

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Phytochemical Optimisation of Blueberry Juice

A research report presented in partial fulfilment of the requirements for the
degree of

Master of Food Technology

Massey University, Turitea Campus, Palmerston North, New Zealand

Natasha Birt

2011

Abstract

Blueberries contain a high concentration and diversity of anthocyanins which are responsible for the blue/purple pigment of their skin. Like many other fruits and vegetables they also contain a large amount of chlorogenic acid (CGA) within the fruit flesh and seeds. Together these phytochemicals appear to account for most of the high antioxidant activity of the fruit, although within the scientific community a consensus has not been reached as to their effects on human health. Fresh blueberries have a limited season and are perishable unless stored frozen. Processing of blueberries into juice allows year round sale, and importantly less market-driven pricing. Therefore the aim of this research project was to investigate and optimise factors which may be significant in producing a high phytochemical blueberry juice.

The pigment of blueberries is concentrated in the skin, and therefore smaller blueberries have the highest anthocyanin concentrations on a weight basis. Consequently the three highest concentrations were found in cultivars 'Elliot', 'Burlington' and 'Duke'. Conversely chlorogenic acid is not restricted to the blueberry skin; 'Elliot', 'Bluecrop' and 'Burlington' had the three highest concentrations, while 'Duke' had the lowest. The profile of individual anthocyanins was also found to be diverse amongst cultivars. Therefore if individual anthocyanin(s) are shown to be important for specific health conditions further consideration should be given to cultivar selection.

Upon review of the current blueberry juice manufacturing process, large anthocyanin losses were seen at three key steps: after thawing of the fruit, removal of the press cake and holding of the juice between pressing and pasteurisation. Two alternative processes were compared where a slightly higher anthocyanin concentration was achieved for 'hot press juice' but a lower chlorogenic acid concentration than the alternative 'cold press' juice.

Modifications to the manufacturing process were investigated using the pilot plant at Massey University. It was found that blanching of frozen blueberries before pressing was successful in significantly increasing the anthocyanin and chlorogenic acid concentration of the juice (about a 5 fold increase of anthocyanins and a 4 fold increase in chlorogenic acid from previous levels). However, this did alter the sensory properties of the juice significantly, with a more cooked flavour and thicker texture. Other variations that were trialled, such as milling,

variation in holding time and temperature between pressing and pasteurising, had comparatively little effect on the anthocyanin and chlorogenic acid concentration.

Storage tests on blueberry juice showed a clear relationship between the storage temperature and anthocyanin retention, where warmer temperatures resulted in larger anthocyanin degradation. At the end of the six month storage period, juice storage at 5°C gave 63% anthocyanin retention while juice storage at 25°C gave only 8% retention. Some protection was also afforded to juice packed in glass bottles rather than plastic and stored in the dark rather than the light; but this difference was far smaller than the effect of temperature. Chlorogenic acid levels appeared to be comparatively less affected; only relatively small amounts of degradation were observed. It is important to note that when the antioxidant capacity was measured for stored juice at six months under the various treatments, there was only a small degree of degradation for all samples as compared with at time zero. Previously researchers had encountered a similar phenomenon and suggested that unknown anthocyanin degradation products may still be able to contribute to the juice's antioxidant capacity. However, here it is also suggested that chlorogenic acid may have had a more significant contribution to the antioxidant capacity that it is usually credited with, due to the large amount present with relatively little degradation throughout the storage period.

This information may be used to produce and market a juice with high anthocyanin, chlorogenic acid and/or antioxidant properties. Of the health effects evaluated here, currently, research in cardiovascular disease and neuroprotection effects are looking the most promising with regard to dietary blueberry supplementation in humans, although there is still a lack of double blind randomised placebo controlled studies to come to any consensus within the scientific community. Additionally, at the present time, the use of health claims on food products in New Zealand is being revised (Food Standards Authority proposal P293). As the current state of nutrition research surrounding plant polyphenols is inconclusive it may be important to use generic statements such as 'high in antioxidants' rather than statements about specific compounds.

Table of Contents

CHAPTER 1	1
Introduction	1
1.1 Background.....	1
1.2 Purpose	2
1.3 Objectives	2
CHAPTER 2	3
Literature Review	3
2.1 Chemistry of Phytochemicals	3
2.1.1 Classification	3
2.1.2 Structure	4
2.1.3 Occurrence of Phytochemicals	7
2.1.4 Phytochemical Diversity in Berries	9
2.1.5 Phytochemical Diversity of Blueberries	10
2.1.6 Chemical composition of blueberries	13
2.1.7 Summary for Phytochemicals in Blueberries	15
2.2 Potential Health Benefits of Blueberry Consumption	17
2.2.1 Antioxidant Power	18
2.2.2 Absorption and Metabolism	20
2.2.3 Evidence for Potential Health Benefits	24
2.2.4 Recommended Intake	36
2.2.5 Summary of Potential Health Benefits.....	37
2.3 Juice Manufacture and Storage.....	39

2.3.1 Chemical Stability of Anthocyanins.....	39
2.3.2 Unit Operations in Juice Manufacturing	42
2.3.3 Retention of Phytochemicals during Processing	47
2.3.4 Storage Trials – Retention of Phytochemicals	55
2.3.4 Bioactivity of Blueberry Juice	57
2.3.5 Summary of the Effects of Juice Processing	59
2.4 Chromatographic techniques	60
2.4.1 Extraction.....	61
2.4.2 Separation.....	61
2.4.3 Detection & Quantification	62
2.5 Summary	63
CHAPTER 3	64
Materials & Methods.....	64
3.1 Research Plan	64
3.2 Material collection	65
3.3 Experimental design.....	67
3.4 Analytical Methods	73
3.4.1 Anthocyanin and Chlorogenic acid analysis	73
3.4.2 Spectrophotometric analysis (Total polyphenol content & antioxidant power)	77
3.4.3 Physical Parameters	79
CHAPTER 4	81
Results & Discussion	81
4.1 Varietal and Material Differences in Eight Blueberry Cultivars	81

4.1.2 Varietal Differences.....	81
4.1.2 Maturational differences.....	90
4.1.3 Implications for juice manufacture.....	92
4.2 Juice Production Analysis.....	93
4.2.1 Standard Manufacturing Analysis.....	93
4.2.2 Alternative Processes.....	105
4.2.3 Implications for juice manufacture.....	113
4.3 Changes during Juice Storage.....	114
4.3.1 Stored Blueberry Juice.....	114
4.3.2 Storage Experiment.....	115
4.3.3 Implications for juice manufacture.....	120
4.4 Analytical Improvements.....	122
CHAPTER 5.....	124
Conclusions & Recommendations.....	124
5.1 Blueberry Juice Manufacture.....	124
5.2 Further Research.....	125
References.....	127
Appendix.....	138

List of Tables

Table 2-1: Phenolic diversity of various berry fruits. Adapted from Beattie et al (2005).....	9
Table 2-2: Total contents of various phenolic compounds found in blueberries (mg/100g) (Howard and Hager, 2007).	11
Table 2-3: Antioxidant capacity (ORAC), total phenolic and anthocyanin content for eight highbush blueberry cultivars (Ehlenfeldt & Prior 2001).	12
Table 2-4: Chemical composition of fresh blueberry.....	13
Table 2-5: Types of nutrition research (Rolfes et al, 2009).	24
Table 2-6: Blueberry Juice Manufacture; Reported Methods.....	49
Table 2-7: Anthocyanin recovery during processing as reported by various research groups.....	49
Table 3-1: Stored juice details	66
Table 3-2: Comparison of different solid extraction methods.	73
Table 4-1: Anthocyanin Contents Reported by Various Researchers (mg/kg FW).....	82
Table 4-2: Individual anthocyanins for various blueberry cultivars measured using HPLC, based on elution order.	84
Table 4-3: Anthocyanin concentrations at various points of blueberry juice processing as seen in juice produced using different methods and measured using HPLC.....	106
Table 4-4: Properties of Juice stored under varying conditions.....	115

List of Figures

Figure 2-1: Classification of major phytochemicals (Erdman et al, 2007).	5
Figure 2-2: Structures of anthocyanidins occurring in grapes and berry fruits (Skrede & Wrolstad, 2002).....	6
Figure 2-3 The skeletal structure of an acylated anthocyanin malvidin-3-(6''acetoyl)glucoside (Adapted from Barnes et al, 2009).....	6
Figure 2-4: Potential cardiovascular benefits of blueberry phytochemicals (Meskin et al, 2002)	29
Figure 2-5: Variation in the anthocyanin structure and pigmentation with changes in pH of a solution (Barnes et al, 2009).....	40
Figure 3-1 Juice production using alternative processes, experimental details in text.	69
Figure 3-2: Overview of treatments in 6 month storage experiment - storage temperature, packaging type & light exposure.....	71
Figure 4-1: Total anthocyanin concentrations of eight different Highbush Blueberry cultivars measured using HPLC. Cultivars labelled with different letters are significantly different. ..	81
Figure 4-2: MINITAB score plot of individual anthocyanins of eight blueberry varieties (with duplicate data).	85
Figure 4-3: Excel scatter plot of latent vector coordinates produced by MINITAB.....	86
Figure 4-4: Total anthocyanin concentration for different cultivars with different extraction solutions, measured using HPLC	89
Figure 4-5: Chlorogenic acid contents for various highbush blueberry varieties measured using HPLC.....	89
Figure 4-6: Cold press blueberry juice production - flow diagram with chemical and material losses.	94
Figure 4-7: Hot press blueberry juice production - flow diagram with chemical and material losses.	96
Figure 4-8: Anthocyanin concentrations of berry and press cake samples during 'hot press' processing.....	99
Figure 4-9: Anthocyanin concentrations in blueberry juice during processing as measured by HPLC.....	100

Figure 4-10: Changes to individual anthocyanins before and after thawing of blueberries101

Figure 4-11: Changes to individual anthocyanins in blueberry juice at different stages of processing102

Figure 4-12: Changes to the concentration of chlorogenic acid during juice processing.....103

Figure 4-13: Antioxidant activity of blueberry juice samples immediately after pressing, as measured by DPPH inhibition105

Figure 4-14: Anthocyanin levels in juice held between pressing and pasteurisation for an extended period of time; TOP: Juice produced using slow defrost; BOTTOM: Juice produced using fast defrost.....109

Figure 4-15: Individual anthocyanins in juice produced using alternative processes; TOP: juice produced in trial 1 using slow defrost. BOTTOM: Juice produced in trial 5 using fast defrost.111

Figure 4-16: Total anthocyanin concentrations of five juice samples measured using HPLC.....114

Figure 4-17: Blueberry juices stored under different conditions for six months LEFT HAND SIDE: Anthocyanin concentrations of blueberry juice RIGHT HAND SIDE: Chlorogenic acid concentrations of blueberry juice.116

Figure 4-18: The profile of individual anthocyanins as seen as seen in juice packaged in glass bottles and stored for 6 months at 25 °C in the light.118

Figure 4-19: 'Cold press' juice stored at 15 °C in plastic bottles LEFT HAND SIDE: Anthocyanin concentrations of blueberry juice RIGHT HAND SIDE: Chlorogenic acid concentrations of blueberry juice.118

Figure 4-20: TOP: Total phenolic content of blueberry juice measured in Gallic acid equivalents; MIDDLE: FRAP antioxidant measured in FeSO₄ equivalents; BOTTOM: DPPH antioxidant measure as % inhibition.119

Figure 4-21: Anthocyanin contents of juice and juice sediment, from juice stored for six months at different temperatures.....120

ACKNOWLEDGMENTS

To the client Mamaku Blue, for their fiscal support and never-ending enthusiasm. Thank you for accepting me as part of the Mamaku family.

To Technology New Zealand for the scholarship which made this project possible.

To my supervisors Julian Heyes and Alistair Carr for their guidance and wisdom along the way and many great learning opportunities. The lab technicians, particularly Michelle McGrath who were always patient and willing to help. Alongside this all the local and visiting students I worked with, particularly Giorgio Tibaldi and Khairul Kasim.

To my family and other friends who make life one worth living no matter how stressful it gets, thank you all for your love and support.

CHAPTER 1

Introduction

1.1 Background

Blueberries of the genus *Vaccinium* are well known for their large array of potentially beneficial compounds, and are often referred to as a 'super food' or 'functional food' because of this (Szajdek & Borowska, 2008). As well as containing common antioxidants such as vitamin C, blueberries also contain a large number of phytochemicals, particularly phenolic compounds. Phenolic compounds are required for the reproduction and growth of plants as well as acting as a defence mechanism against pathogens, parasites and predators (Liu, 2004). They are also commonly referred to as phytochemicals which comes from the Greek word '*phyto*' meaning plant. The specific phenolics which blueberries are notable for are anthocyanin compounds, which provide the purple/blue pigment in the fruit's skin, and chlorogenic acid. As well as having a high concentration of these compounds, blueberries have the most diverse anthocyanin profile of any common berry fruit (Howard & Hager, 2007). Anthocyanins have been a popular area of research for some time, and have been investigated for use in the prevention and/or treatment of a large number of diseases. Although no consensus has been reached as to their health effects they continue to be an active area of nutrition research.

The market for products with health benefits has grown sustainably over the past decade alongside a growth in consumer awareness. Consequently this has resulted in increased consumer demand for blueberries worldwide, which continues to stimulate scientific research.

The orchard sells fresh blueberries for 4 months of the year; however, production of blueberry juice allows year-long retail sales. Additionally juice can be sold as a value-added product and is less susceptible to market driven pricing.

1.2 Purpose

The purpose of this master's project was to provide a local blueberry grower with information which would help them understand the latest developments in blueberry research, particularly from a health perspective, and to work with them to optimise potentially beneficial compounds in their blueberry juice. Although a vast amount of information is available on blueberries within the scientific community, it is extremely difficult for blueberry growers to get a hold of and make sense of this information. The grower's enthusiasm for such a project stemmed from numerous customer anecdotes regarding the positive health benefits of their products.

1.3 Objectives

The aim of this project was to research the evidence for the popular association between health and the phytochemicals found in blueberries, and to use this information to investigate processing factors which may improve the phytochemical profile of blueberry juice. The first of these objectives was addressed by a literature review; the second was investigated using a series of experiments. Areas initially identified for experimental investigation included:

- 1) Berry selection
 - Varietal and maturational phytochemical differences between eight Highbush blueberry cultivars grown in one orchard
- 2) Juice Manufacturing
 - Quantification of phytochemical profile in the current juice manufacturing process
 - Investigation of alternative operations in juice manufacturing process for phytochemical optimisation
- 3) Juice storage
 - Effect of storage on blueberry juice phytochemicals

The final outcome of this research is a recommendation on methods that could be used to enhance the phytochemical composition of blueberry juice.

CHAPTER 2

Literature Review

2.1 Chemistry of Phytochemicals

2.1.1 Classification

Phytochemical is a broad term used to describe compounds found in plants which are believed to benefit human health but are not yet recognised as essential nutrients by regulatory bodies (Liu, 2004). In Greek the word for plant is *Phyto*, therefore the translation of the word is plant chemical (Liu, 2004). A majority of these compounds are plant secondary metabolites and are synthesised for a number of functions in the plant including growth and protection (Kahkonen et al, 1999). Several thousand compounds have been identified and classified as phytochemicals and of these several hundred exist in commonly consumed foods (Erdman et al, 2007). Figure 2-1 by Erdman et al (2007) summarises the major classes of phytochemicals, which are defined according to their chemical structure. However, the number of compounds classed as phytochemicals is continually growing as scientists discover more and more plant compounds which may have an impact on human health.

A phenolic compound is one in which the carbons are arranged in a ring structure. Arguably the largest group of phytochemicals is the phenolic compounds, comprising several subclasses including phenolic acids and flavonoids. The class flavonoids is further segmented into a diverse range of subclasses including flavanols, flavonols, and anthocyanidins. Some of these classes of compounds exist exclusively in one plant family such as isoflavones which are found in soy beans and some other legumes, while others such as anthocyanins are found throughout the plant kingdom (see common food sources in Figure 2-1). Berry fruits are characterised by a high content and wide variety of phenolic compounds, namely the anthocyanin pigments which provide the berries with their distinct purple and red colours (Kahkonen et al, 1999; Szajdek & Borowska, 2008).

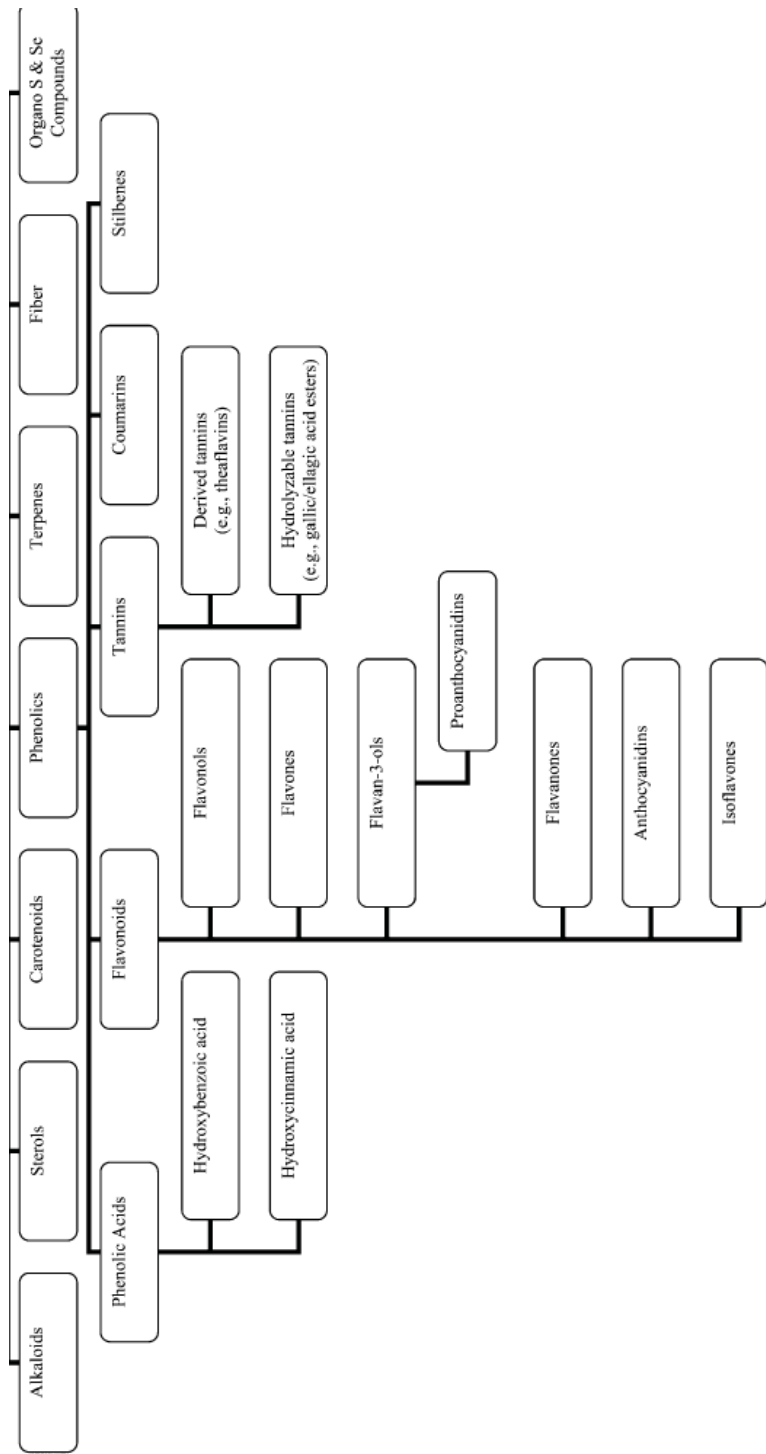
2.1.2 Structure

Phenolic Acids

The most common phenolic acids in plants are classified as either hydroxybenzoic acids which are derivatives of benzoic acids or hydroxycinnamic acids which are derivatives of cinnamic acids (Rice-Evans et al 1996). Some of the major hydroxybenzoic acids include salicylic acid, gallic acid and ellagic acid, and some major cinnamic acids include p-coumaric acid, caffeic acid and ferulic acid (Szajdek & Borowska, 2008). Commonly these compounds are present in esterified forms (Rice-Evans et al 1996). For example, ellagic acid is commonly esterified to glucose forming ellagitannins, which are also classified as hydrolyzable tannins. Caffeic acid is commonly esterified to quinic acid forming chlorogenic acid (Szajdek & Borowska, 2008; Rice-Evans et al, 1996). In berry fruits glycosides and esters account for 57% and 40% of total phenolic acid derivatives respectively, while whole free acids only account for around 3% (Howard & Hager, 2007).

Flavonoids

Flavonoids are a chemical class of secondary plant metabolites which are defined as having fifteen carbons, comprised of two phenolic compounds connected by a three carbon bridge C₆-C₃-C₆ (Crozier et al, 2006). Because they contain more than one phenolic ring they can also be classed as polyphenols. Within flavonoids are several major sub-classes each with its own distinct chemical structures. Figure 2-1 by Erdham et al (2007) outlines the main structural differences between these compounds and their major dietary sources.









Flavonoid subclass	Chemical structure	Prominent food flavonoids	Common food sources
Flavonols		Isohammetin, kaempferol, myricetin, quercetin	Onions, curly kale, leeks, broccoli, blueberries, cherry tomatoes, red wine, tea
Flavones		Apigenin, luteolin	Green leafy spices (e.g., parsley)
Flavanones		Eriodictyol, hesperetin, naringenin	Citrus fruits
Isoflavones		Daidzein, genistein, glycitein	Soybeans, soy foods, legumes
Flavan-3-ols		(+)-Gallocatechin, (+)-gallocatechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin 3-gallate, (-)-epigallocatechin 3-gallate	Teas, red grapes, red wines, cocoa, chocolate, apricots
Anthocyanidins		Cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	Red, purple, and blueberries; black grapes, leafy and root vegetables

Figure 2-1: Classification of major phytochemicals (Erdman et al, 2007).

There are only six anthocyanidins which are found commonly in nature, as shown in Figure 2-2. Furthermore, most anthocyanidins occur as glycosides in plants, where the anthocyanidin unit (the aglycone) is attached to one or more sugars (mono, di and tri saccharides) (Skrede & Wrolstad, 2002; Wu et al, 2006). This glycosylated form of the anthocyanidin is called an anthocyanin (see figure 2-3). The most prevalent sugars are glucose, galactose, rhamnose, arabinose, rutinose, sambubiose, and sophorose (Szajdek & Borowska, 2008). Additionally the sugar may be acylated by organic acids, in which case the compounds are considered acylglycosides of anthocyanidins, or acylated anthocyanins (Wang et al, 1997). These derivations are important to note as research suggests that different anthocyanins have different responses in terms of their stability, bioavailability and potential health effects, including acylation vs. non-acylation (Wu et al, 2006).

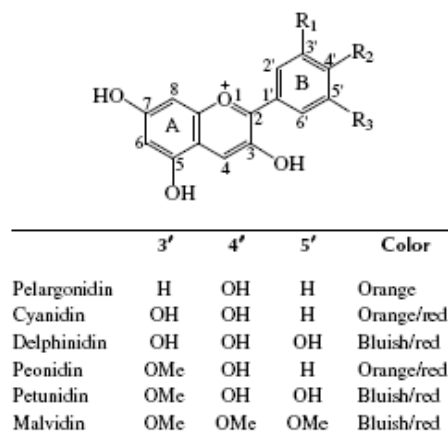


Figure 2-2: Structures of anthocyanidins occurring in grapes and berry fruits (Skrede & Wrolstad, 2002).

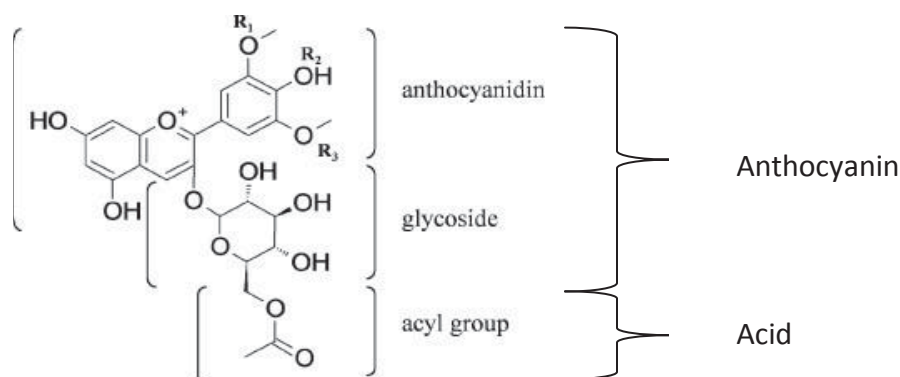


Figure 2-3 The skeletal structure of an acylated anthocyanin malvidin-3-(6''acetoyl)glucoside (Adapted from Barnes et al, 2009).

The two chemically simplest flavan-3-ols are the optical isomers catechin and epicatechin. In fact, the molecule contains two chiral carbons, resulting in four diastereoisomers; (+) and (-) catechin in the trans configuration and (+) and (-) epicatechin in the cis configuration. In nature the most common catechin isomer is (+)-catechin and conversely the most common epicatechin is (-)-epicatechin; the other two isomers are comparatively rare (Crozier et al, 2006).

Not listed separately in this table but worth highlighting are the procyanidins which sit within the class flavan-3-ols. These compounds are of much higher molecular weight than the other flavonoids as they can comprise anywhere between two and fifty units, which are primarily the flavan-3-ols epicatechin and catechin. An alternative classification for procyanidins is tannins as they are used by the leather industry to tan animal hides. However, they are referred to as condensed tannins as unlike hydrolysable tannins these compounds cannot be separated by acid hydrolysis.

Stilbenes are a class of phenolic compounds separate from flavonoids with a C₆-C₂-C₆ structure (Crozier et al, 2006). One particularly important stilbene is resveratrol which was initially found in grapes and has been associated with the potential health benefits from drinking red wine (Gould et al, 2009; Szajdek & Borowska, 2008).

2.1.3 Occurrence of Phytochemicals

Role in Plants

As mentioned previously phytochemicals have a diverse range of important physiological functions in plants. For example all colours found in the plant kingdom are derived from four pigment groups – chlorophylls, carotenoids, betalains and anthocyanins (Gould et al, 2009). Anthocyanins are responsible for the red, blue and black hues found in flowers, fruits vegetables. The word anthocyanin is derived from Greek dialect where the word *anthos* is flower and *kyanos* is blue. The pigments exist primarily in the skin of fruits and therefore play a definitive role in pollination and seed dispersal by attracting animals (Wu et al, 2006). Furthermore, they can protect the fruit tissue from damage caused by exposure to high levels of visible light and may have a role in plant resistance to insect attack (Gould et al, 2009). Due

to their phenolic structure they can also act as an antioxidant within the plant, scavenging free radicals.

Another type of phytochemical common throughout the plant kingdom is tannins. They have an astringent, bitter plant taste and are therefore attributed to providing a defence against predators (Crozier et al, 2006). Within the plant they are able to precipitate proteins and various other organic compounds including amino acids and alkaloids, a function which is required upon cellular death.

Synthesis in Plants

The synthesis of phytochemicals is of course dependent on the biological attributes and maturity of the plant. However, in addition agronomical factors such as sun/wind exposure and soil quality can have an influence on the phytochemical profile (Pascual-Teresa & Sanchez-Ballesta, 2008). This makes sense when considering the physiological roles of the compounds. For example the synthesis of anthocyanins is particularly dependent on light, where high levels of exposure have been shown to produce higher anthocyanin levels in a number of different fruits (Gould et al, 2009). Low temperatures have long been considered to promote, and high temperatures to reduce, anthocyanin synthesis. Furthermore, nutrient deficiencies, especially of phosphorus and nitrogen, commonly induce the accumulation of anthocyanins in many plant species (Pascual-Teresa & Sanchez-Ballesta, 2008).

The synthesis of flavonoids, the largest class of phenolic phytochemicals, is complex and involves a number of different plant enzymes. Crozier et al (2006) outlines the known synthetic pathways of flavonoids. The C₆-C₃-C₆ skeleton structure is a product of reactions between compounds from two separate biosynthesis pathways, malonyl-Co A and coumaroyl-Co A. An important intermediary in the pathway is the flavanone naringenin. From the production of this compound several side branches exist, resulting in the synthesis of flavones, isoflavones, and flavonols, the latter which progress to produce anthocyanins and flavon-3-ols.

2.1.4 Phytochemical Diversity in Berries

Table 2-1 adapted from Beattie et al (2005) summarises the main phytochemicals found in various berry fruits; anthocyanins are displayed in red, flavonols in blue and other flavonoids including phenolic acid derivatives are listed in black. In addition any compounds which are found to be comparably abundant are highlighted in bold. It can be seen that there is a large variation in the phytochemical profile amongst different berry fruits. However this is not surprising from a genetic perspective, as the genus and species of berries is also seen to vary greatly.

Table 2-1: Phenolic diversity of various berry fruits. Adapted from Beattie et al (2005).

<i>Common name</i>	<i>Family</i>	<i>Genus and species</i>	<i>Phenolics</i>
Blackcurrant	Grossulariaceae	<i>Ribes nigrum</i>	Del-3-Rut; Cy-3-Rut; Del-3-Glc; Cy-3-Glc Peo-3-Rut, Mal-3-Rut, Mal-3-Rut, Mal-3-Glc, Myr-3-Rut, Myr-3-Glc, Q-3-Rut, K-3-Glc
Redcurrant	Grossulariaceae	<i>Ribes rubrum</i>	Cy-3-Glc-Rut, Cy-3-Soph, Cy-3-Glc, Cy-3-Xyl-Rut, Cy-3-Rut
Strawberry	Rosaceae	<i>Fragaria x ananassa</i>	Pel-3-Glc, Cy-3-Glc, Pel-3-Ara Ellagic acid
Blackberry	Rosaceae	<i>Rubus spp.</i>	Cy-3-Sop, Cy-3-Glc-Rut, Cy-3-Glc, Cy-3-Rut, Q-3-Gal, Q-3-Glc, Q-3-Rut, Q-3-XylGlcAC, lambertianin C
Red raspberry	Rosaceae	<i>Rubus idaeus</i>	Cy-3-Sop, Cy-3-Glc-Rut, Cy-3-Rut, Cy-3-Glc, Pel-3-Sop, Pel-3-Glc-Rut, Cy-3,5-DiGlc, Cy-3-Samb, Pel-3-Glc, Pel-3-Rut, Q-3-Rut, Q-3-Glc, Q-3-GlcAC, sanguin H-6, lambertianin C
Blueberry	Ericaceae	<i>Vaccinium corymbosum</i>	Del-3-Gal, Del-3-Glc, Cy-3-Gal, Del-3-Ara, Cy-3-Glc, Pet-3-Gal, Cy-3-Arab, Pet-3-Glc, Peo-3-Gal, Pet-3-Arab, Peo-3-Glc, Mal-3-Gal, Peo-3-Arab, Mal-3-Glc, Mal-3-Arab, caffeoylquinic acids, Q-3-Gal, Q-3-Glc, Q-3-Rut
Cranberry	Ericaceae	<i>Vaccinium macrocarpum</i>	Cy-3-Gal, Cy-3-Ara, Cy-3-Gal, Cy-3-Ara, Myr-3-Gal, Q-3-Gal, Q-3-Rham
Elderberry	Caprifoliaceae	<i>Sambucus nigra</i>	Cy-3-Samb-5-Glc, Cy-3,5-DiGlc, Cy-3-Samb, Cy-3-Glc

This summary is not completely comprehensive; it reflects the majority of phytochemicals consistently reported for various berry fruits. For example some cultivars of blueberry are known to contain a number of acylated variations of the anthocyanins listed here. However, it

does illustrate that not all berries are created equal. Blueberries are reported to contain high levels of anthocyanins, procyanidins and chlorogenic acid (Howard & Hager, 2007). Most notably in comparison with other foods, and even other berry fruits, blueberries contain an extremely diverse range of different anthocyanins. Including acylated derivatives, twenty five anthocyanin compounds have been found in blueberries, the largest variety known for any berry fruit (Howard & Hager, 2007). They also contain a higher level of flavonols as compared with raspberries and strawberries but similar levels to blackberries. Howard & Hager (2007) report quercetin is the most abundant flavonol in blueberries; fourteen different quercetin derivatives have been identified along with three myricetin derivatives. The hydroxycinnamic acid derivative chlorogenic acid (the ester formed between caffeic acid and (L)-quinic acid) is also found abundantly. Blueberries have been shown to contain some procyanidins (condensed tannins) consisting of exclusively (epi)catechin units. Blueberries are reported to have much higher procyanidin levels than blackberries and raspberries but similar levels to strawberries (Howard & Hager, 2007). However, unlike raspberries, strawberries and blackberries, blueberries do not usually contain measurable levels of ellagitannins (hydrolysable tannins) and hence contain very low levels of ellagic acid. It is believed that they lack genetic capacity to synthesize these compounds (Howard & Hager, 2007). Additional phytochemicals detected in blueberries include very small amounts of lignins, and some amounts of sterols and stilbenes (Howard & Hager, 2007).

The level of chlorogenic acid in blueberries is particularly high as compared with other food sources. The online database Polyphenol Explorer (French National Institute for Agricultural Research. (2009). *Polyphenol Explorer*. Retrieved from <http://www.phenol-explorer.eu/compounds>) reports that blueberries have the fourth highest level of chlorogenic acid on a weight basis from foods within their database, with 1,312 mg/kg, as compared with other plant derived foods in the database.

2.1.5 Phytochemical Diversity of Blueberries

Over the past century many different varieties of blueberry have been cultivated in different parts of the world. There are four species of blueberry cultivars including Lowbush blueberries (*Vaccinium angustifolium*) which are traditionally considered 'wild' blueberries,

Northern Highbush (*V. corymbosum*) berries which constitute the majority of commercial plantations and Rabbiteye Highbush (*V. ashei*) and Southern Highbush (*V. darrowii*) blueberries which are valued crops in warm climates where other blueberries types fail to thrive (Trehane, 2004). Table 2-2 summarises the range of phytochemical concentrations reported in the four main blueberry types as reviewed by Howard & Hager (2007).

Table 2-2: Total contents of various phenolic compounds found in blueberries (mg/100g) (Howard and Hager, 2007).

Blueberry Type	Total Phenolics	Total Anthocyanins	Total Flavonols	Chlorogenic Acid	Number of Studies	Total number genotypes
<i>Lowbush</i>	295-495	91-260	NA	59-11	2	16
<i>Northern Highbush</i>	106-435	20-269	9-40	27-158	10	84
<i>Southern Highbush</i>	116-586	13-515	17-33	36-108	7	20
<i>Rabbiteye Highbush</i>	295-961	35-823	3-17	NA	3	57

From the data reported by Howard & Hager (2007) it appears that Rabbiteye blueberries and potentially Southern Highbush blueberries have much higher levels of anthocyanins and phenolics than Lowbush or Northern Highbush blueberries. This may be related to a smaller berry size. Conversely though, Northern Highbush berries are shown to have the largest range of chlorogenic acid concentrations. Other studies have also shown mixed results. Lohachoompol et al (2008) reported a statistically higher level of anthocyanins in Rabbiteye berries as compared with Northern Highbush. However, Prior et al (1998) tested various blueberry species and found that although phenolic levels were statistically different for Rabbiteye and Highbush blueberries there was no significant difference for anthocyanin levels or antioxidant capacity.

As indicated by the large range for each of the classes above, significant genetic diversity must exist within each species. This is particularly so within the Northern Highbush species which has been cultivated in many different parts of the world and therefore contains hundreds of different cultivars.

Ehlenfeldt & Prior (2001) carried out a screening exercise using eighty seven different blueberry cultivars. Phenolic contents and antioxidant capacities were measured. The values for eight Northern Highbush blueberry cultivars grown at Mamaku Blue, are shown in Table 2-3.

Table 2-3: Antioxidant capacity (ORAC), total phenolic and anthocyanin content for eight highbush blueberry cultivars (Ehlenfeldt & Prior 2001).

Highbush Cultivar	Berry Weight (g)	ORAC ($\mu\text{mol/g}$)	Phenolics (mg/100g)	Anthocyanins (mg/100g)
<i>Bluecrop</i>	1.9	10.4	48	182
<i>Brigitta Blue</i>	1.4	17.7	93	165
<i>Burlington</i>	1.8	26	700	175
<i>Dixi</i>	2.7	11.3	90	90
<i>Duke</i>	1.8	16.1	130	216
<i>Elliott</i>	1.8	30.5	184	311
<i>Jersey</i>	2.3	19.3	106	205
<i>Reka</i>	1.1	15.5	78	149

Blueberry cultivars can vary greatly in size. Therefore it would be logical that since anthocyanins are concentrated in the skin of the fruit that the total anthocyanins would increase in proportion to the calculated surface area/volume ratio. Prior et al (1998) demonstrated that this relationship was true for several blueberry cultivars. Conversely a more recent study by Kalt et al (2001) reported a lack of any relationship between fruit size and anthocyanin content.

In terms of the influence of agronomical factors, Howard et al (2003) documented significant differences in phenolic content for thirteen blueberry cultivars grown in three different locations during two different seasons. However, they determined that the environmental factors were much less influential than the biological variation observed between cultivars. Prior et al (1998) investigated agronomical effects using three different growing locations (Oregon vs Michigan vs New Jersey) for a single cultivar and determined that it did not have a significant impact on their measurements.

Furthermore, berry maturity has also been shown to have a large influence on the blueberry phytochemical profile. Prior et al (1998) reported an increase in antioxidant activity, total anthocyanin, and total phenolics during berry maturation.

2.1.6 Chemical composition of blueberries

The chemical composition of blueberries can be found in a number of text books. However, here the data was taken from the USDA Food Composition Database (United States Department of Agriculture. *Food Composition Database Release 22. Retrieved from <http://ndb.nal.usda.gov/ndb/foods/list>*).

This database is available online free to the public with over 7,000 foods listed. Data are based on standardised measurements performed in their own laboratories and outside laboratories where the methods meet certain quality assurance standards. Here the search term ‘blueberries’ was inputted and the option ‘blueberries – raw’ was selected.

