The Role of the Human Gut Microbiota in the Metabolism of Dietary Anthocyanins

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Abstract

Background: Anthocyanin consumption has been linked to a variety of health benefits. However, anthocyanins have poor bioavailability, and consequently any biological effect of anthocyanin consumption is hypothesised to be attributed to their metabolites. Recently, there has been evidence for a bi-directional relationship between anthocyanins and the gut microbiota whereby the microbiota largely determines how anthocyanins are metabolised while anthocyanins and/or their metabolites may modulate gut microbial populations. However, research in this field is in its infancy and much remains to be understood.

Objective: To investigate the human *in vivo* metabolism of two different types of anthocyanin, black rice and bilberry anthocyanins, and explore the role of the gut microbiota in anthocyanin metabolism.

Approaches: Apply optimised UHPLC-MS/MS methods to quantify anthocyanins and their metabolites in biological samples from a randomised placebo-controlled crossover trial, where volunteers consumed capsules of bilberry or black rice anthocyanin extract; and profile the gut microbiota of these same individuals using 16S and whole-genome shotgun metagenomics.

Results: Anthocyanins derived from bilberry and black rice extracts are extensively metabolised *in vivo*. Catechol and its phase 2 conjugates were reported for the first time as *in vivo* metabolites of black rice anthocyanins, and 5-hydroxyferulic acid was reported for the first time as a confirmed bilberry anthocyanin metabolite. Metabolism of black rice and bilberry anthocyanins was subject to high inter-individual variation, but there was a clear relationship in the overall urinary excretion of anthocyanin metabolites for participants between treatments (R^2 = 0.259 p = 0.0008). Furthermore, the gut microbiota was associated with the production of anthocyanin metabolites including: catechol, phloroglucinol, and dihydrocaffeic acid, and the specific microbiota anthocyanins were exposed to was observed as a source of inter-individual variation in anthocyanin metabolism.

Conclusion: This thesis supports the notion that anthocyanins are extensively metabolised *in vivo* to a diverse range of metabolites that are present at higher concentrations than the parent compound and thus are likely implicated in any biological effects of anthocyanin consumption. Furthermore, the data presented supports that the gut microbiota has a critical role in this metabolism and is at least partly responsible for observed inter-individual variation in anthocyanin metabolite profiles.

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Abbreviations

ADME	Absorption, distribution, metabolism and excretion
C3A	Cyanidin-3-*O*-arabinoside
C3G	Cyanidin-3-*O*-glucoside
C3Rut	Cyanidin-3-*O*-rutinoside
CCA	Canonical correlation analysis
CLR	Centre-log ratio
CM	Colon model
Cmax	Maximum concentration
D3A	Delphinidin-3-*O*-arabinoside
D3G	Delphinidin-3-*O*-glucoside
D3Rut	Delphinidin-3-*O*-rutinoside
DAD	Diode array detection
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTW	Dynamic time warping
FA	Formic acid
GI	Gastrointestinal
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
ISTD	Internal standard
m/z	Mass-to-charge ratio
M3A	Malvidin-3-*O*-arabinoside
M3G	Malvidin-3-*O*-glucoside
M3Rut	Malvidin-3-*O*-rutinoside
MRM	Multiple reaction monitoring
NaOH	Sodium hydroxide
PCA	Protocatechuic acid
Pel3A	Pelarginidin-3-*O*-arabinoside
Pel3G	Pelargonidin-3-*O*-glucoside
Pel3Rut	Pelargonidin-3-*O*-rutinoside

Peo3A	Peonidin-3-*O*-arabinoside
Peo3G	Peonidin-3-*O*-glucoside
Peo3Rut	Peonidin-3-*O*-rutinoside
Pet3A	Petunidin-3-*O*-arabinoside
Pet3G	Petunidin-3-*O*-glucoside
Pet3Rut	Petunidin-3-*O*-rutinoside
PGA	Phloroglucinaldehyde
PGC	Phloroglucinolcarboxylic acid
QIB	Quadram Institute Bioscience
RNA	Ribonucleic acid
RT	Retention time
SD	Standard deviation
SPE	Solid phase extraction
sPLS-DA	Sparse PLS discriminant analysis
TIC	Total ion current
Tmax	Time of maximum concentration
TOF-MS	Time-of-flight mass spectrometry
UHPLC	Ultra high performance liquid chromatography
UHPLC-MS/MS	Ultra high performance liquid chromatography – with tandem mass spectrometry
XIC	Extracted ion chromatogram

List of Publications

Peer Reviewed Papers

Percival J, Philo M, Jokioja J, Defernez M, Troncoso Rey P, Hollands WJ, Shehata E, Day-Walsh P, Kellingray L, Needs PW, Narbad A, Kroon PA. Combined *In Vivo* and *In Vitro* Assessments of Anthocyanin Metabolism Highlights the Critical Role of the Gut Microbiota (In preparation).

Percival J, Philo M, Jokioja J, Defernez M, Troncoso Rey P, Hollands WJ, Shehata E, Day-Walsh P, Kellingray L, Needs PW, Narbad A, Kroon PA. Identification of Novel Gut Microbiota Derived Metabolites from Anthocyanin-Rich Black Rice and Bilberry Extracts in Humans (In preparation).

Percival J, Shehata E (joint first author), Kroon PA. Spontaneous Anthocyanin Degradation and its Potential Importance for Human Health: A Review (In preparation).

Aboufarrag H, Hollands WJ, **Percival J**, Philo M, Savva GM, and Kroon PA. Isolated anthocyanins from Bilberry Fruit and Black Rice Do Not Reduce LDL Cholesterol or Beneficially Alter Other Biomarkers of Cardiovascular Disease in Adults with Elevated Cholesterol: A Randomized, Placebo-Controlled, Cross-Over Trial (In preparation).

Jokioja J, **Percival J**, Philo M, Yang B, Kroon P A, Linderborg K M. Phenolic Metabolites in the Urine and Plasma of Healthy Men After Acute Intake of Purple Potato Extract Rich in Methoxysubstituted Monoacylated Anthocyanins. Mol. Nutr. Food Res. 2021, 65, 2000898. https://doi.org/10.1002/mnfr.202000898 (see appendix 9).

Posters

Percival J, Philo M, Jokioja J, Defernez M, Troncoso Rey P, Hollands WJ, Shehata E, Day-Walsh P, Kellingray L, Needs PW, Narbad A, Kroon PA. Identification of Novel Human Gut Microbiota Metabolites from Cyanidin- and Delphinidin-Type Anthocyanins. Physiological Society Conference (Online, 12 – 16th July 2021).

Percival J, Philo M, Jokioja J, Day-Walsh P, Kellingray L, Troncoso Rey P, Defernez M, Hollands WJ, Shehata E, Needs PW, Narbad A, Kroon PA. Gut Microbial Metabolism of Bilberry and Black Rice Anthocyanins. Norwich Institute of Health Aging Launch Symposium (Online, 1st February 2021).

Percival J, Philo M, Hollands WJ, Needs PW, Jokioja J, Narbad A, Kroon PA. Human *in vivo* Metabolism of Bilberry and Black Rice Anthocyanins. Norwich Research Park Doctoral Training Partnership Summer Conference (Online, June 2020).

Percival J, Philo M, Hollands WJ, Needs PW, Jokioja J, Narbad A, Kroon PA. Identification of Anthocyanins and their Microbial Metabolites in Humans. Quadram Institute Bioscience Student Science Showcase (Norwich, June 2019).

Percival J, Philo M, Hollands WJ, Needs PW, Jokioja J, Narbad A, Kroon PA. Identification of Novel Gut Microbial Trihydroxy B-ring and Dihydroxy B-ring Anthocyanin Metabolites in Humans. Bioavailability Conference (Norwich, September 2018).

Oral Presentations

Percival J, Philo M, Jokioja J, Defernez M, Troncoso Rey P, Hollands WJ, Shehata E, Day-Walsh P, Kellingray L, Needs PW, Narbad A, Kroon PA. The Relationship Between Anthocyanin Metabolism and the Gut Microbiota. Quadram Institute Bioscience Coffee Break Science (Online, April 2021).

Percival J, Philo M, Jokioja J, Defernez M, Troncoso Rey P, Hollands WJ, Shehata E, Day-Walsh P, Kellingray L, Needs PW, Narbad A, Kroon PA. Anthocyanin Metabolism and the Gut Microbiota. Quadram Institute Bioscience Food and Health Programme meeting (Norwich, March 2020).

Percival J, Philo M, Jokioja J, Defernez M, Troncoso Rey P, Hollands WJ, Shehata E, Day-Walsh P, Needs PW, Narbad A, Kroon PA. Anthocyanin Metabolism and Human Health. Quadram Institute Bioscience Student Seminar. (Norwich, December 2019).

Percival J, Philo M, Jokioja J, Defernez M, Troncoso Rey P, Hollands WJ, Shehata E, Day-Walsh P, Kellingray L, Needs PW, Narbad A, Kroon PA. Identification of Novel Microbial Anthocyanin Metabolites in Human Samples. Knowledge Exchange Trip at the Technical University of Munich (Munich, September 2019).

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know. Dad, thank you for your constant support and laughter, you may have only been here for the very beginning of this journey but somehow still managed to get me through it, your sayings and advice have helped me through the challenges and I wish that I could tell you that I actually managed to do this. I appreciate everything you have both done for me and the sacrifices you have made to help me get to this point, you are the best parents, my biggest inspiration, and this would not have been possible without either of you. Adele, thank you for helping me get through this with laughter, being able to chat about all of this to you has helped me more than you know. Nan and Grandad, you always supported me and I am grateful to both of you, I think back to the many words of encouragement you have given and that has helped me through. George, you have been my rock these last 4 years (and more) through the many ups and downs. Being able to talk about it all with you as we both try to become Dr's has helped me so much, there's nobody I'd rather have gone on this journey with, and I can't wait for our next journey together.

Contributions

The work presented in this thesis is original research by the author. Several pieces of work described in this thesis were collaborative and hence significant contributions were made by other researchers and are summarised as follows.

Chapter 2 describes the development of optimised methods for the quantification of anthocyanins and their metabolites in biological samples. The work described in this chapter was done in collaboration with Dr Johanna Jokioja with a 50:50 division of experimental work between the author and Dr Johanna Jokioja with the help of Mark Philo under the supervision of Dr Paul Kroon. However, the data analysis and interpretation presented in this thesis is the work of the author.

Chapter 3 describes the application of these methods to human urine and plasma samples to identify a range of structurally diverse anthocyanin metabolites produced *in vivo.* The human study from which these samples were derived was conceptualised and organised by Dr Paul Kroon, Wendy Hollands and Dr Hassan Aboufarrag. However, the data reported in this thesis was collected, analysed and interpreted by the author.

Chapter 4 investigates the role of the gut microbiota in anthocyanin metabolism using an *in vitro* colon model, particularly highlighting how this *in vitro* microbial metabolism of anthocyanins is subject to considerable inter-individual variation and looks at relationships with *in vivo* data on anthocyanin metabolism. All experimental work was completed by the author, dynamic time warping and principal component analysis of data was completed by George Oastler, all other data analysis and interpretation is the work of the author.

Chapter 5 explores the postulated bidirectional relationship between anthocyanins and the gut microbiota and investigates both whether anthocyanin consumption can alter gut microbiota profiles, and whether gut microbiota profiles themselves seem to influence anthocyanin metabolism. Experimental work was completed by the author, samples were sent to Novogene for sequencing, and bioinformatics analysis was done by Dr Perla Rey and Dr Marianne Defernez in collaboration with the author. However, the interpretation of the data presented in this thesis is the work of the author.

Chapter 6 explores spontaneous cyanidin degradation. This work was completed collaboratively with Emad Shehata with a 50:50 division of experimental work. However, the data analysis and interpretation presented here is the work of the author.

Chapter 1

General Introduction

Chapter 1 : General Introduction

1.1 Structure of the Thesis

The data presented in this thesis relates to anthocyanin metabolism and the involvement of the gut microbiota in this process. Chapter 1 introduces anthocyanins and gives an overview of their sources, stability, bioavailability and metabolism; as well as providing an overview of the gut microbiota and its relationship with anthocyanins. Furthermore, this chapter highlights gaps in our knowledge in the field of anthocyanin metabolism, the relationship between anthocyanins and the gut microbiota and details the aims, objectives and hypotheses of the thesis. Chapter 2 describes the development of methods for the quantification of anthocyanins and their metabolites in biological samples, and Chapter 3 describes the application of these methods to human urine and plasma samples to identify a range of structurally diverse anthocyanin metabolites produced *in vivo*. Chapter 4 focuses on the role of the gut microbiota in anthocyanin metabolism using an *in vitro* colon model, particularly highlighting how this *in vitro* microbial metabolism of anthocyanins is subject to considerable inter-individual variation and looks at relationships with *in vivo* data on anthocyanin metabolism. Chapter 5 explores the postulated bidirectional relationship between anthocyanins and the gut microbiota and investigates both whether anthocyanin consumption can alter gut microbiota profiles, and whether gut microbiota profiles themselves seem to influence anthocyanin metabolism. Chapter 6 on the other hand takes a closer look at spontaneous anthocyanin degradation and explores how this may be affected by different conditions and may contribute to the overall metabolites detected in urine and plasma.

1.2 What are Polyphenols?

Polyphenols are a family of naturally occurring organic compounds characterised by multiple phenol units (Nijveldt *et al.*, 2001). They are the subject of a wide-range of epidemiological and bioavailability studies due to evidence that they hold several properties that may mean that they can prevent several pathologies including but not limited to: cardiovascular disease, cancer, neurodegenerative disease and obesity (Cory *et al.*, 2018; Pascual-Teresa *et al.*, 2010; Rio *et al.*, 2013; Vauzour *et al.*, 2008). Polyphenols can be divided into three categories: tannins, lignins and flavonoids. Flavonoids are the largest and most renowned class of polyphenols and are consumed as part of an everyday diet through their presence in many plant foods. Flavonoids themselves can

be divided into six main sub-categories (pictured in Figure 1-1) based on structural variations of their heterocyclic C-ring: anthocyanidins, flavan-3-ols, flavanols, flavones, flavanones and isoflavonoids (Nijveldt *et al.*, 2001; Zamora-Ros *et al.*, 2011). This chapter focuses on the current literature around anthocyanins (the glycosylated form of anthocyanidins found in plants) with particular focus on their metabolism, chemical degradation, bioavailability, bioactivity and interplay with the gut microbiota.
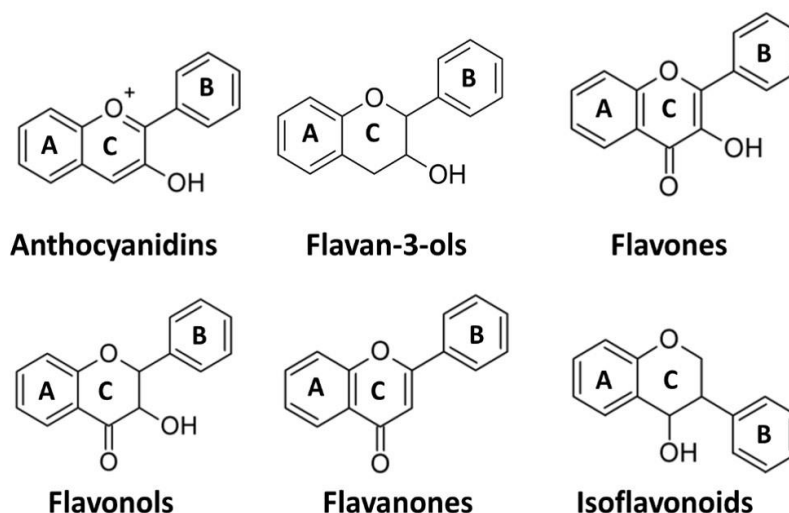
Figure 1-1- General chemical structures of the common flavonoids: anthocyanidins, flavan-3-ols, flavones, flavonols, flavanones and isoflavonoids.

1.3   What are Anthocyanins?

Anthocyanins are a class of water-soluble flavonoids found in an extensive range of fruits and vegetables, and are responsible for giving many plants their signature red-purple pigmentation (Tanaka *et al.*, 2008). Common dietary sources of anthocyanin include: blueberries, strawberries, raspberries, red cabbage, apples, bilberry, black rice and grapes (Braga *et al.*, 2018; Markakis, 1974; Martín *et al.*, 2017). In plants anthocyanins have several functional roles including attracting pollinators and protecting against UV-induced damage (González-Aguilar *et al.*, 2009; Lev-Yadun and Gould, 2008; Liu *et al.*, 2018; Passeri *et al.*, 2016). Consumption of anthocyanin rich foods has been linked to many different health benefits such as being: cardioprotective, anti-inflammatory, neuroprotective, and anti-diabetic, amongst many other health claims (Cassidy *et al.*, 2013; Olivas-Aguirre *et al.*, 2016; Yi *et al.*, 2006; Zafra-Stone *et al.*, 2007). For example, there is evidence that anthocyanins are able to reduce systolic blood pressure in individuals with higher than normal systolic blood pressure, thus decreasing their risk of cardiovascular disease (Matsusima *et al.*,

2013). Whilst another study found that increasing anthocyanin intake from 6 mg to 19 mg per day was associated with an 8% reduction in risk of hypertension (Cassidy *et al.*, 2011). However, anthocyanins are not stable molecules and have low bioavailability, and therefore any health effect of dietary anthocyanins is hypothesised to be attributed to their metabolites rather than the parent compound (Kay *et al.*, 2009).

1.3.1   The Chemistry of Anthocyanins

Anthocyanins consist of an aglycone, known as the anthocyanidin, bound to at least one sugar moiety. Anthocyanidins comprise three aromatic rings, the A-, B- and C- rings; the A- and C- ring are fused together and are joined to the B-ring by means of a carbon-carbon bond. Anthocyanins differ according to the degree of hydroxylation and methylation of the B-ring as well as the type and position of glycosylation and acylation (Cabrita *et al.*, 2000). Over 500 anthocyanins and 17 anthocyanidins are known, among these 17 anthocyanidins only 6 are common in food: pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Pereira *et al.*, 2009). The 6 main anthocyanidins are depicted in Figure 1-2. Glycosylation most commonly occurs with the conjugation of either: glucose, galactose, rhamnose, arabinose and xylose; with anthocyanins frequently being found in nature as 3-monosides, 3-biosides, 3-triosides and 3,5-diglucosides. Cyanidin-3-glucoside (C3G) is the most abundant anthocyanin in Western diets and cyanidin accounts for ~50% of anthocyanidins in fruit and vegetables (Castañeda-Ovando *et al.*, 2009).
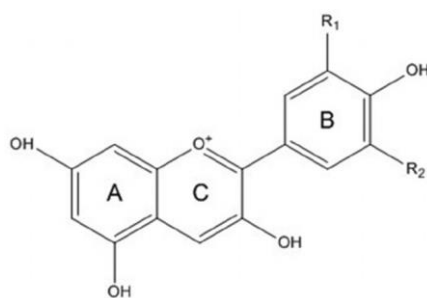
Anthocyanidin	R1	R2	Distribution
Pelargonidin	H	H	12%
Cyanidin	OH	H	50%
Delphinidin	OH	OH	12%
Peonidin	OCH_3	H	12%
Petunidin	OCH_3	OH	7%
Malvidin	OCH_3	OCH_3	7%

Figure 1-2- The chemical structure of the 6 main anthocyanidins found in nature and their approximate distribution according to Castaneda-Ovando et al, 2009.
The pigmentation of each compound is largely dictated by their degree of hydroxylation and methylation and is depicted here by the colour of the text.

1.3.2 Dietary Anthocyanins

Anthocyanins are naturally abundant components of our diets and are most frequently consumed in red-purple fruits and vegetables. The anthocyanin content of such foods is highly variable depending on a range of factors such as: environmental conditions, ripeness, processing and storage (Tanaka *et al.*, 2008). Elderberries can contain 200 – 1816 mg/100g anthocyanins, whilst bilberry can contain 300 – 698 mg/100g and strawberries can contain 13-36 mg/100g fresh weight (Prior and Wu; Sandhu *et al.*, 2016; Wu *et al.*, 2006). Furthermore, it is not only the total anthocyanin content that varies between sources, but also the type of anthocyanin. For example, black raspberry is a particularly rich source of cyanidin type anthocyanins (669 mg/100g), whilst blackcurrant is high in delphinidin type anthocyanins (333 mg/100g), and blueberries are a plentiful source of petunidin and malvidin type anthocyanins (87.6 and 154.6 mg/100g respectively) (Wu *et al.*, 2006).

An individual's daily intake of dietary anthocyanins is highly variable and dependent on a range of factors such as cultural dietary habits, season and availability of produce. Studies have shown that in Europe average anthocyanin intake varies from 18.4 - 64.9 mg/day, with most of this intake coming from consumption of fruit. Furthermore, within Europe Italy tends to be among the highest consumers of anthocyanins, likely due to their largely Mediterranean diet comprising many fruits and vegetables as well as red wine (Cassidy, 2018; Wu *et al.*, 2006). Contrasting with this, anthocyanin consumption in the USA was estimated to be only 12.5 mg/day based on the concentration and food intake data from NHANES 2001 -2002. However, since then there has been criticism that studies such as this have largely underestimated anthocyanin intake due to their reliance on questionnaires and dietary recall, and actually anthocyanin intake is likely to lie anywhere between 3 – 215 mg/day for US citizens (Wu *et al.*, 2006).

1.3.3 Health Effects of Anthocyanins

Many *in vitro*, animal and human studies have evaluated the biological and pharmacological potential of anthocyanins and have reported that they possess many bioactive properties. These include the capacity to counteract oxidative stress, to act as antimicrobial substances, and to counteract the onset and progression of numerous diseases such as neurodegenerative, cardiovascular, metabolic diseases and cancer (Khoo *et al.*, 2017; Mattioli *et al.*, 2020).

In vitro studies have highlighted several potential mechanisms of action for the health effects of anthocyanins. For example, anthocyanin rich blackberry extract has been shown to have anti-proliferative effects on colon cancer, breast cancer, lung cancer and leukaemia cells. Whilst anthocyanin rich blueberry extract has been reported as anti-inflammatory through its inhibition of NF-kB in Caco-2 cells (Taverniti *et al.*, 2014). Other reports have suggested that anthocyanins may protect against cardiovascular disease through improving endothelial function through the

inhibition of mitochondria-mediated apoptotic signalling pathway in human umbilical vein endothelial cells and bovine aortic endothelial cells as well as the activation of cAMP-PKA-eNOS signaling pathways in human aortic endothelial cells (J *et al.*, 2011; Liu *et al.*, 2014; Zapolska-Downar *et al.*, 2008). There is also evidence for anthocyanin metabolites exerting beneficial effects in *in vitro* studies. For example, gallic acid, 3-O-methylgallic acid, and PGA, have been reported to decrease cell viability and cause cell cycle arrest and apoptosis in colon cancer caco-2 cells (Forester *et al.*, 2014).

There is a vast amount of animal studies that have linked anthocyanin consumption to protective health effects. C3G has been observed to suppress the zymosan-induced inflammatory response in rats by reducing the level of iNOS, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 (Tsuda *et al.*, 2002). Additionally, bilberry anthocyanin extract exerted neuroprotective effects by reducing glial scar formation, axonal loss, inflammation and promoting remyelination and neuron survival in a rat model of spinal cord injury (Wang *et al.*, 2012). Bilberry anthocyanins have also been reported to modulate the expression of aortic genes related to increased inter-cellular adhesion, decreased monocyte recruitment, cellular contractility and vascular permeability thus decreasing the risk of endothelial dysfunction, which is an early marker of the development of atherosclerosis in apoE$^{-/-}$ mice (Mauray *et al.*, 2012). Additionally, the anthocyanin metabolite PCA has been reported to inhibit the development of oesophageal cancer in rats (Peiffer *et al.*, 2014).

Several epidemiological studies have reported beneficial effects associated with anthocyanin consumption. A study that followed 43 380 healthy men for 24 years reported that anthocyanin intake was inversely related with non-fatal myocardial infarction (Cassidy *et al.*, 2016). A similar study reported this same effect on women aged between 25 – 42 years (Cassidy *et al.*, 2013). Meanwhile, other epidemiological studies have reported that anthocyanin consumption is associated with improved arterial stiffness, reduced blood pressure and reduced blood insulin concentration (Cassidy *et al.*, 2011; Jennings *et al.*, 2012, 2014). However, some epidemiological studies report no association between anthocyanin consumption and health (Cassidy *et al.*, 2012; Mursu *et al.*, 2008).

Many dietary intervention studies have investigated the effects of anthocyanin consumption on biomarkers of health. A recent study reported that consumption of a high anthocyanin blueberry drink for over 28 days significantly reduced 24 hour ambulatory systolic blood pressure (Rodriguez-Mateos *et al.*, 2019). Several studies have linked anthocyanin consumption to anti-oxidant effects, for example, in one study where healthy male volunteers consumed an anthocyanin-rich juice for 8 weeks, the activity of superoxide dismutase was significantly increased (Bakuradze *et al.,* 2019). Marniemi et al have shown that daily consumption of a portion of 100 g deep-frozen berries (bilberries, lingonberries, or blackcurrants) for 8 weeks increased serum ascorbate concentrations

and produced a slight decrease in LDL diene conjugation along with a slight increase in serum antioxidant capacity (Marniemi *et al.,* 2000). Further to this it has recently been shown that daily consumption of blackcurrant anthocyanins for 5 weeks increased protective anti-oxidant/anti-inflammatory cellular events that promote exercise recovery (Hurst *et al.*, 2020). It has also been reported that blueberry juice consumption improved endothelial function in obese and overweight participants (Curtis *et al.*, 2019).

Although there is lots of evidence for anthocyanins being beneficial to health, anthocyanins are unstable and extensively metabolised *in vivo*. Therefore, any health effect of anthocyanins is hypothesised to be mediated by their metabolites *in vivo* rather than the parent compound (De Ferrars, Czank, Zhang, *et al.*, 2014a). The remainder of this chapter discusses anthocyanin stability and metabolism.

1.3.4 Anthocyanin Stability

Anthocyanins are very unstable and susceptible to degradation; however, it should be noted that the glycosylated anthocyanin is considerably more stable than the anthocyanidin with further stability being conferred by acylation. The colour and stability of anthocyanins is effected by many factors including, but not limited to: pH, concentration, temperature, light, oxygen and proteins (Cabrita *et al.*, 2000; Castañeda-Ovando *et al.*, 2009; Kay *et al.*, 2009; Woodward *et al.*, 2009).

Anthocyanins are extremely sensitive to changes in pH and will undergo conformational changes in response to the pH they are subjected to, which also results in pigmentation changes. At a highly acidic pH (~pH 1) the flavylium cation is the predominant anthocyanin form, which holds an intense red colour. As pH increases to between 4 and 6, proton transfer may occur to give rise to the quinonoidal base which has a bluer colour. Or alternatively in this same pH range, hydration may occur causing the anthocyanin to take the form of the colourless carbinol base. As pH becomes more alkali (between 7 and 8) the quinonoidal base may receive a proton to become ionised and take on a purple colour. Alternatively, if in the form of a carbinol base this may undergo tautomerization to take the form of the chalcone base which is a colourless open C ring form of the anthocyanin which often acts as a precursor to full degradation (Cabrita *et al.*, 2000; Castañeda-Ovando *et al.*, 2009; Oren-Shamir, 2009; Remini *et al.*, 2018).

Temperature has a large baring on anthocyanin stability and has a well-documented inverse relationship. However, sugar conjugation allows anthocyanins to better withstand increases in temperature than anthocyanidins (Castañeda-Ovando *et al.*, 2009). Furthermore, oxygen has been repeatedly shown to have deleterious effect on anthocyanin stability. One of the key characteristics of anthocyanins is their ability to absorb visible light and emit a red-purple pigmentation as a result (Cabrita *et al.*, 2000). However, anthocyanins are also susceptible to light-induced degradation, however, this is also largely affected by concentration and molecular oxygen. Building on this,

anthocyanins are more stable at high concentrations due to their ability to associate with themselves, and concentration has been shown to be more important in conferring stability than structural differences between anthocyanins (Remini *et al.*, 2018).

Upon exposure to neutral pH, high temperatures or enzymes (β-glucosidase), anthocyanins will degrade into their phenolic acid and phloroglucinaldehyde (PGA) components. Due to variations in the B-ring of the 6 main anthocyanins, the phenolic acid degradant is different in each case, illustrated in Figure 3. However, the A-ring of the 6 main anthocyanins is constant and therefore PGA is a degradant of all 6 compounds. There is some discrepancy in the literature regarding whether anthocyanins spontaneously degrade to give just their phenolic acid and PGA constituents or if indeed further reactions take place to give rise to more compounds, this is discussed in more detail in Chapter 6 (Goszcz *et al.*, 2017; Kay *et al.*, 2009; Keppler and Humpf, 2005; Woodward *et al.*, 2009).

Anthocyanidin	Phenolic acid	R1	R2
Pelargonidin	4-Hydroxybenzoic acid	H	H
Cyanidin	PCA	OH	H
Delphinidin	Gallic acid	OH	OH
Peonidin	Vanillic acid	OCH_3	H
Petunidin	3-O-Methylgallic acid	OCH_3	OH
Malvidin	Syringic acid	OCH_3	OCH_3
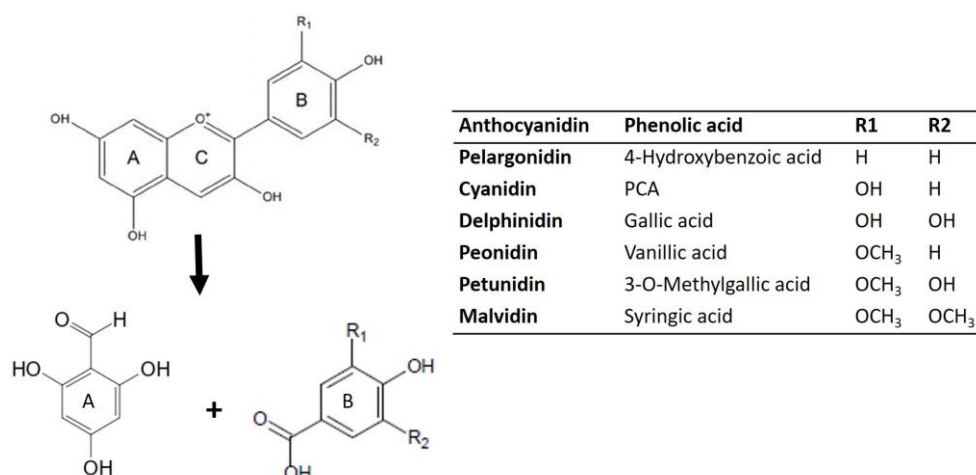
Figure 1-3- Degradation of the 6 main anthocyanins into their phenolic acid and phloroglucinaldehyde constituents.

1.3.5 Anthocyanin Bioavailability

For any bioactive compound to exert an effect *in vivo* it must be bioavailable. Bioavailability refers to the amount of the compound that is absorbed and reaches circulation intact so that it can act on target sites; however, some alternative definitions include both intact and metabolised forms of the bioactive compound. Intact anthocyanins have particularly poor bioavailability and are generally only present in circulation at nanomolar concentrations. Studies have shown that anthocyanins are rapidly absorbed and reach maximum concentrations in plasma between just 30 minutes and 4 hours in humans depending on the contents of the stomach and precise compounds

consumed (De Ferrars, Czank, Zhang, *et al.*, 2014a; Jokioja *et al.*, 2021; Nurmi *et al.*, 2009). Furthermore, recovery of anthocyanins in urine and faeces has also been consistently low in reports, highlighting the very poor bioavailability of these compounds (Czank *et al.*, 2013; Felgines *et al.*, 2003; De Ferrars, Czank, Zhang, *et al.*, 2014a; Jokioja *et al.*, 2021).

Due to their unstable nature and poor recovery in biological samples it is widely hypothesised that anthocyanins are present in circulation as chemical degradants, intermediates and metabolites. This idea has been reinforced by studies where labelled anthocyanins have been fed and metabolites have been present at a 42-fold level higher than the parent compound in biological samples. This indicates that whilst the parent compounds are poorly bioavailable, they are metabolised *in vivo* to give rise to a diverse range of metabolites with higher bioavailability and potential bioactivity (Czank *et al.*, 2013; De Ferrars, Czank, Zhang, *et al.*, 2014a). Nevertheless, it is likely that plasma and urine may provide an inaccurate assessment of anthocyanin bioavailability due to the rapid removal of anthocyanins from circulation and/or the requisition of anthocyanins by tissues in the body. Furthermore, the large range of anthocyanins present in foods and the tendency for anthocyanins to be converted to another anthocyanin through methylation or dehydroxylation adds further complexities to establishing the true bioavailability of anthocyanins (Crozier *et al.*, 2010). Nevertheless, reports have clearly shown that anthocyanins are subjected to extensive metabolism *in vivo*. It remains a major challenge to understand the range of compounds that may arise from this process and to determine both their bioavailability and any bioactivity that may explain the many health effects that have been linked to anthocyanin consumption (De Ferrars, Czank, Zhang, *et al.*, 2014a).

1.4   Anthocyanin Metabolism

For anthocyanins to exert any effect on health sufficient levels of the bioactive compound must be present in circulation for a sufficient length of time, even if the bioactive compound(s) are not the parent compound itself (Del Rio *et al.*, 2013). Although considerable work has investigated the metabolism of anthocyanins, large gaps in our knowledge remain regarding the spectrum of compounds that may be obtained. After ingestion, anthocyanins transit through the GI tract and enter the stomach, followed by the small intestine and colon where they can undergo extensive metabolism (Ichiyanagi *et al.*, 2006; Nurmi *et al.*, 2009). This initial metabolic process may be followed by absorption and phase 1 and 2 metabolism within sites such as the liver and kidneys before being excreted through urinary (kidney) and biliary (liver) routes (Fang, 2014).

1.4.1   Anthocyanin Metabolism in Saliva

Upon consumption anthocyanins enter the oral cavity where they are mixed with saliva. Some studies have suggested that anthocyanin metabolism begins in the oral cavity and is mediated by

salivary enzymes. One human study has shown that in saliva samples from healthy participants collected after black raspberry rinses, several metabolites of black raspberry anthocyanins were detected including their aglycone, glucuronidated conjugates and low levels of PCA. Furthermore, through enzyme assays they were able to show the role of β-glucosidase activity in these biotransformation's, mediated by both bacterial and oral epithelial cells (Mallery *et al.*, 2011). Building on this, another study incubated a number of different anthocyanin-rich extracts with human saliva *ex vivo* and showed that all anthocyanins were partially degraded in saliva. They found that delphinidin and petunidin glycosides were more susceptible to salivary degradation than cyanidin, peonidin, pelargonidin or malvidin glycosides. Rather interestingly, they found that neither PCA or PGA were primary degradation products of cyanidin glycosides or the aglycone in saliva, but they did preliminary detect chalcone glycosides. Furthermore, they showed that anthocyanin degradation was significantly reduced after rinsing with antibacterial chlorhexidine, indicating that the oral microbiota is critical to the first stages of anthocyanin metabolism in the oral cavity (Kamonpatana *et al.*, 2014).

1.4.2 Anthocyanin Metabolism in the Stomach

In the stomach, anthocyanins are met by highly acidic conditions that are favourable for their stability (Khoo *et al.*, 2017; McDougall *et al.*, 2005). Anthocyanin absorption is likely to begin in the stomach and allow anthocyanins to reach circulation, this idea has been reinforced by the rapid appearance of anthocyanins in plasma after ingestion (De Ferrars, Czank, Zhang, *et al.*, 2014a; Talavéra *et al.*, 2003). Anthocyanin absorption from the stomach has been investigated in animal studies. When grape anthocyanins were directly injected into the stomach of rats, after just 6 minutes M3G was detected in both portal and systemic plasma, but neither aglycones or conjugated derivatives were present indicating that the acidic conditions preserve the stability of anthocyanins and they are not rapidly or extensively metabolised at this site (Passamonti *et al.*, 2003). Further to this, it has been shown that when bilberry and black rice anthocyanin extracts are infused into the stomachs of rats, approximately 25% anthocyanin mono-glycosides (including glucoside and galactoside) were absorbed from the stomach, although the rutinoside was poorly absorbed. Interestingly, in bile several unknown anthocyanin metabolites at levels too low to be quantified were also detected, suggesting that some anthocyanin metabolism does occur in the stomach (Talavéra *et al.*, 2003). Additionally, using *in situ* models in rats Felgines et al showed that ~20% red orange anthocyanins were absorbed from the stomach (Felgines *et al.*, 2006).

It has been suggested that anthocyanin absorption in the stomach is mediated by the bilitranslocase transporter (an anion carrier) which is found in the gastric mucosa (Passamonti *et al.*, 2003). However, absorption of nutrients in the stomach is rare, and anthocyanins reside as cations in the stomach. Therefore, it cannot be excluded that the rapid appearance of anthocyanins in circulation post-consumption is simply due to absorption in the small intestine, especially when taking into

account that many studies require volunteers to be fasted (Fang, 2014). Furthermore, although several studies in rats have reported anthocyanin absorption in the stomach, these studies have been performed under simulated laboratory conditions that would not occur in nature. For example, by administering high doses of anthocyanins to the sealed stomach of anaesthetised rats, or through injection, and therefore we cannot be certain how applicable these findings are to human anthocyanin metabolism when anthocyanins are consumed as one component of a wider diet (Passamonti *et al.*, 2003; Talavéra *et al.*, 2003).

1.4.3   Anthocyanin Metabolism in the Small Intestine

Anthocyanins reach the small intestine after the stomach, which is the major site for absorption of intact anthocyanins (Riaz *et al.*, 2016). Absorption of anthocyanins in the small intestine has been estimated using rat models. One study, which used an *in situ* perfusion method found that absorption rate of anthocyanins ranged from 10.7 – 22.4% for M3G and C3G respectively (Talavéra *et al.*, 2004). This high estimation is not consistent with the low levels of anthocyanins reported in a circulation in both animal and human studies (Crozier *et al.*, 2010; De Ferrars, Czank, Zhang, *et al.*, 2014a; Matsumoto *et al.*, 2006). However, a limitation of this study is that it calculated the absorption rate based on the disappearance of the effluent, and consequently this may have only indicated that some of the anthocyanin was taken up into the tissue of the small intestine, but does not necessarily mean that this was then transported to circulation. Further to this, another study showed that although up to 7.5% black raspberry anthocyanins could be taken up by the tissue of the small intestines, only very small amounts were found in urine (He *et al.*, 2009).

In humans, absorption of anthocyanins in the small intestine has been investigated using ileostomy volunteers. One study fed 300 g raspberries to ileostomy patients and obtained an average recovery of 40% of the ingested dose within the ileal fluid. However, recovery of Pel3G was substantially higher (75%) than C3G (6%), highlighting the impact of additional hydroxylation of the B-ring on anthocyanin stability *in vivo* (González-Barrio *et al.*, 2010). This adverse effect of hydroxylation and methylation of the B-ring on anthocyanin stability was reinforced by Kahle *et al* who found that for ileostomy volunteers fed 300 g blueberries anthocyanins with little B-ring hydroxylation/methylation and conjugated to complex sugar moieties were more likely to remain intact until they reach the large intestine (Kahle *et al.*, 2006). However, in the case of anthocyanins such as C3G both of these studies indicate that a high level of degradation/metabolism or absorption takes place before transit to the large intestine. Notably both of these studies only measured disappearance of the anthocyanin and therefore its fate is unknown (González-Barrio *et al.*, 2010; Kahle *et al.*, 2006). Furthermore, studies using ileostomy patients have several limitations. For example, ileostomy patients frequently have increased gut permeability, lower urine production and the final portion of the small intestine often tends to develop its own microflora.

Therefore, it may be questionable as to how findings in these study populations may relate to small intestine anthocyanin absorption in healthy individuals (Hubbard *et al.*, 2017).

Transit from the stomach to the small intestine involves transition to a more neutral pH which is known to adversely affect the stability of anthocyanins (Castañeda-Ovando *et al.*, 2009). A small number of studies have investigated the degradation of anthocyanins in the small intestine. Using ileostomy patients, one study found that after feeding bilberry extract, gallic acid, PCA and PGA were the main anthocyanin degradants present in ileostomy fluid, whilst 3-O-methylgallic acid, syringic acid and vanillic acid were minor components. Interestingly, these products correspond to the known spontaneous breakdown products of the anthocyanins that constitute the bilberry extract fed. This suggests that the neutral pH of the small intestine kick-starts the spontaneous degradation of anthocyanins, giving rise to chemical degradants before they reach the large intestine (Mueller *et al.*, 2017). In addition to these pH dependent transformations anthocyanins may be hydrolysed by several enzymes in the small intestine. For example, cleavage of the sugar moiety to leave the aglycone can occur through the action of lactase phloridzin hydrolase, notably this deglycosylation makes the aglycone more lipophilic and facilitates absorption into the epithelium through passive diffusion (Rodriguez-Mateos *et al.*, 2014). Furthermore, due to the early appearance of 4-hydroxyhippuric acid and ferulic acid derivatives in plasma after raspberry consumption Ludwig *et al* suggested that these metabolites may be formed in and absorbed from the upper GI tract without any involvement of the gut microbiota on their metabolism (Ludwig *et al.*, 2015). Due to the invasiveness of studies that investigate metabolism in the small intestine little is known regarding its precise role in anthocyanin metabolism, particularly in healthy individuals, however, this would be an important point of future work to understand the compounds generated here and that reach the colon where they can be microbially metabolised to a wide range of compounds (Kastl *et al.*, 2020).

1.4.4 Colonic Anthocyanin Metabolism

Anthocyanins that are not absorbed or metabolised in the stomach or small intestine reach the large intestine where they are exposed to a large and diverse community of microorganisms collectively known as the gut microbiota (Aura *et al.*, 2005; Faria *et al.*, 2014; Igwe *et al.*, 2019; Williamson and Clifford, 2010). The relationship between anthocyanins and the gut microbiota is discussed in more detail later in this chapter, but a summary of the colonic metabolism of anthocyanins is given here.

Several *ex vivo* faecal fermentation studies have been performed using both faecal samples derived from humans and animals to investigate colonic anthocyanin metabolism. Several studies have shown that anthocyanins can be deglycosylated in the colon and then further metabolised to PGA and their corresponding B-ring degradation products at quantities much larger than observed when

faecal samples were heat-inactivated. This highlights that the colon is an important site for anthocyanin metabolism and many of the important products observed in biological samples are likely produced by the gut microbiota (Aura *et al.*, 2005; Fleschhut *et al.*, 2006; Keppler and Humpf, 2005).

Several studies investigating colonic metabolism of anthocyanins have reported that the gut microbiota is able to metabolise anthocyanins beyond their known A- and B-ring constituents, giving rise to a range of compounds. This suggests that the colon is perhaps the primary site of anthocyanin metabolism and is may be the reason why we see a large structurally diverse range of metabolites in biological samples. For example, pyrogallol and 4-hydroxybenzoic acid have been shown to be colonic metabolites of raspberry anthocyanins in *ex vivo* human faecal incubations (González-Barrio *et al.*, 2011). Interestingly, no study investigating the colonic metabolism of anthocyanins has been able to account for the total fed dose in recoveries of the parent compound and/or metabolites. Therefore, it is highly likely that further unknown intermediates are involved in the metabolic process and/or unknown metabolites are produced that may have biological relevance.

1.4.5   Phase 2 Anthocyanin Metabolism

Phase 2 metabolism refers to the conjugation of a compound to another molecule, such as a glucuronide or sulfate mediated through human transferase enzymes. Before entering portal circulation from the small intestine anthocyanins and/or their metabolites may be subject to phase 2 metabolism mediated by uridine-5'-diphospho-glucuronosyltransferase (UPDG) and sulfotransferase enzymes amongst others, that are present in the epithelial cells lining the GI tract (Crozier *et al.*, 2010). Once anthocyanins and/or their metabolites have passed into portal circulation they are often subjected to further phase 2 metabolism in the liver, and notably once they enter systemic circulation they may be metabolised further within body tissues (Riaz *et al.*, 2016).

After anthocyanin consumption in humans' phase 2 conjugates of both the parent compound and its metabolites have been detected in biological samples. A study that fed 200 g strawberries found several phase 2 anthocyanin conjugates in urine, including three mono-glucuronides and one sulfate conjugate (Felgines *et al.*, 2003). Animal studies have also shown that phase 2 metabolism is important in anthocyanin metabolism, with glucuronides of cyanidin and peonidin being detected in urine and plasma after rats consumed blackberry anthocyanins (Talavéra *et al.*, 2004). Furthermore, many studies have shown that phase 2 conjugates of phenolic metabolites are abundant after anthocyanin consumption. For example, using caco-2 cells Kay et al found that PCA was metabolised to glucuronide and sulfate conjugates *in vitro* (Kay *et al.*, 2009). *In vivo* tracer studies have shown that the C3G metabolite PCA was more abundant in its sulfate and glucuronide

conjugated forms than the parent metabolite, whilst phase 2 conjugates of vanillic acid and benzoic acid were also reported (Czank *et al.*, 2013; De Ferrars, Czank, Zhang, *et al.*, 2014a). Reinforcing the importance of phase 2 metabolism *in vivo*, phase 2 conjugates of gallic acid, PCA and isoferulic acid were detected in urine in a recent study where healthy males consumed purple potato anthocyanins (Jokioja *et al.*, 2021).

Despite the importance of phase 2 conjugates in anthocyanin metabolism, as major metabolites that reach circulation they have been overlooked by many studies. One of the reasons for this is that in most cases authentic standards are not available for phase 2 conjugates which means verifying their identity can be particularly difficult in analysis of complex biological samples. To validate methods for identification of phase 2 conjugates in-house synthesis of authentic standards is often required, however, this is often expensive, and the resources required to do this are not always available (De Ferrars, Czank, Saha, *et al.*, 2014c). Furthermore, anthocyanin metabolites are most commonly studied using LC-MS/MS, often in MRM mode to reliably quantify small molecules, and instrumentation does not have an infinite capacity for MRM transitions that may be included in one analysis method. Therefore, a refined list of analytes is often selected for analysis to maximise sensitivity, and due to the large number of phase 2 conjugates they are often left out of such methods. Nevertheless, phase 2 conjugates have been proven to comprise a major component of anthocyanin metabolism *in vivo* and expanding studies to include these compounds is important to further our understanding of the metabolite profile derived from dietary anthocyanins and the compounds that may have bioactivity (De Ferrars, Czank, Zhang, *et al.*, 2014a; Jokioja *et al.*, 2021; Kay *et al.*, 2009).

1.4.6 Anthocyanin Distribution and Excretion

Following their consumption, absorption and metabolism anthocyanins are eliminated through both the urinary and biliary routes, however, some studies have shown that a small amount of anthocyanins can also be eliminated through breath (Czank *et al.*, 2013; Felgines *et al.*, 2003; De Ferrars, Czank, Zhang, *et al.*, 2014a; Ichiyanagi *et al.*, 2006). Compounds excreted through bile often tend to be large and lipophilic, consequently phase 2 conjugates most likely enter enterohepatic circulation, where they can be modified, secreted back into the small intestine and potentially reabsorbed. Conversely, small polar metabolites tend to be excreted through urine (Fang, 2014; Ichiyanagi *et al.*, 2006; Jakobsdottir *et al.*, 2013; Talavéra *et al.*, 2003).

Several studies have measured anthocyanins and their metabolites in biological samples to aid our understanding of the complex metabolite profiles that arise from their consumption *in vivo* with the ultimate aim of understanding which compounds are responsible for any bioactivity. A limited number of ^{13}C tracer studies have been employed to facilitate our understanding of how anthocyanins and their metabolites are distributed and their primary routes of excretion following

consumption. These studies are particularly valuable, as although anthocyanins are only present in red-purple pigmented plant foods that are easy to identify, their metabolites are found in a wide array of foods and beverages and consequently pinpointing the compounds derived from anthocyanins can be difficult, particularly when they are only produced at a low level. However, administering a labelled compound allows metabolites to be identified with certainty regarding their origin. One study, which fed 500 mg ^{13}C labelled C3G reported a total recovery of ~43% in urine, breath and faeces with 0.18% accounted for in blood, 5.37% in urine, 6.91% in breath and 32.13% in faeces. Notably, there was very large inter-individual variation in recovery between participants ranging from 15.1 – 99.3%, highlighting that anthocyanin metabolism and excretion is not a uniform process. In serum, C3G reached its peak serum concentration in the first 2 hours after consumption, whereas metabolites peaked between 6 and 24 hours post-consumption. In urine, C3G peaked at 2 hours post-consumption, and its metabolites peaked at 6-24 hours after consumption. In faeces, total metabolites reached their maximum concentration at 24 hours after consumption, but some compounds such as phase 2 conjugates of PCA did not peak until 48 hours (Czank *et al.*, 2013). Of the metabolites identified, hippuric acid was found to be a major metabolite in both urine and serum, whilst vanillic acid and its phase 2 conjugates were also major metabolites in serum and urine, but were only detected in a subset of participants. Crucially, differences were seen in the metabolites reported in different routes of excretion, for example, methyl-vanillate was only detected in faeces suggesting that di-methylation of PCA results either from biliary elimination or as a product of microbial metabolism in the large intestine. Additionally, caffeic acid was abundant in faeces but absent from plasma and urine suggesting that the catechol group of caffeic acid is rapidly methylated to yield ferulic acid, either during intestinal absorption, prior to entry into the systemic circulation or by the liver. Furthermore, only a limited number of phase 1 PCA metabolites were reported, suggesting that phase 1 metabolism may not be highly utilised in anthocyanin metabolism. The route of elimination of A-ring derived metabolites is still poorly understood with only PGA, 2-hydroxy-4-methoxybenzoic acid and ferulic acid detected as A-ring metabolites in this tracer study (De Ferrars, Czank, Zhang, *et al.*, 2014a).

Studies in which unlabelled anthocyanins were administered are more abundant in the existing literature and show that anthocyanins and their metabolites are excreted at different time intervals after anthocyanin consumption. For example, a recent study that fed purple potatoes, rich in anthocyanins, to healthy men showed that ferulic acid, 4-hydroxybenzoic acid, isovanillic acid, 2-methoxybenzoic acid, PGA, phloroglucinol and vanillic acid were increased in the first 4 hours after anthocyanin consumption. Whereas caffeic acid, catechol, chlorogenic acid, p-coumaric acid, gallic acid, protocatechualdehyde, PCA, PCA-4-glucuronide, PCA 3-sulfate and sinapic acid were elevated at 4 – 8 hours after anthocyanin consumption, whilst hippuric acid and 4-hydroxyphenylacetic acid were elevated between 8 – 12 hours after anthocyanin consumption (Jokioja *et al.*, 2021). This

highlights that anthocyanin metabolism takes place over a long period and multiple reactions take place generating a structurally diverse metabolite profile.

Understanding the tissue distribution of anthocyanins and their metabolites is critical to evaluate any health effects of eating anthocyanins. Although many *in vitro* studies have investigated the effects of anthocyanins and their metabolites on several tissue based diseases using cell lines, these systems do not always translate their effects *in vivo* and a criticism of several studies is the use of doses that are not physiologically relevant. Although there are some *in vivo* studies on this they are extremely limited. In male rats fed blackberry extract containing 370 nmol anthocyanin/day for 15 days and sacrificed 3 hours after their last meal, total anthocyanins were 605 nmol g^{-1} in jejunum, 68.6 nmol g^{-1} in stomach, 3.27 nmol g^{-1} in kidney, 0.38 nmol nmol g^{-1} in liver and 0.25 nmol g^{-1} in brain (Talavera *et al.*, 2005). In pigs fed diets supplemented with blueberries for 4 weeks and fasted before sacrifice, 1.30 pmol g of anthocyanins were reported in the liver, 1.58 pmol g^{-1} in eyes, 0.878 pmol g^{-1} in cortex, and 0.664 pmol g^{-1} in cerebellum, suggesting that dietary anthocyanins can cross the blood-brain and blood-retinal barrier (Kalt *et al.*, 2008). Given the overwhelming evidence that anthocyanins are extensively metabolised *in vivo* and that these metabolites are likely to exert bioactivity it would be interesting to investigate tissue distribution in animal models fed labelled anthocyanins. This would facilitate investigation of tissue distribution of metabolites as well as the parent compound to improve our understanding of how eating anthocyanins can exert any health effect.

1.5 Microbial Metabolism of Polyphenols

The gut microbiota is critical to the metabolism of many polyphenols, and several unique microbial metabolites are derived from this process. For example, the conversion of daidzein to equol, and ellagitannins to urolithins are exclusively microbial (Kawabata *et al.*, 2019).

Equol has been endorsed as having numerous beneficial effects on human health, including prevention of cardiovascular and neurodegenerative diseases. The conversion of the isoflavone daidzein to equol takes place in the intestine, mediated by reductase enzymes that belong to incompletely characterised members of the gut microbiota. Although all animal species studied to this point produce equol *in vivo*, only a fraction of human subjects are able to, ostensibly those that possess equol producing microbes. These subjects tend to be the ones that experience the most health benefits from isoflavone consumption. This highlights that the microbiota of an individual is critical in the metabolism of flavonoids and the health effects of consuming flavonoids at the individual level (Mayo *et al.*, 2019).

Ellagitannins are associated with several health benefits and are found in foods such as walnuts and pomegranate. However, much like anthocyanins they have low bioavailability. *In vivo* ellagitannins

are transformed by intestinal bacteria to urolithins which are more efficiently absorbed. Urolithins have specifically been implicated as anti-inflammatory, cardioprotective and anti-carcinogenic compounds (Muku *et al.*, 2018). However, the capacity of individuals to produce urolithins varies substantially, and this variability has been attributed to dissimilarities in the intestinal gut microbiota. Some individuals are only capable of producing specific urolithins, whilst some individuals do not have the capacity to produce urolithins at all. These differences in the metabolising phenotype of individuals to metabolise ellagitannins to urolithins have been termed metabotypes, with the metabotype of an individual dictating the metabolite signature produced and consequently the health benefits associated with ellagitannin consumption (Selma *et al.*, 2017). Three major ellagitannin metabotypes (A, B and 0) have been described. Metabotype A is characterised by the production of urolithin A and its related conjugates. The production of urolithin B and/or isourolithin A, in addition to urolithin A production, is the main biomarker that characterises metabotype B, while metabotype 0 is characterised by the lack of production of any of the aforementioned urolithins.   Evidently, such inter-individual variability in urolithin metabotypes is associated with differences in the gut microbial composition. For example, the presence of *Gordonibacter* is associated with urolithin A production *in vivo* (García-Mantrana *et al.*, 2019). Higher levels of *Gordonibacter* were found in individuals with metabotype A than in those with metabotype B. Interestingly, metabotype B has been reported to be dominant in subjects with colorectal cancer and metabolic syndrome. This pattern suggests a correlation between metabotype B and gut dysbiosis, and hence suggests negative health effects, however, further research required in this area (Sallam *et al.*, 2021).

1.6   Metabotypes

Metabotypes are groups of individuals defined on the basis of their similarities in metabolic profile, which results from an interaction between lifestyle, gut microbiome, genes and environmental factors. Metabotypes have been successfully associated with diet-related diseases and differential responses to dietary nutrients, this has supported the use of metabotyping to deliver personalised nutrition advice (Hillesheim *et al.*, 2020).

The success of the metabotype approach in determining how someone may respond to a particular diet is partly due to the fact that metabotypes are influenced by a combination of genetic and environmental factors such as use of vitamins/supplements and the gut microflora and therefore encompasses a range of important biological processes. Several studies have linked the specific levels of metabolites in urine and faecal water with the gut microbiota, highlighting that the microbiome composition is critical in assigning metabotypes to individuals (Claesson *et al.*, 2012; Nicholson *et al.*, 2012; Wijeyesekera *et al.*, 2012). In recent years, the immense role of the gut microbiota in metabolic diseases has been highlighted. The impact of the gut microbiota on human

health could partly be mediated through the ability of the gut microbiota to metabolise dietary compounds into new metabolites that impact disease risk. For example, the bacterial metabolism of dietary L-carnitine into the metabolites TMA and TMAO presents a mechanistic link of the relationship between red meat consumption and cardiovascular disease risk (Janeiro *et al.*, 2018). Interestingly, diet–microbiota interactions have been shown to vary amongst individuals. Moreover, even when a diet seemingly induces more systematic effects on the phenotype, a large part of the residual variability is associated with the microbiota in terms of composition and functionality. Therefore, understanding the role of the gut microbiota in the metabolism of dietary nutrients is likely to be critical in determining metabotypes that can be used to inform personalised nutrition to maximise health benefits from the diet (Palmnäs *et al.*, 2020). Although several reports have shown that anthocyanin metabolism is variable among individuals and in recent years' research has begun to explore the role of the gut microbiota in this metabolism, work in this area is still in its infancy. Furthermore, no current reports have established metabotypes relating to anthocyanin metabolism.

1.7 Anthocyanins and the Gut Microbiota

Studies on human and animal anthocyanin metabolism have consistently shown that anthocyanin metabolism is subject to high levels of inter-individual variation (Czank *et al.*, 2013; Felgines *et al.*, 2006; De Ferrars, Czank, Zhang, *et al.*, 2014a; Jokioja *et al.*, 2021; Nurmi *et al.*, 2009). One of the many factors thought to contribute to this is the gut microbiota. The gut microbiota is considered a metabolic organ and contributes to the metabolism of anthocyanins that reach the gut intact as well as its degradants and metabolites (Eker *et al.*, 2019). Biotransformation of anthocyanins and their metabolites by the gut microbiota is thought to be key in the absorption and biological activity of several anthocyanin metabolites. However, there is also evidence for anthocyanins being able to modulate microbial populations of the gut microbiota by exerting pre-biotic effects (Faria *et al.*, 2014). Dysbiosis of the microbiota has been linked to several pathologies, and its modulation is a possible mechanism considered regarding how anthocyanins and/or their metabolites exert their beneficial effects. However, research on the possible effects of anthocyanins on the gut microbiota is in its infancy. Consequently, conclusions and generalisations cannot be made because of the limited evidence base and varied techniques employed in the existing literature (Igwe *et al.*, 2019).

1.7.1 The Gut Microbiota

The gut microbiota comprises a vast collection of microorganisms that have colonised the GI tract including: bacteria, archaea, viruses, fungi and eukarya. This microbial community has co-evolved with the host to form an intricate and symbiotic relationship (Bäckhed *et al.*, 2005). Several estimates have been made regarding the ratio of microorganisms that inhabit the gut microbiota. Old estimates suggest that the gut microbiota has 10 times more bacterial cells than the number of

human cells of the host, however, more recent estimates suggest that the ratio of human to bacterial cells is closer to 1:1 (Bäckhed *et al.*, 2005; Sender *et al.*, 2016). The gut microbiota has a central role in health. It metabolises dietary nutrients (including polyphenols, fatty acids and glucose), drugs, and synthesises vitamins and bioactive molecules. It also has the capacity to ferment non-digestible materials such as dietary fibres; this fermentation supports the production of short-chain fatty acids that are known to have beneficial effects on health. The gut microbiota also contributes to intestinal barrier function and has co-evolved with the immune system to prevent the colonisation of pathogenic microorganisms (Jandhyala *et al.*, 2015; Valdes *et al.*, 2018). However, dysbiosis of the gut microbiota has been linked to several pathologies including, Chrohn's' disease, ulcerative colitis, and type 2 diabetes amongst others (Durack and Lynch, 2019).

The human gut microbiota develops from birth, although a narrow selection of publications suggest that this may begin *in utero* due to the detection of microbes in uterine structures such as the placenta (Aagaard *et al.*, 2014). Interestingly, depending on the mode of delivery the gut microbiota of infants is substantially different. Vaginally delivered infants have a high relative abundance of *Lactobacilli* in their first weeks of life, reflecting the high presence of *Lactobacilli* in the vaginal microbiota. Conversely, the microbiota of infants delivered by C-section exhibits delayed colonisation, but becomes colonised by facultative anaerobes such as *Clostridium* (Salminen *et al.*, 2004). By around 2 – 3 years of age the composition and diversity of the gut microbiota resembles that of an adult. Although throughout life the gut microbiota is reasonably stable, it is heavily influenced by a range of factors such as: dietary habits, geographical location, age, medication, genetics, and health conditions. The diverse gastrointestinal microbiota is predominantly composed of bacteria from three major phyla, namely *Firmicutes, Bacteroidetes*, and *Actinobacteria*. Suggestions have been made of the presence of a 'core microbiota', proposed to be a set of the same abundant organisms present in all individuals. However, more similarity can be observed in the repertoire of microbial genes present between individuals than the taxonomic profile, suggesting that the core microbiota may be better defined at a functional rather than organism level (Thursby and Juge, 2017). In elderly individuals ≥ 65 years of age, studies have shown that the microbiota shifts to an increased relative abundance of *Bacteroidetes* and *Clostridium* (cluster IV) compared to younger individuals (Claesson *et al.*, 2011). However, another study showed that the gut microbiota profiles of subjects ≥ 70 years of age and younger participants were comparable, although subjects ≥ 100 years of age had significantly less diversity in their gut microbiota. Furthermore, in elderly populations a relationship has been shown between gut microbial diversity and living arrangements, such as residential care or living independently (Claesson *et al.*, 2012). Additionally, compared to younger people, elderly populations tend to have a shift in their microbiota profile that has reduced capacity for metabolic processes such as short-chain fatty acid production and amylolysis, but increased deleterious proteolysis with the likelihood

to produce N-nitroso compounds, and heterocyclic amines such as p-cresol, highlighting the structural and functional changes undertaken by the gut microbiota throughout life (Biagi *et al.*, 2013; Thursby and Juge, 2017; Winter *et al.*, 2011).

Until recent decades our knowledge of the gut microbiota has been constrained by reliance on labour-intensive culture based methods and limitations of these techniques such as non-cultivable microbes of interest, and lack of population-scale data. However, advances in sequencing has allowed researchers to develop culture-independent methods that can provide both a broader and deeper view into the microbial communities present (Kho and Lal, 2018). 16S rRNA sequencing is a popular technique due to this gene being ubiquitous between all known bacteria and archaea and comprising nine hyper-variable regions which allow different organisms to be distinguished at the genus level. Although species level differentiation can be achieved with 16S sequencing this is much less reliable and is generally not recommended (Durazzi *et al.*, 2021). More reliable estimates of microbiota composition and diversity may be provided by whole-genome shotgun metagenomics due to the higher resolution and sensitivity of these techniques. Whole-genome shotgun metagenomics also has the advantage of sequencing to a high enough resolution to provide metabolic reconstruction, to facilitate maximal capture of organismal and functional data of the microbiota (Thursby and Juge, 2017). The vast majority of studies investigating the composition of the gut microbiota have used faecal samples which are easy to obtain. However, rectal mucosal biopsies have been proposed as a better alternative in terms of gaining an accurate assessment of the gut microbiota profile (Igwe *et al.*, 2019). A comparative study between faecal samples and rectal mucosal biopsies showed a significant difference in bacterial diversity between samples derived from the same participant (Durbán *et al.*, 2010). It is likely that differences exist in the microbial communities of the six major subdivisions of the human colon (caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum). However, the extent of this remains unclear. One study observed significant inter-individual variability and differences between faecal and mucosa community composition (Eckburg *et al.*, 2005a). This discrepancy highlights the importance of sampling sites and suggests that rectal mucosal biopsy samples are more appropriate than faecal samples and should be used instead, or together with faecal samples for the most representative analysis. Nevertheless, faecal samples are still favoured over rectal mucosa biopsy in the vast majority of studies as a result of their ease of collection and being less invasive for participants (Igwe *et al.*, 2019).

1.7.2   The Effect of Anthocyanins on the Gut Microbiota

A proportion of ingested anthocyanins reach the gut intact, along with their chemical degradants and some of their metabolites. Studies on the effects of these compounds on the composition of the gut microbiota are limited, but there is some evidence that anthocyanin consumption has a pre-

biotic effect, with anthocyanins promoting the proliferation of beneficial anaerobes and inhibiting the growth of pathogenic species.

In vitro fermentation studies have shown that M3G can promote the growth of beneficial *Bifidobacterium* and *Lactobacillus* species in human faecal slurries (Hidalgo *et al.*, 2012). However, animal studies have shown mixed evidence regarding the ability of anthocyanins to modulate the gut microbiota. In a study where mice were supplemented with different sources of anthocyanins in addition to either a high or low fat diet, neither blackberry or black raspberry anthocyanins were able to alter the gut microbiota. However, concord grape anthocyanins were able to increase *Actinobacterial* species. Whilst blueberry and blackcurrant anthocyanins were able to increase both *Bacteroidetes* and *Actinobacteria* species (Overall *et al.*, 2017). Furthermore, a study in which rats were fed blueberries showed significant reductions in *Lactobacillus* and *Enterococcus,* alongside increases in *Bifidobacteriaceae* and *Coriobacteriaceae* (Lacombe *et al.*, 2013). Some human studies have also suggested that anthocyanins are able to modulate the gut microbiota. In a study where healthy males received red wine, dealcoholized red wine or gin for 20 days, those that had both types of red wine exhibited increased faecal concentrations of *Bifidobacterium, Enterococcus* and *Eggerthellalenta.* Interestingly, the lowest to the highest changes in *Bifidobacteria* tertiles were associated with a higher excretion of four phenolic metabolites related to anthocyanin metabolism in participants (Boto-Ordóñez *et al.*, 2014). However, some studies report an absence of effects of anthocyanins on gut microbial populations (Flores *et al.*, 2015; Zhang *et al.*, 2016).

Across *in vitro*, animal and human studies, increases in beneficial *Bifidobacterium* and *Lactobacillus* species has been repeatedly shown (Boto-Ordóñez *et al.*, 2014; Hidalgo *et al.*, 2012; Lacombe *et al.*, 2013). These species have been shown to exert beneficial effects in the treatment of diarrhoea, including inflammatory bowel disease, and colorectal cancer (Igwe *et al.*, 2019). However, how anthocyanins may exert these effects is not understood. Research on the gut microbiota and characterisation of the bacteria species present in the gut is ongoing, and our understanding of whether anthocyanins can truly affect these populations is limited. Anthocyanins are not consumed in isolation and are ingested as part of a more complex food which is generally a small part of a much broader diet. Drawing conclusions from the existing literature is difficult due to the differences in anthocyanin sources, metabolism and populations between studies. The complete effect and exact mode of action of anthocyanins on gut microbiota populations needs much more clarification, and this might be achieved partly by conducting more well-designed human clinical trials, reaching a consensus on anthocyanin dose and form, as well as by a uniform approach to control diets to account for inter-individual variability that may affect the results obtained.

1.7.3 The Effect of the Gut Microbiota on Anthocyanin Metabolism

The gut microbiota is recognised as a metabolic organ and is known to have an important role in the metabolism of dietary nutrients, including anthocyanins. The metabolism of anthocyanins is subject to high levels of inter-individual variation and given that considerable amounts of anthocyanins and their degradants reach the lower gut intact it has been postulated that this variability can largely be attributed to differences in the gut microbiota of individuals. Studies that directly investigate gut microbiota mediated anthocyanin metabolism are scarce and have mainly been performed using *in vitro* fermentation models with human or animal faeces.

Early *in vitro* studies of gut microbial anthocyanin metabolism have shown that gut microbes are involved in the cleavage of glycosidic linkages through β-glycosidase activity and the breakdown of the anthocyanin heterocycle (Aura *et al.*, 2005). Although anthocyanin stability is affected by many factors and at a neutral pH at least some degradation will occur spontaneously. However, it has been repeatedly shown that several metabolites are produced either at higher levels or exclusively in the presence of a live gut microbiota. These include, but are not limited to: PCA, PGA, 3-O-methylgallic acid, pyrogallol, p-coumaric acid and gallic acid (Forester and Waterhouse, 2008; González-Barrio *et al.*, 2011; Hidalgo *et al.*, 2012). The microbial metabolism of anthocyanins is discussed in more detail in Chapter 4. So far studies that have been conducted in this area have not delved into the specific microbial species that are responsible for different biotransformation's of anthocyanins and phenolics. Nor has there been any focus on how microbial metabolism of anthocyanins takes different paths in different individuals. Furthermore, a limitation in understanding the microbial metabolism of anthocyanins is that many of the enzymes involved in these chemical conversions have not been characterised (Eker *et al.*, 2019).

1.8 Thesis Aims

In light of the literature summarised in this chapter there are several elements of anthocyanin metabolism and the involvement of the gut microbiota in this process that need to be investigated. Firstly, although there have been several studies on anthocyanin metabolism many of these have either focused on the parent compounds or metabolites and few have looked at both in detail. Furthermore, studies have often combined analysis of anthocyanins and their metabolites in one analytical method, and given the diversity of physiochemical properties of these compounds this compromises some sensitivity and limits the number of compounds that can be included (De Ferrars, Czank, Saha, *et al.*, 2014a; Nurmi *et al.*, 2009). In addition to this, previous studies have often overlooked phase 2 conjugates due to lack of authentic standards and/or limits in the capacity of targeted analysis methods. Therefore, it is necessary to extend analysis methods to include a wide range of parent compounds, metabolites and phase 2 conjugates to further our understanding of how anthocyanins are metabolised *in vivo*, as without this knowledge investigation into the

bioactivity of anthocyanin rich diets is limited (Kay *et al.*, 2005; Woodward *et al.*, 2011). Furthermore, although many reports have acknowledged that the gut microbiota is likely to be a key player in anthocyanin metabolism *in vivo* few have investigated its specific role (Eker *et al.*, 2019; Faria *et al.*, 2014; Igwe *et al.*, 2019). Although some reports have studied the relationship between anthocyanins and the gut microbiota, no study has done this in the context of a large human study and compared *in vivo* data to *in vitro* microbial metabolism. The current literature contains discrepancies regarding whether anthocyanins are able to exert a pre-biotic effect in the gut microbiota and the precise role of the gut microbiota in anthocyanin metabolism and the compounds that are derived from this process (Ávila *et al.*, 2009; Boto-Ordóñez *et al.*, 2014; Flores *et al.*, 2015; Hidalgo *et al.*, 2012; Igwe *et al.*, 2019; Zhang *et al.*, 2016). To further knowledge in these areas and progress from the current literature the overall aims and objectives of the work described in this thesis are outlined below:

Overall Aim

To report the anthocyanins and their metabolites produced *in vivo* from two different dietary sources of anthocyanin (black rice extract, mainly cyanidin-type anthocyanins; and bilberry extract, mainly delphinidin-type anthocyanins), and investigate any bidirectional relationship between anthocyanin metabolism and the gut microbiota.

Overall Hypothesis

Anthocyanin metabolites produced *in vivo* will differ according to the source of dietary anthocyanins (black rice or bilberry extract), and based on previous studies, will also display high inter-individual variation. It is hypothesised that several metabolites will be produced by the gut microbiota and that variability in metabolism can be explained, at least in part, by differences in the microbiome.

To meet this aim and test the proposed hypothesis the following objectives were set.

1. **Develop methods to quantify a wide range of anthocyanins and their metabolites (including phase 2 conjugates) in biological samples.**

Existing published methods for the analysis of anthocyanins and their metabolites in biological samples have been limited in terms of the spectrum of compounds they have included. Therefore, in this thesis two methods were developed for the study of anthocyanins and their metabolites separately in human samples, going beyond the current literature by extending the number of known and predicted metabolites (encompassing phase 2 conjugates) included. Chapter 2 describes the method development process and how this method goes beyond the state-of-the art.

2. **Quantify anthocyanins and their metabolites produced *in vivo* from human urine and plasma samples.**

To enhance our understanding of *in vivo* human anthocyanin metabolism, anthocyanins from two different sources (black rice extract, mainly cyanidin-type anthocyanins; and bilberry extract, mainly delphinidin-type anthocyanins), were fed to participants over a 28-day intervention. 24-hour urine collections and plasma samples were taken pre- and post-intervention and analysed using the method developed in Chapter 2, to understand the differences in metabolites derived from these two different types of anthocyanin, and to what extent this was effected by inter-individual variation. Chapter 3 describes the quantification of a structurally diverse range of anthocyanins and metabolites in urine and plasma derived from bilberry and black rice anthocyanins.

3. **Investigate the role of the gut microbiota in anthocyanin metabolism**

It is important to understand the role of the gut microbiota in anthocyanin metabolism and the metabolites that are microbially derived. To investigate this, faecal slurries from a subset of volunteers were incubated with black rice extract in *in vitro* fermentation colon models to measure the microbial metabolism of black rice anthocyanins. Unlike previous studies in this field, metabolite concentrations measured during *in vitro* fermentation could be compared with urinary levels after a dietary intervention to advance our understanding of how findings regarding the microbial metabolism of anthocyanins *in vitro* translates *in vivo*. Furthermore, having samples from several individuals facilitated investigation of how microbial metabolism of anthocyanins may differ between individuals. The microbial metabolism of anthocyanins is investigated in Chapter 4.

4. **Explore any bi-directional relationship between gut microbiota profiles and anthocyanin consumption/metabolism.**

Literature on the effect of anthocyanins on the gut microbiota, and the influence of microbiota profiles on anthocyanin metabolism is scarce and findings are conflicting regarding whether effects are apparent. For a subset of participants from the human intervention study described in Chapter 3, faecal samples were taken pre- and post-intervention. These samples were profiled using both 16S and whole-genome shotgun metagenomics. Effects of anthocyanins on microbial profiles and associations between anthocyanin metabolism and features of the gut microbiota are explored in Chapter 5.

5. **Investigate spontaneous anthocyanin degradation *in vitro*.**

Anthocyanin stability is adversely affected by a wide range of factors, and under physiological conditions anthocyanins degrade without the involvement of host enzymes or microbes. Therefore, it is important to understand how anthocyanins degrade under physiological conditions as knowledge of how this process takes place and the degradants that form is likely to impact how

anthocyanins are metabolised *in vivo*. The spontaneous degradation of anthocyanins is explored in Chapter 6.

Chapter 2

Detection of Anthocyanins and their Metabolites in Urine and Plasma

Chapter 2 : Detection of Anthocyanins and their Metabolites in Urine and Plasma

2.1 Abstract

Background: Anthocyanin rich diets have been linked to several health benefits. However, the bioavailability of intact anthocyanins is very low and it is known they are chemically unstable. Therefore, it is hypothesised that any protective effects of eating anthocyanins can be attributed to their metabolites rather than the parent compound (Kay *et al.*, 2009). Despite this, large gaps remain in the literature regarding the detailed metabolic fate of anthocyanins encompassing their microbial metabolites and phase 2 conjugates (De Ferrars, Czank, Zhang, *et al.*, 2014a).

Objective: The aim of the present work was to establish a comprehensive method for the extraction and analysis of anthocyanins and their metabolites including phase 2 conjugates and predicted compounds in urine and plasma.

Approach: A previously reported analytical method from De Ferrars *et al* was used as a basis for method development. This method was adapted and extended to include high-throughput analysis of a large number of anthocyanins and metabolites, expanding on those reported previously (De Ferrars, Czank, Saha, *et al.*, 2014c). UHPLC-MS/MS was employed to provide reliable quantification for a broad spectrum of anthocyanins and their metabolites, whilst solid phase extraction (SPE) was used to extract and concentrate anthocyanins and their metabolites in urine and plasma samples. The impact of different SPE sorbents, LC columns and solvents were explored to produce an optimised method with maximal recovery, sensitivity and accuracy.

Results: The final SPE method provided an average recovery of 98.4 ± 18.1% for urine, and 89.4 ± 27.4% for plasma across the range of anthocyanins and phenolics. Unlike several other published methods, the method developed in this chapter provided chromatographic resolution of positional

sulfate isomers. The final method consisted of separate UHPLC-MS/MS gradients and solvents for analysis of anthocyanins and of their metabolites to provide optimal sensitivity for analysis of both sets of compounds and maximise the number of metabolites that could be analysed including phase 2 conjugates.

Conclusion and Future Work: The final method is considered suitable for the analysis of clinical samples derived from a human intervention study where a large number of compounds, including novel anthocyanin metabolites, need to be quantified. The methods described in this chapter provide versatility for the quantification of an extensive range of anthocyanins and their metabolites that may be used to study absorption, distribution and metabolic fate of anthocyanins from a variety of sources in future clinical studies.

2.2 Introduction

An anthocyanin rich diet is associated with numerous health benefits. Reported activities include but are not limited to cardioprotective, anti-diabetic, anti-carcinogenic, anti-microbial and anti-inflammatory effects (Khoo *et al.*, 2017). Anthocyanins are extremely unstable with a wide range of factors causing them to degrade such as: pH, temperature, light, oxygen, metal ions, and enzymes (Remini *et al.*, 2018). It has been hypothesised that any biological effect of an anthocyanin rich diet is likely attributed to the spontaneous and microbial metabolites produced *in vivo*, rather than a direct effect of the parent compound (Kay, 2006; Kay *et al.*, 2009; Keppler and Humpf, 2005). Due to their susceptibility to degradation, anthocyanins are rapidly metabolised when consumed and thus the bioavailability of the parent compound is extremely low (Kay *et al.*, 2009). Therefore, it is important to understand the metabolites produced *in vivo* from an anthocyanin rich diet. Understanding the profiles of metabolites generated, and how these may differ between individuals is likely to reveal key information on which compounds may have bioactivity, and may inform dietary information in the future.

Whilst there have been some excellent and robust studies of anthocyanin metabolism, such as those reported by De Ferrars *et al*. and Nurmi *et al*. (De Ferrars, Czank, Zhang, *et al.*, 2014a; Nurmi *et al.*, 2009), it has been suggested that some anthocyanin metabolites have probably been overlooked in some studies because of the limitations of conventional methods targeting anthocyanins. Furthermore, work by Kay *et al*. has shown that anthocyanins share metabolic characteristics with other flavonoids, whereby they are subject to extensive methylation and glucuronidation (Kay, 2006). To date, much remains to be understood regarding the detailed metabolism of anthocyanins in humans, taking into account spontaneous, microbial, and human derived metabolites. This may be partly attributed to differences in doses used in clinical studies, and how saturated the metabolic pathways become. Furthermore, inadequate extraction and identification protocols have hindered anthocyanin metabolism research as this limits

quantification of metabolites, a particular problem when many metabolites are likely to only be present at extremely low levels, pushing the detection limits of instrumentation, and ultimately hindering our understanding of anthocyanin absorption and bioactivity (Murkovic *et al.*, 2000). These factors contribute to not only the conflicting findings within the literature regarding anthocyanin metabolism and bioactivity, but also the many unanswered questions that remain (Felgines *et al.*, 2003).

Extraction of anthocyanins and their metabolites is most commonly performed using solvent extraction, with anthocyanins being polar compounds, the most common solvents used are acidified methanol or ethanol, with it being reported that use of methanol is 20% more effective than ethanol (Metivier *et al.*, 1980). Crucially, according to extraction methods reported in the literature, to successfully extract acylated anthocyanins and 3,5-diglucosides it is necessary to use weak acid solvents to avoid their hydrolysis. A wide variety of techniques may be used for anthocyanin extraction including: solid phase extraction (SPE), solvent extraction, liquid-liquid extraction (LLE), and countercurrent chromatography (CCC) (Castañeda-Ovando *et al.*, 2009). In 2014, a report by De Ferrars *et al* (2014) identified solid-phase extraction (SPE) as the optimum method to extract anthocyanins and an extensive range of phenolic metabolites. They explored several methods but found that SPE gave superior recovery and consistency, with an added benefit of SPE being that it removes salts that may affect ionisation during LC-MS, thus improving the ability to detect low level compounds. They tested a range of SPE cartridges and found that reversed-phase DSC-18 cartridges with a polymerically bonded endcapped sorbent (for extraction of structurally diverse analytes) were optimal for extraction of anthocyanins and metabolites from urine. Meanwhile, reversed-phase Strata-X cartridges with a functionalised polymeric sorbent utilising three mechanisms of retention (pi-pi bonding, hydrogen bonding and hydrophobic interactions) were superior for extraction of these compounds from serum (De Ferrars, Czank, Saha, *et al.*, 2014c).

The unique spectral characteristics of anthocyanins (i.e. their red-purple pigmentation resulting in high absorbance at 460 - 550 nm on the light spectrum) provides valuable quantitative and qualitative information, and due to its low cost and simplicity UV-visible spectroscopy is the main technique used to quantify anthocyanins (Wrolstad *et al.*, 2005). However, there can be considerable difficulty in obtaining reference compounds particularly when studying uncommon anthocyanins, and spectral similarities between anthocyanins make it difficult to distinguish between compounds in complex mixtures, particularly within a complicated food matrix. Furthermore, UV-visible spectroscopy is not optimal for detecting the phase 2 and microbial metabolites of anthocyanins, which has led to mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy becoming the preferred methods of identification of anthocyanins and metabolites. NMR is particularly useful for gaining structural information on anthocyanins and

their metabolites, providing information on the position of glucuronide and sulfate conjugations. However, NMR is only able to detect compounds present at a relatively high concentration which can be particularly difficult in a complex matrix, and is therefore not usually applied to biological samples. Due to the limitations in sensitivity of NMR, LC-MS/MS is the preferred method to identify anthocyanins and their metabolites, with a combination of diode-array detection (DAD) and multiple-reaction-monitoring (MRM) being applied to identify compounds in complex samples. MRM is particularly useful when identifying structurally similar compounds, where specific transitions and retention times are integral to accurately identifying metabolites, many of which have small masses which are not always unique within the metabolite profile (Castañeda-Ovando *et al.*, 2009).

The vast majority of studies that have used LC-MS/MS for anthocyanin analysis have utilised reverse phase HPLC with C18 packing materials. However, more recent reports, such as the De Ferrars *et al* (2014) penta[13C]-C3G tracer study, have used newer Kinetex PFP phases which incorporate fluorine atoms on the periphery of a phenyl ring which enables chromatographic separation via dipole-dipole interactions, hydrogen bonding and pi-pi interactions, in addition to the hydrophobic and shape selectivity of more traditional C18 columns. Despite this, many studies have been unable to efficiently separate isomers of phase 2 conjugates, with sulfates presenting a particular problem (De Ferrars, Czank, Saha, *et al.*, 2014c). Since then, manufacturers have further refined PFP phases and have produced F5 phases which are able to provide superior chromatographic separation of positional isomers. Development of HSS T3 columns utilizes a trifunctional C18 alkyl phase which enhances retention of polar compounds, with the low-ligand density enabling analytes to access the pore structure balancing the retention of polar and hydrophobic molecules.

One of the many barriers faced when developing extraction and analysis methods for anthocyanins and their metabolites is the diversity of metabolites, with differences in structure and physiochemical properties making extraction and quantification in biological matrices complicated. Consequently, many published methods have focused on the analysis of parent forms of metabolites or phase 2 conjugates with only limited reports showing extensive analysis of both (De Ferrars, Czank, Saha, *et al.*, 2014c). This is partly due to the vast majority of phase 2 conjugates being unavailable commercially for use as reference compounds, limiting the ability to develop a suitable method without bespoke in-house synthesis. Due to the different properties of anthocyanins and their metabolites it may be recommended that multiple methods are used for optimal quantification of anthocyanins and their metabolic by-products. For example, highly acidic solvents improve the chromatographic resolution and stability of anthocyanins but have deleterious effect on a subset of other phenolic metabolites. Therefore, it can be difficult to develop a method that can provide optimal chromatography and sensitivity for both anthocyanins and phenolics concurrently, and it may be more appropriate to optimise LC-MS/MS conditions for both

sets of compounds separately to increase limits of detection. The work in this chapter describes the development and partial validation of protocols for the extraction and quantification of an extensive range of anthocyanins, phenolic metabolites and phase 2 conjugates, specifically developed for the analysis of clinical samples.

2.3 Objectives

The overall aim of the work presented in this chapter was to develop one or more methods appropriate for the analysis of an extensive range of anthocyanins and their phenolic metabolites, including compounds for which there are commercial standards, and others where there are not, such as, predicted metabolites and phase 2 conjugates. The specific objectives were:

- Develop an LC-MS method to accurately detect and quantify phenolic compounds.
- Develop an LC-MS method to accurately detect and quantify anthocyanins.
- Develop an extraction method to 'clean-up' and concentrate human urine samples, where anthocyanins and their phenolic metabolites are recovered efficiently and consistently, for subsequent LC-MS/MS analysis.
- Develop an extraction method to 'clean-up' and concentrate human plasma samples, where anthocyanins and their phenolic metabolites are recovered efficiently and consistently, for subsequent LC-MS/MS analysis.

2.4 Methods

2.4.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Dorset, United Kingdom) unless stated otherwise. All water used was 18 MΩ/cm Milli-Q water, and solvents were LC/MS grade.

Anthocyanin standards used during method development were: cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, delphinidin-3-O-glucoside, delphinidin-3-O-rutinoside, pelargonidin-3-O-glucoside, pelargonidin-3-O-rutinoside, and malvidin-3-O-glucoside (Extrasynthese, Genay, France).

Internal standards and phenolic standards used during method development were: caffeine-(trimethyl-d9) (99 atom-% D, later D9-caffeine), 4-hydroxybenzaldehyde, 3-O-methyl-gallate, methyl vanillate, phloroglucinol, phloroglucinaldehyde, pyrogallol, protocatechualdehyde, taxifolin, caffeic acid, chlorogenic acid, catechin, epicatechin, quercetin, scopoletin, phloridzin, ferulic acid, gallic acid, hippuric acid, homoprotocatechuic acid, homovanillic acid, rutin, 4-hydroxybenzoic acid, 5-hydroxyferulic acid, 4-hydroxyphenylacetic acid, isovanillic acid, p-coumaric acid, protocatechuic acid, sinapic acid, syringic acid, trans-cinnamic acid, and vanillic acid (Sigma-Aldrich, Dorset, United Kingdom); gallic acid-3-O-glucuronide, gallic acid-4-O-glucuronide, quercetin-3-O-sulfate, isoferulic acid-3-O-glucuronide, protocatechuic acid-3-O-glucuronide,

protocatechuic acid-4-O-glucuronide, protocatechuic acid-3-O-sulfate, and protocatechuic acid-4-O-sulfate were kindly synthesized by Dr Paul Needs at The Quadram Institute Bioscience, Norwich, UK.

2.4.2   LC-MS/MS Detection of Phenolic Compounds in Urine and Plasma

A mobile phase (A) of 10 mM ammonium acetate in water, adjusted to pH 5 with acetic acid was used with a mobile phase (B) of 10 mM ammonium acetate in LC-MS grade acetonitrile (Merck, Dorset, United Kingdom) adjusted to pH 5 with acetic acid. The gradient used was as follows: 1% B between 0 and 1 minute, 5% B at 3 minutes, 60% B at 8 minutes, 99% B at 8.5 minutes, 1% B at 9 minutes and 1% B at 12 minutes. Samples were kept at 4°C in an auto-sampler and injected at a volume of 5 µl for urine and 1 µl for plasma onto an ACQUITY UPLC HSS T3 1.8 µm (2.1 x 100 mm) column equipped with an ACQUITY UPLC HSS T3 VanGuard pre-column (100Å, 1.8 µm, 2.1 mm i.d. x 5 mm) (Waters, Wilmslow, United Kingdom) at 37°C, with flow rate kept at 0.4 ml/min. The attached mass spectrometer was operated in negative mode for all compounds except phosphatidyl choline and D9-caffeine (volume control) on Agilent's 6490 Triple Quad LC-MS/MS system (Agilent Technologies, Santa Clara, CA, USA). The MRM parameters used for compound detection of all compounds included in the analysis method can be found in appendix 1, standards included in method development are given in section 2.4.1. Collision gas temperature was 220 °C, gas flow 14 L h^{-1}, nebulizer gas pressure was 25 psi, and capillary voltage 3500 V. Collision energies were optimized for each compound available.

Note, three other columns were tested during method development: a PFP column (100 x 2.1, 1.7µ) by Phenomenex (PFP), a C18 column by Phenomenex (100 x 2.1, 2.6µ) (C18) and an F5 column by Phenomenex (100 x 2.1, 2.6µ) (F5) (Phenomenex, Macclesfield, United Kingdom). Solvents of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) were also tested, along with two other gradients. The gradient reported by De Ferrars at al (De Ferrars, Czank, Saha, *et al.*, 2014c) and a 14-minute gradient as follows: 1% B at 0 minutes, 1% B at 1 minute, 4% B at 4 minutes, 50% B at 10 minutes, 95% B at 11 minutes, 1% B at 11.1 minutes and 1% B at 14 minutes. Method development began on an Agilent 1200 series HPLC-DAD, attached to an ABSciex 4000 series Qtrap MS/MS, before a transition was made to the Agilent 6490 Triple Quad LC-MS/MS system.

2.4.3   LC-MS/MS Detection of Anthocyanins in Urine and Plasma

Samples were kept at 4°C in an auto-sampler and injected at a volume of 5µl for urine and 1 µl for plasma onto an ACQUITY UPLC HSS T3 1.8 um (2.1 x 100 mm) which was fitted with an ACQUITY UPLC HSS T3 VanGuard Pre-column (100Å, 1.8 µm, 2.1 mm X 5 mm (Waters,Wilmslow, United Kingdom)), with the column temperature kept at 37°C. Mobile phases (A) of 5% formic acid in water and (B) of 5% formic acid in acetonitrile (Merck, Dorset, United Kingdom) were used with the following gradient: 5% B between 0 - 1 minutes, 10% B at 5 minutes, 25% B at 30 minutes, 95% B

at 31 minutes, 95% B at 32 minutes, 5% B at 32.1 minutes and 5% B at 36 minutes. Flow rate was constant at 0.4 ml/min. Anthocyanins were detected in positive mode on Agilent's 6490 Triple Quad UHPLC-MS/MS system (Agilent Technologies, Santa Clara, CA, USA) and MRM transitions for all compounds included can be found in appendix 1. Collision gas temperature was 220 °C, gas flow 14 L min−1, nebulizer pressure 25 psi, capillary voltage 3.5 kV and collision energy was 5 eV for phosphatidyl choline and anthocyanidins, and 28 eV for anthocyanins. Note phosphatidyl choline was included to check the success of SPE in removing impurities from urine and plasma.

2.4.4 Solid Phase Extraction (SPE) of Urine

Several modifications of SPE protocols were performed during method development, with methods being adapted from that reported by De Ferrars *et al* (De Ferrars, Czank, Saha, *et al.*, 2014c). Micro-elution was conducted using both a 3 mg HLB micro-elution plate (Waters, Wilmslow, United Kingdom), and a 2 mg Strata-X micro-elution plate (Phenomenex, Macclesfield, United Kingdom). Pre-conditioning was carried out with 200 µl methanol containing 0.25% formic acid (although this step was not required for Waters micro-elution plates). Urine was loaded at a volume of 200 µl (although load volumes 100 – 500 µl were tested) and was diluted 1:1 with water containing 0.1% formic acid. Two separate washes were performed with 2% methanol in water, adjusted to pH 2.4 with formic acid. Elution of compounds from the sorbent was achieved by applying two separate 25 µl volumes of 0.25% formic acid in methanol, with the sorbent 'soaked' for 10 minutes before vacuum elution each time. Post-elution samples were reconstituted 1:1 with water (0.1% formic acid), and spiked with a volume control (phloridzin or D9-caffeine) at 2 µg/ml before being subjected to LC-MS/MS as described above.

SPE cartridges were trialled in the following formats: Strata-X reverse-phase sorbent 500mg 6ml catridges (Phenomenex, Macclesfield, United Kingdom), DSC-18 1 g 6 ml cartridges, and HLB-prime 30 mg 1 ml cartridges (Waters, Wilmslow, United Kingdom). Strata-X and DSC-18 cartridges were pre-conditioned with 6 ml methanol (0.25% formic acid), then 1ml urine spiked with 2 µg/ml internal standard (scopoletin or taxifolin) and diluted with a further 1 ml water (0.1% formic acid) was loaded. Cartridges were washed with two separate fill volumes of 2% methanol in water (adjusted to pH 2.4 with formic acid). Elution was then performed with 7 ml methanol (0.25% formic acid) with a 10 minute 'soak' prior to vacuum elution. Eluted samples were then subjected to evaporation in a Speedvac centrifugal evaporator to a volume of ~200 µl, reconstituted with 200 µl water (0.1% formic acid) and spiked with a volume marker (phloridzin or D9-caffeine) at a concentration of 2 µg/ml. HLB cartridges were treated in the same way but without pre-conditioning and with smaller fill volumes of 1 ml. A Strata-X 30 mg SPE 96-well plate (Phenomenex, Macclesfield, United Kingdom) was also tested. This was done the same way as described for SPE cartridges, however, fill volumes were 1 ml rather than 6 ml, and samples were eluted with 2 ml

methanol (0.25% formic acid). Samples were then evaporated to ~200 µl with a nitrogen drier rather than centrifugal evaporation.

Note that urine used during method development was sourced from human volunteers that took part in the human study described in Chapter 3. Volunteers were asked to restrict their dietary anthocyanin intake and collect all urine passed in a 24 hour period. For method development, urine from volunteers was pooled

2.4.5   SPE of Plasma

Two methods of plasma 'clean-up' were trialled during method development.

A 'plasma-crash' where 1 ml plasma was mixed with 2 ml methanol and shaken for 5 minutes before being left to stand for 15 minutes at 4°C. Samples were then centrifuged at 10 000 xg for 5 minutes. The supernatant was removed and put through an HLB SPE cartridge to remove any remaining proteins, lipids and salts, without compounds being held in the sorbent due to the methanol matrix. Samples were then subjected to centrifugal evaporation until a volume of ~200 µl was reached. Evaporated samples were then reconstituted with 200 µl water (0.1% formic acid), and a volume marker of 2 µg/ml phloridzin or D9-caffeine was added. Samples were then subjected to LC-MS/MS as described previously.

SPE with Strata-X reverse-phase sorbent 500 mg 6 ml (Phenomenex, Macclesfield, United Kingdom), was done in the same way as described for urine previously. However, wash steps were performed with water (0.1% formic acid). Strata-X were pre-conditioned with 6 ml methanol (0.25% formic acid), then 1ml plasma spiked with 2 µg/ml internal standard (scopoletin or taxifolin) and diluted with a further 1 ml water (0.1% formic acid) was loaded. Cartridges were washed with two separate fill volumes of water (0.1% formic acid). Elution was then performed with 7 ml methanol (0.25% formic acid) with a 10 minute 'soak' prior to vacuum elution. Eluted samples were then subjected to evaporation in a Speedvac centrifugal evaporator to a volume of ~200 µl, reconstituted with 200 µl water (0.1% formic acid) and spiked with a volume marker (phloridzin or D9-caffeine) at a concentration of 2 µg/ml.

2.4.6   SPE Recoveries in Urine and Plasma

Samples of acidified urine and plasma (1% final concentration of hydrochloric acid (HCl)) were spiked with standards to give a concentration of 2 µg/ml for each compound in the final sample and subjected to SPE as described above. Pre-spike samples were spiked with standards prior to SPE whilst post-spike samples were spiked with standards post - SPE and evaporation steps. Internal standards scopoletin or taxifolin (pre-spike process control) and phloridzin or D9-caffeine (post-spike volume control) were also added to be at a final concentration of 2 µg/ml.

Peak areas for each compound in every sample were obtained using Agilent's Masshunter Quantitative Analysis software B.06.00 (Agilent Technologies, Santa Clara, CA, USA). Matrix matched standard curves were plotted for each compound (listed in the chemicals and reagents section) and were used to find the response factor for individual compounds, the concentration of each compound was then calculated using formula 1.

$$R = \frac{A_x}{A_{IS}} \div \frac{C_x}{C_{IS}}$$

Formula 1- Calculation of concentrations following UHPLC-MS/MS
Peak area values obtained by UHPLC-MS/MS were subjected to this formula to obtain concentration values for each compound in biological samples. R, response factor; A_x, peak area of the compound of interest; A_{IS}, peak area of the internal standard; C_x, concentration of the compound of interest; C_{IS}, concentration of the internal standard.

Recoveries of each compound were calculated as a percentage following concentration determination according to formula 1, and were normalised according to the volume control to account for differences in concentration due to variation in the evaporation step.

2.5   Results

2.5.1   LC-MS/MS detection of Phenolic Compounds in Urine

Before developing a method for extraction of phenolic metabolites from urine and plasma, it was vital to establish a high sensitivity method of analytical detection and quantification. Due to the vast number of compounds that were intended to be searched for, as well as the low concentrations expected, LC-MS/MS in MRM mode was the method chosen. Using MRM facilitated selective detection of compounds, based on specific ion transitions, to minimise the likelihood of obtaining false positive results. In the publication from De Ferrars *et al*, which extensively investigated extraction and detection methods of anthocyanins and metabolites, an LC-MS/MS method was described in detail which was used as the starting point of this analysis. This method reported use of a Kinetex pentafluorophenol (PFP) reverse phase (RP) (2.6 µm, 100 × 4.6 mm, 100 Å) column, on a 37-minute gradient, with solvents of 0.1% formic acid in water and acetonitrile for the mobile phases A and B respectively on an Agilent 1200 series HPLC-DAD, attached to an ABSciex 3200 series Qtrap MS/MS. Initially, to establish whether compounds could be reliably detected, this method was applied to an ABSciex 4000 series Qtrap to analyse a mix of authentic standards in a matrix of water (0.1% formic acid). The standards run initially were: pyrogallol, PCA-3-O-glucuronide, gallic acid, PCA, PCA-4-O-glucuronide, PCA-3-O-sulfate, PCA-4-O-sulfate, gallic acid-3-O-glucuronde, gallic acid-4-O-glucuronide, hippuric acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 5-hydroxyferulic acid, vanillic acid, catechin, caffeic acid, syringic acid, p-coumaric acid, epicatechin, ferulic acid, sinapic acid, quercetin and quecertin-3-O-sulfate. Pending success of this run, in the

form of high quality chromatography, it was intended to run these standards again in a matrix of urine and then plasma to determine whether the matrix of the samples would dampen the LC-MS/MS signals of compounds of interest below the limits of detection of the instrument. When tested, this reported method provided high resolution chromatography for the vast majority of compounds, however, consistent with the report by De Ferrars *et al* positional sulfate isomers of PCA could not be resolved, as shown in Figure 2-1 (De Ferrars, Czank, Saha, *et al.*, 2014c).
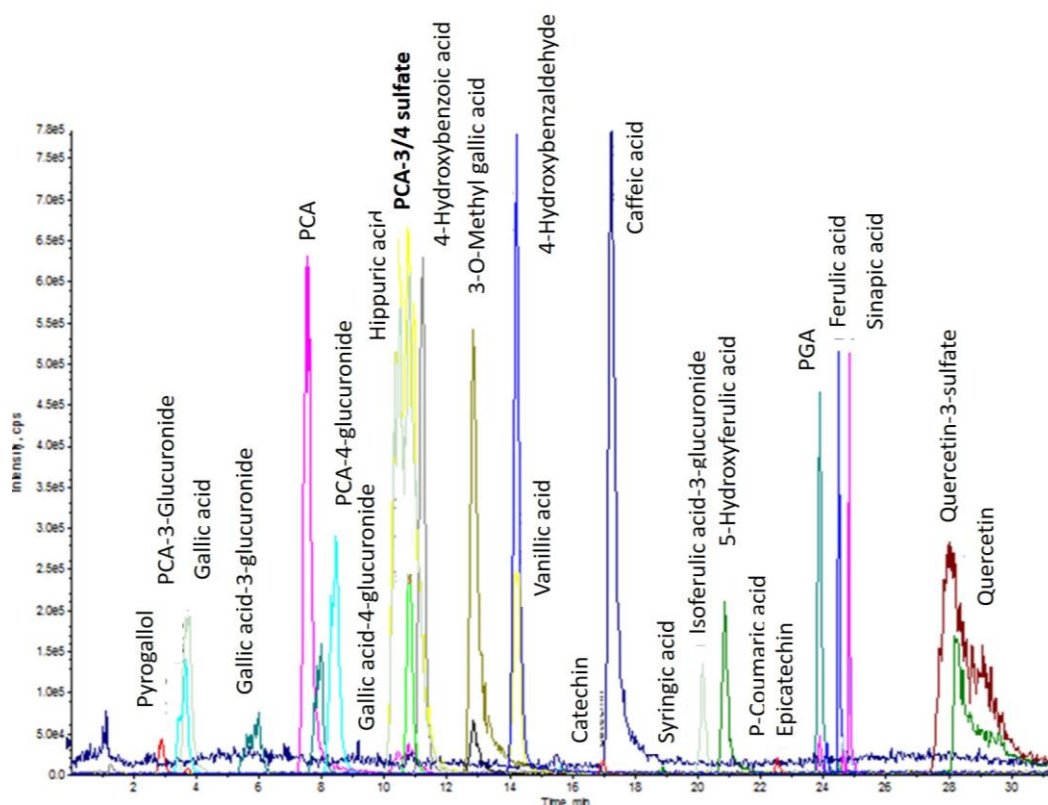
Figure 2-1- Chromatogram of phenolic standards run using the method reported by De Ferrars et al, 2014

Chromatogram obtained from running a mixture of phenolic standards each at 10 µg/ml in a matrix of 0.1% FA in water on the ABSciex 4000 series Qtrap MS/MS, applying the LC-MS/MS method described by De Ferrars et al, 2014.

It was therefore decided to retry the method described by De Ferrars *et al*, but with a different column in an attempt to achieve chromatographic resolution of PCA sulfate isomers. The column selected, was a Kinetex 2.6 µm F5 100 x 2.1 mm column which also uses a pentafluorophenyl stationary phase, and was selected because the manufacturers state that the F5 column provides refinements over PFP columns to overcome reproducibility and performance issues that provide superior resolution of positional isomers. A chromatogram of phenolic standards run with this

method is shown in Figure 2-2. With the F5 column, there was an overall increase in sensitivity and compounds eluted in a narrower time frame. But, positional PCA sulfate isomers were not resolved.
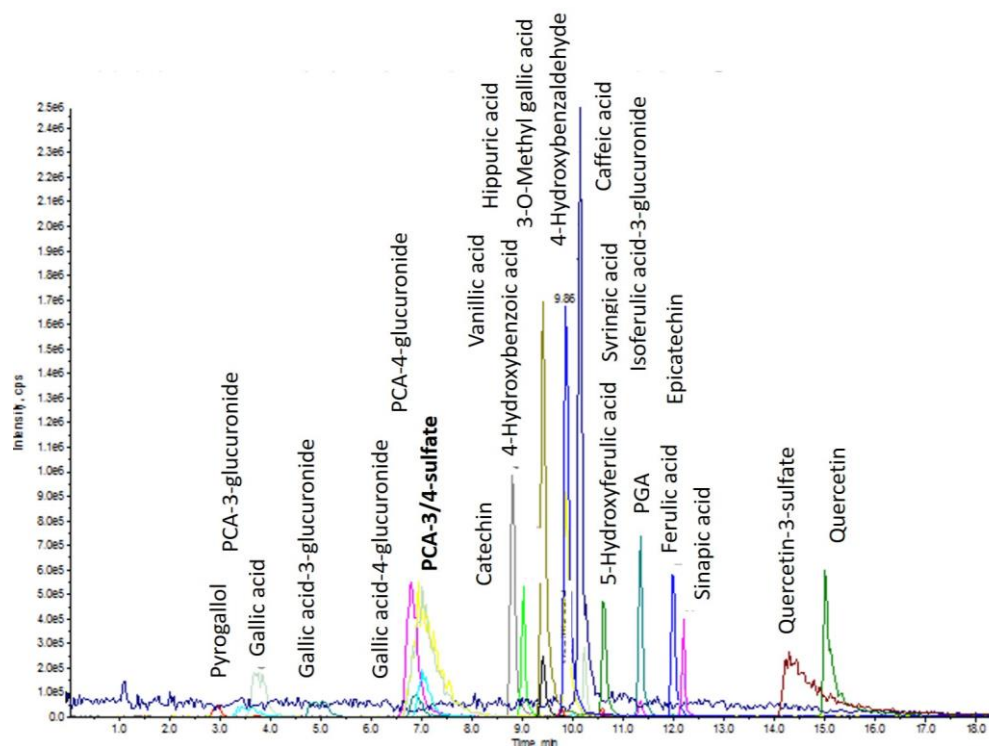
Figure 2-2- Chromatogram of phenolic standards run on the F5 column
Chromatogram obtained from running a mixture of phenolic standards each at 10 μg/ml in a matrix of 0.1% FA in water on the ABSciex 4000 series Qtrap MS/MS, using a Kinetex 2.6 μm F5 100 x 2.1 mm column, applying the LC-MS/MS method described by De Ferrars et al, 2014.

The next parameter to be altered in an effort to provide chromatographic resolution of positional sulfate isomers was the LC solvents. There are reports in the literature that use of ammonium salts in LC solvents can aid the chromatographic separation of positional isomers (Fenner and Kerns, 2011). It was therefore decided to trial solvents of 10mM ammonium acetate in water (mobile phase A) and acetonitrile (mobile phase B), both adjusted to pH 5 with acetic acid. Although the F5 column had shown superior chromatography when applying the method developed by De Ferrars *et al*, any change in solvents drastically changes the LC conditions and therefore may alter the quality of chromatography achieved with each column, and could result in a different column being

optimal for the analysis. Consequently, three different columns were tested with ammonium acetate buffered solvents on the same gradient used by De Ferrars *et al*, as shown in Figure 2-3.
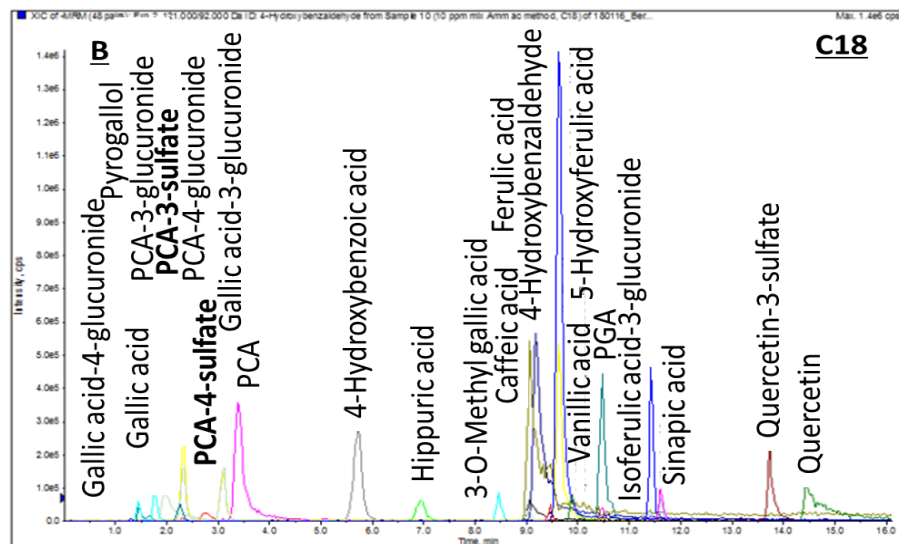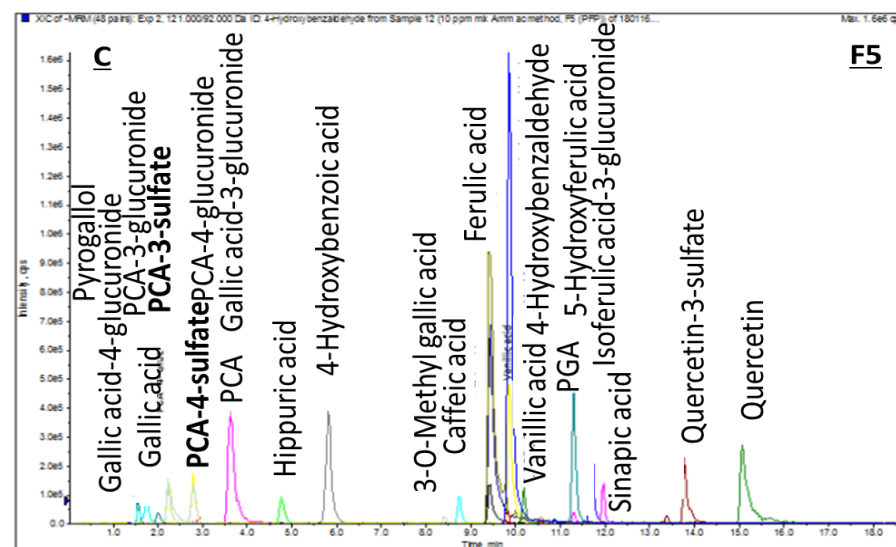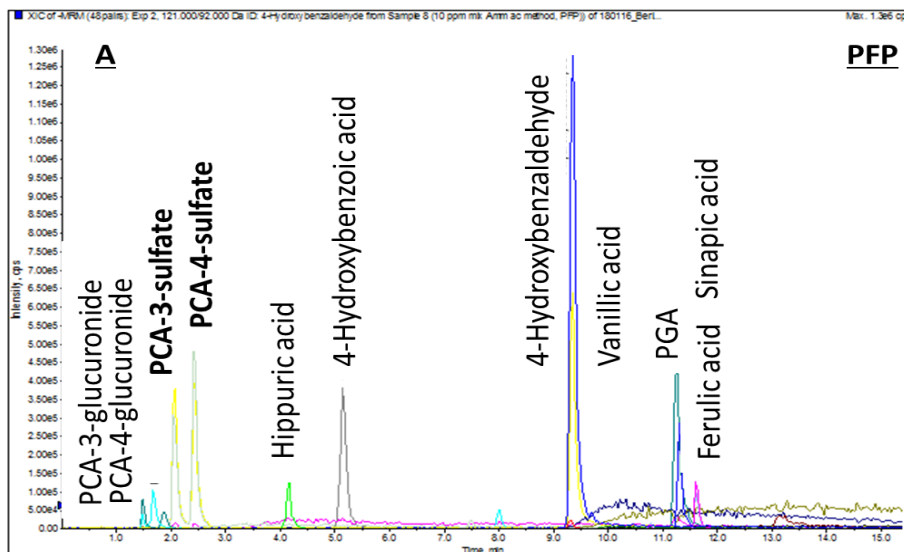
Figure 2-3- Chromatograms of phenolic standards run on the PFP, C18 and F5 columns in 10 mM ammonium acetate buffered solvents (pH5)

Chromatograms produced from running a mixture of phenolic standards each at 10 µg/ml in a matrix of 0.1% FA in water on the ABSciex 4000 series Qtrap MS/MS, with solvents of 10 mM ammonium acetate in water (A) and acetonitrile (B) each adjusted to pH 5 with acetic acid. The following columns were trialled: Kinetex PFP reverse phase (2.6 µm, 100 × 4.6 mm, 100 Å) column (A), Kinetex C18 column (100 x 2.1, 2.6µ) (B) and a Kinetex 2.6 µm F5 100 x 2.1 mm column (C), applying the gradient described by De Ferrars et al, 2014.

The PFP column gave disappointing results (Figure 2-3). Although, there was success in resolving positional PCA sulfate isomers, there were now many compounds missing from the chromatogram, showing this column was unsuitable for use under the revised mobile phase conditions. A C18 column was also tested with ammonium acetate solvents and like the PFP column, this column provided resolution between positional sulfate isomers, but with the added benefit of still providing clear chromatography of all other injected compounds. The F5 column gave very similar results to those achieved with the C18 column, but in several cases the F5 column was able to provide 'cleaner' peak quality. Therefore, the F5 column was selected for further method development with ammonium acetate solvents as a substantial improvement on those reported by De Ferrars *et al*.

Unfortunately, although the F5 column in combination with ammonium acetate buffered solvents provided the best chromatography of the methods trialled up to this point, issues were uncovered when running phenolic standards at lower concentrations (1 – 5 μg/ml) and those that were matrix matched in urine. In these instances, chromatography became corrupted, with poorly defined peaks which had a large amount of tailing (data not shown). Troubleshooting potential issues with the column and samples eventually pointed to the LC-MS instrument being the cause of the problems. Consequently, the method was transferred to an Agilent 6490 UHPLC-MS/MS system. This system provided superior chromatography in matrix matched and low concentration samples compared to what was achieved on the ABSciex 4000 series Q-Trap. However, there was still some tailing present in matrix matched samples. Since the Agilent 6490 LC-MS system allowed the use of UHPLC columns, the potential benefits of using a UHPLC column was explored. An ACQUITY UPLC HSS T3 1.8 μm (2.1 x 100 mm) column was trialled by running a mix of authentic standards each at 10 μg/ml, namely: 3-O-methyl gallic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 5-hydroxyferulic acid, caffeic acid, catechin, chlorogenic acid, cinnamic acid, epicatechin, ferulic acid, gallic acid, gallic acid-3-O-glucuronide, gallic acid-4-O-glucuronide, hippuric acid, isoferulic acid-3-O-glucuronide, isovanillic acid, methyl vanillate, PCA, PCA-3-O-glucuronide, PCA-3-O-sulfate, PCA-4-O-glucuronide, PCA-4-O-sulfate, p-coumaric acid, PGA, phloroglucinol, phloridzin, pyrogallol, quercetin, quercetin-3-O-sulfate, rutin, scopoletin, sinapic acid, syringic acid and vanillic acid. This resulted in a substantially cleaner chromatogram than could be achieved with the F5 column, with sharper peaks that were clearly defined with minimal tailing, shown in Figure 2-4. It was therefore decided to proceed with the Agilent 6490 UHPLC-MS system, using the T3 column with ammonium acetate buffered solvents to facilitate clean chromatography, high sensitivity and separation of positional sulfate isomers.
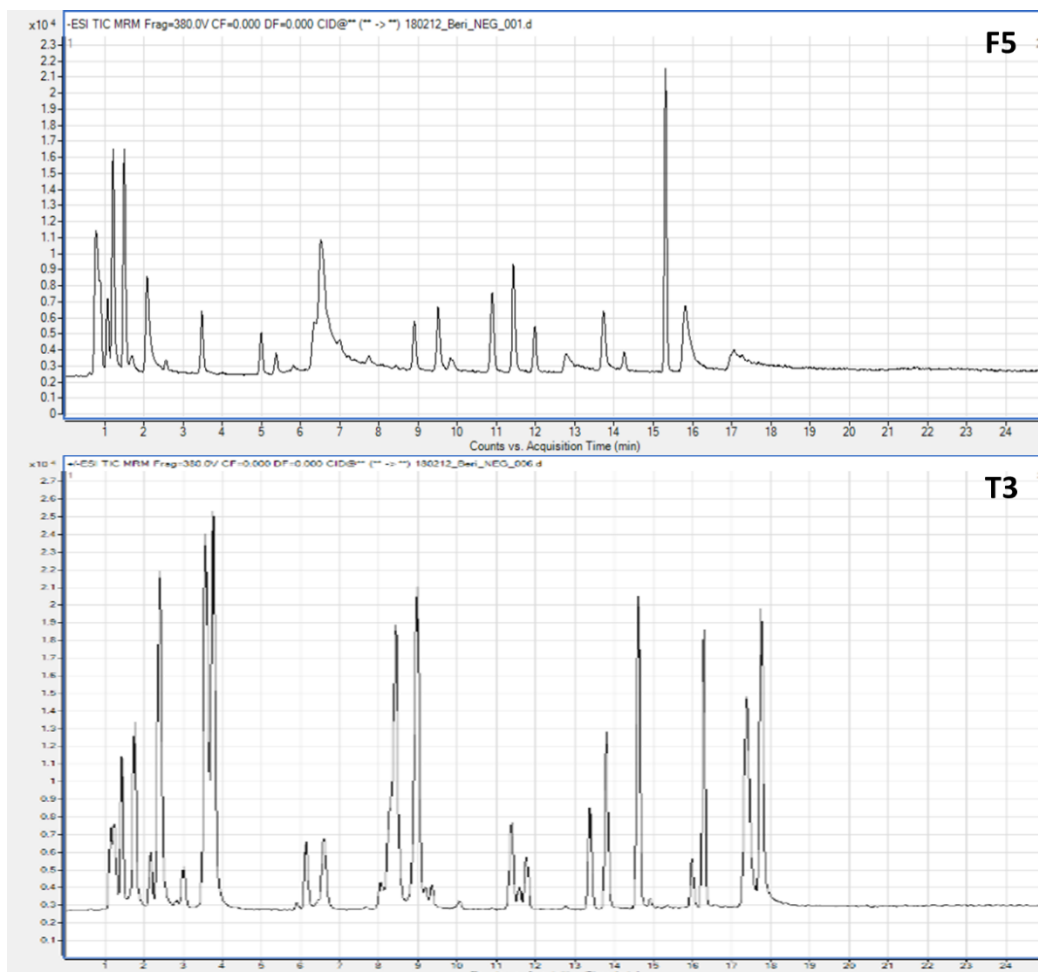
Figure 2-4- Comparison of the F5 and T3 columns
TIC chromatograms produced by the Agilent 6490 UHPLC-MS/MS when running a mixture of authentic phenolic standards at 10ug/ml in a matrix of urine, with solvents of 10mM ammonium acetate in water (A) and acetonitrile (B) adjusted to pH5 with acetic acid. Using a Kinetex 2.6 μm F5 100 x 2.1 mm column (top), and an ACQUITY UPLC HSS T3 1.8 μm (2.1 x 100 mm) column (bottom), applying the gradient described by De Ferrars et al, 2014.

As illustrated in Figure 2-4, all standards elute within the first 20 minutes of the gradient spanning 37 minutes. Therefore, the LC gradient was condensed (20-37 minutes) and further shortened to the final run time of 14 minutes, offering a higher throughput method that would facilitate the analysis of large quantities of clinical samples. With this shortened gradient, high quality chromatography was achieved (Figure 2-5). However, there was a considerable break in elution between 3.5-5.5 minutes and the gradient was condensed further to just 12 minutes with excellent chromatographic separation of compounds retained (Figure 2-5).
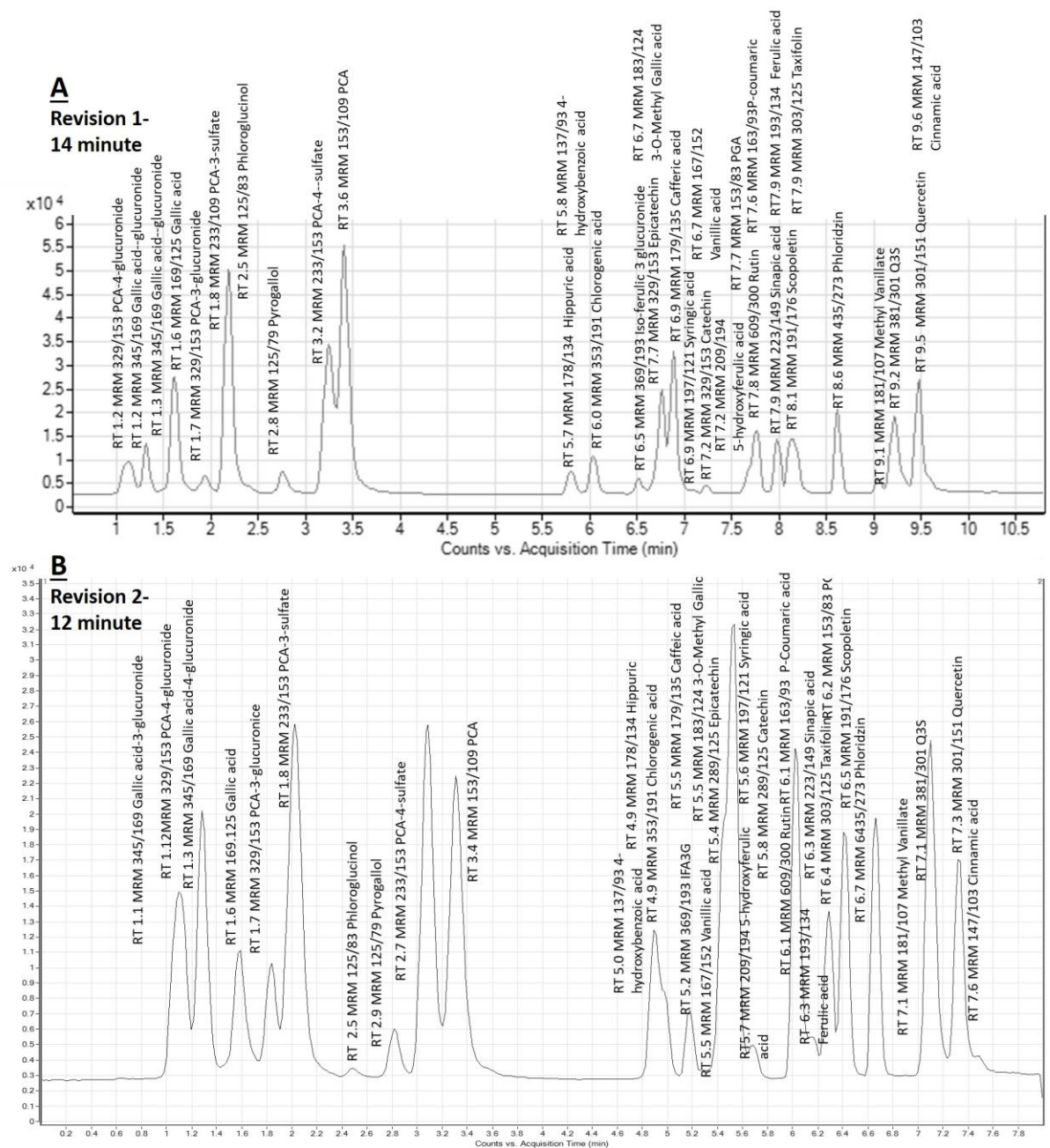
Figure 2-5- Comparison of 14 and 12 minute gradients for LC-MS/MS detection of phenolic standards
TIC chromatograms produced under different revisions of the LC gradient, when running a mixture of phenolic standards in a matrix of urine, using LC solvents of 10 mM ammonium acetate in water and acetonitrile for A and B respectively, each adjusted to pH 5 with acetic acid. A 14 minute (A) and a 12 minute (B) LC gradient were trialled (as described in the methods section) on the Agilent 6490 UHPLC-MS/MS. Compounds are labelled with their name, retention time (RT) and MRM transition.

