University of East Anglia

Anthocyanin Stability, Metabolic Conjugation and in vitro Modulation of Endothelial Superoxide Production

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Abstract

Anthocyanins are thought to possess significant cardio-protective properties and have been proposed as potent modulators of vascular disease. However, their true health benefits have been thrown into question, since the current literature poorly describes their stability in foods, metabolism and mode of biological activity. Therefore, this thesis aimed to evaluate (i) the stability of anthocyanins during commercial juice processing, (ii) the formation of phenolic degradation products under simulated physiological conditions and recovery from clinical samples, (iii) the *in vitro* hepatic conjugation (metabolism) of anthocyanins and their phenolic acid degradation products and (iv) the *in vitro* cardiovascular related bioactivities of anthocyanins and their free or conjugated metabolites.

Using commercial and bench-scale processing models, no significant loss in anthocyanins was observed throughout blackcurrant juice processing and only sodium bisulphite improved anthocyanin yield in juices. Using *in vitro* stability studies, it was demonstrated that B-ring hydroxylation status mediated the degradation of anthocyanins to phenolic acids under simulated physiological and gastrointestinal conditions. Subsequently, it was shown that following microsomal metabolism, pelargonidin and cyanidin formed aglycone, aryl and acyl glucuronides of 4-hydroxybenzoic acid and protocatechuic acid, respectively; thus indicating that anthocyanins may be found in the systemic circulation as free or conjugated phenolic acids.

Finally, it was shown that the attenuation of Ang II induced endothelial superoxide production by anthocyanins was enhanced following degradation and metabolism to phenolic acid metabolites. Furthermore, this biological effect was inversely associated with the number of aryl hydroxyls, which is in express opposition to their antioxidant potential. Therefore, it is likely that the biological effects of these compounds, at physiological concentrations, are not due to their direct radical scavenging properties.

Overall, the findings of this thesis emphasize the importance of metabolism, bioavailability and structure-activity relationships when conducting or interpreting past and future research associated with the health benefits of flavonoids.

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Chapter 1

Review of the literature: Anthocyanin occurrence, stability, metabolism and bioactivity

1.1 Introduction

In recent years it has been established that oxidative stress, vascular inflammation, and endothelial dysfunction play a central role in the development of cardiovascular diseases. As such, advancements in the knowledge of these disease processes have allowed the development of novel pharmaceutical and/or dietary strategies to prevent the development of vascular diseases. Epidemiological observations have played a significant part in the development of such dietary strategies and the identification of numerous cardio-protective phytochemicals. Of these phytochemicals, anthocyanins (natural polyphenols found in many purple to red coloured plant and fruit species; **Figure 1.1**) are thought to possess significant cardio-protective properties.

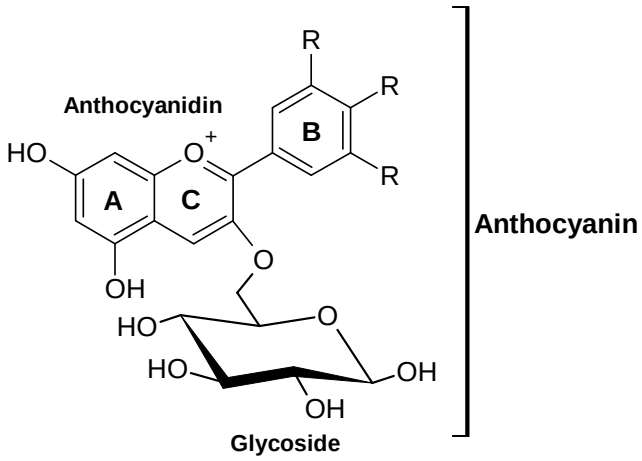


Figure 1.1: Common anthocyanin structure.

Since the 1970s, many studies have investigated the occurrence and utility of anthocyanins in plants and in food production, whilst research concerning their potential health benefits has become prominent only since the late 1990s. *In vitro*, *in vivo* and meta-analysis studies have shown that anthocyanins prevent or reduce the risk factors associated with a multitude of diseases, including those associated with cardiovascular (Mink, Scrafford et al. 2007; Hooper, Kroon et al. 2008) and inflammatory diseases (Oak, Bedoui et al. 2006; Xia, Ling et al. 2006; Yamamoto, Naemura et al. 2006; Sorrenti,

Mazza et al. 2007; Xia, Ling et al. 2007; Leblais, Krisa et al. 2008; Toufektsian, de Lorgeril et al. 2008), carcinogenesis (Feng, Ni et al. 2007; Rossi, Garavello et al. 2007; Elisia and Kitts 2008) and obesity (Tsuda, Horio et al. 2003; Tsuda, Ueno et al. 2004; Jayaprakasam, Olson et al. 2006; Sasaki, Nishimura et al. 2007). Indeed, anthocyanins are thought to act directly on the different cells involved in the development of vascular pathologies. For example, anthocyanins exert a protective effect against TNF-α induced monocyte chemoattractant protein 1 (MCP-1; involved in the recruitment of marophages to sites of inflammation) secretion from the endothelium (Garcia-Alonso, Rimbach et al. 2004). More recently, anthocyanins have been shown to inhibit CD40 induced proinflamatory signalling and cholesterol distribution (Xia, Hou et al. 2005; Xia, Ling et al. 2007).

To date, current literature evidence poorly describes the stability and human metabolism of anthocyanins, with no evidence regarding the biological effect of their metabolites. Thus, in order to fully appreciate the health related benefits of ingested anthocyanins, an understanding of their occurrence, biochemistry, metabolism and final bioactive mechanisms is essential. Within this review, our current understanding of these issues is collated, with some narrative highlighting the gaps in our present knowledge.

1.2 Anthocyanin occurrence in the diet

Anthocyanins occur widely in our diets, with the average human consumption estimated to be 12.5 mg/person/day (Wu, Beecher et al. 2006). Of the many fruits and vegetables that contain anthocyanins, berry species possess the highest concentration of anthocyanins and thus represent a major vehicle for the delivery of these compounds (Maatta-Riihinen, Kamal-Eldin et al. 2004). In plants, anthocyanins are almost exclusively found as 3-*O*-glycosides of anthocyanidin, the aglycone form of the anthocyanin. There are approximately 17 anthocyanidins found in plants, including the six common species (cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg) and

malvidin (Mv) (**Figure 1.2**). They are characterised by their many structural deviations, including differences in the number and position of attached sugars, the number and position of hydroxyl and/or methoxyl groups on the B-ring and the extent of sugar acylation and the identity of the acylating agent (Wu, Beecher et al. 2006). Furthermore, anthocyanins may exist in at least four different structural conformations (**Figure 1.3**) namely, flavylium cation, quinonoidal base, hemiketal and chalcone, which are pH dependant (Asenstorfer, Iland et al. 2003).

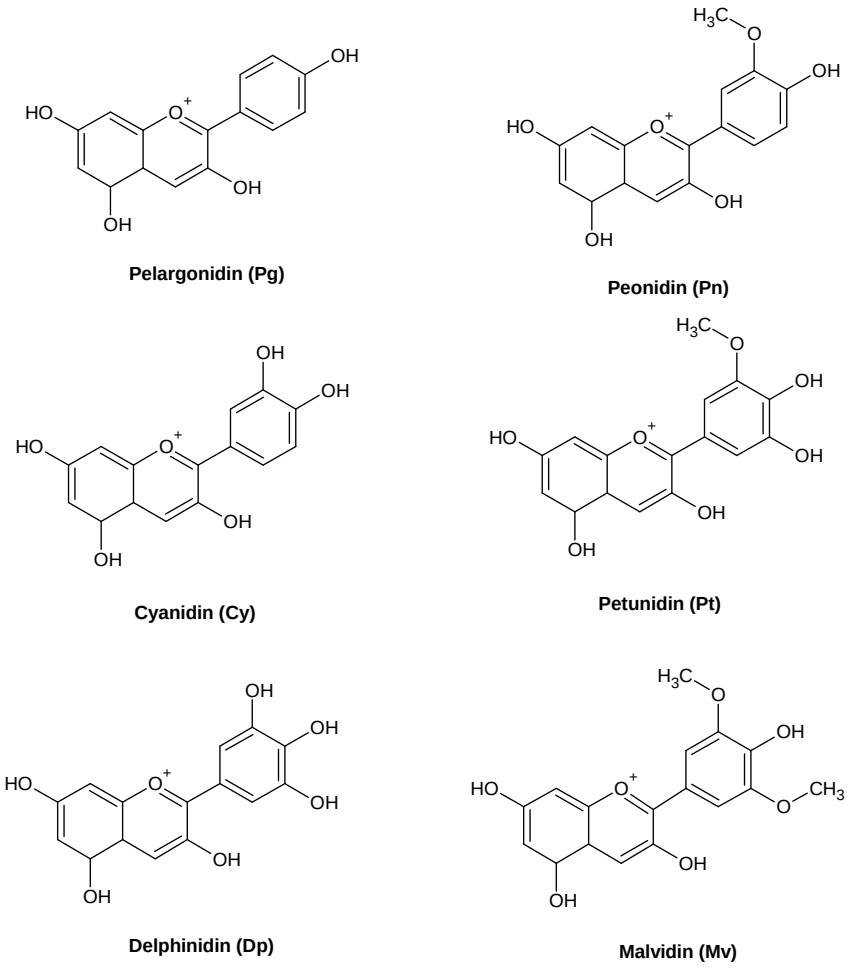


Figure 1.2: Structures of common anthocyanidin species.

When considering the potential health benefits of anthocyanins, an appreciation of their occurrence in human populations is essential. A survey published by the U.S. Department of Agriculture (USDA) (USDA 2006) showed that berries accounted for the majority of all the frozen fruit products

consumed in 2005 . In addition, berry fruits contain the highest concentrations of anthocyanins when compared with most other dietary sources (Mazza and Miniati 1993)(**Table 1.1**). Therefore, berry fruits represent the most abundant and feasible dietary source of anthocyanins in human populations and provide an excellent vehicle for the delivery of these health promoting compounds.

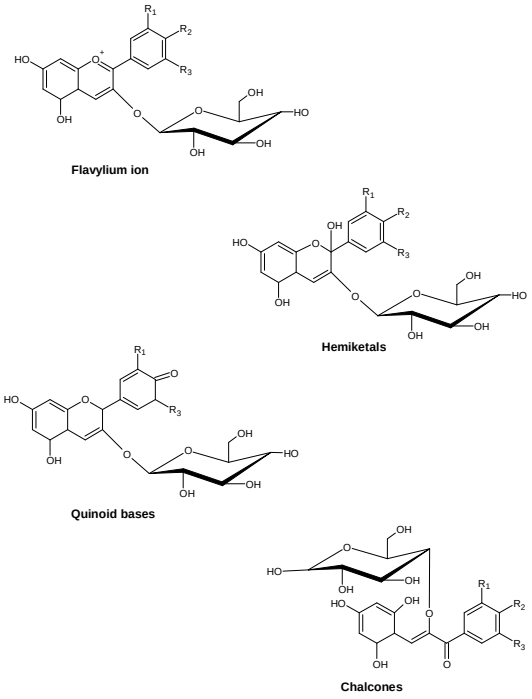


Figure 1.3: The four most common pH dependent structural conformations of anthocyanins.

Table 1.1. **Anthocyanin content in berry species**.

Berry Species	Total Anthocyanin content (mg/100g dry/fresh weight)	
	Fresh weight	Dry Weight
Gooseberry[1-2,7,8]	15	123
Chokeberry[2-4,7,8]	1053	1041
Elderberry[4,7,8]	1027	No Data
Raspberry[2,5,6]	1037	230
Black currant[3,4,7,8]	385	500*
Strawberry[2,3,8]	31	203
Cranberry[2-4,8]	98	397
Black berry[8]	272	No Data

Weight reported as mg anthocyanin/100g dry or fresh weight. *Anthocyanin content reported in freeze dried pomace. [1]M. Jordheim, F. Mage, O. M. Andersen, *J. Agric. Food Chem.* **55**, 5529 (2007). [2]M. P. Kahkonen, A. I. Hopia, M. Heinonen, *J. Agric. Food Chem.* **49**, 4076 (2001). [3]P. G. Kapasakalidis, R. A. Rastall, M. H. Gordon, *J. Agric. Food Chem.* **54**, 4016 (2006). [4]K. R. Maatta-Riihinen, A. Kamal-Eldin, P. H. Mattila, A. M. Gonzalez-Paramas, A. R. Torronen, *J. Agric. Food Chem.* **52**, 4477 (2004). [5]R. A. Moyer, K. E. Hummer, C. E. Finn, B. Frei, R. E. Wrolstad, *J. Agric. Food Chem.* **50**, 519 (2002). [6]W. Mullen *et al.*, *J. Agric. Food Chem.* **50**, 5197 (2002). [7]X. Wu *et al.*, *J Agric Food Chem* **52**, 4026 (Jun 16, 2004). [8]X. Wu *et al.*, *J. Agric. Food Chem.* **54**, 4069 (2006).

Table 1.2. Anthocyanin profiles of various berry species[1-8]

Anthocyanin species	Visual Representation of Anthocyanin Abundance within each berry species*							
	Gooseberry	Chokeberry	Elderberry	Raspberry	Black current	Cranberry	Strawberry	Blackberry
Cy-3-Glucoside	+++++	+	++	++	++	++++	+	++
Cy-3-Rutinoside	+++			+++++	++++			+
Cy-3-Galactoside		+++++				+++		
Cy-3-Arabinoside		+++				+++		+++++
Cy-3-xyloside		+						+
Cy-3-Sambudioside			+++++					
Cy-3-Sophoroside				+++++				
Cy-3-malonylglucoside								+
Cy-3-dioxaloylglucoside								+
Cy-3-Sambudioside-5-Glucoside			++					
Cy-3-(2-glucosylrutinoside)								
Cy-3-Sophoroside-5-Rhamnoside				++				
Cy-3(6-coumarroyl)-Glucoside					+			
Pn-3-Glucoside						+++		++
Pn-3-Rutinoside					+			
Pn-3-Galactoside						+++++		
Pn-3-Arabinose						+++		
Pg-3-Glucoside							+++++	+
Pg-3-sophoroside								
Pg-3-Rutinoside				++	+		++	
Pg-3-glucose-rutinoside								
Pt-3-Rutinoside					+			
Pt-3(6-coumarroyl)-Glucoside					+			
Dp-3-Rutinoside					+++++			
Dp-3-Glucoside					+++			
Dp-3-Arabinose						++		
Other	+++							

*Visual representation of anthocyanincontent from chromatographs: *(Trace)* +<++<+++<++++<+++++ *(Abundant).*
[1]M. Jordheim, F. Mage, O. M. Andersen, J. Agric. Food Chem. 55, 5529 (2007). [2]M. P. Kahkonen, A. I. Hopia, M. Heinonen, J. Agric. Food Chem. 49, 4076 (2001). [3]P. G. Kapasakalidis, R. A. Rastall, M. H. Gordon, J. Agric. Food Chem. 54, 4016 (2006). [4]K. R. Maatta-Riihinen, A. Kamal-Eldin, P. H. Mattila, A. M. Gonzalez-Paramas, A. R. Torronen, J. Agric. Food Chem. 52, 4477 (2004). [5]R. A. Moyer, K. E. Hummer, C. E. Finn, B. Frei, R. E. Wrolstad, J. Agric. Food Chem. 50, 519 (2002). [6]W. Mullen et al., J. Agric. Food Chem. 50, 5197 (2002). [7]X. Wu et al., J Agric Food Chem 52, 4026 (Jun 16, 2004). [8]X. Wu et al., J. Agric. Food Chem. 54, 4069 (2006).

Of the many anthocyanin species that may occur in berry fruits, cy, pg and pn are the most abundant anthocyanidins species, with cy-3-glu, cy-3-rut, cy-3-gal, cy-3-ara and pn-3-glu accounting for the most profuse anthocyanins (**Table 1.2**) (Maatta-Riihinen, Kamal-Eldin et al. 2004; Wu, Gu et al. 2004; Tian, Giusti et al. 2005; Wu and Prior 2005; Netzel, Netzel et al. 2006; Seeram, Adams et al. 2006; Lin and Harnly 2007). A detailed catalogue of anthocyanin containing foods and the respective quantities can be found in the recent Phenol-Explorer database (Scalbert, Neveun et al. 2010) launched by Scalbert *et al*.

Chokeberry, elderberry and strawberry have the smallest variation of anthocyanin species and structural deviations, consisting almost exclusively of either cy or pg derivatives (Chandra, Rana et al. 2001; Maatta-Riihinen, Kamal-Eldin et al. 2004; Nakajima, Tanaka et al. 2004; Wu, Gu et al. 2004). The most abundant anthocyanin in chokeberry is cy-3-gal, with concentrations up to 989.7 mg/100g fresh weight (FW) (Wu, Gu et al. 2004), followed by cy-3-ara, cy-3-xyl and cy-3-glu at concentrations of 399.3, 51.5 and 37.6 mg/100g FW respectively (Wu, Gu et al. 2004), with only trace amounts of pg-3-ara (2.3 mg/100g FW) (Chandra, Rana et al. 2001; Kay, Mazza et al. 2004; Maatta-Riihinen, Kamal-Eldin et al. 2004; Nakajima, Tanaka et al. 2004; Wu, Gu et al. 2004). Elderberries contain an abundance of cy-3-glu and cy-3-sam (739.8 and 545.9 mg/100g FW, respectively) (Wu, Gu et al. 2004), with small to trace amounts of cy-3-sam-5-glu, cy-3-rut and cg-3-glu (82.6, 4.4 and1.8 mg/100g FW, respectively) (Chandra, Rana et al. 2001; Nakajima, Tanaka et al. 2004; Wu, Gu et al. 2004). Strawberries contain predominantly pg-3-glu and pg-3-rut, with some studies identifying varying concentrations of cy-glycosides (Wu, Gu et al. 2004; Seeram, Adams et al. 2006). Interestingly, while blackberries may contain up to 9 anthocyanin species, cy-3-glu represents the vast majority, with only small to trace amounts of other cy-3-glycosides, pn-3-glycosides and pg-3-glycosides (Moyer, Hummer et al. 2002; Wu, Beecher et al. 2006). Of the most commonly occurring berry fruits, chokeberry, elderberry, strawberry and blackberry have the simplest anthocyanin profiles, which make them ideal candidates for assessing anthocyanin chemistry in fruit and their metabolism in human studies. However, when considering structural complexity it should be noted that elderberry contains a high amount of cy-3-sam (Nakajima, Tanaka et al. 2004; Wu, Beecher et al. 2004), a disaccharide, and small amounts of cy-3-sam-5-glu (Nakajima, Tanaka et al. 2004; Wu, Beecher et al. 2004), a trisaccharide, which may significantly vary in absorption and degradation characteristics in comparison to less complex anthocyanin-sugar moieties.

In addition to the anthocyanin profile of berry fruits, it is important to consider their phenolic acid content, particularly those associated with anthocyanins. The significance of the anthocyanin:phenolics ratio (**Table 1.3**) becomes

evident when discerning the bioavailability of berry-derived anthocyanins, where distinguishing anthocyanin derived phenolic acids from indigenous berry phenolic constituents may become problematic, as misidentification is possible during clinical sample analysis. Furthermore, changes in berry anthocyanin:phenolics ratio during processing may serve as a means of identifying anthocyanin degradation. Therefore, it may be shown that the true beneficial properties of berry fruits produces are not solely related to their anthocyanin content, but also to their phenolic acid content. Further study is required to assess the stability and occurrence of anthocyanins throughout commercial fruit (berry) processing (which is a focus of the present thesis; Chapter 2).

Table 1.3. Total anthocyanin to total phenolic acid ratios

Berry species	Total anthocyanin:phenolic ratio
Gooseberry[1,3,5]	1:0.35
Chokeberry[1,3,5]	1:1.56
Elderberry[3,5]	1:3.76
Raspberry[1,4]	1:0.22
Black current[2,3,5]	1:1.05
Strawberry[1]	1:0.01
Cranberry[1,3]	1:0.72
Black berry[4]	1:0.03

[1]M. P. Kahkonen, A. I. Hopia, M. Heinonen, *J. Agric. Food Chem.* **49**, 4076 (2001). [2]P. G. Kapasakalidis, R. A. Rastall, M. H. Gordon, *J. Agric. Food Chem.* **54**, 4016 (2006). [3]K. R. Maatta-Riihinen, A. Kamal-Eldin, P. H. Mattila, A. M. Gonzalez-Paramas, A. R. Torronen, *J. Agric. Food Chem.* **52**, 4477 (2004). [4]R. A. Moyer, K. E. Hummer, C. E. Finn, B. Frei, R. E. Wrolstad, *J. Agric. Food Chem.* **50**, 519 (2002). [5]X. Wu *et al*., *J Agric Food Chem* **52**, 4026 (Jun 16, 2004),

Whilst the anthocyanin and phenolic acid content of berry fruits is of priority when considering their potential health benefits, account for berry species most consumed in the population should also be made, as these species offer the greatest potential for the delivery of anthocyanins within the human diet. A survey published by the U.S. Department of Agriculture (USDA) (USDA 2006) showed that berries represent 67% of all the frozen fruit products consumed in the U.S. in 2005, with strawberries representing a major class of these berry products. However, while raspberry, cranberry and strawberry have been shown to have an estimated daily consumption of 0.2, 0.12 and 1.95 g, respectively (Wu, Beecher et al. 2006), little published data exists regarding

direct berry consumption statistics. The U.S. Department of Agriculture Economic Research Services (USDAERS) *per capita* food availability data serves as an indirect measure of trends in food use, providing an indication of population food consumption over time. A summary for the *per capita* cultivation of commonly consumed berry products, compiled by the USDAERS is shown in **Table 1.4** (USDAERS 2007). Based on this data, it is evident that strawberries are the most highly cultivated berry species in the U.S. This trend may also be true for Europe, where marketing information compiled by TNS Worldpanel (UK) state that strawberries and raspberries represent 63% and 19% of the European berry market, respectively (Garner 2008).

Table 1.4. *Per capita* berry cultivation in the U.S. in 2005[1]

Berry species	Cultivation (lbs FW *per capita*)
Cranberry	2.00
Raspberry	0.30
Strawberry	7.70
Blackberry	0.10
Blueberry	0.80
Cranberry juice	0.21*

Data stated as lbs fresh weight per capita. *Measured in gallons per capita. [1]Data taken from USDAERS (2007) [www.ers.usda.gov/Data/FoodConsumption/FoodAvailFaqs.htm.].

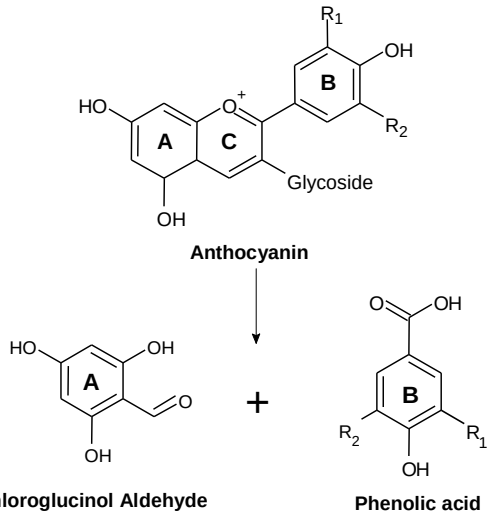
1.3 Anthocyanin stability under physiological conditions

On entry into the body, anthocyanins experience many physiochemical conditions that may lead to their structural modification. Regions of the GIT are characterized by their differential pH, microbial and cellular characteristics, each posing different molecular altering pressures. It is currently unknown whether the majority of anthocyanins remain intact during gastrointestinal tract (GIT) digestion or whether any anthocyanins pass intact to the colon, where further biotransformation by colonic microflora may occur. Many bioavailability studies, performed *in vivo* following the consumption of anthocyanin-rich food sources, have consistently shown that less than 1% of their ingested dose reaches the systemic circulation (Manach, Williamson et al. 2005). In addition, no conclusive evidence has yet accounted for their disappearance following

ingestion. Currently, the chemical and biological degradation and metabolism of anthocyanins needs to be fully characterised in order to sufficiently interpret the findings of *in vivo* bioavailability.

1.3.1 Anthocyanin degradation to phenolic acids

A number of chemical factors affect the structural nature of anthocyanins, including pH and temperature. As anthocyanins exist in at least four pH-dependent structural iso-forms, anthocyanins exhibit differential chemical properties. More importantly, at alkaline pH's, anthocyanins degrade to form free phenolic acids, where dp, cy, pg, pn and mv degrade to form gallic acid (GA), protocatechuic acid (PA), 4-hydroxybenzoic acid (HBA), vanillic acid (VA) and syringic acid (SA) respectively (**Figure 1.4**) (Seeram, Bourquin et al. 2001; Keppler and Humpf 2005; Fleschhut, Kratzer et al. 2006; Sadilova, Stintzing et al. 2006; Kern, Fridrich et al. 2007).



Anthocyanin	Phenolic Acid	R_1	R_2
Cyanidin-3-gly	Protocatechuic acid	OH	H
Pelargonidin-3-gly	4-hydroxybenzoic acid	H	H
Delphinidin-3-gly	Gallic Acid	OH	OH
Peonidin-3-gly	Vanillic Acid	H	OCH_3
Malvidin-3-gly	Syringic Acid	OCH_3	OCH_3

Figure 1.4: Degradation of anthocyanins to their corresponding phenolic acid and aldehyde constituents.

In addition, it has been shown that anthocyanins degrade to an additional phenolic aldehyde, with the most commonly reported constituent being 2,4,6-

trihydroxybenzaldehyde (PGA) (Keppler and Humpf 2005; Sadilova, Stintzing et al. 2006; Kern, Fridrich et al. 2007).

Due to the steric demand of the glycoside structures, a distinction between anthocyanin and anthocyanidin stability is evident, where anthocyanins (glycososides) show a greater pH stability than their respective aglycons, (Fleschhut, Kratzer et al. 2006). However, little has been reported regarding the influence of B-ring hydroxylation on the stability of anthocyanins under experimental or biological conditions. A study by Nielsen *et al.* (Nielsen, Haren et al. 2003) investigated the stability of pure anthocyanins at different pH values in aqueous solutions and found that cy and dp glycosides appeared to be most stable at pH 2, with a 10% loss at a pH value up to 3.3. A local minima in stability occurred at pH 3.8, where it was proposed that a less stable intermediate (pseudo-base) exists between the flavylium and hemiketal structural forms. The stability of these anthocyanins increased between pH 3.8-4.5, followed by an increased degradation at pH values greater than 4.5 (Nielsen, Haren et al. 2003).

A study by Keppler *et al.* (Keppler and Humpf 2005) suggested that ring fission and the formation of phenolic acids may take place at neutral pH and proposed that this was a minor degradation pathway, where anthocyanins form chalcone and coumarin glycosides as final degradation products (Markakis 1974). However, no studies have yet demonstrated the rate of anthocyanin degradation and phenolic acid formation under physiological conditions (which is a focus of the present thesis; Chapter 3). Nonetheless, some attempts have been made to determine the stability of anthocyanins under particular experimental conditions. Two studies by Fleschut *et al.* (Fleschhut, Kratzer et al. 2006) and Kern *et al*. (Kern, Fridrich et al. 2007) showed that at pH 7.4 and 37 ^{o}C, anthocyanidins cy, dp, pn and mv completely degrade within 60 minutes, with dp characterized as the least stable anthocyanidin, followed by mv, cy, pn and pg. This would suggest that the chemical breakdown of anthocyanidins increases with the number of methoxy and hydroxyl groups on the polyphenolic structure. It remains apparent that anthocyanins and anthocyanidins are greatly affected by pH

conditions in aqueous solutions and are unstable at physiological pH. Under cell culture conditions, it is reported by Keppler *et al.* (Keppler and Humpf 2005) that the phenolic acid degradation products of anthocyanins appear to be stable, although PGA was shown to undergo extensive degradation. This was thought to occur due to condensation with amine groups on amino acids or proteins to form imines, with the nucleophilic addition of an amine to the aldehyde followed by the elimination of water.

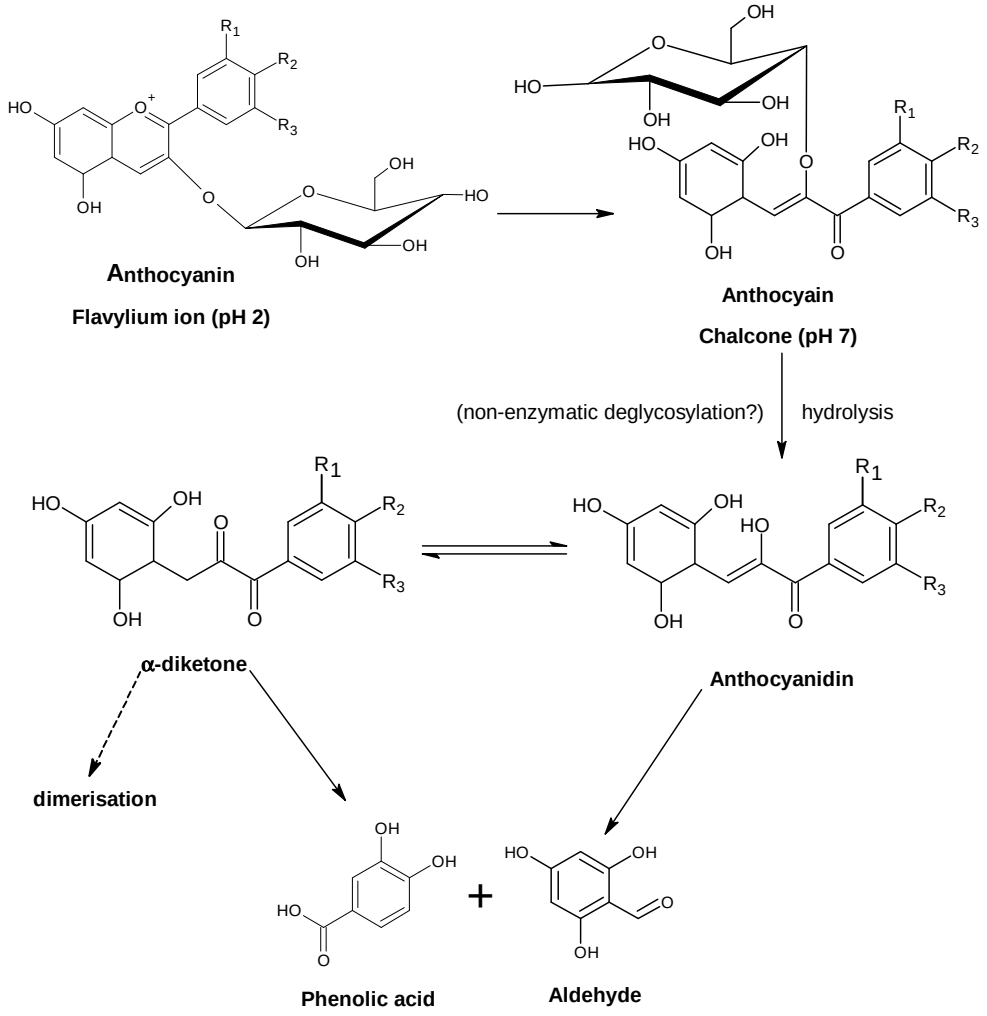


Figure 1.5: Proposed pathway for anthocyanin degradation at pH 7.

Most anthocyanin degradation studies show that the formation of phenolic acid degradation products do not fully account for the loss of their parent anthocyanidins (Keppler and Humpf 2005; Fleschhut, Kratzer et al. 2006; Kern, Fridrich et al. 2007). Therefore, it is postulated that anthocyanidins form

a more stable, intermediary such as a polymerized chalcone or α-diketone, which may undergo dimerisation or degradation to constituent phenolic acids (**Figure 1.5**). However, this should be the focus of future research, as the characterising of anthocyanin degradation intermediates may provide insights into the anomalous data reported during degradation and bioavailability studies.

A number of studies have reported the thermal degradation of anthocyanin pigments at increased temperatures. Still, only a small number of studies have reported the degradation of anthocyanins and the formation of constituent phenolic acids over time under thermal conditions. Studies by Sadilova *et al*. (Sadilova, Stintzing et al. 2006; Sadilova, Florian et al. 2007) investigated the thermal degradation of acylated and non-acylated anthocyanins of strawberry, elderberry and blackcarrot at 95 ^{o}C over 6-7 hours. At pH 1 it was shown that the half life of pg-3-glu, cy-3-glu and cy-3,5-diglu was 2.12, 1.95 and 0.98 hours respectively, with an 81%, 93% and 68% loss of anthocyanins over 7 hours. At pH 3.5, anthocyanins were shown to be completely lost after 6 hours at 95 ^{o}C. In both studies, the degradation of anthocyanins correlated with the formation of HBA, PCA and PGA. From these studies it has been proposed that the first step of thermal degradation is not anthocyanin de-glycosylation, but opening of the pyrylium ring and chalcone glycoside formation, followed by cleavage of anthocyanidin in chalcone form, which may then rapidly degrade to phenolic acids and aldehydes, respectively (Sadilova, Florian et al. 2007). Furthermore, a study by Seeram *et al.* (Seeram, Bourquin et al. 2001) demonstrated that anthocyanidins are degraded in pH 7 and 10 solutions at ambient temperature within 1 hour.

1.3.2 Degradation during GIT digestion

To understand anthocyanin bioavailability, information regarding their absorption, metabolism and excretion is paramount. Although complex animal and human studies have been utilized to determine the digestion, metabolism and excretion of anthocyanins, many studies have made use of *in vitro* procedures that mimic human physiochemical conditions. These include *in vitro* digestions, microsomal incubations and cell culture studies. *In vitro*

digestions generally consist of peptic and pancreatic-bile salt digestions, followed by dialysis diffusion, using cellulose dialysis tubing to simulate the mucus membrane of the lower gastrointestinal tract (GIT) and represent serum (diffused) and colonic (non-diffused) compartments (Perez-Vicente, Gil-Izquierdo et al. 2002; McDougall, Dobson et al. 2005; McDougall, Fyffe et al. 2005). Using these *in vitro* models, studies by McDougall *et al.* (McDougall, Dobson et al. 2005; McDougall, Fyffe et al. 2005) showed that the recovery of pure phenolic acids following peptic digestion is near 100% (McDougall, Dobson et al. 2005), while the recovery of anthocyanins was between 70-90%. This indicates that anthocyanins remain fairly stable during peptic digestion in the stomach (pH ~2) and are passed intact to the upper intestinal tract. Poor anthocyanin recovery is reported following pancreatic and bile salt digestion (pH 6-7), with only a ~38% recovery (Perez-Vicente, Gil-Izquierdo et al. 2002; McDougall, Dobson et al. 2005; McDougall, Fyffe et al. 2005). To account for this poor recovery, further characterisation of anthocyanin degradation during simulated digestion is necessary (and is a focus of the present thesis; Chapter 4), since the loss of anthocyanins has not yet been fully accounted for (**Figure 1.6**).

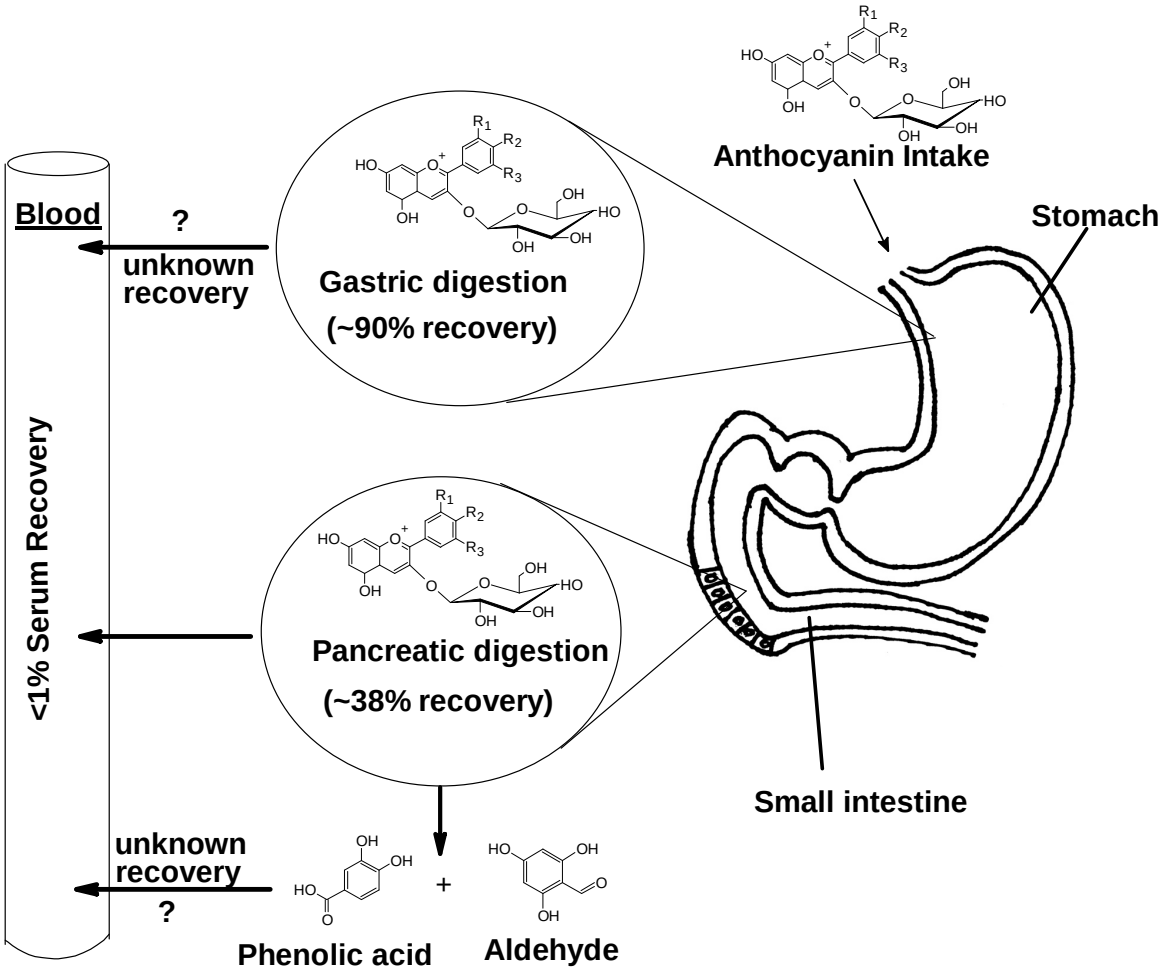


Figure 1.6: Current knowledge of anthocyanin absorption and degradation in the upper GIT.

The absence of aglycone structures and the low recovery of anthocyanins following peptic and pancreatic-bile salt digestions would suggest that anthocyanins are de-glycosylated and degraded during digestion (Seeram, Bourquin et al. 2001; Keppler and Humpf 2005; Fleschhut, Kratzer et al. 2006; Sadilova, Stintzing et al. 2006; Kern, Fridrich et al. 2007). However, a study by Perez-Vicente *et al.* (Perez-Vicente, Gil-Izquierdo et al. 2002) demonstrated that glycoside hydrolysis does not occur during gastric digestion, reporting complete anthocyanin recovery. Thus, the enzymatic and non-enzymatic hydrolysis of anthocyanins under intestinal digestion should be a focus for further research (and is a focus of the present thesis; Chapter 4).

1.4 Anthocyanin metabolism and bioavailability

1.4.1 Absorption

The elucidation of xenobiotic metabolism requires information about their absorption, distribution, metabolism and excretion (ADME). Gastrointestinal metabolism and absorption of many compounds, including flavonoids, is site specific and under the control of numerous physiological factors. Fluid pH and compound ionization (pK_a) alters passive diffusion across physiological barriers, including those of the gastrointestinal tract and mucosal surface area. Specialized transport systems, the permeability of macromolecules and glycosylation also play an important role in site specific absorption of xenobiotics. Aglycones, such as anthocyanidins, are predominantly more hydrophobic and may be absorbed via passive diffusion, while glycosides, such as anthocyanins, are more hydrophilic and either requires an active transport system or hydrolysis before absorption, suggesting the absorption primarily takes place in the intestine. Nevertheless, the exact mechanism of anthocyanin absorption is yet to be established, including whether they may enter the enterocyte as intact molecules or are cleaved and absorbed as aglycones or metabolites.

Evidence from *in vitro* studies suggest that anthocyanins are able to cross cell membranes and are detectable within the cytosol, where the glycosides have a higher transport efficiency than the aglycones (Sichel, Corsaro et al. 1991; Youdim, Martin et al. 2000; Yi, Akoh et al. 2006). Initially it was believed that the mechanism of anthocyanin absorption was similar to that of other flavonoids, where transport is facilitated by either the activity of sodium glucose transporter co-transporter (SGLT) or extracellular hydrolysis via lactate phlorizin hydrolase (LPH) followed by passive diffusion. However, studies by Hollman *et al.* and Arts *et al.* (Hollman, Bijsman et al. 1999; Arts, Sesink et al. 2004) demonstrated that inhibition of both SGLT and LHP did not significantly reduce the absorption of cy-3-glu by jejunal tissue. Therefore, some other mechanism may be responsible for anthocyanin absorption.

Recently a plant homologue to the mammalian bilirubin transporter, bilitranslocase has been identified (Passamonti, Vrhovsek et al. 2002; Sabina, Alessandra et al. 2005). Bilitranslocase operates as a uniporter, shown to catalyze the electrogenic transport of bromosulfophthalein (Sabina, Alessandra et al. 2005), a structural mimic of flavonoids. Indeed, the current body of evidence (Passamonti, Vanzo et al. ; Passamonti, Vrhovsek et al. 2002; Sabina, Alessandra et al. 2005; Karawajczyk, Drgan et al. 2007) suggests that bilitranslocase is a flavonoid membrane transporter with phylogenetic origins. Considering the physiological implications, anthocyanin interactions with bilitranslocase suggests that it could promote the transport of anthocyanins from the lumen into the epithelial layers of the gastric mucosa. This potential aspect of anthocyanin absorption should be the focus of future research.

1.4.2 Microfloral metabolism

Colonic microflora are thought to play a major role in flavonoid metabolism, where heterocyclic ring fission is wholly mediated by microorganisims in the colon, although some studies have shown that methoxy groups on the B-ring structure infer resistance to bacterial ring fission (Hollman and Katan 1998). Ring fission of flavonoids generates phenolic acids which may subsequently be absorbed and excreted or further degraded. *In vitro* studies have shown that phenolic acids are susceptible to microbial metabolism, including dehydroxylation, demethylation, reduction and decarboxylation. Although it is clear that many flavonoids undergo microfloral metabolism, few studies report the bacterial transformations of anthocyanins. Studies investigating the colonic metabolism of anthocyanins show that the anthocyanins cy-3-glu, mv-3-glu, cy-3,5-diglu, mv-3,5-diglu, cy-3-rut and pn-3-glu are hydrolysed within 20 minutes to 2 hours of incubation with colonic fermentations and that mono-glycosides are hydrolysed faster than di-glycosides (Aura, Martin-Lopez et al. 2005; Keppler and Humpf 2005; Fleschhut, Kratzer et al. 2006). Interestingly, cy-3-rut was metabolised at a greatly decreased rate, with significant amounts still detectable after 2 hours, in a study by Keppler and Humpf (Keppler and Humpf 2005). However, the breakdown of anthocyanins and their degradation products (phenolics) by enteric microflora is very similar to that observed in

sterile incubations (Keppler and Humpf 2005), suggesting that spontaneous pH dependent degradation was responsible. It was shown that PGA was degraded to phloroglucinolate only in the presence of enteric microflora, suggesting that this biotransformation does not occur spontaneously. The microfloral biotransformation of syringic acid and vanillic acid was accompanied by the formation of demethylated gallic acid and protocatechuic acid in Keppler's study. However, these finding are contradicted in a study by Fleschut *et al.* (Fleschhut, Kratzer et al. 2006), showing that phenolic acid transformation was not observed, although decarboxylation and dehydroxylation of protocatechuic acid was identified. Nonetheless, these studies have been conducted in systems which do not account for intestinal metabolism or absorption, and consisted of *in vitro* fermentation experiments.

1.4.3 Phase I and II metabolism

Absorption of most compounds from the GIT is usually associated with intestinal and/or hepatic metabolism, where they undergo biotransformation, such as hydroxylation, methylation, glucuronidation or conjugation by phase I and phase II enzymes in the liver. In many early studies, only un-metabolised anthocyanins were identified in the systemic circulation, possibly mistaking glucuronides for glycosides due to their similar polarities and UV-vis characteristics (Lapidot, Harel et al. 1998; Cao and Prior 1999; Matsumoto, Inaba et al. 2001). More recent studies using mass spectrometry techniques have identified glucuronides and sulphates of anthocyanins in plasma and urine samples, suggesting that anthocyanins, like other flavonoids, are extensively conjugated *in vivo*.

Glucuronidation is the foremost conjugating pathway in mammals, during which, hydroxyl, carboxyl or amino groups are conjugated with glucuronic acid via UDP-glucuronyl transferase (UGT). Glucuronidation is also the principal conjugation pathway of many flavonoids (O-glucuronidation) in a wide range of tissues, including liver, lung, kidney, skin, brain and intestine. Markedly, glucuronides do not appear to be readily available for biliary excretion (Dutton 1980), suggesting that intestinal glucuronidation may facilitate the rapid transfer of anthocyanins into the systemic circulation.

Methylation, another common metabolic conjugation pathway for flavonoids, occurs via the actions of methyltransferases, such as catecholmethyltransferase (COMT), found primarily in the liver and intestine. Studies have demonstrated that low doses of flavonoids are extensively methylated in humans and animals (Kuhnle, Spencer et al. 2000; Williamson, Day et al. 2000). Flavonoids may also undergo sulphation and amino acid conjugation (Williamson, Day et al. 2000). Sulphation is catalysed by the sulphotransferases, which are cytosolic enzymes found throughout the body. It should be noted that sulphation is an ATP and sulphate dependant reaction, and thus is highly saturatable, which yields low levels of conjugates.

1.4.4 Pharmacokinetics

Following GIT digestion and metabolism, anthocyanins and their metabolites may be released into the systemic circulation and eventually excreted. There have been many studies over the past decade investigating the serum recovery of anthocyanins, which all demonstrate that ingested anthocyanins are absorbed and excreted intact as glycosylated or glucuronidated compounds (Miyazawa, Nakagawa et al. 1999; Bub, Watzl et al. 2001; Cao, Muccitelli et al. 2001; Matsumoto, Inaba et al. 2001; Netzel, Strass et al. 2001; Mazza, Kay et al. 2002; Mulleder, Murkovic et al. 2002; Rechner, Kuhnle et al. 2002; Wu, Cao et al. 2002; Felgines, Talavera et al. 2003; Nielsen, Dragsted et al. 2003; Bitsch, Netzel et al. 2004; Kay, Mazza et al. 2004; Kay, Mazza et al. 2005; Manach, Williamson et al. 2005; Tian, Giusti et al. 2006; Charron, Clevidence et al. 2007; Vitaglione, Donnarumma et al. 2007). *In vivo* anthocyanin bioavailability studies usually consist of volunteers consuming single doses of anthocyanins, ranging from 150mg to 2g, in the form of berries, berry extracts or concentrates. Anthocyanin absorption has been shown to be very low, with the total anthocyanins (parent and metabolised compounds) plasma recovery in the order of 1-200nmol/l (<0.1% of ingested dose), reaching the systemic circulation within 1.5 hours (usually between 0.7-4 hours) of ingestion. Likewise, urine concentrations have been shown to reach similar levels within 2.5 hours, demonstrating rapid absorption and elimination (Miyazawa, Nakagawa et al. 1999; Bub, Watzl et al. 2001; Cao, Muccitelli et al. 2001; Matsumoto, Inaba et al. 2001; Netzel, Strass et al. 2001;

Mazza, Kay et al. 2002; Mulleder, Murkovic et al. 2002; Rechner, Kuhnle et al. 2002; Wu, Cao et al. 2002; Felgines, Talavera et al. 2003; Nielsen, Dragsted et al. 2003; Bitsch, Netzel et al. 2004; Kay, Mazza et al. 2004; Kay, Mazza et al. 2005; Manach, Williamson et al. 2005; Tian, Giusti et al. 2006; Charron, Clevidence et al. 2007; Vitaglione, Donnarumma et al. 2007). In addition to the parent compounds, anthocyanins are found in the circulation and excreted as glucuronide, methyl and sulphate conjugates. Studies by Kay *et al.* (Kay, Mazza et al. 2004; Kay, Mazza et al. 2005) demonstrated that following the ingestion of anthocyanins, the parent compounds represents only 32% of the total anthocyanins detected in serum, with 68% identified as conjugated metabolites. Similar to the serum availability, only 32% of the anthocyanins excreted in the urine were as parent structures where 67% were shown to be conjugated metabolites. Of these metabolites, glucuronidation was the major metabolic pathway observed for anthocyanins (representing 59% and 57% of the total anthocyanins detected in the blood and urine, respectively), followed by methylation (representing 43% and 51% of the total anthocyanins detected in the serum and urine, respectively).

Whilst these studies show that less than 1% of the ingested anthocyanin dose is detectable in urine or plasma, no conclusive explanation for their disappearance has been agreed. It remains to be shown whether this disappearance is due to degradation of anthocyanins to constituent phenolic acids and aldehydes and subsequent conjugation, or due to poor absorption. As the appearance of anthocyanins in serum is rapid (Hollman and Katan 1998), it is unlikely that the disappearance of anthocyanins is due to poor absorption. Rather, it is likely that anthocyanins form intermediate or end stage degradation products during digestion, absorption and metabolism that have yet to be identified *in vitro* (this is a focus of the present thesis; Chapter 4) or *in vivo*. Markedly, PCA and HBA have been identified in the plasma of rats following high doses of cy and pg glucosides, although very little research has attempted to identify these phenolic acids in the plasma of humans. Currently, a study by Vitaglione (Vitaglione, Donnarumma et al. 2007) was the first to partially account for the low systemic availability of anthocyanins in humans, where almost 73% of ingested cy was accounted for by the formation

of PCA. This suggested that ingested anthocyanins are primarily availably to the systemic circulation as phenolic acid derivatives. Further research is necessary to establish the extent to which anthocyanins are degraded and subsequently metabolised in humans, in order to account for their loss during *in vivo* studies (which is a focus of the present thesis; Chapter 4).

1.5 Anthocyanins in the prevention of cardiovascular disease

It has long been thought that diets rich in anthocyanins and flavonoids may confer a protective effect against cardiovascular disease and mortality. Indeed, this hypothesis has been driven by findings from numerous epidemiological and experimental studies. Whilst the epidemiological evidence for the protective effects of flavonoid rich diets on cardiovascular protection is mixed, the majority of studies are in support of their positive effects (Hertog, Feskens et al. 1993; Hertog, Kromhout et al. 1995; Knekt, Jarvinen et al. 1996; Rimm, Katan et al. 1996; Hertog, Sweetnam et al. 1997; Hertog, Feskens et al. 1997; Yochum, Kushi et al. 1999; Arts, Jacobs et al. 2001; Geleijnse, Launer et al. 2002; Knekt, Kumpulainen et al. 2002; Sesso, Gaziano et al. 2003; Lin, Rexrode et al. 2007; Mink, Scrafford et al. 2007). Furthermore, a study by Mink *et al*. (Mink, Scrafford et al. 2007), a large prospective study of postmenopausal women over a 16 year period, showed that dietary intakes of anthocyanins were associated with a lower risk of mortality and cardiovascular disease. At the experimental levels, anthocyanins have been shown in numerous animal (and some human) studies to prevent or reduce factors associated with cardiovascular and inflammatory disease (Oak, Bedoui et al. 2006; Xia, Ling et al. 2006; Yamamoto, Naemura et al. 2006; Sorrenti, Mazza et al. 2007; Xia, Ling et al. 2007; Leblais, Krisa et al. 2008; Toufektsian, de Lorgeril et al. 2008), carcinogenesis (Feng, Ni et al. 2007; Rossi, Garavello et al. 2007; Elisia and Kitts 2008) and obesity (Tsuda, Horio et al. 2003; Tsuda, Ueno et al. 2004; Jayaprakasam, Olson et al. 2006; Sasaki, Nishimura et al. 2007).

Initially, the beneficial effects of anthocyanins had been linked to their antioxidant potential and radical scavenging activities. However, current research suggests a mechanism of action that is not related to their direct radical scavenging properties, where the primary mechanism for their observed health benefits remains unclear. The following sections of this review provide an account for both the antioxidant and non-radical scavenging mechanisms by which anthocyanins are thought to modulate the development of oxidative diseases, with particular focus on cardiovascular disease and endothelial dysfunction.

1.5.1 Anthocyanins as antioxidants

Since the 1980s, it has been thought that the partial reduction of oxygen was a deleterious reaction leading to the non-specific modification of biological molecules and toxicity. This idea later became known as the “oxidative theory of disease” fuelled by the production of free radicals. Free radicals are defined as any chemical species possessing an un-paired electron, which in a biological context may be considered a “fragment of a molecule”. They are formed via three fundamental mechanisms, namely, through the homolytic cleavage of a covalent bond, loss of a single electron or gain of a single electron. The latter is referred to as electron transfer and is the most common in biological systems.

Radical formation by electron transfer: $\mathbf{A + e^- = A^{-\cdot}}$

Radical formation by homolytic fission: $\mathbf{X{:}Y = X^{\cdot} + Y^{\cdot}}$

Perhaps the most important free radicals in biology are those derived from oxygen, dubbed “reactive oxygen species” (ROS), where a one electron reduction of oxygen results in the formation of the superoxide free radical anions and a two-electron reduction results in the formation of hydrogen peroxide.

$$\mathbf{O_2 + e^- = O_2^{-\cdot}}$$

$$\mathbf{O_2 + 2e^- = H_2O_2}$$

Within a biological context, hydrogen peroxide is commonly produced via superoxide, where two superoxide molecules may react together to form hydrogen peroxide and oxygen.

$$2O_2^{\cdot-} + 2H^+ = H_2O_2 + O_2$$

Hydrogen peroxide is an important molecule in biology because it is easily broken down to produce the most reactive of the oxygen free radicals, the hydroxyl radical ($OH^{\cdot}$). Although the production of ROS from oxygen is reviewed in great depth elsewhere (Cheeseman and Slater 1993), a description of the key ROS in human vasculature (oxygen, superoxide, hydrogen peroxide and the hydroxyl radical) is warranted. *S*uperoxide may not, itself, be considered a particularly detrimental ROS. However, its main significance as a damaging ROS is as a source of hydrogen peroxide and as a reductant of transition metal ions. Superoxide is particularly significant in cardiovascular disease as a derivative reactant with nitric oxide. Once superoxide is converted to hydrogen peroxide, it may quickly react with transition metal ions to produce hydroxyl radicals. Hydroxyl radicals may then react with most biological molecules, at a diffusion limited rate, to cause many detrimental alterations to cellular mechanisms (Cheeseman and Slater 1993).

It had first been proposed that anthocyanins, as with other flavonoids, exert their biological effects through a ROS scavenging mechanism (Kuhnau 1976; Bors, Heller et al. 1990; Sichel, Corsaro et al. 1991). Although a balanced level of oxidation is vital for the homeostasis of living processes, it is clear that an excess of free radicals is detrimental to biological organisms, causing oxidative damage to DNA, lipids, proteins and many other organic components (Halliwell 1994). However, there has been considerable interest in the therapeutic uses of exogenous antioxidant supplementation, including dietary derived phytochemicals in the safeguarding of correct redox homeostasis. There exists some controversy in the field regarding the true benefit of many dietary derived antioxidants, considering the efficiency and abundance of endogenous antioxidants and the availability of molecules such as vitamin C.

It should be noted that, in order to balance free radical production in the cellular environment (redox), cells produce a number of endogenous antioxidants. The redox status of cells are primarily maintained by glutathione (GSH) and thiol redox balance. GSH is a tripeptide and major low molecular weight thiol in cells, which controls the cellular thiol-disulfide redox state (Jones 2002). A cellular redox balance is achieved through the maintenance of the thiol to di-sulphide status of reduced GSH and its oxidized form GSSG (dimerised GSH).

The role of ROS generation in human pathologies is well illustrated in atherosclerosis. Many of the pathologies associated with cardiovascular disease, including coronary artery disease, hypertension, heart failure and stroke, are attributed to oxidative mechanisms and the formation of atherosclerotic plaques and plaque rupture, dubbed 'the oxidative theory of atherosclerosis" (**Figure 1.7**) (Schissel, Tweedie-Hardman et al. 1996; Tabas 1999; Skalen, Gustafsson et al. 2002; Medeiros, Khan et al. 2004; Stocker and Keaney 2004; Williams and Fisher 2005). Briefly, it is believed that oxidation of LDL at the sub-endothelial level (Schissel, Tweedie-Hardman et al. 1996; Tabas 1999; Skalen, Gustafsson et al. 2002; Medeiros, Khan et al. 2004; Stocker and Keaney 2004; Williams and Fisher 2005) due to ROS generation by major cells of the arterial wall, including endothelial cells, smooth muscle cells, monocytes and macrophages, is primarily responsible for the exacerabation of CVD. However, there is no consensus on the predominant mechanism of LDL oxidation, although it is known that superoxide anions produced by vascular and immune cells mediate the progression of vascular disease via a mechanism that is attenuated by superoxide dismutase and metal chelators (Heinecke, Baker et al. 1986). Enzymes such as NAD(P)H oxidase, xanthine oxidase, lipoxygenase and un-coupled nitric oxide synthase are thought to be the major sources of superoxide generation in vascular tissue (discussed below).

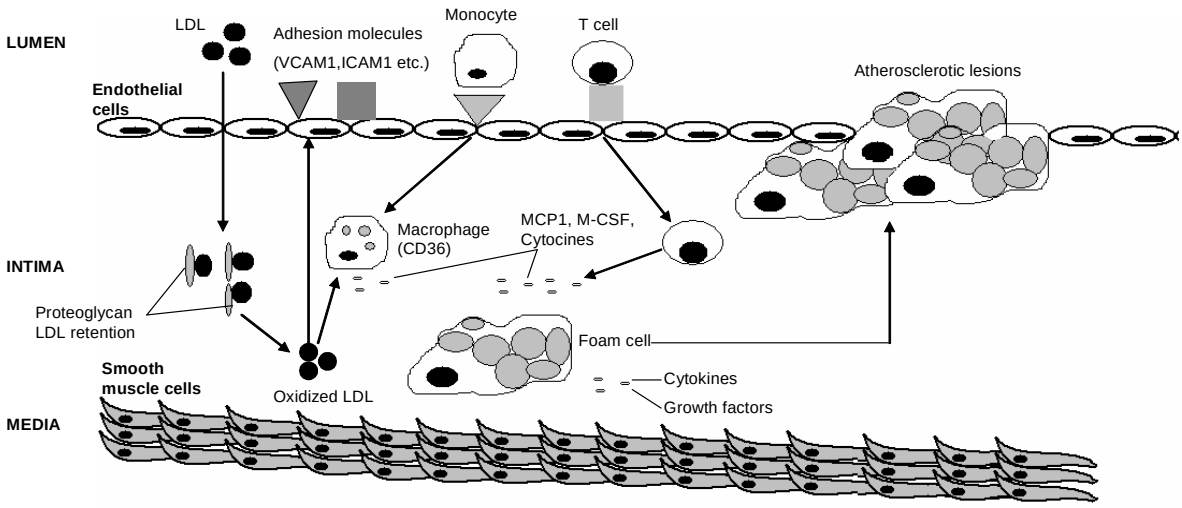


Figure 1.7: The oxidative theory of atherosclerosis[1].

Anthocyanins have consistently been shown to possess novel and potent antioxidant properties (Igarashi, Takanashi et al. 1989; Costanino, Albasini et al. 1992; Tamura and Yamagami 1994; Tsuda, Watanabe et al. 1994) where a number of studies have reported the antioxidant activity of berry extracts and pure anthocyanins by several methods, including the common total antioxidant activity (TAA), Trolox equivalent antioxidant activity (TEAC) (**Table 1.5**), oxygen radical absorbance capacity (ORAC) and DPPH radical scavenging capacity assays. In 1994, the antioxidant capacity of cyanidin-3-glucoside and cyanidin was investigated by Tsuda et al (Tsuda, Watanabe et al. 1994), using linoleic acid auto-oxidation, liposomes, erythrocyte membranes and liver microsomes. In all systems, cyanidin-3-glucoside and cyanidin showed antioxidant activity, where cyanidin possessed a greater antioxidant capacity than its glucoside. Subsequently, the antioxidant capacity of a number of other anthocyanin species were studied and it has been shown

[1] Adapted from Stocker, R. and Keaney, JF. (2004). *Physiol Rev* 84(4):1381-1478.

that the scavenging effect on hydroxyl and superoxide radicals is influenced by their structural characteristics. For example, delphinidin and cyanidin glycosides have relatively strong antioxidant activities compared with other anthocyanins. Indeed, the structure-antioxidant activity of many flavonoids, including anthocyanins, has been extensively studied and is reviewed in Rice-Evans *et al.* (Rice-Evans, Miller et al. 1996). In this review and in studies by Bors *et al.* (Bors, Heller et al. 1990) and Sichel *et al.* (Sichel, Corsaro et al. 1991), key structural characteristics have been identified as important antioxidant components in aqueous phases, using the TAA and TEAC assays. The three polyphenolic structural criteria for effective radical scavenging include, 1) a *O*-dihydroxy structure on the B-ring, conferring stability to the radical form and electron delocalization, 2) a 2,3 double bond in addition to a 4-oxo function on the C-ring, where this structure reacts with free radicals to produce stable phenoxyl radicals via electron delocalization from the B-ring and 3) where maximal radical scavenging potential is achieved when 3- and 5-OH groups on the A-ring are found in conjunction with a 4-oxo function on the C-ring. The effects of these structural characteristics on antioxidant capacity are clearly illustrated in studies comparing cyanindin, pelargonidin, delphinidin and malvidin derivatives (with antioxidant activity in the order of dp=cy>pg), in addition to other flavonoids, including quercetin (Q), morin (M), kaempferol (K) and myricetin (My) (**Figure 1.8 and Table 1.5**) (with antioxidant activity in the order Q>My>M>K).

