Chapter 7
Raspberry Fruit Chemistry in Relation to Fruit Quality and Human Nutrition

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7.1 Introduction

In recent years raspberry fruit breeding has shifted its focus from traits associated with agronomic performance towards those associated with fruit sensory quality (Jennings et al. 2016) and potential health benefits (Mazzoni et al. 2016). Simultaneously, significant advancements have been made in raspberry genetics and genomics as well as analytical chemistry in soft fruit. These new tools are generating knowledge that has the capacity to significantly accelerate the development of new varieties that meet consumer expectations in terms of sensory experience and health benefits of fruit consumption. Significant research in recent years has identified the environmental, biochemical and genetic controls underlying the accumulation of specific compounds in raspberry fruit. Furthermore, increasing information is becoming available regarding the mechanisms of action of specific phytochemicals in relation to human health outcomes. This information is now providing the underpinning science for the development of new cultivars. In this chapter, we outline current understanding of the biosynthetic pathways associated with the accumulation of significant fruit phytochemicals and describe what is presently known regarding the influence of crop genetics and the growing environment on the accumulation of specific phytochemicals. Finally we outline the latest knowledge regarding how fruit phytochemicals modulate human health outcomes. It is anticipated that the work outlined here will guide molecular breeding targets for future crop improvement.

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7.2 Primary Metabolites as Drivers of Fruit Flavour

Sugar-acid balance is considered a key driver of flavour in a range of fruits (Klee 2010) and achieving an appropriate sugar-acid balance is therefore essential for the sensory quality of the fruit. Few studies have been undertaken specifically in raspberry, however in a study of sensory preference in five cultivars soluble solids were positively correlated with fruitiness, sweetness and overall impression, while titratable acidity was positively correlated with sourness and astringency (Shamaila et al. 1993). Furthermore, a detailed sensory and preference analysis of several cultivars grown in the North-Western United States revealed three groups of consumers with different preferences. "Likers" showed a clear preference for raspberry aroma and flavour that was not shared with "nonlikers" and "nondistinguishers". However, what united all three groups was the frequency with which preference was associated with high scores for sweetness, while high scores for sourness and bitterness were associated with a disliking (Villamor et al. 2013). These data indicate the importance of sugars and acids in driving consumer preference in red raspberry.

The main sugars found in raspberry fruit are the monosaccharides glucose and fructose, and the disaccharide sucrose (Famiani et al. 2005; Lee 2015; Mazur et al. 2014a; Stavang et al. 2015). Smaller amounts of xylose, trehalose, myo-inositol (Dincheva et al. 2013a), sorbitol and mannitol (Lee 2015) have also been reported. As different sugars have different perceived levels of sweetness (Moskowitz 1970), the ratios of the major sugars in ripe fruit could influence the perception of sweetness in the fruit. Sugar content of fruit rises throughout development and at commercial harvest fructose and glucose are present at similar concentrations of around 15–30 mg g^{-1} FW, while sucrose has been reported at lower concentrations of approximately 10 mg g^{-1} FW (Mazur et al. 2014a; Stavang et al. 2015; Wang et al. 2009; Famiani et al. 2005).

Sugar accumulation in raspberry fruit is a complex process that proceeds *via* remobilization of starch reserves from root and canes as well as *via* direct translocation of sugars synthesized in leaves (Alvarado-Raya et al. 2007; de Oliveira et al. 2007). Interestingly, immature raspberry fruit can continue to accumulate soluble solids and sugars following detachment from the mother plant. Given that raspberry fruit does not accumulate starch, it has been proposed that the source of such sugars is the solubilization of cell wall components (Wang et al. 2009).

Sugars lie at the heart of central metabolism and participate in a broad range of both anabolic and catabolic biochemical pathways. Like other primary metabolites, varietal differences in fruit sugar content are strongly influenced by environment. For example, Mazur et al. (2014a) observed a significant impact of growing location on sugar content of 'Glen Ample' raspberry that was correlated with growing temperature and precipitation. Similarly, day length had a significant impact on fruit sucrose with fruit grown under long days (22 h) having lower sucrose content and soluble solids to titratable acidity ratio than fruit grown under shorter (10 h) days (Mazur et al. 2014b). Furthermore, in a study on heritability of pomological and quality traits in progenies derived by Meeker's yellow clone, heritability traits of reducing sugars were low and large genotype × year interactions were observed (Fotirić Akšić et al. 2011), suggesting that agronomic rather than genetic intervention may be the preferred method for optimising sugar content in raspberry fruit.

The primary organic acids found in raspberry fruit are citrate (10–15 mg g^{-1} FW) and malate (1.0–1.5 mg g^{-1} FW) (Famiani et al. 2005; Mazur et al. 2014a; Wang et al. 2009). Lower amounts of succinate, maleate, fumarate and glucarate have also been identified in ripe raspberry fruit (Dincheva et al. 2013a). Both malate and citrate levels decline during ripening, associated with an increase in the abundance and activity of phosphoenolpyruvate carboxykinase (PEPCK). This suggests that citrate and malate are diminished in ripening raspberry fruits by the activity of PEPCK leading to the formation of phosphoenolpyruvate which could be subsequently metabolized *via* gluconeogenesis, ethanol fermentation or other biosynthetic pathways as illustrated in Fig. 7.1, (Famiani et al. 2005).

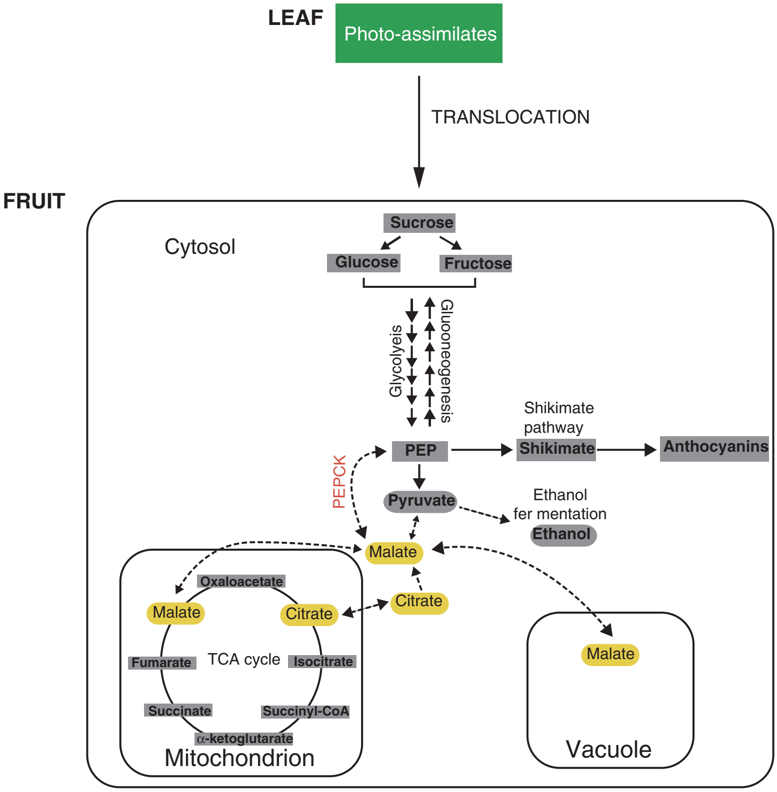


Fig. 7.1 Malate and citrate metabolism in developing raspberry fruit. At early stages of fruit development malate and citrate derive from the metabolism of sugars that translocate from the leaves to the developing fruit. During ripening, malate and citrate levels decline and several metabolic pathways are involved in their catabolism, including oxidation via Krebs cycle, gluconeogenesis, ethanol fermentation and biosynthesis of secondary metabolites such as anthocyanins. Malate and citrate can be found in different compartments within a cell. PEP phospoenolpyruvate, PEPCK phospoenolpyruvate carboxykinase

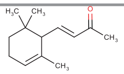
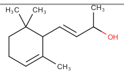
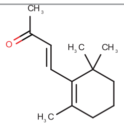
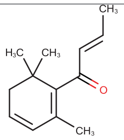
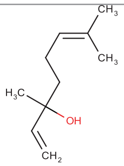
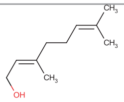
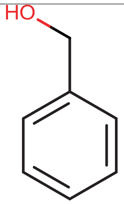
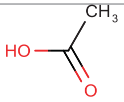
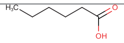
Similar to the sugars present in raspberry fruit, citrate and malate sit at the centre of primary metabolism being key components of the tricarboxylic acid (TCA) cycle. Besides its role in providing substrates (NADH, $FADH_2$) for ATP synthesis by mitochondrial oxidative phosphorylation, the TCA cycle provides substrates and intermediates for amino acid biosynthesis, secondary metabolite biosynthesis, ammonia assimilation, photorespiration, the glyoxylate cycle and purine nucleotide biosynthesis (Sweetlove et al. 2010). Like the sugars, total fruit acidity in raspberry fruits exhibited a low coefficient of heritability and a high phenotypic coefficient of variation indicating strong environmental effects on fruit acid content (Fotirić Akšić et al. 2011). This is confirmed by several studies that indicate that although there are significant differences in fruit acid content of different cultivars (Krüger et al. 2011; Mazur et al. 2014c), the growing environment also has a significant impact (Mazur et al. 2014a, c).