The optimised 12-minute gradient using the T3 column and ammonium acetate solvents provided high quality chromatography for phenolics at a range of concentrations, and crucially also when they were in plasma and urine matrices. Up to this point an injection volume of 1 µl had been used. However, larger injection volumes raise the probability of detecting low abundance compounds of interest, although, this also comes with a risk of corrupting chromatography as the injection volume

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increases. Injection volumes of 1-5 µl were tested, and an injection volume of 5 µl gave the highest response to injected standards without compromising chromatography as shown in Figure 2-6.
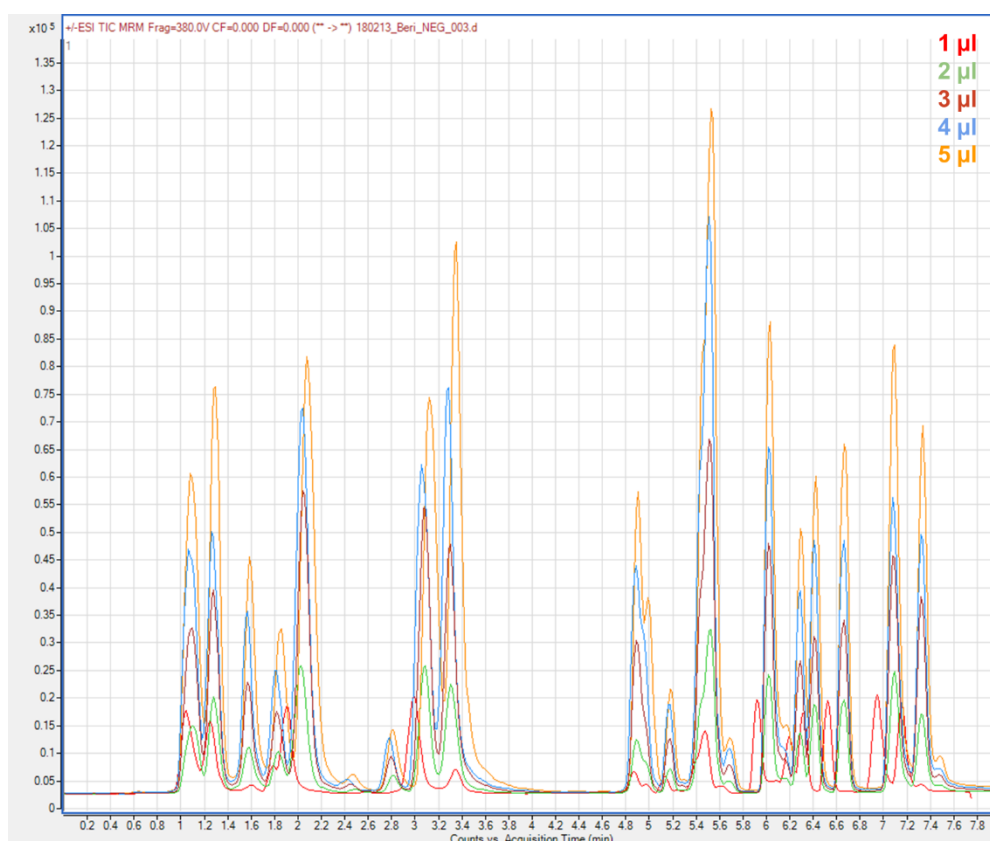
Figure 2-6- Injection volume test for phenolic standards
Overlaid chromatograms testing injection volumes from 1 - 5 µl when injecting a mixture of phenolic standards at 10 µg/ml in a matrix of SPE processed urine.

With SPE being used as the extraction/clean-up method and methanol as the eluent, which had been previously reported as the optimal SPE eluent for anthocyanins and phenolics (De Ferrars *et al.*, 2014c), the final variable to test was the composition of the sample injected onto the UHPLC column. Methanol may not be a suitable matrix for UHPLC if the injection volume is too large and as a consequence the compounds of interest remain in the methanol mobile phase and do not enter the column stationary phase. This can be circumvented by reducing the injection volume and/or reducing the methanol content (%). Three solvent compositions were tested, namely 30, 50 and 75% v/v methanol, with the remainder of each sample comprising 0.1% FA in water and a mixture of phenolic standards at 10 µg/ml. Chromatography was consistently of high quality at both 30%

and 50% methanol, as illustrated in Figure 2-7, however, at 75% the chromatogram produced was severely corrupted (not shown).
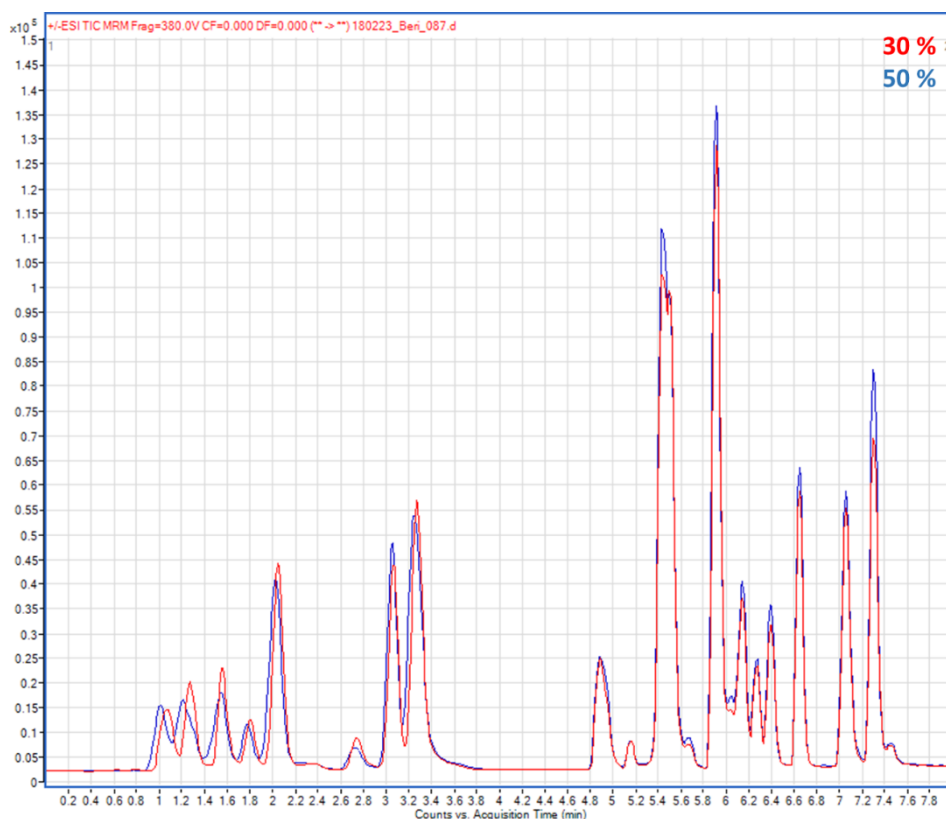
Figure 2-7- LC-MS/MS methanol tolerance test for samples containing phenolic standards
Chromatograms produced with varying amounts of methanol in samples containing a mixture of phenolic standards at 10ug/ml. The red line shows the chromatogram produced at 30% methanol and the blue line represents the chromatogram produced at 50% methanol.

2.5.2 LC-MS/MS Detection of Anthocyanins in Urine

An LC-MS/MS method to detect anthocyanins, using a UPLC T3 HSS column by Waters (as used in the phenolics method), and a mobile phase of 5% formic acid in water (A) and a stationary phase of 5% formic acid in acetonitrile (B) over a 36 minute gradient detailed in the methods section was developed. Whilst it was attempted to run a mixture of anthocyanin standards (C3G, D3G, C3Rut, D3Rut, M3G, Pel3G and Pel3Rut) on the LC-MS/MS method developed for phenolic compound analysis this was unsuccessful, and attributed to pH5 being too high to maintain anthocyanin stability. This theory was tested crudely by spiking the two mobile phases, 10mM ammonium acetate in water (adjusted to pH5 with acetic acid) and 5% formic acid in water with 0.5 µg/ml of a mixture of 7 authentic anthocyanin standards. This is depicted in Figure 2-8 and clearly shows an absence of pigmentation when the solvent is pH 5 compared to when it is acidified, highlighting the need for acidified solvents to maintain anthocyanin stability for LC-MS/MS detection.
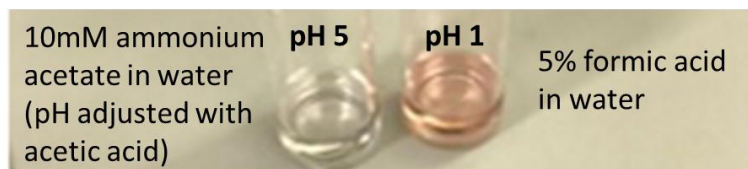
Figure 2-8- The pigmentation difference between solvents of pH 5 and pH1 spiked with 0.5 µg/ml mixed anthocyanin standards.

Use of acidified solvents allowed reliable detection of anthocyanin standards, with high quality chromatography. As for phenolic metabolites, an injection volume test from 1-5µl was performed to assess whether, a higher injection volume of 5µl could be tolerated to increase sensitivity without compromising chromatography. This showed that a 5 µl injection volume would be suitable for this analysis, as illustrated in Figure 2-9.
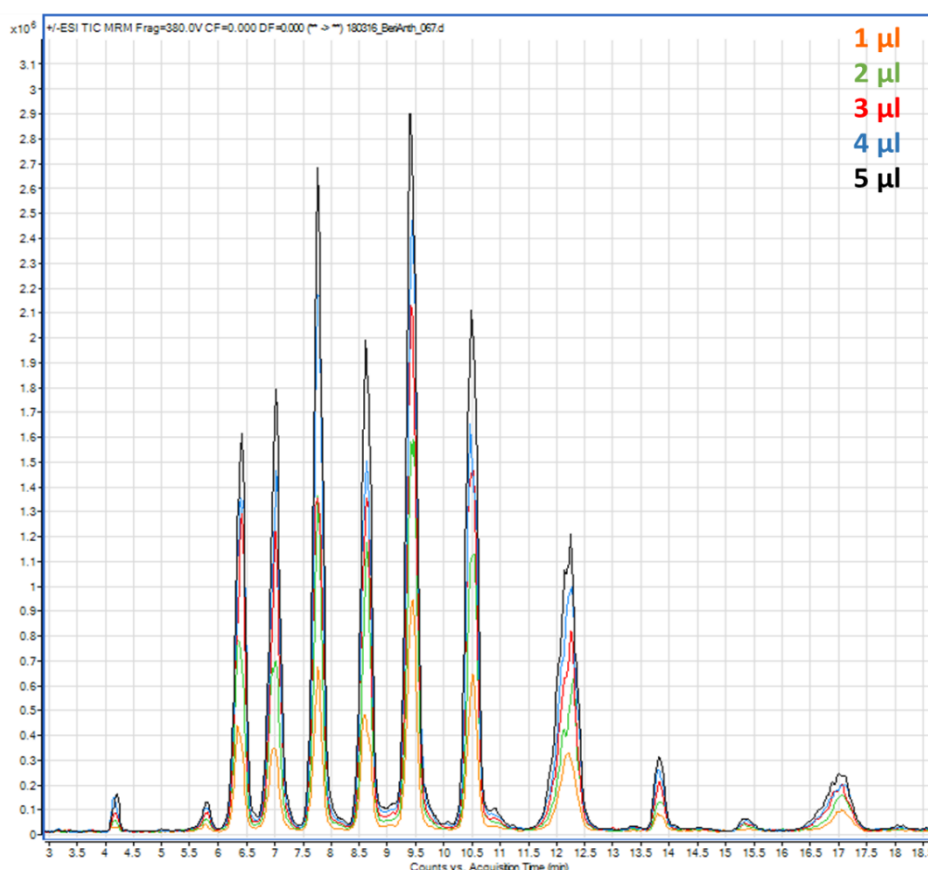
Figure 2-9- Injection volume test for samples containing anthocyanins
Overlaid chromatograms testing injection volumes from 1 - 5 µl when injecting a mixture of anthocyanin standards at 10ug/ml in an SPE processed urine matrix (urine from volunteers who had been asked to exclude anthocyanins from their diet was pooled and subjected to SPE before being spiked with anthocyanin standards).

The methanol content of samples containing anthocyanins was tested to investigate whether 50% methanol content adversely affected LC-MS detection of anthocyanins to ensure the final sample matrix was suitable for the measurement of both phenolics and anthocyanins. 75% methanol was

not tested in this instance as it had already been shown to be unsuitable for phenolics. The results obtained were similar to those for phenolic compounds, i.e. there was no chromatographic difference between a sample matrix of 30% and 50% methanol, as shown in Figure 2-10.
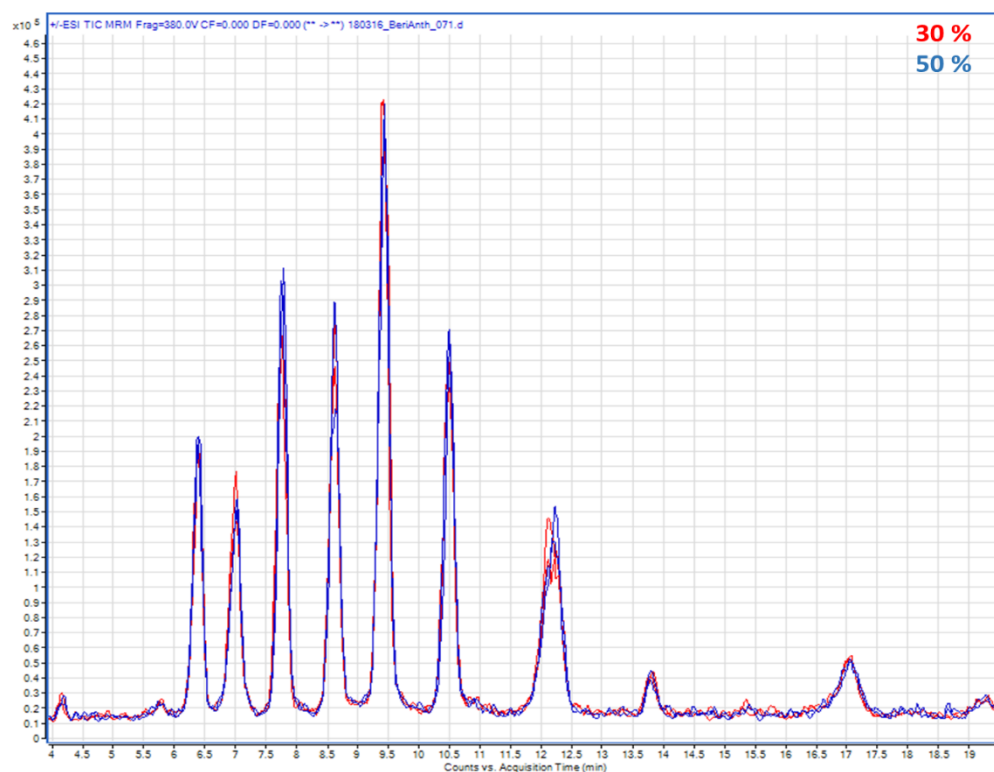
Figure 2-10- LC-MS/MS methanol tolerance test for samples containing anthocyanin standards
Chromatograms produced with varying concentrations of methanol in samples containing a mixture of anthocyanin standards at 10 µg/ml. The red line shows the chromatogram produced with 30% methanol and the blue line represents the chromatogram produced with 50% methanol.

The results described above provide two methods for the detection of anthocyanins and phenolics using UHPLC-MS/MS. The next step was to optimise the extraction method of these compounds from clinical samples to maximise the likelihood of reliably identifying compounds of interest.

2.5.3 SPE of Anthocyanins and their Phenolic Metabolites in Urine

SPE has been identified previously as the optimal extraction method for anthocyanins and their metabolites in biological samples. De Ferrars *et al* tested DSC-18 and Strata-X SPE cartridges and reported that DSC-18 were superior for urine samples, whilst Strata-X were best suited to extracting these compounds from serum (De Ferrars, Czank, Saha, *et al.*, 2014c). Method development was

started with urine samples because urine provides a much simpler matrix, is likely to contain metabolites at higher concentrations and was readily available, whereas plasma is far more complex, metabolites are likely to be at lower concentrations and it is expensive to purchase. The method reported by De Ferrars *et al* was performed using urine samples spiked with a set of authentic phenolic standards to establish if similar recoveries could be achieved (De Ferrars *et al.*, 2014c). Rather surprisingly, because it was contradictory to the report from De Ferrars *et al*, recoveries were far superior for Strata-X cartridges compared to DSC-18, with the most polar compounds showing extremely poor recovery when subjected to SPE using DSC-18; these results are shown in Table 2-1. These data showed that Strata-X cartridges were the most suitable for urine extraction. A drawback of both Strata-X and DSC-18 SPE cartridges was the laborious method required for sample processing involving a long period of centrifugal evaporation (~8 hours) which limited throughput. Furthermore, both of these formats required large amounts of SPE solvents with pre-conditioning required before sample processing can take place Therefore, alternative methods were investigated to try and provide equivalent recoveries but increase sample throughput which would be instrumental when analysing a large number of clinical samples. Oasis HLB sorbent is chemically very similar to Strata-X and is claimed to provide nearly universal SPE material for acidic, basic and neutral analytes. An advantage of Oasis HLB sorbent is that it does not require pre-conditioning and consequently samples can be loaded immediately to increase throughput. Oasis HLB-sorbent is also available in micro-elution format which removes any need for evaporation, further increasing sample throughput by removing the most time-consuming step in a 96 well format. Although this provided a streamlined method with high recovery for the majority of compounds, it was disappointing to see that the most polar compounds were not recovered at acceptable levels, with several key compounds having a recovery below 10% (see Table 2-2).

Table 2-1- Recoveries of phenolic standards spiked in urine achieved with the SPE method described by De Ferrars et al 2014. Data is shown as mean ± standard deviation and n = 3.

Compound	Strata-X Recovery (%)	DSC-18 Recovery (%)
3-*O*-Methyl gallic acid	96.9 ± 20.9	116.5 ± 5.6
4-Hydroxybenzaldehyde	97.1 ± 16.1	109.7 ± 29.8
4-Hydroxybenzoic acid	94.0 ± 8.4	114.3 ± 7.6
5-Hydroxyferulic acid	111.6 ± 23.8	108.5 ± 17.4
Caffeic acid	94.2 ± 15.3	106.0 ± 9.5
Catechin	76.7 ± 0.8	84.1 ± 18.8
Chlorogenic acid	93.1 ± 15.7	108.5 ± 11.6
Cinnamic acid	104.5 ± 20.5	113.9 ± 32.7
Epicatechin	65.8 ± 1.2	83.2 ± 13.6
Ferulic acid	88.2 ± 24.4	109.3 ± 11.4
Gallic acid	96.6 ± 14.4	9.5 ± 0.5

Compound	Strata-X Recovery (%)	DSC-18 Recovery (%)
Gallic Acid-3-O-glucuronide	84.6 ± 9.9	6.9 ± 2.4
Gallic Acid-4-O-glucuronide	96.8 ± 19.5	71.9 ± 8.3
Hippuric acid	102.9 ± 1.6	111.3 ± 18.3
Isoferulic acid-3-O-glucuronide	100.1 ± 11.0	113.1 ± 13.4
Isovanillic acid	98.8 ± 28.6	108.2 ± 13.1
Methyl vanillate	108.4 ± 24.1	123.1 ± 13.7
PCA	97.4 ± 13.9	56.3 ± 5.0
PCA-3-O-glucuronide	98.6 ± 13.7	25.7 ± 5.3
PCA-3-O-sulfate	84.5 ± 9.0	9.2 ± 0.8
PCA-4-O-glucuronide	103.7 ± 9.6	60.5 ± 0.2
PCA-4-O-sulfate	92.1 ± 7.3	10.7 ± 2.1
p-Coumaric acid	86.1 ± 13.5	110.7 ± 17.3
PGA	106.6 ± 22.6	118.2 ± 12.2
Phloroglucinol	77.7 ± 3.2	5.2 ± 1.1
Pyrogallol	90.4 ± 5.9	4.0 ± 0.6
Quercetin	15.8 ± 4.8	85.5 ± 2.7
Quercetin-3-O-sulfate	7.7 ± 3.1	97.3 ± 5.1
Rutin	90.0 ± 10.4	128.4 ± 18.8
Scopoletin (internal standard)	105.1 ± 3.6	110.4 ± 13.1
Sinapic acid	101.7 ± 9.8	112.3 ± 19.7
Syringic acid	110.4 ± 12.1	133.4 ± 64.8
Vanillic acid	84.1 ± 9.8	117.1 ± 13.8

Table 2-2- Recoveries of phenolic standards spiked in urine achieved with Waters Oasis HLB micro-elution SPE according to manufacturer guidelines. Data is shown as mean ± standard deviation and n = 3.

Compound	Recovery (%)
3-O-Methyl gallic acid	46.0 ± 4.4
4-Hydroxybenzaldehyde	70.7 ± 7.4
4-Hydroxybenzoic acid	57.3 ± 8.3
5-Hydroxyferulic acid	64.8 ± 3.9
Caffeic acid	65.9 ± 4.4
Catechin	61.1 ± 7.8
Chlorogenic acid	41.8 ± 5.6
Cinnamic acid	86.2 ± 6.3
Epicatechin	57.3 ± 11.9
Ferulic acid	80.4 ± 11.7
Gallic acid	8.6 ± 1.1
Gallic acid-3-O-glucuronide	1.1 ± 0.2
Gallic acid-4-O-glucuronide	5.9 ± 0.8
Hippuric acid	87.5 ± 8.9
Isoferulic acid-3-O-glucuronide	45.7 ± 5.0
Isovanillic acid	50.8 ± 7.7
Methyl vanillate	90.8 ± 10.8
PCA	30.8 ± 1.3
PCA-3-O-glucuronide	3.6 ± 0.8
PCA-3-O-sulfate	3.0 ± 0.4
PCA-4-O-glucuronide	5.2 ± 0.9
PCA-4-O-sulfate	3.0 ± 0.5

Compound	Recovery (%)
p-Coumaric acid	82.9 ± 10.4
PGA	82.9 ± 11.9
Phloroglucinol	4.2 ± 1.2
Pyrogallol	4.8 ± 0.8
Quercetin	47.6 ± 9.4
Quercetin-3-O-sulfate	24.4 ± 4.8
Rutin	72.9 ± 10.3
Scopoletin (internal standard)	92.0 ± 21.5
Sinapic acid	72.7 ± 2.9
Syringic acid	61.7 ± 11.4
Vanillic acid	66.8 ± 8.7

Initially, micro-elution had been performed according to the manufacturer guidelines with a 200 µl sample load volume, and without soaking prior to vacuum elution. It was thought that perhaps the sorbent was being overloaded in the initial sample loading step. Wash steps were analysed (data not shown) using UHPLC-MS/MS which showed that low recovery compounds were not being lost during the wash process and therefore were either being held on the cartridge or were not being binding to the sorbent at all and were lost in the initial loading step. Load volumes were tested ranging from 100 – 500 µl, however, this did not appear to exert a large effect on recovery, and reducing the sample load volume did not provide any large increase in recovery of polar analytes (data shown in appendix 2). Therefore, in addition to testing load volume, a soak step was also introduced to investigate if this could elute compounds with poor recovery, if they were being held in the sorbent. Soak times of 10 and 30 minutes were tested, and this data is shown in Table 2-3.

Table 2-3- Recoveries of phenolic standards spiked in urine achieved with Waters Oasis HLB micro-elution SPE with varying methanol soaks prior to elution. Data shown as mean recovery (%) ± standard deviation and n = 3.

Compound	0 Minute	10 minute	30 minute
3-O-Methyl gallic acid	37.9 ± 2.4	75.0 ± 3.6	80.5 ± 0.1
4-Hydroxybenzaldehyde	57.8 ± 7.2	97.7 ± 0.1	88.0 ± 15.6
4-Hydroxybenzoic acid	61.0 ± 4.4	89.5 ± 6.0	86.9 ± 2.7
5-Hydroxyferulic acid	49.2 ± 9.8	88.3 ± 2.4	83.7 ± 18.7
Caffeic acid	48.6 ± 2.9	101.0 ± 7.8	98.4 ± 19.1
Catechin	56.2 ± 4.9	87.4 ± 4.9	76.7 ± 21.5
Chlorogenic acid	37.3 ± 3.1	78.2 ± 0.9	81.7 ± 1.2
Cinnamic acid	62.0 ± 15.1	109.2 ± 0.2	82.0 ± 22.3
Epicatechin	45.0 ± 3.2	77.3 ± 4.9	77.2 ± 1.5
Ferulic acid	61.1 ± 0.7	92.8 ± 9.6	80.9 ± 9.9
Gallic acid	8.1 ± 0.6	9.5 ± 1.9	12.3 ± 0.6
Gallic acid-3-O-glucuronide	2.1 ± 0.6	1.5 ± 0.3	2.0 ± 0.7

Gallic acid-4-*O*-glucuronide	5.9 ± 0.7	6.8 ± 0.1	11.9 ± 3.3
Hippuric acid	81.7 ± 6.6	99.6 ± 2.7	99.2 ± 10.1
Isoferulic acid-3-*O*-glucuronide	38.5 ± 4.5	66.9 ± 10.2	65.8 ± 4.3
Isovanillic acid	45.9 ± 2.1	82.3 ± 2.2	76.2 ± 1.6
Methyl vanillate	46.0 ± 0.5	127.6 ± 5.6	102.4 ± 26.8
PCA	27.3 ± 1.9	41.7 ± 0.7	47.1 ± 2.8
PCA-3-*O*-glucuronide	4.0 ± 1.2	5.0 ± 0.9	9.2 ± 2.9
PCA-3-*O*-sulfate	1.7 ± 0.1	3.0 ± 0.8	4.0 ± 0.5
PCA-4-*O*-glucuronide	7.4 ± 0.3	6.5 ± 0.1	12.4 ± 1.5
PCA-4-*O*-sulfate	2.6 ± 0.1	3.0 ± 0.8	4.3 ± 0.5
p-Coumaric acid	66.6 ± 0.6	108.7 ± 9.5	92.7 ± 23.6
PGA	45.1 ± 9.0	109.8 ± 5.7	82.8 ± 14.5
Phloroglucinol	3.5 ± 3.3	4.0 ± 0.5	3.5 ± 0.5
Pyrogallol	7.0 ± 0.6	4.2 ± 1.3	4.1 ± 0.2
Quercetin	19.5 ± 3.8	38.9 ± 9.6	21.3 ± 14.6
Quercetin-3-sulfate	13.2 ± 1.1	18.9 ± 2.4	9.0 ± 0.2
Rutin	64.3 ± 7.9	113.1 ± 8.0	99.9 ± 7.4
Scopoletin (internal standard)	72.1 ± 4.1	131.7 ± 12.1	115.6 ± 17.6
Sinapic acid	57.1 ± 1.8	110.4 ± 4.9	103.6 ± 17.8
Syringic acid	50.6 ± 10.2	84.6 ± 2.0	106.1 ± 4.1
Vanillic acid	51.5 ± 2.2	81.9 ± 2.2	75.9 ± 0.8

In most instances, soaking the sorbent with acidified methanol prior to vacuum elution resulted in a substantial increase in recovery. For example, with no soak step caffeic acid was recovered at 48.6%, but with a soak step introduced of either 10 or 30 minutes nearly 100% recovery was achieved. Notably, recoveries achieved with a 10 or 30 minute soak were extremely similar for nearly all compounds and subsequently a 10 minute soak was used to facilitate increased sample throughput and recovery. However, soaking the sorbent did not lead to any great improvement in the recovery of particularly polar compounds such as: PCA sulfates and glucuronides, phloroglucinol or pyrogallol. Given that several modifications to the micro-elution protocol had not been able to provide high recovery of all phenolic standards available, it was decided to test traditional HLB SPE cartridges to establish whether HLB sorbent itself was appropriate for the extraction of this range of phenolics or if the micro-elution adaptation of SPE was the source of the problems experienced.

Recoveries achieved with 30 mg HLB SPE cartridges were high overall with near 100% recovery achieved for the vast majority of phenolic compounds. However, recoveries were still extremely low for the same subset of polar compounds with < 10% recovery being achieved in many of these cases (data shown in appendix 2). Given the high recoveries of the majority of compounds achieved with Strata-X cartridges and the need to analyse a broad spectrum of phenolics and anthocyanins,

the Strata-X sorbent was preferable to the HLB sorbent. Strata-X is available in micro-elution format and given that the format of SPE had not been the source of issues experienced with HLB, it seemed reasonable that this would provide an effective higher throughput method than the large volume cartridges that require > 8 hours evaporation post processing. Micro-elution was performed according to the manufacturer guidelines, with the addition of a 10 minute soak at the elution step, testing 3 different load volumes to establish the effect on recovery. Disappointingly, the same polar compounds that had poor recovery with HLB sorbent were also poorly recovered with the Strata-X micro-elution protocol (appendix 2). This was surprising given that it is claimed by the manufacturers that micro-elution should reproduce recoveries achieved with full SPE whilst increasing throughput by removing the need for evaporation. However, this appeared not to be the case given the high recoveries of polar compounds achieved with 500 mg Strata-X sorbent cartridges that could not be replicated in micro-elution format. Whilst it was clear that micro-elution would not be suitable for the most polar phenolics included in the analysis, the next step was to test Strata-X SPE cartridges ranging in sorbent amounts, including an SPE plate to increase throughput as using less sorbent requires less elution solvent and consequently less evaporation time which is the rate limiting step for sample processing (data shown in appendix 2).

It should be noted that for the remainder of method development, taxifolin was used as the internal standard instead of scopoletin. This was due to scopoletin being present in many of the urine samples used for method development, and the benefit of taxifolin being a compound that would be less likely to be found in human samples. Furthermore, as a compound of mid-range polarity this provided a suitable internal standard to assess the success of the SPE process. Likewise, the volume control was changed from phloridzin to D9-caffeine as deuterated compounds are not naturally occurring and would not be found in clinical samples, meaning that this compound would facilitate more accurate normalisation of data. As the volume control is not used to gauge the success of the SPE process and is merely used to correct for volume changes during evaporation this compound did not need to be structurally related to the analytes being measured. Table 2-4 shows recoveries achieved in the final revised SPE method for urine samples, including a 10 minute soak prior to elution and changes in internal standards.

Testing a variety of Strata-X sorbent volumes made it clear that for the most polar compounds, a high quantity (500 mg) sorbent was required to hold these compounds as presumably lower amounts of sorbent are unable to retain high levels of extremely polar compounds. For example, PCA sulfates were recovered consistently at > 80% with 500 mg Strata-X SPE cartridges, whilst 60 mg and 30 mg cartridges, were only capable of providing < 40% recovery (appendix 2). Despite, the large requirements for elution solvent and subsequent evaporation, it was clear that 500 mg Strata-X SPE cartridges were the only suitable SPE format out of those tested, to provide consistent high recovery without sacrificing the extraction of the most polar compounds of interest (Table 2-4).

Table 2-4- Recoveries of phenolic standards spiked in urine achieved with Strata-X 500 mg SPE cartridges applying the final protocol. Data shown as mean recovery (%) ± standard deviation and n = 3.

Compound	Recovery (%)
3-*O*-Methyl gallic acid	102.7 ± 19.7
4-Hydroxybenzaldehye	102.8 ± 12.8
4-Hydroxybenzoic acid	103.0 ± 18.9
5-Hydroxyferulic acid	114.6 ± 1.7
Caffeic acid	97.7 ± 6.7
Catechin	29.8 ± 0.1
Chlorogenic acid	100.8 ± 17.0
Cinnamic acid	97.5 ± 7.3
Epicatechin	20.6 ± 3.7
Ferulic acid	82.6 ± 5.3
Gallic acid	100.5 ± 9.7
Gallic acid-3-*O*-glucuronide	93.5 ± 15.8
Gallic acid-4-*O*-glucuronide	114.1 ± 15.4
Hippuric acid	107.9 ± 15.3
Isoferulic acid-3-*O*-glucuronide	97.7 ± 8.8
Isovanillic acid	116.4 ± 15.3
Methyl vanillate	106.1 ± 18.8
PCA	109.0 ± 5.1
PCA-3-*O*-glucuronide	110.8 ± 24.7
PCA-3-*O*-sulfate	114.4 ± 14.9
PCA-4-*O*-glucuronide	93.4 ± 12.5
PCA-4-*O*-sulfate	88.8 ± 8.7
p-Coumaric acid	89.5 ± 7.6
PGA	116.8 ± 13.8
Phloroglucinol	91.0 ± 17.8
Pyrogallol	108.7 ± 1.9
Quercetin	19.1 ± 9.5
Quercetin-3-*O*-sulfate	84.3 ± 7.3
Rutin	51.1 ± 2.8
Sinapic acid	118.3 ± 13.3
Syringic acid	133.5 ± 22.0
Taxifolin (internal standard)	115.0 ± 0.3
Vanillic acid	83.2 ± 2.9

Having established that Strata-X 500 mg SPE cartridges provided the best extraction protocol for urine samples in terms of the recovery of phenolics, the next step was to investigate the recovery of anthocyanins in this extraction method. Of the 7 anthocyanin standards tested, all showed high and consistent recovery (Table 2-5) confirming that the 500 mg Strata-X SPE method was the most appropriate for the analysis of urine samples following an anthocyanin dietary intervention to facilitate that analysis of a comprehensive range of metabolites and parent compounds. One of the factors taken into account is that standards were only available for a subset of compounds being targeted with UHPLC-MS/MS (the full list of targeted compounds is given in appendix 1). Therefore,

it was vital to ensure the method developed was versatile in terms of being able to provide high recovery of compounds ranging in polarity, due to the broad range of the analysis being undertaken to avoid the loss of potentially important metabolites as a result of an inadequate extraction method.

Table 2-5- Recoveries of anthocyanin standards spiked in urine after SPE using Strata-X 500 mg cartridge applying the final protocol. Data is shown as mean ± standard deviation and n = 3.

Compound	Recovery (%)
Cyanidin-3-*O*-glucoside	82.4 ± 9.9
Cyanidin-3-*O*-rutinoside	87.9 ± 6.2
Delphinidin-3-*O*-glucoside	89.5 ± 7.5
Delphinidin-3-*O*-rutinoside	93.9 ± 3.6
Malvidin-3-*O*-glucoside	89.5 ± 2.6
Pelargonidin-3-*O*-glucoside	89.9 ± 6.9
Pelargonidin-3-*O*-rutinoside	93.5 ± 13.2

The final stage was to investigate the stability of urine samples post-processing. The SPE process causes a change in the composition of the urine sample, removing any salts, lipids and proteins, whilst concentrating a number of compounds. This change in the constitution of the sample may also lead to a change in stability, and it was vital to assess this before analysing samples from the human intervention study to ensure that the concentration of analytes would not change during storage. Due to the nature of the SPE protocol where extensive evaporation is required for ~ 8 hours, sample throughput was limited to the capacity of the centrifugal evaporator. This matched with requirements of UHPLC-MS/MS analysis meant that samples would need to be stored for several days' post-extraction before analysis could take place. Due to the uncertainty of equipment availability it was decided to test the stability of processed samples for a period of 3 weeks to establish whether storage at -20°C would be adequate to keep processed samples stable. This was done by processing samples in triplicate, storing for 3 weeks, and on the day of analysis processing a further 3 samples spiked with the same concentration of anthocyanin and phenolic standards to compare responses between fresh and stored samples. Overlaid chromatograms in Figure 2-11 and Figure 2-12 show that these samples produce similar chromatograms, indicating that -20°C storage for a period of up to 3 weeks was not associated with substantial loss of peaks corresponding to phenolics or anthocyanins, meaning that samples could be processed and stored before analysis, if necessary. There were some peaks for anthocyanins that were smaller post-storage compared to freshly analysed (Figure 2-12; 20-24 minutes' elution time) but these were minor components and these changes were not apparent in the most abundant anthocyanin peaks.
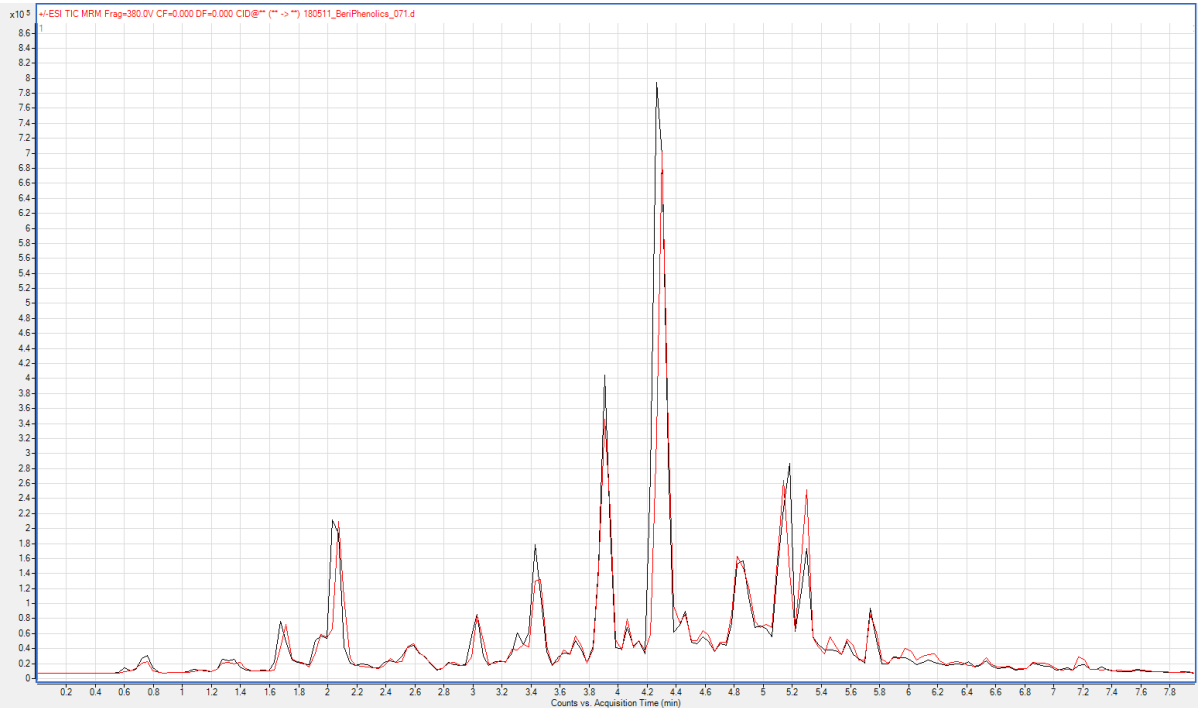
Figure 2-11- Stability test for phenolics in SPE processed urine samples
Overlaid chromatograms for the analysis of phenolics: 3-O-methyl gallic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 5-hydroxyferulic acid, caffeic acid, catechin, chlorogenic acid, cinnamic acid, epicatechin, ferulic acid, gallic acid, gallic acid-3-O-glucuronide, gallic acid-4-O-glucuronide, hippuric acid, isoferulic acid-3-O-glucuronide, isovanillic acid, methyl vanillate, PCA, PCA-3-O-glucuronide, PCA-3-O-sulfate, PCA-4-O-glucuronide, PCA-4-O-sulfate, p-coumaric acid, PGA, phloroglucinol, phloridzin, pyrogallol, quercetin, quercetin-3-O-sulfate, rutin, scopoletin, sinapic acid, syringic acid and vanillic acid (each at 2 µg/ml) in urine samples processed with SPE and stored for 3 weeks at -20°C (red) and freshly SPE processed samples (black).
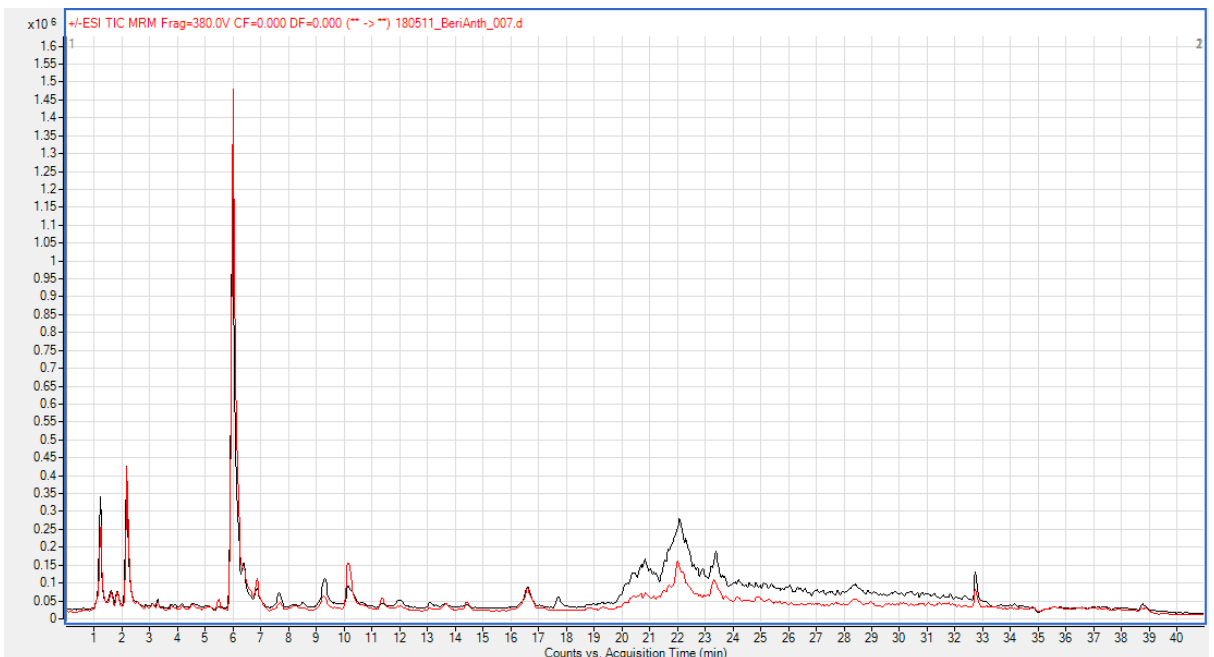
Figure 2-12- Stability test for anthocyanins in SPE processed urine samples
Overlaid chromatograms for the analysis of anthocyanins, C3G, C3Rut, D3G, D3Rut, M3G, Pel3G and Pel3Rut (each at 2 µg/ml) in urine samples processed with SPE and stored for 3 weeks -20°C (red) and freshly SPE

2.5.4 SPE of Anthocyanins and their Phenolic Metabolites in Plasma

To investigate human anthocyanin metabolism, both anthocyanins and their phenolic metabolites would be quantified in plasma as well as urine. Although it would be expected for metabolite profiles to be broadly similar between urine and plasma within individuals, it is also likely that some metabolites may be detected in plasma that have not been excreted in urine. Plasma provides a far more complex matrix than urine, containing large amounts of diverse proteins, which must be removed before reverse-phase UHPLC-MS/MS analysis. Three methods were trialled for the analysis of anthocyanins and phenolics in plasma. The SPE method developed for urine was applied to plasma using Strata-X SPE cartridges, as well as the DSC-18 which had been used by De Ferrars *et al* in urine analysis (De Ferrars, Czank, Saha, *et al.*, 2014c). The protein crash method is commonly used for the processing of serum and plasma samples before LC-MS/MS analysis to remove large quantities of protein by denaturing them, and causing them to crash out of solution. A flaw with protein crash methods is that they fail to remove phospholipids and lysophospholipids, therefore, an additional step was added where the supernatant from the protein crash would be passed through an HLB SPE cartridge which would allow the analytes of interest to pass straight through (because they remained in the methanol mobile phase rather than binding to the stationary phase) while lipids and salts would be removed (by binding the stationary phase). Although the protein crash/HLB method gave high recovery for most compounds, the variability was remarkably high making it unsuitable. DSC-18 cartridges gave poor recoveries which were the lowest of the three methods tested. As with urine, Strata-X cartridges provided the best results overall as shown in Table 2-6 and Table 2-7. High recovery was seen for the vast majority of compounds, with relatively low variability compared to the alternative methods tested (although more variable than urine), indicating that this would be a robust and reliable method to apply to clinical samples. However, sulfates had very low recovery. Interestingly, the non-sulfated forms of compounds were not elevated suggesting that the sulfate was not being removed during processing, and LC-MS/MS analysis of wash steps showed an absence of sulfates (data not shown). Additional elution steps and soaks were also trialled to test whether sulfates were being retained by the sorbent (data not shown), however no improvement in PCA sulfate elution was achieved. Therefore the elution steps and soak times were not increased as they did not provide any improvement in the recovery of these compounds.

Table 2-6- Recoveries of phenolic standards spiked into plasma using 3 different methods: Strata-X SPE, DSC-18 SPE, and a protein crash followed by HLB clean-up.
Data is shown as mean recovery (%) ± standard deviation and n = 3.Strata-X data is shown in bold as this was the method selected to be taken forward for extraction of anthocyanin metabolites in plasma samples from a human intervention study.

Compound	Strata-X	DSC-18	Protein Crash/HLB
3-O-Methyl gallic acid	**109.9 ± 11.4**	92.4 ± 17.6	96.8 ± 23.4
4-Hydroxybenzaldehyde	**90.4 ± 7.5**	94.3 ± 14.7	96.8 ± 56.3
4-Hydroxybenzoic acid	**95.5 ± 10.9**	81.3 ± 2.5	75.7 ± 8.3
5-Hydroxyferulic acid	**113.5 ± 23.2**	141.0 ± 20.4	65.3 ± 6.5
Caffeic acid	**104.8 ± 19.9**	73.5 ± 5.5	108.7 ± 64.0
Catechin	**78.4 ± 13.6**	24.2 ± 11.2	118.2 ± 27.8
Chlorogenic acid	**99.0 ± 15.1**	82.5 ± 2.0	111.6 ± 6.5
Cinnamic acid	**98.6 ± 11.1**	95.6 ± 36.3	181.5 ± 153.2
Epicatechin	**72.4 ± 14.8**	16.4 ± 0.3	125.3 ± 72.4
Ferulic acid	**105.4 ± 14.4**	85.0 ± 6.3	106.9 ± 47.0
Gallic acid	**108.2 ± 15.0**	6.8 ± 0.9	100.5 ± 60.2
Gallic acid-3-*O*-glucuronide	**58.9 ± 5.7**	11.3 ± 1.1	64.5 ± 15.5
Gallic acid-4-*O*-glucuronide	**113.6 ± 34.5**	2.7 ± 0.4	63.0 ± 25.9
Hippuric acid	**97.4 ± 6.9**	84.5 ± 17.6	69.1 ± 35.8
Isoferulic acid-3-*O*-glucuronide	**96.1 ± 21.7**	143.4 ± 105.1	96.8 ± 15.3
Isovanillic acid	**73.7 ± 23.5**	79.4 ± 11.3	98.5 ± 25.4
Methyl vanillate	**91.8 ± 15.4**	98.4 ± 8.8	136.0 ± 70.5
PCA	**113.0 ± 20.1**	26.4 ± 4.6	82.3 ± 28.3
PCA-3-*O*-glucuronide	**113.3 ± 16.3**	6.7 ± 5.1	55.0 ± 17.2
PCA-3-*O*-sulfate	**19.7 ± 1.2**	7.1 ± 3.6	232.1 ± 106.3
PCA-4-*O*-glucuronide	**86.8 ± 7.4**	7.5 ± 2.6	50.7 ± 15.7
PCA-4-*O*-sulfate	**12.2 ± 2.0**	5.4 ± 1.3	151.7 ± 91.2
p-Coumaric acid	**101.2 ± 12.1**	93.3 ± 10.1	105.6 ± 51.1

Compound	Strata-X	DSC-18	Protein Crash/HLB
PGA	**99.8 ± 22.9**	143.4 ± 68.8	132.2 ± 93.1
Phloroglucinol	**24.0 ± 5.2**	26.1 ± 2.6	140.0 ± 36.9
Pyrogallol	**99.5 ± 18.3**	7.1 ± 1.3	92.3 ± 30.3
Quercetin	**106.3 ± 5.1**	83.1 ± 9.2	132.9 ± 138.0
Quercetin-3-*O*-sulfate	**32.9 ± 19.1**	107.6 ± 47.8	78.6 ± 75.5
Rutin	**97.2 ± 15.6**	92.9 ± 5.8	96.8 ± 37.5
Sinapic acid	**113.7 ± 13.9**	112.8 ± 38.3	113.4 ± 36.8
Syringic acid	**119.6 ± 17.7**	71.9 ± 16.9	72.3 ± 39.0
Taxifolin (internal standard)	**110.1 ± 14.1**	142.1 ± 47.7	89.9 ± 16.5
Vanillic acid	**115.4 ± 18.2**	119.1 ± 9.3	100.0 ± 28.6

Table 2-7- Recoveries of anthocyanin standards spiked in plasma using 3 different methods: Strata-X SPE, DSC-18 SPE, and a protein crash followed by HLB clean-up.
Data is shown as mean recovery (%) ± standard deviation and n = 3. Strata-X data is shown in bold as this was the method selected to be taken forward for extraction of anthocyanins in plasma samples from a human intervention study.

Compound	Strata-X	DSC-18	Protein Crash/HLB
Cyanidin-3-*O*-glucoside	**82.6 ± 6.3**	49.2 ± 14.9	95.6 ± 56.5
Cyanidin-3-*O*-rutinoside	**91.0 ± 20.8**	53.7 ± 5.1	87.6 ± 54.7
Delphinidin-3-*O*-glucoside	**87.0 ± 13.0**	49.1 ± 5.5	95.0 ± 53.1
Delphinidin-3-*O*-rutinoside	**93.1 ± 17.1**	53.7 ± 10.4	92.2 ± 60.1
Malvidin-3-*O*-glucoside	**75.8 ± 8.4**	58.4 ± 6.2	81.3 ± 87.5
Pelargonidin-3-*O*-glucoside	**78.4 ± 14.2**	50.7 ± 4.2	105.6 ± 75.4
Pelargonidin-3-*O*-rutinoside	**76.6 ± 14.6**	63.7 ± 11.4	99.5 ± 80.0

2.5.5 Summary of Final Methods

The final methods developed for the analysis of anthocyanins and their phenolic metabolites in urine and plasma were as follows.

Urine and plasma samples were subjected to SPE with Strata-X reverse-phase sorbent 500mg 6ml cartridges. SPE cartridges were pre-conditioned with 6 ml methanol (0.25% formic acid) before samples were loaded at a volume of 1ml spiked with 2 µg/ml internal standard (taxifolin) and diluted with a further 1 ml water (0.1% formic acid). Cartridges were washed with two separate fill volumes of 2% methanol in water (adjusted to pH 2.4 with formic acid) for urine, or water (0.1% formic acid) for plasma. Elution was then performed with 7 ml methanol (0.25% formic acid) with a 10 minute 'soak' prior to vacuum elution. Eluted samples were then subjected to evaporation in a Speedvac centrifugal evaporator to a volume of ~200 µl, reconstituted with 200 µl water (0.1% formic acid) and spiked with a volume marker (D9-caffeine) at a concentration of 2 µg/ml.

Phenolics were analysed using LC-MS/MS with a mobile phase (A) of 10 mM ammonium acetate in water, adjusted to pH 5 with acetic acid and a mobile phase (B) of 10 mM ammonium acetate in LC-MS grade acetonitrile adjusted to pH 5 with acetic acid. The gradient used was as follows: 1% B between 0 and 1 minute, 5% B at 3 minutes, 60% B at 8 minutes, 99% B at 8.5 minutes, 1% B at 9 minutes and 1% B at 12 minutes. Samples were kept at 4°C in an auto-sampler and injected at a volume of 5 µl for urine and 1 µl for plasma onto an ACQUITY UPLC HSS T3 1.8 µm (2.1 x 100 mm) column equipped with an ACQUITY UPLC HSS T3 VanGuard pre-column (100Å, 1.8 µm, 2.1 mm i.d. x 5 mm) at 37°C, with flow rate kept at 0.4 ml/min. The MRM parameters used for compound detection of all compounds included in the analysis method can be found in appendix 1, standards included in method development are given in section 2.4.1.

Anthocyanins were analysed using a separate LC-MS/MS method to phenolics, with samples kept at 4°C in an auto-sampler and injected at a volume of 5µl for urine and 1 µl for plasma onto an ACQUITY UPLC HSS T3 1.8 um (2.1 x 100 mm) which was fitted with an ACQUITY UPLC HSS T3 VanGuard Pre-column (100Å, 1.8 µm, 2.1 mm X 5 mm)), with the column temperature kept at 37°C. Mobile phases (A) of 5% formic acid in water and (B) of 5% formic acid in acetonitrile were used with the following gradient: 5% B between 0 - 1 minutes, 10% B at 5 minutes, 25% B at 30 minutes, 95% B at 31 minutes, 95% B at 32 minutes, 5% B at 32.1 minutes and 5% B at 36 minutes. Flow rate was constant at 0.4 ml/min. Anthocyanins were detected in positive mode on Agilent's 6490 Triple Quad UHPLC-MS/MS system and MRM transitions for all compounds included in the analysis method can be found in appendix 1, along with details of compounds for which authentic standards were held.

2.6   Discussion

The objective of the work described in this chapter was to develop methods suitable for the extraction and quantification of anthocyanins and their metabolites. Consistently high recoveries were achieved for a broad range of anthocyanins and phenolic metabolites in urine (98.4 ± 18.1%) and plasma (89.4 ± 27.4%). By developing separate analysis methods for anthocyanins and their metabolites the total number of compounds included in this targeted analysis was maximised to facilitate comprehensive analysis of anthocyanins and their metabolites in urine and plasma samples, going beyond the range of metabolites targeted in previously published methods (the full range of compounds included in the method is given in appendix 1) (De Ferrars, Czank, Saha, *et al.*, 2014c). The methods described provide versatility for the quantification of an extensive range of anthocyanins and their metabolites that may be used to study absorption, distribution and metabolic fate of anthocyanins from a variety of sources in future clinical studies.

One of the major challenges faced in the development of the LC-MS/MS analysis method described in this chapter, and within the literature, is achieving suitable chromatographic separation of large mixtures of analytes in a complex matrix, further complicated by the presence of isomers such as PCA sulfates and other phase 2 conjugates. The use of ammonium acetate buffered solvents enabled the separation of PCA sulfate isomers, highlighting the importance of LC solvents in achieving chromatographic separation (Fenner and Kerns, 2011). This provided a considerable improvement over the method described by De Ferrars *et al* which did not separate PCA sulfate isomers under any of the conditions explored (De Ferrars, Czank, Saha, *et al.*, 2014c). Being able to separate positional isomers such as this is advantageous to understand the precise metabolic fate of anthocyanins *in vivo* so that we can investigate the bioactivity of the most abundant isomers to increase our knowledge of how eating anthocyanins can propagate any health effects. Although the use of ammonium acetate buffered solvents meant that two chromatographic methods were required for differential analysis of phenolic metabolites and anthocyanins at a cost to analysis time and throughput, the benefits of being able to quantify such a broad range of analytes made it worthwhile. Having two separate analysis methods meant that each was optimised for its particular set of compounds, maximising sensitivity and chromatographic resolution. A combined method would have had the consequence of compromising sensitivity for some phenolics and anthocyanins due to their different physiochemical properties which would have reduced the number of analytes that could be accurately quantified in clinical samples. For example, the use of highly acidified solvents as required for anthocyanin analysis is deleterious to the detection of some phenolics such as gallic acid. Furthermore, any MRM LC-MS/MS method is limited in the number of compounds that can be reliably searched for according to the hardware of the specific instrumentation, and having two methods greatly increases the number of analytes that can be included in the analysis

facilitating extension to phase 2 conjugates. Consequently, MRM transitions were included for sulfate and glucuronide conjugates of all major and predicted anthocyanin metabolites (appendix 1), unlike any known previously reported study. A limitation of many methods developed for these purposes is that they tend to focus either on anthocyanins or metabolites, compromising the analysis of a selection of potentially important compounds. Therefore, it would be recommended to optimise methods for each set of compounds to perform the most extensive analysis possible. Here methods for extraction and analysis of an extensive range of metabolites have been described, going beyond the current state of the art, that can be applied to clinical samples in the future to inform our understanding on the metabolic fate of anthocyanins.

SPE has previously been criticised for the time consuming nature of sample preparation, large quantities of biological samples required as well as some evidence suggesting that the requirement of evaporation can lead to degradation of anthocyanins. The method developed here shows that centrifugal evaporation did not lead to substantial anthocyanin degradation and was able to provide consistently high recovery for all anthocyanins tested, providing an improvement over previous methods (Liu *et al.*, 2017; Woodward *et al.*, 2009). Some reports have favoured simple 'dilute-and-shoot' methods for urinary analysis of anthocyanins and metabolites, for better reproducibility. However, although these methods may bypass variability derived from extraction protocols, this is at the sacrifice of sensitivity. Many anthocyanins and their metabolites are only excreted at low levels in urine and diluting samples risks reducing their concentration below the limits of detection and compromising our understanding of the absorption, distribution and metabolic fate of anthocyanins. Furthermore, these methods do not remove salts and other impurities within the urine matrix which may adversely affect ionisation in LC-MS/MS analysis reducing the sensitivity of analytical methods. Some reports have favoured protein precipitation for the preparation of plasma samples for anthocyanin analysis, however, recoveries of 55 – 89% are reported which while acceptable are lower than what has been achieved with the method described in this chapter (Liu *et al.*, 2017). However, where only very low volumes of plasma are available this may provide a suitable alternative. Here we provide a method which concentrates analytes and removes impurities for anthocyanins and metabolites with a range of physiochemical properties to facilitate comprehensive, sensitive and reproducible analysis of biological samples.

Unexpectedly, a finding of the work presented in this chapter is that contrary to manufacturer claims micro-elution formats of SPE are not able to fully reproduce the recoveries that can be achieved with traditional SPE. Although micro-elution was conducted according to manufacturer guidelines, and despite several communications with the manufacturers in an attempt to troubleshoot the issues experienced, this proved unsuccessful. Whilst high recovery was obtained for the majority of phenolic compounds, micro-elution was unable to provide acceptable recoveries for the most polar compounds included in this analysis. Therefore, although micro-elution may be

suitable for providing a high-throughput extraction method in some instances this is dependent on the compounds being analysed. These findings are reinforced by Cuervo *et al* who found that micro-elution gave inferior recoveries compared to traditional larger scale SPE (Cuervo *et al.*, 2017). Therefore, it may be recommended that whilst micro-elution may have useful applications in high-throughput methods where acceptable recoveries can be obtained for some analytes, these methods are not suitable for all compounds. In this work it was possible to directly test the efficacy of micro-elution due to our use of standards covering a broad range of polarities and molecular weights, which showed that for the most polar compounds micro-elution was unsuitable. This emphasises the importance of testing the full range of polarities of target analytes in the validation of any analytical method to prevent oversights that may result in the loss of some compounds. Furthermore, in applications where compounds are expected to only be present at low levels traditional SPE may be favoured to increase recovery and consequently sensitivity of the analysis method.

As expected, recovery of anthocyanins and phenolics in plasma was lower than urine, with higher variability, attributed to the more complex matrix provided by plasma compared to urine. However, unexpectedly PCA sulfates were poorly recovered in plasma (19.7 ± 1.2 and 12.2 ± 2.0 for PCA-3-*O*-sulfate and PCA-4-*O*-sulfate respectively). Due to the lack of commercially available phase 2 conjugates we cannot be certain that this poor recovery would be obtained with all sulfate conjugates. However, the high recovery of the parent compound PCA and its glucuronide conjugates suggests that this poor recovery can be attributed to the sulfate conjugation. Notably, De Ferrars *et al* did not report any issues in recovering sulfate conjugates in their reported extraction method using Strata-X sorbent as was done here. However, it should be acknowledged that De Ferrars *et al* used serum whereas we used plasma which has several differences for example, the addition of an anti-coagulant and the presence of fibrinogen and clotting factors (Yu *et al.*, 2011). It was hypothesised that PCA-sulfates were binding to an element of the plasma and thus were not being recovered in the extraction process. While it is possible that PCA sulfates remained bound to the sorbent, which was partly tested by conducting an additional elution step, no PCA sulfates were detected in UHPLC-MS/MS analysis (data not shown) indicating that PCA sulfates may have passed through the sorbent at earlier stages of the SPE process. However, this seemed unlikely as Strata-X sorbent had provided excellent recovery for PCA sulfates in urine, and in theory should provide similar recoveries in plasma unless these compounds interact differently with the matrix. If time had permitted, it would have been interesting to look at PCA sulfate recovery in serum to provide further evidence that this phenomenon was due to an interaction between PCA sulfates and plasma. Having said this, the work presented in this chapter combined with the findings reported by De Ferrars *et al* led to the conclusion that a large proportion of PCA

sulfates were binding to a component of plasma and thus were being lost in the sample loading step.

At the time method development commenced the methods described by De Ferrars *et al* included the vastest range of anthocyanins and phenolics, with the high recoveries seen in urine and plasma, therefore this was the method taken as a starting point, given the large range of compounds intended to be studied. However, shortly after development of the methods described in this Chapter a publication by Mueller *et al* was released which used a combination of Strata-X and Strata C18 U sorbent for SPE processing of plasma samples containing a range of anthocyanins and metabolites (including phase 2 conjugates). With this method high plasma recoveries were observed of 91 ± 15% for anthocyanins and 84 ± 15 % for phenolic metabolites (including phase 2 conjugates) (Mueller *et al.,* 2017). Had time permitted it would have interesting to revisit method development to trial this method to investigate whether this method yielded higher recoveries of PCA sulfates, and indeed higher plasma recoveries overall, this would be an important point of future work for studies that aim to investigate the plasma metabolite profiles following anthocyanin consumption.

The methods described here offer an alternative to that described by De Ferrars *et al* with several improvements over this published method. De Ferrars describe an excellent method for the extraction and analysis of a range of anthocyanins and their metabolites which is widely cited due to its extensive applications for clinical samples (De Ferrars, Czank, Saha, *et al.*, 2014c). However, the method reported here goes beyond this by extending the number of anthocyanins, known/predicted metabolites and phase 2 conjugates included in the analytical methods (appendix 1) whilst enhancing sensitivity through division of analytes between two optimised chromatographic methods, as well as providing resolution between positional sulfate isomers. This provides essential improvements to further our understanding of the detailed absorption, distribution and metabolic fate of anthocyanins. As more authentic standards become available for predicted metabolites and phase 2 conjugates it is hoped that this method can be further validated and continually applied to the analysis of biological samples post-anthocyanin consumption. Furthermore, in the future as micro-elution technologies are further developed to tolerate a more diverse range of polarities it may be possible to re-optimise the method for this format to increase throughput. Furthermore, this would facilitate the extensive targeted analysis described here when only small volumes of sample are available, bypassing the loss in sensitivity suffered in alternative 'dilute-and-shoot' and protein precipitation methods of sample preparation (Liu *et al.*, 2017).

2.7 Conclusion

The work described in this chapter describes the development of SPE-based sample processing and LC-MS/MS-based analytical methods suitable for the quantification of a large number of structurally

diverse anthocyanins and their metabolites in human urine and plasma samples. In terms of covering a broad range of anthocyanins and their metabolites, the consistency of analyte recoveries, and the generally high absolute recoveries, these methods are state-of-the-art and are suitable for application to clinical trials generating several hundreds of samples for analysis.

Chapter 3

Human Metabolism of Black Rice and Bilberry Anthocyanins

Chapter 3 : Human Metabolism of Black Rice and Bilberry Anthocyanins

3.1 Abstract

Background: Anthocyanins are a class of polyphenol reported to have bioactivity that can promote good health. However, due to their instability at a neutral pH it is hypothesised that upon consumption anthocyanins undergo extensive degradation and subsequent biotransformation. Whilst there have been studies on the metabolism of anthocyanins, there are still large gaps in our knowledge regarding the extensive range of metabolites that can be derived from different types of anthocyanin and how these may differ between individuals.

Objective: The aim of the work presented in this chapter was to perform extensive analysis of the metabolites derived *in vivo* from black rice (di-hydroxy B-ring) and bilberry (tri-hydroxy B-ring) anthocyanins in order to assess the differences in metabolite profiles between these different types of anthocyanin and the extent of human inter-individual variation.

Methods: In a randomised crossover trial, volunteers consumed capsules of bilberry extract (mainly delphinidin-type tri-hydroxy B-ring anthocyanins) or black rice extract (mainly cyanidin-type di-hydroxy B ring anthocyanins) for 28 days. Urine and plasma samples were collected pre- and post-intervention and were analysed using the solid-phase extraction (SPE) and UHPLC-MS/MS analytical methods described in Chapter 2.

Results: Fifty metabolites were identified and quantified in urine and plasma samples. Total anthocyanin metabolites displayed considerable inter-individual variation across the study population and were 510 ± 262 and 354 ± 219 µmol/24 hours post black rice and bilberry anthocyanin extract consumption respectively in urine. Furthermore, there was a clear positive correlation (R^2 = 0.259 p = 0.0008) between total urinary excretion of anthocyanin metabolites from black rice and bilberry anthocyanins for participants. For the first time, 5-hydroxyferulic acid was identified as a bilberry anthocyanin metabolite. Additionally, several other phenolic

metabolites were identified that were not reported in a study of human metabolism of penta[^{13}C]-C3G (De Ferrars, Czank, Zhang, *et al.*, 2014a). Notably, catechol and its phase 2 conjugates were shown to be the major metabolites of black rice anthocyanins, which has not been reported previously.

Conclusions and Future work: The metabolites produced from black rice and bilberry anthocyanins are distinct in both the range of compounds generated and their relative concentrations. Furthermore, the metabolism of both types of anthocyanin are subject to considerable inter-individual variation, presumably dictated by biological factors of the individual. Investigating the factors that may determine the metabolic pathways involved in anthocyanin metabolism would be an important point of future work to understand why people metabolise anthocyanins differently and the implications this may have for health.

3.2 Introduction

For anthocyanins to exert the protective effects that have been reported from multiple epidemiological studies and human dietary intervention trials, it is necessary for them to be absorbed post-ingestion and reach target sites in a bioactive form (Cassidy *et al.*, 2013; Li *et al.*, 2017; Mattioli *et al.*, 2020). However, there is ambiguity in the literature regarding the absorption, distribution, metabolism and elimination (ADME) of anthocyanins as their recovery in clinical samples has often been extremely low (Kay, 2006; Kay *et al.*, 2005; Milbury *et al.*, 2002, 2010; Mullen *et al.*, 2008). This suggests that anthocyanins are extensively metabolised post consumption and that any health effect would likely therefore be mediated by metabolites, rather than the parent compound. However, much still remains to be understood regarding the precise metabolic fate of anthocyanins and the compounds that may be responsible for any beneficial effect of an anthocyanin rich diet.

Due to their chemical instability, it is hypothesised that anthocyanins are primarily present in circulation and eliminated as degradants and metabolites. It is well known that anthocyanins will spontaneously degrade into their phenolic acid and aldehyde constituents (Kay *et al.*, 2009; Woodward *et al.*, 2009). Despite this, some human studies have not detected these chemical degradants whilst others have found them at much lower levels than other phenolic compounds, indicating that anthocyanins and their chemical degradants are metabolised extensively *in vivo* to generate an extensive range of metabolites that are potentially bioactive (Czank *et al.*, 2013; De Ferrars, Czank, Zhang, *et al.*, 2014a; Jokioja *et al.*, 2021; Nurmi *et al.*, 2009; Vitaglione *et al.*, 2007). *In vitro* and human studies indicate that phenolic acids derived from anthocyanins undergo extensive phase 2 conjugation *in vivo*, whilst there is also clear evidence for the major metabolites

of anthocyanins being derived from bacterial fermentation and absorption from the colon (Williamson and Clifford, 2010; Woodward *et al.*, 2011).

C3G is the most abundant anthocyanin in nature and incidentally is also the most extensively studied, with several studies looking at its metabolism in animals and humans. A landmark study from De Ferrars *et al* gave a detailed view into the human metabolism of C3G by feeding participants penta-[^{13}C]-C3G. This allowed metabolites to be traced back to the parent compound for conclusive evidence regarding their origin and whether they were derived from the A- or B-ring of the anthocyanin. Of the 35 metabolites identified in this study, only PGA, ferulic acid, and 4-methoxysalicylic acid were identified as A-ring-derived metabolites. The vast majority of metabolites were shown to have originated from the B ring, with compounds such as vanillic acid, hippuric acid, ferulic acid and 4-hydroxybenzaldehyde being the most abundant metabolites. Of the metabolites detected, 17 were found in serum, 31 in urine and 28 in faeces. Notably the sum of the traceable quantified metabolites did not account for 100% of the dose of penta-[^{13}C]-C3G fed, and therefore whilst this study provides invaluable information on the metabolism of C3G much remains to be understood. For example, it is highly likely that A-ring metabolites were generated that were not quantified here due to being either at levels below the limits of detection, or because they were not predicted and were therefore not being looked for using targeted LC-MS/MS methods. In keeping with the hypothesis that it is likely that degradants/metabolites are more likely to be the bioactive forms of anthocyanins, a 42-fold higher abundance of ^{13}C$_5$-metabolites relative to ^{13}C$_5$-C3G itself was reported for serum, highlighting the rapid and extensive metabolism of anthocyanins upon consumption (De Ferrars, Czank, Zhang, *et al.*, 2014a). Furthermore, it should be acknowledged that the lack of knowledge regarding the full range of anthocyanin metabolites that reach circulation has hindered our understanding of the mechanisms through which they may confer any beneficial effects (De Ferrars, Czank, Zhang, *et al.*, 2014a; Jokioja *et al.*, 2021; Kay *et al.*, 2005, 2009).

There are distinct gaps in our knowledge of the metabolism of delphinidin type tri-hydroxy B-ring anthocyanins. Goszcz *et al*, reported that delphinidin spontaneously degrades to form gallic acid (derived from the B-ring) and PGA (derived from the A-ring) (Goszcz *et al.*, 2017). Supporting this, Chen *et al* have reported that gallic acid is the main degradation product of delphinidin-3-rutinoside (D3Rut). They also demonstrated differences between cyanidin and delphinidin metabolism, with p-coumaric acid being a product of C3G and C3Rut but not D3Rut, whilst syringic acid was formed in D3Rut metabolism but not from cyanidin type anthocyanins (Chen *et al.*, 2017). Bilberries are particularly high in delphinidin-type anthocyanins, but despite gallic acid being identified as a product of delphinidin glucosides in multiple studies, it was not detected in the urine of volunteers fed a bilberry-lingonberry puree. In the same study, only a small quantity of syringic acid (a

predicted product of malvidin glucosides, also found in bilberry) was detected. This is possibly due to these products being further metabolised, giving rise to microbial metabolites that were not looked for in this study, however, other phenolic acids including vanillic, caffeic and ferulic acid were detected (Nurmi *et al.*, 2009).

It is clear from the literature that anthocyanins undergo extensive metabolism post-consumption, however, much remains to be understood regarding the full range of metabolites that may be produced. The work in this chapter covers an in depth targeted metabolite analysis (spanning both known and predicted anthocyanin metabolites) of clinical samples post consumption of two different sources of anthocyanin; black rice extract (mainly cyanidin-type anthocyanins) and bilberry extract (mainly delphinidin-type anthocyanins) (Figure 3-1), and highlights a range of metabolites produced *in vivo* that may have bioactivity.
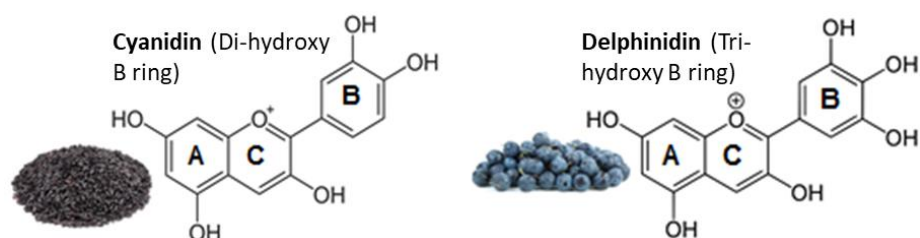
Figure 3-1- A schematic of the main types of anthocyanin (aglycone) found in black rice and bilberry; cyanidin (di-hydroxy B ring) and delphinidin (tri-hydroxy B ring) respectively.

3.3   Objectives

The overall aim of the work in this chapter was to quantify anthocyanins and their metabolites in biological samples to provide insights into the metabolic fate of dietary anthocyanins. The optimised methods described in Chapter 2 were applied to analyse urine and plasma samples from a human intervention study, in which participants had consumed different sources of anthocyanin; black rice extract (mainly cyanidin-type anthocyanins), and bilberry extract (mainly delphinidin-type anthocyanins). The specific objectives of this work were:

- Expand the metabolites included in the analytical method described in Chapter 2 to include phase 2 conjugates and other known and predicted metabolites from the literature beyond those described by De Ferrars *et al* (De Ferrars *et al.*, 2014c).

- Report the anthocyanins and their metabolites present in human urine and plasma samples after consumption of cyanidin based black rice anthocyanins and delphinidin based bilberry anthocyanins.

- Explore differences in the metabolite profiles arising from black rice anthocyanin extract versus bilberry anthocyanin extract.

- Report the degree of inter-individual variation in the absorption and urinary excretion of two sources of anthocyanins and of their phenolic metabolites.

- Investigate if there are correlations between the amounts of anthocyanins and their phenolic metabolites that are excreted in urine after consumption of black rice and bilberry anthocyanin extracts.

3.4 Methods

3.4.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Dorset, United Kingdom) unless stated otherwise. All water used was 18 MΩ/cm Milli-Q water, and solvents were LC/MS grade. Anthocyanin standards were: cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, delphinidin-3-O-glucoside, delphinidin-3-O-rutinoside, pelargonidin-3-O-glucoside, pelargonidin-3-O-rutinoside, and malvidin-3-O-glucoside (Extrasynthese, Genay, France). Internal standards and phenolic standards were: caffeine-(trimethyl-d9) (99 atom-% D, later D9-caffeine), catechol, 4-methyl catechol, 4-hydroxybenzaldehyde, 3-O-methyl-gallate, methyl vanillate, phloroglucinol, phloroglucinaldehyde, pyrogallol, quercetin, quercetin-3-O-sulfate, protocatechualdehyde, taxifolin, caffeic acid, dihydrocaffeic acid, chlorogenic acid, 2,4-dihydroxybenzoic acid, ferulic acid, dihydroferulic acid, dihydroisoferulic acid, gallic acid, hippuric acid, homoprotocatechuic acid, homovanillic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 5-hydroxyferulic acid, 3-hydroxyphenyl acetic acid, 4-hydroxyphenylacetic acid, 4-methoxysalicylic acid, isovanillic acid, 2-methoxybenzoic acid, 3-methyl hippuric acid, 4-methyl hippuric acid, , 4-hydroxyhippuric acid, p-coumaric acid, protocatechuic acid, sinapic acid, syringic acid, phloroglucinol carboxylic acid, trans-cinnamic acid, isovanillic acid and vanillic acid (Sigma-Aldrich, Dorset, United Kingdom); methyl-3,4-dihydroxybenzoate was purchased from Alfa Aesar, Haverhill, MA.; gallic acid-3-O-glucuronide, gallic acid-4-O-glucuronide, isoferulic acid-3-O-glucuronide, protocatechuic acid-3-O-glucuronide, protocatechuic acid-4-O-glucuronide, protocatechuic acid-3-O-sulfate, and protocatechuic acid-4-O-sulfate were kindly synthesized by Dr Paul Needs at The Quadram Institute Biosciences, Norwich, UK.

3.4.2 Human Study

Ethics

The study protocol was approved by both the Human Research Governance Committee of the Quadram Institute Bioscience and the East of England Cambridge and Hertfordshire Research Ethics

Committee (17/WM/0154). The trial was conducted at the Quadram Institute Bioscience, Human Nutrition Unit, Norwich, UK from September 2017 to August 2018 in compliance with the guidelines laid down in the Declaration of Helsinki. The trial is registered on a publicly-accessible database (https://clinicaltrials.gov/show/NCT03213288). All participants gave written informed consent before participating in the study. The study protocol can be found in Appendix 3 and was written by Wendy Hollands and Dr Paul Kroon at The Quadram Institute Bioscience.

Contributions to the human study were as follows. Dr Paul Kroon, Wendy Hollands and Dr Hassan Aboufarrag conceptualised the study and organised the trial. The recruitment process was managed by Wendy Hollands. Participant study days were managed by Wendy Hollands and Dr Hassan Aboufarrag. Participant health screening and blood samples were taken by Aliceon Blair. Encapsulation of bilberry and black rice extracts was carried out by Wendy Hollands and Natalia Perez. Data was collected by Dr Hassan Aboufarrag, Dr Priscilla Day-Walsh, Mark Philo and Jasmine Percival. The data presented in this chapter was collected and processed by Jasmine Percival with the help of Dr Paul Kroon, Mark Philo, Dr Paul Needs and Dr Priscilla Day-Walsh.

Study Aims:

The primary aim of the study was to investigate the effects of 28 days consumption of two types of anthocyanin-rich extract on LDL-cholesterol, along with other biomarkers of cardiovascular disease, including, PON1 genotype, HDL cholesterol, total cholesterol, bile acid profiles and triglyceride profiles. To meet this aim only individuals ≥ 45 years of age were studied who had total cholesterol ≥ 5.0 mmol/L. 24 hour urine collections were taken pre- and post-intervention along with plasma samples for all volunteers whilst, a subset (n = 24) also provided faecal samples. The structure of the study is shown in Figure 3-2.

Measurements of anthocyanins and their phenolic metabolites were secondary outcomes of the study, consequently the original study design was not powered based on these measurements, and the study population and sampling times were not optimised for measuring anthocyanin metabolism.

Study Population:

A study population of males and females were recruited from in and around Norwich, UK from either: the volunteer database held at the Quadram Institute Bioscience, GP surgeries with the help of the co-ordinators from the Primary Research Network (PCRN) – East of England, or from poster and e-mail advertisements placed on the Norwich Research Park and other suitable places. A letter of invitation (Appendix 4) along with a participant information sheet (Appendix 5) were sent to volunteers. The potential subjects were then screened for eligibility by a research nurse following

a 10 hour overnight fast, health screening and completion of an eligibility questionnaire. Basic inclusion criteria were:

- Men and women ≥ 45 years of age.
- Total cholesterol ≥ 5.0 mmol/L.

Basic exclusion criteria are given below:

- Smokers.
- Pre-existing medical conditions judged to affect the primary outcome of the study or well-being of the participant.
- Prescribed and non-prescribed medication that may affect the primary outcome of the study e.g. statins.
- Dietary supplements that may affect the study data.
- Consumption of cholesterol lowering foods e.g benecol.
- Regular/recent use of bowel cleansing techniques.
- Intent to change normal use of pro/prebiotics during the study.
- Irregular bowel movements < 3 times per week.
- Parallel participation in another study.
- Donation of blood during the study or in the 16 weeks leading up to the study.
- Relation to any member of the study team.
- Lack of capacity to provide written informed consent.
- Pregnancy during the study or within the 12 months leading up to the study.

Study Design

The study was conducted as a randomised, double-blind, placebo-controlled three-arm cross-over trial; a schematic of the study design is shown in Figure 3-2. The primary aim of the study was to investigate the effects of 28 days consumption of two types of anthocyanin-rich extract on biomarkers of cardiovascular disease. The three treatment arms were as follows:

- Bilberry extract, providing 320 mg/day anthocyanins (mainly tri-hydroxy-B ring type)
- Black rice extract, providing 320 mg/day anthocyanins (mainly di-hydroxy-B ring type)
- A placebo control of microcrystalline cellulose

The capsules in which each treatment was delivered were: opaque, cellulose-based, suitable for oral consumption, had identical appearance and released their contents within 15 min of reaching the stomach (K-caps vegetarian capsules; GoCap). Each treatment was allocated a letter (A, B or C) by a member of staff not involved with the trial, to ensure all members of the study team remained

blinded for the duration of the trial. The randomization sequences list for treatment order were generated using computer-generated block randomization (randomization.com). Each time a participant was successfully recruited onto the study, the order of intervention was assigned by allocating the participant to the next sequence of letters from the list generated previously. All those involved in the study including: researchers, research nurses and the participants were blinded to the identity of each treatment.

The subjects were asked to restrict their intake of berry fruits to a combined maximum of 3 portions per week for 2 weeks prior to the start of each treatment period and for the 28 day treatment period, including the washout period (minimum 4 weeks). Additionally, they were asked to completely exclude berry fruits from their diet for the 24 hours preceding study days at the start and end of each treatment period. A list of restricted fruits was provided to each participant (Appendix 5). The participants were asked to fast overnight for a minimum of 10 hours and only drink water before undergoing the baseline and post intervention assessment. On day 1 of the treatment period, the baseline assessment was conducted, and a 35 ml blood sample was collected from fasted participants. Participants then consumed 4 capsules (which equated to the daily dose of each treatment) once daily in the morning for 28 days. To assist with compliance, participants were provided with a capsule checklist to complete daily and were asked to return the unused capsules with the record sheet at the end of each treatment period. Participants were also contacted 2-weeks into each treatment to check-up on progress. Compliance was measured using the record sheet and by counting the unused capsules returned at the end of each treatment. Participants who ingested less than 80% of the capsules over the entire treatment period were to be withdrawn from the study. Compliance regarding dietary restriction was assessed through measuring anthocyanins and their metabolites in urine at the baseline. On day 29, the day after the final capsules were consumed, a further 35 ml blood was taken from fasted participants. Following the end of each phase, a minimum 4 weeks washout period was required before starting the next phase, an overview of the study design is shown in Figure 3-2. It should be noted that the primary outcome measure for this study was the change of LDL-cholesterol over the course of each treatment period, and this is the measurement the study was powered on. Measurements of anthocyanins and their phenolic metabolites were secondary outcomes of the study, and the original study design was not powered based on these measurements.
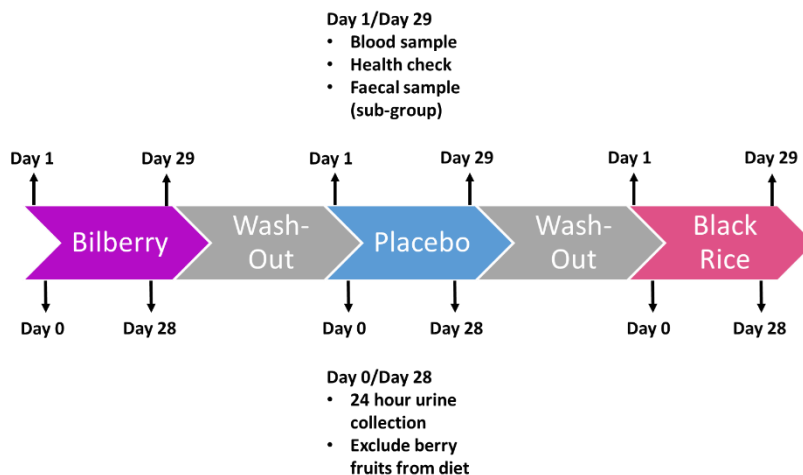
Figure 3-2- Schematic of the human intervention study.

Capsule Preparation:

The anthocyanins chosen to be studied were derived from bilberry and black rice extracts. Black rice extract was chosen as a rich source of cyanidin based anthocyanins (the most common dietary anthocyanins). Bilberry (*Vaccinium myrtillus*) extract was chosen a rich source of delphinidin based anthocyanins, to facilitate investigation of different effects between di- and tri-hydroxy anthocyanins. Bilberry and black rice extract powders were supplied by the Beijing Gingko Group (BGG) and produced commercially. The BGG product specification sheets for both types of extract can be found in Appendix 6. The anthocyanin content of bilberry and black rice extracts were analysed by Hassan Aboufarrag using HPLC-DAD, and used to calculate the dose of each extract required per capsule (Aboufarrag, 2019). The extracts were encapsulated in a food grade kitchen by Wendy Hollands and Natalia Perez. A subset of sealed bottles containing filled capsules for each treatment and placebo arm were sent to an accredited laboratory for microbial safety analysis prior to human consumption.

Analysis of Bilberry and Black Rice Anthocyanin Extracts

The determination of anthocyanins, phenolics and procyanidins in the bilberry and black rice extracts was carried out by a QIB PhD student Hassan Aboufarrag under the supervision of Dr Paul Kroon and has been reported elsewhere (Aboufarrag, 2019). A summary of the methods and results of the compositional analysis is provided here because of the importance of this information in the human dietary intervention and the analysis of anthocyanin metabolites described in this chapter. Anthocyanins in bilberry and black rice extracts were analysed by reverse phase HPLC/MS. 20 µl of sample was injected onto Kinetex XB-C18 column (100× 4.6 mm; particle size 2.6 µm) at 40°C. The

separation method was carried out using Agilent 1100 series system (HP1100 Agilent Technologies, Waldbronn, Germany) equipped with G1956B single quadrupole mass spectrometer. The mobile phase consisted of 5% aqueous formic acid (eluent A) and 5% formic acid in acetonitrile (eluent B). Samples were eluted with a gradient consisting of 5% B at 0 min, 7% B at 10 min, 10% B at 15 min, 13% B at 16:50 min, 20% B at 18 min, 5% B at 20 min to 25 min and flow rate was 1 ml per min. The anthocyanins were detected by diode array detection (DAD) at 520 nm. Detected anthocyanins are shown in Table 3-1 and Table 3-2 for bilberry and black rice extracts, respectively.

The levels of other phenolics was also analysed for both extracts. In bilberry extract, non-anthocyanin phenolic content was very low, accounting for only 3.5% powder, with this mainly consisting of chlorogenic acid; although gallic acid, caffeic acid, catechin, epicatechin, phloridzin, myricetin, quercetin, quercetin-3-glucoside and quercetin-3-rhamanoside were also detected. In black rice extract only trace amounts of phenolics including PCA and PGA were detected and were not quantified. Procyanidins were also analysed, using normal-phase HPLC. Weak signals for procyanidins with their degree of polymerisation from 2 - 6 were detected in bilberry extract, accounting for 27.6 mg (g powder)$^{-1}$. Whilst only traces of monomeric procyandins and no oligomeric procyanidins were detected in black rice extract. Details of this analysis can be found in the PhD thesis of Hassan Aboufarrag (Aboufarrag, 2019).

Table 3-1- Anthocyanin content in bilberry extract
Data shown as mean ± standard deviation and n = 3. The percentage of total anthocyanins was calculated using the mean for each analyte. Table reproduced with permission from (Aboufarrag, 2019).

Compound	mg (g powder)$^{-1}$	% of Total Anthocyanins
Cyanidin-3-*O*-galactoside	40.2 ± 0.1	9.2
Cyanidin-3-*O*-glucoside	41.8 ± 0.1	9.6
Delphinidin-3-*O*-arabinoside	60.7 ± 0.1	13.9
Delphinidin-3-*O*-galactoside	65.8 ± 0.1	15.1
Delphinidin-3-*O*-glucoside	65.3 ± 0.1	15.0
Peonidin-3-*O*-arabinoside	2.3 ± 0.0	0.5
Peonidin-3-*O*-galactoside	3.6 ± 0.0	0.8
Peonidin-3-*O*-glucoside	13.9 ± 0.1	3.2
Petunidin-3-*O*-arabinoside	11.8 ± 0.0	2.7
Petunidin-3-*O*-galactoside	55.3 ± 0.1	12.7
Petunidin-3-*O*-glucoside	37.9 ± 0.1	8.7
Malvidin-3-*O*-arabinoside	7.4 ± 0.1	1.7
Malvidin-3-*O*-galactoside	7.8 ± 0.0	1.8
Malvidin-3-*O*-glucoside	23.2 ± 0.1	5.3
Total Anthocyanins	436 ± 0.8	-

Table 3-2- Anthocyanin content in black rice extract
Data shown as mean ± standard deviation and n = 3. The percentage of total anthocyanins was calculated using the mean for each analyte. Table reproduced with permission from (Aboufarrag, 2019).

Compound	mg (g powder)$^{-1}$	% of Total Anthocyanins
Cyanidin-3,5-*O*-diglucoside	3.87 ± 0.0	1.2
Cyanidin-3-*O*-glucoside	278.6 ± 0.6	89.3
Cyanidin-3-*O*-(6"-*O*-*p*-coumaryl glucoside	4.4 ± 0.0	1.4
Peonidin-3-*O*-glucoside	22.4 ± 0.1	7.2
Peonidin-3-*O*-(6"-*O*-*p*-coumaryl) glucoside	5.9 ± 0.1	1.9
Total Anthocyanins	312.2 ± 0.8	-

Urine Sample Collection

Participants were asked to collect all urine passed in the 24 hours before starting each treatment and for 24 hours after their last capsules of each treatment into a pooled container. Each container had 1 g ascorbic acid added as an antioxidant. 2 ml aliquots of pooled urine samples were stored at -80°C with 1% HCl to maintain anthocyanin stability until samples were thawed for analysis.

Plasma Sample Collection

Whole blood was collected into EDTA tubes and centrifuged immediately at 2500 x g for 10 minutes to obtain plasma. 1 ml aliquots of plasma were stored with 1% HCl at -80°C prior to thawing for analysis.

3.4.3 Anthocyanin and Metabolite Analysis in Urine and Plasma

Urine and plasma samples were processed using the final optimised SPE methods described in Chapter 2, before being analysed via two separate UHPLC-MS/MS methods, one for anthocyanins, and one for phenolics, both described in Chapter 2. Matrix matched standard curves were plotted for each compound (listed in the chemicals and reagents section) and were used to find the response factor for individual compounds, the concentration of each compound in the final concentrated post-SPE sample was calculated according to formula 1 (see Chapter 2), adjusted according to the peak area of the volume control standard, and then the total volume of urine was used to calculate the total urinary amount excreted in 24 hours.

Expansion of Anthocyanins and Phenolics in Analysis

In addition to the compounds listed in Chapter 2, the number of anthocyanins and their metabolites included in the analytical methods were extended to include more compounds and phase 2 conjugates following literature searches on previous studies on anthocyanin metabolism and discussions with Dr Paul Kroon, Mark Philo, Dr Paul Needs and Dr Johanna Jokioja regarding the

potential metabolic routes of these compounds (Chen *et al.*, 2017; He *et al.*, 2009; López De Las Hazas *et al.*, 2017; Ludwig *et al.*, 2015; Mueller *et al.*, 2017; Nurmi *et al.*, 2009; Overall *et al.*, 2017; Vanzo *et al.*, 2013). Notably both of these analytical methods were also applied to another study on purple potato anthocyanins and therefore, particularly in the case of anthocyanins, several compounds were included in the MRM method that were not necessarily expected in this analysis (Jokioja *et al.*, 2021).

The anthocyanins added to the analytical method were: cyanidin, cyanidin-arabinoside, cyanidin-arabinoside-sulfate, cyanidin-arabinoside-glucuronide, cyanidin-diglucoside, cyanidin-diglucoside-sulfate, cyanidin-diglucoside-glucuronide, cyanidin-caffeoyl-rutinoside, cyanidin-caffeoyl-rutinoside-glucoside, cyanidin-caffeoyl-rutinoside-glucoside-glucuronide, cyanidin-caffeoyl-rutinoside-glucoside-sulfate, cyanidin-coumaroyl-rutinoside, cyanidin, coumaroyl-glucoside, cyanidin-coumaroyl-rutinoside-glucoside, cyanidin-coumaroyl-rutinoside-glucoside-glucuronide, cyanidin-coumaroyl-rutinoside-glucoside-sulfate, cyanidin-feruloyl-rutinoside, cyanidin-feruloyl-rutinoside-glucoside, cyanidin-feruloyl-rutinoside-glucoside-glucuronide, cyanidin-feruloyl-rutinoside-glucoside-sulfate, cyanidin-glucoside-glucuronide, cyanidin-glucoside-sulfate, cyanidin-glucuronide, cyanidin-rutinoside-glucoside, cyanidin-sulfate, methyl-cyanidin-glucoside, methyl-cyanidin-glucuronide, methyl-cyanidin-glucuronide-glucoside, delphinidin, delphinidin-arabinoside, delphinidin-arabinoside-sulfate, delphinidin-arabinoside-glucuronide, delphinidin-diglucoside, delphinidin-diglucoside-sulfate, delphinidin-diglucoside-glucuronide, delphinidin-caffeoyl-rutinoside, delphinidin-caffeoyl-glucoside, delphinidin-caffeoyl-rutinoside-glucoside, delphinidin-caffeoyl-rutinoside-glucoside-sulfate, delphinidin-coumaroyl-rutinoside, delphinidin-coumaroyl-rutinoside-glucoside, delphinidin-coumaroyl-rutinoside-glucoside-glucuronide, delphinidin-coumaroyl-rutinoside-glucoside-sulfate, delphinidin-feruloyl-rutinoside, delphinidin-feruloyl-rutinoside-glucoside, delphinidin-glucoside-glucuronide, delphinidin-glucoside-sulfate, delphinidin-glucuronide, delphinidin-rutinoside-glucoside, delphinidin-sulfate, methyl-delphinidin-glucoside, methyl-delphinidin-glucoside-glucuronide, methyl-delphinidin-glucuronide, malvidin, malvidin-arabinside, malvidin-caffeoyl-rutinoside, malvidin-caffeoyl-rutinoside-glucoside, malvidin-coumaroyl-rutinoside, malvidin-coumaroyl-rutinoside-glucoside, malvidin-coumaroyl-rutinoside-glucoside-glucuronide, malvidin-coumaroyl-rutinoside-glucoside-sulfate, malvidin-feruloyl-rutinoside, malvidin-feruloyl-rutinoside-glucoside, malvidin-feruloyl-rutinoside-glucoside-glucuronide, malvidin-feruloyl-rutinoside-glucoside-sulfate, malvidin-glucoside-glucuronide, malvidin-glucoside-sulfate, malvidin-glucuronide, malvidin-rutinoside, malvidin-rutinoside-glucoside, malvidin-sulfate, methyl-malvidin-glucoside, methyl-malvidin-glucoside-glucuronide, methyl-malvidin-glucuronide, pelargonidin, perlargonidin-arabinoside, pelargonidin-caffeoyl-rutinoside, pelargonidin-caffeoyl-rutinoside-glucoside, pelargonidin-coumaroyl-rutinoside,

pelargonidin-coumaroyl-rutinoside-glucoside, pelargonidin-coumaroyl-rutinoside-glucoside, pelargonidin-coumaroyl-rutinoside-glucoside-glucuronide, pelargonidin-feruloyl-rutinoside, pelargonidin-feruloyl-rutinoside-glucoside, pelargonidin-glucoside-glucuronide, pelargonidin-glucoside-sulfate, pelargonidin-glucuronide, pelargonidin-rutinoside-glucoside, pelargonidin-sulfate, methyl-pelargonidin-glucoside, methyl-pelargonidin-glucoside-glucuronide, methyl-pelargonidin-glucuronide, peonidin, peonidin-arabinside, peonidin-caffeoyl-rutinoside, peonidin-caffeoyl-rutinoside-glucoside, peonidin-caffeoyl-rutinoside-glucoside-glucuronide, peonidin-caffeoyl-rutinoside-glucoside-sulfate, peonidin-coumaroyl-rutinoside, peonidin-coumaroyl-rutinoside-acetyl-glucoside, acetyl-peonidin-coumaroyl-rutinoside-glucoside, peonidin-coumaroyl-rutinoside-glucoside, peonidin-coumaroyl-rutinoside-glucoside-glucuronide, peonidin-coumaroyl-rutinoside-glucoside-sulfate, peonidin-feruloyl-rutinoside, peonidin-feruloyl-rutinoside-glucoside, peonidin-feruloyl-rutinoside-glucoside-glucuronide, peonidin-feruloyl-rutinoside-glucoside-sulfate, peonidin-glucoside, peonidin-glucoside-glucuronide, peonidin-glucoside-sulfate, peonidin-glucuronide, peonidin-rutinoside, peonidin-rutinoside-glucoside, peonidin-sulfate, methyl-peonidin-glucoside, methyl-peonidin-glucoside-glucuronide, methyl-peonidin-glucuronide, petunidin, petunidin-arabinside, petunidin-caffeoyl-rutinoside, petunidin-caffeoyl-rutinoside-glucoside, petunidin-caffeoyl-rutinoside-glucoside-glucuronide, petunidin-caffeoyl-rutinoside-glucoside-sulfate, petunidin-coumaroyl-rutinoside, acetyl-petunidin-coumaroyl-rutinoside-glucoside, petunidin-coumaroyl-rutinoside-glucoside, petunidin-coumaroyl-rutinoside-glucoside-glucuronide, petunidin-coumaroyl-rutinoside-glucoside-sulfate, petunidin-feruloyl-rutinoside, petunidin-feruloyl-rutinoside-glucoside, petunidin-feruloyl-rutinoside-glucoside-glucuronide, petunidin-feruloyl-rutinoside-glucoside-sulfate, petunidin-glucoside, petunidin-glucoside-glucuronide, petunidin-glucoside-sulfate, petunidin-glucuronide, petunidin-rutinoside, petunidin-rutinoside-glucoside, petunidin-sulfate, methyl-petunidin-glucoside, methyl-petunidin-glucoside-glucuronide and methyl-petunidin-glucuronide.

The phenolics added to the analytical method in addition to those reported in Chapter 2 were: benzoic acid, 2,4-dihydroxybenzoic acid, dihydroxybenzoic acid glucuronide, dihydroxybenzoic acid sulfate, dimethoxyphenyl glucuronide, gallic acid sulfate, 3/4-hydroxybenzoic acid glucuronide, 3/4-hydroxybenzoic acid sulfate, isovanillic acid glucuronide , 2-methoxybenzoic acid, methoxysalicylic acid, methyl syringate, methyl-3,4-dihydroxybenzoate, methyl-3,4-dimethoxybenzoate, methyl-gallic acid glucuronide, methyl-gallic acid sulfate, syringic acid glucuronide, syringic acid sulfate, trimethoxygallic acid, vanillic acid glucuronide , vanillic acid sulfate, caffeic acid glucuronide, caffeic acid sulfate, chlorogenic acid glucuronide, chlorogenic acid sulfate, cinnamic acid glucuronide, cinnamic acid sulfate, p-coumaric acid glucuronide, p-coumaric acid sulfate, dimethoxycinnamic acid, feruilc acid glucuronide, 5-hydroxyferulic acid glucuronide, 5-

hydroxyferulic acid sulfate, isoferulic acid, isoferulic acid sulfate, sinapic acid glucuronide, sinapic acid sulfate, trihydroxycinnamic acid, trihydroxycinnamic acid glucuronide, trihydroxycinnamic acid sulfate, trimethoxycinnamic acid, hydroxyhippuric acid, 3-methylhippuric acid, 4-methylhippuric acid, catechol, catechol glucuronide, catechol sulfate, dimethoxyphenol, dimethoxyphenyl sulfate, methoxycatechol glucuronide, methoxycatechol sulfate, methoxyphenol, methoxyphenyl glucuronide, methoxyphenyl sulfate, methoxycatechol, 4-methylcatechol, methylcatechol glucuronide, methylcatechol sulfate, methylpyrogallol, methylpyrogallol glucuronide, methylpyrogallol sulfate, pyrogallol glucuronide, pyrogallol sulfate, 3,5-dihydroxy-4-methoxybenzaldehyde, methoxybenzaldehyde, syringaldehyde, vanillaldehyde, vanillaldehyde glucuronide, vanillaldehyde sulfate, dihydroxymandelic acid , 3,4-dihydroxy-5-methoxyphenyl acetic acid, homoprotocatechuic acid glucuronide, homoprotocatechuic acid sulfate, homosyringic acid, homovanillic acid glucuronide , homovanillic acid sulfate, hydroxymandelic acid, 4-hydroxy-3-methoxy-mandelic acid, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, hydroxyphenylacetic acid glucuronide, hydroxyphenylacetic acid sulfate, mandelic acid, trihydroxyphenyl acetic acid, dihydrocaffeic acid, dihydrocaffeic acid glucuronide, dihydrocaffeic acid sulfate, dihydrocoumaric acid glucuronide , dihydrocoumaric acid sulfate, dihydro(iso)ferulic acid, dihydro(iso)ferulic acid glucuronide, dihydro(iso)ferulic acid sulfate, dihydrosinapic acid, dihydrosinapic acid glucuronide, dihydrosinapic acid sulfate, 3,4-dihydroxy-5-methoxyphenylpropanoic acid, dihydroxyphenylpropionic acid, dihydroxyphenylpropionic acid glucuronide, dihydroxyphenylpropionic acid sulfate, hydroxyphenyl propionic acid , hydroxyphenyl propionic acid glucuronide, hydroxyphenyl propionic acid sulfate, trihydroxyphenylpropanoic acid and phloroglucinolcarboxylic acid.

Details of MRM transitions for both anthocyanins and their metabolites, along with their retention times under the LC-MS/MS conditions described in Chapter 2 are given in Appendix 1.

3.4.4 Statistics

Statistical analysis was performed in GraphPad Prism (version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Repeated measures ANOVA was used with a Tukey post-hoc test to compare means for all phases of the study, values of $p \leq 0.05$ were considered statistically significant.

Heat-maps displaying the within-subject data, centred and standardised, for all samples included in the analysis were produced in R (v.3.6.2) [https://CRAN.R-project.org], using the mixOmics package (v 6.10.9) by Dr Marianne Defernez. Hierarchical clustering of the variables was carried out using the Ward method and variables were ordered accordingly in the heat-map. Samples were ordered

by group indicating time-point and diet. For phenolic metabolites, Box-Cox transformation was applied using the caret package.

3.5 Results

3.5.1 Anthocyanins in Plasma and Urine Samples

Anthocyanins were not detected in any of the 306 fasted blood samples analysed of volunteers that had consumed anthocyanin capsules for the past 28 days, whether collected on day 0 (pre-intervention) or day 29 (post-intervention). This was not surprising since (i) the day 0 samples were not expected to contain anthocyanins as the participants had been asked to exclude anthocyanin-rich foods from their diets, and (ii) the day 29 blood samples were taken almost 24 hours after the participants had consumed the anthocyanin capsules on day 28, and it has been consistently shown that anthocyanins are rapidly eliminated from circulation and would not be expected to be present at detectable concentrations after this time period (De Ferrars, Czank, Zhang, *et al.*, 2014a).

Anthocyanins were detected in all urine samples both pre- and post- intervention, however, anthocyanins were significantly increased post black rice and bilberry anthocyanin extract consumption, an example chromatogram of urine post bilberry anthocyanin extract consumption is shown in Figure 3-3. Table 3-3 shows the mean and standard deviation of anthocyanins quantified in all phases (black rice, bilberry and placebo) pre-and post- intervention. Total anthocyanins detected in urine post-black rice and post bilberry anthocyanin extract consumption was 639 ± 539 and 299 ± 305 nmol/24hrs respectively, whilst pre-intervention and post-placebo urines had a mean anthocyanin content of just 22.2 – 30.2 nmol/24hours. This clearly shows that the consumption of each extract elevated urinary anthocyanin levels considerably in participants, as expected. Furthermore, the anthocyanins reported in Table 3-3 largely mirror the anthocyanins in each of the fed anthocyanin extracts shown in Table 3-1 and Table 3-2. Urine samples after black rice anthocyanin extract consumption only show elevations in cyanidin and peonidin based anthocyanins (the only two type of anthocyanins present in the extract). In contrast, a far more diverse profile of anthocyanins were elevated in urine after bilberry anthocyanin extract consumption, reflecting the more diverse anthocyanin profile of the extract: Peo3A, M3A, Pet3G, M3G, petunidin glucuronide, delphinidin glucuronide, cyanidin glucuronide, D3G, D3A, Peo3G and C3G were all substantially increased in urine post-bilberry consumption relative to samples taken in all other phases of the study.

Table 3-4 highlights the compounds that were significantly different post bilberry or black rice consumption compared to pre-intervention and post placebo levels shown in Table 3-3. Here pre-intervention levels of compounds have been subtracted from post-intervention levels to illustrate the quantities of each compound derived from the treatment. Clearly the vast majority of

anthocyanins were derived from the extracts consumed with only small amounts originating from the background diet.
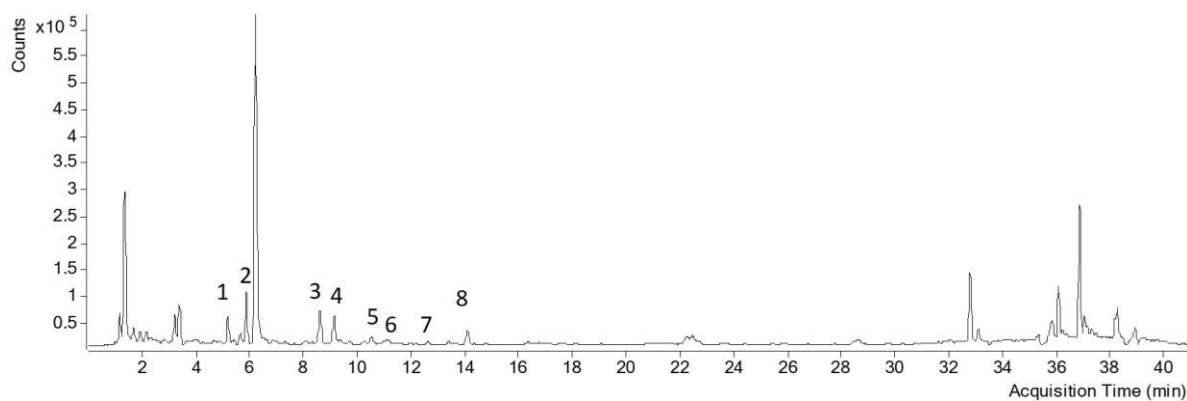
Figure 3-3- An example of a total ion current (TIC) chromatogram obtained for urinary anthocyanin analysis from one participant post-bilberry anthocyanin extract consumption.

Peaks are labelled as follows: (1) D9-caffeine (2) delphinidin-3-O-glucoside, (3) petunidin-3-O-glucoside, (4) taxifolin, (5) cyanidin glucuronide, (6) peonidin-3-O-glucoside, (7) malvidin-3-O-gluoside and (8) malvidin-3-O-arabinoside.

Table 3-3- Anthocyanins quantified in urine samples pre- and post- all three treatment phases in the human intervention study: placebo, black rice extract and bilberry extract. Data is shown as nmol/24hrs with standard deviation and n = 52.

Compound	Pre-Placebo	Post-Placebo	Pre-Black Rice	Post-Black Rice	Pre-Bilberry	Post-Bilberry
Cyanidin-3-*O*-glucoside	2.4 ± 6.0	2.1 ± 3.9	2.5 ± 4.1	**215 ± 159 *****	2.6 ± 4.4	**18.1 ± 14.1**
Cyanidin-3-*O*-rutinoside	0.5 ± 0.6	0.7 ± 2.0	0.4 ± 0.7	**4.7 ± 6.5 *****	0.9 ± 3.3	**0.8 ± 1.8**
Delphinidin-3-*O*-glucoside	2.9 ± 2.8	2.9 ± 3.9	2.8 ± 2.8	**5.0 ± 9.1**	3.4 ± 4.1	**19.2 ± 13.1 *****
Delphinidin-3-*O*-arabinoside[a]	1.9 ± 2.9	1.8 ± 2.1	1.6 ± 1.9	**1.7 ± 1.6**	2.2 ± 2.9	**11.3 ± 8.2 *****
Malvidin-3-*O*-glucoside	8.1 ± 17.1	4.6 ± 11.0	5.5 ± 14.5	**12.6 ± 61.6**	9.0 ± 29.4	**37.2 ± 29.4 ****
Malvidin-3-*O*-arabinoside[b]	0.7 ± 1.0	0.5 ± 0.5	0.6 ± 0.5	**1.3 ± 5.1**	0.5 ± 0.4	**9.8 ± 7.6 *****
Petunidin-3-*O*-glucoside[a]	1.3 ± 2.0	0.9 ± 1.1	1.0 ± 1.1	**1.5 ± 3.2**	1.4 ± 3.5	**16.5 ± 12.2 *****
Peonidin-3-*O*-glucoside[c]	5.9 ± 8.4	4.8 ± 5.8	4.7 ± 4.9	**374 ± 284 *****	9.0 ± 26.1	**53.7 ± 38.2**
Peonidin-3-*O*-arabinoside[c]	0.5 ± 0.5	0.6 ± 0.8	0.6 ± 0.7	**0.7 ± 1.3**	0.5 ± 0.3	**26.5 ± 19.1 *****
Pelargonidin-3-*O*-glucoside	3.3 ± 10.1	2.2 ± 6.4	2.0 ± 3.9	**13.8 ± 74.6**	1.3 ± 1.1	**4.1 ± 15.5**
Cyanidin-*O*-glucuronide[c]	0.4 ± 2.0	1.1 ± 4.7	0.3 ± 1.8	**trace**	0.1 ± 0.8	**59.0 ± 43.4 *****
Delphinidin-*O*-glucuronide[a]	trace	0.1 ± 0.4	trace	**0.1 ± 0.8**	0.1 ± 0.3	**19.0 ± 14.1 *****
Petunidin-*O*-glucuronide[a]	0.2 ± 1.0	trace	0.6 ± 2.9	**0.3 ± 1.7**	trace	**24.1 ± 17.6 *****
Total Anthocyanins	28.0 ± 36.2	22.2 ± 20.9	22.5 ± 25.0	**631 ± 539 *****	30.2 ± 61.6	**299 ± 205 *****

Statistical analysis carried out by repeated measures ANOVA with a Tukey's post-hoc test to compare all pairs of means *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$

[a]Quantifified with delphinidin-3-*O*-glucoside

[b]Quantified with malvidin-3-*O*-glucoside

[c]Quantified with cyanidin-3-*O*-glucoside

Only compounds detected with a signal to noise ratio ≥ 5 were quantified, compounds with a trace amount were present at levels above the limits of detection (signal to noise ration ≥ 3) but below the limits of quantification.

Table 3-4- Difference in urinary anthocyanins (pre- and post- intervention) following a dietary intervention with bilberry and black rice anthocyanins.

The mean difference in anthocyanins quantified in urine (pre-and post- intervention) following black rice and bilberry anthocyanin extract consumption for 28 days, for anthocyanins with statistically significantly different concentrations between pre-intervention and post-placebo samples. Pre-intervention samples were subtracted from post-intervention for every participant, the mean and standard deviation is presented for all 52 participants in nmol/24hrs.

Compound	Black Rice	Bilberry
Cyanidin-3-*O*-glucoside	212 ± 159	-
Cyanidin-3-*O*-rutinoside	4.3 ± 6.7	-
Peonidin-3-*O*-glucoside[c]	369 ± 284	-
Delphinidin-3-*O*-glucoside	-	15.8 ± 11.8
Delphinidin-3-*O*-arabinoside[a]	-	9.1 ± 7.7
Malvidin-3-*O*-glucoside	-	28.2 ± 29.4
Malvidin-3-*O*-arabinoside[b]	-	9.3 ± 7.5
Petunidin-3-*O*-glucoside[a]	-	15.1 ± 11.6
Peonidin-3-*O*-arabinoside[c]	-	26.0 ± 19.2
Cyanidin-*O*-glucuronide[c]	-	58.9 ± 43.8
Delphinidin-*O*-glucuronide[a]	-	19.0 ± 14.2
Petunidin-*O*-glucuronide[a]	-	24.1 ± 17.7
Total Anthocyanins	608 ± 538	269 ± 186

[a]Quantifified with delphinidin-3-*O*-glucoside
[b]Quantified with malvidin-3-*O*-glucoside
[c]Quantified with cyanidin-3-*O*-glucoside

Figure 3-4 shows a heat-map of the data presented in Table 3-3, here the data for every volunteer is shown and coloured according to the relative concentration of each anthocyanin quantified in urine samples, with orange-red indicating high concentrations. This figure visually shows the close similarity between pre-intervention and post-placebo urine samples, highlighting that any low levels of anthocyanins detected are likely to be consistent artefacts of the background diet. Interestingly, Figure 3-4 exposes inter-individual variation in urinary anthocyanin content post-black rice and post-bilberry anthocyanin extract consumption, whereby the extent to which specific anthocyanins are elevated is not uniform across the study population. There are several factors that may explain this variability amongst the study population such as: differences in anthocyanin absorption, differences in metabolism and differences in when/how capsules were taken.
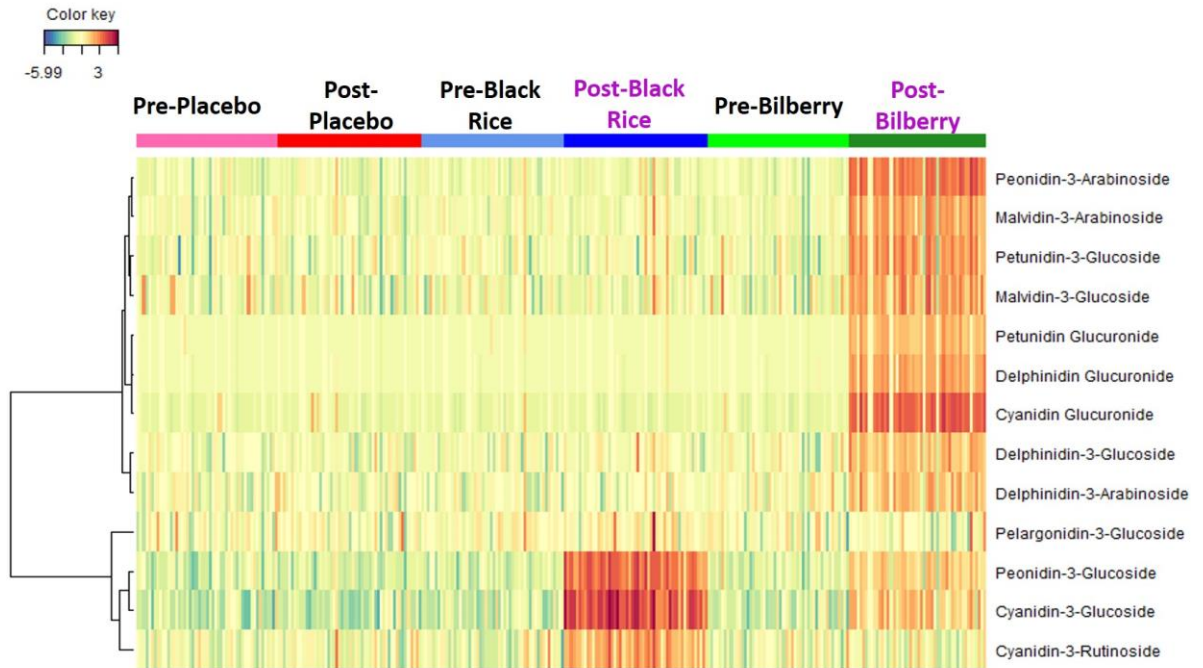
Figure 3-4- Heatmap of urinary anthocyanins
Scaled and centred data showing the within subject urinary anthocyanins before and after each treatment phase. Hierarchical clustering of the variables was carried out using the Ward method. Samples are ordered by study phase indicating time-point and diet, post black rice and bilberry treatments are highlighted in purple. The heat-map describes the concentration of each compound in urine standardised to the range of [-5.99, +5.99]. Red colour indicates high amounts of the compound were detected and blue indicates only low levels were present.

