EFFECTS OF IMMEDIATE POST-HARVEST FREEZING CONDITIONS AND STORAGE TEMPERATURE ON THE COMPOSITION OF NORTON GRAPES

A Thesis

Presented to

The Faculty of the Graduate School

At the University of Missouri-Columbia

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

By

XIAO FENG

Dr. Ingolf Gruen, Thesis Supervisor

MAY 2014

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

EFFECTS OF IMMEDIATE POST-HARVEST FREEZING CONDITIONS AND STORAGE TEMPERATURE ON THE COMPOSITION OF NORTON GRAPES

Presented by XIAO FENG,

A candidate for the degree of Master of Science,

And hereby certify that, in their opinion, it is worthy of acceptance.

Ingolf Gruen, Ph.D., Department of Food Science

Misha T. Kwasniewski, Ph.D., Department of Food Science

Wenping Qiu, Ph.D., Center for Grapevine Biotechnology,

Missouri State University

ACKNOWLEDGEMENTS

I would like to give my sincere thanks to my advisor, Dr. Ingolf Gruen, for his guidance in the research and academic study during these three years in University of Missouri-Columbia. Thank you for your patience and encouragement. In addition, I would like to thank Dr. Misha Kwasniewski for his assistance in this research and Dr. Wenping Qiu who took great interests in my research.

I would like to thank Lakdas Fernando, Jackie Harris and Trudi Black for their crucial supports and assistance in helping me figure out different problems. I would like to thank all the staffs in Grape and Wine Institute and my lab mates I worked with. Without your great support and collaboration, it could not have been possible for me to finish this research.

I really want to thank all of my family, friends and my boyfriend. Thank you for your encouragements and great comfort in these three years during my study.

I also want to give my thanks to Dr. Anthony Peccoux, who passed away one and half years ago. I will remember this knowledgeable professor and good, reliable friend forever.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS....ii

LIST OF FIGURES....vi

LIST OF TABLES....vii

ABSTRACT....viii

CHAPTER

INTRODUCTION....1

LITERATURE REVIEW....3

2.1 History of Norton Grapes....3

2.2 Berries of Norton Grapes....4

2.3 Phenolic Compounds....5

2.4 Anthocyanins....6

2.4.1 Structure and Chemical Properties....6

2.4.2 Biological Properties of Anthocyanins....7

2.4.3 Malvidin....9

2.5 Phenolic Acids....10

2.5.1 Gallic Acid....12

2.5.2 Ferulic Acid....13

2.6 Stilbenes....14

2.6.1 Distribution and Structure of *trans*-Resveratrol....15

2.6.2 Biological Activity of *trans*-Resveratrol....16

2.7 Changes of Chemical Profiles of Grapes in Frozen Storage after Harvest 17

MATERIALS AND METHODS....19

3.1 Chemicals....19

3.2 Experimental Design....19

3.3 Norton Grape Samples Selection and Preparation 20
3.3.1 Grape Harvest 20
3.3.2 Preparation of Grape Skins and Juice 20
3.4 Chemical Analyses 21
3.4.1 pH Measurement 21
3.4.2 Titratable Acidity Measurement 21
3.4.3 Brix Measurement 21
3.5 HPLC Analysis of Anthocyanins 22
3.5.1 Grape Skin Sample Preparation 22
3.5.2 HPLC Conditions 22
3.5.3 Quantitation of Anthocyanins 23
3.6 HPLC analysis of phenolic acids and *trans*-resveratrol 23
3.6.1 Grape Skin Sample Preparation 23
3.6.2 HPLC Conditions 24
3.6.3 Quantitation of Phenolic Acids and *trans*-Resveratrol 24
3.7 Statistical Analysis 24
RESULTS AND DISCUSSION 26
4.1 Basic Chemical Analyses of Grape Juice 26
4.1.1 Results of pH Measurement 26
4.1.2 Results of Titratable Acidity 31
4.1.3 Brix Measurement 34
4.2 HPLC Analysis of Anthocyanins of Norton Grape Skin 37
4.2.1 Results of Malvidin-di-glucoside 37
4.2.2 Results of Malvidin-glucoside 40
4.3 HPLC Analysis of Phenolic Acids of Norton Grape Skin 42

4.3.1 Results of Gallic Acid 42
4.3.2 Results of Ferulic Acid 44
4.4 HPLC Analysis of *trans*-Resveratrol of Norton Grape Skins...................... 46
CONCLUSION AND FUTURE RESEARCH.. 50
5.1 Conclusion 50
5.2 Future Research... 51
REFERENCES 52
APPENDIX 57

LIST OF FIGURES

Figure Page

2-1 Classification of phenolic compounds based on their structures 6

2-2 Structures of main anthocyanidins in grapes.. 7

2-3 Structure of Malvidin 3- glucoside 9

2-4 Structure of Malvidin 3, 5- diglucoside.. 10

2-5 The structures of Hydroxybenzoic acids compounds 11

2-6 The structures of Hydroxycinnamic acids compounds 12

2-7 The structure of trans-resveratrol 16

4-1 pH value of grape juice on regular ice (R) transportation method 28

4-2 pH value of grape juice on dry ice (DI) transportation method...................... 29

4-3 pH value of grape juice on liquid nitrogen (LN) transportation method......... 29

4-4 Titratable acidity of grape juice on regular ice (R) transportation method 32

4-5 Titratable acidity of grape juice on dry ice (DI) transportation method.......... 33

4-6 Titratable acidity of grape juice on liquid nitrogen (LN) transportation method33

4-7 Brix value of grape juice on regular ice (R) transportation method............... 35

4-8 Brix value of grape juice on dry ice (DI) transportation method.................... 36

4-9 Brix value of grape juice on liquid nitrogen (LN) transportation method 36

4-10 Malvidin-di-glucoside in Norton grape skins 38

4-11 Malvidin-glucoside in Norton grape skins 41

4-12 Gallic acid in Norton grape skins 43

4-13 Ferulic acid in Norton grape skins 45

4-14 *trans*-Resveratrol in Norton grape skin.. 47

LIST OF TABLES

Table Page

4-1 Linear mixed model analysis of pH value 26

4-2 Pairwise Comparisons of dry ice, liquid nitrogen and regular ice group 27

4-3 Results of pH value in regular ice group 27

4-4 Results of pH value in dry ice group 27

4-5 Results of pH value in liquid nitrogen group 28

4-6 Linear mixed model analysis of TA 31

4-7 Results of TA in regular ice group 31

4-8 Results of TA in dry ice group 31

4-9 Results of TA in liquid nitrogen group 32

4-10 Pairwise Comparisons of different frozen storage time on Brix (0 month, 1-month, 3-month, 6-month) 35

4-11 Linear mixed model analysis of malvidin-di-glucoside 38

4-12 Results of malvidin-di-glucoside (mg/g FW) in Norton grape skins 38

4-13 Linear mixed model analysis of malvidin-glucoside 40

4-14 Results of malvidin-glucoside (mg/g FW) in Norton grape skins 40

4-15 Two-way ANOVA analysis of gallic acid 42

4-16 Results of gallic acid (mg/g DW) in Norton grape skins 42

4-17 Two-way ANOVA analysis of ferulic acid 44

4-18 Results of ferulic acid (mg/g DW) in Norton grape skins 44

4-19 Two-way ANOVA analysis of trans-resveratrol 46

4-20 Results of *trans*-resveratrol (mg/g DW) in Norton grape skins 46

EFFECTS OF IMMEDIATE POST-HARVEST FREEZING CONDITIONS AND STORAGE TEMPERATURE ON THE COMPOSITION OF NORTON GRAPES

Xiao Feng

Dr. Ingolf Gruen, Thesis Supervisor

ABSTRACT

Norton is one of the most famous grapes widely planted in the state of Missouri. This research focused on optimizing the storage conditions and transportation methods in preserving frozen grapes for medium to long term academic research.

Composition changes of Norton grapes in juice and skins were analyzed for optimizing the transportation methods and frozen conditions. Three different transportation methods (on Regular Ice, Dry Ice and Liquid Nitrogen) and two different storage conditions (at -80 °C and -20 °C) were used to preserve grape samples. In this research, the grape quality characters (pH, titratable acidity, Brix) in grape juice were analyzed for the different treatments mentioned above over time (Fresh, 1 month, 3 months and 6 months). The phenolic compounds in grape skins: anthocyanins (Malvidin-di-glucoside, Malvidin-glucoside), phenolic acids (gallic acid, ferulic acid) and a stilbene (trans-resveratrol) were analyzed by High Pressure Liquid Chromatography (HPLC). The results indicate the composition and quality parameters were changed both in grape juice and skins under different conditions. The best condition for transporting and preserving Norton grapes was the treatment of transporting on regular ice and storing at

-80 °C. This method is also practical for academic research, because it is economical and easily obtainable.

CHAPTER 1

INTRODUCTION

As the official grape of the state of Missouri, Norton (*Vitis aestivalis*) is famous for its high disease resistance, high anthocyanin content, and being a late ripening variety. In addition, Norton wine is produced as the premium red wine in Missouri and has also become more and more popular in other Midwest states of the US. This is due to its unique flavor and taste. In *The Economic Impact of Wine and Grapes in Missouri 2010* (Stone bridge Research 2010), Norton was the largest grape crop in Missouri, and the growth of Norton increased from 213.5 acres in 2005 to 307.9 acres in 2009; this growth contributed to Norton being almost 20% of the entire Missouri harvest. Norton plantings grew faster than other grape varieties, thus growing its share of the industry. Because of the great commercial value of Norton grapes, the Missouri wine industry continues to invest funds and energy to do research on improving viticultural techniques, improving winemaking processes, and improving sensory analysis of Norton grapes and wines.

Vine physiologists, viticulturists and enologists performed a large amount of chemical analyses of grapes and must during harvest for numerous research projects. However, because of facility limitations, the amount of time and labor in the lab, it is impossible for researchers to deal with hundreds of samples fast enough to avoid potential chemical changes on grapes after harvest. Therefore,

freezing is widely used as a convenient and effective method to preserve a large amount of grapes for future analyses.

While this is the preferred method of storage, it can have an adverse effect on the composition of the grapes resulting in differing analytical results compared to freshly harvested berries. Research has shown that pH and titratable acidity might change due to freezing in different varietals of grapes, but effect on other more complex compounds, such as polyphenols and anthocyanins are rarely reported (Garcia and others 2011). In addition, the frozen storage time and methods for sample preservation also varies a lot between different research projects. Therefore, it is important to optimize the frozen conditions during freezing and frozen storage. (Santesteban and others 2013; Cynkar and others 2004; Garcia and others 2011).

CHAPTER 2

LITERATURE REVIEW

2.1 History of Norton Grapes

The exact origin of Norton is controversial. The most persuasive explanation is in Ambers and Ambers (2004) literature about the history of Norton grapes. Norton grape was first mentioned in *Treatise on the Vine* written by William Prince in 1830. This book indicated Norton was a hybrid of Bland, a cross of *labrusca* × *vinifera* with a white parent, and a wild vine of *Vitis aestivalis*. The mother vine Bland is now extinct. This vine was described by the "foxy" characters of *Vitis labrusca*. Some characters in Norton may support this explanation. The slightly foxy flavor that occasionally occur in Norton grapes, and white and pale fruit color in one- third of self-pollinated Norton seedlings also indicate its heritage of a white grape (Ambers and Ambers 2004).

The vines of Norton are highly resistant of fungus disease (Fung and others 2008). Norton can accumulate high amounts of endogenous salicylic acid (SA) in the leaves regulated by SA-associated defense genes, which plays an important role in transmitting signals across pathways to initiate a defense response (Raymond and others 2008). The extraordinary disease resistance of Norton helps viticulturists reduce the usage of pesticides. However, the summer climate in Missouri is usually warm and humid, so Norton grapes are still very vulnerable to many diseases, insects and molds.

Norton grapes are widely planted in Arkansas, Illinois, Indiana, Kansas, Louisiana, Maryland, Missouri, Oklahoma, New Jersey, Pennsylvania, Tennessee, Texas, Virginia, and West Virginia (Ortinau 2009). As the state grape of Missouri, Norton wine (also called Cynthiana) represented almost 20% of wine production in 2010 (Stone bridge research 2010). Norton produces a medium to full bodied dry red wine with hints of spice and berry. Other wine chemical characters are: pH (> 3.5); titratable acidity (up to 15 g/liter); malate (up to 6 g/liter); and potassium (up to 6 g/liter) (Smiley 2008)

2.2 Berries of Norton Grapes

Norton is one of the last grape crops to be harvested in Missouri. The clusters are inclined from medium to small, usually single- shouldered. The ripe Norton berries are small to medium, roundish to oblate, blue- black, and covered with heavy blue bloom. The flesh is described as greenish, translucent, juicy with spicy, tart and astringent characters. Sugar content of fully ripened berries exceeds 20°Brix. Ripe Norton berries contain two to six small, brownish seeds that separate easily from the pulp (Hedrick and others 1908).

The skin of Norton are described as tough, astringent and easily separated from the pulp, which contains a large amount of anthocyanins, polyphenols and flavonoids. According to the literature, anthocyanins in Norton grapes are much higher than other varieties in Middle America, such as Marechal Foch and Concord (Munoz-Espada 2004). The anthocyanins is 258±37 mg/100 g of wet weight in Foch, 888±78 mg/100 g for Norton, and 326±5.9 mg/100 g for Concord grapes (Munoz-Espada 2004). The major anthocyanins in Norton

grapes are malvdin 3, 5-diglucoside and malvidin-glucoside, which contribute to 45% and 31% to total anthocyanins, respectively (Hogan and others 2009, Ali 2011). The abundant anthocyanins in skins substantiate the dark blue characters of Norton grapes.

Phenolic acids and *trans*-resveratrol are subdivisions of the category of non-flavonoid phenols. The primary phenolic acid in Norton skins and seeds is gallic acid, which accounts for 55% of total phenolic acids. Other phenolic acids include vanillic acid, chlorogenic acid, caffeic acid, coumaric acid, and ferulic acid.

2.3 Phenolic Compounds

Phenolic compounds are a group of secondary metabolites in plants, regulating the growth and reproduction of plants, producing defending responses to pathogens and disease attack. As one of the anti-oxidation ingredients in food, phenolic compounds may decrease oxidation rates and extend the shelf-life of fruits and vegetables (Karakaya 2004). In addition, the phenolic compounds show other functions in plants: the protection of organs from UV radiation and pigmentation, defense against pathogens, and attraction of pollinators and seed dispersers (Ramirez-Lopez 2011). There are four thousand phenolic compounds in nature. In grapes, polyphenolic compounds can be divided into two large groups: non-flavonoids and flavonoid compounds. They are mainly responsible for flavor, color, bitterness and astringency (Vilanova and others 2009).

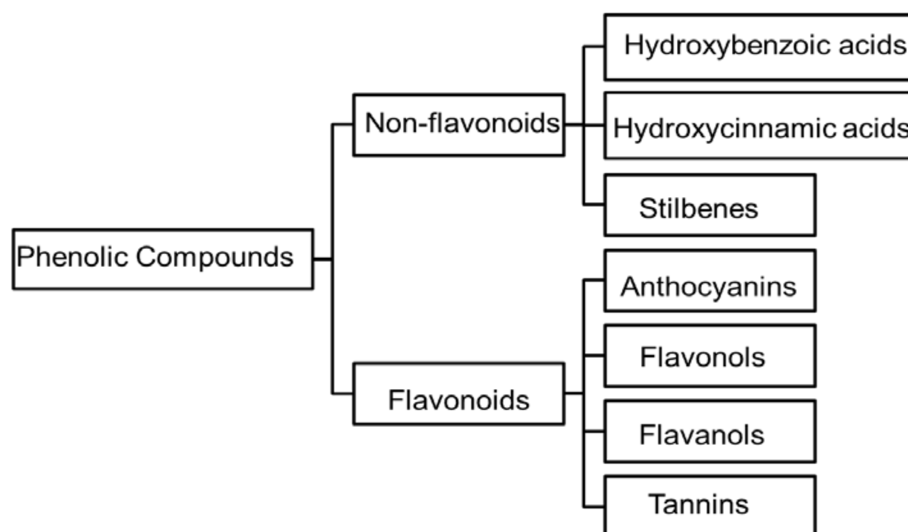


Figure 2-1. Classification of phenolic compounds based on their structures

2.4 Anthocyanins

Anthocyanins are abundant in existing plants, fruits and flowers as a group of natural pigments. They contribute to a broad range of colors in plants from red to blue. As one of the phenolic compounds, anthocyanins fulfill great biological functions, such as enhancing radical oxygen scavenging ability and reducing the risks of chronic diseases (Prior and Wu 2006).