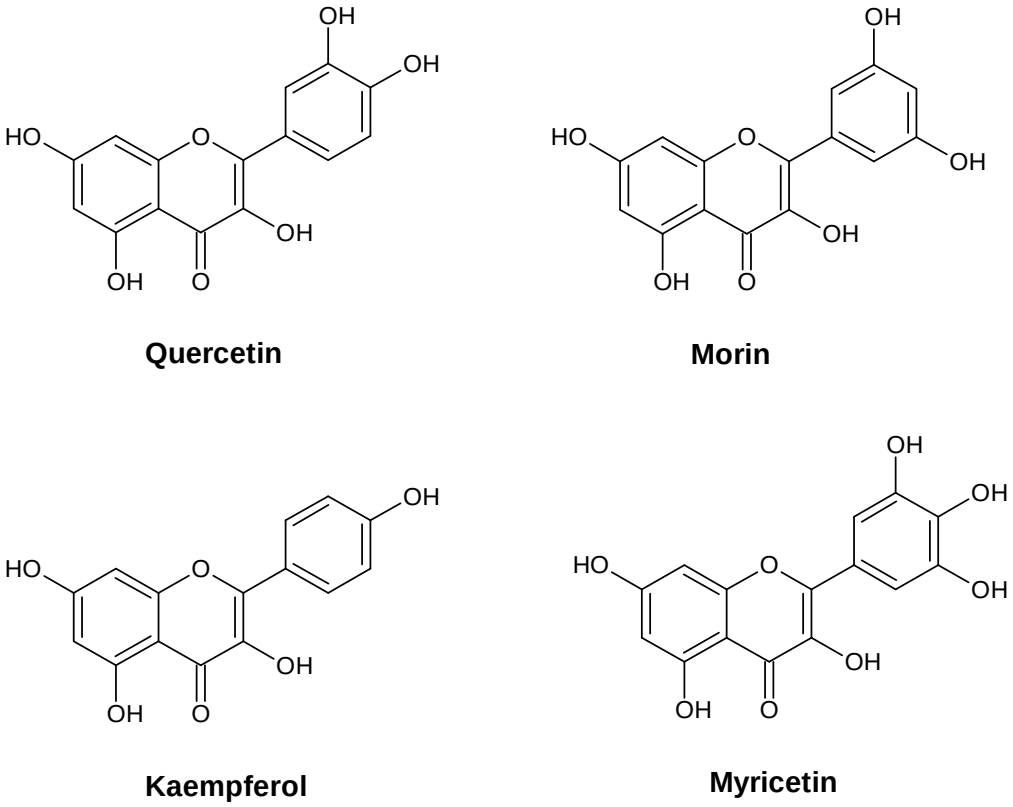


Figure 1.8: B-ring hydroxylation patterns of orthodiphenolic structures.

In a study by Youdim et al. (Youdim, Martin et al. 2000) it was shown that anthocyanins are incorporated into endothelial plasma membranes and cytosols, where these enriched endothelial cells confer significant protection against oxidative stress. In addition, several human trials (Netzel, Strass et al. 2001; Mazza, Kay et al. 2002) and epidemiological studies (Mink, Scrafford et al. 2007) have demonstrated the beneficial role of anthocyanins and other flavonoids in cardiovascular disease prevention. However, due to the low bioavailability of anthocyanins and the controversy regarding the failure of some antioxidant supplementation trials (Fruebis, Steinberg et al. 1994; Witting, Pettersson et al. 1999; Stocker and Keaney 2004), it remains to be shown whether anthocyanin associated cardiovascular protection is due to direct radical scavenging capacity or via some other mechanism (a focus of this thesis; chapter 5).

Table 1.5. Flavonoid structure and free radical scavenging activity*

Flavonoid	OH-Substitution	Antioxidant activity (TEAC**)
Pelargonidin	3, 5, 7, **4'**	1.3
Cyanidin	3, 5, 7, **3'**, **4'**	4.4
Delphinidin	3, 5, 7, **3'**, **4'**, **5'**	4.4
Kaempferol	3, 5, 7, **4'**	1.3
Quercetin	3, 5, 7, **3'**, **4'**	4.7
Myricetin	3, 5, 7, **3'**, **4'**, **5'**	3.1
Morin	3, 5, 7, **3'**, **5'**	2.5

*Data taken from Rice-Evans *et al.* (1996). *Free Rad. Biol. Med.* 20(7): 933-956. **Measured in mM. Bold text refers to B-ring hydroxylated carbons. TEAC, Trolox equivalent antioxidant activity.

1.5.2 Vascular dysfunction, reactive oxygen generation and anthocyanins.

Early researchers of cardiovascular disease observed that the rupture of the atherosclerotic plaque led to, or was preceded by, the recruitment of inflammatory cells (monocytes/macrophages), suggesting that cardiovascular disease and inflammation were inextricably linked and that atherosclerosis was not simply a degradative disease of the elderly. Subsequent work has sought to more precisely define the pathways involved in the development of cardiovascular disease and vascular inflammation. To date, an overwhelming body of evidence now clearly indicates that cardiovascular disease and more particularly, atherosclerosis, is a multi-factorial disease that may commence from childhood and clinically manifest later in life. These inflammatory diseases therefore represent viable targets for therapeutic intervention and are particularly amenable to low cost, long term and sustainable dietary interventions, through the actions of biologically active phytochemicals. Therefore it remains crucial that the cardiovascular effects of dietary phytochemicals, including anthocyanins, are established.

Although the development of atherosclerosis is a multifactorial process, there is accumulating evidence that suggests the vascular production of reactive

oxygen species plays an important role in disease development, particularly since vascular superoxide production has been demonstrated to be associated with atherosclerotic risk factors. Although vascular superoxide may affect the vasculture in many different ways, perhaps their most significant effect occurs through their rapid reactivity with nitric oxide. Indeed, the bioactivity of eNOS is dependent on vascular levels of superoxide and other ROS. In particular, the rapid reaction of superoxide with NO results in the formation of peroxynitrite anions and loss of NO function. Furthermore, increased vascular superoxide promotes the oxidative degradation of tetrahydrobiopterin (BH_4), leading to eNOS “un-coupling” and the increased production of superoxide. Nonetheless, there are many alternative sources of superoxide within the human vasculature, some of which have been implicated in endothelial associated pathologies. These superoxide generation systems include the mitochondria, xanthine/xanthine oxidase couple, uncoupled nitric oxide synthases, endothelial cytochrome P450 enzymes, lipoxygenases and NADPH oxidases (Droge 2002).

Numerous studies have recently suggested that anthocyanins could modulate oxidative diseases through mechanisms auxiliary to radical scavenging. The redox status of cells is an important factor in cellular homeostasis. This is illustrated by the induction of cells from quiescent states to proliferative or apoptotic states by sub-toxic levels of oxidative stress and mild shifts in cellular redox state. Furthermore, oxidants have been shown to trigger phosphorylation cascades that lead to the activation of mitogen activated protein kinases (MAPKs) and nuclear factor kB (NF-kB). Existing evidence suggests a possible link between cellular thiol redox status and NF-kB mediated gene expression, where altered GSH to GSSG ratio have been shown to activate NF-kB (Kokura, Wolf et al. 1999). Recent studies by Xia et al (Xia, Ling et al. 2003; Xia, Hou et al. 2005; Xia, Ling et al. 2006; Xia, Ling et al. 2007), have reported novel mechanisms by which plant pigments, including anthocyanins, may attenuate atherosclerosis development through the modulation of various redox sensitive signalling pathways. It was shown that in the absence of anthocyanins, ligation of the CD40 receptor by CD40L triggers the recruitment of TRAF-2 to lipid rafts and binding to CD40. This then

led to the activation of NFκB, which in turn initiated the transcription of IL-6, IL-8 and MCP-1 (Frolov and Hui 2007). The significance of these pathways is demonstrated through the link between cholesterol metabolism and inflammatory response, where evidence suggests that the activation of CD40L and TLR are significantly involved in the cholesterol dependant development of atherosclerosis. Xia et al. (Xia, Ling et al. 2007) demonstrate that cy-3-glu and pn-3-glu renders the CD40-CD40L inflammatory pathway inactive through the inhibition of TRAF-2 recruitment and up regulates ABCA1-mediated cholesterol efflux to ApoA-1, reducing raft cholesterol content (**Figure 1.9**).

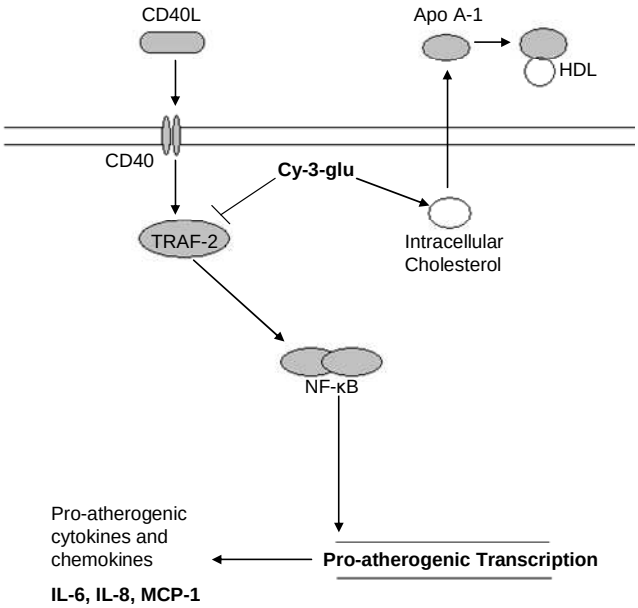


Figure 1.9: Anthocyanin regulation of CD40-CD40L inflammatory pathway[2].

Heme catabolism to carbon monoxide, Fe^{2+} and biliverdin (converted to bilirubin by biliverdin reductase) is catalyzed by heme-oxygenase-1 (HO-1). These reaction products have been shown to modulate cell signal transduction pathways relevant to inflammation (Stocker, Yamamoto et al. 1987), where Fe^{2+} induces ferritin expression and ATPase Fe^{2+} secreting pumps to decrease oxidation, increase vasodilatation and inhibit platelet aggregation. Similar to HO-1, It has been shown that flavonoids are able to inhibit the activity of NADPH oxidases (Tsuda, Ueno et al. 2006; Steffen,

[2] Adapted from Xia *et al.* (2007). *Arterioscler. Thromb. Vasc. Biol*. 27(3): 519-524.

Gruber et al. 2008) (discussed below), although no conclusive evidence has been presented regarding the effects of anthocyanins on NADPH oxidase activity or expression.

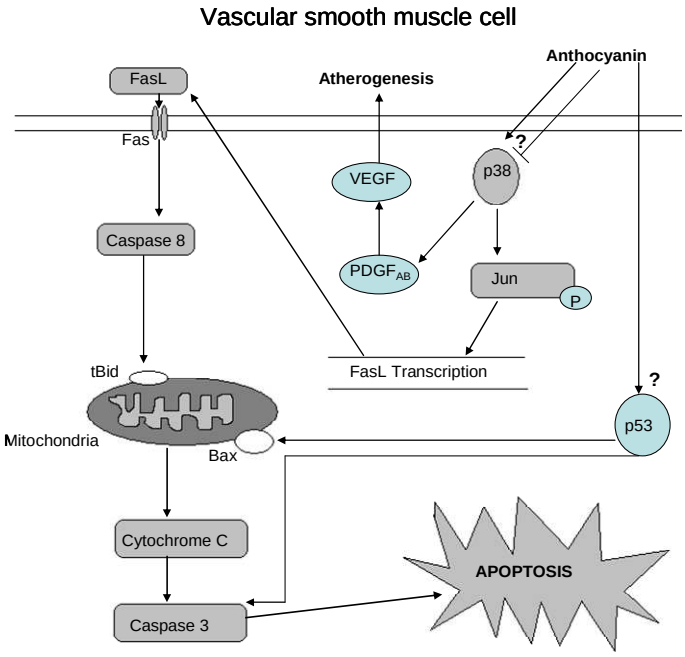


Figure 1.10: Anthocyanin regulation of FasL, P38 and P53 mediated atherogenesis in vascular smooth muscle cells[3].

Ultimately, the many signal transduction pathways already described, lead not only to the development of atherosclerotic plaques, but also to the abnormal proliferation of vascular smooth muscle cells which play an important role in the pathogenesis of atherosclerosis and restenosis (Rivard and Andres 2000). In a study by Lo et al. (Lo, Huang et al. 2007), it has been demonstrated that anthocyanin rich berry extracts may induce apoptosis in proliferating smooth muscle cells, where it was suggested that anthocyanins promote smooth muscle cell apoptosis through the FasL mediated pathway, by inducing the expression of FasL and tBid (components of the mitochondrial associated apoptotic pathway) on the activation of p38 MAP kinase and phosphorylation of target c-Jun proteins, cleaving caspase-3 and 8 and releasing cytochrome c from the mitochondria (**Figure 1.10**). However, a study by Oak et al. (Oak,

[3] Adapted from Oak *et al*. (2006). *Br. J. Pharmacol.* 149(3):283-290.

Bedoui et al. 2006), contradicts these findings, where it was shown that anthocyanins with a 3' hydroxyl are able to inhibit platelet derived growth factor (PDGF) induced vascular endothelial growth factor (VEGF) expression by preventing the activation of p38 MAPK and JNK in vascular smooth muscle cells. In addition, a study by Martin et al. (Martin, Favot et al. 2003) showed that Dp exerted anti-proliferative effects on basal and VEGF induced proliferation of bovine aortic endothelial cells through the transient activation of ERK 1 and 2.

Overall, it is clear that whilst anthocyanins do possess significant radical scavenging capacities, they may also act via additional, or even superior, mechanisms not directly associated with their antioxidant activity. Therefore, the observed effects of anthocyanins on inflammation and cardiovascular disease may be due to their potential duality, both inhibiting inflammation and preventing its induction via free radical damage. Whilst anthocyanins may exert many biological affects associated with cardiovascular disease, few studies have investigated the effect of anthocyanin metabolites on human health (investigated here in chapter 5), despite one study by Rechner and Kroner (Rechner and Kroner 2005) which showed that anthocyanins and their colonic metabolites effect various aspects of platelet function and aggregation. Therefore, the study of anthocyanin metabolism and its effects on human disease is of great importance to this research field.

1.5.3 Endothelial Nitric oxide (NO) and vascular function/dysfunction

While many risk factors affect endothelial phenotype, a consistently identified and defining determinant of endothelial dysfunction is insufficiency of nitric oxide (NO). NO functions as a profound vasodilator that is produced within the endothelium by type II (cytokine inducible) and type III (endothelial) nitric oxide synthase (eNOS/NOS3), reviewed extensively by Moncada *et al*. and Bredt *et al*. (Moncada, Palmer et al. 1991; Bredt and Snyder 1994) and described here in brief.

NOS enzymes catalyze the conversion of L-arginine to nitric oxide that subsequently perform vasodilatory functions. Pro-inflammatory cytokines also

increase the activity of GTP-cyclohydrolase, the rate limiting enzyme in tetra-hydrobiopterin production, which is a cofactor for NOS. Interestingly, NFκB, a redox sensitive transcription factor which regulates the expression of many pro-inflammatory cytokines, is stabilized by constitutively expressed NO through the maintenance of the NFκB subunit IκB, thereby attenuating pro-inflammatory responses. Over the past three decades, NO has been identified as a principle mediator of vaso-protection by controlling vasodilatation, suppressing inflammatory cytokine and adhesion molecule release and limiting vascular smooth muscle proliferation, intimal fibrosis and platelet aggregation. Thus, its synthesis in appropriate amounts sustains vascular health and its loss is characteristic in atherothrombogenesis.

The eNOS enzyme is constitutively expressed by the gene *NOS3* and post-transcriptional modification of target eNOS to the plasma membrane of vascular endothelial cells. The enzyme is rapidly activated by a number of antagonists, including acetylcholine, bradykinin, vascular endothelial growth factor (VEGF), shear stress and estrogens. A number of co-factors are also required for NOS generation of NO, including tetrahydrobiopterin (BH_4), NADPH and flavins. Once produced, NO diffuses into the vessel lumen and underlying smooth muscle tissue. Platelet activity and smooth muscle relaxation occurs as NO activates guanylyl cyclase and raises cyclic guanosine monophosphate (cGMP) levels. NO also causes smooth muscle relaxation through calcium dependent potassium channels.

Unfortunately, many potential sites for NO dysregulation exist. While many pathological situations may alter the amount, activity and cellular localization of eNOS or the availability of NO and its cofactors, this thesis was particularly focused on the mechanism of NO dysregulation through the excess production of ROS, particularly via the actions of endothelial NOX activity (described below).

Many lines of evidence support a critical role for increased oxidative stress in atherosclerosis associated endothelial dysfunction. Furthermore, it has now been well established that endothelial dependent vasodilatation is impaired by

superoxide production, which is reversible with specific superoxide scavengers. However, it remains important to consider that endothelial dysfunction is not always reversible with short-term antioxidant infusion. For example, a study by Widlandsky *et al*. (Widlansky, Biegelsen et al. 2004) showed no effect of high concentration ascorbic acid on endothelial dysfunction in atherosclerotic coronary arteries. This, and other findings, is consistent with the understanding that ROS have complex effects on endothelial cell function beyond the direct inactivation of NO and oxidation of LDL and explain the failure of simple antioxidant strategies to reduce cardiovascular disease risk in many studies. Therefore, it remains essential to evaluate the effect of many potential dietary therapeutics, such as flavonoids, for biological actions beyond their simple radical scavenging capacity. Many studies have thus focused on strategies that act to reduce oxidative stress in the vasculature by inhibiting cellular sources of ROS, such as the NADPH oxidases (NOX).

1.5.4 NADPH oxidases, vascular oxidation and flavonoids

NAD(P)H oxidases (NOX) were first thought to be a superoxide generating enzymes found only in phagocytic cells. However, in recent years it has been established that endothelial cells express a superoxide generating enzyme analogous to phagocytic NADPH oxidase. Specifically, endothelial and vascular smooth muscle cell NADPH oxidase expression has been implicated as a major contributor to CVD development and has been extensively reviewed by Bedard *et al*. (Bedard and Krause 2007), described here in brief.

Endothelial NADPH oxidases, like many other enzymes, are proteins that transfer electrons across biological membranes. However, in the case of NOX, the primary electron acceptor is oxygen which subsequently is reduced to form super oxide. It may therefore be said that NOX enzymes are potent generators of vascular superoxide. All NOX family members are trans-membrane proteins which usually possess an NADPH binding site, a FAD binding site, six conserved trans-membrane regions and heme-binding histidines. Currently, seven NOX family members have been identified (NOX 1, 2, 3, 4 and 5, DUOX 1 and 2) which each possesses slightly different

structures, tissue distributions and cellular localizations. NOX 2, otherwise known as gp91phox, was the first NOX enzyme identified in phagocytes and was later identified in endothelial cells. The activation of NOX2 is reliant on a number of complex protein/protein interactions with membrane and cytosolic subunits. Functionally, NOX2, as with many NOX enzymes, are unstable in the absence of the membrane subunit $p22^{phox}$. Furthermore, activation of NOX2 requires the translocation of cytosolic factors to the NOX2/$p22^{phox}$ complex (also known as flavocytrochrome b_{558}). Current evidence suggests that the phosphorylation of $p47^{phox}$ (the 'organizer' subunit) allows for the interaction with $p22^{phox}$ which promotes the recruitment of an 'activator' subunit, called $p67^{phox}$, to the NOX2 complex. This in turn allows for the recruitment of $p40^{phox}$. Finally, NOX interaction with GTPase RAC, via a two step mechanism, allows the transport of electrons from NADPH in the cytosol, across the NOX2 complex, to oxygen on the luminal or extracellular space. Of note, the NOX isoform, NOX4, found in kidney, endothelial and vascular smooth muscle cells, is the most abundantly expressed isoform in vascular endothelial cells and is the primary catalytic unit for vascular ROS generation.

At present, there is mounting evidence that dietary derived polyphenolic compound may modulate the production of vascular superoxide by NOX enzymes. Recent studies by Davalos *et al*. (Davalos, de la Pea et al. 2009) and Sarr *et al*. (Sarr, Chataigneau et al. 2006) have demonstrated that red grape polyphenols decrease phagocyte NOX expression and superoxide overproduction in humans and rats. However, their remains no published data regarding anthocyanins and vascular NOX expression or activity (a focus of this thesis; chapter 5). This should be a primary focus for future research, since the proposed anthocyanin metabolites (described in chapter 4) bare a striking resemblance to the well established NOX inhibitor, apocynin (review at length in Stefanska *et al*. (Stefanska and Pawliczak 2008)). Indeed, it has been demonstrated that apocynin undergoes dimerization under physiological conditions, which allows it to inhibit $p47^{phox}$ binding to $gp91^{phox}$ (NOX2) in a dose dependent manner. Further evidence for the potential role of dietary polyphenols and NOX enzyme modulation may be observed in quercetin and ursolic acid. To date, there have been numerous studies investigating the

effect of quercetin on NOX enzyme superoxide production and vascular health. Overall these studies indicate that quercetin and other similar compounds (e.g. 3,4-dihydroxyflavonol, chrysin and isorhamnetin), prevent vascular superoxide production by reducing overexpression of $p47^{phox}$ and subsequent loss of NO production though eNOS "uncoupling" (Sanchez, Galisteo et al. 2006; Sanchez, Lodi et al. 2007; Romero, Jiménez et al. 2009). Some suggest that this effect is related to protein kinase C (PKC) inhibition (which is necessary for $p47^{phox}$ phosphorylation and activation). In a study by Steinkamp-Fenske *et al*. (Steinkamp-Fenske, Bollinger et al. 2007) it was shown that ursolic acid from the Chinese herb Danshen down regulated NOX4 and increased eNOS promoter activity in human endothelial cells.

Since it is evident that phenolic acids may modulate NOX activity, flavonoids and their metabolites should be the focus of future research in the flavonoids field. To this end, little published data exists regarding the effects and flavonoid metabolites on NOX and/or eNOS activity. However, research conducted by Steffen *et al*. (Steffen, Schewe et al. 2007; Steffen, Gruber et al. 2008) suggest that the methylated metabolites of (-)-epichatechin mediates the activity of endothelial NOX superoxide production and downstream NO availability. Future work should thus include structure-activity relationship studies for flavonoids, their metabolites and NOX activity (investigated in chapter 5).

Overall, our understanding of vascular NADPH oxidases and its impact on vascular dysfunction and NO activity has increased substantially in recent years. However, much remains to be learned, not only about the function and stimuli of these enzyme systems, but also the elucidation of targeted therapeutic agents. The discovery of specific inhibitors of NOX-based oxidases, particularly deriving from the diet, could provide invaluable tools for the prevention and treatment of a broad range of oxidative diseases. Indeed it may be found that anthocyanins and their metabolites represent viable agents for the dietary prevention or treatment of cardiovascular and oxidative diseases. However, before this claim can be made, further knowledge of anthocyanin stability, metabolism and mechanisms of action is required.

1.6 Analytical methods for assessing anthocyanins

1.6.1 Analytical analysis of anthocyanins

As berry fruits represent an important dietary source of anthocyanins, they have been used in many studies of assessing anthocyanin metabolism and bioactivity. This usually requires the extraction of anthocyanins from raw fruit matrixes and subsequent purification. Initially, berry samples are homogenized (Gao and Mazza 1994) or frozen with subsequent grinding in liquid nitrogen (Labarbe, Cheynier et al. 1999). Following homogenization, centrifugation may be employed to recover juices, whilst solid phase extraction (SPE) (Dugo, Mondello et al. 2001) and/or liquid-liquid extractions (LLE) (Rodriguez-Saona, Giusti et al. 1998) may be used to isolate anthocyanins from sample homogenates or juices. However, it should be noted that due to the unstable nature of anthocyanin, these common extraction techniques are usually conducted under acidified conditions, in order to prevent the degradation of anthocyanins. To avoid chemical modification during extraction with solvents (commonly methanol, ethanol or acetone), mixtures should be acidified with organic acids (e.g. acetic of formic acid) (Goto 1987). In addition, high temperatures should be avoided during the extraction of anthocyanins as anthocyanins are known to be unstable in temperature exceeding 30 ℃ (Goto 1987). Various solvent-to-sample ratios have been used for the extraction of anthocyanin from plant material, where two or more re-extractions of solid residues may be required in order to achieve maximum anthocyanin recovery.

SPE has been extensively used for the purification of anthocyanins from crude extracts, allowing for the removal of large amount of undesirable products, such as sugars, acids, amino acids and proteins. Usually, pre-conditioned SPE cartridges (C18 cartridges conditioned with methanol) are washed with several column volumes of acidified water (commonly 0.01% HCl or 3% formic acid) (Garcia-Viguera, Zafrilla et al. 1998; Wang, Kalt et al. 2000; Kahkonen, Hopia et al. 2001), followed by elution with acidified solvents. Furthermore, improved sample purity and reduced interference with less polar

polyphenolics may be achieved by washing with ethyl-acetate (Kondo, Ohnishi et al. 1999). Besides SPE, paper, thin layer and column chromatography have been used for the purification of anthocyanins. Preparative liquid chromatography (prep-LC) is also a widely used technique for the isolation of anthocyanins from a variety of plant matrices.

No standard method for the isolation of anthocyanins from biological fluids have been established; although there are several important factors that should be considered. As anthocyanins are polar structures, they should be extracted in polar solvents, such as water, methanol or ethanol. Due to their poor stability, it is recommended that temperatures during extraction remain below 30 ℃ and that the pH be maintained at 1-2 (Mazza, Cacace et al. 2004). The most common extraction procedures include filtration, centrifugation and collection of the subsequent supernatants or reversed-phase SPE using C8 or C18 cartridges (Mazza, Cacace et al. 2004). Following these procedures, samples may be directly injected onto an LC column for quantification.

The most widely employed method for the quantification of anthocyanins is UV-Vis spectroscopy, as anthocyanins possess unique UV absorption spectra, with maximum absorbance at about 520 nm. Since the 1960s, a pH differential method has been employed for the rapid, non-specific, quantification of anthocyanins, and does not require the use of any elaborate analytical equipment. This method is normally used to quantify the total anthocyanin content of a fluid and takes advantage of the anthocyanins ability to shift in colour, moving from deep red/blue, at pH 1, to near colourless, at pH 4.5. The total monomeric anthocyanin content may be calculated based on their extinction coefficients and difference in optical densities at pH 1 and pH 4.5 (Markham 1982). However, more specific methods are required to identify and characterise the anthocyanin profiles of complex solutions.

Liquid chromatography (LC) is the most widely used technique for anthocyanin quantification today, with many different methods having been developed (Markham 1982; Doerge, Chang et al. 2000; Wang, Kalt et al.

2000; Chandra, Rana et al. 2001; Nyman and Kumpulainen 2001; Nielsen, Haren et al. 2003; Nakajima, Tanaka et al. 2004; Wu and Prior 2005; Aaby, Ekeberg et al. 2007; Cristina Alcalde-Eon 2007). Briefly, reversed-phase columns with binary aqueous acidified solvents (water, methanol or acetonitrile acidified with acetic, trifluoracetic or formic acid) are commonly used, with detection on a photo-diode array detector (DAD) recording at 280nm, 320nm and 520nm. Anthocyanin analytical standards are commonly used as references for identification and quantification. However, the limited availability of many anthocyanin standards is a significant factor hindering the characterisation of anthocyanin metabolism and bioavailability. In the absence of relevant analytical standards, anthocyanin quantification requires extraction, isolation, purification, and identification using LC-MS and NMR techniques. Recently, capillary zone electrophoresis (CZE) has been employed for the analysis of anthocyanins. This technique combines aspects of chromatography and electrophoresis, separating compounds based on their electrophoretic motility (Issaq 1999; Issaq, Chan et al. 1999; Ichiyanagi, Tateyama et al. 2000). However, this technique cannot offer the separation of complex mixtures that can be achieved with LC-DAD (da Costa, Horton et al. 2000).

Although LC is a reliable and relatively simple technique for the quantification of anthocyanins, the identification and quantification of anthocyanin and their metabolites in biological fluids is inherently difficult. The instability of anthocyanins at higher pH values makes the identification and quantification of metabolites via enzymatic methods particularly problematic. In addition, the liberation of aglycone products greatly increases the rate of degradation. For this reason, identification of anthocyanins is not always possible by enzymatic methods and requires further methods of identification.

Several methods are employed for the identification and characterisation of anthocyanins, exploiting various aspects of their physical and chemical properties. UV-vis spectroscopy is extensively used to identify anthocyanins based on their unique spectral characteristics, described at length in reviews by Harboune (Harborne 1958) and Markham (Markham 1982). Cleaving of glycosides by acid hydrolysis with subsequent identification and quantification

of resultant aglycone structures and individual sugars is a common means of identifying anthocyanins. In addition, enzyme hydrolysis, mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) have been extensively used for the identification of anthocyanins and their biological constituents and metabolites (Markham 1982; Doerge, Chang et al. 2000; Wang, Kalt et al. 2000; Chandra, Rana et al. 2001; Nyman and Kumpulainen 2001; Nielsen, Haren et al. 2003; Nakajima, Tanaka et al. 2004; Wu and Prior 2005; Aaby, Ekeberg et al. 2007; Cristina Alcalde-Eon 2007). However, whilst the qualitative and quantitative analysis of anthocyanins have successfully been achieved using the methods described above, little or no reported data exists regarding many aspects of anthocyanin analysis, including robust techniques for the identification of metabolites and their stability or recovery under analytical conditions, such as extraction, isolation or purification (a focus in this thesis; chapter 3).

1.7 Concluding remarks

It has here been illustrated that anthocyanins potentially possess significant biological activities that could prove beneficial to human health. However, it has been highlighted that their true health benefits are unclear; due to gaps in the current literature. The most important of these gaps include a full account for the very poor bioavailability of anthocyanins and the incorporation of this information into bioactivity studies (i.e. use of appropriate metabolites and physiological concentration in *in vitro* studies). Therefore, in order to fully appreciate the health related benefits of ingested anthocyanins, an understanding of their occurrence, biochemistry, metabolism and final bioactive mechanisms is essential, and was the focus of the current thesis. By enhancing our knowledge of the afore mentioned gaps in our knowledge, a fuller appreciation for the importance of investigating the health related benefits of flavonoid consumption and the significance of metabolism and structural characterisation in elucidating their mechanisms of action might be achieved.

Chapter 2

Anthocyanin occurrence in blackcurrants and their stability during juice processing

This study fulfilled the Collaborative Awards in Science and Engineering (CASE) portion of this BBSRC-CASE studentship

2.1 Introduction

Within the food industry anthocyanins play an important role in food quality, where they are extensively used as “natural” food colorants (Robert L, Rickey Y et al. 1987). In addition to this role, it has been shown in numerous studies (both epidemiologically and experimentally) that anthocyanins may be protective against chronic diseases, including cardiovascular disease and cancer (Galvano, La Fauci et al. 2004; Hooper, Kroon et al. 2008). Berry fruits generally contain high concentrations of anthocyanins; however, their consumption as fresh produce is restricted due to their short seasonal availability and relatively high cost. Therefore, only small proportions of berries are consumed fresh, with most of their intake coming from processed foods, such as frozen products, juices, jams, jellies etc (Caswell 2009). Understanding the potential health benefits of anthocyanins therefore requires the establishment of their degradation or alteration during commercial processing, which will ultimately impact the final polyphenolic profile of processed foods.

The processing of fresh berries to juice concentrates requires many processing stages, including milling, pressing, pasteurization, filtration, clarification and concentration. In addition, enzymatic depectinization is often employed to facilitate the pressing process, since endogenous sugars readily form pectin based gels at low pH levels (Buchert, Koponen et al. 2005; Wang, Xu et al. 2008), which may severely influence pressing efficiency and final product yields. As these enzymes possess glycosidase activities, they may represent an important influence on anthocyanin stability, since the hydrolysis of anthocyanins to aglycones severely decreases their stability in aqueous media (Markakis 1974). Sodium bisulphite is also frequently added during fruit processing to minimize the oxidation of colour components (such as anthocyanins), flavour and ascorbic acid (Markakis 1974). In addition, in order to bring thermally sensitive colloidal substances out of solution, pressed juices are commonly heated to a holding temperature in excess of 100 ℃, known as a ‘hot break’ which is thought to improve final juice yields during pressing and

aid depectinization and pasteurization (Goodman, Fawcett et al. 2002). Currently, the influences of many of these processes on anthocyanins throughout commercial juice processing are unknown.

Prior studies have assessed the recovery of anthocyanins during bench scale (pilot) processing (Skrede, Wrolstad et al. 2000; Lee, Durst et al. 2002; Zhongxiang, Min et al. 2005), however, data on 'large-scale' commercial processing is limited. Previous reports have thus far failed to establish if losses of anthocyanins are the result of poor recovery of parent compounds during processing or degradation, although two studies by Landbo and Meyer *et al* have described the loss of anthocyanins and phenols during different enzyme treatments (Landbo and Meyer 2001; Landbo and Meyer 2004). Establishing the amounts of anthocyanins and their degradation products throughout the manufacturing of commercial products remains important for ascertaining their dietary occurrence and potential health benefits.

In the present investigation we established the recovery and stability of anthocyanins throughout the commercial processing of blackcurrants to blackcurrant juice concentrate using an industrial scale process. Furthermore, several modifiable processing factors were assessed for their influence on anthocyanin yield in final concentrated juice, using a "bench-scale" processing model to simulate industrial processes. The modifiable factors assessed included milling, depectinaze enzyme treatment, sodium bisulphite concentration and evaporation temperature and were investigated using a full-factorial Design of Experiment (DOE) analysis.

2.2 Materials and Methods

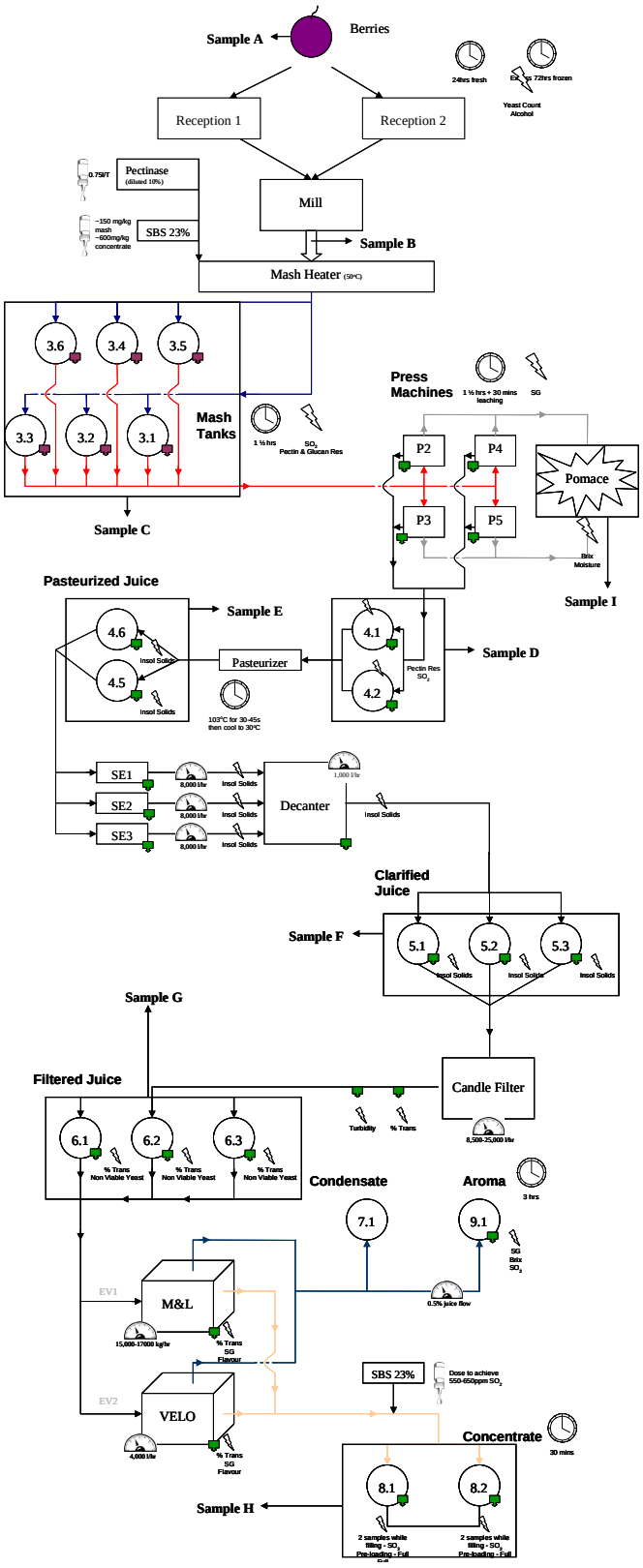
Sodium bisulphite, 4-hydroxybenzoate methyl ester and methyl 3,4-dimethoxybenzoate were purchased from Sigma-Aldrich (UK). Cyanidin-3-glucoside and delphinidin-3-glucoside were purchased from Extrasynthese (Genay, France). A mixed blackcurrant analytical standard (containing cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-glucoside and

delphinidin-3-rutinoside) was provided by Nutritional Healthcare R&D, GlaxoSmithKline. (Coleford, UK). Pectinase enzymes were provided by GlaxoSmithKline. (Proprietary information). Blackcurrants (spp. *Ribes nigrum* cv. 'Ben Hope' and 'Ben Gairn') were harvested in the UK and used within 24 h for industrial processing experiments or were frozen within 24 h and stored at -18 ℃ until use in bench-scale processing. All other chemicals and reagents were from Sigma-Aldrich (UK) and all solvents were of HPLC grade.

2.2.1 Commercial blackcurrant processing and sampling (**scheme 2.1**)

To evaluate the influence of industrial processing on the anthocyanin content of blackcurrant juice concentrate, representative samples were taken at different stages throughout commercial blackcurrant processing. Briefly, freshly harvested blackcurrants (*Ribes nigrum* 'Ben Hope' and 'Ben Gairn') were milled by roller mills and the comminute fed directly into temperature controlled mash heaters. The mash was heated to a controlled temperature of 50℃ for pectolytic enzyme hydrolysis. Sodium bisul phite was added as an aqueous solution to achieve a sulphur dioxide level to 600 mg/kg. Once the enzyme and sodium bisulphite was added, the mash was maintained at 50℃ for three hours. Juice was extracted from the enzyme treated mash by pressing with a Bucher HPX5005i hydraulic press using Bucher berry socks on the press tubular elements. On completion of the initial press, subsequent leaching was performed with evaporator condensate at 60 ℃. Pressed juice was then heated through a tubular pasteurizer at a holding temperature of 103℃ for 30-45 sec, and then cooled to 35℃. Paste urised juice was then clarified by sedicantation and filtration (Flottweg sedicanter S4D -3/408 and Flottweg decanter centrifuge Z4D). The clarified juice was filtered through a Keiselguhr filter aid (Filtrox FILTROjet 1100/1850 candle filter and Securox B 40/16 cartridge filters). Clarified and filtered juice was passed through a Velo evaporator (Velo TSE4000) and chilled to approximately 10 ℃ for storage. Samples were taken throughout the processing procedure (whole berry, post-milling, post-depectinization, post-pressing, post-pasteurization, post-clarification, post-filtration and post-concentration) and stored at -18℃ until extraction and UPLC analysis for anthocyanins and their degradation products, including protocatechuic acid and gallic acid, using authentic

analytical standards. The final analyte concentrations were normalized to a total soluble solids ratio of the initial pressed juice to account for any intrinsic dilutions.



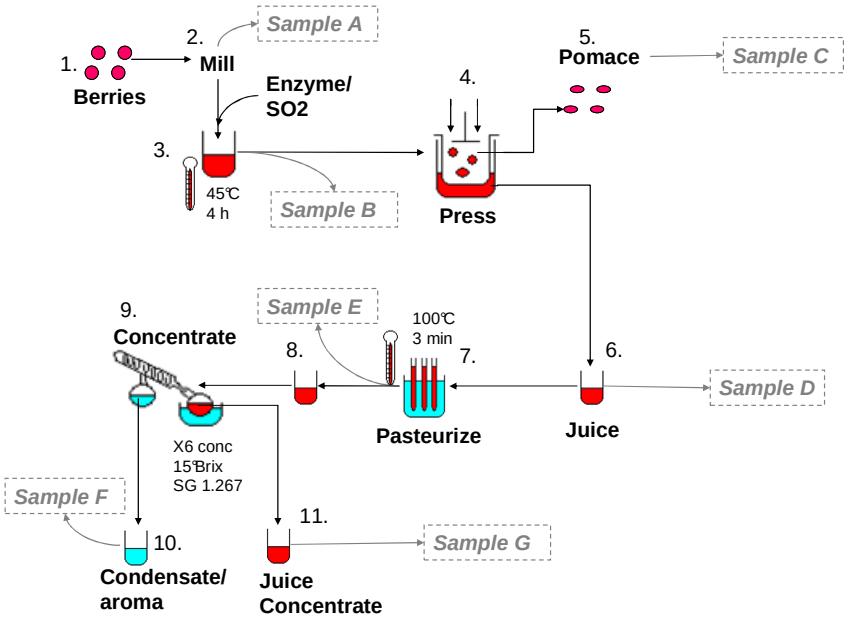
Scheme 2.1. Representation of industrial blackcurrant juice processing with indicated sampling points.

2.2.2. Simulated ‘bench-scale’ blackcurrant processing (scheme 2.2 and 2.3)

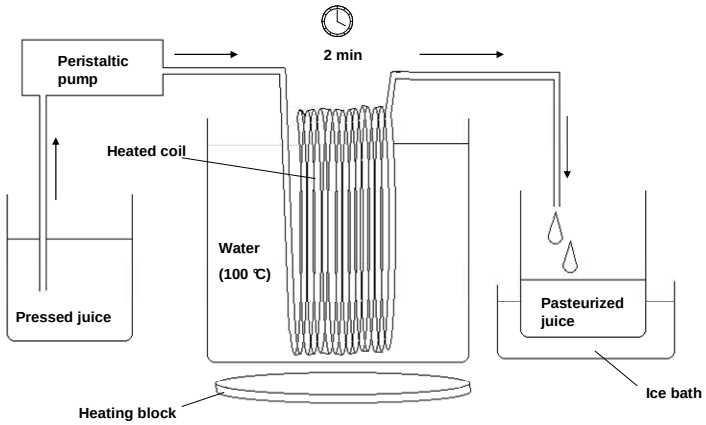
In order to evaluate the commercial processing line under more controlled conditions, a ‘bench-scale’ processing model was developed. Briefly, 250 g portions of fresh blackcurrants (*Ribes nigrum* ‘Ben Hope’) were milled using a bench top blender and incubated for 3 h at 45 ℃ in the presence of pectolytic enzyme and sodium bisulphite. Following enzyme treatment, the blackcurrant mash was pressed through a muslin cloth under vertical pressure by hand, washed with 150 ml water to simulate leaching and re-pressed. To ensure all the juice was extracted, the muslin cloth was tightly twisted several times into a beaker until the resulting pomace was free of residual liquid. The pressed blackcurrant juice was spiked with 500 mg p-hydroxybenzoate methyl ester (dissolved in 10 mL methanol) as an internal dilution control. The juice was then passed though a pasteurizer, comprising a heated (101 ℃) copper coil attached to a peristaltic pump which emptied into an ice-cooled beaker, allowing the juice to be heated to 100℃ and cooled to 40℃ within 2.5 min. Following pasteurization, the juice was filtered through a finely sintered glass funnel and concentrated to a total soluble solids ratio of 65 ℃Brix by rotary evaporation (90℃ *in vacuo*). The blackcurrant concentrate was diluted to its initial internal standard concentration (which corresponded to their initial total soluble solids ratio) prior to extraction. Samples were taken throughout this process and stored at -18 ℃ until analysis for anthocyanins and their degradation products (protocatechuic acid and gallic acid).

The relationship between anthocyanin concentration and various processing factors (milling, enzyme treatment, SO_2 and evaporation) was assessed using a full-factorial Design of Experiment analysis (see appendix A for further details). Blackcurrants were processed as described above (‘bench-scale’) with the following alterations: berries were coarsely or finely milled using a bench top blender (altering blending time and speed); enzyme concentrations were varied from 0.1 to 1.5 ml/kg fruit; enzyme incubation times were varied from 0.5-6 h; sodium bisulphite concentrations were varied from 0 to 300 mg/kg fruit; evaporation temperatures were varied from 40 to 90 ℃. The final

concentrate anthocyanin concentration was analysed by Ultra-Performance Liquid Chromatography (UPLC).



Scheme 2.2. Representation of "bench scale" blackcurrant juice processing with indicated sampling points.



Scheme 2.3. Representation of a novel "bench scale" pasteuriser developed for "bench scale" blackcurrant juice processing.

2.2.3 Sample extraction

Whole blackcurrants and processed samples were stored in the dark at -18 ℃ until extraction. Frozen blackcurrants and pre-press samples were milled in liquid nitrogen with a pestle and mortar to form a homogonous powder. Subsequently, 1 g fresh weight (FW) of the frozen powder was accurately weighed and extracted in 5 ml 5% formic acid in methanol with ultrasound assistance (sonication at 40 ℃ for 15 min) and cen trifuged at 4000 rpm for 20

min. The pellet was re-extracted in an additional 5 ml of 5% formic acid in 80% aqueous methanol with ultrasound assistance and centrifuged at 4000 rpm for 20 min. The combined supernatants were concentrated by rotary evaporation to a 1 ml volume and spiked with 0.5 mg methyl 3,4-dimethoxybenzoate as an internal volume adjuster and filtered through a 0.45 µm syringe filter. All post-press samples and concentrates were diluted 1:1 or 1:3 (post-press samples and final concentrates, respectively) in acidified methanol (5% formic acid), spiked with 0.5 mg methyl 3,4-dimethoxybenzoate as an internal standard and filtered through a 0.45 µm syringe filter.

2.2.4 UPLC analysis

Extracted samples were analysed by UPLC using a Waters Acquity UPLC equipped with an Acquity BEH C18 1.7 µm (2.1 x 50mm) column (Waters) and Van guard BEH C18 guard column (Waters). The column temperature was set to 40℃ and the mobile phase consisted of 5 % aqueous formic acid (A) and acetonitrile (B). The flow rate was 0.49 ml/min and solvent gradient was as follows: 0% B at 0 min, 1% B at 0.26 min, 2.5% B at 2.85 min, 3% B at 3-4 min, 4% B at 4.5 min, 6% B at 5.1 min 8% B at 6 min, 20% B at 7-8 min, 50% B at 8.5 min and 100% B at 9 min. The injection volume was 2 µL. The weak wash consisted of water:acetonitile (95:5) and the strong wash consisted of acetonitrile:iosopropanol:water (7:2:1). Absorbance was recorded at 260nm, 320nm and 500nm. Refer to appendix A for further chromatographic data on juice samples.

2.2.5 Total soluble solids, specific gravity and acidity analysis

For the determination of total soluble solids ratio (°Brix) of the processed blackcurrant samples, 1 mL of processed blackcurrant juice was directly analysed on a auto-refractometer (Bellingham and Stanley Ltd., RFM 90) calibrated for sugar quantification at 24 ℃. The specific gravity was established by applying 2 mL of processed blackcurrant juice to a density meter (Anton Paar, DMA 4500 M) at a cell temperature of 20℃. To measure acidity, 1-3 g of sample was titrated to pH 8.15 with 0.1M NaOH and acidity was calculated as % w/v citric acid mono-hydrate (CAMH) using the following formulae: [Titre volume (ml) x 0.7 x SG]/sample weight = % w/v CAMH]

2.2.6 Statistical analysis

Differences in anthocyanin concentrations in processed samples were evaluated by one-way repeated measures analysis of variance (Windows SPSS, version 14). Significance was determined at $p<0.05$. For commercial scale processing, sample replicates (4 independent experimental replicates) consisted of samples taken at each processing step (i.e. milling, pressing, pasteurisation, clarification and concentration). For bench-scale processing, independent experiments were performed in triplicate. The relationship between processing factors (milling, enzyme treatment and time, SO_2 and evaporation condition) and final concentrate anthocyanin content was assessed using a full-factorial Design of Experiment (DOE) model using Design Expert® Software (Stat-Ease, Inc., Minneapolis). Twenty two independent experimental runs were performed for the DOE analysis and five factors assessed. All analytical procedures were performed in duplicate for each sample (i.e., technical replicates).

2.3 Results and Discussion

2.3.1 Characterization of anthocyanins in whole blackcurrants and juices

Two cultivars of blackcurrants were used in the commercial processing, *Ribes nigrum* cv. 'Ben Gairn' and 'Ben Hope'. The total anthocyanin content of *Ribes nigrum* cv. 'Ben Gairn' was 164±3.4 mg/100 g and for *Ribes nigrum* cv. 'Ben Hope' was 220±8 mg/100 g. These concentrations were consistent with previously reported data for blackcurrants (Mazza and Miniati 1993). The separation of blackcurrant anthocyanins by UPLC is shown in **Figure 1.1**. Peak assignments and identification were made according to UV-vis spectra and retention time in comparison to authentic analytical standards. There were four major anthocyanin species identified in the blackcurrants, namely, cyanidin-3-rutinoside (57±1 and 74±3 mg/100 g FW), cyanidin-3-glucoside (8±0.3 and 11±0.3 mg/100 g FW), delphinidin-3-rutinoside (79±1.1 and 108±3.9 mg/100 g FW) and delphinidin-3-glucoside (18±0.5 and 25±0.6 mg/100 g FW)(for Ben Gairn and Ben Hope, respectively). Although previous

studies have identified low levels of other anthocyanins in blackcurrants, namely pelargonidin, peonidin and malvidin glycosides (Slimestad and Solheim 2002), the present study focused only on establishing the stability and recovery of the major anthocyanins present; namely dephinidin-rutinoside, delphinidin-glucosinde, cyanidin-rutinoside and cyanidin-glucoside. Following commercial processing the raw pressed juice had an anthocyanin concentration of 96±12 mg/100 ml juice and the final juice concentrate had a total anthocyanin concentration of 80±3 mg/100 ml (when re-diluted to their initial raw-pressed juice total soluble solids levels).

Table 2.1. Chemical profile of blackcurrant juice during processing

Processing step	Soluble solids (°Brix)	Specific Gravity	Acidity (% w/v CAMH)	pH
Pressed Juice	10.1±4.4	1.0387±0.0217	2.6±1.1	2.84
Pasteurized Juice	9.8±4.1	1.0402±0.0164	2.5±1.0	2.91
Clarified Juice	7.3±3.8	1.0299±0.0157	2.0±1.0	2.91
Concentrated Juice	66.6±1.5	1.3322±0.0103	18.5±9.0	2.55

Data represented as mean±SD (n=4). CAMH, citric acid mono-hydrate.

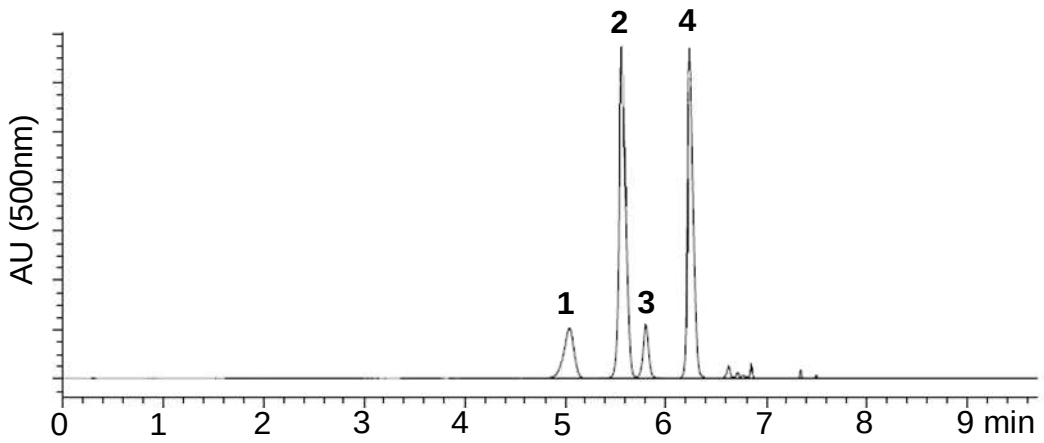


Figure 2.1. Representative chromatograph of blackcurrant anthocyanins at 500nm. AU, absorbance units; 1, delphinidin-3-glucoside; 2, delphinidin-3-rutinoside; 3, cyanidin-3-glucoside; 4, cyanidin-3-rutinoside.

2.3.2 Anthocyanin stability during commercial juice processing

For extraction and analysis purposes, the processing experiments were separated into two phases consisting of pre-press (whole berry, berry mash, pectinaze and sodium bisulphite treatment) and post-press (pasteurized, decanted, filtered and concentrate) juice samples. The relative anthocyanin

content of blackcurrants during the two phases of commercial processing is illustrated in **Figure 2.2**, while chemical and physical characteristics (i.e. pH, specific gravity, acidity and total soluble solids ratio) are described in **Table 2.1**.

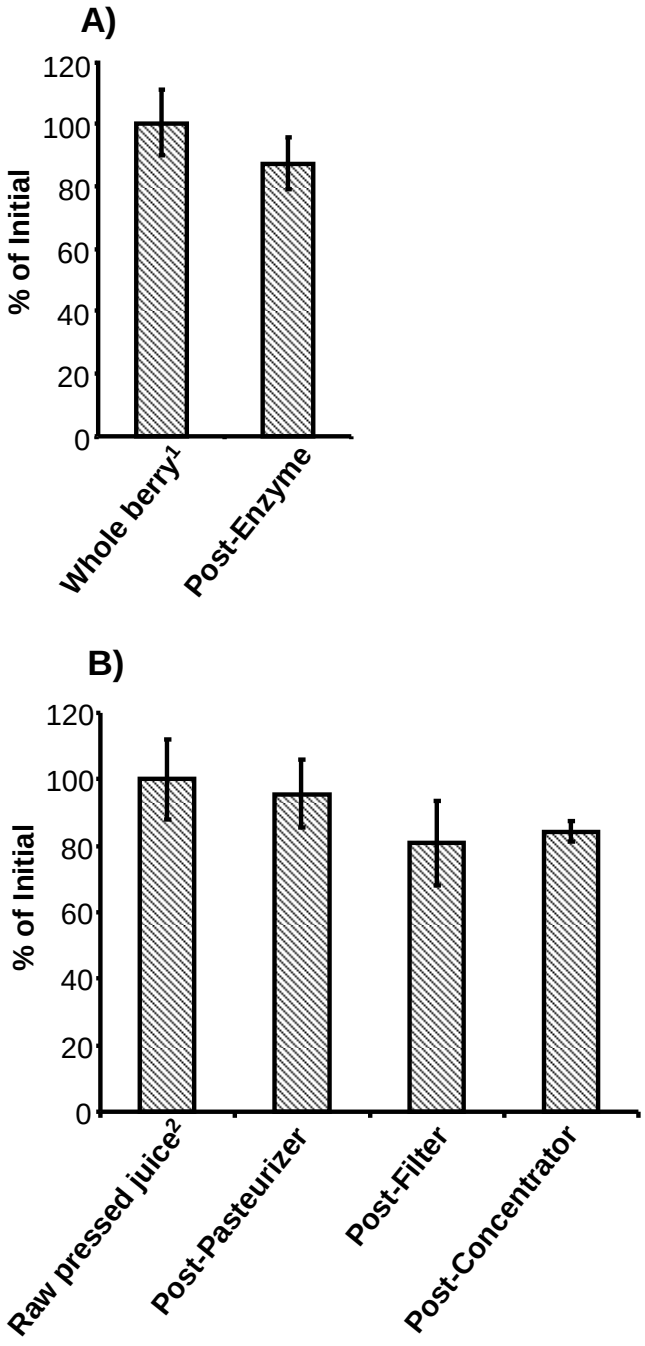


Figure 2.2. Recovery of anthocyanins during commercial juice processing. A, anthocyanin extraction from whole berries (i.e., pre-press). B, anthocyanins during juice processing (i.e., post-press). Data represented as mean±SD (n=4). Significance was assessed by repeated measures ANOVA ($p<0.05$). [1]Refers to initial whole berry anthocyanin content prior to pre-press berry processing. [2]Refers to initial raw pressed juice anthocyanin content prior to post-press juice processing.

Pre-press processing. The pre-press blackcurrant processing (i.e., solid matrix) did not contribute to a significant loss in total anthocyanins (n=4, sampled over four days), from whole berry through milling (p=0.7) to pectin hydrolysis and sodium bisulphite addition (p=1.0), with less than 13% loss across the pre-press processing stages as compared with the initial whole berry (**Figure 2.2A**). No anthocyanin degradation products (anthocyanin derived protocatechuic acid or gallic acid aglycones) were detected during commercial blackcurrant juice pre-press processing samples.

Post-press juice processing. Following pressing, the subsequent processing of the pressed juice did not contribute to a significant loss in total anthocyanins, through pasteurization (p=1.0), decantation (p=1.0), filtration (p=0.9) and concentration (p=1.0); with 17% total anthocyanin lose across the post-press processing steps (**Figure 2.2B**). It should be emphasised that all samples were normalized to their initial raw pressed juice total soluble solids value in order to account for dilutions throughout commercial juice processing. Overall, this study demonstrated that no significant anthocyanin degradation occurred during commercial scale blackcurrant pre and post-press processing. However, it should be noted that there was a significant loss of anthocyanins between the pre-press mash and the post-press juice samples which was likely due to sub-optimal extraction from the berry pomace, as some anthocyanins will remain in the post press berry skins, as reported by Fang *et al*. (Fang, Zhang et al. 2006). Losses were minimal at all other stages of commercial processing. This is consistent with previously reported data, where a study by Skrede et al. (Skrede, Wrolstad et al. 2000) showed no losses of anthocyanins during milling and pectin hydrolysis (pre-press processing) using a bench-scale method.

2.3.3 Assessing the impact of processing on anthocyanins using a bench-scale processing model

In order to assess and modulate the potential impact of commercial juice processing factors/steps on blackcurrant juice anthocyanin content, a “bench-scale” method was developed to simulate the commercial process. The development of a bench-scale model allowed for the alteration of experimental

variables in order to perform factorial analysis of various processing factors. Furthermore, the development of a bench-scale model allowed for greater control over inherent processing dynamics, such as uncontrolled intrinsic dilutions or sample losses that occur throughout industrial scale processes. These dynamics were controlled for by spiking juice samples with *p*-hydroxybenzoic acid methyl ester prior to post press processing and using this as an internal control standard to back-calculate processing volumes *post-hoc*.

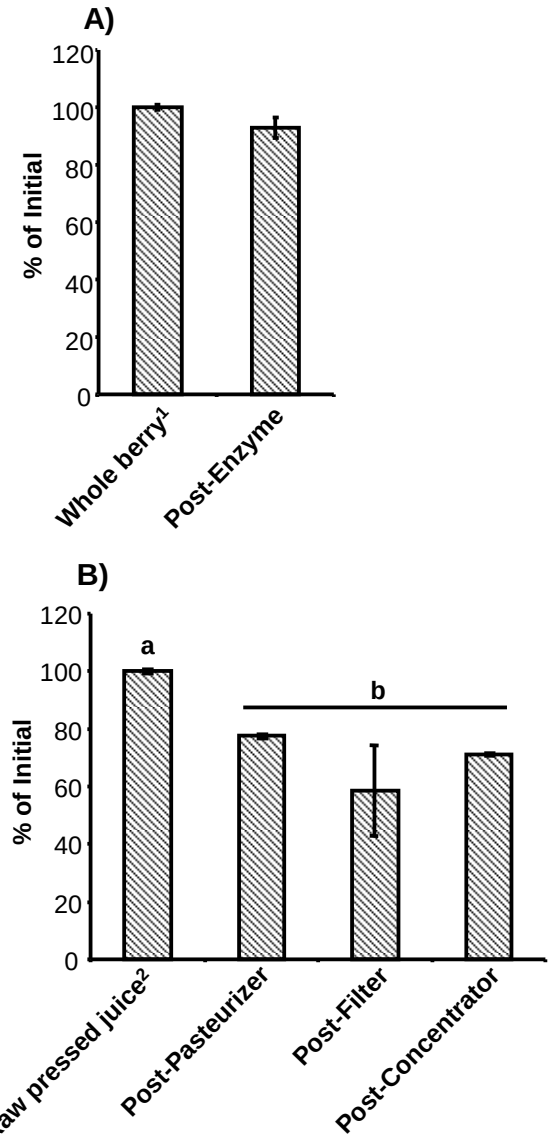


Figure 2.3. Recovery of anthocyanins during bench-scale juice processing. A, anthocyanin extraction from whole berries (i.e, pre-press). B, anthocyanins during juice processing (i.e., post-press). Data represented as mean±SD (n=3). Significance was assessed by repeated measures ANOVA (n=3, $p<0.05$). Like letters (a and b) represent statistical similarity. [1]Refers to initial whole berry anthocyanin content prior to pre-press berry processing. [2]Refers to initial raw pressed juice anthocyanin content prior to post-press juice processing.

Similar to the commercial scale process, the bench-scale model showed no significant losses in anthocyanin content following sequential milling and enzyme treatment (**Figure 2.3A**). In general, post-press juice processing showed no significant anthocyanin losses, which is also consistent with the commercial scale observations (**Figure 2.3B**). However, unlike the commercial scale processing, a significant loss of 22±0.7% anthocyanins ($P<0.001$) was observed during pasteurization. This was likely a result of the difference in pasteurization holding time, since the commercial process consisted of a 30 sec heating period, while the bench scale process took 2.5 min to reach 100 ℃ and lower down to 40 ℃. This o bservation is consistent with the findings of Rubinskiene et al. (Rubinskiene, Viskelis et al. 2005), who assessed the effect of pasteurization times on aqueous anthocyanins concentrations. No phenolic acid degradation products were observed.

