ABSTRACT

CARLSON, JOSHUA STEVEN. Processing Effects on the Antioxidant Activities of Blueberry Juices. (Under the direction of Dr. Leon Boyd.)

The inverse relationship between antioxidant intake and many disease states has been seen repeatedly. The importance of antioxidants has led blueberry processors to look for ways to modify processing techniques to optimize antioxidants in the final product. With the availability of new processing methods such as microwaving, it is important to determine the effects of emerging process technologies on antioxidant retention and product quality. The goal of this research was to determine the best processing method available to produce blueberry juice with the highest antioxidant activity.

One experiment examined the effect of cold (22°C) versus hot processing (43°C) of seven cultivars of blueberries using the oxygen radical absorbance capacity (ORAC) and total phenols assays. Compared to cold processing, hot processing yielded a product with 50% more total phenols and antioxidant capacity. A second experiment used ORAC and total phenols to evaluate the effects of cold (22°C), hot (47°C), and pasteurization (90°C) of blended blueberries heated in a steam-jacketed kettle versus microwave blended blueberries heated (85°C, 92.5°C, and 95°C). Pasteurized blueberries retained the highest total phenols and ORAC values, followed by hot processed and microwaved juices having similar values, with cold processed blueberries having the lowest values. The final study used ORAC, total phenols, and total anthocyanins to evaluate a one month refrigerated (3-5°C) time study on pasteurized (90°C) and microwaved blueberry juices (85°C, 92.5°C, and 95°C). Pasteurized

blueberries retained antioxidant activity, as noted by total phenols and ORAC values, that was equal to or better than microwaved treatments over the 30-day trial. Addition of heat, without sustained boiling, resulted in even higher levels of total phenols and ORAC in blueberry juices. The implication of this research was that the addition of heat increased the levels of total phenols and ORAC of juices.

PROCESSING EFFECTS ON THE ANTIOXIDANT ACTIVITIES OF BLUEBERRY JUICES

by,
JOSHUA STEVEN CARLSON

A thesis submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
Requirements for the Degree of
Master of Science

FOOD SCIENCE

Raleigh

2003

APPROVED BY:

Jonathan C. Allen Daniel E. Carroll

Leon Ford
Chair of Advisory Committee

DEDICATION

I would like to dedicate this thesis to my parents and Kit San (Shirley) Chan who all supported me throughout this endeavor. Without their support I never would have made it.

BIOGRAPHY

The author was born and raised in Wisconsin. He graduated with a B.S. in Food Science from the University of Wisconsin-Madison in August 2001. Upon completion of this thesis, he will be earning his M.S. in Food Science from North Carolina State University in May 2003.

ACKNOWLEDGEMENTS

I would like to thank everyone who helped me during my time and NCSU. I would like to thank Dr. Leon Boyd for giving me the opportunity to work in his laboratory, and for helping me to put this thesis together. I would like to thank Ruth Watkins who helped me numerous times with laboratory equipment. I would like to thank Dr. Josip Simunovic who let me use the microwave, as well as JoAnna Foegeding who taught me how to make juices. I would like to thank Solo Foods Inc. who supplied me with blueberries and also to Industrial Microwave Systems Inc. whose microwave I used in my experiments.

TABLE OF CONTENTS

LIST OF FIGURES ..... *vii*

LIST OF TABLES ..... *viii*

LITERATURE REVIEW *1*

I. Production and Processing of Blueberries **1**
A. Application of Emerging Process Technologies 3
B. Nutritional Benefits of Blueberries 4

II. What are Anthocyanins **5**

III. The Role of Anthocyanins as Antioxidants in Plants **6**

IV. Historical Perspective on Anthocyanins **7**

V. Potential Health Benefits of Blueberries **8**

VI. Effects of Processing on Anthocyanins **12**
A. Natural Enzymes 12
B. Fining Agents 13
C. Ultraviolet Light 13
D. pH 13
E. Temperature 14
F. Free Radicals 14
G. Microwaving 14

VII. Extraction and Measurement of Antioxidant Activity **15**
A. Extraction of Anthocyanins 15
B. Purification of Anthocyanins 16
C. Total Phenols 17
D. The Application of High Performance Liquid Chromatography to Anthocyanin Analysis 17
E. Analysis of Total Anthocyanins 18
F. Capillary Electrophoresis 18
G. Oxygen Radical Absorbance Capacity (ORAC) 19

VIII. Roles of Antioxidants in Controlling Free Radicals **19**

IX. Present Research Objectives **23**

MATERIALS AND METHODS *25*

I. Chemicals **25**

II. Blueberry Samples **25**

III. Experiment #1: Comparison of Extraction Method Efficiency **26**
Extraction Methods 26

V. Experiment #2: Effects of Cold Versus Hot Processing on the Antioxidant Activity of Seven Different Cultivars of Blueberries **27**

A. Cold Processed Blueberry Juices 27
B. Hot Processed Blueberry Juices 28
C. Wine Making 28

VI. Experiment #3: Effects of Microwave Pasteurization on the Antioxidant Activity of Blueberry Juices..... 28

VII. Experiment #4: Effects of Microwaving, Cold Processing, Hot Processing, and Pasteurization on the Initial Antioxidant Retention in Blueberry Juices..... 30
A. Cold Processed Blueberries..... 30
B. Hot Processed Blueberries..... 30
C. Pasteurization of Blueberries..... 31
D. Microwaving Processing Blueberries..... 31

VIII. Analytical Procedures..... 32
A. Total Phenols Assay 32
B. Oxygen Radical Absorbance Capacity (ORAC) Assay 32
C. Analysis of Individual Anthocyanins 33
D. Estimating Total Anthocyanins 33
E. Statistical Analysis..... 34

RESULTS AND DISCUSSION 34

I. Experiment #1: Comparison of Extraction Method Efficiency 34

II. Experiment #2: Effects of Cold Versus Hot Processing on the Antioxidant Activity of Seven Different Cultivars of Blueberries..... 35
A. Total Phenols:..... 35
B. ORAC Values:..... 36

III. Experiment #3: Time Trial on Changes in the Antioxidant Activity as Measured by Changes in Total Phenols, Anthocyanins, and Antioxidant Activity. 37
A. Anthocyanin Level Changes Over Time 37
B. ORAC Values Over Time..... 39
C. Polyphenols Over Time 40

IV. Experiment #4: Effects of Microwaving, Cold Processing, Hot Processing, and Pasteurization on the Antioxidant Retention in Blueberry Juices. 41
Comparing Traditional and Non-Traditional Processing Methods 41

CONCLUSION..... 42

REFERENCES..... 62

LIST OF FIGURES

Figure 1. Structures of Commonly Consumed Anthocyanins……...48

Figure 2. Four States of Anthocyanins…….49

Figure 3. Polyphenols - structure and biosynthesis……...50

Figure 4. Degradation of Cyanidin 3-Glucoside by Blueberry Polyphenol Oxidase……51

Figure 5. Oxidation States of Vitamin E……52

Figure 6. Oxidation States of Ascorbic Acid…… 53

Figure 7. Comparison of Extraction Methods Over Time…….54

Figure 8. Total Phenols Values for Cold versus Hot Processed Blueberry Wines……...55

Figure 9. ORAC Values for Cold versus Hot Processed Blueberry Juices and Wines... 56

Figure 10. Changes in Total Anthocyanins of Microwaved and Pasteurized Blueberries During Refrigerated Storage……..57

Figure 11. ORAC Changes in Microwaved and Pasteurized Blueberry Juices Over Time……...58

Figure 12. Total Phenol Changes in Microwaved and Pasteurized Blueberry Juices Over Time…….. 59

Figure 13. ORAC Values for Different Processing Methods of Blueberry Juices……...60

Figure 14. Total Phenol Values for Different Processing Method of Blueberry Juices…61

LIST OF TABLES

Table 1. Blueberry Composition……44

Table 2. 1998 FAO World Blueberry Production Statistics…..……45

Table 3. Thermal Degradation Kinetics of Anthocyanins: t1/2 for Malvidin-3-glucoside for corresponding temperatures and pHs……..…….46

Table 4. Individual Anthocyanin Composition of Blueberry Juices...……..47

LITERATURE REVIEW

I. Production and Processing of Blueberries

Blueberries are native to the United States. Their European counterparts are called bilberries (Eck 1988). The three main categories of blueberries are Rabbiteye, Highbush and Lowbush (Eck 1988). A blueberry is considered ripe if the following conditions are met (Table 1):

1) pH 3.25-4.25 (Eck 1970)
2) total acid (citric) 0.3-1.3% (Eck 1988)
3) soluble solids (sugar) above 10% (Eck 1970)
4) soluble solids to acid ratio 10:33 (Ballinger and Kushman 1970)
5) anthocyanin content below .5%, but stem scar should be purple (Sapers et al. 1984)

Blueberries are from the same family, Ericaceae, as cranberries, lingonberries, and bilberries. Blueberries can be grown where temperatures stay below 7.2°C without getting lower than –24°C for at least one month straight each year (Eck 1988). This temperature requirement has led Michigan and New Jersey to become the largest producing states in the U.S. Other states where blueberries are grown include North Carolina, Georgia, Arkansas, and Texas (Moore 1994). Blueberries are also grown in Canada (Villata 1998), Australia, New Zealand, Chile, and parts of Europe (Moore 1994). According to the 1998 Food and Agriculture Organization (FAO) statistics, the United States leads the world in the production of blueberries (Table 2).

Blueberries are picked by hand or mechanically through shaking. After picking they are cooled to 40°F in tubs which hold 30-40 lbs. of blueberries and are sorted by color, size, and density. Blueberries are dumped onto a conveyor belt and extraneous, foreign matter is removed by hand. The berries are washed in chlorinated water at 100ppm (Hazen et al. 2003) to kill bacteria. The berries are conveyed through a color detector, which removes unripe blueberries, leaves, and remaining foreign matter. Blueberries are then sent on small rollers that remove any remaining stems. The berries are sized by machine into small and large.

Only 15% of large USDA grade A berries are packaged for fresh market. Only a small percentage of blueberries reach the fresh market, and most berries end up frozen or canned (Moore 1994). Frozen berries can be freeze dried and put into cereals, granola bars, pre-made blueberry pancakes, and pre-made blueberry muffins. Berries that are undeveloped or have visual defects are used to make wines, juices, purees, and jams. Puree is added to many different food sources including spreads, ice cream, and yogurt.

Traditionally, blemished and undesirable blueberries that go into making wines and juices can be processed in one of two ways. Blueberries are either hot or cold processed. The first step in both methods is to crush the blueberries. In hot processing, crushed blueberries are heated to between 47°C and 90°C using superheated steam in a jacketed kettle. The berries are then pressed. In cold processing crushed berries are pressed at room temperature (approximately 22°C). In cases where the temperature treatment was low, juices are pasteurized at 90°C.

A. Application of Emerging Process Technologies

New technologies are used to produce quality products. High-hydrostatic-pressure (HHP) (Kimura et al. 1994, Gimenez et al. 2001) has been used and compared to hot processing. Generally, high pressure yields a product with better short term qualities, including higher volatile retention and higher anthocyanin content (Kimura et al. 1994). When the study time was extended for a month, the quality of the traditionally hot processed samples was higher. When making jams using HHP with mild heat to destroy enzymes, HHP produced a pinker, stickier, and softer jam than traditional processing (Gimenez et al. 2001). The color difference was not attributed to anthocyanin levels. HHP produced jams with higher levels of anthocyanins. The color difference was attributed to the textured surface of the HHP jam. The softer and stickier traits were hypothesized to be a different type of gel resulting from lower levels of caramelisation.