2.4.1 Structure and Chemical Properties

Anthocyanins are a subdivision of flavonoids- a kind of heterosides whose aglycone or anthocyanidin is derived from the flavylium or 2-phenylbenzopyrilium (Eugeane and others, 2007). There are 21 different anthocyanidins according to the literature, but six of them are most common: pelargonidin, cyanidin, malvidin, delphinidin, petunidin, and peonidin. Their distribution in fruits and vegetable is 50% cyanidin, 12 % delphinidin, 12% pelargonidin, 12% peonidin, 7% Petunidin and 7 % malvidin. The most common glycoside widespread forms found in

nature are 3-monosides, 3-biosides, 3,5- and 3,7-diglucosides. The cyanidin- 3-glucoside has the largest proportion in nature (Araceli and others 2009).

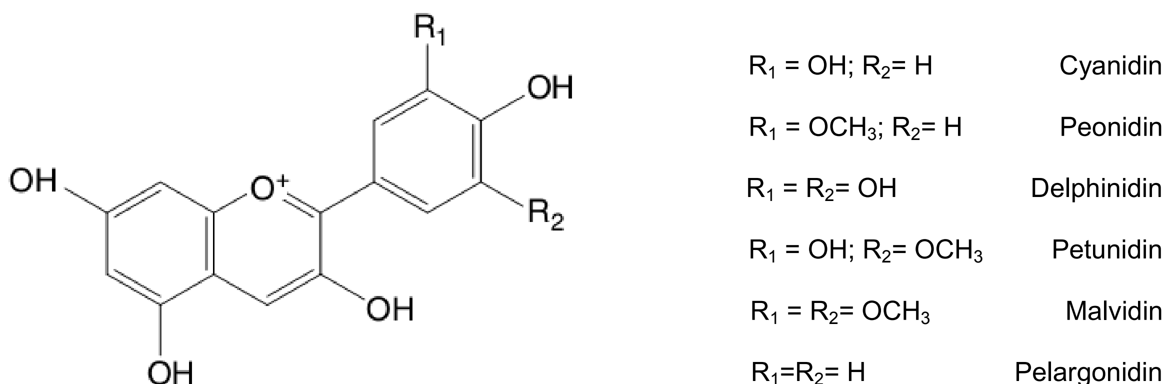


Figure 2-2. Structures of main anthocyanidins in grapes (Flamini 2008)

The structures of anthocyanins indicate their good dissolving abilities in polar solvents, such as- water, ethanol, acetone, and methanol. Considering the safety of solvent extraction in the food industry, ethanol is often used (Eugeane and others 2007). However, in this research, methanol was chosen as an extraction solvent, because of its higher extraction efficiency and easy separation.

Many factors will influence the stability of anthocyanins such as pH, solvents, temperature, anthocyanin concentration, oxygen, light and enzymes. Strong acids, such as hydrochloric acid, cannot be used as the extraction solvents, because they may hydrolyze acylated anthocyanins. To minimize the breakdown of anthocyanins, weak organic acids, such as formic, citric and acetic acids are preferred in acidic extraction.

2.4.2 Biological Properties of Anthocyanins

In recent years, research found that anthocyanins exhibit positive effects in preventing and curing many diseases. They show anti-inflammatory, anti-oedema, anti-carcinogenic activities and cardiovascular disease seem to be

effective against in both *in-vitro* and *in-vivo* studies (Wagner 1985; Ghiselli and others 1998). The literature indicates cancer cell proliferation *in-vitro* was effectively inhibited by anthocyanins. The mechanism of anthocyanins decreasing the cancer cells proliferation is based on blocking regulator proteins in different stages of cell cycles. The whole cell proliferation was stopped. In addition, more investigation has been done on comparing the effects of anthocyanins on normal vs. cancer cells. Interestingly, anthocyanins were very effective in inhibiting the growth of cancer cells, but had almost little or no influence on normal cells. This result indicated that anthocyanins can be used as a potential innovative approach for cancer treatment (Zhang and others 2005; Hakimuddin and others 2004).

In *in-vivo* studies, anthocyanins are very effective in inhibiting cancers, such as esophageal cancer, colon cancer, skin cancer and lung cancer (Wang and Stoner 2008). In the research of Wang and Stoner, Fischer 344 rats were regularly injected with N-nitrosamine carcinogen for 20-25 weeks until esophageal tumors appeared. The researchers utilized freeze-dried berries and berry extract to inhibit the development of tumor cells. The results indicated cancer of the rodent esophagus can be inhibited by 30–60% and tumor of the colon can be reduced by up to 80%. The mechanism is that anthocyanins are effective in inhibiting the initiation and promotion/progression stages of tumor development. Anthocyanins influence the carcinogen metabolism in the initiation of the tumor cell cycle and lead to less DNA damage caused by carcinogens.

2.4.3 Malvidin

Malvidin is widely distributed in many vegetables and fruits, such as red grapes, cranberries, blueberries and black rice. In an acidic solution, malvidin exhibits a red color. In an alkaline solution, malvidin shows a blue color. Malvidin is not the most common anthocyanin in nature, but in grapes and wine, malvidin anthocyanins are the primary anthocyanins. Malvidin 3-glucoside, malvidin 3-glucoside acetate and malvidin 3- glucoside coumarate are the most abundant pigments in red wine (Lapidot and others 1999). In Norton grape berries, the two most abundant Malvidin anthocyanins were Malvidin 3- glucoside and Malvidin- 3, 5- diglucoside. The structures of these two malvidin anthocyanins are listed below.

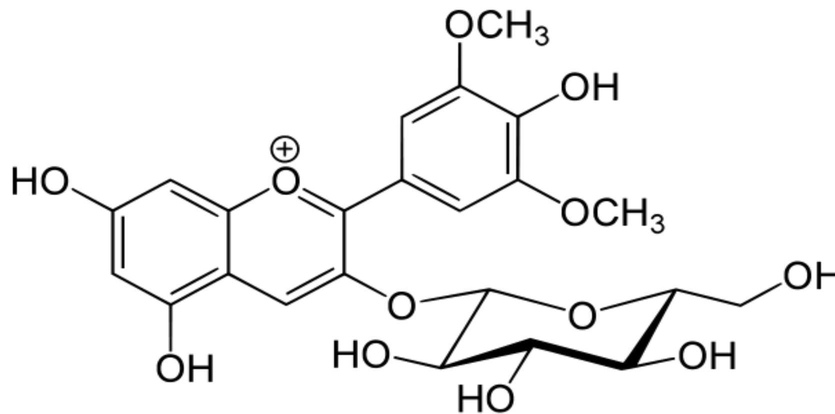


Figure 2-3. Structure of Malvidin 3- glucoside (Yikrazuul 2009)

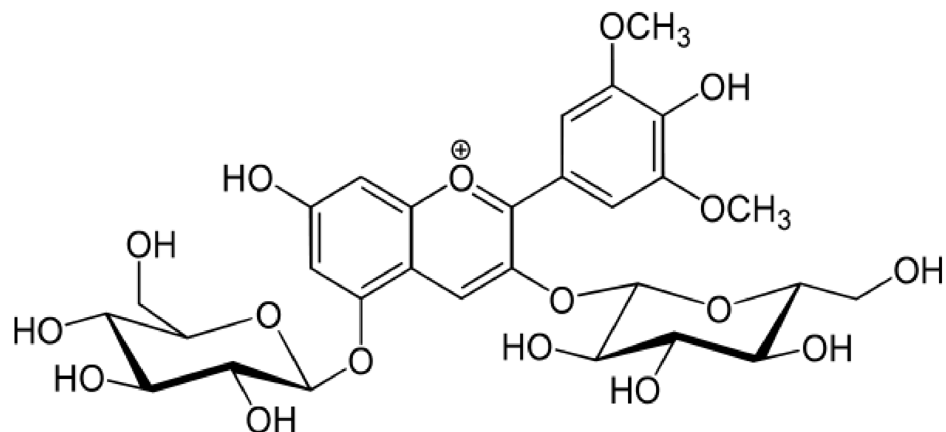


Figure 2-4. Structure of Malvidin 3, 5- diglucoside (Yikrazuul 2011)

Malvidin can prevent cardiovascular and inflammatory diseases as one of the major phenol compounds in red wine. A lot of medical and molecular research on pharmaceutical effects of malvidin has shown that malvidin have preventive and curative effects on these diseases. In an *in-vitro* study, malvdin showed cytotoxic properties to human leukemia cells and HT-29 colon cancer cells. The mechanism is that malvidin stops the G (2)/M phase of cell cycle and induces cellular apoptosis (Hyun and Chung 2004; Patterson and others 2008).

2.5 Phenolic Acids

Phenolic acids are aromatic secondary metabolites that exist widely in plants and which are classified as non-flavonoids. The major functions of phenolic acids in plants are related to nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components, and allelopathy (Saxena and others 2012). In food systems, the carboxylic acid group also contributes other important properties to vegetables such as taste, flavor, mouthfeel and health promoting effects.

In regard to biological properties, phenolic acids and flavonoids show strong anti-oxidation activity in protection of cells from oxidative stress. Too much ROS (reactive oxygen species) and a decrease of antioxidant levels may cause damage to cells and tissues. This process is called oxidative stress. Phenolic acids show a strong ability to scavenge free radicals and enhance antioxidant levels to prevent damage (Tian and others 2007). According to the structures, there are two major classifications: hydroxybenzoic acids and hydroxycinnamic acids.

Hydroxybenzoic acids and hydroxycinnammic acids consist of benzene with a carboxylic group (benzoic acids) or a propenoic acid (cinnamic acids). In nature, the concentrations of hydroxybenzoic acids are low in plants. Coffee has a very high concentration of hydroxycinnammic acids. The common compounds of hydroxybenzoic acids were gallic, vanillic, syringic and p-hydroxybenzoic acid. The derivatives of hydroxycinnamic acids are mainly caffeic, ferulic, sinapic and p- coumaric acid. Gallic acid and caffeic acid are the most common benzoic and cinnammic acid. The caffeic acids account for in 70% of cinnammic acids. The structures of these compounds are shown below.

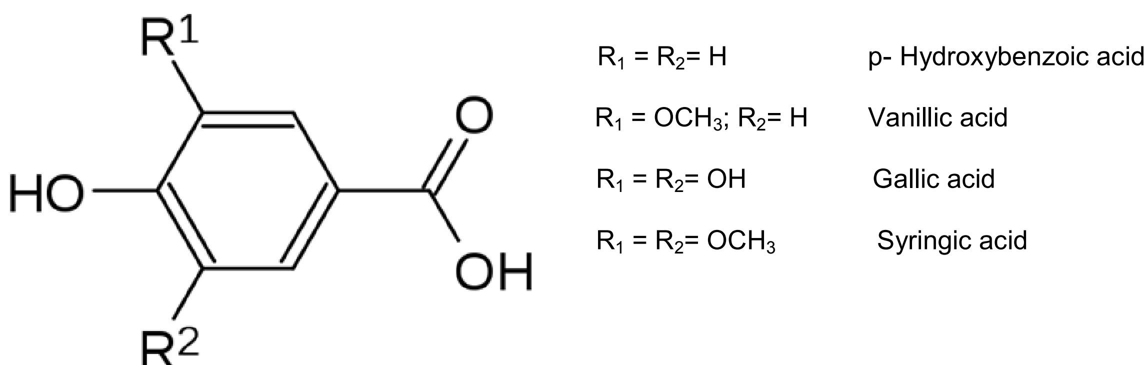


Figure 2-5. The structures of Hydroxybenzoic acids compounds (Mattern 2009)

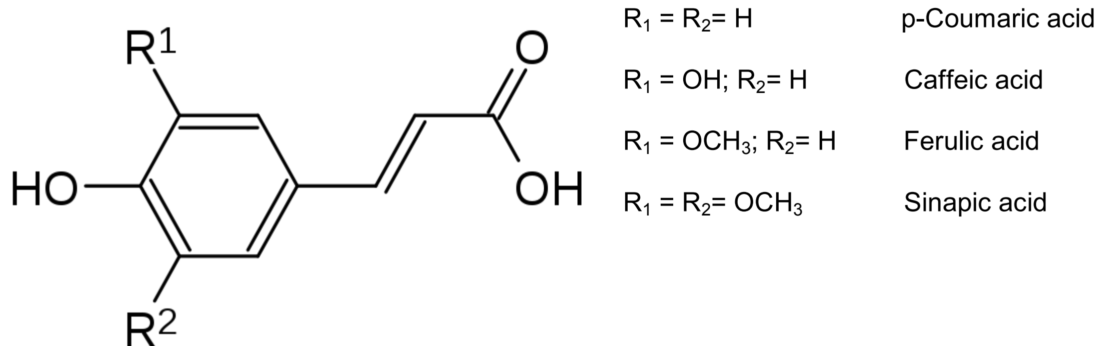


Figure 2-6. The structures of Hydroxycinnamic acids compounds (Mattern 2009)

2.5.1 Gallic Acid

Gallic acid (3, 4, 5-trihydroxybenzoic acid) is widely distributed in different varieties of vegetables, plants, fruits and nuts, such as tea leaves, oak bark, strawberries, pineapples, lemons and gallnuts (Kaur and others 2009). There are two major forms of gallic acids: free molecule form and compound form as part of tannins. It can be obtained by hydrolysis of hydrolysable tannins in acidic conditions (Locatelli and others 2013). In red wine, gallic acid accounts for 46% (g/g) of total phenolic acids (Monagas and others 2005). Many derivatives of gallic acid, such as methyl, propyl, octyl and dodecyl gallates are also broadly used in the food, pharmaceutical and cosmetic industries (Locatelli and others 2013).

Regarding the biological properties, the anti-cancer properties of gallic acid have been widely studied in recent years. In addition, the recent research on gallic acid shows positive effects against high fat diet- induced dyslipidaemia, hepatic steatosis, anti-allergic and anti-ulcer abilities (Locatelli and others 2013). Multiple studies indicated gallic acid has a very strong absorption and metabolic

rate. In an *in-vivo* study, the highest concentration of absorption reached 0.71 μM in 60mins in rats (Konishi and others 2004a). The digestive system (such as the stomach and small intestine) is the main absorption site. Additionally, the absorption of gallic acid might not be influenced by the food matrix. The literature indicated that gallic acid in Assam black tea and gallic acid in the pure molecule form (4-*O*-methylated gallic acid) have similar results in regard to absorption rate (Shahrzad 1998, 2001).

2.5.2 Ferulic Acid

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is broadly distributed in fruits (citrus fruits, banana, grapes), vegetables (eggplant, bamboo shoots, beetroot, cabbage, spinach and broccoli), and especially in rice bran pitch, whole grain, and peels of fruits and vegetables with relatively high concentrations. People can also absorb ferulic acid from natural extracts of food, such as coffee, herbs and spices (Zhao and others 2008). Derived as one of the metabolites of biosynthesis of lignin from phenylalanine and tyrosine, ferulic acid exists in plants in two forms: free and conjugated form. Ferulic acd is rarely found in its free form in nature and is generally esterified with quinic, glucaric, galactaric, malic and tartaric acids or mono-, di-, and polysaccharides (Zhao and others 2008; Baumann 2005)

As one of the phenolic acids in plants, ferulic acid has strong anti- oxidant, anti- cancer capabilities. In addition, ferulic acid can prevent other oxidative stress diseases, such as diabetes (Ramar and others 2012), Alzheimer’s disease (Kanaya 2010), hypertension (Suzuki and others 2007), and atherosclerosis (Ou

and others 2004). One of the most important applications of ferulic acid is in the cosmetic industry for cutaneous benefits. Alpha-tocopheryl ferulate (alpha-TF), a vitamin E/ferulic acid compound, has the capacity to scavenge free radicals introduced by UV radiation and inhibited melanin formation. The research by Funasaka indicated alpha-TF showed stronger abilities than other chemical compounds, such as arbutin, kojic acid, ascorbic acid, and tranexamic acid in *in-vitro* studies. His further research indicated alpha- TF also inhibited melanin formation in normal human melanocytes. A solution of 30 μg/mL of alpha- TF in 150 μg/mL of lecithin effectively inhibited melanization without influencing the growth of cells. Thus, ferulic acid may be a good whitening agent and improve UV-induced facial hyperpigmentation (Funasaka and others 2000).