To visualise the global variation across the study population frequency distributions were plotted to show how participants varied in terms of total anthocyanins quantified in 24 hour urine collections for each treatment. Both treatments show a normal distribution, with some individuals having particularly low or high levels of anthocyanins measured in urine. It was hypothesised that there would be a correlation between the amount of anthocyanins excreted by each participant after consumption of the black rice anthocyanin extract and the bilberry anthocyanin extract, i.e. if a participant exhibited a relatively high excretion of black rice anthocyanins, they would also excrete a relatively high amount of anthocyanins after bilberry extract consumption. This was investigated by means of a linear regression analysis of total anthocyanins between treatments, shown in Figure 3-7.
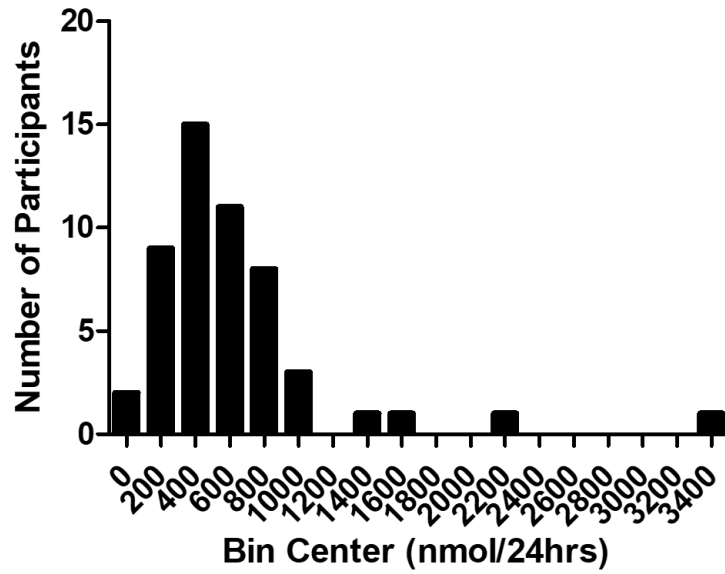
Figure 3-5- Frequency distribution of total urinary anthocyanins post black rice anthocyanin extract consumption for 52 participants.
Each bin spans 200 nmols, where for example, 200 on the X-axis covers the range 100 – 300 nmols.
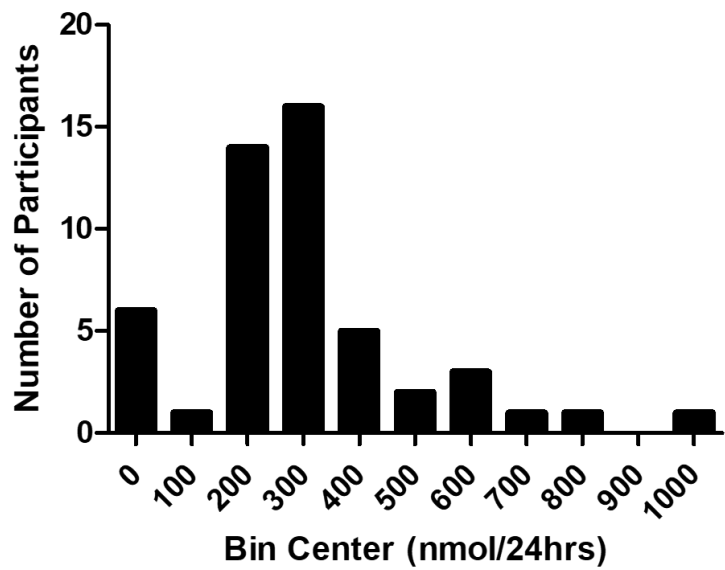
Figure 3-6- Frequency distribution of total urinary anthocyanins post bilberry anthocyanin extract consumption for 52 participants.
 Each bin spans 100 nmol, where for example, 100 on the X-axis covers the range 50 – 150 nmols.
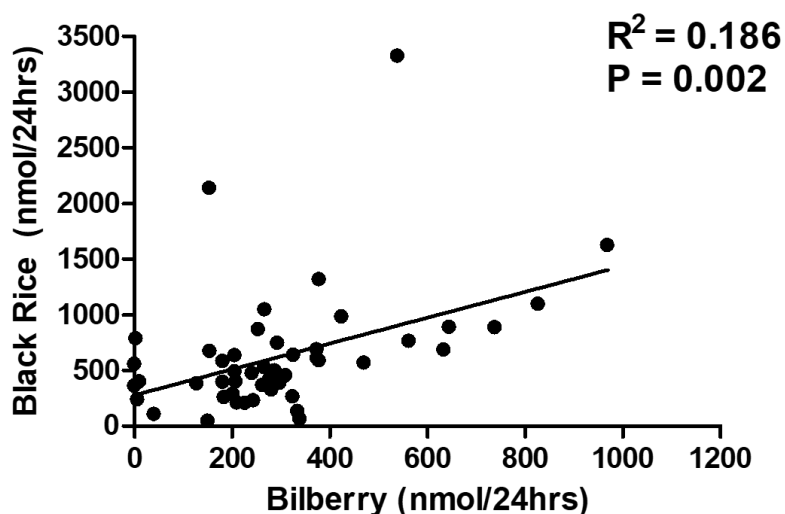
Figure 3-7- Linear regression of total urinary anthocyanins post consumption of black rice and bilberry anthocyanin extracts.
Data is shown as nmol/24hrs for all 52 participants, with each individual represented by one data point.

Figure 3-7 shows a clear positive correlation of total urinary anthocyanins between black rice and bilberry extract treatments. There are some outliers, with two participants exhibiting particularly high levels of urinary anthocyanins post-black rice consumption without similar high levels being observed post-bilberry consumption. Overall, there is a trend in the data whereby if an individual had relatively high levels of urinary anthocyanins after one anthocyanin treatment then they had relatively high levels of urinary anthocyanins after the other treatment, with the bioavailability of the specific anthocyanins consumed dictating their absolute levels. It is also important to note the very high inter-individual variation in this data, with there being a wide range in amounts of total anthocyanins excreted via urine post anthocyanin consumption.

3.5.2 Anthocyanin Metabolites in Urine Samples

The phenolic metabolites of anthocyanins were analysed using a specific UHPLC-MS/MS method, i.e. a separate one to that used for anthocyanins, in order to maximise the number of compounds included in the method and the sensitivity of the analysis (see Chapter 2). A diverse range of phenolic metabolites were quantified in urine samples pre- and post- all three treatments. Notably phenolic compounds can be derived from a wide-range of dietary sources and not only anthocyanins, consequently, the vast majority of phenolics detected were seen in all samples regardless of treatment. However, there were clear elevations in the amounts of several of the excreted compounds post anthocyanin consumption. And some compounds were only detected in

a subset of samples such as 5-hydroxyferulic acid whilst others such as PCA and hippuric acid were identified in all samples.

The reader should note that for several of the compounds included in this analysis MRM may not be able to distinguish between isomers. For example, authentic standards of 3-hydroxybenzoic acid and 4-hydroxybenzoic acid were shown to co-elute under the LC-MS conditions used. Furthermore, several of the compounds included in this analysis were not available as authentic standards at the time of analysis, this was a particular problem for phase 2 conjugates. Therefore, it is highly likely that for these compounds several isomers may have been detected in a single ion current, however, this cannot be known for sure. The details of compounds that may be present as several isomers are given in Table 3-5. An example total ion chromatogram from urine analysis post black rice extract consumption for one participant is depicted in Figure 3-8, notably some low level compounds were only clearly visible in the extracted ion chromatograms which are not shown here.

Table 3-5- MRM transitions of compounds included in the analysis of anthocyanin metabolites that may be present as multiple isomers.
For the compounds in question MRM transition (Q1 and Q3) [M-H]⁻, number of peaks detected, retention time, and the likely compounds that may account for the peaks detected are shown. Note that authentic standards were not held for the compounds highlighted below (with the exception of 3- and 4-hydroxybenzoic acid), here only compounds that are potential candidates for the MRM signals detected are given pending experimental confirmation.

Compound Searched by MRM	Q1 [M-H]⁻	Q3	Number of Peaks	Retention Time(s)	Likely Compounds
Hydroxybenzoic acid	137	93	1	4.9	3-Hydroxybenzoic acid 4-Hydroxybenzoic acid
Dihydroxybenzoic acid glucuronide	153	109	1	1.9	3,5-Dihydroxybenzoic acid-3-*O*-glucuronide 3,5-Dihydroxybenzoic acid-5-*O*- glucuronide 2,3-Dihydroxybenzoic acid-2-*O*-glucuronide 2,3-Dihydroxybenzoic acid-3-*O*-glucuronide
Gallic acid sulfate	249	169	1	3.9	Gallic acid-3-*O*-sulfate Gallic acid-4-*O*-sulfate Gallic acid-5-*O*-sulfate
Gallic acid-*O*-methyl-*O*-sulfate	263	183	1	4.1	Gallic acid-3-*O*-methyl-4-*O*-sulfate Gallic acid-3-*O*-methyl-5-*O*-sulfate Gallic acid-4-*O*-methyl-3-*O*-sulfate

						Gallic acid-4-*O*-methyl-4-*O*-sulfate Gallic acid-5-*O*-methyl-3-*O*-sulfate Gallic acid-5-*O*-methyl-4-*O*-sulfate
(Iso)vanillic glucuronide/homoPCA glucuronide	343	167	2	2.7 7.0		Vanillic acid-4-*O*-glucuronide Isovanillic acid-3-*O*-glucuronide HomoPCA-3-*O*-glucuronide HomoPCA-4-*O*-glucuronide
(Iso)vanillic acid sulfate/homoPCA sulfate	247	167	1	3.9		Vanillic acid-4-*O*-sulfate Isovanillic acid-3-*O*-sulfate HomoPCA-3-*O*-sulfate HomoPCA-4-*O*-sulfate
Caffeic acid glucuronide	355	179	1	5.1		Caffeic acid-3-*O*-glucuronide Caffeic acid-4-*O*-glucuronide
Trihydroxycinnamic acid glucuronide	371	195	1	3.2		3,4,5-Trihydroxycinnamic acid-3-*O*-glucuronide 3,4,5-Trihydroxycinnamic acid-4-*O*-glucuronide 3,4,5-Trihydroxycinnamic acid-5-*O*-glucuronide
Catechol glucuronide	285	109	1	3.6		Catechol-3-*O*-glucuronide Catechol-4-*O*-glucuronide
Catechol sulfate	189	109	1	4.3		Catechol-3-*O*-sulfate Catechol-4-*O*-sulfate
Dihydrocaffeic acid sulfate	261	181	1	5.6		Dihydrocaffeic acid-3-*O*-sulfate Dihydrocaffeic acid-4-*O*-sulfate
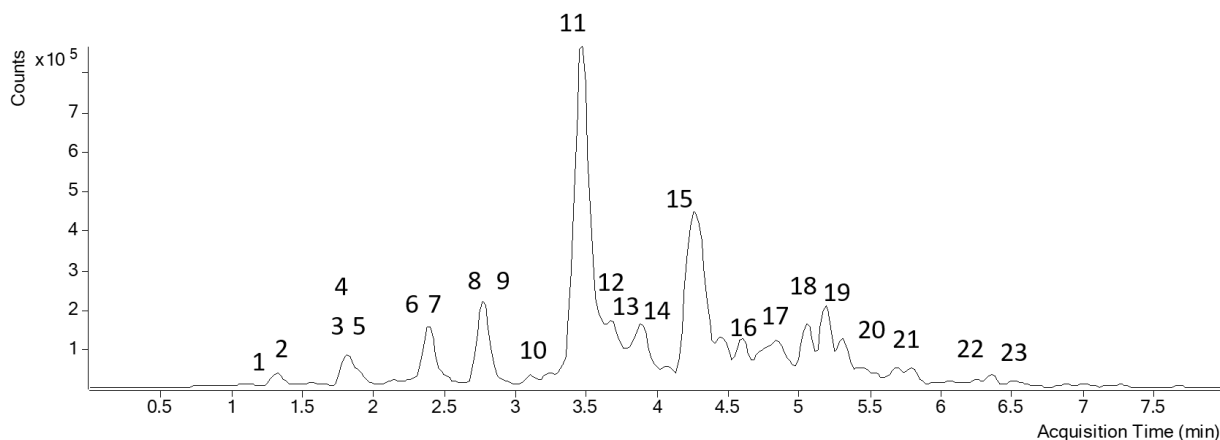
Figure 3-8- An example of a total ion current (TIC) chromatogram obtained for urinary anthocyanin metabolite analysis from one participant post-black rice anthocyanin extract consumption.

Peaks are labelled as follows: (1) gallic acid-3-O-glucuronide, (2) PCA-4-O-glucuronide, (3) gallic acid, (4) PCA-3-O-glucuronide, (5) PCA-4-O-sulfate, (6) phloroglucinol, (7) PCA-3-O-sulfate, (8) pyrogallol, (9) (iso)vanillic acid/homoPCA glucuronide, (10) PCA, (11) hippuric acid, (12) catechol glucuronide, (13) 4-hydroxyhippuric acid, (14) (iso)vanillic acid/homoPCA sulfate, (15) catechol sulfate, (16) dihydroferulic acid, (17) catechol, (18) hydroxyphenylacetic acid, (19) protocatechualdehyde, (20) D9-caffeine, (21) 4-hydroxybenzaldehyde, (22) PGA and (23) taxifolin.

Table 3-6 shows the phenolic compounds quantified in urine samples, as mean μmol/24hrs ± standard deviation. Overall, levels of phenolics remained consistent across pre-treatment and post placebo samples with no statistically significant differences in these measurements detected with repeated measures ANOVA and a Tukey post-hoc test. As predicted, the greatest concentrations of phenolics were seen post-black rice extract consumption, with total phenolics (excluding hippuric acid) being statistically significantly increased. The phenolic metabolites that were increased by statistically significant levels in the study population following black rice extract consumption were: dihydroxybenzoic acid glucuronide, methyl gallate, methyl gallate-sulfate, methyl-3,4-dihydroxybenzoate, PCA, PCA-3-*O*-glucuronide, PCA-3-*O*-sulfate, PCA-4-*O*-glucuronide, PCA-4-*O*-sulfate, vanillic acid, (iso)vanillic acid glucuronide/homoPCA glucuronide, (iso)vanillic acid sulfate/homoPCA sulfate, sinapic acid sulfate, PGA, catechol, catechol glucuronide, catechol sulfate, homoPCA, homovanillic acid sulfate and dihydrocaffeic acid sulfate.

Although increases in phenolics were observed post-bilberry extract consumption this was to a much lesser extent compared to black rice extract. Few compounds were shown to be elevated to statistically significant levels compared to pre-treatment and post-placebo measurements; those that were are as follows: methyl gallate sulfate, 5-hydroxyferulic acid, caffeic acid glucuronide, p-coumaric acid and PGA. Of these compounds, 5-hydroxyferulic acid is reported here for the first time as a confirmed anthocyanin metabolite. In addition to this, increases were seen in other compounds such as: p-coumaric acid glucuronide, isoferulic acid-3-*O*-glucuronide, (iso)vanillic acid/homoPCA glucuronide, and 3/4-hydroxybenzoic acid, however, these changes were modest and not statistically significant, as shown in Table 3-6. The lack of statistical significance of this data is likely due to the modest size of the increases which in combination with very high inter-individual variation (see Figure 3-9), substantially limited the power to detect differences.

A number of phenolic compounds detected in urine samples did not increase after either black rice or bilberry anthocyanin consumption (Table 3-6). These compounds included: gallic acid, 3-methylhippuric acid, 4-hydroxybenzaldehyde, protocatechualdehyde, phloroglucinol, pyrogallol, and 4-hydroxyphenylacetic acid.

Table 3-6- Anthocyanin metabolites quantified in urine samples pre- and post- all three treatment phases: placebo, black rice extract and bilberry extract in the human intervention study. Data is shown as mean µmol/24hrs ± standard deviation and n = 52.

Compound	Pre-Placebo	Post-Placebo	Pre-Black Rice	Post-Black Rice	Pre-Bilberry	Post-Bilberry
___Benzoic acid derivatives___						
3/4-Hydroxybenzoic acid	7.5 ± 5.5	6.0 ± 3.7	7.5 ± 4.7	**8.2 ± 5.4**	7.1 ± 4.8	**9.1 ± 6.8**
3-*O*-Methyl gallic acid	0.02 ± 0.03	0.03 ± 0.05	0.03 ± 0.05	**0.03 ± 0.04**	0.03 ± 0.03	**0.03 ± 0.03**
Dihydroxybenzoic acid glucuronide	0.1 ± 0.1	0.08 ± 0.08	0.10 ± 0.09	**0.4 ± 0.3 ****	0.08 ± 0.07	**0.1 ± 0.1**
Gallic acid	0.5 ± 0.6	0.4 ± 0.4	0.7 ± 1.0	**0.7 ± 1.2**	0.5 ± 0.6	**0.5 ± 0.5**
Gallic acid-3-*O*-glucronide	0.03 ± 0.04	0.05 ± 0.15	0.05 ± 0.07	**0.04 ± 0.06**	0.04 ± 0.05	**0.03 ± 0.04**
Gallic acid-4-*O*-glucronide	1.2 ± 0.8	1.1 ± 0.9	1.3 ± 1.1	**1.5 ± 1.2**	1.2 ± 0.9	**1.4 ± 1.1**
Gallic acid sulfate[a]	2.0 ± 1.6	2.1 ± 1.7	2.2 ± 2.0	**2.6 ± 1.6**	2.2 ± 1.8	**2.8 ± 2.2**
Methyl gallate[b]	0.1 ± 0.0	0.04 ± 0.04	0.06 ± 0.06	**0.1 ± 0.1 ****	0.05 ± 0.06	**0.1 ± 0.1**
Gallic acid-*O*-methyl-*O*-sulfate[c]	0.8 ± 1.3	0.6 ± 1.0	0.5 ± 0.7	**3.6 ± 3.1 *****	0.7 ± 1.4	**1.7 ± 1.5 ****
Methyl-3,4-dihydroxybenzoate	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	**0.3 ± 0.2 ***	0.2 ± 0.1	**0.2 ± 0.2**
PCA	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.13	**1.2 ± 0.8 *****	0.3 ± 0.2	**0.4 ± 0.2**
PCA-3-*O*-glucuronide	0.2 ± 0.2	0.3 ± 0.4	0.3 ± 0.5	**1.5 ± 1.1 *****	0.2 ± 0.2	**0.3 ± 0.4**
PCA-3-*O*-sulfate	9.1 ± 9.6	6.9 ± 5.5	7.6 ± 7.8	**15.4 ± 12.2 *****	7.9 ± 9.0	**9.2 ± 9.3**
PCA-4-*O*-glucuronide	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	**0.4 ± 0.3 *****	0.1 ± 0.1	**0.2 ± 0.2**
PCA-4-*O*-sulfate	16.7 ± 20.6	13.2 ± 9.4	14.9 ± 14.1	**46.7 ± 28.8 *****	13.3 ± 8.4	**17.1 ± 14.6**
Syringic acid	0.04 ± 0.06	0.03 ± 0.03	0.04 ± 0.04	**0.04 ± 0.04**	0.03 ± 0.03	**0.1 ± 0.1**
Vanillic acid	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	**0.2 ± 0.1 ***	0.1 ± 0.1	**0.2 ± 0.2**
(Iso)vanillic acid glucuronide/ HomoPCA glucuronide[c]	0.6 ± 0.5	0.5 ± 0.4	0.5 ± 0.4	**1.7 ± 1.9 *****	0.5 ± 0.4	**0.7 ± 0.5**
(Iso)vanillic acid glucuronide/ HomoPCA glucuronide[c]	5.6 ± 11.5	4.1 ± 5.1	4.5 ± 6.7	**4.8 ± 8.9**	4.7 ± 8.4	**6.4 ± 11.0**
(Iso)vanillic acid sulfate/HomoPCA sulfate[c]	50.0 ± 49.4	45.5 ± 32.6	47.1 ± 30.7	**86.1 ± 52.5 ****	43.4 ± 31.6	**54.1 ± 37.8**

___Cinnamic acid derivatives___

Compound	Pre-Placebo	Post-Placebo	Pre-Black Rice	Post-Black Rice	Pre-Bilberry	Post-Bilberry
5-Hydroxyferulic acid	0.5 ± 1.0	0.3 ± 0.7	0.5 ± 0.9	**0.6 ± 1.4**	0.3 ± 0.7	**1.1 ± 1.5 ***
Caffeic acid	0.6 ± 0.5	0.6 ± 0.4	0.6 ± 0.3	**0.7 ± 0.4**	0.5 ± 0.4	**0.7 ± 0.5**
Caffeic acid glucuronide[d]	1.9 ± 2.4	1.9 ± 2.1	2.3 ± 3.0	**2.0 ± 2.1**	1.7 ± 1.6	**4.0 ± 7.4 ***
Ferulic acid	1.3 ± 1.1	1.2 ± 1.1	1.3 ± 1.3	**1.5 ± 0.9**	1.2 ± 0.8	**1.6 ± 1.2**
Isoferulic acid-3-*O*-glucuronide	13.0 ± 11.2	10.2 ± 6.6	12.4 ± 7.3	**11.7 ± 7.9**	10.4 ± 7.7	**14.3 ± 11.4**
p-Coumaric acid	2.3 ± 2.2	2.1 ± 1.9	2.8 ± 2.7	**3.1 ± 2.6**	2.2 ± 1.8	**3.4 ± 2.4 ***
p-Coumaric acid glucuronide[e]	0.3 ± 0.5	0.6 ± 1.3	0.4 ± 1.1	**0.3 ± 0.6**	0.3 ± 0.5	**0.8 ± 2.7**
Sinapic acid	0.3 ± 0.3	0.3 ± 0.3	0.3 ± 0.3	**0.4 ± 0.5**	0.3 ± 0.3	**0.4 ± 0.5**
Sinapic acid sulfate[f]	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.2	**0.3 ± 0.4 ***	0.1 ± 0.2	**0.2 ± 0.3**
Trihydroxycinnamic acid glucuronide[g]	0.3 ± 0.4	0.3 ± 0.3	0.3 ± 0.3	**0.3 ± 0.4**	0.3 ± 0.9	**0.4 ± 0.7**
Hippuric acid derivatives						
3-Methylhippuric acid[h]	0.3 ± 0.4	0.2 ± 0.3	0.2 ± 0.3	**0.2 ± 0.2**	0.2 ± 0.2	**0.2 ± 0.3**
4-Hydroxyhippuric acid	10.9 ± 10.0	9.9 ± 8.6	11.4 ± 11.0	**12.3 ± 9.5**	8.7 ± 7.1	**13.3 ± 12.0**
Hippuric acid	2997 ± 2939	2847 ± 3872	3207 ± 3478	**3637 ± 3530**	2750 ± 3026	**3027 ± 2857**
Phenolic aldehydes						
4-Hydroxybenzaldehyde	1.9 ± 1.5	1.5 ± 0.1	1.6 ± 0.9	**1.9 ± 1.1**	1.7 ± 1.2	**1.8 ± 1.2**
PGA	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	**0.1 ± 0.1 ****	0.01 ± 0.01	**0.1 ± 0.1 ** **
Protocatechuicaldehyde	0.03 ± 0.03	0.03 ± 0.02	0.03 ± 0.02	**0.04 ± 0.03**	0.02 ± 0.01	**0.03 ± 0.03**
Phenol derivatives						
Catechol	1.7 ± 1.4	1.5 ± 1.0	1.6 ± 1.1	**2.4 ± 1.3 ***	1.5 ± 1.0	**1.8 ± 1.2**
Catechol glucuronide[i]	4.9 ± 3.6	5.2 ± 7.7	5.4 ± 5.1	**11.7 ± 8.0 ****	4.2 ± 3.8	**6.4 ± 4.4**
Catechol sulfate[i]	150 ± 125	126 ± 87	137.2 ± 93.2	**223 ± 122 ****	128 ± 84	**164 ± 113**
Methoxyphenyl sulfate[j]	8.8 ± 12.0	7.3 ± 7.0	7.6 ± 8.5	**10.3 ± 11.6**	6.6 ± 6.8	**8.6 ± 8.2**
Phloroglucinol	0.05 ± 0.05	0.05 ± 0.05	0.05 ± 0.04	**0.05 ± 0.05**	0.1 ± 0.1	**0.1 ± 0.1**
Pyrogallol	0.1 ± 0.1	0.07 ± 0.07	0.1 ± 0.1	**0.08 ± 0.08**	0.1 ± 0.1	**0.1 ± 0.1**
Phenylacetic acid derivatives						
HomoPCA	4.1 ± 4.3	3.6 ± 2.7	3.7 ± 2.4	**7.2 ± 4.3 ****	3.5 ± 2.7	**4.6 ± 3.3**

Compound	Pre-Placebo	Post-Placebo	Pre-Black Rice	Post-Black Rice	Pre-Bilberry	Post-Bilberry
Homovanillic acid sulfate[k]	3.7 ± 4.7	3.2 ± 4.2	3.5 ± 4.3	**12.8 ± 14.1 *****	3.0 ± 3.7	**3.8 ± 4.4**
4-Hydroxyphenylacetic acid	2.0 ± 1.4	1.6 ± 0.9	2.0 ± 1.5	**2.0 ± 1.1**	1.9 ± 1.1	**1.9 ± 1.2**
Phenylproanoic acid derivatives						
Dihydrocaffeic acid sulfate[d]	6.0 ± 6.9	5.6 ± 4.9	6.2 ± 5.0	**21.7 ± 22.9 *****	5.1 ± 4.8	**7.4 ± 7.0**
Dihydroferulic acid	3.7 ± 2.4	3.3 ± 2.5	3.7 ± 2.5	**3.9 ± 2.4**	3.5 ± 2.2	**4.0 ± 2.9**
Dihydro(iso)ferulic acid	0.5 ± 0.6	0.5 ± 0.5	0.5 ± 0.7	**0.6 ± 0.9**	0.4 ± 0.4	**0.6 ± 0.9**
Totals						
Total Phenolics	3315 ± 3042	3119 ± 3978	3504 ± 3579	**4148 ± 3675**	3022 ± 2880	**3381 ± 2898**
Total Phenolics (excluding hippuric acid)	318 ± 263	271 ± 169	297 ± 180	**510 ± 262 *****	272 ± 170	**354 ± 219**

Statistical analysis carried out by repeated measures ANOVA with a Tukey's post-hoc test to compare all pairs of means *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$

[a]Quantified with gallic acid

[b]Quantified with vanillic acid

[c]Quantified with 3-*O*-methyl gallic acid

[d]Quantified with caffeic acid

[e]Quantified with p-coumaric acid

[f]Quantified with sinapic acid

[g]Quantified with cinnamic acid

[h]Quantified with hippuric acid

[i]Quantified with catechol

[j]Quantified with methoxyphenol

[k]Quantified with homovanillic acid

Only compounds detected with a signal to noise ratio ≥ 5 were quantified, compounds with a trace amount were present at levels above the limits of detection (signal to noise ration ≥ 3) but below the limits of quantification.

The compounds that were shown to be increased at statistically significant levels after both anthocyanin treatments are highlighted in Table 3-7. The pre-intervention concentration has been subtracted from the post-intervention concentration for every participant (where this resulted in a negative value it was manually corrected to 0) and the mean and standard deviation has been calculated. This shows that a reasonable proportion of metabolites are derived from the treatment rather than the background diet of participants. The reader should note that the 2 columns on the right of Table 3-7 show the same data where negative values have been included and not manually corrected to 0 to highlight the effect of this process on the data. For individual compounds that were significantly increased post-intervention generally this did not cause any large changes in the data, however, for hippuric acid (as indicated in the totals) this can be seen to have a large effect as several participants exhibited higher hippuric acid levels pre-intervention rather than post, likely due to the wide range of dietary sources of hippuric acid. When hippuric acid is removed from the total phenolics, this has much less of an effect, however the effect is more pronounced post-bilberry rather than black rice, most likely due to the variability in the metabolites that were increased post intervention, with participants not all showing increases in the same compounds, this variation is best shown in Figure 3-9.

Table 3-7- Difference in urinary anthocyanin metabolites pre- and post intervention
The difference in anthocyanin metabolites (pre- and post- intervention) following black rice and bilberry anthocyanin extract consumption for 28 days, for metabolites with statistically significantly different concentrations between pre-intervention and post-placebo samples. For the "negatives zeroed" columns pre-intervention samples were subtracted from post-intervention samples for every participant, and negative values were zeroed, the mean and standard deviation is presented for all 52 participants in μmol/24hrs. For the "including negatives" columns in grey, on the right of the table, pre-intervention samples were subtracted from post-intervention for every participant, and negative values were included in the calculation of the means and standard deviations for all 52 participants.

Compound	Black Rice (negatives zeroed)	Bilberry (negatives zeroed)	Black Rice (including negatives)	Bilberry (including negatives)
Dihydroxybenzoic acid glucuronide	0.3 ± 0.3	-	0.3 ± 0.3	-
Methyl gallate[b]	0.1 ± 0.1	-	0.1 ± 0.2	-
Methyl gallate sulfate[c]	3.0 ± 2.8	1.3 ± 1.4	3.0 ± 2.8	1.1 ± 2.0
Methyl-3,4-dihydroxybenzoate	0.2 ± 0.2	-	0.2 ± 0.2	-
PCA	1.0 ± 0.7	-	1.0 ± 0.7	-
PCA-3-*O*-glucuronide	1.2 ± 0.9	-	1.2 ± 0.9	-
PCA-3-*O*-sulfate	8.5 ± 10.2	-	7.8 ± 11.1	-
PCA-4-*O*-glucuronide	0.3 ± 0.2	-	0.3 ± 0.3	-
PCA-4-*O*-sulfate	32.0 ± 24.5	-	32.0 ± 24.7	-
Vanillic acid	0.1 ± 0.1	-	0.1 ± 0.1	-
(Iso)vanillic acid glucuronide[c]	1.2 ± 1.7	-	1.2 ± 1.7	-
Vanillic acid sulfate/HomoPCA sulfate[c]	41.0 ± 37.6	-	38.9 ± 40.3	-
Sinapic acid sulfate[g]	0.2 ± 0.3	-	0.2 ± 0.4	-
PGA	0.1 ± 0.1	0.04 ± 0.05	0.1 ± 0.1	0.04 ± 0.05
Catechol	0.8 ± 0.9	-	0.8 ± 0.9	-

Compound	Black Rice (negatives zeroed)	Bilberry (negatives zeroed)	Black Rice (including negatives)	Bilberry (including negatives)
Catechol glucuronide[i]	6.5 ± 6.4	-	6.2 ± 6.8	-
Catechol sulfate[i]	92.1 ± 93.6	-	86.0 ± 101.3	-
HomoPCA	3.6 ± 3.0	-	3.6 ± 3.1	-
Homovanillic acid sulfate[k]	9.4 ± 11.3	-	9.4 ± 11.3	-
Dihydrocaffeic acid sulfate[d]	15.5 ± 20.5	-	15.5 ± 20.6	-
5-hydroxyferulic acid	-	1.0 ± 1.5	-	0.8 ± 1.7
Caffeic acid glucuronide[d]	-	3.0 ± 7.0	-	2.4 ± 7.1
p-Coumaric acid	-	1.9 ± 2.3	-	1.1 ± 3.3
Total Phenolics	1630 ± 2387	1644 ± 2581	643 ± 3054	347 ± 3897
Total Phenolics (excluding hippuric acid)	238.9 ± 195.6	171 ± 195	212 ± 213.6	81.9 ± 286.6

[a]Quantified with gallic acid
[b]Quantified with vanillic acid
[c]Quantified with 3-O-methyl gallic acid
[d]Quantified with caffeic acid
[e]Quantified with p-coumaric acid
[f]Quantified with sinapic acid
[g]Quantified with cinnamic acid
[h]Quantified with hippuric acid
[i]Quantified with catechol
[j]Quantified with methoxyphenol
[k]Quantified with homovanillic acid

To gain some understanding of the recovery of the fed dose of anthocyanins the urinary yield of parent compounds and metabolites was estimated. For every compound the post-intervention concentration was subtracted from the pre-intervention concentration, for instances where this resulted in a negative value this was adjusted to 0, concentrations were converted to µg/24 hours and the percentage of which they accounted for the daily dose of anthocyanins (320 mg) was calculated and summed to establish the urinary yield, shown in Table 3-8. Urinary yield of all compounds was very high for both treatments at ~94%, however, when hippuric acid was removed the urinary yield drops dramatically to 16.7 and 11.9% for black rice and bilberry respectively. Anthocyanins accounted for less than 1% of urinary yield in both treatments. The reader should note that adjusting negative values to zero causes a large effect on the estimated yield of the fed anthocyanins. When negative values were included in the calculation, urinary yield of all compounds was lower at 38.9 and 20.6% for black rice and bilberry respectively. However, when hippuric acid was removed this had much less of an effect on urinary yield, with this calculated as 14.8 and 7.8% for black rice and bilberry respectively; the urinary yield of anthocyanins was not

affected. It is not surprising that urinary yield was affected so drastically by hippuric acid as this compound is derived from many dietary sources other than anthocyanins, and therefore it was expected that some participants would have higher urinary excretion of hippuric acid pre-anthocyanin extract consumption. Hippuric acid appears to be problematic due to the combination of its very high concentrations relative to other compounds and the high variance, which will mean that any estimate can have a substantial effect on the calculated yield (positive or negative) even if it is quite close to the true mean value.

Table 3-8- Estimated yield of daily anthocyanin dose recovered in urine
Mean yield of anthocyanins and phenolics in urine as a percentage of the daily anthocyanin dosage (320 mg/day). For the columns on the left (negatives zeroed), pre-intervention samples were subtracted from post-intervention for every participant, negative values were zeroed and percentage of the daily dose was calculated for each compound and summed, the mean and standard deviation is presented for all 52 participants. For the grey columns of the right (negatives included), pre-intervention samples were subtracted from post-intervention for every participant, and negative values were included in the calculation of the percentage of the daily dose accounted for by each compound, these values were summed and the mean and standard deviation is presented for all 52 participants.

Yield	Black Rice (negatives zeroed)	Bilberry (negatives zeroed)	Black Rice (negatives included)	Bilberry (negatives included)
All Compounds	94.6 ± 135	94.3 ± 145	38.9 ± 194	20.6 ± 216
All Compounds (excluding hippuric acid)	16.7 ± 13.3	11.9 ± 13.2	14.8 ± 14.6	7.8 ± 18.7
Phenolics (all)	94.5 ± 135	94.3 ± 145	38.8 ± 194	20.6 ± 216
Anthocyanins (all)	0.08 ± 0.07	0.03 ± 0.03	0.08 ± 0.07	0.03 ± 0.03

Urinary phenolics data is also shown as a heat-map in Figure 3-9. Like Table 3-6 this highlights that phenolics were increased after consumption of both black rice and bilberry extract anthocyanins. What is shown clearly here, but not in Table 3-6, is the inter-individual variation between participants. This is particularly apparent in post-bilberry extract urine samples where increases in individual compounds are modest and not homogenous across the study population, highlighting that anthocyanin metabolism differs between people. Having said this, there are clear patterns whereby particular metabolites are elevated to some extent in all participants, for example, PCA was clearly elevated in all participants' post-black rice extract consumption, however, the extent of this increase was not equal for all individuals. Despite this, consistency is seen between the low levels of phenolics in urine pre-intervention and post-placebo among all members of the study population, highlighting the effect of both anthocyanin treatments on urinary phenolics.
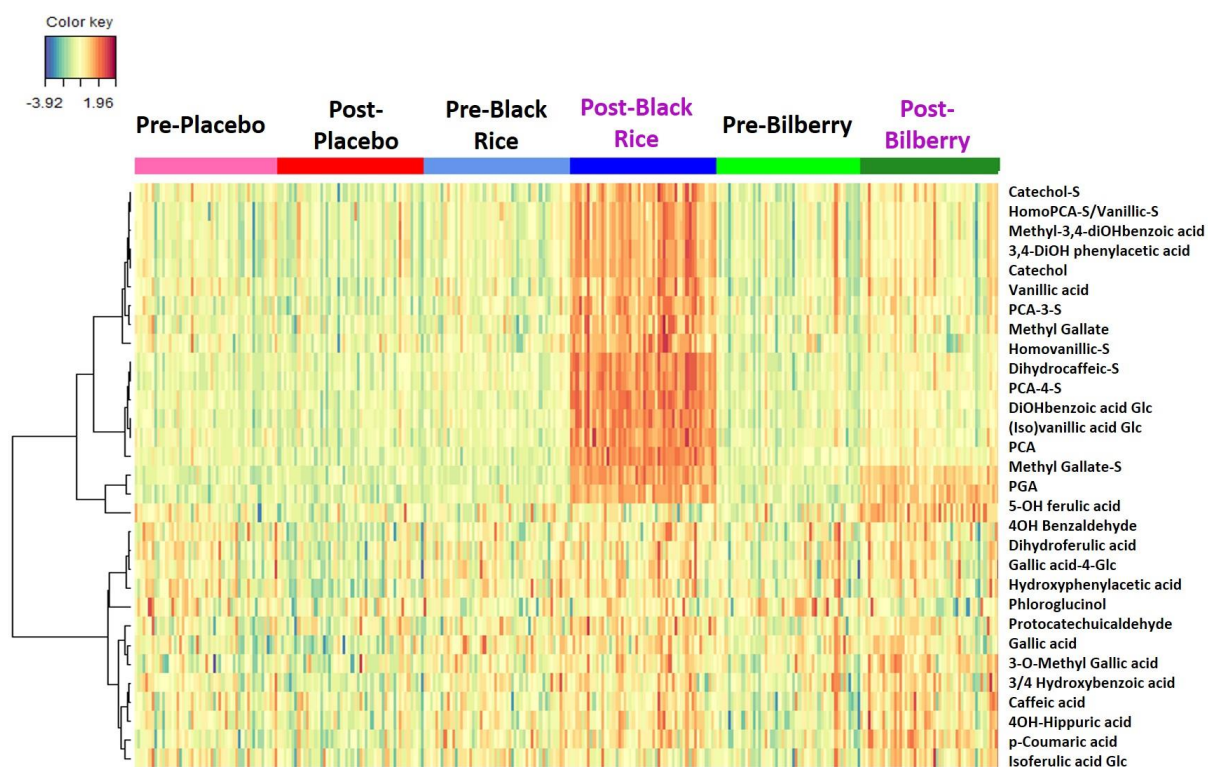
Figure 3-9- Heatmap of the top 30 urinary anthocyanin metabolites
Scaled and centred data showing the within subject urinary levels of the top 30 phenolics (excluding hippuric acid) before and after each treatment phase using Box-Cox transformed data for 52 participants. Hierarchical clustering of the variables was carried out using the Ward method. Samples are ordered by study phase indicating time-point and diet, post black rice and bilberry treatments are highlighted in purple. The heatmap describes the concentration of each compound in urine standardised the range of [-3.92, +3.92]. Red colour indicates high amounts of the compound were detected and blue indicates only low levels were present.

To visualise inter-individual variation across all compounds, total phenolics data was used to plot a frequency distribution of urine samples post black rice (Figure 3-10) and bilberry anthocyanin extract (Figure 3-11) consumption. Both treatments show a normal distribution, with some individuals having particularly low or high levels of urinary phenolics. It was hypothesised that there would be a positive correlation between the amount of phenolics excreted by each participant after consumption of the black rice and the bilberry anthocyanin extract, i.e. if a participant exhibited a relatively high excretion of black rice phenolics, they would also excrete a relatively high amount of phenolics after bilberry extract consumption. This was investigated by means of a linear regression analysis of total anthocyanin metabolites between treatments, shown in Figure 3-12.
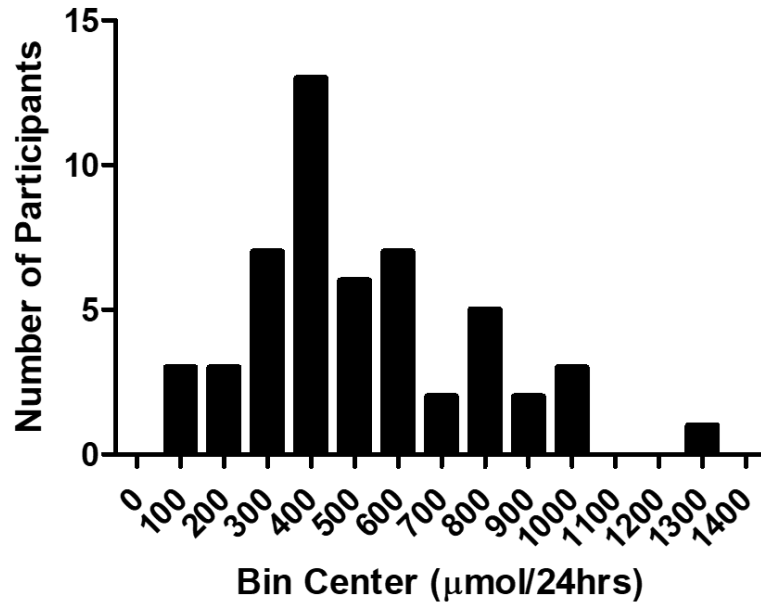
Figure 3-10- Frequency distribution of total urinary anthocyanin metabolites (excluding hippuric acid) post-black rice consumption for 52 participants.
Each bin spans 100 μmols, where for example, 100 on the X-axis covers the range 50 -150 μmols.
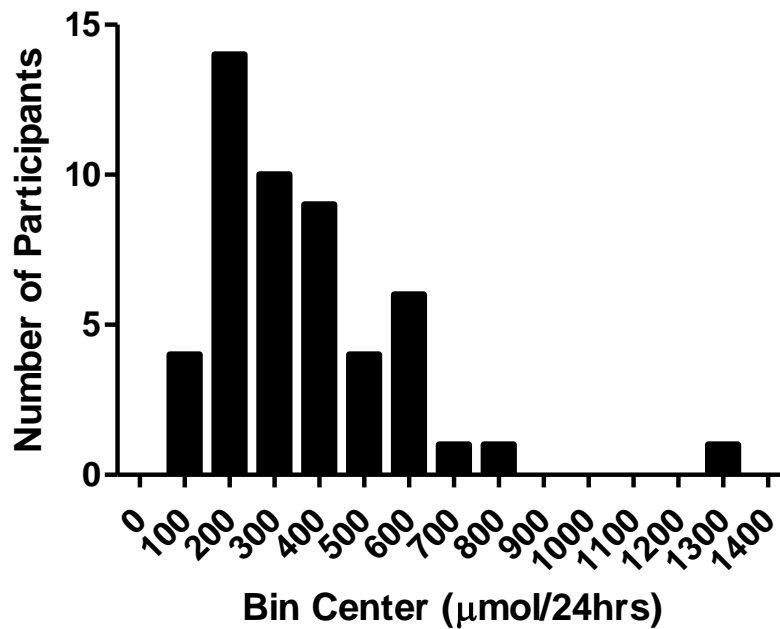
Figure 3-11- Frequency distribution of total urinary anthocyanin metabolites (excluding hippuric acid) post-bilberry consumption for 52 participants.
Each bin spans 100 μmols, where for example, 100 on the X-axis covers the range 50 – 150 μmols.
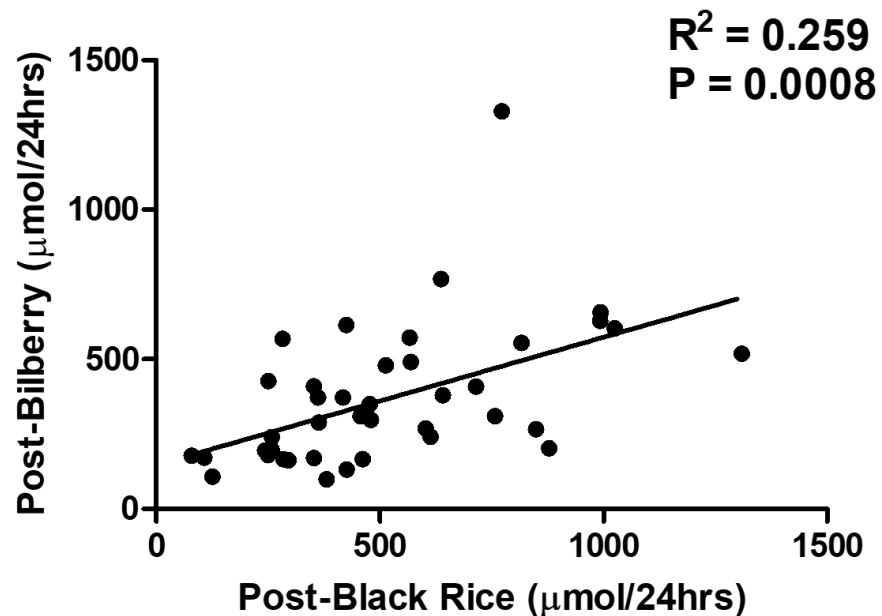
Figure 3-12- Linear regression showing the relationship between total anthocyanin metabolite excretion (excluding hippuric acid) post consumption of black rice anthocyanin extract and post consumption of bilberry extract (µmol/24hrs).
Data is shown for all 52 participants, with each individual represented by one datapoint.

Figure 3-12 shows a clear positive correlation between treatments for individuals regarding the total amount of urinary phenolics after each anthocyanin treatment. Although one participant exhibits a particularly high level of phenolics post-bilberry anthocyanin extract consumption, the vast majority of participants fit the trend that if they have high levels of urinary phenolics after one treatment then this is also seen in the other. The slope of the linear regression analysis could potentially be used to help estimate the relative bioavailability of phenolics derived from bilberry versus black rice anthocyanins, however, it would need to be taken into account there are likely several metabolites present that have not been quantified in this analysis.

3.5.3 Anthocyanin Metabolites in Plasma Samples

Plasma samples for all volunteers were analysed using UHPLC-MS/MS, an example chromatogram obtained post black rice anthocyanin extract consumption is shown in Figure 3-13. Sixteen potential anthocyanin metabolites were quantified among the study population ~24 hours after anthocyanin consumption, shown in Table 3-9. Of the 16 phenolic metabolites detected in plasma, all except vanillin were also detected in urine. This was particularly interesting as vanillin was one of the most abundant compounds observed in plasma. However, it did not appear to increase in response to anthocyanin consumption and was relatively consistent in its concentration among the study population, as indicated by the small standard deviation values for this compound in Table 3-9,

suggesting that it is not likely derived from the fed anthocyanins. Furthermore, it should be noted that an authentic standard was not used at the time of analysis and therefore identification was only putative, and quantification may not be accurate, however, the concentrations given in Table 3-9 can be used to compare accurately between treatment but not between compounds. Of the phenolics detected in plasma, PCA-4-*O*-glucuronide, (iso)vanillic acid glucuronide/homoPCA glucuronide, phloroglucinol and 4-hydroxyphenylacetic acid appeared to increase post-black rice extract consumption. Meanwhile, 4-Hydroxyhippuric acid, 4-hydroxybenzaldehyde, phloroglucinol, and 4-hydroxyphenylacetic acid were increased post-bilberry extract consumption. However, these increases were not statistically significant, likely due to the large inter-individual variation in the data making it difficult to detect significant differences.
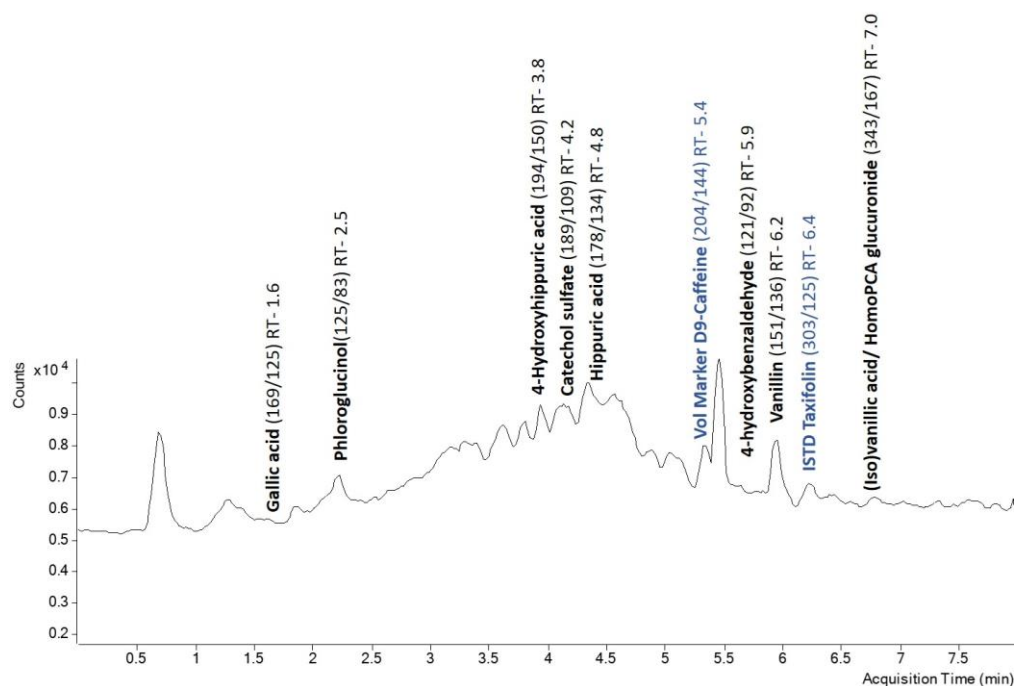
Figure 3-13- An example of a total ion current (TIC) chromatogram obtained for plasma anthocyanin metabolite analysis from one participant post-black rice anthocyanin extract consumption.
Peaks are labelled with their identity, MRM transition [M-H] and retention time. Those coloured in blue correspond to the internal standard (ISTD) taxifolin, and the volume marker D9-caffeine.

Table 3-9- Anthocyanin metabolites quantified in plasma samples pre- and post- all three treatment phases: placebo, black rice extract and bilberry extract in the human intervention study. Data shown as mean in nM ± standard deviation and n = 52.

Compound	Pre-Placebo	Post-Placebo	Pre-Black Rice	Post-Black Rice	Pre-Bilberry	Post-Bilberry
Benzoic acid derivatives						
Gallic acid	3.9 ± 11	8.9 ± 13.5	9.3 ± 18.2	**10.6 ± 18.0**	7.1 ± 12.6	**7.8 ± 16.7**
Gallic acid-4-*O*-glucuronide	1.4 ± 1.6	1.9 ± 2.3	Trace	**1.3 ± 1.6**	Trace	**1.7 ± 1.8**
PCA-3-*O*-glucuronide	6.2 ± 8.2	5.7 ± 7.2	5.0 ± 5.6	**8.4 ± 14.3**	4.3 ± 1.2	**6.7 ± 6.7**
PCA-4-*O*-glucuronide	6.9 ± 10.3	6.4 ± 9.4	4.7 ± 7.5	**12.1 ± 22.5**	4.1 ± 6.3	**7.9 ± 10.5**
(Iso)vanillic acid glucuronide/ homoPCA glucuronide[a]	2.8 ± 12.4	5.7 ± 16.0	4.1 ± 9.5	**8.1 ± 22.1**	5.9 ± 17.6	**6.0 ± 12.9**
Cinnamic acid derivatives						
Trihydroxycinnamic acid glucuronide[b]	10.3 ± 14.0	13.9 ± 13.0	14.8 ± 14.2	**14.4 ± 15.6**	12.3 ± 12.5	**14.5 ± 13.8**
Hippuric acid derivatives						
4-Hydroxyhippuric acid	52.9 ± 80.7	64.9 ± 71.6	54.5 ± 60.0	**64.8 ± 58.0**	45.9 ± 59.9	**76.0 ± 69.1**
Hippuric acid	647 ± 800	705 ± 431	572 ± 300	**699 ± 385**	660.6 ± 430.6	**693 ± 571**
Phenolic aldehydes						
Vanillin[a]	1145 ± 227	1174 ± 165	1195 ± 148	**1189 ± 229**	1148.4 ± 148.2	**1182 ± 164**
4-Hydroxybenzaldehyde	53.6 ± 21.6	55.3 ± 22.4	53.1 ± 16.2	**57.7 ± 23.4**	50.5 ± 18.0	**62.6 ± 25.3**
Phenol derivatives						
Catechol sulfate[c]	1431 ± 1077	2315 ± 6787	1074 ± 1092	**2134 ± 2190**	1419 ± 2242	**2467 ± 4987**
Phloroglucinol	3.9 ± 9.9	5.3 ± 11.1	2.8 ± 9.3	**7.5 ± 17.9**	4.9 ± 14.5	**7.3 ± 14.1**
Pyrogallol	Trace	Trace	Trace	**Trace**	Trace	**Trace**
Phenylacetic acid derivatives						
4-Hydroxyphenylacetic acid	12.2 ± 50.5	6.1 ± 28.4	14.1± 55.9	**43.3 ± 93.3**	27.3 ± 95.2	**40.1 ± 110**

Compound	Pre-Placebo	Post-Placebo	Pre-Black Rice	Post-Black Rice	Pre-Bilberry	Post-Bilberry
Phenylproanoic acid derivatives						
Dihydro(iso)ferulic acid	6.4 ± 21.1	12.7 ± 27.7	13.0 ± 26.9	**14.1 ± 31.4**	12.0 ± 25.2	**12.1 ± 25.4**
Total Phenolics	4585 ± 2110	5757 ± 7413	4267 ± 2181	**5998 ± 3150**	4789 ± 3804	**6114 ± 6117**

Statistical analysis carried out by repeated measures ANOVA with a Tukey's post-hoc test to compare all pairs of means, no statistically significant differences were found.

[a]Quantified with vanillic acid; [b]quantified with cinnamic acid; [c]quantified with catechol.

Only compounds detected with a signal to noise ratio ≥ 5 were quantified, compounds with a trace amount were present at levels above the limits of detection (signal to noise ration ≥ 3) but below the limits of quantification.

3.6 Discussion

A total of fifty metabolites were identified in urine and plasma samples post-bilberry and black rice anthocyanin extract consumption across the study population, with intact anthocyanins only being detected at very low levels. Diversity was seen in the range of metabolites detected and their concentrations, spanning hydroxybenzoic acids, methoxybenzoic acids, cinnamic acids, hippuric acids, phenolic aldehydes, phenol derivatives, phenylacetic acids and phenylproanoic acids. Of these metabolites, 5-hydroxyferulic acid was identified for the first time as a metabolite of bilberry derived anthocyanins, whilst catechol and its phase 2 conjugates were highlighted here and in a collaboration with Jokioja *et al* for the first time as important human anthocyanin metabolites (Jokioja *et al.*, 2021). Distinct differences were seen in the precise metabolite profiles arising in plasma and urine between bilberry and black rice anthocyanins in terms of not only the compounds detected but also their relative concentrations. Furthermore, the data obtained showed high inter-individual variation whereby participants responded differently to the anthocyanins they consumed in terms of their specific metabolite signature.

There have been several studies that have investigated the human *in vivo* metabolism of anthocyanins that have found that the parent compound is extensively metabolised to give rise to an wide range of potentially bioactive metabolites (Bresciani *et al.*, 2020; Felgines *et al.*, 2003; De Ferrars, Czank, Zhang, *et al.*, 2014a Mueller *et al.*, 2017; Nurmi *et al.*, 2009). The work presented in this chapter goes beyond what has been reported previously by extending the analysis method to include an extensive range of known and predicted anthocyanin metabolites including phase 2 conjugates (appendix 1). Several phenolics were statistically significantly increased post-black rice anthocyanin extract consumption. Amongst these were catechol and its phase 2 conjugates (catechol sulfate and glucuronide). These metabolites were not reported in previous studies of *in vivo* anthocyanin metabolism, until our collaboration with Jokioja *et al* (Jokioja *et al.*, 2021). In particular catechol sulfate was increased to very high levels in urine. Little is known regarding the bioactivity of catechol and its phase 2 conjugates, however, this would be an important point of further research to establish whether these major di-hydroxy B-ring derived metabolites are linked to the biological effects of anthocyanin rich diets. Increases in phenolics post-bilberry extract consumption were far more modest than those seen post-black rice extract consumption. Few statistically significant increases were seen in individual phenolics (Table 3-6), likely due to both the modest increases but also the large differences between participants whereby different metabolites were increased in some individuals as illustrated in Figure 3-9. Perhaps the most interesting finding was that 5-hydroxyferulic acid was identified in urine samples and the mean urinary excretion was clearly elevated by bilberry extract consumption, although this metabolite was only detected in 33 out of the 52 study participants. This metabolite has not been reported in

previous studies on anthocyanin metabolism and consequently its bioactivity has not been explored, and would be an important point of future work.

The vast majority of phenolic metabolites detected in this study support the existing literature. However, there are some differences. The human metabolism of bilberry anthocyanins has been investigated in a previous report, which detected largest increases in vanillic and homovanillic acid. Although the data presented in this chapter reports these metabolites they were not increased after consumption of bilberry anthocyanin extract. However, it should be noted that the study in question fed an unpurified bilberry-lingonberry puree which contained many polyphenols in addition to anthocyanins and therefore it is possible these metabolites were not only derived from the anthocyanin content of the study meal. Nevertheless, like the data presented here, this study reported p-coumaric acid, and hydroxybenzoic acid as metabolites detected in urine, suggesting that these are common metabolites of bilberry anthocyanins (Nurmi *et al.*, 2009). Furthermore, the levels of metabolites reported in this chapter differ from those found in other studies in some cases. For example, whilst vanillic acid was detected as a metabolite of di-hydroxy B-ring black rice anthocyanins this was only measured at relatively low levels here. Vanillic acid was detected as one of the major metabolites of C3G (the dominant anthocyanin in black rice extract) by De Ferrars et al, with high levels of urinary excretion. However, this metabolite was only seen in a subset of the participants studied and was maximally excreted at 1-2 hours after anthocyanin consumption. This indicates that although in some individuals vanillic acid is a major metabolite of di-hydroxy B-ring anthocyanins this is not true in all cases, highlighting the inter-individual variation in anthocyanin metabolism. Furthermore, with the knowledge that vanillic acid is excreted soon after anthocyanin consumption this would have been diluted by the later urine samples which it was pooled with in 24-hour urine collections, which may explain why such lower concentrations were observed in this study. Additionally, this study delivered anthocyanins in a highly purified extract containing several anthocyanins (Table 3-2) whilst De Ferrars et al fed pure penta[^{13}C] labelled C3G and took separate urine samples at regular time intervals after consumption (Eker et al., 2019; De Ferrars, Czank, Zhang, et al., 2014a). This difference in the format of anthocyanin fed, and sampling method is likely to explain why we did not see some metabolites reported in other studies such as 4-methoxysalicylic acid (De Ferrars, Czank, Zhang, et al., 2014a). It is plausible that there may have been a matrix effect resulting from feeding anthocyanins in a highly purified extract (containing multiple compounds) as opposed to a pure isolated compound that may have impacted the trajectory of metabolism, although this would need to be tested experimentally. Furthermore, although a 24-hour urine collection paired with a prolonged dietary intervention, as was done in this study, provides an excellent overview of anthocyanin metabolism, approximately accounting for all urinary excretion of anthocyanins and metabolites, by theoretically collecting compounds

excreted early after consumption from the final capsule, but also collecting late excreted compounds from the previous days consumption. The downside is that the pharmacokinetic profiles of compounds cannot be established and there is a risk of diluting low abundance compounds below the limits of detection by pooling large volumes of urine. Collecting urine at time intervals means smaller volumes of urine are collected, reducing the risk of diluting compounds below the limits of detection, and facilitates insights into the pharmacokinetic profiles of anthocyanins and their metabolites. The differences between these sampling methods may account for some of the differences in the findings between these studies.

PCA and PGA were both significantly elevated post black rice consumption (with PGA also being significantly increased post bilberry consumption). However, consistent with the literature they were not among the most abundant metabolites detected (De Ferrars *et al,.* 2014a; Mueller *et al*., 2017). This supports the notion that although PCA and PGA are important chemical degradants of di-hydroxy B-ring anthocyanins they are further metabolised *in vivo* giving rise to a diverse range of metabolites that vary considerably between individuals (Woodward *et al.*, 2009). Phase 2 conjugates of PCA were increased considerably following black rice extract consumption, with sulfates being present at higher concentrations than glucuronides, highlighting the importance of including the analysis of phase 2 metabolites in studies on anthocyanin metabolism, which have been overlooked by some previous reports (Woodward, Needs and Kay, 2011; De Ferrars *et al.*, 2014c). Studies on spontaneous anthocyanin degradation have shown that PGA is a common A-ring product of all anthocyanins at a neutral pH. Therefore, it is not surprising that PGA was a common metabolite elevated after consumption of both black rice and bilberry anthocyanin extracts. However, the B-ring chemical degradant (gallic acid) of delphinidin type anthocyanins which are the most abundant anthocyanin type in bilberry was not increased in plasma or urine samples after bilberry extract consumption. Although in some ways this may be surprising it does reinforce what has been shown in previous studies. Gallic acid has been shown to disappear over time in studies on the spontaneous degradation of delphinidin, whilst gallic acid was not detected in clinical samples after consumption of bilberry-lingonberry puree (Goszcz *et al.*, 2017; Nurmi *et al.*, 2009; Woodward *et al.*, 2009). Furthermore, although phase 2 conjugates of gallic acid were detected in urine samples they did not appear to be increased after bilberry extract consumption, with the exception of putative gallic acid-*O*-methyl-*O*-sulfate, indicating that gallic acid is extensively metabolised *in vivo.*

There were distinct differences in the urinary metabolites elevated post-black rice and bilberry anthocyanin extract consumption. The majority of black rice anthocyanin metabolites were dihydroxy-, including: PCA and its phase 2 conjugates, vanillic acid, catechol and its phase 2 conjugates homoPCA and dihydrocaffeic acid sulfate. Whereas the hydroxylation of metabolites

elevated in urine post-bilberry anthocyanin extract consumption was much more varied, encompassing single hydroxy- compounds such as p-coumaric acid and 3/4-hydroxybenzoic acid; dihydroxy- compounds such as isoferulic acid-3-*O*-glucuronide and caffeic acid glucuronide and trihydroxy- compounds such as gallic acid-O-methyl-O-sulfate and 5-hydroxyferulic acid. This contrast in the hydroxylation of the metabolites observed between treatments reflects the hydroxylation of the anthocyanins fed, whereby black rice anthocyanins are mainly dihydroxy-, whilst bilberry anthocyanins are far more diverse and vary in their hydroxylation of the B-ring.

Hippuric acid has frequently been shown to be an important metabolite of anthocyanins, polyphenols and proteins. In this analysis hippuric acid was the most abundant phenolic detected in urine regardless of treatment. Hippuric acid can be derived from a large variety of dietary sources making it particularly difficult to trace its origin in dietary intervention studies, unless a labelled compound is delivered. In a study where 500 mg penta[13C]-C3G was fed, 2.42 ± 2.22 mg of [13C]-labelled hippuric acid was recovered in urine, whereas here an estimated 76.8 ± 574.1 mg - 249.1 ± 402.1 mg of total hippuric acid was quantified (pre-levels subtracted; negatives included and negatives zeroed respectively) post black rice extract consumption containing 320 mg anthocyanins (De Ferrars, Czank, Zhang, *et al.*, 2014a). This is much higher than the recovered levels reported for the penta[13C]-C3G study, however, this is likely due to the lack of a labelled compound in this study matched with the large number of potential metabolic sources of hippuric acid leading to an overestimation of its concentration. Furthermore, levels of hippuric acid did not appear to significantly change as a result of treatment, although it may be presumed that some of the hippuric acid detected post anthocyanin consumption would be derived from the treatment this is difficult to establish without a labelled compound due to high background levels of hippuric acid that vary greatly between individuals, which makes it difficult to establish the extent to which this compound accounted for the fed anthocyanin dose. This reinforces what has been seen in other studies where increases in hippuric acid have not been seen post anthocyanin consumption due to being predominantly derived from the background diet in high levels (de Ferrars *et al.*, 2014b). Therefore, when looking at the general spread of the data and total anthocyanin metabolites it can be beneficial to exclude hippuric acid as its extremely high levels that are mainly derived from sources other than the phase of the study may mask overall patterns in the data.

The overall urinary yield was calculated based on the daily dose of anthocyanins (320 mg/day), with hippuric acid accounting for largest proportion of estimated yield post bilberry and black rice anthocyanin consumption. However, as discussed previously due to its wide range of metabolic sources we cannot be sure precisely how much of this hippuric acid was derived from the fed anthocyanins. When hippuric acid was not included in the yield calculation, urinary yield of the daily anthocyanin dose was 16.6% (range 1.9 – 72.9%) for black rice and 11.9% (range 1.2 – 60.6%)

for bilberry extract when negatives were zeroed; and 14.8% (range -10.1 – 72.9%) for black rice and 7.8% (range -33.5 – 60.6%) for bilberry extract when negatives were included. These urinary yields are low but higher than those reported in other studies, likely due to the increased range of metabolites included in this analysis compared to similar studies, and that participants had been consuming each anthocyanin extract for 28 days. Furthermore, the high inter-individual variability observed in the range of urinary yield is representative of other reports where labelled anthocyanins have been fed to facilitate traceability. This variation is likely to be due to variation in gastric and intestinal transit times, composition and catabolic activity of colonic flora and the ability to take up and excrete catabolites and metabolites (Czank *et al.*, 2013; De Ferrars, Czank, Zhang, *et al.*, 2014a). There are several reasons why urinary yield of anthocyanins (and their metabolites) tends to be low. Firstly, urine is not the only excretion route of anthocyanins and their metabolites, and reasonable proportions of these compounds will be expelled via alternative routes, i.e. in faeces and through the breath (Czank *et al.*, 2013). Secondly, the analysis here, although extensive, still may not have covered the entire range of compounds that may be metabolically derived from anthocyanins and therefore it is likely that several important metabolites were not accounted for. In addition, many anthocyanin metabolites are only present at very low levels and may be below the limits of detection, hindering the assessment of the recovery of the fed dose of anthocyanins in human samples (De Ferrars *et al.*, 2014a; Eker *et al.*, 2019). Furthermore, the SPE protocol employed for sample clean-up did not provide 100% recoveries for anthocyanins or metabolites and consequently some losses would have occurred that would have impacted the estimated urinary yield. This could have been a problem for predicted metabolites and phase 2 conjugates included in the analysis for which an authentic standard was not available and consequently which precluded obtaining accurate estimates of their recoveries.

Plasma samples only showed low levels of phenolics, with far less compounds being detected than in urine samples. 4-hydroxyphenylacetic acid and phloroglucinol were detected in both plasma and urine but only appeared to be increased in plasma post bilberry and black rice anthocyanin extract consumption (although not at statistically significant levels). Meanwhile, vanillin was the only compound detected in plasma that was not seen in urine, which was intriguing as this was one of the most abundant plasma compounds detected. But, as an authentic standard was not used at the time of analysis, identification was only putative and quantification may be inaccurate; the estimate provided can be used to reliably compare between treatments but should be taken with caution when comparing to other compounds in the analysis. However, it should be noted that since analysis of plasma samples an authentic standard of vanillin was run in matrix matched conditions and eluted at the same retention time as putative vanillin from this analysis. The appearance of any compound in plasma but not urine suggests that some metabolites may be subjected to

biotransformation in plasma, or eliminated by the biliary route and either metabolised further by colonic bacteria and reabsorbed or excreted. Given that vanillic acid is an important urinary anthocyanin metabolite, it may be suggested that reduction of vanillic acid to vanillin is a major process, however, vanillin was not increased by either of the anthocyanin treatments and therefore is likely not to be derived from the fed anthocyanins. No statistically significant changes in phenolics were detected in plasma, and inter-individual variation was high. There are several reasons why statistically significant changes in phenolics were not seen in plasma. Firstly, it may be that compounds were present in plasma but at levels below the limits of detection of the analysis method but became detectable as they were concentrated by the kidneys and excreted in urine. Alternatively, this may be due to the time of plasma sampling, 24 hours after anthocyanin consumption. Phenolic metabolites tend to only circulate in plasma transiently after anthocyanin consumption with the vast majority of phenolics reaching their T_{max} in the first 4 hours after anthocyanin intake (de Ferrars *et al.*, 2014a; Jokioja *et al.*, 2021). Nevertheless, PCA-glucuronides, (iso)vanillic acid-glucuronide, phloroglucinol and 4-hydroxyphenylacetic acid were noticeably increased after black-rice extract consumption, whilst 4-hydroxyhippuric acid, 4-hydroxybenzaldehyde, phloroglucinol and 4-hydroxyphenylacetic acid were all increased post bilberry extract consumption.

In keeping with previously published reports of anthocyanin metabolism, phenolic metabolites were present in much higher concentrations than their parent compounds (De Ferrars, Czank, Zhang, *et al.*, 2014a; Jokioja *et al.*, 2021; Mueller *et al.*, 2017; Nurmi *et al.*, 2009; Schön *et al.*, 2018). This provides further evidence for the notion that any biological effects of an anthocyanin rich diet are likely to be caused by one or more of the extensive range of metabolites generated *in vivo* (Kay *et al.*, 2009). It is important to recognise that phenolic compounds are found in a wide range of dietary sources, and consequently were also seen in clinical samples pre-intervention and post-placebo. Having said this, concentrations of phenolics were broadly consistent across pre-intervention and post-placebo samples indicating that they are merely an artefact of the background diet of volunteers (see Figure 3-9 and Table 3-6). Crucially, following black rice and bilberry anthocyanin extract consumption, a number of phenolics were clearly elevated, as is indicated by Figure 3-9 and Table 3-6. Phenolic metabolites were highest post-black rice extract consumption. This in part may be due to the anthocyanin composition of the extracts; black rice extract is almost exclusively made up of cyanidin based anthocyanins (91.9%), whilst bilberry extract has a far more diverse anthocyanin profile as shown in Table 3-1. Therefore, the origin of phenolic metabolites from black rice extract will almost all be derived from cyanidin, meaning that we would expect to see less metabolites in total but with high increases relative to pre-treatment levels. Whereas with bilberry, where the anthocyanin content is more varied, we would expect to

see a greater variety of metabolites but with smaller relative increases due to the greater potential sources of these compounds. The findings of this study support this hypothesis and is best shown by Figure 3-9. Levels of phenolics post black rice extract are high and are limited to particular compounds. On the other hand, levels of phenolic metabolites post bilberry extract consumption are far more modest (although clearly higher than pre-intervention and post-placebo samples), and are spread across a large number of metabolites with changes not being homogenous across the study population, also highlighting inter-individual variation in anthocyanin metabolism. Furthermore, the higher levels of urinary phenolics observed post-black rice anthocyanin extract consumption relative to bilberry may be in part due to the bioavailability of the metabolites derived from the respective anthocyanins. It is well known that cyanidin based anthocyanins are more bioavailable than delphinidin with increased hydroxylation of the B-ring reducing bioavailability (González-Barrio *et al.*, 2010; Hollands *et al.*, 2008; McDougall *et al.*, 2005; Mullen *et al.*, 2008; Rodriguez-Mateos *et al.*, 2014; Schön *et al.*, 2018). Therefore, it may be suggested that the metabolites of delphinidin type anthocyanins have reduced bioavailability compared to cyanidin, however, this would need to be tested experimentally and existing studies tend to focus on the bioavailability of anthocyanins rather than their metabolites (Braga *et al.*, 2018).

Overall anthocyanin profiles reflected what was expected in light of the literature. It was not surprising that anthocyanins could not be detected in plasma due to the time of sampling (~24 hours after anthocyanin consumption). Previous work has shown that anthocyanins are only detectable in circulation for a few hours post-consumption, for example, De Ferrars *et al* showed that C3G could not be detected in serum 6 hours after consumption, whilst Nurmi *et al* report that anthocyanins derived from bilberry-lingonberry puree could only be detected in plasma between 1.5 – 6 hours post consumption (De Ferrars, Czank, Zhang, *et al.*, 2014a; Nurmi *et al.*, 2009). Anthocyanins were substantially increased in urine after black rice and bilberry anthocyanin extract consumption with the anthocyanin profiles detected largely reflecting the anthocyanin content of the extracts fed. Unsurprisingly, higher levels of anthocyanins were seen post black rice- compared to post bilberry anthocyanin extract consumption, reflecting the higher bioavailability of di-hydroxy B-ring anthocyanins relative to tri-hydroxy B-ring anthocyanins. Interestingly, post black rice anthocyanin extract consumption, urinary Peo3G was higher than C3G (the most abundant anthocyanin in black rice extract), and other studies have reported that C3G is subjected to methylation *in vivo* explaining this finding (De Ferrars, Czank, Zhang, *et al.*, 2014a; Vanzo *et al.*, 2013). Unexpectedly, C3Rut was putatively increased in several individuals after black rice consumption despite not being detected in an analysis of the black rice extract powder. Although still at very low levels (4.74 ± 6.47 nmol/24hrs) this was a statistically significant increase compared to measurements from all other phases of the study. One possible explanation for this is the

difference in sensitivity of the method used to quantify anthocyanins in the extracts and the optimised analysis method applied to clinical samples. The method employed to quantify anthocyanins in extracts used DAD which is less sensitive than the MRM method used in the analysis of clinical samples. Therefore, if only present at extremely low levels, as may be assumed by the low concentration detected in urine, it is plausible this may have been below the limits of detection and consequently not accounted for in the analysis of the extract and would explain why low levels of C3Rut were observed post black rice extract consumption. Alternatively, although the peak observed in analysis matched the retention time of an authentic C3Rut standard, it is possible that the compound may have been misidentified and could have merely been an artefact picked up during analysis, given the known low bioavailability of anthocyanin rutinosides. However, the ion fragmentation matched what has been observed in the literature and gave a response similar to what was seen for other low level anthocyanins, and therefore this compound has been included in the quantification of urinary anthocyanins (Ling *et al.*, 2009). Somewhat unexpectedly, cyanidin-3-glucuronide was the most abundant anthocyanin detected in urine post bilberry extract consumption. This suggests the possibility that tri-hydroxy B-ring anthocyanins are subjected to dehydroxylation and subsequently glucuronidation. Alternatively, cyanidin based anthocyanins are present in bilberry extract (Table 3-1) and cyanidin glucuronide may therefore be derived from these and its high abundance in urine may be explained by the higher bioavailability of cyanidin type anthocyanins relative to delphinidin. However, it should be noted that commercial standards for phase 2 conjugates of anthocyanins were not available and therefore quantification may not be accurate. Consistently low levels of anthocyanins were detected in numerous volunteer samples pre-intervention and post-placebo, most likely from the background diet of individuals, however, the effect of anthocyanin consumption on urinary anthocyanin excretion was pronounced.

Urinary anthocyanin excretion was subject to considerable inter-individual variation as is shown in Figure 3-5 and Figure 3-6. However, what is most interesting in the variability of the data is that there is a clear relationship between both anthocyanin treatments for total urinary anthocyanin excretion, and total anthocyanin metabolite excretion (Figure 3-7 and Figure 3-12). This indicates that there must be biological variation between individuals that determines their anthocyanin metabolising capacity which may include: gut enzyme and receptor levels and the gut microbiota. Time of anthocyanin and/or metabolite absorption from the gut is also likely to vary considerably between individuals. There is evidence in the literature for a role of the gut microbiota in anthocyanin metabolism. The gut microbiome is incredibly diverse and its composition varies enormously between individuals based on a large number of factors including: age, diet, medication and health conditions (Eckburg *et al.*, 2005b). It is hypothesised that the bioavailability of

anthocyanins largely depends on an individual's gut microbiota and its biotransformation mechanisms, however, little research has been done in this area (Eker *et al.*, 2019).

The data presented in this chapter provides insights into the range of metabolites that may be derived from anthocyanins *in vivo,* building on the current literature, indicating that anthocyanins undergo extensive metabolism to produce a diverse profile of phenolic acids, mainly present as phase 2 conjugates, some of which have not been reported previously. There are a number of strengths and weaknesses of this analysis. One of the main strengths was splitting analysis of samples between two optimised LC-MS/MS methods to maximise both sensitivity and the number of compounds included, going beyond what is reported in previous studies. Additionally, the format of a randomised cross-over trial meant that the same individuals were measured in each study phase facilitating comparisons between anthocyanin treatments to provide insights into whether there are correlations in an individual's metabolic response to dietary anthocyanins of different types. Another strength of this analysis was feeding anthocyanins in the matrix of a purified extract meant that phenolics were not at high levels reducing interference in the data obtained from background compounds that may be present in a food extract. However, as the anthocyanins fed were not labelled this meant that the parent compound and its metabolites were not traceable meaning that we cannot be entirely sure what has originated from the treatment in biological samples. Furthermore, although participants were asked to exclude anthocyanin rich foods from their diet, it would have been beneficial to ask participants to consume the same low phenolic meal prior to study days to provide consistency between baseline samples, however, this would have been difficult to enforce. Despite this, there are clearly differences between treatments with regards to anthocyanins and phenolic compounds detected in clinical samples, providing valuable information on anthocyanin metabolism and how this can vary among individuals. It is important to acknowledge that because urine samples were collected as 24-hour urine collections they merely give an overview of the metabolites produced post anthocyanin consumption and pharmacokinetic profiles cannot be established from this information. Whilst high and low level metabolites have been quantified, it should be acknowledged that some very low level metabolites may be missing from this analysis as a result of being diluted below the levels of detection by pooling all urine in a 24-hour period. To gain knowledge of the pharmacokinetic profiles of the fed anthocyanins and their metabolites *in vivo* it may be of interest to take urine and plasma samples at time intervals from just after anthocyanin consumption up to 72 hours to provide snapshots of the metabolite profile in the period after consumption.

3.7 Conclusions

Overall the work presented in this chapter shows that anthocyanins are extensively metabolised *in vivo* giving rise to a diverse range of metabolites, suggesting that it is the accumulation of multiple low molecular weight phenolic metabolites that are responsible for the reported bioactivity of anthocyanins. The metabolites derived from black rice and bilberry anthocyanin extracts display key differences in both the specific compounds produced and their relative concentrations. Catechol and its phase 2 conjugates are important metabolites of black rice anthocyanins, whilst compounds such as 5-hydroxyferulic acid and caffeic acid glucuronide are key metabolites of bilberry anthocyanins. However, there are similarities between the *in vivo* human metabolism of these two different types of anthocyanin, for example PGA is an important metabolite of both. What is evident in the data presented in this chapter is that the *in vivo* metabolism of anthocyanins is subject to large inter-individual variation in terms of not only the specific metabolites produced but also their relative concentrations. Here it has been reported that the urinary recovery of ingested anthocyanins (in both parent compound and metabolites) is vastly different between individuals and metabolite signatures are not consistent among the study population. Despite this, a clear relationship is shown between urinary excretion of anthocyanins and their metabolites for participants between different types of dietary anthocyanin, indicating that biological factors of individuals dictate the metabolic response to dietary anthocyanins. It is anticipated that one of the biological factors at play is the gut microbiota and that the microbiome profile of individuals has a large bearing on the metabolites produced, their relative concentrations and the time at which they are excreted following anthocyanin consumption, and this will be an important point of future work to aid our understanding of inter-individual variation in anthocyanin metabolism and how this may impact any health effects of eating anthocyanins. Therefore, in the next chapter variability in metabolism driven by the gut microbiota will be investigated through *in vitro* fermentation of faecal slurry samples with black rice anthocyanin extract, to establish the role of the gut microbiota in this metabolism and how this may vary between individuals.

Chapter 4

Investigating the Role of the Gut Microbiota in Anthocyanin Metabolism

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4.1 Abstract

Background: In Chapter 3 it was shown that anthocyanins have low bioavailability and are extensively metabolised *in vivo*. These metabolites may be derived from spontaneous degradation, microbial metabolism and/or human metabolism. Few studies have investigated the precise role of the gut microbiota in the biotransformation of anthocyanins and their phenolic degradants, and much remains to be understood regarding the range of microbial derived anthocyanin metabolites. It is hypothesised that the gut microbiota is responsible for a great degree of anthocyanin metabolism *in vivo* and the variability in metabolite profiles seen between individuals.

Objective: The aim of the work presented in this chapter was to study the associations between *in vitro* microbial dependent metabolism of C3G derived from black rice extract, and the *in vivo* anthocyanin metabolite profiles from the same participants who had taken part in a dietary intervention study in which they consumed encapsulated black rice anthocyanin extract.

Methods: Faecal samples were collected from volunteers in a human study before they started a dietary intervention where black rice and bilberry anthocyanin extracts were consumed. Faecal samples were preserved as glycerol stocks and used in *in vitro* fermentation experiments to measure the gut microbial metabolism of black rice anthocyanins over 48 hours. Anthocyanins and their metabolites were quantified using a combination of the optimised UHPLC-MS/MS analytical method described in Chapter 2 and untargeted single quadrupole mass spectroscopy.

Results: 18 anthocyanin metabolites were confirmed to be microbially derived including: catechol, dihydrocaffeic acid and pyrogallol amongst others. The rate of anthocyanin catabolism and the generation of metabolites was subject to considerable inter-individual variation. But statistically significant positive correlations were seen between the Cmax of *in vitro* microbial metabolites PCA ($R^2 = 0.13$ p = 0.02), pyrogallol ($R^2 = 0.34$ p = 0.003) and phloroglucinol ($R^2 = 0.49$ p = 0.0002), and

their urinary concentration after a dietary intervention, highlighting the crucial role of the gut microbiota in anthocyanin metabolism.

Conclusion and Future work: The gut microbiota is vital for the generation of a number of potentially bioactive anthocyanin metabolites. The specific metabolite profile generated and their relative concentrations varied depending on the volunteer from which faecal samples were derived, most likely due to differences in the microbial species present. Investigating the relationship between gut microbiota profiles and anthocyanin metabolism will be important in future work to understand the microbial species that may be important in the production of metabolites that may have bioactivity.

4.2   Introduction

Anthocyanins have low bioavailability and are extensively metabolised *in vivo* to give rise to several compounds with potential bioactivity. However, anthocyanin metabolite profiles are subject to high inter-individual variation as shown in Chapter 3, and several other studies (Nurmi *et al.*, 2009; Czank *et al.*, 2013; de Ferrars *et al.*, 2014a; Bresciani *et al.*, 2020; Jokioja *et al.*, 2021). Variability in anthocyanin metabolism may arise from several biological factors concerning an individual such as, enzyme and receptor levels, rate of absorption and the gut microbiota (Eker *et al.*, 2019; Fang, 2014; Nishioka *et al.*, 2021). A reasonable proportion of ingested anthocyanins are known to reach the gut intact, where they are met by a large community of microorganisms, collectively known as the gut microbiota, which has important roles in the metabolism of dietary nutrients, including polyphenols, such as anthocyanins (Eckburg *et al.*, 2005a; Jandhyala *et al.*, 2015). Microbial metabolism of anthocyanins is hypothesised to be critical in the generation of numerous metabolites with potential bioactivity and thus is key to understanding any potential health effects of consuming anthocyanins (Eker *et al.*, 2019; De Ferrars, Czank, Zhang, *et al.*, 2014a; Igwe *et al.*, 2019).

The gut microbiota comprises at least 1000 species of known bacteria, the majority of which come under the phyla: *Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria and Actinobacteria* (Eckburg *et al.*, 2005a). The relative abundance of these phyla and the many species within them varies greatly between individuals, with factors such as: age, gender, BMI and disease all being known to effect the population of the gut microbiota. The precise microbial communities within the microbiota of an individual are likely to be one of the major sources of inter-individual variation in anthocyanin metabolism and consequently one of the dictating factors of an individuals' response to dietary anthocyanins (Igwe *et al.*, 2019).

Studies specifically investigating the gut microbial metabolism of anthocyanins are scarce and have several limitations. Human dietary intervention studies reporting metabolites following ingestion

of pure anthocyanins are useful in providing information on the compounds derived from specific anthocyanins and their relative concentrations, but it is difficult to distinguish between gut microbial and human metabolism (de Ferrars *et al.*, 2014a). Furthermore, studies that feed participants anthocyanin-rich foods such as berries or extracts suffer from additional problems, most importantly that these sources of anthocyanin typically contain several phenolic acids which can compromise the reliable detection of low level metabolites. Whilst all *in vivo* studies are affected by inter-individual variability that cannot be avoided in humans and animals which complicates the interpretation of results (Faria *et al.*, 2014; Jokioja *et al.*, 2021). *In vitro* incubation of anthocyanins with human faecal matter has been performed in some studies to investigate microbial catabolism, bypassing difficulties in differentiating between human and gut microbial metabolism *in vivo*. Aura *et al*, found that protocatechuic acid (PCA) is a major colonic metabolite of cyanidin-3-glucoside (C3G) along with two other unidentified compounds (Aura *et al.*, 2005). Whilst Hanske *et al* found PCA, phloroglucinaldehyde (PGA) and gallic acid to be microbial products of C3G, in addition to 4 unidentified compounds (Hanske *et al.*, 2013).

Early *in vitro* studies have shown that bacterial metabolism of anthocyanins results in the cleavage of glycosidic linkages and the breakdown of the anthocyanidin heterocycle. Consequently, a spectrum of new metabolites are generated in the gut (Eker *et al.*, 2019). Colonic fermentation studies have shown that different gut microbial metabolites are generated from different anthocyanins. For example, colonic fermentation of cyanidin based anthocyanins resulted in the production of p-coumaric acid, gallic acid and 3-hydroxycinnamic acid (Chen *et al.*, 2017; Khanal *et al.*, 2014). Whilst malvidin based anthocyanins gave rise to syringic acid, 4-hydroxybenzoic acid and homovanillic acid (Forester and Waterhouse, 2008). Furthermore, recent unpublished work in the Kroon group has shown that anthocyanins are subject to both microbial and spontaneous degradation with PCA, PGA and catechol as the main anthocyanin metabolites (Emad Shehata, QIB, upublished).

Studies of microbial metabolism of anthocyanins within a food matrix are of higher physiological relevance as pure anthocyanins are not consumed as part of the diet. However, studies that use a whole food have limitations in determining which metabolites are anthocyanin derived due to their complex and varied composition, which can ultimately hinder our understanding of anthocyanin metabolite profiles. Berries are one of the most anthocyanin rich foods. *In vitro* fermentation studies utilising human or rat faeces have shown that PCA, 3-hydroxyphenylpropionic acid and pyrogallol are important microbial metabolites of berries (Chen *et al.*, 2017; González-Barrio *et al.*, 2011). Cheng *et al* studied 5 intestinal bacteria incubated with mulberry anthocyanins under anaerobic conditions and identified caffeic acid and ferulic acid as microbial metabolites formed by bacterial β-glucosidase action (Cheng *et al.*, 2016). There is evidence in the literature that bacterial

enzyme activity leads to the catabolism of anthocyanins into more bioavailable forms and this in turn may regulate the colonic microbiota composition (Eker *et al.*, 2019).

Most studies analyse anthocyanin metabolites in *in vitro* fermentation models or in *in vivo* digestion assays 8 hours after anthocyanin consumption or more. Faria *et al* showed that the highest faecal recovery of anthocyanin metabolites is seen 24 hours after ingestion, whilst Czank *et al* have reported that many C3G metabolites did not reach maximal concentrations in faeces until 48 hours of interaction. They suggest that prolonging the faecal sampling period, perhaps even to 72 hours may show better recoveries (Czank *et al.*, 2013; Eker *et al.*, 2019; Faria *et al.*, 2014).

The work described in this chapter seeks to go beyond the current state of the art in understanding the role of the gut microbiome in anthocyanin metabolism. This was achieved by (i) combining assessments of the anthocyanin metabolites produced following consumption of highly purified anthocyanin extracts in a randomised, placebo controlled dietary intervention study (data presented in Chapter 3), with (ii) the metabolic capacity of faecal samples collected from the same participants to metabolise the same highly purified anthocyanin extract and produce the metabolites observed in urine.

4.3 Objectives

The overall aim of the work presented in this chapter was to measure the black rice derived C3G metabolic capacity of 23 individuals using a batch *in vitro* fermentation model inoculated with faecal samples from individuals that took part in the human intervention study described in Chapter 3. The specific objectives of this work were:

- Measure the capacity of the gut microbiota of 23 individuals to degrade black rice derived C3G and compare this to spontaneous anaerobic C3G degradation in the same individuals.
- Measure the initial rate of the appearance of microbial metabolites of black rice derived C3G and their final concentration after 48 hours and compare gut microbial metabolism of C3G between these 23 individuals
- Determine the associations between metabolites produced in the *in vitro* fermentation model and those produced *in vivo* using urinary metabolite data from Chapter 3.

4.4 Methods

4.4.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Dorset, United Kingdom) unless stated otherwise. All water used was 18 MΩ/cm Milli-Q water, and solvents were LC/MS grade. The list of compounds included in standard curves for LC-MS/MS analysis of samples is given in Chapter 3.

4.4.2 Human Study

The human study was performed according to the protocol described in Chapter 3. For a subset of 23 participants, faecal samples were collected pre- and post-intervention for each phase of the study. Participants were asked to provide a faecal sample within 3 days preceding the start of the treatment period or ± 3 days of finishing the treatment period. Participants were asked to deliver faecal samples to the study team within 6 hours of production. Samples were kept on ice until processed.

4.4.3 Faecal Slurry Processing

Faecal samples were provided by volunteers within 6 hours of production, and were kept on ice until processed by the study team (prior to, and during processing, samples were exposed to an aerobic environment). To preserve the microbial composition of faecal samples, faecal slurries were produces as follows. Approximately 50 g faeces was weighed into a stomacher bag containing a filter which was then diluted 1:1 with 0.5 mM potassium phosphate buffer containing 0.05% L-cysteine (Thermo-fisher, Kent, United Kingdom) at pH 6.6. This was then processed in a stomacher at 230 rpm for 30 seconds. The filter insert was then removed and analytical grade glycerol (Thermo-fisher, Kent, United Kingdom) was added to give a final concentration of 25% glycerol. This was then mixed thoroughly before being aliquoted and stored at -80°C until use for a period of 1 – 3 years (O'Donnell *et al.*, 2016). Notably only pre-intervention slurry samples were used for the *in vitro* fermentation colon model experiments described in this chapter.

4.4.4 Nutritive Culture Media Preparation

Nutritive culture media was made containing: 2g/L peptone water, 2g/L yeast extract, 2g/L sodium bicarbonate, 0.5g/L cysteine hydrochloride, 0.5g/L bile salts, 0.1g/L sodium chloride, 0.04g/L dipotassium phosphate, 0.04g/L monopotassium phosphate, 0.01g/L magnesium sulfate heptahydrate, 0.01g/L calcium cholorde, 0.2% tween80, 0.02g/L hemin and 0.0001% Vitk1 dissolved in MilliQ-grade water (Parmanand *et al.,* 2019). Media was autoclaved for sterilisation prior to 10g/L glucose (final concentration) being added the day before the experiment and filtered to prevent any microbial contamination.

4.4.5 Total Anaerobe Viability

Nutritive media was made as described above, and kept in an anaerobic cabinet 24 hours prior to the experiment taking place. Faecal slurries were thawed at room temperature for 30 minutes and transferred to an anaerobic cabinet where they were incubated at a concentration of 1% faecal material in nutritive media. Samples were taken at 0, 4, 8, 12 and 24 hours and were serially diluted to 10^{-7} in sterile PBS and plated on Wilkins-Chalgren agar. Plates were kept in the anaerobic cabinet for 24 hours before being removed for colony counting. Note, as a negative control one faecal slurry

sample was autoclaved prior to incubation to kill any live microbes present, and one faecal slurry sample was incubated with 50 µg/ml (111 µM) C3G in black rice extract (Beijing Gingko Group, China) to assess whether this effected total anaerobe viability.

4.4.6 *In vitro* Batch Fermentation Model

The working volume of each vessel was set at 150 ml, containing nutritive media, 1% faecal material in the matrix of a processed glycerol stock and 50 µg/ml (111 µM) C3G in black rice extract (Beijing Gingko Group, China) which was dissolved directly into the media at the beginning of the experiment. The pH was controlled and maintained between 6.6 – 7.0 using Fermac 260 pH control units (Electrolab, United Kingdom), connected to 0.5M NaOH and 0.5M HCl solutions (Thermo-fisher, Kent, United Kingdom). Vessels were kept at 37 °C by a circulating water jacket and anaerobiosis was maintained by continuous bubbling of the system with oxygen-free nitrogen gas (Parmanand *et al*., 2019). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours from the vessels and diluted 1:1 with 5% formic acid in water (MilliQ-grade) to stabilise C3G and prevent any further degradation, samples were stored at -20°C prior to analysis. In addition to vessels containing live faecal sample, vessels were also run containing heat inactivated autoclaved faecal sample to account for spontaneous degradation, whilst blank vessels with live faecal sample but without black rice extract were also run to account for any products of *in vitro* fermentation of live faecal samples that may not be derived from black rice anthocyanin extract.

4.4.7 LC-MS/MS Analysis of Anthocyanins and Phenolics

Samples from the batch fermentation model were thawed on ice and centrifuged at 15 000 rpm for 10 minutes at 4°C to pellet bacterial cells for their removal and then spiked with a final concentration of 10 µM taxifolin as in internal standard. For targeted phenolic analysis the same method was applied as was used for the analysis of urine and plasma samples and is described in the methods section of Chapter 2.

For anthocyanin analysis, and untargeted detection of metabolites, the same samples were kept at 4°C and injected onto a Kinetex XB-C18 100A, 2.6µ 100 x 4.6 mm column (Phenomenex, Macclesfield, United Kingdom) linked to an Agilent 1100 series system (HP1100 Agilent Technologies, Waldbronn, Germany) equipped with G1956B single quadrupole mass spectrometer operating in negative mode, and DAD channels at 520 nm, 280 nm, 250 nm and 320 nm. The mobile phase A was 1% formic acid in water, and the mobile phase B 1% formic acid in acetonitrile. The gradient used was: 2% B at 0 minutes, 10% B at 15 minutes, 15% B at 20 minutes, 30% B at 25 minutes, 50% B at 30 minutes, 50% B at 34 minutes, 2% B at 35 minutes and 2% B at 37 minutes.

Matrix matched standard curves were used to find the response factor of identified compounds and the concentration of each compound was calculated according to formula 1 (Chapter 2), and corrected according to the volume change of the vessel.

4.4.8 Statistical Analysis

Statistical analysis was performed in GraphPad Prism (version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). A Mann-Whitney U Test was used to compare means between autoclaved and live faecal slurry samples due to datasets being non-parametric and violating the assumption of having equal variances', values of $p \leq 0.05$ were considered statistically significant.

4.4.9 Distance Measures and Principle Component Analysis

Time series data for each compound and participant was processed using dynamic time warping (DTW) by George Oastler (University of East Anglia) (https://github.com/uea-machine-learning/tsml/blob/master/src/main/java/tsml/classifiers/distance_based/distances/dtw/DTWDistance.java), with the DTW window set to 100% to compare time series. DTW produced distance measures indicating how similar each time series was between participants for each compound, with larger distances indicating more dissimilarity. The similarity matrix produced was then used to perform a principal component analysis (per compound) in Python v3.9.6. The principal component analysis was configured to use full singular value decomposition, identifying all principal components. The first two principal components were used in scatter plots to visualise the similarity of data between individuals.

For global analysis of similarity of all compounds between participants, multivariate DTW was performed with the window set to 100%. Each dimension of the multivariate instance was an individual compound. DTW compared every instance (participant) by conducting multivariate DTW across the dimensions (compounds) and produced a similarity matrix reflecting the data for all compounds for each participant and was used for principal component analysis as described previously.

4.5 Results

4.5.1 Total Anaerobe Viability in Stored Glycerol Stocks

Throughout the human intervention study (described in the methods section of Chapter 3), faecal samples were collected from a subset of participants (n = 23) and were preserved as glycerol stocks to be used in *in vitro* fermentation experiments. The human intervention study spanned a prolonged period of time and some glycerol stocks had been in -80°C storage for 2-3 years before *in vitro* fermentation model experiments took place. Although glycerol stocks have been reported

to be an effective preservation technique for bacteria, a subset of samples were subjected to plating and counting to verify viability of total anaerobes to ensure the samples were fit for the purpose they were intended (O'Donnell *et al.*, 2016; Parkar *et al.*, 2019). Five samples were selected to be plated that were collected at early, middle and late phases of the human study, in addition to a quality control (QC) sample that had been in storage for just 5 months. One sample (BERI 23) was also incubated in anaerobic conditions with black rice extract to check if its addition affected growth of anaerobes. As a negative control, a portion of one sample (BERI 67) was autoclaved to kill any live bacteria. The results from this experiment are shown in Figure 4-1.
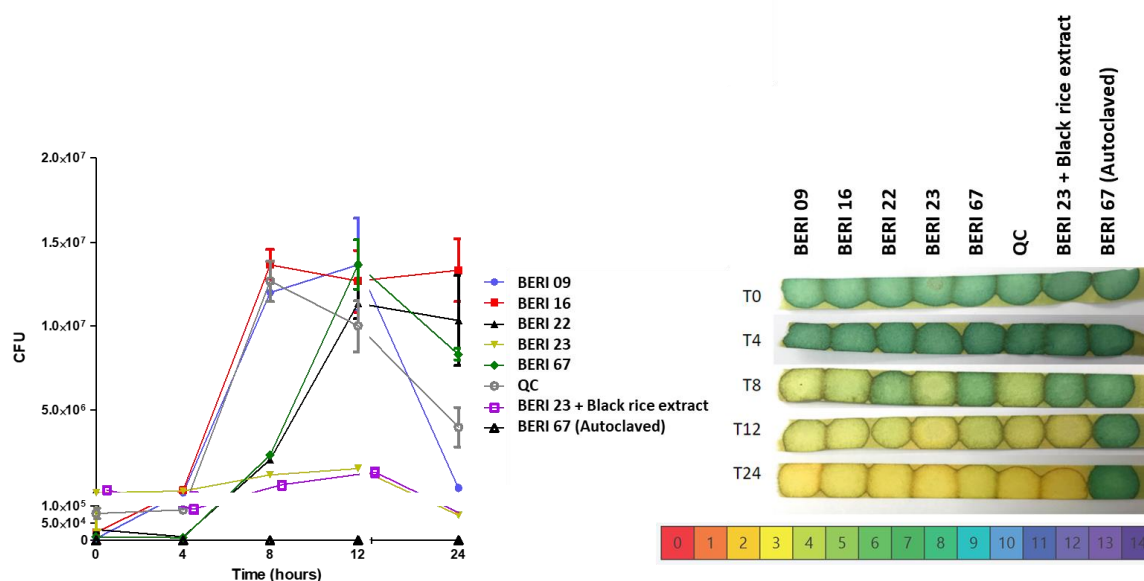
Figure 4-1-Total anaerobe viability, CFU over 24 hours.
Faecal slurries from 5 volunteers from the human intervention study, along with a recently prepared QC sample (stored for 5 months at -80°C) were incubated in nutritive media under anaerobic conditions for 24 hours and plated at 0, 4, 8, 12 and 24 hours on wilkins-chalgren agar, and CFUs were counted. One sample (BERI 23) was incubated with the addition of black rice extract to give a final concentration of 50 µg/ml C3G to establish if this affected bacterial growth, whilst a negative control of heat inactivated autoclaved faecal sample from one volunteer (BERI 67) was also included. Mean ± SD n = 3. The right shows the pH changes that took place over the 24 hours, assessed using litmus paper.

Figure 4-1 shows that most samples had good bacterial growth (BERI 09, BERI 16, BERI 22, BERI 67 and QC). Surprisingly, sample BERI 23 gave considerably fewer total anaerobes (CFU) compared to the other samples plated. This was also the case when incubated with black rice extract, but importantly black rice extract did not appear to cause any inhibition of bacterial growth. The low counts of total anaerobes for sample BERI 23 were particularly surprising as this was not the oldest of the samples plated. Excluding BERI 23, all other samples plated showed bacterial growth comparable to that which was seen with the QC sample, suggesting that up to 3 years in -80°C storage did not adversely affect the viability of total anaerobes. In several samples (BERI 09, BERI 67 and QC) a reduction in total anaerobes was observed between 12 and 24 hours. This reduction

in CFU's was coupled to a reduction in pH, likely caused by waste products produced by bacteria in the media and the lack of pH regulation in the anaerobic cabinet. This idea was reinforced by the lack of pH change in the autoclaved negative control (Figure 4-1). The main finding from this experiment was that faecal slurries preserved as glycerol stocks generally showed high counts of viable total anaerobes and would be suitable to assess the anthocyanin metabolising capacity of an individual's gut microbiota in an *in vitro* batch fermentation model.

4.5.2 Reproducibility of the *In Vitro* Batch Fermentation Model

The *in vitro* batch fermentation model has limited throughput and only small amounts of faecal slurry were available for this series of experiments. Therefore, the reproducibility of the model was tested to assess whether experiments could be justifiably performed in singlet. To test this, C3G metabolism was measured for two individuals in triplicate and the coefficient of the variation was calculated.

Table 4-1- Coefficient of Variation (%) for BERI 36 (n = 3).
Faecal slurry sample from BERI 36 (1% faeces) was subjected to in vitro fermentation with 50 µg/ml C3G in black rice extract for 48 hours in nutritive media under anaerobic conditions, metabolites were quantified using UHPLC-MS/MS and the coefficient of variation was calculated at each sampling time.

Compound	0 Hours	4 Hours	8 Hours	12 Hours	24 Hours	30 Hours	36 Hours	48 Hours
Cyanidin-3-*O*-glucoside	0	3.5	6.9	27.1	0	0	0	0
PCA	2.7	2.2	13.1	8.2	26.1	28.8	0	0
PGA	8.4	9.6	9.0	8.1	19.5	28.8	6.5	17.0
Catechol	0	0	0	24.0	16.2	5.2	3.9	9.4
Gallic acid	7.9	17.3	5.4	9.4	10.9	13.4	0	0
Pyrogallol	0	0	0	0	0	23.4	22.9	9.7
PGC	0	1.4	5.1	16.0	6.9	4.9	8.9	8.3
4-Hydroxybenzoic acid	0	0	7.1	7.5	11.2	24.6	0	0
Dihydroferulic acid	0	1.4	5.1	16.0	6.9	4.9	8.9	8.3
4-Methyl Catechol	0	0	0	0	0	0	34.4	6.7

Table 4-2- Coefficient of Variation (%) for BERI 62 (n = 3).
Faecal slurry sample from BERI 62 (1% faeces) was subjected to in vitro fermentation with 50 µg/ml C3G in black rice extract for 48 hours in nutritive media under anaerobic conditions, metabolites were quantified using UHPLC-MS/MS and the coefficient of variation was calculated at each sampling time.

Compound	0 Hours	4 Hours	8 Hours	12 Hours	24 Hours	30 Hours	36 Hours	48 Hours
Cyanidin-3-*O*-glucoside	0	11.2	28.3	20.8	0	0	0	0
PCA	8.6	20.9	3.5	4.3	9.2	20.1	16.0	6.1
PGA	8.6	14.5	18.9	7.8	9.8	20.1	23.4	13.6
Catechol	0	0	0	12.3	13.9	19.8	9.4	5.1
Gallic acid	12.3	6.3	9.0	13.6	9.6	9.9	0	0
Pyrogallol	0	0	0	0	0	7.3	0	5.8
PGC	0	0	0	0	23.9	45.9	21.4	8.6

Compound	0 Hours	4 Hours	8 Hours	12 Hours	24 Hours	30 Hours	36 Hours	48 Hours
4-Hydroxybenzoic acid	0	0	17.2	13.6	3.7	7.4	17.9	13.5
Dihydroferulic acid	0	0	23.5	18.9	5.8	8.6	6.4	8.7

Overall, the data in Table 4-1 and Table 4-2 show low variability, indicating that the batch *in vitro* fermentation model provides a robust system for measuring the microbial metabolism of anthocyanins. In the vast majority of cases the coefficient of the variation is < 20%, suggesting consistency between vessels. The coefficient of variation is inversely related to the concentration of compounds, i.e. as concentrations are lower the coefficient of variation tends to increase, this relationship was best illustrated by C3G whereby as it degraded the coefficient of variation increased until this compound had completely disappeared. In instances where variability became high, for example at 30 hours for phloroglucinol carboxylic acid (PGC) in BERI 62 where the coefficient of variation leapt to 45.9%, this increase was only transient with the following time points showing reduced variability, down to 8.6% by the final 48 hour time-point. This shows that although the rate at which metabolic reactions are taking place may vary between vessels slightly, they will effectively 'catch-up' with each other and the overall picture of the metabolism taking place is very consistent in terms of the concentrations reached. Accordingly, only one vessel was used to establish the anthocyanin metabolising capacity of individual gut microbiotas.

4.5.3 Anthocyanin Metabolising Capacity of the Gut Microbiota

In vitro fermentation of black rice anthocyanin extract with faecal slurries from 23 individuals resulted in the detection of 18 compounds, with only 2 out of these 18 compounds also being generated spontaneously. Disappearance of C3G occurred in both live and heat inactivated samples, however, this occurred at a faster rate in live samples and resulted in C3G completely disappearing from all vessels incubated with live bacteria before 48 hours. In contrast, in heat inactivated samples even after 48 hours ~20% C3G remained. Crucially, C3G disappearance varied considerably amongst individual live faecal samples, but this was far more consistent in heat inactivated samples, as indicated by the error bars in Figure 4-2, up to 24 hours, where C3G concentration plateaued in vessels with both live and heat inactivated faecal sample. Peo-3G is a low level anthocyanin found in black rice extract accounting for ~7% total anthocyanin content (Chapter 3), however, in all samples tested (live faecal samples and heat inactivated) this was below the limits of quantification after 4 hours (this was also the case for other low level black rice anthocyanins, listed in Chapter 3) and therefore this data is not presented.

Figure 4-2- C3G metabolism in the in vitro fermentation colon model
*C3G concentration was monitored over 48 hours in an in vitro fermentation model containing 1%
faecal material and 111 µM (50 µg/ml) C3G black rice extract in nutritive media over 48 hours for the
23 individuals. Data are shown as mean ± SD, the black line shows data for heat inactivated samples,
and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and
48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and
**** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.*

PCA and PGA were identified during *in vitro* fermentation of heat inactivated faecal samples,
indicating that these metabolites are produced spontaneously, i.e. do not require an active
microbiota. This reinforces findings from Triebel *et al* who showed that PCA is a cell free
anthocyanin degradation product (Triebel *et al.,* 2012). Interestingly, despite PCA and PGA being
the only spontaneous products of black rice anthocyanins detected they only accounted for a very
small proportion of observed spontaneous C3G degradation < 20%. Furthermore, both of these
compounds were produced at higher levels in the presence of a live microbiota, with high variability
between individuals, emphasising an important role also for microbes in the production of these
compounds; this data is shown in Figure 4-3 and Figure 4-4. Both PCA and PGA are present at 0
hours, with PCA increasing until 4 hours and then decreasing until 48 hours, whereas PGA increased
over the first 12 hours and then decreased between 12 and 48 hours, highlighting that these initial
metabolites are likely precursors for other microbial anthocyanin metabolites.

Figure 4-3- PCA concentration in the in vitro fermentation colon model.
PCA concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 μM C3G in black rice extract in nutritive media for the 23 individuals. Data are shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.
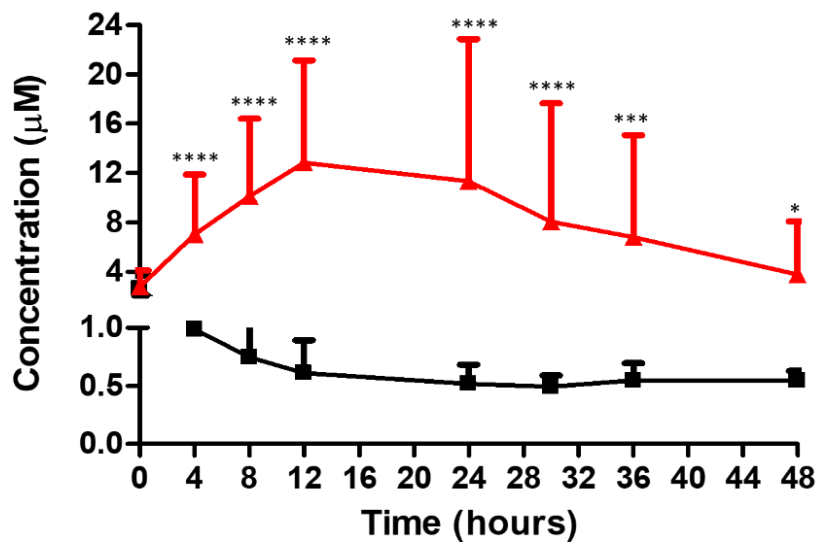
Figure 4-4- PGA concentration in the in vitro fermentation colon model
PGA concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 μM C3G in black rice extract in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.

All other compounds that were identified were only observed in the presence of a live microbiota. Most of these metabolites exhibited a gradual increase in concentration over the 48 hour time course of the experiment. Catechol, was identified in all faecal samples tested, however, the observed concentrations of catechol were subject to considerable inter-individual variation, as indicated by the large standard deviation values shown in Figure 4-5. Catechol was not present at 0 hours in any faecal sample tested, but could be detected at low concentrations at 4, 8 and 12 hours with much larger increases in concentration between 12 – 48 hours, reaching 13.9 ± 12 µM by 48 hours. Unlike catechol, other metabolites that showed this gradual increase over the entire 48 hours of *in vitro* fermentation were only produced by a subset of faecal samples. 4-methyl catechol was identified in 13 of the 23 samples (details of the faecal samples that produced each compound when incubated with black rice extract are given in Table 4-3). Although only reaching low concentrations (0.2 ± 0.3 µM at 48 hours), 4-methyl catechol presented a similar trajectory to catechol whereby it first appeared at very low levels over 4 – 24 hours but showed much greater increases between 24 – 48 hours, shown in Figure 4-6. Pyrogallol (Figure 4-8) shared a very similar trajectory to 4-methyl catechol with only very low levels observed and largest increases in concentration between 24 - 48 hours but was produced by slightly more samples (14 out of 23). Like 4-methyl catechol and pyrogallol, dihydrocaffeic acid was also a low level metabolite only produced by a small subset of samples (8 out of 23), however, this was first produced later (not until 12 hours) but also showed greatest increases in the latest time-points of *in vitro* fermentation (36 and 48 hours), shown in Figure 4-7. 3-hydroxyphenylacetic acid was not detected in any faecal sample until 24 hours of *in vitro* fermentation, and displayed a much steadier increase between 24 and 48 hours than other compounds produced, and reached 17.6 ± 29.2 µM by 48 hours (Figure 4-9), however, this metabolite was not common across all samples and was only detected in 11 out of the 23 faecal samples tested. Homovanillic acid was first detected at low levels after 4 hours of *in vitro* fermentation, however, the most rapid increases were between 4 – 24 hours with only modest increases seen thereafter, reaching 1 ± 1.6 µM at 48 hours (Figure 4-10) and only being observed in 12 of the 24 faecal samples analysed. Of the metabolites that increased over the whole 48 hour time course 2,4-hydroxybenzoic acid was produced by the smallest subset of samples (4 out of 23), and was extremely variable reaching 0.7 ± 2.6 µM by 48 hours across all samples tested (Figure 4-11), with BERI 09 exhibiting much higher concentrations of this metabolite than faecal samples from all other volunteers as shown in Table 4-4.
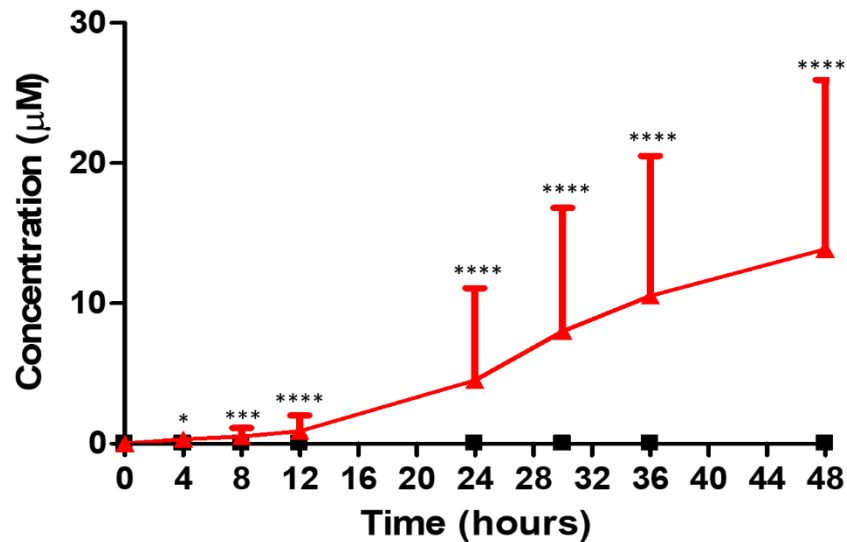
Figure 4-5- Catechol concentration in the in vitro fermentation colon model.
Catechol concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 µM C3G in black rice extract in nutritive media for the 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.

Figure 4-6- 4-Methyl catechol concentration in the in vitro fermentation colon model.
4-Methyl catechol concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 µM C3G in black rice extract, in nutritive media for the 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.

Figure 4-8- Pyrogallol concentration in the in vitro fermentation colon model.
Pyrogallol concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 μM C3G in black rice extract, in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.
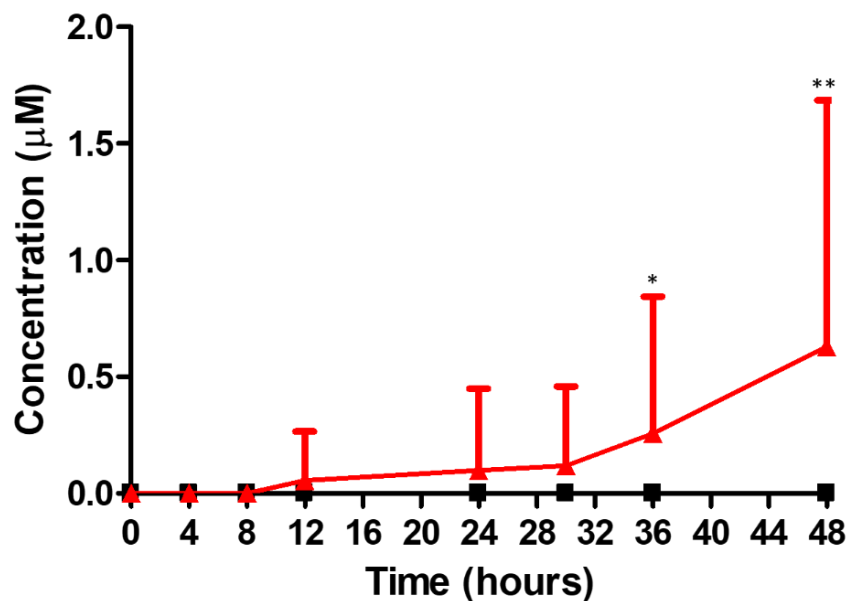
Figure 4-7- Dihydrocaffeic acid concentration in the in vitro fermentation colon model.
Dihydrocaffeic acid concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 μM C3G in black rice extract, in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23) Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.
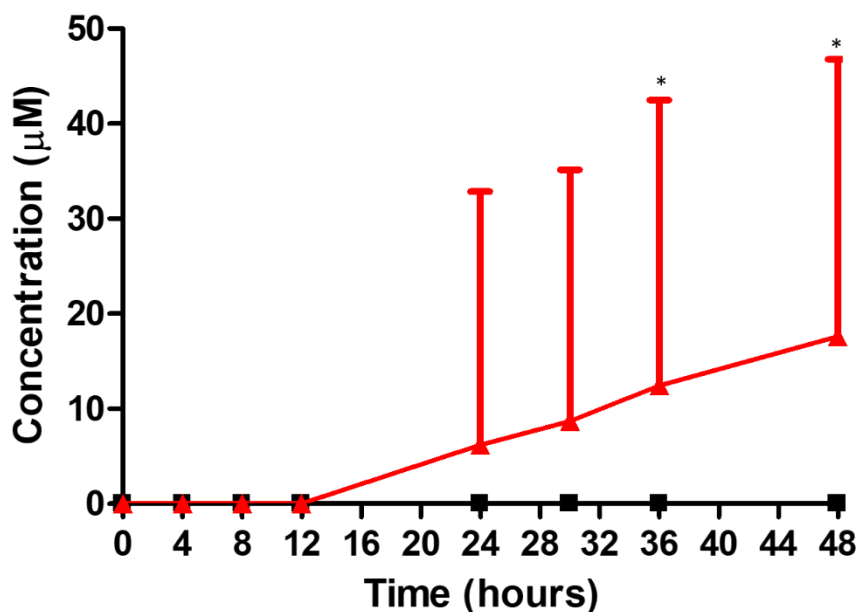
Figure 4-9- 3-Hydroxyphenylacetic acid concentration in the in vitro fermentation colon model.
3-Hydroxyphenylacetic acid was monitored concentration over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 μM C3G in black rice extract, in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.
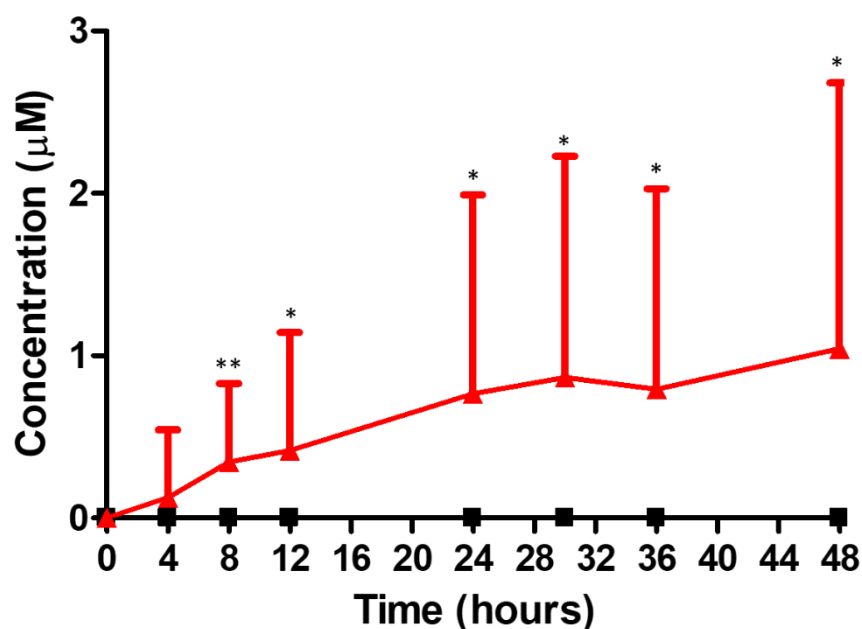
Figure 4-10- Homovanillic acid concentration in the in vitro fermentation colon model.
Homovanillic acid concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 μM C3G in black rice extract, in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.

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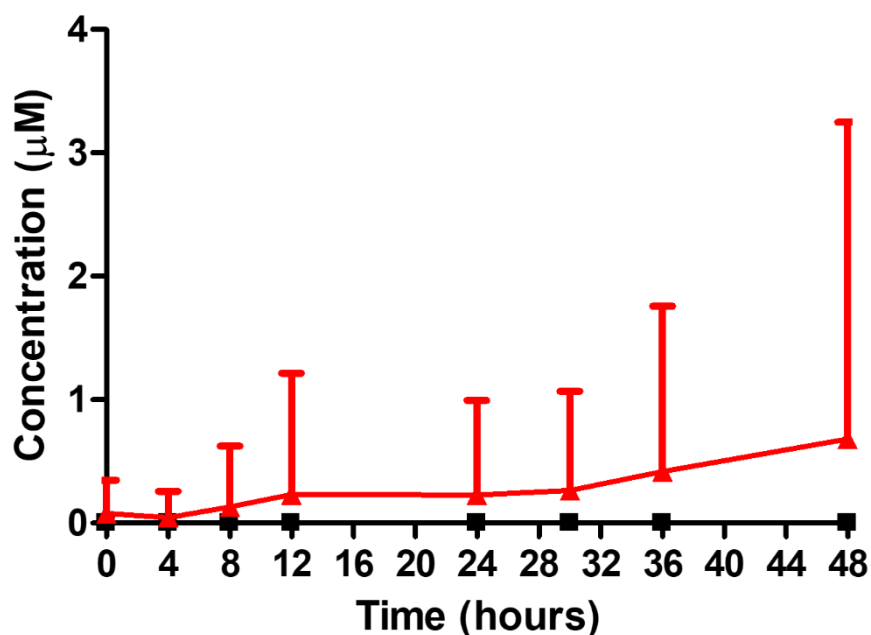
Figure 4-11- 2,4-Dihydroxybenzoic acid concentration in the in vitro fermentation colon model.
2,4-Dihydroxybenzoic acid concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 μM C3G in black rice extract, in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.