Attempts have been made to heat blueberries using microwaves instead of conduction heating in a steam jacketed kettle. Using microwaves to extract samples, microwave assisted extraction (MAE), was done as early as 1986 (Ganzler et al.), and is still used (Eskilsson and Bjorklund 2000). MAE uses crushed food in extracting solvents, and heated in solvents to just below boiling. "The ability to rapidly heat the sample solvent mixture is inherent to MAE and the main advantage of this method" (Eskilsson and Bjorklund 2000). MAE is a 15-30 minute process. After MAE, the use of microwaves instead of a steam jacketed kettle to heat crushed blueberries was tried (de Ancos et al. 1999). In this method, 50g samples were microwaved in a household microwave at 850W for 15, 30, 45, and 60 seconds then cooled in an ice waterbath for 5 minutes. de Ancos et al. (1999) concluded microwaving could inactivate polyphenol

oxidase (PPO) and peroxidase enzymes, and did not significantly alter anthocyanin levels. Trials have been done on the effects of using microwave heating on PPO from mushrooms (Rodriquez-Lopez et al. 1999). Results indicate that microwave heating had faster inactivation kinetics for PPO than did the traditional heating.

Other possible reasons for the difference in inactivation kinetics between microwaving and traditional heating include the hypothesis that microwaves break hydrogen bonds between proteins and water. The theory is that an interaction of the electrical component of the microwave field, and the polar and/or charged moieties associated with the protein molecule, occurred (Kermasha et al. 1993). This could explain why wheat germ lipase was inactivated faster using microwaving than conventional heating. Another possibility is that faster heating through microwaving stabilizes different enzyme intermediates (Rodriquez-Lopez et al. 1999). The theory is that intermediates may have faster inactivation rates than enzymes normally present.

The difficulty of each of these microwave experiments was that they were batch preparations, done in 850W microwave ovens, and not in a single pass using a continuous flow unit. Cooling was done in waterbaths, which are less efficient than mechanical heat exchangers. These experiments could have measured the effects of microwaving more accurately using a plate or tube heat exchanger.

B. Nutritional Benefits of Blueberries

Blueberries have been traditionally used for either fresh consumption or processed into jams, jellies, and juices. Despite centuries of folklore revolving around the healthful properties of blueberries (Mazza and Oomah 2000), the beneficial effects associated with these fruits were not seriously entertained by scientists. Until recently the scientific

community saw few health benefits associated with the consumption of blueberries. In the 1960's, blueberries were reported to be a source of fiber, calcium, iron, and limited vitamin C (Watt and Merrill, 1963). Due to the fact that the levels of these components were not as high as other fruits, blueberries were not pushed for public consumption.

The only other potential use seen for blueberries was to supply a natural coloring agent (anthocyanins). This idea has interested the food industry for quite some time. Anthocyanins are present in the epidermal and hypodermal cells of blueberries (Eck 1988). The interest in anthocyanins as a coloring agent, has continued to this day.

There is limited use of anthocyanins as a natural coloring agent because of problems in extraction and shelf stability. To use an extract in food, the extracting solvents must be food grade, this was and still is a hurdle. Shelf stability is the larger problem. Depending on factors including pH, temperature, and oxygen levels, natural coloring agents degrade more quickly and completely than synthetic antioxidants.

II. What are Anthocyanins

Anthocyanins are a subclass of flavonoids. They can be in the form of anthocyanosides, which are anthocyanins bound to glucose. They can also be found in proanthocyanidins, which are polymers of anthocyanins. For the purpose of this discussion, only free and unbound anthocyanins (anthocyanidins) will be addressed.

The meaning of the word anthocyanin comes from Greek *anthos* = flower, and *kyanos* = blue. Anthocyanins are polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium. Different anthocyanins are identified based on functional groups present on the **A** 5,7 **B** 3',4',5', and **C** 3 locations (Figure 1). Anthocyanins are formed through photosynthesis and glycolysis (Mazza, and Miniati 1993). Anthocyanins are

susceptible to pH changes. At pH values below 2, they exist primarily in the form of red or yellow. At pH values between 2 and 4, anthocyanins exist at different proportions as red, yellow, blue, and purple. At pH values between 4 to 4.5, anthocyanins exist as blues and purples. If the pH rises above 4.5 anthocyanins are colorless. Anthocyanins are responsible for providing color many plants (Figure 2).

Anthocyanins are found in many species of plants such as blueberries, cranberries, blackberries, strawberries, dark skinned grapes, red cabbage, tradescantia, ajuga, and plums. Only six anthocyanins are commonly found in plants; malvidin, cyanidin, petunidin, peonidin, delphinidin, and pelargonidin. The most commonly consumed anthocyanins are cyanidin and malvidin (Scalbert and Williamson 2000).

III. The Role of Anthocyanins as Antioxidants in Plants

Antioxidants serve the same function in plants as they do in humans. Plant produce anthocyanins (Figure 3) to deal with oxidative stresses from physiological stress or environmental insults. Production of free radicals in plants occurs during photosynthesis. When photosynthesis II and photosynthesis I (light and dark photosynthesis) occur, oxygen radicals are produced which is exacerbated by extended exposure to light during cold temperatures, or sudden extended exposure to light without a gradual adaptation time (Krause 1994). "In these times, the supply of reducing power in the plant, generated by the light reactions, exceeds the demands by the dark reactions. This causes chloroplasts to have oxidative stress, which in turn leads to pigment bleaching and lipid peroxidation" (Inze and Montagu 2002). Plants need antioxidants when exposed to herbicides or fungal toxins. These compounds cause accumulation of chlorophyll intermediates, which are free radicals (Daub and Briggs 1983).

Atmospheric pollution causes free radical formation within plants. (Cross et al. 1998). Ozone (Melhorn 1990) and sulfur dioxide (Cross et al. 1998) both seem to lead to the formation of free radicals, with ozone being the harsher (Heagle 1989). Ultraviolet light B (290-320nm) also increases free radical production (Teramura and Sullivan 1994). Research has shown stresses, such as drought and heat shock (Price and Hendry 1991) and chilling and freezing (Wise and Naylor 1987) lead to increased free radical production. Environmental conditions effect free radical levels in plants.

Plants intentionally produce some free radicals to deal with problems. One such instance is when the plant responds to pests. A common defense by plants to pathogens Figure 2. Four States of Anthocyanins (Haslam, 1989) is to produce hydrogen peroxide (Kauss et al. 1994). Superoxide radicals have been linked to programmed cell death (Jabs et al. 1996). Plants also produce nitric oxide radicals (Delledonne et al. 1998). Because external and internal circumstances alter the level of free radicals in plants, they are able to produce many antioxidants, including anthocyanins.

Other antioxidants produced by plants include Vitamin C, Vitamin E, flavonoids, and carotenoids. The production of these compounds makes some plants good dietary sources for antioxidants.

IV. Historical Perspective on Anthocyanins

Early study on anthocyanins began in the 1960's (Durkee and Jones 1969). Testing was done because of the market for natural colorants for the food industry, and the predicted growth of the blueberry industry. The main method of extraction of anthocyanins involved the use of alcohols. The gist of this method was to extract the anthocyanins with methanol (Durkee and Jones 1969) and measure the absorbance at

specific wavelengths. Some tentative identifications of anthocyanins in blueberries were made. There was some use of Folin-Ciocalteau reagent to test total phenols as well (Singleton and Rossi 1965).

Research led to the identification of many of the anthocyanins present in blueberries (Ballinger et al. 1970, Makus and Ballinger 1973). This initial identification was done primarily using paper chromatography methods (Francis et al. 1966).

In the 1980's, use of high performance liquid chromatography (HPLC) and mass spectrometry (Goto 1987) identified specific anthocyanins present in blueberries. Little attention was paid to the possibility that health benefits could be tied to anthocyanins. The goal of the research was to evaluate naturally occurring colorants.

The main emphasis in blueberry and anthocyanin research was related to anthocyanin levels at different points in the harvest, stability of the anthocyanins at different temperatures, stability of anthocyanins at different pH's, and the development of better extraction methods.

V. Potential Health Benefits of Blueberries

In the early 1990's having realized the health value of antioxidants, Dr. Ronald Prior and others at the United States Department of Agriculture developed a new assay called ORAC (oxygen radical absorbance capacity). This procedure measures the total capacity of many different fruits and vegetables to absorb free radicals. These scientists observed that blueberries had one of the highest free radical absorbing capacities (Prior et al. 1998). Because free radicals contribute to many health problems (Gutteridge and Halliwell 1994), this was a major discovery.

The discovery of potential health benefits tied to blueberries led to new products for blueberries called dietary supplements. Dietary supplements, under the Dietary Supplement Health and Education Act of 1994 can be marketed without going through the rigorous trials that drugs must overcome to be put on the market. Today, there are pills containing ground dried blueberries, ground dried blueberry leaves, and combinations of blueberries and leaves as well as extracts of blueberries available on the market at health food stores. Companies that produce their own vitamin lines have begun to incorporate blueberries and other sources of anthocyanins into other products as well.

Blueberry supplements contain claims of health benefits including enhanced eye adaptation to light, improved vascular retinal flow, prevention of cataracts, reduction in diabetic-induced glaucoma, and myopia, reduced eye strains. Other reported health claims include improvement of the body's circulatory system, support of the production of healthy connective tissues, and reduction of the potential for diseases (Herbal Remedies, Apollo Herbs, General Nutrition Center).

Anthocyanins were shown to be protective against oxidation of low density lipoproteins (LDL) in experiments with copper catalysts (Satue-Gracia et al. 1997). In *in vitro* aqueous studies, LDL in the presence of anthocyanins retarded oxidation (Laplaud et al. 1997).

Anthocyanins have been shown to reduce cardiovascular disease (CVD). Hypertension and atherosclerosis reduce flexibility of capillary walls causing poor blood flow (Hertog et al. 1993). Delphinidin, found in blueberries, induces a similar vasorelaxation to that induced by red wine polyphenols (Andriambeloson et al. 1998, Andriambeloson et al. 1996). In one study, Wistar rats (12-14 weeks old), were fed 3-

18mg of anthocyanins, tannins, or red wine polyphenol compounds and the effect on vasorelaxation was studied on the aortic rings. The results found that delphinidin had a vasorelaxation effect 89% as efficient as red wine polyphenolic compounds. Malvidin and cyanidin did not exhibit vasorelaxation effects.

Anthocyanins have been found to reduce platelet aggregation (Demrow et al. 1995). Dogs were fed wines and fruit juices. The effects of the ethanol alone and that of the wines and juices were evaluated. They determined red wines and juices had a significant effect on decreasing platelet aggregation greater than white wines. This was attributed to anthocyanins, tannins, and phenolic flavonoids present in higher concentrations in the red wines and fruit juices.

Anthocyanins inhibit the enzyme aldose reductase that converts sugars to sugar alcohols. This pathway has been tied to diabetic complications including neuropathy, retinopathy and heart disease (Varma, 1986) Blueberry leaf extracts have been shown to lower plasma glucose levels in diabetic rats by 26% (Cignarella et al. 1996).

Blueberries improve night vision (Jayle et al. 1965) and blueberries may relieve eyestrain from staring at a computer screen (Kalt and Dufour 1997). These are tied to increased blood circulation to the eye due to vasorelaxation, and reduced damage suffered by the eye due to exposure to free radicals. Similarly, antioxidants keep eye lens protein radicals from cross-linking potentially leading to cataracts (Kalt and Dufour 1997).

Anthocyanins reduce urinary tract infections (Howell et al. 1998, Ofek et al. 1991) by inhibiting *Escherichia coli* from adhering to epithelial cells in the urinary tract (Ofek et al. 1996). To date, research studies done on the urinary tract have focused on

the effects of cranberry juice. Anthocyanins also have anti-microbial properties (Cristoni and Magistretti 1987) through interference with microbial enzymes.

