

2015

# Juice Processing Effects on Small Scale not from Concentrate Rabbiteye Blueberry Juice Production: The Evaluation of Juice Recovery and Identification of Anthocyanins and Anthocyanidins through Processing Steps

Rebecca Elaine Stein-Chisholm

Louisiana State University and Agricultural and Mechanical College, rstein07@gmail.com

Follow this and additional works at: [https://digitalcommons.lsu.edu/gradschool\\_theses](https://digitalcommons.lsu.edu/gradschool_theses)



Part of the [Life Sciences Commons](#)

---

## Recommended Citation

Stein-Chisholm, Rebecca Elaine, "Juice Processing Effects on Small Scale not from Concentrate Rabbiteye Blueberry Juice Production: The Evaluation of Juice Recovery and Identification of Anthocyanins and Anthocyanidins through Processing Steps" (2015). *LSU Master's Theses*. 3271.

[https://digitalcommons.lsu.edu/gradschool\\_theses/3271](https://digitalcommons.lsu.edu/gradschool_theses/3271)

This Thesis is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Master's Theses by an authorized graduate school editor of LSU Digital Commons. For more information, please contact [gradetd@lsu.edu](mailto:gradetd@lsu.edu).

JUICE PROCESSING EFFECTS ON SMALL SCALE NOT FROM  
CONCENTRATE RABBITEYE BLUEBERRY JUICE PRODUCTION: THE  
EVALUATION OF JUICE RECOVERY AND IDENTIFICATION OF  
ANTHOCYANINS AND ANTHOCYANIDINS THROUGH PROCESSING  
STEPS

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in Partial Fulfillment of the  
requirements for the degree of  
Master of Science

in

The Department of Nutrition and Food Science

by  
Rebecca Elaine Stein-Chisholm  
B.S., Texas A&M University-College Station, 2007  
December 2015

Dedicated to my Beloved.

## **ACKNOWLEDGEMENTS**

The completion of my Master's was a long venture which challenged and enriched me along the way. I could not have done this without the guidance, advice, and encouragement of those who supported and helped me. For without this group of people, I would not been able to complete this research and degree.

First and foremost, I would like to thank God for giving me my gifts and talents to be able to pursue the wonders of plants and research.

I would like to thank my committee members, Drs. John Beaulieu, Jack Losso, and John Finley. Without their enthusiasm and knowledge of food science, I would not have known where to start. Thank you for sticking with me to see me through the end. I would especially like to thank Dr. Beaulieu for giving me the opportunity to work with him on this research and learn from such a great plant physiologist mentor. It has been an honor and privilege working with him. His ability to focus on the important things and the bigger picture helped me to keep pushing to the finish.

I would like to thank the USDA-ARS Southern Regional Research Center in New Orleans, Louisiana for giving me the opportunity to complete my research at the facility and utilize their expertise and resources to continue the objectives and missions of which the USDA was founded upon. Thank you to Dr. Casey Grimm and Steve Lloyd for being such wonderful co-workers and mentors. Also, thank you to them for helping me run samples and allowing me to use (and take over) the LC-MS/MS to complete this

data. To Maureen Tully, thank you for being a great co-worker and assisting me in the laboratory at the USDA. I would like to give a special thank you to Dr. Donna Marshall-Shaw and Lavonne Stringer at the Thad Cochran Southern Horticultural Laboratory, USDA-ARS in Poplarville, Mississippi for their help with the blueberries. I would also like to express thanks to Pete Whitehead, Goodnature Products, Inc., for his advice regarding hydraulic pressing and the supplemental pressing data reported.

I would like to thank Lenny Zichichi for being a great study partner and helping me get back into the college frame of mind, as well as being a great and positive friend. To Lauren Navarre and Kaci Fisher, thank you to them for being such supportive and awesome friends and roommates while I lived in Baton Rouge.

To my parents, family and friends, many thanks to them for all the support, encouragement, and faith it took to complete this adventure. The phone calls, emails, texts, and words of encouragement were and are much appreciated and needed.

Lastly, I would like to thank my devoted husband, Adam, for the love, encouragement, and support he gave me especially when I moved to Baton Rouge without him. This is our degree and thesis.

# TABLE OF CONTENTS

ACKNOWLEDGEMENTS .....	iii
LIST OF TABLES .....	vii
LIST OF FIGURES.....	viii
LIST OF SUPPLEMENTAL IMAGES, DOCUMENTS AND FIGURES.....	ix
LIST OF ABBREVIATIONS AND ACRONYMS.....	x
ABSTRACT .....	xii
CHAPTER 1 LITERATURE REVIEW.....	1
1.1. Introduction .....	1
1.2. Literature Review .....	3
1.2.1. Blueberries.....	3
1.2.2. Blueberry Fruit .....	5
1.2.3. Industry.....	12
1.2.4. Juice Processing and Anthocyanin Characterization Methods .....	16
1.3. Conclusion .....	30
CHAPTER 2 PRESS EFFICIENCY AND PILOT SCALE UP EXPERIMENT – PRESSING TEMPERATURE AND ENZYME TREATMENT EFFECTS ON SMALL SCALE BLUEBERRY JUICE RECOVERY .....	32
2.1. Abstract.....	32
2.2. Introduction .....	33
2.3. Materials and Methods.....	36
2.3.1. Preliminary Press Weight.....	36
2.3.2. Bench Top Press Regimes .....	36
2.3.3. Rapid Qualitative Appraisals.....	38
2.3.4. Pilot Scale Press.....	39
2.4. Results and Discussion.....	40
2.4.1. Preliminary Press Weight.....	40
2.4.2. Bench Top Presses .....	41
2.4.3. Pilot Plant Press Juice and Press Cake Recovery.....	48
2.4.4. Juice Loss and Total Juice Recovery for Bench Top and Pilot Presses .....	49
2.4.5. Supplementary Pilot Plant Press Data.....	52
2.5. Conclusion .....	54

CHAPTER 3 INTEGRATED PILOT EXPERIMENT – EVALUATION OF ANTHOCYANINS AND ANTHOCYANIDINS AS JUICE QUALITY MARKERS THROUGH PROCESSING STEPS OF RABBITEYE BLUEBERRIES BY LC-MS/MS AND UPLC-UV .....	55
3.1. Abstract.....	55
3.2. Introduction .....	56
3.3. Materials and Methods.....	59
3.3.1. Juice Processing.....	59
3.3.2. Anthocyanin Analysis.....	61
3.3.3. Anthocyanidin Analysis.....	62
3.4. Results and Discussion.....	64
3.4.1. Identification of Anthocyanins in Tifblue Blueberry and Juices .....	64
3.4.2. Anthocyanin Changes during Blueberry Juice Processing .....	71
3.4.3. Identification of Anthocyanidins in Tifblue Blueberry and Juices.....	77
3.4.4. Anthocyanidin Changes during Blueberry Juice Processing.....	78
3.5 Conclusion .....	85
CHAPTER 4 .....	87
CONCLUSION .....	87
REFERENCES.....	91
APPENDIX .....	109
SUPPLEMENTAL IMAGES.....	109
VITA .....	119

## LIST OF TABLES

Table 1.1. Survey of blueberry anthocyanin extraction methods found in literature.....	28
Table 2.1. Average percent free juice recovery in heat and enzyme treated blueberry bench top presses.....	42
Table 2.2. Average physical properties of bench top press free juice recovery in heat and enzyme treated blueberry presses.....	47
Table 2.3. Goodnature X-1 manufacturer blueberry press testing information.....	53
Table 3.1. Major and minor anthocyanin compound descriptions and mass spectrometry variables found in blueberries.....	65
Table 3.2. Anthocyanins in blueberries from literature.....	67
Table 3.3. Average semi-quantitative LC-MS/MS anthocyanin abundance in RAB blueberry during various juice processing steps.....	72
Table 3.4. Average UPLC-UV anthocyanidin content in RAB blueberry juice processing steps in mg/100 g sample.....	79



## LIST OF FIGURES

Figure 1.1. Principal anthocyanin chemical structure and flavylum cation backbone....	10
Figure 1.2. Anthocyanidins and structures commonly found in blueberries.....	10
Figure 1.3. Structure of malvidin-3-galactoside.....	11
Figure 1.4. Structure of delphinidin-3-(6-p-coumaroyl) glucoside.....	25
Figure 2.1. Average percent free juice recovery comparisons between SHB and RAB blueberry bench top presses.....	44
Figure 2.2. Average percent total juice recovery comparisons between SHB and RAB blueberry for bench top.....	51
Figure 2.3. Free and total juice recovery comparison between bench top and pilot experiments in RAB blueberry presses.....	52
Figure 3.1. Overlay of [M] <sup>+</sup> ions of major anthocyanins in raw berry sample.....	68
Figure 3.2. Chemical structure of delphinidin-3-(p-coumaroyl-glucoside), cyanidin-3-(p- coumaroyl-glucoside), and petunidin-3-(p-coumaroyl-glucoside) identified in processed 'Tifblue' blueberries.....	69
Figure 3.3. MS/MS spectrum scan of delphinidin-3-(p-coumaroyl-glucoside) in unfiltered (NCJ) rabbiteye blueberry juice.....	70
Figure 3.4. MS/MS spectrum scan of cyanidin-3-(p-coumaroyl-glucoside) in unfiltered (NCJ) rabbiteye blueberry juice.....	70
Figure 3.5. MS/MS spectrum scan of petunidin-3-(p-coumaroyl-glucoside) in unfiltered (NCJ) rabbiteye blueberry juice.....	70
Figure 3.6. Anthocyanidins found in rabbiteye blueberry press cake as detected by UPLC-UV.....	78
Figure 3.7. Percent anthocyanidin loss through unfiltered and filtered RAB blueberry juice processing.....	83

## LIST OF SUPPLEMENTAL IMAGES, DOCUMENTS, AND FIGURES

Supplemental Image A.1. Stainless steel press showing nylon press cloth and spigot for juice extraction.....	109
Supplemental Image A.2. Bench top juice experiment water bath used to heat blueberries to 95 °C.....	110
Supplemental Image A.3. Pilot scale steam jacketed kettle mashing of rabbiteye ‘Tifblue’ blueberries heated to 95 °C with constant stirring.....	110
Supplemental Image A.4. Pilot scale pressing of rabbiteye ‘Tifblue’ blueberries in a Goodnature X-1 press, showing the juice flowing out the bottom of the press into a stainless steel pan.....	111
Supplemental Image A.5. Press cake removed from mashed and enzyme treated ‘Tifblue’ rabbiteye blueberries.....	111
Supplemental Image A.6. Pilot scale ultrafiltration of rabbiteye ‘Tifblue’ blueberries from a PCI B-1 pilot ultrafiltration unit.....	112
Supplemental Document 1.1. AB Enzymes Rohapect 10L Description and Specification Sheet.....	113
Supplemental Document 1.2. Novozymes Pectinex BEXXL Product Data Sheet.....	116
Supplemental Figure 1.1. Temperature plots recorded during Tifblue blueberry mash treatments in steam jacketed kettles.....	118

## LIST OF ABBREVIATIONS AND ACRONYMS

ACN	Acetonitrile
BIL	Bilberry
CJP	Clarified (ultrafiltered) pasteurized blueberry juice
CUF	Clarified ultrafiltered (un-pasteurized) blueberry juice
Cya-3-ara	Cyanidin-3-arabinoside
Cya-3-cou	Cyanidin-3-(p-coumaroyl-glucoside)
DAD	Diode-array detector
Del-3-ara	Delphinidin-3-arabinoside
Del-3-cou	Delphinidin-3-(p-coumaroyl-glucoside)
Del-3-pyr	Delphinidin-3-pyranside
ESI	Electrospray Ionization
EtOH	Ethanol
FAC	Frozen Absolute Control (raw berry)
FDA	Food and Drug Administration
H <sub>2</sub> O	Water
HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
HTST	High temperature short time
LTLT	Low temperature long time
Mal-3-ara/Peo-3-pyr	Malvidin-3-arabinoside/Peonidin-3-pyranside
Mal-3-pyr	Malvidin-3-pyranside
MeOH	Methanol

min	Minute(s)
MS	Mass Spectrometry
MW	Molecular weight
NCJ	Non-clarified (unfiltered, un-pasteurized) blueberry juice
NCP	Non-clarified (unfiltered) pasteurized blueberry juice
NFC	Not from concentrate
NHB	Northern highbush blueberry
nm	Nanometer
SHB	Southern highbush blueberry
Peo-3-ara	Peonidin-3-arabinoside
Pet-3-ara/Cya-3-pyr	Petunidin-3-arabinoside/ Cyanidin-3-pyranoside
Pet-3-pyr	Petunidin-3-pyranoside
Pet-3-cou	Petunidin-3-(p-coumaroyl-glucoside)
PRC	Press cake
PVDF	Polyvinylidene difluoride
RAB	Rabbiteye blueberry
s	Second(s)
TFA	Trifluoroacetic acid
UF	Ultrafiltration
UHT	Ultra high temperature
UPLC	Ultra performance liquid chromatography
USDA	United States Department of Agriculture
UV-Vis	Ultra violet-visible

## ABSTRACT

The order and combination of juicing steps can change a blueberry's bioactive phytochemicals and effect juice recovery. In addition to physical treatments such as pressing, recovery is also affected by heat and enzymes steps. Not optimizing juicing methods affect juice quality and leave many bioactive components in the press cake. To evaluate pre-press treatments, southern highbush and rabbiteye blueberries were individually pressed in a bench top press at varying temperatures. The temperature treatments included fresh, thawed, frozen and heating to 95 °C. Two pectinase enzymes, Rohapect 10L and Pectinex BEXXL, were individually used to assess impact on juice extraction. Juice recovery was averaged from three press replications for each treatment. Frozen berries which were heated to 95 °C and treated with enzyme had the highest recovery at  $68.6 \pm 1.1\%$ . This process was then transferred to a pilot scale press. Pilot scale juice recovery was calculated at  $74.0 \pm 0.9\%$ . Using data from the initial pilot presses, a full pilot scale experiment was triplicated. 'Tifblue' rabbiteye blueberries were heated in a steam jacketed kettle to 95 °C for three minutes followed by a Rohapect 10L enzyme treatment before pressing. The resulting juice from the pilot press was then filtered and pasteurized. Unfiltered juice was also pasteurized. Press cake was collected and frozen. Samples of raw berries, filtered and unfiltered juice, pasteurized juice, and press cake were taken to identify anthocyanin compounds and changes caused by juice processes using LC-MS/MS. Samples were also hydrolyzed for anthocyanidin quantification using UPLC-UV. Ten major anthocyanins were identified, including 5 arabinoside and 5 pyranoside anthocyanins and three minor anthocyanins. The five anthocyanidins, cyanidin, delphinidin, malvidin, peonidin, and

petunidin, were quantified. Raw berries and press cake contained the highest anthocyanidin contents with 85.1 mg/100 g and 265.6 mg/100 g respectively. Decreases of 67% loss after pressing and 10% loss after pasteurization were determined for anthocyanins and anthocyanidins in juices. However, three new conjugated anthocyanins were found in processed juices which have not previously been reported in rabbiteye. This contributes to the value and interest of press cake for use in other food and non-food products.

# CHAPTER 1

## LITERATURE REVIEW

### 1.1. Introduction

Blueberries have been a part of the human diet for centuries. Folklore that surrounds blueberries includes remedies related to illness and beauty (Hutchens, 1992).

Blueberries deliver sweet flavors and health benefits, so consequently they are often eaten fresh, baked, juiced, and preserved (Brownmiller et al., 2008). There are many research studies reporting multiple beneficial phytochemicals in blueberries which has increased consumer demand for the fruit in various forms (Folmer et al., 2014; Kalt and Dufour, 1997; Soto-Vaca et al., 2012). Blueberries have become popular ingredients within the juice and functional beverage categories because they deliver nutritional benefit such as vitamins, minerals, and antioxidants into the diet. Research has demonstrated anti-inflammatory, anti-carcinogenic, and anti-mutagenic properties of blueberry compounds and suggested that blueberries may help protect the brain, cardiovascular and central nervous system, and reduce type 2 diabetes (Basu et al., 2010; Soto-Vaca et al., 2012). The potential of blueberry health benefits has resulted in a surge in production and consumption of the berries (Hummel et al., 2012). In 2012, there were more than 1,400 new products introduced in the U.S. market containing blueberries (Anonymous, 2013). Of those products, 17% were beverages. Just a few percentage points behind the largest growth category of baked goods (Anonymous, 2013). Products featuring blueberries have projected increases of 10-20% every year (Mellentin and Crawford, 2008).

Juices labeled 'Not From Concentrate' are single strength juices which are pasteurized after extraction (Bates et al., 2001). Not from concentrate (NFC) juices are gaining in niche markets with an 8% growth each year through 2014 and are creating economically attractive alternatives for juice products (Barkla, 2011). While there are not many public sources for NFC industry information, trends in the orange juice segment show that NFC juices are increasing in sales while concentrates are decreasing (Santos et al., 2013). This increased demand calls for blueberry research to understand blueberry cultivar characteristics and variability, processing effects and improved processing technology, use of by-products, understanding of nutrient and phytonutrient stability, and maximizing opportunities for use of the entire fruit. While much of the blueberry juicing research has focused on northern highbush (NHB) cultivars, southern highbush (SHB) and rabbiteye (RAB) blueberries are gaining interest as these species become more popular with growers in the southeast United States, including the Gulf Coast region due to their adaption to less acidic soils and longer production (Ballington, 2007). The objective of this study is to enhance our understanding pertaining to NFC blueberry juice using small, pilot scale processing and to increase juice recovery percentages by exploring process steps which maintain quality attributes while attempting to limit anthocyanin degradation in the juice. Local small scale producers can benefit from knowledge of how processing steps affect the phytochemicals in their RAB and SHB products. Identification of anthocyanins and analysis of their degradation through juice processing creates NFC blueberry juices which have better color



attributes, greater antioxidant properties, and improved transfer of health properties from raw blueberries to processed blueberry products. Small scale juicers can take this information and apply it to their production processes for improved juice products.

## 1.2. Literature Review

### 1.2.1. Blueberries

Blueberries are members of the *Ericaceae* family, which also includes azaleas and heathers (Kron, Powell, & Luteyn, 2002). Members of this family are perennial woody shrubs that favor acidic, well drained soils in temperate climates (Sellmer et al., 2004). Blueberries are part of the *Vaccinae* tribe, which includes 20 other genera of plants that produce blue colored berries. Further categorization places them into the *Vaccinium* genus (Ballington, 2007). The *Vaccinium* genus contains more than 400 species (Lyrene et al., 2003). While there is still a debate as to where some of the native species of *Vaccinium* belong, many of the cultivated species and well known wild species are further divided up into sections within the *Vaccinium* genus. Each section includes important berries consumed by humans. Blueberries belong to the *Cyanococcus* section and bilberries (*V. myrtillus*) are in the *Myrtillus* section (Lyrene et al., 2003). *Vaccinium* species are naturally found on all continents except for Antarctica and Australia (Lyrene et al., 2003). However, Australia has begun to develop an increasing blueberry industry (Retamales and Hancock, 2012).

#### 1.2.1.1. Species

Blueberries can be diploids, tetraploids, and hexaploids, meaning they have 2, 4, or 6 sets of chromosomes (Bruederle et al., 1991; USDA, 2001). Most commercial varieties of blueberries are traditionally propagated by cuttings and are tetraploids and hexaploids, while wild species are naturally occurring diploids (Boches et al., 2006; Lyrene et al., 2003). NHB are mostly tetraploids while RAB are hexaploids (Boches et al., 2006; Lyrene et al., 2003). The following species are the most important and relevant for the industry in the Southeast and Southern U.S.

##### Rabbiteye - *V. ashei*

Rabbiteye blueberries are native to the southeastern U.S. (Strik et al., 2014). Most hexaploid RAB varieties have common parental lineages, going back to the original wild type northern FL and southern GA native selections that were named 'Black Giant', 'Clara', 'Ethel' (a.k.a. 'Satilla') and 'Myers' (Lyrene, 1981). *V. ashei* also include synonyms *V. ashei* 'Reade' and *V. virgatum*. (Boches et al., 2006; Wang et al., 2011). Cultivation of RAB berries began in the 1940's and are especially adapted for heat and dry soils with higher pH (Ballington, 2007; Lyrene, 1981; Strik and Yarborough, 2005). RAB are great for fresh market because they have good post-harvest fruit quality and are larger than the average SHB berries (Ballington, 2007). They are often more vigorous and will produce good harvests for longer than SHB in addition to their sweeter tasting berries (Ballington, 2007). Commercial production of blueberries began in Louisiana in the late 1950's with the introduction of RAB berries (Puls Jr., 1999). These berries make up 90% of the Gulf Coast region's production acreage (Stringer et al.,

2009). 'Tifblue' at one point was the most widely planted RAB in the world (Brooks and Olmo, 1997). It is a cross of 'Ethel' and 'Clara' parents, introduced in 1955, and accounts for over 50% of RAB varieties planted today (USDA, 2014). 'Tifblue' is considered the standard variety to which RAB are compared (Marshall et al., 2006).

#### Southern Highbush - *V. corymbosum* x *V. darrowii*

Southern highbush blueberries are a cross between *V. corymbosum* x *V. darrowii* (Fonsah et al., 2013; Kalt et al., 2001). Interspecific hybrids of *V. ashei*, *V. corymbosum*, and *V. darrowii* have also lead to various SHB varieties (Du et al., 2011). Other genetics that have been bred into SHB include the *V. virgatum* and *V. tenellum*, which are native to the southern U.S. (Ballington, 2007). The addition of these genetics allowed for shorter chilling requirements and heat tolerance (Ballington, 2007). Synonyms of SHB include *V. australe* and *V. formosum* (USDA, 2014). Southern highbush blueberries were first planted in the 1970's and have since grown in acreage in the southern U.S. (Strik and Yarborough, 2005). Evaluation of SHB varieties in Louisiana began in the late 1990's when the state's blueberry production was around 500 acres (Johnson and Huang, 1999). Some cultivars of SHB bloom at the same time as RAB, but develop ripe fruit up to three weeks sooner than some cultivars of RAB (Spiers et al., 2002).

#### 1.2.2. Blueberry Fruit

Blueberry fruit are true berries, meaning they are simple fruits with seeds and pulp produced from a single inferior ovary (Janick, 1986). Generally, *Vaccinium* species have self-incompatible flowers and need bees to cross-pollinate their flowers, however, there

are some species which are self-compatible (Ballington, 2007; Lyrene et al., 2003). Many cultivars have larger yields when cross pollinated and with better pollination, comes larger fruit (Williamson et al., 2012). Blueberry fruit has a protective coating of powdery epicuticular wax which is known as the bloom and depending on the variety, the bloom can be more pronounced and give a powdery blue color to the berry (Sapers et al., 1984). This edible wax protects the fruit against water loss and fruit deterioration, but also causes the blue color of the berry (Albrigo et al., 1980). Anthocyanins give the berry the dark color under the bloom. Anthocyanins are mainly found in the skin of the berries, but can be found throughout the berry depending on the berry section. Berries belonging to the *cyanococcus* section only have pigmentation in the skin (Kalt et al., 2001). The skin consists of epidermal and subepidermal layers (Allan-Wojtas et al., 2001). The content of anthocyanins in the subepidermal layers varies based on the cultivar. The highbush species 'Coville', has 3 layers with even distribution of medium sized cells with anthocyanins spread between all three layers (Allan-Wojtas et al., 2001). Another highbush species, 'Elliott', has two layers of medium and large cells, with the large cells containing the most anthocyanins (Allan-Wojtas et al., 2001). Berries have around 80 seeds depending on the cultivar (Lyrene et al., 2003). The greater the number of seeds, the larger the fruit (Williamson et al., 2012). Blueberry seed oil is a rich source of the essential fatty acid, linoleic acid (Parry et al., 2005).

### 1.2.2.1. Fruit and Juice Characterization

Berry and juice characterization is affected by many factors including genetics, sunlight, temperature, water levels, plant nutrients, and plant and berry maturity (Sapers et al., 1984). The following characteristics are noteworthy due to their importance in juice quality.

#### Sugars

The sugar content and total soluble solids varies based on genetics and environmental factors. Rabbiteye 'Tifblue' berries range from 11.5 °Brix total soluble solids in early harvest to 17.1 °Brix in later harvests (de Moraes et al., 2007; Prior et al., 1998). Berry maturity has a direct effect on sugars, as it was found in *V. myrtillus*. Sucrose levels are not detected until the mid-ripe and ripe stages, while fructose and glucose increase significantly during the later stages of ripening (Ayaz et al., 2001). Analysis of total sugars in *V. myrtillus* determined they contain around 64% sugar based on dry weight (Ayaz et al., 2001).

#### Titrateable Acidity (TA)

Much like sugars, titrateable acidity in berries is affected by genetics and environmental factors. The titrateable acids decrease as the berries ripen. Often the acid to sugar ratio is used to determine ripeness, with total soluble solids increasing while titrateable acids decrease (Sapers et al., 1984). Rabbiteye 'Tifblue' from early harvests have around 0.84 milliequivalent acid per gram dry matter and decrease to 0.35 milliequivalent acid per gram dry matter as they ripen (Prior et al., 1998).

## pH

Blueberries and blueberry juices are known to be very acidic. Blueberry fruit pH ranges between 3.1 and 3.9 (Anonymous, 1962). Rabbiteye blueberry juice pH values were between 2.56 and 2.67 using blueberries grown in Brazil (de Moraes et al., 2007). Northern highbush berry pH ranges from 2.85 to 3.49 while RAB berries range from 2.5 to 3.0 (Saftner et al., 2008; Sapers et al., 1984). The acidic ranges of blueberry juice pH is also beneficial for producers as an inherent microbial deterrent against spoilage and harmful bacteria and mold (Brul and Coote, 1999).

## Color

The co-evolution of plants and birds is believed to have contributed to the colors of fruits and flowers. Anthocyanins are more visible to birds than other plant pigments and a major benefit of birds is seed dispersal (Duan et al., 2014). While berry surface color is determined by the waxy bloom content, juice color is determined by anthocyanin content and pH (Sapers et al., 1984). Blueberry juices blanched before pressing had more blue hued juices (Rossi et al., 2003).

## Anthocyanins

Anthocyanins are just one of the groups of compounds that are found in fruits and vegetables that contribute to consumer health benefits (Khanal et al., 2010). The health benefits of consuming blueberries are well documented and include anti-inflammatory and anti-carcinogenic properties as well as the ability to reduce obesity and cardiovascular disease (Basu et al., 2010; Soto-Vaca et al., 2012). It is this factor that

contributes to the importance of juice processing to preserve these compounds. In plants, anthocyanins are important secondary metabolites and are used as defense mechanisms, coloration, and are results of environmental factors (Brambilla et al., 2008; Howard et al., 2003; Tomás-Barberán and Espín, 2001). Anthocyanins are composed of an anthocyanidin flavylum backbone with varying combinations of glycosides. Flavylum ions are pyrylium salts containing a conjugated 6-member carbon ring system with a positively charged oxygen atom replacing one of the ring's carbons atoms (Pina et al., 2012). A diagram of a flavylum cation and a general anthocyanin can be seen in Figure 1.1 (Pina et al., 2012). In plants, 19 different anthocyanidins have been identified, but there are six most commonly found in many plants (Tanaka et al., 2008). These six are cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin (Tanaka et al., 2008). Cyanidin is the most common anthocyanidin found in plants and can have 76 different glycoside combinations (Baxter et al., 1998). Combinations of these 19 anthocyanidins have contributed to the identification of over 600 naturally occurring anthocyanins identified and reported in plants (Wu et al., 2006). The five major anthocyanidins in blueberries are cyanidin, delphinidin, malvidin, peonidin, and petunidin (Figure 1.2) (Gao and Mazza, 1994). The major anthocyanins in blueberries include 3-glycosidic derivatives of these major anthocyanidins; with glucose, galactose, and arabinose as the most abundant sugars (Routray and Orsat, 2011). Regardless of species, malvidin-3-galactoside is the most abundant anthocyanin in blueberries (Figure 1.3) (Routray and Orsat, 2011; Skrede et al., 2000). The lesser anthocyanins consist of acetoyl, malonoyl and coumaroyl compounds (Barnes et al., 2009).

