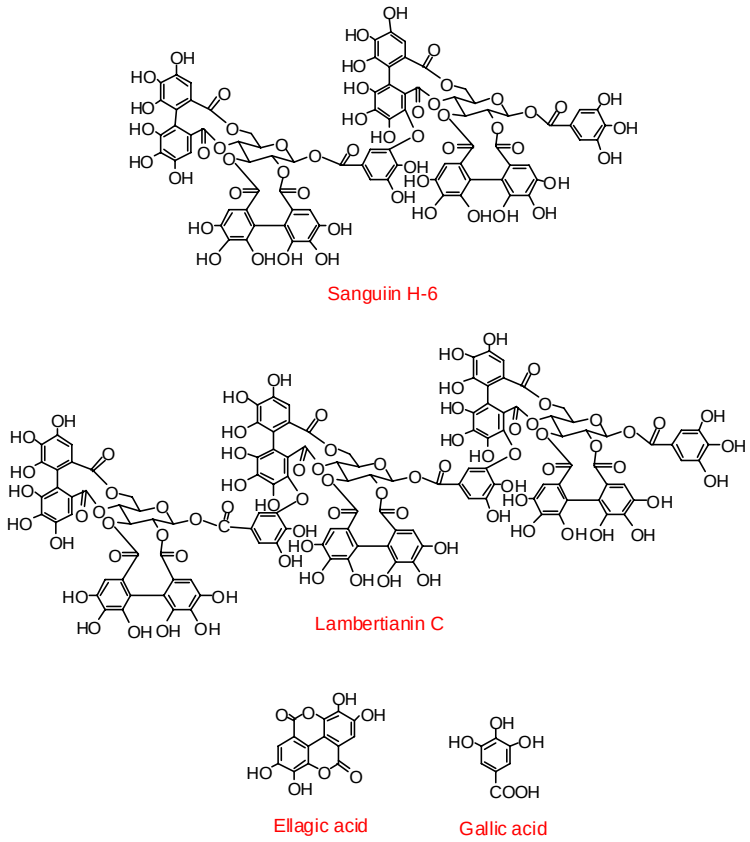


Figure 1.9. Chemical structures of some phenolic acids and ellagitannins.

1.1.4.2.3. Hydroxycinnamates

The most common hydroxycinnamates are caffeic, *p*-coumaric, ferulic and sinapic acids (Figure 1.10) which are produced by the phenylpropanoid pathway via a

series of hydroxylation and methylation reactions. Chlorogenic acid (5-*O*-caffeoylquinic acid) and neochlorogenic acid (3-*O*-caffeoylquinic acid) are derivatives of caffeic acid and are common in food and beverages including lettuce, apples, tea, coffee and maté (Ferreres *et al*., 1997). Hydroxycinnamic acids are rarely found in their free form, tending instead to accumulate as glycosylated conjugates in most plant tissues including pollen (Harborne, 1993).

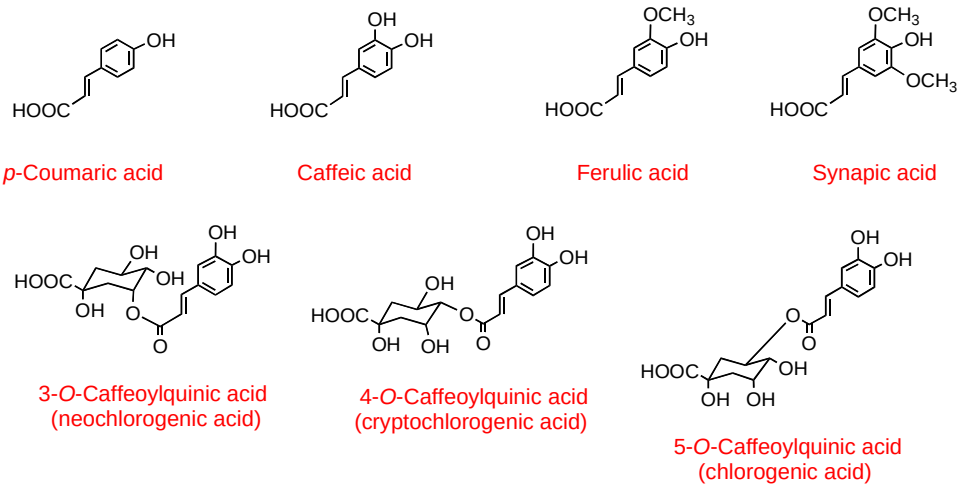


Figure 1.10. Chemical structures of some hydroxycinnamates.

1.1.4.2.4. Other categories

The remaining compounds which make-up the non-flavonoids are from other minor families such as acetophenones, coumarins, chalcones, xanthones and lignans. There are numerous structural variations within these flavonoid sub-classes, which depend on the degree of hydrogenation, hydroxylation, methylation and sulphation of the molecules.

1.2. Bioavailability of phenolics

Bioavailability can be defined in different ways. The commonly accepted definition is the proportion of the ingested nutrient that is absorbed and metabolised through

normal normal pathways and distribution to target tissue, sometimes, the effect that it produces in a tissue or organ, is included in the definition. In addition to absorption, activity *in vivo* depends on the extent and manner of metabolism by the small intestine, liver, kidney and colonic microflora, and on the rate of excretion and the degree of retention in body tissues. Holst and Williamson, (2004) extended the classical pharmacological definition of bioavailability, and referred to it as "a linked and integrated processes: liberation, absorption, distribution, metabolism and excretion (LADME)".

Bioavailability can be determined experimentally by orally administering a single-dose of a phenolic rich food or a pure compound to experimental animals or humans, and then calculating the peak plasma levels (C_{max}), the time to reach the peak plasma concentration (T_{max}) and the area under the curve of plasma concentration *vs* time (*AUC*). It is thought that the latter is the most reliable parameter because it reflects the entire response over time in contrast to C_{max}, which is a one-point measurement (Hoppe and Krennrich, 2000). However, this is debatable, as recent evidence has demonstrated that phenolic metabolites are rapidly turned over in plasma and as a consequence, AUC values do not accurately reflect absorption.

The route of absorption and metabolism of phenolics is shown in Figure 1.11. The first stage corresponds to the mouth where some polymeric polyphenols can bind to salivary proteins, possibly limiting their availability to some degree. This interaction is thought to be part of animal and human defence mechanisms against tannins which as they can have a variety of harmful effects especially in grazing animals (Mehansho *et al*., 1987; Butler *et al*., 1986). They are also responsible for the astringency sensation of tea, wine and fruits, such as grapes, peaches, persimmon (kaki), by precipitation of polyphenol/proline-rich protein complexes (Charlton *et al*., 2002). Reports on effects of saliva on phenolics are rare, however, catechol-containing compounds such as chlorogenic acid and (+)-catechin, have been shown to increase nitric oxide bioavailability when exposed to saliva (Peri *et al*., 2005).

Dietary phenolics pass then through the stomach and enter the small intestine where some are absorbed, either with or without structural modifications such as glycosylation, glucuronidation and sulphation. On entering the circulatory system, they are transported to the liver via the portal vein.

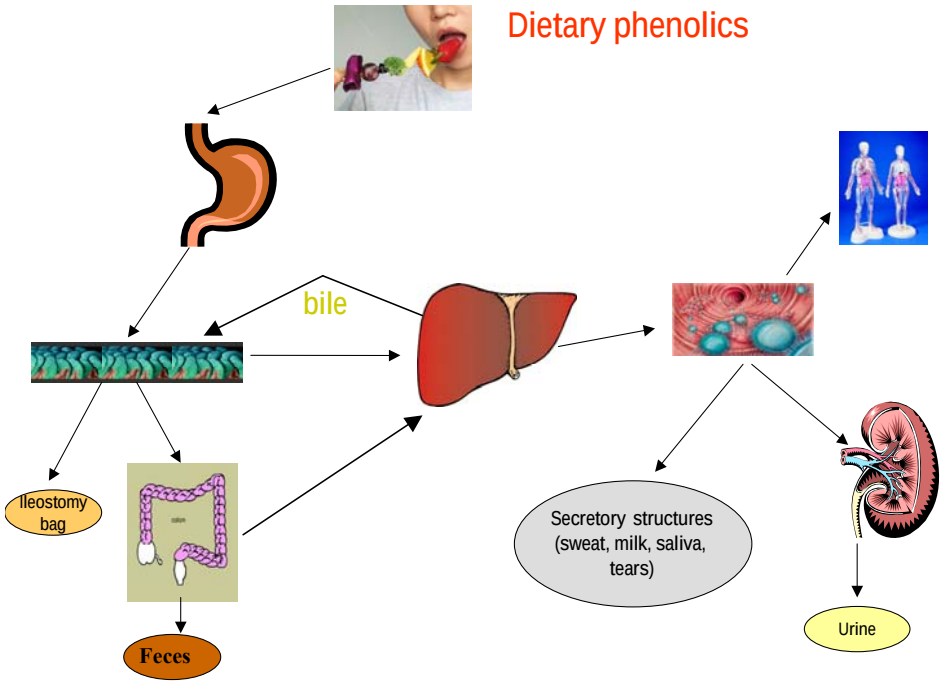


Figure 1.11. The fate of phenolic compounds following ingestion.

Bile acts to emulsify fats, allowing greater absorption. From the liver, phenolics can be transported to body tissues or to the kidneys, where they are excreted in urine. Compounds not absorbed in the small intestine enter thecolon, where they are subjected to the action of colonic bacteria with the catabolic products being either expelled in faeces or absorbed into the blood stream and ultimately excreted in urine (Donovan *et al.*, 2006).

The presence of glycosides and esters of polymer form of phenolics influences their chemical, physical and biological properties and have an effect on absorption into the bloodstream. They can diffuse across a biological membrane and enter in a cell depending on their partition coefficient. For example quercetin-3′-*O*-rhamnoglucoside has a lower coefficient than quercetin (0.37 vs 1.20) showing a higher hydrophilicity (Brown *et al*., 1998).

Since most dietary phenolics are too hydrophilic to penetrate the gut wall by passive diffusion, it has been proposed that membrane carriers are involved in their absorption. Hollman *et al*. (1999) suggested that flavonol glucosides could be transported into enterocytes by the sodium-dependent glucose transporter SGLT1.

While this may facilitate entry of flavonol glucosides into enterocytes, it is not involved in their transport into the circulatory system where flavonols appear not as glucosides but as their glucuronide, sulphated and methylated metabolites (Day *et al*., 2001; Mullen *et al*., 2004). The production of methylated, sulphated and glucuronide metabolites in the wall of the small intestine and the liver is probably a detoxification process common to many xenobiotics that restrict their potential toxic effects and facilitates their biliary and urinary elimination by increasing their hydrophilicity.

Studies with rats indicate that hydrolysis of flavonol glucosides, releasing the aglycone to form metabolites prior to passage into the bloodstream, involves two different β-glycosidases, namely lactase phoridzin hydrolase (LPH) in the brush border of the lumen of the small intestine and cytosolic β-glucosidase (CBG) which is found in the enterocyte. The enzymes exhibit different substrate specificities. Quercetin-4′-glucoside is hydrolysed by both LPH and CBG while quercetin-3-glucoside is cleaved by LPH and not CBG (Gee and Johnson, 2001).

The quercetin disaccharide, rutin, which has a rhamnose-glucose sugar moiety, appears not to be hydrolysed by either LPH or CBG (Jaganath *et al.,* 2006). Subjects with an ileostomy, fed tomato juice containing this flavonol, excreted more than 80% of the ingested rutin in the ileal fluid. In healthy subjects this passes from the small to the large intestine where the rutin is broken down to 3,4-dihydroxyphenylacetic acid which is absorbed into the bloodstream and methylated, probably in the liver, forming 3-methoxy-4-hydroxyphenylacetic acid which together with 3,4-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid is excreted in urine in amounts corresponding to 22% of rutin intake.

Gallic acid and isoflavones are the most well absorbed phenolics, followed by flavan-3-ols, flavanones and quercetin glucosides with the different plasma C_{max} values ranging from 0.4 to 4 μM when 50 mg of aglycone equivalents are consumed (Manach *et al*., 2005). In comparison anthocyanins, procyanidins and galloylated flavan-3-ols are poorly absorbed but, as mentioned above in the case of rutin, these compounds may become bioavailable as phenolic acids after being subjected to the action of colonic bacteria in the large intestine. A recent study by Vitaglione *et al*., (2007) showed that when 6 healthy volunteers ingested 71 mg of cyanidin-3-glucoside contained in “blood orange juice”, 44% of the cyanidin ingested was

recovered as protocatechuic acid (3,4-dihydroxybenzoic acid) in urine. However, the protocatechuic acid appeared in plasma after 2 hours of consumption, which is seemingly a too short time for colonic fermentation to occur in humans.

An increasing number of studies have now detected selected flavonoids and their metabolites in plasma and urine of human volunteers following the consumption of pure compounds, and phenolic-rich fruit, vegetables or beverages. The phenolic urinary excretion differs depending on the type of compound investigated. Manach *et al.,* (2005) reviewed the results of 97 studies of absorption and excretion of phenolics in humans. The urinary excretion of anthocyanins ranged from 0.004% to 0.1% of the ingested dose while for flavonols it was 0.9% for rutin and 6.4% for quercetin glycosides from onion. Urinary excretion of flavanones was 4-30% and higher still for isoflavones at 16-66% of intake (Figure 1.12).

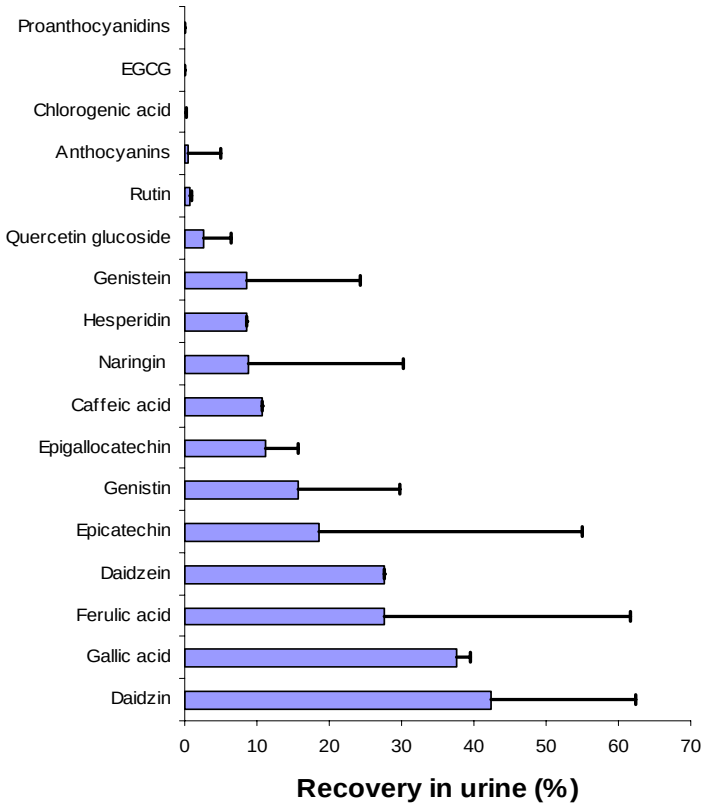


Figure 1.12. Recoveries of different groups of phenolics in urine (Manach *et al.*, 2005).

Radiolabelled compounds are a useful tool with which to study the metabolic fate of these polyphenolics. Mullen *et al*, (2002b) fed [2-^{14}C]quercetin-4'-glucoside to rats and demonstrated a limited absorption and low levels of metabolites in plasma and peripheral tissues, with 93.6% of the ingested radioactivity remaining in the gastrointestinal tract 60 min after ingestion. After a further 4 h 85% of the radioactivity was still in the digestive tract principally as metabolites, with only 6% being detected in blood and internal tissues (Graf *et al.,* 2005). A subsequent study suggests that after 48 h most of the radioactivity is excreted in urine as phenolic acids (Mullen *et al.,* 2008). More studies of this type would give much clear view of the fate of dietary phenolics.

The potential importance of phenolic acids produced as flavonoid degradation products by the action of colonic bacteria is being acknowledged more and more in the area of bioavailability. 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, and once absorbed in to the circulatory system may elicit protective effects.

Better knowledge of bioavailability is essential for investigating the health properties of polyphenols. There is no single approach recommended but techniques like radiolabelled compounds, microflora composition, *in vitro* fermentation, intervention studies with ileostomy volunteers, or subjects with kidney failure who require dialysis could provide new insights into the metabolism of polyphenols. Investigation into biomarkers for diseases is another important task in order to correlate directly or indirectly with levels of phenolics in plasma, tissues or organs and to measure efficiency or potency of the compounds.

1.3. Health effect of fruits and vegetables in the diet

Despite the controversy surrounding the optimal ingredients of a healthy diet, there is little disagreement regarding the importance of fruits and vegetables. The results of literally hundreds of epidemiological studies and recent trials provide strong and consistent evidence that diets rich in fruits and vegetables can reduce the risk of chronic disease (Steinmetz and Potter, 1996; Law and Morris, 1998; Riboli

and Norat, 2001; Arts and Hollman, 2005). Fruits and vegetables contain thousands of potentially bioactive phytochemicals that are likely to interact in a number of ways to prevent disease and promote health. For example, the potential systemic biological effects of pomegranate juice ingestion appear to be attributed to urolithins, colonic microflora metabolites, rather than to the high antioxidant polyphenols present in juices (Cerdá *et al*., 2004).

While screening foodstuffs to identify high antioxidant produce is useful, caution should be exercised in relating such *in vitro* data to direct claims of *in vivo* antioxidant bioactivity as the mechanism of action is unclear.

1.3.1 Cardiovascular diseases

Dietary patterns characterized by relatively high intakes of fruits and vegetables are consistently associated with reductions in the risk of coronary heart disease (CHD) and stroke. Law and Morris, (1998), carried out a meta-analysis combining the results of 11 prospective cohort studies and found that people eating ~ 5 servings/day or more of fruit and vegetables had a risk of myocardial infarction that was about 15% lower that those eating less than 5 portions/day. Among more than 126000 men and women participating in the Health Professionals Follow-up Study and the Nurses' Health Study (Joshipura *et al.,* 2001), those who consumed 8 or more servings of fruits and vegetables daily had a risk of developing CHD over the next 8 to 14 years that was 20% lower than those who consumed less than 3 servings daily. In the same population group the risk of ischemic stroke was 30% lower in those who consumed at least 5 servings of fruits and vegetables daily than in those who consumed less than 3 servings daily (Joshipura *et al.*, 1999). In a more recent meta-analysis study designed to estimated the global burden of disease attributable to low fruit and vegetable consumption, Lock *et al*, (2005) concluded that increasing individual fruit and vegetable consumption to 600 g/d (ca. 7 servings/d) could decrease the risk of CHD by 31% and the risk of ischemic stroke by 19%. The supplementation of individual phytochemicals has not generally resulted in a significant decrease in the incidence of cardiovascular events in randomized controlled trials (Higdon, 2007). Thus, in the case of fruits and vegetables, "the benefit of the whole may be greater than the sum of its parts".

1.3.2. Cancer

The interest in the potential cancer preventive properties of dietary phytochemicals has increased in recent years. Numerous case-control studies indicate that eating a diet rich in fruits and vegetables decreases the risk of developing several different types of cancer, particularly cancers on the digestive tract and lung (WCRF/AICR, 2007; Tsugane and Sasazuki, 2007). Dietary constituents like folate (Childers *et al*., 1995), β-carotene plus vitamin A and E (Omenn *et al*., 1996), calcium plus vitamin D (Grau *et al*., 2003) have or are currently undergoing phase III cancer chemoprevention studies. However, no polyphenolic compounds have reached this level. (–)-Epigallocatechin gallate from tea, quercetin from onions, genistein from soya, curcumin in curry spice and resveratrol from red wine or grapes have shown efficacy in preclinical carcinogenesis models. *In vitro* studies with these compounds have been shown to affect signal transduction pathways leading to inhibition of cell growth and transformation, enhanced apoptosis and reduced metastasis (Lambert *et al.*, 2005; Thomasset *et al.*, 2007; Goel *et al.*, 2008). Phenolics with a low (systemic) bioavailability have a potential to prevent cancers of the colon, skin or the oral cavity. For example Perkins *et al,* (2002) examined the pharmacokinetics of curcumin administered either in the diet or injected as ^{14}C-labeled form to Min/+ mouse, a model of familial adenomatous polyposis (intestinal adenoma). Though detected in only trace amounts in the plasma, they concluded that the equivalent daily dose of 1.6 g of curcumin is required for efficacy in humans.

When epidemiological studies together with cancer biomarkers studies, toxicity and potency research, bioavailability, *in vitro* effects, animal model trials and human preclinical trials, indicate that certain polyphenols have a potential as a chemo-preventive drugs then they might be candidate for phase III clinical interventions

1.3.3. Age related deficits in cognitive and motor functions

J.A. Joseph and collaborators at USDA-ARS, Human Nutrition Research Centre of Aging at Tufts University, Boston, USA have carried out extensive research on cognitive effect of diets rich in fruits and vegetables and specific phenolics in

animals and humans. Although it is not clear whether a diet rich in fruits and vegetables decreases the risk of neurodegenerative aging or diseases such as Parkinson's and Alzheimer in humans, studies with animal models suggest that the phenolic compounds found in fruits, may exert their beneficial effects through their ability to lower oxidative stress and anti-inflammatory properties or by altering directly the signaling involved in neuronal communication, calcium buffering ability, stress signaling pathways among other. (Shukitt-Hale *et al*., 2008; Shukitt-Hale *et al*., 2006). Blueberry, strawberry or spinach extracts fed to Fisher rats for 8 weeks, all reversed age-related deficits measured by different methods including motor function (Joseph *et al*., 1999). It was proposed that blueberry supplementation, in addition to enhancing mitogen-activated protein kinase signalling and GTPase activity in the striatal pathway, is involved in the conversion of short-term to long-term memory as well as planning and modulation of movement and some cognitive processes (Joseph *et al*., 2005). The relevance of this research area is enormous as age related diseases are becoming more common in developed countries where the life expectancy has increased but with a high social and economic costs.

1.4. Mechanisms of action

Inflammatory disease, cardiovascular disease, cancer, and other chronic diseases are associated with increased oxidative stress. On the other hand, polyphenols present in fruit and vegetables are strong antioxidants and as such are free radical scavengers and can inactivate other pro-oxidants. Together with epidemiological evidence demonstrating that consumption of polyphenol-rich foods is associated with a decreased incidence of these diseases, it is not difficult to put together all these associations and conclude that dietary polyphenols may exert health benefits through an antioxidant mechanism. This has been in part demonstrated by many studies that have found transient increases in the total antioxidant capacity of human plasma (Serafini *et al.*, 2000; Vinson *et al.*, 2001). However, accumulating evidence indicates that not all polyphenolics are readily bioavailable, reaching only low μM concentrations in human plasma, even after a large intake. Most of these compounds are also extensively metabolized *in vivo* (see Section 1.2) and this can adversely

affect their "natural" antioxidant activity (Lottito and Frei, 2006). Based on this evidence we can ask the question: If there is any effect of polyphenols in the human body, is the physiological molecular mechanism of action based on their antioxidant activity, or are other mechanisms involved?

1.4.1. The Antioxidant Hypothesis

The antioxidant hypothesis has its origins in 1954 when Harman published his free radical theory of aging (FRTA) and the simultaneous discovery of the free radicals in endogenous metabolic reactions. The FRTA suggests that the rate of aging changes can be slower by measures to decrease the length of the free radicals reactions with antioxidants like such as vitamin E and/or their rates of initiation by minimazing oxidant catalyst like iron and copper. In 1987 a cross-cultural comparison of population groups demonstrated that plasma levels of vitamin C, β-carotene, vitamin E and selenium were significantly higher in men, 40-49 years of age from Switzerland and Italy compared to their counterparts in Finland and Scotland. An arbitrary index calculated by dividing the cholesterol level by the "antioxidant potential" (the combined concentration of vitamin C, E, selenium and β-carotene), was inversely related to mortality rates for heart disease (Gey *et al.*, 1987). The antioxidant hypothesis evolved from these studies with the proposal that high intakes of dietary antioxidants prevent oxidation of plasma and thereby protect against disease induced by oxidative stress. Now, more than forty years later, we can evaluate the evidence for the "antioxidant hypothesis" based on the data obtained in numerous intervention studies.

The antioxidant properties of plant phenolics are due to the hydrogen of the phenoxyl groups that is prone to be donated to a radical, and by the ensuing structure that is chemically stabilized by resonance. Bors *et al.* (1997) postulated three criteria that should define the best free radical scavenging activity for phenolics: 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. The flavonol quercetin fulfils these criteria and they are partially met by flavan-3-ols such as (+)-catechin (Figure 1.13).

Iron, copper, manganese and other metals can catalyze the decomposition of hydrogen peroxide into hydroxyl radicals, which are one of the most powerful oxidant species. Some polyphenols with dihydroxy groups have the capacity to conjugate such metals. They can also activate antioxidant enzymes, inhibit oxidases and reduce α-tocopherol radicals. The antioxidant activity of phenolics is then a multidimensional effect that has been demonstrated in many *in vitro* biological systems (Heim *et al.*, 2002) including studies with synthetic liposomes (Verstraeten

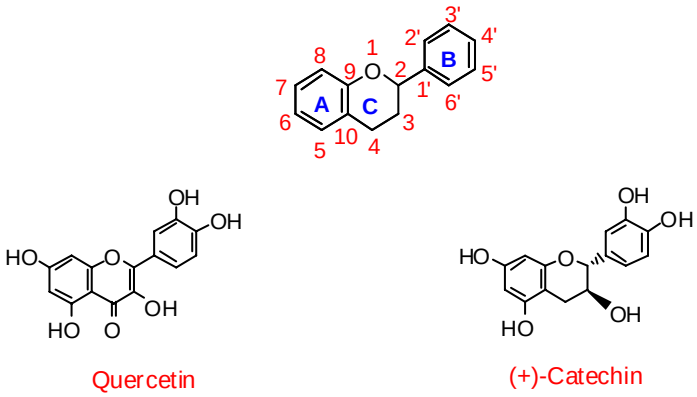


Figure 1.13. Quercetin and (+)-catechin chemical structures.

et al., 2005), *ex vivo* human plasma (Lotito and Fraga, 2000) and isolated low-density lipoproteins (LDL) (Lotito *et al.*, 2000). However these potential multidimensional effects of antioxidants in biological systems, pose difficulties in delineating structure-activity relationships.

As mentioned previously, most polyphenols are modified during absorption from the small intestine, through conjugation and metabolism, and by the large intestine, mainly through the action of colonic bacteria and by subsequent hepatic metabolism. 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 polyphenols following absorption is still unclear, their antioxidant protective effect could occur within the gastrointestinal tract itself as discussed in Section 1.2. Garsetti *et al.* (2000) reported the level of antioxidants in the human large intestine was 26.6 ± 10.5 mM Trolox eq. in faeces

compared to 1.5 ± 0.1 mM Trolox eq. in plasma. The gastrointestinal tract represents a critical defence barrier against luminal toxic agents and is constantly challenged by diet oxidants, mutagens, carcinogens, as well as endogenous reactive oxygen species and reactive nitrogen species. Polyphenols passing through the tract could neutralize these compounds.

1.4.2. Cellular signalling cascade hypothesis

Cellular signal transduction refers to any process by which a cell convert one kind of signal or stimulus into another, most often involving ordered sequences of biochemical reactions inside the cell, that are carried out by enzymes and linked through second messengers resulting in what is known as a "second messenger pathway". Such processes are usually rapid, lasting approximately milliseconds in the case of ion flux, to minutes for the activation of protein- and lipid-mediated kinase cascades. In many signal transduction processes, the number of proteins and other molecules participating in these events increases as the process progresses from the initial stimulus, resulting in a "signal cascade" and often results in a relatively small stimulus producing a large response e.g cell growth, proliferation and death (apoptosis).

There is now evidence that polyphenols may play a role as modulators of cascade signaling mechanisms (Williams *et al.*, 2004). Intracellular concentrations of phenolics required to affect cell signalling pathways are considerably lower than those required to impact on cellular antioxidant capacity, and their metabolites may still retain an ability to interact with cell signalling proteins, even if their antioxidant activity is diminished (Spencer *et al.*, 2003a and 2003b; Williams *et al.*, 2004). Some reports indicate that phytochemicals can selectively regulate multiple signalling pathways at the level of transcription, especially those involving nitrogen-activated protein kinases (Frigo *et al.*, 2002)

The number of reports claiming that polyphenols have *in vitro* effects in biological systems is enormous. However, if physiological studies with cell culture and *in vitro* test systems, investigating potential protective effects are to be of any relevance, they should be making use of genuine *in vivo* metabolites, such as glucuronide and sulphate conjugates with or without methylation of the catechol group, and not aglycones or glycosides which do not leave the gastrointestinal tract.

There are some examples of the bioactivity of polyphenol metabolites in *in vitro* cellular test systems. Koga and Meydani. (2001) reported that unidentified *in vivo* metabolites of (+)-catechin decreased the adhesion of monocytes to human aortic endothelial cells (HAEC), whereas the (+)-catechin itself did not. The mechanism is not known but it could be by acting as ROS scavengers and/or inhibition of interleukin IL-1β activation. Lotito and Frei. (2006) found that metabolism of apigenin and kaempferol in rat hepatocytes *in vitro* diminished and even abolished their activity in HAEC. Although Schroeter *et al.* (2001) have reported that (–)-epicatechin and its metabolite 3′-methyl-(–)-epicatechin were equally effective in to attenuating oxLDL-mediated neuronal death neither of these compounds appears in the circulatory system (Stalmach *et al*., 2008).

Other studies are reported in the literature involving metabolites for *in vitro* studies. Quercetin-3-glucuronide, a metabolite that appears in plasma after ingestion of onions (Mullen *et al.*, 2006), inhibits Angiotensin II, a potent vasopressor peptide, which induces cellular events by changing intracellular signalling molecules, generating ROS and producing vascular smooth muscle cell (VSMC) hypertrophy in rat aortic smooth cells (Yoshizumi *et al.,* 2002; Kyaw *et al.*, 2004).

(–)-Epigallocatechin-3-gallate, a flavan-3-ol that accumulates in plasma, albeit transiently and in low concentrations, after the consumption of green tea (van Amelsvoort *et al.,* 2001; Stalmach *et al*., 2008) is one of several compounds that have been reported to have insulin-like glucose-lowering properties in mammals. (–)-Epigallocatechin gallate induces phosphorylation of insulin-sensitive residues on the transcription factor FOX1a, similar to insulin thus it may be a useful anti-diabetic agent (Anton *et al.*, 2007).

In summary, it appears that polyphenolics might exert their cellular effect by binding to enzymes that activate or deactivate key pathways leading to changes that consequently produced a physiological response, but more studies needs to be carried out.

1.5. Soft fruits (e.g. berries and currants)

Scottish men and woman have the highest premature mortality from coronary heart disease and one of the lowest fruit and vegetable intakes in the world (Scottish Office, 1993). For such populations, berries locally grown are a potentially important source of vitamins, fibre and phytochemicals. However, a change in their eating habits is needed.

Soft fruits make up only a tiny part of the diet in the UK but are more important in Nordic countries (Saltmarsh *et al.*, 2003). Additional health benefits have been claimed for berries as unlike most other fruits and vegetables they are rich in a wide range of phenolic compounds including anthocyanins, flavonols, flavan-3-ols and ellagitannins as well as vitamin C, minerals and fibre (Beattie *et al*., 2005).

This section includes those fruits referred to by the public as berries and not by the classical definition of berries, "fruit which have more than one seed within the fruit and that seed is not compartmentalised" (Stewart *et al.*, 2007). Such fruits include cranberry (*Vaccinium oxycoccoc*), elderberry (*Sambucus nigra*), blueberry (*Vaccinium corymbosum*), blackcurrant (*Ribes nigrum*), strawberry (*Fragaria x ananassa*), raspberry (*Rubus idaeus*) and blackberry (*Rubus spp*.). Edible berries have been part of the human diet for millenia but despite the historical longevity, some types of berries have never been developed beyond local markets, reflecting in part their susceptibility to post-harvest decay. However, as increasing evidence of their phytochemicals attributes is reported as well as their stability in frozen conditions, new products will be developed making them more available to the public. In this context, there is enormous potential to use genetical engineering not only to control fruit ripening and extend shelf life by blocking ethylene biosynthesis in fruit but also to increase the levels of phytochemicals. Unfortunately, such biotechnology gets unwarranted bad publicity in the UK press and as a result, it is not something that the general public currently consider acceptable for use with commercial products.

1.5.1 Flavonoids and phenolic compounds in berries

1.5.1.1. Anthocyanins

The average intake of anthocyanins in the USA was estimated to be 180-215 mg/ day by Kühnau, (1976) although recently values were recalculated to 12.5 mg/day/person in 2001-2002 by Wu *et al*. (2006) using different food intake data. Estimations for women aged 20-30 years in the UK are much lower at ca. 5 mg per day (Gosnay *et al.*, 2002). In comparison, the daily intake for flavones and flavonols in the Netherlands was reported by Hertog *et al.* (1992) as 23 mg/day. The available evidence suggests that consumers can more readily increase their consumption of anthocyanins than other flavonoids as their concentrations in red wines of 120 mg/L are not unusual (Timberlake, 1988), a single serving of some berries can contain >100 mg (McGhie *et al.*, 2003) and there is a report of 200 mL of Austrian elderberry juice containing 2 g of anthocyanins, principally as cyanidin-3-*O*-glucoside and cyanidin-3-*O*-sambubioside (Murkovic *et al.*, 2001).

Data obtained with *in vitro* studies indicate that anthocyanins found in berries have a range of potentially anti-cancer and anti-heart disease properties including antioxidant activity (Wang *et al.*, 1999), inhibition of the growth of cancerous cells (Ding *et al.,* 2006), anti-inflammatory (Seeram *et al.*, 2001) and antiobesity effects (Tsuda, 2003). The therapeutic properties of anthocyanins have been recently reviewed by Prior and Wu. (2006) and Zafra-Stoone *et al*. (2007).

Anthocyanins exhibit unusual chemical behaviour *in vitro* and this can result in a complex behaviour *in vivo*. At pH 1-3, the red flavylium cation is the most abundant molecular form (Figure 1.14). As the pH increases, there is a rapid loss of a proton to generate the blue quinonoidal structure. At the same time, a much slower hydration of the flavylium cation occurs to yield the colourless hemiketal form that tautomerises through an opening of the C-ring to generate *cis* and *trans* chalcone structures. The relative composition of the different molecular forms of anthocyanins coexisting in aqueous solution at any given time will be dependant on pH, temperature and time (McGhie and Walton, 2007).

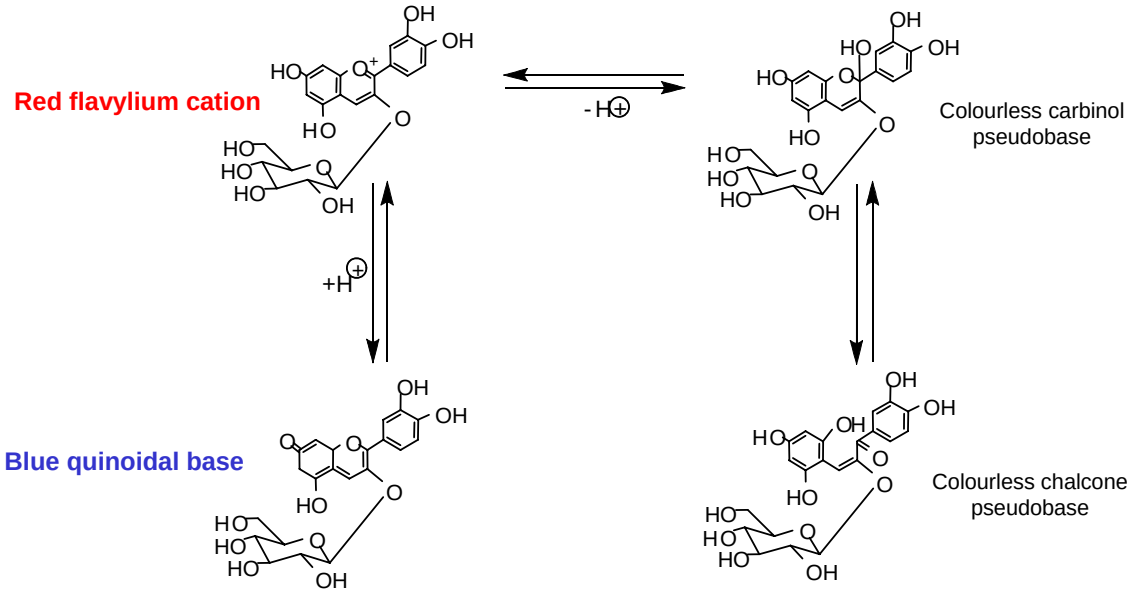


Figure 1.14. Chemical pH changes on the structure of anthocyanins.

Anthocyanins are naturally present as the red flavylium in berries and most foods, however during passage through the gastrointestinal tract the molecular structure may vary. This variation in chemical structure is one of the key factors that almost certainly affect the absorption, metabolism, bioavailability and consequently, the biological responses to anthocyanins. In practice, little is known about the *in vivo* structure of anthocyanins as typically prior to analysis samples are acidified with the anthocyanins reverting to the flavylium cation. Current analytical methodology is such that any metabolic event that prevents ring closure and conversion of the chalcone form back to the flavylium structure will go unobserved.

Anthocyanins have been shown to be a strong antioxidants and may exert a wide range of health benefits through antioxidant or other mechanisms, however without a clear knowledge on the rate and extent of their absorption, metabolism and tissue or cell distribution, the mechanism of anthocyanins in disease prevention remains an enigma.

1.5.1.1.1. Absorption and metabolism of anthocyanins

A report on the excretion of pigmented urine by rabbits fed red grapes was published as long ago as 1933 by Horwitt. However, it was not until the late 1990's that information on anthocyanin bioavailability began to emerge when it was reported that anthocyanins were absorbed intact without deglycosylation (Cao and Prior, 1999; Miyazawa *et al.*, 1999). Subsequently, many studies have been conducted in which rats, rabbits, pigs and human have been fed extracts and foods containing a variety of anthocyanins. The data obtained have recently been reviewed by Prior and Wu. (2006) and McGhie and Walton. (2007).

As mentioned earlier, the phytochemicals in food are exposed to a number of different environments following ingestion and entry into the body. The pH of the stomach is acidic (<2) which ensures that anthocyanins are present as the flavylium cation, which is the most stable form of anthocyanins. In contrast to the stomach, the environment of the small and large intestines is almost at neutral pH where anthocyanins are less stable. In the colon, the microflora can further alter anthocyanins by fermentation to phenolic acids through the cleavage of the C3-ring. Studies to date show that anthocyanins are little modified by gastric conditions but extensively modified by the gut microflora (Keppler and Humpf, 2005; Fleschhut *et al.*, 2006).

After oral administration of berries and berry extracts, glycosylated anthocyanins have been detected in the blood stream within minutes with T_{max} values ranging from 0.5 to 3 h (Cao *et al.,* 2001; Bub *et al.,* 2001; Kay *et al.,* 2005; Kay, 2006). The levels of absorption and excretion in urine are low, typically <0.1% of intake. Maximum levels in human plasma are in the range of 1-100 nmol/L with doses of 0.7- 10.9 mg/kg. After 6 h, anthocyanins are clear of the circulatory system (Cao *et al.*, 2001; Bub *et al.*, 2001). In contrast to quercetin glucosides, most anthocyanins glucosides are not hydrolysed by either LPH or CBG (Passamonti *et al.*, 2003). Studies with rats indicate that anthocyanin absorption occurs in the stomach as well as the small intestine (Passamonti *et al.,* 2003; Talavera *et al.,* 2003 and 2004). In urine, some methylated, glucuronidated and sulphated metabolites have been detected but usually unmodified glycosides are also present (Felgines *et al.*, 2005; Ichiyanagi *et al.,* 2005a; Kay, 2006). For instance, after consumption of a chokeberry (*Aronia sp.*) extract by human subjects, plasma and urine were found to

contain cyanidin-3-galactoside and cyanidin-3-arabinoside, together with more substantial quantities of methylated and glucuronidated metabolites (Kay *et al.,* 2004). Sulphate conjugates have been detected in a few investigations but in very low amounts (Felgines *et al.,* 2003 and 2005). The glucuronidation mechanism for anthocyanins is still not clear. Two pathways have been proposed for the formation of cyanidin glucuronides (Prior and Wu. 2006). The first possibility is that the glucuronide is formed directly from cyanidin-3-glucoside by uridine diphosphate (UDP) glucuronyldehydrogenase. The second pathway involves the hydrolysis of cyanidin-3-glucoside releasing cyanidin, which is then glucuronidated directly through the action of an UDP-glucuronyltransferase. Evidence from the ratio of cyanidin-3-glucoside to cyanidin monoglucoronide in urine supports the second route as the most likely pathway (Wu *et al.*, 2005) as does the detection of the anthocyanidins cyanidin and peonidin in plasma of rats by Talavera *et al.* (2005). The formation of the methylated anthocyanin metabolites is thought to occur in the liver being catalysed by a catechol-*O*-methyl transferase. In keeping with this possibility, there are reports of the presence of methylated cyanidin-3-glucoside in the liver of rats following the consumption of red fruit anthocyanins (Miyazawa *et al.*, 1999; Tsuda *et al.*, 1999).

It is unclear why plasma and urine levels of anthocyanins are usually so much lower than those of other flavonoids after supplementation. Two possible factors may have an influence in absorption. As mentioned earlier anthocyanins are not hydrolysed by β-glucosidases in the gastrointestinal tract and this may substantially reduce the amounts that are able to pass through the gut wall into the bloodstream. However, a study in which anthocyanin absorption was investigated after *in situ* rat perfusion of the jejunum and ileum found that absorption ranged from 10.7% for malvidin-3-glucoside to 22.4% for cyanidin-3-glucoside (Talavera *et al.*, 2004). This is much higher than indirect estimates of anthocyanin absorption based on either plasma content or urinary excretion levels. There is one report in which cyanidin-3-glucoside was injected intravenously into rats and there was a 36% recovery of the glycoside and its metabolites in urine within 4 h (Ichiyanagi *et al.,* 2005b). This implies that the perfusion experiments may have over-estimated the extent of anthocyanin absorption for the gastrointestinal tract because if 10-20% of the ingested anthocyanins entered the circulatory system this would be accompanied by

the appearance of substantially more than ca. <0.1% of intake that is routinely obtained in anthocyanin feeding studies.

Vitaglione *et al*. (2007) reported the detection of protocatechuic acid in human plasma as the major metabolite of glycosilated cyanidins representing 44% of the ingested amount (Figure 1.15). This is in keeping with a report by Aura *et al*. (2005) in which it was hypothesized that deglycosilation of cyanidin-3-glucoside took place in the small intestine via the action of β-glycosidase while degradation of the aglycone to protocatechuic acid occurred in the colon through the action of colon bacteria. This possible metabolism of cyanidin needs more evidence.

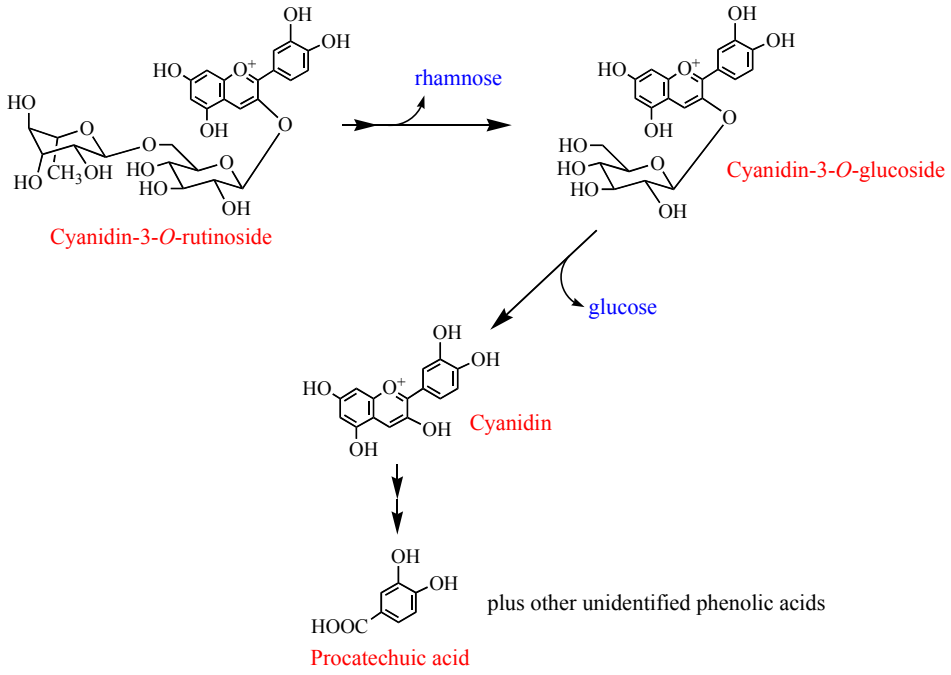


Figure 1.15 Possible cetabolism of cyanidin glycosides to protocatechuic acid.

1.5.1.2. Flavonols

Flavonols and other flavonoids are commonly quantified as the aglycone after acid or enzyme hydrolysis to remove sugar residues. Using this approach the myrecitin, quercetin and kaempferol content of edible berries have been estimated (Hakkinen *et al.*, 1999). Quercetin was found to be highest in bog whortleberry (*Vaccinium uliginosum*) (15.8 mg/100g) and bilberry (*Vaccinium myrtillus*) (1.7-3.0 mg/100g).

Myricetin was the most abundant flavonol in blackcurrant (8.9-20.3 mg/100g) followed by quercetin (7.0-12.2 mg/100g) and kaempferol (0.9-2.3 mg/100g) (Mikkonen *et al.*, 2001). Specific flavonol glycosides that have been identified in berries include quercetin-3-glucoside, quercetin-3-rutinoside, quercetin-3-galactoside (Mullen *et al.*, 2003b).

1.5.1.2.1. Absorption and metabolism of flavonols

This topic was discussed in Section 1.2.

1.5.1.3. Flavan-3-ols and procyanidins

Berries can contain substantial amounts of the flavan-3-ols monomers (+)-catechin and (–)-epicatechin as well as dimers, trimers and polymeric procyanidins. The concentration of polymers is usually greater than the monomers, dimers and trimers. Cranberries are a particularly rich source of polymeric procyanidins (Gu *et al.*, 2004).

1.5.1.3.1. Absorption and metabolism of flavon-3-ols

Studies, mainly with green tea and cocoa indicate that (–)-epicatechin and (–)-epigallocatechin are readily absorbed with glucuronide, methyl and sulphate metabolites appearing in plasma in μmolar concentrations and being excreted in urine in relatively high amounts (Manach *et al.,* 2005). Interestingly, unmetabolised (–)-epigallocatechin gallate accumulates in plasma but neither it nor any of its metabolites are excreted in urine (Feng *et al.,* 2006). In rats, (–)-epigallocatechin gallate appears to be removed from the bloodstream and undergo enterohepatic recirculation being returned to the duodenum in the bile (Kohri *et al*., 2001). To what extend such enterohepatic recirculation occurs in humans is difficult to determine. A recent study showed glucuronides of (–)-epicatechin and methyl-(–)-epicatechin were detected in the brains of mice fed an (–)-epicatechin diet for six weeks. The animals also exhibited enhanced angiogenesis and retention of spatial memory (van Praag *et al.,* 2007). There is also a report that (–)-epigalloacatechin gallate crosses the blood-brain barrier of rats (Lin *et al.,* 2007). The only study with

humans to date detected flavan-3-ol metabolites in plasma but not cerebrospinal fluid after acute supplementation with 300 mL of green tea (Zini *et al.,* 2006).

There is limited information of the bioavailability of procyanidins. Dimers have been detected in rat and human plasma and urine after feeding chocolate (Baba *et al.,* 2002) and dimers and trimers have been detected in rat urine after ingestion of a grape seed extract (Tsang *et al.*, 2005). However, the amounts detected were extremely small compared to the quantities ingested indicating that passage from the small intestine may be restricted especially for high molecular weight procyanidins, which are known to bind to proteins. However, a recent study in which apple procyanidins were fed to rats was able to detect procyanidin dimers through to pentamers in plasma by extracting with 8 M urea, which prevented irreversible procyanidin binding to proteins, which is a feature when plasma is extracted with methanol or acetonitrile (Shoji *et al*., 2006). More evidence on absorption of these compounds are needed.

1.5.1.4. Ellagitannins

The hydroxybenzoate, ellagic acid has been reported to be present in berries, particularly in raspberries, strawberries and blackberries (Amakura *et al.*, 2000). The levels are generally low, although substantial quantities are detected along with gallic acid after acid treatment of extracts results in ellagitannin breakdown. For instance raspberries, the health benefits of which are often promoted based on high ellagic acid content, contain ca. 100 μg/100 g of ellagic acid compared to ca. 30 mg/100 g of ellagitannins, mainly in the form of sanguiin H-6 and lambertianin (Mullen *et al.*, 2002c). The occurrence of ellagitannins in common foodstuffs is limited to a few fruit and nut species. Dietary intake of ellagitannins is largely explained by the consumption of strawberries, raspberries, blackberries and pomegranate. There are apparently conflicting claims for beneficial and toxic effects caused by ellagitannins and tannins in general in various animal species (Singh et al., 2003; Wastendarp, 2006).

1.5.1.4.1. Metabolism of ellagitannins

Several recent studies have recently investigated ellagic acid bioavailability. After consumption of pomegranate juice providing ellagic acid and ellagitannins, intact ellagic acid was detected in human plasma, with a C_{max} 1 h after intake (Seeram *et al.*, 2004). In contrast, ellagic acid was not recovered in urine from volunteers 24 h after feeding strawberries, raspberries, walnuts or oak-aged red wine (Cerdá *et al.*, 2005). However, a microbial metabolite urolithin B conjugated with glucuronide acid was found in the urine of volunteers 32 to 56 h after ingestion and has been suggested as a biomarker of exposure to ellagitannins and ellagic acid. The structures of these molecules and the possible pathways for conversion into urolithin B by the microflora action are shown in Figure 1.16.

Most of the ellagitannins present in food have a molecular weight in excess of 1000 Da and have to be hydrolysed by intestinal enzymes or by colonic bacteria before they can be absorbed. In further study with humans subjects who consumed pomegranate juice, Cerdá *et al.* (2004) detected a urolithin B glucuronides in urine 24 h after supplementation, suggesting that the metabolites were produced in the distal part of the intestine by the action of the colon bacteria. There were large variations in the quantity of urolithin excreted by the individual volunteers indicating substantial person-to-person differences in the composition of the colonic microflora, a point also noted by Jaganath *et al.,* (2006) when investigating colonic degradation of rutin. The concentration of urolithin B detected in plasma after ingestion of pomegranate juice was as high as 18.6 μM, but this was not accompanied by increases in either plasma or urine antioxidant activity (Cerdá *et al.*, 2004).

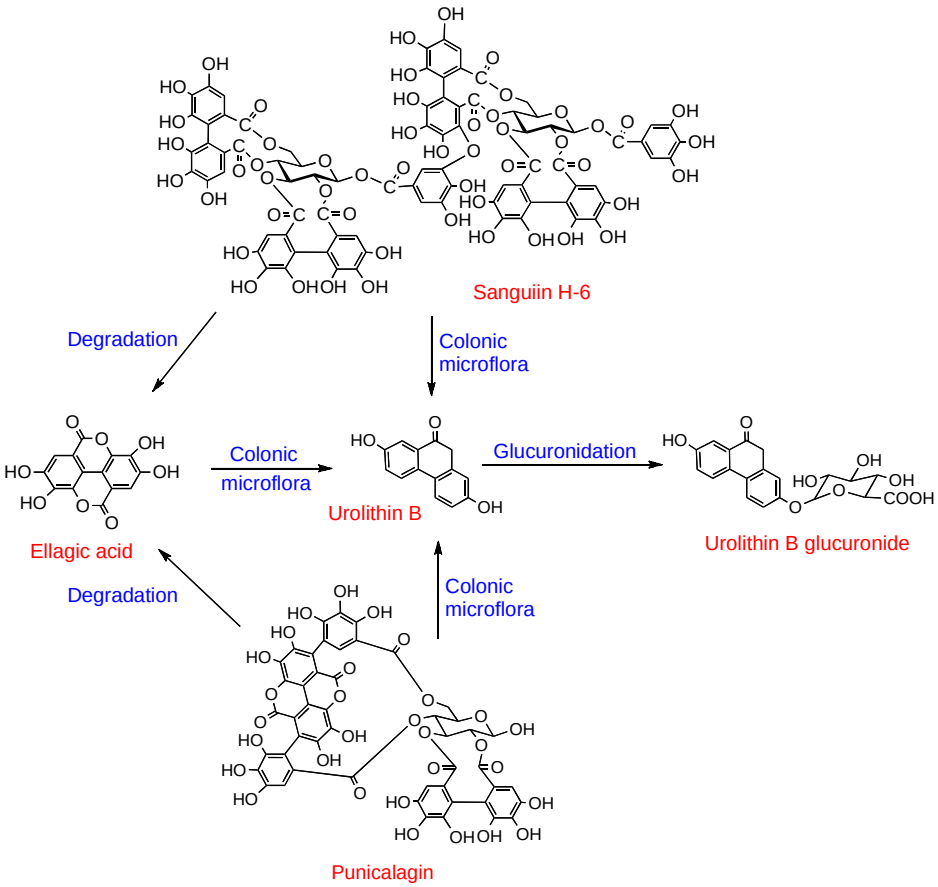


Figure 1.16. Possible cetabolism of ellagitannins to yield ellagic acid and urolithin B (Cerdá *et al*., 2005).