As sugar-acid balance is such a key component of raspberry fruit flavour, it is desirable for breeders and growers to have the capacity to predict and control the sugar-acid balance. However, these metabolites sit at the centre of fruit metabolism in highly branched and interconnected metabolic pathways. Whilst some minor QTL have been detected for sugars, these are not stable in different years and environments (J Graham, personal communication) highlighting the strong influence of the growing environment. It therefore appears that, in the short-term at least, management of the growing environment is the most promising opportunity to optimize fruit sugars and acids for quality.

7.3 Volatile Components of Flavour and Aroma Quality

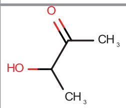
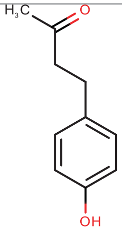
Almost 300 volatile compounds have been reported in raspberry fruit belonging to a range of chemical classes including acids, phenols, aldehydes, ketones, terpenoids, isoprenoids, esters, ethers and hydrocarbons (Aprea et al. 2015). However, only a fraction of these compounds have been demonstrated to be significant for fruit aroma and sensory properties. Larsen et al. (1991) identified ten compounds contributing to raspberry flavour by combining gas chromatographic analysis of solvent extracted aroma concentrates with sensory analysis of jams made from different cultivars. These comprised products of carotenoid cleavage, monoterpenes, fatty acid breakdown products and the key raspberry aroma compound *p*-hydroxyphenylbutan-2-one (raspberry ketone), which derives from the phenylpropanoid biosynthetic pathway (Table 7.1). Subsequent work expanded this list using a combination of simulated retronasal aroma generation in combination with gas chromatography-mass spectrometry (GC/MS) and GC-olfactometry (GCO). Using these techniques, a number of additional fatty acid derivatives, the phenylpropanoid vanillin, the amino acid derivative sotolon that imparts maple notes and the key strawberry aroma compound furaneol were identified as significant for raspberry aroma (Roberts and Acree 1996). This list of compounds was further expanded to 75 compounds following solvent extraction of fruit in combination with aroma

Table 7.1 Major raspberry volatiles and their sensory characteristics

Aroma compound	Chemical structure	Metabolic pathway	Sensory description
α-Ionone		Carotenoid	Woody, floral, balsamic and sweet tones, violet
α-Ionol		Carotenoid	Floral
β-Ionone		Carotenoid	Cedar wood, floral, fruity
β-Damascenone		Carotenoid	Floral, fruity, sweet
Linalool		Monoterpenes	Floral, spicy, woody
Geraniol		Monoterpenes	Sweet, fruity, floral
Benzyl alcohol		Fatty acids	Faint aromatic, sharp burning taste
Acetic acid		Fatty acids	Pungent, vinegar-like
Hexanoic acid		Fatty acids	Cheese, goat-like
(Z)-3-hexenol		Fatty acids	Intense grassy green

(continued)

Table 7.1 (continued)

Aroma compound	Chemical structure	Metabolic pathway	Sensory description
Acetoin		Fatty acids	Buttery, fatty creamy
Raspberry ketone		Phenylpropanoid	Raspberry, sweet fruity

Larsen and Poll (1990), Klesk et al. (2004), Paterson et al. (2013)

extract dilution analysis, a method for estimating the odour potency of volatile compounds (Klesk et al. 2004). It should be noted that while this greatly expanded list is useful for identifying components that may contribute to raspberry aroma, the extraction and concentration methods used might result in chemical transformations that do not normally occur during the ingestion of fresh fruit. Furthermore, some of the compounds identified in the concentrated extract might be below the limits of human detection in fresh fruit. Hence, Table 7.1 only lists the major volatile components.

Raspberry volatiles are synthesized from a range of metabolic pathways and here we describe only the metabolic pathways associated with some of the key volatile compounds. Raspberry ketone (*p*-hydroxyphenylbutan-2-one) is described as imparting the characteristic raspberry aroma and it exists both as the free ketone and as the glucoside (Borejsza-Wysocki and Hradzina 1994). The compound is synthesised in a two-step biosynthetic pathway from the precursors malonyl-CoA and *p*-coumaryl-CoA formed from acetyl-CoA and phenylalanine respectively (Fig. 7.2). The first step is a condensation reaction catalyzed by an atypical aromatic polyketide synthase (benzalacetone synthase, BAS) to form the intermediate *p*-hydroxybenzalacetone (Borejsza-Wysocki and Hrazdina 1996). In the second step, raspberry ketone/zingerone synthase catalyses the NADPH-dependent reduction of *p*-hydroxybenxalacetone to raspberry ketone (Koeduka et al. 2011). Genes encoding both enzymes have been cloned and kinetically characterized. BAS is encoded by *RiPKS4* and has both BAS and chalcone synthase activity – the latter activity producing precursors for flavonoid and anthocyanin biosynthesis. Raspberry ketone/zingerone synthase is encoded by *RZS1* and exhibits activity against hydroxybenzalacetone and to a lesser extent 3-methoxy-4-hydroxybenzalacetone. The reaction product of the latter compound with RZS1 is zingerone, which is not present in raspberry fruit. The abundance of both *RiPKS4* and *RZS1* transcripts increases during fruit ripening consistent with their function in raspberry ketone biosynthesis (Zheng and Hrazdina 2008).

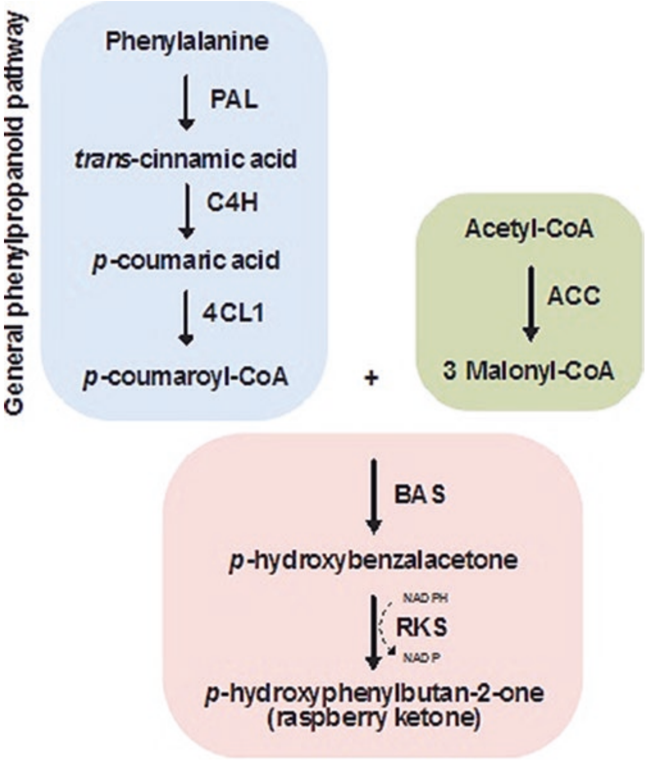


Fig. 7.2 Outline of the raspberry ketone biosynthetic pathway. Raspberry ketone is synthesised via a two-step reaction pathway using *p*-coumaroyl-CoA and malonyl-CoA as precursors. These compounds derive from the phenylpropanoid pathway and acetyl-CoA respectively. The first step of raspberry ketone synthesis is catalysed by benzalacetone synthase (BAS), leading to formation of *p*-hydroxybenzalacetone. The second step includes the NADPH-dependent reduction of *p*-hydroxybenzalacetone to raspberry ketone, which is catalysed by raspberry ketone/zingerone synthase (RKS). PAL phenylalanine ammonia lyase, C4H cinnamate-4-hydroxylase, 4CL1 4-coumarate:CoA ligase1, ACC acetyl-CoA carboxylase

In addition to its well established role in generating important raspberry aroma notes, raspberry ketone has also been shown to have anti-obesogenic properties. When supplied as a supplement to mice supplied with a high fat diet, raspberry ketone either alone (Morimoto et al. 2005) or in combination with ellagitannins (Luo et al. 2016) significantly inhibited weight gain and the accumulation of adipose tissue. Furthermore, raspberry ketone promoted lipolysis and fatty acid oxidation in adipocyte cell cultures that was associated with increased accumulation of adiponectin, an adipocytokine known to regulate lipid and glucose metabolism (Morimoto et al. 2005; Park 2010).