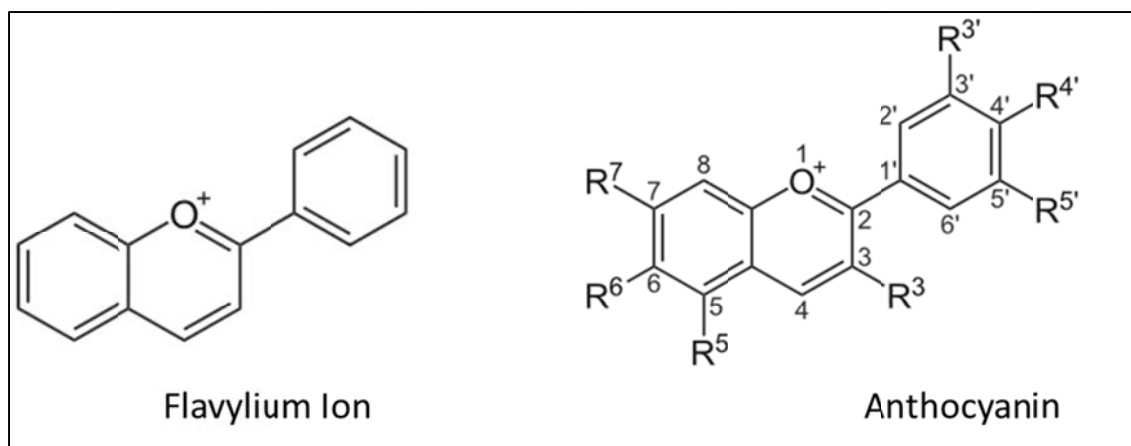


Figure 1.1. Principal anthocyanin chemical structure and flavylium cation backbone (R can be any glycoside). Figure obtained from (Neveu et al., 2012).

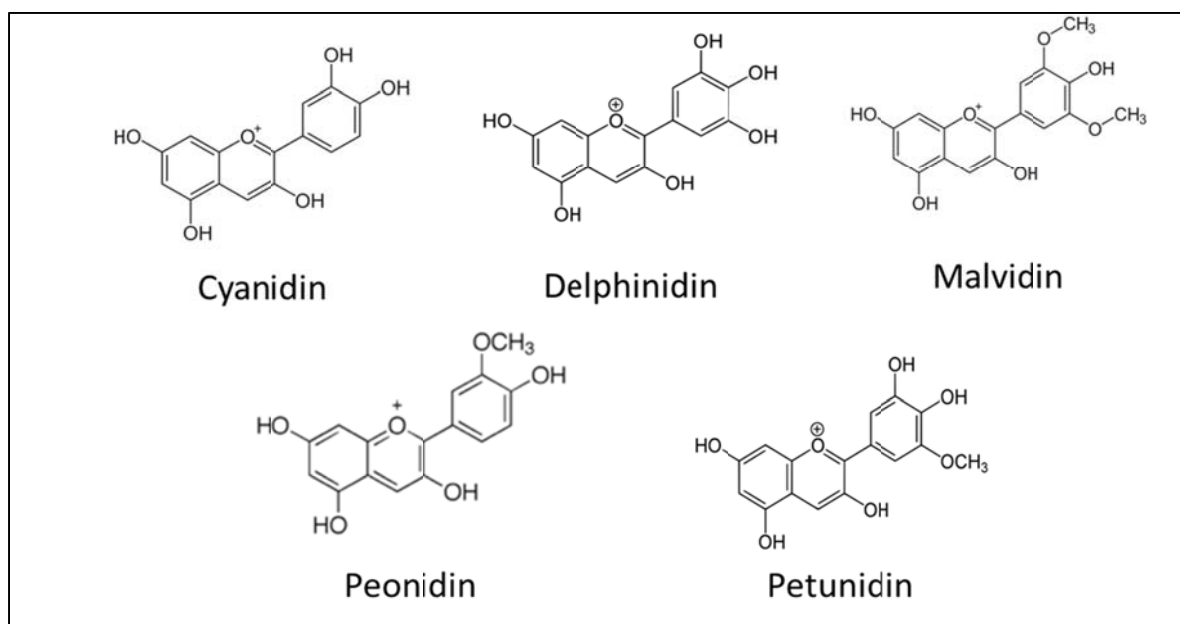


Figure 1.2. Anthocyanidins and structures commonly found in blueberries. Figure obtained from (Neveu et al., 2012).

The synthesis of anthocyanins in berries is affected by many factors including temperature, sunlight, pathogens, and pests (Kalt et al., 2001; Khanal et al., 2009). Anthocyanins are produced even as the berry becomes overripe, after harvest, and during storage (Castrejón et al., 2008). They are mainly found in the skin of the berries,



but can be found throughout the berry depending on the berry section (Brambilla et al., 2008). A study by Brambilla et al. (2008) showed that different *V. corymbosum* cultivars had differences in anthocyanin glycosidic derivatives. Cultivars 'Bluegold' and 'Bluecrop' were more abundant in glucoside containing anthocyanins; while 'Coville', 'Berkeley', and 'Bluechip' had more galactoside containing anthocyanins (Brambilla et al., 2008). Differences in amounts of glycosides between these cultivars is believed to be due to differences in their genetic backgrounds (Cho et al., 2004).

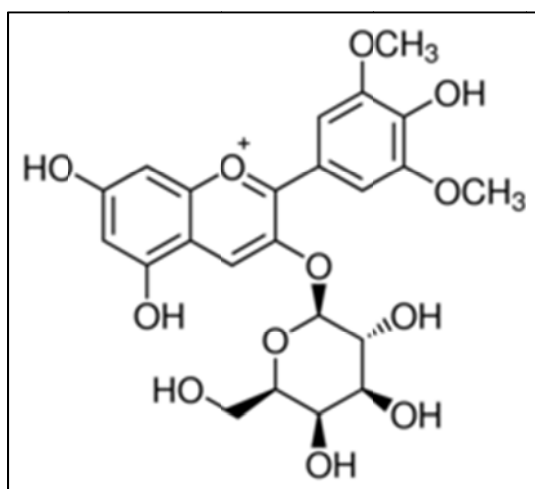


Figure 1.3. Structure of malvidin-3-galactoside. Figure obtained from (Neveu et al., 2012).

Anthocyanins can be degraded by many factors including heat, pH, light,  $H_2O_2$ , and duration of time exposed to these conditions (Khanal et al., 2010). Their stability in juice processing can be determined by many factors. *In vivo*, anthocyanin stability can be attributed to self-association, inter- and intramolecular co-pigmentation and creating complexes with metal ions (Barnes et al., 2009; Holzwarth et al., 2012). These co-pigmentations have been observed over a wide range of pH in fruits and vegetables (Barnes et al., 2009). High sugar concentrations are also reported to enhance

anthocyanin stability due to water immobilization with the reduction in water activity (Holzwarth et al., 2012). Processing can cause blueberry anthocyanins to be oxidized due to the presence of native polyphenol oxidases (PPO) (Buckow et al., 2010; Skrede et al., 2000). However, some anthocyanins are not oxidized by PPO due to their structure, and it is believed that the sugar moiety of these anthocyanins causes steric hindrance against PPO activation (Buckow et al., 2010). This steric hindrance can be overcome in the presence of  $\beta$ -glucosidase, which removes the sugar moiety, converting the anthocyanin into its aglycone anthocyanidin, which is easily oxidized by PPO (Buckow et al., 2010; Wightman and Wrolstad, 1996). Developing a process which can limit the activity of these endogenous enzymes and reduce exposure to detrimental physical factors (i.e. high pH or temperature) may help prevent anthocyanin degradation in juices.

### 1.2.3. Industry

Blueberries are native to North America, so naturally they are grown all over the United States. Northern highbush are grown in the Pacific Northwest, Michigan, North Carolina, and New Jersey (Retamales and Hancock, 2012). Southern highbush are mostly grown in California, Georgia, and Florida while RAB are grown in Texas, Louisiana, and Mississippi as well as parts of California, Georgia and Florida (Retamales and Hancock, 2012). Fruits harvested in Florida, Mexico, California, New Jersey, and North Carolina are mostly sold as fresh; while only half of the fruits harvested in Georgia, Mississippi, Louisiana, British Columbia, and Oregon are sold as fresh (Muhammad and Allen, 2000; Retamales and Hancock, 2012). Much of the berries harvested in Michigan and

Washington, 60% to 75%, are used for processing or freezing; while 10% of berries grown in California are used for processing (Retamales and Hancock, 2012). Wild berries are mainly used for processed and baked goods (Retamales and Hancock, 2012). As of 2012, Northern highbush (*V. corymbosum*) was the predominantly cultivated blueberry species followed by RAB and wild *V. angustifolium* (Retamales and Hancock, 2012).

#### Acreage and Production

North American U.S. blueberry production acreage has increased significantly over the past few years in response to high blueberry demand and prices. From 1992 to 2003, U.S. production increased 15%, growing from 48,790 to 55,898 acres (Strik and Yarborough, 2005). Blueberry production in the U.S. was estimated to increase by 33% in the next 10 years (Strik and Yarborough, 2005). In 1989, only around 700 acres of blueberries were planted in Louisiana and Mississippi (Press, 1989). As of 2010, Louisiana and Mississippi farmers cultivated more than 3,850 acres of blueberries (Anonymous, 2013).

From 2002 to 2012 in the U.S. there has been a 2.2-fold increase in cultivated and wild blueberry production and utilization from 113.8 million kg (251.1 million lbs.) to 250.8 million kg (552.9 million lbs.) (USDA, 2013c). Likewise, during this same period, the southeastern U.S. (AL, AR, FL, GA, MS and NC) has seen a 3.6-fold increase in cultivated blueberry production and utilization from 17.1 million kg (37.6 million lbs.) to 62.2 million kg (137.2 million lbs.) (USDA, 2013c). In 2014, the U.S. cultivated yield and

value for fresh and processed blueberries was 255.5 million kg (563.2 million lbs.) (USDA, 2015). Recently, the southeast blueberry industry in Georgia has experienced phenomenal growth with over 42,000 kg (92,000 lbs.) produced in 2014 (Eklund, 2014). Within the U.S. industry, production is soon to outpace fresh product demand (Brazelton, 2011). There is need for alternative markets and opportunities to keep up with production. Studies involving the processing effects on blueberries for NFC juices are needed to continue influencing the market of niche products.

### Economics

Due to their recent growth in popularity, blueberries have increased in value over the last few decades. The delicate nature of the fruit itself in addition to the bush size and shape, make mechanical harvest difficult, contributing to the price of fresh blueberries (Takeda et al., 2008). However, blueberry prices have not slowed industry growth. The U.S. market value for blueberries increased four-fold over the last decade (2002 - 2012) to \$781.8 million, up from just \$194.6 million and has increased to \$824.9 million in 2014 (USDA, 2013b; USDA, 2015). In 1997, blueberry production in Louisiana was valued at \$1.5 million and has increased to \$5.1 million in 2014 (Johnson and Huang, 1999; LSU, 2014). The price of berries per pound is reflected in this increase as well. In 2007, fresh berries were selling at \$1.45/lb. and \$0.48/lb. for processed berries. In 2009, the price increased to \$2.00/lb. for fresh and \$0.75/lb. for processed (Fonsah et al., 2013). Retail prices for fresh berries in 2013 were around \$4.75/lb, while frozen berries were \$3.65/lb. (USDA, 2013a).

Fruit juice sales in the U.S. in 2010 were \$32.3 billion and is estimated to be worth around \$33.3 billion in 2015 (AAFC, 2012). Products containing 100% juice captured \$8.8 billion in sales in 2010, with sales of NFC fruit juices increasing from \$3.04 billion in 2005 to \$3.35 billion in 2010 (AAFC, 2011). Information and data showing Louisiana and other small scale juice production and prices are scarce, but internet searches show local juice bar type restaurants opening across the state and region. A local Baton Rouge juice bar sells mixed blueberry juice with other fruits and vegetables for \$5 per 4oz. serving or \$10 for a liter of single fruit type juice (<http://thebigsqueezy.com/> 9/10/2015). A Georgia based blueberry juice producer sells a NFC 100% blueberry juice product for \$2.91 per 10oz. bottle (<http://southernpresspacking.storenvy.com/> 10/15/2015). Juices for health detoxes and cleanses are increasing juice prices as well (Nartea, 2014). The same Baton Rouge juice company has juice cleanse packages which include blueberry juice for \$49.00 per day.

One of the by-products of the juicing industry is press cake, which may be another opportunity for juice producers to benefit from processing. Several studies have determined that press cake contains the highest concentration of anthocyanins due to anthocyanins being located in the skins of the berries (Howard et al., 2012). Utilizing anthocyanins from press cake in other products is gaining interest outside of the food industry, as pharmaceutical companies look for sources of nutraceuticals to develop supplements and natural drugs (Thomasset et al., 2009). An internet vitamin and

supplement company sells blueberry extract for \$4.99 a bottle containing 0.6 g of extract (<http://www.puritan.com/> 9/10/2015). More refined extracts could sell for more as developed drug and supplement products.

#### 1.2.4. Juice Processing and Anthocyanin Characterization Methods

Juice is a popular way of berry preservation due to its widespread consumption (Rossi et al., 2003). Using less than perfect berries which cannot be sold in fresh markets to make juice decreases waste and allows blueberry products to easily be shipped across the world while still maintaining their nutrients and flavor (Bates et al., 2001). Substantial amounts of blueberry fruit is processed into juice and juice concentrate and used in mixed beverages, syrups, and other food products (Lee and Wrolstad, 2004). Juice processing studies in many fruits and vegetables, including blueberries, have shown that the steps of juicing cause oxidative degradation of nutrients and phytochemicals. Much of the degradation is due to the disorganization of tissues after mashing and high temperatures of processing, as well as physical loss to pressing (Brambilla et al., 2008).

##### 1.2.4.1. Extraction of Juice and Juice Processing

It is well documented that pretreating fruit with heat and enzymes increases juice yield (Jaeger et al., 2012; Landbo et al., 2007; Mieszczakowska-Frąć et al., 2012). In an experiment by Gerard and Roberts (2004), apples were heated by microwave to improve juice recovery. When not heated before pressing, a bench top scale press of 'Fuji' and 'McIntosh' apples resulted in juice recovery of 72%. By heating the apples before pressing, the juice recovery increased to 80.3%. Juice recovery in elderberry

juices utilizing an enzyme (Pectinex BE 3L) similar to the enzyme used in our blueberry juice production, showed a juice recovery of 77.5% (Landbo et al., 2007). An experiment with plums and currants using Pectinex BEXXL enzyme, a commonly used enzyme for blueberry juicing, delivered a juice recovery of 75% for currants and 95% for plums (Mieszczakowska-Frac et al 2012). Skrede et al.,(2000), produced an 83% juice yield after milling the blueberries and heating before pressing in a Wilmes bag press. A study on berries blanched before pressing and treated with a depectinizing enzyme, yielded juice recoveries of 68% to 72% in a filter bag press (Brambilla et al 2008). Juice yield in multiple cultivars of pressed and enzyme treated NHB berries ranged from 68%-83% (Brambilla et al., 2008; Skrede et al., 2000).

The right combination of pre-press treatments is important to the success of juice processing because without pre-treatments, pressed berries generally produce highly viscous and light colored juice with less flavor and very low juice recovery. On the other hand, hot-pressed, enzyme treated juice has stronger flavors and greater color (Bates et al., 2001; Grassin and Fauquembergue, 1996; Hilz et al., 2005; Nindo et al., 2005). The following information therefore includes descriptions and effects of process steps including pre-press treatments options and equipment to consider in juice processing.

### Temperatures

Freezing of fruit is a beneficial way to store fruit and maintain quality before processing into juice and allows production to continue after the harvest season is over. How long berries are kept in freezing temperatures affects the quality of the berry and the

subsequent juice. Freezing temperatures have been proven to have minimal effects on anthocyanins in red and black raspberries over storage of 6 months (de Ancos et al., 2000; Hager et al., 2008). Total anthocyanin content in blueberries stored at -20 °C for three months was not significantly different from fresh berries (Lohachoompol et al., 2004). While antioxidant capacity is preserved in cold storage, anthocyanin content decreases after 6 months of blueberry and juice product storage (Brownmiller et al., 2008; Reque et al., 2014). In red raspberries frozen at -20 °C for 1 year, an 18% decrease in anthocyanins was observed (de Ancos et al., 2000). It is possible that proanthocyanidin polymers are irreversibly bound to cell wall polysaccharides when stored at -20 °C, decreasing anthocyanin amounts (Khanal et al., 2010; Le Bourvellec et al., 2004). While anthocyanin degrading endogenous enzymes found in blueberries are affected by temperatures below 0 °C, enzyme activity is slowed down by freezing temperatures prolonging berry quality (Bello and Sule, 2012; Chisari et al., 2007; Pedrolli et al., 2009). However, enzymatic activities are not completely stopped. Issues with water loss factor into decreases in quality of frozen berries as well, causing dehydrated berries (Basiouny and Chen, 1988).

Heating steps are one of the most common methods of processing in today's food products (Khanal et al., 2010). As a juice processing step, blanching of blueberries before pressing improves phenolic recoveries including anthocyanins and hydroxycinnamic acids (Brambilla et al., 2007; Brambilla et al., 2008; Kader et al., 1997; Rossi et al., 2003). Blanching works by inactivating endogenous enzymes, including PPO, before they can degrade phytochemicals during processing, as well as improve



permeability of compounds through the pericarp cell membranes which contain the anthocyanins (Brambilla et al., 2008; Rossi et al., 2003). A study by Brambilla et al., (2008), showed that a blanching treatment of 85 °C for 3 minutes improved anthocyanin content by 32%. In the juice processing experiments in this thesis, a mash treatment was performed in a steam-jacketed kettle which blanched berries at 95 °C for 3 minutes. This time and temperature was chosen because results from Brownmiller et al., (2009) reported greater anthocyanin retention in juices blanched in this manner in comparison to other studies using lower blanch temperatures. Another benefit of blanching berries before pressing is that heat denatures endogenous enzymes, including PPO (Bello and Sule, 2012). These enzymes can decrease the quality of products by degrading anthocyanins and other phenolic compounds causing enzymatic browning of juice (Skrede et al., 2000).

Long term exposure to heat can be detrimental to juice quality and it is well documented that heat destroys anthocyanins, but the extent of damage varies depending on temperature and time of exposure to heat (Khanal et al., 2010). The most anthocyanin degradation occurs when temperatures are above 70 °C in purified anthocyanin extracts (Markaris et al., 1957). Anthocyanins in juice can be heated up to 40 °C with low levels of degradation (Kechinski et al., 2010). Anthocyanins in press cake are also not affected by heating to 40 °C for 72 hours. However, heating anthocyanins from 60 °C to 125 °C resulted in significant degradation of total anthocyanins, up to 52% (Khanal et al., 2010). The sugar-aglycone bond has been determined to be more labile to heat than any other

bond in the anthocyanin molecule (Sadilova et al., 2006). In berry matrices, the combination of co-pigmentation, sugar concentrations and water activity help to protect anthocyanins during heating and blanching steps (Barnes et al., 2009; Holzwarth et al., 2012).

### Pectinase Enzymes

Pectinase is a group of enzymes utilized by many bacteria and fungi to break down the pectin polysaccharide molecules found in plant tissues (Pedrolli et al., 2009). While pectin provides structure and strength to cell walls, fragmented pectin molecules cause gelling in fresh pressed juice, as well as heated juice, which decreases juice recovery (Pedrolli et al., 2009). Utilizing pre-press pectinase treatments in juice processing may increase juice yield by 20% and increase the press capacity as well as aid in improving liquefaction, clarification and filtration of juice (Demir et al., 2001). Pectinase treatments increased juice recovery by degrading pectin in the middle lamella of the cell wall and decreased the water binding capacity of pectin (Bagger-Jorgensen and Meyer, 2004; Grassin and Fauquembergue, 1996). Extensive degradation of pectin molecules during enzymatic mash treatments is facilitated with combinations of pectinase enzymes including polygalacturonase, pectinmethylesterase, and pectin lyase (Demir et al., 2001). Enzyme treatments can range from 40 °C to 60 °C for 1 to 4 hours depending on the enzyme (Hilz et al., 2005). Some specific berry-related enzymes used in juicing were previously mentioned (see “Extraction of Juice and Juice Processing” section).

Commercial enzyme production from bacterial or fungal extracts may produce isolated enzymes of certain enzymes (i.e. pectin lyase) or extracts containing multiple enzymes all with pectin degrading properties to increase enzyme activity. Proteases, whether incidental or formulated, are often added to help with clarification of juices (Pedrolli et al., 2009). Enhanced degradation of cell walls from combination enzyme treatments also decrease the amount of press cake (Demir et al., 2001).

### Pressing

Juice recovery from fruits and vegetables depends greatly on the mass energy transfer of the press (Jaeger et al., 2012). There are several factors that affect this transfer including the press design and operation, ripeness of the fruit, texture, degree of milling, mash treatments and number of outlets for drainage of juice during pressing (Jaeger et al., 2012). Additional pressing factors which can affect the juice yield and quality include pressure applied, duration of applied pressure, pressing aids, and press cake redistribution (Bates et al., 2001). Depending on the size of the operation, presses can range from manual hand-cranked to fully automated mechanical presses (Bates et al., 2001). Rack and cloth presses are made to increase the surface area to volume ratio to extract juices and may use wooden or plastic racks with cotton or synthetic cloths to hold press cakes as well as utilize hydraulics to further close the press (Bates et al., 2001). Disadvantages to this type of pressing are the quantity of fruit that can be pressed each time as well as the difficulty of cleaning and sanitizing cloths after pressing (Bates et al., 2001). Screw presses continuously crush and press fruit to remove juice which is great for large quantities of fruit. However, shear forces can

cause any seeds and stems remaining in the press to be crushed along with the pulp, allowing undesirable compounds to be mixed with the juice (Bates et al., 2001). Willmes presses are pneumatic presses which press fruit with an inflatable bladder or membrane and use compressed air or water to remove juice. This type of press is best for soft fruits and is one of the most common presses used in wine production (Kinzer and Schreier, 1980). Large scale production may incorporate a belt press which presses fruit between a porous belt and rollers (Bates et al., 2001).

### Filtration

Fresh pressed fruit juices are naturally cloudy due to the presence of polysaccharides, proteins, tannins, and metals (Vaillant et al., 2001). Consumers often perceive that clearer juices are of better quality (Sandri et al., 2011). While pulp and other large cellular pieces can be filtered out using settling techniques or centrifugation, the macromolecules, such as proteins and polysaccharides, need to be removed for clearer products. The use of pectinases to decrease viscosity of juices also aids in clarification (Pinelo et al., 2010; Sandri et al., 2011). By breaking up the large pectin molecules, which can form clusters, pectinases increases pectin removal when the juice is centrifuged or ultrafiltered. Blueberry juice treated with a commercial pectinase had 40% decreased turbidity (Sandri et al., 2011). Pectin fragments, as well as other polysaccharides and proteins, that cause turbidity can further be removed from juice by pairing filtration with bentonite. Bentonite powder removes compounds from juice by unspecific binding of the compound molecules (Pinelo et al., 2010).

Ultrafiltration (UF) can be utilized to exclude certain sized molecules from the juice and remove proteins and polysaccharides, increasing the clarity (Bates et al., 2001).

Ultrafiltration uses varying pore sizes to create a sieve effect and separates particles at the molecular cut-off weight of the membrane (Nakao, 1994). Membrane pore sizes can be modified to fit particular juice quality parameters. A positive side effect of UF is the concentration of certain compounds in the juice. Studies have shown that UF can be used to increase the anthocyanin content in juices and other products (Chung et al., 1986; Patil and Raghavarao, 2007). Other benefits to UF include low energy consumption, operation temperature variability, and scale up to commercial production rates with little adjustment (Sant'Anna et al., 2012).

### Pasteurization

The importance of pasteurization is to protect the consumer from harmful microorganisms and to increase product shelf life through the destruction of spoilage microorganisms and enzymes. Successful pasteurization is based on a 5-log reduction of harmful microorganisms using a combination of times and temperatures to achieve this reduction (FDA, 2001). Pasteurization can be divided into two categories, low temperature long time (LTLT) and high temperature short time (HTST) (Rupasinghe and Yu, 2012). LTLT pasteurization is batch processing at temperatures ranging from 60-65 °C or higher for long times from 30 minutes up to an hour or more (Rupasinghe and Yu, 2012). This temperature and time length kills microorganisms, but also produces undesirable quality changes in the process. Pasteurization utilizing HTST parameters varies from LTLT by increasing the temperature at which the juice is pasteurized and

markedly decreasing the time the juice stays at the raised temperature. HTST pasteurization minimizes poor quality issues of LTLT while still achieving affective reduction of microorganisms. This has become the most commonly used method for fruit juice (Rupasinghe and Yu, 2012). Recommendations for HTST time and temperature combinations include heating juice to 71 °C for at least 6 seconds or increasing heating to 82 °C for at least 0.3 seconds (FDA, 2001). Literature and commercial operations seem to favor higher temperatures due to food safety risk assessment and liability issues (IFT, 2001).

#### 1.2.4.2. Characterization of Anthocyanins

Anthocyanins get their name from the Greek words, *anthos* and *cyan*, meaning flower and blue, respectively (Valls et al., 2009). Created from yellow flavonoids by the loss of oxygen, anthocyanins contribute the reds, blues, and purples to plant flowers, leaves, and fruits and are one of the many classes of flavonoids (Merken and Beecher, 2000; Zhang et al., 2004). The study of anthocyanins has contributed to determining the health benefits of blueberries and other plants. The use of these water soluble plant-derived color compounds as natural food colorants is gaining interest as well (Merken and Beecher, 2000; Zhang et al., 2004). These compounds have been studied for hundreds of years, but their characterization did not begin until after 1914 (Barnes et al., 2009). Anthocyanins are easily distinguished from other colored compounds because they undergo re-arrangements in response to pH (Crozier et al., 2010). They can have one or more sugar moieties attached to the molecule (generally the 3 position in the C-ring or R3) including arabinose, galactose, and glucose (Figure 1.1) (Zhang et al.,

2004). The anthocyanin can increase in complexity when the sugars are acylated by acetic acid and coumaric acid (Figure 1.4) (Barnes et al., 2009). Anthocyanidins are the sugarless-backbones of anthocyanins and there are five found in blueberries (Figure 1.2) (Barnes et al., 2009). The stability and water solubility of the molecule increases with the addition of sugar moieties (Fanali et al., 2011).

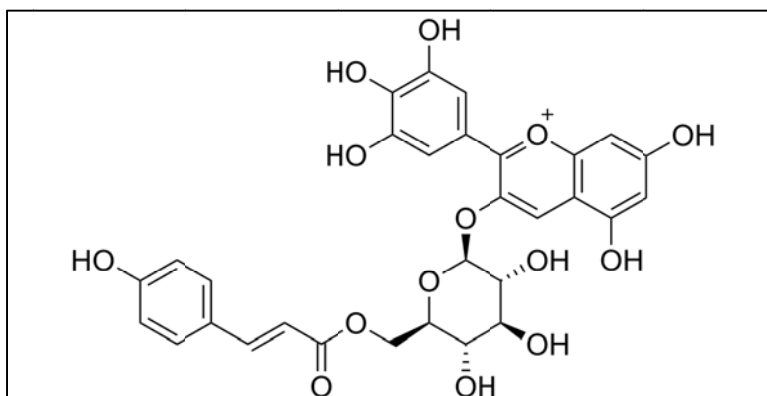


Figure 1.4. Structure of delphinidin-3-(6-p-coumaroyl) glucoside. Figure obtained from (Neveu et al., 2012).

### Anthocyanin Extraction in Fruits

Many studies have been performed to determine the optimal method of extraction in fruit, including blueberries (Kalt and Dufour, 1997). Anthocyanins are typically extracted using acidic solvents where the red flavylium cation is the stable arrangement of the molecule (Crozier et al., 2010). Polar organic solvents, such as methanol (MeOH), acetone, ethanol (EtOH), or acetonitrile (ACN) can be used, but MeOH is by far the most used solvent (Table 1.1). The percentage of organic solvent content can vary from 50% to 100% and the use of strong acids including trifluoroacetic acid (TFA) and hydrochloric acid (HCl), or weaker acids, like formic acid and acetic acid, can be added

to the extraction solution (Barnes et al., 2009). Caution should be utilized with strong acids since hydrolysis of acylated compounds and branched diglucosides can occur and decrease the ability to identify anthocyanins (Castañeda-Ovando et al., 2009).

Barnes et al., (2009), performed an extensive study on anthocyanin extractions of blueberries to determine how solvents affect recovery. The authors tested the effect of the type of organic solvent and acid used in the extraction solution. To evaluate organic solvents for extractions, combinations of 70:30:1 MeOH, water (H<sub>2</sub>O) and either HCl, TFA, formic acid, or acetic acid were assessed. Organic solvents evaluated were MeOH, EtOH, isopropanol, acetone, and ACN. The combination of 70:30:1 MeOH:H<sub>2</sub>O:TFA extracted the greatest total anthocyanins. The TFA concentration was determined to have minimal effect on the extraction when the acid concentration varied between 0.1 and 10 parts acid. Review of other methods in the literature outlined in Table 1.1, illustrates the variation of techniques utilized in anthocyanin extractions of blueberries.

