Effect of storage and freezing on the phenolic and antioxidant activities of raspberries

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Abstract

A method was developed to analyze anthocyanins in red raspberries (*Rubus idaeus* L.) cv. Glen Ample. The anthocyanins were extracted from the fruit by homogenizing in acidified methanol. The methanolic extract was centrifuged and the supernatant analyzed by reverse-phase HPLC. The eluent was monitored at 371 and 520 nm before being introduced into a single quadrupole mass spectrometer through an atmospheric pressure chemical ionization probe operating in positive ion mode. This method facilitated the identification of eight anthocyanins in the raspberry matrix.

Further analysis of raspberry extracts by gradient, reverse-phase HPLC with diode array detection and tandem mass spectrometry identified eleven anthocyanins, including cyanidin-3-sophoroside, cyanidin-3-(2^G-glucosylrutinoside), cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-sophoroside, pelargonidin-3-(2^G-glucosylrutinoside) and pelargonidin-3-glucoside. Significant quantities of an ellagitannin, sanguiin H-6, with a M_r of 1870, were detected along with lower levels of a second ellagitannin, lambertianin C which has a M_r of 2804. Other phenolic compounds that were detected included trace levels of ellagic acid and its sugar conjugates along with one kaempferol and four quercetin-based flavonol conjugates. Fractionation by preparative HPLC revealed that sanguiin H-6 was a major contributor to the antioxidant capacity of raspberries together with vitamin C and the anthocyanins. Vasodilation activity was restricted to fractions containing lambertianin C and sanguiin H-6.

The antioxidant capacity of the fresh fruit and the levels of vitamin C and phenolics were not affected by freezing. When fruit were stored at 4 °C for 3 days then at 18 °C for 24 h, mimicking the route fresh fruit takes after harvest to the supermarket and onto the consumer's table, anthocyanin levels were unaffected while vitamin C levels declined and elligitannins increased, while the antioxidant capacity of the fruit remained unchanged. It is concluded, therefore, that as far as antioxidant phytochemicals are concerned, freshly picked, commercial and frozen raspberries are all equally beneficial.

Acknowledgements

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Last, but not least, to my dear Mum who passed away last year. She had always wanted me to go to University, almost as much as she wanted me to go to school, better late than never!

Table of Contents

1. List of Papers

This thesis is based on the following papers, which have been submitted for publication.
Paper 1 was accepted for publication on the 21st of May 2002
Papers 2 and 3 were accepted for publication on the 4th of June 2002

1.1   **Characterisation of anthocyanins in red raspberries by high performance liquid chromatography coupled to a single quadrupole mass spectrometer. Mullen *et al.* (2002)** *Journal of Chromatography A,* **in press.**

1.2   **Ellagitannins, flavonoids and other phenolics: their contribution to the antioxidant capacity and vasorelaxation properties of red raspberries. Mullen *et al.* (2002)** *Journal of Agricultural and Food Chemistry,* **in press.**

1.3   **Effect of freezing and storage on the phenolics, ellagitannins, flavonoids and antioxidant capacity of red raspberries. Mullen *et al.* (2002)** *Journal of Agricultural and Food Chemistry,* **in press.**

2. Introduction

The findings of "The Scottish Health Survey (Shaw *et al.* 1998) confirmed what has long been suspected. Scotland has one of the highest death rates due to coronary heart disease (CHD), strokes and colon cancer, and the indications are that this is due, in no small measure to our national diet, which is high in saturated fats and low in fresh fruit and vegetables.

Finland was once the twin of Scotland in terms of mortality rates from CHD, strokes and cancer. The joke in Finland was: Question - "What is the favourite fruit of Finland"? Answer - "A sausage". However, epidemiological studies carried out in Finland have shown that changes in the diet and lifestyle of the population have had a dramatic affect on the mortality rate. There was a 60 % reduction in heart disease and strokes over the 20 year period when these changes were introduced (Knekt *et al.* 1996). As well as reduced smoking and salt intake one of the dietary changes that occurred included a 2-3 fold increase in the amount of fruit and vegetables eaten (Puska *et al.* 1995). A major part of this was from the consumption of locally-grown berries. In addition to vitamins, minerals and fiber, fruits, berries and vegetables are a source of phytochemicals, most notably flavonoids and phenolic compounds, that act as antioxidants and are believed to provide protection against CHD, strokes and cancer. (Hertog *et al.* 1992a; 1993a).

3. Classification of phenolic compounds

Phenolic compounds are secondary plant metabolites, characterized by having at least one aromatic ring with one or more hydroxyl groups attached. In excess of 8000 phenolic structures have been reported, and they are widely dispersed throughout the plant kingdom (Strack *et al.* 1992). They are synthesized via the shikimate and phenylpropanoid pathways (Fig. 1). Phenolics range from simple, low molecular weight, single aromatic-ringed compounds, like gallic acid to large and complex tannins, such as the polymeric proanthocyanidins (Lairon *et al.* 1999). They can be classified by the number and arrangement of their carbon atoms (Table. 1) and are commonly found conjugated to sugars and organic acids. Phenolics can be classified into two groups, the flavonoids and the non-flavonoids.
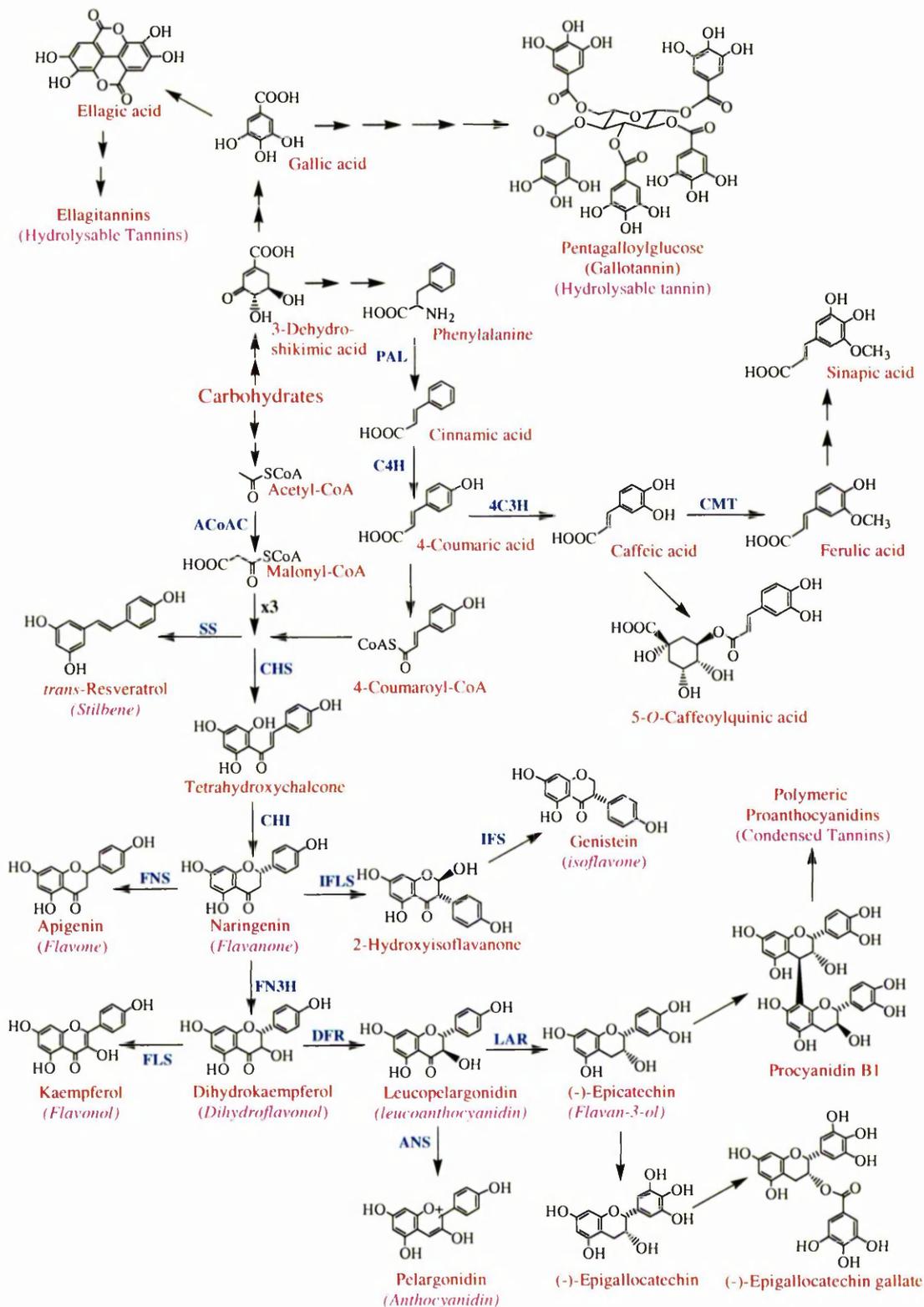
Figure 1. Main routes for the production of phenolic and polyphenolic compounds via the shikimic acid, phenylpropanoid and flavonoid biosynthesis pathways. Enzyme acronyms for biosynthetic pathway: PAL, Phenylalanine ammonia-lyase; C4H, Cinnamate 4-hydroxylase; 4C3H, 4-Coumarate 3-hydroxylase; CMT, Caffeate methyl transferase, 4CL, 4-Coumarate:CoA ligase; SS, Stilbene synthase; CHS, Chalcone synthase; CHI, Chalcone isomerase; IFLS, Isoflavanone synthase; IFS Isoflavone synthase FS, Flavone synthase; FN3H, Flavanone 3-hydroxylase; FLS, Flavanol synthase; DFR, Dihydroflavonol 4-reductase; ANS, Anthocyanidin 4-reductase; LAR, Leucoanthocyanidin 4-reductase.

Table 1. Basic structures of the main groups of phenolic and polyphenolic compounds

Carbon no.	Skeleton	Classification	Example	Basic Structure
7	C_6-C_1	**Phenolic acids**	Gallic acid	
8	C_6-C_2	**Acetophenones**	Xanthoxylin	
8	C_6-C_2	**Phenylacetic acid**	*p*-Hydroxyphenyl-acetic acid	
9	C_6-C_3	**Hydroxycinnamic acids**	Caffeic acid	
9	C_6-C_3	**Coumarins**	Esculetin	
10	C_6-C_4	**Naphthoquinones**	Juglone	
13	C_6-C_1-C_6	**Xanthones**	Gentisin	
14	C_6-C_2-C_6	**Stilbenes**	Resveratrol	
15	C_6-C_3-C_6	**Flavonoids**	Quercetin	
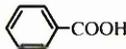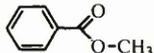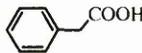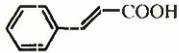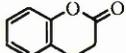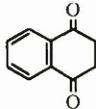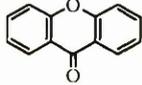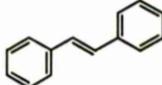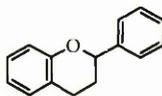
3.1   Flavonoids.

Flavonoids are polyphenolic compounds comprising 15 carbons, with two aromatic rings connected by a three carbon bridge (Fig. 2). They are the most numerous of the phenolics and are found throughout the plant kingdom. They are concentrated mainly in the epidermis of leaves and the skin of fruits, and they have many important and varied roles as secondary plant metabolites. Their location in plants associates well with their functions, which include UV protection, pigmentation, stimulation of nitrogen-fixing nodules and disease resistance (Koes *et al*. 1994) (Pierport. 2000).
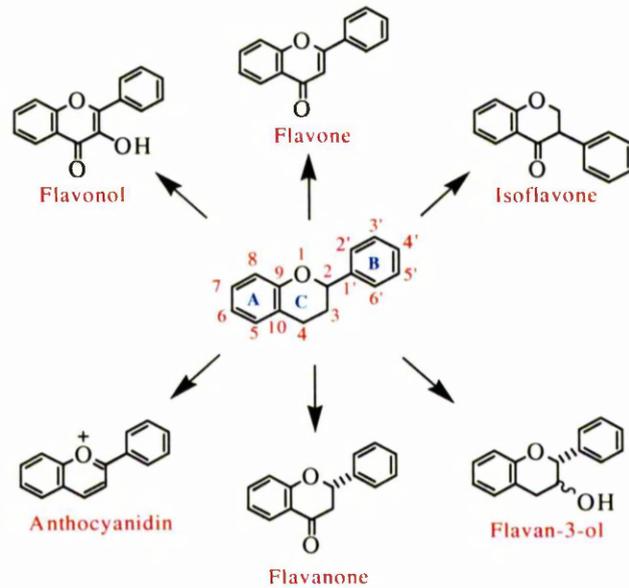
Figure 2. Generic structures of the major flavonoids.

The C_6-C_3-C_6 flavonoid structure is the product of two separate biosynthetic pathways (Figs. 1 & 3). The bridge and one aromatic ring, ring B, constitute a phenylpropanoid unit synthesized from phenylalanine, which itself is a product of the shikimic acid pathway. The six carbons of ring A originate from the condensation of three acetate units via the malonic acid pathway. The fusion of these two parts involves the stepwise condensation of a phenylpropanoid, 4-coumaryl CoA, with three malonyl-CoA residues, each of which donates two carbon atoms, in a reaction catalysed by chalcone synthase. Tetrahydroxychalcone, the product of this reaction, gives rise to all the other types of flavonoids via the flavonoid biosynthetic pathway (Fig. 1).
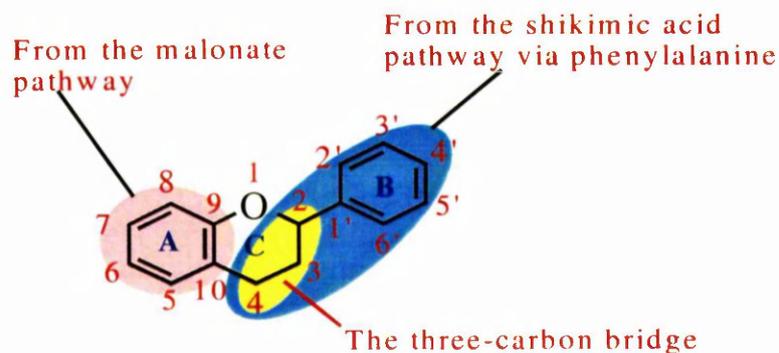
Figure 3. Basic structure and biosynthetic origin of the flavonoid skeleton.

The main sub-classes of flavonoids are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins (Fig. 2). The basic flavonoid skeleton can have numerous substituents. Hydroxyl groups are usually present at the 4, 5 and 7 positions. Sugars are very common with the majority of flavonoids existing naturally as glycosides. Whereas both sugars and hydroxyl groups increase the water solubility of flavonoids, other substituents, such as methyl ethers and isopentyl units give flavonoids lipophilic properties.

3.2   Flavonols

Flavonols are arguably the most widespread of the flavonoids, being dispersed throughout the plant kingdom with the exception of fungi and algae. Flavonol distribution and the wide structural variations have been comprehensively described (Williams & Harborne 1992; Wollenweber 1992). Within the plant, flavonols such as myricetin, quercetin, isorhamnetin and kaempferol (Fig. 4) are most commonly found as O-glycosides. Conjugation occurs most frequently at the 3 position of the C ring but 5, 7, 4', 3' and 5' substitutions also occur (Herrmann 1976). Although the number of aglycones are limited there are numerous flavonol conjugates with more than 200 different sugar conjugates of kaempferol alone (Strack & Wray 1992). There is extensive information on the levels of flavonols found in commonly consumed fruits, vegetables and beverages (Hertog 1992b; 1993b). However, large differences are often found in the amounts present in seemingly similar produce, possibly due to seasonal variation and varietal differences (Crozier *et al.* 1997).
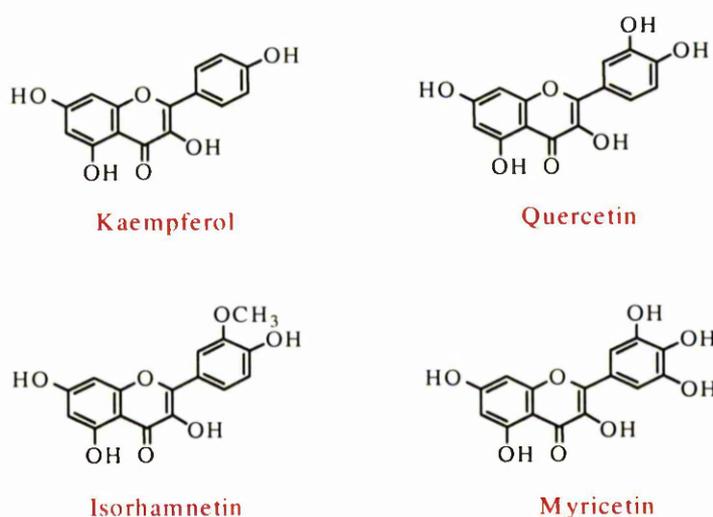
Figure 4. The flavonols, kaempferol, quercetin, isorhamnetin and myricetin.

3.3   Flavones

Flavones are structurally very similar to flavonols (see Fig. 2). Flavones have A and C ring substitutions, as in luteolin and apigenin (Fig. 5). However, they lack oxygenation at C3. A wide variety of substitutions are also possible with flavones, including hydroxylation, methylation, *O*- and *C*-alkylation and *O* and *C*-glycosylation. Most flavones occur as 7-*O*-glycosides (Bohm 1998). Unlike flavonols, flavones are not widely distributed. The only significant occurrences of flavones are reported in celery and parsley. It has also been reported that polymethoxylated flavones have been found in citrus (Kühnau 1976).
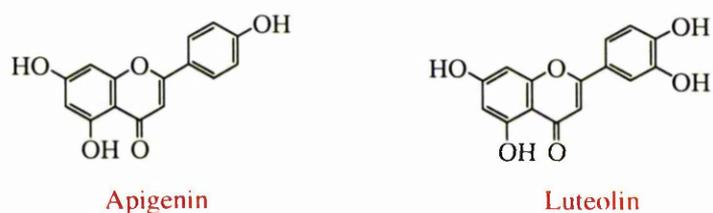
Figure 5. The flavones, apigenin and luteolin.

3.4   Flavan-3-ols

Flavan-3-ols are the most complex subclass of flavonoids. The simplest are the monomers (+)-catechin and its isomer (-)-epicatechin. The most complex being the oligomeric and polymeric proanthocyanidins, which are also known as condensed tannins (Fig. 6). Proanthocyanidins are formed from (+)-catechin and (-)-epicatechin with oxidative coupling occurring between the C-4 of the heterocycle and the C-6 or C-8 positions of the adjacent unit. Proanthocyanidins can occur as polymers of up to 50 units. In addition to forming such complexes, flavan-3-ols are hydroxylated to form gallocatechins, and also undergo esterification with gallic acid (Fig. 6).
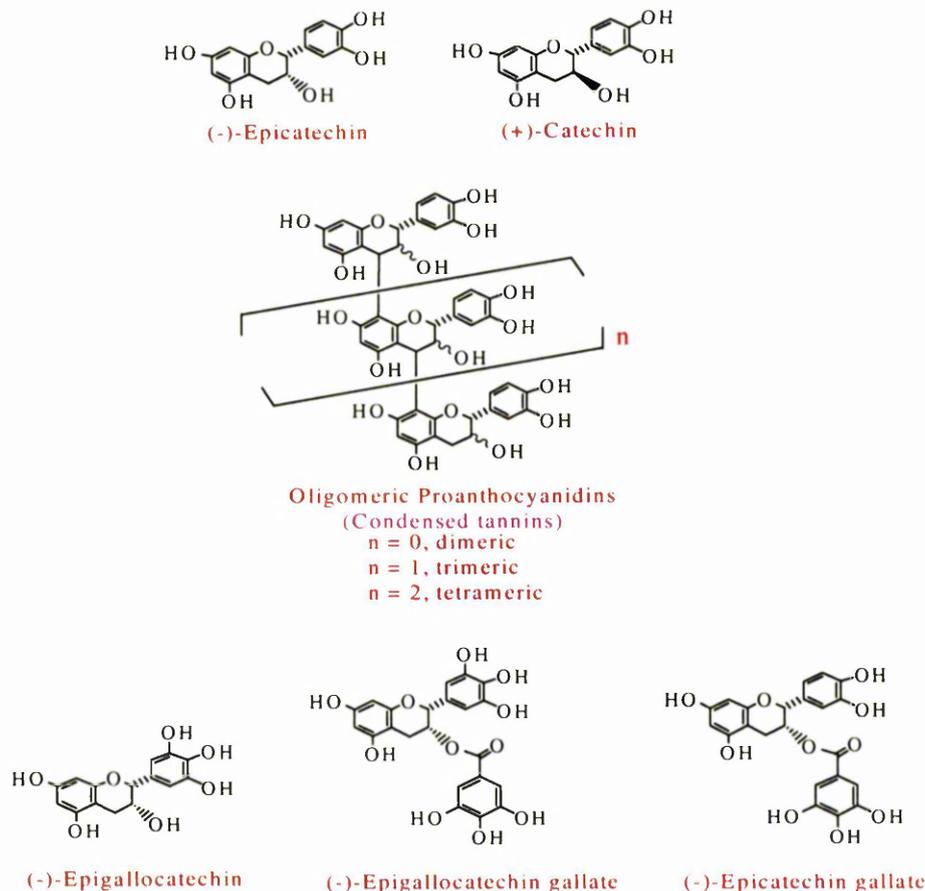
Figure 6. Flavan-3-ol structures.

Red wines contain oligomeric proanthocyanidins derived mainly from the seeds of black grapes. Green tea is also a rich source of flavan-3-ols, principally (-)-epigallocatechin, (-)-epigallocatechin gallate and (-)-epicatechin gallate (Fig. 6). The levels of catechins decline during fermentation of the tea leaves and the main components in black tea are high molecular weight thearubigins (Balentine *et al.* 1997). While these can reasonably be described as flavonoid-derived, their structures are unknown.