2.6 Stilbenes

Stilbenes are a class of plant secondary metabolites derived from phenylpropanoid. When plants face biotic and abiotic stresses in nature, some of them produce anti- microbial compounds in response to a pathogen or herbivore attack. These compounds are classified as phytoalexins (Chong and others 2009). Stilbenes are a type of phytoalexin that is not widely distributed in the plant kingdom. Natural stilbenes exist in a few edible plants like grape, pine, peanut and sorghum (Chong and others 2009). The concentration of stilbenes increase in grapevines when attacked by grey mold, downy mildew and berry rot (Dai and others 2012). The structural feature of stilbene is a 1,2-diphenylethylene nucleus and the classification of stilbenes is very complex, stilbenes can be generally divided into two categories: monomeric and oligomeric stilbenes (Shen

and others 2009). Oligomeric stilbenes are produced with homogeneous or heterogeneous monomeric stilbenes and can be classified into several groups according to their diverse skeletons and configurations. One of the oligomeric stilbenes is *trans*-resveratrol.

In recent years, researchers have developed more interest in stilbenes due to their biological activity and clinical potentials. Stilbenes have very good healthful properties, such as preventing cardiovascular diseases and reducing carcinogenic and tumor possibilities. In addition, stilbenes also show antibacterial and antifungal capacities in *in-vitro* studies, for example, Pinosylvin and its derivatives are very active against two wood-destroying fungi, *Coriolus versicolor* and *Gloeophyllum trabeum* (Schultz and others 1992).

2.6.1 Distribution and Structure of *trans*-Resveratrol

One of the phytoalexins is *trans*-resveratrol (*trans*-3, 5, 4’-trihydroxystilbene) abundant in grapes, wines, grape juices, blueberries, peanuts and peanut products (Shyamal and others 2009; Romero-Pe´rez and others 2001). The production of resveratrol is a consequence of environmental stress, botrytis infections, and/or UV Irradiation. In addition, climatic factors, such as temperature, relative humidity and sun-light also influence the accumulation of *trans*-resveratrol. In grapes, *trans*-resveratrol increases more in interspecific hybrid varieties upon UV-irradiation than *Vitis vinifera* grape varieties (Dai and others 2012). The grape leaves and stems produce *trans*-resveratrol, and in the later status, the highest concentration of *trans*-resveratrol in grapes occurs in

grape skins, which is much higher than other parts of grapes (Shyamal and others 2009; Chong and others 2009).

In grapes, the major grape stilbenes are *cis*- and *trans*-resveratrol, resveratrol-3-O-β-D-glucopyranoside (piceid), piceatannol (3,4,3',5'-tetrahydroxy-*trans*-stilbene) and resveratrol dimers (viniferins) (Flamini and others 2013). *trans*--Resveratrol is the basic structure of other compounds and its structure is listed below. Under UV-irradiation, *trans*-resveratrol can transform to *cis*-resveratrol, and this process is called photoisomerization (Lamuela-Raventos and others 1995).

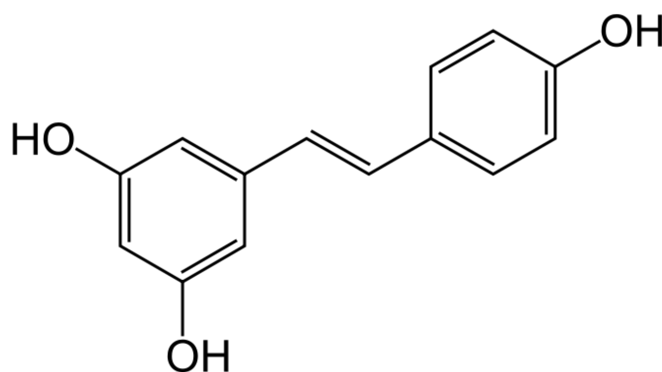


Figure 2-7. The structure of *trans*-resveratrol (Polimerek 2005)

2.6.2 Biological Activity of *trans*-Resveratrol

There is a huge amount of literature about the beneficial effects of *trans*-resveratrol, such as anti-tumor, anti-aging, anti-viral, cardiovascular and neuroprotective effects. However, in 2012, Dr. Dipak K. Das, the director of the Cardiovascular Research Center at the University of Connecticut Health Center, was found to have fabricated more than 100 articles about *trans*-resveratrol studies. Because of this, the biological activities and functions of *trans*-resveratrol are being studied more deeply now. From Walle's article (2004), the

chemopreventive activity of resveratrol against cardiovascular disease and cancers were effective in *in-vitro* study, however, in *in-vivo* study, especially when *trans-* resveratrol was absorbed after oral ingestion by human, only a small amount of unchanged resveratrol (<5 ng/mL/ 25 mg oral dose) can be detected in plasma because of its high metabolism rate. Therefore, the oral bioactivity of *trans*-resveratrol was extremely low (Walle and others 2004). Based on the great interests and limited *in-vivo* studies in humans concerning cardiovascular disease and cancer treatments, scientists are currently doing more clinical trials for humans.

2.7 Changes of Chemical Profiles of Grapes in Frozen Storage after Harvest

During the harvest season, grape researchers need to harvest enough samples for laboratory analyses in the future. However, because of the limited labor, time restriction and the facilities in the lab, researchers have to store a large amount of samples. Freezing is a very common method widely used in labs. However, the frozen conditions could change the components of grapes. In freezing the whole berries, frozen condition increased the color and acids of juice (Flora 1976; Threlfall and others 2006). In Cynkar's study (2004), the total anthocyanins in grapes increased after freezing, and pH was also higher after 24 hours of frozen storage than in the fresh samples. But frozen storage up to 3 months at -18 °C had no significant effects on total anthocyanins, total soluble solids and total phenolics.

Grape freezing changed the intracellular liquids, disrupting cell membranes and providing an easy exit for the phenolic compounds and

anthocyanins, so grape researchers also studied low temperature prefermentative techniques (cold maceration, superficially frozen grapes and dry-ice frozen must) to enhance the color in wine. The result showed all low temperature prefermentative techniques enhanced anthocyanin extraction in Cabernet Sauvignon and Syrah wines. (Alvarez and others 2005; Gil-Munoz and others 2009).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Phosphoric acid, formic acid, HPLC grade acetonitrile, glacial acetic acid, 0.1 N sodium hydroxide were obtained through Fisher Scientific (St. Louis, MO). Gallic acid, *trans*-ferulic acid, *trans*-resveratrol, malvidin chloride (≥95.0%), malvidin-3-glucoside chloride (≥90%), HPLC grade water were obtained from Sigma-Aldrich (St. Louis, MO).

3.2 Experimental Design

In this project, grape skins and grape pulps were manually separated. The anthocyanins analysis and non- colored phenolic compounds analysis were conducted on grape skins. The grape juice was used in basic chemical analyses.

For anthocyanin analysis, grape sample preparation treatments was conducted with three different transportation methods (liquid nitrogen, dry ice and regular ice), two different storage temperatures (-20°C and -80 °C) and three storage times (Fresh, 1 months and 3 months).

For the phenolic acids and trans- resveratrol analyses, the treatments included three different transportation methods (liquid nitrogen, dry ice and regular ice), two different storage temperature (-20 °C and -80 °C) but no time variable.

The basic chemical analyses investigated in grape juice included pH, Brix and titratable acidity. The juice was extracted from frozen grapes. Different

treatments included three transportation methods, two storage temperatures, and four storage times (Fresh, 1 month, 3 months and 6 months).

3.3 Norton Grape Samples Selection and Preparation

3.3.1 Grape Harvest

Norton grapes were collected from University of Missouri's Horticulture and Agroforestry Research Station in New Franklin, MO (39.0161° N, 92.7383° W) on October 9th, 2012. The harvest date was determined by the ripeness of grapes. Norton grapes were harvested randomly by hand-picking and then placed in a standard 50mL polypropylene centrifuge tube. Each tube contained 27 to 30 grapes. Once Norton grapes were collected, tubes were frozen under different transportation methods, namely on regular ice (R), dry ice (DI), and liquid nitrogen (LN). The total time for transportation was one hour. After arrived at the university, the grapes transported on regular ice, dry ice and liquid nitrogen were put into storage at temperature -20 °C and -80 °C, and one set was placed into a refrigerator overnight for juice analysis the next day, called "fresh" sample. The rest of the samples were separately preserved under -20°C and -80 °C. All treatments were replicated in triplicate.

3.3.2 Preparation of Grape Skins and Juice

Tubes with grapes were placed into an ice-box container filled with dry ice. Grape berries were peeled by hand in a nitrogen atmosphere. Skins were preserved in the centrifuge tubes with nitrogen inside and frozen in liquid nitrogen immediately. The pulp was collected in polyethylene bags and crushed by hand.

Grape juice was squeezed from the pulp and preserved overnight in refrigerator for further analysis.

3.4 Chemical Analyses

3.4.1 pH measurement

The pH value of grape juice was measured by a Orion 3 STAR model pH meter (Thermo Scientific Inc.) with a Corning Model 476086 electrode (Corning, NY) and replicated three times.

3.4.2 Titratable Acidity Measurement

Titratable acidity (TA) was expressed as g/L tartaric acid equivalent. Grape juice (5 mL) was pipetted into a beaker with 100 mL degassed distilled water. Juice samples were titrated with 0.1N NaOH (freshly opened) while stirred with a magnetic bar. The pH value of titration end point at 8.2 was measured by the same pH meter mentioned above. Titratable acidity of the grape juice was calculated by the formula:

$$\text{TA (g/L tartaric acid equivalents)} = \frac{\text{(mL base)(N base)(0.075)(1000)}}{\text{mL sample}}$$

3.4.3 Brix Measurement

Distilled water was used as a control to calibrate the refractometer. Brix of grape juice was measured using a Mark II Plus model Abbe refractometer (Reichert, NY) at room temperature.

3.5 HPLC Analysis of Anthocyanins

3.5.1 Grape Skin Sample Preparation

Norton grape skins were pulverized in liquid nitrogen with a mortar and pestle. Aliquots of 5g of grape skins were weighed and extracted with 100 mL of methanol/ 0.1% HCl (v/v) for 3 hours in VWR ds500 orbital shaker after flushing with nitrogen in order to prevent oxidation during extraction. The grape skin extracts were then cleaned by vacuum filtration and preserved in a refrigerator until further analysis (Kammerer and others 2004).

3.5.2 HPLC Conditions

Phenolic acids, anthocyanins and *trans*- resveratrol were evaluated by reverse-phase high-performance liquid chromatography (RP- HPLC). Anthocyanins analysis was carried out with an Agilent HPLC series 1100 (Agilent, Santa Clara, CA) equipped with ChemStation software, a model G1379A degasser, a model G1311A quaternary pump, a model G1329A thermo-autosampler, a model G1316A column oven, and a model G1315B diode array detector. Separation was conducted in Phenomenex Aqua C18 column (250 mm* 4.6 mm, particle size 5 mm) (Phenomenex, Torrance, CA). The mobile phase was 87:10:3 (v/v/v) water/ formic acid/ acetonitrile solution (solvent A) and 40:10:50 (v/v/v) water/ formic acid/ acetonitrile (solvent B) at a flow rate of 0.8 mL/min. The linear gradient of phase B was as follows: 12% B, 0 min; 22% B, 10 min; 26% B, 15 min; 31% B, 20 min. The volume of sample injection was 10 µL with needle wash. The column temperature was set at 35℃ and DAD detector wavelength was set at 520 nm. The method for preparing the grape skin sample

was adapted with small changes from Kammerer and others (2004). The column temperature, gradient program and injection volume were changed in this research.

3.5.3 Quantitation of Anthocyanins

Malvidin chloride and malvidin-3-glucoside chloride were used to make two standard curves. The standards were precisely reweighed because of the small size. The total weight of the chemicals and package was taken. The chemical was then dissolved by 1 mL methanol in its original package. The stock solution was transferred to a 1.5mL centrifuge tube. The glass vial was weighed after drying in oven. The actual weight of the Malvidin choride and Oenin chloride were calculated by the following formula:

Actual Weight (mg) =Total weight – package weight

The concentrations of solutions for the standard curve were made based on the actual weight. The concentrations of standard solutions were listed in appendix A1 and A2

3.6 HPLC analysis of phenolic acids and *trans*-resveratrol

3.6.1 Grape Skin Sample Preparation

Grape skins were freeze dried (Labconco, Kansas City, MO) overnight. Dried grape skins in a mount of 250 mg were pulverized in liquid nitrogen and extracted with 5 ml of ethanol/ water (80:20, v/v). Samples were incubated at 60°C in a water bath for 30 minutes with gentle stirring. They were then preserved in brown tubes to prevent light exposure. After extraction, the sample was stabilized for 5 minutes at room temperature. 1 mL of supernatant extract

was centrifuged using Centrifuge 5804R at 16,000× g for 20 minutes (Eppendorf, IL) and preserved in the refrigerator temporarily until analysis could be performed. The method for preparing the grape skin sample was adapted with small changes from Dai and others (2012).

3.6.2 HPLC Conditions

The same HPLC system for the anthocyanin analyses was used. The mobile phase was 2% phosphoric acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.8 mL/min. The linear gradient of solvent B was as follows: 16% B, 0 min; 17.5% B, 13 min; 20% B, 15 min; 25% B, 17 min; 30% B, 21 min; 35% B, 27 min; 50% B, 28 min; 70% B, 30; 100% B, 33 min. The volume of sample injection was 20 µL with needle wash. The column temperature was set at 40 °C and DAD detector wavelength was set at 270 nm, 306 nm and 320 nm (Dai and others 2012).

3.6.3 Quantitation of Phenolic Acids and *trans*-Resveratrol

The commercial gallic acid and *trans*-ferulic acid were used as standards. The concentrations of gallic acid solution for the standard curve were 100 µg/mL, 50 µg/ mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL. The concentrations of *trans*-ferulic acid were 20 µg/mL, 10 µg/mL, 5 µg/mL, 2.5 µg/mL and 1.25 µg/mL. The concentration of *trans*- Resveratrol for the standard curve was 20 µg/mL, 10 µg/mL, 5 µg/mL, 1 µg/mL and 0.5 µg/mL.

3.7 Statistical Analysis

All Statistical analyses were performed using SPSS software (SPSS Inc., Ver. 24, Chicago, IL). The linear mixed model analysis was conducted to analyze

the effect of temperature, time, transportation method and the interaction of them on pH, TA and anthocyanins. A two-way ANOVA was used to analyze the influence of temperature and transportation method on penolic acids and *trans*-resveratrol. The significance level was set at $p < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Basic Chemical Analyses of Grape Juice

4.1.1 Results of pH Measurement

As shown in Table 4-1 and 4-2, time, temperature, transportation method and the interaction of time and temp had the significant influence on pH of grape juice. The pH value in the treatment of regular ice, dry ice and liquid nitrogen are showed in Table 4-3 to 4-5.

Table 4-1 Linear mixed model analysis of pH value

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	48.000	328330.5	.000
Time	3	48.000	51.447	.000
Transmeth	2	48.000	30.239	.000
Temp	1	48.000	24.849	.000
Time * Transmeth	6	48.000	.663	.680
Time * Temp	3	48.000	6.168	.001
Transmeth * Temp	2	48.000	1.222	.304
Time * Transmeth * Temp	6	48.000	2.092	.072

Table 4-2 Pairwise Comparisons[a] of dry ice, liquid nitrogen and regular ice group

Transmeth (I)	Transmeth (J)	Mean	Mean Difference (I-J)	Std. Err	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
DI	LN	3.652	.047*	.016	48.000	.005	.015	.078*
	R		-.074*	.016	48.000	.000	-.106	-.043*
LN	DI	3.605	-.047*	.016	48.000	.005	-.078	-.015*
	R		-.121*	.016	48.000	.000	-.152	-.089*
R	DI	3.726	.074*	.016	48.000	.000	.043	.106*
	LN		.121*	.016	48.000	.000	.089	.152*

Based on estimated marginal means[a]

*. The mean difference is significant at the .05 level.

a. Dependent Variable: pH.

c. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 4-3 Results of pH value in regular ice group*

	-20 °C	-80 °C
0 month	3.84[a]	3.84[a]
1-month	3.71[c]	3.73[bc]
3-month	3.82[ab]	3.67[c]
6-month	3.70[c]	3.50[d]

*. Means with same letters are not significantly different at P< 0.05 by Duncan's Multiple Range Test.