In summary, there is considerable observational evidence indicating that adequate fruit and vegetable consumption has a role in reducing the incidence of some chronic diseases. There is also an increasing body of evidence that phenolic compounds in fruits and vegetables have a key role in mediating those protective effects. Knowledge of the composition of phenolic constituents in different fruits and vegetables would be an important factor for the consumers when deciding on the plant products they purchase as it could have a positive impact in their long term health. More research on the absorption, distribution and excretion of these compounds in the body needs to be done in order to further elucidate the mechansim(s) of action.

1.6. Aims of the study

Given the need for more detailed knowledge of the polyphenolic content of fruits and vegetables and for more investigation on their bioavailability, the work in this thesis aimed to achieved the following objectives:

1. To screen the antioxidant activity of common fruits, vegetables and nuts available in supermarkets in Scotland to identify products that are rich in phenolics. Such information would provide the general public with a ready means of increasing the potential protective effects of fruit and vegetable consumption.
2. To analyse the phenolic composition of raspberries, blueberries, blackcurrant, redcurrants and cranberries as they constitute a group of fruits with a very high antioxidant capacity. This detailed analysis will reveal not only the nature and concentration of individual phenolic compounds but also their contribution to the overall antioxidant activity of the berries.
3. To evaluate the bioavailability of anthocyanins, ellagitannins and ellagic acid occurring in raspberries by an intervention study with human subjects.
4. To investigate the distribution of anthocyanins, ellagitannins and their metabolites in the gastrointestinal tract and their presence in, plasma, urine and other organs and tissues following ingestion of raspberry juice by rats.

Chapter 2. MATERIALS AND METHODS

In this chapter analytical methods for polyphenols, metabolites and antioxidant assays are present. Other methods are described in individual chapters.

2.1. Collection, processing and storage of fresh plant product samples for antioxidant analysis

In order to screen the antioxidant activity of plant products available to the public in a supermarket in Scotland a selection of 41 vegetables, 21 fruits and seven dry fruits, seed and nuts were purchased from a local food shop in the West End of Glasgow. Details of the products are shown in Tables 2.1, 2.2 and 2.3. Depending on the size of the product 3 to 6 units or sections of the different products were cleaned, weighed, chopped, mixed, immediately frozen in liquid nitrogen, freeze-dried, ground to powder and stored at -20°C until analyzed. Samples in triplicate were further subject to analysis for antioxidant activity, total phenolic content, flavonol analysis and vitamin C content.

2.2. Extraction of phenolics

Ten (10) mg of lyophilised tissues were weighed, placed on ice and homogenised in 1 mL methanol/water (1:1, v/v) using an Ultra-Turrax T25 homogeniser (IKA Werke, Staufen, Germany) for 1 minute. The mixture was then centrifuged at 13000 g at 4°C for 20 minutes. The supernatant was separated and a second extraction of the pellet was made pooling the supernatants at the end. Solutions were stored at – 80^{o}C until analysis.

Table 2.1. Details of the vegetables purchased in a local supermarket and analyzed in this study.

VEGETABLES	Scientific name	Country of origin
1. Kale	*Brassica oleracea*	Russia
2. Curly kale	*Brassica oleracea* var. Winter bore	Spain
3. Spinach	*Spinacea oleracea* var. Chica	UK
4. Broccoli - florets	*Brassica oleracea*	Spain
5. Broccoli – stalks	*Brassica oleracea*	Spain
6. Purple Broccoli		UK
7. Green cabbage - heart	*Brassica oleracea* var. Primo	UK
8. Green cabbage – outerleaves	*Brassica oleracea* var. Primo	UK
9. Sweetheart cabbage	*Brassica oleracea* var. Duchy	UK
10. Savoy cabbage	*Brassica oleracea* var. Mardora	n.s
11. Red cabbage	*Brassica oleracea* var. Mardora	n.s
12. Mangetout	*Pisum sativum*	Kenya
13. Sugarsnap	*Pisum sativum*	n.s
14. Broad beans - whole	*Vicia faba major*	n.s
15. Broad beans - beans only	*Vicia faba major*	n.s
16. Fine beans	*Phaseolus vulgaris*	Kenya
17. Green beans	*Phaseolus vulgaris*	Egypt
18. Cherry tomatoes	*Lycopersicon esculentum* var. Cerasiforme	Spain
19. Vine cherry tomatoes	*Lycopersicon esculentum*	Italy
20. Lollo Rosso Lettuce	*Lactuca sativa*	UK
21. Iceberg lettuce	*Lactuca sativa* var. Brandon	UK
22. Beetroot - cooked	*Beta vulgaris*	UK
23. Raddichio	*Cichorium intybus*	UK
24. Chicory	*Cichorium intybus*	UK
25. Fennel	*Foeniculum vulgare* var. Zefa Tardo	Holland
26. Yellow onion	*Allium cepa*	n.s
27. White onion	*Allium cepa*	n.s
28. Red onion	*Allium cepa* var. Baron	n.s
29. Radishes	*Raphanus sativus*	UK
30. Salad onion - green part	*Allium cepa*	Mexico
31. Salad onion - white part	*Allium cepa*	Mexico
32. Red pepper	*Capsicum annum*	Spain
33. Yellow pepper	*Capsicum annum*	Spain
34. Green pepper	*Capsicum annum*	Spain
35. White potatoes	*Solanum tuberosum* var. Cara	UK
36. Organic potatoes	*Solanum tuberosum* var. Ditta	Germany
37. Courgettes	*Cucurbita pepo* var. Store Green	Spain
38. Baby courgettes	*Cucurbita pepo*	Kenya
39. Carrots	*Daucus carota* var. Nairobi	UK
40. Minicarrots	*Daucus carota*	USA
41. Sweetcorn	*Zea mayz* var. Supersweet	USA

n.s – non specified.

Table 2.2. Details of fruits and fruit products analyzed**.**

FRUITS	Scientific name	Country of origin
42. Mango	*Mangifera indica*	Brasil
43. Lychees	*Litchi chinensis*	Israel
44. Kids range kiwi slices	*Actinidia chinensis* var. Hayward	New Zealand
45. Kids range melon pieces	*Cucumis melo* var. Honeydew/Canteloup	Spain
46. Blueberries	*Vaccinium corymbosum*	n.s
47. Kids range strawberries	*Fragaria vesca* var. Elsanta	Belgium
48. Mini strawberries	*Fragaria vesca* var. Elsanta	Holland
49. Giant strawberries	*Fragaria vesca* var. Longstem	USA
50. Strawberries	*Fragaria vesca* var. Tamar	Israel
51. Raspberries	*Rubus ideaus* Var. Autumm Bliss	Chile
52. White grapes	*Vitis vinifera* var. Superior Seedless	South Africa
53. Red grapes	*Vitis vinifera* var. Seedless	South Africa
54. Clementines	*Citrus reticulata* Var. Fortuna	Spain
55. Plums	*Prunus domestica* agg. var. Ruby Nell	South Africa
56. Melon	*Cucumis melo* var. Honeydew	Peru
57. Apples	*Malus pumila* var. Royal gala	France
58. Apples	*Malus plumila* var. Pink lady	USA
59. Peaches	*Prunus persica* var. California Re	USA
60. Nectarines	*Prunus persica* var. Flavortop	South Africa
61. Pears	*Pyrus communis* var. Conference	Holland
62. Bananas	*Musa paradisiaca* var. Banana 3	Panama

n.s – non specified.

Table 2.3. Details of dried seeds products analyzed.

Dried products	Scientific name	Country of origin
63. Coconut	*Cocos mucifera*	Sri Lanka
64. Cashew nuts	*Anarcardium occidentale*	India
65. Almonds	*Prunus dulcis*	USA
66. Kids range raisins	*Vitis vinifera* var. California Seedless	USA
67. Nuts and raisins assortment		USA
68. Raw peanuts	*Arachis hypogea*	China
69. Roasted peanuts	*Arachis hypogea*	China

n.s – non specified.

2.3. Collection, processing and storage of five different fresh berries samples for identification, quantification of phenolics and on line antioxidant analysis

Five varieties of berries were selected, based on their high antioxidant capacity, for a detailed analysis of phenolics by HPLC with on-line antioxidant detection. Blueberries (*Vaccinium corymbosum)*, cranberries (*Vaccinium oxycoccus*), raspberries (*Rubus idaeus)*, blackcurrants (*Ribes nigrum*) and redcurrants (*Ribes rubrum*) were purchased in local fruit shops in Byres Road., Glasgow. Freeze-dried samples were extracted with 20 mL of acidified methanol using an Ultra-Turrax T25 homogeniser (IKA Werke, Staufen, Germany) for 1 min and centrifuged for 20 min at 4000 g. The pellets were extracted twice and supernatants were pooled before being concentrated using a rotovapor (Buchi R-200, Flawil, Switzerland). The residues were resuspended in 10 mL of acidified methanol. Aliquots were stored at –80ºC until analysis.

2.4. Colorimetric assays

2.4.1. Analysis of total phenol content (TPC)

The TPC of the different fruits and vegetables was determined using the Folin-Ciocalteu method of Singleton and Rossi, (1965). This method determines phenols and oxidised substances which produce a blue colour as a result of reducing yellow phosphomolibdate-tungstate anions. In brief, 1 mL of 1:10 diluted Folin-Ciocalteu reagent was added to 20 µL of an appropriate diluted sample and 180 µL of water. After 5 min, 700 µL of a 0.74 M Na_2CO_3 was added; the solutions were mixed and after a 2 h incubation at room temperature, the absorbance was read at 765 nm using a Unicam UV500 UV-visible spectrophotometer (Thermo Spectronic, Cambridge, United Kingdom). Absorbance was calculated in gallic acid equivalents by reference to a standard calibration curve obtained with 50-800 µg/mL gallic acid. The total phenolic content was expressed on a fresh weight basis as mg of gallic acid equivalent (GAE)/g. Samples were analyzed in triplicate.

2.4.1.1. Analysis of antioxidant activity (AOC) using Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay, described by Benzie and Strain (1996), was used to estimate the antioxidant capacity of fruit and vegetable samples. This method measures the ability of a solution to reduce a ferric-tripyridyl-triazine complex (Fe^{3+}-TPTZ) to the ferrous form Fe^{2+}, producing a blue colour with λ_{max} at 593 nm. A 1.5 mL volume of freshly prepared FRAP reagent (containing the Fe^{3+}-TPTZ in excess at pH 3.6), was added to 50 µL of sample and 150 µL water and the absorbance at 593 nm was measured after 4 min of reaction. The absorbance was compared to a 0 to 1 mM Fe^{2+} standard curve. The results are expressed as µmoles Fe^{+3}/g or mM in the case of liquids.

2.5. Analysis of the antioxidant capacity using Electron-Spin Resonance (ESR) assay

The ability of plant extracts to reduce the Fremy's salt (potassium nitrosulphonate) was measured as described by Gardner *et al.* (1998). The extracts were diluted with ethanol/water (12:88 v/v). Three mL aliquots were reacted with an equal volume of 1mM Fremy's radical in ethanol/water (12:88, V/V). The ESR spectra of the low field resonance of the Fremy's radical were obtained after 20 min by which time the reaction was complete. Signal intensity was obtained by double integration and concentration calculated by comparison with a control reaction using ethanol/water without sample. Spectra were obtained at 21ºC on a Bruker ECS 106 spectrometer equipped with a cylindrical (TM110 mode) cavity and operating at ca. 9.5 GHz (X-band frequency). The microwave power and modulation amplitude were set at 2 mW and 0.01 mT, respectively.

2.6. High Performance Liquid Chromatography (HPLC) methods

2.6.1. Analysis of flavonols

Quercetin, kaempferol and isorhamnetin were analysed in samples eluted using a 150 x 3.0 mm i.d., 4 μm C18 Genesis column (Jones Chromatography, Mid-Glamorgan, UK) at a flow rate of 0.5 mL/min with a gradient of 20-40% acetonitrile in water adjusted to pH 2.5 with trifluoroacetic acid or formic acid. After passing through the photodiodearray detector (PDA) operating at 365 nm, column eluate was mixed with 0.1 M methanolic aluminium nitrate in 7.5% acetic acid pumped at a flow rate of 0.5 mL/min and fluorescent flavonol complexes detected with a fluorimeter (excitation 425 nm, emission 480 nm) as described by Aziz *et al.* (1998). Post column derivatization was optimised from the method published by Hollman *et al*. (1996) decreasing the 1.0 M aluminium nitrate this to 0.1 M which which enabled the use of a low pressure pump (Crozier *et al*, 2000).

2.6.2. Analysis of ascorbic acid

The ascorbic acid content of fruits and vegetables was assessed as described by Ross (1994). The samples were analysed on a HPLC system comprising of a HPLC pump, a PDA detector and an autosampler cooled to 4°C (Surveyor, Thermo Finnegan, San Jose, USA). Separation was carried out using a 5 μm 250 x 4.6 mm i.d. Nucleosil ODS column (Jones Chromatography, Henygoed, Mid Glamorgan, UK) fitted with Supelco C_{18} guard cartridge (Anachem Ltd, Luton, UK). The column was eluted isocratically with a mobile phase comprising 0.05 mM sodium hydroxide, 25 mM myristyltrimethylammonium bromide, 0.06 M acetic acid, 7.5 % acetonitrile mobile phase containing 100 mg/L homocysteine and 200 mg/L EDTA. The system was operated at 30°C with a flow-rate of 0.6 mL/min and PDA detection at 262 nm. The amount of ascorbic acid was calculated by reference to a calibration curve obtained with 0-500 μM ascorbic acid in 5 % metaphosphoric acid.

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

The $ABTS^{\bullet+}$ assay was based on the method of Pellegrini *et al.* (2003) where the capacity of antioxidant molecules to reduce the blue-green radical cation 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) to ABTS which is

colourless. The antioxidant activity of some berries to discussed in Chapter 3 was measured using an on-line HPLC antioxidant detection initially described by Koleva *et al.* (2001) and Dapkevicius *et al*. (2001) and subsequently optimized by Stewart *et al*. (2005). The instrumental set-up is schematised in Figure 2.1. Briefly, antioxidants in the HPLC eluate react post-column with pre-formed $ABTS^{\bullet+}$ and the induced bleaching is measured as a negative peak at 720 nm. The stock solution of $ABTS^{\bullet+}$ was made by adding 0.5 mL of a 70 mM $K_2S_2O_8$ solution to 50 mL of ABTS 2 mM. This mixture was stored overnight in the dark at room temperature to convert ABTS to $ABTS^{\bullet+}$. The $ABTS^{\bullet+}$ stock solution was mixed in a 0.1 M phosphate-buffer solution adjusted to pH 8 in the ratio 1:8 (v/v).

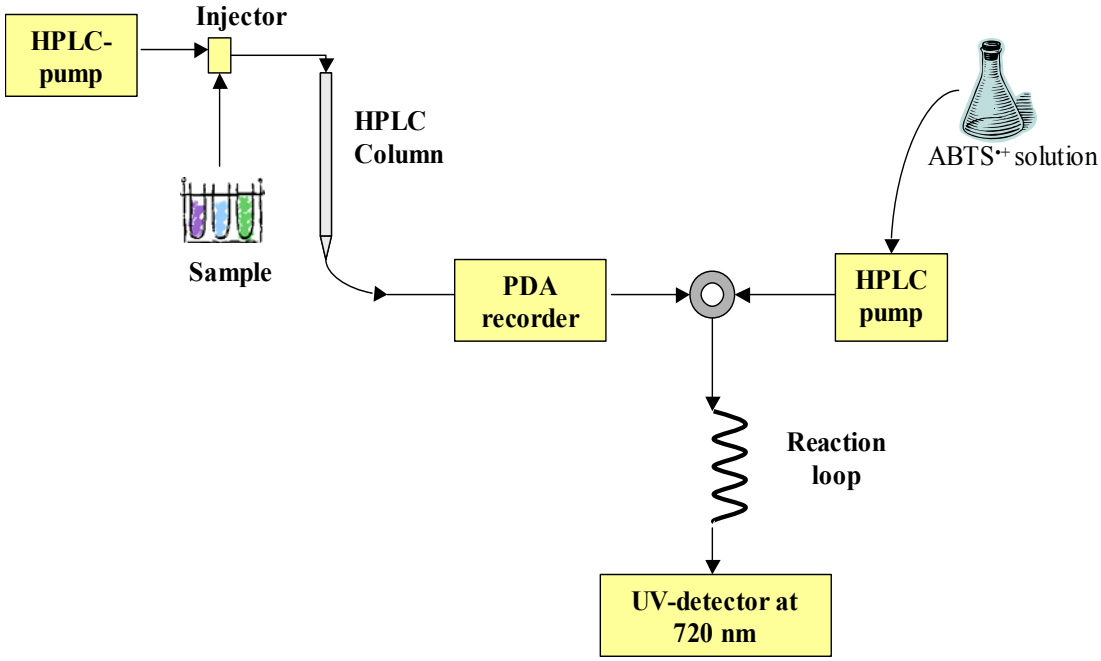


Figure 2.1 Instrument set-up for the HPLC analysis of antioxidant compounds using an on-line post column reaction with $ABTS^{\bullet+}$.

The HPLC system comprised an HPLC pump, an auto-injector with sample cooler, a column oven linked to a PDA detector (Thermo Finnegan, San Jose, USA). The separation was carried on a HPLC system comprising an pump, a PDA detector scanning from 250 to 700 nm and an autosampler cooled to 4°C (Thermo Finnegan, San Jose, USA). Samples were run using a 250 x 4.6 mm (i.d.), 4 µm Synergie RP-

Max column (Phenomenox, Macclesfield, UK) maintained at 40°C with a flow rate of 1 mL/min using different acetonitrile and formic acid 1% or 0.1% gradients. $ABTS^{\bullet+}$ solution was delivered by a LC-10-AD *VP* pump, connected to a GT-154 Vacuum Degasser (Shimadzu, Kyoto, Japan) at a flow rate of 0.5 mL/min. After mixing through a 1.5 m x 0.4 mm (i.d.) loop, absorbance was measured using a detector operating at 720 nm (Nemphlar Bioscience, Lanark, UK). Quantification of the antioxidant activity of the HPLC peaks was achieved by comparing the area of the peak to a standard curve obtained with 10-400 μmol/L of Trolox. Results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC).

2.7. Analysis of phenolic compounds by HPLC-MS^2

The phenolic profile of fruits and vegetables was analysed on a Surveyor HPLC system comprising an HPLC pump, a PDA detector scanning from 250 to 700 nm and an autosampler cooled to 4°C (Thermo Finnegan, San Jose, USA). Samples were run using a 250 x 4.6 mm (i.d.) Synergi RP-Max column (Phenomenex, Macclesfield, UK) maintained at 40°C. Samples were analysed at a flow rate of 1 mL/min using different solvent gradients. After passing through the flow cell of the PDA detector, the column eluate was split and 0.3 mL/min was directed to a LCQ Decamp ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnegan, San Jose, USA). All samples were analysed in negative and/or positive ion mode. A wide variety of compounds was identified based on their molecular ion and MS^2 fragmentation pattern. In addition, use of a PDA detector also provided absorbance spectra, which give useful information in locating the presence of anthocyanins (λ_{max} 520 nm), flavonols (λ_{max} 365 nm) and phenolic acids, flavanols and procyanidins (λ_{max} 280 nm). The majority of these compounds were found conjugated with various sugars and organic acids. As few standards are available to allow identification by co-chromatography, a variety of different analyses was carried out to confirm their identities. First analysis was by full scan MS^2 which provided information on the molecular ion, type of conjugation and in some case provided information on the aglycone. However, in many instances MS^2 of the aglycone was necessary to aid identification. Samples were analysed with the mass spectrometer operating in full scan, negative and positive ionisation modes. In the

negative ionization mode, acidic hydroxybenzoic and hydroxycinnamic acids deprotonate and dimeric and trimeric proanthocyanidin chains, depending on the chromatographic conditions favour protonation to positive ions and deprotonation to negative ions (Tomás-Barberán *et al*., 2001). Flavonol glycosides also show response in both positive and negative ions. Anthocyanins are identify in positive ion mode in their native forms as flavylium cations stabilized by acidifying extracts (Giusti *et al*., 1999).

2.8. Chemicals

HPLC solvents were obtained from Rathburn Chemicals (Walkerburn, Scotland). (+)-Catechin, quercetin-3-glucoside, malvidin-3-glucoside, myricetin, ellagic acid, kaempferol-3-glucoside, hydroxybenzoic acid, β-carotene, α-tocopherol, trolox, gallic acid, ascorbic acid, metaphosporic acid, ferrous sulphate, ferrous chloride, ferric chloride, TPTZ, Folin reagent, homocysteine, sodium acetate, myristyltrimethylammonium bromide, sodium hydroxide, ferric ammonium sulphate were obtained from Sigma (Poole, Dorset, UK). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt was provided by Merck (Dormstadt, Germany). EDTA and acetic acid were from BDH Chemicals Ltd (Poole, UK), sodium carbonate from Riedel de Haehn GmbH (Seelze, Germany). Naringenin, hesperitin and lutein were purchased from AASC Ltd (Southampton, England). Sodium diethyldithiocarbamate, Aluminium nitrate, SPE columns from Phenomenex, (Macclesfield, UK), diclomethane, methanol, ethanol, hydrochloric acid.

2.9. Statistics

Each sample was analysed in triplicate and data are presented as mean values ± standard error. Where appropriate data were subject to statistical analysis performed using Minitab software (Minitab Inc., Addison-Wesley publishing Co, Reading, MA).

Chapter 3. Screening of antioxidant activity and total phenolics in fruits, vegetables and other dietary products.

3.1. Introduction

The beneficial effects of regular consumption of fresh fruit and vegetables on human health have been discussed in Chapter 1. Phenolic compounds, which possess antioxidant properties, together with vitamins E and C, have been identified as being responsible for at least part of the protective effects of fresh fruit and vegetables. From a local perspective, Scotland has one of the highest rates of premature death from chronic diseases, most notably coronary heart disease, strokes and colon cancer (Scottish Office, 1993). This, in part, may reflect traditional dietary patterns due to the cost and/or lack of availability of fresh fruit and vegetables. One way to reduce this problem is to encourage the consumption of, not just fruits and vegetables, but fruits and vegetables containing elevated levels of phenolics compounds.

This study screened 69 different fruits, vegetables and nuts available in a supermarket chain in UK in order to identify products that are rich in phenolic antioxidants. Such information would provide the public with a ready means of increasing the potential protective effects of fruit and vegetable consumption. Details of the fruits, vegetables and other products selected are presented in Table 2.1, 2.2 and 2.3 in Chapter 2 of Materials and Methods.

The selection of the products analyzed was based on their availability in a supermarket chain. For comparative purposes different varieties, parts of the plant and/or commercial presentation of the same product were selected. For instance, kale and curly kale, broccoli and purple broccoli, green, sweetheart and Savoy cabbages, broad, fine and green beans, cherry and vine cherry tomatoes, broccoli stalk and florets, green cabbage heart and outer leaves, broad beans and pods, green and white parts of salad onions, kids range fruit products and raw and roasted peanuts. Nuts, cashew nuts, peanuts and almonds were also analysed as they are known to be good sources of vitamin E (Chen *et al*., 2005; Ryan *et al*., 2006).

Two different assays were used to assess antioxidant activity, electron-spin resonance spectroscopy (ESR) and Ferric Reducing Antioxidant Potential (FRAP).

The equipment and technical assistance for ESR were available at the Rowett Institute, Aberdeen. The total phenolic content of products was determined using the Folin-Ciocalteau assay (Singleton and Rossi, 1965). Individual flavonols like quercetin, isorhamnetin and kaempferol were also analyzed. Flavonols have long held an interest for nutritionists due to a Zutphen study in the early 1990's that showed that dietary intake of flavonols was inversely correlated with the incidence of coronary heart disease (Hertog *et al*., 1993a). The analysis of flavonols has been also improved by using a sensitive and selective HPLC procedure developed by Crozier *et al.,* (2000). Vitamin C, another source of antioxidant activity, was also measured by HPLC (Ross 1994). Correlations between all these parameters were investigated. For further details of all methods and techniques used in this section, see Chapter 2.

3.2. Results

3.2.1. Measurement of antioxidant capacity by FRAP and ESR assays.

Both assays revealed large differences in antioxidant content among the vegetables analysed (Table 3.1). With the FRAP assay values ranged from 23.5 (broad beans) to 0.3 μmole Fe^{+2}/g fresh weight (iceberg lettuce) while figures from 20.4 (broad beans) to 0.1 x 10^{18} radicals reduced/g (courgettes, radishes and white onion) were obtained with the ESR assay. Vegetables with a low antioxidant capacity in both methods included courgettes, radishes, iceberg lettuce, fine beans, green beans and white onions. Among the three beans analyzed broad bean had a much higher antioxidant content than fine and green beans. Vine cherry tomatoes were higher than cherry tomatoes. Values were similar for courgette and baby courgette, carrot and baby carrots and white and organic potatoes.

With fruits, values obtained with the ESR assay ranged from 9.1 x 10^{18} radicals reduced/g for mini strawberries to 0.3 for melon honeydew while the FRAP values varied from 22.4 μmoles Fe^{+2}/g for blueberries to 0.9 for peaches (Table 3.2). The four different samples of strawberries that were analysed had similar antioxidant

Table 3.1. ESR and FRAP antioxidant activity of different vegetables.

	TISSUE	H_2O (%)	ESR (rad red x10^{18}/g)	Rank	FRAP (µmol Fe^{+2}/g)	Rank
1	Kale	83.1	1.4 ± 0.0	11	4.3 ± 0.0	10
2	Curly kale	85.2	2.3 ± 0.0	7	8.3 ± 0.1	4
3	Spinach	92.3	0.5 ± 0.0	28	2.3 ± 0.1	18
4	Broccoli - florets	86.2	1.3 ± 0.0	12	2.4 ± 0.1	16
5	Broccoli - stalks	90.4	1.0 ± 0.0	18	1.5 ± 0.1	24
6	Purple Broccoli	79.9	4.3 ± 0.1	5	14.7 ± 0.6	3
7	Green cabbage - heart	91.8	1.0 ± 0.0	19	1.65 ± 0.1	22
8	Green cabbage - outerleaves	93.0	0.6 ± 0.0	26	1.2 ± 0.0	28
9	Sweetheart cabbage	89.2	0.8 ± 0.0	20	2.1 ± 0.1	19
10	Savoy cabbage	91.2	0.4 ± 0.0	33	1.5 ± 0.0	23
11	Red cabbage	91.4	1.2 ± 0.0	13	4.1 ± 0.1	11
12	Mangetout	87.1	1.2 ± 0.0	14	2.3 ± 0.1	17
13	Sugarsnap	85.2	1.1 ± 0.0	17	2.1 ± 0.2	20
14	Broad beans - whole	86.2	20.4 ± 0.4	1	19.6 ± 1.2	2
15	Broad beans - beans only	81.8	9.6 ± 0.5	2	23.5 ± 0.7	1
16	Fine beans	90.8	0.3 ± 0.0	35	0.6 ± 0.0	38
17	Green beans	84.9	0.7 ± 0.0	24	0.5 ± 0.1	39
18	Cherry tomatoes	92.0	1.8 ± 0.2	9	3.2 ± 0.1	13
19	Vine cherry tomatoes	91.8	2.3 ± 0.3	8	4.3 ± 0.2	12
20	Lollo Rosso Lettuce	94.8	1.6 ± 0.1	10	7.3 ± 0.7	6
21	Iceberg lettuce	95.3	0.2 ± 0.0	38	0.3 ± 0.0	41
22	Beetroot - cooked	85.0	3.9 ± 0.2	6	7.2 ± 0.3	7
23	Raddichio	93.2	1.1 ± 0.0	16	2.8 ± 0.1	15
24	Chicory	94.0	1.2 ± 0.0	15	1.7 ± 0.1	21
25	Fennel	92.3	0.4 ± 0.0	32	1.0 ± 0.1	32
26	Yellow onion	88.2	0.3 ± 0.1	36	2.9 ± 0.1	14
27	White onion	90.4	0.1 ± 0.0	40	0.8 ± 0.0	35
28	Red onion	88.3	0.5 ± 0.1	29	5.6 ± 0.0	9
29	Radishes	94.6	0.1 ± 0.0	39	0.9 ± 0.1	33
30	Salad onion - green part	93.7	0.5 ± 0.0	30	1.2 ± 0.0	27
31	Salad onion - white part	90.8	0.8 ± 0.0	21	0.8 ± 0.0	34
32	Red Pepper	88.3	7.0 ± 0.2	4	6.8 ± 0.2	8
33	Yellow Pepper	89.9	7.3 ± 0.1	3	8.1 ± 0.0	5
34	Green Pepper	94.0	0.7 ± 0.1	22	1.2 ± 0.0	29
35	White potatoes	79.3	0.3 ± 0.5	34	1.1 ± 0.1	30
36	Organic potatoes	79.6	0.4 ± 0.7	31	1.4 ± 0.0	25
37	Courgettes	94.2	0.1 ± 0.1	41	0.4 ± 0.0	40
38	Baby courgettes	92.9	0.2 ± 0.2	37	0.7 ± 0.0	37
39	Carrots	90.4	0.6 ± 0.0	27	1.0 ± 0.2	31
40	Minicarrots	90.3	0.6 ± 0.0	25	0.7 ± 0.0	36
41	Sweetcorn	83.0	0.7 ± 0.0	23	1.3 ± 0.1	26

Values represent mean ± SE (n=3) per g of fresh weight of edible portion. ESR units in radicals reduced x 10^{18}/g

Table 3.2. ESR and FRAP antioxidant activity of different fruits

.

	TISSUE	H_2O (%)	ESR (Rad red x10^{18}/g)	Rank	FRAP (µmol Fe^{+2}/g)	Rank
42	Mango	83.2	3.3 ± 0.0	10	6.6 ± 0.8	10
43	Lychees	82.9	1.2 ± 0.0	17	2.2 ± 0.4	17
44	Kids range kiwi slices	86.6	4.0 ± 0.3	8	6.5 ± 0.5	11
45	Kids range melon pieces	89.1	1.2 ± 0.0	16	2.4 ± 0.4	16
46	Blueberries	86.0	8.5 ± 0.0	4	22.4 ± 1.7	1
47	Kids range strawberries	88.3	8.9 ± 0.2	2	18.7 ± 3.9	3
48	Mini strawberries	90.9	9.1 ± 0.2	1	18.0 ± 1.9	5
49	Giant strawberries	88.5	7.5 ± 0.2	5	15.1 ± 1.4	6
50	Strawberries	90.5	8.8 ± 0.3	3	18.3 ± 0.9	4
51	Raspberries	84.8	7.4 ± 0.6	6	20.1 ± 0.1	2
52	White grapes	83.8	0.4 ± 0.5	20	1.5 ± 0.3	19
53	Red grapes	82.6	0.9 ± 0.5	18	3.5 ± 1.0	14
54	Clementines	87.2	2.6 ± 0.4	13	8.6 ± 0.4	8
55	Ruby Nell plums	83.9	5.9 ± 0.6	7	7.2 ± 0.3	9
56	Melon Honeydew	90.9	0.3 ± 0.2	21	0.1 ± 0.0	20
57	Royal gala apples	83.8	3.7 ± 0.1	8	8.6 ± 1.4	7
58	Pink lady apples	81.3	2.8 ± 0.0	11	5.7 ± 2.3	12
59	Peaches	80.3	0.6 ± 0.0	19	0.9 ± 0.2	21
60	Nectarines	84.7	2.7 ± 0.0	12	5.4 ± 0.7	13
61	Pears	85.1	1.6 ± 0.0	15	2.7 ± 0.2	15
62	Bananas	74.5	2.0 ± 0.0	14	2.1 ± 0.6	18

Values represent mean ± SE (n=3) per g of fresh weight of edible portion. ESR units in radicals reduced x 10^{18}/g

levels, 15.1 to 18.7 µmol Fe^{+2}/g, in the FRAP assay. The "kids range fruits" a mixture of fresh melon, red grapes and apple product, contained "intermediate" antioxidant activity compared to melon, white grapes and peaches contained low levels.

The AOC of raisins and nuts is relatively high with levels between 1.5 to 7.8 x 10^{18} radicals reduced/g by ESR and between 0.9 and 9.1 µmoles Fe^{+2}/g for FRAP (Table 3.3). Coconut was the lowest in AOC by both methods whilst raisins, peanuts and almonds were among the highest.

3.2.2. Total phenolic content (TPC)

Total phenolic content (TPC) of vegetables ranged from 3.1 to 6.8 mg GAE/g (Table 3.4). Purple broccoli, broad beans and curly kale were the highest while spinach, red cabbage, red onion, broccoli, beetroot, mangetout and radicchio had a low TPC with levels of around 2 mg GAE/g. Analysis of fruits (Table 3.5) showed that the

Table 3.3. ESR and FRAP antioxidant activity of different dried fruits and nuts.

	TISSUE	H_2O (%)	ESR (rad red x10^{18}/g)	Rank	FRAP (µmol Fe^{+2}/g)	Rank
63	Coconut	46.6	1.5 ± 0.0	7	0.9 ± 0.0	7
64	Cashew nuts	5.6	3.3 ± 0.0	5	3.7 ± 1.5	5
65	Almonds	1.7	4.0 ± 0.0	2	4.7 ± 1.9	4
66	Kids range raisins	14.4	2.0 ± 0.0	6	9.1 ± 0.9	1
67	Nuts and raisins assortment	9.0	7.8 ± 0.0	1	3.1 ± 1.7	6
68	Raw peanuts	8.6	2.6 ± 3.0	5	5.2 ± 3.4	3
69	Roasted peanuts	1.6	3.7 ± 3.7	3	7.8 ± 4.9	2

Values represent mean ± SE (n=3) per g of fresh weight of edible portion. ESR units in radicals reduced x 10^{18}/g

TPC ranking was plums > strawberries > raspberries > blueberries = bananas > apples with values of 1.9 - 3.6 mg GAE/g being obtained. The TPC of the products with a low water content range between 1.1 mg GAE/g for coconuts to 8.8 mg GAE/g for roasted peanuts (Table 3.6).

3.2.3. Flavonol content

The total flavonol content is represented by the sum of the three main flavonols individually analysed: quercetin, kaempferol and isorhamnetin. There was a high total flavonol content in Lollo Rosso lettuce, mangetout, kale, curly kale, red and yellow onion, and fine beans with concentrations of between 205 and 775 µg/g fresh weight while the remainder of the products contain less than 80 µg/g (Table 3.4). The total flavonol content of purple broccoli at 2242 µg/g was especially high with quercetin (1312 µg/g) being the main contributor. Blueberries, strawberries, apples and banana had the highest flavonol content of the fruits analysed (22.3 to 54 µg/g) (Table 3.5). Once again, quercetin was the main contributor in most instances. Exceptions were strawberries and bananas where, kaempferol and isorhamnetin, respectively, were the main flavonols. With nuts and raisins, the highest total flavonol contents ranged from 54.3 µg/g for kids range raisins to 23.1 µg/g for roasted peanuts. The levels in coconut, cashew nuts and almonds were relatively low (Table 3.6).

Table 3.4. Levels of total phenolics, total flavonol, quercetin, kaempferol, isorhamnetin and ascorbic acid content of vegetables based on fresh weight.

	TISSUE	TPC (mg GAE/g)	Total Flavonol (µg/g)	Quercetin (µg/g)	Kaempferol (µg/g)	Isorhamnetin (µg/g)	Ascorbic acid (µg/g)
1	Kale	6.3 ± 0	415 ± 20	169 ± 11	127 ± 8	119 ± 5	158 ± 8
2	Curly kale	5.2 ± 0	391 ± 27	244 ± 28	132 ± 8	15.1 ± 1	208 ± 0
3	Spinach	2.3 ± 0	5.2 ± 0	4.8 ± 0.2	n.d.	0.4 ± 0	231 ± 13
4	Broccoli - florets	2.0 ± 0	59.4 ±2	21.6 ± 1.0	33.1 ± 1	4.7 ± 0	305 ± 14
5	Broccoli - stalks	0.7 ± 0	3.7 ± 0	2.1 ± 0.2	1.5 ± 0	0.1 ± 0	475 ± 0
6	Purple Broccoli	6.8 ± 0	2242 ± 106	1312 ± 74	787 ± 0	143 ± 10	636 ± 9
7	Green cabbage - heart	0.7 ± 0	3.1 ± 0	0.7 ± 0	2.4 ± 0	n.d.	933 ± 0
8	Green cabbage - outerleaves	0.7 ± 0	6.0 ± 0	1.2 ± 0	4.8 ± 0	n.d.	116 ± 6
9	Sweetheart cabbage	0.7 ± 0	5.5 ± 0	0.6 ± 0	4.9 ± 0	n.d.	149 ± 3
10	Savoy cabbage	0.8 ± 0	23.3 ± 1	2.4 ± 0	20.9 ± 1	n.d.	112 ± 4
11	Red cabbage	2.3 ± 0	6.4 ± 0	5.7 ± 0	0.3 ± 0	0.4 ± 0	185 ± 0
12	Mangetout	2.0 ± 0	205 ± 5	199 ± 4	5.4 ± 0	0.6 ± 0	173 ± 8
13	Sugarsnap	1.1 ± 0	159 ± 24	155 ± 4	4.2 ± 0	0.0 ± 0	344 ± 0
14	Broad beans - whole	5.1 ± 0	37.3 ± 3	21.1 ± 1	15.7 ± 2	0.5 ± 0	259 ± 11
15	Broad beans - beans only	5.0 ± 0	11.8 ± 0	10.2 ± 0	1.5 ± 0	0.1 ± 0	na
16	Fine beans	0.4 ± 0	49.5 ± 1	37.9 ± 1	9.1 ± 0	2.5 ± 0	12 ± 0
17	Green beans	0.7 ± 0	41.0 ± 1	27.2 ± 1	11.9 ± 0	1.9 ± 0	1.6 ± 0
18	Cherry tomatoes	0.6 ± 0	20.7 ± 1	19.6 ± 1	1.0 ± 0	0.1 ± 0	215 ± 4
19	Vine cherry tomatoes	0.7 ± 0	5.9 ± 0	5.0 ± 0	0.6 ± 0	0.3 ± 0	243 ± 26
20	Lollo Rosso Lettuce	3.1 ± 0	775 ± 44	770 ± 39	4.5 ± 0	0.7 ± 0	58 ± 8
21	Iceberg lettuce	0.3 ± 0	2.4 ± 0	2.2 ± 0	0.1 ± 0	0.1 ± 0	7 ± 0
22	Beetroot - cooked	1.9 ± 0	0.8 ± 0	0.6 ± 0	0.1 ± 0	0.1 ± 0	20 ± 0
23	Raddichio	2.0 ± 0	6.9 ± 2	4.4 ± 1	2.1 ± 0	0.4 ± 0	7 ± 0
24	Chicory	0.6 ± 0	0.9 ± 0	0.1 ± 0	0.7 ± 0	0.1 ± 0	3 ± 0
25	Fennel	0.5 ± 0	0.7 ± 0	0.4 ± 0	0.2 ± 0	0.1 ± 0	8 ± 0
26	Yellow onion	1.4 ± 0	387 ± 35	360 ± 33	2.7 ± 0	24.8 ± 2	29 ± 1
27	White onion	0.8 ± 0	0.1 ± 0	0.1 ± 0	0.0 ± 0	0.0 ± 0	86 ± 12
28	Red onion	2.1 ± 0	614 ± 72	561 ± 65	1.4 ± 0	52.2 ± 7	57 ± 4
29	Radishes	0.9 ± 0	5.9 ± 0	0.4 ± 0	5.5 ± 0	n.d.	61 ± 3
30	Salad onion – green part	0.9 ± 0	80.8 ± 2	54.2 ± 1.1	22.5 ± 1	4.1 ± 0	109 ± 8
31	Salad onion – white part	1.0 ± 0	11.6 ± 1	7.3 ± 0.6	3.7 ± 0	0.6 ± 0	134 ± 0
32	Red Pepper	1.5 ± 0	1.6 ± 0	1.4 ± 0	n.d	0.2 ± 0	962 ± 0
33	Yellow Pepper	1.8 ± 0	2.9 ± 0	2.7 ± 0	n.d.	0.2 ± 0	1216 ± 0
34	Green Pepper	1.2 ± 0	5.6 ± 0	5.2 ± 1	0.1 ± 0	0.3 ± 0	401 ± 39
35	White potatoes	1.4 ± 0	0.6 ± 0	0.5 ± 0	0.1 ± 0	n.d.	70 ± 0
36	Organic potatoes	1.6 ± 0	0.8 ± 0	0.6 ± 0	0.1 ± 0	0.1 ± 0	71 ± 0
37	Courgettes	0.6 ± 0	8.3 ± 1	4.3 ± 0	2.6 ± 0	1.4 ± 0	6 ± 0
38	Baby courgettes	1.1 ± 0	17.5 ± 1	9.1 ± 1	4.8 ± 0	3.6 ± 0	47 ± 3
39	Carrots	0.5 ± 0	0.6 ± 0	0.6 ± 0	n.d.	n.d.	20 ± 0
40	Minicarrots	0.5 ± 0	0.4 ± 0	0.4 ± 0	n.d.	n.d.	97 ± 28
41	Sweetcorn	1.0 ± 0	2.6 ± 0	2.3 ± 0	0.2 ± 0	0.1 ± 0	24 ± 4

Values represent mean ± SE (n=3) per g of fresh weight of edible portion. GAE stands for gallic acid equivalents

Table 3.5. Levels of total phenolics, total flavonol, quercetin, kaempferol, isorhamnetin and ascorbic acid content of vegetables based on fresh weight.

TISSUE	TPC (mg GAE/g)	Total Flavonol (µg/g)	Quercetin (µg/g)	Kaempferol (µg/g)	Isorhamnetin (µg/g)	Ascorbic acid (µg/g)
42 Mango	1.1 ± 0	6.7 ± 0.8	4.6 ± 1	1.6 ± 0	0.5 ± 0	236 ± 6
43 Lychees	1.1 ± 0	7.0 ± 0.4	5.2 ± 0	1.3 ± 0	0.5 ± 0	83 ±3
44 Kids range kiwi slices	0.9 ± 0	5.8 ± 0.5	3.5 ± 0	2.1 ± 0	0.2 ± 0	463 ± 0
45 Kids range melon pieces	0.7 ± 0	3.4 ± 0	3.3 ± 0	0.1 ± 0	n.d.	258 ± 0
46 Blueberries	2.5 ± 0	33.2 ± 2	26.3 ± 1	0.8 ± 0	6.1 ± 0	2 ± 0
47 Kids range strawberries	3.4 ± 0	30.8 ± 4	5.0 ± 0	25.4 ± 4	0.4 ± 0	690 ± 26
48 Mini strawberries	2.9 ± 0	38.6 ± 2	13.4 ± 0	24.8 ± 2	0.4 ± 0	784 ± 14
49 Giant strawberries	2.8 ± 0	25.5 ± 1	10.1 ± 0	14.9 ± 1	0.5 ± 0	673 ± 23
50 Strawberries	2.9 ± 0	25.2 ± 1	12.8 ± 0	12.0 ± 0	0.4 ± 0	469 ± 11
51 Raspberries	3.0 ± 0	7.7 ± 0	6.6 ± 0	1.0 ± 0	0.1 ± 0	84 ± 0
52 White grapes	1.0 ± 0	5.8 ± 0	4.9 ± 0	0.6 ± 0	0.3 ± 0	11 ± 0
53 Red grapes	1.5 ± 0	13.3 ± 1	12.3 ± 1	0.5 ± 0	0.5 ± 0	25 ± 1
54 Clementines	1.4 ± 0	6.8 ± 0	3.6 ± 0	0.4 ± 0	2.8 ± 0	302 ± 0
55 Ruby Nell plums	3.6 ± 0	4.6 ± 0	4.5 ± 0	0.1 ± 0	n.d.	17 ± 1
56 Melon Honeydew	0.6 ± 0	0.3 ± 0	0.3 ± 0	n.d.	n.d.	14 ± 0
57 Royal gala apples	1.9 ± 0	22.3 ± 1	21.8 ± 1	0.3 ± 0	0.2 ± 0	8 ± 0
58 Pink lady apples	1.4 ± 0	25.1 ± 2	24.8 ± 2	0.2 ± 0	0.1 ± 0	40 ± 2
59 Peaches	1.2 ± 0	2.9 ± 0	2.2 ± 0	0.3 ± 0	0.4 ± 0	13 ± 0
60 Nectarines	1.5 ± 0	9.6 ± 1	8.2 ± 1	1.4 ± 0	n.d.	50 ± 4
61 Pears	1.0 ± 0	1.4 ± 0	0.8 ± 0	0.3 ± 0	0.3 ± 0	4 ± 0
62 Bananas	2.5 ± 0	54.8 ± 6	12.1 ± 1	0.3 ± 0	42.4 ± 5	4 ± 1

Values represent mean ± SE (n=3) per g of fresh weight of edible portion. GAE, gallic acid equivalents

Table 3.6. Levels of total phenolics, total flavonol, quercetin, kaempferol, isorhamnetin and ascorbic acid content of low humidity plant products based on fresh weight.

TISSUE	TPC (mg GAE/g)	Total Flavonol (µg/g)	Quercetin (µg/g)	Kaempferol (µg/g)	Isorhamnetin (µg/g)	Ascorbic acid (µg/g)
63 Coconut	1.1 ± 0	0.8 ± 0	0.7 ± 0	n.d.	0.1 ± 0	4 ± 0
64 Cashew nuts	1.9 ± 0	8.9 ± 1	5.2 ± 0	3.5 ± 0	0.2 ± 0	11 ± 0
65 Almonds	2.0 ± 0	9.8 ± 1	3.3 ± 0	4.6 ± 0	1.9 ± 0	90 ± 3
66 Kids range raisins	6.6 ± 0	54.3 ± 9	48.8 ± 9	4.7 ± 2	0.8 ± 0	9 ± 0
67 Nuts and raisins assortment	5.4 ± 0	47 ± 2	19.3 ± 2	3.8 ± 1	24.1 ± 2	81 ± 3
68 Raw peanuts	7.5 ± 0	27.8 ± 2	16.0 ± 1	10.8 ± 1	1.0 ± 0	15 ± 0
69 Roasted peanuts	8.8 ± 0	23.1 ± 1	21.3 ± 3	0.8 ± 0	1.0 ± 1	26 ± 0

Values represent mean ± SE (n=3) per g of fresh weight of edible portion. GAE, gallic acid equivalents

3.2.4. Vitamin C content

Levels of vitamin C were measured in order to identify its contribution and correlation with antioxidant capacity data. Vegetables with a high vitamin C content were peppers (between 400 and 1216 μg/g) broccoli and purple broccoli (475 and 637 μg/g respectively) (Table 3.4). Among the fruits strawberries (469 - 784 μg/g), clementine (302 μg/g), kids kiwi slices (463 μg/g) and melon pieces (258 μg/g) were the highest (Table 3.5).

3.2.5. Portion size and antioxidant activity

The "average portion size" of fruits and vegetables consumed in UK were obtained from a dietary analysis software package, Diet 5™ for windows (Robert Gordon University, Aberdeen, UK). This made it possible to calculate the potential antioxidant capacity available in a portion of a given product. Comparing the fruits and vegetables based on serving size can be useful because there are differences in the amounts that are normally consumed. For instance, the smallest serving size in the group of products analyzed was 25 g for cashew nuts and almonds and the largest was 180 g for melon and pears. The top five vegetables that provide both high levels of antioxidant activity and total phenolics per serving were broad beans, purple broccoli, yellow and red pepper and curly kale (Table 3.7). The top five fruits for antioxidant activity were strawberries, blueberries, raspberries, royal gala apples and nectarines with a similar ranking for TPC except for the addition of bananas to the list (Table 3.8). A portion of peanuts (raw or roasted) or nuts and raisins provides a similar level of phenolics (TPC) as a portion of berries or broad beans although not levels by FRAP (Table 3.9). A portion of Iceberg lettuce contains *ca.* 200-fold less antioxidant activity than a portion of broad beans.

Table 3.7. TPC and FRAP antioxidant activity of different vegetables calculated per portion size*.

	TISSUE	Portion size (g)	FRAP (µmoles Fe^{+2} /portion)	Total Phenolic (mg/portion)
1	Kale	90	390	**566**
2	Curly kale	90	751	**464**
3	Spinach	90	205	**206**
4	Broccoli - florets	90	212	182
5	Broccoli - stalks			
6	**Purple Broccoli**	90	**1320**	**612**
7	Green cabbage - heart	90	149	66
8	Green cabbage - outerleaves			
9	Sweetheart cabbage	90	189	65
10	Savoy cabbage	90	137	73
11	Red cabbage	90	367	**208**
12	Mangetout	75	175	148
13	Sugarsnap	75	155	85
14	**Broad beans - whole**	75	**1469**	**383**
15	**Broad beans - beans only**	75	**1761**	**378**
16	Fine beans	75	47	33
17	Green beans	75	34	54
18	Cherry tomatoes	85	265	53
19	Vine cherry tomatoes	85	343	57
20	Lollo Rosso Lettuce	30	220	92
21	Iceberg lettuce	30	8	9
22	Beetroot - cooked	35	251	68
23	Raddichio	30	84	59
24	Chicory	150	249	92
25	Fennel	120	121	56
26	Yellow onion	90	257	123
27	White onion	90	72	72
28	Red onion	90	500	186
29	Radishes	30	26	26
30	Salad onion - green part	100	123	88
31	Salad onion - white part	100	81	104
32	Red Pepper	130	888	198
33	**Yellow Pepper**	130	**1052**	**239**
34	Green Pepper	130	157	161
35	White potatoes	120	128	166
36	Organic potatoes	120	168	196
37	Courgettes	90	39	54
38	Baby courgettes	90	59	97
39	Carrots	80	82	42
40	Minicarrots	80	57	39
41	Sweetcorn	90	117	90

*Bold fonts indicates high values

Table 3.8. TPC and FRAP antioxidant activity of different fruits calculated by portion size*.

TISSUE	Portion size (g)	FRAP (µmoles Fe^{+2}/portion)	Total Phenolic (mg/portion)
42 Mango	100	661	110
43 Lychees	100	216	110
44 Kids range kiwi slices	80	522	75
45 Kids range melon pieces	80	190	54
46 Blueberries	65	**1456**	163
47 Kids range strawberries	100	**1868**	**337**
48 Mini strawberries	100	**1802**	**292**
49 Giant strawberries	100	**1514**	**282**
50 Strawberries	100	**1829**	**285**
51 Raspberries	65	**1307**	196
52 White grapes	50	76	50
53 Red grapes	50	175	73
54 Clementines	60	517	83
55 Ruby Nell plums	55	398	198
56 Melon Honeydew	180	178	101
57 Royal gala apples	100	864	189
58 Pink lady apples	100	567	143
59 Peaches	110	101	132
60 Nectarines	155	834	**237**
61 Pears	180	486	176
62 Bananas	120	246	**299**

*Bold fonts indicates high values

Table 3.9. TPC and FRAP antioxidant activity of different dried fruits and nuts calculated by portion size*.

TISSUE	Portion size (g)	FRAP (µmol Fe^{+2}/ portion)	Total Phenol (mg/portion)
63 Coconut	30	27	33
64 Cashew nuts	25	93	48
65 Almonds	25	118	50
66 Kids range raisins	25	228	165
67 Nuts and raisins assortment	45	140	**243**
68 Raw peanuts	50	260	**375**
69 Roasted peanuts	50	390	**440**

*Bold fonts indicates high values

3.2.6. Fractionation of fruit and vegetables with high antioxidant capacity

In order to further investigate the phenolics in products with high antioxidant activity methanolic extracts were fractionated using a solid phase C_{18} extraction cartridge that was eluted sequentially with acidified water (F-1), ethyl acetate (F-2) and acidified methanol (F3). The products selected for fractionation were broad beans, yellow, green and red pepper, purple and green broccoli, Lollo Rosso lettuce, curly kale, red and yellow onion, blackberries, blackcurrant, redcurrants, strawberries and blueberries. The three fractions from each product were analyzed for antioxidant activity using the FRAP assay.

Broad beans, yellow, red and green pepper had highest activity in F-1 contributing 63, 74, 44 and 78% to the total antioxidant activity respectively (Figure 3.1A). In contrast, most of the antioxidant activity in red onion, purple broccoli, Lollo Rosso lettuce and curly kale, 71, 76, 64 and 62% respectively, eluted in F-3. This fraction was also the major contributor to the antioxidant activity of berries with 45% for strawberries, 87% for blueberries and 96% with blackcurrant (Figure 3.1B). Studies with reference compounds have shown that phenolic acids elute in F-1, flavan-3-ol monomers in F-2 and anthocyanins in F3 while flavonols appear principally in F-2 but also elute in F-3.

All these fractions were to be further analyzed to define the composition of flavonoids and phenolics but in October 2001 a fire destroyed the laboratory and all the samples were lost. One year later when a refurbished laboratory became available, the approach changed and it was decided that the analyses by HPLC-PDA-MS^2 and HPLC with PDA and on-line antioxidant detection would focus on berries. The results obtained are presented in Chapter 4.