Anthocyanins possess anti-mutagenic activity (Edenharder et al. 1994). This study examined 28 fruit and 34 vegetable juices commonly consumed in Germany using *in vitro* studies for anti-mutagenic activity using the *Salmonella*/reversion assay. The results for fruit juices found strong anti-mutagenic activity in banana, blackberry and blueberry juices.

Anthocyanins are anti-inflammatory (Boniface et al 1986). Macrophages view free radicals as invaders. When macrophages engulf invaders, it can cause inflammation. Anthocyanins, through the elimination of free radicals, keep macrophages from engulfing free radicals, and reduce inflammation.

Besides opening new markets for blueberries, the research findings of Dr. Prior et al. made the antioxidant properties of blueberries more common knowledge in both the scientific and lay communities. The ORAC showed blueberries had antioxidant ability, and that blueberries rank among the highest antioxidant quenching ability of any fruit. All studies have supported these findings. Anthocyanins are found in the skins of blueberries. Smaller blueberries have higher surface area to volume ratio yielding higher antioxidant value per weight compared to larger berries.

The Food and Nutrition Board defined dietary antioxidants as "a substance in foods that significantly decreases the adverse effects of reactive oxygen species, reactive nitrogen species, or both on normal physiological function in humans." (Institute of Medicine 1998). Numerous research studies correlate health benefits to antioxidants. Anthocyanins exhibit beneficial effects in animal models so it is important to maximize

their levels in the human diet. Therefore, understanding what conditions are advantageous and what conditions are deleterious to maintaining anthocyanin levels in foods is quite important.

VI. Effects of Processing on Anthocyanins

A. Natural Enzymes

Polyphenol oxidase (PPO) enzymes, present in most fruits, are detrimental towards anthocyanins. PPO does not act directly on anthocyanins. PPO acts on acids with structures similar to caffeic and coumaric acid, forming o-quinones. Phenolic compounds, including anthocyanins are degraded by o-quinones through oxidation or condensation reactions (Figure 4) (Wesche-Ebeling and Montgomery 1990, Kader et al. 1998). Studies done without acid intermediates show no anthocyanin degradation in the presence of PPO, but showed degradation when caftaric acid was present (Sarni-Manchado et al. 1997). Studies showed chlorogenic acid in the presence of PPO and anthocyanins has a similar effect (Yokotsuka and Singleton 1997). This relationship has been seen in other anthocyanin containing fruits including strawberries (Wesche-Ebeling and Montgomery 1990), and grape must (Prieur 1994). PPO is most active in a pH range of 3-5 (Kader et al. 1999). Anthocyanin degradation can be retarded by reducing the pH or by keep fruit products cold (3-5°C) (Kalt et al. 1999). PPO is 50% denatured above 90°C in 30-45 seconds. Anthocyanins are 50% destroyed at 90°C in 60-90 minutes (Table 3) (Tanchev 1974, Tanchev and Yoncheva 1974). Addition of L-cysteine and sodium metabisulfite was shown to inhibit PPO activity (Siddiq et al. 1994). Coating blueberries with sodium carboxymethylcellulose to retard the leakage of berries would

also slow the activity of PPO (Zhang et al. 1997). by delaying leakage of anthocyanins and PPO.

B. Fining Agents

The addition of pectinolytic enzymes increases total phenolic levels in juices and wines (Revilla and Gonzalez-SanJose 2002, Landbo and Meyer 2001). Landbo and Meyer (2001) examined Grindamyl pectinase, Macer8 FJ, Macer8 R, and Pectinex BE, with Novozym 89 protease. The results showed pectinase increases cell wall breakdown of pomace and all enzyme preparations, except Grindamyl pectinase, increased phenols extracted. Deleterious effects were seen on anthocyanin levels. Macer8 FJ and Macer8 R reduced levels of anthocyanins while Pectinex BE and Novozym 89 protease showed no effects. Revilla and Gonzalez-SanJose (2002) examined the effects of Zimopec PX1, Rapidase CX, Pectinase WL, and Rapidase Ex. Colour. Higher levels of phenols were present in the final products, with mixed results on the anthocyanin levels.

C. Ultraviolet Light

Anthocyanins are susceptible to ultraviolet light. Exponential degradation of anthocyanins was observed when subjected to intense UV light (Baublis et al. 1994). Extracts were placed in a 30°C waterbath and surrounded by eight 40W round fluorescent lamps. HPLC analysis on samples exposed between 0 and 16 days found greatest anthocyanin loss occurred during the first four days.

D. pH

pH affects anthocyanin stability. In acidic solutions, four anthocyanin species exist; quinonoidal base, flavvylium cation, hemiacetal pseudobase and chalcone

(Brouillard 1982). At low pH levels -1-3, the stable flavvylium cation is predominant with minor amounts of the colorless hemiacetal form present (Mazza 1997, Brouillard 1982, Sarni-Manchado et al. 1997). The flavvylium cation is the most stable form of anthocyanin, so low pH levels are preferable for anthocyanin retention.

E. Temperature

Temperature affects anthocyanin stability. Heating kinetics of anthocyanin degradation were shown by Tanchev (1974) (Table 3). Boiling had the greatest rate of anthocyanin destruction (Garcia-Viguera et al. 1998, Garcia-Viguera et al. 1999). Kalt et al. (1999) showed freezing (-20°C) to have little effect on anthocyanin levels. Temperatures between freezing and boiling allow gradual loss of anthocyanins (Kalt et al. 1999, Seeram et al. 2001).

F. Free Radicals

Anthocyanins degrade when exposed to free radicals (Prior et al. 1998). Others have found that pectinase and other enzymes can cause a decrease in total anthocyanins (Neubeck and Seidel 1975, Landbo and Meyer 2001)), while addition of sulfur dioxide increases anthocyanin extraction (Markakis 1982). Growing environment and practices affect the amount of anthocyanins present (Wang et al. 2002).

G. Microwaving

One study examined the effects of microwaving on PPO (Rodriguez-Lopez et al. 1999). Browning reactions of foods by polyphenol oxidase was studied. Comparisons were done between autoclaving, blanching, and microwaving with a 2450MHz

microwave. Microwave treatment was cheaper, faster, and resulted in a higher antioxidant and lower browning product.

A second study focused on the extraction of phenolic compounds from grape seeds using a 300W microwave (Hong et al. 2001). Berries and solvents were heated together simultaneously. The results found heating solvents with higher water ratios led an increase in anthocyanin extractions.

A third study was conducted on the effects of heating on PPO, peroxidase (POD), pigment composition, and color of fruit purees using an 850W microwave (de Ancos et al. 1999). Heating in microwaves for 30 seconds inactivated PPO and POD by different amounts, based on type of fruit and time microwaved. Little change was observed in strawberry anthocyanins resulting from microwaving, but significant antioxidant losses from kiwi fruit was seen.

None of these groups had access to microwaves capable of delivering as much energy as the Industrial Microwave Systems. Inc. (IMS) (Morrisville N.C.) microwave. The IMS microwave is capable of delivering 5kW as opposed to 300W or 850W. These previous studies also had to heat batches. The IMS System allowed heating of products in a continuous flow operation.

VII. Extraction and Measurement of Antioxidant Activity

A. Extraction of Anthocyanins

The extraction methods used on blueberries have evolved greatly since the 1960's. Originally, acetone powder was used (Durkee and Jones 1969). Since then many different methods of extraction were attempted. Generally, simple alcohols (MeOH, EtOH), acetone, and sometimes water, were combined with small amounts of

concentrated acids (generally HCl or glacial acetic) to optimize specific extraction procedures (Prior et al. 2001, de Ancos et al. 2000). Sometimes chemicals such as chloroform were used (Wrolstad 2000). EtOH, the safest for human consumption, was not nearly as effective as acetone and MeOH. In the early 1990's procedures calling for EtOH and glacial acetic acid became more common. These procedures used maceration and overnight extraction in the solvent. A recent extraction procedure uses acetone, water, and glacial acetic acid solvent with heat (50°C) (Prior et al. 2001). The heat was kept below 60°C to avoid destruction of anthocyanins. Gao and Mazza (1996) found that the addition of 1000-5000mg SO_2/L was also an effective method of extracting anthocyanins.

B. Purification of Anthocyanins

Purified anthocyanins are difficult to obtain commercially. Isolation and purification of authentic individual anthocyanins has been done by researchers over 20 years. The model for purification is to use a Whatman filter on a Buchner funnel to remove the larger particulate matter, followed by the use of a conditioned silica column, or Sep-Pak filter, to separate the sugars and phenols from the anthocyanins (Rodriguez-Saona and Wrolstad 2001). Conditioning is done by pre-rinsing the column with methanol. The sample, reconstituted in water, is run through the column and rinsed with water to remove unwanted constituents. The column is rinsed with 0.01% hydrochloric acid in methanol to remove the anthocyanins and proanthocyanidins and the effluent is collected.

C. Total Phenols

Many different procedures have been used to determine the phytochemicals present in blueberry cultivars. Total phenols content of prepared blueberry extracts is done through the use of a spectrophotometer at 765nm (Singleton and Rossi 1965). Currently, the most commonly used total phenol assay comes from Singleton and Rossi (1965) and employs the use of the Folin- Cocialteu reagent. The spectrophotometer reading is compared to the standard, gallic acid.

The main limitation of this assay is that it does not differentiate between different phenol types. All phenols are summed. In order to use this method a standard curve, using gallic acid, must first be made. The curve should span the sensitive range of the spectrophotometer employed by the lab.

D. The Application of High Performance Liquid Chromatography to Anthocyanin Analysis

High Performance Liquid Chromatography (HPLC) is used to assay for individual anthocyanins. HPLC works by separating different chemical structures through chemical interactions. Chemical separation can be based on a number of factors, including compound size, charge, and polarity. The pH of the column is very important. Anthocyanins are most stable as flavvyium cations which require a low pH so it is imperative to select a column that is able to handle acidic pH's. Anthocyanins have the same carbon backbone, so the retention time will differ primarily based upon number and location of both the hydroxyl groups and methyl groups. Reversed-phase C_{18} columns are commonly used (Rodriguez-Saona and Wrolstad, R. 2001). Reversed-phase C_{18} column use nonpolar silica with polar mobile phases (Rodriguez-Saona and Wrolstad

2001). The intermolecular interactions within the column include van der Waals, dipole-dipole, hydrogen bonding, and possible electron donor-acceptor considerations. The detection wavelength of 520nm is because the maximum absorbance for most anthocyanins is located between 514-528nm (Mazza and Miniati 1993, Mazza 1997).

E. Analysis of Total Anthocyanins

Total anthocyanins can be measured without an HPLC. Total anthocyanins are analyzed with absorbance in an alcoholic extract at 520nm (Mazza 1997) or by using anthocyanins as an indicator and titrating to a color change with a known quantity of base (Mazza and Oomah 2000). The absorbance value of an alcoholic extract or the amount of base required to induce a color change is proportional to the total amount of anthocyanins present. The limitation of this assay is that the precise quantity of individual anthocyanins is not determined. When determining the total concentration of anthocyanins present, spectrophotometry is recommended over other techniques, such as paper chromatography (Francis 1966) or thin layer chromatography (Petri et al. 1997).

F. Capillary Electrophoresis

Recently, capillary electrophoresis has been employed for separating anthocyanins (Bridle et al. 1996, Ichiyanagi et al. 2000). This procedure applies an electrical current to separate large molecules. Anthocyanins can be separated because their charge at a given pH is known. A slight difference in charge allows them to be separated using electricity. Two advantages to this new separation method are that the separation time is about 10 minutes when optimized (Ichiyanagi et al. 2000), and that

capillary electrophoresis allows significantly increased peak resolution and improves detection limits by several orders of magnitude.

