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CHANGES IN SELECT PHENOLIC COMPOUNDS DURING ETHANOL FERMENTATION AND ACETIFICATION OF RASPBERRY, BLUEBERRY AND PERSIMMON

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 Food Science

by
Yen-Ping Tan
B.S., Louisiana State University, 2007
August, 2011

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my major professor, Dr. Paul Wilson for his expertise, guidance, and patience. He has been a wonderful and dedicated mentor in helping me complete my research and thesis. I thank Dr. Zhimin Xu and Dr. Subramaniam Sathivel for allowing me to use their laboratory equipment for analysis and providing their help and advice throughout my master program.

I would also like to extend my appreciation to Dr. Charles Johnson for providing me fresh blueberries and persimmons and sharing with me the knowledge of horticulture. I also thank Dr. David Picha for his kind consideration by allowing me to work in his lab. Many thanks to my classmates and labmates: Sungjoon Jang, Huaixia Yin, Waheeda Mohideen and Ling Tian for their suggestions and help in my research.

Without the support of my family member, I would not have successfully completed my study abroad. I thank my parents, brothers and sisters for their support and encouragement throughout my education in United States. My special thanks go to my husband, Elun Hsu, for his understanding, kindness and encouragement. His support and unconditional love means the whole world to me.

Last but not the least, I thank God for giving me strength and wisdom in everything I do.

TABLE OF CONTENT

ACKNOWLEDGEMENTS ii

LIST OF FIGURES vii

ABSTRACT ix

CHAPTER 1. INTRODUCTION ..... - 1 -

CHAPTER 2. LITERATURE REVIEW ..... - 5 -

2.1 Fruit Substrates ..... - 5 -
2.1.1 Introduction ..... - 5 -
2.1.2. Total Phenolics and Anthocyanins in Berries Species ..... - 5 -
2.1.3. Health Benefits of Berries ..... - 7 -
2.1.4 Phenolics in Persimmons ..... - 8 -
2.1.5. Health Benefits of Persimmons ..... - 8 -
2.2 Anthocyanins ..... - 9 -
2.2.1. Introduction ..... - 9 -
2.2.2 Chemical Structures and Properties ..... - 9 -
2.3. Wine ..... - 12 -
2.3.1. Background ..... - 12 -
2.3.2. Wine Fermentation ..... - 15 -
2.3.2.1. Alcoholic Fermentation – Primary Fermentation ..... - 15 -
2.3.2.1.1. Yeasts ..... - 15 -
2.3.2.2. Malolactic Fermentation – Secondary Fermentation ..... - 17 -
2.3.2.2.1. Lactic Acid Bacteria ..... - 17 -
2.3.2.2.2. Effects of Malolactic Fermentation ..... - 17 -
2.3.3. Health Benefits of Wine ..... - 19 -
2.4. Vinegar ..... - 20 -
2.4.1. Background ..... - 20 -
2.4.2. Acetification Process ..... - 21 -
2.4.2.1. Acetic Acid Bacteria ..... - 21 -
2.4.2.2. Mother of Vinegar ..... - 22 -
2.4.3. Understanding the Vinegar Making Process ..... - 22 -
2.4.3.1. Orleans Process ..... - 24 -
2.4.3.2. Generator Fermentation ..... - 25 -
2.4.3.3. Submerged Fermentation ..... - 26 -
2.4.4. Health Benefits of Vinegar ..... - 26 -

CHAPTER 3. MATERIALS AND METHODS ..... - 28 -

3.1. Wine Fermentation (Alcohol Fermentation) ..... - 28 -
3.2. Vinegar Fermentation (Acetification) ..... - 28 -
3.3. Black Raspberries ..... - 29 -
3.3.1. Wine Fermentation Treated with Proteases ..... - 29 -

3.3.2. Determination of Total Selected Anthocyanin - 30 -
3.4. Persimmons - 32 -
3.4.1. Alcoholic Fermentation - Primary Fermentation - 32 -
3.4.2. Malolactic Fermentation - Secondary Fermentation - 34 -
3.4.3. Vinegar Fermentation - Orleans Process - 34 -
3.4.4. Physicochemical Analysis - 35 -
3.4.4.1. pH, Titratable Acidity & Brix - 36 -
3.4.4.2. Total Phenolics - 36 -
3.4.4.3. Antiradical Activity - 37 -
3.5. Blueberries - 38 -
3.5.1. Alcoholic Fermentation – Primary Fermentation - 38 -
3.5.2. Malolactic Fermentation - Secondary Fermentation - 40 -
3.5.3. Vinegar Fermentation - Orleans Process - 41 -
3.5.4. Physicochemical Analysis - 41 -
3.5.4.1. pH, Titratable Acidity & Brix - 42 -
3.5.4.2. Total Selected Anthocyanins and Epicatechin Profile - 42 -
3.5.4.3. Total Phenolics - 43 -
3.5.4.4. Antiradical Activity - 44 -
3.5.4.5. EPA and DHA in Salmon Oil-in-water Emulsion - 44 -
3.6. Statistical Analyses - 46 -

CHAPTER 4. RESULTS AND **DISCUSSION - 47 -**

4.1. Black Raspberry - 47 -
4.2. Persimmon - 52 -
4.2.1. pH, Titratable Acidity and Brix - 52 -
4.2.2. Total Phenolics and Antiradical Activity - 53 -
4.3. Blueberries - 55 -
4.3.1 pH, Titratable Acidity, and Brix - 55 -
4.3.2 Total Anthocyanin - 56 -
4.3.3. Epicatechin - 58 -
4.3.4. Antiradical Activity - 59 -
4.3.5. Total Phenolics - 60 -
4.3.6. Overall Changes of Phenolics and Antioxidant Activity - 61 -
4.3.7. Changes in Concentration of EPA and DHA in Salmon Oil-in-water Emulsion - 62 -

CHAPTER 5. SUMMARY, CONCLUSION AND FUTURE RESEARCH - 66 -

REFERENCES - 68 -

APPENDIX A. CHROMATOGRAM OF ANTHOCYANIN IN BLACK RASPBERRY FERMENTATION PRODUCTS AFTER TREATED WITH 1% PROTEASES IN OPENED BEAKER - 76 -

APPENDIX B. CHROMATOGRAM OF ANTHOCYANIN IN BLACK RASPBERRY FERMENTATION PRODUCTS AFTER TREATED WITH 0.1% AND 0.01% PROTEASES IN VOLUMETRIC FLASK COVERED WITH AIR-LOCK .... - 77 -

APPENDIX C. SUMMARY OF PH, TA AND BRIX OF BLUEBERRY JUICE AND FERMENTATION PRODUCTS .... - 78 -

APPENDIX D. SUMMARY OF PH, TA AND BRIX OF ASTRINGENT AND NON-ASTRINGENT PERSIMMON JUICE AND FERMENTATION PRODUCTS .... - 79 -

APPENDIX E. ANTHOCYANIN CHROMATOGRAM OF TIFBLUE RABBITEYE BLUEBERRY JUICE AND FERMENTATION PRODUCTS .... - 80 -

APPENDIX F. EPICATECHIN (EPC) CHROMATOGRAM OF TIFBLUE RABBITEYE BLUEBERRY JUICE .... - 81 -

APPENDIX G. CHROMATOGRAM OF EPA AND DHA IN SALMON OIL-IN-WATER EMULSION WITH 1% AND 10% TREATMENT AT 0 DAY AND 3 DAY OF INCUBATION .... - 82 -

APPENDIX H. SAS PROGRAM OF STATISTICAL ANALYSES .... - 84 -

VITA .... - 89 -

LIST OF TABLES

Table 2.1. Structures of Naturally Occuring Anthocyanidins …………………………………..11

Table 4.1. Anthocyanins in Black Raspberry After Treated with 1% Proteases……………......48

Table 4.2. Anthocyanin in Black Raspberry Fermentation Products After Treated with 1% Proteases…………………………………………………………………………………......49

Table 4.3. Anthocyanin in Black Raspberry Fermentation Products After Treated with 0.1% and 0.01% Proteases ……………………………………………………………………………51

Table 4.4. Summary of Total Phenolics and Antiradical Activity of Astringent and Non-astringent Persimmon Juice and Fermentation Products ………………………………………..55

Table 4.5. Summary of Total Anthocyanin Content, Total Epicatechin, Total Phenolics and Antiradical Activity of Blueberry Juice and Fermentation Products ………………………......62

Table 4.6. Summary of Eicosapentaenoic Acid and Docosahexaenoic Acid Remained in Salmon Oil-in-water Emulsion After 3 days of Incubation ……………………………………………...64

LIST OF FIGURES

Figure 2.1. Basic Structure of Anthocyanidins ……………………………………………………10

Figure 2.2. Chemical Structures of Anthocyanin Chromophores ………………………………12

Figure 2.3. World Wine Production and Wine Consumption Countries ………………………14

Figure 2.4. Acetic Acid Bacteria ………………………………………………………………..22

Figure 2.5. Double Fermentation ………………………………………………………………23

Figure 2.6. Triple Fermentation ………………………………………………………………...23

Figure 2.7. Conversion of Alcohol to Acetic Acid Reaction …………………………………...24

Figure 3.1. Alcohol and Vinegar Fermentation Flow Chart……………………………………31

Figure 3.2. Astringent Persimmon Tree and Fruits Grown at Burden Research Center ……….32

Figure 3.3. Astringent and Non-astringent Persimmon Fruits ………………………………….33

Figure 3.4. Primary Fermentation of Astringent and Non-astringent Persimmons …………….33

Figure 3.5. Secondary Fermentation of Astringent and Non-astringent Persimmons …………34

Figure 3.6. Acetification of Astrigent and Non-astringent Persimmons ……………………….35

Figure 3.7. Folin-ciocalteau Microscale Colorimetry Method …………………………………37

Figure 3.8. Free Radical Scavenging Activity by DPPH Solution ……………………………..38

Figure 3.9. Tree and Fruits of Tifblue Rabbiteye Blueberry Grown at Bob R. Jones – Idlewild Research Station, Louisiana ……………………………………………………………………39

Figure 3.10. Stalk Seperator and the Procedure of Seperating the Branches and Leaves from the Fruits …………………………………………………………………………………………...39

Figure 3.11. Primary Fermentation of Blueberries in a Plastic Fermenting Vessel ……………40

Figure 3.12. Secondary Fermentation of Blueberries in Glass Fermenting Vessel …………….40

Figure 3.13. Acetification of Blueberry Wine in a Beaker ……………………………………..41

Figure 3.14. High Performance Liquid Chromatography (HPLC) ……………………………..43

Figure 3.15. Preparing Salmon Oil-in-water Emulsion by Using Sonication …………………..45

Figure 4.1. Anthocyanin in Black Raspberry Fermentation Products After Treated with 1% Proteases ………………………………………………………………………………...48

Figure 4.2. Anthocyanin in Black Raspberry Fermentation Products After Treated with 0.1% and 0.01% Proteases …………………………………………………………………………50

Figure 4.3. Figure 4.3. pH, titratable acidity and brix of astringent and non-astringent juice and fermentation products……………………………………………………………………52

Figure 4.4. Total Phenolics in Astringent and Non-astringent Persimmon Juice and Fermentation Products …………………………………………………………………………….....54

Figure 4.5. Antiradical Activity in Astringent and Non-astringent Persimmon Juice and Fermentation Products ……………………………………………………………………..54

Figure 4.6. pH, TA and Brix of Blueberry Juice and Fermentation Products …………………..56

Figure 4.7. Total Anthocyanin Content of Fresh Blueberry Juice and Fermentation Products …57

Figure 4.8. Epicatechin of Blueberry Juice and Fermentation Products ……………………..….58

Figure 4.9. Antiradical Activity of Blueberry Juice and Fermentation Products ……………….59

Figure 4.10. Total Phenolics of Blueberry Juice and Fermentation Products …………………..60

Figure 4.11. Eicosapentaenoic Acid (EPA) Remained in 1% Salmon Oil-in-water Emulsion After 3 days of Incubation ……………………………………………………………………….63

Figure 4.12. Docosahexaenoic Acid (DHA) Remained in 1% Salmon Oil-in-water Emulsion After 3 days of Incubation …………………………………………………………………64

ABSTRACT

Polyphenols are major dietary components in fruits and vegetables. Many research and epidemiological studies have reported that phenolic compounds, such as anthocyanins, may have a protective effect against various degenerative diseases. Phenolics in grape wine have been extensively studied but information on changes of phenolics in wine and vinegar made from other fruit crops is limited, however. This research started with the objective of determining the effect of proteases in preventing phenolic and, specifically, anthocyanin degradation in the ethanolic fermentation of black raspberries to reduce sugar content. The results of this study led to investigations of changes of other polyphenols in persimmons and blueberries during both ethanolic (vinification) and vinegar (acetification) fermentations.

Initial results showed that a 1% neutral bacterial protease could retain anthocyanin in open vinification of black raspberries but the protease treatment provided no benefit when a closed fermentation was used. Additional research using closed vinification followed by acetification of persimmons indicated that phenolics were significantly affected by these fermentations. Phenolics in astringent persimmons were significantly degraded during both vinification and acetification while phenolics in non-astringent persimmons actually increased during vinification but decreased during acetification. In blueberries, anthocyanin and epicatechin were increased during vinification but decreased during acetification.

The lipid-protective properties of fruit wine and vinegar was also investigated. In this study of antioxidant properties, blueberry vinegar was more effective than blueberry juice or blueberry wine in preventing EPA and DHA degradation in salmon oil-in-water emulsion model system. This thesis research provides informative insights concerning changes in polyphenols, such as anthocyanin, during vinification and acetification of some fruit crops. The information is

especially beneficial to the juice, wine and vinegar industries since polyphenols are one of the major health promoting constituents in fruit juices, wines and vinegars.

CHAPTER 1. INTRODUCTION

Phytochemicals in fruits, vegetables and grains have been extensively studied throughout decades. Many of them have been identified and associated with the prevention of cancer, heart disease and other diseases. Black raspberries have been long known to contain substantial amount of anthocyanins and total phenolics (Torre and others 1977; Hong and others 1990; Moyer and others 2002). The high antioxidant capability of black raspberries is attributed to the high anthocyanin and phenolics content. Black raspberries have been linked to many possible health benefits such as acting as angiogenesis inhibitors, preventing inflammatory effects, protecting against DNA damage and exhibiting anti-cancer activity (Harris and others 2001; Lazze and others 2003; Liu and others 2005; Kresty and others 2006). Persimmons, though not popular in Western countries, are extensively studied in Asian countries. Persimmons contain high amounts of biologically active compounds such as ascorbic acid, polyphenols and tannins (Gorinstein and others 1994; Bubba and others 2009). Because of the high bioactive compounds, persimmons have been associated with various health benefits such as anticarcinogenic and antidiabetic effects, and prevention against rise in plasma lipids. (Katwase and others 2003; Lee and others 2006; Park and others 2008).

Blueberries are also one of the most popular fruits that are widely known to be rich in phytochemicals such as phenolic compounds and flavonoids. Blueberry phenolics exhibit various protective effects which include antioxidant, anti-inflammatory and anti-carcinogenic properties (Wedge and others 2001; Sellapan and others 2002; Zheng and others 2003; Neto 2007). Several phenolic acids such as gallic acid, caffeic acid, and others have been identified in rabbiteye blueberry and Southern highbush blueberries. Some anthocyanins have also been identified in lowbush, highbush, rabbiteye and Tifblue blueberries. The major anthocyanins in lowbush and

Tifblue blueberries were cyanidin-3-arabinoside, malvidin-3-glucoside and others (Shahidi and others 2004). These phenolic acids and anthocyanins are the major phytonutrients in blueberries and have significant health promoting effects both *in vitro* and *in vivo*.

Phenolic compounds are very important components in fruits, juices and wine because they contribute to the sensory characteristics such as color, astringency, bitterness, flavor, and taste. Furthermore, the phenolic compounds in juice products and wine are purported to have distinct health benefits. According to several epidemiologic studies with wine, regular moderate consumption reduces the risk of coronary heart disease, type 2 diabetes, cancers and lowers the risk of overall mortality (German and others 2000; Jackson 2008).

Vinegar has been used as a seasoning in cooking since ancient times. However, consuming fruit or wine vinegar as a drink on a regular basis is becoming increasingly popular, especially in Asia, because of the potential health benefits. According to the Nielson Company, vinegar sales increased 1.6% in 2006 with specialty vinegars, such as red wine vinegar and balsamic vinegar, leading the way (Vinegar Institute 2010). Also, the Japanese market for vinegar drinks was estimated at $559.6 million and is expected to grow 15% annually (Berry 2007). Recent research has reported that vinegar has anti-glycemic effects on type 2 diabetes adults, anti-obesity effects, anti-hypertensive effects, and anti-bacterial activity. It reduces cholesterol and triacyglycerols, inhibits cancer cells, and serves as a refreshing drink after exercise. (Kondo S and others 2001; Fushimi and others 2002; Shimoji and others 2004; Fushimi and others 2006; Medina E and others 2007; Kondo T and others 2009; Johnson and others 2010).

Wine vinegar beverages could provide potential health benefits from both the phenolic compounds and acetic acid (vinegar) in the drink. However, little is known about the changes of individual phenolic compounds during alcohol fermentation and acetification processes in fruit

crops other than grapes. Also, the potential protective effect of wine vinegar is currently limited. This research began with an industry supported project intended to produce black raspberry juice with reduced sugars and high antioxidant content using a brief ethanolic fermentation. During production, it was found that antioxidant content decreased and polyphenoloxidase was thought responsible. Polyphenoloxidase (PPO) is found naturally in most plants including most fruits that have high commercial value. It accounts for catalyzing some undesirable enzymatic browning reactions and also degrading anthocyanins in some fruit (Labuza and others 1986; Francis 1989). Many inhibitors such as sulfite and ascorbic acid have been used to inhibit the PPO activity; however, some research has indicated a more novel approach. The protein structure of PPO may be susceptible to protease enzymes which could alter PPO activity but information about using proteases to prevent anthocyanin degradation is still limited. Therefore, the initial project was designed to investigate the effect of proteases in preserving anthocyanins in a sugar-reduced black raspberry juice. Unfortunately, the total amount of anthocyanins in black raspberry juice reduced during the brief fermentation process exceeded any positive benefit that could be provided by proteases.

The black raspberry project provided inspiration for the subsequent research. Upon completion of the black raspberry project, it was clear that significant changes of phenolic compounds could occur with alcoholic fermentation. Furthermore, it was suspected that aerobic acetification of the wine to vinegar would affect phenols even more. Due to limited access to appropriate fresh fruit at the time, persimmons were used for the next phase of the research. In this persimmon research, total phenolics and antiradical activity were investigated during the persimmon wine and vinegar fermentation processes, and a comparison was made between astringent and non-astringent persimmons.

The persimmon project improved skills and provided ideas for a more thorough investigation of changes in phenolic compounds in blueberries during wine and vinegar fermentation later in the summer when fruit was plentiful.

The major objectives of this research includes:

1) Investigating the effect of proteases in reducing PPO activity and retaining anthocyanins during ethanolic fermentation of black raspberry juice

2) Screening changes of total phenolics and antiradical activity in astringent and non-astringent persimmons during ethanolic wine and subsequent acetification fermentation to vinegar.

3) Determining the changes in a select group of phenolic compounds in blueberries during wine and vinegar production.

CHAPTER 2. LITERATURE REVIEW

2.1 Fruit Substrates

2.1.1 Introduction

Blueberries (*Vaccinium sp*) and black raspberries (*Rubus occidentalis*) are widely consumed around the world, especially in the Western countries while persimmons are more popular in Asia . The most common blueberries produced in the commercial scale include lowbush (wild), highbush and rabbiteye (cultivated) blueberries (Kalt 2001). Blueberries are rich in anthocyanins, proanthocyanidins, phenolic acids, flavonols, and catechin (Kader and others 1996; Skrede and others 2000; Smith and others 2000). The total contents of phenolics in blueberries are affected by the degree of maturity at harvest, pre-harvest environmental condition, postharvest environmental condition, and cultivars (Sahidi 2004).

Black raspberries are one of the richest sources of anthocyanins and polyphenols among fruits and vegetables (Tian and others 2006). The content of phenolic compounds in raspberries is affected by cultivar, maturity, processing and geographic area of origin (Rommel and others 1993; Wang and others 2000).

Persimmons have been studied and used in Asian countries for centuries. They have been found to contain high amounts of biologically active compounds that have been associated with various health benefits.

2.1.2. Total Phenolics and Anthocyanins in Berries Species

After investigated 87 highbush blueberries (*Vacciunium corymbosum L.)* and species-introgressed highbush blueberry cultivars, Ehlenfeldt and other (2001) reported that the total content of phenolics in the fruit ranged between 430-1990 mg/kg of fresh weight expressed in gallic acid equivalents. The total anthocyanin contents ranged from 890-3310mg/kg fresh weight

expressed as cyanidin-3-glucoside equivalents. Sellapan and others (2002) investigated various Georgia-grown rabbiteye blueberry cultivars (*Vaccinium ashei Reade*) and reported that total phenolics in these blueberries were between 127 – 1973.4 mg/kg fresh weight expressed in gallic acid equivalents and total anthocyanins were between 2700.2 - 6690.1 mg/kg fresh weight expressed in cyanidin-3-glucoside equivalents. Gallic, caffeic, p-coumaric, ferulic and ellagic acids were identified in rabbiteye and Northern highbush blueberries (*V. corymbosum L.*). Also, Sahidi (2004) found rabbiteye blueberries contained higher levels of catechin (145.3 – 3874.8 mg/kg fresh weight) than southern highbush (*V. darrowii*) blueberries (98.7 292.8mg/kg fresh weight).