As with commercial scale processing, it was shown that the post-press juice contained a lower concentration of anthocyanins than the pre-press mash (or whole berry) as some anthocyanins remain in the post press berry skins (pomace), as reported by others (Fang, Zhang et al. 2006). This previously identified aspect of juice processing was not the focus of the present investigation which aimed to establish the degradation of anthocyanins during juice processing and not the extraction efficiency of anthocyanins from berry skins, which is a known limitation in the processing of berry juice.

Factorial analysis of processing and anthocyanin yield. The association between juice processing stages (i.e. milling, enzyme concentration and incubation time, sodium bisulphite concentration and evaporation conditions) and final juice anthocyanin yield was assessed using a factorial DOE model. A DOE model approach was chosen in order to assess the multi-factor interactions between various processing factors and final juice anthocyanin yield (synergistic effects). An overview of the association between final anthocyanin yield and various common processing influences is shown in **Figure 2.4**, displayed as a two factor interaction plot. The only processing event which had an impact on anthocyanin recovery in this analysis was the concentration of sodium bisulphite used. Sodium bisulphite concentration

produced a marked increase ($p<0.001$) in final anthocyanin concentration (**Figure 2.4a-c**) when altered in conjunction with other processing factors: pectinase incubation time, pectinase concentration, evaporation time or milling method. This would indicate that sodium bisulphite concentration may be an important factor to consider during commercial blackcurrant juice processing, where it is likely that sodium bisulphite improves the extraction of anthocyanins from the fruit skin, as previously described (Cacace and Mazza 2002).

Throughout commercial blackcurrant processing, no significant anthocyanin degradation was observed and no anthocyanin derived degradation products (protocatechuic acid and gallic acid aglycones) were identified. This would suggest that anthocyanins are not degraded to any significant extent during commercial processing, most likely as a result of the short residence times at high temperature and low pH of blackcurrant fruits and juice concentrate (pH 2-3), which would have a stabilizing effect on the anthocyanin flavylium iso-form (Markakis 1974). Losses of anthocyanins were observed following pressing which was likely due to incomplete anthocyanin recovery from the pressed berries/pomace as described above and by others previously (add references from above).

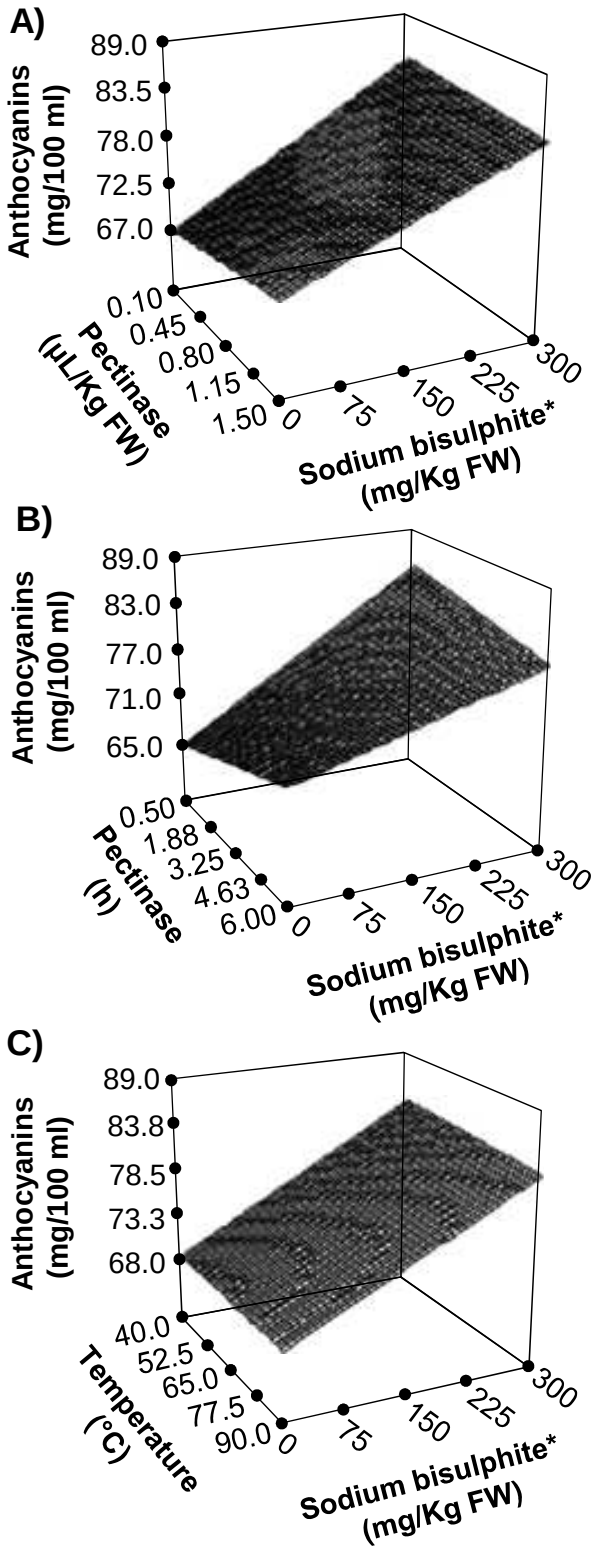


Figure 2.4. Two factor relationship plots of various juice processing factors on final anthocyanin content in blackcurrant juice concentrate. A, the effect of pectinase enzyme concentration and sodium bisulphite concentration on juice anthocyanin content; B, the effect of pectinase enzyme incubation time and sodium bisulphite concentration on juice anthocyanin content; C, the effect of evaporation temperature and sodium bisulphite concentration on juice anthocyanin content. Sodium bisulphate concentration significantly effected anthocyanin concentration ($p<0.001$). *Pectinase concentration, pectinase incubation time and evaporator temperature did not demonstrate a significant effect on anthocyanin concentration ($p>0.05$). FW, fresh weight.

It has been reported that under the described processing conditions, anthocyanin condensation may occur, where anthocyanin species may be conjugated to other flavonoids and aldehyde structures (*17, 18*). It may therefore be hypothesised that anthocyanins may form protein bound or condensation products during commercial processing. This represents a potential area for future research, where novel anthocyanin condensation products could be targets for bioactivity studies examining the health benefits of processed berry products. However, this is merely speculation; as there was no significant loss of anthocyanins observed in the present study except during the pressing step.

In a recent study by Hollands *et al*. (Hollands, Brett et al. 2008) the anthocyanin content of several processed blackcurrant products was assessed. The study reported that the total anthocyanin level in commercial blackcurrant products was substantially lower (0.05–10.3% of the levels in fresh fruit) than that found in fresh fruits. This would indicate that processed blackcurrant products are not rich sources of anthocyanins in the diet, when compared to fresh produce. We propose that the apparent loss of anthocyanin content in processed juices is not likely the result of processing, but rather due to degradation during product storage. This was confirmed in a pilot study in which the anthocyanin concentration was monitored in various commercial juice preparations over twelve weeks under different storage conditions (some pilot data is represented in **Figures 2.5, 2.6** and **2.7**). Briefly, commercial black currant juices were formulated (with/without sugar; with/without ascorbate; with/without SBS;–*formulation was proprietry to GlaxoSmithKline*) and stored at 4 ℃ and 21 ℃ (representing storage in a fridge or ambient temperature) for 12 weeks. At 0, 6 and 12 weeks aliquots of the stored formulations were analysed by HPLC for anthocyanins and the degradation products gallic acid and protocatechuic acid. From this pilot data it is evident that anthocyanins are rapidly degraded during storage (both at 4 ℃ and 21 ℃) although the formation of phenolic acid degrada tion products was not consistent.

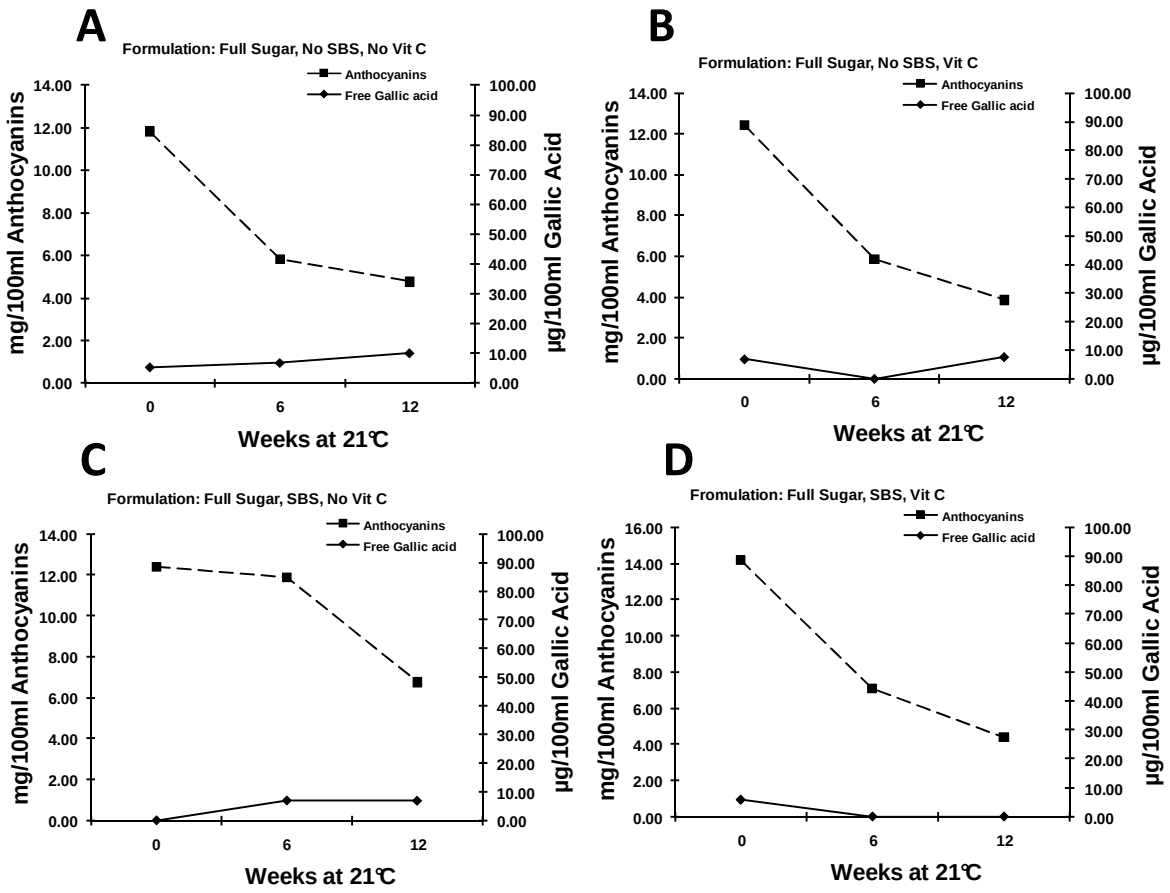


Figure 2.5. Total anthocyanin loss in different blackcurrant juice preparations when stored at 21 °C over 12 weeks. A, full sugar formulation (proprietary to GSK) without SBS and vitamin C; B, full sugar formulation (proprietary to GSK) without SBS but with vitamin C; C, full sugar formulation (proprietary to GSK) with SBS but without vitamin C; D, full sugar formulation (proprietary to GSK) with SBS and vitamin C. SBS, sodium bisulphite; Vit C, vitamin C/ascorbate.

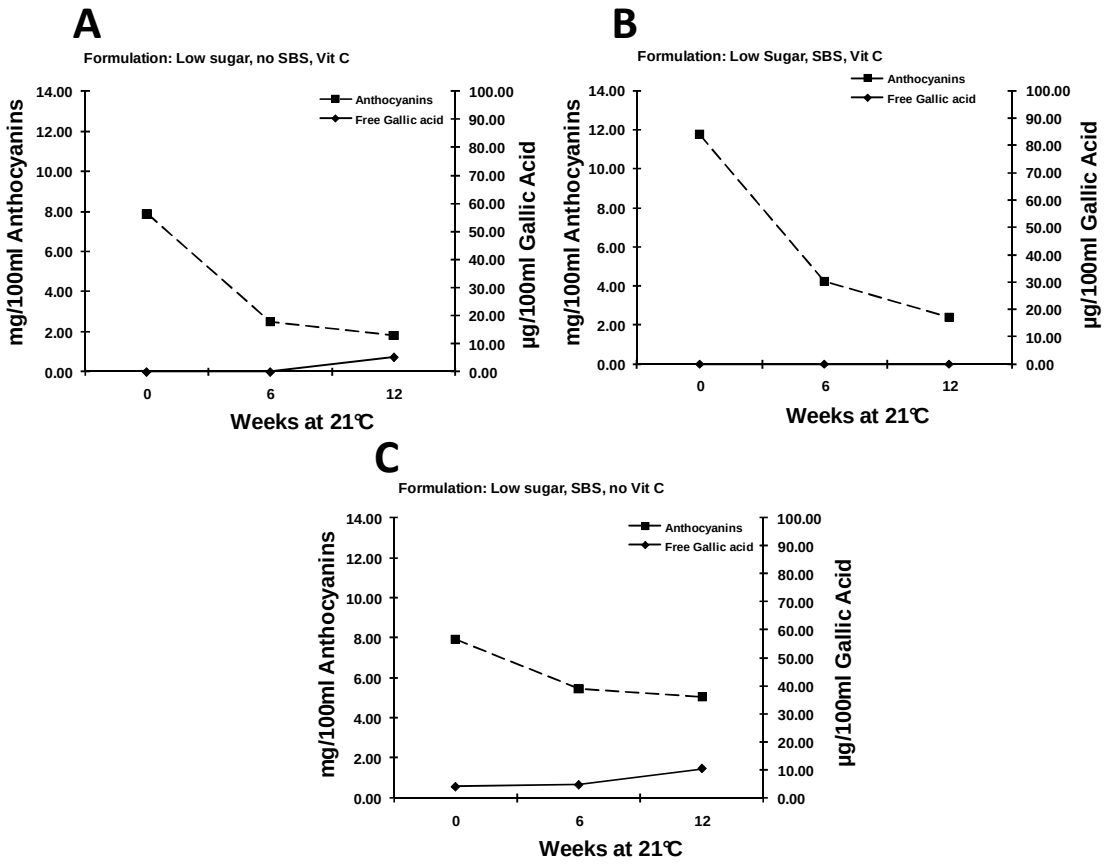


Figure 2.6. Total anthocyanin loss in different blackcurrant juice preparations when stored at 21 °C over 12 weeks. A, low sugar formulation (proprietary to GSK) without SBS but with vitamin C; B, low sugar formulation (proprietary to GSK) with SBS and vitamin C; C, low sugar formulation (proprietary to GSK) without SBS but with vitamin C. SBS, sodium bisulphite; Vit C, vitamin C/ascorbate.

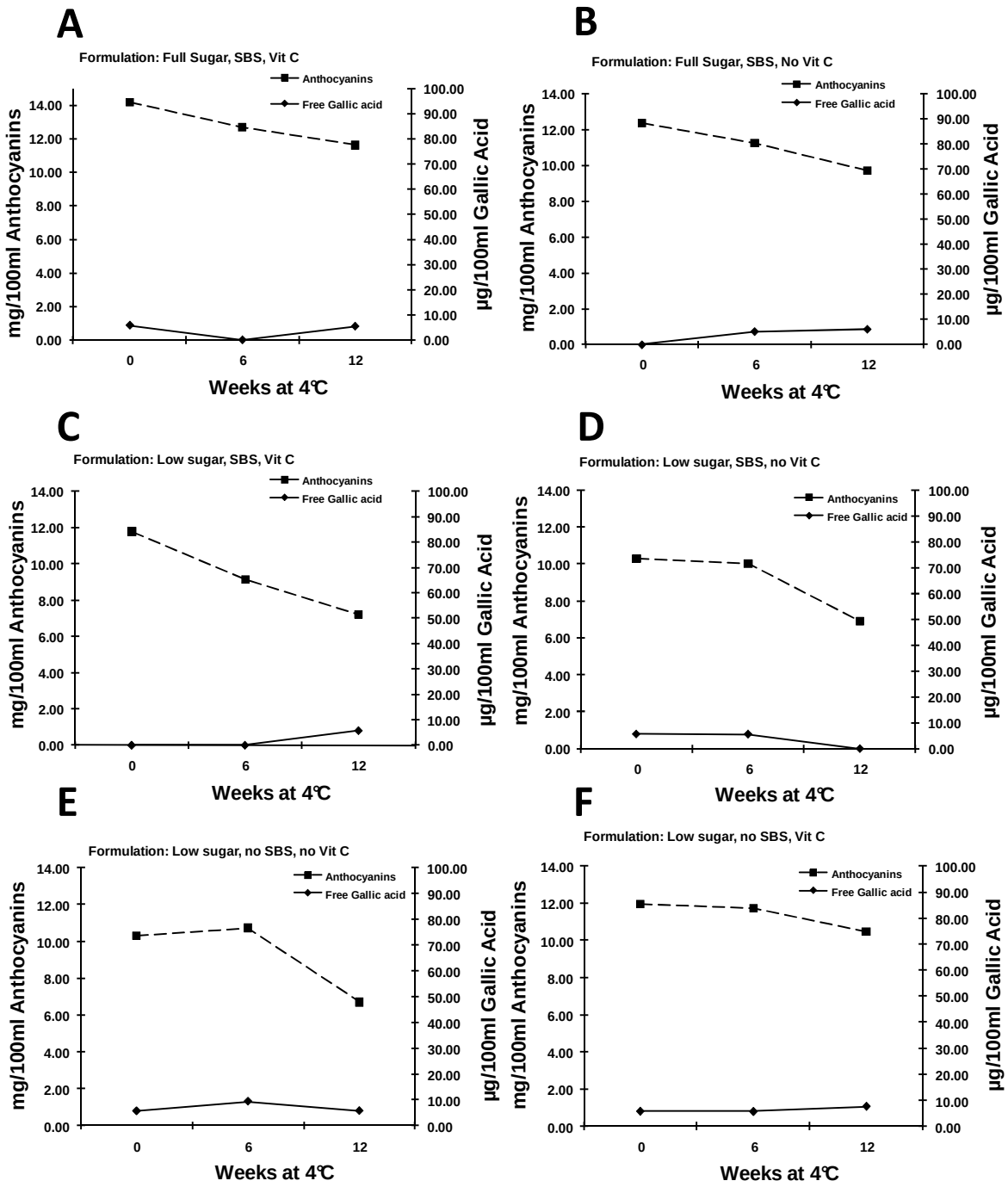


Figure 2.7. Total anthocyanin loss in different blackcurrant juice preparations when stored at 4 °C over 12 weeks. A, full sugar formulation (proprietary to GSK) with SBS and vitamin C; B, full sugar formulation (proprietary to GSK) with SBS but without vitamin C; C, low sugar formulation (proprietary to GSK) with SBS and vitamin C; D, low sugar formulation (proprietary to GSK) with SBS but without vitamin C; E, low sugar formulation (proprietary to GSK) without SBS and vitamin C; F, low sugar formulation (proprietary to GSK) without SBS but with vitamin C. SBS, sodium bisulphite; Vit C, vitamin C/ascorbate.

In conclusion, the results of this study demonstrated that of the five processing factors assessed only sodium bisulphite had any significant impact on the final anthocyanin yield of the blackcurrant juice. This study also showed that during commercial processing no significant anthocyanin degradation was observed and anthocyanins were not fully recovered from the press pomace. Further study should be conducted to establish the degradation characteristics of anthocyanins during processed juice storage.

Chapter 3

Anthocyanin stability and recovery under simulated physiochemical conditions

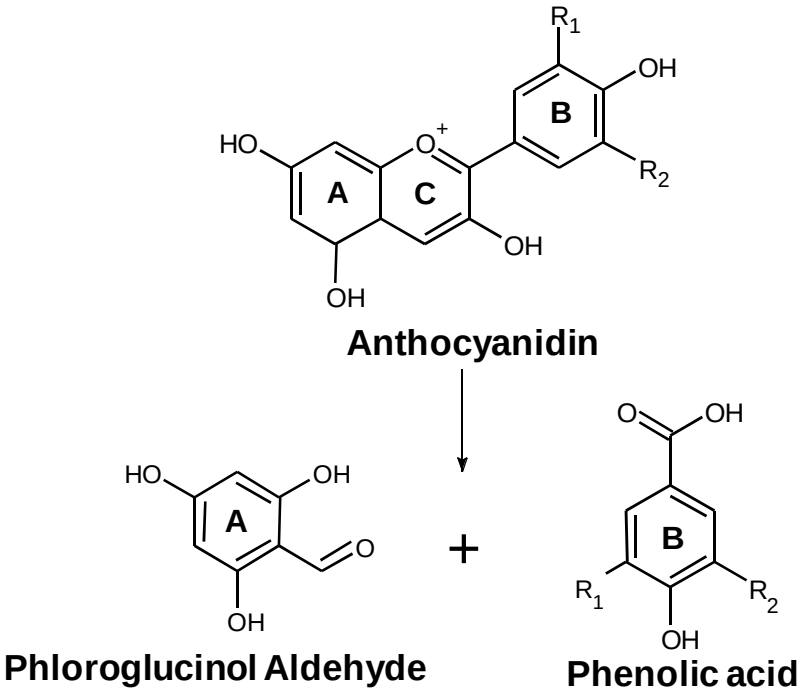
Data published in *J. Agric. Food Chem.* (2009) 57: 12, 5271-5278.

3.1 Introduction

Over the past decade, interest in anthocyanins has grown considerably, as evidence of their beneficial effects on health continues to increase, particularly regarding their role in reducing cardiovascular disease risk (Xia, Ling et al. 2006; Mink, Scrafford et al. 2007; Xia, Ling et al. 2007), cancer (Feng, Ni et al. 2007; Rossi, Garavello et al. 2007) and obesity (Tsuda, Horio et al. 2003). In plants, anthocyanins are almost exclusively found as glycosides of the aglycone form (anthocyanidins) (**Figure 3.1**), where three common structures, cyanidin, delphinidin and pelargonidin exist, differing only in the number and position of hydroxyl groups on their B-ring. Anthocyanins may exist in at least four different pH-dependent structural isoforms, namely, flavylium anion, hemiketals, quinoid bases and chalcones, arising at pH 1-3, 4-5, 6-8 and 7-8, respectively (Markakis 1974). At more alkaline pH values, anthocyanins have consistently been shown to degrade to their constituent phenolic acids, where delphinidin, cyanidin and pelargonidin degrade to form gallic acid, protocatechuic acid and 4-hydroxybenzoic acid, respectively (Seeram, Bourquin et al. 2001; Fleschhut, Kratzer et al. 2006) (**Figure 3.1**). In addition, it is known that all species of anthocyanins degrade to a common phenolic aldehyde, with the most frequently reported constituent being phloroglucinol aldehyde (Keppler and Humpf 2005; Sadilova, Stintzing et al. 2006).

Since the 1970s, many studies have investigated the occurrence and utility of anthocyanins in plants and in food production, whilst research concerning their potential health related benefits has become prominent only since the late 1990s. It has since been established that anthocyanins are poorly available to the systemic circulation (Kay, Mazza et al. 2004; Kay, Mazza et al. 2005; Vitaglione, Donnarumma et al. 2007). We postulate that this is a result of their reported instability, as it has been shown that, under certain conditions (e.g. food processing (Hollands, Brett et al. 2008)), anthocyanins are subject to significant degradation (McDougall, Fyffe et al. 2005; Fleschhut, Kratzer et al. 2006; Sadilova, Florian et al. 2007). Therefore, it is paramount that the chemical stability of anthocyanins is assessed under physiological and experimental conditions in order to determine the contribution of

degradation to their reported bioavailability. Whilst a great deal of studies have reported on aspects of anthocyanin stability, there is no single investigation fully describing their recovery and degradation following the routine extraction, pre-analytical preparation, and storage of clinically and experimentally derived samples. Thus, the aim of this study was to assess the degradation and recovery of selected anthocyanins (aglycones and mono-glucosides), with mono-, di- and tri-hydroxylated B-rings, under simulated (*in vitro*) physiological conditions (i.e., *in vitro* models designed to simulate aspects of *in vivo* conditions) and routine experimental conditions.



Anthocyanin	Phenolic Acid	R_1	R_2
Cyanidin	Protocatechuic acid	OH	H
Pelargonidin	4-hydroxybenzoic acid	H	H
Delphinidin	Gallic Acid	OH	OH

Figure 3.1. Structure of anthocyanidins and their respective phenolic acid and aldehyde constituents.

3.2 Materials and Methods

Cyanidin-3-glucoside (kuromanin chloride), delphinidin-3-glucoside (myrtillin chloride), pelargonidin-3-glucoside (callistephin chloride), cyanidin chloride, delphinidin chloride, pelargonidin chloride and 4-hydroxybenzoic acid were purchased from Extrasynthese (Genay, France) and dissolved in DMSO. Protocatechuic acid, gallic acid, *trans*-cinnamic acid, 4-hydroxycinnamic acid and phloroglucinol aldehyde were purchased from Sigma-Adlrich (UK) and dissolved in DMSO. Phenol free Dulbecco's Modified Eagle's Medium (DMEM) and bovine calf serum were purchased from Invitrogen (UK). All water used was 18MΩ/cm milliQ water and solvents were of HPLC grade.

3.2.1 Influence of B-ring hydroxylation on the rate of anthocyanin degradation and the formation of phenolic acids

To assess the effect of B-ring hydroxylation on anthocyanin degradation and the formation of phenolic acid and aldehyde constituents under physiological conditions, pelargonidin-3-glucoside, cyanidin-3-glucoside, delphinidin-3-glucoside, pelargonidin, cyanidin and delphinidin were individually made up to 150 *μ*M in pre-incubated physiological buffer (10 mM Na/K phosphate, pH 7.4) or water (titrated to pH 7 with the drop wise addition of dilute NaOH) and incubated at 37 ℃ (final DMSO concentration was 1. 5%). After 0, 2, 6, 12 and 24 h, 150 *μ*L aliquots were acidified with HCl (2% final concentration) and injected onto an HPLC-DAD system. Control samples consisted of acidified (2% HCl) buffer matrices that were filtered and then spiked with anthocyanins and anthocyanidins to a final concentration of 150 *μ*M. An automated method was utilised to further assess the rate of anthocyanin degradation in the buffer matrix, where samples were incubated in an HPLC auto-sampler at 37 ℃ with injections at 0, 2, 4, 8 and 12 h, using the same conditions as above.

3.2.2 Anthocyanin recovery following routine pre-analytical treatments

Sample preparation: To determine the recovery of anthocyanins with different B-ring hydroxyl moieties under common experimental conditions, 0.5 M Na/K phosphate buffer, foetal calf serum (Invitorogen, UK) and cell culture media

(phenol free DMEM) were acidified to pH ~2 with the drop wise addition of HCl and spiked to a final concentration of 25 *μ*g/mL with either pelargonidin-3-glucoside, cyanidin-3-glucoside or delphinidin-3-glucoside. The final serum and cell culture medium concentrations were 99.5% in a 1 mL volume. In addition, to establish the recovery of anthocyanin degradation products, protocatechuic acid, 4-hydroxybenzoic acid, gallic acid and phloroglucinol aldehyde were spiked to 100 *μ*M in buffer and serum samples. Recoveries were established following filtration, centrifugation and SPE (as outlined below) and were quantified using HPLC/DAD.

Filtration: Sample preparations (1 mL) were filtered through 17mm Cronus PVDF 0.45 *μ*m syringe filters (SMI-Labhut Ltd, Maisemore, UK). The subsequent filtrates were stored at -80 °C until HPLC analysis. Control samples consisted of pre-filtered, acidified, buffer matrices, spiked to 25 *μ*g/mL with either pelargonidin-3-glucoside, cyanidin-3-glucoside or delphinidin-3-glucoside, respectively (1 mL final volume) and exposed to the same thermal conditions as the extracted samples.

Centrifugation. Sample preparations were centrifuged at 10,000 rpm for 20 min and the supernatants stored at -80 ℃ until HPLC analysis. Sample controls consisted of spiked buffer preparations, exposed to the same thermal conditions as the extracted samples, but not exposed to centrifugation.

Solid phase extraction method 1: Solid phase extraction (SPE) was adapted from previously reviewed methods (Mazza, Cacace et al. 2004). Briefly, Strata-X 6 mL/500 mg solid phase extraction cartridges (Phenomenex, Macclesfield, UK) were preconditioned with 1 column volume (6 mL) of methanol followed by 1 column volume of 0.5% HCl in water. Prior to extraction, samples and controls were spiked with 25 *μ*g/mL tran-cinnamic acid as an internal standard. The recovery of the internal standard was established to be 94±3%. SPE cartridges were loaded with 1 mL of 0.5% HCl followed by 1mL of spiked sample preparation. Sample tubes were further rinsed with 1 mL 0.5% HCl and loaded onto the SPE cartridges. Samples were eluted using a peristaltic pump at approximately 1 drop/s, washed with 2

column volumes of 0.5% HCl under a stream of nitrogen. Extraction cartridges were then loaded with 3 ml 0.5% HCl in methanol and allowed to soak for 10 min. Samples were eluted with 7 mL 0.5% HCl in methanol and the eluate evaporated to dryness on a rotary evaporator, re-dissolved in 1 mL initial HPLC mobile phase (details below) and stored at -80 ℃ until HPLC analysis. Sample controls consisted of spiked buffer preparations, exposed to the same thermal conditions as the extracted samples, but not subjected to SPE.

Solid phase extraction method 2 (minimalised-evaporation method). Solid phase extraction was performed as previously stated, with the following alterations; prior to extraction, samples and controls were spiked to 25 *μ*g/mL with 4-hydroxycinamic acid as an internal standard. Samples were eluted with 8 mL acidified methanol and the eluate evaporated to approximately 500 *μ*L on a rotary evaporator. Sample eluates were spiked to 25 *μ*L with 1 mg/mL trans-cinnamic acid as a second internal standard. Sample volumes were adjusted to 1 mL with 0.5% HCl. Actual sample volumes were calculated from the two internal standard concentrations determined by HPLC-DAD.

3.2.3 Anthocyanin freeze-thaw stability

To determine the storage stability of various anthocyanins, acidified buffer solutions (10 mM Na/K phosphate buffer in 2% HCl) were spiked with 150 *μ*M delphinidin-3-glucoside, cyanidin-3-glucoside, pelargonidin-3-glucoside, delphinidin, cyanidin or pelargonidin (individually). Samples were immediately injected onto an HPLC system and subsequently frozen at -80 °C. Samples were re-analysed sequentially for six freeze-thaw cycles. One freeze-thaw cycle consisted of a 24 h storage at -80 ℃ followed by a 20 min defrost at ambient room temperature prior to HPLC analysis.

3.2.4 HPLC/DAD/MS conditions

HPLC analysis was performed on an Agilent 1100 series HPLC equipped with a diode array detector (DAD) and a 4 *μ*m, 250 x 4.6 mm Synergi Max-RP reverse phase column (Phenomenex, Macclesfield, UK) with a SecurityGuard guard cartridge (Phenomenex AJO-6074) for samples from anthocyanin recovery and freeze-thaw experiments and a 4 *μ*m, 250 x 4.6 mm Synergi

Polar-RP (polar-N-capped) reverse phase column (Phenomenex, Macclesfield, UK) with a SecurityGuard guard cartridge (Phenomenex AJO-6074) for samples from anthocyanin degradation experiments. The column temperature was set at 37 ℃ with an injection volu me of 50 μL and mobile phases consisting of 5% formic acid (v/v) in water (solvent A) and 5% formic acid (v/v) in acetonitrile (solvent B). For anthocyanin recovery and freeze-thaw stability experiments the solvent gradient consisted of 2.5% B at 0 min, 25% B at 15 min, 45% B at 20 min and 100% B at 25-30 min; at a flow rate of 1 mL/min. For anthocyanin degradation experiments, the solvent gradient consisted of 0% B at 0 min 0.5% B at 5 min, 8% B at 20 min, 8% B at 28 min, 10% B at 39 min, 13% B at 40 min, 20% B at 44 min, 25% B at 50 min, 50% B at 65 min and 100% B at 75-80 min; at a flow rate of 1 ml/min. Absorbance was recorded at 520, 360 and 280 nm for both HPLC methods.

3.2.4 LC-MS conditions. Following HPLC/DAD analysis, a subset of samples were then injected onto an HPLC-ESI-MS system for structural elucidation, using an Agilent 1100 series HPLC-DAD coupled to an Agilent 1100 series LC/MSD SL single quadrupole mass spectrometer. Electrospray ionization was performed in full scan mode (Mass range: 100-1000 Da) with the following spray chamber conditions: drying gas flow of 13 L/min; nebulizer pressure of 50 Psi; drying gas temperature of 350 °C. Positive mode ionization was used to analyse all anthocyanin species at a capillary voltage of 4000v and a fragmentor setting of 100. Negative mode ionization was used to analyse phenolic acids, aldehyde and anthocyanin intermediates at a capillary voltage of -3000v and a fragmentor setting of 70. HPLC conditions were as stated above with the following alterations. Levels of formic acid in the mobile phases were reduced to prevent ion suppression. Solvent A consisting of 0.1% formic acid in water and solvent B consisting of 0.1% formic acid in acetonitrile. Anthocyanins, phenolic acids and PGA were identified by retention times, optical density ratios ($\lambda_{440}/\lambda_{max}$ for anthocyanins and $\lambda_{280}/\lambda_{max}$ for phenolic acids) and UV/mass spectra in comparison to analytical standards and previously reported data (Harborne 1958; Markham 1982; Mazza and Miniati 1993). Quantification was by HPLC-DAD using

standard curves generated from pure analytical standards with R^2 values greater than 0.98.

3.2.5 Statistical analysis

Differences within anthocyanin species were evaluated by one way ANOVA with Tukey post-hoc tests (Windows SPSS, version 15) on concentration data derived from standard curves. Significance was determined at $P<0.05$. In order to evaluate significant differences between species, all recovery and degradation data were transformed to percent of control values and analysed using one-way ANOVA with Tukey post hoc tests. Differences between anthocyanin degradation within water and buffer matrices were analysed by independent Student's *t*-Tests with a significance threshold of $P<0.05$. All treatments were performed in triplicate, and data were obtained from three independent experiments.

3.3 Results and Discussion

3.3.1 Influence of B-ring hydroxylation on anthocyanidin (aglycone) stability

Anthocyanidin degradation: To assess anthocyanidin stability under simulated physiological conditions, their degradation was determined in both water and in buffered matrices (**Figure 3.2** and **3.3**). In both matrices an instantaneous loss of >50% of the initial anthocyanidins was observed, with 100% degradation occurring by the first time point measurement (2 h); therefore rates of degradation were not established. The loss of anthocyanidins over 24 h was associated with the formation of their respective phenolic acids.

Phenolic acid formation: In water the formation of phenolic acids accounted for 30-40% of initial anthocyanidin molar concentrations, while in buffer the phenolic acid formation accounted for only 8-18% of initial anthocyanidin molar concentrations. In water, pelargonidin [λ_{max} 512nm; *m/z* 271], cyanidin [λ_{max} 524nm; *m/z* 287] and delphinidin [λ_{max} 528nm; *m/z* 303] degraded to yield 53±4.2 *μ*M 4-hydroxybenzoic acid [λ_{max} 254nm; *m/z* 138] (accounting for

38±0.1% pelargonidin loss), 58±1.2 *μ*M protocatechuic acid [λ_{max} 260nm, 294nm; *m/z* 154] (accounting for 39±0.0% of cyanidin loss) and 53±2.0 *μ*M gallic acid [λ_{max} 270nm; *m/z* 170] (accounting for 31±0.0% of delphinidin loss), respectively.

In buffered samples, pelargonidin, cyanidin and delphinidin degraded to yield 25±0.7 *μ*M 4-hydroxybenzoic acid (accounting for 18±0.0% of pelargonidin loss), 32±0.3 *μ*M protocatechuic acid (accounting for 21±0.0% of cyanidin loss) and 14±0.3 *μ*M gallic acid (accounting for 8±0.0% of delphinidin loss), respectively. It should be noted that the formation of gallic acid (associated with delphinidin degradation) increased linearly in water, while in buffer gallic acid showed first an increase in formation followed by a decrease (loss) in concentration between 6-24 h.

Phenolic aldehyde formation: All anthocyanin species degraded to their common A-ring constituent, tentatively identified as phloroglucinol aldehyde [λ_{max} 294nm; *m/z* 154]. After 24 h, phloroglucinol aldehyde formation was 46±1.6 *μ*M in water and 5±1.2 *μ*M in buffered samples (±SD for pelargonidin, cyanidin and delphinidin) accounting for 30% and 3% of initial anthocyanin concentrations, respectively.

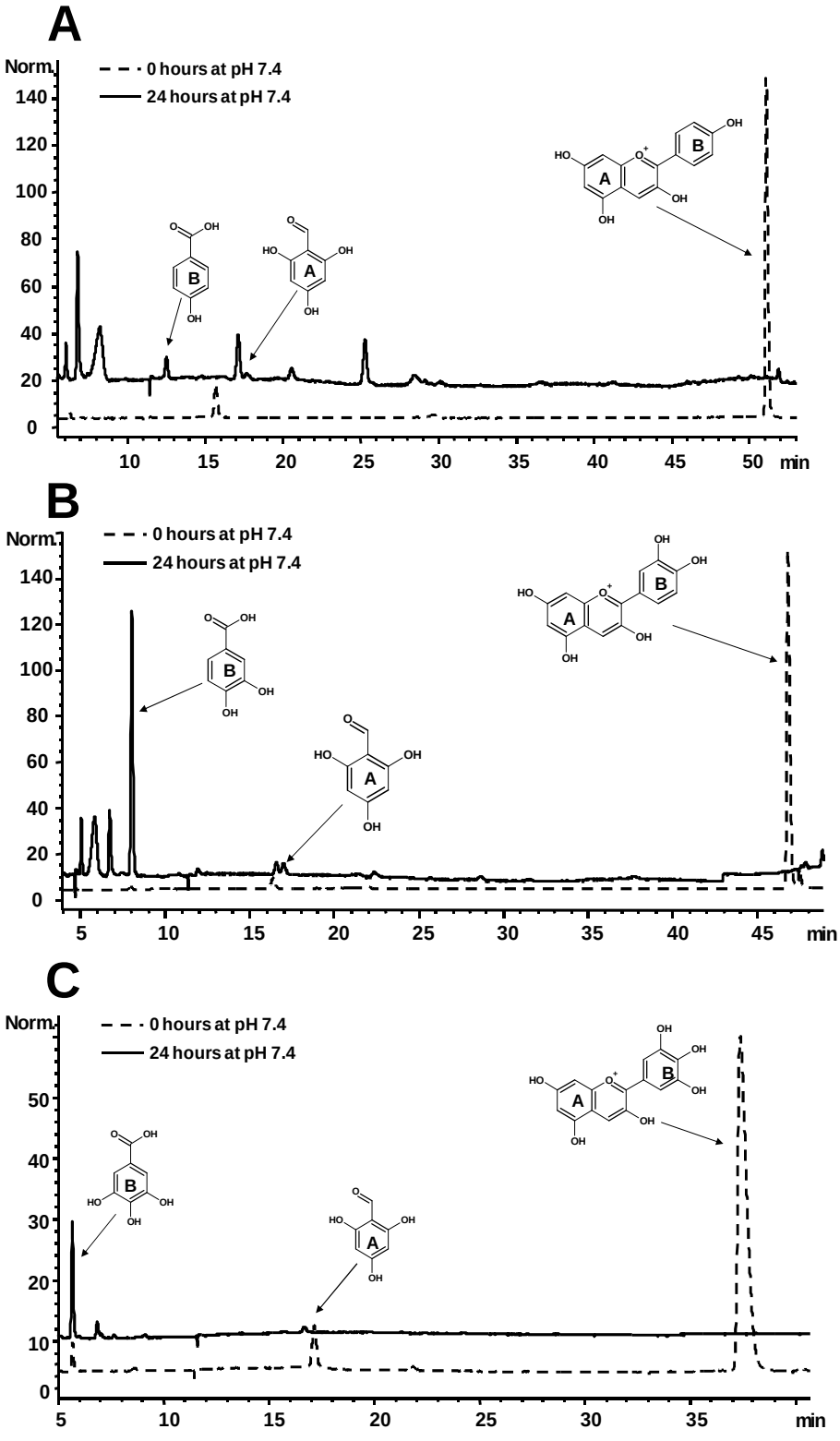


Figure 3.2. Representative chromatograph of anthocyanins and the phenolic degradation products following 24 h incubations at pH 7.4 and 37 °C. A, pelargonidin degradation to 4-hydroxybenzoic acid and phlorogluceraldehyde; B, cyanidin degradation to protocatechuic acid and phlorogluceraldehyde; C, delphinidin degradation to gallic acid and phlorogluceraldehyde.

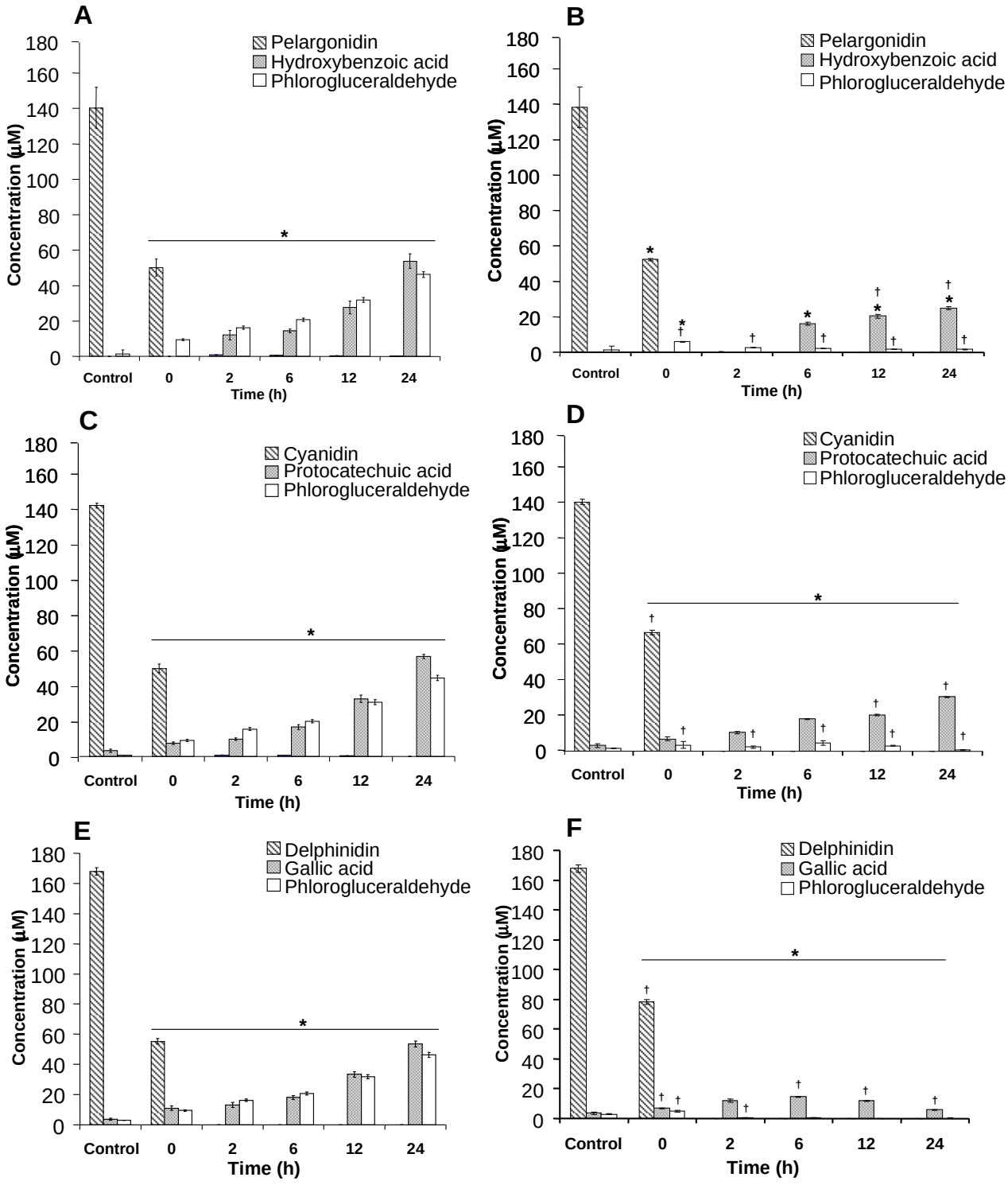


Figure 3.3. Anthocyanidin degradation over 24 h at 37 °C in water (pH 7 with dilute NaOH) and in physiological buffer (10 mM Na/K phosphate buffer, pH 7.4). (A) pelargonidin degradation in water; (B) pelargonidin degradation in buffer; (C) cyanindin degradation in water; (D) cyanidin degradation in buffer; (E) delphinidin degradation in water; (F) delphinidin degradation in buffer. Results expressed as mean±SD (n=3). *Significance from controls ($P<0.05$), which consisted of analytical standards spiked directly into acidified water or buffer matrices at t=0. †Significant difference ($P<0.05$) between samples incubated in water and buffer matrices within a given anthocyanin species and incubation time.

3.3.2 Influence of B-ring hydroxylation on anthocyanin (glycoside) stability

Anthocyanin degradation: The influence of B-ring hydroxylation on the rate of anthocyanin degradation and the degree of phenolic acid formation was assessed under simulated physiological conditions (**Figure 3.4**). The anthocyanins were shown to degrade at a rate of between 3-11 *μ*M per h over the first 12 h, representing a total loss of 20%, 40% and 100% for pelargonidin-3-glucoside [λ_{max} 502nm; *m/z* 433], cyanidin-3-glucoside [λ_{max} 514nm; *m/z* 449] and delphinidin-3-glucoside [λ_{max} 524nm; *m/z* 465], respectively. Individual rates of degradation were 3.3 *μ*M/h for pelargonidin-3-glucoside, 4.5 *μ*M/h for cyanidin-3-glucoside and 11.4 *μ*M/h for delphinidin-3-glucoside.

Phenolic acid formation: In water, the loss of anthocyanins over 24 h was associated with the formation of their respective phenolic acids, which accounted for 12%, 8% and 0% of the initial cyanidin-3-glucoside, delphinidin-3-glucoside and pelargonidin-3-glucoside concentrations, respectively. cyanidin-3-glucoside degraded to yield 15±14.4 *μ*M protocatechuic acid (accounting for 65±27.3% of cyanidin-3-glucoside loss) and delphinidin-3-glucoside degraded to yield 12±9.5 *μ*M gallic acid (accounting for 56±41.7% of delphinidin-3-glucoside loss). There was no significant degradation of pelargonidin-3-glucoside or respective formation of phloroglucinol aldehyde over the 24 h incubation.

In buffer, the appearance of phenolic acids accounted for 10%, 8% and 2% of initial pelargonidin-3-glucoside, cyanidin-3-glucoside and delphinidin-3-glucoside concentrations, respectively. pelargonidin-3-glucoside, cyanidin-3-glucoside and delphinidin-3-glucoside degraded to yield 16±2.5 *μ*M 4-hydroxybenzoic acid (accounting for 12±1.8% of pelargonidin-3-glucoside loss), 11±1.8 *μ*M protocatechuic acid (accounting for 9±1.3% of cyanidin-3-glucoside loss) and 3±0.4 *μ*M gallic acid (accounting for 2±0.0% of delphinidin-3-glucoside loss), respectively.

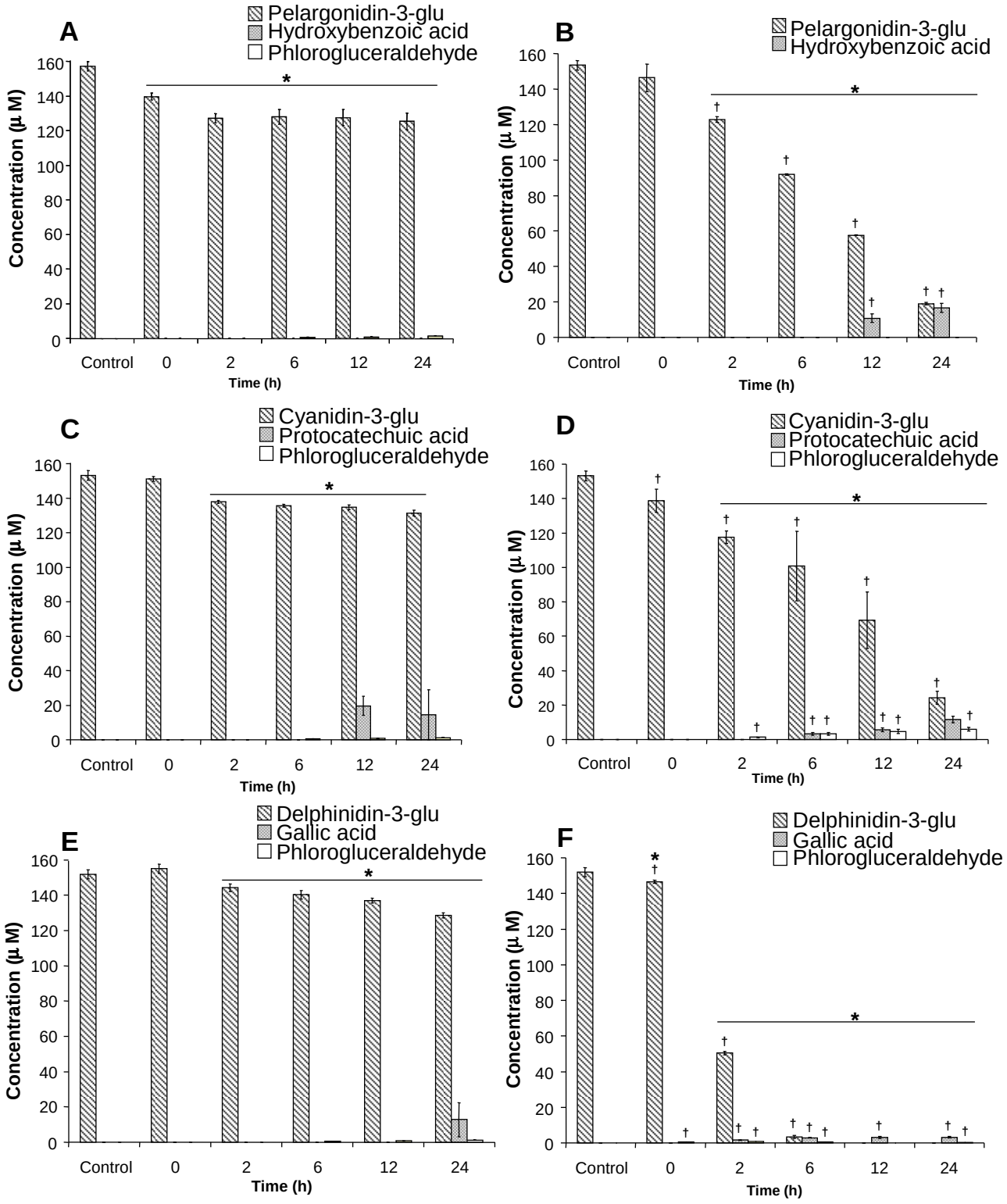


Figure 3.4. Anthocyanin degradation over 24 h at 37 °C in water (pH 7 with dilute NaOH) and in physiological buffer (10 mM Na/K phosphate buffer, pH 7.4). (A) pelargonidin-3-glucoside degradation in water; (B) pelargonidin-3-glucoside degradation in buffer; (C) cyanindin-3-glucoside degradation in water; (D) cyanidin-3-glucoside degradation in buffer; (E) delphinidin-3-glucoside degradation in water; (F) delphinidin-3-glucoside degradation in buffer. Results expressed as mean±SD (n=3). *Significance from controls ($P<0.05$), which consisted of analytical standards spiked directly into acidified water or buffer matrices at t=0. †Significant difference ($P<0.05$) between samples incubated in water and buffer matrices within a given anthocyanin species and incubation time.

Phenolic aldehyde formation: The degradation of cyanidin-3-glucoside, delphinidin-3-glucoside and pelargonidin-3-glucoside was associated with the appearance of a common constituent in buffered matrices, however, its formation was not significant in pelargonidin-3-glucoside samples. The maximum phloroglucinol aldehyde formation was 5±1.1 *μ*M for cyanidin-3-glucoside and 1±0.1 *μ*M for delphinidin-3-glucoside, accounting for 3% and 0.6% of initial anthocyanin concentrations. No significant phloroglucinol aldehyde formation was observed for any of the anthocyanin species in water.

The degradation of the three anthocyanin species as described above was associated with the appearance of a common constituent, which was identified as phloroglucinol aldehyde, based on its retention time, UV-vis and MS characteristics. It is to be noted that phloroglucinol aldehyde appeared as a split peak, which could have been the result of the ionization characteristic of the aldehyde structure under acidic aqueous conditions, where the dynamic equilibrium of the hydrated form and hetero-dimmers are likely to occur (Bell and McDougall 1960; King, Woodside et al. 1974).

The degradation of all anthocyanin aglycone and glucoside species was also associated with the appearance of three unidentified compounds, eluting at similar retention times. Since these compounds were common to all anthocyanin species in this study, they are likely to be derived from the common anthocyanin A-ring. However, mass fragmentation ions could only be obtained for one of the three unidentified compounds [R_t 6.6 min; *m/z* 198], since the remaining two unidentified compounds showed no ion fragments in both positive and negative ionization modes.

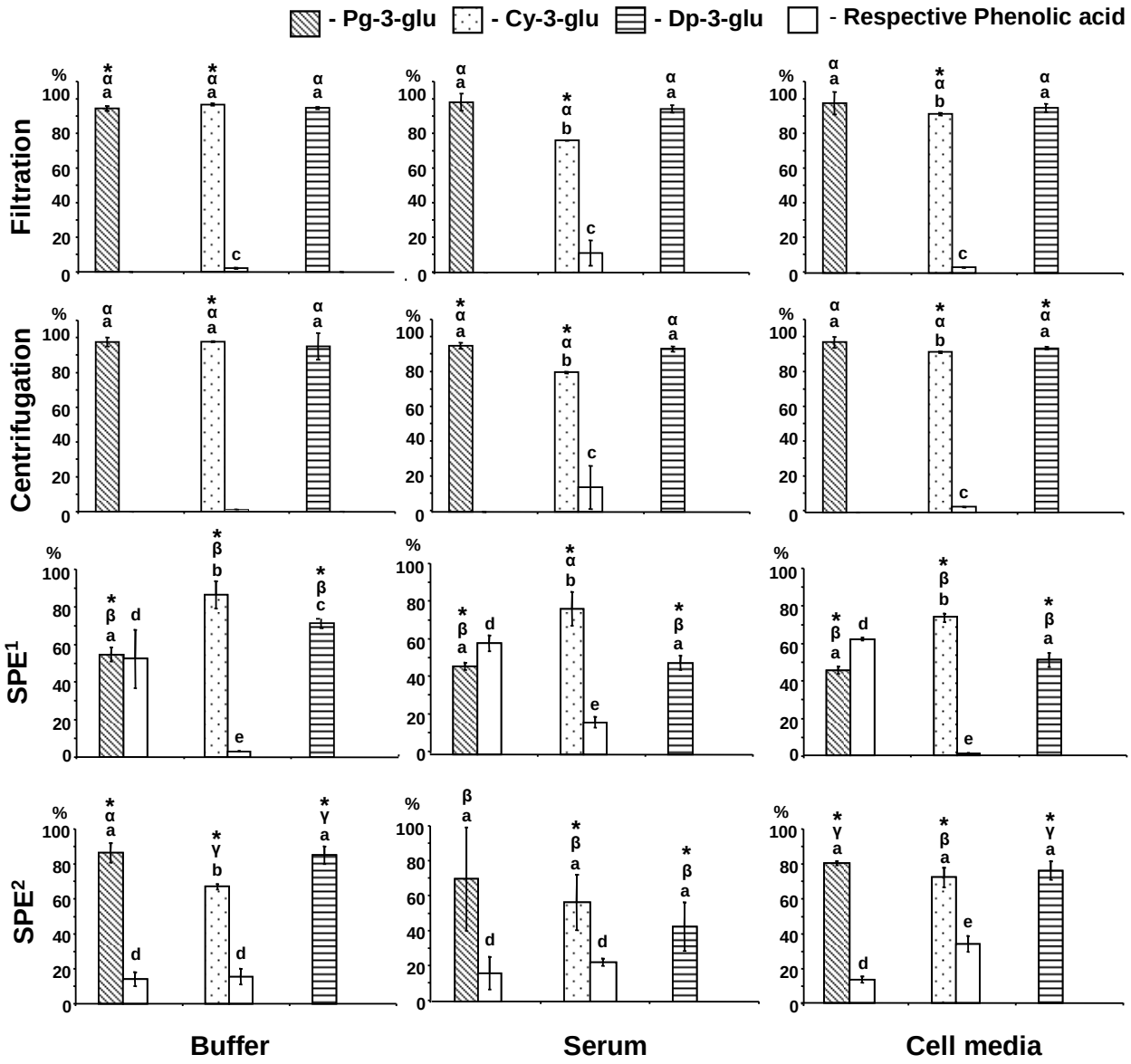


Figure 3.5. Anthocyanin recovery (%) and the formation of their respective phenolic acid constituents following filtration, centrifugation and SPE in phosphate buffer (pH 2), foetal calf serum (pH 2) and cell culture media (pH 2). Values expressed as means±SD (n=3). *Significance from controls ($P<0.05$). [a-e] Like letters represent statistically similar recoveries between anthocyanin species within a given preparation technique and matrix. [α,β,γ]Like symbols represent statistically similar recoveries between preparation techniques for a given anthocyanin species within a given matrix. [1]Solid phase extraction method with complete evaporation. [2]Solid phase extraction method with minimalised-evaporation. Controls consisted of anthocyanins spiked into pre-extracted buffer matrices.

3.3.3 Anthocyanin recovery following routine sample preparation techniques

To assess the effects of B-ring hydroxylation on the recovery of anthocyanins during routine pre-analytical sample preparation, pelargonidin-3-glucoside, cyanidin-3-glucoside and delphinidin-3-glucoside recovery and degradation was measured following filtration, centrifugation and SPE in buffer, serum and cell culture matrices (**Figure 3.5**). No significant differences in recovery were observed between pelargonidin-3-glucoside and delphinidin-3-glucoside following filtration and centrifugation, although cyanidin-3-glucoside did show significantly lower recovery (relative to pelargonidin-3-glucoside and delphinidin-3-glucoside) in serum and cell media matrices. No significant difference in anthocyanin recovery was observed between filtered and centrifuged samples, for a given anthocyanin species. All anthocyanin species showed significant loss following routine SPE, while SPE with an incomplete concentration/evaporation step (i.e. not taken to dryness) significantly improved their stability by 32%, 29% and 18% for pelargonidin-3-glucoside (in buffer, serum and cell media, respectively), and 14%, 19% and 0% for delphinidin-3-glucoside (in buffer, serum and cell media, respectively). Cyanidin-3-glucoside did not show an improvement in recovery. We speculate that this is likely due to the chelating and/or protein binding properties of the catechol group on the anthocyanin B-ring (Ito, Kato et al. 1988).

The losses of anthocyanins following sample preparation were accounted for by a stoichiometric appearance of their respective phenolic acid constituents, where pelargonidin-3-glucoside and cyanidin-3-glucoside were recovered as 4-hydroxybenzoic acid and protocatechuic acid, respectively (**Figure 3.4**). delphinidin-3-glucoside degradation to gallic acid was not observed following SPE. To assess the recovery of the phenolic acids following SPE, buffer and serum samples were spiked with 100 μM standards of protocatechuic acid, 4-hydroxybenzoic acid and phloroglucinol aldehyde, respectively. The recovery of protocatechuic acid, 4-hydroxybenzoic acid and phloroglucinol aldehyde standards were established to be 92±2.8%, 92±2.5% and 78±2.6% in buffered samples and 89±2.3%, 115±2.5% and 68±2.8% in serum samples, respectively. Note that the recovery of 4-hydroxybenzoic acid in serum was

>100%, which may have resulted from additional endogenous 4-hydroxybenzoic acid liberated from serum samples following methanol treatment during SPE, as recoveries were compared to spiked buffer controls.

Whilst the loss of anthocyanins could be accounted for by the recovery of their respective B-ring phenolic acid constituents, phloroglucinol aldehyde (the A-ring constituent) was not consistently identified in post-extraction samples (i.e., post SPE). In addition, the disappearance of anthocyanins was associated with the formation of an additional unidentified peak [λ_{max} 272nm; R_t 21.5 min; *m/z* 189, 226.9, 248.8] which was similar to the compound formed when phloroglucinol aldehyde was incubated in the presence of acidified methanol [λ_{max} 272 nm; T_R 22.4 min; *m/z* 187,189] (**Figures 3.6 and scheme 3.1**).