G. Oxygen Radical Absorbance Capacity (ORAC)

The ORAC works as follows. In any individual test, there is a blank, a standard (trolox), and samples. They are all tested together. Each has a free radical producer, such as a peroxyl radical generating agent, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). Each must also have an indicator detector that decays in the presence of free radicals. In this case the detector of choice is fluorescein that degrades in the presence of free radicals. After the fluorescein, AAPH, and blank, standard, or samples are mixed, the assay begins, and the fluorescein decays over time. The sample's antioxidant quenching ability is directly proportional to the level of fluorescence. Fluorescence is measured with a fluorometer or fluorescence plate reader, the cytofluor. The fluorometer excites the indicator and measures the emission given off by the excited indicator. It is also capable of recording the emission levels at regular intervals over time. When the emission becomes less than 5% of the original amount, the test is considered to be finished. In the end, the results are expressed in terms of ORAC units or μMole of Trolox equivalents.

VIII. Roles of Antioxidants in Controlling Free Radicals

Given the right conditions, many substances can generate free radicals. Free radicals are produced in the body with every breath and meal. The human body has adapted with the changes in the atmosphere over time to develop defense mechanisms to reduce the damage done by free radicals. The tools for the body's defense system against free radicals are antioxidants that act as free radical scavengers.

Antioxidants produced within the body include dismutase, peroxidase, and catalase enzymes, as well as glutathione (GSH) and cytochrome P450. Antioxidants made by plants and ingested include vitamin C, vitamin E, carotenoids, and anthocyanins. Synthetic antioxidants used as food additives include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-Butylhydroquinone (TBHQ). Antioxidants work to prevent damage done by free radicals.

There are three classes of antioxidant capabilities in the human body. The first is work done by enzymes to control initial free radical production. When oxygen is taken in and used during respiration, superoxide, hydrogen peroxide, and hydroxyl radicals are all commonly formed (Gutteridge and Halliwell 1994). Catalase and dismutase enzymes decrease the formation of hydroxyl radicals (McCord and Fridovich 1969). Glutathione peroxidases remove all peroxides, including hydrogen peroxides (Foyer and Halliwell 1976). The enzymes all require metal co-factors in order to work. Dismutases require copper, zinc, or manganese, depending on the pH (McCord and Fridovich 1969). Catalase requires iron as a co-factor (Halliwell and Gutteridge 1989). Glutathione peroxidases may require selenium (Halliwell and Gutteridge 1989).

The second class of antioxidants come from the diet, focus on fixing any problems created by free radicals. This class ends the chain reaction of free radicals through donation of a proton. Two common sources would be vitamin E and vitamin C, but include beta-carotene, and flavonoids. Because it is fat soluble, Vitamin E (tocopherol) works in lipid tissues (Combs Jr. 1998). Vitamin E can donate one or two protons (Figure 5). The first proton donated comes from a hydroxyl group, but does not affect the structure of vitamin E (Combs Jr. 1998). Because of vitamin E's resonance

ability, it is un-reactive with its free radical, and can be regenerated if another proton is donated. Vitamin E also has the ability to donate a second proton without the first proton having been replenished leading to the breaking of the chromanol ring that is irreversible (Diplock 1985). Vitamin C (ascorbic acid) works in aqueous phases. Vitamin C is able to donate one or two protons (Figure 6). Donation of one proton leaves semidehydroascorbate radical. Donation of a second proton leaves deyhydroascorbate which is unstable and can break down to oxalic and threonic acid (Seib and Tolbert 1982). Dehydroascorbate can be replenished and returned to ascorbic acid with the addition of glutathione:

Dehydroascorbate + 2GSH $\rightarrow$ Ascorbate + GSSG

The third class of antioxidants is replenishers. Vitamin C and vitamin E are both the primary antioxidants and are susceptible to breaking down if their donated protons are not replenished. The source of protons comes from another structure that can readily donate a proton, while remaining stable so as not to become a free radical. Examples of replenishers include carotenoids, coenzyme Q, glutathione, and flavonoids. Both beta-carotene and coenzyme Q work in lipid material and have a synergistic relationship with vitamin E (Ernster and Nelson 1981). Glutathione and flavonoids are tied more to the aqueous environment where they can work both as proton donors to free radicals, and act to replenish vitamin C. Uric acid also contributes protons to antioxidants (Davies et al. 1986).

Other methods to control the impact of free radicals include the body's handling of metals, and its ability to correct damage done through oxidation. When metals such as copper, iron, and manganese are not being used in the mitochondria to turn superoxide

back into oxygen, or as co-factors to enzymes they are always bound to a carrier (eg. iron has ferritin or transferrin). They would be far too catalytic in the formation of new free radicals if not bound because of their ability to donate and accept an electron.

Antioxidants have some ability to repair damage done by free radicals. Repair of DNA is done by glutathione. Glutathione donates protons to mildly damaged DNA. Repair of lipids is done by phopholipase enzymes which catalyze the cleavage of peroxidized fatty acid side chains from the membrane and replaces them with a new, undamaged fatty acids (Gutteridge and Halliwell 1994).

In cases where free radical damage can not be repaired, damaged tissues are destroyed. This is one reason why a double-helix strand of DNA is preferable. Damaged DNA is cut and rebuilt 5'→ 3' with the second strand as a template. Damaged fatty acid side chains of membranes are replaced through rotating in new fatty acid side chains (Gutteridge and Halliwell 1994).

The growth in understanding the usefulness of antioxidants may be tied to reducing the high incidence of heart disease in the United States. A major breakthrough in heart disease occurred in 1974 when aspirin was shown to slow platelet aggregation. Future heart disease research revolved around vitamin E. In one study, 87,245 female nurses between the ages of 34-59 participated in a questionnaire study of vitamin E intake (Stampfer et al. 1993). Over an 8-year period, it was shown that the nurses in the top 20 percent of vitamin E intake had 23-50 percent less heart disease. A second study involving vitamin E, examined 38,910 health professionals between 40-75 who filled out questionnaires to assess their intake of vitamin C, beta-carotene, and vitamin E (Rimm et al. 1993). Over a 4-year follow-up period it was observed that men with higher intakes of

vitamin E had a lower risk for coronary disease. In 1991 it was observed that French people who eat fatty foods, suffered from less heart disease than did their American counterparts. It was determined that the wines, especially red wines, drunk during meals by the French, provided protection to their cardiovascular system through antioxidant activity, similar to that of vitamin E (Renaud and de Lorgeril 1992). This is presently referred to as the "French Paradox". The French Paradox increased interest in colored fruits.

EFFECTS OF PROCESSING ON THE ANTIOXIDANT ACTIVITIES OF BLUEBERRY JUICES

IX. Present Research Objectives

The purpose of this research was to determine the effects of selected processing techniques on antioxidant activity in blueberry juices. Pasteurization, hot, and cold processing of blueberries was compared with an emerging technology, the use of microwave heating. Recent work has shown that extraction using heat (Prior et al. 2001) leads to an increase in anthocyanins extracted. Pasteurization and hot processing blueberries use conduction heating (steam kettle) with constant agitation. While the entire puree is heated to between 47-90°C, some parts of the puree may slightly exceed temperatures of 90°C. Not only does heating crushed blueberries in pasteurization and hot processing have harsh temperature effects but also take time to heat berries from their initial temperature up to 90°C, making microwaving more economical.

The advantage of pasteurization and hot processing is that they are known technologies. Blueberry products that required heat existed long before microwaving was

available. One major drawback is the uneven treatment (heating) of the blueberries. Crushed blueberries on the exterior are exposed to extreme heat while those in the middle of the "batch" have much less heat exposure. A second major drawback is that the hot processing involves gradual temperature increase whereas microwaving involves a more rapid temperature increase.

Another advantage to microwaving was that crushed blueberries were treated more uniformly. All parts get similar heating. Another advantage found in previous studies was that microwaving was found to be cheaper than present heating methods. In traditional, conduction heating, superheated steam must be supplied whereas microwaving only requires an electrical source. It can be turned on and off like a light switch.

The drawbacks of microwaving are substantial. Unlike hot processing, microwaving is a relatively new technology, and with lack of understanding comes apprehension to its use. Hot processing can be done by nearly everyone, but microwaving requires someone with a degree of knowledge on how to run the microwave. One of the largest drawbacks to microwaving is the initial purchasing cost. For smaller processors this can be very prohibitive.

Unlike previous studies that examined the effects of processing on anthocyanin retention, this study will compare the effects of hot processing and microwaving on anthocyanin retention and antioxidant activity. As anthocyanins represent a form of antioxidants, the measurement of ORAC will give a more effective evaluation of the effects of processing on free radical scavenging activities of all antioxidant type compounds contained within the juices, including anthocyanins.

MATERIALS AND METHODS

I. Chemicals

All chemicals, solvents, and reagents were of analytical grade and were purchased from Fisher Scientific in Pittsburg, PA. These reagents included phosphoric acid, acetonitrile, methanol, hydrochloric acid, and glacial acetic acid.

II. Blueberry Samples

In the first experiment Pender blueberries from the 2002 harvest were obtained from Castle Hayne Research Station, Wilmington N.C. Fruit was harvested when judged commercially ripe, frozen, and held at –20°C until analyzed.

In the second experiment, seven different cultivars of blueberries (Reveille, Bladen, Tifblue, Powder Blue, Croatan, Premiere, and Pender) were examined. All blueberries were obtained harvested when judged commercially ripe in the 2000 harvest from the Castle Hayne Research Station (Wilmington, NC). All blueberries were frozen and held at –23°C until processed and analyzed.

In the third and fourth experiments Tifblue blueberries from the 2002 harvest were obtained from Solo Foods Inc. in Wilmington N.C. Fruit was harvested when judged commercially ripe, frozen, and held at –20°C until processed and analyzed.

III. Experiment #1: Comparison of Extraction Method Efficiency

Extraction Methods

Three extraction techniques were selected for comparison of their efficiency to remove anthocyanins from blueberries. The techniques were those of Hong and Wrolstad (1990); Gao and Mazza (1996); and Prior et al. (2001);

An aliquot of 100 grams of Pender blueberries were thawed overnight at refrigeration temperatures (3-5°C). After thawing, all 100 grams was blended in a Waring blender until liquefied. Ten grams of the initial 100 gram pool was extracted in each procedure. This procedure was repeated three additional times from start to finish.

1) The Hong and Wrolstad Extraction Procedure:

   This procedure involved the use of 1% hydrochloric acid in methanol and was made at a solvent to sample ratio of 10:1 (100ml solvent to 10 grams of blended blueberries). To determine the effectiveness of the extraction technique, length of extraction was for periods of 30 minutes, 60 minutes, 8 hours, and 18 hours. The overall effectiveness of the extraction was determined through the use of the total anthocyanins assay (Rodriguez-Saona and Wrolstad 2001).

2) The L. Cao and G. Mazza Extraction Procedure:

   This procedure involved the use of an ethanol:glacial acetic acid: water solvent at a ratio of 50:1:49 v/v/v. The solvent to sample ratio run was 10:1 (100ml solvent to 10 grams of blended blueberries). To determine the effectiveness of the extraction technique, length of extraction was for periods of 30 minutes, 60 minutes, 8 hours, and 18 hours. The overall effectiveness of the extraction was

determined through the use of the total anthocyanins assay (Rodriguez-Saona and Wrolstad 2001).

3) The Prior et al. Extraction Procedure:

This extraction procedure involved the use of an Acetone:Water: Glacial Acetic Acid solvent at a ratio of 70:29.5:0.5 v/v/v heated to 50°C. The solvent to sample ratio run was 10:1 (100ml solvent to 10 grams of blended blueberries). To determine the effectiveness of the extraction technique, the sample was extracted for 30 and 60 minutes. The overall effectiveness of the extraction was determined through the use of the total anthocyanins assay (Rodriguez-Saona and Wrolstad 2001).