Table 2-4: Chemical composition of fresh blueberry

Nutrient	Units	Value per 100 g	No. data points	Std. error
Water	G	84.21	12.00	0.67
Energy	kJ	240.00	0.00	0.00
Protein	G	0.74	12.00	0.02
Total lipid (fat)	G	0.33	12.00	0.02
Carbohydrate, by difference	g	14.49	0.00	0.00
Sugars, total	g	9.96	8.00	0.55
Fiber, total dietary	g	2.40	4.00	0.12
Ash	g	0.24	12.00	0.01
<i>Minerals</i>				
Calcium, Ca	mg	6.00	12.00	0.79
Iron, Fe	mg	0.28	12.00	0.01
Magnesium, Mg	mg	6.00	12.00	0.20
Phosphorus, P	mg	12.00	12.00	0.51
Potassium, K	mg	77.00	6.00	5.45
Sodium, Na	mg	1.00	6.00	0.35
Zinc, Zn	mg	0.16	12.00	0.02
Copper, Cu	mg	0.06	12.00	0.01

Manganese, Mn	mg	0.34	8.00	0.03
Selenium, Se	mcg	0.10	2.00	0.00
<i>Vitamins</i>				
Vitamin C, total ascorbic acid	mg	9.70	4.00	0.89
Thiamin	mg	0.04	12.00	0.01
Riboflavin	mg	0.04	12.00	0.00
Niacin	mg	0.42	12.00	0.09
Pantothenic acid	mg	0.12	12.00	0.01
Vitamin B-6	mg	0.05	12.00	0.00
Folate, total	mcg	6.00	12.00	0.12
Folic acid	mcg	0.00	0.00	0.00
Folate, food	mcg	6.00	12.00	0.12
Folate, DFE	mcg_DFE	6.00	0.00	0.00
Choline, total	mg	6.00	0.00	0.00
Betaine	mg	0.20	1.00	0.00
Vitamin B-12	mcg	0.00	0.00	0.00
Vitamin B-12, added	mcg	0.00	0.00	0.00
Vitamin A, RAE	mcg_RAE	3.00	0.00	0.00
Retinol	mcg	0.00	0.00	0.00
Carotene, beta	mcg	32.00	16.00	3.29
Carotene, alpha	mcg	0.00	12.00	0.00
Cryptoxanthin, beta	mcg	0.00	12.00	0.00
Vitamin A, IU	IU	54.00	0.00	0.00
Lycopene	mcg	0.00	6.00	0.00
Lutein + zeaxanthin	mcg	80.00	6.00	5.65
Vitamin E (alpha-tocopherol)	mg	0.57	4.00	0.10
Vitamin E, added	mg	0.00	0.00	0.00
Tocopherol, beta	mg	0.01	4.00	0.00
Tocopherol, gamma	mg	0.36	4.00	0.05
Tocopherol, delta	mg	0.03	4.00	0.01
Vitamin D (D2 + D3)	mcg	0.00	0.00	0.00
Vitamin D	IU	0.00	0.00	0.00
Vitamin K (phylloquinone)	mcg	19.30	8.00	1.55

This table gives an indication of the levels of macronutrients and micronutrients found in a blueberry and may be useful for rough comparisons with other fruits and vegetables. For instance from the same database it can be seen than a carrot contains approximately 7 mg/kg of vitamin C (data not shown) while blueberries contain approximately 10 mg/100 g. However, comparison should be made with caution; due to relatively small sample sizes these values will not be truly representative of each population.

2.1.7 Summary for Phytochemicals in Blueberries

The word phytochemical is a broad term used to represent any plant compound which may be beneficial to human health. Blueberries are abundant in two types of phytochemicals - anthocyanins of which there are up to fifteen unique anthocyanins and a further then acetylated variants and chlorogenic acid. This anthocyanin profile of the blueberry is dependant on variety. All anthocyanins and chlorogenic acid are classified as phenolic compounds, which refers to the carbon ring structures within the molecule. Anthocyanins are further classified as flavonoid due to their specific C₆-C₃-C₆ structure. The diversity of anthocyanin compounds is unique to the blueberry, even compared with other berry fruits which generally contain only 3 – 8 different anthocyanins. However, one common berry phytochemical which blueberries do not contain is ellagitannins - also referred to as hydrolysable tannins.

Several agronomical factors have been shown to influence anthocyanin levels in different plants. Light exposure has been shown to produce higher anthocyanin levels in a number of different fruits. Low temperatures have long been considered to promote, and high temperatures to reduce, anthocyanin synthesis. Furthermore, nutrient deficiencies, especially of phosphorus and nitrogen, commonly induce the accumulation of anthocyanins in many plant species. However, this information should be considered with caution since the findings do not relate specifically to blueberries.

Furthermore the evidence suggests that environmental factors are much less influential than the biological variation observed between blueberry cultivars. Hundreds of different blueberry cultivars exist throughout the world, which actually belong to different species of blueberry. The two most commercially common species are Northern Highbush (*V. corymbosum*) and Rabbiteye Highbush (*V. ashei*) with some areas also growing Lowbush

blueberries (*Vaccinium angustifolium*) (traditionally considered 'wild' blueberries) and Southern Highbush (*V. darrowii*) blueberries.

In accordance with a large amount of genetic diversity the anthocyanin and chlorogenic acid levels reported for different species of blueberry are varied significantly. Overall it does appear that the Rabbit-eye species have the highest anthocyanin levels on a weight basis. Some studies have reported a correlation between berry size and anthocyanin level, which seems logical and may relate to anthocyanin differences between species, where the average berry size may be different.

2.2 Potential Health Benefits of Blueberry Consumption

There has been a substantial amount of information in the public arena suggesting that the compounds found in blueberries are able to act against a wide variety of diseases such as cancer, cardiovascular disease, Alzheimer's disease, and urinary tract infections. These claims have been made on the basis of various pieces of scientific research which have been conducted *in vitro* (in a test tube) and *in vivo* (in the body). Such pieces of research may have many strengths and weaknesses; therefore health authorities are usually reluctant to support such claims without extensive evidence. Additionally more regulation has been developed in most western countries with regard to marketing and health claims. However, this should not deter the food industry from producing products which may potentially benefit the health of consumers. This section of the report attempts to summarise relevant research, discuss some of the strengths and weaknesses of this research and identify what is required in order to move towards a scientific consensus.

From a historical perspective the European blueberry (now referred to as the bilberry) was used as treatment for diarrhoea in adults, small children and animals, was believed to improve night vision and was used as a gargle for treating sore throats (Skreke & Wrolstad, 2002).

The phenomenon which led scientists to investigate potentially bioactive compounds in fruits and vegetables was the significant inverse association between the intake of fruits and vegetables and illnesses such as cardiovascular disease and cancer (Hocman, 1988; Cao et al, 1998; Szajdek & Borowska, 2008). Most fruit and vegetables are low in energy and fat while providing an abundant source of essential nutrients, fibre and antioxidants. In addition it was suspected that specific phytochemicals such as anthocyanins could protect individuals from degenerative diseases, in most part due to the French paradox; low incidences and mortality rates from heart disease and cancer were documented in France despite the fact that their saturated fat intakes, serum cholesterol, blood pressure, and prevalence of smoking were no lower there than elsewhere (Rice Evans et al, 1996). Scientists attributed this phenomenon to high red wine consumption, since the red skin pigments contained relatively high levels of anthocyanins. Later it was discovered that red wine also contains a large amount of the non-

phenolic phytochemical resveratrol (a stilbenoid) which may also have protection potential (Erdman et al, 2007).

The increasing incidence of cardiovascular disease and cancer prompted researchers to probe into additional high flavonoid fruits and vegetables, with inherently high antioxidant capacities. Developments in technology allowed researchers to identify compounds that contributed to antioxidant capacity apart from well-established antioxidants (vitamins C and E), such as flavonoids and resveratrol in the example above. If dietary antioxidants have the potential to play a large role in disease prevention then various berries which contain high levels of a diverse range of flavonoids and vitamins may contribute significantly. In a blueberry the major contributors to its high antioxidant capacity are vitamin C and phenolic compounds, predominantly chlorogenic acid and a diverse range of anthocyanins. Plant polyphenols are able to act as antioxidants through a number of mechanisms, including acting as reducing agents, hydrogen donating antioxidants, single oxygen quenchers and potentially metal chelators (Kahkonen et al, 1999; Rice-Evans et al, 1996). In addition scientists have documented other disease prevention mechanisms, such as anti-inflammatory and cell regulatory effects for many polyphenols (Beattie et al, 2005). Through the activation of genes containing “antioxidant response elements” the production of protective enzymes (for instance phase II enzymes which remove toxins from the bloodstream) could be increased. In this instance some specific phytochemicals may literally switch on the body’s natural defence systems. Importantly this “dietary hormone” effect is likely to occur at concentrations several orders of magnitude lower than concentrations required for “antioxidant” effects. The ultimate goal for scientists is to establish a dose dependent relationship between specific compounds within a food and treatment and/or prevention of a disease.

2.2.1 Antioxidant Power

Human cells are continuously exposed to a wide array of oxidising species, some of which are necessary for life. However, oxidative stress can cause damage to large biomolecules such as lipids, proteins, and subsequently DNA. Over time damage may result in development of risk of various conditions, including cardiovascular disease and cancer (Erdman et al, 2007). Therefore, in order to maintain optimal physiological condition the body strives to maintain a

balance of oxidants and antioxidants (Liu et al, 2004). The relationship between the two is as follows. Molecules such as free radicals (oxidants) are missing an electron and want to "steal" electrons from compounds. When a free radical (oxidant) steals an electron from another compound, that compound then becomes a free radical (oxidant), and a chain reaction occurs leading to cellular damage. However, antioxidants are able to donate electrons to oxidants such as free radicals, thereby stopping the chain reaction.

Antioxidant capacity can be measured in either aqueous (water) or lipid environments. Additionally different types of radicals are used in different types of antioxidant tests. Generally speaking in an antioxidant test the more radicals a food can absorb the higher its antioxidant value. For example the Oxygen Radical Absorbance Capability (ORAC) test uses an oxygen radical. The more oxygen radicals a food can absorb, the higher its ORAC value. From a human health perspective the usefulness of these measurements are limited, since they are an *in vitro* assay and therefore do not take bioavailability in account.

However, the measurements still are popular particularly for making comparisons between different foodstuffs. Blueberries are reported to have one of the highest antioxidant activities in a number of studies comparing different fruits and vegetables such as that of Cao et al (1998). Many different compounds can contribute to the antioxidant capacity. For blueberries various research groups have shown a significant correlation between antioxidant capacity and both total phenolic content and total anthocyanin content (Cao et al, 1998). For example Prior et al (1998) reported R values of 0.77 and 0.92 and 0.83 between ORAC and anthocyanins and ORAC and phenolics respectively while Kalt et al (1999) reported R values of 0.90 and 0.83 between ORAC and anthocyanins and ORAC and phenolics respectively. Although fresh blueberries can also contain reasonable amounts of vitamin C, a much lower contribution to antioxidant capacity is usually reported as compared with the phenolic compounds (Prior et al, 1998; Kalt et al 1999, Borges et al 2010).

In terms of the antioxidant activity of individual anthocyanins a number of different relationships are reported. Firstly the radical scavenging activity of the anthocyanidins (aglycones) is reported to be superior to that of their respective anthocyanins (glycosides). Additionally radical scavenging activity decreases as the number of sugar moieties attached to

the aglycone increases (Wang & Stoner, 2008). However, these relationships are not true in all cases. For example in a study by Wang et al (1997) glycosylation of the constituent anthocyanidin to their respective anthocyanin, increased, decreased, or did not have a significant effect on the ORAC activity of the aglycones in their study. Additionally the importance of the hydroxyl groups on both the 3' and 4' positions of the B ring in contributing to a high antioxidant capacity has been reported by various researchers (Rice-Evans et al, 1996; Wang et al 1997). For example Wang et al (1997) observed that cyanidin which contains two hydroxyl groups at these positions had a much higher ORAC activity as compared with pelargonidin, malvidin, and peonidin. However, this particular study found that delphinidin, which also contains two hydroxyl groups at these positions was the exception to this rule. Furthermore, different sugar molecules have different effects on the antioxidant capacity depending on the constituent anthocyanin. Wang et al (1997) studied fourteen isolated anthocyanins using the ORAC assay. Kuromanin (cyanidin-3-glucoside) had the highest antioxidant capacity and pelargonin had the lowest. Borges et al (2010) who studied anthocyanin extracts from whole blueberries attributed most of the antioxidant activity to delphinidin-3-galactoside, cyanidin-3-galactoside, delphinidin-3-arabinoside, petunidin-3-galactoside, malvidin-3-galactoside and malvidin-3-arabinoside.

Wang et al (1997) highlighted the fact that other research groups had reported a different order of antioxidant activity of anthocyanidins and anthocyanins, which is likely due to the nature of the anthocyanins being tested (i.e. their source), concentration and the different types of antioxidant assays which were employed. One paper which highlights this well is Kähkönen & Heinonen (2003) who studied the antioxidant capacity contributions of anthocyanins at varied concentrations in different lipid antioxidant systems and found conflicting results within each.

2.2.2 Absorption and Metabolism

Understanding the absorption and metabolism of components is crucial in understanding their potential to benefit the human body. Food components can be absorbed intact, modified during absorption or pass through the gastrointestinal tract, unabsorbed, and be excreted. In order for biological changes to take place the compounds must be available in sufficient

concentrations within the blood (Walton, 2006). Furthermore, even when compounds are absorbed they may not necessarily be utilised before being removed from the blood and excreted, depending on other biological processes. The one potential exception to the requirement for sufficient absorption is dietary modification of populations of gut microflora, which may influence human health, as the compounds only need to be present in the gastrointestinal tract. Variations in absorption and metabolism will occur from person to person due to differences in diet and genetics (Koli et al, 2010). Ideally absorption in the gastrointestinal tract, metabolism, utilization, excretion, and a measure of efficacy should be incorporated into the definition of bioactivity. However, many researchers simply define it as the blood plasma concentration of a compound, which gives little indication of its impact on the human body (McGhie & Walton, 2007).

During the late 1990s and early 2000s a number of studies were conducted on fasting polyphenol absorption, particularly of anthocyanins, where subjects consumed a berry meal (after fasting) and the concentrations in their blood and/or urine were measured during subsequent hours. Reported levels of intact anthocyanins were extremely low, in many cases less than 0.01% of the ingested concentration (Cao & Prior, 1999; Mazza et al, 2002). However, the absorption of anthocyanins was seen to be relatively rapid, as concentrations peaked in blood and urine samples within a few hours of consumption (Del Bo et al, 2010).

Studies also began to report apparent metabolic conversion of anthocyanins (Erham et al, 2007) such as glucuronidation. The conversion of compounds to glucuronides increases their water solubility, thereby allowing them to more easily be excreted from the body. Glucuronides of anthocyanins were detected in rat blood plasma by Ichiyanagi (2005), who suggested that these metabolites were mainly produced in the liver, rather than by intestinal flora. Studies also reported the occurrence of methylated anthocyanins which had not been present in the original samples, indicating that methylation could occur in the body. These two metabolic pathways are both commonly reported for other flavonoids. Kay et al (2004) identified both methylated and glucuronidated anthocyanins in the blood plasma and urine of human subjects who consumed a chokeberry extract. These studies were also able to confirm that the kinetics of anthocyanin absorption in rodents were similar to humans, meaning that in theory rodents

are good candidates as a model biological system. Ultimately the results obtained in rodent studies would be directly transferable to the expected effects in the human population.

More recent animal studies have investigated the metabolism of berries after long term consumption, not just a single dose. Some researchers investigated whether anthocyanins could accumulate in tissues, including crossing the blood-brain barrier. Kalt et al (2008) found intact anthocyanins in liver, eye, and brain tissue of pigs supplemented with a moderate blueberry diet. Anthocyanins were also detected in tissues of the control group and therefore a clear relationship between dosage and accumulation could not be established. Additionally Talavera et al (2005) reported anthocyanin accumulation in the brain tissue of rats. This later observation may support suggestions that anthocyanins have neuroprotective effects (Kalt et al, 2008). Research by Del Bro et al (2010) provided a more in-depth investigation into the metabolism of anthocyanins, feeding rats a blueberry enriched diet for either four or eight weeks. In their study anthocyanins were detected in both urine and faeces samples of the supplemented groups, with highest levels at eight weeks as compared with the four week mark. No anthocyanins were detected in the blood plasma or other tissues, but the group suggested this could have been due to the fast metabolism of anthocyanins and the delay of three to four hours between consumption of the last berry meal and euthanization. Phenolic acids were also identified in urine and faeces samples. Interestingly the urinary excretion of hippuric acid increased significantly in blueberry supplemented groups after both four and eight weeks as compared with the control. Therefore the authors suggested that anthocyanin metabolism by the intestinal microflora to respective phenyl-alkyl acids, which were further metabolized to benzoic acid, could be a possible metabolism mechanism.

A limited number of long-term human intervention studies have been performed. In a study by Koli et al (2010) human subjects consumed a diet containing two servings of various berries every day for eight weeks, with an average berry intake of 160 g/day. The levels of phenolic acids and quercetin in blood plasma samples increased significantly in the berry group, as compared with the control group and baseline.

Prior & Wu (2006) summarised five major attributes that appear to characterise anthocyanin bioavailability specifically:

1. Anthocyanins can be absorbed and excreted as intact molecules
2. Anthocyanins are rapidly absorbed and depleted from blood plasma and urine
3. The chemical structure of anthocyanins influences their absorption
4. Anthocyanins can be converted in the lower GI tract
5. Anthocyanin absorption can be affected by the food matrix

In their review of 97 flavonoid bioavailability studies Manach et al (2005) highlighted that fact that anthocyanins have a relatively low bioavailability as compared with other flavonoids. The mean maximum blood plasma anthocyanin concentration across studies was 0.03 $\mu\text{mol/L}$, whereas the blood plasma concentrations for other common phenolics was 0.26 $\mu\text{mol/L}$ for chlorogenic acid, 1.46 $\mu\text{mol/L}$ for quercetin glycosides, and 0.4 $\mu\text{mol/L}$ for (epi)catechin (based on an adjusted 50-mg dose of the aglycone). However, as has already been established, there are several probable conversions occurring to flavonoids in the body (including anthocyanins), such as methylation, glucuronidation and degradation to various phenolic acids. Unfortunately the biological properties of such phenolic metabolites have rarely been examined (Scalbert et al, 2002). It is still unclear what all the potential metabolites are, as there are likely to be more, what their relative proportions are or whether they are utilised by the human body. Therefore, it is not currently possible to establish whether the consumption of phenolic rich foods can have biologically significant outcomes.

More research needs to focus specifically on how effectively flavonoids, most importantly for blueberries anthocyanins, are extracted from the food matrix during transit through the gastrointestinal tract (Kalt et al, 2008; Walton, 2006). One potential solution is the use of isotopically labeled anthocyanins to better track their movements (McGhine & Walton, 2007; Manach et al, 2005). Such information will eventually help to establish a better relationship between phenolic consumption, bioavailability and biological interactions.

2.2.3 Evidence for Potential Health Benefits

Types of Nutrition Research

A large number of observational human studies have documented the fact that a diet high in fruit and vegetables can protect individuals against a number of age related diseases including cardiovascular disease, cancer, diabetes, Parkinson’s or Alzheimer’s disease (Szajdek & Borowska, 2008). The challenge for researchers has been determining whether individual components within fruits and vegetables such as antioxidants or fibre are responsible for this effect or whether other associated factors such as a lower energy, saturated fat, and meat intake are considerably more significant.

The process of nutrition research involves first studying disease distribution in human populations. Then, using this information in combination with laboratory based studies, potential dietary interventions and treatments can be identified. Rolfes et al (2009) outlines the different types of nutrition research along with their strengths and weaknesses Table 2-5.

Table 2-5: Types of nutrition research (Rolfes et al, 2009).

Type of Research	Strengths	Weaknesses
<i>Epidemiological (observational) studies</i> Cross sectional, case control and cohort. Determine the incidence and distribution of a disease in a population and match that incidence to broad variations in diet.	<ul style="list-style-type: none"> ○ Can narrow down the list of possible causes ○ Can raise questions to pursue through other types of research 	<ul style="list-style-type: none"> ○ Cannot control variables that may influence the development or the prevention of a disease ○ Cannot prove cause and effect
<i>Laboratory-based studies</i> In vitro (within the glass) or in vivo (in the living) animal. Explore the effects of a specific variable on a tissue, cell or molecule.	<ul style="list-style-type: none"> ○ Can control conditions ○ Can determine effects of a variable 	<ul style="list-style-type: none"> ○ Cannot always apply results from test tubes or animals directly to human beings
<i>Human intervention or clinical trials</i> Human beings are directed to follow a specified regimen and outcomes are measured or observed.	<ul style="list-style-type: none"> ○ Can control some conditions ○ Can apply findings to human beings 	<ul style="list-style-type: none"> ○ Cannot generalise findings to all human beings ○ Cannot use certain treatments for clinical or ethical reasons ○ Can be very expensive

The three main types of studies used to investigate the effects of foods and food components on biological systems are *in vitro* cell culture studies, *in vivo* animal model systems

and *in vivo* human intervention or trial studies (Wang & Stoner, 2008). *In vitro* cell culture tests are effectively 'test tube' studies which allow scientists to test specific biological mechanisms. However, these potential mechanisms are only the first part of the picture; researchers then need to demonstrate that their effect occurs in the body. *In vivo* animal model systems allow scientists to observe dietary intervention effects in a comparatively inexpensive model biological system while being able to submit the subjects to experiments which would be unethical in human studies. However, a rat or pig's biological processes are not exactly the same as a humans and their life span is significantly shorter. Furthermore animal experiments often accelerate disease progression using chemicals or specially bred transgenic variants which may involve mechanism that are to equivalent to those of disease development in humans. Therefore, *in vivo* human studies are required to prove that any effects observed in the preceding studies translate to real, relevant outcomes, and can ultimately be used to establish a dose dependant relationship using well documented biomarkers.

These tests may be carried out using either isolated compounds, or the whole food. Different fruits and vegetables contain a diverse range of different phytochemicals which are likely to interact with each other. For example berry extracts have been reported to display higher antioxidant activities than pure constituent phenolic compounds and vitamins (Szajdek & Borowska, 2008). Testing with isolated compounds has the advantage of being able to distinguish which compounds are responsible for a specific biological effect. On the other hand isolating a compound from its original food source means studies do not take into account any synergistic effects. Furthermore, measurements observed *in vitro* do not necessarily translate to *in vivo* studies. For example Wang & Stoner (2008) estimate that *in vitro* studies expose cells to anthocyanin concentrations in the range of 10^{-6} to 10^{-4} M, whereas levels of anthocyanins reported in human blood are generally in the order of 10^{-8} to 10^{-7} M. Therefore anthocyanins which contribute significantly to antioxidant power of berry fruits *in vitro* may not be well absorbed in the digestive tract and may therefore not be able to significantly influence antioxidant status *in vivo* (Hocman, 1989). However, Stevenson & Hurst (2007) suggest that additional protection mechanisms such as cell signaling effects may occur at concentrations much lower than those required for effective radical scavenging.

The most extensively studied high phytochemical foods are tea and grapes/wine, due to their commonality across cultures (Neto, 2007). However the mechanisms of action for individual phytochemicals in these foods are still far from clear. Many other foods have been researched by the scientific community but are even less well understood, including blueberries.

Cancer

It has been documented that the risk of cancer mortality in populations who consume large amounts of fruits and vegetables and small amounts of meat can be up to half that seen in populations with opposing lifestyles (Hocman, 1989). It is likely that this is due to both to a decrease in dietary carcinogens from meat and an increase in dietary phytochemicals and antioxidants from fruits and vegetables. Cancer arises from mutations in genes that control cell division which accelerate cellular growth or prevent cellular death, thereby allowing the proliferation of cells with genetic defects. There are many potential mechanisms of DNA damage including oxidative damage, chronic inflammation, and environmental chemical carcinogens (Rolfes et al, 2009). The human genome is made up of approximately 25,000 genes, of which at the present time more than 350 genes have been linked with any given cancer (Prasad et al, 2010). These genes may both up-regulate and down-regulate the production of undesired protein products. Combined with an incubation period of 20–30 years, cancer is an incredibly difficult disease to study (Prasad et al, 2010).

Wang & Stoner (2008) outline the different types of anti-carcinogenic mechanisms including antioxidant effects, which anthocyanins have demonstrated *in vitro*. This includes directly scavenging reactive oxygen species (ROS), increasing the oxygen-radical absorbing capacity of cells, stimulating the expression of Phase II detoxification enzymes, reducing the formation of oxidative adducts in DNA, decreasing lipid peroxidation, inhibiting mutagenesis by environmental toxins and carcinogens, and reducing cancer cell proliferation (by modulating signal transduction). Other phenolic compounds found in blueberries have also been reported to demonstrate the mechanisms described above. One of the fastest growing areas of cancer research is dietary influence on gene expression and subsequent cell signaling pathways. A

review paper by Prasad et al (2010) provides an outline of the specific cell signalling pathways which each of the flavonoids (including anthocyanins) have been shown to affect.

The consumption of extracts from various berry fruits has been shown to inhibit the development of cancer in carcinogen-treated animals and in animals with a genetic predisposition to cancer (Seeram, 2008). Reduction of cancer cell growth in animals consuming high anthocyanin diets was documented for skin, lung and throat cancer by various researchers (Wang & Stoner, 2008). In a study by Boateng et al (2007) rats were fed a diet containing either berry fruits (blueberries, blackberries, or cranberry juice) or other high-antioxidant fruits (pomegranate, watermelon, mangoes, and plum), and injected with a carcinogen (azoxymethane) three weeks into the trial. Rats on a berry supplemented diet had lower levels of cancerous colon cells compared to rats fed the control diet. Furthermore, among the rats fed selected fruits, those consuming blueberries showed a significant reduction in the formation of colon cancer cells compared with those consuming blackberries, plum or mango. Conversely a more recent study by Stoner et al (2010) reported that seven berry/fruit types (black raspberries, blueberries, red raspberries, strawberries, noni, goji berry and acai) were equally capable of inhibiting tumor progression in the rat esophagus in spite of the phytochemical diversity, suggesting some generic benefit rather than a link between specific compounds and chemoprotective action.

Results from some human observational studies on flavonoid intake and cancer have been mixed. Beattie et al (2005) reviewed the outcomes of seven large prospective cohort studies and four case-control studies which investigated the intake of flavonoids on cancer. Two observational studies observed a significant protective effect against lung cancer and one study for colorectal cancer. No evidence for an effect of flavonoid intake was seen for cancer of the stomach, urinary tract, prostate, or breast. Additionally the case-control studies of prostate, lung, testicular, and ovarian cancer did not identify significant associations for flavonoid intake (Beattie et al, 2005). A recent study was conducted by Rossi et al (2007) to determine if there was a relationship between flavonoid intake and oral or head and neck cancer risk, using data from a case-control study conducted in Italy between 1992 and 2005. Within the study there were 805 cases of oral, head or neck cancer and 2,081 patients with no incidence of cancer. The

researchers reported that an increased flavonoid intake showed an inverse association to oral and pharyngeal cancer which remained significant after adjustment for fruit and vegetable consumption. However, of the six major flavonoid classes tested no significant relationship was seen for anthocyanins specifically, only flavanones. In a recent, large prospective study, the European Prospective Investigation into Cancer and Nutrition (EPIC), carried out in 23 centers from 10 European countries with 519,978 subjects, found consumption of fruit was negatively associated with cancer of the lung but probably not with prostate cancer and breast cancer (Gonzalez & Riboli, 2006). A more recent analysis of EPIC data by Boffetta et al (2010) reported a very weak association between high fruit and vegetable consumption and decreased cancer risk overall.

Intervention studies investigating the effect of berry supplementation on cancer, including blueberries, have been inconsistent. A small intervention study was conducted by Duthie et al (2006) where twenty healthy middle aged women consumed 750 ml/day of either cranberry juice or a placebo drink for two weeks. The authors concluded that cranberry juice consumption did not alter blood or cellular antioxidant status and had no effect on base line or induced oxidative DNA damage. Conversely a study in Germany showed that dialysis patients who consumed 200 ml per day of mixed berry juice over several weeks, had reduced oxidative DNA damage and a significant increase in the antioxidant glutathione in blood plasma when compared to a control group (Spormann et al, 2008).

In review of these studies it appears that consumption of high flavonoid foods including blueberries may be effective against cancer. Although human intervention studies are difficult to perform for a disease with such a long incubation time and complex nature, many more are necessary if a dose dependent relationship is to be established between the consumption of specific high flavonoid fruits and cancer prevention or treatment.

Cardiovascular Disease

Cardiovascular disease is a broad term describing diseases of the heart and blood vessels. Atherosclerosis is the term referring specifically to disease of blood vessels, which can reduce and/or obstruct blood flow in the body. Blood deprivation suffered by the heart can result in a heart attack and similarly blood deprivation to the brain can result in a stroke (Rolfes et al,

2009). The main biological markers associated with cardiovascular disease which can be potentially influenced by the diet are the balance of low density lipoprotein (LDL) cholesterol and high density lipoprotein (HDL), regulation of blood flow (endothelial function), anti-inflammatory and antioxidant effects, platelet aggregation, blood pressure and gene expression. In cell culture studies anthocyanins have been demonstrated to be capable of influencing cholesterol oxidation and to act on different cells involved in the development of atherosclerosis (Pascual-Teresa & Sanchez-Ballesta, 2008).

Shaughnessy et al (2009) found that spontaneously hypertensive stroke-prone rats fed a 3% blueberry supplemented diet for eight weeks showed slower development of hypertension (high blood pressure) and reduced markers of renal oxidative stress as compared with a control group. In pigs, Kalt et al (2008) demonstrated that blueberry supplementation with 1, 2 and 4% blueberries resulted in a decrease in total, LDL and HDL cholesterol, the greatest reduction being observed at 2%. However, no effect on blood platelet activity or on DNA oxidation was observed for any of the treatments. The authors suggested that the 1-4% daily supplementation of blueberries would be achievable in a human diet as it would be equivalent to 1-4 cup servings. A diagram (Figure 2-4) presented by Meskin et al (2002) shows all the current suggestions for the actions which blueberries may have in preventing and treating cardiovascular disease.

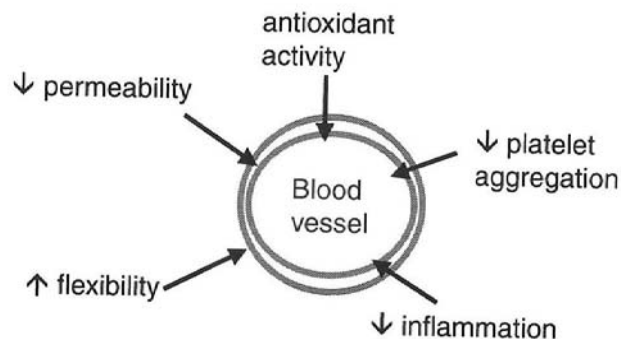


Figure 2-4: Potential cardiovascular benefits of blueberry phytochemicals (Meskin et al, 2002)

There is a lack of epidemiological studies relating consumption of anthocyanins or berries to disease risk specifically (Beattie et al, 2005). However, several large, long term observational studies, conducted throughout the US and Europe, have found an association between a high

intake of flavonoids and a decreased risk of cardiovascular disease. These studies were reviewed by Arts & Hollman (2005) and Beattie et al (2005) and include the 7-Countries study, Zutphen Elderly Study, Finnish Mobile Clinic Health Examination Survey, Iowa Women's Health Study, and Rotterdam Study. However, of the six major classes of flavonoids, comprehensive data were collected for only three of them - the flavonols, flavones and catechins. Many foods contain the flavonol quercetin, including blueberries which contain moderate amounts. In 2008, Hooper et al conducted a meta-analysis which aimed to review evidence for the effectiveness of different flavonoid subclasses and flavonoid-rich food sources on cardiovascular disease using information from the Cochrane Library, MEDLINE, and EMBASE. Such databases have been established to make information from randomised control trials free and available for researchers around the world. The risk factors that were evaluated in the randomised controlled trials included balance of lipoproteins, blood pressure, and flow-mediated dilatation and mortality. From the one hundred and thirty seven studies included in the review Hooper et al (1998) identified that dark chocolate, soy protein isolates and green tea consistently demonstrated statistically significant benefits to the various risk factors described above. For other food and flavonoid groups including anthocyanins and red wine there was insufficient evidence to draw conclusions. Similarly the group suggested that more research should focus on anthocyanins and flavanones specifically. Weaknesses in many of the current observational studies highlighted by both Hooper et al (2008) and Erdman et al (2007) are a lack of information relating to dose-response effects and the length of many studies not being long enough to allow assessment of clinically relevant endpoints. More recently a randomized placebo controlled intervention was published (Basu et al, 2010) which observed an improvement to some cardiovascular disease risk factors in 25 patients with metabolic syndrome who consumed a blueberry beverage daily, both at 4 and 8 weeks. The supplementation was seen to be effective in reducing hypertension (there was also some reduction for the placebo group but the blueberry group was statistically different) and lowering of baseline serum LDL cholesterol concentration. However, the supplementation did not affect fasting serum glucose, insulin, lipid profiles, body weight or waist circumference. The

dose administered to the blueberry group was equivalent to 50 g of freeze dried blueberry per day, or 350 g of fresh berries.

In addition to direct biomarkers of cardiovascular disease some research also suggests that anthocyanins may have the ability to combat other cardiovascular disease risk factors such as obesity and type 2 diabetes. These conditions alone can both have a severely negative impact on quality and duration of life. Only a small number of studies have been conducted on diabetes and blueberry supplementation. A study by Nettleton et al (2006) tracked over 35,000 post-menopausal women, five of whom developed type 2 diabetes over the eighteen year study period. No significant association was seen between berry consumption and the risk of developing type 2 diabetes. Conversely a recent human intervention study by Stull et al (2010) showed that daily consumption of whole blueberry bioactives for 6 weeks improved insulin sensitivity in a high risk population (for type 2 diabetes) as compared with a control group. The researchers were careful to prevent changes to participants' weight during the study, as adiposity can greatly alter whole-body insulin sensitivity. Although on average a beneficial effect was seen for the supplemented group the researchers did highlight significant variation within treatment groups. Interestingly, a slight increase in insulin sensitivity was also seen for the placebo treatment group.

Several studies using animal models have shown that long term blueberry consumption can reduce body fat in animals as compared with non-supplemented animals. Kalt et al (2008) found that pigs had a significant reduction in body fat when consuming a diet supplemented with 4% blueberries but not 1 or 2%. Prior et al (2008) found no effect on the weight gain of mice using 10% blueberry supplementation in combination with either a low fat or high fat diet. However, weight gain of mice fed a high fat diet was significantly lower when anthocyanin extract supplementation was employed rather than blueberry supplementation. In a subsequent study by the same group (Prior et al, 2010) blueberry juice and anthocyanin supplementation both produced a significant weight decrease in mice, but again anthocyanin supplementation was more effective. Additionally Prior et al (2010) suggested that research in this area appeared to exhibit a phenomenon where low doses of anthocyanins were effective in weight control but high doses were not. A paper published by Cho et al (2010) demonstrated

that mice on a high fat diet supplemented with chlorogenic acid, the second most abundant phytochemical in blueberries, had a lower body weight and improved regulation of lipid metabolism. This occurred even though the energy consumption of the supplemented mice was the same as the control group, suggesting that chlorogenic acid may be able to play an important role in weight loss.

Again, much relevant research has been conducted on consumption of flavonoid rich foods and cardiovascular disease. However, in most cases not enough information has been obtained to distinguish between specific high flavonoid foods. Currently there is insufficient information to determine whether consumption of blueberries can have a beneficial effect against cardiovascular disease.

Neuroprotection

Ageing related decline in cognitive performance and specific neurodegenerative disorders including Parkinson's disease, Alzheimer's and dementia are becoming a serious concern for health professionals as the world's ageing population continues to expand. Currently there is no cure for these diseases, and it is not clear when or if effective therapies will be developed (Krikorian et al, 2010). Again human observational studies suggest that a high consumption of fruits and vegetables presents a lower risk of neurodegenerative disorders and better cognitive performance in later life, most likely due to a decrease in the level of oxidative stress in brain regions (Krikorian et al, 2010; Galli et al, 2006). In animal and cell models, changes in neuronal lipid membranes, metabolic pathways, cellular Ca^{2+} homeostasis and neuronal signal transduction all appear to be affected by age and oxidative stress, which can result in neuronal damage and functional deficits (Joseph et al, 2010). Anthocyanins have been demonstrated to increase neuronal signaling in brain centers, mediate memory function and improve glucose disposal, benefits that would be expected to help prevent neurodegeneration in the human population (Krikorian et al, 2010).

A significant number of studies using blueberry supplementation in rodent models have shown reduced impairment to neurochemistry, synaptic transmission and behaviour (Malin et al, 2010). Galli et al (2006) demonstrated that blueberry supplementation in aged rats improved the response of heat shock proteins in an *in vitro* inflammatory challenge and concluded that

supplementation could result in protection against a number of neurodegenerative processes in the brain. Duffy et al (2008) found that an eight week dietary supplementation with blueberries in rats significantly protected against neurotoxicity caused by central injections of kainic acid. In this study rats consuming the blueberry enriched diet scored significantly better in a 14-unit T-maze test and were found to have reduced neuron loss upon examination of brain tissue. Similar improvements were seen by Joseph et al (2003) when transgenic mice consuming a blueberry supplemented diet displayed Y-maze performance similar to those seen in non-transgenic mice and significantly greater than that observed in non-supplemented transgenic group. More recently Malin et al (2010) published results comparing different lengths of blueberry supplementation on object recognition memory of aging rats. They reported that improved performance of rats was almost identical for either 1 month or 2 months of supplementation and also notably similar to the performance of rats maintained for 4 months on a blueberry diet in a previous study (Goyarzu et al, 2004), suggesting no increased benefit during supplementation depending on duration. However, after diet termination the researchers did observe a more prolonged performance benefit for rats supplemented for two months as compared with 1 month. Also notably Malin et al (2010) concluded that the blueberry supplementation had not just prevented age-related object memory decline in the rats but had actually reversed it.