The composition of black raspberry anthocyanins has been extensively studied. The anthocyanins that have been previously identified and quantified include: cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-rutinoside, cyanidin 3-xylosylrutinoside and pelargonidin 3-rutinosdie (Torre and others 1977; Tian and others 2006). Cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside are the predominant anthocyanins, comprising 24-40 and 49-58% of total anthocyanins in black raspberries (Tulio and others 2008). The total anthocyanin in black raspberries has been reported to be about 228 ±54 mg/kg of fresh weight and as high as 1770mg/100g of freeze-dried black raspberries (Wang and others 2000; Harris and others 2001). The total phenolics in black raspberry have also been reported to be 1900±35 mg/kg fresh weight expressed in gallic acid equivalents (Wang and others 2000). Gallic acid, protocatechuic acid, p-Coumaric acid, ferulic acid, and hydrobenzoic acid are the phenolic acids that have been identified in black raspberries (Liu and others 2005; Wu and others 2009).

2.1.3. Health Benefits of Berries

Blueberries are rich in phenolics and anthocyanins as discussed in the previous section. Berry phenolics and anthocyanins are widely known to improve human health because of their high antioxidant properties. Intake of anthocyanins from black currents resulted in significantly improved night adaptation in human subjects, and similar benefits were also observed after administration of anthocyanins from bilberries (Nakaishi and others 2000; Muth and others 2000). *In vivo* and *in vitro* studies have shown that anthocyanins can reduce cancer cell proliferation, inhibit tumorigenesis, reduce inflammation and enhance capillary strength (Koide and others 1997; Folts 1998; Hou 2003; Kang and others 2003). Other phytochemical compounds from blueberries can reduce cancer risk by inhibiting cancer cell proliferation and inducing apoptosis (Yi 2005; Seeram 2006). Consumption of blueberries can promote cardiovascular health by lowering blood cholesterol and lipid level (Kalt and others 2008).

Black raspberries have been extensively investigated and reported to be the chemopreventive dietary constituents. Liu and others (2005) reported that black raspberry extract and fractions contain angiogenesis inhibitors that can potentially inhibit tumor growth. Consumption of black raspberries can help to prevent cancer development in colon, esophagus, and liver (Harris and others 2001; Kresty and others 2001; Reen and others 2006).

In summary, the phenolics and anthocyanins in blueberries and black raspberries prevent proliferation of cancer cells, exhibit antioxidant effects and many other biochemical functions, such as regulating the activities of metabolizing enzymes, repairing DNA oxidative damage and modulating nuclear receptors, gene expression and subcellular signaling pathway (Seeram 2008).

2.1.4 Phenolics in Persimmons

Persimmon is one of the most popular fruit crops in Asian countries. China, Korea and Japan are the leading world persimmons producers, with production of approximately 2.5 million, 0.5 million, and 250,000 metric tons, respectively, in 2008 (FAOSTAT 2011). Persimmon is generally categorized into two categories: astringent and non-astringent. The major difference between astringent and non-astringent persimmons is that astringent persimmons contain large amounts of soluble tannins at maturity (Macheix and others 1990; Suzuki and others 2005) while non-astringent persimmons do not. The pulp of the astringent persimmon is almost unpalatable before maturity but that of the non-astringent persimmon is sweet even when the fruit is still firm. Persimmons contain a rich source of nutrients such as ascorbic acid, condensed tannins, carotenoids, and other polyphenols that are beneficial to human health (Homnava and others 1990; Gorinstein and others 1994; Gu and others 2008).

Persimmons are known to contain high amounts of phenolic compounds such as p-coumaric (425-615µg/g), gallic acid (159-221µg/g), protocatechuic (63-241 µg/g), proanthocyanidins and tannins consisted of catechin (0.8-3.33mg/100g dry weight), epicatechin (0.5-1.5mg/100g dry weight), epigallocatechin (0.45-2.25mg/100g dry weight), catechin-gallate, gallocatechin and gallocatechin-gallate (Matsuo and other 1978; Haslam and other 1988; Gorinstein and others 1994; Suzuki and others 2005).

2.1.5. Health Benefits of Persimmons

Several researchers have reported the potential health benefits of persimmon due to its high antioxidant properties. Studies show that persimmons possess antitumor and multidrug resistance reversal properties (Kawase and others 2003), antidiabetic effects (Lee and others 2006), hypocholesterolemic and antioxidant effects (Gorinstein and others 1998), and prevent the rise in

plasma lipids (Matsumoto and others 2006). Persimmon vinegar also exhibits antitumor effects (Mishima and others 2000), antioxidative effects (Takeshita and others 2007) and prevents metabolic disorders induced by chronic alcohol administration (Moon and others 2008). Two flavonol glucosides, isolated from persimmon, have been shown to have a hypotensive action in rats (Funayama and other 1979).

2.2 Anthocyanins

2.2.1. Introduction

Anthocyanins constitute a special class of flavonoids. They are water-soluble pigments that give most plants the red, purple, and blue color. Blackberries, blueberries, red and black raspberries, bilberries, cherries, currents, grape, pomegranates and cranberries contain substantial anthocyanin. However, anthocyanins are not present in certain other red fruits, such as tomato and hot pepper, in which carotenoids are the predominant pigments. The total content of anthocyanins vary among fruits depending upon, fruit cultivar, growing temperature and light, pulp pH, sugar content, and the presence of enzymes, ascorbic acid, oxygen, condensation products, metals, and copigmentation (Francis 1989; Sahidi and others 2004).

2.2.2 Chemical Structures and Properties

About 260 anthocyanins have been identified and approximately 70 have been found in fruits (Sahidi and others; 2004; Francis 1989). Six major anthocyanidins are commonly found in nature: (1) pelargonidin, (2) cyanidin, (3) peonidin, (4) delphinidin, (5) petunidin and (6) malvidin. Of these, cyanidin is the most common anthocyanidin (Heywood 1972).

Anthocyanins are glycosides of anthocyanidins with a typical A-ring benzoyl and B-ring hydroxycinnamoyl as shown in Figure 2.1 (Harborne 1989). In other words, they are

hydroxylated and methoxylated derivatives of phenyl-2-benzopyrylium (flavylium salt structure, Figure 2.1). Anthocyanin molecules usually consist of an aglycone base on the flavylium nucleus, a group of sugars and, sometimes, a group of acyl acids (Francis 1989). They are cations in acid media and their structure is stabilized by resonance with many mesomeric forms (Macheix and others 1990). Table 2.1 shows the structures of some naturally occurring anthocyanidins.

Anthocyanins are unstable compounds. Their stability depends on many factors such as pH, heat, storage temperature, contaminants, etc. Loss of color can occur during food processing of plant products. Among the factors mentioned above, anthocyanins are especially sensitive to pH. Anthocyanins exist in four different forms in solution: neutral or ionized quinonoidal base, flavylium cation or oxonium salt, the colorless pseudobase and chalcone.

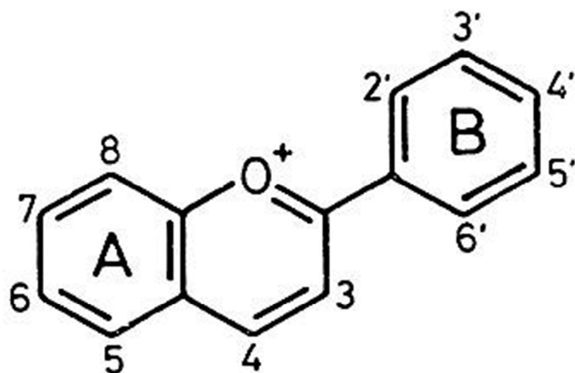


Figure 2.1. Basic structure of anthocyanindins
Source: Harborne 1989

Table 2.1. Structures of naturally occuring anthocyanidins.

	Substitution pattern[a]						
Anthocyanidin	**3**	**5**	**6**	**7**	**3'**	**4'**	**5'**
Common Structures							
Pelargonidin	OH	OH	H	OH	H	OH	H
Cyanidin	OH	OH	H	OH	OH	OH	H
Peonidin	OH	OH	H	OH	OMe	OH	H
Delphinidin	OH	OH	H	OH	OH	OH	OH
Petunidin	OH	OH	H	OH	OMe	OH	OH
Malvidin	OH	OH	H	OH	OMe	OH	OMe
Rarer Structures							
Aurantinidin	OH	OH	OH	OH	H	OH	H
6-Hydroxycyanidin	OH	OH	OH	OH	OH	OH	H
5-Methylcyanidin	OH	OMe	H	OH	OH	OH	H
Rosinidin	OH	OH	H	OMe	OMe	OH	H
Pulchellidin	OH	OMe	H	OH	OH	OH	OH
Europinidin	OH	OMe	H	OH	OMe	OH	OH
Hirsutidin	OH	OH	H	OMe	OMe	OH	OMe
Capensinidin	OH	OMe	H	OH	OMe	OH	OMe

[a] Numbering according to the anthocyanidin C-Numbering system in the structures scheme

Source: Harborne 1989

Figure 2.2 shows the pH effect on the predominant forms of anthocyanins. In terms of visual color, the flavylium cation which appears red is the most important. Most intense red color of anthocyanins occurs between pH 1 – 3 under the equilibrium condition between the flavylium cation and colorless carbinol base. This is the reason most anthocyanin colorants can only be used under pH 4

(Shahidi 2004; Francis 1989).

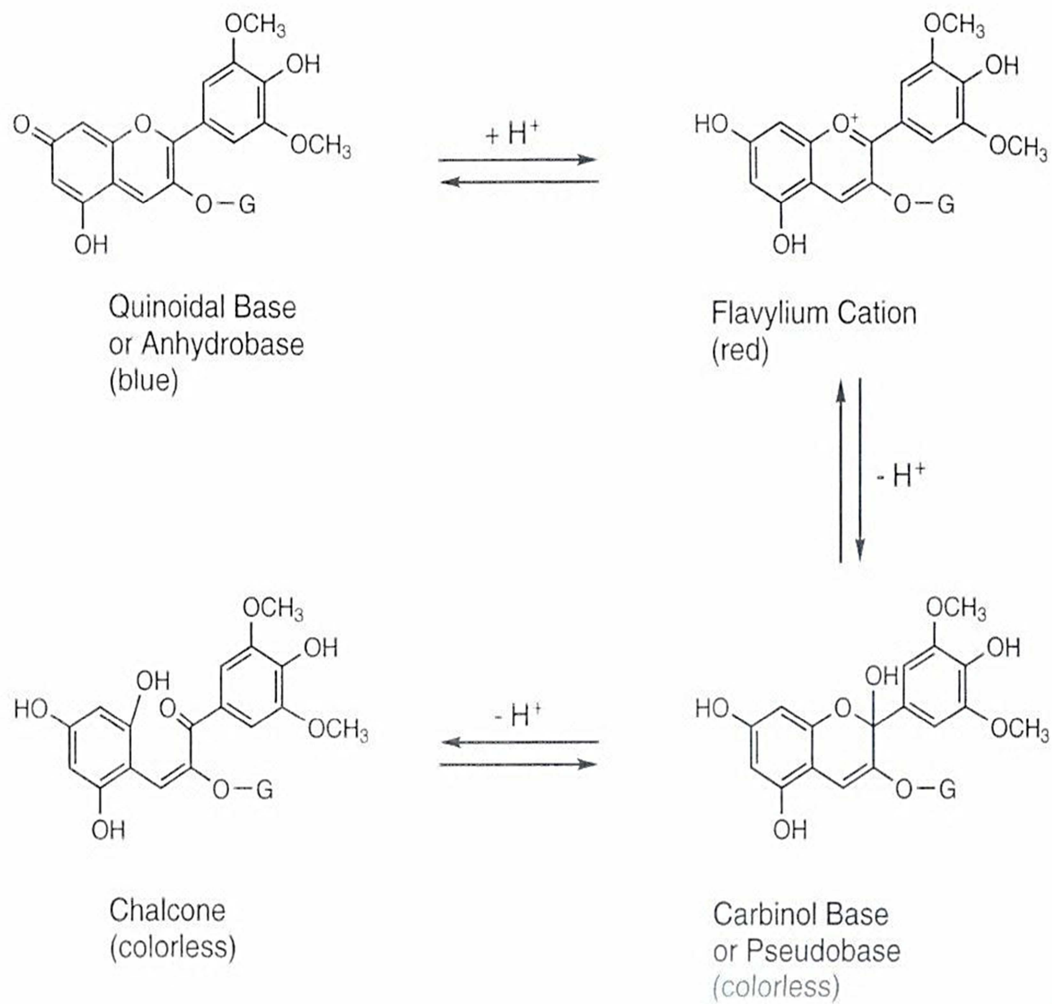


Figure 2.2 Chemical structures of anthocyanin chromophores.
Source: Shahidi 200

2.3. Wine

2.3.1. Background

Wine is an alcoholic beverage made from fermented juices, typically grape juice. The history of wine can be traced back 7,500 years ago, with the earliest residues of wine dating from early to mid-fifth millennium B.C. (McGovern and others 1996). Many researchers believe that the

discovery and development of wine originated in southern Caucasia, which includes present-day northwestern Turkey, northern Iraq, Azerbaijian and Georgia. Most ancient and medieval wine resemble today's dry to semidry table wine. In these early years, wines turned vinegary by spring because the knowledge regarding methods of protecting wine from oxidation and microbes was scarce. Therefore, prolonged shelf life of wine was rare in ancient times. Nevertheless, the modern expression of wine started in the seventeenth century when the use of sulfur as a disinfectant in barrel treatment was recognized and widely adapted by the wine makers. This innovation increased the shelf life and quality of wine, allowing us to enjoy a wide variety of wine throughout the year (Jackson 2000).

Many types of wines are available today. However, there is no generally accepted classification system for wine. They may be categorized by alcohol content, sweetness, carbon dioxide content, grape variety, fermentation or maturation process, and geographic origin. Wines can also be divided into categories such as still table wine, sparkling wines and fortified wine for taxation purposes. Table wine has 9-14% alcohol content while fortified wine has 17 – 22% alcohol by volume (Jackson 2000).

Wine is consumed in many countries worldwide. However, Europe is the region that produces and consumes the largest amount of wine by volume in the world. According to the Wine Institute (2005), Unites States, Italy, France and Spain are the top wine producers while France, Italy and United States were the top three wine consumers in 2008. Statistics on wine production and consumption for several countries are given in Figure 2.3.

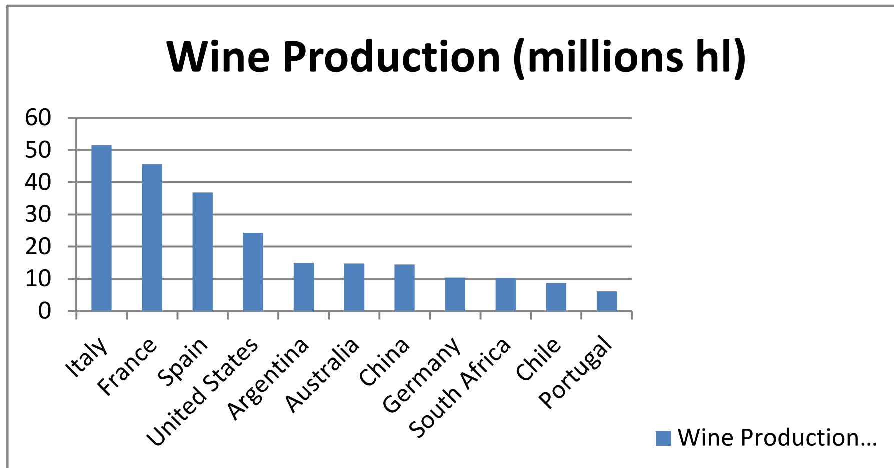
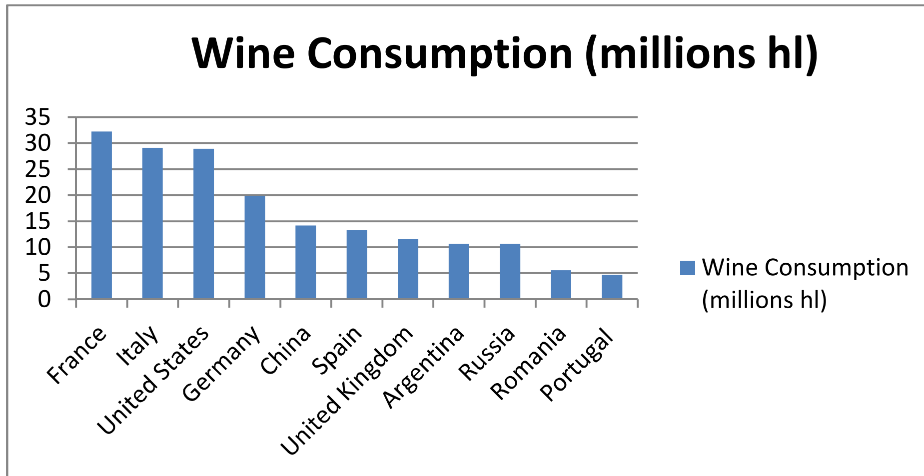


Figure 2.3. World wine production and wine consumption countries (2008).
Source: Wine Institute (2010)

Continued from Figure 2.3.



The process of wine making or vinification incurs multiple steps of fermentation that is monitored in a well-controlled environment. Primary fermentation (alcohol fermentation) and secondary fermentation (malolactic fermentation) are the two major processes in wine making. During primary fermentation, sugars in fruit must are rapidly transformed into alcohol by yeasts. Most of the sugars are converted into alcohol in the primary stage. Secondary fermentation takes

place anaerobically often in different fermenting barrels. The principal effect of secondary fermentation is the reduction in acidity since lower acidity gives wine a desirable “mellow” and “fat” characteristics (Peynaud 1984).

2.3.2. Wine Fermentation

2.3.2.1. Alcoholic Fermentation – Primary Fermentation

Alcoholic fermentation is the primary stage of the wine fermentation process where sugar is converted to alcohol and carbon dioxide by yeasts. Gay-Lussac gave a chemical formula to explain the reaction:

Sugar (100) = Alcohol (51.34) + Carbon Dioxide (48.66)

However, Pasteur later showed that the Gay-Lussac equation is only valid for 90% of the sugar transformed because the rest is converted into other organic compounds such as glycerol, succinic acid, and acetic acid. More research was conducted and other by-products were identified in the reaction: lactic acid, butyleneglycol, acetaldehyde, pyruvic acid, higher alcohols, ethyl acetate and some other substances present in minute quantities (Peynaud 1984).

Yeasts are very important in the alcoholic fermentation because they play an active role in converting sugar into alcohol. Therefore, several important factors, such as temperature, air and nutrients, must be controlled and monitored in order to keep yeasts alive.

2.3.2.1.1. Yeasts

Yeasts are a collection of fungi that possess a particular unicellular growth habit. Yeasts in grape wine fermentation can be found in many shapes: elliptical or ovoid, elongate, spherical and apiculate. They reproduce in two ways: (1) vegetative reproduction by budding (2) formation of spores, which produces more yeasts after germination (Peynaud 1984; Ruf 2003).

During natural fermentation of grapes, different species of yeasts predominate over each other at different stages. For example, apiculate start the fermentation in sulfited must and produce the initial alcohol. However, apiculate yeasts can only tolerate 3% - 4% alcohol, so *Saccharomyces ellipsoideus* quickly takes over at this time and, by mid-fermentation, the initiation yeasts will have disappeared. Toward the end of fermentation when higher alcohol content is achieved, *Saccharomyces oviformis*, the less sensitive yeast becomes predominant and can tolerate up to 18% of alcohol (Peynaud 1984).

Temperature, air and nutrients are the three major factors that affect the growth of yeasts. Temperature is the predominant factor that influences the yeast metabolism. Yeasts only develop properly in a narrow range covering a maximum of twenty degrees. At warmer temperatures (>20°C), the cells rapidly decline at the end of the fermentation; at colder temperatures (<14°C), the start of fermentation is almost impossible. Yeasts require air or oxygen initially to multiply. Without oxygen, they only reproduce a few generations and their growth stops. Also, yeasts need oxygen biologically to synthesize sterol and to assimilate fatty acids with long chain molecules which they need. Therefore, oxygen is of great importance for sterol synthesis and the initiation of fermentation. In most fruit, yeasts normally have abundant sugars during fermentation as a carbon source. However, their need for nitrogen is poorly satisfied because of the limited resources in many common fruit musts. Yeasts consist of 25-60% nitrogenous substances. They need nitrogen to reproduce and form new cells. Often the addition of nitrogenous substances such as ammonium nitrate into the fermentation process is vital for the survival, growth and multiplication of yeasts (Peynaud 1984).

2.3.2.2. Malolactic Fermentation – Secondary Fermentation

Malolactic fermentation is the secondary fermentation process following alcoholic fermentation in grape wine. Malolactic fermentation is also called the concluding or refining fermentation. It is the biochemical deacidification or degradation of malic and other acids. This fermentation process promotes the quality and improves microbial stability of grape wine. It appears to be the critical point that distinguishes the production of good wine from the production of less premium wine (Peynaud 1984).

2.3.2.2.1. Lactic Acid Bacteria

Lactic acid bacteria play an important role in malolactic fermentation. They provoke the reduction of malic acid to lactic acid, creating a less acidic and better drinkability characteristic in grape wine. Lactic acid bacteria can be found in various shapes which include round or oval, pelleted, long or short and sometimes sinuous. The genera of wine bacteria are *Leuconostoc* (heterofermentive cocci), *Pediococcus* (homofermentive cocci), and *Lactobacillus* (bacilli). There are two basic kinds of lactic acid bacteria in wine. The first type is desirable and predominantly decomposes malic acid and, secondarily, sugar, citric acid but not tartaric acid and glycerol. They are the normal malolactic fermentation agents and form only few volatile acids. The second type is the noxious bacteria that decompose pentoses, tartaric acid and glycerol. They raise volatile acidity and usually cause wine spoilage.

2.3.2.2.2. Effects of Malolactic Fermentation

The effects of malolactic fermentation include reduction of acidity, influencing stability and affecting sensory characteristics of the wine (Peynaud 1984; Ruf 2003).