#### Instrumental Detection

A simple low cost way of determining total anthocyanins is the pH differential method (Wrolstad, 1993). The pH differential method is a spectrophotometric method and has been validated to measure the total monomeric anthocyanin content of samples (Lee et al., 2005). By lowering the pH to 1.0, anthocyanin molecules take on the flavylium arrangement which creates highly colored samples, while raising the pH to 4.5 rearranges the anthocyanins into the colorless carbinol form (Wrolstad, 1993). Utilizing this physical property of anthocyanins, two samples are prepared at pH 1.0 and 4.5 and



the difference in the absorbance between the maximum wavelength is proportional to the anthocyanin content (Giusti and Wrolstad, 2001; Wrolstad, 1993). The speed and accuracy of this method in addition to cost make this method beneficial to producers and researchers alike, however this method was not utilized in this thesis because interest in the individual compounds was desired. While generally blueberry results reported from spectrophotometric methods are higher in the same sample than results from methods quantifying anthocyanins, the opposite has been reported as well (Cho et al., 2004; Lee et al., 2005; Wang et al., 2008; You et al., 2011). Although the two methods report differing results, blueberry and other berry data tend to be closely correlated which indicates both approaches are reliable (Nicoué et al., 2007).

Detection of anthocyanins is most commonly accomplished using HPLC with UV-Vis or diode array detectors (DAD) (Lee et al., 2008). However, standards for some anthocyanins are expensive and/or difficult to source (Fanali et al., 2011; Zhang et al., 2004). The use of mass spectrometry (MS) and tandem mass spectrometry (MS/MS) are becoming an alternate source for detection and identification using product ion analysis (Fanali et al., 2011; Tian et al., 2005). Nano-LC methods have been developed to increase efficiency, shorten analysis time, and minimize sample and mobile phase quantities (Fanali et al., 2011). Anthocyanins and anthocyanidins can be detected with UV-Vis using the 520-525 nm wavelength (Fanali et al., 2011).

Table 1.1. Survey of blueberry anthocyanin extraction methods found in literature.

Species	Berry Pre-Extraction Sample Treatment	Extraction Solvent	Detection Method	Authors
SHB	Berries were lyophilized and ground, mixed with extraction solvent, sonicated, centrifuged, filtered	70:30:1 MeOH:H <sub>2</sub> O:TFA	HPLC-PDA	(Barnes et al., 2009)
NHB, SHB, RAB	Berries were homogenized with extraction solvent, centrifuged, supernatant was sampled	4% Acetic Acid in ACN	pH Differential Method	(Ehlenfeldt and Prior, 2001)
Not Identified	Commercial blueberry juice was diluted 1:10 in extraction solvent, centrifuged, supernatant was sampled	5% Formic Acid in H <sub>2</sub> O	HPLC-UV-Vis and nano-LC-ESI-MS	(Fanali et al., 2011)
NHB	Berries were frozen, mixed with acidified acetone, sonicated, centrifuged, supernatant was mixed with extraction solvent and filtered	99:1 Acetone:Acetic Acid; 20% MeOH in H <sub>2</sub> O	HPLC-DAD-ESI-MS	(Gavrilova et al., 2011)
NHB	Berry skins were blended with extraction solvent, filtered, evaporated, washed, and aqueous phase concentrated	99:1 MeOH:HCl; Wash-Ethyl Acetate	pH Differential Method	(Kader et al., 2002)
NHB, SHB	Berries were blended, frozen, ground in extraction solvent, centrifuged, supernatant was sampled	88% MeOH:12% H <sub>2</sub> O: 0.1% Formic acid	pH Differential Method	(Kalt et al., 2001)
Wild, NHB	Berries were blended, homogenized with extraction solvent, then filtered	70:2:28 MeOH:Formic Acid:H <sub>2</sub> O	HPLC-UV-Vis	(Kalt et al., 1999)
Wild, RAB, NHB	Berries were frozen, blended, washed with extraction solvents, supernatants were pooled and partitioned with chloroform, polar phase was sampled	Acetone; 70:30 Acetone/H <sub>2</sub> O; Chloroform	pH Differential Method	(Moyer et al., 2002)

(Table 1.1. continued next page)

(Table 1.1. continued)

Species	Berry Pre-Extraction Sample Treatment	Extraction Solvent	Detection Method	Authors
BIL, NHB	Juices were diluted in extraction solvent and filtered. Berries were lyophilized, grinded and mixed with extraction solvent, centrifuged, and filtered	87% ACN:3% H <sub>2</sub> O: 10% Formic Acid	HPLC-UV-Vis	(Muller et al., 2012)
RAB, BIL	Berries were homogenized with extraction solution, centrifuged, supernatants filtered	90% EtOH with 0.1% H <sub>2</sub> SO <sub>4</sub>	HPLC-PDA-ESI-MS	(Nakajima et al., 2004)
Wild	Juice was diluted 1:5 in extraction solvent	100% H <sub>2</sub> O	HPLC-PDA	(Obón et al., 2011)
NHB, RAB, BIL, Wild	Berries were homogenized with extraction solvent, centrifuged, supernatant was sampled	4% Acetic Acid in H <sub>2</sub> O; 4% Acetic Acid in ACN	pH Differential Method	(Prior et al., 1998)
RAB, SHB	Berries were frozen and ground, mixed with extraction solvent, filtered	4% Acetic Acid in ACN	pH Differential Method	(Sellappan et al., 2002)
RAB	Berries were dried, delipidated, extracted	Delipidation Solution - 1:1 Hexane:EtOH; Extraction Solvent - 1:1 EtOH:H <sub>2</sub> O	MALDI-MS	(Yoshimura et al., 2012)
BIL	Berry extract powder was mixed with extraction solvent, sonicated, filtered	1:1 MeOH:H <sub>2</sub> O with 2% HCl	HPLC-DAD	(Zhang et al., 2004)

BIL = Bilberry; RAB = Rabbiteye; NHB = Northern Highbush; SHB = Southern Highbush; Wild = Wild Native berries;

Based on the physiochemical properties of the anthocyanins, the anthocyanidin and glycosidic derivatives can be determined based on polarity to determine the elution order (Wu and Prior, 2005). Anthocyanidins elute in the order of delphinidin, cyanidin, petunidin, peonidin, and malvidin (Hynes and Aubin, 2006). The order in which the glycosidic derivatives elute is galactoside, glucoside, and arabinoside (Brambilla et al., 2008). Differences in the methods, column type, and complexity of fruit anthocyanin compounds may change the elution order based on the attached aglycone (Wu and Prior, 2005). Methods of detection found in the literature can be seen in Table 1.1. Columns most commonly used for separation of anthocyanins are C<sub>18</sub> packed (Brambilla et al., 2008; Brownmiller et al., 2008; Wu and Prior, 2005).

### 1.3. Conclusion

Blueberries have become popular ingredients in juice and functional beverages due to their nutritional benefits such as vitamins, minerals, and antioxidants. This increased demand calls for blueberry research to understand blueberry cultivar characteristics and variability, processing effects and improved processing technology, use of by-products, understanding of nutrient and phytonutrient stability, and maximizing opportunities for use of the entire fruit. Understanding the differences in blueberry types, such as SHB and RAB, and how each juice processing step can impact anthocyanins can help producers tailor their processes to maximize juice extraction and protect the quality of

the juice and open opportunities to expand on product options. The objectives are to enhance our understanding pertaining to NFC rabbiteye blueberry juices and optimize juice recovery in a pilot scale NFC process by evaluating the processing effects on anthocyanins.

## CHAPTER 2 PRESS EFFICIENCY AND PILOT SCALE UP EXPERIMENT – PRESSING TEMPERATURE AND ENZYME TREATMENT EFFECTS ON SMALL SCALE BLUEBERRY JUICE RECOVERY

### 2.1. Abstract

Juice production is multibillion dollar industry and an economical way to utilize fruit past seasonal harvests. To evaluate how production steps influence blueberry juice recovery, bench top and pilot scale experiments were performed. In bench-top, Southern highbush (*Vaccinium darrowi* x *Vaccinium corymbosum*) and rabbiteye (*Vaccinium ashei*) blueberries were pressed at varying temperatures. Press treatments included ambient, frozen then thawed, and frozen then heated berries. Additionally, two commercial pectinase enzymes were evaluated as well. Three batches were pressed and average juice recovery was calculated. The highest average free juice recovery ( $68.8 \pm 1.1\%$ ) was attained by heating frozen berries and treating with enzyme. Comparing berry type, Southern highbush presses produced significantly more juice than rabbiteye presses. However, differences between enzymes were not significantly different between berry types. Using this preliminary data, the optimum juice recovery method was then transferred to pilot scale processing. Rabbiteye blueberries were heated and treated with enzyme then pressed. Free juice recovery from the pilot scale was  $74.0 \pm 0.9\%$ . Total juice recovery was calculated to be  $87 \pm 0.6\%$ . With this information, further development of juice processes will increase juice production output for small scale producers and give new outlets for growers to market their crops.

## 2.2. Introduction

Due to recent research involving health benefits of phytochemicals, development of the functional foods segment, and keen marketing, certain fruits have been portrayed as “superfruits” causing an increase in demand (Folmer et al., 2014; Khurana et al., 2013). Blueberries have seen much of this popularity in the recent years and have been incorporated into various food products including baked goods, beverages, and snacks for every market segment. The demand for blueberries has subsequently resulted in increased production and crop value over the last decade. Blueberry production in the southeast U.S. (AL, AR, FL, GA, MS, NC) has increased from 17.1 million kg in 2002 to 58.2 million kg in 2012 with the value of the crop increasing to \$781.8 million, up from just \$194.6 million, over the same period (USDA, 2013b; USDA, 2013c). In addition to fresh berry consumption, blueberry juice has increased as a popular ingredient in mixed fruit beverages as well as a 100% single strength fruit juice options (Sun-Waterhouse, 2011). Availability of fresh-squeezed juices in restaurants, supermarkets, and coffee shops are increasing consumer demands for more local juice options (Watrous, 2014). Public awareness of the benefits of blueberries and the development of functional food products is opening opportunities for alternative markets and small scale producers to get involved in the market with residual high quality fruit not sold as fresh. To aid in development of these opportunities in the southeastern U.S., especially in Louisiana and Mississippi, studies involving the processing effects on local types of blueberries for the small scale niche producer are needed. With the LA and MS blueberry production increasing in the last decade, small scale juice producers have an abundance of berries to develop their place in the market.

Processing fruit into juice is a popular product option and a way to extend marketing windows and shelf life of the berries (Perera and Smith, 2013). Recent research studies make blueberries a great choice for not from concentrate (NFC) juices and oftentimes, offers an economically attractive alternative to fresh sales with an 8% average annual growth through 2014 (Barkla, 2011). NFC juice products are gaining popularity as an alternative niche market. Producing juice from fresh berries is only possible around three months out of the year for producers using local fruit (Puls Jr., 1999).

Concentrating juice can be an expensive step and removing this from the process can save time and money for small scale producers. This makes NFC processes more attractive to small scale producers (Bates et al., 2001). Studies and literature providing information on RAB and SHB blueberry juice recovery and NFC juice production is scarce and is needed for small scale producers to benefit from.

The process of producing juice varies and may include freezing, heating, and enzyme treatments (Howard et al., 2012). Berries can be frozen after harvesting to reduce the effects of pectin and gelling, as well as to extend utilization beyond the narrow harvest season (Gössinger et al., 2009). To improve juice recovery, berries can be treated with elevated temperatures. Heating or blanching the berries before pressing is a common step used in juicing fruits (Brownmiller et al., 2008). Pressing heated berries extracts more juice and color pigments while cold pressing results in lower yield, less-bodied, lighter character juices (Gerard and Roberts, 2004; Lee et al., 2002). In addition to increased juice recovery, the elevated temperature also denatures native enzymes that can otherwise cause degradation of flavor, color, and phytochemical levels (Patras et



al., 2010). Use of pectolytic enzymes from bacteria, such as *Aspergillus niger* and *Aspergillus aculeatus*, can be used to increase juice yield, improve liquefaction, clarification, and filtration of juices and improve extraction from plant tissues (Landbo et al., 2007). Pectinase enzymes increase juice yield by breaking down the pectin in the cell walls and in the middle lamellae between the cells (Grassin and Fauquembergue, 1996). Enzymes, along with the heat, can contribute to the breakdown of associated pectin, phenolic, and lipid components that are known to create bitter/astringent flavors and lead to precipitation and color instability during storage (Horváth-Kerkai, 2007). It is important to evaluate the optimum extraction temperature, time, and enzymatic treatments to maximize the juice recovery as well as desired color and flavor attributes (Bates et al., 2001; Howard et al., 2012). The importance of juice recovery is essential for small scale producers to maximize their product output and profits.

This experiment evaluated two types of blueberries, Southern highbush (*Vaccinium darrowi* x *Vaccinium corymbosum*) (SHB) and rabbiteye (*Vaccinium ashei*) (RAB) berries, commonly grown in the southern United States, including Louisiana and Mississippi. This experiment establishes a general baseline for small scale juice recovery in SHB and RAB blueberries treated with heat and enzymes before pressing. By evaluating how mash temperature and enzymatic processing can affect the amount of juice recovered, combinations of temperatures and enzyme processing steps were applied to determine their influence on juice recovery.

## 2.3. Materials and Methods

### 2.3.1. Preliminary Press Weight

To optimize the efficiency of the press, an initial berry weight experiment was performed with a 2.2 L bench top stainless steel screw fruit press (Ferrari Group, Parma, Italy, Model #11065) fitted with a custom-cut nylon mesh fabric pressing sack (Goodnature Products, Inc., Orchard Park, NY, #2623; Supplemental Image A.1). Tifblue RAB blueberries were harvested from the Louisiana State University Bob R. Jones-Idlewild Research station in Clinton, LA in June of 2011 and stored at -20 °C until sampled. Thawed RAB berry samples of 500, 750, and 1000 g were tested for juicing ease in the bench top press. Three repetitions for each weight were pressed separately and the juice, press cloth, and press cake weights were recorded. The juice and press cake recovery averages were calculated and the highest average yielding weight was chosen as the optimal sample size to be used for the bench top experiment.

### 2.3.2. Bench Top Press Regimes

Four blueberry varieties were harvested from the Thad Cochran Southern Horticultural Laboratory blueberry plots in Poplarville, MS in June of 2011 and 2012. Two varieties of SHB blueberries, 'Magnolia' and 'Biloxi', and two varieties of RAB, 'Columbus' and 'Montgomery' were utilized. After harvesting, all berries were transported on ice, washed, dried in 4 °C, frozen in large plastic freezer bags, and stored at -20 °C until sampling. A small amount of fresh 2012 SHB berries were stored at 4 °C for 2 days. For each berry type (SHB or RAB), the two varieties from each year were mixed together in equal amounts as frozen berries to create the berry type samples. This was

accomplished because generally secondary fruit (not primary fresh market fruit) ends up mixed and aggregated for other food uses (freezing, canning, juicing, etc.). The steel screw fruit press (Ferrari) was used with a custom-cut nylon mesh fabric pressing sack to press 1000 g of berries.

### Process Treatments

Based on initial presses made to evaluate press function, it was determined that pressing fresh berries with no other processing did not render adequate juice due to gelling and lack of liquefaction. Therefore, only one berry type was chosen to make a fresh versus frozen pressing comparison. Using only SHB berries, three repetitions of fresh 2012 berries were individually pressed at ambient temperature as the fresh control. Another three repetitions of fresh 2012 SHB berries were individually heated to 95 °C for 3 minutes and pressed hot for the heated control. Treatments for the remaining frozen berries for both berry types were as follows. Berries to be pressed at ambient temperature were removed from the freezer and allowed to warm for 30 minutes in a beaker placed in an ambient water bath (25 °C). Berries that received heat treatments were placed, still frozen, in a beaker and heated on a hot plate to 95 °C for 3 minutes maintaining temperature with constant stirring (Supplemental Image A.2). For the enzyme treatments, two commercial enzymes that are routinely used for berry operations were utilized as suggested by correspondence with enzyme manufacturers. Rohapect 10 L (AB Enzymes, Darmstadt, Germany) and Pectinex BEXXL (Novozyme, Bagsvaerd, Denmark) were the chosen enzymes (Supplemental documents 1.1 and 1.2). Rohapect 10 L is a pectinase (IUB 3.2.1.15, CAS 9032-75-1;

<http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/2/1/15.html>) which is recommended for low pH juices. Aside from the source (*Aspergillus niger*), the specific type of pectinase was not labeled by the manufacturer. The Pectinex BEXXL is a pectin lyase (IUB 4.2.2.10; CAS 9033-35-6; <http://www.chem.qmul.ac.uk/iubmb/enzyme/EC4/2/2/10.html>) which is a specific enzyme that catalyzes the random cleavage of the pectin molecule, especially in highly esterified pectin chains, in acidic fruits. Enzyme treated berries were heated to 95 °C for 3 minutes and cooled to 50 °C. While stirring, 200 ppm of enzyme was added to the mash and stirred on a stir plate for 1 hour before hot pressing. Each resulting mash was pressed and to mimic commercial press forces, the press was opened and the press cake was manually broken up, redistributed and repressed. All process sequences were repeated three times. Free juice, press cakes, and nylon press bags were weighed and recoveries of free juice, press cake, juice loss, and total juice were calculated and averaged. The highest free juice recovery treatment method was then transferred to the pilot scale press experiment.

### 2.3.3. Rapid Qualitative Appraisals

Physical measurements of soluble solids, pH, and titratable acidity (TA) were taken for each replicate in each treatment sequence. Soluble solids were measured in °Brix with a digital Pocket PAL-1 hand held refractometer (Atago, Tokyo, Japan). Measurements for pH and TA were performed with 10 mL juice on an 836 Titrand (Metrohm, Herisau, Switzerland) and titration endpoint was pH 8.2, reached using 0.1 N sodium hydroxide. Titratable acidity was calculated as percent citric acid (wt/wt).

#### 2.3.4. Pilot Scale Press

Pilot scale processing used 'Tifblue' RAB berries harvested in June 2012 from the Louisiana State University Bob R. Jones-Idlewild Research station in Clinton, LA and stored at -20 °C until sampled. 'Tifblue' RAB berries were used for the pilot scale press since a large supply could be harvested. 'Tifblue' is considered the standard variety to which RAB are compared (Marshall et al., 2006). Each ~27 kg batch of frozen berries was heated in a 37.9 L stainless steel steam jacketed kettle (Groen, Jackson, MS) with constant stirring until a mash temperature of 95° C was attained and held for 3 minutes (Supplemental Image A.3). Mash was poured into stainless steel vessels and cooled to 50 °C and enzyme treated with 200 ppm of Rohapect 10 L (AB Enzymes, Darmstadt, Germany) for 1 hour. The treated mash was poured into an X-1 single-layer hydraulic press (Goodnature, Orchard Park, NY) and pressed warm (~45 °C) at 124.1 MPa using a medium-weave polyester mesh press bag (Goodnature, #2636) for 1 minute (Supplemental Image A.4). The entire juice process was replicated three times. Free juice, press cakes, and press bags were weighed and recoveries of free juice, press cake, juice loss, and total juice were calculated and averaged. Free juice was considered the free flowing juice that was collected. Press cake was the pulp, skins, and seeds that remained in the nylon fabric press bag (Supplemental Image A.5). Juice loss was the amount of juice lost in the mashing and pressing stages, including transfer losses and within the nylon press bags. The total juice recovery was the free juice plus the juice loss.

## Statistical Analysis

Results are presented as mean  $\pm$  standard deviation. Values obtained by experimentation are presented as the mean of three repetitions. Statistical analysis was carried out using the Statistical Analysis System (SAS 9.4, SAS Institute Inc., Cary, NC). Data were analyzed using analysis of variance (ANOVA). The means and standard deviations were compared using Tukey's studentized range method with  $P < 0.05$ .

## 2.4. Results and Discussion

### 2.4.1. Preliminary Press Weight

The preliminary juicing sample size experiment indicated that regardless of the amount of berries pressed, the Ferrari press extracted the same percentage of free juice from the berries with no statistical difference. The average free juice weight from the 500 g press weight was  $365.7 \pm 9.6$  g with a  $73.1 \pm 1.9\%$  free juice recovery. For the 750 g press weight,  $545.7 \pm 12.3$  g with a  $72.7 \pm 1.7\%$  free juice recovery was produced. The 1000 g press weight produced  $744.3 \pm 4.0$  g of free juice on average with  $74.4 \pm 0.4\%$  recovery for free juice. Looking to factors in addition to free juice, the 1000 g presses produced uniform press cakes with lower visible moisture content than the smaller weight presses and resembled pilot scale presses. Utilizing this preliminary data, the 1000 g weight was chosen to be used for the heat and enzyme press experiments.

## 2.4.2. Bench Top Presses

### 2.4.2.1. Free Juice Recovery from Bench Top Presses

Fresh SHB berries were pressed at ambient and heated to serve as control presses to see the influence of temperature on free juice recovery. The SHB fresh ambient presses resulted in the lowest average free juice recovery with  $42.1 \pm 4.6\%$ . Fresh SHB berries that were heated to  $95\text{ }^{\circ}\text{C}$  produced the most juice of the entire experiment. With  $71.1 \pm 1.8\%$  average free juice recovery, the SHB fresh heated presses were significantly greater than the SHB fresh ambient presses. Thawed SHB berries pressed at ambient temperature produced  $61.4 \pm 7.4\%$  average free juice recoveries; a 19.3% improvement over the SHB fresh ambient presses. There was no significant difference between the SHB fresh heated and the SHB frozen heated presses, without enzyme treatment. The effect of freezing was minimal, with average free juice recoveries of  $71.1 \pm 1.8\%$  for the fresh SHB heated presses and  $68.6 \pm 2.6\%$  for thawed SHB heated presses. This demonstrates short term freezing of berries prior to processing does not significantly change free juice recovery. The process in which berries are frozen is important to maintain the quality and quantity of the juice (Reque et al., 2014). Results from the RAB presses were similar to the frozen SHB presses (Table 2.1). Thawed RAB ambient presses had the next lowest recovery of the experiment with  $45.9 \pm 6.0\%$  average free juice recovery. Heating the frozen RAB berries before pressing improved the average free juice recovery by almost 15% over the thawed RAB ambient presses. Pressing thawed ambient berries confirmed that pectin was not degraded by freezing temperatures alone and can affect juice recovery if not properly addressed. Heating berries before pressing degrades pectin molecules to varying degrees depending on the

degrees of methylation, pH range, and temperature (Diaz et al., 2007). However, this heat step does not completely prevent pectin from forming gel matrixes, since demethylation of some pectin chains can still create uninhibited chains and form strong matrixes (Diaz et al., 2007). This causes gelling problems and decreased free juice recovery, as seen in the ambient presses (Table 2.1).

Table 2.1. Average percent free juice recovery in heat and enzyme treated blueberry bench top presses.

Process	Free juice recovery	Press cake recovery	Total juice loss	Total juice recovery
SHB, frozen, ambient	61.4 ± 7.4 b	31.3 ± 4.1 c	7.3 ± 4.9 f	68.7 ± 4.1 e
SHB, frozen, 95 °C	68.6 ± 2.6 ab	15.9 ± 2.6 ef	15.5 ± 0.9 bcd	84.1 ± 2.6 bc
SHB, frozen, 95 °C, 10 L	68.6 ± 1.1 ab	10.6 ± 0.7 g	20.8 ± 1.2 a	89.4 ± 0.7 a
SHB, frozen, 95 °C, BEXXL	67.3 ± 3.8 ab	11.3 ± 2.1f g	21.4 ± 2.2 a	88.7 ± 2.1 ab
RAB, frozen, ambient	45.9 ± 6.0 d	43.2 ± 4.4 b	10.9 ± 1.6 ef	56.8 ± 4.4 f
RAB, frozen, 95 °C	60.8 ± 6.3 c	24.1 ± 4.4 d	15.1 ± 2.1 cd	75.9 ± 4.4 d
RAB, frozen, 95 °C, 10 L	60.7 ± 2.4 c	19.7 ± 3.0 de	19.3 ± 0.4 ab	80.3 ± 3.0 cd
RAB, frozen, 95 °C, BEXXL	62.5 ± 4.2 bc	19.7 ± 0.2 de	17.8 ± 4.0 abc	80.3 ± 0.2 cd

Significant differences (Tukey's method with  $P < 0.05\%$ ) per parameter are designated by letters in each column. Means not connected by the same letter are significantly different at  $P < 0.05$ . Reported data is the means of 3 repetitions of each weight.

Comparing 10 L and BEXXL enzymes to each other within the same berry type, there was no significant difference in free juice recovery. SHB presses had  $68.6 \pm 1.1\%$  free juice recovery using 10 L enzyme and  $67.3 \pm 3.8\%$  with the BEXXL enzyme. However a significant difference in comparing 10 L and BEXXL recoveries between berry types was observed (Table 2.1). SHB enzyme treated presses produced 10% more free juice



than RAB presses. This could be due to the fact that RAB berries contain more complex polysaccharides than SHB berries, which limit the enzyme activity (Deng et al., 2013). Regardless of berry type (SHB vs. RAB) or pre-pressing storage temperature (fresh vs. frozen), ambient pressed berries had the lowest average percent juice recoveries (49.8%) of the experiment (Figure 2.1). The heat and enzyme treatment free juice recovery results from this experiment are similar to the recoveries of Lee and others (2002), where a juice recovery of 75%-83% on northern highbush blueberries was reported using similar procedures. Overall, SHB presses produced higher free juice recoveries than RAB presses. This could also be due to the difference in the genetic background of the two berry types and/or variety. Differences may include varying pectin content, native enzymes, sugar and water content, skin thickness as well as the effect pectinase enzymes have on the intercellular structures of the berries (Boches et al., 2006; Gerard and Roberts, 2004; Pedrolli et al., 2009; Silva et al., 2005).

Juice recovery from fruits and vegetables depends greatly on the mass energy transfer of the press (Jaeger et al., 2012). There are several factors that effect this transfer including the press design and operation, the ripeness of the fruit, the degree of milling, the mash treatments, and the number of outlets for drainage of juice during pressing (Jaeger et al., 2012). It is well documented that pretreating fruit with heat and enzymes increases juice yield (Jaeger et al., 2012).

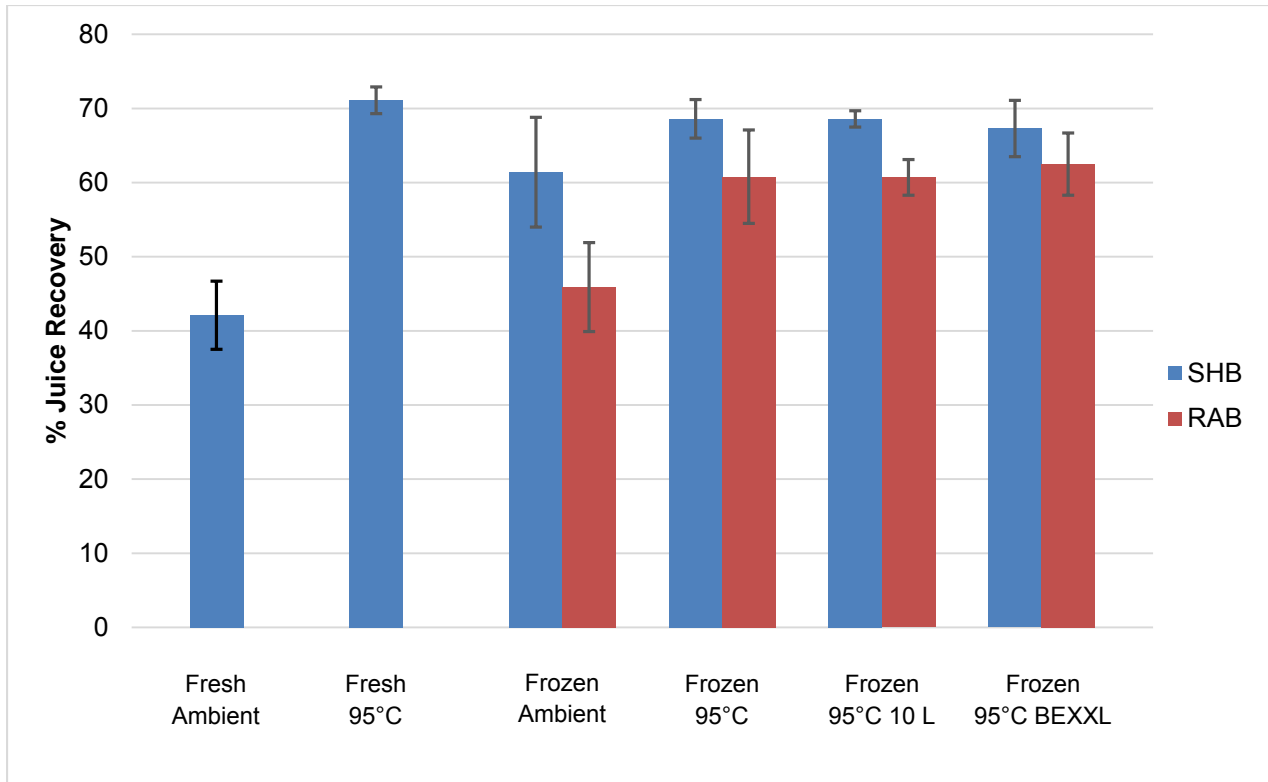


Figure 2.1. Average percent free juice recovery comparisons between SHB and RAB blueberry bench top presses.