It is still not clear specifically what parts of the blueberry may be responsible for these functions. Joseph et al (2010) investigated whether different fractions of blueberry could have a greater or lesser impact on recovery of Ca^{2+} , a mechanism which is thought to be related to the decreased in cognitive functions. However, the group reported that the whole blueberry extract was more effective than any fractions of the blueberry, where the chlorogenic acid fraction was found to be the least effective.

Letenneur et al (2007) reported that results of several human observation studies have found that a high flavonoid intake (not anthocyanins specifically) is associated with a reduced risk of Alzheimer's disease. In their own ten year study using 1,640 French males, 65 years and older, a high flavonoid intake was significantly associated with better cognitive performance throughout the ten year period. Potential memory enhancement using blueberry

supplementation was investigated by Krikorian et al (2010) on nine older adults presenting early memory changes. Participants consumed between 444 ml and 621 ml of blueberry juice per day depending on body weight, for twelve weeks. Significant improvements in paired associate learning and word list recall were observed. In addition, there were trends suggesting reduced depressive symptoms and lower blood glucose levels. However, interestingly, when an identical experiment was conducted using a berry placebo drink similar improvements were observed for the paired associate learning measurement.

Again, there does appear to be positive evidence that a high flavonoid intake can exert a protective effect against age related neurodegenerative disorders. However, it is not possible from this evidence to identify which specific compound(s) may be responsible for this effect, or consequently what specific foods are most beneficial.

Muscle Recovery and Performance

A recent article by Hurst et al (2010) reported a protective effect of blueberry extracts on oxidative stress on muscle cells. During exercise reactive oxygen species (ROS) are produced as a consequence of increased metabolism. These have the potential to contribute to tissue destruction which can result in decreased physical performance, particularly over long term accumulation (Hurst et al, 2010). Fractionation of the blueberry extract and identification of polyphenolic components suggested that the anthocyanin malvidin was primarily responsible for the observed protective effects *in vitro*. The researchers concluded the results warranted human intervention studies evaluating effects of blueberry supplementation in sports nutrition.

Alongside this, unpublished data from an honours project School from the School of Sport and Exercise, Massey University found that short term dietary supplementation with large quantities of blueberries (approximately 1kg per day) could improve muscle performance in human subjects (S. Stannard, personal communication, 2009). The information from this thesis may become available in the near future. This particular area of research appears to be worth further investigation as it may be a realistic target market for blueberry supplementation. However, currently there doesn't appear to be any well designed human intervention studies using blueberries specifically for this purpose.

Urinary Tract Infections

Cranberries have been used to prevent and treat urinary tract infections for decades and, like blueberries are members of the *Vaccinium* genus. The term urinary tract infection (UTI) is defined as the presence of a threshold number of bacteria in the urine (usually greater than 100,000/mL) causing pain and discomfort (Stothes, 2002; Jepson & Craig, 2007). Symptomatic infection of the bladder has been estimated to occur in up to 30% of women at some stage during their lives (Jepson & Craig, 2007).

One potential mechanism of prevention by consuming cranberries is the reduced adhesion of bacteria to cell walls, as without adhesion the infectious bacteria are flushed out and will not cause problems. Specifically fructose and procyanidins have been shown to reduce the adhesion of bacteria in cell adhesion tests (Stothes, 2002; Jepson & Craig, 2007). They also appear particularly effective against *E. coli* which is found in the urinary tract. Fructose is the sugar commonly found in fruits in combination with glucose, whereas procyanidins are high molecular weight flavanols consisting of polymer chains of smaller flavonoids (such as catechins). Schmidt et al (2004) fractionated a wild blueberry extract and demonstrated that the two high procyanidin fractions were able to significantly decrease adhesion of *E. coli*. They also reported that higher molecular weight procyanidins (tetramers through to higher polymers) are more potent in bacterial anti-adhesion assays than lower molecular weight procyanidins (dimers and trimers).

Unfortunately although reasonable evidence using human intervention studies exists for cranberries to prevent UTI, trials investigating blueberry supplementation are scarce. A meta-analysis to review the evidence from randomised controlled trials for cranberries and blueberries in UTI prevention was performed by Jepson & Craig (2007). To be considered in the analysis studies needed to use supplementation of either cranberries or blueberries for at least one month and include a comparison group, which could be placebo, no intervention, or any other intervention (e.g. antibiotics). There was sufficient evidence to conclude that cranberry supplementation could prevent UTI in women but no studies on blueberry met the inclusion criteria. Therefore, currently there is insufficient evidence to determine whether blueberries do have a significant protective effect against UTI.

Anti-nutritional Effects

There is a small concern that food components consumed in large quantities may have anti-nutritional effects such as inhibition of protein break down within the gut, reduced glucose uptake, impaired food utilisation, and impaired vitamin and mineral absorption. While some of these effects may be useful for parts of the population, such as reduced glucose uptake for weight control, effects such as reduced vitamin and mineral absorption would likely be an issue for a majority of the population. Erdman et al (2007) reported that myricetin, quercetin, and catechin have been demonstrated to have some of these effects when fed in large amounts to animals. Additionally drug interactions are possible, which may have serious consequences for individuals on medication to treat conditions such as cancer. Further research is required in this area, particularly if the scientific community moves to recommend that consumption of specific high flavonoid foods are beneficial for the treatment and prevention of various health conditions.

2.2.4 Recommended Intake

As outlined above, researchers have not yet established a dose dependent relationship between phytochemical consumption and the various health benefits that have been demonstrated. This is primarily because it is unclear what proportion of the phytochemicals in blueberries, particularly anthocyanins, which are most abundant, can be absorbed and metabolised by the human body upon consumption. Furthermore potential anti-nutritional or drug interaction effects of high flavonoid consumption have not been well researched by the scientific community (Erdman et al, 2007). Therefore dietary advice for the consumption of blueberries should currently be made on the basis of what is regarded as a high to average daily intake of anthocyanins.

Two pieces of information are crucial in establishing the dietary intake of individual food components in populations. Firstly, a large number of foods need to be chemically profiled to determine the average levels of various components. Secondly, detailed food frequency questionnaires need to be obtained from considerable numbers of individuals, with information specific enough to distinguish between the compounds of interest, taking into account additional factors such as seasonal variability where possible.

Hertog et al (1992) reported that total flavonoid intake had been estimated to be 1 g/day. However, more recent reports suggest that intake of all flavonoids is anywhere between a few hundred milligrams to 650 mg/day (Liu, 2004). Erdman et al (2007) presented a comprehensive review of estimated intakes for individual flavonoids including flavonols, flavones, flavanones, isoflavones flavan-3-ols, anthocyanins and procyanidins in Europe, US and Asia. The data reported an estimated anthocyanin intake of 6.5 mg/day in Germany and 1.3 mg/day in the US. However, the most recent review, by Wu et al (2006) estimated that the daily anthocyanin intake in the US was 12.5 mg/day. This calculation was based on anthocyanin concentrations they had measured in common foods along with food intake data from the US National Health and Nutrition Examination Survey (NHANES). With a majority of blueberry cultivars presenting anthocyanin levels between 100-250 mg/100 g the value presented by Wu of 12.5 mg/day would be easily exceeded by consuming less than ¼ of cup blueberries per day. Based on one cup being equivalent to 145 g, ¼ of cup blueberries a day would be almost 3-7 times the 12.5 mg of anthocyanins estimated as the US daily intake.

2.2.5 Summary of Potential Health Benefits

Even in this modern age many challenges exist for researchers in the field of human nutrition. When a complex chemical matrix (food) is introduced into an extremely complex biological system (the human body) it is difficult to study the interaction between these two variables without confounding effects. In human nutrition it is important that both a cause and an effect is established. Epidemiological (observation) studies alongside human intervention studies are used to establish an effect exists within a population while laboratory based studies demonstrate the cause at a cellular level. However, notably, the first two are comparatively much more expensive to carry out than the later.

Scientists became focused on investigating the compounds within blueberries after the development of antioxidant tests, which showed blueberries had a high antioxidant capacity *in vitro*. However, their *in vivo* effects continue to elude scientists. Even with a significant number of published papers there is still no coherent evidence that the compounds in blueberries have any significant benefits to the human body, other than that provided by most fruits or vegetables.

One of the cornerstone challenges in nutrition research is measuring the 'bioavailability' of a compound group, which is important for determining what is 'available' to interact with the body. Some researchers determine how much of a compound group is absorbed intact into the bloodstream over a period of time. In this respect scientists report the bioavailability of anthocyanins to be relatively poor, with the bioavailability of chlorogenic acid faring somewhat better. In this regard, the ability of anthocyanins to have any significant effect on the antioxidant status of the human body is unlikely. However, such a simplistic measurement does not account for any chemical transformations which may occur to anthocyanins or chlorogenic acid prior to, or during absorption. Most flavonoids, including anthocyanins, have been reported to undergo methylation, glucuronidation and breakdown to respective phenolic acids. To this day little work has been done to investigate the cellular effects of such resulting chemical products, although such research is difficult owing to the compounds being present at even smaller concentrations than their constituents.

A growing area in nutrition research is cell signalling effects, whereby dietary compounds may influence gene expression. The human genome is made up of approximately 25,000 genes, hundreds of which may be associated with any given disease, therefore again the task is problematic as many confounding effects arise.

Of the main disease areas evaluated here those which appear to show increasing potential are dietary supplementation with blueberries for neuroprotection and protection against cardiovascular disease, although again more human studies are required. Some potentially under-researched areas include protection from urinary tract infections and enhanced muscle performance from blueberry supplementation. Additionally these two areas are possibly more 'claimable' than other potential health benefits.

Nutrition research is far from providing a complete picture of the effects, positive and negative, which food can have on the human body. Although expensive, it appears imperative that more human intervention studies, specifically human randomised double blind control trials, are carried out to reach a scientific consensus for each potential health benefit of blueberry consumption.

2.3 Juice Manufacture and Storage

2.3.1 Chemical Stability of Anthocyanins

As a result of their antioxidant properties polyphenols (including anthocyanins, flavan-3-ols and phenolic acids) are highly reactive and therefore susceptible to degradation in different chemical environments (Skrede et al, 2000). From a processing point of view it is important to identify what factors may be the most critical to stability during various unit operations. Literature commonly focuses on the stability of anthocyanins in different chemical environments, particularly relating to changes in colour. Additionally, their degradation is usually reported to follow first order kinetics (Wang & Xu, 2007 & Patras et al, 2010).

Some of the specific factors that potentially influence anthocyanin degradation are discussed in the following sections.

Effects of Molecular Structure and Co-pigmentation

Blueberries are unique because in comparison to other berry fruits they contain a large number of different anthocyanin compounds. Degradation rates of individual anthocyanins have been seen to vary greatly, which is understandably related to their structure. Glycosylation confers increased stability and water solubility to the anthocyanins compared with the anthocyanidins, which is why anthocyanins are found more readily in nature (Bridle & Timberlake, 1997). An increasing number of sugar residues on the anthocyanin provide further stability. Additionally acylation (such as by caffeic acid) stabilises the anthocyanins against pH, heat and light (Skrede & Wrolstad, 2002).

Anthocyanins can also form intermolecular structures with flavonols, quercetin and rutin, other anthocyanins and metal ions, which are generally more chemically stable than the constituent anthocyanin (Skrede & Wrolstad, 2002; Patra et al, 2010). Furthermore, at high concentrations, the anthocyanins may associate with themselves, increasing their stability in solution. In some cases this effect has been shown to be more important than differences in anthocyanin structure (Skrede & Wrolstad, 2002).

Effect of pH

In isolation, anthocyanins have little colour above pH 3.5, but in natural media they become much more coloured by copigmentation with other plant components (Bridle & Timberlake, 1997). The primary, pH-dependent, anthocyanin equilibrium structure is the flavylium cation, from which secondary structures are derived either by proton transfer or hydration. Proton transfer results in the production of bluish quinonoidal bases and corresponding ionised quinonoidal bases whereas hydration produces colourless carbinol pseudobases (Bridle & Timberlake, 1997). When colour is a concern in anthocyanin pigmented products the food should have a pH value below 2-5 (Skrede & Wrolstad, 2002).

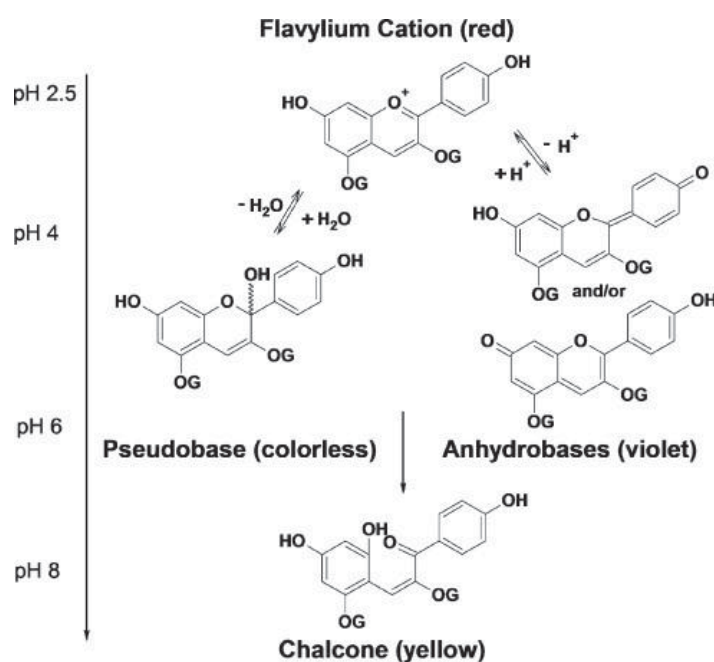


Figure 2-5: Variation in the anthocyanin structure and pigmentation with changes in pH of a solution (Barnes et al, 2009).

As described above acylation can improve the pH stability of the anthocyanin. Bridle & Timberlake (1997) report that acylation hinders hydrolysis of the red flavylium cationic structure to the colourless carbinol base, allowing preferential formation of the blue quinonoidal bases, thereby resulting in pigments that are less sensitive to pH changes.

Effect of Oxygen

Oxygen has been shown to have an adverse effect on anthocyanins. For example it is known that anthocyanins stored under vacuum or in nitrogen are more stable than when exposed to oxygen (Skrede & Wrolstad, 2002). The oxygen may degrade anthocyanins either directly or indirectly by oxidizing compounds, which in turn, may degrade the anthocyanins. For example when both oxygen and vitamin C levels are high more anthocyanin degradation has been documented. It is thought that hydrogen peroxide is formed by the oxidation of ascorbic acid which can then react with the anthocyanins (Skrede & Wrolstad, 2002).

Effect of Light

Although exposure to light enhances biosynthesis in nature, outside the plant both visible and UV light are detrimental to anthocyanins and have been shown to increase the rate at which anthocyanins undergo thermal degradation (Jackson & Smith, 1996). For example Palamidis & (1975) reported that anthocyanins in grape beverages had a half-life of 416 days in the dark as compared with 197 days in daylight at 20°C. Additionally, Skrede & Wrolstad (2002) report that shorter wavelengths, such as UV light, are more detrimental than longer wavelengths such as visible light.

Effect of Temperature

It is commonly reported that temperature has a negative impact on anthocyanin stability, both during processing and during storage. For strawberry preserves, it was shown that the half-lives of anthocyanins were 1 h at 100°C, 240 h at 38°C and 1300 h at 20°C (Skrede & Wrolstad, 2002). Therefore high-temperature short time processing is recommended for maximum anthocyanin retention of foods containing anthocyanins (Jackson & Smith, 1996). Various experiments have also reported that anthocyanins are also better retained at cooler temperatures during storage.

Effect of Plant Enzymes

Blueberries are known to contain native plant enzymes which can oxidise phenolic compounds, including anthocyanins. These reactions are commonly referred to as enzymic

browning reactions, as the resulting compounds are brown in colour. Using sensitive detection methods Kader et al (1997a) isolated the enzyme polyphenol oxidase (PPO) from blueberries. In the plant this enzyme functions to polymerise phenolic compounds (such as flavonoids and chlorogenic acid) in the presence of oxygen which of course occurs in the event of injury to tissue. The polymers seal the damaged tissue and deter insect feeding, as the compounds are brown and unpalatable (Stevenson & Hurst, 2007). The two main polyphenol oxidases are catechol oxidase and laccase (Adams, 1991). Polyphenol oxidase does not react with anthocyanins directly, but first reacts with chlorogenic acid to give a p-quinone which has been shown to be responsible for the degradation of anthocyanins to brown pigments (Kader et al, 1997b). Kader et al (1997a) reported that the activity of the enzyme was high at low pH with an optimal pH of 4.0. Therefore the enzyme has the potential to have a significant impact on blueberries, which are inherently low pH foods. Adams et al (1991) report that the most extensively studied polyphenol oxidase, catechol oxidase, only exhibits significant inactivation rates at temperatures greater than 65°C and that degradation of PPO generally follows first order kinetics.

Blueberries are also known to contain the enzyme peroxidase (POD), which can also oxidise phenolic compounds in the presence of hydrogen peroxide (Kader, 1997b). Kader et al (2002) demonstrated that the addition of hydrogen peroxide to blueberries led to anthocyanin degradation. Furthermore, the researchers found that PDO, like polyphenol oxidase, oxidise chlorogenic acid to give a p-quinone which can react with anthocyanins (Kader et al, 2002). However, due to the low levels of hydrogen peroxide in most foods, its activity is usually limited (Kader et al, 1997b).

2.3.2 Unit Operations in Juice Manufacturing

Juice production involves the extraction of water and water soluble compounds resulting in a liquid product. The basic steps for manufacturing juice are common across a wide range of fruits and vegetables:

- ***Pre-treatments*** - Breakdown of the cell tissue to release water and obtain maximum juice recovery, through combination of heating, crushing and enzymes.

- **Juicing** - Separation of solid material from juice, achieved through pressing or centrifugation.
- **Pasteurisation** - Thermal treatment of the juice to destroy pathogenic and spoilage micro-organisms.

Each of these operations is covered in the following sections as they relate to blueberry juice manufacture.

Pre-treatments – Freezing, Blanching, Milling and Enzyme treatment

Pre-treatments can be employed to optimise juice extraction and improve final product quality. There is no strict definition for describing a processing step as a pre-treatment; generally any operation employed before juicing can be described as one.

Freezing & Thawing

Temperature adjustment is probably the most commonly employed pre-treatment, most significantly freezing of fruit. This has two potential advantages: firstly the fruit can be stored for an indefinite period and juice produced as required and secondly the formation of ice causes destruction of fruit tissue and results in significantly higher juice extraction (Stewart, 2005). Ashurst et al (1995) classified freezing into four groups according to the method employed and the relative amount of time taken to completely freeze the fruit. These are outlined below;

- **Stored freezing:** Full trays of fruit are frozen at a temperature of approximately -22°C. Depending on factors such as field heat and volume of fruit, surface freezing will usually occur within 24-36 hours and complete freezing of the fruit will take 3-4 days.
- **Blast freezing:** Similar to above, but air is directed around the trays, meaning the fruit can freeze completely within 10 hours.
- **Belt Freezing:** The fruit moves along a perforated belt which takes 20-30 mins to pass through a refrigeration unit. The surface is frozen by the time the fruit emerges.
- **Nitrogen Freezing:** This method is reserved for high value crops at low volume as it is significantly more expensive. However, due to the much faster freezing there is less opportunity for enzymic breakdown.

Milling

Physical size reduction of fruit before juicing through milling, crushing or mashing is another method to achieve a higher juice extraction. Since blueberries are a soft fruit, milling equipment is usually a simple set of rollers or blades that basically crush the fruit (Stewart, 2005). Alternatively some form of crushing may be incorporated into other pre-treatments depending on the process design.

Heating

Heating can contribute to tissue breakdown, as high temperatures decrease the microbiological load during subsequent processing and most significantly deactivate plant enzymes which are known to contribute to fruit browning (Kader et al, 2002). In wine production, the process of milling and heating the grape mash is common practice and is described as hot breaking (McLellan & Padilla-Zakour, 2005). The grapes are milled and then heated to between 50 – 60°C using large bore, tubular heat exchanger before being pressed (Skrede & Wrolstad, 2002; McLellan & Padilla-Zakour, 2005). This process results in a higher juice recovery and better extraction of flavonoids. Another possible temperature treatment is blanching, where fruit is subjected to high temperatures, 95°C, for no more than a few minutes and then cooled quickly. In recent publications where blanching during juice manufacture was employed only 2-20 kg of blueberries were processed, therefore a steam jacketed vessel could be used to heat the fruit. However, this equipment could not achieve a high temperature in a short enough time on a large scale. Commercial blanching equipment which is generally designed for intact fruit could be employed. However equipment such as a traditional steam blanching tunnel would add water to the final product. Alternatively a shell and tube heat exchanger as described in hot breaking with a sufficient heating source could be used to heat the fruit to 95°C.

Enzyme Treatments

Commercial enzyme treatments can be employed to break down various structural components of fruits, such as pectin, cellulose and starch. However, the additional recovery must outweigh the cost of the enzyme. Cellulose and pectin are the primary components of cell

walls, consisting of long chain polysaccharides which are not known to contribute to human nutrition. High pectin levels are common in berry fruit which is useful in jam making but can gel and hinder extraction during juice manufacture, significantly reducing the yield of juice (Hohn et al, 2005). Therefore depectinisation enzymes are commonly employed. Conversely cellulose is tightly associated to cell walls and so is easily removed from the juice processing stream (Whitehurst & Oort, 2010). Additionally starch, which is the form of energy storage in unripe fruits, can be high in fruits such as apples but in berries it is not found in sufficient levels to be of concern (Whitehurst & Oort, 2010).

Commercial depectinisation enzymes are added to the blueberry mash on a percentage weight basis and the mash is then heated to the optimal reaction temperature. In wine making enzyme treatments are normally combined with the hot breaking process (McLellan & Padilla-Zakour, 2005). The treatment time is determined using a negative alcohol precipitation test. Hohn et al (2005) reported that for blackcurrant or cranberry pulp a one hour enzyme treatment at 55°C is widely practiced. However, as the anthocyanins are primarily located in the skin of the fruits it may also be advantageous to employ a cellulase enzyme, which would result in more breakdown of the fruit skin and potentially release more anthocyanins

Juicing

Juicing is defined as the initial physical separation of the insoluble solid material from the aqueous phase. The two common commercial methods of juicing are pressing and centrifugation.

The most common form of juicing is pressing. The basic principle is that a force is applied to the mash to extract as much liquid as possible from the fruit, while a physical filter is incorporated to separate a majority of the solid material from the juice. Press aids such as rice hulls or cellulose wood fibre may also be employed to improve juice recovery (Stewart, 2005). Ideally, a press aid should have relatively long fibres that can be separated with a minimum breakage and should neither impart off-flavours to the juice nor remove the fruit's flavour (McLellan & Padilla-Zakour, 2005). Grapes and berries are soft fruit and so require comparatively little force. Therefore a simple mechanical press such as a screw press which

incorporates plates on a rotating screw is able to provide satisfactory pressure for juicing (Ashurst, 1995).

Clarification

After juicing the liquid will still contain small solid particulates. These particulates cause cloud in some fruit juice which is considered undesirable in many markets. However, this is not such an issue with blueberry juice as the dark non-transparent colour is part of its attraction. Clarification can be employed either before or after pasteurisation to remove these smaller particulates. Ashurst (1995) outlines several different clarification methods which fall into three broad categories: decantation, centrifugation, and filtration.

Decantation simply involves holding the juice in a tank until solid particulates settle and then tipping off the juice above. Fining agents may be employed to settle the particulates at a faster rate. Centrifugation was one of the first methods suitable for a continuous process. The juice is passed through a spinning conical bowl and centrifugal forces separate the solids which are denser from the lighter liquid component. Filtration methods involve the use of a physical barrier with pores of a defined size through which only particles of a minimum size can pass. This allows for the separation of small solid particulates from the aqueous phase. There are a number of different types of filtration methods employed in the fruit juice industry, including earth filtration, rotary vacuum filtration, sheet filtration, cartridge filtration and membrane filtration. Refer to Ashurst (1995) for further detail.

Pasteurisation and Packaging

Berry juices are considered relatively acidic food products; the pH of whole blueberries is around 3-3.2 (Kader et al, 1997a). A minority of common food pathogens cannot proliferate in high acidic environments; only yeasts and moulds are of a significant concern. Therefore blueberry juices do not require extensive thermal processing from a microbiological point of view. However, if no prior heat treatment has been employed then pasteurisation is important for deactivation of fruit enzymes.

Pasteurisation conditions for fruit juices are generally between 85 - 95°C for 15 to 60 seconds, commonly referred to as flash pasteurisation (Ashurst, 2005). The heat exchanger

design can be either plate or tubular depending on the amount of solid material and typically incorporates heat recovery (Ashurst, 2005). Plate heat exchangers are generally preferable as tubular heat exchangers are less efficient because of their lower surface:volume ratios and therefore are more expensive to run in addition to their higher capital cost. The pasteurised product can either be cooled and packaged aseptically or hot filled. Aseptic filling requires specialised equipment which is able to fill the bottles under sterile conditions. Hot filling of bottles requires less capital expense but the types of suitable packaging are more limited, as the packaging needs to withstand filling at high temperatures. An alternative option is to use in-pack pasteurisation; however, again the right type of packaging needs to be used. In-pack pasteurization is achieved by simple immersion of the filled product into a series of tanks containing heated water, or the use of a commercial pasteurization tunnel. Typical pasteurization conditions are usually 70-75°C for up to 20 minutes (Ashurst, 2005).

Commonly large scale processors will also choose to concentrate the juice post pasteurization (although this does not seem to be the case for blueberry juice). Previous operations may have resulted in the addition of water to the system. Concentrate can be adjusted to a standardised solids concentration (°Brix) which is likely to be a requirement for wholesale. Furthermore, concentration can reduce transportation and storage costs when large quantities are handled.

2.3.3 Retention of Phytochemicals during Processing

This section of the report evaluates literature investigating whether juice manufacture affects the levels of the major blueberry phytochemicals and identifies which operations are the most influential. Subsequently the effect of post-manufacture storage conditions on the retention of these phytochemicals is also evaluated.

Types of Loss

There are two main types of losses to consider when investigating the effect of processing on individual phytochemicals. Firstly there is the potential for physical material losses when the process stream separates into two or more streams and some of the components of interest pass out of the primary product stream. Secondly there may be chemical losses when

compounds either are modified or degrade entirely. However, it should be remembered that potentially modified or degraded compounds may have different biological interactions in the human body than their parent compound. The most abundant phytochemicals in blueberries are phenolic compounds, namely the anthocyanins along with chlorogenic acid.

Juice Manufacture Trials – Retention of Phytochemicals

Over the past decade several researchers have carried out small scale manufacture of their own blueberry juice and measured the levels of various phytochemicals during processing. Manufacturing methods are summarised in Table 2-7. However, each group employed different processing methods and variations, making it difficult to assess what the optimal processing conditions are for phytochemical retention. Variations included chilled thawing and heated thawing, blanching and no blanching, sulphur dioxide addition, clarification and no clarification, different extraction and pressing methods and finally the use of a wide variety of non-commercial and semi-commercial equipment. Furthermore, many of the researchers failed to provide their mass balance data, so in some cases it is unclear whether they were reporting the measured concentration of their sample, or the concentrations were adjusted according to the mass ratios in different processing streams. However, for the purposes of the presented table (2-7) it was assumed that no adjustment had been made.

Nevertheless the data does present some clues as to which operations are potentially the most critical; the following sections look at the retention of key phytochemicals.

Anthocyanins

All research groups evaluated levels of anthocyanins using either the pH differential method (spectrophotometric method) or High Performance Liquid Chromatography (HPLC). The latter method is more time consuming and expensive particularly with regard to the equipment. However, it allows for characterisation of individual anthocyanin compounds which the pH differential method does not (Wu et al, 2006). Therefore, those who employed the pH differential method were able to only report the total levels of anthocyanins. Conversely those who used HPLC were able to identify individual anthocyanins and observe how the anthocyanin profile changed.

Table 2-6: Blueberry Juice Manufacture: Reported Methods

Paper	Operations									
	Sample	Thawing	SO ₂	Blanching	Blanching Equipment	Extraction	Pressing	Clarifying	Pasteurisation	Pasteurisation Equipment
Skrede et al 2000	26 kg	5 °C				2 h 43°C	5 Bar	Filter + water	1 min 90 °C	HTST Heat Exchanger
Lee et al 2002	20 kg	Heated 32 °C	~50 ppm			~27°C	5 Bar		1.5 min 90 °C	HTST Heat Exchanger
Lee et al 2002	20 kg	Heated 32 °C		2 min 95°C	Steam Kettle	~27°C	5 Bar		1.5 min 90 °C	HTST Heat Exchanger
Lee et al 2002	20 kg	Heated 32 °C				~27°C	5 Bar		1.5 min 90 °C	HTST Heat Exchanger
Rossi et al 2003	5 kg	12 h 5 °C		3 min 100 °C	Steam Blanching Tunnel	1 h ~22°C	8.8 Bar		1 min 90 °C	HTST Tubular Heat Exchanger
Rossi et al 2003	5 kg	12 h 5 °C				1 h ~22°C	8.8 Bar		1 min 90 °C	HTST Tubular Heat Exchanger
Srivastava et al 2007 ¹	2.1 kg	12 h 5 °C		3 min 100 °C	Boiling Water	1 h 22°C		20 min 9740 G	>2 min 85 °C	Unknown - Jacketed Vessel
Srivastava et al 2007 ²	2.1 kg	12 h 5 °C		3 min 100 °C	Boiling Water	1 h 22°C		20 min 9740 G	>2 min 85 °C	Unknown - Jacketed Vessel
Brownmiller et al 2008	Unknown	Heated to 95 °C		3 min 95 °C	Steam Kettle	1 h 40°C	Unknown		90 °C	Bottles in Steam Box
Brownmiller et al 2008	Unknown	Heated to 95 °C		3 min 95 °C	Steam Kettle	1 h 40°C	Unknown	10 min 6000 G	90 °C	Bottles in Steam Box
Sablani et al 2010 ³	Unknown	Fresh		2 min 95°C	Unknown					Stove Top
Sablani et al 2010 ³	Unknown	Fresh					Juicer			Stove Top
Sablani et al 2010 ⁴	Unknown	Fresh		2 min 95°C	Unknown		Juicer			Stove Top
Sablani et al 2010 ⁴	Unknown	Fresh					Juicer			Stove Top

¹ Tifblue cultivar, ² Powderblue cultivar, ³ Duke cultivar, ⁴ Reka cultivar

Table 2-7: Anthocyanin recovery during processing as reported by various research groups.

Paper	Operations					
	Sulfur dioxide	Blanching	Press Cake	Pressed Juice	(Total recovery)	Pasteurised Juice
Skrede et al 2000			18.2%	28.0%	46.2%	32.0%
Lee et al 2002	-		18.9%	20.2%	39.1%	20.8%
Lee et al 2002		-	22.0%	20.2%	42.2%	22.4%
Lee et al 2002			4.7%	4.7%	9.4%	12.6%
Rossi et al 2003		-	-	-	-	23.0%
Rossi et al 2003		-	-	-	-	12.0%
Srivastava et al 2007 ¹			-	-	-	29.5%
Srivastava et al 2007 ²			-	-	-	29.9%
Brownmiller et al 2008		99.90%	15.1%	80.2%	95.3%	41.2%
Brownmiller et al 2008		99.90%	15.1%	80.2%	95.3%	72.5%
Sablani et al 2010 ³		-	-	-	-	55.6%
Sablani et al 2010 ³		-	-	-	-	10.0%
Sablani et al 2010 ⁴		-	-	-	-	40.9%
Sablani et al 2010 ⁴		-	-	-	-	7.3%

¹ Tifblue cultivar, ² Powderblue cultivar, ³ Duke cultivar, ⁴ Reka cultivar

Table 2-6 and 2-7 summarises the findings of investigations into total anthocyanin levels during juice processing.

Generally the biggest potential loss of anthocyanins was seen during pressing. This is understandable when considering that pressing separates the aqueous phase from solid particulates and that the anthocyanins are primarily located in the skin of fruits. However, blanching was consistently reported to improve the recovery of anthocyanins in both the pressed juice (Lee et al, 2002) and subsequently pasteurised juice (Lee et al, 2002; Rossi et al, 2003; Sablani et al, 2010). Similarly sulphur dioxide treatment was shown to improve the recovery of anthocyanins in pressed and subsequently pasteurised juice (Lee et al, 2002). However, one marketing practicality with the use of sulphur dioxide is that it requires labelling on juice packaging and is commonly considered undesirable by consumers.

The scientific consensus was that blanching could contribute to a breakdown of particulates during extraction, but that more significant heating inhibited the activity of enzymes such as PPO before the berries were crushed. Kader et al (1997b) demonstrated that PPO is deactivated upon treatment with heat or with sulphur dioxide. According to Skerek et al (2002) addition of blanched blueberry pulp to their juice resulted in no additional degradation whereas unblanched blueberry pulp caused a 50% loss of anthocyanins, demonstrating the large potential contribution by such enzymes to anthocyanin degradation. Lee et al (2002) found that a pre-treatment with sulphur dioxide increased the level of anthocyanins and assumed that this was also due to inhibition of enzyme activity. Additionally, Lee & Wrolstad (2004) suggest that SO₂ addition may reduce the dielectric constant of water thereby increasing the solubility of phenolics.

Further research which supports the benefits of various pre-treatments, particularly during extraction includes papers by Kalt et al (2000) and Lee & Wrolstad (2004). Neither group attempted small scale juice production but simply investigated how variations in temperature, pH and SO₂ concentration during extraction affected measurements of interest such as antioxidant capacities, total anthocyanins and total phenolics. Kalt et al (2000) demonstrated that the extraction of blueberries at 60°C compared with 25°C proved extremely beneficial in maximizing the antioxidant, phenolic and anthocyanin content. Similar findings were reported

by Lee & Wrolstad (2004) who found that heating to 80°C as compared with 50°C gave significantly higher anthocyanin and phenolic levels. Both groups also investigated the effect of a pH adjustment and found a more acidic pH gave slightly higher levels for all measurements. Additionally Lee & Wrolstad (2004) investigated sulphur dioxide treatment with concentrations of 0, 50 and 100 ppm. The highest levels appeared to be achieved at 50 ppm for total anthocyanins and 100 ppm for total phenolics. Lee & Wrolstad (2004) also analysed the interactions between their treatments as they had employed a factorial design. Of the two-way interactions they found that a combination of heat and sulphur dioxide had a significant effect on both total anthocyanins and total phenolics. The interaction of sulphur dioxide and pH reduction was significant for total anthocyanins only and conversely the interaction between heat and pH reduction was significant for total phenolics only. (The addition of sulphur dioxide did not affect pH). There was a significant three-way interaction for total phenolics but not total anthocyanins. In this experiment the highest level of total anthocyanins was achieved with conditions of 80°C, 50 ppm SO₂ and pH ~ 3 whereas the highest level of total phenolics was achieved with 80°C, 100 ppm SO₂ and a pH ~ 3.

Lee et al (2002) reported the lowest recovery of total anthocyanins after pressing. Compared with their starting material only 4.66% of the anthocyanins were present in their pressed control juice (no pre-treatment) and slightly higher levels, 12.7% and 12.6%, were obtained for blanched and sulphur dioxide treatments, respectively. The press cakes were found to contain similar levels of anthocyanins to the pressed juices, leaving large percentages unaccounted for which were presumed to have been lost through degradation. Skrede et al (2000) achieved a recovery which was somewhat higher; their pressed juice contained 28% of the original anthocyanin content. However, after accounting for the levels in the press cake a similar proportion of anthocyanins remained unaccounted for, approximately 54%. Rossi et al (2003) and Sablani et al (2010) did not sample throughout processing, therefore the levels in their pressed juice cannot be compared. Brownmiller et al (2008) managed to achieve a significantly higher retention of anthocyanins throughout processing, particularly after pressing. They recovered 80% of the anthocyanins in the pressed juice and only 15% into the press cake, giving a total recovery of 95%. Brownmiller et al (2008) compared their research methods to

those published earlier and attributed their high recovery to two factors, firstly faster thawing and secondly their use of a blanching pre-treatment. However, it appears that they may have adjusted their results according to the mass of juice obtained after pressing, which would also explain why their recovery appears higher. On closer inspection of the various methods it is difficult to distinguish between the different heat treatments employed by the different research groups in thawing, blanching and extraction. However, in general it seemed that those who used some form of heating during the initial processing steps achieved both a higher total recovery of anthocyanins and a higher proportion of that in the juice after pressing. As suggested by Brownmiller et al (2008) it may also be preferable to thaw over the shortest time possible, although exactly how critical this is remains unclear.

Two of the groups chose to clarify the juice before pasteurisation which involved removing the finer sediment from the juice. Brownmiller et al (2008) observed that clarification resulted in a significant reduction in anthocyanins in the juice whereas Lee et al (2002) found there was only a small loss of anthocyanins from clarification. Brownmiller et al (2008) attributed this loss to physical separation of solid material from the juice and suggested that a higher retention was obtained by Lee et al (2002) because a continuous centrifugal separator was employed, as compared with the less effective standard batch centrifuge which they used.

The effect of pasteurisation on anthocyanin recovery was inconsistent. On the one hand pasteurisation may concentrate the juice slightly if some water is evaporated or additional heating may favour the release of additional anthocyanins from skin pigments, particularly if a significant amount of solids are present. Conversely heating may result in thermal degradation of phenolic compounds including anthocyanins. Lee et al (2002) observed minimal changes in levels after pasteurisation, Skrede et al (2000) observed a small increase and Brownmiller et al (2008) found that levels in their clarified juice only slightly decreased while levels in their non-clarified sample substantially decreased. However, on closer inspection of the various methods Brownmiller et al (2008) did not use a commercial heat exchanger and this is potentially why they observed the largest reduction in total anthocyanins in this operation. In general though, from the small amount of variation seen before and after pasteurisation it appears that anthocyanins may be reasonably heat stable in fresh blueberry juice.