V. Experiment #2: Effects of Cold Versus Hot Processing on the Antioxidant Activity of Seven Different Cultivars of Blueberries.

Blueberry cultivars were thawed to room temperature (22°C), then processed through a Fitz Mill Comminuting Machine on high speed with 12-mm screen.

A. Cold Processed Blueberry Juices

Crushed berries were treated with RapidaseTM Super BE pectinase (Gist-brocades, Charlotte, NC) at a rate of 50ml per 45.5 kg and held under refrigerated conditions of 3°C overnight prior to pressing. Blueberries were pressed in an Enrossi (Calzolaro, Italy) 13.2L stainless shell bladder basket press lined with polypropylene press cloth. Juice yields ranged from 78.8% and 81.9%.

B. Hot Processed Blueberry Juices

Crushed blueberries were treated with RapidaseTM Super BE pectinase (Gist-brocades, Charlotte, NC) at a rate of 50ml per 45.5 kg and held at 22°C for 2 hours, followed by rapidly heating the fruit to 43°C and pressed immediately in an Enrossi (Calzolaro, Italy) 13.2L stainless shell bladder basket press lined with polypropylene press cloth. Juice yeilds ranged from 78.8% and 81.9%.

C. Wine Making

The hot and cold processed rabbiteye cultivar blueberry juices (Tifblue and Powder Blue) were further processed into wines. Montrachet yeast and yeast nutrient were added and fermentation allowed to proceed for 5 days.

*For all processes, samples were taken an placed in a test tube which was flushed with nitrogen and frozen at –23C until testing.

VI. Experiment #3: Effects of Microwave Pasteurization on the Antioxidant Activity of Blueberry Juices.

Frozen Tifblue blueberries(120kg at -20°C) were thawed to room temperature (22°C).

In 10 kg portions, the Tifblue blueberries were blended approximately 1kg at a time in a Waring blender until liquefied then poured into the reservoir of the 5kW IMS System (Morrisville, NC). When the reservoir was full, the pump was turned to speed setting 6 and the blended blueberries filled the system. After the system was full of liquefied blueberries, the microwave was turned on to heat the liquefied blueberries to 85°C, 92.5°C or 95°C. Blueberries were heated in a single pass from room temperature to microwaved temperature, then held at to 85°C, 92.5°C or 95°C for 30 seconds before

being collected, placed in an icebath, and the icebath with heated liquefied blueberries was placed in a –20°C freezer until reaching an internal temperature of 15°C (about 30 minutes). After reaching 15°C, the samples were moved to a cooler at 3-5°C for eight hours. Each treatment was done in triplicate.

In a second treatment 10 kg. of Tifblue blueberries were liquefied via blending and weighed. Conduction heating was done with a steam jacketed kettle (TDC/2-20 Sterling Power Systems Inc. Elk Grove Village, IL), and continuously agitated while heat was applied to the liquefied blueberries up to 90°C. After reaching 90°C, the samples were immediately removed and re-weighed, with water added to replace the lost weight. After 30 seconds elapsed, the heated liquefied berries were placed in an icebath, and the icebath and liquefied berries were placed in a –20°C freezer. After reaching an internal temperature of 15°C, the samples were moved to a cooler at 3-5°C for eight hours. This treatment was done in triplicate.

After 8 hours at 3-5°C, the microwaved and conduction heated blueberries were pressed in an Enrossi (Calzolaro, Italy) 13.2L stainless shell bladder basket press lined with polypropylene press cloth. The 3.8 Liters of juice was obtained and collected from each 10 kg. sample (36% yield) and was stored at 3-5°C. Aliquots were taken at days 1, 4, 7, 11, 13, 19, and 31 to evaluate the changes in total phenols, antioxidant capacity. Samples were evaluated for changes in anthocyanin levels over time at days 1, 18, 31, and 60.

VII. Experiment #4: Effects of Microwaving, Cold Processing, Hot Processing, and Pasteurization on the Initial Antioxidant Retention in Blueberry Juices.

Frozen Tifblue blueberries (110kg at -20°C) were thawed to room temperature (22°C).

A. Cold Processed Blueberries

An aliquot of 10 kg. of thawed Tifblue blueberries (22°C) was liquefied using a Waring blender, collected, and stored in a cooler for 8 hours at 3-5°C. After 8 hours the liquefied Tifblue blueberries were pressed using an Enrossi (Calzolaro Italy) 13.2L stainless shell bladder basket press lined with polypropylene press cloth. A quantity of 3.8 Liters of juice was obtained and collected from each 10 kg. sample (36% yield) and collected. This procedure was done in triplicate. Juice samples were taken immediately in test tubes filled to the top, and frozen at –20°C.

B. Hot Processed Blueberries

An aliquot of 10 kg. of thawed Tifblue blueberries (22°C) was liquefied using a Waring blender. The liquefied blueberries were weighed and then transferred to a steam jacketed steam kettle (TDC/2-20 Sterling Power Systems Inc. Elk Grove Village, IL), and continuously agitated during heating. The liquefied Tifblue blueberrries were heated to 47°C and then removed from the kettle. The heated liquid was then re-weighed and the lost weight was added back as distilled water. After 30 seconds the heated liquefied blueberries were placed in an icebath, and the icebath and liquefied blueberries were placed in the freezer until reaching an internal temperature of 15°C and then transferred to a cooler (3-5°C) for 8 hours. After 8 hours the liquefied Tifblue blueberries were pressed using an Enrossi (Calzolaro Italy) 13.2L stainless shell bladder basket press lined with polypropylene press cloth. A quantity of 3.8 Liters of juice was obtained and

collected from each 10 kg. sample (36% yield) and collected. This procedure was done in triplicate. Juice samples were taken immediately in test tubes filled to the top, and frozen at –20°C.

C. Pasteurization of Blueberries

An aliquot of 10 kg. of thawed Tifblue blueberries (22°C) was liquefied using a Waring blender. The liquefied blueberries were weighed and then transferred to a steam jacketed steam kettle (TDC/2-20 Sterling Power Systems Inc. Elk Grove Village, IL), and continuously agitated during heating. The liquefied Tifblue blueberrries were heated to 90°C and then removed from the kettle. The heated liquid was then re-weighed and the lost weight was added back as distilled water. After 30 seconds the heated liquefied blueberries were placed in an icebath, and the icebath and liquefied blueberries were placed in the freezer until reaching an internal temperature of 15°C and then transferred to a cooler (3-5°C) for 8 hours. After 8 hours the liquefied Tifblue blueberries were pressed using an Enrossi (Calzolaro Italy) 13.2L stainless shell bladder basket press lined with polypropylene press cloth. The 3.8 Liters of juice was obtained and collected from each 10 kg. sample (36% yield) and collected. This procedure was done in triplicate. Juice samples were taken immediately in test tubes filled to the top, and frozen at –20°C.

D. Microwaving Processing Blueberries

The juice samples evaluated for the initial value of antioxidant retention in the final product were collected immediately after pressing from the microwaved products made in experiment 2. The juice samples were taken immediately in test tubes filled to the top, and frozen at –20°C.

VIII. Analytical Procedures

A. Total Phenols Assay

Total phenols were determined as described by Singleton and Rossi (1965). This involved the addition of Folin-Ciocalteau reagent (Sigma Aldrich Milwaukee, WI) to an aliquot of each treatment, allowing the mixture to sit in the dark for 2 hours, followed by reading the absorption at 765nm and comparing to a standard curve using gallic acid (Sigma Aldrich, Milwaukee WI).

B. Oxygen Radical Absorbance Capacity (ORAC) Assay

The antioxidant capacity of all treatments was determined using the ORAC assay as described by Ou et al. (2002). The samples were prepared by combining appropriate dilutions with sodium monophosphate: sodium diphosphate buffer (pH 7.4), a free radical producing source of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (Chemicals USA Inc. Richmond, VA), and a fluorescing indicator, fluorescein (Riedel-de Haen through Sigma Aldrich, Milwaukee, WI). Trolox (6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) (Sigma Aldrich Milwaukee, WI), was used as the reference standard. The blank contained buffer, AAPH, and fluorescein. The assay was run at 37°C for 70 minutes on a Cytofluor II (PerSeptive Biosystems Foster City, CA) for 15 cycles at 5.0 minutes per cycle and excitation/emission of 485/530nm. Over the 70 minute span, the fluorescent indicator is degraded by the free radicals produced, and when the indicator fluoresces at 5% or less of the initial amount it is complete. If the final reading is greater than 5%, the results are unusable. Trolox equivalents (TE) are

determined by calculating the ratio of area under the curve (AUC) of the standard versus the sample less the blank.

C. Analysis of Individual Anthocyanins

The protocol for the HPLC used in these experiment comes from Rodriguez-Saona and Wrolstad (2001). Acetonitrile and 4% phosphoric acid in distilled water were used as the mobile phases, with the sample diluted in 4% phosphoric acid, using the profile listed below.

Time (minutes)	Percent A (acetonitrile)	Percent B (4% phosphoric acid in water)
0	6	94
55	25	75
65	25	75
10	6	94

Anthocyanins were separated on a C_{18} polymetric support reverse phase column (5µm x 250 mm x 4.6mm) (PLRP-S 100, Polymer Labs Amherst, MA) and read at an absorption of 520nm . A Waters Automated Gradient Controller (Milford, MA) as well as Waters Model 510 pumps (Milford, MA), in conjunction with a Spectroflow 757 detector (Kratos Analytical Systems, Kyoto, Japan) were used.

D. Estimating Total Anthocyanins

The method explained by Rodriquez-Saona and Wrolstad (2001) was used. This method involves the use of two buffers a 0.025M potassium chloride buffer (pH =1) and 0.4M sodium acetate buffer (pH =4.5). Samples were extracted using the listed extraction procedures, and fractions were suspended in the buffers. The absorbance in each buffer was taken at 520nm and 700nm using a spectrophotometer (Shimadzu Corporation, Kyoto, Japan), using the formula:

$A = (A_{520nm}-A_{700nm})_{pH=1.0} - (A_{520nm}-A_{700nm})_{pH=4.5}$ where A = absorbance.

E. Statistical Analysis

Samples were run in quadruplicate for experiment number 1, only one trial was available for experiment 2, and in triplicate for experiments 3 and 4. Standard error of sample means was calculated for experiments 1, 3, and 4 by assuming a normally distributed source population and a normally distributed sampling distribution.

RESULTS AND DISCUSSION

I. Experiment #1: Comparison of Extraction Method Efficiency

In determining the best extraction procedure for the experiments to follow, the best extraction method had to have the highest amount of anthocyanins in the final extract. The Gao and Mazza extraction method which was ethanol: acetic acid: water at a ratio of 50:49:1 (v/v/v) was the least effective at extracting anthocyanins (Figure 7). After 30 minutes, the Hong and Wrolstad procedure of 1% HCl in methanol was statistically better than the highest yield seen in the Gao and Mazza method. Figure 7 also shows the results from extracting using the Prior et al. method of acetone: water: glacial acetic acid 70: 49.5: 0.5 at 50°C. Statistically, the Hong and Wrolstad procedure at 60 minutes and after 8 hours was equal to the Prior method after 30 minutes. Because the Prior et al method was quicker, it did not allow the blueberries to be in contact with PPO at an elevated temperature nor did it allow the samples to be exposed to oxygen for as long, it was determined to be the most effective extraction procedure and ws used throughout other experiments presented here.