Table 4-4 Results of pH value in dry ice group*

	-20 °C	-80 °C
0 month	3.74[a]	3.74[a]
1-month	3.72[a]	3.59[b]
3-month	3.72[a]	3.60[b]
6-month	3.58[b]	3.52[b]

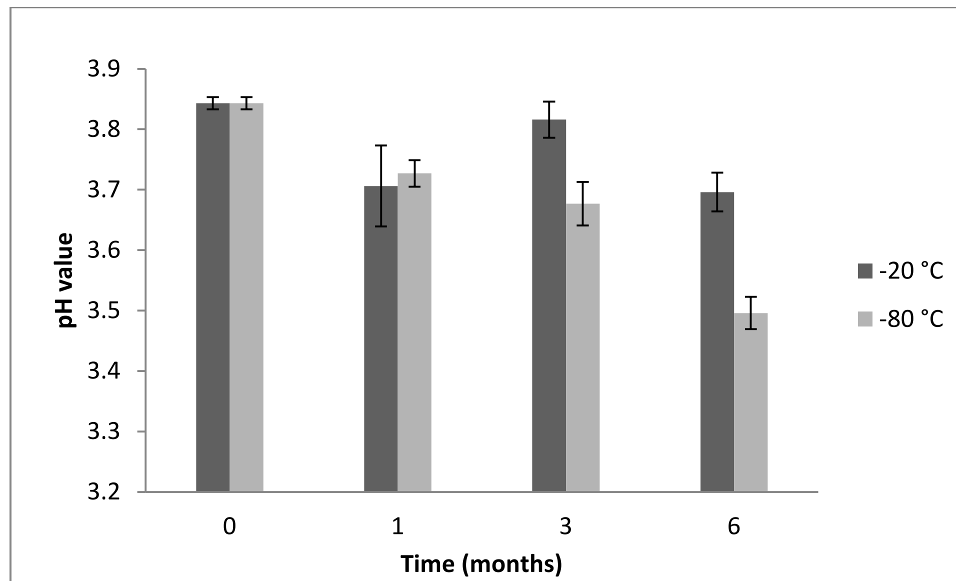
*. Means with same letters are not significantly different at P< 0.05 by Duncan's Multiple Range Test.

Table 4-5 Results of pH value in liquid nitrogen group*

	-20 °C	-80 °C
0 month	3.73[a]	3.73[a]
1-month	3.58[b]	3.63[ab]
3-month	3.64[ab]	3.54[bc]
6-month	3.54[bc]	3.45[c]

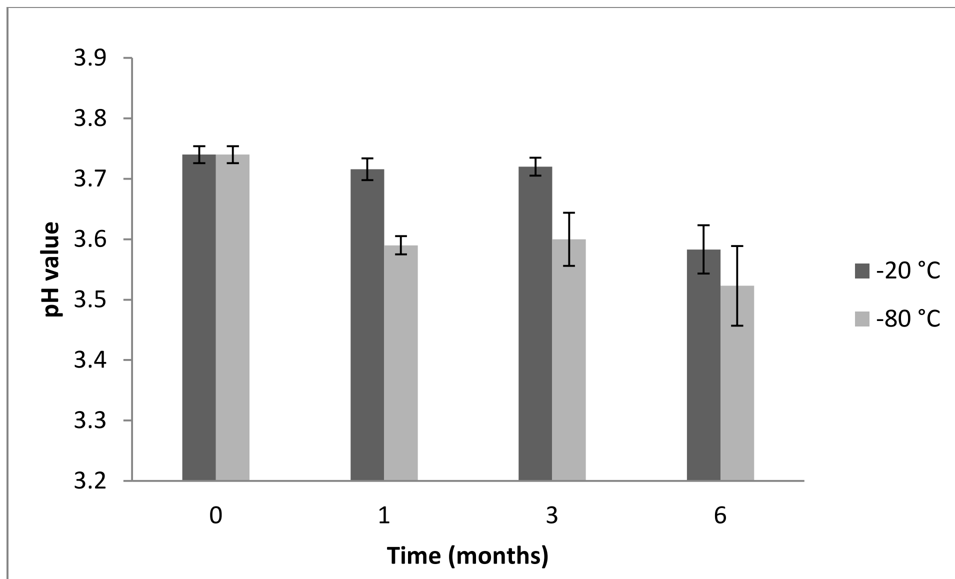
*. Means with same letters are not significantly different at P< 0.05 by Duncan's Multiple Range Test

The pH value of grape juice is shown in Figure 4-1, 4-2 and 4-3.



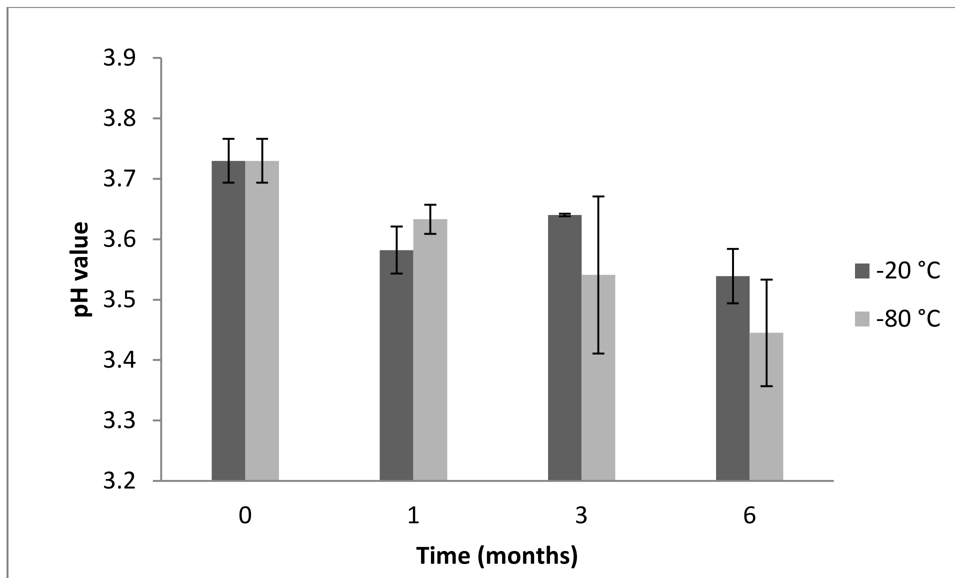
Storage temperature: -20 °C= storage at -20 °C, -80 °C= storage at -80 °C

Figure 4-1. pH value of grape juice on regular ice (R) transportation method



Storage temperature: -20 °C= storage at -20 °C, -80 °C= storage at -80 °C

Figure 4-2. pH value of grape juice on dry ice (DI) transportation method



Storage temperature: -20 °C= storage at -20 °C, -80 °C= storage at -80 °C

Figure 4-3. pH value of grape juice on liquid nitrogen (LN) transportation method

The data indicate pH of grape juice decreased during storage for both storage temperatures (-20 °C and -80 °C) over time. As shown in Figure 4-1 to 4-3, the pH value of Norton grape juice stored at -80 °C decreased for all three transportation methods over time. pH was significantly higher at -20 °C in regular

ice (R) group at 3 and 6 months and in dry ice (DI) group at 1and 3 months. pH showed no significant difference in liquid nitrogen (LN) group at -20 °C and -80 °C. The pH of grapes stored immediately after harvest on regular ice decreased from 3.84 to 3.50. While the LN group showed changes from 3.74 to 3.45. The DI group decreased from 3.73 to 3.52.

In this research, the pH value changes at -20°C storage generally matched the information shown in earlier work. In a previous study, the pH value of Cabernet Sauvignon, Grenache and Tempranillo stored at -18°C showed a pH increase in the first and third month of storage, but decreased after 6 months of storage. In the study, the reason to explain this phenomena is that acid salification likely occurred during the freezing and thawing, in which tartaric acid formed potassium hydrogen tartrate and calcium tartrate, so that the pH value increased in the 1st- and 3rd- month storage. After 6 months, the pH decreased since the protons were released into the grape juice as a result of polyphenol oxidase- mediated degradative reactions of phenolics (Santesteban and others 2013). In my research, the TA at -80 °C decreased at the first month and after that TA increased at the three months. However, TA showed no significant difference at -20 °C storage in three transportation methods with storage. The pH of Norton grape juice is significantly influenced by the ripeness and the climate of different harvest years (Jogaiah and others 2012). The decrease of the pH value was partially caused by the increase of titratable acidity.

4.1.2 Results of Titratable Acidity

The statistical analyses of TA under different treatments are showed in Table 4-6. As shown in Table 4-6, time as well as the interaction of time and temperature had significant influence on TA. TA (g/L) in the treatment of regular ice, dry ice and liquid nitrogen are showed in Table 4-7 to 4-9.

Table 4-6. Linear mixed model analysis of TA

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	12	9659.852	.000
Time	3	36.000	10.473	.000
Transmeth	2	12	1.943	.186
Temp	1	12	.401	.538
Time * Transmeth	6	36.000	1.123	.369
Time * Temp	3	36.000	6.341	.001
Transmeth * Temp	2	12	.212	.812
Time * Transmeth * Temp	6	36.000	1.680	.154

Table 4-7 Results of TA (g/L) in regular ice group*

	-20 °C	-80 °C
0 month	4.42^{bc}	4.42^{bc}
1-month	4.18^{cd}	4.02^{d}
3-month	4.35^{bc}	4.44^{bc}
6-month	4.6^{ab}	4.77^{a}

*. Means with same letters are not significantly different at P< 0.05 by Duncan's Multiple Range Test

Table 4-8 Results of TA (g/L) in dry ice group*

	-20 °C	-80 °C
0 month	4.71^{ab}	4.71^{ab}
1-month	4.23^{ab}	4.15^{b}
3-month	4.37^{ab}	5.01^{a}
6-month	4.73^{ab}	4.81^{ab}

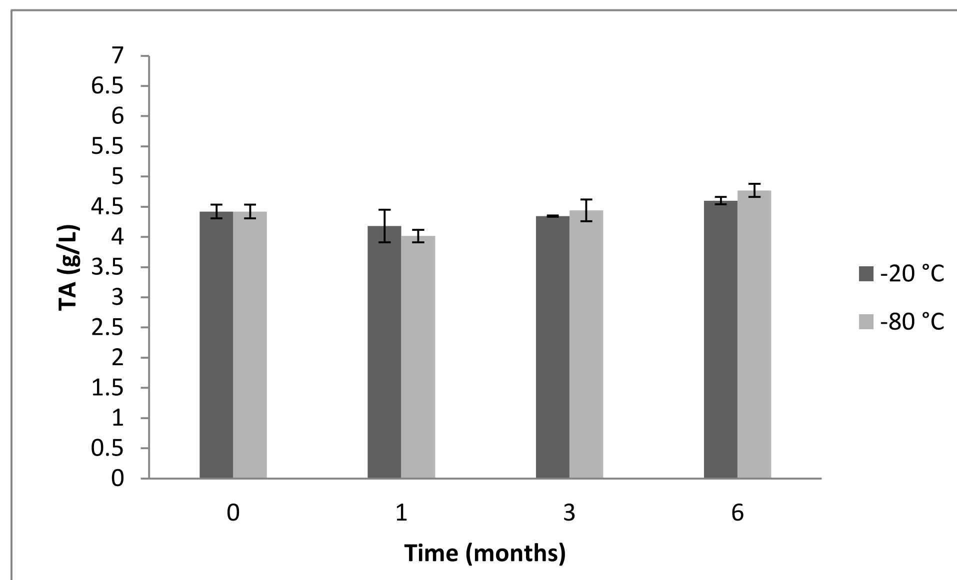
*. Means with same letters are not significantly different at P< 0.05 by Duncan's Multiple Range Test

Table 4-9 Results of TA (g/L) in liquid nitrogen group*

	-20 °C	-80 °C
0 month	4.84^{b}	4.84^{b}
1-month	4.74^{b}	3.88^{b}
3-month	4.47^{ab}	4.84^{b}
6-month	4.29^{ab}	4.81^{b}

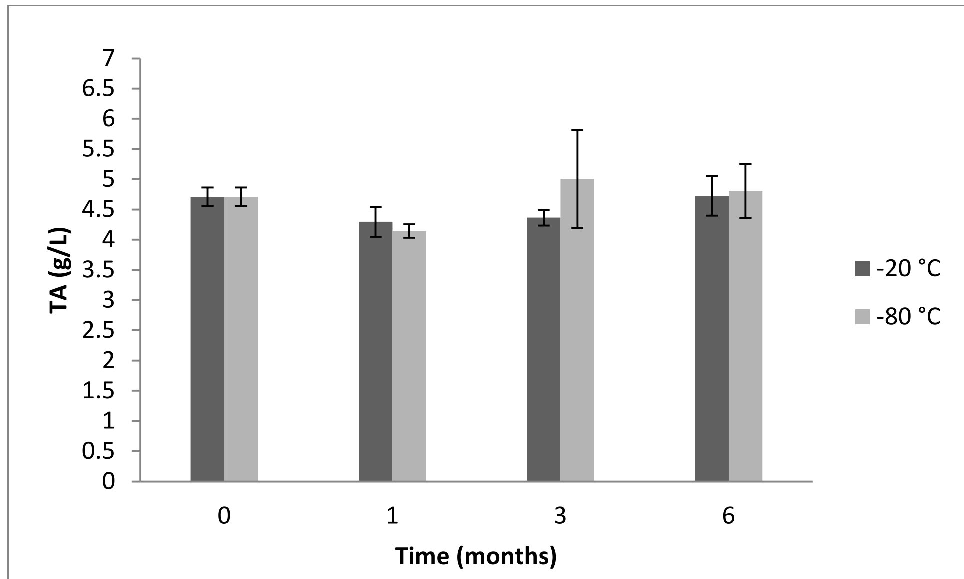
*. Means with same letters are not significantly different at P< 0.05 by Duncan's Multiple Range Test

The TA of grape juice can be seen in Figure 4-4, 4-5 and 4-6.



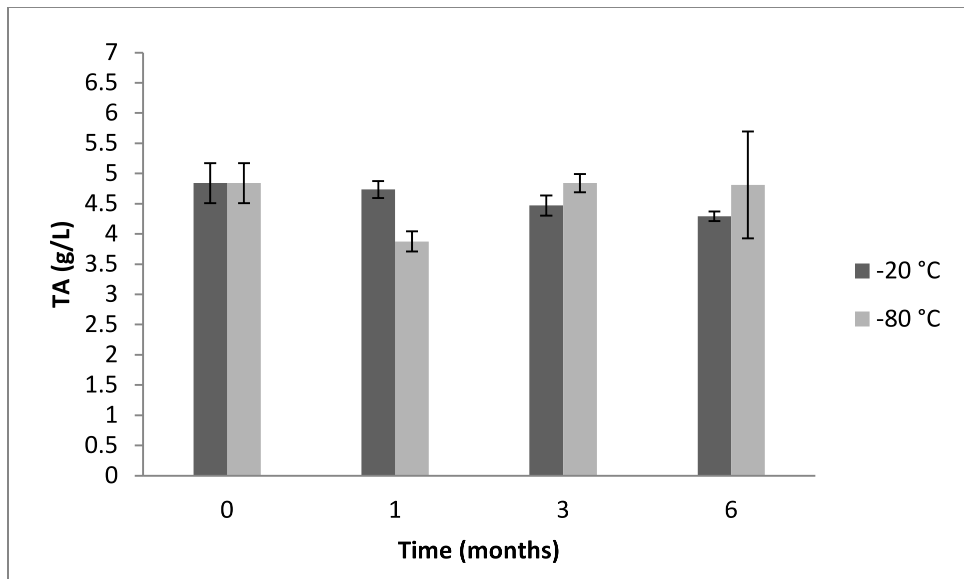
Storage temperature: -20 °C= storage at -20 °C, -80 °C= storage at -80 °C

Figure 4-4. Titratable acidity of grape juice on regular ice (R) transportation method



Storage temperature: -20 °C= storage at -20 °C, -80 °C= storage at -80 °C

Figure 4-5. Titratable acidity of grape juice on dry ice (DI) transportation method



Storage temperature: -20 °C= storage at -20 °C, -80 °C= storage at -80 °C

Figure 4-6. Titratable acidity of grape juice on liquid nitrogen (LN) transportation method

From Figure 4-4 to 4-6 and Table 4-6, there is no significant difference for TA between -20 °C and -80 °C in three transportation methods except DI at 3 months and LN at 1 month. As mentioned before, TA stored at -80 °C in one month was significantly lower than other groups. The highest amount was

5.01g/L (DI, -80 °C, three months) and the lowest amount was 3.88 g/L (LN, -80 °C, one month).