As well as total levels of anthocyanins being affected by heat treatment some researchers also demonstrated that the relative levels of individual anthocyanins change. As already discussed different anthocyanin structures may be more or less stable in different chemical environments. Skrede et al (2000) and Lee et al (2002) reported high concentrations of malvidin glycosides in the berry fruit which decreased proportionally less than delphinidin derivatives during juice processing. This resulted in a higher percentage of malvidin and a lower percentage of delphinidin in the juice. Lee et al (2002) also reported an abundance of petunidin glycosides which also proportionally decreased during juice manufacturing. Srivastava et al (2007) documented similar results; in their starting material the concentrations of malvidin and peonidin anthocyanins were highest. However, in this case the peonidin anthocyanins were slightly more stable than the malvidin. Again the percentage of delphinidin and petunidin glycosides decreased in the juice as compared with the berry. Although two of the research groups also sampled throughout processing it is difficult to establish if consistent changes were observed over specific operations.

The large losses apparent were commonly attributed to polymerisation of anthocyanins which were measured using polymeric colour measurements (Brownmiller et al, 2008; Lee et al, 2002). Brownmiller et al (2008) suggested this could occur through reactions with other phenolic compounds including flavan-3-ols, tannins, and potentially phenolic acids, or products of enzyme modification such as chlorogenic acid modified by the enzymes PPO and POD.

Additionally, some other relevant investigations have been carried out on factors which may contribute to anthocyanin degradation during processing. Kalt et al (2000) investigated the presence of oxygen on the stability of anthocyanins, phenolics and antioxidant capacity in blueberry juice extracts. Test tubes were filled or half filled with juice, and tested after six hours of exposure. No losses were observed for full test tubes but 76% of anthocyanins, 30% of the phenolic content and 46% of the antioxidant capacity was lost in half filled test tubes over the same period. Several researchers have also investigated the possibility of anthocyanin degradation by the commercial depectinisation enzyme commonly employed before juice extraction. Most commercial pectolytic enzymes contain certain anthocyaninase side activity, which can degrade anthocyanins to anthocyanidins (Hohn et al, 2005). However, these affects

are usually only seen when the enzyme is employed in excessive concentrations. Wightman & Wrolstad (1996) evaluated the glucosidase activity of twenty-six different commercial enzymes, applied at two different concentrations. Only one preparation produced a decrease in total monomeric anthocyanins at the recommended dose. However, when higher levels were used there was more anthocyanin loss. Furthermore Skrede et al (2000) eliminated their depectinisation enzyme as a possible source of degradation during juice manufacturing as it appeared to exhibit no glycosidase activity or anthocyanin degradation activity.

Other Phenolic compounds

Various research groups also measured the levels of other flavonoids during juicing, including cinnamic acid derivatives or chlorogenic acid, flavonols and in some cases procyanidins. Different flavonoid classes have large variations in their functions in plants and are likely recovered more or less readily than anthocyanins. For example compared to anthocyanins chlorogenic acid is more water soluble and like anthocyanins is primarily found in the cell vacuole.

Lee et al (2000) found no variation across their three treatments (control, blanching and SO₂) for cinnamic acid derivatives or flavonols. For cinnamic acids approximately 33% of the original berry content was found in each of the pressed juice and presscake, leaving another 33% unaccounted for. Slightly more was recovered for flavonols with 40% in each of the pressed juice and press cake, leaving 20% unaccounted for. The levels in pressed juice remained relatively unchanged during subsequent processing steps. Lee et al (2000) assumed that the compounds were lost through degradation. Conversely Rossi et al (2003) reported a significant increase for levels of cinnamic acid derivatives using a blanching pre-treatment, as compared with their control. This is not unexpected since blanching potentially deactivates polyphenol oxidase for which chlorogenic acid is the preferential substrate. Skrede et al (2000) did not employ any pre-treatment variations but did measure the levels of chlorogenic acid, flavonols and procyanidins. For chlorogenic acid 40% was recovered in the pressed juice and only 1% was retained by the press cake. The concentration increased slightly upon subsequent pasteurisation, suggesting the chlorogenic acid is fairly heat stable. However, 59% of the berry chlorogenic acid was still unaccounted for which was assumed to have degraded. Similar

percentages of extraction were seen for procyanidins in the pressed juice and cake, and like chlorogenic acid their levels were retained upon pasteurisation. Again the recovery of flavonols in pressed juice was somewhat higher than chlorogenic acid at 53%, with 7% in the presscake. However, a substantial amount of flavonols was lost upon subsequent pasteurisation, suggesting they are not as susceptible to enzyme degradation and potentially more heat sensitive than the other flavonoids. These findings are in slight contrast to Brownmiller et al (2009) who reported they achieved a higher recovery for flavonols and chlorogenic acid than anthocyanins and procyanidins during juice manufacture.

2.3.4 Storage Trials – Retention of Phytochemicals

It is also necessary to consider how well the phytochemicals are retained during storage and whether the processing method or storage conditions influence this at all. Some of the researchers discussed previously extended their investigations of blueberry juice manufacture to include a shelf life study.

Brownmiller et al (2008) evaluated storage effects on blueberry juice stored in 6 oz (168 ml) glass bottles at 25°C over a six month period. Measurements at 1, 3 and 6 months showed that monomeric anthocyanin levels decreased significantly. This degradation was linear over the storage period, and the clarified juice had consistently lower levels of anthocyanins than the non-clarified juice. After six months non-clarified juice contained 23% of the original anthocyanin content and clarified juice contained 15%. However, the antioxidant capacity was practically completely retained in the stored samples and polymeric colour values increased. Therefore, Brownmiller et al (2008) assumed that the monomeric anthocyanin loss was due to the polymerisation of anthocyanins which were still able to contribute to the antioxidant capacity. An additional conference paper by Brownmiller et al (2009) reported that during storage at 25°C better retention was seen for flavonols and chlorogenic acid as compared with anthocyanins and procyanidins.

Srivastava et al (2007) stored samples of two blueberry cultivars in 30 ml glass bottles for sixty days at four different temperatures (-20, 6, 23, and 35°C). At the end of the storage period samples stored in frozen and chilled temperatures (-20 and 6°C) had consistently higher antioxidant capacities and anthocyanin and phenolic levels than samples stored in ambient and

higher temperatures (23 and 35°C). For example at day zero juice produced from the Tifblue cultivar was reported to have an antioxidant capacity of 17,000 $\mu\text{mol/L}$, an anthocyanin content of 340 mg/L and a phenolic content of 900 mg/L. At the end of the storage period at 35°C these levels dropped to 13,500 $\mu\text{mol/L}$, 180 mg/L and 400 mg/L whereas juice stored at -20°C had much higher levels: 16,800 $\mu\text{mol/L}$, 270 mg/L and 650 mg/L. As can be seen from these data during storage the antioxidant activity did not decrease proportionally as much as anthocyanin or phenolic levels, again supporting the suggestion that products from anthocyanin degradation are in fact polymerised anthocyanins. The researchers also suggest that oxidation could have contributed to some of this degradation on the basis of the paper by Kalt et al (2000).

The stability of individual anthocyanin compounds during storage reported by Srivastava et al (2007) was consistent with chemical structure. Malvidin and petunidin glycosides which have a single hydroxyl group and two methoxy groups on their B phenolic ring, making them less reactive, appeared to be the most stable upon storage. Conversely the delphinidin glycosides with three hydroxyl groups were seen to be the most unstable. Degradation of phenolic acids and flavonoids were also reported by Srivastava et al (2007). Of the eight flavonoids and phenolic acids measured only the catechins were present in amounts comparable to the anthocyanins at 350 mg/kg. However, their levels had decreased by 85 – 90% at the end of the storage period for all storage temperatures. The absolute decrease of the other compounds was much less significant as the concentrations of these compounds were ten fold lower initially. However, quercetin and ellagic acid did appear to benefit from a low storage temperature. The authors concluded that there was a significant advantage in storing the juice at low temperatures.

Vitamin C Retention

Vitamin C is one of the most abundant antioxidants in blueberries aside from the phenolic phytochemicals. Harb et al (2010) reported that fresh blueberries contained 6.3 mg/g of vitamin C. Although it is documented to contribute relatively insignificantly to the antioxidant capacity of blueberries (Prior et al, 1998) it is still a recognised nutrient and therefore potentially important to maintain, particularly from a marketing perspective. Vitamin C is readily oxidised, especially in the presence of metal ions such as copper and iron. Heat and light

accelerate the process, while additional factors such as oxygen concentration, pH and water activity strongly influence the rate of the reaction (Fennema, 1996). Fennema (1996) outlines several possible mechanisms of vitamin degradation but states that vitamin C activity is compromised in all resulting degradation compounds.

Harb et al (2010) evaluated changes in vitamin C in fresh blueberries during storage. They reported that a dramatic loss occurred under all storage conditions, which was attributed to changes in gene expression between freshly harvested and stored fruit. Degradation ranged between 60 and 40% after 3 weeks chilled storage for the various treatments. The least degradation of vitamin C was observed with low O₂ and high CO₂ level (up to 18%). Conversely Kalt et al (1999) found no significant change in vitamin C content for highbush blueberries stored at 0, 10, 20, and 30°C for up to 8 days.

From a processing perspective Kalt et al (2005) reported that because of its high water solubility the largest losses of vitamin C generally occur in processes involving water. For example blanching was shown to decrease vitamin C levels by 50% in some fresh fruits and vegetables, although the remaining vitamin C was then more stable upon subsequent storage. They suggested that, again, the heat treatment could inactivate native plant enzymes, such as ascorbate oxidase which is capable of breaking down vitamin C. The high water solubility of vitamin C would also mean that leaching of water during thawing could be particularly detrimental to levels in the juice. In terms of the effect of heat, Villota & Hawkes (2007) reported that although an increasing temperature is generally shown to increase the breakdown of vitamin C, sub-freezing temperatures may also accelerate degradation. However, unfortunately none of the blueberry juice experiments discussed here included a measurement of vitamin C. Therefore it is difficult to evaluate the potential overall effect of juice processing. However, vitamin C is easily added back into processed foods in order to meet consumer expectations.

2.3.4 Bioactivity of Blueberry Juice

From a biological perspective even a slight modification in structure of a compound may result in a loss of bioactivity. However, on the other hand, as with metabolic conversion of food components, the products of degradation during juice manufacture may be equally or more

biologically active. The purpose of investigating the retention of individual phytochemicals during processing and storage was to ascertain what effect juice manufacture has on the compounds which are believed to have an impact on health. However, it is equally important to evaluate the juice in its entirety as it is unknown what the effect of additional degradation compounds may have on the human body.

Several of the blueberry juice research groups included a measurement of antioxidant capacity. In general the antioxidant capacities were seen to decrease throughout processing. However, any pre-treatments which were able to increase the levels of phenolics inherently increased the antioxidant capacity of the resulting juice. Kalt et al (2000) reviewed the antioxidant capacity of a number of thermally processed blueberry food products and reported that in general, products that had experienced more processing (such as juice and pie filling) had lower antioxidant capacities than fresh/frozen berries. The study by Brownmiller et al (2008) observed the same trend in the levels of phenolics: a large drop was seen in the antioxidant capacity after pressing, but pasteurisation did not appear to have a large effect. Subsequent storage of blueberry juice did not result in a large reduction in antioxidant capacity in experiments by Brownmiller (2008) and Lee et al (2002) although phenolic levels decreased. This was believed to be due to the formation of polymerised anthocyanins which were able to retain their antioxidant power. However, it has been established that the antioxidant power *in vitro* may not relate to a biologically significant effect *in vivo*, as anthocyanins are potentially poorly absorbed. Furthermore, it is unknown how well polymeric anthocyanins are absorbed or what effect they may have on the human body.

Schmidt et al (2005) measured antioxidant capacity, and antiproliferation effect on liver cancer cells for different blueberry products including frozen whole blueberries and heat processed products such as jam. They found that although the antioxidant capacity was retained in heat processed products (as compared with frozen berries) the bioactivity of heat processed products appeared to be compromised when the samples were tested on liver cancer cells. Srivastava et al (2007) also observed that juice processing and storage led to significant reduction of inhibition of cancer cells when they tested their stored juice on colon cancer cells.

Research in this area is far from complete. Interestingly much of the research presented for various health claims employed supplementation with blueberry juice rather than fresh berries, particularly since this allows for the use of a placebo. However, it appears that many chemical changes take place both during manufacture and storage. If these relationships are established then it is important that the health benefits of blueberry fruit and juice should be investigated separately and compared using well designed experiments.

2.3.5 Summary of the Effects of Juice Processing

In chemistry studies a number of factors are shown to influence anthocyanin stability. Low pH and low temperatures can enhance stability while light, high temperatures, oxygen and plant enzymes have been shown to contribute to anthocyanin degradation. However, as these studies were performed on isolated compounds they may or may not be relevant to a far more complex chemical system such as blueberry juice.

The fundamental operation in blueberry juice manufacturing is pressing of the fruit incorporating physical separation of solid material from liquid. However, a number of operations can be utilised in pre and post pressing. One of the most common pre-treatments is the use of pectinase enzymes to reduce gelling of the blueberry mash during pressing. Usually the directions for enzyme usage will require the mash to also be heated mildly for 1-2 hours once the enzyme is added. Once the juice is pressed it is commonly pasteurised and then either hot filled or aseptically filled into juice bottles. Sometimes large juice manufacturers also employ a clarification operation to remove sediment from the juice and a concentration operation to standardise the °Brix concentration for wholesale.

Researchers found that one of the key places of anthocyanin loss during the manufacturing process was removal of the press cake which still contained a large amount of blueberry skin. However, many reported that a heat pre-treatment before pressing could greatly enhance phytochemical recovery, particularly for anthocyanins. A number of publications suggested that a blanching pre-treatment (85 - 90°C for 2-3 minutes) was the most successful temperature for enhancing juice anthocyanin levels, and that one reason for this was inactivation of the enzyme polyphenol oxidase (PPO). However none of the researchers used a full experimental design

with a good range of temperatures for comparison. Therefore from the information reviewed here an optimal heating temperature cannot be reported with complete confidence.

Storage experiments on blueberry juice showed a consistent and significant degradation of anthocyanin levels with time, with more deterioration occurring at higher temperatures. Specifically the profile of individual anthocyanins showed slightly more degradation of delphinidin derivatives and slightly less degradation of malvidin derivatives, which could be attributed to the variation of the substituents in their B phenolic rings. However, researchers who measured juice antioxidant capacity noted surprisingly that the antioxidant capacity of stored juice was relatively unaffected. Therefore they deduced that the lost anthocyanins had formed products which could still contribute to the juice's antioxidant capacity. However, so far no researchers have identified what these compounds might be and consequently no assumption can yet be made about the biological impact of storing blueberry juice.

2.4 Chromatographic techniques

Wu and Prior (2005) discuss the evolution of chromatographic techniques for anthocyanin detection;

“With the advancement of analytical technology, analysis and identification of anthocyanins have varied from thin-layer chromatography (TLC) and paper chromatography (PC) in early times to HPLC with photodiode array detector (PDA; or photo diode array detector, DAD), and, then, HPLC or CE with mass spectrometry or with tandem mass spectrometry. In recent years, HPLC coupled with mass spectrometry has become the standard and most powerful method for routine anthocyanin analysis..... Among them (MS methods) electrospray ionization mass spectrometry has been preferred by the majority of investigators”

As indicated above HPLC with different forms of mass spectrometry (MS) have become the most popular flavonoid quantification method over the past 20 years. It has been used in anthocyanin research by groups such as Hong & Wrolstad (1990), Gao & Mazza (1994), Smith et al (2000), Wang et al (2000), Brambilla et al (2008), Barnes et al (2009), Borges et al (2010), and many others. However, still no standardised method exists for using HPLC methods to quantify

anthocyanins. Instead review tables of different methods can be found in some articles/texts such as Horbowicz et al (2008), Naczk & Shahidi (2004) and Valls et al (2009).

Three main steps exist in HPLC methods, which can all be done in different ways – extraction, separation, detection and quantification. Some of the most commonly practiced methods are described in the following sections.

2.4.1 Extraction

Extraction methods should be optimised to uniformly extract all components of interest, remove interfering components and prevent of degradation during extraction. The latter is particularly an issue in plants which inherently have high enzyme activity (Robards, 2003).

Anthocyanins are usually extracted from plant materials with an acidified organic solvent. This solvent system dissolves the cell membranes while the reduced pH stabilizes anthocyanins (Naczk & Shahidi, 2004). Review tables show the most popular solvents to be methanol and acetone; acetone is an especially effective solvent for phenolic materials (Smith et al, 2000).

Fractionation of the extract may be utilised to provide a crude separation of phenolic types such as that employed by Smith et al (2000). Some researchers, such as Hong & Wrolstad (1990), also advocate the use of solid-phase extraction to remove potentially interfering compounds such as sugars which may interfere with anthocyanin detection. Conversely Ehlenfeldt & Prior (2001) suggest that such an extraction step is unnecessary and only compromises accurate quantification of anthocyanins.

In regards to extracts being prepared directly from fresh vs. frozen fruits, Smith et al (2000) reported that fruit stored at -20°C or lower appeared to possess a relatively complete anthocyanin profile as compared with that seen in fresh fruit.

2.4.2 Separation

Again, review tables give the best indication of column and mobile phase selections. The most popular column for anthocyanin analysis is a C18 reverse phase column. Wu & Prior (2005) give the following explanation as to why this column is most suitable:

“For anthocyanin analysis, the most important aspect is the column’s stability in a low pH mobile phase. From our experiences, a C18 column is quite stable for a mobile phase

containing 5% formic acid. The column efficacy does not show significant decreases even after several years of usage. Cleaning and storing the column with a neutral solvent immediately after anthocyanin analysis are highly recommended to prolong the life of the column.”

Generally mobile phases are seen to be performed as a gradient which consist of two mobile phases containing organic solvent but acidified to different degrees or an organic solvent run against acidified water. One issue with acid selection is that strong acids may induce anthocyanin degradation via hydrolysis, obscuring a realistic profile (Barnes et al, 2009). Therefore formic acid is frequently chosen for acidification, while the organic solvent used is commonly acetonitrile and/or methanol.

Duration of separation is usually quite lengthy, of the order of 60-80 minutes. However, a longer elution allows for better separation of co-eluting compounds (Wu & Prior, 2005).

2.4.3 Detection & Quantification

There is a wide range of identification methods possible through different types of detectors attached to the HPLC. A simple UV-visual detector, particularly a photo diode array detector (PDA) may be suitable in some instances. However, the lack of calibration standards and the issue of co-eluting compounds which cannot be resolved, often makes this method unsuitable (Barnes et al, 2009; Valls et al, 2009).

As described in the first passage above by Wu & Prior (2005) more sophisticated detection methods have become available which are more sensitive and specific. Currently, one of the most popular methods for anthocyanin detection and quantification is HPLC coupled with electrospray ionization mass spectrometry (MS), particularly using a tandem mass spectrometer (MS/MS) (Valls et al, 2009; Wu & Prior, 2005).

2.5 Summary

The most commonly studied compounds in the blueberry are the highly abundant anthocyanins. These compounds are the most significant contributors to blueberries' antioxidant capacity and additionally the anthocyanin diversity of blueberries appears unique. The other appreciably abundant phytochemical is chlorogenic acid, a compound which is common to many other fruits and vegetables along with coffee. Although these compounds are not substantiated as biologically significant there is still growing evidence that they have a role to play in prevention and treatment of various diseases. Therefore they were still considered compounds of interest in the research described in this thesis.

This review has shown that the genetic diversity seen in blueberries is large. Therefore it was considered important to profile the phytochemical fingerprint of each cultivar. Additionally maturational changes are not widely reported therefore they were also considered worth investigating.

A number of chemical changes appear to take place to the phytochemical profile during juice manufacture from blueberries. A number of alternative processes can be used in blueberry juice manufacture. Although some studies tried to compare a number of alternative operations and a few included storage trials there were no comprehensive studies which compared the whole range of variations for raw materials, processing conditions and storage conditions. Therefore the usefulness of this information was somewhat limited as each group only investigated part of the process. Additionally many of the papers did not provide a clear mass balance which made interpretation difficult. Therefore this review has shown that as well as the current process being evaluated a number of alternative processes need to be trialled and quantified.

Although widely studied, blueberries are still not proven to have any nutritional benefits besides those that fruits and vegetables normally provide. However, there are a significant number blueberry studies presenting positive results for beneficial cellular mechanisms and physiological effects. Hopefully with an increasing number of studies and further evolutions in technology scientists will one day establish dose dependent relationships, for which the information contained in this thesis will be valuable.

CHAPTER 3

Materials & Methods

3.1 Research Plan

Before commencement of the project a research plan was compiled in collaboration with Mamaku Blue, Technology New Zealand and Massey University. All experimental work was focused on identifying and evaluating compounds found in blueberries which were recognised as being potentially beneficial to human health. The main areas of investigation were any varietal and maturation differences across the eight blueberry cultivars grown at Mamaku Blue and optimisation of blueberry juice phytochemicals during manufacturing and storage. The research year was split into these three major areas of work in order to provide information relevant to both the grower and the wider scientific community. These three areas were: Berry Analysis, Juice Manufacture and Juice Storage.

3.2 Material collection

All blueberry material was obtained from Mamaku Blue blueberry orchard (Rotorua, NZ).

Berry Analysis

A visit was made to the orchard in Feb 2010. Samples of approximately 2 kg from 10 year old plants of 'Dixi', 'Burlington' 'Bluecrop', 'Brigitta', 'Duke' and 'Reka', 15 year old plants of 'Elliot' and 30 year old 'Burlington', 'Dixi' and 'Jersey' plants, were hand-picked randomly across an entire block, ensuring berries were at the commercially ripe stage (100% blue, picking time determined by the grower's knowledge). Additionally approximately 2 kg of 'green' and 'red' berries from 30 year old 'Dixi' cultivars were picked, where green was 100% green, red was >75% red (all colours assessed by eye). The material was transported to Massey University on ice within 24 hours of harvesting, frozen using liquid nitrogen immediately on arrival, then stored at -30°C until analysis.

Standard Juice Manufacture

Juice was produced on site at Mamaku Blue in June 2010, using berries that had been machine harvested in Feb-March 2010 and stored frozen at -20°C. The material included 80% 'Dixi' commercially ripe, 10% overripe or damaged 'Duke' and 10% overripe or damaged 'Dixi'. Approximately 250 kg of blueberries were used to produce 200 L of 'hot press' juice and 120 kg were used to produce 91 L of 'cold press' juice. Samples were collected during the production processes and stored immediately in a -20°C freezer. Later they were transported back to Massey University on ice and stored at -30°C until analysis.

Alternative Juicing Strategies

The 'Burlington' cultivar was selected for juice production experiments in the Pilot Plant at Massey University. Approximately 10 kg of fully ripe 'Burlington' berries were transported frozen on an overnight courier, and arrived at Massey University at a temperature of -7°C. These berries were re-frozen in a -30 °C freezer until they were required for juice production.

Juice Storage

Blueberry juice previously produced by Mamaku Blue was sent via courier to Massey University, Palmerston North in Dec 2009. The juices had been prepared at various times during the previous two years, as per table 3-2, and stored at Mamaku Blue. When the juices arrived at Massey they were stored at room temperature as per the previous storage conditions.

Table 3-1: Stored juice details

Juice Type	Production Dates
Green Juice	Unknown
Standard Juice	Nov 09, March 09*, June 08
Drips n Dregs	Nov 09

* Berries were heated approximately 5-10°C hotter than during normal manufacture

For the storage experiment sixty bottles of juice (30 x 375 ml PET and 30 x 250 ml glass) which had been produced and bottled on site during the standard manufacturing experiment were utilised. The bottled juice was transported back to Palmerston North straight after production (at ambient temperature) and immediately placed under experimental conditions.

3.3 Experimental design

Berry Analysis

In order to investigate varietal differences two 15 g aliquots for each blueberry cultivar (30 year old 'Dixi', 'Burlington' Jersey; 15 year old 'Elliot'; 10 year old 'Bluecrop', 'Brigitta', 'Duke' and 'Reka') were extracted and analysed for total anthocyanins, individual anthocyanins and chlorogenic acid using methods described in section 3.4, within 2-3 months of collection.

One 15 g aliquot of each of 30 year old 'red' and 'green' 'Dixi' berries were prepared and analysed for total anthocyanins, individual anthocyanins and chlorogenic acid using methods described in section 3.4. One 15 g aliquot of each of 10 year old 'Dixi' and 10 year old 'Jersey' were prepared and analysed for total anthocyanins in order to investigate a potential difference caused by plant maturity.

A sample of approximately 50 commercially ripe berries of each cultivar were used to determine the average berry weight and approximately 25 were used to determine the height and diameter - in order to calculate an average surface area:volume ratio for each cultivar.

Standard Juice Manufacture

Two small batches of blueberry juice were produced at Mamaku Blue, with samples collected for analysis during processing, so that the effect of processing on anthocyanin and chlorogenic acid levels during manufacture could be quantified. In addition the process streams were tracked and weighed so that mass and chemical balances could be completed to quantify total losses. This information was then used to determine where the greatest losses occurred and consequently what areas should be focused on when trying to improve the yield and/or retention of juice components.

Initially a mass of blueberries (~390 kg) was removed from the freezer and thawed in 20 L buckets over a three day period in the pack house, reaching a final temperature of 2°C.

For 'hot pressing', 256 kg of thawed berries were divided between two 2,000 L vats so each vat contained ~126 kg of berries. The vats were heated to between 20 - 30°C for 2-3 hours, with a pectinase - Lallzyme HC enzyme (Lallemand, Montreal, Canada) - dosed at 1 g per 100 kg of fruit and diluted with 100 ml water per g of enzyme, added half way through heating. The slurry

was then pumped out of the vats and pressed in a series of basket presses over baths. The vat and pump were rinsed out, adding some additional water (a few L) to the processing stream. The press cakes were removed from the wire baskets and pressed a second time in a SPAGNI stainless steel 120 L hydraulic press (Reggio Emilia Italy) with an internal bladder which was filled with water using a pump. The second press of the press cakes was successful in recovering additional juice. The combined juice was stored in a plastic pod, before transportation to a contract pasteuriser ~ 40 hours later.

The remaining thawed berries, approximately 135 kg, were used to produce 'cold press' juice. Here the berries were simply pressed once in the SPAGNI hydraulic press. However the berries had to be split into two batches, ~68 kg, due to the capacity of the press. No enzyme was added. The pressed juice was stored in two 60 L plastic barrels before transportation to a contract pasteuriser ~ 40 hours later.

All juice was then transported to a contract pasteuriser in a refrigerated truck. Pasteurisation conditions were 90°C for thirty seconds using an Alpha Lavall plate heat exchanger (Lund, Sweden) and hot fill bottling on site at either 72°C for plastic bottles (375 ml PET) or 85°C for glass bottles (250 ml).

All juice samples and solid material (frozen berries, thawed berries and press cakes) collected during processing were analysed for total anthocyanins & chlorogenic acid according to methods outlined in section 3.4 Analysis was delayed due to equipment break-down. Consequently samples were analysed approximately 3 months after collection.

Alternative Juicing Strategies

The following alternative juicing strategies were used to produce blueberry juice using Pilot Plant facilities at Massey University in Palmerston North:

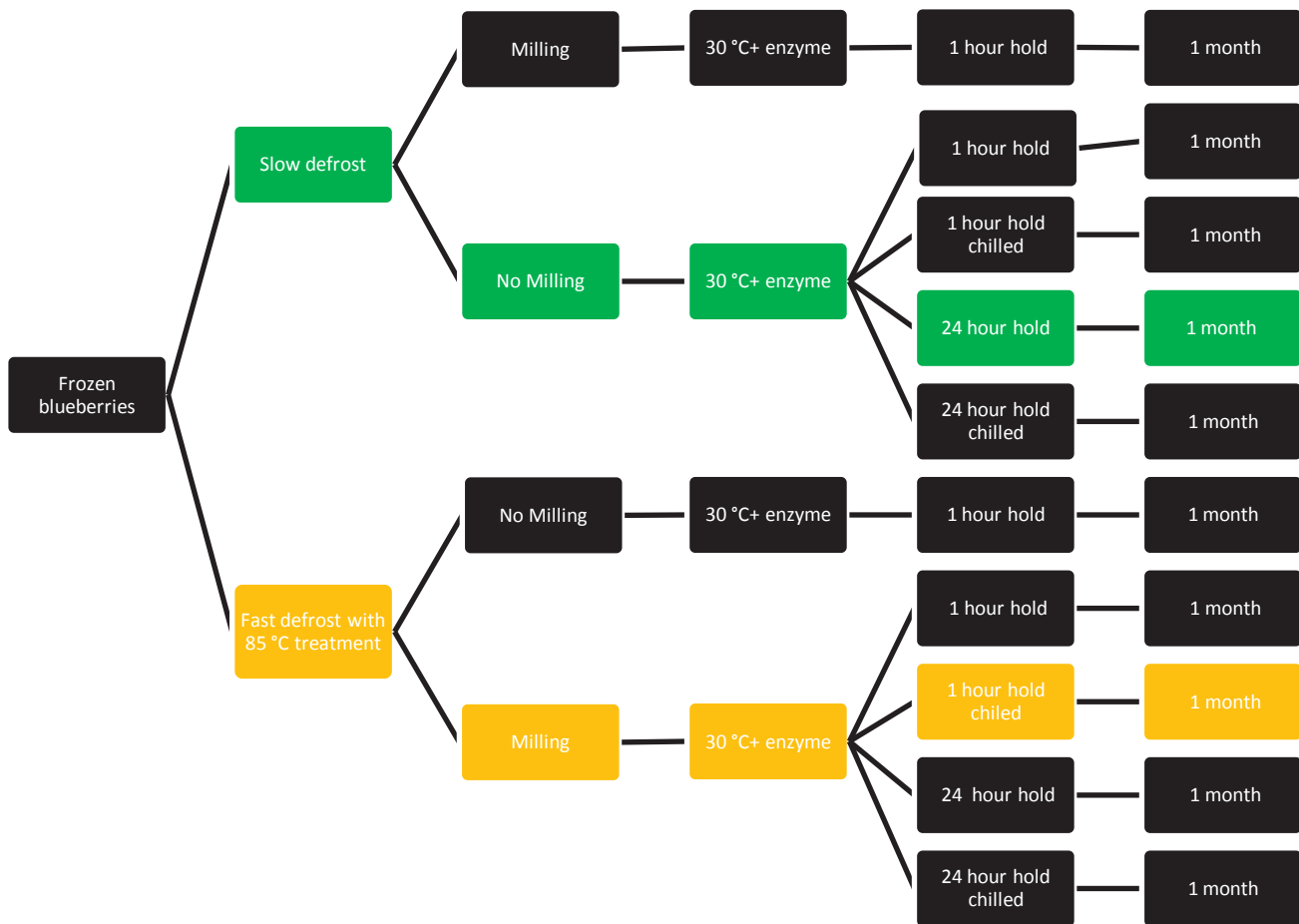


Figure 3-1 Juice production using alternative processes, experimental details in text.

Slow defrost berries were removed from the freezer and thawed in ice cream containers over a 24-25 hour period at ambient temperature.

Fast defrost berries were removed from the freezer, placed in a mesh cone and seated inside a 100 L steam blanching pan with a stainless steel bowl underneath. A digital temperature probe (Dick Smith Electronics, types J,K meter, Model Q1437) was placed within the mass of berries. The steam blanching pan was turned on full (steam dryness approximately 85%) and the berries were heated and held for five minutes once the temperature probe

reached 85°C (reaching this temperature took 2-3 minutes). The berries were then tipped into the stainless steel bowl which had been used in the steam blanching pan and placed on ice to cool as quickly as possible to 30°C or below (achieved within 3-4 minutes).

If berries were milled they were placed in a food processor for 1 minute on full (we used a Magimix cuisine systeme 5100, 1000 W, France).

In all experiments the blueberry mash was enzyme treated. The mash was placed in a stainless steel bowl within a water bath which had been preheated to 30°C. Zymus Celluzyme enzyme (Auckland, New Zealand) was prepared at a dosage rate of 0.1 ml/ 100 g of mash and diluted nine fold with water. The diluted enzyme was added to the mash which was held for one hour at 30°C.

The berry mash was then pressed in a small bench-top screw press (diameter 23 cm) with a chiffon cloth to keep all the mash directly under the screw plate. The mash was pressed twice with the screw being tightened as far as possible each time. Juice was collected in a 2 L plastic jug.

The juice was split into 300 ml LDPE plastic bottles (similar to cream bottles). Ambient samples were left on the bench while chilled samples were retained in a 4°C fridge.

Due to the small sample volume the pasteurising was completed using a steam jacketed kettle, consisting of a 12 L stainless steel bucket held in boiling water within a 30 L steam jacketed kettle. The pastueriser was pre-heated, juice was poured into the bucket and held for one minute once the juice had reached 90°C (reaching this temperature took 2-3 minutes, which was again measured with the Dick Smith Electronics temperature probe).

The pasteurised juice was immediately 'hot filled' into two glass Schott bottles for duplicate assessment, which varied in size between 50 – 80 ml. This juice was held for one month in controlled storage at 15°C.

All juice samples and solid material (frozen berries, thawed berries and press cakes) were analysed for total anthocyanins & chlorogenic acid as per the methods in section 3.4.

Juice Storage

The following storage conditions were used the storage experiment:

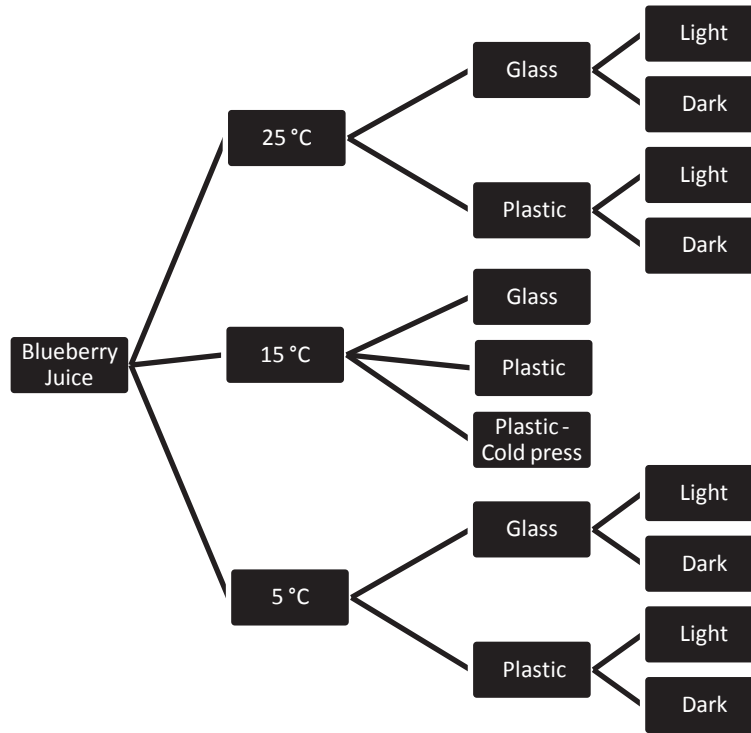


Figure 3-2: Overview of treatments in 6 month storage experiment - storage temperature, packaging type & light exposure.

Juice was produced at Mamaku Blue, packaged into 250 ml glass or 375 ml PET plastic bottles and transported back to storage facilities at Plant and Food Research in Palmerston North. The storage facilities were three individually temperature controlled 1 m x 2.5 m rooms, each containing a stand of mesh racks. As described by Solomon et al (1995), fluorescent lights were hung above the bottles at a distance which corresponded to a light exposure of 2000 lux, which was measured with a Li-Cor, LI-250 Light Meter (Lincoln, Nebraska U.S.A.). ‘Cool white’ fluorescent bulbs were used since these are the most common types of bulb used in commercial fridges. Bottles which were to be stored in the dark were wrapped in tin foil and stored on the same racks as the bottles stored in the light. Due to the slight difference in light exposure across the length of the rack all bottles were randomly positioned and rotated randomly every month.

The total storage period of the juice was six months. Sampling occurred every month beginning after two months of storage (i.e. in total five samples were analysed for each treatment by the end of the storage experiment). A new bottle of juice was opened for sampling each month for each treatment. All bottles of juice were analysed for total anthocyanins & chlorogenic acid according to methods outlined in section 3.4.

3.4 Analytical Methods

3.4.1 Anthocyanin and Chlorogenic acid analysis

Phenolic analysis (anthocyanins and chlorogenic acid) was carried out using high performance liquid chromatography (HPLC) with a photo diode array detector (PDA) and standards for quantification. However, first extracts of the materials of interest needed to be prepared for this purpose. These extraction methods are outlined in the following sections.

Solid material extract methods

Table 3-2: Comparison of different solid extraction methods.

Method	Sample Size	Description	Sections used
Method S	15 g	Single extraction using 30 ml solvent, filtered once. Rinsed with extra 20 ml.	Whole Berry
Method W	15 g	Single extraction using 30 ml acidified water, filtered once. Rinsed with extra 20 ml.	Whole Berry
Method S2	2 – 6 g (depending on concentration)	Multiple extractions (5-6 washes) centrifuged and poured off each time, totalling 26 -30 ml.	Juice solids

Table 4-3 summaries the various extraction methods used in the subsequent discussion to extract the compounds of interest (anthocyanins and chlorogenic acid) from solid blueberry material for analysis.

Extracts of whole berry material

Initially two different extraction methods were used to evaluate the anthocyanin concentration of highbush blueberry varieties. Solvent extraction with methanol/acetone/formic acid (method S) was compared with an acidified water extraction (method W) for all the cultivars. The methods previously reported by Wang et al (2000) and Rossi et al (2003) were merged to obtain the current methods used in this study.