3.3. Discussion

The screening of 69 plant products divided into three major food groups, i) vegetables, ii) fruits and iii) nuts, seeds and dry fruits revealed large differences by FRAP, ESR and TPC values among the groups and also within the groups. The top ten vegetables for AOC measured by ESR were:

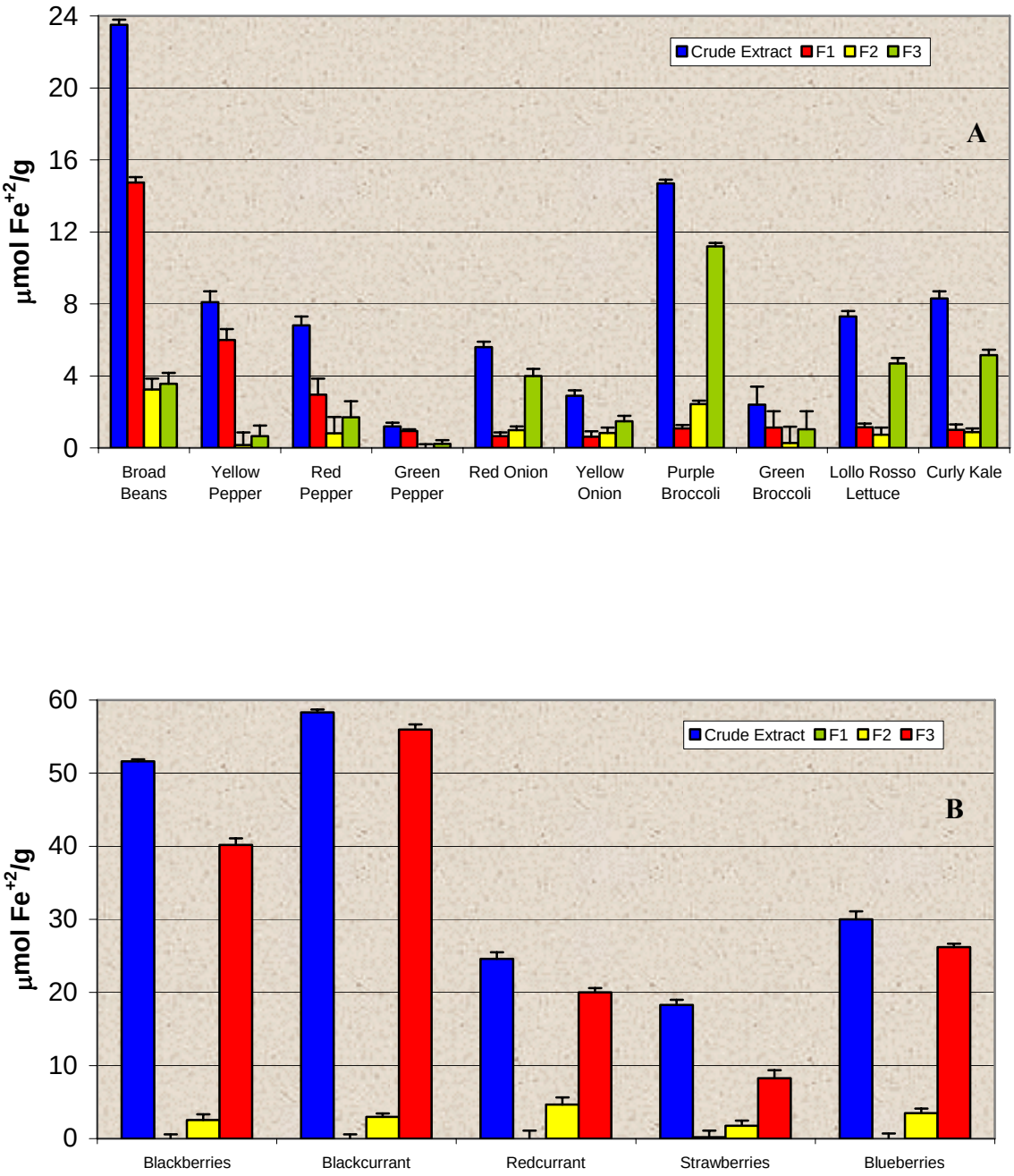


Figure 3.1. FRAP antioxidant activity of the fractionated vegetables (Panel A) and fruits (Panel B) on C_{18} Sep-Pak cartridges. F1 = water eluted fraction, F2 = ethyl acetate eluted fraction, F3 = acidified methanol eluted fraction (n=3).

broad beans > yellow pepper > red pepper > purple broccoli > beetroot >curly kale > vine cherry tomatoes > cherry tomatoes > Lollo Rosso lettuce > kale. The order for FRAP activity was broad beans > purple broccoli > curly kale > yellow pepper > Lollo Rosso lettuce > beetroot > red pepper >red onion > kale >red cabbage; similar to that obtained with ESR.

Comparison of FRAP and ESR results in this study with the data in the literature is not straightforward. The amount of data published on AOC of fruits and vegetables in the last ten years is substantial but the lack of standardisation of the assays, definition of produce, collection, storage conditions, variety etc. are all important confounding issues. For example Halvorsen *et al.* (2002) found levels for FRAP of >50 μmol Fe^{+2}/g in products like strawberry and blueberry while in the current study values of 22 and 19 μmol Fe^{+2}/g, respectively, were obtained, while Proteggente *et al*. (2002) reported 33 μmol Fe^{+2}/g for strawberry. Although there are differences in number and composition of the group of products analyzed by different investigators, it is noticeable that Pellegrini *et al.* (2003) reported that spinach yielded the highest FRAP antioxidant capacity followed by red pepper and beetroot. This is a similar ranking to that obtained in the present study and by Proteggente *et al*. (2002). The actual levels of AOC were however, lower in the current study than the values obtained by these two groups. This is probably a direct consequence of the reaction time used for the FRAP assay in the three studies. In this study, a 4 min reaction period was used as the original report of Benzie and Strain, (1996). Proteggente *et al*. (2002) used a 7.5 min reaction time and Pellegrini *et al.* (2003) a lengthy 30 min. Prior *et al*. (2005) explain that some phenolics do react fast within 4 min but others can take even several hours to complete the reaction. The lack of standardization for the reaction time of FRAP produce a tremendous difference in results.

There are no previous reports on the AOC of broad beans, purple broccoli and Lollo Rosso lettuce, all of which are readily available in Scotland and showed high antioxidant activity in both the FRAP and ESR assays. It is known that Lollo Rosso lettuce is a rich source of flavonols (Crozier *et al.,* 2000) and also contains anthocyanins and the hydroxycinnamate derivatives caffeoyltartaric acid, dicaffeoyltartaric acid, 5-*O*-caffeoylquinic acid and 3,5-*O*-dicaffeoylquinic acid

(Ferreres *et al.,* 1997) and that broad beans can contain high levels of flavan-3-ol monomers (Pascual-Teresa *et al*., 2000).

When different varieties of the same product were analyzed, the results showed an interesting pattern (Table 3.1). The coloured varieties were always higher than their green counterpart, examples being red and yellow peppers compared to green pepper; purple vs. green broccoli, red vs. green cabbage, Lollo Rosso and iceberg lettuce, and red and yellow vs. white onion. Depending upon the vegetable, anthocyanins or carotenoids typically contribute to the colour differences so they may be responsible for the higher antioxidant capacity although, without further investigation, the possibility that other secondary metabolites may play a role cannot be ruled out.

With broccoli, cabbage, broad beans and spring onion different parts of the vegetable were analysed. The results were not very clear. Broccoli florets contained higher activity than the stalks while the heart of the green cabbage was a richer source of antioxidants than the outer leaves (Table 3.1). The ESR for broad beans showed higher AOC for the intact pod than for beans while the white section of salad onion was higher than the green tissue. Paradoxically, the opposite trend was obtained with the FRAP assay. *In planta* phenolic compounds play an important role in UV protection and disease resistance (Gould and Lister, 2006) and their levels may be influenced by these factors. For instance, cherry tomatoes exhibit an increased flavonol content compared to regular tomatoes because of the concentration of flavonols in the skin and the increased skin-to-volume ratio of cherry tomatoes. Because they are field grown and exposed to sunlight Spanish cherry tomatoes contain higher levels of flavonols that their English and Scottish grown counterparts which are cultivated under glass filtering out the UV light and thereby reduces flavonol biosynthesis (Stewart *et al.,* 2000). For this reason one would expect higher AOC in those parts of the plant that are exposed to sunlight. The data obtained on this point, albeit indirectly, are not conclusive and further investigation is required before firm conclusions can be drawn.

Strawberries, blueberries and raspberries, as well as plums and apples, exhibited the highest AOC while $\geq$25-fold lower levels occurred in melon, peaches,

white grapes and lychees. Wang *et al.* (1996) used the ORAC assay to analyze the AOC of ten fruits and the order of activity for the group they analyzed was strawberries > plum > oranges > red grapes > kiwi > pink grapefruit > white grapes > banana > pear > melon; a sequence similar to that obtained with the FRAP and ESR assays in the present study. The high AOC of plums is in agreement with data obtained in other studies (Gil *et al.,* 2002; Szeto *et al.,* 2002; Pellegrini *et al.,* 2003; Halvorsen *et al.*, 2006). Consistently, higher AOC has been reported for berries than for other fruits, regardless the assay used (Lichtenhaler and Marx, 2005; Halvorsen *et al.*, 2006). Berries, have recently received much attention with many studies on their flavonoid and phenolic content and the bioavailability of these compounds when ingested by humans and animal models (Prior *et al*., 2001; Mullen *et al.,* 2002a and 2002b; Crozier *et al*., 2006; Prior and Wu, 2006 and McGhie *et al.,* 2007).

The AOC of nuts is in keeping with the data of Halvorsen *et al.* (2002). A factor that contributes to the relatively high values of this group is the water content of nuts – *ca.* 10% compared to *ca.* 90% for many fruits and vegetables. Peanuts contain isoflavones and procyanidins (Crozier *et al.,* 2006) and produce the phytoalexin resveratrol in response to fungal infection. There are a number of reports, based on animal studies and *in vitro* test systems, on the potential protective effects of resveratrol and its 3-glucoside (Young *et al*., 2005; Young and Martin, 2006; Hu *et al.,* 2007). However, the levels of resveratrol in peanuts, red wine and other foodstuffs are extremely low (Burns *et al,* 2002) and, as a consequence, normal dietary intake might be insufficient to elicit similar effects in humans.

The FRAP assay estimates the reduction capacity of Fe^{+3} to Fe^{+2} in the presence of antioxidants. Because the ferric to ferrous iron reduction occurs with all reductants with half-reaction reduction potentials above that of Fe^{+3}/Fe^{+2}, the values of the FRAP assay will express the corresponding concentration of electron-donating antioxidants. The assay, therefore, quantifies the total reduction power of a sample, which is not necessarily the physiological antioxidant activity. Many plant secondary metabolites are redox-active compounds (Demming-Adams and Adams, 2002) and will therefore react in the FRAP assay. However, other secondary metabolites, such as phytoalexins and phytoanticipins, with no redox potential will be inactive in the FRAP assay although they may still have reducing stress effect *in*

vivo (Halvorsen *et al.*, 2006). The synthetic Fremy's radical used in the ESR assay requires very strong protons donors which is a capacity necessary for *in vivo* antioxidant activity in a biological system. That makes ESR a very sensitive and reliable method and, moreover, it can be used with turbid and coloured samples (Gardner *et al.,* 1998). However, it is a more indirect assay than FRAP because it measures the inhibition of reactive species generated in the reaction mixture and the results depend on the type of free radical that is used. Physiologically, reactive oxygen species, including $O_2^{-\bullet}$, $HO^{\bullet}$, $ONOO^-$ and oxygen singlet, have different mechanisms of action *in vivo*. An assay that can measure the "total antioxidant activity" which includes all or most of the ROS has not been developed and is neither an easy nor a realistic approach.

Based on the data in Tables 3.1, 3.2 and 3.3, Table 3.10 shows the correlation coefficient (r) between the ESR and FRAP data calculated for each group and for a combination of all the products. The linearity between the methods when considered for all 69 samples is positive and significant (r = 0.726, Figure 3.2). It is also significant when the 41 vegetables (r = 0.825) and it is even higher for the 21 fruits (r=0.956) analyzed, but not for the 7 low humidity products are considered (r = -0.161)

Table 3.10. Coefficient correlation (r) for ESR and FRAP antioxidant data.

	Correlation coefficient (r) ESR-FRAP
Vegetables (n = 41)	0.825[a]
Fruits (n = 21)	0.956[a]
Nuts and raisins (n = 7)	-0.161
All products (n = 69)	0.665[a]

[a] p = 0.005

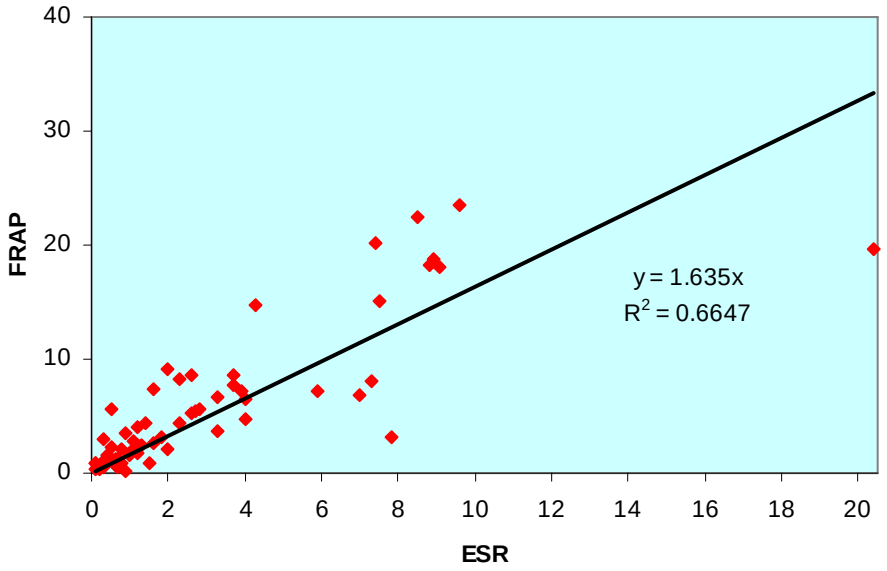


Figure 3.2. Trend line and distribution of values for FRAP and ESR for 69 plant foods.

In Figure 3.3 the difference between the FRAP and ESR rankings and their average are plotted. The values are distributed around the zero showing good agreement between the methods.

As shown above, there is good correlation between the data obtained with the FRAP and ESR assays. ESR requires an NMR spectrometer and a skilled operator. In contrast, the FRAP assay uses a spectrophotometer and is a much simpler and more economic technique. It is, therefore, well suited for routine analysis although its limitations, like those of other assays, should always be borne in mind.

In addition to the antioxidant capacity, the total phenolic content (TPC) of the samples was analysed. The basic mechanism of the Folin-Ciocalteau assay of TCP is an oxidation/reduction and as such, is considered another antioxidant assay by some researchers (Chun *et al*., 2005; Prior *et al.,* 2005). In this study, those products with high antioxidant capacity also exhibited a high TPC (Table 3.1). Among the top ranked TPC vegetables were purple broccoli > kale > curly kale > broad beans > Lollo Rosso lettuce (Table 3.45). Chun *et al.* (2005) in a study of phenolic content in

popular fruits and vegetables in the USA diet did not include any of our top five vegetables and their top ranked vegetables were pepper, sweet corn, cabbage, potatoes and spinach with TPC values between 0.3 and 0.5 mg GAE/g.

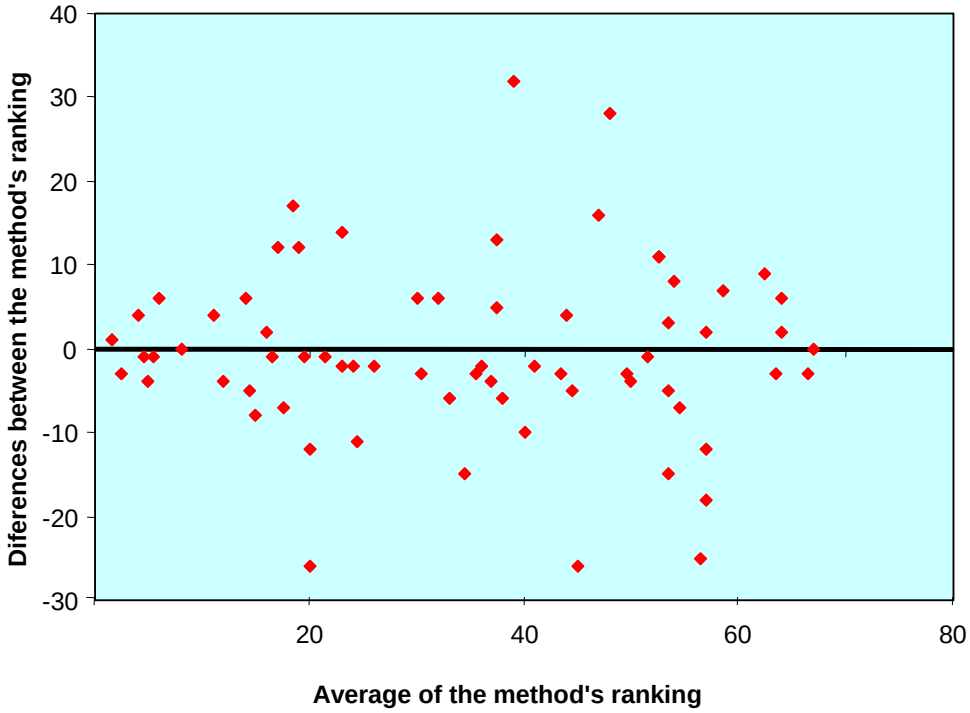


Figure 3.3. Scatter plot of differences for the antioxidant estimates obtained with ESR and FRAP for 69 plant products.

These figures are low compared to the UK produce, which ranged from 0.3 – 6.8 mg GAE/g. Vinson *et al.* (1998) analyzed the TPC of 23 vegetables in the USA and in this study beetroot, red onion, broccoli, asparagus and kidney bean where among the highest with values 0.7 – 9.1 mg catechin eq/g. Pandjaitan *et al.* (2005) reported levels of phenolics ~1.7 to 4.9 mg GAE/g range on eight commercial cultivars of spinach. In the present study, the TPC of spinach was 2.3 mg GAE/g that is within that range.

Among the fruits, the berries, plum, banana and apples had high TPC (3.6 – 1.9 mg GAE/g with melon, kiwi and pears having the lowest (< 0.9 mg GAE/g, Table 3.5). Although when examined in detail there are some differences with the values obtained in other studies, in general there is broad agreement especially with regard

to products with a high TPC. Gil *et al.* (2002) found that the TPC of five different cultivars of nectarines, peaches and plum ranged from 0.1 and 1.1 mg GAE/g while Chun *et al.* (2005) reported that the top ranking USA fruit were plums, strawberries, bananas and apple with TPC values of 1.1 to 3.6 mg GAE/g.

For the dried products group the TPC levels were relatively high. Milbury *et al.* (2006) report levels of TPC for almonds similar to those obtained in this study. The levels of vitamin E of these products were not measured and its contribution to the antioxidant and phenolics content cannot be ruled out.

It should be noted there are variations in the extraction procedures used in different reports that might produce differences in the results. The current study used extraction in 50% methanol/water by homogenizing with an Ultraturrex while Vinson *et al*. (2001) extracted phenolics in 1.2 M HCl in 50% methanol/water followed by heating for 2 or 3 h at 90ºC. For the TPC assay the Vinson study used a reaction time of 10 min compared to the more traditional 2 h (Singleton and Rossi, 1965) employed in the current study. They also used (+)-catechin as a standard, which yields numerical values *ca.* 50% lower than when data are calculated in gallic acid equivalents. Chun *et al.* (2005) used 80% methanol as the extraction solvent followed by ultrasound-assisted extraction for 40 min at 40ºC. Brat *et al.* (2006) homogenized the samples with 70% acetone/water followed by solid phase extraction to eliminate water-soluble reducing interferences.

The correlation factors between ESR, FRAP and TPC values obtained in the current study are shown in the Table 3.11. It is positive and significant for ESR when n=69 (0.361) and more significant for FRAP (0.716). For vegetables alone (n=28) the correlation is higher for both methods and even more so for fruits (n=21).

FRAP showed a significant correlation with TPC for the low water content products but not with ESR.

Table 3.11. Correlation factor (r) between FRAP, ESR and TPC.

Correlation factor (r)	All products (n = 69)	Vegetables (n = 28)	Fruits (n = 21)	Low water content products (n = 7)
ESR-TPC	0.361[c]	0.531[a]	0.842[a]	0.127
FRAP-TPC	0.716[a]	0.751[a]	0.753[a]	0.731[a]

[a] $p<0.000$ [b] $p<0.001$ [c] $p<0.002$

To investigate the possible influence of flavonols on the AOC and TPC the levels of quercetin, isorhamnetin and kaempferol in the 69 samples of fruits, vegetables and raisins and nuts were quantified. Quercetin was the major flavonol present in the majority of vegetables analyzed like purple broccoli (1312 μg/g), Lollo Rosso lettuce (770 μg/g), red onion (561 μg/g) however, in green broccoli and sweetheart and savoy cabbage, kaempferol predominated (Table 3.4). The levels of quercetin and total flavonols as a result, were high for purple broccoli, Lollo Rosso lettuce, red and yellow onion, kale and curly kale, products with a high AOC. None of the fruits analyzed contained total flavonol levels > 55 μg/g corresponding to banana, where 77% was attributable to isorhamnetin (Table 3.5). Strawberries and blueberries contain around 25 and 33 μg/g of flavonols, respectively, with kaempferol being the main contributor. Raspberries contained 7.7 μg/g of total flavonol with 86% corresponding to quercetin. For the nuts and dry products raisins was the top product (54.3 μg/g) with quercetin as the main flavonol (Table 3.6). For almonds there is a discrepancy between the levels of flavonols in this study and those reported by Milbury *et al*, (2006) probably a reflection of different methods of analysis.

The main dietary flavonols in nature are quercetin glycosides. Its aglycone, quercetin, has been the subject of numerous *in vitro* and animal based studies some of them relating to protection against cancer and other chronic diseases (Brisdelli *et al*., 2007; Giuliani *et al*., 2007). The relevance of such studies is in serious doubt as after ingestion quercetin glucosides appear in the blood stream not as the aglycone but as metabolites such as quercetin-3'-sulphate and quercetin-3-glucuronide (Mullen

et al., 2006).

The correlation between FRAP and flavonols is low and not significant for any of the groups analyzed, only for low water products the quercetin levels have a significant correlation (Table 3.12).

Table 3.12. Correlation factor (r) for FRAP antioxidant activity and total flavonols and quercetin content.

Correlation factor (r)	All products (n = 69)	Vegetables (n = 28)	Fruits (n = 21)	Low water content products (n = 7)
FRAP - total flavonols	0.020	0.374	0.463	0.5871
FRAP - quercetin	0.012	0.359	0.422	0.8109[a]

[a] $p<0.000$

This indicates, albeit indirectly, that the proposed health benefit attributed to quercetin relates to a mode of action other than the antioxidant activity for most of the fruits and vegetables. In reality, the interpretation of data obtained in the Zutphen study, that first linked high flavonol intake to a reduced incidence of heart disease (Hertog *et al.,* 1993a), is open to question. The major sources of dietary flavonols in Hertog's study were black tea, apples and onions. While onions are a rich source of flavonols (Aziz *et al.,* 1998), black tea also contains very high levels of thearubigins (Del Rio *et al.*, 2004) while apples contain 5-*O*-caffeoylquinic acid, hydroxychalcones such as phloretin-2'-*O*-glucoside, (–)-epicatechin and procyanidins in more substantial quantities than flavonols (Marks *et al*., 2007). To identify flavonols as the causative agent for the reduced incidence of heart disease is, therefore not justified. A more appropriate interpretation of the data obtained in the Zutphen study is that regular consumption of apples, onions and tea is linked to a lowering of heart disease.

Vitamin C (ascorbic acid) can contribute to the AOC of plant products. Levels of vitamin C were measured in order to investigate its contribution to the FRAP antioxidant activity. Among the vegetables rich in vitamin C were yellow

pepper, red pepper and green cabbage with 1216, 962, 933 μg/g respectively (Table 3.4). The levels in fruits varied between 2 μg/g for blueberry to 690 μg/g for kids range strawberries (Table 3.5). The correlation factors between FRAP and vitamin C were positive but not significant (Table 3.13). Proteggente *et al.* (2002) report a good correlation between FRAP and vitamin C levels. In contrast, the results in the present study reveal several examples of a lack of correlation, with products such as beetroot, plums and blueberries having relatively high antioxidant capacity (7.2, 22.4, 7.2 μmoles Fe^{+2}/g respectively) but contain low levels of vitamin C (20, 17 and 11.5 μg/g) or like green cabbage with a high content of vitamin C (1216 μg/g) and low FRAP (1.7 μmoles Fe^{+2}/g). With berries the vitamin C content varied greatly (11.5-784 mg μg/g) while the FRAP antioxidant content was consistently high (*ca*. 20 μmol Fe^{+3} reduced/g). This is in keeping with data obtained by Wang *et al.* (1996) and Proteggente *et al.* (2002) who suggested this was because citrus fruits are rich in flavanones, which have a much lower antioxidant activity than anthocyanins, and flavonols, which are present in high amount in berries.

Table 3.13. Correlation factor (r) for FRAP antioxidant activity and vitamin C content.

Correlation factor (r)	All products (n = 69)	Vegetables (n = 28)	Fruits (n = 21)	Nuts and raisins (n = 7)
FRAP-vitamin C	0.035	0.417	0.554	-0.136

A "minimum daily requirement" (MDR) for phenolic antioxidants in human diet is difficult to define, as they are not associated with any short-term symptoms of deficiency. However, it is estimated that at least 20% of coronary heart disease is attributable to diet, and dietary factors are considered responsible for 40-60% of cancer incidence and 35% of cancer deaths (National Research Council, 1989). A knowledge of the phenolics content of fruits and vegetables would help the general public make more informed choices as to what produce to eat in order to increase their intake of phenolic compounds. A potentially confounding factor in this regard, is the lack of a universally accepted assay to analyze and quantify either antioxidants in general or phenolic compounds *per se*. There is much debate on the topic of

antioxidant assays with investigators in the USA appearing to favour the ORAC assay (Cao *et al.,* 1996; Wang *et al.,* 1996; Wu *et al.,* 2004) while in Europe the FRAP assay has it supporters (Szeto *et al*., 2002; Nilsson *et al.,* 2005; Halvorsen *et al.,* 2002 and 2006). A further complication, that prevents nutritionists coming to a consensus on a recommended antioxidant intake, is the fact that little is known about the metabolism and bioavailability of phenolic antioxidants following ingestion. However, regardless of the lack of human antioxidant/phenolic MDR, or a universally accepted method to quantify antioxidant activity, it is clear from both the ESR and FRAP data in Table 3.1 that consuming vegetables such as broad beans, red pepper, beetroot and red onions will provide a markedly higher antioxidant intake than courgettes, iceberg lettuce, radishes and fine beans. As mentioned previously, many coloured products including strawberries, raspberries, blackberries, plums, yellow and red pepper, purple broccoli, beetroot, tomatoes, Lollo Rosso lettuce, red onion and red cabbage, exhibit high AOC. While chlorophyll is responsible for the wide spread green colour of plants, the huge variety of other colours in flower, fruits and vegetables is due principally to carotenoids and anthocyanins and, in some instances, betalains (Crozier *et al.,* 2006). The main difference between these compounds is that carotenoids are lipophilic and anthocyanins are hydrophilic. This not only implies a possible different mechanism of action at a cellular level both in plants and humans but also the use different solvents to extract these compounds from plant tissues for analytical purposes. In the current study, a mixture of 50% methanol in water was selected for the extraction of plant tissues, as it would include as many phenolic compounds as possible as they are the principle contributors to antioxidant activity. This, however, results in only limited extraction of carotenoids and tocopherols, therefore, when presents their contribution will be under-estimated.

An appropriate combination of fruits and vegetables with high AOC can be a good way to achieve an enhanced antioxidant intake. In the UK, the recommended intake is five 80 g portions of fruits and vegetables per day. This is based on the premise that all fruits and vegetables contain bioactive compounds such as vitamins, minerals and dietary fibre with potential to decrease the risk of a range of diseases and improve health (Johnsen *et al*., 2003; Rissanen *et al*., 2003; WCRF, 2007). In practice, the actual levels of fruits and vegetables consumed in the UK are lower than is recommended. According to a survey published in 2004, only 13% of men and

15% of women met the five-a-day recommendation (NDNS, 2004). In Scotland despite recommendations, the actual daily consumption between 1993 and 2000 only rose from 190 g to 200 g (MAFF, 2000). Availability, access and affordability may be responsible (Anderson *et al.*, 1998; Beattie *et al.,* 2005). Data on the FRAP antioxidant activity of fruits and vegetables presented in Tables 3.1 – 3.2 (excuding the low water content product) have been used in a study conducted in collaboration with the Centre for Public Health Nutrition Research at the University of Dundee and a manuscript has been submitted for publication (Haleen *et al*., 2008, Appendix 1). The current antioxidant activity intake from fruits and vegetables in the UK population was assessed as well as different consumption models for dietary antioxidant intake from fruit and vegetables in Scotland. The antioxidant activity levels were applied to consumption and portion size from the National Diet and Nutrition Survey (NDNS) records. Taking as an example some of the data reported by Haleem *et al.* (2008), in Scotland the mean daily intake of products with an evaluated antioxidant/phenolic content in 2000-2001 (National Diet and Nutrition Survey, NDNS) was 48 g of pears, 16.4 g of purple broccoli and 23 g of strawberries, quantities much lower than the recommended 80 g portion size.

Knowledge of the levels of antioxidants and phenolic compounds in fruits and vegetables could help people to increase or optimize their consumption. However, nutritionists inevitably prefer to be on the side of caution and it is unlikely that there will be any official recommendations in this regard until there is overwhelming evidence on the ability of phenolic compounds/antioxidants in plant-derived dietary products to prevent the risk of certain diseases.

The effect of different cooking conditions and food matrix effect on the antioxidant capacity and phenolic content of fruits and vegetables are important considerations about which very little research has been conducted and are topics that needs to be addressed.

An appropriate way of communicating information on antioxidants, flavonoids, phenolics and/or phytochemicals to the general public has to be found as coverage on TV and in newspapers and magazines can be over simplistic and misleading.

3.4. Conclusions

In summary, the results presented in this chapter demonstrate that there are differences of up to 758-fold in the total FRAP antioxidant capacity of the three groups of dietary plant products analyzed: vegetables, fruits and products with low water content. Those especially rich in antioxidants included broad beans, blueberries, raspberries, strawberries and purple broccoli. All three groups contained products that contained only small quantities of antioxidants. When the FRAP data are calculated in relation to the average portion, the ranking of the products is strawberries > broad beans > blueberries > purple broccoli > raspberries > yellow pepper > red pepper. The FRAP antioxidant measurements are well correlated to values obtained with the ESR assay. The former assay is much cheaper and, with an automated plate reader, much easier to adapt for the processing of large numbers of samples. Measurement of TPC by the Folin-Ciocalteau assay can also be used as a predictor of the flavonoids content in fruits and vegetables. It is necessary to address that the chemical mechanism of reaction for any antioxidant method available at the moment is not relevant to *in vivo* conditions, however, it is a property that appears to be common to most of the flavonoids and phenolic compounds. The vitamin C and flavonol concentrations were not correlated with either TPC nor to the antioxidant activity measured by FRAP. The data obtained in the current study can be used to evaluate the overall intake of flavonoids in the UK although a more standarized method of selection would be recommended including more products like cereals, juices, nuts and popular beverages such as tea, coffee and wine, from different areas of the country and at different seasons, to produce a more complete flavonoid and phenolic content database. Similar databases are being developed in Turkey, Italy and USA using different methodologies (Karakaya *et al.*, 2001; Pellegrini *et al.*, 2006; Wu *et al*., 2004). If these could be standardised it would permit not only a meaningful comparison of foods but also a provision of quality standards for regulatory issues and health claims once they are validated.

There is a growing realisation that the wide-ranging protective effects of a fruit and vegetable-based diet are not based exclusively on antioxidant effects. The levels of antioxidants in the bloodstream and tissues are generally too low to adequately supplement endogenous antioxidant systems and the protective effects, including inhibition of endothelin-1 synthesis (Corder *et al*., 2006), reduced platelet

aggregation, inhibition of LDL oxidation (Meyer *et al*., 1997), lowering of cholesterol, anti-inflammatory (Middleton and Kandaswami, 1992), anti-bacterial and anticarcinogenic effects are too wide ranging for all to be the result of a simple lowering of free radicals in the body (Halliwell, 1994). None-the-less there is substantial evidence supporting the view that it is phenolic compounds and, in some instances, carotenoids or possible a synergistic effect with other compounds in the fruits, vegetables and selected beverages, that are responsible for their diverse array of protective effects. The FRAP, Folin-Ciocalteu together with ORAC assays principally measure phenolic compounds and flavonoids and as a consequence databases using information from a these assays do provide a useful "broad brush" guide as to the potential protective effects of dietary components. Much more research remains to be done on topics including i) the effects of cooking and storage on potentially protective compounds in plant-derived products, ii) the fate of dietary phenolics within the body, iii) matrix effects, and iv) the mechanisms by which they ellicit protective effects.

Chapter 4. Analysis and identification of antioxidants in berries

4.1. Introduction

As mentioned in Chapter 3, among the products with high AOC, berries were chosen for a more detailed analysis of their phenolic constituents. Raspberries (*Rubus idaeus*), blueberries (*Vaccinium corymbosum*), blackcurrants (*Ribes nigrum*), redcurrants (*Ribes rubrum*) and cranberries (*Vaccinium oxycoccus*), which had not been previously analysed, were included in the study. The importance of a detailed quantitative and qualitative analysis of phenolics from any specific source is considerable because their chemical structure has an impact on the absorption and bioavailability although the mechanisms involved are still not clear. Changes in the chemical structure of dietary phenolics following ingestion by humans and animal models can in part help unravel this puzzle.

Berries, in general, are also of interest as a result of a study in Finland by Knekt *et al.,* (1996) in which their intake was associated, alongside other factors, with a 60% decline in heart disease and stroke. A high consumption of berries is a particular feature of the Finnish diet (Kahkonen *et al*., 2001).

Scotland is an important producer of a number of berries, including strawberries, raspberries and blackcurrants, and bodies such as Berry Scotland Programme (http://www.berryscotland.com/), actively promote the potential health benefits of increased consumption as a simple and effective means of improving the much criticised Scottish diet.

Interest in natural dietary antioxidants has also increased in other areas of food science and technology. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been used in foods for years. However, these compounds are suspected of causing liver damage and cancer (Ito *et al*., 1992) and hence there is growing interest in the use of naturally occurring phenolic compounds with antioxidant activity.

In this chapter, a description of the analytical identification and quantification of phenolic compounds in five different berries will be discussed.

4.2. Materials and Methods

Berries for this study were obtained from local supermarkets in the West end of Glasgow. Extraction method, HPLC conditions, on-line detection of antioxidants and mass spectrometric analysis are described in Chapter 2. Samples were analysed with the mass spectrometer operating in full scan, negative and positive ionisation modes.

Identifications were based on mass spectra, absorbance spectra and co-chromatography with standards. Where standards were not available identifications were assisted by the data of Baldo *et al*. (1995), Goiffon *et al.* (1991 and 1999), Prior *et al.* (2001), Maatta, *et al.* (2003 and 2004), Mullen *et al.* (2002b, 2003a and 2003b) and Degenevé (2004) among others. These publications provided information on the reverse-phase HPLC elution order of anthocyanidins, anthocyanins with different conjugated sugar moieties, flavonols and other phenolics.

Some of general rules for reversed phase HPLC elution order related to structure have been established although there will be variations depending upon the support and mobile phase used. They are:

i) The elution order of the anthocyanidins is delphinidin > cyanidin > pelargonidin > petunidin > peonidin followed by malvidin (Hong and Wrolstad *et al.,* 1990; Goiffon *et al*., 1991; Burns *et al.,* 2002).

ii) The order of elution of anthocyanins conjugated with different sugars at the 3-position with different conjugated sugars is generally galactoside > glucoside > rutinoside > arabinoside (Goiffon *et al*., 1991).

iii) In general, as the degree of glycosylation of the conjugate increases, the anthocyanins are less well retained and have shorter retention times. The presence of a hydrophobic methyl group in the rhamnose molecules affects the chromatographic behaviour as cyanidin-3-rutinoside (cyanidin-3-glucosyl-rhamnoside) elutes after cyanidin-3-glucoside (Goiffon *et al*., 1991) and cyanidin-3(2^{G}-glucosylrutinoside) elutes after cyanidin-3-sophoroside and likewise the equivalent pelargonidin derivatives (Mullen *et al.,* 2002c). A very marked increase in retention time is observed with acetylated anthocyanins such as delphinidin-3-*O*-acetyl glucoside (Baldi *et al.,* 1995).

iv) For flavonol conjugates the sequence of elution has been reported by Rommel and Wrolstad (1993), Zafrilla *et al.* (2001), Mullen *et al.* (2002b,c; 2003a,b) and Marks *et al*. (2007). In summary, the order of elution for quercetin conjugates is galactosylrhamnoside > rutinoside > galactoside > glucoside > pentoside. For kaempferol conjugates, the order is similar however, in this case the galactoside seemingly elutes before the rutinoside.

For quantification purposes, all phenolic acids were expressed as 4-hydroxybenzoic acid, caffeic acid or chlorogenic acid equivalents; all flavan-3-ols and their polymers as (–)-epicatechin or (+)-catechin equivalents; anthocyanins conjugates in cyanidin-3-glucoside equivalents, quercetin conjugates in quercetin-3-glucoside or quercetin-3-rutinoside equivalents, kaempferol conjugates in kaempferol-3-glucoside equivalents; myricetin conjugate in myricetin equivalents, ellagic acid conjugates in ellagic acid equivalents and ellagitannins in gallic acid equivalents. For each compound, the antioxidant activity (TEAC in μmol Trolox/g) and the percentage contribution to the total antioxidant activity (% TEAC) were calculated. The Trolox Equivalent Antioxidant Ratio (TEAR), which is the ratio of individual antioxidant activity per nmole present in the sample, was calculated as it is a useful indicator of relative antioxidant potency. The total antioxidant activity of the unchromatographed extracts was also measured by using the FRAP and TEAC assays.

Vitamin C was quantified in a separate HPLC system described in Chapter 2.

4.3. Results

4.3.1. Total antioxidant activities

The total antioxidant activities values were high for all five berries and similar relative activities were obtained in both assays with the exception of blueberries which were higher than raspberries in the FRAP assay but were lower in the TEAC assay (Table 4.1). Overall, blackcurrants, blueberries and raspberries had the highest antioxidant capacity with lower values obtained for redcurrants and cranberries.

Table 4.1. Total antioxidant activity of berries measured by FRAP and TEAC assay.

Berry	FRAP (μmol Fe^{+2}/g)	TEAC (μmol Trolox/g)
Blackcurrant	51.6 ± 1.2	47.9 ± 0.1
Blueberries	30.0 ± 1.9	31.9 ± 0.6
Raspberries	27.7 ± 1.1	36.3 ± 0.6
Redcurrant	24.6 ± 0.5	24.8 ± 0.8
Cranberries	18.6 ± 0.3	14.4 ± 0.5

Values represent mean ± SE (n=3) per g fresh weight.

Comparison of these values with those reported in the literature is difficult as already discussed in Chapter 3, as numerous factors such as variety, degree of ripeness, season, storage conditions, as well as the solvents used for extraction, can influence the levels of total phenolics and antioxidant activity. For example Moyer *et al.* (2002) report FRAP values ranging from 18.5-61.4 and 13.1-45.2 μmol Fe^{2+}/g for blueberries and raspberries, respectively. The levels obtained by FRAP assay for blueberries and raspberries that are presented in Table 4.1, 30.0 and 27.7 μmol Fe^{2+}/g, respectively, are in keeping with these figures. However, in the earlier FRAP analysis of blueberries and raspberries reported in Chapter 3, much lower values, 11.5 and 20.1 μmol/g Fe^{2+}, respectively, were obtained. The extraction and assay procedures used were identical so these variations are likely to be due to different varieties, degree of ripeness, growing conditions and/or season.

4.3.2. Blackcurrant analysis

4.3.2.1. Identification of phenolic compounds

HPLC-PDA-MS^2 analysis facilitated the identification of 18 phenolic compounds in blackcurrant. The HPLC profiles at three different wavelengths (280, 520 and 365 nm) as well as the 720 nm antioxidant trace are shown in Figure 4.1 and details of the identification in Table 4.2. The basis of the identification each compound is summarised as follows:

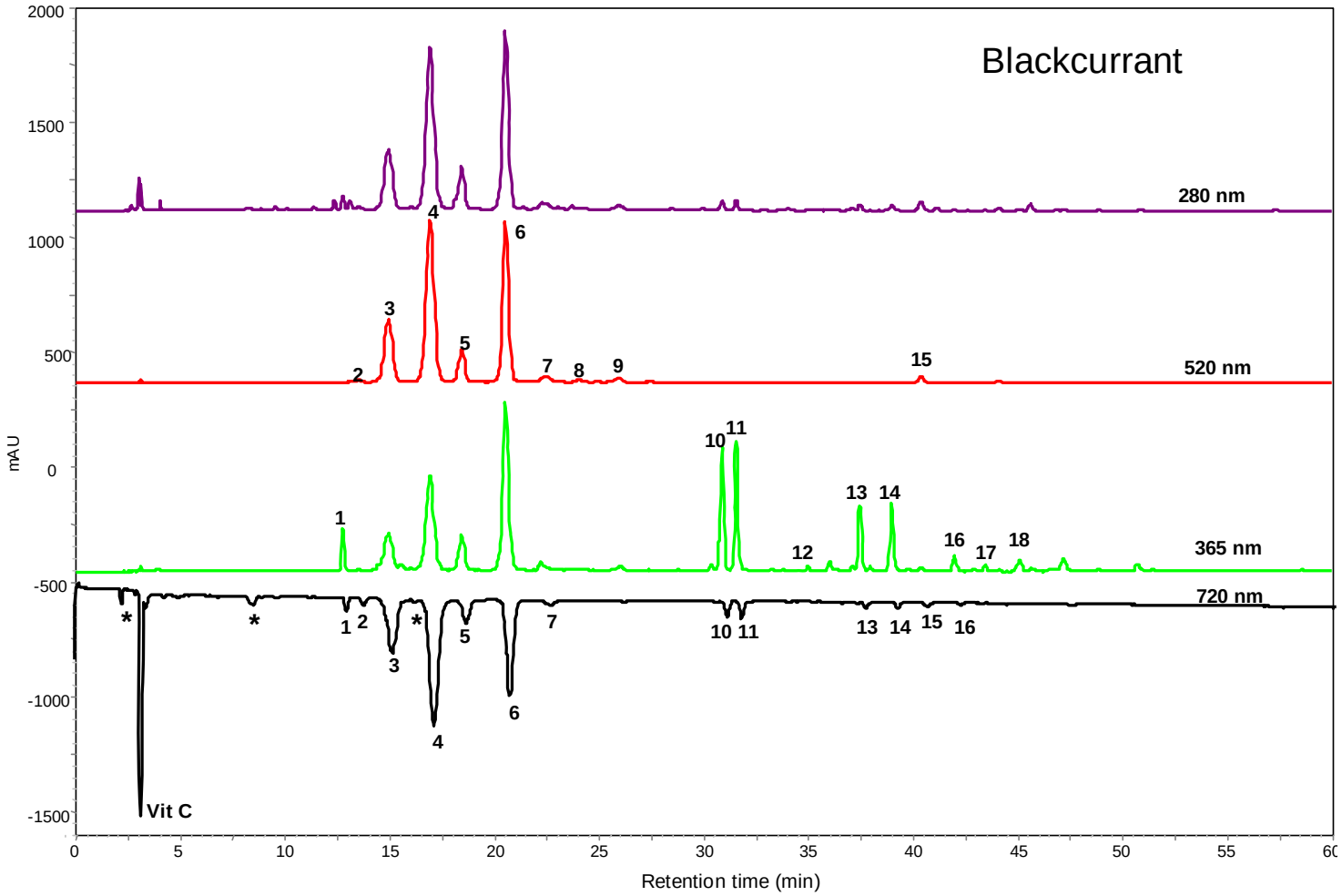


Figure 4.1. HPLC-PDA traces and on-line antioxidant detection (720 nm) of a blackcurrant extract. For identity of peaks refer to Table 4.2. * unidentified peaks

Table 4.2. Phenolic compounds identified in blackcurrant by HPLC with diode array and MS^3 detection[a]

Peak	t_R	Compound	λ_{max} (nm)	[M-H] (*m/z*)	MS^2 ions (*m/z*)	MS^3 ion (*m/z*)
1	12.9	caffeic acid-glucoside	330	341	179 [CafAc]([M-H]$^-$ - Hex)	
2	13.2	delphinidin-3-galactoside	520	465*	303 [Del]([M+H]$^+$- Gal)	
3	14.8	delphinidin-3-glucoside	520	465*	303 [Del]([M+H]$^+$- Glc)	
4	17.0	delphinidin-3-rutinoside	520	611*	465 ([M+H]$^+$- Rham), 303 [Del]([M+H]$^+$- Glc-Rham)	
5	18.4	cyanidin-3-glucoside	520	449*	287[Cyan]([M+H]$^+$- Glc)	
6	20.5	cyanidin-3-rutinoside	520	595*	449 ([M+H]$^+$- Rham), 287 [Cyan]([M+H]$^+$- Glc-Rham)	
7	22.4	peonidin-3-galactoside	520	463*	301 [Peon]([M+H]$^+$- Gal)	
		petunidin-3-rutinoside		625*	479 ([M+H]$^+$- Rham), 317 [Pet]([M+H]$^+$- Rham-Glc)	
8	24.0	malvidin-3-galactoside	520	493*	331 [Malv]([M+H]$^+$- Gal)	
		peonidin-3-glucoside		463*	301 [Peon]([M+H]$^+$- Glc)	
9	25.9	peonidin-3-rutinoside	520	609*	463 (M^+- Rham), 301 ([Peon]([M+H]$^+$- Glc-Rham)	
		malvidin-3-arabinoside		463*	331 [Malv]([M+H]$^+$- Arab)	
10	30.7	myricetin-3-rutinoside	355	625	317 [Myr]([M-H]$^-$- Rut)	
11	31.5	myricetin-glucuronide	355	493	317 [Myr]([M-H]$^-$- GlcUA)	
12	35.0	myricetin-3-malonylglucoside	355	521	317 [Myr]([M-H]$^-$- Glc-((Mal-COOH))	
13	36.9	quercetin-3-rutinoside	350	609	301 [Q]([M-H]$^-$- Rut)	
14	39.1	quercetin-3-glucoside	350	463	301 [Q]([M-H]$^-$- Gal)	271, 255, 179
15	40.5	delphinidin-3-*O*-6″-coumaroylglucoside	525	611*	303 [Del]([M+H]$^+$- Coum-Glc)	
16	42.2	quercetin-3-malonylglucoside	350	505	301 [Q]([M-H]$^-$- Glc-(Mal-COOH))	271, 255, 179
17	43.9	kaempferol-3-rutinoside	315	593	285 [K]([M-H]$^-$- Rut)	
18	45.2	kaempferol-3-galactoside	340	477	301 [K]([M-H]$^-$- Glc)	

CafAc, caffeic acid; Del, delphinidin; Hex, hexosyl; Gal, galactosyl; Glc, glucosyl; Rut, rutinosyl; Arab, arabinosyl; Cyan, cyanidin; Pet, petunidin; Peon, peonidin; Malv, malvidin; Myr, myricetin; GlcUA, glucuronyl; Pent, pentosyl; Mal, malonyl; Q, quercetin; Coum, coumaroyl; K, kaempferol; t_R, retention time, [a] Peak numbers and retention times refer to HPLC traces in Figure 4.1. * positive ionisation molecular ion ([M+H]$^+$

Peak 1 (t_R – 12.9 min, λ_{max} - 330 nm) had a $[M-H]^-$ at *m/z* 341 and a MS^2 ion at *m/z* 179. The *m/z* 179 fragment corresponds to the aglycone caffeic acid produced by a 162 *amu* cleavage of a hexose moiety. Based on the mass spectral and absorbance data as well as previously published reports (Schuster and Herrmann, 1985; Maata *et al*., 2003), this compound is a caffeic acid glucoside

Peak 2 (t_R – 13.2 λ_{max} - 520 nm) had a $[M+H]^+$ at *m/z* 465, which yielded a MS^2 fragment at *m/z* 303 (delphinidin, $[M+H]^+$-162, loss of a hexose moiety). This was identified as delphinidin-3-galactoside, which elutes before delphinidin-3-glucoside, peak 3.

Peak 3 (t_R – 14.8 λ_{max} - 520 nm) had also a $[M+H]^+$ at *m/z* 465, which yielded a MS^2 fragment at *m/z* 303 (delphinidin, $[M+H]^+$-162, loss of a hexose moiety). This peak was identified as delphinidin-3-glucoside and this was confirmed by co-chromatography with a standard. This glycoside is a known component of blackcurrants (Goiffon *et al*., 1991; Froytlog *et al*., 1998; Slimestadt *et al*., 2002).

Peak 4 (t_R – 17.0 min, λ_{max} - 520 nm) had a $[M+H]^+$ at *m/z* 611 which fragmented to produce MS^2 ions at *m/z* 465 ($[M+H]^+$-146, loss of a rhamnosyl moiety) and *m/z* 303 ($[M+H]^+$-308, cleavage of rhamnosyl and glucosyl units). Based on the fragment pattern it was identified as delphinidin-3-rutinoside, a known major anthocyanin in blackcurrants (Slimestad *et al*., 2002; Maatta *et al*., 2003).

Peak 5 (t_R – 18.4 min, λ_{max} - 520 nm) yielded a MS containing a ($[M+H]^+$ at *m/z* 449, which fragmented on MS^2 to produce a cyanidin ion at *m/z* 287 ($[M+H]^+$-162, loss of a hexose moiety). This peak is cyanidin-3-glucoside and the identification was confirmed by co-chromatography with a cyanidin-3-glucoside standard.

Peak 6 (t_R – 20.5 min, λ_{max} - 520 nm) was identified as cyanidin-3-rutinoside. It had a $[M+H]^+$ at *m/z* 595 which fragmented to produce MS^2 ions at *m/z* 449 ($[M+H]^+$-146, loss of a rhamnosyl moiety) and *m/z* 287 ($[M+H]^+$-308, cleavage of rhamnosyl and glucosyl units).

Peak 7 (t_R – 22.4 min, λ_{max} - 520 nm) contained two anthocyanins. One had a $[M+H]^+$ at *m/z* 463, which on MS^2 yielded a fragment ion at *m/z* 301 (peonidin, $[M+H]^+$-162, loss of an hexose moiety). As this was the earlier eluting of three

peonidin hexose conjugates (see peak 8 and 9) it was identified as peonidin-3-galactoside. The second compound had a $[M+H]^+$ at *m/z* 625, which yielded MS^2 fragments at *m/z* 479 ($[M+H]^+$-146, loss of rhamnose) and *m/z* 317 (petunidin, $[M+H]^+$-308, loss of rhamnose and glucose moieties). This corresponded with the fragmentation pattern of petunidin-3-rutinoside, previously reported to occur in blackcurrants by Slimestadt *et al*. (2002).

Peak 8 (t_R – 24.0 min, λ_{max} - 520 nm) also contained two anthocyanins. The MS of this peak contained an ion at *m/z* 493 which fragmented on MS^2 to produce an ion at *m/z* 331 (malvidin, $[M+H]^+$-162, loss of an hexose unit), tentatively identified as malvidin-3-galactoside because it elute earlier than a second malvidin conjugate, malvidin-3-arabinoside (peak 9). The second compound in peak 8 was identified as peonidin-3-glucoside based on its mass spectrum ($[M+H]^+$ at *m/z* 463 and a MS^2 ion at *m/z* 301), its elution order and previous published data (Slimestadt *et al*., 2002).

Peak 9 (t_R – 25.9 min, λ_{max} - 525 nm) contained two components. One compound was identified as peonidin-3-rutinoside, a known component of blackcurrants (Slimestadt *et al.,* 2002), on the basis of a $[M+H]^+$ at *m/z* 609, which yielded a MS^2 peonidin fragment ion at *m/z* 301 formed by the successive cleavage of a rhamnosyl and a glucosyl unit. The second compound had a $[M+H]^+$ at *m/z* 463, which with a loss of a 132 *amu* pentose unit yielded an MS^2 fragment at *m/z* 331. It was therefore a malvidin pentose conjugate, which, as it co-elutes with peak 10 in blueberries, identified as malvidin-3-arabinoside**.**

Peak 10 (t_R – 30.7 min, λ_{max} - 355 nm) yielded a $[M-H]^-$ at *m/z* 625 and MS^2 fragments at *m/z* 317 (myricetin, $[M-H]^-$-308, loss of a rutinosyl moiety). It was, therefore, identified as myricetin-3-rutinoside, previously identified in blackcurrant extract by Maatta *et al*. (2003).