There are a combination of factors that may help to explain why the Prior et al. (2001) extraction method was most efficient. First, the addition of heat has been shown to increase the permeability of blueberry cell membranes (Sapers et al. 1985). The addition of heat also has been shown to decrease the solubility of oxygen in juice (Sapers et al. 1985), so heating would reduce the oxygen that stayed in the solution. Polarities of the methanol versus acetone and water may have also contributed to the extraction ability of the solvents. All extraction methods had a pH adjustment so that was probably not a significant factor. It is noteworthy that the extraction with ethanol, a food grade solvent, was consistent with previous work that found it to be a less efficient extracting solvent.

II. Experiment #2: Effects of Cold Versus Hot Processing on the Antioxidant Activity of Seven Different Cultivars of Blueberries.

The composition of different blueberries varies greatly both between types as well as within a type based on growing conditions. While these inherent variations make comparisons between differing crops difficult, comparisons done within one crop of a single cultivar is informative.

A. Total Phenols:

Each cultivar had different total phenols values. The order highest to lowest gallic acid equivalents showed the Rabbiteye varieties had the highest total phenol values, followed by the Highbush. The order of hot processed samples (from highest to lowest) was: Powder Blue > Tifblue > Croatan > Bladen > Premiere > Reveille > Pender. For cold processed samples, the order (highest to lowest) was: Powder Blue > Tifblue > Bladen > Croatan > Premiere > Pender > Reveille. Comparing total phenols values for

hot and cold processing of the different cultivars of blueberries suggested that the addition of heat in processing caused an increase in the final product (figure 8). The processing of one cultivar incorporating heating always resulted in a higher total phenols value (figures 8). The addition of heat (Sapers et al. 1985) has been shown to increase permeability of cells likely leading to these results. The trend was that the hot processed products had higher values than seen in literature and the cold processed products had lower values than seen in the literature (Mazza and Miniati 1993, Gao and Mazza 1994, Ehlenfeldt and Prior 2001).

Inter-cultivar comparison of total phenol values correlates well with prior studies as well (Mazza and Miniati 1993, Gao and Mazza 1994, Ehlenfeldt and Prior 2001). The Rabbiteye varieties (Tifblue and Powder Blue) had a higher total phenol and antioxidant activity than did the Highbush varieties, irregardless of the fact that they were made into wines after treatment and pressing. This is consistent with other studies where Highbush blueberries have been found to contain lower anthocyanin, total phenols, and ORAC values than Lowbush (Kalt et al. 2001). Rabbiteye blueberries are a result of plant breeding efforts and are not naturally occuring (Eck 1988) and generally incorporate at least one type of Lowbush plant. Therefore, it is not surprising to have higher total phenols values in Rabbiteye blueberriess than Highbush cultivars.

B. ORAC Values:

While total phenols measures hydroxyl groups and ORAC measures peroxyl radical quenching ability, both tests have been found to correlate well to each other in previous studies (Ou et al. 2002). This study found similarity as well. Each cultivar again had different ORAC values. The order highest to lowest ORAC values, like the total

phenols test, showed the Rabbiteye varieties had the highest values, followed by the Highbush. The order of hot processed samples (from highest to lowest) was: Powder Blue > Tifblue > Croatan > Bladen > Reveille > Premiere > Pender. For cold processed samples, the order (highest to lowest) was: Powder Blue > Tifblue > Bladen > Croatan > Premiere > Pender > Reveille. Heat used in processing was shown to produce a more desirable final product as valued in this study (figure 9).

Also as seen in the total phenols assay, ORAC values determined in this study correlated well with previous work (Mazza and Miniati 1993, Gao and Mazza 1994, Ehlenfeldt and Prior 2001).

III. Experiment #3: Time Trial on Changes in the Antioxidant Activity as Measured by Changes in Total Phenols, Anthocyanins, and Antioxidant Activity.

As fully processed blueberry products may often be stored under refrigerated conditions for an extended period of time, this experiment was designed to determine the effects of storage time and temperature. This study was conducted on pasteurized and microwaved blueberry juices following refrigerated storage at temperatures 3-5°C.

A. Anthocyanin Level Changes Over Time

All samples had an increase in total anthocyanins from the first sampling to the second sampling (Figure 10). The increases in overall anthocyanin levels over time has been seen in other studies on fruits during storage (Kalt et al. 1999, Mazza and Miniati 1993, Connor et al. 2002). A possible explanation for this phenomena in this study could be that anthocyanins continued to be released from particulates during storage, as well as some contribution of chalcones that re-equilibrated to their anthocyanin forms within 12-

48 hours. The level of anthocyanins present in the blueberry juices was consistent with previous studies. Mazza and Miniati (1993) found 25-495mg of anthocyanins present in average blueberries for every 100g sample. Gao and Mazza (1994) have seen levels between 100-250mg of anthocyanins per 100g sample in Tifblue blueberries.

While the total anthocyanins levels increased, the proportions of Delphinidin-3-glucoside (13%), Cyanidin-3-glucoside (15%), Petunidin-3-glucoside (12%), Peonidin-3-glucoside (38%), and Malvidin-3-glucoside (22%) (table 4) did not change significantly in their relative amounts (Table 4). These proportions of individual anthocyanins contained in the juices processed in this study were different from previous research. Some studies (Sato et al. 2001) have found delphinidin to be the highest, followed by cyanidin, petunidin, malvidin, and peonidin. Other studies (Durst et al. 2002) have found malvidin the highest followed by delphinidin, petunidin, cyanidin, and lastly peonidin. It is interesting that the anthocyanin composition in the juices produced in this study reflected the stability of anthocyanins. Knowing that the amount of anthocyanins present is greatly affected by environmental and processing conditions, the cause of various proportions of anthocyanins present was likely based on the most advantageous conditions for the plant, such as pests, pollution, fungicides, pesticides, temperature, moisture, and many others. These results also show that pasteurizing liquefied blueberries in a steam jacketed kettle retained the highest levels of anthocyanins in the final product. A possible explanation for this is that when the blueberry skin cells, which hold the anthocyanins, came into contact with the kettle walls, they were heated to a higher temperature than the microwaved cells. Since the addition of heat increases the permeability of the cells, the higher temperature that the cells were exposed to may have

caused more water soluble constituents (including anthocyanins) to be released. These constituents were subsequently transferred into the juice when pressed. While the entire mixture only reached a temperature of 90°C, the steam jacketed kettle walls were significantly hotter to the point where boiling on the edges did occur. In the microwaved samples, there was no boiling, so the cells were exposed to milder heat so permeability was not as high resulting in fewer water soluble compounds (including anthocyanins) being released from the epithelial cells. Only cells that were weakened by the freezing, thawing, blending, transfer, pumping, and microwaving process were released. Perhaps a more efficient way to force anthocyanins from epithelial cells would be to do larger batches so that higher pressure could be applied to the blueberries during juicing that a larger press would better facilitate.

B. ORAC Values Over Time

The ORAC values over time are fairly stable (Figure 11), given the fact that an ORAC value is accurate with +/- 15% between samples (Ou et al. 2001) run from the same standard in different trials. Even when the entire system is fully automated, the +/- 15% does not go down significantly (Huang et al. 2002). When the same sample is run multiple times on the same plate, the accuracy varies between 91-107% (Ou et al. 2001) for manual sampling, where all pipetting and transfers are done by hand. Even when the system is fully automated, having no human factor, there is still an acceptable range of 99-109% (Huang et al. 2002). Due to the fact that the literature accepts such a wide span in value as being equal, and since the data collected have a wide standard deviation inherent to this assay as it is presently run, these data suggest there is no difference in any individual treatment level over time. While the ORAC does have some variability, it was

the assay that most directly measures the capacity of extracts to scavenge radicals and the one chosen for this study.

The values for the ORAC found in this study are consistent with previous studies. While variation of up to 2.4 fold for blueberries harvested from the same field in different years has been observed (Kalt et al. 2001), the ORAC values for Tifblue blueberries are generally found to be around 38,000 TE/L (Mazza and Miniati 1993). The average value in the present study for an extracted bluberry was 52,000 TE/L.

C. Polyphenols Over Time

The polyphenol values over time, like the ORAC values seem to be fairly stable with the pasteurized (90°C) and 92.5°C microwaved sample having values around 3,750 mg gallic acid equivalents per liter and the 95°C and 85°C microwaved treatments having values close to 3,000 mg gallic acid equivalents per liter. The variation between the microwaved samples is likely due to sample variability as well as experimental variation in the pump. While the speed was always returned to speed "6" after cleaning, there still may have been slight differences in the actual pumping speeds (Figure 12). The values determined were in line with previous studies where 1kg of Tifblue blueberries were found to contain 3,916mg (Sellappan et al 2002). Since the antioxidant capacity, which represents the free radical scavenging activity of all compounds did not change, one would expect that total phenols would also remain stable over time, as was the case. An small increase in anthocyanins was observed. Perhaps no correlating increase in total phenols or antioxidant capacity was seen during that same period due to the anthocyanin content not increasing greatly.

IV. Experiment #4: Effects of Microwaving, Cold Processing, Hot Processing, and Pasteurization on the Antioxidant Retention in Blueberry Juices.

Comparing Traditional and Non-Traditional Processing Methods

In order to evaluate the efficiency of the processing methods, relative to what an extraction could do, the resulting data was compared to that of unprocessed, extracted blueberries (Prior et al. 2001). Other blueberries from the same bulk lot of Tifblue blueberries used to make the microwaved samples were processed into juices using cold, hot, or pasteurizing conditions. Each treatment was analyzed for changes in total phenols, and antioxidant capacity (ORAC) (Figure 13). These traditional processing values of cold processing, hot processing, and pasteurization were compared to the total phenols and ORAC values obtained for blueberry juices that had been produced through microwaving at 85°C, 92.5°C, and 95°C (Figure 14).

The data show the same trends exist for the total phenols as they do for the ORAC data (Figures 15 and 16). As expected, the extracted sample had the highest values of all the samples. Of the processed samples the pasteurized sample had the highest average values. The microwaved and hot processed (47°C) values were similar for both evaluation methods. The cold processed values were significantly lower than the other processing methods. Since the standard errors of all the ORAC values overlap, except for the cold processed blueberry juice, the average values for each processing method follow the total phenol trend. The trends suggest that the cold processing of blueberries was not as efficient at transferring antioxidants or phenols to the final juice as were the methods where heat was used. A possible explanation for this is that the addition of heat: increased cell permeability (Sapers et al. 1985), reduced the solubility of oxygen in the

juice (Sapers et al. 1985), inactivated enzymes present in the juice (Tanchev 1974), and may have contributed more general stresses, such as water expansion, causing more skin cells to be damaged and release their contents. These rationale would help to explain why the method of heating affected the final value in the various trials when the final temperature did not clarify this issue. The steam jacketed kettle had higher temperatures than did the microwave at the point of heating, which would explain why the hot processed (47°C) was similar in total phenols and antioxidant capacity (ORAC) to the microwaved samples, and why the pasteurized sample was the highest in total phenols and antioxidant activity.

CONCLUSION

Tests, both initially and over time, show that the blueberries processed in the steam kettle (pasteurized) have the highest antioxidant capacity, and total phenol values. While the statistical reliability of the ORAC method is not yet accurate enough to allow strict conclusions to be drawn, the total phenol data and anthocyanin data do suggest that pasteurization using a steam kettle is presently the most efficient at retaining antioxidant activity as measured by total phenols and ORAC in the final product. One likely reason for this significantly higher level of antioxidant retention is due to the exposure to higher heating temperatures resulting in increased permeability of water soluble substances to move out of the cells. The higher heat also degraded the enzymes (PPO and glucosidases) present that would be harmful to the anthocyanins as well as lowered the levels of oxygen present in the final product which contribute to antioxidant destruction.