3.5   Anthocyanidins

Anthocyanidins, principally as their conjugated derivatives, the anthocyanins, are widely distributed in the plant kingdom. They are most noticeably in fruit and flowers where they are responsible for the red, blue and purple colours. Leaves, stems, seeds and root tissue are also a source of these compounds (Strack & Wray 1992). They are involved in the protection of plants

against excessive light by shading of the leaf mesophyll cells. In addition, they have an important role to play in the attraction of pollinating insects.

The most common anthocyanidins are cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (Fig. 7). However, in plant tissues these compounds are almost always found as anthocyanins, conjugated primarily to sugars. They also form conjugates with hydroxycinnamates and organic acids such as malic and acetic acids. Although conjugation can take place on carbons 3, 5, 7, 3′ and 5′ it occurs most often on C3. Studies in model wine solutions have shown that they can also form complexes with pyruvic acid and flavonols (Bakker *et al.* 1997).
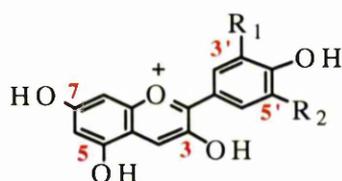
Anthocyanidin	R_1	R_2	Colour
Pelargonidin	H	H	orange-red
Cyanidin	OH	H	red
Delphinidin	OH	OH	pink
Peonidin	OCH_3	H	bluish purple
Petunidin	OCH_3	OH	purple
Malvidin	OCH_3	OCH_3	redish purple

Figure 7. Structures of major anthocyanidins.

3.6 Flavanones

The flavanones are formed from tetrahydroxychalcone in a reaction catalysed by chalcone isomerase, which is the first step in the flavonoid biosynthetic pathway (Fig. 1). They have a chiral center at C2 (Fig. 2) and are noted for the absence of the C2-C3 double bond. In most naturally occurring flavanones, ring C is attached to the B ring at C2 in the α-configuration. They are highly reactive and can undergo hydroxylation, glycosylation and *O*-methylation reactions. Flavanones are found mainly in citrus fruits. The most common flavanone glycoside is hesperidin (hesperetin-7-*O*-rutinoside), which is found in citrus peel (Fig. 8). Flavanone rutinosides are tasteless. In contrast, flavanone neohesperidoside conjugates such as neohespridin (hesperetin-7-*O*-neohesperidoside) from bitter orange (*Citrus aurantium*) and

naringin (naringenin-7-*O*-neohesperidoside) (Fig. 8) from grapefruit peel (*Citrus paradisi*) have an intensely bitter taste (Bohm 1998).

Hesperidin
(hesperetin-7-*O*-rutinoside)

Neohesperidin
(hesperetin-7-*O*-neohespidoside)

Naringin
(naringenintin-7-*O*-neohespidoside)
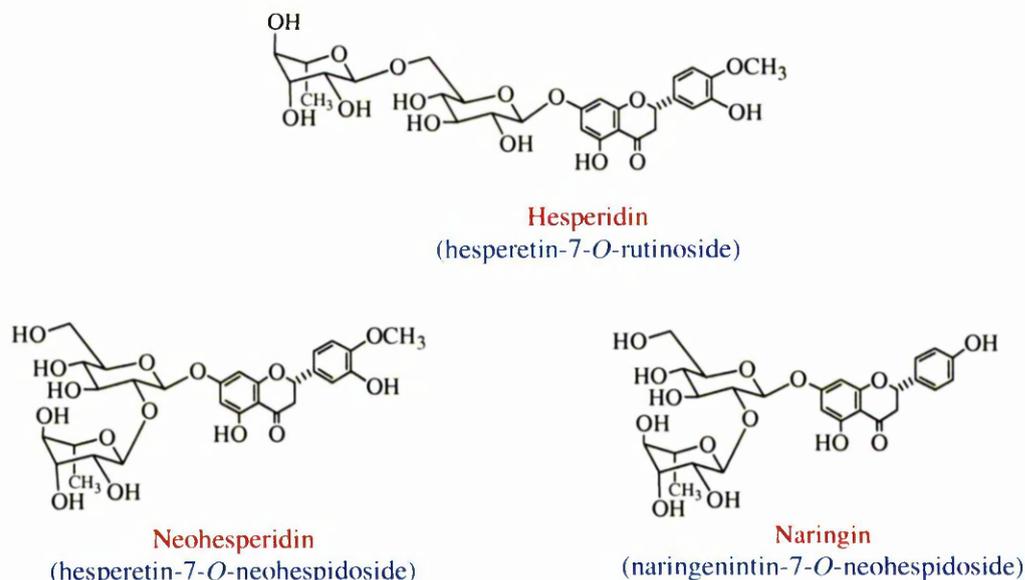
Figure 8. Structures of the flavanones, hesperidin, neohesperidin and naringin.

3.7   Isoflavones

Isoflavones are another step along the flavonoid biosynthetic pathway, with naringenin being converted to genistein via 2-hydroxyisoflavanone (Fig. 1). Hydroxylation of genistein at C5 then yields daidzein (Fig. 9). Isoflavones are characterised by having the B ring attached at C3 rather than the C2 position. (Fig. 2) They are found throughout the plant kingdom, but the principal source is legumes such as soya bean (*Glycine max*) (Bohm 1998). As with the rest of the flavonoid family, isoflavones such as genistein undergo hydroxylation and methylation reactions, in addition to prenylation, which yields a range of isoflavonoids including coumestans, rotenoids and pterocarpins (Fig. 9) (Dewick 2002).
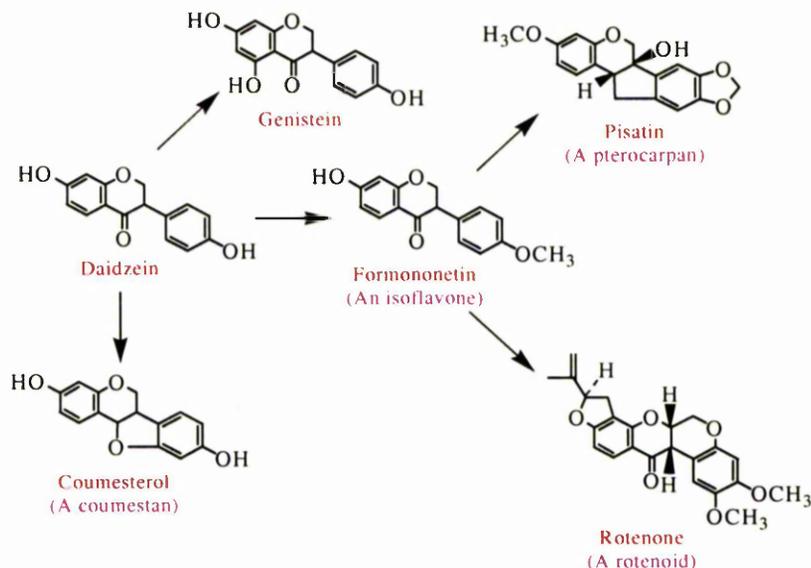
Figure 9. The isoflavones daidzein and genistein and related isoflavonoid structures.

The soya-derived isoflavones, genistein and daidzein, and the coumestan coumestrol from lucerne and clovers (*Trifolium* spp) have sufficient oestrogenic activity to seriously affect the reproduction of grazing animals, such as cows and sheep, and are termed phyto-oestrogens. The structure of these isoflavonoids is such that they appear to mimic the steroidal hormone oestradiol (Fig. 10), which blocks ovulation. The consumption of legume fodder by animals must therefore be restricted or low isoflavonoid producing varieties selected. This is clearly an area where it would be beneficial to produce genetically modified isoflavonoid-deficient legumes. This could be achieved with transgenic plants carrying the isoflavone synthase gene (see Fig. 1) in antisense orientation, thereby blocking the conversion of naringenin to isoflavones.

Dietary consumption of genistein and daidzein from soya products is thought to reduce the incidence of prostate and breast cancers in humans. However, the mechanisms involved are different. Growth of prostate cancer cells is induced by and dependent upon the androgen testosterone (Fig. 10), the production of which is suppressed by oestrodiol. When natural oestradiol is insufficient, the isoflavones can lower androgen levels and, as a consequence inhibit tumour growth. Breast cancers are dependent upon a supply of oestrogens for growth especially during the early stages. Isoflavones compete with natural oestrogens, restricting their availability thereby suppressing the growth of the cancerous cells.
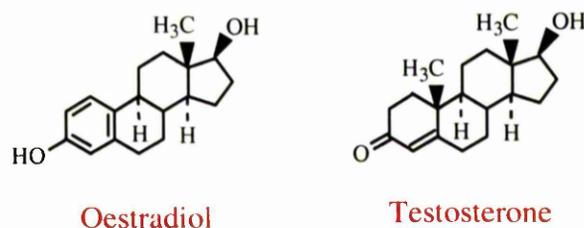
Figure 10. Structure of the oestrogen, oestradiol and the androgen, testosterone.

3.8   Non-flavonoids.

The principle non-flavonoids of dietary significance are the C_6-C_1 phenolic acids, most notably gallic acid and its dimer ellagic acid, which are the source of the hydrolysable tannins, the C_6-C_3 hydroxycinammates and their conjugated derivatives, and polyphenolic C_6-C_2-C_6 stilbenes (Fig. 12).

3.9   Phenolic acids and condensed tannins

Phenolic acids are also known as hydroxybenzoates, the principle components being gallic acid. Its name comes from the French word galle, the French term for a swelling in the tissue of a plant after an attack by parasitic insects. The swelling is from a build up of carbohydrate and other nutrients that support the growing insect larvae. It has been reported that the phenolic composition of the gall consists of up to 70% gallic acid esters (Gross 1992).

Although alternative routes exist from hydroxybenzoic acids, gallic acid appears to be formed primarily via the shikimic acid pathway from 3-dehydroshikimic acid (Fig.1). Gallic acid is converted to its dimer ellagic acid and to pentagalloylglucose and a range of gallotannins. Ellagic acid is the basic unit of the ellagitannins, which together with the gallotannins are referred to as hydrolysable tannins (Fig. 1). Hydrolysable tannins, as their name suggests, are more readily hydrolysed by treatment with dilute acid than condensed tannins.

Condensed tannins and hydrolysable tannins are capable of binding to and precipitating collagen proteins in animal hides. This changes the hide into leather making it resistant to putrefaction. Plant-derived tannins have, therefore, for many years formed the basis of the tanning industry (Bohm 1998).

Tannins bind to salivary proteins, producing a taste which humans recognise as astringency. Mild astringency enhances the taste and texture of a number of foods and beverages,

most notably tea and red wines. Many tannins are extremely astringent and render plant tissues inedible. Mammals such as cattle, deer and apes, characteristically avoid eating plants with high tannin contents. Many unripe fruits have a very high tannin content, typically concentrated in the outer cell layers. Tannin levels decline as the fruits mature and the seeds ripen. This may have been an evolutionary benefit, delaying the eating of the fruit until the seeds were capable of germinating.

It has been suggested that lack of tolerance to tannins may be one reason for the demise of the red squirrel. The grey squirrel is able to consume hazelnuts before they mature, and to survive on acorns. In contrast, the red squirrel has to wait until hazelnuts are ripe before they become palatable, and is much less able to survive on a diet of acorns which are the only thing left after the grey squirrels have eaten the immature hazelnuts (Haslam 1998).

Tannins can bind to dietary proteins in the gut and this process can have a negative impact on herbivore nutrition. The tannins can inactivate herbivore digestive enzymes creating aggregates of tannins and plant proteins that are difficult to digest. Herbivores that regularly feed on tannin-rich plant material appear to possess some interesting adaptations to remove tannins from their digestive systems. For instance, rodents and rabbits produce salivary proteins with a very high proline content (25-45%) that have a high affinity for tannins. Secretion of these proteins is induced by ingestion of food with a high tannin content and greatly diminishes the toxic effects of the tannins (Butler 1989).

3.10 Hydroxycinnamates

4-Cinnamic acid is a C_6-C_3 compound produced by phenylalanine ammonia lyase-catalysed deamination of the amino acid phenylalanine (Fig. 1), this reaction is believed to be common to all plants. 4-Coumaric acid is then produced by hydroxylation of 4-cinnamic acid. The most common hydroxycinnamates are caffeic, 4-coumaric, ferulic and sinapic acids. These are produced by a series of hydroxylation and methylation reactions (Fig. 1) and they often accumulate as their respective tartarate esters, coutaric, caftaric and fertaric acids (Fig. 11). In addition to being found in their free form, hydroxycinnamates are also found esterified to choline (Fig. 11), sugars and organic acids (Strack 1997). Cinnamic acid and its hydroxycinnamate derivatives are products of the phenylpropanoid pathway and are referred to collectively as phenylpropanoids.
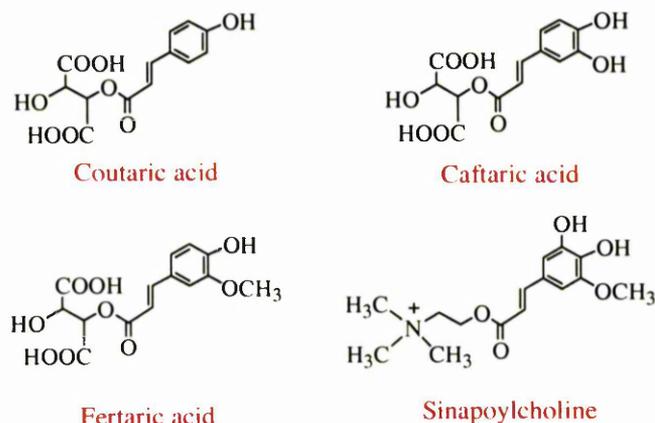
Figure 11. Structures of conjugated hydroxycinnamates.

3.11 Stilbenes

The stilbene family is characterized by a C_6-C_2-C_6 structure (Table 1) and, thus, like flavonoids are polyphenolic compounds. Stilbenes are phytoalexins, compounds produced by plants in response to attack by fungal, bacterial and viral pathogens. *Trans*-resveratrol is synthesised by condensation of 4-coumaroyl CoA with three units of malonyl CoA, each of which donates two carbon atoms, in a reaction catalysed by stilbene synthase (Fig. 1). The same substrate yields tetrahydroxychalcone, the immediate precursor of flavonoids, when catalysed by chalcone synthase. Stilbene synthase and chalcone synthase have been shown to be structurally very similar and it is believed that both are members of a family of polyketide enzymes (Soleas *et al.* 1997). Chalcone synthase is constitutively present in tissues, while stilbene synthase is induced only by a range of stresses including UV-radiation, trauma and infection. Resveratrol is found as both the *cis* and *trans* isomers and is present in plant tissues primarily as *trans*-resveratrol-3-*O*-glucoside which is know as piceid (Fig. 12).
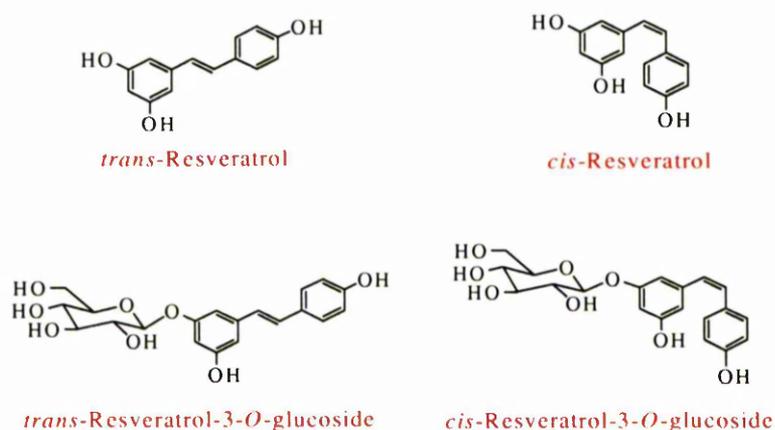
Figure 12. Structures of *cis* and *trans* resveratrol and their glucosides

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4. Protective effects of dietary phenolics

The protective effects of dietary phenolic compounds are thought to be due primarily to their role as antioxidants although they may also operate though other mechanisms.

Antioxidant activity

Reactive oxygen species (ROS) or free radicals can be defined as compounds with an unpaired electron in the outer orbit producing a highly reactive chemical (Stols 1995). These ROS molecules and molecular fragments such as $OH^{\bullet}$ and $O_2^{2-\bullet}$ can oxidise DNA, lipids, proteins and other vital biomolecules and, as a consequence, can induce a wide range of common human conditions linked to vascular function, CHD and some cancers. While the body has an array of endogenous antioxidant defences, such as superoxide dismutase and glutathione transferase, that quench ROS, thereby preventing damage, it is believed that dietary antioxidants also have a vital role to play. As well as the common antioxidants, vitamins C and E, other phytochemicals that occur in fruits and vegetables appear to be involved in these processes. Flavonoids and phenolic compounds are of particular interest in this regard because they are especially powerful antioxidants. There is a growing body of evidence in the chemistry, biochemistry and pharmacology literature supporting the view that they have a key biological role in scavenging ROS (Halliwell *et al.* 1992).

Chemically there are three features that confer antioxidant properties on phenolic and polyphenolic compounds (Rice-Evans *et al.* 1997):

- The hydrogen donating substituents (hydroxyl groups), attached to the aromatic ring structures enable phenolics to under go a redox reaction that helps them to scavenge free radicals more easily.

- A stable delocalisation system, consisting of aromatic and heterocyclic rings as well as multiple unsaturated bonds, which helps to delocalise the resulting free radicals.

- The presence of certain structural groups which are capable of forming transition metal-chelating complexes that can regulate the production of reactive oxygen species such as $OH^{\bullet}$ and $O_2^{2-\bullet}$.

4.2 Anti-atherogenic effects

The death rate from CHD in England and Wales is 448 and 167 per 100,000 for men and women respectively, aged 35-74. This contrasts with 101 and 32 per 100, 000 for men and women in the South of France (Renaud *et al.* 1992). This huge difference between the two populations may be due to cultural variations, genetic predisposition, lifestyle factors, climate and diet. However, there was a positive association with intake of saturated fats and a negative association with red wine intake. The active ingredient was thought to be the polyphenols in the red wine.

Atherosclerosis is due to a combination of hyperlipidemia and the oxidation of lipoproteins. These conditions may arise due to genetic predisposition. Lipid metabolism may be abnormal and/or the processes controlling and preventing excessive oxidation may be at fault. Reducing dietary fat intake can act to lower plasma lipid levels and decreases the occurrence of CHD. The dietary antioxidant vitamin E is known to prevent the oxidation of lipoproteins and phenolic antioxidants may have a similar role.

The exact chemical nature of the pathogenesis of atherosclerosis remains unknown. However the major mechanisms have been described (see Fig. 13). Low density lipoproteins (LDL) present in blood bind to glycoproteins on the arterial walls. They are internalised, trapped and begin to accumulate in the vessel cell wall. At this stage LDL is oxidised through lipid peroxidation. Oxidised LDL binds to monocytes and is accumulated internally which leads to the production of cytokines. These stimulate the influx of monocytes into the intima leading to further uptake of oxidised LDL. The monocytes gradually become lipid laden and transform into foam cells. These cells build up leading to the formation of a fatty streak and to vascular occlusion.
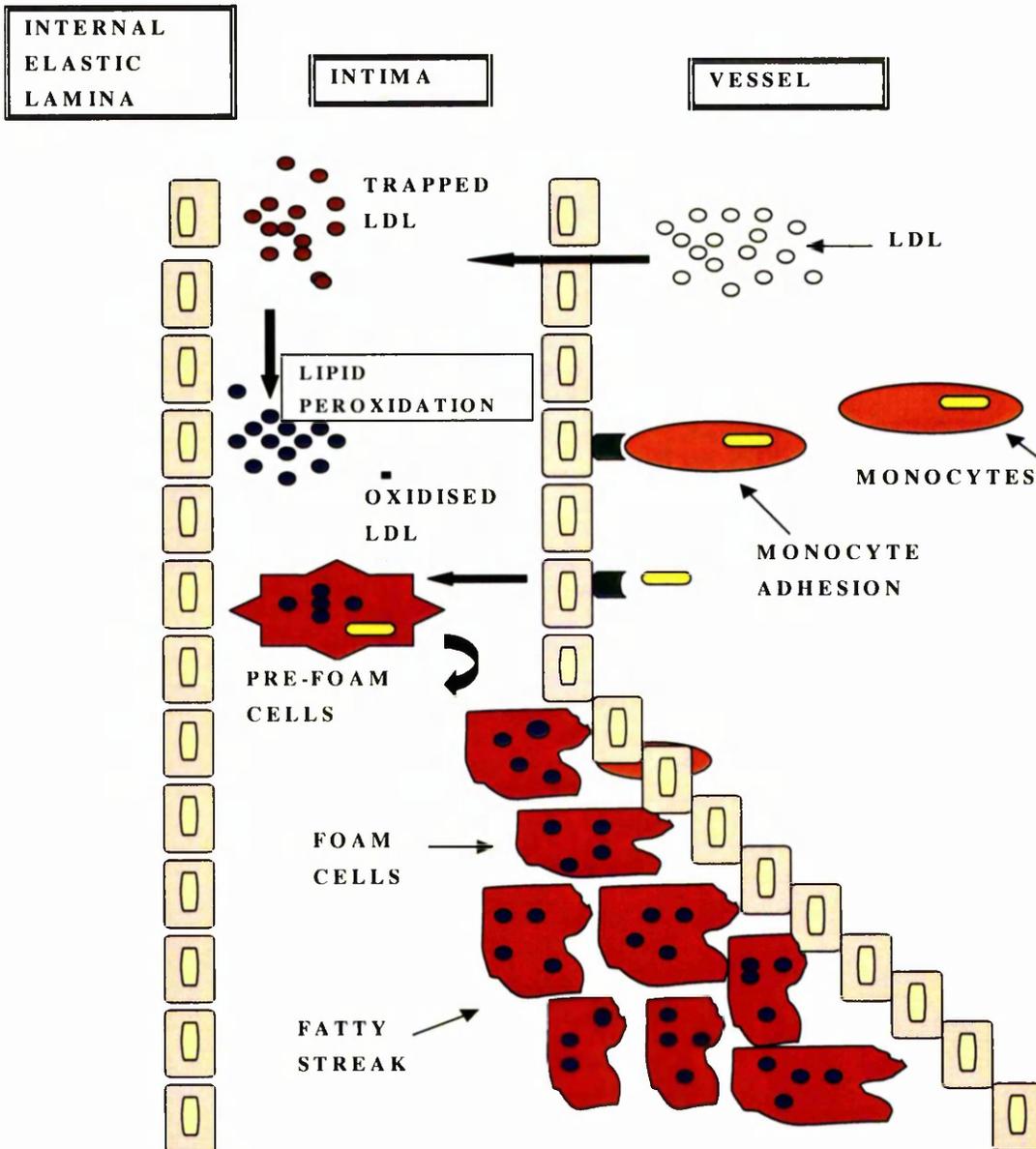
Figure 13. Pathogenesis of atherosclerosis (by kind permission Dr. J. Burns)

Evidence for the attenuation of LDL oxidation from the dietary intake of high phenolic containing beverages has been conflicting. While two studies with red wine have found that the lag phase for LDL oxidation is prolonged (Fuhrman *et al.* 1995), a similar investigation found no effect (De Rijke *et al.* 1996). A comparison of the influence of grape and orange juice on LDL oxidation, found that while the intake of grape juice increased the lag phase of LDL oxidation, orange juice did not (Vinson *et al.* 1995).