Juice recovery in elderberry juices with a similar enzyme used in this study from Novozyme (Pectinex BE 3L), showed a recovery of 77.5% (Landbo et al., 2007). Another experiment with currants and plums using the same enzymes as this experiment, delivered a juice recovery of 75% in blackcurrants and 95% in plums (Mieszczakowska-Fraç et al., 2012). Skrede et al. (2000) performed a study using Northern highbush (NHB) blueberry juice that attained an 83% juice yield after milling and heating the berries before pressing in a Wilmes bag press. On the other hand, a study using 5 mixed NHB blueberry varieties blanched before pressing and treated with

a depectinizing enzyme, yielded juice recoveries of 68% to 72% in a filter bag press (Brambilla et al., 2008). Variety and pressing protocols are important to maximize juice recovery whilst minimizing transfer and pressing losses which will increase profitability for the producer.

#### 2.4.2.2. Physical Measurements of Free Juice from Bench Top Presses

Differences in °Brix between berry types were not significant (Table 2.2). Comparing the differences between the enzyme and temperature press data indicates that both enzymes resulted in 22% more soluble solids than the ambient pressed juices. This is due to the ability of the enzymes to break down the cell walls, liberating more lower molecular weight solutes and thus better liquefying the mash, compared to pressing alone (Landbo et al., 2007). This same pattern could be seen in the RAB berries with the frozen ambient presses compared to the heated enzyme presses. The heated enzyme presses had 24% more soluble solids. The enzyme presses had significantly lower pH and higher TA compared to ambient treatments. These results can relate back to the breakdown of cell walls and releasing cell contents.

#### 2.4.2.3. Press Cake from Bench Top Presses

While juice recovery is the main product of processing, press cake is a by-product of the juicing process. It contains the skins and seeds left in the press after removing the juice. Initially, press cake has been regarded as a waste product but new studies are expanding on press cake uses and proving that press cake may be just as valuable as the juice (Thomasset et al., 2009). Theoretically, samples with the highest juice

recoveries should have the lowest press cake amounts. Fresh SHB berries pressed at ambient temperatures produced the greatest average press cake amounts of  $49.7 \pm 5.2\%$ . These presses resulted in low juice recovery and produced a viscous juice. Juices with high viscosity cause decreases in free juice recovery because the thick juice sticks to the press cake, as well as equipment and press cloth. To a producer, this reflects money lost if they do not further process the cake in-house. Frozen SHB presses produced 25% less press cake than the fresh presses when pressed at ambient temperatures, but produced still 43% more press cake than the heated presses (Table 2.1). Heated fresh SHB presses resulted in  $13.3 \pm 0.7\%$  press cake. Thawed SHB ambient presses resulted in 49% more press cake than heated SHB frozen presses. This may be attributed to the pectin gelling the juice since there was no heating. Heated SHB presses had 31% more press cake than the enzyme treated presses. SHB enzyme treated presses had significantly less juice lost in the press cake than SHB presses heated without enzyme. Percentage of press cake in all RAB presses paralleled results of the free juice recovery, with significant differences between the thawed RAB ambient and frozen heated presses, as well as 10 L enzyme and BEXXL enzyme (Table 2.1). As with the free juice recovery, there were no significant differences between the frozen heated RAB presses and the enzyme press cake recoveries. The thawed RAB ambient presses produced 44% more press cake than the frozen heated presses and 55% more than the enzyme treated press. All SHB presses had less press cake than all RAB presses, which is congruent with the free juice recovery percentages and polysaccharide differences found between these berry types (Table 2.1).

Table 2.2. Average physical properties of bench top press free juice recovery in heat and enzyme treated blueberry presses.

	°Brix	pH	TA (mg/ml)
SHB, fresh, ambient	12.4 ± 0.5 cd <sup>z</sup>	3.25 ± 0.02 b	0.45 ± 0.08 cd
SHB, fresh, 95 °C	15.1 ± 0.3 abc	3.40 ± 0.03 ab	0.55 ± 0.07 abcd
SHB, frozen, ambient	13.8 ± 0.5 bcd	3.36 ± 0.05 ab	0.46 ± 0.04 bcd
SHB, frozen, 95 °C	16.3 ± 3.2 ab	3.45 ± 0.04 ab	0.46 ± 0.05 bcd
SHB, frozen, 95 °C, 10L	16.9 ± 0.7 ab	3.31 ± 0.09 ab	0.61 ± 0.02 abc
SHB, frozen, 95 °C, BEXXL	17.4 ± 0.3 a	3.26 ± 0.01 ab	0.60 ± 0.02 abc
RAB, frozen, ambient	11.5 ± 1.4 d	3.31 ± 0.03 ab	0.42 ± 0.01 d
RAB, frozen, 95 °C	13.0 ± 1.1 bcd	3.26 ± 0.04 cd	0.62 ± 0.06a b
RAB, frozen, 95 °C, 10L	15.2 ± 0.7 abc	3.19 ± 0.03 b	0.61 ± 0.08a b
RAB, frozen, 95 °C, BEXXL	15.6 ± 0.7 abc	3.13 ± 0.03 b	0.66 ± 0.07 a

<sup>z</sup>Significant differences (Tukey's method with  $P < 0.05\%$ ) per parameter are designated by letters in each column. Means not connected by the same letter are significantly different at  $P < 0.05$ . Reported data is the means of 3 repetitions of each weight.

There were also significant differences between the berry types and the two enzymes.

This was likely caused by the way the enzymes affect the different types of pectins in the two berry types. The Rohapect 10 L is a pectinase, but not specifically labeled by the manufacturer. Pectinase includes many enzymes that break down pectic molecules and are mainly produced from plant pathogenic microbes (Pedrolli et al., 2009). Many commercial pectinase mixes are not pure sources of enzyme, but may include other enzymes including proteases (Pedrolli et al., 2009). The Novozyme Pectinex BEXXL is a pectin lyase which is recommended for acidic fruits. Pectin lyase is a specific enzyme that catalyzes the random cleavage of the pectin molecule, especially in highly esterified pectin chains (Pedrolli et al., 2009). The pectins in the press cake from RAB berries are

more complex than the SHB berries (Deng et al., 2013). Pectins are affected by temperature, the degree of esterification and acetylation, pH, sugar types and quantities, and other solutes (Pedrolli et al., 2009). Subsequently, based on enzyme function, the two enzymes used will degrade pectin in different ways. The pectins in the SHB berries were apparently more easily degraded by the enzymes, allowing smaller, less active fragments of pectin to be removed from the press cake with the juice during pressing (Table 2.1). In the RAB berries, the enzymes were likely limited by the branches of the pectin molecules, creating similar amounts of juice as the SHB berries, but keeping more pectin in the press cake. Enzymatic activity was not specifically tested in this experiment since preliminary tests using these enzymes were determined to be effective. This activity was observed via gel precipitation (Bates et al., 2001; Rommel et al., 1990) by adding juice to ethyl alcohol and observing no gel formation (therefore, data *per se* not collected).

#### 2.4.3. Pilot Plant Press Juice and Press Cake Recovery

Using the data from the bench top experiment, it was determined that both enzyme presses produced statistically similar results. Therefore, Rohapect 10 L enzyme was randomly chosen to scale up to the pilot plant on frozen heated berries. The average free juice recovery for the pilot presses was  $74.0 \pm 1.0\%$  and had  $13.0 \pm 0.6\%$  of press cake. Resulting free juices produced from the pilot presses had soluble solids contents similar to the other free juices ( $14.2 \pm 0.2$  °Brix). Amounts for TA and pH averages were  $0.40 \pm 0.04$  mg/ml and  $3.2 \pm 0.04$ , respectively.

#### 2.4.4. Juice Loss and Total Juice Recovery for Bench Top and Pilot Presses

Juice loss is based on processing factors including the size of the press cloth between the bench top and the pilot scale press, surface area of the containers, and size of the pressing equipment used. Residual juice remaining in the containers after progressing through juicing steps, as well as the juice trapped in the woven strands of the press cloth, all contribute to juice loss and total juice recovery. Using data from single presses in clean holding vessels and dry press cloths, press cloth juice was calculated and added to the juice loss and total juice recovery. Since juice producers would make several presses before changing out the cloth, and presumably reuse holding vessels repeatedly, we have overestimated the “loss”. Commercially, the only major loss would come from stopping the production to clean and sanitize the equipment. Juice loss varied throughout the treatments (Table 2.1). Ambient presses produced significantly higher amounts of press cake (*e.g.* 49.7%), but resulted in lower juice loss since the berries did not press well. Fresh SHB ambient presses resulted in only  $8.3 \pm 2.3\%$  total juice loss since much of the pressed juice not recovered in the free juice was accounted for in the press cake. Heated fresh SHB presses had higher amounts of juice loss with  $15.5 \pm 1.2\%$ . Frozen heated presses without enzyme from both berry types produced similar losses of about 15%. Enzyme presses resulted in significantly more juice loss than the ambient presses (Table 2.1). This is explained by the enzymes ability to increase juice extraction from the berries. The enzyme treated juices have the greatest relative loss due to a small amount of water evaporation from the mash during the heating step and spillage from less viscous mashes in the pressing step. Difference in juice loss from non-enzymatically treated juices was attributed to the juice viscosity.

Viscus juice can become trapped in the press cake or remain within the press, press cloth, and containers. Ultimately, if a producer was making juice in a continuous process, the juice loss in the press and containers could be recovered as the next batch/press is produced; reducing the loss to much less than the calculated 13% from the press and containers. The average  $13.0 \pm 0.5\%$  loss from the pilot press therefore seems high compared to the smaller bench top press results. To compare loss as a relative percentage of free juice, the total juice loss was divided by the free juice and a percentage was calculated. With this comparison between the bench top RAB 10 L enzyme treatment to the pilot RAB 10 L enzyme treatment, the pilot treatments had only 17.6% loss compared to 31.8% loss in the average bench top press.

Subsequently, the total juice recovery was calculated by adding the total juice loss to the free juice. This is designated separately since depending on the size and actual process of juicing, loss is a highly important factor. Fresh SHB ambient presses still had the lowest amount with  $50.3 \pm 5.2\%$  total juice recovery, while Fresh heated SHB presses produced  $86.7 \pm 0.7\%$  (Figure 2.2). This amount recovered from fresh heated SHB presses was not drastically different from the enzyme treated presses for both berry types, including the pilot presses.

The ambient presses had 30% less total juice recovery than the heated presses. For both berry types, the fresh heated (SHB only) and frozen heated average total juice recoveries were significantly greater than the ambient pressed berries. There was an average of 22% more total juice recovered from enzyme treatments compared to those



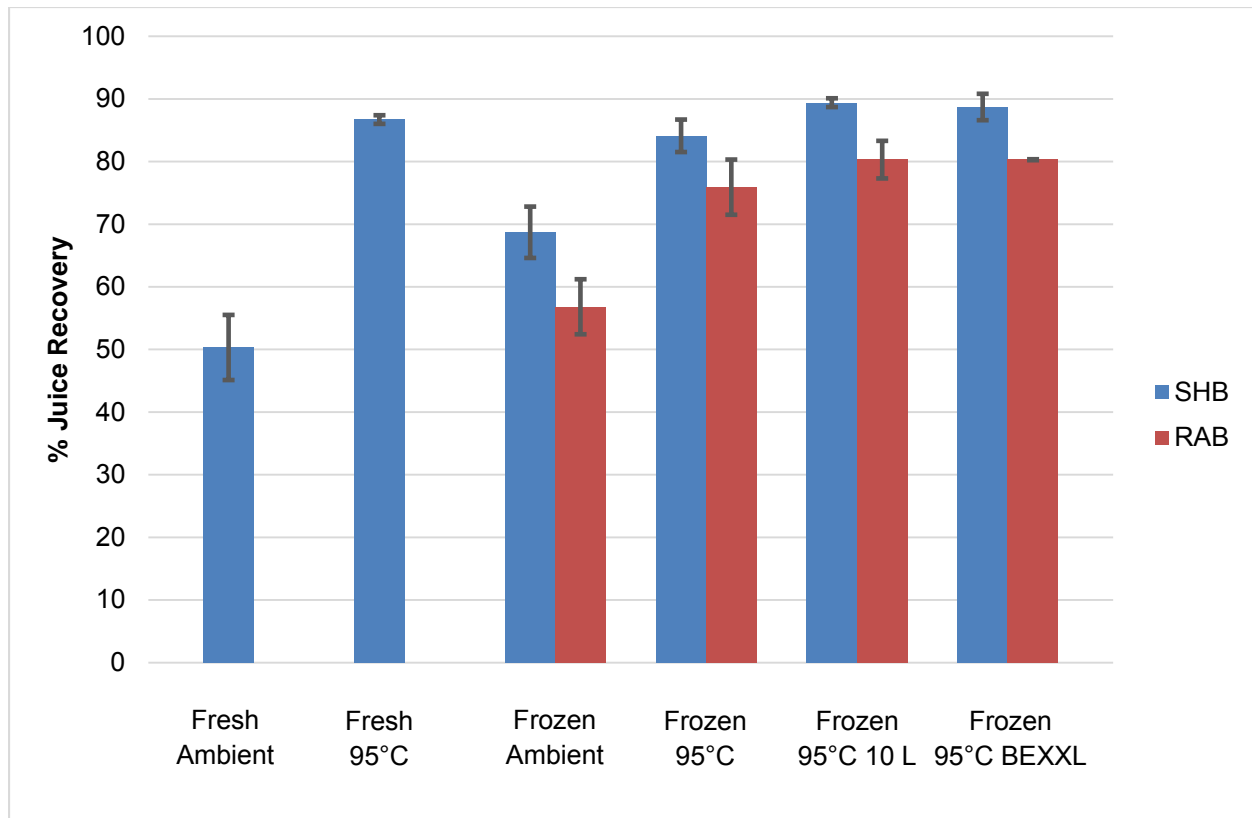


Figure 2.2. Average percent total juice recovery comparisons between SHB and RAB blueberry for bench top presses.

heated treatments without enzymes. The use of enzymes therefore delivered significantly increased juice recovery for both berry types, demonstrating the effectiveness and benefit of using enzymes. The resulting average pilot free juice recovery of  $87.0 \pm 0.6\%$  was comparable to other juice recovery experiments in the literature (Brambilla et al., 2008; Lee et al., 2002; Skrede et al., 2000). Comparing the pilot presses to the bench top RAB Rohopect 10 L enzyme presses, the pilot press delivered an average of 18% more juice from the berries and had 34% less press cake (Figure 2.3). We attribute significantly less press cake recovery to the fact that a commercial-like hydraulic press was implemented (see next paragraph).

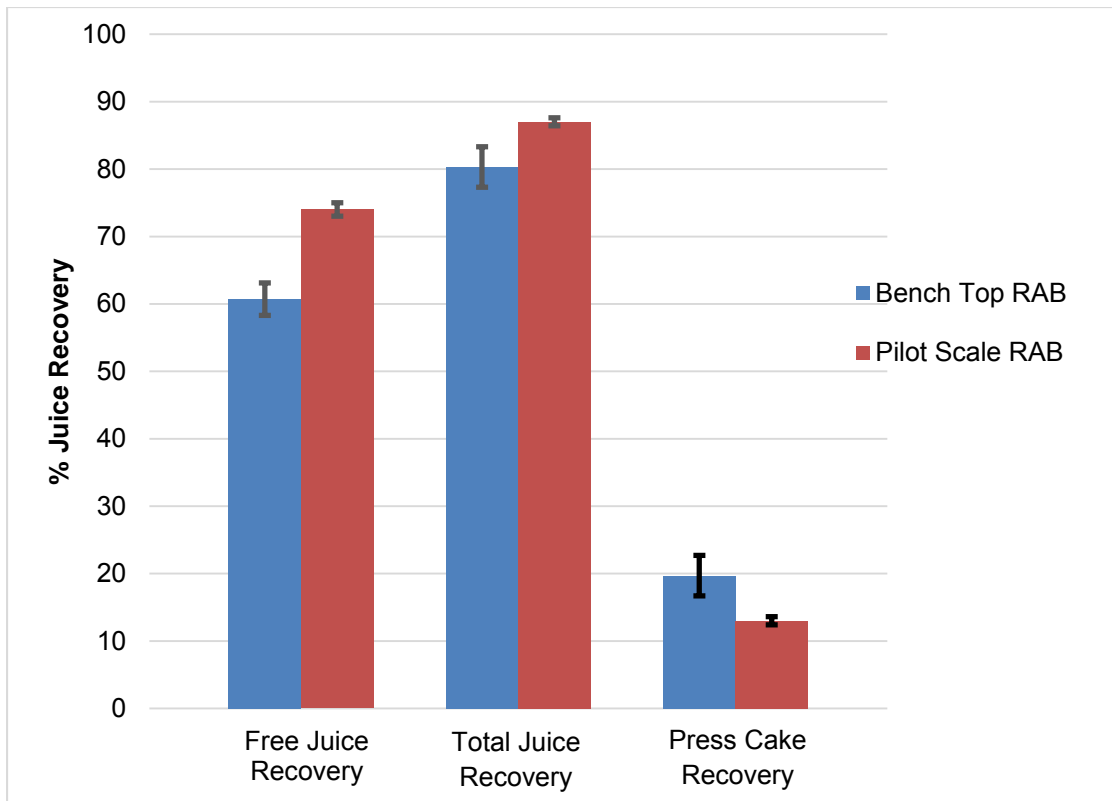


Figure 2.3. Free and total juice recovery and residual press cake comparisons between bench top and pilot experiments in RAB blueberry presses.

#### 2.4.5. Supplementary Pilot Plant Press Data

Manufacturer’s hydraulic press results delivered to owners or potential owners of the Goodnature X-1 press, show four press processes using thawed berries (Table 2.3).

The first press was blueberries without enzyme, with 2% rice hulls added as a pressing aid which yielded 82.6% juice. The hulls help break berry skins and create more surface area to press the berries within the press bag. Another press without enzyme yielded 72.4% juice recovery. The third press was heated to 50 °C for 1 hour with an unspecified enzyme which yielded 83.7% juice (unpublished data). This comparison helps to confirm that by applying heat and enzyme to break up the berries, whether physically or enzymatically, juice recovery is increased.

Table 2.3. Goodnature X-1 manufacturer blueberry press testing information.

Date <sup>z</sup>	Location	Berry <sup>y</sup>	Juice Yield	Notes
2/24/2009	Alma, Georgia, United States	Cultivated	82.6%	11.0 kg Berries mixed with 225 g (2%) rice hulls as a press-aid. Pressed for approximately 5 minute cycles with material #2636 (55 Mesh).
2/25/2009	Quebec, Canada	Wild	75.7%	23.6 kg Berries (no press-aids). Pressed for approximately 5 minute cycles with material #2636 (55 Mesh).
3/2/2009	Corvallis, Oregon, United States	Cultivated	72.4%	6.9 kg Berries (no press-aids). Pressed for approximately 5 minute cycles with material #2636 (55 Mesh).
3/2/2009	Corvallis, Oregon, United States	Cultivated	83.7%	6.4 kg Berries – Skins were broken and mixed with 2 mL enzyme, heated to 122 °F and held for 1 hr. before pressing. Pressed for approximately 3 minute cycles with material #2636 (55 Mesh).

<sup>z</sup>Supplemental information provided by Pete Whitehead, Goodnature, Orchard Park, NY.

<sup>y</sup>All tests utilized thawed berries.

## 2.5. Conclusion

A viable juicing process for production of blueberry juice was determined by evaluating variations in the juice process itself. Properly freezing berries to extend the juicing period does not affect juice recovery. This is an important result for small-scale or local commercial juicers who are trying to produce juice for year round production and consumption. Heating the berries before pressing, as well as an added pectinase enzyme resulted in greatly increased juice recovery. In the comparison of berry types, the SHB berries produced more juice in all treatments than RAB berries, likely due to the complexity of the pectins in the RAB berries. On a pilot scale, results were comparable to the bench top experiment, with a 74% juice recovery. However, in an industrial scale process, one could expect up to  $87\% \pm 0.6\%$  total juice recovery using pilot plant procedures outlined herein. Further experiments testing variations on temperatures and pressing aids, such as the rice hulls, could be used to further optimize and increase juice recovery. Likewise, expanded evaluations of cell wall and membrane degrading enzyme combinations beyond pectinases (i.e. proteases or lipases) to increase anthocyanin and juice extraction also may elucidate processing regimes to further increase juice recovery as well as juice phytochemical content.

## **CHAPTER 3 INTEGRATED PILOT EXPERIMENT – EVALUATION OF ANTHOCYANINS AND ANTHOCYANIDINS AS JUICE QUALITY MARKERS THROUGH PROCESSING STEPS OF RABBITEYE BLUEBERRIES BY LC-MS/MS AND UPLC-UV**

### 3.1. Abstract

Blueberry juice processing has multiple steps with each one effecting the chemical composition of the berries. Decreases in anthocyanins are often seen in thermal processing of juice. Not from concentrate blueberry juice was made by heating and enzyme treating berries before pressing, filtering, and pasteurizing the juice. Using LC-MS/MS, major and minor anthocyanins were identified and semi-quantified. Ten anthocyanins were identified, including 5 arabinoside and 5 pyranoside anthocyanins. Three minor anthocyanins were also identified. These were delphinidin-3-(p-coumaroyl-glucoside), cyanidin-3-(p-coumaroyl-glucoside), and petunidin-3-(p-coumaroyl-glucoside). These minor anthocyanins did not significantly change after pressing. The five known anthocyanidins, cyanidin, delphinidin, malvidin, peonidin, and petunidin, were quantitated with standard curves using UPLC-UV. Raw berries and press cake contained the highest anthocyanidin contents with 85.1 mg/100 g and 265.6 mg/100 g respectively. This contributes to the value and interest of press cake for use in other food and non-food products. Anthocyanidins decreased 67% after pressing and 10% after pasteurization.

### 3.2. Introduction

Blueberries are a well-known source of health promoting phytochemicals (Kalt and Dufour, 1997; Soto-Vaca et al., 2012). These phytochemicals can be divided into different classes based on their chemical structures. Of the major classes of phytochemicals, flavonoids are becoming popular for studies focusing on their health benefits. One of the most unique classes of flavonoids is the anthocyanins.

Anthocyanins give fruits and vegetables their vibrant red, blue, and purple colors (Zhang et al., 2004). These compounds are unique because they exist in five configurations and various colors based on pH. These configurations include the blue colored anionic quinonoidal base, the violet colored quinonoidal base, the red colored flavylum cation, the colorless carbinol base, and the yellow colored (*E*) or (*Z*) chalcone (Gould et al., 2009). At lower pH values anthocyanins are red in color, but as the pH shifts to more neutral, they turn clear. As pH rises further, anthocyanins will be more blue in color, until pH above 10, when the alkaline pH will destroy the compound (Pina et al., 2012).

Currently there are over 600 naturally occurring anthocyanins reported in plants (Wu et al., 2006). Anthocyanins are composed of an anthocyanidin backbone with varying glycosides (Figure 1.1). The five major anthocyanidins in blueberries are cyanidin, delphinidin, malvidin, peonidin, and petunidin (Figure 1.2) (Gao and Mazza, 1994). Cyanidin is the most common anthocyanidin found in plants and can have 76 different glycoside combinations (Baxter et al., 1998). The major anthocyanins in blueberries include 3-glycosidic derivatives of cyanidin, delphinidin, malvidin, peonidin, and

petunidin; with glucose, galactose, and arabinose as the most abundant sugars (Routray and Orsat, 2011). The lesser anthocyanins consist of acetoyl, malonoyl, and coumaroyl conjugated compounds (Barnes et al., 2009).

Consumer demand for food and beverage products which are made from locally or regionally grown raw materials are driven by the belief that these products help local communities, provide healthier alternatives, as well as decrease carbon footprints (Tropp, 2014). With increased demand for natural and less processed food options, local small scale juice producers are looking at not from concentrate (NFC) juice products to meet demand and create niche markets (Barkla, 2011). In Europe, the NFC juice market segment was up 6.7% in 2012 from 2011 (AIJN, 2012). In the United States, NFC juices had an annual average growth of 8% through 2014 (Barkla, 2011). Utilizing NFC juices as a compromise between unpasteurized fresh juices and highly processed evaporated reconstituted juices allows for small scale local producers to expand beyond farmers markets (Bates et al., 2001; Nikdel et al., 1993). The ability to process berries into juice is an efficient way to extend their shelf life and extend the profitability of a berry harvest for growers (Perera and Smith, 2013). Processing can affect the anthocyanins and other phytochemicals, as well as the macronutrients in the berries (Lee et al., 2002). Evaluating these effects builds information and awareness to help juice producers to develop juice processes that can maximize profit and quality.

The process of making juice includes heating berries before pressing as well as enzyme treatments to increase juice recovery and minimize anthocyanin loss (Brambilla et al., 2011). It is proposed that one of the pathways of degradation of anthocyanins is caused by native enzymes, mainly polyphenol oxidase (PPO), breaking down other polyphenols to form quinones. These in turn react with the anthocyanins, forming brown pigments (Lee et al., 2002). Heating the berries before pressing denatures native enzymes and reduces enzymatic browning in juice (Brambilla et al., 2008). The use of pectinases increases juice recovery by degrading pectin in cell walls, improving liquefaction and clarification, and aiding in filtration processes (Landbo et al., 2007). Heated mash is then pressed, removing remaining skins and seeds, resulting in an unfiltered juice. Filtration is an optional step to reduce sedimentation to clarify the juice and remove polymeric compounds which can affect overall color and turbidity (Alper et al., 2005). Pasteurization is a safety step used to decrease spoilage and contamination.

The 'Tifblue' variety at one point was the most widely planted rabbiteye (RAB) blueberry in the world (Brooks and Olmo, 1997). It is still a very popular RAB cultivar today and is highly regarded for its appearance, productivity, harvesting and shipping qualities, as well as a standard for comparison to other selections and cultivars (Marshall et al., 2006; USDA, 2014). Many studies have been conducted on the processing effects on blueberry juice anthocyanins, but to our knowledge, only one has been carried out on RAB (*Vaccinium ashei*) blueberries (Srivastava et al., 2007). In this thesis experiment, RAB blueberry anthocyanins and their anthocyanidin backbones were identified in each



step and the changes were evaluated. This NFC juice evaluation contributes to the knowledge of RAB blueberry juice properties and pilot plant process parameters affecting polyphenolics during various juice processing steps.

### 3.3. Materials and Methods

#### 3.3.1. Juice Processing

Commercially harvested 'Tifblue' RAB blueberries (*V. ashei*) were purchased for juice processing (Blue River Farms, Hattiesburg, MS). Commercial packaging included sorting, grading, washing, air-drying, and forced air rapid freezing of berries (FAC) before packaging and commercial storage at -20 °C (Nordic Cold Storage, Hattiesburg, MS). 'Tifblue' RAB berries were used for the pilot scale press since a large commercial supplier could assure delivery of commercially frozen boxes of this pure variety, without SHB berries mixed in. The most common RAB varieties grown through 2005 in Louisiana and Mississippi were 'Climax', 'Premier' and 'Tifblue' (Marshall et al., 2006), and 'Tifblue' is considered the standard variety to which RAB are compared.

Using a 37.9 L steam jacketed kettle (Groen-A Dover Industries Co., Byram, MS), 27 kg of frozen berries (two individual 13.5 kg boxes) were quickly heated to 95 °C using steam and constant stirring with a large wooden paddle (Supplemental Image A.3). Temperature was monitored with thermal probes and the mash was held at 95 °C for 3 minutes (Supplemental Figure 1.1). The crude mash was then poured into a 37.9 L

stainless steel vessel and allowed to cool to 50 °C for addition of pectinase enzyme.