Deacidification is the principal effect of malolactic fermentation. The reduction in acidity and rise in pH increase the smoothness and drinkability of wine, making the wine more supple. The

reduction in total acidity in grape wine is not explained by the tartaric precipitation during clarification but by the disappearance of malic acid. With the disappearance of malic acid, the level of lactic acid increases and the acidity decreases because malic acid possesses two acid functions but lactic acid carries only one. This explains the decrease in acidity when malic acid is transformed into lactic acid by lactic acid bacteria. The following shows the overall reaction of malolactic fermentation (Peynaud 1984):

Malic acid (1g) = Lactic acid (0.67g) + Carbon dioxide (0.33g)

During malolactic fermentation, malic and citric acids are consumed. The more microbially stable tartaric and lactic acids are the only acids left after this fermentation process. Thus, secondary fermentation is thought to promote microbial stability because of the production of more stable tartaric and lactic acids. On the other hand, malolactic fermentation can actually decrease microbial stability when the initial pH of the must is high. Spoilage organisms will start growing above pH 3.5. Therefore, it is important to keep the initial fermentation pH under 3.5 to prevent undesirable growth of spoilage microbes (Ruf 2003).

Deacidification decreases the acidity of the wine by replacing the malic acid that is more aggressive on the tongue with the lactic acid that is milder and more palate-pleasing. Other than the acids, many different volatiles are synthesized during fermentation. For example, diacetyl compounds that are formed under an appropriate environment often provide a desirable fragrance to the wine at the threshold of 1-4mg/liter. Above this threshold, the fragrance is too strong and is often considered as an undesirable off-odor. Temperature, pH, and variability of lactic acid bacteria are the factors that influence the taste of the wine. Different fermenting temperatures, variable pH and species of lactic acid bacteria will create different volatiles thereby influencing the sensory characteristics of wine. For instance, *Leoconostoc oenos* often predominates during

malolactic fermention at pH under 3.5 and this lactic acid bacteria species is less likely to produce undesirable off-odors (Ruf 2003).

2.3.3. Health Benefits of Wine

Excessive alcohol consumption (alcoholism) has long been associated with various detrimental health effects such as liver damage, hypertension, stroke, digestive tract cancers, fetal alcohol syndrome. However, recent studies have concluded that moderate wine consumption could lower the risk of all-cause mortality by 20-30% (Ruf 2003).

Phenolic compounds are an important constituent in wine. Not only do they contribute to the sensory characteristics such as flavor, color and astringency in wine, they also provide some potential health benefits to the consumers. The composition of phenolics in wine varies among the types and cultivars of the fruits used, the extraction methods and the vinification processes. For instance, longer primary fermentation favors greater extraction of phenolics from the fruit by the ethanol produced during the fermentation process. In general, young wine contains low to medium molecular weight phenolics while aged wine contains relatively higher molecular weight of polymerized phenolic compounds (Sahidi and others 2004).

The medicinal uses of wine can be dated back to the ancient Egyptians. Even though wine had been widely used for medicinal purposes for thousands of years, the benefits of wine consumption was reviewed and investigated in the late 1900s because of the serious alcoholism at that period of time. It was not until recently that scientists became interested in the medicinal uses of wine and started to investigate the potential health benefits in depth. Phenolic compounds in wine are the major components that have been extensively studied by scientists for potential health benefits such as cancer and heart disease etc. Scientists have concluded that the antioxidant effects of wine phenolics have a positive influence in preventing various chronic

diseases, for instance limiting LDL oxidation and improving lipoprotein metabolism thereby reduce the risk of heart disease (Maxwell and others 1994; German and other 2000). Wallerath and others (2002) reported that resveratrol, which is a polyphenolic compound in red wine, accounted for the cardiovascular protective effects *in vivo*. Recent studies have also shown that moderate consumption of wine could significantly reduce various expressions of cardiovascular disease such as hypertension, stroke, and heart attack (Sahidi and others 2004; Corder and others 2006). Ebeler and others (1996) have also shown that while moderate consumption of wine can reduce the risk of certain cancers, excessive consumption can cause cancers. In short, moderate consumption of wine is encouraged for the potential health benefits.

2.4. Vinegar

2.4.1. Background

Vinegar is defined by Cruess (1958) as a condiment made from starchy and sugary materials obtained by alcoholic and subsequent acetic fermentation. Vinegar has been in the human diet for thousands of years. The earliest descriptions of vinegar were made by Babylonians in about 5000 BC. The Babylonians used dates, which are high in sugar content, to make their first vinegar. The sugar in dates can be turned first into alcohol and then into vinegar. Besides use as a condiment, vinegar was also prescribed by Hippocrates, "the father of modern medicine", for all kinds of ailments in Greece (Diggs 2000).

Vinegar is a popular condiment, which is used as a flavoring ingredient and preservative in cooking and food processing. Many types of vinegar can be produced using various types of source materials such as rice, fruit musts, wine (red and white), barley, distilled alcohols, etc. Vinegar plays an important role in salad dressings, pickles, ketchup, hot sauces, mayonnaise and other sauces. Acetic acid is the predominant flavoring ingredient and antimicrobial component in

vinegar. As described by Diggs (2000), from a chemical standpoint, vinegar is, in fact, nothing but a weak acid with small amounts of soluble extractives and mineral salts obtained from the raw materials. These extractives and salts give the vinegar its distinctive flavor and quality. Therefore, different kinds of vinegars have their own flavor and characteristics.

2.4.2. Acetification Process

Acetification is the fermentation process where alcohol is converted into vinegar by acetic acid bacteria in the presence of air. *Acetobacter*, or so called acetic acid bacteria, are the major bacteria involved in the acetification process. The methods of producing vinegar range from the traditional to the most current industrialized methods. To date, three types of acetification processes, which include the Orleans process, submerged fermentation and generator fermentation, are used to produce many types of vinegar (Morales and others 2001). The Orleans method, which is also called the slow process, is the oldest way of producing vinegar. The generator process and the submerged culture acetification are the quick processes that are used for commercial vinegar production today (Tan 2005). Details of the Orleans process, submerged fermentation and generator fermentation will be discussed later in sections 2.4.3.2 to 2.4.3.3 respectively.

2.4.2.1. Acetic Acid Bacteria

Vinegar is primarily made by acetic acid bacteria called *Acetobacters*. *Acetobacters* produce vinegar by converting alcohol, which is produced by yeasts, to acetic acid. These microorganisms are biologically defined as aerobic and non-sporiferous microbes. They are gram negative, pleomorphic and reproduce by duplicating themselves. They are usually rod-shaped but they also exist in round, thread and other forms. *Acetobacters* are motile and often use their

flagella as the mean to move. However, their motility depends on age, oxygen supply, medium, etc. (Gonzalez and others 2004; Diggs 2000).

Figure 2.4. Acetic acid bacteria.
Source: Vinegar Connoisseurs International.

2.4.2.2. Mother of Vinegar

Mother of vinegar is also called the zoogloea, vinegar bees, vinegar mat and mycoderma. It appears as a mat floating at the top of the fermenting vinegar when using the "Orleans" method. It can be either thick or thin and the surface may be smooth or textured. The colors vary from transparent to dark brown to black. The mother of vinegar is composed of cellulose and the acetic acid bacteria. It is useful in converting the alcohol into acetic acid; however, it is sometimes undesirable in commercial production because it clogs up machinery and slows down the process (Diggs 2000).

2.4.3. Understanding the Vinegar Making Process

Vinegar making is a biological process where carbohydrate is turned into acetic acid through a multi-step fermentation processes. In general, a double fermentative process (Figure 2.5) is used, which starts with a sugar source which is converted first to ethyl alcohol and then to acetic acid. However, in some cases, a triple fermentative process (Figure 2.6) is used such as in rice wine

vinegar, where the rice starch is first broken down into fermentable sugars which are then fermented to alcohol and then to acetic acid or vinegar.

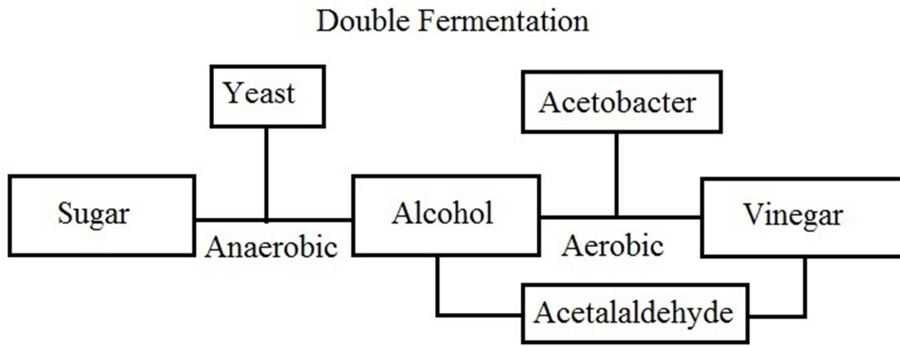


Figure 2.5. Double Fermentation

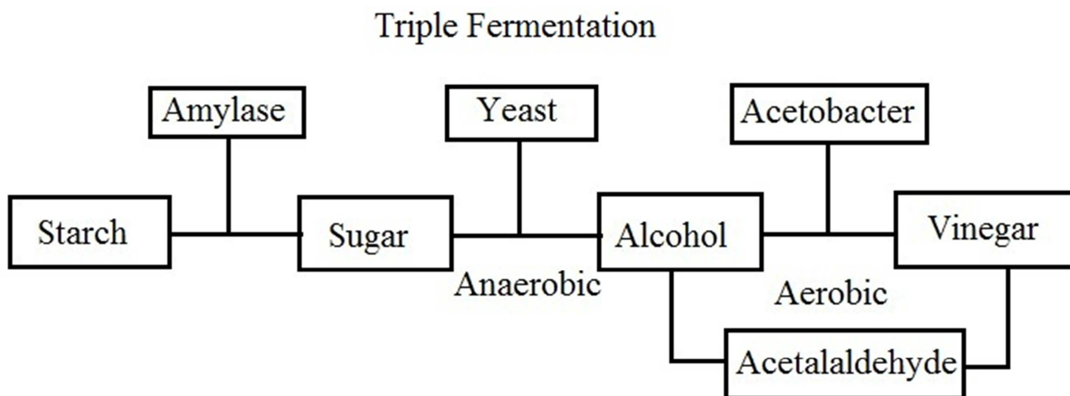


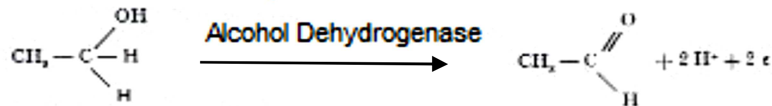
Figure 2.6. Triple Fermentation.

Double or triple fermentation involve a three-step or four-step reaction involving the conversion of starch to sugar by enzymes, conversion of sugar to ethanol by yeast fermentation under anaerobic conditions, conversion of ethanol to hydrated acetaldehyde, and finally dehydrogenation to acetic acid by aldehyde dehydrogenase and the aid of *Acetobacter* under aerobic conditions (Nichol 1979; Canning 1985). Kehrer (1921) established the detailed

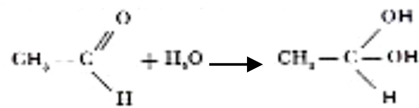
acetification reactions as shown below (Figure 2.7).

Theoretically, every 100 parts of sugar will produce approximately 50 – 55 parts of acetic acid under favorable conditions. In other words, to achieve 5.2% acetic acid, the fermentation should start with a 10% sugar solution (Diggs 2000). The remaining sugar metabolites are either lost through volatilization or converted to other compounds (Ghommidh and others 1986).

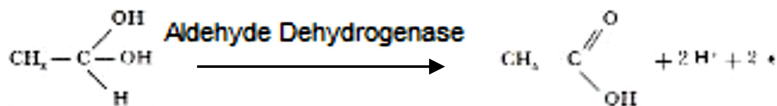
1. Formation of Acetaldehyde



2. Hydration of Acetaldehyde



3. Foramtion of Acetic Acid



4. Electron Transfer

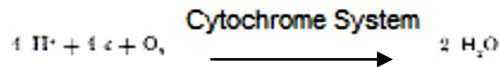


Figure 2.7. Conversion of alcohol to acetic acid reaction.
Source: Kehrer 1921

2.4.3.1. Orleans Process

The Orleans process is the slowest and oldest method. It is named after the French city famous for creating good quality vinegars for many generations. The Orleans process is also the best method of producing a good quality vinegar in large quantity compared to submerged fermentation and generator fermentation (Diggs 2000). In this process, a wine solution greater

than 5% alcohol is used for successful acetification. Phosphates and nitrogeneous nutrients must be added to the mash if the alcohol content in the fermenting liquid is less than 5% (Mitchell 1916). Wood barrels are usually used as the converter in this process and the alcoholic fermenting liquid is filled to approximately ¾ full of the barrel. Holes are drilled at the end of the barrel. They are left opened for air exposure but are covered with a screen to avoid flies and other insects. About 20-25% of fresh vinegar is added to the fermenting liquid to increase the acidity strength that provides optimum growth for the acetic acid bacteria. The mash is then fermented in the wood barrel at 21°C - 29°C for about 1 – 3 months. In this period, a slimy, leathery and gelatinous layer of “mother of vinegar” is formed at the top of the liquid (Peppler and others 1967). About 1/3 of the vinegar is drawn for bottling purpose and an equivalent amount of alcohol liquid is added back to the fermenting liquid (Cruess 1958).

2.4.3.2. Generator Fermentation

Generator fermentation, also called quick process, was developed by the German chemist Schutzenbach in 1832. Vinegar generators are large barrel like containers made in different sizes, shapes and wood materials. The generators have a false bottom with holes, designed for the entrance of air. Near the top of the generator, is a false top or perforated plate with a rotating sparger that provides uniform distribution of the vinegar stock. In this process, wood shavings are usually used as a packed bed media where the acetic acid bacteria can grow and form a thick slimy layer around the wood shavings (Peppler and others 1967). The re-circulated fermenting liquid trickles toward the bottom of the barrel while the air moves upward toward the top of the generator. The process takes about 3-7 days under the optimum temperature of 29°C - 32°C. Upon completion, only 2/3 of the vinegar is drawn for bottling and an equivalent amount of the mash is added back to the generator (Hickey and Vaughn 1954; Diggs 2000; Tan 2005).

2.4.3.3. Submerged Fermentation

Submerged fermentation is the most common vinegar-making method in commercial practice today (Hickey and others 1954). In this process, the fermenting liquid is constantly stirred and aerated for the optimum growth of the acetic acid bacteria (DeLey and others 1984). The temperature in the fermenter is also controlled and maintained at its optimum level by refrigeration. It works more efficiently than generator fermentation because of the improved aeration, stirring and temperature control. Semi-continuous is a typical operation mode in the industrial scale. In this operation, a certain volume of the vinegar is discharged and a like amount of ethanolic mash is replenished at the end of each cycle (De Ory and others 2004).

2.4.4. Health Benefits of Vinegar

Vinegar has been used as a medicine since ancient times. Hippocrates, the "father of modern medicine", prescribed vinegar as an antibiotic to treat patients. He also told his patients that oxymel (a combination of honey and vinegar) was a good remedy to clear up phlegm and congestion. Hippocrates also prescribed it for other ailments such as respiratory disorders and others (Orey 2009). Even though many medicinal properties have been ascribed to vinegar over thousands of years, very few of these properties have been scientifically proven through medical trials to be effective for human health.

Today, the most common medical research about vinegar includes blood glucose control or diabetic management, possible cholesterol and triacyglycerol effects and antitumor activity. According to Ostman and others (2005), vinegar could lower glucose and insulin responses in healthy subjects. The Fushimi (2006) research group reported that dietary acetic acid can reduce serum cholesterol and triacyglycerols in rats fed with a cholesterol-rich diet. Shimoji and others

(2004) also showed that vinegar made from unpolished rice can inhibit Azoxymethane-induced colon carcinogenesis in rodents.

CHAPTER 3. MATERIALS AND METHODS

3.1. Wine Fermentation (Alcohol Fermentation)

Primary fermentation and secondary fermentation are two major fermentation steps in wine-making process. Primary fermentation, also called alcoholic fermentation, focuses on the multiplication of yeasts and the conversion of sugar to alcohol. Temperature, aeration, yeasts nutritional needs, and acidity are the factors that influence the quality of wine during primary fermentation. The optimum temperature for fermentation of red wine is between 26°C to 30°C. For making white wine, a lower temperature is preferred, in the range of 18°C to 20°C. Appropriate oxygen and nitrogenous supply are important for the multiplication of the yeasts. The optimum pH should be controlled between pH 3 to 4. Secondary fermentation or malolactic fermentation is another important fermentation step continued after primary fermentation. The principle effect of malolactic fermentation is to reduce the acidity level while improving the drinkability and quality of wine. The conditions for malolactic fermentation include the influence of pH, temperature, ethanol and other chemical and biological factors. During secondary fermentation, the preferable pH and temperature are between pH 3 to 4 and 20°C to 25°C (Peynaud 1984; Ruf 2003).

3.2. Vinegar Fermentation (Acetification)

The Orleans process, generator process and submerged acetification are the three main vinegar fermentation processes in the vinegar industry. The Orleans process is the most traditional and slowest process among these. It takes up to 3 months for the completion of a bath of vinegar. Nevertheless, it is also considered the best vinegar processing method for producing good quality vinegars. Compared to the Orleans process, the generator process and submerged acetification

are less time consuming. These processes take only 3-7 days under optimum conditions (Diggs 2000; Tan 2005).

In the persimmon and blueberry experiments, the Orleans process was chosen because it is the most non-destructive and natural method compared to the generator process and submerged acetification. Since we were interested in examining the anthocyanins profile and antioxidant properties of the persimmons and blueberries during the acetification process, it was preferable to eliminate any unnecessary ingredients and harsh processing methods. The generator process and submerged acetification both require a large amount of oxygen incorporation in a short period of time and special cooling, both of which may greatly influence the quality of the vinegar due to excessive oxidation; therefore, the Orleans process was the most preferred method in this experiment. An overall process flow diagram can be seen in Figure 3.1.

3.3. Black Raspberries

3.3.1. Wine Fermentation Treated with Proteases

Frozen black raspberries were supplied by a grower in Washington state. The fruits were thawed overnight at 4°C and then ground into mash. Commercial proteases such as Neutral Bacterial Protease, Ficin, Liquipanol were obtained from EDC (New York, NY) and Bromelain, Acid Protease, Fungal Protease, Neutral Protease were obtained from Bio-Cat Inc. (Troy, VA). To screen for the proteases that had the potential to retain anthocyanin in black raspberry, proteases were mixed with black raspberry mash and incubated at 47° for 2 hours. Total selected anthocyanin was quantified by HPLC and the most effective proteases (top three) were chosen for the black raspberry fermentation experiment designed to remove sugar. The black raspberry mash was mixed thoroughly with 1%, 0.1% and 0.01% of Acid Protease and Neutral Bacterial

Protease. In a volumetric flask, appropriate amount of dry yeast (Lallemand Inc, Canada) and yeast energizer (L.D. Carlson, Ohio) were added to the treated black raspberry mash and the mixture was mixed thoroughly. The volumetric flask was then covered with cheese cloth and the mash was subjected to primary fermentation for 2 days under room temperature. After 2 days of fermentation, cheese cloth used to cover the volumetric flask was substituted with an air-lock and the mash was subjected to further fermentation at room temperature for another 5 days. Black raspberry wine for chemical analysis was obtained by squeezing and filtering the mash through four-layers of cheesecloth.

3.3.2. Determination of Total Selected Anthocyanin

An appropriate amount of wine obtained from each treated sample was diluted 20 times with methanol. An HPLC system was employed to isolate and quantify anthocyanins. The HPLC system consisted of a Supelco (Bellefonte, PA) Discovery C18 column (id 3 mm x 25 cm), a Waters 2690 separation module, a 996 photodiode array detector, and a Millennium32 chromatography manager. The mobile phase was a mixture of A: 10 % acetic acid in water and B: acetonitrile, with percentage of A: 10% acetic acid in water ramped from 100% to 55% in 45 min with a constant flow rate of 0.8 ml/min. The chromatogram obtained at a wavelength of 520 nm was used to quantify the anthocyanins. The total selected anthocyanins was the summation of the three detected peaks area, with the retention time 12.7, 13.2 and 15.2 minute, as shown in the HPLC chromatogram (see Appendix A and B). The concentration of total anthocyanin was calculated based on a standard curve.