Peak 11 (t_R – 31.5 min, λ_{max} - 355 nm) is a myricetin glucuronide conjugate, having a $[M-H]^-$ at *m/z* 493, which with neutral loss of 176 *amu* yielded a MS^2 fragment corresponding to myricetin at *m/z* 317.

Peak 12 (t_R – 35.0 min, λ_{max} - 355 nm) produced a $[M+H]^+$ at *m/z* 567 which on MS^2 yielded a fragment at *m/z* 319 via a loss of 248 *amu*. According to Maatta *et al.* (2003) this corresponds to the loss of a malonyglucosyl unit with the fragmentation pattern being indicative of the occurrence of myricetin-3-

malonylglucoside. When analysed with negative ionisation no $[M-H]^-$ was apparent instead an ion at *m/z* 521 was observed which fragmented in MS^2 to produce myricetin ion at *m/z* 317, through a loss of 204 *amu*. The loss of 45 *amu* to produce the *m/z* 521 ion equates with the loss of the carboxyl function from the malonyl unit.

Peak 13 (t_R – 36.9 min, λ_{max} - 350 nm) had a $[M-H]^-$ at *m/z* 609 which yielded a MS^2 ion at *m/z* 301 (quercetin, $[M-H]^-$-308, loss of a rutinosyl unit). Based on this MS spectrum and co-chromatography with a reference compound, this compound was identified as quercetin-3-rutinoside, previously detected in blackcurrants by Maatta *et al.* (2003).

Peak 14 (t_R – 39.1 min, λ_{max} - 350 nm) produced a typical quercetin hexoside mass spectrum ($[M-H]^-$ at *m/z* 463, MS^2 at 301). Peak 14 was identified as quercetin-3-glucoside through co-chromatography with a reference compound.

Peak 15 (t_R – 40.5 min, λ_{max} - 525 nm) was a minor anthocyanin with a $[M+H]^+$ at *m/z* 611 and a MS^2 delphinidin fragment ion at *m/z* 303 produced by cleavage of a 308 *amu*. This compound was strongly retained on the column. Slimestadt *et al.* (2002) reported the presence of delphinidin conjugate with similar properties (MS^2, absorption and late t_R). This compound was, therefore, identified as delphinidin-3-*O*-(6"-coumaroylglucoside).

Peak 16 (t_R – 42.2 min, λ_{max} - 350 nm) yielding the same $[M-H]^-$ and MS^2 fragments as myricetin-3-malonylglucoside (peak 12), but at *m/z* values 16 *amu* lower indicative of the presence of quercetin-3-malonylglucoside. The occurrence of this compound has been reported in blackcurrants by Maatta *et al.* (2003 and 2004).

Peak 17 (t_R – 43.9 min, λ_{max} - 315 nm) produced a $[M-H]^-$ at *m/z* 593 and MS^2 fragment at *m/z* 285. The *m/z* 285 ion, produced by a 308 *amu* loss of rhamnose and glucose, corresponded to the aglycone kaempferol. This compound was identified as kaempferol-3-rutinoside, which has been reported to occur in blackcurrants (Maatta *et al*., 2003).

Peak 18 (t_R – 45.2 min, λ_{max} - 340 nm) was identified as kaempferol-3-galactoside. It yielded a $[M-H]^-$ at *m/z* 447 and an MS^2 fragment at *m/z* 285 after a 162 *amu* loss corresponding to cleavage of a glucose unit but did not co-chromatograph with kaempferol-3-glucoside.

4.3.2.2. Phenolic quantification and individual antioxidant activity of phenolics in blackcurrant

The quantity in nmol/g, antioxidant activity (TEAC) in nmol trolox/g, the percentage of contribution to the AOC and the TEAR of the peaks obtained for blackcurrants are presented in Table 4.3. Figure 4.1 illustrates the HPLC 280, 520 and 365 nm absorbance profiles and the 18 peaks that were identified. The negative 720 nm profile is the on-line antioxidant detection the areas of the peaks are equivalent to their antioxidant activity. The major phenolic compound was the anthocyanin delphinidin-3-rutinoside (peak 4). It was present in high and similar amounts to vitamin C (2233 and 2328 nmol/g respectively). The anthocyanin, however, had double the TEAC of vitamin C (2049 vs. 1094 nmol trolox/g) and therefore its contribution to the AOC was higher (32.8% vs. 17.5%). The total amount of anthocyanins present (5459 nmoles/g) was the highest of all the five berries analyzed, contributing with 73% of the AOC. This seems to be a characteristic of the anthocyanins present in blackcurrants, their TEAR was around 1.0 in comparison to 0.5 for vitamin C which means that for each nmol of anthocyanin or vitamin C present in the fruit, 1.0 and 0.5 units of TEAC are produced, respectively however, in vivo, the bioavailability of vitamin C is higher than anthocyanins. Among the flavonols present, the glycosides of myricetin were predominant, however, along with quercetin and kaempferol they are minor components (515 nmoles/g) and as such made only 6% contribution to the total AOC. The major phenolics in blackcurrant, delphinidin and myricetin both have a trihydroxylated B ring, which seems to be an important characteristic for antioxidant capacity as mentioned in Chapter 1.

4.3.3. Blueberries

4.3.3.1. Identification of phenolic compounds

HPLC-PDA-MS^2 analysis resulted in the identification of 18 phenolic compounds in blueberries (Figure 4.2). The data are summarised in Table 4.4. Unlike the earlier published analysis of blackcurrants that aided identification, a number of studies with

Table 4.3. Levels of phenolic compounds and Trolox equivalent antioxidant activity (TEAC) in blackcurrants.

Peak	t_R	Compound	Quantity (nmol/g)	TEAC (nmol Trolox/g)	TEAC (%)	TEAR
	3.2	Vitamin C	2328 ± 99	1094 ± 101	18 ± 2	0.5
1	12.9	caffeic acid glucoside conjugate	80 ± 0	76 ± 13	1.2 ± 0.2	0.9
2	13.2	Delphinidin-3-galactoside	53 ± 0	61 ± 14	1.0 ± 0.2	1.1
3	14.8	Delphinidin-3-glucoside	839 ± 7	886 ± 158	14 ± 3	1.0
4	17.0	Delphinidin-3-rutinoside	2233 ± 37	2049 ± 336	33 ± 5	0.9
5	18.4	cyanidin-3-glucoside	327 ± 5	261 ± 61	4.2 ± 1.0	0.8
6	20.5	cyanidin-3-rutinoside	1693 ± 1	1181 ± 236	19 ± 4	0.7
7	22.4	petunidin-3-rutinoside peonidin-3-galactoside	104 ± 2	77 ± 15	1.2 ± 0.2	Δ
8	24.0	malvidin-3-galactoside peonidin-3-glucoside	72 ± 0	n.d.	-	Δ
9	25.9	peonidin-3-rutinoside malvidin-3-arabinoside	126 ± 17	n.d.	-	Δ
10	30.7	myricetin-3-rutinoside	135 ± 3	119± 17	1.9 ± 0.3	0.9
11	31.5	myricetin-glucuronide	138 ± 2	116 ± 21	1.9 ± 0.3	0.8
12	35.0	myricetin-3-malonylglucoside	30 ± 1	n.d.	-	0
13	36.9	quercetin-3-rutinoside	77 ± 2	40 ± 8	0.6 ± 0.1	0.5
14	39.1	quercetin-3-glucoside	83 ± 3	40 ± 9	0.6 ± 0.1	0.5
15	40.5	Delphinidin-3-O-6″-coumaroylglucoside	77 ± 0	43 ± 8	0.7 ± 0.1	0.6
16	42.2	quercetin-3-malonylglucoside	17 ± 1	19 ± 3	0.3 ± 0.1	1.1
17	43.9	kaempferol-3-rutinoside	12 ± 0	n.d.	-	-
18	45.2	kaempferol-3-galactoside	23 ± 0	n.d.	-	-
*		non identified peaks			3.0 ± 0.1	

Quantitative estimates expressed as mean values ± standard error (n=3). Peak numbers and retention times refer to HPLC traces in Figure 4.1. dentification by MS^3 see Table 4.2. n.q.- not quantified; n.d.- not detected; * the contribution of the non identified peaks at 720 nm to the of antioxidant capacity. Δ not calculated due to coelution of compounds.

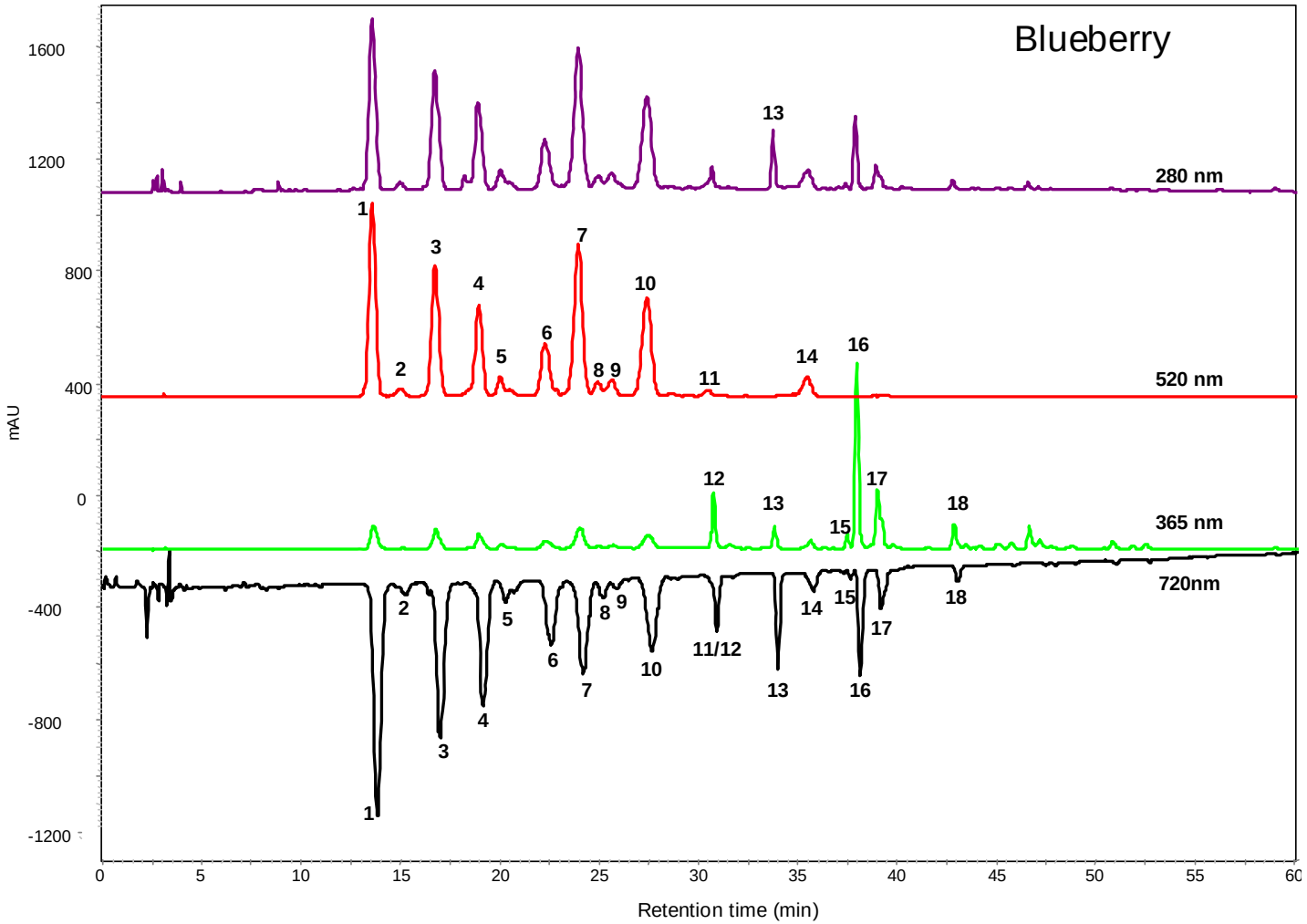


Figure 4.2 HPLC-PDA traces and on-line antioxidant detection (720 nm) of a blueberry extract. For identity of peaks refer to Table 4.4. * unidentified peaks.

Table 4.4. Phenolic compounds identified in blueberries by HPLC with diode array and MS^3 detection[a].

Peak	t_R	Compound	λmax (nm)	[M-H] (*m/z*)	MS^2 ions (*m/z*)	MS^3 ions (*m/z*)
1	13.5	Delphinidin-3-galactoside	520	465*	303 [Del]([M+H]$^+$- Gal)	
2	14.9	Delphinidin-3-glucoside	520	465*	303 [Del]([M+H]$^+$- Glc)	
3	16.7	cyanidin-3-galactoside	520	449*	287 [Cyan]([M+H]$^+$- Gal)	
		Delphinidin-3-arabinoside		435*	303 [Del]([M+H]$^+$- Arab)	
4	19.4	petunidin-3-galactoside	520	479*	317 [Pet]([M+H]$^+$- Gal)	
5	20.2	cyanidin-3-arabinoside	515	419*	287 [Cyan]([M+H]$^+$- Arab)	
6	22.4	petunidin-3-arabinoside	520	449*	317 [Pet]([M+H]$^+$- Arab)	
		peonidin-3-galactoside		463*	301 [Peon]([M+H]$^+$- Gal)	
7	23.8	malvidin-3-galactoside	520	493*	331 [Malv]([M+H]$^+$- Gal)	
8	24.7	malvidin-3-glucoside	525	493*	331 [Malv]([M+H]$^+$- Glc)	
		Delphinidin-6-acetyl-3-glucoside		507*	303 [Del]([M+H]$^+$- AcGlc)	
9	25.7	peonidin-3-arabinoside	525	433*	301 [Peon]([M+H]$^+$- Arab)	
10	27.4	malvidin-3-arabinoside	525	463*	331 [Malv]([M+H]$^+$- Arab)	
11	30.4	petunidin-6-acetyl-3-glucoside	525	521*	317 [Pet]([M+H]$^+$- AcGlc)	
12	30.7	myricetin-3-galactoside	355	479	317 [Myr]([M-H]$^-$-Gal)	
13	33.8	quercetin di glucoside	355	625	301 [Q]([M-H]$^-$Glc-Glc)	
		5-feruloylquinic acid	325	367	191, 179, 135	
14	35.5	malvidin-6-acetyl-3-glucoside	530	535*	331 [Malv]([M+H]$^+$- AcGlc)	
15	36.9	quercetin-3-rutinoside	355	609	301 [Q] ([M-H]$^-$ -Rut)	179, 151
16	37.8	quercetin-3-galactoside	355	463	301 [Q] ([M-H]$^-$ -Gal)	
17	39.2	quercetin-3-glucoside	355	463	301 [Q] ([M-H]$^-$ -Glc)	
18	43.0	quercetin-3-arabinoside	355	433	301 [Q] ([M-H]$^-$ -Arab)	271, 255, 179
		quercetin-6-acetyl-3-glucoside	355	505	301 [Q]([M+H]$^-$- AcGlc)	

Del, delphinidin; Glc, glucosyl; Cyan, cyanidin; Arab, arabinosyl; Pet, petunidin; Peon, peonidin; Malv, malvidin; Gal, galactosyl; AcGlc, acetylglucosyl; Myr, myricetin; Hex, hexosyl; Rut, rutinosyl; Q, quercetin; Pent, pentosyl; t_R, retention time. [a] Peak numbers and retention times refer to HPLC traces in Figure 4.2. * positive ionisation molecular ion ([M+H]$^+$)

blueberry have analysed samples as aglycones after acid/enzyme hydrolysis (Hakkinen *et al*., 1999; Justesen *et al*., 1998; Sellapan *et al*., 2002). As such they were of limited value, in contrast to the investigations of Goiffon *et al.* (1999) and Prior *et al.* (2001) which analysed unhydrolysed blueberry extracts.

Peak 1 (t_R – 13.5 min, λ_{max} 520 nm) had $[M+H]^+$ at *m/z 4*65, which on MS^2 yielded a fragment ion at *m/z* 303 (delphinidin, $[M+H]^+$-162, loss of a hexosyl unit). Based on the mass spectra data and HPLC elution properties, peak 1 was identified as delphinidin-3-galactoside, a major anthocyanin in blueberry (Goiffon *et al*., 1999; Prior *et al*., 2001).

Peak 2 (t_R – 14.9 min, λ_{max} 520 nm) had $[M+H]^+$ at *m/z 4*65, which on MS^2 yielded a fragment ion at *m/z* 303 (delphinidin, $[M+H]^+$-162, loss of a hexosyl unit). Based on the mass spectral data and co-chromatography with standard, peak 2 was identified as delphinidin-3-glucoside**.**

Peak 3 (t_R – 16.7 min, λ_{max} – 520 nm) contained two components. One had a $[M+H]^+$ at *m/z* 449 which on MS^2 produced a fragment at *m/z* 287 (cyanidin, $[M+H]^+$-162, corresponding to the cleavage of an hexose unit). This compound, which eluted before a cyanidin-3-glucoside standard, was identified as cyanidin-3-galactoside, a known component of blueberries (Goiffon *et al*., 1999; Prior *et al*., 2001). The second compound had a $[M+H]^+$ at *m/z* 435, which yielded on MS^2 fragment at *m/z* 303 (delphinidin, $[M+H]^+$-132, loss of a pentosyl group). This compound was therefore delphinidin-3-arabinoside, also previously identified in blueberries by Goiffon *et al.* (1999) and Prior *et al.* (2001).

Peak 4 (t_R – 19.4 min, λ_{max} – 520 nm) had a $[M+H]^+$ at *m/z* 479 which on MS^2 produced a fragment ion corresponding to petunidin at *m/z* 317 via a 162 *amu* loss of a hexosyl unit. The presence of both petunidin-3-galactoside and petunidin-3-glucoside has been reported in blueberry (Goiffon *et al.,* 1999), but as this compound eluted before cyanidin-3-arabinoside (peak 5), peak 4 was more likely to be petunidin-3-galactoside.

Peak 5 (t_R – 20.2 min, λ_{max} – 515 nm) had a $[M+H]^+$ at *m/z* 419 and a MS^2 cyanidin fragment at *m/z* 287 produced by cleavage of a pentose moiety. This peak was, therefore, identified as cyanidin-3-arabinoside a known blueberry anthocyanin (Goiffon *et al*., 1999; Prior *et al*., 2001).

Peak 6 (t_R – 22.4 min, λ_{max} – 520 nm) contained two anthocyanins. One was identified as petunidin-3-arabinoside, which has been identified in blueberry in previous studies (Goiffon *et al*., 1999; Prior *et al*., 2001). It had a $[M+H]^+$ at *m/z* 449 which fragmented to produce MS^2 ions at *m/z* 317 (petunidin, $[M+H]^+$-132, loss of a pentosyl unit). The MS of this peak also contained an ion at *m/z* 463, which on MS^2 produced a peonidin fragment at *m/z* 301 ($[M+H]^+$-162, loss of an hexosyl unit). This compound was identified as peonidin-3-galactoside, rather than peonidin-3-glucoside by comparison with a standard.

Peak 7 (t_R – 23.8 min, λ_{max} – 520 nm) had a $[M+H]^+$ at *m/z* 493 which on MS^2 produced an ion at *m/z* 331 (malvidin, $[M+H]^+$-162, loss of an hexosyl). On the basis of this mass spectral data, elution prior to malvidin-3-glucoside (see peak 8) and previous studies (Goiffon *et al*., 1999; Prior *et al*., 2001) this compound was identified as malvidin-3-galactoside**.**

Peak 8 (t_R – 24.7 min, λ_{max} – 525 nm) contained two anthocyanins. One, like peak 7, had a $[M+H]^+$ at *m/z* 493 and yielded a similar MS^2 fragmentation pattern. This compound was identified as malvidin-3-glucoside, a known anthocyanin in blueberries (Goiffon *et al*., 1999; Prior *et al*., 2001). The identification was confirmed by co-chromatography with a reference compound. The other component had a $[M+H]^+$ at *m/z* 507 which on MS^2 produced a fragment ion corresponding to delphinidin at *m/z* 303. The 204 *amu* loss equates with cleavage of acetyl and glucosyl units. This mass spectral fragmentation pattern is in keeping with the presence of delphinidin-6-acetyl-3-glucoside, an endogenous anthocyanin of low bush blueberry (Prior *et al*., 2001).

Peak 9 (t_R – 25.7 min, λ_{max} – 525 nm) was a minor anthocyanin with a $[M+H]^+$ at *m/z* 433 and a MS^2 peonidin fragment at *m/z* 301 produced by the cleavage of a pentosyl unit. On the basis the mass spectrum and previously published data (Goiffon *et al*., 1999; Prior *et al*., 2001), this peak is identified as peonidin-3-arabinoside.

Peak 10 (t_R – 27.4 min, λ_{max} – 525 nm) which had a $[M+H]^+$ at *m/z* 463 that fragmented to produce a MS^2 ion at *m/z* 331 ($[M+H]^+$-132, loss of a pentosyl unit) was identified as malvidin-3-arabinoside, which has been detected in blueberries in earlier studies (Goiffon *et al*., 1999; Prior *et al*., 2001).

Peak 11 (t_R – 30.4 min, λ_{max} – 525 nm) was a minor anthocyanin. It had a $[M+H]^+$ at *m/z* 521 which fragmented to produce an MS^2 ion at *m/z* 317 ($[M+H]^+$-204, loss of an acetyl and glucosyl unit). This mass spectrum indicated the presence of petunidin-6-acetyl-3-glucoside, a known low bush blueberry anthocyanin (Prior *et al*., 2001).

Peak 12 (t_R – 30.7 min, λ_{max} – 355 nm) had a $[M-H]^-$ at *m/z* 479 which with neutral loss of a 162 *amu* hexose unit yielded a MS^2 fragment corresponding to myricetin at *m/z* 317. It was therefore a myricetin-hexose conjugate, probably myricetin-3-galactoside as this compound have been reported previously in blueberries extract (Maatta *et al*., 2004).

Peak 13 (t_R – 33.8 min, λ_{max} – 325-355 nm) contained two compounds. One had a $[M-H]^-$ at *m/z* 625 with neutral loss of a 324 *amu* (cleavage of two hexose units) yielded a MS^2 fragment corresponding to quercetin at *m/z* 301. It is, therefore, a quercetin-di-glucoside. The other component had a $[M-H]^-$ at *m/z* 367 which on MS^2 produced fragment at *m/z* 191, 179 and 135. Based on MS data, in agreement with the mass spectral key of Clifford *et al.* (2003), this compound was identified as 5-feruloylquinic acid.

Peak 14 (t_R – 35.5 min, λ_{max} – 530 nm) was a minor anthocyanin. It had a $[M+H]^+$ at *m/z* 535 and the same loss of a 204 *amu* as in peak 8 and 11. It was therefore identified as malvidin-6-acetyl-3-glucoside, as its presence having been detected previously in lowbush blueberry by Prior *et al*. (2001).

Peak 15 (t_R – 36.9 min, λ_{max} – 355 nm) had a $[M-H]^-$ at *m/z* 609, which on MS^2 yielded a quercetin-like fragment at *m/z* 301. The 308 *amu* loss corresponds to cleavage of a hexose and rhamnose unit. This indicates the presence of quercetin-3-rutinoside, which was confirmed by co-chromatography with a standard.

Peak 16 (t_R – 37.8 min, λ_{max} – 355 nm) contained an ion at *m/z* 463, which produced on MS^2 a quercetin fragment at *m/z* 301. This compound, which eluted before a quercetin-3-glucoside standard, was identified as quercetin-3-galactoside, a known constituent of blueberries (Maatta-Riihinen *et al*., 2004).

Peak 17 (t_R – 39.2 min, λ_{max} – 355 nm) is a flavonol with a $[M-H]^-$ at *m/z* 463, which with loss of 162 *amu* yielded a MS^2 fragment at *m/z* 301 corresponding to quercetin. It was identified as quercetin-3-glucoside, as it co-eluted with a standard.

Peak 18 (t_R – 43.0 min, λ_{max} – 355 nm) contained two compounds. One had a $[M-H]^-$ at *m/z* 433, which yielded a MS^2 fragment at *m/z* 301 ($[M-H]^-$-132, loss of a pentose unit) and MS^3 at *m/z* 271, 255 and 179. It is, therefore, a quercetin pentose conjugate, possibly quercetin-3-arabinoside, as this compound has previously been identified in blueberries by Maatta-Rihinen *et al.* (2004). The second compound had $[M+H]^+$ at *m/z* 505 and the same loss of a 204 *amu* as in peak 8, 11 and 14. It was therefore identified as quercetin-6-acetyl-3-glucoside.

4.3.3.2. Phenolic quantification and individual antioxidant activity of phenolics in blueberries

The TEAC, %AOC and TEAR values of the peaks are shown in Table 4.5. The major phenolic compounds were once again anthocyanins, this time malvidin-3-galactoside (peak 7), malvidin-3-arabinoside (peak 10), delphinidin-3-galactoside (peak 1) together with minor quantities of cyanidin, petunidin, and peonidin glycosides. The overall concentration of the anthocyanins was 4699 nmoles/g, which contributed 84% of the total TEAC. The quercetin and myricetin glycosides at 751 nmoles/g contributed 14% of the total TEAC. The highest TEAC values were for delphinidin-3-galactoside (peak 1) and peak 3 (cyanidin-3-galactoside and delphinidin-3-arabinoside). The TEAR for delphinidin-3-galactoside and petunidin-3-galactoside were 1.6 and 1.7 respectively, which is higher than that of any of the blackcurrant anthocyanins.

4.3.4. Raspberries

4.3.4.1 Identification of phenolics

HPLC-PDA- MS^3 analysis of raspberries resulted in the identification of 15 phenolic compounds (Figure 4.3, Table 4.6). Where reference compounds were not available,

Table 4.5. Levels of phenolic compounds and Trolox equivalent antioxidant activity (TEAC) in blueberries.

Peak	t_R	Compound	Quantity (nmol/g)	TEAC (nmol Trolox/g)	TEAC (%)	TEAR
	3.2	vitamin C	115 ± 5	n.d	-	
1	13.5	delphinidin-3-galactoside	729 ± 64	1135 ± 44	20 ± 1	1.6
2	14.9	delphinidin-3-glucoside	67 ± 1	61 ± 2	1.1 ± 0.0	0.9
3	16.7	cyanidin-3-galactoside delphinidin-3-arabinoside	590 ± 55	860 ± 39	15 ± 1	Δ
4	19.4	petunidin-3-galactoside	402 ± 35	680 ± 41	12 ± 1	1.7
5	20.2	cyanidin-3-arabinoside	119 ± 5	143 ± 4	2.6 ± 0.4	1.2
6	22.4	petunidin-3-arabinoside peonidin-3-galactoside	282 ± 19	421 ± 42	7.5 ± 0.8	Δ
7	23.8	malvidin-3-galactoside	996 ± 100	654 ± 96	12 ± 2	0.7
8	24.7	malvidin-3-glucoside delphinidin-6-acetyl-3-glucoside	212 ± 8	90 ± 19	1.6 ± 0.3	Δ
9	25.7	peonidin-3-arabinoside	96 ± 5	25 ± 1	0.5 ± 0.0	0.3
10	27.4	malvidin-3-arabinoside	808 ± 77	510 ± 33	9.1 ± 0.6	0.6
11	30.4	petunidin-6-acetyl-3-glucoside	81 ± 6	164 ± 4	2.9 ± 0.1	Δ
12	30.7	myricetin-3-galactoside	114 ± 11			
13	33.8	quercetin di glucoside 5-feruloylquinic acid	16 ± 3.0	239 ± 0	4.3 ± 0.0	Δ
14	35.5	malvidin-6-acetyl-3-glucoside	317 ± 13	83 ± 2	1.5 ± 0.0	0.3
15	36.9	quercetin-3-rutinoside	31 ± 0	18 ± 3	0.3 ± 0.0	0.6
16	37.8	quercetin-3-galactoside	368 ± 36	303 ± 18	5.4 ± 0.3	0.8
17	39.2	quercetin-3-glucoside	155 ± 15	144 ± 6	2.6 ± 0.1	0.9
18	43.0	quercetin-3-arabinoside quercetin-6-acetyl-3-glucoside	75 ± 2	45 ± 2	0.8 ± 0.0	0.6

Quantitative estimates expressed as mean values ± standard error (n=3). Peak numbers and retention times refer to HPLC traces in Figure 4.2. Identification by MS^3 see Table 4.4. n.q.- not quantified; n.d.- not detected; . * The contribution of the unidentified peaks at 720 nm to the of antioxidant capacity. Δ not calculated due to coelution of compounds.

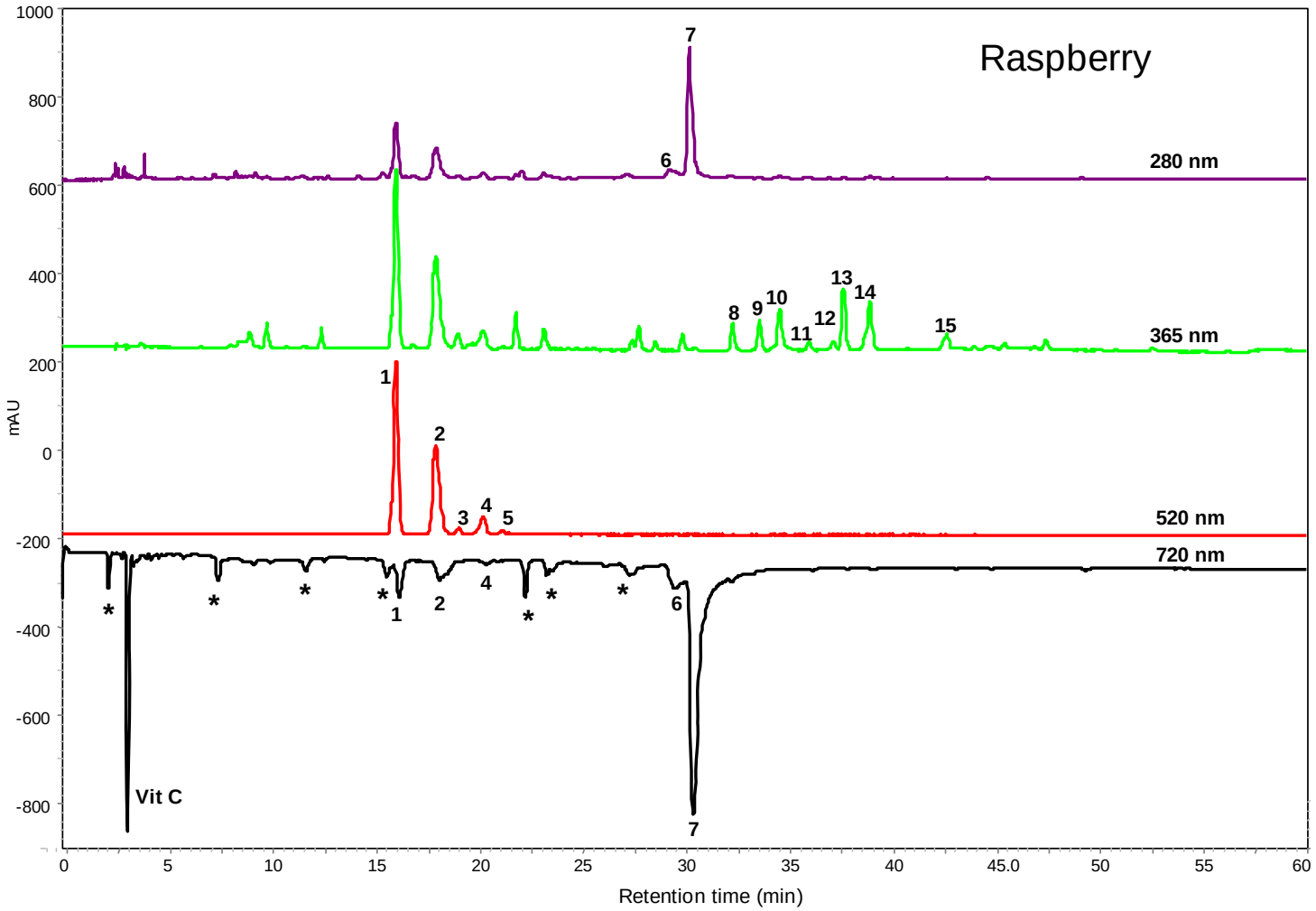


Figure 4.3 HPLC-PDA traces and on-line antioxidant detection (720 nm) of a raspberry extract. For identity of peaks refer to Table 4.6. * unidentified peaks.

Table 4.6. Phenolic compounds identified in raspberries by HPLC with diode array and MS^3 detection[a].

Peak	t_R	Compound	λmax (nm)	$[M-H]^-$ *(m/z)*	MS^2 ions *(m/z)*	MS^3 ion *(m/z)*
1	16.1	cyanidin-3-sophoroside	515	611*	287 [Cyan]($[M+H]^+$-Soph)	
2	17.9	cyanidin-3-(2^G-glucosylrutinoside)	515	757*	611 ($[M+H]^+$-Rham), 287 [Cyan]($[M+H]^+$-Rham-Glc-Glc)	
		cyanidin-3-sambubioside		581*	287 ($[M+H]^+$-Samb)	
		cyanidin-3-glucoside		449*	287 [Cyan]($[M+H]^+$-Glc)	
3	18.9	pelargonidin-3-sophoroside	500	595*	271 [Pel]($[M+H]^+$-Soph)	
4	20.2	cyanidin-3-rutinoside	510	595*	449 (M^+-Rham), 287 [Cyan](M^+-Rham-Glc)	
5	21.2	pelargonidin-3-glucoside	500	433*	271 [Pel]($[M+H]^+$-Glc)	
		pelargonidin-3-(2^G-glucosylrutinoside)		741*	271 [Pel](M^+- Rham-Glc-Glc)	
6	29.6	lambertianin C	250	1401^2 (2801)	1869, 1567 (1869-HHDP), 1265 (1869-HHDP-HHDP), 1251, 935 (1869-HHDP-HDDP-Glc-galloyl), 633 (1869-HHDP-HHDP-Glc-galloyl-HHDP)	
7	30.8	sanguiin H-6	250	1869	1567 ($[M-H]^-$-HHDP), 1265 ($[M-H]^-$-HHDP-HHDP), 1235, 933 ($[M-H]^-$-HHDP-HHDP-Glc-galloyl), 631 ([M-H]-HHDP-HHDP-Glc-galloyl-HHDP)	
8	32.7	ellagic acid-pentose conjugate	360	433	301 [HHDP]($[M-H]^-$-pent)	257
9	33.5	ellagic acid-pentose conjugate	360	433	301 [HHDP]($[M-H]^-$-pent)	257
10	34.4	ellagic acid	360	301	257	
11	35.8	quercetin-galactosylrhamnoside	340	609	301 [Q]($[M-H]^-$-Gal-Rham)	
12	37.1	quercetin-3-glucosylrutinoside	340	609	301 [Q]($[M-H]^-$-Glc-Rham)	
13	37.8	quercetin-3-galactoside	340	463	301 [Q]($[M-H]^-$-Gal)	
14	39.4	quercetin-3-glucoside	340	463	301 [Q]($[M-H]^-$-Glc), 179	
15	42.5	ellagic acid-4-acetylxyloside	360	475	301 [HHDP]($[M-H]^-$-XylAc)	

Cyan, cyanidin; Pel, pelargonidin; Soph, sophorosyl; Samb, sambubiosyl; Q, quercetin; Glc, glucosyl; Gal, galactosyl; Rham, rhamnosyl; Pent, pentosyl; Xyl, xylosyl; XylAc, acetylxylosyl; AraAc, acetylarabinosyl; GlcUA, glucuronyl; HHDP, hexahydroxydiphenoyl; t_R, retention time [a] Peak numbers and retention times refer to HPLC traces in Figure 4.3. * positive ionisation molecular ion ($[M+H]^+$)

identifications were made by comparison with earlier data on raspberry anthocyanins and data on the MS fragmentation patterns of anthocyanins and the *m/z* losses associated with cleavage of various sugars and other substituent groups presented by Goiffon *et al*. (1999) and Giusti *et al.* (1999). Similarly, the data of Rommel and Wrolstad (1993) and Zafrilla *et al*. (2001) aided the MS-based identifications of flavonols and ellagic acid sugar conjugates. Ellagitannin identification was based on Mullen *et al.* 2002a, 2002c and 2003b.

Peak 1 (retention time [t_R] – 16.1 min, λ_{max} – 515 nm) had a positively charged molecular ion ($[M+H]^+$) at *m/z* 611, which on MS^2 produced a fragment ion corresponding to cyanidin at *m/z* 287. The 324 *amu* loss equates with cleavage of a sophorosyl unit, which consists of two glucose moieties. This peak, therefore was identified as cyanidin-3-sophoroside, which is the major anthocyanin in raspberries.

Peak 2 (t_R – 17.9 min, λ_{max} – 515 nm) contained three anthocyanins. One was identified as cyanidin-3-(2^G-glucosylrutinoside), which has been detected in a previous study with raspberries (Mullen *et al*., 2002a). It had a $[M+H]^+$ at *m/z* 757 which fragmented to produce MS^2 ions at *m/z* 611 ($[M+H]^+$-146, loss of a rhamnosyl moiety) and *m/z* 287 ($[M+H]^+$-470 corresponding to the cleavage of one rhamnosyl and two glycosyl units). The MS of this peak also contained an ion at *m/z* 581 which fragmented on MS^2 to produce a minor ion at *m/z* 449 ($[M+H]^+$-132, loss of a xylosyl group) and a major fragment at *m/z* 287 ($[M+H]^+$-132-162, loss of xylosyl and glucosyl units). This compound is cyanidin-3-sambubioside, a known raspberry anthocyanin (Mullen *et al*., 2002c). The third anthocyanin was cyanidin-3-glucoside which had a $[M+H]^+$ at *m/z* 449 which on MS^2 yielded a cyanidin fragment ion at *m/z* 287 formed by the cleavage of a 162 *amu* glucosyl unit. A cyanidin-3-glucoside standard co-chromatographed with the 520 nm peak 2. Cyanidin-3-glucoside is a known constituent of raspberries (Mullen *et al*., 2002c).

Peak 3 (t_R – 18.9 min, λ_{max} – 500 nm) was a minor anthocyanin with a $[M+H]^+$ at *m/z* 595 and a MS^2 pelargonidin fragment ion at *m/z* 271 produced by cleavage of a 324 *amu* sophorosyl unit. This compound was therefore identified as pelargonidin-3-sophoroside detected in raspberries in earlier studies (Goiffon *et al*., 1999; Mullen *et al*., 2002c).*Peak 4* (t_R – 20.2 min, λ_{max} – 510 nm) was an anthocyanin with a $[M+H]^+$ at *m/z* 595 which on MS^2 produced a minor fragment at *m/z* 449 ($[M+H]^+$-146, loss of rhamnosyl) and a major fragment at *m/z* 287 (cyanidin,

$[M+H]^+$-308, corresponding to cleavage of a rhamnosyl unit and a glucosyl moiety). This compound was, therefore, cyanidin-3-rutinoside.

Peak 5 (t_R – 21.2 min, λ_{max} - 500 nm) contained two compounds. One had a $[M+H]^+$ at *m/z* 433 which on MS^2 produced a pelargonidin fragment ion at *m/z* 271 via a 162 *amu* cleavage of a glucosyl unit. This compound was pelargondin-3-glucoside, a known endogenous raspberry anthocyanin (Mullen *et al.*, 2002c). The second compound was also an anthocyanin. It had a $[M+H]^+$ at *m/z* 741, which after a 470 *amu* loss corresponding to cleavage of xylosyl and rutinosyl moieties, yielded a pelargonidin MS^2 ion at *m/z* 271. This compound was, therefore pelargonidin-3-2^G-glucosylrutinoside (Mullen *et al*., 2002c).

Peak 6 (t_R – 29.6 min, λ_{max} - 250 nm) had a $[M-H]^-$ at *m/z* 1401, reported to be doubly charged, with a true mass of 2804 (Mullen *et al*., 2003). Again MS^2 of the doubly charged ion produced a range of fragments at *m/z* 1869, *m/z* 1567 (1869-302, loss of HHDP), *m/z* 1265, *m/z* 935 and *m/z* 633. This peak was the ellagitannin lambertianin C, which has previously been detected in extracts of raspberry leaves (Tanaka *et al*., 1993) and fruit (Mullen *et al.,* 2003b).

Peak 7 (t_R – 30.8 min, λ_{max} - 250 nm), the major component in the raspberry extract had a $[M-H]^-$ at *m/z* 1869 which on MS^2 yielded fragment ions very similar to those produced by lambertianin C with ions at *m/z* 1567, 1265, 933 and 631. The mass spectrum and retention properties match those of sanguiin H-6 (Mullen *et al*., 2003b).

Peak 8 (t_R – 32.7 min, λ_{max} - 360 nm) had a $[M-H]^-$ at *m/z* 433 and MS^2 yielded an ion at *m/z* 301 ($[M-H]^-$-132, loss of a pentosyl unit) and MS^3 produced a *m/z* 257 fragment indicating the presence of ellagic acid rather than quercetin (Mullen *et al.*, 2003b). This compound was, therefore, an ellagic acid pentose conjugate.

Peak 9 (t_R – 33.5 min, λ_{max} - 360 nm) had a $[M-H]^-$ at *m/z* 433 and MS^2 yielded an ion at *m/z* 301 ($[M-H]^-$-132, loss of a pentosyl unit) and MS^3 produced a *m/z* 257 fragment indicating the presence of ellagic acid. This compound was, therefore, another ellagic acid pentose conjugate.

Peak 10 (t_R – 34.4 min, λ_{max} - 360 nm) produced a $[M-H]^-$ at *m/z* 301 and MS^2 ionisation yielded a fragment at *m/z* 257, which matches the mass spectrum of

ellagic acid. This identification was confirmed by co-chromatography with a reference compound.

Peak 11 (t_R – 35.8 min, λ_{max} - 340 nm) had a mass spectra with a $[M-H]^-$ at *m/z* 609, which yielded a MS^2 fragment at *m/z* 301 which in this instance corresponds to loss of hexose and rhamnose units ($[M-H]^-$-308). Peak 11 was, therefore, the quercetin galactosylrhamnoside previously detected in raspberries by Mullen *et al.* (2002c).

Peak 12 (t_R – 37.1 min, λ_{max} - 340 nm) had identical mass spectra than peak 11, a $[M-H]^-$ at *m/z* 609, which yielded a MS^2 fragment at *m/z* 301. In keeping with this fragmentation, pattern this peak co-chromatographed with a quercetin-3-rutinoside (aka rutin) standard. Previous analytical work with raspberries has shown that quercetin-3-rutinoside elutes after the quercetin galactosylrhamnoside peak (peak 9) (Mullen *et al.,* 2002a).

Peak 13 (t_R – 37.8 min, λ_{max} - 340 nm) is a quercetin hexose, having a $[M-H]^-$ at *m/z* 463, which with neutral loss of 162 *amu* yielded a MS^2 fragment corresponding to quercetin at *m/z* 301. This compound, which eluted before a quercetin-3-glucoside standard, was identified as quercetin-3-galactoside.

Peak 14 (t_R – 39.4 min, λ_{max} - 340 nm) had a $[M-H]^-$ at *m/z* 463 which on MS^2 yielded an ion at *m/z* 301 which with neutral loss of 162 *amu* yielded a MS^2 fragment corresponding to quercetin at *m/z* 301. The MS data, and co-chromatography with a standard, indicate the presence of a quercetin-3-glucoside.

Peak 15 (t_R – 42.5 min, λ_{max} - 360 nm) had both a $[M-H]^-$ at *m/z* 475, which yielded MS^2 fragments at *m/z* 301 ($[M-H]^-$-174, loss of acetylpentose). Mullen *et al.* (2003b) have tentatively identified it as ellagic acid-4-acetylxyloside. These compound previously have been detected in raspberries by Zafrilla *et al.* (2001).

4.3.4.2. Phenolic quantification and individual antioxidant activity of phenolics in raspberries

The TEAC, % TEAC and TEAR values of the peaks obtained for raspberries are shown in Table 4.7. Five anthocyanin peaks were detected at 520 nm (Figure 4.3). Peak 1, cyanidin-3-sophoroside is the major anthocyanin followed by the three

Table 4.7. Levels of phenolic compounds and Trolox equivalent antioxidant activity (TEAC) in raspberries**.**

Peak	t_R	Compound	Quantity (nmoles/g)	TEAC (nmoles Trolox/g)	TEAC (%)	TEAR
	3.1	vitamin C	1014 ± 30	455 ± 27	11 ± 0	0.5
1	16.1	cyanidin-3-sophoroside	375 ± 28	454 ± 41	6.9 ± 0.6	1.2
2	17.9	cyanidin-3-(2^G-glucosylrutinoside) cyanidin-3-glucoside cyanidin-3-sambubioside	308 ± 11	526 ± 15	8.1 ± 0.23	Δ
3	18.9	pelargonidin-3-sophoroside	44 ± 3	n.d	-	-
4	20.2	Cyanidin-3-rutinoside	85 ± 4	93 ± 9	1.4 ± 0.1	1.1
5	21.2	pelargonidin-3(2^G-glucosylrutinoside) pelargonidin-3-glucoside	74 ± 0	n.d	-	Δ
6	29.6	lambertianin C	322 ± 41	886 ± 12	13.6 ± 0.2	2.8
7	30.8	Sanguiin H-6	1030 ± 107	2905 ± 360	45 ± 6	2.8
8	32.7	ellagic acid pentose	7.9 ± 1.2	n.d.	-	-
9	33.5	ellagic acid pentose	10 ± 1	n.d.	-	-
10	34.4	ellagic acid	11 ± 1	n.d.	-	-
11	35.8	quercetin-galactosylrhamnoside	7.5 ± 0.1	n.d.	-	-
12	37.1	quercetin-3-rutinoside	6.7 ± 0.1	n.d.	-	-
13	37.8	quercetin-3-galactoside	25 ± 2	n.d.	-	-
14	39.4	quercetin-3-glucoside	28 ± 4	n.d.	-	-
15	42.5	ellagic acid-4-acetylxyloside	23 ± 2	n.d.	-	-
*		other unidentified peaks			14.6 ± 0.3	

Quantitative estimates expressed as mean values ± standard error (n=3). Peak numbers and retention times refer to HPLC traces in Figure 4.3. Identification by MS^3 see Table 4.6. n.q.- not quantified; n.d.- not detected; * the contribution of the non identified peaks at 720 nm to the of antioxidant capacity. Δ not calculated due to coelution of compounds.

compounds contained in peak 2, cyanidin-3-(2^G-glucosylrutinoside), cyanidin-3-sambubioside and cyanidin-3-glucoside. In this case, anthocyanins present at total concentration of 886 nmols/g contribute 16.4% to the total TEAC, while vitamin C (1014 nmoles/g) contributes with 11%. The ellagitannins lambertianin C (peak 6) and sanguiin H-6 (peak 7) are the main contributors to the AOC being responsible for more than 58% of the total. The TEAR value of both ellagitannins is 2.8 which is the highest value detected in the berries analyzed in this study.

4.3.5 Redcurrants

4.3.5.1 Identification of phenolic compounds

The HPLC traces obtained at 520, 365 and 280 nm indicate the 13 compounds identified in redcurrants (Figure 4.4). When standard compounds were not available, identifications were assisted by previous analyses of anthocyanins (Oydvin, 1974; Goiffon *et al*., 1991 and 1999; Maatta *et al*., 2001 and 2003) and hydroxycinnmate conjugates in redcurrants (Schuster and Herrmann, 1985). The identification of phenolic compounds are summarised below and presented in Table 4.8.

Peak 1 (t_R – 9.1 min, λ_{max} - 260 nm) had a $[M-H]^-$ at *m/z* 299 and MS^2 yielded an ion at *m/z* 137 ($[M-H]^-$-162, loss of an hexose unit). This compound was tentatively identified as a 4-hydroxybenzoic acid hexose conjugate, in agreement with Matta *et al.* (2001) who identified 4-hydroxybenzoic acid after acid hydrolysis of a redcurrant extract.

Peas 2 (t_R – 12.6 min, λ_{max} - 330 nm) had both a $[M-H]^-$ at *m/z* 341 which on MS^2 yielded a fragment releasing an ion at *m/z* 179. The *m/z* 179 ion is the aglycone caffeic acid produced by a loss of 162 *amu*, which corresponds with the cleavage of a hexose moiety. Based on the mass spectral and PDA data and previously published data (Schuster *et al*., 1985), this compounds was identified as caffeic acid hexose conjugate. *Peak 3* (t_R – 18.6 min, λ_{max} - 515 nm) contained two compounds. One had a $[M+H]^+$ at *m/z* 581 which on MS^2 produced a cyanidin fragment ion at *m/z* 287. The loss of 294 *amu* corresponds to the cleavage of xylosyl and glucosyl units. The mass spectrum and the HPLC retention correspond to that of cyanidin-3-

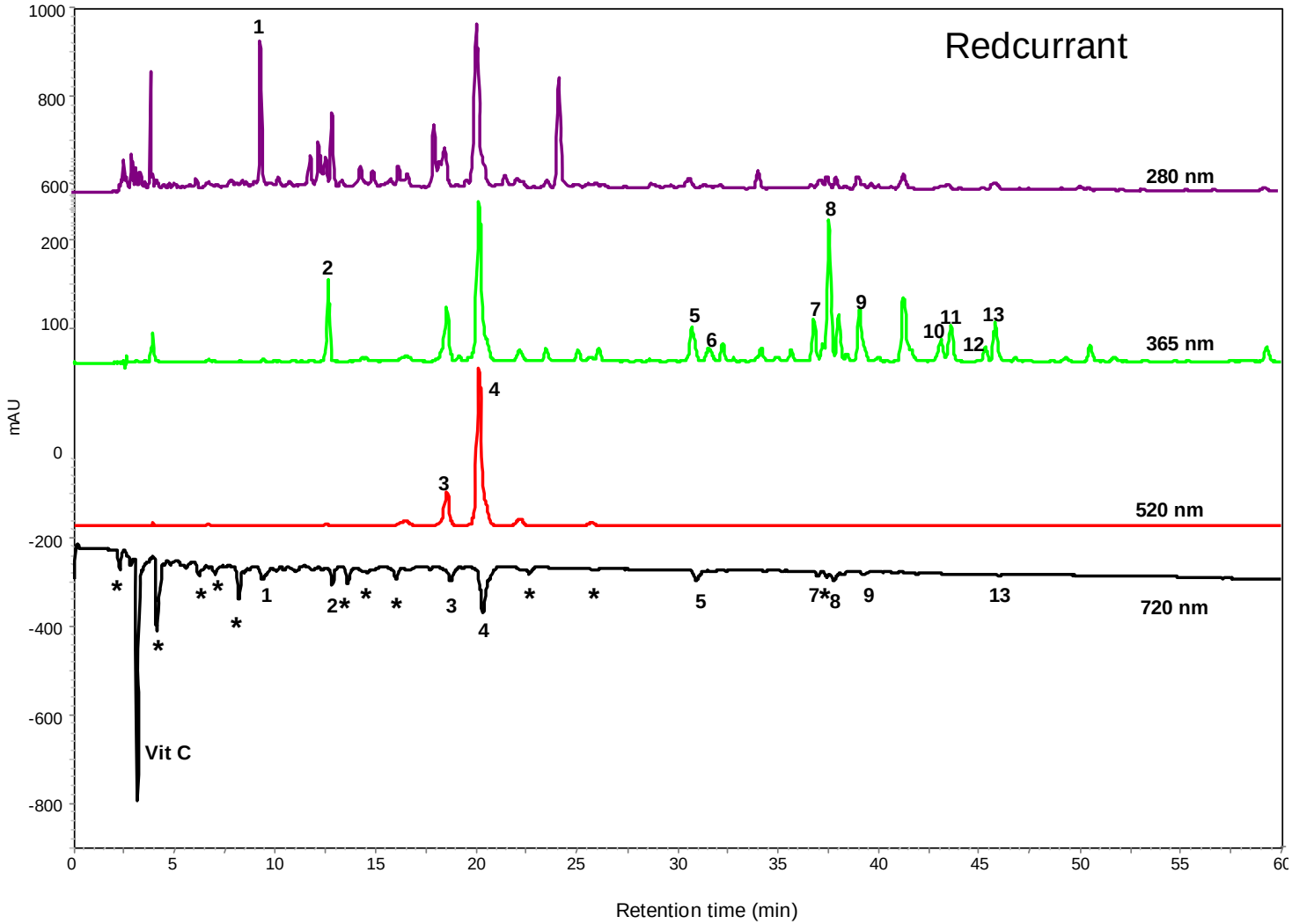


Figure 4.4 HPLC-PDA traces and on-line antioxidant detection (720 nm) of a redcurrant extract. For identity of peaks refer to Table 4.8. * unidentified peaks.

Table 4.8. Phenolic compounds identified in redcurrants by HPLC with diode array and MS^3 detection[a].