Several compounds increased in early stages of *in vitro* fermentation before reaching a plateau. 4-hydroxybenzoic acid was produced by faecal samples from all 23 individuals when incubated with black rice anthocyanin extract over 48 hours. Increases were observed between 0 – 24 hours, with the steepest increase between 8 – 12 hours. However, after 24 hours concentrations of 4-hydroxybenzoic acid plateaued with a modest decrease observed between 24 and 48 hours, illustrated in Figure 4-12. Dihydroferulic acid (Figure 4-13) showed a similar trajectory to 4-hydroxybenzoic acid and reached a similar concentration with a Cmax of 1.3 ± 0.5 μM at 24 hours, and was also produced by all faecal samples measured during *in vitro* fermentation (Table 4-3). Phloroglucinol on the other hand was only produced by 14 of the 23 faecal samples, and was only seen at low concentrations. Like dihydroferulic acid and 4-hydroxybenzoic acid this increased until 24 hours and then plateaued with a slight decrease from 24 – 48 hours, shown in Figure 4-15. Phloroglucinol carboxylic acid (PGC) was detected in just 10 of the 23 faecal samples subjected to *in vitro* fermentation with black rice extract and was not seen until 8 hours, and increased until 30 hours but then remained at an average of 0.23 μM from 30 – 48 hours, as shown in Figure 4-14.
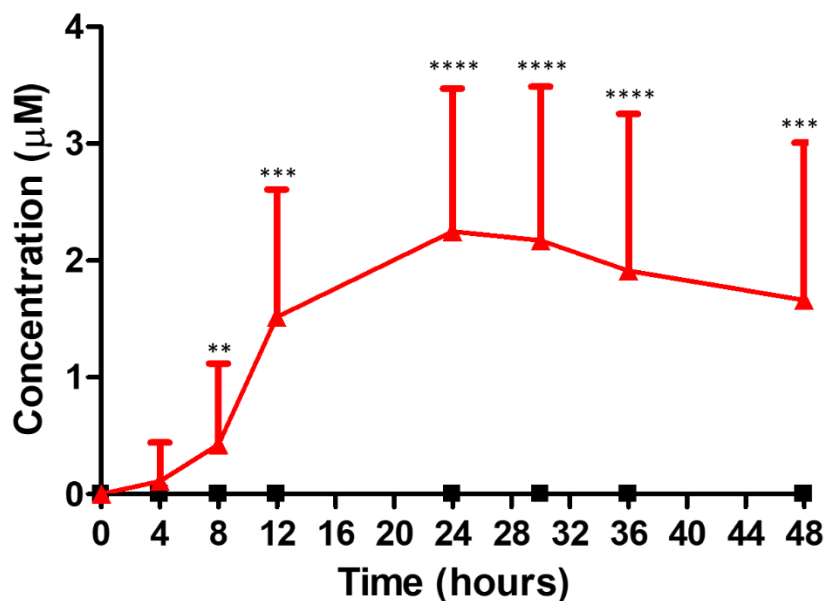
Figure 4-12- 4-Hydroxybenzoic acid concentration in the in vitro fermentation colon model.
4-Hydroxybenzoic acid concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 μM C3G in black rice extract, in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.
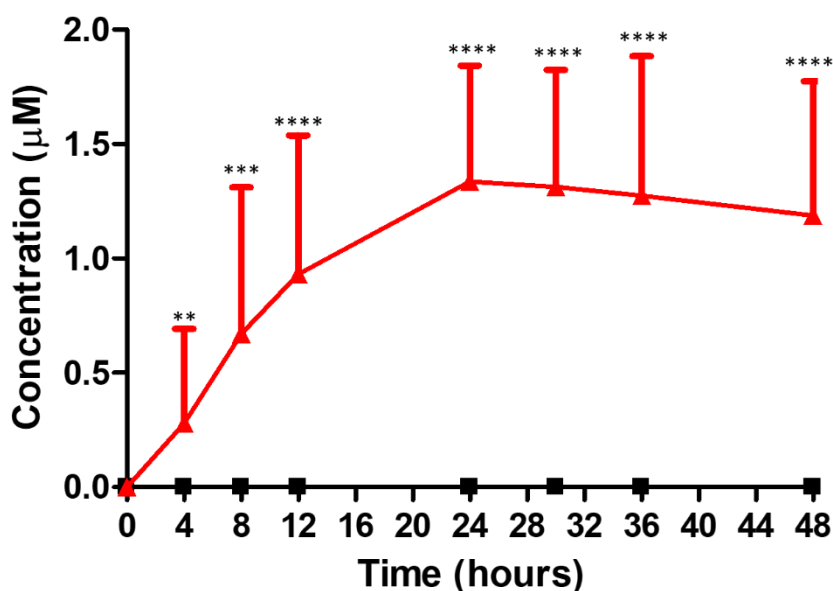
Figure 4-13- Dihydroferulic acid concentration in the in vitro fermentation colon model.
Dihydroferulic acid concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 μM C3G in black rice extract, in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.
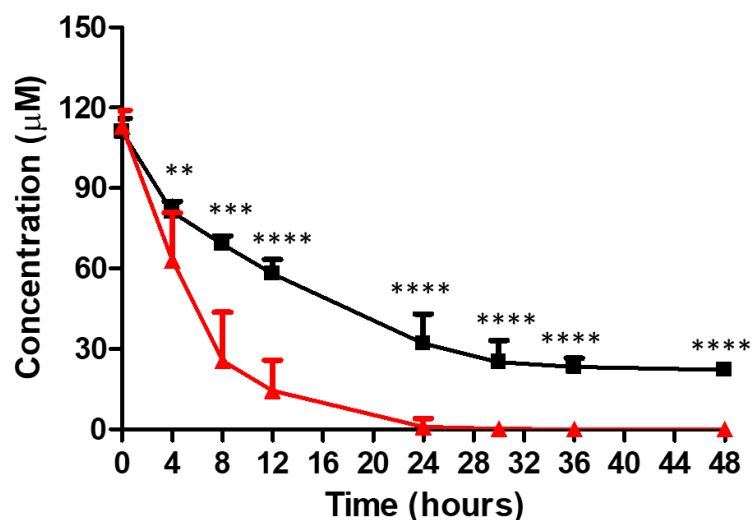
Figure 4-15- Phloroglucinol concentration in the in vitro fermentation colon model.
Phloroglucinol concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 µM C3G in black rice extract, in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** $p < 0.0001$; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.
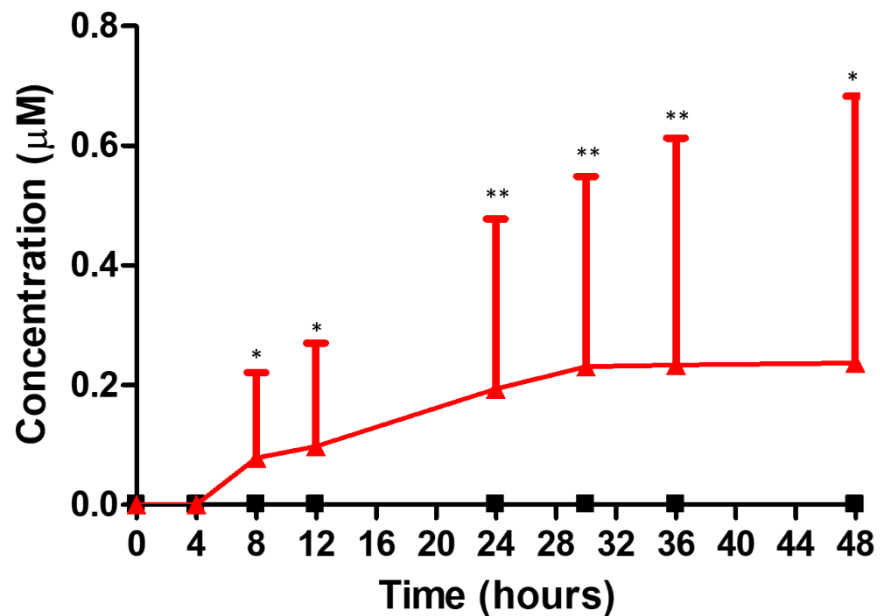
Figure 4-14- Phloroglucinol carboxylic acid (PGC) concentration in the in vitro fermentation colon model.
Phloroglucinol carboxylic acid concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 µM C3G in black rice extract, in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** $p < 0.0001$; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

p-Coumaric acid was the only compound to appear transiently and then completely disappear in all the faecal samples where it was produced. A steep increase was seen between 0 – 4 hours of *in vitro* fermentation with a Cmax of 0.3 ± 0.2 µM being reached at 8 hours, however, after 8 hours the concentration decreased until 48 hours where p-coumaric acid had completely disappeared in all participants, as shown in Figure 4-16. However, some participants no longer had any detectable p-coumaric acid at much earlier time-points with the majority of participants exhibiting an absence of p-coumaric acid after 24 hours as shown in Table 4-5.
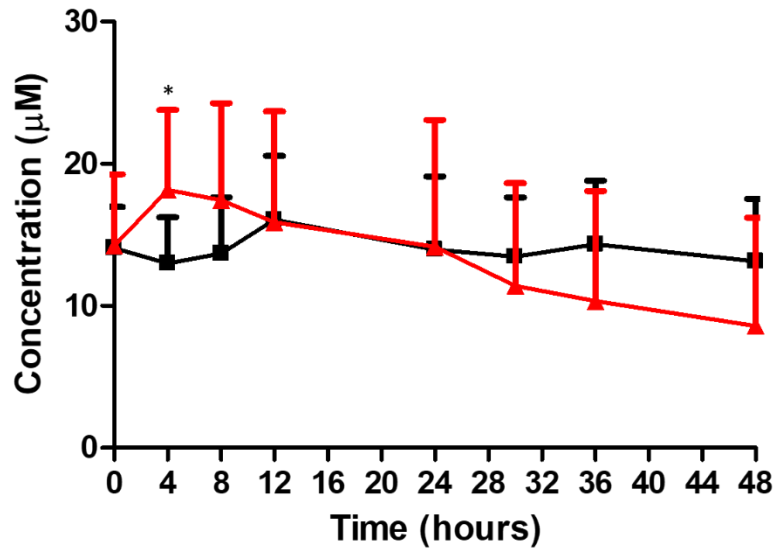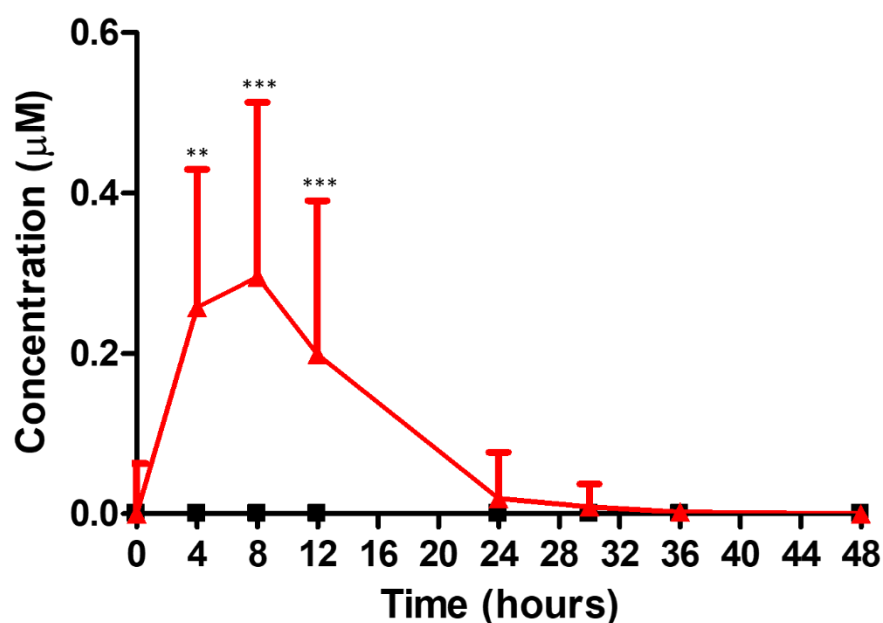

Figure 4-16- p-Coumaric acid concentration in the in vitro fermentation colon model.
*p-Coumaric acid concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 µM C3G in black rice extract, in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.*

Gallic acid was detected in the vast majority of vessels incubated with live faecal sample and black rice extract (21 out of 23). However, this compound exhibited an interesting observation (first made by Emad Shehata (Kroon Group, QIB, unpublished)), whereby autoclaving faecal slurry for heat inactivation purposes resulted in the production of gallic acid, shown in Figure 4-17. This phenomenon was not seen for any other compound included in this analysis. Although it was unclear why autoclaving faecal sample resulted in an increase in gallic acid it is possible that gallic

acid may have been bound to proteins in the faecal sample and when said faecal sample is autoclaved, causing protein to denature some of this bound gallic acid may be released, which may explain the findings in Figure 4-17.
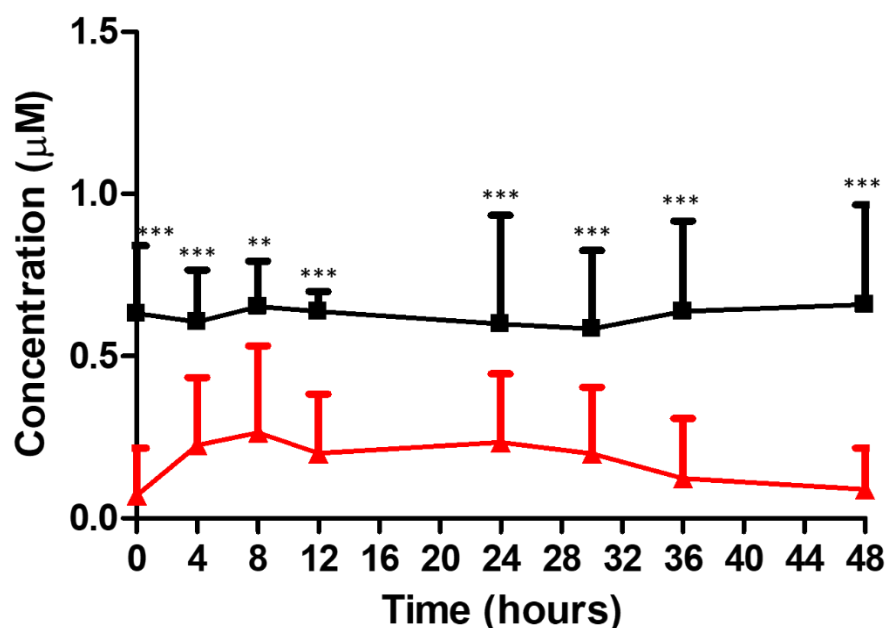
Figure 4-17- Gallic acid concentration in the in vitro fermentation colon model.
Gallic acid concentration was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 µM C3G in black rice extract, in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05.

In addition to these known metabolites two compounds were also detected that could not have their identities confirmed. One of these compounds (unknown X) gave a strong signal in the MRM channel for hippuric acid but ran at a different retention time by approximately 1 minute indicating this compound cannot be hippuric acid but may have a similarly structured identity. Interestingly, this compound was not detected until 24 hours and then increased steadily until 48 hours (Figure 4-18) and was only produced in the faecal samples of 7 individuals (Table 4-3) highlighting that this metabolite is not a common product of black rice anthocyanins. Likewise, a particularly strong signal was detected in the MRM channel for homoPCA (unknown Y), however, this ran at a retention time 20 seconds later than an authentic matrix matched standard of homoPCA suggesting that this could not be the identity of the compound detected. Quantification of this compound with homoPCA also gave concentrations much higher than what would be expected as this exceeded the concentration of black rice C3G seeded in the *in vitro* fermentation model, likely due to the true

identity of the compound having a higher response factor than homoPCA that cannot be accounted for in the absence of an authentic standard. Nevertheless this compound was detected in the faecal samples of all individuals tested and showed a trajectory of increasing between 8 and 24 hours before decreasing slightly until 48 hours, depicted in Figure 4-19. Furthermore, unknown Y was not detected in either heat-inactivated or blank samples suggesting that this compound must be a microbial product of black rice anthocyanins, most likely, C3G.
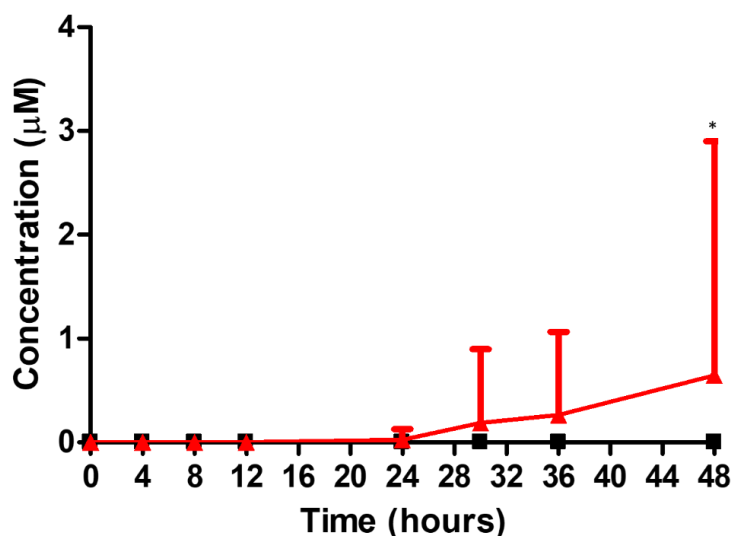
Figure 4-18- Estimated concentration of Unkown X in the in vitro fermentation colon model.
*Unknown X (found in the hippuric acid MRM channel) was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 µM C3G in black rice extract, in nutritive media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** p < 0.0001; *** p < 0.001, ** p < 0.01, * p < 0.05. Note due to lack of an authentic standard for this compound the concentration shown is merely an estimation based on quantification with hippuric acid.*
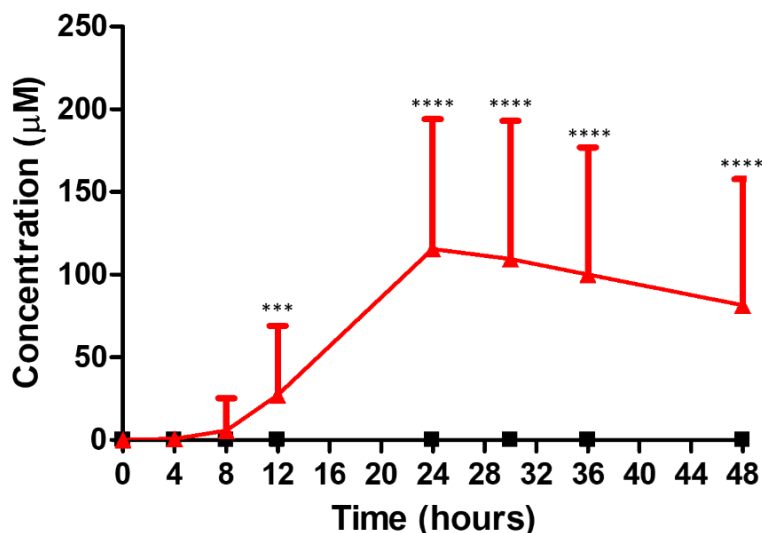
Figure 4-19- Estimated concentration of Unkown Y in the in vitro fermentation colon model
Unknown Y (found in the homoPCA MRM channel) was monitored over 48 hours in an in vitro fermentation model containing 1% faecal material and 111 µM C3G in black rice extract, in nutritive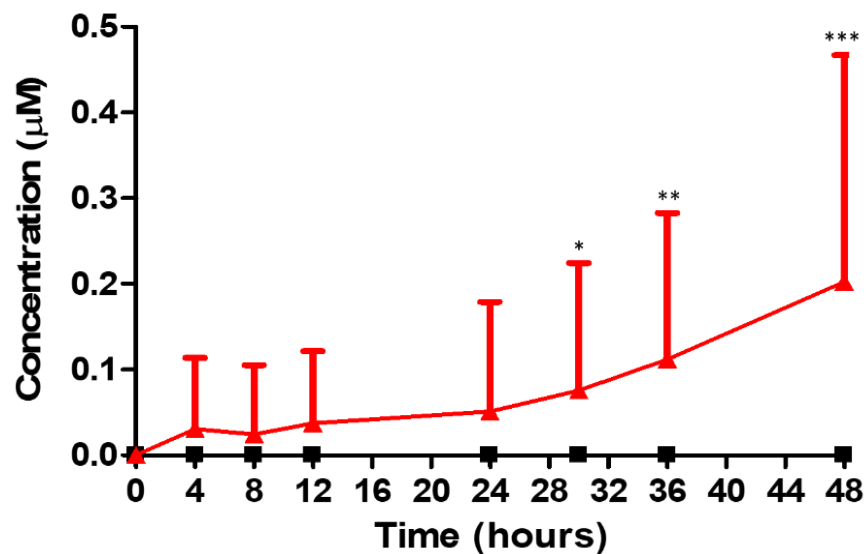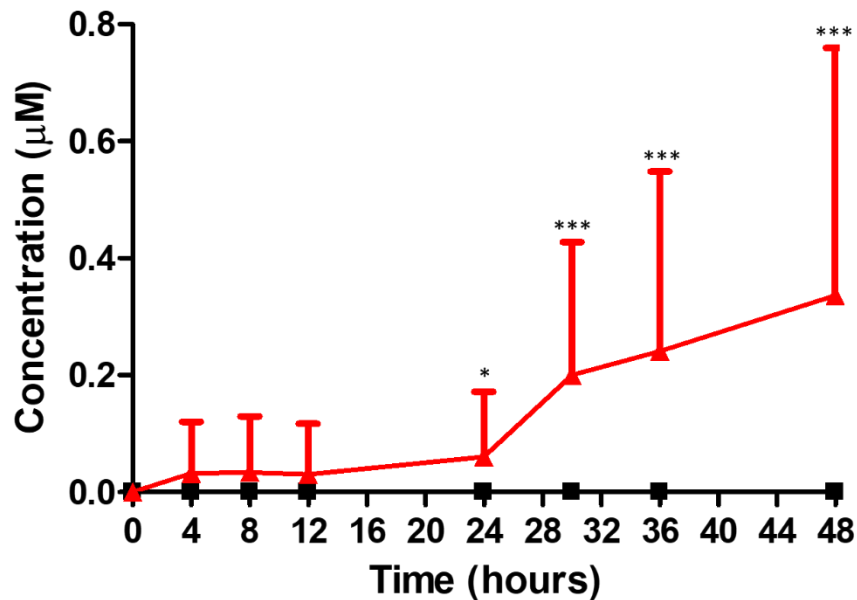 media for 23 individuals. Data is shown as mean ± SD, the black line shows data for heat inactivated samples, and the red line shows data for live samples (n = 23). Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Statistical analysis was carried out with a Mann-Whitney U-test for each time point and **** $p < 0.0001$; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Note due to lack of an authentic standard for this compound the concentration shown is merely an estimation based on quantification with homoPCA.

Overall the data presented here show an important role for the gut microbiota in anthocyanin metabolism, with a large number of compounds only generated in the presence of a live microbiota. An important observation from the figures presented is that standard deviation values are high, indicating that there is considerable inter-individual variation in anthocyanin metabolism, most likely due to differences in the gut microbiota of individuals. This inter-individual variation is highlighted in Table 4-3 - Table 4-5. Table 4-3 shows the time-point at which microbial anthocyanin metabolites were first produced for each individuals faecal sample (excluding PCA and PGA as these were also produced spontaneously and present at baseline). Catechol, was produced by faecal samples from all individuals tested, however, the time at which this was first detected varied considerably between individuals. Nine samples had quantifiable catechol after just 4 hours, four samples after 8 hours, nine after 12 hours and one slow metaboliser only after 24 hours. This variation in the time at which metabolites were first measured was shown in all microbial metabolites identified. Furthermore, several metabolites were only detected in a subset of volunteer samples during *in vitro* fermentation of black rice derived C3G, these were: 2,4-dihydroxybenzoic acid, 3-hydroxyphenylacetic acid, 4-methyl catechol, dihydrocaffeic acid, homovanillic acid, p-coumaric acid, phloroglucinol, PGC, pyrogallol and unknown X. Of the metabolites detected, only PCA, PGA, 4-hydroxybenzoic acid, catechol, dihydroferulic acid and unknown Y were produced in faecal samples from all individuals.

Table 4-3- Time-point at which microbial anthocyanin metabolites were first detected during in vitro fermentation of black rice derived C3G.

111 µM C3G was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours and the time-point at which microbial metabolites were first detected was recorded.

Volunteer	BERI 07	BERI 08	BERI 09	BERI 16	BERI 20	BERI 22	BERI 23	BERI 25	BERI 26	BERI 34	BERI 36	BERI 43	BERI 44	BERI 47	BERI 56	BERI 57	BERI 58	BERI 61	BERI 62	BERI 63	BERI 64	BERI 67	BERI 72
2,4-Dihydroxybenzoic acid	n.d	n.d	8	n.d	n.d	n.d	n.d	n.d	4	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	24	36
3-Hydroxyphenylacetic acid	n.d	n.d	n.d	n.d	30	48	36	n.d	n.d	n.d	48	24	n.d	30	36	36	n.d	36	24	30	n.d	n.d	n.d
4-Hydroxybenzoic acid	12	8	8	24	12	24	30	8	4	24	8	8	12	12	12	12	4	12	8	8	24	4	4
4-Methyl Catechol	12	n.d	n.d	48	30	n.d	4	n.d	24	n.d	36	36	36	30	n.d	n.d	n.d	4	n.d	n.d	48	4	12
Catechol	12	12	8	8	4	12	4	24	4	12	12	4	12	4	12	8	8	4	12	12	4	4	4
Dihydrocaffeic acid	n.d	48	12	n.d	48	n.d	n.d	48	36	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	48	12	30
Dihydroferulic acid	4	8	12	8	4	8	4	12	4	24	4	4	4	8	12	8	12	8	8	12	4	4	12
Gallic acid	n.d	0	0	4	n.d	0	24	0	4	4	4	4	0	30	4	4	0	4	0	4	8	4	4
Homovanillic acid	4	8	n.d	n.d	4	8	8	n.d	n.d	n.d	n.d	8	8	8	8	8	n.d	8	n.d	8	n.d	n.d	n.d
p-Coumaric acid	4	4	4	4	4	4	4	4	4	4	n.d	4	4	4	4	4	4	4	n.d	4	4	4	4
Phloroglucinol	4	n.d	4	8	4	n.d	4	8	4	8	n.d	n.d	n.d	4	n.d	n.d	4	4	n.d	n.d	4	24	24
Phloroglucinol carboxylic acid	n.d	n.d	8	8	n.d	n.d	n.d	8	8	8	24	n.d	n.d	n.d	n.d	n.d	8	n.d	24	n.d	n.d	24	24
Pyrogallol	n.d	n.d	4	48	36	n.d	30	48	n.d	n.d	30	24	24	30	n.d	n.d	36	n.d	30	n.d	30	24	4
Unknown X[a]	n.d	n.d	n.d	48	n.d	30	n.d	36	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	24	30	n.d	n.d	48	n.d	24
Unknown Y[b]	24	12	12	12	24	4	24	12	8	8	12	12	24	12	8	24	24	12	24	12	24	4	4

n.d- Compound not detected; [a]quantified with hippuric acid, [b] quantified with homoPCA.

Unknown X (unidentified compound observed in the MRM channel of hippuric acid); Unknown Y (unidentified compound observed in the MRM channel of homoPCA).

Table 4-4 shows highest concentration (Cmax) of each metabolite quantified for each participant during *in vitro* fermentation of black rice derived C3G. The Cmax across the 48 hour time course varies considerably between individuals as did the time-point at which compounds were first detected. Catechol and PGA show particular variation between subjects, with the Cmax of catechol ranging from 0.37 – 43.83 µM and PGA ranging from 3.39 – 44.82 µM. However, some metabolites showed much less variation such as phloroglucinol, pyrogallol and dihydroferulic acid, as shown in Table 4-4.

A number of compounds were only present transiently. C3G was catabolised at different rates by different individuals and this is exemplified by the data in Table 4-5 which shows the time-point at which C3G was no longer present at detectable levels for each individuals faecal sample. The vast majority of individuals had fully degraded C3G after 24 hours, however one particularly fast metaboliser (BERI 26) had fully degraded C3G after just 8 hours and one particularly slow metaboliser (BERI 22) did not completely metabolise C3G until 36 hours. Some metabolites, such as PGC and phloroglucinol, were only detected transiently in some individuals, but were either not detected at all or remained at the final 48 hour time-point in others, showing that anthocyanin metabolism is a variable process dependent on biological factors of the individual that consumes them.

Table 4-4- Maximum concentration (Cmax) of microbial anthocyanin metabolites detected during in vitro fermentation of black rice extract.

111 µM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours and the Cmax of each metabolite was recorded and is shown as µM.

Volunteer	BERI 07	BERI 08	BERI 09	BERI 16	BERI 20	BERI 22	BERI 23	BERI 25	BERI 26	BERI 34	BERI 36	BERI 43	BERI 44	BERI 47	BERI 56	BERI 57	BERI 58	BERI 61	BERI 62	BERI 63	BERI 64	BERI 67	BERI 72
PCA	17.15	19.76	24.51	26.93	12.85	17.27	14.32	26.93	23.70	35.33	12.15	10.92	19.32	11.93	18.90	26.05	31.39	14.08	13.13	18.18	32.56	29.77	29.22
PGA	35.72	5.56	14.93	13.25	13.86	6.18	23.80	27.86	26.92	17.82	13.57	13.00	14.81	17.57	10.40	3.39	19.39	19.91	12.55	18.04	44.82	14.06	17.08
2,4-Dihydroxybenzoic acid	n.d	n.d	12.32	n.d	n.d	n.d	n.d	n.d	1.38	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	2.04	0.54
3-Hydroxyphenylacetic acid	n.d	n.d	n.d	n.d	88.71	11.61	16.03	n.d	n.d	n.d	41.90	42.47	n.d	56.14	7.76	13.15	n.d	39.67	135.49	15.67	n.d	n.d	n.d
4-Hydroxybenzoic acid	3.32	1.49	3.57	2.69	2.03	0.96	1.79	5.01	4.13	1.00	3.62	1.68	1.79	3.54	2.07	2.55	3.21	5.04	3.31	2.14	3.88	2.98	3.71
4-Methyl Catechol	0.07	n.d	n.d	0.15	0.17	n.d	0.46	n.d	0.64	n.d	0.47	0.61	0.84	0.37	n.d	n.d	n.d	0.41	n.d	n.d	0.15	0.44	0.13
Catechol	0.37	2.84	19.70	10.41	15.37	2.24	16.44	20.83	24.25	2.43	19.53	13.38	20.60	9.53	1.85	1.71	10.59	0.70	20.72	0.55	18.28	43.83	36.75
Dihydrocaffeic acid	n.d	3.78	1.22	n.d	1.49	n.d	n.d	1.57	3.07	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	1.38	1.73	1.18
Dihydroferulic acid	1.98	1.75	1.42	1.68	2.04	2.35	2.14	1.06	0.81	0.38	1.65	1.30	2.32	1.60	2.07	1.62	1.04	1.33	1.68	1.93	2.25	1.25	1.49
Gallic acid	n.d	0.37	1.25	0.40	n.d	0.19	0.23	0.22	0.23	0.49	0.52	0.22	0.37	0.18	0.14	0.33	0.59	0.32	0.56	0.13	0.22	0.08	0.20
Homovanillic acid	1.60	3.14	n.d	n.d	1.28	3.24	0.50	n.d	n.d	n.d	n.d	3.51	3.49	0.47	3.99	3.31	n.d	0.53	n.d	4.11	n.d	n.d	n.d
p-Coumaric acid	0.41	0.65	0.28	0.29	0.27	0.58	0.22	0.31	0.05	0.61	n.d	0.24	0.67	0.30	0.68	0.50	0.32	0.33	n.d	0.43	0.41	0.06	0.13
Phloroglucinol	0.30	n.d	0.38	0.37	0.26	n.d	0.65	0.38	0.58	0.54	n.d	n.d	n.d	0.27	n.d	n.d	0.26	0.21	n.d	n.d	0.35	0.23	0.22
Phloroglucinol carboxylic acid	n.d	n.d	0.71	0.72	n.d	n.d	n.d	0.43	0.49	0.92	0.18	n.d	n.d	n.d	n.d	n.d	0.58	n.d	0.21	n.d	n.d	1.65	1.08
Pyrogallol	n.d	n.d	1.19	0.22	0.21	n.d	0.52	0.27	n.d	n.d	1.0	0.35	0.44	0.57	n.d	n.d	0.25	n.d	0.95	n.d	0.38	0.63	1.43
Unknown X[a]	n.d	n.d	n.d	0.01	n.d	10.70	n.d	0.04	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.12	3.39	n.d	n.d	0.95	n.d	0.60
Unknown Y[b]	159.63	20.08	181.47	212.02	125.30	40.84	110.33	292.15	142.86	264.30	95.31	8.84	15.89	94.93	26.64	26.54	193.51	128.41	135.49	26.42	161.76	206.97	208.38

n.d - Compound not detected; [a] quantified with hippuric acid, [b] quantified with homoPCA.

Table 4-5- Time of disappearance for transient compounds detected during in vitro fermentation of black rice extract
111 µM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours and the time-point at which compounds were no longer detected was recorded.

Volunteer	BERI 07	BERI 08	BERI 09	BERI 16	BERI 20	BERI 22	BERI 23	BERI 25	BERI 26	BERI 34	BERI 36	BERI 43	BERI 44	BERI 47	BERI 56	BERI 57	BERI 58	BERI 61	BERI 62	BERI 63	BERI 64	BERI 67	BERI 72
Cyanidin-3-*O*-glucoside	30	12	24	24	24	36	24	24	8	24	24	12	24	12	24	24	24	24	30	24	24	12	24
PCA	-	-	-	-	-	-	-	-	-	-	36	48	-	-	-	-	-	-	-	-	-	-	-
Gallic acid	n.d	-	-	-	n.d	-	30	48	36	-	36	36	24	48	12	-	-	-	36	36	36	36	-
Pyrogallol	n.d	n.d	-	-	-	n.d	-	-	n.d	n.d	-	-	-	-	n.d	n.d	48	n.d	-	n.d	-	-	-
Phloroglucinol carboxylic acid	n.d	n.d	-	-	n.d	n.d	n.d	48	36	-	48	n.d	n.d	n.d	n.d	n.d	-	n.d	-	n.d	n.d	-	-
4-Hydroxybenzoic acid	-	-	-	-	24	30	-	-	-	-	36	-	48	-	-	-	-	-	-	-	36	48	-
Dihydroferulic acid	-	-	-	-	-	-	-	48	-	-	-	-	-	-	-	-	-	-	-	-	-	48	-
p-Coumaric acid	24	24	30	24	24	24	24	24	8	36	n.d	12	12	24	24	24	24	48	n.d	12	24	24	24
Phloroglucinol	-	n.d	36	-	-	n.d	-	48	36	-	n.d	n.d	n.d	-	n.d	n.d	-	48	n.d	n.d	48	-	-
Unknown Y[b]	-	-	-	-	-	-	-	-	-	-	30	36	-	-	-	-	-	-	-	-	-	-	-

n.d compound not detected; - compound still present at the final 48 hour time-point; [b] quantified with homoPCA.

Unknown Y (unidentified compound observed in the MRM channel of homoPCA).

4.5.4 Inter-Individual Variation in Gut Microbiota Dependent Anthocyanin Metabolism

In order to summarise the global view of the data for all compounds between participants, time series data for all compounds for each participant was processed using multivariate dynamic time warping (DTW) to provide distance measures between participants according to their similarity during *in vitro* fermentation, taking all compounds into account. This data was used for principal component analysis, shown in Figure 4-20. This data illustrates that when looking globally at the metabolic behaviour of participant faecal samples during *in vitro* fermentation of black rice derived C3G there are clear clusters of participants who behaved similarly and groups of individuals who were differentiated in their metabolism. BERI 43, BERI 44, BERI 63, BERI 08, BERI 56, BERI 57, BERI 36 and BERI 22 show similar anthocyanin metabolism; whilst BERI 67, BERI 58, BERI 16, BERI 47, BERI 20, BERI 26, BERI 64, BERI 09, BERI 07, BERI 61 and BERI 23 (who tended to produce a higher concentration of metabolites targeted in this analysis overall) form a separate cluster, clearly differentiated from other participants but similar to one another. A small cluster is seen between BERI 34 and BERI 25 who show very similar black rice anthocyanin metabolism to each other (both exhibiting a particularly high estimated concentration of unknown Y), whilst BERI 62 (the highest 3-hydroxyphenylacetic acid producer) and BERI 72 (the highest pyrogallol producer) do not cluster with any other participants and show clearly distinguished metabolism when taking a global view of the data collected in these experiments for all compounds (data processed with this model can be seen for each individual compound in Appendix 7). This highlights that the course of microbial anthocyanin metabolism is variable according to the individual faecal sample anthocyanins are exposed to, and suggests that individuals may fall into different metabotypes according to how they metabolise anthocyanins and that there must be characteristics of an individual's microbiome that can define these metabotypes.

Overall, the data presented in this results section to this point show that microbes are critical for the metabolism of C3G with many metabolites only being generated in the presence of a live microbiota, and the microbiota being a source of variability in this metabolism. Crucially, many of the metabolites detected during faecal *in vitro* fermentation were also detected in urine and plasma from a human intervention (shown in Chapter 3) concerning the same individuals. Of note, what is highlighted here is that the concentration of metabolites, time at which they are produced and indeed the specific metabolites generated varies between individuals, most likely as a result of their specific gut microbiota.
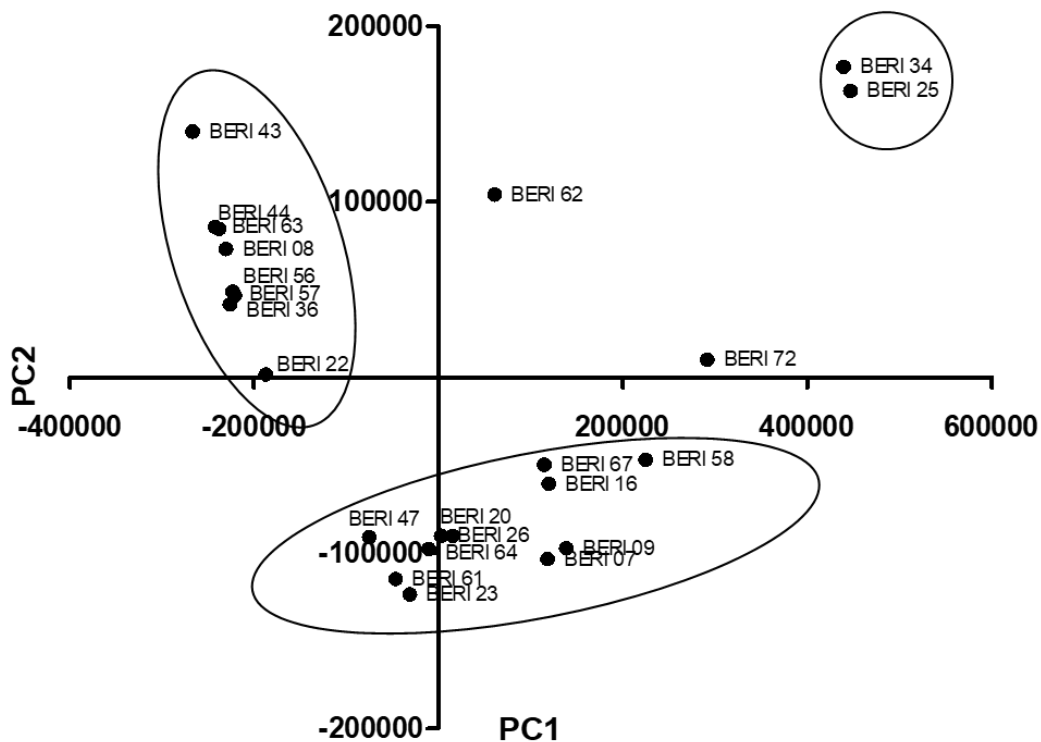
Figure 4-20- Principal component analysis of multivariate DTW data for all compounds measured during in vitro fermentation of black rice extract.

111 μM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. This data was processed using multivariate dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation, taking all compounds into account for the entire time series of the experiment. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.

4.5.5 Relationships Between *In Vitro* Microbial and *In Vivo* Anthocyanin Metabolism

The gut microbiota was shown to play a crucial role in anthocyanin metabolism. Several of the metabolites observed in urine samples in Chapter 3 were also formed in the presence of a live microbiota during *in vitro* fermentation, but were not generated spontaneously, including catechol and pyrogallol. Considerable inter-individual variations were seen in urine and plasma metabolite profiles in terms of which metabolites were observed and their relative concentrations (shown in Chapter 3). This variability was also seen in the *in vitro* fermentation colon model data where some metabolites such as 4-methyl catechol were only produced by a subset of participants. Figure 4-20 shows that faecal samples derived from different individuals will metabolise black rice anthocyanins differently. These observations suggest that the microbiota of an individual will define the amounts and types of anthocyanin metabolites produced, and since several of these compounds appear to

be absorbed and excreted in urine, correlations between *in vitro* colonic fermentation and urinary excretion were expected. Urinary and *in vitro* fermentation colon model metabolic data was analysed by means of linear regression for metabolites observed in both systems to establish any correlations between these datasets that may be explained by differences in the gut microbiota.

The Cmax of metabolites from the *in vitro* fermentation colon model was plotted against the urinary μmol/24hrs of the same metabolite. Where phase 2 conjugates were detected in urine, for example PCA sulfates and glucuronides were identified in addition to the parent compound, these were summed together due to the lack of absorption and passage through the liver in the *in vitro* fermentation model, meaning that phase 2 metabolism cannot take place in this system. A statistically significant (p = 0.02) positive correlation was seen between urinary PCA levels (including phase 2 conjugates) and the Cmax of PCA observed in the *in vitro* fermentation colon model, shown in Figure 4-21. This strongly suggests that the gut microbiota has an important role in PCA production from black rice anthocyanins.
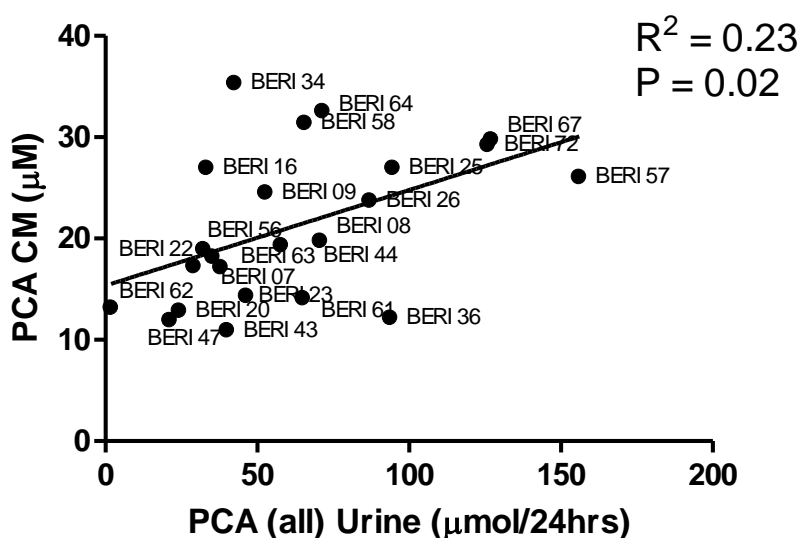
Figure 4-21- Linear regression analysis of PCA levels in in vitro fermentation of black rice anthocyanins with faecal samples and urinary excretion of PCA in a dietary intervention where black rice anthocyanin extract was consumed by the same individuals.
Volunteers consumed black rice anthocyanin extract for 28 days (285 mg/day C3G) and urinary excretion of PCA and its phase 2 conjugates were measured in 24 hour urine collections, these were summed and are shown as PCA (all) on the X axis in μmol/24 hours. Faecal samples from these same volunteers were subjected to incubation with the same black rice anthocyanin extract (50 μg/ml C3G) in an in vitro fermentation colon model (CM) for 48 hours and the Cmax was recorded and is shown in μM. Linear regression was applied to these datasets to assess whether any relationship was observed, the R^2 and p values are shown to the right of the figure.

Of the B ring metabolites detected in both systems, only PCA showed a relationship of statistical significance. A weak positive correlation $(R^2 = 0.13)$ was also seen for catechol levels in the *in vitro* fermentation colon model and catechol (and phase 2 conjugates) in urine, however, this was not of statistical significance, as depicted in Figure 4-22. The lack of statistical significance of this relationship can be attributed to a subset of individuals (including BERI 57, BERI 08, BERI 61) that only produced very low levels of catechol in the *in vitro* fermentation model but had high levels of catechol and its phase 2 conjugates in urine, as shown in Figure 4-22. Although 4-hydroxybenzoic acid, dihydroferulic acid, p-coumaric acid and dihydrocaffeic acid were also formed during both *in vitro* fermentation and *in vivo* there was no correlation in concentrations of these compounds between the two systems (data not shown).
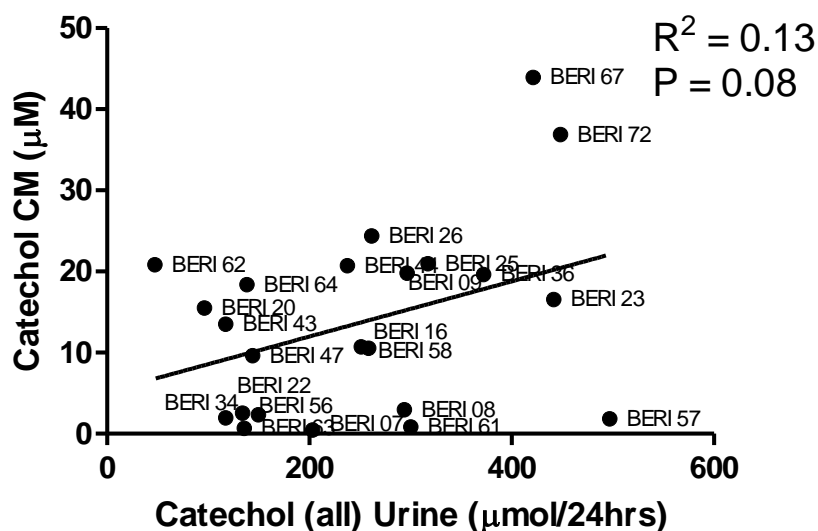
Figure 4-22- Linear regression analysis of catechol levels in in vitro fermentation of black rice anthocyanins with faecal samples and urinary excretion of catechol in a dietary intervention where black rice anthocyanin extract was consumed by the same individuals.
Volunteers consumed black rice anthocyanin extract for 28 days (285 mg/day C3G) and urinary excretion of catechol and its phase 2 conjugates were measured in 24 hour urine collections, these were summed and are shown as catechol (all) on the X axis in μmol/24 hours. Faecal samples from these same volunteers were subjected to incubation with the same black rice anthocyanin extract (50 μg/ml C3G) in an in vitro fermentation colon model (CM) for 48 hours and the Cmax of catechol was recorded and is shown in μM. Linear regression was applied to these datasets to assess whether any relationship was observed, the R^2 and p values are shown to the right of the figure.

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Stronger positive correlations were seen between *in vitro* fermentation and urinary excretion concentrations of A ring metabolites, with phloroglucinol and pyrogallol exhibiting statistically significant relationships between datasets, as shown in Figure 4-23 and Figure 4-24. However, no

correlation was seen between the urinary μmol/24 hours and *in vitro* fermentation Cmax for PGA, most likely due to PGA being a precursor for other metabolites (data not shown).
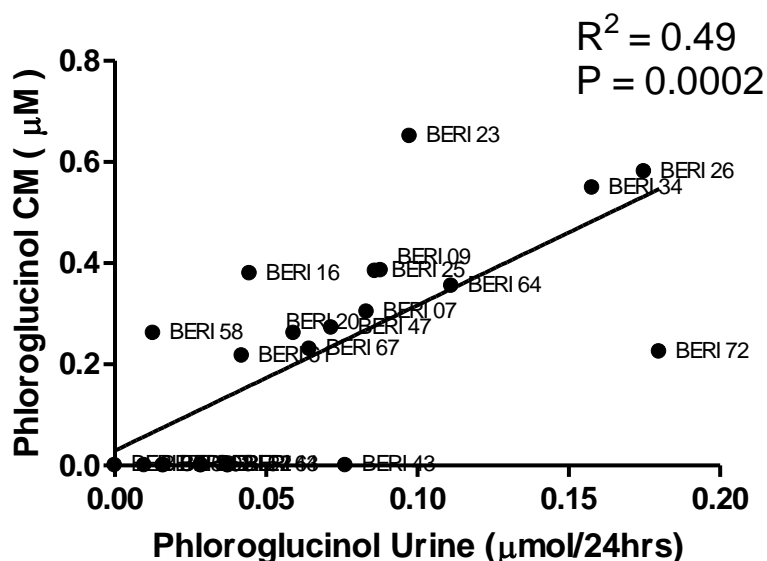
Figure 4-23- Linear regression analysis of phloroglucinol levels in in vitro fermentation of black rice anthocyanins with faecal samples and urinary excretion of phloroglucinol in a dietary intervention where black rice anthocyanin extract was consumed by the same individuals.
Volunteers consumed black rice anthocyanin extract for 28 days (285 mg/day C3G) and urinary excretion of phloroglucinol was measured in 24 hour urine collections, and is shown on the X axis in μmol/24 hours. Faecal samples from these same volunteers were subjected to incubation with the same black rice anthocyanin extract (50 μg/ml C3G) in an in vitro fermentation colon model (CM) for 48 hours and the Cmax of phloroglucinol was recorded and is shown in μM. Linear regression was applied to these datasets to assess whether any relationship was observed, the R^2 and p values are shown to the right of the figure.
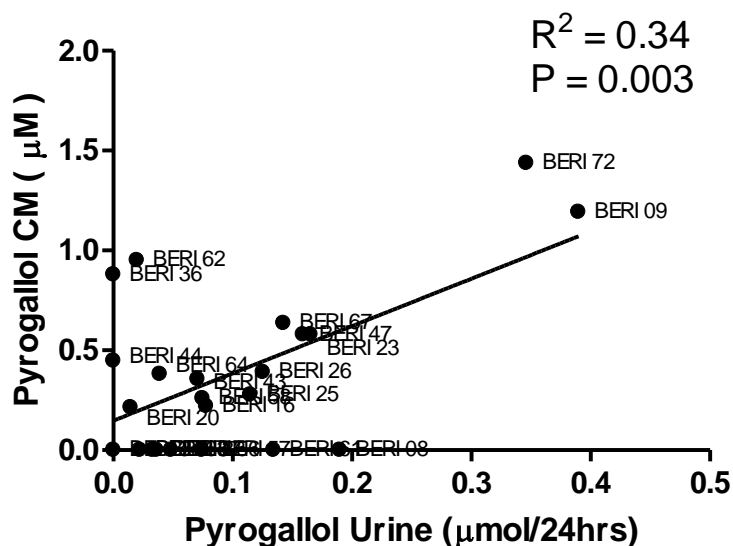
Figure 4-24- Linear regression analysis of pyrogallol levels in in vitro fermentation of black rice anthocyanins with faecal samples and urinary excretion of pyrogallol in a dietary intervention where black rice anthocyanin extract was consumed by the same individuals.
Volunteers consumed black rice anthocyanin extract for 28 days (285 mg/day C3G) and urinary excretion of pyrogallol was measured in 24 hour urine collections, and is shown on the X axis in μmol/24 hours. Faecal samples from these same volunteers were subjected to incubation with the same black rice anthocyanin extract (50 μg/ml C3G) in an in vitro fermentation colon model (CM) for 48 hours and the Cmax of pyrogallol was recorded and is shown in μM. Linear regression was applied to these datasets to assess whether any relationship was observed, the R^2 and p values are shown to the right of the figure.

Although other microbial metabolites were detected in *in vitro* fermentation colon model experiments, such as 4-methyl catechol and PGC these compounds were not detected in urine or plasma samples and therefore comparisons between datasets could not be explored for these compounds.

4.6 Discussion

The aim of the work presented in this chapter was to establish the role of the gut microbiota in anthocyanin metabolism and how this varies between individuals, with a particular focus on relationships between *in vitro* microbial metabolism and *in vivo* metabolites. A total of 18 metabolites were detected during *in vitro* fermentation of black rice anthocyanin extract with faecal samples from 23 individuals, of these metabolites only PCA and PGA were also produced in the absence of a live microbiota. The rate of C3G degradation (which accounted for ~ 89% of the anthocyanin content) varied according to the individual from which the faecal sample was derived, likewise the specific metabolites observed and their relative concentrations showed high inter-individual variation. Furthermore, significant positive correlations were observed between the Cmax of PCA, phloroglucinol and pyrogallol produced during *in vitro* fermentation of faecal samples with black rice anthocyanins, and urinary excretion of these compounds after consumption of black rice anthocyanin extract in a dietary intervention.

The work presented in this chapter shows a previously undescribed relationship between human *in vivo* metabolite profiles and *in vitro* fermentation of black rice C3G using faecal samples derived from the same individuals, highlighting the importance of the gut microbiota in *in vivo* metabolism and anthocyanin bioavailability. PCA has been previously reported as both a spontaneous and microbial metabolite of C3G (Aura *et al.*, 2005; Kay *et al.*, 2009; Triebel *et al.*, 2012). However, for the first time the data presented in this chapter demonstrates a statistically significant relationship between *in vitro* microbial PCA production mediated by faecal samples and urinary µmol/24hrs of PCA (and its phase 2 conjugates) after consumption of black rice anthocyanins in a dietary intervention for the same individuals. This highlights that the gut microbiota is critical in the production of PCA *in vivo,* and spontaneous degradation only contributes to small amounts PCA detected in biological samples. Several studies have reported bioactivity of PCA, and therefore understanding why some individuals produce more of this metabolite than others may be an important point of future work to maximise the beneficial effects of anthocyanin consumption (Kakkar and Bais, 2014; Semaming *et al.*, 2015). The data presented in this chapter suggests that the gut microbiota is likely to contribute in some way to the concentration of PCA that reaches

circulation. The strongest positive correlations between *in vitro* fermentation of faecal samples with black rice extract and 24 hour urine collections following consumption of black rice extract in a dietary intervention for the same individuals were observed for the A-ring metabolites, pyrogallol and phloroglucinol, indicating that microbial metabolism is important for the production of A-ring metabolites beyond PGA. It is hypothesised that the strongest relationships between datasets was seen for these A-ring products due to the reduced diversity of compounds known to be derived from the A-ring compared to the wide range of known B-ring metabolites (de Ferrars *et al.*, 2014a). In addition to these significant positive correlations between faecal *in vitro* fermentation and *in vivo* metabolism, Figure 4-22 shows a trend whereby those that had higher concentrations of catechol (and its phase 2 conjugates) in 24 hour urine collections after consumption of black rice extract also produced higher levels of catechol during *in vitro* fermentation. Although the positive trend in this data is not of statistical significance, it does reinforce that the gut microbiota has a role in anthocyanin metabolism *in vivo*. It should be noted that for both catechol and PCA, linear regression analysis has been performed on the sum of the primary metabolite and its phase 2 conjugates in urine against the primary metabolite produced during *in vitro* fermentation. Due to *in vitro* fermentation keeping the anthocyanin and its downstream metabolites in an artificial colon model for the duration of the experiment, once formed, compounds cannot be absorbed and reach the liver for phase 2 metabolism and consequently phase 2 conjugates are not produced during *in vitro* fermentation. Therefore, comparison between the sum of the parent metabolite and phase 2 conjugates with the metabolite observed in the colon model was deemed the most representative way to gauge any potential relationship between datasets.

Although several microbial metabolites of black rice anthocyanins detected during faecal *in vitro* fermentation were also observed in 24 hour urine collections (following consumption of encapsulated black rice extract) from the same individuals, this was not the case for all compounds. PGC, 2,4-dihydroxybenzoic acid and 4-methyl catechol were all detected during *in vitro* fermentation but were not observed in the urine of any participant. This may be attributed to a variety of reasons. Firstly, these metabolites were all detected at low levels during *in vitro* fermentation, therefore, it is plausible that these metabolites may have also been generated at low levels *in vivo* and were excreted in urine at very low concentrations. Due to the method of urine collection, pooling urine in a 24 hour period, it is possible that these compounds may have become diluted below the limits of detection. If urine had been collected as smaller samples at time intervals after anthocyanin consumption it is possible low levels of these metabolites may have been quantified. Alternatively, these differences in metabolites may be an artefact of the mode of study. During *in vitro* fermentation the treatment administered is kept in an artificial colon model environment, exposed to microbes in an anaerobic atmosphere for the full time-course of the experiment, without absorption taking place. *In vivo* the treatment transits through the GI tract and

absorption of the parent compound and its metabolites takes place over time, whilst some metabolism may be carried out by human enzymes rather than by microbes, which may explain why we don't see correlations between all compounds produced in both systems, and why there are some differences in metabolite profiles between systems.

The data presented in this chapter shows that anthocyanin metabolism is highly variable between faecal samples derived from different individuals. The variability in the capacity of individual faecal samples to metabolise anthocyanins is best depicted in Figure 4-20. There are clear groups of individual samples that behaved similarly during *in vitro* fermentation and others that behaved very differently, and this is most likely due to differences in the microbial composition of these samples. Chapter 3 also showed high levels of inter-individual variation for urine and plasma metabolite profiles after dietary consumption of anthocyanin rich extracts in the same individuals. This therefore suggests that the gut microbiota of individuals is likely to have an important role in dictating anthocyanin metabolism *in vivo* and individuals may be able to be assigned a metabotype based on their gut microbiota profile (Igwe *et al.*, 2019; Mayta-Apaza *et al.*, 2018; Nishioka *et al.*, 2021). Although other studies have reported high inter-individual variation in anthocyanin metabolism, none have established anthocyanin metabotypes that can be used to define how an individual metabolises dietary anthocyanins *in vivo.* The metabolism of several other flavonoids has been metabotyped to define the metabolic signature of individuals. For example, metabolism of ellagitannins to urolithins has been described with 3 metabotypes, A, B and 0 according to whether or not an individual produces urolithins and, if so, the types of urolithins they produce. These metabotypes are attributed to specific characteristics of the gut microbiota and have been linked to the health effects of eating ellagitannins (García-Mantrana *et al.*, 2019). The data presented in Figure 4-20 suggests that anthocyanin metabolism may also be able to described by metabotypes. Investigating the metabolites and biological characteristics of individuals that may be able to define anthocyanin metabotypes would be an important point of future work. This would allow us to better understand how anthocyanin metabolism and any biological response to dietary anthocyanins may differ between people, and the impact this may have on health.

Overall, the work in this chapter shows a distinct role for the gut microbiota in anthocyanin metabolism. Although there is a spontaneous element to anthocyanin metabolism, highlighted in Figure 4-2 - Figure 4-4, with PCA and PGA shown to be chemical degradants of C3G here, and within the literature (Kay *et al.*, 2009; Woodward *et al.*, 2009; Triebel *et al.,* 2012). This is clearly not the only route through which these metabolites can be generated, with both products also being formed microbially, reinforcing what has been shown in unpublished work from Shehata *et al* (Kroon group, QIB) and in previous studies on colonic C3G metabolism (Aura *et al.*, 2005; Hanske *et al.*, 2013). For example, Figure 4-4 shows that PGA is present at much higher levels in samples with live bacteria compared to heat-inactivated samples. This suggests that despite being produced

spontaneously, bacteria are able to accelerate the production of PGA and facilitate higher levels of PGA production overall. Additionally, both PCA and PGA concentrations show some decline over the 48-hour time course during *in vitro* fermentation of faecal samples containing live bacteria, highlighting that these two primary metabolites serve as precursors for a wide range of microbial metabolites. An interesting finding from this series of experiments is that PCA and PGA did not account for the total amount of spontaneous black rice derived C3G degradation observed during *in vitro* fermentation of heat-inactivated samples (Figure 4-2). This disputes what was found by Kay *et al*, and suggests that PCA and PGA are not the only spontaneous products of the chemical degradation of C3G (Kay *et al.*, 2009). Although untargeted analysis was performed using single-quad mass spectroscopy, no clear spontaneous products of C3G were identified. This was mainly attributed to the high level of background signals obtained by the media which contains a complex mixture of compounds, making it extremely difficult to accurately identify unknown compounds of interest in untargeted analysis.

The majority of C3G metabolites detected in this analysis were exclusively microbial. One of the major metabolites produced in faecal samples from all individuals (and observed in urine post dietary intervention) was catechol, although its concentration and time at which it was first detected varied considerably between individuals. Some fast metabolisers had quantifiable levels of catechol after just 4 hours of *in vitro* fermentation, slower metabolisers at 12 hours, and one particularly slow metaboliser after 24 hours. Variability was also seen in the Cmax of catechol with this ranging from 0.37 - 43.83 µM, highlighting how microbial metabolism of anthocyanins is subject to high inter-individual variation. Catechol is most likely derived from the microbial decarboxylation of PCA, mediated by the enzyme protocatechuate decarboxylase, however other enzymes such as 4-hydroxybenzoate decarboxylase are also known to be capable of catalysing this reaction (Selmer and Andrei, 2001). As the microbiota composition of every individual is different this ultimately means that each individual will have different amounts of microbes capable of performing this bio-transformation, which is likely to explain why the concentration of catechol, and the time at which quantifiable levels are first measured varies so drastically amongst the faecal samples from the study population. This is also likely to explain why in Chapter 3 large inter-individual variation was seen in the concentration of catechol, and its phase 2 conjugates, in urine. This study, and our collaboration with Jokioja *et al* are the first to show catechol as an important human *in vivo* anthocyanin metabolite (Jokioja *et al.*, 2021). Whilst the work in this chapter, and unpublished work from Shehata *et al*, is the first to confirm the origins of catechol as a microbially derived metabolite. Catechol has been overlooked in most studies on anthocyanin metabolism, furthermore, *in vivo* catechol is largely present in its phase 2 conjugated forms which are often missing from analytical methods and difficult to verify due to lack of authentic standards (De Ferrars, Czank, Zhang, *et al.*, 2014c; Nurmi *et al.*, 2009).

A subset of faecal samples produced 4-methyl catechol during *in vitro* fermentation, which has not previously been identified as a microbial product of black rice anthocyanin metabolism. Generally, this metabolite was not detected until later time-points (Table 4-3), however, a minority of participants faecal samples produced low levels after just 4 hours of *in vitro* fermentation. Interestingly, there was no tendency for this metabolite to preferentially be seen in high or low catechol producers, despite this compound seemingly being derived from microbial methylation of catechol. It is possible that 4-methyl catechol was simply produced by faecal samples containing microbes capable of performing this methylation that may not reside in the gut microbiota of all individuals. Alternatively, it is possible that 4-methyl catechol was produced by more participants than reported but was not detected due to being present at levels below the limits of detection. In all samples where it was detected, this was a very low-level metabolite and it is therefore plausible that limits of instrumentation may have prevented detection in some samples. Furthermore, its low level suggests that this may have been a microbial metabolite of Peo3G (which accounts for ~7% black rice anthocyanin content), however, the origin of this compound cannot be known for certain without fermenting pure anthocyanins. Nevertheless, this is the first study to report 4-methyl catechol production from anthocyanins by the faecal microbiota during *in vitro* fermentation.

Dihydrocaffeic acid was present in a subset of participants, as a late metabolite not detected in any sample before 12 hours. It is postulated that this compound could be a microbial metabolite of C3G. Interestingly, dihydroferulic acid was detected in all individuals, initially it was assumed that this compound would be derived by microbial methylation of dihydrocaffeic acid. However, inspection of the data obtained showed that this metabolite was frequently detected before dihydrocaffeic acid during *in vitro* fermentation. Furthermore, it has the same methylation pattern as the B-ring of peonidin indicating that dihydroferulic acid may be a microbial metabolite of Peo-G (which makes up ~7% anthocyanin content in black rice extract). This could mean that dihydrocaffeic acid may alternatively be derived from microbial demethylation of dihydroferulic acid which would explain the sequence in which these compounds were detected (Table 4-3) and their relative concentrations during *in vitro* fermentation. It would be interesting to investigate *in vitro* fermentation of pure C3G and pure Peo3G to conclusively determine the origin of these metabolites.

Several A-ring anthocyanin metabolites were identified during faecal *in vitro* fermentation of black rice derived C3G, with PGA being the only A-ring metabolite produced by all faecal samples tested. Phloroglucinol and pyrogallol were detected at low levels in the vast majority of samples, and in some samples these compounds had disappeared by the end of the experiment, highlighting that the trajectory of metabolism of the A-ring is not consistent among individuals despite there being less known A-ring products described in the literature (De Ferrars, Czank, Zhang, *et al.*, 2014a). These findings suggest that the production and bio-transformation of A-ring metabolites is largely

dictated by the gut microbiota of an individual. Furthermore, PGC was detected as a suspected A-ring product thought to be enzymatically produced from PGA, however, this was not detected in all *in vitro* fermentation samples, and was not seen in urinary metabolite analysis. This finding is reinforced by unpublished work from Shehata *et al*, who first found PGC to be a microbial metabolite of C3G produced by subsets of individuals. This may imply that some individuals exhibit common bacterial species and/or functional profiles of their gut microbiota that dictates whether PGC can be produced during anthocyanin metabolism. However, the overwhelming majority of compounds detected during *in vitro* fermentation are known to be derived from the B-ring as has been established in a study that fed penta[^{13}C]-C3G (De Ferrars, Czank, Zhang, *et al.*, 2014a).

Several metabolites were produced by all faecal samples subjected to *in vitro* fermentation of C3G, such as: PCA, PGA, 4-hydroxybenzoic acid, catechol and dihydroferulic acid. This shows that there is some consistency in anthocyanin metabolism between individuals in the production of key metabolites, however, the concentrations at which these metabolites were produced did vary considerably as highlighted by the high standard deviations in Figure 4-2, Figure 4-3, Figure 4-4, Figure 4-5, Figure 4-13, and the Cmax values in Table 4-4. Other metabolites observed in this analysis including: PGC, pyrogallol, 4-methyl catechol and homovanillic acid were only produced by a subset of faecal samples. Whilst little is known regarding the bioactivity of many of these compounds this reinforces what has been suggested by previous studies, that the capacity of an individual to produce bioactive metabolites determines their response to dietary anthocyanins (Igwe *et al.*, 2019; Kay, 2006). This also suggests that if we can gain a better understanding of which anthocyanin metabolites can exert a biological effect then we may be able to develop supplements to provide these compounds to individuals that do not produce them at high levels endogenously.

Overall the microbial metabolites identified in this work support what has been shown in the literature, with key metabolites such as PCA, PGA, pyrogallol and 4-hydroxybenzoic acid having consensus amongst this study and previous reports (Aura *et al.*, 2005; Czank *et al.*, 2013; Hanske *et al.*, 2013; Khanal *et al.*, 2014). However, some previous reports on microbial anthocyanin degradation report metabolites not detected in this analysis. For example, in another study on microbial metabolism of black rice anthocyanins compounds such as: benzoic acid, phenylacetic acid, and hydroxyphenylpropionic acid were detected during 48 hour anaerobic fermentation experiments, but were not detected here (Zhu *et al.*, 2018). Likewise, the analysis presented in this chapter found compounds such as: catechol, phloroglucinol and dihydroferulic acid, that were not observed in this publication. The most likely reason for the difference in these findings is that Zhu *et al* only looked at *in vitro* fermentation of black rice anthocyanins by *Bifidofacteria* and *Lactobacilli* species, whereas we looked at *in vitro* fermentation by human faecal samples that would contain a diverse and variable population of microbial species (Eker *et al.*, 2019; Faria *et al.*, 2014; Zhu *et al.*, 2018). This implies that the species of bacteria present is critical to the pathways taken in

anthocyanin metabolism and deducing the bacteria responsible for biotransformation of anthocyanins and their metabolites is an important point for future work.

Gallic acid has previously been reported as a microbial metabolite of C3G (Hanske *et al.*, 2013; Khanal *et al.*, 2014). Whilst gallic acid was detected in this analysis in some samples, it was only present at a low level and did not show large increases throughout the duration of *in vitro* fermentation. What was most interesting regarding gallic acid was not its production or degradation during *in vitro* fermentation of live faecal samples, but was a phenomenon whereby gallic acid was produced through autoclaving faecal sample. This has not been reported in the literature, however, was first shown by Shehata *et al* in unpublished work. How and why gallic acid is produced in this process is unclear but is important to acknowledge as the gallic acid detected in these samples is not a product of spontaneous anthocyanin breakdown. One possibility is that gallic acid is bound to proteins contained in faeces and autoclaving faecal material, denaturing these proteins may cause this gallic acid to be released, resulting in an increase in the gallic acid concentration of the sample. This idea is reinforced by studies that have shown that gallic acid binds to proteins in serum, however, this hypothesis would need to be tested experimentally (Zhang *et al.*, 2017).

Two unidentified compounds were detected through *in vitro* fermentation. Unknown Y, which was detected in the MRM channel for homoPCA but ran at a different retention time to matrix matched standards. This combined with the very high concentrations quantified, exceeding the starting concentration of C3G suggested that this compound must have a different identity despite giving a strong signal in the MRM channel of homoPCA. Although vanillic acid, isovanillic acid, 3,4-dihydroxymandelaldehyde, 4-methoxysalicylic acid, and 3/4-hydroxymandelic acid have the same mass as unknown Y they were excluded, as authentic matrix matched standards ran at different retention times to the compound in question (data not shown). Other compounds highlighted by Human Metabolome Database (HMDB) searches that could potentially be candidates for this mass are: 3,5-dihydroxyphenylacetic acid and 3-hydoxy-5-methoxybenzoic acid, however, authentic standards were not available at the time of analysis. A further unidentified compound, unknown X, was detected that gave a signal in the same MRM channel as hippuric acid but the retention time was approximately 1 minute ahead of matrix matched standards highlighting that this compound must have a different identity. However, no clear candidates were established from HMDB searches regarding the identity of this compound.

Before investigating gut microbial dependent anthocyanin metabolism, a subset of samples were tested for viability of total anaerobes. Although the preservation technique of processing faecal samples as glycerol stocks has been demonstrated as an effective method of preserving live bacteria, samples had been in storage for a considerable length of time, in some cases up to 3 years

(O'Donnell *et al.*, 2016; Parkar *et al.*, 2019). This led to the concern of whether samples would still contain high counts of viable total anaerobes. To assess the viability of faecal slurry samples anaerobic plating was conducted and showed that most samples had high numbers of viable total anaerobes. However, it was surprising that one sample (BERI 23) showed low growth indicating low viability, particularly considering this was not the oldest sample tested. Despite this, BERI 23 was not the slowest C3G metaboliser and was among the highest producers of several microbial metabolites such as catechol and 4-methyl catechol. This is likely due to the microbial composition of this sample having a high capacity to metabolise anthocyanins. Furthermore, it is likely not to be the number of total anaerobes that is most important but rather the type of anaerobes present that will dictate the capacity of a microbiota to metabolise anthocyanins. There are several factors that may explain why some samples may have poor viability of total anaerobes relative to others irrespective of the time in storage (Eckburg *et al.*, 2005). Firstly, participants were asked to provide faecal samples within 6 hours of excretion, and the time at which the sample was produced was not recorded. Samples that had been delivered later in this time period may be liable to reduced anaerobe viability due to prolonged exposure to an aerobic environment which would result in the death of some anaerobes. Building on this, the time taken for samples to be processed was variable and samples that took longer to process would have been exposed to an aerobic environment and room temperature for longer and this is likely to have had some baring in the survival of anaerobes residing in the sample. A further factor that may have effected counts of viable anaerobes is the microbial composition of the faecal sample. Some species may be more tolerant to a short time exposed to an aerobic atmosphere than others and depending on the profile of bacteria this may have led to increases in the total anaerobe survival in samples from some participants compared to others. For example, it has been demonstrated that *Eubacterium*, *Coproccocus*, and *Peptostreptococcus* species are particularly intolerant to atmospheric oxygen exposure (Brusa *et al.*, 1989).

Demonstrating that the *in vitro* fermentation model was able to provide consistency between technical replicates was extremely important to facilitate the analysis of anthocyanins metabolism in all participants for which faecal slurry samples were available. Biological replicates were anticipated to exhibit considerable variation as it has been repeatedly shown in human studies that anthocyanin metabolism differs between individuals, this is highlighted by the data presented in Chapter 3 and in the literature available on anthocyanin metabolism (de Ferrars *et al.*, 2014a; de Ferrars *et al.*, 2014b; Jokioja *et al.*, 2021). Although the same experiment was performed several times using the same batch fermentation system, media and anthocyanin source, the trajectory of black rice anthocyanin metabolism varied according the volunteer from which faecal samples were derived. The rate of metabolism and the relative concentrations of compounds within the metabolite profile were very different, highlighting that anthocyanin metabolism is a variable

process and any biological effect of eating anthocyanins is likely to vary according to an individual's capacity to metabolise anthocyanins, which is likely at least in part dictated by the profile of the gut microbiota (Eker *et al.*, 2019; De Ferrars, Czank, Zhang, *et al.*, 2014a; Igwe *et al.*, 2019).

Studying gut microbiota dependant anthocyanin metabolism via *in vitro* fermentation has several strengths and weaknesses. Firstly, *in vitro* fermentation using live and heat inactivated faecal samples allows discrimination between microbial metabolism and spontaneous degradation, whilst the *in vitro* nature of experiments eliminates the possibility of metabolism mediated by human enzymes. *In vitro* fermentation also allows the concentration of compounds to be monitored over a time-course, however, due to the need to maintain anaerobic conditions sampling times should be minimised and consequently compounds that are produced only transiently may be missed. Furthermore, although *in vitro* fermentation provides invaluable information on which metabolites are microbially derived we are unable to infer the specific microbial species and/or enzymes responsible for anthocyanin metabolism. Finally, the series of experiments described in this chapter rely on faecal samples which are easily obtained from study volunteers, however, there is an argument that faecal samples are not representative of the microbial composition of all parts of the gut and that rectal mucosal biopsies provide a more accurate assessment of gut microbiota profiles (Igwe *et al.*, 2019).

This work provides insights into the role of microbes in anthocyanin metabolism and shows that a number of key metabolites seen in human samples from the analysis in Chapter 3 (and within the literature) are microbially derived (De Ferrars, Czank, Zhang, *et al.*, 2014a; Jokioja *et al.*, 2021). Although from this work we cannot establish the specific microbial species responsible for the production of these metabolites, understanding how anthocyanin metabolism may vary between individuals according to their gut is important in understanding how to maximise the biological effects of an anthocyanin rich diet. This chapter demonstrates that some anthocyanin metabolites may only be produced by particular individuals, and relative quantities of the same metabolite can be vastly different, most likely due to differences between individual gut microbiotas, which will ultimately affect any potential bioactivity of the consumed anthocyanins.

4.7   Conclusion

Overall the work in this chapter highlights a critical role of the gut microbiota in anthocyanin metabolism, whilst showing that this is subject to inter-individual variation according to the microbiota to which it is exposed. For the first time, a clear, but imperfect, relationship between *in vitro* microbial anthocyanin metabolism and *in vivo* human metabolism has been shown, demonstrating that the gut microbiota is pivotal to the generation of metabolite profiles *in vivo*, and likely any biological effect associated with eating anthocyanins. From the work presented in this chapter it is hypothesised that inter-individual variation in anthocyanin metabolism can be

attributed to differences in microbiome profiles which are known to vary considerably between individuals due to many factors (Eckburg *et al.*, 2005). A further conclusion from this work investigating the microbial metabolism of anthocyanins is that spontaneous degradation of C3G does not yield just PCA and PGA and likely gives rise to a spectrum of compounds, as these experiments showed that PCA and PGA did not account for total spontaneous anthocyanin degradation as has been suggested by previous reports (Kay *et al.*, 2009). Accordingly, the remaining chapters of the thesis will investigate the relationship between anthocyanin metabolism and the gut microbiota (Chapter 5) and the spontaneous degradation of cyanidin (Chapter 6).

Chapter 5

Is there a Relationship Between Anthocyanin Metabolism and the Microbiome?

Chapter 5 : Is there a Relationship Between Anthocyanin Metabolism and the Microbiome?

5.1 Abstract

Background: Chapter 4 showed that a large number of anthocyanin metabolites are generated microbially by the gut microbiota, and there is considerable inter-individual variation in anthocyanin metabolite profiles from human studies, as demonstrated in Chapter 3 (and in the literature). Therefore, it is hypothesised that the gut microbiota of an individual largely dictates how they metabolise anthocyanins which may also determine their biological response to dietary anthocyanins. Furthermore, there is evidence that anthocyanins themselves alter the composition of the gut microbiota, and this bi-directional relationship is hypothesised to be critical to the physiological effects of consuming anthocyanins.

Objective: The aims of the work presented in this chapter were to determine whether consuming anthocyanins during a human dietary intervention study exerted any changes on the microbiome. In addition to assessing whether there were any associations between how anthocyanins were metabolised and taxonomic features of the gut microbiota of individuals.

Methods: Faecal samples were collected from volunteers (n = 23) pre- and post- intervention for every phase of the human study, where a placebo, bilberry or black rice anthocyanins were consumed for 28 days (Chapter 3). Faecal samples were subjected to DNA extraction and were sequenced using 16S and metagenomic sequencing to determine the microbiota profile of samples.

Results: Consumption of bilberry or black rice anthocyanins did not exert any large effects on the gut microbiota profiles of the study population. However, in some individuals, *Anaerostipes hadrus and Eubacterium rectale* were increased post bilberry anthocyanin consumption ($\uparrow$ 0.4% ± 1.3 and 1.1% ± 3.6 respectively), whilst *Roseburia faeces* and *Blautia obeum* were decreased post black rice

anthocyanin consumption ($\downarrow$ 1.6% ± 3.4 and 0.26% ± 1.4 respectively), although, these changes were not observed across all participants. Furthermore, relationships were observed between C3G metabolites produced in the *in vitro* colon model and gut microbiota profiles, for example, *Lachnospira* and *Ruminococcus_1* were positively associated with the microbial production of phloroglucinol and dihydrocaffeic acid. Relationships were also observed between gut microbiota profiles and the majority of anthocyanin metabolites excreted in urine, including catechol, p-coumaric acid, homoPCA and gallic acid, after bilberry and black rice anthocyanin consumption. However, correlations were not observed between the abundance of microbial genes known to be implicated in anthocyanin metabolism such as β-glucosidase and protocatechuate decarboxylase and the concentrations of their known substrates and/or products produced *in vivo* or during *in vitro* fermentation.

Conclusion and Future Work: From the data presented in this chapter it is not possible to establish whether human anthocyanin consumption is able to modulate the gut microbiota as a result of high inter-individual variation in the microbiome of individuals limiting the ability to reliably detect changes. Nevertheless, clear relationships are shown between characteristics of the microbiota and anthocyanin metabolites produced *in vivo* and in an *in vitro* colon model, highlighting that the gut microbiota is critical in determining precisely how anthocyanins are metabolised. However, further work is required to confirm the importance of these relationships in wider populations. Furthermore, to aid our understanding of variability in anthocyanin metabolism and the health effects of eating anthocyanins, it would be recommended that future work seeks to characterise the microbial enzymes involved in these biotransformation's.

5.2 Introduction

Dietary anthocyanins have poor bioavailability, with only a small proportion of intact anthocyanins being able to pass through the gastrointestinal wall. Anthocyanins absorbed in the gastrointestinal tract, are mainly found in methylated, sulfated forms or as intact glycosides at extremely low levels in plasma and urine 10 – 2000 nM (De Ferrars, Czank, Zhang, *et al.*, 2014a). Despite their limited bioavailability, considerable levels of matrix-bound anthocyanins are able to enter the large intestine and colon, where they are subjected to extensive metabolism to generate a wide range of potentially bioactive metabolites that are absorbed into circulation (Fang, 2014; Igwe *et al.*, 2019; Keppler and Humpf, 2005). Human studies as described in Chapter 3, and by others, highlight that the absorption, metabolic fate, distribution and excretion of anthocyanin metabolites is variable between individuals (De Ferrars, Czank, Zhang, *et al.*, 2014a; Jokioja *et al.*, 2021). There has been recent interest in the role of the gut microbiota in determining the pathways taken during anthocyanin metabolism but also some evidence for anthocyanins themselves modulating gut microbial populations. This proposed bidirectional relationship between anthocyanin metabolism

and the gut microbiota has implications for understanding any potential health benefits of eating anthocyanins (Igwe *et al.*, 2019).

The gut microbiota comprises a vast amount of microorganisms including at least 1000 identified bacterial species. The majority of bacteria in the gut come under the phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, *Cyanobacteria* and *Actinobacteria* with *Firmicutes* and *Bacteroidetes* accounting for over 90% in most cases (Eckburg *et al.*, 2005; Tap *et al.*, 2009). The precise composition of the gut microbiota is subject to inter-individual variation due to a wide range of factors, including, but not limited to: age, gender, diet, disease, geographical location and lifestyle. The gut microbiota has many important functions such as: vitamin production, lipid metabolism and short-chain fatty acid production. Irregularities and imbalances in the microbiota (termed dysbiosis) have been linked to poor health. However, the role of the gut microbiota in anthocyanin metabolism, the influence of anthocyanins on gut microbial populations, and how this may affect overall health is not well understood (Faria *et al.*, 2014; Igwe *et al.*, 2019; Tian *et al.*, 2019).

Some studies have suggested that anthocyanins can increase the growth of beneficial gut bacteria and inhibit the growth of some bacterial species that are known to have negative implications for health (Faria *et al.*, 2014). For example, *in vitro* work with bacterial strains showed that black rice anthocyanins increased the growth of *Bifidobacterium* and *Lactobacillus* (Zhu *et al.*, 2018). Furthermore, incubation of M3G with faecal slurry enhanced total bacterial growth, including *Bifidobacterium* species and *Lactobacillus* species, whilst gallic acid (a known microbial anthocyanin metabolite) reduced *Clostridium histolyticum* without negatively affecting beneficial bacteria (Hidalgo *et al.*, 2012). This effect is reinforced by another study which found that *C.histolyticum* in human faeces showed decreased growth when incubated with red wine extract, however, notably red wine comprises a complex mixture of polyphenols so this effect cannot be directly attributed to anthocyanins (Queipo-Ortuño *et al.*, 2012).

There are relatively few animal and human studies that investigate the effects of anthocyanin consumption on the gut microbiota. One mouse study found that a black raspberry anthocyanin supplement increased the proliferation of *Eubacterium rectale, Faecalibacterum prausnitzii,* and *Lactobacillus* whilst inhibiting the growth of *Desulfovibrio* species and *Enterococcus* species (Chen, Jiang, *et al.*, 2018). Another mouse study reported that dietary supplementation with apples genetically modified to have a high anthocyanin content increased the relative abundance of *Bifidobacterium* species (Espley *et al.*, 2014). Likewise, a human study has shown that 6 week consumption of a blueberry drink (which has a similar anthocyanin profile to bilberry) significantly increased *Bifidobacterium* species (Vendrame *et al.*, 2011). In addition to this, a very recent study showed that consumption of an anthocyanin rich juice influenced the composition of the gut

microbiota. *Adlercreutzia, Lachnospira, Alistipes* and unclassified *Lachnospiraceae* were increased following consumption of an anthocyanin rich juice for 56 days whilst *Dorea* and a different unclassified *Lachnospiraceae* genus were decreased. Crucially no known potentially harmful taxa were enriched after juice consumption, suggesting that consumption of an anthocyanin rich juice promotes a healthy microbiome. Further to this, after 56 days of anthocyanin rich juice consumption participants' microbiomes were enriched for genes encoding enzymes involved in the metabolism of carbohydrates, lipids and amino acid and in pathways involved in DNA repair (Groh *et al.,* 2020). One factor to consider in human and animal studies is that it is difficult to establish whether anthocyanins exert a direct effect, or if they act indirectly via their microbial metabolites (Faria *et al.*, 2014). It should also be noted that some studies have reported no effect of anthocyanins on gut microbiota profiles, whilst Flores *et al* did not observe any increases in *Bifidobacterium* or *Lactobacillus* species as is reported by others (Flores *et al.*, 2015; Igwe *et al.*, 2019; Zhang *et al.*, 2016).

A common motif between *in vitro*, human and animal studies is that anthocyanins are frequently associated with increases in *Bifidobacterium* and *Lactobacillus* species. Interestingly, many of these species have β-glucosidase activity and therefore can metabolise phenolic compounds and enrich the bacterial medium with the release of glucose. Furthermore, these bacterial groups are associated with an antimicrobial effect against pathogenic organisms through the production of short-chain fatty acids as well as by competition for growth substrates and adhesion sites (Gibson and Wang, 1994).

Although there is some evidence for anthocyanins modulating the gut microbiota, there is also evidence for the microbiota being critical to anthocyanin catabolism. Studies have shown that many anthocyanin metabolites are microbially derived. Several metabolites have been reported as gut microbial products of cyanidin derivatives such as: PCA, vanillic acid, p-coumaric acid, PGA, gallic acid and 4-hydroxybenzoic acid (Chen *et al.*, 2017; Hanske *et al.*, 2013; Khanal *et al.*, 2014). Furthermore, the work presented in Chapter 4, and unpublished work from Emad Shehata (Kroon group, QIB) highlights that numerous black rice anthocyanin metabolites are only produced in the presence of a live microbiota, and that microbial anthocyanin metabolism can vary considerably between different faecal microbiota samples. Very little work has looked at the microbial species responsible for specific biotransformation's in the catabolism of anthocyanins. However, a recent study incubated elderberry anthocyanin extract with three single gut microbiota strains (*Enterobacter cancerogenous, Bifidobacterium dentium, and Dorea longicatena*). Whilst all strains degraded the elderberry anthocyanins, the metabolic pathways followed were different. Among the phenolic acids detected throughout the duration of the experiment, PCA and caffeic acid were significantly higher in *Dorea longicatena* compared to *Bifidobacterium dentium* and *Enterobacter cancerogenous* cultures. An unknown compound was observed at higher concentrations in

Bifidobacterium dentium cultures and only *Enterobacter cancerogenous* was able to yield pyrogallol from cyanidin derivatives (Bresciani *et al.*, 2020). Furthermore, another study found that PCA, PGA, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, benzoic acid, phenylacetic acid, and hydroxyphenylpropionic acid were microbial products of black rice anthocyanins produced by *Bifidofacteria* and *Lactobacilli* species (Zhu *et al.*, 2018). However, one factor to consider is that *in vivo* anthocyanins are exposed to a large and diverse community of microorganisms rather than single strains. Therefore, it is likely that combinations of microbes with according functional properties are responsible for differences in metabolism rather than specific strains in isolation. Furthermore, it is likely that different microbes play more of a central role at different points in anthocyanin metabolism where different substrates may be utilised by microbial enzymes. A barrier in understanding the precise role of the gut microbiota in anthocyanin metabolism is that studies tend to focus on microbial communities rather than the enzymes present that may be responsible for anthocyanin metabolism. This is partly due to the fact that few of the enzymes involved in anthocyanin metabolism have been characterised. Nevertheless, some microbial enzymes such as β-glucosidase, protocatechuate decarboxylase and 4-hydroxybenzoate decarboxylase are known to play important roles in the metabolism of anthocyanins and their derivatives (Eker *et al.*, 2019; Grant and Patel, 1969; Selmer and Andrei, 2001). Although metagenomic data does provide some insights into the enzymes encoded in the genomes of the microbial species present, this does not provide any insight into the expression or activity of these enzymes so has limited validity in providing any explanation for the role of particular enzymes in anthocyanin metabolism. Furthermore, as far as literature searches have shown, no current reports have focused on the functional capabilities of the gut microbiota and anthocyanin metabolism, leaving this as a major area for future research.

Although there is clearly some evidence for a bidirectional relationship between anthocyanins and the gut microbiota, the strength of this relationship is unclear, and not all studies have observed clear effects of anthocyanins on gut microbial populations (Flores *et al.*, 2015; Igwe *et al.*, 2019; Zhang *et al.*, 2016). Likewise, we do not have an understanding of the specific gut microbiota profiles that may characterise how an individual metabolises dietary anthocyanins and the implications this may have for health. This chapter aims to address this by studying the gut microbiota profiles of 23 individuals before and after consumption of different sources of anthocyanin (bilberry and black rice) for 28 days, and comparing these profiles to *in vivo* metabolite data from both sources of anthocyanin as well as data on the capacity of faecal samples from these same individuals to metabolise black rice anthocyanins in an *in vitro* fermentation colon model.

5.3 Objectives

The overall aim of the work presented in this chapter was to profile the gut microbiota for all faecal collectors (n = 23) from the human study described in Chapter 3, and establish if there was any relationship between these profiles and anthocyanin metabolism. The specific aims were:

- Establish whether consuming either black rice or bilberry anthocyanin extracts influenced the composition of the gut microbiota.

- Compare and contrast the taxonomic microbiota profiles of individuals who metabolised anthocyanins differently during *in vitro* microbial fermentation of black rice anthocyanins, and *in vivo* after a dietary intervention where bilberry and black rice anthocyanins were consumed.

- Assess whether any functional features of the gut microbiota are associated with anthocyanin metabolising characteristics of individuals from *in vitro* microbial metabolism and *in vivo* metabolic data.

5.4 Methods

5.4.1 Human Study Sample Collection

The human study was performed according to the methods described in Chapter 3. For a subset of participants (n = 23) faecal samples were collected pre- and post-intervention for each phase of the study (placebo, bilberry and black rice anthocyanin extracts). Participants were asked to provide a faecal sample within 3 days preceding the start of the treatment period or ± 3 days of finishing the treatment period. Participants were asked to deliver faecal samples to the study team within 6 hours of production, samples were kept on ice and hand-homogenised before being frozen in 5 g aliquots and stored at -80°C.

5.4.2 DNA Extraction

Faecal samples were thawed on ice and approximately 200 mg (± 5 mg) was weighed into a sterile tube and subjected to DNA extraction using the FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA) according to manufacturer guidelines, with an additional bead beating step using FastPrep (MP Biomedicals, USA) as previously described by Kellingray et al (Kellingray et al., 2017). DNA samples were then quantified using Qubit broad range reagents according to manufacturer guidelines (Thermo-fisher, Cambridge, UK), samples were kept on ice throughout DNA extraction and quantification protocols. Samples were then stored at -80°C until use.

5.4.3 16S Sequencing

DNA extracted from faecal samples was diluted to 50 ng/μl in DNAase free water and sent to Novogene for library preparation and 16S rDNA sequencing (Novogene, Cambridge, UK). The 16S

rRNA genes of distinct regions (V3–V4) were amplified using specific primers (341F: CCTAYGGGRBGCASCAG; 806R: GGACTACNNGGGTATCTAAT) Sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA). The library was sequenced on an Illumina HiSeq2500 platform, and paired-end reads were generated.

5.4.4 Shotgun-Metagenomics

DNA extracted from faecal samples was diluted to 0.5 ng/μl in DNAase free water and sent to the in-house sequencing department (Quadram Institute Bioscience, Norwich, UK) for library preparation according to the method described by Foster-Nyarko *et al* (Foster-Nyarko *et al.*, 2020). The pooled library was then sent to Novogene for shotgun-metagenomic sequencing at a sequencing depth of 10 Gb per sample, on the Illumina Novaseq 6000 platform, and paired-end reads were generated (Novogene, Cambridge, UK).

5.4.5 Bioinformatics

Bioinformatics analysis was completed by Dr Perla Rey and Dr Marianne Defernez.

Bioinformatics Analysis of 16S Data (Dr Perla Rey)

For 16S data, the usearch v11 pipeline (https://drive5.com/usearch/manual/ex_miseq.html) was used for: quality control, chimera removal, OTU classification and assigning taxonomy. All sequences were filtered to meet the following criteria: read length within 200 - 1000 bp; a maximum of 6 ambiguous bases; a minimum average quality score of 25 within a 50 bp window; and exact match to primer sequences; for bacterial taxonomy assignment a confidence value threshold of 50% was used with trimmed reads clustered into operational taxonomic units (OTUs) at 97% identity level. MEGAN v6 was used to generate figures from the OTU table produced by usearch (https://software-ab.informatik.uni-tuebingen.de/download/megan6/welcome.html).

Bioinformatics of Metagenomics Data (Dr Perla Rey)

For metagenomics data human sequence removal was performed using Kraken2 v2.0.8 (https://github.com/DerrickWood/kraken2) with a confidence level of 0.3 using the human reference genome (GRCh38.p12) (Wood *et al.*, 2019; Wood and Salzberg, 2014). Quality control was then performed using fastp v0.20.1 (https://github.com/OpenGene/fastp) with default options for paired-end data to remove adapter sequences, low quality reads based on their PHRED score (q < 30) and short reads (length < 60 bp). High quality and trimmed reads were used for downstream analyses (Chen *et al.*, 2018).

High quality and trimmed reads filtered with fastp were used to estimate the microbial taxonomic composition profiles using MetaPhlAn v3.0.2 (https://github.com/biobakery/MetaPhlAn) (Beghini *et al.*, 2021; Segata *et al.*, 2012). MetaPhlAn identifies the microbes and their abundance from

metagenomics reads by mapping them to the ChocoPhlAn v296 database of unique clade-specific marker genes. Clades are groups of organisms and clade-specific markers are coding sequences that are strongly conserved within the clade's genomes and are sufficiently different to any sequence outside the clade. The marker genes in the database were identified from over 17,000 reference genomes of bacteria, archaea, viruses and eukaryotes (Beghini *et al.*, 2021). Statistical analysis of community profiling was done using MaAsLin2 (https://github.com/biobakery/Maaslin2) for differential abundance and multivariate association testing for taxonomic and functional profiles with the centre-log-ratio (CLR) normalisation method and a prevalence threshold of 1% (Mallick *et al.*, 2021).

For functional profiling, HUMAnN3 (https://github.com/biobakery/humann) was used to evaluate the abundance of microbial metabolic pathways and other molecular functions from metagenomic sequencing data using KEGG Orthology to estimate abundance of each orthologous gene family in the community. Orthologous families are groups of genes that perform roughly the same biological roles. Pathways are sets of two or more genes with a similar function. The run with HUMAnN3 was done using nucleotide mapping and translated search to provide organism-specific gene and pathway abundance profiles from the quality-controlled shotgun metagenomics dataset. Gene families are annotated using UniRef90 definitions (protein database – The UniProt Reference Clusters, provides clustered sets of sequences) (Suzek *et al.*, 2015). UniRef90 is built such as each cluster is composed of sequences that have at least 90% sequence identity to and 80% overlap with the longest sequence of the cluster. Pathways are annotated using MetaCyc (Caspi *et al.*, 2013, 2020; Karp and Caspi, 2011). MetaCyc is a curated database of metabolic pathways from all domains of life. It contains 2937 pathways from over 3000 different organisms. HUMAnN3 was used with the default options (minimum percentage of reads matching a species: 0.01, identity threshold for nucleotide alignments: 0.0). To be able to compare gene families and pathway abundances between samples with different sequencing depths, the normalised values using "copies per million", CPM, or sum-normalisation to relative abundance was used.

Bioinformatics Analysis of the Effect of Anthocyanin Consumption on 16S/Metagenomic Profiles (Dr Marianne Defernez)

To assess the effect of anthocyanin consumption on the microbiome a multivariate approach was taken using a multi-level sparse PLS discriminant analysis (sPLS-DA) for centre-log-ratio (CLR) transformed data in R 3.6.2 using the mixOmics package (v 6.10.9). sPLS-DA enables the selection of the most predictive or discriminative features in the data that help classify the samples (Lê Cao *et al.*, 2011). For 16S the data was filtered to 68 taxa (criterion: 'either all groups have 12 or more zeros, or the maximum for all samples is below 0.5%') The summed relative abundance that was captured was between 94.8- 99% of the original coverage. For the metagenome data the analyses

were run with 3 different filters. The options were to filter to 147, 85, or 33 taxa (criteria being: at least 0.1% in 6 samples, 0.5% in 8 samples, 1% in 22 samples) the summed relative abundance that was captured over the 136 samples was (respectively) between 93.0- 99.9, 84.3- 99.2, and 27.4- 95.2% of the original coverage.

Bioinformatics Analysis of Relationships Between 16S/Metagenomic Data and Anthocyanin Metabolism Data (Dr Marianne Defernez)

For multivariate analysis of relationships between the *in vitro* fermentation colon model and microbiome data, and between urinary metabolite and microbiome data a sparse canonical correlation analysis was used in R 3.6.2 with the mixOmics package (v 6.10.9). Canonical correlation analysis is used to identify and measure the associations among two sets of variables. Canonical correlation is appropriate in the same situations where multiple regression would be, but where there are multiple inter-correlated outcome variables. Canonical correlation analysis determines a set of canonical variates, orthogonal linear combinations of the variables within each set that best explain the variability both within and between sets (Parkhomenko *et al.*, 2009). For 16S and metagenomics data centre-log-ratio (CLR) transformed data was used; for 16S the data was filtered to 68 taxa, and for metagenomics data was filtered to 147 taxa as described above. A tuning step was run to establish the number of variables to be retained to define each canonical correlation axis, and permutation tests were used to calculate the probability of obtaining the same correlation coefficient by chance. Samples scores were then plotted for each dataset on the corresponding axes to establish whether the scores between datasets were linearly related. Correlation circles were then produced to highlight the variables in each dataset responsible for any observed relationship. Heatmaps were then produced which show a measure of the correlation between variables from two datasets, this was a derived measure taken from coordinates in the correlation circle plot for each pair of variables i.e. a urinary metabolite and a taxonomic group, and calculating their scalar product. A scalar product threshold of 0.4 was used to only show the most correlated variables between datasets; variables were then clustered and visualised as a heatmap.

5.5 Results

5.5.1 Effect of Anthocyanin Consumption on Gut Microbiota Profiles (16S)

To profile the microbiota of individuals from the human study initially a 16S sequencing approach was taken. Sequencing the hypervariable regions of the highly conserved 16S rRNA gene allows discrimination of microorganisms such as bacteria and archaea to the genus level. This provides an excellent overview of the gut microbiota and allows comparison between the profiles observed at each phase of the human study. Rarefaction analysis (Figure 5-1) showed that species coverage was high as indicated by the levelled off curves for each sample suggesting that only the scarcest species

remain to be sampled. Whilst the rank abundance curve (Figure 5-2) indicates that species richness of samples was high.
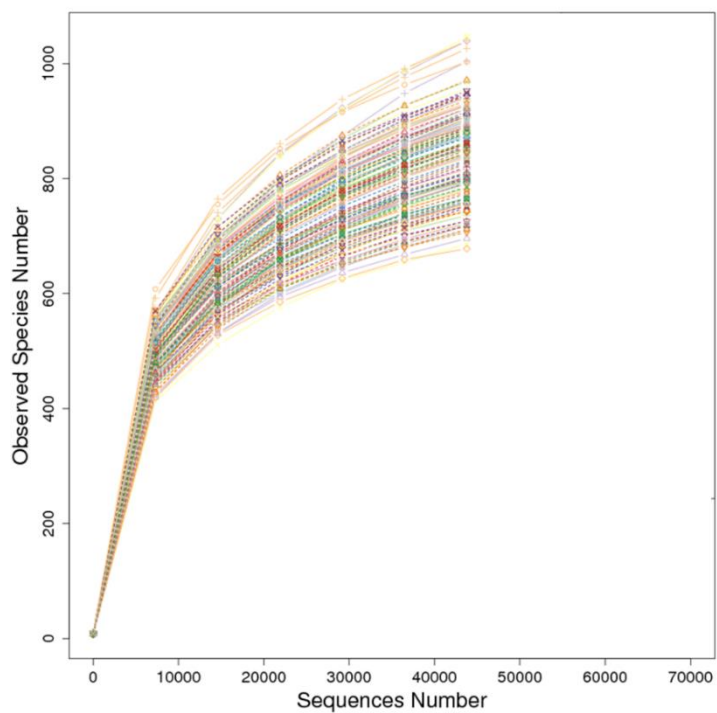
Figure 5-1- Rarefaction curve for 16S samples (n = 136)
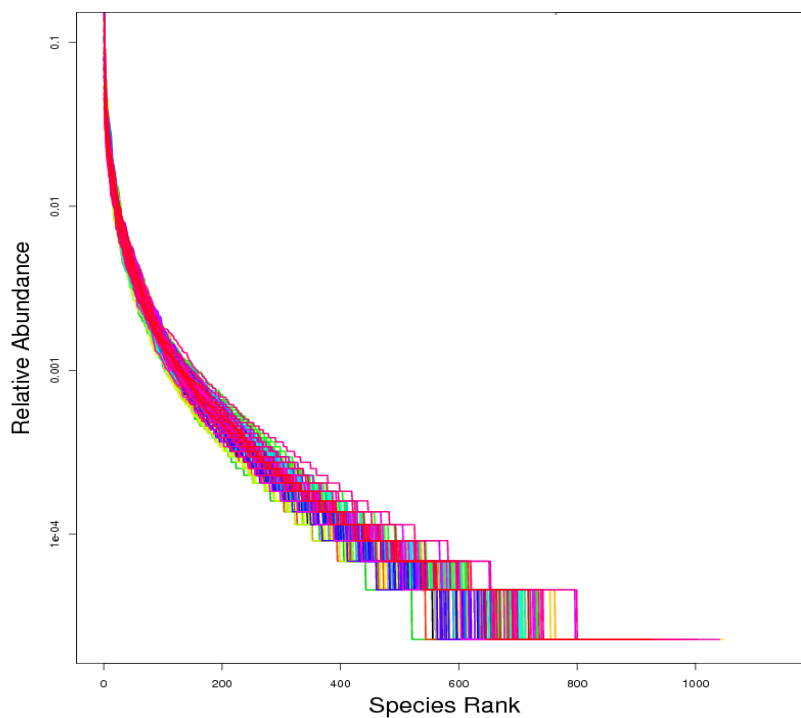
Figure 5-2- Rank abundance curve for 16S samples (n = 136).

The top 10 genera were plotted for each study phase and did not show any clear differences between treatments, shown in Figure 5-3. The other category which encompassed all genera not included in the top 10 comprised the highest proportion of microbes in samples and it was considered that perhaps there were subtle changes contained within this category. Therefore, the entire OTU table was taken to produce a neighbour joining (NJ) tree to plot all of the samples according to their similarity, with closer samples having more similar microbiota profiles. Samples are colour coded according the phase of the study from which they were derived but there was no association between samples from the same study phase (Figure 5-4). However, when the same NJ tree was colour coded according to volunteer (Figure 5-5), clear clustering could be seen indicating that inter-individual variation in gut microbiota profiles was the distinguishing factor of these samples and was potentially masking any effect of treatment.
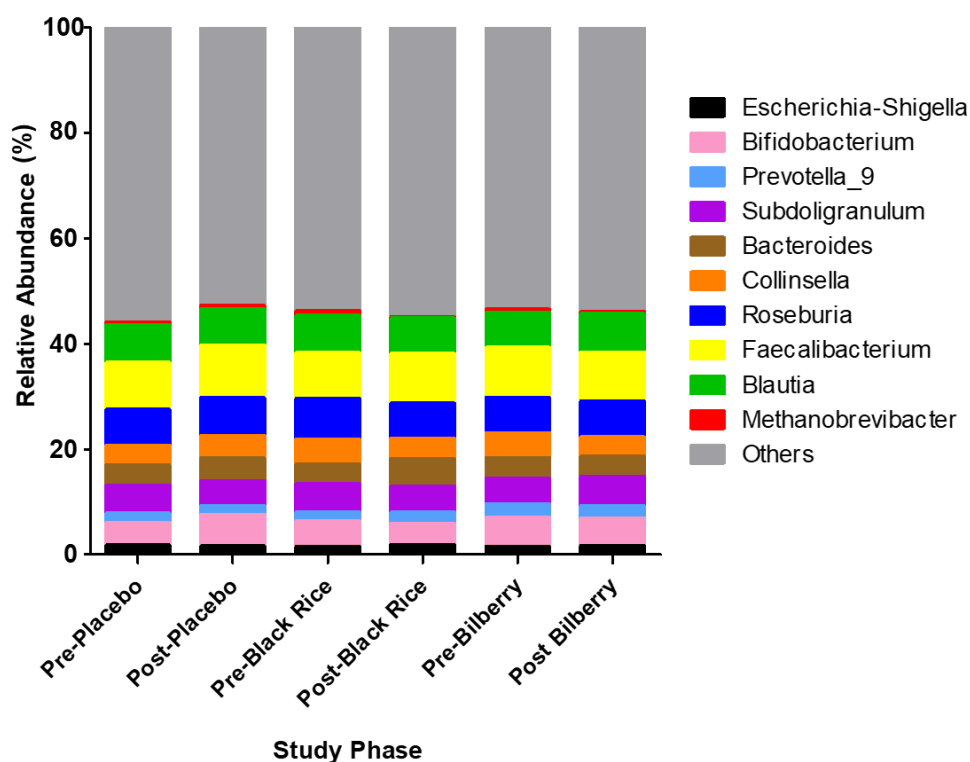
Figure 5-3- Top 10 genera
The top 10 genera across all 16S sequenced faecal samples shown as relative abundance (%)
(n = 136) for each phase of the human intervention study: pre-placebo (n = 22), post placebo
(n = 22), pre-black rice (n = 23), post-black rice (n = 23), pre-bilberry (n = 23) and post-bilberry
(n= 23).
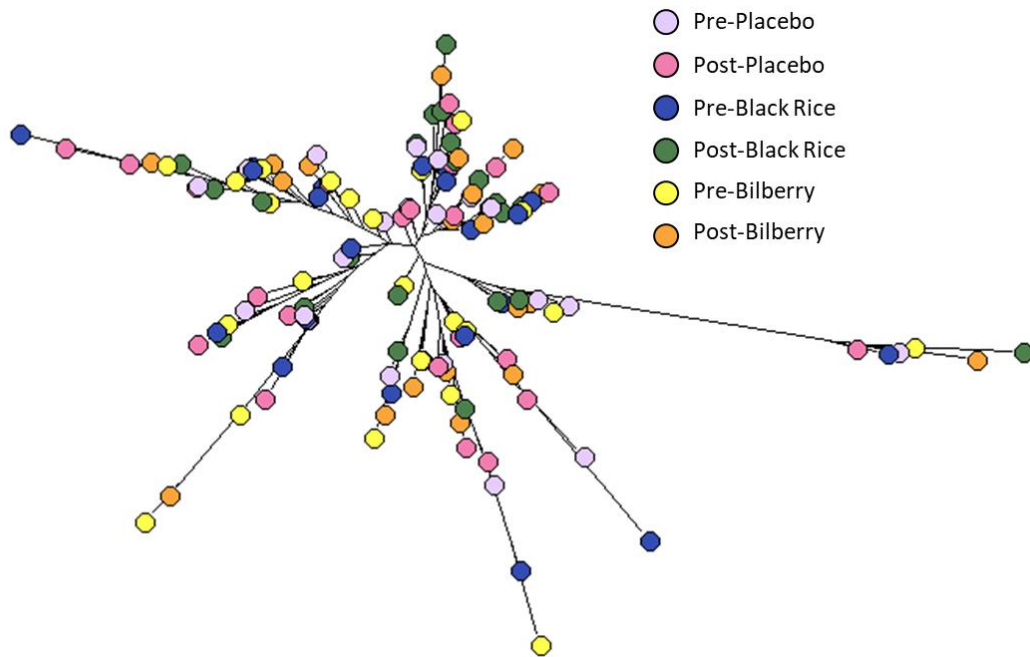
Figure 5-4- NJ Tree of 16S samples colour coded according to study phase
NJ tree of all 16S samples (n = 136), taking into account the relative abundance of all genera identified, colour coded according to study phase, detailed by the key on the right of the figure. Each circle represents one sample.
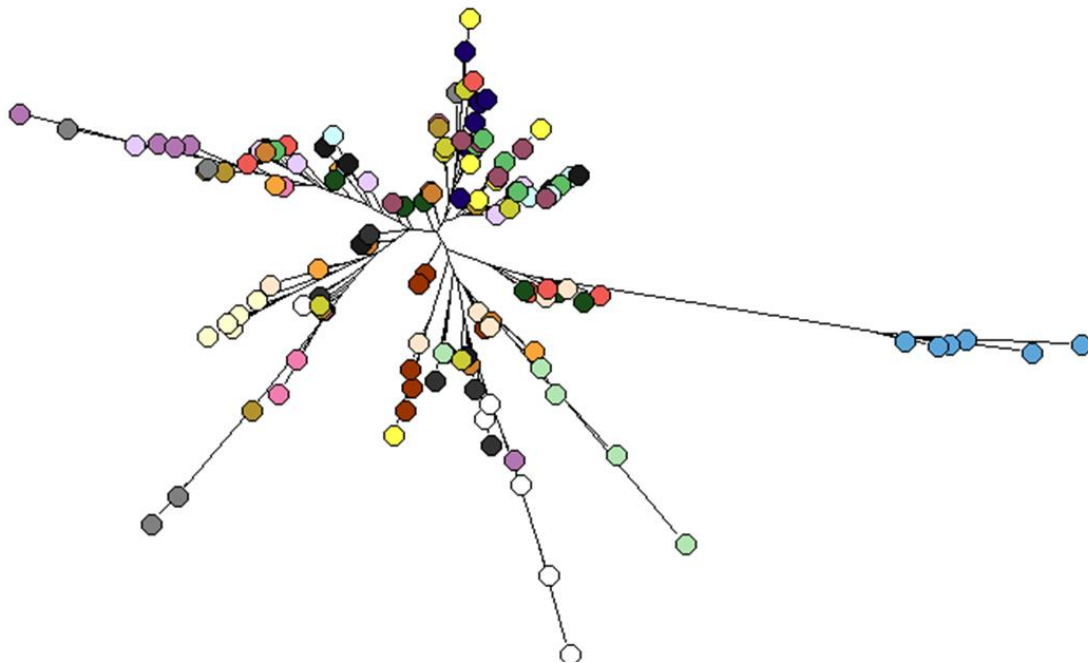
Figure 5-5- NJ Tree of 16S samples colour coded according to volunteer
NJ tree of all 16S samples (n = 136), taking into account the relative abundance of all genera identified, colour coded according to volunteer. Each circle represents one sample.

The NJ trees shown in Figure 5-4 *and* Figure 5-5 suggested that differences between individuals could be large compared to those between treatments, and therefore analysis was required that focused on intra-individual variation. Therefore, a multi-level sPLS-DA was employed to take into account the study design. With this approach centre-log-ratio (CLR) transformed data was used as an alternative to relative abundance. CLR data is argued to be more appropriate for the analysis of compositional data such as genomic profiles and increases the validity of correlations calculated between taxa (Gloor *et al.*, 2016; Gloor and Reid, 2016). The 16S data was filtered to 68 taxa using the criteria that 'either all groups have 12 or more zeros, or the maximum for all samples is below 0.5%' and the summed relative abundance captured was between 94.8 - 99% of the original coverage. This data is shown graphically in Figure 5-6. There is some separation between day 1 and day 29 (pre- and post- treatment) for black rice and bilberry extract treatments (B and C respectively). However, there is also some separation between day 1 and day 29 profiles for the placebo (A). Taxa that were picked up as being associated with this separation were: *Alistipes*, *Paraprevotella*, *Coprococcus_2, Alloprevotella* and *Prevotella_2.* Of these genera, *Coprococcus_2, Alistipes* and *Paraprevotella* tended to be increased in all day 29 (post-treatment) samples relative to day 1 (pre-treatment), whilst *Alloprevotella* and *Prevotella_2* were generally decreased in day 29 samples relative to day 1, irrespective of treatment, indicating that the placebo may be having some small effect on the gut microbiota. The genera that were more specifically highlighted in the separation of post black rice samples (*_29_B), and to some extent post bilberry samples (*_29_C) were: *Ruminococcus_torques_group, Fusicalenibacter, Erysipelotrichaceae_UCG_003, Romboutsia, Anaerostipes* and *Ruminococcus_1. Ruminococcus_torques_group* were decreased after both bilberry and black rice extract consumption, whilst *Fusicalenibacter* and *Romboutsia* were only decreased after black rice and *Ruminococcus_1* was increased by only black rice. *Erysipelotrichaceae_UCG_003* was increased post bilberry but decreased post black rice, and *Anaerostipes* was increased only post bilberry. However, all of these changes were very subtle and did not constitute clear effects exerted by any treatment for the study population. Box plots for the within subject variation of each of the genera highlighted in this analysis are shown in Appendix 8, and illustrate the modesty in the changes observed.
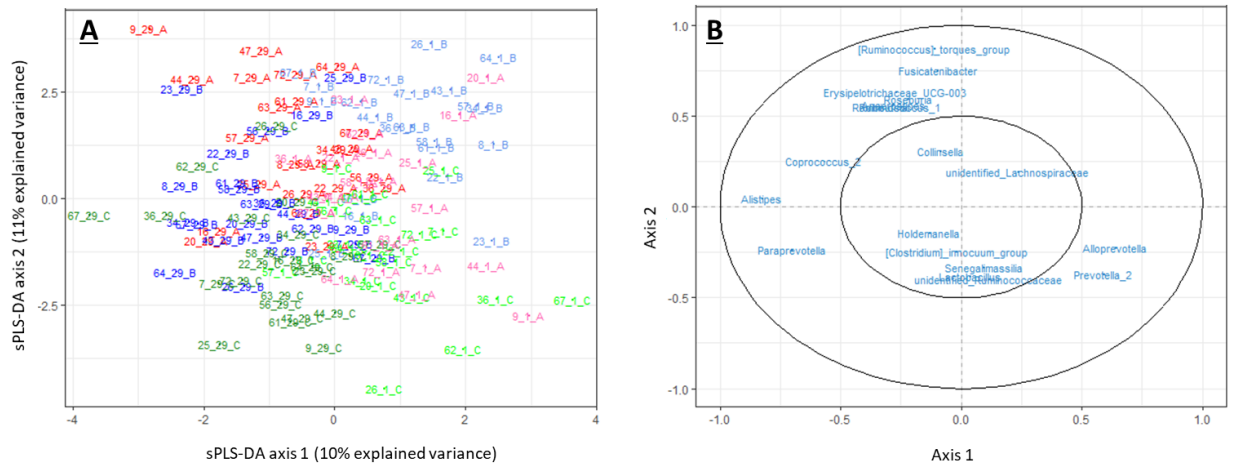
Figure 5-6- Multilevel sPLS-DA on 16S CLR-transformed data
A: Scores plot from multilevel sPLS-DA on 16S CLR-transformed data filtered to 68 taxa described by axes 1 and 2 (criterion: 'either all groups have 12 or more zeros, or the maximum for all samples is below 0.5%') The summed relative abundance that was captured was between 94.8- 99% of the original coverage. Data is shown as participant-ID_study-day_treatment, where 1 is pre-treatment and 29 is post-treatment; A corresponds to placebo, B to black rice and C to bilberry. Pink shows pre-placebo, red shows post-placebo, light blue shows pre-black rice, dark blue shows post-black rice, light green shows pre-bilberry and dark green shows post-bilberry. Samples that are closest together had more similar 16S profiles. B: Correlation circle highlighting which taxa may be relevant to the separation of samples in the scores plot on the left.

It was surprising that there were no clear substantial effects of anthocyanin consumption on gut microbiota profiles, it was hypothesised that there would be an increase in *Bifidobacteria* post-black rice and bilberry anthocyanin extract consumption in keeping with other reports, however, this effect was not shown. One flaw of 16S sequencing is that it can only give a reliable profile at the genus level. Given that anthocyanin extracts were being consumed as part of a wider diet it seemed unlikely that they would exert such a large effect on the microbiota and that any observed effect was likely to be modest, perhaps only at the species level. Furthermore, multi-level sPLS-DA analysis suggested there may be some low level changes pre- and post- treatment for some genera, and it was postulated that these changes may be more pronounced at the species level. Therefore, the decision was taken to send samples for metagenomics sequencing. Unlike 16S, metagenomics sequences all genomic DNA in a sample and can identify microorganisms accurately at the species level and can also be used to profile microbial genes to provide functional information. It was hypothesised that any changes in the microbiota would have been subtle and low level, and therefore would be far more likely to be detectable from metagenomics analysis.

5.5.2 Effect of Anthocyanin Consumption on Gut Microbiota Profiles (Metagenomics)

The first step in the analysis of metagenomic data was to remove human reads using Kraken2. The quality of the remaining reads was then assessed using fastp, which showed that the vast majority

of samples had over 90% reads pass with only a small proportion being removed in filtering, as shown in Figure 5-7.
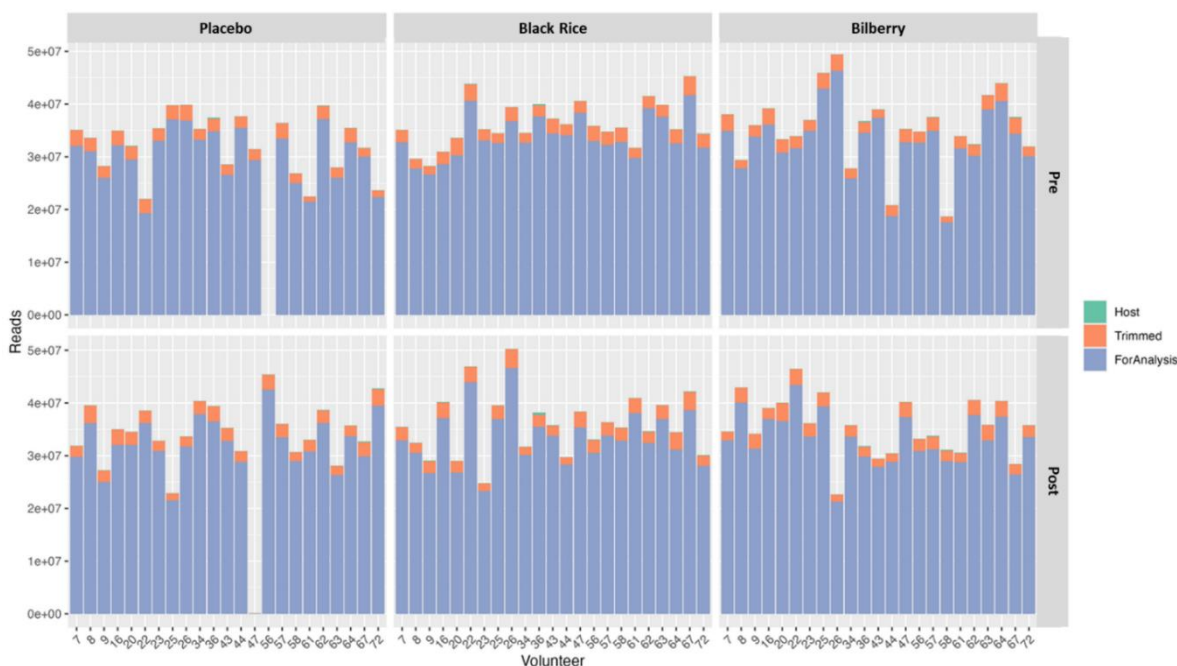
Figure 5-7- Quality report of whole-genome shotgun metagenomics data
Quality report of whole-genome shotgun metagenomic reads determined using fastp after removal of human sequences with kraken2 for all samples (n = 136). The number of reads is given on the Y axis and the volunteer number on the X axis. The top panels of the figure presents samples pre-intervention and the bottom panels of the figure show samples post-intervention for placebo, black rice and bilberry anthocyanin extracts from left to right. Bars are colour coded according to whether reads were removed due to being derived from the host, trimmed due to low quality or were high quality and taken forward for analysis, this colour coding is described by the key on the right of the figure. Note gaps are present for two samples (56, pre-placebo and 47, post-placebo) due to non-submission of faecal samples from study volunteers.

Filtered reads were profiled using MetaPhlAn3, which is able to provide species-level identification for bacteria, archaea, viruses and eukaryotes by comparing shotgun metagenomics data to sequences held in the ChocoPhlAn database. 384 species were detected across all of the samples sequenced and initially this profiling was taken as a whole and used to produce a PCoA plot to establish if there were any apparent clustering between treatment or volunteer, shown in Figure 5-8. No clustering was evident according treatment or whether samples were taken pre- or post-intervention. Indicating that from an initial global view treatment did not exert any effect on the gut microbiota profile of the study population. What is clear in Figure 5-8 is that samples from the same participant tend to be clustered, irrespective of treatment or time-point, mirroring what is shown in Figure 5-4 and Figure 5-5. This indicates that inter-individual variation is the key distinguishing factor between samples and likely masks any potential effect of treatment. It is unlikely the effects of anthocyanin consumption would transform the microbiota profile of individuals and effects are likely to be subtle and only concern a small number of species.