Although Norton grapes usually demonstrate high pH and high titratable acidity, the titratable acidity in this research was quite low. In the previous study, the TA was up to 15 g/L in Norton wine (Main 2005). The possible reason is due to the low level of malic acid in the grapes, which is used for respiration. When temperature increases, the rate of respiration increases and the amount of malic acid drops significantly (Responses and others 1975). Since the harvest time for this research was in October and the weather was very warm, the malic acid may have dropped dramatically and the titratable acidity was therefore lower than average.

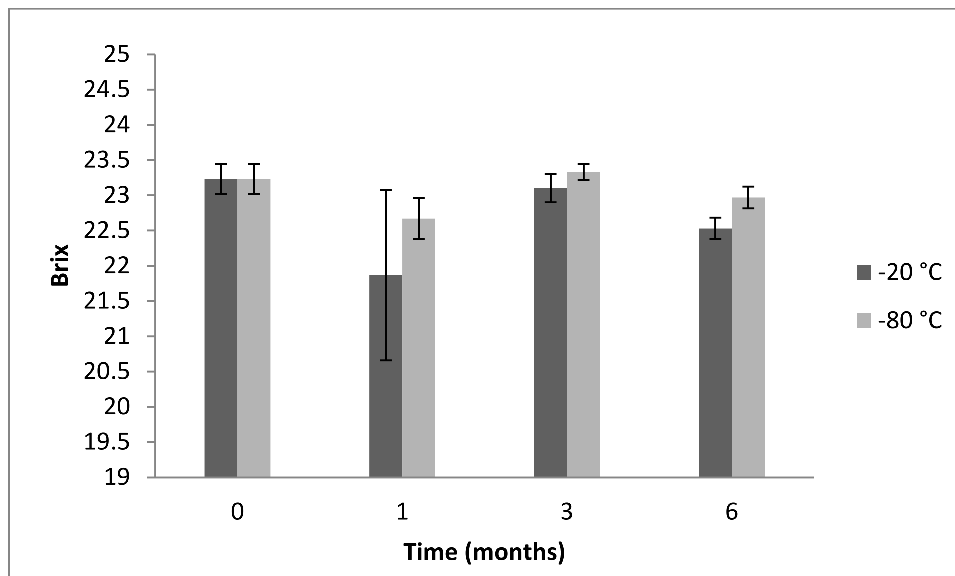
4.1.3 Brix Measurement

From the linear mixed model analysis of Brix, only time and temperature had significant influence on Brix, and no interactions among time, temperature and transportation methods had significant influence on Brix. The statistical analyses are showed in Table 4-10. Brix value of grape juice on different transportation methods can be seen from Figure 4-7 to 4-9.

Table 4-10 Pairwise Comparisons[a] of different frozen storage time on Brix (0 month, 1-month, 3-month, 6-month)

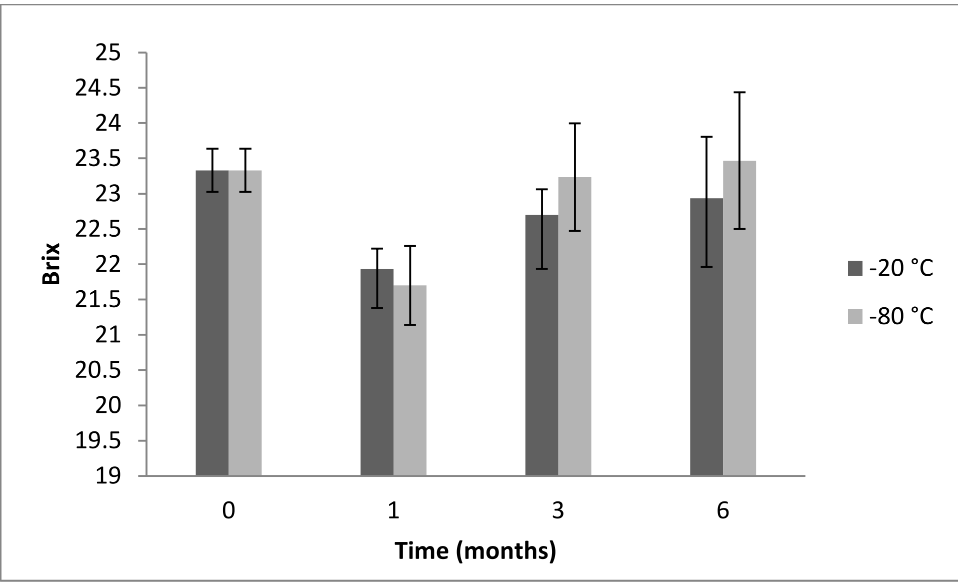
(I) Time	(J) Time	Mean	Mean Difference (I-J)	Std. Error	Sig.[c]	95% Confidence Interval	
						Lower Bound	Upper Bound
0.00	1.00	23.33	1.20000*	.18836	.000	.8241	1.5759
	3.00		.28889	.18836	.130	-.0870	.6648
	6.00		.43889*	.18836	.023	.0630	.8148
1.00	0.00	22.13	-1.20000*	.18836	.000	-1.5759	-.8241
	3.00		-.91111*	.18836	.000	-1.2870	-.5352
	6.00		-.76111*	.18836	.000	-1.1370	-.3852
3.00	0.00	23.04	-.28889	.18836	.130	-.6648	.0870
	1.00		.91111*	.18836	.000	.5352	1.2870
	6.00		.15000	.18836	.429	-.2259	.5259
6.00	0.00	22.89	-.43889*	.18836	.023	-.8148	-.0630
	1.00		.76111*	.18836	.000	.3852	1.1370
	3.00		-.15000	.18836	.429	-.5259	.2259

*. The mean difference is significant at the 0.05 level.
a. Dependent Variable: Brix.
c. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).



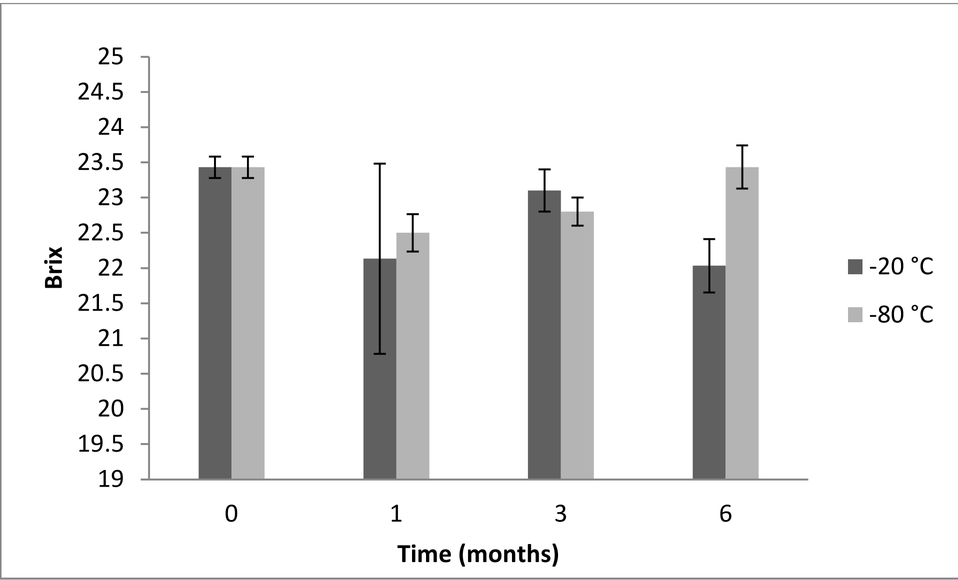
Storage temperature: -20 °C= storage at -20 °C, -80 °C= storage at -80 °C

Figure 4-7. Brix value of grape juice on regular ice (R) transportation method



Storage temperature: -20 °C= storage at -20 °C, -80 °C= storage at -80 °C

Figure 4-8. Brix value of grape juice on dry ice (DI) transportation method



Storage temperature: -20 °C= storage at -20 °C, -80 °C= storage at -80 °C

Figure 4-9. Brix value of grape on liquid nitrogen (LN) transportation method

Brix values for -80 °C storage were higher than those for -20 °C storage. The possible reasons are that almost all the chemical reactions were stopped at

the lower temperature storage condition, including enzymatic reactions and cell respiration. Therefore, no glucose and fructose were decomposed in the grapes at -80 °C. The concentration of unfrozen water was still high in fruit at -20 °C. So the chemical reactions, such as the decrease of pectin and the invertase of sucrose, which might lead to the change of sugar content very possible to happen (Sigita and others 2013, Skrede 1983).

For the time treatment (one month, three months, and six months), Brix increased slightly during storage at -80 °C most likely because the water in the grapes sublimated slowly and the sugars in grapes concentrated. The changes in Brix of grape berries stored at -20 °C did not show a clear trend, because the water loss rate and chemical reactions rate probably balanced each other out and contributed little effect on Brix, meaning that the decrease in soluble solids (glucose, etc) due to enzymatic activities was off-set by the loss of water due to sublimation.

4.2 HPLC Analysis of Anthocyanins of Norton Grape Skin

4.2.1 Results of Malvidin-di-glucoside

From previous studies, malvidin-containing anthocyanins contribute the highest proportion to the total anthocyanins in Norton grape skins. Malvidin-di-glucoside and malvidin-glucoside are the major anthocyanins in the berry skins, which account for 45% and 31% of total anthocyanins, respectively (Hogan and others 2009). The contents of malvidin-di-glucoside in Norton grape skins can be seen in Table 4-11 and Figure 4-10. The means of malvidin-di-glucoside in each treatment are showed in Table 4-12.

Table 4-11. Linear mixed model analysis of malvidin-di-glucoside

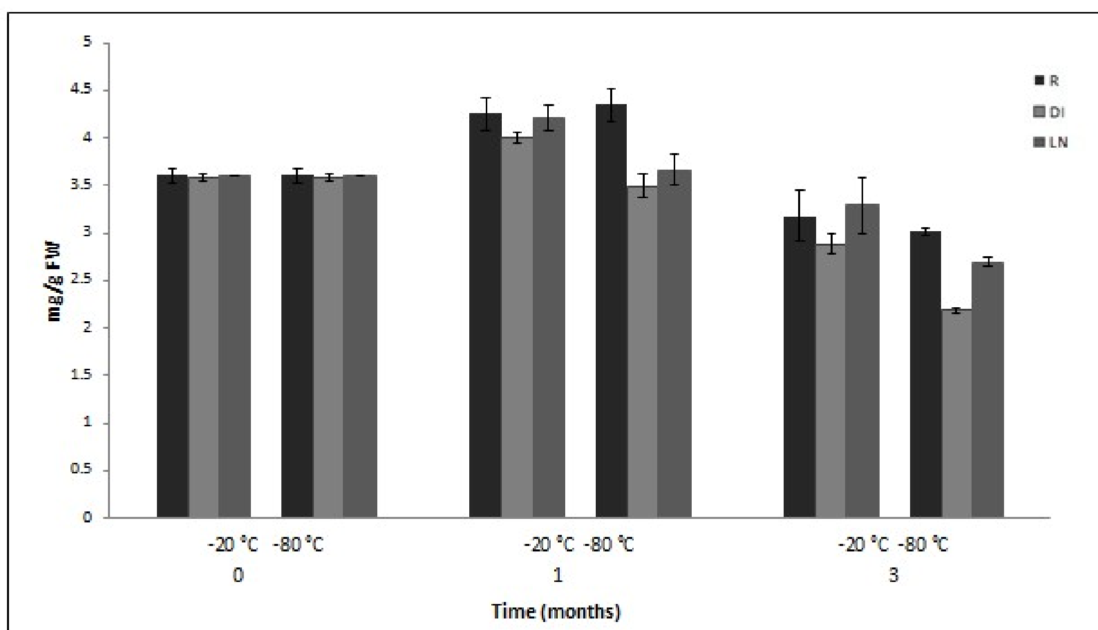
Source	Numerator df	Denominator df	F	Sig.
Intercept	1	36	39965.625	.000
Time	2	36	353.297	.000
Transmeth	2	36	39.153	.000
Temp	1	36	59.266	.000
Time * Transmeth	4	36	10.811	.000
Time * Temp	2	36	16.754	.000
Transmeth * Temp	2	36	12.366	.000
Time * Transmeth * Temp	4	36	3.347	.020

Table 4-12 Results of malvidin-di-glucoside (mg/g FW) in Norton grape skins*

	-20 °C			-80 °C		
	0 month	**1-month**	**3-month**	**0 month**	**1-month**	**3-month**
R	3.60^{c}	4.25^{a}	3.18^{def}	3.60^{c}	4.35^{a}	3.01^{efg}
DI	3.58^{c}	4.01^{ab}	2.88^{gf}	3.58^{c}	3.50^{cd}	2.19^{h}
LN	3.60^{c}	4.21^{a}	3.29^{cde}	3.60^{c}	3.66^{bc}	2.70^{g}

Immediate postharvest treatment: R= Regular Ice, DI= Dry Ice, LN= Liquid Nitrogen; storage temperature -20 °C= storage at -20 °C, -80 °C= storage at -80 °C; FW= Fresh Weight;

*. Means with same letters are not significantly different at P< 0.05 by Duncan's Multiple Range Test.



Immediate postharvest treatment: R= Regular Ice, DI= Dry Ice, LN= Liquid Nitrogen; storage temperature -20 °C= storage at -20 °C, -80 °C= storage at -80 °C; FW= Fresh Weight;

Figure 4-10. Malvidin-di-glucoside in Norton grape skins

The interaction of time, temperature and transportation methods all had significant influences on the changes of malvidin-di-glucoside. The amounts of malvidin-di-glucoside in Norton grape skins had increased after one month of storage and then decreased after three month storage. For the transportation methods, the amount of malvidin-di-glucoside in the dry ice (DI) group was lower than that in both, the regular ice (R) and liquid nitrogen (LN) groups. The amount in the R group was higher than that in the LN group for both -20 °C and -80 °C, except for the treatment of -20 °C at three months. In this study, the highest amount of malvidin-di-glucoside in berry skin was 4.35 mg/g (one month, regular ice and at -80 °C). The lowest amount was 2.19 mg/g (three months, DI and at -80 °C). The amount of malvidin-di-glucoside after one month was slightly higher than that in the fresh samples or after treatments. The malvidin-di-glucoside was higher than previously reported for Norton grape skin at 1.01mg/g FW (Munoz-Espada 2004). However, the Virginia- Norton grapes had a similar amount of malvidin-di-glucoside, which was 0.42 mg/g in the whole grape, and since the berry skin usually accounts for one-tenth of the total weight, it was equivalent to almost 4.2 mg/g in skins (Hogan and others 2009). A previous study showed that grapes accumulated higher levels of malvidin-di-glucoside than malvidin-glucoside. The content of five major anthocyanins increased steadily until harvest time at 127 DAB (Day After Bloom), which was longer than for Cabernet Sauvignon (Ali and others 2011). In our research, the harvest time was at the beginning of October, which might be the reason that the accumulation of anthocyanins was higher than in previous studies.

4.2.2 Results of Malvidin-glucoside

As shown in Table 4-13 and Figure 4-11, the interaction of time, temperature and transportation method had significant influences on the amount of malvidin-glucoside in grape skins. The means of malvidin-di-glucoside in each treatment are showed in Table 4-14. The amount of malvidin-glucoside reached a peak after one month.