Alongside raspberry ketone, the norisoprenoids α- and β-ionone and the monoterpenes linalool and geraniol are considered to be significant aroma compounds in raspberry fruit (Larsen et al. 1991). Both groups of compounds share common precursors synthesized via the mevalonate (MVA) pathway in the cytosol or the methylerythritol phosphate (MEP) pathway in the plastid (Paterson et al. 2013). Both pathways produce the precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are condensed to geranyl pyrophosphate (C10, GPP), the precursor of the terpenoid aroma volatiles geraniol and linalool (Chen et al. 2011), or to geranylgeranyl pyrophosphate (C20, GGPP), the precursor of carotenoids (McQuinn et al. 2015) that are subsequently cleaved to α- or β-ionone (Fig. 7.3). However, labelling studies using deuterated pathway intermediates revealed selectivity concerning cytosolic or plastidial biosynthetic routes with linalool being synthesized solely *via* the cytosolic MVA pathway, while the ionones were labelled when fruit was provided with precursors from either pathway (Hampel et al. 2007). These data implied a cytosolic

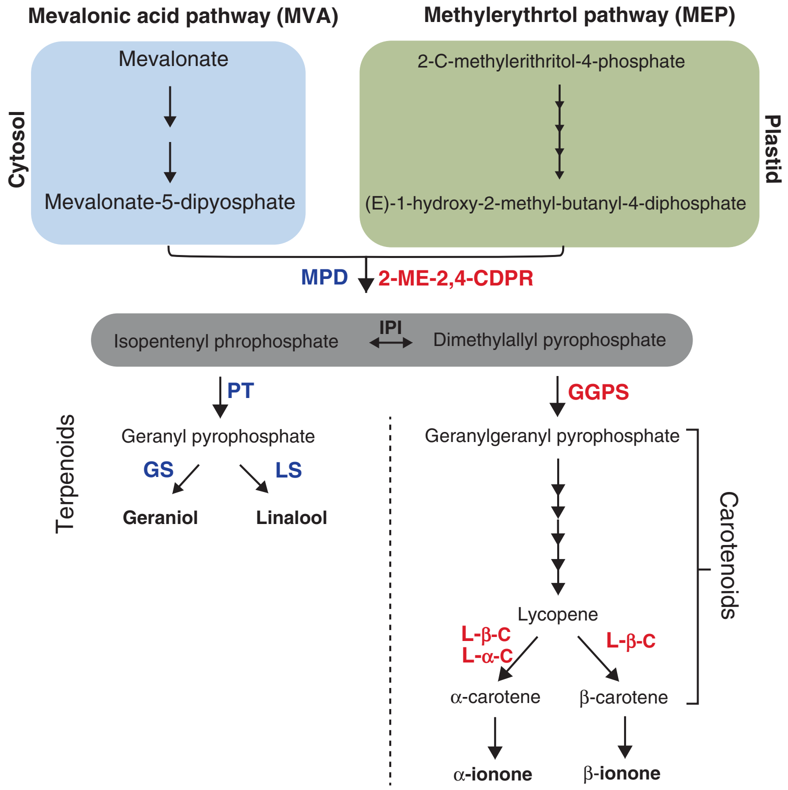


Fig. 7.3 Biosynthesis of major isoprenoids (geraniol, linalool, α-ionone and β-ionone) found in raspberry fruit. Precursors for the formation of these metabolites are isopentenyl pyrophosphate and dimethyallyl pyrophosphate that can be either formed through mevalonic acid (MVA) pathway in the cytosol or methylerythritol pathway (MEP) in the plastid. MPD mevalonate pyrophosphate decarboxylase, 2-ME-2,4—CDPR 2-C-methyl-D-erythritol-2,4-cyclodiphosphate reductase, IPI isopentenyl pyrophosphate isomerase, PT prenyltransferase, GGPS geranylgeranyl pyrophosphate synthase, GS geraniol synthase, LS linalool synthase, L-β-C lycopene-β-cyclase, L-ε-C lycopene-ε-cyclase

location for the monoterpene synthase required for linalool biosynthesis, contrary to the finding that the majority of known monoterpene synthases have been assigned as plastid localized enzymes (Sun et al. 2016). However, Aharoni et al. (2004) identified a dual function terpene synthase in strawberry fruit cytosol that was capable of synthesizing both linalool from geranyl disphosphate as well as nerolidol from C15 farnesyl diphosphate, indicating a cytosolic location for monoterpene synthases in the fruit of other rosaceous species. Carotenoid cleavage dioxygenases (CCDs), responsible for the synthesis of α- and β-ionone among other apocarotenoids, are found in both the plastid and cytosol (Rubio et al. 2008; Simkin et al. 2004), although it has been suggested that cytoplasmic enzymes are likely associated with the plastid membrane. In raspberry, a gene with 80% homology to Arabidopsis *CCD1* was cloned and expressed in carotenoid synthesizing *Escherichia coli* strains. The bacteria expressing the gene were found to produce α- and β-ionone from different substrates, while bacteria transformed with the empty vector did not. Furthermore, expression of the *CCD1* gene strongly correlated with the accumulation of ionones in developing raspberry fruit, suggesting that the encoded protein (RiCCD) catalyzed the synthesis of ionones from carotenoids in raspberry fruit (Beekwilder et al. 2008).

Raspberry volatile content depends both upon fruit genotype and growing environment. For example, several studies have shown variation between cultivars in raspberry ketone, α-ionone and β-ionone of up to fourfold, while the monterpenes geraniol and linalool typically vary tenfold and up to 100-fold, respectively (Larsen et al. 1991; Malowicki et al. 2008; Moore et al. 2002; Shamaila et al. 1993). Similarly, variation was observed when the same cultivar was grown at different sites in the same year or when the same cultivar was grown at the same site in different years, although environmental variation tended to be lower than between cultivars (Malowicki et al. 2008; Moore et al. 2002). This indicates the potential for developing molecular markers for fruit volatile content. Indeed, Paterson et al. (2013) were able to identify quantitative trait loci (QTL) for 11 of 12 volatiles mapped in a ‘Glen Moy’ × ‘Latham’ population. These loci exhibited a reasonable degree of consistency across years and cultivation sites, such as open field or polytunnels, indicating their potential utility. A number of candidate genes were identified underlying markers for fruit volatiles. One gene encoding for a deoxy-D-xylulose-5-phosphate reductase, a key enzyme of the MEP pathway, underlies a QTL for β-ionone, whereas genes encoding for terpene synthases underlie QTLs for linalool.

7.4 Lipophilic Components: Carotenoids, Tocopherols and Seed Oils

Raspberries exhibit an exceptional lipophilic antioxidant capacity having the highest value of 32 surveyed fruits, with the sole exception of avocado pears, and also scoring higher than a wide range of vegetables (Wu et al. 2004). Key lipophilic antioxidants in raspberries are the tocopherols and carotenoids.The latter group provides precursors for the key aroma compounds α- and β-ionone and play an important role in the colouration of yellow fruit. Tocopherols and carotenoids share a common biosynthetic precursor (geranylgeranyl pyrophosphate, GGPP), but despite their significance they have been little studied in raspberry fruit. Immature fruits are reported to contain relatively high contents of β-carotene and lutein that decline during fruit ripening (Beekwilder et al. 2008; Carvalho et al. 2013a). This was associated with a decline in chlorophyll-derived pheophytins (Carvalho et al. 2013a) and is consistent with dismantling of light harvesting complexes as green fruit starts to change colour. On a fresh weight (FW) basis tocopherols also declined during ripening (Beekwilder et al. 2008) although it was not clear whether this was due to degradation or dilution as the fruit size increased. In saponified extracts there was a decline in total lutein/FW during ripening (Beekwilder et al. 2008); however, a significant increase in lutein esters, primarily dilauroyl lutein, was observed during ripening. This was associated with an increase in the carotenoid precursor phytoene at the later stages of fruit development consistent with active lutein biosynthesis in ripening raspberry fruit (Carvalho et al. 2013a). Some variation in lutein and esterified lutein content was observed in yellow and red fruited raspberry cultivars, although there was no correlation with fruit colour. More variation was observed in the levels of different tocopherols indicating significant genetic diversity in this trait.