4.3 Anti-carcinogenic properties

A wide range of large-scale epidemiological studies have investigated the relationship between cancer and diet. A number of foods and their components have been highlighted as being of particular interest. As part of a multi-cultural study, 12,763 people in 16 cohorts over seven countries were followed over 25 years. The association between the intake of flavonols and mortality from all cancer causes was investigated, and no relationship was observed (Hertog *et al.* 1995). The relationship between dietary flavonol intake and the development of cancer at various sites has been investigated (Knekt *et al.* 1997). A cohort study of nearly 10,000 Finnish men and women compared their intake of flavonols and their risk of cancer over a period of 24 years (Knekt *et al.* 1996). An inverse relationship was observed between dietary flavonol intake and the development of cancer at all sites. This relationship was primarily attributed to a protection against lung cancer. In a recent Spanish study the risk of lung cancer was not observed to be attenuated by flavonol intake (Garcia-Closas *et al.* 1998). This study investigated only 103 cases (all women) and 206 controls, matched by age and residence. However a further case-controlled study with 354 cases of gastric cancer found that the intake of kaempferol offered protection (Garcia-Closas *et al.* 1998) High quercetin intake was noted to be associated with a lower risk of stomach cancer. A substantial number of studies have looked at the consumption of tea, and the risk of cancer. Overall no clear picture has emerged. While some studies do suggest a lowered risk of digestive cancers among tea drinkers (Blot *et al*1997) further research is required to clarify the situation. However, despite the investigations listed above, it is a mute point as to whether epidemiological studies are a sufficiently refined tool to enable protective effects to be linked to a specific group of phytochemicals.

Although the epidemiological evidence for the prevention of cancer from flavonols is at best contradictory. *In vitro* studies, in contrast, suggest that polyphenols can exert their anti-carcinogenic activity at various stages of tumour development (Stavric 1994). A study using transgenic HTLV-1 (human T-lymphocyte virus type-1) mice has shown that red wine extracts significantly delayed tumour onset (Ebeler *et al.* 1997), although, as yet, the active components have not been identified. It has also been reported that rats fed a diet containing 5% quercetin had a 48% lower incidence of mammary cancer induced by 7,12-dimethylbenz[α]anthracene (Verma *et al.* 1988).

4.4 Anti-inflammatory properties

Polygonum cuspidatum Sieb. et Zucc., is a noxious weed that was introduced into the UK over a century ago and is now only too well known to British gardeners as Japanese knotweed (Burns *et al.* 2002). In Japan it is known as the Itadori plant. Itadori means "well-being", and tea prepared from itadori root has been used for centuries in Japan and China as a herbal remedy for many diseases including arteriosclerosis, hyperlipidemia, allergic and inflammatory conditions (Kimura *et al.* 1985). The active medicinal ingredient is believed to be the stilbene *trans*-resveratrol and its glucoside (Fig. 12). Resveratrol inhibits cyclo-oxygenase and lipoxygenase enzymes. These enzymes catalyse the formation of inflammatory mediators and produce a range of compounds including the prostaglandins, thromboxanes and leukotrienes, each acts as a starting point for a general inflammatory response. Other phenolic compounds including quercetin and myricetin are also known to inhibit the cyclo-oxygenase and lipoxygenase enzymes (Koshihara *et al.* 1983). The mechanism by which this inhibition occurs has yet to be fully elucidated. It has also been proposed that flavonoids may suppress inflammatory signals by decreasing the permeability of vessels or through inhibition of hyaluronidase or inhibition of histamine release (Robak *et al.* 1996).

4.5 Dietary sources and intake of phenolics.

The ubiquitous nature of phenolic compounds in plants ensures that they are found in significant levels in the diet. However, there is a lack of information available on the levels of phenolics in foodstuff. Unlike well-known dietary components, such as vitamin C and E, comprehensive food tables are not available for phenolic compounds, and as a consequence, there is no recommended daily allowance. A wide range of methods have been used to quantify food phenolics but few studies have examined more than one food group. This inconsistency in analysis and the inaccurate methodology used in older studies have combined to leave the estimated dietary intake of phenolics open to question.

Another contributor to the inaccuracy in estimates of dietary intake of phenolics lies in the nature of the foods analysed. Seasonal variations can dramatically influence the phenolic contents of fruits and vegetables, as can varietal differences (Crozier *et al.* 1997; 2000). None-the-less, estimates have been made of the total dietary intake of flavonoids although the figures vary greatly. (Hertog *et al.* 1993a) estimated flavonoid consumption was 23 mg/day in the Netherlands. Although widely quoted, this figure applies not to flavonoids but is restricted to

only three flavonols and two flavones, and the calculation is based on aglycones rather than conjugates. It is, therefore, almost certainly a serious under estimate of flavonoid ingestion. At the other end of the scale (Kuhnau 1976) estimated the intake in the USA as being over 1000 mg per day. This figure is based on all phenols being glycosides/conjugates and is likely to be an over estimate of flavonoid consumption. Berries, along with other fruits, vegetables and beverages, can contribute significantly to the dietary consumption of phenolics and a regular daily consumption can readily increase the intake of a variety of phenolics.

4.6   Raspberries

As mentioned in Section 2, increased consumption of berries was one of the associated factors that resulted in a decline in CHD in Finland. Red raspberries (*Rubus idaeus*), together with blackcurrants (*Ribes nigrum*) and strawberries (*Fragaria* x *ananassa*) are grown extensively on a commercial basis around the east coast of Scotland. Raspberries are rich in vitamins, minerals and phenolic compounds that can act as antioxidants (de Ancos *et al.* 2000). A potential problem with Scottish raspberries is that fresh berries are available only during a short summer season and little is known about how freezing and storage processes affect the potentially protective phytochemicals and antioxidant properties of the fruit. If the changes are minimal then fresh and frozen raspberries could be used as "an all year round" rich and reliable source of dietary antioxidants.

The major phytochemicals in raspberries are the anthocyanins (Torre *et al.* 1977). They are most noticeable as they give raspberries their characteristic dark red colour. Over the years, a total of eleven anthocyanins have been identified in a range of studies with different varieties of raspberries. Some of the earlier studies utilised laborious thin-layer chromatography procedures (Barritt & Torre 1973; 1975), while more recent investigations have employed HPLC-tandem MS (Giusti *et al.* 1999). The major component is cyanidin-3-sophoroside (Fig. 14) which is accompanied by smaller quantities of cyanidin-3-(2^G-glucosylrutinoside), cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-(2^G-xylosylrutinoside), cyanidin-3,5-diglucoside, cyanidin-3-sambubioside, pelargonidin-3-sophoroside, pelargonidin-3-(2^G-glucosylrutinoside), pelargonidin-3-glucoside and pelargonidin-3-rutinoside.
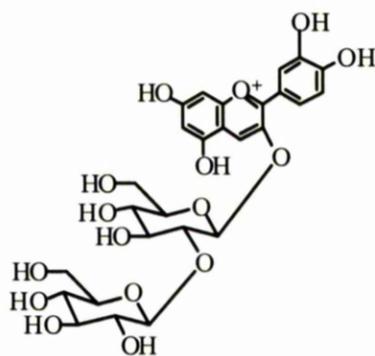
Cyanidin-3-*O*-sophoroside

Figure 14. Structure of cyanidin-3-sophoroside, the main anthocyanin in raspberries.

There are also reports that several berries including raspberries contain substantial amounts of ellagic acid which is a dimer of gallic acid (Fig. 15) (Daniel *et al*. 1989). Ellagic acid is a powerful antioxidant and has been reported to have antiviral activity and provide protection against cancers of the colon, lung and oesophagus (Dixit *et al*. 1985; Stavric 1994; Knekt *et al* 1997)
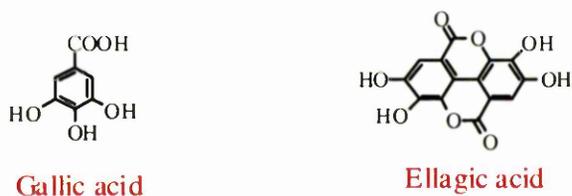
Gallic acid   Ellagic acid

Figure 15. Gallic acid and its dimer ellagic acid.

Raspberries also contain a wide variety of quercetin and kaempferol-based flavonol conjugates with the major components being quercetin-3-glucuronide and quercetin-3-glucoside (Henning 1981) . The hydroxycinnamates, coumaric acid and caffeic acid and derivatives such as 5-*O*-caffeoylquinic acid are also present, albeit it in relatively low amounts (Rommel *et al*. 1993). (+)-Catechin and catechin dimers have also been detected in raspberry juice (Arts *et al*. 2001).

5. Aims of the Project

There were three main aims.

- To develop a method that involved minimal sample preparation for the accurate analysis of anthocyanins in Glen Ample raspberries, using HPLC-MS. This work was carried out with a Shimadzu single quadrupole mass spectrometer and a dual wavelength absorbance monitor. When these instruments were replaced with a more sophisticated Finnigan Duo ion trap tandem mass spectrometer and a diode array detector (DAD) it became possible to analyse a much wider range of phenolic compounds, which had previously represented unidentified peaks on HPLC traces.

- Having identified more than twenty compounds in raspberries, the next step was to determine to what extent these components contributed to the antioxidant activity and vasodilation capacity of raspberries.

- Finally, an investigation of the effects of storage and freezing on the antioxidant capacity and levels of phenolic compounds and vitamin C in raspberries was carried out.

6.   Results and Discussion

6.1   Identification of phenolic compounds in Glen Ample raspberries

The instrumentation available in the laboratory at the beginning of this study, namely a gradient HPLC with a dual wavelength absorbance monitor and a single quadrupole MS proved to be adequate to determine the levels of anthocyanins in raspberry extracts without the need to carry out any sample processing. Using a 5 μm Novapac C18 column fitted with a 20 x 4.6 mm (i.d.) Novapac C18 guard column (Waters Associates, Milford, MA) eluted at 0.8 ml min^{-1} with a 30 min gradient of 8-18 % acetonitrile in water containing 1% formic acid it was possible to detect and identify eight anthocyanins, namely cyanidin-3-sophoroside, cyanidin-3-(2^G-glucosylrutinoside), cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-sophoroside, pelargonidin-3-(2^G-glucosylrutinoside), pelargonidin-3-glucoside and pelargonidin-3-rutinoside (see page 47, 48).

That short paragraph, however, does not tell the full story of the development of the analytical method. Mass spectrometers have long been thought to be the ultimate analytical instruments for compound identification. They provide the molecular weight of a compound to

the nearest atomic mass unit and its fragmentation pattern. However, MS instruments used with HPLC have a relatively gentle method of ionisation, and this limits to some extent the information that is generated. For example, the flavonol quercetin-3-glucoside has a molecular weight of 464 amu and HPLC-MS would produce a positively charged molecular ion (M^+) at 465 m/z and an M-162 fragment ion (loss of glucose) at m/z 303 which corresponds to the aglycone quercetin. This, so called fingerprint is not unique as the anthocyanin delphinidin-3-glucoside produces the same M^+ and fragment ion (see page 50). These two compounds do however, have a different λ_{max} and can therefore be readily distinguished from each other. A further complicating factor can arise as anthocyanins also form conjugates with organic acids and delphinidin conjugated with caffeic acid would have the same mass spectrum and λ_{max} as delphinidin-3-glucoside. These two compounds can be distinguished by their HPLC properties as delphinidin-3-glucoside is more polar than the caffeic acid derivative and elutes much earlier in a reverse-phase HPLC system.

Thus by using HPLC to separate these compounds on the basis of their polarity and by monitoring two wavelengths simultaneously, it is possible to selectively detect and quantify various groups of flavonoid compounds within a matrix that could not be distinguished solely on the basis of mass spectral data from a single quadrupole instrument.

The ability to differentiate between the above mentioned compounds was a straight forward analytical problem to solve compared to the difficulties of dealing with the sugar content of raspberries. In an HPLC-MS system, the compounds are analysed in a liquid, which on leaving the chromatographic part of the system is dried with nitrogen gas, ionised by a high voltage source and drawn into the vacuum system of the mass spectrometer through a heated capillary tube. The atmospheric pressure chemical ionization (APCI) interface of the Shimadzu QP8000 mass spectrometer was unable to cope with samples with a high sugar content and rapidly became coated with a caramelised material, which blocked the heated capillary tube. Due to these and other problems the mass spectrometer was replaced during the course of the project.

The replacement system, a Finnigan LCQ Duo tandem mass spectrometer, was not only far more reliable, but provided a greater degree of certainty in the analysis due to its ability to carry out data dependant mass spectrometry. This means the detector selects the major ion from a full scan spectrum, typically the molecular ion, isolates it and then fragments it and records the fragmentation pattern of that ion. In addition, the UV absorbance detector was also upgraded to a DAD, which enabled absorbance spectra to be automatically obtained for HPLC peaks. The new instrumentation was, therefore a much more powerful analytical tool.

When aliquots of a raspberry extract were analysed by HPLC tandem MS, twenty three different polyphenolic compounds were eventually identified (see page 69). These included eleven anthocyanins. The main components were cyanidin-3-sophoroside, cyanidin-3-(2^G-glucosylrutinoside), and cyanidin-3-glucoside with smaller amounts of cyanidin-3-rutinoside, pelargonidin-3-sophoroside and pelargonidin-3-(2^G-glucosylrutinoside) and trace levels of cyanidin-3,5-diglucoside, cyanidin-3-sambubioside, cyanidin-3-xylosylrutinoside, pelargonidin-3-sophoroside, pelargonidin-3-glucoside and pelargonidin-3-rutinoside (Fig.15). Although all of these anthocyanins have been previously identified (Barritt & Torre 1973; 1975) this was the first report of all eleven being identified in single sample from a single cultivar of raspberries.

Cyanidin-3-O-sophoroside

Cyanidin-3-O-(2^G-O-glucosylrutinoside)

Cyanidin-3-O-glucoside

Cyanidin-3-O-rutinoside

Cyanidin-3-O-(2^G-O-xylosylrutinoside)

Cyanidin-3-O-sambubioside

Cyanidin-3,5-O-diglucoside

Pelargonidin-3-O-sophoroside

Pelargonidin-3-O-glucoside

Pelargonidin-3-O-rutinoside

Pelargonidin-3-O-(2^G-O-glucosylrutinoside)
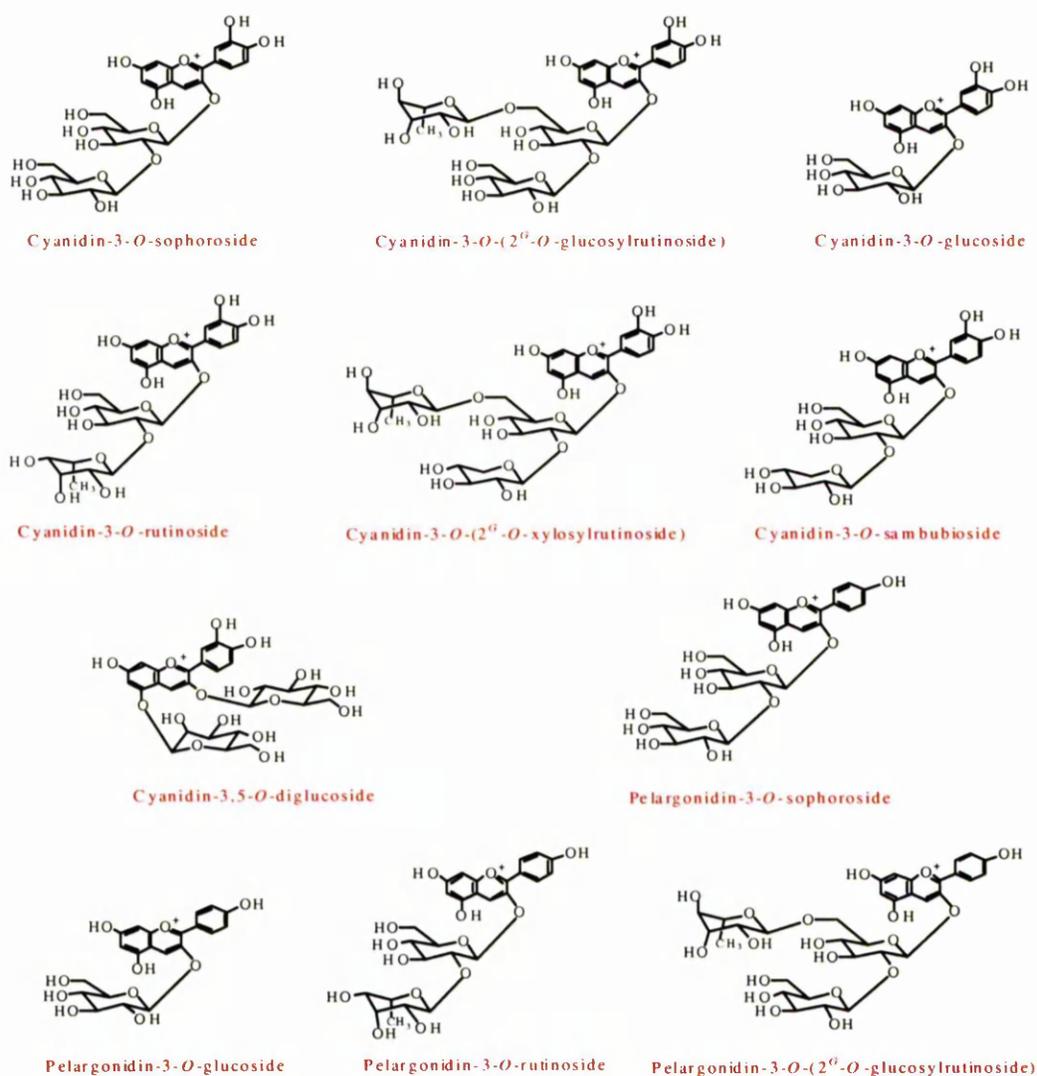
Figure 15. Structures of anthocyanins detected in Glen Ample raspberries.

The flavonols quercetin-3-rutinoside, quercetin-3-glucoside, quercetin-3-glucuronide (Fig.16) were also identified along with a kaempferol glucuronide conjugate and a putative xyloside conjugate of methylquercetin (see page 67 peaks 17, 18, 19, 20 and 22 respectively).
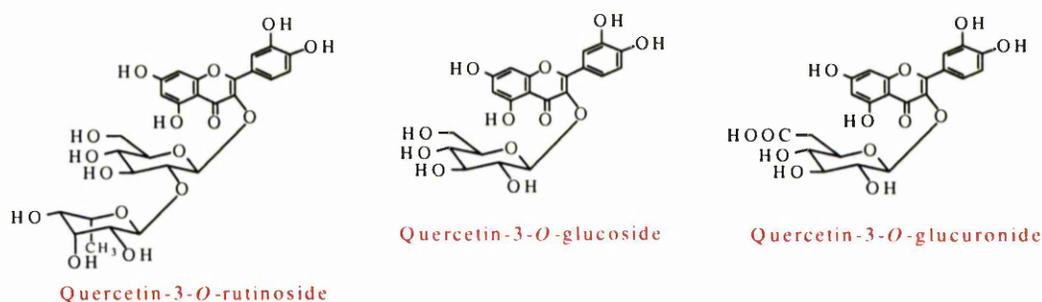
Figure 16. Quercetin sugar conjugates detected in raspberries.