Table 4-13. Linear mixed model analysis of malvidin-glucoside

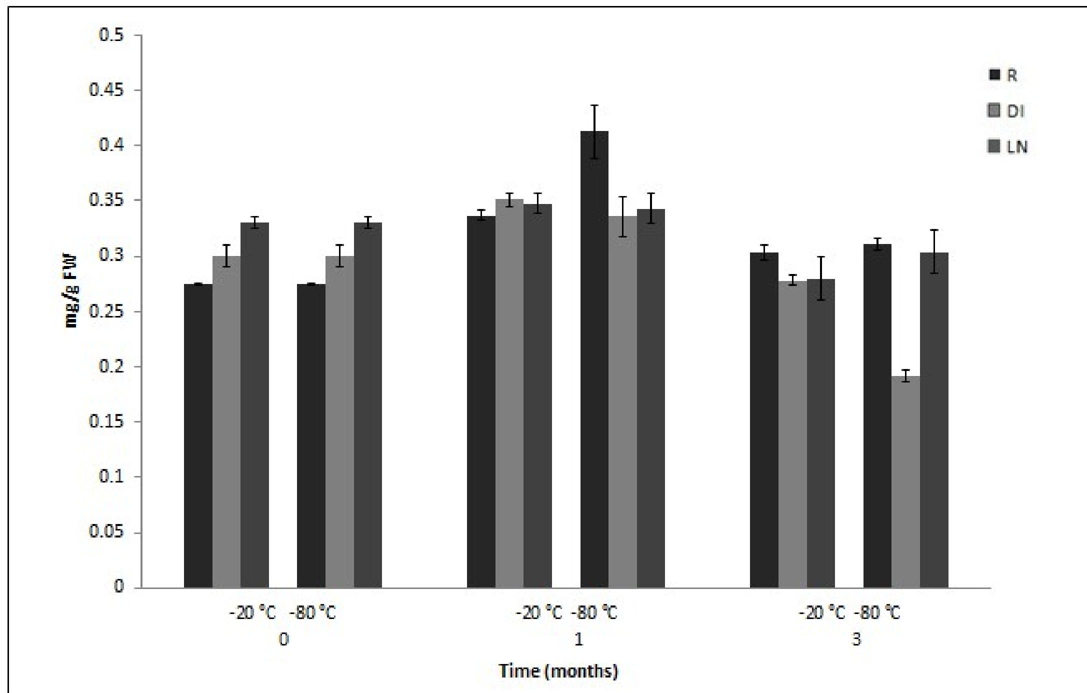
Source	Numerator df	Denominator df	F	Sig.
Intercept	1	33	38300.753	.000
Time	2	33	207.594	.000
Transmeth	2	33	34.753	.000
Temp	1	33	.001	.972
Time * Transmeth	4	33	40.094	.000
Time * Temp	2	33	11.671	.000
Transmeth * Temp	2	33	33.612	.000
Time * Transmeth * Temp	4	33	17.683	.000

Table 4-14 Results of malvidin-glucoside (mg/g FW) in Norton grape skins*

	-20 °C			-80 °C		
	0 month	**1-month**	**3-month**	**0 month**	**1-month**	**3-month**
R	0.275^{g}	0.337^{bcd}	0.303^{defg}	0.275^{g}	0.413^{a}	0.311^{cdef}
DI	0.301^{efg}	0.351^{b}	0.278^{gf}	0.301^{efg}	0.336^{bcde}	0.192^{h}
LN	$0.330bc^{de}$	0.3475^{b}	0.280^{gf}	0.330^{bcde}	0.343^{bc}	0.303^{defg}

Immediate postharvest treatment: R= Regular Ice, DI= Dry Ice, LN= Liquid Nitrogen; storage temperature -20 °C= storage at -20 °C, -80 °C= storage at -80 °C; FW= Fresh Weight;

*. Means with same letters are not significantly different at P< 0.05 by Duncan's Multiple Range Test.



Immediate postharvest treatment: R= Regular Ice, DI= Dry Ice, LN= Liquid Nitrogen; storage temperature -20 °C= storage at -20 °C, -80 °C= storage at -80 °C; FW= Fresh Weight

Figure 4-11. Malvidin-glucoside in Norton grape skins

Malvidin-glucoside is significantly lower in regular ice group than the liquid nitrogen group at the beginning of storage. The amount of Malvidin-glucoside at -20 °C and -80 °C in 1 month storage was significantly higher than that in 3 month storage. The "one month-R-at -80 °C group" had the highest amount of malvidin-glucoside (0.41mg/g). The "three month-DI- at -80 °C group" had the lowest amount of malvidin-glucoside (0.19 mg/g). In a previous study, Virginia-Norton grapes had 0.2 mg/g malvidin-glucoside in the total weight of grape (Hogan and others 2009), which is about 10-20 times more than in our study. But compared with malvidin-di-glucoside, malvidin-glucoside always accounts for a smaller proportion in the Norton grape skins. However, large variations exist among grape varieties; for example, malvidin-glucoside reached 1.82 mg/g FW in

Cabernet Sauvignon, which accounted for 67% of the total anthocyanins in grape skin (Ali and others 2011).

In Romero's study (2007), the low temperature increased contents of anthocyanins in Cardinal table grapes after short term storage. The effect of cold stress on anthocyanins production has been assessed only for total anthocyanin content, rather than for specific anthocyanins. However, the explanation of this mechanism is still unclear.

4.3 HPLC Analysis of Phenolic Acids of Norton Grape Skin

4.3.1 Results of Gallic Acid

The statistical analysis is shown in Table 4-15 and the amount of gallic acid in Norton grape skins based on dry material is shown in Figure 4-12. The means of gallic acid in each treatment are showed in Table 4-16.

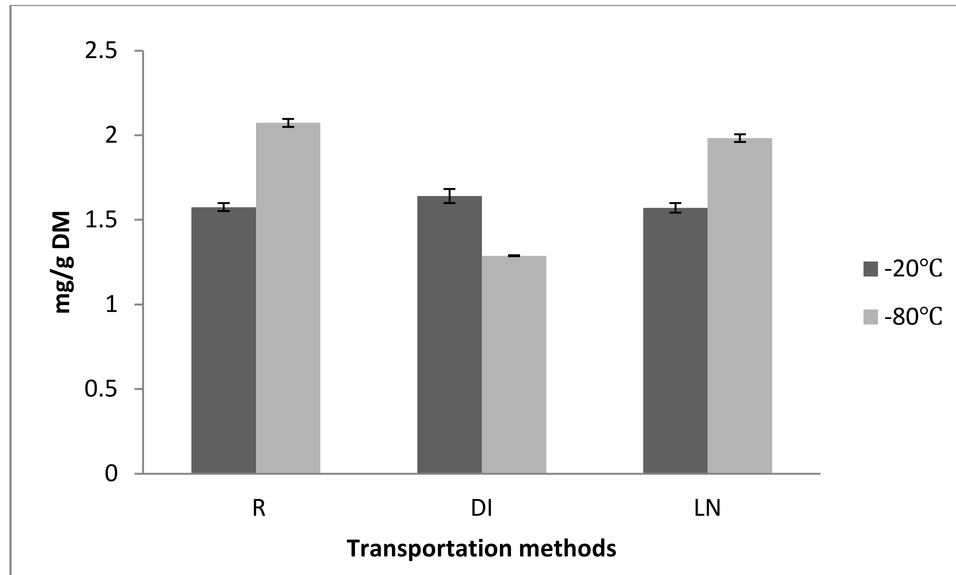
Table 4-15 Two-way ANOVA analysis of gallic acid

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	51303087.993	1	51303087.993	71760.196	.000
Temp	154887.680	1	154887.680	216.649	.000
Transmeth	458483.329	2	229241.665	320.652	.000
Temp * Transmeth	657829.681	2	328914.840	460.070	.000

Table 4-16 Results of gallic acid (mg/g DW) in Norton grape skins*

	-20 °C	-80 °C
R	1.575^{c}	2.073^{a}
DI	1.640^{c}	1.288^{d}
LN	1.570^{c}	1.982^{b}

Immediate postharvest treatment: R= Regular Ice, DI= Dry Ice, LN= Liquid Nitrogen; storage temperature -20 °C= storage at -20 °C, -80 °C= storage at -80 °C; DW= Dry Weight;
*. Means with same letters are not significantly different at P< 0.05 by Duncan's Multiple Range Test.



Immediate postharvest treatment: R= Regular Ice, DI= Dry Ice, LN= Liquid Nitrogen; storage temperature: -20 °C= storage at -20 °C, -80 °C= storage at -80 °C; DM= Dry Materials

Figure 4-12. Gallic acid in Norton grape skins

From the HPLC analysis of gallic acid in Norton grape skins, transporting on Regular Ice and storing at -80 °C for six months resulted in the highest amount of gallic acid (2.07 mg/g Dry Material), whereas on dry Ice at -80 °C resulted in the lowest amount of gallic acid (1.29 mg/g DM). Based on a two-way ANOVA statistical analysis, the interaction of transportation methods and storage temperatures had significant influence on the results. The amounts of gallic acid at -20 °C storage showed little change. However, at -80 °C, the DI group contained 35% less gallic acid than the R and LN groups. Gallic acid is the main phenolic acid in grape seeds and skins, especially in Norton grapes (Yilmaz and others 2004). According to the literature, gallic acid in Norton fresh grape samples was 72.6 μg/g, which is higher than that found in Cabernet Franc (16.8 μg/g) (Hogan and others 2009). In Chardonnay and Merlot, the amounts of gallic acid are 0.05 mg/g of DM and 0.03 mg/g of DM respectively. However,

location, climate, and temperature greatly influence the composition of the grapes; therefore, literature data can only a general indication of the contents of gallic acid in different grape varieties. Grape seeds have a much higher amount of gallic acid than skins. In Chardonnay and Merlot, the amount of gallic acid was around 3 fold higher in grape seeds than in skins (Yilmaz and others 2004).

4.3.2 Results of Ferulic Acid

The statistical analysis of ferulic acid in Norton grape skins based on dry materials are shown in Table 4-17 to 4-18 and the amount of ferulic acid is shown in Figure 4-13

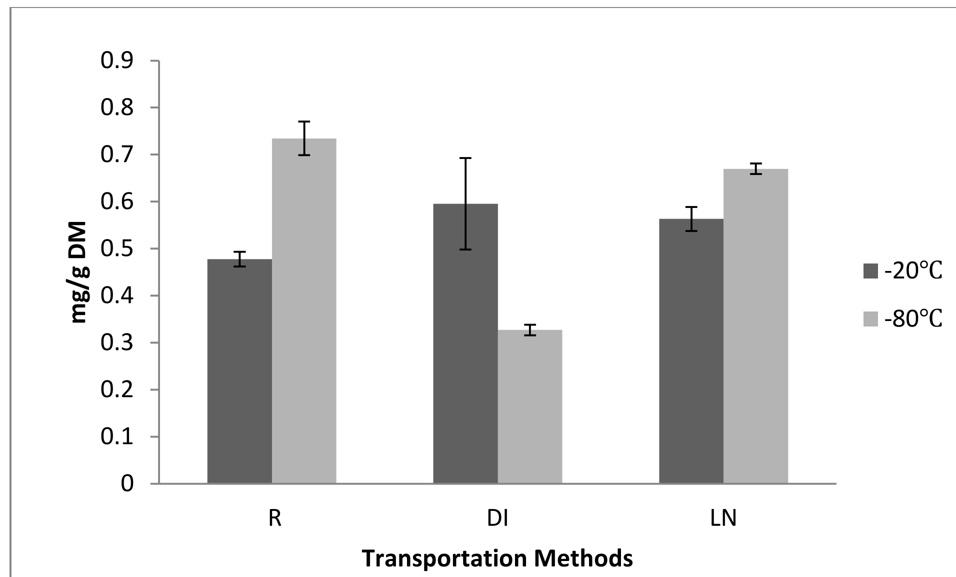
Table 4-17. Two-way ANOVA analysis of ferulic acid

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	5669545.460	1	5669545.460	2862.523	.000
Temp	4514.590	1	4514.590	2.279	.157
Method	90518.924	2	45259.462	22.851	.000
Temp * Method	219414.931	2	109707.465	55.391	.000

Table 4-18 Results of ferulic acid (mg/g DW) in Norton grape skins*

	-20 °C	-80 °C
R	0.478^{c}	0.734^{a}
DI	0.595^{bc}	0.327^{d}
LN	0.563^{bc}	0.670^{ab}

Immediate postharvest treatment: R= Regular Ice, DI= Dry Ice, LN= Liquid Nitrogen; storage temperature -20 °C= storage at -20 °C, -80 °C= storage at -80 °C; DW= Dry Weight;
*. Means with same letters are not significantly different at P< 0.05 by Duncan's Multiple Range Test.



Immediate postharvest treatment: R= Regular Ice, DI= Dry Ice, LN= Liquid Nitrogen; storage temperature: -20 °C= storage at -20 °C, -80 °C= storage at -80 °C; DM= Dry Materials

Figure 4-13. Ferulic acid in Norton grape skins

The ferulic acid analysis showed a similar trend to that of gallic acid. The possible reason was that ferulic acid and gallic acid, a hydroxycinnamic acid and a hydroxybenzoic acid, respectively, are classified as non-flavonoids according to their structures; therefore, their chemical properties are close to each other. The highest amount of ferulic acid in grape skins was 0.73 mg/g (Regular Ice, at -80 °C). The lowest amount was 0.33 mg/g (Dry ice, at -80°C). In a previous study, in fresh Norton berries (including skins and pulp), the ferulic acid content was 0.6 µg/g, and in other fresh red grapes (*Vitis vinifera L*), the content was 4.3 µg/g (Hogan and others 2009). The white grape Merzling (*V. vinifera* L. Cv. Merzling) had 2.6 µg/g Dry Material in grape skins (Kammerer and others 2004). However, the accumulation of phenolic acid is very complex and affected by many biotic and abiotic stresses. The influence of low temperature stress on phenolic changes of freshly harvested fruits is still not clear.

4.4 HPLC Analysis of *trans*-Resveratrol of Norton Grape Skins

The statistical analysis of *trans*-resveratrol is showed in Table 4-19 to 4-20. As can be seen in this table, the interaction of temperature and transportation methods has significant influence on *trans*-resveratrol.

Table 4-19. Two-way ANOVA analysis of trans-Resveratrol

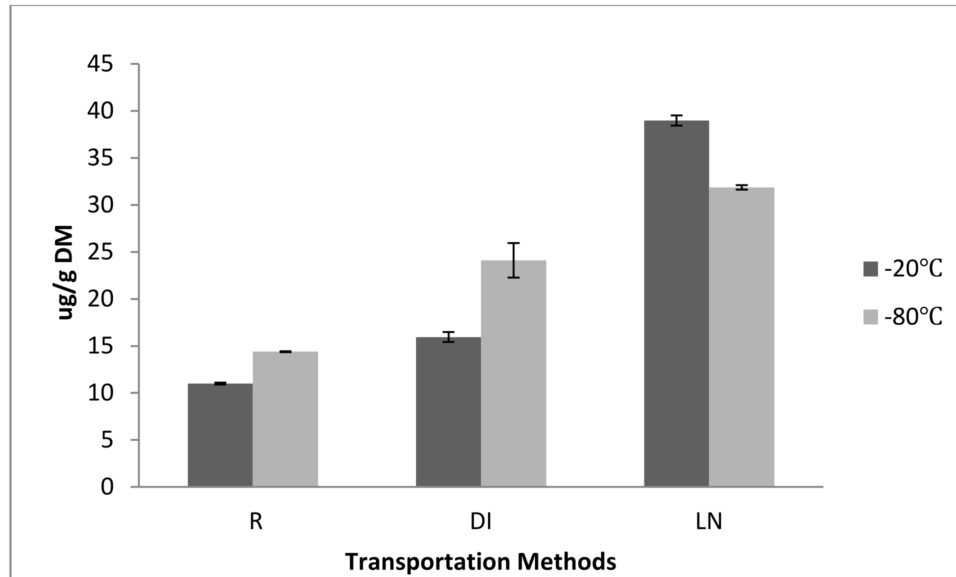
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	9286.029	1	9286.029	13832.591	.000
Temp	9.875	1	9.875	14.709	.002
Method	1615.814	2	807.907	1203.469	.000
Temp * Method	183.144	2	91.572	136.407	.000

The amounts of *trans*-resveratrol in Norton grape skins are showed in Figure 4-14.

Table 4-20 Results of *trans*-resveratrol (mg/g DW) in Norton grape skins*

	-20 °C	-80 °C
R	10.99^{e}	14.40^{d}
DI	15.94^{d}	24.09^{c}
LN	38.98^{a}	31.87^{b}

Immediate postharvest treatment: R= Regular Ice, DI= Dry Ice, LN= Liquid Nitrogen; storage temperature -20 °C= storage at -20 °C, -80 °C= storage at -80 °C; DW= Dry Weight;
*. Means with same letters are not significantly different at P< 0.05 by Duncan's Multiple Range Test.