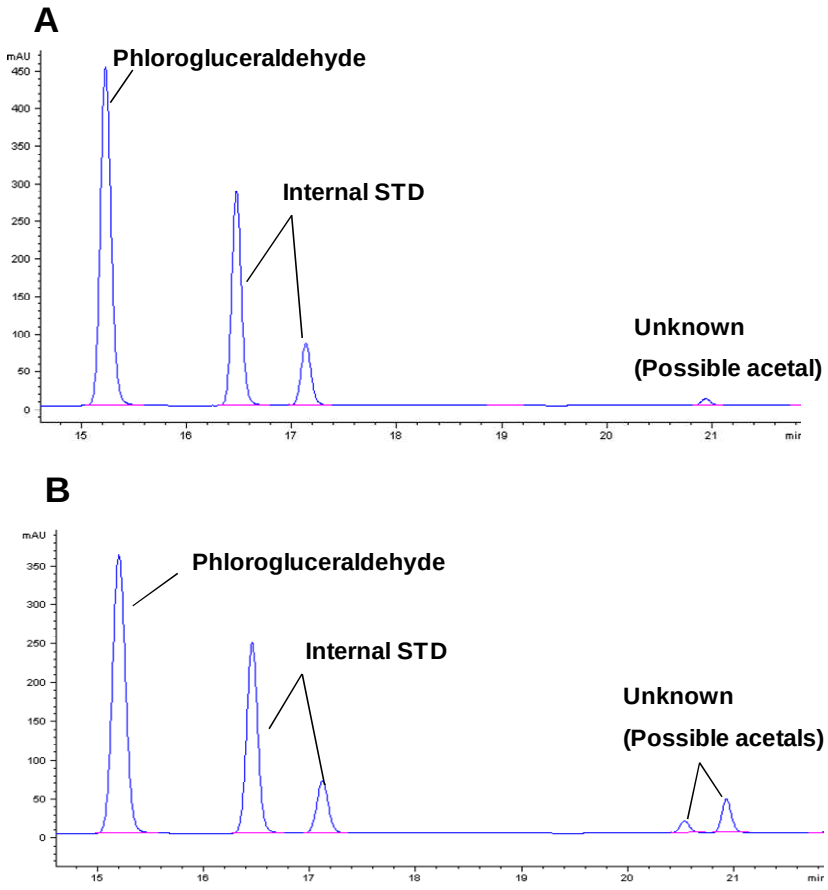
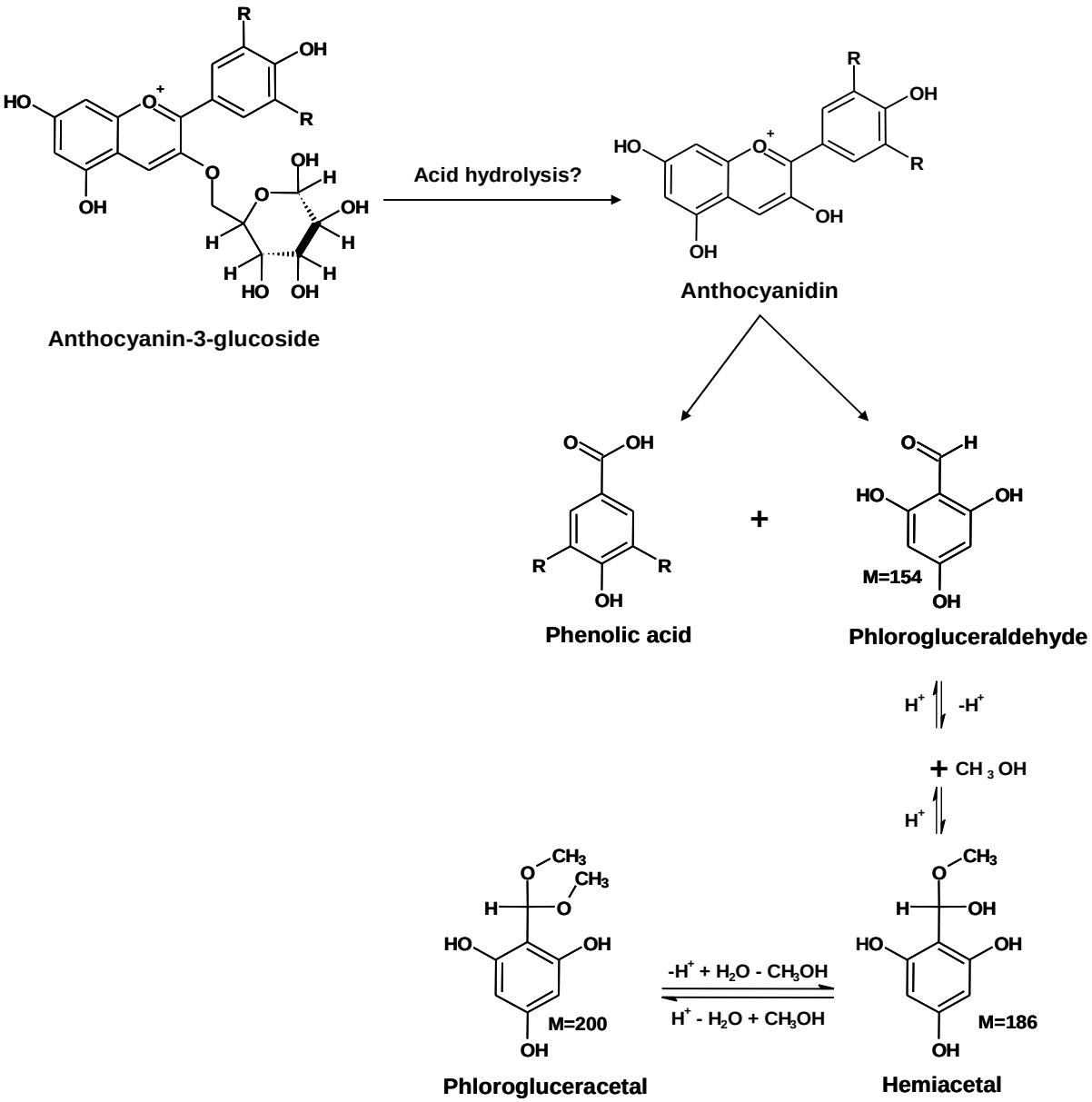


Figure 3.6. Representative chromatograph for the formation of additional phlorogluceraldehyde derived peaks under non-acidified (A; water:methanol) and acidified (B; 1% formic acid in water:methanol) SPE extraction conditions (post-SPE chromatographs). SPE, solid phase extraction.



Scheme 3.1. Proposed scheme for the formation of phlorogluceraldehyde derived hemiacetals and acetals under acidified solvent extraction conditions.

3.3.4 Anthocyanin and anthocyanidin freeze-thaw stability

To assess the influence of B-ring hydroxylation on anthocyanin freeze-thaw stability, anthocyanin species, with mono-, di- and tri-hyroxylated B-rings were frozen and thawed six times and their degradation assessed by HPLC/DAD (**Figure 3.7**). It was found that anthocyanin glucosides were stable during storage and freeze-thaw treatment, with no significant losses observed. Of the three aglycone species, both pelargonidin (P=0.001) and cyanidin (P=0.026) showed a significant loss following freeze-thaw cycling, while Del remained stable. pelargonidin showed a linear rate of degradation, with significant

losses demonstrated at four freeze-thaw cycles and a total reduction of 17 μM (11±0.0%) at six freeze-thaw cycles (representing an average loss of 1.8% per cycle). cyanidin also showed a linear rate of degradation, with a maximum reduction of 10 μM (6±0.0%) at six freeze thaw cycles (representing an average loss of 1.2% per cycle).

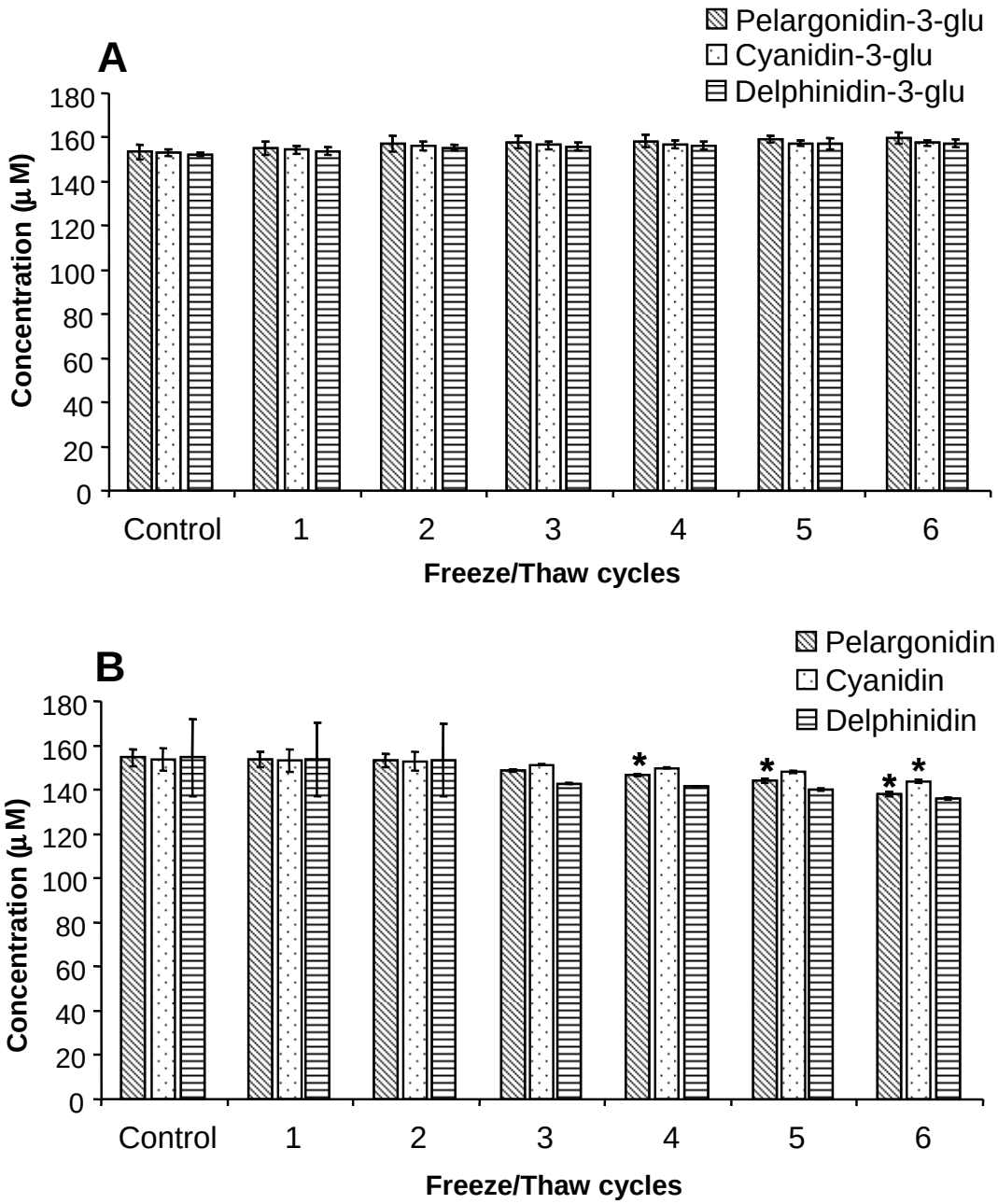


Figure 3.7. Anthocyanin (A) and anthocyanidin (B) freeze thaw stability. * represents significance ($p<0.05$) with respect to control. Data represents mean±SD (n=3).

On the whole, anthocyanins have consistently been shown to possess a very low bioavailability (Mazza, Kay et al. 2002; Kay, Mazza et al. 2004; Kay, Mazza et al. 2005; Vitaglione, Donnarumma et al. 2007). We propose that the poor systemic availability of anthocyanins is due to their instability and physio-chemical degradation *in vivo* or during routine clinical and laboratory sample processing. Therefore this study aimed to quantitatively investigate the effect of structure on the degradation of anthocyanins under simulated (*in vitro*) physiological conditions and assess the degradation and recovery of selected anthocyanins following pre-analytical sample treatment and storage.

Evidence presented here shows that under simulated (*in vitro*) physiological conditions (pH and temperature), anthocyanidins degraded at much higher rates than their glucoside counterparts; which was consistent with previously reported data (Markakis 1974; Seeram, Bourquin et al. 2001; Keppler and Humpf 2005; Fleschhut, Kratzer et al. 2006). Indeed, the rate of degradation varied according to the B-ring hydroxylation pattern such that an increase in hydroxylation was associated with a decrease in stability. Furthermore, this study demonstrates that anthocyanin degradation under physiological conditions is associated with the stoichiometric formation of phenolic acid constituents. As the observed rates of anthocyanidin degradation to phenolic acids was rapid, it is likely that these compounds are present at far higher concentrations than anthocyanidins/anthocyanins in processed foods, the gut and the systemic circulation. Although it is likely that pH and temperature are the chief contributors to anthocyanin degradation under biological conditions, the extent to which other physiological forces, such as protein interactions and enzymatic or microbial transformation, contribute to anthocyanin degradation is yet to be fully elucidated.

Phloroglucinol aldehyde represented the A-ring constituent of anthocyanins in the present investigation; however, phloroglucinol aldehyde may also represent the A-ring of other similarly structured flavonoids, although their occurrence in human-flavonoid studies is yet to be determined. Due to the instability of anthocyanins relative to other flavonoids in the diet, it is likely that the majority of phloroglucinol aldehyde in clinically derived samples would be

derived from anthocyanins. Further study is however required to fully establish the extent to which phloroglucinol aldehyde formation is derived from the degradation of anthocyanins and other flavonoids within the diet.

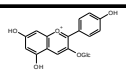
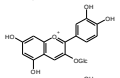
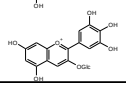
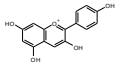
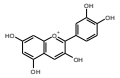
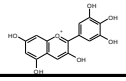
An apparent lack of appreciation for the rate and extent of anthocyanin degradation under physiological conditions may explain the almost complete absence of data aimed at quantifying anthocyanin breakdown products in human samples (tissue, urine, plasma) and the current lack of reports concerned with the biological activities of these compounds and their impact, as dietary constituents on human health. We contend that anthocyanins may be regarded as “pro-drugs” for the delivery of bioactive phenolic intermediates and this should be a focus for future research.

In this report we provide evidence to show that anthocyanins degrade at a faster rate in buffered solution than in water. However, the rate of end stage degradation product (phenolic acids) formation was relatively slower in buffered solution than in water. These observations imply that the buffered environment confers a greater stability to the pH dependent anthocyanin isoforms (including chalcone, hemiketal and α-diketone intermediate structures). However, it is possible that slight differences in sample pH could account for some of the differences in anthocyanin stability as observed in the present investigation. As biological samples (i.e. serum, urine, and cell culture media) possess a degree of buffering agents, the degradation characteristics of anthocyanins in these matrices should be considered in light of the current finding, where anthocyanins may demonstrate differential degradation rates in other matrices such as foods, which will be dependent on their individual buffering characteristics.

The data presented here show that B-ring hydroxylation significantly affected the recovery of anthocyanins following routine SPE. This observation suggests that an underestimation of anthocyanin levels in clinically derived samples is possible when employing SPE. Further, we observed that the disappearance of anthocyanins was accompanied by the stoichiometric formation of their respective phenolic acid breakdown products, demonstrating

that degradation rather than poor recovery was the foremost cause of anthocyanin loss following SPE, particularly in the case of cyanidin-3-glucoside. This highlights the importance of establishing method accuracy and precision when estimating anthocyanin levels in clinical studies. Our data suggested that the degradation of anthocyanins observed during SPE may be the result of sample evaporation. We provide direct evidence that evaporation of SPE eluents to dryness contributes significantly to loss of anthocyanins, and that recoveries can be substantially improved by partially evaporating SPE eluents. This may be the result of acid concentration and eventual hydrolysis in non-neutralized samples during post-SPE evaporation. Thus, it is clear that both sample preparation technique and anthocyanin structure contribute to their experimental recovery. Consequently, these factors should be considered when analysing clinical or biological samples (such as cell culture medium, serum/plasma or urine) in order to avoid the underestimation of anthocyanin bioavailability.

Table 3.1. Summary of Anthocyanin stability and recovery characteristics.

Compound	Structure	Physiological stability[a,d]	Freeze/Thaw stability[a,e]	Recovery[b]	Extraction method	Degradation products	Clinical implications
Pelargonidin-3-O-glucoside		++++	+++++	+++++ +++++ ++++	Filtration Centrifugation SPE[c]	4-Hydroxybenzoic acid Phloroglucinol aldehyde	1. Degrades to phenolic acids in clinical samples prior to acidification. 2. Risk of underestimating bioavailability following SPE.
Cyanidin-3-O-glucoside		+++	+++++	+++ ++++ +++	Filtration Centrifugation SPE[c]	Protocatechuic acid Phloroglucinol aldehyde	
Delphinidin-3-O-glucoside		+	+++++	+++++ ++++ +++	Filtration Centrifugation SPE[c]	Gallic acid Phloroglucinol aldehyde	
Pelargonidin		+	+++	— — —	Filtration Centrifugation SPE[c]	4-Hydroxybenzoic acid Phloroglucinol aldehyde	1. Quickly degrades to phenolic acids in clinical samples prior to acidification. 2. Care required during extraction/analysis to avoid phenolic acid clinical samples
Cyanidin		+	++++	— — —	Filtration Centrifugation SPE[c]	Protocatechuic acid Phloroglucinol aldehyde	
Delphinidin		+	+++++	— — —	Filtration Centrifugation SPE[c]	Gallic acid Phloroglucinol aldehyde	

[a]Representation of stability: *(unstable)* +<++<+++<++++<++++ *(stable).* [b]Representation of compound recovery from serum following pre-analytical extraction techniques: *(low recovery)* +<++<+++<++++<+++++ *(high recovery).* [c]Solid phase extraction method 2. [d]Stability determined at 37ºC and pH 7.4. [e]Stability determined following 6 freeze/thaw cycles (-80ºC and pH 2). (—), not determined. Refer to results section for further details.

Whilst some studies have evaluated the storage stability of anthocyanins in various food matrices (Kalt, Forney et al. 1999; de Ancos, Ibanez et al. 2000; Eiro and Heinonen 2002), no studies have formally reported their storage stability under clinically relevant conditions. Thus, this study has shown that

anthocyanins are stable when stored at -80 ℃ and ≤pH 2, whilst anthocyanidins are relatively less stable. This indicates that the poor recovery of anthocyanins following clinical feeding trials is not likely to be the result of degradation in stored samples but the result of degradation *in vivo* or during initial sample processing.

In conclusion, this report demonstrates that anthocyanins are rapidly degraded to their phenolic acid and aldehyde constituents under simulated (*in vitro*) physiological conditions and that increased anthocyanin B-ring hydroxylation is associated with a decrease in stability; these observations have major implications for the design and interpretation of dietary intervention studies utilising anthocyanins (**Table 3.1**). Furthermore, anthocyanin losses during sample preparation were accounted for by the stoichiometric appearance of their phenolic acid degradation products, particularly during SPE. Hence we suggest that the degradation of anthocyanins and the representative formation of their phenolic acid constituents be of important consideration when investigating anthocyanins within clinical and laboratory settings, and that these degradation products be targeted in future bioavailability and bioactivity studies in order to establish their true occurrence and influence on human health and disease.

Chapter 4

Anthocyanin derived phenolic acids form glucuronides following simulated gastrointestinal digestion and microsomal glucuronidation

Data published in *Mol. Nutr. Food Res.* (in press).

4.1 Introduction

In recent years it has been accepted that inflammation plays a central role in the development of chronic diseases. As such, novel pharmaceutical and dietary strategies are being developed to prevent the progression of chronic inflammatory conditions, such as atherosclerosis. Among these strategies, dietary polyphenols, including anthocyanins, have been shown to be associated with reduced chronic disease risk and mortality (Rimm, Katan et al. 1996; Lin, Rexrode et al. 2007; Mink, Scrafford et al. 2007). However, current evidence in the literature poorly describes the human metabolism of anthocyanins, with no information on the biological effects of their metabolites. Thus, a full appreciation of the health-related benefits of ingested anthocyanins has yet to be achieved and requires a greater understanding of their metabolism and associated mechanisms of action.

It has been demonstrated that less than 1% of ingested anthocyanins are detectable in urine or plasma (Kay, Mazza et al. 2004) and it has been suggested that anthocyanins form intermediate or end stage degradation products during digestion, absorption and metabolism that have yet to be sufficiently established *in vitro* or *in vivo* (Kay, Kroon et al. 2009). To this end the anthocyanin degradation products, protocatechuic acid and 4-hydroxybenzoic acid (Tsuda, Horio et al. 1999; Ichiyanagi, Rahman et al. 2004) (**Figure 4.1**), have been identified in the plasma of rats following high dose consumption of cyanidin and pelargonidin (**Figure 4.1**). Furthermore, data from a recent study by Vitaglione *et al.* explains the low systemic presence of anthocyanins in humans, as almost 73% of ingested cyanidin was accounted for by the formation of protocatechuic acid. This suggests that ingested anthocyanins are primarily available to the systemic circulation as phenolic acid derivatives. However, the fate of these derivatives remains unknown. Indeed, current opinion suggests that anthocyanins are likely metabolised to their phenolic acid constituents (Tsuda, Horio et al. 1999; Vitaglione, Donnarumma et al. 2007; Han, Ryu et al. 2009; Kay, Kroon et al. 2009). We propose that the low recovery of anthocyanins post-consumption

may not only be accounted for by the formation of free phenolic acids but also their conjugated metabolites following absorption and/or first pass metabolism. Human studies have yet to demonstrate the phase II conjugation of the anthocyanin derived phenolic acids, protocatechuic acid and 4-hydroxybenzoic acid, but a recent study has reported the *in vivo* metabolic conjugation of protocatechuic acid to monoglucuronide, methyl-monoglucuronide and methyl-monoglycine conjugates in rats (Cao, Zhang et al. 2009).

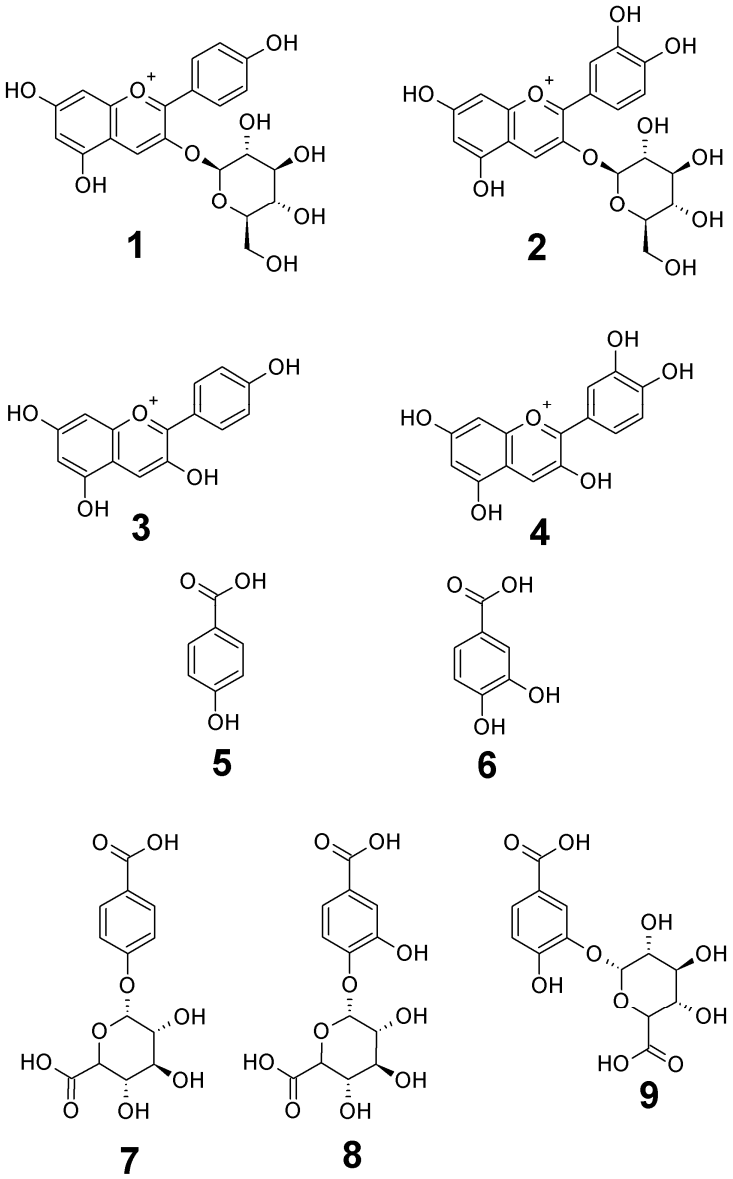


Figure 4.1. Chemical structures of anthocyanins and their potential metabolites. 1, pelargonidin-3-glucoside; 2, cyanidin-3-glucoside; 3, pelargonidin; 4, cyanidin; 5, 4-hydroxybenzoic acid; 6, protocatechuic acid; 7, 4-(1-carboxyphenyl) β-D-glucuronic acid; 8, 4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid; 9, 3-(1-carboxy-4-hydroxyphenyl) β-D-glucuronic acid.

In the current study the potential degradation of anthocyanins (pelargonidin, cyanidin, pelargonidin-3-glucoside and cyanidin-3-glucoside) during the early stages of gastro-intestinal digestion was explored using a simulated *in vitro* GIT digestion model. The potential for anthocyanin derived degradation products, 4-hydroxybenzoic acid and protocatechuic acid, to undergo hepatic glucuronidation, both as free phenolic acids and as constituents of their precursor anthocyanidin structures was also evaluated. Furthermore, in order to assess the conjugation position of the phenolic acid metabolites, the glucuronide derivatives of protocatechuic acid and 4-hydroxybenzoic acid, namely 4-(1-carboxyphenyl) β-D-glucuronic acid, 4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid and 3-(1-carboxy-4-hydroxyphenyl) β-D-glucuronic acid (**Figure 4.1**), were chemically synthesised and compared to the biologically derived metabolites.

The focus of the present study was to establish the potential sites of metabolic conjugation of anthocyanins and their products of degradation. Therefore, the utilised experimental system was designed and optimised for maximal conjugation efficiency of each of the phenolic substrates. The system was not designed to mimic in vivo situations, but to establish potential targets for searching for biological metabolites of anthocyanins in future human trials and/or cell studies aimed at unravelling the mechanisms behind anthocyanins' proposed bioactivity.

4.2 Materials and Methods

For metabolism and digestion studies, pooled human liver microsomes (male), pepsin, panceatin, bile extract, alamethicin, saccharolactone and 4-uridine 5′-diphosphoglucuronic acid trisodium salt (UDP-GA) were purchased from Sigma Aldrich (UK). The phenolic compounds 4-hydroxybenzoic acid, protocatechuic acid, kaempferol, cyanidin chloride, pelargonidin chloride, cyanidin-3-glucoside chloride and pelargonidin-3-glucoside chloride were purchased from Extrasynthese (France) or Sigma Aldrich (UK). For chemical synthesis, methyl 2,3,4-tri-O-acetyl-β-D-glucopyranosylurate bromide was

purchased from Apollo Scientific Ltd. (UK) and methyl 4-hydroxybenzoate and methyl 3,4-dihydroxybenzoic acid was purchased from Alfa Aesar (UK). All water used was 18MΩ/cm milliQ water and solvents were of HPLC grade.

4.2.1 Anthocyanin degradation during simulated gastrointestinal digestion

To assess the degradation of anthocyanins during gastrointestinal digestion, cyanidin-3-glucoside, pelargonidin-3-glucoside, cyanidin and pelargonidin were, individually, subjected to a simulated gastro-intestinal digestion. Simulated digestions were based on previously established methods with modifications (Glahn, Lee et al. 1999). Briefly, to purify pepsin for gastric phase digestion, 0.1 g of pepsin was made up to 2.5 ml in 0.1 M HCl, added to 1.25 g Chelex-100 and shaken gently on a platform tabletop shaker (EMLI, Sky Line) for 30 min to remove any residual iron that may chelate the catechol anthocyanins. The solution was then poured into a 1.6 cm diameter glass column (acid washed) and the eluent collected. A further 2.5 ml of 0.1 M HCl was added to the column and the filtrate collected. The final pepsin solution (filtrate) was 20 mg/mL pepsin. To purify pancreatin for duodenal (intestinal) digestion, 0.0125 g of pancreatin and 0.075 g of bile extract was made up to 6.25 ml with 0.1 M $NaHCO_3$ and shaken gently with 3.125 g Chelex-100 for 30 min. The pancreatin/bile solution was then poured into a 1.6 cm diameter glass column to filter out the chelex. An additional 2.5 ml of 0.1 M $NaHCO_3$ was added to the column and the eluent collected. The final pancreatin/bile solution contained 1.4 mg/mL pancreatin and 8.6 mg/mL bile extract. Both enzyme solutions were stored at 4 °C and used within 24 hours.

To simulate gastric phase digestion, the individual anthocyanins (final concentrations of 200 µM) were added to a 140 mM NaCl, 5 mM KCl buffer solution (pH 2) containing 0.15 ml pepsin solution and 25 µg trans-cinnamic acid as an internal standard (3.15 ml final volume). The individual solutions were incubated at 37 °C for 30 min on a platform oscillating tabletop shaker (EMLI, Sky Line) and at 0, 15 and 30 min, 500 µl aliquots were taken, acidified with 0.2 M HCl in ethanol (1:1 v/v final sample dilution) and stored at -80 °C

until HPLC analysis. To simulate duodenal (intestinal) digestion, the pH of the gastric phase digest was titrated to pH 6.7 by the addition of 1 M $NaHCO_3$, followed by the addition of 0.417 ml pancreatin/bile solution (0.6 mg pancreatin and 3.6 mg bile extract). The digest was made up to a final volume of 2.5 ml with 140 mM NaCl, 5 mM KCl buffer solution (pH 6.7, degassed under vacuum for 30 min prior to use) and incubated for 120 min at 37 °C on a platform oscillating tabletop shaker. At 0, 60 and 120 min, 500 µl aliquots were removed from the incubation mixture and acidified with 0.2 M HCl in ethanol (1:1 v/v final sample dilution) and stored at -80 °C until HPLC analysis. Control samples consisted of initial samples (0 min) spiked with 0.1 M HCl and stored at -80 °C until HPLC analysis. Sample concentrations were normalised to the final incubation volumes for the gastric and intestinal phases respectively. For quantification, samples were thawed at room temperature, vortexed and centrifuged at 10,000 rpm for 20 min at room temperature and the supernatants analysed by HPLC.

4.2.2 Microsomal conjugation of anthocyanidins and phenolic acids

Microsomal conjugation was performed as previously described (Day, Bao et al. 2000; Oliveira and Watson 2000) with some adjustments. Substrate concentration and incubation times were optimized for each respective compound (cyanidin, pelargonidin, 4-hydroxybenzoic acids and protocatechuic acid) to achieve optimal concentrations and chromatographic clarity of phenolic acid glucuronides. Briefly, within a 0.1 M potassium phosphate buffer (pH 7.4), 0.25 mg human liver microsomes and 12.5 µg alamethicin were vortexed and placed on ice for 15 min. $MgCl_2$ (1mM), saccharolactone (5 mM) and the respective test compounds (1-2.5 mM) were added (as individual treatments) and each mixture was pre-incubated for 3 min at 37 ℃. To initiate the reaction, uridine 5′-diphosphoglucuronic acid (UDP-GA; 5 mM) was added to give a final incubation volume of 0.1 mL. The reaction mixtures were then incubated at 37 ℃ for 4-12 h. Control incubations were performed without the addition of UDP-GA. Incubations were also performed with 0.3 mM kaempferol under the above stated conditions as a reaction control, since its microsomal conjugation has previously been

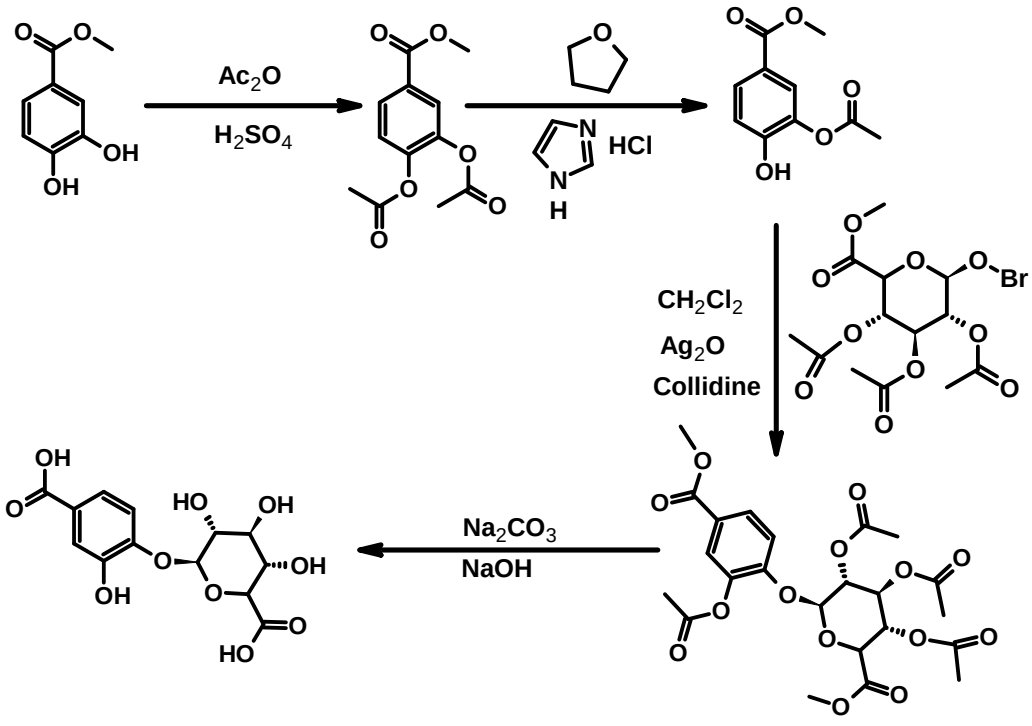
characterized. The reactions were stopped with the addition of 100 µL ice cold 1% HCl in acetonitrile. After equilibration on ice for 15 min, stopped reaction mixtures were centrifuged (13,000 rpm for 15 min at room temperature) to pellet precipitate proteins and the supernatants analysed by HPLC.

4.2.3 Chemical synthesis of phenolic acid glucuronides

In general, all solvents were dried over freshly activated 3Å molecular sieves. Evaporations were performed *in vacuo* at 50℃ and solids were dried overnight *in vacuo* over P_2O_5 before use. TLC was performed on Macherey-Nagel Silica Gel 60/UV254 plates using UV light, or 50% sulphuric acid and charring for visualisation. Flash chromatography used pre-packed silica cartridges (Isolute Flash Si, BiotageTechnologies) and UV detection. The final reaction products were characterised by ^{1}H NMR, run on a JEOL GX-400 spectrometer at room temperature. NMR data interpretation was conducted by Dr. Paul Needs (Institute of Food Research, Norwich, UK).

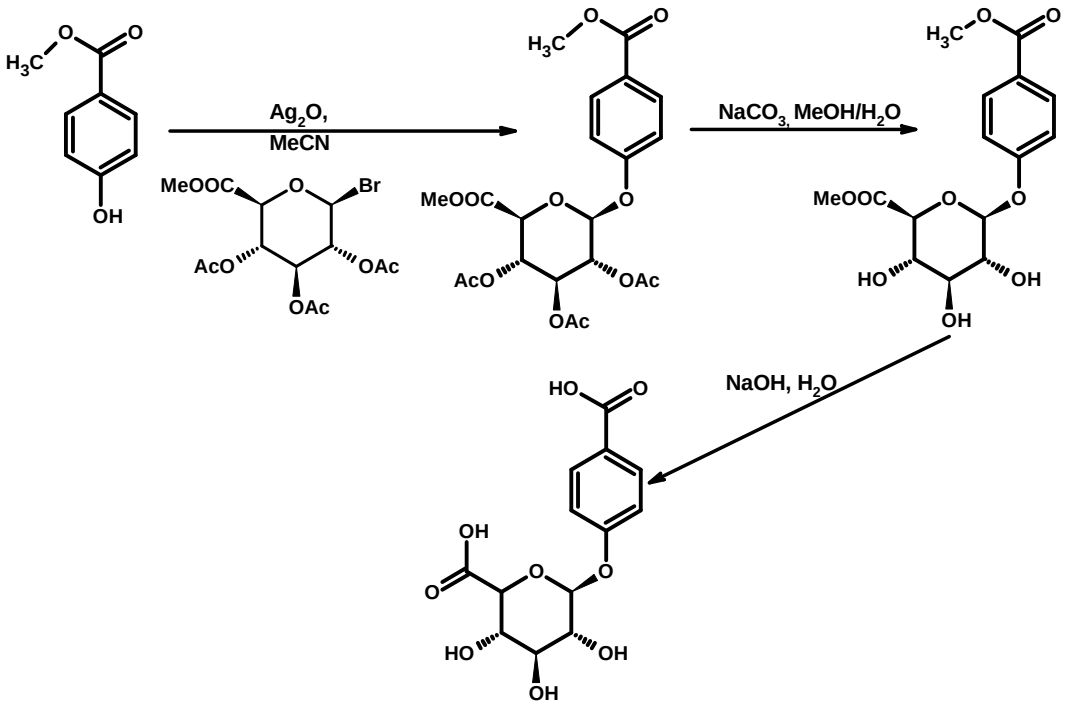
4-(1-Carboxy-3-hydroxyphenyl) β-D-glucuronic acid synthesis (**Scheme 4.1**). Briefly, methyl 3,4-dihydroxybenzoate (3 g, 17.8 mmol) was stirred in acetic anhydride (50 ml) at room temperature and H_2SO_4 (5 ml) was added drop wise. After 40 min the mixture was poured into ice-water (250 ml) and extracted with EtOAc (2 x 200 ml). The combined organic extracts were washed with water (2 x 100 ml) and dried ($MgSO_4$). Evaporation, followed by co-evaporation with toluene produced methyl 3,4-diacetoxybenzoate [^{1}H NMR: ($CDCl_3$) 2.30 (6H, s, 2 x $COCH_3$), 3.90 (3H, s, OCH_3), 7.29 (1H, d, J =10.4 Hz, H-5), 7.87 (1H, d, J = 1.8 Hz, H-2), 7.94 (1H, dd, H-6)]. Subsequently, methyl 3,4-diacetoxybenzoate (1 g, 3.96 mmol) was dissolved in a mixture of THF (50 ml) and imidazolium hydrochloride (pH 7; 50 ml) at room temperature. After 30 min the mixture was extracted with CH_2Cl_2 (2 x 200 ml) and the combined extracts were washed with water (100 ml), $NaHCO_3$ (100 ml) and water (100 ml), dried ($MgSO_4$) and evaporated. Purification by flash chromatography (100 % CH_2Cl_2 to 5% MeOH in CH_2Cl_2) gave pure methyl 3-acetoxy-4-hydroxybenzoate [^{1}H nmr: ($CDCl_3$) 2.37 (3H, s, $COCH_3$), 3.87 (3H, s, OCH_3), 7.02 (1H, d, J =8.6 Hz, H-5), 7.82 (1H, d, J = 1.8 Hz, H-2), 7.84 (1H, dd, H-6)]. Methyl 3-acetoxy-4-hydroxybenzoate (0.50 g,

2.38 mmol) was dissolved in dry CH_2Cl_2 (25 ml) under Ar gas at room temperature. Subsequently, powdered 3Å molecular sieves (0.5 g), methyl 2,3,4-tri-O-acetyl-α-D-glucopyranosyluronate bromide (1.04 g, 1.1 eq.), silver (I) carbonate (0.72 g, 1.1 eq.) and collidine (0.34 ml, 1.1 eq.) were added to the methyl 3-acetoxy-4-hydroxybenzoate solution. The reaction was stirred for 7 d in the dark and then filtered through filter aid powder. The latter was washed with acetone (30 ml) and CH_2Cl_2 (30 ml) and the combined organics evaporated. The resulting red oil was dissolved in CH_2Cl_2 (50 ml) and washed with 1 M HCl aq. (2 x 50 ml), and water (1 x 50 ml), and dried ($MgSO_4$). Flash chromatography (100 % CH_2Cl_2 to 4% MeOH in CH_2Cl_2) produced 4-(1-methoxycarboxy-3-acetoxyhydroxyphenyl)-2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate methyl ester, which was de-protected as previously described (Needs and Kroon 2006; He, Shi et al. 2008) and purified by preparative HPLC to yield 4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid [^{1}H nmr (dmso-d_6)- 3.34-3.40 (m, partially obscured by HOD peak, H-2', H-3', H-4'), 3.92(1H,d, J = 9.8 Hz, H-5'), 5.06 (1H, J = 7.3 Hz, 1H'), 7.09 (1H, d, J = 9.2 Hz, H-5), 7.36 - 7.38 (2H, m, H-2, H-6)]. Refer to appendix B for purity data and NMR spectrum.



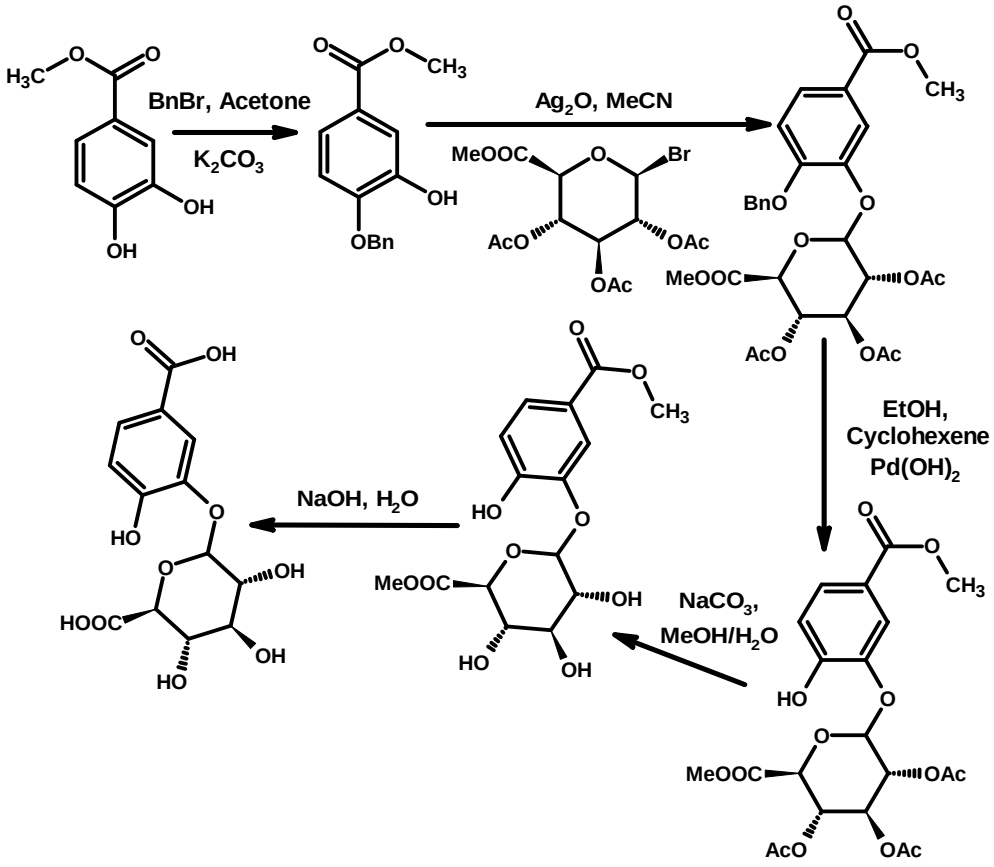
Scheme 4.1. Synthesis of *4-(1-Carboxy-3-hydroxyphenyl) β-D-glucuronic acid*.

4-(1-carboxyphenyl) β-D-glucuronic acid synthesis (**Scheme 4.2**). 4-(1-carboxyphenyl) β-D-glucuronic acid was synthesised as described previously (Ghosh and Farquhar 1997) for the glucuronidation of 4-hydroxybenzaldehyde. Briefly, methyl 4-hydroxybenzoate (100 mg), methyl 2,3,4-tri-O-acetyl-β-D-glucopyranosylurate bromide (234 mg, 1 equiv.), Ag_2O (200mg, 2 equiv.) and acetonitrile (0.66 ml) were stirred in the dark under Ar gas. After 16 h the reaction product was purified by MPLC (20g silica, 60% ethylactetate/40% hexane isocratic elution) and de-protected as previously described (Needs and Kroon 2006). The final product was precipitated in 50% MeOH aq at 4 ℃ to yield 4-(1-carboxyphenyl) β-D-glucuronic acid as a yellow powder [^{1}H NMR (dmso d_6)- [δH 7.89 (2H, d, J=8.4Hz, H-2, H-6), 7.09 (2H, d, J= 8.4Hz, H-3, H-5), 4.97 (1H, d, J=7.2Hz, H-1'), 3.48 (1H, d, J=10.0Hz, H-5'), 3.15- 3.40 (3H, m, H-2, H-3, H-4)]. Refer to appendix B for purity data and NMR spectrum.



Scheme 4.2. Synthesis of 3-hydroxy-4-*O*-glucuronosylbenzoate.

3-(1-Carboxy-4-hydroxyphenyl) β-D-glucuronic acid synthesis (**Scheme 4.3**). The reaction precursor, methyl 4-benzyloxy-3-hydroxybenzoate was synthesised as previously described [14]. Subsequently, methyl 4-benzyloxy-3-hydroxybenzoate (100 mg), methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosylurate bromide (153 mg, 1 equiv.), acetonitrile (1 ml) and Ag_2O (179 mg, 2 equiv.) were stirred continually for 24 h on ice under Ar gas. The mixture was then purified by MPLC (20g silica, 20% ethylactetate/80% hexane isocratic elution) and the main reaction product de-protected as previously described (Needs and Kroon 2006; He, Shi et al. 2008). Due to poor reaction yields, synthesised metabolite, 4-hydroxy-3-O-glucuronosyl benzoic acid was tentatively identified based on ESI-MS and UV-vis spectral data following comparison with biologically chemically derived glucuronide conjugates. Refer to appendix B for purity data.



Scheme 4.3. Synthesis of *3-(1-Carboxy-4-hydroxyphenyl) β-D-glucuronic acid*.

4.2.4 HPLC conditions

HPLC analysis was performed on an Agilent 1100 series HPLC equipped with a diode array detector (DAD). Simulated GIT digestion samples were analysed on a 4 µm, 250 x 4.6 mm Synergi Max-RP reverse phase column (Phenomenex, Macclesfield, UK) with a SecurityGuard guard cartridge (Phenomenex AJO-6074) and microsmal samples on a 5 µm, 250 x 4.6 mm Lunar C18 column (Phenomenex, Macclesfield, UK) with a SecurityGuard guard cartridge (Phenomenex AJO-6074) at a column temperature of 37 ℃. The mobile phase consisted of 1% formic acid (v/v) in water (A) and 1% formic acid (v/v) in methanol (B). The solvent gradient consisted of 2.5% B at 0 min, 25% B at 15 min, 45% B at 20 min and 100% B at 25-30 min for simulated GI digestion samples and 0% B at 0 min, 3% B at 4 min, 3% B at 10 min, 15% B at 20 min, 18% B at 25 min, 35% B at 30 min, 40% B at 35 min, 50% B at 40 min and 100% B at 45-50 min for microsomal samples. The flow rate was 1 mL/min and absorbance was recorded at 520, 360, 280 and 265nm. Quantification and identification of known compounds was performed using authentic analytical standards.

4.2.5 ESI-MS and MS/MS conditions

Full scan LC/MS analysis of all microsomal samples and synthesised metabolites was conducted on an Agilent 1100 series LC/MSD SL single quadrupole mass spectrometer coupled to an Agilent 1100 series HPLC with DAD detector (refer to appendix B for ESI-MS method development data). Electrospray ionization was performed in full scan mode (Mass range: 100-1000Da) with the following spray chamber conditions: drying gas flow 13 l/min; nebulizer pressure 50 psi; drying gas temperature 350 ℃. Negative mode ionization was performed at a capillary voltage of -3000 V and a fragmentor setting of 100. For further structural elucidation of a selected number of samples MS/MS analysis was conducted on an Applied Biosystems 3200 Q-Trap LC/MS/MS system coupled to an Agilent 1200 series HPLC with DAD detector. Source parameters were as follows: electrospray flow rate 1 mL/min, source temperature 500 ℃, ion spray voltage -3500 V, curtain gas 10 psi, collision gas medium, ion source gas 1, 65 psi and ion source gas 2, 65 psi. For 4-hydroxybenzoic acid glucuronide

metabolites negative mode product ion scanning parameters were as follows: Q1 mass 313.3, declustering potential -61 V, entrance potential -4 V, collision energy -19 V and collision cell exit potential -2 V. For protocatechuic acid glucuronide metabolites: Q1 mass 329.2, declustering potential -55 V, entrance potential -3 V, collision energy -18 V and collision cell exit potential -2 V. HPLC conditions were as previously described.

4.2.6 Statistical Analysis

Simulated GIT digestion samples were compared by one-way analysis of variance (ANOVA) with Tukey post-hoc tests (Windows SPSS, version 15) on concentration data derived from standard curves of authentic analytical standards. Significance was determined at $p<0.05$ across triplicate experiments.

4.3 Results and Discussion

4.3.1 In vitro gastrointestinal digestion of anthocyanins. To assess the degradation of pelargonidin-3-glucoside, cyanidin-3-glucoside, pelargonidin and cyanidin during GIT digestion, the anthocyanins were exposed to simulated gastric and duodenal digestion (**Figures 4.2 and 4.3**). During the simulated gastric digestion (0-30 min; **Figure 4.2A**) no significant anthocyanin degradation was observed. However, significant loss of anthocyanin aglycones was observed following 15 min of gastric digestion ($p<0.05$; **Figure 4.3A**). During post-gastric duodenal digestion (0-120 min), a significant loss of cyanidin-3-glucoside was observed (**Figure 4.2B**), while significant losses of both anthocyanin aglycones were observed across 0-120 min ($p<0.05$; **Figure 4.3B**) with complete loss of the aglycones within 60 min of digestion. No phenolic acid degradation products were identified within the 120 min digestion.

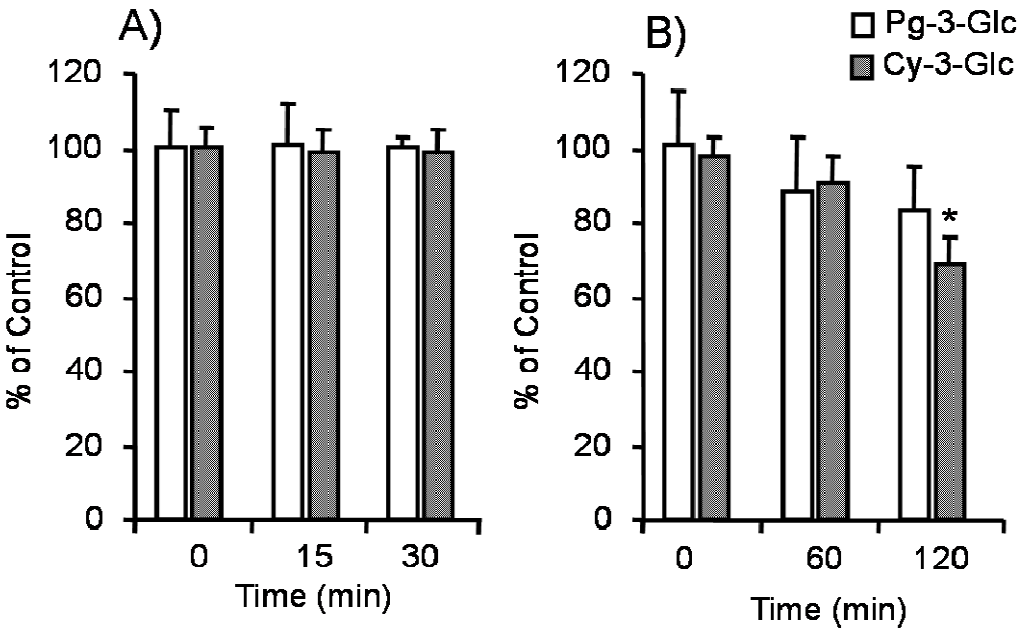


Figure 4.2. Anthocyanin (glycoside) loss during *in vitro* gastrointestinal digestion. A) Gastric phase digestion in the presence of pepsin (pH 2). B) Duodenal phase digestion in the presence of pancreatin and bile salts (pH 6.7). *Significance from control (initial anthocyanin spiked samples maintained at pH 2 (0.1 M HCl) throughout digestion; t=0; $p<0.05$). Pg-3-Glc, pelargonidin-3-glucoside; Cy-3-Glc, cyanidin-3-glucoside. Data represents mean±SD (n=3).

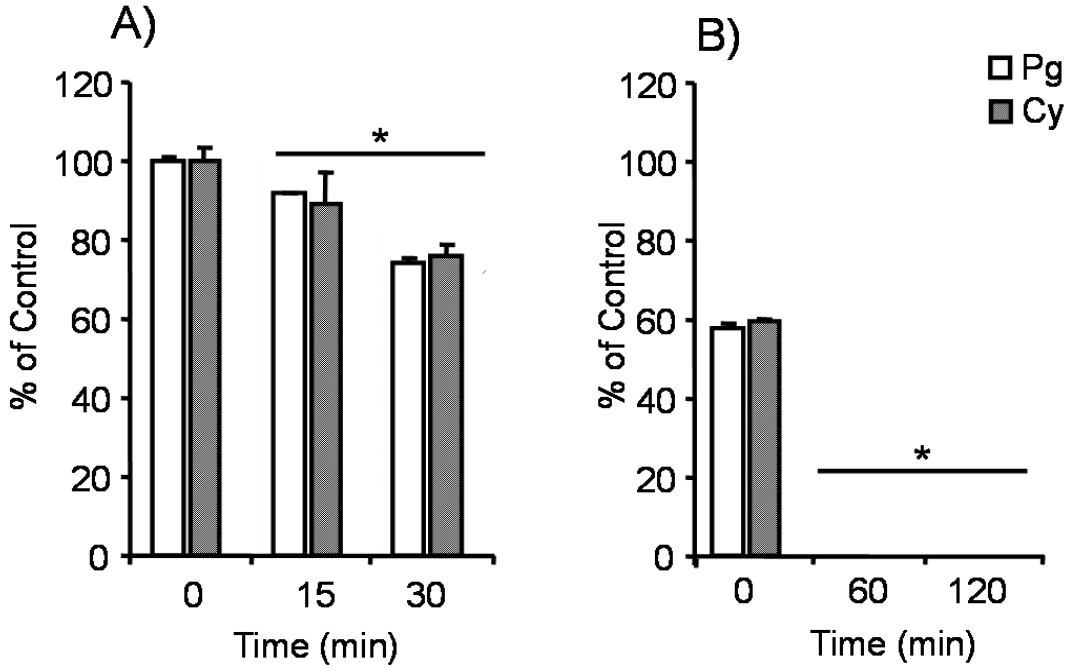


Figure 4.3. Anthocyanidin (aglycone) loss during *in vitro* gastrointestinal digestion. A) Gastric phase digestion in the presence of pepsin (pH 2). B) Duodenal phase digestion in the presence of pancreatin and bile salts (pH 6.7). *Significance from control (initial anthocyanin spiked samples maintained at pH 2 (0.1 M HCl) throughout digestion; t=0; $p<0.05$). Pg, pelargonidin; Cy, cyanidin. Data represents mean±SD (n=3).

4.3.2 Microsomal glucuronidation of anthocyanin derived phenoic acids. To assess the potential for anthocyanins to form glucuronidated phenolic acid metabolites, pelargonidin and cyanidin; and their reported degradation products, 4-hydroxybenzoic acid and protocatechuic acid, were incubated with human liver microsomes in the presence of UDP-GA. As a reaction control, kaempferol was subjected to the incubation conditions described for anthocyanins and phenolic acids. Following 30 min, kaempferol demonstrated complete conjugation to form two glucuronide metabolites in a manner consistent with previously reported data (Oliveira and Watson 2000) (**Figure 4.4**). Following microsomal metabolism, pelargonidin formed two phenolic acid metabolite peaks (**Figure 4.5A**) which were identified as 4-hydroxybenzoic acid (138 *m/z*) and its monoglucuronide metabolite **M1** (314 *m/z*; demonstrating a loss of 176 *m/z* under ESI-MS and MS/MS conditions). In addition cyanidin formed one phenolic acid metabolite peak (**Figure 4.6B**) which was identified as protocatechuic acid (154 *m/z*). No protocatechuic acid glucuronide metabolites were observed using UV-vis, however, LC/MS/MS analysis using product ion scanning parameters optimised for the synthesised standards revealed (based on MS^2 fragmentation patterns) the presence of multiple protocatechuic acid glucuronides (Table 1; M4-7).

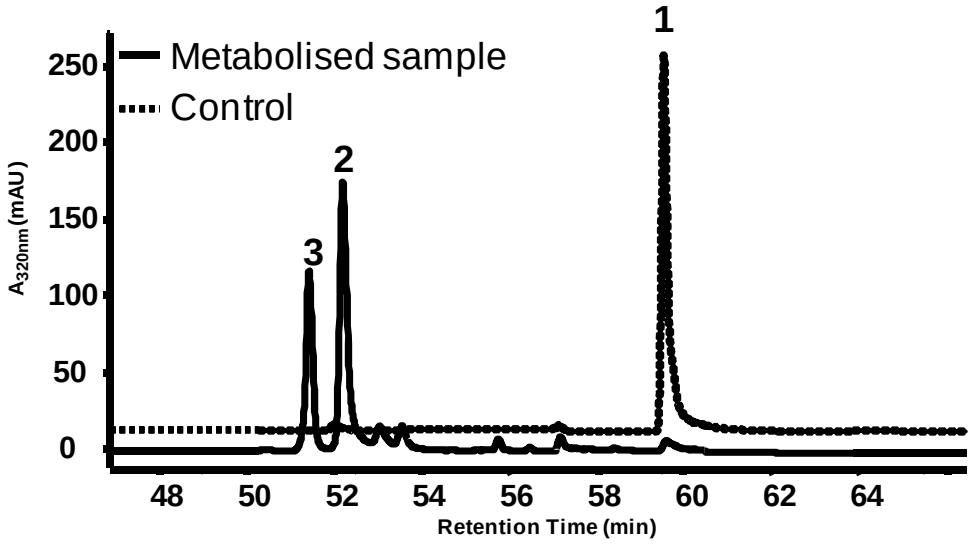


Figure 4.4. Kaempferol glucuronidation by liver microsomes. Chromatograph showing kaempferol (1) and its microsomal glucuronide metabolites (2 and 3).

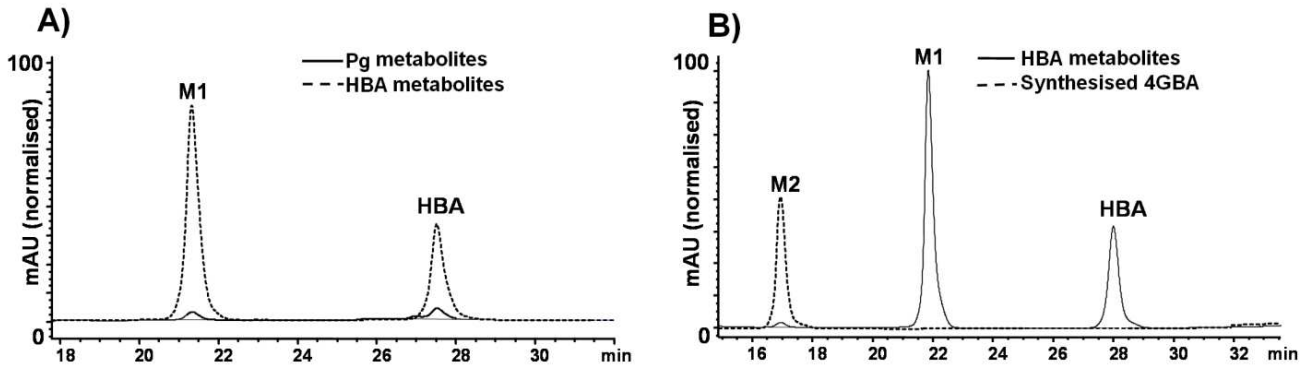


Figure 4.5. Representative chromatograms of pelargonidin and 4-hydroxybenzoic acid post incubation with human liver microsomes in the presence of UDP-GA. A) Individual incubations with pelargonidin (Pg) or its degradation product 4-hydroxybenzoic acid (HBA) (with chromatograms overlaid). B) Incubations with 4-hydroxybenzoic acid overlaid with the chromatogram of the chemically synthesised metabolite, 4-(1-carboxyphenyl) β-D-glucuronic acid (4GBA). M1 and M2 refers to microsomal metabolites 1 (4-hydroxybenzoyl glucuronide) and 2 (4GBA). For metabolite and synthetic standard spectral data refer to Table 1.

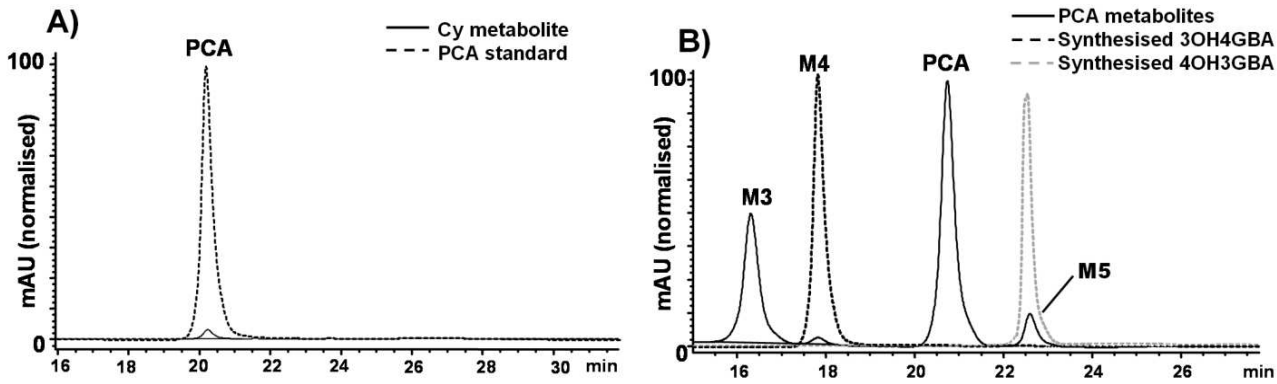


Figure 4.6. Representative chromatographs of cyanidin and protocatechuic acid post incubation with human liver microsomes in the presence of UDP-GA. A) Incubation with cyanidin (Cy) overlaid with the chromatogram of the commercial standard protocatechuic acid (PCA). B) Incubation with PCA overlaid with the chromatograms of the chemically synthesised metabolites, 3-(1-carboxy-4-hydroxyphenyl) β-D-glucuronic acid (4OH3GBA) and 4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid (3OH4GBA). M3-5 refers to microsomal metabolites 3 (protocatecuic acid-acyl-glucuronide), 4 (protocatechuic acid-3'-glucuronide) and 5 (protocatechuic acid-4'-glucuronide). For metabolite and synthetic or commercial standard spectral data refer to Table 1.