Rohapect 10L (AB Enzymes, Darmstadt, Germany) was added to the mash at 200 mL ton<sup>-1</sup> and allowed to activate with occasional stirring for 1 hour.

The enzyme treated mash was poured into an X-1 single-layer hydraulic press (Goodnature, Orchard Park, NY) and pressed warm (~45 °C) at 124.1 MPa using a medium-weave polyester mesh press bag (Goodnature, #2636) for 1 minute (Supplemental Image A.4). Pressed juice from each batch was individually collected in a stainless steel vessel and cooled overnight at 4 °C. Press cake (PRC) samples were collected and stored at -20 °C (Supplemental Image A.5). The experiment was repeated three times.

Half of the chilled pressed juice was portioned off as unfiltered non-clarified juice (NCJ) and samples were collected at this point and stored at -20 °C. The remaining pressed juice was filtered using an ultrafiltration pilot unit with a 100-L hopper tank (Membrane Specialists, Hamilton, OH, USA) (Supplemental A.6). The unit consisted of an in-line membrane filtration module (PCI B-1 Module Series, Aquious PCI Membrane, Hamilton, OH) and a heat exchanger fed by a 7.5 hp screw pump. Filtration occurred with a 200,000 molecular weight cut-off (0.2 µm) XP-201 polyvinylidene fluoride (PVDF) membrane (ITT PCI Membrane Systems, Zelienople, PA, USA), with the heat exchanger run at ambient (~25 °C). After equilibrating the ultrafiltration unit, filtered not from concentrate blueberry juice was collected for sampling (CUF) and pasteurization.

NCJ and CUF samples were pasteurized using a MicroThermics Electra UHT/HTST Lab-25EDH pasteurization unit (Raleigh, NC) at 90 °C for 10 s., at 1.2 L m<sup>-1</sup>, followed by hot-filling at 85 °C into pre-sterilized 250 mL glass media bottles followed by inversion and ice water bath chilling. Unfiltered pasteurized juice (NCP) and filtered pasteurized juice (CJP) samples were frozen at -20 °C before anthocyanins and anthocyanidins analysis.

### 3.3.2. Anthocyanin Analysis

Berry and juice samples were analyzed for anthocyanins using a LC-MS/MS method. For the FAC and PRC samples, 5 g of raw berries were thawed and homogenized using a Tekmar Tissumizer (SDK-1810, IKA-Werke, Staufen, Germany). Using 2 g of sample, all triplicated samples were lyophilized in a VirTis Genesis 25ES freeze dryer (SP Scientific, Warminster, PA). After lyophilization, 100 mg of the powder was weighed into 2 mL centrifuge tubes and 1 mL of an extraction solvent [70:30:1 methanol (MeOH): water (H<sub>2</sub>O): trifluoroacetic acid (TFA)] was added. The tubes were vortexed for 15 s and left undisturbed for 60 min. Following extraction, the tubes were sonicated for 20 min. and centrifuged (IEC CL, International Equipment Company, Needham Heights, MA) for 15 min. at 1,200 rpm. The supernatant was filtered through a 0.2 µm syringe filter into a HPLC vial then stored at -20 °C.

Extracted samples were analyzed on an Agilent 1200 HPLC with an Agilent Small Molecule Chip Cube interface and Agilent 6520 Q-TOF MS/MS. The chip contained a 40 µl enrichment column and a C<sub>18</sub> (43mm x 75µl, 80Å) column. The eluents were acidified H<sub>2</sub>O with 0.1% formic acid (A) and 90% acetonitrile with 9.9% H<sub>2</sub>O and 0.1%

formic acid (B). The gradient was held at 2% B then raised to 20% over 10 min., and then increased to 40% B to 18 min. The MS fragmenter was set to 175V, and the VCap at 1800V. Capillary temperature was 300 °C with N<sub>2</sub> as carrier gas with a flow rate of 5 L min<sup>-1</sup>. The MS scan rate was 1 scan s<sup>-1</sup> (10,000 Transients). Auto MS/MS had selected m/z ranges (Table 3.1) and the scan rate was 1 scan s<sup>-1</sup>. Using the characteristic anthocyanin parent molecular weights ( $[M]^+$ ) along with the fragmented MS/MS backbone anthocyanidin molecular weight, likewise confirmed by the residual sugar (generally the 3 position in the C-ring or R3; the R-O-sugar group) moiety fragment(s), a semi-quantified abundance was utilized as a way to measure and compare process changes in the anthocyanins in juices. Confirmation of anthocyanin identification was verified by the MS/MS scan of selected parent ion fragments and the sugar moiety molecular weight using the MassHunter Workstation 6.00 software (Agilent, Santa Clara, CA) (Table 3.1) (Barnes et al., 2009). Anthocyanin standards are difficult to find and the few that are available are expensive, so literature was utilized to determine ions.

### 3.3.3. Anthocyanidin Analysis

Berry samples were analyzed for anthocyanidins using a modified UPLC method (Barnes et al., 2009; Hynes and Aubin, 2006), as modified by Beaulieu et al. (2015). For the FAC samples, 10 g of raw berries were thawed and homogenized using a Tekmar Tissumizer (SDK-1810, IKA-Werke, Staufen, Germany) and a 2 g sample was collected. Press cake was lyophilized in 40 g samples from each batch and a 2.5 g sample was utilized for extraction. Using 2ml of juice for processing step samples, raw

berry and juice vials were lyophilized (VirTis Genesis 25ES, SP Scientific, Warminster, PA). The samples were then removed and weighed before being stored at -20 °C. Extraction of the juice and FAC samples was done using the frozen lyophilized powder in 2 mL of an extraction solvent comprised of 70:30:1 MeOH: H<sub>2</sub>O: TFA. Press cake samples were extracted with 25 ml of extraction solvent. Each sample, including PRC samples, was vortexed for 15 s and left undisturbed for 60 min. Following the extraction, the samples were sonicated 20 min then centrifuged (IEC CL, International Equipment Company, Needham Heights, MA) for 20 min at 7,000 rpm and the supernatant stored at -20 °C.

The extracted samples (2 ml) were then hydrolyzed in a 4 mL vial, in which was mixed 200 µl of 12N HCl. After purging vials with nitrogen gas, the vials were sealed and vortexed for 5 s and heated at 95 °C for 20 min. Immediately after heating, the samples were stored at -20 °C. Hydrolysates were filtered using a 0.2 µm polyvinylidene difluoride (PVDF) syringe filter into autosampler vials.

Samples were analyzed on an Acquity ultra performance liquid chromatography system equipped with an ultra violet detector (UPLC-UV) using an Acquity BEH C<sub>18</sub> column (50 mm x 2.1 mm x 1.7 µm) (Waters Corporation, Milford, MA). The flow rate was 1.0 mL/min. The eluents consisted of acidified water with 3% phosphoric acid (A) and 100% acetonitrile (ACN) (B). The gradient started at 10% B, ramped to 20% B at 2 min., increased to 100% B at 2.1 min., then held at 100% B until 2.7 min., and returned to 10% B at 2.8 min. Detection was done with single wavelength UV at 20 points/min. at

525 nm. Anthocyanidin standards cyanidin, delphinidin, malvidin, peonidin, and petunidin (Chromadex, Santa Ana, CA) were run at a concentration gradient of 0.001, 0.003, 0.010, 0.030 and 0.100 mg mL<sup>-1</sup> ( $r^2 \geq 0.995$ ) to report anthocyanidins (mg 100 g<sup>-1</sup>).

### Statistical Analysis

Results are presented as mean  $\pm$  standard deviation. Anthocyanin and anthocyanidin values obtained by HPLC-MS/MS and UPLC-UV experimentation are presented as the mean of three batches with each batch sampled three times, for a total of 9 data points for each treatment. Data were analyzed using analysis of variance (ANOVA), using the Statistical Analysis System (SAS 9.4, SAS Institute Inc., Cary, NC). The means and standard deviations were compared using Tukey's studentized range method with  $P < 0.05$ .

## 3.4. Results and Discussion

### 3.4.1. Identification of Anthocyanins in Tifblue Blueberry and Juices

Anthocyanins were identified to determine the effects of processing on juice quality due to their heat sensitivity and as a marker of health benefits in blueberries (Havlíková and Míková, 1985). Using literature to focus in and narrow down specific compounds, 10 major and 10 minor anthocyanins were evaluated, including 5 coumaroyl-glucosides and 5 acetyl-pyranoside anthocyanins (Tables 3.1 and 3.2). Of these 20 compounds, 10 major and 3 minor anthocyanins were identified in the various blueberry juices and were used to visualize changes in juice quality through each processing step.

Table 3.1. Major and minor anthocyanin compound descriptions and mass spectrometry variables found in blueberries.

Major Anthocyanin	Sugar Moiety	Molecular Formula	[M] <sup>+</sup> (m/z) <sup>z</sup>	MS/MS <sup>y</sup> (m/z)	RT <sup>x</sup>
Delphinidin-3-arabinoside	Arabinose	C <sub>20</sub> H <sub>19</sub> O <sub>11</sub>	435.0922	303.0500	10.94
Cyanidin-3-arabinoside	Arabinose	C <sub>20</sub> H <sub>19</sub> O <sub>10</sub>	419.0928	287.0550	11.47
Petunidin-3-arabinoside	Arabinose	C <sub>21</sub> H <sub>21</sub> O <sub>11</sub>	449.1078	317.0700	11.66
Peonidin-3-arabinoside	Arabinose	C <sub>21</sub> H <sub>21</sub> O <sub>11</sub>	433.1129	301.0700	12.20
Malvidin-3-arabinoside	Arabinose	C <sub>22</sub> H <sub>23</sub> O <sub>11</sub>	463.1235	331.0800	12.34
Delphinidin-3-pyranoside	Galactose/Glucose	C <sub>21</sub> H <sub>21</sub> O <sub>12</sub>	465.1027	303.0500	10.38
Cyanidin-3-pyranoside	Galactose/Glucose	C <sub>21</sub> H <sub>21</sub> O <sub>11</sub>	449.1078	287.0550	10.99
Petunidin-3-pyranoside	Galactose/Glucose	C <sub>22</sub> H <sub>23</sub> O <sub>12</sub>	479.1184	317.0700	11.29
Peonidin-3-pyranoside	Galactose/Glucose	C <sub>22</sub> H <sub>23</sub> O <sub>11</sub>	463.1235	301.0700	12.99
Malvidin-3-pyranoside	Galactose/Glucose	C <sub>23</sub> H <sub>25</sub> O <sub>12</sub>	493.1340	331.0800	11.98
Minor Anthocyanin	Sugar Moiety	Molecular Formula	[M] <sup>+</sup> (m/z)	MS/MS (m/z)	RT
Delphinidin-3-(p-coumaroyl-glucoside)	Glucose	C <sub>30</sub> H <sub>27</sub> O <sub>14</sub>	611.1395	303.0500	13.11
Cyanidin-3-(p-coumaroyl-glucoside)	Glucose	C <sub>30</sub> H <sub>27</sub> O <sub>13</sub>	595.1446	287.0550	13.86
Petunidin-3-(p-coumaroyl-glucoside)	Glucose	C <sub>31</sub> H <sub>29</sub> O <sub>14</sub>	625.1552	317.0700	14.02
Peonidin-3-(p-coumaroyl-glucoside)	Glucose	C <sub>31</sub> H <sub>29</sub> O <sub>13</sub>	609.1603	301.0700	ND <sup>w</sup>
Malvidin-3-(p-coumaroyl-glucoside)	Glucose	C <sub>32</sub> H <sub>31</sub> O <sub>14</sub>	639.1708	331.0800	ND
Delphinidin-3-(6"-acetyl-pyranoside)	Galactose/Glucose	C <sub>23</sub> H <sub>23</sub> O <sub>13</sub>	507.1133	303.0500	ND
Cyanidin-3-(6"-acetyl-pyranoside)	Galactose/Glucose	C <sub>23</sub> H <sub>23</sub> O <sub>12</sub>	491.1184	287.0550	ND
Petunidin-3-(6"-acetyl-pyranoside)	Galactose/Glucose	C <sub>24</sub> H <sub>25</sub> O <sub>13</sub>	521.1289	317.0700	ND
Peonidin-3-(6"-acetyl-pyranoside)	Galactose/Glucose	C <sub>24</sub> H <sub>25</sub> O <sub>12</sub>	505.1340	301.0700	ND
Malvidin-3-(6"-acetyl-pyranoside)	Galactose/Glucose	C <sub>25</sub> H <sub>27</sub> O <sub>13</sub>	535.1446	331.0800	ND

<sup>z</sup>[M]<sup>+</sup> = Molecular ion weight. Values corroborated by several literature sources, principally listed in Table 3.2.

<sup>y</sup>MS/MS = Fragmented anthocyanidin molecular weight; Subsequently, this fragment ion (the backbone anthocyanidin), is free from the sugar moiety cleavage product.

<sup>x</sup>RT = retention time from the HPLC.

<sup>w</sup>ND = not detected.

Conformation of anthocyanins was determined by the anthocyanin molecular weight ion ( $[M]^+$ ) and the backbone anthocyanidin molecular weight ion (MS/MS), as verified in literature (Barnes et al., 2009; Burns et al., 2002). With the exception of peonidin-3-arabinoside and cyanidin-3-(p-coumaroyl-glucoside) in the press cake, all 13 major and minor anthocyanidins were identified in each processing step. This corroborates with other studies identifying anthocyanins in blueberries, including RAB berries (Table 3.2) (Lohachoompol et al., 2008; Nakajima et al., 2004; Prior et al., 2001; Yoshimura et al., 2012). Several studies on SHB and NHB as well as bilberry blueberry types identified minor 5 acetyl-pyranoside anthocyanins, which were not identified in this study (Table 3.2). This may be due to varietal and species differences between RAB berries and other highbush blueberry types (Howard et al., 2003; Prior et al., 2001). A 'Tifblue' RAB juice study by Srivastava et al (2007) created juice by thawing, boiling, and blending the berries in a household blender before treating them with a pectinase enzyme. The berry slurry was then centrifuged and batch pasteurized to 85 °C for 2 mins before bottling. Results of their bench top experiment identified 8 anthocyanins using HPLC-UV (Table 3.2). While their study was able to separate the glucosides and galactosides, Srivastava et al. did not identify as many arabinoside compounds as our experiment. An LC-MS overlay of the major anthocyanin abundances in a raw berry sample (FAC) chromatogram shows how closely the compounds elute to each other and how MS/MS can help to differentiate some of the different compounds (Figure 3.1).



Table 3.2. Anthocyanins in blueberries from the literature.

Anthocyanin	Literature
Delphinidin-3-arabinoside	1, 2, 3, 4, 5, <b>6</b> , 7, 8, 9, <b>10</b> , <b>11</b> , 12, <b>13</b> , 14, 15
Cyanidin-3-arabinoside	1, 2, 3, 5, <b>6</b> , 7, 8, 9, <b>10</b> , <b>11</b> , 12, 14, 15
Petunidin-3-arabinoside	1, 2, 4, 5, <b>6</b> , 7, 8, 9, <b>10</b> , <b>11</b> , 12, <b>13</b> , 14, 15
Peonidin-3-arabinoside	1, 5, <b>6</b> , 9, <b>10</b> , <b>11</b> , 12, <b>13</b> , 14, <b>16</b>
Malvidin-3-arabinoside	2, 4, 5, <b>6</b> , 7, 8, 9, <b>10</b> , <b>11</b> , 12, <b>13</b> , 14, 15
Delphinidin-3-pyranoside	1, 2, 3, 4, 5, <b>6</b> , 7, 8, 9, <b>10</b> , 12, <b>13</b> , 14, 15, <b>16</b>
Cyanidin-3-pyranoside	1, 2, 3, 4, 5, <b>6</b> , 7, 8, 9, <b>10</b> , 12, <b>13</b> , 14, 15, <b>16</b>
Petunidin-3-pyranoside	1, 2, 3, 4, 5, <b>6</b> , 7, 8, 9, <b>10</b> , 12, <b>13</b> , 14, 15, <b>16</b>
Peonidin-3-pyranoside	1, 2, 3, 4, 5, <b>6</b> , 7, 8, 9, <b>10</b> , 12, <b>13</b> , 14, 15, <b>16</b>
Malvidin-3-pyranoside	1, 2, 3, 4, 5, <b>6</b> , 7, 8, 9, 12, <b>13</b> , 14, 15, <b>16</b>
Delphinidin-3-(p-coumaroyl-glucoside)	12
Cyanidin-3-(p-coumaroyl-glucoside)	12
Petunidin-3-(p-coumaroyl-glucoside)	12
Peonidin-3-(p-coumaroyl-glucoside)	12
Malvidin-3-(p-coumaroyl-glucoside)	12
Delphinidin-3-(6"-acetyl-pyranoside)	2, 4, 5, 7, 12, 13
Cyanidin-3-(6"-acetyl-pyranoside)	2, 7, 12, 13
Petunidin-3-(6"-acetyl-pyranoside)	2, 5, 7, 12, 13
Peonidin-3-(6"-acetyl-pyranoside)	2, 7, 12, 13
Malvidin-3-(6"-acetyl-pyranoside)	2, 4, 5, 7, 12, 13

1 = (Wu and Prior, 2005); 2 = (Gao and Mazza, 1994); 3 = (Zhang et al., 2004); 4 = (Skrede et al., 2000); 5 = (Cho et al., 2004); **6 = (Lohachoopol et al., 2008)**; 7 = (Kalt et al., 1999); 8 = (Lee et al., 2002); 9 = (Lee et al., 2004); **11 = (Nakajima et al., 2004)**; **12 = (Yoshimura et al., 2012)**; 12 = (Barnes et al., 2009); **13 = (Prior et al., 2001)**. 14 = (Wang et al., 2000); 15 = (Fanali et al., 2011) **16 = (Srivastava et al., 2007)**. **Bold face references include rabbiteye blueberry samples.**

Of the major anthocyanins identified, separation issues were identified between the glucoside and galactoside containing anthocyanins. These two sugar moieties proved difficult to separate the 6-carbon structures with identical  $[M]^+$  and MS/MS ions as well as similar elution times (Figure 3.1 – Peaks 1,3,4,6). Peonidin-3-arabinoside was not separated from cyanidin-3-pyranoside as well as malvidin-3-arabinoside from peonidin-3-pyranoside (Figure 3.1 – Peaks 7 and 8). These compound's elution times, almost

identical  $[M]^+$  fragment ions MW, and difficult to separate pyranoside compounds made separation impossible without extensive LC method development for peak separation, as previously reported (Fanali et al., 2011). Therefore the unresolved peaks were evaluated as one cluster of compounds and not individual anthocyanins.

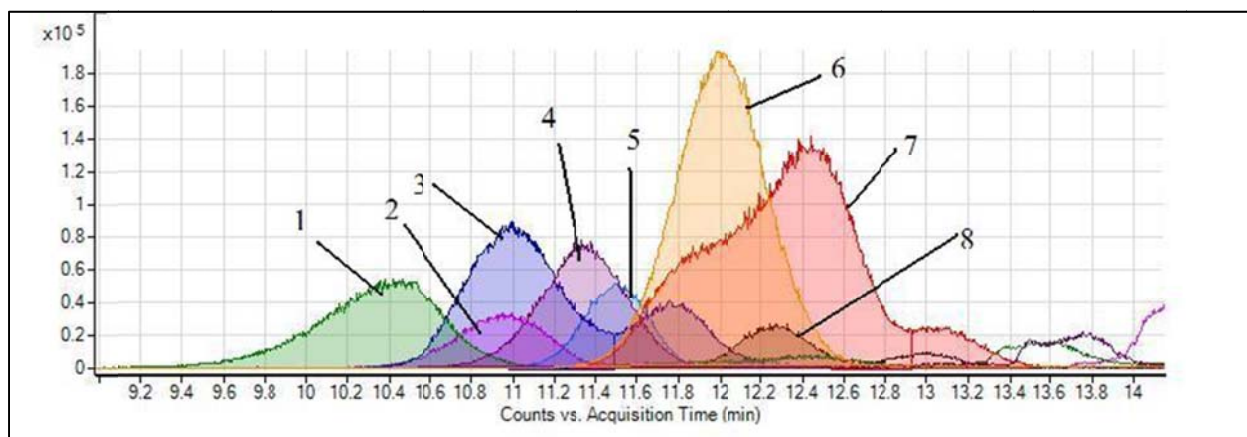


Figure 3.1. Overlay of  $[M]^+$  ions of major anthocyanins in raw berry sample. 1 = Delphinidin-3-pyranoside (465); 2 = Delphinidin-3-arabinoside (435); 3 = Cyanidin-3-pyranoside (449); 4 = Petunidin-3-pyranoside (479); 5 = Cyanidin-3-arabinoside (419); 6 = Malvidin-3-pyranoside (493); 7 = Malvidin-3-arabinoside/Peonidin-3-pyranoside (463); 8 = Peonidin-3-arabinoside (433);

As part of the complexing and/or co-pigmentation of anthocyanins, they can also form esters with hydroxycinnamates and organic acids (Burns et al., 2002). The lesser anthocyanins may consist of acetyly, malonyl and coumaroyl compounds (Barnes et al., 2009). In this study, 5 coumaroyl-glucosides and 5 acetyl-pyranoside anthocyanins were searched for and three minor coumaroyl anthocyanin compounds were identified using MS/MS ions as confirmed in literature (Figure 3.2 and Table 3.1) (Burns et al., 2002; Cho et al., 2004; Neveu et al., 2012).

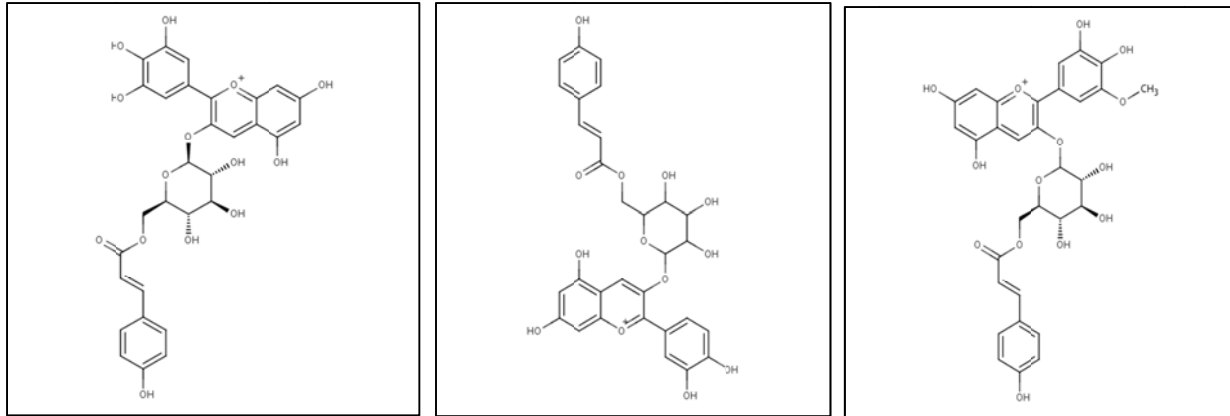


Figure 3.2. Chemical structure of delphinidin-3-(p-coumaroyl-glucoside), cyanidin-3-(p-coumaroyl-glucoside), and petunidin-3-(p-coumaroyl-glucoside) identified in processed 'Tifblue' blueberries. Figure obtained (Neveu et al., 2012).

The compounds were delphinidin-3-(p-coumaroyl-glucoside), cyanidin-3-(p-coumaroyl-glucoside), and petunidin-3-(p-coumaroyl-glucoside). These compounds have not yet been reported in RAB blueberries (Barnes et al., 2009). These three coumaroyl anthocyanins have been reported in grapes, radishes, red cabbage, and black carrots (Burns et al., 2002; Giusti and Wrolstad, 2003). A possibility for not identifying the other acetyl-pyranoside anthocyanins as identified by other studies in RAB samples may be due to the inadequate LC separation before MS detection of the compounds including phase gradients, length of gradient, and run times. Figures 3.3, 3.4, and 3.5 illustrate the MS/MS scan of the 3-coumaroyl compounds and the ion fragments of each compound. Literature references and LC-MS/MS results were used to identify individual anthocyanins as juice quality markers. Further LC-MS/MS method development is needed to better separate and further confirm identification of the individual anthocyanins in RAB berries which can be applied as a possible tool to monitor processing variables and juice quality.

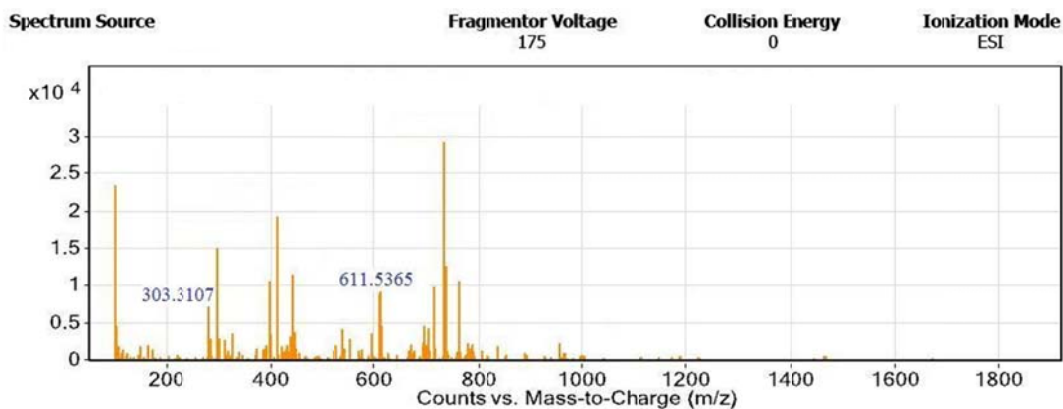


Figure 3.3. MS/MS spectrum scan of delphinidin-3-(p-coumaroyl-glucoside) in unfiltered (NCJ) rabbiteye blueberry juice.

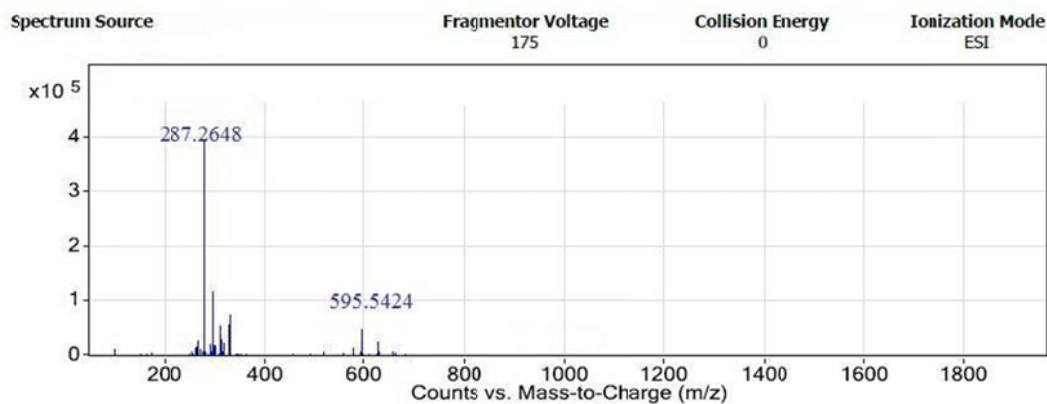


Figure 3.4. MS/MS spectrum scan of cyanidin-3-(p-coumaroyl-glucoside) in unfiltered (NCJ) rabbiteye blueberry juice.

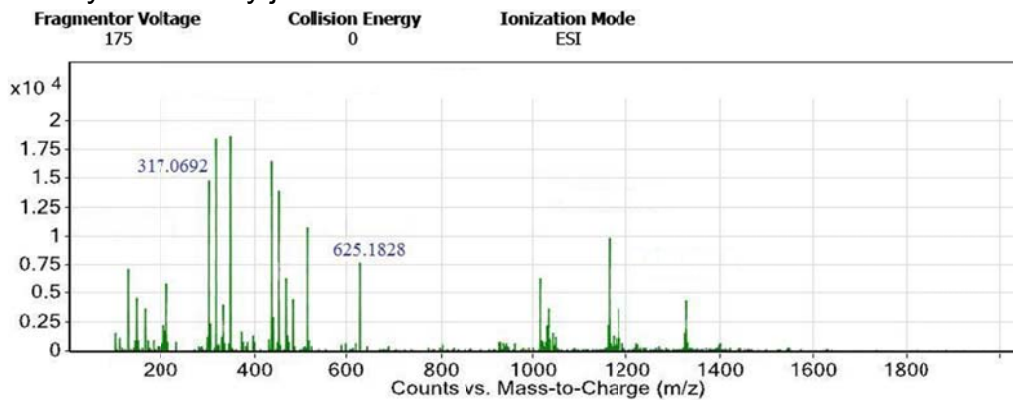


Figure 3.5. MS/MS spectrum scan of petunidin-3-(p-coumaroyl-glucoside) in unfiltered (NCJ) rabbiteye blueberry juice.