PREVIEW OF ALCOHOL AND VINEGAR FERMENTATION

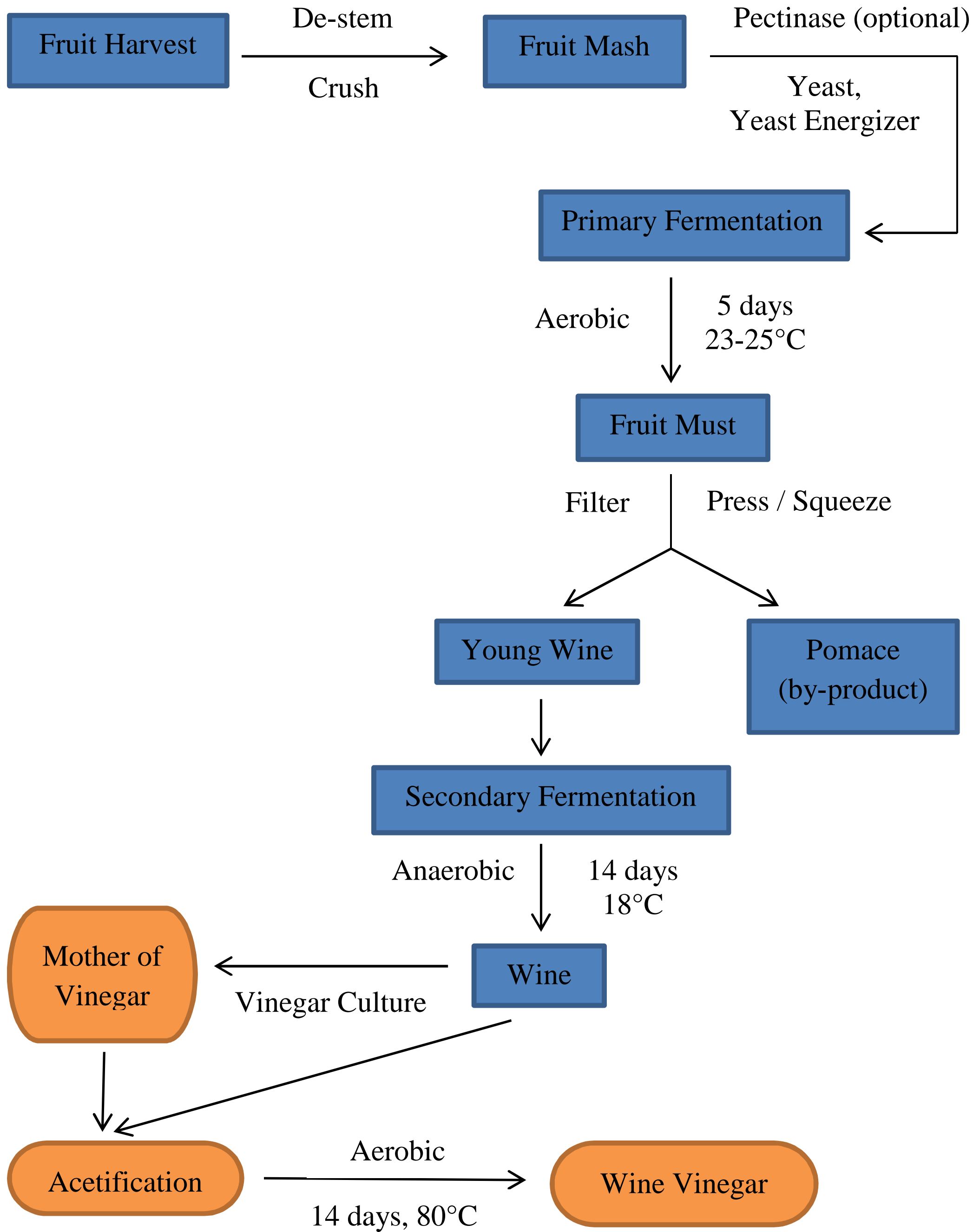


Figure 3.1. Alcohol fermentation and acetification flow chart

3.4. Persimmons

3.4.1. Alcoholic Fermentation - Primary Fermentation

Astringent (*Diospyros kaki c.v. Tamopan*) and non-astringent (*Diospyros kaki c.v. Fuyu Imoto*) persimmons were harvested from Burden Research Center, LSU Agcenter, Louisiana during Fall 2010. Figures 3.2 and 3.3 contain images of the persimmon cultivars used. The fruit were cleaned and sepals were removed. Persimmons were cut and crush into mash by a commercial grinder (W.J. Fitzpatrick Company, Chicago, IL).

Figure 3.2. Astringent persimmon tree (left) and fruit (right) grown at Burden Research Center, Louisiana.

Figure 3.3. Astringent and non-astringentpersimmon fruits.

The pH, titratable acidity and Brix of the fruit must were measured before primary fermentation. The appropriate amount of dry wine yeast (Lallemand Inc, Canada) and yeast energizer (L.D. Carlson, Ohio) were added to the persimmon mash, stirred and mixed thoroughly (Figure 3.4). The fermenting vessel was then covered with four-layers of cheesecloth and the must was fermented at room temperature (23-25°C) for 5 days.

Figure 3.4. Primary fermentation of astringent (left) and non-astringent (right) persimmon.

3.4.2. Malolactic Fermentation - Secondary Fermentation

After 5 days of primary fermentation, young persimmon wine was obtained by pressing and filtering the must through four-layered cheesecloth. The fermenting liquid was then transferred to a clean glass fermenting vessel and the opening of the flask was covered with an air-lock. The fermenting liquid was fermented at 18°C for another 2 weeks.

Figure 3.5. Secondary fermentation of astringent (left) and non-astringent (right) persimmon.

3.4.3. Vinegar Fermentation - Orleans Process

The initial pH, titratable acidity, total phenolics and total radical scavenging activities of persimmon wine were measured. An active mother of vinegar was produced in a 1000ml beaker (Figure 3.6) by adding 400ml of the persimmon wine mixed with the instructed amount of vinegar culture - “Natural Mother of Vinegar, white wine” (Beer & Winemaking Supplies Inc. Northampton, MA). The beaker was covered with a 4-layers of cheesecloth and then fermented under 24°C for 2 weeks or until the mother of the vinegar was formed as a floating layer at the top of the solution. The mother produced was then used to produce a new batch of vinegar by

transferring the mother to another 1000ml beaker and 400ml of persimmon wine was added. The beaker was again covered with a 4-layers of cheesecloth and then fermented under 24°C for 2 weeks or until the desired acidity was achieved. This batch of persimmon vinegar made with the in-house mother of vinegar was later analysed by several physicochemical analyses. All other vinegars were produced in like manner using a self generated mother of vinegar to prevent any possible interference that could later intervene with the analyses.

Figure 3.6. Acetification of astringent (left) and non-astringent (right) persimmon.

3.4.4. Physicochemical Analysis

Titratable acidity, pH, brix, and alcohol content are the important parameters in wine and vinegar fermentation process. These parameters are the critical factors that contribute to the quality of the wine and vinegar.

A spectrophotometer is a device that can measure the intensity of the lights at different wavelengths. Total phenolics can be quantified by spectrophotometer through the Folin-

Ciocalteau method. Free radical activity can also be determined by this device through DPPH radical scavenging assay.

3.4.4.1. pH, Titratable Acidity & Brix

Titratable acidity (TA) and pH were measured using an Orion EA920 pH meter and Thermo Orion 915600 (Orion, MA) pH probe. Titratable acidity was determined as ml of 0.1N NaOH used to obtain an endpoint of pH= 8.20. Malic acid is the major acid in fresh persimmon fruit and acetic acid is the major acid in persimmon vinegar (Daood and others 1991; Lee and others 2009). The formula to calculate %TA as malic acid and acetic is as below:

$$\%TA\ (\text{Malic Acid}) = \frac{\text{ml of NaOH x N of NaOH x 134.09}}{\text{10 x sample weight}}$$

$$\%TA\ (\text{Acetic Acid}) = \frac{\text{ml of NaOH x N of NaOH x 60.05}}{\text{10 x sample weight}}$$

Percentage of sugar (brix) was measured using BS RFM 80 digital refractometer (Bellingham Stanley Limited, England).

3.4.4.2. Total Phenolics

The total phenolics were measured by Folin-Ciocalteau microscale colorimetry method (Waterhouse 2002). A 20 µl of sample or standard solution (gallic acid) was mixed with 100 µl of Folin-Ciocalteau reagent (Sigma-Aldrich, St Louis, MO) and 1.58 ml of distilled water. The mixture was mixed thoroughly by vortex and incubated under room temperature for 5 minutes. Three hundred µl of sodium carbonate solution (200g in 800ml of distilled water) was then added to the mixture and the result was incubated at 40°C in a waterbath for 30 minute. Sample

was measured at 765 nm by Lambda 35 uv/vis spectrometer (Perkin Elmer,CT, USA). Standard curve was calculated using 0, 5, 10, 15, 25 and 50 mg/100ml gallic acid solutions. Results were expressed as mg gallic acid (GAE) per 100ml of sample (mg GAE/100ml). Figure 3.7 shows the intensity of the blue color is proportional to the concentration of total phenolics in samples.

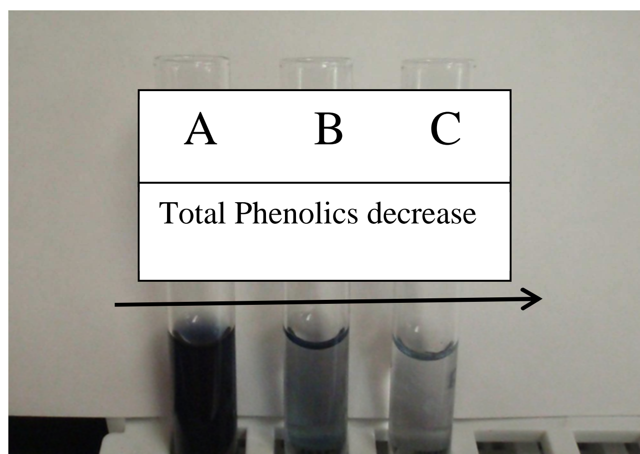


Figure 3.7. Folin-ciocalteau microscale colorimetry method. Test tube A has the highest amount of total phenolics while test tube C has the lowest.

3.4.4.3. Antiradical Activity

Free radical scavenging activity or antiradical activity was determined with DPPH (1,l-diphenyl-2-picrylhydrazil radical) assay. The antiradical activity was evaluated according to Burda and others (2001) and Heimler and others (2006) with slight modification. All products were diluted 5 times before preparing for analysis. A 20 µl of sample was mixed with 2 ml of DPPH solution (0.025g/l methanol). The mixture was then incubated under room temperature for 20 minutes. The absorbance was measured at 516 nm by Lambda 35 uv/vis spectrometer (Perkin Elmer,CT, USA). The antiradical activity was calculated as a percentage of DPPH solution decoloration versus methanol (blank). Figure 3.8 shows the color disintegration of the DPPH solution after 30 minutes. The lighter the color of the solution after 30 minutes of incubation, the more powerful is

the free radical scavenging activity.

% of Inhibition = {[Absorbance of DPPH solution at t = 0min - Absorbance of DPPH solution at t = 30min] / [Absorbance of DPPH solution at t = 0min] x 100

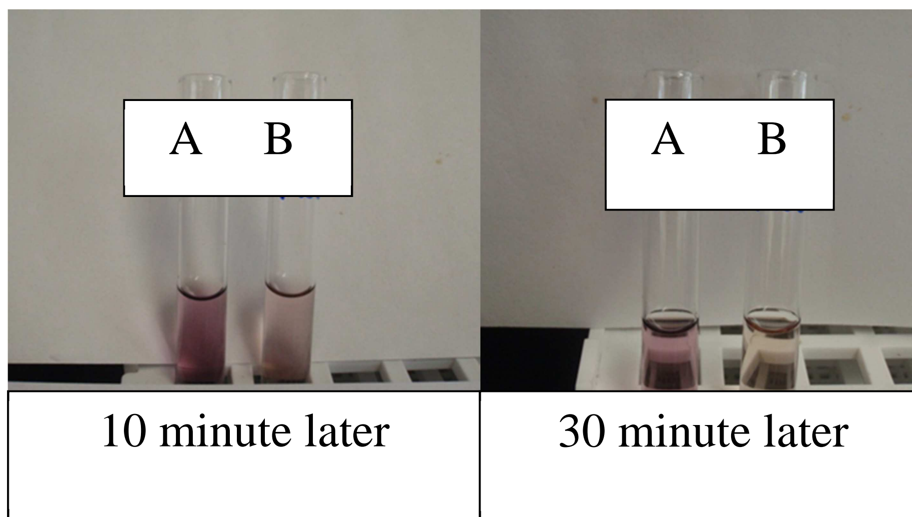


Figure 3.8. Free radical scavenging activity by DPPH solution.

3.5. Blueberries

3.5.1. Alcoholic Fermentation – Primary Fermentation

Fresh blueberries, *Vaccinium ashei*, (Tifblue Rabbiteye Blueberry) were harvested from Bob R. Jones-Idlewild Research Station, Louisiana, during the summer of 2010 (Figure 3.9). A stalk separator (BEI Incorporated, MI) was used to de-stalk the blueberries in bunches (Figure 3.10). Without any washing, the blueberries were packed into 1-gallon Ziploc® bags after de-stalking and kept frozen under -30°C until further use.

The frozen blueberries were thawed under 4°C overnight before proceeding to primary fermentation (Figure 3.11). After thawing, the blueberries were lightly crushed by hand and transferred to a plastic fermenting vessel. The pH, titratable acidity and brix of the fruit must were measured. The blueberry mash was subjected to primary fermentationas indicated in 3.4.1.

Figure 3.9. Tree (left) and fruits and Tifblue rabbiteye blueberry grown at Bob R. Jones - Idlewild Research Station, Louisiana.

Figure 3.10. Stalk separator (left) and the procedure of separating the branches and leaves from the fruits.

Figure 3.11. Primary fermentation of blueberries in a plastic fermenting vessel.

3.5.2. Malolactic Fermentation - Secondary Fermentation

After five days of primary fermentation, young blueberry wine was obtained by squeezing the must by hand and filtering through four-layered cheesecloth. Secondary fermentation (Figure 3.12) was performed as indicated in 3.4.2.

Figure 3.12. Secondary fermentation in glass fermenting vessel. Transferring and filtering blueberry must (left) into a cleaned glass fermenting vessel and the vessel was covered with an air-lock (right).

3.5.3. Vinegar Fermentation - Orleans Process

The initial pH, titratable acidity, anthocyanins, epicatechin, total phenolics and total radical scavenging activities of blueberry wine were measured. Blueberry vinegar (Figure 3.13) was made according to the method described in 3.2.3.

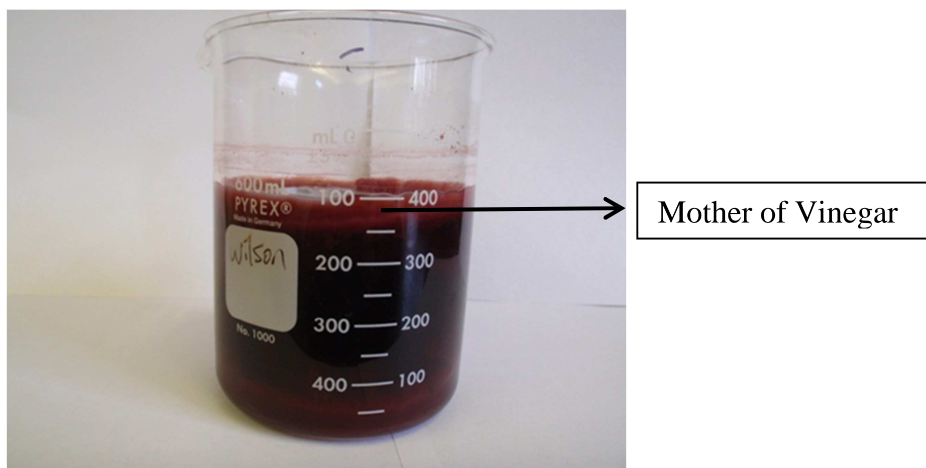


Figure 3.13. Acetification of blueberry wine in a beaker.

3.5.4. Physicochemical Analysis

High pressure liquid chromatography or high performance liquid chromatography (HPLC) is a commonly used chromatographic method that can separate, identify and quantify chemical compounds. The amount of anthocyanins and epicatechin in blueberries can be quantified by using HPLC.

Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), components of marine fish oil, are the essential omega-3 polyunsaturated fatty acids (PUFA) that provides profound health benefits such as preventing cardiovascular disease and cancer (Siddiqui and others 2004). However, the use of marine oil is limited by the oxidative susceptibility. Lipid oxidation is a major problem in food, neutraceutical and pharmaceutical industry. In this experiment, EPA and DHA in the salmon oil-in-water emulsion (SOE) were measured at 0 day and after 3 days to

observe the oxidative degradation effects. A gas chomatrograph with flame ionizing detector (GC-FID) was used to determine the EPA and DHA in SOE. Gas chromatography is a sensitive instrument that is commonly used in chemical analysis. It can effectively separate the compounds in the sample through the column and detect the compounds by its detector.

3.5.4.1. pH, Titratable Acidity & Brix

Titratable acidity (TA), pH and Brix were measured according to the method as indicated in 3.2.4.1. Malic acid is the major acid in blueberry (Ashurts 2005) and acetic acid is the major acid in blueberry vinegar.

The formula to calculate %TA as malic acid and acetic is as below:

$$\%TA\ (\text{Malic Acid}) = \frac{\text{ml of NaOH x N of NaOH x 134.09}}{\text{10 x sample weight}}$$

$$\%TA\ (\text{Acetic Acid}) = \frac{\text{ml of NaOH x N of NaOH x 60.05}}{\text{10 x sample weight}}$$

3.5.4.2. Total Selected Anthocyanins and Epicatechin Profile

Appropriate amount of sample were centrifuged and supernatant was obtained in a 2ml vial. A sample was then subjected to HPLC analysis for anthocyanins and epicatechin isolation and quantification. The HPLC system (Figure 3.14) consisted of a Waters 2690 Separation Module equipped with a Waters 996 Photodiode Array Detector (Milford, MA), Supelco Discovery C18 column (id 3 mm x 250mm) and a Millennium 32 chromatography manager. The mobile phase consisted of solvent A: 10% acetic acid (concentrated acetic acid/distilled water, 10/90, v/v) and solvent B: acetonitrile, with percentage of acetic acid in water ramped from 100% to 0% in 90 min at 2.0 ml/min. The injection volume was 100 µl. The chromatograms obtained at a

wavelength of 520nm and 287nm were used to quantify anthocyanins and epicatechin respectively. Total selected anthocyanins was the summation of the seven detected peaks area, with the retention time 13.3, 14.8, 16.2, 18.0, 19.4, 20.7 and 22.2 minute, as shown in the HPLC chromatogram (see Appendix D). Epicatechin was detected at 12.0 minute of the retention time (see Appendix E). The concentration of anthocyanins and epicatechin were calculated based on a standard curve.

Figure 3.14. High Performance Liquid Chromatography (HPLC).

3.5.4.3. Total Phenolics

Total phenolics of the blueberry samples were measured according to the method as indicated in 3.2.4.2.

3.5.4.4. Antiradical Activity

DPPH antiradical activity and % of inhibition were measured and calculated according to the method as indicated in 3.2.4.3.

3.5.4.5. EPA and DHA in Salmon Oil-in-water Emulsion

Unrefined salmon oil was produced by processing salmon byproducts (viscera, heads, skins, frame, and discarded fish) obtained from a commercial plant in Alaska. To prepare the oil-in-water emulsion, a 1% of salmon oil-in-water emulsion was made using 0.1% of Tween20 (Sigma-Aldrich, St Louis, MO) as the emulsifier. Four gram of salmon oil was mixed with 396ml of distilled water and 0.4g of Tween20. To create the oil-in-water emulsion, the mixture was sonicated (Figure 3.15) (Cole Parmer Ultrasonic Processor, Vernon Hills, IL) for 10 minutes in a water bath filled with ice water. For each sample, 15ml of the emulsion was added to a 50ml glass vial with a stir bar at the bottom. One percent and 9.1% of blueberry juice, wine and vinegar were added to the emulsion. The samples were oxidized in a magnetic-stirring water bath (Lauda-Brinkmann, Delran, NJ) at 40°C for 3 days.

The docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were determined using the method of Yue and others (2010) by GC-FID. Heptadecanoic acid (C17:0) was used as an internal standard at 100ppm (0.1mg/ml) in hexane. One ml of the emulsion was mixed and vortexed (Thermolyne Corporation, Dubuque, IA) with 2ml of C17:0 hexane solution. The supernatant was then transferred to another clean test tube. This step was repeated twice for better oil extraction. The supernatant was then dried by nitrogen gas flow until all supernatant evaporated. Two ml of BCl_3-methanol and 1 mL 2, 2'-dimethoxypropane were added to the dried test tube. The mixture was vortexed and heated in a water bath at 60°C for 30min for the

derivatization of fatty acid methyl esters. After that, the sample was incubated in tap water at room temperature for 5 min. After cooling, 2ml of hexane and 1ml of distilled water were then added to the mixture. The mixture was vortexed and centrifuged (Clay Adam, Sparks, MD). The upper layer of hexane was transferred to another test tube, dried with sodium sulfate and then transferred to a 2ml GC vial.

A GC with a FID detector (Hewlett Packard 5890, Agilent Technologies, Palo Alto, CA) was used to determine the EPA and DHA concentration. The column was a Supelco SP2380 (30m × 0.25mm) (Bellefonte, PA). Helium was used as a carrier gas at the flow rate of 1.2ml/min. The injection volume was 1 ul and the split ratio was 1:100. The injector and detector temperature was 250 and 270°C respectively. The oven temperature was held at 50 ^{o}C for 3 min and then increased to 250°C at 4.0 ^{o}C /min. The retention time of EPA and DHA were 43 and 50 minute, respectively (see Appendix F). The concentration of EPA and DHA were calculated using C17:0 (Sigma-Aldrich, St Louis, MO), the internal standard, as the reference.

Figure 3.15. Preparing salmon oil-in-water emulsion by using sonication.

3.6. Statistical Analyses

All data was analyzed with the Statistical Analysis System (SAS, Cary, NC). The result of anthocyanin, epicatechin, total phenolics and antiradical activity were evaluated statistically for differences using one-way analysis of variance (ANOVA). The EPA and DHA value were also statistically analyzed for differences between the control and treated samples using two-way ANOVA. Significance of all tests was set at $P \leq 0.05$. All results reported were expressed as mean ± standard error.

CHAPTER 4. RESULTS AND DISCUSSION

4.1. Black Raspberry

Total anthocyanin content in the black raspberry juice was 207.72 mg/100ml as compared to the reported range of 244.8 to 541.3mg/100ml (Dossett and others 2010). Many reasons could be attributed to the variation found, for example the cultivars and other environmental differences (Ozgen and others 2008).