Future studies in this area involving microwaving would be quite interesting. Perhaps the addition of pressure, which is presently possible, would be beneficial at transferring more of these antioxidants into the final product by causing the epithelial cells to release more of their contents, without exposing the anthocyanins to boiling temperatures. The use of a more rapid heat exchanger to cool the heated macerate in the set-up could also have significant impact in this area. Finally, it would be beneficial if in future studies larger batches were examined. The individual batch sizes used in this study were representative of 2-4 blueberry plants. With the large variability seen in the antioxidant and anthocyanin content of different plants on the same field the same year, as well as the variability seen in the same plant year to year, starting all batches from aliquots from one larger batch would be a worthwhile experiment.

Table 1. Typical Blueberry Composition (North American Blueberry Council, 2003).

Nutrient Measurement Units of Blueberries	**Amount/100g**
Moisture	85.15 g
Protein	1.12 g
Fat	0.02 g
Ash	0.19 g
Carbohydrates	13.51 g
Sugars*	10.65 g*
Total Dietary Fiber	2.86 g
Vitamin C	1.40 mg
Sodium	10.00 mg
Potassium	56.40 mg
Calcium	8.80 mg
Iron	0.186 mg

Composition by Percent	**Percent %**
Water	85.15
Protein	1.12
Ash	0.19
Carbohydrates	13.51

Total Sugars Breakdown	**Percent %**
Fructose	49
Glucose	48
Sucrose	3

Table 2. 1998 FAO World Blueberry Production Statistics (Mazza and Oomah 2000).

Producing Country	**Production (metric tons)**
United States	95,000
Canada	33,000
Eastern Europe	17,000
Western Europe	5,900
New Zealand	1,000

Table 3. Thermal Degradation Kinetics of Anthocyanins: t1/2 for Malvidin-3-glucoside for Corresponding Temperatures and pHs.

Temperature (°C)	Buffer pH=2.5	Buffer pH=3.5	Buffer pH=4.5
78	266	450	378
88	106	161	131
98	37	49	43
108	14	23	18

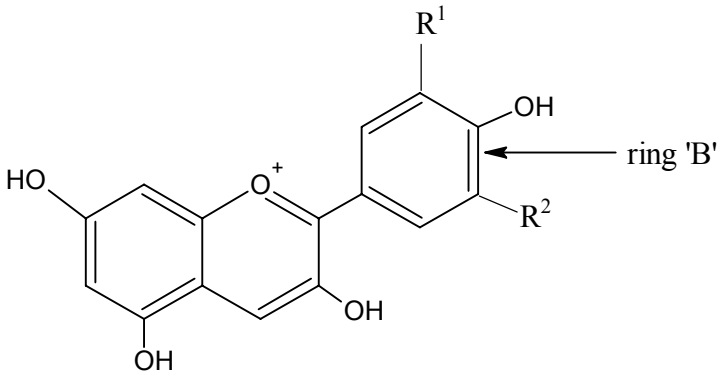
Temperature (°C)	Juice pH=2.5	Juice pH=3.5	Juice pH=4.5
78	276	260	260
88	117	107	107
98	42	47	50
108	20	21	23

(Chart taken from "Kinetics of Thermal Degradation of the Anthocyanins Delphinidin-3-rutinoside and Malvidin-3-glucoside", S. Tanchev and N. Yoncheva 1974, Die Nahrung 18; 747-752)

Table 4. Individual Anthocyanin Composition of Blueberry Juices

Individual Anthocyanins	Percent Composition of Total Anthocyanins
Peonidin-3-glucoside	38%
Malvidin-3-glucoside	22%
Cyanidin-3-glucoside	15%
Delphinidin-3-glucoside	13%
Petunidin-3-glucoside	12%

Figure 1. Structures of Commonly Consumed Anthocyanins. (Haslam, 1998, Practical Polyphenols)



Anthocyanidin	**R^1**	**R^2**	**λ_{max} nm***
Pelargonidin	H	H	520
Cyanidin	OH	H	535
Peonidin	OMe	H	532
Delphinidin	OH	OH	546
Petunidin	OMe	OH	543
Malvidin	OMe	OMe	542

* λ_{max} in 0.01% HCl/MeOH (v/v).
Data taken from Harborne (1967).

Figure 2. Four States of Anthocyanins. (Haslam, 1998, Practical Polyphenols)

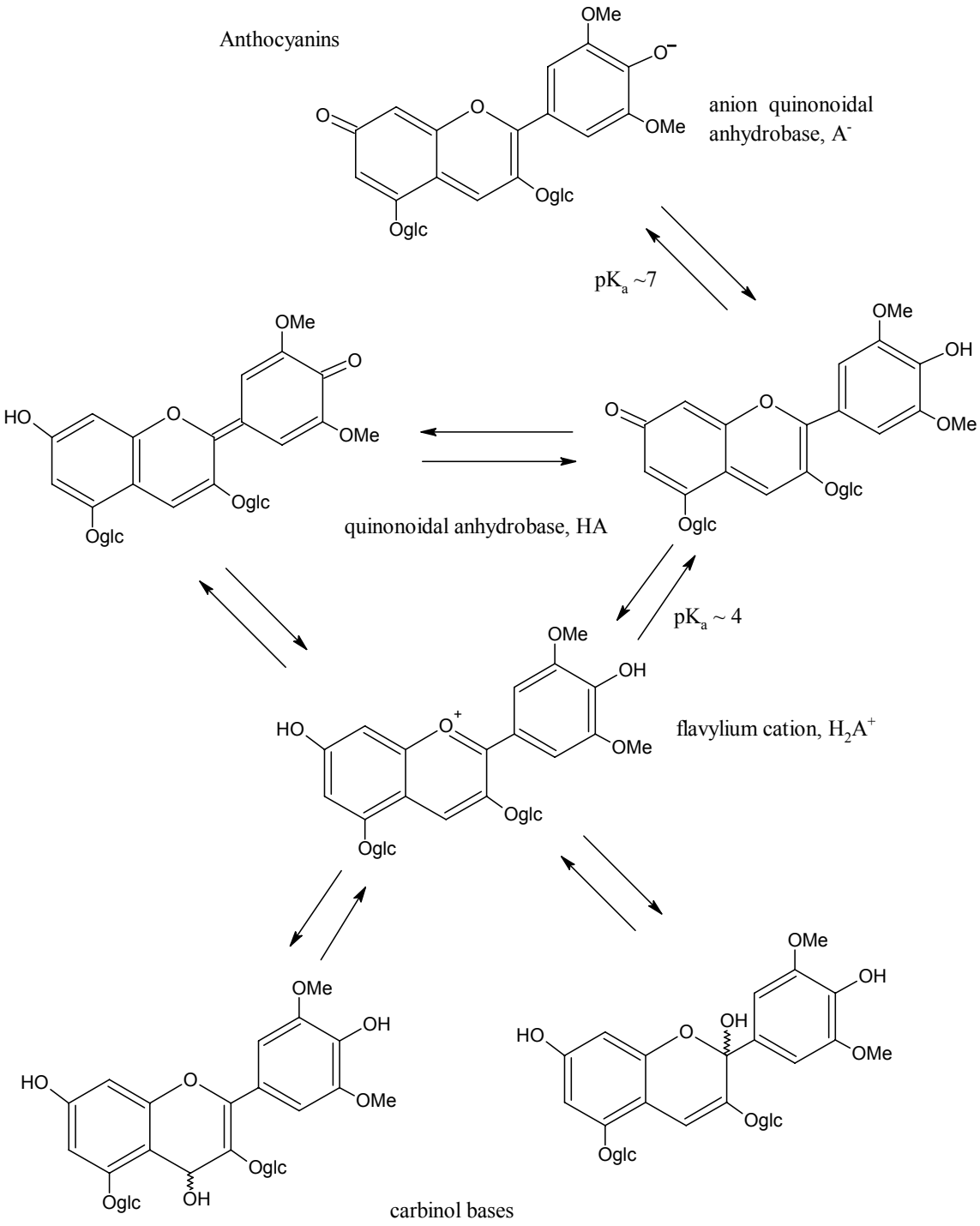


Figure 3. Polyphenols – structure and biosynthesis. (Haslam, 1998, Practical Polyphenols)

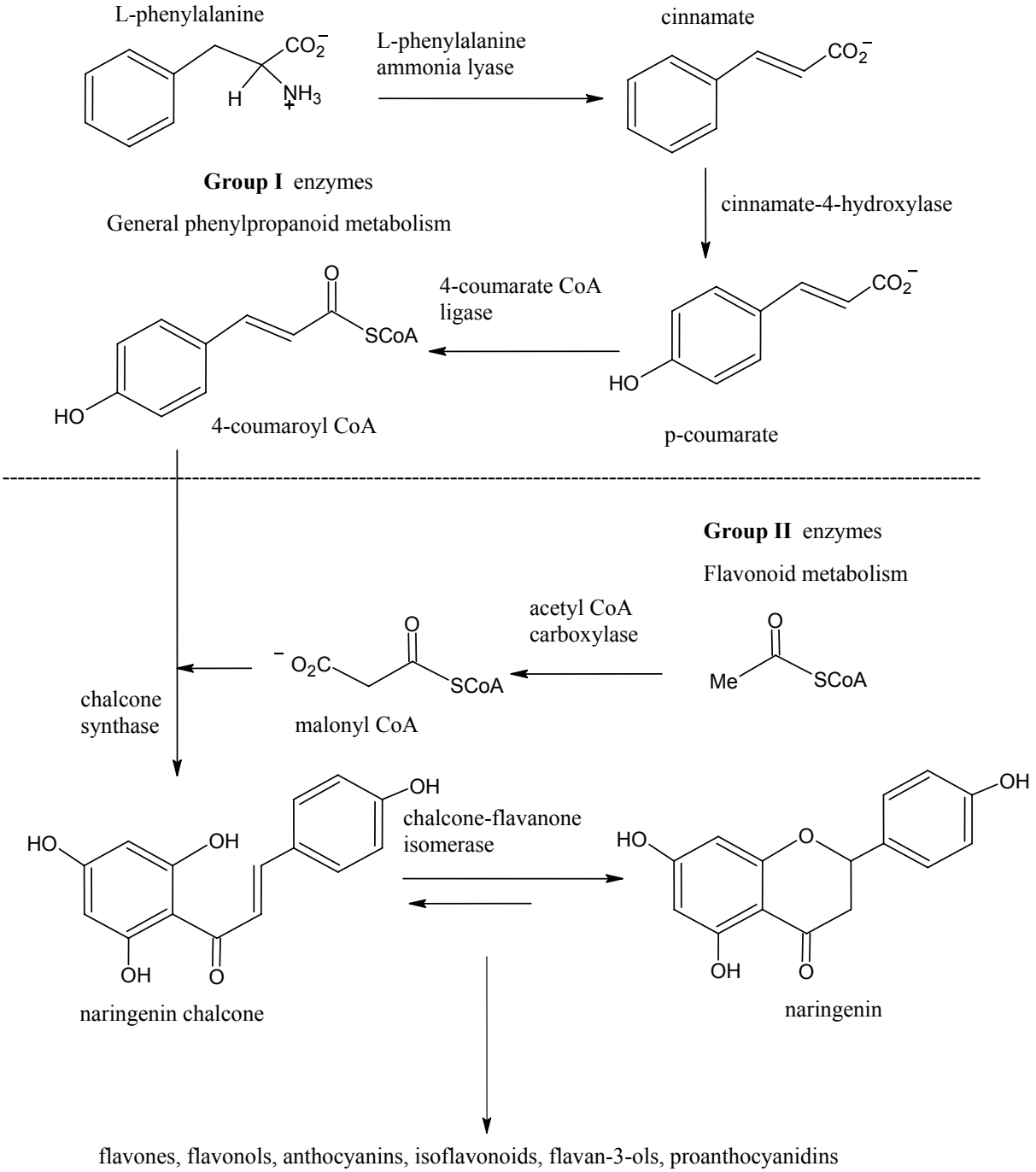


Figure 4. Degradation of Cyanidin 3-Glucoside by Blueberry Polyphenol Oxidase. (Kader F., Haluk J.P., Nicolas J.P., and Metche M., 1998)

$$\text{CG} + 0.5\text{moles } O_2 \xrightarrow{\text{PPO}} \text{CG } o\text{-Q} + H_2O \qquad (1)$$

$$\text{CG } o\text{-Q} + \text{Cy 3-glu} \longrightarrow \text{CG} + \text{Cy 3-glu } o\text{-Q} \qquad (2)$$