Raspberry seed oils represent a potential value-added byproduct of fruit processing with dried raspberry seeds yielding in the region of 10–20% oil (Oh et al. 2007; Oomah et al. 2000). The oil primarily comprises neutral lipids (94% w/w) with small amounts of free fatty acids and phospholipids (Oomah et al. 2000). In terms of fatty acid composition, raspberry seed oils contain high levels of polyunsaturated fatty acids which exceed 80% of the total fatty acid content. The primary fatty acids are linoleic and α-linolenic acids that are present in a ratio of less than two to one. Oleic acid is the other significant fatty acid present at around 10–12% of total fatty acids, while smaller amounts of stearic (1–2%) and palmitic acids (2–3%) are also recorded alongside trace (<0.5%) amounts of longer chain (C20-C22) fatty acids (Bushman et al. 2004; Oh et al. 2007; Oomah et al. 2000; Parry et al. 2005; Radočaj et al. 2014; Yang et al. 2011). In addition to high levels of polyunsaturated and monounsaturated fatty acids, raspberry seed oils contain high levels of carotenoids (~25 mg/100 g) (Oomah et al. 2000). Similarly, raspberry seed oils contain high levels of tocopherols (up to 400 mg/100 g), higher than many other berry species (Bushman et al. 2004; Parry et al. 2005; Yang et al. 2011) and − sixfold higher than safflower and grape seed oils (Oomah et al. 2000). Very little information is available regarding environmental or varietal influences on raspberry seed oil composition. In one study fatty acid composition was similar between seed oils from yellow and red fleshed varieties, although seed oil yield was about 30% higher in the red variety (Oh et al. 2007).

7.5 Polyphenols, Important Components of Flavour, Appearance and Potential Health Benefits

Raspberry fruit contains a broad range of polyphenolic compounds that include phenolic acids, flavonols, anthocyanins, proanthocyanidins and ellagitannins (Dincheva et al. 2013b). These compounds have been subject to a great deal of research both in terms of their sensory properties, particularly in relation to wine, and for the potential health benefits associated with their consumption where they contribute the majority of the antioxidant capacity of raspberry fruit (Deighton et al. 2000).

Numerous studies have examined the influence of genotype and environment on the total polyphenolic and antioxidant content of raspberry fruit. According to Freeman et al. (2011), oxygen radical absorbance capacities (ORAC) ranged between 34 and 48 μmol trolox equivalents (TE) per g FW among raspberry cultivars, representing <1.5-fold variation between the lowest ('Caroline') and highest ('Autumn Bliss') cultivars. 'Autumn Bliss' was also shown to have a relatively high ORAC value compared against three other cultivars in a previous study, although again the variance in antioxidant capacity remained small with the highest cultivar being only 1.25 higher than the lowest cultivar (Wang and Lin 2000). Since differences in antioxidant capacity between cultivated varieties are low, the introduction of new germplasm into breeding programmes is required to boost antioxidant capacity in cultivated varieties further. However, in a study comparing wild varieties harvested in several Turkish provinces against the widely cultivated varieties 'Heritage' and 'Tulameen' both of the cultivated varieties scored favourably using the ferric reducing antioxidant potential (FRAP) and trolox equivalent antioxidant capacity (TEAC) methods. In particular, 'Heritage' scored higher than 12 out of 14 wild accessions in both measures, while 'Tulameen' also exhibited a greater antioxidant capacity than the majority of wild accessions (Çekiç and Özgen 2010), indicating that germplasm for breeding higher fruit antioxidant capacity might be difficult to obtain. Total polyphenol content is often closely correlated with total antioxidant capacity (Deighton et al. 2000; Dobson et al. 2012; Remberg et al. 2010) and like antioxidant capacity there is some variation in the total polyphenol content of different raspberry cultivars grown under similar environmental conditions. In the majority of reported studies, total polyphenol levels vary less than twofold between cultivars (Anttonen and Karjalainen 2005; Freeman et al. 2011; Krüger et al. 2011; Mazur et al. 2014c; Wang and Lin 2000) and only in a comparison of wild and cultivated genotypes did the variation exceed twofold (Çekiç and Özgen 2010). As with total antioxidant capacity, cultivated raspberry genotypes compared favourably with wild accessions and yellow fruited varieties did not have a conspicuously different phenolic content than red fruited varieties (Anttonen and Karjalainen 2005). In general, total phenolics were reported to be in the range of 200–400 mg/100 g FW, although there was some variation between studies. While environment does have a significant impact on fruit polyphenol content, studies have demonstrated a reasonable maintenance of hierarchy in fruit harvested in different years (Mazur et al. 2014c). Furthermore, several QTL for fruit phenolic content have been demonstrated to exhibit stability in multiple harvest years (Dobson et al. 2012).

Several studies have examined the influence of environment on fruit antioxidant content and phenolics. Mazur et al. (2014c) demonstrated significant variation in both FRAP activity and phenolics in fruit of 'Glen Ample' grown at three different sites in southern Norway that differed primarily in their altitude. Fruit grown at higher altitudes had significantly lower phenolic content and FRAP activity than fruit grown at lower altitudes and this was strongly correlated with the air temperature in the immediate 2 weeks prior to harvest with higher temperatures promoting greater polyphenol accumulation. These data were consistent with previous work in controlled environment chambers that showed a significant increase in FRAP and phenolic content as plant growth temperatures increased between 12 and 24 °C (Remberg et al. 2010). Regarding photoperiod, experiments conducted in controlled environments at 18 °C indicated that extended photoperiods had little impact on either polyphenols or antioxidant capacity (Mazur et al. 2014b).

Taking into consideration (1) the limited genetic diversity in terms of total phenol content and antioxidant activity, and (2) the stability of QTLs for these traits across different environments, there is limited potential for improvement through breeding. However, as outlined below, different polyphenolic compounds have specific impacts on fruit quality and therefore the polyphenolic composition of the fruit might be more important than the absolute amount of polyphenols in the fruit. Indeed, while the consumer may associate antioxidant capacity with potential health benefits, numerous studies indicate that this measurement does not provide meaningful information regarding the health benefits of consumption of specific fruits and any health benefits are likely to be imparted by specific polyphenolic components (Hancock et al. 2007) or their metabolites (Williamson and Clifford 2010).

7.5.1 Phenolic Acids

A number of simple phenolic acids have been detected in raspberry fruits including *p*-coumaric acid, caffeic acid and caffeoyl quinic acid (chlorogenic acid) (Kula et al. 2016; Maksimović et al. 2013). Sugar conjugates of *p*-coumarate and caffeate have also been reported (Dincheva et al. 2013b). Phenolic acids are found at low concentrations, usually less than 10 μg g^{-1} FW (Maksimović et al. 2013), which is at least an order of magnitude lower than concentrations of the more abundant ellagitanins (Gasperotti et al. 2010) and anthocyanins (Scalzo et al. 2008). As minor components they do not contribute significantly to the antioxidant capacity of raspberry fruit (Beekwilder et al. 2005; Mullen et al. 2002).

Phenolic acids represent the entry point into the general phenylpropanoid pathway and are synthesized by deamination of phenylalanine by phenylalanine ammonia lyase (PAL) to form cinnamic acid, which is further converted to *p*-coumaric acid by cinnamate-4-hydroxylase (C4H) (Yu and Jez 2008). *p*-Coumaric acid may serve as substrate for hydroxylation on the 3- position of the benzyl ring to form caffeic acid. The conversion of *p*-coumaric acid to caffeic acid is catalyzed by *p*-coumarate 3-hydroxylase (C3H). Less clear is the situation regarding caffeoyl quinate biosynthesis, where three possible routes have been proposed; in the most probable route (route 1), caffeoyl quinate is synthesised *via* caffeoyl CoA and quinate through the action of hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (HQT). In route 2, hydroxycinnamoyl D-glucose/quinate hydroxycinnamoyl transferase converts caffeoyl D-glucose to caffeoyl quinate, while in route 3 cacaffeoyl quinate is formed through a two-step reaction involving hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) and C3H. However, the importance of the route 3 for plants has been questioned since *Arabidopsis thaliana* plants are unable to synthesise caffeoyl quinate, although HCT and C3H are normally expressed. (Niggeweg et al. 2004; Ferrer et al. 2008; Payyavula et al. 2015, Fig. 7.4).