Peak	t_R	Compound	Amax (nm)	$[M-H]^-$ (*m/z*)	MS^2 ions (*m/z*)	MS^3 ions (*m/z*)
1	9.1	4-hydroxy-benzoic acid-hexose	260	299	137 [HbenzAc][([M-H]$^-$ - Hex)	
2	12.6	caffeic acid-hexose conjugate	330	341	179 [CafAc]([M-H]$^-$ -Hex)	
3	18.6	cyanidin-3-sambubioside	515	581*	287[Cyan]([M+H]$^+$-Samb)	
		cyanidin-3-glucoside		449*	287 [Cyan]([M+H]$^+$-Glc)	
4	20.4	cyanidin-3-rutinoside	515	595*	449([M+H]$^+$- Rham), 287[Cyan]([M+H]$^+$- Glc-Rham)	
		cyanidin-3-xylosyl-rutinoside		727*	581([M+H]$^+$- Rham), 287 [Cyan]([M+H]$^+$- Xyl-Rut)	
5	30.7	myricetin-3-rutinoside	355	625	317 [Myr]([M-H]$^-$- Rut)	
6	31.6	myricetin-rhamnoside	330	463	317 [Myr]([M-H]$^-$- Rham)	
7	36.7	quercetin-3-rutinoside	355	609	301 [Q]([M-H]$^-$- Gal),	271, 179, 255
8	37.6	quercetin-3-galactoside	355	463	301 [Q]([M-H]$^-$- Rut)	271, 179, 255
9	39.1	quercetin-3-glucoside	355	463	301 [Q]([M-H]$^-$- Glc),	271, 179, 255
10	43.2	quercetin-malonyl-hexose	345	505	463([M-H]$^-$- Rut), 301 [Q]([M-H]$^-$- Hex-(Mal-COOH)), 179	
11	43.7	kaempferol-rutinoside	345	593	285 [K]([M-H]$^-$- Rut)	
12	45.2	kaempferol-3-galactoside	340	447	285[K]([M-H]$^-$- Gal)	
13	45.9	kaempferol-3-glucoside	340	447	285[K]([M-H]$^-$- Glc)	

HbenzAc, 4-hydroxybenxoic acid; Hex, hexosyl; CafAc, Caffeic acid; Cyan, cyanidin; Samb, sambubiosyl; Rut, rutinosyl; Xyl, xylosyl; Myr, Myricetin; Rham, rhamnosyl; Q, quercetin; Glc, glucosyl; Mal, malonyl; K, kaempferol; t_R, retention time. [a] Peak numbers and retention times refer to HPLC traces in Figure 4.4. * positive ionisation molecular ion ($[M+H]^+$)

sambubioside, identified in redcurrants in earlier studies (Oydvin, 1974; Maatta *et al*., 2003). The other compound had a $[M+H]^+$ at *m/z* 449 which on MS^2 produced a cyanidin fragment ion at *m/z* 287. The loss of 162 *amu* corresponds to the cleavage of a glucosyl unit. It was identified as cyanidin-3-glucoside**.**

Peak 4 (t_R – 20.4 min, λ_{max} - 515 nm) was the main peak present in the redcurrant extract and contained two compounds. The first had a $[M+H]^+$ at *m/z* 595 which on MS^2 produced a minor fragment at *m/z* 449 ($[M+H]^+$-146, loss of rhamnosyl unit) and a major fragment at *m/z* 287 (cyanidin, $[M+H]^+$-308 corresponding to the cleavage of rhamnosyl and glucosyl moieties). This compound was, therefore, cyanidin-3-rutinoside. The second compound had a $[M+H]^+$ at *m/z* 727, which yielded MS^2 fragments at *m/z* 581 ($[M+H]^+$-146, loss of rhamnose) and *m/z* 287 (cyanidin, $[M+H]^+$-440, loss of rhamnosyl, glucosyl and xylosyl groups). This corresponded to the fragmentation of cyanidin-3-xylosylrutinoside, which has previously been detected in redcurrants (Oydvin, 1974; Goiffon *et al*., 1999; Maatta *et al*., 2003).

Peak 5 (t_R – 30.7 min, λ_{max} – 355nm) had a $[M-H]^-$ at *m/z* 625 which with the loss of 308 *amu* yielded an MS^2 fragment corresponding to myricetin at *m/z* 317. These spectra indicated the presence of a myricetin-3-rutinoside conjugate.

Peak 6 (t_R – 31.6, λ_{max} – 330 nm) yielded a $[M-H]^-$ at *m/z* 463 and MS^2 fragment at 317 ($[M-H]^-$-146, loss of a rhamnosyl moiety). It was, therefore, identified as a myricetin rhamnoside.*Peak 7* (t_R – 36.7 min, λ_{max} – 355 nm) was identified as quercetin-3-rutinoside. It yielded a $[M-H]^-$ at *m/z* 609, which with neutral loss of 308 *amu* yielded a MS^2 fragment corresponding to quercetin at *m/z* 301. The identification was confirmed by co-elution with a standard.

Peak 8 (t_R – 37.6 min, λ_{max} – 355 nm) produced a typical quercetin hexose mass spectrum ($[M-H]^-$ at *m/z* 463, MS^2 at *m/z* 301 and MS^3 at *m/z* 271, 255 and main ion at *m/z* 179). Earlier elution than quercetin-3-glucoside (Peak 9) indicates that this compound could be quercetin-3-galactoside.

Peak 9 (t_R – 39.1 min, λ_{max} – 355 nm) produced a typical quercetin glucoside mass spectrum ($[M-H]^-$ at *m/z* 463, MS^2 at *m/z* 301 and MS^3 at *m/z* 271, 255 and main ion at *m/z* 179). Co-chromatography established the presence of quercetin-3-glucoside.

Peak 10 (t_R – 43.2 min, λ_{max} 345 nm) had a $[M-H]^-$ at *m/z* 505 and MS^2 fragments at *m/z* 463 ($[M-H]^-$-42, loss of a malonyl-COOH group) and *m/z* 301 (quercetin, $[M-H]^-$–204). As in peak 16 in blackcurrants (see Table 4.2), the 204 amu loss corresponds to the loss of hexose and malonyl groups, which in negative ion mode lost the 45 *amu* carboxylic function from the pseudomolecular ion. This is indicative of the presence of a quercetin malonyl hexose conjugate. The presence of such a compound has previously been reported in redcurrants by Maatta *et al*. (2003).

Peak 11 (t_R – 43.7 min, λ_{max} - 345 nm) yielded a $[M-H]^-$ at *m/z* 593 and an MS^2 fragment at *m/z* 285. The *m/z* 285 ion (kaempferol) was produced by a 308 *amu* cleavage, which corresponded to the loss of a rutinose unit. Peak 11 was therefore identified as a kaempferol rutinoside.

Peak 12 (t_R – 45.2 min, λ_{max} - 340 nm) had a $[M-H]^-$ at *m/z* 447 which with a loss of 162 *amu* (cleavage of hexose) produced a MS^2 fragment at *m/z* 285 (kaempferol). Earlier elution than kaempferol-3-glucoside (peak 13) established that this compound could be kaempferol-3-galactoside.

Peak 13 (t_R – 45.9 min, λ_{max} - 340 nm) had a $[M-H]^-$ at *m/z* 447 which with a loss of 162 *amu* (cleavage of hexose) produced a MS^2 fragment at *m/z* 285 (kaempferol). Co-elution with a standard established that this compound was kaempferol-3-glucoside.

4.3.5.2. Phenolic quantification and individual antioxidant activity of phenolics in redcurrants

The quantification of phenolics in redcurrants is shown in Table 4.9 together with the TEAC, percentage of contribution to the AOC and the TEAR. The two main peaks at 520 nm (Figure 4.4) corresponding to cyanidin-3-sambubioside/cyanidin-3-glucoside (peak 3) and cyanidin-3-xylosyl-rutinoside/cyanidin-3-rutinoside (peak 4) are the main phenolics present in a concentration of 329 nmoles/g. They contribute 29 % of the total AOC. A similar amount of vitamin C was detected (313 nmol/g of vit C) which was responsible for 25.7% overall AOC. At 1.5, the caffeic acid conjugate (peak 2) had the highest TEAR.

Table 4.9. Levels of phenolic compounds and Trolox equivalent antioxidant activity (TEAC) in redcurrants.

Peak	t_R	Compound	Quantity (nmol/g)	TEAC (nmol Trolox/g)	TEAC (%)	TEAR
	3.2	Vitamin C	313 ± 41	156 ± 5	26 ± 1	0.5
1	9.1	4-hydroxy-benzoic acid hexose	73 ± 1	17 ± 0	2.7 ± 0.0	0.2
2	12.6	caffeic acid hexose conjugate	16 ± 1	25 ± 0	4.1 ± 0.1	1.5
3	18.6	cyanidin-3-sambubioside cyanidin-3-glucoside	81 ± 1	23 ± 1	3.8 ± 0.1	Δ
4	20.4	cyanidin-3-xylosyl-rutinoside cyanidin 3-rutinoside	248 ± 1	156 ± 7	26 ± 1	Δ
5	30.7	myricetin-rutinoside	4.6 ± 0.0	2.5 ± 0.0	0.5 ± 0.0	0.5
6	31.6	myricetin-rhamnoside	3.7 ± 0.1	n.d	-	-
7	36.7	quercetin-3-rutinoside	8.6 ± 0.1	8.3 ± 0.1	1.4 ± 0.0	1.0
8	37.6	quercetin-3-galactoside	23 ± 0	9.4 ± 0.2	1.6 ± 0.0	0.4
9	39.1	quercetin-3-glucoside	11 ± 0	4.9 ± 0.3	0.8 ± 0.1	0.5
10	43.2	quercetin-hexoside-malonate	4.6 ± 0.0	n.d	-	-
11	43.7	kaempferol-rutinoside	5.1 ± 0.1	n.d	-	-
12	45.2	kaempferol-3-galactoside	3.2 ± 0.1	n.d	-	-
13	45.9	kaempferol-3-glucoside	5.4 ± 0.2	4.2 ± 1.1	0.7 ± 0.2	0.8
*		unidentified peaks			33 ± 1	

Quantitative estimates expressed as mean values ± standard error (n=3). Peak numbers and retention times refer to HPLC traces in Figure 4.4. Identification by MS^3 see Table 4.8. n.q.- not quantified; n.d.- not detected; . * % contribution of the non identified peaks at 720 nm to the of antioxidant capacity. Δ not calculated due to coelution of compounds.

Flavonols were present in small amounts (69 nmol/g) the main component being quercetin-3-rutinoside. A number of peaks detected at 280 nm and which were responsible for 33% of the total AOC could not be identified.

4.3.6. Cranberries

4.3.6.1. Identification of phenolic compounds

The HPLC traces obtained at 520, 365 and 280 nm facilitated the detection of 18 phenolic compounds (Figure 4.5). Where standard compounds were unavailable, identifications were based on previously published analysis of cranberry extracts (Prior *et al*., 2001; Maatta *et al*., 2004; Vvedenskaa *et al*., 2004). The identifications of phenolic compounds are summarised in Table 4.10.

Peaks 1, 11 and 13 (t_R – 13.1, 32.7 and 37.7 min, λ_{max} - 290 nm) yielded a $[M-H]^-$ at *m/z* 577 and MS^2 fragments at *m/z* 425 and *m/z* 289 ($[M-H]^-$-288, loss of a catechin/epicatechin unit), characteristic of a procyanidin dimer.

Peak 2 (t_R – 16.8 min, λ_{max} - 515 nm) had a $[M+H]^+$ at *m/z* 449 which on MS^2 produced a fragment ion corresponding to cyanidin at *m/z* 287. The 162 *amu* loss equates with cleavage of a hexose unit. This compound was, therefore, identified as cyanidin-3-galactoside, one of the major anthocyanins in cranberries (Prior *et al*., 2001). *Peak 3* (t_R – 18.4 min) in negative ionisation revealed the presence of a $[M-H]^-$ at *m/z* 325, which on MS^2 yielded an *m/z* 163 fragment (coumaric acid, $[M-H]^-$-162, loss of an hexose moiety). This compound was, therefore, a *p*-coumaric acid hexose conjugate, which has been reported previously in cranberries (Maatta *et al*., 2004)

Peak 4 (t_R – 20.3 min, λ_{max} - 515 nm) had a $[M+H]^+$ at *m/z* 419 which on MS^2 produced a fragment ion corresponding to cyanidin at *m/z* 287 via a 132 *amu* cleavage of a pentose unit. This compound was identified as cyanidin-3-arabinoside, a major anthocyanin present in cranberries (Prior *et al*., 2001).

Peak 5 (t_R – 21.7 min, λ_{max} - 280 nm) had a $[M-H]^-$ at *m/z* 289 which on MS^2 yielded a fragments of *m/z* 245. This fragmentation pattern is that of a flavan-3-ol

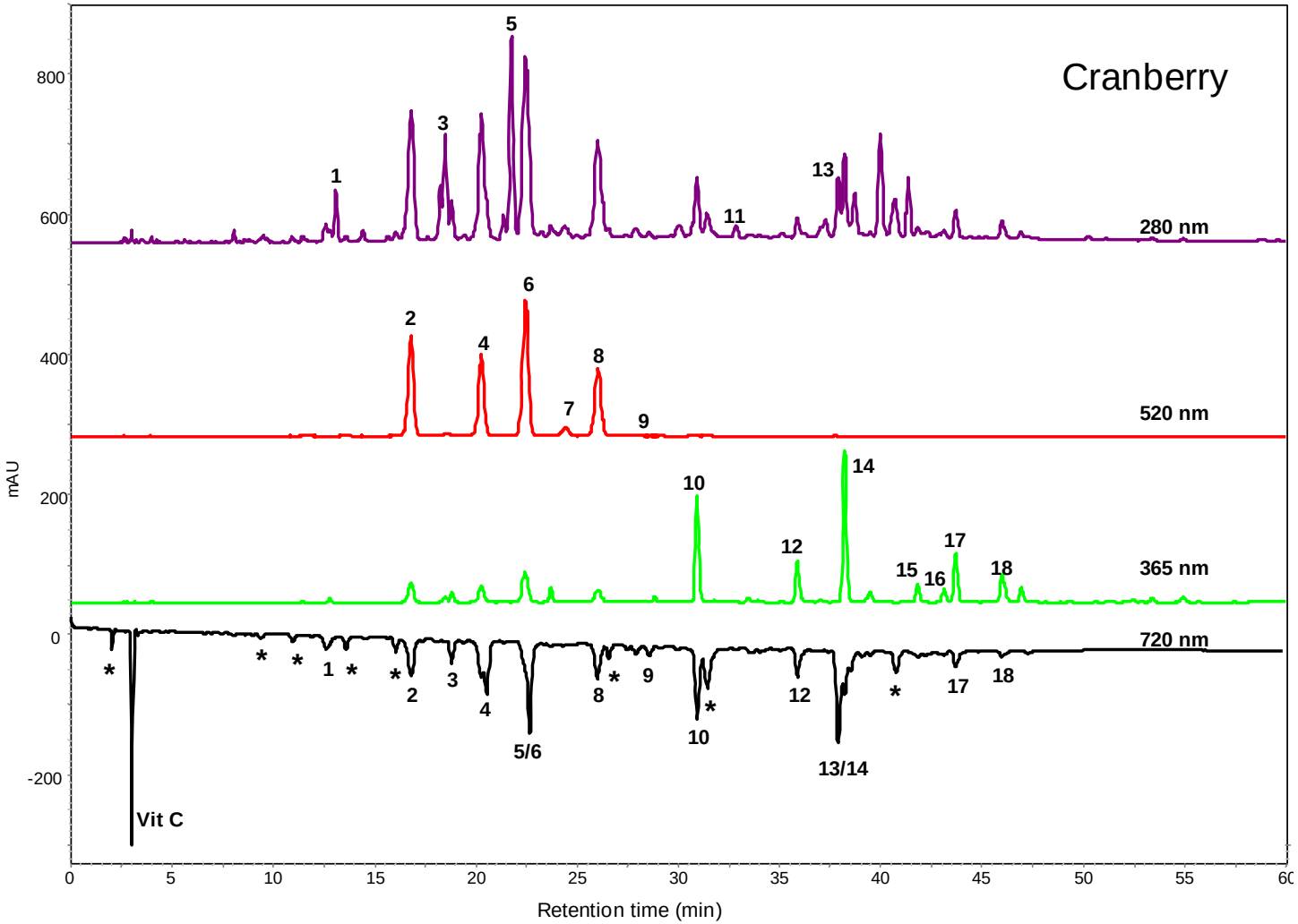


Figure 4.5. HPLC-PDA traces and on-line antioxidant detection (720 nm) of a cranberry extract. For identity of peaks refer to Table 4.10. * unidentified peaks.

Table 4.10. Phenolic compounds identified in cranberries by HPLC with diode array and MS^3 detection[a].

Peak	t_R	Compound	Λmax (nm)	[M-H] (*m/z*)	MS^2 ions (*m/z*)	MS^3 ion (*m/z*)
1	13.1	procyanidin dimmer	290	577	425, 289 [Cat] ($[M-H]^-$ - Cat)	
2	16.8	cyanidin-3-galactoside	515	449*	287 [Cyan] ($[M+H]^+$-Gal)	
3	18.4	*p*-coumaric acid hexose conjugate		325	163 [CoumAc]($[M-H]^-$ -Hex),145,187	
4	20.3	cyanidin-3-arabinoside	515	419*	287 [Cyan] ($[M+H]^+$- Arab)	
5	21.7	(–)-epicatechin	280	289	245	
6	22.5	peonidin-3-galactoside	515	463*	301 [Peon] ($[M+H]^+$- Gal)	
7	24.4	peonidin-3-glucoside	515	463*	301 [Peon] ($[M+H]^+$- Glc)	
8	26.1	peonidin-3-pentoside	515	433*	301 [Peon] ($[M+H]^+$- Arab)	
9	27.8	malvidin-3-arabinoside	520	463*	331 [Malv] ($[M+H]^+$- Arab)	
10	30.9	myricetin-3-galactoside	355	479	317 [Myr] ($[M-H]^-$ -Gal)	271, 179, 151
11	32.7	procyanidin dimmer	280	577	425, 289 [Cat] ($[M-H]^-$ - Cat)	
12	35.8	myricetin-3-arabinoside	355	449	317 [Myr]($[M-H]^-$ - Arab)	271, 179, 151
13	37.7	procyanidin dimmer	280	577	425, 289 [Cat] ($[M-H]^-$ - Cat)	
14	38.2	quercetin-3-galactoside	355	463	301 [Q] ($[M-H]^-$ - Gal)	271, 255, 179
15	41.4	quercetin-3-xylopyranoside	355	433	301[Q] ($[M-H]^-$ - Xyl)	271,255,179
16	43.2	quercetin-3-arabinopyranoside	350	433	301[Q] ($[M-H]^-$ - Arab)	
17	43.8	quercetin-3-arabinofuranoside	350	433	301[Q] ($[M-H]^-$ - Arab)	271,255,179
18	46.1	quercetin-3-rhamnoside	345	447	301 [Q] ($[M-H]^-$ - Rham)	271,255,179

Cat, catechin; CafAc, caffeic acid; CoumAc, *p*-coumaric acid; Cyan, cyanidin; Mal, malvidin; Peon, peonidin; Myr, myricetin; Q, quercetin; Arab, arabinosyl; Glc, glucosyl; Gal, galactosyl; Rham, rhamnosyl; Pent, pentosyl; Hex, hexose; t_R, retention time [a] Peak numbers and retention times refer to HPLC traces in Figure 4.5. * positive ionisation molecular ion ($[M+H]^+$)

monomer which co-chromatography with a standard identified, therefore as **(–)-**epicatechin.

Peak 6 (t_R –22.5 min, λ_{max} - 515 nm) was a major anthocyanin with a $[M+H]^+$ at *m/z* 463 and a MS^2 peonidin fragment at *m/z* 301 produced by cleavage of a hexose moiety. This fragmentation pattern was in keeping with the presence of peonidin-3-galactoside, a known constituent of cranberries (Prior *et al*., 2001) that elutes before peonidin-glucoside (peak 7).

Peak 7 (t_R –24.4 min, λ_{max} - 280 and 520 nm) had a $[M+H]^+$ at *m/z* 463, which on MS^2 produced a fragment ion corresponding to peonidin at *m/z* 301 and a $[M+H]^+$-162, loss of a glucosyl unit. It was identified as peonidin-3-glucoside.

Peak 8 (t_R –26.1 min, λ_{max} - 515 nm) had a $[M+H]^+$ at *m/z* 433, which yielded MS^2 fragments at *m/z* 301 (peonidin, a $[M+H]^+$-132, loss of a pentosyl unit). It was therefore identified as a peonidin-pentoside.

Peak 9 (t_R –27.8 min, λ_{max} - 520 nm) was a minor anthocyanin and had a $[M+H]^+$ at *m/z* 463 which with neutral loss of 162 *amu* yielded a MS^2 fragment corresponding to malvidin at *m/z* 331. This compound was a malvidin-pentose conjugate, possibly malvidin-3-arabinoside based on its retention time being the same as peak 10 in blueberries (see Table 4.4).

Peak 10 (t_R –30.9 min, λ_{max} - 355 nm) produced a $[M-H]^-$ at *m/z* 479 which with a loss of 162 *amu* yielded a MS^2 at *m/z* 317. It was therefore a myricetin hexose conjugate, probably myricetin-3-galactoside a known cranberry flavonol (Vvedenskaya *et al*., 2004).

Peak 12 (t_R –35.8 min, λ_{max} - 355 nm) was a myricetin pentose conjugate having a $[M-H]^-$ at *m/z* 449 and a MS^2 ion at *m/z* 317. This compound might be myricetin-3-arabinoside, which has been identified in cranberries by Vvedenskaya *et al*. (2004).

Peak 14 (t_R – 38.2 min, λ_{max} - 355 nm) was the major flavonol present in cranberries and produced a mass spectrum characteristic of a quercetin hexose conjugate ($[M-H]^-$ at *m/z* 463, MS^2 ion at *m/z* 301 and MS^3 of this ion produced a major fragment at *m/z* 179). In view of this mass spectrum, the high concentration

and HPLC order of elution, this compound was quercetin-3-galactoside**,** previously detected in cranberries by (Vvedenskaya *et al*., 2004).

Peaks 15, 16 and 17 (t_R – 41.4, 43.2 and 43.8 min, λ_{max} - 355 and 350 nm) all had a $[M-H]^-$ at *m/z* 433 which with neutral loss of a 132 *amu* pentose unit yielded a MS^2 fragment corresponding to quercetin at *m/z* 301. These three flavonols are, therefore, all quercetin pentose conjugates. Based on their elution order they were identified as quercetin-3-xylopyranoside, quercetin-3-arabinopyranoside and quercetin-3-arabinofuranoside respectively, previously identified in a cranberry extract by HPLC-MS and NMR (Vvedenskaya *et al*., 2004).

Peak 18 (t_R – 46.1 min, λ_{max} - 345 nm) had a $[M-H]^-$ at *m/z* 447 which yielded an MS^2 fragment at *m/z* 301 (quercetin), the $[M-H]^-$-146 loss corresponding to cleavage of a rhamnose unit. The mass spectral data and HPLC retention are in keeping with this peak being quercetin-3-rhamnoside, which was previously detected in cranberries by Vvedenskaya *et al*. (2004).

4.3.6.2. Phenolic quantification and individual antioxidant activity of phenolics in cranberries

The quantification of phenolics in cranberries, their TEAC, percentage of contribution and TEAR are shown in Table 4.11. The main antioxidant peak at 720 nm (Figure 4.5) corresponds to vitamin C, which was responsible for 22.6% of the AOC. (–)-Epicatechin (peak 5) is the major phenolic compounds at 1121 nmol/g but it only contributes 14% of the overall AOC along with peonidin-3-galactoside. The anthocyanins constitute the second major group with peaks 2, 4, 6, 7, 8 and 9 adding up to 726 nmoles/g and contributing 32% of the AOC of cranberries. A total of 456 nmoles of flavonols were present and they were responsible for 16% of the overall AOC. Cyanidin-3-arabinoside had the highest TEAR value at 1.9 among all the anthocyanins identified in the berries.

Table 4.11. Levels of phenolic compounds and Trolox equivalent antioxidant activity in cranberries.

Peak	t_R	Compound	Quantity (nmol/g)	TEAC (nmol Trolox/g)	TEAC (%)	TEAR
	3.0	vitamin C	1107 ± 3	487 ± 10	23 ± 0	0.4
1	13.1	procyanidin dimmer	405 ± 59	38 ± 2	3.8 ± 0.1	0.1
2	16.8	cyanidin-3-galactoside	160 ± 24	154 ± 6	7.1 ± 0.3	1.0
3	18.4	*p*-coumaric acid hexose conjugate	119 ± 13	36 ± 2	1.7 ± 0.1	0.3
4	20.3	cyanidin-3-arabinoside	138 ± 20	262 ± 6	12 ± 0	1.9
5	21.7	(–)-epicatechin	1121 ± 185	310 ± 16	14 ± 1	Δ
6	22.5	peonidin-3-galactoside	243 ± 34			
7	24.4	peonidin-3-glucoside	34 ± 5	n.d.	-	-
8	26.1	peonidin-3-arabinoside	124 ± 15	103 ± 2	4.8 ± 0.1	0.8
9	27.8	malvidin-3-arabinoside	26 ± 1	18 ± 4	0.8 ± 0.2	0.7
10	30.9	myricetin-3-galactoside	112 ± 17	139 ± 7	6.5 ± 0.3	1.2
11	32.7	procyanidin dimmer	91 ± 14	n.d.	-	-
12	35.8	myricetin-3-arabinoside	42 ± 6	40 ± 3	1.8 ± 0.1	0.9
13	37.7	procyanidin dimer	498 ± 64	226 ± 20	11 ± 1	Δ
14	38.2	quercetin-3-galactoside	184 ± 28			
15	41.4	quercetin-3-xylopyranoside	23 ± 3	n.d.	-	-
16	43.2	quercetin-3-arabinopyranoside	19 ± 2	n.d.	-	-
17	43.8	quercetin-3-arabinofuranoside	58 ± 11	32 ± 0	1.5 ± 0.0	0.6
18	46.1	quercetin-3-rhamnoside	18 ± 3	15 ± 0	0.7 ± 0.0	0.8
*		unidentified peaks			12 ± 1	

Quantitative estimates expressed as mean values ± standard error (n=3). Peak numbers and retention times refer to HPLC traces in Figure 4.5. Identification by MS^3 see Table 4.10. n.q.- not quantified; n.d.- not detected; . * the contribution of the non identified peaks at 720 nm to the of antioxidant capacity. Δ not calculated due to coelution of compounds.

4.4. Discussion

Blackcurrant had the highest antioxidant activity followed by raspberries, blueberries, redcurrant and the lowest was cranberries (Table 4.1). Detailed analysis of the compounds in each berry revealed that blackcurrant contains highest levels of anthocyanins with 5446 nmoles/g while cranberries contained 726 nmoles/g and redcurrants 329 nmoles/g (Table 4.12). In blackcurrant, they were responsible for 73% of the total AOC, followed by vitamin C, which contributed 18% (Table 4.12).

Table 4.12. Total content and contribution to the antioxidant capacity of different groups of phenolics detected in berries by HPLC-MS^3.

	Redcurrant	Cranberries	Raspberries	Blueberries	Blackcurrant
Vitamin C	313 (26)	1107 (23)	1014 (11)	115 (0)	2328 (18)
Anthocyanins	329 (28)	726 (32)	885 (16)	4908 (86)	5446 (73)
Ellagitannins	-	-	1353 (58)	-	-
Ellagic acid	-	-	35 (0)		
Epi/catechin	-	1121 (7)	-	-	-
Procyanidins	-	993 (7)	-	-	-
Chlorogenic acid	89 (7)	119 (2)	-	8 (2)	80 (1)
Flavonols	68 (5)	546 (16)	67 (0)	1099 (12)	514 (5)
Unidentified	(33)	(14)	(15)	-	(3)

Values are in nmoles/g and in parenthesis are the percentages of contribution to the antioxidant activity.

Raspberries contained also a relatively lower quantity of anthocyanins (845 nmoles/g) that contribute only 16% of the AOC with 58% being due to the presence of the ellagitannins (ETs), lambertianin C and sanguiin H-6. Beekwilder *et al.* (2005) analyzed antioxidants on-line in extracts of raspberries var. Tulamen and reported similar results, a 17% contribution from anthocyanins and 54% from ellagitannins.

Strawberries, walnuts, pomegranate, oak-aged red wine are also sources of ellagitannins. Both, ellagic acid and ellagitannins are of interest from a dietary point

of view as they have antiviral effects (Fukuchi *et al*., 1989), are anticarcinogenic, induce apoptosis in tumour cells (Rao *et al*., 1991; Stoner and Morse, 1997) and possess antioxidant activity (Kalt *et al*., 1999) among other properties. Ellagic acid and its conjugates in the raspberry extract were present at a total concentration of 42 nmoles/g, which was not sufficient to induce a measurable response in the on-line ABTS antioxidant detector system (Figure 4.3). However if we consider that breakdown of lambertianin and sanguiin H-6 is the primary source of ellagic acid, as a result of acid-induced cleavage, the bioavailable levels of ellagic acid may increase after ingestion, because of beakdown of ellagitannins in stomach and/or metabolism by colonic bacteria.

Lambertianin and sanguiin H-6 both had a TEAR of 2.8, the highest of any of the compounds identified in the five berry extracts. Anthocyanins glycosides had TEAR values ranging from 0.2 for malvidin-6-acetyl-3-glucoside in blueberry to 1.6 for delphinidin-3-galactoside in blueberries and 1.9 for cyanidin-3-arabinoside in cranberries values still lower than ellagitannins. The cellular mechanism of action for the ellagitannins and anthocyanins appears to be different. Ellagitannins have been shown to have *in vitro* vasorelaxing effects, while, in contrast, anthocyanins do not induce vasodilation (Mullen *et al.,* 2002a). However, the high molecular weight of ellagitannins restrict their presence in the bloodstream.

The antioxidant activity of flavonoids is due to the phenolic hydroxyl groups attached to carbon ring structure. Although there is evidence of the importance of antioxidants in conferring stability towards, or protection from, oxidation, the relationship between antioxidant activity and chemical structure is far from clear. The TEAC method used in this study reflects the ability of hydrogen-donating antioxidants to scavenge the $ABTS^{\bullet+}$ radical cation compared with that of the trolox. Rice-Evans *et al*. (1996) reported that the similar Trolox values of quercetin and cyanidin aglycones was due to their structural similarities as they are both hydroxylated at the 3-, 5-, 7-, 3′- and 4′-positions. These investigators reported that glycosylation of the 3-hydroxyl group at on the C-ring reduced antioxidant activity. However, is not supported by the TEAR values obtained in the present study. Blueberry cyanidin-3-arabinoside had a TEAR value of 1.9, raspberry cyanidin-3-rutinoside 0.7, while for quercetin-3-glucoside in redcurrant and raspberry the TEAR was 0.5.

The TEAR values of 2.8 obtained with the raspberry ellagitannins is similar to those reported by Stewart *et al.* (2005) for (–)-epicatechin gallate and (–)-epigallocatechin gallate in green tea, while a lower value of 1.3 was obtained for (–)-epicatechin, which has fewer hydroxyl groups.

Blueberries, like blackcurrant, contain a high concentration of anthocyanins (4908 nmoles/g) and, because the berries lacked vitamin C, anthocyanins are responsible for 86% of the total AOC. Redcurrants and cranberries are good sources of vitamin C, and are low in anthocyanins, which therefore contribute only 29 and 31%, respectively to the total AOC. The vast majority of flavonoids identified in the berries were anthocyanins (delphinidin, cyanidin, malvidin, pelargonidin and peonidin glucosides) with flavonols (quercetin, myricetin and kaempferol glycosides) present in minor amounts. As both types of flavonoids occurred as glycoside conjugates, they would be more water-soluble than their glycones, a property permitting storage in the vacuole *in planta*. Raspberries are an exception to this rule as the main phenolics present were the ellagitannins.

Some flavan-3-ol monomers and procyanidin dimmers, but not higher molecular weight polymeric procyanidins, were identified by HPLC-PDA-MS^3 in blueberry extract (Table 4.10). Reverse phase HPLC is not the most appropriate for the analysis of polymeric proanthocyanidins as they either are retained by the column or elute as a broad unresolved band. According to Gu *et al*. (2004) raspberries contain 0.3 mg/g polymeric proanthocyanidins and cranberries 4.2 mg/g. Special analytical techniques were utilised by these investigators. The analysis of proanthocyanidins comprising up to ten flavan-3-ol units, involved the use of normal phase HPLC with fluorescence/PDA and MS^2 detection. Higher molecular weight procyanidins are also retained by this column so they have to be quantified indirectly and the average degree of polymerisation determined by subjecting samples to degradation by thiolysis prior to analysis by reverse-phase HPLC (Gu *et al.*, 2003). Although polymeric procyanidin standards for normal phase HPLC have been isolated by investigators working for Mars plc in the USA, they are not available to other groups. The fluorescence/absorption response on procyanidins eluting from the normal phase HPLC column change with increasing polymerisation and this complicates accurate quantification. A conversion factor relating the response of the various polymers to that of (–)-epicatechin is available but has not been published.

Although the situation has now changed, at the time this study was carried out the normal phase and thiolysis procedures were not in use in the laboratory and thus could not be utilised.

4.5. Conclusions

The HPLC system with on-line ABTS assay and mass spectrometric analysis used on berry extracts allowed the identification and quantification of the phenolics and their contribution to the total AOC. The phenolic compounds that are present in major quantities and are principally responsible for the antioxidant capacity in blackcurrant and blueberries are the anthocyanins. Blackcurrant contained a large amount of anthocyanins (5446 nmol/g) and also vitamin C (2328 nmol/g) having consequently the major activity of the five berries. Blueberries are the second highest with anthocyanins levels of 4908 nmol/g but contained no vitamin C. Raspberry, redcurrant and cranberries contain anthocyanins but in lower amounts. Ellagitannins like sanguiin H-6 were responsible of 58% of the AOC in raspberries. Flavonols (16%) and vitamin C (23%) are important antioxidants in cranberries while in redcurrants a number of unidentified peaks were the major contributors for the AOC (33%) along with the 28% from anthocyanins. Levels of proanthocyanidins in these berries have to be analyzed in order to evaluate their contribution to the total AOC.

Chapter 5. Evaluation of bioavailability of the major phenolics from raspberries in healthy humans and ileostomy volunteers.

5.1. Introduction

Berries can be a particular interesting source of phenolics to investigate as they have a high antioxidant capacity on a per gram fresh weight basis and are also a rich source of anthocyanins and other phenolic compounds as was discussed in detail in Chapter 4. Anthocyanins have been reported to be strong antioxidants (Rice Evans *et al.,* 1996), to inhibit the growth of cancerous cells (Cooke *et al*., 2005), inhibit inflammation (Seeram *et al*., 2001), and have anti-obesity effects (Tsuda *et al*., 2003).

In view of the fact that anthocyanins have a variety of potential protective effects and that sizable intakes are feasible as part of a normal diet, the bioavailability of anthocyanins becomes an important issue. Compared with other flavonoid groups such as flavonols, relatively little is known about the fate of anthocyanins following ingestion by either humans or animals. The majority of evidence supporting a therapeutic effect of anthocyanins is *in vitro* or mechanistic in nature and there is a lack of *in vivo* evidence from animal or human intervention studies (Donovan *et al*., 2006; Prior and Wu, 2006). Research on the fate of anthocyanins following absorption as discussed in Chapter 1 has shown that a variety of anthocyanins appear in urine after supplementation with berries or berry extracts but the amounts excreted are typically < 0.1% of the ingested dose (McGhie *et al*., 2003; Talavera *et al*., 2005; Felgines *et al*., 2005).

Anthocyanins exhibit complex chemical behaviour *in vitro* and this will result in complex behaviour *in vivo*. At acidic pHs in berries and other foods they are present as a red flavylium cation but the molecular structures present at higher pH, the quinoidal bases, hemiketals and chalcones (Figure 1.14, Chapter 1) may be responsible for the *in vivo* bioactivity observed following ingestion.

Raspberries were chosen in this study for further bioavailability analysis because earlier research had shown that they contained high levels of phenolic antioxidants and were rich in a range of anthocyanins containing 30-50 mg/100 g fresh weight. These compounds and the antioxidant activity were stable when the berries were stored frozen for 12 months or more (Mullen *et al*., 2002a). Also through contacts with Scottish Soft Fruit Grower, who had funded earlier studies in the laboratory, frozen raspberries were readily available in quantity throughout the year and in the summer fresh berries could be obtained in quantity *gratis* from growers near Blairgowrie. As discussed in Chapter 4, raspberries, as well as anthocyanins, also contain substantial amount of the ellagitannins lambertianin C and sanguiin H-6 which are potent antioxidants and potential sources of ellagic acid, an anticancer agent (Mandal and Stoner, 1990; Rao *et al*., 1991; Boukharta *et al*., 1992). Free ellagic acid levels in raspberries are generally low (100 μg/100g) but substantial amounts are detected after acid hydrolysis of extracts as a product of ellagitannin breakdown (Mullen *et al*., 2002a). Lambertianin C and sanguiin H-6 with a molecular weight in excess of 1000 *Da* would appear to be too large to be absorbed into the circulatory system but they could exert protective effect as they pass through the GI-tract.

In this chapter, raspberries were chosen from the berries analysed in the previous chapter for a further intervention study with human subjects. The bioavailability of anthocyanins, ellagitannins and ellagic acid occurring in raspberries will be described. Plasma and urine were collected from six healthy human subjects and plasma, urine and ileal fluid from three volunteers with an ileostomy, were analyzed using HPLC-PDA-MS^2. The samples were taken at a series of time points over a 24 h period after the consumption of 300 g raspberries.

5.2. Materials and Methods

5.2.1. Study design

Six healthy human subjects, (4 females and two male), and three volunteers with an ileostomy (one female, two male) non-smokers and not on medication participated in this study. They were aged between 24 and 46 years with a mean body mass index of 23.5. Three (3) ileostomy subjects volunteered for this study. They all had their

operation at least 5 years before the study and had minimal resection of the small intestine, establishing that they had as close to normal small intestine function as possible. Subjects were required to follow a low flavonoid diet for two days prior to the study, which involved avoiding fruits, vegetables and beverages such as tea, coffee, fruit juices and wine. On the morning of the study after an overnight fast, the volunteers ate 300 g of raspberries (previously defrosted). Ten mL samples of venous blood were collected in heparinised tubes at 0, 1.0, 1.5, 2.0, 3.0, 5.0, 6.5 and 7.5 h post-ingestion. The blood was immediately centrifuged at 4000 *g* for 10 min at 4°C, the plasma was decanted and 1 mL aliquots were acidified to pH 3 with 30 μL of 50% aqueous formic acid. Ascorbic acid (100 μL, 10mM) was added to prevent oxidation. The samples were stored at –80°C prior to analysis. Urine and ileal fluid were collected before and after the feeding over 0-4, 4-7 and 7-24 h periods. After recording the volumes of ileal fluid and urine collected, aliquots were acidified to pH 3 with 50% aqueous formic acid before being stored at –80°C. The study protocol was approved by the Glasgow Royal Infirmary Local Research Ethics Committee.

5.2.2. Materials

The raspberries fruits, *Rubus idaeus* variety Glen Ample used for this study were collected fresh at a farm near Blairgowrie, Angus, Scotland, transported to the laboratory in Glasgow where they were frozen and stored at –20°C. HPLC grade methanol, acetonitrile and ethyl acetate were obtained from Rathburn Chemicals (Walkerburn, Scotland). Formic acid was purchased from Riedel-DeHaen (Seeize, Germany) and acetic acid from BDH (Poole, UK). Cyanidin-3-glucoside, quercetin-3-glucoside, gallic acid standards and sodium diethyldiothiocarbamate were obtained from Sigma-Aldrich (Poole, UK). Ellagic acid was supplied by AASC Ltd. (Southampton, UK). [2-^{14}C]Quercetin-4'-glucoside was available within the group having been synthesized "in house" by Caldwell *et al*. (2002).

5.2.3. Extraction, identification and quantification of phenolics in raspberries. HPLC-PDA-MS^2 analysis

Details were described in Chapter 2 and 4.

5.2.4. Extraction of phenolics from plasma

The method of Day *et al.* (2001) was used to extract plasma. The internal standard used was [2-^{14}C]quercetin-4'-glucoside. Five hundred μL of plasma was added drop wise to 1200 μL of acetonitrile, containing 143000 dpm of the internal standard, to precipitate proteins. The mixture was vortexed for 30 sec every 2 min over a 10 min period and centrifuged at 16,000 *g* at 4°C for 20 min. The pellet was re-extracted with methanol. The supernatants were combined and dried *in vacuo*. Extracts were re-suspended in 25 □L methanol plus 225 □L of 1% formic acid and centrifuged prior analysis by HPLC-PDA-MS^2.

5.2.5. Extraction of phenolics from ileal fluid

Ileal fluid was collected at 0, 4, 7 and 24 h after ingestion and stored at –80°C. To minimize bacterial growth and contamination for the 7-24 h time point, the pouch was emptied and frozen at -20°C every 3 h except when volunteers were asleep. Before extraction, the ileal fluid was defrosted and mixed. Triplicate 0.5 g samples were homogenised in 3 mL of 95% methanol containing 1% formic acid and 20 mM sodium diethyldiothiocarbamate for 1 min using an Ultra-Turrex T25 homogeniser after which they were centrifuged for 20 min at 4000 *g*. The pellets were re-extracted twice and supernatants were pooled before before being reduced to dryness *in vacuo.* The residues were re-suspended in 250 μL of acidified methanol and 4750 μL of 1% formic acid and aliquots were analyzed by HPLC-PDA-MS^2.

5.2.6. Urine analysis

Urine samples were defrosted, vortexed and centrifuged at 13,000 *g* at 4°C for 2 min. Two hundred μL aliquots of the supernatant were analysed by HPLC-PDA-MS^2. For detection of ellagic acid glucuronide, 5 mL of urine was loaded on to a 2 g C_{18} Sep-pak cartridge that was eluted with 10 mL of water followed by 10 mL of ethyl acetate and 10 mL of methanol. The three fractions were reduced to dryness and analyzed by HPLC-PDA-MS^2.

5.2.7. Statistical analysis

Each sample was analysed in triplicate for each volunteer and data were presented as mean values ± standard error (n=6 for volunteers without ileostomy and n=3 for volunteers with an ileostomy).

5.3. Results

5.3.1. Analysis of raspberries

The HPLC-PDA-MS^2 analysis of a raspberry extract detected nine peaks at 520 nm (Figure 5.1A) corresponding to the anthocyanins listed in Table 5.1. The MS^2-based identification of anthocyanins, flavonols, ellagic acid and ellagitannins was in keeping with previous reports on phenolic compounds in raspberries (Mullen *et al.,* 2002a; 2002c and 2003b). The three main anthocyanins were cyanidin-3-sophoroside (peak 1), cyanidin-3-(2^G-glucosyrutinoside) (peak 2) and cyanidin-3-glucoside (peak 3). Other minor cyanidin and pelargonidin conjugates (peaks 4-9) were also identified (Table 5.1). In addition to lambertianin C (peak 12) and sanguiin H-6 (peak 13), previously detected in the raspberries analyzed in chapter 3, this batch of raspberries also contained sanguiin H-10, (peak 11) (Figure 5.1B) which had been previously identified in raspberries by Mullen *et al*. (2003) and in *Sanguisorba officinalis* by Tanaka *et al.* (1993). Peak 10 could not be fully identified but produced a similar MS^2 fragmentation pattern to that of sanguiin H-10. Quercetin conjugates and ellagic acid were monitored at 325 nm (Figure 5.1C). The raspberries contained a total of 681.9 nmoles of anthocyanins/g, 390.5 nmoles of ellagitannins/g of which 73% was sanguiin H-6 (Table 5.2). The total ellagic acid and ellagic acid conjugate content was 75.5 nmoles/g and the total level of quercetin conjugates was 10.3 nmoles/g . A total of 204.6 μmoles of anthocyanins were ingested by the human volunteers along with 117.1 μmoles of ellagitannins, 22.3 of ellagic acid and 3.1 of total flavonols correpondant to 300 g of raspberries (Table 5.3).

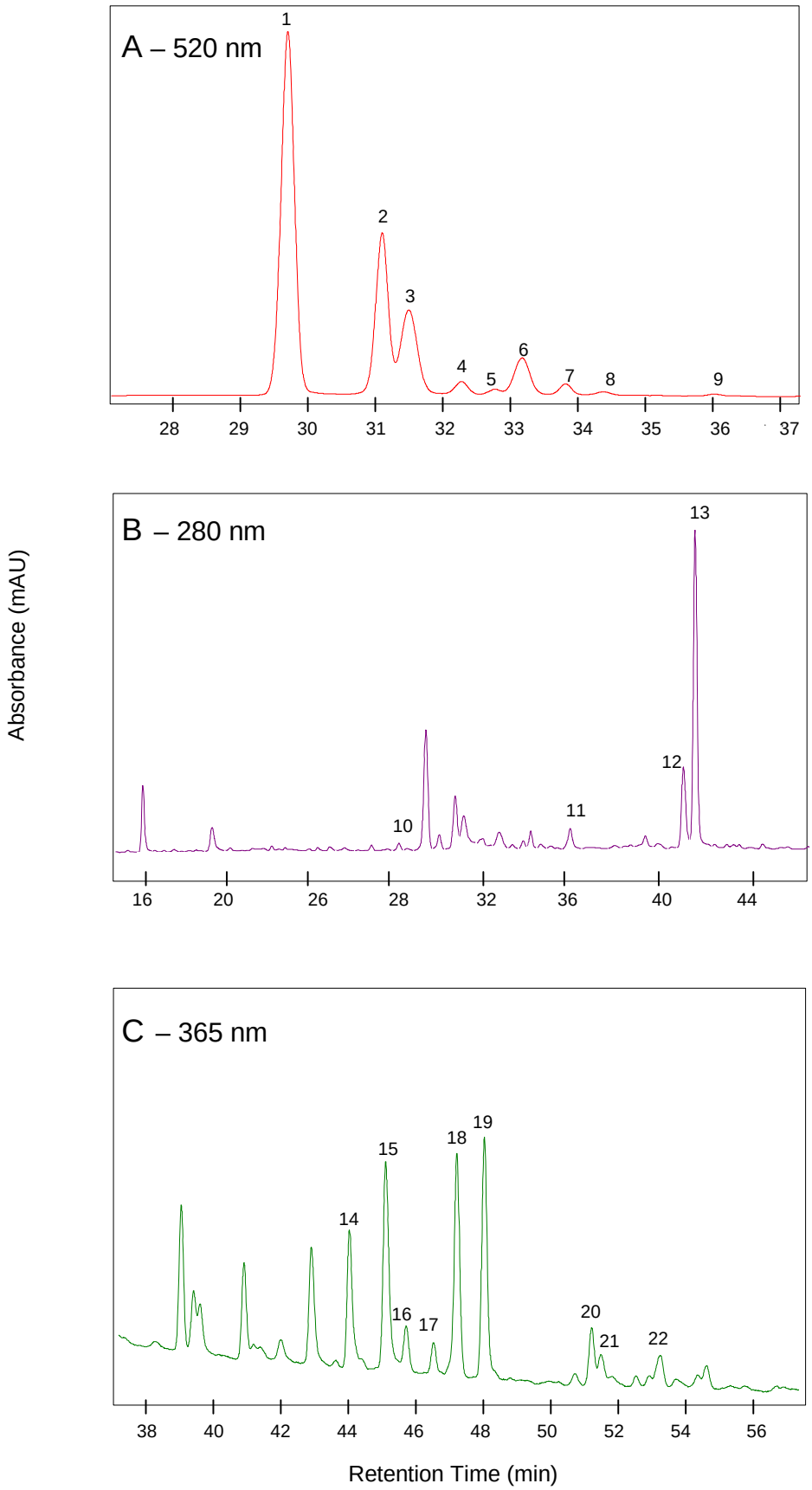


Figure 5.1. Gradient reverse phase HPLC of an extract of Glen Ample raspberries. (A) anthocyanins at 520 nm; (B) ellagitannins at 280 nm and (C) ellagic acid and flavonols at 365 nm. For identification of numbered peaks see Table 5.1.

Table 5.1. Compounds identified in raspberries (*Rubus ideaus* L, variety Glen Ample) by HPLC-MS^{2*}.

Peak	Compound	R_t (min)	$[M-H]^+$ *(m/z)*	MS^2 ions *(m/z)*
1	Cyanidin-3-sophoroside	29.9	611	449, 287
2	Cyanidin-3-(2^G-glucosylrutinoside)	31.3	757	287
3	Cyanidin-3-glucoside	31.8	449	287
4	Pelargonidin-3-sophoroside	32.4	595	271
5	Cyanidin-3-xylosylrutinoside	33.0	727	581, 287
6	Cyanidin-3-rutinoside	33.4	595	287
7	Pelargonidin-3-(2^G-glucosylrutinoside)	34.0	741	595, 271
8	Pelargonidin-3-glucoside	34.4	433	271
9	Pelargonidin-3-rutinoside	36.1	579	271
10	Sanguin H-10 like	28.2	[783]2*	1265, 933, 617
11	Sanguin H-10	36.3	[783]2*	1265, 1103, 933, 63
12	Lambertianin C	41.9	[1401]2*	1250, 935
13	Sanguin H-6	42.2	1869*	1567, 1265, 935
14	Ellagic acid pentose	44.6	433*	301
15	Ellagic acid	45.6	301*	301
16	Quercetin-galactosylrhamnoside	46.1	609*	301
17	Quercetin-3-rutinoside	46.9	609*	301
18	Quercetin-3-glucoside	47.6	463*	301
19	Quercetin glucoronide	48.4	477*	301
20	Methyl ellagic acid pentose	51.5	447*	315
21	Ellagic acid-4-acetylxyloside	51.8	475*	301
22	Ellagic-4-acetylarabinoside	53.7	475*	301

*$[M-H]^+$, positively charged molecular ion. ion; R_t, retention time; *, negatively charged molecular ion.

5.3.2. Identification and quantification of anthocyanins in plasma, urine and ileal fluid

The purpose of the study was to identify anthocyanins and their metabolites in human fluids in order to obtain a better understanding of their bioavailability. Although different methods were tested for recovering anthocyanins from plasma, no anthocyanins were detected at any time point analyzed. Considering that after extraction the recovery of the internal standard was > 90 %, anthocyanins were not present in the plasma samples in quantities greater than the 25 ng/mL plasma limit of detection.

Analysis of extracts of ileal fluid collected 0-4 h after supplementation revealed the presence of all the anthocyanins originally present in the fruit and no metabolites (Table 5.4, Figure 5.2). A 36.5 % overall recovery was obtained between 0-4 h and a further 2.7 and 0.7 % were detected in the 4-7 h and 7-24 h ileal fluid samples respectively for an overall recovery of 81 μmoles which represents almost 40 % of the original intake.

Table 5.2. Compounds identified and quantified in raspberries (*Rubus ideaus* L. variety Glen Ample) by HPLC-PDA-MS^2*.

Compound	nmoles/g	μg/g
Cyanidin-3-sophoroside	375.9 ± 0.2	168.4 ± 0.1
Cyanidin-3-(2^G-glucosylrutinoside)	150.4 ± 0.0	67.2 ± 0.0
Cyanidin-3-glucoside	90.6 ± 0.3	40.6 ± 0.2
Pelargonidin-3-sophoroside	11.6 ± 0.4	5.2 ± 0.2
Cyanidin-3-xylosylrutinoside	4.5 ± 0.0	2.0 ± 0.0
Cyanidin-3-rutinoside	37.1 ± 0.1	16.6 ± 0.1
Pelargonidin-3-(2^G-glucosylrutinoside)	7.8 ± 0.1	3.5 ± 0.0
Pelargonidin-3-glucoside	2.7 ± 0.1	1.2 ± 0.0
Pelargonidin-3-rutinoside	1.8 ± 0.0	0.8 ± 0.0
Total anthocyanins	***681.9 ± 0.9***	***305.5 ± 0.4***
Sanguin H-10 like	7.8 ± 0.4	12.2 ± 0.6
Sanguin H-10	25.9 ± 0.4	40.6 ± 0.6
Lambertianin	56.9 ± 0.1	159.3 ± 0.3
Sanguin H-6	299.9 ± 1.2	560.9 ± 2.2
Total ellagitannins	***390.5 ± 1.9***	***773.0 ± 2.8***
Ellagic acid pentose	30.5 ± 4.6	9.2 ± 1.4
Ellagic acid	26.8 ± 0.7	8.1 ± 0.2
Methyl ellagic acid pentose	3.0 ± 0.0	0.9 ± 0.0
Ellagic acid-4-acetylxyloside	7.3 ± 2.3	2.2 ± 0.7
Ellagic-4-acetylarabinoside	7.9 ± 0.0	2.4 ± 0.0
Total ellagic acid	***75.5 ± 4.9***	***22.8 ± 1.5***
Quercetin-3-galacosylrhamnoside	0.9 ± 0.0	0.4 ± 0.0
Quercetin-3-rutinoside	0.9 ± 0.0	0.4 ± 0.0
Quercetin-3-glucoside	4.3 ± 0.0	2.0 ± 0.0
Quercetin glucoronide	4.3 ± 0.0	2.0 ± 0.0
Total flavonols	***10.3 ± 0.0***	***4.8 ± 0.0***

*Data expressed in μg/g and nmol/g of fresh fruit ± standard error (n=3)

Table 5.3. Amounts of phenolics in 300 g of raspberries (*Rubus ideaus* L. variety Glen Ample) ingested by human volunteers*.