Plant proteases such as ficin, papain and bromelain have been reported to be effective in preventing enzymatic browning. Ficin was reported to be effective in preventing black spot formation in shrimp (Taoukis and others 1990). Papain and ficin were shown to be as effective as sulfilte in slowing the rate of enzymatic browning in apple and potato slices (Labuza and others 1992). In this experiment, seven different proteases were screened in an attempt to control destruction of anthocyanins in black raspberry: Acid Protease, Liquipanol, Neutral Bacterial Protease, Ficin, Bromelain, Fungal Protease, and Neutral Protease. According to table 4.1, Acid Protease (AP), Liquipanol (L) and Neutral Bacterial Protease (NBP) were the top three proteases that could potentially retain total selected anthocyanins in black raspberry; therefore AP, L and NBP were selected to study the effect of proteases in preventing anthocyanin degradation in ethanolic fermentation of black raspberries to reduce sugar content.

4.1.1. Total Selected Anthocyanins

Black raspberry mash was treated 1% Acid Protease (AP), Liquipanol (L) and Neutral Bacterial Protease (NBP) before the ethanol fermentation began. Figure 4.1 and table 4.2 shows the total selected anthocyanins remaining in black raspberry after 1 week of open fermentation with and without protease treatments compared with fresh juice. The open fermentation is the common

industry practice for removal of sugars without accumulating appreciable ethanol. The data of Liquipanol treatment was dropped because of huge variation.

Table 4.1. Anthocyanins in Black Raspberry After Treated with 1% Proteases.

Products	**Total Selected Anthocyanins (mg/100ml of juice)**
Control	207.72±3.18
Acid Protease	237.09±8.37
Liquipanol	198.99±10.75
Neutral Bacterial Protease	197.04±6.98
Ficin	163.37±7.92
Bromelain	177.28±6.83
Fungal Protease	173.48±7.17
Neutral Protease	169.30±20.75

No protease was added in Control (black raspberry mash). 1% of the proteases were added to fresh ground black raspberry fruit mash and incubated at 47°C for 2 hrs. The mash was later squeezed and filtered through 4-layered cheese clothes to obtain juice for analysis.

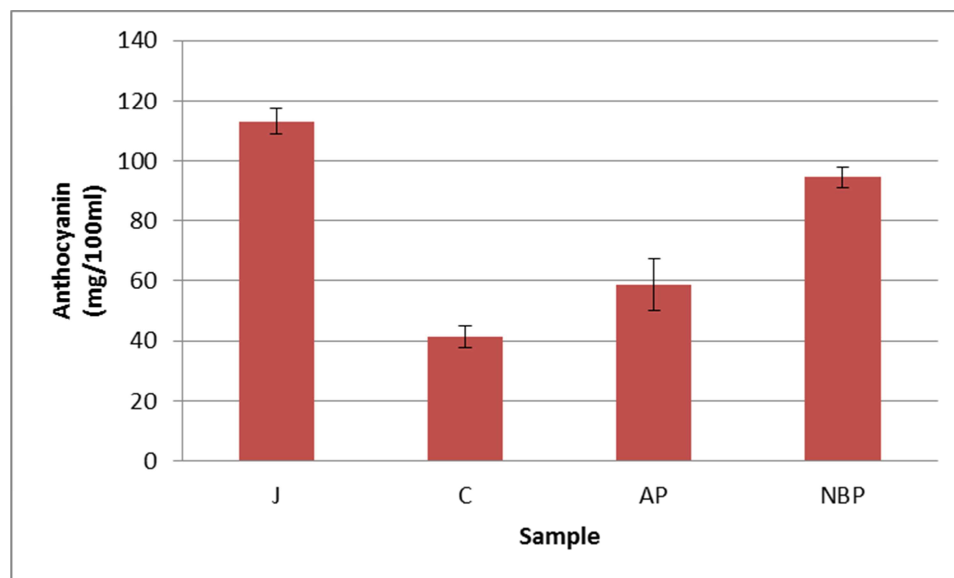


Figure 4.1.Selected Anthocyanins in Black Raspberry (BRB) Ferments After Treatments with 1% Proteases. J: Fresh juice - no protease – no fermentation; C: Control – no protease; AP: Acid Protease; NBP: Neutral Bacterial Protease. Products were fermented for 1 week in a beaker, with the opening covered with cheesecloth. Values are expressed as mean ± standard error.

Results showed that NBP retained more anthocyanins than AP and control (C) – fermented juice with no protease added. One percent NBP and AP retained 128% and 42% more anthocyanins, respectively, compared to C; however, only NBP was statistically different from AP and C. Fresh black raspberry juice contained the most anthocyanins among all samples. While anthocyanins in C were degraded by 63%, anthocyanins in black raspberry mash treated with 1% NBP were only degraded by 16% compared to J. It is clear that processing and open ethanolic fermentation significantly affected the black raspberry anthcyanins and protease treatments could alleviate some of the destruction. A 1% NBP treatment was effective in retaining anthocyanins in fermented black raspberry mash.

Table 4.2.Anthocyanin (ACN) in Black Raspberry Fermentation Products After Treated with 1% Proteases.

Sample	**ACN (mg/100ml)**
J	113.07±4.32b
C	41.39±3.60c
AP	58.71±8.81c
NBP	94.61±3.34b

J: Fresh juice with no treatment; C: Fermentation with no protease; AP: Acid Protease; NBP: Neutral Bacterial Protease. Products were fermented for 1 week in a beaker, with the opening covered with cheesecloth. Values are expressed as mean ± standard error. Within the same column, means followed by different letters are significantly different at $P \leq 0.05$.

Figure 4.2 and table 4.3 shows the results of total selected anthocyanins remaining in black raspberry after 2 days of aerobic (primary) fermentation continued by 5 days of anaerobic (secondary) fermentation. The samples were treated with lower concentrations than the previous test, 0.1% and 0.01% of AP and NBP, respectively, before fermentation.

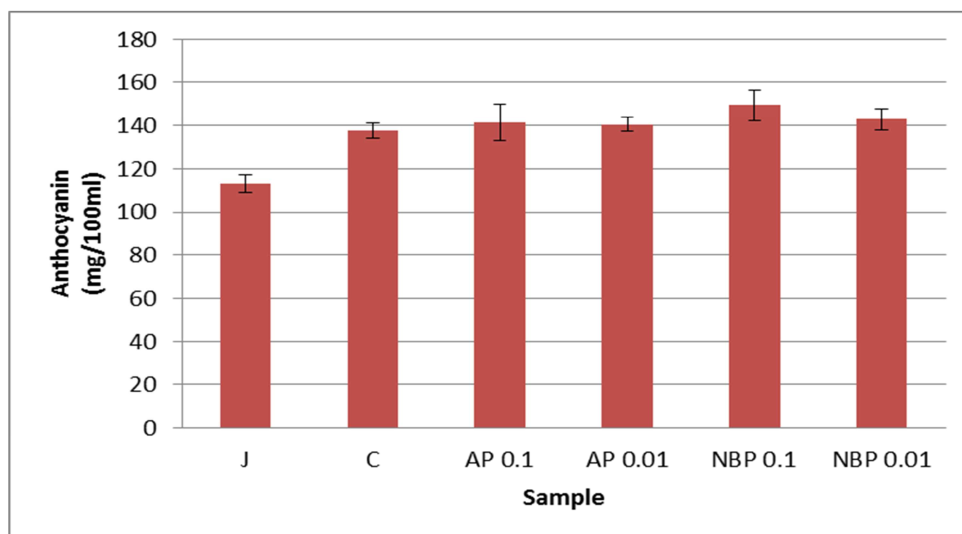


Figure 4.2.Anthocyanin in Black Raspberry Ferments After Treating with 0.1% and 0.01% Proteases. J: Fresh juice - no protease – no fermentation; C: Control – no protease; AP: Acid Protease; NBP: Neutral Bacterial Protease. Products were fermented for 1 week in a flask, with the opening covered with air-lock. Values are expressed as mean ± standard error.

Clearly, closed fermentation helped retain more anthocyanins than the open fermentation used previously. Results showed that 0.1% NBP retained the most anthocyanins among all. It preserved 8% more anthocyanins than the control (C). Though AP and NBP at 0.1% and 0.01% were effective in retaining more anthocyanins, the samples were not statistically different from one another as shown in table 4.2.

Results in table 4.2 and 4.3 reveal that differences in fermentation process critically affect the total anthocyanin remained after 1 week of fermentation. Fermentation that involved 2 days of aerobic continued by 5 days of anaerobic fermentation was more effective in retaining anthocyanin than the one-week aerobic fermentation carried out in an opened beaker. Two hundred and thirty two percent more anthocyanin (compared C in table 4.2 and 4.3) was retained while shorter period (2 days instead of 7 days) of aerobic fermentation was introduced to the fermentation process. Also, with 2 days of aerobic and 5 days of anaerobic fermentation, the

fermented black raspberry juice (C) contained 22% more anthocyanin than the fresh black raspberry juice (J) (table 4.3).

Table 4.3.Anthocyanin (ACN) in Black Raspberry Fermentation Products After Treated with 0.1% and 0.01% Proteases.

Sample	**Concentration (%)**	**ACN (mg/100ml)**
J	-	113.07±4.32b
C	-	137.44±3.53a
AP	0.1	141.31±8.52a
AP	0.01	140.58±3.51a
NBP	0.1	149.37±7.10a
NBP	0.01	142.91±5.02a

J: Fresh juice with no treatment; C: Control; AP: Acid Protease; NBP: Neutral Bacterial Protease. Products were fermented for 1 week in a flask, with the opening covered with air-lock. Values are expressed as mean ± standard error.Within the same column, means followed by different letters are significantly different at $P \leq 0.05$.

In conclusion, while 1% of neutral bacterial protease effectively retained total selected anthocyanin in open fermented black raspberry juice by 128%, the results for closed fermentation were much less dramatic. None of the 0.1% and 0.01% of protease treatments significantly differed from the control in closed fermentation. While comparing the fermentation process that involved 2 days of aerobic fermentation followed by 5 days of anaerobic fermentation to the process that involved 7 days of aerobic fermentation, shorter aerobic fermentation retained 232% more anthocyanin than the longer aerobic fermentation process. The results from this study indicated that while proteases may have a positive effect preventing PPO activity and preserving anthocyanins, the fermentation process also had a greater impact on anthocyanins. For this reason, further studies involving alcoholic and acetic fermentations impact on fruit anthocyanins were warranted.

4.2. Persimmon

4.2.1. pH, Titratable Acidity and Brix

Titratable acidity (TA) of persimmon juice and wine were expressed in % malic acid since malic acid is the predominant acid in persimmon (Daood and others 1991; Lee and others 2009). TA of persimmon vinegar was expressed in % acetic acid because acetic acid is the major acid in vinegar. pH of astringent and non-astringent persimmon juice decreased 42.9% and 45.6%, respectively, after wine and vinegar fermentation. Wine and vinegar fermentation increased titratable acidity of astringent and non-astringent persimmon by 14 and 11 times respectively. Brix of astringent and non-astringent persimmon juice decreased 81.7% and 81.4%, respectively, after primary wine fermentation. Detailed data was shown in Appendix D.

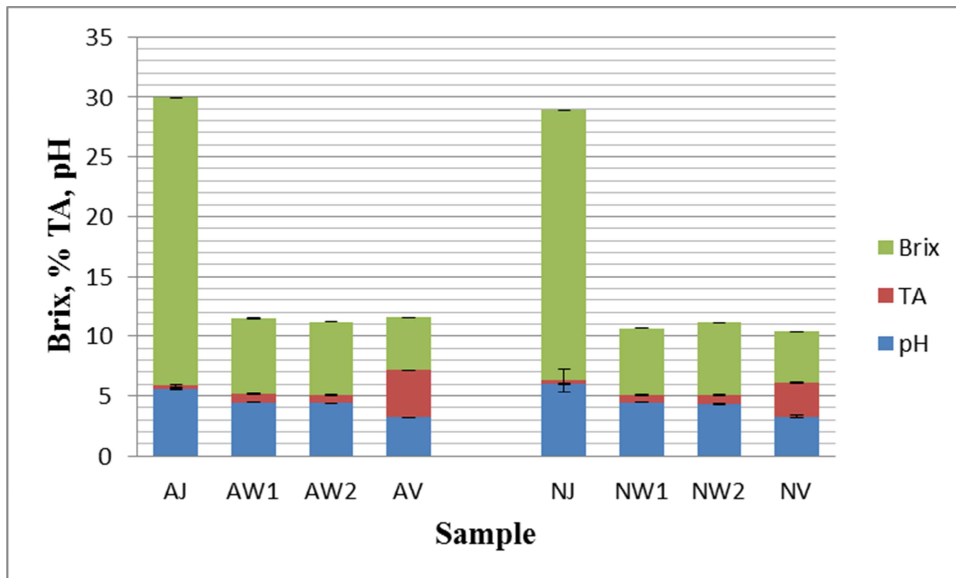


Figure 4.3. pH, titratable acidity and brix of astringent and non-astringent juice and fermentation products. AJ & NJ: astringent and non-astringent juice; AW1 & NW1: astringent and non-astringent persimmon after primary wine fermentation; AW2 & NW2: astringent and –non-astringent persimmon after secondary wine fermentation; AV & NV: astringent and non-astringent persimmon vinegar. Values are expressed as mean ± standard error.

4.2.2. Total Phenolics and Antiradical Activity

Figure 4.3, 4.4 and table 4.4 show the total phenolics (TPH) and antiradical activity (AR) of astringent and non-astringent persimmons with both ethanolic and acetic acid fermentations. Astringent persimmon unfermented juice contained the most total phenolics and possessed the strongest antiradical activity among all treatments. Total phenolics in astringent persimmon juice were about 90 times more than that in non-astringent. Antiradical activity was 73 times stronger than non-astringent juice. Total phenolics in astringent persimmon juice were significantly degraded during the primary and secondary wine fermentation while antiradical activity was only significantly degraded during the primary wine fermentation. Acetification process also degraded total phenolics and antiradical activity; however, TPH and AR of astringent persimmon wine were not statistically different from astringent persimmon vinegar.

The detected level of TPH and AR in non-astringent persimmon juice was lower than that in non-astringent persimmon wine and vinegar. This was probably because TPH in non-astringent persimmon was susceptible to alcoholic extraction when alcohol was produced during the primary wine fermentation; thereby significantly increased TPH in NW1.

According to our results, comparing astringent to non-astringent persimmon juice and fermentation products, only astringent persimmon juice was significantly different from non-astringent juice. Astringent persimmon wine and vinegar were not statistically different from non-astringent persimmon wine and vinegar in terms of TPH and AR determined.

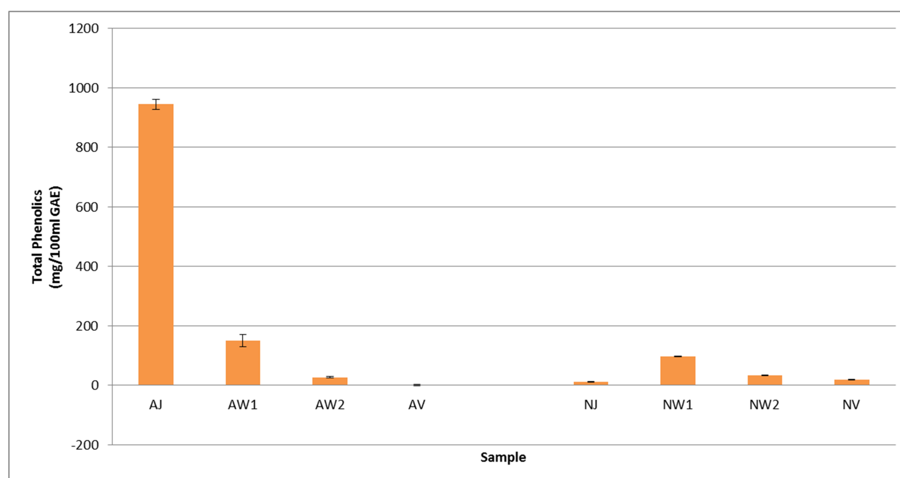


Figure 4.4.Total phenolics in astringent and non-astringent persimmon juice and fermentation products. AJ & NJ: astringent and non-astringent juice; AW1 & NW1: astringent and non-astringent persimmon after primary wine fermentation; AW2 & NW2: astringent and –non-astringent persimmon after secondary wine fermentation; AV & NV: astringent and non-astringent persimmon vinegar. Only AJ and AW1 were diluted 100 and 10 times respectively, others were not diluted. Values are expressed as mean ± standard error.

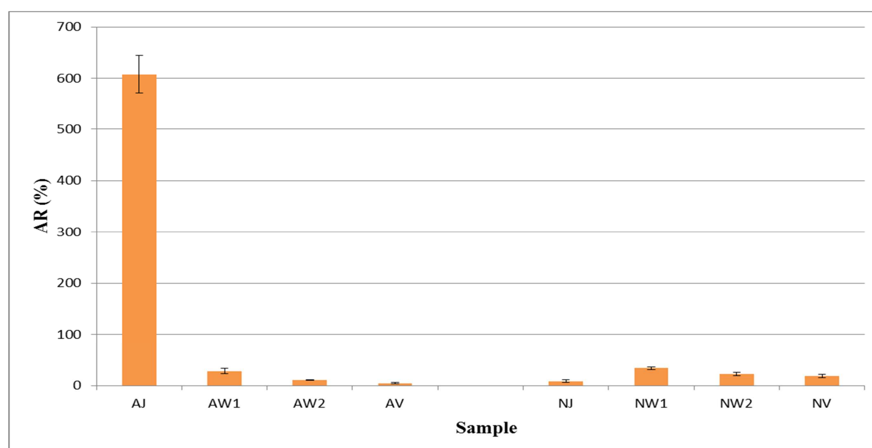


Figure 4.5.Antiradical activity in astringent and non-astringent persimmon juice and fermentation products. AJ & NJ: astringent and non-astringent juice; AW1 & NW1: astringent and non-astringent persimmon after primary wine fermentation; AW2 & NW2: astringent and –non-astringent persimmon after secondary wine fermentation; AV & NV: astringent and non-astringent persimmon vinegar. Values are expressed as mean ± standard error.

Overall, in astringent persimmon, TPH was significantly degraded in primary and secondary wine fermentation but not in acetification process. However, AR was only significantly degraded during primary wine fermentation but not secondary wine fermentation and acetification. As for

non-astringent persimmon, primary wine fermentation significantly increased TPH. Secondary wine fermentation and vinegar fermentation degraded TPH; however, they were not statistically different. AR had the similar trend as TPH in non-astringent persimmon but the activity was not statistically different. Determination of specific phenolic compounds which are abundant in persimmon, such as tannins and polyphenols (Piretti 1991; Gu and others 2008) is needed to confirm either elevation or degradation of phenolic compounds was incurred during the wine and vinegar fermentation in astringent and non-astringent persimmon.

Table 4.4.Summary of total phenolics (TPH) and antiradical activity (AR) of astringent and non-astringent persimmon juice and fermentation products.

Products	**TP (mg/100ml GAE)**	**AR (%)**
AJ	945.00±16.82a	607.77±36.56a
AW1	151.20±19.77b	27.88±5.05b
AW2	26.83±1.90cd	10.07±0.72b
AV	1.15±2.11d	4.25±1.40b
NJ	10.50±1.50d	8.25±2.46b
NW1	97.94±2.22c	33.54±2.62b
NW2	32.66±0.48cd	22.44±2.91b
NV	17.62±0.95cd	18.69±3.34b

AJ & NJ: astringent and non-astringent juice; AW1 & NW1: astringent and non-astringent persimmon after primary wine fermentation; AW2 & NW2: astringent and –non-astringent persimmon after secondary wine fermentation; AV & NV: astringent and non-astringent persimmon vinegar. Values are expressed as mean ± standard error. Within the same column, means followed by different letters are significantly different at $P \leq 0.05$.

4.3. Blueberries

4.3.1 pH, Titratable Acidity, and Brix

The pH of fresh blueberry juice was 3.10 (Figure 4.5 and Appendix C) and it decreased to 2.62 after wine and vinegar fermentation. Titratable acidity (TA) of blueberry juice and wine were expressed in % malic acid since malic acid is the predominant acid in blueberry (Ashurts 2005).

The TA of blueberry vinegar was expressed in % acetic acid because acetic acid is the major acid in vinegar. Titratable acidity in fresh blueberry juice was about 0.8% but it increased to 1.5% during primary and secondary wine fermentation. During acetification process, TA in blueberry must quickly multiplied 3.2 times in about 2 weeks. The Brix of the fresh blueberries decreased 43%, from 9.8% sugars to 5.6% sugar at the end of primary wine fermentation in 5 days.

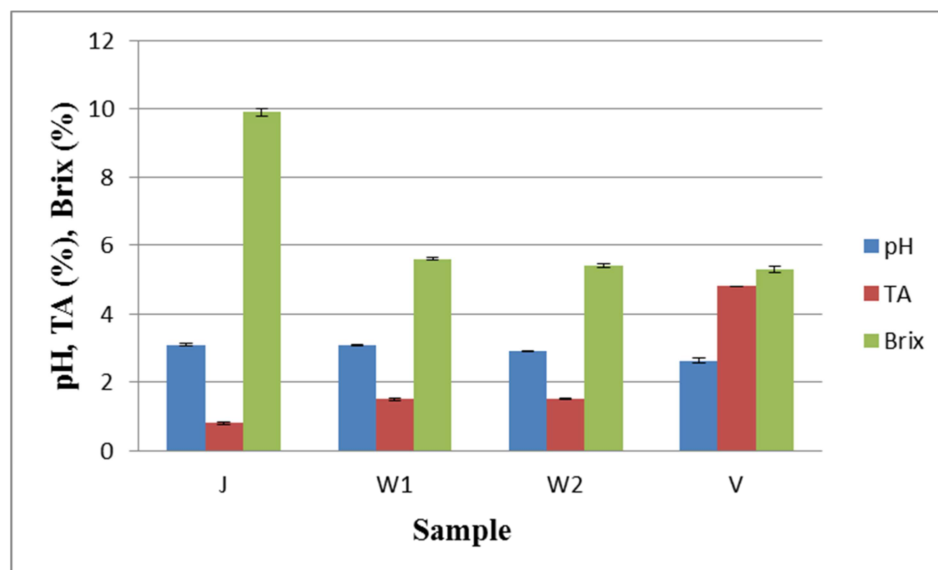


Figure 4.6. pH, TA and Brix of Tifbluerabbiteye blueberry juice and fermentation products. TA is expressed in % of malic acid for J, W1, W2 and % of acetic acid for V. Brix is expressed in % of sugars. J: fresh juice; W1: fermentation product after primary wine fermentation; W2: fermentation product after secondary wine fermentation; V: fermentation product after acetification. Values are expressed as mean ± standard error.