Consequently, in a variable population it is difficult to establish whether changes are occurring and to what extent, as high variation may mask the effects of treatment.
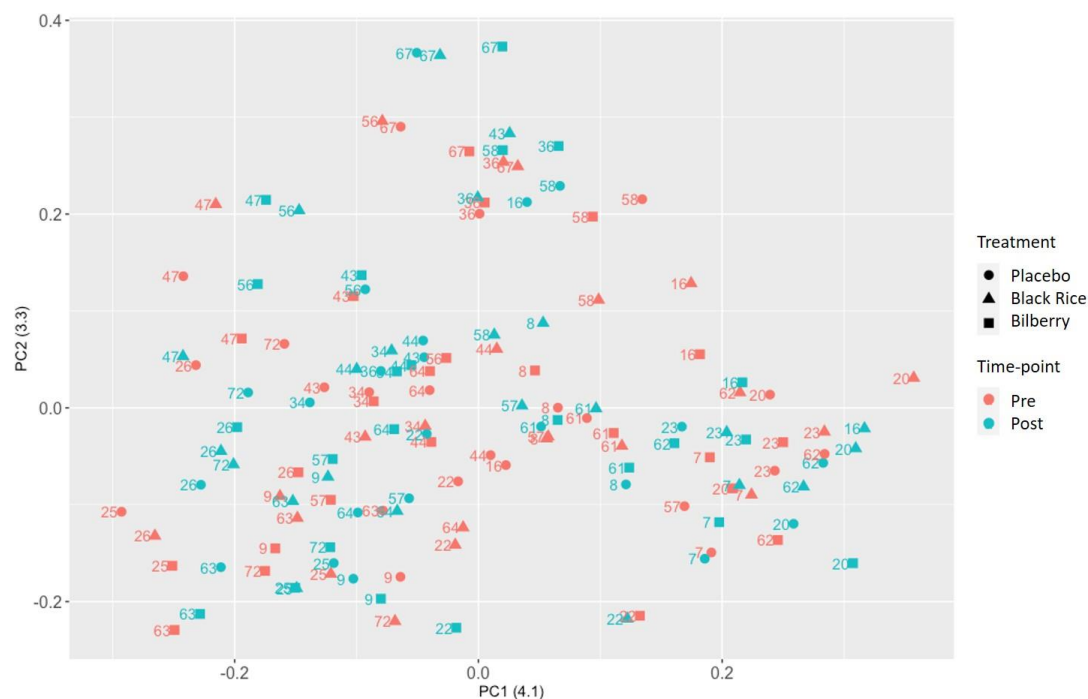
Figure 5-8- PCoA plot using Bray Curtis dissimilarity distances for metagenomic data
PCoA plot using Bray Curtis dissimilarity distance for all metagenomic samples profiled (n = 136) using MetaPhlAn 3. Data points are numbered according to the volunteer from which they were derived and shaped and coloured according the phase of the study, described by the key right of the figure. Data points are arranged so distances among each pair of points represents the dissimilarity between those two samples, i.e. the further away two data points are the less similarity between them.

Due to the observed high inter-individual variation making it difficult to establish whether any changes were occurring according to treatment globally, a heat-map was produced showing the top 50 species present in all samples. This clustered samples according to their taxonomic similarity and resulted in samples from the same volunteer generally being clustered together, shown in Figure 5-9. Information regarding the volunteer, treatment and study day are also included on the figure. From first glance, no clear patterns can be seen in the relative abundance of species between treatments, much like in the case of the 16S relative abundance data presented in Figure 5-3 and Figure 5-4. However, echoing what is shown by the PCoA plot in Figure 5-8, in Figure 5-9 the main similarities are seen between samples from the same volunteer, overshadowing any small effect that may be resulting from treatment. For example, BERI 26 has extremely high relative abundance of *E.coli* in all samples, much higher than any other volunteer and this does not appear to be effected by treatment. Figure 5-10, Figure 5-11 and Figure 5-12 show the top 20 species present in stacked bar plots pre-and post each treatment; placebo, black rice and bilberry respectively.

As expected, no consistent differences were observed between pre- and post-placebo samples, however, the relative abundance of *Prevotella copri* was reduced for BERI 57, whilst *Roseburia hominis* decreased in BERI 23 post-placebo *(*Figure 5-10*).* However, these changes were not seen in other participants and are therefore likely to be due to other factors outside of the study rather than the placebo, particularly considering the delicate nature of the gut microbiota and many factors that will affect its composition. Black rice consumption did not appear to exert any clear effects on microbiota profiles across the study population. In a subset of participants*, Roseburia faecis* appeared to decrease after black rice consumption (BERI 07, BERI 08, BERI 16, BERI 26, BERI 43, BERI 57, BERI 63 and BERI 72). However, this was not observed in all participants. Other changes were subtler and did not occur in enough participants to constitute a pattern *(*Figure 5-11*).* Likewise, bilberry extract did not seem to exert any major influence on gut microbiota profiles of the study population. Some participants appeared to show reduced levels of *Bifidobacterium adolescentis* post bilberry consumption (BERI 25, BERI 62, BERI 63, BERI 64), whilst others showed an increase (BERI 08, BERI 09, BERI 16, BERI 22, BERI, 34, BERI 56 BERI 57). This highlights that there were no clear uniform changes in the gut microbiota observed as a result of consumption of either black rice or bilberry anthocyanins, when looking at the most abundant species across the study population. And the data presented does not support the hypothesis that one month of daily anthocyanin consumption from black rice or bilberry extract would alter the microbiome. Furthermore, what is clear between Figure 5-9,Figure 5-10, Figure 5-11 and Figure 5-12 is that there are considerable differences in the relative abundance of microbial species between individuals, and that over the 6 sampling points of the study major changes were not observed for any individual and any changes that were observed tended to be small and were not consistent across the study population. This much like the other data presented so far in this chapter indicates that the overarching factor determining the gut microbiota profile is inter-individual variation (derived from outside of the study) and any low level changes that may be occurring due to treatment are likely to be masked by this variability across the study population.
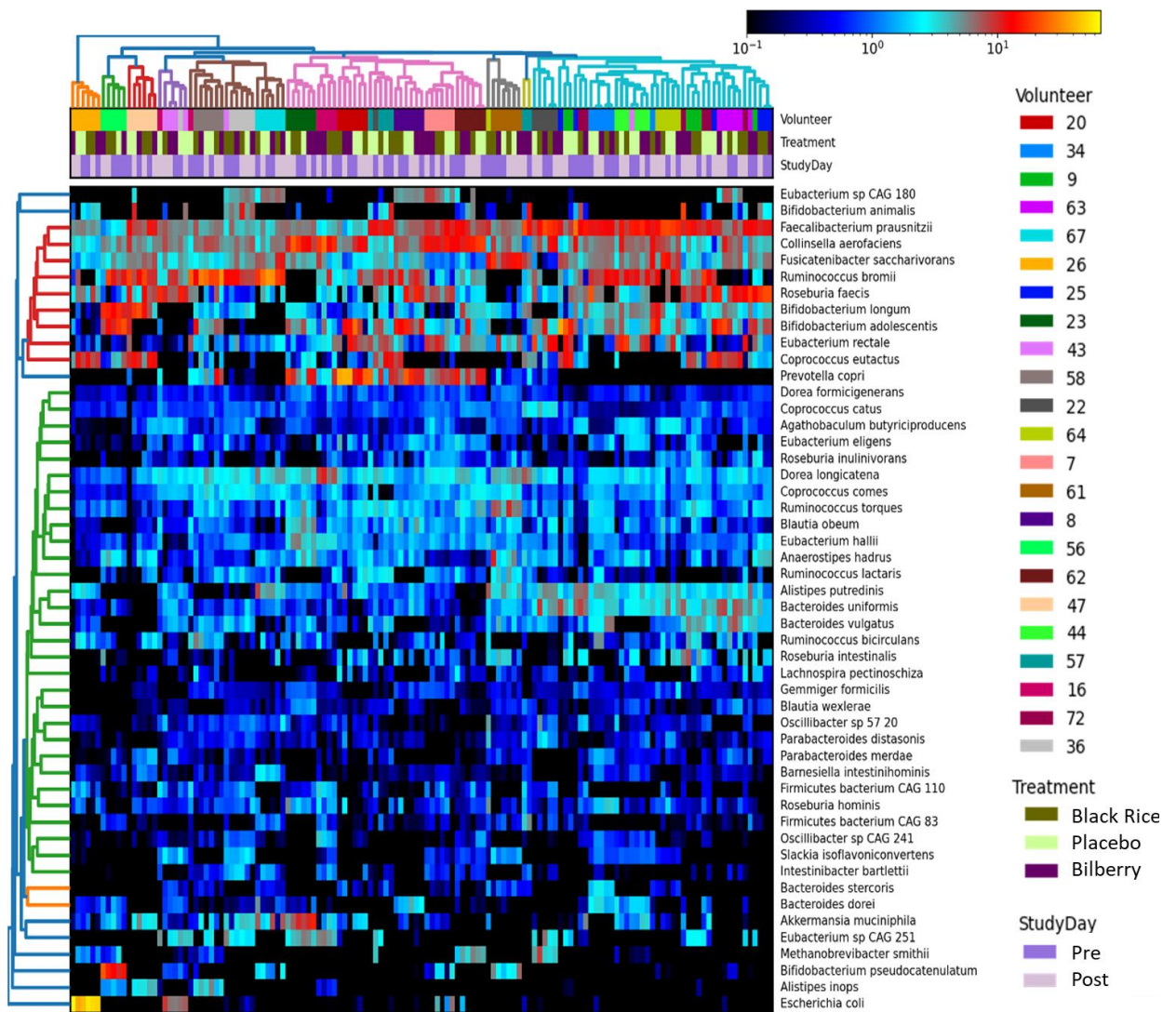
Figure 5-9- Relative abundance heatmap for the taxonomic profiles of metagenomics data
Relative abundance heatmap for the taxonomic profiles of all metagenomics samples (n = 136). Heatmap produced with hierarchical clustering (samples clustered by taxonomic similarity) for the top 50 species using Bray-Curtis distance measures for samples and species. The heatmap describes the relative abundance of each species in faeces standardised to the range of $[10^{-1}, 10^{2}]$. Yellow-red colour indicates high relative abundance of a species and black-blue indicates only low levels were present.
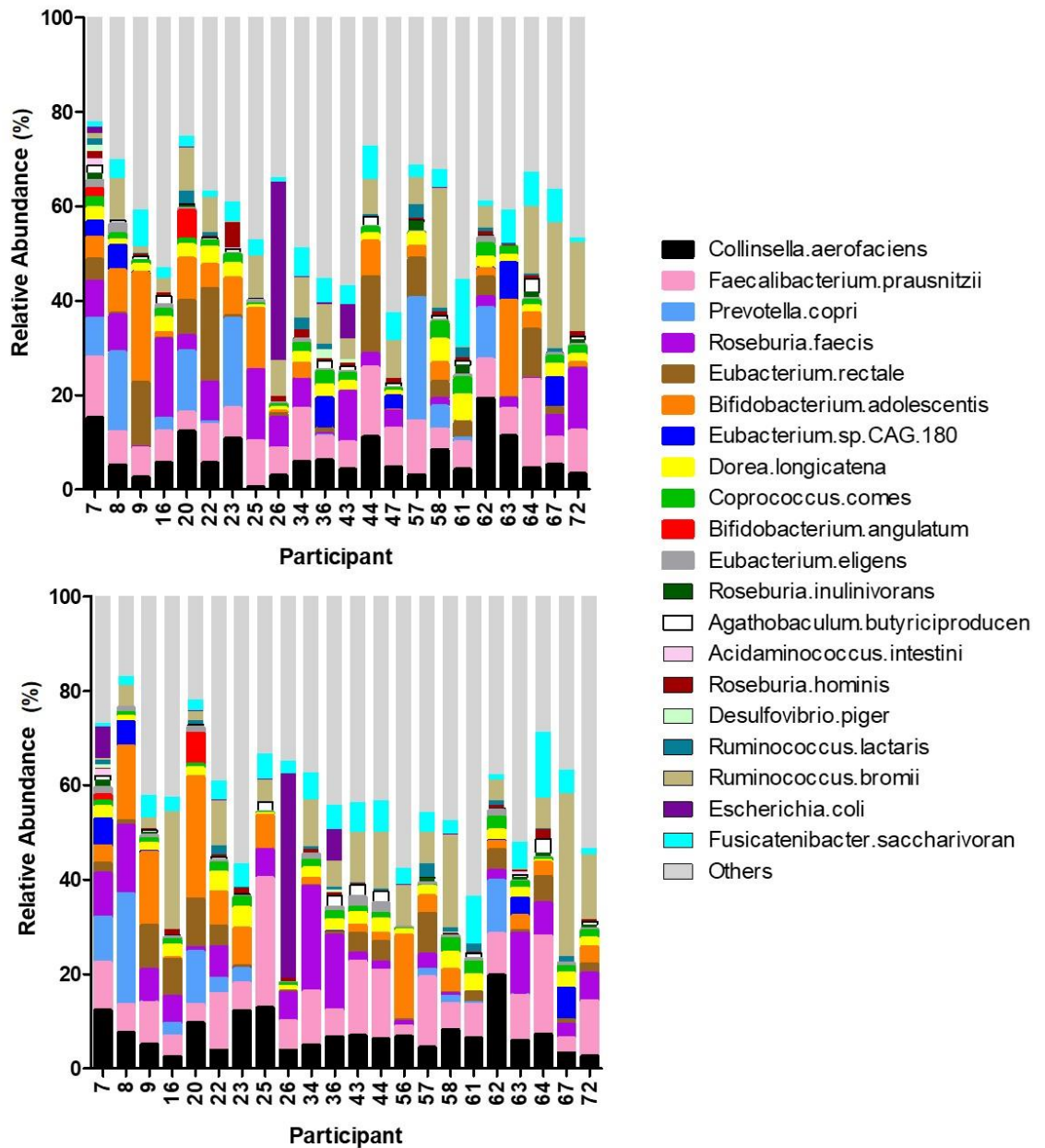
Figure 5-10- Top 20 species pre- and post- placebo
Relative abundance (%) of the top 20 microbial species identified in metagenomics samples pre-placebo (top) and post-placebo (bottom), data is shown per participant.
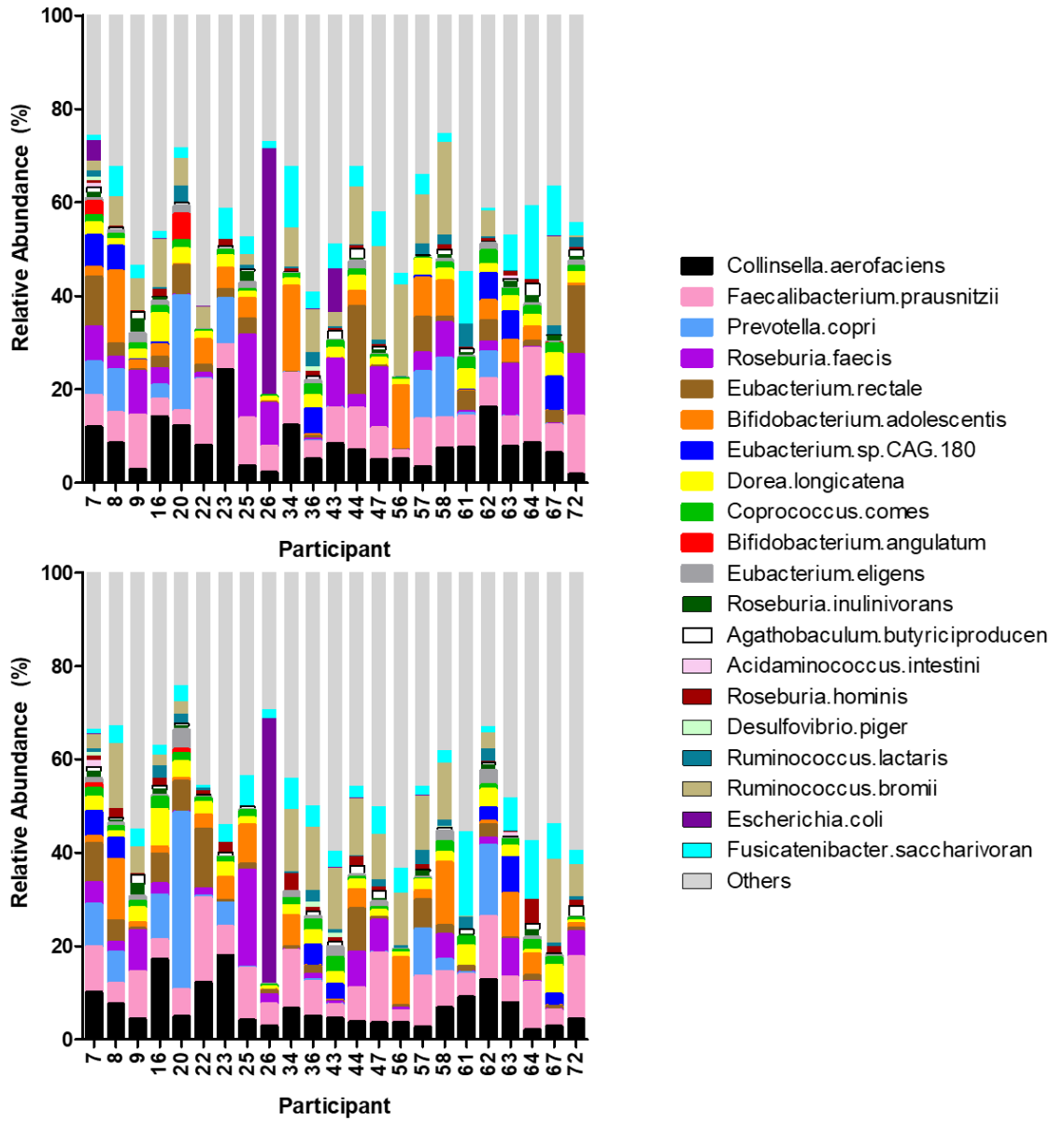
Figure 5-11- Top 20 species pre- and post- black rice anthocyanin extract
Relative abundance (%) of the top 20 microbial species identified in metagenomics samples pre-black rice extract (top) and post-black rice extract (bottom), data is shown per participant.
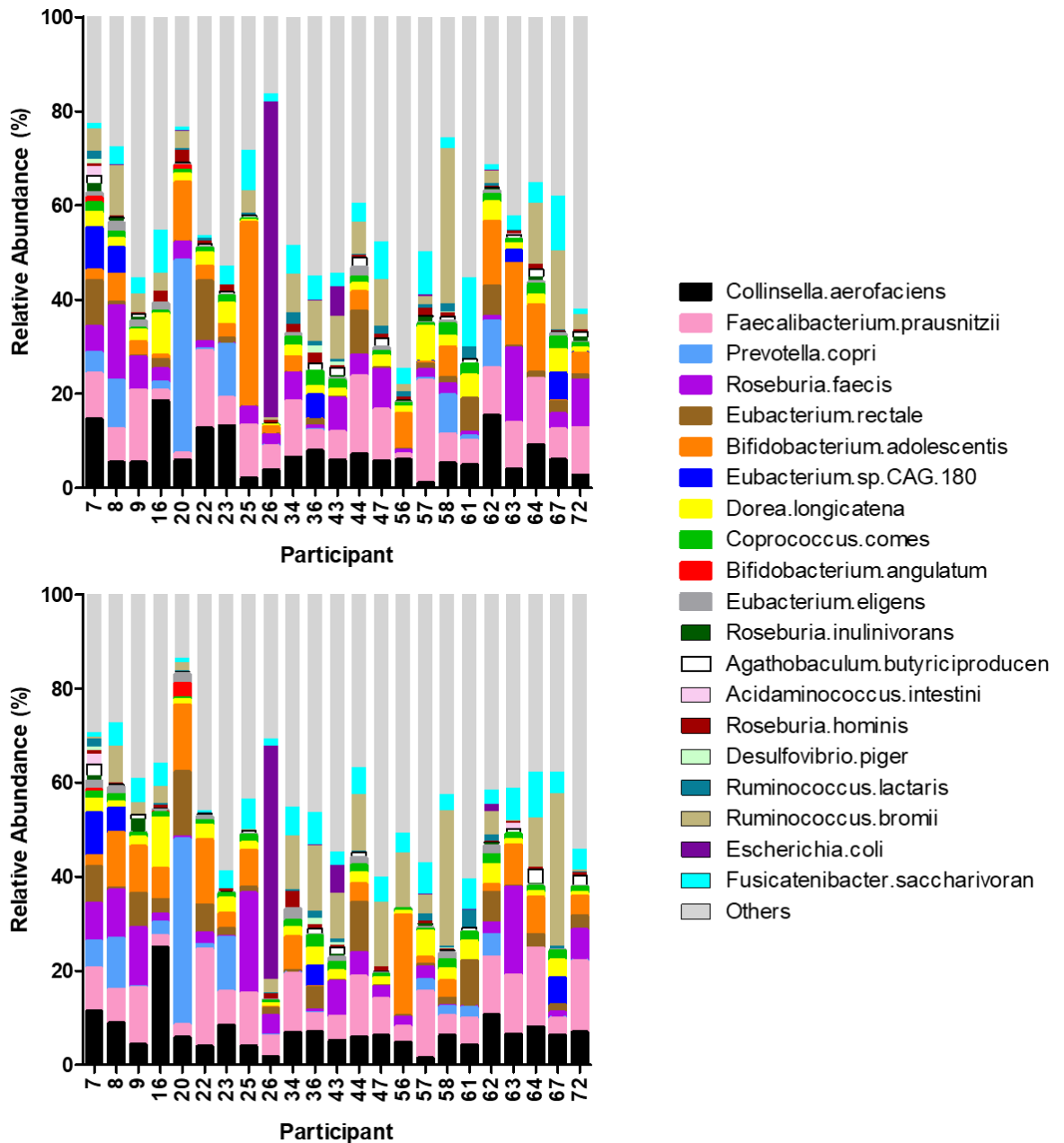
Figure 5-12- Top 20 species pre- and post- bilberry extract

Relative abundance (%) of the top 20 microbial species identified in metagenomics samples pre-bilberry extract (top) and post-bilberry extract (bottom), data is shown per participant.

The Shannon Diversity Index was calculated for all samples and is shown in Figure 5-13. No clear diversity changes were observed following any of the study treatments. Although some participants exhibited large diversity changes in some instances, i.e. BERI 47 had a large reduction in diversity post-placebo whilst BERI 25 and BERI 26 had a large increase in diversity following bilberry anthocyanin extract consumption, these changes were not consistent across the study population and therefore are unlikely to be a result of the treatments given. BERI 26 consistently had the lowest Shannon Diversity Index, however, this is not surprising given the extremely large relative abundance of E.coli in the gut of this volunteer observed in all 6 time-points of the study, as shown

in Figure 5-9. Furthermore, the medians across all 6 time-points (pre-and post- all treatment phases) were very consistent and indicate that none of the treatments administered exerted any consistent effect on gut microbial diversity, shown in Figure 5-13B.
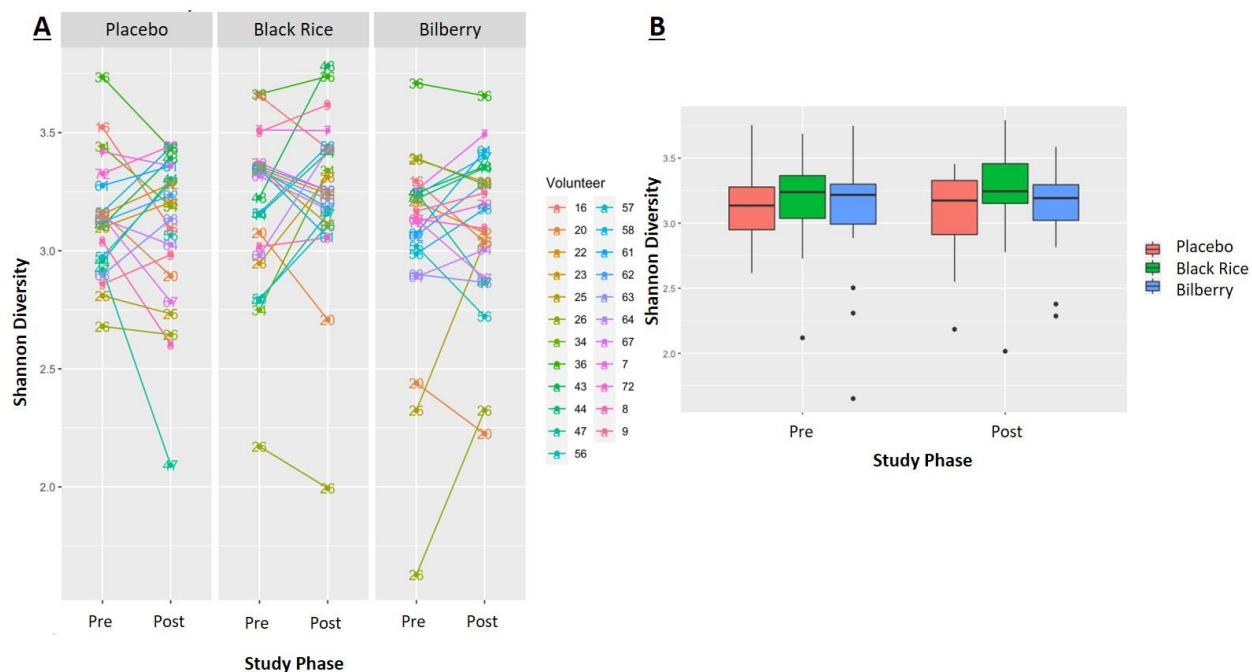
Figure 5-13- Shannon Diversity Index.
A: Shannon Diversity changes per participant pre- and post- intervention for all three phases of the study: placebo, black rice and bilberry extract. Data points are colour coded according to the volunteer from which they were derived as described by the key on the right of the figure. B: Box plots showing the median Shannon diversity pre- and post- each treatment with 25% and 75% quantiles, data that exceeds 1.5 X the interquartile range are represented by individual data points.

Following the lack of effects seen in the microbiota profiles of the study population after consumption of either bilberry or black rice anthocyanins, MaAsLin2 was employed to look for microbiome multivariate association with linear models, taking into account random effects of the volunteer from which the sample was derived. No statistically significant changes were detected, although, some patterns could be highlighted with this approach. For example, the median relative abundance of *Bifidobacterium longum* appeared to be slightly elevated post bilberry extract consumption (Figure 5-14), whilst the mean of *Bifidobacterium adolescentis* appeared slightly decreased by black rice anthocyanin consumption (Figure 5-15).
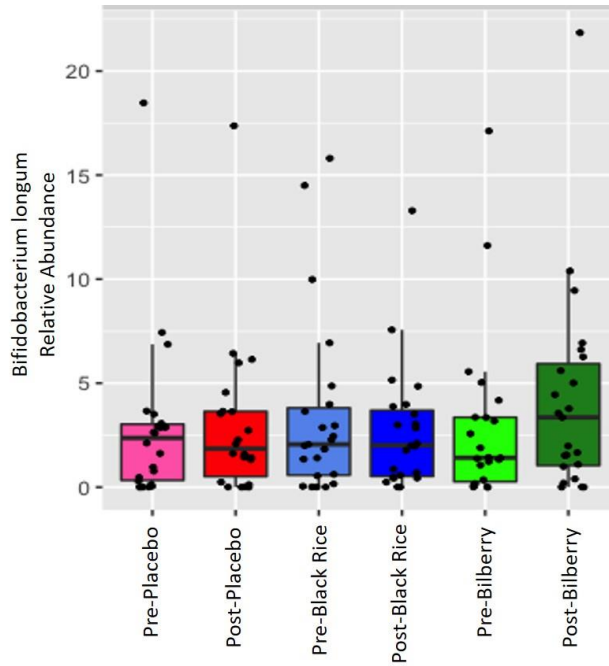
Figure 5-14- Box plots for the relative abundance of Bifidobacterium longum
Box plots produced using MaAslin2 for the relative abundance of Bifidobacterium longum in all metagenomics samples (n = 136) for each phase of the human intervention study: pre-placebo (n = 22), post placebo (n = 22), pre-black rice (n = 23), post-black rice (n = 23), pre-bilberry (n = 23) and post-bilberry (n= 23). The 25% and 75% quantiles are shown; data that exceeds 1.5 X the interquartile range are represented by individual data points beyond the whiskers.
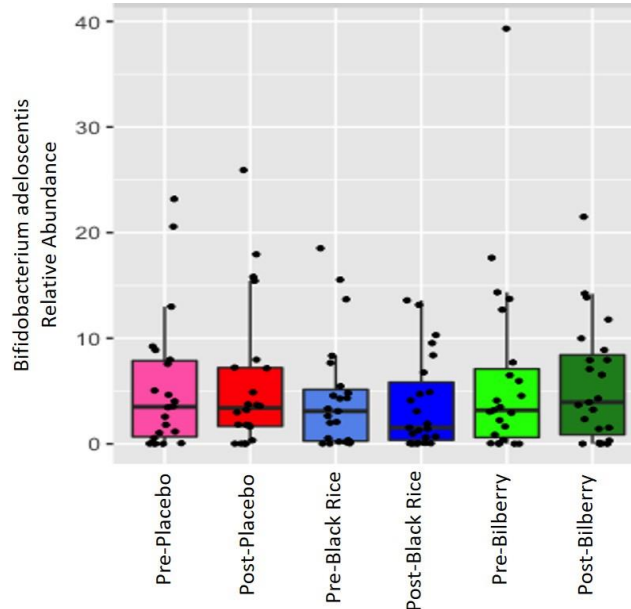
Figure 5-15- Box plots for the relative abundance of Bifidobacterium adolescentis
Box plots produced using MaAslin2 for the relative abundance of Bifidobacterium adolescentis in all metagenomics samples (n = 136) for each phase of the human intervention study: pre-placebo (n = 22), post placebo (n = 22), pre-black rice (n = 23), post-black rice (n = 23), pre-bilberry (n = 23) and post-bilberry (n= 23). The 25% and 75% quantiles are shown; data that exceeds 1.5 X the interquartile range are represented by individual data points beyond the whiskers.

Although MaAslin2 exposed some potential minor changes in the microbiota of the study population, these were very small and not across the whole study population, consequently there was not enough evidence to conclude that anthocyanins were responsible for these changes. The common factor observed in all of the data presented in this chapter is that inter-individual variation in the study population creates a large spread in data which makes it extremely difficult to establish if there is any overall effect of anthocyanin consumption on gut microbiota profiles, or if changes seen are merely artefacts of natural variation according to lifestyle factors outside of the study.

Given the lack of effects observed the same approach was adopted as was taken for 16S data, taking CLR transformed data and applying a multivariate sPLS-DA to assess whether any small effects of bilberry or black rice extract consumption could be seen when taking into account the study design. Given the increased complexity of the metagenomics data compared to 16S three filters were trialled for this analysis. Species were filtered to 147, 85, or 33 taxa with the criteria being that included taxa accounted for: at least 0.1% in 6 samples, 0.5% in 8 samples, or 1% in 22 samples; this accounted for 93 – 99.9, 84.3 – 99.2 and 27.4 – 95.2% of the original coverage respectively.

When species were filtered to include just 33 species there was some evidence for separation between metagenomic profiles of pre- (*_1_C) and post-bilberry extract (*_29_C) consumption samples. Species on axis 2 *(*Figure 5-16*)* that appeared to contribute to this separation were *Roseburia hominis* and *Ruminococcus birculans* which were both decreased after bilberry anthocyanin extract consumption; whilst *Anaerostipes hadrus* and *Eubacterium rectale* were both increased after bilberry extract consumption. Box plots for all species highlighted in this analysis can be found in appendix 8.
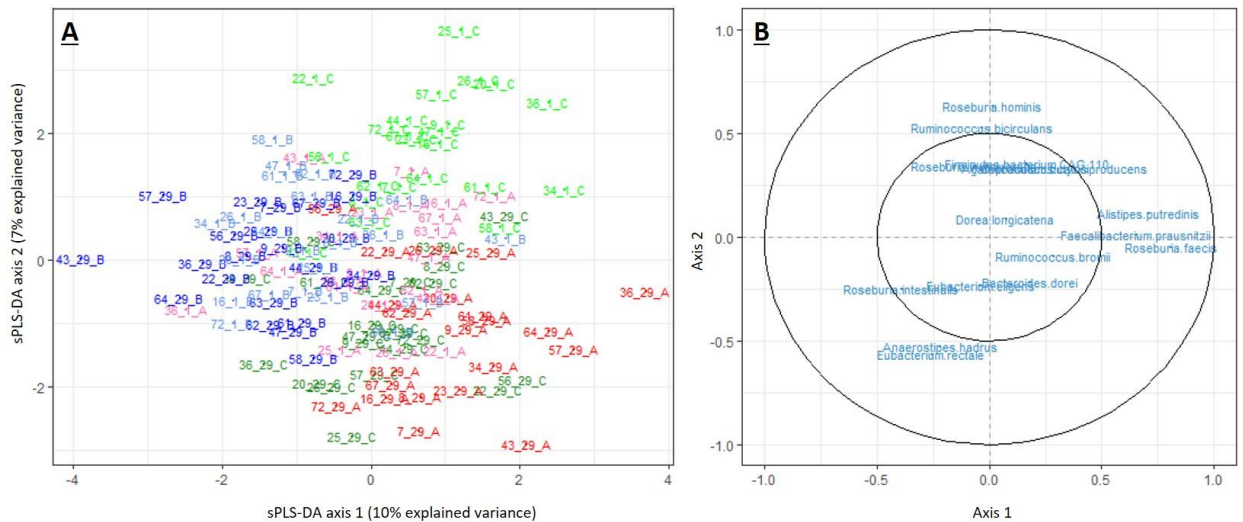
Figure 5-16- Multilevel sPLS-DA on metagenomics CLR-transformed data filtered to 33 taxa
A: Scores plot from multilevel sPLS-DA on metagenomics CLR-transformed data filtered to 33 taxa described by axes 1 and 2 (criterion 'taxa account for at least 1% in 22 samples) The summed relative abundance that was captured was between 27.4 – 95.2% of the original coverage. Data is shown as participant-ID_study-day_treatment, where 1 is pre-treatment and 29 is post-treatment; A corresponds to placebo, B to black rice and C to bilberry. Pink shows pre-placebo, red shows post-placebo, light blue shows pre-black rice, dark blue shows post-black rice, light green shows pre-bilberry and dark green shows post-bilberry. Samples that are closest together had more similar metagenomic profiles. B: Correlation circle highlighting which taxa may be relevant to the separation of samples in the scores plot on the left.

When species were filtered to include 85 species tuning suggested that the data was best described by 3 axes rather than 2. When taking the scores plotted on axes 1 and 2 there was evidence of separation between metagenomic profiles pre- (*_1_B) and post- black rice (*_29_B) consumption. On axes 2 and 3 there was also evidence of separation between metagenomic profiles pre- (*_1_C) and post- bilberry (*_29_C) consumption; furthermore, interestingly post bilberry and black rice samples are clustered with one another on the scores plot in Figure 5-18A. The species that appeared to be involved in the separation of post-black rice extract consumption samples on axis 2 included *Blautia obeum* and *Eubacterium ramulus* which were both decreased post-black rice extract consumption. Based on axis 3, *Roseburia hominis* and *Ruminococcus birculans* appeared decreased and *Anaerostipes hadrus, Eubacterium rectale* and *Parabacteroides distasonis* appeared increased after bilberry extract consumption (Figure 5-18). Box plots for all species highlighted in this analysis can be found in appendix 8.

Figure 5-17- Multilevel sPLS-DA on metagenomics CLR-transformed data filtered to 85 taxa (Axes 1 and 2)
A: Scores plot from multilevel sPLS-DA on metagenomics CLR-transformed data filtered to 85 taxa described by axes 1 and 2 (criterion 'taxa account for at least 0.5% in 8 samples) The summed relative abundance that was captured was between 84.3 – 99.2% of the original coverage. Data is shown as participant-ID_study-day_treatment, where 1 is pre-treatment and 29 is post-treatment; A corresponds to placebo, B to black rice and C to bilberry. Pink shows pre-placebo, red shows post-placebo, light blue shows pre-black rice, dark blue shows post-black rice, light green shows pre-bilberry and dark green shows post-bilberry. Samples that are closest together had more similar metagenomic profiles. B: Correlation circle highlighting which taxa may be relevant to the separation of samples in the scores plot on the left.

Figure 5-18- Multilevel sPLS-DA on metagenomics CLR-transformed data filtered to 85 taxa (Axes 2 and 3)
A: Scores plot from multilevel sPLS-DA on metagenomics CLR-transformed data filtered to 85 taxa described by axes 2 and 3 (criterion 'taxa account for at least 0.5% in 8 samples) The summed relative abundance that was captured was between 84.3 – 99.2% of the original coverage. Data is shown as participant-ID_study-day_treatment, where 1 is pre-treatment and 29 is post-treatment; A corresponds to placebo, B to black rice and C to bilberry. Pink shows pre-placebo, red shows post-placebo, light blue shows pre-black rice, dark blue shows post-black rice, light green shows pre-bilberry and dark green shows post-bilberry. Samples that are closest together had more similar metagenomic profiles. B: Correlation circle highlighting which taxa may be relevant to the separation of samples in the scores plot on the left.

When samples were filtered to include 147 species, the data was best described by axes 1 and 2

and there was clear separation between metagenomic profiles pre- (*_1_B) and post-black rice

extract (*_29_B), but no evidence of separation pre- (*_1_C) and post-bilberry extract (*_29_C) consumption, shown in Figure 5-19. Species that stood out on axis 1 as contributing to this separation were *Roseburia faecis* and *Phascolarctobacterium succinatutens* which were both decreased after black rice anthocyanin extract consumption. Looking at axis 2 highlighted that *Alstipes indistinctus, Parabacteroides merdae, Oscilibacter.sp.CAG.241* and *Flavonifractor plauti* were all increased after black rice consumption and *Eubacterium ramulus, Firmicutes bacterium CAG 95,* and *Blautia obeum* were decreased after black rice extract consumption. Box plots for all species highlighted in this analysis can be found in appendix 8.
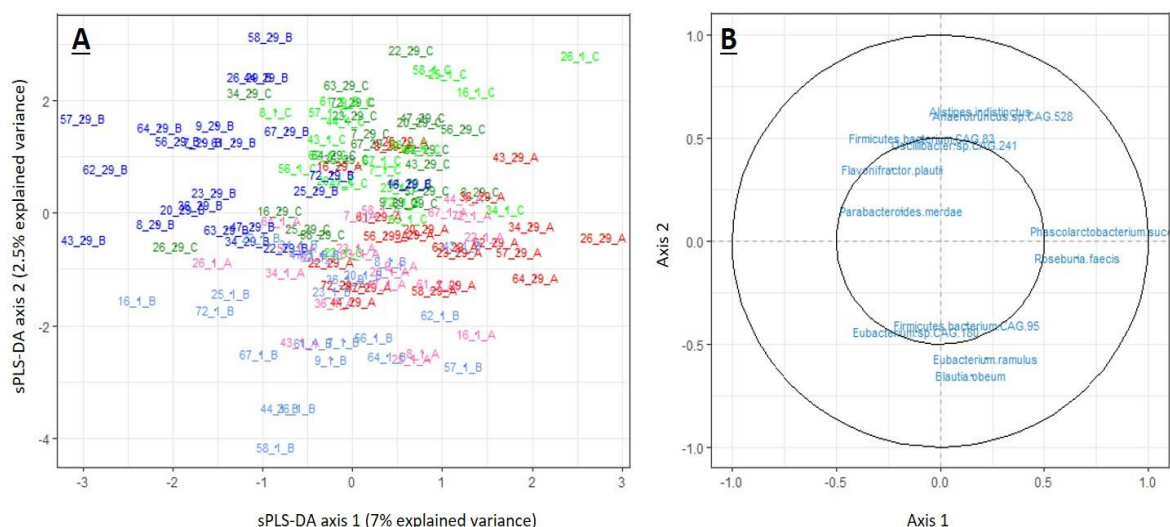
Figure 5-19- Multilevel sPLS-DA on metagenomics CLR-transformed data filtered to 147 taxa
A: Scores plot from multilevel sPLS-DA on metagenomics CLR-transformed data filtered to 147 taxa described by axes 1 and 2 (criterion 'taxa account for at least 0.1% in 6 samples) The summed relative abundance that was captured was between 93 – 99.9% of the original coverage. Data is shown as participant-ID_study-day_treatment, where 1 is pre-treatment and 29 is post-treatment; A corresponds to placebo, B to black rice and C to bilberry. Pink shows pre-placebo, red shows post-placebo, light blue shows pre-black rice, dark blue shows post-black rice, light green shows pre-bilberry and dark green shows post-bilberry Samples that are closest together had more similar metagenomic profiles. B: Correlation circle highlighting which taxa may be relevant to the separation of samples in the scores plot on the left.

Although multi-level sPLS-DA analysis uncovered some taxa that differentiated post bilberry and black rice metagenomic profiles these differences were very subtle and did not constitute clear characteristics of the microbiome that could be conclusively associated with consumption of either anthocyanin source. The subtlety of the changes in the taxa identified in this multivariate analysis is best depicted in the box-plots shown in appendix 8. It is likely that high inter-individual variation in combination with modest changes in the relative abundance of taxa made it difficult to establish any clear patterns in microbial changes in the study population in response to either anthocyanin treatment. If the study population had more similar microbiome profiles prior to the intervention,

it would have been easier to establish whether the anthocyanin extracts given were able to exert any effect on these profiles.

5.5.3 Is there a Relationship Between *In Vitro* Microbial Anthocyanin Metabolism and Gut Microbiota Profiles?

Data from *in vitro* fermentation experiments (Chapter 4) was analysed alongside gut microbiota profiles to establish if any links could be made between these datasets. A number of different metabolites were found at different concentrations during *in vitro* fermentation between different samples, and it was shown that many metabolites were only produced microbially and not spontaneously. Chapter 4 showed that people behaved differently in terms of their overall microbial metabolism of black rice derived C3G. Therefore, it was hypothesised that these differences between individuals could be explained by their microbiome profiles. Figure 5-20 shows a heat map of the top 50 most abundant species identified in metagenomic profiles, clustered according to their *in vitro* microbial metabolism of black rice anthocyanins in the *in vitro* fermentation colon model (taking all detected compounds into account, using the groupings from the DTW data presented in Chapter 4). There are no clear differences between groups in terms of the profiles of species present, suggesting a lack of association between microbial species and overall microbial anthocyanin metabolism. The Shannon diversity index was also studied in relation to anthocyanin metabolism in the *in vitro* fermentation colon model, but no clear differences could be seen between participants who metabolised anthocyanins differently in terms of the diversity of their gut microbiota and therefore this data is not shown.
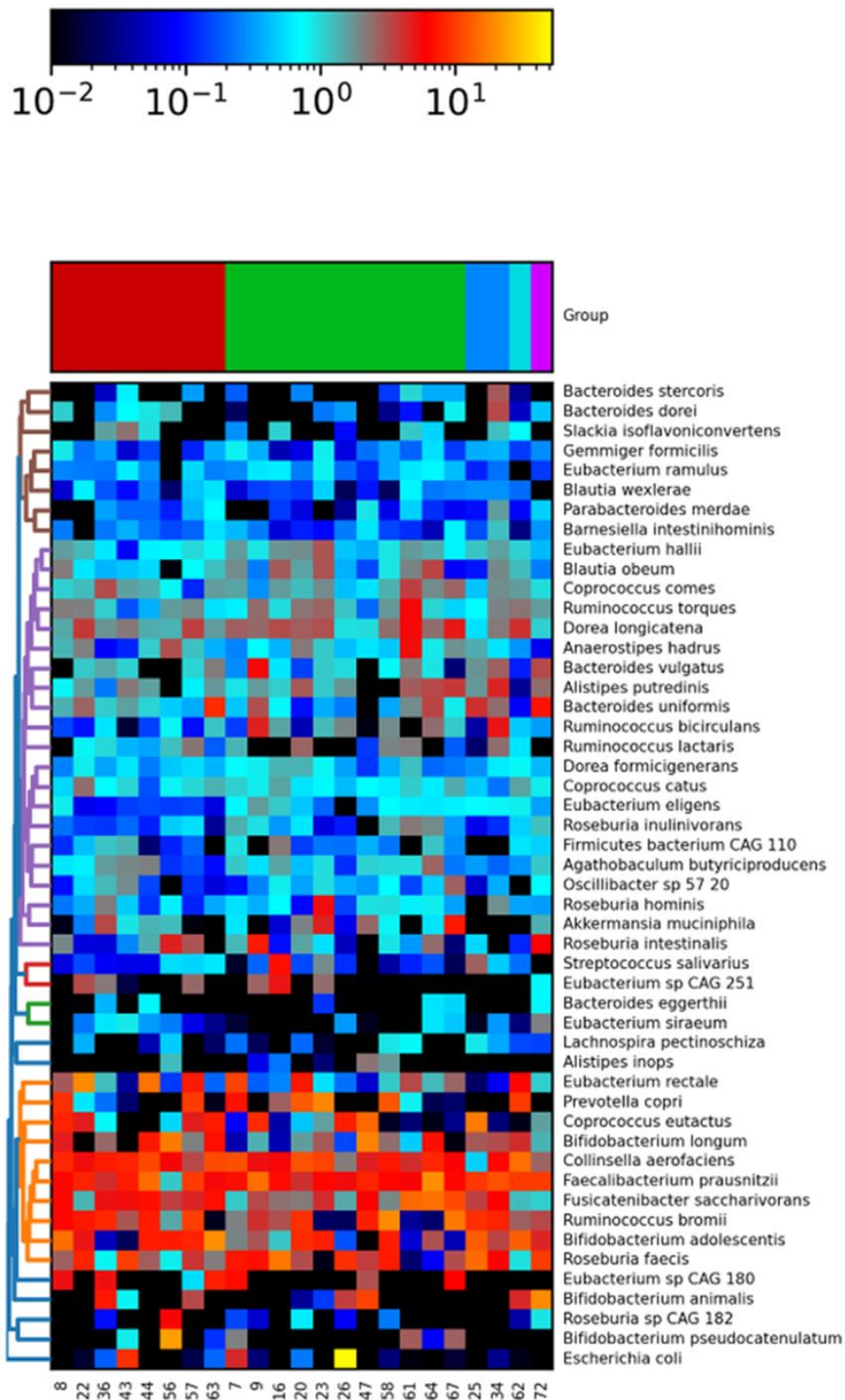
Figure 5-20- Relative abundance heatmap of the top 50 species in metagenomics data grouped according to their in vitro microbial metabolism of black rice anthocyanins in the colon model

Relative abundance heatmap for the taxonomic profiles of metagenomics samples subjected to in vitro fermentation of black rice anthocyanin extract (n = 23). Heatmap produced with hierarchical clustering (samples clustered according to their microbial metabolism of black rice anthocyanin extract (from DTW data presented in Chapter 4)) for the top 50 species using Bray-Curtis distance measures for samples and species. The heatmap describes the relative abundance of each species in faeces standardised the range of $[10^{-1}, 10^{2}]$. Yellow-red colour indicates high amounts of the compound were detected and black-blue indicates only low levels were present.

Given that no clear differences were observed in the metagenomic profiles of individuals according to their overall metabolism of C3G during the *in vitro* fermentation colon model, deeper analysis was required to establish if there were any relationships between the two data sets. Therefore, multivariate analysis by means of a sparse canonical correlation analysis (CCA) was performed to identify any variables that may explain a relationship between *in vitro* microbial metabolism (colon model) of C3G and either 16S genus level data or metagenomic species level data. For the colon model data, a large number of variables were included in the analysis, including the overall metabolic behaviour of each compound but also specifically taking into account factors such as time first produced, Cmax and Tmax so that this dataset could be considered in relation to gut microbial profiles in multiple ways. Furthermore, 16S and metagenomic sequencing both also consisted of a large number of variables with many genera and species making up these datasets respectively. Therefore, due to the large number of variables in both the sequencing and colon model datasets a tuning step was carried out to determine the number of variables that should be taken forward in each dataset to provide the best correlation, this was first performed for the 16S data in relation to the colon model data. Tuning showed that models based on a very low number of variables led to a lack of linearity between colon model and 16S sequencing CCA scores. However, linearity improved in models when more variables were taken into account with R^2 values of ~ 0.8 between 16S and CM scores on the first CCA axis, when at least 10 variables from each dataset were included. Based on this, the results were visualised for 10, 15 and 30 variables on each axis. To validate the observed results a permutation test was performed with the same data, whereby the datasets were matched randomly (rather than according to volunteer) 500 times to assess how likely it was that the results obtained would occur by chance. Where 10, 15 or 30 variables were taken into account there was a 21, 25 and 13% probability respectively of obtaining the same correlation coefficient on axis 1 by chance. Anything over 10% was considered particularly high and therefore these results are not shown. However, if 30 variables were taken into account for the colon model and 40 variables from the 16S data then this reduced the probability of obtaining the same correlation coefficient by chance to 10%. The canonical correlation analysis (CCA) scores plot can be seen for axis 1 and 2 in Figure 5-21, and the correlation circles highlighting the variables that define these axes are shown in Figure 5-22. For the variables that most strongly define a relationship between the data sets, a heat-map is shown in Figure 5-23 for the visualisation of these specific relationships.
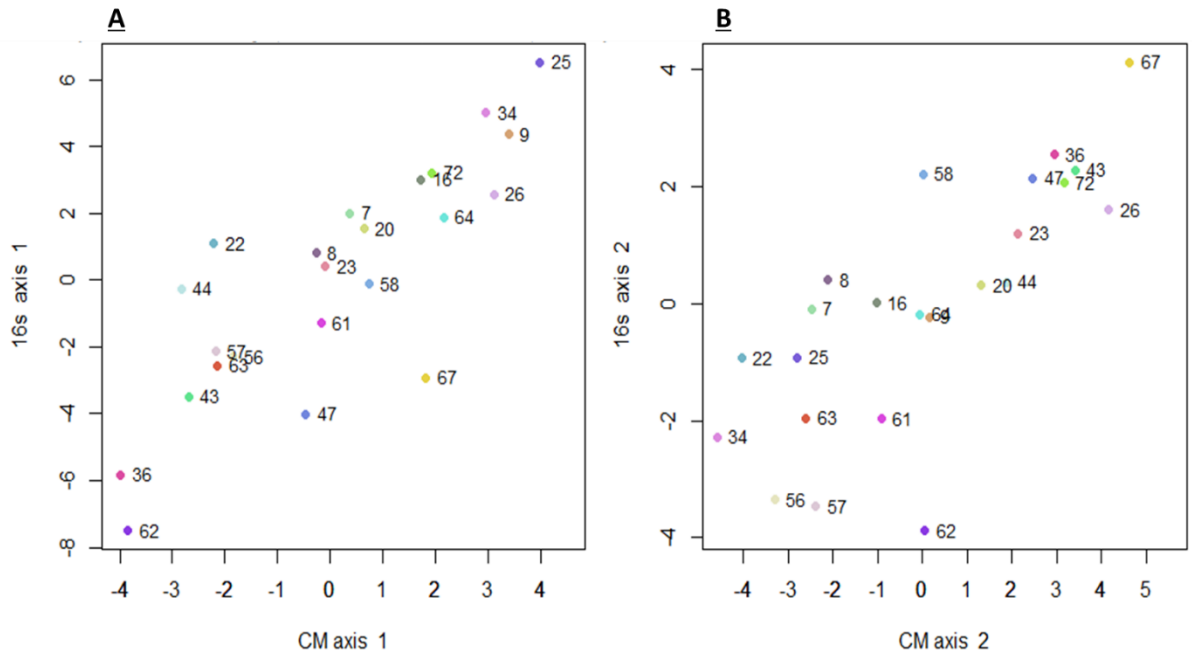
Figure 5-21- Canonical correlation analysis (CCA) scores plots for colon model and 16S data.
A: CCA scores on axis 1 for 30 colon model (CM) variables and 40 16S variables. B: CCA scores on axis 2 for 30 colon model (CM) variables and 40 16S variables. Data points are labelled according the volunteer they represent.
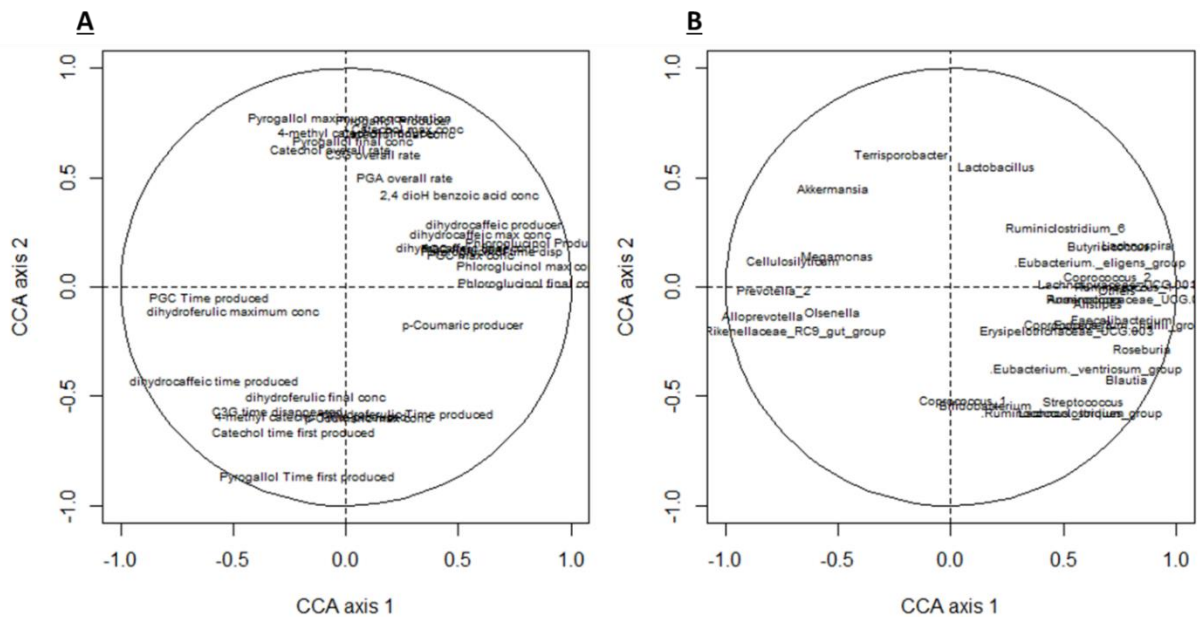
Figure 5-22- Correlation circles highlighting the variables that define each axis from canonical correlation analysis of 16S and colon model data.
A: The colon model (CM) variables that define axis 1 and 2. B: The 16S variables that define axis 1 and 2. Note that CM and 16S variables have been shown separately to improve readability but otherwise would be shown on the same plot.

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Figure 5-23- Heat-map of the relationships between variables identified in canonical correlation analysis of colon model and 16S data.

Heat-map visualising the relationships between the most strongly correlated variables (correlation scalar product ≥ 0.4) from canonical correlation analysis between the colon model data and 16S genera. Hierarchical clustering of the variables was carried out to show the similarity in the genera (top) and similarity in the behaviour of compounds measured in the colon model (left). The heat-map describes the relationship between variables standardised to the range of [-0.62, +0.62]. Red colour indicates a strong positive correlation between variables and blue indicates a strong negative relationship between variables. Yellow indicated no correlation between variables.

Canonical correlation analysis showed a relationship between several variables from the colon model and 16S data. Notably a high abundance of *Blautia, Roseburia, Ruminoclostridium_6, Anaerostipes, Faecalibacterium, Eubacterium_halli_group, Alistipes, Ruminococcaceae_UCG.013, Butyricicoccus, Lachnospira, Ruminococcus_1, Lachnospiraceae_UCG.001, Eubacterium_eligens_group* and *Coprococcus_2* were all positively associated with the production of phloroglucinol and dihydrocaffeic acid in the colon model. These genera were associated with not only whether or not faecal samples derived from a participant produced these metabolites but also the concentration that was observed. However, it should be noted that these genera were more strongly associated with phloroglucinol production than dihydrocaffeic acid.

Akkermansia and *Terrisporobacter* were both positively associated with the production of pyrogallol, 4-methyl catechol, catechol, and overall C3G metabolism. Whereas *Coprococcus_1,*

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Bifidobacterium, Lachnoclostiridium, Ruminococcus_torques_group, and *Streptococcus* were positively associated with the time at which pyrogallol was first detected in the colon model but not its concentration, highlighting that different taxa may be associated with different metabolic parameters of the same compound. Similarly, *Alloprevotella, Rilenellaceae_RC9_gut_group, Prevotella_2* and *Cellulosilyticum* were all positively associated with the time at which PGC was first detected in the colon model but not with its concentration.

Further positive associations were observed whereby *Coprococcus_1, Bifidobacterium, Lachnoclostiridium, Ruminococcus_torques_group, Streptococcus, Blautia, Roseburia, Faecalibacterium, Eubacterium_halli_group, Alistipes,* and *Ruminococcaceae_UCG.013* were all positively associated with the Cmax of p-coumaric acid and time at which dihydroferulic acid was first detected in the colon model.

Negative associations were also observed with several metabolic parameters from the *in vitro* colon model and microbial genera. In particular, there was a strong negative association between the abundance of *Alloprevotella* and the production of phloroglucinol. Phloroglucinol production was also negatively associated with the abundance of *Rilenellaceae_RC9_gut_group* and *Prevotella_2* but to a lesser extent. These negative associations suggest that these genera may be important in the biotransformation of phloroglucinol to other compounds.

The next step was to complete the same multivaraite cannonical correlation analysis for the metagenomic and colon model data to see if relationships were also observed at the species level. Tuning was carried out as decribed previously to establish the number of variables to include for each dataset in CCA. Contrary to what was observed with the 16S data, in this instance the probability of obtaining a similar correlation by chance was lower when less variables were included. Therefore the results were visualised when taking into account 10 variables from each dataset. The canonical correlation analysis (CCA) scores plot can be seen for axis 1 and 2 in Figure 5-24, and the correlation circle highlighting the variables that define these axes is shown in Figure 5-25. For the variables that most strongly define a relationship between the data sets, a heat-map is shown in Figure 5-26 for the visualisation of these specific relationships.
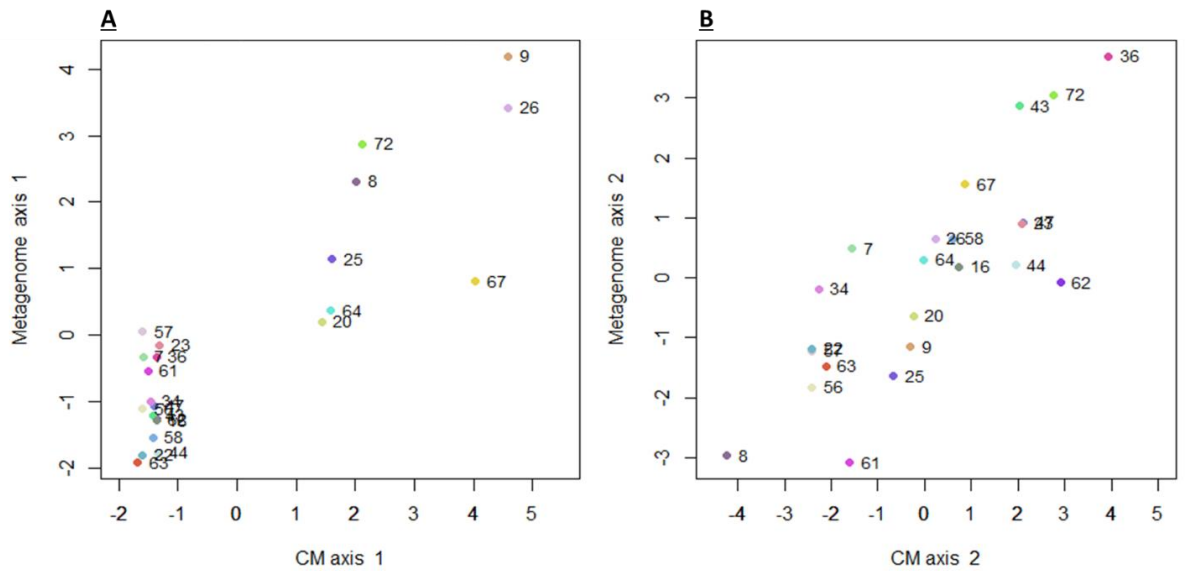
Figure 5-24- Canonical correlation analysis (CCA) scores plots for colon model and metagenomics data.
A: CCA scores on axis 1 for 10 colon model (CM) variables and 10 metagenome variables. B: CCA scores on axis 2 for 10 colon model (CM) variables and 10 metagenome variables. Data points are labelled according the volunteer they represent.
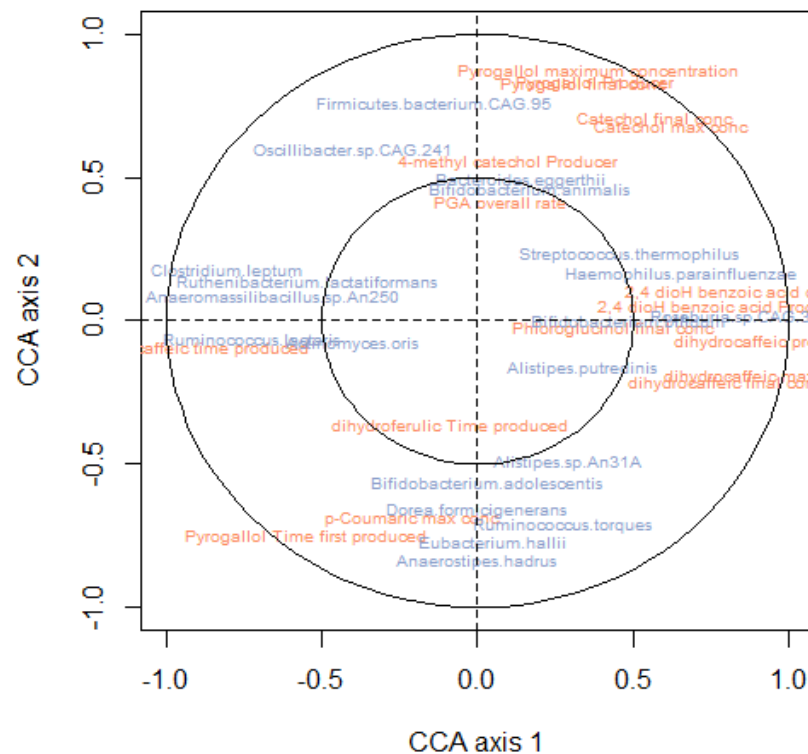
Figure 5-25- Correlation circle highlighting the variables that define each axis in CCA of colon model and metagenomics data.
Purple highlights variables from the metagenomics data and orange highlights variables from the colon model data.
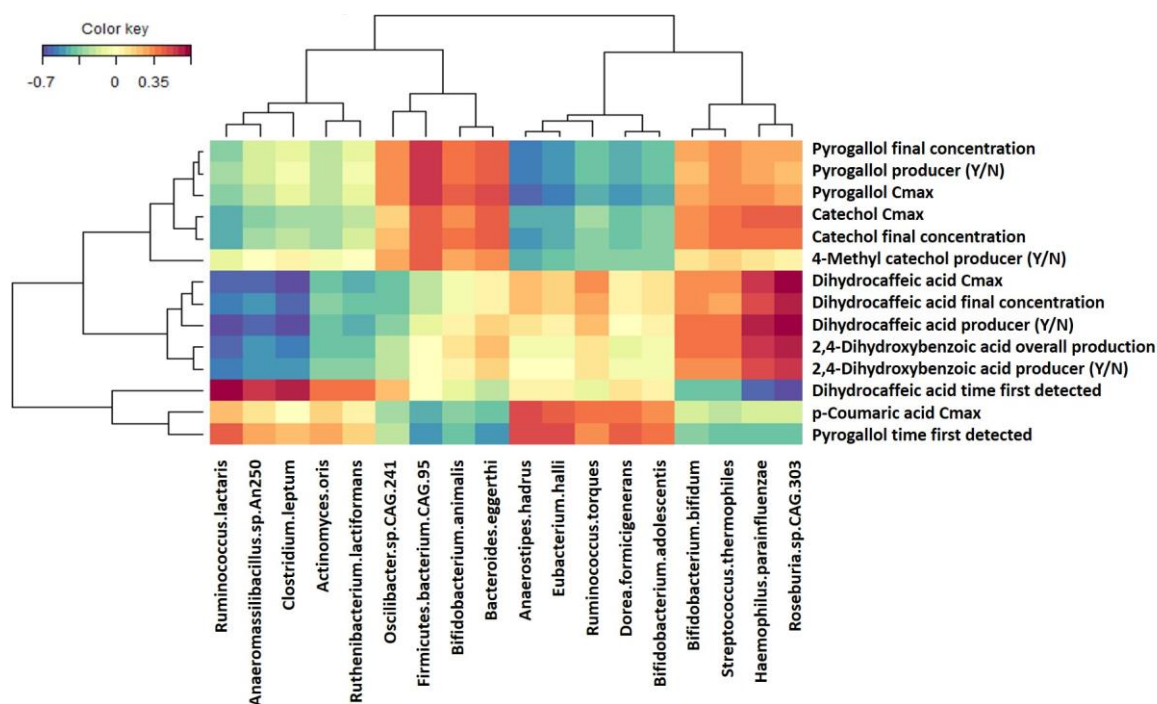
Figure 5-26- Heat-map showing the relationships identified in canonical correlation analysis of colon model and metagenomics data

Heat-map visualising the relationships between the most strongly correlated variables (correlation scalar product ≥ 0.4) from canonical correlation analysis between the colon model and metagenomics data. Hierarchical clustering of the variables was carried out to show the similarity in the species (top) and similarity in the behaviour of compounds measured in the colon model (left) between samples. The heat-map describes the relationship between variables standardised to the range of [-0.7, +0.7]. Red colour indicates a strong positive correlation between variables and blue indicates a strong negative relationship between variables. Yellow indicates no correlation between variables.

Canonical correlation analysis showed a relationship between several variables from the colon model and metagenomics data. A positive association was observed between the abundance of *Firmicutes bacterium.CAG.95, Bifidobacterium animalis, Bacteroides eggerthi, Streptococcus thermophiles, Haemophilus parainfluenzae, Roseburia sp.CAG.303* and the production of pyrogallol and catechol in the colon model, with *Firmicutes bacterium.CAG.95* giving the strongest association, notably *Firmicutes bacterium.CAG.95* was also positively associated with 4-methyl catechol production. Strikingly, dihydrocaffeic production in the *in vitro* colon model from black rice anthocyanins was negatively associated with *Ruminococcus lactaris, Anaeromassilibacillus sp.An250,* and *Clostridium leptum*. Meanwhile, the production of dihydrocaffeic acid showed a positive association with *Haemophilus parainfluenzae* and *Roseburia sp.CAG.303*. These species were also positively correlated with the production of 2,4-dihydroxybenzoic acid in the colon model, however, this association is only based on 4 samples producing this compound versus 19 not producing it, meaning that this result may be spurious and would require further investigation. *Anaerostipes hadrus, Eubacterium halli, Ruminococcus torques, Dorea formicigenerans,* and *Bifidobacterium adolescentis* were all positively associated with the Cmax of p-coumaric acid in the

colon model as well as the time at which pyrogallol was first detected (with *Anaerostipes hadrus, Eubacterium halli* showing the strongest association), however, this relationship was not observed with the concentration of pyrogallol in the colon model.

Overall, multivariate canonical correlation analysis showed that there were some relationships between gut microbes (at both the genus and species level) and the *in vitro* microbial metabolism of C3G in the colon model. Relationships were only observed with parameters concerning some of the compounds measured in the colon model, and not for others such as PCA and PGA, most likely due to compounds such as this not being exclusively microbially derived. Nevertheless, there were clearly some links between these datasets.

5.5.4 Relationships Between *In Vivo* Anthocyanin Metabolism and the Gut Microbiota

In addition to *in vitro* microbial metabolism of anthocyanins (Chapter 4), *in vivo* metabolism of both black rice and bilberry anthocyanin extracts was investigated in Chapter 3. This *in vivo* metabolism showed high inter-individual variation in not only the metabolites produced but also their relative concentrations. It was hypothesised that this inter-individual variation was at least in part due to differences in the gut microbiota profiles of individuals. Therefore, this data was analysed using the same multivariate canonical correlation model, as was used for *in vitro* fermentation colon model data, to look for relationships with microbiome profiles at both the genus (16S) and species (metagenomics) level. This analysis highlighted a number of relationships between the urinary excretion of anthocyanin metabolites and the microbiome at both the genus and species level.

Tuning was carried out as decribed previously to establish the number of variables to include for each dataset in CCA. However, the correlation coefficient between urinary metabolite and microbiome scores on axis 1 did not vary with different choices of parameters. Therefore, the analysis was performed with two choices of parameters; 15 variables on each axis for both metabolites and microbiome, and 35 and 50 variables (respectively). However, in practice when more variables were included these were not relevant and were not shown on graphs where a scalar product threshold of 0.4 was used to show only the strongest relationships. Therefore, only results where 15 variables were included for both datasets are shown here. Analysis was first performed for urinary metabolites post black rice extract consumption and 16S genus data. The canonical correlation analysis (CCA) scores plot can be seen for axis 1 and 2 in Figure 5-27, and correlation circles highlighting the variables that define these axes are shown in Figure 5-28. For the variables that most strongly define a relationship between the data sets, a heat-map is shown in Figure 5-29 for the visualisation of these specific relationships.

Figure 5-27- Canonical correlation analysis (CCA) scores plots for black rice urinary metabolite and 16S data.
A: CCA scores on axis 1 for 15 black rice urinary metabolite variables (phenolics) and 15 16S variables. B: CCA scores on axis 2 for 15 black rice urinary metabolites variables (phenolics) and 15 16S variables. Data points are labelled according the volunteer they represent.

Figure 5-28- Correlation circles highlighting the variables that define each axis from canonical correlation analysis of black rice urinary metabolite and 16S data.
A: The black rice urinary metabolite variables that define axis 1 and 2. B: The 16S variables that define axis 1 and 2. Note that black rice urinary metabolite and 16S variables have been shown separately to improve readability but otherwise would be shown on the same plot.
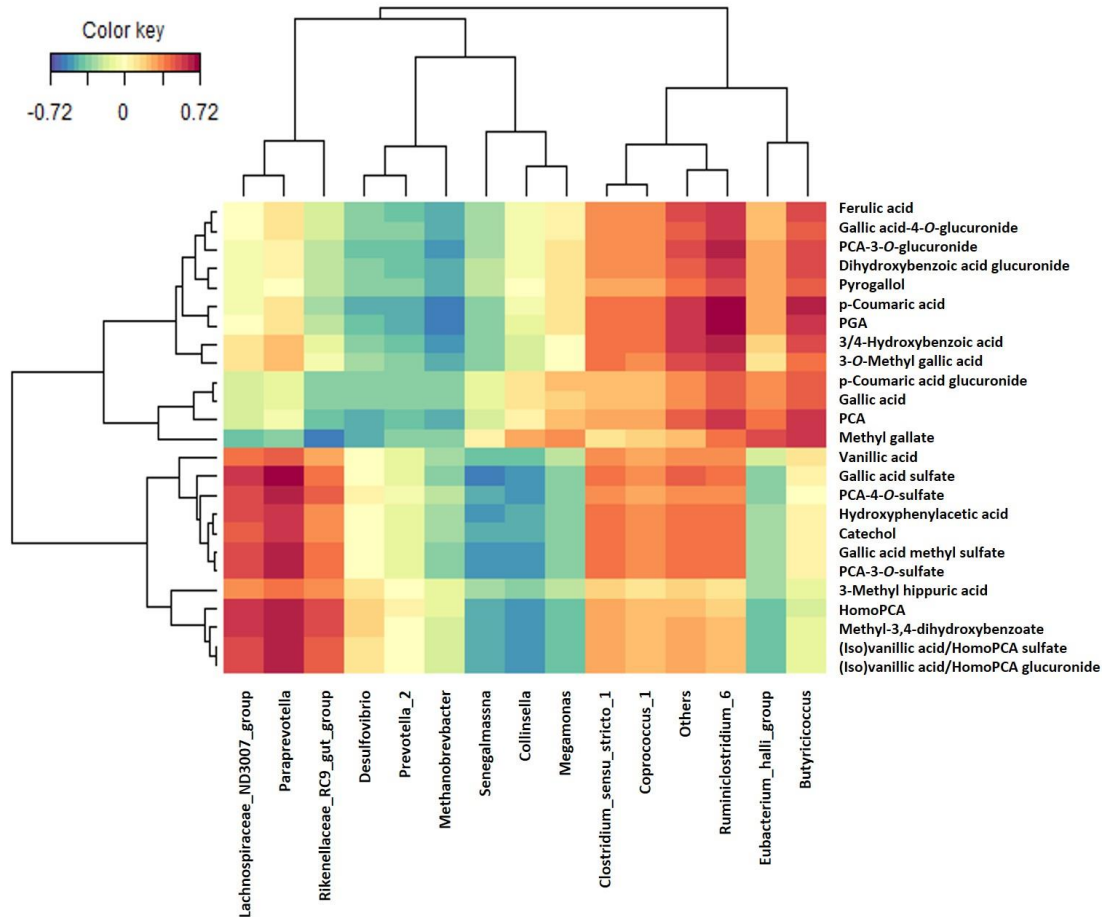
Figure 5-29- Heat-map showing relationships identified in canonical correlation analysis of black rice urinary metabolite and 16S data

Heat-map visualising the relationships between the most strongly correlated variables (correlation scalar product ≥ 0.4) from canonical correlation analysis between black rice urinary phenolics and 16S data. Hierarchical clustering of the variables was carried out to show the similarity in the species (top) and similarity in the behaviour of compounds measured in urine post black rice consumption (left) between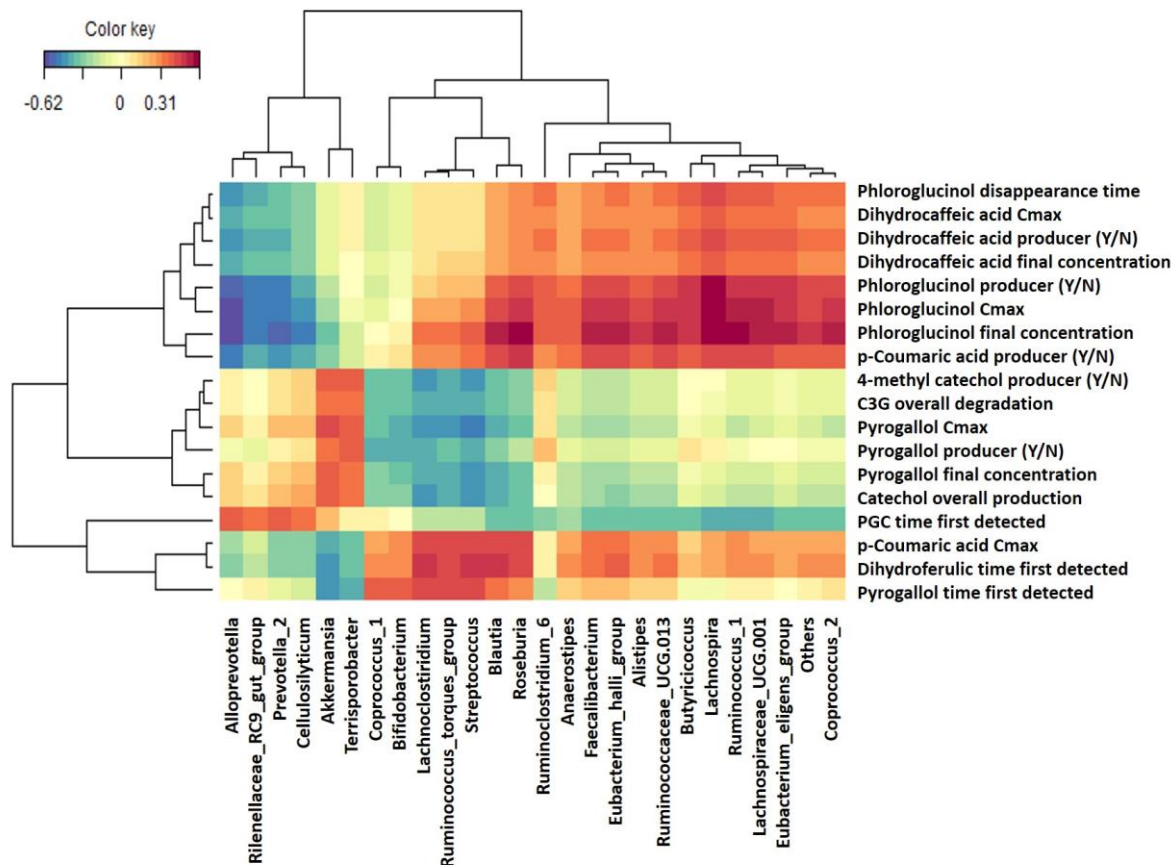 samples. The heat-map describes the relationship between variables standardised to the range of [-0.72, +0.72]. Red colour indicates a strong positive correlation between variables and blue indicates a strong negative relationship between variables. Yellow indicates no correlation between variables.

Canonical correlation analysis showed a relationship between several variables from the black rice urine metabolite and 16S data. Ferulic acid, gallic acid-4-O-glucuronide, PCA-3-O-glucuronide, dihydroxybenzoic acid glucuronide, pyrogallol, p-coumaric acid, PGA, 3/4-hydroxybenzoic acid, 3-O-methyl gallic acid, p-coumaric acid glucuronide, gallic acid, PCA and methyl gallate were all positively associated with the abundance of the genera *Ruminiclostridium_6* and *Butyricicoccus*. Meanwhile, vanillic acid, gallic acid sulfate, PCA-4-O-sulfate, hydroxyphenylacetic acid, catechol, gallic acid methyl sulfate, PCA-3-O-sulfate, homoPCA, methyl-3,4-dihydroxybenzoate, (iso)vanillic acid/homoPCA sulfate and (iso)vanillic acid/homoPCA glucuronide were all positively associated with the abundance of *Lachnospiraceae_ND3007_group* and *Paraprevotella*. Conversely, *Rikenellaceae_RC9_gut_group* was negatively associated with urinary methyl gallate excretion, and *Methanobrevbacter* was negatively associated with p-coumaric acid and PGA. *Senegalmassna* was

negatively associated with many urinary anthocyanin metabolites, but showed the strongest negative association with gallic acid sulfate. These negative associations suggest that these bacteria may have role in the biotransformation of these metabolites to further compounds.

Canonical correlation analysis for bilberry urine metabolites and 16S genus data is shown in Figures 30 - 32. The canonical correlation analysis (CCA) scores plot can be seen for axis 1 and 2 in Figure 5-30, and the correlation circle highlighting the variables that define these axes is shown in Figure 5-31. For the variables that most strongly define a relationship between the data sets (bilberry urine metabolites and 16S genera), a heat-map is shown in Figure 5-32 for the visualisation of these specific relationships.
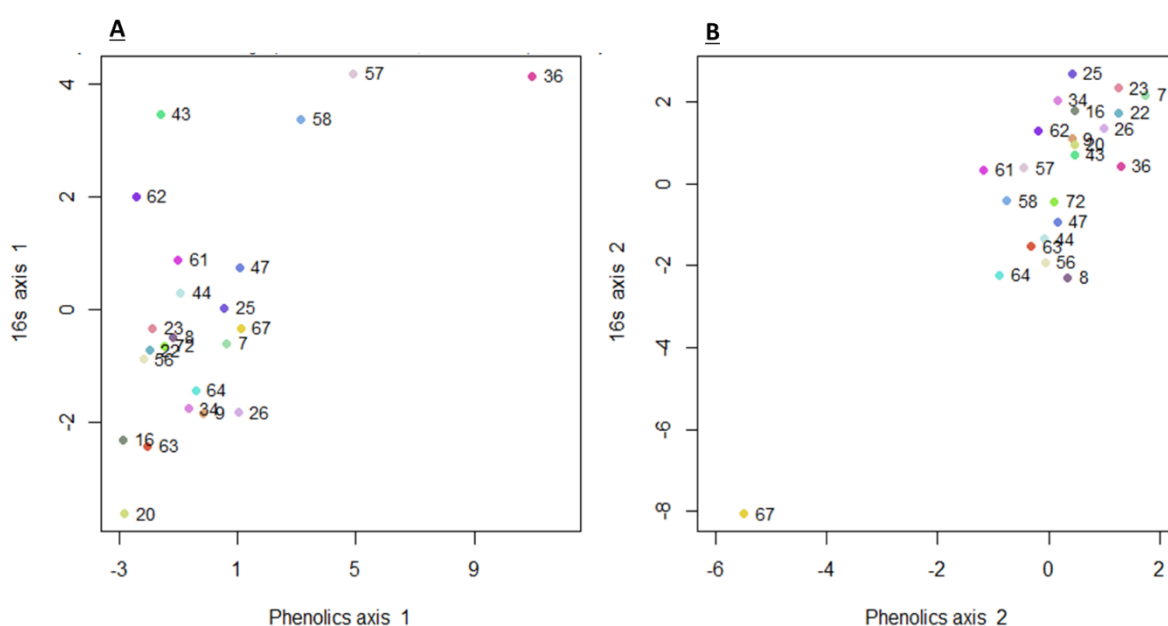
Figure 5-30- Canonical correlation analysis (CCA) scores plot for bilberry urinary metabolites and 16S data.
A: CCA scores on axis 1 for 15 bilberry urinary metabolite variables (phenolics) and 15 16S variables. B: CCA scores on axis 2 for 15 bilberry urinary metabolites variables (phenolics) and 15 16S variables. Data points are labelled according the volunteer they represent.
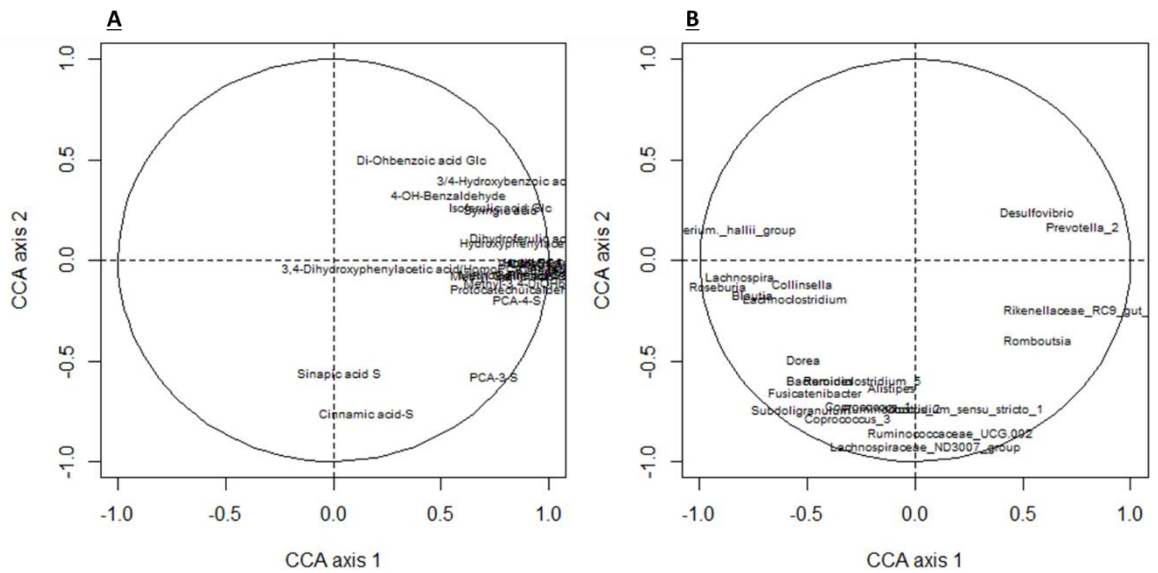
Figure 5-31- Correlation circles highlighting the variables that define each axis from canonical correlation analysis of bilberry urinary metabolite and 16S data.

A: The bilberry urinary metabolite variables that define axis 1 and 2. B: The 16S variables that define axis 1 and 2. Note that bilberry urinary metabolite and 16S variables have been shown separately to improve readability but otherwise would be shown on the same plot.
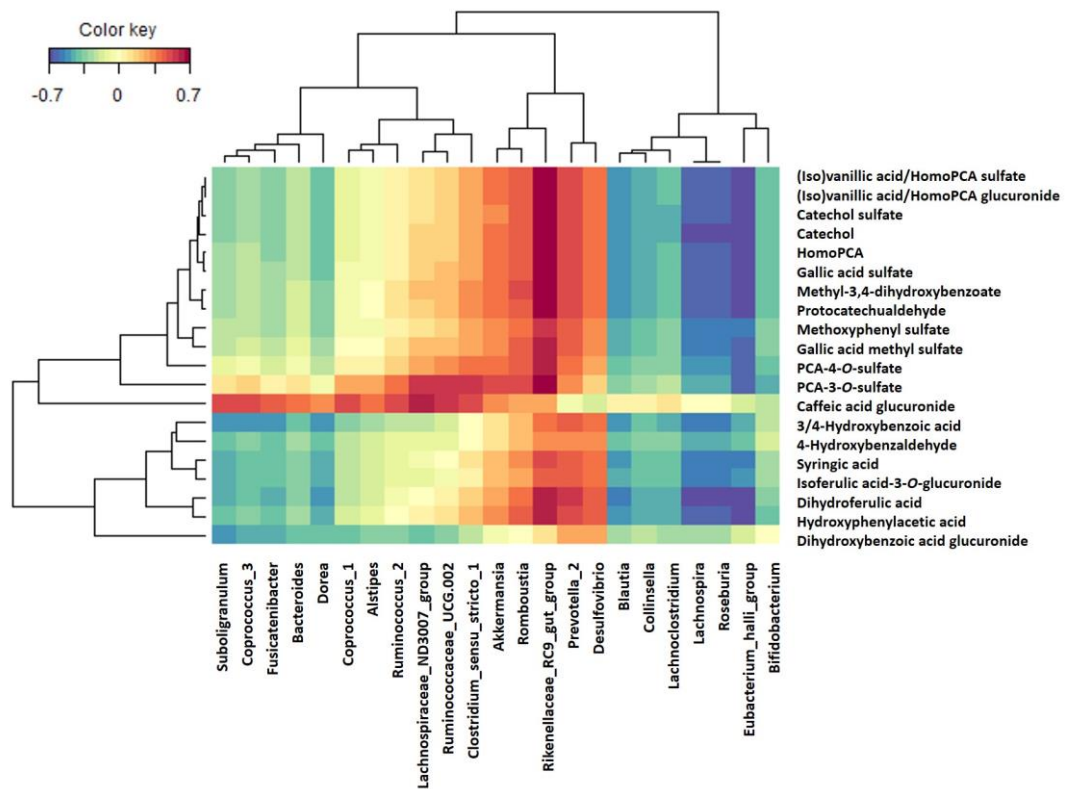
Figure 5-32- Heat-map showing relationships identified in canonical correlation analysis of bilberry urinary metabolite and 16S data

Heat-map visualising the relationships between the most strongly correlated variables (correlation scalar product ≥ 0.4) from canonical correlation analysis between bilberry urinary phenolics and 16S data. Hierarchical clustering of the variables was carried out to show the similarity in the species (top) and similarity in the behaviour of compounds measured in urine post bilberry consumption (left) between samples. The heat-map describes the relationship between variables standardised to the range of [-0.7, +0.7]. Red colour indicates a strong positive correlation between variables and blue indicates a strong negative relationship between variables. Yellow indicates no correlation between variables.

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Canonical correlation analysis of urinary excretion of bilberry anthocyanin metabolites and 16S genus data highlighted several relationships between these datasets. However, the overall relationship between the two datasets is not as linear post-bilberry as post black rice (see Figure 5-27 and Figure 5-30), most likely due to the higher variability in urinary metabolite profiles post bilberry extract consumption relative to black rice, as shown in Chapter 3. Nevertheless, *Romboustia, Rikenellaceae_RC9_gut_group* and *Prevotella_2* were positively associated with the production of (iso)vanillic acid/homoPCA sulfate, (iso)vanillic acid/homoPCA glucuronide, catechol sulfate, catechol, homoPCA, gallic acid sulfate, methyl-3,4-dihydroxybenzoate, protocatechualdehyde, methoxyphenyl sulfate, gallic acid methyl sulfate and PCA-4-O-sulfate, with *Rikenellaceae_RC9_gut_group* being most strongly associated with the excretion of all of these metabolites. Meanwhile, *Ruminococcus_2, Lachnospiraceae_ND3007_group, Ruminococcaceae_UCG.002, Clostridium_sensu_stricto_1, Akkermansia, Romboustia* and *Rikenellaceae_RC9_gut_group* were positively associated with urinary excretion of PCA-3-O-sulfate following consumption of bilberry extract. *Suboligranulum, Coprococcus_3, Fusicatenibacter, Coprococcus_1, Ruminococcus_2, Lachnospiraceae_ND3007_group, Ruminococcaceae_UCG.002*, and *Clostridium_sensu_stricto_1* were associated with high urinary excretion of caffeic acid glucuronide. The genera *Rikenellaceae_RC9_gut_group, Prevotella_2* and *Desulfovibrio* were positively associated with urinary excretion of 3/4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, syringic acid, isoferulic acid-3-O-glucuronide, dihydroferulic acid and hydroxyphenylacetic acid post bilberry anthocyanin extract consumption. Meanwhile, *Blautia, Collinsella, Lachnoclostridium, Lachnospira, Roseburia, Eubacterium_halli_group,* and *Bifidobacterium* were negatively associated with the post bilberry urinary excretion of: (iso)vanillic acid/homoPCA sulfate, (iso)vanillic acid/homoPCA glucuronide, catechol sulfate, catechol, homoPCA, gallic acid sulfate, methyl-3,4-dihydroxybenzoate, protocatechualdehyde, methoxyphenyl sulfate, gallic acid methyl sulfate, PCA-4-O-sulfate, PCA-3-O-sulfate, 3/4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, syringic acid, isoferulic acid-3-O-glucuronide, dihydroferulic acid and hydroxyphenylacetic acid, however, it should be noted that *Lachnospira, Roseburia* and *Eubacterium_halli_group* showed the strongest negative associations with all of these compounds.

Next, canonical correlation analysis was performed for urinary metabolites and metagenomic data to establish whether relationships were also seen at the species level. Canonical correlation analysis for black rice urine metabolites and metagenomic species data is shown in Figures 33 - 35. The canonical correlation analysis (CCA) scores plot can be seen for axis 1 and 2 Figure 5-33, and the correlation circle highlighting the variables that define these axes is shown in Figure 5-34. For the variables that most strongly define a relationship between the data sets (black rice urine

metabolites and gut microbial species), a heat-map is shown in Figure 5-35 for the visualisation of these specific relationships.

Figure 5-33- Canonical correlation analysis (CCA) scores plots for black rice urinary metabolite and metagenomics data.
A: CCA scores on axis 1 for 15 black rice urinary metabolite variables (phenolics) and 15 metagenomics variables. B: CCA scores on axis 2 for 15 black rice urinary metabolites variables (phenolics) and 15 metagenomics variables. Data points are labelled according the volunteer they represent.

Figure 5-34- Correlation circles highlighting the variables that define each axis from canonical correlation analysis of black rice urinary metabolite and metagenomics.
A: The black rice urinary metabolite variables that define axis 1 and 2. B: The metagenomics variables that define axis 1 and 2. Note that black rice urinary metabolite and metagenomics variables have been shown separately to improve readability but otherwise would be shown on the same plot.

Figure 5-35- Heat-map showing relationships identified in canonical correlation analysis of black rice urinary metabolite and metagenomics data

Heat-map visualising the relationships between the most strongly correlated variables (correlation scalar product ≥ 0.4) from canonical correlation analysis between black rice urinary phenolics and metagenomics data. Hierarchical clustering of the variables was carried out to show the similarity in the species (top) and similarity in the behaviour of compounds measured in urine post black rice consumption (left) between samples. The heat-map describes the relationship between variables standardised to the range of [-0.62, +0.62]. Red colour indicates a strong positive correlation between variables and blue indicates a strong negative relationship between variables. Yellow indicates no correlation between variables.

Canonical correlation analysis of urinary excretion of black rice anthocyanin metabolites and metagenomics species data highlighted several relationships between these datasets. *Bacteroides eggerthi, Alistipes shahii, Agathobaculum butyriciproducens, Oscillibacter sp.57.20, Bacteroides intestinalis, Roseburia sp.CAG.303,* and *Eubacterium sp.CAG.251* were all positively associated with urinary excretion of; gallic acid-3-O-glucuronide, dihydroxybenzoic acid glucuronide, 3/4-hydroxybenzoic acid, phloroglucinol, pyrogallol, ferulic acid, PGA, p-coumaric acid and PCA-3-O-glucuronide. *Paraprevotella xylanphila, Bifidobacterium animalis, Bacteroides finegoldii* and *Bacteroides salyersiae* were positively associated with the post black rice urinary concentrations of sinapic acid sulfate, trihydroxycinnamic acid glucuronide, 3-O-methyl gallic acid, homoPCA, methyl-

3,4-dihydroxybenzoate, (iso)vanillic acid/homoPCA sulfate, (iso)vanillic acid/homoPCA glucuronide, PCA-4-O-sulfate, PCA-3-O-sulfate, hydroxyphenylacetic acid, catechol sulfate, catechol and gallic acid sulfate. Meanwhile, *Oscillibacter sp.57.20, Bacteroides intestinalis* and *Bacteroides salyersiae* were all positively associated with urinary excretion of gallic acid methyl sulfate and dihydroferulic acid.

Eubacterium sp.CAG.38, Ruminococcus birculans and *Roseburia faecis* were negatively associated with post-black rice urinary excretion of sinapic acid sulfate, trihydroxycinnamic acid glucuronide, 3-O-methyl gallic acid, homoPCA, methyl-3,4-dihydroxybenzoate, (iso)vanillic acid/homoPCA sulfate, (iso)vanillic acid/homoPCA glucuronide PCA-4-O-sulfate, PCA-3-O-sulfate, hydroxyphenylacetic acid, catechol sulfate, catechol and gallic acid sulfate. Furthermore, *Ruminococcus lactaris, Intestinibacter bartlettii, Streptococcus salivarius* and *Ruminococcus callidus* were all negatively associated with the urinary excretion of gallic acid-3-O-glucuronide, dihydroxybenzoic acid glucuronide, 3/4-hydroxybenzoic acid, phloroglucinol, pyrogallol, ferulic acid, PGA, p-coumaric acid, and PCA-3-O-glucuronide post black rice anthocyanin extract consumption, with *Intestinibacter bartlettii, Streptococcus salivarius* and *Ruminococcus callidus* also being negatively associated with urinary excretion of gallic acid methyl sulfate, dihydroferulic acid, PCA-4-O-sulfate, PCA-3-O-sulfate, hydroxyphenylacetic acid, catechol sulfate, catechol and gallic acid sulfate, but to a slightly lesser extent.

Canonical correlation analysis for bilberry urine metabolites and metagenomic species data is shown in Figure 5-36 - Figure 5-38. The canonical correlation analysis (CCA) scores plot can be seen for axis 1 and 2 in Figure 5-36 and the correlation circle highlighting the variables that define these axes is shown in Figure 5-37. For the variables that most strongly define a relationship between the data sets (bilberry urine metabolites and gut microbial species), a heat-map is shown in Figure 5-38 for the visualisation of these specific relationships.
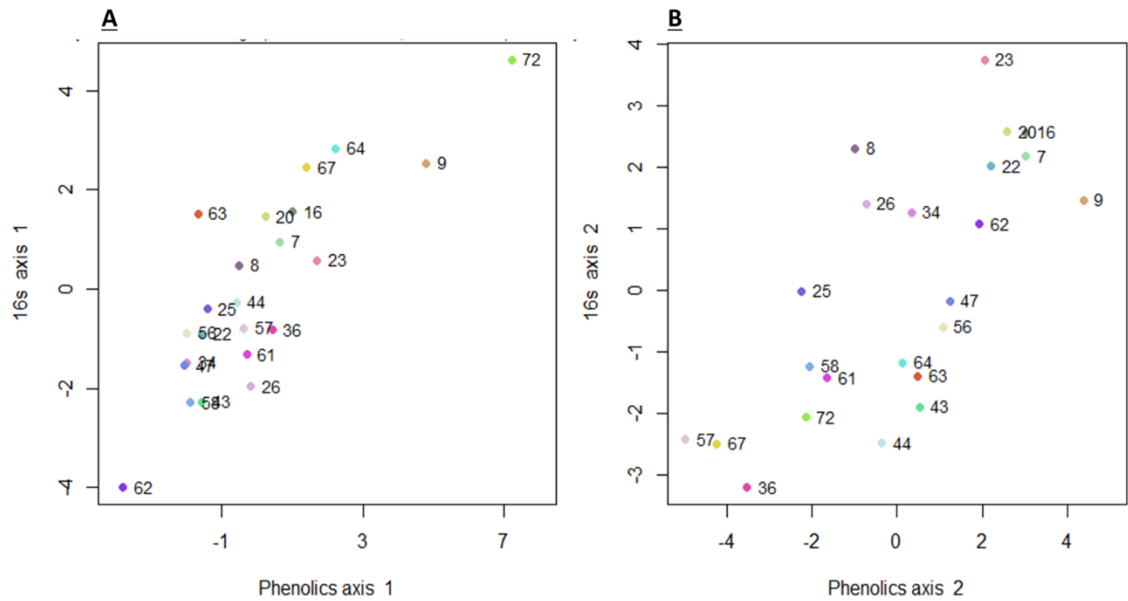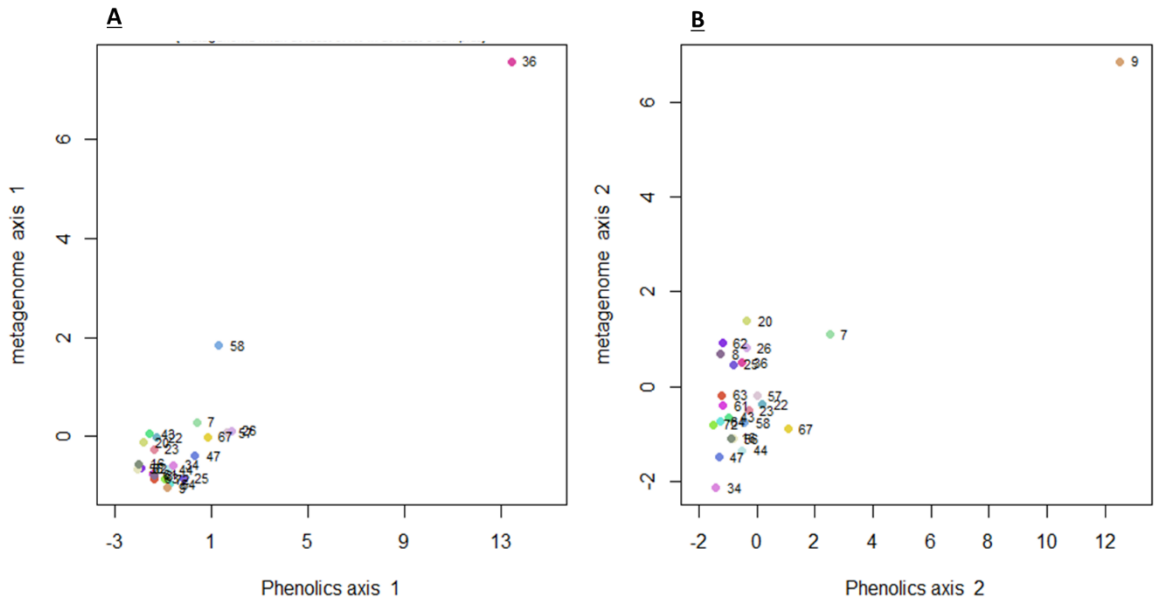
Figure 5-36- Canonical correlation analysis (CCA) scores plot for bilberry urinary metabolite and metagenomics data.

A: CCA scores on axis 1 for 15 bilberry urinary metabolite variables (phenolics) and 15 metagenomics variables. B: CCA scores on axis 2 for 15 bilberry urinary metabolites variables (phenolics) and 15 metagenomics variables. Data points are labelled according the volunteer they represent.
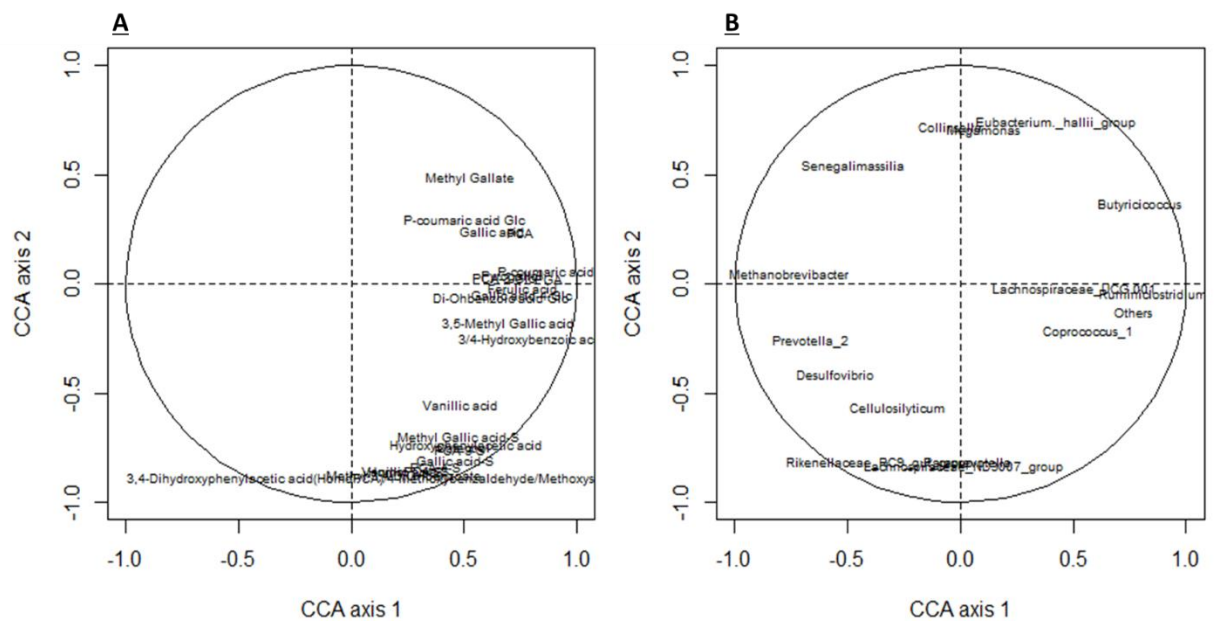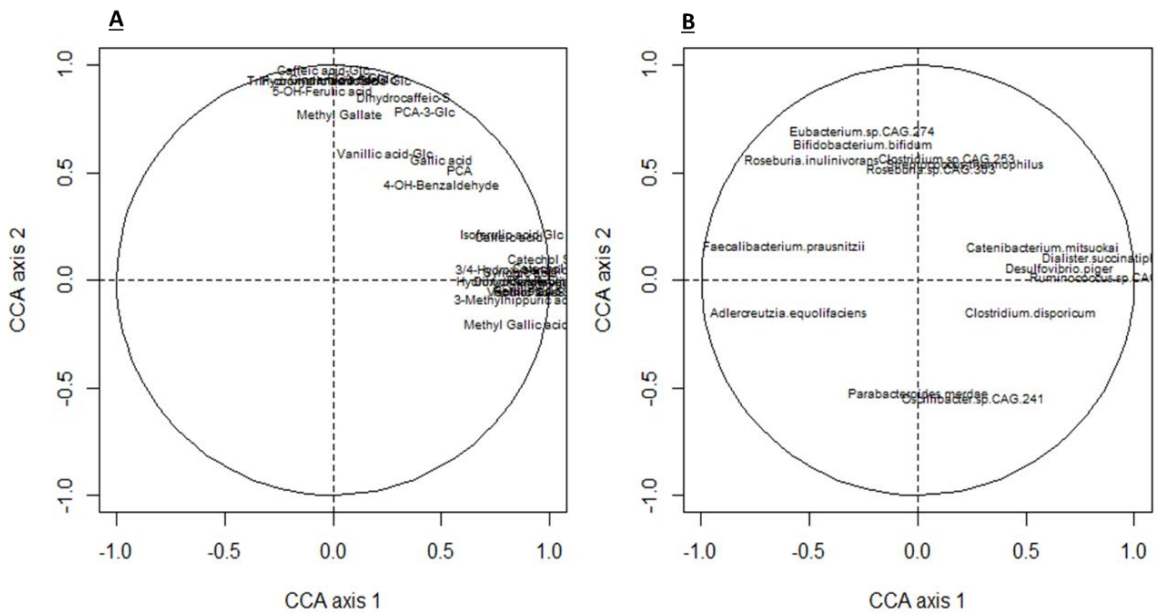

Figure 5-37- Correlation circles highlighting the variables that define each axis from canonical correlation analysis of bilberry urinary metabolite and metagenomics data.

A: The bilberry urinary metabolite variables that define axis 1 and 2. B: The metagenomics variables that define axis 1 and 2. Note that bilberry urinary metabolite and metagenomics variables have been shown separately to improve readability but otherwise would be shown on the same plot.
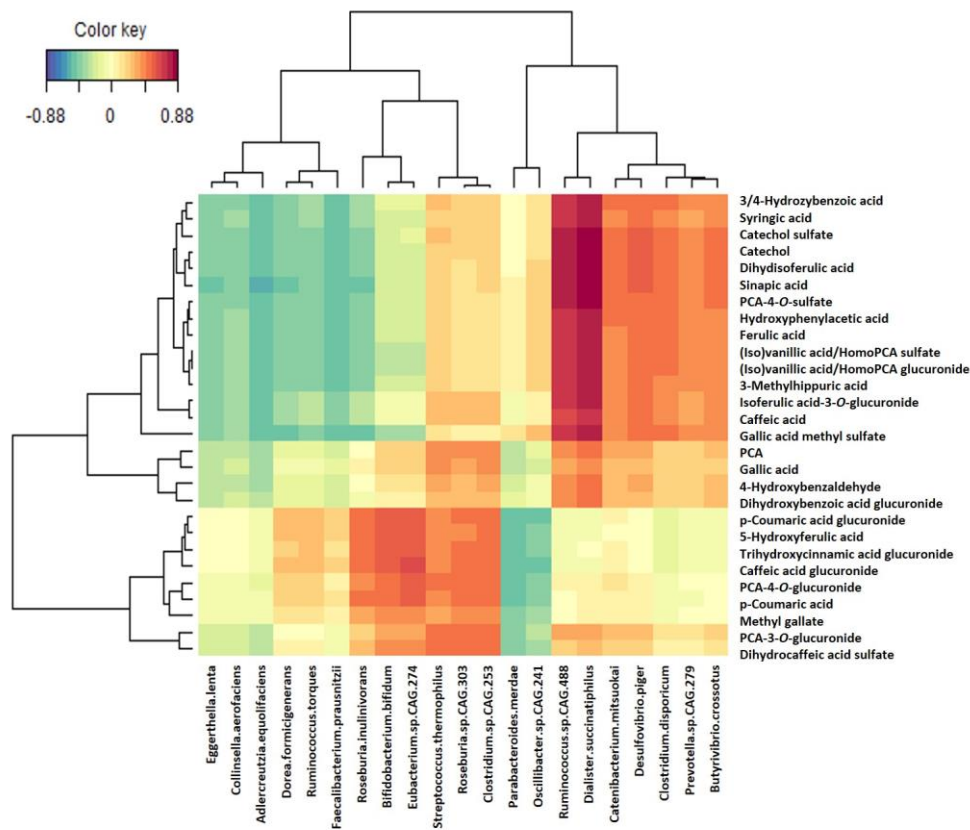
Figure 5-38- Heat-map showing relationships identified in canonical correlation analysis of bilberry urinary metabolite and metagenomics data
Heat-map visualising the relationships between the most strongly correlated variables (correlation scalar product ≥ 0.4) from canonical correlation analysis between bilberry urinary phenolics and metagenomics data. Hierarchical clustering of the variables was carried out to show the similarity in the species (top) and similarity in the behaviour of compounds measured in urine post bilberry consumption (left) between samples. The heat-map describes the relationship between variables standardised to the range of [-0.88, +0.88]. Red colour indicates a strong positive correlation between variables and blue indicates a strong negative relationship between variables. Yellow indicates no correlation between variables.

Canonical correlation analysis of urinary excretion of bilberry anthocyanin metabolites and metagenomic data showed several relationships between the urinary levels of anthocyanin metabolites and gut microbial species. *Ruminococcus sp.CAG.488* and *Dialister succinatiphilus* had a relatively strong positive association with urinary excretion of 3/4-hydroxybenzoic acid, syringic acid, catechol sulfate, catechol, dihydisoferulic acid, sinapic acid, PCA-4-O-sulfate, hydroxyphenylacetic acid, ferulic acid, (iso)vanillic acid/homoPCA sulfate, (iso)vanillic acid/homoPCA glucuronide, 3-methylhippuric acid, isoferulic acid-3-O-glucuronide, caffeic acid, and gallic acid methyl sulfate. These metabolites were also positively associated with the abundance of *Catenibacterium mitsuokai, Desulfovibrio piger, Clostridium disporicum, Prevotella sp.CAG.279* and *Butyrivibrio crossotus*, but to a lesser extent. *Streptococcus thermophilus, Roseburia sp.CAG.303, Clostridium sp.CAG.253, Ruminococcus sp.CAG.488* and *Dialister succinatiphilus* were positively associated with post bilberry anthocyanin consumption urinary excretion of PCA, gallic acid, 4-hydroxybenzaldehyde, and dihydroxybenzoic acid glucuronide.

Meanwhile, *Roseburia inulinivorans, Bifidobacterium bifidum, Eubacterium sp.CAG.274, Streptococcus thermophilus, Roseburia sp.CAG.303* and *Clostridium sp.CAG.253* were positively associated with post bilberry urinary excretion of p-coumaric acid glucuronide, 5-hydroxyferulic acid, trihydroxycinnamic acid glucuronide, caffeic acid glucuronide, PCA-4-O-glucuronide, p-coumaric acid, methyl gallate, PCA-3-O-glucuronide and dihydrocaffeic acid sulfate. Conversely, urinary excretion of these compounds was negatively associated with *Parabacteroides merdae* and *Oscillibacter sp.CAG.241.* Meanwhile, *Eggerthella lenta, Collinsella aerofaciens, Adlercreutzia equolifaciens, Dorea formicigenerans, Ruminococcus torques, Faecalibacterium prausnitzii* and *Roseburia inulinivorans* were all negatively associated with 3/4-hydroxybenzoic acid, syringic acid, catechol sulfate, catechol, dihydisoferulic acid sinapic acid, PCA-4-O-sulfate, hydroxyphenylacetic acid, ferulic acid, (iso)vanillic acid/homoPCA sulfate, (iso)vanillic acid/homoPCA glucuronide, 3-methylhippuric acid, isoferulic acid-3-O-glucuronide, caffeic acid, and gallic acid methyl sulfate urinary excretion post bilberry anthocyanin extract consumption.

Canonical correlation analysis of microbiome data in relation to *in vivo* and *in vitro* colon model anthocyanin metabolism data highlights several relationships between microbial taxa and the production of anthocyanin metabolites that have not been previously described. This data highlights several microbes that may work in concert synergistically to metabolise anthocyanins and their derivatives, however, it should be noted that this chapter only shows associations and not causal links between variables, and therefore further work would be required to establish whether these microbes are directly responsible for anthocyanin metabolism. It would be particularly important to explore the gene products of these microbes to establish their functional capacity to metabolise anthocyanins. Although the correlations described in the chapter provide insights into some of the microbes that may be required in the multi-step metabolism of anthocyanins, it is the functional capacity of any microbe that will ultimately determine its capacity to metabolise anthocyanins. Therefore, the relationship between some genes known to be involved in anthocyanin metabolism was studied in relation to anthocyanin metabolites *in vivo* and in the *in vitro* colon model to explore whether any associations were observed at the functional level.

5.5.5 Functional Profiling of the Gut Microbiota

Unlike 16S sequencing, whole-genome shotgun metagenomics has the advantage of sequencing to a high enough resolution to provide metabolic reconstruction, to facilitate maximal capture of organismal and functional data of the microbiota (Thursby and Juge, 2017). HUMAnN3 was employed for profiling the abundance of microbial metabolic pathways and other molecular functions. Approximately 300 pathways were found in each participants' microbiome, and of these pathways those involved in starch degradation and amino acid biosynthesis were the most abundant, which was not surprising as these constitute some of the primary functions of the gut

microbiota. There was no evidence of any treatment (placebo, black rice or bilberry extract) exerting any change in the abundance of any of the pathways identified (data not shown).

In addition to identifying pathways, enzymes were also categorised, and 2226 enzymes were found in the gut microbiotas of the study population. As with pathways, there was no apparent changes in the abundance of any enzyme encoding gene in response to treatment (data not shown). One of the barriers in terms of understanding the role of the gut microbiota in anthocyanin metabolism is that few of the enzymes known to be involved in anthocyanin metabolism have been characterised. Furthermore, it is highly likely that several of the reactions involved in anthocyanin metabolism can be carried out by multiple enzymes. It should be acknowledged that the metagenomic dataset can only be used to see if genes are present in the profiled microbiota, but cannot provide any information relating to gene expression.

β-glucosidase activity is key in anthocyanin metabolism, specifically the initial deglycosylation step to release the aglycone from the sugar moiety(s). Many microbial species genomes encode the β-glucosidase enzyme and unsurprisingly this enzyme was one of the most abundant, ranging from 183 – 719 copies per million (CPM). It was present in many of the species sequenced, but was most abundant in *Fusicatenibacter saccharivorans,* as well as being abundant in *Bifidobacteria* and *Roseburia* species, as shown in Figure 5-39. The abundance of the β-glucosidase gene did not change across the study population in response to either anthocyanin treatment or placebo. Nor were there any relationships between the CPM of this gene and the concentration of anthocyanins or their metabolites *in vivo,* or during *in vitro* fermentation (data not shown). However, we cannot infer from this whether or not expression of this gene was implicated.
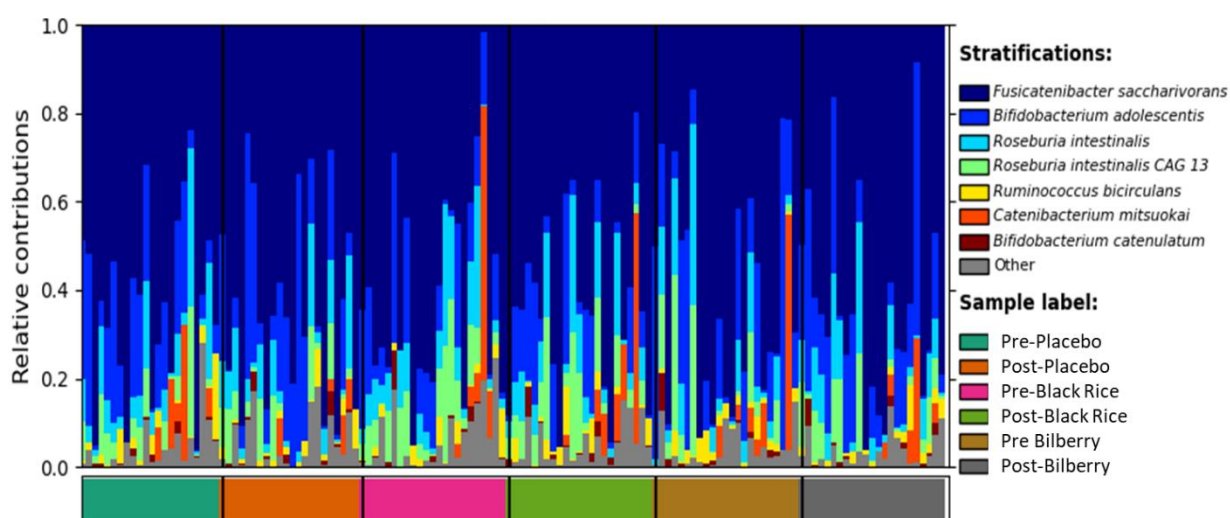
Figure 5-39- Relative contributions of microbial species to the abundance of the β-glucosidase gene in metagenomics samples.
Samples are grouped according to study phase: pre-placebo (n = 22), post placebo (n = 22), pre-black rice (n = 23), post-black rice (n = 23), pre-bilberry (n = 23) and post-bilberry (n= 23). Species that contribute to the abundance of β-glucosidase are colour coded in the stacked bar plots detailed in the key on the right of the figure.

One of the most notable findings in Chapter 3 and Chapter 4 was the importance of catechol and its phase 2 conjugates as metabolites of black rice anthocyanins. Catechol can be derived from the enzymatic decarboxylation of PCA, known to be mediated by protocatechuate decarboxylase. It was hypothesised that protocatechuate decarboxylase would be most abundant in volunteers where high levels of catechol and its phase 2 conjugates were seen in urine, and where catechol was produced at high levels during *in vitro* fermentation. However, surprisingly this gene was only identified in the microbiome of one sample (BERI 09, pre-black rice). This suggests that this metabolic conversion may also be performed by other enzymes. Another enzyme known to partake in this conversion is 4-hydroxybenzoate decarboxylase, however, this enzyme was only found in 27 of the 136 sequenced samples. Nevertheless, this enzyme was consistently most abundant in samples derived from BERI 26 where it ranged from 9.3 – 16.9 CPM whilst in other volunteers this reached a maximum of 2.2 CPM in BERI 16. It was somewhat surprising that BERI 26 had such high levels of this gene given that they were not among the participants with the highest concentration of catechol and its phase 2 conjugates in urine. However, BERI 26 was among the earliest producers of catechol during *in vitro* fermentation, with the second highest levels of catechol detected after 4 hours (the first time-point after inoculation). Although BERI 26 did not have the highest Cmax of catechol measured during *in vitro* fermentation, catechol may serve as a precursor to other microbial metabolites and there may be some link in the high abundance of the gene encoding 4-hydroxybenzoate decarboxylase in this participant and their early high production of catechol. Another enzyme that may be able to catalyse the decarboxylation of PCA to catechol (although this has not been reported previously) is 4-hydroxyphenylacetate decarboxylase (which is known to act on homoPCA to produce 4-methylcatechol, as well as being able to act on 4-hydroxybenzoic acid to produce phenol) (Lihua Yu *et al.*, 2006; Selmer and Andrei, 2001). This enzyme was present in all microbiota samples however, was at substantially higher levels in BERI 26. This is particularly interesting as BERI 26 was among the highest 4-methyl catechol producers in the colon model. BERI 26 had much higher levels of *E.coli* than any other participant and this suggests that *E.coli* are likely to be important in the production of catechol and 4-methyl catechol during anthocyanin metabolism. However, no correlation was seen between the levels of substrates or products of this enzyme (during *in vitro* fermentation or in 24 hour urine collections) and 4-hydroxyphenylacetate decarboxylase abundance (data not shown), perhaps due to gene abundance not necessarily correlating with gene expression, or enzyme activity.

Other genes known to be involved in anthocyanin metabolism that were identified in the microbiotas of the study population were phloretin hydrolase and pyrogallol hydroxytransferase. The former is able to produce phloroglucinol from phloretin and other phenolic compounds. Whilst pyrogallol hydroxytransferase is able to yield phloroglucinol from pyrogallol (Brune and Schink,

1990). However, it should be noted that these genes were only found in unclassified species and genera. Correlational analysis was performed for the abundance of both of these enzymes with phloroglucinol and pyrogallol concentrations from 24 hour urine collections and from *in vitro* fermentation experiments, but no relationship was found and therefore this data is not shown.