Compound	μmoles	mg
Cyanidin-3-sophoroside	112.8 ± 0.1	50.5 ± 0.0
Cyanidin-3-(2^G-glucosylrutinoside)	45.0 ± 0.0	20.2 ± 0.0
Cyanidin-3-glucoside	27.2 ± 0.1	12.2 ± 0.1
Pelargonidin-3-sophoroside	3.4 ± 0.1	1.5 ± 0.1
Cyanidin-3-xylosiylrutinoside	1.4 ± 0.0	0.6 ± 0.0
Cyanidin-3-rutinoside	11.1 ± 0.0	5.0 ± 0.0
Pelargonidin-3-(2^G-glucosylrutinoside)	2.3 ± 0.0	1.0 ± 0.0
Pelargonidin-3-glucoside	0.8 ± 0.0	0.4 ± 0.0
Pelargonidin-3-rutinoside	0.5 ± 0.0	0.2 ± 0.0
Total anthocyanins	***204.6 ± 0.9***	***91.7 ± 0.4***
Sanguin H-10 like	2.3 ± 0.1	3.7 ± 0.2
Sanguin H-10	7.8 ± 0.1	12.2 ± 0.2
Lambertianin	17.1 ± 0.0	47.8 ± 0.1
Sanguin H-6	89.9 ± 0.3	168.3 ± 0.6
Total ellagitannins	***117.1 ± 0.6***	***232.0 ± 2.8***
Ellagic acid pentose	9.1 ± 1.0	2.8 ± 0.3
Ellagic acid	7.9 ± 0.2	2.4 ± 0.1
Methyl ellagic acid pentose	0.9 ± 0.0	0.3 ± 0.0
Ellagic acid-4-acetylxyloside	2.2 ± 0.5	0.7 ± 0.1
Ellagic-4-acetylarabinoside	2.2 ± 0.2	0.7 ± 0.1
Total ellagic acid	***22.3 ± 4.9***	***6.9 ± 1.5***
Quercetin-3-galacosylrhamnoside	0.3 ± 0.0	0.1 ± 0.0
Quercetin-3-rutinoside	0.2 ± 0.0	0.1 ± 0.0
Quercetin-3-glucoside	1.3 ± 0.0	0.6 ± 0.0
Quercetin glucoronide	1.3 ± 0.0	0.6 ± 0.0
Total flavonols	***3.1 ± 0.0***	***1.4 ± 0.0***

Data expressed in μg/g and nmol/g of fresh fruit ± standard error (n=3)

Table 5.4. Recovery of anthocyanins in ileal fluid collected 0-4 h, 4-7 h and 7-24 h after the consumption of 300 g of raspberries*.

Anthocyanin	0-4 h	4-7 h	7-24 h	Total
Cyanidin-3-sophoroside	40.6 ± 1.4 *(36.0 ± 1.3)*	3.3 ± 1.0 *(3.4 ± 0.4)*	0.4 ± 0.2 *(0.4 ± 0.2)*	44.3 ± 2.2 *(39.8 ± 1.4)*
Cyanidin-3-(2^G-glucosylrutinoside)	22.0 ± 0.6 *(48.8 ± 1.3)*	1.5 ± 0.4 *(3.3 ± 0.8)*	0.2 ± 0.1 *(0.5 ± 0.2)*	23.7 ± 0.9 *(52.6 ± 1.9)*
Cyanidin-3-glucoside	1.5 ± 0.2 *(5.5 ± 0.6)*	0.1 ± 0.0 *(0.5 ± 0.1)*	0.0 ± 0.0 *(0.2 ± 0.2)*	1.7 ± 0.2 *(6.2 ± 0.5)*
Pelargonidin-3-sophoroside	1.9 ± 0.2 *(55.0 ± 6.0)*	0.1 ± 0.0 *(4.1 ± 1.0)*	0.0 ± 0.0 *(0.8 ± 0.8)*	2.1 ± 1.0 *(59.9 ± 6.1)*
Cyanidin-3-xylosylrutnoside	1.2 ± 0.1 *(85.0 ± 7.3)*	0.1 ± 0.0 *(5.6 ± 1.8)*	0.0 ± 0.0 *(1.9 ± 1.9)*	1.3 ± 0.1 *(92.8 ± 7.1)*
Cyanidin-3-rutinoside	5.6 ± 0.2 *(50.5 ± 2.1)*	0.2 ± 0.1 *(1.6 ± 0.8)*	0.1 ± 0.1 *(0.7 ± 0.7)*	5.9 ± 0.4 *(52.8 ± 3.7)*
Pelargonidin-3-(2^G-glucosylrutinoside)	1.1 ± 0.1 *(49.8 ± 4.7)*	0.2 ± 0.1 *(6.1 ± 2.8)*	n.d. -	1.3 ± 0.2 *(55.9 ± 7.5)*
Pelargondin-3-glucoside	0.6 ± 0.0 *(75 ± 2.2)*	0.0 ± 0.0 *(0.0 ± 0.0)*	n.d. -	0.6 ± 0.0 *(75 ± 0.8)*
Total	**74.5 ± 2.6** ***(36.5 ± 1.3)****	**5.5 ± 1.4** ***(2.7 ± 0.7)****	**0.8 ± 0.5** ***(0.4 ± 0.3)****	**81.0 ± 3.5** ***(39.6 ± 1.7)****

*Data presented as means values of anthocyanins in μmoles ± standard error (n=3) and in italicised parentheses as a percentage of the individual amount ingested. * percentage of the total amount ingested.

There was a 6.2 % recovery for cyanidin-3-glucoside compared to values of 39.8 - 92.8 % for the other anthocyanins. This indicates the different susceptibility of conjugated anthocyanins absorption to degradation and metabolism in the GI tract.

Several studies have reported that unmetabolised anthocyanins are recovered in urine (Kay *et al*., 2005; Talavera *et al*., 2005; Wu *et al*., 2005; Felgines *et al*., 2006). In the current investigation, the three main raspberry anthocyanins were detected in their intact unmetabolised form in urine after ingestion of a single dose of 300 g of berries (Figure 5.3). Although other minor 520 nm HPLC peaks were present only the main three main anthocyanins, cyanidin-3-sophoroside, cyanidin-3-(2^G-glucosylrutinoside) and cyanidin-3-glucoside, could be identified by HPLC-PDA-MS^2

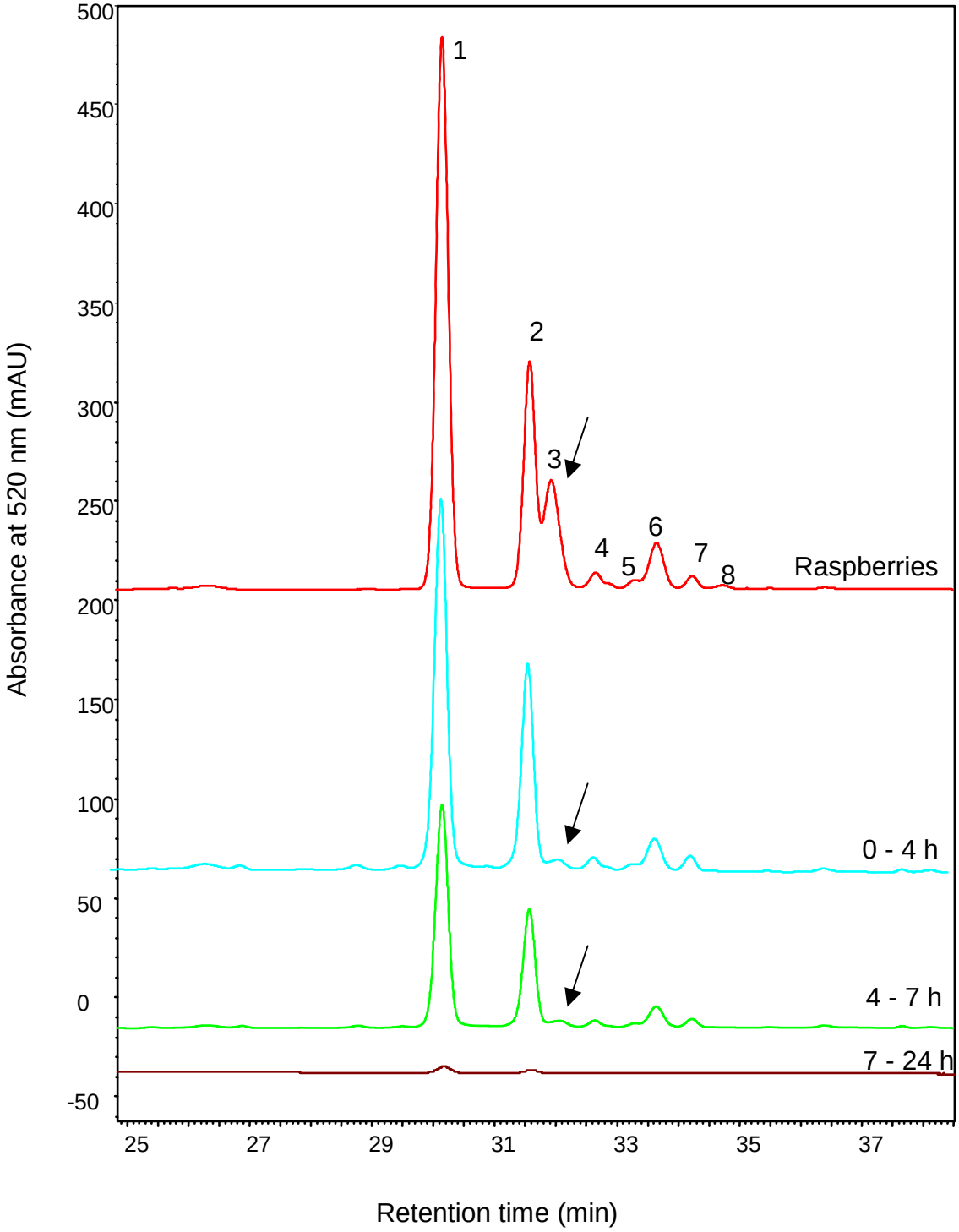


Figure 5.2. Changes on the anthocyanins profile of human ileal fluid extracts at different time points after ingestion of 300 g of raspberries. For numbered peaks see Table 5.4. Cyanidin-3-glucoside is peak 3 which is indicated by an arrow.

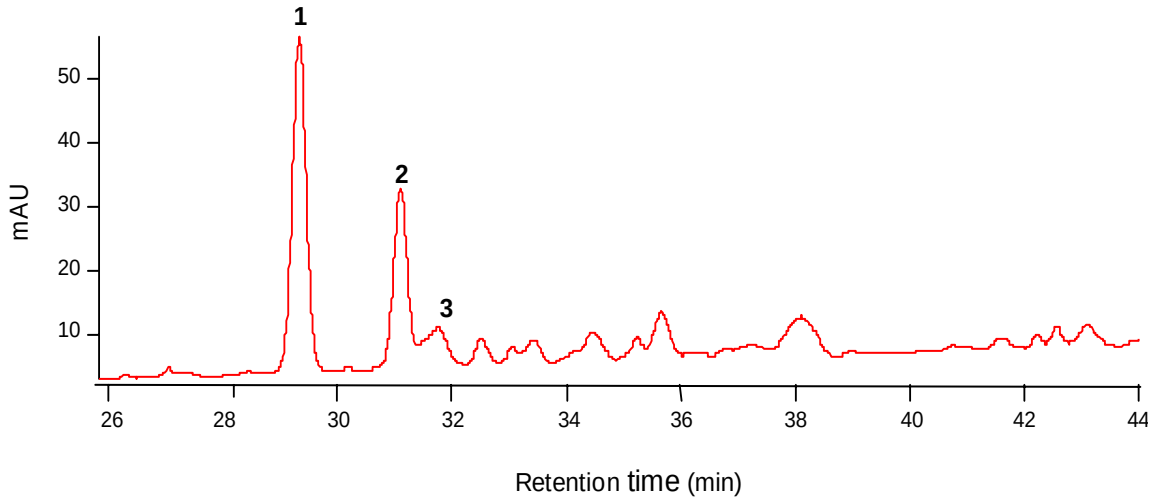


Figure 5.3. Gradient reverse phase HPLC with detection at 520 nm of human urine collected 0-4 h after the ingestion of 300 g of red raspberries. Peak 1 – cyanidin-3-sophoroside, peak 2 – cyanidin-3-(2^G-glucosylrutinoside), peak 3 – cyanidin-3-glucoside.

The total mean urinary anthocyanin for the six healthy volunteers over 24 h was 0.038 % of intake (Table 5.5) with the maximum excretion occurred between 0-4 h. The 0-24 h excretion of cyanidin-3-sophoroside varied among the healthy volunteers from 16.1 to 51.8 nmoles between 0-4 h and 2.6 to 16.3 nmoles from 4-7 h. No anthocyanins were detected in urine collected 7-24 h after ingestion of the raspberries. The total amount of anthocyanins excreted by the individual volunteers ranged from 30.2 to 112.6 nmoles (Table 5.5).

For the subjects without a colon the overall urinary recovery of the three anthocyanins was 0.066% with peak excretion over the 0-4 h collection period. The total amounts excreted over the 24 h collection period varied between individuals from 100.4 to 168.9 nmoles (Table 5.6).

Although the percentages recovered of the individual anthocyanins in urine of healthy volunteers and ileostomy volunteers were very low indeed, the recoveries of cyanidin-3-glucoside were slightly less (0.03 and 0.04%) that those of cyanidin-3-sophoroside (0.04 and 0.07%) and cyanidin-3-(2^G-glucosylrutinoside) (0.06 and 0.09%) (Table 5.7 and 5.8).

Table 5.5. Recovery of anthocyanins in urine collected from six healthy human subjects 0-4 h, 4-7 h and 7-24 h after the consumption of 300 g of raspberries*.

Subject	Anthocyanin		0-4 h	4-7 h	7-24 h	Total
1	C3S		19.7 ± 0.8	3.3 ± 0.2	n.d.	23.0 ± 0.2
	C3GRt		10.2 ± 0.4	2.4 ± 0.1	n.d.	12.6 ± 1.0
	C3G		6.6 ± 0.5	0.6 ± 0.0	n.d.	7.2 ± 0.1
		Total	**36.5 ± 1.2**	**6.4 ± 0.1**	**n.d.**	**42.9 ± 1.2**
		%	***0.018 ± 0.001***	***0.003 ± 0.000***	-	***0.021 ± 0.001***
2	C3S		33.7 ± 1.9	16.2 ± 0.5	n.d.	49.8 ± 1.6
	C3GRt		18.5 ± 0.9	14.5 ± 1.0	n.d.	33.0 ± 1.7
	C3G		8.5 ± 0.2	4.2 ± 0.0	n.d.	12.7 ± 0.1
		Total	**60.7 ± 2.8**	**34.9 ± 1.4**	**n.d.**	**95.5 ± 2.4**
		%	***0.028 ± 0.001***	***0.017 ± 0.000***	-	***0.045 ± 0.000***
3	C3S		35.3 ± 0.7	4.5 ± 0.1	n.d.	39.8 ± 0.9
	C3GRt		17.9 ± 0.5	4.0 ± 0.4	n.d.	21.9 ± 1.3
	C3G		10.6 ± 1.2	1.6 ± 0.1	n.d.	12.2 ± 0.4
		Total	**63.8 ± 1.0**	**10.1 ± 0.5**	**n.d.**	**73.9 ± 1.4**
		%	***0.031 ± 0.001***	***0.005 ± 0.000***	-	***0.036 ± 0.001***
4	C3S		49.6 ± 2.4	12.3 ± 0.2	n.d.	61.9 ± 2.6
	C3GRt		31.2 ± 1.6	9.2 ± 0.1	n.d.	40.4 ± 1.7
	C3G		8.4 ± 0.4	1.8 ± 0.0	n.d.	10.2 ± 0.4
		Total	**89.2 ± 4.4**	**23.2 ± 0.3**	**n.d.**	**112.6 ± 4.64**
		%	***0.044 ± 0.002***	***0.011 ± 0.000***	-	***0.055 ± 0.002***
5	C3S		51.8 ± 1.5	12.1 ± 0.1	n.d.	63.9 ± 1.5
	C3GRt		27.5 ± 1.2	8.6 ± 0.1	n.d.	36.1 ± 1.3
	C3G		9.9 ± 0.1	n.d.	n.d.	9.9 ± 0.1
		Total	**89.2 ± 2.4**	**20.7 ± 0.3**	**n.d.**	**109.9 ± 2.5**
		%	***0.044 ±0.001***	***0.010 ± 0.000***	-	***0.054 ± 0.001***
6	C3S		16.1 ± 0.1	2.6 ± 0.0	n.d.	18.7 ± 0.1
	C3GRt		9.6 ± 0.1	1.8 ± 0.1	n.d.	11.5 ± 0.1
	C3G		n.d.	n.d.	n.d.	0
		Total	**25.7 ± 0.1**	**4.5 ± 0.1**	**n.d.**	**30.2 ± 0.2**
		%	***0.012 ± 0.000***	***0.002 ± 0.000***	-	***0.015 ± 0.000***
Mean	C3S		34.4 ± 5.9	8.5 ± 2.3	n.d.	42.9 ± 7.7
	C3GRt		19.2 ± 3.5	6.8 ± 2.0	n.d.	25.9 ± 5.0
	C3G		7.3 ± 1.5	1.4 ± 0.6	n.d.	8.7 ± 1.9
		Mean	**60.9 ± 10.5**	**16.6 ± 4.7**	**n.d.**	**77.5 ± 13.9**
		%	***0.030 ± 0.005***	***0.008 ± 0.002***	-	***0.038 ± 0.007***

*Data for individual subjects are expressed as nmoles ± standard error (n=3). Figures in italic represent the percentage of the total amount of anthocyanins ingested. C3S = cyanidin-3-sophoroside, C3GRt = cyanidin-3-(2^G-glucosylrutinoside), C3G = cyanidin-3-glucoside. n.d. – not detected.

Table 5.6. Recovery of anthocyanins in urine collected from three human volunteers with an ileostomy 0-4 h, 4-7 h and 7-24 h after consumption of 300 g of raspberries*.

Subject	Anthocyanin	0-4 h	4-7 h	7-24 h	Total
1	C3S	87.5 ± 1.0	12.1 ± 0.7	n.d.	99.6 ± 1.7
	C3GRt	42.3 ± 3.3	8.7 ± 0.4	n.d.	51.0 ± 2.9
	C3G	17.2 ± 0.6	1.2 ± 0.1	n.d.	18.4 ± 0.7
	Total	**147.0 ± 2.9**	**21.9 ± 1.0**	**n.d.**	**168.9 ± 1.9**
	%	***0.072 ± 0.001***	***0.011 ± 0.001***	**-**	***0.083 ± 0.001***
2	C3S	63.7 ± 3.4	19.2 ± 0.8	n.d.	82.9 ± **4**.1
	C3GRt	30.4 ± 2.0	11.8 ± 0.3	n.d.	42.2 ± 1.8
	C3G	8.1 ± 0.7	n.d.	n.d.	8.1 ± 0.7
	Total	**102.1 ± 5.5**	**31.0 ± 0.6**	**n.d.**	**133.2 ± 5.9**
	%	***0.050 ± 0.003***	***0.015 ± 0.000***	**-**	***0.065 ± 0.003***
3	C3S	56.1 ± 1.3	7.1 ± 0.1	n.d.	31.3 ± 0.9
	C3GRt	27.1 ± 0.1	4.4 ± 0.1	n.d.	26.4 ± 1.3
	C3G	5.7 ± 0.5	n.d.	n.d.	5.7 ± 0.5
	Total	**88.9 ± 1.4**	**11.5 ± 0.2**	**n.d.**	**100.4 ± 1.6**
	%	***0.043 ± 0.001***	***0.006 ± 0.000***	**-**	***0.049 ± 0.001***
Mean	C3S	69.1± 6.6	12.8 ± 2.4	n.d.	81.9 ± 7.3
	C3GRt	33.3 ± 3.2	8.3 ± 1.5	n.d.	41.6 ± 3.9
	C3G	10.3 ± 2.4	0.4 ± 0.3	n.d.	10.7 ± 2.7
	Mean	**112.7 ± 12.2**	**21.5 ± 3.9**	**n.d.**	**134.2 ± 13.7**
	%	***0.055 ± 0.012***	***0.011 ± 0.019***	**-**	***0.066 ± 0.001***

*Data for individual subjects are expressed as nmoles ± standard error (n=3). Figures in italic represent the percentage of the total amount of anthocyanins ingested. C3S = cyanidin-3- sophoroside, C3GRt = cyanidin-3-2 G-glucosylrutinoside, C3G = cyanidin-3-glucoside. n.d. – not detected.

Table 5.7. Recovery of individual anthocyanins in urine collected from healthy human volunteers 0-4 h, 4-8 h and 8-24 h after the ingestion of 300 g of raspberries*.

Anthocyanin	0-4 h	4-7 h	7-24 h	Total
Cyanidin-3-sophoroside	34.4 ± 5.9 *(0.03 ± 0.01)*	8.5 ± 2.3 *(0.01 ± 0.00)*	n.d. -	42.9 ± 7.61 *(0.04 ± 0.01)*
Cyanidin-3-(2^G-glucosylrutinoside)	19.2 ± 3.5 *(0.04 ± 0.01)*	6.8 ± 2.0 *(0.02 ± 0.00)*	n.d. -	25.9 ± 4.6 *(0.06 ± 0.01)*
Cyanidin-3-glucoside	7.3 ± 1.5 *(0.03 ± 0.01)*	1.4 ± 0.6 *(0.01 ± 0.00)*	n.d. -	8.7 ± 1.9 *(0.03 ± 0.01)*

*Data presented as means values in nmoles ± standard error (n=6 volunteers) and in italicised parentheses as a percentage of the individual amounts of anthocyanins ingested. n.d. – not detected.

Table 5.8. Recovery of individual anthocyanins from urine of volunteers with ileostomy 0-4 h, 4-8 h and 8-24 h after the ingestion of 300 g of raspberries*.

Anthocyanin	0-4 h	4-7 h	7-24 h	Total
Cyanidin-3-sophoroside	69.1 ± 6.6 *(0.06 ± 0.01)*	12.8 ± 2.4 *(0.01 ± 0.00)*	n.d	81.9 ± 7.3 *(0.07 ± 0.01)*
Cyanidin-3-(2^G-glucosylrutinoside)	33.3 ± 3.2 *(0.07 ± 0.01)*	8.3 ± 1.5 *(0.02 ± 0.00)*	n.d	41.6 ± 3.9 *(0.09 ± 0.01)*
Cyanidin-3-glucoside	10.3 ± 2.4 *(0.04 ± 0.01)*	0.4 ± 0.3 *(0.00 ± 0.00)*	n.d	10.7 ± 2.7 *(0.04 ± 0.01)*

*Data presented as means values in nmoles ± standard error (n=3 volunteers) and in italicised parentheses as a percentage of the individual amounts of anthocyanins ingested. n.d. – not detected.

5.3.3. Identification and quantification of ellagic acid and ellagitannins in plasma, urine and ileal fluid of healthy and ileostomy volunteers

Neither ellagic acid, gallic acid, ellagitannins nor their putative metabolites were detected in plasma collected from healthy and ileostomized volunteers after ingestion of 300 g of raspberries. However, ellagic acid and ellagic acid glucuronide were detected in urine from both groups (Figure 5.4).

This was achieved when samples were fractionated on a C_{18} Sep-Pak prior to analysis by HPLC-PDA-MS^2 using both selective reaction monitoring and in-source fragmentation. Urine collected from the six healthy volunteers 0-4 h after ingestion contained 13.1 nmoles of ellagic acid and its glucuronide conjugate corresponding to 0.2% of the ellagic acid ingested (Table 5.9). For the ileostomy volunteers the 33.6 nmoles excreted represents a 0.4% recovery (Table 5.10). More than 60% of the total ellagic acid excreted was in the non-conjugated form.

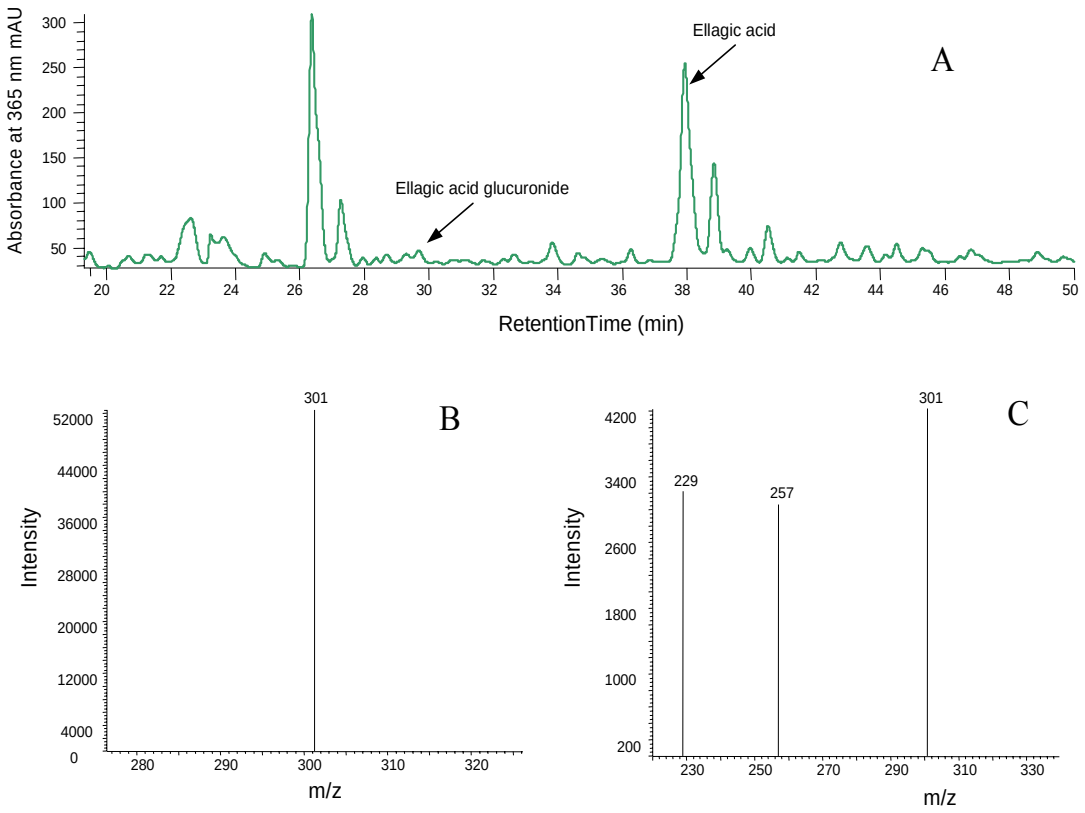


Figure 5.4. (A) HPLC-PDA-MS^2 analysis at 325 nm of C_{18} Sep-pak purified urine from a healthy volunteer collected 0-4 h after ingesting 300 g of raspberries. (B) Fragmentation of the *m/z* 477 $[M-H]^-$ in the 29.9 min HPLC peak. The *m/z* 301 fragment ion corresponds to ellagic acid and the 176 *amu* loss equates with cleavage of glucuronic acid, indicating that the peak is an ellagic acid glucuronide conjugate. (C) Fragmentation of the 38.2 min HPLC peak produces a spectrum indicative of the presence of ellagic acid with a $[M-H]^-$ at *m/z* 301 and additional ions at *m/z* 257 and 229.

Table 5.9. Recovery of ellagic acid and metabolites from urine collected from human volunteers 0-4 h, 4-7 h and 7-24 h after the consumption of 300 g raspberries***.**

Compound	0-4 h	4-7 h	7-24 h	Total
Ellagic acid	6.7 ± 1.6 *(0.1 ± 0.0)*	1.2 ± 0.4 *(0.0 ± 0.0)*	n.d. -	7.9 ± 1.4 *(0.1 ± 0.0)*
Ellagic acid glucuronide	5.2 ± 1.1 *(0.1 ± 0.0)*	n.d	n.d. -	5.2 ± 1.1 *(0.1 ± 0.0)*
Total	**11.9 ± 2.2** ***(0.2 ± 0.0)***	**1.2 ± 0.4** ***(0.0 ± 0.0)***	**n.d.** -	**13.1 ± 0.2** ***(0.2 ± 0.0)***

*****Data presented as means values in nmoles ± standard error (n=6 volunteers) and in italicised parentheses as a percentage of the total amounts of ellagic acid ingested. . n.d. – not detected.

Table 5.10. Recovery of ellagic acid and metabolites from urine collected from volunteers with an ileostomy 0-4 h, 4-7 h and 7-24 h after consumption of 300 g of raspberries*.

Compound	0-4 h	4-7 h	7-24 h	Total
Ellagic acid	25.0 ± 4.5 *(0.3 ± 0.1)*	0.9 ± 0.2 *(0.0 ± 0.0)*	n.d. -	25.8 ± 4.6 *(0.3 ± 0.1)*
Ellagic acid glucuronide	7.8 ± 3.4 *(0.2 ± 0.0)*	n.d	n.d. -	7.8 ± 3.4 *(0.2 ± 0.0)*
Total	**32.8 ± 7.5** ***(0.4 ± 0.1)***	**0.9 ± 0.2** ***(0.0 ± 0.0)***	**n.d.** **-**	**33.6 ± 7.5** ***(0.4 ± 0.1)***

*Data presented as means values in nmoles ± standard error (n=3 volunteers) and in italicised parentheses as a percentage of the total amounts of ellagic acid ingested. n.d. – not detected.

Analysis of ileal fluid revealed a 26.2% recovery of sanguiin H-6 over a 24 h period but the other ellagitannins, lambertianin C and sanguiin H-10 were not present in detectable amounts (Table 5.11). Ellagic acid, but no ellagic acid sugar conjugates, was also detected in ileal fluid. The 19 μmoles of ellagic acid that were recovered over the 24 h period corresponds to 239.4% intake (Table 5.11).

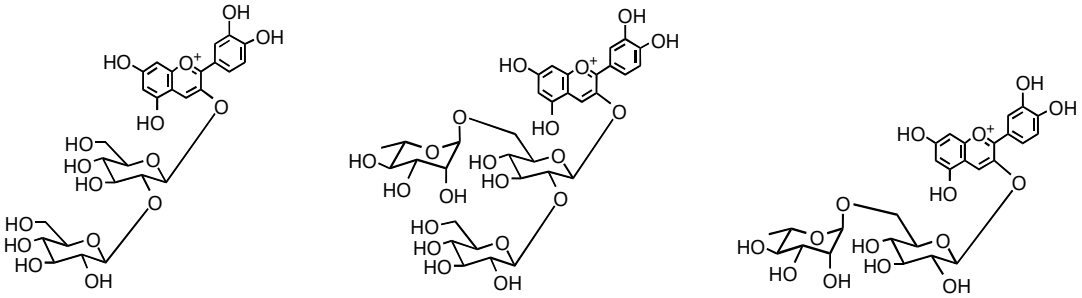
Table 5.11. Recovery of ellagic acid, sanguiin H-6, lambertianin C and sanguiin H-10 from ileal fluid of volunteers collected at 0-4 h, 4-7 h and 7-24 h after the consumption of 300 g of raspberries*.

	0-4 h	4-7 h	7-24 h	Total
Ellagic acid	12.9 ± 3.3 *(162.9 ± 41.2)*	4.3 ± 0.2 *(53.6 ± 3.0)*	1.8 ± 0.5 *(22.9 ± 6.0)*	19.0 ± 3.0 *(239.4 ± 37.9)*
Sanguiin H-6	16.3 ± 0.4 *(18.1 ± 0.5)*	5.1 ± 0.7 *(5.7 ± 0.8)*	2.1 ± 2.1 *(2.32 ± 0.2)*	23.5 ± 0.9 *(26.2 ± 1.0)*
Lambertianin C	n.d.	n.d.	n.d.	n.d.
Sanguiin H-10	n.d.	n.d.	n.d.	n.d.

*Data presented as means values in μmoles ± standard error (n=3 volunteers) and in italicised parentheses as a percentage of the total amount ingested. . n.d. – not detected.

5.4. Discussion

The analysis of raspberries revealed the presence of a disaccharide conjugate of cyanidin, cyanidin-3-sophoroside, which accounted for more than 55% of the total anthocyanin content. The other two main anthocyanins were the trisaccharide conjugate, cyanidin-3-(2^G-glucosylrutinoside) and the monosaccharide cyanidin-3-glucoside (Fig. 5.5), which accounted for 22% and 13%, respectively, of the anthocyanins. The other six cyanidin and pelargonidin conjugates were present in much lower amounts (Fig. 5.1, Table 5.2). The 300 g of raspberries ingested by the human volunteers in this study was equivalent to 4.6 portions of berries as mentioned in Chapter 3 and contained a total of 204.6 µmoles of anthocyanins (Table 5.3).



Cyanidin-3-*O*-sophoroside | Cyanidin-3-*O*-(2^G-*O*-glucosylrutinoside) | Cyanidin-3-*O*-rutinoside

Figure 5.5. Structures of the three main anthocyanins in raspberries.

No anthocyanins were detected in plasma at any time point over the 24 h collection period. The trace quantities of anthocyanins excreted in urine were in their native, unmetabolised form. The level of the three main anthocyanins excreted 0-4 h after supplementation was 60.9 ± 10.5 nmoles which represents a mere 0.03 % of intake. A further 16.6 nmoles (0.008%) were excreted between 4 and 7 h and no anthocyanins were detected in the 7-24 h urine. The total 0-24 h anthocyanin excretion, therefore, was 77.5 nmoles which represents a recovery of 0.038 % (Table 5.5). The urinary excretion of anthocyanins by ileostomy volunteers was slightly higher but still low, reaching 134.2 nmoles after 24 h, which corresponds to 0.066% of the amount consumed (Table 5.6). No anthocyanins, or their glucuronide or sulphate metabolites were detected at any time point for any of the volunteers.

The albeit low but seemingly higher urinary excretion of anthocyanins by the ileostomy volunteers implies that the presence of a colon lowers the absorption of anthocyanins. Absorption of anthocyanins in the stomach has been proposed by Passamonti *et al*. (2002) and Talavera *et al*. (2004), but it is difficult to see how this could account for the differential levels of excretion. It is possible that it is a consequence of a slower gastric transit time, which increases absorption in the small intestine of the volunteers with an ileostomy. No references could be found in the literature comparing gastric transit times for healthy subjects and those with an ileostomy. However, there are person to person variations in the level of urinary excretion of phenolics and it is likely probable that a larger number of ileostomy volunteers would have reflected a more closer and similar levels between the two groups.

Most studies have shown that urinary excretion of anthocyanins is very low, <0.1% of intake following the ingestion of foods or extracts (Matsumoto *et al.,* 2001; Wu *et al*., 2002; Nielsen *et al*., 2003). This indicates a poor absorption and excretion of these compounds in rats, human or pigs compared with other polyphenols (Manach *et al*., 2005). However, Felgines *et al*. (2002) and Mullen *et al*. (2008) who fed strawberries and Ohnishi *et al*. (2006) who fed a cranberry supplement to humans, have reported urinary excretion of anthocyanins of 1-5%, still low but substantially higher than what has been observed in other studies. These differences can have various explanations.

It is possible that some anthocyanins are more readily absorbed than others. The available data suggest that the absorption and excretion of anthocyanins is influenced by the nature not only of the sugar moiety but also of the aglycone (McGhie *et al*., 2003; Wu *et al*., 2005). Pelargonidin-3-glucoside from strawberry is better absorbed than other anthocyanins being excreted in urine, principally as a pelargonidin glucuronide metabolite, in quantities equivalent to ca. 1.8% of intake (Felgines *et al*., 2002; Mullen *et al*., 2007). In contrast, feeding studies with blackberries indicate that cyanidin-3-glucoside, the 3'-hydroxy derivative of pelargonidin-3-glucoside, is much less readily absorbed as it appears in urine in only trace amounts corresponding to <0.1% of intake (Felgines *et al*., 2005).

Ohnishi *et al*. (2006) speculated that their high recovery of anthocyanins in urine (5% of intake) was due to the absorption of cranberry anthocyanins being

accelerated by other components in the fruit. However, neither the potential compounds nor the mechanism involved was discussed. There are reports on the effect of the other components on the food like proteins and carbohydrates on the absorption/excretion of anthocyanins by humans (Bub *et al*., 2001; Frank *et al*., 2003; Mullen *et al.,* 2008) but there is nothing in the literature on other phytochemicals in fruit having an impact on anthocyanin absorption. Other analytical issues have been reported as possible contributors to the different levels of anthocyanin recoveries. Felgines *et al*. (2002) and Mullen *et al*. (2008) emphasize that in feeding studies with strawberries, analysis has to be carried out as soon as possible after collection of samples as pelargonidin glucuronide in urine decreases and can even disappear during storage at –80^{o}C. A study has been carried in our laboratory on the stability of raspberry anthocyanins in urine stored at –80^{o}C for up to 60 days. However, no change in anthocyanin content was detected (W. Mullen, unpublished data).

With hindsight, it is evident that the more heterogeneous anthocyanin profile of raspberries, blackcurrants (Wu *et al.,* 2004), blueberries (Cho *et al.,* 2004) and many fruits used in feeding studies the more difficult to explain the results obtained in any detail. In this regard, strawberries and blackberries are better material as they contain one principal anthocyanin, pelargonidin-3-glucoside and cyanidin-3-glucoside, respectively, and studies with these berries clearly demonstrate that pelargonidin-3-glucoside is absorbed more readily than cyanidin-3-glucoside (Felgines *et al*., 2002 and 2005; Mullen *et al*., 2008).

Although the overall levels of raspberry anthocyanins in urine was low, examination of the data obtained with individual anthocyanins shows that there was a much lower recovery of cyanidin-3-glucoside than cyanidin-3-sophoroside and cyanidin-3-(2^{G}-glucosylrutinoside) with both healthy subjects and ileostomy volunteers (Tables 5.7 and 5.8). The comparatively low recovery of cyanidin-3-glucoside was also evident in the studies with ileal fluid where there was an overall anthocyanin recovery of 48% compared with 6.2% for cyanidin-3-glucoside (Table 5.4, Figure 5.3). The loss of cyanidin-3-glucoside from ileal fluid is unlikely to be due to preferential absorption as the data of Felgines *et al.* (2005) with blackberries indicate that it is poorly absorbed, at least in rats. It may therefore be more prone to breakdown, to as yet undetected products, in the stomach and/or small intestine. The

data obtained with ileal fluid suggests that other anthocyanins are less susceptible to such breakdown and pass intact, in substantial amounts, from the small to the large intestine. This would certainly appear to be the case with the trisaccharide, cyanidin-3-xylosylrutinoside, for which there was a 92.8% recovery in ileal fluid (Table 5.4).

The fate of the sizable quantities of raspberry anthocyanins that enter the large intestine could be studied by *in vitro* incubations with faecal slurries with the formation of phenolic acids being monitored. The data obtained could then be compared with the urinary excretion of phenolic acid after raspberry ingestion as 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. Time constraints prevented the analysis of phenolic acids in urine as potential indicators of degradation of anthocyanins by colonic bacteria in the present study. However, an appropriate GC-MS protocol has now been established and was used in the study described in Chapter 6 where the bioavailability of raspberry anthocyanins in rats was investigated (Borges *et al*., 2007). A number of phenolics were identified and quantified in urine as potential breakdown products of anthocyanins but firm conclusions could not be drawn because of the high background level of phenolic acids in the urine collected from control rats that did not receive the raspberry supplement. Rather than increasing the intake of anthocyanins by feeding concentrated extracts in amounts that would be well in excess of normal dietary consumption, an alternative approach would be to feed radiolabelled anthocyanins to rats in the same way that [^{14}C]quercetin-4'-glucoside has been used to investigate flavonol metabolism, absorption and bioavailability (Mullen *et al.,* 2003a; Graf *et al.,* 2005).

In vivo studies have shown anthocyanin localization in rat's peripheral tissues including brain, liver, kidney, and intestinal regions (Talavera *et al*., 2005). *In vitro* studies of endothelial cells incubated with anthocyanins from elderberry extract have shown incorporation into the membrane and cytosol of the cells, where they enhanced resistance to oxidative stress (Youdin *et al*., 2000). Uptake appeared to be structure dependent with monoglycosides concentrations higher than di-glucosides in both the cytosol and the membrane. Although these are results from a cell model, and the extrapolation to *in vivo* conditions is not straight forward, the information

may be of value in helping to understand the fate of anthocyanins in the body and delineate some of the protective activities observed in human studies.

Research on the bioavailability of anthocyanins has been carried using different animal models (rats, pigs, humans) or different sources of anthocyanins, including fresh fruit and purified extracts with or without other foods. Different methods have been used to introduce the anthocyanins into the body such as perfusion, injection and gavage using liquids and solids in single or multiple doses, which in humans have varied from 1.4 - 960 μmoles. The doses used in some animal studies, when calculated in human equivalents, are clearly well in excess of what could possibly be consumed in a single dietary supplement. For example, when El Mohsen *et al*. (2006) injected pelargonidin into rats at a dose of 50 mg/kg, which is equivalent to a 70 kg human ingesting *ca.* 14 kg of strawberries and all the native anthocyanins being absorbed into the bloodstream. The data obtained have, therefore, limited physiological relevance and a further complication is the fact that pelargonidin occurs in plant foods as a variety of sugar conjugates not the aglycone that was injected to the rats.

The other important phenolic compounds present in raspberries are ellagic acid and ellagitannins. The health benefits of raspberries are often promoted on the basis of high ellagic acid content (Mandal and Stoner, 1990; Rao *et al*., 1991; Boukharta *et al*., 1992). However, the current study showed that the levels of ellagic acid and conjugates are low, 75.5 nmoles/g compared to 390.5 nmoles/g of ellagitannins, mainly in the form of sanguiin H-6 and lambertianin C (Figure 1.9, Chapter 1). Potential cardiovascular protective effects of ellagitannins have been reported as vasorelaxation assays on rabbit aorta found that sanguiin H-6 and lambertianin C, were the active components of raspberry extracts responsible for vasorelaxation activity (Mullen *et al*., 2002c). The molecular weight of these compounds is in excess of 1000 *Da* which would appear to preclude the possibility of them crossing intact to the circulatory system. However, they could have an impact on the health of the GI tract and their potential breakdown products where ellagic acid, gallic acid and other phenolic acids might be absorbed and exert also protective effects.

In order to obtain information on the bioavailability of ellagic acid and ellagitannins in humans their presence in plasma, urine and ileal fluid was

investigated. No intact or conjugated forms of ellagitannins, ellagic acid or gallic acid were detected in plasma of human volunteers at any time after ingestion of 300 g of raspberries. However, ellagic acid and ellagic acid glucuronide were detected in urine (Figure 5.4). The levels excreted 0-4 h after ingestion was 11.9 nmoles (0.2%) for healthy subjects and 32.8 nmoles (0.4%) for ileostomy volunteers (Tables 5.9 and 5.10). No further significant excretion occurred after 4 h. As with anthocyanins, higher urinary recoveries of ellagic acid and ellagic acid glucuronide were obtained with the ileostomy volunteers. The metabolite 3,8-dihydroxy-6H[b,d]pyran-6-one (urolithin B) (Figure 1.16, Chapter 1) was not detected in urine or plasma. This metabolite has been previously reported as a colonic bacterial metabolite of ellagic acid in rats by Doyle and Griffith (1980). Cerdá *et al*. (2004) also found urolithin B in human urine collected after volunteers began ingesting 1 L of pomegranate juice per day for a period of 5 days. Pomegranate juice contains the ellagitannin punicagalin. Urolithin B was first detected in urine, in the free form and as a glucuronide, 24 h after feeding began. Levels peaked after 3-4 days, but there was a high inter-individual variability with the amounts of urolithin B excreted corresponding to 1-50% of punicalin intake. In contrast to urolithin, ellagic acid and punicagalin were not detected in any of the urine samples. Cerdá *et al.* (2004) suggest that the colonic microflora may need time to adapt in order to be able to metabolize punicalin to urolithin. In keeping with this possibility, Cerdá *et al.* (2005) fed humans 225 g of raspberries in a single dose and detected urolithin B and its glucuronide in human urine, in quantities of ellagitannin intake equivalent to 0.2% after 40 h to 7.6% after 3 days of the ingestion of 225 g of raspberries. This delay could explain why urinary urolithin B was not detected in the present study.

In a further study with pomegranate, another research group detected urolithin B glucuronide in the urine of 3 of 18 subjects 24 h after feeding 180 mL of juice and in the urine of 5 of 18 volunteers after 48 h (Seeram *et al.,* 2006). These investigators, unlike Cerdá *et al*. (2004) detected ellagic acid in plasma (T_{max} = 0.98 + 0.06 h, C_{max} = 0.06 + 0.01 mM, $T_{1/2}$ = 0.71 + 0.09 h).

The fate of ellagitannins following absorption requires further more detailed study covering, among other topics, the systemic effects of urolithin B and similar metabolites and the nature of the inter-individual differences which may be a consequence of differences in colonic microflora which are evident when the

degradation of quercetin-3-rutinoside in the large intestine was investigated (Jaganath *et al.,* 2006).

The only ellagitannin in ileal fluid collected 0-4 h after ingestion of raspberries was sanguiin H-6 in amounts corresponding to 18.1 % of intake. A further *ca.* 9% was detected in the 4-7 h and 7-24 h samples making a total recovery of 259 μmoles (26.2%) (Table 6.11). Over the 24 h collection period, the ellagic acid recovered in ileal fluid was 239.4% of intake. This indicates a possible breakdown of the ellagitannins in the stomach and/or small intestine releasing ellagic acid. In theory each mole of lambertianin C can breakdown yielding 6 moles of ellagic acid and 3 moles of gallic acid whilst sanguiin H-6 can produce 4 moles of ellagic acid and one mole gallic acid. The increased quantity of ellagic acid in ileal fluid would be released by a 5.3% degradation of sanguiin H-6 when in reality there was a 73.8% loss. Ellagitannin breakdown in the stomach and small intestine could release ellagic acid, which is absorbed into the circulatory system and so does not appear in ileal fluid. The excretion of only trace quantities of ellagic acid and its glucuronide in urine (Table 5.9) indicates this is an unlikely occurrence. This is in keeping with the data of Daniel *et al.* (1991) who monitored ellagic acid levels in raspberry extracts incubated at different pHs, with digestive enzymes and small intestine and caecum contents from rats. Boukharta *et al.* (1992) and Stoner *et al.* (2005) reported that the uptake of ellagic acid into plasma from ellagitannins is minimal in animals. Whitley *et al.* (2003) and Seeram *et al.* (2004) suggest the poor absorption of ellagic acid can be explained by its poor solubility in water, its ionization at physiological pH to form poorly soluble complexes with calcium and magnesium ions in the intestine, and its ability to bind to epithelium.

The chemo preventive value of dietary anthocyanins, ellagitannins and ellagic acid contained in raspberries is likely to depend not only on their absorption and systemic effects but also their impact on the lumen of the GIT during the transit. In addition to antioxidant activity, other potential mechanisms of cancer chemoprevention, such as induction of apoptosis in tumour cells and the endothelial cell as a target for cardiovascular disease in humans, need to be investigated. Raspberries may prevent oesophageal cancer (Kresty *et al*., 2001), colon adenomas in rats (Harris *et al*., 2001) and reduce oral cancer in hamsters (Casto *et al*., 2002). They also may prevent fatty acid streak being deposited in the arterial wall of

hamsters which develop atherosclerosis in a similar manner to humans (Rouanet *et al.,* 2008). Exactly what compounds are absorbed into the circulatory system after ingestion to raspberries to exert this key protective effect is as yet unclear. The current studies with humans and rats (Chapter 6) have revealed that, at best, only trace quantities of anthocyanins and ellagic acid derivatives enter the bloodstream. Possibly, phenolic acids, produced by degradation of anthocyanins and ellagitannins in the colon, may have a much larger impact on cardiovascular health than is currently realised. They are not readily analysed by HPLC-PDA-MS^2 and so were not detected in plasma in the current study. However, as mentioned previously more sensitive GC-MS procedures have now been developed and could be used for further study in this area.

5.5. Conclusions

In this study, the absorption of raspberry anthocyanins and ellagitannins into the human circulatory system and their excretion in urine was investigated along with the fate of unabsorbed material passing from the small to the large intestine. Six subjects healthy subjects with an intact colon (*H*) and three subjects with an ileostomy (*IL*) consumed 300 g of raspberries containing 204.6 μmoles of anthocyanins and 117.1 μmoles of ellagitannins. Samples of plasma, urine and ileal fluid were collected over a 0-24 h period after ingestion. No anthocyanins, ellagitannins or their metabolites or breakdown products were detected in the plasma of any of the volunteers. Eight of the anthocyanins identified in raspberries were detected and quantified in ileal fluid in their native form by HPLC-PDA-MS^2. They reached a maximum level of 36.5% of intake in samples collected 0-4 h after supplementation and after 24 h there was an overall anthocyanin recovery of 39.6%. The recovery of individual anthocyanins varied with 92.8% for cyanidin-3-xylosylrutinoside to 6.2% for cyanidin-3-glucoside. Only the three main raspberry anthocyanins were present in detectable quantities in urine with recoveries of 42.9 nmoles for *H* and 81.9 nmoles for *IL* of cyanidin-3-sophoroside, 25.9 nmoles for *H* and 41.6 nmoles for *IL* with cyanidin-3-(^{2}glucosylrutinoside) and 8.7 nmoles for *H* and 10.7 nmoles for *IL* with cyanidin-3-glucoside. The overall urinary excretion of anthocyanins was 0.038% and 0.066% of intake for *H* and *IL* respectively. Urinary

excretion of anthocyanins occurred principally 0-4 h after ingestion of the raspberries.

With regard to ellagitannins, 16.3 μmoles of sanguiin H-6, was detected in ileal fluid after 0-4 h with 26.2% of intake being detected over the 0-24 h collection period. No lambertianin or sanguiin H-10 were found. The levels of ellagic acid in 0-24 h ileal fluid corresponded to 239.4% of intake 24 h with 162.9% being collected 0-4 h after ingestion. This coincided with the peak levels of ellagic acid in urine although the levels, 13.1 nmoles and 33.6 nmoles for *H* and *IL* respectively, were low and equivalent to no more that 0.4% of intake. No ellagic acid or metabolites were detected in plasma at any point.

This study, therefore, found a low absorption and excretion of anthocyanins and ellagic acid in human subjects. The *ca*. 40% recovery of these compounds in ileal fluid indicates that in healthy subjects with a colon substantial quantities pass from the small to the large intestine where they will be catabolised by the gut microflora.

Chapter 6. Evaluation of bioavailability of the major phenolics from raspberries in a rat model.

6.1. Introduction

The purpose of studies on the bioavailability of a nutrient is to obtain information on the proportion of a bioactive compound that is absorbed in the gastrointestinal tract including the metabolism, excretion and distribution in the circulatory system, organs, and tissues (McGhie and Walton, 2007). If anthocyanins are responsible for health effects, tissue distribution of the parent anthocyanin and its metabolites becomes important in understanding the mechanism underlying the effects. Human studies produce limited information on organs and tissues distribution and that is why animal models are used. As it was discussed in the previous chapters, anthocyanins have shown a rapid and apparent high absorption from the stomach and small intestine (Talavera *et al*., 2003 and 2004) but questions on their disposition once they are absorbed still remains. Studies with rats have shown that anthocyanins reach the brain (Joseph *et al*., 1999; Andres-LaCueva *et al*., 2005; Passamonti *et al*., 2005), kidney (Talavera *et al*., 2005), liver and lung (El Mohsen *et al.*, 2006).

The present study investigated the distribution of anthocyanins, ellagitannins and their metabolites in the GI tract and their presence in other tissues of rats fed 2.77 mL of raspberry juice by gavage.

6.2. Materials and Methods

6.2.1. Study design

Sprague–Dawley male rats (*n* 24), weighing 276.6 ± 20.7 g, were housed individually in metabolic cages allowing the collection of 24 h urine and faecal samples. Rats were handled according to the guidelines of the Committee on Animal Care at the University of Montpellier and NIH guidelines (National Research Council, 1985). Rats were deprived of food for 16 h before being fed raspberry juice (10 ml/kg body weight) by gavage. The raspberry juice contained 1.2 g raspberries/mL juice. Three animals were terminally anaesthetised with pentobarbital at 0, 1, 2, 3, 4, 6, 12

and 24 h after administration of the juice. Blood was removed by cardiac puncture with heparin-moistened syringes and plasma was obtained by centrifugation at 2000 *g* for 10 min at 4°C. Liver, kidney and brain tissues were perfused in situ with chilled 0.15 M KCl and then removed along with stomach, duodenum/jejunum, ileum, caecum, and colon with their contents at each time point. All the organs were immediately weighted and frozen in liquid N_2 and stored at -80°C prior to lyophilised and analyzed.

6.2.2. Materials

The raspberries fruits, *Rubus idaeus* variety Glen Ample used for this study were obtained from a commercial grower in Angus, Scotland and were the same batch of berries that were used in the human study described in Chapter 5. HPLC grade methanol, acetonitrile and ethyl acetate were obtained from Rathburn Chemicals (Walkerburn, UK). Formic acid, gallic acid, cyanidin-3-glucoside and quercetin-3-glucoside standards were purchased from Sigma-Aldrich (Poole, UK). Ellagic acid was supplied by AASC Ltd. (Southampton, UK). Cyanidin-3-sambubioside-glucopyranoside was obtained from Polyphenols, (Sandnes, Norway).