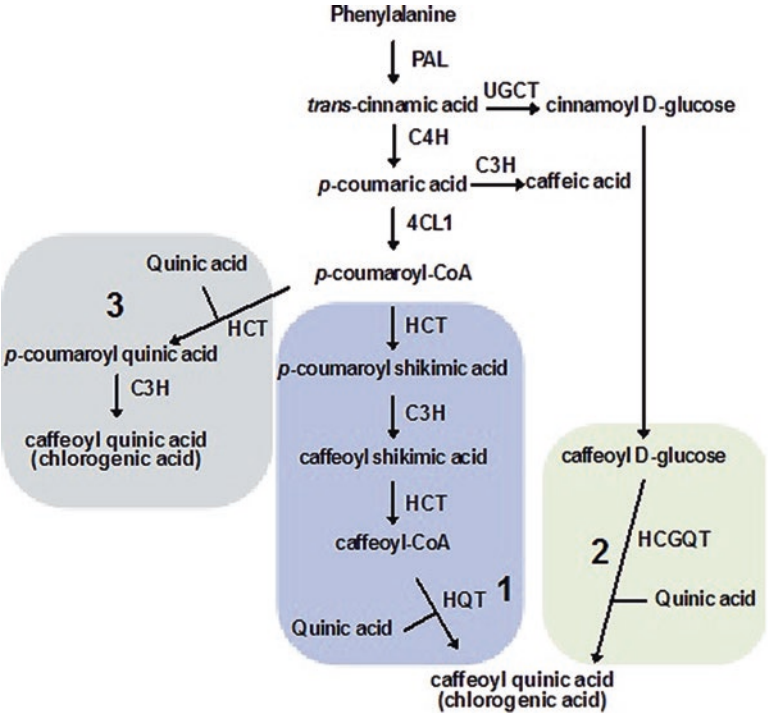


Fig. 7.4 Phenylpropanoid pathway and proposed routes leading to chlorogenic acid synthesis. The three possible routes are marked with different colours and numbered as 1, 2 and 3. PAL phenylalanine ammonia lyase, C4H cinnamate-4-hydroxylase, 4CL1 4-coumarate:CoA ligase, C3H *p*-coumarate 3-hydroxylase, UGCT UDP glucose:cinnamate glucosyl transferase, HCT hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase, HQT hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase, HCQQT hydroxycinnamoyl CoA D-glucose:quinate hydroxycinnamoyl transferase

PAL is considered to be a key regulator of flux into the phenylpropanoid pathway. Two genes, designated *RiPAL1* and *RiPAL2*, encoding PAL proteins have been isolated from raspberry that exhibit 81% amino acid identity. Both genes were expressed to differing degrees in leaves, shoots, flowers, fruits and roots. In fruits, the pattern of expression depended on fruit development. *RiPAL1* transcripts were most abundant at early fruit stages, whilst *RiPAL2* transcripts were more abundant in fruits that were accumulating colour (Kumar and Ellis 2001). Thus, it was suggested that the two encoded enzymes may be involved in channeling of substrates into flavonoids and anthocyanins, respectively.

Activation of phenolic acids for subsequent synthesis of downstream phenylpropanoids occurs via acylation catalyzed by 4-coumarate:CoA ligase (4CL). Three genes encoding 4CL were cloned from different raspberry tissues with the predicted amino acid sequence for *Ri4CL1* and *Ri4CL2* showing greater similarity (approximately 72% amino acid identity) than the more divergent *Ri4CL3*, with approximately 60% amino acid identity to both *Ri4CL1* and *Ri4CL2*. Recombinant proteins of the three 4CLs exhibited different catalytic properties with hydroxycinnamic and benzoic acids as substrates. Ri4CL1 exhibited the greatest substrate specificity for *p*-coumaric acid, but also accepted caffeic, cinnamic and ferulic acids albeit at 10–30% of the activity exhibited against *p*-coumarate. Ri4CL2 was most active against cinnamic acid, but also accepted *p*-coumarate, ferulate and caffeate. Ri4CL3 exhibited high activity using *p*-coumarate and could also utilize caffeic acid, but not cinnamate or ferulate. In addition, the expression profiles of the three genes were different with *Ri4CL1* most highly expressed in leaves, whilst *Ri4CL2* transcripts most abundant in shoots and roots, suggesting a potential role in providing lignin precursors. *Ri4CL3* was highly expressed in early developing and ripening fruits and taken in conjunction with the high specificity of the enzyme for *p*-coumarate, it was suggested that this enzyme played a key role in the activation of substrates for flavonoid biosynthesis (Kumar and Ellis 2003).

7.5.2 Flavonoids

Anthocyanins play a critical role in raspberry fruit quality being the key compounds providing fruit colour in red-fruited varieties. Alongside the ellagitannins, anthocyanins are described as contributing significantly to the antioxidant capacity of ripe raspberry fruit (Beekwilder et al. 2005; Mullen et al. 2002). On the contrary, other flavonoids, such as flavonols, are present only in low amounts in raspberry fruit and hence their contribution to antioxidant capacity is negligible (Borges et al. 2010). However, as they share a common biosynthetic pathway with the anthocyanins (Fig. 7.5) they will be discussed here.

In red raspberry, the dominant flavonols are quercetin and kaempferol, derivatives of the latter generally being present at concentrations approximately tenfold lower than the former (Carvalho et al. 2013b). Although small amounts of free flavonols have been reported (Carvalho et al. 2013b), they are mainly found as

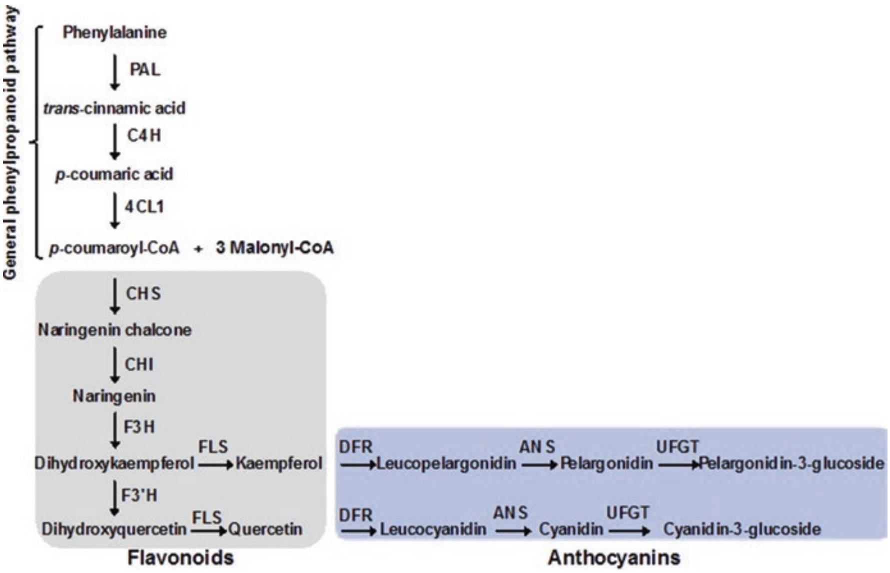


Fig. 7.5 Flavonoid and anthocyanin biosynthetic pathway. PAL phenylalanine ammonia lyase, C4H cinnamate-4-hydroxylase, 4CL1 4-coumarate:CoA ligase, CHS chalcone synthase, CHI chalcone isomerase, F3H flavanone 3-hydroxylase, F3'H flavonoid 3-hydroxylase, FLS flavonol synthase, DFR dihydroflavonol 4-reductase, ANS anthocyanidin synthase, UFGT UDP-glucose flavonoid 3-O-glucosyltransferase

conjugates of mono- and disaccharides, such as glucose, galactose, glucuronate, rutinose and sophorose (Borges et al. 2010; Carvalho et al. 2013b; Dincheva et al. 2013b; Kula et al. 2016). In total, flavonol content in raspberry fruit is generally lower than 0.1 mg g^{-1} FW (Carvalho et al. 2013b).

For anthocyanins, only cyanidin and pelargonidin derivatives have been described in red raspberry with cyanidin derivatives being dominant (Beekwilder et al. 2005; Borges et al. 2010; Chen et al. 2014; Kula et al. 2016; Scalzo et al. 2008). The anthocyanidin moieties are present as glucosides, sophorosides, glucosylrutinosides, rutinosides and xylosylrutinosides. Concentrations of cyanidin derivatives range from approximately 20 mg kg^{-1} fresh weight for the less abundant components, such as cyanidin-xylosylrutinoside, up to hundreds of mg kg^{-1} for the more abundant components, such as cyanidin-sophoroside and cyanidin-glucoside (Scalzo et al. 2008). Pelargonidin derivatives tend to represent less than a tenth of the total anthocyanins (Borges et al. 2010; Chen et al. 2014; Kula et al. 2016).