Microbial anthocyanin metabolism is a complex multi-step process and consequently many enzymes are involved. However, few of the enzymes involved in this process have been characterised. The functional analysis of metagenomic data presented in this chapter highlights a number of genes known to contribute to anthocyanin metabolism *in vivo,* however, no relationships could be identified between the abundance of these genes and levels of their known substrates and/or products. This is likely due to gene abundance not providing any indication of gene expression in combination with the fact that the same reaction may be carried out by multiple enzymes from different species and not all of these have been characterised to be taken into account in this analysis.

5.6   Discussion

The aim of the work presented in this chapter was to profile the gut microbiota of 23 individuals and establish whether there was any relationship between these profiles and anthocyanin consumption and/or metabolism. No large effects were observed in terms of anthocyanins changing the composition of the gut microbiota at either the genus or species level, with observed changes being minor and not consensus across the study population. Nevertheless, some relationships were observed between the gut microbiota profile of individuals and the *in vitro* colon model microbial metabolism of black rice anthocyanins using faecal samples derived from the same individuals. *Haemophilus parainfluenzae* and *Roseburia.sp.CAG.303* were both positively correlated with the production of 2,4-dihydroxybenzoic acid, whilst *Butyricicoccus* and *Lachnospira* were associated with the production of phloroglucinol and dihydrocaffeic acid. Correlations were also observed between *in vivo* metabolism of black rice and bilberry anthocyanins and the microbiome at both the genus and species level for the majority of observed urinary metabolites, including catechol, p-coumaric acid, caffeic acid, 5-hydroxyferulic acid and many more suggesting that several microbes may work synergistically in the multi-step process of anthocyanin metabolism. Several genes known to be implicated in anthocyanin metabolism such as β-glucosidase and 4-hydroxybenzoate decarboxylase were detected in metagenomic data, however, no correlations were observed between their relative abundance and the levels of either their products or substrates in *in vitro* fermentation colon model or *in vivo* metabolite data. The work presented in this chapter goes beyond the current literature by investigating the gut microbiota profiles of individuals in the context of their *in vivo* metabolism of anthocyanins and the capacity of their microbiota *in vitro* to metabolise anthocyanins.

The data presented in this chapter highlights that the microbial composition of faecal samples is associated with the production of a number of microbial metabolites. In particular, this relationship was shown for compounds such as phloroglucinol, pyrogallol and dihydrocaffeic acid. Several taxa were highlighted in a canonical correlation analysis as being positively associated with the production of these metabolites during *in vitro* fermentation of black rice anthocyanins with faecal samples derived from the same individuals. *Akkermansia* and *Terrisporobacter* were both positively associated with the production of pyrogallol in the *in vitro* colon model, but were negatively associated with the time point at which pyrogallol was first detected in this system. Instead, *Lachnoclostiridium, Ruminococcus_torques_group* and *Streptococcus* were positively associated with time of first detection of this metabolite. This constitutes a potential example of metabolic cross-feeding whereby metabolic products produced from dietary components by some microbes may then provide substrates to support growth of other populations (Henriques *et al.*, 2020). Moreover, this indicated that although some microbes may partake in the production of microbial anthocyanin metabolites early on, they are not necessarily the greatest producers of these compounds. This may be due to a variety of reasons. Firstly, when a metabolite is produced early in anthocyanin metabolism it may then serve as a substrate for other microbial enzymes and be converted into other compounds, so early production may not correspond to high concentrations. Secondly, microbes associated with high production of pyrogallol may be able to survive in an environment with high pyrogallol concentrations better than those that are associated with early production, rather than directly contributing to production themselves, however, this would need to be tested experimentally. Additionally, metabolites that are produced at particularly high concentrations are not necessarily produced early and microbes that produce compounds such as pyrogallol at their highest concentrations are not necessarily those that produce them first. This is because metabolites such as pyrogallol may arise from several precursors with different enzymes being able to yield pyrogallol in different reactions. Therefore, pyrogallol (and other metabolites) may be produced at various stages in anthocyanin metabolism by different microbes utilising different substrates (i.e. pyrogallol may be derived from decarboxylation of gallic acid or from phloroglucinol in a hydroxytransferase reaction) which would explain why we see different taxa correlated with different metabolic parameters of this compound, as different microbes may become more central to the production of pyrogallol at different stages of anthocyanin metabolism (Brune and Schink, 1990; Yoshida and Yamada, 1985). This is the first study to report some of the microbes that may be important in the production of these specific anthocyanin metabolites. Furthermore, this is also the first study to report that different microbes may be important for different metabolic parameters of the same anthocyanin metabolite i.e. how quickly metabolites are produced and the concentration they reach during anthocyanin metabolism.

In some instances, relationships were detected between the same microbial metabolites of anthocyanins from the *in vitro* fermentation colon model and microbiome profiles at both the species and genus level as expected. However, this was not the case for all compounds. At the species level canonical correlation analysis highlighted a positive relationship between catechol production and several bacterial species including, *Firmicutes bacterium.CAG.95, Bacteroides eggerthii* and *Haemophilus parainfluenzae*. However canonical correlation analysis did not show any relationship between catechol production and the microbiome at the genus level. This may be attributed to a genus consisting of several species and therefore unless several of the included species are correlated with catechol production, or at least the most dominant species in the genera then an effect is unlikely to be detected at this high level. This is reinforced by the fact that the species associated with catechol production during *in vitro* fermentation belong to different genera, explaining why a correlation is only observed at the species level. What was more surprising was that in some cases a relationship was detected at the genus level but not at the species level. For example, the overall metabolism of C3G in the colon model was positively correlated with the relative abundance of *Akkermansia* and *Terrisporobacter* but no relationships were detected at the species level within these genera. This may be due to many species in each of these genera contributing to these relationships at a low level with no particular species being the major player in the relationship, meaning that at the species level relationships are too weak to be detected independently by canonical correlation analysis (Eckburg *et al.*, 2005). These differences in the relationships between anthocyanin metabolism and the microbiome at different taxa levels have not been explored by previous studies and highlights that both the genus and species should be considered when investigating the role of the microbiome in anthocyanin metabolism. However, in some cases relationships observed at the genus level were reinforced at the species level. For example, *Ruminococcus_torques_group* (genus) along with *Ruminococcus lactaris* and *Ruminococcus torques* (species) were shown to be positively correlated with the time of first pyrogallol detection during *in vitro* fermentation. And *Paraprevotella* and *Paraprevotella xylanphila* were positively correlated with urinary excretion of catechol, homoPCA and gallic acid sulfate at the genus and species level respectively post black rice anthocyanin consumption.

In Chapter 4 it was shown that several anthocyanin metabolites are microbially derived, and therefore it was expected that all of these metabolites would show some relationship with the gut microbiota at either the genus or species level. It was surprising that no correlation was observed between 3-hydroxyphenylacetic acid production and the microbiome, or unknown Y and the microbiome as the extent of production of these metabolites was a distinguishing factor of some individuals in the colon model, and these compounds were widely produced. However, this may be attributed to many microbes being capable of producing these compounds in anthocyanin metabolism and gene expression and/or enzyme activity best defining the metabolic parameters of

individuals with regards to these metabolites, although this would need to be tested experimentally. Furthermore, although PCA and PGA are not exclusively produced microbially, with spontaneous degradation contributing to their production, in Chapter 4 it was shown that these compounds are largely produced by microbes. However, multivariate canonical correlation analysis did not show any relationship between *in vitro* microbial production of PCA and PGA with the microbiome. This may be due to several species being involved in the production of these compounds, paired with the contribution of factors outside of the microbiome in the production of these compounds making it difficult to elucidate any relationship between specific species and/or genus in the behaviour of these metabolites in individuals. Furthermore, it should be acknowledged that in Chapter 4 it was demonstrated that PCA and PGA both serve as precursors to multiple microbial anthocyanin metabolites and the tendency of these compounds to increase and then decrease during *in vitro* fermentation may have made it difficult to establish the microbes that may be involved in producing these compounds. A negative correlation may have been expected between these compounds and features of the gut microbiota which would provide insights into the microbes that metabolise these compounds further. However, this was not observed for *in vitro* colon model data, likely due to these compounds being substrates for multiple enzymes encoded in the genomes of several microbes that may metabolise these compounds differently, as would be suggested from the wide range of anthocyanin metabolites observed in Chapter 4 and Chapter 3. Despite this, both positive and negative correlations were observed between urinary excretion of PCA and PGA after anthocyanin consumption in the human intervention study. It was surprising that canonical correlation analysis highlighted correlations between the microbiome and urinary excretion of PCA and PGA, but not their production in the *in vitro* fermentation colon model. This may be due to differences in experimental models. Firstly, these two models measure different parameters of anthocyanin metabolism, the colon model measures the production of compounds in an *in vitro* colon, whereas urinary metabolites are a measure of the excretion of compounds; this difference is likely to partly explain why the same relationships are not observed between datasets. Furthermore, it is possible that processing of faecal samples prior to *in vitro* fermentation may have led to the death of some anaerobes and consequently changes the composition of the microbiota. This may have meant that the microbes correlated with urinary excretion of PCA and PGA may have not had such a dominant role during *in vitro* fermentation as other microbes capable of the same biotransformation may have accounted for a higher proportion of the microbiota than they had *in vivo* (O'Donnell *et al.*, 2016). Furthermore, the *in vitro* fermentation colon model does not perfectly replicate the *in vivo* conditions of the gut and this may mean that some microbes behave differently between the two systems. These factors may explain why there are differences in whether the microbiome is associated with anthocyanin metabolites such as PCA and PGA as well as others including catechol and phloroglucinol between the colon model and urinary excretion.

Surprisingly, different taxa were associated with the urinary excretion of PCA and PGA between bilberry and black rice anthocyanin consumption. Negative correlations were observed between the urinary excretion of PCA and PGA and the microbiome, with *Methanobrevbacter* and *Intestinibacter bartlettii* being implicated at the genus and species level respectively, post black rice anthocyanin extract consumption (but not post-bilberry). These negative correlations suggest that these taxa may be important in the biotransformation of PCA and PGA to a range of potentially bioactive metabolites *in vivo*. Moreover, positive correlations were also detected between the urinary excretion of PCA and PGA and the microbiome post-black rice and bilberry anthocyanin extract consumption. Post-black rice anthocyanin extract consumption *Butyricicoccus* and *Ruminiclostridium_6* were positively correlated with the urinary excretion of PCA and PGA at the genus level, with *Bacteroides* species implicated at the species level. Whereas post-bilberry anthocyanin consumption PGA was not correlated with any genus or species, whilst PCA and its phase 2 conjugates were positively associated with *Rikenellaceae_RC9_gut_* at the genus level and *Ruminococcus sp.CAG.488* and *Dialister succinatiphilus* at the species level. It was unexpected that different taxa were correlated with the production of PCA and PGA between bilberry and black rice anthocyanin consumption as these metabolites are common between the two anthocyanin treatments. PGA is derived from the A-ring of both di- and tri-hydroxy B-ring anthocyanins which is structurally the same between these compounds. Therefore, it was hypothesised that the same taxa would be correlated with PGA production between these two different anthocyanin treatments, especially given that PGA was found at a mean of 0.1 μmol/24 hours in urine after both black rice and bilberry anthocyanin extract consumption (Chapter 3). It is possible that taxa were correlated with urinary excretion of PGA post-black rice extract but not post-bilberry due to differences in the parent anthocyanins. Although the A-ring of di- and tri-hydroxy B ring anthocyanins is structurally the same, structural variations in the B-ring mean that di-hydroxy B-ring anthocyanins are more stable than tri-hydroxy B-ring anthocyanins (Castañeda-Ovando *et al.*, 2009). Therefore, tri-hydroxy (bilberry) B-ring anthocyanins spontaneously degrade more rapidly than di-hydroxy-B ring (black rice) anthocyanins (Woodward *et al.*, 2009). Given that spontaneous degradation contributes to anthocyanin metabolism *in vivo* it is plausible that lack of relationship between the microbiome and PGA urinary excretion post bilberry anthocyanin consumption may be due to the increased contribution of spontaneous degradation in the case of bilberry anthocyanins relative to black rice. Furthermore, different taxa were highlighted in the urinary excretion of PCA (and its phase 2 conjugates) post black rice and bilberry anthocyanin consumption. This was unexpected, but may be attributed to different enzymes being implicated in the production of PCA due to the different types of anthocyanins present in bilberry and black rice. PCA can be derived directly from the B-ring of di-hydroxy B-ring (black rice) anthocyanins, whereas gallic acid is the direct B-ring metabolite of tri-hydroxy B-ring (bilberry) anthocyanins. However, *in vivo*

gallic acid is not an abundant bilberry anthocyanin metabolite, and PCA may be derived from the microbial dehydroxylation of gallic acid. This would suggest that this same metabolite may be derived from different precursors depending on the specific anthocyanin consumed, and would explain why different taxa are highlighted between bilberry and black rice consumption in cases such as this.

In Chapter 3 it was shown that a large profile of metabolites are produced *in vivo* after consumption of bilberry and black rice anthocyanins with the specific metabolites generated and their relative concentrations being subject to high inter-individual variation. It was hypothesised that this inter-individual variation was largely attributed to differences in the gut microbiota of individuals. Relationships were observed for many of these metabolites and the microbiome at both the genus and species level. This is the first report to highlight some of the specific taxa that may be implicated in the production of *in vivo* anthocyanin metabolites such as: catechol, caffeic acid, 5-hydroxyferulic acid, methyl gallate, p-coumaric acid, ferulic acid and dihydrocaffeic acid amongst others. Full details of the species and genera that have been putatively associated with the urinary excretion of *in vivo* anthocyanin metabolites are given in the results section, and these relationships are best described by: Figure 5-29, Figure 5-32, Figure 5-35, and Figure 5-38. What is observed here is that several microbes are associated with the urinary excretion of multiple anthocyanin metabolites. For example: *Paraprevotella xylanphila, Bifidobacterium animalis, Bacteroides finegoldii* and *Bacteroides salyersiae* were all positively associated with the post black rice urinary excretion of sinapic acid sulfate, trihydroxycinnamic acid glucuronide, 3-O-methyl gallic acid, homoPCA, methyl-3,4-dihydroxybenzoate, (iso)vanillic acid/homoPCA sulfate, (iso)vanillic acid/homoPCA glucuronide, PCA-4-O-sulfate, PCA-3-O-sulfate, hydroxyphenylacetic acid, catechol sulfate, catechol and gallic acid sulfate. This suggests that several microbes may work in concert synergistically in the multi-step process of anthocyanin metabolism, however, the precise mechanisms of this are yet to be understood. Furthermore, it should be noted that although several microbes have been associated with the urinary excretion of anthocyanin metabolites, the data presented is merely correlational and it cannot be concluded that these microbes are directly involved in the metabolic conversions that produce these compounds, and it is not clear what features of these microbes may implicate them in anthocyanin metabolism if indeed they are involved. Moreover, it is possible that some of these microbes survive better in the presence of particular anthocyanin metabolites but may not be involved in their production, and this may explain some of the positive associations observed. Likewise, it is plausible that some of the negative associations observed may be due to the presence of particular metabolites being deleterious to the survival of some microbes rather than these microbes being implicated in their biotransformation (Henriques *et al.*, 2020). Therefore, although the work in this chapter provides previously undescribed associations between the microbiome and anthocyanin metabolites produced *in vivo* it is important that future work looks to deduce whether

these microbes are directly involved in anthocyanin metabolism or are merely effected by the presence of anthocyanin metabolites. Nevertheless, this chapter provides insights into some of the microbes that may be important in defining anthocyanin metabotypes in the future, pending further investigation.

What was observed at the genus and species level is that the same taxa tended to correlate with the urinary excretion of a parent metabolites, such as gallic acid and its phase 2 conjugates. This was not surprising as phase 2 metabolism occurs in the liver and is not microbial (Phang-Lyn and Llerena, 2020). Therefore, if the parent metabolite is associated with taxa of the microbiome we would expect to observe the same relationship for its phase 2 conjugates as the microbiota would not have been responsible for phase 2 conjugation, and would have only had a role in the production of the parent metabolite. However, in some instances this was not the case, for example, post bilberry anthocyanin extract consumption *Ruminococcus sp.CAG.488* and *Dialister succinatiphilus* were positively associated with urinary excretion of PCA and its sulfate conjugates but not its glucuronides. This may be due to PCA being produced at different stages of anthocyanin metabolism by different microbes utilising different precursors, and PCA being subjected to sulfation or glucuronidation at different stages in metabolism *in vivo*, resulting in different microbes being associated with the urinary levels of these compounds. To test this, it would be interesting to measure anthocyanin metabolites in urine at different time intervals to obtain pharmacokinetic profiles for metabolites and analyse these in relation to the microbiome. This data could be used to explore whether different bacteria appear to be associated with the production of a parent metabolite and its phase 2 conjugates at different time-points and whether this is linked to the reduction in different potential precursors to the metabolite in question, as this may help to deduce the precise pathways of anthocyanin metabolism.

Although relationships were observed between many urinary anthocyanin metabolites and the microbiome, for some compounds such as hippuric acid and vanillic acid no correlations were observed between urinary excretion and the microbiome after either bilberry or black rice anthocyanin consumption. This lack of correlation between datasets in these instances may be attributed to several factors. Firstly, although *in vivo* the gut microbiota does contribute to anthocyanin metabolism it is not the only factor at play, with human enzymes also metabolising anthocyanins. This interplay between human and gut microbial metabolism results in a diverse and variable metabolite profile, and ultimately makes it difficult to establish precisely which metabolism is microbial (Braga *et al.*, 2018). Nevertheless, lack of association between the microbiome and these metabolites may suggest that they could be produced predominantly by human enzymes, however, this would need to be tested experimentally. Additionally, although microbes metabolise anthocyanins, it is the enzymes encoded in their genomes that are ultimately responsible for this metabolism. Therefore, it may be expected anthocyanin metabolism is best correlated with gut

microbial enzyme expression/activity, rather than taxonomic composition. This idea is reinforced by the lack of a 'core' microbiota with high variability in the microbial communities present in the gut of individuals, but consensus in the repertoire of microbial genes present between individuals, suggesting that the core microbiota may be better defined at a functional rather than organism level (Thursby and Juge, 2017). It is therefore likely that individuals who metabolise anthocyanins similarly are likely to be better associated with the functional capabilities of their microbiome rather than taxonomic profiles. However, few studies have investigated the relationship between the microbiota and anthocyanin metabolism at taxonomic level and there is an absence of reports on the relationship between the functional capabilities of the gut microbiota and anthocyanin metabolism (Igwe *et al.*, 2019). This would be an incredibly important point of future work to establish how and why individuals metabolise anthocyanins differently, and the impact this may have on the health effects of eating anthocyanins.

Although relationships were observed between anthocyanin metabolism and the gut microbiota, at the genus and species level, no relationships were found at the functional level for genes known to be implicated in the metabolism of anthocyanins and biotransformation of their metabolites. This was particularly surprising in the case of catechol. Catechol and its phase 2 conjugates were major metabolites of black rice anthocyanin excreted in urine, and catechol was shown to be microbially derived in the *in vitro* fermentation colon model. Catechol and its sulfate conjugate were positively correlated with microbial species *Bacteroides finegoldii* and *Bacteroides salyersiae* post black rice extract; and *Ruminococcus sp.CAG.488* and *Dialister succinatiphilus* species post bilberry extract consumption. However, it was surprising that the same species were not highlighted post bilberry and black rice anthocyanin consumption and therefore it was hypothesised that perhaps relationships would be observed at the functional level. Enzymes known to be involved in the production of catechol (protocatechuate decarboxylase and 4-hydroxybenzoate decarboxylase) were detected in the microbiomes of the study population but were not observed in all participants and did not correlate with the concentration of catechol either in urine or in the colon model (Grant and Patel, 1969). Although 4-hydroxybenzoate decarboxylase was most abundant in the microbiome of BERI 26, who was also among the fastest catechol producers in the colon model, they did not have the highest concentration of catechol in either urine or the colon model. Furthermore, another gene, 4-hydroxyphenylacetate decarboxylase, which is postulated to be capable of catalysing the enzymatic conversion of PCA to catechol was found in all participants but was also must abundant in BERI 26 with *E.coli* being the largest contributor to the abundance of this gene. This would imply that these enzymes are important in the early production of catechol, although, it was surprising that BERI 26 did not have the highest concentration of catechol in either urine or the colon model given the high abundance of both of these genes. However, catechol may be further metabolised to other compounds and this may

explain why the abundance of these enzymes did not correlate with the concentration of catechol in urine or the colon model. Nevertheless, no correlation was observed between the time catechol was first detected in the colon model and the abundance of these enzymes. The lack of correlation in this data is likely due to two main reasons. Firstly, samples were taken with large time intervals between them in the colon model (minimum 4 hours) and this meant that we could not monitor the precise time at which metabolites were first produced making it difficult to establish if this can truly be related to the genes present in the microbiome. Additionally, abundance of a gene in the genome does not provide any information on gene expression or enzyme activity, therefore just because a gene has high abundance levels in the genome it may not be expressed, and abundance data cannot be used to establish the contributions of a gene to any function *in vivo* (Pereira *et al.*, 2018). It would be interesting to perform transcriptomics to investigate whether the expression of these important genes are associated with anthocyanin metabolism and can explain some of the inter-individual variation observed. It would be recommended that future studies investigate the expression of genes involved in anthocyanin metabolism. Furthermore, little is known regarding the range of genes that may be involved in this process and characterising these genes would be particularly important to deepen our understanding of how anthocyanins are metabolised *in vivo*.

There is conflicting evidence in the literature regarding whether anthocyanins are able to modulate the gut microbiota. No large effects were exerted on the gut microbiota by the consumption of either bilberry or black rice anthocyanins in this study. However, some very minor changes were observed, *Roseburia* species appeared slightly decreased after both bilberry and black rice anthocyanin consumption. *Roseburia* species are butyrate producers that have been associated with several health benefits such as prevention of type 2 diabetes and colon cancer, however, this disagrees with a study that reported *Schisandra chinesis* increased the relative abundance of *Roseburia* species in human volunteers, however, this study fed an unpurified food extract containing many polyphenols besides anthocyanins and therefore this effect cannot be directly attributed to the anthocyanin content (Hillman *et al.*, 2020; Song *et al.*, 2015). *Eubacterium rectale* were increased post bilberry anthocyanin consumption but were not affected by black rice anthocyanin consumption. Another study reported that black raspberry anthocyanins increased the proliferation of *Eubacterium rectale,* which exerts beneficial effects through the production of butyrate (Chen et al., 2018). It is therefore surprising that we did not see an increase in *Eubacterium rectale* after black rice anthocyanin consumption as both black rice and black raspberry are predominantly comprised of cyanidin based anthocyanins. However, the report from Chen *et al* used a mouse model whereas this study used human volunteers and this difference in the mode of study may explain the difference in these findings. In agreement with a report by *Pan et al* bilberry anthocyanin consumption was associated with increase in Anaerostipes hadrus (Pan et al., 2017). Furthermore, Ruminococcus birculans was decreased after bilberry anthocyanin consumption,

Ruminococcus species have also been reported to be decreased by *Schisandra chinensis* in humans and blackberry in rats (Marques *et al.*, 2018; Song *et al.*, 2015). High abundance of *Ruminococcus* species has been associated with diseases of the gut such as inflammatory bowel disease and Crohn's disease, therefore evidence that relative abundance of *Ruminococcus* may be reduced by anthocyanins in this report and others supports that anthocyanins may in part exert beneficial effects through modulation of the gut microbiota, however, further work would be needed to test this hypothesis.

A recent study reported that *Adlercreutzia, Lachnospira and Alistipes* were increased following consumption of an anthocyanin rich juice for 56 days whilst *Dorea* were decreased, however, these effects were not observed here, possibly due to differences in the way anthocyanins were fed i.e. in a juice or in a capsule (Groh *et al.,* 2020). In addition to this, several studies that have investigated the effect of anthocyanins on the gut microbiota have reported that anthocyanins increase the abundance of beneficial *Bifidobacteria* and *Lactobacilli* species (Espley *et al.*, 2014; Hidalgo *et al.*, 2012; Vendrame *et al.*, 2011; Zhu *et al.*, 2018). However, no substantial effect was seen on either of these genera after either bilberry or black rice anthocyanin consumption in this study. Analysis with MaAslin2 showed a very slight elevation of *Bifidobacterium longum* post bilberry extract consumption, however this was not consistent in the entirety of the study population and was not observed post black rice anthocyanin extract consumption (Figure 5-14). Furthermore, in contrast to what has been shown in other studies MaAslin2 revealed a very slight decrease in the relative abundance of *Bifidobacteria adolescentis* post black rice anthocyanin consumption, however this was very small, not consistent across the study population, and not statistically significant. It was surprising that neither *Bifidobacteria* or *Lactobacilli* were increased after consumption of either bilberry or black rice anthocyanins, however, not all reports have shown this, for example, Flores *et al* did not observe any effects on either of these genera and (Flores *et al.*, 2015). Similar to these reports, no substantial effects were observed on these genera or species in this study, however, some minor effects were observed that have not been reported in the literature. *Eubacterium ramulus* and *Blautia obeum* both appeared slightly decreased post black rice anthocyanin consumption, with the former being shown previously to be involved in the gut microbial metabolism of a variety of flavonoids so it is somewhat surprising that it appeared decreased post black rice anthocyanin consumption (Braune *et al.*, 2019; Tomás-Barberán *et al.*, 2009). Furthermore, the *Blautia* genus has been associated with visceral fat accumulation in adults which is a major risk factor of metabolic diseases, therefore reducing species belonging to this genus would support that anthocyanins are able to protect against metabolic syndrome (Ozato et al., 2019). *Flavonifractor plautii* relative abundance was slightly elevated post black rice anthocyanin consumption, this species is known to be involved in the microbial degradation of flavonoids. Some studies have reported beneficial effects of this species in relation attenuating metabolic syndrome,

however, other studies have linked this to increased risk of colorectal cancer, although more work is needed in this area, this suggests that in some cases flavonoid metabolites may negatively affect health (Gupta et al., 2019; Mikami et al., 2020). Furthermore, *Phascolarctobacterium succinatutens* were decreased in some participants post black rice anthocyanin consumption, this species is important in the production of short-chain fatty acids acetate and propionate which are important regulators of metabolic health and therefore downregulation of these species may be considered unfavourable (Ariefdjohan et al., 2017). Nevertheless, it should be noted that all effects observed on the gut microbiota in this study were incredibly small and not consensus across the whole study population. Therefore, although the minor effects observed here would be interesting points of future investigation the evidence presented in this chapter is not strong enough to conclude that anthocyanins truly exert these effects.

Neither bilberry or black rice anthocyanins were associated with any effect on the diversity of the gut microbiota (Figure 5-13). This disagrees with several other studies that have reported that anthocyanins increase the overall diversity of the gut microbiome. However, it should be noted that data from these reports was generated from mouse and rat models and delivered a higher anthocyanin dose than the human intervention study described in Chapter 3 (Gu *et al.*, 2019; Jakobsdottir *et al.*, 2013; Liu *et al.*, 2017; Petersen *et al.*, 2019). It is not surprising that no effect on gut microbial diversity was observed in this study where a modest dietary relevant dose of anthocyanins (320 mg/day) was given as a small part of participants regular diets that would have varied considerably between individuals. Therefore, if anthocyanins were to exert any effect on gut diversity it would be difficult to detect them using this study design. Animal studies are far more likely to detect changes in gut diversity as all subjects are fed the same background diet reducing inter-individual variation and anthocyanin doses tend to be much higher than those given in human studies, and therefore the effects seen in mice and rats may not translate to humans *in vivo* (Eckburg *et al.*, 2005; Wankhade *et al.*, 2019; Beghini *et al.*, 2021). It should be noted that although generally no effects were observed for participants in terms of their gut diversity, for two participants a sharp increase in the Shannon diversity index was observed post bilberry extract consumption. However, this was not true for the other 21 participants. Furthermore, the two participants where this diversity increase was observed (BERI 25 and 26) happen to be husband and wife. Given that no large change in diversity was seen for other participants this led to the conclusion that the increase in diversity for these participants was due to a lifestyle factor outside of the study. This also highlights that many factors affect gut diversity and studies need to control these factors as best possible and record lifestyle changes of study participants so that effects on gut diversity unrelated to study treatments can be accounted for.

There are several strengths and weaknesses of the work presented in this chapter. This chapter brings together data on gut microbiota profiles, *in vitro* microbial anthocyanin metabolism and *in*

vivo anthocyanin metabolism for the same participants, previous studies have either focused on the effect of anthocyanins on the gut or the effect of the gut on anthocyanin metabolism, and no previous reports have brought together data in this way. By studying the same volunteers for all parameters this chapter has provided new insights into how these variables may relate to one another. A strength of this study is that it used human subjects which provides more representative findings that can be used to inform future dietary advice that may be needed in relation to the interplay between anthocyanins and the gut microbiota. However, a fundamental problem with human studies is that the lifestyles of participants tend to vary considerably and this can sometimes interfere with the effect of treatment and/or the results obtained. Although less representative, animal studies are often more likely to detect significant effects of a treatment due to the increased levels of control over the study population, where less factors outside of the study are able to cause an effect. It may be beneficial for some future studies that investigate the bidirectional relationship between anthocyanin metabolism and the gut microbiota to provide a controlled diet to study participants to avoid differences in dietary habits from masking effects of any treatment given, although this would be impractical and difficult to enforce. What may be an easier measure would be to ask participants to record a food diary and any lifestyle changes including increased exercise or stress that may affect the microbiome so that these factors can be taken into account. However, arguably the biggest challenge of the work presented in this chapter (and indeed previous chapters of this thesis) is the very high inter-individual variation observed between participants and the effect this has on being able to detect changes that may be due to the anthocyanins given in the study. The data presented here shows that the gut microbiota profile of individuals was vastly different at both the genus and species level, and the stark differences between participants made it particularly difficult to detect differences associated with anthocyanin consumption and/or metabolism. To overcome this problem in the future it may be recommended to screen participants according to their microbiome profile so that participants with a similar microbiome can be studied to make it easier to reliably establish whether anthocyanins exert any effect on the microbiome. Alternatively, it may be of interest to perform a large study where individuals can be clearly grouped according to their microbiome to investigate how individuals with different microbiota profiles metabolise anthocyanins and the effect of anthocyanin consumption on the microbiome. Another limitation of this study is the relatively small sample size of 23 individuals that were included in this analysis. This matched with high inter-individual variability in anthocyanin metabolism meant that in some cases participants that exhibited a particular metabolic characteristic was only present in very small proportion of the study population and this may have led to some spurious results. For example, only 4 out of 23 participants were observed to produce 2,4-dihydroxybenzoic acid in the *in vitro* colon model. Although production of this metabolite was associated with taxonomic features of the microbiome this dataset is too small to reach any reliable conclusion. Furthermore,

this study, like many others relied on faecal samples to profile the gut microbiota of individuals and although this is undoubtedly the easiest and least invasive option for participants, there is evidence that the microbial composition of the gut can vary considerably in different sections. Therefore, it would be interesting to use gut mucosal biopsies to investigate the relationship between anthocyanins and the gut microbiota and to compare this to faecal samples (Durbán *et al.*, 2010).

5.7 Conclusion

This chapter highlights the complexity of investigating the hypothesised bidirectional relationship between anthocyanin metabolism and the gut microbiota. Overall, the data presented does not support the hypothesis that one month of daily anthocyanin consumption from black rice or bilberry extract alters the gut microbiota. Although some minor changes were observed at the genus and species level, these were very small and not consensus among the study population with very high inter-individual variation in participant microbiomes and extremely small effect sizes compromising the power to reliably detect differences. Therefore, from the data presented in this chapter, it is not possible to draw a reliable conclusion with respect to whether anthocyanin consumption modulates the microbiome. Despite this, clear relationships were observed for the first time between several urinary and *in vitro* microbial anthocyanin metabolites such as, pyrogallol, phloroglucinol, catechol and dihydrocaffeic acid with the microbiome at both the genus and species level. This indicates that the microbiome has a considerable bearing on the trajectory of anthocyanin metabolism both *in vivo* and *in vitro*. What was particularly interesting is that in some cases different microbes appeared to be associated with different metabolic parameters of the same compound, highlighting the complexity of microbial anthocyanin metabolism. However, it should be acknowledged that the data presented in this chapter is correlational and no causal links have been demonstrated between features of the microbiome and anthocyanin metabolism, and further work is required to establish whether the taxa highlighted in this chapter are directly involved in anthocyanin metabolism. It was particularly surprising that relationships were not highlighted between the microbiome at the functional level and the concentration of microbial metabolites such as catechol. However, it is likely that this is due to functional metagenomics data only providing information regarding the abundance of a gene in the metagenome and not its expression, limiting the conclusions that can be drawn from this data. Furthermore, few of the enzymes involved in anthocyanin metabolism have been characterised. Therefore, it would be recommended that future work may focus on characterising the microbial enzymes involved in the biotransformation of anthocyanins and their metabolites and the expression and/or activity of these enzymes in the gut in relation to anthocyanin metabolism *in vivo*. This may provide important information on why some individuals metabolise anthocyanins differently, how this may affect an

individuals' response to dietary anthocyanins, and could provide foundations for defining anthocyanin metabotypes.

Chapter 6

Spontaneous Degradation of Cyanidin

Chapter 6 : Spontaneous Degradation of Cyanidin

6.1 Abstract

Background: In Chapter 4 it was shown that anthocyanin breakdown in an *in vitro* model of the human colon was partly due to spontaneous chemical degradation. There is consensus within the literature that anthocyanidins will chemically degrade to form an A-ring and a B-ring product, in the case of cyanidin, phloroglucinaldehyde (PGA) and protocatechuic acid (PCA) respectively. However, there is conflicting evidence on whether these two compounds account for the full chemical breakdown, or if other compounds are produced in this process (Kay *et al.*, 2009; Woodward *et al.*, 2009). It is crucial to understand how anthocyanins degrade spontaneously as these products may have implications for the *in vivo* metabolism of anthocyanins and consequently any effects on human health.

Objective: The aim of the work presented in this chapter was to study the spontaneous degradation of cyanidin to establish whether PCA and PGA are the only spontaneous products obtained or if further compounds are produced as products of this reaction.

Methods: The spontaneous degradation of cyanidin was studied in phosphate buffer at a neutral pH at various concentrations using untargeted single-quad and TOF mass spectroscopy to identify the masses of products produced in this reaction.

Results: PCA and PGA were shown to be chemical breakdown products of cyanidin in agreement with the literature, accounting for up to 10% and 2% of cyanidin degradation in phosphate buffer respectively. However, six additional products many of which could not be identified were consistently detected, with the most abundant products having masses of 301 and 303 [M-H]. Additionally, factors such as buffer strength were shown to impact the extent of anthocyanin chemical degradation and the concentrations of products formed. Perhaps most interestingly, a product with a mass of 301 [M-H] that shared many chromatographic similarities with ellagic acid, however, this requires additional analytical work for accurate identification.

Conclusion and Future work: Although PCA and PGA were shown to be important chemical degradants of cyanidin, they did not fully account for its spontaneous breakdown. Spontaneous cyanidin degradation gives rise to a complex mixture of compounds, many of which remain

unknown. An important point of future work would be to determine the identity of these compounds, to understand if they may have bioactivity *in vivo,* and to investigate how they may be further metabolised post-consumption as this may have biological relevance to the health effects of eating anthocyanins.

6.2 Introduction

In Chapter 4 it was shown that degradation of C3G occurs partly spontaneously. Both PCA and PGA were produced as spontaneous products of C3G, however, these compounds did not account for the total C3G degradation observed, suggesting that additional compounds must be produced in this process as either intermediates or end-products. It is widely known that anthocyanins are a particularly unstable class of polyphenol with many factors leading to their degradation both *in vitro* and *in vivo*. These include light, pH, concentration, temperature, metal ions, enzymes, and oxygen, amongst others (Remini *et al.*, 2018). This instability means that anthocyanins per se have low bioavailability and understanding their chemical degradants is important to understand how anthocyanins can exert any biological effects, both directly through these products but also from their further metabolism *in vivo* (Kay, 2006; Kay *et al.*, 2005, 2009; Remini *et al.*, 2018; Woodward *et al.*, 2011).

Anthocyanins comprise a common 3-ring structure depicted in Figure 6-1. The specific type of anthocyanin is determined by the number and position of hydroxyl and/or methyl groups as well as the nature, number, position and acylation of the sugar moieties conjugated to the aglycone. In the absence of a linked sugar, the aglycone is known as an anthocyanidin. These variations in the structure of the anthocyanin and its aglycone dictate the stability of the compound (Castañeda-Ovando *et al.*, 2009; Keppler and Humpf, 2005; Remini *et al.*, 2018). Anthocyanins are stabilised by their sugar conjugation, and deglycosylation is the first step in anthocyanin catabolism, which most often occurs enzymatically but may also occur non-enzymatically (Cheng *et al.*, 2016; Kay *et al.*, 2009; Keppler and Humpf, 2005). Therefore studies on spontaneous degradation generally focus on anthocyanidins which are chemically volatile and rapidly degrade under physiological conditions (Kay *et al.*, 2009; Woodward *et al.*, 2011).
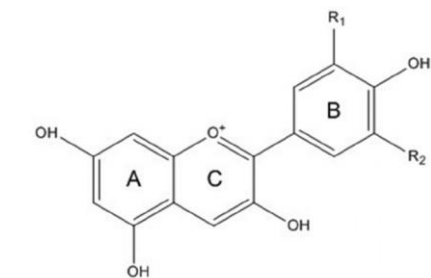
Anthocyanidin	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 6-1- The general chemical structure of anthocyanidins with substitutions on the B-ring highlighted that constitute the most common anthocyanidins in nature.

There is consensus in the literature that at a neutral to alkali pH anthocyanidins will degrade to form a common phenolic aldehyde derived from the A-ring (PGA) and a constituent phenolic acid, dictated by the hydroxylation and methylation pattern of the B-ring, (Keppler and Humpf, 2005; Woodward *et al.*, 2011). However, there is conflicting evidence regarding whether these two products account for spontaneous anthocyanidin degradation in full or if they are only one element of a more complex profile of chemical degradants (Kay *et al.*, 2009; Woodward *et al.*, 2011).

Cyanidin is the most extensively studied anthocyanidin in human, animal and spontaneous degradation studies, and is the most common anthocyanidin in nature. Kay *et al* reported that in phosphate buffer at physiological pH and temperature (pH 7.4 and 37°C), cyanidin rapidly degraded to produce PCA and PGA with the recovery of these two degradation products being equivalent for the loss of the parent compound (Kay *et al.*, 2009). Conversely, Woodward *et al* did not observe this equimolar recovery to PCA and PGA. Although they did observe PCA and PGA as spontaneous degradation products of cyanidin, they only accounted for ~ 24% loss of cyanidin in phosphate buffer and ~ 69% in water. Interestingly, a matrix effect dictated the course of degradation, whereby in water PGA increased continuously, but in buffer it only increased up to 6 hours before falling. They also investigated degradation of C3G under the same conditions and reported that it was considerably slower than the aglycone, with a small amount of C3G still present after 24 hours. Further to this, Woodward *et al* observed 3 unidentified compounds (in both matrices, for both the anthocyanin and the aglycone), contrasting with Kay *et al* who only report detection of PCA and PGA as cyanidin break-down products. Although these two publications describe similar experiments there are some notable differences. Kay *et al* used a far higher starting concentration of cyanidin and a stronger phosphate buffer and analysed degradation over a shorter time course (13.5 hours vs 24 hours) in comparison to Woodward *et al*. It is plausible that the difference in the

findings of these publications could be explained by PCA and PGA subsequently degrading after reaching a maximum concentration at 13.5 hours (where they total the starting material in the report from Kay *et al*). However, this can be excluded as Woodward *et al* took a measurement at 12 hours, and at this point PCA and PGA were at low levels, not equalling the proportion seen by Kay *et al*. Despite the differences in the methodologies, it is somewhat surprising that such similar reports state such startlingly different results. However, as anthocyanidins are unstable and affected by a large range of factors, including concentration, it is plausible that subtle differences such as these, could account for their drastically different findings, and highlights how small modifications in conditions can change the course of chemical breakdown (Kay *et al.*, 2009; Woodward *et al.*, 2011).

Fleshhut *et al* investigated cyanidin degradation in similar conditions to Woodward *et al*, at a similar concentration (160 µM) in a phosphate buffer (pH 7.4) at 37 °C. As observed by both Woodward *et al* and Kay *et al*, cyanidin disappeared quickly (within 60 minutes), and PCA was reported as a spontaneous product of both cyanidin and C3G. However, several unknown products with masses of 589, 605 and 587 were detected and assumed to be dimerisation products of two cyanidin units, although, this was not confirmed experimentally. These reports highlight that cyanidin degradation is complex, and the presence of masses greater than the parent compound indicates that there is likely dimerisation of cyanidin units and/or its breakdown products, which may have important implications for human and microbial metabolism (Fleschhut *et al.*, 2006).

Susceptibility of cyanidin based anthocyanins and the aglycone to spontaneous breakdown is strongly affected by glycosylation which considerably increases stability. This was highlighted by Hanske *et al* who observed C3G to be chemically more stable than cyanidin. However, similar to other reports they did observe PCA and PGA to be chemical degradants of both compounds (Hanske *et al.*, 2013). Triebel *et al* reported similar findings whereby they found that cyanidin based anthocyanins were far more stable than the aglycone in cell culture media with degradation taking place in both the presence and absence of cells. This study, like others, showed that PCA was formed at higher concentrations than PGA, they also reported the formation of unidentified products at 260, 290 and 520 nm, suggesting that PCA and PGA are not the only chemical degradants of cyanidin (Triebel *et al*., 2012). In addition to this it has been demonstrated that anthocyanin B-ring hydroxylation and methylation influences susceptibility to chemical degradation; and it has been observed that cyanidin (which only has two hydroxyl groups on the B-ring), has higher chemical stability than delphinidin and petunidin which have three hydroxyl groups, with one methylated in the case of petunidin (Kuntz *et al.*, 2015).

Cyanidin has been shown to degrade in saliva both enzymatically and spontaneously. Kamanpotana *et al*, showed that cyanidin glycosides in chokeberry extract degraded in heat-inactivated, cell-free

and enzyme-free saliva; although degradation was greatest in intact saliva. This shows that whilst there is an important role of chemical degradation in humans, enzymes and cellular activity are also involved in human anthocyanin metabolism. Interestingly, neither cyanidin, PCA or PGA were observed as products of cyanidin glycosides in this report. However, in agreement with other studies they showed the intrinsic instability of the aglycone, with this being rapidly degraded by > 90%, and similar to Woodward *et al*, PCA only accounted for a small amount of the aglycone (9.6% after 60 minutes). Furthermore, by obtaining accurate mass information, high resolution quadrupole time-of-flight (TOF) analysis suggested that chalcone glucosides of cyanidin are formed as open-ring intermediates, prior to hydrolysis of the glycosidic bond in agreement with the alternative pathway proposed by Markaskis in 1974 (Kamonpatana *et al.*, 2012; Markakis, 1974).

Studies investigating the contrast between microbial and spontaneous anthocyanin metabolism have exposed similarities and differences between these two modes of anthocyanin breakdown. For example, Hanske *et al*, demonstrated that in the absence of the microbiota C3G remains stable even up to 24.5 hours incubation. Similar to other studies, PCA was identified as a spontaneous product of C3G degradation, but at a lower concentration than was observed in the presence of a live microbiota (in the form a faecal slurry). They also identified both PCA and PGA as chemical degradants of cyanidin in the absence of a microbiota, however, again this was at a lower concentration than observed when C3G was incubated in the presence of a microbiota. Unlike other reports, they detected a further two chemical degradants of cyanidin and C3G with masses of 304 and 300. They proposed that the α-hydroxychalcone was the mass of 304 and that the mass of 300 may be the result of an oxidation of PCA with the A-ring forming a quinone methide and the B-ring a 1,2-quinone (Hanske *et al.*, 2013). These findings show that direct chemical degradants of cyanidin and its glycoside are likely subjected to further spontaneous reactions.

Whilst there is consensus between reports on spontaneous cyanidin anthocyanin/aglycone degradation in that PCA and PGA are undoubtedly products, and that the anthocyanin has higher chemical stability than the anthocyanidin, there are several inconsistencies in the literature. Different studies report detecting a different number of products, whilst some only report PCA and PGA, others report a range of products, often with masses greater than that of the parent compound, suggesting there is polymerisation of cyanidin and/or its products. Additionally, there is also variability in the proportion of starting cyanidin/cyanidin glycoside recovered after degradation, in product and parent material. There are subtle differences in how all of these reports were conducted, and given the instability of cyanidin it is likely that modest alterations in experimental conditions may change the path of degradation. In this chapter, the spontaneous degradation of cyanidin was investigated using untargeted mass spectroscopy to gain some understanding of the extent to which PCA and PGA are produced as chemical degradants and whether there are other masses that may be obtained from chemical degradation of cyanidin.

6.3 Objectives

The overall aim of the work in this chapter was to measure the spontaneous degradation of cyanidin to establish how much chemical degradation can be accounted for by PCA and PGA and to investigate the masses obtained in this process following findings in Chapter 4 highlighting that PCA and PGA are not the only spontaneous products of cyanidin based anthocyanin degradation. This was investigated in phosphate buffer at a physiological pH and temperature to provide a simple matrix to facilitate reliable detection of cyanidin degradants. The specific objectives of this work were:

- Investigate the extent of spontaneous cyanidin degradation accounted for by PCA and PGA, in phosphate buffer at varying concentrations, and in water.
- Characterise the spontaneous degradation products of cyanidin using untargeted mass spectroscopy and attempt to identity these metabolites.

6.4 Methods

6.4.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Dorset, United Kingdom) unless stated otherwise. All water used was 18 MΩ/cm Milli-Q water, and solvents were LC/MS grade.

6.4.2 Spontaneous Cyanidin Degradation (Full Scan Analysis):

Cyanidin degradation experiments were performed at 37°C in potassium phosphate buffer at pH 7.4 (100 mM or 10 mM) or water titrated to pH 7.4 with the dropwise addition of dilute NaOH. Cyanidin (Extrasynthese, Genay, France) degradation was investigated at three different concentrations, 1 mg/ml, 100 µg/ml and 43 µg/ml. Cyanidin was buffered to pH 7.4 in potassium phosphate buffer 1 minute prior to initial HPLC injection.

Sequential 10 µl HPLC injections were performed every hour for 15 – 24 hours onto a Kinetex XB-C18 100A, 2.6µ 100 x 4.6 mm column (Phenomenex, Macclesfield, United Kingdom) using an auto-sampler at 37°C connected to an Agilent 1100 series system (HP1100 Agilent Technologies, Waldbronn, Germany) equipped with G1956B single quadrupole mass spectrometer in negative mode and DAD with channels at 520 nm, 280 nm, 250 nm and 320 nm. The mobile phase (A) was 1% formic acid in water, and the mobile phase (B) was 1% formic acid in acetonitrile (Fisher Scientific, Loughborough, United Kingdom). The gradient used was: 2% B at 0 minutes, 10% B at 15 minutes, 15% B at 20 minutes, 30% B at 25 minutes, 50% B at 30 minutes, 50% B at 34 minutes, 2% B at 35 minutes and 2% B at 37 minutes. Collision gas temperature was 220 °C, gas flow 14 L min−1, nebulizer pressure 25 psi, and capillary voltage 3.5 kV. A control adjusted to pH2 with 1% HCl was run alongside all experiments controlled for temperature, time and pH. Cyanidin, PCA and PGA were

identified and quantified relative to pure authentic standards, unknown masses were identified in total ion current (TIC) chromatograms produced in single quadrupole mass spectroscopy but were not quantified.

6.4.3 Spontaneous Cyanidin Degradation (Targeted MRM and TOF analysis):

Cyanidin degradation experiments were performed as described previously. Sequential 1 µl UHPLC injections were performed every 15 minutes for 15 – 24 hours onto an ACQUITY UPLC HSS T3 1.8 µm (2.1 x 100 mm) column, equipped with a ACQUITY UPLC HSS T3 VanGuard Pre-column, 100Å, 1.8 µm, 2.1 mm X 5 mm (Waters, Wilmslow, United Kingdom) using an auto-sampler at 37°C connected to a Waters Acquity TQS-micro UHPLC-MS/MS system for targeted MRM analysis or a Waters Synapt G2si (TOF) coupled to a Waters Acquity UHPLC in negative mode and DAD with channels at 520 nm, 280 nm, 250 nm and 320 nm for untargeted analysis. The mobile phase (A) was 1% formic acid in water and the mobile phase (B) was 1% formic acid in acetonitrile. The gradient used was 3% B at 0 minutes, 3% B at 2 minutes, 20% B at 7 minutes, 95% B at 11 minutes, 3% B at 11.1 minutes and 3% B at 14 minutes. On both systems collision gas temperature was 220 °C, gas flow 14 L min−1, nebulizer pressure 25 psi, and capillary voltage 3.5 kV. A control adjusted to pH2 with 1% HCl was run alongside all experiments controlled for temperature, time and pH. Cyanidin, PCA and PGA were identified relative to pure authentic standards, unknown masses were identified in total ion current chromatograms produced in full scan mass spectroscopy and high resolution TOF mass spectroscopy, but were not quantified.

Note for targeted MRM analysis using the Waters Acquity TQS-micro UHPLC-MS/MS system the following MRM transitions were used. For cyanidin; 287/287 (CE − 5) in positive mode. For PCA; 153/109 and 153/91 (CE − 22) in negative mode. For PGA; 153/83 and 153/135 (CE − 26) in negative mode. For ellagic acid; 300/228, 300/200, 300/184, 300/144 and 300/116 (CE − 28) in negative mode.

6.4.4 Statistical Analysis

Statistical analysis was performed in GraphPad Prism (version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Independent samples T-tests were used to compare the means of the control and experimental samples due the parametric nature of the data and $p < 0.05$ was considered statistically significant.

6.4.5 Searches for Unidentified Masses

The human metabolome database (HMDB) version 4.0 (https://hmdb.ca/) was used to search for unknown masses detected in untargeted LC-MS protocols (Wishart *et al.*, 2018).

6.5 Results

Initially cyanidin degradation was investigated using a starting concentration of 100 μg/ml, under the same degradation conditions as reported by Kay *et al,* in 100 mM phosphate buffer at 37°C. Untargeted single quadrupole HPLC-MS/MS and DAD was used to quantify cyanidin, PCA and PGA against authentic standards over the 15 hour time course of the experiment. Untargeted HPLC-MS/MS facilitated the identification of additional masses with unknown identities and their peak area was monitored, however, quantification could not be performed for these compounds.

Several masses were detected during the spontaneous degradation of cyanidin beyond PCA and PGA. Furthermore, PCA and PGA only accounted for ~ 5.5% of cyanidin degradation. Figure 6-2 shows a chromatogram that was produced when a sample from the incubation solution containing cyanidin after 13 hours was injected onto the HPLC column, and the eluent monitored with a DAD detector in line with a MS detector. The chromatogram in Figure 6-2 is from the DAD output at 280 nm and shows multiple peaks. For every peak observed via DAD a full scan MS was generated (data not shown) which aided in peak identification, the masses identified are annotated on the corresponding peaks in Figure 6-2. Peaks corresponding to PCA (RT = 3.7 minutes) along with the substrate cyanidin (RT = 20.2 minutes) were clearly identified in the DAD. On the basis of relative DAD response, the PGA peak was a very minor component of the total non-cyanidin peaks observed in the sample, and its presence could only be verified using the XIC which separates ions of interest from the full mass spectrum improving the resolution of low level compounds. Several other peaks were identified at 6.9, 11.0, 12.5, 14.8 and 18.3 minutes with masses of 319, 569, 167, 303 and 301 respectively. Many of these peaks had peak areas larger than that of PCA or PGA indicating that they may be important intermediates and/or additional products produced in cyanidin degradation. Further to this, Figure 6-3 shows the concentration of cyanidin, PCA and PGA over 15 hours. Although PCA and PGA increase gradually as cyanidin degrades they remain at low concentrations not exceeding 20 μM, suggesting that the unknown products shown in Figure 6-2 may account for much of the loss of cyanidin observed over the 15 hours at physiological pH and temperature.
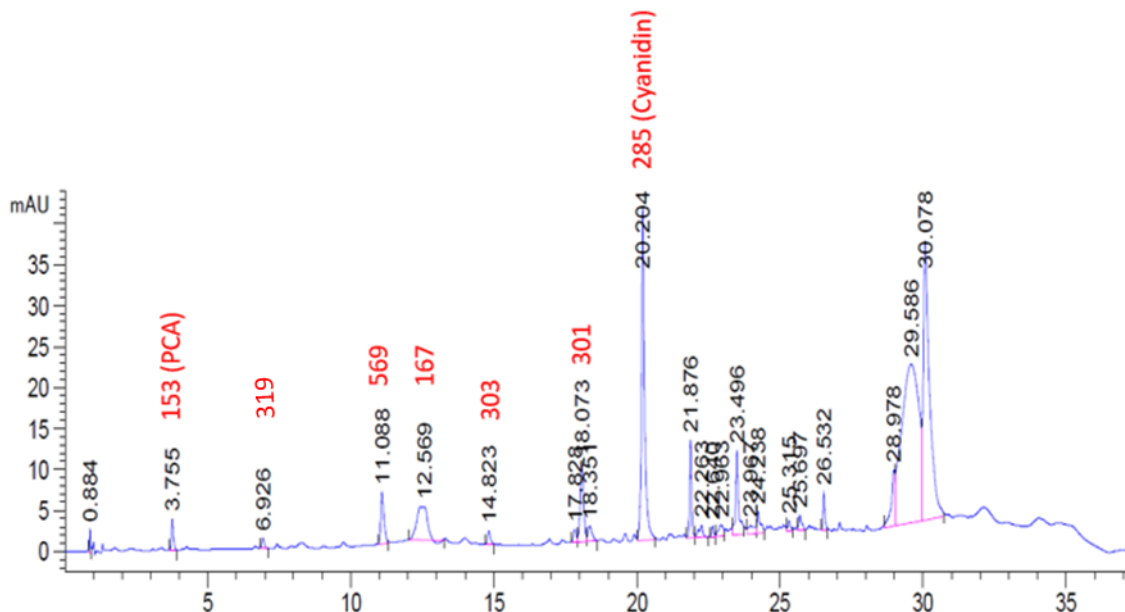
Figure 6-2- Typical chromatogram (DAD 280 nm) after 13 hours of 100 µg/ml spontaneous cyanidin degradation

Cyanidin 100 µg/ml (348 µM) was incubated in 100 mM phosphate buffer (pH 7.4) at 37°C. This chromatogram shows the masses present after 13 hours of degradation in the DAD 280 nm channel. Peaks with identifiable masses from the full-scan mass spectrum are labelled as M-H, those that are unlabelled gave many signals in the mass spectrum and could not have their mass established with certainty.
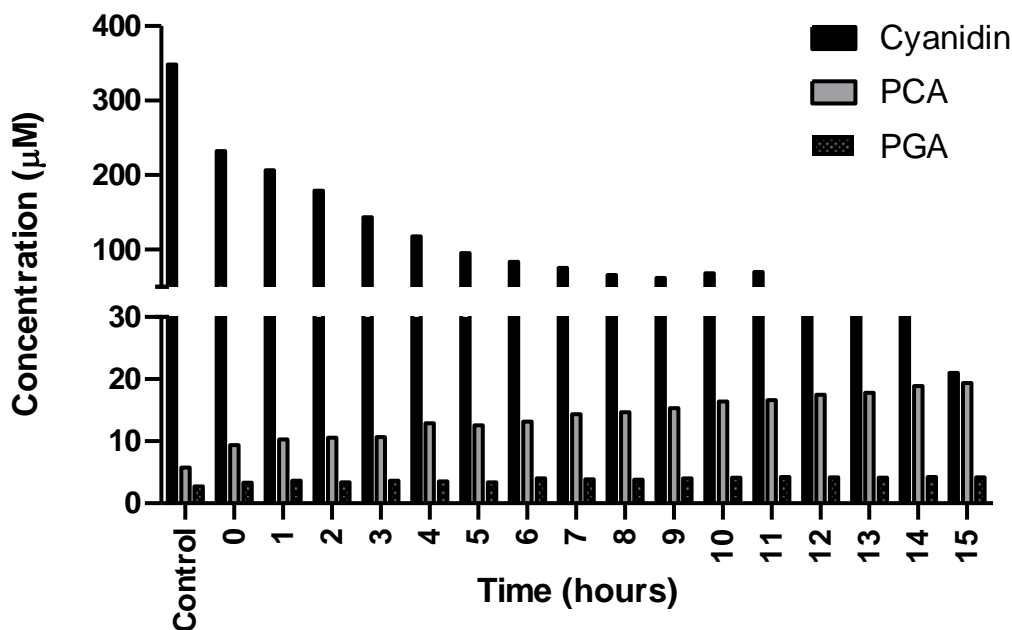
Figure 6-3- Concentration of cyanidin, PCA and PGA over 15 hours of 348 µM (100 µg/ml) cyanidin degradation

Cyanidin 100 µg/ml (348 µM) was incubated in 100 mM phosphate buffer (pH 7.4) at 37°C for 15 hours, PCA, PGA and Cyanidin were quantified with authentic standards each hour. The control constitutes 348 µM cyanidin in phosphate buffer acidified to pH 2.

Since Kay *et al* only reported the appearance of PCA and PGA when cyanidin was incubated in phosphate buffer under very similar conditions, the experiment was repeated with cyanidin at 1 mg/ml (the same concentration reported by Kay *et al*). Like at 100 µg/ml, cyanidin degradation starting at 1 mg/ml showed that although PCA and PGA are products of cyanidin, they only accounted for a small amount of the starting material. Again, many compounds were detected, most of which had a larger mass than cyanidin, contradicting Kay *et al*s report. Therefore, the experiment was repeated 5 times, with all materials (including HPLC vials) autoclaved to ensure that there were no bacteria present which could cause the formation of a complex mixture of compounds as was being observed. All replicates crucially yielded the same results, showing that spontaneous cyanidin breakdown is more complex than is currently reported in the literature.

Figure 6-4 shows typical TIC chromatograms at various time points throughout the experiment, and the reader should note that the masses displayed are not the only ones present, but are the only masses that gave strong enough signals to be shown in the TIC. An important observation here is that at 0 hours there is a large peak of 303 (M-H) at a RT of 14.1 minutes which disappeared from the TIC as known spontaneous breakdown products PCA and PGA appeared. This suggests that this compound with a mass of 303 (M-H) is an intermediate that is formed before cyanidin can form PCA and PGA. An unknown peak with a mass of 301 (M-H) with a RT of 18.3 minutes was also observed which initially increased and then decreased suggesting that this too may be an intermediate in the formation of PCA and PGA. Figure 6-4 shows that PCA (RT = 3.8 minutes) gradually increases over the duration of the experiment as cyanidin (RT = 20.2 minutes) progressively degrades, confirming that PCA is an important spontaneous product formed from cyanidin. Although PGA (RT = 9.4 minutes) was observed as a product of cyanidin it is only present at a low level, and consequently is not seen in the majority of TIC images presented in Figure 6-4. Furthermore, unlike PCA, PGA does not increase steadily throughout the experiment, instead an initial increase is observed followed by a decrease.

Consistent with earlier experiments that used 100 µg/ml cyanidin, with a 10-fold higher concentration (1 mg/ml), PCA and PGA only accounted for a small amount of cyanidin degradation, as shown in Figure 6-5. In both cases, PCA accounts for approximately 5% of the starting amount of cyanidin, and PGA even less (approximately 0.5%). This suggests that there are likely to be several important intermediates involved in the reaction and possibly more than two spontaneous end products.

Figure 6-4- Snapshots of typical HPLC-MS/MS TICs obtained in negative mode at various time-points during spontaneous cyanidin degradation

A starting concentration of 3.48 mM (1 mg/ml) cyanidin was incubated in 100 mM phosphate buffer (pH 7.4) at 37°C. A, shows a typical TIC at 0 hours; B, shows a typical TIC at 1 hour; C, shows a typical TIC at 3 hours; D shows a typical TIC at 6 hours; E, shows a typical TIC at 9 hours and F, shows a typical TIC at 12 hours. Peaks of known compounds (cyanidin (Cy), PCA and PGA) are annotated with their and mass [M-H] and identity which was confirmed with authentic standards; unknown peaks are annotated with their mass [M-H].

Figure 6-5- Concentration of cyanidin, PCA and PGA over 15 hours of 3.48 mM (1 mg/ml) cyanidin degradation

Cyanidin 1 mg/ml (3.48 mM) was incubated in 100 mM phosphate buffer (pH 7.4) at 37°C for 15 hours, PCA, PGA and Cyanidin were quantified with authentic standards each hour. The control constitutes 3.48 mM cyanidin in phosphate buffer acidified to pH 2. Data shown is mean ± standard deviation and n = 5.. The mean of the control was compared to the mean of the experimental cyanidin degradation vial using an independent samples t-test and p < 0.05 (*) was considered statistically significant.

An interesting observation was that these experiments gave rise to a purple precipitate that fell out of solution overtime and accumulated around the bottom of the tube. At the beginning of the experiment cyanidin appeared to be fully dissolved. However, this solution was a very deep colour and it was therefore difficult to determine if any undissolved particulate matter was present. Nevertheless, cyanidin is hydrophilic and highly soluble and consequently would be expected to be soluble in a phosphate buffer. Why this precipitate formed was unclear. Consequently, both supernatant and precipitate were analysed separately to establish any differences in composition, particularly with regards to amounts of PCA and PGA as it was possible that PCA and PGA were more abundant in the precipitate and may account for more of the starting material than previously thought.

LC-MS analysis of the precipitate, once it had been dissolved in DMSO, showed its composition (Figure 6-6) and the supernatant (Figure 6-2) had some similarities, but also multiple differences. Most notably, the precipitate had a very large peak of m/z 289 (M-H) which dwarfed the other peaks detected and was not present in the supernatant. This was only 3 mass units larger than

cyanidin, and due to its purple colour it is likely cyanidin underwent a reaction to make it insoluble and fall out of solution throughout the experiment. Crucially, PCA and PGA were not abundant in the precipitate (with PGA not being detected at all), showing that other intermediates and products are formed during spontaneous cyanidin degradation, meaning PCA and PGA are only minor products of cyanidin breakdown. The common peaks seen between all experiments, regardless of starting concentration are listed in Table 6-1.
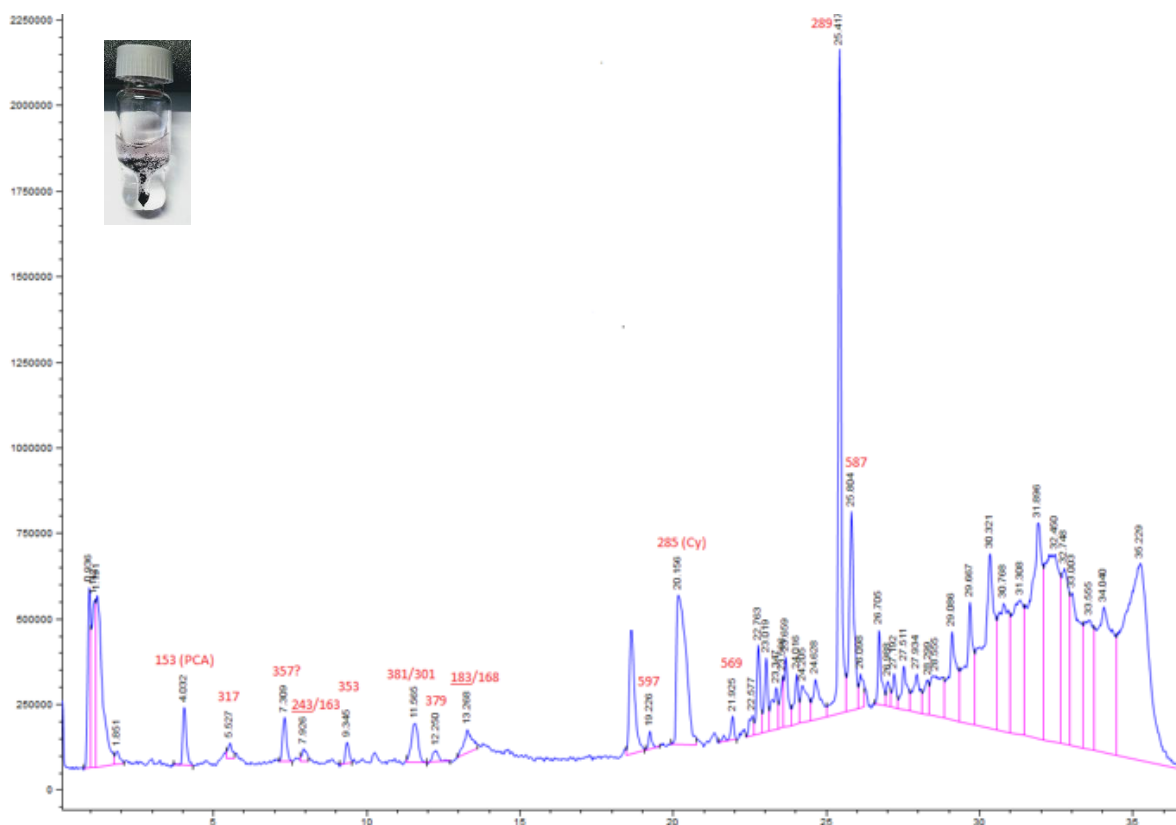
Figure 6-6- TIC of precipitate from cyanidin degradation
TIC of the precipitate (dissolved in DMSO) resulting from cyanidin degradation 3.48 mM (1mg/ml) in 100 mM phosphate buffer (pH 7.4) at 37°C, 24 hours after cyanidin degradation was initiated. Peaks are annotated with masses [M-H] and identity where this is known.

Table 6-1- Common masses identified during spontaneous degradation of cyanidin at 100 µg/ml and 1 mg/ml in 100 mM phosphate buffer (pH 7.4) at 37°C

M – H	Daughter Ion	Retention Time (minutes)	Name
285 (M – 2H)	-	20.2	Cyanidin
153	109	3.8	PCA
153	129	9.4	PGA
303	195	14.4	Unknown
319	165	6.9	Unknown
569	543	11.2	Unknown
167	130	12.7	Unknown
301	-	18.8	Unknown

Upon finding a much more complex mixture of compounds than anticipated based on the existing reports, further searches of the literature were conducted regarding work on spontaneous cyanidin degradation. The publication by Woodward *et al* was of interest, which also investigated the spontaneous degradation of cyanidin but at a much lower concentration, (150 μM; 43 μg/ml). Woodward *et al* investigated degradation in two mediums, 10 mM phosphate buffer and pH adjusted water (both at pH 7.4 and 37°C). They also saw PCA and PGA as products of spontaneous cyanidin degradation but at much lower concentrations than observed by Kay *et al*, and with a clear matrix effect between water and buffer. In phosphate buffer they showed that PCA and PGA accounted for 21% and 3% cyanidin degradation respectively, whilst in water they accounted for 39% and 30% for PCA and PGA respectively. This experiment was repeated and showed similar findings. In both matrices PCA and PGA were observed as products of cyanidin, with PCA accounting for ~ 10% and PGA accounting for ~ 2% cyanidin degradation in buffer and ~ 17% and ~ 12% in water respectively. Although the percentages are not identical to those reported by Woodward *et al* they show a similar pattern whereby PCA and PGA account for a higher percentage of cyanidin degradation in water than in buffer (Figure 6-8 and Figure 6-7). An interesting observation was that PGA increased progressively in water over the time course of the experiment but increased and then decreased in buffer. This matrix effect may be due to the lack of pH control in water meaning that throughout the duration of the experiment the pH reverted to being more acidic as demonstrated in Figure 6-9. Furthermore, unidentified products with masses of 319, 301 and 303 (M − H) were identified, however, the other unidentified products listed in Table 6-1 were not observed here, most likely due to the low concentration of cyanidin used in these experiments causing these compounds to be below the limits of detection.
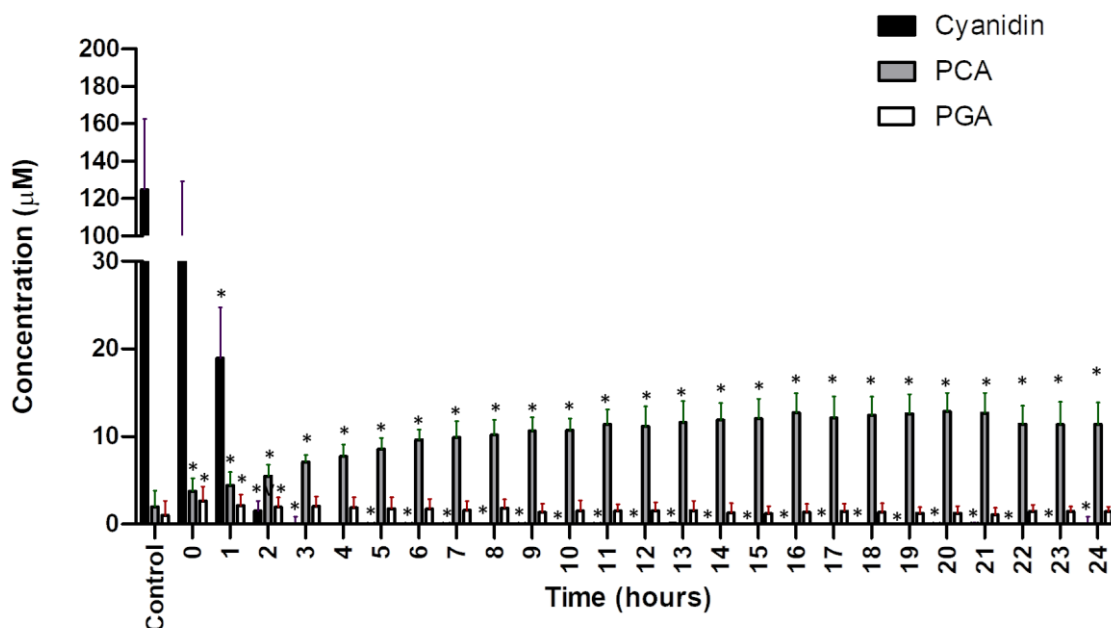
Figure 6-7- Concentration of cyanidin, PCA and PGA over 15 hours of 150 μM (43 μg/ml) cyanidin degradation in phosphate buffer

Cyanidin 150 μM (43 μg/ml) was incubated in 10 mM phosphate buffer (pH 7.4) at 37°C for 24 hours, PCA, PGA and Cyanidin were quantified with authentic standards each hour. The control constitutes 150 μM cyanidin in phosphate buffer acidified to pH 2. Data shown is mean ± standard deviation and n = 3. The mean of the control was compared to the mean of the experimental cyanidin degradation vial using an independent samples t-test and p < 0.05 (*) was considered statistically significant.
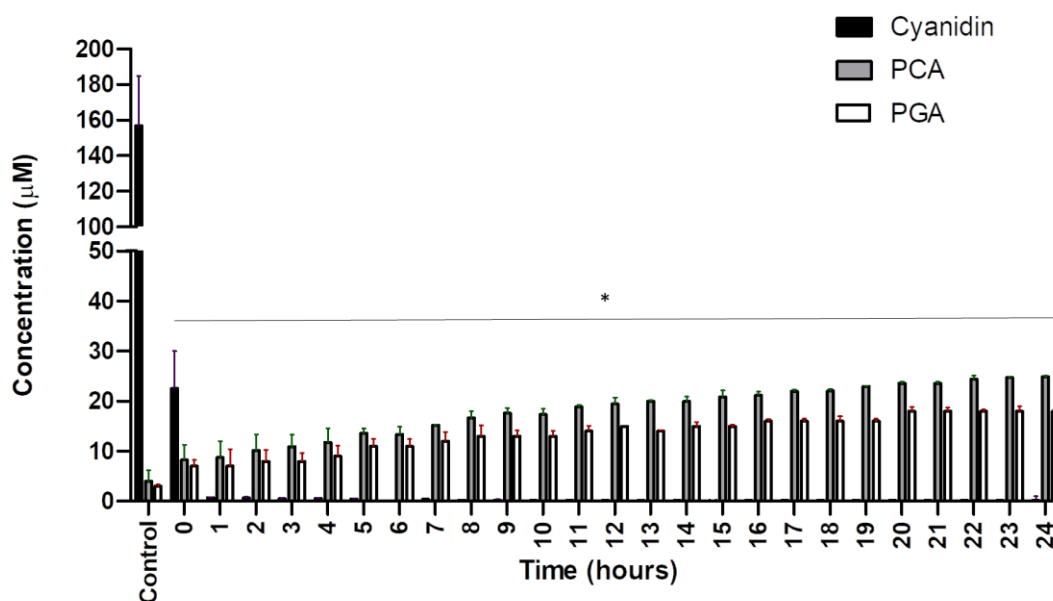
Figure 6-8- Concentration of cyanidin, PCA and PGA over 15 hours of 150 μM (43 μg/ml) cyanidin degratation in water

Cyanidin 150 μM (43 μg/ml) was incubated in water (adjusted to pH 7.4 with NaOH) at 37°C for 24 hours, PCA, PGA and Cyanidin were quantified with authentic standards each hour. The control constitutes 150 μM cyanidin in water acidified to pH 2. Data shown is mean ± standard deviation and n = 3. Mean concentrations of cyanidin, PCA and PGA were significantly different from the control for all time points. The mean of the control was compared to the mean of the experimental cyanidin degradation vial using an independent samples t-test and p < 0.05 (*) was considered statistically significant.
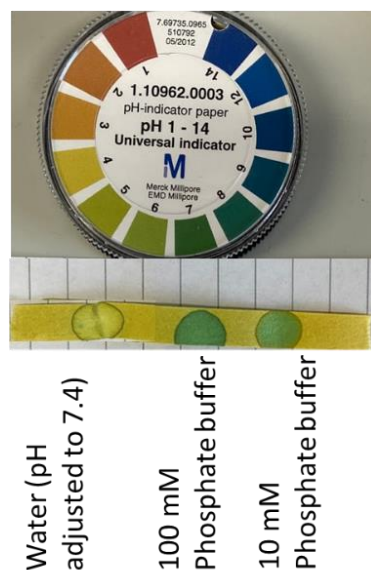
Figure 6-9- pH after 24 hours of 150 µM (43 µg/ml) cyanidin being incubated in water (adjusted to pH 7.4 with NaOH at 0 hours), 100 mM phosphate buffer, and 10 mM phosphate buffer at 37°C.

Having conducted a number of experiments to characterise cyanidin breakdown it was clear that (i) in all of these PCA and PGA were not the only products formed, (ii) PCA and PGA were relatively minor products of spontaneous breakdown and (iii) that there were a significant number of other compounds formed, several of which were of a higher molecular mass than cyanidin. However, the identity of the majority of these remained unknown. To obtain more accurate mass information on these compounds the experiment was repeated but using a TOF-MS. A drawback of this technique is that its sensitivity is significantly reduced. Therefore, TOF-MS was performed using 1 mg/ml cyanidin to maximise the likelihood of obtaining accurate mass information for as many of the compounds seen in the previous experiments as possible. Use of the TOF-MS meant changing from HPLC to UHPLC which provides better chromatographic separation of compounds and can be used with a shorter gradient. This meant that sample injections could be made more frequently over the time-course of the experiment, allowing closer monitoring of the masses produced throughout the spontaneous degradation process.

The TIC chromatograms only showed a limited number of compounds and did not detect either PCA or PGA, likely due to them not being at high enough concentrations; however, they could be seen in the XIC, with retention times of 3.8 and 6.9 minutes respectively (not shown). Figure 6-10 shows the TIC over three time points of the experiment (0, 9 and 15 hours). There are differences between

the time-points reflecting the progression of cyanidin breakdown. As cyanidin disappears over the course of the experiment, a peak appears with a retention time of 8.06 minutes and a mass of 301.03. Interestingly, this peak was not observed in previous experiments on the Agilent single quadrupole MS. Although an ion current for a mass of 301 had been observed previously, this eluted before cyanidin, meaning that it was a different compound, or the same compound was behaving differently between the two systems. Another interesting observation is that there was a peak with a mass of 303.05 eluting shortly before cyanidin, which is seen in the very first injection but then disappears by the end of the experiment (Figure 6-10). This mass matches that of the ion current of 303 seen in experiments performed on the Agilent single quadrupole MS (Figure 6-2 and Figure 6-4). In addition to masses depicted in TICs from the TOF-MS (Figure 6-10), several others were detected. A peak with a mass of 319.04 increased gradually throughout the experiment. This is of particular interest as a small peak with a mass of 319 appeared gradually throughout the time course of cyanidin degradation (Table 6-1) on the Agilent single quadrupole MS. Additional signals were detected, but were very weak and did not increase throughout the experiment and therefore are not reported here. PCA and PGA were only present at low levels, consistent with all previous experiments. PGA increased in the first few hours of the experiment but then decreased and was below the limit of detection by the end of the experiment. PCA was detected, however, issues with the detector for low level compounds meant that the concentration appeared to oscillate over time; this issue was observed with multiple weak-signal compounds, and for these it would be unreliable to draw conclusions regarding whether they increase or decrease as cyanidin degrades. It is likely that this oscillation was a result of these compounds being below the limits of quantification of the TOF-MS.

Following TOF-MS analysis, the human metabolome database (HMDB) was used to characterise candidates for key masses that were detected. This search was extensive but was limited to known compounds. The peak of 303.05 was putatively identified as the chalcone intermediate, an open C ring form of cyanidin which acts as an intermediate to its full breakdown, pictured in Figure 6-11. During the search for the compound with an accurate mass of 301.03 (M-H), which increased over the duration of the experiment, ellagic acid was highlighted which has a mass of 302.01. This compound immediately stood out as its chemical structure looked like two molecules of PCA which had dimerised, despite informally being known as di-gallic acid and consequently a reasonably unlikely candidate (Figure 6-12). Despite this, given that an authentic standard was readily available this was analysed using the waters TQS micro LC-MS/MS, under the same conditions used on the TOF-MS. The previously used TOF-MS was not available at the time, so instead a sister system manufactured by the same company with the same LC system was used, and the conditions used on the TOF-MS were closely reproduced. Because of the change in LC-MS/MS instrument it was essential to run ellagic acid alongside other standards with known retention times to give a reliable

indication of whether retention times had shifted as a result of the instrument change. PCA and PGA, which gave retention times of 3.8 and 6.9 minutes respectively on the TOF-MS were run alongside an ellagic acid standard on the TQS-micro. A direct infusion of ellagic acid gave 5 MRM transitions in negative mode. Notably, ellagic acid did not run in positive mode. Promisingly, ellagic acid gave a well-defined peak at 8.05 minutes (illustrated in Figure 6-13), matching the retention time of the peak with a mass of 301.03 from the TOF-MS analysis (Figure 6-10), and was positive for all five transitions providing convincing evidence that this compound could be ellagic acid. PCA and PGA also gave perfectly matched retention times to those observed on the TOF-MS, confirming that the chromatography from the TOF had been reproduced accurately on the Waters TQS-Micro UHPLC-MS/MS (Figure 6-13). Furthermore, if this compound was ellagic acid it was possible that the compound of 319 that forms throughout the experiment (although stays at a low level) may be ellagic acid monohydrate, a product of the hydration of ellagic acid, however this seemed unlikely. It should be noted that on the single quad MS the authentic standard of ellagic acid did not elute at the same retention time as the unknown compound of 301 which had been seen consistently eluting at 18.3 minutes (Figure 6-2, Figure 6-3 and Table 6-1), but, the ellagic acid standard did co-elute with cyanidin at 20.2 minutes. Inspection of the mass spectra for cyanidin from earlier experiments showed a signal of 301 [M-H] along with the signal of 285 [M-2H] for cyanidin (Figure 6-14), indicating that these two masses were co-eluting under the HPLC conditions used and only UHPLC could separate these masses adequately. Furthermore, this suggests that there are two different products with a mass of 301 [M-H] that may be produced during spontaneous cyanidin degradation.
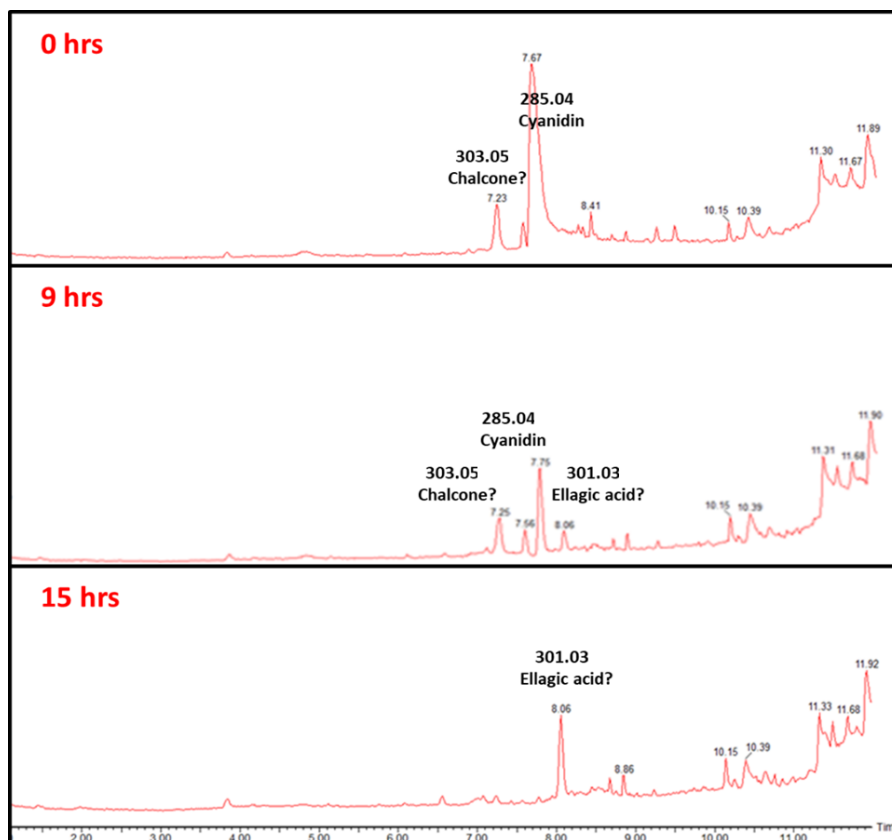
Figure 6-10- TIC of 1mg/ml cyanidin incubated in 100mM phosphate buffer (pH 7.4) at 37°C over 15 hours on the TOF-MS.
Peaks are labelled with their accurate mass [M-H] and proposed identity.
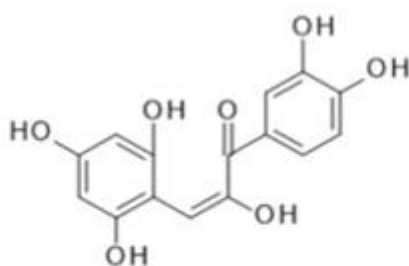
Formula Weight: 304.25
Exact Mass: 304.06
Molecular Formula: $C_{15}H_{12}O_7$

Figure 6-11- Chemical structure of cyanidin chalcone
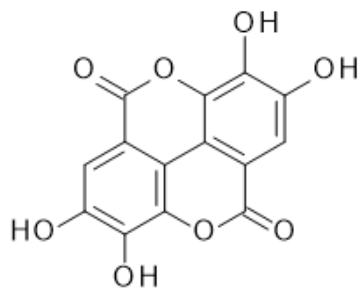
Formula Weight: 302.19
Exact Mass: 302.01
Molecular Formula: $C_{14}H_6O_8$

Figure 6-12- Chemical structure of ellagic acid
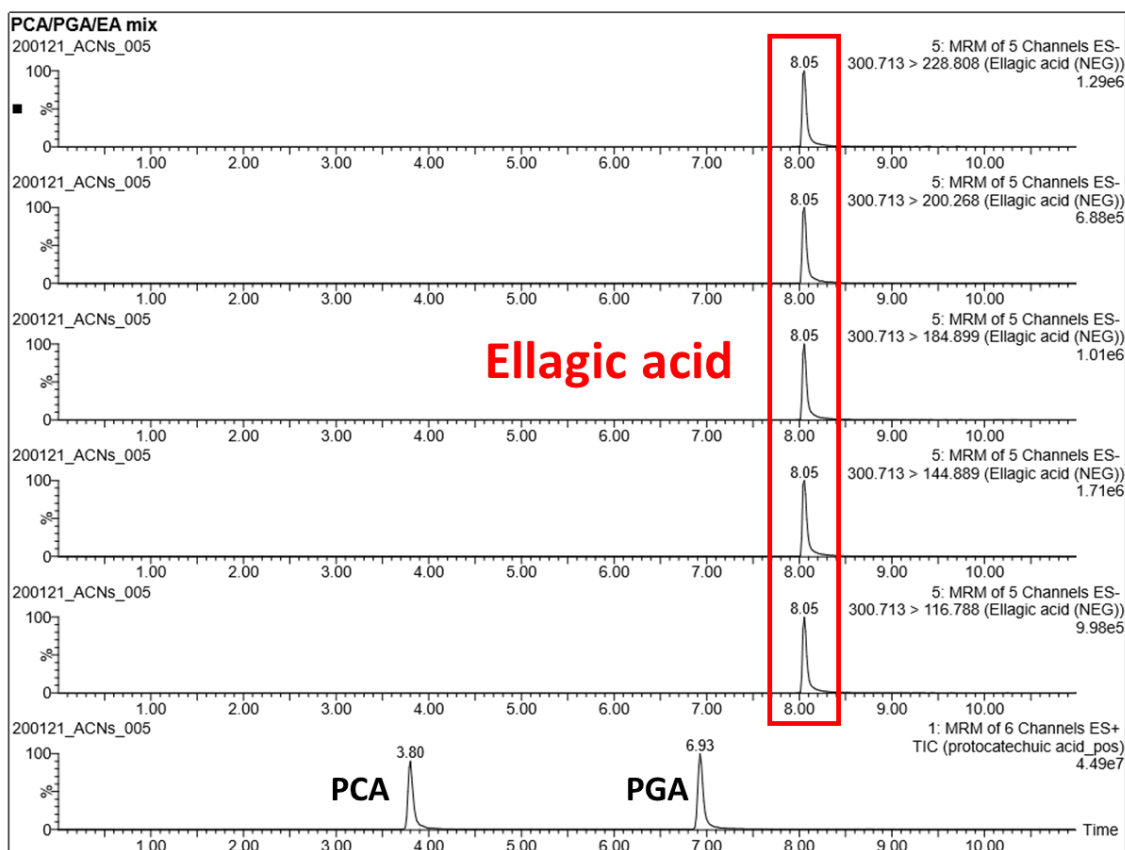
Figure 6-13- Chromatograms from the Water TQS-micro when running authentic standards of ellagic acid, PCA and PGA.

PCA, PGA and ellagic acid were run at 10 µg/ml in 100 mM phosphate buffer (pH 7.4). The chromatograms show the normalised detector response on the Y axis and time along the X axis. The retention times observed matched up exactly to those obtained on the Waters TOF-MS, with ellagic acid eluting at 8.05 minutes, PCA at 3.8 minutes and PGA at 6.9 minutes.
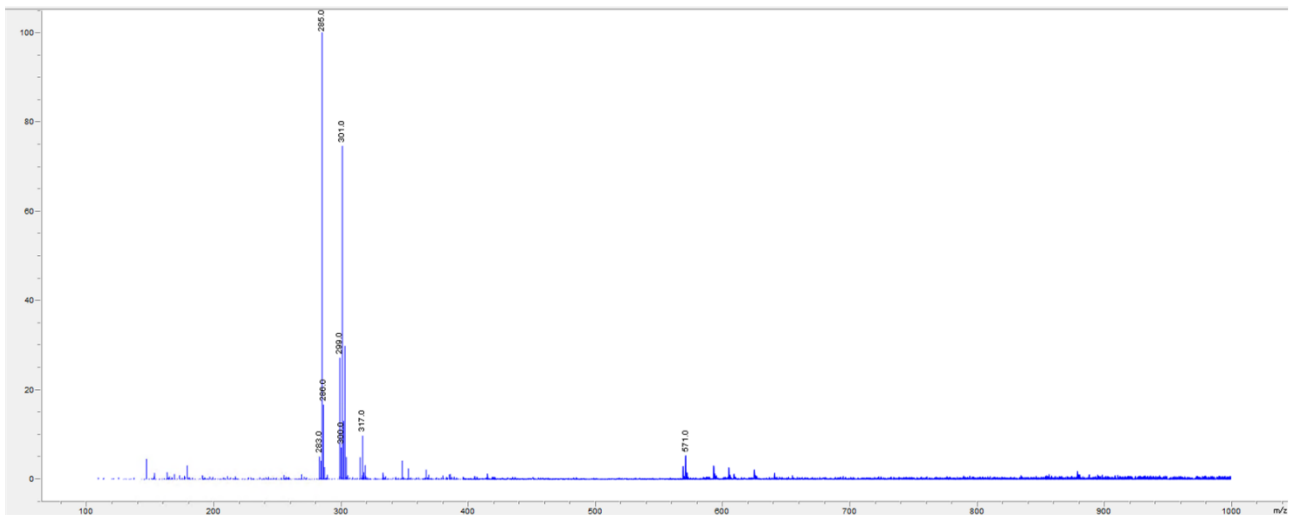
Figure 6-14- Typical mass spectra from the Agilent single quad MS for the peak with a retention time of 20.2 minutes that corresponds to cyanidin (285 [M-2H]), showing a signal of 301 after 3 hours of spontaneous cyanidin degradation (1 mg/ml) in 100 mM phosphate buffer (pH 7.4) at 37°C.

Overall, these results show that although PCA and PGA are chemical degradants of cyanidin in all of the conditions tested, they do not fully account for the spontaneous breakdown of cyanidin and other compounds are produced in this process either as intermediates or as end-products. This may include compounds such as chalcone and a major product which has several LC-MS features similar to ellagic acid. Although the evidence from the data provided in this chapter suggests that ellagic acid is a product of cyanidin degradation it is not obvious how this compound would be produced chemically from cyanidin or PCA and therefore further evidence would be required to test this hypothesis.

6.6 Discussion

The objective of this chapter was to study the spontaneous degradation of cyanidin under physiological temperature and pH to establish the extent to which PCA and PGA account for the degradation observed and whether other compounds are produced in this process, following findings in Chapter 4 highlighting that PCA and PGA are not the only spontaneous products of cyanidin based anthocyanin degradation. Under the various conditions tested, PCA and PGA only accounted for a minor fraction (~ 5.5 – 29% depending on matrix) of the starting cyanidin concentration and several unknown masses were repeatedly detected as either intermediates or end-products. Of these products, those of 303 and 301 [M-H] were the most abundant in terms of the peak areas obtained during LC-MS/MS. The data presented in this Chapter highlights some potential candidates for these unidentified masses, however, further experimental confirmation is required.

The data reported here show that under several incubation conditions PCA and PGA only accounted for a small amount of cyanidin degradation, and that the matrix cyanidin is in effects the extent to which PCA and PGA account for cyanidin breakdown. In 100 mM phosphate buffer PCA accounted for just ~ 5% cyanidin breakdown, ~ 10% in 10 mM buffer and ~ 17% in water, whilst PGA accounted for ~ 0.5%, 2% and 12% respectively indicating that buffer strength affects the path of cyanidin degradation. This is supported by Woodward *et al* who also reported that PCA and PGA accounted for a larger proportion of cyanidin degradation in water compared to buffer. It is proposed that this difference in the trajectory of cyanidin degradation is due to the lack of pH control in non-buffered pH-adjusted water meaning that the pH is not stabilised and can revert to being more acidic throughout the course of the experiment (Figure 6-9). Although this reversion in pH is not reported by Woodward *et al*, they also do not state that they measured pH during or after degradation had taken place and therefore it is likely that this change was not considered (Woodward *et al.*, 2009). Ultimately, this suggests that the environment of an anthocyanidin has a large bearing on the precise path of degradation and the concentration of PCA and PGA produced, in agreement with previous reports highlighting the fragility of anthocyanidin stability (Castañeda-Ovando *et al.*, 2009; Keppler and Humpf, 2005).

Under all the conditions tested, as expected, PCA increased gradually over the time course of the experiment as cyanidin degraded. However, PGA increased initially and then decreased, in phosphate buffer (pH 7.4), but increased over the entire time course in water. This suggests that PGA is unstable at a neutral pH and degrades and/or reacts with other products/intermediates in cyanidin degradation. This disputes findings from Kay *et al* who report PGA to increase progressively over 13.5 hours of cyanidin degradation, but is in agreement with Woodward *et al* who report PGA increasing and then decreasing in phosphate buffer and only increasing continuously in water (Kay *et al.*, 2009; Woodward *et al.*, 2009). It would be interesting to investigate the stability of PGA under physiological conditions, however, during anthocyanin/anthocyanidin degradation PGA does not exist in isolation and it is likely that PGA may require other compounds present in this process to react with.

Several unidentified masses were repeatedly detected, under all the incubation conditions trialled. In every experiment, no matter the starting concentration of cyanidin, strength of buffer, or instrumentation used for analysis, a mass of 303 (M-H) was detected which appeared and then disappeared over the time-course of the experiment. The trajectory of this mass suggests that it must be an intermediate formed in the process of cyanidin degradation before the formation of PCA and PGA (and/or other degradation products). This is in agreement with Hanske *et al* who detected a product of the same mass which they putatively identified as α-hydroxychalcone during the chemical breakdown of both cyanidin and C3G. Presumably this is the same compound detected in this analysis whereby an open C-ring form of cyanidin is produced as an intermediate prior to its

full breakdown (Hanske *et al.*, 2013). The general concept of the chalcone intermediate being an important step in anthocyanidin breakdown is reinforced by Goszcz *et al* who report observing, this 30 minutes into the spontaneous degradation of delphinidin (Goszcz *et al.*, 2017). Furthermore, it is known that chalcone is formed as pH becomes more neutral-alkali due to the nucleophilic hydration reaction of the flavylium ion, reinforcing that this a likely candidate for the mass of 303 (M-H) observed in cyanidin degradation experiments presented here (Fossen *et al.*, 1998).

Arguably the most interesting finding from this series of experiments was the consistent detection of an unknown compound that several pieces of evidence suggested was ellagic acid. TOF-MS experiments showed a large peak of 301 (M-H) increasing throughout the duration of the experiment as cyanidin decreased. This was particularly surprising as this mass was larger than that of the starting compound. Searches using the human metabolome database drew attention to ellagic acid depicted in Figure 6-12. This compound was particularly striking as at first glance it is somewhat like a dimer of PCA. However, ellagic acid is informally known as di-gallic acid, and it is indeed a dimer of gallic acid. This information made it seem unlikely that ellagic acid could be the unknown compound. However, an authentic standard of ellagic acid ran at the exact same retention time as the mass of 301 (M-H) observed during TOF-MS experiments, under the same chromatographic conditions. This provided compelling evidence that ellagic acid could potentially be a product of spontaneous cyanidin degradation, a concept that had not been considered previously in the available literature. Further supporting this evidence was the formation of insoluble material in HPLC vials during experiment using high (1 mg/ml) concentrations of cyanidin, as ellagic acid is a very insoluble compound that is consequently particularly difficult to work with. Nevertheless, it should be pointed out that the precipitate observed was deep purple in colour, did not comprise the mass of ellagic acid, and ellagic acid does not have this colour. Other compounds were flagged in HMDB searches such as quercetin, but, an authentic standard of this compound eluted from the UHPLC column much earlier than the mass identified during TOF-MS and this was consequently excluded as a candidate. A further compound presents a likely candidate for this mass. It is very plausible that this mass of 301 [M-H] is the anionic form of the chalcone intermediate, however, this could not be tested experimentally due to the lack of an authentic standard. One of the strange characteristics of this analysis is that when an authentic standard of ellagic acid was run on the single quad mass spectrometer it did not run at the same retention time as the peak of 301 (M-H) seen in early experiments (Figure 6-2) despite corresponding to the compound observed in TOF-MS. This left many questions as it indicated that either two different compounds were being detected in the different modes of analysis or that the same unidentified compound was behaving differently between the two systems. On the single quad mass spectrometer an authentic standard of ellagic acid eluted at the same retention time as cyanidin (20.2 minutes). This led to closer inspection of chromatograms from earlier experiments which

showed that in the mass spectra of cyanidin there was a strong signal of 301 [M-H] present in addition to cyanidin at later time-points. This highlights the importance of UHPLC which was able to separate this mass of 301 from cyanidin and bring this potentially important product to attention, where standard HPLC was unable to provide adequate separation. Furthermore, this co-elution may explain why this mass was not identified in previous studies investigating the spontaneous degradation of cyanidin, as these have often used HPLC rather than UHPLC. The presence of two masses of 301 suggests that both ellagic acid and the anionic form chalcone are potentially formed during the spontaneous degradation of cyanidin. If indeed ellagic acid was the product formed through the chemical degradation of cyanidin this may also facilitate identification of the mass of 319 [M − H] as a hydrated form of ellagic acid, however, this seems unlikely. Having said this, further evidence for ellagic acid as a product of cyanidin degradation was given by preliminary work on the spontaneous degradation of delphinidin (not presented) using the same TOF-MS protocol which identified a mass of 637 (M-H) which corresponds to gallagic acid, which is chemically very similar to ellagic acid, suggesting a common chemical pathway of the spontaneous degradation of these two closely related anthocyanidins. However, it should be acknowledged that these compounds have only been identified putatively and further experimental work would be required for full confirmation. It is therefore important that future work on cyanidin degradation uses NMR to obtain structural information on the products formed to achieve a full understanding of this process and the chemical degradants produced. In summary, evidence to suggest the unknown compound of 301 [M-H] was ellagic acid was (i) an authentic standard of ellagic acid gave the same retention time as this unknown compound on the TOF-MS, (ii) PCA is very similar to gallic acid and it is possible that PCA undergoes hydroxylation to form gallic acid which then dimerises to form ellagic acid, and (iii) the insolubility of unknown compounds formed during cyanidin degradation and of ellagic acid. On the other hand, evidence that does not support the notion that this unknown compound is ellagic acid include (i) no detection of gallic acid during cyanidin degradation, (ii) lack of plausible chemical mechanism for the formation of ellagic acid from PCA (or PGA) and (iii) no prior description of ellagic acid as a product of cyanidin breakdown in the scientific literature. It would be particularly interesting to deduce whether ellagic acid is a product that can be formed during the spontaneous breakdown of cyanidin as ellagic acid itself has poor bioavailability but is known to be metabolised *in vivo* to urolithins which have been linked to many health benefits such as being cardioprotective and anti-carcinogenic (Muku *et al.*, 2018). Therefore, if ellagic acid were to be a product of this reaction it would be of interest to search for urolithins in human samples following consumption of cyanidin based anthocyanins as this may have implications for any health effects of cyanidin consumption.

Although the work presented in this chapter agrees with the literature in that PCA and PGA are spontaneous products of cyanidin, and larger masses than the parent compound are detected

throughout the duration of degradation, there are several differences in the findings presented outside of those already discussed. Firstly, masses of 167 and 569 [M − H] were repeatedly identified which have not been reported previously. It was presumed that mass of 167 must be a small molecule formed through the degradation process however, no likely candidate compounds were found. It was presumed that the mass of 569 [M-H] was most likely a dimer of cyanidin units, however this was not confirmed experimentally. Masses of 589 and 605 were not observed in this series of experiments but have previously been reported by Fleschhut *et al* (Fleschhut *et al.*, 2006). Furthermore, among the three unidentified compounds detected by Woodward *et al* the only mass they were able to establish was of 198, and this mass was not observed in any of the conditions of cyanidin degradation trialled in this chapter(Woodward *et al.*, 2009). This highlights that the spontaneous degradation of anthocyanidins is a sensitive process and minor differences in conditions can alter not only the relative concentrations of core breakdown products but can also mean that different products are seen, explaining why different reports show differences in their findings. In future work it would be important to purify these products to establish their true chemical identities, however, there are significant barriers in this process due to the high commercial cost of anthocyanidins and instability of intermediates such as chalcone and possibly of some end-products that may make purification for NMR analysis difficult. Nevertheless, this would provide important insights into the spectrum of compounds that may be generated from spontaneous anthocyanidin degradation which could be investigated *in vivo* to gain a more comprehensive understanding of the full range of compounds that may be responsible for any health effects of an anthocyanin rich diet. It would also be of interest to investigate in detail the spontaneous degradation of the other 5 main anthocyanidins. Although preliminary work on delphinidin this was carried out, this was without replicates and consequently this data is not presented here. Overall this chapter provides some new insights into the spontaneous degradation of cyanidin building on previous reports, postulating new ideas of how this process may occur under the tested conditions.

6.7 Conclusion

Overall the data in this chapter shows that (i) PCA and PGA are important chemical degradants of cyanidin, in agreement with other reports (Fleschhut *et al.*, 2006; Hanske *et al.*, 2013; Kay *et al.*, 2009; Woodward *et al.*, 2009). But that (ii) PCA and PGA only account for a small fraction of the cyanidin that has been transformed/degraded to give other compounds, and (iii) that these other compounds include masses of 303, 301 and 319 [M-H]. The work presented in this chapter provides some offering of potential compounds that may be involved in this process, however, further experimental confirmation is required. Future work should focus on obtaining an unequivocal

identification of the various unknown compounds of cyanidin degradation, and expanding this to other types of anthocyanin and their aglycones.

Chapter 7

General Discussion

Chapter 7 : General Discussion

7.1 Summary of Findings

The overall aim of the research presented in this thesis was to investigate the *in vivo* metabolism of two different sources of anthocyanins (black rice extract, mainly dihydroxy B-ring cyanidin-type anthocyanins; and bilberry extract, mainly trihydroxy B-ring delphinidin-type anthocyanins) and explore any bidirectional relationship between anthocyanin metabolism and the gut microbiota. The main findings of the work presented are summarised as follows.

- Anthocyanins are extensively metabolised *in vivo* to give rise to a structurally diverse range of compounds including hydroxybenzoic, hydroxyphenylacetic and hydroxycinnamic acids, hydroxybenzaldehydes and hydroxybenzenes, all of which are also found as phase-2 conjugates (O-sulphates, O-glucuronides, and O-methylated). To date the largest number of human metabolites arising from black rice and bilberry anthocyanins are reported here.

- Catechol and its phase 2 conjugates are major metabolites of black rice anthocyanins, and 5-hydroxyferulic acid has been identified for the first time as a human metabolite of bilberry anthocyanins.

- There is high inter-individual variation in both *in vivo* anthocyanin metabolism and in *in vitro* microbial metabolism of anthocyanins, whereby individuals fall into different metabotypes according to the specific metabolites they produce, and their relative concentrations. Furthermore, individuals tend to display a similar trajectory of anthocyanin metabolism in terms of their total excreted anthocyanins and metabolites (in urine) after consumption of different types of anthocyanins.

- Several anthocyanin metabolites are produced microbially and are not generated spontaneously, including catechol, phloroglucinol, pyrogallol, dihydrocaffeic acid, dihydroferulic acid, 4-methyl catechol and 4-hydroxybenzoic acid. However, the observed concentration of these compounds largely depends on the individual microbiota they are exposed to.

- PCA and PGA are important products of cyanidin that are formed chemically, however, these compounds are mainly produced microbially *in vivo*. Furthermore, PCA and PGA are not the only spontaneous products of cyanidin and several other compounds are produced, either as intermediates or end products, that may be metabolised enzymatically *in vivo* to give rise to metabolites that are not yet known.

- At the taxonomic level, features of the gut microbiota are associated with anthocyanin metabolism both *in vivo* and *in vitro*. The data in this thesis highlights several microbial genera and species that may act synergistically in the multi-step process of anthocyanin metabolism, and highlights some of the metabolites they may have a role in producing.
- Different characteristics of the microbiome may be associated with different metabolic parameters of the same compound i.e. different species of microbes may be associated with the time at which a metabolite is first detected and its Cmax.
- Four weeks of daily anthocyanin consumption was only associated with very minor changes in the composition of the microbiota and observed high inter-individual variability in the microbiome of individuals made it difficult to establish whether anthocyanins had any significant impact on gut microbial populations.

7.2 Novelty of the Thesis

There are several reports on the metabolism of anthocyanins, however, the work presented in this thesis goes beyond previous reports through its development of analysis methods that covered the most extensive range of known and predicted anthocyanins metabolites and their phase 2 conjugates reported to date. Previous reports tend to focus on either the analysis of anthocyanins or their metabolites, and frequently combine these analyses in one method which compromises the sensitivity of the analytical method for multiple compounds due to the vastly different physiochemical properties of anthocyanins and their metabolites. Furthermore, MRM which is the preferred LC-MS/MS application for quantification of anthocyanin metabolites has a finite capacity for compounds that may be included in any analytical method, and consequently phase 2 conjugates have frequently been neglected in studies on anthocyanin metabolism. This thesis describes the optimisation of state of the art methods for the identification of anthocyanins and their metabolites in biological samples, including phase 2 conjugates of both anthocyanins and metabolites. Furthermore, unlike previous methods, the method described in this thesis facilitated the chromatographic separation of positional sulfate isomers (De Ferrars *et al.*, 2014c). The application of this comprehensive analysis method facilitated the identification of a large number of anthocyanin metabolites some of which have not been reported previously. Catechol has frequently been overlooked by studies on anthocyanin metabolism but here (and in a collaboration with Jokioja *et al*) has been shown to be a major anthocyanin metabolite produced *in vivo* (Chapter 3) with microbial origin demonstrated by Chapter 4 (Jokioja *et al.*, 2021). Furthermore, this is the first study to report 5-hydroxyferulic acid as an anthocyanin metabolite *in vivo*. In addition to this, phase 2 conjugates of previously reported anthocyanin metabolites have been putatively identified in this analysis for the first time, and highlight that phase 2 metabolism is a major process

undergone by anthocyanins and their metabolites *in vivo*. No other study has reported the large number of phase 2 conjugates putatively identified in this analysis.

Other reports have investigated the microbial metabolism of anthocyanins *in vitro* using faecal slurries (Aura *et al.*, 2005; Fleschhut *et al.*, 2006; Hanske *et al.*, 2013). However, no previous reports have done this in the context of a large human intervention study where *in vitro* microbial metabolism of anthocyanins by faecal samples has been compared to *in vivo* metabolite data of the same individuals. This thesis uncovers a previously undescribed relationship between the capacity of an individuals' faecal microbiota to metabolise anthocyanins and their urinary excretion of the same metabolites after a dietary intervention where the same anthocyanin source was consumed. This demonstrates for the first time that *in vitro* microbial metabolism of anthocyanins mediated by faecal samples has biological relevance to the metabolites produced *in vivo*. Furthermore, what is demonstrated here but not in other reports, is the distinct differences in the metabolism of anthocyanins by different faecal samples, demonstrating that the specific microbiota that anthocyanins are exposed to largely dictates the metabolite profile obtained and likely any health effect of anthocyanin consumption.

In recent years' interest has grown in the relationship between anthocyanins and the gut microbiota. Reports have suggested that anthocyanin consumption can modulate gut microbial populations and that the composition of the gut microbiota largely dictates how anthocyanins are metabolised. However, research in this field is still in its infancy and much remains to be understood regarding the extent of this relationship and how it may influence the health effects of anthocyanins. The work in this thesis goes beyond the current literature by investigating the microbiome in relation to *in vivo* anthocyanin metabolism and *in vitro* microbial anthocyanin metabolism for the same individuals. Chapter 5 highlights several microbial taxa that are associated with the urinary excretion of major anthocyanin metabolites, and suggests that numerous microbes may act in concert synergistically in the multi-step process of anthocyanin metabolism. This is the first report to provide associations between features of the gut microbiota and the production of anthocyanin metabolites *in vivo* such as catechol, pyrogallol and dihydrocaffeic acid amongst many others. This provides insights beyond the current literature into how the microbiome may contribute to inter-individual variation in anthocyanin metabolism. In addition to this, Chapter 5 shows that different taxa are associated with different metabolic parameters of the same metabolite in some cases, for example, *Akkermansia* and *Terrisporobacter* were positively associated with the concentration of pyrogallol produced in the *in vitro* colon model but not its early production, instead *Lachnoclostiridium, Ruminococcus_torques_group* and *Streptococcus* were positively associated with time of first detection. This is the first report to show that different microbes may be related to the production of anthocyanin metabolites at different stages in metabolism, and emphasises the importance of considering anthocyanin metabolite profiles over

a time course to provide the most comprehensive understanding of how the microbiome may influence anthocyanin metabolism.

No large effects of either bilberry or black rice anthocyanin consumption on the gut microbiota profile of individuals were observed in this study. And there is conflicting evidence in the literature regarding whether anthocyanins modulate the composition of the gut microbiota (Faria *et al.*, 2014; Gu *et al.*, 2019; Igwe *et al.*, 2019; Zhang *et al.*, 2016; Zhu *et al.*, 2018). Nevertheless, microbiome analysis did show some very minor effects of black rice anthocyanin consumption on the composition of the gut microbiota that have not been reported previously. For example, *Eubacterium ramulus, Phascolarctobacterium succinatutens* and *Blautia obeum* were slightly decreased post black rice anthocyanin consumption, whilst *Flavonifractor plautii* was slightly increased. However, it should be noted that these effects were very small, and not consensus across the study population. Consequently, there is insufficient evidence in this thesis to conclude whether or not anthocyanins were responsible for these minor changes, however, these would be important points of further investigation.

Overall the work presented in this thesis provides new insights into the human metabolism of anthocyanins *in vivo*, highlighting the role of the gut microbiota in this process and how this may give rise to inter-individual variation. This thesis identifies the most extensive range of bilberry and black rice anthocyanin metabolites derived from humans *in vivo* compared to any other report known by the author, and shows that several of these metabolites are microbially derived, this information can be taken forward to predict additional metabolites and to investigate mechanisms of potential bioactivity. This is the first study to explore the association of specific anthocyanin metabolites with features of the microbiome *in vivo*. The data presented provides new insights into the specific taxa that may be involved in the production of anthocyanin metabolites. This provides valuable information to be taken forward to investigate the specific microbial biotransformation's of anthocyanins and their metabolites that take place *in vivo,* and will facilitate the future understanding of microbial metabolic pathways that are critical to the complex multi-step process of anthocyanin metabolism. Ultimately this thesis advances knowledge in the field by increasing our knowledge on the metabolites that are derived from bilberry and black rice anthocyanins *in vivo* and providing new insights into the role of the gut microbiota in this metabolism beyond previous reports. The data presented highlights several previously undescribed relationships between the microbiome and anthocyanin metabolites, which may form the basis of future research in this area.

7.3   Limitations of the Thesis

Despite the many strengths of the work presented in this thesis and new insights provided regarding anthocyanin metabolism and the role of the gut microbiota in this process, there are

several limitations. Firstly, although the analytical method for the quantification of anthocyanins and their metabolites in biological samples goes beyond previously published methods by its inclusion of a large number of phase 2 conjugates, it must be acknowledged that authentic standards are not available for most of these compounds. Consequently, identification of these compounds is only putative and quantification may not be accurate as we do not know the response factor of such compounds in the absence of authentic standards. Furthermore, due to limitations of LC-MS/MS instrumentation not all possible phase 2 conjugates could be included in the analytical methods, sulfates and glucuronides for all compounds were included, but, unfortunately di-sulfates, di-glucuronides and sulfo-glucuronides could not be included without compromising the MS scan time and reducing the data-points per peak which would have led to unreliable quantification.

A limitation of the human study is that volunteers did not consume a controlled diet over the dietary intervention or on their study days. Although participants were asked to restrict their anthocyanin intake over the duration of the study the background diet of participants would have varied considerably among individuals and was not accounted for. Many anthocyanin metabolites can be derived from several dietary sources other than anthocyanins and this can make it particularly difficult to establish the extent to which metabolites may have originated from the fed anthocyanins. This was a particular problem for hippuric acid which presented at high levels in all urine samples irrespective of treatment due to its wide range of metabolic sources. It would have been beneficial for participants to consume the same low phenolic meal preceding study days to reduce the background level of phenolics in biological samples and to reduce inter-individual variation, however, this would have been difficult to enforce and would have likely hindered recruitment of volunteers. Alternatively, it may have been beneficial to ask participants to complete a food diary over the duration of the study to gauge their consumption of phenolic compounds in their habitual diet. Although this would not have mitigated inter-individual variability in the habitual diets of participants it would have been helpful information to consider, particularly in cases where some phenolics were present at high levels in pre-intervention samples. Furthermore, despite clear increases in anthocyanins and their metabolites in biological samples, not taking habitual diet into consideration when recruiting participants and the lack of dietary control during the intervention were weaknesses of the study.

A further limitation of the human study was that urine samples were only taken as 24 hour urine collections. Although this provides an excellent overview of anthocyanin metabolism whereby late metabolites are collected from the previous study days and early metabolites are collected on the final study day there are several weaknesses to this method. Firstly, pooling urine over a 24 hour period can result in low level metabolites becoming very dilute and below the limits of detection. Secondly, 24 hour urine collections do not allow data to be collected regarding the pharmacokinetic

profiles of anthocyanin metabolites. Another limitation of the human study is that plasma samples were taken 24 hours after final anthocyanin consumption, anthocyanins and their metabolites only reside in circulation transiently and at low concentrations, and this likely explains why anthocyanins were not detected in plasma and why no statistically significant increases in anthocyanin metabolites were observed. The anthocyanin extracts fed in the human study, although highly purified, did not contain labelled anthocyanins. This ultimately means that anthocyanins and the metabolites derived from them were not traceable. This was a particular problem for metabolites that were also present at high levels from the habitual diet such as hippuric acid, and for bilberry where the anthocyanins fed were less bioavailable and a more diverse anthocyanin profile meant more metabolites were observed with smaller relative increases. This made it difficult in some instances to know exactly what metabolites, and the proportion, that was derived from the fed anthocyanins, although estimations could be made by subtracting pre-intervention samples this was imperfect due to the lack of a controlled meal being given preceding study days. Although anthocyanins and their metabolites were increased by both bilberry and black rice anthocyanin extracts, lack of traceability of these compounds was a limitation of the data reported in this thesis.

A challenge faced in this study was that the study population exhibited very high inter-individual variation in the taxonomical composition of their microbiomes which ultimately made it very difficult to establish whether any modest changes in the microbiome could be attributed to the fed anthocyanins. This is not the only study to report that differences in the microbiome of study participants compromised the ability to detect differences (Healey *et al.*, 2017; Zhang *et al.*, 2016). Screening participants based on their baseline microbiome may have been beneficial to obtain a less variable study population, that would have facilitated more reliable detection of whether anthocyanin treatments exerted effects on the microbial composition of the gut microbiota. Although this would not have altered the size of observed effects it may have increased the power to detect statistically significant differences.

The work presented in this thesis provides important insights into microbial metabolism of anthocyanins and how this may vary between individuals. However, a limitation of this work is that it used faecal samples that were collected up to 6 hours before processing and preservation. This meant that samples were exposed to an aerobic atmosphere for varying amounts of time which would have been detrimental to the survival of some anaerobes (Brusa *et al.*, 1989). Ultimately, this would have led to some compositional change in the microbial communities present (compared to *in vivo*) and may have caused some changes in the microbial metabolism observed during *in vitro* fermentation compared to *in vivo*. However, combating this issue would have been extremely difficult and would have led to logistical issues. It may have been possible to request that participants bring faecal samples in shorter time-frame, however, due to limited availability of microbiology safety cabinets it would not have always been possible to process samples instantly,

additionally, it would be impossible to avoid any aerobic exposure of samples and any length of time in an aerobic atmosphere may affect the survival of anaerobic microbes.

7.4 Recommendations for Future Research

This thesis provides new insights into the metabolism of bilberry and black rice anthocyanins and makes new observations on the role of the gut microbiota in this process and how it may vary between individuals. However, much still remains to be understood regarding the hypothesised bi-directional relationship between anthocyanin metabolism and the gut microbiota, and in the authors opinion the research topics discussed in the proceeding section must be at the forefront of future research.

The present thesis describes optimised SPE and UHPLC-MS/MS methods for the quantification of a vast number of anthocyanins and their metabolites. However, it would be naïve to consider the methods put forward here as final to be taken forward for all future studies on anthocyanin metabolism. As discussed previously, a limitation of the method developed in Chapter 2 is the lack of authentic standards for phase 2 conjugates, as more standards become available it would be recommended to revisit method development for the validation of these compounds. Furthermore, as LC-MS/MS technology advances and more MRM transitions can be included in analytical methods the number of compounds included would preferably be expanded to include more variations of phase 2 conjugates and to include compounds that may be identified in future studies on anthocyanin metabolism that were not considered in this thesis. Furthermore, method development showed problems with current micro-elution platforms when working with compounds that have a diverse range of polarities. However, as micro-elution sorbents are improved to tolerate a wider range of polarities it would be beneficial to re-optimise the extraction method for micro-elution to increase throughput and reduce the requirements for large ≥ 1 ml sample volumes.

There is an abundance of literature linking anthocyanins to a range of health benefits, an overview of this is given in Chapter 1. However, the data presented in Chapter 3 and 4 (in addition to other reports in the literature) shows that anthocyanins have poor bioavailability and are extensively metabolised to give a diverse profile of metabolites which have potential bioactivity (De Ferrars, Czank, Zhang, *et al.*, 2014a). Early studies that have sought to investigate how anthocyanins exert any health effects have frequently treated cells with high concentrations of anthocyanins, however, these studies are not physiologically relevant. This is because, although very low levels of anthocyanins do reach circulation this is for a very short period of time ≤ 6 hours and at very low concentrations (De Ferrars, Czank, Zhang, *et al.*, 2014a; Jokioja *et al.*, 2021; Nurmi *et al.*, 2009). Therefore, studies that treat cell lines with high concentrations of anthocyanins cannot be used to aid our understanding of how anthocyanins may exert any physiological effect *in vivo*. In more

recent years, it has been accepted that due to their poor bioavailability, and extensive metabolism, anthocyanins are mostly found in circulation as their metabolites and it is these metabolites that are likely responsible for any health effect of anthocyanin consumption (Czank *et al.*, 2013; De Ferrars, Czank, Zhang, *et al.*, 2014a; Kay, 2006; Kay *et al.*, 2005, 2009; Rio *et al.*, 2013; Rodriguez-Mateos *et al.*, 2014; Woodward *et al.*, 2011). Some studies have highlighted PCA as an anthocyanin metabolite and subsequently many studies have investigated the effects of PCA on cell lines (Vitaglione *et al.*, 2007). However, many studies have done this using high concentrations that are not physiologically relevant and therefore have limited validity in providing any understanding of bioactivity of PCA *in vivo* (Kakkar and Bais, 2014; Youdim *et al.*, 2000). Furthermore, the work presented in this thesis, and from other studies, suggests that although PCA is an anthocyanin metabolite, it is predominantly present in its sulfate-conjugated form, and other metabolites account for a higher proportion of the overall metabolite profile (De Ferrars, Czank, Zhang, *et al.*, 2014a; Jokioja *et al.*, 2021). For example, catechol sulfate was highlighted in Chapter 3 as a major anthocyanin metabolite. However, studies on the bioactivity of catechol are scarce, and some reports show that catechol may be cytotoxic and have deleterious effects on health, suggesting that anthocyanin consumption may in some cases exert negative biological effects (Pereira *et al.*, 2004; Schweigert *et al.*, 2001). However, to the best of my knowledge no studies have investigated the bioactivity of catechol's phase 2 conjugates. It would be recommended that future studies which focus on deducing the potential mechanisms through which anthocyanins exert any biological activity investigate metabolites such as catechol sulfate which have been highlighted as some of the most abundant anthocyanin metabolites produced in humans *in vivo*. Although authentic standards of phase 2 conjugates are scarce and this remains a barrier in studying their bioactivity, as more standards become commercially available it is important that this is investigated, as this thesis has demonstrated that phase 2 conjugates of anthocyanin metabolites are the dominant form *in vivo*. Furthermore, it is important that future research uses physiologically relevant doses of metabolites of interest to ensure findings are as representative as possible, to prevent reports of biological effects being made of the basis of data obtained under conditions that cannot be replicated *in vivo*. Additionally, future work should aim to investigate the bioactivity of anthocyanin metabolite profiles rather than single metabolites. Few studies in the current literature have investigated the role of multiple anthocyanin metabolites synergistically, despite this being the most physiologically relevant mode of study (Aboufarrag *et al.*, 2019). *In vivo* cells do not come into contact with one singular metabolite, they are met with a complex range of compounds at varying concentrations and any biological effects are likely to be the result of multiple compounds acting synergistically. Therefore, future work should use the anthocyanin metabolite data reported here, and from other studies, to work with mixtures of metabolites that are seen *in vivo* at their relative concentrations to study the bioactivity of anthocyanin metabolites in physiologically relevant

models. Furthermore, as it is known that anthocyanin metabolism is subject to high inter-individual variation, it may be of interest to study different mixtures of metabolites that are associated with different metabotypes to gain an understanding of the physiological implications of differences in anthocyanin metabolism.