### 3.4.2. Anthocyanin Changes during Blueberry Juice Processing

Anthocyanin amounts were measured to determine the effects of processing on juice quality. The most abundant major anthocyanin identified in the FAC blueberry sample was malvidin-3-pyranoside (Table 3.3). Malvidin-3-galactoside is the most abundant anthocyanin found in blueberries (Skrede et al., 2000). Although peak resolution did not allow for the separation of the two 6 carbon sugars, malvidin-3-galactoside likely contributed to the large recovery of malvidin-3-pyranoside. The least abundant major anthocyanin identified was peonidin-3-arabinoside. Comparing these results to a study completed using 'Tifblue' RAB juice, amounts were slightly different. While malvidin-3-pyranoside was also the largest anthocyanin recovered, cyanidin-3-pyranoside was the least abundant anthocyanin in 'Tifblue' RAB juice (Srivastava et al., 2007).

After pressing, a large percentage of anthocyanins were not transferred to the NCJ from the whole berry (FAC). With one exception, there was an approximate 70% decrease between FAC and NCJ in all anthocyanins (Table 3.3). However, malvidin-3-pyranoside (Mal-3-pyr) only had a 50% loss. Peonidin-3-arabinoside (Peo-3-ara) had the greatest loss (74%), decreasing from  $5.1 \pm 0.9 \times 10^5$  to  $1.3 \pm 0.2 \times 10^5$  counts  $\text{min}^{-1}$ . The anthocyanin decreases between the NCJ and the pasteurized unfiltered juice (NCP) were minimal and not significantly different. This may be explained by the increased heat stability caused by co-pigmentation of the anthocyanins.

Table 3.3. Average semi-quantitative LC-MS/MS anthocyanin abundance in RAB blueberry during various juice processing steps.

Treatment <sup>z</sup>	Del-3-ara <sup>y</sup>	Cya-3-ara	Pet-3-ara/Cya-3-pyr	Peo-3-ara
PRC	0.2 ± 0.05 d <sup>xw</sup>	0.04 ± 0.03 d	0.2 ± 0.03 d	ND
FAC	8.8 ± 1.8 a	8.4 ± 1.2 a	22.8 ± 2.9 a	5.1 ± 0.9 a
NCJ	2.8 ± 0.5 b	2.4 ± 0.4 bc	7.0 ± 1.0 b	1.3 ± 0.2 bc
NCP	2.7 ± 0.9 bc	2.6 ± 0.8 bc	7.3 ± 1.0 b	1.6 ± 0.5 ab
CUF	3.6 ± 0.7 b	3.1 ± 0.6 b	8.3 ± 0.8 b	1.9 ± 0.2 b
CJP	1.7 ± 0.4 c	1.8 ± 0.4 c	4.4 ± 0.4 c	0.9 ± 0.2 c
	Mal-3-ara/Peo-3-pyr	Del-3-pyr	Pet-3-pyr	Mal-3-pyr
PRC	0.3 ± 0.1 d	0.5 ± 0.09 d	0.3 ± 0.03 d	0.5 ± 0.1 d
FAC	49.2 ± 8.3 a	20.5 ± 4.1 a	18.8 ± 3.8 a	49.5 ± 9.7 a
NCJ	16.5 ± 2.8 bc	7.1 ± 1.5 b	7.1 ± 1.6 b	24.7 ± 4.4 bc
NCP	17.7 ± 5.6 b	6.0 ± 1.7 bc	7.0 ± 2.2 b	25.6 ± 7.9 b
CUF	22.1 ± 2.3 b	8.4 ± 1.3 b	8.7 ± 1.9 b	30.5 ± 4.6 b
CJP	12.2 ± 3.6 c	4.0 ± 0.6 c	4.3 ± 0.9 c	18.2 ± 4.6 c
	Del-3-cou	Cya-3-cou	Pet-3-cou	Total Abundance
PRC	0.05 ± 0.01 c	ND	0.1 ± 0.05 c	2.2 ± 0.4 d
FAC	0.5 ± 0.1 b	0.3 ± 0.05 b	1.4 ± 0.5 b	185.2 ± 34.0 a
NCJ	1.1 ± 0.3 a	0.4 ± 0.1 a	2.1 ± 0.7 ab	72.5 ± 13.4 b
NCP	0.9 ± 0.4 a	0.4 ± 0.1 ab	2.2 ± 1.1 a	74.0 ± 21.2 b
CUF	1.1 ± 0.4 a	0.5 ± 0.2 a	2.9 ± 1.1 a	85.2 ± 21.2 b
CJP	1.0 ± 0.3 a	0.4 ± 0.08 ab	2.1 ± 0.4 ab	50.9 ± 11.5 c

<sup>z</sup>FAC = frozen absolute control; CUF = ultrafiltration-filtered juice; NCJ = unfiltered juice; NCP = unfiltered pasteurized juice; CJP = filtered pasteurized juice; PRC = press cake.

Table 3.3 (continued)

---

<sup>y</sup>Del-3-ara = Delphinidin-3-arabinoside; Cya-3-ara = Cyanidin-3-arabinoside; Pet-3-ara/Cya-3-pyr = Petunidin-3-arabinoside/ Cyanidin-3-pyranoside; Peo-3-ara = Peonidin-3-arabinoside; Mal-3-ara/Peo-3-pyr = Malvidin-3-arabinoside/Peonidin-3-pyranoside; Del-3-pyr = Delphinidin-3-pyranoside; Pet-3-pyr = Petunidin-3-pyranoside; Mal-3-pyr = Malvidin-3-pyranoside; Del-3-cou = Delphinidin-3-(p-coumaroyl-glucoside); Cya-3-cou = Cyanidin-3-(p-coumaroyl-glucoside); Pet-3-cou = Petunidin-3-(p-coumaroyl-glucoside).

<sup>x</sup>Abundance values in table are reported as average counts min<sup>-1</sup> ± standard deviations, x 100,000.

<sup>w</sup>Significant differences (Tukey's method with  $P < 0.05\%$ ) per parameter are designated by letters in each column. Means not connected by the same letter are significantly different at  $P < 0.05$ .

Co-pigmentation of anthocyanins with other anthocyanins and polyphenols increases the molecule's heat stability (Castañeda-Ovando et al., 2009). Concentration of anthocyanins by UF ranged from 15% to 30% in the ultrafiltered juice (CUF) (Table 3.3). After the initial decrease from pressing the berries, the second greatest decrease in anthocyanins happened when ultrafiltered juice (CUF) was pasteurized. The major anthocyanins significantly decreased 40% to 53% after pasteurization in the CJP samples (Table 3.3). Major anthocyanins containing delphinidin had the greatest anthocyanin degradation, with delphinidin-3-pyranoside (Del-3-pyr) decreasing from  $8.4 \pm 1.3 \times 10^5$  to  $4.0 \pm 0.6 \times 10^5$  counts  $\text{min}^{-1}$ . Delphinidin is less stable in elevated temperatures which resulted in greater decreases in relation to the other anthocyanins due to its greater heat lability (Srivastava et al., 2007).

The minor anthocyanin trends after pressing were the opposite of the major anthocyanins. Both cyanidin-3-(p-coumaroyl-glucoside) (Cya-3-cou) and petunidin-3-(p-coumaroyl-glucoside) (Pet-3-cou) increased 33% in NCJ samples from their initial FAC amounts. Delphinidin-3-(p-coumaroyl-glucoside) (Del-3-cou) increased 50% from  $0.5 \pm 0.1 \times 10^5$  to  $1.1 \pm 0.3 \times 10^5$  counts  $\text{min}^{-1}$  after pressing (Table 3.3). These increases may be due to the heat degradation process by which anthocyanins break down into chalcone structures which can then undergo transformation into a coumarin glucoside (Sadilova et al., 2006). One possible cause of the recoveries for the minor anthocyanins through processing involves the acylation of the anthocyanin molecule and its ability to increase stability by preventing hydration when exposed to pH changes and heat (Giusti and Wrolstad, 2003; Patras et al., 2010).



The difference in anthocyanin degradation between the CJP and NCP juices may be directly related to the role that co-pigmentation and anthocyanin polymers play in the heat stability of the compounds themselves. Ultrafiltration removes most of the co-pigment and polymer molecules from the juice which are over the 200,000 molecular weight cut off size of the filtration membrane. It can be assumed that the larger the polymer or co-pigment, the greater the increase in heat stability of the compounds included in the polymer (Castañeda-Ovando et al., 2009). The PRC samples had unusually low abundance amounts reported overall than the remaining processing steps (Table 3.3). The low reports of anthocyanins in the PRC are likely due to problems with the extraction method itself (discussed further below) and not reflective of the actual amounts of anthocyanins found in the PRC. Anthocyanidin recoveries from the PRC support the supposition that the majority of anthocyanins remained in the PRC.

There was a significant 61% decrease in total anthocyanins between the FAC and NCJ samples in the juice processing steps (Table 3.3). The FAC berries contained  $185.2 \pm 34.0 \times 10^5$  counts  $\text{min}^{-1}$ , while the NCJ juice contained only  $72.5 \pm 13.4 \times 10^5$  counts  $\text{min}^{-1}$  (Table 3.3). As seen in the individual anthocyanins, this was expected due to the large percentage of anthocyanins remaining in the press cake (Brownmiller et al., 2008).

There was an insignificant concentration increase in the amounts of anthocyanin content of the CUF samples, as compared to the NCJ (Table 3.3). A concentrating effect regarding monomer anthocyanins has been observed in grape juice filtration using varying sizes of membranes (Kalbasi and Cisneros-Zevallos, 2007). Utilization of

filtration to increase the concentration of beneficial compounds in juices is a concept that is being explored in other fruits and can increase juice nutritional value (Chung et al., 1986; Cissé et al., 2011; Pap et al., 2012; Patil and Raghavarao, 2007). The retentate from the filtration process was not evaluated in this experiment, but also adds another possible value added by-product for the producer to capitalize on in addition to the press cake. Removal of the anthocyanin polymers could be utilized as more stable natural food colorings (Cissé et al., 2011). Filtered juices (CJP) had significantly greater anthocyanin degradation from pasteurization than the NCP juice. The 40% decrease in total anthocyanins after pasteurization of CUF juices may be attributed to anthocyanin degradation from pasteurization due to the removal of the co-pigmentation of the anthocyanins by ultrafiltration (Rwabahizi and Wrolstad, 1988).

In regards to other literature working with blueberry juice anthocyanins losses, significant losses occurred in NHB blueberry juice and products (Brownmiller et al., 2008; Lee et al., 2002; Rossi et al., 2003; Sablani et al., 2010; Skrede et al., 2000). There was 58.8 % loss of monomeric anthocyanins in enzyme treated, non-clarified, batch pasteurized NHB blueberry juice prepared from frozen blanched berries, but only 27.5 % loss in centrifuge clarified juice (Brownmiller et al., 2008). A slightly different process using thawed, blanched NHB blueberries treated with pectinase, pressed then clarified and followed by HTST (90 °C) pasteurization resulted in higher losses of 77.6 % and 84.3 % anthocyanin glycosides and anthocyanins, respectively (Lee et al., 2002). There was a 68.0 % total anthocyanin loss in juice created with IQF NHB blueberries that were partially thawed, heated to 43 °C, treated with pectinase, pressed then HTST

pasteurized (90 °C) (Skrede et al., 2000). In puree/juice made with NHB that were manually crushed and machine juiced, not filtered or clarified, then batch pasteurized at 92 °C, there was a massive 95.7 % loss of total anthocyanins (Sablani et al., 2010). In addition, there was a 76.2 % and 79.1 % anthocyanin loss in 'Powderblue' and 'Tifblue' RAB varieties, respectively, that were thawed 12 h at 5 °C, hot water blanched, homogenized in a blender, pectinase added, centrifuged then batch pasteurized (85 °C) (Srivastava et al., 2007). Overall, massive anthocyanin "losses" occur in processed blueberry juices and we did not prevent losses using blanching and UF with NFC juices.

#### 3.4.3. Identification of Anthocyanidins in Tifblue Blueberry and Juices

The five major anthocyanidins found in blueberries (cyanidin, delphinidin, malvidin, peonidin, and petunidin) were all identified in every step of the juice process (Table 3.4). A chromatogram of the anthocyanidins found in a press cake sample shows the elution order for the anthocyanidins in RAB blueberries (Figure 3.6). Acid hydrolysis of anthocyanins produces anthocyanidins which are identified with UV detectors. Sugar moieties have extremely similar molecular weights which can complicate identification (Hynes and Aubin, 2006). By removing the glycoside from the molecule, the anthocyanidin backbones can be evaluated and precisely quantified to illustrate the type of anthocyanin molecule classes present in the sample.

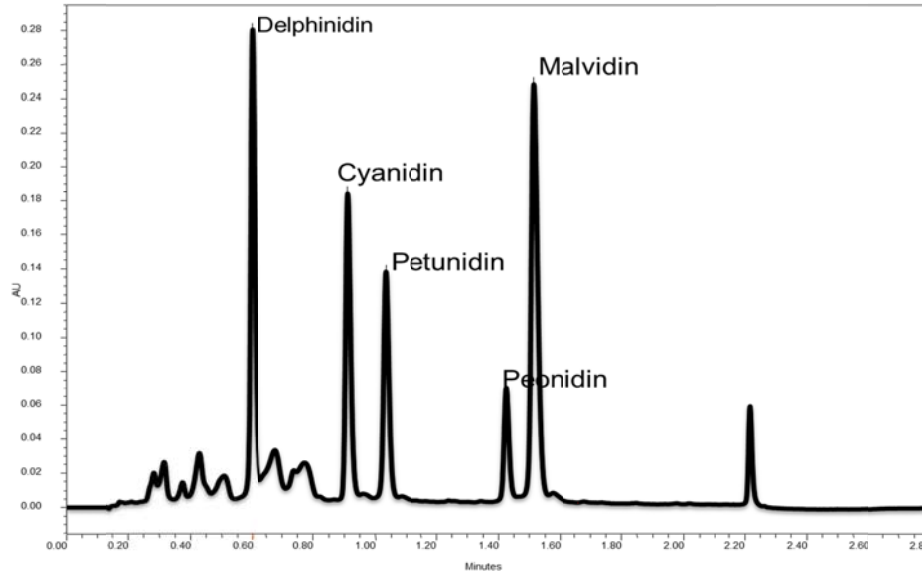


Figure 3.6. Anthocyanidins found in rabbiteye blueberry press cake as detected by HPLC-UV.

#### 3.4.4. Anthocyanidin Changes during Blueberry Juice Processing

To better define the changes caused by juice processing on anthocyanins, anthocyanidins were also evaluated. Malvidin and petunidin were the most abundant anthocyanidins in the FAC samples with  $26.6 \pm 6.7$  mg/100 g and  $25.9 \pm 6.6$  mg/100 g, respectively (Table 3.4). Peonidin was the least abundant anthocyanidin recovered in the initial berries ( $4.1 \pm 1.2$  mg/100 g). Pressing mashed berries decreased anthocyanidin content the greatest of all the processing steps with losses from 50% to 80% (Table 3.4). Petunidin decreased 78% and had the greatest amount lost during pressing of any anthocyanidin (Table 3.4). Ultrafiltration of NCJ juices removed 20% to 30% of each anthocyanidin from the CUF juice samples. Differences in the rates of degradation of anthocyanidins caused by pasteurization varied between the CJP and NCP juices (Table 3.4).

Table 3.4. Average UPLC-UV anthocyanidin content in RAB blueberry juice processing steps in mg/100 g sample.

Treatment <sup>z</sup>	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin	Total
PRC	55.7 ± 7.0 a <sup>x</sup>	77.7 ± 1.3 a	56.5 ± 8.6 a	16.0 ± 2.4 a	59.7 ± 10.4 a	265.6 ± 34.6 a
FAC	13.7 ± 3.8 b	14.7 ± 2.8 b	25.9 ± 6.6 b	4.1 ± 1.2 b	26.6 ± 6.7 b	85.1 ± 19.2 b
NCJ	6.7 ± 1.2 c	6.7 ± 0.5 c	5.8 ± 0.6 cd	1.6 ± 0.2 cd	7.2 ± 1.3 c	28.0 ± 2.7 c
NCP	5.3 ± 0.7 cd	6.1 ± 1.2 c	5.3 ± 0.7 d	1.6 ± 0.2 cd	7.0 ± 1.3 c	25.3 ± 3.0 cd
CUF	4.7 ± 0.8 cde	4.9 ± 1.3 cd	4.3 ± 1.1 d	1.2 ± 0.3 d	5.7 ± 1.2 c	20.9 ± 4.6 cd
CJP	4.8 ± 1.5 cd	4.3 ± 1.8 cd	3.7 ± 1.5 d	1.0 ± 0.5 d	4.6 ± 1.8 c	18.4 ± 6.9 cd

<sup>z</sup> FAC = frozen absolute control; PRC = press cake; NCJ = unfiltered juice; NCP = unfiltered pasteurized juice; CUF = ultrafiltration-filtered juice; CJP = filtered pasteurized juice.

<sup>x</sup>Significant differences (Tukey's method with  $P < 0.05\%$ ) per parameter are designated by letters in each column. Means not connected by the same letter are significantly different at  $P < 0.05$ .

Significant losses (20%) of anthocyanidins occurred in NCJ juices after the pasteurization step (NCP). Delphinidin had the greatest degradation with amounts decreasing from  $6.7 \pm 1.2$  mg/100 g to  $5.3 \pm 0.7$  mg/100 g in the NCJ pasteurized samples. Malvidin in the CJP juices decreased 20% after pasteurization. Delphinidin on the other hand remained steady at  $4.7 \pm 0.8$  mg/100 g after pasteurization.

Cyanidin was the most abundant anthocyanidin recovered from PRC with  $77.7 \pm 1.3$  mg/100 g. Delphinidin, petunidin, and malvidin were all recovered at over 50 mg/100 g in the PRC samples (Table 3.4). Recoveries of petunidin were only  $16.0 \pm 2.4$  mg/100g, the least abundant anthocyanidin through all pressing steps. Markedly increased amounts of anthocyanidins detected in the press cake are due to the majority of anthocyanins being located in the skins of the berries (Skrede et al., 2000).

Differences in the recoveries of LC-MS/MS anthocyanins compared to UPLC anthocyanidins may be due to the difference in extraction method. Anthocyanins were extracted using 1% TFA acidified methanol, which is a weak acid compared to the 0.1% concentrated HCl acidified methanol extraction used for the UPLC anthocyanidin analysis. Extraction of anthocyanidins using concentrated HCl removed the sugar moiety from the compound and allowed for a more complete removal of compounds from the extracted sample matrix (Barnes et al., 2009). Method extraction problems, possibly due to the 1% TFA acidified MeOH not effectively extracting anthocyanins from the PRC samples, is evident by the abnormally low LC-MS/MS abundance across all processing steps and individual anthocyanins in the PRC samples, as compared to the

anthocyanidin and literature recoveries from press cake (Lee and Wrolstad, 2004; Skrede et al., 2000). This is possibly due to the complexity of blueberry skin cell walls and incomplete cell wall degradation by pectinase and the ability of acidified MeOH to remove the anthocyanins from cell wall components (Ortega-Regules et al., 2006). In addition, the low moisture and high fiber content of the press cake may attribute to difficulty extracting the anthocyanins since it absorbs much of the extract liquid and inhibits part of the acid extraction.

The greatest loss of anthocyanidins in the whole juice process occurred at pressing with a 67% loss of total anthocyanins (Table 3.4). The total anthocyanidin amount in the FAC was  $85.1 \pm 19.2$  mg/100 g and were relatively low compared to amounts found in RAB berries, however press cake amounts were comparable to amounts found in literature (Sellappan et al., 2002; Skrede et al., 2000; You et al., 2011). The PRC samples contained  $265.6 \pm 34.6$  mg/100 g and were much higher than all other processing steps (Table 3.4). Press cake samples contained 3 fold greater amounts of anthocyanidins than the FAC samples. Since berry skins account for 10% to 20% of the berry weight and as the main location for anthocyanins in the berry, the majority of anthocyanins were left behind in the press cake, as previously demonstrated (Brownmiller et al., 2008; Lee et al., 2002; Skrede et al., 2000). Comparing the losses of anthocyanidins from filtration and pasteurization, there is a significant loss from pasteurization, but filtration does not significantly increase the loss of anthocyanidins (Table 3.4).

Juice producers are more concerned with total anthocyanin changes than individual compounds and the relation to how steps in the juice process affect the compounds that change clarity and cause sedimentation. Since the anthocyanins are only semi-quantified and the PRC samples were not adequately extracted for a good representation of the amounts, the total anthocyanins could not be utilized to track the percent of anthocyanins that proceeded forward from the whole berry. This was however calculated with the anthocyanidins.

To determine how much of the juice and press cake come from the raw berries for each sample, relative percentages were determined by utilizing the pilot press data from Chapter 2. The percentage of the whole berries broken down into relative free juice and press cake amounts were 74.0% and 13.0%, respectively. The percent of anthocyanidins that remained in the juice and press cake after pressing, as well as percentage lost after each processing step were calculated using these estimations (Figure 3.7). The sequential degradation or loss/polymerization of anthocyanins begins with loss to pressing, which removed the majority of anthocyanidins. There was a 40.6% loss of anthocyanidins to the press cake and 35.1% was lost to mashing and juice loss in the press cloth and equipment. Of the total anthocyanidins in raw berries, only 24.3% were transferred to the juice (NCJ) (Figure 3.7).



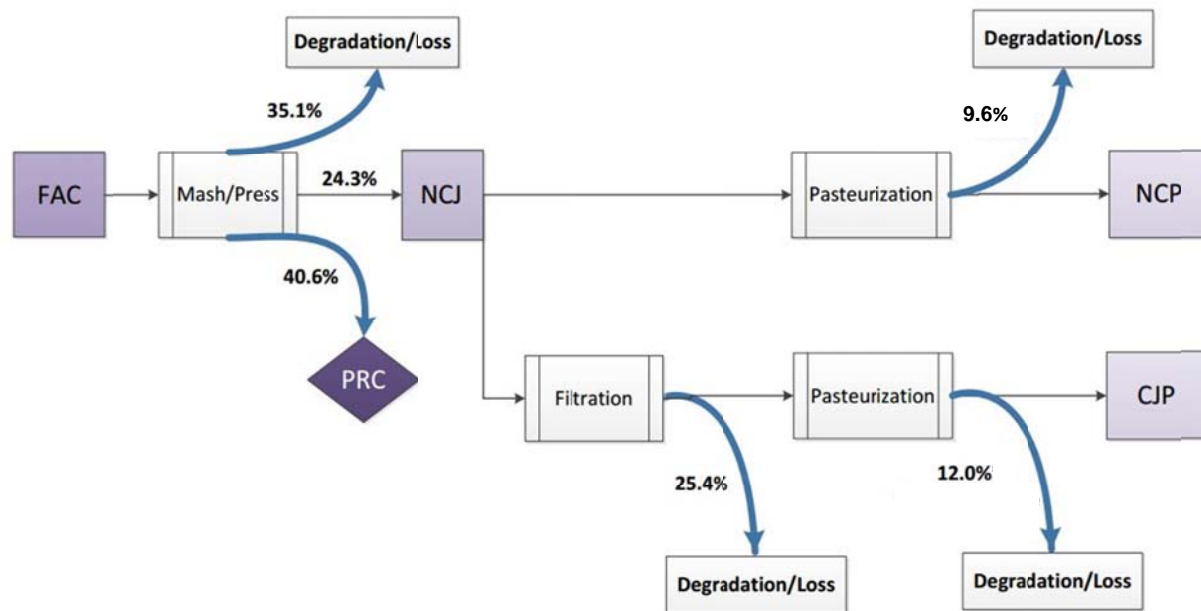


Figure 3.7. Percent anthocyanidin loss through unfiltered and filtered RAB blueberry juice processing

Unfiltered juice then loses another 9.6% in pasteurization. This translates to only 22% of the original amount of anthocyanidins being transferred to a non-filtered pasteurized juice product. Filtered juice removes an additional 25.4% of the anthocyanidins in the NCJ before pasteurization (Figure 3.7). Pasteurized filtered juice (CJP) had a decrease of 12.0% of anthocyanidins compared to unpasteurized filtered juice (CUF). The pasteurized filtered juice contained only about 16% of the raw berry anthocyanidins after processing.

Beyond heat degradation or loss to press cake, anthocyanin loss through processing may be due to anthocyanin polymerization which, our methods used during extraction may not remove from the juice matrix. Reports have indicated that processing and

storage of blueberry juices increase polymeric color (Brownmiller et al., 2008). While color was not reported in this study, significant color changes were not observed throughout processing (data to be reported elsewhere). Findings in blackberry juices, showed 75% anthocyanin losses due to polymerization (Hager et al., 2008). Another possible explanation is the difference in calculating total anthocyanidins by summation of individual anthocyanidin amounts compared to using the pH differential method which calculates total anthocyanidins as one group of compounds based on UV spectra (Giusti and Wrolstad, 2001; Lee et al., 2008). In general, blueberry results reported from a spectrophotometric method are higher in the same sample than results from methods quantifying the individual anthocyanidins (Cho et al., 2004; Lee et al., 2002; You et al., 2011), yet the reverse situation has also been reported wang (Wang et al., 2008). Although the pH differential method versus LC analyses can deliver differing results from the same sample, blueberry data tend to be closely correlated which, indicates both approaches are reliable (Lee et al., 2008; Nicoué et al., 2007; You et al., 2011). The loss to press cake is similar to the percentages found in literature (Lee et al., 2002; Skrede et al., 2000). Highlighting the amount of anthocyanidins remaining in the press cake again confirms that economic opportunities abound for producers to further utilize their waste stream. Minimizing waste from the juice production scheme should be accomplished as value-added polyphenolic products could be developed from the press cake. Greener technologies, like environmentally friendly hot water extraction, is an option for removing the remaining beneficial compounds from press cake (Plaza and Turner, 2015). The seeds and skins which remain in the press cake can be separated

and utilized for seed oil, natural coloring, and confectionary products (Bates et al., 2001).

### 3.5 Conclusion

Evaluation of RAB blueberry juice processing steps identified 10 major and three minor anthocyanins, with the three minor anthocyanins not having been reported in RAB blueberries. All five of the anthocyanidins found in blueberry were identified as well.

Comparing the trends of the individual anthocyanins, the major individual compounds all significantly decreased after pressing and pasteurization. The three minor anthocyanins, while increased slightly, did not have significant changes after juice processing.

Acylation of the minor anthocyanins may increase stability when exposed to pH changes and heat and this may explain why different trends were observed in minor coumaroyl anthocyanins compared with the major anthocyanins. Raw berries and press cake contained the highest anthocyanidin contents with 81.5 mg/100 g and 265.6 mg/100 g respectively. Anthocyanidins decreased 67% after pressing and 10% after pasteurization. Herein, we too realized that ultrafiltration concentrated anthocyanins slightly and ultrafiltration may be further evaluated and optimized to increase anthocyanins in NFC juices. These findings contribute to the value and interest of press cake for use in other food and non-food products as value-added ingredients to boost health benefits and product quality. In juice processing, the more steps in the process, the greater the loss of anthocyanins in the end product. Higher processing temperatures with shorter overall thermal exposures resulting in less anthocyanin loss illustrates the efficacy of pilot scale mash and HTST temperatures used in our studies. Nonetheless,

further studies evaluating the storage effects on juice quality between unfiltered and filtered juices, taking into account lower molecular weight anthocyanins and polymers associated with increased health benefits, would contribute to the importance of filtration in juice processing. Information regarding this NFC blueberry juice allows local small scale producers to better choose processes that help create niche market products.

## CHAPTER 4 CONCLUSION

As blueberries gain in popularity in the marketplace, further characterization, descriptions and uses are needed for local berries, as well as processing knowledge. A juicing process for production of fresh not from concentrate (NFC) blueberry juice was evaluated and optimized based upon examining variations in the juice process itself. Freezing the berries to extend the juicing period did not affect juice recovery. This is an important result for small scale or local commercial juicers who are trying to produce juice for year round production and consumption. As previously found in Northern highbush (NHB) berries, heating the Southern highbush (SHB) and rabbiteye (RAB) berries before pressing as well as an added pectinase enzyme resulted in greatly increased juice recovery. With small scale trials, this processing method produced 68% free juice in SHB berries and 62% free juice in RAB berries. Two pectinase enzymes, Rohopect 10 L and Pectinex BEEEXL, were evaluated in both SHB and RAB berries. In terms of free juice recovery, both enzymes resulted in similar recoveries for both types of berries. The addition of enzymes proved to be beneficial to the process. In the comparison of berry types, the SHB berries produced about 10% more juice in all treatments compared to RAB berries. This is possibly due to the differences in complexity of the pectin structures between the two berry types. In the pilot scale up trials, juice recovery was comparable to the bench top experiment, with a 74% free juice recovery. However, if we include all juice amounts lost during weighing, transfers, and within press bags, the protocol could result in up to 87% juice recovery. This shows that the pilot press was also a more efficient pressing regime than the bench top presses.