Immediate postharvest treatment: R= Regular Ice, DI= Dry Ice, LN= Liquid Nitrogen; storage temperature: -20 °C= storage at -20 °C, -80 °C= storage at -80 °C; DM= Dry Materials

Figure 4-14. *trans*-Resveratrol in Norton grape skins

In Figure 4-14, the *trans*-resveratrol varied for the different treatments. The highest amount of *trans*- resveratrol was 38.98 μg/g DM grape skin (-20°C, Liquid Nitrogen), and the lowest amount was 10.99 μg/g DM grape skin (-20°C, Regular ice). Few references are available in regard to the *trans*-resveratrol content of Norton grapes. However, the *trans*-resveratrol for other grape skins is available. For the red grapes, Cabernet Mitos had 123.0±5.1 μg/g DM in 2001 and 11.1±1.6 μg/g DM in 2002; Lemberger had 22.7±1.0 μg/g DM in 2001 and 22.4 ±1.5 μg/g DM in 2002; Trollinger had 50.0±3.5 μg/g DM in 2001and 37.9 ±4.7 μg/g DM in 2002. While Weisser Riesling, a white grape, had 86.4±4.5 μg/g DM in 2002(Kammerer and others 2004). In our research, the content of *trans*-resveratrol in Norton grape skins was LN＞DI＞R. The possible reason was the *trans*-resveratrol was photosensitive and easily oxidized, so *trans*-resveratrol in grape skins without any protection was easily exposed to oxygen and invert to

cis-resveratrol. Therefore, in this research, the DI and LN groups had higher amounts of *trans*-resveratrol than the R group.

In this research, the combinations of immediate post-harvest freezing conditions (R, DI and LN) and storage temperatures (-20 °C and -80 °C) showed varied results in Norton grapes. The temperature of each freezing condition is -15 °C to -20 °C for regular ice, −78.5 °C for dry ice and -196 °C for liquid nitrogen. The total time that the grapes were exposed to each freezing condition is around one hour, for example, the time it took from harvest to transporting the grapes to the laboratory. As a result of that, grapes placed on regular ice just chilled down but did not freezing completely. In the dry ice group, grapes are gradually frozen and intercellular water may have formed large ice crystals. These ice crystals would then have destroyed the membrane of cells and cause chemical and enzymatic reactions during storage (A´lvarez and others 2006). Because of the extremely cold temperature of liquid nitrogen, grapes were frozen very fast and the intercellular water just formed small crystals and the cell membrane received little damage.

As mentioned before, quite a few reactions still can happened at -20 °C, such as decrease of pectin and the invert sugar formation of sucrose. However, compared with -20 °C, there are few or no chemical and enzymatic activities in grapes at -80°C. When the different freezing temperatures and storage temperatures are combined together, grapes are either “warmed up” or “cooled down” during storage. This kind of fluctuation of temperature leads to the diverse

results in grape analyses. But because of lack of published literature on frozen grapes, it is hard to explain some results in this research.

CHAPTER 5

CONCLUSION AND FUTURE RESEARCH

5.1 Conclusion

Regarding the basic chemical analyses of Norton grapes, pH and TA were influenced by the interaction of temperature and time. Brix was influenced by time and temperature but not affected by their interaction. Transportation methods only influenced pH but had no effect on TA and Brix. However, the phenolic compounds were more easily influenced by temperature, time and transportation methods. These factors had significant influence on the amounts of anthocyanins, phenolic acids and *trans*-resveratrol. In regard to transportation methods, regular ice may keep the frozen grape quality more close to that of the fresh grape quality than the other two methods for subsequent storage. The grapes are preserved better at -80 °C because the chemical and enzymatic activities are minimized at this temperature.

For the anthocyanin and phenolic acid analyses, using dry ice generally decreased the content of these compounds. The regular ice group had slightly higher values than the liquid nitrogen group. Comparing the two different temperatures, -80 °C may keep more phenolic compounds than -20 °C. However, for the *trans*-resveratrol analysis, using liquid nitrogen and storing at -20 °C contributes to a higher amount of *trans*-resveratrol.

In summary, there were distinct differences and changes in grape compounds for the different storage treatments; therefore, it is very difficult to

choose the optimal conditions for all compounds. However, considering the cost of liquid nitrogen and the undesirable results of the dry ice treatment, transportation on regular ice is a more practical and reasonable method for dealing with a large amount of samples. Grape samples can be preserved at a higher quality and for a longer time at -80 °C.

5.2 Future Research

As mentioned before, there is little research about the influence of immediate post-harvest freezing conditions and storage temperatures on grapes. The biological mechanisms in frozen grapes are still unclear at the molecular levels. More studies are needed to fully understand these mechanisms and find the better ways to preserve grapes. In addition, compositional changes of grape berries during frozen storage are very important for academic research. However, data for one harvest year is not enough to obtain accurate results. Future research should replicate the analyses of grape berry compounds for different harvest years to get consistent results.

Other phenolic compounds of Norton grapes, such as flavonols, flavanols, and tannins may also be studied. Knowing the changes of these compounds under different storage conditions and transportation methods may help identify the best grape sample preservation method for academic research. In addition, the frozen Norton grapes stored under optimal conditions should be used to make wine. The successful application of frozen grape technology may increase Norton wine production because it may allow winemaking throughout the year.

REFERENCES

Ali MB, Howard S, Chen SW, Wang YC, Yu O, Kovacs LG, Qiu WP. 2011. Berry skin development in Norton grape: Distinct patterns of transcriptional regulation andflavonoid biosynthesis. BMC Plant Biology 11:7

Alvarez I, Aleixandre JI, Garcı´a MJ, Lizama V. 2005. Impact of prefermentative maceration on the phenolic and volatile compounds in Monastrell red wines. Analytica Chimica Acta 563:109–115

Ambers RKR, Ambers CP. 2004. Dr. Daniel Norborne Norton and the Origin of the Norton Grape. American Wine Society Journal: 36(3): 77- 87

Chong J, Poutaraud A, Hugueney P. 2009. Review: Metabolism and roles of stilbenes in plants. Plant Sci 177: 143–155.

Cynkar WU, Cozzolino D, Dambergs RG, Janik L, Gishen M. 2004. The effects of homogenization method and freezing on the determination of quality parameters in red grape berries of *Vitis vinifera*. Australian Journal of Grape and Wine Research 10: 236-242.

Dai R, Ge H, Howard S, Qiu W. 2012. Transcriptional expression of Stilbene synthase genes are regulated develop mentally and differentially in response to powdery mildew in Norton and Cabernet Sauvignon grapevine. Plant Sci 197: 70–76.

Flora LF.1976. Juice quality from whole muscadine grapes held in frozen storage. American J of Enology and Viticulture 27:84–87.

Flamini R, Mattivi F, De Rosso M, Arapitsas P, Bavaresco L. 2013. Advanced knowledge of three important classes of grape phenolics: anthocyanins, stilbenes and flavonols. Inter J Mol Sci 14(10): 19651–19669.

Funasaka Y, Komoto M, Ichihashi M. 2000. Depigmenting effect of alpha-tocopheryl ferulate on normal human melanocytes. Pigment Cell Society13 (8): 170–174.

Fung RWM, Gonzalo M, Fekete C, Kovacs LG, He Y, Marsh E, McIntyre LM, Schachtman DP, Qiu WQ. 2008. Powdery Mildew Induces Defense-Oriented Reprogramming of the Transcriptome in a Susceptible But Not in a Resistant Grapevine. Plant Physiology 146: 236-249.

Garcia S, Santesteban LG, Miranda C, Royo JB. 2011. Variety and storage time affect the compositional changes that occur in grape samples after frozen storage. Australian Journal of Grape and Wine Research 17: 162–168.

Gil-Munoz R, Ana Moreno-Perez A,Rosario Vila-Lopez R, Fernandez-Fernandez JI, Martinez-Cutillas A, Gomez-Plaza E. 2009. Influence of low temperature prefermentative techniques on chromatic and phenolic characteristics of Syrah and Cabernet Sauvignon wines. Eur Food Res Technol 228:777–788

Hakimuddin F, Paliyath G, Meckling K. 2004. Selective cyto-toxicity of a red grape wine flavonoid fraction against MCF-7 cells. Breast Cancer Res. Treat 85: 65–79.

Hogan S, Zhang L, Li JR, Zoecklein B, Zhou KQ. 2009. Antioxidant properties and bioactive components of Norton (*Vitis aestivalis*) and Cabernet Franc (*Vitis vinifera*) wine grapes. Food Sci and Techy 42: 1269–1274.

Hyun JW, Chung HS. 2004. Cyanidin and Malvidin from Oryza sativa cv. Heugjinjubyeo mediate cytotoxicity against human monocytic leukemia cells by arrest of G(2)/M phase and induction of apoptosis. J Agri Food Chem 52(8):2213-2220.

Joaiah S, Striegler KR, Bergmeier E, Harris J. 2012. Influence of cluster exposure to sun on fruit composition of “Norton” grapes (Vitis estivalis Michx) in Missouri. International J Fruit Sci 12: 410-426.

Kammerer D, Claus A, Carle R, Schieber A. 2004. Polyphenol Screening of Pomace from Red and White Grape Varieties (*Vitis vinifera L.*) by HPLC-DAD-MS/MS. J Agric Food Chem 52: 4360 −4367.

Kanaya K. 2010. Effects of ferulic acid and Angelica archangelica extract (Feruguard) in patients with Alzheimer's disease. J Alzheimer's Association 6(4): 548.

Karakaya S. 2004. Bioavailability of phenolic compounds. Critical Reviews in Food Science and Nutrition 44: 453–464.

Konishi Y, Kobayashi S. 2004a. Transepithelial transport of chlorogenic acid, caffeic acid, and their colonic metabo-lites in intestinal Caco-2 cell monolayers. J Agric Food Chem 52:2518–2526.

Lamuela-Raventos RM, Romero-Perez AI, Waterhouse AL, de la Torre-Boronat MC. 1995. Direct HPLC Analysis of cis- and trans-Resveratrol and Piceid Isomers in Spanish Red Vitis vinifera Wines. J Agri Food Chem 43 (2): 281–283.

Lapidot T, Harel S, Granit R, Kanner J. 1999. Anthocyanins in red wines: antioxidant acitivity and bioavailability in human. In: Kumpulainen JT, Salonen JT. Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease. Cambridge: The Royal Society of Chemistry. p 151-153.

Locatelli C, Filippin-Monteiro FB, Creczynski-Pasa TB. 2013. Alkyl esters of gallic acid as anticancer agents: A review. J Europ Med Chem 60: 233 -239.

Main, G. 2005. Growing and vinting Cynthiana/Norton grapes. In Proceedings of the 24th Annual Horticulture Industries Show. January 14-15, 2005. Available from: http://www.uark.edu/depts/ifse/grapeprog/articles/ahis05wg.pdf. Accessed 2014 March 30.

Mattern R. 2009. File:Hydroxybenzoic acids.svg. Available from: http://commons.wikimedia.org/wiki/File:Hydroxybenzoic_acids.svg. Accessed 2014 March 30.

Mattern R. 2009. File:Hydroxycinnamic acids.svg. Available from: http://commons.wikimedia.org/wiki/File:Hydroxycinnamic_acids.svg?uselang=zh-cn. Accessed 2014 March 30.

Monagas M, Hernandez-Ledesma B, Garrido I, Martin-Alvarez PJ, Gomez-Cordoves C, Bartolome B. 2005. Quality assessment of commercial dietary antioxidant products from *Vitis vinifera l*. grape seeds. Nutr Cancer 53(2): 244–254.

Munoz-Espada AC, Wood KV, Bordelon B, Watkins BA. 2004. Anthocyanin Quantification and Radical Scavenging Capacity of Concord, Norton, and Marechal Foch Grapes and Wines. J Agric Food Chem 52: 6779−6786.

Ortinau L. 2009. Resveratrol and Procyanidin content in select Missouri Red Wines. [MSc thesis]. Columbia, MO: Missouri Univ- Columbia. on: https://mospace.umsystem.edu/xmlui/handle/10355/5366

Ou SY, Kwok KC. 2004. Ferulic acid: pharmaceutical functions, preparation and applications in foods. J Sci Food Agric 84 (11): 1261 – 1269.

Patterson SJ, Fischer JG, Dulebohn RV. 2008. DNA damage in HT-29 colon cancer cells is enhanced by high concentrations of the anthocyanin malvidin. Faseb J 22.

Polimerek. 2009. File:Resveratrol trans.png. Available from: http://commons.wikimedia.org/wiki/File:Resveratrol_trans.png?uselang=zh-cn. Accessed 2014 April 2

Responses EI, Lakso AN, Kliewer WM. 1975. The Influence of Temperature on Malic Acid Metabolism in Grape Berries Plant. Physiol 56: 370-372.

Ramara M, Manikandanb B, Ramanc T, Priyadarsinib A, Palanisamya S, Velayudamb M, Munusamyb A, Prabhua NM, Vaseeharana B. 2012. Protective effect of ferulic acid and resveratrol against alloxan-induced diabetes in mice. J Europ Pharm 690 (1–3): 226–235.

Romero-Pe´rez AI, Lamuela-Ravento´s RM, Andre ´s-Lacueva C, de la Torre-Boronat MC. 2001. Method for the Quantitative Extraction of Resveratrol and

Piceid Isomers in Grape Berry Skins. Effect of Powdery Mildew on the Stilbene Content. J Agric Food Chem 49: 210−215.

Romero I, Sanchez-Ballesta MT, Escribano MI, Merodio C. 2008. Individual anthocyanins and their contribution to total antioxidant capacity in response to low temperature and high CO2 in stored Cardinal table grapes. Postharvest Biology and Technology 49: 1–9.

Santesteban LG, Miranda C, Royo JB. 2013. Influence of the freezing method on the changes that occur in grape samples after frozen storage. J Sci Food Agric 93: 3010– 3015.

Saxena M, Saxena J, Pradhan A. 2012. Flavonoids and phenolic acids as antioxidants in plants and human health. Int. J. Pharm. Sci. Rev. Res., 16(2): 130-134.

Schultz TP, Boldin WD, Fisher TH, Nicholas DD, McMurtrey KD, Pobanz K. 1992. Structure-fungicidal properties of some 3- and 4-hydroxylated stilbenes and bibenzyl analogues. Phytochemistry 31: 3801–3806.

Shahrzad S, Aoyagi K, Winter A, Koyama A, Bitsch I. 2001. Pharmacokinetics of gallic acid and its relative bioavail-ability from tea in healthy humans. J Nutr 131:1207–1210.

Shahrzad S, Bitsch I. 1998. Determination of gallic acid and its metabolites in human plasma and urine by high-perfor-mance liquid chromatography. J Chromatogr B 705:87–95.

Shen T, Wang XN, Lou HX. 2009. Natural stilbenes: an overview. Natural Product Reports 26: 916–935.

Shyamal K. Goswami, Dipak K. Das. 2009. Resveratrol and chemoprevention. Cancer Letters 284: 1–6.

Sigita B, Krasnova I, Seglina D, Skrupskis I.2013. Changes of Pectin Quantity in Fresh and Frozen Apple Products. J Chem Chem Eng : 64-69.

Skrede G. 1983. Changes in Sucrose, Fructose and Glucose Content of Frozen Strawberries with Thawing. J Food Sci 48 (4): 1094-1096.

Smiley. 2008. ‘Cynthiana’ / ‘Norton’. Available from: http://viticulture.hort.iastate.edu/cultivars/Cynthiana.pdf . Accessed 2014 April 2.

Stonebridge Research Group. 2010. The Economic Impact of Wine and Grapes in Missouri 2010. In: Board MGaW, editor. Jefferson City, Missouri.

Suzuki A, Yamamoto M, Jokura H, Fujii A, Tokimitsu I, Hase T, Saito I. 2007. Ferulic acid restores endothelium-dependent vasodi-lation in aortas of spontaneously hypertensive rats. J American Soc Hypertension 20: 508–513.

Tian Y, Jiang B, An L, Bao Y.2007. Neuroprotective effect of catalpol against MPP+-induced oxidative stress in mesencephalic neurons. J Europ Pharma. 568, 2007; 142-148.