Flavonol and anthocyanin biosynthetic pathways are well established in many species, including raspberries. In the initial step in the pathway, the polyketide synthase, chalcone synthase, catalyses the conjugation of 4-coumaroyl-CoA with three molecules of malonyl-CoA to produce naringenin chalcone. Zheng et al. (2001) identified three polyketide synthase genes that were amplified by PCR-based cloning from cv. 'Royalty'. Heterologous expression in *E. coli* revealed that only one of the genes, *RiPKS1*, encoded a chalcone synthase, whilst the other two encoded a

4-coumaroyltriacetic acid lactone synthase and a non-functional protein, respectively. Subsequent work by the same group identified two further polyketide synthases in the 'Royalty' genome (Zheng and Hrazdina 2008). As discussed above, *RiPKS4* encoded an enzyme with both benzylacetone synthase activity, important for the production of a key aroma compound, and chalcone synthase activity. *RiPKS5* exclusively used 4-coumaryl-CoA as substrate synthesizing only naringenin chalcone. A recent RNA-seq transcript analysis of cv. 'Nova' confirmed multiple sequences encoding polyketide synthases and 9 different unigenes encoding chalcone synthases were identified (Hyun et al. 2014). This confirmed a previous report in which 11 genes were cloned from cv. 'Meeker' (Kumar and Ellis 2003). Detailed expression analysis of 3 out of the 11 CS genes revealed that they were differentially expressed leaves, shoots, roots, flowers, and fruit. However one gene exhibited strong developmental control in ripening fruit that was consistent with a function in anthocyanin accumulation. All three of these genes encoded proteins exhibiting typical chalcone synthase activity.

Downstream of naringenin chalcone, chalcone isomerase is responsible for the formation of naringenin, which is further converted to dihydrokaempferol by the action of the P450 flavanone 3-hydroxylase. A second P450 flavonoid 3′-hydroxylase converts dihydrokaempferol to dihydroquercetin and these compounds can be reduced to the flavonols kaempferol and quercetin, respectively by the action of 2-oxoglutarate-dependent flavonol synthase. The observation that these compounds are minor components in raspberry fruit, with respect to anthocyanins, suggests that this enzyme is relatively inactive in this tissue. Many species additionally contain a flavonoid 3′,5′-hydroxylase that synthesises dihydromyricetin, the precursor of the flavonol myricetin and the anthocyanidin delphinidin. However the absence of these components suggests that raspberries lack an active enzyme, despite the identification of a gene potentially encoding this activity (Hyun et al. 2014). The anthocyanidins cyanidin and pelargonidin are synthesised from dihydroquercetin and dihydrokaempferol, respectively, by the combined actions of dihydroflavonol 4-reductase and leucoanthocyanbidin dioxygenase (anthocyanidin synthase). Subsequent glycosylation for the formation of mature anthocyanins is catalyzed by a series of glycosyltransferases.

Despite the detailed analysis of genes and proteins involved in the early parts of the biosynthetic pathway in raspberries, very little work has been reported concerning the later steps of biosynthesis. RNA sequencing indicates that all steps are encoded by multiple transcripts and differential expression analysis has indicated candidates significant for anthocyanin accumulation in fruit (Hyun et al. 2014). In a more targeted analysis in red and yellow fruited varieties, there was significantly higher expression of chalcone synthase, flavanone 3-hydroxylase, dihydroflavanol 4-reductase and glycosyltransferase in both varieties during fruit ripening than at earlier stages of development. However, anthocyanidin synthase expression remained low at both developmental stages in the yellow fruited variety. Furthermore, the yellow fruited variety had a 5 bp insertion in the coding region of anthocyanidin synthase resulting in an early stop codon. It was demonstrated by complementation of an Arabidopsis anthocyanin mutant that the inserted gene resulted in the synthesis of an inactive protein indicating that the mutation led to the yellow fruit colour, at least in the cultivar examined (Rafique et al. 2016).

The role of environment and genotype on fruit anthocyanin content has been examined in detail and at least two groups have identified quantitative trait loci associated with colour and anthocyanin content. Several studies have indicated a seasonal effect on the content of both total and individual anthocyanins (e.g. Mazur et al. 2014c) and specific environmental parameters, such as temperature (Bradish et al. 2012) and light (Wang et al. 2009) have been correlated with the content of specific anthocyanins. Although environment does have an influence on anthocyanin content, there is also a high potential for successful breeding of desired fruit anthocyanin content. Indeed, detailed work to investigate the heritability of total and individual anthocyanins in 42 families derived from 13 raspberry cultivars indicated high narrow sense heritability estimates both for individual and total anthocyanins, suggesting that breeding could be a powerful means towards increasing anthocyanin content in raspberry (Connor et al. 2005). Several studies have further enhanced the potential for breeding for bespoke anthocyanin content by developing molecular markers and identifying quantitative loci underlying the anthocyanin trait. Kassim et al. (2009) examined anthocyanin content of a raspberry population comprising a cross between the North American cultivar ‘Latham’ and the European cultivar ‘Glen Moy’. The parents of this population exhibited significant differences in total and individual anthocyanin content, with ‘Latham’ containing almost double the quantity of total anthocyanins. QTL analysis revealed two major chromosomal regions on linkage group 1 and 4 that were highly associated with the content of eight and seven individual anthocyanins, respectively. These QTL were consistent over two separate years and explained between 10% and 65% of the variation dependent on the specific anthocyanin, year of analysis and marker. Analysis of genes associated with the markers failed to identify any genes encoding biosynthetic enzymes; however, several genes encoding transcription factors were identified. In a separate study, a population comprising a cross between black (*R. occidentalis*) and red (*R. idaeus*) raspberry was phenotyped for anthocyanin content across three growing seasons (Bushakra et al. 2013). Linkage analysis in this population identified a total of 27 QTL of which 6 were stable in all 3 years of the analysis. This analysis not only confirmed some of the candidate transcription factors identified by Kassim et al. (2009),but additionally identified markers associated with several biosynthetic pathway genes.

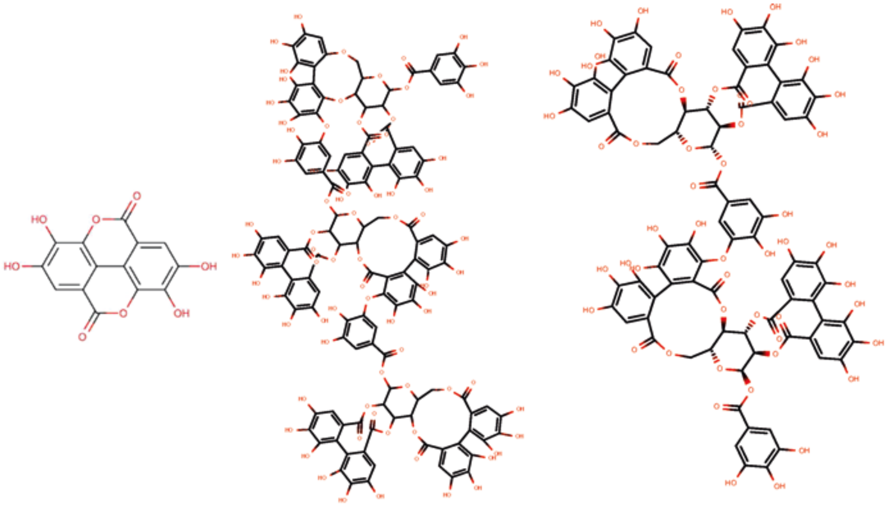
7.5.3 Tannins: Ellagitanins and Proanthocyanidins

A distinguishing feature of raspberries is their high level of ellagitannins in relation to other berry fruits. For example, the major ellagitannins, lambertianin C and sanguiin H-6, were reported at ~300–1000 nmol g^{-1} FW in raspberry fruits and represented >50% of the total fruit antioxidant capacity (Beekwilder et al. 2005; Borges et al. 2010). By comparison, anthocyanins totaled <900 nmol g^{-1} FW and represented less than 20% of antioxidant capacity. In addition to providing a significant proportion of fruit antioxidant capacity, the ellagitannins and proanthocyanidins impart important astringency, thereby having significant impact on the sensory properties of the fruit (He et al. 2015).

The levels of proanthocyanidins in raspberry fruit are considerably lower than the ellagitannins (Gu et al. 2003) and may be closely associated with seeds (Godevac et al. 2009; McDougall et al. 2014). Proanthocyanidins are polymers of flavan-3-ols synthesised from leucoanthocyanindins, in the case of raspberry leucocyanidin or leucopelargonidin, or anthocyanidins catalysed by leucoanthocyanidin or anthocyanidin reductase, respectively (He et al. 2015). Despite significant research effort, mechanisms of polymerization and the genes and enzymes required for these reactions remain largely unknown.