4.3.2 Total Anthocyanin

Total anthocyanin content (ACY) of the blueberry juice was lower than that of the blueberry wine and vinegar (Figure 4.6). Blueberry must, after primary and secondary ethanolic fermentation, had 266% and 169% more anthocyanin than the fresh juice, respectively, while the blueberry vinegar contained only 27% more anthocyanin than the juice. While total anthocyanins were increased during the primary fermentation, it was later decreased 27% (W2) and then

decreased another 53% (V) during the secondary fermentation and acetification process. During primary wine fermentation, the anthocyanin content peaked at 8.54mg/100ml of blueberry must. This tremendous increase may be due to the ethanol produced during the fermentation assisting in extracting the anthocyanins from the fruit pulp; thereby increasing the amount of anthocyanins in the wine. Various solvents, such as ethanol, methanol, acetone, trifluoroacetic, hydrochloric and phosphoric acid, have been successfully used to increase anthocyanin extraction (Mazza and others 2003; Nicoue and others 2007; Mane and others 2007). Therefore, the ethanol produced during the primary fermentation could be the potential extraction agent that is accountable for the anthocyanin elevation. Our findings are similar to the results of Mazza and others (1999) who found that total anthocyanins in grape wine increased during the early stages of ethanolic fermentation, peaking at 2-3 days after the start of fermentation and decreasing during malolactic fermentation.

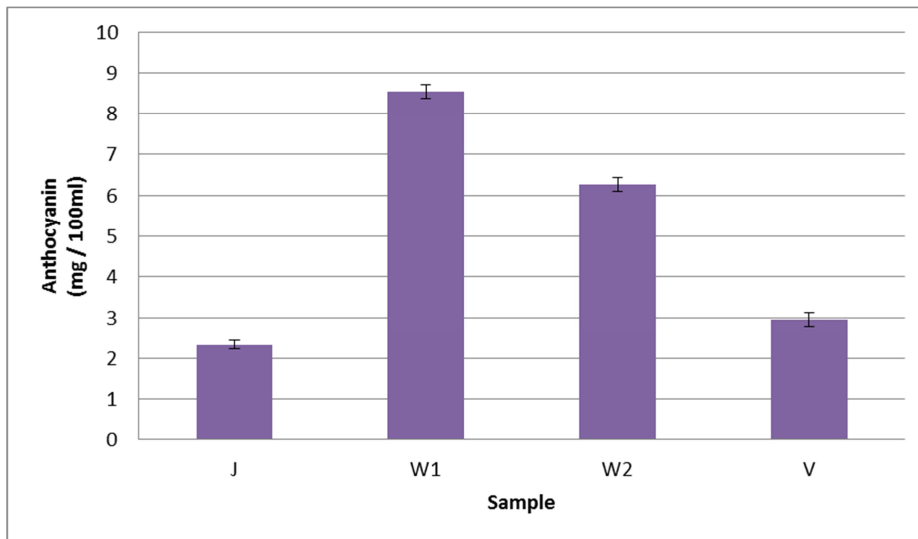


Figure 4.7. Total anthocyanin content of blueberry juice and fermentation products. J: fresh juice; W1: fermentation product after primary wine fermentation; W2: fermentation product after secondary wine fermentation; V: fermentation product after acetification. Values are expressed as mean ± standard error.

4.3.3. Epicatechin

According to Zhao (2007), epicatechin is the major flavan-3-ol in blueberries and it is present at a concentration of 1mg/100g fresh weight. Therefore, other than anthocyanin, it is also important to determine and quantify the concentration of epicatechin throughout the fermentation processes. Epicatechin concentration (EPC) was highest in the blueberry must after primary fermentation and lowest in the fresh blueberry juice (Figure 4.7). After primary and secondary wine fermentation, epicatechin in blueberry wine increased by 227% (W1) and 157% (W2), respectively, compared to that of the fresh juice. The epicatechin content in blueberry vinegar after acetification was 78% more than that in blueberry juice. Although epicatechin was significantly increased during primary fermentation, it was later degraded during secondary wine fermentation and acetification.

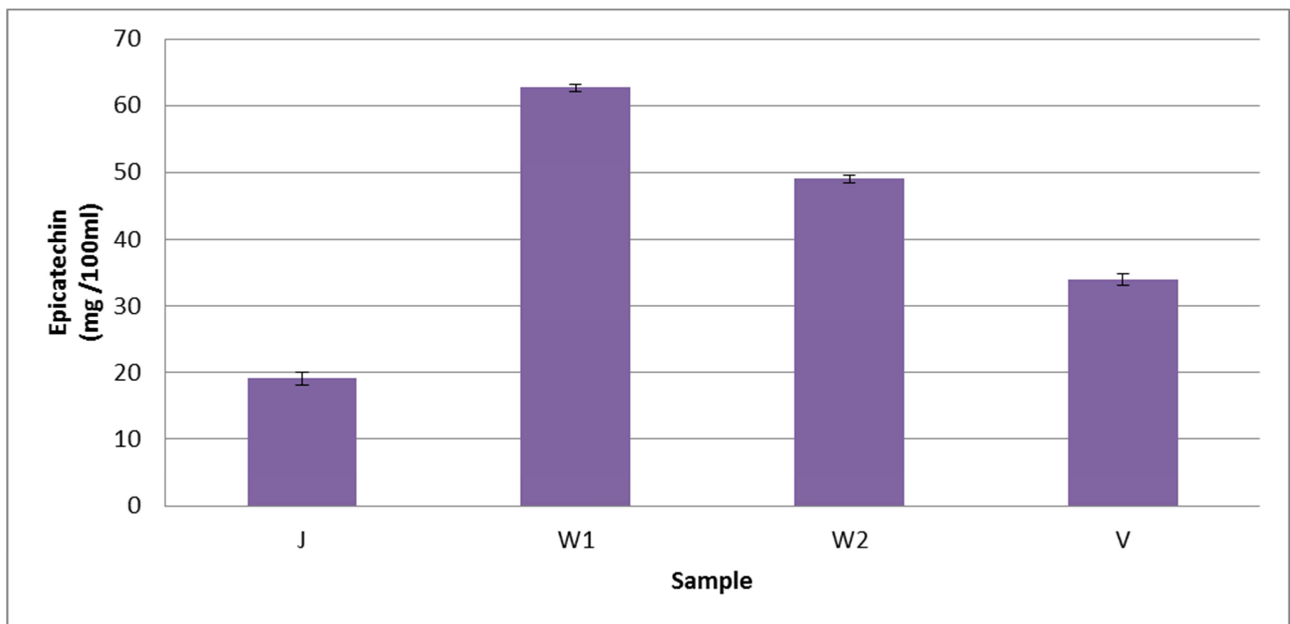


Figure 4.8. Epicatechin of fresh Tifbluerabbiteye blueberry juice and fermentation products. J: fresh juice; W1: fermentation product after primary wine fermentation; W2: fermentation product after secondary wine fermentation; V: fermentation product after acetification. Values are expressed as mean ± standard error.

4.3.4. Antiradical Activity

Antiradical activity (AR) of the blueberries was represented in percentage of inhibition of the decoloration of DPPH solution. The free radical scavenging activity was the highest in blueberry must during primary wine fermentation and the lowest in blueberry vinegar (Figure 4.8). Antiradical activity increased by 28% during primary fermentation and then decreased by16% and 28%, respectively, during secondary wine fermentation and acetification compared to that of the fresh blueberry juice. Even though the overall trend was in line with the trend of total anthocyanin and epicatechin, differences in antiradical activity throughout the fermentation processes were not significant.

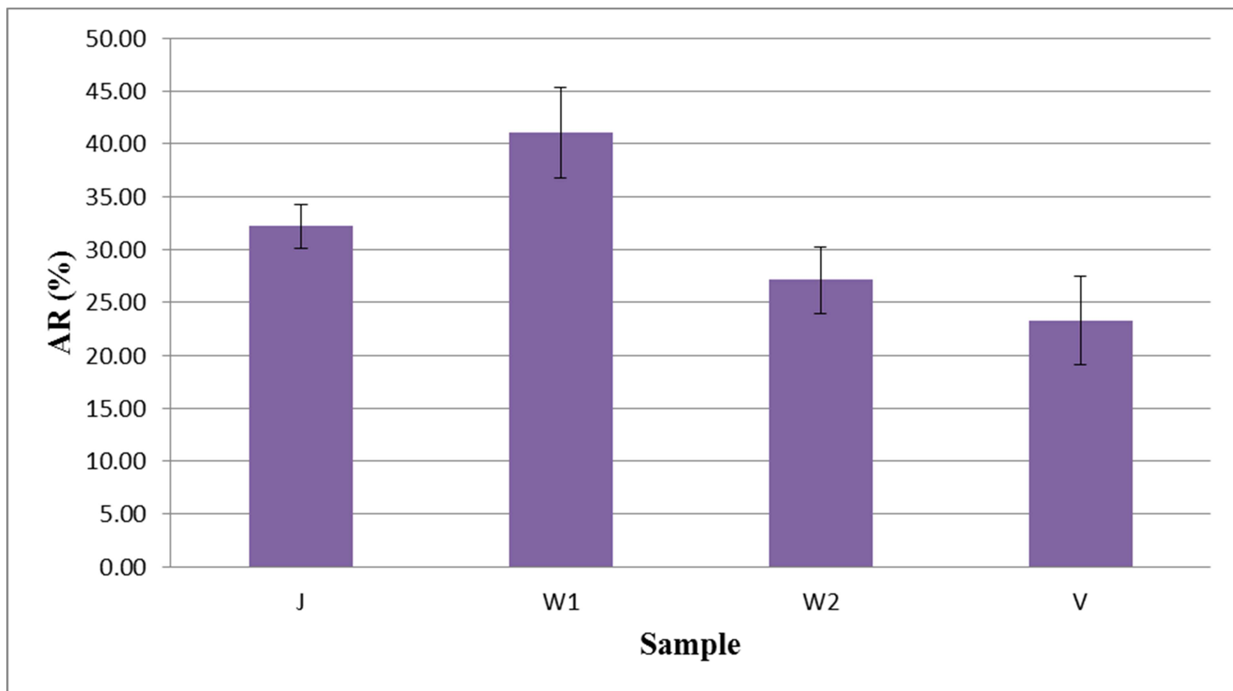


Figure 4.9.Antiradical activity of Tifbluerabbiteye blueberry juice and fermentation products. J: fresh juice; W1: fermentation product after primary wine fermentation; W2: fermentation product after secondary wine fermentation; V: fermentation product after acetification. All of the samples were diluted 5 times. Values are expressed as mean ± standard error.

4.3.5. Total Phenolics

Total phenolics were the highest in fresh blueberry juice (Figure 4.9). Throughout the wine fermentation and acetification process, only primary wine fermentation significantly reduced the total phenolics in blueberry by 21%. The total phenolics in W1, W2 and V were not statistically different from one another. Also, the overall trend of the total phenolics did not agree with that of the total anthocyanin, epicatechin and antiradical activity during the fermentation processes. On the other hand, antiradical activity and total phenolics of W1, W2 and V shared some similarity and were not statistically different from one another. Other than anthocyanin and epicatechin, this experiment did not account for other phenolic compounds that could also be part of the total phenolics in blueberry. Therefore, this could partly explain the disagreement stated above. In addition, Folin-Ciocalteau colorimetry method that was used in this experiment has the disadvantage of responding to sugar and sulfur dioxide (Waterhouse 2002). This could elevate the apparent phenolic content in the fresh juice which contained sugars.

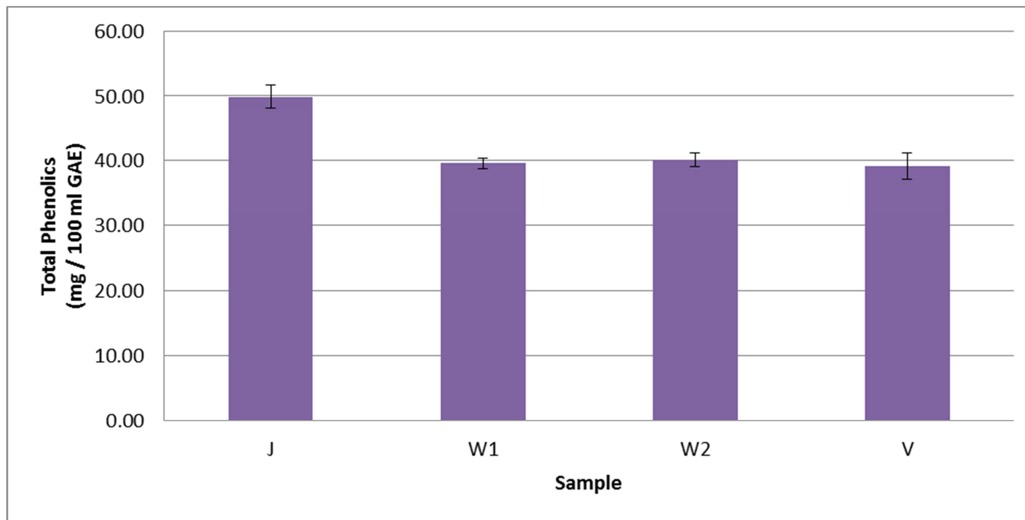


Figure 4.10.Total phenolics of fresh Tifbluerabbiteye blueberry juice and fermentation products. J: fresh juice; W1: fermentation product after primary wine fermentation; W2: fermentation product after secondary wine fermentation; V: fermentation product after acetification; GAE: gallic acid equivalent. Values are expressed as mean ± standard error.

4.3.6. Overall Changes of Phenolics and Antioxidant Activity

In general, the results of total anthocyanin (ACY), epicatechin (EPC) and antiradical activity (AR) of blueberry juice and fermentation products were in line with one another. ACY, EPC, and AR were increased during primary wine fermentation process but were later decreased during secondary wine fermentation process. After that, they further decreased during the acetification process. During wine fermentation, most of the alcohol is produced during the primary fermentation (alcohol fermentation). The increase of alcohol content during alcohol fermentation significantly raised the ACY, EPC and AR of the fermentation products, by escalating the extraction from the fruit pulp. Initial alcoholic fermentation increased ACY, EPC and AR while acetification responded oppositely. Our findings are in accordance with Su and others (2007) in that blueberry wine contained more total anthocyanin and possessed stronger antiradical activity than that in blueberry juice and vinegar. Andlauer and others (2000) investigated the influence of acetification process on phenolic compounds in cider, red and white wine and vinegar. They concluded that acetification process decreased the total phenolics in cider vinegar (40%), red wine vinegar (13%) and white wine vinegar (8%). Also, Su and others (2006) compared the antioxidant properties of blueberry juice, wine and vinegar pomace. They reported that wine pomace has the highest antioxidant activites while vinegar pomace has the lowest. They also concluded that acetification significantly decreased TPH, ACY and antiradical activity. In short, our conclusion is in line with that of Andlauer and others (2000) and Su and others (2006, 2007).

Table 4.5.Summary of total anthocyanin content (ACY), total epicatechin (EPC), total phenolics (TPH), and antiradical activity (AR) of blueberry juice and fermentation products.

Sample	ACY (mg/100ml)	EPC (mg/100ml)	TPH (mg/100ml)	AR (%)
J	2.33±0.10c	19.13±0.93d	49.89±1.80a	32.17±2.07a
W1	8.54±0.16a	62.65±0.64a	39.60±0.84b	41.03±4.32a
W2	6.26±0.17b	49.07±0.60b	40.12±1.07b	27.09±3.14a
V	2.96±0.17c	33.99±0.89c	39.15±1.99b	23.27±4.13a

J: fresh juice; W1: fermentation product after primary wine fermentation; W2: fermentation product after secondary wine fermentation; V: fermentation product after acetification. All values are expressed as mean ± standard error. Within the same column, means followed by different letters are significantly different at $P \leq 0.05$.

4.3.7. Changes in Concentration of EPA and DHA in Salmon Oil-in-water Emulsion

Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) contents in salmon oil are reported to be in the range of 6.2-7.9% and 9.1-10.5%, respectively (Frankel and others 2002; Barrow and others 2008). Salmon oil used in this experiment contained 9.20±0.75 (%) of EPA and 8.10±0.60 (%) of DHA by GC-FID. The slight variation is probably because of the varieties of the salmon species used and the discrepancy of the testing method applied in the experiments. Nevertheless, the slight discrepancies can be considered insignificant in this case while we are comparing the salmon oil-in-water emulsion (SOE) internally instead of externally.

Figures 4.10 to 4.11 and table 4.5 show the EPA and DHA remaining in SOE after treatment with 1% and 9.1% of blueberry juice, wine and vinegar. After 3 days of incubation in a warm water bath, EPA and DHA in the control SOE (C3) degraded 87% and 91% respectively while compared to that in the control at 0 days (C0). One percent of juice (J1), wine (W1) and vinegar (V1) effectively prevented the degradation of EPA by 57%, 70% and 77%, respectively, and DHA by 57%, 72% and 79%, respectively. In addition, 9.1% of juice (J10), wine (W10) and vinegar (V10) prevented the degradation of EPA by 73%, 77% and 75%, respectively, and DHA

by 80%, 84% and 88% respectively. Even though 9.1% treatment was more effective than the 1% treatment in preventing the degradation of EPA and DHA, both the treatments were not statistically different in terms of EPA. As for DHA, although the results showed that 9.1% and 1% treatments were statistically different with the calculated p-value of 0.034, the p-value was very close to our significant difference level which was at $P \leq 0.05$. Therefore, 9.1% treatment was close to having the same effect as the 1% in preventing DHA degradation. In other words, 1% of treatment was sufficient enough to prevent EPA and DHA degradation in SOE. A similar finding was reported by Luther and others (2007). They investigated the inhibitory effect of black raspberry seed extract on lipid oxidation in fish oil and reported that black raspberry seed extract significantly reduced the degradation of n-3 polyunsaturated fatty acid.

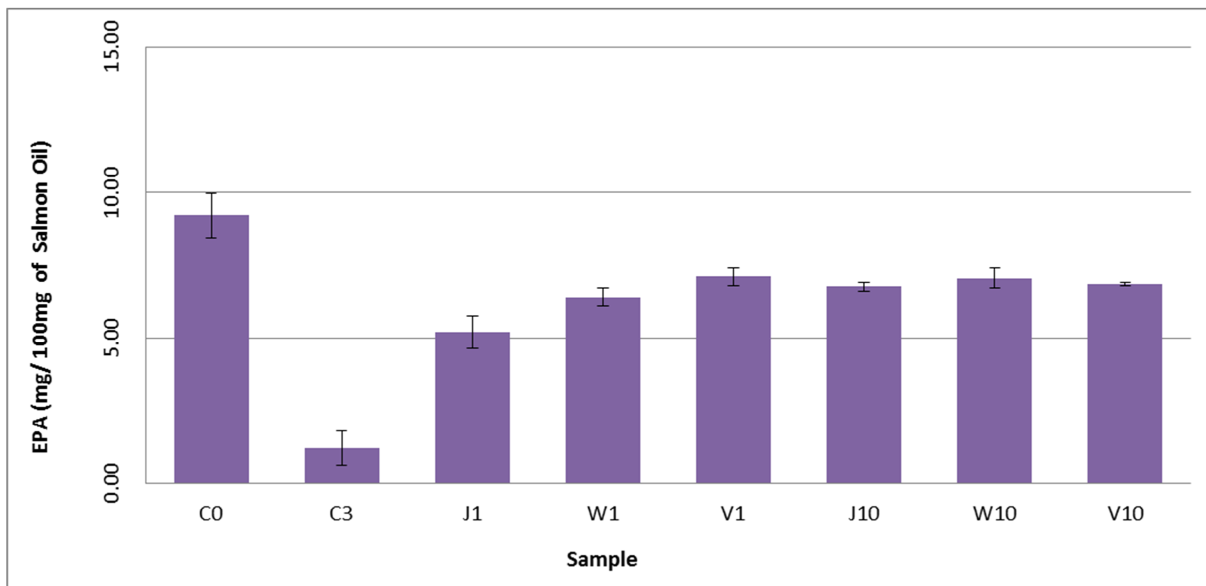


Figure 4.11. Eicosapentaenoic acid (EPA; C20:5n-3) remained in 1% salmon oil-in-water emulsion (SOE) after 3 days of incubation. C0: control at 0 day; C3: control after 3 days; J1, W1, V1: blueberry juice, wine, vinegar at 1%; J10, W10, V10: blueberry juice, wine, vinegar at 9.1%. Values are expressed as mean ± standard error.