Threlfall R, Main G, Morris J. 2006. Effect of freezing grape berries and heating must samples on extraction of components and composition parameters of red wine grape varieties. Australian J of Grape and Wine Research 12: 161–169

Top Cultures. Phytochemicals. Available from: http://www.phytochemicals.info/phytochemicals/malvidin.php. Acessed 2014 March 22.

Walle T, Hsieh F, Delegge MH, Oatis JE, Jr., Walle UK. 2004. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos. 32(12):1377-1382.

Prior RL, Wu X. 2006. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities, Free Radic. Res. 40: 1014–1028.

Yikrazuul. 2009. File:Malvidin-3-glucoside.svg. Available from: http://en.wikipedia.org/wiki/File:Malvidin-3-glucoside.svg. Accessed 2014 March 29

Yikrazuul. 2011. File:Malvidin-3, 5-diglucoside.svg. Available from: http://en.wikipedia.org/wiki/File:Malvin.svg. Accessed 2014 March 29

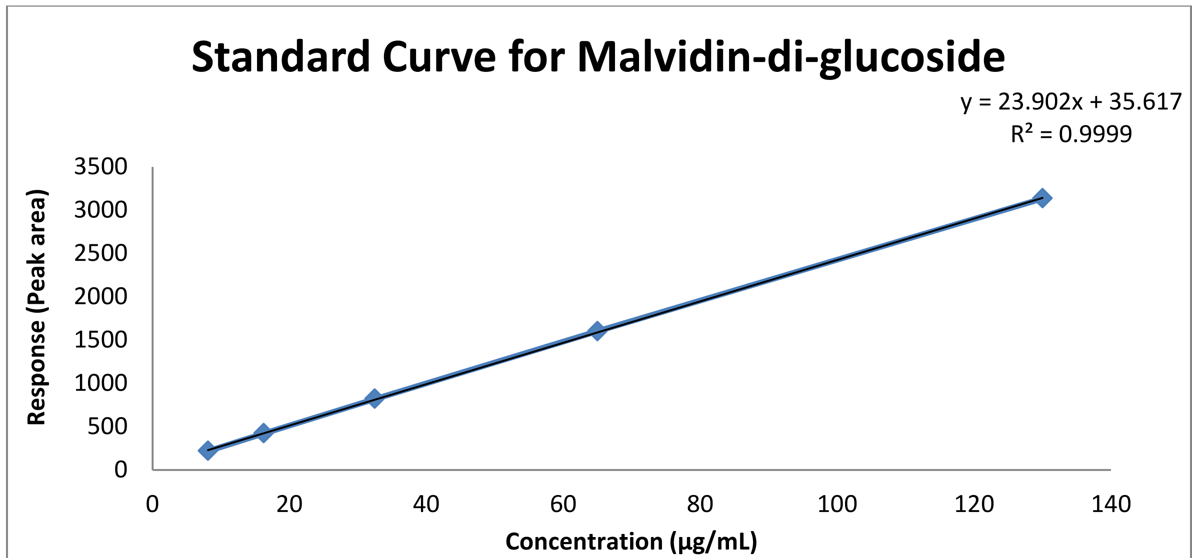
Yilmaz Y, Toledo RT. 2004. Major flavonoids in grape seeds and skins: anti-oxidant capacity of catechin, epicatechin, and gallic acid. J Agri Food Chem 52(2): 255–260.

Zhang Y, Vareed SK, Nair MG. 2005. Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. Life Sci 76: 1465–1472.

Zhao ZH, Moghadasian MH. 2008. Chemistry, natural sources, dietary intake and pharmacokinetic properties of ferulic acid: A review. Food Chem 109: 691–702.

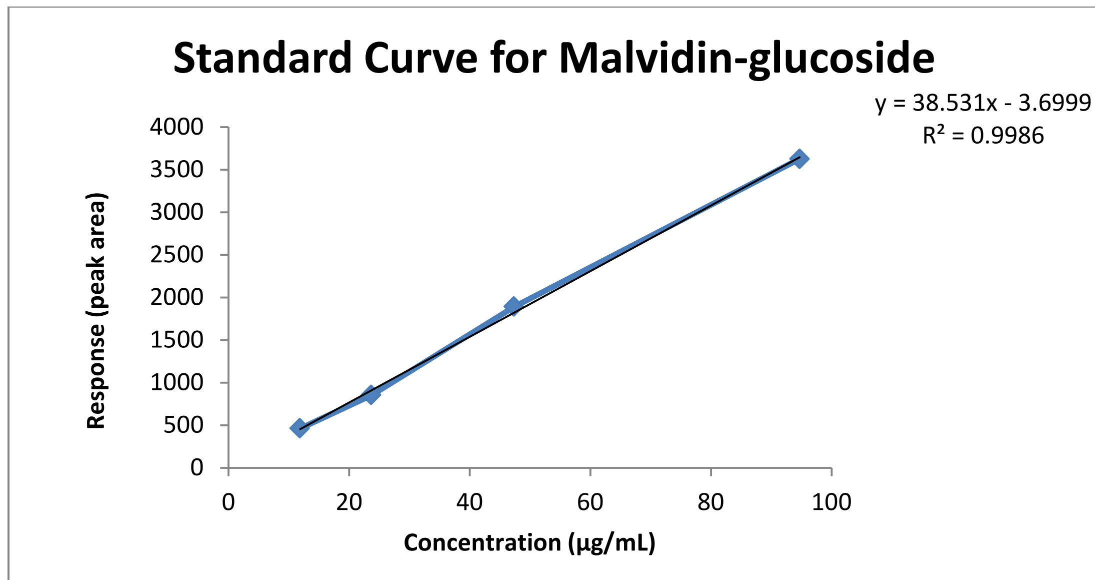
APPENDIX

A-1 Malvidin-di-glucoside Standard Curve and Concentration used



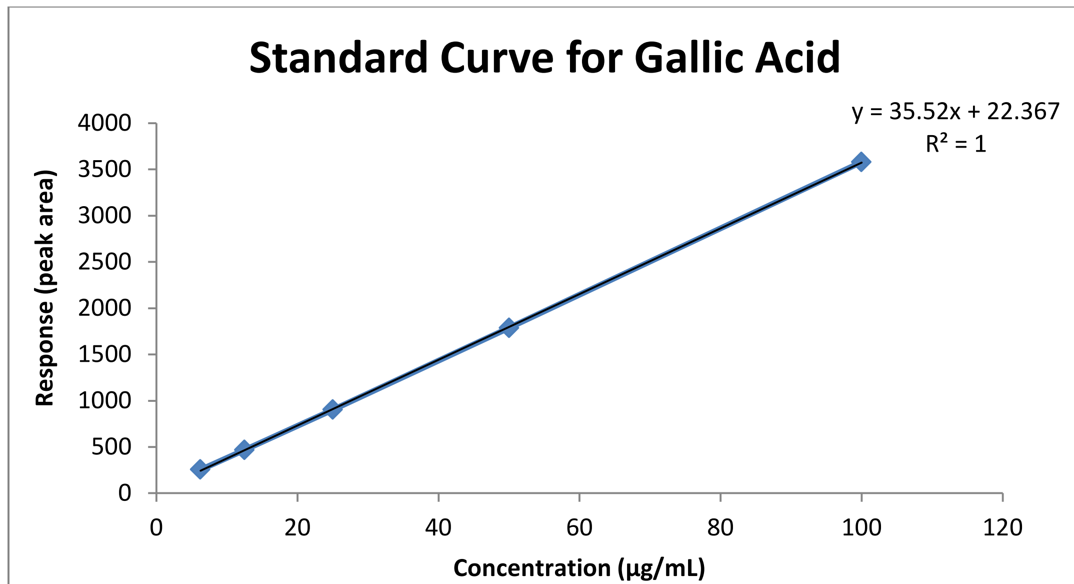
Malvidin-di-glucoside	
Concentration (ug/mL)	**Peak area**
130	3135.9
65	1601.4
32.5	820.2
16.25	421.3
8.125	219.7

A-2 Malvidin-glucoside Standard Curve and Concentration used



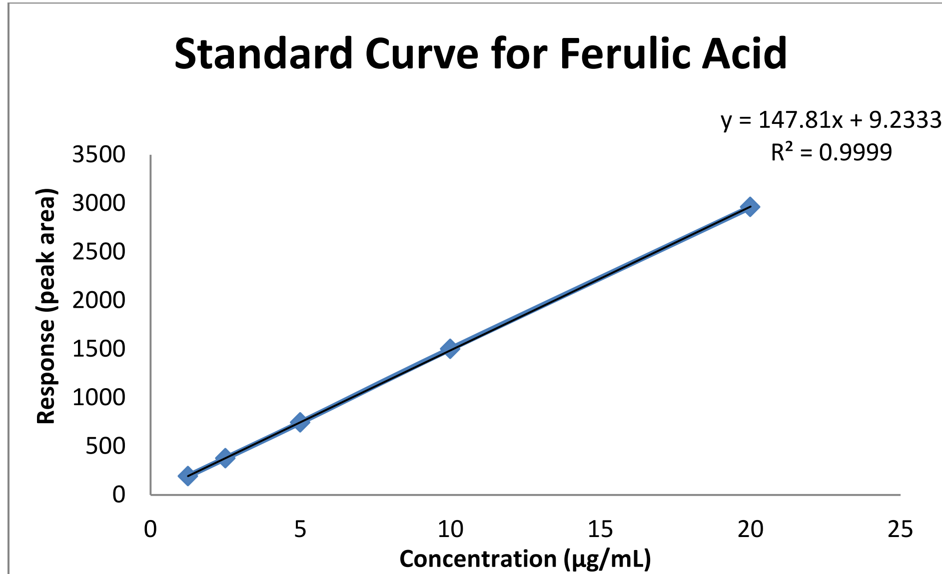
Malvidin-glucoside	
Concentration (µg/mL)	**Peak area**
94.74	3625.3
47.37	1890.3
23.68	852.5
11.84	461.4

A-3 Gallic acid Standard Curve and Concentration used



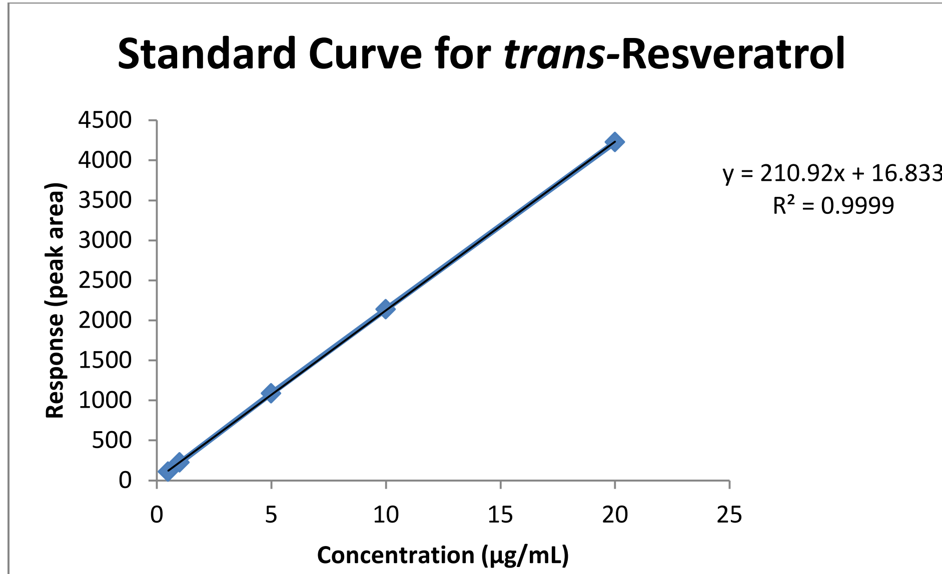
Gallic Acid	
Concentration (µg/mL)	**Peak area**
100	3580.7
50	1787.7
25	902.9
12.5	468.1
6.25	254.4

A-4 Ferulic acid Standard Curve and Concentration used



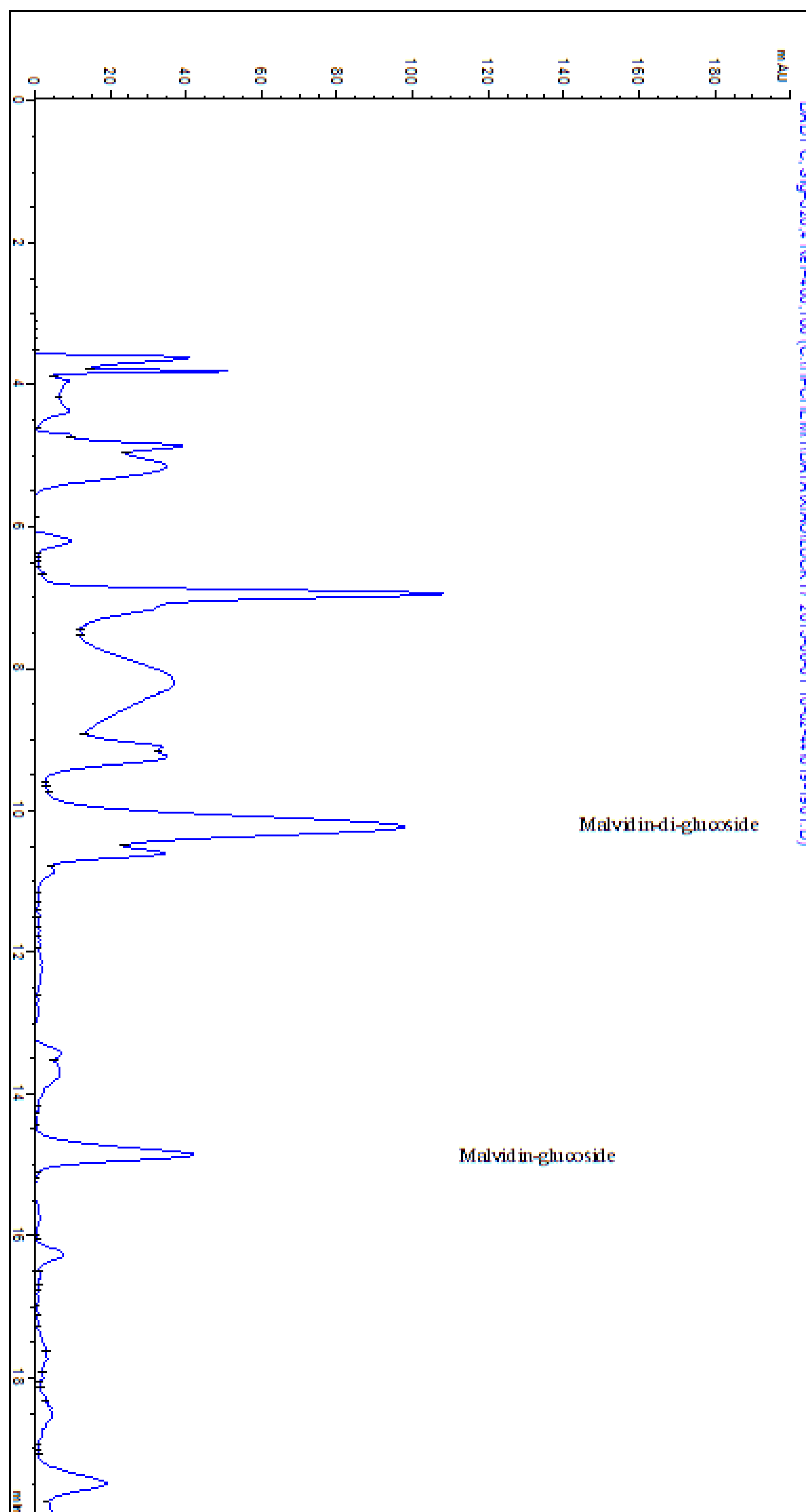
Ferulic Acid	
Concentration (µg/mL)	**Peak area**
20	2959.9
10	1501.4
5	744.4
2.5	375.9
1.25	192.3

A-5 *trans*-Resveratrol Standard Curve and Concentration used



trans-Resveratrol	
Concentration (µg/mL)	**Peak area**
20	4225.8
10	2138
5	1087.8
1	222.5
0.5	108.7

A-5 Chromatography Example of Anthocyanins in Norton Grape Skins (Liquid nitrogen, -80°C, 3 month)



A-6 Chromatography Example of Penolic Acids and *trans*- Resveratrol in Norton Grape Skins (Regular ice, -80°C)