Ellagitannins are also polymeric polyphenolic compounds, in this case of ellagic acid. Raspberry fruits have been reported to contain free ellagic acid, ellagic acid glycoconjugates and a range of di- and tri-meric ellagitannins (Fig. 7.6). The most abundant compounds are the ellagitannins sanguiin H6 and lambertianin C, which were reported at concentrations in the ranges of 0.4–0.7 and 0.2–0.6 mg g^{-1} FW, respectively, in a number of raspberry cultivars harvested in northern Italy (Gasperotti et al. 2010). Other sanguiin and lambertianin derivatives were present at lower concentrations. Free ellagic acid and several ellagic pentosides were also abundant being reported at concentrations up to 0.16 mg gFW^{-1}.

Although the latter stages of ellagitannin biosynthesis remain obscure, progress has been made in the early parts of the biosynthetic pathways in recent years.



Ellagic acid Sanguiin H-6 Lambertianin C

Fig. 7.6 Structure of major ellagitannins found in raspberry fruit

Labelling experiments indicate that gallic acid is the precursor to ellagic acid in strawberries and raspberries (Schulenburg et al. 2016). Gallic acid has been demonstrated to be a product of the shikimate pathway, where atypical dehydroquinate dehydrogenases/shikimate dehydrogenases produce significant amounts of gallic acid both *in vivo* and in grapevine hairy root cultures (Bontpart et al. 2016). Further conversion of gallic acid to ellagic acid requires the glucosylation of gallic acid, with β-glucogallin as a key intermediate. Recent work identified five genes encoding putative gallic acid:UDP-glucose glucosyltranseferases in the genomes of *Fragraria vesca*, *Fragraria x ananassa* and *Rubus idaeus* based upon sequence homology to grapevine genes. Kinetic analysis of the five glycosyltransferases (GTs) revealed that FaGT2 had the highest catalytic efficiency (k_{cat}/k_m) against gallic acid (Schulenburg et al. 2016). Instead, RiGT2 showed higher affinity for cinnamic acid, coumaric acid derivatives and benzoic acid derivatives, similar to FvGT2. A better understanding of the genes involved in this key catalytic step would improve the prospects for breeding for high or low ellagitannin content in the future.

7.6 Potential Health Benefits Associated with Raspberry Polyphenol Consumption

Raspberries are dominated by high levels of polyphenols (total polyphenol contents of >200 mg/100 g $FW^{-1}$are common; Deighton et al. 2000) and these components have been a specific focus of attention with respect to understanding health benefits associated with raspberry consumption.

A concept has developed that the high antioxidant capacity of polyphenol components could contribute to health benefits by ameliorating the detrimental effects of reactive oxygen species (ROS) generated in the body through oxygen metabolism (Halliwell 2007). It was proposed that they could act as chain-breaking agents preventing the ROS from instigating free radical cascades that could damage cells, DNA and membranes and through accumulation cause diseases. However, this simple attractive precept is not generally tenable and high antioxidant capacity in the test-tube does not automatically translate into *in vivo* effectiveness. Although berry polyphenols may have high antioxidant capacity, their effectiveness is limited by their often low (μM – nM) uptake into the blood stream. Indeed, the effectiveness of even the small proportion taken up in to the serum is exacerbated by further metabolism in the liver and excretion through the bile or urine (Koli et al. 2010). In many cases, the original components are effectively absent and their circulating metabolites may differ greatly in structure and potential function (Williamson and Clifford 2010). In fact, different polyphenols have different stabilities, bioavailability and therefore potential effectiveness.

Berry polyphenols that are not taken up into the blood stream could still have beneficial functions in the gastrointestinal tract (GIT) as they pass through the digestive system such as preventing the formation of oxidation products from foods in the stomach and GIT (Gorelik et al. 2005) or by influencing food digestion, glucose levels and calorie usage (McDougall et al. 2008a). Berry polyphenols that pass through the upper digestive tract and reach the colon are subject to fermentation by bacteria and this produces a range of long-lasting phenolic metabolites that recirculate in the bloodstream, which could be the causative agents for some beneficial effects (Williamson and Clifford 2010).

The importance of understanding component bioavailability is neatly illustrated by the fate of ellagitannins, the major polyphenol components of raspberries. These large components remain in the GIT and break down to release ellagic acid (McDougall et al. 2014), which is subsequently metabolised by colonic bacteria to urolithin degradation products (Tomas-Barberan et al. 2017). These urolithins and their phase II metabolites reach significant levels in the serum (Ludwig et al. 2015) and these have been shown to have anti-cancer, cardiovascular and neuroprotective effects (Gonzalez-Sarrias et al. 2017a, b; Savi et al. 2017) at physiologically-relevant levels. Therefore, these microbiota-derived derivatives may deliver some of the health benefits associated with ellagitannin intake from raspberries.

7.6.1 Anti-Cancer Effects

There is considerable evidence that components from berries of *Rubus* species can influence the proliferation of human cancer cell models. Evidence has been obtained from a range of cancer lines originating from different organs and body locations from prostate to lung, breast to colon and blood to cervical cancers. Many studies have not adequately defined their extract composition so making assumptions about effective components is impossible and sometimes certain components are proposed to be causative without sufficient evidence. Within the polyphenols, many studies have highlighted the possible role of anthocyanins (Bowen-Forbes et al. 2010) but previous work suggested that ellagitannins from raspberry were more effective against cervical cancer cells than anthocyanins (Ross et al. 2007). Indeed, extracts from berry species rich in ellagitannins, such as the *Rubus* species, have been found to be particularly effective in cell line studies (McDougall et al. 2008b; Seeram et al. 2006) and individual ellagitannin components, such as Sanguiin H6, have potent effects in cancer cell models (e.g. Sakai et al. 2016; Lee et al. 2016; Lee and Lee 2005) or models of cellular inflammation (Sangiovanni et al. 2013), perhaps through degradation and release of ellagic acid *in situ* (Whitley et al. 2006).

Considering the issues of bioavailability outlined above, the use of cell lines derived from GIT cancers which could be in contact with active components in the digestive tract could be more physiologically-relevant models (Brown et al. 2012). For example, red raspberry extracts were found to inhibit the migration and invasion ability of oral cancer cells and altered metastasis by suppression of MMP-2 expression through the FAK/Scr/ERK signaling pathway (Hsieh et al. 2013). Modelling the effects of gastrointestinal digestion using *in vitro* digestion (IVD) systems provided a clearer picture of the physiological stability of red raspberry polyphenol components and confirmed that these gut-accessible components were protective in models of colon cancer (Coates et al. 2007). Other studies have suggested that anticancer effects of blackberry and wild *Rubus* extracts in colon cell models were enhanced by IVD treatment (e.g. Chen et al. 2016a, b) and again effectiveness was correlated with the survival of specific components. Furthermore, subsequent fermentation of IVD extracts using faecal bacteria provided a range of faecal metabolites and confirmed that anti-cancer effects survive bacterial degradation in the colon (Brown et al. 2014) although the original polyphenol components had been extensively metabolised to simpler phenolics.

7.6.2 Diabetes

The incidence of type 2 diabetes has reached near-epidemic proportions in the Western world. Following the outline of Hanhineva et al. (2010), there are four main areas where *Rubus* berries could beneficially influence glycemic control (i) protection of pancreatic β-cells from glucose-induced toxicity and oxidative stress, (ii) inhibition of starch digestion and absorption to control blood glucose levels, (iii) suppression of glucose release from the liver and (iv) improvement of glucose uptake in peripheral tissues such as muscles.

In the pre-diabetic state, the pancreatic β-cells (which secrete insulin) become dysfunctional and decline in number due to glucose-induced toxicity and oxidative stress (Hanhineva et al. 2010). Considerable evidence has accrued that polyphenols found in raspberries can maintain insulin secretion in β-cells grown in culture (Martineau et al. 2006), protect against oxidative damage induced by elevated glucose in rats (Rodrigo et al. 2011), and modulate insulin secretion and function in humans (Seymour et al. 2011; Stull et al. 2010). However, none of these studies used *Rubus* extracts.

Many dietary polyphenols are retained in the gastrointestinal tract and pass through to the colon without substantial absorption. These components can interact and modulate the digestion of glycaemic carbohydrates, namely starch and sucrose. Polyphenol-rich extracts from berries inhibit α-amylase and α-glucosidase *in vitro* (McDougall et al. 2005), the key enzymes involved in glucose production from starch. The degree of inhibition differed between the berries and was linked to their polyphenol composition. Fractionation studies suggested that tannins (ellagitannins and proanthocyanidins) were potent inhibitors of amylase (Grussu et al. 2011). Therefore, raspberry extracts were effective against amylase but less so against glucosidase. Indeed, a range of polyphenols may be capable of inhibition of glucosidase (Lo Piparo et al. 2008; Boath et al. 2012). Depending on their phytochemical composition, it is therefore possible that specific berries could inhibit both amylase and glucosidase and synergistically reduce the pool of glucose available for uptake into the blood. This raises the possibility of breeding to enhance the content of specific polyphenols that inhibit α-glucosidase further enhancing the capacity of raspberry fruit consumption for glycaemic control.