Two ellagitannins, lambertianin C (M_r = 2804) and sanguiin H-6 (M_r = 1870) (Fig. 17), which could not be identified with the original mass spectrometer because they were outside its upper molecular weight range of 1500 amu, were also detected (see page 67 peaks 12 and 13)
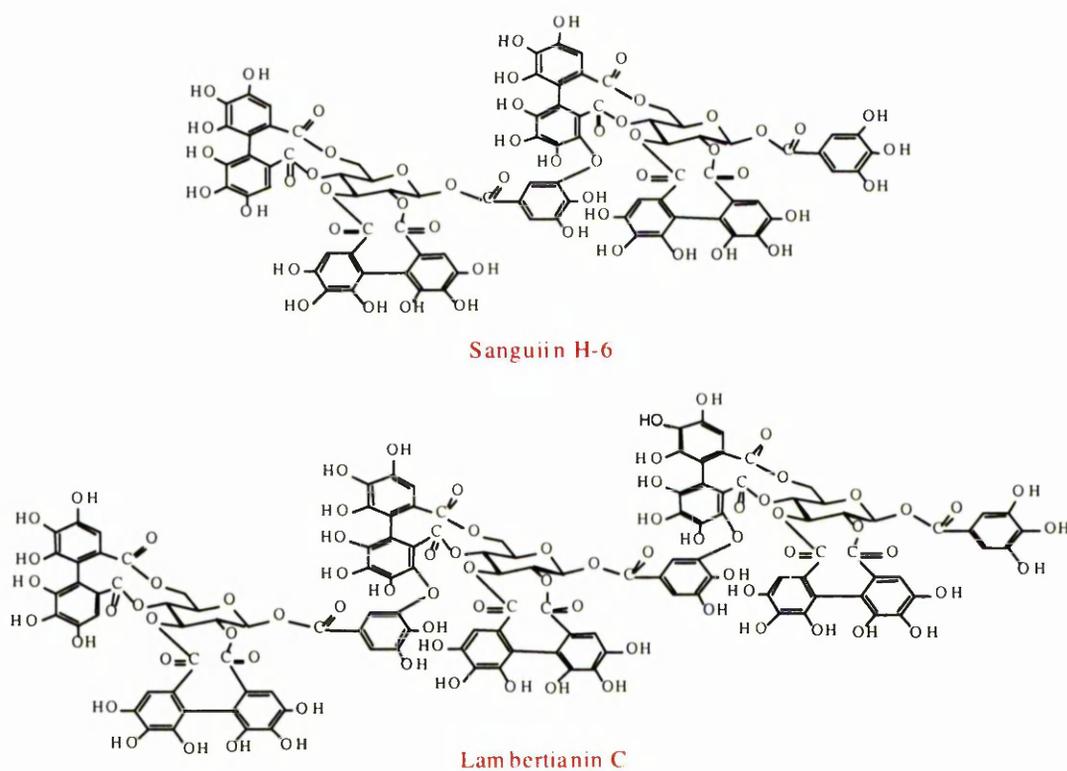
Figure 17. Structures of the raspberry ellagitannins, sanguiin H-6 and lambertianin C.

27

Ellagic acid-4-arabinoside   Ellagic acid-4-acetylarabinoside   Ellagic acid-4-acetylxyloside
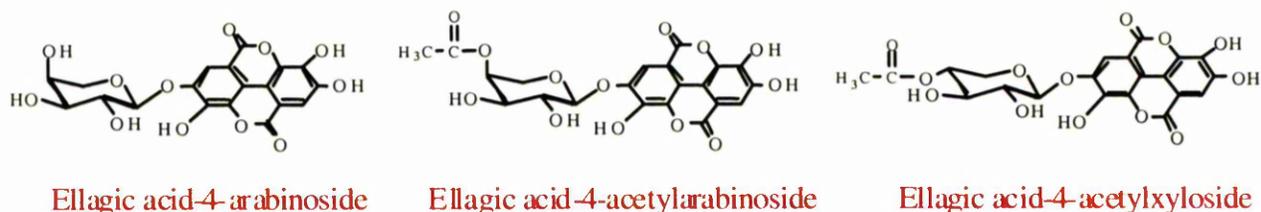
Figure 18. Ellagic acid sugar conjugates detected in raspberries.

The raspberry extract was also found to contain trace levels of ellagic acid and two pentose conjugates of ellagic acid, one of which may be ellagic acid-4-arabinoside, which has been detected in low levels in raspberry jams (Zafrilla *et al.* (2001). The raspberry jams also contain ellagic acid-4-acetylxyloside and ellagic acid-4-acetylarabinoside (Fig. 18) both of which were detected in the Glen Ample extracts (see page 67 peaks 14, 15, 16 and 21).

6.2   Identification of compounds responsible for the antioxidant activity and vasodilation capacity of raspberries

In order to identify the components in raspberries, which contribute to the antioxidant activity and vasodilation capacity of the fruit, a large scale extract was subjected to preparative scale reverse-phase HPLC. Sixty successive fractions were collected and analysed colorimetrically for total phenols and anthocyanins and, in addition assayed, for antioxidant activity by electron spin resonance spectroscopy and vasodilation capacity. Individual fractions were also analysed by HPLC-tandem-MS while the vitamin C content was determined by HPLC.

The raspberry extract separated into three zones of antioxidant activity (Fig. 19). The first, in fractions 2-4, contained vitamin C. The second in fractions 23-32 contained anthocyanins, while lambertianin C and sanguiin H-6 were detected in the third zone in fraction 36-39. The major peak of antioxidant activity was in fraction 38, which contained sanguiin H-6. However, calculation of the overall antioxidant activity in each of the three zones revealed that the vitamin C zone in fractions 2-4 reduced 1.6×10^{17} radicals g^{-1} f.w., the anthocyanins in fractions 23-32, 3.8×10^{17} radicals g^{-1} f.w., and the ellagitannins in fractions 36-39, 3.2×10^{17} radicals g^{-1} f.w. Thus, although no single anthocyanin makes a contribution to the antioxidant capacity of the raspberries equivalent to that of sanguiin H-6, the combined contribution of the 11 anthocyanins in fractions 23-32 is slightly higher than that of the ellagitannins in fractions 36-

39. The antioxidant activity in fractions 2-4, which contain vitamin C, is half that of the ellagitannins.

The flavonols, ellagic acid and its conjugates eluted after fraction 40. The lack of antioxidant activity after fraction 40 indicates that these compounds do not make a significant contribution to the antioxidant capacity of Glen Ample raspberries. This is especially interesting as Zafrilla *et al.* (2001) have reported that ellagic acid and its sugar conjugates are strong antioxidants in raspberry jams. The data presented in Figure 19 would suggest that this is not the case unless of course sanguiin H-6 and lambertianin C are degraded to ellagic acid during the manufacture of the jams. Neither compound was reported by Zafrilla *et al* (2001), who used a mobile phase composition that would have resulted in lambertianin C and sanguiin H-6 eluting either with or shortly after the void volume peak.

There are a number of reports on the high ellagic acid content of raspberries and strawberries in both the scientific literature (Maas *et al.* 1991; Rommel *et al* 1993, Hakkinen *et al.* 2000) and the "nutritional" columns in the popular press. This is a myth, high ellagic acid levels are in fact detected only after extracts have been treated with HCl at 90°C for 2 h or more. In raspberries this procedure hydrolyses both sanguiin H-6 and lambertianin C releasing substantial quantities of ellagic acid.

(W. Mullen, unpublished)

Vasodilation assays were carried out on fractions 20-40. The anthocyanins in fractions 23-32 exhibited little activity while a major zone of activity in fractions 35-39 was closely associated with the presence of lambertianin C and sanguiin H-6. Many plants tissues, especially fruits and vegetables, contain extractable compounds that cause endothelium-dependent vasorelaxation *in vitro* (Fitzpatrick *et al.* 1995). In this study, raspberry fractions associated with the presence of lambertianin C and sanguiin H-6, have been shown to be potent vasodilators of rabbit aortic vessels with intact endothelium.
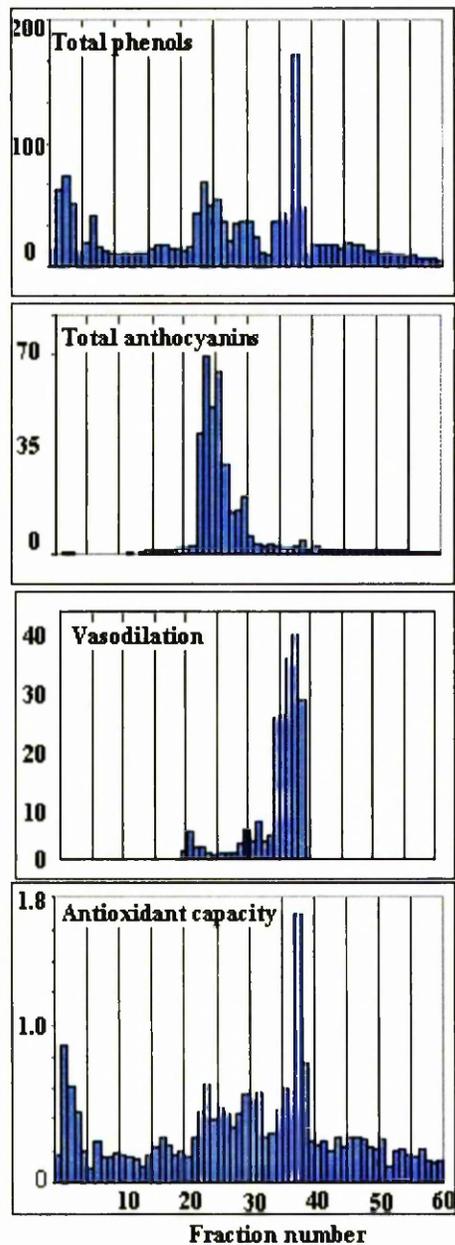
Figure 19. Preparative reversed phase HPLC of an extract of Glen Ample raspberries. Column eluted with a 30 min gradient of 5-25% acetonitrile in water containing 1% formic acid followed by an 80% acetonitrile wash. Successive 30 s fractions collected after 3 min, concentrated and aliquots assayed for total phenolics, anthocyanins, antioxidant capacity and vasodilation activity. Data expressed as follows: total phenolics (μg gallic acid equivalents g^{-1} f.w.); anthocyanins (μg cyanidin-3-glucoside equivalents g^{-1} f.w.); vasodilation activity (% relaxation); antioxidant capacity (number of Fremy's radicals reduced [x 10^{17}] g^{-1} f.w.).

6.3 Effects of freezing and storage on the phenolics, vitamin C and antioxidant capacity of raspberries

In order to investigate the effects of freezing and storage on phenolics, vitamin C and antioxidant capacity of raspberries, ripe field-grown Glen Ample berries were hand picked at Blairgowrie, Perthshire, UK. The fruit was divided into four lots which were treated in the following manner: *fresh* – extracted with methanol within 3 h of picking; *frozen* - within 3 h of picking, frozen at – 30°C in a commercial plant operated by Scottish Soft Fruit Growers plc, Blairgowrie; *store* – maintained at 4°C for three days prior to freezing in liquid nitrogen - equivalent to arrival in the supermarket; and *home* – as *store* but kept at 18°C for a further 24 h before freezing in liquid nitrogen - equivalent to keeping the raspberries in the kitchen for one day prior to eating at home. The four samples were extracted with methanol and the phenolics, vitamin C and antioxidant capacity of the extracts determined.

Free and conjugated 4-coumaric acid were detected in the raspberries and although present in higher concentrations in frozen fruit, the levels were very low indeed at 1.9 ± 0.4 nmoles g^{-1} fresh weight (see page 85 Table 1). Anthocyanins were present in far higher amounts with the main component cyanidin-3-sophoroside being detected at concentrations >500 nmoles g^{-1} and overall anthocyanin levels being >1000 nmoles g^{-1} (see page 85 Table 3). Analysis of *fresh, frozen, shop* and *home* fruit revealed no significant difference in the levels of the individual anthocyanins. The level of total phenolics were significantly higher in the *shop* and *home* raspberries than in the *fresh* and *frozen* fruit. The low levels of ellagic acid also rose during storage of raspberries and there were small but significant increases in the much higher concentrations of lambertianin C in *home* and sanguiin H-6 in *shop* and *home* fruit. The vitamin C concentration declined from 672 for *fresh* to 622 nmoles g^{-1} for *home* berries with the levels in the *shop* and *home* raspberries being significantly lower than those in the *fresh* and *frozen* samples. In contrast, to these changes, the antioxidant capacity in *fresh, frozen, shop* and *home* raspberries did not alter significantly (see page 86 Table 4).

The data in Figure 19 demonstrate that the main contributors to the antioxidant capacity of raspberries are the ellagitannins, anthocyanins and vitamin C. It is, therefore, likely that the increases in lambertianin C and sanguiin H-6 during storage, and the small but significant increase in total phenolics, were not accompanied by increases in antioxidant capacity because they were either relatively minor and/or were off-set by the decline in vitamin C in *shop* and *home* fruit (Table 4). The explanation for these post-harvest changes lies presumably in the fact

that secondary metabolism in berries remains active under *home* and *shop* conditions but not in *frozen* tissues.

It is concluded that raspberries are a rich source of antioxidants whether they are eaten either immediately after being picked or after purchase from a supermarket as fresh or frozen produce.

7 Future strategies

The finding of the preparative HPLC experiment that sanguiin H-6 is the most potent antioxidant in raspberries is of potential significance. Coupled to the vasodilation data presented in Figure 19, it points to the possibility of sanguiin H-6 and lambertianin C being potentially very important dietary components. Because of their high molecular weight it is most unlikely that either compound will be absorbed intact. However, they may be slowly degraded as they pass through the gastrointestinal tract releasing gallic acid and ellagic acid, which are more likely to pass through the gut wall and into the blood stream. It would be interesting to determine the pattern of accumulation of these ellagitannin-derived phenolic acids in the blood after the consumption of raspberries. If their levels were to rise quickly it would indicate that the ellagitannins are being broken down and the phenolic acids absorbed in the stomach and or small intestine. As gallic acid and ellagic acid are both good antioxidants it would be expected that their appearance in the circulatory system would have a beneficial effect. The longer the lag time before the appearance of gallic acid and/or ellagic acid in the bloodstream, the further the ellagitannins would have travelled through the gastrointestinal tract. If, however, there was no absorption this could also be of interest, as it could be a consequence of sanguiin H-6 and lambertianin C having passed through the digestive tract and the colon without being degraded. It could be argued that the presence of potent antioxidants in the gastrointestinal tract may provide beneficial action against free radical damage which, in theory, could have a protective effect against colon cancer.

From an analytical perspective, the development of the preparative HPLC system was an important step. However, analysis of sixty fractions in triplicate using five different assays is time consuming and is not the type of experiment that be carried out on even a weekly basis. The laboratory has recently purchased a liquid handling machine, which will be able to carry out repetitive pipetting and mixing of solutions on an automatic basis. A new spectrophotometer has also been purchased with automation in mind. The design of the equipment will enable the liquid handling system to pump the final solution directly into the spectrophotometer flow cell. The

absorbance data obtained will be passed directly to a computer and processed by a software package such as Excel. This would not only be less tedious than manual processing but should also eliminate many potential sources of error.

Another avenue being investigated is the development of an on-line HPLC antioxidant detection system along the lines of recently published reports by Dapkevicius (2001) and Bandoniene (2002). This would allow rapid screening of fruit and vegetables for their antioxidant properties and make it practical to build up a reference data base containing the "HPLC-antioxidant finger prints" of commonly consumed fruit and vegetables. What is more, when coupled to HPLC-tandem MS or HPLC-Time of Flight MS it would enable the compounds with antioxidant activity to be directly identified.

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9 Development of analytical techniques to determine the levels of anthocyanins in raspberries.

Characterisation of anthocyanins in red raspberries by high performance liquid chromatography coupled to a single quadrupole mass spectrometer. Mullen *et al.* (2002) *Journal of Chromatography A,* in press.

Rapid characterization of anthocyanins in red raspberry fruit by high performance liquid chromatography coupled to a single quadrupole mass spectrometer.

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Abstract

Anthocyanins from red raspberries were extracted from the fruit by homogenizing in acidified methanol. The methanolic extract was centrifuged and the supernatant analyzed by reverse-phase HPLC. The eluent was monitored at 371 and 520 nm before being introduced into a single quadrupole mass spectrometer through an atmospheric pressure chemical ionization probe operating in positive ion mode. This method allowed the identification of eight anthocyanins. In the absence of readily available reference compounds approaches that can be taken to analyse anthocyanins by HPLC with absorbance and mass spectrometric detection are discussed.

1. Introduction

Anthocyanins are best known as the compounds responsible for the red color of many fruits. A number of chromatographic techniques, including thin layer chromatography (TLC) [1] and high performance liquid chromatography (HPLC) [2], have been employed to investigate the distribution and anthocyanin content of raspberries. However, it is the choice of detector that has received most attention. Hong and Wrolstad [3] used HPLC coupled to a photo diode array detector (DAD) to analyse anthocyanins in a range of fruits and berries. The diode array

absorbance spectra can be used to distinguish not only between different anthocyanins, but also whether they are glycosylated at the 3- or 3,5-positions. Furthermore information regarding the presence of acylation by hydroxylated aromatic organic acids can also be obtained from absorbance spectra. However, DAD detection is unable to distinguish between compounds with close retention times and similar absorbance spectra [4]. Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) has also been used to identify anthocyanins [5]. This too, has limitations, as it cannot distinguish between diverse structures such as the anthocyanin delphinidin-3-glucoside and the flavonol, quercetin-3-glucoside, which have the same molecular weight (M_r = 464). However, this problem can be overcome by fractionating and purification of samples prior to analysis by HPLC [6]. Following the use of solid phase extraction techniques, Giutsi et al. [7] were able to identify glycosylated and acylated anthocyanin derivatives by HPLC with DAD detection coupled to a triple quadrupole mass spectrometer. The anthocyanins were selectively fractionated and thereby separated from potential interfering compounds such as flavonols.

The aim of this investigation was to assess whether the anthocyanins present in unpurified extracts of Glen Ample raspberries could be rapidly characterized by HPLC with a dual wavelength absorbance detector coupled to a single quadrupole mass spectrometer.

2. Experimental

2.1. Extraction of plant material

Red raspberries (*Rubes idaeus* L. var. Glen Ample) were supplied by the Scottish Soft Fruit Growers (Blairgowrie, Perthshire, UK). Forty grams of fruit were macerated in a glass homogeniser. The resultant juice were decanted and remaining seeds and tissue were further extracted with methanol containing 0.1% HCl. The material was combined with the initial extract and centrifuged at 2,000 g for 30 min. The supernatant was removed and stored as 2 ml aliquots in Eppendorf tubes at -80°C. The contents of individual tubes were thawed as required and centrifuged at 15,800 g for 20 min prior to analysis by gradient elution reversed-phase HPLC.

2.2. High Performance Liquid Chromatography

Use was made of a Shimadzu (Kyoto, Japan) LC10ADvp series automated liquid chromatograph comprising a SCL-10Avp system controller, two LC-10ATvp pumps, a SIL-10ADvp auto-injector with a sample cooler and a CTO-10Avp column oven operating at 40 °C. Data were collected and processed via a Gateway 2000 G6-400 PC running Shimadzu QP8000 software. Anthocyanins in 5 μl aliquots of raspberry extract were separated using a 250 x 4.6 mm (i.d.) 5 μm Novapac C18 column fitted with a 20 x 4.6 mm (i.d.) Novapac C18 guard column (Waters Associates, Milford, MA) eluted at 0.8 ml min^{-1} with a 30 min gradient of 8-18% acetonitrile in water containing 1% formic acid. Column eluate was passed to a Shimadzu LC10AVvp uv-vis detector operating at 371 and 520 nm (maximum wavelength separation possible on this detector) before being directed to a Shimadzu QP8000 quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface probe at a voltage of 4KV, gas flow 2.5 L min^{-1} and a temperature of 400 °C. The mass spectrometer was operated in positive ion mode, scanning from 250 to 800 amu.

2.3. Reference compounds

Quercetin-3-glucoside was purchased from Extrasynthese, Genay, France. Cyanidin-3-glucoside and delphinidin-3-glucoside were obtained from Polyphenols Laboratories, Sandnes, Norway. All other chemicals were supplied by Sigma Aldrich, Dorset, England. HPLC solvents were purchased from Rathburn Chemicals, Walkerburn, Scotland.

3. Results and discussion.

Gradient reverse-phase HPLC with absorbance detection and mass spectra analysis was used to rapidly identify the main anthocyanins in Glen Ample raspberries. An A_{520nm} HPLC trace is illustrated in Figure 1A, mass spectral fragmentation patterns are shown in Figure 3 and identifications are summarized in Table 1. With the exception of cyanidin-3-glucoside, anthocyanin reference compounds were unavailable so identifications were assisted by the findings of earlier studies [1,2,8,9] and mass spectral fragmentation patterns reported by Giutsi et al. [7].

Peak 1 (t_R – 15.1 min) had a molecular ion (M^+) at m/z 611and a fragment ion at m/z 287 (M-324, loss of sophorosyl unit), which corresponds with cyanidin. On the basis of this evidence, it is concluded that peak 1 is cyanidin-3-sophoroside (**I**, Fig. 2) which is a major anthocyanin in raspberries [2].

Peak 2 (t_R – 16.5 min) was also a major raspberry anthocyanin and was identified as cyanidin-3-(2^G-glucosylrutinoside) (**II**) having a mass spectrum with an M^+ at m/z 757 and a cyanidin base peak at m/z 287 which involves a loss of M-470 corresponding to cleavage of one rhamnosyl and two glucosyl units.

Peak 3 (t_R – 17.1 min) was cyanidin-3-glucoside (**III**). It co-chromatographed with an authentic standard and the mass spectrum obtained comprised an M^+ at m/z 449 and an m/z 287 cyanidin base peak formed by loss of a single glucosyl unit (M-162).

Peak 4 (t_R – 17.7 min) had an M^+ at m/z 595 and a fragment ion at m/z 271 (pelargonidin, M-324, cleavage of sophorosyl group) and is therefore pelargonidin-3-sophoroside (**IV**).