Following microsomal incubations, the pelargonidin degradation product 4-hydroxybenzoic acid (138 *m/z*) was metabolised to form two glucuronide conjugates (**M1** and **M2**; 314 *m/z*; **Figure 4.5B**) which both demonstrated a loss of 176 *m/z* under ESI-MS and MS/MS conditions. It should be noted that the microsomal metabolite **M1** was observed during the incubation of both pelargonidin and 4-hydroxybenzoic acid (individually). Protocatechuic acid (154 *m/z*), when incubated in the presence of liver microsomes, was

metabolised to form three glucuronide conjugates (**M3**, **M4** and **M5**; 330 *m/z*; **Figure 4.6B**) which all demonstrated a loss of 176 *m/z* under ESI-MS and MS/MS conditions. The spectral and chromatographic data for metabolites **M1-5** are described in **Table 4.1** and were confirmed using synthetic standards.

Table 4.1. Summary of HPLC-MS/MS characteristics of phenolic acid metabolites[a]

Compound	Rt (min)	Mass ion spectrum $[M-H]^-$, (m/z)[b]		UV maximum (nm)	Relative abundance[c] (% peak area)	Identification[d]
		Precursor	Fragment (product)			
Metabolites						
M1[e]	21.8	313	193, 175, 137, 113	262	99±18	HBA-acyl-GlcA[f]
M2[e]	16.9	313	175, 137, 113	248	1±1	HBA-4'-GlcA
M3[e]	16.3	329	193, 175, 153, 113	266, 298	81±3	PCA-acyl-GlcA[f]
M4[e]	17.8	329	193, 175, 153, 113	252, 292	3±0	PCA-3'-GlcA
M5[e]	22.5	329	175, 153, 113	258, 288	15±0	PCA-4'-GlcA
M6[g]	27.5	329	193, 175, 153, 113	-	-	Unknown
M7[g]	28.8	329	193, 175, 153, 113	-	-	Unknown
Commercial Standards						
HBA	27.9	137	93, 75, 65	254	-	-
4GBA	16.9	313	175, 137, 113	248	-	-
PCA	20.7	153	109, 91, 65	260, 294	-	-
Synthesised Standards						
4OH3GBA	17.8	329	175, 153, 113	252, 292	-	-
3OH4GBA	22.5	329	175, 153, 113	258, 288	-	-

[a]Spectral and chromatographic data for phenolic metabolites derived following microsomal incubations in the presence of UDP-GA. [b]Mass fragmentation patterns as determined by full scan MS analysis and confirmed by product ion MS/MS scanning (refer to methods section). [c]Tentative abundance based on peak areas relative to controls. [d]Tentative identification based on comparisons with synthesised and commercial standards. [e]M1-5, microsomal metabolites 1-5 as illustrated in figures 4 and 5. [f]Metabolite identification based on plausible remaining conjugation site and indirect comparisons with aryl-synthetic standards and HPLC-MS/MS data. [g]Microsomal metabolites 6 and 7 were identified in cyanidin microsomal incubations based on product ion HPLC-MS/MS scans only. Rt, retention time; HBA, 4-hydroxybenzoic acid; 4GBA, synthesised 4-(1-carboxyphenyl) β-D-glucuronic acid; HBA-acyl-GlcA, acyl glucuronide of 4-hydrocybenzoic acid; HBA-4'-GlcA, 4' glucuronide of 4-hydroxybenzoic acid; PCA, protocatechuic acid; 4OH3GBA, synthesised 3-(1-carboxy-4-hydroxyphenyl) β-D-glucuronic acid; 3OH4GBA, synthesised 4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid; PCA-acyl-GlcA, acyl glucuronide of protocatechuic acid; PCA-3'-GlcA, 3' glucuronide of protocatechuic acid; PCA-4'-GlcA, 4' glucuronide of protocatechuic acid; GlcA, glucuronic acid; UDP-GA, uridine 5′-diphosphoglucuronic acid.

4.3.3 Structural elucidation of phenoic acid metabolites. To aid structural elucidation, the synthesised glucuronide conjugates of 4-hydroxybenzoic acid and protocatechuic acid were chemically synthesised and compared with the biologically derived anthocyanin and phenolic acid metabolites. It was

confirmed that the synthesised metabolite 4-(1-carboxyphenyl) β-D-glucuronic acid had the same retention time, UV maxima and mass fragmentation pattern as 4-hydroxybenzoic acid's microsomal derived metabolite **M2** (**Figure 4.5B**). Synthesised metabolites 4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid and 3-(1-carboxy-4-hydroxyphenyl) β-D-glucuronic acid were also confirmed to have the same retention time, UV maxima and mass fragmentation pattern as protocatechuic acid's microsomal derived metabolites **M4** and **M5**, respectively (**Figure 4.6B**).

Although anthocyanins possess a number of beneficial bioactivities (Mazza 2007), their apparent systemic bioavailability has been shown to be very low (Manach, Williamson et al. 2005). Previous studies have suggested that this is likely due to spontaneous degradation under physiological conditions (Woodward, Kroon et al. 2009) or following microbial metabolism (Keppler and Humpf 2005; Han, Ryu et al. 2009). In this study the aim was to identify the plausible alternative occurrences of anthocyanins following metabolism and it was hypothesised that anthocyanins are degraded to form phenolic acids and subsequently conjugated via phase II metabolism.

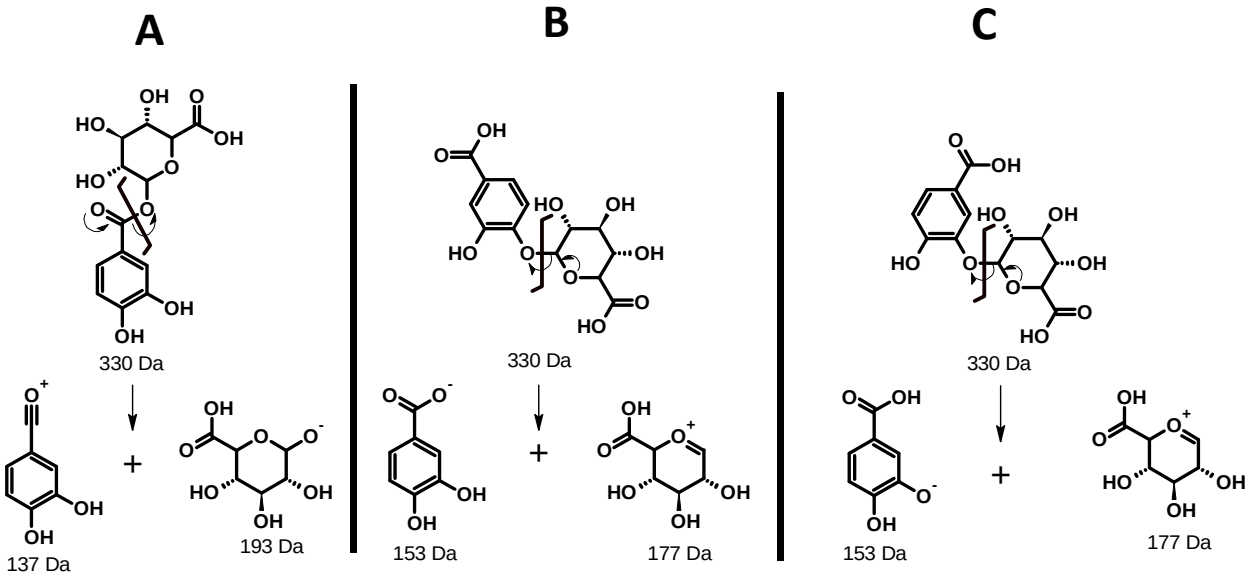
Here it was observed that anthocyanins were stable during gastric digestion, but were degraded during intestinal digestion. Although this is consistent with previous studies (Perez-Vicente, Gil-Izquierdo et al. 2002; McDougall, Dobson et al. 2005; McDougall, Fyffe et al. 2005), little account for phenolic acid formation or spontaneous degradation has been previously given. Importantly, the loss of anthocyanins and anthocyanidins during GIT digestion in this study did not result in the formation of their representative phenolic acid degradation products. This was likely due to the short incubation time (2h) at neutral pH (Keppler and Humpf 2005; Woodward, Kroon et al. 2009), where we have previously described an initial lag in phenolic acid formation following anthocyanin degradation at neutral pH and 37 ℃; po tentially as a result of the formation of relatively stable 'ring-open' transition products (Woodward, Kroon et al. 2009).

Degradation of anthocyanins was similar both in the presence and absence of digestive enzymes, which was consistent with previous observations regarding anthocyanin degradation under simulated physiological conditions and suggests that anthocyanin degradation during digestion occurred spontaneously. It remains to be established the extent to which anthocyanins are hydrolysed *in vivo* by the human intestinal brush-boarder enzymes (Day, DuPont et al. 1998; Day, Cañada et al. 2000), however the efficient deglucosylation of pelargonidin has been previously reported (Kroon, Clifford et al. 2004); suggesting significant degradation following deglycosylation should occur *in vivo*.

This study demonstrated that both the degradation products of anthocyanins (pelargonidin and cyanidin) 4-hydroxybenzoic acid and protocatechuic acid were efficiently metabolised by human liver microsomes to form monoglucuronide conjugates. Indeed, 4-hydroxybenzoic acid was metabolised to form two monoglucuronide species and protocatechuic acid was metabolised to form three monoglucuronide species. This pattern of mono-glucuronidation is similar to that demonstrated by Cao et al. [10] in rats and is consistent with the number of free hydroxyl groups present on these phenolic structures. Comparison of the microsomal metabolites for 4-hydroxybenzoic acid with the chemically synthesised glucuronides confirmed that metabolite **M2** was the 4'-glucuronide conjugate of 4-hydroxybenzoic acid. This evidence suggests that pelargonidin and its 4-hydroxybenzoic acid metabolite **M1** is an acyl glucuronide. Similarly, comparison of the protocatechuic acid microsomal metabolites with the chemically synthesised glucuronides confirmed that metabolites **M4** and **M5** were the 4' and 3' glucuronide conjugate of protocatechuic acid. This indicates that the protocatechuic acid metabolite **M3** is an acyl glucuronide. Interestingly, the fragmentation of these phenolic acyl glucuronides gives a characteristic mass fragmentation ion at 193 *m/z*, which may prove useful in distinguishing these conjugation sits (acyl vs aryl) in future human studies (**Scheme 4.4**).

Herein we provide evidence that suggests acyl and aryl mono-glucuronides of 4-hydroxybenzoic acid and protocatecuic acid may be common in vivo

biological conjugates following anthocyanin consumption. Further research should be conducted to evaluate the extent to which acyl glucuronide products of anthocyanin derived carboxylates are formed *in vivo*. Indeed, the occurrence of acyl glucuronides may be of particular biological interest, since they may cause covalent modifications to endogenous proteins and other macromolecules (Boelsterli 2002) and may impact pharmaceutical drug metabolism/bioactivity (Boelsterli 2002). In addition, future work is needed to establish the sites of methyl and glycine conjugation as indicated in recent rodent studies (Cao, Zhang et al. 2009).



Scheme 4.4. Proposed mechanism for phenolic glucuronide MS fragmentation under the current MS conditions where acyl glucuronides (A) may yield a characteristic mass ion at 193 *m/z* in comparison to its aryl glucuronide counterparts (B and C).

It should be noted that the focus of the utilised experimental system in this study was developed to establish potential sites of metabolic conjugation and therefore the methods were developed to produce a system optimised for conjugation efficiency of each of the phenolic substrates. Consequently, the results cannot be implied or translated relative to physiological events such as systemic absorption, bioavailability, or excretion. Therefore, future research should aim to identify these metabolic target *in vivo*, in order to determine their systemic absorption, bioavailability and excretion.

In conclusion, evidence is presented to suggest that anthocyanins can be degraded during gastrointestinal digestion and hepatic metabolism to form several mono-glucuronide metabolites. Hence, it is proposed that anthocyanins may additionally be found in the systemic circulation as free or conjugated phenolic acids.

Chapter 5

Structure-activity relationship study: Modulation of endothelial superoxide production by anthocyanins and their phenolic metabolites

5.1 Introduction

The endothelial generation of reactive oxygen species (ROS) is important both for normal physiology and in the progression of cardiovascular diseases (Droge 2002). ROS generated by the endothelium include superoxide, hydrogen peroxide, nitric oxide (NO), peroxynitrite and hydroxyl radicals (Droge 2002). Superoxide radicals, the focus of the current investigation, may have several effects on the vasculature, either directly or indirectly. These effects include rapid inactivation of the potent signalling molecule and endothelial vasodilator, NO, leading to endothelial dysfunction, the mediation of oxidative signal transduction ("redox signalling") and oxidative damage (Munzel and Harrison 1999; Cai and Harrison 2000). Multiple enzyme systems are responsible for the generation of superoxide within the endothelium; notably, xanthine oxidase, un-coupled nitric oxide synthase and the mitochondria. Recent studies have indicated that the major source of endothelial superoxide is a multi-component NADPH oxidase or NOX (particularly NOX4) that is subject to specific regulatory stimuli such as oscillatory shear stress, hypoxia, angiotensin II, cytokine, growth factors and hyperlipidaemia (Ago, Kitazono et al. 2004; Bedard and Krause 2007). Depending on the level of oxidants generated and the relative balance between pro- and antioxidant systems, ROS may be involved in the progression and modulation of cardiovascular diseases such as atherosclerosis (Kojda and Harrison 1999; Taniyama and Griendling 2003).

Of particular interest, the rennin-angiotensin system is a central component of the physiological and pathological responses of the vasculature (Dzau 1988; Brasier, Recinos et al. 2002). Its primary effector hormone, angiotensin II (Ang II), not only mediates its acute haemo-dynamic effects through the renal system, but is also implicated in inflammation, endothelial dysfunction and atherosclerosis through its chronic exposure to the endothelium (Mehta and Griendling 2007). The effect of Ang II on the vasculature is dependent on time (chronic vs. acute) and cell/tissue specific factors. In addition to G protein and non-G protein related signalling pathways, Ang II acting via the angiotensin II

receptor 1 (AT1R) carries out its functions through the MAP kinases (ERK1/2, JNK, p38MAPK), receptor tyrosine kinases (PDGF, EGFR) and non-receptor tyrosine kinases (Src, JAK/STAT). Importantly, AT1R stimulation also leads to the activation of NOX in the endothelium and subsequent generation of superoxide and other ROS (Rueckschloss, Quinn et al. 2002).

Perhaps the most documented role of superoxide in the development of vascular disease is its ability to disable endothelial nitric oxide synthase (eNOS also known as ecNOS or NOS3) generated NO (Munzel and Harrison 1999; Cai and Harrison 2000). eNOS is a calcium-dependent flavoprotein that generates NO in a process that involves the oxidation of L-arginine by the reduction of molecular oxygen (Moncada, Palmer et al. 1991; Bredt and Snyder 1994). The essential eNOS cofactor tetrahydroxybiopterin (BH4) appears to have a key role in regulating NOS function by “coupling” the reduction of oxygen to L-arginine oxidation, as well as maintaining the stability of NOS dimmers. BH4 is itself highly susceptible to oxidative degradation and the initial oxidative loss of BH4 in response to increased ROS production has been shown to amplify oxidative stress through the resulting loss of NO production and increased NOS-dependent superoxide production (eNOS “un-coupling”) (Moncada, Palmer et al. 1991; Bredt and Snyder 1994). Furthermore, the rapid reaction of superoxide with NO results in the formation of peroxynitrite anions and loss of NO function. This appears to play an important role in cardiovascular disease development.

It has long been thought that diets rich in anthocyanins and other flavonoids may confer protection against cardiovascular disease and mortality. Indeed, this hypothesis has been driven by finding from numerous epidemiological, clinical and experimental studies (Hertog, Feskens et al. 1993; Hertog, Kromhout et al. 1995; Knekt, Jarvinen et al. 1996; Rimm, Katan et al. 1996; Hertog, Sweetnam et al. 1997; Hertog, Feskens et al. 1997; Yochum, Kushi et al. 1999; Arts, Jacobs et al. 2001; Geleijnse, Launer et al. 2002; Knekt, Kumpulainen et al. 2002; Sesso, Gaziano et al. 2003; Tsuda, Horio et al. 2003; Xia, Ling et al. 2003; Lin, Rexrode et al. 2007; Mink, Scrafford et al. 2007; Sasaki, Nishimura et al. 2007; Xia, Ling et al. 2007; Hooper, Kroon et

al. 2008; Leblais, Krisa et al. 2008; Toufektsian, de Lorgeril et al. 2008). Whilst the epidemiological evidence for the protective effects of flavonoid rich diets, including anthocyanins, on cardiovascular protection are mixed, the majority of clinical and animal studies are in support of their positive effects (Andriambeloson, Magnier et al. 1998; Benito, Lopez et al. 2002; Mendes, Desgranges et al. 2003; Serraino, Dugo et al. 2003; Matsubara, Kaneyuki et al. 2005; Mink, Scrafford et al. 2007). Although early research did not attribute the beneficial effects of anthocyanins to their antioxidant potential and radical scavenging activities, considerable research has described the antioxidant properties of anthocyanins (Vinson, Dabbagh et al. 1995; Prior, Cao et al. 1998; Wang, Nair et al. 1999; Kalt, McDonald et al. 2000; Mazza, Kay et al. 2002; Zafra-Stone, Yasmin et al. 2007). Recent research suggests a mechanism of action that is un-related to their direct radical scavenging properties (Zhang, Lian et al. ; Martin, Favot et al. 2003; Lamy, Blanchette et al. 2006; Oak, Bedoui et al. 2006; Xia, Ling et al. 2007; Guo, Ling et al. 2008; Williams, El Mohsen et al. 2008; Min, Ryu et al. 2010), however, the primary mechanism for this observation still remains unclear. To this end, the present study aimed to assess the effect of different anthocyanin derived structures (anthocyanins and their phenolic metabolites as determined in chapter 4) on the production of superoxide in the endothelium.

In order to evaluate the structure-activity relationship of several hydroxylated and conjugated anthocyanin derived compounds (*mono-hydroxyl derivatives*: pelargonidin-3-glucoside, 4-hydroxybenzoic acid and the 4' glucuronide of 4-hydroxybenzoic acid; *catechol derivatives*: cyanidin-3-glucoside, protocatechuic acid, vanillic acid, 3-hydroxy-4-methoxybenzoic acid and the 4' glucuronide of protocatechuic acid; *tri-hydroxyl derivatives*: delphinidin-3-glucoside, gallic acid and 2,4,6-trihydroxybenzaldehyde (phlorogluceraldehyde)), a number of experimental methods were developed and optimised based on previously reported techniques. Using these developed assays, structure-activity studies were sequentially conducted to assess both the antioxidant and non-antioxidant properties of anthocyanins, pertaining specifically to superoxide production.

Firstly, the effect of anthocyanins and their metabolites and cell treatment medium conditions on endothelial cell viability was assessed in order to ascertain the correct treatment dose and conditions. Secondly, the reduction of cytochrome c by anthocyanins and their metabolites was investigated, since it has been suggested that the ability for compounds to reduce cytochrome c is associated with their direct iron-ion reducing/electron donation capacity. It remained essential to determine the ability for anthocyanins and their metabolites to reduce cytochrome c, since cytochrome c was used as an indicator for superoxide production in subsequent structure-bioactivity assays. Thirdly, the structure-activity relationship of anthocyanins and their metabolites as inhibitors of xanthine oxidase was investigated. In order to measure the inhibition of xanthine oxidase, a cytochrome c reduction assay was developed and optimised. Subsequently, the ability for anthocyanins and their metabolites to modulate superoxide release from intact endothelial cells was investigated under Ang II stimulated conditions. To achieve this, a cytochrome c reduction assay was developed and optimised for incubation time, cell treatment medium conditions, Ang II concentration and cytochrome c concentration. Lastly, the structure-activity effects of anthocyanins and their metabolites on endothelial NO production was assessed. Thus, the findings of these studies may provide some evidence in support of current epidemiological and animal study observations, and provide us with mechanistic insights that might be taken forward in human trials.

5.2 Materials and Methods

Large vessel endothelial basal medium, endothelial supplementation pack and trypsin/EDTA solution were purchased from *TCS Cellworks*, UK. Foetal calf serum, Medium 199 and phenol free Medium 199 were purchased from *Invitrogen*, UK. Bovine serum albumin (BSA) was purchased from *Sigma-Aldrich*, UK. Equine cytochrome c, xanthine, xanthine oxidase, superoxide dismutase and human angiotensin II (Ang II) were purchased from *Sigma-Aldrich*, UK. Rabbit anti-NOX4 was purchased from *Abcam*, UK, goat anti-β-actin was purchased from *Santa Cruz Biotechnologies*, USA and donkey anti-

rabbit, donkey anti-goat, instant blue staining solution and Odyssey blocking buffer were purchased from *Li-Corr Biosciences*, UK. Protease inhibitor cocktail and PCR nucleotide mix were purchased from *Roche*, UK. Random hexamers and BioScript were purchased from *Promega*, UK. PCR reaction buffer was purchased from *Bioline*, UK. Rnase out ribonuclease inhibitor was purchased from *Invitrogen*, UK. All PCR primers and probes were purchased from *Sigma-Aldrich*, UK. Precision MasterMix was purchased from *Primer Design*, UK. Cyanidin-3-glucoside (kuromanin chloride), delphinidin-3-glucoside (myrtillin chloride), pelargonidin-3-glucoside (callistephin chloride), and 4-hydroxybenzoic acid were purchased from *Extrasynthese*, France and dissolved in DMSO at an initial concentration of 40 mM. Protocatechuic acid, gallic acid, vanillic acid, 3-hydroxy-4-methoxybenzoic acid and 2,4,5-trihydroxybenzaldehyde were purchased from *Sigma-Adlrich* (UK) and dissolved in DMSO. All water used was 18MΩ/cm milliQ water.

5.2.1 General cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from TCS Cellworks (UK) and used between passages 2-4. The cells were cultured in a humidified atmosphere at 37 ℃ and 5% CO_2 in large vessel endothelial cell basal medium supplemented with hydrocortisone, human epidermal growth factor, human fibroblast growth factor with heparin, foetal calf serum (FCS; 2% v/v) and an antibiotic supplement containing 25 mg/ml gentamicin and 50 µg/ml amphotericin B (final supplement concentrations were proprietary to TCS Cellworks, UK). Typically, cells were cultured on Cell+™ 75 cm^2 culture flasks (Sarstedt, UK) and subcultured at 80% confluence using a 0.025% trypsin/0.01% EDTA solution.

5.2.2 Method development and optimisations

Cell viability under different treatment conditions: To determine the viability of HUVECs under different treatment media conditions, endothelial cell viability was assessed using the WST-1 cell proliferation reagent. Briefly,

Cells were seeded into 96-well plates (NunclonΔ surface, Nunc, UK) at 5000 cells.cm^{-2} in 200 µL culture medium and incubated at 37 ℃ under a humidified atmosphere containing 5% CO_2. At confluence, the medium was removed and replaced with 100 µL cell treatment medium (phenol free Medium 199); medium optimisation was tested with 0, 1, 5 and 10% FCS supplementation and 0.1, 0.2 and 0.4% BSA supplementation. Control incubations consisted of cells cultured in growth medium as previously described. Negative control consisted of cell incubated in PBS. Cell viability was determined after 24 and 48 h using WST-1 cell proliferation reagent as described below.

Effect of treatment compounds on cellular viability: To assess the effect of different anthocyanin derived treatment compounds and DMSO vehicle controls (0.05% DMSO) on HUVEC cell viability and to determine optimal treatment ranges, WST-1 cell proliferation reagent was used to assess cellular respiration under different treatment condition. Cells were seeded into 96-well plates (NunclonΔ surface, Nunc, UK) at 5000 cells.cm^{-2} in 200 µL culture medium and incubated at 37 ℃ under a humidified at mosphere containing 5% CO_2 until confluence. The medium was removed and replaced with 100 µL Medium 199 containing 1% FCS and 0, 0.5, 1, 10, 50 and 100 µM of structure-activity test compounds (*mono-hydroxyl derivatives*: pelargonidin-3-glucoside, 4-hydroxybenzoic acid and the 4' glucuronide of 4-hydroxybenzoic acid; *catechol derivatives*: cyanidin-3-glucoside, protocatechuic acid, vanillic acid, 3-hydroxy-4-methoxybenzoic acid and the 4' glucuronide of protocatechuic acid; *tri-hydroxyl derivatives*: delphinidin-3-glucoside, gallic acid and 2,4,6-trihydroxybenzaldehyde (phlorogluceraldehyde)). Cell viability was determined after 24 h using WST-1 cell proliferation reagent. Briefly, 10 µL of tetrazolium salt WST-1 reagent was added to each well following treatment and the soluble formazan dye produced by metabolically active cells was monitored every 30 min for 2 h at 440nm on a microplate reader (Fluostar, BMG Labtech, UK).

The iron-ion reducing capacity of anthocyanins and their metabolites: To assess the direct reduction of cytochrome c by anthocyanin metabolites, 2, 20, 200 and 2000 µM of structure-activity test compounds (*mono-hydroxyl derivatives*: pelargonidin-3-glucoside and 4-hydroxybenzoic acid; *catechol derivatives*: cyanidin-3-glucoside, protocatechuic acid, vanillic acid, 3-hydroxy-4-methoxybenzoic acid and the 4' glucuronide of protocatechuic acid; *tri-hydroxyl derivatives*: delphinidin-3-glucoside and gallic acid) were co-incubated with 20 µM cytocrome c in PBS at 37 ℃ as described in previously for catechols and quinols (Saleem and Wilson 1982). The spontaneous reduction of cytochrome c was monitored as a kinetic assay at 550nm (Fluostar, BMG Labtech, UK) over 2 h. Cytocrome c reduction was quantified using the millimolar extinction coefficient for reduced cytochrome c (29.5 $mM.cm^{-1}$). Control/blank samples consisted of the above reaction in the absence of phenolic compounds or in the absence of cytochrome c.

Cytochrome c reduction assay for xanthine/xanthine oxidase activity: Superoxide production by xanthine/xanthine oxidase was measured using a cytochrome c reduction assay optimised from a previously described method (Steffen, Gruber et al. 2008). Briefly, the reaction mixture contained xanthine (as the primary substrate), cytochrome c (which acted as an indicator of superoxide production) and xanthine oxidase (the enzyme source) in a 50 mM sodium phosphate buffer (pH 7.4). To optimise the reaction the three primary variables were sequentially altered as follows: xanthine oxidase (0, 0.025, 0.05 and 0.1 U/ml), xanthine (0, 50, 100, 200 and 400 µM) and cytochrome c (0, 50, 100 and 200 µM). The reaction was started with the addition of xanthine oxidase and incubated at 25 ℃. The reduction kinetics of cytochrome c was followed spectrophotometrically (Fluostar, BMG Labtech, UK) at 550 nm in a kinetic assay until the reaction plateaued. Following optimisation, the specificity of the reaction for superoxide was ascertained by incubating 80 µM cytochrome c, 0.1 U/ml xanthin oxidase and 200 µM xanthine both in the presence and absence of SOD (330 U/ml) in a 50 mM sodium phosphate buffer (pH 7.4). The superoxide generated was determined by subtracting the rate of cytochrome c reduction (increase in absorbance at 550 nm) in the presence of SOD from those in the absence of SOD. Blanks

consisted of samples with heat inactivated xanthine oxidase (heated to 100 ℃ for 5 min).

Ang II induced superoxide production by cultured endothelial cells: To measure the release of superoxide from intact endothelial cells, a cytochrome c reduction assay was developed and optimised from a previously reported method (Steffen, Gruber et al. 2008). Briefly, cells were seeded into 6 well culture plates (NunclonΔ surface, Nunc, UK) at 5000 cells.cm^{-2} and grown to confluence. Confluent cells were equilibrated in 2% FCS supplemented Medium 199 for 24 h. Following equilibration, cells were washed in warm PBS and re-suspended in phenol free Medium 199 containing 2% (as per the findings of method development and optimisation I, above) or 20% FCS, 50 μM cytochrome c with or without (basal condition) the addition of SOD (400 U). Blanks consisted of treatment in the absence of cells or in the absence of cytochrome c. Cells were incubated for a total of 6 h at 37 ℃ under a humidified atmosphere containing 5% CO_2. At 2 and 6 h, 200 ul of cell supernatant was transferred to a 96-well plate and the reduced cytochrome c measured at 550 nm (Fluostar, BMG Labtech, UK).

To establish a model for vascular oxidative stress (Rueckschloss, Quinn et al. 2002), HUVECs were treated with various concentrations of Ang II (0.05, 0.1 or 1 μM; dissolved in 1 mg/ml BSA) and the superoxide production assessed as described above with some alterations. Briefly, cells were seeded into 24 well culture plates (NunclonΔ surface, Nunc, UK) at 5000 cells.cm^{-2} and grown to confluence. Confluent cells were equilibrated in 2% FCS supplemented Medium 199 for 24 hours. Following equilibration, cells were washed in warm PBS and re-suspended in phenol free Medium 199 containing 2% FCS, 20 μM cytochrome c and 0.05, 0.1 and 1 μM Ang II, with or without the addition of SOD (400 U). Blanks consisted of treatment in the absence of cells or in the absence of cytochrome c. Cells were incubated for a total of 6 h at 37 ℃ under a humidified atmosphere containing 5% CO_2. At 6 h, 200 μl of cell supernatant was transferred to a 96-well plate and the reduced cytochrome c measured at 550 nm (Fluostar, BMG Labtech, UK). The superoxide generated was determined by subtracting the absorbance at

550 nm in the presence of SOD from those in the absence of SOD. Control (basal) condition consisted of cells incubated in the absence of Ang II. All cell treatment assays were normalised to total protein concentrations. Protein from individual treatment wells were harvested and quantified as described below.

In addition to the above optimised conditions, the following experimental design controlled for Ang II binding to plastics, control and basal consistency during experimentation; edge effects common to microplate assays; biological and technical replicate variation; in plate cell effects on absorbance; path-length and low volume assay conditions and assay sensitivity. To address the afore mentioned assay considerations, the experiment was conducted under the following conditions: Ang II losses/inaccuracy due to binding to plastics was accounted for by dissolving Ang II and washing all plastics in 1 mg/ml BSA solutions; experimental consistency was assessed by including six biological replicates for each sample and 3 technical replicates per samples (i.e. 6 biological replicates analysed 3 times); edge effects were controlled for by plating samples at least one well from the edge of the plate with media in the outer most wells; effects of cells on the absorbance variation of samples was accounted for by transferring cell free supernatants to a cell-free 96-well plate prior to spectroscopic measurement; insensitivity due to shorter light path lengths were negated by aliquoting 200 ul of sample into 96 well plate wells (6.6 mm path length) and assay sensitivity was assessed by comparing optical density between SOD treated and untreated cells with respect to inter-sample and technical variation (i.e. whether the replicate standard deviations were significant and whether this difference was sufficiently great enough to measure differences upon compound treatments in later experiments).

NOX4 protein expression. To investigate whether the increase in endothelial superoxide release induced by Ang II treatment was due to NOX4 induction, protein expression was analysed by Western blot analysis. Briefly, cells were seeded into 6 well culture plates (NunclonΔ surface, Nunc, UK) at 5000 cells.cm^{-2} and grown to confluence. Confluent cells were equilibrated in 2% FCS supplemented Medium 199 for 24 h. Following equilibration, cells were washed in warm PBS and re-suspended in Medium 199 containing 2% FCS

and 0.05, 0.1 or 1 μM Ang II. Control treatments were conducted in the absence of angiotensin II. Cells were incubated for 6 h at 37 ℃ and 5% CO_2, whereupon total protein was collected and stored at -80 ℃ until analysis.

Protein was harvested using NP-40 lysis buffer and ulta-sonication. Cell treatment media was aspirated and the cell monolayer washed three times in cold PBS. Cells were suspended in NP-40 lysis buffer (150 mM NaCl, 20mM Tris-EDTA, 10% v/v glycerol, 2% v/v NP-40 and 1x protease inhibitor cocktail) at 6.25 $\mu l.cm^2$ and incubated at 4 ℃ for 30 min with constant shaking. Cells were then scraped off using a rubber policeman and the cell lysate suspension stored at -80 ℃ for 24 h. Cell lysates were thawed at room temperature and sonicated on ice for 3x10 sec using a probe sonicator. The cell lysates were then centrifuged at 13,000 rpm for 15 min at 4 ℃ and the supernatant retained. Total protein was quantified using a BCA protein assay as per the manufacturer's instructions. Western blot analysis was conducted on equal amounts of protein (20 μg), run on 10% SDS polyacrylamide resolving gels and the protein transferred to PVDF membranes by semi-dry blotting (1h at 15-25v). Equal rates of transfer were confirmed by staining the gel with Instant Blue as per the manufacturer's instructions. Non-specific binding was blocked by incubating in Odyssey blocking buffer at room temperature for 1 h or overnight at 4 ℃. Membranes were then incubated with anti-NOX4 (1/500 dilution) and anti-β-actin (1/10,000 dilution) antibody overnight at 4 ℃. After washing in 0.1% PBST, membranes were incubated with anit-rabbit (1/10,000 dilution) and anti-goat (1/10,000 dilution) for 1 h at room temperature. Membranes were then imaged using an Odyssey infrared imager. Band densities were determined using the Odyssey infrared imager software.

NOX4 mRNA expression. To investigate whether the increase in endothelial superoxide release induced by Ang II was due to NOX4 induction, mRNA expression was measured by qRT-PCR. Briefly, cells were seeded into 6 well culture plates (NunclonΔ surface, Nunc, UK) at 5000 $cells.cm^{-2}$ and grown to confluence. Confluent cells were equilibrated in 2% FCS supplemented Medium 199 for 24 h. Following equilibration, cells were washed in warm PBS

and re-suspended in Medium 199 containing 2% FCS and 0.05, 0.1 or 1 µM Ang II. Control treatments were conducted in the absence of angiotensin II. Cells were incubated for 6 h at 37 ℃ and 5% CO_2, whereupon mRNA was collected and analysed. Total RNA was extracted using a GeneElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, UK) as per the manufacturer's instructions and quantified using a nanodrop RNA spectrophotometer (Thermo Scientific, UK). First strand cDNA was synthesised from the total RNA. Briefly, 1 mg of total RNA was incubated with 1 ml of random hexamers at 70 ℃ for 10 min. Samples were then transferred to ice, allowing the primers to anneal, followed by the addition of master mix containing 50 U BioScript, 4 ml 5x reaction buffer (Bioline, UK), 1 ml PCR nucleotide mix and 1ml Rnase outTM ribonuclease inhibitor in a final volume of 20 ml. Samples were mixed by pipetting and returned to the PCR 200 Thermal Cycler (MJ Research, UK) for 1 hour at 42 ºC.

NOX4 mRNA quantification was determined by TaqMan using the ABI 7300 Fast Real-Time PCR System. Primers and the fluorogenic TaqMan probes were designed using Primer Express according to human NOX4 sequence (**Table 5.1**). The primers were designed to detect all four NOX4 isoforms. The probes were labelled with a 5´ reporter dye (FAM) and 3´ quencher dye (TAMRA). Real-time RT-PCR reactions were carried out in a 96-well plate in a total volume of 25 µl per well consisting of 5 ng of cDNA, 10 µl Precision MasterMix, 100 nM probe, 100 nM forward and 100 nM reverse primers for NOX4. Following a 10 min hot start at 95 ℃, PCRs were performed for 45 cycles of denaturing at 95 ℃ for 15s and annealing/extension at 60 ℃ for 60 sec using an ABI 7300 machine (Applied Biosystems, Warrington, UK). Reactions were carried out in triplicate. Data was normalized against an invariant endogenous control, 18S ribosomal RNA. The reaction efficiency slope for 18S was determined to be -3.6 and for NOX4 was -3.3. Quantification was performed by the standard curve method and ΔΔCT method (Nolan, Hands et al. 2006), for comparison.

Table 5.1 Primers and Probes Sequences used for qRT-PCR

Gene	Direction	Primers (5' – 3')
NOX4	F	TTCTGGTATACTCATAACCTCT
	R	AGCAGCCCTCCTGAAACATG
	Probe	CTACATGCTGACGTT
18S	F	GGCTCATTAAATCAGTTATGGTTCCT
	R	GTATTAGCTCTAGAATTACCACAGTTATCCA
	Probe	TGGTCGCTCGCTCCTCTCCCA

5.2.3 Structure-activity studies

Inhibition of xanthine/xanthine oxidase by anthocyanins and their metabolites: Superoxide production by xanthine/xanthine oxidase was measured using the optimised method described above. Briefly, to a reaction mixture containing 200 µM cytochrome c, 0.1 U/ml xanthine oxidase and 200 µM xanthine, was added 1, 10, 100 and 1000 µM of structure-activity test compounds (*mono-hydroxyl derivatives*: pelargonidin-3-glucoside, 4-hydroxybenzoic acid and the 4' glucuronide of 4-hydroxybenzoic acid; *catechol derivatives*: cyanidin-3-glucoside, protocatechuic acid, vanillic acid and the 4' glucuronide of protocatechuic acid) in 50 mM sodium phosphate buffer (pH 7.4). The reaction was started with the addition of xanthine oxidase and the reduction kinetics of cytochrome c followed kinetically at 550 nm (Fluostar, BMG Labtech, UK) at 25 ℃ over 15 min (r eaction plateau). The superoxide generated was determined by subtracting the rate of cytochrome c reduction (increase in absorbance at 550 nm) in the presence of SOD from those in the absence of SOD. Blanks consisted of representative samples with heat inactivated xanthine oxidase (heated to 100 ℃ for 5 min). Controls for anthocyanin degradation and direct reaction with cytochrome c consisted of samples without the addition of test compounds but with an equivalent proportion of DMSO (not exceeding 0.5 % v/v) and incubations with the addition of anthocyanins but without cytochrome c and with or without SOD.

Modulation of angiotensin II stimulated superoxide release from intact cells by anthocyanins and their metabolites: The modulation of Ang II induced superoxide production in HUVECs by various anthocyanin metabolites (*mono-hydroxyl derivatives*: pelargonidin-3-glucoside, 4-hydroxybenzoic acid and the 4' glucuronide of 4-hydroxybenzoic acid; *catechol derivatives*: cyanidin-3-glucoside, protocatechuic acid, vanillic acid and the 4' glucuronide of protocatechuic acid) was assessed using the above optimised assay condition with the addition of treatment compounds. Briefly, cells were seeded into 24 well culture plates (NunclonΔ surface, Nunc, UK) at 5000 cells.cm^{-2} and grown to confluence. Confluent cells were equilibrated in 2% FCS supplemented Medium 199 for 24 hours. Following equilibration, cells were washed in warm PBS and re-suspended in phenol free Medium 199 containing 2% FCS, 20 µM cytochrome c, 0.1 uM Ang II and 0.1 and 1 µM structure-activity test compounds (mentioned above), with or without (basal condition) the addition of SOD (400 U). Blanks consisted of treatment in the absence of cells or in the absence of cytochrome c. Cells were incubated for a total of 6 h at 37 ℃ under a humid ified atmosphere containing 5% CO_2. At 6 h, 200 µl of cell supernatant was transferred to a 96-well plate and the reduced of cytochrome c measured at 550 nm (Fluostar, BMG Labtech, UK). The superoxide generated was determined by subtracting the absorbance at 550 nm in the presence of SOD from those in the absence of SOD. Control conditions consisted of cells incubated in the presence of Ang II but absence of anthocyanin derived treatment compounds.

Modulation of NO release from intact cells by selected anthocyanin derived phenolic acid metabolites: Since some of the compounds used in the previously described structure-activity relationship studies demonstrated some significant effects on cellular superoxide production, effect of these anthocyanin metabolites (4-hydroxybenzoic acid, protocatecuic acid, 4' glucuronide of 4-hydroxybenzoic acid and 4' glucuronide of protocatechuic acid) on endothelial cell nitric oxide (NO) production was assessed. Induced

NO production was estimated from the primary and non-volatile breakdown products of NO, nitrite (NO_2) and nitrate (NO_3), as per previously reported studies (Cheung, Siow et al. 2001; Pergola, Rossi et al. 2006; Wattanapitayakul, Suwatronnakorn et al. 2007). Initially, cells were cultured to confluence as previously described and subsequently incubated with 0.1 and 1 µM 4-hydroxybenzoic acid, protocatecuic acid, 4' glucuronide of 4-hydroxybenzoic acid or 4' glucuronide of protocatechuic acid, respectively, for 24 h in 2% FCS supplemented Medium 199 (phenol free). Control treatments consisted of cells treated in 2% FCS supplemented Medium 199 (phenol free) only. Positive control incubations consisted of cells treated with 10 nM PMA or 100 µM resveratrol, as previously described (Leikert, F. et al. 2002; Wallerath, Deckert et al. 2002). Following cell treatments, the culture media was collected and cleared by centrifugation at 2,000 rpm for 5 min. The cleared supernatant was then assayed for nitrite (NO_2) and nitrate (NO_3) levels using a commercially available kit (R&D Systems, UK), as per the manufacturer's instructions. Briefly, 50 µl of cell supernatant was added to, 50 µl reaction buffer and 100 µl of Griess reagent, mixed and incubated at room temperature for 10 min. Following incubation, the endogenous nitrite levels were determined at 540 nm. To determine total nitrite and nitrate levels, 50 µl of cell supernatant was added to 25 µl nitrate reductase and 25 µl NADH, mixed well and incubated at 30 ℃ for 30 min. Following incubation, 100 µL of Griess reagent was added the total nitrite and nitrate (converted to nitrite by nitrate reductase) levels were determined at 540 nm. Since very little or no NO_2 was found in the cell supernatants (relative to blank samples), differentiation between NO_2 and NO_3 levels were not attempted. Rather, all NO_3 was enzymatically converted to NO_2, where NO was reported as total NO_2 and NO_3. Following the collection of cell supernatants, total cellular protein was collected and quantified (as previously described), and all results normalised to total protein levels.

5.2.4 Statistical analysis

Statistical significance was evaluated by one way ANOVA with Tukey post-hoc tests (Windows SPSS, version 15) on raw or transformed data (i.e. blank corrected and normalisation to total protein where applicable). Significance was determined at $P<0.05$ against control or basal conditions. Unless otherwise stated, all experiments were conducted in triplicate and the data expressed as % of controls (mean±SD).

5.3 Results and discussion

5.3.1 Method development and optimisations

Cell viability under different treatment conditions: Since previously reported cell based flavonoid studies have used either low serum or BSA supplemented medium in cell treatment assays (Zhang, Schmeisser et al. 1999; Steffen, Schewe et al. 2007; Steffen, Gruber et al. 2008), it was important in this study to determine whether endothelial cells remained viable under different treatment conditions. To assess the effect of reported cell treatment conditions on endothelial cell viability, the respiration of confluent cells were assessed over 24 and 48 hours under low serum (1, 5 and 10% FCS) or BSA (0.1, 0.2, 0.4% BSA) supplemented conditions, without additional growth factors (**Figure 5.1**). All treatment conditions showed a significant increase (up to two fold) in cell viability within the first 24 h of cell incubation. However, following 48 h of cell incubation, all treatment conditions showed a significant loss ($p<0.001$) in viability compared to complete growth medium controls. In the case of serum supplemented media, a dose dependent loss in viability was observed, such that an increase in serum supplementation inferred an increase in viability. The loss of viability in serum supplemented media was 40-85% (1-10% FCS, respectively), while BSA supplemented media showed a 60% loss (0.1-0.4% BSA, respectively) in viability, across all concentration ranges. This indicated that low levels of serum supplementation was the most appropriate treatment medium condition for subsequent bioactivity assays. However, microscopic observations

showed that at a serum concentration of 1% or less, cells became rounded and detached from 6 and 24 well culture plates. This did not occur at 2% serum supplementation with 24 h. For these reasons, a serum concentration of 2% was adopted in all subsequent bioassays and cell treatments, since this was the minimum serum concentration which allowed for adequate cell adherence to the culture plates and maintenance of cellular viability.

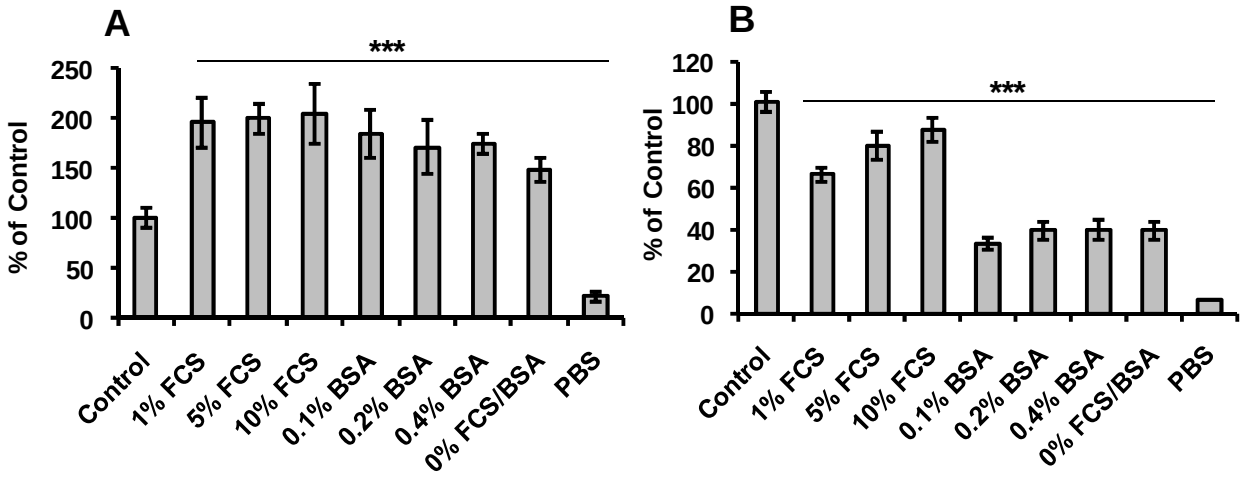


Figure 5.1. Cell viability under different treatment medium conditions (FCS or BSA supplementation). To assess the effect of different culture conditions on cell viability, HUVECs were treated with low serum (1-10%) or BSA (0.1-0.4%) supplemented medium 199 for 24 (**A**) and 48 (**B**) h. Controls consisted of cells treated with complete growth medium (basal medium supplemented with hydrocortisone, EGF, FGF with heparin and 2% FCS). Negative controls consisted of cells treated with PBS. FCS, Foetal calf serum; BSA, bovine serum albumin. *** represents significance at $P<0.001$ with respect to control. Data represented as mean±SD (n=3).

Effect of treatment compounds on cell viability: In order to determine the structure-activity reationship between different anthocyanins and their metabolites (*mono-hydroxyl derivatives*: pelargonidin-3-glucoside, 4-hydroxybenzoic acid and the 4' glucuronide of 4-hydroxybenzoic acid; *catechol derivatives*: cyanidin-3-glucoside, protocatechuic acid, vanillic acid, 3-hydroxy-4-methoxybenzoic acid and the 4' glucuronide of protocatechuic acid; *tri-hydroxyl derivatives*: delphinidin-3-glucoside, gallic acid and 2,4,6-trihydroxybenzaldehyde (phlorogluceraldehyde)) and cell survival, endothelial cell respiration was assayed using WST-1 reagent (**Figures 5.2-5.5**). In addition to structure-activity relationships, this study also determined the

optimal compound concentrations to be used in subsequent cell based bioassays.

Of the anthocyanins tested, cyanidin-3-glucoside (catechol B-ring) induced a significant loss in cell viability at ≥50 µM (P=0.01) (**Figure 5.3**) and pelargonidin-3-glucoside induced a significant loss in cell viability at 100 µM (P=0.013) (**Figure 5.2**). Delphinidin-3-glucoside did not show any significant effect on cell viability at ≤100 µM (p>0.05) (**Figure 5.4**). Of the phenolic metabolites tested, only protocatechuic acid (a catechol) induced a significant loss in cell viability at 100 µM (p<0.05). All other phenolic compounds did not show an effect on cell viability at ≤100 µM (p>0.05). Conjugation of protocatechuic acid on the aryl position (3' or 4' hydroxyl) with either a methoxy or glucuronide conjugate abolished the effect observed for the parent (catechol) structure (i.e. loss of cell viability when treated with protocatechuic acid). The compounds that most significantly affected cell viability under the present assay conditions were shown to be protocatechuic acid and cyanidin-3-glucoside which both possess a catechol on their benzene ring structure. However, since pelargonidin-3-glucoside was more active than delphinidin-3-glucoside, it cannot be said that an increase in B-ring hydroxyls infers greater cellular activity with regards to the loss of cell viability. Although, the poor activity observed for delphinidin-3-glucoside may have been due to its increased instability under physiochemical conditions, as described in chapter 3.

It was established that the optimal concentration for structure-activity test compounds was between 0 and 10 µM, since no significant effects were observed on cell viability at these concentration across all tested compounds. To this end, all test compounds did not affect cell viability (respiration) at the levels used in subsequent bioactivity assays or at physiologically relevant concentrations (0.1-1 µM).

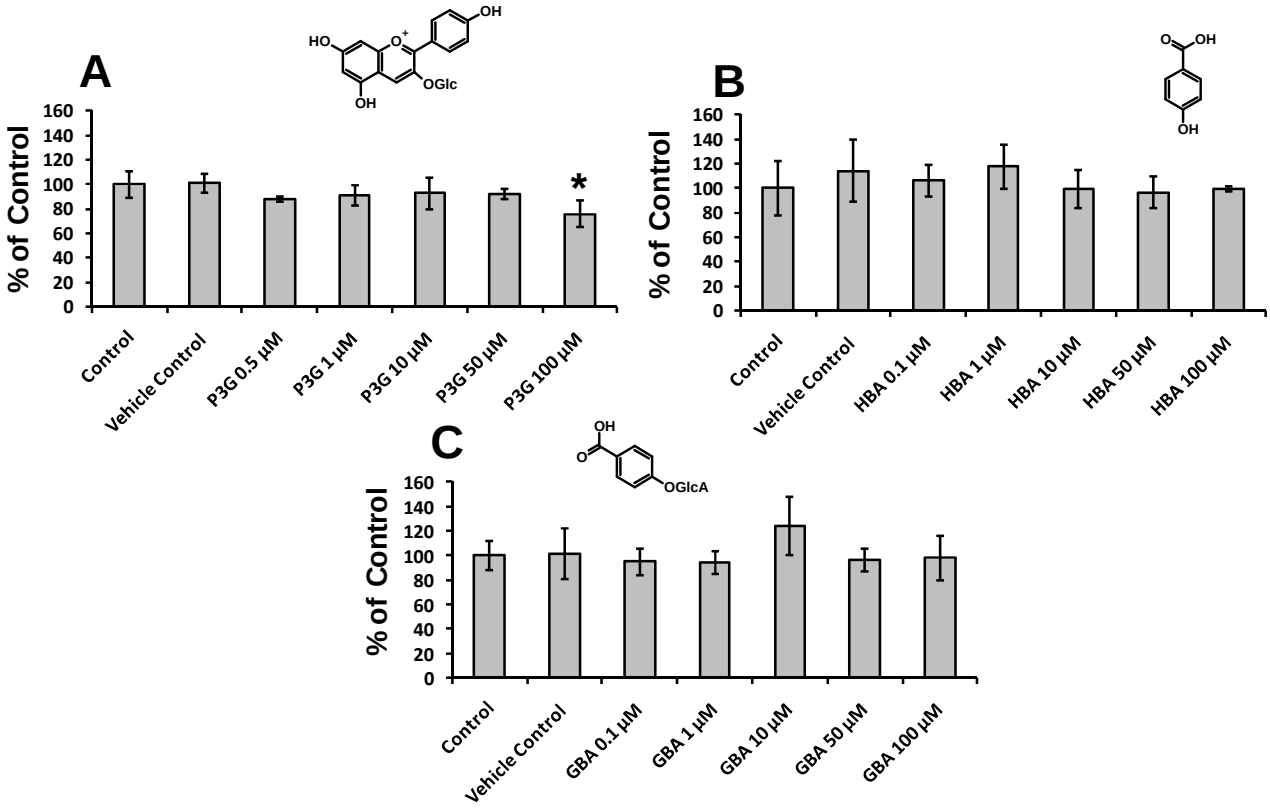


Figure 5.2. The effect of different mono-hydroxyl derivatives on endothelial cell viability. To assess the effect of different anthocyanin derived structures on cell viability, HUVECs were treated with P3G (**A**; pelargonidin-3-glucoside), HBA (**B**; 4-hydroxybenzoic acid) and GBA (**C**; 4' glucuronide conjugate of HBA) for 24 h in 2% FCS in medium 199 and the cellular respiration assayed using WST-1 reagent. Controls consisted of cells treated with 2% FCS in medium 199. Vehicle control consisted of cells treated with 0.05% DMSO in 2% FCS in medium 199. FCS, Foetal calf serum; OGlc, O-glucose; OGlcA, O-glucuronic acid. *represents significance at $P<0.05$ with respect to control. Data represented as mean±SD (n=3).

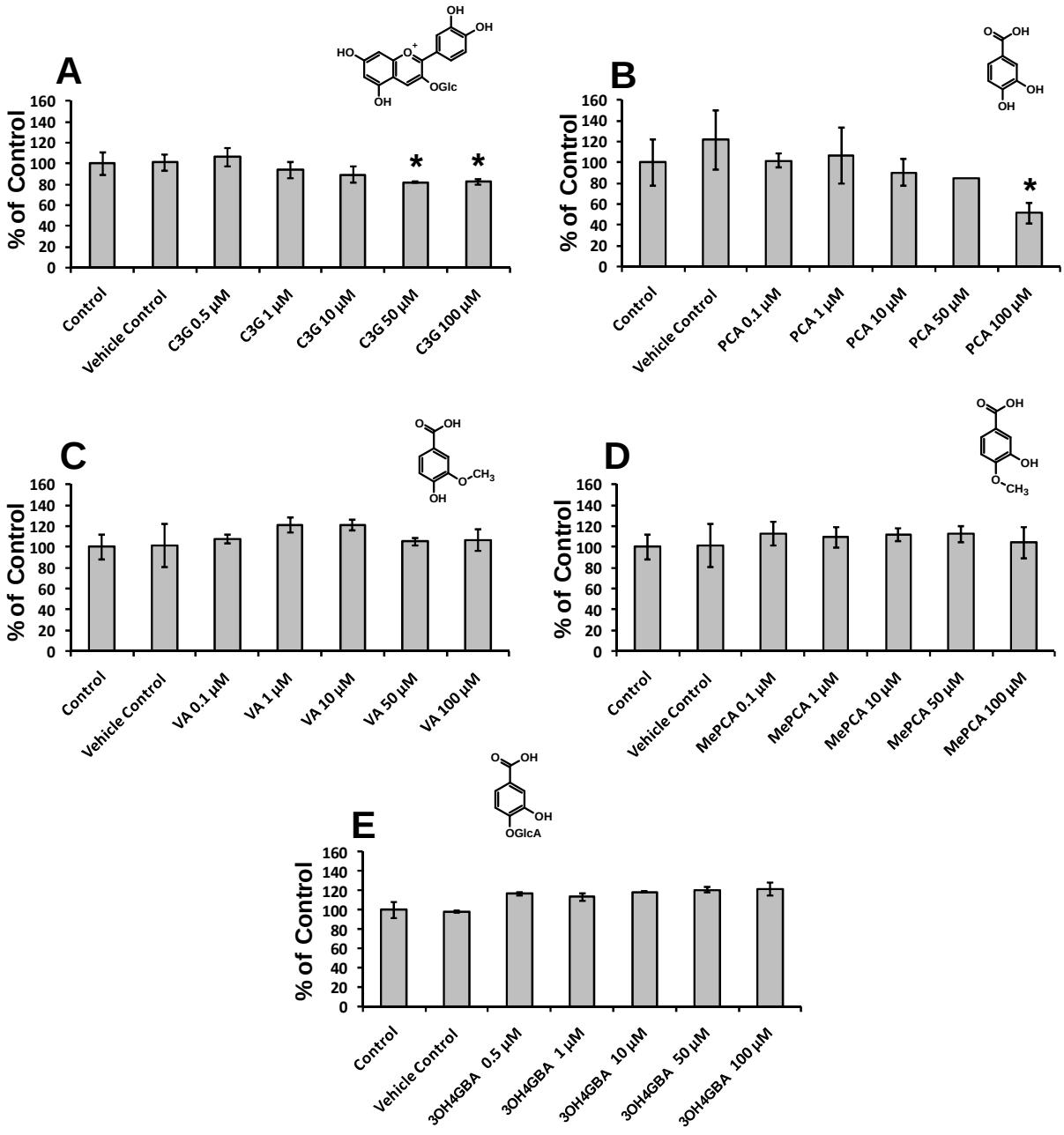


Figure 5.3. The effect of different catechol derivatives on endothelial cell viability. To assess the effect of different anthocyanin derived structures on cell viability, HUVECs were treated with C3G (**A**; cyanidin-3-glucoside), PCA (**B**; protocatechuic acid), VA (**C**; vanillic acid), MePCA (**D**; 3-hydroxy-4-methoxybenzoic acid) and 3OH4GBA (**E**; 4' glucuronide conjugate of protocatechuic acid) for 24 h in 2% FCS in medium 199 and the cellular respiration assayed using WST-1 reagent. Controls consisted of cells treated with 2% FCS in medium 199. Vehicle control consisted of cells treated with 0.05% DMSO in 2% FCS in medium 199. FCS, Foetal calf serum; OGlc, O-glucose; OGlcA, O-glucuronic acid. *represents significance at $P<0.05$ with respect to control. Data represented as mean±SD (n=3).

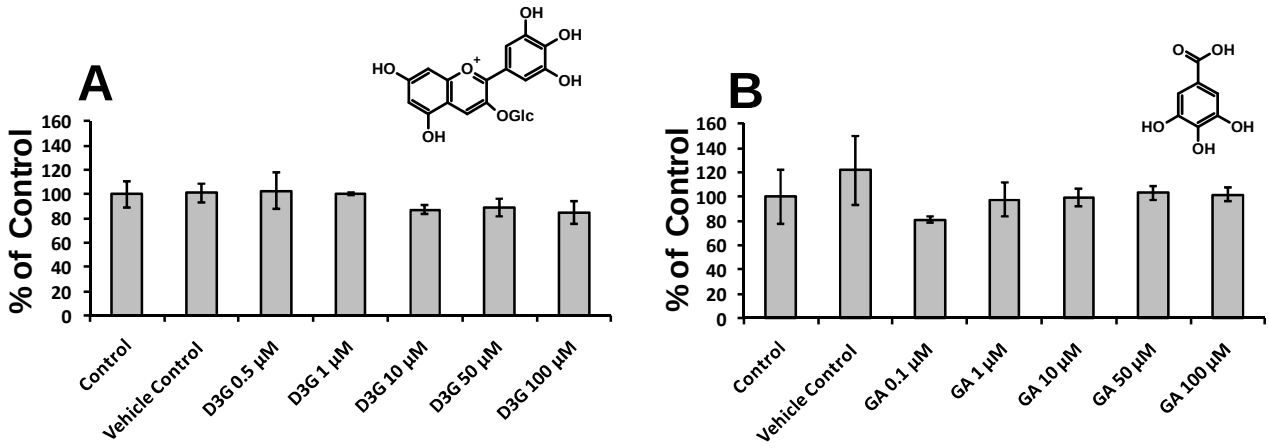


Figure 5.4. The effect of different trihydroxyl derivatives on endothelial cell viability. To assess the effect of different anthocyanin derived structures on cell viability, HUVECs were treated with D3G (**A**; delphinidin-3-glucoside) and GA (**B**; Gallic acid) for 24 h in 2% FCS in medium 199 and the cellular respiration assayed using WST-1 reagent. Controls consisted of cells treated with 2% FCS in medium 199. Vehicle control consisted of cells treated with 0.05% DMSO in 2% FCS in medium 199. FCS, Foetal calf serum; OGlc, O-glucose; OGlcA, O-glucuronic acid. Data represented as mean±SD (n=3).

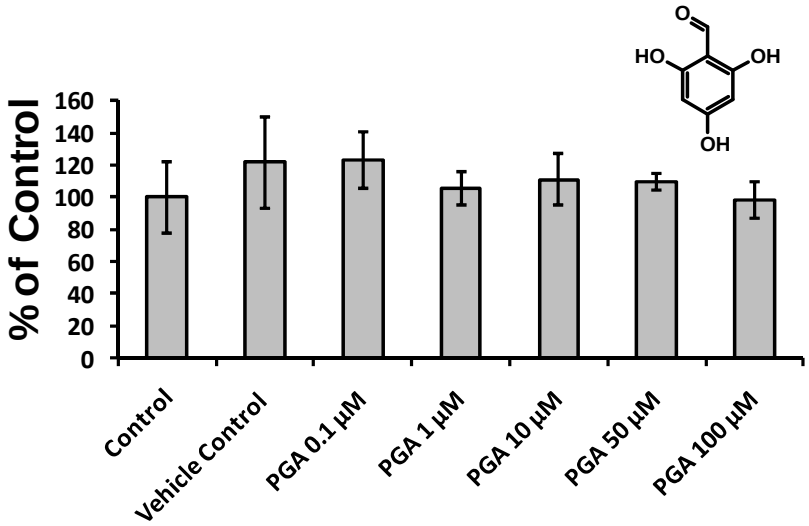


Figure 5.5. The effect of 2,3,4-trihydroxybenzaldehyde acid (PGA) on endothelial cell viability. To assess the effect of PGA on cell viability, HUVECs were treated with for 24 h in 2% FCS in medium 199 and the cellular respiration assayed using WST-1 reagent. Controls consisted of cells treated with 2% FCS in medium 199. Vehicle control consisted of cells treated with 0.05% DMSO in 2% FCS in medium 199. FCS, Foetal calf serum. Data represented as mean±SD (n=3).