Method S consisted of grinding 60 g of frozen blueberries in a food processor for 3 min. After that, a 15 g aliquot of the sample was combined with ½ teaspoon of food-grade diatomaceous earth (Celite 545 - VWR, Prolabo, Paris, France) and 30 ml of solvent mix

(acetone/methanol/water/formic acid, 40:40:20:0.1 in volume) and homogenised using an Ultra Turrax T18 (IKA, Petaling Jaya, Malaysia) for 2 min at 20,500 rpm. The homogenised sample was filtered in a Büchner funnel under vacuum using 2 sheets of Whatman No. 4 filter paper (Whatman International Ltd, Maidstone, UK) and the residue was rinsed with the same solvent mix until the filtrate reached 50 ml. The solvent mix was removed using a Rotavapor R-215 with vacuum controller V-850 (Global Science & Technology Ltd., NZ - Büchi Labortechnik AG, Flawil, Switzerland) operating at 50°C and 72 mBar. The dried extract was re-solubilised with 50 ml of the starting eluent phase used in the HPLC analysis, consisting of 5% formic acid/methanol 86:14 in volume. Preliminary studies on the purification of the blueberry extract using Sep-Pak C18 cartridge (Waters Scientifics, Mississauga, Canada) were conducted according to the method described by Wang et al (2000). However it was determined that this extraction step did not improve the detection of anthocyanins upon HPLC analysis, as was also previously reported by Barnes et al (2009). In fact the Sep-Pak cartridge was shown to result in a loss of anthocyanins, as was evident by the colour of the waste and confirmed by analysis of this waste. Thereafter Sep-Pak cartridges were not used in extract preparation.

Method W consisted of grinding 60 g of frozen blueberries in a food processor for 3 min. An aliquot of 15 g was combined with ½ teaspoon of food-grade Celite 545 diatomaceous earth and 30 ml of water-based solvent (water/5% formic acid, 95:5 in volume) and homogenised using the Ultra Turrax T18 for 2 min at 20,500 rpm. The homogenised sample was filtered in a Büchner funnel under vacuum using 2 sheets of Whatman No. 4 filter paper and the residue was rinsed with the same water-based solvent until the filtrate reached 50 ml.

All extracts were filtered with a 0.22 µm regenerated cellulose syringe filter (Grace Davison) using a 1 ml luer lip syringe, into the previously described RAM HPLC vials.

Extracts of solid material in juice processing

During juicing experiments the frozen berry, thawed berry and presscake samples were extracted using a new solvent extraction method (method S2) since a single wash and filter could not remove all the red pigment from the most concentrated presscake samples.

Approximately 20 g of material was blended in a food processor for 3 min. 2-6 g of sample was weighed out accurately and placed in a 50 ml centrifuge tube (Grace Davison Discovery Sciences Maryland, U.S.). Initially 10 ml of the solvent mix (acetone/methanol/water/formic acid, 40:40:20:0.1 in volume) was added to the blueberry material which was then vortexed for 30 seconds. After 5 minutes extracting at room temp the tube was centrifuged at 710 g for 10 min using a Thermo Scientific Heraeus multifuge IS-R centrifuge (Massachusetts, U.S.A.). The supernatant was then poured off into a 100 ml rotary evaporator flask. Washing of the blueberry material and pouring off of the supernatant was repeated every 6 ml until the supernatant ran clear. The original solvent mix was then removed using a Rotavapor R-215 with vacuum controller operating at 50 °C and 72 mBar. The dried extract was subsequently re-solubilised in 20 ml of the starting eluent phase used in HPLC analysis, consisting of 5% formic acid/methanol 86:14 in volume. All extracts were filtered with a 0.22 µm regenerated cellulose syringe filter (Grace Davison) using a 1 ml luer lip syringe, into the previously described HPLC vials.

Extracts of juice

All juice samples were diluted 1:1 with a 5% formic acid solution (2 ml + 2 ml). Sediment was removed from the juice to prevent blocking of the analysis equipment. For storage samples this involved syringing the diluted juice into 1 ml luer slip syringes and filtering the diluted juice into HPLC vials (12 x 32 mm clear RAM (robotic arm machine) vial with 9 mm Black open-hole cap and 40 mm polytetrafluoroethylene/silicone septa (Grace Davison Discovery Sciences, Maryland, U.S.A.)) using 0.22 µm regenerated cellulose syringe filters (Grace Davison Discovery Sciences, Maryland, U.S.A.). However, it became obvious that this method was not sufficient for removing juice sediment as by the end of the juice manufacturing experiments the guard column was severely blocked. Therefore for juice samples collected during the alternative manufacturing experiments the samples were centrifuged as well as filtered. The diluted juice was first pipetted into 2 ml centrifuge tubes and centrifuged at 12,500 rpm for 7 minutes using a Thermo Scientific microCL 17 centrifuge (Massachusetts, U.S.A.). The juice was then syringed into 1 ml luer slip syringes (avoiding the sediment at the bottom) and syringe-filtered into HPLC vials as previously described.

Measurement of Anthocyanins and Chlorogenic acid using High Performance Liquid Chromatography (HPLC)

Anthocyanins were measured using the method previously reported by Wang et al (2000). The HPLC system consisted of a Shimadzu CTO-20A (Shimadzu Corp., Kyoto, Japan) instrument coupled to an auto-sampler SIL-20AC and a photodiode array (PDA) detector (SPD-M20A). Anthocyanins were separated on a Phenomenex Luna C18 (2) 150 × 4.6 mm (5 µm) reverse phase column fitted with a Phenomenex guard cartridge system (part no KJ0-4284) with an AQ C18 4 × 3 mm safety cartridge (part no AJ0-4287) (Phenomenex, North Shore City, NZ).

The solvent gradient was made with 5% aqueous formic acid (solvent A) and 100% HPLC grade methanol (solvent B). The flow rate was 1.00 mL min⁻¹, with a linear gradient profile consisting of the following proportions (v/v) of solvent B: 0.00 – 1.00 min, 14% B; 1 - 10.24 min, 14 – 17% B; 10.24 – 35.28 min, 17 – 23% B; 35.28 – 64.59 min, 23 – 47% B; 64.59 – 66.59 min, 47 – 14% B. The total running time was 70 min. The column was operated in an air conditioned room at 22 ± 3 °C, not exceeding 25 °C. Anthocyanin and chlorogenic acid identification was based on the retention time of standards and on the order of elution reported by other researchers for highbush blueberries. Quantification of anthocyanins was based on peak areas determined at 520 nm and compared to the absorbance of cyanidin 3-O-glucoside chloride concentrations of 200, 20 and 2 ppm. Quantification of chlorogenic acid was based on the peak area of a chlorogenic acid standard at 320 nm at concentrations of 100, 10 and 1 ppm. Replicate standard samples were included within each analysis to check the machine reproducibility.

Acetone, HPLC grade methanol and formic acid were purchased from J.T. Baker B.V. (Deventer, The Netherlands). The cyanidin 3-O-glucoside chloride (0915S ≥96%) standard and malvidin 3-O-glucoside chloride (0911S ≥95%) standard were purchased from Extrasynthese SA (Genay Cedex, France). The chlorogenic acid standard (C3878 ≥95%) was supplied by Sigma (St. Louis, Missouri, USA). All water used was ultra-pure water for HPLC usage (Milli-Q water purification system, Millipore Corp., Bedford, MA).

The calculations used to determine the anthocyanin or chlorogenic acid concentration of the original material using the corresponding standard curve were as follows:

Equation 3-1: Conversion of detector intensity reading (HPLC) to component concentration in berry juice

$$\frac{\text{Standard Concentration} \left(\frac{mg}{L}\right)}{\text{Standard area } (\mu A)} \times \text{dilution ratio} \times \text{Sample area } (\mu A)$$

$$= \frac{mg}{L} \text{ solution} \times \text{juice ratio} = \frac{mg}{L \text{ juice}}$$

Equation 3-2: Conversion of detector intensity reading (HPLC) to component concentration in berry fresh weight

$$\frac{\text{Standard Concentration} \left(\frac{mg}{L}\right)}{\text{Standard area } (\mu A)} \times \frac{\text{Extracted sample}(mg)}{\text{Original berry sample } (mg *)} \times \text{Sample area } (\mu A)$$

$$= \frac{mg}{L} \text{ solution} \times \text{berry ratio} = \frac{mg}{kg \text{ berry FW}}$$

* assumed 1L equivalent to 1 kg

Note that the first term of this equation is equivalent to the inverse gradient of the standard curve.

3.4.2 Spectrophotometric analysis (Total polyphenol content & antioxidant power)

All three spectrophotometric assays presented here (Total Polyphenol Content (TPC), Ferric Reducing Antioxidant Power (FRAP) and 2,2-Diphenyl-1-Picrylhydrazyl antioxidant power (DPPH)) were based on the methods described by Molan et al (2009). These assays were carried out only on blueberry juice samples. Therefore no preliminary extraction was required.

Total Polyphenol Content

Initially juice samples were diluted 1:1 with water to ensure they would be within the range of detection for the spectrophotometer (microplate reader). For each diluted juice sample six wells of a 96-well microplate were filled with 12.5 μ L of diluted juice solution. Additionally gallic acid solutions with concentrations of 200 μ g/ ml to 2500 μ g/ ml were prepared so that a standard curve could be generated for post-run quantification. For each gallic acid

concentration 3 wells were filled with an aliquot of 12.5 μl per well. The first reaction solution, 2% sodium carbonate, was then pipetted into each well at 250 μl . The plate was left to react for 5 minutes at room temperature. The second reaction solution, Folin-Ciocalteu phenol reagent (diluted 50%), was then added with 12.5 μl pipetted into each well. The plate was then left to stand for 30 min at room temperature to ensure the reaction would progress to completion. The plate was then measured at an absorbance of 650 nm using an ELX 808 Ultra Microplate Reader (Bio-tek Instruments. Inc, U.S.A.). The standard curve was generated from the data for gallic acid solutions which was used to calculate the polyphenol content for each sample in mg of gallic acid equivalent. The following calculation was used, also correcting for the dilution of the juice:

Equation 3-3: Conversion of spectrophotometric absorbance of juice to equivalent standard concentration

$$\begin{aligned} \text{absorbance of juice} \times \frac{\text{standard concentration } \mu\text{g/ml}}{\text{absorbance of standard}} \times \text{dilution factor} \\ = \text{equivalent anitradical activity } \frac{\mu\text{g}}{\text{ml}} \end{aligned}$$

Ferric Reducing Antioxidant Power

Again juice samples were diluted 1:1 with water to ensure they would be within the range of detection for the spectrophotometer (microplate reader). For each diluted juice sample, six wells of a 96-well microplate were filled with 8.5 μL of diluted juice sample. Additionally ferrous sulfate heptahydrate solutions with concentrations of a 500 to 5,000 $\mu\text{mol/L}$ were prepared so that a standard curve could be generated for post-run quantification. For each ferrous sulfate heptahydrate concentration 3 wells were filled with an aliquot of 12.5 μl per well. The reaction solution was then prepared by combining 10 volumes of 300 mmol/L acetate buffer, pH 3.6, with 1 volume of 10 mmol/L 2,4,6-tripyridyl s-triazine in 40 mmol/L hydrochloric acid and 1 volume of 20 mmol/L ferric chloride. 275 μL of this solution was then added to every well. The microplate was incubated at 37°C for 30 minutes in the dark to ensure the reaction would progress to completion. The plate absorbances were measured at 595 nm using an ELX 808 Ultra Microplate Reader (Bio-tek Instruments. Inc, U.S.A.). The standard curve was generated from the data for the ferrous sulfate heptahydrate solutions and this was used to calculate the

antioxidant power for each sample in ferrous sulfate heptahydrate equivalents (as per equation 3-3).

2,2-Diphenyl-1-Picrylhydrazyl Antioxidant Power

For this assay all juice samples were diluted 1:4 with water to ensure that the final measurement would be within the sensitivity of the spectrophotometer (microplate reader). For each diluted juice sample six wells of a 96-well microplate were filled with 25 μ L of diluted juice solution. The reaction solution, 4 mg DPPH per 50 ml (95%) ethanol, was then pipetted into each well at 275 μ L, additionally ensuring that three 'blank' wells were also filled (wells without any juice sample). Here no standard solution was required as instead the 'blank' wells became the value for comparison. The plate was incubated at room temperature for 30 minutes in the dark to allow the reaction to progress to completion. The plate absorbances were measured at 550 nm using an ELX 808 Ultra Microplate Reader (Bio-tek Instruments. Inc, U.S.A.). The antiradical activity was calculated as a percentage of discoloration relative to the blank sample using the following calculation;

$$\begin{aligned} & \text{Equation 3-4: Antioxidant activity of juice in DPPH assay calculation} \\ & \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance control}} \times 100 \\ & = \% \text{ DPPH antiradical activity} \end{aligned}$$

3.4.3 Physical Parameters

Berry size & weight

To quantify the average blueberry weight of each cultivar an aliquot of approximately 25 berries was randomly selected and weighed together, with the average value calculated by dividing by the number of berries.

The calculation of the surface area:volume ratio was based on the shape of a prolate ellipse. First the equatorial and longitudinal diameters of 25 berries for each cultivar were measured using digital callipers. These two measurements were used to calculate the surface area and

volume of each berry based on the prolate ellipse formula. In order to obtain the ratio the surface area was divided by the volume and consequently an average value of specific surface area was calculated for each cultivar.

Juice °Brix

Juice °Brix was measured directly using a Pocket Pal-1 pocket refractometer (Atago, Japan). Initially 2-3 drops of distilled water were used to check the calibration. Then 2-3 drops of juice were pipetted on to the sensor and the measurement was taken. Usually duplicate samples were measured. The sensor was wiped dry between each sample.

Juice pH

Juice pH was measured using a Thermo Scientific Orion 3 star benchtop pH meter (Massachusetts, U.S.A.). A calibration curve was established using pH 7 and pH 4 buffer solutions (Ajax Finechem, Auckland, New Zealand). The probe was placed into the samples and the measurement was recorded once the pH icon had stopped flashing. Between samples the probe was rinsed with distilled water and blotted dry.

Juice Sediment

Juice sediment was quantified using a centrifuge method. 2 ml centrifuge tubes were first weighed on a four decimal place balance. Each tube was then filled with 2 ml of juice and re-weighed. The tubes were centrifuged at 12,500 rpm for 7 minutes using a Thermo Scientific microCL 17 centrifuge (Massachusetts, U.S.A.). The supernatant in the tubes was discarded, leaving behind just the sediment in the tube. The tube was weighed again, so that the final weight of sediment could be quantified. Sediment was calculated as a percentage of the original weight of juice in each 2 ml tube.

CHAPTER 4

Results & Discussion

4.1 Varietal and Material Differences in Eight Blueberry Cultivars

4.1.2 Varietal Differences

The total contents of anthocyanins for the eight cultivars can be seen below in Figure 4-1, with substantial varietal differences. The highest anthocyanin concentrations were seen for cultivars ‘Elliot’ and ‘Burlington’ with 2,650 mg/ kg fresh weight (FW) and 2,170 mg/ kg FW respectively. Conversely cultivars ‘Bluecrop’ and ‘Brigitta’ contained the lowest levels of anthocyanins with 790 mg/ kg FW and 910 mg/kg FW respectively.

In regards to the three cultivars ‘Dixi’, ‘Jersey’ and ‘Burlington’ which had both ten and thirty year old plants on the property the values shown in figure 4-1 are based on extracts taken from thirty year old plants, since these constituted the majority of the harvest.

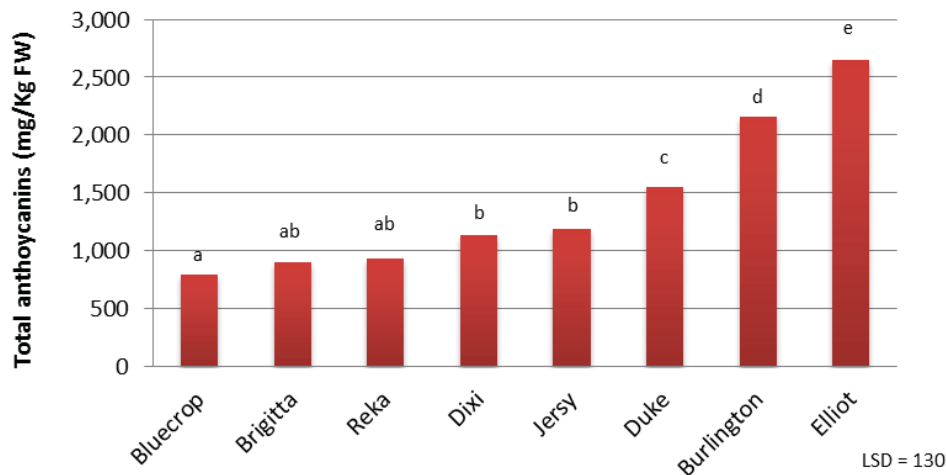


Figure 4-1: Total anthocyanin concentrations of eight different Highbush Blueberry cultivars measured using HPLC. Cultivars labelled with different letters are significantly different.

Data reported by other researchers showed similar trends to the data presented here (see Table 4-1). What is important to note is that even though significant variation is evident the top

three varieties identified here ('Elliot' 'Burlington' and 'Duke') have been consistently reported to have the highest anthocyanin contents by other researchers.

Table 4-1: Anthocyanin Contents Reported by Various Researchers (mg/kg FW)

	Birt & Tibaldi (2010)	Cho et al (2004)	Conner et al (2002)	Moyer et al (2002)	Ehlenfeldt & Prior (2001)	Prior et al (1998)	Gao & Mazza (1994)
Bluecrop	794	1,435	1,230	840	1,820	930	1,000
Brigitta	909		1,320	1,030	1,650		
Reka	935				1,490		
Dixi	1,137				1,640		
Jersey	1,193		1,210		2,050	1,166	
Duke	1,552			1,730	2,160	1,274	
Burlington	2,168				2,530		
Elliot	2,650		1,910		3,110		

There are hundreds of blueberry varieties worldwide. However, with the interest in blueberries for potential health benefits a reasonable amount of literature is available on anthocyanin contents in different blueberry varieties, particularly for 'Bluecrop' which is a commercially common Northern Highbush cultivar. In 2001 a large study was conducted by Ehlenfeldt & Prior and was the only paper found to contain all eight varieties studied here. Only two of the eight varieties analysed by Ehlenfeldt & Prior (2001) - 'Burlington' and 'Dixi' had similar anthocyanin concentrations, while all other varieties were reported to have concentrations 20-50% higher. However, this is probably due to a difference in methods as the pH differential method which was used by Ehlenfeldt & Prior (2001) is reported to estimate total anthocyanins higher than the HPLC methods utilised here. Spectrophotometric data on monomeric anthocyanin content are always higher than chromatographic data on glycosylated anthocyanin content. In support of this, an earlier publication by Prior et al (1998) using HPLC methods reported the total anthocyanin content for three of the same varieties with values closer to those reported here.

The variation seen in this Table 4-1 is likely to be the result of many differences such as growing conditions, berry maturities, and as already mentioned, experimental methods.

Growing conditions can have a reasonable influence but are not easily controlled in an experiment. For example a paper by Howard et al (2003) reported the level of total anthocyanins changed by as much as 63% across two growing seasons. Although many experimental methods are similar, small changes to experimental procedures such as holding temperatures during extraction can result in differences. Additionally some researchers prefer to perform their analysis on dried material (e.g. freeze dried blueberries). However, the values for the analysis of such material is reported as a concentration per unit dried weight and can therefore not be directly compared with those reported as a concentration per unit fresh weight unless conversion data is available. Another concern for biological material is the inability of a small number of samples to be representative of a large population. For example in HPLC anthocyanin quantification (the method used here) only micro litres (0.001 ml) were used for each analysis, which is only a fraction of a single fruit. In order to minimise this issue, large samples were utilised and well homogenised before extraction and sub sampling. Furthermore replicate extracts should always be prepared, for example Ehlenfeldt & Prior (2001) prepared six extracts for each cultivar in their experiment. Here the consistency of results across duplicate extracts for two extraction types provided confidence in the analysis.

Total anthocyanin concentrations were calculated as the sum of individual anthocyanins. Therefore the profile of individual anthocyanins could also be compared for each variety. As research on the health properties of blueberries continues to progress it is likely that individual anthocyanins will be identified as being useful for specific health conditions, and therefore this quantification may become important. Generally speaking there are 15 anthocyanins found in blueberries and in some cases additional acylated anthocyanins (anthocyanins with acids attached). The Table 4-2 provides a breakdown of anthocyanin compounds for each cultivar as measured here, along with the proportion of each anthocyanin relative to the total anthocyanin content.

Table 4-2: Individual anthocyanins for various blueberry cultivars measured using HPLC, based on elution order.

Anthocyanins	Highbush Blueberry (<i>V. corymbosum</i>) Cultivar															
	Blucrop	Brigitta	Reka	Dixi	Jersey	Duke	Burlington	Elliot								
	mg/ kg	% total antho	mg/ kg	% total antho	mg/ kg	% total antho	mg/ kg	% total antho	mg/ kg	% total antho						
Delphinidin-3- galactoside	99	12%	252	28%	137	15%	98	9%	216	18%	340	22%	563	26%	657	25%
Delphinidin-3- glucoside	57	7%	4	0%	51	6%	88	8%	102	9%	5	0%	9	0%	11	0%
Cyanidin-3- galactoside	22	3%	31	3%	31	3%	30	3%	35	3%	75	5%	63	3%	83	3%
Delphinidin-3- arabinoside	104	13%	174	19%	135	14%	92	8%	189	16%	171	11%	351	16%	414	16%
Cyanidin-3- glucoside	12	1%	1	0%	13	1%	29	3%	15	1%	2	0%	2	0%	3	0%
Petunidin-3- galactoside	48	6%	121	13%	66	7%	55	5%	100	8%	205	13%	287	13%	311	12%
Cyanidin-3- arabinoside	17	2%	16	2%	25	3%	23	2%	23	2%	30	2%	33	1%	49	2%
Petunidin-3- glucoside	42	5%	3	0%	35	4%	70	6%	79	7%	4	0%	8	0%	9	0%
Peonidin-3- galactoside	4	1%	6	1%	7	1%	8	1%	6	0%	25	2%	16	1%	23	1%
Petunidin-3- arabinoside	40	5%	66	7%	50	5%	39	3%	70	6%	85	5%	148	7%	163	6%
Peonidin-3- glucoside	12	2%	0	0%	11	1%	17	2%	19	2%	0	0%	0	0%	0	0%
Malvidin-3- galactoside	78	10%	144	16%	104	11%	95	8%	120	10%	410	26%	443	20%	592	22%
Peonidin-3- arabinoside	2	0%	2	0%	4	0%	4	0%	3	0%	9	1%	5	0%	8	0%
Malvidin-3- glucoside	78	10%	4	0%	62	7%	121	11%	104	9%	13	1%	13	1%	19	1%
Malvidin-3- arabinoside	78	10%	81	9%	90	10%	80	7%	95	8%	172	11%	224	10%	293	11%
Total non-acylated	694	87%	906	100%	822	88%	850	75%	1176	99%	1544	99%	2164	100%	2636	99%
Total acylated antho	100	13%	2	0%	76	8%	287	25%	17	1%	9	1%	4	0%	9	0%
Total anthocyanins (mg/ kg)	794		909		935		1,137		1,193		1,552		2,168		2,650	

Note: Shading indicates common sugar constituent

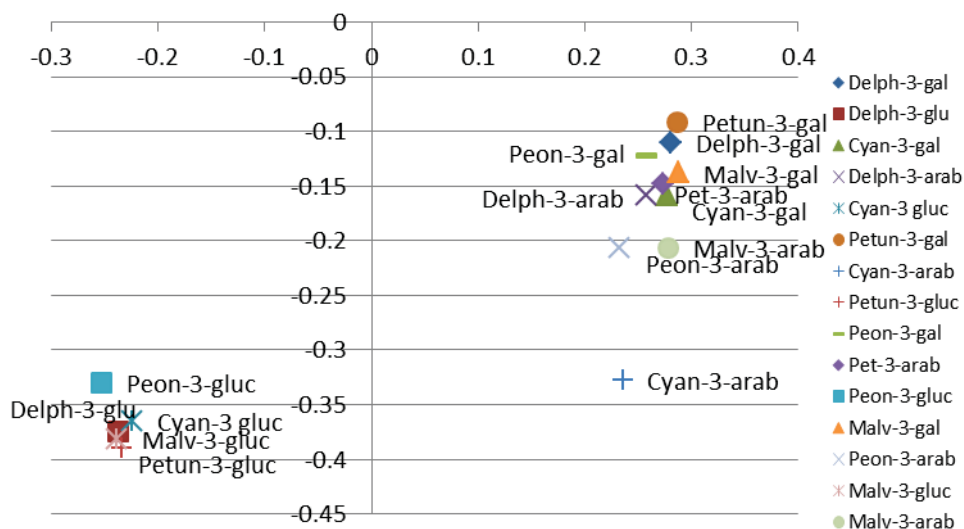


Figure 4-3: Excel scatter plot of latent vector coordinates produced by MINITAB

Upon examination of the raw data cultivars on the left hand side of the PCA score plot ('Reka', 'Dixi', 'Jersey' and 'Bluecrop') all had reasonable amounts of the anthocyanidins with glucose attached, as compared with those on the right hand side ('Burlington', 'Duke', and 'Elliot') which had practically none. Other scientists have also reported a consistent distribution of anthocyanin glycosidic fractions, and suggest that this phenomenon is related to the particular enzymatic background (glycosyl transferase) that each cultivar has, likely to be a result of common ancestors (Brambilla et al, 2008).

Another varietal difference was that some cultivars appeared to have a large number of acylated anthocyanins present, which eluted after the major anthocyanins. Cultivars 'Bluecrop', 'Reka' and 'Dixi' all appeared to have significant levels of acylated compounds with 13%, 25% and 8% of total anthocyanins. Again though, it is difficult to find data in the literature for comparison. Some examples were found for 'Bluecrop' berries where papers by Cho et al (2004), Kalt et al (1999) and Gao & Mazza (1994) reported 6.4%, 11.3% and 13.5% acylated anthocyanins respectively. Acylation may make the anthocyanins more stable against pH, heat and light changes (Skrede & Wrolstad, 2002). Also, as research on the health properties of blueberries continues to progress it is likely that individual anthocyanins will be identified as being useful for specific health conditions. Therefore this quantification may become important.

The identification of anthocyanins was based on the retention times of two external anthocyanin standards (cyanidin-3-glucoside and malvidin-3-glucoside) and the order of elution reported in the literature for UV-spectrophotometric detection. The order of anthocyanin elution in literature was extremely consistent for the first ten peaks (delphinidin-3-galactoside – petunidin-3-arabinoside) and there is a general pattern that the glycosides always elute in the order galactoside, glucoside, arabinoside. However, the order of the last five anthocyanins varied considerably. For instance peonidin-3-glucoside and peonidin-3-arabinoside were sometimes reported as eluting before malvidin-3-galactoside and malvidin-3-glucoside respectively, and other times eluting after. In addition two researchers (Kalt et al 1999, and Gao & Mazza 1994) detected acylated delphinidin-3-galactoside in their samples. However, Kalt et al (1999) reported delphinidin-3-galactoside as eluting before malvidin 3-glucoside while Gao & Mazza (1994) reported it as eluting after.

In the results presented here the order of these last five anthocyanins was selected based on the retention time of the standard malvidin-3-glucoside and the contrast of proportions commonly observed in the literature. Malvidin-3-galactoside and malvidin-3-glucoside were always reported to be present in much higher amounts than peonidin-3-glucoside or peonidin-3-arabinoside. However, the order cannot be finalised with complete confidence due to the limited identification methods employed. Ideally mass spectrometry would have been used in conjunction with the HPLC to accurately identify the compounds.

Total anthocyanin contents of each variety were correlated against berry weight and specific surface area. Correlations are a statistical tool, used to determine the likelihood of a relationship existing between two or more variables. In this case a correlation was often reported by researchers where the smaller fruit had higher anthocyanin contents on a weight basis (Prior et al, 1998; Moyer et al, 2002; Castrejon et al, 2008). This is simply because anthocyanins are primarily located in the skin of the blueberry fruit. Correlations were run separately for total anthocyanin content vs weight and total anthocyanin concentration vs specific surface area. The total anthocyanin concentrations were almost significantly correlated to the berry weight (Pearson correlation index ($P = 0.055$)) but not the specific surface area ratio. This appears to support the supposition that the smallest berries contained the highest

anthocyanin levels on a weight basis. Therefore varieties which produce the smallest berries should be selected to produce a crop with high anthocyanin concentrations.

As mentioned previously, often experimental variation will result in measurement variation. Data presented to this point were based on solvent extracts of each blueberry variety, the most common method reported by researchers. Solvents such as acetone and methanol can break down organic material, as they are able to destroy cell membranes (Naczka & Shahid, 2004), therefore allowing a very efficient extraction. However, water extracts were also prepared, which, from the point of bioavailability, are perhaps more consistent with the environment of the human digestive system. Acidified water (5% formic acid) was found to be preferable over pure water as it prevented the extract turning brown. It was assumed this was due to the lower pH stabilising the anthocyanins. Additionally it was decided that acidified water was probably more comparable with the pH of the digestive system. The anthocyanin concentrations obtained using the two types of extraction can be seen in Figure 4-4.

It was apparent during the acidified water extraction that not all the anthocyanins would be removed from the blueberry material; unlike when acetone and methanol were used it took the acidified water a long time to filter and the remaining press cake was still very coloured. The two types of extracts were shown to be significantly different when a two way ANOVA was used ($P \leq 0.01$), with the factors extract type and variety. On average the anthocyanin concentration was 30% higher in solvent extractions. Brambilla et al (2008) carried out a similar methodical comparison which gave a 32% higher anthocyanin content when blueberries were extracted with an acidified organic solvent, as compared with an acidified water extract.

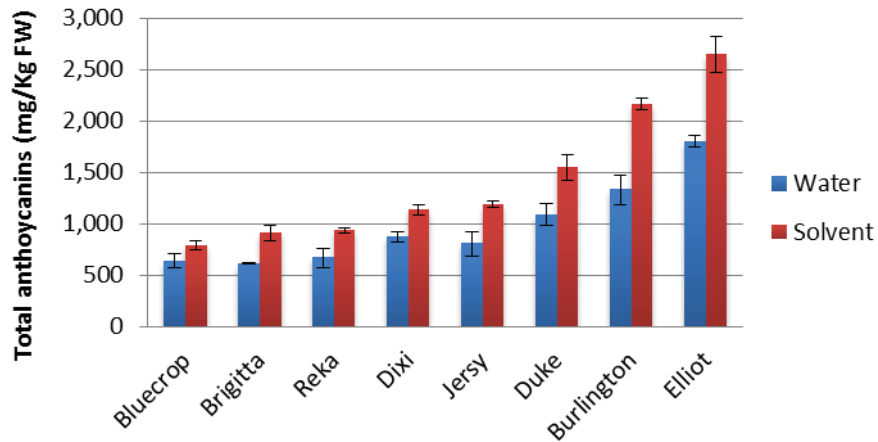


Figure 4-4: Total anthocyanin concentration for different cultivars with different extraction solutions, measured using HPLC

Chlorogenic acid was also measured using HPLC for the eight cultivars as it is the only other blueberry polyphenol known to be present in significant levels. As can be seen in Figure 4-5 the concentration range was similar to that of total anthocyanins. However, the hierarchy of values was somewhat different.

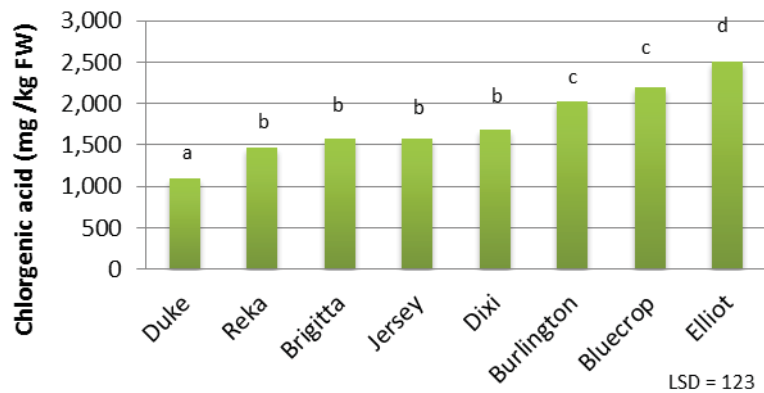


Figure 4-5: Chlorogenic acid contents for various highbush blueberry varieties measured using HPLC

Again cultivars ‘Elliot’ and ‘Burlington’ were amongst the top three highest concentrations. However, ‘Bluecrop’, which had one of the lowest anthocyanin concentrations, had the second highest chlorogenic acid content. Conversely ‘Duke’ which had one of the highest anthocyanin concentrations, here, had one of the lowest chlorogenic acid concentrations. Data for

comparison with other researchers is not as abundant as it is for anthocyanins and reported concentrations vary considerably. For example levels reported for 'Bluecrop' cultivar include 29 – 68 mg/ kg FW (Wang et al, 2008), 214 mg/kg FW (Skrede et al, 2000), 415 mg/ kg FW (Cho et al, 2004), 1,000 mg/ kg FW (Gao & Mazza, 1994) and 1,850 mg/kg (Schuster & Herrmann, 1985). The value presented here for 'Bluecrop', over 2,000 mg/kg FW, is much higher than any of these other experimental values. The wide range of chlorogenic acid values is surprising considering similar experimental methods were employed. One possibility is that its synthesis is perhaps more sensitive to growing conditions than that of anthocyanins. However, these data do appear valid since comparisons were made with a local external laboratory that had tested several hundred genotypes.

From a method perspective it was not known which extraction type would be most suitable for chlorogenic acid quantification, as some literature suggested that chlorogenic acid was more heat sensitive than anthocyanins. The solvent method required the acetone to be evaporated at 50°C before HPLC analysis using a rotary evaporator whereas the acidified water method did not. However, the values from the solvent extract were significantly higher than those obtained for the water extract (data not shown). Therefore the solvent method was still preferable for obtaining the maximum chlorogenic acid value.

4.1.2 Maturation differences

In order to investigate changes during berry maturation samples of green and red berries from thirty year old 'Dixi' plants were analysed using HPLC. Not surprisingly green berries had very low concentrations of anthocyanins with 11 mg/ kg FW. Red berries contained some anthocyanins 211 mg/ kg FW, which was still only 1/6 of the 1,137 mg/ kg FW seen in ripe 'Dixi' berries.

The concentrations of chlorogenic acid in green and red berries, with 1,700 mg/ kg FW and 1,520 mg/ FW respectively, were very similar to the level of chlorogenic acid seen in ripe 'Dixi' berries, an average of 1,684 mg/ kg FW.

In terms of production of individual anthocyanins it appeared the proportions were similar at both red and blue stages of maturity, suggesting that the maturity of the berry do not affect the proportions of individual anthocyanins present.

Only one cultivar was tested, so there may be differences during maturation depending on the variety. Only a single sample of each berry maturity was extracted, therefore a statistical analysis could not be done and there is a risk that significant variation within a cultivar was not quantified. Lastly, in terms of material selection the sample for each level of maturity was selected visually rather than by colour measurement, so the experiment is not precisely replicable.

These results are a little different from those reported by Castrejon et al (2008) who assessed four highbush blueberry varieties over five stages of maturation, two unripe, and three harvests once commercially ripe. They reported that chlorogenic acid was present in the highest concentrations in unripe berries, for all cultivars, and then steadily degraded during maturation along with the small amounts of flavonols which were measured. Conversely anthocyanins were detected only in ripe berries. Interestingly though, the anthocyanin concentration was seen to increase upon consequent harvests of these same plants. Therefore the potential for maturational differences once berries are commercially ripe is an issue which should be considered carefully when quantifying such compounds across different cultivars, since this study observed that changes in anthocyanin and chlorogenic acid levels continue to take place post commercial maturity. However, the chlorogenic acid concentration remained higher than the anthocyanin concentration throughout maturation, even after ripening, up until the third harvest (last harvest). Another significant finding from this study was that changes in anthocyanins were not seen to correlate with the changes in antioxidant activity; instead, “decreasing contents of hydroxycinnamic acids during maturation and ripening mirrored more or less the pattern of antioxidant activity” (Castrejon et al 2008). This indicates that perhaps chlorogenic acid may contribute more significantly to the total antioxidant capacity of a blueberry than is generally recognised.

For plant maturity a comparison of berries from ten year old ‘Dixi’ and ‘Burlington’ plants vs thirty year old plants was analysed using the HPLC. For 10 year old ‘Dixi’ the anthocyanin concentration was 1,345 mg / kg FW, similar to the 1,137mg/ kg FW that was obtained for 30 year old plants. However the concentration for young Burlington plants, 3,054 mg/ kg FW was much higher than was obtained for thirty year old plants at 2,168 mg/ kg FW. Rather than being

a difference caused by plant maturities, potentially this could have been a result of slightly different berry maturities as samples were picked during the same visit but it is known that young plants ripen earlier than older plants. This is another issue which should be taken into account for any blueberry sampling: both different cultivars and plants of different ages will ripen at different times. The other conclusion that could be drawn from this analysis was that the proportions of individual anthocyanins for the young plants and old plants were comparable, suggesting that the maturity of the plant does not influence the proportion of individual anthocyanins present.

Again in this experiment only one sample of each maturity was extracted so a statistical analysis could not be done and there is a risk that significant variation within the cultivar has not been quantified.

4.1.3 Implications for juice manufacture

It was confirmed that the smallest blueberries had the highest anthocyanin concentrations on a weight basis. These were 'Elliot', 'Burlington' and 'Duke'. The hierarchy of values seen for the concentration of chlorogenic acid did not completely match that seen for anthocyanin concentration. Instead 'Elliot', 'Bluecrop' and 'Burlington' had the three highest concentrations, while 'Duke' had the lowest. Therefore in order to obtain maximum levels of both anthocyanins and chlorogenic acid in blueberry juice ideally 'Elliot' or 'Burlington' would be selected as the raw material. Additionally, using ripe berries would ensure maximum concentrations of anthocyanin and chlorogenic acid in the juice. It is believed that the age of the plant does not affect anthocyanin or chlorogenic acid concentrations so berries from any age of plant could be used.