The data presented in this thesis (Chapter 4 and 6) highlights that PCA and PGA are not the only spontaneous products of cyanidin degradation produced under physiological conditions *in vitro*. This thesis goes beyond previous reports by providing new insights into the potential compounds that may be produced in this process. However, a barrier faced in the work described in Chapter 5, and in previous reports, is that anthocyanins and their aglycones are extremely expensive and thus it can be difficult to obtain high yields of degradation products which could be taken forward for identification using NMR due to limited materials (Goszcz *et al.*, 2017; Kay *et al.*, 2009; Woodward *et al.*, 2009). However, two approaches may be taken to increase the yield of degradation products, either through increasing the starting concentration or volume of the reaction. Given that the concentration of cyanidin can affect its degradation, it would be inappropriate to increase the starting concentration to increase product yield, and instead it would be more suitable to increase reaction volumes (Castañeda-Ovando *et al.*, 2009). Nevertheless, neither the work in this thesis or in previous reports has been able to provide conclusive identification of the spontaneous products of cyanidin beyond PCA and PGA (Kay *et al.*, 2009; Woodward *et al.*, 2009). Therefore, it would be recommended that future research focuses on identifying these compounds using techniques such as NMR and that the spontaneous degradation of the other five main anthocyanidins is studied comprehensively. This is of great importance as the spontaneous products of anthocyanins are likely subjected to extensive metabolism *in vivo* by human and/or microbial enzymes, and if we understand these spontaneous products then this may provide insights into additional anthocyanin metabolites that may be produced *in vivo* that are yet to have been considered.

Research on the interplay between anthocyanin metabolism and the gut microbiota is in its infancy, and current reports provide conflicting evidence (Eker *et al.*, 2019; Faria *et al.*, 2014; Gu *et al.*, 2019; Igwe *et al.*, 2019; Zhang *et al.*, 2016; Zhu *et al.*, 2018). The work presented in this thesis does not support the notion that regular anthocyanin consumption affects the structure and diversity of the gut microbiota. One of the main challenges in the data reported in this thesis, and in other studies, is the high inter-individual variability in the microbiome of individuals. This high variation in the taxa present makes it extremely difficult to reliably detected differences (Healey *et al.*, 2017). Therefore, moving forward, it may be recommended that studies screen participants based on their microbiome to reduce this variability and study the effect of anthocyanin consumption on certain demographics based on their microbiome. This would help to provide more conclusive evidence for whether anthocyanins truly effect the microbial composition of the gut and it would be of particular interest to perform large studies where participants are stratified according to their microbiome to

investigate whether anthocyanins exert different effects on different microbiome profiles (Murga-Garrido *et al.*, 2021). In addition to this, longitudinal studies may be more appropriate to assess whether anthocyanins can modulate the gut microbiota as effects may take a considerable length of time to become apparent (Eckburg *et al.*, 2005a).

This thesis identifies several relationships between the gut microbiota and microbial anthocyanin metabolism at both the genus and species level. Other studies have tended to either focus on anthocyanin metabolism by isolated bacterial strains or by faecal samples without profiling the species present (Faria *et al.*, 2014; Igwe *et al.*, 2019). Although many relationships were observed between taxa and anthocyanin metabolites this data is only correlational and no causal links are provided, consequently further investigation is required to establish if the taxa highlighted in this thesis are truly important in anthocyanin metabolism. To enhance our understanding of the specific microbes that may be responsible for anthocyanin metabolism it may be recommended to perform enrichment experiments. This would involve incubating live faecal samples with anthocyanins, removing some of the culture, and re-incubating with anthocyanins and sequencing samples to identify the species that have the highest relative abundance after this process as these are likely the microbes that efficiently metabolise anthocyanins. Alternatively, to improve our understanding of the microbes that are important for particular biotransformation's of anthocyanin metabolites it may be of interest to incubate faecal samples and/or strains of microbes with intermediates in anthocyanin metabolism such as PCA and PGA to monitor the metabolites that may be produced. This could be done in an enrichment style experiment as described previously to elucidate the communities of microbes likely to be important in this process. Furthermore, current work, including this thesis, focuses on the relationship of microbial taxa with anthocyanin metabolism. However, it is the enzymes encoded by the genomes of such microbes that are responsible for this metabolism and multiple species may encode the same enzyme, or different enzymes that are capable of performing the same reaction. Few of the enzymes involved in anthocyanin metabolism have been characterised and this is a major barrier in understanding how and why anthocyanins are metabolised differently between individuals. Therefore, it would be beneficial for future work to focus on characterising the enzymes responsible for anthocyanin metabolism and studying not only which species encode these enzymes in their genomes but also their expression and activity as this likely to be what best distinguishes different anthocyanin metabotypes. Furthermore, work in this area would also be important in humans as well as microbes and would potentially help us understand the relative contributions of microbial and human metabolism of anthocyanins *in vivo*.

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Appendix 1

MRM transitions for the analysis of anthocyanins and their metabolites in biological samples using LC-MS/MS

Appendix 1

These tables are with reference to the methods applied in Chapter 2, 3 and 4.

Table S1- LC-MS/MS parameters used for the detection of anthocyanins in biological samples
The multiple reaction monitoring (MRM) parameters for anthocyanins and their conjugates are given including: MS polarity (mode), Q1 and Q3 masses, collision energy (CE) and retention time (RT). Retention times italicized and in grey were not validated with an authentic standard but were putatively identified in biological samples. Note galactosides were not included separately in the MRM list due to anthocyanin galactosides having the same MRM parameters as glucosides.

Anthocyanins	Mode	Q1	Q3	CE (eV)	RT (min)
Cyanidin derivatives					
Cyanidin	+	287	287	5	
Cyanidin-arabinoside	+	455	287	28	
Cyanidin-arabinoside-sulfate	+	535	287	28	
Cyanidin-arabinoside-glucuronide	+	631	287	28	
Cyanidin-diglucoside	+	611	287	28	
Cyanidin-diglucoside-sulfate	+	691	287	28	
Cyanidin-diglucoside-glucuronide	+	787	287	28	
Cyanidin-caffeoyl-rutinoside	+	757	287	28	
Cyanidin-caffeoyl-rutinoside-glucoside	+	919	287	28	
Cyanidin-caffeoyl-rutinoside-glucoside-glucuronide	+	1095	287	28	
Cyanidin-caffeoyl-rutinoside-glucoside-sulfate	+	999	287	28	
Cyanidin-coumaroyl-glucoside	+	593	287	28	
Cyanidin-coumaroyl-rutinoside	+	741	287	28	
Cyanidin-coumaroyl-rutinoside-glucoside	+	903	287	28	
Cyanidin-coumaroyl-rutinoside-glucoside-glucuronide	+	1079	287	28	
Cyanidin-coumaroyl-rutinoside-glucoside-sulfate	+	983	287	28	
Cyanidin-feruloyl-rutinoside	+	771	287	28	
Cyanidin-feruloyl-rutinoside-glucoside	+	933	287	28	
Cyanidin-feruloyl-rutinoside-glucoside-glucuronide	+	1109	287	28	
Cyanidin-feruloyl-rutinoside-glucoside-sulfate	+	1013	287	28	
Cyanidin-3-*O*-glucoside	+	449	287	28	7.7
Cyanidin-glucoside-glucuronide	+	625	287	28	
Cyanidin-glucoside-sulfate	+	529	287	28	
Cyanidin-glucuronide	+	463	287	28	*10.5*
Cyanidin-3-*O*-rutinoside	+	595	287	28	8.6
Cyanidin-rutinoside-glucoside	+	757	287	28	

Anthocyanins	Mode	Q1	Q3	CE (eV)	RT (min)
Cyanidin-sulfate	+	367	287	28	
Methyl-cyanidin-glucoside	+	463	301	28	
Methyl-cyanidin-glucuronide	+	477	301	28	
Methyl-cyanidin-glucuronide-glucoside	+	639	301	28	
Delphinidin derivatives					
Delphinidin	+	303	303	5	
Delphinidin-arabinoside	+	471	303	28	*7.5*
Delphinidin-arabinoside-sulfate	+	551	303	28	
Delphinidin-arabinoside-glucuronide	+	647	303	28	
Delphinidin-diglucoside	+	662	303	28	
Delphinidin-diglucoide-sulfate	+	722	303	28	
Delphinidin-diglucoside-glucuronide	+	838	303	28	
Delphinidin-caffeoyl-rutinoside	+	773	303	28	
Delphinidin-caffeoyl-rutinoside-glucoside	+	935	303	28	
Delphinidin-caffeoyl-rutinoside-glucoside-sulfate	+	1015	303	28	
Delphinidin-coumaroyl-rutinoside	+	611	303	28	
Delphinidin-coumaroyl-rutinoside	+	757	303	28	
Delphinidin-coumaroyl-rutinoside-glucoside	+	919	303	28	
Delphinidin-coumaroyl-rutinoside-glucoside-glucuronide	+	1095	303	28	
Delphinidin-coumaroyl-rutinoside-glucoside-sulfate	+	999	303	28	
Delphinidin-feruloyl-rutinoside	+	787	303	28	
Delphinidin-feruloyl-rutinoside-glucoside	+	949	303	28	
Delphinidin-3-*O*-glucoside	+	465	303	28	6.4
Delphinidin-glucoside-glucuronide	+	641	303	28	
Delphinidin-glucoside-sulfate	+	545	303	28	
Delphinidin-glucuronide	+	479	303	28	*8.5*
Delphinidin-3-*O*-rutinoside	+	611	303	28	7.0
Delphinidin-rutinoside-glucoside	+	773	303	28	
Delphinidin-sulfate	+	383	303	28	
Methyl-delphinidin-glucoside	+	479	317	28	
Methyl-delphinidin-glucoside-glucuronide	+	655	317	28	
Methyl-delphinidin-glucuronide	+	493	317	28	
Malvidin derivatives					
Malvidin	+	331	331	5	
Malvidin-arabinoside	+	499	331	28	*13.5*
Malvidin-arabinoside-sulfate	+	579	331	28	
Malvidin arabinoside-glucuronide	+	675	331	28	
Malvidin-diglucoside	+	655	331	28	
Malvidin-diglucoside-sulfate	+	735	331	28	
Malvidin-diglucoside-glucuronide	+	831	331	28	
Malvidin-caffeoyl-rutinoside	+	801	331	28	
Malvidin-caffeoyl-rutinoside-glucoside	+	963	331	28	
Malvidin-coumaroyl-glucoside	+	639	331	28	

Anthocyanins	Mode	Q1	Q3	CE (eV)	RT (min)
Malvidin-coumaroyl-rutinoside	+	785	331	28	
Malvidin-coumaroyl-rutinoside-glucoside	+	947	331	28	
Malvidin-coumaroyl-rutinoside-glucoside-glucuronide	+	1123	331	28	
Malvidin-coumaroyl-rutinoside-glucoside-sulfate	+	1027	331	28	
Malvidin-feruloyl-rutinoside	+	815	331	28	
Malvidin-feruloyl-rutinoside-glucoside	+	977	331	28	
Malvidin-feruloyl-rutinoside-glucoside-glucuronide	+	1153	331	28	
Malvidin-feruloyl-rutinoside-glucoside-sulfate	+	1057	331	28	
Malvidin-3-O-glucoside	+	493	331	28	12.2
Malvidin-glucoside-glucuronide	+	669	331	28	
Malvidin-glucoside-sulfate	+	573	331	28	
Malvidin-glucuronide	+	507	331	28	
Malvidin-rutinoside	+	639	331	28	
Malvidin-rutinoside-glucoside	+	801	331	28	
Malvidin-sulfate	+	411	331	28	
Methyl-malvidin-glucoside	+	507	345	28	
Methyl-malvidin-glucoside-glucuronide	+	683	345	28	
Methyl-malvidin-glucuronide	+	521	345	28	
Pelargonidin derivatives					
Pelargonidin	+	271	271	5	
Pelargonidin-arabinoside	+	404	271	28	
Pelargonidin-arabinoside-sulfate	+	351	271	28	
Pelargonidin-arabinoside-glucuronide	+	580	271	28	
Pelargonidin-diglucoside	+	631	271	28	
Pelargonidin-diglucoside-sulfate	+	711	271	28	
Pelargonidin-diglucoside-glucuronide	+	807	271	28	
Pelargonidin-caffeoyl-rutinoside	+	741	271	28	
Pelargonidin-caffeoyl-rutinoside-glucoside	+	903	271	28	
Pelargonidin-coumaroyl-glucoside	+	579	271	28	
Pelargonidin-coumaroyl-rutinoside	+	725	271	28	
Pelargonidin-coumaroyl-rutinoside-glucoside	+	967	271	28	
Pelargonidin-coumaroyl-rutinoside-glucoside	+	887	271	28	
Pelargonidin-coumaroyl-rutinoside-glucoside-glucuronide	+	1063	271	28	
Pelargonidin-feruloyl-rutinoside	+	755	271	28	
Pelargonidin-feruloyl-rutinoside-glucoside	+	917	271	28	
Pelargonidin-3-O-glucoside	+	433	271	28	9.4
Pelargonidin-glucoside-glucuronide	+	609	271	28	
Pelargonidin-glucoside-sulfate	+	513	271	28	
Pelargonidin-glucuronide	+	447	271	28	
Pelargonidin-3-O-rutinoside	+	579	271	28	10.5
Pelargonidin-rutinoside-glucoside	+	741	271	28	

Anthocyanins	Mode	Q1	Q3	CE (eV)	RT (min)
Pelargonidin-sulfate	+	351	271	28	
Methyl-pelargonidin-glucoside	+	447	285	28	
Methyl-pelargonidin-glucoside-glucuronide	+	623	285	28	
Methyl-pelargonidin-glucuronide	+	461	285	28	
Peonidin derivatives					
Peonidin	+	301	301	5	
Peonidin-arabinoside	+	469	301	28	*12.0*
Peonidin-arabinoside-sulfate	+	549	301	28	
Peonidin-arabinoside-glucuronide	+	645	301	28	
Peonidin-diglucoside	+	625	301	28	
Peonidin-diglucoside-sulfate	+	705	301	28	
Peonidin-diglucoside-glucuronide	+	801	301	28	
Peonidin-caffeoyl-rutinoside	+	771	301	28	
Peonidin-caffeoyl-rutinoside-glucoside	+	933	301	28	
Peonidin-caffeoyl-rutinoside-glucoside-glucuronide	+	1109	301	28	
Peonidin-caffeoyl-rutinoside-glucoside-sulfate	+	1013	301	28	
Peonidin-coumaroyl-glucoside	+	769	301	28	
Peonidin-coumaroyl-rutinoside	+	755	301	28	
Peonidin-coumaroyl-rutinoside-acetyl-glucoside	+	959	301	28	
Acetyl-peonidin-coumaroyl-rutinoside-glucoside	+	957	301	28	
Peonidin-coumaroyl-rutinoside-glucoside	+	917	301	28	
Peonidin-coumaroyl-rutinoside-glucoside-glucuronide	+	1093	301	28	
Peonidin-coumaroyl-rutinoside-glucoside-sulfate	+	997	301	28	
Peonidin-feruloyl-rutinoside	+	785	301	28	
Peonidin-feruloyl-rutinoside-glucoside	+	947	301	28	
Peonidin-feruloyl-rutinoside-glucoside-glucuronide	+	1123	301	28	
Peonidin-feruloyl-rutinoside-glucoside-sulfate	+	1027	301	28	
Peonidin-glucoside	+	463	301	28	*11.1*
Peonidin-glucoside-glucuronide	+	639	301	28	
Peonidin-glucoside-sulfate	+	543	301	28	
Peonidin-glucuronide	+	477	301	28	
Peonidin-rutinoside	+	609	301	28	
Peonidin-rutinoside-glucoside	+	771	301	28	
Peonidin-sulfate	+	381	301	28	
Methyl-peonidin-glucoside	+	477	315	28	
Methyl-peonidin-glucoside-glucuronide	+	653	315	28	
Methyl-peonidin-glucuronide	+	491	315	28	
Petunidin derivatives					
Petunidin	+	317	317	5	
Petunidin-arabinoside	+	485	317	28	

Anthocyanins	Mode	Q1	Q3	CE (eV)	RT (min)
Petunidin-arabinoside-sulfate	+	565	317	28	
Petunidin-arabinoside-glucuronide	+	661	317	28	
Petunidin-diglucoside	+	677	317	28	
Petunidin-diglucoside-sulfate	+	757	317	28	
Petunidin-diglucoside-glucuronide	+	853	317	28	
Petunidin-caffeoyl-rutinoside	+	787	317	28	
Petunidin-caffeoyl-rutinoside-glucoside	+	949	317	28	
Petunidin-caffeoyl-rutinoside-glucoside-glucuronide	+	1125	317	28	
Petunidin-caffeoyl-rutinoside-glucoside-sulfate	+	1029	317	28	
Petunidin-coumaroyl-glucoside	+	625	317	28	
Petunidin-coumaroyl-rutinoside	+	771	317	28	
Acetyl-petunidin-coumaroyl-rutinoside-glucoside	+	975	317	28	
Petunidin-coumaroyl-rutinoside-glucoside	+	933	317	28	
Petunidin-coumaroyl-rutinoside-glucoside-glucuronide	+	1109	317	28	
Petunidin-coumaroyl-rutinoside-glucoside-sulfate	+	1031	317	28	
Petunidin-feruloyl-rutinoside	+	801	317	28	
Petunidin-feruloyl-rutinoside-glucoside	+	963	317	28	
Petunidin-feruloyl-rutinoside-glucoside-glucuronide	+	1139	317	28	
Petunidin-feruloyl-rutinoside-glucoside-sulfate	+	1043	317	28	
Petunidin-glucoside	+	479	317	28	*9.3*
Petunidin-glucoside-glucuronide	+	655	317	28	
Petunidin-glucoside-sulfate	+	559	317	28	
Petunidin-glucuronide	+	493	317	28	*11.3*
Petunidin-rutinoside	+	625	317	28	
Petunidin-rutinoside-glucoside	+	787	317	28	
Petunidin-sulfate	+	397	317	28	
Methyl-petunidin-glucoside	+	493	331	28	
Methyl-petunidin-glucoside-glucuronide	+	669	331	28	
Methyl-petunidin-glucuronide	+	507	331	28	
Phospholipids					
Phosphatidyl choline	+	184	184	5	
Internal standards					
Caffeine-(trimethyl-*d*₉)	+	204	144	27	5.7
Taxifolin	+	303	125	30	10.7

Table S2- LC-MS/MS parameters used for the detection of anthocyanin metabolites in biological samples

The multiple reaction monitoring (MRM) parameters for anthocyanin metabolites are given including: MS polarity (mode), Q1 and Q3 masses, collision energy (CE), and retention time (RT) in minutes. Retention times italicized and in grey were not validated with an authentic standard but were putatively identified in biological samples.

Phenolic metabolites	Mode	Q1	Q3	CE (eV)	RT (min)
Benzoic acid derivatives					
Benzoic acid	-	121	108	20	
2,4-Dihydroxybenzoic acid	-	153	109	22	2.9
Dihydroxybenzoic acid glucuronide	-	329	109	50	*1.9*
Dimethoxyphenyl glucuronide	-	329	153	20	
Gallic acid	-	169	125	22	1.6
Gallic acid sulfate	-	249	169	26	*3.8*
Gallic acid-3-*O*-glucuronide	-	345	169	20	1.1
Gallic acid-4-*O*-glucuronide	-	345	169	20	1.3
3-Hydroxybenzoic acid	-	137	93	30	4.9
3-Hydroxybenzoic acid glucuronide	-	313	137	20	
3-Hydroxybenzoic acid sulfate	-	217	137	26	
4-Hydroxybenzoic acid	-	137	93	30	4.9
4-Hydroxybenzoic acid glucuronide	-	313	137	20	
4-Hydroxybenzoic acid sulfate	-	217	137	26	
Isovanillic acid	-	167	152	22	5.5
(Iso)vanillic acid glucuronide	-	343	167	20	*2.8/7.0*
2-Methoxybenzoic acid	-	151	107	15	
4-Methoxysalicylic acid	-	167	123	20	5.3
3-*O*-Methyl gallate	-	183	100	20	5.4
3-*O*-Methyl gallate	-	183	124	30	5.4
Methyl syringate	-	211	180	30	
Methyl vanillate	-	181	107	32	7.1
Methyl-3,4-dihydroxybenzoate	-	167	108	20	6.2
Methyl-3,4-dimethoxybenzoate	-	197	165	17	
Methyl-4-hydroxybenzoate	-	151	93	30	
Methyl-gallic acid glucuronide	-	359	183	23	
Methyl-gallic acid sulfate	-	263	183	23	*4.0*
Phloroglucinol carboxylic acid	-	169	152	20	4.3
Protocatechuic acid	-	153	109	22	3.2
Protocatechuic acid-3-*O*-glucuronide	-	329	153	20	1.7
Protocatechuic acid-4-*O*-glucuronide	-	329	153	20	1.2
Protocatechuic acid-3-*O*-sulfate	-	233	153	26	2.7
Protocatechuic acid-3-*O*-sulfate	-	233	109	26	2.7
Protocatechuic acid-4-*O*-sulfate	-	233	153	26	1.8
Protocatechuic acid-4-*O*-sulfate	-	233	109	26	1.8
Syringic acid	-	197	121	26	5.6
Syringic acid glucuronide	-	374	197	20	
Syringic acid sulfate	-	277	197	26	
Trimethoxygallic acid	-	211	167	20	

Vanillic acid	-	167	152	22	5.4
Vanillic acid glucuronide	-	343	167	20	*2.8/7.0*
(Iso)vanillic acid sulfate	-	247	167	26	*3.9*
Cinnamic acid derivatives					
Caffeic acid	-	179	135	22	5.5
Caffeic acid glucuronide	-	355	179	20	*5.1*
Caffeic acid sulfate	-	259	179	26	
Chlorogenic acid	-	353	191	31	4.9
Chlorogenic acid glucuronide	-	529	353	31	
Chlorogenic acid sulfate	-	433	353	31	
Cinnamic acid	-	147	103	15	7.5
Cinnamic acid glucuronide	-	323	147	20	
Cinnamic acid sulfate	-	227	147	26	
p-Coumaric acid	-	163	119	46	6.1
p-Coumaric acid glucuronide	-	339	163	20	*6.8*
p-Coumaric acid rutinoside	-	471	163	30	
p-Coumaric acid sulfate	-	243	163	26	
Dimethoxycinnamic acid	-	207	191	20	
Ferulic acid	-	193	134	22	6.3
Ferulic acid-4-*O*-glucuronide	-	369	193	20	
Feruloyl rutinoside	-	501	193	22	
Ferulic acid sulfate	-	273	193	26	
5-Hydroxyferulic acid	-	209	194	24	5.6
5-Hydroxyferulic acid glucuronide	-	385	209	22	
5-Hydroxyferulic acid sulfate	-	289	209	22	
Isoferulic acid	-	193	134	22	
Isoferulic acid sulfate	-	273	193	26	
Isoferulic acid-3-*O*-glucuronide	-	369	193	20	5.1
Sinapic acid	-	223	149	28	6.3
Sinapic acid glucuronide	-	399	223	20	
Sinapic acid sulfate	-	303	223	26	*4.0*
Trihydroxycinnamic acid	-	195	151	22	
Trihydroxycinnamic acid glucuronide	-	371	195	20	*3.2*
Trihydroxycinnamic acid sulfate	-	275	195	24	
Trimethoxycinnamic acid	-	237	133	20	
Flavonol derivatives					
Quercetin	-	301	151	26	7.3
Quercetin-3-*O*-sulfate	-	609	300	55	6.0
Hippuric acid derivatives					
Hippuric acid	-	178	134	18	4.8
4-Hydroxyhippuric acid	-	194	150	18	3.8
3-Methylhippuric acid	-	192	148	18	5.9
4-Methylhippuric acid	-	192	148	18	5.7
Phenol derivatives					
Catechol	-	109	91	24	5.1
Catechol glucuronide	-	285	109	31	*3.6*
Catechol sulfate	-	189	109	31	*4.2*
Dimethoxyphenol	-	153	107	20	

Dimethoxyphenyl sulfate	-	233	153	26	*2.7*
Methoxycatechol glucuronide	-	315	139	20	
Methoxycatechol sulfate	-	219	139	25	
Methoxyphenol	-	123	93	20	
Methoxyphenyl glucuronide	-	299	123	29	
Methoxyphenyl sulfate	-	203	123	29	*6.0*
Methoxycatechol	-	139	125	20	
4-Methylcatechol	-	123	108	24	6.4
Methylcatechol glucuronide	-	299	123	29	
Methylcatechol sulfate	-	203	123	29	
Methylpyrogallol	-	139	124	20	
Methylpyrogallol glucuronide	-	315	139	20	
Methylpyrogallol sulfate	-	219	139	25	
Phloroglucinol	-	125	83	15	2.5
Pyrogallol	-	125	79	26	2.8
Pyrogallol glucuronide	-	301	125	26	
Pyrogallol sulfate	-	205	125	26	*4.4*
Phenolic alcohols					
Homovanillyl alcohol	-	167	151	20	
4-Hydroxybenzyl alcohol	-	123	105	16	
Phenolic aldehydes					
3,5-Dihydroxy-4-methoxybenzaldehyde	-	167	151	20	
4-Hydroxybenzaldehyde	-	121	92	35	5.9
Methoxybenzaldehyde	-	167	123	20	
Phloroglucinaldehyde	-	153	83	26	6.1
Protocatechualdehyde	-	137	108	32	5.3
Syringaldehyde	-	181	165	20	
Vanillaldehyde	-	151	136	18	6.2
Vanillaldehyde glucuronide	-	327	151	28	
Vanillaldehyde sulfate	-	231	151	20	
Phenylacetic acid derivatives					
Dihydroxymandelic acid	-	183	139	20	
3,4-Dihydroxy-5-methoxyphenyl acetic acid	-	197	100	20	
Homoprotocatechuic acid	-	167	123	14	4.5
Homoprotocatechuic acid glucuronide	-	343	167	20	*2.8/7.0*
Homoprotocatechuic acid sulfate	-	247	167	26	*3.9*
Homosyringic acid	-	211	167	20	
Homovanillic acid	-	181	137	15	5.6
Homovanillic acid glucuronide	-	357	181	21	
Homovanillic acid sulfate	-	261	181	25	*5.8*
Hydroxymandelic acid	-	167	123	20	
4-Hydroxy-3-methoxy-mandelic acid	-	197	137	20	
3-Hydroxyphenyl acetic acid	-	151	107	14	5.6
4-Hydroxyphenyl acetic acid	-	151	107	15	5.2

Hydroxyphenyl acetic acid glucuronide	-	327	151	28	
Hydroxyphenyl acetic acid sulfate	-	231	151	20	
Mandelic acid	-	151	107	15	
Trihydroxyphenyl acetic acid	-	183	138	20	
Phenylpropanoic acid derivatives					
Dihydrocaffeic acid	-	181	137	15	5.2
Dihydrocaffeic acid glucuronide	-	357	181	21	
Dihydrocaffeic acid sulfate	-	261	181	25	*5.6*
Dihydrocoumaric acid glucuronide	-	341	165	20	
Dihydrocoumaric acid sulfate	-	245	165	21	
Dihydroferulic acid	-	195	136	18	4.9
Dihydroisoferulic acid	-	195	136	18	6.2
Dihydro(iso)ferulic acid glucuronide	-	371	195	20	
Dihydro(iso)ferulic acid sulfate	-	275	195	24	
Dihydrosinapic acid	-	225	59	20	
Dihydrosinapic acid glucuronide	-	401	225	20	
Dihydrosinapic acid sulfate	-	305	225	20	
3,4-Dihydroxy-5-methoxyphenylpropanoic acid	-	211	193	20	
Dihydroxyphenylpropionic acid	-	181	137	15	
Dihydroxyphenylpropionic acid glucuronide	-	357	181	21	
Dihydroxyphenylpropionic acid sulfate	-	261	181	25	
Hydroxyphenyl propionic acid	-	165	121	16	
Hydroxyphenyl propionic acid glucuronide	-	341	165	20	
Hydroxyphenyl propionic acid sulfate	-	245	165	21	
Trihydroxyphenylpropanoic acid	-	197	138	20	
Phospholipids					
Phosphatidyl choline	+	184	184	5	
Internal standards					
Caffeine-(trimethyl-d_9)	+	204	144	27	5.4
Taxifolin	-	303	125	30	6.4
Phloridzin	-	435	273	25	6.7
Scopoletin	-	191	176	22	6.5

Appendix 2

Method development for the extraction and quantification of anthocyanin metabolites in biological samples

Appendix 2

The following tables are with reference to Chapter 2.

Table S3- Recoveries of phenolic standards in urine achieved with Waters Oasis HLB micro-elution SPE at varying sample load volumes from 100 – 500 µl. Data shown as mean recovery (%) ± standard deviation and n = 3.

Compound	100 µl	200 µl	300 µl	400 µl	500 µl
3-*O*-Methyl gallic acid	45.99 ± 3.61	37.93 ± 2.40	36.07 ± 4.62	39.57 ± 2.74	39.97 ± 2.36
4-Hydroxybenzaldehyde	64.46 ± 5.15	57.83 ± 7.22	58.17 ± 4.17	55.39 ± 4.60	52.68 ± 5.50
4-Hydroxybenzoic acid	63.79 ± 12.11	61.06 ± 4.43	57.65 ± 0.14	54.55 ± 1.79	54.39 ± 1.81
5-Hydroxyferulic acid	56.19 ± 13.56	49.20 ± 9.84	38.54 ± 11.90	48.15 ± 13.80	45.40 ± 4.57
Caffeic acid	56.34 ± 4.73	48.66 ± 2.90	45.30 ± 4.15	46.80 ± 1.25	50.80 ± 4.15
Catechin	56.92 ± 9.00	56.24 ± 4.96	43.37 ± 5.86	46.00 ± 5.75	47.01 ± 1.43
Chlorogenic acid	51.93 ± 51.93	37.33 ± 3.17	33.48 ± 2.47	34.61 ± 3.57	33.50 ± 3.15
Cinnamic acid	56.14 ± 56.14	62.07 ± 15.17	57.91 ± 7.50	57.44 ± 13.76	63.22 ± 7.00
Epicatechin	51.21 ± 3.59	45.02 ± 3.26	34.49 ± 2.45	36.73 ± 1.09	36.84 ± 5.23
Ferulic acid	65.13 ± 7.58	61.12 ± 0.78	59.99 ± 7.65	66.17 ± 5.63	64.91 ± 7.84
Gallic acid	9.82 ± 2.47	8.11 ± 0.60	7.13 ± 0.01	4.21 ± 0.33	4.69 ± 1.41
Gallic acid-3-*O*-glucuronide	2.45 ± 0.45	2.14 ± 0.69	1.24 ± 0.04	0.76 ± 0.03	0.98 ± 0.58
Gallic acid-4-*O*-glucuronide	9.13 ± 0.91	5.93 ± 0.71	4.29 ± 1.10	2.76 ± 0.61	2.34 ± 1.04
Hippuric acid	81.70 ± 3.49	81.77 ± 6.62	86.47 ± 5.03	82.80 ± 2.45	86.94 ± 9.32
Isoferulic acid-3-*O*-glucuronide	47.30 ± 1.72	38.53 ± 4.55	38.67 ± 5.66	36.47 ± 4.39	38.78 ± 0.17
Isovanillic acid	51.11 ± 4.27	45.95 ± 2.18	46.48 ± 1.39	45.74 ± 0.49	38.33 ± 1.87
Methyl vanillate	39.83 ± 7.87	46.03 ± 0.55	50.46 ± 9.53	56.54 ± 3.27	68.29 ± 14.65
PCA	35.98 ± 7.38	27.34 ± 1.98	25.50 ± 2.08	18.72 ± 0.42	18.08 ± 0.19
PCA-3-*O*-glucuronide	6.39 ± 0.78	4.02 ± 1.20	2.97 ± 0.16	1.61 ± 0.47	1.42 ± 0.69
PCA-3-*O*-sulfate	1.98 ± 0.56	1.78 ± 0.13	1.31 ± 0.05	0.86 ± 0.10	0.92 ± 0.24
PCA-4-*O*-glucuronide	9.88 ± 1.59	7.46 ± 0.32	5.14 ± 0.13	2.76 ± 0.86	2.82 ± 0.89
PCA-4-*O*-sulfate	3.93 ± 0.66	2.66 ± 0.17	1.96 ± 0.08	1.08 ± 0.05	1.27 ± 0.45
p-Coumaric acid	74.85 ± 4.39	66.69 ± 0.65	62.79 ± 9.43	65.38 ± 2.03	70.69 ± 4.35

Compound	100 µl	200 µl	300 µl	400 µl	500 µl
PGA	44.71 ± 2.29	45.12 ± 9.01	42.97 ± 0.36	51.63 ± 7.56	62.35 ± 6.51
Phloroglucinol	3.74 ± 1.14	3.52 ± 3.35	3.24 ± 0.90	1.63 ± 0.20	2.17 ± 1.34
Pyrogallol	8.80 ± 1.82	7.08 ± 0.65	5.12 ± 0.79	3.74 ± 0.15	3.91 ± 0.94
Quercetin	17.16 ± 8.50	19.55 ± 3.85	19.65 ± 10.25	32.41 ± 7.29	42.88 ± 14.82
Quercetin-3-*O*-sulfate	4.84 ± 2.85	13.28 ± 1.12	16.17 ± 4.18	22.03 ± 5.27	32.05 ± 0.19
Rutin	66.81 ± 7.19	64.34 ± 7.98	56.91 ± 4.47	63.83 ± 2.93	67.67 ± 4.45
Scopoletin (internal standard)	78.70 ± 5.11	72.17 ± 4.12	66.07 ± 3.00	72.43 ± 5.27	76.45 ± 3.59
Sinapic acid	67.38 ± 10.15	57.16 ± 1.87	55.84 ± 0.35	58.03 ± 1.10	57.18 ± 1.33
Syringic acid	50.59 ± 1.38	50.64 ± 10.25	44.42 ± 0.44	35.54 ± 9.07	39.07 ± 2.65
Vanillic acid	58.30 ± 2.22	51.56 ± 2.23	50.06 ± 6.66	52.27 ± 1.11	50.45 ± 2.41

Table S4- Recoveries of phenolic standards in urine achieved with Waters Oasis HLB 30mg SPE tubes. Data is shown as mean ± standard deviation and n = 3.

Compound	Recovery (%)
3-O-Methyl gallic acid	96.71 ± 17.45
4-Hydroxybenzaldehyde	106.75 ± 9.44
4-Hydroxybenzoic acid	105.39 ± 14.08
5-Hydroxyferulic acid	101.71 ± 0.15
Caffeic acid	95.13 ± 11.42
Catechin	93.84 ± 5.22
Chlorogenic acid	104.27 ± 23.92
Cinnamic acid	99.72 ± 3.45
Epicatechin	92.85 ± 10.03
Ferulic acid	93.39 ± 9.80
Gallic acid	40.79 ± 9.76
Gallic acid-3-O-glucuronide	1.49 ± 0.41
Gallic acid-4-O-glucuronide	8.11 ± 0.78
Hippuric acid	92.25 ± 9.07
Isoferulic acid-3-O-glucuronide	86.66 ± 13.97
Isovanillic acid	119.95 ± 23.51
Methyl vanillate	102.06 ± 1.76
PCA	85.78 ± 10.13
PCA-3-O-glucuronide	2.33 ± 0.19
PCA-3-O-sulfate	3.50 ± 1.15
PCA-4-O-glucuronide	6.83 ± 1.35
PCA-4-O-sulfate	3.86 ± 0.66
p-Coumaric acid	100.84 ± 1.91
PGA	90.58 ± 4.08
Phloroglucinol	10.04 ± 0.11
Pyrogallol	8.63 ± 3.32
Quercetin	90.45 ± 6.57
Quercetin-3-O-sulfate	13.24 ± 9.40
Rutin	93.93 ± 10.20
Scopoletin (internal standard)	113.45 ± 0.72
Sinapic acid	107.43 ± 37.97
Syringic acid	109.89 ± 37.28
Vanillic acid	93.44 ± 8.32

Table S5- Recoveries of phenolic standards in urine achieved with Strata-X micro-elution SPE at various sample load volumes from 200 – 500 µl. Data shown as mean recovery (%) ± standard deviation and n = 3.

Compound	200 µl	350 µl	500 µl
3-O-Methyl gallic acid	54.21 ± 7.79	36.69 ± 2.04	38.10 ± 13.04
4-Hydroxybenzaldehyde	70.84 ± 7.30	51.14 ± 3.38	50.45 ± 5.17
4-Hydroxybenzoic acid	66.55 ± 5.23	52.42 ± 1.33	53.36 ± 7.04
5-Hydroxyferulic acid	72.00 ± 4.73	55.21 ± 3.39	51.81 ± 13.52
Caffeic acid	72.70 ± 1.35	59.79 ± 1.03	47.58 ± 7.67
Catechin	75.80 ± 2.18	56.68 ± 2.51	48.60 ± 1.99
Chlorogenic acid	58.57 ± 6.92	47.33 ± 6.27	36.79 ± 6.32
Cinnamic acid	83.34 ± 1.87	63.46 ± 10.38	63.86 ± 0.56
Epicatechin	75.41 ± 10.32	53.10 ± 6.22	48.48 ± 10.59
Ferulic acid	76.74 ± 19.46	62.60 ± 0.86	66.52 ± 4.66
Gallic acid	4.14 ± 0.22	2.98 ± 0.73	3.00 ± 0.70
Gallic acid-3-O-glucuronide	0.88 ± 0.08	0.83 ± 0.01	0.84 ± 0.31
Gallic acid-4-O-glucuronide	5.99 ± 0.02	3.63 ± 0.27	3.86 ± 2.14
Hippuric acid	94.60 ± 5.95	83.95 ± 5.63	92.44 ± 6.43
Isoferulic acid-3-O-glucuronide	96.10 ± 1.21	143.42 ± 0.05	96.89 ± 7.55
Isovanillic acid	67.92 ± 14.14	44.47 ± 10.43	55.57 ± 0.86
Methyl vanillate	79.72 ± 9.59	54.22 ± 5.50	64.32 ± 3.79
PCA	29.86 ± 2.20	23.04 ± 0.51	19.92 ± 5.38
PCA-3-O-glucuronide	3.38 ± 0.55	2.90 ± 0.23	1.84 ± 0.80
PCA-3-O-sulfate	1.56 ± 0.37	1.79 ± 0.05	2.54 ± 1.08
PCA-4-O-glucuronide	8.31 ± 0.58	6.16 ± 0.89	5.10 ± 3.02
PCA-4-O-sulfate	2.47 ± 0.34	2.83 ± 0.19	3.41 ± 1.34
p-Coumaric acid	76.69 ± 11.17	57.40 ± 3.51	71.81 ± 6.58
PGA	75.80 ± 14.17	53.89 ± 5.07	56.51 ± 3.37
Phloroglucinol	2.06 ± 0.52	1.59 ± 0.02	1.26 ± 0.14
Pyrogallol	2.76 ± 0.01	2.59 ± 0.10	2.60 ± 0.23
Quercetin	51.02 ± 5.99	25.76 ± 2.11	28.13 ± 4.25
Quercetin-3-O-sulfate	31.54 ± 7.86	19.17 ± 1.63	40.05 ± 7.05
Rutin	80.42 ± 2.71	73.39 ± 12.13	58.72 ± 5.31
Scopoletin (internal standard)	94.36 ± 11.43	100.04 ± 7.18	96.38 ± 0.65
Sinapic acid	94.25 ± 24.97	58.75 ± 6.22	60.74 ± 3.75
Syringic acid	81.73 ± 4.04	44.09 ± 9.71	54.23 ± 1.02

Compound	200 µl	350 µl	500 µl
Vanillic acid	91.41 ± 0.97	59.34 ± 2.48	57.88 ± 3.12

Table S6- Recoveries of phenolic standards in urine achieved with Strata-X SPE cartridges containing varying amounts of sorbent: 60 mg cartridges, 30 mg cartridges and a 30 mg 96 well SPE plate. Data shown as mean recovery (%) ± standard deviation and n = 3.

Compound	60 mg	30 mg	30 mg Plate
3-O-Methyl gallic acid	96.79 ± 3.45	85.40 ± 10.27	107.92 ± 6.71
4-Hydroxybenzaldehye	94.96 ± 9.27	93.04 ± 9.40	113.74 ± 8.18
4-Hydroxybenzoic acid	57.97 ± 5.83	83.63 ± 19.32	108.46 ± 9.55
5-Hydroxyferulic acid	128.68 ± 12.75	85.31 ± 0.34	88.59 ± 14.38
Caffeic acid	95.80 ± 1.37	100.91 ± 9.75	104.27 ± 13.42
Catechin	103.92 ± 87.92	72.30 ± 49.78	147.66 ± 27.36
Chlorogenic acid	116.07 ± 17.77	108.98 ± 6.98	100.11 ± 12.49
Cinnamic acid	74.50 ± 9.31	87.75 ± 10.77	115.99 ± 37.94
Epicatechin	63.30 ± 42.06	55.50 ± 29.04	142.36 ± 44.4
Ferulic acid	84.50 ± 5.71	86.97 ± 7.87	110.72 ± 10.80
Gallic acid	49.15 ± 0.13	7.52 ± 0.88	50.29 ± 12.54
Gallic acid-3-O-glucuronide	10.57 ± 1.23	1.90 ± 0.15	11.46 ± 0.80
Gallic acid-4-O-glucuronide	62.50 ± 4.90	27.18 ± 6.97	51.83 ± 6.74
Hippuric acid	95.22 ± 1.76	110.27 ± 14.33	99.65 ± 17.10
Isoferulic acid-3-O-glucuronide	97.25 ± 3.93	127.44 ± 28.41	103.08 ± 16.28
Isovanillic acid	65.72 ± 8.99	78.98 ± 4.98	89.60 ± 17.72
Methyl vanillate	72.05 ± 7.45	97.33 ± 5.29	108.11 ± 25.79
PCA	86.15 ± 1.19	58.92 ± 8.08	95.79 ± 13.99
PCA-3-O-glucuronide	50.10 ± 0.77	16.15 ± 2.98	40.31 ± 4.72
PCA-3-O-sulfate	18.89 ± 5.97	3.01 ± 0.83	22.01 ± 4.57
PCA-4-O-glucuronide	70.82 ± 0.70	31.89 ± 7.83	46.34 ± 26.31
PCA-4-O-sulfate	36.06 ± 9.82	5.40 ± 1.07	26.44 ± 2.98
p-Coumaric acid	102.71 ± 5.42	90.87 ± 10.12	100.43 ± 15.45
PGA	71.97 ± 3.54	81.88 ± 10.81	117.02 ± 28.39
Phloroglucinol	44.82 ± 7.05	6.19 ± 1.61	21.51 ± 29.56
Pyrogallol	27.38 ± 3.21	3.00 ± 1.14	49.85 ± 19.99
Quercetin	241.23 ± 18.43	108.82 ± 73.58	107.58 ± 15.34
Quercetin-3-O¬-sulfate	83.96 ± 0.42	85.09 ± 6.87	66.95 ± 9.04
Rutin	72.83 ± 0.60	83.03 ± 4.39	99.39 ± 16.40
Sinapic acid	88.46 ± 17.11	74.33 ± 5.70	151.57 ± 37.33
Syringic acid	87.58 ± 10.50	74.37 ± 14.56	114.21 ± 18.37
Taxifolin (internal standard)	102.51 ± 0.70	102.35 ± 3.31	109.23 ± 15.36

Compound	60 mg	30 mg	30 mg Plate
Vanillic acid	90.42 ± 13.05	79.25 ± 5.61	104.59 ± 4.77

Appendix 3

Study protocol for the human intervention study described in Chapter 3, known as the BERI study

Appendix 3

The effects of bilberry fruit and black rice derived anthocyanins on lipid status in adults

Short Title: BERI study

STUDY PROTOCOL

Contents

1. Summary

Human intervention trials with anthocyanin-rich berry fruits/extracts such as whole strawberries, elderberry juice and whortleberry extracts, and purified anthocyanins from a mixture of blackcurrant and bilberry have been shown to beneficially alter biological markers for CVD risk, with beneficial effects on lipid profiles common across these studies. The primary aim of the current study is to directly compare the two major types of anthocyanins found in the diet; cyanidin-type and delphinidin-type. In a double-blind, randomized, 3-arm crossover trial we aim to recruit 50 participants to consume capsules containing (i) a bilberry extract providing 320 mg anthocyanins (delphinidin-type), (ii) a black rice extract providing 320 mg anthocyanins (cyanidin-type) and (iii) a placebo control. Capsules for each treatment arm will be consumed for a period of 28 days with a washout period of at least 4 weeks in-between. Samples will be collected at the start (day 1) and end (day 29) of each treatment period. Changes in circulating LDL-Cholesterol will be evaluated as a primary outcome measure in this study. In addition, total/HDL_cholesterol & triglycerides, other markers for cardio-metabolic risk (blood glucose, fructosamine and insulin), markers for lipid metabolism (cholesterol efflux capacity & PON-1 activity) and circulating bile acids and bile acid derivatives will be evaluated. In a subset of participants (n= 24) we will also investigate the relationship between bilberry and black rice anthocyanin ingestion on RNA expression profiles that are known to be involved in atherosclerosis, non-alcoholic fatty liver disease and reverse cholesterol transport as well as faecal excretion of bile acids and lipids. Should more resources become available we will also determine the effects of the interventions on cardio-metabolic disease related microRNA's and gut microbiota profiles in this subset of participants. Dr Paul Kroon (a Quadram Institute Bioscience Research Leader) is Chief Investigator for this study. The study will be conducted and managed at the Quadram Institute Bioscience (QIB) by Wendy Hollands (research scientist/study manager). Other members of the research group (Dr Priscilla Day, Dr Natalia Perez, Mark Philo, Jasmine Percival, Emad Shehata, Dr Supriya Yadav and Hassan Aboufarrag) will be required to assist. This study is funded through the Food and Health Institute Strategic Programme grant to QIB.

2. Scientific Background

Dietary anthocyanins

Flavonoids are phenolic compounds present in plant based foods and beverages. Anthocyanins are a sub-class of flavonoid that exist in a variety of berry fruits such as blueberry, bilberry, raspberry, strawberry and other foods such as aubergine skins and black rice. Anthocyanins are what confer the red, black, purple and blue colours of anthocyanin rich foods. The daily consumption of anthocyanins is reported to range from 29 – 153 mg/d in European countries [1]. More than 90% of our intake of anthocyanins is derived from soft fruit and is therefore readily incorporated into the habitual diet. There are three main types of anthocyanins based on the number of hydroxyl groups on the B-ring; pelargonidin-type (monohydroxy), cyanidin-type (dihydroxy) and delphinidin-type (trihydroxy) (see Figure 1). Although there are numerous substitutions (e.g. glycosylation, methylation, acylation) that give rise to a much larger number of structures, all anthocyanins are derived from these three structures, and the important B-ring metabolites will also be derived from these three structures. The major anthocyanins in human diets are the cyanidin-type and delphinidin-type.

Epidemiological evidence indicates that those consuming the highest quantities of anthocyanins are at a lower risk of developing cardiovascular disease (CVD) [2-4]. These observations are substantiated by several published reports from

randomized controlled trials (RCT's) providing evidence that consumption of anthocyanin rich foods/extracts such as whole strawberries [5, 6], elderberry juice [7] and whortleberry extracts [8] have beneficial effects on biomarkers of CVD with beneficial effects on lipid profiles common across these studies.

Figure 1. Chemical structures of the two major dietary anthocyanin types, cyanidin and delphinidin. In plant foods, anthocyanins are always present as O-linked conjugates with sugars. For example, the major anthocyanin in black rice is cyanidin-3-O-glucoside. The B-ring is also important because it is the main source of gut microbiota-derived metabolites that have been shown to be accumulate in peripheral blood.

Cyanidin (dihydroxy B-ring)   Delphinidin (trihydroxy B-ring)
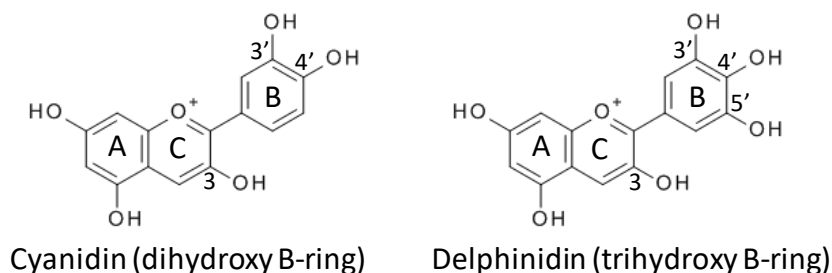
Dyslipidaemia and atherosclerosis

Atherosclerosis is an underlying pathophysiological cause of CVD and is characterized, in part at least, by dyslipidaemia; an elevation in LDL cholesterol and triglycerides and a reduced conc. HDL cholesterol, either singly or in combination. Cholesterol is predominantly produced in the liver. LDL particles transport cholesterol away from the liver and deliver it into the peripheral tissues. Once inside the vessel wall, LDL cholesterol is susceptible to oxidation by free radicals that are produced by endothelial cells, and oxidized LDL drives the atherosclerotic process. HDL on the other hand is known to protect against the development of atherosclerosis by mediating a process called reverse cholesterol transport; a process by which cholesterol is transported from the peripheral tissues back to the liver for conversion to bile acids and excretion via the bile, thus inhibiting atherosclerosis. A critical part in the reverse cholesterol transport process is the transfer of cholesterol from cells to circulating HDL's and this function can be measured as the cholesterol efflux capacity (CEC) of serum. It is not only the levels of HDL that are important, its quality is also important. For example, paroxinase-1 (PON-1) is a protein which, in serum, is almost exclusively located on HDL. PON-1 has been the focus of research activities because of its capacity to stimulate cholesterol efflux and protect HDL and LDL from oxidative modifications, thus inhibiting atherosclerotic development. In fact, there is a suggestion that PON-1 might be a better marker for risk of atherosclerotic CVD compared with HDL cholesterol [9]. PON1 genotype is also crucial, since it has been shown that the single nucleotide polymorphisms that alter amino acids located at position 55 (leucine/L or methionine/M) and 192 (arginine/Q or glutamine/R) of PON1 have a profound effect on the lipid peroxide hydrolytic activity of PON1 that is important in protecting LDL from oxidation, and that people with 55-M/M and 192-Q/Q genotypes gain greater protection against CVD [10]. MicroRNA's are small non-coding RNA molecules that have been shown to regulate gene expression and have been implicated in atherosclerosis and CVD risk. Several microRNA's are known to correlate with disease states such as atherosclerosis (mirs 33 and 758) and non-alcoholic fatty liver disease (mirs122, 192, 19a and 19b) [11, 12]. Other microRNA's

(mirs 223, 24, 342p, 17 and 624) have been directly associated with familial hypercholesterolemia [13, 14].

Effects of anthocyanin consumption on lipids/lipoproteins and CVD risk

Berry fruit extract and anthocyanin supplementation has been shown to increase HDL cholesterol concentrations [8, 15], enhance HDL-associated PON-1 activity [16] and promote cholesterol efflux capacity [16] in subjects with hypercholesterolemia. Similarly, in a recent systematic review of RCT's [17] assessing the impact of anthocyanins on biomarkers of CVD risk, nine studies included LDL cholesterol as an outcome evaluated for statistical significance in the intervention group compared with a control group. Of these nine studies, four reported improvements in LDL cholesterol. Of note, is that these four studies were conducted in a population of individuals already hyperlipidaemic with a total cholesterol conc. ≥ 5.17mmol/L upon enrolment to the study. For example, in the trial conducted by Zhu et al [18], upon which the power calculations for the proposed study has been based, the mean total and LDL cholesterol conc. was 6.5 and 3.5 mmol/L respectively, derived from a study population recruited with a total cholesterol conc. ranging between 5.17 – 8 mmol/L at eligibility assessment.

Bile acids are synthesised from cholesterol in the liver and later transported to the small intestine for re-absorption or subsequent excretion in the faeces. The conversion of cholesterol into bile acids and subsequent faecal excretion is the final step in the reverse cholesterol transport process described above. The initial products of the bile acid synthetic pathway are referred to as primary bile acids. The action of intestinal bacterial flora on primary bile acids results in the formation of secondary bile acids. As such, the quantity and composition of bile acids is determined by the intestinal microbiota which in turn has been shown to correlate with the risk of atherosclerotic development [19, 20]. Thus it is considered important to determine how intestinal microbiota relates to atherosclerotic disease markers such as bile acids, LDL/HDL cholesterol and whether their profile can be modulated by anthocyanins.

Glycaemic control and CVD risk

Epidemiological evidence also supports an association between glycaemic control and CVD risk [21]. Fructosamine is a glycosylated protein that reflects cumulative glycaemic control in humans over the short term. There is evidence that glycated proteins are increased in non-diabetic subjects [22, 23] and that the increase is associated with insulin resistance [22]. As insulin resistance progresses, increased blood glucose levels ensue. Insulin resistance is associated with abnormal lipid profiles [24]. It is well documented that ingestion of berry anthocyanins has an acute beneficial effect on blood glucose status by delaying carbohydrate digestion and inhibiting the rate of glucose absorption across the intestine [25]. However, the literature evidence for the long term effects of ingestion of berry anthocyanins on glucose and insulin status is not entirely clear.

Anthocyanins from bilberry and black rice

Bilberry fruit (*Vaccinium myrtillus L*) and black rice (*Oryza sativa L.*) are particularly rich sources of anthocyanins. Bilberry fruit is related to (albeit distinct from) the blueberry. Bilberries are reported to contain ~ 285 mg anthocyanins/100g fresh fruit and the majority of the anthocyanins are of the delphinidin-type [26]. Black rice is a special cultivar of rice and is reported to contain ~ 300 mg anthocyanins/100g [27]. Black rice contains exclusively cyanidin-type anthocyanins, mainly cyanidin (92 %) with a small amount of peonidin (8 %) which is 3-O-methyl-peonidin [28]. Thus, the

anthocyanins in black rice are largely di-hydroxy B-ring anthocyanins whilst those in bilberry fruit are largely tri-hydroxy anthocyanins (see Figure 1). The relationship between anthocyanin structure and biological activity is not well understood but there is evidence to indicate that it may influence both absorption [29] and subsequent bioactivity [30].

Purpose of the proposed intervention trial

In the proposed study we aim to investigate the ability of each of the two major dietary types of anthocyanins to improve biomarkers of cardiovascular health in hyperlipidaemic individuals by assessing the effects on circulating cholesterol/lipids (total/HDL/LDL cholesterol & triglycerides) and markers of cardio-metabolic risk (blood glucose, fructosamine and insulin). We also aim to determine the effects of supplementing diets with these two anthocyanin types on bile acids and markers for lipid metabolism (CEC & PON-1 activity) and determine the relationship between diet-mediated changes in PON-1 activity and PON-1 genotype. In a subset of participants (n=24) we will also investigate the effects of dietary supplementation with bilberry (trihydroxy) and black rice (dihydroxy) anthocyanins on the expression of microRNAs that are known to be involved in atherosclerosis, non-alcoholic fatty liver disease and reverse cholesterol transport processes, and on faecal excretion of bile acids and lipids. *Should more resources become available during the course of the trial, we will also determine the effects of the interventions on cardio-metabolic disease related microRNA's as well as gut microbiota profiles in this subset of participants*.

3. Study hypotheses

- Daily consumption for 28 days of a bilberry extract delivering a 320 mg dose of anthocyanins will significantly reduce LDL cholesterol compared with a placebo control in individuals with higher than optimal plasma cholesterol concentrations.

- Daily consumption for 28 days of a black rice extract delivering a 320 mg dose of anthocyanins will significantly reduce LDL cholesterol compared with a placebo control in individuals with higher than optimal plasma cholesterol concentrations.

4. Study Objectives

Primary objective

To determine the effects of 28 days dietary supplementation with bilberry fruit and black rice-derived anthocyanins on circulating levels of LDL cholesterol.

Secondary objectives

To determine the effects of 28 days dietary supplementation with bilberry fruit and black rice derived anthocyanins on other biomarkers related to vascular function (e.g. total cholesterol, HDL cholesterol and triglycerides), and glycaemic control (glucose, insulin, fructosamine).

To investigate the effects of bilberry fruit and black rice derived anthocyanin ingestion on circulating bile acids and bile acid derivatives, PON-1 activity and

cholesterol efflux capacity, and determine the relationship between diet mediated changes in Pon-1 activity and PON-1 genotype affecting PON-1 protein at positions 55 and 192.

To investigate in the sub-set of participants the relationship between bilberry fruit and black rice derived anthocyanin ingestion on (i) expression of microRNAs that have been implicated in atherosclerosis, non-alcoholic fatty liver disease and reverse cholesterol transport and (ii) faecal excretion of bile acids and lipids.

Should more resources become available during the course of the trial we will also determine the effects of the interventions on gut microbiota profiles and cardio-metabolic disease related microRNA's in this subset of participants.

5. Study Design

A randomized, double blind, placebo-controlled, three-arm crossover trial (see fig 2 for overview) will be undertaken to assess the effects of (i) a bilberry extract providing 320 mg/d anthocyanins, (ii) a black rice extract providing 320 mg/d anthocyanins and, (iii) a placebo control. The test materials will be encapsulated. Each test arm will comprise a 28-day treatment period which will be identical in nature (except for the test compound consumed) and separated by a washout period of at least 4 weeks. For 2 weeks prior to commencing the first study arm and then for the duration of the entire study participants will be required to restrict berry fruit consumption to a combined maximum of 3 portions per week. However, berry fruits will be completely excluded from the diet for the 24-h period immediately preceding the pre and post intervention assessment days.

Figure 2 - Overview of study
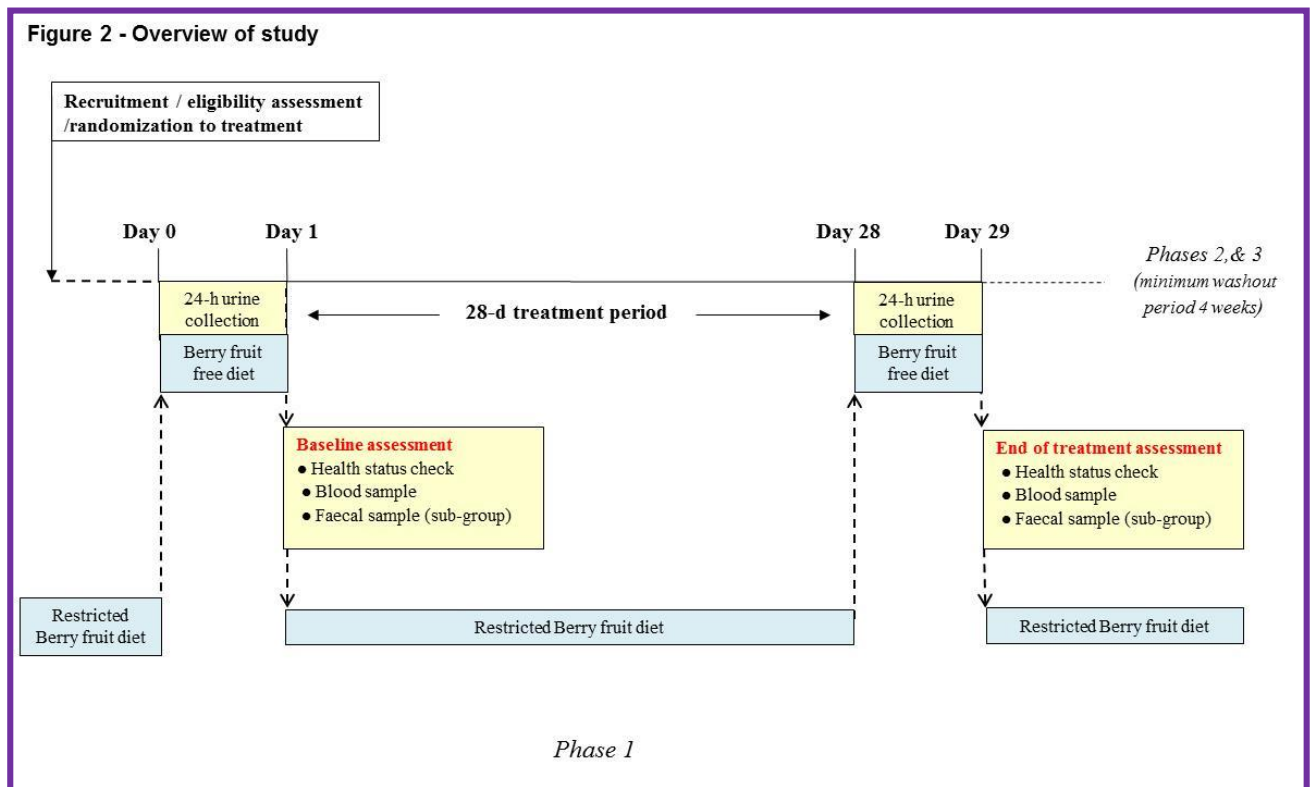
5.1 Basic inclusion criteria

- Men and women ≥ 45 years of age
- Total cholesterol ≥ 5.0 mmol/L

5.2 Basic exclusion criteria
- Smokers (or stopped smoking for less than 3 months)
- Medical conditions that are judged to affect the primary outcome measure for this study or which may compromise the well-being of the participant e.g. diabetes. This will be assessed on a case by case basis.
- Prescribed and non-prescribed medications that may affect the primary outcome measure for this study e.g. lipid lowering therapy (this will be assessed on a case by case basis).
- Dietary supplements judged to affect the study data unless the participant is willing to discontinue them for 4 weeks preceding the start of the study and for the duration of the study. (This will be assessed on a case by case basis)
- Use foods for lowering cholesterol e.g. benecol, flora proactive (this will be assessed on a case by case basis).
- Regular/recent use of colonic irrigation or other bowel cleansing techniques.
- Intend to change the normal use of pre or probiotics during the study e.g. if participants do not take these products then we do not want them start but equally if they do take them regularly we do not want them to stop during the study. (only applies to those collecting faecal samples)
- Bowel movements ≤ 3 times per week (only applies to those collecting faecal samples)
- Gastro-intestinal diseases (excluding hiatus hernia unless symptomatic or study intervention/procedure is contra-indicated).
- Parallel participation in another research project which involves dietary intervention
- Participation in another research project which has involved blood sampling within the last four months unless the total amount of combined blood from both studies does not exceed 470 ml.
- Has donated or intends to donate blood within 16 weeks prior to or during the study period.
- Any person related to or living with any member of the study team
- Lack of capacity to provide written informed consent
- Are pregnant or have been pregnant within the last 12 months

5.3 Clinical screening exclusion criteria
- Clinical screening results deemed by the HNU Medical Advisor to be indicative of a health problem, which may compromise the well-being of the participant, or which could affect the study data.
- Depressed or elevated blood pressure measurements (<90/50 or 95/55 if symptomatic or ≥160/100 mmHg)
- BMI (Kg/m^2) <19.5 or > 40

6. Recruitment strategy
A total of 50 participants aged ≥ 45 years with a total cholesterol concentration ≥ 5.0 mmol/L at clinical screening are required to complete this trial. Based on screening data obtained from two previous trials we expect ~ 70% of otherwise eligible participants aged 45+ years to have a total cholesterol conc. ≥ 5.0 mmol/L. However, recruitment for this study will only cease once we are confident that the required number of participants will complete the study.

Potential participants meeting the basic criteria for this study will be identified using the Quadram Institute Bioscience Human Nutrition Unit (HNU) volunteer database. The HNU database contains names and contact details of people who have registered an interest in volunteering for human studies. Identification of participants from the database is carried out by the HNU senior research nurse. Those identified from the database will be sent a participant information sheet (Annex 1) supported by a letter of invitation (Annex 2). A response slip and pre-paid envelope will be included.

We also aim to recruit potential participants via GP surgeries. We intend to seek the adoption of this study onto the National Institute of Health Research (NIHR) portfolio which will enable us to recruit via GP surgeries with the help of the co-ordinators from the Primary Research Network (PCRN) – East of England. **Identification of potential participants from** GP surgeries will be carried out by the surgery administrative staff and/or research nurses. The list containing the names of eligible patients identified on the database search will be checked by the GPs in that practice before mailing the participant an invitation letter (Annex 3), participant information sheet (Annex 1) and a pre-paid response envelope. A pack containing information about the study and copies of the study approval letters will be sent to all GP surgeries who indicate an interest in assisting with recruitment. If permitted, we will place an electronic copy of the study advertisement (Annex 4) on the website of participating GP surgeries.

In addition, poster and E-mail advertisements (Annex 4) will be placed across the Norwich Research Park (University of East Anglia (UEA), John Innes Centre (JIC), The Earlham institute, QI and other suitable locations such as social clubs, supermarkets, fitness centres etc. within the vicinity of QI (approx. 40 mile radius), inviting anyone who is interested in receiving information about the study to contact named researchers or the HNU senior research nurse. The local media (e.g. radio, newspapers/magazines etc.) may also be approached to advertise the study. We will also take advantage of social networking sites such as Facebook and twitter which will display either the ethically approved advert or be used to direct potential participants to a website where the approved advert is already displayed. Those responding to advertisements will be sent a letter of invitation (Annex 5) along with a participant information sheet (Annex 1). A response slip and pre-paid envelope will also be included.

Those participants responding positively after reading the information sheet will be invited to the HNU for an informal meeting with a member of the research team who will explain the study fully, focusing on the participant's involvement. Participants will be encouraged to ask questions at this stage prior to making any commitment. At the end of the meeting, participants will be given a minimum of 24 hours to consider whether they wish to take part in the study. During the consideration period, participants will not be contacted. If, following the consideration period they wish to participate in the study, they will be asked to contact the study scientist or HNU senior research nurse to arrange an appointment at the HNU for the pre-study clinical screening. For those individuals who are very keen to participate in the study and request to book a date for their clinical screening whilst at this meeting, this may be arranged. However, the screening appointment will be arranged for some time after the 24-hour consideration period and there will be no contact during this time.

6.1 Clinical screening

Prior to the clinical screening, participants will be reminded to bring details of any prescribed medication, herbal remedies or dietary supplements. Participants

will attend the HNU following a 10 hour overnight fast but will be advised that they should drink water during the fasting period. All potential participants will be assessed for eligibility on the basis of the inclusion/exclusion criteria, eligibility questionnaire and the results of clinical laboratory tests.

On arrival at the HNU the study scientist will go through the consent form (Annex 6) with the participant and answer any questions that may arise at this stage. He/she will then be asked to sign a consent form agreeing to participate in the study. Participants will also be asked to sign a medication declaration form (Annex 7) agreeing to inform the study scientist of any medication (prescribed or not) that they ingest once they have started the trial. A copy of these forms will be given to the participant to keep. An HNU research nurse will then complete a basic eligibility questionnaire (Annex 8) measure and record blood pressure, pulse, height and weight and calculate body mass index. A 12 mL blood sample (equivalent to ~ 2 teaspoons) will be obtained and sent to Spire hospital for FBC, lipid profile (Total cholesterol, HDL & LDL cholesterol & triglycerides) and HbA1c analysis. Upon completion of the clinical screening, participants will be offered a standard HNU breakfast.

Results of the screening blood test that fall outside the standard reference ranges will be assessed by the HNU Medical Advisor (or in her absence a doctor from the UEA medical centre) who will advise whether inclusion, re-screen or exclusion is appropriate. It is possible that some minor deviations from the normal reference range will neither affect the study data nor have health implications for the participant. In this case, the medical advisor may deem inclusion the appropriate course of action and write 'satisfactory' on the blood result form. These participants will be included in the study without being informed that the results of the blood test fell outside the standard reference range. If however, a re-screen is deemed the appropriate course of action then the participant will be contacted and offered an appointment for a further blood test. This will be after an appropriate period of time as advised by the medical advisor. Those participants who display screening parameters outside the standard reference ranges on both occasions may be excluded from the study depending upon the results flagged. Participants who do not wish to be re-screened will be excluded from the study. Participants who are excluded from the study will be informed of 'flagged' results by telephone and a letter advising them to contact their GP (Annex 9). Study scientists will not inform the participant which results are flagged or advise on any course of action. If the blood test results indicate a medical problem requiring immediate attention (e.g. anaemia, raised blood glucose) the HNU medical advisor may advise the HNU senior research nurse to contact the participant directly to inform them of the results and enable them to make an appointment with their GP. The test results will be sent to the GP within 24 hours of comment by the medical advisor. Participants who meet the study criteria and whose clinical screening results are deemed satisfactory will be advised that they may continue with the study. All clinical screening results will be forwarded to the respective GP (Annex 10) supported by an appropriate letter (Annex 11; flagged results and Annex 12; normal results). Additionally, the respective GP of those participants successfully recruited onto the study will be informed by letter of their patient's participation (annex 13).

7. Study procedure

Once successfully recruited onto the study, participants will commence the trial no later than 12 weeks from the screening date.

7.1 Participant Randomization

Participants in this study will act as their own controls in a 3-arm randomised cross-over study involving the daily ingestion of capsules containing (i) a bilberry extract providing 320 mg anthocyanins, (ii) a black rice extract providing 320 mg anthocyanins and (iii) a placebo control over a 28 day period. Each treatment will be assigned a letter (A-C) by a staff member not involved with the trial. For emergency purposes only, a member of QI staff will receive a sealed envelope containing the above letters and corresponding treatment. In the absence of an emergency the envelope will only be opened upon completion of the data analysis by the study statistician. Randomization to treatment order will be assigned as follows: There are 6 possible sequences of the letters A-C. Each sequence of letters will be listed 1-6. Each time a participant is successfully recruited onto the study, the order of intervention will be assigned by allocating the participant to the next sequence of letters from the list. Once all 6 sequences have been used the process will be repeated. The randomization sequences will be generated by the computer programme, randomization.com. The study scientist will be responsible for the randomization process and subsequent allocation of participant code to treatment sequence. However, both the study scientists, HNU research nurses and the participants will be blinded to the treatments.

7.2 Capsule preparation

Bilberry fruit and black rice extract powders will be supplied by the Beijing Gingko Group (BGG). The BGG product specification sheets for both types of extract have been supplied with this submission (Annexes 14 & 15). The bilberry and black rice extracts will be analysed at the QI to confirm the anthocyanin content of the material. This information will be used to determine the correct mass of 'active' material required to deliver the daily dose of treatments (i) and (ii). Once established, the extracts will be encapsulated in the food grade kitchen in the HNU by appropriately trained members of the study team holding certificates in Level 2 Food Safety and Catering. Members of the research team have experience in encapsulating test materials for human consumption acquired as part of the EU BACCHUS project (REC ref No: 13/EE/0393). Product specification sheets for the capsules to be used have been included with this submission (Annexes 16 & 17). Each capsule will contain a total mass not exceeding 500 mg of material which will comprise the active ingredient (bilberry or black rice extract) and pharmaceutical grade microcrystalline cellulose (*Vivapur 112*); a biologically inactive and commonly used filler in tablets and capsules (product specification sheet attached; Annex 18). The placebo capsules will contain microcrystalline cellulose only. Filled capsules for each treatment arm will be counted into moisture resistant HDPE bottles with an integrated desiccant cap and labelled accordingly. A subset of sealed bottles containing filled capsules for each treatment and placebo arm will be sent to an accredited laboratory for microbial safety analysis prior to human consumption. All capsules will look the same. Preliminary analysis at QI of a small sample of the two extracts indicates that participants will be required to ingest four capsules per day for each of the three treatment phases. The capsules will be stored according to the manufacturer's instructions.

NOTE: As stated previously bilberries contain ~ 285 mg anthocyanins per 100 g fresh weight (USDA). Therefore, to achieve the 320mg daily dose of anthocyanins proposed in this trial, an equivalent 112 g fresh bilberry would be required. Based on

an average portion size of 80 g (FSA) this is equivalent to ingesting ~ 1.5 portions of bilberries per day.

7.3 Intervention procedure

Participants will be required to restrict their intake of berry fruits to a combined maximum of 3 portions per week. The restrictions will commence 2 weeks before starting the first study phase and then continue until he/she has completed the study in its entirety (inclusive of wash-out phases). In addition, for the 24-h preceding test days at the start and end of each treatment period, berry fruits will be completely excluded from the diet. Participants will be provided with a list of restricted fruits.

Each of the three study phases will comprise a 28-day treatment period which will be identical in nature (except for the treatment consumed) and separated by a washout period of at least 4 weeks. Each treatment period is described below.

Day 0 – As mentioned above participants will already be restricted in terms of berry fruit consumption. For this 24-h period preceding day 1 they will be required to collect all urine passed and to completely exclude berry fruits from the diet. They will also be asked to fast overnight (minimum 10 hours) but will be advised to drink as much water as they need during the fasting period.

Day 1 – Fasted participants will arrive at the HNU on the morning of day 1 to undergo a baseline assessment. The sub-set of participants (n= 24) will be required to arrive with a faecal sample. Instructions on how to do this (Annex 19) will have been given. Prior to starting any procedures, participants will be asked some basic questions to establish whether or not any changes in medical conditions/medications etc. have occurred since their last visit and whether they have experienced any adverse events not already disclosed by telephone, E-mail or letter (Annexe 20). Next, the participant will be weighed, after which a 35 mL blood sample will be obtained. Participants will then consume 4 capsules (equivalent to the daily dose) containing the allocated treatment. The capsules will be ingested with 150 mL water. Participants will be required to remain in the nutrition unit for at least 20 minutes after ingestion of the capsules. A standard HNU breakfast consisting of toast with spread and/or cereals, tea/coffee will then be served.

Prior to leaving the HNU participants will be instructed on the correct administration/storage of the capsules and supplied with the required number of capsules to be consumed over the ensuing 28 day period. Participants will also be reminded to continue restricting berry fruit consumption to a maximum of 3 portions per week. Participants will be contacted 2-weeks into the treatment period to check on progress. Regardless, they will be advised to contact a member of the study team should they become unwell or experience any problems with taking the capsules.

Day 28 – As per day 0, participants will be required to exclude all berry fruits from the diet and collect all urine passed for the 24-h period preceding day 29. They will also be asked to fast overnight (minimum 10 hours) but will be advised to drink as much water as they need during the fasting period.

Day 29 – Fasted participants will arrive at the HNU on the morning of day 29 to undergo post intervention assessment as described for D1. The sub-set of participants (n= 24) will be required to arrive with a faecal sample. A further 35 mL blood sample will be obtained. On completion of the post intervention assessment, participants will be given a standard HNU breakfast.

In the unlikely event that a participant is unable to attend the pre-scheduled d29 assessment day, a lee-way of ± 3 days is considered an acceptable time period for the purposes of re-scheduling the appointment. In the event that the original appointment is delayed, the participant will remain on the treatment until the day preceding the final assessment day. If the participant is unable to re-schedule within ± 3 days or due to unforeseen circumstances is required to interrupt the treatment period (e.g. minor illness) they will be given the opportunity to repeat the treatment at a later date.

The blood volume obtained from a participant during a single test phase is 70 ml. The total blood volume taken over the entire study is 222 ml (incl. screening sample). This will rise to 234 mL if a participant requires a repeat screening blood test. The container to be used for each of the 24-hour urine collections will contain ~ 1 g ascorbic acid (vitamin C) as an antioxidant.

Donation of faecal samples by the sub-group of participants on this trial will be done so on a voluntary basis. It will be made clear to participants that if he/she is willing to donate the sample then it is a requirement to do so at the start and end of all three treatment phases. Participants will be given the option of providing a faecal sample within 3 days preceding the start of the treatment period (D1) or ± 3 days of finishing the treatment period (D29). If they have been unable to produce a faecal sample within the time period preceding or on D29, the assessment day will continue as scheduled but the participant will be asked to continue consuming capsules until the faecal sample has been produced. Any faecal samples provided outside the d1 and d29 assessment days will be delivered to QI within 6 hours of production. Since we will be excluding participants from this aspect of the study if their habitual daily bowel movements are ≤ 3 days per week, we do not anticipate faecal sample collection to be a problem.

The number of participants required to provide faecal samples is twenty four. To ensure that we finish the trial with the required number of faecal samples we will review the situation part way through. Should there be doubt that we will reach the target number, we will take the decision to only enrol those participants willing to donate faecal samples. This is reflected in the participant information sheet. We adopted a similar approach to recruitment in a previous study (REC ref: 13/EE/03930).

7.4 Treatment compliance

Participants will consume <u>four</u> capsules <u>once</u> daily for 28-days. They will be instructed to consume the capsules in the morning time. However, in the event that they forget, the capsules may be consumed later in the day. To assist participants with compliance to treatment they will be provided with a capsule checklist (Annex 21). Each time the capsules are consumed, participants will record this on the checklist. Participants will be contacted 2-weeks into each of the treatment periods to assess progress. Compliance to treatment will be assessed from the record sheet and the number of unused capsules returned at the end of the treatment period. Participants who ingest less than 80% of the capsules over the entire treatment period may be withdrawn from the study. This will be assessed on a case-by-case basis.

7.5 Participant expenses/payments

An inconvenience payment of £125 will be given to each participant who completes the study or pro rata for non-completion; this will increase to £155 for those participants collecting faecal samples. Participants will also be reimbursed

travel expenses to and from the QI (at the current QI mileage rate) or bus/train fares on production of a receipt. Participants will be notified that such payments are liable to tax. QI staff will be taxed at source.

7.6 Participant travel during treatment periods

Participants will not be able to travel during a 28-day treatment period. However, they may travel in-between treatment periods (e.g. during the washout phases of the study). The length of time between returning from abroad and commencing the next treatment will be determined on a case-by-case basis depending upon where travelled and whether or not they developed any gastro-intestinal symptoms.

8. Analytical methods

8.1 Processing and analysis of blood samples

Whole blood will be collected into EDTA, and serum separating tubes. EDTA samples will be immediately centrifuged at 2500 x g for 10 mins to obtain plasma. Samples collected into serum separating tubes will be allowed to clot for 30 min before centrifugation at 2000 x g for 10 mins. Sub-samples of plasma and serum will aliquoted into appropriate storage tubes, frozen on dry ice and then subsequently stored at – 80 ^{0}C until analysis.

8.1.1. Analysis of lipids, glucose, fructosamine and insulin

Total/HDL/LDL cholesterol, triglycerides glucose and fructosamine will be analysed using an automated bench top clinical chemistry analyser (RX Daytona plus) according to the manufacturer's instructions. Plasma levels of apolipoprotein A1 (ApoA1) will be quantified using an ELISA kit (Cusabio Biotech Co., Wuhan, P.R. China). We will separate and quantify HDL sub-fractions using a commercial product that is based on polyacrylamide gel electrophoresis separation of the sub-fractions, such as the Lipoprint® HDL Subfractions product from Quantimetrix (http://quantimetrix.com/lipoprint-2/product-information-2/lipoprint-hdl-subfractions-100-tests/).   Insulin concentrations will be determined by enzyme-linked immunosorbent assay (Elisa) according to the manufacturer's instructions.

8.1.2 Analysis of PON-1 and cholesterol efflux capacity

PON-1 activity will be measured using spectrophotometric assays to determine the rate of cleavage of a general PON1 substrate such as para-nitrophenol-acetate. In addition, the activity will be measured using a physiologically relevant lipid peroxide substrate as described by Aviram et al [31]. PON1 protein will be quantified using ELISA or western blotting using commercially available ELISA kits or anti-PON1 antibodies. The PON1 genotypes for the major single nucleotide polymorphisms at positions 192 (glutamine/Q versus arginine/R) and 55 (leucine/L versus methionine/M) and other known SNPs in PON1 will be determined using PCR reactions with specific primers with DNA obtained from whole blood as described by Browne et al. [32] and Mackness et al [10] and sequencing of the PCR products using standard sequencing techniques.

The effects of the diets on the cholesterol efflux capacity (CEC) of blood will be quantified by measuring the rate of efflux of labelled cholesterol from pre-loaded human macrophages (phorbol myristate-treated THP-1 cells) to serum samples obtained before and after the 4-week dietary intervention. Assays will be conducted using a cholesterol efflux assay available from Abcam (Product:

ab196985). By quantifying the CEC of minimally processed plasma and also plasma that has been pre-processed to remove ApoB1, we will also be able to determine if the anthocyanin-mediated changes in CEC are due to changes in ApoB1, HDL-cholesterol or both. Ethylene glycol precipitation (MW=8,000; Sigma) will be used to strip serum of apoB lipoprotein according to a protocol published by Asztalos et al. [33] CEC values will also be correlated with HDL concentrations (determined in 8.1.1).

8.1.3 Analysis of bile acids (and derivatives)
Bile acids and bile acid derivatives will be analysed using an established in-house LC-MS technique. In brief, aliquots of plasma will be mixed with methanol prior to centrifugation at 17,000 x g for 10 min. The resultant supernatant will be rotary evaporated, almost to dryness, before the addition of 1 mL aqueous methanol. Samples will be passed through an SPE cartridge before applying to LC-MS.

8.1.4 Analysis of anthocyanins
Plasma anthocyanins and anthocyanin metabolites will be analysed using an established in-house extraction procedure and LC-MS technique. In brief, trifluoroacetic acid will be added to aliquots of plasma to precipitate the proteins. Post centrifugation (16,000 x g; 4°C; 15 minutes) samples of plasma will be transferred into auto-sampler vials for HPLC analysis.

8.1.5 microRNA analysis
MicroRNA's will be extracted using a miRNAeasy serum/plasma kit (Qiagen, UK) according to the manufacturer's instructions. cDNA for individual microRNA will be synthesised using a Taqman MicroRNA reverse transcription kit (Life technologies) and quantitative PCR will be carried out using Taqman MicroRNA assays for individual microRNAs (Life technologies).

8.1.6 Plasma metabolite profiling
Plasma metabolite profiles will be determined using a protocol/platform such as the Absolute/DQ® p180 kit marketed by Biocrates Life Sciences (Innsbruck, Austria) or similar. In addition, other metabolites of interest such as those in the L-carnitine / trimethylamine (TMA) / trimethylamine-N-oxide (TMAO) pathway that are prognostic of cardiovascular and metabolic diseases will be measured using targeted LC-MS or other methods as appropriate.

8.2 Processing and analysis of faecal and urine samples
Participants will be provided with a faecal collection kit consisting of an insulated container, a plastic pot, bag and clip, and ice cube bags. A description of the collection procedure is given in Annex 19. Urine will be collected in containers with ascorbic acid as previously described. Aliquots of fresh faecal matter and urine will be placed into appropriate vials and stored at – 80 ^{0}C until analysis. Portions of faecal matter will also be used to prepare faecal slurry glycerol stocks and these will be stored at -80 ^{0}C until used.

8.2.1 Analysis of bile acids and bile acid derivatives in faeces
Bile acids and bile acid derivatives from faecal samples will be analysed using established in-house LC-MS protocols.

8.2.2 Analysis of cholesterol in faeces

Faecal cholesterol will be measured using a colorimetric assay (Cell Biolabs Inc) according to the manufacturer's instructions.

8.2.3 Microbiota profiling (resources permitting)

DNA will be extracted from the faecal samples using FastDNA™ spin kits according to the manufacturer's instructions. Gut microbiota composition will be done under contract at the Earlham Institute (Norwich Research Park, UK) using Illumina MiSeq technology to undertake 16S-based rRNA gene profiling metagenomics. In addition, we will use faecal slurries to seed simple anaerobic batch colon models which will be used to (a) measure the anthocyanin metabolic capacity of the individual faecal microbiota samples which can be compared with the appearance of anthocyanin metabolites in plasma and urine samples, and (b) identify polyphenol metabolites that alter the gut microbiota profile and metabolic capacity.

8.2.4 Analysis of anthocyanins in urine

Urinary anthocyanins and anthocyanin metabolites will be analysed using an established in-house extraction procedure and LC-MS technique. In brief, an internal standard will be added to aliquots of urine before centrifugation (16,000 x g; 4°C; 15 minutes). Samples of urine will be transferred into auto-sampler vials for HPLC analysis.

8.2.5 Analysis of microbial metabolites in faeces and urine

Metabolites in faeces and urine will be determined using NMR and / or LC-MS methods. In addition, other metabolites of interest such as those in the L-carnitine / trimethylamine (TMA) / trimethylamine-N-oxide (TMAO) pathway that are prognostic of cardiovascular and metabolic diseases will be measured using targeted LC-MS or other methods as appropriate.

8.3. *Analysis of anthocyanins in berry fruit and black rice extracts*

Berry fruit and black rice powder (5 mg), will be dissolved in 1 mL acidified aqueous methanol and centrifuged at 17,000 x g for 5 mins. The supernatant will be applied to LC-MS. Chromatographic anthocyanin peak identification will be achieved by comparison of retention times with analytical standards and mass spectral data. Anthocyanins will be quantified against reference standards over the range 0-50 µg/mL.

9. Ethical considerations

9.1 Confidentiality

Participants will be assigned a unique code which will be used on all samples/data arising from this study. Only the study scientist named in the approved documentation will be able to link the code with the participant. However, the confidential code may be broken in the event of a medical emergency as deemed appropriate and necessary by the HNU senior research nurse or medical advisor. Coded information will be kept in locked cabinets at the QI.

All personal information will be kept confidential and known only to the Chief Investigator or project scientist, HNU nurses and medical advisor and the participants GP. Personal information will be kept separately to coded information in locked cabinets at the QI.

9.2 Informed consent

To ensure participants can make an informed decision as to whether or not they wish to take part on the trial they will be provided with a PIS and invited to an informal discussion about the study. Consent will be obtained by a member of staff who is GCP trained and experienced in conducting human intervention trials.

9.3 Risks and burdens

This study involves blood sampling and as such there is a risk of minor discomfort and/or bruising at the site of venepuncture. The research nurses at QI are experienced in this procedure thus minimizing this risk. The total volume of blood collected over the entire duration of the study is 222 mL, which is less than that donated at a single session with the blood transfusion service. Regardless, iron status will be measured as part of the eligibility assessment to exclude anaemia prior to participation.

This trial also involves overnight fasting. Participants will be advised to drink water during the fasting. All study sessions will be scheduled for the morning time to reduce the length of fast and participants will be offered breakfast prior to leaving the nutrition unit. At the eligibility assessment, we will also undertake an HbA1c test to exclude undiagnosed diabetic participants

As part of the eligibility assessment exercise it is possible that some blood test results are abnormal. The procedure for this eventuality is described in section 6.1. All blood results (normal or otherwise) will be forwarded to the respective GP.

On this trial we will be recruiting participants with a higher than optimal total cholesterol. Participants who are subsequently treated with lipid lowering therapies will be excluded/removed from the trial. This information is provided in the PIS.

10. Power calculations & statistical analysis

LDL Cholesterol: The primary outcome measure for this study is LDL-cholesterol. Sample size was determined based on data from a study reported by Zhu et al [18]. We have chosen to use a conservative effect size of 0.14 mmol/L for the change in LDL cholesterol. In order to detect a change (relative to placebo) in LDL cholesterol of 0.14 mmol/L at the 0.05 level of significance and with 90% power in at least one of the treatment groups, 50 participants would be needed to complete a cross-over trial. This assumes that the standard deviations in the treatment and placebo groups are similar to that observed in the data from Zhu et al (approximately 0.5mmol/L).

MicroRNA: Sample size for the sub-set of participants was based on a study conducted by Kim et al [34]. In order to detect a change (relative to the athero-risk group) in Mir33a of 2.24 units at the 0.05 level of significance and with 90% power in the treatment group, 24 participants would be needed to complete a crossover trial. This assumes the standard deviations in the treatment and athero-risk groups are similar to that observed by Kim et al (approximately 3.24 units).

Data will be checked for normality and transformed if necessary to allow parametric statistical techniques (e.g. paired t-test, repeated measures ANOVA) to be used for analysis. Tests will be reported as significant if $P < 0.05$.

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Appendix 4

Invitation letters for participants of the BERI study

Appendix 4

Letter of Invitation for Non-Registered Participants

Quadram Institute Bioscience
Norwich Research Park
Colney Lane
Norwich
NR4 7UA

Dear

Thank you for your response to the advertisement regarding a study at the Quadram Institute Bioscience (formerly **Institute of Food Research**). Please find enclosed the details of the study.

If, after reading the information, you are still interested in participating please complete the reply slip and return in the freepost envelope provided. Alternatively, you can contact the scientist/study manager **Wendy Hollands (01603) 255051** or the HNU Senior Research Nurse **Aliceon Blair (01603) 255305** as stated on the study information sheet.

Expressing an interest does not commit you to taking part.

Thank you very much for your time

Yours sincerely,

Wendy Hollands
Study Manager

Letter of Invitation for GP Registered Participants

GP Practice Headed Paper

Date

Dear (name and address),

Research study: The effects of bilberry fruit and black rice derived anthocyanins on lipid status in adults

Recently, doctors at the practice have been approached to ask if any of our patients would be interested in taking part in a research study at the Quadram Institute Bioscience in Norwich (formerly **Institute of Food Research**).

The aim of the study is to determine whether a compound in berry fruits and black rice, called anthocyanins, can have an effect on some 'risk' factors for cardiovascular disease. One main example being investigated is whether anthocyanins can lower blood cholesterol.

Our records show that you might be suitable for this research. The **Participant Information Sheet (PIS),** included with this letter, will tell you all about what taking part in the study would mean. If, after reading the PIS you would like to find out more about the study please return the reply slip (at the back of the PIS) to the study team at the Quadram Institute in the freepost envelope supplied. A member of the study team will contact you once they have received the reply slip. You can also contact a member of the study team if you wish to ask any questions about the study before you return the reply slip. The contact details for the study team are on the front of the PIS.

The study will take place in the **Human Nutrition Unit at the Quadram Institute Bioscience** (near the Norfolk and Norwich University Hospital).

Please do not send any reply slips to your GP surgery

Yours sincerely

Dr…………………………………

Letter of Invitation for Quadram Institute Bioscience Database Participants

Quadram Institute
Colney Lane
Norwich
NR4 7LU

Dear

Thank you for your interest in studies at the Quadram Institute (formerly **Institute of Food Research)**. Please find enclosed information on a study in progress at present entitled: The effects of bilberry fruit and black rice derived anthocyanins on lipid status in adults (short title: BERI study)

Details held on the QI Human Nutrition Unit (HNU) volunteer database suggest that you may fit the criteria for this study. If, after reading the information, you are interested in participating in this study, please complete the reply slip and return in the freepost envelope provided. Alternatively, you can contact the scientist concerned Wendy Hollands (01603) 255051.

Should your details have changed or you would prefer to no longer remain on the database please could you inform the Human Nutrition Unit on (01603) 255305.

Expressing an interest does not commit you to taking part.

Thank you very much for your time.

Yours sincerely,

Aliceon Blair
Senior Research Nurse

Appendix 5

Participant information sheet for the BERI study

Appendix 5

Quadram Institute Bioscience
Norwich Research Park
Colney
Norwich
NR4 7UA

PARTICIPANT INFORMATION SHEET

The effects of bilberry fruit and black rice derived anthocyanins on lipid status in adults

Short title: BERI Study

You are being invited to take part in a research study at the Quadram Institute Bioscience **(formerly Institute of Food Research**). Before you decide to take part it is important for you to understand why the research is being done and what it will involve for you. Please take time to read the following information carefully and discuss it with others if you wish.

<u>Part 1</u> of this information sheet tells you the purpose of the study and what will happen if you decide to take part.

<u>Part 2</u> gives you more detailed information about the conduct of the study.

Please ask if there is anything contained in the information sheet that is not clear, or if you would like more information. Take time to decide whether or not you would like to take part. This information is yours to keep. Thank you for reading this.

Study contact details:

Wendy Hollands (study manager)
(01603) 255051
Wendy.hollands@quadram.ac.uk

Aliceon Blair (HNU senior research nurse) (01603) 255305
aliceon.blair@quadram.ac.uk

Dr Paul Kroon (Chief Investigator)
(01603) 255236
Paul.kroon@quadram.ac.uk

Part 1

What is the purpose of the study?

Regular consumption of fruit and vegetables has been shown to reduce the risk of cardiovascular disease (CVD). The protective effect may be because of compounds contained within these foods, called flavonoids. Anthocyanins are a particular group of flavonoids and are found in berry fruits (e.g. blueberry, bilberry, raspberry, and strawberry) and other foods such as aubergine skins and black rice. Anthocyanins are what give the red, black, purple and blue colours to such foods and different foods may contain different types of anthocyanins. For example, bilberry fruit contains a different type of anthocyanin to that of black rice.

There are several 'risk' factors for CVD which can be easily measured. One of the measurements that most people know about is blood cholesterol; that is good cholesterol (otherwise called HDL cholesterol), bad cholesterol (otherwise called LDL cholesterol) and triglycerides. A small number of studies have found improvements in blood cholesterol after consumption of foods rich in anthocyanins. In this study we want to compare the effects of two different types of anthocyanins (one from bilberry fruit and the other from black rice) on cholesterol status in people with higher than optimal blood

cholesterol levels. We also want to investigate the effects of the different anthocyanin types on how the body processes cholesterol (called transport and metabolism) and relate this information to a specific gene involved in the cholesterol metabolism process. How we achieve all this is describe throughout this information sheet. This study will involve the consumption of capsules that contain anthocyanins that have been removed from bilberry fruit and black rice and not the whole food. It will also involve the collection of blood and urine samples and for a small sub-group of people, the collection of faecal samples.

Why have I been invited?

You have received this information because you have responded to an advertisement about the study OR you have been identified as a potentially suitable candidate either from the volunteer database held at the QI (formerly **Institute of Food Research**) human nutrition unit (HNU) or via your GP surgery.

We are aiming to recruit a total of 50 volunteers (male and female), who are aged 45 years or more with a cholesterol level at or greater than 5 mmol/L.

You will not be able to volunteer if you:

● Are a smoker (or stopped smoking for less than 3 months)
● are diabetic
● are taking medication for high cholesterol (e.g. statins)
● have a medical condition or are taking
  other medications (prescribed or otherwise) which may affect the study
  outcome (we will advise you accordingly)
● have gastro-intestinal disease (except
  hiatus hernia)

- take supplements judged to affect the study data (we will advise you accordingly).
- Use foods for lowering cholesterol e.g. benecol, flora proactive (this will be assessed on an individual basis).
- Intend to change your normal use of pre or probiotics during the study e.g. if you don't take these products we don't want you to start taking them but equally if you take them regularly we don't want you to stop taking them during the study.
- Regular/recent use of colonic irrigation or other bowel cleansing techniques.
- are pregnant or have been pregnant within the last 12 months
- are related to someone in the study team (e.g. spouse, partner, immediate family member)
- have donated or intend to donate blood within 16 weeks of the first and last study samples.
- have participated in another study which has involved blood sampling within the last four months unless the total amount of combined blood from both studies does not exceed 470 mL
- are currently involved in a study which involves dietary intervention.
- the results of our screening test indicate that you are not suitable to take part in this study
- Are unable to provide written informed Consent
- Have a BMI less than 19.5 or more than 40 kg/m^2

Do I have to take part?

It is up to you to decide. We will describe the study in this information sheet. If, after reading it you are interested in taking part in the study, you should contact one of the researchers named on the first page of this information sheet

OR

You can complete the attached response form and return it using the pre-paid envelope enclosed. Please feel free to say no by not responding to this information. Do not worry, nobody will contact you to try and persuade you to join the study.

After you have replied to tell us you are interested in participating, a member of the study team will contact you, and you will be invited to the HNU at the QI for a talk with the study scientist/manager. An expression of interest does not commit you to taking part.

If you are on the QI HNU volunteer database, a decision to withdraw or not to take part will not affect your participation in future studies. Similarly, if you have been recruited via your GP surgery, a decision to withdraw or not to take part will not affect your care.

What will happen to me if I do take part?

If you decide to take part, your involvement in the study will last about 6 months depending upon your availability for the study visits. You will be required to visit the HNU on 8 separate occasions (2 visits before the study starts and 6 visits during the study). Each of these visits is described below. Where possible appointments will be made at your convenience but will take place on a weekday (Mon-Fri).

Pre-study talk (**Visit 1**)

This meeting will last for about one hour. The study scientist will go through this information sheet with you and answer any questions you may have. At the end of the meeting you

will be given as much time as you need to decide whether or not to take part, but this will be at least 24 hours. You will not be contacted during this time. After the 24 hours if you decide to take part you will need to contact the study scientist to arrange an appointment for the next visit.

Elibility assessment /informed consent **(Visit 2)**.

This visit will last up to one and three quarter hours. You will be asked to come to the HNU between 07.30 and 10.30 hours. You will be asked to arrive having fasted for at least 10 hours. Fasting means you must not eat anything or drink anything other than plain water. During the fasting period you may drink as much water as you need.

Before we complete the eligibility assessment you will be asked to sign a consent form agreeing to take part in the study. You will also be asked to sign a medical declaration form. These forms will also be signed by the scientist/nurse and you will be given a copy of the forms to keep. After signing the consent form you are still free to withdraw from the study at any time without giving a reason.

An HNU research nurse will then complete a brief eligibility questionnaire with you and also measure and record your blood pressure, pulse rate, weight, height and body mass index (BMI). Your height and weight measurements will be used to work out your BMI. BMI is an expression of body weight which takes into account your height. A simple formula is used to calculate your BMI. If your BMI falls outside the range for this study you will be excluded from taking part.

The HNU research nurse will then take a 12ml blood sample (equivalent to 2 teaspoons) from a vein in one of your arms. The blood sample will be used to test Full Blood Count (FBC), cholesterol (total, HDL, LDL and triglycerides) and HbA1c (blood sugar). These tests will check for anything outside of the standard reference ranges, which may affect your wellbeing or the study data if you participate. However, we are looking to recruit people with a higher than optimal total cholesterol level (at or greater than 5 mmol/L). After this you will be encouraged to have breakfast (cereal, toast, tea, coffee) before you leave the HNU.

If any of your clinical results are outside the standard reference ranges, we may recommend that you speak to your GP about the results. All results outside the reference ranges are checked by the HNU medical advisor. The medical advisor will decide whether we may include you in the study, offer you the opportunity of a second screening (re-screen) or exclude you from taking part in the study. If your results fall outside the standard reference ranges on the second occasion you may be excluded from the study depending on the results flagged. We cannot tell you what your results may mean as we are not medically qualified to do so. You should not worry if your results are flagged as it may be a one off result or it may be perfectly normal for you and your GP will be able to explain them to you. Please remember these tests are performed to determine if you are suitable for the study not to find out if you are healthy.

You will be excluded from the study if the results of the test are judged by the medical advisor to be unsuitable for your participation or your cholesterol level is less than 5 mmol/L.

The HNU research nurses are experienced in carrying out clinical procedures, and if you have any

questions about any of the tests, please ask one of the nurses.

You will be invited to take part in this study if the blood test from the eligibility assessment is satisfactory and you meet all the listed criteria for participation.

The study

There are three phases to this study each of which will last for a period of 29 days. The only difference between each of the three test phases is the content of the capsules consumed. These are described here.

a) 4 capsules/ day containing 320 mg bilberry anthocyanins
b) 4 capsules/day containing 320 mg black rice anthocyanins
c) 4 capsules/day containing no anthocyanins (placebo)

The dose of anthocyanins is equivalent to eating about one and a half standard portions of bilberries per day.

The order in which you consume the capsules containing each of the three test products will be 'randomly' assigned by a computer programme. When we say "randomly assigned" this means that the order is assigned by a method similar to being picked out of a hat. You will not be able to choose your order. Neither the scientist in charge of the project or you will know in which order you are consuming them. The capsules will all look the same.

Two weeks before starting the first phase of the study you will be required to restrict your intake of berry fruits to a combined maximum of 3 portions per week. A list of fruits to restrict is provided at the end of this information sheet. The restrictions will continue until you have completed the study in its entirety. A test phase is described below:

Day 0 – You will be asked to collect all urine passed (in containers which we will provide) for 24 hours before the visit on day 1. Please note that this urine bottle will contain a white powder called ascorbic acid (vitamin C) which is needed to preserve the urine. The urine container should be kept away from children and animals. You will also be asked to completely avoid eating berry fruits.

Day 1 - (**Visit 3**)
This visit will last for approximately 1 hour. You will be asked to come to the HNU between 07.30 and 10.30 hours. You will be asked to arrive having fasted for at least 10 hours. During the fasting period, you may drink as much water as you need.

After arrival and before consumption of the test capsules a nurse will weigh you and take a 35mL blood sample (equivalent to about 3 tablespoons). You will then be given 4 capsules containing one of the test products to consume. The capsules will be taken with 150 mL water (equivalent to about ½ a glass). You will be required to remain in the nutrition unit for at least 20 minutes after consuming the capsules. Breakfast (toast, cereal and tea/coffee) will then be offered.

For the next 28 days you will be asked to continue taking 4 capsules once daily. Capsules should be taken some time during the morning. However, if you forget, they can be taken in the afternoon. Capsules may be taken with water but not at the same time as food (please allow 15 minutes either side of food). If you become unwell or experience any problems with taking the capsules, then please contact a

member of the study team. You will be provided with a capsule checklist and asked to tick the checklist each day the capsules are taken. Remember, you will also be restricting your berry fruit intake.

Day 28 - You will again be required to collect all urine passed for this 24-h period immediately preceding the day 29 visit. You will also be asked to completely avoid eating all berry fruits.

Day 29 (**Visit 4)**
This visit will last about 1 hour. You will be asked to come to the HNU between 07.30 and 10.30 hours. You will be asked to arrive having fasted for at least 10 hours. On this visit another 35mL blood sample will also be obtained. Afterwards, breakfast (tea/coffee, toast and/or cereal) will be provided. This completes phase 1 of the study.

The remaining 2 test phases will each be separated by a minimum of 4 weeks and there are 2 visits per test phase (**Visits 5-8)**. You will be asked to restrict your berry fruit intake to 3 portions per week during these wash-out phases and completely avoid berry fruits for 24-hours before each of the visits to the nutrition unit.

Please note: If you intend to holiday/ travel whilst taking part on the study, we will require you to remain in the UK during each of the three 28-day test phases. However, you may holiday/travel outside the UK in-between test phases.

A flowchart giving a brief outline of the study can be found on page 10.

A small sub-group of participants (24 people) will be required to provide a faecal sample at the start and end of each of the three test phases. You will be given a choice as to whether or not you donate faecal samples but you will

not be able to donate if your normal bowel movements are equal to or less than 3 times per week. If, part way through the trial there is doubt that we will reach the required number of participant donations, we will take the decision to only enrol onto the study those willing to donate faecal samples. If you do volunteer to collect faecal samples you will be provided with a collection kit and instructions on how to do this.

The total amount of blood we take in a single test phase (day 1 and day 29 inclusive) is 70 ml (about 6 tablespoons). The total amount of blood we take over the whole study, including the screening sample is 222 ml. If you need to have a repeat screening blood test this will rise to 234 mL). This is about half of that which you would give during a single blood donor session.

What we do with the blood and faecal samples is described in part 2 of this information sheet.

Should you become unwell whilst on a test phase we may ask you to stop taking the capsules and repeat that test phase once you are better. This will depend upon the nature of the illness and whether or not it will affect the study outcome.

Access to personal information
Once recruited onto the study you will be given a code number. This number will be unique to you and is used to protect your information and make your samples anonymous. Access to your personal information is restricted to the research team, HNU research nurses, HNU medical advisor and your GP. Further details about this are given in part 2 of this information sheet.

Expenses and payments
Participation in these studies is done on a voluntary basis. However, we do

recognize that being involved in the study can cause you some inconvenience and there are costs associated with visiting the HNU. You will receive an inconvenience payment of £125 for participation. This will be increased to £155 if you collect faecal samples. If you are excluded from the study or you withdraw from the study the inconvenience payment you receive will be adjusted according to how much of the study you completed before exclusion/withdrawal. You will also be reimbursed travelling expenses to and from HNU on production of a receipt for buses and trains or at the current QI mileage rate for private cars.

Please note that payments are liable to tax and you are personally liable for your own tax assessment. If you are claiming state benefit, your entitlement may be affected by payments made for participating in the study. QI employees will be taxed via the payroll.

What are the risks and side effects from taking part in this study?

There can be a small amount of discomfort associated with taking blood. This may affect some people more than others, but the discomfort occurs generally only on insertion of the needle.

You may develop a small bruise at the site of the blood sample, but this will fade as with any bruise.

The HNU research nurses will be happy to answer any questions you may have about any procedures involved.

What are the potential benefits of taking part?

There are no direct benefits for you taking part in this study. However, the information we find out from this study will contribute to our understanding of the effects of anthocyanins on risk for CVD.

Will my taking part be kept confidential?

Yes. We follow Ethics and Research Governance requirements. All information about you will be handled in confidence. More details about this are included in part 2 of this information sheet.

This concludes part 1 of the information sheet.

If the information in part 1 has interested you and you are considering taking part, then it is important that you read the additional information in part 2 before making any decision.

Part 2

What if relevant new information becomes available or changes to the study are made?

If this happens we will let you know. If changes to the study are made and they impact on your participation, you will be given the new amended participant information sheet to read before being asked to sign another consent form.

What will happen if I don't want to carry on with the study?

You are free to withdraw from the study at any time without giving a reason. However, the study scientist will need to be informed of your decision to withdraw. You will receive payment pro rata for samples given up to the point of withdrawal. Samples collected will be kept until the study has finished and any data collected until the point of withdrawal will still be used.

What if there is a problem?

If you have a concern about any aspect of the study you should ask to speak to a member of the study team who will do their best to answer your queries. You can telephone Wendy Hollands on (01603) 255051 or the HNU senior research nurse on (01603) 255305.

If you require independent advice about the study or you are unhappy about any aspect of the study and/or wish to complain formally, you can do this through the chairperson of the QI Human Research Governance Committee (Dr Linda Harvey) on (01603) 255000.

The QI accepts responsibility for carrying out trials, and as such will give consideration to claims from participants for any harm suffered by them as a result of participating in the trial, with the exception of those claims arising out of negligence by the participant. The QI has liability insurance in respect of research work involving human volunteers.

Please note that the Institute will not fund any legal costs arising from any such action unless awarded by a court.

Will my taking part on this study be confidential?

Only personal information which is needed for the study will be collected and this will be held in the strictest confidence. Access to your personal records is restricted to the study team, the HNU nurses, HNU medical advisor and your GP. All personal information is kept in locked cupboards at the QI.

Once recruited onto the study you will be issued with a volunteer code number. This number will be used on all your personal information and samples so that nobody else will know or be able to work out that they are yours. Coded information is also kept in a locked filing cabinet at the QI but separately to your personal information.

The data generated from study samples will be stored in a secure archive for up to fifteen years after the end of the study. It will then be destroyed.

All research is subject to inspection and audit and although your records may be accessed for this purpose any personal information remains confidential.

Please note that the QI has CCTV cameras in use for security purposes. However, provision has been made so that volunteers attending HNU will not be identified.

Will my GP be informed?

Yes. It is routine practice to inform your GP that you are taking part in a study at QI. We will also send your GP

details of all your clinical screening results (blood tests, blood pressure weight and BMI measurements). Your permission will be sought and this is one of the things you are agreeing to when you sign the consent form. If any of these blood results fall outside the standard reference ranges we may recommend you speak to your GP about it. We are unable to discuss your blood results with you.

What will happen to the samples I provide?

For all participants: The 12mL blood sample you provide at visit 2 (eligibility assessment) will be sent to Spire hospital for Full Blood Count, HbA1c (blood sugar) and lipid profile (total cholesterol, HDL and LDL cholesterol and triglycerides). The purpose of this is to check for anything outside the reference ranges which may affect your well-being if you took part but also to make sure you fit the criteria for the study.

The blood samples you provide during the course of the study will be used to investigate the differences in effects between the two anthocyanin types in the capsules you consumed on (i) components associated with CVD 'risk'. These include blood sugar status (glucose and fructosamine), levels of insulin and also lipids (total cholesterol, HDL and LDL cholesterol and triglycerides); (ii) components of blood associated with how we metabolize and transport lipids within the body and the specific gene (called PON-1) associated with the cholesterol metabolism process; and (iii) bile acids (these are breakdown products of cholesterol found in blood). Blood and urine samples you provide will also be analysed for anthocyanins and the breakdown products of anthocyanins to check how much has been absorbed by the body after consumption.

The PON-1 gene is important because it is involved in preventing atherosclerosis (a cause of CVD). We want to determine if there is a relationship between the effects of consuming anthocyanins with PON-1 activity and PON-1 genotype. There are differences between people in their PON-1 gene (called genotype). We can find out which PON-1 genotype you are, and then we will determine if there is a relationship between different PON-1 genotypes and how they responded to the anthocyanins (e.g. changes in good cholesterol). The result of this gene test has no significance for you or your family, and you will not be told your genotype.

For the sub-group (24 people): In the faecal samples you provide, we will measure the amount of bile acids and lipids that are excreted. We will also make additional measurements of something called RNA (Ribonucleic acid) in the blood samples you have already provided. In short, when your body naturally produces new proteins your DNA (Deoxyribonucleic acid) is the code used to make RNA which in turn is the code for making the new protein. By looking at the RNA we can get an idea of the proteins that were especially made as a result of the anthocyanins you consumed from the study. The RNA's we measure will be some of those specifically related to atherosclerosis (a cause of CVD) and cholesterol metabolism.

NOTE: Should more resources become available during the trial, we will determine the effect of the two anthocyanin types on other RNA's related to CVD. By using the faecal samples you provided we may also investigate the relationship between the natural gut bacteria and faecal bile acids and lipids to determine if they can be modulated by anthocyanins.

What will happen to the results of the research study?

As a volunteer you are valuable to us but we are unable to tell you any of your individual results. The general findings of the study however, will be fed back to you in the form of a talk or in the form of a letter.

Results may be published in scientific journals or presented at meetings. It is also possible that data arising from this study will be shared with other researchers to support future research.

Please note that data is presented as a whole and is anonymous. Your name will not appear anywhere in any of the results presented, shared or published.

Who is funding the study?
This study is being funded through the Food and Health Strategic Programme grant to QI.

Who has reviewed this study?
Human studies research carried out at QI is reviewed by the Human Research Governance Committee (HRGC) and a group of independent people called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity.

This study has been reviewed and approved by HRGC and a Research Ethics Committee.

What you need to tell us?
We do need you to tell us some things for your safety and for the success of the study. Some medication may affect the information we are collecting so you need to tell us if you take any medication. You will also need to tell us if you become pregnant whilst on the study. Should you become unwell during the study or after a visit to the HNU then you need to tell us.

Taking part in this study is entirely voluntary. You are free to withdraw at any time and without giving a reason.

For further information or to arrange a study appointment, please contact:

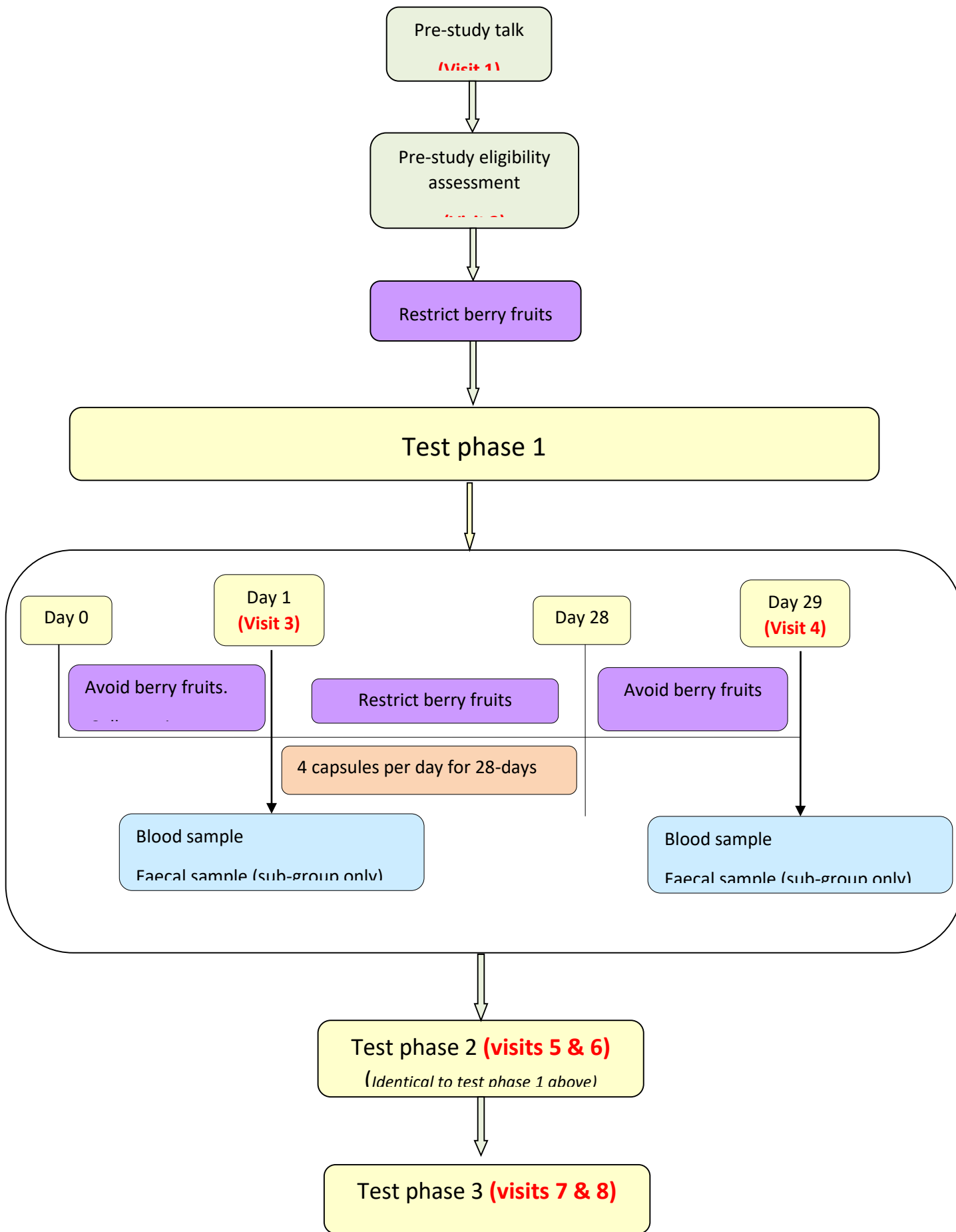Wendy Hollands (01603) 255051 E-mail: wendy.hollands@quadram.ac.uk

OR

Aliceon Blair HNU senior research nurse (01603) 255305

Alternatively you can complete the attached response slip and return this to us in the prepaid envelope provided.

Outline of study

Pre-study talk

(Visit 1)

↓

Pre-study eligibility assessment

(Visit 2)

↓

Restrict berry fruits

↓

Test phase 1

↓

Day 0

Day 1 **(Visit 3)**

Day 28

Day 29 **(Visit 4)**

Avoid berry fruits.

Restrict berry fruits

Avoid berry fruits

4 capsules per day for 28-days

Blood sample

Faecal sample (sub-group only)

Blood sample

Faecal sample (sub-group only)

↓

Test phase 2 **(visits 5 & 6)**

(Identical to test phase 1 above)

↓

Test phase 3 **(visits 7 & 8)**

Fruits to exclude/restrict

No more than **3 portions per week** (total) of the fruits listed below.

Strawberries
Blackberries
Blueberry
Raspberries
Bilberry
Blackcurrants
Red currants
Whortleberry
Cherry
Chokeberry
Cranberry
Elderberry
Goji Berry
Black/red grapes
Black plums

Please remember to completely exclude these for 24 hours before your study day assessments.

The effects of bilberry fruit and black rice derived anthocyanins on lipid status in adults

Short title: BERI Study

I am interested in taking part and/ or finding out more information about this study *(please complete the personal details below).*

Name: ……………………………………………………

Address: ……………………………………………………

…………………………………………………

…………………………………………………

Daytime telephone no: ………………………………………..

Evening telephone no: ………………………………………..

I am happy for a message to be left via my daytime/evening* no: **YES/NO**
please delete as applicable

Email address:……………………………………………….

Please return this form in the FREEPOST envelope provided to:

Wendy Hollands
Quadram Institute Bioscience
FREEPOST NC 252
Norwich Research Park
Colney
NORWICH
NR4 7BR

Expressing an interest does not commit you to taking part in the study

Appendix 6

Product specifications of the bilberry and black rice extracts fed in the BERI study

Appendix 6

BGG

PRODUCT SPECIFICATION SHEET FOR BLACK RICE EXTRACT

PRODUCT: Black Rice Extract Powder PRODUCT NO.: BR-30
ISSUE DATE: Jan. 16, 2017
SPECIFICATION NO.: 000
STARTING HERBAL MATERIAL:
BOTANICAL NAME: Oryza sativa L. PART OF PLANT USED: Seed

CULTIVATED/WILD: Cultivated

PRODUCTION:
SOLVENT USED: Ethanol COUNTRY OF PROCESSING: China

DRUG-TO-EXTRACT RATIO: 150-250:1 EXCIPIENTS/OTHER COMPONENTS: None
ANTIOXIDANTS/PRESERVATIVES: None

PHYSICAL/CHEMICAL SPECIFICATIONS

ITEMS	SPECIFICATIONS	METHOD
CHARACTERISTICS:	Purple black powder	Visual inspection
※HEAVY METALS:	20ppm Max.	According to Eur. Ph. 2.4.8
※CADMIUM:	1ppm Max.	According to Eur. Ph. 2.4.27
※LEAD:	3ppm Max.	According to Eur. Ph. 2.4.27
※MERCURY:	0.1ppm Max.	According to Eur. Ph. 2.4.27
LOSS ON DRYING:	5.0% Max.	According to Eur. Ph. 2.8.17
ASH:	3.0% Max.	According to Eur. Ph. 2.4.16
※PESTICIDES:	In accordance to EC Regulation No. 396/2005 and modifications	
ANTHOCYANINS:		
Content of Anthocyanins by HPLC (on dry basis, expressed as Cyanidin 3-O-glucoside)	30.0% Min.	In-house method

CYANIDIN 3-O-GLUCOSIDE:

Content of Cyanidin 3-O-glucoside by HPLC (on dry basis)	20.0% Min.	In-house method
MICROBIOLOGY		According to Eur. Ph. 2.6.12 and 2.6.13
TOTAL PLATE COUNT:	< 5,000cfu/g	
YEAST & MOLD:	< 500cfu/g	
BILE-TOLERANT GRAM-NEGATIVE BACTERIA:	< 100cfu/g	
ESCHERICHIA COLI:	Absence/g	
SALMONELLA:	Absence/10g	
※**AFLATOXINS:**		According to Eur. Ph. 2.8.18
SUM OF AFLATOXIN B1, B2, G1 AND G2:	< 4ppb	
AFLATOXIN B1:	< 2ppb	
※**BENZO(a)PYRENE:**	< 10ppb	In-house method
※SUM OF BENZO(a)PYRENE, BENZO(a)ANTHRACENE, BENZO(a)FLUORANTHENE, CHRYSENE:	< 50ppb	In-house method

Not from Japan (Regulation (EC) No 297/2011) Not irradiated

※Periodic test (not carried out batch by batch) for which BGG tests batches randomly every year. Complies: BGG can provide a Certificate of Compliance for this test with historical data from tested batches.