6.2.3. Extraction of phenolics from rat tissues

Lyophilised tissue samples from three individual rats were combined and extracted by homogenizing 200 mg with 2 mL of methanol 50% in formic acid 1% using an Ultraturrax homogeniser and extracted by continuous shaking for 30 min. The mixture was centrifuged at 2000 *g* for 20 min. The supernatant was decanted and the pellet re-extracted twice. The three supernatants were combined and reduced to dryness *in vacuo*. The extract was dissolved in 50 μL methanol in 950 μL aqueous 1 % formic acid before analysis by HPLC–PDA-MS^2.

6.2.4. Extraction of phenolics from plasma, brain and liver

Samples of the tissue extracts and plasma were loaded on to a 2 g Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA). The cartridge was washed with acidified water (pH 3.0) before elution of polar compounds with 4 mL of methanol containing 1%

formic acid. The methanolic eluates were reduced to dryness under vacuum and resuspended in 50 μL methanol in 950 μL aqueous 1 % formic acid before analysis using HPLC-PDA-MS^2. Cyanidin-3-sambubioside-glucopyranoside was used as an internal standard.

6.2.5. HPLC with photo diode array and tandem mass spectrometry detection

All samples were analysed by HPLC-PDA-MS^2 in triplicate on a Surveyor HPLC system as described in Chapters 2 and 3. The elution, this time consisted on a 30 min gradient of 8-18 % acetonitrile in 1 % formic acid before splitting and passing to a LCQ Deca XP ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan). The criteria for identification were the same as described in previous chapters.

6.2.6. Analysis of the raspberry extract

Raspberries (200 g) were blended at a low speed and sieved to obtain a juice that was fed to the rats as described above. A 5 mL sample of the juice was extracted with 25 mL methanol 1% formic acid using an Ultraturrax homogeniser and continuous shaking for 30 min. The mixture was centrifuged at 2000 *g* for 20 min. The supernatant was decanted and the pellet re-extracted twice. The three supernatants were combined and reduced to dryness under vacuum. The extract was dissolved in 50 μL methanol in 950 μL aqueous 1 % formic acid before analysis by HPLC-PDA-MS^2.

6.3. Results and discussion

6.3.1. Analysis of raspberry juice

Although the raspberries used in this study were analyzed previously, the fact that they were blended and sieved in order to obtain the juice meant that the composition of the juice had to be established. The HPLC-PDA-MS^2 analysis of the raspberry juice resulted in the identification and quantification of nine anthocyanins, two ellagitannins and ellagic acid. The HPLC profiles of the extract at 280, 365 and 520 nm are shown in Figure 6.1. Identifications, which are summarised below, as in previous chapters,

were based on published data on the MS^2 fragmentation of raspberry phenolics (Mullen *et al.,* 2002b and 2003b).

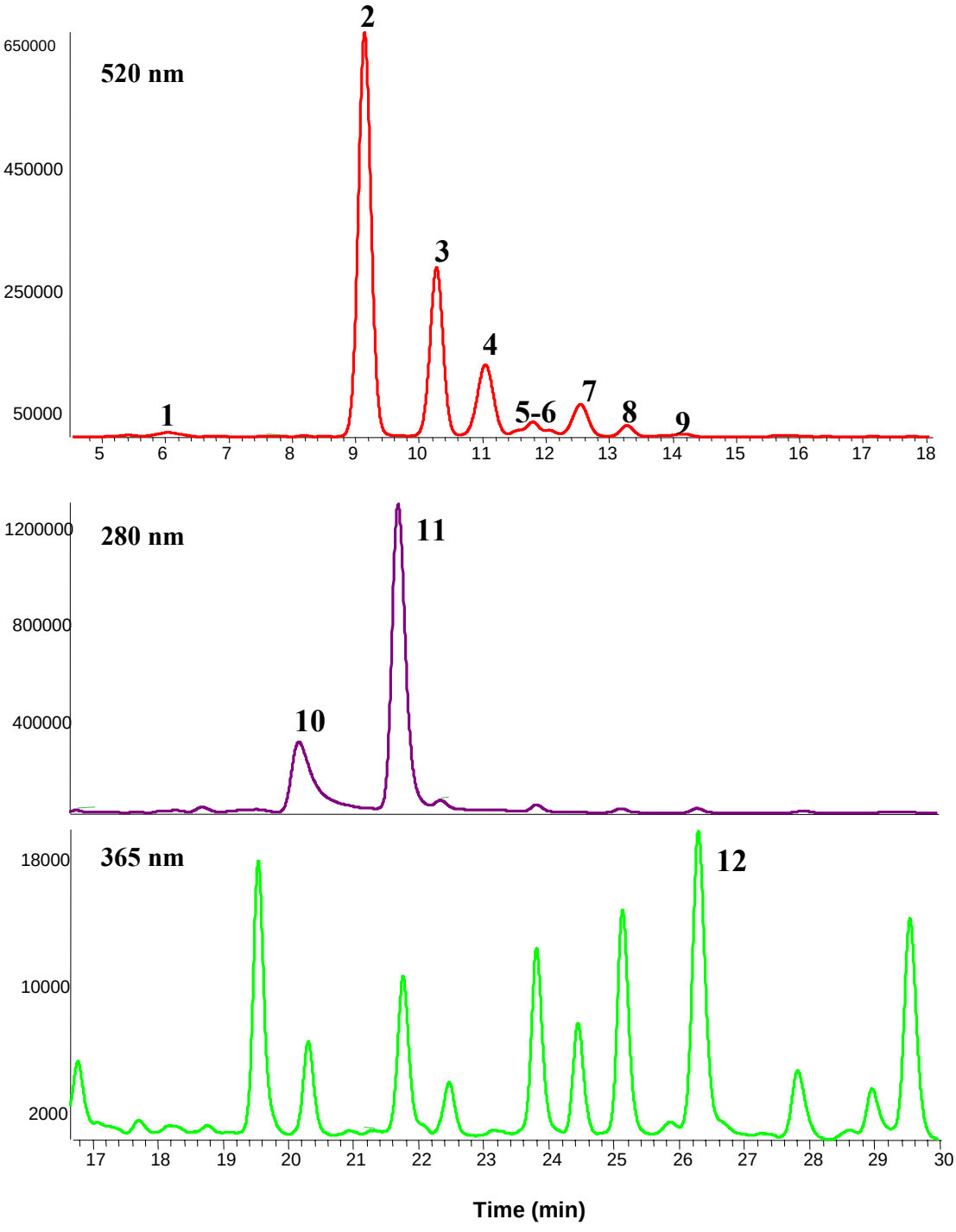


Figure 6.1. Reverse phase HPLC of raspberry juice with detection at 520, 280 and 365 nm. For MS^2 data and identification and quantification of peaks 1-12 see Table 6.1.

Peak 1 correspond to cyanidin-3,5-diglucoside (*m/z* 625→ 449, 287); *peak 2* correspond to cyanidin-3-sophoroside (*m/z* 611→287); *peak 3* was cyanidin-3-(2^{G}-glucosylrutinoside) (*m/z* 757→ 611, 287); *peak 4* was cyanidin-3-glucoside (*m/z* 449→287); *peak 5* was pelargonidin-3-sophoroside (*m/z* 595→271); *peak 6* was cyanidin-3-xylosylrutinoside (*m/z* 727→ 581, 287); *peak 7* was cyanidin-3-rutinoside (*m/z* 595→287); *peak 8* correspond to pelargonidin-3-(2^{G}-glucosylrutinoside) (*m/z* 741→595, 271); *peak 9* was pelargonidin-3-glucoside (*m/z* 433→271); *peak 10* was the ellagintannin lambertianin C (*m/z* 2801→ 1869, 1567, 1265, 1251, 935, 633); *peak 11* is also an ellagitannin, sanguiin H-6 (*m/z* 1869→ 1567, 1265, 1235, 933, 631) and *peak 12* is ellagic acid (*m/z* 301→ 257).

The main raspberry anthocyanins are di- and tri-glycosides (Table 6.1) with disaccharide cyanidin-3-sophoroside being the major anthocyanin (56% of the total anthocyanins) in the juice followed by the trisaccharide cyanidin-3-(2^{G}-glucosylrutinoside) (23%) and the monosaccharide cyanidin-3-glucoside (11.1%). The ellagitannins lambertianin C and sanguiin H-6 were also present along with ellagic acid as reported previously (Mullen *et al.,* 2003b). In addition to the compounds listed in Table 6.1 the raspberry juice also contained a number of mono-, di- and tri-saccharide flavonol conjugates which appeared as HPLC peaks on the 365 nm trace (Figure 6.1). However, these compounds were present in very low concentrations, which made it impractical to monitor their fate in rats.

6.3.2. Ingestion of raspberry juice by rats

Each rat ingested 2.77 mL of raspberry juice containing 8.7 nmoles cyanidin-3,5-diglucoside, 513 nmoles cyanidin-3-sophoroide, 211 nmoles cyanidin-3-(2^{G}-glucosylrutinoside), 102 nmoles cyanidin-3-glucoside, 14 nmoles pelargonidin-3-sophoroside, 5.5 nmoles of cyanidin-3-xylosylrutinoside, 46 nmoles cyanidin-3-rutinoside, 14 nmoles pelargonidin-3-(2^{G}-glucosylrutinoside) and 3.7 nmoles of pelargonidin-3-glucoside. The 2.77 mL of juice, thus, contained a total of 918 nmoles of anthocyanins as well as 355 nmoles of ellagitannins and 36 nmoles of ellagic acid (Table 6.1). This supplement is equivalent to a human of 70 kg weight and 170 cm height consuming 797 mL of raspberry juice based on the US Food and Drug Administration Centre for Drug Evaluation and Research dose calculator (http://www.fda.gov/cder/cancer/animalframe.htm).

Table 6.1. Summary of phenolic compounds identified and quantified by HPLC with diode array and MS^2 detection in 2.77 mL of squeezed raspberries fed to rats by gavage*.

Peak	R_t	Compound	μg/mL	nmoles/rat
1	6.1	cyanidin-3,5-glucoside	1.4 ± 0.0	8.7 ± 0.3
2	9.2	cyanidin-3-sophoroside	83.0 ± 0.9	513.2 ± 5.5
3	10.3	cyanidin-3-(2^G-glucosylrutinoside)	34.1 ± 0.4	210.8 ± 2.5
4	11.1	cyanidin-3-glucoside	16.5 ± 0.2	101.9 ± 1.4
5	11.8	pelargonidin-3-sophoroside	2.3 ± 0.2	14.1 ± 1.4
6	12.1	cyanidin-3-xylosylrutinoside	0.9 ± 0.0	5.5 ± 0.0
7	12.6	cyanidin-3-rutinoside	7.4 ± 0.1	45.7 ± 0.6
8	13.3	pelargonidin-3-(2^G-glucosylrutinoside)	2.3 ± 0.1	14.1 ± 0.6
9	14.2	pelargonidin-3-glucoside	0.6 ± 0.0	3.7 ± 0.2
		Total anthocyanins	**148.5**	**918.2**
10	20.3	lambertianin C	72.3 ± 4.0	71.3 ± 3.9
11	21.8	sanguiin H-6	192.1 ± 0.4	284.2 ± 0.6
		Total ellagitannins	**264.4**	**355.5**
12	26.3	ellagic acid	4.0 ± 0.1	36.6 ± 0.9

*For HPLC traces and peak number see Figure 6.1.

6.3.3. Fate of anthocyanins

Figure 6.2 provides an over view of the fate of raspberry juice anthocyanins after being fed to rats by gavage. The HPLC-520 nm anthocyanin profiles of plasma, urine, faeces and the gastrointestinal organs were very similar to that of the ingested juice, containing principally cyanidin-3-sophoroside, cyanidin-3-(2^G-glucosylrutinoside) and cyanidin-3-glucoside. Putative metabolites were also present, but in trace amounts which, in most instances, precluded identification. Mass spectral information was, however, obtained on two minor urinary metabolites. The larger, earlier eluting 520 nm peak had a positively charged molecular ion ($[M+H]^+$) at *m/z* 625 which on MS^2 fragmented with a loss of 324 *amu* (cleavage of a sophorosyl unit) to yield an ion at 301 *m/z* which corresponds with methyl-cyanidin (Figure 6.3). In view of the presence of cyanidin-3-sophoroside in raspberries, this minor metabolite is probably its 3′-methylated derivative peonidin-3-sophoroside. The second metabolite in urine had a $[M+H]^+$ at *m/z* 463 which ionised with a 164 *amu* loss to produced an MS^2 fragment at *m/z* 301. This again is in keeping with a 3′-methylation resulting in the conversion of cyanidin-3-glucoside to peonidin-3-glucoside (Figure 6.3).

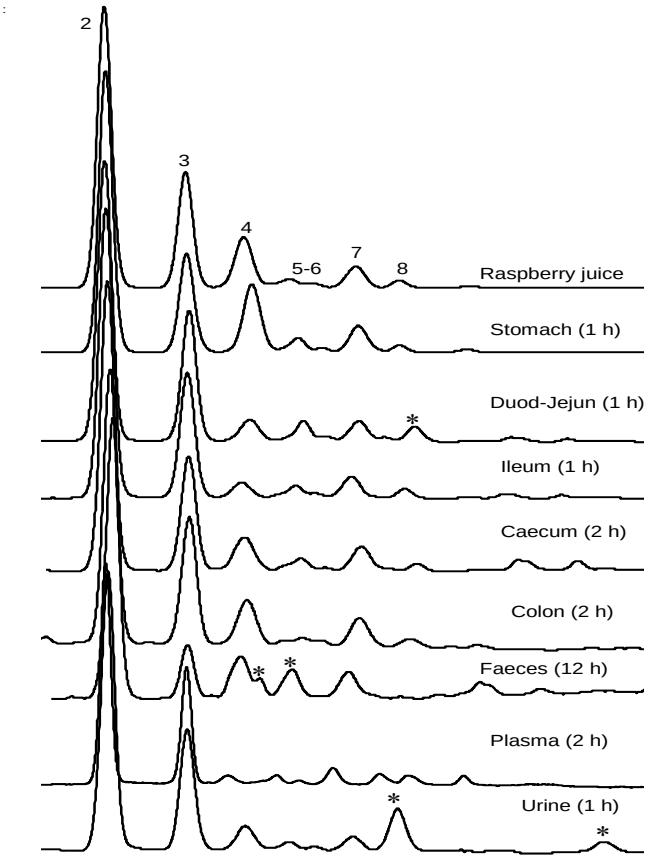


Figure 6.2. HPLC traces of raspberry juice and rat tissues and fluids after ingestion of raspberry juice with detection at 520 nm. For peak numbers see Table 6.1. Asterix indicates a metabolite. Duoden-Jejun, duodenum-jejunum.

Quantitative estimates of the overall levels of anthocyanins in the rat tissues and fluids over a 24 h period after ingestion are presented in Table 6.2. Within the first hour 59.4 % of the ingested anthocyanins were found in the ileum with 31.5 % remaining in the stomach. After 2 h, almost all the anthocyanins had left the stomach and moved to the ileum (85.9%). At the 1 and 2 h time points, the overall recoveries of anthocyanins, almost exclusively from the gastrointestinal tract, were high at 99.6% and 97.0%, respectively, of intake. Three hours after ingestion more than 50% of the anthocyanins had disappeared and, of the original dose, 40.3% was still in the ileum with 6.5% distributed in the rest of the digestive tract. After 6 h, only 2% of the anthocyanins remained principally in the caecum and colon in their native forms native forms. Over a 24 h post-ingestion period, 1.5% of the anthocyanins appeared in faeces, 1.2% was excreted in urine and only traces were detected in plasma.

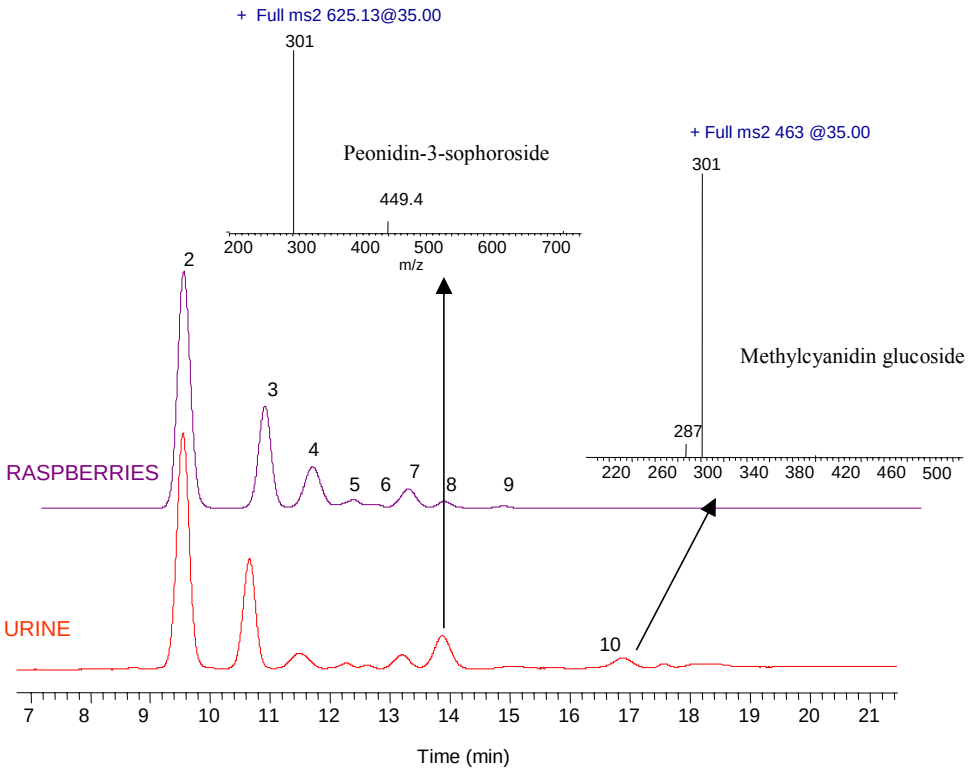


Figure 6.3. Traces of anthocyanins in urine and metabolites fragmentation pattern by HPLC-MS^2.

The percentage recoveries of the three main raspberry anthocyanins, cyanidin-3-sophoroside, cyanidin-3-(2^G-glucosylrutinoside) and cyanidin-3-glucoside in rat tissues, plasma, faeces and urine over a 24 h post-gavage period are shown in Table 6.3.

After 2 h there were only trace losses of the sophoroside and the glucosylrutinoside while cyanidin-3-glucoside declined by almost 40%. A similar observation was made in the previous chapter describing a human feeding study. Arguably, this suggests that the monosaccharide may be more readily metabolised and/or absorbed that the di- and trisaccharide. However, there was no evidence of preferential increases in the levels of cyandin-3-glucoside in either plasma or urine compared to those of cyanidin-3-sophoroside and cyanidin-3-(2^G-glucosylrutinoside) (Table 6.4) implying that the initial decline in the glucoside in the gastrointestinal tract may due to breakdown or metabolism, possibly conversion to peonidin-3-glucoside which is excreted in urine (see Table 6.5), rather than preferential absorption into the circulatory system.

Table 6.2. Overall recovery of anthocyanins from organs, faeces, urine and plasma of rats after the ingestion of raspberry juice[a)].

Tissue/Fluid	1 h	2 h	3 h	4 h	6 h	12 h	24 h
Stomach	289.6 ± 8.0 *(31.5 ± 0.9)*	36.7 ± 3.9 *(4.0 ± 0.4)*	33.3 ± 7.5 *(3.6 ± 0.8)*	1.3 ± 0.0 *(0.1 ± 0.0)*	0.1 ± 0.0 *(0.0 ± 0.0)*	n.d. -	n.d. -
Duodenum/ jejunum	78.7 ± 2.1 *(8.6 ± 0.2)*	34.7 ± 2.3 *(3.8 ± 0.2)*	8.8 ± 0.2 *(1.0 ± 0.0)*	2.0 ± 0.1 *(0.2 ± 0.0)*	n.d. -	n.d. -	n.d. -
Ileum	545.3 ± 12.5 *(59.4 ± 0.5)*	788.7 ± 12.3 *(85.9 ± 1.4)*	369.6 ± 25.1 *(40.3 ± 2.8)*	65.5 ± 7.4 *(7.1 ± 0.8)*	0.9 ± 0.0 *(0.1 ± 0.0)*	0.6 ± 0.0 *(0.1 ± 0.0)*	0.8 ± 0.0 *(0.1 ± 0.0)*
Caecum	0.2 ± 0.0 *(0.0 ± 0.0)*	24.8 ± 0.9 *(2.7 ± 0.1)*	1.8 ± 0.1 *(0.2 ± 0.0)*	21.3 ± 3.6 *(2.3 ± 0.4)*	9.0 ± 2.2 *(1.0 ± 0.2)*	1.0 ± 0.2 *(0.1 ± 0.0)*	n.d. -
Colon	0.4 ± 0.2 *(0.0 ± 0.0)*	1.5 ± 0.2 *(0.2 ± 0.0)*	17.1 ± 1.0 *(1.9 ± 0.1)*	10.2 ± 0.3 *(1.1 ± 0.0)*	7.6 ± 1.5 *(0.8 ± 0.2)*	0.3 ± 0.0 *(0.0 ± 0.0)*	n.d. -
Faeces	n.d. -	n.d. -	n.d. -	n.d. -	n.d. -	6.1 ± 2.7 *(0.7 ± 0.3)*	6.9 ± 1.3 *(0.8 ± 0.1)*
Urine	0.8 ± 0.2 *(0.1 ± 0.0)*	4.3 ± 1.9 *(0.5 ± 0.2)*	0.5 ± 0.4 *(0.0 ± 0.0)*	1.8 ± 0.5 *(0.2 ± 0.1)*	1.1 ± 0.1 *(0.1 ± 0.0)*	1.4 ± 0.4 *(0.2 ± 0.1)*	0.7 ± 0.1 *(0.1 ± 0.0)*
Plasma[b)]	0.3 ± 0.1 *(0.0 ± 0.0)*	0.1 ± 0.0 *(0.0 ± 0.0)*	0.1 ± 0.0 *(0.0 ± 0.0)*	n.d. -	n.d. -	n.d. -	n.d. -
Total	915.3 ± 12.5 *(99.6 ± 0.1)*	890.8 ± 12.3 *(97.0 ± 1.4)*	431.2 ± 25.1 *(47.0 ± 2.8)*	102.1 ± 7.4 *(11.1 ± 0.8)*	18.7 ± 0.0 *(2.0 ± 0.0)*	9.4 ± 0.0 *(1.0 ± 0.0)*	8.4 ± 0.0 *(0.9 ± 0.0)*

[a)]Data presented as mean values in nmoles ± standard error n=) and in italicised parentheses as a percentage of total anthocyanins ingested. [b)]Data for plasma calculated based on 12 mL of plasma per rat. n.d., not detected. No anthocyanins detected in brain, liver or kidney.

Three hours after feeding, anthocyanin levels in the ileum had declined without concomitant increases further down the gastrointestinal tract in the caecum and colon (Table 6.3). Between 2 and 3 h there was also a fall in the overall recovery of the individual anthocyanins with values of 50% or less being obtained (Table 6.3). This trend continued with *ca.* 10% recoveries at 4 h, and at 6, 12 and 24 h losses were of the order of 98+ %; a pattern in keeping with bacterial degradation of anthocyanins in the caecum and colon.

Analysis of purified extracts of brain, liver and kidneys of rats did not detect the presence of any anthocyanins or anthocyanin metabolites. Plasma contained low nM concentrations of cyanidin-3-sophoroside and cyanidin-3-(2^G-glucosylrutinoside) and sub-nM levels cyanidin-3-glucoside (Table 6.4). The levels of the cyanidin-3-sophoroside and cyanidin-3-(2^G-glucosylrutinoside) were highest (T_{max}) 1 h after ingestion of the raspberry juice with respective C_{max} values of 16.4 ± 3.4 and 7.7 ± 2.1 nM.

Table 6.3. Recovery of the three main anthocyanins from the gastrointestinal tract, faeces, plasma and urine of rats after ingestion of raspberry juice[a)].

Time	Anthocyanin	Stomach	Duod/jej	Ileum	Caecum	Colon	Faeces	Urine	Plasma	**Total Recovered**
1 h	Cyan-3-Soph	29.9± 0.0	8.4 ± 0.0	61.6 ± 0.0	n.d.	0.1 ± 0.0	n.d.	0.09 ± 0.02	0.038 ± 0.008	**100**
	Cyan-3-Glc-Rut	26.5± 0.0	9.6 ± 0.0	66.5 ± 0.3	n.d.	n.d.	n.d.	0.10 ± 0.02	0.044 ± 0.012	**103**
	Cyan-3-Glc	43.8± 0.1	4.0 ± 0.0	19.1 ± 0.1	n.d.	n.d.	n.d.	0.04 ± 0.01	0.004 ± 0.001	**67**
2 h	Cyan-3-Soph	3.4 ± 0.0	3.5 ± 0.0	87.5 ± 0.5	2.6 ± 0.0	0.2 ± 0.0	n.d.	0.48 ± 0.21	0.019 ± 0.002	**98**
	Cyan-3-Glc-Rut	2.6 ± 0.0	3.5 ± 0.0	89.3 ± 0.5	2.6 ± 0.0	0.1 ± 0.0	n.d.	0.52 ± 0.23	0.022 ± 0.012	**99**
	Cyan-3-Glc	7.1 ± 0.0	3.1 ± 0.0	49.1 ± 0.2	1.9 ± 0.0	0.1 ± 0.0	n.d.	0.15 ± 0.07	0.000 ± 0.000	**61**
3 h	Cyan-3-Soph	3.4 ± 0.1	1.0 ± 0.0	42.4 ± 2.9	0.2 ± 0.0	2.5 ± 0.1	n.d.	0.06 ± 0.04	0.014 ± 0.004	**50**
	Cyan-3-Glc-Rut	2.4 ± 7.2	0.8 ± 0.0	39.7 ± 2.7	0.1 ± 0.0	2.7 ± 0.1	n.d.	0.06 ± 0.04	0.011 ± 0.001	**46**
	Cyan-3-Glc	7.2 ± 0.1	1.0 ± 0.0	28.8 ± 1.6	0.3 ± 0.0	3.0 ± 0.3	n.d.	0.02 ± 0.02	0.005 ± 0.005	**40**
4 h	Cyan-3-Soph	0.2 ± 0.0	0.3 ± 0.0	7.3 ± 0.8	2.2 ± 0.0	1.4 ± 0.1	n.d.	0.19 ± 0.06	n.d.	**12**
	Cyan-3-Glc-Rut	0.1 ± 0.0	0.2 ± 0.0	6.6 ± 0.7	1.8 ± 0.0	0.5 ± 0.0	n.d.	0.22 ± 0.08	n.d.	**9.4**
	Cyan-3-Glc	0.2 ± 0.0	0.1 ± 0.0	6.5 ± 0.8	2.6 ± 0.0	1.2 ± 0.1	n.d.	0.05 ± 0.02	n.d.	**11**
6 h	Cyan-3-Soph	n.d.	n.d.	0.1 ± 0.0	1.2 ± 0.0	1.0 ± 0.1	n.d.	0.13 ± 0.01	n.d.	**2.4**
	Cyan-3-Glc-Rut	n.d.	n.d.	0.1 ± 0.0	0.5 ± 1.9	0.4 ± 0.1	n.d.	0.15 ± 0.01	n.d.	**1.1**
	Cyan-3-Glc	n.d.	n.d.	0.1 ± 0.0	n.d.	1.3 ± 0.2	n.d.	0.06 ± 0.01	n.d.	**1.5**
12 h	Cyan-3-Soph	n.d.	n.d.	0.1 ± 0.0	0.1 ± 0.0	n.d.	0.7 ± 0.0	0.16 ± 0.08	n.d.	**1.1**
	Cyan-3-Glc-Rut	n.d.	n.d.	n.d.	n.d.	n.d.	0.3 ± 0.0	0.16 ± 0.08	n.d.	**0.5**
	Cyan-3-Glc	n.d.	n.d.	n.d.	0.2 ± 0.0	0.1 ± 0.0	1.5 ± 0.5	0.04 ± 0.02	n.d.	**1.8**
24 h	Cyan-3-Soph	n.d.	n.d.	0.1 ± 0.0	n.d.	n.d.	0.8 ± 0.0	0.08 ± 0.02	n.d.	**1.0**
	Cyan-3-Glc-Rut	n.d.	n.d.	n.d.	n.d.	n.d.	0.4 ± 0.0	0.09 ± 0.01	n.d.	**0.5**
	Cyan-3-Glc	n.d.	n.d.	n.d.	n.d.	n.d.	0.7 ± 0.4	0.04 ± 0.01	n.d.	**0.7**

[a)]Data for the three individual anthocyanins presented as a percentage of the amount ingested ± standard error (n = 3). Figures for plasma based on a total of 12 mL of plasma per rat. Cyan-3-Soph, cyanidin-3-sophoroside; Cyan-3-Glc-Rut, cyanidin-3-(2^G-glucosylrutinoside); Cyan-3-Glc, cyanidin-3-glucoside; Duod/jej, duodenum/jejunum; n.d., not detected.

Table 6.4. Concentration of anthocyanins in plasma of rats 0-24 h after the ingestion of raspberry juice[a)].

Anthocyanin	0 h	1 h	2 h	3 h	4, 6, 12 and 24 h
Cyan-3-Soph	n.d.	16.4 ± 3.4 *(0.04 ± 0.008)*	8.2 ± 1.1 *(0.019 ± 0.002)*	5.8 ± 1.5 *(0.014 ± 0.004)*	n.d. -
Cyan-3-Glc-Rut	n.d.	7.7 ± 2.1 *(0.044 ± 0.012)*	3.8 ± 2.1 *(0.022 ± 0.012)*	2.0 ± 0.2 *(0.011 ± 0.001)*	n.d. -
Cyan-3-Glc	n.d.	0.3 ± 0.1 *(0.004 ± 0.001)*	n.d. -	0.4 ± 0.4 *(0.005 ± 0.005)*	n.d. -
Total	**n.d.**	**24.4 ± 6.5** ***(0.04 ± 0.009)***	**12.0 ± 2.8** ***(0.017 ± 0.004)***	**8.2 ± 1.3** ***(0.012 ± 0.002)***	**n.d.** **-**

[a)]Data presented as mean values in nM ± standard error (n=3). Figures in italicised parentheses are data expressed as a percentage of the amount ingested calculated based on a total of 12 mL of plasma in the circulatory system of each rat. Cyan-3-Soph, cyanidin-3-sophoroside; Cyan-3-Glc-Rut, cyanidin-3-(2^G-glucosylrutinoside); Cyan-3-Glc, cyanidin-3-glucoside; n.d., not detected.

As the highest plasma anthocyanin concentrations occurred at the first time point, if earlier samples had been collected it is possible that a T_{max} of less than 1 h would have been obtained and that the C_{max} values may have been slightly higher. After 1 h the concentration of both anthocyanins declined rapidly. The elimination half-life ($T_{1/2}$) values were 1.33 h for cyanidin-3-sophoroside and 1.03 h for cyanidin-3-(2^G-glucosylrutinoside), with no anthocyanins being detected in plasma at the 4, 6, 12 and 24 h time points. The amounts of anthocyanins detected in plasma were very low indeed. On the basis of each rat containing a total of 12 mL of plasma, the 16.4 nM concentration of cyanidin-3-sophoroside present at T_{max} is equivalent to 0.038% of the amount ingested and the figure for cyanidin-3-(2^G-glucosylrutinoside) is 0.044%. The level of cyanidin-3-glucoside was *ca.*10-fold lower (Table 6.4).

In keeping with the pharmacokinetic data obtained with plasma, the highest level of anthocyanin excretion in urine, 4.34 ± 1.90 nmoles, occurred 1-2 h after feeding the raspberry juice (Table 6.5). The total urinary excretion over a 24 h period was 9.7 ± 2.1 nmoles corresponding to 1.2 ± 0.2 % of the anthocyanins ingested, a figure somewhat higher than that obtained in many anthocyanin-feeding studies. As mentioned previously, the urinary anthocyanin profile was very similar to that of raspberries with the exception that three minor components, cyanidin-3,5-diglucoside,

pelargonidin-3-(2^G-glucosylrutinoside) and pelargonidin-3-glucoside which were not detected, while the methylated metabolites, peonidin-3-sophoroside and peonidin-3-glucoside were present in quantifiable amounts (Table 6.5). Overall, however, absorption and excretion was not associated with extensive metabolism of the raspberry anthocyanins.

It is of note that the levels of anthocyanins in plasma were highest 1 h after supplementation with the raspberry juice (Table 6.4) at which point most of the anthocyanins in the gastrointestinal tract were in the stomach (31.5%) and the ileum (59.4%) (Tables 6.2 and 6.3). Over the next hour the levels declined in the stomach and rose in the ileum to 85.9% of intake while the low concentrations in the bloodstream declined. This suggests that the albeit low absorption of anthocyanins occurs before reaching the ileum. This is in keeping with evidence obtained in other studies with rats and mice indicating that the stomach (Passamonti *et al*., *2003;* Talavera *et al*., 2003) and the jejunum (Matuschek *et al.,* 2006) are sites of anthocyanin absorption.

As noted above, anthocyanins were not detected in the brain of rats following the ingestion of the 2.77 mL of raspberry juice. Other studies have detected anthocyanins in rat brains after supplementation with berry and grape extracts. Andres-Lacueva *et al.* (2005) daily fed rats a blueberry extract, containing an uncited amount of anthocyanins, for 8-10 weeks after which the animals exhibited enhanced special learning and memory in the Morris water maze test. Trace levels of anthocyanins, which could not be quantified, were detected in the cerebellum, cortex and hippocampus, regions of the brain important for learning and memory. Extremely low concentrations of anthocyanins, 0.25 nmoles/g, were also detected by Talavera *et al*. (2005) in rat brains after feeding a blackberry extract for 15 days. The daily dose of anthocyanins was of the order of 318 μmoles per rat which is equivalent to a human weighing 70 kg and 170 cm height eating a daily serving of *ca.* 115.8 kg of blackberry extract. It has also been reported that within 10 min of the introduction of a red grape extract, containing 3.8 μmol of anthocyanins (2 mg), into the stomach of rats, unmetabolised anthocyanins were detected in the brain at 192 ng/g (Passamonti *et al.,* 2005).

Table 6.5. Recovery of anthocyanins in the urine of rats 0-24 h after the ingestion of raspberry juice.

Anthocyanin	0-1 h	1-2 h	2-3 h	3-4 h	4-6 h	6-12 h	12-24 h	Total
Cyan-3,5-diGlc	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**n.d.**
Cyan-3-Soph	0.44 ± 0.10 *(0.09 ± 0.02)*	2.39 ± 1.04 *(0.48 ± 0.21)*	0.25 ± 0.21 *(0.06 ± 0.04)*	0.95 ± 0.29 *(0.19 ± 0.06)*	0.55 ± 0.04 *(0.13 ± 0.01)*	0.81 ± 0.22 *(0.16 ± 0.08)*	0.36 ± 0.05 *(0.08 ± 0.02)*	**5.24 ± 1.17** ***(1.17 ± 0.16)***
Cyan-3-Glc-Rut	0.20 ± 0.04 *(0.10 ± 0.02)*	1.07 ± 0.47 *(0.52 ± 0.23)*	0.11 ± 0.09 *(0.03 ± 0.01)*	0.44 ± 0.16 *(0.22 ± 0.08)*	0.25 ± 0.01 *(0.15 ± 0.01)*	0.32 ± 0.09 *(0.16 ± 0.08)*	0.16 ± 0.01 *(0.09 ± 0.01)*	**2.43 ± 0.55** ***(1.25 ± 0.22)***
Cyan-3-Glc	0.04 ± 0.01 *(0.04 ± 0.01)*	0.15 ± 0.07 *(0.15 ± 0.07)*	0.03 ± 0.02 *(0.02 ± 0.02)*	0.06 ± 0.02 *(0.05 ± 0.02)*	0.05 ± 0.00 *(0.06 ± 0.01)*	0.05 ± 0.01 *(0.04 ± 0.02)*	0.04 ± 0.01 *(0.04 ± 0.01)*	**0.39 ± 0.07** ***(0.40 ± 0.04)***
Pel-3-Soph	0.01 ± 0.00 *(0.05 ± 0.02)*	0.04 ± 0.02 *(0.27 ± 0.11)*	0.01 ± 0.01 *(0.10 ± 0.07)*	0.02 ± 0.00 *(0.11 ± 0.02)*	0.02 ± 0.01 *(0.13 ± 0.06)*	0.01 ± 0.01 *(0.11 ± 0.06)*	n.d.	**0.10 ± 0.02** ***(0.74 ± 0.07)***
Cyan-3-Xylo-Rut	0.00 ± 0.00 *(0.08 ± 0.06)*	0.01 ± 0.01 *(0.21 ± 0.23)*	n.d.	0.01 ± 0.00 *(0.19 ± 0.04)*	n.d.	0.00 ± 0.01 *(0.09 ± 0.22)*	n.d.	**0.01± 0.01** ***(0.36 ± 0.14)***
Cyan-3-Rut	0.03 ± 0.01 *(0.05 ± 0.02)*	0.12 ± 0.05 *(0.23 ± 0.10)*	0.01 ± 0.01 *(0.03 ± 0.02)*	0.05 ± 0.02 *(0.10 ± 0.03)*	0.03 ± 0.00 *(0.07 ± 0.01)*	0.03 ± 0.01 *(0.07 ± 0.03)*	n.d.	**0.25 ± 0.06** ***(0.54 ± 0.08)***
Pel-3-Glc-Rut	n.d.	n.d.	n.d.	n.d	n.d.	n.d	n.d.	**n.d.**
Peon-3-Soph (M)	0.07 ± 0.02	0.41 ± 0.18	0.05 ± 0.04	0.20 ± 0.04	0.17 ± 0.02	0.16 ± 0.04	0.11 ± 0.01	**1.11 ± 0.21**
Pel-3-Glc	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**n.d.**
Peon-3-Glc (M)	0.026 ± 0.007	0.150 ± 0.074	0.070 ± 0.056	0.045 ± 0.009	0.015 ± 0.011	0.006 ± 0.012	n.d.	**0.308 ± 0.040**
Total	**0.81 ± 0.18** ***(0.10 ± 0.02)***	**4.34 ± 1.90** ***(0.48 ± 0.21)***	**0.52 ± 0.37** ***(0.04 ± 0.04)***	**1.76 ± 0.53** ***(0.20 ± 0.06)***	**1.07 ± 0.07** ***(0.14 ± 0.01)***	**1.40 ± 0.39** ***(0.18 ± 0.06)***	**0.67 ± 0.07** ***(0.08 ± 0.01)***	**9.71 ± 2.07** ***(1.22 ± 0.17)***

[a)]Data presented as means values in nmoles ± standard error (n = 3) and in italicised parentheses as a percentage of the amounts of anthocyanin ingested. Cyan-3-Soph, cyanidin-3-sophoroside; Cyan-3-Glc-Rut, cyanidin-3-(2^G-glucosylrutinoside; Cyan-3-Glc, cyanidin-3-glucoside; Cyan-3,5-diGlc, cyanidin-3,5,-diglucoside; Cyan-3-Rut, cyanidin-3-rutinoside; Cyan-Xylo-Rut, cyanidin-3-xylosylruinoside; Pel-3-Glc, pelargonidin-3-glucoside; Pel-3-Glc-Rut, pelargonidin-3-(2^G-glucosylrutinoside); Peon-3-Glc, peonidin-3-glucoside; M, metabolite; n.d., not detected.

This intake corresponds to a 70 kg weight, 170 cm height human consuming *ca*. 300 g of red grapes, an amount that is in keeping with a normal diet.

6.3.4. Fate of ellagitannins

Along with the anthocyanins, the raspberry juice fed to rats contained substantial amounts of the ellagitannins lambertianin C and sanguiin H-6 together with a smaller quantity of ellagic acid (Fig. 6.1 and Table 6.1). The ellagitannins disappeared rapidly and were not detected in plasma, urine, faeces, the stomach, duodenum/jejunum, ileum, and other parts of the gastrointestinal tract, 1 h after ingestion and at all later time points. This contrasts with the anthocyanins, which at 1 h were recovered intact, mainly in the stomach and ileum (Table 6.2). The absence of ellagitannins in the rats implies that breakdown of sanguiin H-6 and lambertianin C may have occurred in the acidic conditions of the stomach to such an extent that they had disappeared after 1 h probably converted to products that went undetected by the HPLC-PDA-MS^2 conditions employed in the current study. HPLC-PDA-MS^2 analysis of urine demonstrated an absence of urolithin B, hydroxyurolithin B and their glucuronide conjugates, compounds excreted by rats after a 37-day intake of a pomegranate husk extract containing very high quantities of the ellagitannin punicalagin (Cerdá *et al.,* 2003).

Ellagic acid recovered in the stomach after 1 h was 9.6% of the amount present in the raspberry juice and after 2 h, only traces were detected (Fig. 6.4). Except for these small amounts in the stomach, no ellagic acid was detected in any of the rat organs/tissues or fluids collected over a 24 h period after consumption of the raspberry juice. Degradation of the ellagitannins has the potential to release substantial amounts of ellagic acid. In theory, the ingested 71 nmoles of lambertianin C and 284 nmoles of sanguiin H-6 could give rise to 426 and 1136 nmoles of ellagic acid, respectively. This would appear not to have occurred in the rats.

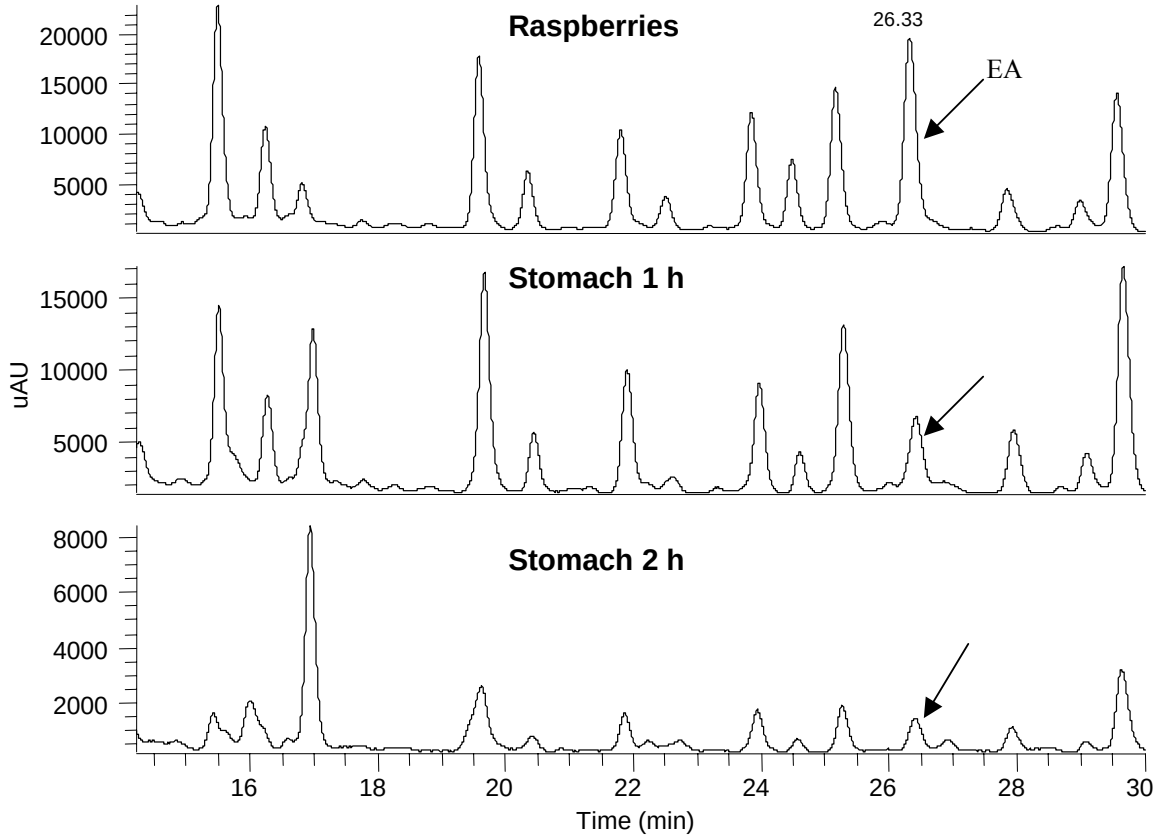


Figure 6.4. HPLC traces at 365 nm of raspberries and rat's stomach extracts after 1 and 2 h of raspberry juice ingestion. EA, ellagic acid.

An accurate picture of the fate of both ellagitannins and anthocyanins is unlikely to be ascertained until radiolabelled forms of these compounds become available. This would enable their fate to be monitored in animal test systems in much the same way as [2-^{14}C]quercetin-4′-glucoside has been used to provide a detailed insight of flavonol glucoside bioavailability in rats (Mullen *et al.,* 2002b, 2003a; Graf *et al.,* 2005)

6.3.6. Stability of anthocyanins in the gastrointestinal tract

Anthocyanins are readily distinguished from other flavonoids as they undergo re-arrangements in response to pH. Anthocyanins are traditionally extracted and

analysed in acidic medium as the red flavylium cation as this is the most stable form. However, it is not known what forms predominate *in vivo*. The limited available experimental evidence indicates that in the acidic conditions that prevail in the stomach, anthocyanins are in the red flavylium form but once they enter more basic conditions in the small intestine the carbinol pseudobase is likely to predominate (McGhie *et al.,* 2003). To what extent this occurs and what influence it has on absorption remains to be determined. In the present study, extracting the gastrointestinal tract and its contents with acidic methanol resulted in high recoveries of the flavylium cation from the duodenum, jejunum and ileum in the initial two hours after ingestion of the raspberry juice (Tables 6.2 and 6.3). However, it does not follow that *in vivo* the anthocyanins were in this form and, due to an absence of appropriate analytical procedures, nothing is known about either the metabolism or absorption of the pseudo bases or the quinoidal base in the gastrointestinal tract. It could be that after anthocyanins leave the stomach, the colourless carbinol pseudobase becomes the main form, in the small intestine where it undergoes very limited absorption. Consequently, significant amounts pass into the large intestine where degradation, to yet undetermined products, occurs due to the action of colonic bacteria. This would be in keeping with the data obtained in this study but other more subtle scenarios may exist.

6.4. Conclusions

Rats were fed by gavage a single 2.77 mL supplement of raspberry juice, containing anthocyanins and ellagitannins. One hour after feeding the ellagitannins, sanguiin H-6 and lambertianin C had disappeared with only traces of ellagic acid being detected in the stomach. Up to 2 h after supplementation, there was a very high recovery of unmetabolised anthocyanins, principally cyanidin-3-sophoroside, cyanidin-3-(2^{G}-glucosylrutinoside) and cyanidin-3-glucoside, as they passed from the stomach to the duodenum/jejunum and ileum. After 3 h, less than 50% was recovered, after 4 h this declined to 11% of intake and after 6 h only 2% remained. Only trace quantities of

anthocyanins were detected in the caecum, colon and faeces and they were absent in extracts of liver, kidneys and brain.

After 1 h low nM concentrations of cyanidin-3-sophoroside, cyanidin-3-(2^{G}-glucosylrutinoside) and cyanidin-3-glucoside were detected in plasma but these declined by 2 h and were not present in detectable quantities 4 h after feeding. Excretion of the three main raspberry anthocyanins in urine over a 24 h period after feeding was equivalent to 1.2% of the amounts ingested which is unexpectedly high for unmentabolised anthocyanins.

These findings imply anthocyanins are poorly absorbed and that this occurs before they reach the ileum, in keeping with evidence indicating that the stomach (Talavera *et al*., 2003; Passamonti *et al.,* 2003) and the jejunum (Matuschek *et al.,* 2006) are sites of anthocyanin absorption in mice and rats. Because anthocyanins are poorly absorbed substantial amounts pass from the small to the large intestine where their rapid disappearance suggests they are degraded by faecal bacteria.

These results have been published in 2007 (Borges *et al*., 2007), see Appendix 2.

Chapter 7. General conclusions and future.

There is a considerable body of evidence indicating that inclusion of fruits and vegetables in the human diet has impact on health and the prevention of diseases. Dietary antioxidants including polyphenols, vitamins E and C, and carotenoids are the compounds that have been associated with these benefits either through their reducing capacities or through their reducing possible influence on the intracellular redox status or signalling. Although the exact mechanism(s) of action of the compounds involved is not clear, numerous researches in the area are developing and revealing more and more data that contribute to the understanding of the process involved. In this study, polyphenols were investigated and the antioxidant capacity and total phenolic content of a number of common fruits and vegetables, available from the local supermarkets were screened. There were major differences between individual products with broad beans, purple broccoli, lollo rosso lettuce, berries and nuts being the richest in phenolics and having highest AOC. An important task to achieve in the future will be the identification of those plant varieties, which produce high levels of a particular group of polyphenols, and to what degree this can be enhanced by factors like growing conditions and genetical modifications. Cooking and processing are also variables that can affect the levels of phenolics in food and not much research has been published in this area.

There is much debate as to what antioxidant assay is the most appropriate to monitor AOC both *in vitro* and *in vivo*. Opinions vary considerable and at present, there are diverse views in this complex area. In the mean time data based on ORAC, TRAP, FRAP, DPPH, ABTS, ESR, TPC (Folin-Ciocalteau) assays are cited in the literature making the comparison of results from different groups a very difficult task. FRAP is a versatile method, easy, inexpensive and fast that correlated significantly in this study with ESR and TPC, so although it does not mimic a biological antioxidant reaction is a practical approach that can be considered for the rapid phenolics screening of plant products.

As berries were identified as a group of fruits rich in phenolic compounds and antioxidants, whose consumption could be promoted locally, a more detail analysis was conducted in this study to identify and quantify the individual phenolic

compounds in five different berries. The phenolic composition of the five berries analysed was interestingly different. Blackcurrants had the highest AOC attributable to anthocyanins, principally delphinidin-3-rutinoside and cyanidin-3-rutinoside, as well vitamin C and small amounts of flavonols. In blueberries, malvidin-3-galactoside and malvidin-3-arabinoside were the main anthocyanins contributing to the AOC and, at least in the sample analysed, vitamin C was absent. Raspberries were rich in ellagitannins that contributed about one third of the total AOC with the anthocyanins and vitamin C being equally responsible for the remained. Redcurrants had the lowest AOC of the berries investigated and the main anthocyanins present were cyanidin-3-xylosyl-rutinoside and cyanidin-3-rutinoside, which occurred along with flavonols and caffeic acid. Cranberries were rich in procyanidins dimers and (–)-epicatechin and contained only minor amounts of flavonols. The anthocyanins peonidin-3-galactoside and cyanidin galactoside were the major contributors to the AOC of cranberries.

The identification of the phenolic compounds in berries by HPLC with mass spectrometric detection in the MS^2 and MS^3 mode demonstrated the versatility of the technique. The combination of parameters like HPLC retention time, UV/Vis spectral information, mass fragmentation patterns and the accurate estimates of mass that can be obtained with high resolution instruments, are beginning to be used to develop data bases on the plant secondary metabolites present in fruits of dietary interest such as tomatoes (Moco *et al*., 2006). From another perspective and in this new post-genoma era mass spectrometry represent one of the pillars of a new series of methodologies developing in nutrition research area which has the potential to help understanding the complex relationship between nutrition and metabolism in humans.

In addition to the investigation on the antioxidants and the phenolic profile of berries, a central part of the project was to investigate the bioavailability of dietary phenolics. Raspberries were chosen for this study as they have an interesting phenolic profile being rich in both anthocyanins and and ellagitannins. The intervention study with six volunteers ingesting a normal sized portion of raspberries showed that the anthocyanins and ellagitannins did not accumulate in plasma in detectable quantities. The three main anthocyanins were excreted in urine in quantities corresponding to 0.038% of intake – indicating an apparent low level of

absorption. In ileostomy volunteers 26.2 % of the ellagitannin sanguiin H-6 and 39.6 % of anthocyanins were recovered in the ileal fluid after 24 h implying that in healthy subjects with a large intestine substantial amounts of these compounds would be available for metabolism by the colonic microflora. However, the potential products of this process, ellagic acid and its urolithin metabolites were not detected in either plasma or urine of the healthy subjects.

When raspberry juice wa fed to rats by gavage in order to investigate the distribution of phenolics in the gastrointestinal tract and other organs, *ca.* 59 % of the anthocyanins were in the ileum after 1 h. Although a detailed comparison with the human study is not straightforward and ultimately was not the intention, the amounts of anthocyanins in the ileal bags were *ca.* 37% after 4 h. Anthocyanins were detected in rat plasma, albeit it in low amounts, *ca.* 0.3 nmoles/L. In contrast, anthocyanins were not detected in human plasma after the ingestion of raspberries. It is important to mention that the phenolic composition of the ingested raspberry supplement was different in the rat and human studies (see Table 5.2 and Table 6.1). With humans intact raspberries were ingested while the rats had a raspberry juice produced by mixing and sieving the berries which would removed phenolics in the pulp and the seeds, the latter being an especially rich source of ellagitannins.