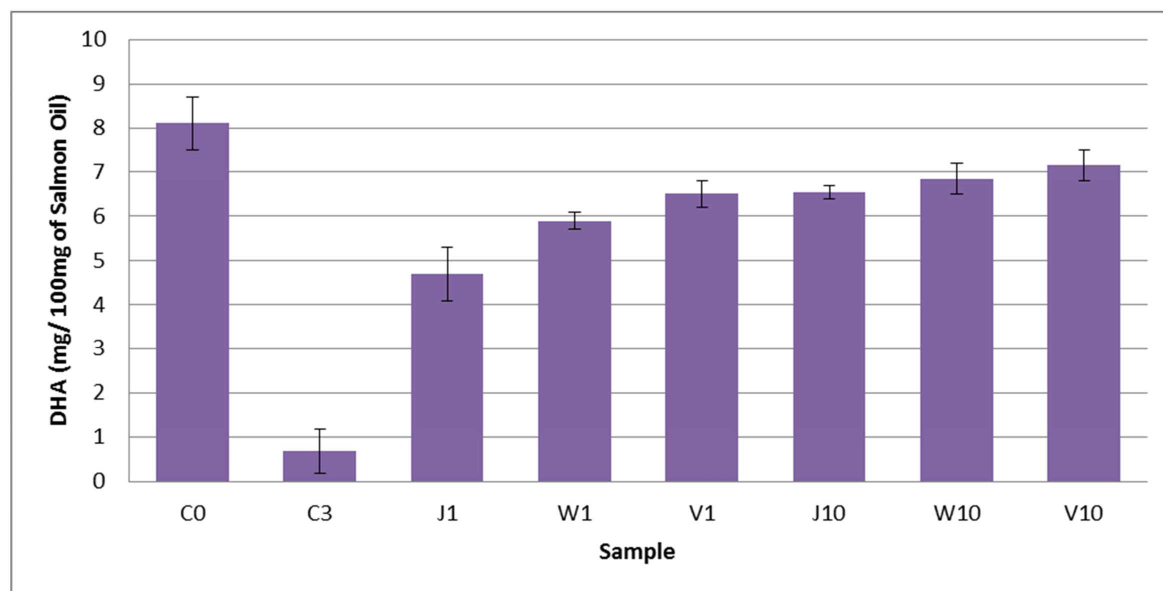


Figure 4.12. Docosahexaenoic acid (DHA; C22:6n-3) remained in 1% salmon oil-in-water emulsion after 3 days of incubation. C0: control at 0 day; C3: control after 3 days; J1, W1, V1: blueberry juice, wine, vinegar at 1%; J10, W10, V10: blueberry juice, wine, vinegar at 9.1%. Values are expressed as mean ± standard error.

Table 4.6. Summary of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) remained in salmon oil-in-water emulsion after 3 days of oxidation.

Sample	**EPA (mg/100mg)**	**DHA (mg/100mg)**
C0	9.20±0.75a	8.10±0.60a
C3	1.20±0.60c	0.70±0.50c
J1	5.20±0.55b	4.70±0.60b
W1	6.40±0.30ab	5.90±0.20ab
V1	7.10±0.30ab	6.50±0.30ab
J10	6.75±0.15ab	6.55±0.15ab
W10	7.05±0.35ab	6.85±0.35ab
V10	6.85±0.05ab	7.15±0.35ab

C0: control at 0 day; C3: control after 3 days; J1, W1, V1: blueberry juice, wine, vinegar at 1%; J10, W10, V10: blueberry juice, wine, vinegar at 9.1%. Values are expressed as mean ± standard error. Within the same column, means followed by different letters are significantly different at $P \leq 0.05$.

In this experiment, blueberry vinegar contained less anthocyanin, epicatechin and total phenolics and possessed weaker antiradical activity; however, it was the strongest agent in preventing EPA and DHA oxidation compared to blueberry juice and wine. Therefore, phenolics compounds, such as anthocyanin and epicatechin, were not the only factor that influenced the degradation of EPA and DHA in this oil-in-water emulsion system. Other factors, such as the physical location of the antioxidant, its interaction with other food components and environmental conditions, have to be considered in a heterogeneous food system as well (Decker 2005).

In this experiment, other than the phenolic compounds, pH could be an important factor that influenced the lipid oxidation in the SOE system. Few studies investigated the effect of pH on antioxidant compounds in oil-in-water emulsions but the results were contradictory. Some show that the rate of lipid oxidation increases with increasing pH in oil-in-water emulsions (Huang and others 1996; Sorensen and others 2008) but others disagree (Shimada and others 1994; Donnelly and others 1998). A food matrix is a very complex system; therefore, a lot of measures should be taken into consideration before an established conclusion is made. Though several studies have shown that pH has an effect in oil-in-water emulsions, it is clear that pH can impact the oxidative stability of the emulsions in various way, such as its effect on the reactivity, solubility, size of the droplets and partitioning of the reactive species involved (Mcclements and other 2000; Sorensen and others 2008). This may explain the inconclusive result of whether lower or higher pH retards lipid oxidation. Therefore, further research has to be done to confirm the impact of pH on specific antioxidant compounds in the complex oil-in-water emulsions.

CHAPTER 5. SUMMARY, CONCLUSION AND FUTURE RESEARCH

"Phytochemicals and functional foods" is one of the hottest terms that have attracted significant attention from scientists, health professionals and food manufacturers in recent decades. Anthocyanin and other phenolic compounds from various fruits and vegetables are the major constituents that are accountable for optimal health. This research started with a curiosity to know if proteases were capable in preventing anthocyanin degradation in sugar-reduced (open ferment to reduce sugars) black raspberry juice. The curiosity led the research group to search and in searching, ideas about investigating the changes of phenolic compounds during fermentation flourished. Though phenolic compounds in grape wine have been extensively studied, the information about the changes in phenolic compounds of other fruit crops during wine and vinegar fermentation is limited, however. Therefore, upon the completion of the black raspberry project, fresh persimmons and blueberries were used to investigate the changes of polyphenols during alcohol and vinegar fermentation.

Our findings concluded that:

1) One percent of neutral bacterial protease can potentially prevent anthocyanin degradation in fermented black raspberries

2) Total phenolics in astringent persimmons was degraded throughout the alcohol and vinegar fermentation processes. However, in non-astringent persimmons, alcohol fermentation increased but acetification decreased the total phenolic

3) In blueberries, alcohol fermentation increased but acetification decreased anthocyanin and epicatechin. While comparing the ability of blueberry juice, wine and vinegar in preventing EPA and DHA degradation, blueberry vinegar was the most effective agent among all.

A more established research that focuses on the anti-oxidative effect of blueberry wine and

vinegar in preventing lipid oxidation of fish oil can be designed and conducted. Other than using GC-FID to detect the concentration of docosahexaenoic acid (DHA) and eicosapentaenoic acid EPA (EPA) in salmon oil-in-water emulsion (SOE) before and after oxidation, thiobarbituric acid (TBA) method could also be used to access the overall lipid oxidation in SOE model system. Though EPA and DHA are the major fatty acids in salmon oil, alpha-linolenic acid (C18:3) is also an important fatty acid in salmon oil. It could be quantified together with EPA and DHA by GC-FID in future research.

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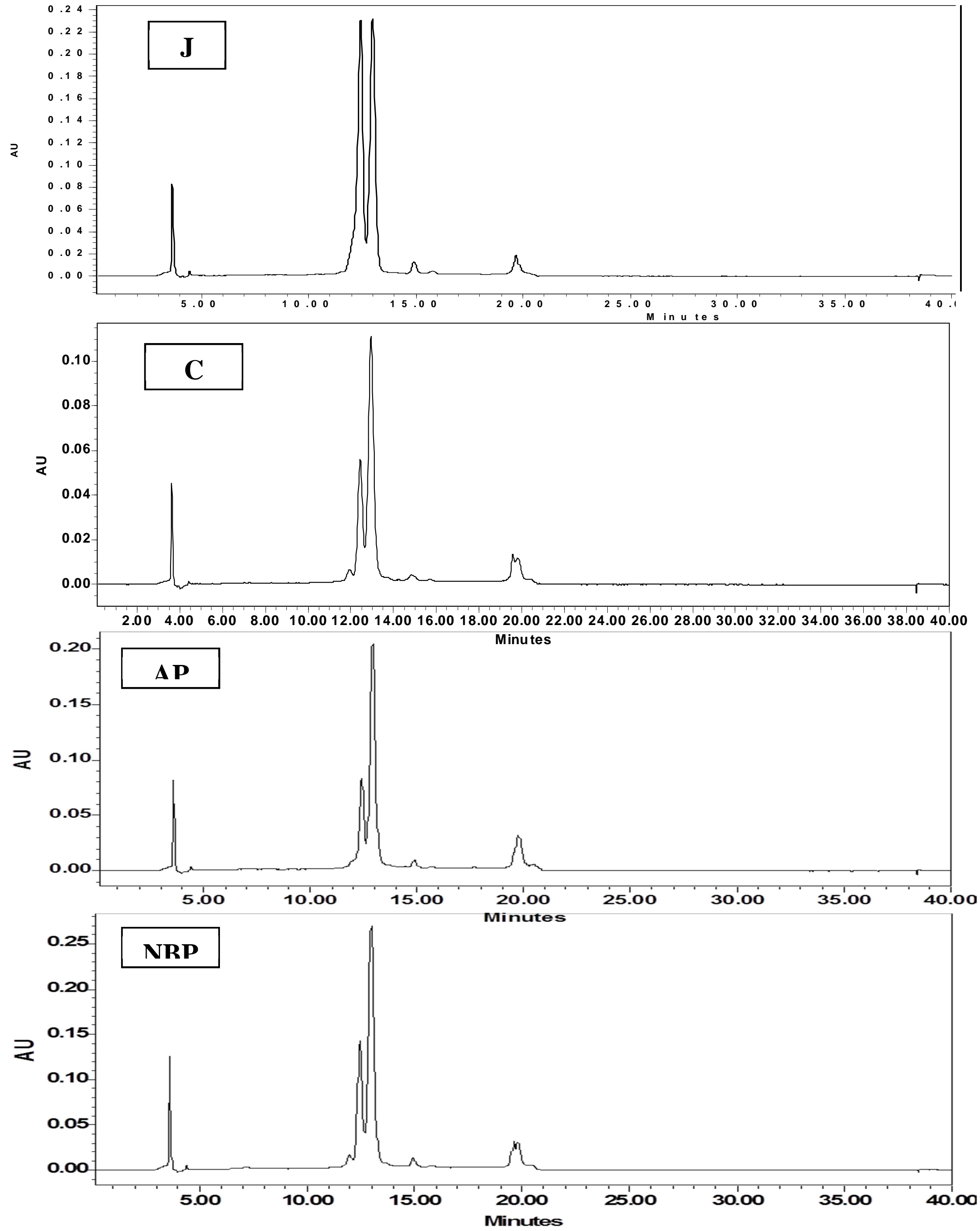
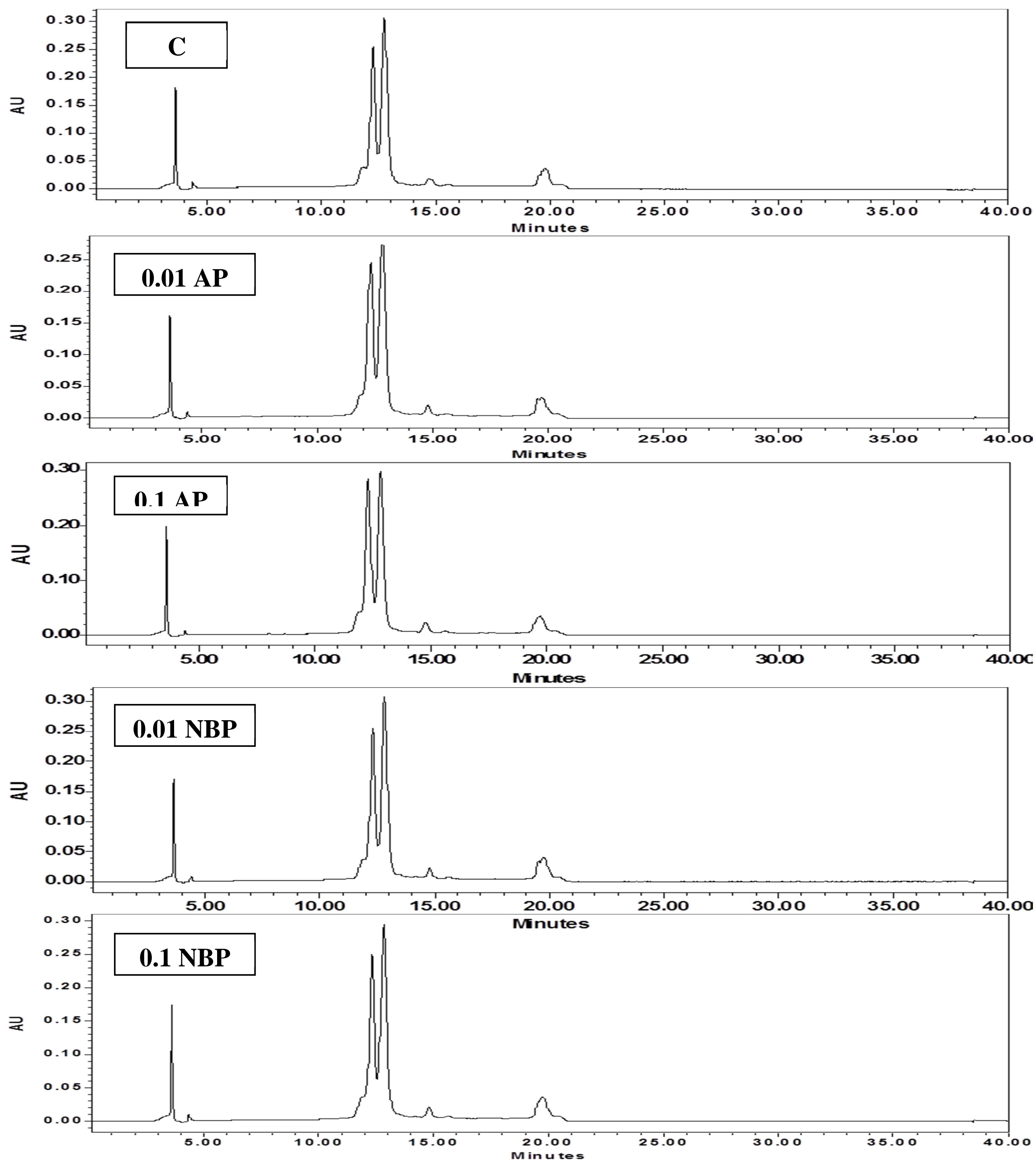
APPENDIX A. CHROMATOGRAM OF ANTHOCYANIN IN BLACK RASPBERRY FERMENTATION PRODUCTS AFTER TREATED WITH 1% PROTEASES IN OPENED BEAKER



J: Juice; C: Control; AP: Acid Protease; NBP: Neutral Bacterial Protease

APPENDIX B. CHROMATOGRAM OF ANTHOCYANIN IN BLACK RASPBERRY FERMENTATION PRODUCTS AFTER TREATED WITH 0.1% AND 0.01% PROTEASES IN VOLUMETRIC FLASK COVERED WITH AIR-LOCK



C: Control; 0.01AP, 0.01NBP: treated with 0.01% acid protease, neutral bacterial protease; 0.1AP, 0.1 NBP: treated with 0.1% acid protease, neutral bacterial protease.

APPENDIX C. SUMMARY OF PH, TA AND BRIX OF BLUEBERRY JUICE AND FERMENTATION PRODUCTS

Products	pH	TA (%)	Brix (%)
J	3.10±0.03	0.80±0.03	9.90±0.10
W1	3.10±0.01	1.50±0.03	5.60±0.05
W2	2.90±0.02	1.50±0.02	5.40±0.05
V	2.62±0.07	4.80±0.01	5.30±0.10

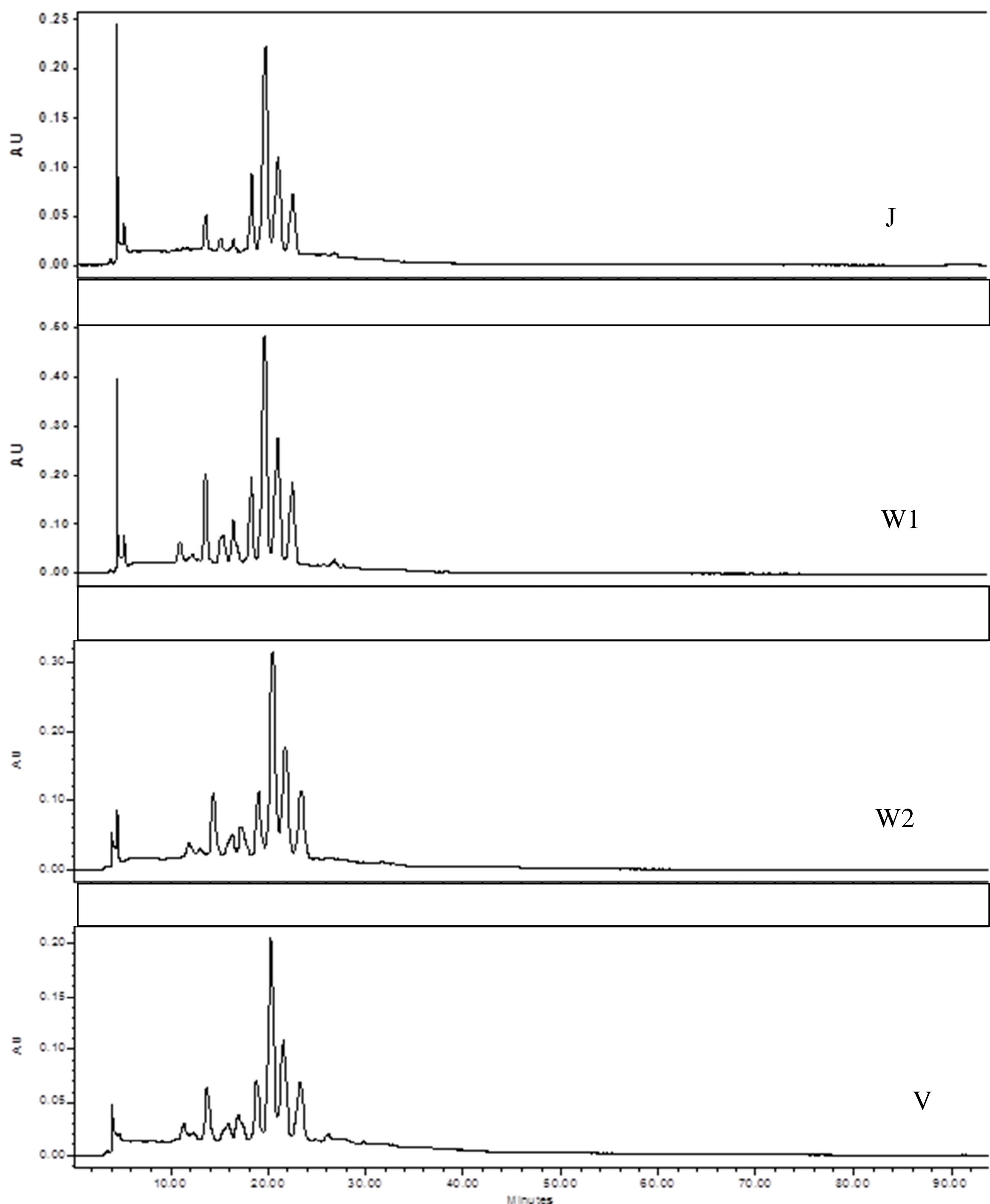
TA is expressed in % of malic acid for J, W1, W2 and % of acetic acid for V. Brix is expressed in % of sugars. J: fresh juice; W1: fermentation product after primary wine fermentation; W2: fermentation product after secondary wine fermentation; V: fermentation product after acetification. Values are expressed as mean ± standard error.

APPENDIX D. SUMMARY OF PH, TA AND BRIX OF ASTRINGENT AND NON-ASTRINGENT PERSIMMON JUICE AND FERMENTATION PRODUCTS

Products	pH	TA (%)	Brix (%)
AJ	5.59±0.05	0.28±0.10	24.10±0.01
AW1	4.44±0.01	0.72±0.06	6.30±0.04
AW2	4.39±0.01	0.69±0.02	6.10±0.01
AV	3.19±0.04	3.95±0.03	4.40±0.02
NJ	6.04±0.06	0.26±0.10	22.6±0.01
NW1	4.47±0.02	0.60±0.06	5.60±0.03
NW2	4.32±0.07	0.72±0.08	6.10±0.01
NV	3.28±0.08	2.88±0.04	4.20±0.02

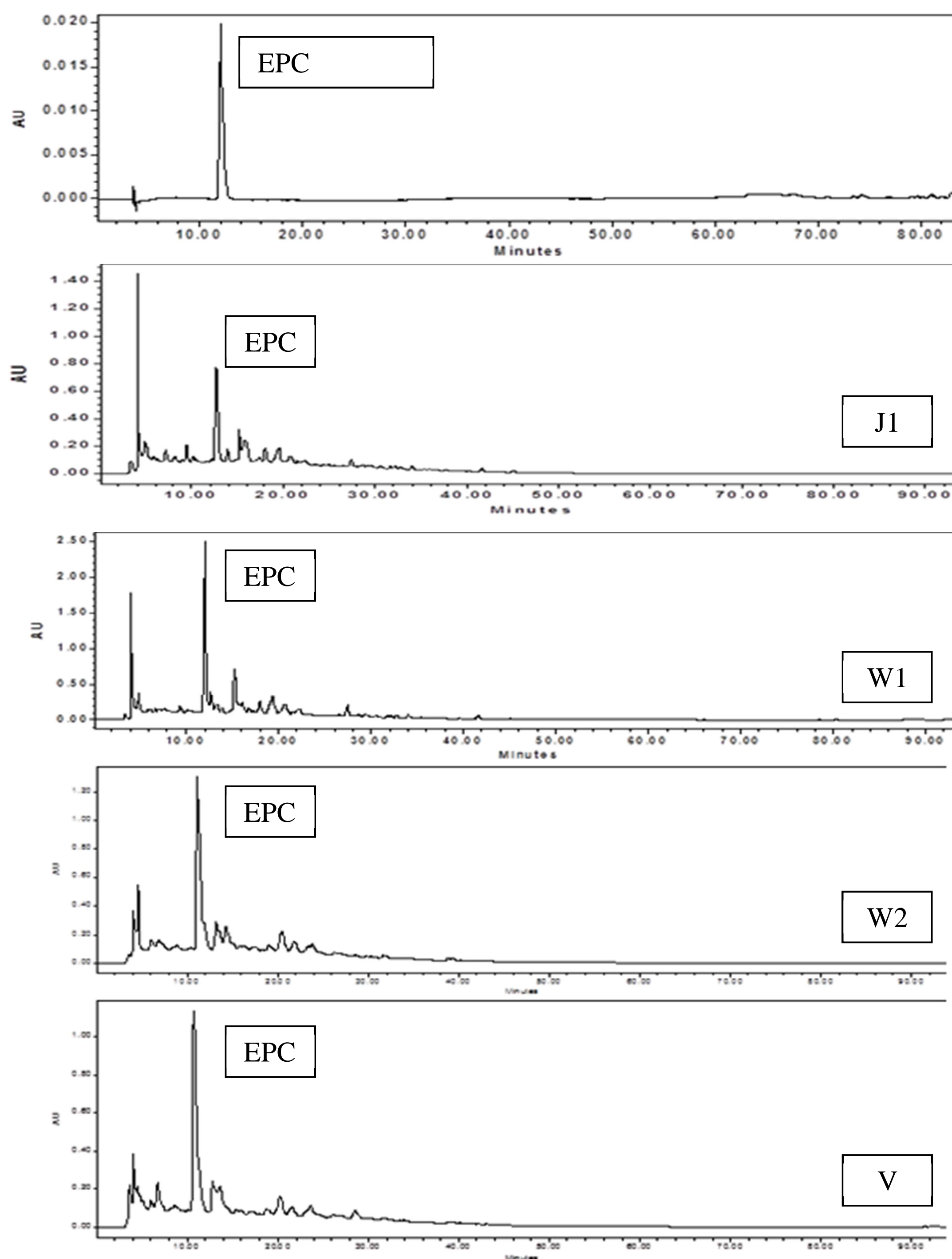
TA expressed in % malic acid for AJ, AW1, AW2, AJ, NJ, NW1, NW2 and % of acetic acid for AV & NV. AJ & NJ: astringent and non-astringent juice; AW1 & NW1: astringent and non-astringent persimmon after primary wine fermentation; AW2 & NW2: astringent and –non-astringent persimmon after secondary wine fermentation; AV & NV: astringent and non-astringent persimmon vinegar. Values are expressed as mean ± standard error.