Peak 5 (t_R – 18.7 min) also yielded a m/z 595 M^+ that yielded a major fragment ion at m/z 287 which equates with cyanidin and a loss of M-308 corresponding to cleavage of a rutinosyl unit. Peak 5 is thus identified as cyanidin-3-rutinoside (**V**).

Peak 6 (t_R – 19.3 min) produced a mass spectrum comprising M^+ at m/z 741 and loss of M-470 to yield a m/z 271 ion. This equates with cleavage of one rhamnosyl and two glucosyl units from M^+ producing a fragment ion corresponding to pelargonidin. Peak 6 is, therefore, identified as pelargonidin-3-(2^G-glucosylrutinoside) (**VI**).

Peak 7 (t_R – 20.2 min) was a minor anthocyanin with an M^+ at m/z 433 and a base peak at m/z 271 (pelargonidin) [M-162, loss a glucosyl group] and was identified as pelargonidin-3-glucoside (**VII**).

Peak 8 (t_R – 21.7 min), another minor anthocyanin, had an M^+ at 579 m/z and a base peak at m/z 271 (pelargonidin) [M-308, loss of a rutinosyl unit] and is, therefore, identified as pelargonidin-3-rutinoside (**VIII**).

These identifications above are in keeping with previously published information on red raspberry anthocyanins [2,8,9]. All eight anthocyanins had previously been detected in raspberries, though not in a single variety, in an elegant TLC-based study by [1].

The elution profile of the anthocyanins provides substantial information on the nature and identity of the compounds under study. Under the chromatographic conditions used in the present study, the anthocyanin reversed-phase HPLC elution profile is consistent with that reported by Hong and Wrolstad [3]. Dephinidin elutes first followed by cyanidin, pelargonidin, petunidin and malvidin. In general as the degree of glycosylation of the conjugate increases, the anthocyanins are less well retained and have shorter retention times [3]. However, when the glycoside moiety contains rutinose, the hydrophobic methyl group increases the retention time, hence cyanidin-3-(2^G-glucosylrutinoside) elutes after cyanidin-3-sophoroside and likewise the

equivalent pelargonidin derivatives (Fig.1A). A much more marked increase in retention time is observed with anthocyanins that have been acylated. For instance, reported gradient reversed phase HPLC retention times for delphinidin-3-glucoside and delphinidin-3-O-(6-O-p-coumaroyl)glucoside were 13.5 and 51.5 min respectively [10].

The detection method of choice for anthocyanins has been an absorbance monitor since this offers the advantage that in the region of maximal absorbance for anthocyanins, 500-520 nm, very few other compounds absorb strongly and, as a consequence, extremely clean HPLC traces are obtained without sample purification. This is evident in the A_{520nm} HPLC trace obtained with a crude raspberry extract illustrated in Figure 1A. Examination of the mass spectrometric total ion current trace obtained with the same sample (Fig. 1B) reveals a very large early eluting multicomponent impurity peak that was not present in the A_{520nm} trace (Fig. 1A). These, and other impurities can adversely affect the signal to noise ratio of the mass spectrometer, reducing sensitivity and adding a degree of uncertainty to the identification of a compound by its mass spectral characteristics. In order to prevent this in the present investigation, HPLC mobile phase conditions were employed that allowed the bulk of the impurities to elute before the anthocyanins. To minimize mass spectrometer contamination, this portion of the chromatogram was diverted to waste.

Good chromatographic separations and retention time information can play an important role in the interpretation of mass spectra data obtained with complex mixtures. As seen in Table 1 both cyanidin-3-rutinoside and pelargonidin-3-sophoroside have an M^+ at m/z 595 and they elute within one minute of each other. These compounds cannot be distinguished solely by molecular ion information, which is what is generated by MALDI-MS [5] and selected ion monitoring (SIM) and as a consequence, with these types of MS, accurate chromatographic information and/or fragmentation data are required to reveal the identity of the anthocyanin conjugate.

The need for further information becomes more evident when an examination of common sugars and acylating groups associated with anthocyanins is undertaken [7]. For instance, the mass spectrum of cyanidin-3-(2^G-glucosylrutinoside) contains two prominent ions, the M^+ at m/z 757 and the aglycone base peak at m/z 287 (Table 1). This mass spectrum could be interpreted as being that of cyanidin-3-sophoroside acetylated with p-coumaric acid. In theory DAD spectra would distinguish between these compounds as the acylated aromatic acid has a characteristic peak at 310 nm [3]. However, in a complex mixture with multiple co-eluting compounds, an increase in absorbance at 310 nm could easily arise from a co-eluting component, thereby adding a degree of uncertainty to the analysis.

Sole reliance on HPLC with DAD detection has already been shown to have caused two misidentifications of alleged anthocyanins, as discussed by Cao and Prior [11]. In both cases the investigators, Paganga and Rice-Evan [12] and Lapidot et al. [13], claimed to have identified anthocyanins solely on the basis of absorbance spectra obtained by HPLC with a DAD detector. However, on closer inspection, the spectrum obtained from a urine sample by Lapidot et al. [13] has a 431 nm peak as well as a peak at 520 nm. This spectrum is not that of an authentic anthocyanin and at best could be a metabolite. The peak detected in plasma by Paganga and Rice-Evans [12] is reported by Cao and Proir [11] to have too broad a 280 nm peak, ranging from 250-350 nm. Further doubt must be cast on this study as the identification was based on a single analysis of a single plasma sample from a single volunteer whose dietary intake of flavonoids was not controlled.

Crude extracts can also contain compounds that have the same fragmentation patterns as anthocyanins. This is the case with delphinidin-3-glucoside and the equivalent flavonol conjugate quercetin-3-glucoside (Fig. 3). After positive ionisation the anthocyanin produces an M^+ at m/z 465 and an aglycone ion at m/z 303 and the fragmentation of the flavonol is similar. This potential problem is readily resolved by monitoring absorbance at 371 and 520 nm as this distinguishes between flavonols, which absorb only at the lower wavelength and anthocyanins which absorb at 520 nm and also, albeit less intensely, at 371 nm.

4. Conclusions

With state of the art technology and minimal sample preparation Gusti et al (4) were able to positively identify anthocyanins from various sources. Barritt and Torre (1) were able to achieve similar results with much simpler, but time-consuming, TLC-based procedures. However, the results obtained by Paganga and Rice–Evans (12), using gradient reverse-phase HPLC coupled to DAD detection, have been questioned, because of the subjective interpretation of an absorbance spectrum (11) allied to poorly controlled experimental procedures.

When analysing complex mixtures it is important to be aware of the limitations of the analytical system. The key information provided by HPLC with absorbance detection is retention time data. As well as determining the retention time of reference standards it is vital that they also be used routinely for co-chromatography to confirm the identity of peaks in extracts with greater surety.

The use of HPLC with mass spectrometric detection can reduce the reliance on retention time data. However, on its own, it does not necessarily provide positive identification of all compounds. For instance, MALDI-TOF and SIM-MS cannot distinguish between flavonol conjugates and anthocyanins with the same molecular weight. Therefore, either separation of these flavonoid groups must be performed before analysis or absorbance spectra must be acquired. However, there are many anthocyanins with the same molecular weight (4) in which case MS fragmentation data must be obtained. If this is not possible, acid hydrolysis could be performed to enable the released anthocyanidin to be identified.

The system in the present study can monitor absorbance at two wavelengths, so by selecting the appropriate wavelengths identification of flavonols and anthocyanins can be achieved in one chromatographic analysis. Furthermore, the mass spectrometric data contains information on the fragmentation of the compounds of interest allowing identification of the conjugate and the aglycone moiety. The method, thus, fits the criteria for the characterization of anthocyanins in raspberry extracts without the need for sample preparation.

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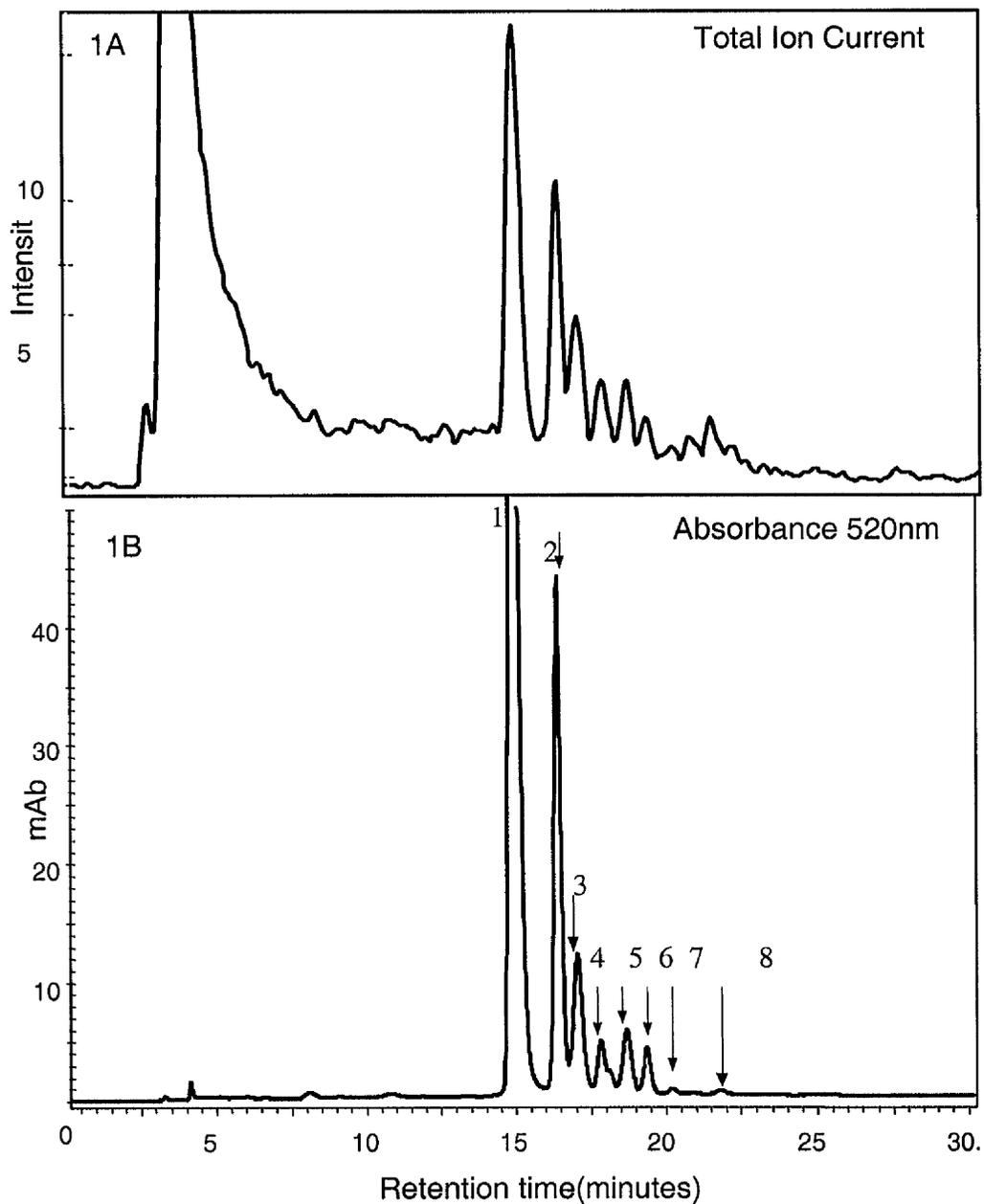
Fig.1 Analysis of a raspberry extract with by gradient reverse-phase HPLC using absorbance and mass spectrometric detection. A 5 μl aliquot of a raspberry extract was analysed using a 250 x 4.6 mm (i.d.) 5 μm novapac C18 column fitted with a 20 x 4.6 mm (i.d.) Novapac C18 guard column eluted at 0.8 ml min^{-1} with a 30 min gradient of 8-18% acetonitrile in water containing 1% formic acid. Column eluate was passed to an absorbance monitor operating at 371 and 520 nm before being directed to a quadrupole mass spectrometer equipped with an APCI interface probe at a voltage of 4KV, gas flow 2.5 L min^{-1} and a temperature of 400 ¢^{a}C. The mass spectrometer was operated in positive ion mode, scanning from 250 to 800 amu.

Trace **A** obtained with an absorbance monitor operating at 520 nm. Trace **B** is the total ion current from the mass spectrometer.
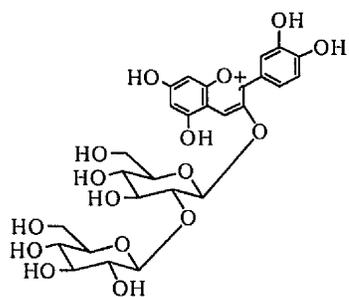
Cyanidin-3-*O*-sophoroside (**I**)
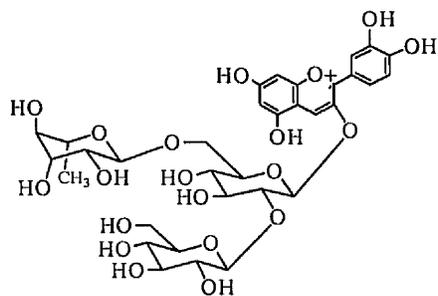
Cyanidin-3-*O*-(2^G-*O*-glucosylrutinoside (**II**)
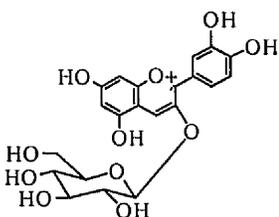
Cyanidin-3-*O*-glucoside (**III**)
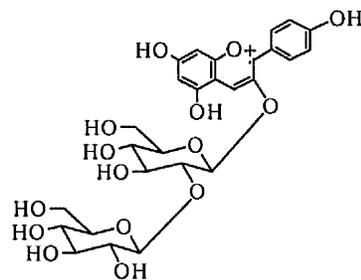
Pelargonidin-3-*O*-sophoroside (**IV**)
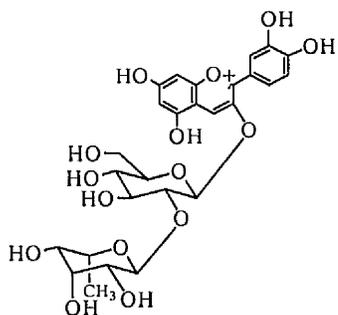
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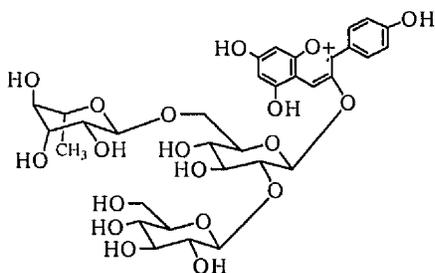
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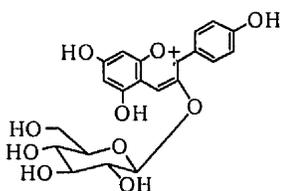
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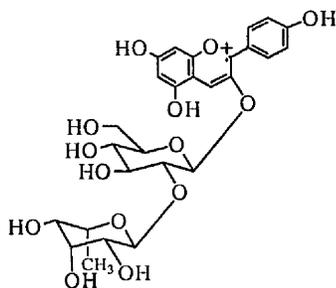
Pelargonidin-3-*O*-rutinoside (**VIII**)

Figure 2 Structures of anthocyanins found in raspberries

48
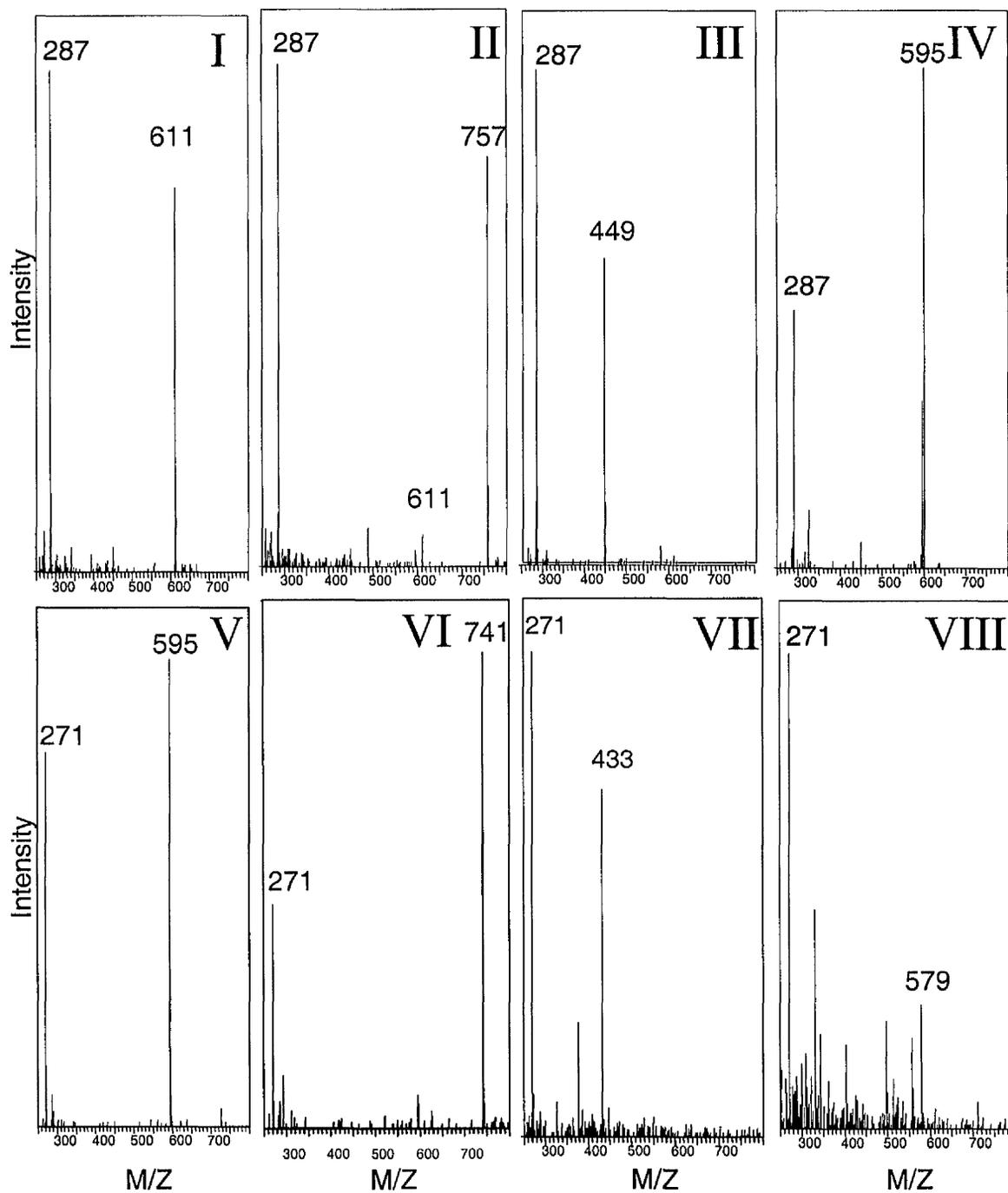
Fig. 3. positive ion APCI mass spectra of anthocyanins detected in red raspberries;
(I) cyanidin-3-sophoroside, (II) cyanidin-3-(2^G-glucosylrutinoside), (III) cyanidin-3-glucoside,
(IV) pelargonidin-3-sophoroside, (V) cyanidin-3-rutinoside, (VI) pelargonidin-3-(2^G-glucosylrutinoside),
(VII) pelargonidin-3-glucoside, (VIII) pelargonidin-3-rutinoside.

49
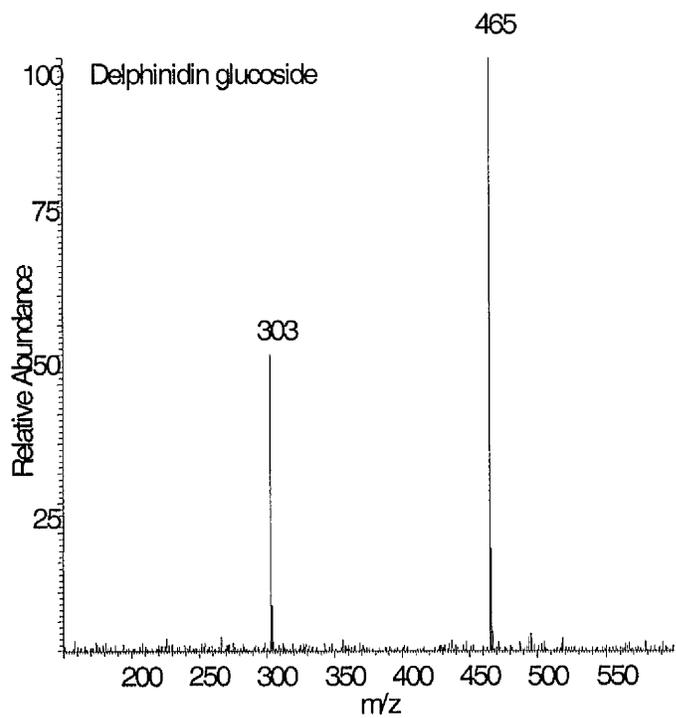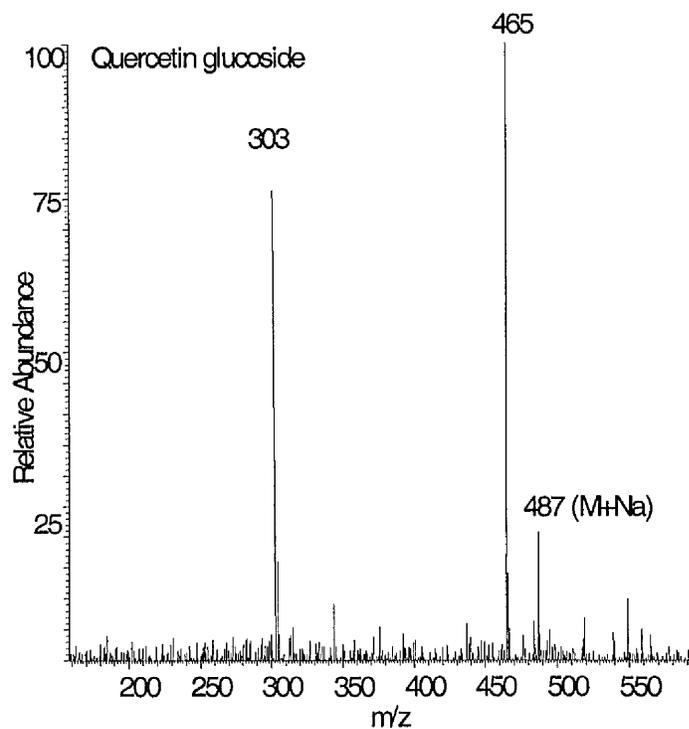
Fig. 4. Positive ion APCI mass spectra of quercetin-3-glucoside and delphinidin-3-glucoside.