Purified polyphenols can additionally influence intestinal absorption of glucose through interaction with sodium-dependent glucose transporter (SGLT1) and the glucose transporter, GLUT2 (Hanhineva et al. 2010) in the GIT. In addition, strawberry polyphenols decreased glucose transport across gut epithelial cells through inhibition of both SGTL1 and GLUT2 (Manzano and Williamson 2010).

Purified polyphenols commonly found in *Rubus* spp. increased basal and insulin-stimulated glucose uptake in muscle cells (Claussnitzer et al. 2011) and basal glucose uptake was increased in muscle cells through activation of the adenosine monophosphate kinase system (Eid et al. 2010). The anthocyanin, cyanidin-3-glucoside, which is common in raspberries (and its metabolite protocatechuic acid) exerted insulin-like effects in a human adipocyte model (Scazzocchio et al. 2011).

Polyphenols may interact with the insulin-sensing pathway and modulate glucose release from the liver through reduction in glucose synthesis. Polyphenols appear to act on signal transduction pathways and influence the phosphorylation status of key transcription factors such as FOXO1a (Cheng and White 2011; Takikawa et al. 2010).

Taken together this research illustrates the capacity of raspberry polyphenols to modulate diabetic control at numerous levels. It is also clear that different components influence different aspects of glucose control opening up the possibility of breeding to enhance specific polyphenolic components to improve the potential benefits of raspberry fruit consumption even further.

7.6.3 Cardiovascular Disease (CVD)

Substantial evidence has been provided that berry components can influence parameters relevant to CVD in *in vitro* cell studies and animal models. For example, berry extracts including *Rubus spp* beneficially modulated function in endothelial cells *in vitro* (Tulio et al. 2012). Polyphenol-rich extracts of red raspberry, black raspberry and blackberry fruits attenuated angiotensin-II-induced senescence in vascular smooth muscle cells through reduction of intracellular reactive oxygen species (ROS). The different extracts operated by both NOx-1-dependent and independent mechanisms (Feresin et al. 2016).

Polyphenols may aid the muscle layer of blood vessels to relax (i.e. vasodilation). Endothelial cells, which make up the inner layer of blood vessels, produce nitric oxide which regulates blood pressure. Polyphenols found in berries can increase the activity of endothelial nitric oxide synthase (eNOS) to stimulate nitric oxide (NO) production and increase vasodilation (Galleano et al. 2010). Indeed, ethyl acetate extracts of red raspberries promoted antihypertensive effects in spontaneously hypertensive rats through enhanced NO production (Jia et al. 2011).

A review of human intervention studies with fruit polyphenols (Chong et al. 2010) found inconsistent but positive effects on CVD risk factors and suggested that the inconsistency was due to differences in experimental design and treatment groups. However, other analyses suggest that common berry components such as anthocyanins may beneficially influence clinical parameters associated with enhanced risk of CVD (Basu et al. 2010). Individuals with elevated cholesterol levels improved their endothelium-dependent vasodilation after berry anthocyanin intake along with improved serum lipid profiles and decreased markers of inflammation (Zhu et al. 2011).

7.6.4 Obesity

Obesity is often associated as an underlying risk factor in CVD, metabolic syndrome and diabetes. Berries have also been implicated in the prevention of obesity perhaps through interference with lipid digestion (McDougall et al. 2009) and/or modulation of lipid metabolism (Prior et al. 2011). Extracts from *Rubus* species were found to be particularly effective in inhibiting pancreatic lipase *in vitro* (McDougall et al. 2009), with ellagitannins particularly effective.

There is substantial evidence for effects of *Rubus* spp. in cell line studies. For example, water extracts of unripe *R. coreanus* caused anti-hyperlipdemic effects in liver cells through inhibition of cholesterol biosynthesis brought about by reduced apolipoprotein release and modulation of FOXO-1 signalling pathways (Bhandary et al. 2012; Jeong et al. 2013). Butanol extracts from unripe *R. coreanus* inhibited the differentiation of adipocytes (Oh et al. 2016) and five components (ellagic acid, erycibelline, 5-hydroxy-2-pyridinemethanol, *m*-hydroxyphenylglycine, and 4-hydroxycoumarin) were identified that could be responsible for this effect.

In an interesting study, a range of freeze dried berry powders (including black raspberry, red raspberry, and blackberry) did not prevent weight gain or hyperlipidemia in high-fat fed rats (Prior et al. 2009). However, purified anthocyanins from blackberries were effective, which suggests that these red pigments were important for lipid-lowering effects (Prior et al. 2010). Other components known to be present in *Rubus* fruits have also been implicated. Ellagic acid attenuated high fat/high sugar (HF/HS) diet effects on hyperlipidemia through antioxidant and anti-inflammatory effects (Panchal et al. 2013).

R. coreanus extracts reduced weight gain in mice whilst reducing white adipose tissue (Jeong et al. 2015) and other studies on brown pre-adipocytes also showed increases in thermogenic gene expression. Boysenberry (a *Rubus* hybrid) juice inhibited weight gain in high-fat fed rats and inhibited pancreatic lipase *in vitro* and triglyceride absorption from the gastrointestinal tract *in vivo*. Fractionation suggested that fractions enriched in ellagitannins and ellagic acid conjugates, and anthocyanins were most effective in inhibiting triglyceride uptake *in vivo* (Mineo et al. 2015). However, pure ellagic acid was not effective.

Raspberry juice and raspberry puree decreased weight gain in HF- fed mice with concomitant significant reductions in resistin levels (Luo et al. 2016). Extracts of *R. crataegifolius* significantly reduced weight gain in HF-fed rats with concomitant reductions in epididymal fat weight, serum cholesterol and triglycerides (Jung et al. 2016). Raspberry seed flour reduced HF/HS-diet-induced hyperlipidemia and biomarkers of inflammation in C57BL/6 male mice (Kang et al. 2016). The researchers suggested that ellagic acid derviatives may be important but raspberry seed flour would contain other phenolic components including proanthocyanidins (Godevac et al. 2009; McDougall et al. 2014) and substantial levels of dietary fibre.

7.6.5 Neuroprotective Effects

A body of evidence has developed that supports a role for berry polyphenol components in neuroprotection (Miller and Shukitt-Hale 2012). The berry components are proposed to protect against damage induced by ROS, which are known to be implicated in the development of neurological conditions such as Alzheimer's disease (Spencer 2010). The brain represents only 2.5% of the body's weight but it receives 15% of the cardiac output, and consumes 20% of total body oxygen and 25% of total body glucose utilization. Consequently this highly oxygenated environment means that the brain is particularly prone to damage induced by ROS and it has innately less-well developed antioxidant mechanisms. Berry components are proposed to mediate in cell signalling pathways that potentiate antioxidant mechanisms and influence inflammatory responses. A recent review has outlined neuroprotective effects of red raspberries (Burton-Freeman et al. 2016) and this section extends this to other *Rubus* species.

Polyphenol-rich *R. coreanus* extracts exhibited protective effects on neuronal PC-12 cells (Im et al. 2013). Extracts from wild, but not cultivated, blackberry varieties provided neuroprotective effects in cell models (Tavares et al. 2012, 2013) through reduction in intracellular reactive oxygen species, modulation of glutathione levels and caspase activation. These effects were potentiated in extracts that had undergone simulated gastrointestinal digestion. Red raspberry extracts protected both neuronal and microglial cells against H_2O_2-induced oxidative stress, lipopolysaccharide-induced inflammation and NO production (Garcia et al. 2012). The anti-inflammatory activity was retained by extracts that had undergone simulated intestinal digestion and appeared to act through inhibition of IBa1 expression and TNF-α release. *In vitro* studies also support the premise that pure polyphenols also found in *Rubus spp* can beneficially remodel amyloid-beta aggregation *in vitro* (Ladiwala et al. 2011), a process implicated in brain damage in Alzheimer's disease.

7.7 Conclusions

In recent years significant advances in our knowledge regarding the pathways of biosynthesis and mechanisms of control of accumulation of important raspberry fruit metabolites has greatly increased. Alongside this, knowledge about the phytochemistry underlying fruit sensory attributes continues to accumulate and mechanistic work to understand the role of specific fruit phytochemicals in modulating human disease status gathers apace. This has been accompanied by an increase in knowledge regarding raspberry genetics, and genes and loci underlying specific fruit phytochemical traits. Taken together, this raises the very real possibility of breeding new raspberry cultivars that deliver the consumer with the sensory experience they seek whilst delivering a fruit rich in phytonutrients that will protect and even reverse common degenerative diseases.

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