APPENDIX E. ANTHOCYANIN CHROMATOGRAM OF TIFBLUE RABBITEYE BLUEBERRY JUICE AND FERMENTATION PRODUCTS



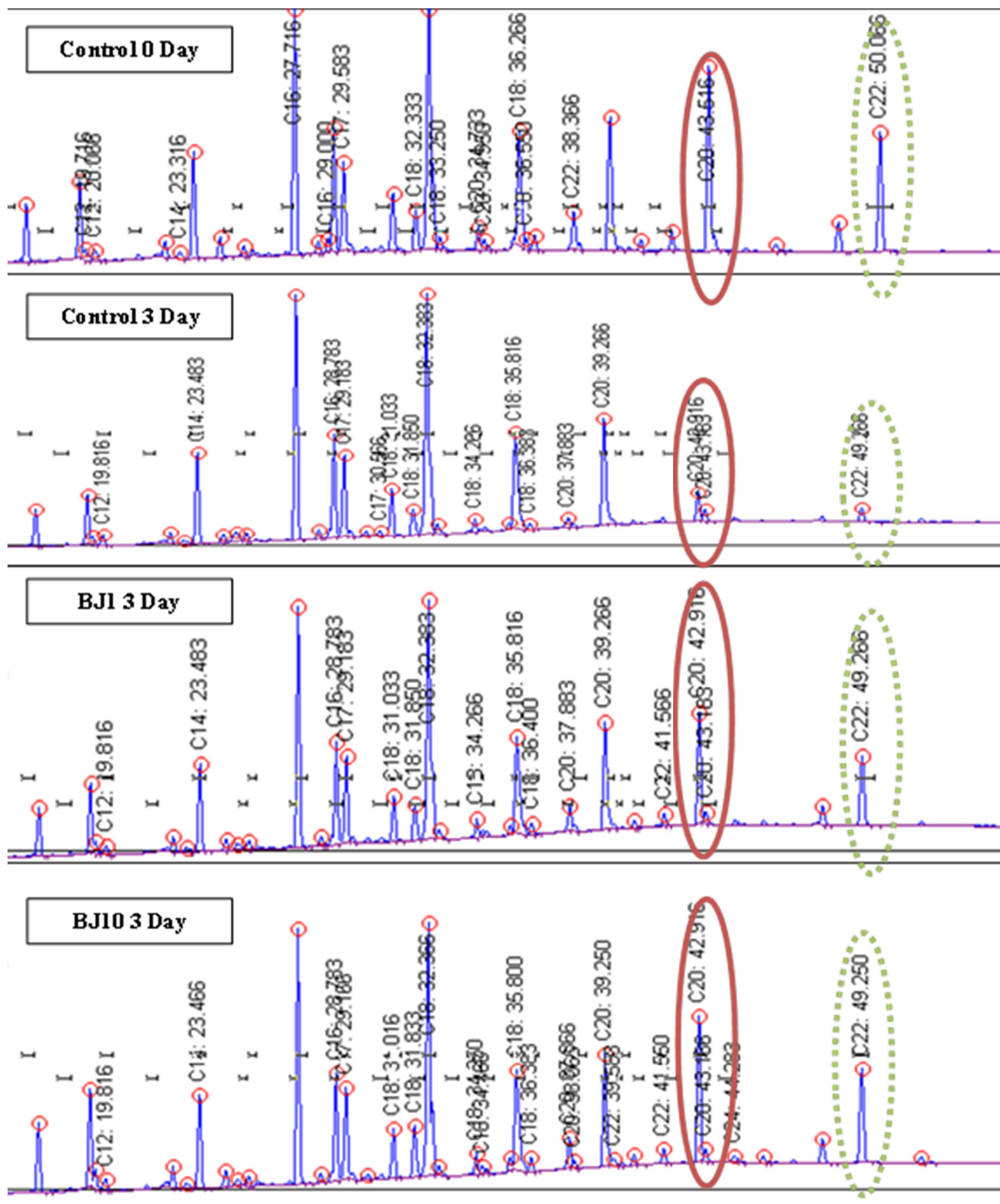
J: fresh juice; W1: fermentation product after primary wine fermentation; W2: fermentation product after secondary wine fermentation; V: fermentation product after acetification.

APPENDIX F. EPICATECHIN (EPC) CHROMATOGRAM OF TIFBLUE RABBITEYE BLUEBERRY JUICE

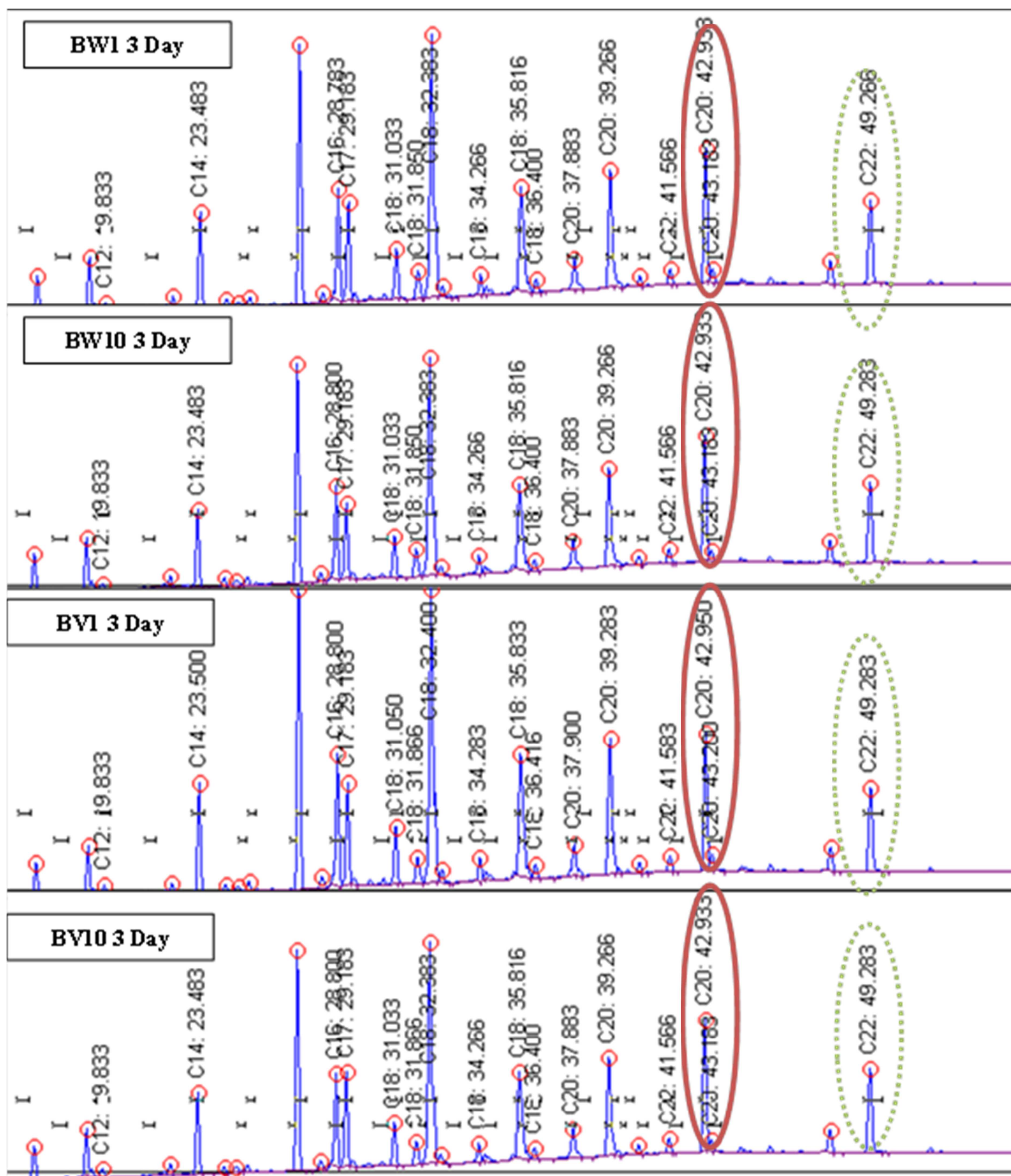


J: Blueberry juice; W1: fermentation product after primary wine fermentation; W2: fermentation product after secondary wine fermentation; V: fermentation product after acetification.

APPENDIX G. CHROMATOGRAM OF EPA AND DHA IN SALMON OIL-IN-WATER EMULSION WITH 1% AND 10% TREATMENT AT 0 DAY AND 3 DAY OF INCUBATION



Continued From Appendix F.



BJ1, BW1, BV1: treated with blueberry juice, wine, vinegar at 1%; BJ10, BW10, BV10; treated with blueberry juice, wine, vinegar at 9.7%; solid red oval: EPA; doted green oval: DHA.

APPENDIX H. SAS PROGRAM OF STATISTICAL ANALYSES

SAS Program for the Analyses of Total anthocyanin Content of Blueberry Juice and Fermentation Products

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.Anthocyanin
            DATAFILE= "C:\Thesis\SAS\Blueberry Data_1.xls"
            DBMS=EXCEL REPLACE;
     SHEET="Antho_SAS";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
     RUN;
DATA Anthocyanin;
set Anthocyanin;
ODS RTF FILE = 'C:\Thesis\SAS\Amanda_Anthocyanin_SAS';

Proc Print;
run;
PROC Mixed data=Anthocyanin;
Class Trt;
Model Anthocyanin = Trt / outp=ResidACY;
LSMEANS Trt / Adjust=Tukey;
ods output diffs=ppp;
ods output lsmeans=mmm;
run;
%include 'C:\Thesis\SAS\pdmix800.sas';
%pdmix800 (ppp, mmm, alpha=0.05, sort=no);

PROC UNIVARIATE data=ResidACY PLOT NORMAL;
Var Resid;
run;
ODS RTF CLOSE;
QUIT;
```

SAS Program for the Analyses of Epicatechin of Blueberry Juice and Fermentation Products

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.Epicatechin
            DATAFILE= "C:\Thesis\SAS\Blueberry Data_1.xls"
            DBMS=EXCEL REPLACE;
     SHEET="Epicatechin_SAS";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
     RUN;
DATA Epicatechin;
set Epicatechin;
ODS RTF FILE = 'C:\Thesis\SAS\Amanda_Epicatechin_SAS';
Proc Print;
run;
PROC mixed data=Epicatechin;
Class Trt;
Model Epicatechin = Trt / outp=ResidACY;
LSMEANS Trt / Adjust=Tukey;
ods output diffs=ppp;
ods output lsmeans=mmm;
run;
%include 'C:\Thesis\SAS\pdmix800.sas';
%pdmix800 (ppp, mmm, alpha=0.05, sort=no);

PROC UNIVARIATE data=ResidACY PLOT NORMAL;
Var Resid;
run;
ODS RTF CLOSE;
QUIT;
```

SAS Program for the Analyses of Total Phenolics of Blueberry Juice and Fermentation Products

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.TotalPhenolic
            DATAFILE= "C:\Thesis\SAS\Blueberry Data_1.xls"
            DBMS=EXCEL REPLACE;
     SHEET="Total Phenolic_SAS";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
     RUN;
DATA TotalPhenolic;
set TotalPhenolic;
ODS RTF FILE = 'C:\Thesis\SAS\Amanda_Total Phenolic_SAS';
Proc Print;
run;
PROC Mixed data=TotalPhenolic;
Class Trt;
Model TotalPhenolic = Trt / outp=ResidACY;
LSMEANS Trt / Adjust=Tukey;
ods output diffs=ppp;
ods output lsmeans=mmm;
run;
%include 'C:\Thesis\SAS\pdmix800.sas';
%pdmix800 (ppp, mmm, alpha=0.05, sort=no);

PROC UNIVARIATE data=ResidACY PLOT NORMAL;
Var Resid;
run;
ODS RTF CLOSE;
QUIT;
```

SAS Program for the Analyses of Antiradical Activity of Blueberry Juice and Fermentation Products

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.DPPH
            DATAFILE= "C:\Thesis\SAS\Blueberry Data_1.xls"
            DBMS=EXCEL REPLACE;
     SHEET="DPPH_SAS";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
     RUN;
DATA DPPH;
set DPPH;
ODS RTF FILE = 'C:\Thesis\SAS\Amanda_DPPH_SAS';
Proc Print;
run;
PROC Mixed data=DPPH;
Class Trt;
Model DPPH = Trt / outp=ResidACY;
LSMEANS Trt / Adjust=Tukey;
ods output diffs=ppp;
ods output lsmeans=mmm;
run;
%include 'C:\Thesis\SAS\pdmix800.sas';
%pdmix800 (ppp, mmm, alpha=0.05, sort=no);

PROC UNIVARIATE data=ResidACY PLOT NORMAL;
Var Resid;
run;
ODS RTF CLOSE;
QUIT;
```

SAS Program for the Analyses of EPA Remained in Salmon Oil-in-water Emulsion after 3 days of Incubation

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.EPA
            DATAFILE= "C:\Thesis\SAS\Blueberry Data_1.xls"
            DBMS=EXCEL REPLACE;
     SHEET="FA_SAS";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
     RUN;
DATA EPA;
set EPA;
ODS RTF File = 'C:\Thesis\SAS\EPA_Output';
Proc Print;
run;
PROC Mixed data=EPA;
Class Group Trt;
Model EPA = Group Trt Group*Trt / outp=ResidACY;
LSMEANS Group Trt Group*Trt / Adjust=Tukey;
ods output diffs=ppp;
ods output lsmeans=mmm;
run;
%include 'C:\Thesis\SAS\pdmix800.sas';
%pdmix800 (ppp, mmm, alpha=0.05, sort=no);
PROC UNIVARIATE data=ResidACY PLOT NORMAL;
Var Resid;
run;
ODS RTF CLOSE;
QUIT;
```

SAS Program for the Analyses of DHA Remained in Salmon Oil-in-water Emulsion after 3 days of Incubation

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.DHA
            DATAFILE= "C:\Thesis\SAS\Blueberry Data_1.xls"
            DBMS=EXCEL REPLACE;
     SHEET="FA_SAS";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
     RUN;
DATA DHA;
set DHA;
ODS RTF File = 'C:\Thesis\SAS\DHA_Output';
Proc Print;
run;
PROC Mixed data=DHA;
Class Group Trt;
Model DHA = Group Trt Group*Trt / outp=ResidACY;
LSMEANS Group Trt Group*Trt / Adjust=Tukey;
ods output diffs=ppp;
ods output lsmeans=mmm;
run;
%include 'C:\Thesis\SAS\pdmix800.sas';
%pdmix800 (ppp, mmm, alpha=0.05, sort=no);
PROC UNIVARIATE data=ResidACY PLOT NORMAL;
Var Resid;
run;
ODS RTF CLOSE;
QUIT;
```

SAS Program for the Analyses of Antiradical Activity of Persimmon Juice and Fermentation Products

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.DPPH
            DATAFILE= "C:\Thesis\SAS\Persimmon Data_1.xls"
            DBMS=EXCEL REPLACE;
     SHEET="DPPH_SAS";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
     RUN;
DATA DPPH;
set DPPH;
ODS RTF FILE = 'C:\Thesis\SAS\Amanda_PersimmonDPPH_SAS';
Proc Print;
run;
PROC Mixed data=DPPH;
Class Trt;
Model DPPH = Trt / outp=ResidACY;
LSMEANS Trt / Adjust=Tukey;
ods output diffs=ppp;
ods output lsmeans=mmm;
run;
%include 'C:\Thesis\SAS\pdmix800.sas';
%pdmix800 (ppp, mmm, alpha=0.05, sort=no);

PROC UNIVARIATE data=ResidACY PLOT NORMAL;
Var Resid;
run;
ODS RTF CLOSE;
QUIT;
```

SAS Program for the Analyses of Total Phenolics of Persimmon Juice and Fermentation Products

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.TotalPhenolic
            DATAFILE= "C:\Thesis\SAS\Persimmon Data_1.xls"
            DBMS=EXCEL REPLACE;
     SHEET="Total Phenolic_SAS";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
     RUN;
DATA TotalPhenolic;
set TotalPhenolic;
ODS RTF FILE = 'C:\Thesis\SAS\Amanda_Persimmon Total Phenolic_SAS';
Proc Print;
run;
PROC Mixed data=TotalPhenolic;
Class Trt;
Model TotalPhenolic = Trt / outp=ResidACY;
LSMEANS Trt / Adjust=Tukey;
ods output diffs=ppp;
ods output lsmeans=mmm;
run;
%include 'C:\Thesis\SAS\pdmix800.sas';
%pdmix800 (ppp, mmm, alpha=0.05, sort=no);

PROC UNIVARIATE data=ResidACY PLOT NORMAL;
Var Resid;
run;
ODS RTF CLOSE;
QUIT;
```

SAS Program for the Analyses of Total Anthocyanin of Black Raspberry Juice and Fermentation Products After Treated with 1% Proteases

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.Anthocyanin
            DATAFILE= "C:\Thesis\SAS\BRB Data_1.xls"
            DBMS=EXCEL REPLACE;
     SHEET="Antho_SAS";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
     RUN;
DATA Anthocyanin;
set Anthocyanin;
ODS RTF FILE = 'C:\Thesis\SAS\Amanda_BRBAnthocyanin_SAS';

Proc Print;
run;
PROC Mixed data=Anthocyanin;
Class Trt;
Model Anthocyanin = Trt / outp=ResidACY;
LSMEANS Trt / Adjust=Tukey;
ods output diffs=ppp;
ods output lsmeans=mmm;
run;
%include 'C:\Thesis\SAS\pdmix800.sas';
%pdmix800 (ppp, mmm, alpha=0.05, sort=no);

PROC UNIVARIATE data=ResidACY PLOT NORMAL;
Var Resid;
run;
ODS RTF CLOSE;
QUIT;
```

SAS Program for the Analyses of Total Anthocyanin of Black Raspberry Juice and Fermentation Products After Treated with 0.1% and 0.01% Proteases

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.Anthocyanin
            DATAFILE= "C:\Thesis\SAS\BRB Data_1.xls"
            DBMS=EXCEL REPLACE;
     SHEET="Antho_SAS1";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
     RUN;
DATA Anthocyanin;
set Anthocyanin;
ODS RTF FILE = 'C:\Thesis\SAS\Amanda_BRBAnthocyanin1_SAS';

Proc Print;
run;
PROC Mixed data=Anthocyanin;
Class Trt;
Model Anthocyanin = Trt / outp=ResidACY;
LSMEANS Trt / Adjust=Tukey;
ods output diffs=ppp;
ods output lsmeans=mmm;
run;
%include 'C:\Thesis\SAS\pdmix800.sas';
%pdmix800 (ppp, mmm, alpha=0.05, sort=no);

PROC UNIVARIATE data=ResidACY PLOT NORMAL;
Var Resid;
run;
ODS RTF CLOSE;
QUIT;
```

VITA

Yen-Ping Tan was born on February, 23, 1985, in Johor, Malaysia. After she graduated from a local high school in Malaysia, she attended an America University Transfer Program in January 2004. A year later, she was transferred to Louisiana State University and continued her undergraduate studies in food science. In May 2007, she earned her Bachelor of Science degree and returned to her home country and worked for a cocoa manufacturing company. In summer of 2009, she decided to pursue a graduate degree in Louisiana State University in the Department of Food Science. She conducted research under the supervision of Dr. Paul Wilson. She is a candidate for the degree of Master of Science in food science in August 2009.