However, if individual anthocyanin(s) become important for specific health conditions further consideration should be given as to which cultivar is selected for juice manufacture. The concentrations of individual anthocyanins seen in each cultivar were extremely diverse. For example 'Bluecrop', 'Dixi' and 'Duke' had delphinidin-3-arabinoside (13%), malvidin-3-glucoside (11%) and malvidin-3-galactoside (22%) as their most prevalent anthocyanins while all other cultivars ('Brigitta', 'Reka', 'Jersey', 'Burlington' & 'Elliot') had delphinidin-3-galactoside as their most prevalent (with proportions ranging from 11-28%).

4.2 Juice Production Analysis

4.2.1 *Standard Manufacturing Analysis*

Blueberries are turned into a value added product by processing them into a natural blueberry juice. The juice is made primarily from the three oldest cultivars which are consequently harvested in the largest volumes – ‘Dixi’, ‘Burlington’ and ‘Jersey’.

Two small batches of blueberry juice were produced at Mamaku Blue using approximately 80% ‘Dixi’ machine-harvested berries & 20% overripe or damaged reject berries. Samples were collected for analysis throughout processing so that changes in the anthocyanin levels during manufacture could be measured. In addition the process streams were tracked and weighed so that mass and chemical balances could be completed to quantify total losses. This information was then used to determine where the greatest losses occurred and consequently what areas should be focused on when trying to improve the yield and/or retention of juice components. The literature review had identified that heating before pressing could improve the extraction of anthocyanins. Therefore, in order to investigate this theory two separate batches of juice were produced from the same thawed blueberries. The first batch was split in two (due to capacity restraints) and was pressed without any prior heating (‘cold press’). The second batch was split into two and heated in two large vats to between 20 – 30°C, then pressed together (‘hot press’). It is important to note that the ‘hot press’ process involved a significantly larger amount of manual work since the berries had to be heated in vats and then pumped out into the presses.

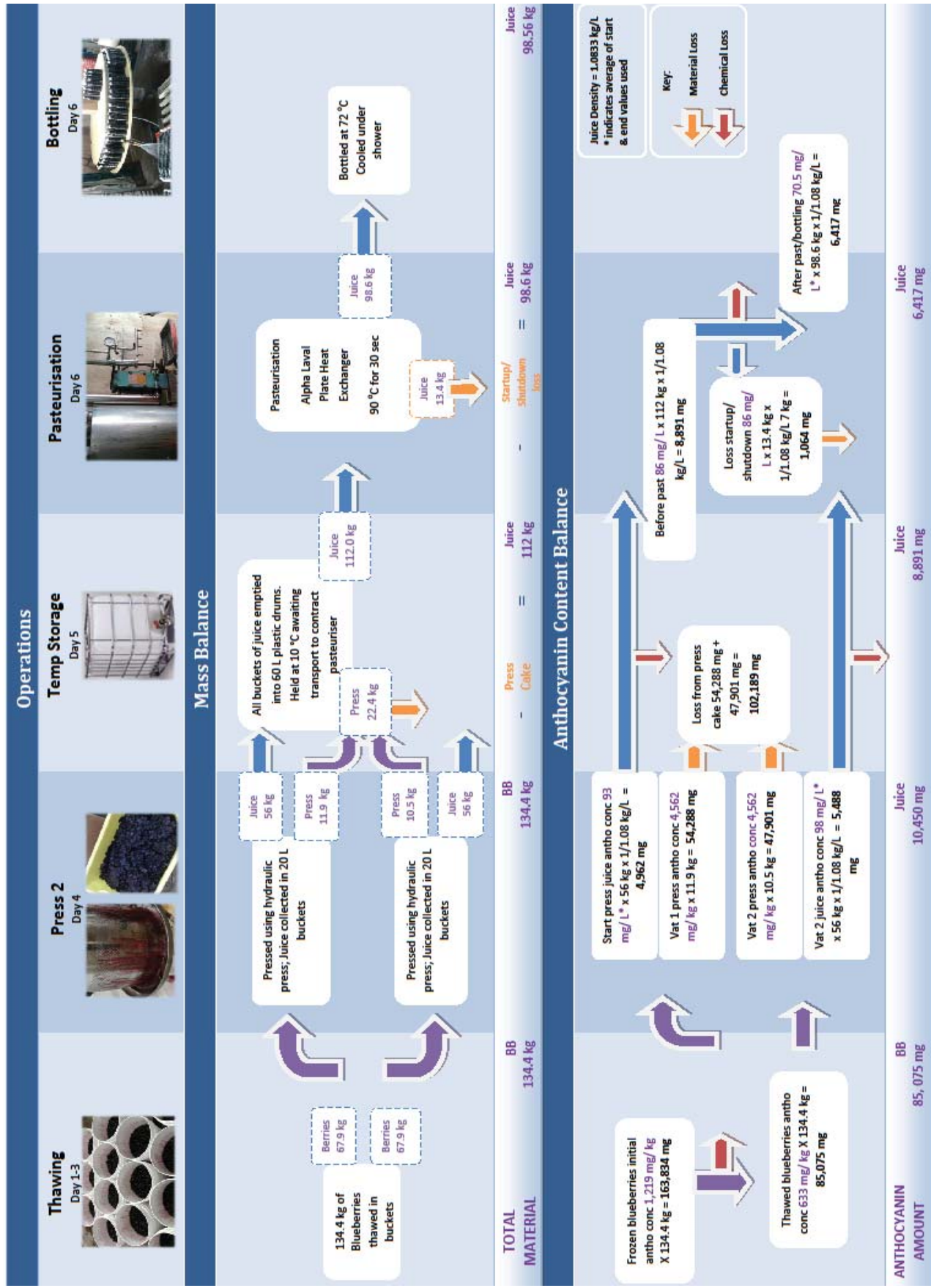


Figure 4-6: Cold press blueberry juice production - flow diagram with chemical and material losses.

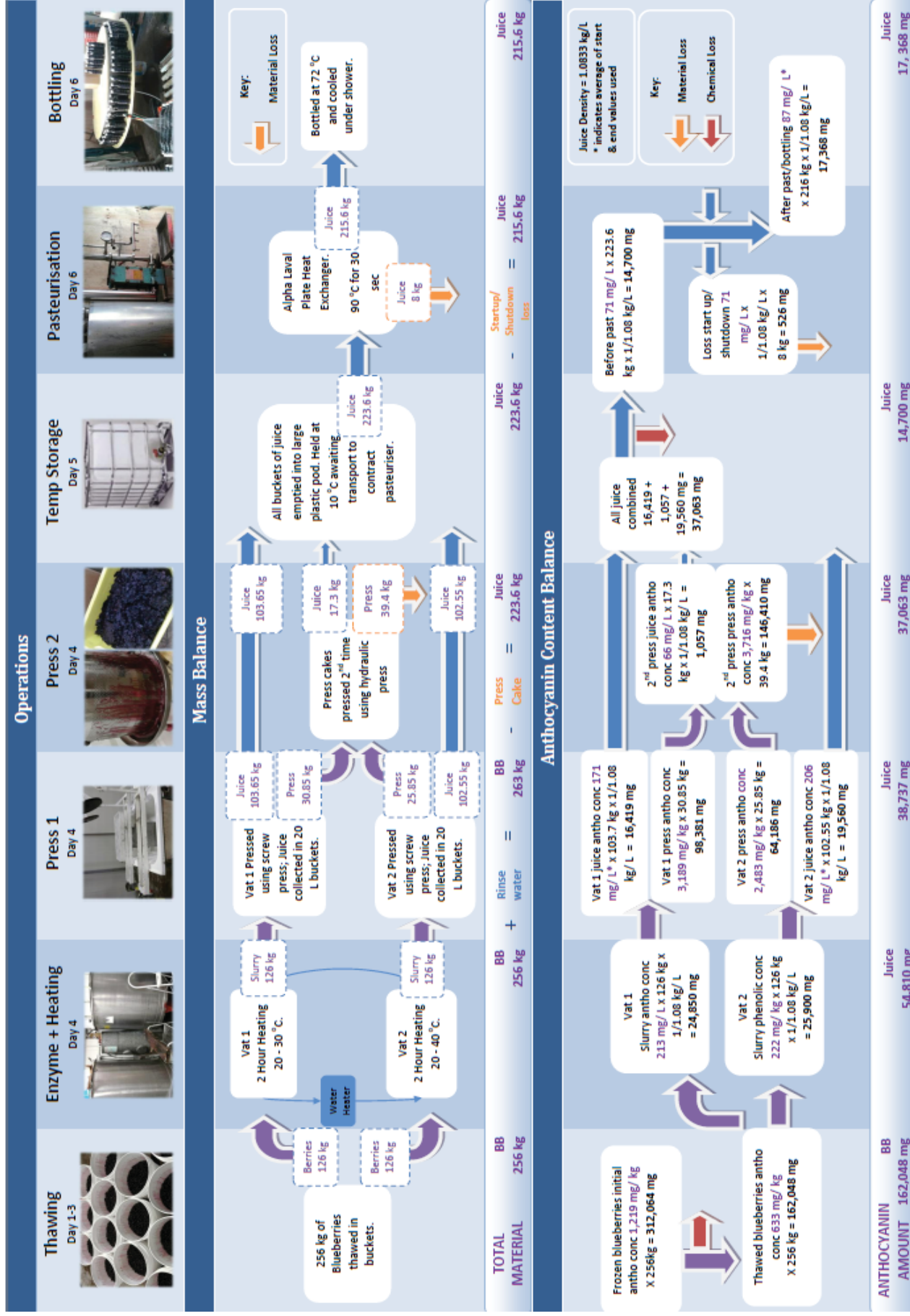


Figure 4-7: Hot press blueberry juice production - flow diagram with chemical and material losses.

Material Recovery

The mass and anthocyanin balances shown in Figures 4-6 and 4-7 depict both the physical and chemical changes that occurred during juice processing. The same two material losses occurred during manufacture for both 'hot press' and 'cold press' batches. The first was removal of the press cake and the second involved small losses during pasteurisation. This pasteurisation loss could have been a result of a combination of two factors, firstly a start-up/shut-down loss within the pasteuriser (believed to be the primary reason) and secondly evaporative losses of water due to heating of the product. If the start-up/shut-down loss was the primary cause then the proportion of juice lost would be minimised by processing as large a batch as possible.

Interestingly, the overall percentage recovery of material into the juice was similar for both types of processes. For the 'hot press' the recovery of original material (250 kg) into the juice after pressing was about 87%. Approximately seven litres of juice was then lost during pasteurisation, giving a final percentage recovery of 84%. For 'cold press' the recovery of juice on a weight basis after pressing was again reasonably high, with an average value of 83%. Approximately eleven litres of juice were lost during pasteurisation, giving a final percentage recovery of 73% for the bottled juice. This final percentage recovery of the 'cold press' juice was only lower than that of 'hot press' because the batch size was smaller. Therefore, these values suggest that essentially the yield of juice was not improved by the 'hot press' method tested here.

Overview of material and chemical losses

The first step in processing was thawing of the berries, where a substantial anthocyanin loss was observed. The anthocyanin concentration of frozen berries was 1,219 mg/ kg which dropped to 633 mg/ kg in berries collected at the end of the three day thawing period, a loss of approximately 50%. The temperature of the berries exiting storage was -20°C. This temperature had risen to 2°C by the end of the three day thawing period in a pack-house with an air temperature of approximately 10°C. The literature suggested that this loss would have been caused by enzymatic degradation, namely by the enzyme polyphenol oxidase. Polyphenol

oxidase reacts with chlorogenic acid which is abundant in blueberries, forming a compound which can polymerize anthocyanins. Thawing over a shorter time period may assist in minimizing this loss. Additionally, briefly exposing the berries to high temperatures (85°C +) at the beginning of processing may deactivate oxidative enzymes and minimise degradation throughout manufacturing (Krader et al, 2002). This has been common practice for a number of researchers investigating the optimisation of blueberry juice manufacture. Some researchers have compared the anthocyanin content of heat treated juice with a control (i.e. non heat treated juice) and showed an increase of anthocyanins in the final product (Lee et al (2002) and Rossi et al (2003): 56% increase and 51% increase respectively), with an increase in chlorogenic acid (34%) also reported by Rossi et al (2003). Other researchers have seen the positive results found by previous researchers so have not used a control for comparison when employing a blanching pre-treatment (Strivastava et al (2007) and Brownmiller et al (2008)). A limited number of researchers have included enzyme assays to actually measure PPO activity and quantify the processing effect. One example of this was Buckow et al (2010) who reported that a berry blanching step resulted in a 34% increase in anthocyanins, a 26% increase in total phenolics and 100% inactivation of PPO.

During pressing, solid material is separated from the liquid (juice). As expected the removal of the press cake, consisting mostly of blueberry skin where the anthocyanins are primarily located, significantly reduced the amount of anthocyanins in the juice processing stream. On the basis of the analysed anthocyanin concentration and the weight of press cake 146,400 mg of anthocyanins were removed with the 'hot press' press cake as compared with the 37,000 mg which remained in the juice. This difference is large. Even though the presscake was much smaller in volume, it contained a total amount of anthocyanins: about 4 fold the total amount of anthocyanins seen in the juice. Therefore breaking down the press cake and incorporating it back into the juice processing stream could significantly increase anthocyanin concentrations in the juice. This might be achieved simply by milling or by the use of additional enzymes to break down the blueberry skin such as cellulase. Alternatively, much more expensive options may be some form of solvent or supercritical fluid extraction. However, due potentially to the large

expense, these latter options may be more suited to an additional high-end nutraceutical product which could be sold for a higher price.

The measured anthocyanin concentration of the solid material was consistent with the treatment and point in processing (Figure 4-8). Initially there was a large loss of anthocyanins and chlorogenic acid upon thawing. Then as more water was removed from the solid material to produce juice the anthocyanin concentration of the press cakes became more and more concentrated. Additionally 'Vat 2 press cake' had a lower concentration of anthocyanins than 'Vat 1 press cake' as it had been heated to a slightly higher temperature, and therefore more anthocyanins had been extracted into the juice. The final level of anthocyanins in the press cake was 4 fold that seen in frozen berries, making the cake a valuable source of anthocyanins. Dissimilarly chlorogenic acid was not significantly concentrated into the solid material by processing, with about 1/3 the level of frozen berries. Skrede et al (2000) make similar observations, pointing out that chlorogenic acid is much more extractable than anthocyanins because it is more water-soluble and is found in the cell vacuole rather than being associated with cell wall material.

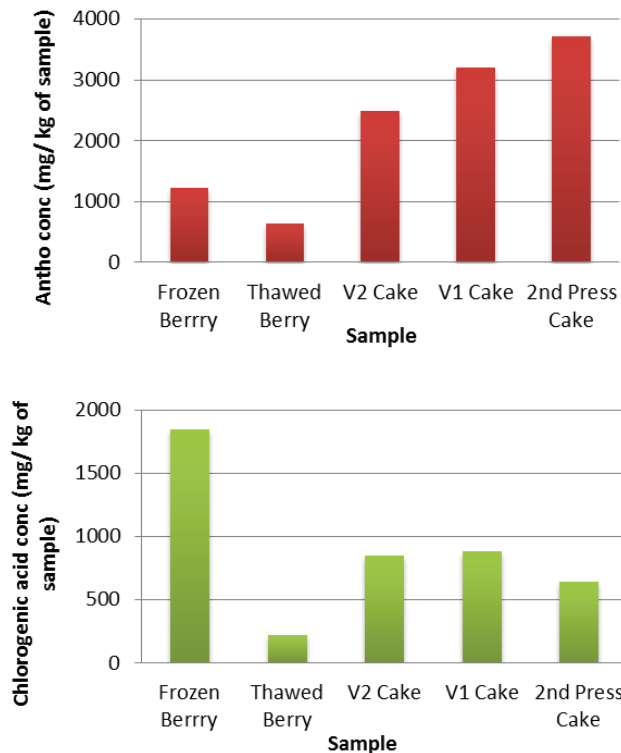


Figure 4-8: Anthocyanin concentrations of berry and press cake samples during 'hot press' processing.

During pressing the juice was collected in the same buckets that the berries had been thawed in. At the end of pressing the ‘hot press’ juice was combined in a large plastic vessel and held for approximately 40 hours before pasteurisation. A large decrease in anthocyanin concentration occurred during this holding period. The total amount of anthocyanins at the end of pressing (start of the holding period) was calculated as 37,000 mg. This had dropped to 14,700 mg by the end of the holding period, a loss of about 50% (see figure 4-9). The juice temperature at the end of pressing (start of holding) was between 15-18°C which would have slowly dropped to the pack-house air temperature of 10°C. Again, it was assumed that anthocyanin degradation might have occurred due to enzymic activity. However, it was surprising that the cold press juice only degraded comparatively slowly over the same holding period. This juice was stored in two 60 L plastic barrels within the same facility. Other options for retaining the anthocyanin levels seen initially for ‘hot press’ juice might be minimising holding time or colder storage of the juice between pressing and pasteurisation. Pasteurisation should then deactivate the enzyme polyphenol oxidase preventing further enzymatic degradation from occurring. However, this may not be nearly as effective as employing enzymatic deactivation right at the beginning of processing. Figure 4-9 below gives the measured anthocyanin concentrations of the juice during processing.

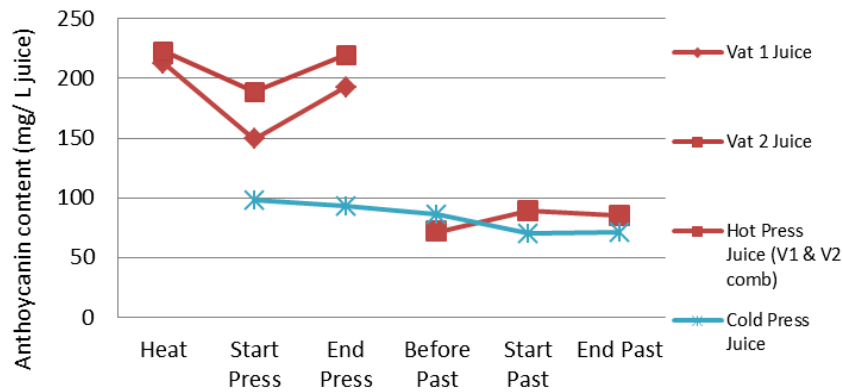


Figure 4-9: Anthocyanin concentrations in blueberry juice during processing as measured by HPLC

The final processing step was pasteurisation. In ‘hot pressing’ a small material loss occurred during pasteurisation (see mass balance) but was counteracted by a slight increase in the

anthocyanin concentration of the bulk juice (as shown in Figure 4-9), giving a net increase in anthocyanins. The increase in anthocyanin concentration after pasteurisation could have occurred because of concentration due to evaporation and additional extraction from sediment remaining in the juice. Perhaps breakdown of the press cake and incorporation into the juice after pressing would allow for further additional extraction at this point of the process. The concentration of anthocyanins in ‘cold press’ juice was seen to slightly decrease throughout processing, including during pasteurisation. This change after pasteurisation was again not large. Perhaps the difference in responses could be attributed to a difference in sediment levels. These findings are consistent with previous reports that the anthocyanins are relatively stable during pasteurisation of freshly processed blueberry juice (Skrede et al, 2000; Lee et al, 2002).

Changes to individual anthocyanins were also quantified. The first comparison to be made is between frozen and thawed berries as seen in Figure 4-10. Both were analysed from a solvent extract.

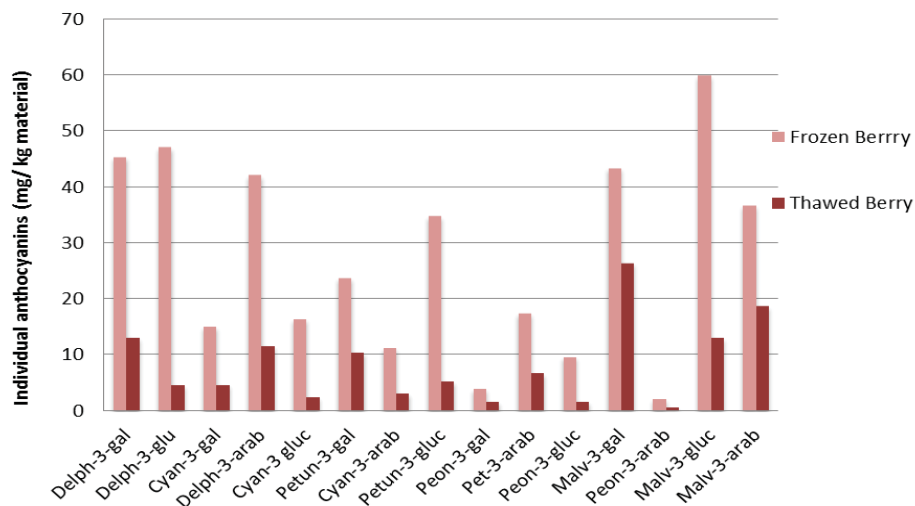


Figure 4-10: Changes to individual anthocyanins before and after thawing of blueberries

Significant changes in the proportions of individual anthocyanins could be observed from this very first step of the juice making process. Most predominantly it was apparent the malvidin glycosides were retained better than all other anthocyanins, with other initially predominant anthocyanins such as delphinidin-3-galactoside and delphinidin-3-glycoside

degrading significantly. These samples were compared with the profile observed for the original solvent extract of ‘Dixi’, since this was the primary cultivar used in the juice. In Dixi extracts malvidin-3-glucoside was identified as the most prevalent anthocyanin with delphinidin-3-galactoside the second most prevalent and delphinidin 3-glucoside only slightly behind. Here the frozen berry sample had a very similar profile suggesting that it was a ‘Dixi’ extract. The only difference was that in this case the delphinidin-3-glucoside content was slightly higher than that of delphinidin-3-glucoside.

A very small increase in the proportion of malvidin derivatives was observed throughout manufacturing. The profiles of selected juice samples during different points of processing can be seen in Figure 4-11. As consistent with the overall increase seen after pasteurisation the concentration of all individual anthocyanins increased slightly here.

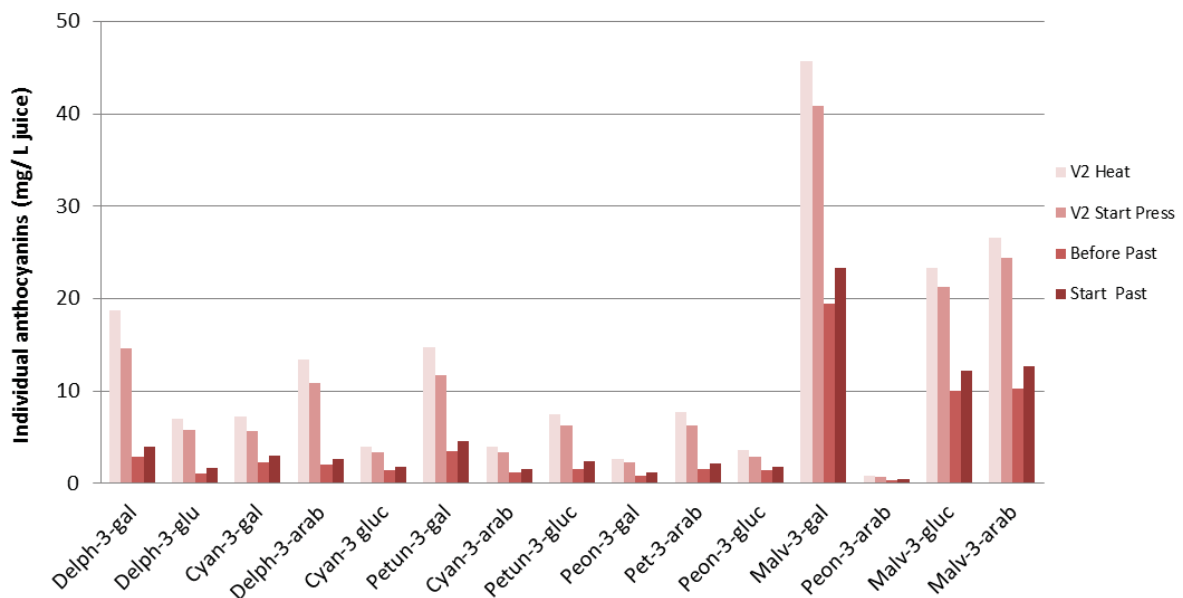


Figure 4-11: Changes to individual anthocyanins in blueberry juice at different stages of processing

Similar trends were observed by Skrede et al (2000), Lee et al (2002) and Brambilla et al (2008) who reported delphinidin derivatives were the least stable as their relative proportion in the juice decreased in favour of malvidin derivatives, which increased proportionally. Additionally Srivastavea et al (2008) reported large amounts of malvidin and peonidin in their blueberry juice, which were more stable than other minor anthocyanins, but they observed

peonidin as being slightly more stable than malvidin. This is reported to be related to the two methoxyl substituents on the B ring which, due to a lesser degree of polarity, are not as reactive as the hydroxyl groups found on delphinidin anthocyanins (Skrede et al, 2000; Rossi et al, 2003; Srivastava, et al 2007).

Chlorogenic acid levels were also quantified during juice manufacture, with varying results observed during processing (Figure 4-12). Again a loss was observed in ‘hot press’ juice between the start and end of the holding period (‘end press’ vs ‘before past’). In comparison ‘cold press’ juices were seen to have consistently higher levels of chlorogenic acid as compared with ‘hot press’ juices, suggesting that chlorogenic acid is fairly heat sensitive. However, surprisingly this level increased after pasteurisation for both types of juice. Again this is potentially the result of two effects, further extraction of some of the sediment such as crushed blueberry seeds and concentration of the juice, due to evaporation of water during pasteurisation.

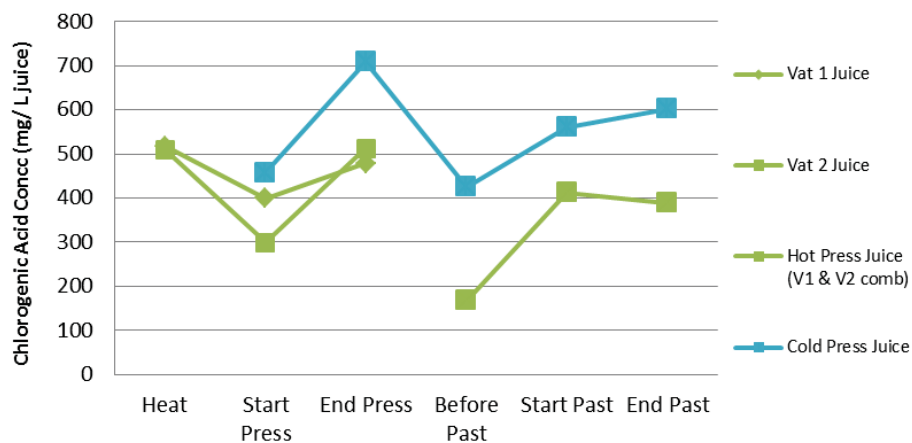


Figure 4-12: Changes to the concentration of chlorogenic acid during juice processing

Discussion

In terms of material recovery, surprisingly similar recovery percentages were achieved for both ‘cold press’ and ‘hot press’ batches of juice. After pressing the material, recovery of ‘hot press juice’ was 87%, as compared with 83% for ‘cold press’ juice. As discussed previously the amount of loss on start-up and shut-down during pasteurisation was a fixed amount. This

meant the recovery for cold press juice after pasteurization was a little smaller as a percentage because the original size of the batch was only half that of the 'hot press' juice.

As was expected the anthocyanin levels in the 'hot press' juice after pressing were higher than those in the 'cold press' juice. Interestingly though, during the 40 hour storage period the anthocyanin levels in the hot press juice dropped dramatically. It is suspected that this degradation could be a result of PPO activity, which will be greater at higher temperatures resulting in more degradation.

The different responses of the two juices to pasteurisation could be related to the level of sediment in each juice. It is feasible 'hot pressing' resulted in more sediment present in the 'hot press' juice after pressing than the 'cold juice'. Therefore pasteurisation slightly increased the anthocyanin concentration of the 'hot press' juice by further extraction from the sediment. However, overall, the relatively small response to pasteurisation does suggest that anthocyanins in freshly processed blueberry juice are relatively stable to heat, even at high temperatures. This is consistent with what was reported in the literature on juice processing. Additionally pasteurisation is expected to have heat deactivated PPO, preventing further degradation by this enzyme.

Overall the 'hot press' processing was physically much more work and did not result in much additional material recovery as compared with 'cold press' processing. Although a significantly higher anthocyanin content was achieved initially with the 'hot press', this degraded during temporary storage to be only slightly higher than 'cold press' juice, making the additional work arguably worthless. However, as per Figure 4-13 this difference in anthocyanins did result in a slightly higher response of 'hot press' juice to antioxidant tests. As is often reported by researchers, anthocyanins usually correlate well with fresh juice antioxidant status (Brambilla et al, 2008). If PPO was the cause of the anthocyanin degradation seen in 'hot press' juice then perhaps blanching berries before pressing may allow for retention of the anthocyanin levels seen upon pressing. Ultimately more work to improve the extraction and retention of anthocyanins using the 'hot press' method may result in better antioxidant properties in the final product.

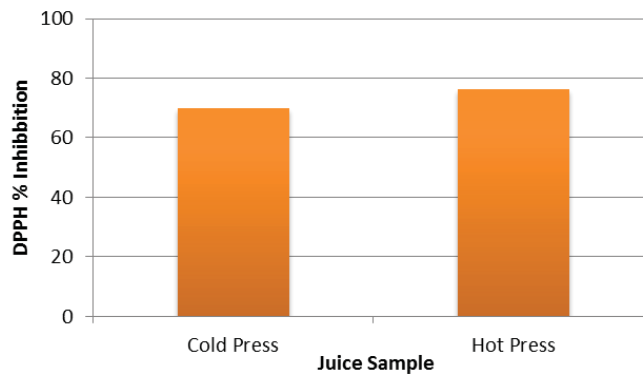


Figure 4-13: Antioxidant activity of blueberry juice samples immediately after pressing, as measured by DPPH inhibition

4.2.2 Alternative Processes

For this set of experiments juice was produced in the pilot plant at Massey University using the 'Burlington' variety, which was selected due to its having the highest anthocyanin levels out of the three machine harvested varieties. Variables used in manufacturing were selected on the basis of the outcome of both the literature review and findings from analysis of juice manufacturing on the orchard. These variables included a slow defrost of blueberries (trials 1-4) vs a fast defrost with an 85°C heat treatment (trials 5-7), milling of berries (trials 3 & 7 only) or no milling and variations in juice holding time and holding temperature between pressing and pasteurisation. A full factorial design was not used due to time restraints (i.e. all possible combinations of variables were not run). Furthermore in the case of fast defrost trials the effects of a fast defrost and 85°C heat treatment were confounded. The only way to separate these two effects would have been to run another set of trials with a combination of a slow defrost and 85°C heat treatment, but it was assumed the former would be more valuable. Additionally the entire experiment was not replicated. Only replicates of trials 1 and 5 were carried out. These were trials 4 and 7 respectively. Data can be found in Table 4-3, which is split into levels to indicate which data can be compared. Any data can be compared with a treatment colour of the same shade and treatments shaded lighter. For example, data for 'thawed berry' can be compared only with 'defrost type', either slow or fast, whereas the 'hold end' and 'after pasteurisation' data can be compared with all the treatments that were used to produce the juices.

Table 4-3: Anthocyanin concentrations at various points of blueberry juice processing as seen in juice produced using different methods and measured using HPLC.

Trial	Treatments					Anthocyanin Concentration							Chloro After Past Juice (mg/ L)
	% Recovery	Defrost Type (Slow/F ast)	Milling (Yes/N o)	Hold Time (Hours)	Hold Temp (°C)	Frozen Berry (mg/ kg)	Thawed Berry (mg/ kg)	Press Cake (mg/ kg)	Press Juice (mg/ L)	Hold End Juice (mg/ L)	After Past Juice (mg/ L)	Month Storage (mg/ L)	
1	66.70%	S	N	45	Amb	1,752	738	2,103	174	84	100	39	477
1	66.70%	S	N	45	chilled	1,752	738	2,103	174	112	150	68	555
2	63.14%	S	N	1	Amb	1,752	885	2,632	174	*	184	93	668
2	63.14%	S	N	1	chilled	1,752	885	2,632	174	141	187	96	630
4 (R1)	62.30%	S	N	35	Amb	1,752	1,086	2,367	150	77	91	118	413
4 (R1)	62.30%	S	N	35	chilled	1,752	1,086	2,367	150	104	144	54	534
3	75.63%	S	Y	1	Amb	1,752	1,034	4,481	123	109	210	44	733
3	75.63%	S	Y	45	Amb	1,752	1,034	4,481	123	104	124	78	587
5	76.76%	F	N	1	Amb	1,752	1,082	3,525	769	779	911	823	1,469
5	76.76%	F	N	48	Amb	1,752	1,082	3,525	769	608	762	687	1,244
5	76.76%	F	N	48	chilled	1,752	1,082	3,525	769	601	914	877	1,379
6 (R5)	74.64%	F	N	1	Amb	1,752	1,066	2,989	745	768	946	855	-
6 (R5)	74.64%	F	N	48	Amb	1,752	1,066	2,989	745	656	767	661	1,327
6 (R5)	74.64%	F	N	48	chilled	1,752	1,066	2,989	745	765	944	890	1,413
7	86.50%	F	Y	1	Amb	1,752	1,137	3,970	656	630	722	618	-
7	86.50%	F	Y	35	Amb	1,752	1,137	3,970	656	581	657	582	1,255
7	86.50%	F	Y	35	chilled	1,752	1,137	3,970	656	573	712	648	1,251

Note: 'R' indicates replicate

* No Sample

Material Recovery

As can be seen in Table 4-3 the material recovery of juice in the first four experiments was acceptable in all instances but not quite as good as that achieved at the orchard. For the last three trials with a fast defrost, an 85°C steam treatment actually added water to the juice. Within the steam blanching pan the berries sat in a mesh cone, with a pan underneath to catch dripped juice. However, it appeared that condensation was also caught in this pan, as after this operation the weight of juice increased by 224 g on average (32%). This meant that after pressing the percentage recovery of juice was artificially inflated. Some practical issues were faced in these trials, most significantly that a mass balance was much more difficult to complete with small scale production, in this case < 10 kg berries compared with the analysis that had been carried out at the orchard using 250 kg of berries. Some of the areas where issues occurred were the removal of material for samples and losses in containers (particularly during milling).

Overview of material and chemical losses

Surprisingly, although a loss of anthocyanins was seen during thawing (column 8, Table 4-3) there appeared to be no significant differences depending on the thawing type - slow thawing (24 – 25 h at 20°C) or fast defrost with heat treatment (3 min to reach temp 85°C, held for 5 min and 3 min to cool to 30°C). Looking down the column it actually appeared that the total anthocyanin level of thawed berries increased through consecutive trials, which could be due to an improving extraction technique upon analysis.

Differences could be observed at the point of pressing (columns 9 & 10, Table 4-3). Most notably a much higher anthocyanin concentration was apparent for all juices produced using an initial 'fast defrost' (85°C steam treatment on frozen berries) (trials 5 – 7) than the concentrations achieved in juice produced from defrosting using 'slow defrost' and juicing without a berry steam treatment (trials 1 – 4). For example the average anthocyanin concentration after immediate pressing in 'slow defrost' trials (without milling) was 166 mg/kg whereas the average value for trials with a 'fast defrost' (without milling) was 757 mg/kg. It is likely that the high temperatures allowed a higher extraction of anthocyanins from the blueberry skin into the juice as well as more physical sediment. The range of values obtained for 'fast defrost' juice was similar to that reported by Brambilla et al (2008), who produced juice from blanched berries using 5 different Northern Highbush varieties.

However, the two different thawing treatments produced juice with quite different sensory properties, in terms of taste and texture. The juice which had been heated to 85°C had a much more astringent, cooked flavour and a much thicker texture. Although an enzyme treatment was used containing pectinase, cellulase and amylase this did not completely break down the gel. Other researchers noted that blueberries contain a significant amount of 'mucilaginous' material which can contribute to the gelling (Bates et al, 2001 as cited in Nindo et al, 2005). Cold press extracted juice has a light blue colour and delicate flavour, while hot press enzyme treated juice has a deep purple-blue colour and stronger flavour. Therefore, although the heat treatment significantly increased the anthocyanin concentration it had what was perceived as a detrimental impact on the sensory properties of the juice. Additionally juice was produced using both thawing treatments where the berries were milled before pressing using a food processor. However, the addition of milling was actually seen to have a slight detrimental impact on the anthocyanin concentration and extra material losses were apparent. However, potentially an alternative method of milling may be more suitable as the method used may have introduced additional air in the system which can stimulate oxidative losses.

Using the 'Burlington' variety to try and increase the anthocyanin level of the juice appeared to be somewhat successful. The juice produced using the orchard's facilities had an anthocyanin concentration of 87 mg/L. The juice produced using an equivalent process here (trial 1 amb and trial 4 amb table 4-3) had an average concentration of 96 mg/L. However, without further replication it is difficult to be certain how big this difference actually is.

Holding freshly pressed juice at two different temps (chilled and ambient) was also investigated, along with the length of holding. The concentration of anthocyanins after one hour of holding were not dissimilar to the concentration measured immediately after pressing, whereas significant levels of degradation were seen in samples held for >35 h. chilling the juice during holding was not particularly effective at preventing degradation, and in one case ambient storage gave concentrations slightly higher than chilled storage.

One of the main reasons for investigating the 85°C heat treatment was to see if it would prevent degradation of anthocyanins in the juice after pressing through inactivation of the enzyme polyphenol oxidase. The same absolute amount of anthocyanin loss occurred in both 'slow defrost' and 'fast defrost' juices over the holding period. Despite this however, if

the degradation of anthocyanins does indeed follow first order kinetics, then the fact that the degradation had proportionally decreased using a ‘fast defrost’ treatment would indicate some success.