The iron-ion reducing capacity of anthocyanins and their metabolites: Eukaryotic cytochrome c is a constituent of the electron-transport chain responsible for transfer of electrons from cytochrome c_1 to the terminal electron acceptor of the mitochondria (Lemberg and Barret 1973). Reduction of cytochrome c by ascorbic acid has been extensively investigated (Al-Ayash and Wilson 1979), where it was suggested that dianionic molecules donate electrons to the exposed haem edge or bind to the arginine-38 residue of cytochrome c, followed by electron transfer to the haem via aromatic residues. Therefore, in the present investigation, the direct reduction of cytochrome c by anthcyanins and their metabolites (structure-activity study: *mono-hydroxyl derivatives*: pelargonidin-3-glucoside, 4-hydroxybenzoic acid and the 4' glucuronide of 4-hydroxybenzoic acid; *catechol derivatives*: cyanidin-3-glucoside, protocatechuic acid, vanillic acid and the 4' glucuronide of protocatechuic acid; *tri-hydroxyl derivatives*: delphinidin-3-glucoside and gallic acid) was investigated. This was important for several reasons. Firstly, it remained essential to determine the ability for these compounds to reduce cytochrome c directly, since it would be used as an indicator for superoxide production in subsequent structure-activity bioassays. It has been suggested that the ability for compounds to reduce cytochrome c is associated with their iron-ion reducing/electron donation capacity and, therefore, their ability to inhibit proteins such as lipoxygenases (Kemal, Louis-Flamberg et al. 1987; Van der Zee, Eling et al. 1989; Laughton, Evans et al. 1991). This property may also serve to indicate their antioxidant potential (Bast, Haenen et al. 1991; Mira, Fernandez et al. 2002), since electron donation potential is crucial to direct radical scavenging activity. Secondly, the anthocyanin derived compounds are structurally similar, in some respects, to ascorbic acid, possessing, in the case of catechols, conjugated enediol-like structures.

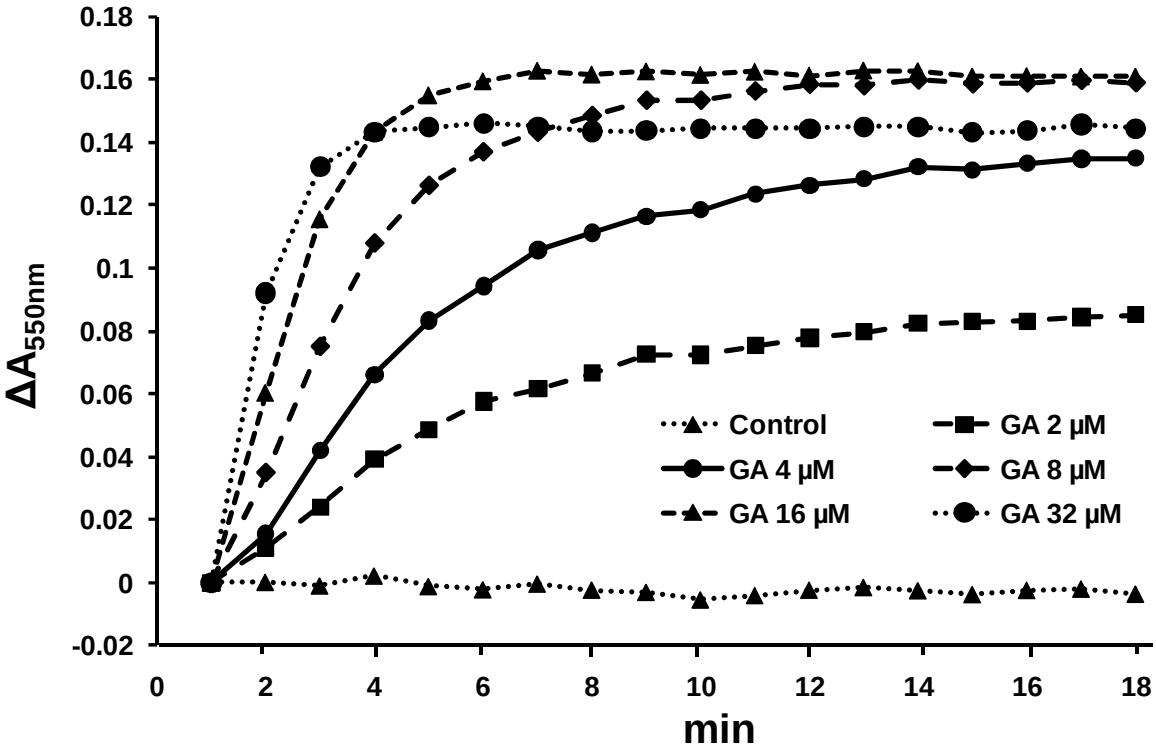


Figure 5.6. Representative kinetic plot of direct phenolic acid reduction of cytochrome c. In this case, gallic acid (GA) was incubated in the presence of cytochrome c in buffer (pH 7.4) at 0-32 µM and the rate of cytochrome c reduction (observed as an increase in absorbance) followed kinetically at 550nm for 18 min.

In **figure 5.6,** a representative kinetic curve for the reduction of cytochrome c by phenolic acids is shown. In the case of anthocyanins, an increase in B-ring hydroxylation inferred a greater potential to oxidise (reduced absorbance at 550nm) cytochrome c (**Figure 5.7**). However, anthocyanin co-incubations in the absence of cytochrome c also demonstrated a loss in absorbance at 550nm at neutral pH. Thus, although the cytochrome c reduction experiments were blanked to co-incubations in the absence of cytochrome c, it is possible that this did not negate the effect of anthocyanin degradation in this experiment. Therefore, further investigation should be conducted to elucidate the direct cytochrome c reduction potential of anthocyanins, where the spectral effects of anthocyanin degradation should be accounted for.

For anthocyanin derived phenolic acids, in keeping with previously reported data (Saleem and Wilson 1982), hydroxylated phenolic compounds rapidly reduced cytochrome c at physiological pH (increase in absorbance at 550nm). The reduction kinetics for hydroxyl substituted benzoic acids (*mono-hydroxyl*

derivatives: 4-hydroxybenzoic acid; *catechol derivatives*: protocatechuic acid; *tri-hydroxyl derivatives*: gallic acid) are shown in **figure 5.7**, where the rate of cytochrome c reduction was dependent on the number of hydroxyls present on the aromatic ring, as an increase in hydroxylation inferred an increase in the rate of cytochrome c reduction. This activity appeared to be dependent on the presence of multiple aryl hydroxyls (two and three B-ring hydroxyls i.e. protocatechuic acid and gallic acid), since the mono-hydroxyl, 4-hydroxybenzoic acid, did not appear to reduce cytochrome c.

In order to further investigate this effect, the direct reduction of cytochrome c by the catechol protocatechuic acid and its metabolic substitutes (methoxy or glucuronide substituted aryl hydroxyls) was investigated (**Figure 5.7**). In this manner it was shown that substitution of the catechol structure with either a methoxy or glucuronide abolished protocatechuic acid's direct cytochrome c reduction potential. Furthermore, substitution with a glucuronide abolished the ability for protocatechuic acid to directly reduce cytochrome c to a greater extent when compared with methoxy substitution. This effect is likely due to differential abilities for methoxy or glucuronide conjugates to withdraw electrons from the adjacent ring system.

The finding of this structure-activity relationship study appear to be in keeping with the reported antioxidant potentials of flavonoids and phenolic acids, where it has been shown that an increase in aromatic hydroxyls infers an increase in antioxidant potential (Rice-Evans, Miller et al. 1996), likely due to their increased ability to donate electrons. With regards to mechanism, these compounds would be de-protonated at neutral pH [with a PK_a of 4.4 for 4-hdroxybenzoic acid, 4.4 for protocatechuic acid and 4.2 for gallic acid (Erdemgil, Sanli et al. 2007)], forming either mono or di-anions, in which the hydroxyl groups are negatively charged. It is well established that catechols and quinols can donate two electrons per molecule, forming stable quinones as final products (Patai 1971; Paltai 1988). As it is reported that cytochrome c is a one electron acceptor (Lemberg and Barret 1973), it follows that an increase in the number of deprotonated hydroxyls would reduce a greater

number of cytochrome c molecules, which is in keeping with the present findings.

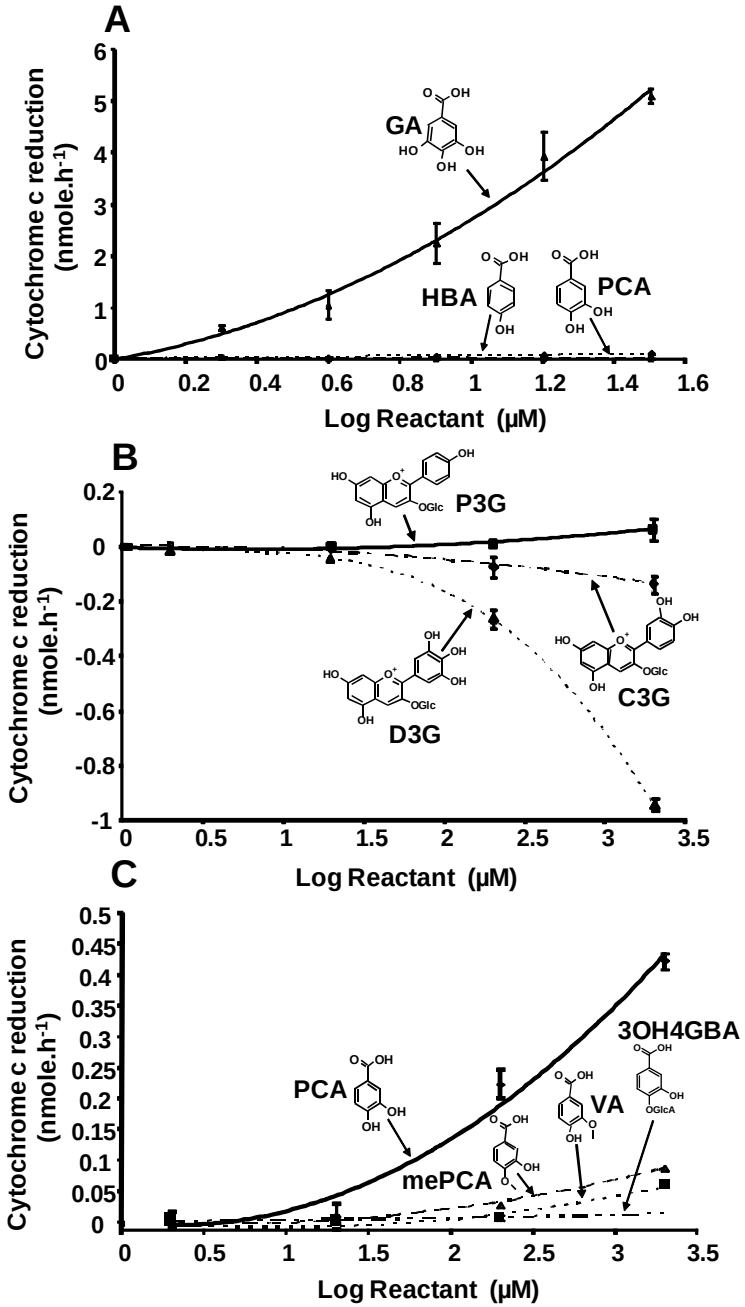


Figure 5.7. The rate of direct cytochrome c reduction by anthocyanins and their metabolites. **A**, the rate of cytochrome c reduction by PCA (protocatechuic acid), HBA (4-hydroxybenzoic acid) and GA (gallic acid), plotted as the rate of reduction (change in cytochrome c concentration over time at the linear range) vs. the log of phenolic acid concentration. **B**, the rate of cytochrome c reduction by P3G (pelargonidin-3-glucoside), C3G (cyanidin-3-glucoside) and D3G (delphinidin-3-glucoside), plotted as the rate of reduction (change in cytochrome c concentration over time at the linear range) vs. the log of phenolic acid concentration. **C**, the rate of cytochrome c reduction by PCA and its aryl conjugates, mePCA (3-hydroxy-4-methoxybenzoic acid), VA (vinallic acid or 4-hydroxy-3-methoxybenzoic acid) and 3OH4GBA (4' glucuronide conjugate of protocatechuic acid PCA), plotted as rate of reduction (change in cytochrome c concentration over time at the linear range) vs. the log of phenolic acid concentration. Rate data was based on individual kinetic assays (refer to **Figure 5.6** for a representation of the individual kinetic assay plots). Data represented as mean±SD (n=3), with a polynomial line of regression.

In summary, anthocyanin derived phenolic acids acted as direct reductants of cytochrome c at physiological pH, under the present assay conditions, where an increase in aryl hydroxylation inferred an increase in iron-ion reducing capacity. Since conjugation of the catechol structure on protocatechuic acid abolished its cytochrome c reduction capacity, it may be surmised that the charge on the de-protonated compound must reside on the conjugated sites (i.e., the oxygen and aromatic ring system). This effect is likely due to withdrawal of electrons from the ring system following substitution of the aryl hydroxyl with a methoxy or glucuronide conjugate, causing them to be unavailable for reduction reactions. Importantly, the assessment for these compounds as reducers of cytochrome c was key to future method/assay development, since cytochrome c was to be used in bioassays aimed at quantifying superoxide production. From these experiments it was evident that all the compounds tested, with the exception of gallic acid, would be suitable for use in subsequent superoxide bioassays, without the occurrence of extensive direct cytochrome c reduction and, therefore, depletion of the assay substrate/marker.

Cytochrome c reduction assay for xanthine/xanthine oxidase activity: The enzyme xanthine oxidase catalyses the oxidation of hypoxanthine and xanthine to uric acid (Parks and Granger 1986). During the re-oxidation of xanthine oxidase, molecular oxygen acts as an electron acceptor, producing superoxide radical and hydrogen peroxide. It has been reported that flavonoids inhibit xanthine oxidase and that this inhibition is due to their direct radical scavenging activities (Cos, Ying et al. 1998). Presently, the structure-activity relationship of anthocyanins and their metabolites (*mono-hydroxyl derivatives*: pelargonidin-3-glucoside, 4-hydroxybenzoic acid and the 4' glucuronide of 4-hydroxybenzoic acid; *catechol derivatives*: cyanidin-3-glucoside, protocatechuic acid, vanillic acid and the 4' glucuronide of protocatechuic acid) as inhibitors of xanthine oxidase and scavengers of superoxide were investigated. In order to measure the inhibition of xanthine oxidase, a cytochrome c reduction assay was developed and optimised from a previously reported system (Steffen, Schewe et al. 2007; Steffen, Gruber et al. 2008).

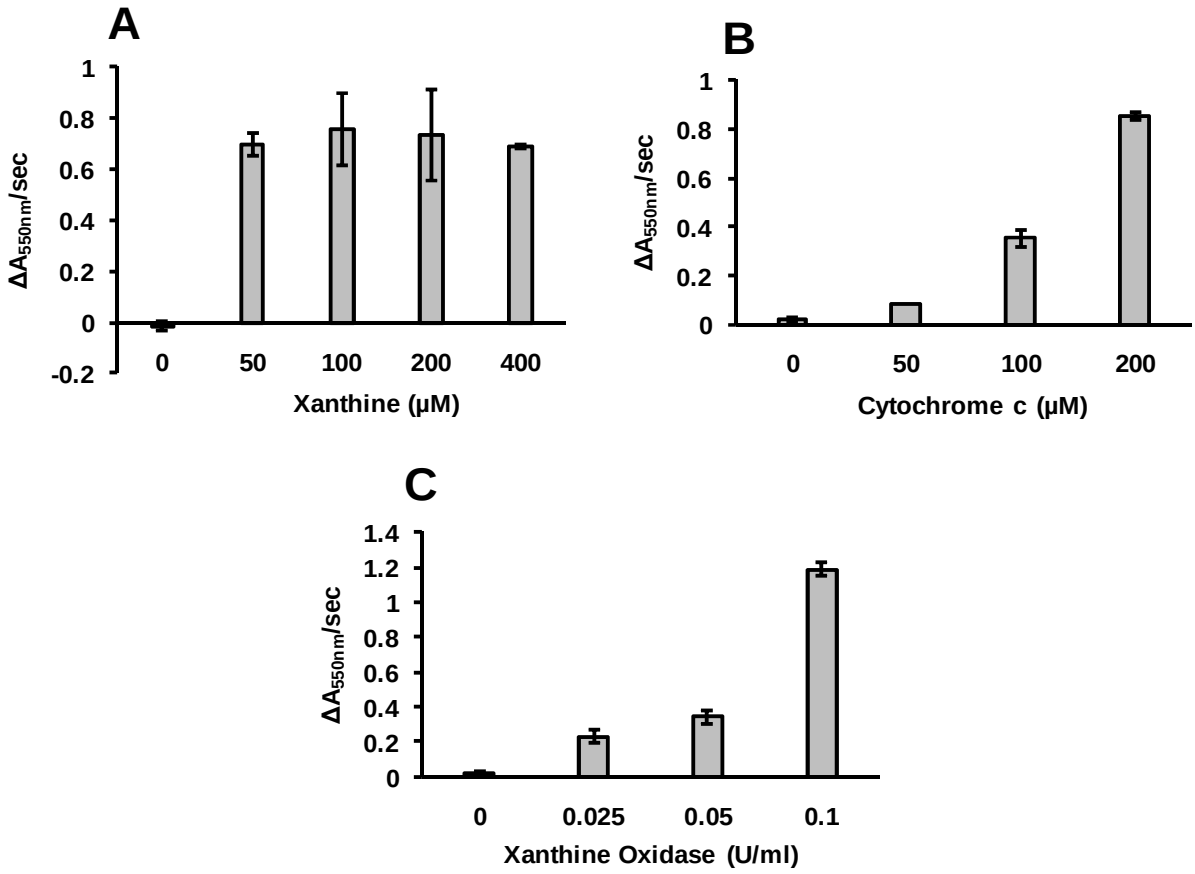


Figure 5.8. Optimisation of a xanthine oxidase catalysed superoxide assay using cytochrome c reduction. First, rate of cytochrome c reduction by xanthine oxidase was assessed at different xanthine (substrate) concentrations (**A**). Next, the rate of cytochrome c reduction by xanthine oxidase was assessed at different cytochrome c (detector molecule) concentrations (**B**). Finally, the rate of cytochrome c reduction by xanthine oxidase was assessed at different enzyme concentrations (**C**). Data represented as mean±SD (n=3).

Initially, the rate of cytocrome c reduction by a xanthine oxidase catalysed reaction was assessed at different concentrations of primary substrate (xanthine at 0, 50, 100, 200 and 400 μM). It was shown that the primary substrate was in excess abundance, since a change in xanthine concentration did not affect the rate of cytochrome c reduction (**Figure 5.8**). Therefore substrate depletion would not be a compounding factor in subsequent structure-activity assays and an economical concentration of 200 μM xanthine was therefore used in all subsequent xanthine oxidase assays. Subsequently, the rate of cytocrome c reduction by a xanthine oxidase catalysed reaction was assessed at different concentrations of cytocrome c (0, 50, 100 and 200 μM). It was shown that a cytochrome c concentration of 200 μM demonstrated

the greatest assay sensitivity, since this concentration had the greatest change in absorbance (**Figure 5.8**). Therefore a cytochrome c concentration of 200 µM was used in all subsequent xanthine oxidase assays. Finally, the rate of cytocrome c reduction by a xanthine oxidase catalysed reaction was assessed at different enzyme concentrations (0, 0.025, 0.05 and 0.1 U/ml). It was shown that a xanthine oxidase concentration of 0.1 U/ml produced the greatest reduction of cytochrome c, since this concentration demonstrated the largest change in absorbance (**Figure 5.8**). Therefore a xanthine oxidase concentration of 0.1 U/ml was used in all subsequent xanthine oxidase assays. Thus, the optimal assay parameters were determined to be at a xanthine oxidase concentration of 0.1 U/ml, cytochrome c concentration of 200 µM and xanthine concentration of 200 µM.

Using the above developed cytochrome c reduction assay it was demonstrated that SOD significantly attenuated the rate of cytochrome c reduction (**Figure 5.9**), indicating that this assay was specific for superoxide. However, SOD did not completely abolish cytochrome c reduction. This was likely due to reduction by hydrogen peroxide produced by SOD. Here it was demonstrated that when including SOD as a control (co-incubation), cytochrome c could be used as an effective assay for xanthine oxidase catalyzed superoxide production, since SOD significantly inhibited the reduction of cytochrome c. Thus, all subsequent xanthine oxidase bioassays included a SOD containing co-incubation (blank), through which the specific superoxide production by the optimised xanthine oxidase reaction could be assessed.

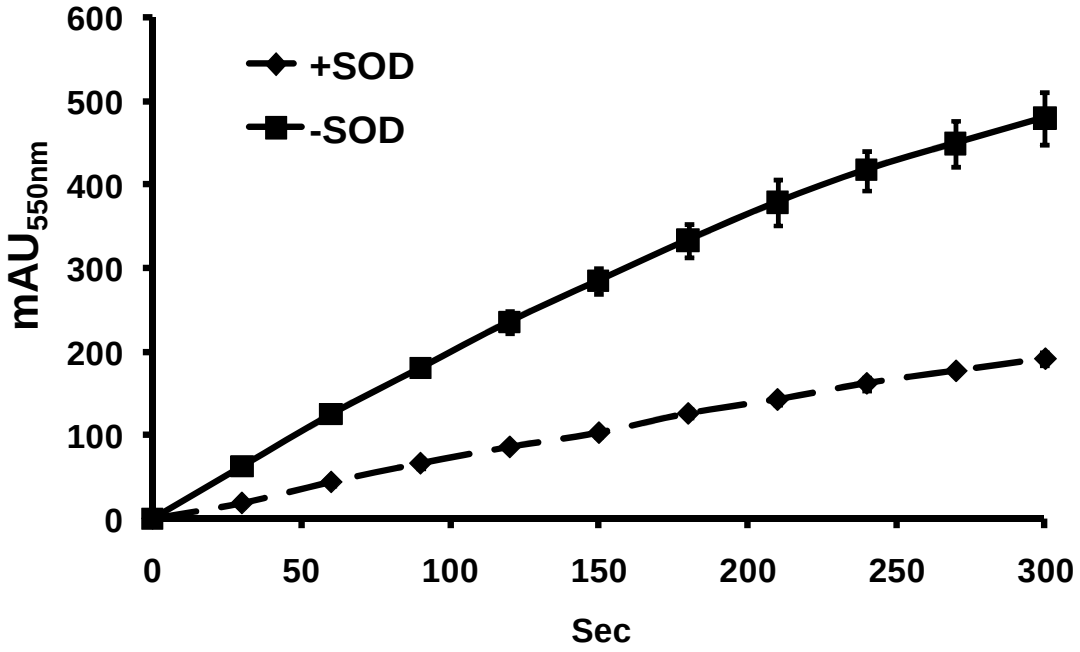


Figure 5.9. SOD inhibition of cytochrome c reduction by xanthine oxidase. The reduction of cytochrome c was assessed in the presence and absence of SOD, using an optimised xanthine oxidase reaction assay. SOD, superoxide dismutase, mAU, milli-absorbance units. Data represented as mean±SD (n=3).

Ang II induced oxidative stress (superoxide production) in cultured endothelial cells: Increased levels of angiotensin II (Ang II) has been shown to be involved in the development and progression of atherosclerosis (Brasier, Recinos et al. 2002; Mehta and Griendling 2007). This hypothesis is supported by recent studies with Ang II receptor type 1 (AT1R) inhibitors, which show anti-atherosclerotic effects (Rueckschloss, Quinn et al. 2002; Gaede, Vedel et al. 2003). A putative risk factor involved in the pro-atherosclerotic effects of Ang II is increased oxidative stress by an elevated formation of ROS, including superoxide. Superoxide rapidly reacts with NO and reduces the bioavailability of this vasoprotective mediator of endothelium-dependent relaxation. Therefore, Ang II stimulated increases in vascular superoxide formation likely contributes to the development of endothelial dysfunction and atherosclerosis. In the present study, the dose-dependent regulation of superoxide production and expression of NAD(P)H oxidase (NOX4) subunit by Ang II in human endothelial cells was investigated and subsequently employed as a bioassay to screen the structure-activity relationship of anthocyanin derived phenolic acids and metabolites. In order to

measure the induction of superoxide by Ang II and conduct a structure-activity relationship study, a cytochrome c reduction assay was developed and optimised, based on previously reported studies (Rueckschloss, Quinn et al. 2002; Steffen, Schewe et al. 2007; Steffen, Gruber et al. 2008) and methods developed above. This assay was subsequently used to evaluate the structure-activity relationship of anthocyanins and their metabolites with respect to Ang II induced superoxide production.

Initially, the optimised cytochrome c reduction assay developed for measuring xanthine oxidase catalysed superoxide production was adapted to cell culture experiments and developed further. First, the optimum incubation time for cytochrome c in the presence of endothelial cells was assessed in 2% serum supplemented medium (as previously optimised in cell viability assays). In this experiment the basal rates of cytochrome c reduction by cultured endothelial cells was assessed. Simultaneously, the ability for SOD to inhibit the endothelial cell induced reduction of cytochrome c was also assessed. This allowed for the evaluation of superoxide specific cytochrome c reduction. Thus, it was demonstrated that cultured HUVECs were capable of reducing cytochrome c in a superoxide dependent manner (**figure 5.10**), since a) the reduction of cytochrome c was greater in the presence of cells than in their absence (blank) and b) the reduction of cytochrome c by cultured endothelial cells was significantly inhibited by SOD. Furthermore, when incubated in the presence of endothelial cells for 2, 6 and 8 h, the degree of cytochrome c reduction was shown to be greatest at 6 h and demonstrated the least degree of variation (**Figure 5.10 and 5.11**).

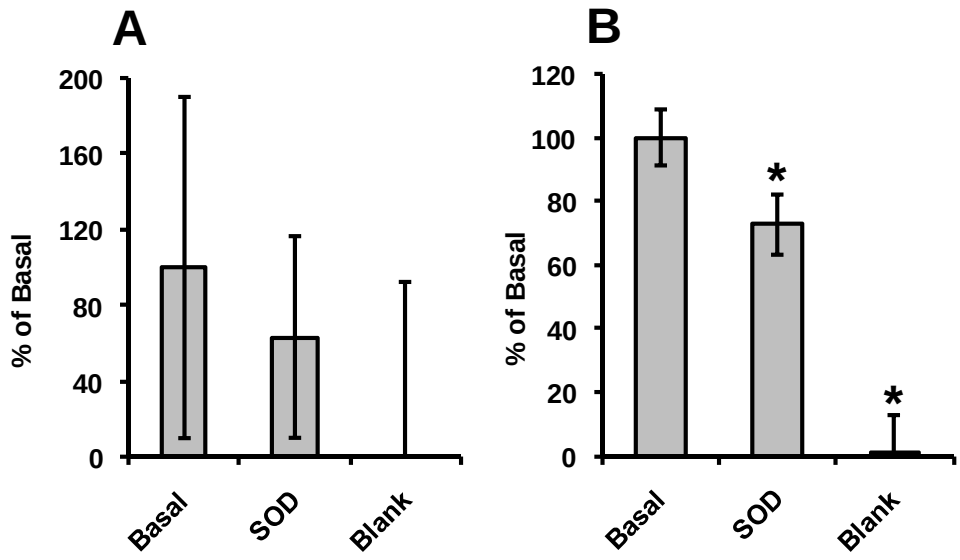


Figure 5.10. The SOD inhibitable reduction of cytochrome c by HUVECs. Cultured endothelial cells were exposed to cytocrome c in 2% FCS containing medium 199 in the absence (basal) and presence (SOD) of SOD. The reduced cytochrome c was measured at 550nm at 2h (**A**) and 6h (**B**). Blanks consisted of treatments in the absence of cells. *represents significance at P<0.05 with respect to basal levels of cytochrome c reductions. Data represented as mean±SD (n=6).

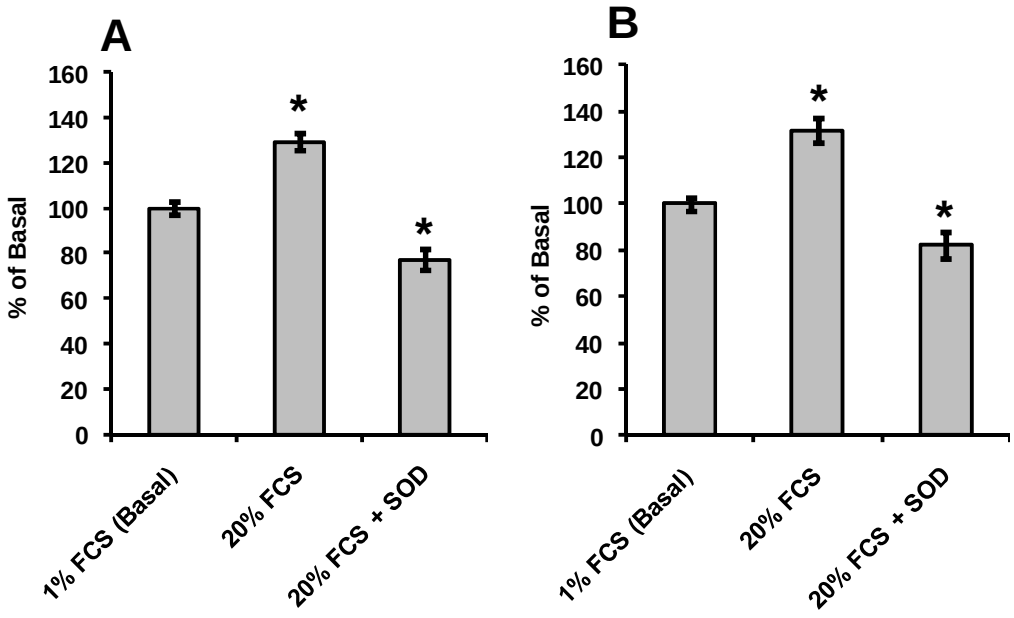


Figure 5.11. The SOD inhibitable reduction of cytochrome c by HUVECs under low and high serum conditions. Cultured endothelial cells were exposed to cytocrome c in 2% FCS containing medium 199 in low (1%) and high (20%) serum supplemented medium 199. The reduced cytochrome c was measured at 550nm at 6h (**A**) and 8h (**B**). *represents significance at P<0.05 with respect to basal levels of cytochrome c reductions. Data represented as mean±SD (n=6).

Since it had previously been reported that serum supplementation increases the production of superoxide production and expression of NOX in cultured cells (Wingler, Wünsch et al. 2001), it was important to establish the degree of serum induced cytochrome c reduction in the current cell culture model. Following the stimulation of endothelial cells with 1 and 20% serum supplemented medium (**Figure 5.11**), it was demonstrated that increased serum concentrations increase the reduction of cytochrome c. Furthermore, the reduction of cytochrome c by serum stimulated endothelial cells was significantly inhibited by the addition of SOD. This was consistent with previously reported data (Wingler, Wünsch et al. 2001) demonstrating that increased serum supplementation induced an increase in basal superoxide production by cultured endothelial cells. In addition, no difference in cytochrome c reduction by cultured endothelial cells was observed between 6 and 8 h incubation. Thus, subsequent structure-activity relationship experiments were performed in 2% serum supplemented medium in accordance with this data and the findings of earlier cell viability experiments.

Following the evaluation of basal superoxide production (above), cultured human endothelial cells were exposed to 0.05, 0.1 and 1 µM Ang II (Rueckschloss, Quinn et al. 2002) over 6 h in 2% serum supplemented medium, as optimised in previously described experiments. It was demonstrated that exposure of the cells to Ang II resulted in a dose dependent induction of endothelial superoxide production (**Figure 5.12**). This was in agreement with previously reported data at concentrations ≤0.1 µM (Rueckschloss, Quinn et al. 2002), although a study by Rueckschloss et al. showed a bimodal response, with a decrease in superoxide production at concentrations ≥0.1 µM (Rueckschloss, Quinn et al. 2002). The difference in Ang II induced superoxide production between this study and that of Rueckschloss et al. could be rationalised by previously reported activation and un-coupling of eNOS by Ang II (Millatt, Abdel-Rahman et al. 1999; Chalupsky and Cai 2005). Thus, it may be hypothesised that methodological differences in this study were unable to detect the bi-modal response or that the cells were less responsive to Ang II induced eNOS expression/activation. If substrates for NO production by eNOS were insufficient under the current

assay condition, it could be hypothesised that 0.1 µM Ang II increased superoxide production due to increased activity/expression of NOX4, while at >0.1 µM, Ang II stimulation resulted in eNOS activity/expression, which was "un-coupled" due to substrate (BH4) depletion as discussed elsewhere (Chalupsky and Cai 2005). Interestingly others have used Ang II at 1 µM to induce superoxide release from HUVECs (Steffen, Schewe et al. 2007; Steffen, Gruber et al. 2008). In order to establish a molecular source for the dose-dependent induction of endothelial superoxide production by Ang II, the expression of a NAD(P)H oxidase (NOX4) was examined in response to Ang II stimulation, both at the protein and mRNA level. Stimulation of HUVECs with Ang II caused a dose-dependent bimodal induction of NOX4 protein and mRNA expression (**Figure 5.13**), which was consistent with previously reported data (Rueckschloss, Quinn et al. 2002), although not statistically significant. Based on these findings and in accordance with previously reported data, subsequent structure-activity relationship studies were conducted using Ang II stimulation at 0.1 µM for 6 h in 2% serum supplemented medium, since these parameters appeared to induce superoxide production from endothelial cells due to NOX4 up-regulation and/or activity.

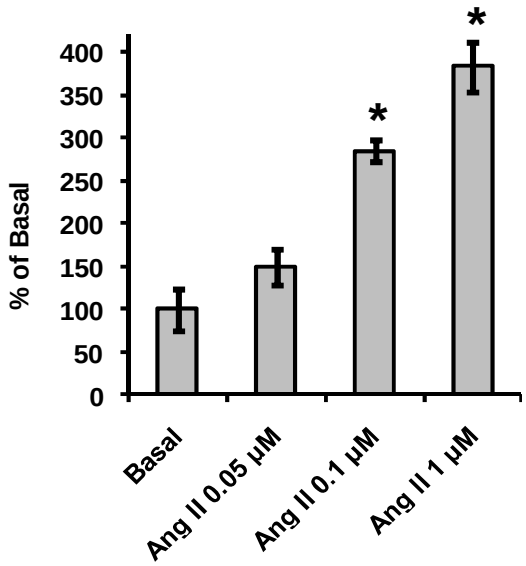


Figure 5.12. The induction of superoxide production by Ang II. Cultured HUVECs were exposed to Ang II (angiotensin II) for 6 h in 2% FCS supplemented medium 199 and the production of superoxide (difference in reduced cytochrome c in treatments in the presence and absence of SOD) assessed using an optimised cytochrome c reduction assay and normalised to total protein content. *represents significance at $P<0.05$ with respect to basal levels of superoxide production (no treatment). Data represented as mean±SD (n=3).

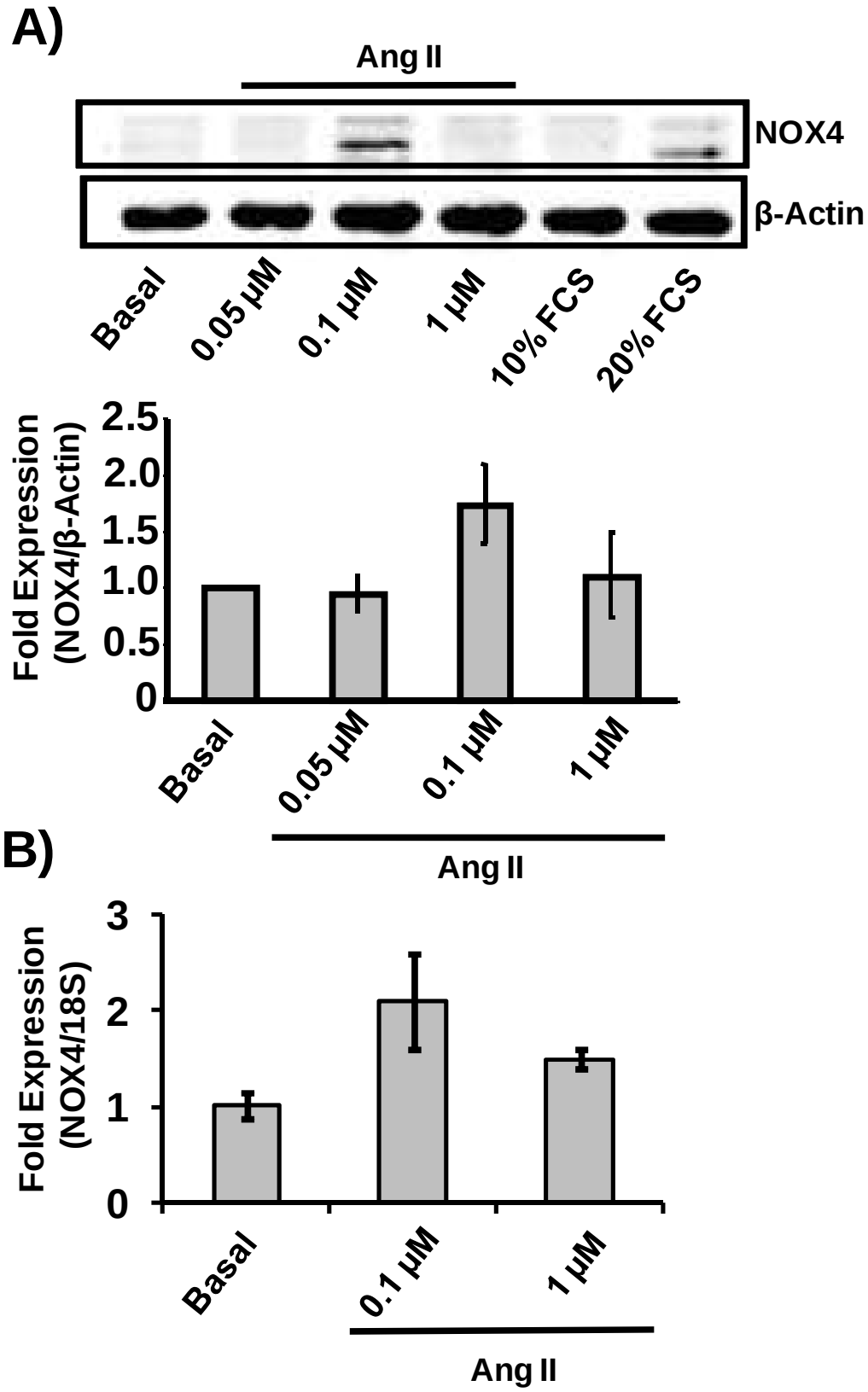


Figure 5.13. The induction of NOX4 protein (**A**) and mRNA (**B**) in HUVECs exposed to Ang II (angiotensin II) for 6 h in 2% FCS supplemented medium 199. Data represented as mean±SD (n=2).

5.3.2 Structure-activity studies

Inhibition of xanthine/xanthine oxidase by anthocyanins and their metabolites: The structure-activity relationship of anthocyanins and their metabolites (*mono-hydroxyl derivatives*: pelargonidin-3-glucoside, 4-hydroxybenzoic acid and the 4' glucuronide of 4-hydroxybenzoic acid; *catechol derivatives*: cyanidin-3-glucoside, protocatechuic acid, vanillic acid and the 4' glucuronide of protocatechuic acid) on xanthine oxidase catalysed superoxide production was assessed using the above optimised xanthine oxidase assay. The B-ring monohydroxyl derived anthocyanin compounds did not appear to inhibit superoxide production by xanthine oxidase (**Figure 5.14**). Interestingly, the glucuronide conjugate of pelargonidin-3-glucoside's primary degradation product, 4-hydroxybenzoic acid, appeared to increase superoxide production (**Figure 5.14C**).

The B-ring catechol derived anthocyanin compounds appeared to inhibit the production of superoxide by xanthine oxidase. However, substitution of the phenolic catechol with a methoxy and glucuronide conjugate appeared to eliminate this inhibitory effect (**Figure 5.15**). This suggests that the inhibition of xanthine oxidase catalysed superoxide production was increased with the number of B-ring hydroxyls (catechols). This was consistent with their antioxidant potential, where radical scavenging capacity has been shown to increase with the number of free hydroxyls on the phenolic structure (Rice-Evans, Miller et al. 1996). This pattern of structure-activity is also consistent with their observed electron donor properties as determined from the direct iron-ion reducing capacity studies (described above). Therefore, it is likely that anthocyanins and their phenolic metabolites inhibit xanthine oxidase through their direct radical scavenging activity. This was consistent with previously reported findings for other flavonoids (Cos, Ying et al. 1998) and indicates that the antioxidant properties of these compounds are retained only as un-metabolised parent compounds. Thus, the ability for anthocyanins to scavenge xanthine oxidase produced superoxide radicals is not likely to occur *in vivo*, since no effect was observed at physiologically relevant

concentrations. Therefore, the reported vascular benefits associated with these compounds must act via an alternative mechanism. To this end, a cellular model was developed in subsequent studies to assess the inhibition of superoxide production by these compounds at physiologically relevant concentrations (0.1-1 µM (Manach, Williamson et al. 2005)).

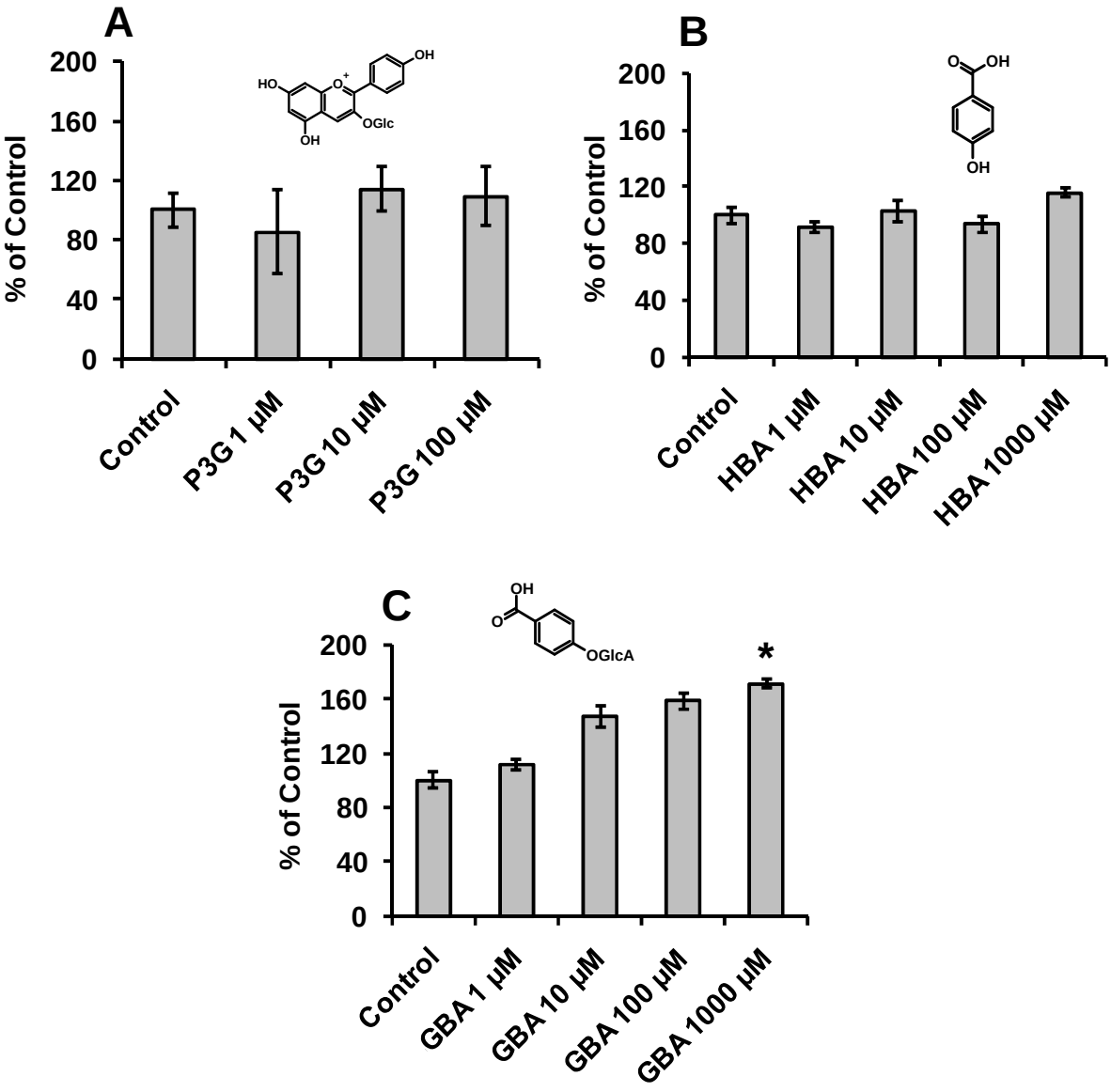


Figure 5.14. The inhibition of xanthine oxidase catalysed superoxide production by the mono-hydroxyl derivatives, P3G (**A**), HBA (**B**) and 4GBA (**C**). The rate of superoxide production (difference between the linear rates of cytocrome c reduction in the presence and absence of superoxide dismutase) by xanthine oxidase was assessed in the presence of P3G (pelargonidin-3-glucoside), HBA (4-hydroxybenzoic acid) and 4GBA (4' glucuronide conjugate of HBA) using an optimised cytochrome c reduction assay. OGlc, O-glucose; OGlcA, O-glucuronic acid. * represents significance at $P<0.05$ with respect to control (no test compounds). Data represented as mean±SD (n=3).

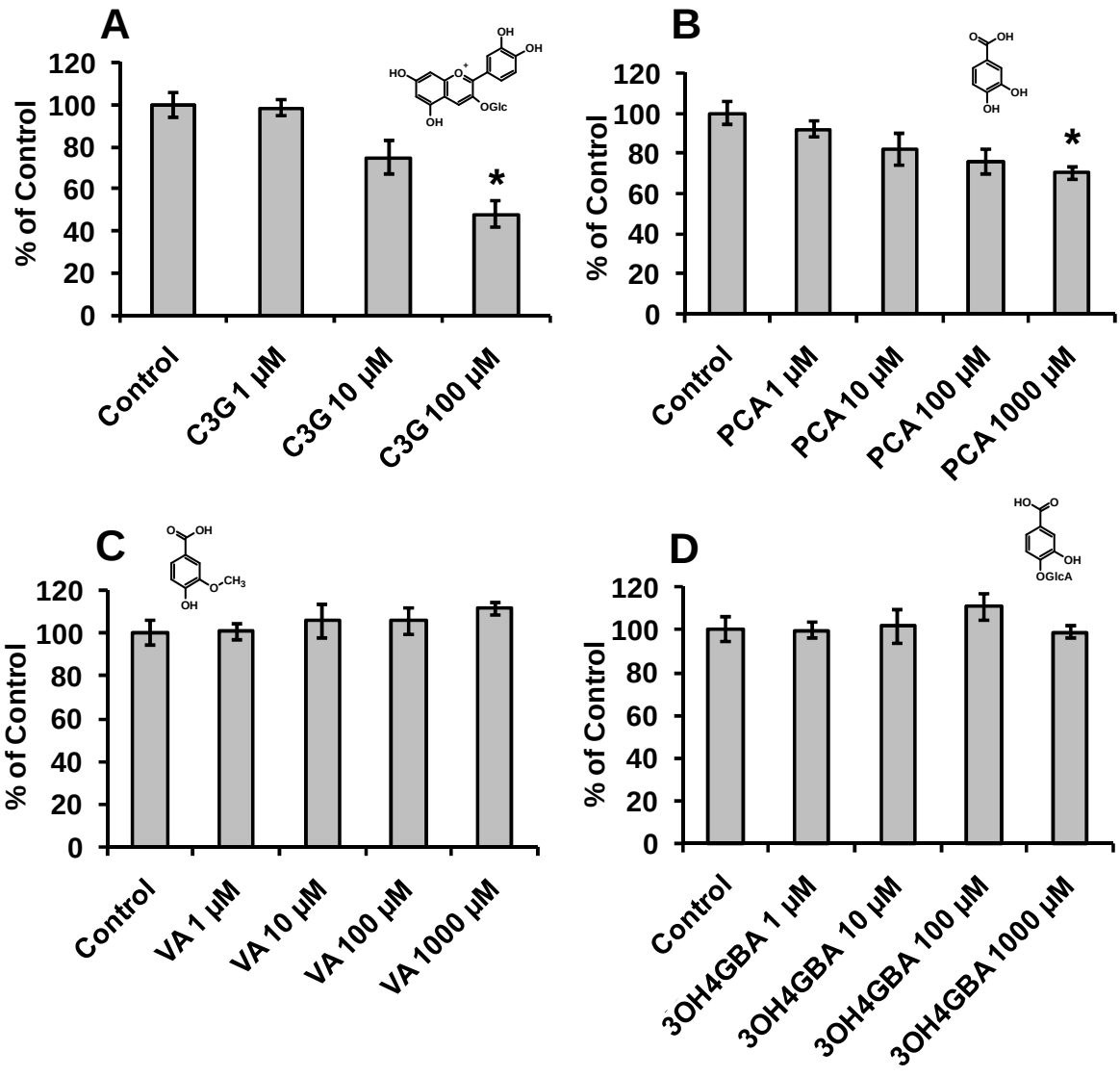


Figure 5.15. The inhibition of xanthine oxidase catalysed superoxide production by the catechol derivatives, C3G (**A**), PCA (**B**), VA (**C**) and 3OH4GBA (**D**). The rate of superoxide production (difference between the linear rates of cytocrome c reduction in the presence and absence of superoxide dismutase) by xanthine oxidase was assessed in the presence of C3G (cyanidin-3-glucoside), PCA (protocatechuic acid), VA (vinallic acid or 4-hydroxy-3-methoxybenzoic acid) and 3OH4GBA (4' glucuronide conjugate of protocatechuic acid) using an optimised cytochrome c reduction assay. OGlc, O-glucose; OGlcA, O-glucuronic acid. *represents significance at $P<0.05$ with respect to control. Data represented as mean±SD (n=3).

Modulation of angiotensin II stimulated superoxide release from intact cells by anthocyanins and their metabolites: It has been shown that numerous compounds from both natural and synthetic sources possess NOX inhibitory effects, or at least reduce the production of superoxide within cellular environments (Steffen, Schewe et al. 2007; Steffen, Gruber et al. 2008). Therefore the ability for novel anthocyanin metabolites (*mono-hydroxyl*

derivatives: pelargonidin-3-glucoside, 4-hydroxybenzoic acid and the 4' glucuronide of 4-hydroxybenzoic acid; *catechol derivatives*: cyanidin-3-glucoside, protocatechuic acid, vanillic acid and the 4' glucuronide of protocatechuic acid) to inhibit Ang II stimulated release of superoxide from endothelial cell was assessed, using the developed and optimised Ang II induced superoxide production assay, described above (optimised primarily for Ang II concentration and treatment duration, SOD inhibition, treatment medium conditions, cytochrome c concentrations, protein normalisation and NOX4 induction).

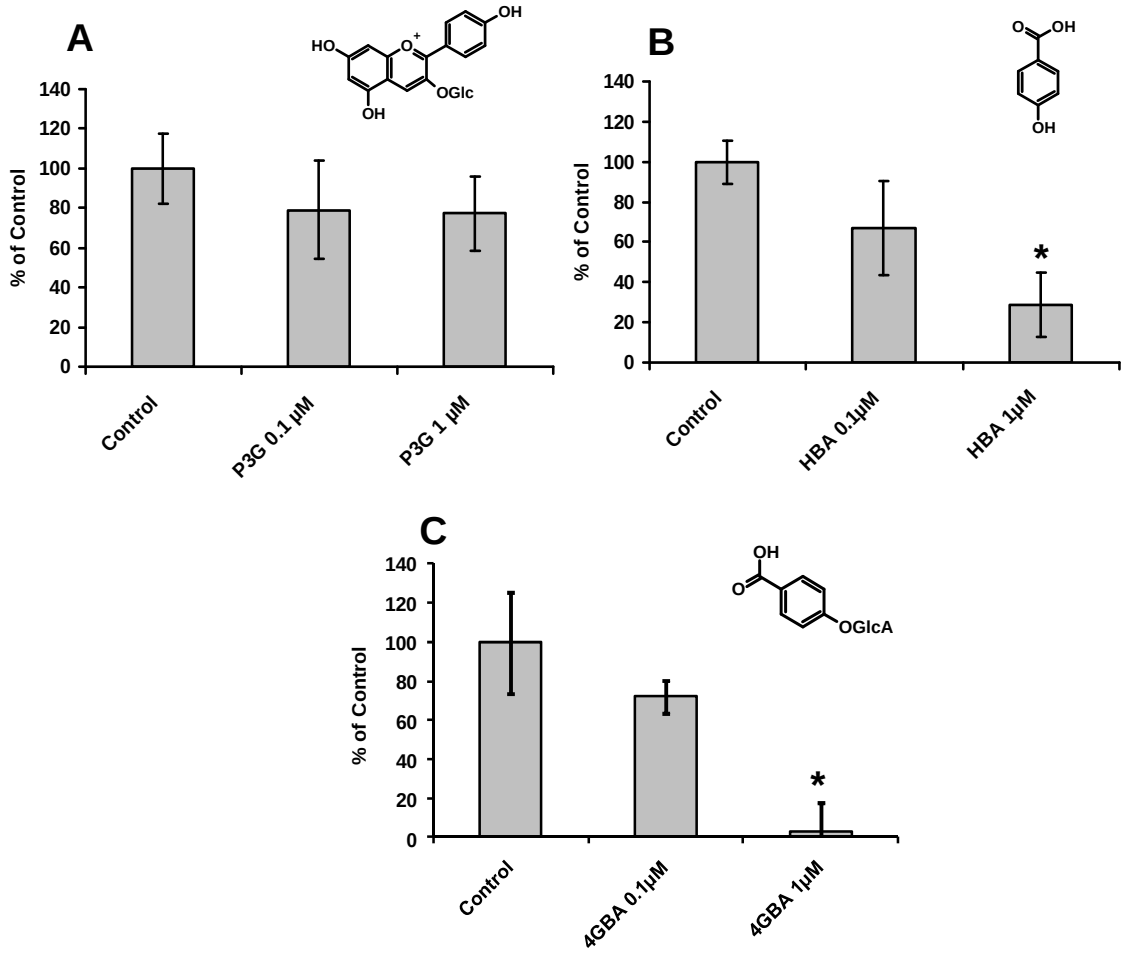


Figure 5.16. The inhibition of Ang II induced superoxide production by the mono-hydroxyl derivatives, P3G (**A**), HBA (**B**) and 4GBA (**C**). Superoxide production (difference in reduced cytochrome c between treatments in the presence and absence of superoxide dismutase) by cultured HUVECs was assessed in the presence of P3G (pelargonidin-3-glucoside), HBA (4-hydroxybenzoic acid) and 4GBA (4' glucuronide conjugate of HBA) using an optimised cytochrome c reduction assay and normalised to total protein content. *represents significance at $P<0.05$ with respect to control (no treatment). Data represented as mean±SD (n=3).

Cells treated with the B-ring mono-hydroxylated anthocyanin derived family (pelargonidin-3-glucoside, 4-hydroxybenzoic acid and its 4' glucuronide conjugate) showed a dose dependent inhibition of Ang II induced superoxide production (**Figure 5.16**). Furthermore, 4-hydroxybezoic acid (33.2±23.3% and 71.4±16.2% inhibition at 0.1 and 1 µM, respectively) had a greater effect than its precursor pelargonidin-3-glucoside (21.0±24.7% and 22.7±18.8% inhibition at 0.1 and 1 µM, respectively). The glucuronide conjugate of 4-hydroxybenzoic acid (27.9±8.4% and 97.2±15.3% inhibition at 0.1 and 1 µM, respectively) showed an even greater effect, at physiologically relevant concentrations. This suggests that anthocyanins become more bioactive following degradation to phenolic acid constituents and metabolic conjugation. Thus, although anthocyanins themselves possess low systemic bioavailability, their vasoactive properties may be mediated by their phenolic acid metabolites. This is consistent with previously reported hypotheses (Kay, Kroon et al. 2009).

Cells treated with the B-ring catechol derived anthocyanin family (cyanidin-3-glucoside, protocatechuic acid and its 4' glucuronide conjugate or 3' methyl conjugate, vanillic acid) showed some bimodal responses (i.e. bioactive at specific concentrations, which does not show a dose response relationship) (**Figure 5.17**). This bimodal response was consistent with the previously reported bioactive characteristics for cyanidin-3-glucoside (Xu, Ikeda et al. 2004), where cyanidin-3-glucoside induced eNOS expression in endothelial cells only at 0.1 µM, with little or no response at 0.01 or 1 µM. The primary end stage degradation product of cyanidin-3-glucoside, protocatechuic acid, did not appear to attenuate Ang II induced superoxide production at physiological concentrations (15.2±16.4% and 16.7±8.8% inhibition at 0.1 and 1 µM, respectively). However, both the glucuronide (87.9±4.1% and -1.4±18.9% inhibition at 0.1 and 1 µM, respectively) and methyl (0.5±17.7% and 67.6±26.3% inhibition at 0.1 and 1 µM, respectively) conjugates of protocatechuic acid did demonstrate significant inhibitory effects. Cyanidin-3-glucoside demonstrated a 35.8±21.0% and -3.8±22.0% inhibition of Ang II induced superoxide production at 0.1 and 1 µM, respectively. Again, this suggests that the vasoactive properties of anthocyanins are mediated by their

phenolic acid metabolites, since they possess greater bioactivity, in this instance, than their anthocyanin precursors. Interestingly, the inhibition of superoxide production by cyanidin-3-glucoside and the 4' glucuronide conjugate of protocatechuic acid showed similar responses (i.e. bimodal). Future studies should be conducted to assess the degree of structural homology between cyanidin-3-glucoside in its chalcone form (at pH 7) and the glucuronide conjugates of protocatechuic acid.

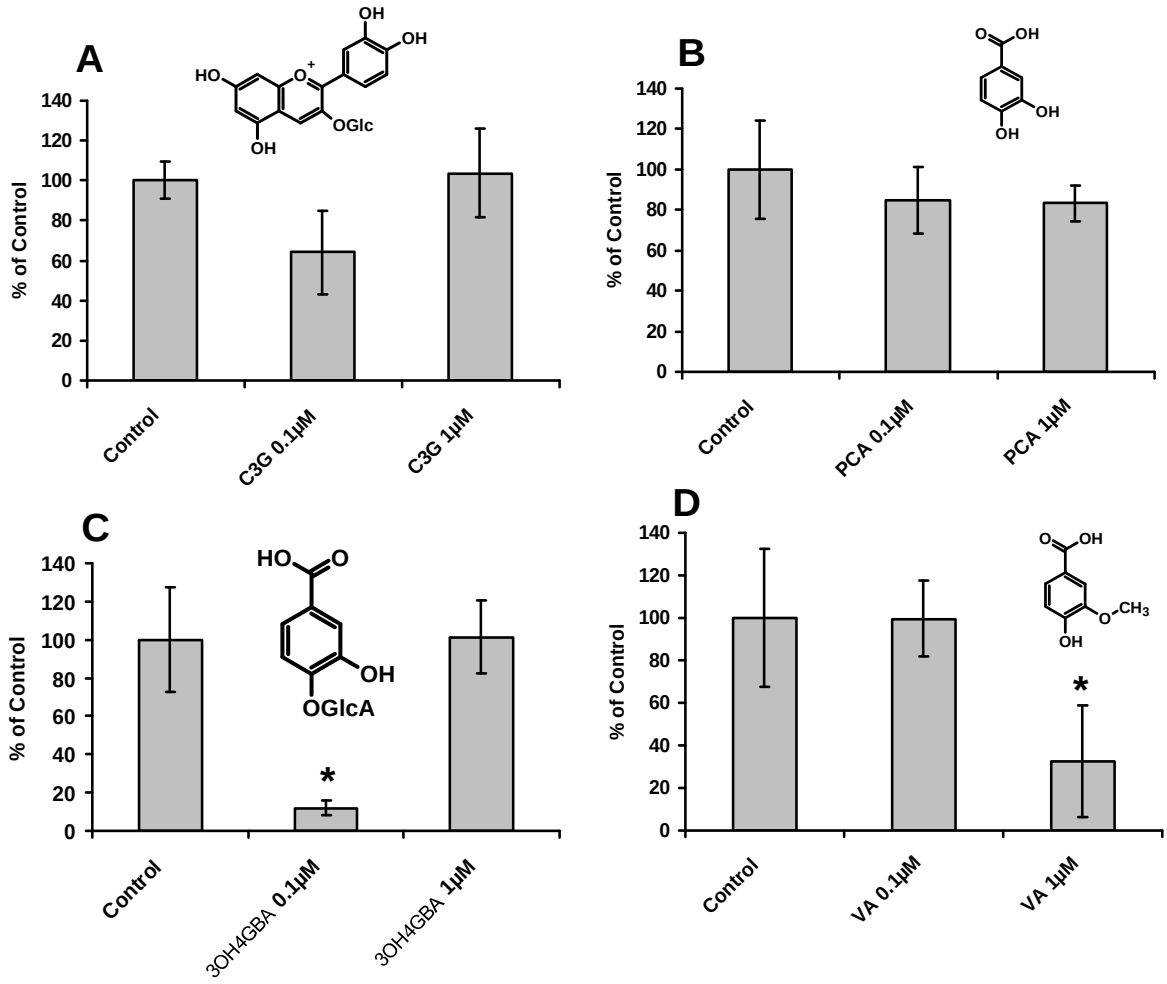


Figure 5.17. The inhibition of Ang II induced superoxide production by the catechol derivatives, C3G (**A**), PCA (**B**), VA (**C**) and 3OH4GBA (**D**). Superoxide production (difference in reduced cytochrome c between treatments in the presence and absence of superoxide dismutase) by cultured HUVECs was assessed in the presence of C3G (cyanidin-3-glucoside), PCA (protocatechuic acid), VA (vanillic acid) and 3OH4GBA (4' glucuronide conjugate of protocatechuic acid) using an optimised cytochrome c reduction assay and normalised to total protein content. *represents significance at $P<0.05$ with respect to control (no treatment). Data represented as mean±SD (n=3).