10 Determination of the major antioxidant and vasodilatory compounds in raspberries

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Ellagitannins, Flavonoids and Other Phenolics: Their Contribution to Antioxidant Capacity and Vasorelaxation Properties of Red Raspberries

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SUMMARY

Analysis of extracts of Glen Ample raspberries (*Rubus idaeus* L.) by gradient, reverse phase HPLC with diode array and tandem mass spectrometry identified eleven anthocyanins, including cyanidin-3-sophoroside, cyanidin-3-(2^G-glucosylrutinoside), cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-sophoroside, pelargonidin-3-(2^G-glucosylrutinoside) and pelargonidin-3-glucoside. Significant quantities of an ellagitannin, sanguiin H-6, with a M_r of 1870 were detected along lower levels of a second ellagitannin, lambertianin C which has a M_r of 2804. Other phenolic compounds that were detected included trace levels of ellagic acid and its sugar conjugates along with one kaempferol- and four quercetin-based flavonol conjugates. Fractionation by preparative HPLC revealed that sanguiin H-6 was a major contributor to the antioxidant capacity of raspberries together with vitamin C and the anthocyanins. Vasodilation activity was restricted to fractions containing lambertianin C and sanguiin H-6.

Keywords: *raspberries; ellagitannins, anthocyanins, vitamin C, flavonols; HPLC; tandem mass spectrometry; antioxidant capacity; vasodilation activity,*

INTRODUCTION

Raspberries (*Rubus idaeus* L.) have a high free radical scavenging capacity and are rich in both vitamin C and total phenolics (1). They contain a distinct spectrum of anthocyanins (2). The major component is cyanidin-3-sophoroside with smaller quantities of other anthocyanins including cyanidin-3-(2^G-glucosylrutinoside), cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-sophoroside, pelargonidin-3-(2^G-glucosylrutinoside) and pelargonidin-3-glucoside (2-4). Some varieties of raspberries contain very high levels of ellagitannins, which on hydrolysis release ellagic acid (5), a compound that has been reported to have antiviral activity (6) and provide protection against cancers of the colon (7) lung and oesophagus (8). Raspberries also contain a wide variety of quercetin and kaempferol-based flavonol conjugates with the major components being quercetin-3-glucuronide and quercetin-3-glucoside (5,9). In addition, raspberry juice is reported to contain catechins (10).

This paper reports on the phenolic compounds in Glen Ample raspberries which are cultivated extensively on a commercial basis in Scotland. Extracts were analysed by gradient, reverse phase HPLC with diode array detection followed by tandem mass spectrometry. Preparative HPLC was carried out in order to identify compounds in raspberries possessing antioxidant activity and

vasorelaxation ability. Natural dietary phenolics can cause endothelium-dependent vasodilation in human and rodent arteries (11), via the release of nitric oxide from vascular endothelium and subsequent increases in cGMP levels (11-13). Previous studies have shown that red wine and grape derived products are potent vasodilators *in vitro*. This cardio-protective effect has been attributed to the phenolic component, especially the high grape skin-derived anthocyanin content, of red wine. To date, there is no published information on the vasodilatory activity of raspberries.

MATERIALS AND METHODS

Chemicals. Cyanidin-3-glucoside was purchased from Apin Chemicals (Abingdon, Oxford, UK). Methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Peebleshire U.K.). All other chemicals and reagents were purchased from Sigma-Aldrich (Poole, Dorset, U.K.).

Plant Material. One kg of ripe field-grown raspberries cv. Glen Ample, hand picked at Blairgowrie, Perthshire, UK were frozen in liquid nitrogen within 3 h of harvest and there after stored at -80°C prior to processing.

Extraction of Raspberries. Forty g of raspberries were macerated in an ice-cold pestle and mortar and the resulting homogenate was centrifuged at 2,000 g for 30 min at 4°C. The supernatant was decanted and the pellet homogenised and extracted with acidified (0.1% HCl) methanol after which it was re-centrifuged. The two supernatants were combined, and made up to a known volume with acidified methanol. This was then sub-divided into 2-ml aliquots and stored in microcentrifuge tubes at -80°C prior to analysis.

For preparative studies the same procedure was used with 150 g of raspberries after which the acidified extract in 1 % methanol was applied to a 50 x 20 mm (i.d.) ion-exchange column (Diaion HP-20) which was eluted with 100 ml acidified methanol/water (1:99, v/v) to remove sugars and other contaminants. It was next washed with 100 ml 100% acidified methanol to elute phenolic and flavonoid compounds. The methanol fraction was then passed through a 50 x 20-mm (i.d.) column containing a 40-μm C_{18} silica gel support that was further eluted with 100-ml acidified methanol. The methanolic eluates were combined and reduced to dryness *in vacuo* prior to preparative HPLC.

Qualitative Analysis of Phenolic Compounds in Raspberries by HPLC with Absorbance and MS Detection. Methanolic extracts of raspberries were analysed on a P4000 liquid chromatograph fitted with an AS 3000 autosampler and with detection by a UV6000 diode

array absorbance monitor scanning from 250 to 700 nm (Thermo-Finnigan, San Jose, CA., USA). Separation was carried out using a RP-MAX 4 μm 250 x 4.6 mm (i.d.) C_{12} reverse phase column (Phenomenex, Torrance, CA., USA) maintained at 40°C and eluted at 1.0 mL min^{-1} with a 60 min gradient of 5-30% acetonitrile in water containing 1% formic acid. After passing through the flow cell of the diode array detector, the column eluate was split and 50% directed to an LCQ Duo mass spectrometer (Thermo-Finnigan) with an electrospray interface operating in full scan data dependant MS-MS mode from 150 to 2000 amu. Each sample was analysed twice, first in positive ion then in negative ion mode.

Analysis of Vitamin C by HPLC with Absorbance Detection. The vitamin C content of fractions collected after preparative HPLC of a raspberry extract were analysed by HPLC (14) using a Nucleosil ODS 5 μm 250 mm x 4.6 mm (i.d.) column (Jones Chromatography, Glamorgan, UK) eluted isocratically at a flow rate of 0.6 mL min^{-1} using a Gilson model 305 liquid chromatograph with a cooled autoinjector and a 231 absorbance detector operating at 263 nm. Data were recorded on a Gilson 715 data system.

Preparative Reverse Phase HPLC. The acidic, partially purified methanolic extract from 150 g of raspberries was redissolved in 100 ml 5% methanol in water containing 1 % formic acid and a 10 ml aliquot fractionated into 60 separate 5 mL samples using a Gilson 305 gradient liquid chromatograph with a Rheodyne preparative injector (CA, USA) and a 10 mL sample loop. Separation was carried out using a 150 x 20-mm (i.d.) 5 μm ODS-H optimal® column (Capital HPLC, Broxburn, UK) maintained at 40°C in a Shimadzu (Kyoto, Japan) CTO-6A column oven. Samples were eluted at a flow rate of 10-mL min^{-1} with a 30-min gradient of 5-25% acetonitrile in 1% formic acid followed by an 80% wash. Column eluate was directed first to a Shimadzu SPD-10Avp UV-vis absorbance monitor operating at 520 nm before being directed to a Gilson FC 203 microfraction collector. Collection of column eluate began 3 min after injection of the sample, with successive fractions collected every 0.5-min thereafter. Signals from the absorbance monitor were processed by a Reeve Analytical 27000 data system. Each fraction was then analysed by HPLC-MS-MS as described above. The antioxidant capacity, total phenol and anthocyanin contents of each fraction were also determined as outlined below. The vitamin C content of fractions 1-6 was determined by HPLC using methodology that is also outlined above.

Determination of Total Phenol Content. The total phenol contents of raspberry extracts were determined in gallic acid equivalents using the Folin-Ciocalteu method (15).

Colorimetric Analysis of Total Anthocyanin Content. The anthocyanin content of raspberry extracts were estimated using a pH shift method (16). Anthocyanins were quantified as cyanidin-3-glucoside equivalents, one of the three major anthocyanin in raspberries, using the extinction coefficient $\varepsilon = 29600$.

Measurement of Antioxidant Capacity by Electron Spin Resonance Spectroscopy. The antioxidant capacity of raspberries extracts was determined by their ability to reduce the Fremy's salt (potassium nitrosodisulphonate) (17). The extracts were diluted to 5% (v/v) with ethanol/water (12:88, v/v). Three 1.0-mL aliquots were reacted with an equal volume of 1-mM Fremy's radical in ethanol/water (12:88, v/v). The electron spin resonance 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 (12:88, v/v) without raspberry extract. Spectra were obtained at 21° C 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 was set at 2 mW and 0.01 mT, respectively.

Vasorelaxation Assay. Male New Zealand rabbits, weighing ca. 3.5 kg, were anaesthetised by intravenous administration of pentobarbitone (100 mg kg^{-1}) with 1000 IU heparin into the marginal ear vein. The descending thoracic aorta was then carefully removed, cleaned of adhering fat and connective tissue and cut into transverse ring segments ca. 3-5 mm in length. The rings were suspended in 10 mL organ baths filled with Krebs buffer solution (composition: 118.4 mM NaCl; 25 mM NaHCO$_3$; 4.7 mM KCl; 1.2 mM KH$_2$PO$_4$; 1.2 mM MgSO$_4$; 2.5 mM CaCl$_2$; 11 mM D-glucose, pH 7.4) continuously oxygenated with 16% O$_2$, 5% CO$_2$ and 79% N$_2$ and maintained at 37 °C to mimic the internal environment. Rings were placed under the optimal resting tension of 2 g. This tension was kept constant throughout the course of the experiment. Vessel contraction/relaxation was measured via an isometric force transducer, linked in turn to a data handling system.

After an equilibration period of 45 min, vessels were maximally contracted twice with KCl (50 mM). After each contraction, vessels were washed out with Krebs solution. Vessel tone was then raised in all vessels with phenylephrine (10^{-7} M), and relaxed with acetylcholine, to test for functional and intact endothelium. Vessels were then washed thoroughly with Krebs solution.

After washing and returning to initial baseline tension, aortic rings with functional endothelium were precontracted submaximally with phenylephrine (10^{-7} M). After precontraction of the aortic rings the effect of the raspberry fractions on vascular tone was

studied. Once a stable plateau had been reached, 25 μL of each raspberry fraction concentrate was added to the organ bath to induce vasodilation in the aortic ring segments. All results were calculated and expressed as the percentage vasorelaxation induced by each raspberry fraction following precontraction with phenylephrine.

RESULTS

HPLC-diode array-MS-MS analysis of raspberry phenolics. An extract of Glen Ample raspberries was analysed by reverse phase HPLC using 60 min, 5-30% acetonitrile gradient with column eluate being directed first to a diode array absorbance monitor then a mass spectrometer with an electrospray interface operating in full scan MS-MS mode. The sample was analysed twice, in positive ion then in negative ion mode. The 10-25 min absorbance trace obtained at 520 nm is illustrated in Figure 1 in which the 11 peaks, on which positive ion MS data were obtained, are labelled. Further peaks were detected when the 25-45 min segment of the chromatogram was examined at 365 nm (Fig. 2). The peaks labelled 12-23 in Figure 2 are those that yielded negative ion MS data. The identifications of peaks 1-23, based on MS-MS data and λ_{max}, are summarised below and outlined in Table 1. Identification of 11 anthocyanins, all of which had previously been detected in raspberries, though not in a single variety, in a TLC-based study by Barritt and Torre (18), was assisted by data on the MS fragmentation patterns of anthocyanins, their aglycones and the m/z losses associated with cleavage of various sugars and other substituent groups presented by Giusti et al (19). Likewise the data of Rommel and Wrolstad (5) and Zafrilla et al. (20) aided the MS-MS-based identifications of flavonols and ellagic acid sugar conjugates, respectively. Additional compounds to those listed in Table 1 were identified by MS-MS but they were invariably present in trace amounts close to or co-chromatographing with larger peaks which precluded accurate analysis by HPLC with diode array detection. Compounds coming into this category included (-)-epicatechin (M^+ - m/z 291) which had a retention time (R_t) of 21.8 min and a catechin dimer (M^+ - m/z 579) which had an R_t of 18.52 min.

Peak 1 (R_t - 12.3 min) was an anthocyanin with a λ_{max} at 519 nm. When analysed by MS-MS it yielded a M^+ at m/z 611 which fragmented to produce an m/z 449 ion (M-162, loss of a glucosyl group) and m/z 287 which equates with cyanidin produced from m/z 449 by cleavage of a second glucosyl fragment. On the basis of this evidence, it is concluded that this compound is cyanidin-3,5-diglucoside.

Peak 2 (R_t - 15.7 min) had a λ_{max} of 519 nm indicating that it was an anthocyanin which on the basis of a M^+ at m/z 611 that produced a fragment ion at m/z 287 (M-324, loss of a sophorosyl unit) was identified as cyanidin-3-sophoroside which is a major anthocyanin in raspberries (see Fig. 2).

Peak 3 (R_t - 17.1min) was another major anthocyanin and was identified as cyanidin-3-(2^G-glucosylrutinoside) on the basis of its λ_{max} at 519 nm and a mass spectrum comprising a M^+ at m/z 757 which fragmented to produce a minor ion at m/z 611 (M-146, loss of a rhamnosyl group) and a major ion at m/z 287 (M-324, loss of two glucosyl moieties).

Peak 4 (R_t - 17.5 min) was cyanidin-3-glucoside. Its properties corresponded with those of an authentic standard with a λ_{max} at 519 nm and an MS-MS spectrum consisting of a M^+ at m/z 449 which on loss of a glucosyl unit (M-162) yielded a secondary fragment at m/z 287 corresponding to cyanidin.

Peak 5 (R_t - 17.7 min) was identified as cyanidin-3-sambubioside on the basis of its λ_{max} at 519 nm and a mass spectrum comprising a M^+ at m/z 581 which fragmented to produce a minor ion at m/z 449 (M-132, loss of a xylosyl group) and a major ion at m/z 287 (M-162, loss of a glucosyl unit).

Peak 6 (R_t - 18.4 min), had a λ_{max} at 503 nm which is 16 nm lower than the λ_{max} of the cyanidin conjugates. The MS-MS spectrum, with a M^+ at m/z 595 that fragments with a loss of m/z 324 to produce an m/z 271 ion, corresponds with pelargonidin-3-sophoroside which has an M_r 16 mass units less than that of cyanidin-3-sophoside in peak 2.

Peak 7 (R_t - 18.8 min) had a λ_{max} at 519 nm and on the basis of its MS-MS spectrum (m/z 727 [M^+], m/z 581 (M-146, loss of a rhamnosyl moiety) and m/z 287) (M-294, loss of xylosyl and glucosyl groups) was identified as cyanidin-3-xylosylrutinoside.

Peak 8 (R_t - 19.3 min) had an absorbance spectrum with a λ_{max} at 519 nm and MS-MS analysis yielded a M^+ at m/z 595 which fragmented to give an m/z 287 ion (M-308, cleavage of a rutinosyl unit). Peak 7 is therefore cyanidin-3-rutinoside.

Peak 9 (R_t - 20.0 min) had a λ_{max} of 503 nm and the MS-MS spectrum was identical to that of cyanidin-3-(2^G-glucosylrutinoside) in peak 3 except that the ions were at m/z values 16 amu lower (M^+ - m/z 741 with fragment ions at m/z 595 [M-146] and 271 [M-324]). Peak 8 is therefore pelargonidin-3-(2^G-glucosylrutinoside).

Peak 10 (R_t - 20.7 min) had a λ_{max} of 503 nm and the mass spectrum contained an M^+ at m/z 433 which fragmented to yield m/z 271 (M-162) which is in keeping with the properties of pelargonidin-3-glucoside.

Peak 11 (R_t - 22.2 min) also had a λ_{max} of 503 nm and on the basis of the MS data obtained, M^+ at m/z 579 giving rise to a secondary fragment at m/z 271 (M-308) is identified as pelargonidin-3-rutinoside.

Peak 12 (R_t - 26.3 min) had a λ_{max} of 250 nm and when subjected to acid hydrolysis yielded ellagic acid. The mass spectrum of this compound was complicated as the M_r was greater than the 2000 amu upper mass limit of the mass spectrometer. The exact mass of 2804 was determined from a doubly charged ion at m/z 1401. Another doubly charged fragment was observed at m/z 1250 (M-302, loss of a hexahydrodiphenoyl [HHDP] unit). The first singly charge ion at m/z 935 corresponds with the ellaginanin isomers casuarictin/potentillin. On the basis of this fragmentation pattern the compound is identified as lambertianin C, which is a trimer of casuarictin/potentillin, previously identified in raspberry leaves (21)

Peak 13 (R_t - 27.8 min) had a λ_{max} of 250 nm and when subjected to acid hydrolysis yielded ellagic acid. The MS-MS spectrum had an M^- at m/z 1869 which fragmented to produce m/z 1567 (M-302, loss of an HHDP unit), m/z 1265 (M-302, a further loss of HHDP), m/z 1103 (M-162, loss of a glucosyl group) and m/z 933 (M-170, loss of a gallic acid), m/z 631 (M-302), loss of HHDP). On the basis of the MS-MS spectrum and previously published data (21,22,23), this compound is identified as sanguiin H-6 which is a dimer of casuarictin/potentillin.

Peak 14 and 15 (R_t - 29.7 and 31.0 min) Both had λ_{max} at 361 nm and produced a mass spectrum with a M^- at m/z 433 which yielded a fragment ion at m/z 301 (M-132), which corresponds with ellagic acid. This is indicative of the presence of pentose conjugates of ellagic acid. One of these compounds may be ellagic acid-4-arabinoside, the presence of which has been reported in raspberries (20).

Peak 16 (R_t - 32.1 min) also had a λ_{max} at 361 nm. It was identified as ellagic acid on the mass spectral data as it produced a M^- at m/z 301, but was in such a low concentration no MS-MS data were obtained. This peak co-chromatographed with an authentic ellagic acid standard.

Peak 17 (R_t - 33.0 min) had a λ_{max} at 365 nm and is quercetin-3-*O*-rutinoside (rutin) on the basis of co-chromatography with an authentic standard and a mass spectrum with ions at m/z 609 (M^-) and 301 (M-308).

Peak 18 (R_t - 34.7 min) had a λ_{max} at 365 nm and was identified as quercetin-3-glucoside on the basis of co-chromatography with an authentic standard and an MS-MS spectrum with a molecular ion at m/z 463 and a fragment ion at m/z 301 (M-162, loss of a glucosyl unit).

Peak 19 (R_t - 35.5 min) was also a flavonol with a λ_{max} at 365 nm. The mass spectrum had a m/z 477 molecular ion that yielded a M-176 (cleavage of a glucuronosyl unit) fragment ion

at m/z 301 indicating the presence of quercetin-3-glucuronide which has been detected previously in raspberries (24)

Peak 20 (R_t - 38.2 min) was characterised by a λ_{max} at 365 nm and an MS-MS with an m/z 447 molecular ion which ionised to produce an M-132 fragment (loss of a pentosyl unit) at m/z 315 suggesting the presence of an methylquercetin pentose conjugate, probably a xyloside.

Peak 21 and 23 (R_t - 38.7 and 41.6min) had a λ_{max} at 360 nm, both mass spectra had a m/z 475 molecular ion and a fragment ion at m/z 301 (M-174, loss of an acetyl pentose unit). Both components are therefore tentatively identified as ellagic acid acetyl pentose conjugates. On the basis of their HPLC elution order and the data of Zafrilla et al. (20) peak 21 may be ellagic acid-4-acetylxyloside and peak 23 ellagic acid-4-acetylarabinoside.

Peak 22 (R_t - 41.1 min) also had a λ_{max} at 365 mn and a mass spectrum with a 461 m/z molecular ion that yielded a MS-MS fragment at m/z 285 (M-176, loss of a glucuronosyl group) indicating the occurrence of a kaempferol glucuronide, the presence of which has previously been reported in raspberries (9,24).

Fractionation of a raspberry extract. After removal of the sugars and having been run through a low-pressure reverse phase column an extract aliquot corresponding to 15 g of raspberries was separated into 60 fractions by preparative, gradient elution reversed phase HPLC. The fractions were then analysed colorimetrically for total phenolics and anthocyanin content as well as for antioxidant and vasodilation activity. The data obtained are presented in Figure 3. The highest concentration of phenolics was centred in fraction 38. There were also smaller peaks in fractions 2-4 and fraction 7 as well as a broad band of phenolics, which corresponded with the anthocyanins in fractions 23-30. There was a close match between profiles obtained for the total phenolics and the antioxidant capacity with zones of antioxidant activity being detected in fractions 2-4, 23-32 and 36-39 with most activity being present in fraction 38. There were no peaks of antioxidant activity eluting after fraction 40 in fractions that contained the ellagic acid derivatives and flavonols listed in Table 1. Vasodilation assays were carried out on fractions 20-40 and this revealed a major peak of activity in fractions 35-39.