Further experiments testing variations on temperatures and processing steps (e.g. using pressing aids such as rice hulls or cellulose fiber) would be beneficial. A more in depth evaluation of endogenous enzymes that produce negative effects in berries such as anthocyanin degradation, gelling, and browning, in concert with processing changes to ameliorate such events, could further benefit the quality of juices. Meanwhile, expanded evaluations of cell wall and membrane degrading enzyme combinations beyond pectinases (i.e. proteases or lipases) to increase anthocyanin and juice extraction could elucidate improvements to the general juicing process. Studies evaluating time spent in freezing temperatures are also needed to determine how long berries can be stored before water loss and polyphenolic degradation affect quality of the berries for juicing purposes.

While identifying anthocyanins through NFC processing steps in 'Tifblue' RAB blueberry juice, three new minor anthocyanins not identified before in RAB berries were tentatively identified in all process steps. This expands on information about anthocyanins present in RAB berries through processing. Quantification of anthocyanidins helped to locate where changes can be made to improve juice quality for producers in the juicing process. The retention of almost 40% of the anthocyanidins in the press cake from the raw berry is an indication that pre-press treatments need to be further evaluated and that press cake should be reconsidered for sources of value-added anthocyanins. The development of innovative steps to extract more anthocyanins, as well as other

phytochemicals, from the berry skins and seeds, is needed for value added juices and blueberry products. Unfiltered juices retained only 22%, while filtered juice retained 16% of the anthocyanidins from the raw berries.

Herein, we too realized that ultrafiltration concentrated anthocyanins slightly and this method may prove to be beneficial in increasing anthocyanins in NFC juices. The retentate from the UF process was not studied in this experiment, but offers another opportunity for value added blueberry products. The filtered juice had greater degradation of anthocyanins than unfiltered juice after pasteurization. This was possibly due to the removal of co-pigmentation of the anthocyanins by ultrafiltration. These anthocyanin polymers help protect anthocyanins from heat. In juice processing, the more steps in the process, the greater the loss of anthocyanins in the end product. In comparison to other literature studies, the higher processing temperatures with shorter overall thermal exposures used in our studies, resulted in less anthocyanin loss compared to literature studies which used lower temperatures and longer times. Nonetheless, further studies evaluating the storage effects on juice quality between unfiltered and filtered juices, taking into account lower molecular weight anthocyanins and polymers associated with increased health benefits, would contribute to the importance of filtration in juice processing.

Maintaining anthocyanin quantities while maximizing juice output in NFC juices is a significant challenge producer's face. Carefully monitoring and evaluating juice processing steps can lead to increased juice recovery. This thesis research evaluated

juice processing inputs and steps, including blanching in steam-jacket kettles and pectinase enzyme pre-press treatments, filtration, and pasteurization and determined the impacts these steps had on juice recovery and anthocyanins in NFC 'Tifblue' RAB blueberry juice. By evaluating the input options of pre-press berry temperature and enzyme use, juice recovery was calculated to be 74% free juice and 87% total juice. Addition of cell wall degrading enzymes proved to be beneficial to increase juice recovery. Identifying and utilizing anthocyanins and anthocyanidins to demonstrate the effect of juice processing has on blueberries, allows for a simplified measurement of juice quality. With increased demand for natural and less processed food options, local small scale juice producers can utilize NFC juice products as a compromise between unpasteurized fresh juices and highly processed evaporated reconstituted juices.

In summary, the importance of optimizing the individual steps in the juice process is vital to increasing juice recovery and maintaining quality in NFC juices. These results in conjunction with the literature provide a basis of information for small scale production of blueberry juices. Streamlining the juice process by evaluating then improving pre-press treatments of berries will allow small scale producers, as well as their customers, to benefit from higher quality products with added value and beneficial health promoting phytochemicals, such as anthocyanins.



## REFERENCES

- AAFC. 2011. Fruit Juices in the United States. Agriculture and Agri-Food Canada, Ottawa, ON Canada. 11655E: 1-9.  
[http://publications.gc.ca/collections/collection\\_2012/agr/A74-1-42-2011-eng.pdf](http://publications.gc.ca/collections/collection_2012/agr/A74-1-42-2011-eng.pdf).
- AAFC. 2012. American Eating Trends Report. Agriculture and Agri-Food Canada, Ottawa, ON Canada. K1A: 1-7. <http://www.agr.gc.ca/eng/industry-markets-and-trade/statistics-and-market-information/by-region/united-states/fruit-juices/?id=1410083148489>.
- AIJN. 2012. 2012 Liquid Fruit Market Report. European Fruit Juice Association, Brussels, Belgium.  
<http://www.aijn.org/pages/main/file.handler?f=AIJNMarketReport2012.pdf>.
- Albrigo, L.G., P.M. Lyrene, and B. Freeman, 1980. Waxes and Other Surface Characteristics of Fruit and Leaves of Native *Vaccinium ellotti* Chapm. J. Am. Soc. Hortic. Sci. 105:230-235.
- Allan-Wojtas, P.M., C.F. Forney, S.E. Carbyn, and K.U.K.G. Nicholas, 2001. Microstructural Indicators of Quality-related Characteristics of Blueberries—An Integrated Approach. LWT-Food Sci. Technol. 34:23-32.
- Alper, N., K.S. Bahceci, and J. Acar, 2005. Influence of Processing and Pasteurization on Color Values and Total Phenolic Compounds of Pomegranate Juice. J. Food Process. Pres. 29:357-368.
- Anonymous, 1962. pH values of food products. Food Eng. 34:98-99.
- Anonymous. 2013. Food Industry Resource. U.S. Highbush Blueberry Council, Date Accessed:03/08/2015. <http://www.blueberry.org/industrial/indust21.jpg>.
- Ayaz, F.A., A. Kadioglu, E. Bertoft, C. Acar, and I. Turna, 2001. Effect of fruit maturation on sugar and organic acid composition in two blueberries (*Vaccinium arctostaphylos* and *V. myrtillus*) native to Turkey. New Zeal. J. Crop Hort. 29:137-141.

- Bagger-Jorgensen, R. and A.S. Meyer, 2004. Effects of different enzymatic pre-press maceration treatments on the release of phenols into blackcurrant juice. *Eur. Food Res. Technol.* 219:620-629.
- Ballington, J.R. 2007. Blueberry Breeding, Duplin County Center, Kenansville, NC.
- Barkla, C., 2011. Fruit Juices, p. 1-21, Market News Service. International Trace Centre, Geneva, Switzerland.
- Barnes, J.S., H.P. Nguyen, S. Shen, and K.A. Schug, 2009. General method for extraction of blueberry anthocyanins and identification using high performance liquid chromatography–electrospray ionization-ion trap-time of flight-mass spectrometry. *J. Chromatogr. A* 1216:4728-4735.
- Basiouny, F.M. and Y. Chen. 1988. Effects of harvest date, maturity and storage interval on postharvest quality of rabbiteye blueberry (*Vaccinium ashei* Reade)101:281-284).
- Basu, A., M. Rhone, and T.J. Lyons, 2010. Berries: Emerging impact on cardiovascular health. *Nutr. Rev.* 68:168-177.
- Bates, R.P., J.R. Morris, and P.G. Crandall, 2001. Principles and Practices of Small- and Medium-scale Fruit Juice Processing. *FAO Ag. Serv. Bul.* 146.
- Baxter, H., J.B. Harborne, and G.P. Moss, 1998. *Phytochemical Dictionary: A Handbook of Bioactive Compounds from Plants*. 2nd ed. CRC Press, Boca Raton, FL.
- Beaulieu, J.C., S.W. Lloyd, J.E. Preece, J.W. Moersfelder, R.E. Stein-Chisholm, and J.M. Obando-Ulloa, 2015. Physicochemical properties and aroma volatile profiles in a diverse collection of California-grown pomegranate (*Punica granatum* L.) germplasm. *Food Chemistry* 181:354-364.
- Bello, A.B. and M.S. Sule, 2012. Optimum temperature and thermal stability of crude polyphenol oxidase from some common fruits. *Nig. J. Basic and Appl. Sci.* 20:27-31.

- Boches, P., N.V. Bassil, and L. Rowland, 2006. Genetic diversity in the highbush blueberry evaluated with microsatellite markers. *J. Am. Soc. Hortic. Sci.* 131:674-686.
- Brambilla, A., R. Lo Scalzo, G. Bertolo, and D. Torreggiani, 2007. Cultivar selection bridged with innovative processing; A new strategy to preserve and maximize antioxidant activity in blueberry juice. *Acta Hortic.* 744:337-342.
- Brambilla, A., R. Lo Scalzo, G. Bertolo, and D. Torreggiani, 2008. Steam-blanching highbush blueberry (*Vaccinium corymbosum* L.) juice: Phenolic profile and antioxidant capacity in relation to cultivar selection. *J. Agr. Food Chem.* 56:2643-2648.
- Brambilla, A., D. Maffi, and A. Rizzolo, 2011. Study of the influence of berry-blanching on syneresis in blueberry purées. *Procedia Food Sci.* 1:1502-1508.
- Brazelton, C. 2011. World Blueberry Acreage and Production. U.S Highbush Blueberry Council. 1-51.
- Brooks, R.M. and H.P. Olmo, 1997. The Brooks and Olmo Register of Fruit & Nut Varieties. ASHS Press, Alexandria, VA USA.
- Brownmiller, C., L.R. Howard, and R.L. Prior, 2008. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed blueberry products. *J. Food Sci.* 73:H72-79.
- Bruederle, L.P., N. Vorsa, and J.R. Ballington, 1991. Population genetic structure in diploid blueberry *Vaccinium* Section *Cyanococcus* (*Ericaceae*). *Am. J. Bot.* 78:230-237.
- Brul, S. and P. Coote, 1999. Preservative agents in foods: Mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology* 50:1-17.
- Buckow, R., A. Kastell, N.S. Terefe, and C. Versteeg, 2010. Pressure and temperature effects on degradation kinetics and storage stability of total anthocyanins in blueberry juice. *J. Agr. Food Chem.* 58:10076-10084.

- Burns, J., W. Mullen, N. Landrault, P.-L. Teissedre, M.E.J. Lean, and A. Crozier, 2002. Variations in the profile and content of anthocyanins in wines made from Cabernet Sauvignon and hybrid grapes. *J. Agr. Food Chem.* 50:4096-4102.
- Castañeda-Ovando, A., M.d.L. Pacheco-Hernández, M.E. Páez-Hernández, J.A. Rodríguez, and C.A. Galán-Vidal, 2009. Chemical studies of anthocyanins: A review. *Food Chem.* 113:859-871.
- Castrejón, A.D.R., I. Eichholz, S. Rohn, L.W. Kroh, and S. Huyskens-Keil, 2008. Phenolic profile and antioxidant activity of highbush blueberry (*Vaccinium corymbosum* L.) during fruit maturation and ripening. *Food Chem.* 109:564-572.
- Chisari, M., R.N. Barbagallo, and G. Spagna, 2007. Characterization of polyphenol oxidase and peroxidase and influence on browning of cold stored strawberry fruit. *J. Agr. Food Chem.* 55:3469-3476.
- Cho, M.J., L.R. Howard, R.L. Prior, and J.R. Clark, 2004. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agr.* 84:1771-1782.
- Chung, M.Y., L.S. Hwang, and B.H. Chiang, 1986. Concentration of perilla anthocyanins by ultrafiltration. *J. Food Sci.* 51:1494-1497.
- Cissé, M., F. Vaillant, D. Pallet, and M. Dornier, 2011. Selecting ultrafiltration and nanofiltration membranes to concentrate anthocyanins from roselle extract (*Hibiscus sabdariffa* L.). *Food Research International* 44:2607-2614.
- Crozier, A., D. Del Rio, and M.N. Clifford, 2010. Bioavailability of dietary flavonoids and phenolic compounds. *Mol. Aspects Med.* 31:446-467.
- de Ancos, B., E. Ibañez, G. Reglero, and M.P. Cano, 2000. Frozen storage effects on anthocyanins and volatile compounds of raspberry fruit. *J. Agr. Food Chem.* 48:873-879.
- de Moraes, J.O., P.B. Pertuzatti, F.V. Corrêa, and M.d.L.M. Salas-Mellado, 2007. Study of rabbiteye blueberry (*Vaccinium ashei* 'Reade') in the process of food products. *Food Sci. Technol. Res.* 27:18-22.

- Demir, N., J. Acar, K. Sarıoğlu, and M. Mutlu, 2001. The use of commercial pectinase in fruit juice industry. Part 3: Immobilized pectinase for mash treatment. *J. Food Eng.* 47:275-280.
- Deng, J., Z.-J. Shi, X.-Z. Li, and H.-M. Liu, 2013. Soluble polysaccharides isolation and characterization from rabbiteye blueberry (*Vaccinium ashei*) fruits. *BioResources* 8:405-419.
- Diaz, J.V., G.E. Anthon, and D.M. Barrett, 2007. Nonenzymatic degradation of citrus pectin and pectate during prolonged heating: effects of pH, temperature, and degree of methyl esterification. *J. Agr. Food Chem.* 55:5131-5136.
- Du, X., A. Plotto, M. Song, J. Olmstead, and R. Rouseff, 2011. Volatile composition of four Southern highbush blueberry cultivars and effect of growing location and harvest date. *J. Agr. Food Chem.* 59:8347-8357.
- Duan, Q., E. Goodale, and R.-c. Quan, 2014. Bird fruit preferences match the frequency of fruit colours in tropical Asia. *Sci. Rep.* 4:1-8.
- Ehlenfeldt, M.K. and R.L. Prior, 2001. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. *J. Agr. Food Chem.* 49:2222-2227.
- Eklund, B., 2014. 2014 Blueberry Statistics, p. 9. National Agricultural Statistics Service, Trenton, NJ.
- Fanali, C., L. Dugo, G. D'Orazio, M. Lirangi, M. Dacha, P. Dugo, and L. Mondello, 2011. Analysis of anthocyanins in commercial fruit juices by using nano-liquid chromatography-electrospray-mass spectrometry and high-performance liquid chromatography with UV-vis detector. *J. Sep. Sci.* 34:150-159.
- FDA, 2001. Hazard Analysis and Critical Control Point (HACCP) Systems, p. 311-320. In: H.a.H. Services (ed.).
- Folmer, F., U. Basavaraju, M. Jaspars, G. Hold, E. El-Omar, M. Dicato, and M. Diederich, 2014. Anti-cancer effects of bioactive berry compounds. *Phytochem. Rev.* 13:295-322.

- Fonsah, E.G., J. Massonnat, L. Wiggins, G. Krewer, D. Stanaland, and J. Smith, 2013. Southern Highbush Blueberry Marketing and Economics, p. 1-10. In: U.C. Extension (ed.), UGA Cooperative Extension Bulletin. UGA Cooperative Extension Bulletin, Athens, GA.
- Gao, L. and G. Mazza, 1994. Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries. *J. Food Sci.* 59:1057-1059.
- Gavrilova, V., M. Kajdžanoska, V. Gjamovski, and M. Stefova, 2011. Separation, characterization and quantification of phenolic compounds in blueberries and red and black currants by HPLC–DAD–ESI-MS. *J. Agr. Food Chem.* 59:4009-4018.
- Gerard, K.A. and J.S. Roberts, 2004. Microwave heating of apple mash to improve juice yield and quality. *LWT-Food Sci. Technol.* 37:551-557.
- Giusti, M.M. and R.E. Wrolstad, 2001. Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy, *Current Protocols in Food Analytical Chemistry*. John Wiley & Sons, Inc.
- Giusti, M.M. and R.E. Wrolstad, 2003. Acylated anthocyanins from edible sources and their applications in food systems. *Biochemical Engineering Journal* 14:217-225.
- Gössinger, M., S. Moritz, M. Hermes, S. Wendelin, H. Scherbichler, H. Halbwirth, K. Stich, and E. Berghofer, 2009. Effects of processing parameters on colour stability of strawberry nectar from puree. *J. Food Eng.* 90:171-178.
- Gould, K., K.M. Davies, and C. Winefield, 2009. *Anthocyanins: Biosynthesis, Functions, and Applications*. 1st ed. Springer-Verlag New York.
- Grassin, C. and P. Fauquembergue, 1996. Fruit Juices, p. 226-264. In: T. Godfrey and S. West (eds.), *Industrial Enzymology*. MacMillan, London, UK.
- Hager, A., L.R. Howard, R.L. Prior, and C. Brownmiller, 2008. Processing and Storage Effects on Monomeric Anthocyanins, Percent Polymeric Color, and Antioxidant Capacity of Processed Black Raspberry Products. *J. Food Sci.* 73:H134-H140.
- Havlíková, L. and K. Míková, 1985. Heat stability of anthocyanins. *Z Lebensm Unters Forch* 181:427-432.

- Hilz, H., E.J. Bakx, H.A. Schols, and A.G.J. Voragen, 2005. Cell wall polysaccharides in black currants and bilberries—characterisation in berries, juice, and press cake. *Carbohydr. Polym.* 59:477-488.
- Holzwarth, M., S. Korhummel, R. Carle, and D.R. Kammerer, 2012. Impact of enzymatic mash maceration and storage on anthocyanin and color retention of pasteurized strawberry purées. *Eur. Food Res. Technol.* 234:207-222.
- Horváth-Kerkai, E., 2007. Manufacturing Fruit Beverages, p. 205-215. In: Y.H. Hui (ed.), *Handbook of Fruits and Fruit Processing*. Blackwell.
- Howard, L.R., J.R. Clark, and C. Brownmiller, 2003. Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. *J. Sci. Food Agr.* 83:1238-1247.
- Howard, L.R., R.L. Prior, R. Liyanage, and J.O. Lay, 2012. Processing and storage effect on berry polyphenols: Challenges and implications for bioactive properties. *J. Agr. Food Chem.* 60:6678-6693.
- Hummel, N.A., A.D. Attaway, E.D. Coneva, J. Braswell, W.O. Cline, D. Marshall, D.M. Ferrin, K.L. Machtmes, and H. Roy, 2012. Creating a community of practice for blueberries. *Int. J. Fruit Sci.* 12:350-359.
- Hutchens, A.R., 1992. *A Handbook of Native American Herbs: The pocket guide to 125 medicinal plants and their uses*. 1 ed. Shambhala Publications Inc., Boston, MA.
- Hynes, M. and A. Aubin, 2006. Acquity UPLC for the rapid analysis of anthocyanidins in berries. Waters Corporation, Milford, MA.
- IFT. 2001. Chapter 4. Analysis of Microbial Hazards Related to Time/Temperature Control of Foods for Safety. Institute of Food Technologists. 223-98-2333:
- Jaeger, H., M. Schulz, P. Lu, and D. Knorr, 2012. Adjustment of milling, mash electroporation and pressing for the development of a PEF assisted juice production in industrial scale. *Innov. Food Sci. Emerg.* 14:46-60.
- Janick, J., 1986. *Horticultural Science*. 4th ed. W.H. Freeman and Company, United States of America.

- Johnson, C.E. and Y. Huang, 1999. New blueberry variety shows promise for expanding production in Louisiana. *La. Agr. Winter* 1999:1-28.
- Kader, F., M. Irmouli, J.P. Nicolas, and M. Metche, 2002. Involvement of blueberry peroxidase in the mechanisms of anthocyanin degradation in blueberry juice. *J. Food Sci.* 67:910-915.
- Kader, F., B. Rovel, M. Girardin, and M. Metche, 1997. Mechanism of browning in fresh highbush blueberry fruit (*Vaccinium corymbosum* L.). Role of blueberry polyphenol oxidase, chlorogenic acid and anthocyanins. *J. Sci. Food Agr.* 74:31-34.
- Kalbasi, A. and L. Cisneros-Zevallos, 2007. Fractionation of monomeric and polymeric anthocyanins from Concord grape (*Vitis labrusca* L.) juice by membrane ultrafiltration. *J. Agric. Food Chem.* 55:7036-7042.
- Kalt, W. and D. Dufour, 1997. Health functionality of blueberries. *HortTechnology* 7:216-221.
- Kalt, W., J.E. McDonald, R.D. Ricker, and X. Lu, 1999. Anthocyanin content and profile within and among blueberry species. *Can. J. Plant Sci.* 79:617-623.
- Kalt, W., D.A.J. Ryan, J.C. Duy, R.L. Prior, M.K. Ehlenfeldt, and S.P. Vander Kloet, 2001. Interspecific variation in anthocyanins, phenolics, and antioxidant capacity among genotypes of highbush and lowbush blueberries (*Vaccinium* Section *cyanococcus* spp.). *J. Agr. Food Chem.* 49:4761-4767.
- Kechinski, C.P., P.V. Guimaraes, C.P. Norena, I.C. Tessaro, and L.D. Marczak, 2010. Degradation kinetics of anthocyanin in blueberry juice during thermal treatment. *J. Food Sci.* 75:C173-176.
- Khanal, R.C., L.R. Howard, C.R. Brownmiller, and R.L. Prior, 2009. Influence of extrusion processing on procyanidin composition and total anthocyanin contents of blueberry pomace. *Journal of food science* 74:H52-58.
- Khanal, R.C., L.R. Howard, and R.L. Prior, 2010. Effect of heating on the stability of grape and blueberry pomace procyanidins and total anthocyanins. *Food Res. Int.* 43:1464-1469.



- Khurana, S., K. Venkataraman, A. Hollingsworth, M. Piche, and T. Tai, 2013. Polyphenols: Benefits to the cardiovascular system in health and in aging. *Nutrients* 5:3779-3827.
- Kinzer, G. and P. Schreier, 1980. Influence of Different Pressing Systems on the Composition of Volatile Constituents in Unfermented Grape Musts and Wines. *American Journal of Enology and Viticulture* 31:7-13.
- Landbo, A.-K., K. Kaack, and A.S. Meyer, 2007. Statistically designed two step response surface optimization of enzymatic prepress treatment to increase juice yield and lower turbidity of elderberry juice. *Inn. Food Sci. Emerg.* 8:135-142.
- Le Bourvellec, C., S. Guyot, and C.M.G.C. Renard, 2004. Non-covalent interaction between procyanidins and apple cell wall material: Part I. Effect of some environmental parameters. *Biochim. Biophys. Acta* 1672:192-202.
- Lee, J., R.W. Durst, and R.E. Wrolstad, 2002. Impact of juice processing on blueberry anthocyanins and polyphenolics: Comparison of two pretreatments. *J. Food Sci.* 67:1660-1667.
- Lee, J., R.W. Durst, and R.E. Wrolstad, 2005. Determination of Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines by the pH Differential Method: Collaborative Study. *J. AOAC Int.* 88:1269-1278.
- Lee, J., C.E. Finn, and R.E. Wrolstad, 2004. Comparison of anthocyanin pigment and other phenolic compounds of *Vaccinium membranaceum* and *Vaccinium ovatum* native to the Pacific Northwest of North America. *J. Agr. Food Chem.* 52:7039-7044.
- Lee, J., C. Rennaker, and R.E. Wrolstad, 2008. Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods. *Food Chemistry* 110:782-786.
- Lee, J. and R.E. Wrolstad, 2004. Extraction of anthocyanins and polyphenolics from blueberry processing waste. *J. Food Sci.* 69:564-573.

- Lohachoopol, V., M. Mulholland, G. Srzednicki, and J. Craske, 2008. Determination of anthocyanins in various cultivars of highbush and rabbiteye blueberries. *Food Chem.* 111:249-254.
- Lohachoopol, V., G. Srzednicki, and J. Craske, 2004. The Change of Total Anthocyanins in Blueberries and Their Antioxidant Effect After Drying and Freezing. *Journal of Biomedicine and Biotechnology* 2004:248-252.
- LSU. 2014. Louisiana Summary Agriculture and Natural Resources. Louisiana State University Agriculture Center, Baton Rouge, LA. 2382: 1-342.  
<http://www.lsuagcenter.com/NR/rdonlyres/6B369793-D285-41FE-9F37-F41AB43050E1/102539/pub2382AgSummary2014FORWEB.pdf>.
- Lyrene, P.M., 1981. Recurrent selection in breeding rabbiteye blueberries (*Vaccinium Ashei Reade*). *Euphytica* 30:505-511.
- Lyrene, P.M., N. Vorsa, and J.R. Ballington, 2003. Polyploidy and sexual polyploidization in the genus *Vaccinium*. *Euphytica* 133:27-36.
- Markaris, P., G.E. Livingston, and C.R. Fellers, 1957. Quantitative aspects of strawberry pigment degradation. *J. Food Sci.* 22:117-130.
- Marshall, D.A., J.M. Spiers, and J.H. Braswell, 2006. Splitting severity among rabbiteye (*Vaccinium ashei* 'Reade') blueberry cultivars in Mississippi and Louisiana. *Int. J. Fruit Sci.* 6:77-81.
- Mellentini, J. and K. Crawford, 2008. Marketing healthy fruit, p. 55-71. In: F.A. Tomas-Barberan and M.I. Gil (eds.), *Improving the Health-Promoting Properties of Fruit and Vegetable Products*. CRC Press, LLC., Boca Raton, FL.
- Merken, H.M. and G.R. Beecher, 2000. Measurement of food flavonoids by high-performance liquid chromatography: A review. *J. Agr. Food Chem.* 48:577-599.
- Mieszczakowska-Frać, M., J. Markowski, M. Zbrzeźniak, and W. Płocharski, 2012. Impact of enzyme on quality of blackcurrant and plum juices. *LWT-Food Sci. Technol.* 49:251-256.

- Moyer, R.A., K.E. Hummer, C.E. Finn, B. Frei, and R.E. Wrolstad, 2002. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: *Vaccinium*, *Rubus*, and *Ribes*. *J. Agr. Food Chem.* 50:519-525.
- Muhammad, S. and A.J. Allen, 2000. Efficient marketing of blueberries in Mississippi and Louisiana. *Journal of Food Distribution Research* 31:152-158.
- Muller, D., M. Schantz, and E. Richling, 2012. High performance liquid chromatography analysis of anthocyanins in bilberries (*Vaccinium myrtillus* L.), blueberries (*Vaccinium corymbosum* L.), and corresponding juices. *J. Food Sci.* 77:C340-345.
- Nakajima, J.-i., I. Tanaka, S. Seo, M. Yamazaki, and K. Saito, 2004. LC/PDA/ESI-MS profiling and radical scavenging activity of anthocyanins in various berries. *J. Biomed. Biotechnol.* 5:241-247.
- Nakao, S.-i., 1994. Determination of pore size and pore size distribution: 3. Filtration membranes. *J. Membr. Sci.* 96:131-165.
- Nartea, T., 2014. Being berry profitable markets and trends for Virginia small growers, 6th Annual Virginia Berry Conference. Virginia State University, Petersburg, Virginia.
- Neveu, V., J. Perez-Jimenez, F. Vos, V. Crespy, L. du Chaffaut, L. Mennen, C. Knox, R. Elisner, J. Cruz, D. Wishart, and A. Scalbert. 2012. Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. <http://database.oxfordjournals.org/content/2010/bap024.full>
- Nicoué, E.É., S. Savard, and K. Belkacemi, 2007. Anthocyanins in wild blueberries of Quebec: Extraction and identification. *J. Agric. Food Chem.* 55:5626-5635.
- Nikdel, S., C.S. Chen, M.E. Parish, D.G. MacKellar, and L.M. Friedrich, 1993. Pasteurization of citrus juice with microwave energy in a continuous-flow unit. *J. Agric. Food Chem.* 41:2116-2119.
- Nindo, C.I., J. Tang, J.R. Powers, and P. Singh, 2005. Viscosity of blueberry and raspberry juices for processing applications. *J. Food Eng.* 69:343-350.