Modulation of NO production by anthocyanin derived phenolic acid metabolites: Since it had been shown that 4-hydroxybenzoic acid, protocatecuic acid, the 4' glucuronide of 4-hydroxybenzoic acid and the 4' glucuronide of protocatechuic acid significantly attenuated the Ang II induced release of superoxide from endothelial cells (structure-activity study IV), these compounds were subsequently assessed for their ability to induce NO release. Of the compounds tested, only the 4' glucuronide of 4-hydroxybenzoic acid showed a significant induction of NO at a concentration of 1 µM (**Figure 5.18**). This appeared similar to the effects of these compounds of endothelial superoxide production described above (i.e. the compound with the least number of hydroxyls has the greatest effect). This may suggest that the vasoactive properties of these compounds are mediated through the attenuation of superoxide production rather that a direct stimulation of NO production. However, this study was conducted under basal conditions (i.e. not stimulated with Ang II) and since these compounds appeared to show bioactivity under stimulated conditions, further structure-activity relationship studies should be conducted to assess the true bioactive properties for these compounds.

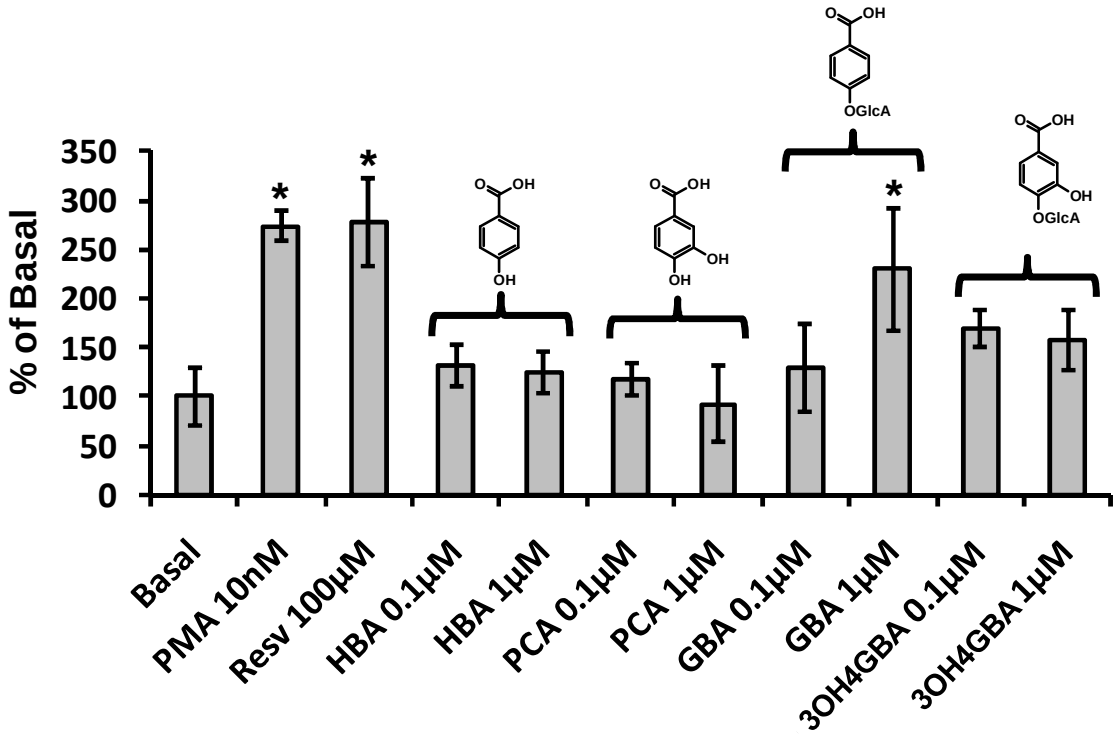


Figure 5.18. The modulation of NO by selected anthocyanin metabolites. Cultured endothelial cells were treated with 0.1 and 1 µM 4-hydroxybenzoic acid (HBA), protocatecuic acid (PCA), 4' glucuronide of 4-hydroxybenzoic acid (GBA) and 4' glucuronide of protocatechuic acid (3OH4GBA), respectively, for 24 h in 2% FCS supplemented medium 199, whereupon NO production was quantified as total nitrite and nitrate in cell supernatants. Basal treatment consisted of cells in 2% FCS supplemented medium 199 only. Positive control incubations consisted of cells treated with 10 nM PMA (phorbol 12-myristate 13-acetate) or 100 µM resveratrol (Resv). *represents significance at $P<0.05$ with respect to basal. Data represented as mean±SD (n=3). NO, nitric oxide.

5.4 Conclusion

In summary, this study showed that anthocyanins and their metabolites do attenuate Ang II induced endothelial superoxide production and that the bioactivity of anthocyanins is enhanced following degradation and metabolism to phenolic acid metabolites. With respect to structure-activity relationships, mono-hydroxylated anthocyanin derived compounds (pelargonidin-3-glucoside, 4-hydroxybenzoic acid and the 4' glucuronide of 4-hydroxybenzoic acid) produce a dose dependent reduction of superoxide production while the catechol anthocyanin derived compounds (cyanidin-3-glucoside, protocatechuic acid, vanillic acid and the 4' glucuronide of protocatechuic acid) show a bimodal inhibition of superoxide production when conjugated to

glucose analogous (i.e. glucose or glucuronic acid). Interestingly, the most bioactive compounds, with respect to inhibition of Ang II induced superoxide production, were those with less B-ring (aryl) hydroxyls. For instance, the mono-hydroxyl, 4-hydroxybenzoic acid, showed a greater bioactivity than the di-hydroxyl, protocatechuic acid. When the di-hydroxyl, protocatechuic acid, was converted to a mono-hydroxyl by substituting one of the aryl hydroxyls with a metabolic conjugate (methoxyl or glucuronide), its bioactivity was increased. Furthermore, when the aryl hydroxyl on 4-hydroxybenzoic acid was substituted with a glucuronide, its bioactivity was further increased.

The ability for phenolic acids to inhibit Ang II induced superoxide production or induce NO production by the endothelium appeared to be **a**) inversely associated with the number of aryl hydroxyls such that ↓OH = ↑bioactivity and **b**) this effect is in express opposition to their direct iron-ion reducing capacity and direct radical scavenging capacity (↑OH = ↑bioactivity).

Based on this dichotomy between points **a** and **b**, it is likely that the biological effects of these anthocyanins on the vasculature, at physiological concentrations, are not due to their direct radical scavenging properties and thus, this study provides evidence in support of epidemiological and animal studies that indicate a positive effect of anthocyanins on vascular health. Thus, the effects of anthocyanin consumption on the vasculature are likely mediated by their phenolic acid degradation products and metabolites.

Chapter 6

General discussion and future research perspectives

6.1 Overview

Oxidative stress, vascular inflammation, and endothelial dysfunction play a central role in the development of cardiovascular diseases. As such, novel pharmaceutical and/or dietary strategies have been proposed to prevent the development and progression of vascular diseases. Epidemiological observations have played a significant part in the development of such dietary strategies, which includes the identification of numerous cardio-protective phytochemicals. In general, research into the health related benefits of flavonoids has been supported by epidemiological studies signifying that foods rich in flavonoids provide cardiovascular protection. Reviews of numerous flavonoid intervention trials show modest effects on cardiovascular risk factors, such as flow mediated dilatation (FMD) and blood pressure (Hooper, Kroon et al. 2008). This is particularly evident in foods such as chocolate, red wine and purple grape juice. However, the evidence is inconsistent for most other biomarkers of cardiovascular disease. This may be due to poor interpretation of findings from observational studies to intervention trials, non-standardised biomarker analysis and/or difficulties in identifying primary bioactive food components. Specifically, anthocyanins are thought to possess significant cardio-protective properties and have been proposed as potent modulators of vascular disease. However, their true health benefit has been thrown into question, since current literature evidence demonstrates that anthocyanins possess poor bioavailability (Manach, Williamson et al. 2005). To date, studies have failed to fully establish the bioavailability of anthocyanins and have been unable to pinpoint their bioactive mechanisms of action. Moreover, little is currently known about anthocyanin absorption (**Figure 6.1**), routes of metabolism and excretion (**Figure 6.2**), formation of phenolic degradation products, pH dependent chemical derivatives and the pharmacokinetics of anthocyanin metabolites. No studies have yet determined their biological mechanisms of action, nor their physiologically relevant metabolites. Indeed, much is still unknown about the major sites and modes of anthocyanin metabolism.

In order to fully appreciate the health related benefits of ingested anthocyanins, an understanding of their occurrence, biochemistry, metabolism and final bioactive mechanisms is essential. Therefore, within this thesis, the experimental gaps in our knowledge of anthocyanin stability, metabolism and bioactivity were addressed in a series of experiments designed to evaluate (i) the stability of anthocyanins during processing from whole berry (anthocyanin rich food source) to commercial juice product (**Chapter 2**), (ii) the formation of phenolic degradation products under simulated physiological and experimental conditions and recovery from clinical samples (**Chapter 3**), (iii) the *in vitro* hepatic conjugation (metabolism) of anthocyanins and their phenolic acid degradation products (**Chapter 4**) and (iv) the *in vitro* cardiovascular related bioactivities of anthocyanins and their novel free or conjugated metabolites (**Chapter 5**).

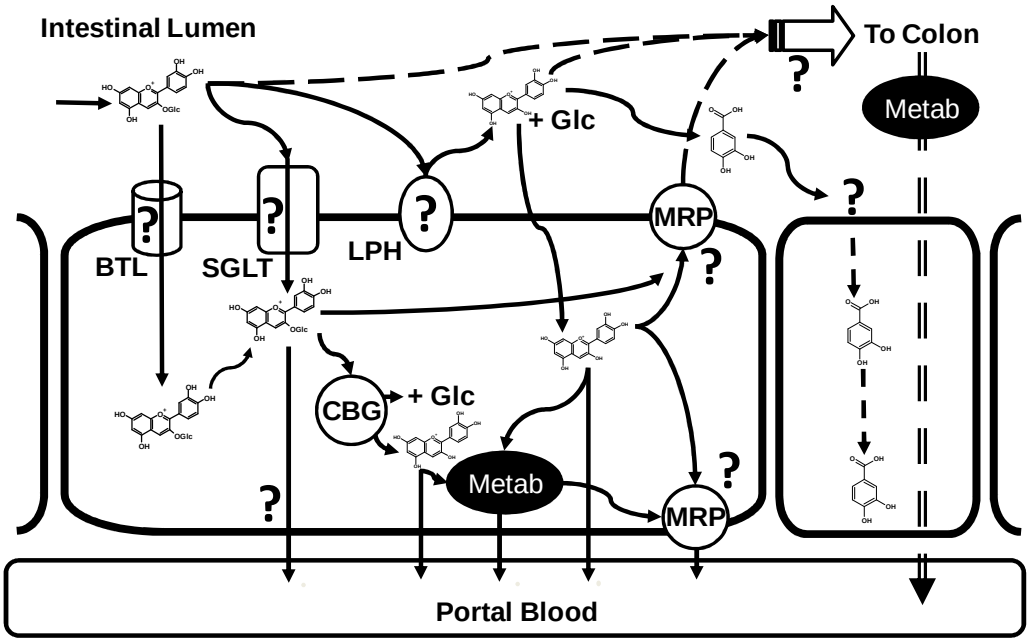


Figure 6.1. Current unknowns for anthocyanin absorption. "?" represent possible sites/modes of absorption that are yet to be confirmed. Image modified from Kay, C. D. (2006) *Nutr. Res. Rev.* 19:137-146. BLT, bilitranslocase; SGLT, sodium dependent glucose transporters; LPH, lactase phloridzin hydrolase; CBG, cytosolic β-glucosidase; Glc, glucose; Metb, metabolism; MRP, multi-drug resistance associated proteins.

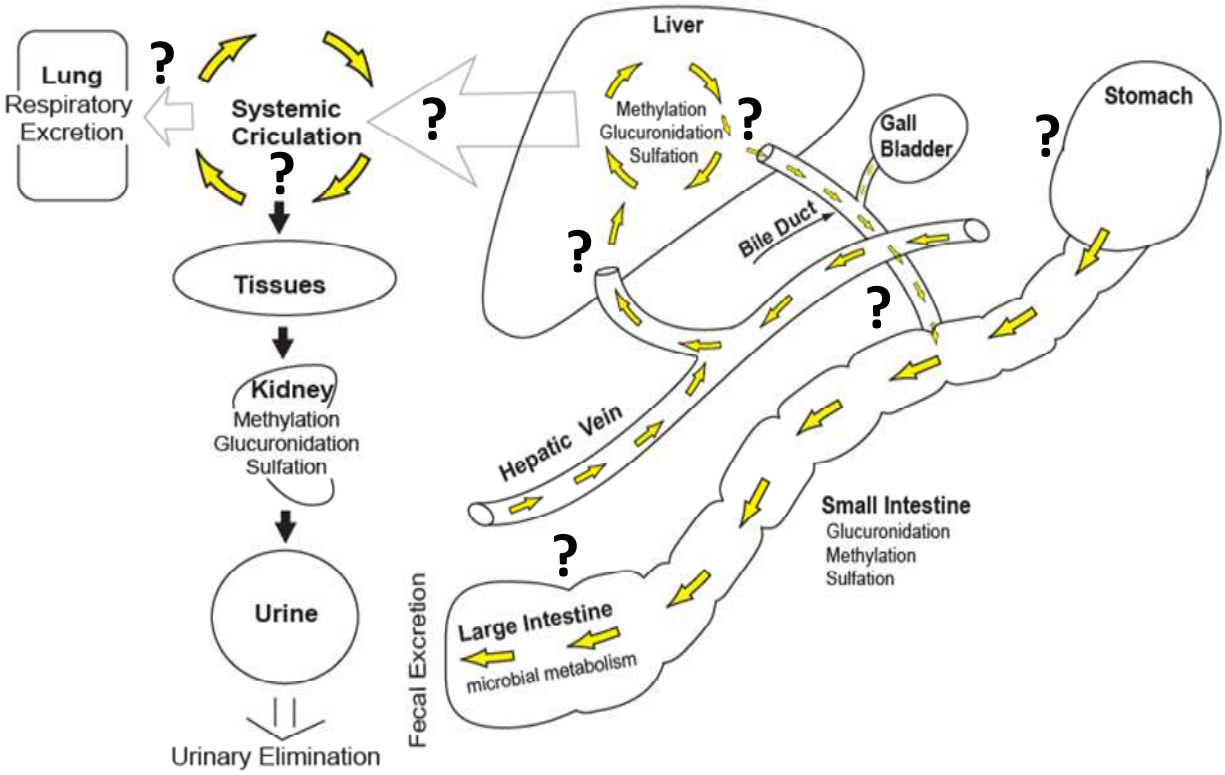


Figure 6.2. Current unknowns for anthocyanin absorption and sites of metabolism. "?" represent possible sites/modes of absorption/metabolism that are yet to be confirmed. Figure modified from Kay, C. D. (2006) *Nutr. Res. Rev.* 19:137-146.

6.2 Anthocyanins and processed foods (remarks on Chapter 2)

With regards to evaluating anthocyanin occurrence in processed foods, it remained important to establish the stability of anthocyanins during commercial juice processing in order to evaluate their availability to the general population and their bioactivity within processed food/s. In this study it was demonstrated that no significant loss in anthocyanins was observed throughout commercial processing. Thus it remains clear that anthocyanins are available for consumption by the population in high concentration, where the evaluation of anthocyanin interactions with human physiology is clearly important. However, from this and other studies (Hollands, Brett et al. 2008) it is shown that the levels of anthocyanins found in commercial juice products are far below that indicated by the manufacturers. Therefore, anthocyanins are likely to degrade during storage of processed juice products and should

be the focus of future research in the food and drinks industry. Particularly, research should aim to evaluate the degradation of anthocyanins and the formation of free phenolic acids or dimerised polymers in processed foods, since these products could significantly modulate health or disease upon consumption. Furthermore, since the work presented in this thesis indicates that the effects of anthocyanins on the vasculature (*in vitro*) are enhanced by their degradation and metabolism, it may prove beneficial to promote the formation of these degradation compounds during fruit processing to commercial products.

6.3 Anthocyanin bioavailability (remarks on Chapters 3 and 4)

A crucial aspect in modern anthocyanin research is demonstrated by past and present bioavailability studies, which confirm that less than 1% of an ingested dose is present in the serum and urine, and thus 99% is un-available to the systemic circulation (Manach, Williamson et al. 2005). Therefore, it remains paramount to this research field that the metabolic fate of the remaining 99% of ingested anthocyanins is determined. To account for the metabolic loss of anthocyanins, protocatechuic acid, as a by-product of cyanidin-3-glucoside degradation has been investigated following (i) thermal or chemical degradation, (ii) microbial catabolism, (iii) simulated digestion, (iv) cell culture metabolism studies and (v) rodent feeding trials. Also, in one human study, protocatechuic acid has been suggested to be the primary degradation product or metabolite of cyaniding-3-glucoside (Vitaglione, Donnarumma et al. 2007). Thus, it remained important to evaluate the physio-chemical stability of anthocyanins and their stability following routine sample analysis in order to fully account for their poor bioavailability. To this end the study presented in **Chapter 3** assessed the effect of anthocyanin B-ring hydroxylation on their stability under simulated (*in vitro)* physiological conditions and their degradation and recovery following routine pre-analytical sample extraction and storage. It was shown that (i) the B-ring hydroxylation status of anthocyanins mediated their degradation to phenolic acid and aldehyde

constituents, (ii) that successful anthocyanin extraction was dependant on both sample preparation technique and anthocyanin structure and (iiI) that anthocyanins remain stable through multiple freeze thaw cycles. Importantly, these data indicated that significant portions of ingested anthocyanins are likely to degrade to phenolic acids and aldehyde and that the poor bioavailability of anthocyanins is not due to inappropriate sample extraction or clinical sample storage.

An apparent lack of appreciation for the rate and extent of anthocyanin degradation under physiological or processing conditions may explain the almost complete absence of data aimed at quantifying anthocyanin breakdown products and metabolites in humans and the current lack of reports concerned with the biological activities of these compounds or their metabolites. The studies presented in this thesis have described the degradation of anthocyanins to phenolic acids under simulated physiological conditions, with the subsequent formation of phenolic acid metabolites, particularly glucuronide conjugates. This would indicate that following ingestion, anthocyanins may be found in the systemic circulation as free or conjugated phenolic acids. However, in order to fully validate this hypothesis, further bioavailability should be conducted with the express aim of identifying both anthocyanins and phenolic acid conjugated metabolites in humans. Further to this, these studies should aim to identify the conjugation position and structure of these metabolites, since this may significantly affect their bioactivity. Particularly, the study presented in **Chapter 4** indicated that the primary phenolic glucuronide conjugates were acyl glucuronides (under the current experimental conditions). This may be of particular research interest since (i) the formation of acyl glucuroides over aryl glucuronides contradicts common pharmacological dogma and (ii) it has been well reported that acyl glucuronides are able to cross-link proteins and cause adverse drug reactions (side affects). These aspects of anthocyanin metabolism require further investigation. In addition, it has been reported that protocatechuic acid and gallic acid form sulphate and methyl conjugates upon metabolism where it may be found that these anthocyanin derived phenolic acids may be found in

the systemic circulation as multiply conjugated metabolites (methyl-glucuronides etc.).

A consistent observation throughout the analysis of anthocyanin degradation and metabolism, was the “lag” time between anthocyanin loss and phenolic acid formation, particularly under physiologically buffered conditions. It may be found that the identification of these intermediate anthocyanin products may serve to account for the poor bioavailability of anthocyanins. To date, no analystical method has been developed to identify these intermediates (pH dependent iso-forms) in buffered/biological samples and should be the focus of further studies aimed at elucidating the degradation or “ring opening” of anthocyanins under physio-chemical conditions.

6.4 Anthocyanin and cardiovascular disease (remarks on Chapter 5)

The vascular endothelium plays a fundamental role in the maintenance of vascular health, and thus organ function. The fact that our daily diet directly and indirectly influences cardiovascular and endothelial function has been widely demonstrated. While there is a large body of knowledge on oxidative risk for vascular disease, rigorous clinical investigations that establish a link between the intake of dietary polyphenols and the improvement of vascular function in humans is scarce. However, it has been observed that human volunteers ingesting beverages rich in polyphenols exhibit an increase in FMD (Hooper, Kroon et al. 2008), which is believed to be largely dependent on eNOS and NO activity. While such reports support the notion that polyphenols are biologically active compounds, a direct causal link has yet to be established. Indeed, which compounds are biologically active at the molecular level remains an open question that requires further investigation. Nonetheless, some mechanistic observations have lead to a better understanding of polyphenol function. The most important of these has lead to a paradigm shift in the flavonoid research field where it is now accepted that

rather than directly acting as antioxidants *per se*, certain flavonoids may be thought of as bioactive compounds with biological specificity and functionality. Indeed, this notion has been demonstrated in the present thesis (**Chapter 5**) where the biological effect of anthocyanins has been shown to be in express opposition to their antioxidant potential. *In vitro* assays clearly demonstrate that flavonoids react with free radicals. These and other chemical properties related to antioxidant activity can be classified as direct actions, and much emphasis has been placed on this in recent years. However, the following considerations argue against a direct antioxidant affect *in vivo*, based on the findings of this and other studies; (i) the low concentration of flavonoids in the systemic circulation compared with endogenous antioxidant compounds and enzymes, (ii) the lack of evidence regarding the efficacy of long term antioxidant supplementation and (iii) the high level of metabolism and biotransformation that flavonoids undergo, which ultimately reduce their antioxidant potential. The latter point represents yet another important paradigm shift in recent flavonoid research, namely, the question as to whether polyphenols or their metabolites mediate their biological effects? In a recent study, an O-methylated metabolite of (-)-epicatechin, 3'-O-methylepicatechin, was shown to inhibit NADPH oxidase. Importantly, it was shown that this (-)-epicatechin metabolite was more effective than its un-metabolised precursor (Steffen, Schewe et al. 2007; Steffen, Gruber et al. 2008). The importance and relevance of metabolism is also highlighted here (**Chapter 5**), where it was demonstrated that changes to anthocyanin structure following degradation or metabolic conjugation elicited divergent effects on endothelial superoxide production. In particular, a reduction in the number of aryl hydroxyls was associated with a greater bioactivity. These results are in general agreement with other flavonoid structure-activity relationship studies. More specifically, this study showed that anthocyanins and their metabolites do attenuate Ang II induced endothelial superoxide production and that the bioactivity of anthocyanins is enhanced following degradation and metabolism to phenolic acid metabolites. Furthermore, the ability for phenolic acids to inhibit Ang II induced superoxide production or induce NO production by the endothelium was inversely associated with the number of aryl hydroxyls (↓OH = ↑bioactivity). This effect is in express

opposition to their antioxidant potential (↑OH = ↑bioactivity). Therefore, it is likely that the biological effects of these compounds on the vasculature, at physiological concentrations, are not due to their direct radical scavenging properties. Others have shown that phenolic acids (including protocatechuic acid and gallic acid) exert a range of bioactivities in addition to their radical scavenging capacity, including the induction of apoptosis. These observations therefore support the hypothesis that anthocyanin degradation products and metabolites are likely to mediate their biological activity. Thus, future research may typify flavonoids as pre-cursors or “pro-drugs” for bioactive metabolites. However, considering that cyanidin-3-glucoside is most bioactive at 0.1 µM, a physiologically achievable concentration, it may be thought that the low bioavailability of anthocyanins may exemplify their biological affects. Therefore, future studies should aim to assess the dose dependant effects of anthocyanins and other phenolic compounds in bioactivity assays at concentration lower than previously considered (i.e. 0.1 µM), in order that a “therapeutic window” for these bi-modally active compounds is established.

Although it has here been demonstrated that anthocyanins and their metabolites modulate vascular functions at physiological concentrations, the mechanisms of this action remains to be assessed. To this end, initial studies should aim to assess the ability for these compounds to modulate the protein and gene expression of superoxide associated enzymes, particularly in the relation to Ang II and other inflammatory stimulators. Among these targets should be included NOX (NOX4) and eNOS. Furthermore, the effects of anthocyanins and their metabolites should be assessed in both acute and prolonged inflammatory environments in which gene/protein expression as well as activation (phosphorylation) and translocation (i.e. the association of NOX4 with its intracellular and membrane bound components including $p22^{phox}$). With regards to protein activation, since NOX and eNOS are primarily subject to the MAP kinase pathway (including Erk and AKT associated pathways) it may prove likely that anthocyanins and their metabolites modulate the production of superoxide and NO through the upstream modulation of the afore mentioned pathways, as has been shown for procyanidins and VEGF receptor signalling.

6.5 Concluding remarks

Overall, the findings of this thesis emphasize the importance of investigating the health related benefits associated with flavonoid consumption and the significance of stability and metabolism in elucidating the mechanisms by which flavonoids may modulate human health and disease. The concepts of metabolism (metabolic structural alterations), bioavailability and mechanisms of action should be regarded as vitally important when conducting and interpreting past and future research associated with the health benefits of flavonoids.

Appendices

Appendix A: Anthocyanins and juice products

A.1. Analysis of commercial juice products my HPLC

Preparation of GSK berry products

Ribena blackcurrant (tetrapack): 10 µl of single strength Ribena (tetrapack) was injected directly on to the HPLC system.

Ribena blackcurrant concentrate: 10 µl of Ribena concentrate was injected directly onto the HPLC system.

Eurovit Anthocyanin (grape skin) additive: An aliquot of Eurovit Anthocyanin additive was diluted 1:9 with 5% aq. formic acid. 10 µl of the diluted sample was injected onto the HPLC system.

Blackcurrant berries: A portion of frozen blackcurrants were extracted (see below) and injected (10 µl) onto the HPLC system.

Anthocyanin/phenolic acid extraction method

Materials

Frozen blackcurrant were provided by GSK and stored at -18^{o}C. Degraded blackcurrants were prepared as described below. Anthocyanin standards were purchased from Extrasynthese (France) or provided by GSK. Phenolic acid standards were purchased from Sigma Aldrich (UK).

Extraction conditions

Anthocyanins and phenolic acids were extracted from a 25g sample of frozen blackcurrants. Berry sample were blended in 100ml 80:20 Acetonitrile:dH_2O and 5% formic acid for 3 min. The resulting 'slurry' (100 ml) was then sonicated in a bath sonicator at 40^{o}C for 30 min. The sonicated sample was then centrifuged at 4000 rpm (4x25 ml samples) for 20 min on a bench top centrifuge. The supernatants were then filtered through 0.45 µm syringe filters, diluted in 5% aq. formic acid (1:4 and 1:3 dilution for fresh and degraded berry samples respectively) and analysed by HPLC.

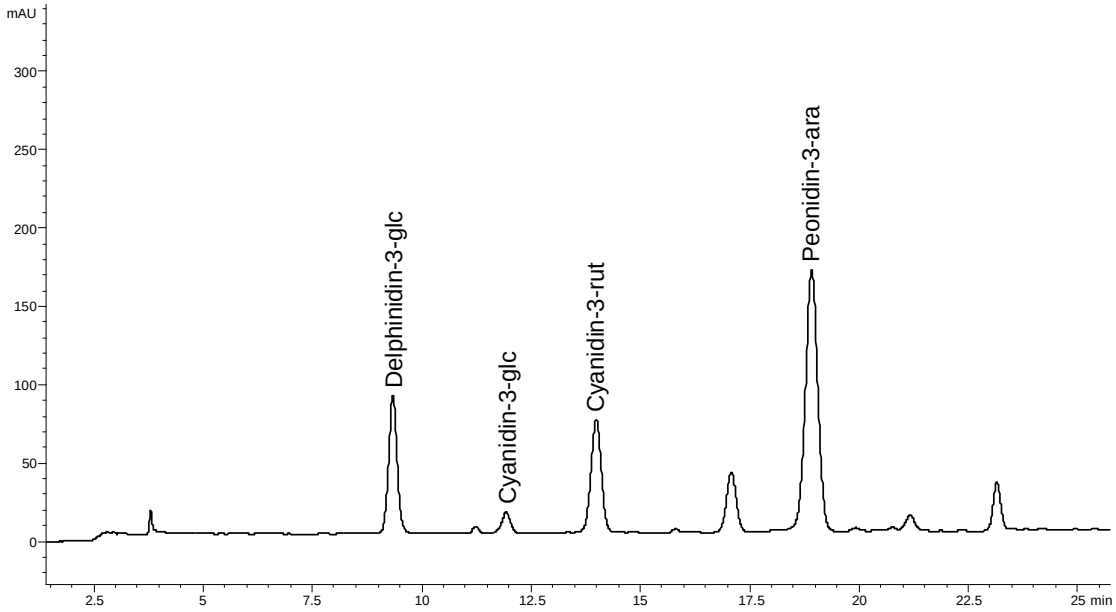
Degradation of whole berries

In order to determine if anthocyanin derived phenolic acids were present and extracted from whole berries, a sample of whole blackcurrants (25g) was submerged in 50 ml dH_2O (titrated to pH 8 with 0.1 M NaOH) and heated to 70^{o}C for 24 h under vacuum). The remaining dH_2O was evaporated on a rotary evaporator and the anthocyanins and phenolic acids extracted as described above.

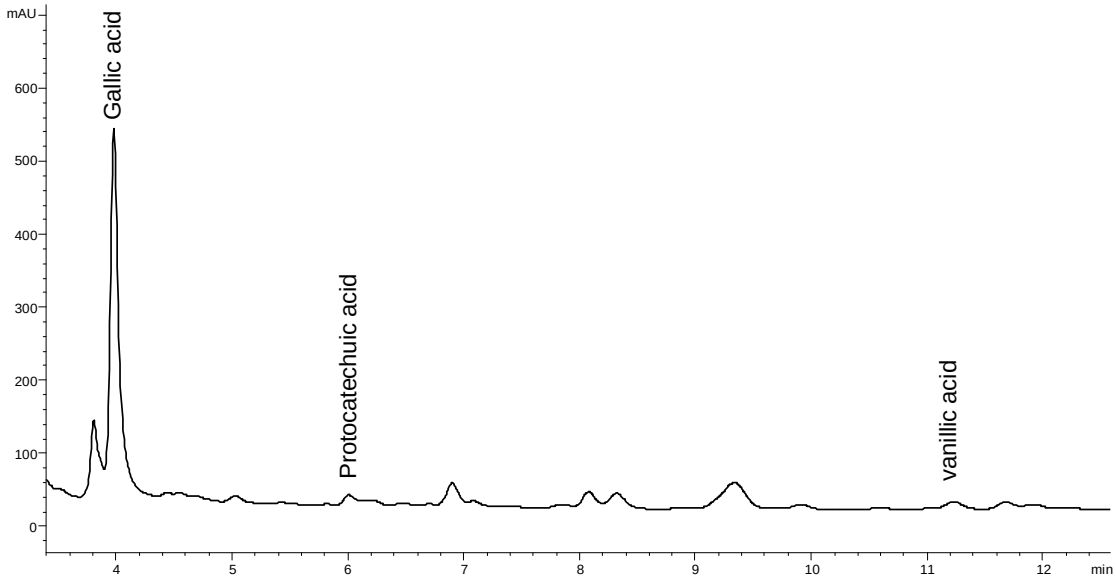
HPLC method

HPLC analysis was performed on an Agilent 1200 series HPLC equipped with a diode array detector (DAD) and a 4 µm, 250 x 4.6 mm Synergi Max-RP reverse phase column (Phenomenex, Macclesfield, UK) with a SecurityGuard guard cartridge (Phenomenex AJO-6074). The column temperature was set at 30 ℃ with an injection volume of 10 µL and mobile phases consisting of 5% formic acid (v/v) in water (solvent A) and 5% formic acid (v/v) in acetonitrile (solvent B). The solvent gradient consisted of 8% B at 0 min, 10% B at 5 min, 18% B at 25 min and 100% B at 27-33 min; at a flow rate of 1 mL/min. Absorbance was recorded at 520, 360 and 260 nm.

Analysis of Eurovit. anthocyanin colouring additive

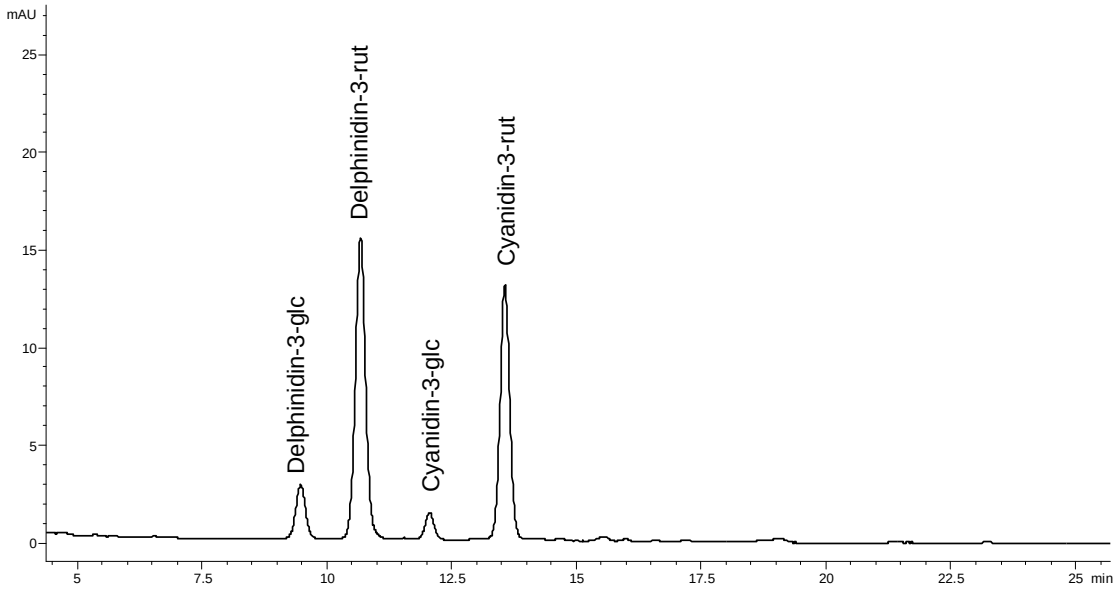


Compound	Rt (min)	Peak Area	Dilution f.	Concentration (g/L)
Delphinidin-3-glucoside	9.3	1181.5	10	1.198
Cyanidin-3-glucoside	11.9	180	10	0.063
Cyanidin-3-rutinoside	13.9	1187	10	1.010
Peonidin-3-arabinoside	18.9	3274.2	10	1.405

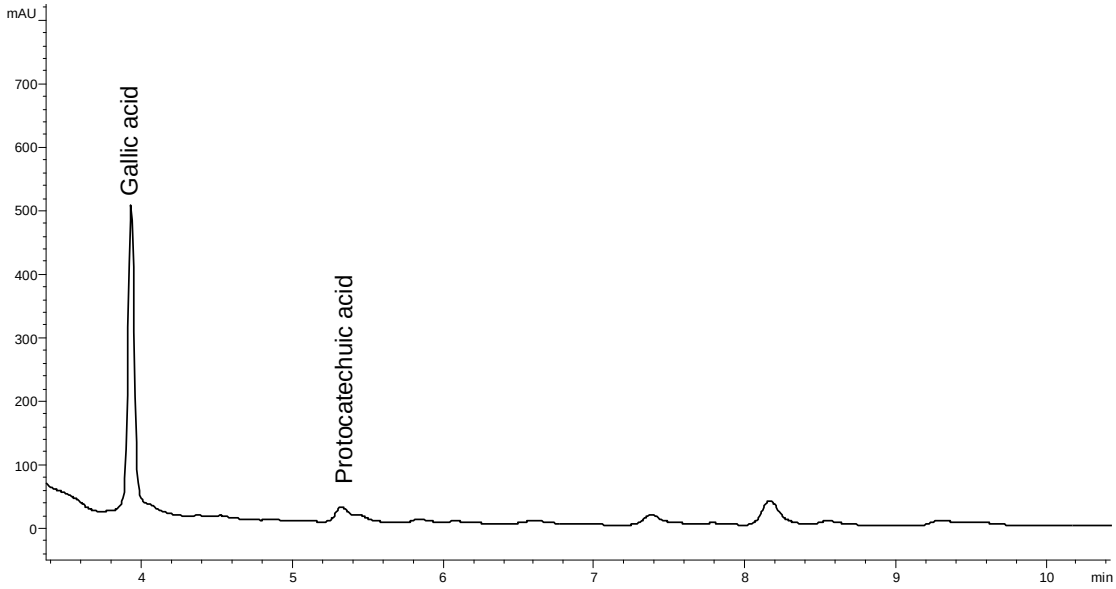


Compound	Rt (min)	Peak Area	Dilution f.	Concentration (g/L)
Gallic acid	3.9	3722	10	1.493
Protocatechuic acid	6.0	427.3	10	0.118
Vanillic acid	11.6	172.8	10	0.049

Analysis of Ribena tetra-pack (no added colour)

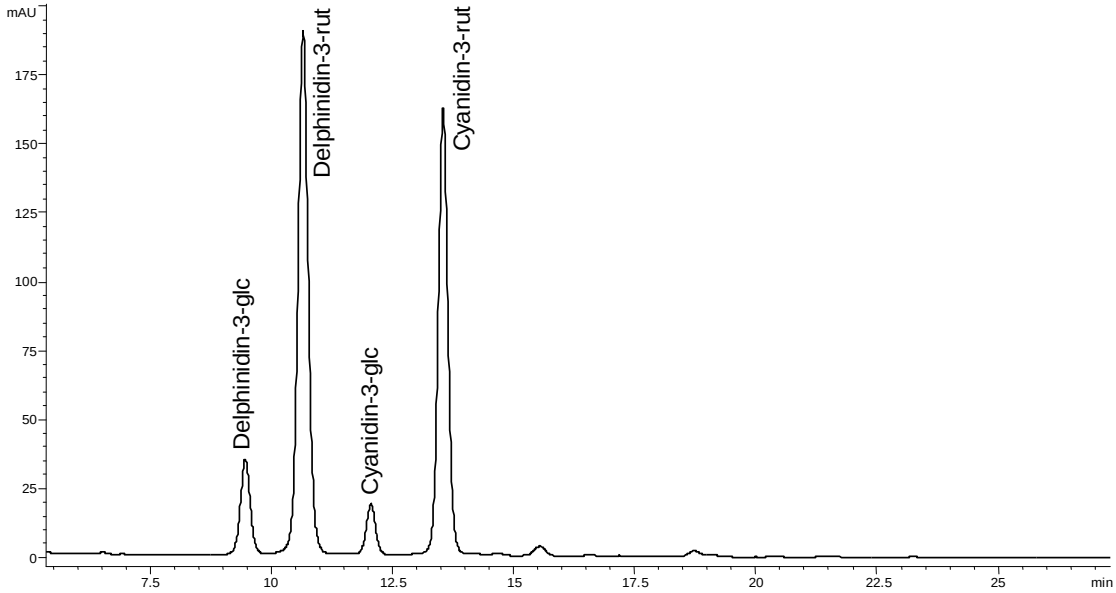


Compound	Rt (min)	Peak Area	Dilution f.	Concentration (ug/mL)
Delphinidin-3-glucoside	9.4	36.7	1	3.721
Delphinidin-3-rutinoside	10.6	219.5	1	19.953
Cyanidin-3-glucoside	12	16.7	1	0.585
Cyanidin-3-rutinoside	13.5	173.1	1	14.731

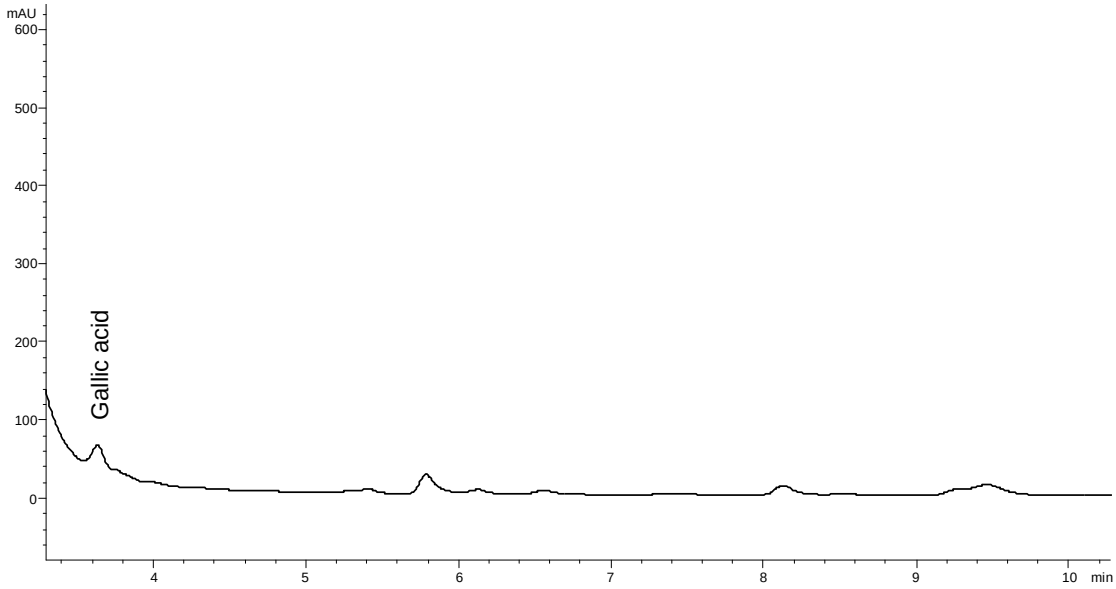


Compound	Rt (min)	Peak Area	Dilution f.	Concentration (ug/mL)
Gallic acid	3.9	56.3	1	2.258
Protocatechuic acid	5.7	9.4	1	0.260

Analysis of Ribena Concentrate

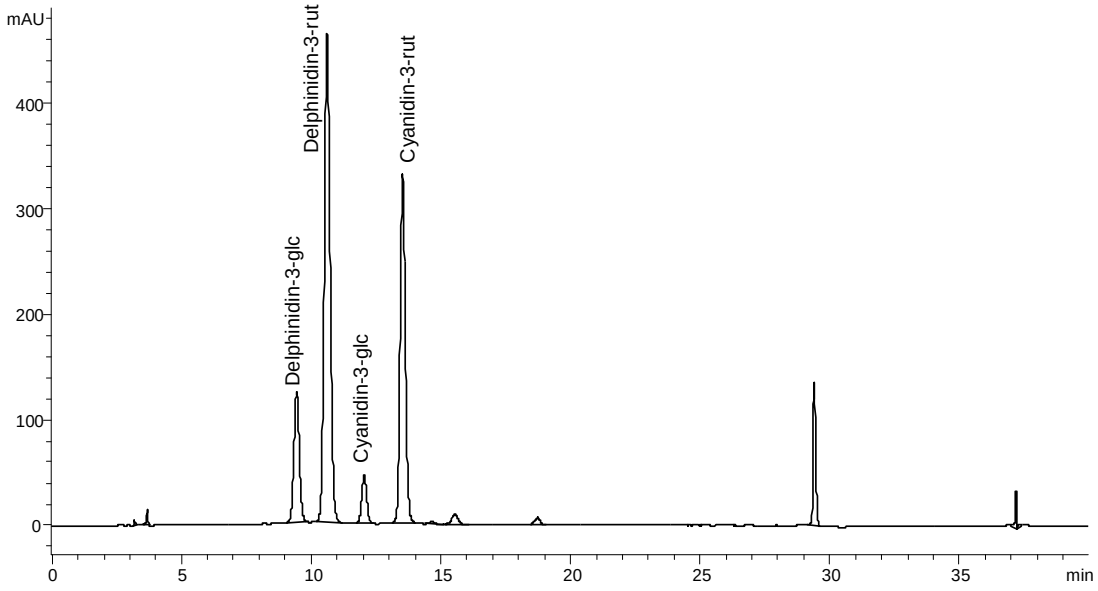


Compound	Rt (min)	Peak Area	Dilution f.	Concentration (ug/mL)
Delphinidin-3-glucoside	9.4	482.0	1	48.875
Delphinidin-3-rutinoside	10.6	2826.0	1	256.883
Cyanidin-3-glucoside	12	238.5	1	8.348
Cyanidin-3-rutinoside	13.5	2228.9	1	189.679

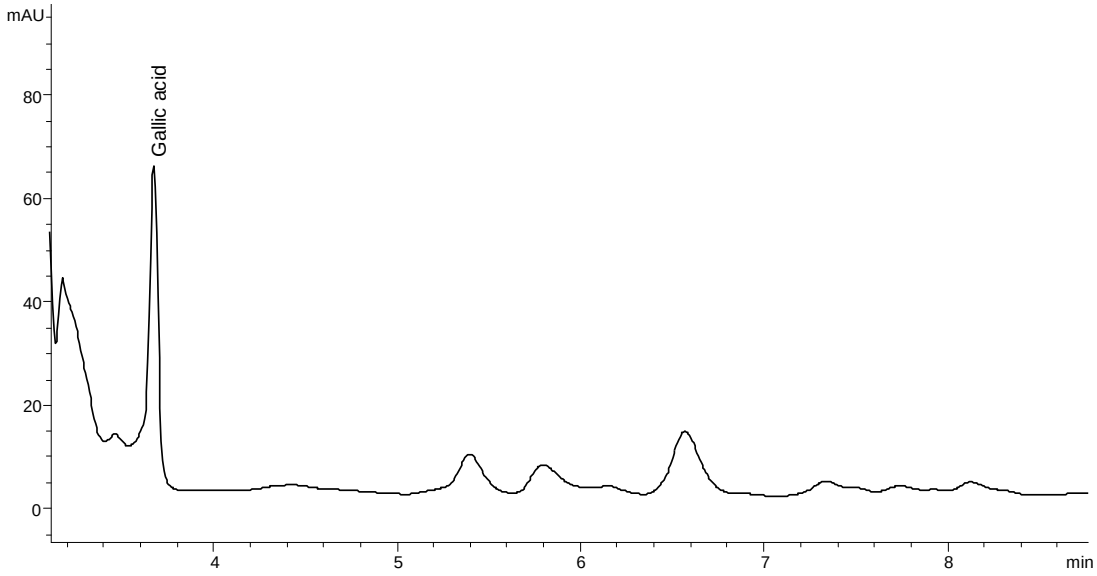


Compound	Rt (min)	Peak Area	Dilution f.	Concentration (ug/mL)
Gallic acid	3.9	292.7	1	11.737

Analysis of frozen blackcurrants

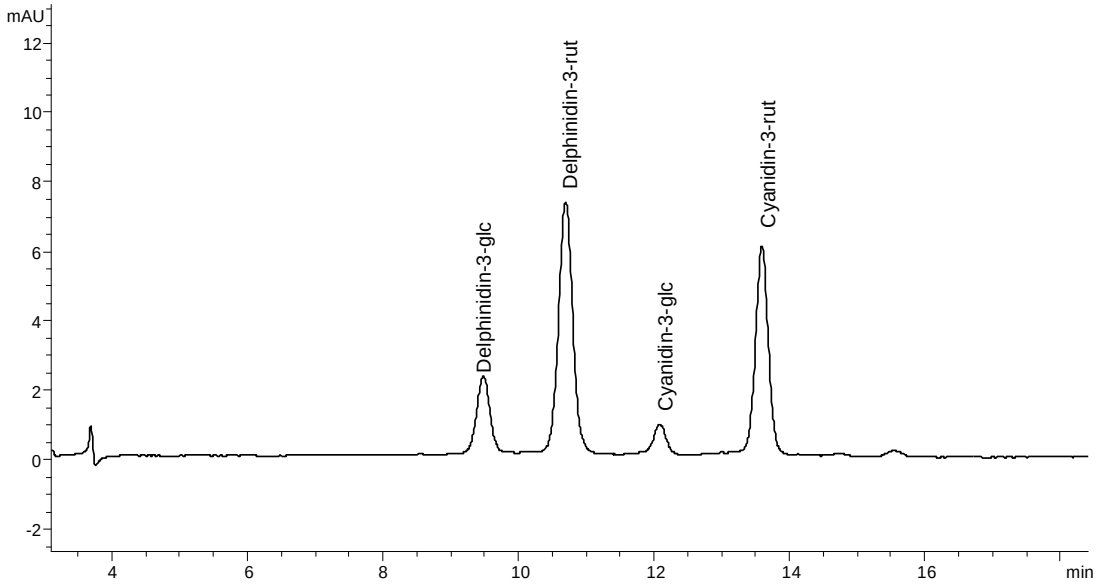


Compound	Rt (min)	Peak Area	Berry content (mg/100g FW)
Delphinidin-3-glucoside	9.4	365.5	74.1234
Delphinidin-3-rutinoside	10.6	1429.5	259.8831
Cyanidin-3-glucoside	12	124.8	8.736
Cyanidin-3-rutinoside	13.5	920.0	156.584

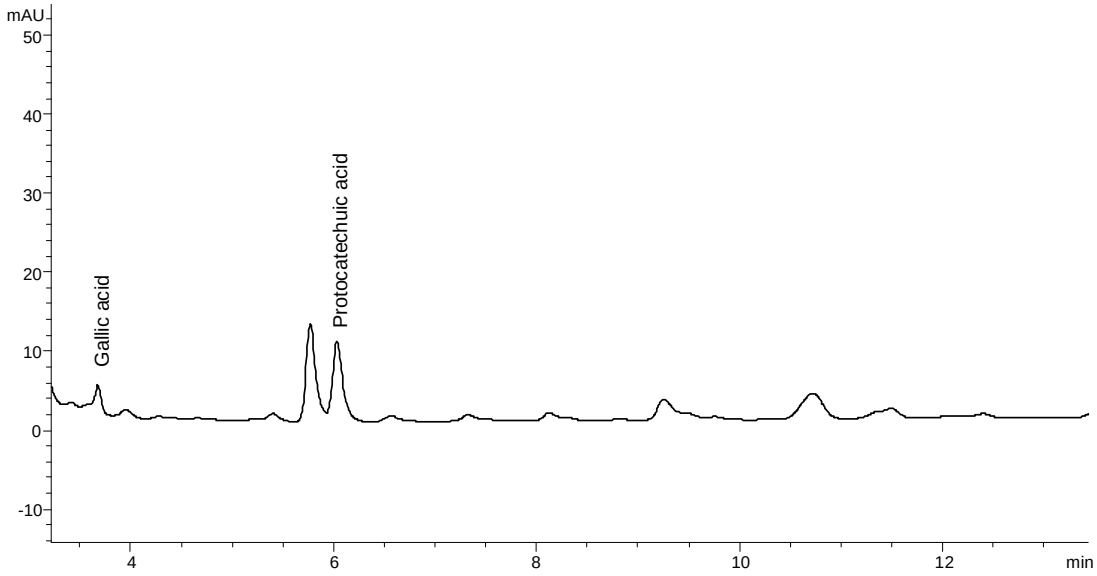


Compound	Rt (min)	Peak Area	Berry content (mg/100g FW)
Gallic acid	3.6	26.0	2.0852
Protocatechuic acid	0	0.0	0

Analysis of degraded blackcurrants



Compound	Rt (min)	Peak Area	Berry content (mg/100g FW)
Delphinidin-3-glucoside	9.4	44.4	5.402592
Delphinidin-3-rutinoside	10.6	158.8	17.321904
Cyanidin-3-glucoside	12	15.4	0.6468
Cyanidin-3-rutinoside	13.6	117.7	12.019524



Compound	Rt (min)	Peak Area	Berry content (mg/100g FW)
Gallic acid	3.6	110.8	5.331696
Protocatechuic acid	6.3	116.5	3.87246

A.2. Design of Experiment (DOE) 1: Factors influencing the loss of anthocyanins during berry concentrate processing.

Overview:

In order to identify key point for anthocyanin losses during routine industrial scale blackcurrant processing, samples of processed blackcurrant were analyse pre and post significant processing steps in a previous experiment. In order to elucidate the relationship between anthocyanin loss and various processing influence and potentially improve processed berry concentrate anthocyanin content, a DOE (design of experiment) was conducted using a simulated blackcurrant processing system. The key influences on anthocyanin content investigated were milling method, SO2 concentration, pectinaze enzyme concentration, enzyme treatment time and concentration temperature.