The total excretion of anthocyanins in rat urine over a 24 h period after feeding raspberry juice was 1.2%, a level unexpectedly high for unmetabolised anthocyanins, certainly much higher than the amounts excreted the human study, and also somewhat higher than obtained in other animal studies reported in the literature and discussed in the corresponding Chapter 5 and 6. Interestingly, the ellagitannins seemingly “disappeared” from the gastrointestinal tract of the rats 1 h after feeding raspberry juice. In contrast there was *ca.* 26 % recovery of ellagitannins in human ileal fluid over a 24 h period after ingestion of raspberries. This is most likely due to most of the ellagitannns being located within the seeds and therefore more difficult get expose to the gastric machinery, enzymes, mucus, acids and also bacteria in the gut. For the rats, the relative smaller amounts of ellagitannins ingested were more available as they were in solution.

In the area of anthocyanins metabolism, at least two points need to be addressed. One is the development of techniques to identify the colourless forms of anthocyanins which would be a major technical advances that would, without doubt

result in knowledge of the fate of dietary anthocyanins being markedly enhance. The second point, related to the first, is the use of radiolabelled anthocyanins that would greatly simply attempts to monitor the fate of anthocyanins within the body of animal models.

More and better design clinical studies need to be carried out to investigate the health effects of flavonoids and related phenolic compounds, provided that biomarkers for specific diseases are reliable and available. The public is becoming more and more aware of health topics and as marketing studies have shown, more interested in health-promoting products. The need to clarify facts from fiction is essential as a lack of scientific evidence is making easier for nutraceutical companies and "herbal doctors"promote products based on largely fraudulent claims.

Since this study was completed, there has been a substantial increase in the technical skills and experience accumulated in the laboratory with HPLC-PDA-MS^2 which has resulted in major advances in our knowledge of the bioavailability of flavanols, flavonols, anthocyanins and flavan-3-ols (Jaganath *et al.*, 2006, Marks *et al.*, 2007, Mullen *et al.*, 2008a, Mullen *et al.*, 2008b, Stalmach *et al.*, 2008 and Roowi *et al.*, 2008). Application of these skills, together with the recent development of GC-MS for the analysis of phenolic compounds, coupled with recent reports in the literature about catabolic products of colonic micro flora, would almost certainly have provided a more complete picture of the bioavailability of the raspberry anthocyanins and ellagitannins.

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APPENDIX 1

Title **Increasing antioxidant intake from fruits and vegetables - practical strategies for the Scottish population (in press)**

Running title **Increasing antioxidant intake**

Keywords: Fruits, Vegetables, FRAP Antioxidant Assay, Antioxidant intake

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Abstract

Background: Increasing intakes of[1] dietary antioxidants may help to reduce oxidative damage caused by free radicals and provide protection against the progression of a number of chronic diseases.

*Objective***:** To estimate the antioxidant intake from fruits and vegetables in the UK and Scottish population and to examine consumption models to identify potential strategies to optimise antioxidant intake from these foods.

*Methods***:** This was a retrospective study of cross-sectional data on fruit and vegetable intake in relation to antioxidant intake. Antioxidant capacity of individual fruits and vegetables was determined by the FRAP assay and data on quantity and frequency of consumption of fruits and vegetables determined from National Diet and Nutrition Survey (NDNS) 2000-2001.

*Results***:** Mean antioxidant intake in UK population from fruits and vegetables varied by region. In the Scottish sample (n=123), mean antioxidant intake was estimated at 680 $\pm$ 689 µmol/d with 92% subjects consuming <400g of fruits and vegetables per day. Consumption data showed that strawberries, apples, orange citrus fruits, purple broccoli and cauliflower were the top five sources of antioxidants from fruits and vegetables in Scots.

*Conclusions***:** Appropriate selection of fruits and vegetables would help to achieve a higher antioxidant intake with the potential to produce significant health benefits.

1

Introduction

Free radicals are routinely formed in the human body as a result of normal metabolic activity but endogenous antioxidants are inadequate to completely remove these substances. Therefore, continuous oxidative damage over a prolonged period of time contributes to the development of chronic diseases such as cancer, cardiovascular, inflammatory and neurodegenerative diseases. It has been suggested that increasing intakes of dietary antioxidants may help reduce oxidative damage caused by free radicals *in vivo* and thereby provide some protection against the onset of these diseases (Buttris *et al.,* 2002).

The main sources of dietary antioxidants are minimally processed plant foods and selected beverages. A wide variety of components in fruits and vegetables, such as vitamins C and E, folate, various carotenoids, isothiocyanates, flavonoids and other phenolic compounds have antioxidant function (Goldberg, 2003; Crozier *et al.,* 2006). Recently, polyphenols, particularly flavonoids, have been of increased interest because of their antioxidant properties and free radical scavenging abilities observed *in vitro* (Ross and Kasum, 2002). A strong correlation has been reported between phenolic compounds and the antioxidant capacity of fruit and vegetables (Cao *et al.,* 1996; Wang *et al.,* 1996). It is, therefore plausible that regular intake of fruits and vegetables rich in phenolic antioxidants may provide enhanced levels of protection against the onset of chronic diseases (Halvorsen *et al.,* 2002, 2006).

The aim of this study was to estimate the current antioxidant intake from fruit and vegetables in the UK population and subgroups in order to identify strategies to optimise antioxidant intake from fruits and vegetables. This is of particular relevance given the continuing trend of low intakes of fruit and vegetable consumption in Scotland (Wrieden et al., 2006). It is recognised that the Scottish diet is notoriously difficult to change and a portfolio of actions are required to optimise nutrient intake and achieve a healthy balanced diet (Scottish Executive 2006). Health promotion and marketing approaches which take account of current nutrient composition offer scope to contribute to improved nutrient status in a population with high rates dietary related diseases (Scottish Government, 2008a, Scottish Government, 2008b)

Methods

Antioxidant intakes of the UK population and sub-groups were estimated using data on the antioxidant content of different fruits and vegetables determined by the Ferric Reducing Antioxidant Power (FRAP) assay and data on frequency and consumption of fruits and vegetables in the UK population. The latter data were obtained from the National Diet and Nutrition Survey (NDNS) records of 2000-2001 (Henderson *et al.,* 2002; UK Data Archive).

In the NDNS survey, dietary data were collected for a total of 1724 people aged between 19-64 years (UK Data Archive). Weighed dietary records were collected for seven consecutive days for all food and drinks consumed both within and outside the home. In the current study estimates of antioxidant capacity utilised data for fruit and vegetables only.

Inclusion criteria:

1. In this study the definition of fruit and vegetables was based on that of 'five-a-day' programme (UK Department of Health, 2003), where most fruit and vegetables are included except starchy produce such as potatoes, yams and cassava.
2. Fruit and vegetable intake data, which recorded inedible or non-fruit and vegetable portions, were adjusted to ascertain actual consumption weight with reference to published conversion factors in order to obtain the weights for 100% fruit and vegetable (Holland *et al.,* 1992).

The intake data of the sample population for fruits and vegetables were linked with the data on the antioxidant capacity of individual fruits and vegetables. All varieties of fruits and vegetables were assigned a single estimated value (e.g. all varieties of oranges were grouped as orange citrus fruits). This approach allowed the antioxidant intakes derived from fruit and vegetables for individuals as well as the whole sample population and sub-groups to be calculated.

The following exclusion criteria were set for the analysis of the antioxidant capacity of fruits and vegetables in the above-mentioned sample.

1. Any fruit or vegetable consumed by <1% of the sample population.
2. Composite fruit and vegetable dishes (due to unknown recipes).
3. Fruit juice and dried fruits.

4. Furthermore, the data for antioxidant capacity were not available for the items consumed infrequently (such as apricots, aubergine, cherries, grapefruits, lemons, cantaloupe melons, pineapples, mushrooms, celery, mustard cress, olive, parsnips, radish-red-raw, turnips and water-cress).

*FRAP assay***.** The antioxidant capacity of fruit and vegetables, purchased from Safeway Stores plc, Byres Road, Glasgow, was determined by the FRAP assay (Benzie & Strain, 1999). The fresh produce were weighted, sliced, frozen with liquid nitrogen, freeze dried, powdered and stored at –20°C prior to analysis. The powder samples (10-50 mg aliquots) were extracted by shaking overnight in 2 mL of 50% aqueous methanol at 4°C. After centrifugation 100 □L aliquots of the supernatant were assayed for antioxidant activity. The FRAP assay is an established method for assessing total antioxidant activity and measures the capacity of the sample to reduce Fe^{+3} to Fe^{+2} and data are expressed as μmol Fe^{+3} reduced/g fresh weight.

The FRAP assay which was used in the present study measures reducing power while the ORAC and TEAC assays measure chain breaking antioxidant potential (Huang et al. 2005). However, the data obtained from the three assays are usually well correlated (Pellegrini et. al. 2003, 2006). There are a number of practical advantages associated with the use of the FRAP assay rather than the ORAC or TEAC assays that have been summarised by Halvorsen *et al.* (2002). In particular, the FRAP assay uses antioxidants as reductants in a redox-linked colorimetric reaction while the other assays use a lag phase type of measurement that is difficult to standardise and has generated varying results among different laboratories.

Statistical analysis.

Statistical analyses were carried out using SPSS version 11.5. The mean and median intake of antioxidants was calculated for the whole UK population and sub-groups. The data for the mean antioxidant intake were not normally distributed so non-parametric Spearman's rank correlation coefficients were used to determine the relationship between antioxidant and fruit and vegetable intake. Non-parametric Mann-Whitney test was used to see the comparisons in antioxidant intakes among the different regions and different social classes.

Results

The FRAP antioxidant capacity of different commonly consumed fruit and vegetables in the UK is presented in Table 1. In general the values are good agreement with figures obtained in a recent detailed survey of the antioxidant activity of foods in the USA (Halvorsen *et al.,* 2006). The ranking of the antioxidant capacity of the various products is also similar to that obtained with Italian foods by Pellegrini *et al*. (2003) although in this study the actual FRAP μmol Fe^{+3} reduced/g values were somewhat higher than those obtained both in the present study and the USA investigation (Halvorsen *et al.,* 2006). This is because the Italian investigators used a 30 min rather than the more traditional 4 min reaction time for the FRAP assay.

The data in Table 1 were used to estimate antioxidant intake for the UK population based on fruit and vegetable consumption (Table 2). The average (mean) value was 670 μmol/d. A significant difference was detected in antioxidant intake between people living in London and South East England (730 μmol/d) compared to Northern England (610 μmol/d) ($p<0.05$). A similar difference was also been found between Central and South West England (660 μmol/d) compared to Northern England ($p<0.05$). In all social classes, the female population was found to have higher antioxidant consumption than the male. Non-parametric Mann-Whitney test showed a statistically significant difference ($p<0.05$) among all social classes except between social classes II and III (non-manual) (Table 3).

The Scottish sample of the NDNS comprised 123 people. The average (mean) estimated antioxidant intake in the Scottish sample from fruit and vegetables was 680 μmol/d (Table 2). A total of 113 people consumed less than 400 g of fruit and vegetables with an average (mean) antioxidant intake of 560 μmol/d. Only 10 people were found to consume ≥400g of fruit and vegetables and with a mean antioxidant intake of 2120 μmol/d (Table 6).

A detailed analysis of fruit and vegetable intake in the Scottish sample population showed that apples, bananas, orange citrus fruits, pears and honeydew melons were the most commonly eaten fruits and tomatoes, baked beans, peas, Iceberg lettuce and carrots were the commonly consumed vegetables. However, the main five fruits contributing to high intakes of antioxidants were apples, strawberries, orange citrus fruits, pears and bananas. For vegetables, purple broccoli, cauliflower, red pepper, tomatoes and yellow onion contributed most antioxidant intake (Table 4). Table 5 presents the different potential combinations of fruits and vegetables in the Scottish diet. The total antioxidant intakes achieved with these combinations were markedly higher in all cases than the

average (mean) intake achieved by those consuming more than 400 g of unspecified fruit and vegetables per day (Table 6).

Discussion:

In this study, the Scottish sample from NDNS was selected to see which fruits and vegetables contribute most to the antioxidant intake in the Scottish diet and to determine combinations of fruits and vegetables that could be used to enhance antioxidant consumption. The NDNS provides the only UK population representative weighed data on fruits and vegetable consumption, although the population size for Scotland is smaller than desirable. This database has been used to estimate dietary intake and trends in food consumption in Scotland for policy purposes (Wrieden et al, 2006) and provides the only weighed dietary methodology data available

The relatively low antioxidant intake from fruits and vegetables was mainly due to two factors: low intakes *per se* and the consumption of fruits and vegetables with a very low antioxidant capacity. From the intake data of commonly consumed fruits and vegetables, it was clear that apart from apples and orange citrus fruits other commonly consumed fruits and vegetables (e.g. bananas, tomatoes, baked beans, peas, Iceberg lettuce and carrots) have a low antioxidant capacity. The mean portion weight (86 g) of strawberries provides 1570 µmol of antioxidants whereas the mean portion weight (96 g) of banana provides only 200 µmol in the sample population. Similarly the mean portion weight (103 g) of broccoli provides 1520 µmol of antioxidants, which is more than twenty times higher than antioxidant capacity of a mean portion weight (64 g) for tomatoes (Table 4).

It has been reported that the antioxidant capacity of fruit and vegetables is mainly due to phenolics and flavonoid compounds (Liu *et al.,* 2002). Fruits and vegetables such as apples, different types of berries, citrus fruits, broccoli, kale and onions are the richest source of flavonoids (Ross and Kasum, 2002). These results serve to remind us that the beneficial effect of fruits and vegetables may be due to the combined action of different bioactive compounds (Halvorsen *et al.*, 2002). In this study fruit juices, dried fruits and vegetable dishes were not included due to unavailability of antioxidant data. This could under-estimate antioxidant consumption but none-the-less it still provides a useful insight

to relate to current fruit and vegetable recommendations. The impact of bioavailability phenolic antioxidants and other influences on cellular uptake, is a complex issue (Kroon et al. 2004, Donovan et al. 2006) and for reasons of practicality was not assessed in the current study.

The main reason for the recommendation of at least 400 g of different fruits and vegetables (World Health Organisation, is based on the fact that it will reduce the early death from coronary heart disease and cancer. Phenolic compounds and flavonoids acting as antioxidants are believed to play a major role in preventing oxidative damage at the cellular level. With current recommendation the average intake of fruit and vegetable in UK is 2.7 portions for men and 2.9 portions in women, with just 13% of males and 15% of females consuming five portions daily (Henderson *et al.* 2002). The reason for this low consumption is complex and intervention studies have found that different factors such as availability, accesses and affordability play an important role (Anderson *et al.,* 1998, Anderson, 2006).

Consuming a combination of fruits and vegetables with a high antioxidant content, as shown in the Table 5, is a convenient way to increase antioxidant intake. A combination of the top three or four sources of fruit and vegetables could provide even more than the mean antioxidant intake achieved by consuming 400g of fruits and vegetables. By consuming the top five fruit and vegetables (strawberries, purple broccoli, apples, orange citrus fruits and cauliflower) the mean intake and mean antioxidant content was found to be 520 g per day and 5970 μmol/d respectively (Table 5). The potential antioxidant consumption with these fruits and vegetables is 3-fold more than the mean intake of people taking ≥400g of unspecified fruits and vegetables (Table 6). Even with top two fruits and one vegetable or the top fruit and top two vegetables, the antioxidant intake is twice that of those consuming ≥400g of fruits and vegetables (Table 5). Therefore, this knowledge of the antioxidant capacity of fruit and vegetables could contribute to achieving a markedly higher antioxidant intake and a more nutrient dense diet. Furthermore, it is clear that total fruit and vegetable intake varies according to social position (Table 3) and where total intake is low it might be particularly useful to promote high antioxidant containing fruits and vegetables.

Lack of availability of fresh fruits is regarded as one the main barrier in achieving current targets. However, the climate of Scotland is extremely favourable for commercial cultivation of wide variety of soft fruits including raspberries, strawberries and

blackcurrants (Berry Scotland, 2006). Therefore, an increase in production of locally grown fruits and vegetables might help to achieve the recommended target for the Scottish people and also increase the antioxidant consumption of the population. Among the vegetables, cauliflower and purple broccoli are very rich in antioxidants and also widely grown in Scotland.

Conclusions:

The antioxidant intake determined by fruit and vegetable intake is low throughout the UK. The main factor behind this is low consumption of fruit and vegetables. However, certain fruits and vegetables contain relatively high levels of antioxidants. Different combinations of these fruits and vegetables showed that marked increased intakes of antioxidants could be achieved by including one top fruit (strawberries) and two top vegetables (broccoli and cauliflower). In Scotland, there is a huge potential for the cultivation of different varieties of berries and vegetables, which contain elevated levels of antioxidants. Therefore, knowledge of the antioxidant capacity of fruits and vegetables might encourage a higher consumption of antioxidant-rich produce. Alternatively, for people who find it difficult to achieve 'five-a-day', selection of these fruits and vegetables would help them to enhance their antioxidant intake and thereby achieve enhanced beneficial health effects without resorting to dietary supplements which may have unknown side effects (Expert Group on Vitamins and Minerals, 2003).

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Conflict of Interest

None

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Table 1 Antioxidant contents of some commonly consumed fruits and vegetables*

Fruits and vegetables	Antioxidant content	Fruits and vegetables	Antioxidant content
Blackcurrant	57.2 ± 3.5	Nectarines	5.4 ± 0.1
Blackberries	50.1 ± 2.5	Kale	4.3 ± 0.2
Broad beans	23.5 ± 1.1	Red grapes	3.5 ± 0.0
Blueberries	22.4 ± 0.3	Cherry tomatoes	3.1 ± 0.0
Raspberries	20.1 ± 0.4	Yellow onion	2.9 ± 0.2
Cranberries	20.0 ± 2.5	Pears	2.7 ± 0.0
Broad beans - whole	19.6 ± 0.5	Melon pieces	2.4 ± 0.1
Strawberries	18.3 ± 0.1	Bananas	2.0 ± 0.0
Purple broccoli	14.7 ± 2.8	Peas (frozen)	1.8 ± 0.1
Cauliflower	9.0 ± 0.9	Peas (boiled)	1.5 ± 0.0
Apples	8.6 ± 0.3	Tomatoes	1.4 ± 0.0
Orange citrus fruits	8.6 ± 0.6	Canned mixed vegetables	1.4 ± 0.1
Curly kale	8.3 ± 0.2	Organic potatoes	1.4 ± 0.0
Yellow pepper	8.1 ± 0.2	Baked beans	1.3 ± 0.1
Ruby Nell plums	7.2 ± 0.3	Green Pepper	1.2 ± 0.1
Red pepper	6.8 ± 0.5	White potatoes	1.1 ± 0.1
Mango	6.6 ± 0.0	Carrots	1.0 ± 0.0
Kiwi slices	6.5 ± 0.2	Honeydew melon	1.0 ± 0.1
Red onion	5.5 ± 0.0	White onion	0.8 ±0.0

* Data expressed in μmol Fe^{+3}/g as mean values ± standard deviation (n = 3)

Table 2 Daily intakes of antioxidants (μmol/d) in the UK population from fruits and vegetables

Region	Sex	Mean	Median	S.D.*
Scotland	Male	748	529	756
	Female	685	407	664
	Total	680	415	689
Northern England	Male	579	381	560
	Female	642	396	721
	Total	610	377	651
Central and South West England and Wales	Male	635	445	650
	Female	691	498	641
	Total	660	481	637
London and South East England	Male	733	526	704
	Female	720	550	662
	Total	730	530	692
Total	Male	660	450	656
	Female	686	487	670
	Total	670	466	663

*S.D. = standard deviation

Table 3 Average daily antioxidant intake from fruits and vegetables (μmol/d) in different social class

Social Class	Sex	Mean	Median	S.D.*
I	Male	877	745	656
	Female	967	909	625
	Total	890	806	647
II	Male	756	595	620
	Female	782	588	724
	Total	765	586	682
III (Non-manual)	Male	611	391	571
	Female	716	515	638
	Total	692	494	628
III (Manual)	Male	633	419	724
	Female	724	648	546
	Total	637	435	671
IV	Male	532	244	696
	Female	581	357	711
	Total	559	319	705
V	Male	300	191	289
	Female	376	241	437
	Total	345	213	385

*S.D. = standard deviation

Table 4 Average daily antioxidant intake from the top five fruit and vegetable contributors in Scottish NDNS consumers, and antioxidant intake per portion

Fruit/vegetable	Mean intake (g/d)*	Mean antioxidant intake (µmol/d)	Mean portion size (g)	Mean antioxidant intake per portion (µmol)
Strawberries	23 (*n*=15)	420	86	1570
Apples	56 (*n*= 55)	480	130	1120
Orange citrus		330		
fruits	38 (*n*=44)		105	900
Pears	48 (*n*=22)	130	157	430
Bananas	44 (*n*=69)	90	97	200
Purple broccoli	32 (*n*= 89)	240	103	1520
Cauliflower	16 (*n*=22)	140	95	860
Red pepper	8 (*n*=9)	60	33	230
Yellow onion	13 (*n* =50)	40	64	180
Tomatoes	30 (*n*=29)	40	61	70

**n*= Number of consumers

Table 5 Impact of different potential combinations of fruits and vegetables on antioxidant intake

Combinations	Potential fruit and vegetable intake (g/day)*	Potential antioxidant intake (µmol/d)
Top five sources	520	5970
Top four sources	424	5112
Top 2 fruit plus top 3 vegetable sources	447	5295
Top 2 fruit plus top 2 vegetable sources	415	5069
Top 2 fruit plus top vegetable sources	319	4208
Top fruit and top two vegetable sources	285	3948

*Using mean portions of fruits and vegetables consumed (Table 4)

Table 6. Comparison of antioxidant intake in people consuming unspecified fruits and vegetables in the Scotland

Fruits and vegetables	Mean Intake (g/d)	Median intake (g/d)	Mean antioxidant intake (µmol)
Unspecified <400g per day	157	135	560
Unspecified >400g per day	505	467	2120

Appendix 2

http://www3.interscience.wiley.com/cgi-bin/fulltext/114274037/PDFSTART

The bioavailability of raspberry anthocyanins and ellagitannins in rats

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Short title: Bioavailability of raspberry phenolics in rats

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Abstract

The fate of anthocyanins and ellagitannins in rats was monitored following ingestion of raspberry juice. After 1 h low nM concentrations of unmetabolised anthocyanins were present in plasma but these declined by 2 h and after 4 h they were no longer detectable. For the first 2 h there was an almost full recovery of anthocyanins as they passed from the stomach through the duodenum/jejunum and into the ileum. After 3 h less than 50% were recovered, and the levels declined rapidly thereafter. Excretion of raspberry anthocyanins in urine over a 24 h period was equivalent to 1.2% of the amount ingested. Trace quantities of anthocyanins were detected in the caecum, colon and faeces and they were absent in extracts of liver, kidneys and brain. Urine also contained a number of phenolic acids but most were present in quantities well in excess of the 918 nmoles of anthocyanins present in the ingested juice. These findings indicate that raspberry anthocyanins *per se* are poorly absorbed, probably prior to reaching the ileum, and that substantial amounts pass from the small to the large intestine where they are degraded by colonic bacteria. Ellagitannins disappeared in the stomach without accumulation of ellagic acid.

1 Introduction

Anthocyanins are glycosylated anthocyanidins, one of the major sub-groups of the C_6-C_3-C_6 flavonoids. They are red and blue coloured pigments and occur widely in the plant kingdom being present in leaves, flowers and fruits [1]. They are normal dietary components, being present in a wide range of fruits, vegetables and beverages [2, 3]. Anthocyanins are antioxidants [4,5] and have ant-carcinogenic [6] and anti-inflammatory properties [7], are reported to improve vision [8] and enhance memory [9] and may also reduce the incidence of coronary heart disease [10]. The average daily intake of anthocyanins in the USA has been estimated to be 12.5 mg per person [3]. However, it is relatively easy for consumers to markedly increase their consumption of anthocyanins as concentrations in red wine of 120 mg/L are not unusual [11], a 100 g serving of some berries can contain >100 mg [3] and there is a report of 200 mL of Austrian elderberry juice containing 2 g of anthocyanins, principally as cyanidin-3-*O*-glucoside and cyanidin-3-*O*-sambubioside [12].

In order to better understand the action and potential protective effects of dietary anthocyanins *in vivo* more needs to be known about their fate following ingestion. Although there are exceptions, unlike other flavonoids that have been absorbed and/or excreted [13], most anthocyanins do not undergo extensive metabolic modification of the parent glycosides to glucurono-, sulpho- and methyl-derivatives [14-17]. In feeding studies with animals and humans, typically *ca.* 0.1% of the quantities ingested, and sometimes much less, have been detected in plasma and urine [14, 15] implying a low level of absorption compared to other flavonoids [13]. However, *in situ* perfusion studies with rat small intestine indicate that absorption may be of the order of 10-20%, depending upon the aglycone moiety [18, 19]. The low concentration of anthocyanins in plasma may, therefore, be a consequence of their rapid removal from the circulatory system. In keeping with this possibility, following injection of delphinidin-3-*O*-rutinoside into the neck vein of rats, plasma concentrations fell from 26 nM, 1 min post-injection, to 5 nM after 15 min and were not detectable after 2 h [20]. However, after intravenous administration of cyanidin-3-*O*-glucoside to rats there was a 36% recovery of the glucoside and its metabolites in urine and 12 % in bile within 4 h [21]. This implies that the perfusion experiments may have over-estimated the extent of anthocyanin absorption from the gastrointestinal tract as if 10-20% of the ingested anthocyanins entered the circulatory system this would be

accompanied by the appearance of substantially higher quantities in urine than the *ca.* <0.1% of intake that is routinely obtained in anthocyanin feeding studies [14, 15].

Excretion of <0.1% of intake was obtained with 15 anthocyanins in the urine of rats and humans after supplementation with blueberry, boysenberry, black raspberry and black currant extracts [15]. The ratio of the levels of some of the individual anthocyanins in urine was different to that found in the berries. For instance, after supplementation with a boysenberry extract, the relative concentrations of cyanidin-3-*O*-sophoroside, cyanidin-3-*O*-(^{2}G-*O*-glucosylrutinoside) and cyanidin-3-*O*-rutinoside were comparable to the berry extract indicating that the three anthocyanins behave similarly with respect to absorption and excretion. In contrast, the relative concentration of cyanidin-3-*O*-glucoside was much lower in urine suggesting that the nature of the sugar moiety has an influence on anthocyanin bioavailability. Overall the data obtained in this study suggest that the determinants of absorption and excretion of anthocyanins are influenced by the nature not only of the sugar moiety but also the anthocyanidin structure [15]. This conclusion is supported by data obtained in a recent study in which weanling pigs were fed acute supplements of chokeberry, blackcurrant and elderberry extracts [22].

This paper reports on a study the bioavailability of anthocyanins and ellagitannins following the ingestion of raspberry juice by rats. The animals were which were fed raspberry juice by gavage after which body tissues and fluids, collected over a 24 h period, were analysed by HPLC with photodiode array (PDA) and tandem MS (MS^2).

2 Materials and methods

2.1 Chemicals

4-Hydroxybenzoic acid was obtained from Aldrich (Poole, Dorset, UK), 4-hydroxyphenylacetic acid from Fluka, (Gillingham, Dorset, UK), hippuric acid and ferulic acid from Sigma, (Poole, Dorset, UK) while other phenolic acids were purchased from AASC (Southampton, Hants, UK). Derivatization reagent *N,O*-bis(trimethylsilyl)acetamide (BSTFA) and 1% trimethylchlorosilane (TMCS) were also purchased from Sigma, as were formic acid, ellagic acid and cyanidin-3-*O*-glucoside. Methanol and ethyl acetate was obtained from Rathburn Chemicals (Walkerburn, Borders, UK). Cyanidin-3-*O*-sambubiosyl-5-*O*-glucopyranoside was purchased from Polyphenols Laboratories AS (Sandnes, Norway).

2.2 Animal and sample preparation

Sprague–Dawley male rats (n = 24), weighing 277 ± 21 g, were housed in metabolic cages allowing the collection of 24 h urine and faecal samples. Rats were deprived of food but did have access to water for 16 h before and 24 h after being fed 2.77 mL of raspberry juice by gavage. The juice was obtained by squeezing raspberries (*Rubus idaeus* var. Glen Ample) and 1 mL of juice corresponded to 1.2 g of raspberries. As a blank, urine was collected for a 3 h period prior to supplementation. Three animals were terminally anaesthetised with pentobarbital at 0, 1, 2, 3, 4, 6, 12 and 24 h after administration of the juice. Blood was removed by cardiac puncture with heparin-moistened syringes and plasma was obtained by centrifugation at 2000 *g* for 10 min at 4°C after which 250 μL aliquots were acidified to pH 3.0 with 7.5 μL 50% aqueous formic acid and 25 μL of ascorbic acid (10 mM). Liver, kidney and brain were prefused *in situ* with chilled 0.15 M KCl, and then removed along with stomach, duodenum/jejunum, ileum, caecum and colon, with their contents intact, at each time point. Urine and faeces were collected and acidified to pH 3.0. All samples were frozen in liquid nitrogen and stored at -80°C. With the exception of plasma and urine, all samples were lyophilised prior to analysis.

2.3 Processing rat tissues, plasma and urine

Tissue samples from three individual rats at each time point were combined, extracted by homogenising 200 mg of lyophilised tissue with 2 mL of 50% aqueous methanol containing 1% formic acid using an Ultra-turrax homogeniser and further extracted by continuous shaking for 30 min. The mixture was centrifuged at 2000 *g* for 20 min, the supernatant decanted and the pellet re-extracted twice. The three supernatants were combined and reduced to dryness *in vacuo*. The extract was dissolved in 50 μL methanol in 1950 μL aqueous 1 % formic acid and loaded on to a 2 g Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) which was washed with 4 mL acidified water (pH 3·0) before elution with 4 mL methanol containing 1% formic acid. The methanolic eluates were reduced to dryness and resuspended in 50 μL methanol in 950 μL aqueous 1 % formic acid before analysis by HPLC–PDA-MS^2. Plasma from individual rats was loaded directly onto the Sep-Pak C_{18} cartridge without extraction while urine from each animal was analysed directly without extraction or purification. Cyanidin-3-*O*-sambubioside-5-*O*-glucoside was used as an internal standard.

2.4 HPLC-PDA-MS^2

All samples were analysed in triplicate on a Surveyor HPLC system comprising of a HPLC pump, diode array detector scanning from 250 to 700 nm, and an autosampler set at 4°C (Thermo Finnigan, San Jose, USA) with separation carried out using a 250 x 4·6 mm internal diameter 4 μm Synergi RP-Max column (Phenomenex, Macclesfield, UK) eluted at a flow rate of 1 mL/min. A mobile phase consisting of a 30 min 8–18 % gradient of acetonitrile in 1 % aqueous formic acid was used for the analysis of all samples. After passing through the flow cell of the diode array detector the column eluate was split and 0.3 mL was directed to a LCQ Deca XP ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan). Analysis was carried out with positive and negative ionisation operating in full-scan mode from 100 to 2000 atomic mass units (*amu*). The tuning of the mass spectrometer was optimised by infusing a standard of cyanidin-3-*O*-glucoside and ellagic acid dissolved in methanol containing 1 % formic acid, into the source at a flow rate of 0.25 mL/min. Capillary temperature was 350°C, sheath gas and auxiliary gas were 80 and 20 arbitrary units, respectively, source voltage was 5 kV. Identification of anthocyanins, their metabolites and other compounds in all samples was carried out using full-scan data-dependent MS^2. Quantitative estimates of the anthocyanins and other phenolic compounds in the raspberries were based on the absorbance response at 520 nm and 280 nm. Anthocyanins were quantified by reference to cyanidin-3-*O*-glucoside as a standard. Ellagitannins were quantified by reference to a gallic acid standard curve and the data expressed in nmoles of sanguiin H-6 and lambertianin C.

2.5 GC-MS analysis of urine

Urine samples from individual rats were prepared as described by Olthof *et al.* [23] with slight modification. After thawing, 1.0 mL aliquots of urine were added to 4.0 mL of 0.2 M hydrochloric acid (HCl) containing 30 μg of 2,4,5 trimethoxycinnamic acid as an internal standard. A styrene divinyl benzene (SDB-L) (Phenomenex, USA) solid phase extraction cartridge was used for purification. Before loading the acidified urine, cartridge was pre-conditioned with 5 mL of ethyl acetate, followed by methanol (5 mL) and finally 5 mL 0.1M HCl. After the addition of the extract the cartridge was washed with 5 mL of 0.1M HCl before elution with 3 mL of ethyl acetate. The upper ethyl acetate phase was separated from the traces of aqueous phase and dried using an activated molecular sieve (Sigma) prior to being reduced to dryness. The extract was then redissolved in ethyl

acetate and transferred to a silylated glass vial and further dried with nitrogen gas. Three hundred μL of BSTFA + 1% TMCS was then added to the vial which was sealed and the sample silylated by heating at 80°C for 80 min. The vials was vortexed every 30 min to ensure complete silylation. Care was taken during preparation as the reagents and silylated derivatives are both highly sensitive to moisture. Samples were cooled in a closed, dry container prior to analysis by GC-MS (Trace DSQ, Thermo Finnigan). Phenolic acids were separated on a ZB-5MS 30 m x 0.25 i.d. X 0.25 μm capillary column (Phenomenex with helium as a carrier gas (1.0 mL/min). The GC-MS conditions were as follows; injection volume (1 μL), initial temperature 80 °C for 5 min then to 160°C at 10°C/min for 10 min and to 235°C at 5°C/min for 10 min; injector temperature (280°C), MS transfer line (290°C), ion source (200 °C), split ratio (1:100). Mass spectra were scanned from *m/z* 50-650 at 0.82 scans/sec. Electron impact energy was 70ev. Identification of phenolic compounds in urine was based on the retention time and mass spectra of authentic standards and NIST98 mass spectral library. Quantifications were based on a standard curve of 2,4,5-trimethoxycinnamic acid (internal standard). All standards and samples were analysed in triplicate.

3.1 Analysis of raspberry juice

3 Results and discussion

The HPLC-PDA-MS^2 analysis of the raspberry juice resulted in the identification and quantification of nine anthocyanins, two ellagitannins and ellagic acid. The HPLC profiles of the extract at 280 nm and 520 nm are shown in Figure 1. Identifications, which are summarised below were based on published data on the MS^2 fragmentation of raspberry phenolics [24, 25].

Peak 1 correspond to cyanidin-3,5-*O*-diglucoside (*m/z* 625 ∧ 449, 287); *peak 2* correspond to cyanidin-3-*O*-sophoroside (*m/z* 611 ∧ 287); *peak 3* was cyanidin-3-*O*-(2^G-*O*-glucosylrutinoside) (*m/z* 757 ∧ 611, 287); *peak 4* was cyanidin-3-*O*-glucoside (*m/z* 449 ∧ 287); *peak 5* was pelargonidin-3-*O*-sophoroside (*m/z* 595 ∧ 271); *peak 6* was cyanidin-3-*O*-xylosylrutinoside (*m/z* 727 ∧ 581, 287); *peak 7* was cyanidin-3-*O*-rutinoside (*m/z* 595 ∧ 287); *peak 8* correspond to pelargonidin-3-*O*-(2^G-*O*-glucosylrutinoside) (*m/z* 741 ∧ 595, 271); *peak 9* was pelargonidin-3-*O*-glucoside (*m/z* 433 ∧ 271); *peak 10* was the ellagintannin lambertianin C (*m/z* 2801 ∧ 1869, 1567, 1265, 1251, 935, 633); *peak 11* is

also an ellagitannin, sanguiin H-6 (*m/z* 1869 ∧ 1567, 1265, 1235, 933, 631) and *peak 12* is ellagic acid (*m/z* 301 ∧ ***3.2 Ingestion of raspberry juice by rats*** 257).

The main raspberry anthocyanins are di- and tri-glycosides (Table 1) with the disaccharide cyanidin-3-sophoroside being the major anthocyanin (56% of the total anthocyanins) in the juice followed by the trisaccharide cyanidin-3-*O*-(2^G-*O*-glucosylrutinoside) (23%) and the monosaccharide cyanidin-3-*O*-glucoside (11.1%). The ellagitannins lambertianin C and sanguiin H-6 were also present along with ellagic acid as reported previously [25]. Ellagitannins are powerful antioxidants in raspberries [24] and acid treatment of extracts results in their breakdown and the release of ellagic acid. In addition to the compounds listed in Table 1 the raspberry juice also contained a number of mono-, di- and tri-saccharide flavonol conjugates which appeared as HPLC peaks on the 365 nm trace. This is in keeping with the data of Mullen *et al.* [25]. However, these compounds were present in very low concentrations which made it impractical to monitor their fate in rats.

Each rat ingested 2.77 mL of raspberry juice containing 8.7 nmoles cyanidin-3,5-*O*-diglucoside, 513 nmoles cyanidin-3-*O*-sophoroide, 211 nmoles cyanidin-3-*O*-(2^G-*O*-glucosylrutinoside), 102 nmoles cyanidin-3-*O*-glucoside, 14 nmoles pelargonidin-3-*O*-sophoroside, 5.5 nmoles of caynidin-3-*O*-xylosylrutinoside, 46 nmoles cyanidin-3-*O*-rutinoside, 14 nmoles pelargonidin-3-*O*-(2^G-*O*-glucosylrutinoside) and 3.7 nmoles of pelargonidin-3-*O*-glucoside. The 2.77 mL of juice, thus, contained a total of 918 nmoles of anthocyanins as well as 355 nmoles of ellagitannins and 36 nmoles of ellagic acid (Table 1). This supplement is equivalent to a 70 kg human consuming 700 mL of raspberry juice.

3.4 Fate of anthocyanins

Figure 2 provides an over view of the fate of raspberry juice anthocyanins after being fed to rats by gavage. The HPLC-520 nm anthocyanin profiles of plasma, urine, faeces and the gastrointestinal organs are very similar to that of the ingested juice, containing principally cyanidin-3-*O*-sophoroside, cyanidin-3-*O*-(2^G-*O*-glucosylrutinoside) and cyanidin-3-*O*-glucoside. Putative metabolites were also present, but in trace amounts which, in most instances, precluded identification. Mass spectral information was, however, obtained on two minor urinary metabolites. The larger, earlier eluting 520 nm peak had a positively

charged molecular ion ($[M+H]^+$) at *m/z* 625 which on MS^2 fragmented with a loss of 324 *amu* (cleavage of a sophorosyl unit) to yield an ion at 301 *m/z* which corresponds with methyl-cyanidin. In view of the presence of cyanidin-3-*O*-sophoroside in raspberries, this minor metabolite is probably its 3′-methylated derivative peonidin-3-*O*-sophoroside. The second metabolite in urine had a $[M+H]^+$ at *m/z* 463 which ionised with a 164 *amu* loss to produced an MS^2 fragment at *m/z* 301. This again is in keeping with a 3′-methylation resulting in the conversion of cyanidin-3-*O*-glucoside to peonidin-3-*O*-glucoside.

Quantitative estimates of the overall levels of anthocyanins in the rat tissues and fluids over a 24 h period after ingestion are presented in Table 2. Within the first hour 59.4 % of the ingested anthocyanins were found in the ileum with 31.5 % remaining in the stomach. After 2 h almost all the anthocyanins had left the stomach and moved to the ileum (85.9%). At the 1 and 2 h time points the overall recoveries of anthocyanins, almost exclusively from the gastrointestinal tract, were high at 99.6% and 97.0%, respectively, of intake. Three hours after ingestion more than 50% of the anthocyanins had disappeared and of the original dose 40.3% was still in the ileum with 6.5% distributed in the rest of the digestive tract. After 6 h only 2% of the anthocyanins remained principally in the caecum and colon in their native forms. Over a 24 h post-ingestion period, 1.5% of the anthocyanins appeared in faeces, 1.2% was excreted in urine and only traces were detected in plasma (Table 2).

The percentage recoveries of the three main raspberry anthocyanins, cyanidin-3-*O*-sophoroside, cyanidin-3-*O*-(2^G-*O*-glucosylrutinoside) and cyanidin-3-*O*-glucoside in rat tissues, plasma, faeces and urine over a 24 h post-gavage period are shown in Table 3. After 2 h there were only trace losses of the sophoroside and the glucosylrutinoside while cyanidin-3-*O*-glucoside declined by almost 40%. Arguably, this suggests that the monosaccharide may be more readily metabolised and/or absorbed that the di- and trisaccharide. However, there was no evidence of preferential increases in the low levels of cyandin-3-*O*-glucoside in either plasma or urine compared to those of cyanidin-3-*O*-sophoroside and cyanidin-3-*O*-(2^G-*O*-glucosylrutinoside) (Table 4) implying that the initial decline in the glucoside in the gastrointestinal tract may due to metabolism, possibly conversion to peonidin-3-*O*-glucoside which is excreted in urine (see Table 5), rather than preferential absorption into the circulatory system.

Three hours after feeding, anthocyanin levels in the ileum had declined without concomitant increases further down the gastrointestinal tract in the caecum and colon (Table 3). Between 2 and 3 h there was also a fall in the overall recovery of the individual anthocyanins with values of 50% or less being obtained (Table 3). This trend continued with *ca.* 10% recoveries at 4 h, and at 6, 12 and 24 h losses were of the order of 98+%; a pattern in keeping with bacterial degradation of anthocyanins in the colon and, possibly, also the caecum.

Analysis of purified extracts of brain, liver and kidneys of rats did not detect the presence of any anthocyanins or anthocyanin metabolites. Plasma contained low nM concentrations of cyanidin-3-*O*-sophoroside and cyanidin-3-*O*-(2^G-*O*-glucosylrutinoside) and sub-nM levels cyanidin-3-*O*-glucoside (Table 4). The levels of the cyanidin-3-*O*-sophoroside and cyanidin-3-*O*-(2^G-*O*-glucosylrutinoside) were highest (T_{max}) 1 h after ingestion of the raspberry juice with respective C_{max} values of 16.4 ± 3.4 and 7.7 ± 2.1 nM. As the highest plasma anthocyanin concentrations occurred at the first time point, if earlier samples had been collected it is possible that a T_{max} of less than 1 h would have been obtained and that the C_{max} values may have been slightly higher. After 1 h the concentration of both anthocyanins declined rapidly. The elimination half-life ($T_{1/2}$) values were 1.33 h for cyanidin-3-sophoroside and 1.03 h for cyanidin-3-(2^G-glucosylrutinoside), with no anthocyanins being detected in plasma at the 4, 6, 12 and 24 h time points. The amounts of anthocyanins detected in plasma were very low indeed. On the basis of each rat containing a total of 12 mL of plasma, the 16.4 nM concentration of cyanidin-3-sophoroside present at T_{max} is equivalent to 0.038% of the amount ingested and the figure for cyanidin-3-(2^G-glucosylrutinoside) is 0.044%. The level of cyanidin-3-glucoside was *ca.*10-fold lower (Table 4).

In keeping with the pharmacokinetic data obtained with plasma, the highest level of anthocyanin excretion in urine, 4.34 ± 1.90 nmoles, occurred 1-2 h after feeding the raspberry juice (Table 5). The total urinary excretion over a 24 h period was 9.7 ± 2.1 nmoles corresponding to 1.2 ± 0.2 % of the anthocyanins ingested, a figure somewhat higher than that obtained in many anthocyanin-feeding studies. As mentioned previously, the urinary anthocyanin profile was very similar to that of raspberries with the exception that three minor components, cyanidin-3,5-*O*-diglucoside, pelargonidin-3-*O*-(2^G-*O*-glucosylrutinoside) and pelargonidin-3-*O*-glucoside which were not detected, while the methylated metabolites, peonidin-3-*O*-sophoroside and peonidin-3-*O*-glucoside were

present in quantifiable amounts (Table 5). Overall, however, absorption and excretion was not associated with extensive metabolism of the raspberry anthocyanins.

It is of note that the levels of anthocyanins in plasma were highest 1 h time after supplementation with the raspberry juice (Table 4) at which point most of the anthocyanins in the gastrointestinal tract were in the stomach (31.5%) and the ileum (59.4%) (Tables 2 and 3). Over the next hour the levels declined in the stomach and rose in the ileum to 85.9% of intake while the low concentrations in the bloodstream declined. This suggests that the albeit low absorption of anthocyanins occurs before reaching the ileum. This is in keeping with evidence obtained in other studies with rats and mice indicating that the stomach [26, 27] and the jejunum [28] are sites of anthocyanin absorption.

As noted above, anthocyanins were not detected in the brain of rats following the ingestion of the 2.77 mL supplement of raspberry juice that is equivalent to a 70 kg human consuming 700 mL of juice. Other studies have detected anthocyanins in rat brains after supplementation with berry and grape extracts. Andres-Lacueva *et al.* [29] daily fed rats a blueberry extract, containing an uncited amount of anthocyanins, for 8-10 weeks after which the animals exhibited enhanced special learning and memory in the Morris water maze test and trace levels of anthocyanins, which could not be quantified, were detected in the cerebellum, cortex and hippocampus, regions of the brain important for learning and memory. Extremely low concentrations of anthocyanins, 0.25 nmoles/g, were also detected in rat brains after feeding a blackberry extract for 15 days [30]. The daily dose of anthocyanins was of the order of 318 μmoles per rat which is equivalent to a 70 kg human eating a daily serving of *ca.* 18 kg of blackberries. It has also been reported that within 10 min of the introduction of a red grape extract, containing 3.8 μmol of anthocyanins (2 mg), into the stomach of rats, unmetabolised anthocyanins were detected in plasma (176 ng/mL) and also in the brain (192 ng/g) [30]. This intake corresponds to a 70 kg human consuming *ca*. 300 g of red grapes, an amount that is an intake in keeping with a normal dietary intake.

3.5 Fate of ellagitannins

Along with the anthocyanins, the raspberry juice fed to rats contained substantial amounts of the ellagitannins lambertianin C and sanguiin H-6 together with a smaller quantity of ellagic acid (Fig. 1 and Table 1). The ellagitannins disappeared rapidly and were not

detected in plasma, urine, faeces, the stomach, duodenum/jejunum, ileum, and other parts of the gastrointestinal tract, 1 h after ingestion and at all later time points. This contrasts with the anthocyanins which at 1 h were recovered intact, mainly in the stomach and ileum (Table 2). The absence of ellagitannins in the rats implies that breakdown of sanguiin H-6 and lambertianin C occurred in the acidic conditions of the stomach to such an extent that they had disappeared after 1 h. HPLC-MS^2 analysis of urine demonstrated an absence of urolithin B, hydroxyurolithin B and their glucuronide conjugates, compounds excreted by rats after a 37-day intake of a pomegranate husk extract containing very high quantities of the ellagitannin punicalagin [32].

Ellagic acid recovered in the stomach after 1 h was 9.6% of the amount present in the raspberry juice and after 2 h only traces were detected (Fig. 3). Except for these small amounts in the stomach, no ellagic acid was detected in any of the rat organs/tissues or fluids collected over a 24 h period after consumption of the raspberry juice. Degradation of the ellagitannins has the potential to release substantial amounts of ellagic acid. In theory, the ingested 71 nmoles of lambertianin C and 284 nmoles of sanguiin H-6 could give rise to 426 and 1136 nmoles of ellagic acid, respectively. This would appear not to have occurred. An accurate picture of the fate of the ellagitannins is unlikely to be ascertained until radio-labelled derivatives become available.

3.6 GC-MS analysis of phenolic acids in urine

In view of the likely involvement of colonic bacteria in the disappearance of anthocyanins after the ingestion of raspberry juice a search was made of urine HPLC profiles within the 240-365 nm range for the accumulation of putative catabolites. Most of the traces were very complex and no clear-cut evidence of the presence of catabolites derived from raspberry anthocyanins was obtained. Protocatechuic acid (3,4-dihydroxybenzoic acid), vanillic acid (3-methoxy-4-hydroxybenzoic acid) and syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid) have been proposed as a colonic breakdown products of anthocyanins [33-36] but HPLC-MS^2 analysis revealed they not present in detectable amounts in any of the urine samples collected in the present study. An alternative analytical strategy was therefore employed, based on the methods of Olthof *et al.* [23], which involved forming trimethylsilyl derivatives and analysing samples by capillary GC-MS. Representative traces obtained are presented in Figure 4, the identification of the

compounds detected is summarised in Table 6, and quantitative estimates of the amounts excreted over a 24 h post-gavage period are presented in Table 7.

Nine compounds were identified in the urine samples but, in most instances, the amounts excreted over the 0-24 h collection period were well in excess of the 0.9 μmoles of ingested anthocyanins (Table 6). While 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid may be anthocyanin catabolites, this is speculative and definitive information will require feeding studies to be carried out using ring-labelled ^{14}C-anthocyanins. As far as ellagitannins are concerned, each rat consumed 71 nmoles of lambertianin C and 284 nmoles of sanguiin H-6 but neither of these compounds entered the large intestine and are, therefore, unlikely to be degraded to phenolic acids by the colonic microflora.

3.7 Stability of anthocyanins in the gastrointestinal tract

Anthocyanins are readily distinguished from other flavonoids as they undergo re-arrangements in response to pH. The red flavylium cation predominates at pH 1-3 but as the pH increases to 4 and above the colourless carbinol pseudobase is the major component along with smaller amounts of the colourless chalcone pseudobase and the blue quinoidal base [37]. Anthocyanins are traditionally extracted and analysed in acidic medium as the red flavylium cation as this is the most stable form. However, it is not known what forms predominate *in vivo*. The limited available experimental evidence indicates that in the acidic conditions that prevail in the stomach anthocyanins are in the red flavylium form but once they enter more basic conditions in the small intestine the carbinol pseudobase is likely to predominate [15]. To what extent this occurs and what influence it has on absorption remains to be determined. In the present study extracting the gastrointestinal tract and its contents with acidic methanol resulted in high recoveries of the flavylium cation from the duodenum, jejunum and ileum in the initial two hours after ingestion of the raspberry juice (Tables 2 and 3). However, it does not follow that *in vivo* the anthocyanins were in this form and, due to an absence of appropriate analytical procedures, nothing is known about either the metabolism or absorption of the pseudobases or the quinoidal base in the gastrointestinal tract. It could be that after anthocyanins leave the stomach, the colourless carbinol pseudobase becomes the main form, in the small intestine where it undergoes very limited absorption. As a consequence, significant amounts pass into the large intestine where degradation, to as yet undetermined products, occurs due to the action

of colonic bacteria. This would be in keeping with the data obtained in this study but other more subtle scenarios may exist.

4 Concluding remarks

Rats were fed by gavage a single 2.77 mL supplement of raspberries juice, containing anthocyanins and ellagitannins – a dose equivalent to a 70 kg human drinking 700 mL of juice. One hour after feeding the ellagitannins, sanguiin H-6 and lambertianin C had disappeared with only traces of ellagic acid being detected in the stomach. Up to 2 h after supplementation there was a very high recovery of unmetabolised anthocyanins, principally cyanidin-3-*O*-sophoroside, cyanidin-3-*O*-(2^{G}-*O*-glucosylrutinoside) and cyanidin-3-*O*-glucoside, as they passed from the stomach to the duodenum/jejunum and ileum. After 3 h, less than 50% was recovered, after 4 h this declined to 11% of intake and after 6 h only 2% remained. Only trace quantities of anthocyanins were detected in the caecum, colon and faeces and they were absent in extracts of liver, kidneys and brain.

After 1 h low nM concentrations of cyanidin-3-*O*-sophoroside, cyanidin-3-*O*-(2^{G}-*O*-glucosylrutinoside) and cyanidin-3-*O*-glucoside were detected in plasma but these declined by 2 h and were not present in detectable quantities 4 h after feeding. Excretion of the three main raspberry anthocyanins in urine over a 24 h period after feeding was equivalent to 1.2% of the amounts ingested. These findings imply anthocyanins are poorly absorbed and that this occurs before they reach the ileum, in keeping with evidence indicating that the stomach [26, 27] and the jejunum [28] are sites of anthocyanin absorption in mice and rats. Because anthocyanins are poorly absorbed substantial amounts pass from the small to the large intestine where their rapid disappearance suggests they are degraded by faecal bacteria. GC-MS analysis of urine detected a number of phenolic acids but it was not possible to determine which were catabolites derived from the raspberry juice as most were present in quantities well in excess of the 918 nmoles of anthocyanins that were ingested.

More complete information on what happens to anthocyanins and ellagitannins after ingestion would be greatly assisted by the availability of radio-labelled derivatives which would enable the fate of these compounds to be monitored in animal test systems in much the same way as [2-^{14}C]quercetin-4′-*O*-glucoside has been used to provide a detailed insight of flavonol glucoside bioavailability in rats [38-40].

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Figure legends

Figure 1. Gradient reversed phase HPLC of raspberry juice with detection at 520 and 280 nm. For identification by MS^2 and quantification of peaks 1-12 see text and Table 1.

Figure 2. Gradient reverse phase HPLC with detection at 520 nm of raspberry juice and of rat tissues and fluids after ingestion of raspberry juice. For peak numbers see Table 1. Asterisk indicates a metabolite. Duoden-Jejun, duodenum-jejunum.

Figure 3. Gradient reversed phase HPLC-365 nm profile of raspberry juice and extracts of rat stomachs collected 1 and 2 h after the ingestion of the raspberry juice .

Figure 4. GC-MS total ion current traces of urine collected from rats prior to feeding raspberry juice by gavage supplementation (blank) and 6-12 h after supplementation. For identity of peaks see Table 6. IS, internal standard.