STORAGE:
Preserve in tight containers, protected from light. Avoid excessive heat.

SHELF LIFE:
Three (3) years when properly stored.

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PRODUCT SPECIFICATION SHEET FOR BILBERRY EXTRACT POWDER

PRODUCT: Bilberry Extract Powder (*MyrtiPRO*)
PRODUCT NO.: GC-021J
ISSUE DATE: Jul. 6, 2016
SPECIFICATION NO.: 000

STARTING HERBAL MATERIAL:
BOTANICAL NAME: *Vaccinium myrtillus* L.
PART OF PLANT USED: Fresh Frozen Fruit
CULTIVATED/WILD: Wild

PRODUCTION:
SOLVENT USED: Ethanol/Water
COUNTRY OF PROCESSING: China
DRUG-TO-EXTRACT RATIO: 80-100:1
EXCIPIENTS/OTHER COMPONENTS: None
ANTIOXIDANTS/PRESERVATIVES: None

PHYSICAL/CHEMICAL SPECIFICATIONS

ITEMS	SPECIFICATIONS	METHOD
CHARACTERISTICS:	Purple black powder	Visual inspection
IDENTIFICATION:	HPLC complies	According to Eur. Ph. monograph 2394
※HEAVY METALS:	20ppm Max.	According to Eur. Ph. 2.4.8
※CADMIUM:	1ppm Max.	According to Eur. Ph. 2.4.27
※LEAD:	3ppm Max.	According to Eur. Ph. 2.4.27
※MERCURY:	0.1ppm Max.	According to Eur. Ph. 2.4.27
PH:	3.0-4.5	According to Eur. Ph. 2.2.3
LOSS ON DRYING:	5.0% Max.	According to Eur. Ph. 2.8.17
ASH:	3.0% Max.	According to Eur. Ph. 2.4.16
※PESTICIDES:	In accordance to EC Regulation No. 396/2005 and modifications	
FREE ANTHOCYANIDINS: Free Anthocyanidins by HPLC	1.0% Max.	According to Eur. Ph. monograph 2394

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ANTHOCYANIDINS:

Content of Anthocyanidins by UV (on dry basis)	25.0% Min.	In-house method

ANTHOCYANINS:

Content of Anthocyanins by HPLC (on dry basis, expressed as Cyanidin 3-O-glucoside chloride)	36.0% Min.	According to Eur. Ph. monograph 2394

MICROBIOLOGY	According to Eur. Ph. 2.6.12 and 2.6.13
TOTAL PLATE COUNT:	< 5,000cfu/g
YEAST & MOLD:	< 500cfu/g
BILE-TOLERANT GRAM-NEGATIVE BACTERIA:	< 100cfu/g
ESCHERICHIA COLI:	Absence/g
SALMONELLA:	Absence/10g
※AFLATOXINS:	According to Eur. Ph. 2.8.18
SUM OF AFLATOXIN B1, B2, G1 AND G2:	< 4ppb
AFLATOXIN B1:	< 2ppb

GMO: This is IP Non-GMO product (Regulation (EC) No 1830/2003)
Not from Japan (Regulation (EC) No 297/2011)
Free from food allergens (Dir. 2000/13/EC and subsequent amendments)
Not irradiated

※Periodic test (not carried out batch by batch) for which BGG tests batches randomly every year. Complies: BGG can provide a Certificate of Compliance for this test with historical data from tested batches.

STORAGE:
Preserve in tight containers, protected from light. Avoid excessive heat.

SHELF LIFE:
Three (3) years when properly stored.

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Appendix 7

Dynamic Time Warping (DTW) data for every compound detected in the *in vitro* fermentation colon model analysed by means of a principle component analysis

Appendix 7

The following figures are with reference to Chapter 4.
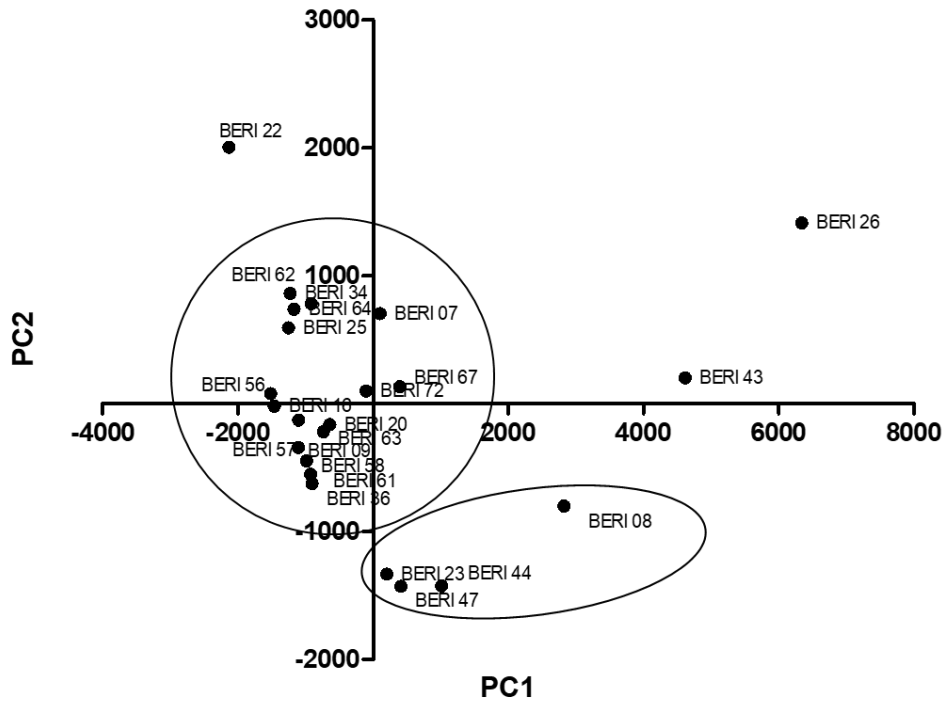
Figure S1- Principal component analysis of DTW data for C3G metabolism during in vitro fermentation of black rice extract.

111 μM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on C3G metabolism for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
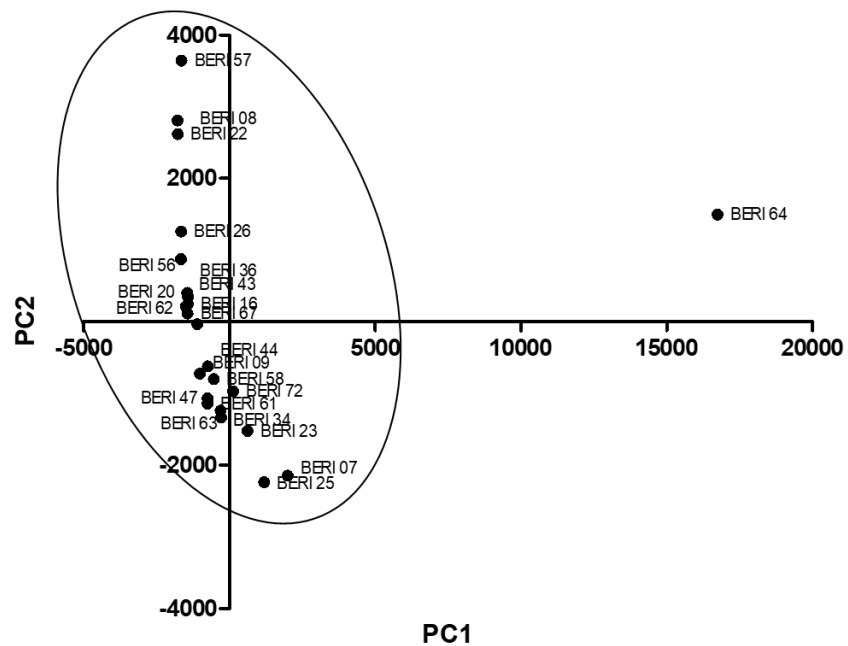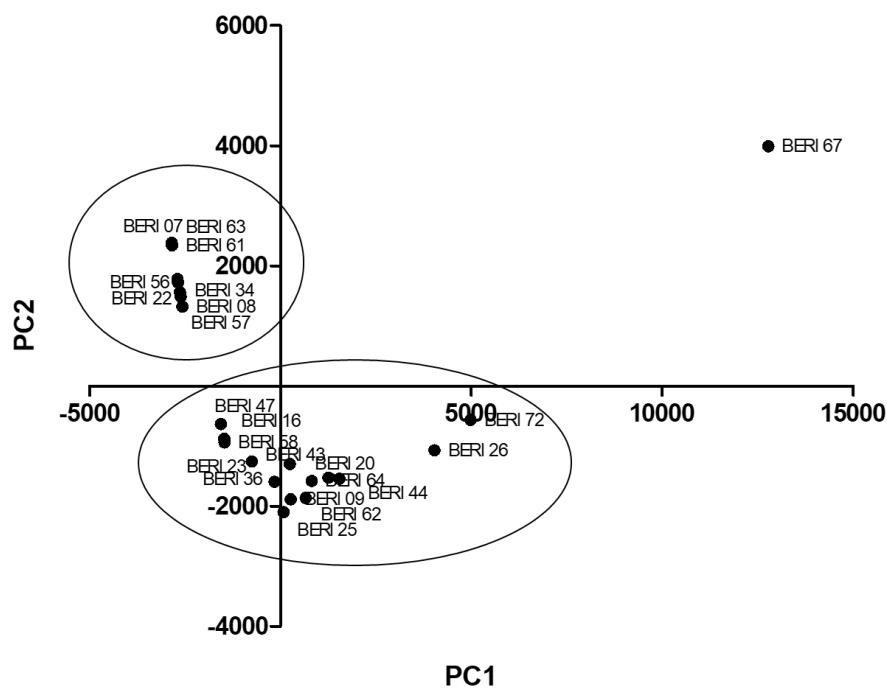
Figure S2- Principal component analysis of DTW data for PGA production during in vitro fermentation of black rice extract.

111 µM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on PGA metabolism for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
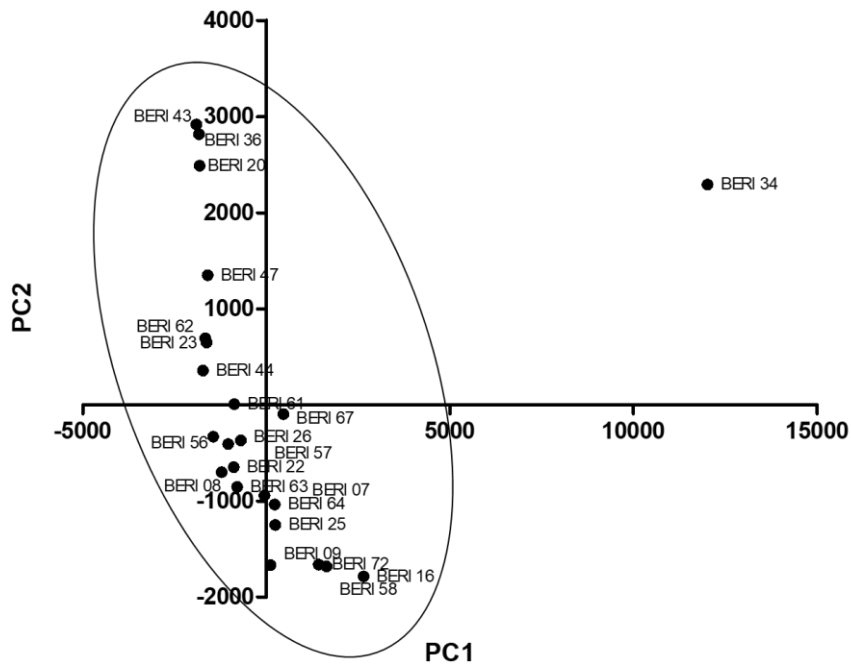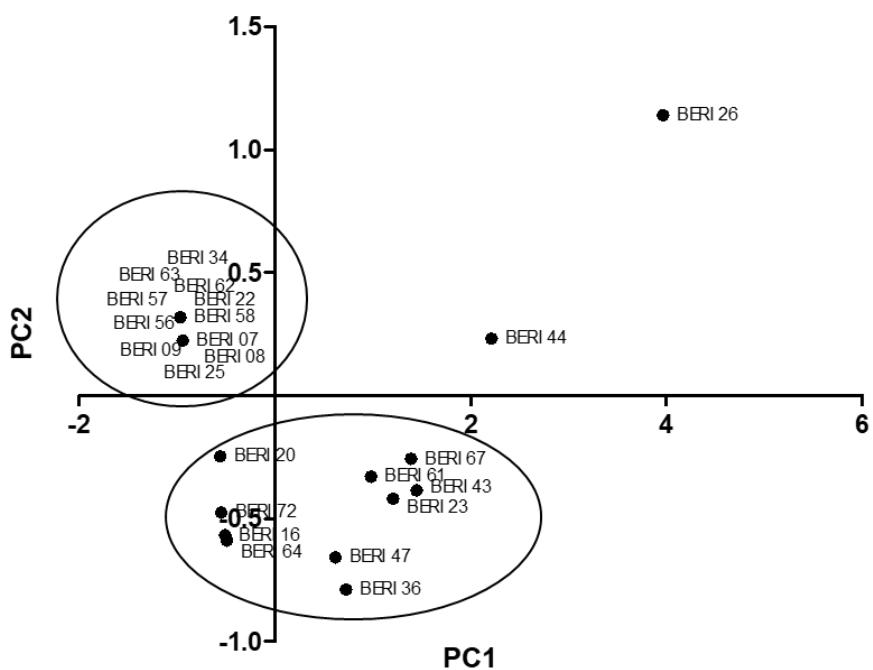
Figure S3- Principal component analysis of DTW data for PCA production during in vitro fermentation of black rice extract.

111 μM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on PCA metabolism for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
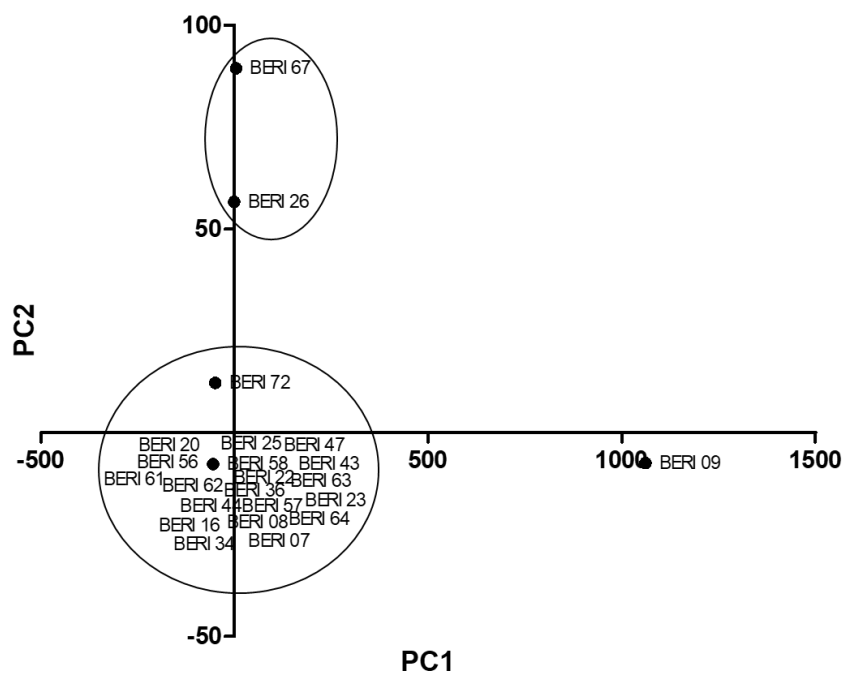
Figure S4- Principal component analysis of DTW data for catechol production during in vitro fermentation of black rice extract.

111 µM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on catechol concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
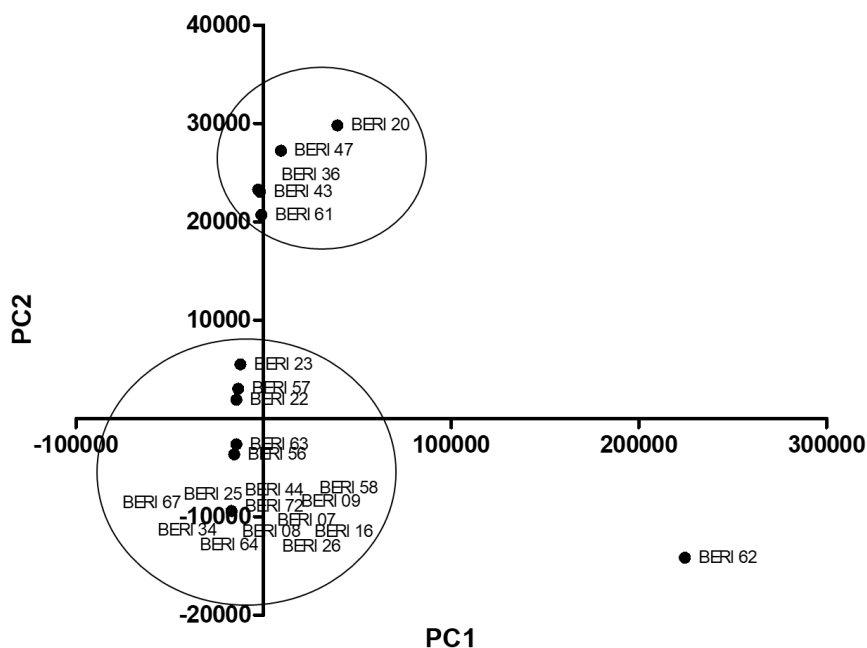
Figure S5- Principal component analysis of DTW data for 4-methyl catechol production during in vitro fermentation of black rice extract.

111 µM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on 4-methyl catechol concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.

Figure S6- Principal component analysis of DTW data for 2,4-hydroxybenzoic acid production during in vitro fermentation of black rice extract.

111 μM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on 2,4-dihydroxybenzoic acid concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
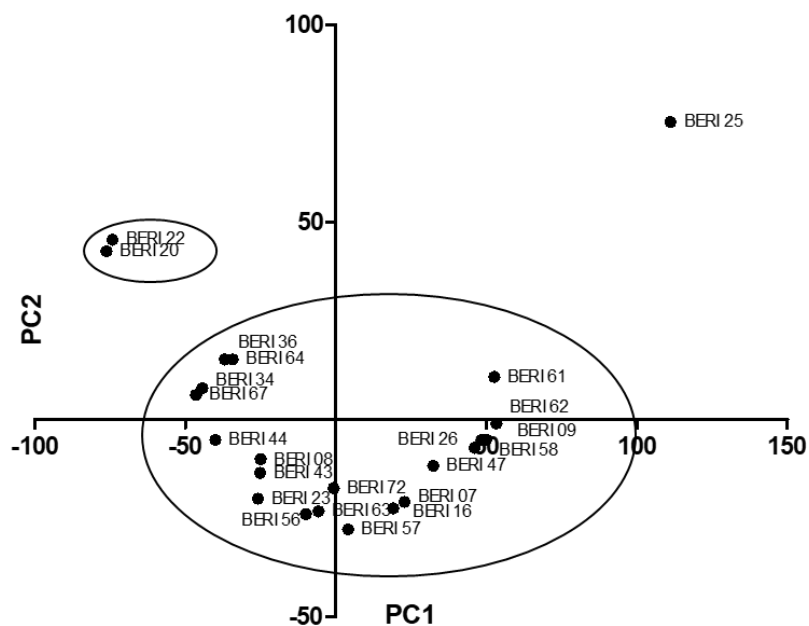
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Figure S7- Principal component analysis of DTW data for 3-hydroxyphenylacetic acid production during in vitro fermentation of black rice extract.

111 µM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on 3-hydroxyphenylacetic acid concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
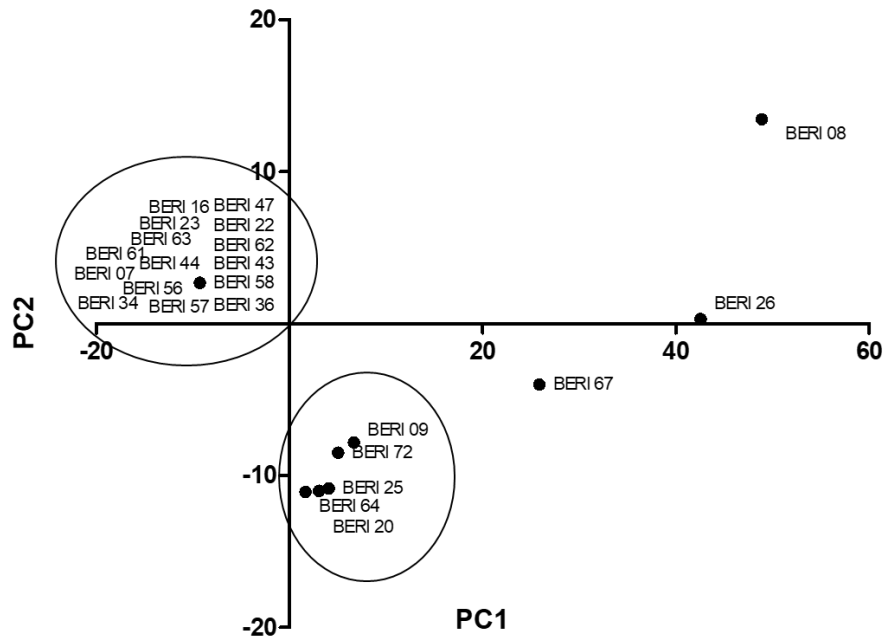
Figure S8- Principal component analysis of DTW data for 4-hydroxybenzoic acid production during in vitro fermentation of black rice extract.

111 µM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on 4-hydroxybenzoic acid concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
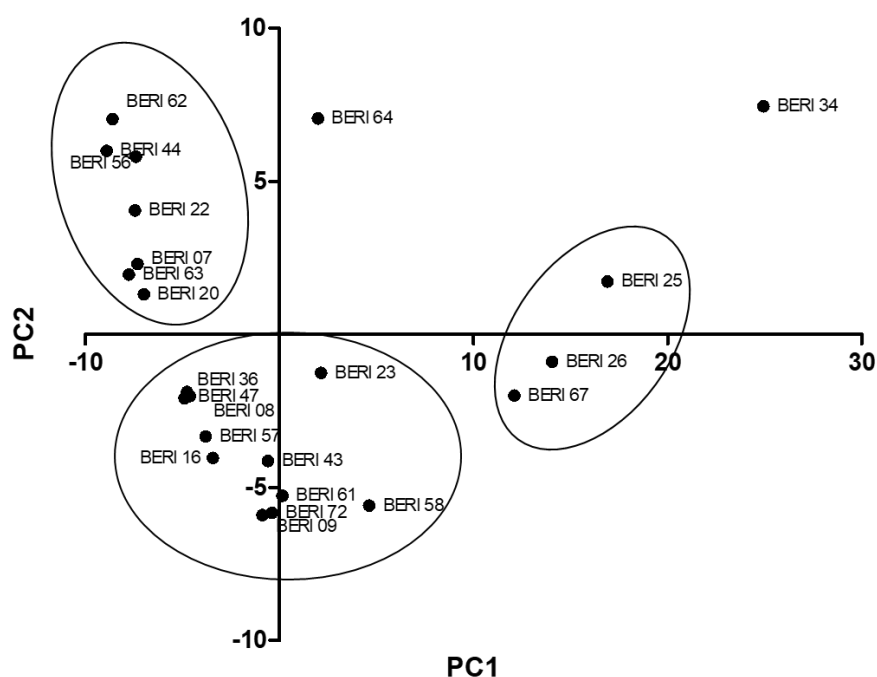
Figure S9- Principal component analysis of DTW data for dihydrocaffeic acid production during in vitro fermentation of black rice extract.

111 μM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on dihydrocaffeic acid concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
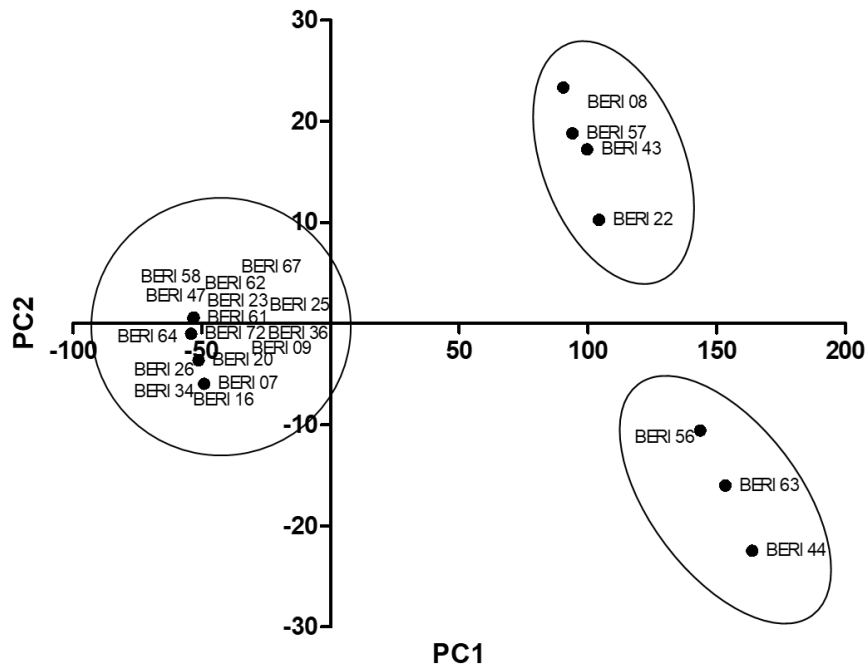
Figure S10- Principal component analysis of DTW data for dihydroferulic acid production during in vitro fermentation of black rice extract.

111 µM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on dihydroferulic acid concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
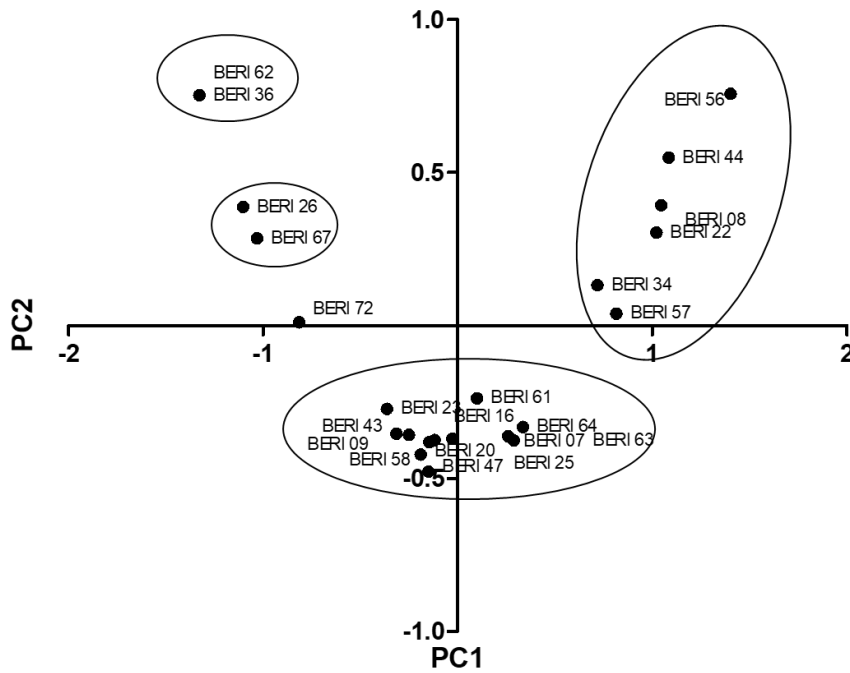
Figure S11- Principal component analysis of DTW data for homovanillic acid production during in vitro fermentation of black rice extract.

111 μM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on homovanillic acid concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
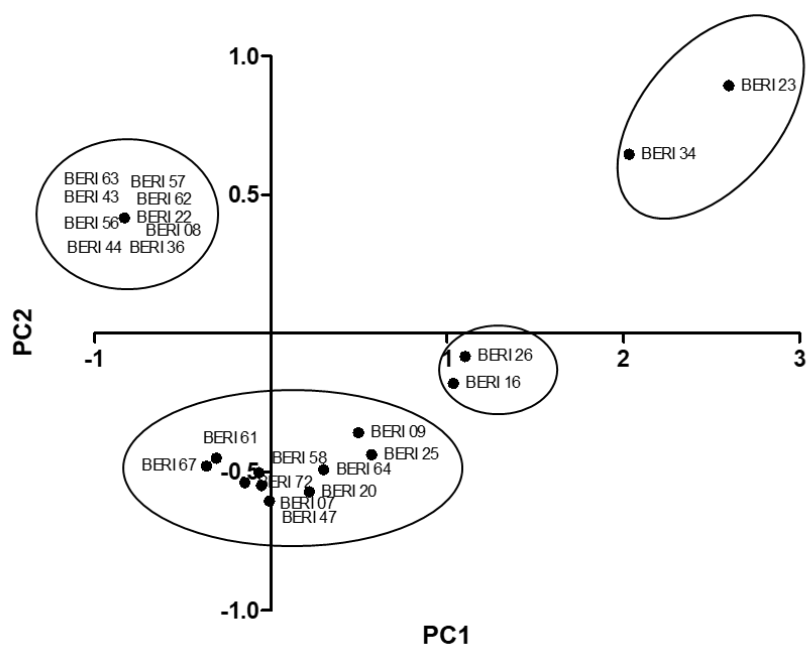
Figure S12- Principal component analysis of DTW data for p-coumaric acid production during in vitro fermentation of black rice extract.

111 µM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on p-coumaric acidl concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
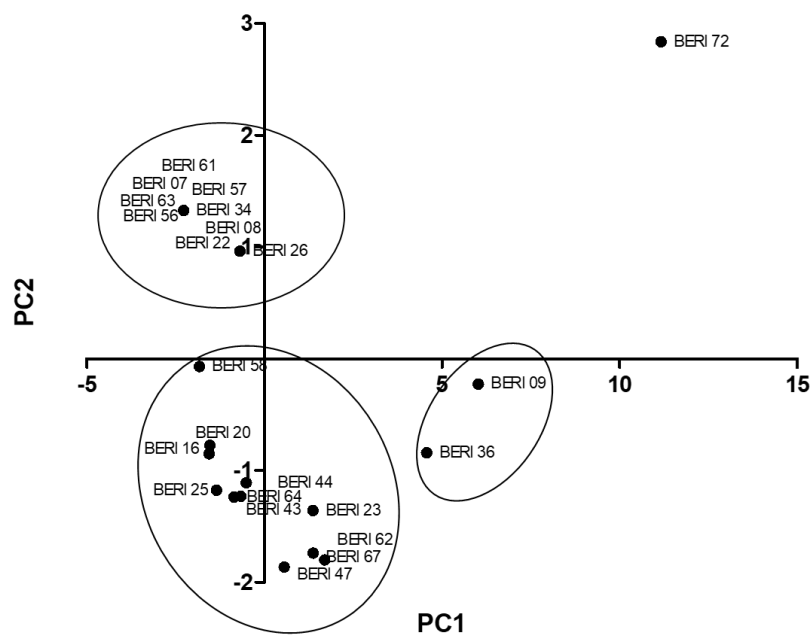
Figure-S13- Principal component analysis of DTW data for phloroglucinol production during in vitro fermentation of black rice extract.

111 μM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on phloroglucinol concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
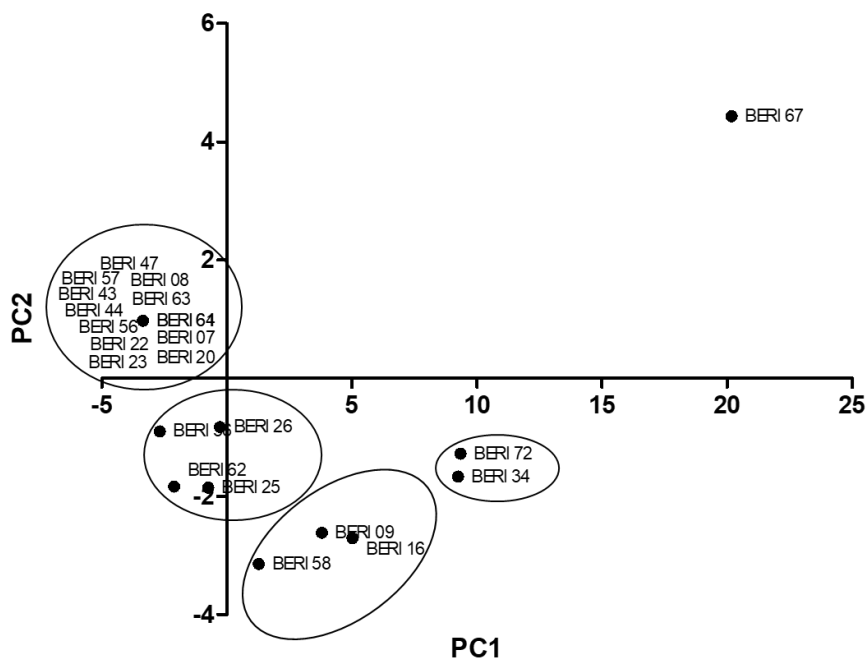
Figure S14- Principal component analysis of DTW data for pyrogallol production during in vitro fermentation of black rice extract.

111 µM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on pyrogallol concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.

Figure S15- Principal component analysis of DTW data for PGC production during in vitro fermentation of black rice extract.

111 µM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on PGC concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
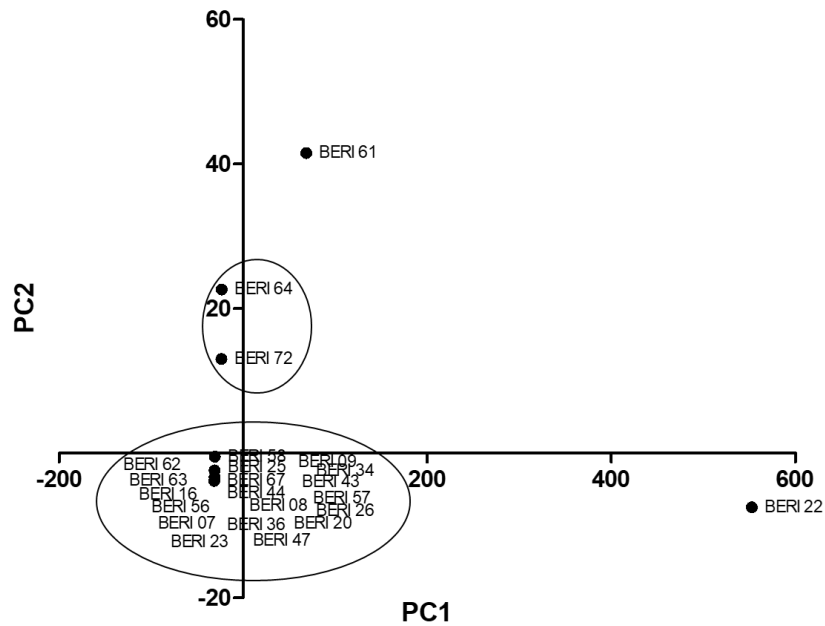
Figure S16- Principal component analysis of DTW data for unknown X production during in vitro fermentation of black rice extract.

111 μM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on unknown X (observed in the same MRM channel as hippuric acid) estimated concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.
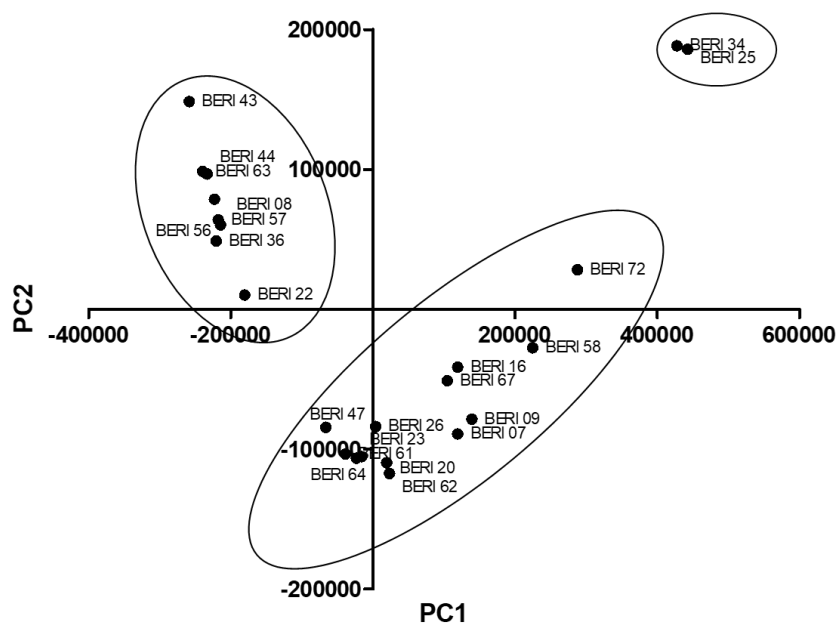
Figure S17- Principal component analysis of DTW data for unknown Y production during in vitro fermentation of black rice extract.

111 µM C3G in a matrix of black rice extract was incubated with faecal slurry samples (1% faeces) from 23 volunteers in nutritive media under anaerobic conditions for 48 hours; samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48 hours. Data on unknown Y (observed in the MRM channel of homoPCA) estimated concentration for the entire time series of the experiment was processed using dynamic time warping (DTW) to provide distance measures between participants according to their similarity during in vitro fermentation. DTW data was then subjected to principal component analysis and PC1 and PC2 are plotted to describe the similarity of data between individuals, whereby samples plotted closer together displayed more similar metabolism of black rice anthocyanins in the in vitro fermentation colon model.

Appendix 8

Box-plots showing the within subject variation for taxa highlighted in sPLS-DA of microbiome data to explore any effect of treatment on microbiome composition

Appendix 8

The following figures are with reference to the data presented in Chapter 5.
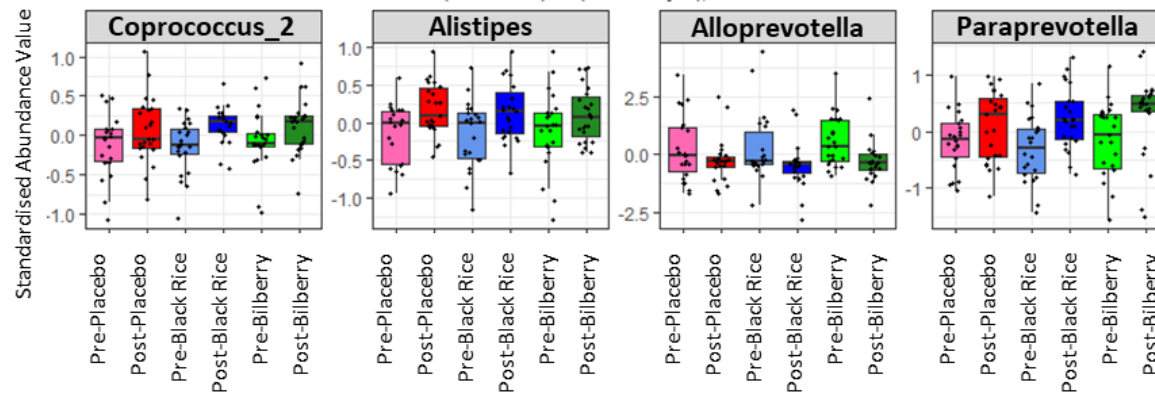

Figure S18- Box plots showing the within-individual variation for genera highlighted on axis 1 in multilevel sPLS-DA of 16S (CLR transformed abundance) data
Standardised abundance (CLR transformed relative abundance, mean centred for each individual) of genera highlighted on axis 1 in multilevel sPLS-DA of 16S CLR-transformed data (filtered to 68 criterion: 'either all groups have 12 or more zeros, or the maximum for all samples is below 0.5%'), The median, 25% and 75% quantiles are shown; data that exceeds 1.5 X the interquartile range are represented by individual data points outside of the whiskers. Each box plot shows data for one genus for every phase of the human intervention study (pre-placebo (n = 22), post-placebo (n = 22), pre-black rice (n = 23), post black rice (n = 23), pre-bilberry and post-bilberry (n = 23).
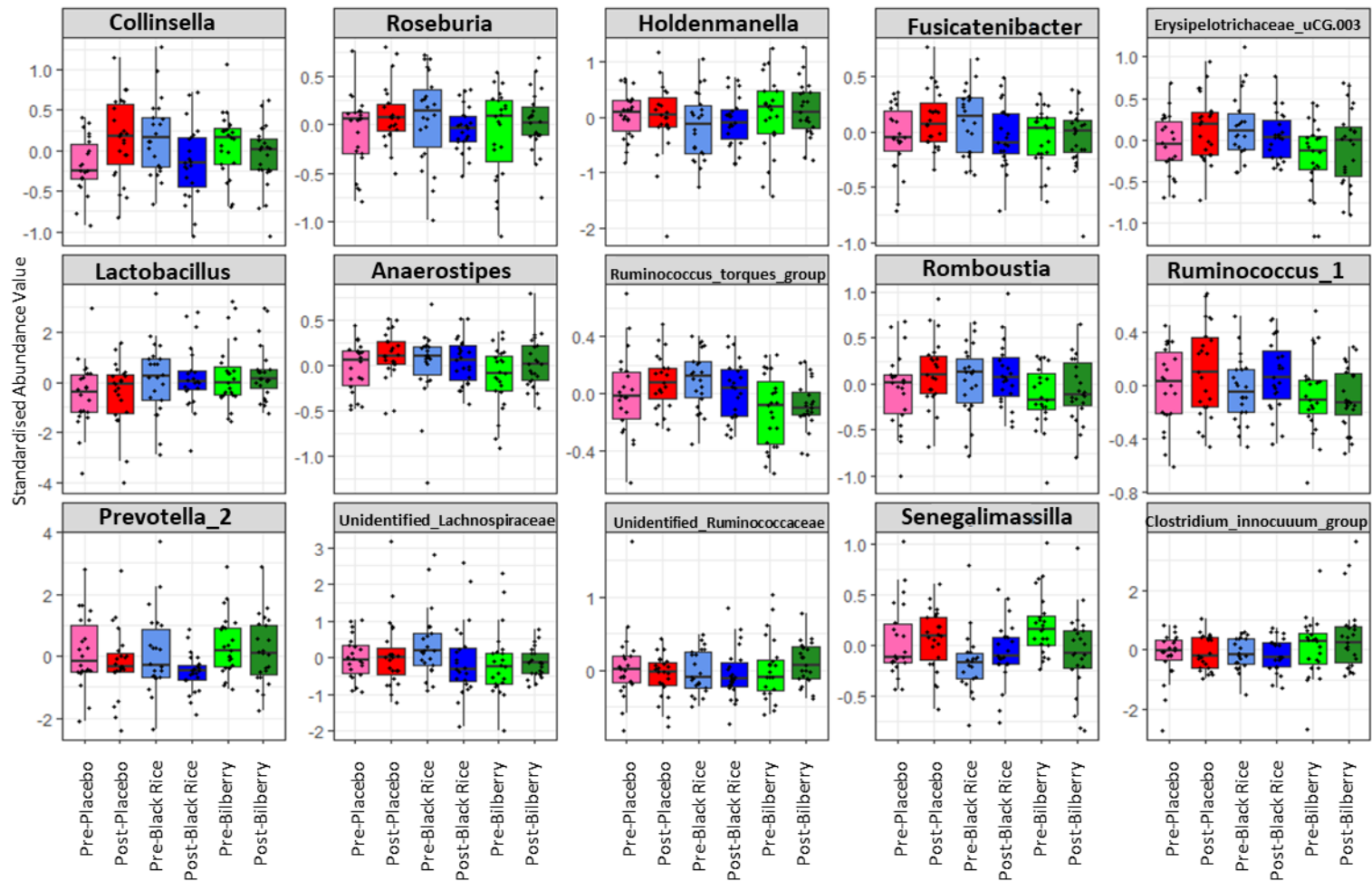
Figure S19- Box plots showing the within-individual variation for genera highlighted on axis 2 in multilevel sPLS-DA of 16S (CLR transformed abundance) data

Standardised abundance (CLR transformed relative abundance, mean centred for each individual) of genera highlighted on axis 2 in multilevel sPLS-DA of 16S CLR-transformed data (filtered to 68 criterion: 'either all groups have 12 or more zeros, or the maximum for all samples is below 0.5%'), The median, 25% and 75% quantiles are shown; data that exceeds 1.5 X the interquartile range are represented by individual data points outside of the whiskers. Each box plot shows data for one genus for every phase of the human intervention study (pre-placebo (n = 22), post-placebo (n = 22), pre-black rice (n = 23), post black rice (n = 23), pre-bilberry and post-bilberry (n = 23).
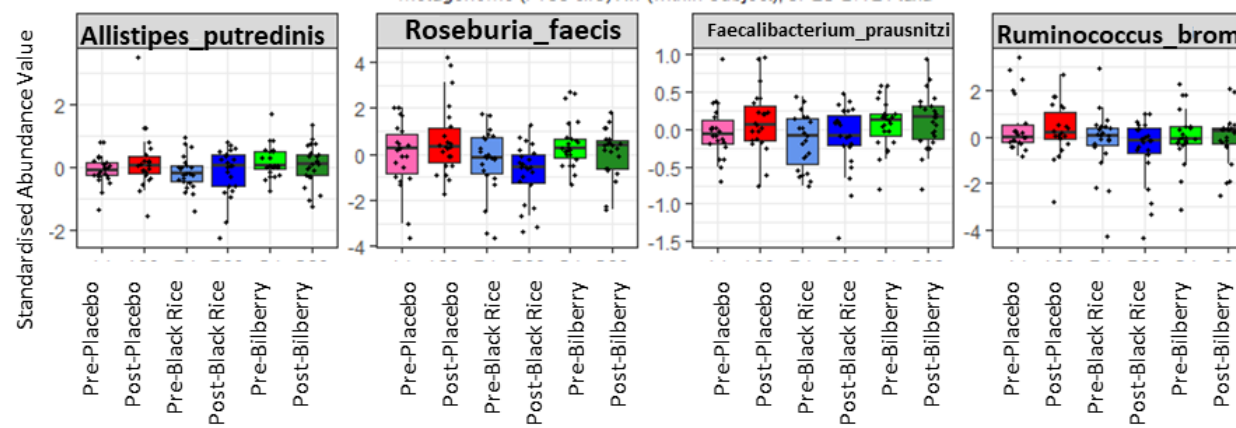
Figure S20- Box plots showing the within-individual variation for species highlighted on axis 1 in multilevel sPLS-DA of metagenomics (CLR transformed abundance) data filtered to 33 taxa

*Standardised abundance (CLR transformed relative abundance, mean centred for each individual) ofspecies highlighted on axis 1 in multilevel sPLS-DA of metagenomics CLR-transformed data (filtered to 33 taxa with the criterion: 'taxa account for at least 1% in 22 samples), The median, 25% and 75% quantiles are shown; data that exceeds 1.5 X the interquartile range are represented by individual data points outside of the whiskers. Each box plot shows data for one species for every phase of the human intervention study (pre-placebo (n = 22), post-placebo (n = 22), pre-black rice (n = 23), post black rice (n = 23), pre-bilberry and post-bilberry (n = 23**).**
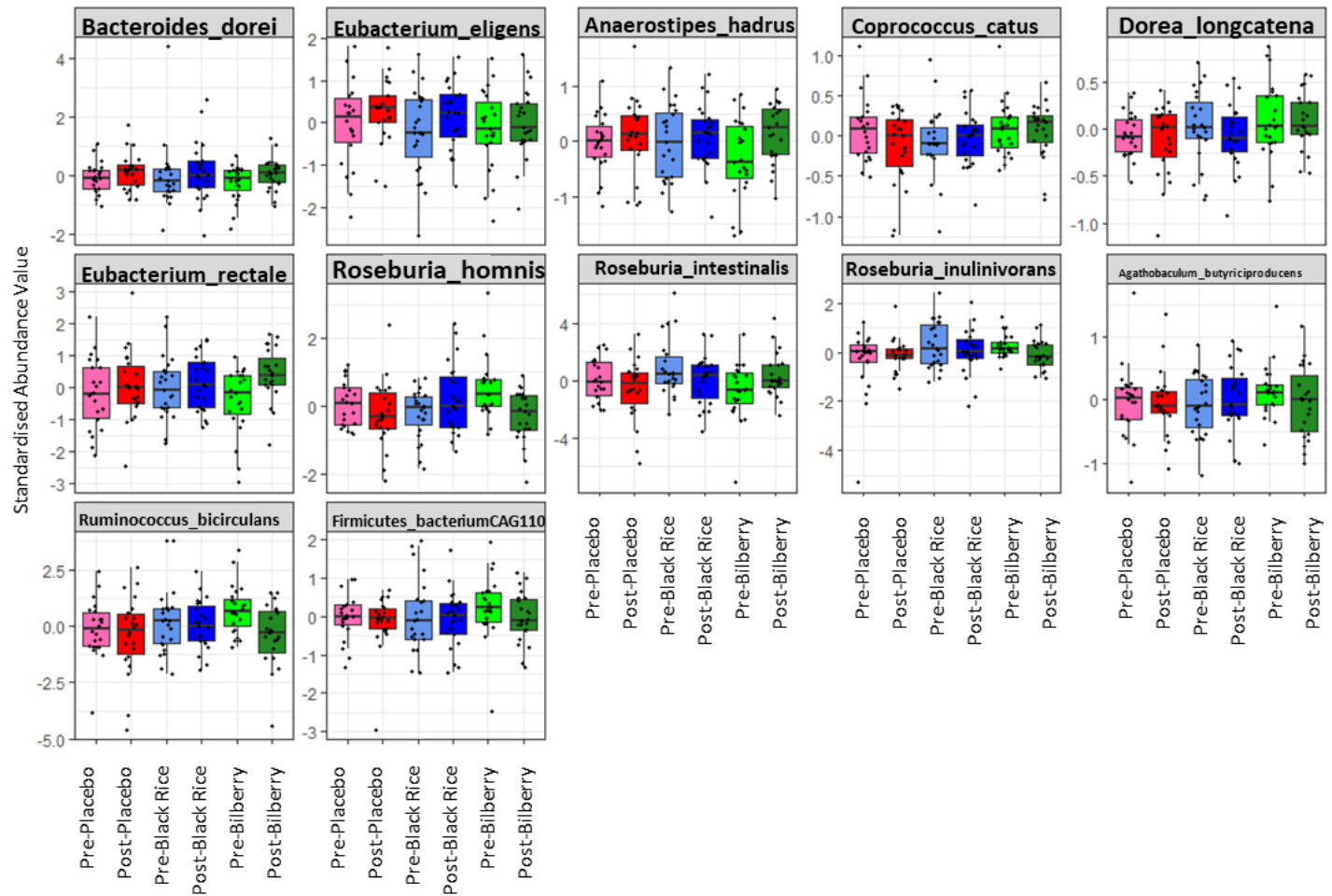
Figure S21- Box plots showing the within-individual variation for species highlighted on axis 2 in multilevel sPLS-DA of metagenomics (CLR transformed abundance) data filtered to 33 taxa

Standardised abundance (CLR transformed relative abundance, mean centred for each individual) of species highlighted on axis 2 in multilevel sPLS-DA of metagenomics CLR-transformed data (filtered to 33 taxa with the criterion: 'taxa account for at least 1% in 22 samples), The median, 25% and 75% quantiles are shown; data that exceeds 1.5 X the interquartile range are represented by individual data points outside of the whiskers. Each box plot shows data for one species for every phase of the human intervention study (pre-placebo (n = 22), post-placebo (n = 22), pre-black rice (n = 23), post black rice (n = 23), pre-bilberry and post-bilberry (n = 23).
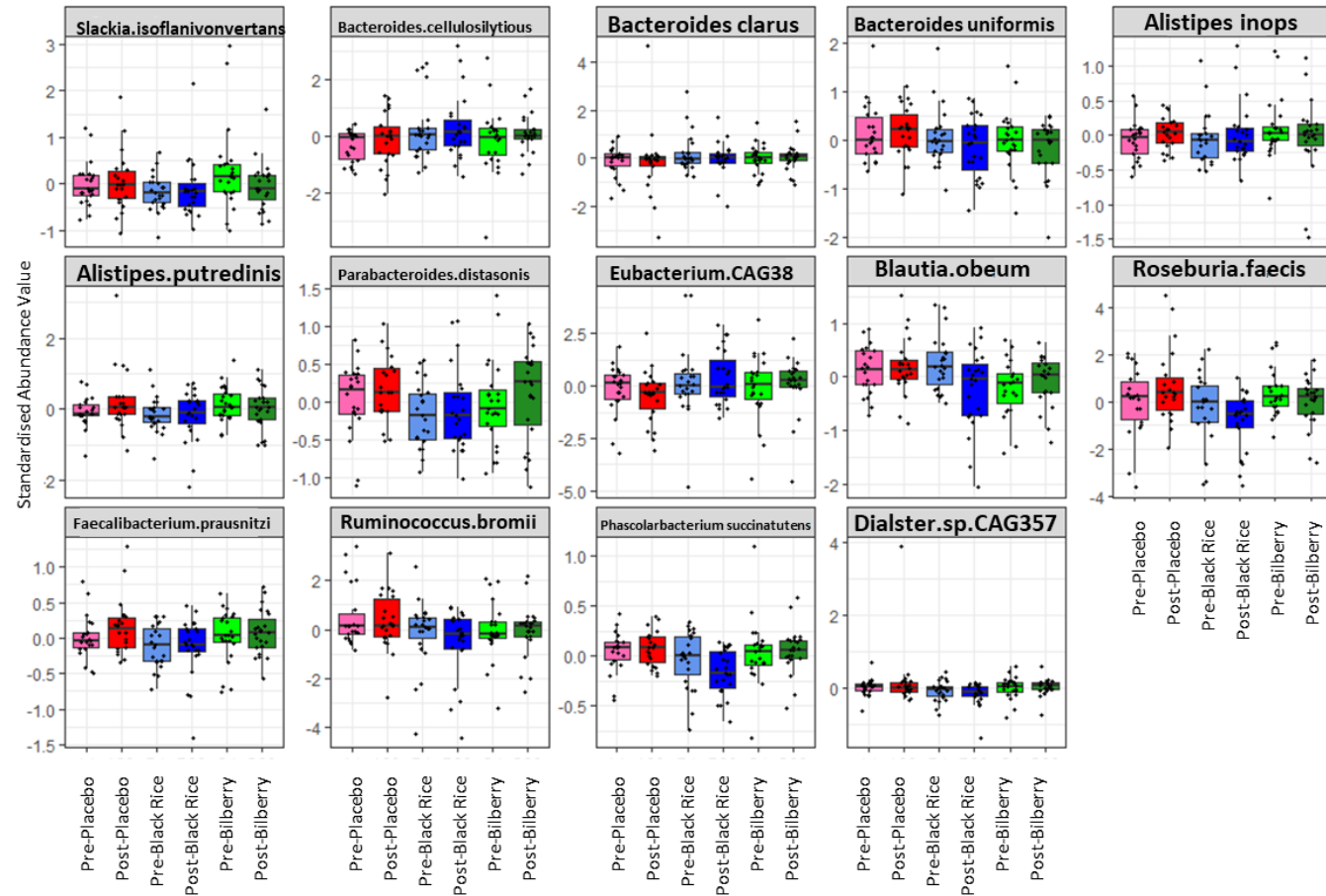
Figure S22- Box plots showing the within-individual variation for species highlighted on axis 1 in multilevel sPLS-DA of metagenomics (CLR transformed abundance) data filtered to 85 taxa

Standardised abundance (CLR transformed relative abundance, mean centred for each individual) of species highlighted on axis 1 in multilevel sPLS-DA of metagenomics CLR-transformed data (filtered to 85 taxa with the criterion: 'taxa account for at least 0.5% in 8 samples'), The median, 25% and 75% quantiles are shown; data that exceeds 1.5 X the interquartile range are represented by individual data points outside of the whiskers. Each box plot shows data for one species for every phase of the human intervention study (pre-placebo (n = 22), post-placebo (n = 22), pre-black rice (n = 23), post black rice (n = 23), pre-bilberry and post-bilberry (n = 23).
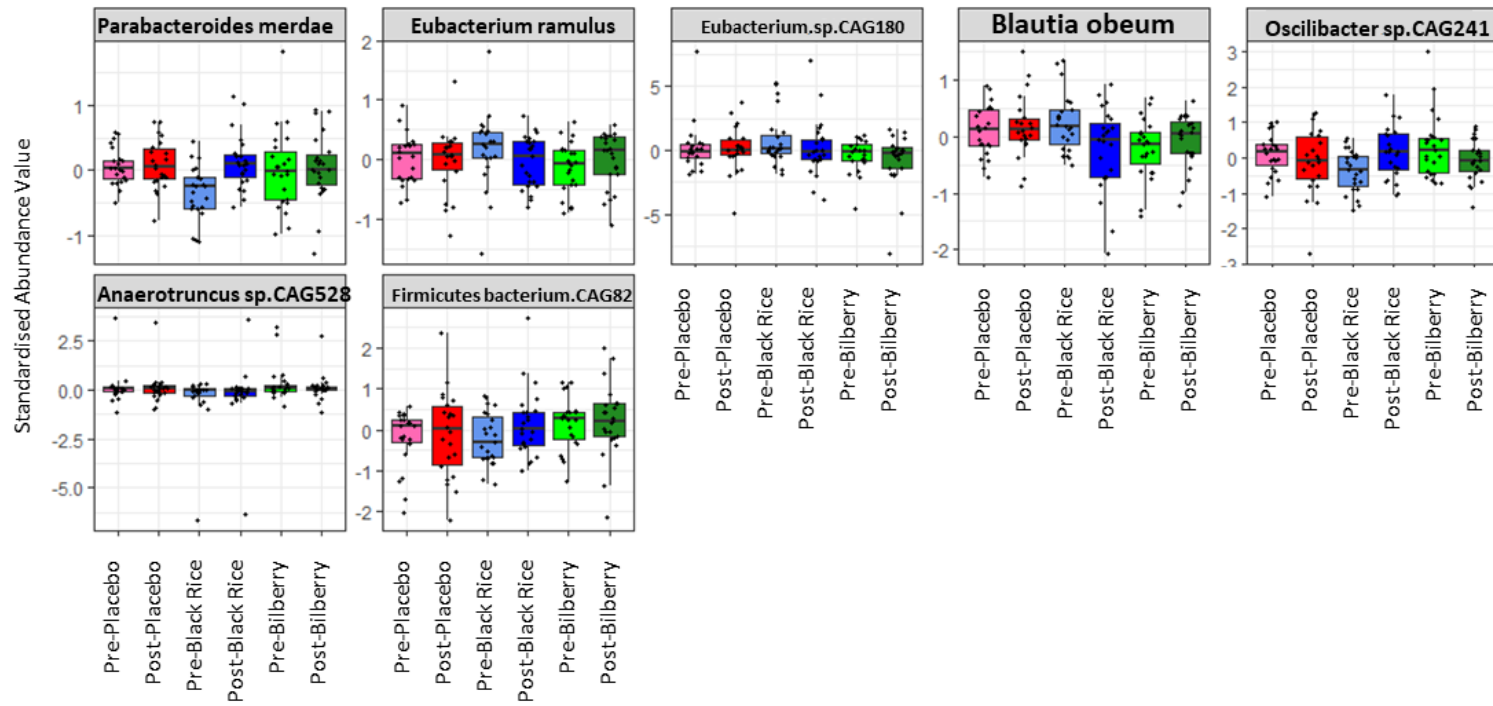
Figure S23- Box plots showing the within-individual variation for species highlighted on axis 2 in multilevel sPLS-DA of metagenomics (CLR transformed abundance) data filtered to 85 taxa

Standardised abundance (CLR transformed relative abundance, mean centred for each individual) of species highlighted on axis 2 in multilevel sPLS-DA of metagenomics CLR-transformed data (filtered to 85 taxa with the criterion: 'taxa account for at least 0.5% in 8 samples'), The median, 25% and 75% quantiles are shown; data that exceeds 1.5 X the interquartile range are represented by individual data points outside of the whiskers. Each box plot shows data for one species for every phase of the human intervention study (pre-placebo (n = 22), post-placebo (n = 22), pre-black rice (n = 23), post black rice (n = 23), pre-bilberry and post-bilberry (n = 23).
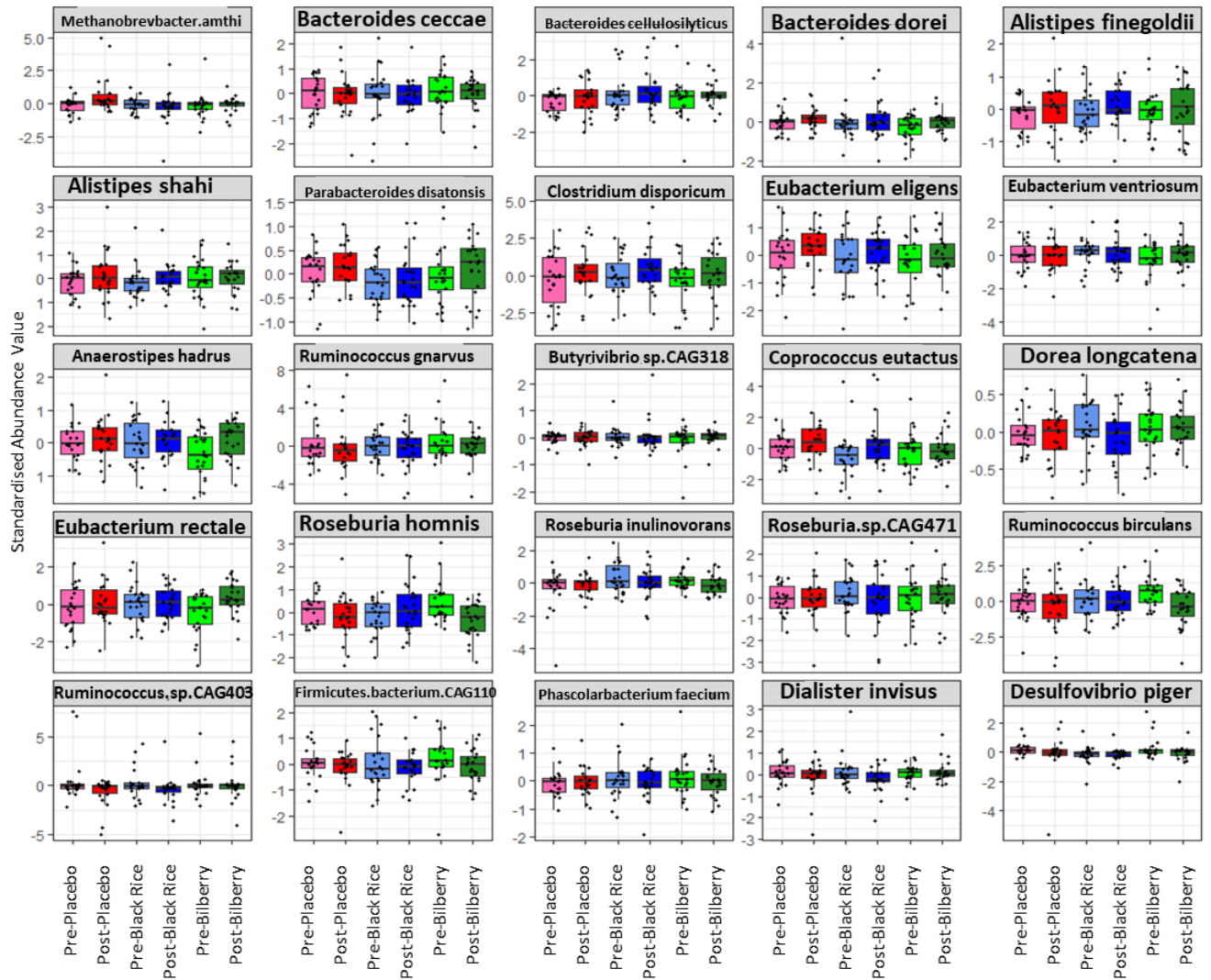
Figure S24- Box plots showing the within-individual variation for species highlighted on axis 3 in multilevel sPLS-DA of metagenomics (CLR transformed abundance) data filtered to 85 taxa

Standardised abundance (CLR transformed relative abundance, mean centred for each individual) of species highlighted on axis 3 in multilevel sPLS-DA of metagenomics CLR-transformed data (filtered to 85 taxa with the criterion: 'taxa account for at least 0.5% in 8 samples'), The median, 25% and 75% quantiles are shown; data that exceeds 1.5 X the interquartile range are represented by individual data points outside of the whiskers. Each box plot shows data for one species for every phase of the human intervention study (pre-placebo (n = 22), post-placebo (n = 22), pre-black rice (n = 23), post black rice (n = 23), pre-bilberry and post-bilberry (n = 23).
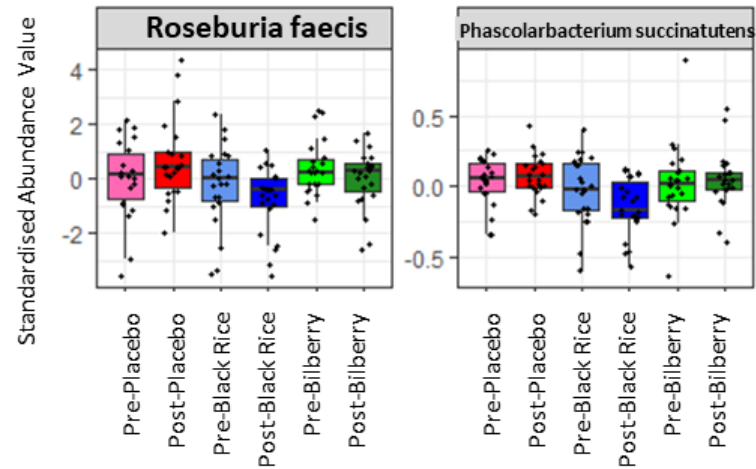
Figure S25- Box plots showing the within-individual variation for species highlighted on axis 1 in multilevel sPLS-DA of metagenomics (CLR transformed abundance) data filtered to 147 taxa

Standardised abundance (CLR transformed relative abundance, mean centred for each individual) of species highlighted on axis 1 in multilevel sPLS-DA of metagenomics CLR-transformed data (filtered to 147 taxa with the criterion: ''taxa account for at least 0.1% in 6 samples'), The median, 25% and 75% quantiles are shown; data that exceeds 1.5 X the interquartile range are represented by individual data points outside of the whiskers. Each box plot shows data for one species for every phase of the human intervention study (pre-placebo (n = 22), post-placebo (n = 22), pre-black rice (n = 23), post black rice (n = 23), pre-bilberry and post-bilberry (n = 23).
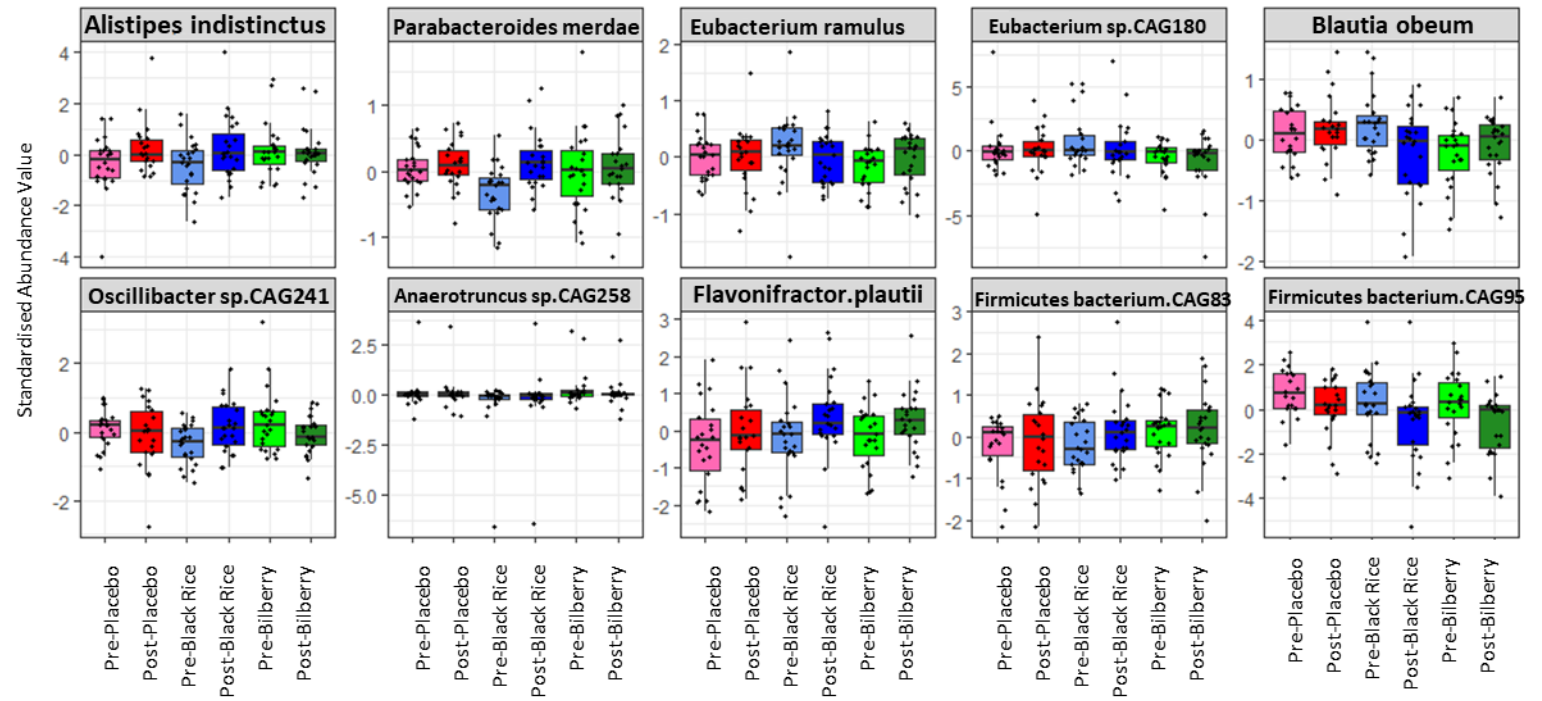
Figure S26- Box plots showing the within-individual variation for genera highlighted on axis 2 in multilevel sPLS-DA of metagenomics (CLR transformed abundance) data filtered to 147 taxa

Standardised abundance (CLR transformed relative abundance, mean centred for each individual) of species highlighted on axis 2 in multilevel sPLS-DA of metagenomics CLR-transformed data (filtered to 147 taxa with the criterion: ''taxa account for at least 0.1% in 6 samples'), The median, 25% and 75% quantiles are shown; data that exceeds 1.5 X the interquartile range are represented by individual data points outside of the whiskers. Each box plot shows data for one species for every phase of the human intervention study (pre-placebo (n = 22), post-placebo (n = 22), pre-black rice (n = 23), post black rice (n = 23), pre-bilberry and post-bilberry (n = 23).

Appendix 9

Phenolic Metabolites in the Urine and Plasma of Healthy Men After Acute Intake of Purple Potato Extract Rich in Methoxysubstituted Monoacylated Anthocyanins

Johanna Jokioja, Jasmine Percival, Mark Philo, Baoru Yang, Paul A. Kroon, Kaisa M. Linderborg

Appendix 9

Phenolic Metabolites in the Urine and Plasma of Healthy Men After Acute Intake of Purple Potato Extract Rich in Methoxysubstituted Monoacylated Anthocyanins

Johanna Jokioja,* Jasmine Percival, Mark Philo, Baoru Yang, Paul A. Kroon, and Kaisa M. Linderborg

Scope: Structurally stable acylated anthocyanins have potential in various food applications but the effects of acylation and methoxysubstitution on anthocyanin metabolism are poorly understood. This is the first study thoroughly investigating phenolic metabolites, their time-wise changes, and pharmacokinetics following an acute intake of methoxysubstituted monoacylated anthocyanins.

Methods and Results: Healthy male volunteers ($n = 17$) consumed a yellow potato meal with and without purple potato extract rich in acylated anthocyanins (152 mg) and hydroxycinnamic acid conjugates (140 mg). Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) is used for identification and quantification of metabolites from serially collected urine and plasma. While the parent anthocyanins are not detected, 28 phenolic metabolites from urine and 14 from plasma are quantified, including hydroxybenzoic and hydroxycinnamic acids and protocatechuic acid sulfates and glucuronides; three (catechol, gallic acid-4-O-glucuronide, and 2-methoxybenzoic acid) are detected for the first time after anthocyanin-rich food. Urinary hippuric acid is the most abundant with an increase of 139 μM mM^{-1} creatinine after the treatment. A large additional set of tentatively identified phenolic metabolites are detected. Late urinary peak time values suggest colonic degradation.

Conclusion: Acylated anthocyanins are more bioavailable than earlier reported after extensive degradation in human and/or colonial metabolism to phenolic metabolites, which may be further conjugated and demethylated.

1. Introduction

Anthocyanins, the red-purple colorants of berries and fruits, are hypoglycemic and decrease the risk of type II diabetes.[1,2] While the health properties of dietary components are typically linked to bioavailability, the bioavailability of anthocyanins is extremely low and affected by their vast structural diversity. For example, the urinary recovery of pure ^{13}C-labeled cyanidin-3-O-glucoside was less than 2%,[3] and 0.03% for bilberry anthocyanins.[4] Acylation of anthocyanins, i.e., linking one or more organic acids to the glycosyl moiety, decreases the bioavailability even further. The mainly diacylated cyanidin diglucosides of red cabbage are recovered four times less, whereas the monoacylated cyanidin xylosides of purple carrots are recovered 14 times less than the nonacylated anthocyanins of these foods.[5,6] Also, acylated anthocyanins are acutely hypoglycemic as detected with both cooked purple potatoes[7] and their extract[8] (99% acylated anthocyanins). Therefore, the health effects of anthocyanins may be mediated by their more bioavailable metabolites.[9,10]

Acylated anthocyanins have more potential for food industrial applications than nonacylated ones due to their enhanced structural stability[11] and they are common in everyday diets as part of pigmented vegetables and tubers.[5,6,8,12,13] However, their degradation in humans has not been studied extensively previously. Earlier clinical trials investigating the metabolites of anthocyanins have focused mainly on the pure common nonacylated anthocyanin glycoside, cyanidin-3-O-glucoside,[3,14] foods rich in nonacylated anthocyanins, such as various berries,[4,15–23] and to some extent on foods with a mix of nonacylated and acylated anthocyanins, such as grapes.[24,25] One study demonstrated that an acute intake of purple potatoes rich in acylated anthocyanins leads to the elevation of the total concentration of phenolics in biofluids of healthy volunteers ($n = 5$). Only a few of the phenolics were tentatively identified due to the lack of standard compounds, and the study did not include

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a control arm.[26] Altogether, these papers suggest that in addition to possible minor absorption, nonacylated anthocyanins undergo fission of the heterocyclic C-ring leading to phenolic metabolites, such as hydroxybenzoic acids, phenyl acetic acids, phenyl propionic acids, hydroxycinnamic acids, phenyl alcohols, phenyl aldehydes, and hippuric acids. After entering enterohepatic circulation, the metabolites may be further conjugated in the phase II metabolism. The in vivo degradation of acylated anthocyanins, however, is still not understood.

The aim of this study was to investigate the postprandial structural changes of the ingested purple potato extract (PPE) from *Solanum tuberosum* L. 'Synkeä Sakari' rich in methoxysubstituted anthocyanin glucosides with a rutinosyl moiety acylated to a caffeic, coumaric, or ferulic acid as our previous clinical trials gave evidence that the potatoes and their extract decrease postprandial glycemia and insulinemia.[7,8] Here, the phenolic metabolites were determined from the collected urine and plasma samples to clarify the molecular structures potentially affecting the observed health effects. The acylated anthocyanins were served as an extract free from potato matrix instead of comparing two potato varieties in order to remove the effect of compositional differences in phenolic compounds, starch, and monosaccharides on the postprandial state and metabolites. According to our knowledge, this is the first study to screen, identify, and quantify the phenolic metabolites of acylated anthocyanins from serially collected urine and plasma samples of humans.

2. Experimental Section

2.1. Solvents

For solid-phase extraction, formic acid (reagent grade, ≥95.0%) and methanol (HPLC grade, ≥99.8%) were obtained from Sigma-Aldrich (St Louis, MO). For LC-MS analyses, LC-MS grade acetonitrile (Thermo Scientific, Rockford, IL, for anthocyanins and CHROMASOLV, Sigma Aldrich, St Louis, MO, for other phenolics), MS-grade formic acid (98%, Honeywell, Muskegon, MI), and UPLC/MS-grade glacial acetic acid (Biosolve B.V., Dieuze, France) were purchased. Ammonium acetate for HPLC buffer preparation (HPLC-grade, ≥99.0%) was obtained from Sigma-Aldrich (St Louis, MO). Water used was Milli-Q grade (Millipore, Burlington, MA).

2.2. Standard Compounds

Anthocyanin standards (cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, pelargonidin-3-*O*-glucoside, pelargonidin-3-*O*-rutinoside, and malvidin-3-*O*-glucoside) were purchased from Extrasynthese (Genay, France). Caffeine-(trimethyl-d_9) (99 atom-% D, later caffeine-d_9), catechol, 4-hydroxybenzaldehyde, 3-*O*-methyl-gallate, methyl vanillate, phloroglucinol, phloroglucinaldehyde, pyrogallol, quercetin, quercetin-3-*O*-rutinoside, protocatechuic aldehyde, taxifolin, as well as caffeic, chlorogenic, ferulic, gallic, hippuric, homoprotocatechuic, homovanillic, 3-hydroxybenzoic, 4-hydroxybenzoic, 5-hydroxyferulic, 4-hydroxyphenylacetic, isovanillic, 2-methoxybenzoic, 3-methyl hippuric, 4-methyl hippuric, *p*-coumaric, protocatechuic, sinapic, syringic, *trans*-cinnamic, and vanillic acids from Sigma-Aldrich

(St Louis, MO). Methyl-3,4-dihydroxybenzoate was purchased from Alfa Aesar, Haverhill, MA. Gallic acid-3-*O*-glucuronide, gallic acid-4-*O*-glucuronide, isoferulic acid-3-*O*-glucuronide, protocatechuic acid-3-*O*-glucuronide, protocatechuic acid-4-*O*-glucuronide, protocatechuic acid-3-*O*-sulfate, and protocatechuic acid-4-*O*-sulfate were kindly synthesized by Dr Paul Needs at The Quadram Institute Biosciences, Norwich, UK.

2.3. Ethics

The study protocol was accepted by the Ethical Committee of the Hospital District of Southwest Finland (ETMK:93/1801:2016) and registered at clinicaltrials.gov as NCT02940080. Each study subject provided written informed consent.

2.4. Study Design

Seventeen healthy men aged between 18 and 45 years consumed 350 g of steam-cooked yellow potato mash with (study meal) and without (control meal) PPE solution rich in acylated anthocyanins as a breakfast after a 12-h fast in a cross-over, single-blinded, and randomized study described previously. The PPE and yellow potato mash were carefully characterized for nutrient composition, sugars, organic acids, anthocyanins, and other phenolic compounds.[8] For 48 h before and 24 h after consuming a test meal, the participants followed a study diet composed of foods and drinks low in flavonoids and dietary fiber. Total voids of urine were collected at fasting state, then pooled at 0–4, 4–8, 8–12, and 12–24 h postprandially. Urine samples (154 in total) were stored as aliquots at −80 °C containing 1/0.2 V/V trifluoroacetic acid (0.44 M). Venous blood samples (268 in total) were collected into lithium-heparin tubes at fasting state, and then 20, 40, 60, 90, 120, 180, and 240 min postprandially. Blood samples were centrifuged for 15 min, 1500 × *g*, to collect plasma, and then acidified with 1/0.2 V/V trifluoroacetic acid (0.44 M) and stored at −80 °C until analyses.

2.5. Dosage Information

The PPE was extracted from purple potatoes (*Solanum tuberosum* L. 'Synkeä Sakari') as described previously using aqueous ethanol acidified with acetic acid and purified with Amberlite XAD-7HP adsorbent.[27] The PPE was used instead of potatoes as such to remove the effect of the compositional differences of potato varieties. The PPE was carefully characterized earlier,[8] providing 152 mg of anthocyanins and 140 mg hydroxycinnamic acid conjugates per meal corresponding to 0.48 kg of fresh purple potatoes. The methoxysubstituted petunidin and peonidin derivatives dominated with minor amounts of cyanidin, malvidin, delphinidin, and pelargonidin. The anthocyanidins were linked to a glucose and a rutinose (a disaccharide of a rhamnose and a glucose), and 99% of the anthocyanins were monoacylated with a caffeic, ferulic, or coumaric acid. Petunidin-coumaroyl-rutinoside-glucoside represented 60% of the anthocyanins (**Figure 1**). The hydroxycinnamic acid derivatives in the PPE were chlorogenic acid (84%), caffeic acid (6%), and cryptochlorogenic acid (3%). Both meals contained 0.7 mg of flavonol glycosides and 4.5 mg of hydroxycinnamic acids from the yellow potatoes.[8] The amount of anthocyanins was based on the previous clinical trial.[7]
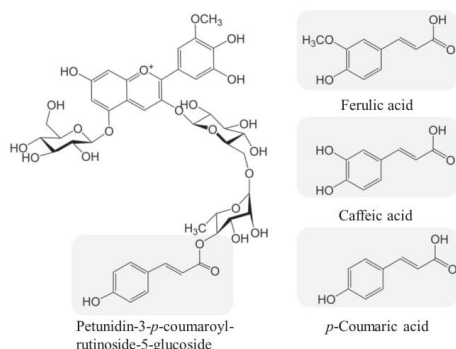
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Figure 1. The major anthocyanin of purple potatoes, petunidin-coumaroyl-rutinoside-glucoside, and the acyl groups of purple potato anthocyanins: caffeic, *p*-coumaric, and ferulic acids.[8] The suggested positions of the acyl and glycosyl groups and the isomer of the coumaric acid are based on a previous nuclear magnetic resonance spectroscopy (NMR) characterization study.[48]

2.6. Sample Randomization

The samples of each volunteer were processed and analyzed as a set of 10 (urine) and 16 (plasma) samples. Within these sample sets, the samples were paired according to the sampling time point with randomized order of the study visit. The order of these pairs was randomized within the set, and the order of the sample sets of the volunteers were randomized. The *randperm* function in Matlab (The Mathworks, Cambridge, UK) was used with the default setting of Mersenne Twister.

2.7. Solid-phase Extraction

The modified extraction method[28] with the chosen StrataX (6 mL, 500 mg) solid-phase cartridges was verified by comparing the analytical responses of matrix-matched blank samples spiked with selected compounds before and after solid-phase extraction (extraction recoveries, Supporting Information S1 and S2). Samples were extracted in a room with yellow light to protect the light-sensitive anthocyanins. The SPE cartridges were activated and stabilized using one load volume of 0.25% formic acid in methanol followed by a load volume of 0.1% formic acid in water. Thawed samples were diluted 2/1 with 0.1% formic acid and spiked with 10 μL of 400 mM a mid-polar and mid-range responder, taxifolin, as an internal SPE process control standard. The cartridges were washed twice with a load volume of 2% methanol in 0.1% formic acid for urine, and 0.1% formic acid for plasma. The cartridges were dried under vacuum for 30 min. Samples were eluted with 5 mL 0.25% formic acid in methanol after 10 min of soaking, followed by an additional 2 mL rinse.

The samples were evaporated to a volume of about 200 μL and reconstituted with 200 μL of 0.1% formic acid. As an internal volume control standard, 10 μL of caffeine-d_9 (200 mM) was added. The samples were shaken after which urine samples were spun for 10 s and plasma samples centrifuged 10 000 × g for 10 min at 4 °C. For anthocyanin analyses, 200 μL of the supernatant

was acidified with 10 μL of strong formic acid. All samples were stored in −80 °C. Prior to analyses, the samples were thawed, shaken carefully, and centrifuged for 10 000 × g for 5 min at 4 °C. Matrix-matched positive (urine) and standard-spiked (urine, plasma) quality control samples were processed daily along with the analytical samples.

2.8. UHPLC-MS/MS Analyses

Phenolic metabolites and anthocyanins were analyzed using 1290 Infinity UHPLC combined with 6490 Triple Quad mass spectrometer equipped with iFunnel (Agilent Technologies, Santa Clara, CA). The analytes were separated in an ACQUITY UPLC HSS T3 1.8 μm (2.1 × 100 mm, Waters, Milford, MA) column at 35 °C. The injection volume was 5 μL for urine and 1 μL for plasma. The sample rack was cooled to 4 °C. Identification and quantification of the phenolic metabolites and anthocyanins were conducted with comprehensive, targeted multiple reaction monitoring (MRM) panels (Supporting Information S1, S2), which contained the degradants, metabolites and conjugates predicted by the research group and presented in the current literature for nonacylated anthocyanins. Authentic standard compounds (Chapter 2.2) were used in identification whenever available.

For anthocyanins, the gradient consisted of 5% formic acid in water (A) and 5% formic acid in acetonitrile (B) at 0.4 mL min⁻¹ followingly: 0–1 min, 5% B; 5 min, 10% B; 30 min, 25% B; 31 min, 95% B; 32 min, 95% B; 32.1 min, 5; % B and 36 min, 5% B. Collision gas temperature was 220 °C, gas flow 14 L min⁻¹, nebulizer pressure 25 psi, capillary voltage 3.5 kV and collision energy 5 eV for phosphatidyl choline and anthocyanidins, and 28 eV for anthocyanins. The retention times for the purple potato anthocyanins were verified using urine spiked with the PPE. For phenolic metabolites, the mobile phase A was 10 mM ammonium acetate in water (pH 5) and B was 10 mM ammonium acetate in acetonitrile (pH 5). The flow rate was 0.4 mL min⁻¹. The concentration of eluent B was 1% for a minute, then at 3 min, 5%; 8 min, 60%; 8.50 min, 99% and 9–12 min, 1%. MRM was used in negative mode except for caffeine-d_9 and phosphatidyl choline. Collision gas temperature was 220 °C, gas flow 14 L h⁻¹, nebulizer 25 psi, and capillary voltage 3500 V. Collision energies were optimized for each compound available. The performance of the mass spectrometer was monitored by analyzing repeatedly a matrix-matched sample containing all available standard compounds.

The data was processed using MassHunter Quantitative Analysis B.06.00 (Agilent Technologies, Santa Clara, CA, USA). The sample volumes were corrected on the basis of the caffeine-d_9 levels in each sample. Then, the collected data was divided into two parts: the quantified metabolite data set, of which identification of the compounds was confirmed using the available standard compounds, and the screened metabolite data set, which was tentatively identified based on the MRM transitions and chromatographic retention. Quantification was performed using matrix-matched external standard curves, and endogenously present target metabolites were subtracted. All quantified metabolites were within the linear range of the standard curves. If a metabolite was detected in the samples of over half of the study volunteers (nine or more), it was interpreted as detected, and further quantified.

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2.9. Urinary Creatinine

Urinary creatinine was analyzed in the laboratory of the Hospital District of Southwest Finland using enzymatic methods (Cobas C702 automatic analyzer, Roche Diagnostics GmbH, Mannheim, Germany).

2.10. Pharmacokinetic and Statistical Analyses

For the separate time points of the quantified data set, statistical analyses were performed using R 3.5.1.[29] For normally distributed data (Shapiro–Wilk test), the paired-samples t-test was used to compare the differences in the metabolite concentrations between the plasma and urine samples at certain time points after the control and the study meal. The nonparametric counterpart, the Wilcoxon signed rank test, was used otherwise. The significance level was set to 0.05. Additionally, the pharmacokinetic incremental area under the curve (iAUC) values were calculated using the trapezoidal rule, and maximum concentration (C_{max}) and time point at the maximum concentration (t_{max}) were determined as the mean of the maximal values of the individual volunteers. For urine, t_{max} was determined on the basis of the most frequent categorical time period variable.

The screened and tentatively identified data set was visualized as heatmaps using R 3.5.1[29] with gplots version 3.0.1.1[30] and RColorBrewer version 1.1.2.[31] The differences between the volume corrected metabolite areas between the study and control meals were calculated resulting in a variable describing change between the meals in certain time point or period (delta). Due to the large differences in the areas of the screened metabolites, the fasting state value of the delta variable was set to zero by subtracting it from the postprandial values. All compounds were normalized to range $[-1, 1]$ by dividing with the absolute maximum delta of the corresponding compound. The time-wise change of the delta variable of the compounds regarding to the fasting state can be observed, but the change is not comparable between the compounds.

3. Results and Discussion

3.1. Anthocyanins

In urine, five anthocyanin degradants were detected: cyanidin-3-O-glucoside, as confirmed with the standard compound, and tentatively identified, malvidin-rutinoside, petunidin-glucoside, peonidin-glucoside, and peonidin-glucuronide (aglycones, **Figure 2**; heatmap **Figure 3**A). The anthocyanin degradants peaked at 8–12 h, and were present in urine samples until 24 h, suggesting enterohepatic circulation of the degradants. These results indicate that the monoacyl group and rutinose are hydrolyzed. Detection of cyanidin-3-O-glucoside refers to the high bioavailability of the minor cyanidin-based anthocyanins present in potato or O-demethylation of the methoxysubstituted peonidin to cyanidin. Detecting peonidin-glucuronide, a conjugate, indicates phase II metabolism. A subset of plasma samples was analyzed for anthocyanins, but none were detected.

This is the first clinical study to detect degradants of monoacylated anthocyanins of purple potatoes in human urine;[26] fragments of cyanidin-derived mono- and diacylated anthocyanins

of red cabbage have been detected in earlier clinical trials.[5,32] The nonacylated monoglycosylated anthocyanins are deglycosylated after ingestion and extensively glucuronidated and methylated, and also sulfated in minor amounts,[22,28] leading to 91% of the total anthocyanins appearing as conjugates of aglycones in urine.[22] On the contrary, our results show that the absorbed potato acylated anthocyanins seem to be resistant for deglycosylation. Nonacylated anthocyanin conjugates have been detected after a meal of red cabbage rich in acylated anthocyanins,[5,32] but according to our knowledge, this is the first time glucuronidation is reported for acylated anthocyanins derived from purple potatoes.[26]

Acylation decreases the bioavailability of anthocyanins[5,6] possibly due to the steric hindrance of several glycosyl and acyl moieties against the gastric absorption mechanisms as detected in vitro.[33,34] Selective structure-dependent absorption of anthocyanins has been suggested,[12] but understanding the dependence requires more investigations as contradictory results have been presented. Previous clinical interventions which fed purple potatoes[26] and Concord grape juice[24] (38% of total anthocyanins were monoacylated, mainly delphinidin, petunidin, and malvidin diglucosides) to healthy volunteers either did not detect acylated anthocyanins[24,26] or any anthocyanins at all[26] in human biofluids. Other clinical interventions have detected selected, structurally different (B-ring, acylation pattern, sugar moiety) acylated anthocyanins of red cabbage (mono- and diacylated cyanidin diglucosides),[5,32] purple carrot (monoacylated cyanidin xylosides),[6] and purple sweet potatoes (diacylated cyanidin and peonidin sophorosides)[12,13] in human plasma and/or urine, and in addition, of eggplant (monoacylated delphinidin rutinosides) in rats.[35] As our methodology was optimized with nonacylated anthocyanins and the level of acylated anthocyanins in biological fluids is low due to their extremely poor bioavailability, more studies are needed to confirm our results.

The B-ring substitution and sugar moieties of the ingested anthocyanins affect the bioavailability in vivo.[4,22,36] In humans, increased hydroxylation of B-ring (delphinidin, cyanidin) may increase the bioavailability of nonacylated anthocyanins[36] but it should be noted that anthocyanidins are readily interconverted to each other in phase I and II reactions via demethylation, methylation, hydroxylation, and dihydroxylation reactions in vivo. For example, the methylation of nonacylated cyanidin to peonidin has been well-recognized,[14,22,35] whereas malvidin may be demethylated to petunidin.[22] The anthocyanidin may also affect the degree of enzymatic conjugation; for malvidin, it may be decreased but for the slightly smaller petunidin, the conjugation is similar compared to cyanidin glycosides.[22] In future, studies feeding pure labeled anthocyanins are ideal for further structural comparisons.

3.2. Quantified Phenolic Metabolites

From urine, 28 phenolic metabolites were identified and quantified (Figure 2, **Figure 4**A, **Table 1**, Supporting Information S3, S5). The mean maximum concentrations (C_{max}) varied from 2 nM mM^{-1} creatinine of quercetin to 290 µM mM^{-1} creatinine of hippuric acid, and the t_{max} values of the detected metabolites were late (4–8, 8–12 h). Of the urinary metabolites, 19 were

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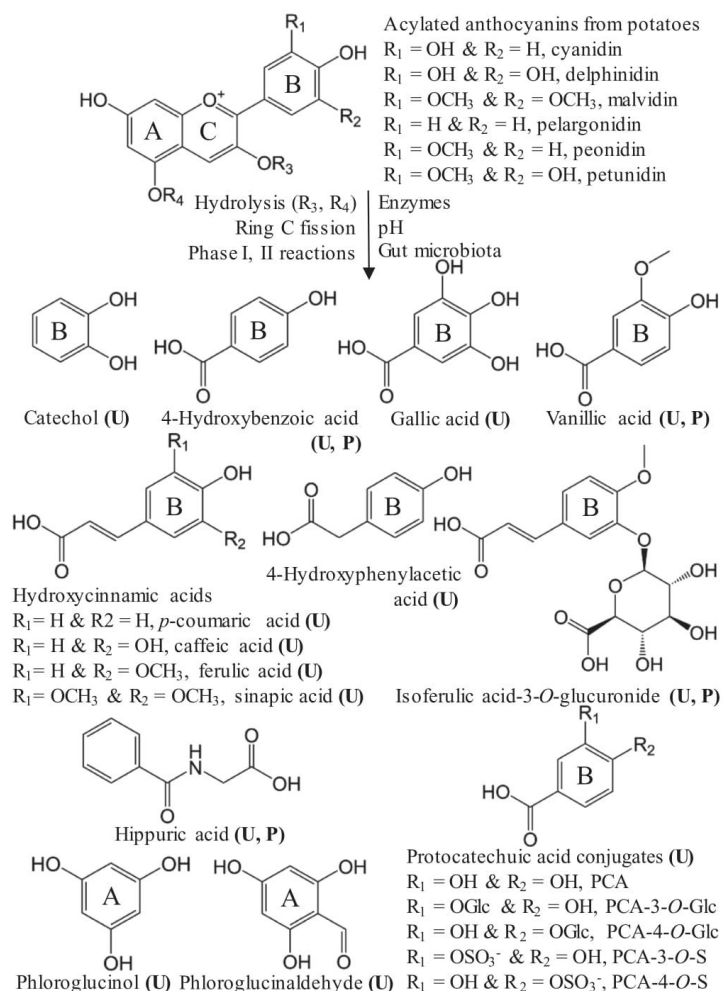Food Research**

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Figure 2. Identified phenolic metabolites detected in urine (U) and plasma (P) after a meal of purple potato extract rich in acylated anthocyanins and their suggested origin. PCA, protocatechuic acid; glc, glucuronide; s, sulfate. R_3 and R_4 may carry a glycoside of which R_3 may be acylated with a hydroxycinnamic acid. The values are presented in the Supporting Information S3 and S4.

elevated statistically significantly after the study meal compared to the control meal. Hippuric acid reached an increase of 139 µM mM^{-1} creatinine from the control levels after the study meal ($p = 0.026$ and 0.006 at 4–8 and 8–12 h). Monomethoxysubstituted vanillic acid ($p = 0.002$ and 0.001 at 0–4 and 4–8 h, respectively) and dimethoxysubstituted sinapic acid ($p = 0.000$ at 4–8 h) were detected. Other elevated abundant metabolites were hydroxycinnamic acids, including caffeic acid ($p = 0.006$

and 0.003 at 4–8 and 8–12 h), p-coumaric acid ($p = 0.014$ at 4–8 h), and ferulic acid ($p = 0.041$, <0.001, and 0.010 at 0–4, 4–8, and 8–12 h). Dihydroxysubstituted protocatechuic acid was increased ($p = 0.003$, 0.003, and 0.001 at 0–4, 4–8, and 8–12 h, respectively). Furthermore, its 3- and 4-sulfates and glucuronides were detected, of which protocatechuic acid-4-O-glucuronide, -3-O-sulfate and -4-O-sulfate were increased ($p = 0.008$, 0.017, 0.025 at 0–4 , 4–8, and 8–12 h; $p = 0.020$, 0.001, and 0.010 at 0–4,

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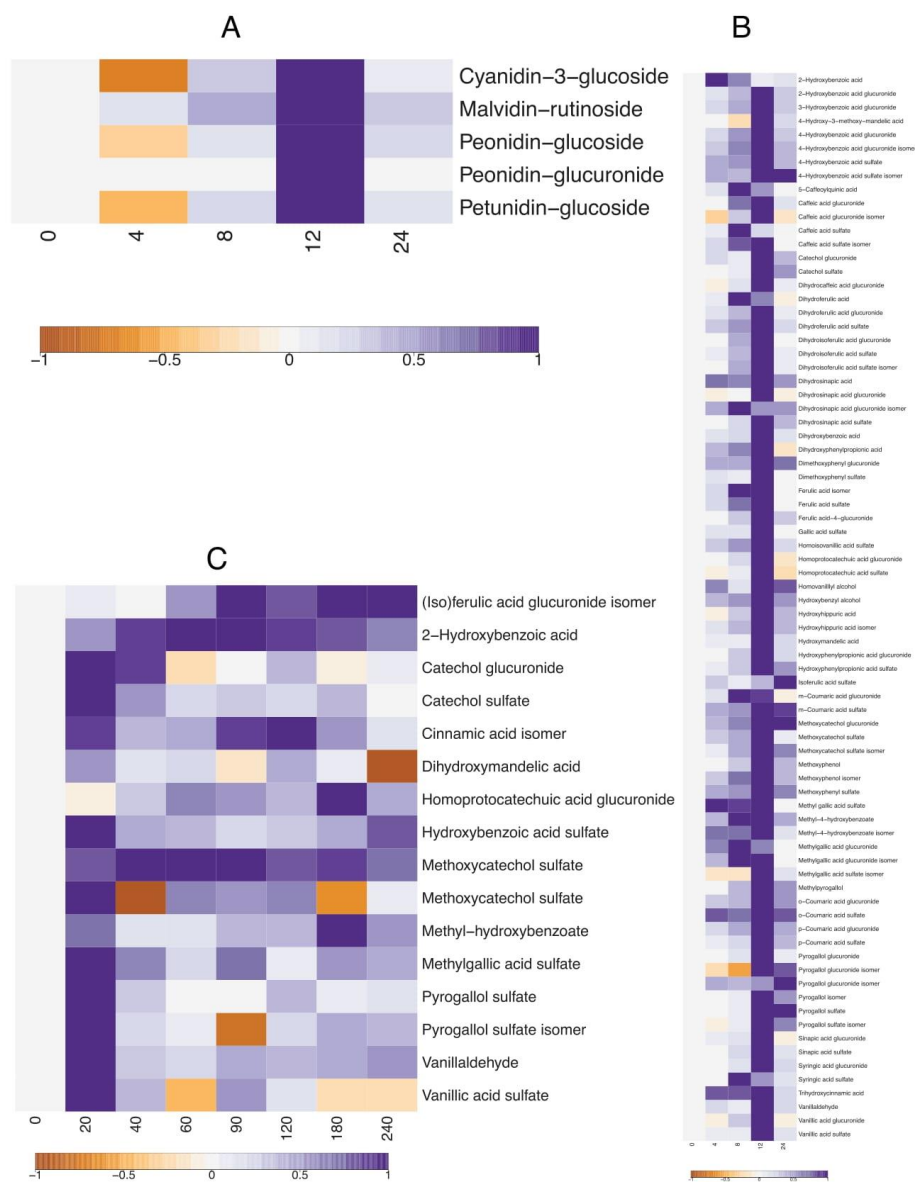**Molecular Nutrition
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Figure 3. Screened A) anthocyanins in urine, B) phenolic metabolites in urine, and C) phenolic metabolites in plasma of 17 healthy men after a potato meal with (study meal) or without (control meal) anthocyanin-rich purple potato extract. Tentative identifications are based on the MRM transition and chromatographic retention. The heatmaps describe change between the samples collected after the study and control meal at certain time points standardized to the range of [−1, 1]. Purple color indicates that larger amount of the metabolites were detected after the study meal, whereas orange indicates the opposite.

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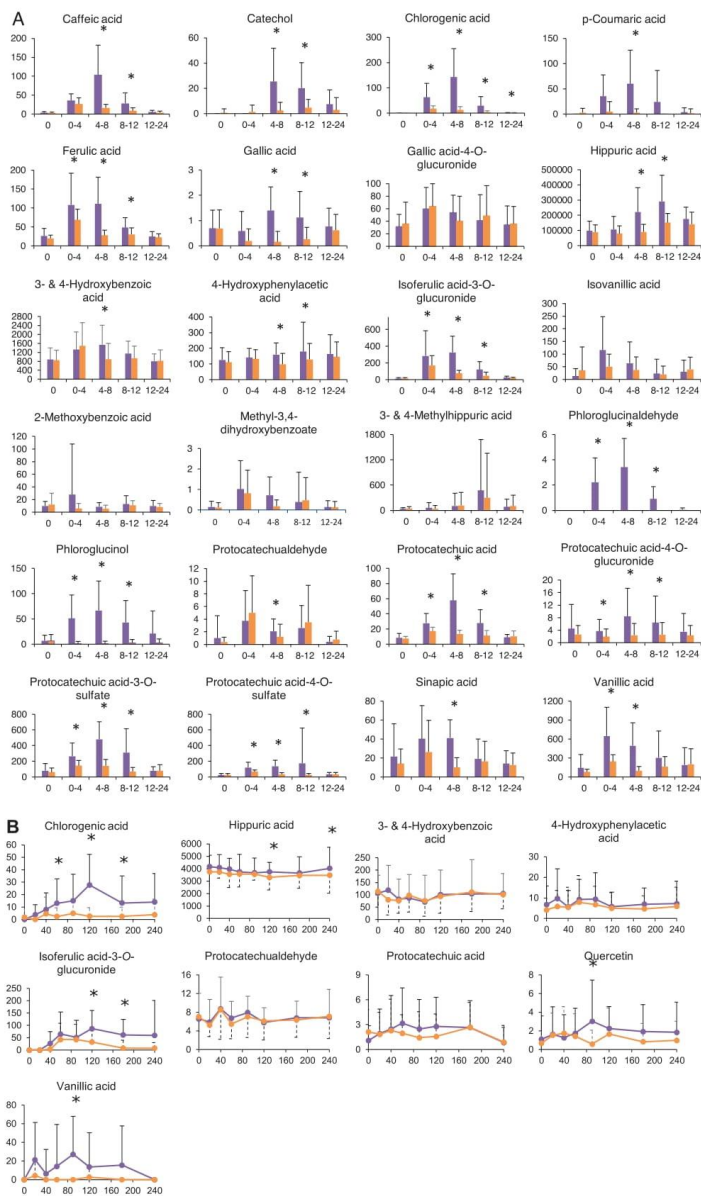**Molecular Nutrition
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Figure 4. The phenolic metabolites identified and quantified in A) urine and B) plasma of 17 healthy men using corresponding standard compounds. Values are given as concentrations (nM mM^{-1} creatinine, urine and nM, plasma) with standard deviations in the fasting state (0-point) and postprandially (Figure A, h; Figure B, min). The purple plots represent the treatment values whereas the orange plots represent the control values. The between-meal significant differences ($p < 0.05$) are shown with asterisks. Supporting information S5 and S6 contain the p-values.

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Table 1. The urinary pharmacokinetic parameters of the phenolic metabolites after an acute intake of a purple potato extract rich in methoxysubstituted, monoacylated anthocyanins.

	n	C_{max} [nM mM^{-1} creatinine]	SD	t_{max} [h]	Detected earlier in
Caffeic acid	17	93.6	71.0	4–8	[16,17,19,21]
Catechol	13	41.7	17.4	4–8	
Chlorogenic acid[a]	17	131.8	99.9	4–8	[19]
p-Coumaric acid	14	85.0	73.9	8–12	[16–19,21,24,28]
Ferulic acid	17	119.9	67.5	4–8	[3,14–18,21,23,28]
Gallic acid	14	1.8	0.8	4–8	[17,21]
Gallic acid-4-O-glucuronide	17	68.6	40.4	0–4	
Hippuric acid	17	288 692.8	166 291.8	8–12	[3,15,17,19,21,23,26,28]
Homoprotocatechuic acid[b]	14	26.6	19.6	4–8	[3,14,16–19,21,23,28]
Homovanillic acid[c]	17	538.1	437.5	4–8	[16–19,21,23,25,28]
4-Hydroxybenzaldehyde	17	200.0	212.6	8–12	[3,17,19,21]
3- & 4-Hydroxybenzoic acid	17	1791.7	840.4	4–8	[3,16–19,21,23,28]
4-Hydoxyphenylacetic acid[d]	17	266.3	170.4	0–4	[3,14,17,19,21]
Isoferulic acid-3-O-glucuronide	17	345.6	255.8	4–8	[17,19,23,24]
Isovanillic acid	15	140.2	117.5	0–4	[3,14,17,19,21]
2-Methoxybenzoic acid[e]	16	34.7	73.5	8–12	
Methyl-3,4-dihydroxybenzoate	12	1.7	1.9	4–8	[3,14,21]
3- & 4-Methylhippuric acid	14	560.4	1273.3	8–12	[28]
Phloroglucinaldehyde	16	3.5	1.9	4–8	[3,18,28]
Phloroglucinol	15	86.4	49.9	0–4	[21]
Protocatechualdehyde	17	5.9	5.0	8–12	[3,19,21]
Protocatechuic acid	17	53.8	32.3	4–8	[3,14–19,21,23]
Protocatechuic acid-4-O-glucuronide	15	8.8	9.1	4–8	[3,14,18,28]
Protocatechuic acid-3-O-sulfate	17	482.3	250.6	4–8	[3,14,18,21,28]
Protocatechuic acid-4-O-sulfate	17	239.4	418.3	0–4	[3,14,18,21,28]
Quercetin[a]	17	1.5	1.9	4–8	[17,19]
Sinapic acid	17	53.7	36.1	4–8	[17–19,28]
Vanillic acid	17	676.7	412.9	0–4	[14,16–19,21,23,25,28]

The values are presented as mean with standard deviations (SD) detected in n study participants with the exception of the categorical t_{max}, which is presented on the basis of the highest frequency. C_{max}, the mean of the maximum metabolite concentrations of the volunteers; t_{max} was determined on the basis of the highest frequency of the categorical time period variable; [a] Chlorogenic acid and quercetin are not likely anthocyanin metabolites but are derived from the meals; Quantified with [b] protocatechuic acid; [c] vanillic acid; [d] caffeic acid; [e] gallic acid.

4–8, and 8–12 h; $p = 0.003$, 0.002, and <0.001 at 0–4, 4–8, and 8–12 h, respectively). Protocatechuic acid-3-O-glucuronide was detected but not quantified due to co-elution.

Of the 14 phenolic metabolites detected and quantified from plasma (Figure 2, Figure 4B, **Table 2**, Supporting Information S4 and S6), five were statistically significantly elevated after the study meal compared to the control meal. Chlorogenic acid was increased at 60, 120, and 180 min postprandially ($p = 0.024$, <0.001, and 0.022, respectively), hippuric acid at 0, 120, and 240 min ($p = 0.039$, 0.001, 0.038), isoferulic acid-3-O-glucuronide at 120 and 180 min ($p = 0.018$ and 0.013), quercetin at 90 min ($p = 0.038$), and vanillic acid at 90 min ($p = 0.036$). The C_{max} ranged between 2 nM of methyl-3,4-dihydroxybenzoate and 4700 nM of hippuric acid, whereas most of the t_{max} values were between 1 and 2 h. Plasma contained fewer metabolites compared to urine, to which the kidneys concentrate metabolites for excretion. Laterise metabolites, however, were not detected from plasma due to the 4-h postprandial sampling.

These results show that the acylated anthocyanins of purple potatoes are degraded into phenolic metabolites in humans. O-Demethylation of the metabolites may occur as the potato anthocyanins are mainly methoxysubstituted, but dihydroxylated catechol and protocatechuic acid and its conjugates were abundant in urine. Detecting conjugated phenolic acids indicates phase II metabolism as suggested in studies regarding berry anthocyanins.[17–19,28] Hippuric acid, the major metabolite of potato anthocyanins, is another phase II metabolite formed by the liver cells from benzoic acid and glycine, and is a recognized biomarker for consumption of polyphenol-rich berries and fruits.[37,38] However, the levels of hippuric acid are affected also by endogenous, such as protein, metabolism. Both urine and plasma contained p-coumaric acid, and in urine, also ferulic acid and caffeic acid were abundant; they might be originated from the A- and B-rings and the acyl groups of the parent anthocyanins. p-Coumaric acid and caffeic acid were both also present in minor amounts in the ingested PPE, and

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Table 2. The plasma pharmacokinetic parameters of the phenolic metabolites after an acute intake of a purple potato extract rich in methoxysubstituted, monoacylated anthocyanins.

	n	C_{max} [nM]	SD	t_{max} [h]	SD	$iAUC_{0-4}$ [nM*h L^{-1}]	SD	Detected earlier in
Caffeic acid	17	27.1	18.9	1.7	1.2	14.4	12.3	[17,19,24]
Chlorogenic acid[a]	16	36.4	24.0	2.5	0.8	59.9	53.9	[17,19]
Hippuric acid	17	4649.2	1293.3	1.7	1.6	370.6	611.6	[3,15,17,19,21,28]
4-Hydroxybenzaldehyde	17	264.2	122.7	1.2	1.0	40.9	42.8	[3,17–19,21,28]
3- & 4-Hydroxybenzoic acid	17	214.3	123.7	1.4	1.3	163.7	175.8	[17,19,21,28]
4-Hydroxyphenylacetic acid[b]	12	23.1	17.0	1.3	1.2	12.8	13.0	[17,19,21]
Isoferulic acid-3-O-glucuronide	15	161.2	130.2	2.2	1.2	240.6	173.8	[17,19,23]
Methyl-3,4-dihydroxybenzoate	17	1.8	1.1	1.7	1.4	1.6	1.7	[3,14,18,21,28]
Phloroglucinaldehyde	12	13.3	7.0	1.6	1.3	14.5	17.7	[4,18,21,28]
Protocatechualdehyde	17	12.3	6.5	1.1	1.1	9.0	9.6	[18,19,21,28]
Protocatechuic acid	16	7.9	2.9	1.8	1.3	7.0	6.9	[3,4,14,15,17–19,21,28]
Protocatechuic acid-3-O-sulfate	16	15.4	8.2	1.6	0.9	13.6	15.8	[3,14,18,28]
Quercetin[a]	15	6.4	4.0	1.8	1.1	7.3	7.5	[17]
Vanillic acid	12	91.5	38.4	1.4	0.7	76.0	71.6	[3,4,14,18,19,28]

The values are presented as mean with standard deviations (SD) detected in n study participants. C_{max}, the mean of the maximum metabolite concentrations of the volunteers; t_{max}, mean of the time point when C_{max} was observed; iAUC, incremental area under the curve calculated using the trapezoidal rule; [a] Chlorogenic acid and quercetin are not likely anthocyanin metabolites but are derived from the meals; [b] Quantified with caffeic acid.

chlorogenic acid, abundant in the PPE, may partially break down to caffeic acid. Ferulic acid may be formed from caffeic acid via O-methylation.[39] Chlorogenic acid and quercetin are most likely derived from the PPE and yellow potatoes[8] and are not metabolites of anthocyanins.

Under physiological conditions, nonacylated anthocyanins are rapidly hydrolyzed leading to the formation of an unstable α-diketone which, without the protecting glycoside, is subjected to degradation leading to a phenolic acid (expected B-ring metabolite) and aldehyde (expected A-ring metabolite).[40] Thereafter, the phenolic metabolites may be conjugated as detected with labeled cyanidin-3-O-glucoside in vivo.[3,14] Acylated anthocyanins, on the other hand, are structurally more stable than nonacylated anthocyanins as the acyl groups protect from the nucleophilic attack of water, and in comparison to hydroxysubstituted anthocyanins, methoxysubstituted are less reactive. Even 72% of the applied acylated anthocyanins of potatoes (but only 45% of the cyanidin-based, acylated anthocyanins of carrot) may reach the colonic vessel in an in vitro gastrointestinal model.[41] In colon, acylated anthocyanins may be degraded by human gut microbiota as detected in vitro with mono- and diacylated pelargonidin sophoroside glucosides.[40] This is substantiated by our in vivo results: in addition to late t_{max} times, urinary protocatechuic acid and phloroglucinaldehyde, the known colonic metabolites of cyanidin-3-O-glucoside,[42] were detected. The proposed degradation mechanism occurs first by enzymatic hydrolysis of the acyl and glycosyl groups followed by enzymatic and/or spontaneous C-ring fission of the unstable anthocyanidin leading to phenolic metabolites, which may be then subjected to further phase I and II reactions (Figure 2).

Considering the earlier reported beneficial health effects of purple potatoes[7] and the PPE[8] on human carbohydrate metabolism, acylated anthocyanins may inhibit pancreatic α-amylase[43] and intestinal maltase[44] as detected in vitro. More bioavailable phenolic metabolites may contribute to these

health effects; for example, protocatechuic acid has insulin-like properties[45,46] and other phenolic metabolites may modulate carbohydrate metabolism by inhibiting salivary α-amylase, enhancing the intestinal uptake of glucose and downregulating sugar transporters as detected in vitro.[47] More investigations of the physiological role of the phenolic metabolites are required to understand the health effects and underlying molecular mechanisms of anthocyanin-rich foods.

Metabolites not previously reported in clinical trials feeding anthocyanin-rich foods were detected: catechol, gallic acid-4-O-glucuronide, and 2-methoxybenzoic acid, of which only catechol was increased statistically significantly in urine after the study meal. The lack of statistical significance may be due to inter-individual variation amongst the volunteers or that the metabolite was derived from the yellow potatoes used both in the control and study meals. Furthermore, phloroglucinol was detected earlier by only one study,[21] which fed raspberries rich in both anthocyanins and other phenolic compounds to healthy human volunteers. Earlier, a meal of purple potatoes led to the tentative identification of homovanillic acid/dihydrocaffeic acid, dihydroferulic acid, (iso)ferulic acid, dihydrocaffeic acid sulfate, and hippuric acid, but the study lacked the control arm.[26] As a next-step suggestion, a trial feeding labelled acylated anthocyanins would further discriminate the potato-derived metabolites from anthocyanin-derived metabolites. Here, large inter-individual variation was detected;[15,17–19] individual metabolism and gut microbiota composition may affect the metabolism of anthocyanins.

3.3. Screening of Tentatively Identified Phenolic Metabolites

A large number of phenolic metabolites and conjugates were detected, tentatively identified and heatmapped (Figure 3B), most of them peaking at 8–12 h. For example, vanillaldehyde

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and hydroxyhippuric acids, and isomers of hydroxybenzoic acid, dihydroferulic acid, and dihydrosinapic acid were detected. Sulfates and glucuronides were detected for hydroxycinnamic acid derivatives (coumaric, caffeic, and ferulic acids), benzoic acid derivatives (hydroxybenzoic, vanillic, syringic, and methylgallic acids), phenol derivatives (catechol and pyrogallol), phenylacetic acid derivatives (homoprotocatechuic acid), and phenylpropanoic acids such as dihydro(iso)ferulic and dihydrosinapic acids. From plasma, 16 tentatively identified metabolites were detected (Figure 3C). 2-Hydroxybenzoic acid showed high analytical response compared to the other ones, peaking at 90 min after the study meal. Several sulfate and glucuronide conjugates were detected: catechol glucuronide, catechol sulfate, homoprotocatechuic acid glucuronide, hydroxybenzoic acid sulfate, (iso)ferulic acid glucuronide isomer, two methoxycatechol sulfate isomers, methylgallic acid sulfate, and vanillic acid sulfate.

The screened data set highlights the vast extent of phenolic metabolites after an acute intake of PPE rich in acylated anthocyanins even though the identification was only tentative due to practical challenges. The number of possible degradants and conjugates to be screened was large as the potato anthocyanins contain all six anthocyanidins varying in the acylation pattern, but the availability of commercial standards is limited. Therefore, in addition to our large compound library, the MRM transitions and chromatographic retention were used for identifying the screened dataset as reported earlier.[22] Additionally, the concentration of anthocyanins and phenolic metabolites is low in physiological samples. Here their levels were concentrated using optimized SPE and evaporation, and the samples were analyzed using a modern state-of-the-art mass spectrometer with a targeted MRM approach providing high instrumental sensitivity and capacity to scan numerous transitions without compromising the amount of data points per peak. Regardless of its high sensitivity, a targeted MRM approach is limited to predicted metabolites, whereas an untargeted approach, which is usually performed with high resolution, could lead to the identification of unpredicted metabolites.

4. Concluding Remarks

This report demonstrates that an acute intake of PPE rich in methoxysubstituted anthocyanins monoacylated with caffeic, coumaric and ferulic acids leads to absorption and excretion of only a small number of anthocyanins degradants and an extensive number of spontaneous and/or colonic phenolic metabolites and their phase II conjugates. The rutinose and acyl groups of anthocyanins are hydrolyzed and the methoxysubstituted anthocyanidins may be *O*-demethylated. Novel phenolic metabolites of acylated potato anthocyanins, such as glucuronyl and sulfonyl conjugates of protocatechuic acid, were detected. Three novel phenolic metabolites not reported earlier in clinical trials after a meal rich in anthocyanins, such as purple potatoes, were detected: catechol, gallic acid-4-*O*-glucuronide, and 2-methoxybenzoic acid, of which only catechol was increased statistically significantly in comparison to the control yellow potato meal without the PPE. These results provide new insights on the bioavailability and metabolism of acylated anthocyanins in humans.

Author Contribution

J.J., K.L., and B.Y. concepted the study and organized the clinical trial. J.J. and J.P. optimized the analysis method and collected the data under supervision of M.P. and P.K., and J.J. processed the data. J.J., B.Y., P.K., and K.L. participated to funding acquisition and B.Y., P.K., and K.L. provided the resources. J.J. drafted the original manuscript with the help of K.L., and all authors commented on and approved the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

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