DOE design matrix:

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Run	A Method of Milling (fine/coarse)	B SO2 (mg/Kg)	C Enzyme (ul/Kg)	D Enzyme time (h)	E Concentration temp. (℃)
1	Method 1	150	0.8	3.25	65
2	Method 1	300	1.5	6	40
3	Method 1	300	1.5	0.5	90
4	Method 1	300	0.1	0.5	40
5	Method 2	0	1.5	6	40
6	Method 2	150	0.8	3.25	65
7	Method 1	150	0.8	3.25	65
8	Method 2	300	0.1	0.5	90
9	Method 2	0	1.5	0.5	90
10	Method 1	150	0.8	3.25	65
11	Method 2	0	0.1	6	90
12	Method 1	0	0.1	0.5	90
13	Method 1	300	0.1	6	90
14	Method 2	300	0.1	6	40
15	Method 2	150	0.8	3.25	65
16	Method 1	0	0.1	6	40
17	Method 2	300	1.5	6	90
18	Method 1	0	1.5	0.5	40
19	Method 1	0	1.5	6	90
20	Method 2	300	1.5	0.5	40
21	Method 2	0	0.1	0.5	40
22	Method 2	150	0.8	3.25	65

Appendix B: Compound purity and structure

B.1. HPLC purity of synthesised compounds

4-(1-carboxyphenyl) β-D-glucuronic acid (HBA-GlcA) = >99% pure

4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid (PCA-GlcA) = 99% pure

3-(1-carboxy-4-hydroxyphenyl) β-D-glucuronic acid (3OH4GlcABA) = 98% pure

(Purity based on % peak area at <33 min. see chromatographs below)

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                                                   Inj Volume : 5.0 µl
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Analysis Method  : C:\CHEM32\1\METHODS\ALLINCIN ANALYSIS 04.M
Last changed     : 1/11/2010 4:24:20 PM by Gary Woodward
Method Info      : Allumax tablet purity analysis
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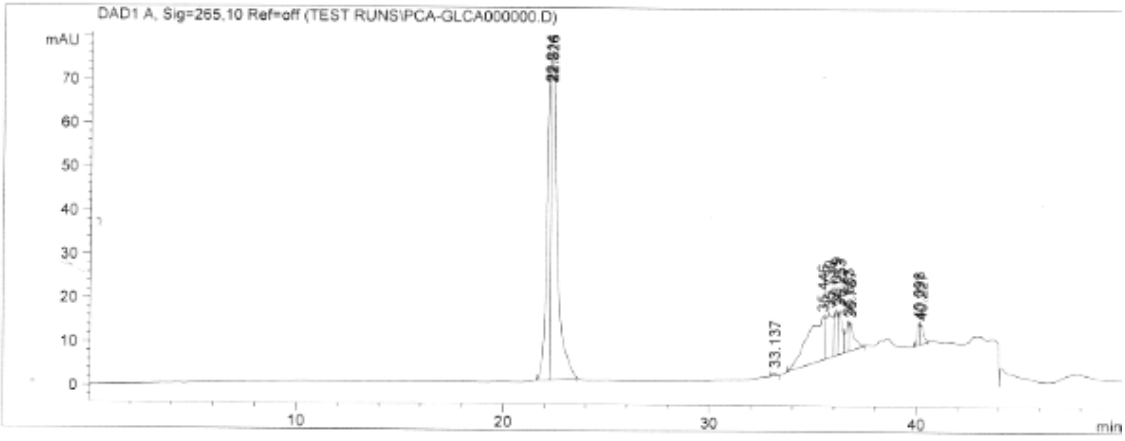

Area Percent Report

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Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs
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2	22.326	VB	0.2994	1646.86938	75.21909	42.5916
3	33.137	BB	0.1583	8.99496	7.48011e-1	0.2326
4	35.446	BV	0.7123	592.00311	10.03514	15.3105
5	35.739	VV	0.2716	222.57500	10.40212	5.7563
6	36.079	VV	0.1492	119.85583	10.65870	3.0997
7	36.253	VV	0.1796	133.41008	10.00507	3.4503
8	36.653	VV	0.1461	77.79664	7.19658	2.0120
9	36.787	VB	0.2054	102.81162	6.54042	2.6589
10	40.098	BV	0.1387	45.57159	4.89156	1.1786

ta File C:\CHEM32\1\DATA\TEST RUNS\PCA-GLCA000000.D
mple Name: PCA-GlcA

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Last changed : 1/11/2010 4:24:20 PM by Gary Woodward
Method Info : Allumax tablet purity analysis
Column: Luna C18 5u

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Totals :				3866.65340	213.67774	

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Different Inj Volume from Sequence !     Actual Inj Volume : 2.0 µl
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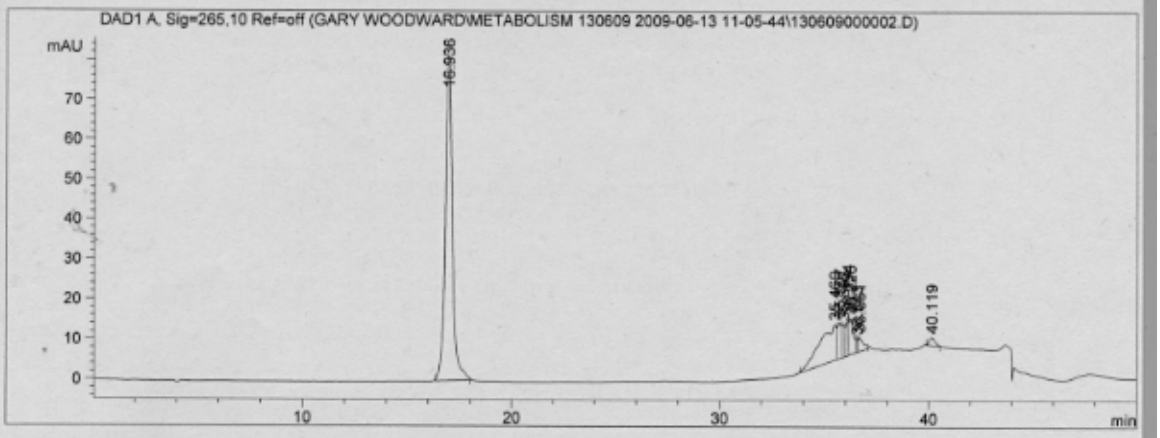

Area Percent Report

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3	35.707	VV	0.1786	117.54580	9.10692	4.0051
4	35.893	VV	0.1347	79.05115	8.18456	2.6935
5	36.094	VV	0.1296	90.32005	9.43991	3.0774
6	36.226	VV	0.1870	120.47104	8.95066	4.1047
7	36.543	VV	0.0835	20.06237	3.36605	0.6836
8	36.661	VB	0.2075	58.92260	3.62802	2.0076
9	40.119	BB	0.2953	37.59480	1.89650	1.2809

ata File C:\CHEM32\...\GARY WOODWARD\METABOLISM 130609 2009-06-13 11-05-44\130609000002.D
ample Name: HBAglcA STD

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   Method Info     : Allumax tablet purity analysis
                     Column: Luna C18 5u


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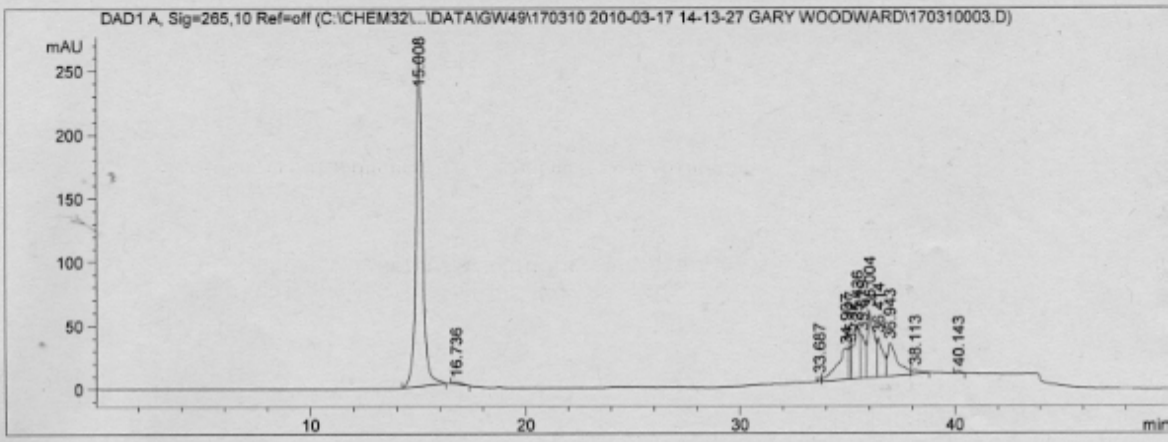

Area Percent Report

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Use Multiplier & Dilution Factor with ISTDs

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4	34.907	VV	0.4397	881.26361	24.49678	8.1694
5	35.127	VV	0.1073	208.77594	26.11142	1.9354
6	35.436	VV	0.2497	835.17822	42.05032	7.7422
7	35.679	VV	0.1850	472.11267	33.79659	4.3765
8	36.004	VV	0.2554	1065.77820	52.36820	9.8799
9	36.414	VV	0.2415	580.57953	31.14141	5.3820

ata File C:\CHEM32\...WARD\DATA\GW49\170310 2010-03-17 14-13-27 GARY WOODWARD\170310003.D
ample Name: 3OH4GlcABA

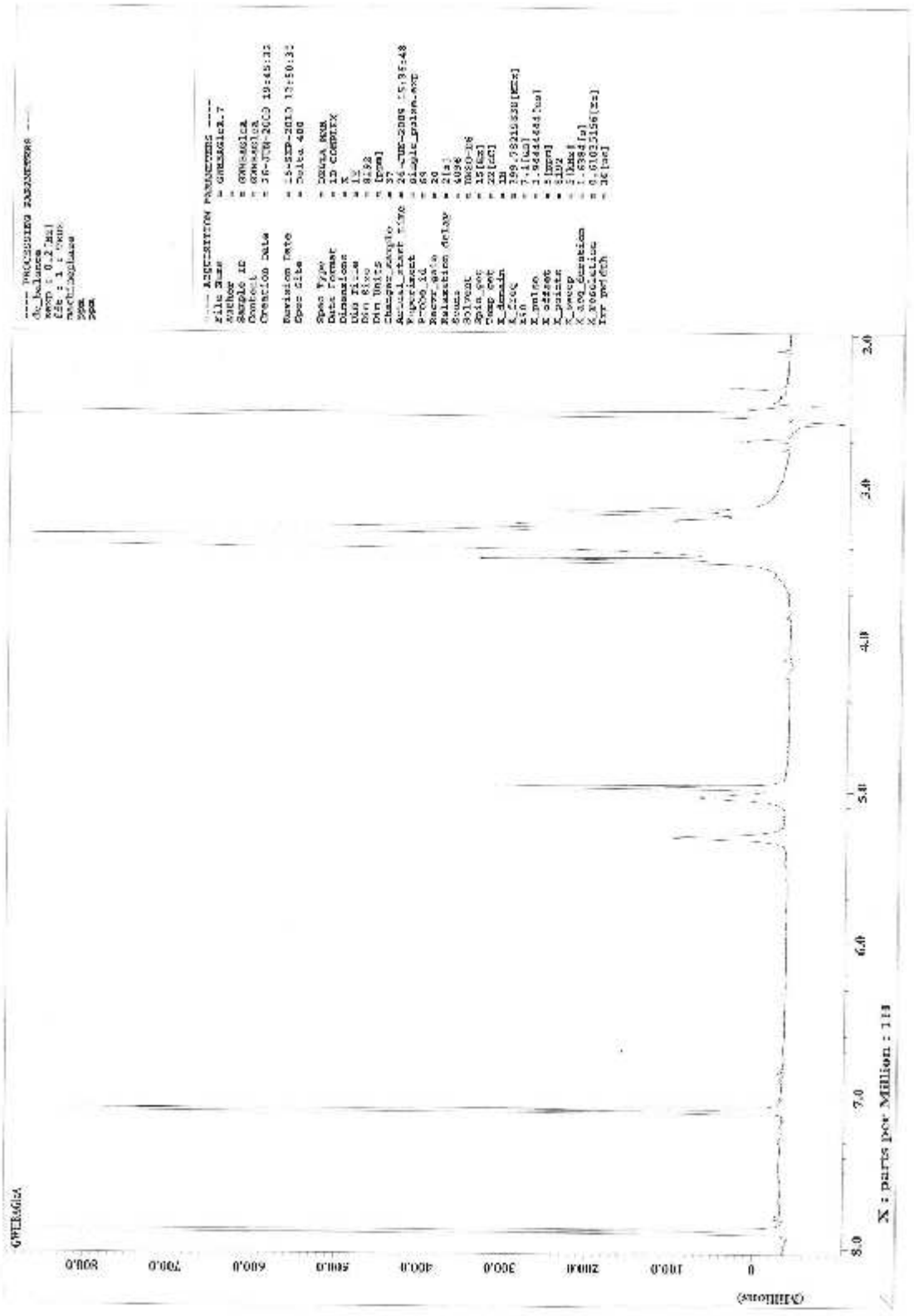
Acq. Operator : Gary Woodward Seq. Line : 3
Acq. Instrument : Instrument 1 Location : P1-A-03
Injection Date : 3/17/2010 3:56:36 PM Inj : 1
Inj Volume : 20.0 µl
Different Inj Volume from Sequence ! Actual Inj Volume : 2.0 µl
Acq. Method : C:\CHEM32\1\GARY WOODWARD\DATA\GW49\170310 2010-03-17 14-13-27 GARY WOODWARD\PHENOLIC ACID GLUCURONIDES 01.M
Last changed : 3/17/2010 12:12:50 PM by Gary Woodward
Analysis Method : C:\CHEM32\1\METHODS\ALLINCIN ANALYSIS 04.M
Last changed : 1/11/2010 4:24:20 PM by Gary Woodward
Method Info : Allumax tablet purity analysis
Column: Luna C18 5u

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
10	36.943	VB	0.3987	828.49182	25.65755	7.6802
11	38.113	BB	0.3529	95.76604	3.48153	0.8878
12	40.143	BB	0.2019	29.06005	2.12052	0.2694
Totals :				1.07873e4	507.92696	

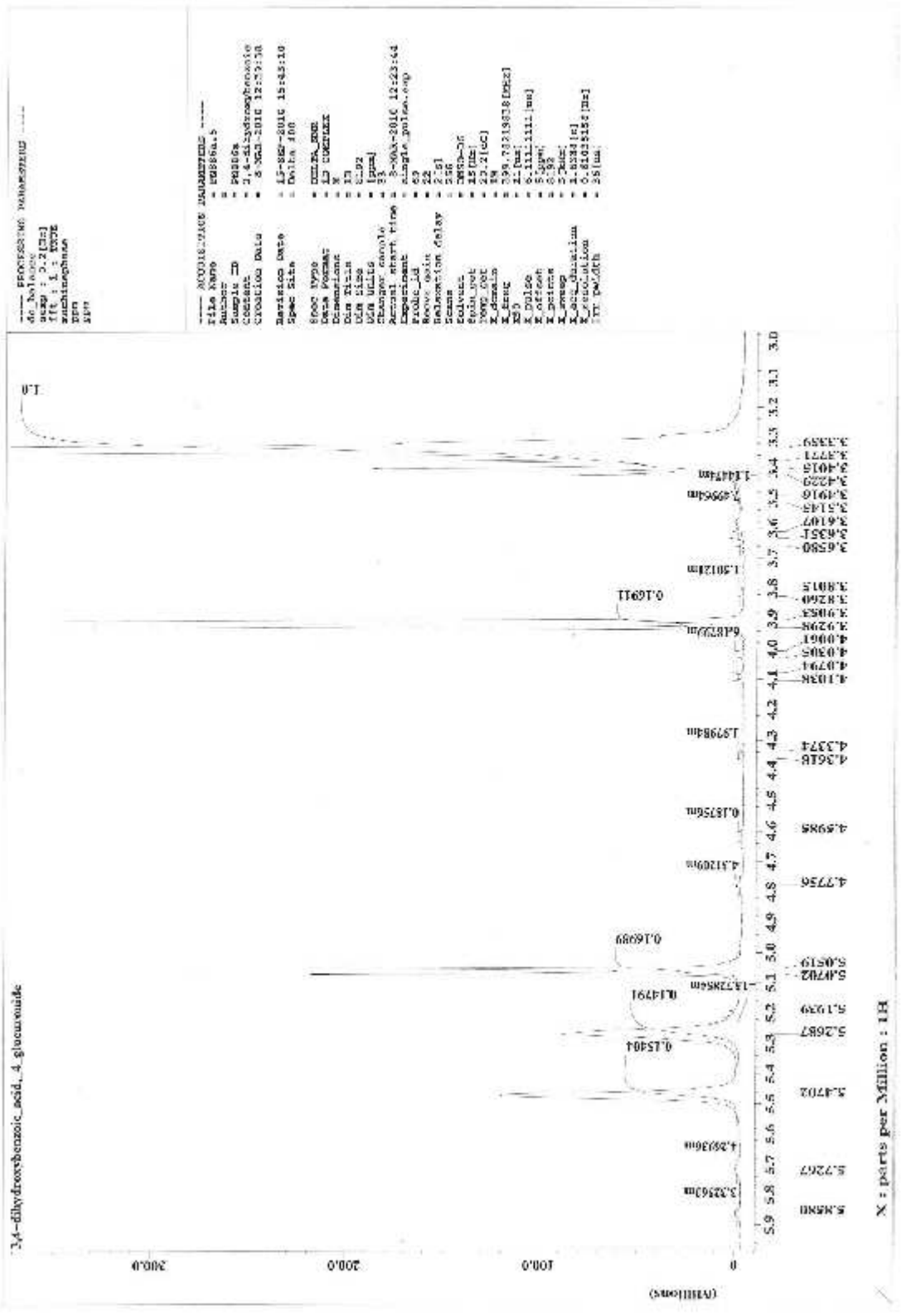
*** End of Report ***

B.2. NMR spectra for synthesised compounds

4-(1-carboxyphenyl) β-D-glucuronic acid



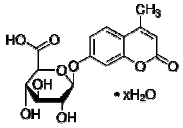
4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid



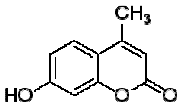
B.3. MS Method development 1: Glucuronide Fragmentation Parameters: Injection Flow Analysis (IFA) of 7-Hydroxy-4-Methylcoumarin-D-Glucuronide (4MUG)

Brief Method:

In order to determine the cone-voltage (fragmentor voltage) required to produce both parent and aglycone (a-glucuronide) fragments an injection flow analysis (IFA) of a common glucuronide standard (4MUG) was performed under various cone-voltage conditions, in both negative and positive ionisation modes (sample and LC/MS conditions for each experiment are described intern in the experimental section below).



7-Hydroxy-4-Methylcoumarin-D-Glucuronide (Mass = 352 Da)

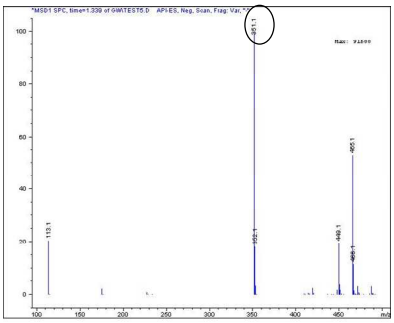


7-Hydroxy-4-Methylcoumarin (Mass = 176 Da)

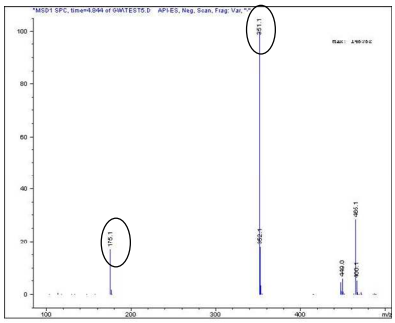
Overall Summery of Results

It was found that cone voltage conditions significantly affect the fragmentation of the glucuronide standard 4MUFGA. Figures A and B illustrate the influence of cone-voltage on 4MUFGA fragmentation, where an increase in cone-voltage yields an increase in the relative abundance of the “aglucuronide” fragment ion and a decrease in the glucuronide fragment ion.

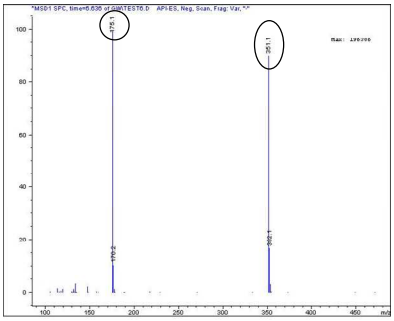
Fragmentor: 110v



Fragmentor: 150v



Fragmentor: 270v



Fragmentor: 300v

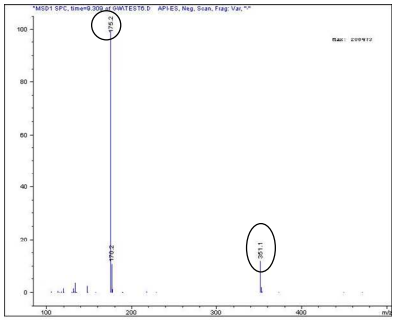


Figure A. Mass fragmentation patterns for 4MUFGA at cone-voltages (fragmentor) of 110v, 150v, 270v and 300v. Ionization performed in negative full scan mode [100-500Da]. Circled are the masses of interest ("aglycone" = 175 Da; Glucuronide = 351 Da [M-1]). Additional fragment ions were observed.

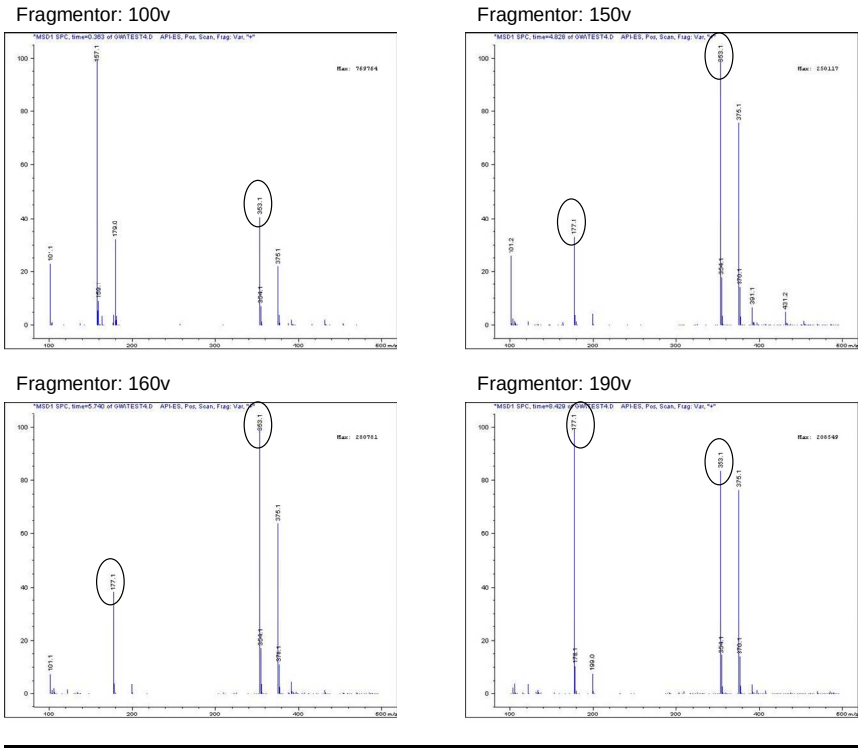


Figure B. Mass fragmentation patterns for 4MUFGA at cone-voltages (fragmentor) of 100v, 150v, 2600v and 190v. Ionization performed in positive full scan mode [100-500Da]. Circled are the masses of interest ("aglycone" = 177 Da; Glucuronide = 353 Da $[M+1]^+$). Additional fragment ions were observed.

Overall Conclusions

The glucuronide 4MUG, representing a phenolic glucuronide structure, may be ionized and fragmented under both positive and negative LC/MS conditions. Should negative mode ionization be used for phenolic glucuronide fragmentation, it is recommended that a cone-voltage of 270v be utilized under these MS conditions, described in the experimental section. Likewise, should positive mode ionization be used for phenolic glucuronide fragmentation, it is recommended that a cone-voltage of 180-190v be utilized under these MS conditions, described in the experimental section. The above stated cone-voltage parameters should yield glucurinide and a-glucuronide fragments in equal relative abundances.

Experimental Section:

The following section describes a series of experiments designed to determine the cone-voltage (fragmentor voltage) required to produce both parent and aglycone (a-glucuronide) fragments from a phenolic glucuronide standard.

Experiment 1:

In order to determine the initial cone voltage required to fragment 4MUFGA, an injection flow analysis was performed, under the below stated MS conditions, with varying cone voltages (50-300v).

Sample concentration: 0.1mg/ml in water

LC Conditions:
Flow rate: 0.4ml/min
Solvent conditions: isocratic run with 75% solvent A (0.1% formic acid in water) and 25% solvent B (0.1% formic acid in actetonitrile).
Injection volume: 20ul

MS Conditions:
Sim mode: positive mode, ion 1 = 353, ion 2 = 177
Gain: 1
Threshold: 150
Drying gas flow: 13 l/min
Nebulizer pressure: 30psig
Drying gas temperature: 350
Capillary voltage: 4000
Ionization mode: API-ES

Results:

As showing in figure 1 below, several 4MUFGA injections were performed (each peak represents an injection at 0.8 min intervals), each at different (increasing) cone-voltages (fragmentor settings). Fragmentation of 4MUFGA appeared to occur between 180 and 190 cone volts, as illustrated below in figures 2 and 3, showing the mass fragmentation patterns for 4MUFGA at fragmentor settings 180v and 190v.

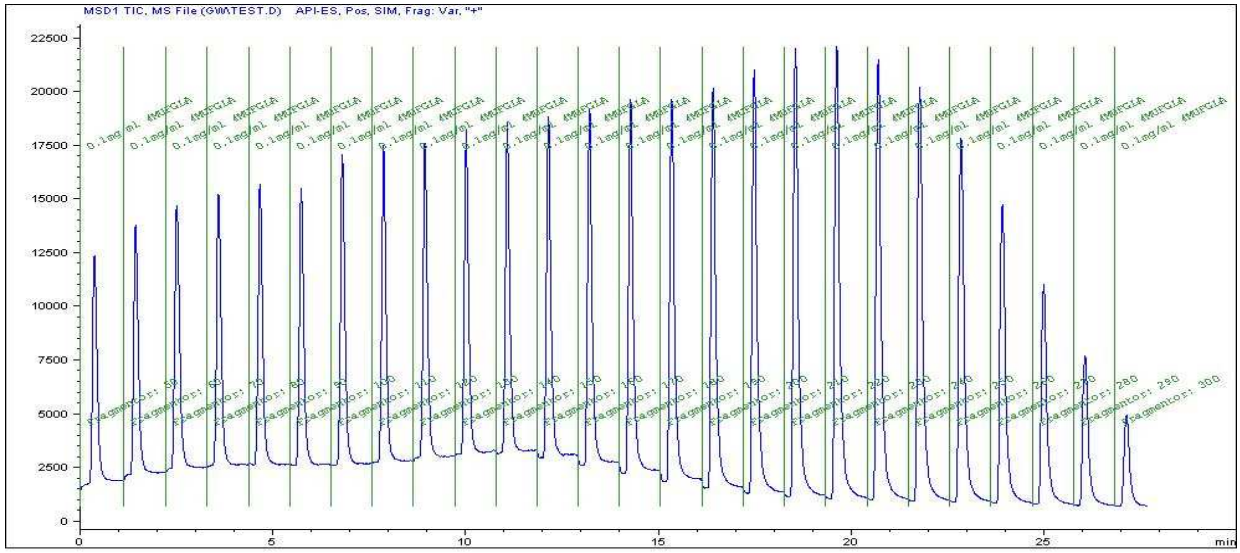


Figure 1. Mass chromatograph of 4MUFGA at cone voltages of 50-300v, increasing in 10v increments (positive sim mode [353Da; 177Da]).

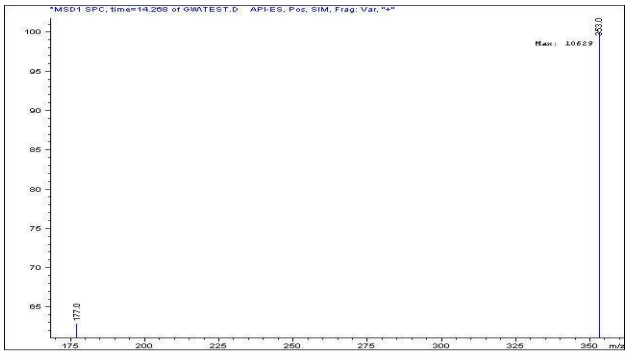


Figure 2. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 180v (positive sim mode [353Da; 177Da]).

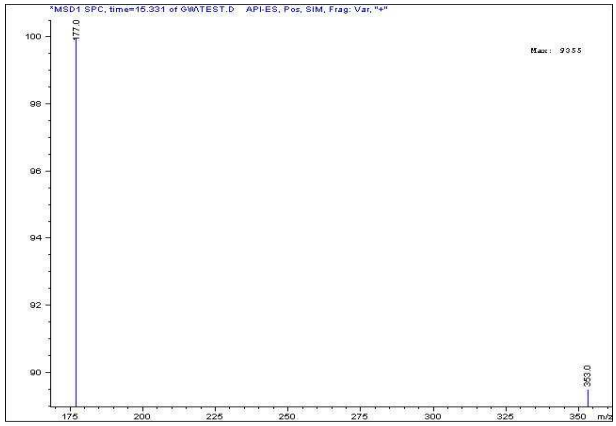


Figure 3. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 190v (positive sim mode [353Da; 177Da]).

Experiment 2:

As it was observed that fragmentation of 4MUFGA occurred between 180-190 cone volts, an injection flow analysis was performed, under the below stated MS conditions, with varying cone voltages (180-190v).

Sample concentration: 0.1mg/ml in water

LC Conditions:
Flow rate: 0.4ml/min
Solvent conditions: isocratic run with 75% solvent A (0.1% formic acid in water) and 25% solvent B (0.1% formic acid in actetonitrile).
Injection volume: 20ul

MS Conditions:
Sim mode: positive mode, ion 1 = 353, ion 2 = 177
Gain: 1
Threshold: 150
Drying gas flow: 13 l/min
Nebulizer pressure: 30psig
Drying gas temperature: 350
Capillary voltage: 4000
Ionization mode: API-ES

Results:

Several 4MUFGA injections between 180 and 190 cone volts were performed (each peak represents an injection at 0.8 min intervals), each at different (increasing) cone-voltages (fragmentor settings), as shown in figure 4. Fragmentation of 4MUFGA appeared to occur between 185 and 186 cone volts, as illustrated below in figures 5 and 6, showing the mass fragmentation patterns for 4MUFGA at fragmentor settings 185v and 186v.

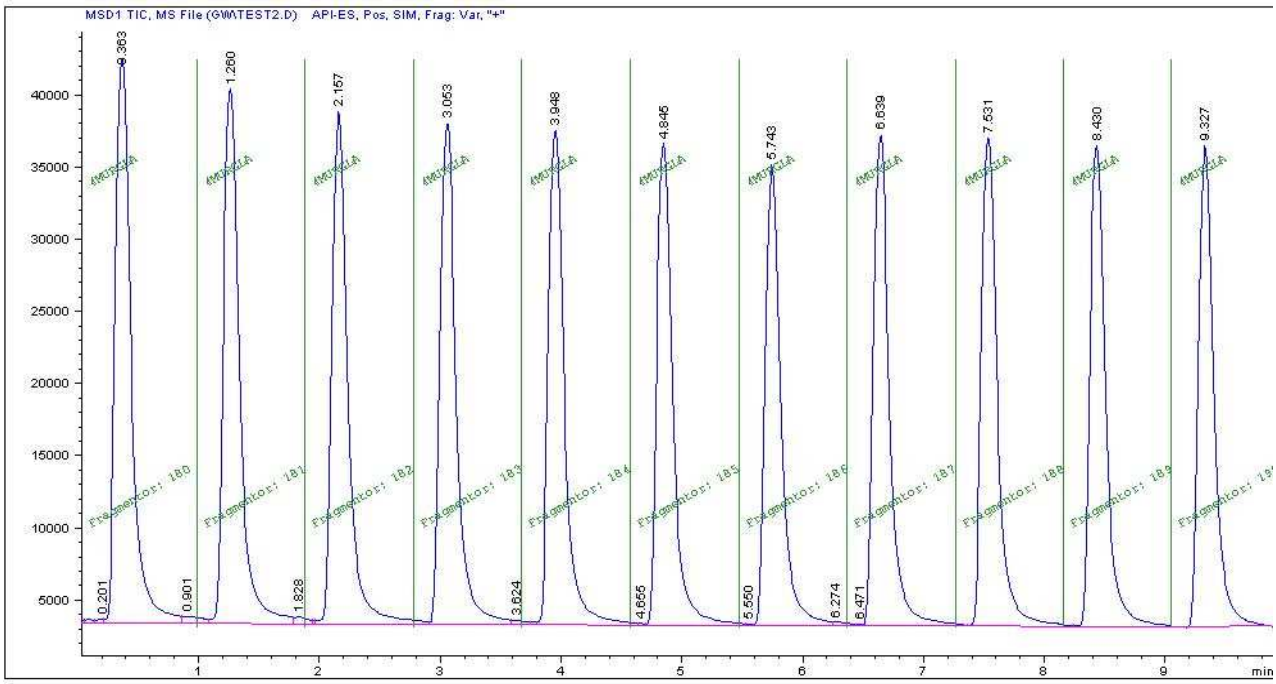


Figure 4. Mass chromatograph of 4MUFGA at cone voltages of 180-190v, increasing in 1v increments (positive sim mode [353Da; 177Da]).

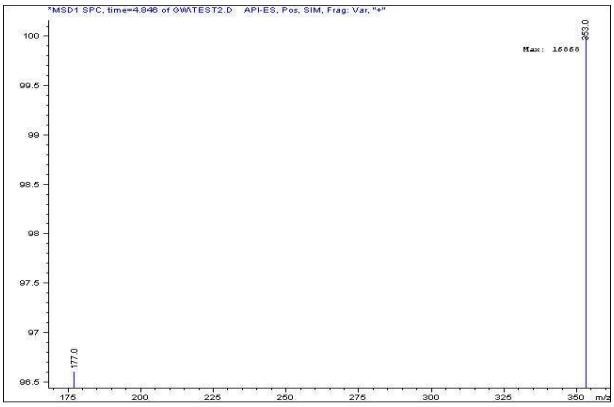


Figure 5. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 195v (positive sim mode [353Da; 177Da]).

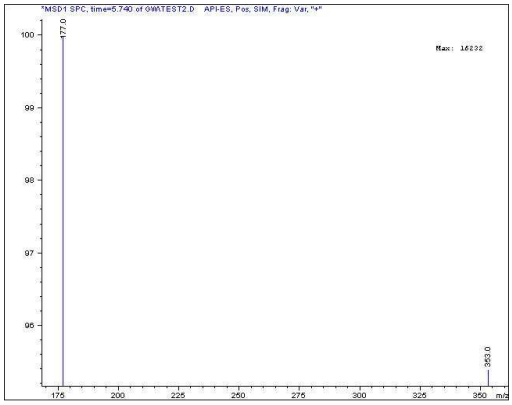


Figure 6. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 186v (positive sim mode [353Da; 177Da]).

Experiment 3

Although fragmentation appeared to occur at 186 cove volts in positive sim mode, it was necessary to assess the fragmentation 4MUFGA in full scan mode (as would be performed for experimental samples). In order to assess varying cone voltages to the fragmentation of 4MUFGA in full scan positive mode, an injection flow analysis was performed, under the below stated MS conditions, with varying cone voltages (170-200v).

Sample concentration: 0.1mg/ml in water

LC Conditions:
Flow rate: 0.4ml/min
Solvent conditions: isocratic run with 75% solvent A (0.1% formic acid in water) and 25% solvent B (0.1% formic acid in actetonitrile).
Injection volume: 10ul

MS Conditions:
Full scan mode: positive mode, 100-1000Da
Gain: 1
Threshold: 150
Drying gas flow: 13 l/min
Nebulizer pressure: 30psig
Drying gas temperature: 350
Capillary voltage: 4000
Ionization mode: API-ES

Results:

Injections of 4MUFGA, fragmented at cone voltages between 180 and 190v in full scan positive mode are shown in figure 7. The fragmentation patterns produced under these conditions are shown in figure 8. Although our masses of interest were identified, their abundance appeared to be significantly lower than the “background” fragments, due to the decreased sensitivity of full scan mode. Subsequent experiments were performed in more concentrated solutions.

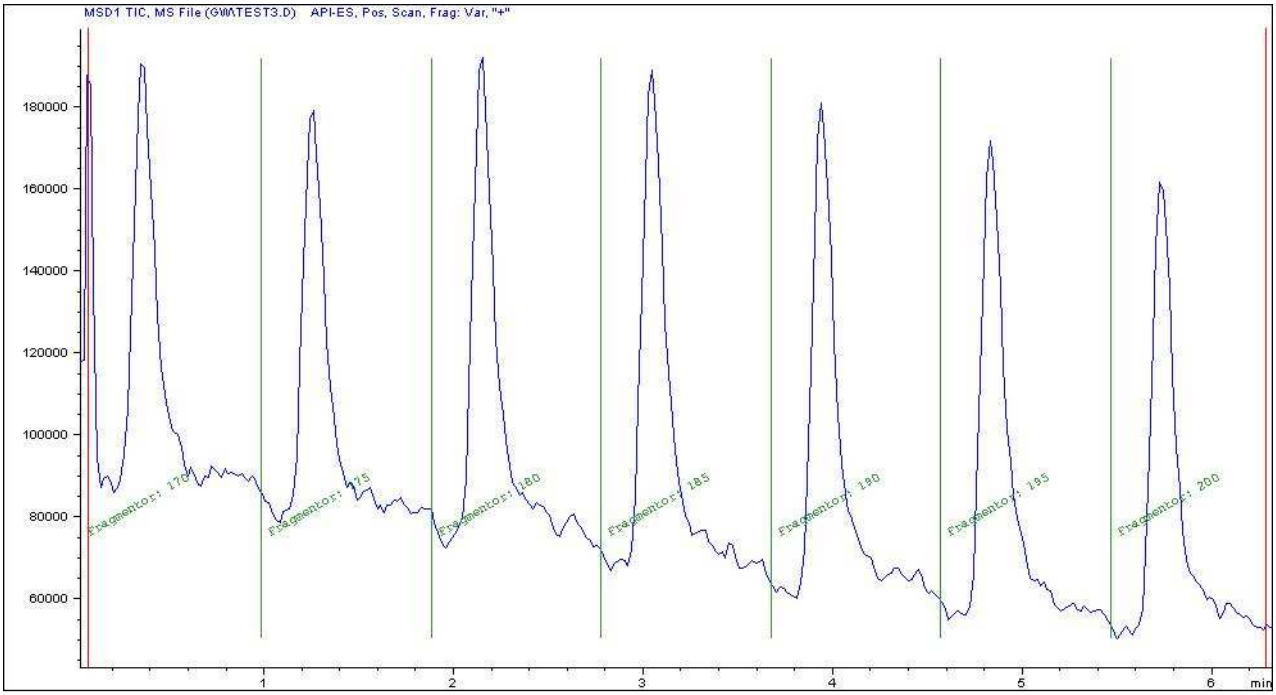


Figure 7. Mass chromatograph of 4MUFGA at cone voltages of 170-200v, increasing in 10v increments (positive full scan mode [100-1000Da]).

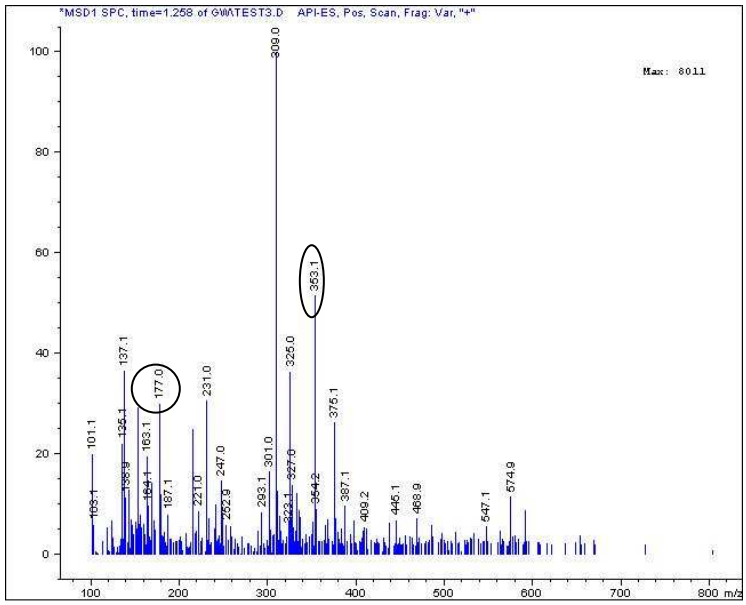


Figure 8. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 170v (positive full scan mode [100-1000Da]). Circled are the masses of interest (aglycone and glucuronide). Significant proportions of additional fragments are observed.

Experiment 4

The fragmentation of 4MUFGA was again assessed in positive full scan mode (100-500Da) with a more concentrated sample. Injection flow analysis was performed under the below stated MS conditions.

Sample concentration: 1mg/ml in water

LC Conditions:
Flow rate: 0.4ml/min
Solvent conditions: isocratic run with 75% solvent A (0.1% formic acid in water) and 25% solvent B (0.1% formic acid in actetonitrile).
Injection volume: 10ul

MS Conditions:
Full scan mode: positive mode, 100-500Da
Gain: 1
Threshold: 150
Drying gas flow: 13 l/min
Nebulizer pressure: 30psig
Drying gas temperature: 350
Capillary voltage: 4000
Ionization mode: API-ES

Results:

Injections of 4MUFGA, fragmented at cone voltages between 100 and 200v in full scan positive mode are shown in figure 9. The fragmentation patterns produced under these conditions are shown in figure 10-13. Increasing cone voltages resulted in an increase in "aglycone" ion formation, were about equal abundances of "aglycone" and glucuronide fragments occurred at 190v.

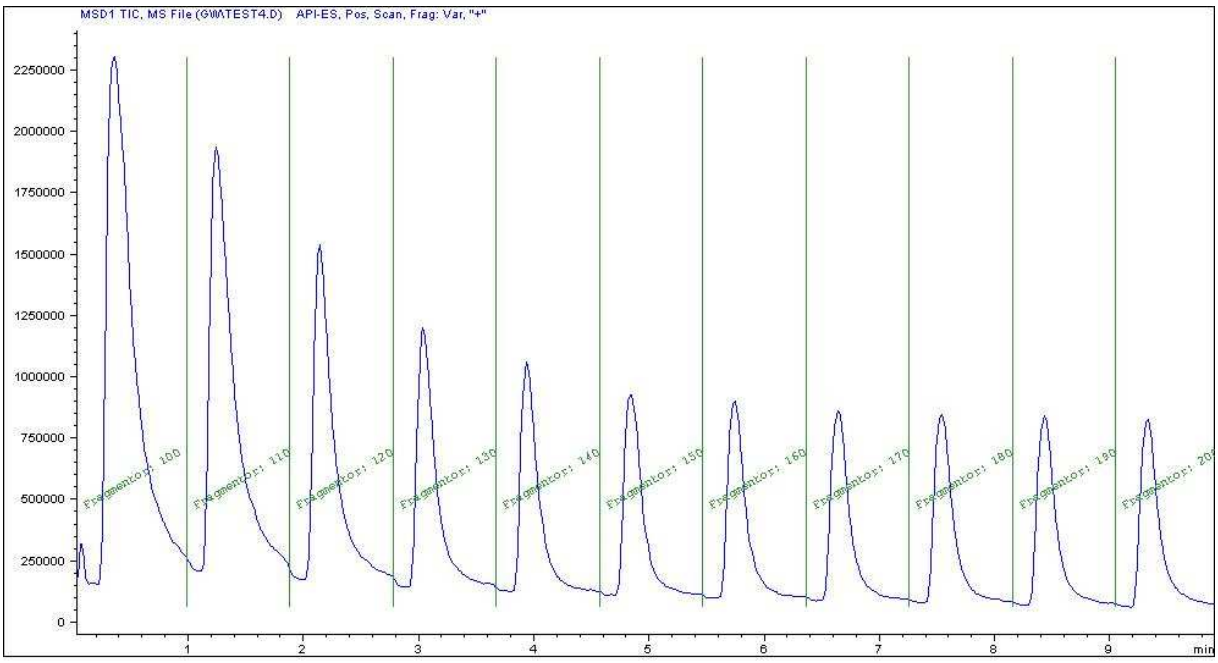


Figure 9. Mass chromatograph of 4MUFGA at cone voltages of 100-200v, increasing in 10v increments (positive full scan mode [100-500Da]).

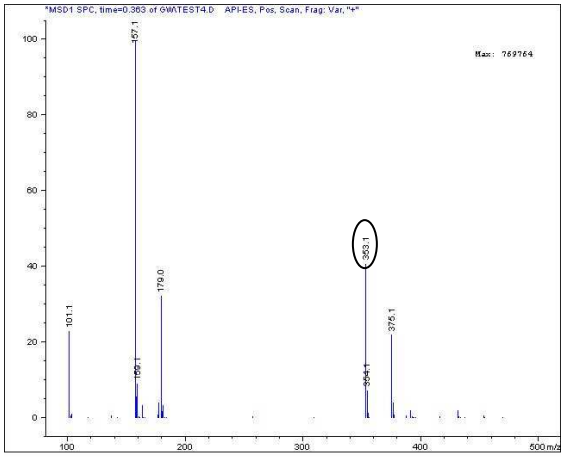


Figure 10. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 100v (positive full scan mode [100-1000Da]). Circled is the glucuronide mass of interest. Additional mass fragments are observed.

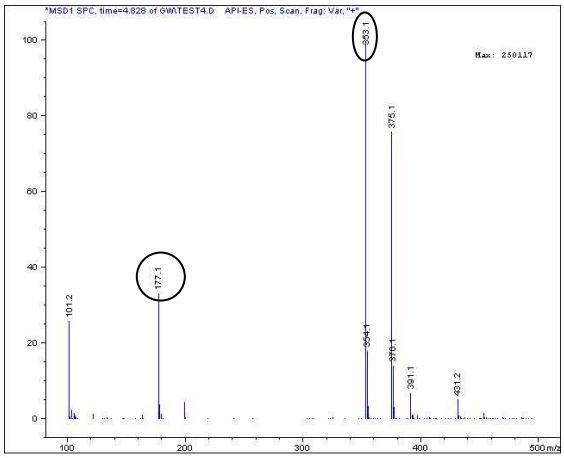


Figure 11. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 150v (positive full scan mode [100-1000Da]). Circled are the glucuronide masses of interest. Additional mass fragments are observed.

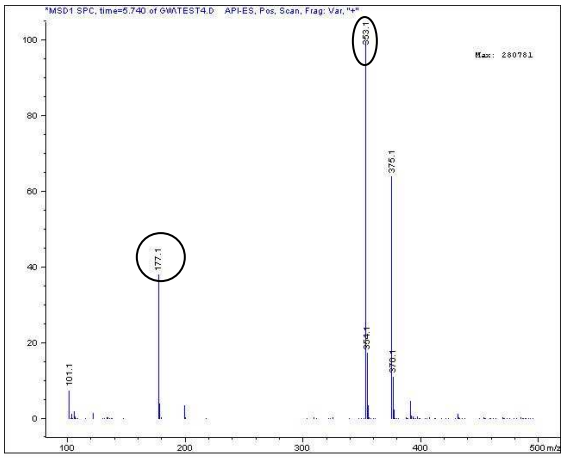


Figure 12. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 160v (positive full scan mode [100-1000Da]). Circled are the glucuronide masses of interest. Additional mass fragments are observed.

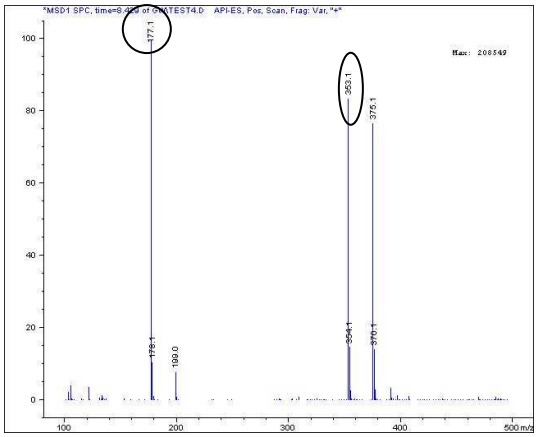


Figure 13. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 190v (positive full scan mode [100-1000Da]). Circled are the glucuronide masses of interest. Additional mass fragments are observed.

Experiment 5

In order to determine the cone voltage required to fragment 4MUFGA in full scan negative mode, an injection flow analysis was performed, under the below stated MS conditions, with varying cone voltages.

Sample concentration: 1mg/ml in water

LC Conditions:
Flow rate: 0.4ml/min
Solvent conditions: isocratic run with 75% solvent A (0.1% formic acid in water) and 25% solvent B (0.1% formic acid in actetonitrile).
Injection volume: 10ul

MS Conditions:
Full scan mode: negative mode, 100-500Da
Gain: 1
Threshold: 150
Drying gas flow: 13 l/min
Nebulizer pressure: 30psig
Drying gas temperature: 350
Capillary voltage: 3000
Ionization mode: API-ES

Results:

Several 4MUFGA injections were performed (each peak represents an injection at 0.8 min intervals), each at different (increasing) cone-voltages (fragmentor settings) (Figure 14). Fragmentation under these conditions occurred at 150 cone volts as shown in figures 15-17.

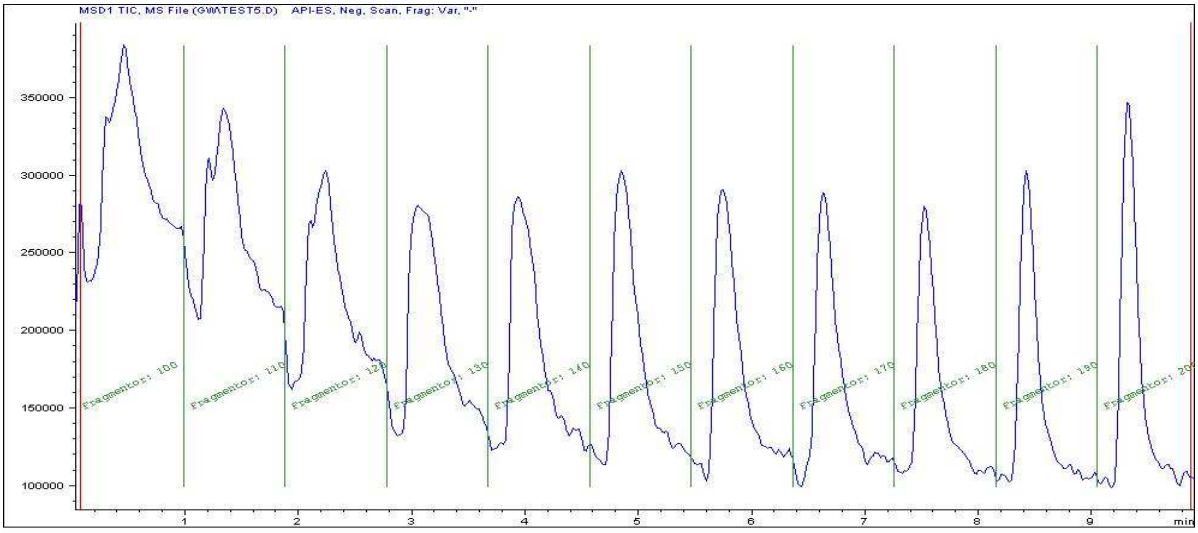


Figure 9. Mass chromatograph of 4MUFGA at cone voltages of 100-200v, increasing in 10v increments (negative full scan mode [100-500Da]).

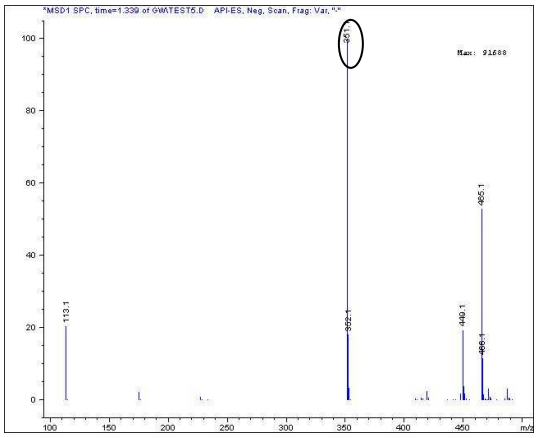


Figure 15. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 110v (negative full scan mode [100-500Da]). Circled is the mass of interest. Additional mass fragments are observed.

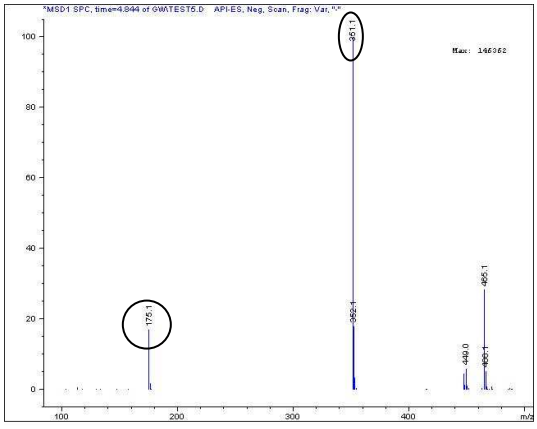


Figure 16. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 150v (negative full scan mode [100-500Da]). Circled are the masses of interest. Additional mass fragments are observed.

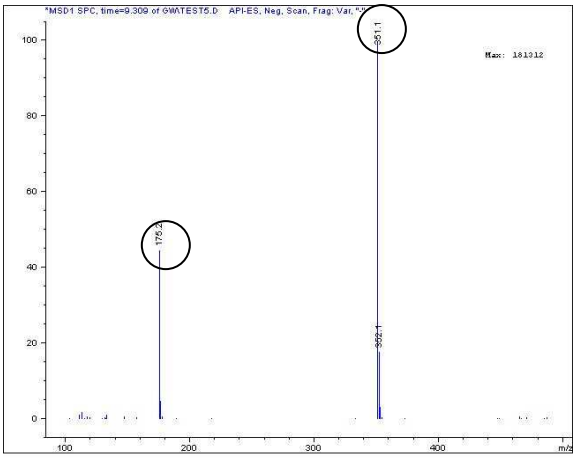


Figure 17. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 200v (negative full scan mode [100-500Da]). Circled are the masses of interest.

Experiment 6

Although fragmentation occurred at >186 cone volts in negative full scan mode, fragmentation did not yield equal "aglycone" and glucuronide ion abundances. Therefore, it was nessesary to assess the fragmentation 4MUFGA at an increased cone voltage (200-300v). An injection flow analysis was performed, under the below stated MS conditions, with varying cone voltages (200-300v).

Sample concentration: 1mg/ml in water

LC Conditions:
Flow rate: 0.4ml/min
Solvent conditions: isocratic run with 75% solvent A (0.1% formic acid in water) and 25% solvent B (0.1% formic acid in actetonitrile).
Injection volume: 10ul

MS Conditions:
Full scan mode: negative mode, 100-500Da
Gain: 1
Threshold: 150
Drying gas flow: 13 l/min
Nebulizer pressure: 30psig
Drying gas temperature: 350
Capillary voltage: 3000
Ionization mode: API-ES

Results:

Several 4MUFGA injections were performed (each peak represents an injection at 0.8 min intervals), each at different (increasing) cone-voltages (fragmentor settings) (Figure 18). Fragmentation patterns under these are shown in figures 19-21.

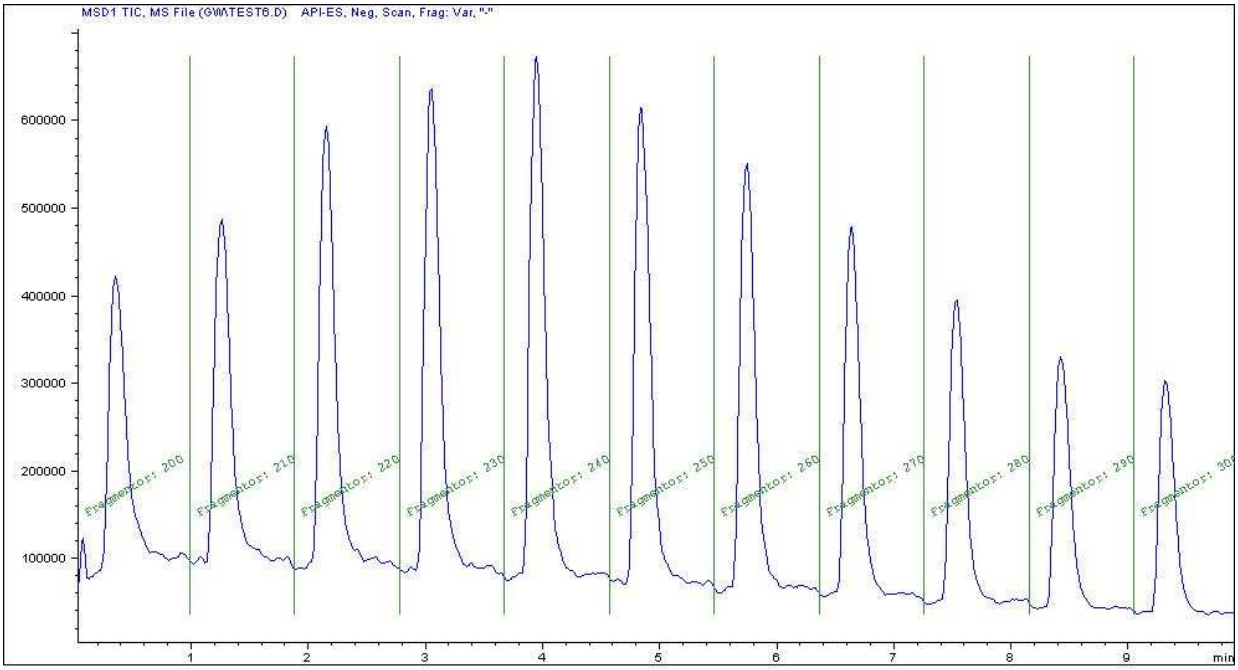


Figure 18. Mass chromatograph of 4MUFGA at cone voltages of 200-300v, increasing in 10v increments (negative full scan mode [100-500Da]).

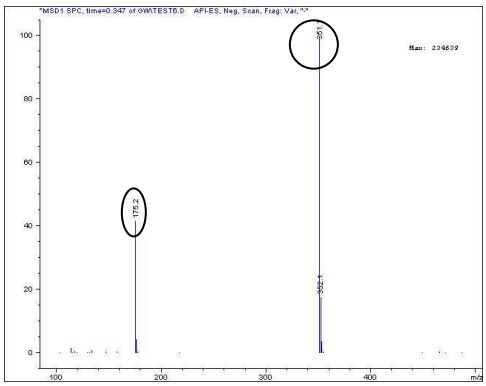


Figure 19. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 200v (negative full scan mode [100-500Da]). Circled are the masses of interest.

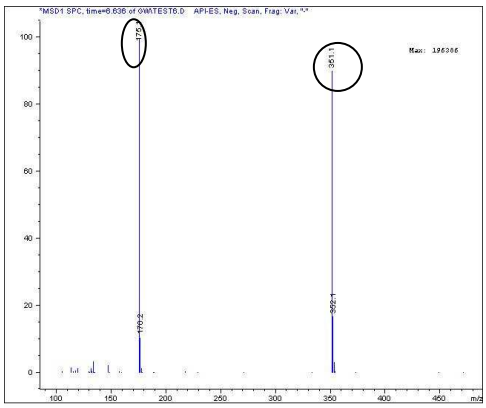


Figure 20. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 270 (negative full scan mode [100-500Da]). Circled are the masses of interest.

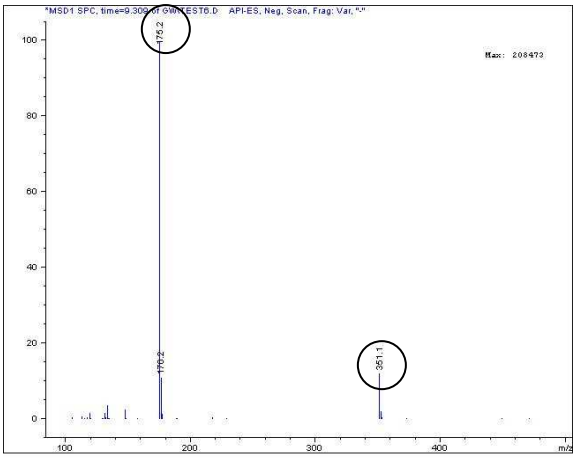


Figure 21. Mass fragmentation pattern for 4MUFGA at a fragmentor setting of 300v (negative full scan mode [100-500Da]). Circled are the masses of interest.

Glossary Definitions

Glossary, Definitions and Abbreviations

°Brix - unit representative of the sugar content of an aqueous solution. One degree Brix corresponds to 1 gram of sucrose in 100 grams of solution and thus represents the strength of the solution as a percentage by weight (% w/w)

^{1}H - common isotope of hydrogen, namely protium.

4GBA - synthesised 4-(1-carboxyphenyl) β-D-glucuronic acid.

3-O-Glucoside – glucose conjugate on the 4' position via an ether bond.

3OH4GBA - synthesised 4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid.

4OH3GBA - synthesised 3-(1-carboxy-4-hydroxyphenyl) β-D-glucuronic acid.

ABCA - ATP-binding cassette transporter.

Acyl – functional group derived by the removal of one or more hydroxyl groups from an acid containing oxygen (oxoacid).

Acylation - process of adding an acyl group to a compound.

ADME - acronym in pharmacokinetics and pharmacology for Absorption, Distribution, Metabolism, and Excretion.

Aglycone - non-sugar compound remaining after replacement of the glycosyl group from a glycoside by a hydrogen atom.

Aldehyde - with the structure R-CHO.

Amine/Amino - functional group that contains a basic nitrogen atom with a lone pair.

Ang II - angiotensin II.

ANOVA – analysis of variance.

ApoA-1 - protein that has a specific role in lipid metabolism.

-ara – arabinose conjugate.

Aryl - functional group or substituent derived from a simple aromatic ring.

AT1R – angiotensin II receptor type 1.

BCA - Bicinchoninic acid assay is a biochemical assay for determining the total level of protein in a solution, similar to Lowry protein assay, Bradford protein assay or biuret reagent.

BH4 – tetrahydrobiopterin.

Bioavailability - Describe the fraction of an administered dose of unchanged xenobiotic that reaches the systemic circulation.

BSA – Bovine Serum Albimin.

Callistephin - pelargonidin-3-O-glucoside chloride.

Carboxyl - functional group consisting of a carbonyl and a hydroxyl, which has the formula –COOH.

Catechol - organic phenol having two adjacent hydroxyl groups attached to a benzene ring.

CD40 - Type I transmembrane protein present on mature B cells, monocytes, dendritic cells, and epithelial cells involved in signal transduction leading to cell activation, proliferation, adhesion, and/or differentiation

CD40L - a ligand for CD40 that induces activation, proliferation, and/or differentiation of CD40-expressing cells.

cGMP - cyclic guanosine monophosphate.

Chalcone - aromatic ketone like compounds.

c-Jun - gene and protein that forms the activator protein 1 (AP-1) early response transcription factor.

COMT - catechol-O-methyltransferase.

CVD – cardiovascular.

Cy – cyanidin

Cytochrome - membrane-bound hemoproteins that contain heme groups and carry out electron transport.

CZE – capillary zone electrophoresis.

DAD – photodiode array detector

DMEM - Dulbecco's modified Eagle's medium.

DMSO - dimethyl sulfoxide.

DMSO d6 - deuterated DMSO.

DOE - design of experiments (DOE) or experimental design is the design of any information-gathering exercises.

Dp – delphinidin.

DPPH - 2,2-diphenyl-1-picrylhydrazyl.

DUOX – NADPH oxidase isoform.

EDTA - ethylenediaminetetraacetic acid.

Electron delocalisation - when electrons are not associated with one atom but are spread over several atoms.

eNOS/NOS3 – endothelial nitric oxide synthase.

Enteric - general term for the intestines.

Enterocyte - simple columnar epithelial cells found in the small intestines and colon.

ER – endoplasmic reticulum.

Erk - extracellular-signal-regulated kinases.

ESI – electrospray ionisation.

ESR - electron spin resonance.

EtOAc – ethylacetate.

FAD - flavin adenine dinucleotide.

FasL - type-II transmembrane protein that belongs to the tumor necrosis factor (TNF) family.

FCS - Foetal Calf Serum.

Ferricytochrome - cytochrome containing oxidized (ferric) iron.

Fission - splitting of something into two parts.

Flavin - organic compounds based on pteridine, formed by the tricyclic heteronuclear organic ring isoalloxazine.

Flavonoid - group of compounds containing a characteristic aromatic nucleus and widely distributed in higher plants, often as pigments.

Flavylium - 2-phenylchromenylium that is the basis of the anthocyanins.

FW – fresh weight.

GA – gallic acid.

-gal – galactose conjugate.

GIT – gastrointestinial tract.

-glu/-glc – glucose conjugate.

Glucuronide - substance produced by linking glucuronic acid to another substance via a glycosidic bond.

Glycoside - molecules in which a sugar is bound to a non-carbohydrate moiety.

GSH – glutathione.

GSK – GlaxoSmithKline.

GSSG - glutathione disulfide.

GTP - guanosine-5'-triphosphate.

HBA – 4-hydroxybenzoic acid.

Hemiketal - carbonyl compound that results from the addition of an alcohol to the carbonyl group of a ketone.

Homolytic/homolysis - chemical bond dissociation of a neutral molecule generating two free radicals.

HPLC – high performance liquid chromatography.

HUVEC – human umbilical vein endothelial cells.

Hydrolysis - chemical process in which a molecule is split into two parts by the addition of a molecule of water.

Hydroxyl - compound containing an oxygen atom bound covalently with a hydrogen atom (-OH).

Imine - functional group or chemical compound containing a carbon–nitrogen double bond, with the nitrogen attached to a hydrogen atom or an organic group.

IFN-γ - Interferon-gamma.

I-κB - inhibitor of nuclear factor kappa-B.

JAK/STAT - signalling pathway that transmits information from chemical signals outside the cell, through the cell membrane, and into gene promoters on the DNA in the cell nucleus.

JNK - c-Jun N-terminal kinases.

K – campferol.

Kuromanin – cyaniding-3-glucoside chloride.

LC – liquid chromatography.

M – morin.

m/z – mass to charge ratio.

MAPK - mitogen-activated protein (MAP) kinases.

MCP - monocyte chemotactic protein.

Metabolite - intermediates and products of metabolism.

Methoxyl - functional group consisting of a methyl group bound to oxygen.

Microsome - vesicle-like artefacts formed from the endoplasmic reticulum (ER) when eukaryotic cells are broken-up in the laboratory.

MPLC – medium pressure liquid chromatography.

MS – mass spectrometry.

Mv - melvidin.

My - myricetin.

Myrtillin – delphinidin-3-glucoside chloride.

NF-κB - nuclear Factor Kappa B.

NMR - nuclear magnetic resonance.

NO – nitric oxide.

NOX – NADPH oxidase.

NP-40 – detergent (nonyl phenoxypolyethoxylethanol).

Nucleophilic/nucleophile - reagent that forms a chemical bond to its reaction partner (the electrophile) by donating both bonding electrons.

OH – hydroxyl.

$OH^{\bullet}$ - hydroxyl radical.

ORAC - oxygen radical absorbance capacity.

- oxo - a ketone functional group.

P38 - p38 mitogen-activated protein kinases, a class of phosphorylating enzymes.

PBS – phosphate buffered saline.

PBST – phosphate buffered saline with tween.

PCA – protocatechuic acid.

PCR – polymerase chain reaction.

PDGF – platelet derived growth factor.

Pectinolytic – degradation of pectin.

PGA – phloroglucinaldehyde (2,4,6-trihydroxybenzaldehyde).

Phenolic acid - consists of a phenyl bonded to a hydroxyl (-OH) and carboxylic acid (-COOH).

Phenotype - observable characteristic or trait of an organism.

Phytochemical - chemical compounds that occur naturally in plants.

PK_a - negative logarithm of the ionization constant (K) of an acid.

PKC – protein kinase C.

Plasma – cell free liquid component of blood containing with fibrinogen present.

PMA - phorbol 12-myristate 13-acetate.

Pn – peonidin.

Polyphenol - chemical substances found in plants characterized by the presence of more than one phenol unit.

prep-LC – preparative liquid chromatography.

Pt – petunidin.

PVDF - polyvinylidene fluoride.

Pyrylium – any derivative of a conjugated 6-membered carbon ring system with one carbon atom replaced by a positively charged oxygen atom.

Q – queretin.

qRT-PCR - qualtitative real-time polymerase chain reaction.

Quinonoidal – pertaining to organic compounds that are formally derived from aromatic compounds by exchanging an even number of –CH= groups with –C(=O)– groups, with any necessary rearrangement of double bonds.

Radical - atoms, molecules, or ions with unpaired electrons on an open shell configuration.

Redox – reduction/oxidation state.

Resinosis - reoccurrence of a narrowing of a blood vessel.

ROS – reactive oxygen species.

RT-PCR – reverse transcription polymerase chain reaction.

-rut – rutinoside conjugate.

SA – salicylic acid.

SBS – sodium bisulphite.

Serum - cell free liquid component of blood containing without fibrinogen present.

SOD – superoxide dismutase.

SPE – solid phase extraction.

Src - family of proto-oncogenic tyrosine kinases.

Stoichiometry - quantitative relationships that exist among the reactants and products in chemical reactions.

TAA - total antioxidant activity.

TEAC - trolox equivalent antioxidant activity.

THF - Tetrahydrofuran

TLC – thin layer chromatography.

TNF-α - tumour necrosis factor.

T_R – retention time.

TRAF - tumour necrosis factor receptor associated factor.

UDP - uridine diphosphate.

UDP-GA - uridine 5'-diphospho-glucuronic acid.

UGT - uridine 5'-diphospho-glucuronosyltransferase.

UPLC – ultra-performance liquid chromatography.

UV – ultraviolet.

VA – vanillic acid.

VEGF - vascular endothelial growth factor.

Vis – visible.

Vit C – vitamin C.

XO – xanthine oxidase.

Diketone - a molecule containing two carbonyl groups.

λ_{max} – maximum absorbance wavelength.

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