Analysis of the individual fractions by LC-MS-MS established that sanguiin H-6 was concentrated in fraction 38, with lower amounts in fractions 37 and 39, while lambertianin C was present in fractions 35-37. In keeping with the data obtained by colorimetric assay, LC-MS-MS showed that fractions 23-30 contained anthocyanins with the major components being distributed as follows: cyanidin-3,5-diglucoside (fractions 22-23); cyanidin-3-sophoroside (fractions 23-25), cyanidin-3-(2^G-glucosylrutinoside) (fractions 25-27); cyanidin-3-glucoside (fractions 27-29);

cyanidin-3-rutinoside (fractions 29-30). No recognisable ions were detected when fractions 2-4 and 7 were analysed by LC-MS-MS. Vitamin C eluted as a void volume peak under the HPLC conditions used for MS-MS. However, analysis by HPLC, using the procedure outlined in the Materials and Methods, revealed the presence of vitamin C in fractions 2-4.

DISCUSSION

LC-MS-MS analysis identified 11 anthocyanins in Glen Ample raspberries (Table 1). The main components were cyanidin-3-sophoroside, cyanidin-3-(2^G-glucosylrutinoside, and cyanidin-3-glucoside with smaller amounts of cyanididn-3-rutinoside, pelargonidin-3-sophoroside and pelargonidin-3-(2^G-glucosylrutinoside) and trace levels of cyanidin-3,5-diglucoside, cyanidin-3-sambubioside, cyanidin-3-xylosylrutinoside, pelargonidin-3-sophoroside, pelargonidin-3-glucoside and pelargonidin-3-rutinoside. The flavonols quercetin-3-rutinoside, quercetin-3-glucoside, quercetin-3-glucuronide were identified along with a kaempferol glucuronide conjugate and a putative xyloside conjugate of methylquercetin. Two ellagitannins were also detected. The first, lambertianin C, which has an M_r of 2804 consists of six HHDP, three galloyl and three glucosyl moieties. The second ellagitannin, sanguiin H-6, which was present in substantial amounts, has an M_r of 1870 and comprises four HHDP, two galloyl and two glucosyl units. Trace levels of ellagic acid were also present as well as two pentose conjugates of ellagic acid that eluted from the HPLC column immediately before ellagic acid. One of these compounds may be ellagic acid-4-arabinoside which was detected in low concentrations in raspberries and raspberry jams by (20). In addition, these investigators also characterised ellagic acid-4-acetylxyloside and ellagic acid-4-acetylarabinoside that was also detected in the present study.

The data obtained in the present study indicate that raspberries do not contain high levels of ellagic acid *per se* and that the high levels of this compound that have been detected after hydrolysis of extracts (1,5) are likely to have been derived primarily from sanguiin H-6 and lambertianin C.

Preparative reversed phase HPLC of a raspberry extract separated three zones of antioxidant capacity in fractions 2-4 which contained vitamin C, fractions 23-32 where anthocyanins were present and fractions 36-39 were lambertianin C and sanguiin H-6 were detected (Fig. 3). The major peak of antioxidant activity was in fraction 38 which contained sanguiin H-6. However, calculation of the overall antioxidant activity in each of the three zones reveals that the vitamin C zone in fractions 2-4 reduced 1.6 x 10^{17} radicals g^{-1} f.w., the

anthocyanins in fractions 23-32, 3.8 x 10^{17} radicals g^{-1} f.w., and the ellagitannins in fractions 36-39, 3.2 x 10^{17} radicals g^{-1} f.w. Thus, although no single anthocyanin makes a contribution to the antioxidant capacity of the raspberries equivalent to that of sanguiin H-6, the combined contribution of the 11 anthocyanins in fractions 23-32 is slightly higher than that of the ellagitannins in fractions 36-39. The antioxidant activity in fractions 2-4, which contain vitamin C, is half that of the ellagitannins. The lack of antioxidant activity after fraction 40 indicates that ellagic acid and its sugar conjugates and flavonols do not make a significant contribution to the antioxidant capacity of Glen Ample raspberries.

Vasodilation assays were carried out on fractions 20-40. The anthocyanins in fractions 23-32 exhibited little activity while a major zone of activity in fractions 35-39 was closely associated with the presence of lambertianin C and sanguiin H-6. Many plants, especially fruits and vegetables, contain extractable compounds that cause endothelium-dependent vasorelaxation *in vitro* (25). In this study, raspberry fractions associated with the presence of lambertianin C and sanguiin H-6, have been shown to be potent vasodilators of rabbit aortic vessels with intact endothelium. However, a previous study has shown that anthocyanins derived from red wine, unlike those from raspberries, induced vasorelaxation (11). These anthocyanins were subsequently found to be more potent dilators than oligomeric condensed tannins that are also present in red wine. Additional work with anthocyanins has demonstrated that delphinidin, but not malvidin or cyanidin, is able to elicit endothelium-dependent vasorelaxation in vascular tissue (12). Delphinidin-induced vasorelaxation, which was comparable to the original red wine polyphenolic extract, was mediated via the release of nitric oxide (11). Therefore, from the data obtained with grapes and other red fruits, it is evident that only specific anthocyanin structures may be able to induce vasorelaxation. The exact anthocyanins from red wine that produce a cardio-protective vasodilatory effect remain to be determined.

ACKNOWLEDGEMENTS

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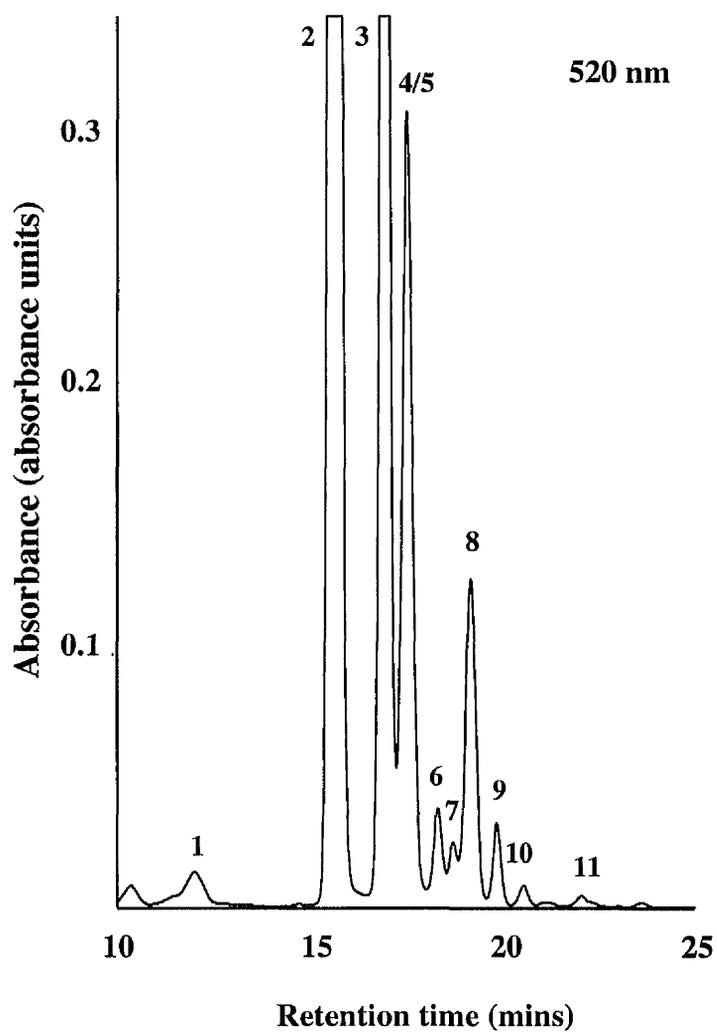
Figure 1. Gradient reversed phase HPLC of an extract of Glen Ample raspberries.
The 10-25 min segment of a 60 min gradient of 5-30% acetonitrile in water containing 0.1% formic acid is illustrated with detection at 520 nm.
Numbering of peaks refers to their subsequent identification by tandem mass spectrometry.
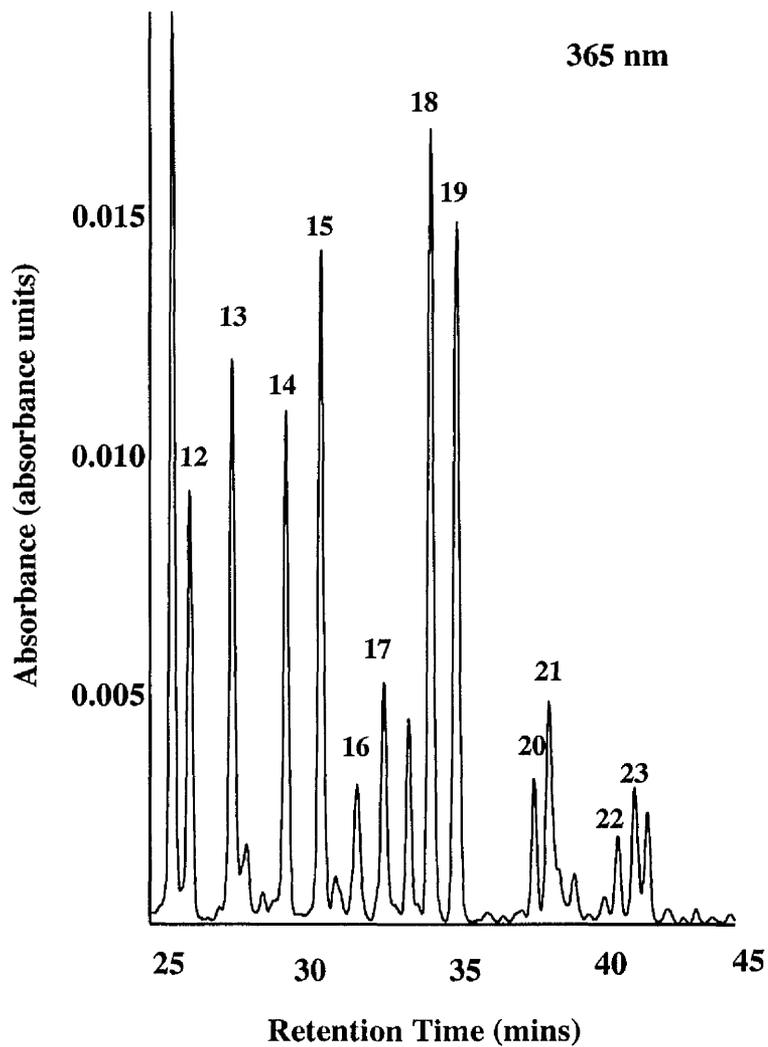
Figure 2. Gradient reversed phase HPLC of an extract of Glen Ample raspberries.
The 25-45 min segment of a 60 min gradient of 5-30% acetonitrile in water containing 0.1% formic acid is illustrated with detection at 365 nm.
Numbering of peaks refers to their subsequent identification by tandem mass spectrometry..
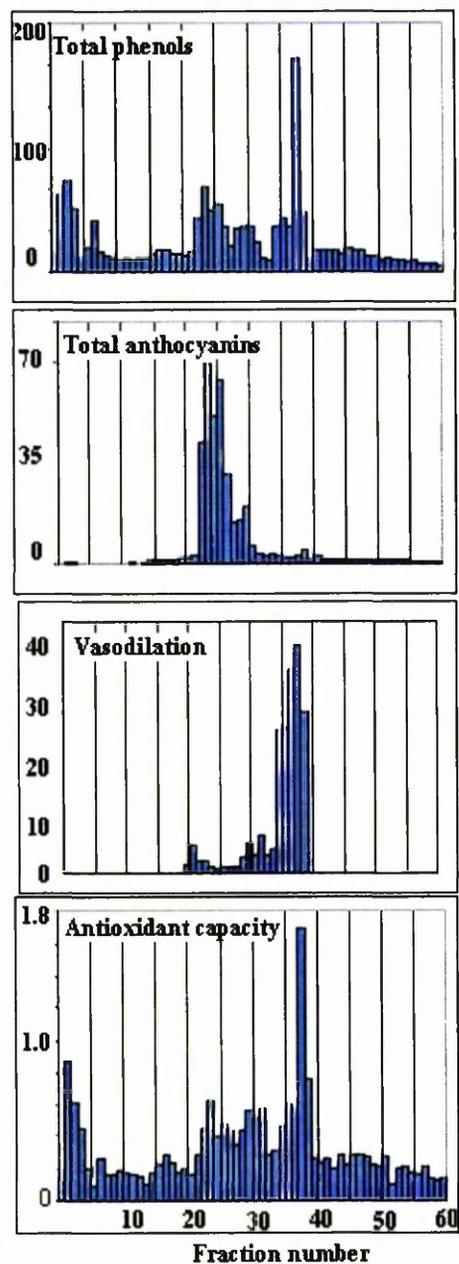
Figure 3. Preparative reversed phase HPLC of an extract of Glen Ample raspberries. Column eluted with a 30 min gradient of 5-25% acetonitrile in water containing 1% formic acid followed by an 80% acetonitrile wash. Successive 30 s fractions collected after 3 min, concentrated and aliquots assayed for total phenolics, anthocyanins, antioxidant capacity and vasodilation activity. Data expressed as follows: total phenolics (μg gallic acid equivalents g^{-1} f.w.); anthocyanins (μg cyanidin-3-glucoside equivalents g^{-1} f.w.); vasodilation activity (% relaxation); antioxidant capacity (number of Fremy's radicals reduced [x 10^{17}] g^{-1} f.w.).

Table 1. Summary of the properties of compounds detected in extracts of Glen Ample raspberries following analysis by HPLC with diode array and MS-MS detection. Peak numbers and retention times refer to numbers given in Figures 1 and 2.

Peak	R_t(min)	λ_{max} (nm)	Compound	$^{+/-}$(m/z)	Fragment ions (m/z)
1	12.3	519	Cyanidin-3,5-diglucoside	625^+	449(M^+-Glc), 287[Cyan](M^+-Glc-Glc)
2	15.7	519	Cyanidin-3-sophoroside	611^+	287[Cyan](M^+-Soph)
3	17.1	519	Cyanidin-3-(2^G-glucosylrutinoside)	757^+	611(M^+-Rham), 287[Cyan](M^+-Rham-Glc-Glc)
4	17.5	519	Cyanidin-3-glucoside	449^+	287[Cyan](M^+-Glc)
5	17.7	519	Cyanidin-3-sambubioside	581	449(M^+-Xyl), 287[Cyan](M^+-Xyl-Glc)
6	18.4	503	Pelagonidin-3-sophoroside	595^+	271[Pel](M^+-Soph)
7	18.8	519	Cyanidin-3-xylosylrutinoside	727^+	581(M^+-Rham), 287[Cyan](M^+-Rham-Xyl-Glc)
8	19.3	519	Cyanidin-3-rutinoside	595^+	287[Cyan](M^+-Rham-Glc)
9	20.0	503	Pelagonidin-3-(2^G-glucosylrutinoside)	741^+	595(M^+-Rham), 271[Pel](M^+-Rham-Glc-Glc)
10	20.7	503	Pelagonidin-3-glucoside	433^+	271[Pel](M^+-Gluc)
11	22.1	503	Pelagonidin-3-rutinoside	579^+	271[Pel](M^+-Rham-Glc)
12	26.3	250	Lambertianin C	$[1401^-]2$	1250 ({$[1401^-]$2-HHDP}/2), 935[Casu/Pot]
13	27.8	250	Sanguiin H-6	1869^-	1567(M^--HHDP), 1265(M^+-HHDP-HHDP), 935(M^+-HHDP-HHDP-Glc-Galloyl)
14	29.7	361	Ellagic acid pentose conjugate	433^-]	301[HHDP](M^--Pent)
15	31.0	365	Ellagic acid pentose conjugate	433^-	301[HHDP](M^--Pent)
16	32.1	n.d.	Ellagic acid	301^-	
17	33.0	365	Quercetin-3-rutinoside (rutin)	609^-	301[Q] (M^--Rham-Glc)
18	34.7	365	Quercetin-3-glucoside	463^-	301[Q] (M^--Glc)
19	35.5	365	Quercetin-3-glucuronide	477^-	301[Q] (M^--GlcAC)
20	38.2	365	Methylquercetin pentose conjugate	447^-	315[Iso](M^--Pent)
21	38.7	360	Ellagic acid-4-acetylxyloside	475^-	301[HHDP] (M^--XylAc)
22	41.1	365	Kaempferol glucuronide	461^-	285[K] (M^--GlcAC)
23	41.6	360	Ellagic acid-4-acetylarabinoside	475^-	301[HHDB] (M^--AraAc)

Cyanidin (Cyan); Pelargonidin (Pel); Quercetin (Q), Isorhamnetin (Iso), Kaempferol (K); Glucosyl (Glc), Sophorosyl (Sop); Rhamnosyl (Rham); Pentosyl (Pent); Acetylxyloside (XylAc); Acetylarabinoside (AraAc); Glucuronosyl (GlcAC); Casuarictin/potentillin (Casu/Pot); Hexahydroxyphenoyl (HHDP); Molecular ion ($M^{+/-}$)

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Effect of Freezing and Storage on the Phenolics, Ellagitannins, Flavonoids and Antioxidant Capacity of Red Raspberries

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SUMMARY

Scottish-grown red raspberries are a rich source of vitamin C and phenolics, most notably the anthocyanins cyanidin-3-sophoroside, cyanidin-3-(2^G-glucosylrutinoside) and cyanidin-3-glucoside, and two ellagitannins, sanguiin H-6 and lambertianin C, which are present together with trace levels of flavonols, ellagic acid and hydroxycinnamates The antioxidant capacity of the fresh fruit and the levels of vitamin C and phenolics were not affected by freezing. When fruit were stored at 4 °C for 3 days then at 18 °C for 24 h, mimicking the route fresh fruit takes after harvest to the supermarket and onto the consumer's table, anthocyanin levels were unaffected while vitamin C levels declined and elligitannins increased, and overall there was no affect on the antioxidant capacity of the fruit. It is concluded, therefore, that freshly picked, fresh commercial and frozen raspberries all contain similar levels of phytochemicals and antioxidants per serving.

Keywords: *freezing; storage; raspberries; phenolics; ellagic acid; ellagitannins, anthocyanins; vitamin C; flavonols; hydroxycinnamates; antioxidant capacity; electron spin resonance spectroscopy*

INTRODUCTION

There is consistent epidemiological evidence linking consumption of a diet rich in fruit and vegetables with reduced incidences of cancer and coronary heart disease (*1-4*). As well as displacing dietary fat, fruits and vegetables contain several health-promoting factors including vitamins, minerals and high concentrations of phenolic compounds and flavonoids. These compounds, while not essential for survival, may over the long term provide protection against a number of chronic diseases (*5*). The phenolic compounds potentially involved in these beneficial effects include gallic acid, its dimer ellagic acid and hydroxycinnamates including coumaric acid, caffeic acid and derivatives such as chlorogenic acid (*6*). The main flavonoids of interest are anthocyanins, flavan-3-ols and their polymeric products, flavanones, flavonols and flavones (*7*). To varying degrees these compounds are potent antioxidants *in vitro,* scavenging $O_2^{\cdot-}$ $OH^{\cdot}$, $ROO^{\cdot}$ (*8*) inhibiting lipid peroxidation (*9*) and protecting low density lipoproteins against oxidation (*10*). They can also reduce platelet aggregation (*11*) and enhance vasodilation (*12*).

Epidemiological studies carried out in Finland between 1970s and 1990s have shown 60% declines in both heart disease and stroke over a period when multi-component programmes were successfully directed at diet and lifestyle (*13*). The health gains can largely be explained by major behavioural changes which resulted in a reduced intake of saturated fat, a lowering of serum cholesterol at a population level, reduced salt intake and blood pressure, a decline in smoking by men and a 2 to 3-fold increase in fruit and vegetable consumption nationally (*14*). A striking feature of the diet in Finland and other Nordic countries is the high consumption of antioxidant-rich berries (*15*), particularly wild bilberry, cloudberry, cranberry and cowberry, which has been maintained and enhanced by Finnish Government Berry project promoting the consumption of cultivated berries such as raspberries, strawberries and blackcurrants.

Scotland has one of the highest rates of premature deaths from chronic disease such as coronary heart disease, strokes and colon cancer. This is ascribed, in part, to a national diet rich in saturated fats but also a habitually low consumption of foods rich in antioxidant micronutrients. The expense and lack of availability of fresh fruit and vegetables are cited as barriers to an improved diet although paradoxically Scotland has an excellent climate and growing conditions for a range of popular fruits and vegetables (*16*)

If the Nordic model is to be followed, a potentially important source of antioxidant-rich food for the Scots could be locally cultivated berries, such as raspberries, which are already grown extensively on a commercial basis primarily for the export market. Most raspberries are sold fresh. Frozen berries are much cheaper but are widely regarded as being nutritionally inferior.

Raspberries have a high antioxidant capacity (*15,17*) which is attributable to ellagitannins, anthocyanins and vitamin C (*18*). The two main ellagitannins are sanguiin H-6 and lambertianin C, (*17-19*). Although raspberries themselves contain only relatively small amounts of ellagic acid and its sugar conjugates when raspberry extracts are treated with acid sanguiin H-6 and lambertianin C are hydrolysed and release substantial quantities of ellagic acid (*6,17,20*). The major raspberry anthocyanin is cyanidin-3-sophoroside with smaller quantities of other anthocyanins including cyanidin-3-(2^G-glucosylrutinoside), cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-sophoroside, pelargonidin-3-(2^G-glucosylrutinoside) and pelargonidin-3-glucoside (*17,21-23*) Raspberries also contain quercetin and kaempferol-based flavonol conjugates (*6,17,24*) and trace levels of (-)-epicatechin and a procyanidin dimer (*17*).