Figure 4-14 shows the anthocyanin concentration for samples obtained during the holding period between pressing and pasteurisation, as intermediate values are not given in Table 4-3. Results are shown for juice produced using the two types of defrost (slow or fast), with duplicate results for each holding temperature.

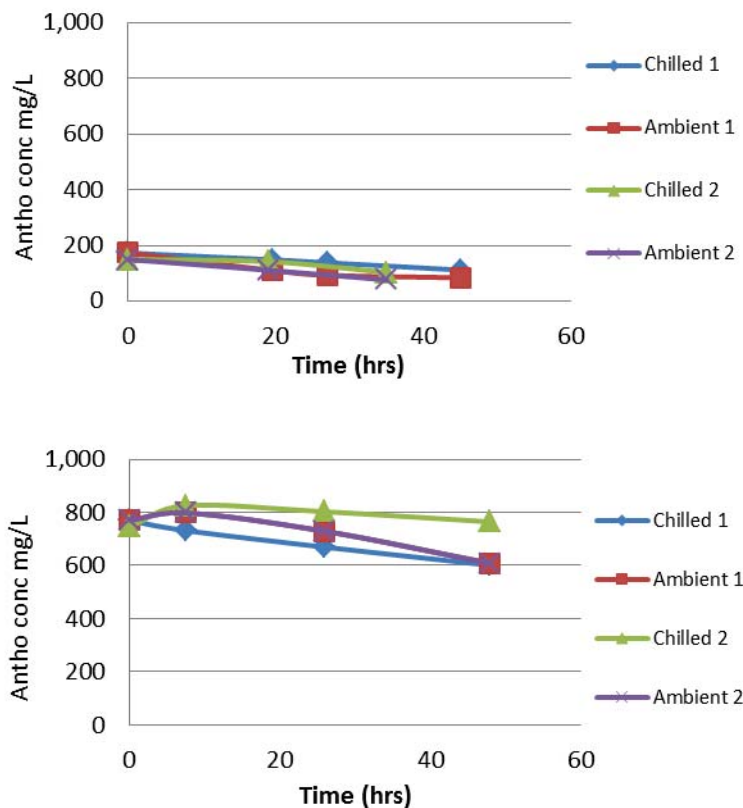


Figure 4-14: Anthocyanin levels in juice held between pressing and pasteurisation for an extended period of time;
TOP: Juice produced using slow defrost; BOTTOM: Juice produced using fast defrost.

As seen in figure 4-14 the initial concentrations of replicate juices produced with a slow were 174 mg/ L (trial 1) and 150 mg/ L (trial 4) which degraded by the end of the holding period to 84 mg/ L (45 h) and 91 mg/ L (36 h) respectively. Unfortunately here the holding times were slightly different, but taking this difference into account the concentrations seem similar. Furthermore, the absolute losses were equivalent to that observed in juice prepared at the orchard (note the scale on graphs in figure 4-14 is larger than that seen in 4-

9). Data are not shown for samples analysed from juice produced using milled material. However, similar rates of degradation were observed.

This information should be compared to the differences seen after pasteurisation of each juice (column 12, Table 4-3). From literature and previous experimentation it was known that anthocyanin levels often increase slightly after pasteurisation, presumably due to concentration and/or further extraction of anthocyanins from sediment. Surprisingly though, juice which had been stored at chilled temperatures gave a much greater increase in anthocyanin concentration after pasteurisation than juice stored at ambient temperatures. This may further support the conclusion that the observed increase in anthocyanin concentration was a result of evaporation, since juice at a lower temperature would require more heating allowing more evaporation to occur. Due to the small amount of material being processed a 'mock' pasteuriser was used, consisting of a stainless steel bucket sitting in a water bath in a steam jacketed kettle. Even though pasteurisation took merely a few minutes, this type of set-up would inherently allow water loss through evaporation. Unfortunately, with the logistics of handling so many treatments and the removal of samples tracking the weight of the juice was overlooked. However, if just the weight of juice before and after pasteurisation had been measured then this speculation could be confirmed.

All juices were stored at 15°C in 80 ml Schott bottles during the one month holding period. At the end of the holding period no large trends were apparent in the data but some small differences were observed across treatment types. For example 'fast defrost' juices stored at chilled temperatures during extended holding fared better after one month than those stored at ambient. However, this difference was not consistent across all the data. Overall the rate of degradation during storage appeared to be similar for all juices.

Individual anthocyanins for some of the alternative processes were also quantified. Figure 4-15 shows the individual anthocyanin concentrations in juice produced using the two different types of defrost. Malvidin derivatives remained dominant in the juice produced using the 'slow defrost' method, most similarly to Mamaku's own product (top). However, the delphinidin and petunidin derivatives were surprisingly dominant in fast defrost juice (bottom). Brambilla et al (2008) reported a similar phenomenon "the anthocyanin profiles of blueberry juices and raw blueberry are more similar if a berry blanching step is introduced in processing." Perhaps then, rather than being destroyed

when exposed to mild heat, delphinidin derivatives are actually just not as easily extracted from the skin of blueberries using mild heat. Also worth noting is the fact that malvidin 3-glucoside was not prevalent in contrast to the juice tested in the current manufacturing testing. However, this difference can be attributed to varietal differences, as ‘Burlington’ has a comparatively low concentration of this particular anthocyanin (see Table 4-2).

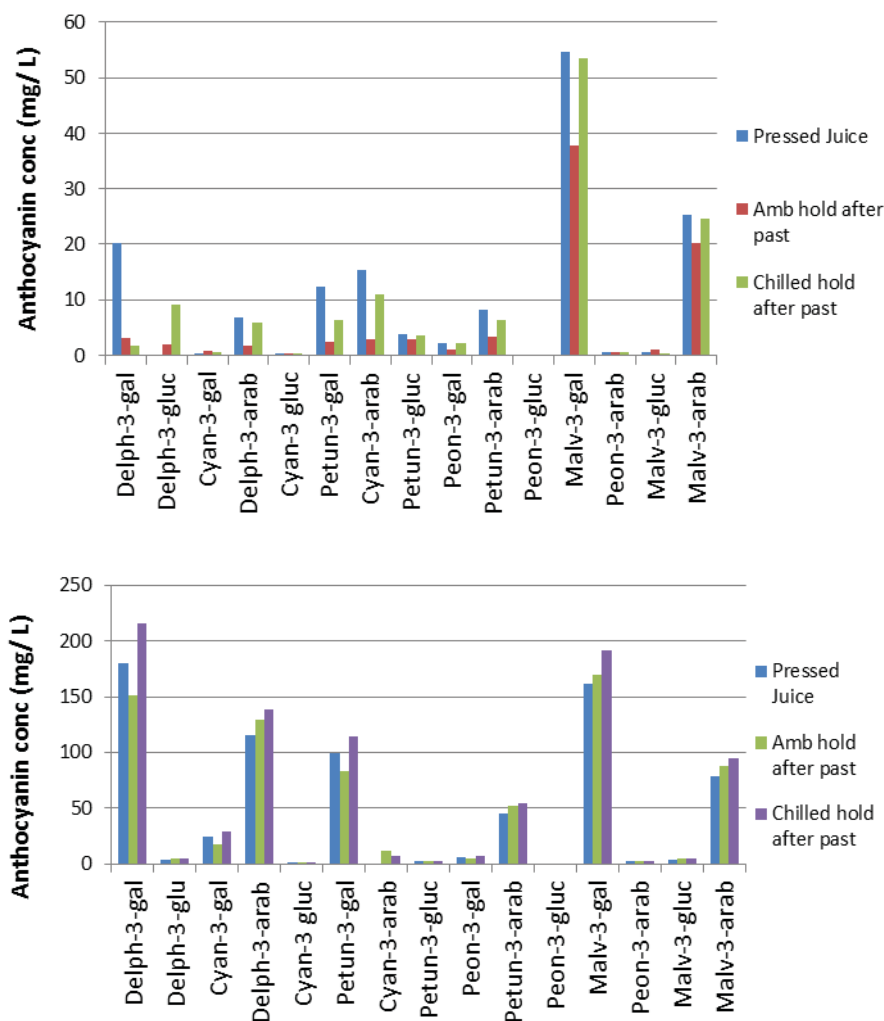


Figure 4-15: Individual anthocyanins in juice produced using alternative processes; TOP: juice produced in trial 1 using slow defrost. BOTTOM: Juice produced in trial 5 using fast defrost.

Chlorogenic acid concentrations were also seen to be significantly affected by the berry thawing process (column 14, Table 4-3). Berries which were defrosted using the 85°C heat treatment, produced juice with a significantly higher chlorogenic acid concentration. The increase seen here is somewhat larger than the 210 – 850 mg/ L reported by Brambilla et al (2008) for their blanched juice produced from 5 different Northern Highbush cultivars.

Additionally reduced holding time resulted in slightly increased concentration, along with a cold holding temperature, particularly for the juice made from slow defrost berries which had the lower starting concentration.

Total polyphenol and antioxidant tests were completed for approximately a quarter of collected samples, including juice produced using the two defrost procedures and juice held under different conditions between pressing and pasteurisation (data not shown). As described previously the anthocyanin content of the fast defrost/85°C heat treatment was about tenfold higher than slow defrost juice. However, surprisingly, the total polyphenol concentrations of the two types of juice were not much different. Overall the fast defrost/85°C heat treatment and juice gave much higher antioxidant values in both FRAP and DPPH assays than juice produced using the slow defrost method. For example slow defrost non-milled juice had a FRAP value of 4,000 µg/ml GA equivalents and 20% DPPH inhibition whereas fast defrost, non-milled juice had a FRAP value of 6,000 µg/ml GA equivalents and 50% DPPH inhibition. As for comparisons between chilled and ambient hold juices (produced using the same methods) only slight and inconsistent differences were observed for both of the antioxidant tests. One interesting point to note is that the loss of antioxidant capacity in the juice appeared to take place within the first half of the holding period between pressing and pasteurisation.

Discussion

Of the alternative juice procedures that were tested in the second experiment, the fast defrost/85°C heat treatment was seen to have the most significant positive impact on anthocyanin concentration while milling of the berries or variation of holding time/conditions between pressing and pasteurisation had comparably little effect. The fast defrost/85°C heat treatment generated a 5 fold increase in anthocyanin concentration compared with juice produced from slow defrost berries. However, one surprising observation was that concentrations of anthocaynins in thawed berry samples from both slow defrost and fast defrost were similar; suggesting that a fast defrost was not effective at ensuring less degradation of anthocyanins. This indicated that the reason for the higher anthocyanin concentration in 'fast defrost' juice was solely from additional extraction of anthocyanin generated by the 85°C heat treatment. Another benefit that was expected but not seen with this treatment was the prevention of anthocyanin degradation during the

holding of juice between pressing and pasteurisation: the rate of degradation observed during holding was similar for both 'fast defrost' and slow 'defrost juice'. It is unlikely that any polyphenol oxidase survived the fast defrost 85°C heat treatment. Therefore it may be a misconception that PPO is the leading cause of anthocyanin degradation.

4.2.3 Implications for juice manufacture

Upon review of the current manufacturing process large anthocyanin losses were seen after thawing of the fruit, removal of the press cake and holding of the juice between pressing and pasteurisation. Additionally the two alternative processes that were compared gave a higher anthocyanin concentration for 'hot press juice' but a much lower chlorogenic acid concentration than the alternative 'cold press' juice.

In order to significantly increase the anthocyanin and chlorogenic acid concentration of the juice (about 5 fold increase of anthocyanins and 4 fold increase chlorogenic acid from current levels) blanching of blueberries before pressing could be employed. However, this would alter the sensory properties of the juice significantly, giving a more cooked flavour and thicker texture. Of the holding options that were trialled a shorter hold gave a better anthocyanin concentration, particularly for slow defrost juice; therefore immediate pasteurisation is likely to confer some benefit if the current practice continues to be used. Additionally juice which was chilled during the hold period showed increased anthocyanin concentrations after pasteurisation, small in the case of slow defrost juice and large in the case of fast defrost juice. However it is suspected that this difference was due to concentration of juice during pasteurisation, as it had to be heated from a lower temperature.

There are still some options which have not been explored in this thesis such as re-introduction of sediment in the form of a tea-bag type system. Such concepts would need to be tested before adoption to verify their efficacy.

4.3 Changes during Juice Storage

4.3.1 Stored Blueberry Juice

Five samples of juice (Figure 4-16), prepared and stored under slightly different conditions at Mamaku Blue, were provided for the purpose of preliminary analysis and to potentially gain some understanding of storage effects. It should be noted that the juices were produced with different berry varieties sourced from different locations.

As this was actually the first anthocyanin analysis to be completed, the following hypotheses were made with regard to the relative anthocyanin levels contained in the juice; 'Nov 09 Drips & Dregs' would have the highest anthocyanin levels since it was thought to contain the most solid material. The remaining juices would have sequentially lower levels dependent on increased storage length and green juice, made from immature berries, would have negligible anthocyanins. The total levels of anthocyanins actually found in the juices can be seen in the following graph (Figure 4-16).

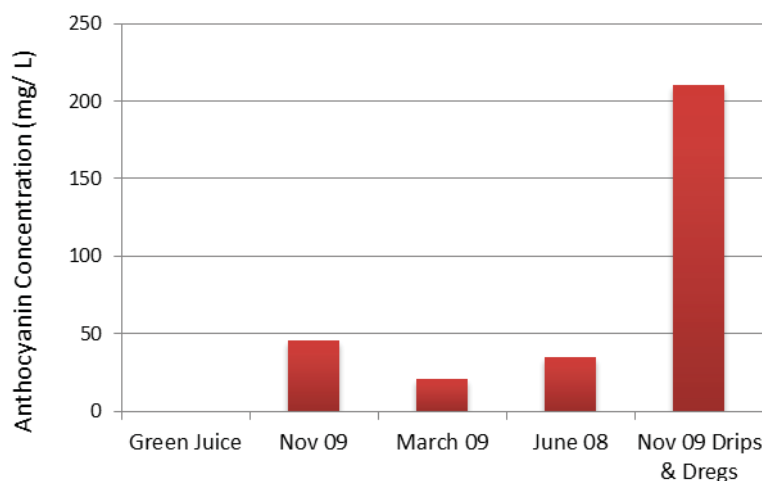


Figure 4-16: Total anthocyanin concentrations of five juice samples measured using HPLC.

As expected there were no measurable anthocyanins in 'Green' juice. Conversely anthocyanin levels were tenfold higher in the 'Nov 09 Drips & Dregs' juice as compared with the other four. There was some variability amongst the other three juices where 'March 09' juice had a lower amount than the older 'June 08' juice. This lack of a consistent trend with storage time may have been due to a difference in varieties and/or different storage conditions.

Other parameters measured for the juices were pH, brix and sediment level as seen in Table 4-4.

Table 4-4: Properties of Juice stored under varying conditions.

	Green Juice	Nov 09	March 09	June 08	Nov 09 Drips & Dreg
Anthocyanins mg/L		46	21	35	211
Dry Cake (% weight)		0.39%	0.82%	0.40%	0.08%
pH	2.3	3	2.9	2.8	3.1
°Brix	4.3	12.2	10.4	10.3	14.2

For blue juices pH and brix appeared to have declined slightly during storage. 'Nov 09 Drips & Dregs' had the highest pH and Brix levels but surprisingly had the lowest levels of measurable sediment (% dry cake in Table 4-4).

Upon inspection of the juices, sediment from 'Nov 09 Drips & Dregs' appeared much finer than that of the other juices. Therefore this apparent decrease may be an inaccurate measurement as possibly some sediment was too small to be detected using the centrifugation method employed. However, there was some discussion about the apparent inverse relationship between sediment and anthocyanin concentration and it was suggested that potentially sediment had the ability to trap anthocyanins. However, this theory was later invalidated using juice from the storage experiment (see end of the storage experiment Figure 4-21).

4.3.2 Storage Experiment

Sixty bottles of juice produced at the orchard were transported back to Massey in order to conduct a long term storage experiment. The treatments were based on a combination of three variables, temperature, light and packaging materials. The literature review had identified that the rate of degradation would be affected particularly by the storage temperature. Here multiple samples were not tested for each treatment due to a limited availability of the juice. Instead it was assumed that establishing trends over time across a number of different variables would support the reliability of the results. Aside from a few slight anomalies within the dataset there appeared to be sufficient evidence for the findings that the various rates of degradation were different for each of the variables, particularly for temperature. The results can be seen in Figure 4-17.

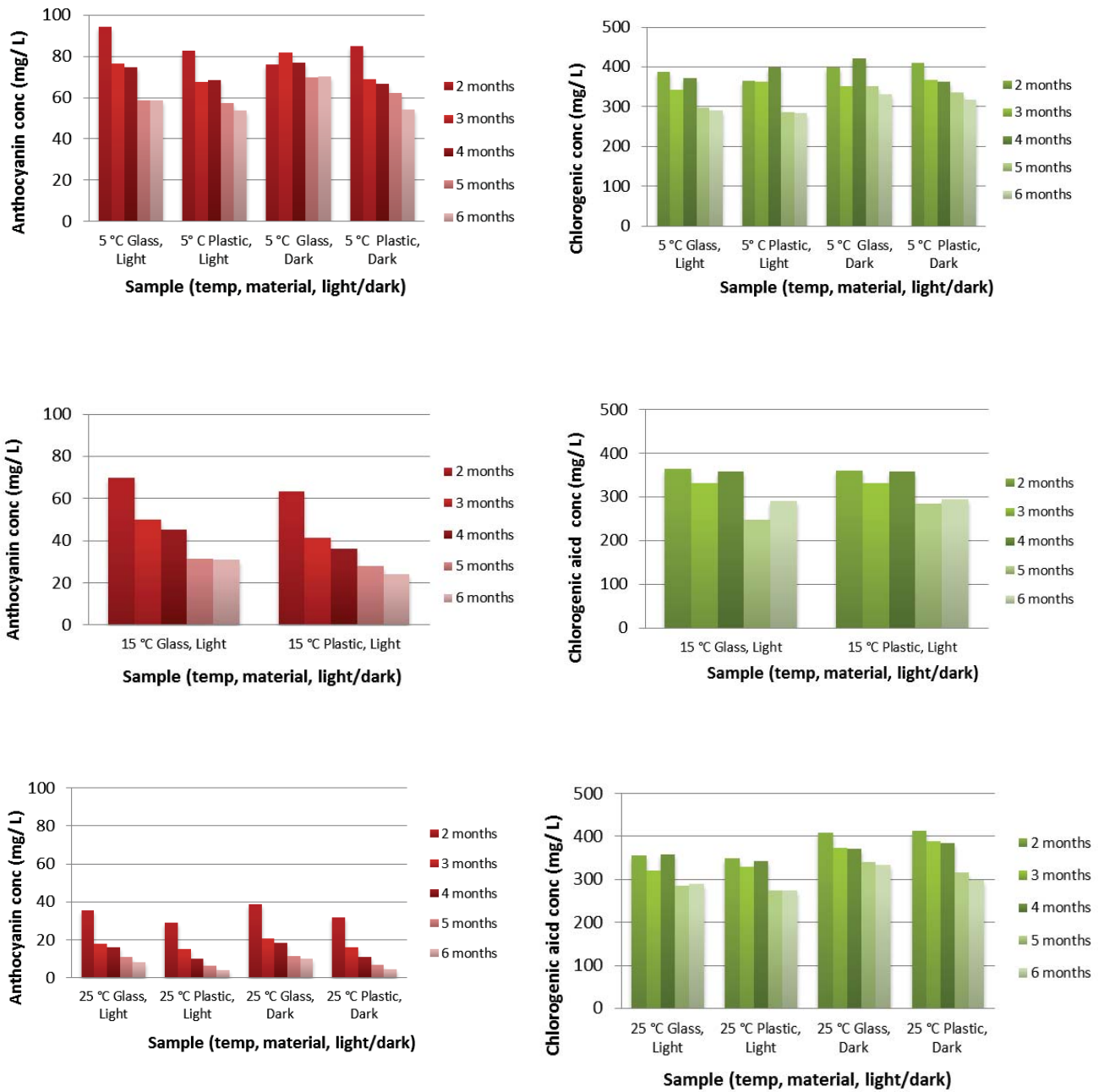


Figure 4-17: Blueberry juices stored under different conditions for six months LEFT HAND SIDE: Anthocyanin concentrations of blueberry juice RIGHT HAND SIDE: Chlorogenic acid concentrations of blueberry juice.

Juice stored at the coldest temperature had the highest levels of anthocyanins remaining after six months and juice stored at the warmest temperature had the lowest levels of anthocyanins remaining after six months. In addition some consistent differences were seen for packaging type and light exposure where glass bottles and dark storage appeared better for the retention of anthocyanins. It should also be noted that although the colour of the juice was not measured, the juice with less anthocyanins was visually much browner.

Srivastava et al (2007) trialled juice storage at a number of different temperatures in glass bottles and found that after two months of storage at 6°C and 23°C there remained 71% and 57% of total anthocyanins respectively. Here at two months 95% of anthocyanins remained in blueberry juice stored in glass bottles at 5°C on average (light and dark) but only 39% remained in the juice stored in glass bottles at 25°C on average (light and dark). Brownmiller et al (2008) also conducted blueberry juice storage trials and reported that non-clarified juice packed in glass bottles held at 25°C contained only 23% of its original anthocyanins at the end of the six month period. In this study the average anthocyanin concentration of juice held in glass bottles (light and dark) at 25°C was only 11% of the original anthocyanin concentration, whereas the average retention for glass bottles (light and dark) at 5°C was 74% at the end of six months.

Conversely the chlorogenic acid concentration remained much higher than that observed for anthocyanins and degraded similarly across all temperatures. At the end of the storage period the only variable which appeared to provide a notable benefit was that dark storage retained the chlorogenic acid better than storage in the light. Similarly significant retention of chlorogenic acid was reported by Brownmiller et al (2009) who observed that after six months storage in the dark non-clarified blueberry juice at 25°C retained 80 – 83% of its chlorogenic acid content.

Individual anthocyanins were also investigated. Figure 4-18 shows the changes observed for juice held at 25°C, which suffered the most degradation of the three storage temperatures. The profiles were similar to that seen in the juice production analysis on the orchard (Figure 4-11) where malvidin glycosides were the dominant anthocyanins in all the juice samples. The percentage of malvidin glycosides increased slightly during the experiment, while the percentage of a number of the other anthocyanins decreased slightly.

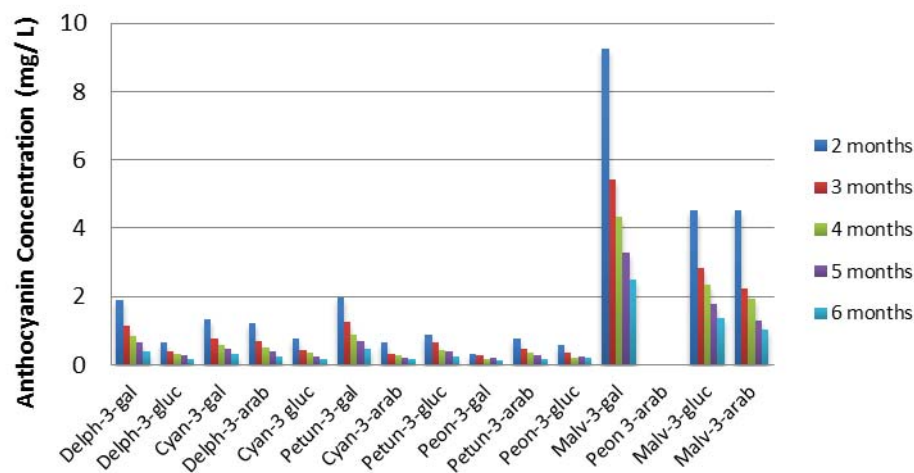


Figure 4-18: The profile of individual anthocyanins as seen in juice packaged in glass bottles and stored for 6 months at 25 °C in the light.

A few bottles of ‘cold press’ juices were also held for analysis during the storage experiment at a single temperature. The anthocyanin and chlorogenic acid concentrations measured in those bottles can be seen in figure 4-19.

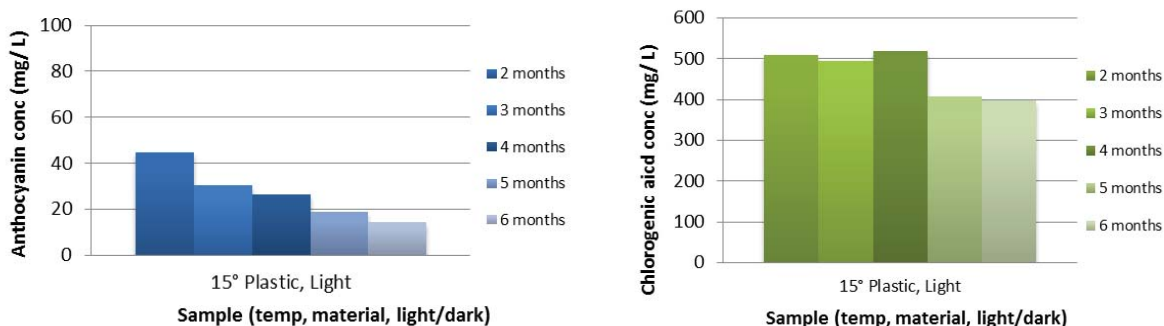


Figure 4-19: ‘Cold press’ juice stored at 15 °C in plastic bottles LEFT HAND SIDE: Anthocyanin concentrations of blueberry juice RIGHT HAND SIDE: Chlorogenic acid concentrations of blueberry juice.

It can be seen in Figure 4-19 that the anthocyanin concentration of ‘cold press’ juice was initially half that seen in ‘hot press’ juice but it degraded at a similar rate over the six months.

Total phenolic and antioxidant tests were also performed for samples of freshly processed blueberry juice and juice at the end of the storage period. It had been noted in the literature review that previous researchers had reported that the antioxidant capacity of blueberry juice did not degrade in accordance with the anthocyanin level (Srivastava et al,

2007; Brownmiller et al, 2008). Therefore these researchers hypothesised that the degradation products of anthocyanins must still possess antioxidant properties. Reasonably good retention of antioxidant capacity was also observed in this experiment, where the total phenolic content showed only a slight degradation and the two antioxidant tests, FRAP and DPPH showed an increase and decrease respectively. The results may have been confounded by a change in sediment level as sediment was not controlled. However, the lack of degradation does still support the general findings of the other researchers, that the antioxidant capacity is not severely compromised by storage even when the anthocyanin content is. However, on noting the retention of chlorogenic acid in the juice, potentially more investigation could be done to consider the role of chlorogenic acid in the antioxidant capacity, as a high contribution towards antioxidant capacity could be another explanation for the trend.

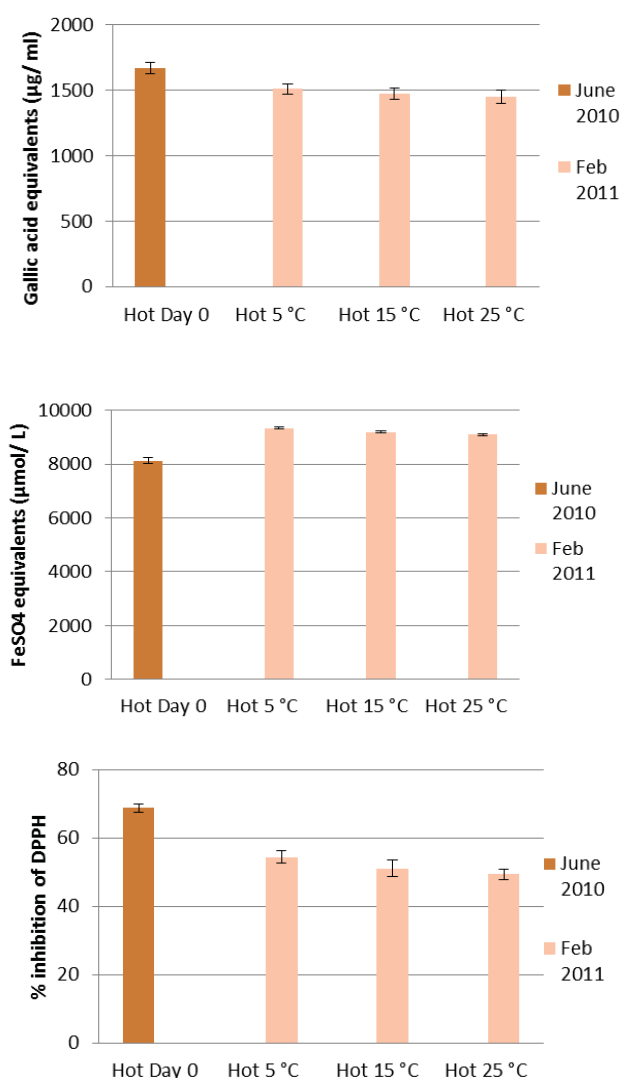


Figure 4-20: TOP: Total phenolic content of blueberry juice measured in Gallic acid equivalents; MIDDLE: FRAP antioxidant measured in FeSO4 equivalents; BOTTOM: DPPH antioxidant measure as % inhibition.

As an extension to this section some investigative work was carried out on juice sediment to determine whether the same decrease, or perhaps increase, of anthocyanin concentration was occurring in the sediment of stored juice. In order to ensure that samples were chosen with different anthocyanin levels within the juice, product from the storage experiment at three different storage temperatures was utilised. The sediment extraction was carried out within two days of measurement of the anthocyanin concentration of the juice. The results from solvent extraction of the sediment alongside the values obtained for the juice stored for four months are shown in Figure 4-21.

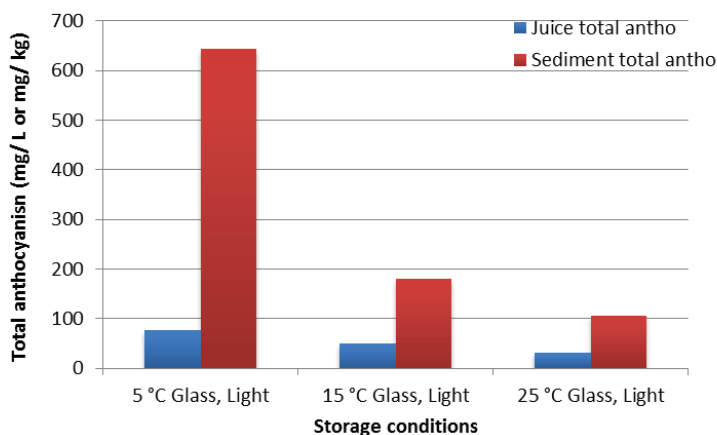


Figure 4-21: Anthocyanin contents of juice and juice sediment, from juice stored for six months at different temperatures.

It is plainly seen here that a lower anthocyanin value in the juice also corresponded to a lower level of anthocyanin in the sediment. Therefore the suggestion that the lost anthocyanins during storage were trapped by sediment in the juice seems very unlikely. Instead, as per the suggestions by other researchers, anthocyanins are more likely lost through degradation reactions, e.g. polymerisation into different compounds.

4.3.3 Implications for juice manufacture

Storage tests showed a clear relationship between the storage temperature and anthocyanin retention, where juice stored at lower temperatures had the highest anthocyanin retention. At the end of the six month storage period juice stored at 5°C had 74% retention while juice stored at 25°C had only 11% retention. By the end of the 6 month storage period it was also apparent that the anthocyanin concentration of juice packed in

glass bottles and stored in the dark fared better than that for plastic bottles stored in the light.

Chlorogenic acid levels appeared to be comparatively less affected, although some degradation was observed. However, this appeared to be much more a function of time, not temperature, although there did appear to be some protection afforded to juice stored in the dark.

Although there was a substantial decrease in anthocyanins the antioxidant capacity was reasonably well maintained and did not appear to be notably affected by storage temperature. Therefore if marketing claims are made pertaining to antioxidant properties of the juice, degradation during storage will be less of a concern than if anthocyanin claims are made.

Overall the recommendation is that blueberry juice be always stored chilled, if not for the protection of anthocyanins, to prevent aesthetic degradation through browning of the juice. Additionally, some protection appears to be afforded to the anthocyanin concentration if juice is packaged in glass bottles and some protection is afforded to the chlorogenic acid concentration when juice is stored in the dark.

Unfortunately at this stage it is not possible to quantify what reaction products of the anthocyanin degradation during storage might be. However, it is important to note that the colour of the juice became increasingly brown with the loss of anthocyanins.

4.4 Analytical Improvements

With the continuous improvement of technology analytical methods are constantly evolving. However, this inherently results in a limited consistency and refinement of techniques. Thereby a number of potential analytical pitfalls were identified in completing this work.

HPLC is well documented for use in analysing a wide range of different chemical compounds and its versatile nature makes it a favourable instrument to have in the laboratory. In the work presented here quantification was carried out using external analytical standards. However, it was discovered during the course of this work that such standards have a very limited shelf-life. Degradation was seen to occur over a matter of weeks even though the standards were stored frozen and thawed under vacuum. Furthermore external standards can only give limited certainty in identification of a spectrum of peaks. Thereby in accordance with the literature discussed previously; mass spectrometry would have been the preferred qualification and quantification method if available.

Extraction techniques for phenolic acids vary greatly in the literature. In order to maximise the extraction from concentrated press cake samples several washes of the samples were required. Additionally questions were raised around the analysis of juice. The majority of researchers simply diluted their samples with water, filtered and then analysed. However, it appeared that the juice sediment contained a significant amount of anthocyanins. Fine solids are easily suspended; therefore when the juice is unclarified this can represent a significant proportion of the juice material. In order to obtain a maximum anthocyanin concentration this sediment would need to be solvent extracted like other solid material.

Antioxidant measurements can be dynamic over a range of concentrations. Therefore it has become common to find the concentration which results in 50% DPPH oxidation as a standard to facilitate comparison of test results (Prior et al, 2005). Unfortunately this was overlooked at the time of the research and therefore the results recorded should only be used for comparison within this thesis.

Additionally it is recognised that in some areas of this work rest upon limited statistical analysis. However, a wide variety of investigations were covered over a short time frame and the results achieved give a good direction for more thorough research into specific and important areas.

CHAPTER 5

Conclusions & Recommendations

5.1 Blueberry Juice Manufacture

This work successfully identified a number of factors which should be considered when producing a high anthocyanin and/or high chlorogenic acid blueberry juice.

Cultivar selection is potentially important. However, the maturity of the plant is not. Further consideration should be given to this if particular anthocyanins are important since the profile of individual anthocyanins varies with cultivar.

In the current blueberry juice process thawing of berries and holding of juice between pressing and pasteurisation resulted in a large amount of anthocyanin degradation and some chlorogenic acid degradation. Removal of the press cake resulted in the greatest loss of anthocyanins from the primary processing stream.

Using alternative manufacturing methods to combat these issues gave mixed results. Fast thawing at 85°C did not induce a higher anthocyanin content for thawed berries or prevent degradation of juice over an extended holding period through enzyme deactivation as expected, but it was extremely successful in producing juice with significantly higher anthocyanin and chlorogenic acid concentrations, presumably due to increased extraction from the blueberry skin. However, its effect on sensory properties of the juice, inducing a thick texture and more cooked flavour, may make it unsuitable for utilisation. In order to increase extraction from the blueberry skins, milling was employed (blending) but was also unsuccessful in improving the juice's anthocyanin concentration. Alternate milling treatments could be tested. Holding juice under chilled conditions between pressing and pasteurisation did not provide a significant reduction of degradation, and reduced holding time also had little effect.

Juice storage conditions are important for retention of anthocyanins but were not as important for chlorogenic acid or antioxidant levels. Storage at low temperatures resulted in much less anthocyanin degradation than storage at high temperatures.

5.2 Further Research

It is difficult to understand the full picture from individual publications. However, this thesis has allowed for the consolidation of the most recent scientific evaluations of blueberries, blueberry juice and health. This consolidation, combined with the experimental investigations carried out have also revealed many interesting areas for further research.

Unfortunately at the present time there is no clear relationship between blueberries or specific blueberry compounds and prevention or treatment of degenerative diseases. The need for well-designed human clinical trials has been highlighted throughout publications within the last part of this decade. Hopefully with the consolidation of resources and improvements in technology more conclusive outcomes between diet and health will be established in the near future.

It was discovered that the sensory profile of blueberry juice can vary greatly depending on the manufacturing operations employed. For instance blueberries have a large amount of mucilaginous material which can gel even with moderate heat; the resulting gel is not destroyed using enzymes such as pectinase or cellulase. Quantification of differences in sensory parameters such as taste, texture and colour are not common in literature. However, with the potential for so much variation such quantifications would be useful when making comparisons. The absence of any sensory quantification or consumer testing is a limitation of this work. Arguably if the focus is a commercial juice product then optimisation of the sensory profile alongside the phytochemical profile is necessary.

Currently it is unclear as to what the anthocyanin degradation products and degradation mechanisms in blueberry juice are. This thesis shows that a large amount of chemical degradation can occur during both processing and long term storage. In literature there is a large emphasis on PPO as the major anthocyanin degradation factor. Therefore it was assumed that blanching of the berries would prevent a significant amount of degradation in the juice. However, we observed that although there was a significant initial increase in anthocyanins in blueberry juice as compared with a control, the same amount of degradation appeared to occur in both blanched and non-blanched juice over a temporary storage period (≥ 35 h). Furthermore, significant amounts of degradation were observed during long term storage, which is consistent with literature. It is apparent that one of the limitations of current literature is that although degradation is thoroughly quantified the

degradation mechanisms or products have not been elucidated by researchers'. Therefore in order to prevent anthocyanin degradation in blueberry juice it is apparent that initially a better understanding of degradation is required. Investigations using NMR would assist in identifying degradation compounds.

The focus of this project was anthocyanins. However, chlorogenic acid was an even more abundant phytochemical in all varieties studied here. However, surprisingly this abundance has not been observed by all researchers. A majority of authors report concentrations 10 fold lower than what was measured during this study. If chlorogenic acid is demonstrated to be important for health then it will be necessary to ascertain exactly why these differences exist. Perhaps the concentration of chlorogenic acid is significantly affected by variations in analysis, its synthesis is heavily dependent on growing conditions or localised genetic differences exist.