- Obón, J.M., M.C. Díaz-García, and M.R. Castellar, 2011. Red fruit juice quality and authenticity control by HPLC. *J. Food Compos. Anal.* 24:760-771.
- Ortega-Regules, A., I. Romero-Cascales, J.M. Ros-García, J.M. López-Roca, and E. Gómez-Plaza, 2006. A first approach towards the relationship between grape skin cell-wall composition and anthocyanin extractability. *Analytica Chimica Acta* 563:26-32.
- Pap, N., M. Mahosenaho, E. Pongrácz, H. Mikkonen, M. Jaakkola, V. Virtanen, L. Myllykoski, Z. Horváth-Hovorka, C. Hodúr, G. Vatai, and R. Keiski, 2012. Effect of ultrafiltration on anthocyanin and flavonol content of black currant juice (*Ribes nigrum* L.). *Food and Bioprocess Technology* 5:921-928.
- Parry, J., L. Su, M. Luther, K. Zhou, M.P. Yurawecz, P. Wittaker, and L. Yu, 2005. Fatty acid composition and antioxidant properties of cold-pressed marionberry, boysenberry, red raspberry, and blueberry seed oils. *J. Agr. Food Chem.* 53:566-573.
- Patil, G. and K.S.M.S. Raghavarao, 2007. Integrated membrane process for the concentration of anthocyanin. *J. Food Eng.* 78:1233-1239.
- Patras, A., N.P. Brunton, C. O'Donnell, and B.K. Tiwari, 2010. Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation. *Trends in Food Sci. Tech.* 21:3-11.
- Pedrolli, D.B., A.C. Monteiro, E. Gomes, and E.C. Carmona, 2009. Pectin and pectinases: Production, characterization and industrial application of microbial pectinolytic enzymes. *Open Biotech. J.* 3:9-18.
- Perera, C.O. and B. Smith, 2013. Technology of Processing of Horticultural Crops, p. 259-315. In: M. Kutz (ed.), *Handbook of Farm, Dairy, and Food Machinery Engineering*. Academic Press.
- Pina, F., M.J. Melo, C.A.T. Laia, A.J. Parola, and J.C. Lima, 2012. Chemistry and applications of flavylum compounds: *a handful of colours*. *Chem. Soc. Rev.* 41:869-908.

- Pinelo, M., B. Zeuner, and A.S. Meyer, 2010. Juice clarification by protease and pectinase treatments indicates new roles of pectin and protein in cherry juice turbidity. *Food and Bioprocess Technology* 88:259-265.
- Plaza, M. and C. Turner, 2015. Pressurized hot water extraction of bioactives. *TrAC Trends in Analytical Chemistry* 71:39-54.
- Press, A., 1989. Mississippi and Louisiana cashing in on blueberries, p. 3B, *The Advocate*, Baton Rouge, LA.
- Prior, R.L., G. Cao, A. Martin, E. Sofic, J. McEwen, C. O'Brien, N. Lischner, M. Ehlenfeldt, W. Kalt, G. Krewer, and C.M. Mainland, 1998. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. *J. Agr. Food Chem.* 46:2686-2693.
- Prior, R.L., S.A. Lazarus, G. Cao, H. Muccitelli, and J.F. Hammerstone, 2001. Identification of procyanidins and anthocyanins in blueberries and cranberries (*Vaccinium* Spp.) using high-performance liquid chromatography/mass spectrometry. *J. Agr. Food Chem.* 49:1270-1276.
- Puls Jr., E.E., 1999. Commercial Blueberry Production, p. 1-36. In: LSU (ed.). Louisiana State University Agricultural Center and Cooperative Extension Service, Baton Rouge, LA.
- Reque, P.M., R.S. Steffens, A. Jablonski, S.H. Flôres, A.d.O. Rios, and E.V. de Jong, 2014. Cold storage of blueberry (*Vaccinium spp.*) fruits and juice: Anthocyanin stability and antioxidant activity. *J. Food Comp. Anal.* 33:111-116.
- Retamales, J.B. and J.F. Hancock, 2012. *Blueberries*. CABI, Cambridge, MA.
- Rommel, A., D.A. Heatherbell, and R.E. Wrolstad, 1990. Red raspberry juice and wine: Effect of processing and storage on anthocyanin pigment composition, color and appearance. *J. Food Sci.* 55:1011-1017.
- Rossi, M., E. Giussani, R. Morelli, R. Lo Scalzo, R.C. Nani, and D. Torreggiani, 2003. Effect of fruit blanching on phenolics and radical scavenging activity of highbush blueberry juice. *Food Res. Int.* 36:999-1005.

- Routray, W. and V. Orsat, 2011. Blueberries and their anthocyanins: Factors affecting biosynthesis and properties. *Compr. Rev. Food Sci. Food Safety* 10:303-320.
- Rupasinghe, H.P.V. and L.J. Yu, 2012. Emerging Preservation Methods for Fruit Juices and Beverages. In: Y. El-Samragy (ed.), *Food Additive*. InTech, Rijeka, Croatia.
- Rwabahizi, S. and R.E. Wrolstad, 1988. Effects of mold contamination and ultrafiltration on the color stability of strawberry juice and concentrate. *J. Food Sci.* 53:857-861.
- Sablani, S.S., P.K. Andrews, N.M. Davies, T. Walters, H. Saez, R.M. Syamaladevi, and P.R. Mohekar, 2010. Effect of thermal treatments on phytochemicals in conventionally and organically grown berries. *J. Sci. Food Agric.* 90:769-778.
- Sadilova, E., F.C. Stintzing, and R. Carle, 2006. Thermal degradation of acylated and nonacylated anthocyanins. *J. Food Sci.* 71:C504-C512.
- Saftner, R., J. Polashock, M. Ehlenfeldt, and B. Vinyard, 2008. Instrumental and sensory quality characteristics of blueberry fruit from twelve cultivars. *Postharvest Biol. Tec.* 49:19-26.
- Sandri, I.G., R.C. Fontana, D.M. Barfknecht, and M.M. da Silveira, 2011. Clarification of fruit juices by fungal pectinases. *LWT-Food Sci. Technol.* 44:2217-2222.
- Sant'Anna, V., L.D.F. Marczak, and I.C. Tessaro, 2012. Membrane concentration of liquid foods by forward osmosis: Process and quality view. *J. Food Eng.* 111:483-489.
- Santos, R.M.d., I.d.A. Nääs, M. Mollo Neto, and O. Vendrametto, 2013. An overview on the Brazilian orange juice production chain. *Revista Brasileira de Fruticultura* 35:218-225.
- Sapers, G.M., A.M. Burgher, J.G. Phillips, S.B. Jones, and E.G. Stone, 1984. Color and composition of highbush blueberry cultivars. *J. Am. Soc. Hortic. Sci.* 109:105-111.

- Sellappan, S., C.C. Akoh, and G. Krewer, 2002. Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *J. Agr. Food Chem.* 50:2432-2438.
- Sellmer, J., R. Bates, G. Hoover, and G. Moorman, 2004. Ericacea (Heath) Family and Their Culture. In: P.S.C.o.A.S.D.o. Horticulture (ed.). Penn State, University Park, PA 16802.
- Silva, J.L., E. Marroquin, F.B. Matta, J.O. Garner, and J. Stojanovic, 2005. Physicochemical, carbohydrate and sensory characteristics of highbush and rabbiteye blueberry cultivars. *J. Agr. Food Chem.* 85:1815-1821.
- Skrede, G., R.E. Wrolstad, and R.W. Durst, 2000. Changes in anthocyanins and polyphenolics during juice processing of highbush blueberries (*Vaccinium corymbosum* L.). *J. Food Sci.* 65:357-364.
- Soto-Vaca, A., A. Gutierrez, J.N. Losso, Z. Xu, and J.W. Finley, 2012. Evolution of phenolic compounds from color and flavor problems to health benefits. *J. Agr. Food Chem.* 60:6658-6677.
- Spiers, J.M., S.J. Stringer, A.D. Draper, and C.L. Gupton, 2002. 'Biloxi' Southern highbush blueberry. *Acta Hortic.* 574:153-155.
- Srivastava, A., C.C. Akoh, W. Yi, J. Fischer, and G. Krewer, 2007. Effect of storage conditions on the biological activity of phenolic compounds of blueberry extract packed in glass bottles. *J. Agric. Food Chem.* 55:2705-2713.
- Strik, B.C., C.E. Finn, and P.P. Moore, 2014. Blueberry Cultivars for the Pacific Northwest, p. 1-13, Pacific Northwest Extension Publication. Oregon State University, Washington State University, and University of Idaho.
- Strik, B.C. and D. Yarborough, 2005. Blueberry production trends in North America, 1992 to 2003, and predictions for growth. *HortTechnology* 15:391-398.
- Stringer, S.J., A.D. Draper, J.M. Spiers, and D.A. Marshall, 2009. Potential new blueberry cultivars for the Gulf Coast region of the U.S. *Acta Hortic.* 810:87-91.

- Sun-Waterhouse, D., 2011. The development of fruit-based functional foods targeting the health and wellness market: A review. *Int. J. Food Sci. Tech.* 46:899-920.
- Takeda, F., G. Krewer, E.L. Andrews, B. Mullinix, and D.L. Peterson, 2008. Assessment of the V45 blueberry harvester on Rabbiteye blueberry and Southern highbush blueberry pruned to V-shaped canopy. *HortTechnology* 18:130-138.
- Tanaka, Y., N. Sasaki, and A. Ohmiya, 2008. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *The Plant Journal* 54:733-749.
- Thomasset, S., N. Teller, H. Cai, D. Marko, D. Berry, W. Steward, and A. Gescher, 2009. Do anthocyanins and anthocyanidins, cancer chemopreventive pigments in the diet, merit development as potential drugs? *Cancer Chemoth. Pharm.* 64:201-211.
- Tian, Q., M.M. Giusti, G.D. Stoner, and S.J. Schwartz, 2005. Screening for anthocyanins using high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry with precursor-ion analysis, product-ion analysis, common-neutral-loss analysis, and selected reaction monitoring. *J. Chromatogr. A* 1091:72-82.
- Tomás-Barberán, F.A. and J.C. Espín, 2001. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *J. Sci. Food Agr.* 81:853-876.
- Tropp, D. 2014. *Why Local Food Matters: The rising importance of locally-grown food in the U.S. food system* Washington D.C.
- USDA, 2001. *Plant Guide: Highbush Blueberry*. In: U.N.R.C. Service (ed.). USDA.
- USDA. 2013a. Blueberries - Average retail price per pound and per cup equivalent. United States Department of Agriculture. SR-26 2013: 1. <http://www.ers.usda.gov/data-products/fruit-and-vegetable-prices.aspx#26369>.
- USDA. 2013b. Cultivated blueberries: Commercial acreage, yield per acre, production, and season-average grower price in the United States, 1980-2009 (table08.xls). Economic Research Service (ERS). USDA Economics, Statistics and Market Information System Date Accessed:03/04/2015. <http://usda.mannlib.cornell.edu/usda/ers/blueberry/table08.xls>.



- USDA. 2013c. U.S. blueberry production and utilization (cultivated and wild), selected States, 1980-2012 (table02.xls). Economic Research Service (ERS). USDA Economics, Statistics and Market Information System, Date Accessed:03/04/2015.  
<http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1765> .
- USDA. 2014. Germplasm Resources Information Network (GRIN) Online Database. 4/1/2015. <http://www.ars-grin.gov/cgi-bin/npgs/acc/>
- USDA. 2015. Noncitus Fruits and Nuts 2014 Summary. 98.
- Vaillant, F., A. Millan, M. Dornier, M. Decloux, and M. Reynes, 2001. Strategy for economical optimisation of the clarification of pulpy fruit juices using crossflow microfiltration. J. Food Eng. 48:83-90.
- Valls, J., S. Millán, M.P. Martí, E. Borràs, and L. Arola, 2009. Advanced separation methods of food anthocyanins, isoflavones and flavanols. J. Chromatogr. A 1216:7143-7172.
- Wang, J., W. Kalt, and P. Sporns, 2000. Comparison between HPLC and MALDI-TOF MS Analysis of Anthocyanins in Highbush Blueberries. J. Agric. Food Chem. 48:3330-3335.
- Wang, S.Y., C.-T. Chen, W. Sciarappa, C.Y. Wang, and M.J. Camp, 2008. Fruit quality, antioxidant capacity, and flavonoid content of organically and conventionally grown blueberries. J. Agric. Food Chem. 56:5788-5794.
- Wang, S.Y., H. Chen, and M.K. Ehlenfeldt, 2011. Variation in antioxidant enzyme activities and nonenzyme components among cultivars of rabbiteye blueberries (*Vaccinium ashei* 'Reade') and *V. ashei* derivatives. Food Chem. 129:13-20.
- Watrous, M. 2014. Juicing: A trend. Food Business News, Date Accessed:9/20/2015.  
[http://www.foodbusinessnews.net/articles/news\\_home/Consumer\\_Trends/2014/04/Slideshow\\_Juicing\\_a\\_trend.aspx?ID=%7B4022ED8D-6FFA-4781-8936-F754871CBD0C%7D](http://www.foodbusinessnews.net/articles/news_home/Consumer_Trends/2014/04/Slideshow_Juicing_a_trend.aspx?ID=%7B4022ED8D-6FFA-4781-8936-F754871CBD0C%7D).
- Wightman, J.D. and R.E. Wrolstad, 1996.  $\beta$ -glucosidase activity in juice-processing enzymes based on anthocyanin analysis. J. Food Sci. 61:544-548.

- Williamson, J.G., J.W. Olmstead, and P.M. Lyrene, 2012. Reproductive Growth and Development of Blueberry. In: UF (ed.). University of Florida; Florida Cooperative Extension Service; Institute of Food and Agricultural Sciences; Gainesville, FL.
- Wrolstad, R.E., 1993. Color and Pigment Analysis in Fruit Products, p. 1-17. Agricultural Experiment Station Oregon State University, Corvallis, OR.
- Wu, X., G.R. Beecher, J.M. Holden, D.B. Haytowitz, S.E. Gebhardt, and R.L. Prior, 2006. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *J. Agr. Food Chem.* 54:4069-4075.
- Wu, X. and R.L. Prior, 2005. Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: Fruits and berries. *J. Agr. Food Chem.* 53:2589-2599.
- Yoshimura, Y., H. Enomoto, T. Moriyama, Y. Kawamura, M. Setou, and N. Zaima, 2012. Visualization of anthocyanin species in rabbiteye blueberry *Vaccinium ashei* by matrix-assisted laser desorption/ionization imaging mass spectrometry. *Anal. Bioanal. Chem.* 403:1885-1895.
- You, Q., B. Wang, F. Chen, Z. Huang, X. Wang, and P.G. Luo, 2011. Comparison of anthocyanins and phenolics in organically and conventionally grown blueberries in selected cultivars. *Food Chemistry* 125:201-208.
- Zhang, Z., X. Kou, K. Fugal, and J. McLaughlin, 2004. Comparison of HPLC methods for determination of anthocyanins and anthocyanidins in bilberry extracts. *J. Agr. Food Chem.* 52:688-691.

## APPENDIX

### SUPPLEMENTAL IMAGES



Supplemental Image A.1 Stainless steel press showing nylon press cloth and spigot for juice extraction.



Supplemental Image A.2 Bench top juice experiment water bath used for heating blueberries to 95°C.



Supplemental Image A.3 Pilot scale steam jacketed kettle mashing of rabbiteye 'Tifblue' blueberries heated to 95 °C with constant stirring.



Supplemental Image A.4 Pilot scale pressing of rabbiteye 'Tifblue' blueberries in a Goodnature X-1 press, showing the juice flowing out the bottom of the press into a stainless steel pan.



Supplemental Image A.5 Press cake resulting from mashed and enzyme treated 'Tifblue' rabbiteye blueberries



Supplemental Image A.6 Pilot scale ultrafiltration of rabbiteye 'Tifblue' blueberries from a PCI B-1 pilot ultrafiltration unit



## ROHAPECT® 10 L

Description and Specification

2009-12-08

Rev. No. 07

### Description

ROHAPECT® 10 L is a pectolytic enzyme preparation for the universal application in fruit juice processing. The pectinase is derived from a "classic" strain of *Aspergillus niger*.

- IUB-No.: 3.2.1.15
- CAS-No.: 9032-75-1

### Properties

The product has the following characteristics:

- a) liquid product
- b) brown coloured with aromatic smell
- c) specific weight: – 1.18 g/ml

### Activity

ROHAPECT® 10 L contains a declared minimum activity of 260 PA.

The PA is the reciprocal value of the enzyme amount in kilogramm, which is required to depectinize 100 liter of standard apple juice under standard conditions ( 50°C, pH 3,2, 1 hr).

### Application

ROHAPECT® 10 L is universally applicable and splits both soluble and insoluble pectin as well arabanes. In fruit processing it is qualified for viscosity reduction, extraction, mash and juice treatment, clarification and total depectinisation.

ROHAPECT® 10 L is recommended for depectinising low pH-juices, like lemon and lime juice.





Supplemental Document 1.1 AB Enzymes Rohapect 10L Description and Specification  
Sheet Page 2

**AB Enzymes GmbH**  
Postfach 101239  
64212 Darmstadt  
Germany  
Tel: +49 (0) 6151 3680 100  
Fax: +49 (0) 6151 3680 120

ROHAPECT® 10 L

**Dosage**

Application	Reaction conditions	Dosage
Pome Fruit Juice Treatment Mash Treatment	50°C / 30 – 60 min 20 – 30°C / 60 min	20 – 30 g / 1000 l 50 – 100 g / t
Raspberries, Strawberries, Blackberries, Currants, Cherries, etc.	mash: 50°C / 1 – 3 h juice: 20 – 45°C / 1 – 6 h	80 – 200 g / t 40 – 80 g / 1000 l
Lemon & Lime Juice	20 – 35°C / 1 – 3 h	60 – 100 g / 1000 l
Tropical Fruits: Guava, Banana, etc.	depending from process 20 – 50°C / 1 – 3 h	30 – 60 g / t

**Specification**

The product fulfils the requirements of the FAO/WHO's Joint Expert Committee on Food Additives (JECFA) and Food Chemicals Codex (FCC) for food grade enzymes.  
The total viable counts are within the upper limit of  $5 \times 10^4 \text{ g}^{-1}$

**Packaging**

ROHAPECT® 10 L is available in 25-kg PE cans.

**Composition**

Water, glycerol qs, pectinase, ammonium sulphate qs, sodium benzoate 0.30 %, potassium sorbate 0.14 %.

**Storage**

Stored in a cool place ( $>10^\circ\text{C}$ ) the activity loss will be less than 10% within one year.


**Safety Handling**

Avoid the formation of aerosol and dust of the product. Repeated inhalation of enzyme aerosol or dust may cause sensitisation and may cause allergic type reactions in sensitised individuals. For detailed information please refer to the Material Safety Data Sheet (MSDS).

2 / 2

Our technical advice on the uses of our products is given without obligation. AB Enzymes is not responsible for the application and processing of the products by the customer or any third party. The customer is solely liable to comply with the applicable laws and regulations and with intellectual property rights of third parties. This information contains product specifications which may be altered without prior notice.

Product Data Sheet



**novozymes**  
Rethink Tomorrow

1 of 2  
Valid from 2012-02-14

---

Pectinex® BE XXL

**In this product the key enzyme activity is provided by**  
pectin lyase catalyzing eliminative cleavage of (1,4)-alpha-D-galacturonan methyl ester giving oligosaccharides with 4-feroxy-5-O-methyl-alpha-D-galact-4-enuronosyl groups at their non-reducing ends

PRODUCT CHARACTERISTICS/PROPERTIES

Declared enzyme	Pectin lyase
Declared activity	16000 PECTU/ml
Color	Brown
Physical form	Liquid
Approximate density (g/ml)	1.17
Odor	Slight fermentation odor
Solubility	Active component is readily soluble in water at all concentrations that occur in normal usage. Standardisation components can cause turbidity in solution.

Color can vary from batch to batch. Color intensity is not an indication of enzyme activity.

ALLERGEN

Allergen	Substance contained <sup>1</sup>	Allergen	Substance contained
Beef	no	Lactose	no
Carrot	no	Legumes	no
Celery	no	Lupin	no
Cereals containing gluten <sup>2</sup>	no	Milk	no
Chicken meat	no	Molluscs	no
Cocoa	no	Mustard	no
Coriander	no	Nuts <sup>3</sup>	no
Corn/maize	no	Peanuts	no
Crustaceans	no	Pork	no
Egg	no	Sesame	no
Fish	no	Soy	no
Glutamate	no	Sulphur dioxide/sulphites, more than 10 mg per kg or l	no

<sup>1</sup>Definition of substances according to LaCia/ALBA and EU Directives 2000/13/EC and 2007/68/EC, as amended  
<sup>2</sup>i.e. wheat, rye, barley, oats, spelt, kamut  
<sup>3</sup>i.e. almond, hazelnut, walnut, cashew, pecan nut, Brazil nut, pistachio nut, macadamia nut and Queensland nut

PRODUCT SPECIFICATION

	Lower Limit	Upper Limit	Unit
Pectinase unit: PECTU	16000		/ml
Total viable count	-	50000	/g
Coliform bacteria	-	30	/g
E.coli	Not Detected		/25 g
Salmonella	Not Detected		/25 g
Heavy metals		Max 30	mg/kg
Lead		Max 5	mg/kg
Arsenic		Max 3	mg/kg
Cadmium		Max 0.5	mg/kg
Mercury		Max 0.5	mg/kg

COMPOSITION

Ingredients	Appr. % (w/w)
Glycerol, CAS no. 56-81-5	45
Water, CAS no. 7732-18-5	43
Pectin lyase, CAS no. 9033-35-6*	7
Potassium chloride, CAS no. 7447-40-7	5

\* Defined as enzyme conc. (dry matter basis)  
No preservatives added

PRODUCTION ORGANISM

Production organism Aspergillus niger

Produced by fermentation of microorganisms which are selected according to the EU definition (further information available upon request). The enzyme proteins are separated and purified from the production organisms.

STORAGE CONDITION

**Recommended storage:** 0-10 °C (32-50 °F)

Packaging must be kept intact, dry, and away from sunlight. Please follow the recommendations and use the product before the best before date to avoid the need for a higher dosage.

**Best before:** You will find the best before date in the certificate of analysis or on the product label.

The product gives optimal performance when stored as recommended and used prior to the best-before date.

© Novozymes A/S

## Pectinex<sup>®</sup> BE XXL

### SAFETY AND HANDLING PRECAUTIONS

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes, and mucous membranes upon prolonged contact. See the MSDS or Safety Manual for further information regarding safe handling of the product and spills.

### COMPLIANCE

The product complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC), and with relevant Chinese food safety and product standards for food-grade enzymes.

Kosher and Halal certificates are available from the Customer Center or sales representative.

### CERTIFICATIONS

Novozymes is a signatory to United Nations Global Compact, United Nations Convention on Biological Diversity and report on our sustainability performance through Global Reporting Initiative (GRI). See all our commitments under sustainability on [www.novozymes.com](http://www.novozymes.com).



### FOOD SAFETY

Novozymes has carried out a hazard analysis and prepared an HACCP plan describing the critical control points (CCPs). The HACCP plan is supported by a comprehensive prerequisite program implemented in Novozymes' GMP practices.

The product is produced according to Novozymes' HACCP plan, GMP practices, and additional requirements controlled by Novozymes' Quality Management System.

The product complies with FAO/WHO JECFA- and FCC-recommended purity requirements regarding mycotoxins. The product complies with EU legislation regarding pesticides.

### PACKAGING

The product is available in different types of packaging. Please contact the sales representative for more information.

Novozymes A/S  
Krogshøjvej 36  
2880 Bagvaerd  
Denmark

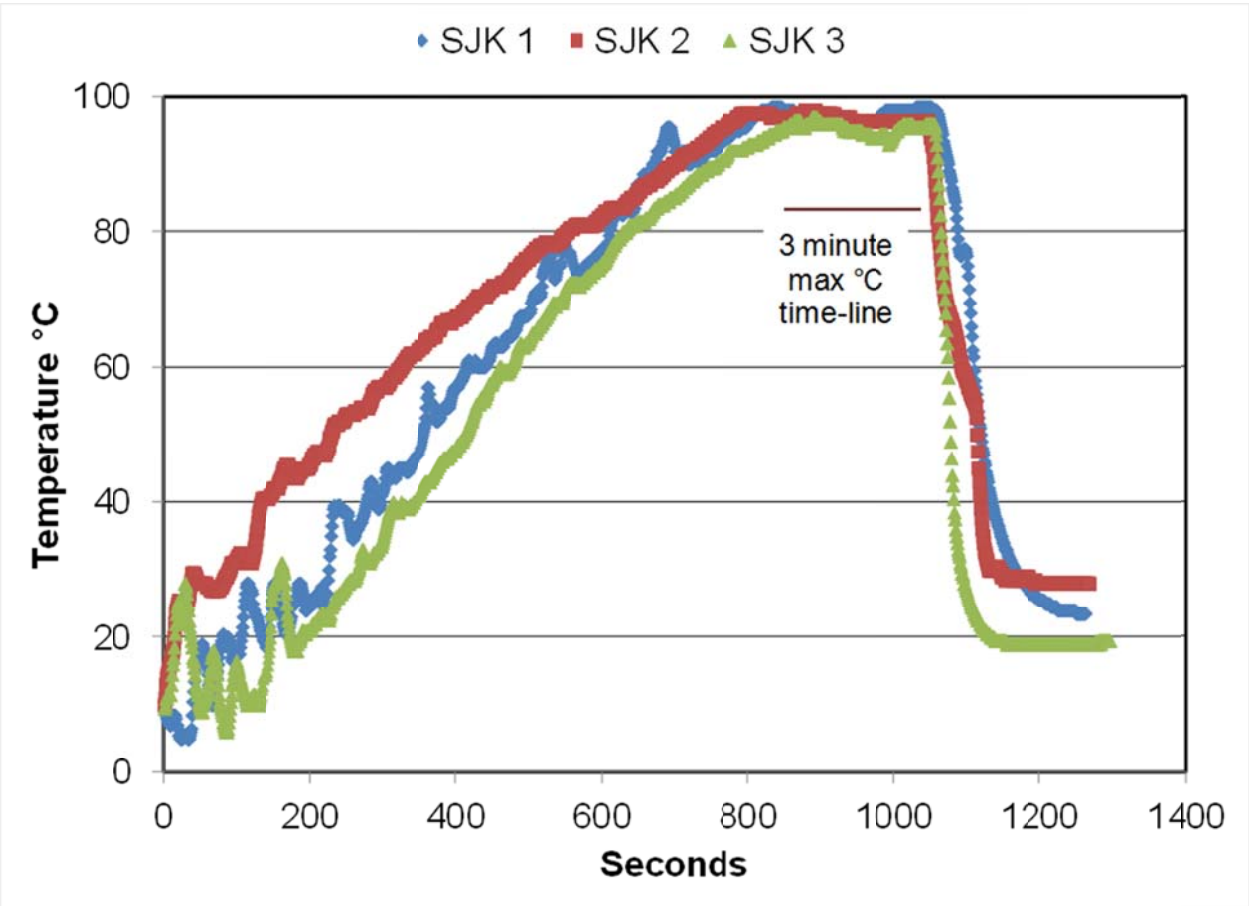
Tel. +45 4446 0000  
Fax +45 4446 9999

For more information, or for more office addresses, visit [www.novozymes.com](http://www.novozymes.com)

Laws, regulations and/or third party rights may prevent customers from importing, using, processing and/or reselling the products described herein in a given manner. Without separate, written agreement between the customer and Novozymes to such effect, this document does not constitute a representation or warranty of any kind and is subject to change without further notice.

© Novozymes A/S

Supplemental Figure 1.1. Temperature plots recorded during Tifblue blueberry mash treatments in steam jacketed kettles.



## VITA

Rebecca Stein-Chisholm is from San Antonio, Texas. In 2007, she graduated from Texas A&M University in College Station, Texas with a Bachelor's of Science in Horticulture with concentrations in Biotechnology and Floral Design. After graduation, Rebecca took a position at the USDA's Southern Regional Research Center in New Orleans, Louisiana to work with Dr. John Beaulieu as a Food Technologist in the Food Processing and Sensory Quality unit. There she worked with fresh cut cantaloupe and honeydew and small fruit juice processing of pomegranates, satsumas, and blueberries. In 2011, Rebecca decided to pursue her Master's degree at Louisiana State University in the Food Science department to work with Drs. Jack Losso and John Finley in collaboration with Dr. John Beaulieu.

Currently, Rebecca works as a Research and Development Chemist at Active Organics in Lewisville, Texas. As the botanical expert for the company, Rebecca helps develop active ingredients for personal care products. Her current research includes finding natural phytochemical ingredient alternatives for skin care products, new product development, and product characterization. Rebecca's hobbies include scrapbooking, gardening, yoga, and making French macaroons. She is a riding member of the Mystic Krewe of Nyx, a Mardi Gras parade in New Orleans, Louisiana. Professional societies include the Society of Cosmetic Chemists, the American Chemist Society as well as a member of Pi Alpha Xi, the horticultural honor society and Alpha Zeta, the agricultural honor society.