The object of the present study was to investigate the effects of freezing and storage on the antioxidant capacity and the levels of phenolics, ellagitannins and flavonoids in Glen Ample raspberries, which are grown commercially on a wide scale in Scotland. The berries were

processed in a manner that simulates the production chains for commercial raspberries from harvest to the consumer's table.

MATERIALS AND METHODS

Chemicals. Cyanidin-3-glucoside was purchased from Apin Chemicals (Abingdon, Oxford, UK). Methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Peebleshire U.K.). All other chemicals and reagents were purchased from Sigma-Aldrich (Poole, Dorset, U.K.).

Plant Material. Ripe field-grown raspberries (*Rubus idaeus* L.) cv. Glen Ample were hand picked at Blairgowrie, Perthshire, UK. The fruit was divided into four lots which were treated in the following manner: *fresh* – extracted with methanol as described below within 3 h of picking; *frozen* - within 3 h of picking, frozen at –30°C in a commercial plant operated by Scottish Soft Fruit Growers plc, Blairgowrie; *store* – maintained at 4°C for three days prior to freezing in liquid nitrogen - equivalent to arrival in the supermarket; and *home* – as *store* but kept at 18°C for a further 24 h before freezing in liquid nitrogen - equivalent to keeping the raspberries in the kitchen for one day prior to eating at home. After freezing in liquid nitrogen all samples were stored at -80°C for less than 24 h before extraction.

Extraction of Raspberries. Forty g of raspberries were macerated in an ice-cold pestle and mortar and the resulting homogenate was centrifuged at 2,000 g for 30 min at 4°C. The supernatant was decanted and the pellet vortexed in ice-cold acidified (0.1% HCl) methanol after which the mixture was re-centrifuged. The two supernatants were combined, and made up to a known volume with acidified methanol. This was then sub-divided into 2-ml aliquots and stored in microcentrifuge tubes at -80°C prior to analysis..

Determination of Total Phenol Content. The total phenol contents of raspberry extracts were determined in triplicate in gallic acid equivalents using the Folin-Ciocalteu method (*25*). This assay also detects vitamin C (*17*) but on mole to mole basis the response is ca. 10% of that of flavonols such as quercetin.

Colorimetric Analysis of Total Anthocyanin Content. The free and polymeric anthocyanin contents of triplicate raspberry extracts were estimated using a pH shift method (*26*). Anthocyanins were quantified as cyanidin-3-glucoside equivalents, one of the three major anthocyanin in raspberries, using the extinction coefficient $\varepsilon = 29600$.

Measurement of Antioxidant Potential by Electron Spin Resonance Spectroscopy. The antioxidant potential of triplicate raspberries extracts was determined by their ability to reduce

the Fremy's salt (potassium nitrosodisulphonate) (27). The extracts were diluted to 5% (v/v) with ethanol/water (12:88, v/v). Three 1.0 mL aliquots were reacted with an equal volume of 1 mM Fremy's radical in ethanol/water (12:88, v/v). The electron spin resonance (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 (12:88, v/v) without raspberry extract. Spectra were obtained at 21° C 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 was set at 2 mW and 0.01 mT, respectively.

Analysis of Hydroxycinnamates by HPLC with Diode Array Detection. Triplicate raspberry extracts were reduced to dryness prior to being re-dissolved in 0.1 M Tris HCl (pH 7.4) and incubated with 0.3 mg of β-glucosidase for 1 h at 37°C to cleave sugars from hydroxycinnamate conjugates. After centrifugation, the hydrolysed and pre-hydrolysed samples were analysed by HPLC using a Waters (Milford, MASS, USA) Alliance liquid chromatograph with a photodiode array detector simultaneously monitoring wavelengths from 200-650 nm. Separation of chlorogenic acid, caffeic acid and *p*-coumaric acid was achieved on a 250 x 4.6 mm (i.d.) 5 μm ODS Hypersil column (Therma-Finnigan, Runcorn, Cheshire, UK) eluted isocratically at a flow rate of 1 mL min^{-1} with 1% acetonitrile in water containing 5% acetic acid.

Quantitative analysis of Flavonols by HPLC with Post-Column Derivatization. Triplicate methanolic extract of the raspberry samples (100 μl) were placed in a 4 mL glass v-vial to which was added 1.6 mL of 60% methanol containing 20 mM sodium diethydithiocarbamate and 600 μl of 6 M HCl. The sample was incubated at 90° C for 2 h after which it was centrifuged at 15,800 g for 5 min. A 100 μl aliquot of the supernatant was removed and added to 150 μl of 0.5% TFA. One hundred and fifty μl of this solution was then analysed on a Gilson (Villiers Le Bel, France) model 305 gradient HPLC system with a Shimadzu 10A*vp* autosampler (Kyoto, Japan) and Shimadzu SPD-10A*vp* UV-vis absorbance monitor (28) and a fluorescence detector in series (29,30). Separation was carried out using a RP-MAX 4 μm 250 x 4.6 mm (i.d.) C_{12} reverse phase column (Phenomenex) maintained at 40°C and eluted at flow rate of 1.0 mL min^{-1} with a 20 min gradient of 20-40% acetonitrile in water containing 0.5% trifluoroacetic acid (TFA). After the column eluate passed through the absorbance monitor operating at 365 nm, 0.1 M methanolic aluminium nitrate was added at a flow rate of 1.0 mL min^{-1} by a Reeve Analytical (Glasgow, UK) post-column reaction pump. The mixture was then

passed through a reaction coil at 40°C before being directed to the fluorimetric detector (excitation 425 nm, emission 480 nm). Signals from the two detectors were processed by a dual channel Reeve Analytical Model 27000 data system (Glasgow, UK).

Quantitative Analysis of Anthocyanins and Ellagic Acid by HPLC with Absorbance and Tandem Mass Spectrometric Detection. Anthocyanins and ellagic acid in triplicate raspberry extracts were analysed on a P4000 liquid chromatograph fitted with an AS 3000 autosampler and with detection by a UV6000 diode array absorbance monitor scanning from 250 to 700 nm (Thermo-Finnigan, San Jose, CA., USA). Separation was carried out using a RP-MAX 4 μm 250 x 4.6 mm (i.d.) C_{12} reverse phase column (Phenomenex, Torrance, CA., USA) maintained at 40°C and eluted at 1.0 mL min^{-1} with a 30 min gradient of 8-18% acetonitrile in water containing 1% formic acid. Quantification of cyanidin-3-sophoroside, cyanidin-3-(2^G-glucosylrutinoside), cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-sophoroside and pelargonidin-3-(2^G-glucosylrutinoside), in cyanindin-3-glucoside equivalents, was based on absorbance at 520 nm. Ellagic acid was monitored at 365 nm and, because of the limited solubility of the reference compound, was quantified in gallic acid equivalents. After passing through the flow cell of the diode array detector, the column eluate was split and 50% directed to an LCQ Duo mass spectrometer (Thermo-Finnigan) with an electrospray interface operating in full scan data dependant MS-MS mode from 150 to 2000 amu. Anthocyanins were analysed in positive ion mode and ellagic acid by negative ionisation. The mass spectral data obtained was used to confirm the identity of the absorbance peaks used for quantitative analysis.

Quantitative Analysis of Vitamin C by HPLC with Absorbance Detection. The vitamin C content of triplicate methanolic raspberry extracts was analysed by HPLC (*31*) using a Nucleosil ODS 5 μm 250 mm x 4.6 mm (i.d.) column (Jones Chromatography, Glamorgan, UK) eluted isocratically at a flow rate of 0.6 mL min^{-1} using a Gilson model 305 liquid chromatograph with a cooled autoinjector and a 231 absorbance detector operating at 263 nm. Data were recorded on a Gilson 715 data system.

Quantitative Analysis of Ellagitannins by HPLC with Absorbance Detection. Methanolic raspberry extracts were analysed in triplicate on a Shimadzu 10A*vp* liquid chromatograph fitted with an autosampler and with detection by a SPD-10A*vp* UV-vis absorbance monitor operating at 280 nm. Separation was carried out using a RP-MAX 4 μm 250 x 4.6 mm (i.d.) C_{12} reverse phase column maintained at 40°C and eluted at flow rate of 1.0 mL min^{-1} with a 30 min gradient of 5-20% acetonitrile in water containing 0.5% TFA. The signal from the detector was processed by a Reeve Analytical 27000 data system. In the absence of

reference compounds, the lambertianin C and sanguiin H-6 peaks, which had retention times (R_t) of 23.9 min and 25.4 min, respectively, were quantified in gallic acid equivalents.

Statistics. Data are represented as mean values ± standard deviation (S.D.) (n=3). Where appropriate data were subjected to statistical analysis using analysis of variance (ANOVA) to determine the significance of the treatment relationships. Statistical analyses were performed using Minitab software, version 12 (Minitab Inc., Addison-Wesley Publishing Co., Reading, MA., USA)

RESULTS

Hydroxycinnamates and flavonols. The hydroxycinnamate content of *fresh, frozen, shop* and *home* raspberries was analysed before and after treatment with β-glucosidase by HPLC conditions that separated chlorogenic acid, caffeic acid and *p*-coumaric acid. Only *p*-coumaric acid was detected. The free acid was present in sub-nmoles quantities g^{-1} fresh weight with slightly higher quantities of conjugated *p*-coumaric acid (Table 1). The highest concentration of total *p*-coumaric acid was detected in frozen raspberries but this was a mere 1.9 ± 0.4 nmoles g^{-1}.

Flavonols were present in higher concentrations than hydroxycinnamates with the conjugated forms again being present in larger amounts that the aglycones. The main component was conjugated quercetin with smaller quantities of conjugated kaempferol (Table 2). The total flavonol content ranged from 22.15 to 26.93 nmoles g^{-1} and the values obtained with *fresh, frozen, shop* and *home* raspberries were not significantly different.

Anthocyanins. The six major anthocyanins in raspberries, cyanidin-3-sophoroside, cyanidin-3-glucosylrutinoside, cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-sophoroside and pelargonidin-3-glucosylrutinoside were analysed quantitatively by HPLC. There were no significant differences in either the levels of the individual anthocyanins or in the overall anthocyanin content the *fresh, frozen, shop* and *home* raspberries (Table 3). The anthocyanins were, however, present in the raspberries in much higher concentrations than either *p*-coumaric acid or flavonols.

Total Phenolics, Polymeric and Free Anthocyanins, Vitamin C, Lambertianin C, Sanguiin H-6, Ellagic Acid and Antioxidant Capacity. The overall level of phenolics in extracts of the *fresh, frozen, shop* and *home* raspberry samples were determined colorimetrically using the Folin-Ciocalteu assay and the data obtained are presented in Table 4. The concentrations of phenolics in the *shop* and *home* raspberries (3510 and 3769 nmoles g^{-1} respectively) were slightly higher that in the *fresh* and *frozen* samples (3383 and 3383 nmoles g$^-$

^{1}) and this difference was statistically significant (p = 0.004). Anthocyanins were also measured colorimetrically which enabled a combined estimate of free and polymeric forms to be obtained (Table 4). The values for total anthocyanins ranged from 770-819 nmoles g^{-1} and there were no statistically significant differences in the amounts found in the *fresh, frozen, shop* and *home* raspberry samples, in keeping with the information obtained by HPLC analysis of anthocyanins (Table 3). The vitamin C concentration, determined by HPLC, declined from 672 for *fresh* to 622 nmoles g^{-1} for *home* berries with the levels in the *shop* and *home* raspberries being significantly lower than those in the *fresh* and *frozen* samples (Table 4). In contrast, to vitamin C, the ellagitannin content rose during storage. The level of lambertianin C was significantly higher in the sample of *home* berries (p = 0.002) while sanguiin H-6 showed a statistically significant rise from *fresh* and *frozen*, 522 and 517 nmoles g^{-1} respectively, to 571 nmoles g^{-1} for *shop* and 639 nmoles g^{-1} for *home* (p = 0.004). The albeit low concenetrations of ellagic acid in *fresh* and *frozen* fruit, 3.5 and 3.9 nmoles g^{-1}, respectively, increased ca. 2-fold in *shop* and 5-fold in *home* raspberries. Unlike the vitamin C, ellagitannin and ellagic acid concentrations, there were no discernible differences in the antioxidant capacity of the four raspberry samples, which ranged from 406 to 420 x 10^{16} Fremy's radicals reduced g^{-1} f.w. (Table 4).

DISCUSSION

Studies on the effects of storage and freezing on raspberries showed that the antioxidant capacity was not significantly different in *fresh, frozen, shop* and *home* berries. Free and conjugated coumaric acid were detected in the raspberries and although present in higher concentrations in frozen fruit, the levels were very low indeed at 1.9 ± 0.4 nmoles g^{-1} (Table 1). The overall flavonol levels, comprised primarily of conjugated quercetin, ranged from 22.1 to 26.9 nmoles g^{-1} were unaffected by freezing and storage (Table 2). Anthocyanins were present in far higher concentrations with the main component cyanidin-3-sophoroside being detected at concentrations >500 nmoles g^{-1} and overall anthocyanin levels being >1000 nmoles g^{-1} (Table 3). Analysis of *fresh, frozen*, shop and home fruit revealed no significant difference in the levels of the individual anthocyanins measured by HPLC (Table 3) or in the free and conjugated anthocyanin content that was determined colorimetrically (Table 4). The level of total phenolics were significantly higher in the *shop* and *home* raspberries than in the *fresh* and *frozen* fruit (Table 4). The low levels of ellagic acid also rose during storage of raspberries and there were small but significant increases in the much higher concentrations of lambertianin C in *home* and sanguiin H-6 in *shop* and *home* fruit (Table 4). In contrast, to these changes, the antioxidant capacity in *fresh, frozen, shop* and *home* raspberries did not alter significantly. Previous

investigation has shown that the main contributors to the antioxidant capacity of raspberries are the ellagitannins, anthocyanins and vitamin C (*17*). It is, therefore, likely that the increases in lambertianin C and sanguiin H-6 during storage, and the small but significant increase in total phenolics, were not accompanied by increases in antioxidant capacity because they were either relatively minor and/or were off-set by the decline in vitamin C in *shop* and *home* fruit (Table 4). The explanation for these post-harvest changes lies presumably in the fact that secondary metabolism in berries remains active under *home* and *shop* conditions but not in *frozen* tissues.

The information obtained in the present investigation is in broad agreement with studies on the effects of freezing and subsequent long-term storage of Spanish raspberries which showed that anthocyanins were more stable in spring fruiting cultivars than in autumn fruiting varieties (*20*). It was also demonstrated that the freezing process had little effect on the ellagic acid released by acid hydrolysis, total phenol, vitamin C content and antioxidant capacity. However, during long-term frozen storage (12 months) ellagic acid declined 14-21% and vitamin levels fell 33-55% although the antioxidant capacity of the berries was unchanged (*20*)

In another study with Canadian-grown raspberries, fruit were stored at 0, 10, 20, and 30 °C for up to 8 days (*32*). There was an increase in the antioxidant capacity of the raspberries at storage temperatures >0 °C which was associated with increases in anthocyanins and total phenolics and a decline in vitamin C. Although the number of components analysed was fewer than in the present study, the findings are broadly similiar to our own, except for the increase in anthocyanins which was not observed in Glen Ample raspberries. This may well be due to the fact that the Canadian berries were, in most instances, stored for longer periods and at higher temperatures than the Glen Ample fruits where the fresh berries were stored at 4 °C for 3 days (*shop*) and then at 18 °C for a further day (*home*).

In conclusion, this investigation has shown that Scottish-grown raspberries are a rich source of vitamin C and phenolics, most notably anthocyanins and ellagitannins. The antioxidant capacity of the fresh fruit and the levels of vitamin C and phenolics were not affected by freezing, which is in keeping with previously published data obtained with Spanish raspberries (*20*). When fruit were stored at 4 °C for 3 days then at 18 °C for 24 h, mimicking the route fresh fruit takes after harvest to the supermarket and onto the consumer's table, anthocyanin levels were unaffected while vitamin C levels declined and elligitannins increased, and overall there was no affect on the antioxidant capacity of the fruit. We conclude, therefore, that freshly picked, fresh commercial and frozen raspberries provide similar levels of phytochemicals and antioxidants per servings.

ACKNOWLEDGEMENTS

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Table 1. Effects of freezing and storage on the free, conjugated and total p-coumaric acid content of Glen Ample raspberries[1].

p-Coumaric acid	*Fresh*	*Frozen*	*Shop*	*Home*
Free	0.07 ± 0.02	0.05 ± 0.01	n.d.	0.19 ± 0.04
Conjugated	0.28 ± 0.04	1.85 ± 0.10	0.52 ± 0.04	0.61 ± 0.05
Total	0.35 ± 0.06	1.90 ± 0.08	0.52 ± 0.04	0.80 ± 0.05

1Data expressed as nmoles g-1 fresh weight ± standard deviation (n = 3). n.d. – not detected.

Table 2. Effects of freezing and storage on the free, conjugated and total flavonol content of Glen Ample raspberries[1].

Flavonols	*Fresh*	*Frozen*	*Shop*	*Home*
Free quercetin	1.0 ± 0.03	0.8 ± 0.03	1.1 ± 0.03	0.9 ± 0.07
Conjugated quercetin	20.2 ± 0.5	24.8 ± 0.4	23.0 ± 0.3	21.6 ± 0.9
Total quercetin	21.2 ± 0.4	25.6 ± 0.4	24.1 ± 0.3	22.5 ± 0.9
Free kaempferol	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.01	0.1 ± 0.00
Conjugated kaempferol	1.0 ± 0.07	1.3 ± 0.03	1.1 ± 0.03	1.1 ± 0.03
Total kaempferol	1.1 ± 0.07	1.4 ± 0.03	1.2 ± 0.03	1.2 ± 0.03
Total flavonols	22.3 ± 0.4	27.0 ± 0.4	25.3 ± 0.4	23.7 ± 0.9

[1]Data expressed as nmoles g-[1] f.w. ± standard deviation (n = 3)

Table 3. Effect of freezing and storage on the anthocyanin content of Glen Ample raspberries[1].

Anthocyanin	*Fresh*	*Frozen*	*Shop*	*Home*
Cyanidin-3-sophoroside	555 ± 4 *(53.5)*	567 ± 8 *(54.1)*	579 ± 7 *(53.6)*	536 ± 6 *(53.2)*
Cyanidin-3-(2^G-glucosylrutinoside)	235 ± 2 *(22.7)*	232 ± 3 *(22.1)*	229 ± 3 *(21.2)*	209 ± 3 *(20.7)*
Cyanidin-3-glucoside	156 ± 1 *(15.0)*	159 ± 2 *(15.2)*	172 ± 2 *(15.9)*	163 ± 2 *(16.2)*
Cyanidin-3-rutinoside	62 ± 0.5 *(6.0)*	62 ± 0.8 *(5.9)*	71 ± 0.9 *(6.6)*	68 ± 0.8 *(6.8)*
Perlargonidin-3-sophoroside	24 ± 0.2 *(2.3)*	23 ± 0.3 *(2.2)*	24 ± 0.3 *(2.2)*	24 ± 0.3 *(2.4)*
Pelargonidin-3-glucose-rutinoside	5.2 ± 0.1 *(0.5)*	5.2 ± 0.1 *(0.5)*	5.4 ± 0.1 *(0.5)*	7.1 ± 0.1 *(0.7)*
Total anthocyanin content	1037 ± 8	1049 ± 14	1081 ± 14	1008 ± 12

[1]Data expressed as nmoles cyanidin-3-glucoside equivalents g-[1] f.w. ± standard deviation (n = 3) and in brackets as a percentage of total anthocyanin content.

Table 4. Effects of freezing and storage on the total phenolics, anthocyanins, vitamin C, lambertianin C, sanguiin H-6, ellagic acid and antioxidant capacity of Glen Ample raspberries*.

	Fresh	Frozen	Shop	Home
Total phenolics	3383 ± 230 (100)	3321 ± 103 (98)	3510 ± 107 (104)	3769 ± 125 (111)
Free anthocyanins	580 ± 25 (100)	600 ± 32 (103)	621 ± 19 (107)	621 ± 19 (107)
Total anthocyanins	770 ± 21 (100)	782 ± 31 (102)	819 ± 25 (106)	794± 30 (103)
Vitamin C	672 ± 11 (100)	671 ± 14 (100)	638 ± 12 (95)	622 ± 22 (93)
Lambertianin C	110 ± 3.1 (100)	116 ± 8.7 (105)	124 ± 4.2 (113)	159 ± 4.5 (145)
Sanguiin H-6	407 ± 1.5 (100)	406 ± 1.5 (100)	447.± 3.6 (110)	480 ± 4.5 (118)
Ellagic acid	3.5 ± 0.1 (100)	3.9 ± 0.1 (111)	6.4 ± 0.1 (190)	18.5 ± 0.2 (528)
Antioxidant capacity	406 ± 9.2 (100)	420 ± 9.1 (103)	406 ± 3.0 (100)	406 ± 6.3 (100)

*Data presented as mean values g^{-1} f.w. ± standard deviation (n = 3) and in italicised parentheses as a percentage of the values for *fresh* raspberries. Total phenolics expressed as nmoles gallic acid equivalents, anthocyanins as nmoles cyanidin-3-glucoside equivalents, lambertianin C, sanguiin H-6 and ellagic acid as nmoles gallic acid equivalents and antioxidant capacity as number of Fremy's radical x 10^{16} reduced.