References

- Adams, J. (1991). Review: Enzyme inactivation during heat processing of food-stuffs. *International Journal of Food Science and Technology*, 26, 1-20.
- Arts, I., & Hollman C. (2005). Polyphenols and Disease Risk in Epidemiologic Studies. *The American Journal of Clinical Nutrition*, 81, 317S-325S.
- Ashurst, P. (Ed.). (1995). *Production and Packaging of Non-Carbonated Fruit Juices and Fruit Beverages*. (2nd ed.). Glasgow: Blackie Academic & Professional.
- Ashurst, P. (Ed.). (2005). *Chemistry and Technology of Soft Drinks and Fruit Juices*. (2nd ed.). Oxford: Blackwell Publishing.
- Barnes, J., Nguyen, H., Shen, S., Schug, K. (2009). General method for extraction of blueberry anthocyanins and identification using high performance liquid chromatography–electrospray ionization-ion trap-time of flight-mass spectrometry. *Journal of Chromatography A*, 23, 4728-4735.
- Basu, A., Du, M., Leyva, M., Sanchez, K., Betts, N., Wu, N. Aston, C. & Lyons, T. (2010). Blueberries decrease cardiovascular risk factors in obese men and women with metabolic syndrome. *The Journal of Nutrition*, 140, 1582-1587.
- Bates, R. P., Morris, J. R., & Crandall, P. G. (2001). Principles and practices of small- and medium-scale fruit juice processing. Agricultural Services Bulletin No. 146. FAO, Rome.
- Beattie, J., Crozier, A., & Duthie, G. (2005). Potential Health Benefits of Berries. *Current Nutrition & Food Science*, 1, 71-86.
- Boateng J., Verghese, M., Shackelford, L, Walker. L., Khatiwada, J., Ogutu, S....Chawan, C. (2007). Selected fruits reduce azoxymethane (AOM)-induced aberrant crypt foci (ACF) in Fisher 344 male rats. *Food and Chemical Toxicology*, 45, 725-732.
- Boffetta, P., Couto, C., Wichmann, J., Ferrari, P., Richopoulos, D., Bueno-de-Mesquita, H. ...Trichopoulou, A. (2010). Fruit and Vegetable Intake and Overall Cancer Risk in the European Prospective Investigation into and Nutrition (EPIC). *Journal of the National Cancer Institute*. Retrieved from <http://jnci.oxfordjournals.org/cgi/reprint/102/8/529?maxtoshow=&hits=10&RESULTFORM AT=&fulltext=EPIC&searchid=1&FIRSTINDEX=0&resourcetype=HWCIT>.
- Borges, G., Degeneve, A., Mullen, W. & Crozier, A. (2010). Identification of Flavonoid and Phenolic Antioxidants in Black Currants, Blueberries, Raspberries, Red Currants, and Cranberries. *Journal of Agricultural and Food Chemistry*, 58, 3901-3909.

- Brambilla, A., Lo Scalzo, R., Bertolo, G. & Torreggiani, D. (2008). Steam-Blanched Highbush Blueberry (*Vaccinium corymbosum* L.) Juice: Phenolic Profile and Antioxidant Capacity in Relation to Cultivar Selection. *Journal of Agricultural and Food Chemistry*, *56*, 2643-2648 .
- Bridle, P., & Timberlake, C. (1997). Anthocyanins as natural food colours - selected aspects. *Food Chemistry*, *58*, 103-109.
- Brownmiller, C., Howard, L., & Prior, R. (2008). Processing and Storage Effects on Monomeric Anthocyanins, Percent Polymeric Color, and Antioxidant Capacity of Processed Blueberry Products. *Journal of Food Science*, *73*, 72-79.
- Brownmiller, C., Howard, L., & Prior, R., (2009). Processing and Storage Effects on Blueberry (*Vaccinium corymbosum* L.) Polyphenolics. *Proc. IInd IS on Human Health Effects of F&V*.
- Buckow, R., Kastell, A., Terefe, S., Versteeg. (2010). Pressure and Temperature Effects on Degradation Kinetics and Storage Stability of Total Anthocyanins in Blueberry Juice. *Journal of Agricultural and Food Chemistry*, *58*, 10076 - 10084.
- Cao, G., Booth, S., Sadowski, J., Prior, R. (1998). Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. *The American Journal of Clinical Nutrition*, *68*, 1081-7.
- Cao, G., Prior, R. (1999). Anthocyanins Are Detected in Human Plasma after Oral Administration of an Elderberry Extract. *Clinical Chemistry*, *45*, 4, 574-576.
- Castrejon, A., Eichholz, I., Rohn S., Kroh, L., Huyskens-Keil, S. (2008). Phenolic profile and antioxidant activity of Highbush blueberry (*Vaccinium corymbosum* L.) during fruit maturation and ripening. *Food Chemistry*, *109*, 564-572
- Cho, A., Jenó, S., Kim, M., Yeo, J., Seo, K., Choi, S. & Lee, M. (2010). Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice . *Food and Chemical Toxicology*, *48*, 937-943.
- Cho, J., Howard, R., Prior, R. & Clack, J. (2004). Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *Journal of the Science of Food and Agriculture*, *84*, 1771-1782.
- Connor, A., Luby J., Tong, B., Finn, C., & Hancock, F. (2002) Genotypic and environmental variation in antioxidant activity, total phenolic content, and anthocyanin content among blueberry cultivars. *Journal of the American Society for Horticultural Science*, *127*, 89–97.
- Crozier, A., Clifford, M., & Ashihara, H. (Eds.). (2006). *Plant Secondary Metabolites: Occurrence, Structure and Role in Human Diet*. Oxford: Blackwell Publishing.
- Del Bo, C., Ciappellano, S., Klimis-Zacas, D., Martini, D., Gardana, C., Riso, P., & Porrini, M. (2010).

- Anthocyanins, Absorption, Metabolism, and Distribution from a Wild Blueberry-Enriched Diet is Affected by Diet Duration in the Sprague - Dawley Rat. *Journal of Agricultural and Food Chemistry*, 58, 2491-2497.
- Duffy, K., Spangler, E., Devan, B., Guob, Z., Bowker, J., Janas, A., ...Ingram, D. (2008). A blueberry-enriched diet provides cellular protection against oxidative stress and reduces a kainate-induced learning impairment in rats. *Neurobiology of Aging*, 29, 1680-1689.
- Duthie, S., Jenkinson, A., Crozier, A., Mullen, W., Pirie, L., Kyle, J...Duthie, G. (2006). The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart disease and cancer in healthy human volunteers. *The European Journal of Nutrition*, 45, 113-122.
- Ehlenfeldt, M., & Prior, R. (2001). Oxygen Radical Absorbance Capacity (ORAC) and Phenolic and Anthocyanin Concentrations in Fruit and Leaf Tissues of Highbush Blueberry. *Journal of Agricultural and Food Chemistry*, 49, 2222-2227.
- Erdman, J., Balentine, D., Arab, L., Beecher, G., Dwyer, J., Folts, J...Burrowes, J. (2007). Flavonoids and Heart Health: Proceedings of the ILSI North America Flavonoids Workshop May 2005. *The Journal of Nutrition*, 137, 718S-737S.
- Fennema, O. (Ed.). (1996). *Food Chemistry*. (3rd ed). New York: Marcel Dekker.
- French National Institute for Agricultural Research. (2009). Polyphenol Explorer, 1.0. Retrieved August 2010, from <http://www.phenol-explorer.eu/>
- Galli R., Bielinski D., Szprengiel A., Shukitt-Hale, B., & Joseph, J. (2006). Blueberry supplemented diet reverses age-related decline in hippocampal HSP70 neuroprotection. *Neurobiology of Aging*, 27, 344-350
- Gao, L. & Mazza, G. (1994). Quantitation and Distribution of Simple and Acylated Anthocyanins and Other Phenolics in Blueberries. *Journal of Food Science*, 59, 1057-1059.
- Gonzalez, C., & Riboli, E. (2006). Diet and Cancer Prevention: Where We Are, Where Are We Going? *Nutrition and Cancer*, 56, 225-231.
- Gould, K., Davies, K., & Winefield, C. (Eds.). (2009). *Anthocyanins: Biosynthesis, Functions, and Applications*. New York: Springer.
- Goyarzu, P., Malin, D., Lau, F., Tagliatela, G., Moon, W., Jennings, R., Moy, E., Lippold, S., Shukitt-Hale, B. & Joseph, J. (2004). Blueberry supplemented diet: effects on object recognition memory and nuclear factor-kappa B levels in aged rats. *Nutritional neuroscience*, 7, 75-83.
- Harb, J., Khraiwesh, B., Streif J., Reskid, R., & Frank, W. (2010). Characterization of blueberry monodehydroascorbate reductase gene and changes in levels of ascorbic acid and the antioxidative capacity of water soluble antioxidants upon storage of fruits under various

- conditions. *Scientia Horticulturae*, 125, 390-395.
- Hertog, M., Hollman, P., & Kahan, M. (1992). Content of Potentially Anticarcinogenic Flavonoids of 28 Vegetables and 9 Fruits Commonly Consumed in The Netherlands. *Journal of Agricultural and Food Chemistry*, 40, 2379-2383.
- Hocman, G. (1989). Prevention of Cancer: Vegetables and Plants. *Comparative Biochemistry and Physiology*, 93B, 201-212.
- Hohn, A., Sun, D., & Nolle, D. (2005). Juice Processing. In M. Barrett, S. Laszlo & H. Ramaswamy (Eds.), *Processing Fruits; Science and Technology* (pp. 97-112). (2nd ed.). Boca Raton: CRC Press.
- Hong, V. & Wrolstad, R. (1990). Use of HPLC Separation/Photodiode Array Detection for Characterization of Anthocyanins. *Journal of Agricultural and Food Chemistry*, 38, 708-715.
- Hooper, L., Kroon, P., Rimm, E., Cohn, J., Harvey, I., Le Cornu, K., ...Cassidy, A. (2008). Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. *The American Journal of Clinical Nutrition*, 88, 38-50.
- Horbowicz, M., Kosson, R., Grezesiuk, A. & Deski, H. (2008). Anthocyanins of Fruits and Vegetables - Their Occurrence, Analysis and Role in Human Nutrition. *Vegetable crops and Research Bulletin*, 68, 5-22.
- Howard, J., Clark, J., & Brownmiller, C. (2003). Antioxidant Capacity and Phenolic Content in Blueberries as Affected by Genotype and Growing Season. *Journal of the Science of Food and Agriculture*, 83, 1238-1247.
- Howard, L., & Hagar T. (2007). Berry Fruit Phytochemicals. In Y. Zhao (Ed), *Berry Fruit, Value added Products for Health Promotion* (pp. 73-105). Boca Raton: CRC Press.
- Hurst, R., Wells, R., Hurst, S., McGhie, T., Cooney, J., & Jensen, D. (2010). Blueberry fruit polyphenolics suppress oxidative stress induced skeletal muscle cell damage in vitro. *Molecular Nutrition & Food Research*, 54, 353-363.
- Ichiyanagi, T., Shida, Y., Rahman, M., Hatano, Y., Matsumoto, H., Hirayama, M., & Konishi, T. (2005). Metabolic Pathway of Cyanidin 3-Glucopyranoside in Rats. *Journal of Agricultural and Food Chemistry*, 53, 145-150.
- Jackson, R., & Smith J. (1996). Anthocyanins and Betalains. In G. Henry and J. Houghton (Eds.), *Natural Food Colorants* (pp. 244-309), (2nd ed.). Glasgow: Blackie Academic and Professional.
- Jepson, R., & Craig, J. (2007). A systematic review of the evidence for cranberries and blueberries in UTI prevention. *Molecular Nutrition & Food Research*, 51, 738-745.
- Joseph, J., Denisova, N., Arendash, G., Gordon, M., Diamond, D., Shukitt-Hale, B., & Morgan, D.

- (2003). Blueberry Supplementation Enhances Signalling and Prevents Behavioural Deficits in an Alzheimer Disease Model. *Nutritional Neuroscience*, *6*, 153-162.
- Joseph, J., Shukitt-Hale, B., Brewer, G., Weikel, K., Kalt, W. & Fisher, D. (2010). Differential Protection among Fractionated Blueberry Polyphenolic Families against DA-, A β 42- and LPS-Induced Decrements in Ca²⁺ Buffering in Primary Hippocampal Cells. *Journal of Agricultural and Food Chemistry*, *58*, 8196-8204.
- Kader, F., Rovel, B., Girardin M., & Metche, M. (1997a) Mechanism of Browning in Fresh Highbush Blueberry Fruit (*Vaccinium corymbosum* L). Partial Purification and Characterisation of Blueberry Polyphenol Oxidase. *Journal of the Science of Food and Agriculture*, *73*, 513-516.
- Kader, F., Rovel, B., Girardin M., & Metche, M. (1997b) Mechanism of Browning in Fresh Highbush Blueberry Fruit (*Vaccinium corymbosum* L). Role of PPO, Chlorogenic acid and Anthocyanins. *Journal of the Science of Food and Agriculture*, *74*, 31-34.
- Kader, R., Irmouli, M., Nicolas, J., & Metche, M. (2002). Involvement of Blueberry Peroxidase in the Mechanisms of Anthocyanin Degradation in Blueberry Juice. *Journal of Food Science*, *67*, 910-915.
- Kahkonen M. & Heinonen M. (2003). Antioxidant Activity of Anthocyanins and Their Aglycons. *Journal of Agricultural and Food Chemistry*, *51*, 628-633.
- Kahkonen M., Hopia, A., Vuorela, H., Rauha, J., Pihlaja, K., Kujala, T., & Heinonen, M. (1999). Antioxidant Activity of Plant Extracts Containing Phenolic Compounds. *Journal of Agricultural and Food Chemistry*, *47*, 3954-3962.
- Kalt, W. (2005). Effects of Production and Processing Factors on Major Fruit and Vegetable Antioxidants. *Journal of Food Science*, *70*, 11-19.
- Kalt, W., Blumberg, J., McDonald, J., Vinqvist-Tymchuk, M., Fillmore, S., Graf, B., O'Leary, J., & Milbury, P. (2008). Identification of Anthocyanins in the Liver, Eye, and Brain of Blueberry-Fed Pigs. *Journal of Agricultural and Food Chemistry*, *56*, 705-712.
- Kalt, W., Foote, F., Fillmore, S., Lyon, M., Van Lunen, T., & McRae, K. (2008). Effect of Blueberry Feeding on Plasma Lipids in Pigs. *British Journal of Nutrition*, *100*, 70-78.
- Kalt, W., Forney, C., Martin, A. & Prior, R. (1999). Antioxidant Capacity, Vitamin C, Phenolics, and Anthocyanins after Fresh Storage of Small Fruits. *Journal of Agricultural and Food Chemistry*, *47*, 4638-4644.
- Kalt, W., McDonald, J., & Donner, H. (2000). Anthocyanins, Phenolics, and Antioxidant Capacity of Processed Lowbush Blueberry Products. *Journal of Food Science*, *65*, 390-393.
- Kalt, W., Ryan, D., Duy, J., Prior, R., Ehlenfeldt, M. & Kloet, V. (2001). Interspecific Variation in Anthocyanins, Phenolics, and Antioxidant Capacity among Genotypes of Highbush and

- Lowbush Blueberries (*Vaccinium* Section *cyanococcus* spp.). *Journal of Agricultural and Food Chemistry*, *49*, 4761-4767
- Kay, C., Mazza, G., Holub, B., & Wang, J. (2004). Anthocyanin Metabolites in Human Urine and Serum. *British Journal of Nutrition*, *91*, 933-942.
- Koli, R., Erlund, I., Julia, A., Marniemi, J., Mattila, P., & Alfthan, G. (2010). Bioavailability of Various Polyphenols from a Diet Containing Moderate Amounts of Berries. *Journal of Agricultural and Food Chemistry*, *58*, 3927-3932.
- Krikorian, R., Shidler, M., Nash, T., Kalt, W., Vinqvist-Tymchuk, M., Shukitt-Hale, B., & Joseph, J. (2010). Blueberry Supplementation Improves Memory in Older Adults. *Journal of Agricultural and Food Chemistry*, *58*, 3996-4000.
- Lee, J. & Wrolstad, R. (2004). Extraction of Anthocyanins and Polyphenolics from Blueberry Processing Waste. *Food Chemistry and Toxicology*, *69*, 564-573.
- Lee, J., Durst, R., & Wrolstad, R. (2002). Impact of Juice Processing on Blueberry Anthocyanins and Polyphenolics: Comparison of Two Pretreatments. *Food Chemistry and Toxicology*, *67*, 1660-1667.
- Letenneur, L., Proust-Lima, C., Le Gouge, A., Dartigues N., & Barberger-Gateau, P. (2007). Flavonoid Intake and Cognitive Decline over a 10-Year Period. *American Journal of Epidemiology*. Retrieved from <http://aje.oxfordjournals.org/cgi/reprint/kwm036v1>.
- Liu, R. (2004). Potential Synergy of Phytochemicals in Cancer Prevention: Mechanism of Action. *The Journal of Nutrition*, *134*, 3479S-3485S.
- Lohachoompol, V., Mulholland, M., Srzenicki, G., & Craske, J. (2008). Determination of Anthocyanins in Various Cultivars of Highbush Blueberries. *Food Chemistry*, *111*, 249-254.
- Malin, D., Lee, D., Goyarzu, P., Chang, Y., Ennis, L., Beckett, E., Shukitt-Hale, B. & Joseph, J., (2010). Short-term blueberry-enriched diet prevents and reverses object recognition memory loss in aging rats. *Nutrition*, *27*, 338-342.
- Manach, C., Williamson, G., Morand, C., Scalbert, A., & Rémésy, C. (2005). Bioavailability and Bioefficacy of Polyphenols in Humans. I. Review of 97 Bioavailability Studies. *The American Journal of Clinical Nutrition*, *81*, 230S-42S.
- Mazza, G., Kay, C., Cottrell, T., & Holub, B. (2002). Absorption of Anthocyanins from Blueberries and Serum Antioxidant Status in Human Subjects. *Journal of Agricultural and Food Chemistry*, *50*, 7731-7737.
- McGhie T., & Walton, M. (2007). The Bioavailability and Absorption of Anthocyanins: Towards a Better Understanding. *Molecular Nutrition Food Research*, *51*, 702-713
- McLellan, M., & Padilla-Zakour, O. (2005). Juice Processing. In In M. Barrett, S. Laszlo & H.

- Ramaswamy (Eds.), *Processing Fruits; Science and Technology* (pp. 73-96). (2nd ed.). Boca Raton: CRC Press.
- Meskin, M., Bidlack, W., Davies, A. & Omaye, S. (Eds). (2002). *Phytochemicals in nutrition and human health*. CRC Press. Florida, USA.
- Molan, A.L., Flanagan, J., Wei, W. and Moughan, P.J. (2009). Selenium-containing green tea has higher antioxidant and prebiotic activities than regular green tea. *Food Chemistry*, *114*, 829-835.
- Moyer, R., Hummer, K., Finn, C., Frei, B. & Wrolstad, R. (2002). Anthocyanins, Phenolics, and Antioxidant Capacity in Diverse Small Fruits: Vaccinium, Rubus, and Ribes. *Journal of Agricultural and Food Chemistry*, *50*, 519-525.
- Naczka, M. & Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A*, *1054*, 95–111.
- Neto, C. (2007). Cranberry and Its Phytochemicals: A Review of In Vitro Anticancer Studies. *The Journal of Nutrition*, *137*, 186S-193S.
- Nettleton, J., Harnack, L., Scrafford, C., Mink, J., Barraj, L., & Jacobs, D. (2006). Dietary Flavonoids and Flavonoid-Rich Foods Are Not Associated with Risk of Type 2 diabetes in Postmenopausal Women. *The Journal of Nutrition*, *136*, 3039–3045.
- Palamidis, N. & Markakis, P. (1975). Stability of grape anthocyanin in a carbonated beverage. *Journal of Food Science*, *40*, 1047-1048
- Pascual-Teresa, S., & Sanchez-Ballesta, M. (2008). Anthocyanins: From Plant to Health. *Phytochemistry Reviews*, *7*, 281-299.
- Patras, A., Bruton, N., O'Donnell, C. & Tiwan, B.K. (2010). Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation. *Trends in Food Science & Technology*. *21*, 3-11.
- Prasad, S., Phromnoi, K., Yadav, V., Chaturvedi, M. & Aggarwal, B. (2010). Targeting Inflammatory Pathways by Flavonoids for Prevention and Treatment of Cancer. *Planta Med*, *76*, 1044-1063
- Prior, R., Wilkes, S., Rogers, T., Khanal, R., Wu, X., & Howard, L., (2010). Purified Blueberry Anthocyanins and Blueberry Juice Alter Development of Obesity in Mice Fed an Obesogenic High-Fat Diet. *Journal of Agricultural and Food Chemistry*, *58*, 3970-3976.
- Prior, R., Wu, X., Gu, L., Hager, T., Hager, A., & Howard, L. (2008). Whole Berries versus Berry Anthocyanins: Interactions with Dietary Fat Levels in the C57BL/6J Mouse Model of Obesity. *Journal of Agricultural and Food Chemistry*, *56*, 647-653.
- Prior, R., Wu, X., & Schaich, K. (2005). Standardized Methods for the Determination of Antioxidant

- Capacity and Phenolics in Foods and Dietary Supplements. *Journal of Agricultural and Food Chemistry*, 53, 4290-4302.
- Prior, R., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., Lischner, N., Ehlenfeldt, M., Kalt, W., Krewer, G., & Mainland, M. (1998). Antioxidant Capacity As Influenced by Total Phenolic and Anthocyanin Content, Maturity, and Variety of Vaccinium Species. *Journal of Agricultural and Food Chemistry*, 46, 2686-2693.
- Rice-Evans C., Miller, N. & Paganga, G. (1996). Structure Antioxidant Activity Relationships of Flavonoids and Phenolic Acids. *Free Radical Biology & Medicine*, 20, 7, 933-956.
- Robards, K (2003). Strategies for the determination of bioactive phenols in plants, fruit and vegetables. *Journal of Chromatography A*, 1000, 657–691.
- Rolfes, S., Pinna, K., Whitney, E., (2009). *Understanding Normal and Clinical Nutrition*. (8th ed.). Belmont: Wadsworth, Cengage learning.
- Rossi, M., Garavello, W., Talamini, R., Negri, E., Bosetti, C., Dal Maso, L...La Vecchia, C. (2007). Flavonoids and the Risk of Oral and Pharyngeal Cancer: A Case-Control Study from Italy. *Cancer Epidemiology, Biomarkers & Prevention*, 16, 1621-1625.
- Rossi, M., Giussania, E., Morellib, R., Lo Scalzoc, R., Nani, R., & Torreggiani, D. (2003). Effect of fruit blanching on phenolics and radical scavenging activity of Highbush blueberry juice. *Food Research International*, 36, 999-1005.
- Sablani, S., Andrews, P., Davies, N., Walters, T., Saez, H., Syamaladevia, R., & Mohekara, P. (2010). Effect of thermal treatments on phytochemicals in conventionally and organically grown berries. *Journal of the Science of Food and Agriculture*, 90, 769-778.
- Scalbert, A., Morand, C., Manach, C., & Rémésy, C. (2002). Absorption and Metabolism of Polyphenols in the Gut and Impact on Health. *Biomedicine and Pharmacotherapy*, 56, 276-282.
- Schmidt, B., Howell, A., Mceniry, B., Knight, C., Seigler, D., Erdman, J., & Lila, M. (2004). Effective Separation of Potent Antiproliferation and Antiadhesion Components from Wild Blueberry (*Vaccinium angustifolium* Ait.) Fruits. *Journal of Agricultural and Food Chemistry*, 52, 6433-6442.
- Schmidt, M., Erdman, J., & Ann Lila, M. (2005). Effects of Food Processing on Blueberry Antiproliferation and Antioxidant Activity. *Journal of Food Science*, 70, 389-394.
- Seeram, N. (2008). Berry Fruits for Cancer Prevention: Current Status and Future Prospects. *Journal of Agricultural and Food Chemistry*, 56, 630-635.
- Shaughnessy, K., Boswalla, I., Scanlana, A., Gottschall-Passb, K., & Sweeneya, M. (2009). Diets containing blueberry extract lower blood pressure in spontaneously hypertensive stroke-prone rats. *Nutrition Research*, 29, 130-138.

- Skrede, G. & Wrolstad, R. (2002). Flavonoids in Berries and Grapes. In G. Mazza (Ed.), *Functional Foods - Biochemical and Processing Aspects*. Boca Raton : CRC Press
- Skrede, G., Wrolstad, R., & Durst, R. (2000). Changes in Anthocyanins and Polyphenolics During Juice Processing of Highbush Blueberries. *Journal of Food Science*, 65, 357-364.
- Smith, M., Marley, K., Seigler, D., Singletary, K. Meline, B. (2000). Bioactive Properties of Wild Blueberry Fruits. *Journal of Food Science*, 65, 352-356.
- Solomon, O., Svanberg, U. & Sahlstrom, A. (1995). Effect of oxygen and fluorescent light on the quality of orange juice during storage at 8 ° C. *Food Chemistry*, 53, 636-668.
- Spormann, T., Albert, F., Rath, T., Dietrich, H., Will, F., Stockis, J., Janzowski, C. (2008). Anthocyanin/Polyphenolic-Rich Fruit Juice Reduces Oxidative Cell Damage in an Intervention Study with Patients on Hemodialysis. *Cancer Epidemiology, Biomarkers & Prevention*, 17, 3371-3380.
- Srivastava, A., Akoh, C., Weiguang, Y., Fischer, J., & Krewer, G. (2007). Effect of Storage Conditions on the Biological Activity of Phenolic Compounds of Blueberry Extract Packed in Glass Bottles. *Journal of Agricultural and Food Chemistry*, 55, 2705-2713.
- Stevenson, D., & Hurst, R. (2007). Polyphenolic Phytochemicals – just antioxidants or much more. *Cellular and Molecular Life Sciences*, 64, 2900-2916.
- Stewart, K. (2005). Processing of Cranberry, Blueberry, Current and Gooseberry. In M. Barrett, S. Laszlo & H. Ramaswamy (Eds.), *Processing Fruits; Science and Technology* (pp. 563-584). (2nd ed.). Boca Raton: CRC Press.
- Stoner, G., Wang, L., Seguin, C., Rocha, C., Stoner, K., Chui, S. & Kinghorn, D. (2010). Multiple Berry Types Prevent N-nitrosomethylbenzylamine-Induced *Esophageal Cancer in Rats*. *Pharmaceutical Research*, 27, 1138-1145.
- Stothers, L. (2002). A randomised trial to evaluate effectiveness and cost effectiveness of naturopathic cranberry products as prophylaxis against urinary tract infections in women. *The Canadian Journal of Urology*, 9, 1558-1562.
- Stull, J., Cash, K., Johnston, W., Champagne, C. & Cefalu, W. (2010). Bioactives in Blueberries Improve Insulin Sensitivity in Obese, Insulin-Resistant Men and Women. *The Journal of Nutrition & Disease*, 140, 1764-1768.
- Szajdek A., & Borowska E. (2008). Bioactive Compounds and Health-Promoting Properties of Berry Fruits: A Review. *Plant Foods for Human Nutrition*, 63, 147-156.
- Talavera, S., Felgines, C., Texier, O., Besson, C., Gil-Izquierdo, A., Lamaisoin, J. & Remesy, C. (2005). Anthocyanin Metabolism in Rats and Their Distribution to Digestive area, Kidney, and Brain. *Journal of Agricultural and Food Chemistry*, 53, 3902-3908.
- Trehane, J. (2004). *Blueberries, Cranberries and other Vacciniums*. Cambridge: Timber Press.

- United States Department of Agriculture. (n.d.). The USDA National Nutrient Database for Standard Reference, Release 22. Retrieved August 2010, from Nutrient Data Laboratory: <http://www.nal.usda.gov/fnic/foodcomp/search/>
- Valls, J. Millana, S., Pilar Martia, M., Borrassa, E. & Arolab, L. (2009). Advanced separation methods of food anthocyanins, isoflavones and flavanols. *Journal of Chromatography A*, 1216, 7143-7172.
- Villota, R., & Hawkes, J. (2007). Reaction Kinetics in Food Systems. In D. Heldman & D. Lund (Eds.), *Handbook of Food Engineering* (pp. 125-286), (2nd ed.). Boca Raton: CRC Press
- Walton, M. (2006). *Berry Fruit Anthocyanins in Human Nutrition: Bioavailability and Antioxidant Effects* (Published doctoral dissertation). Massey University, Palmerston North, New Zealand.
- Wang, H., & Lin, H. (2000). Antioxidant Activity in Fruits and Leaves of Blackberry, Raspberry, and Strawberry Varies with Cultivar and Developmental Stage. *Journal of Agricultural and Food Chemistry*, 48, 140-146.
- Wang, H., & Stoner, G. (2008). Anthocyanins and their role in Cancer Prevention. *Cancer Letters*, 269, 281-290.
- Wang, J., Kalt, W. & Sporns, P. (2000). Comparison between HPLC and MALDI-TOF MS Analysis of Anthocyanins in Highbush Blueberries. *Journal of Agricultural and Food Chemistry*, 48, 3330-3335.
- Wang, S., Chen, C., William, S., Wang, C. & Camp, M. (2008). Fruit Quality, Antioxidant Capacity, and Flavonoid Content of Organically and Conventionally Grown Blueberries. *Journal of Agricultural and Food Chemistry*, 56, 5788-5794.
- Wang, H., Cao, G., & Prior, R. (1997). Oxygen Radical Absorbing Capacity of Anthocyanins. *Journal of Agricultural and Food Chemistry*, 45, 204-309.
- Wang, W. & Xu, S. (2007). Degradation kinetics of anthocyanins in blackberry juice and concentrate. *Journal of Food Engineering*, 82, 271-275.
- Whitehurst, R., & Oort, M. (Eds.). (2010). *Enzymes in Food Technology*. (2nd ed.). Chichester: John Wiley and Sons.
- Wightman, J., & Wrolstad, R. (1996). b-glucosidase Activity in Juice-Processing Enzymes Based on Anthocyanin Analysis. *Journal of Food Science*, 61, 544-548.
- Wu, X. & Prior, R. (2005). Systematic Identification and Characterization of Anthocyanins by HPLC-ESI-MS/MS in Common Foods in the United States: Fruits and Berries. *Journal of Agricultural and Food Chemistry*, 53, 2589-2599.
- Wu, X., Beecher, G., Holden, J., Haytowitz, D., Gebhardt, S. & Prior, R. (2006). Concentrations of

Anthocyanins in Common Foods in the United States and Estimation of Normal Consumption. *Journal of Agricultural and Food Chemistry*, 54, 4069-4075.

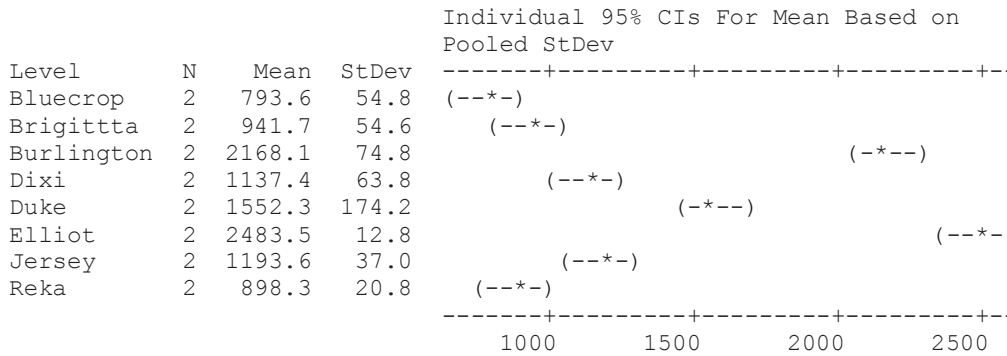
Appendix

ANOVA - Total Anthocyanin

One-way ANOVA: Total antho versus Variety

Source	DF	SS	MS	F	P
Variety	7	5456028	779433	129.97	0.000
Error	8	47975	5997		
Total	15	5504003			

S = 77.44 R-Sq = 99.13% R-Sq(adj) = 98.37%



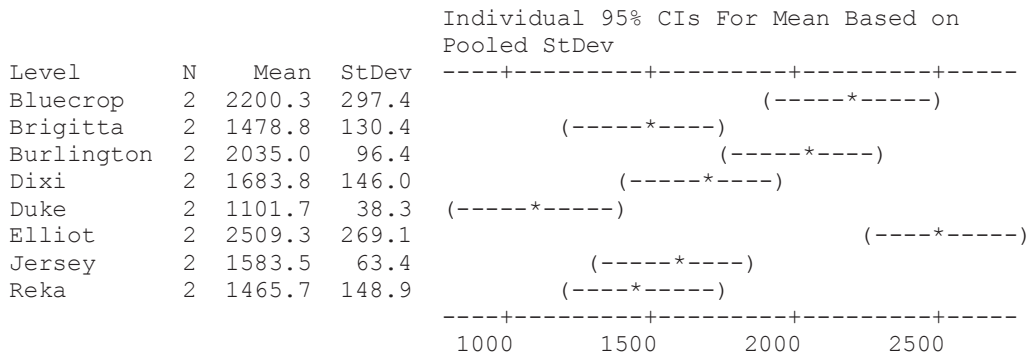
Pooled StDev = 77.4

ANOVA - Chlorogenic acid

One-way ANOVA: CA solvent versus Variety

Source	DF	SS	MS	F	P
Variety	7	2933710	419101	14.20	0.001
Error	8	236097	29512		
Total	15	3169808			

S = 171.8 R-Sq = 92.55% R-Sq(adj) = 86.03%



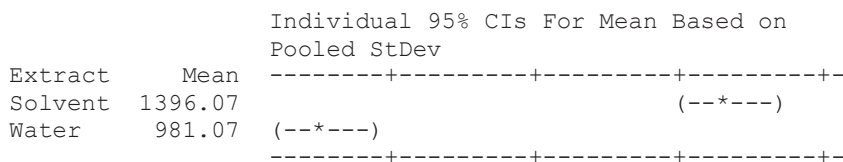
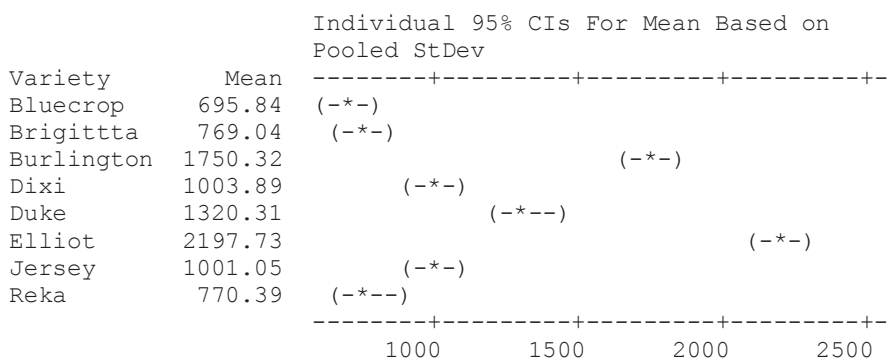
Pooled StDev = 171.8

Two-way ANOVA - Total Anthocyanis vs Variety & Extract

Two-way ANOVA: Antho W versus Variety, Extract

Source	DF	SS	MS	F	P
Variety	7	8056992	1150999	115.51	0.000
Extract	1	1377794	1377794	138.28	0.000
Interaction	7	304887	43555	4.37	0.007
Error	16	159425	9964		
Total	31	9899099			

S = 99.82 R-Sq = 98.39% R-Sq(adj) = 96.88%



PCA - Individual Anthocyanins (first fifteen only)

Principal Component Analysis: Delph-3-gal, Delph-3-glu, Cyan-3-gal, Delph-3-ara

Eigenanalysis of the Correlation Matrix

	1	2	3	4	5	6	7	8	9
Eigenvalue	11.411	2.288	0.907	0.209	0.117	0.035	0.019	0.006	0.005
Proportion	0.761	0.153	0.060	0.014	0.008	0.002	0.001	0.000	0.000
Cumulative	0.761	0.913	0.974	0.988	0.995	0.998	0.999	0.999	1.000

Eigenvalue	0.003	0.001	0.001	0.000	0.000	0.000
Proportion	0.000	0.000	0.000	0.000	0.000	0.000
Cumulative	1.000	1.000	1.000	1.000	1.000	1.000

Variable	PC1	PC2	PC3	PC4
Delph-3-gal	0.281	-0.110	-0.263	-0.087
Delph-3-glu	-0.238	-0.375	-0.094	-0.279
Cyan-3-gal	0.277	-0.158	0.224	-0.284
Delph-3-arab	0.258	-0.158	-0.442	-0.034
Cyan-3 gluc	-0.224	-0.365	0.132	0.487
Petun-3-gal	0.287	-0.092	-0.140	-0.200
Cyan-3-arab	0.236	-0.328	-0.016	0.609
Petun-3-gluc	-0.234	-0.390	-0.087	-0.297
Peon-3-gal	0.258	-0.122	0.467	-0.156
Pet-3-arab	0.273	-0.148	-0.318	-0.075
Peon-3-Gluc	-0.253	-0.330	-0.049	-0.190
Malv-3-gal	0.288	-0.138	0.042	0.007
Peon-3-arab	0.233	-0.207	0.548	-0.105
Malv-3-gluc	-0.239	-0.382	0.021	0.016
Malv-3-arab	0.278	-0.207	-0.088	0.136

Correlation – Total Anthocyanis vs Berry surface area:volume Ratio

Correlations: Total antho, Ratio

Pearson correlation of Total antho and Ratio = 0.606
P-Value = 0.111

Correlation – Total Anthocyanis vs Berry Weight

Correlations: Total antho, Weight

Pearson correlation of Total antho and Weight = -0.697
P-Value = 0.055