CG = Chlorogenic Acid
CG *o*-Q = Chlorogen *o*-quinone
Cy 3-glu = Cyanidin 3-Glucoside
Cy 3-glu *o*-Q = Cyanidin 3-glucoside *o*-quinone
PPO = Polyphenol Oxidase

Figure 5. Oxidation States of Vitamin E. (C.A. Rice-Evans and R.H. Burton, 1994, Free Radical Damage and its Control)

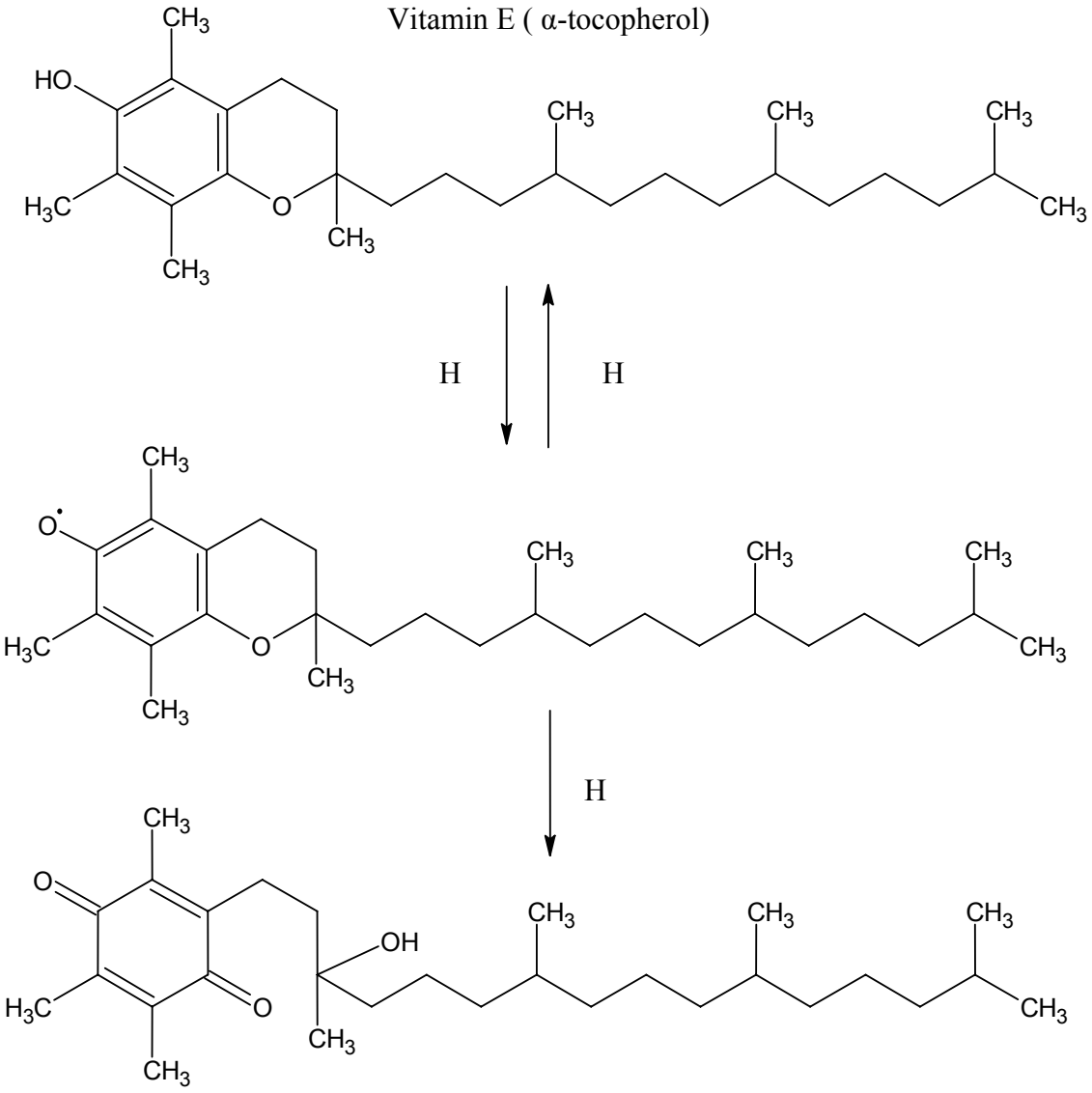


Figure 6. Oxidation States of Ascorbic Acid. (C.A. Rice-Evans and R.H. Burton, 1994, Free Radical Damage and its Control)

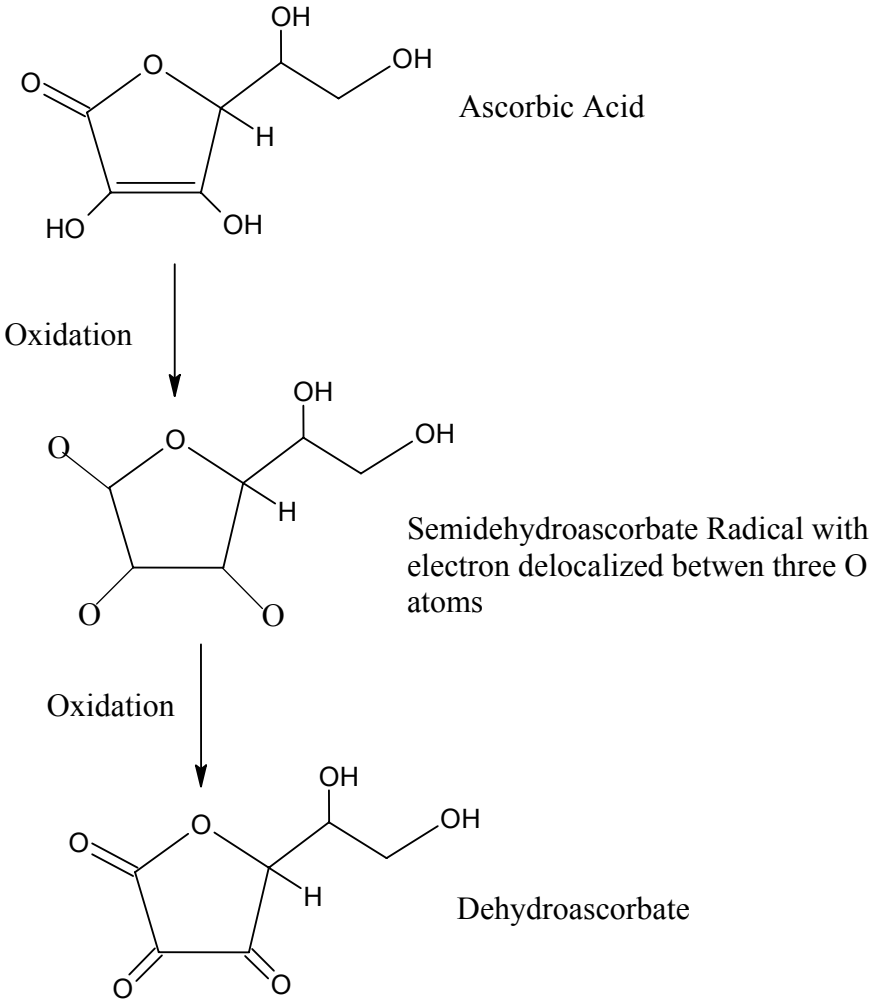


Figure 7. Comparison of Extraction Methods Over Time.

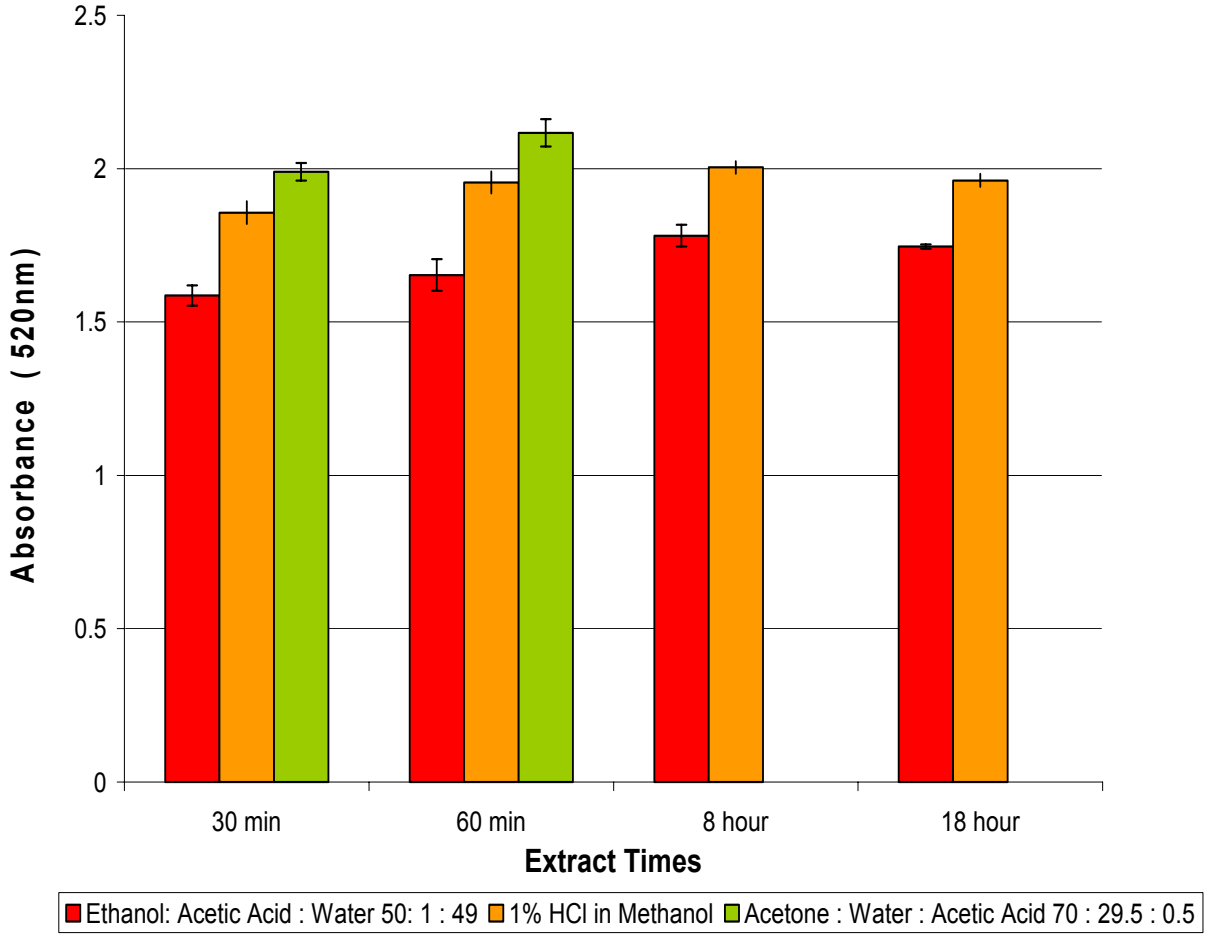


Figure 8. Total Phenol Values for Cold versus Hot Processed Blueberry Wines.

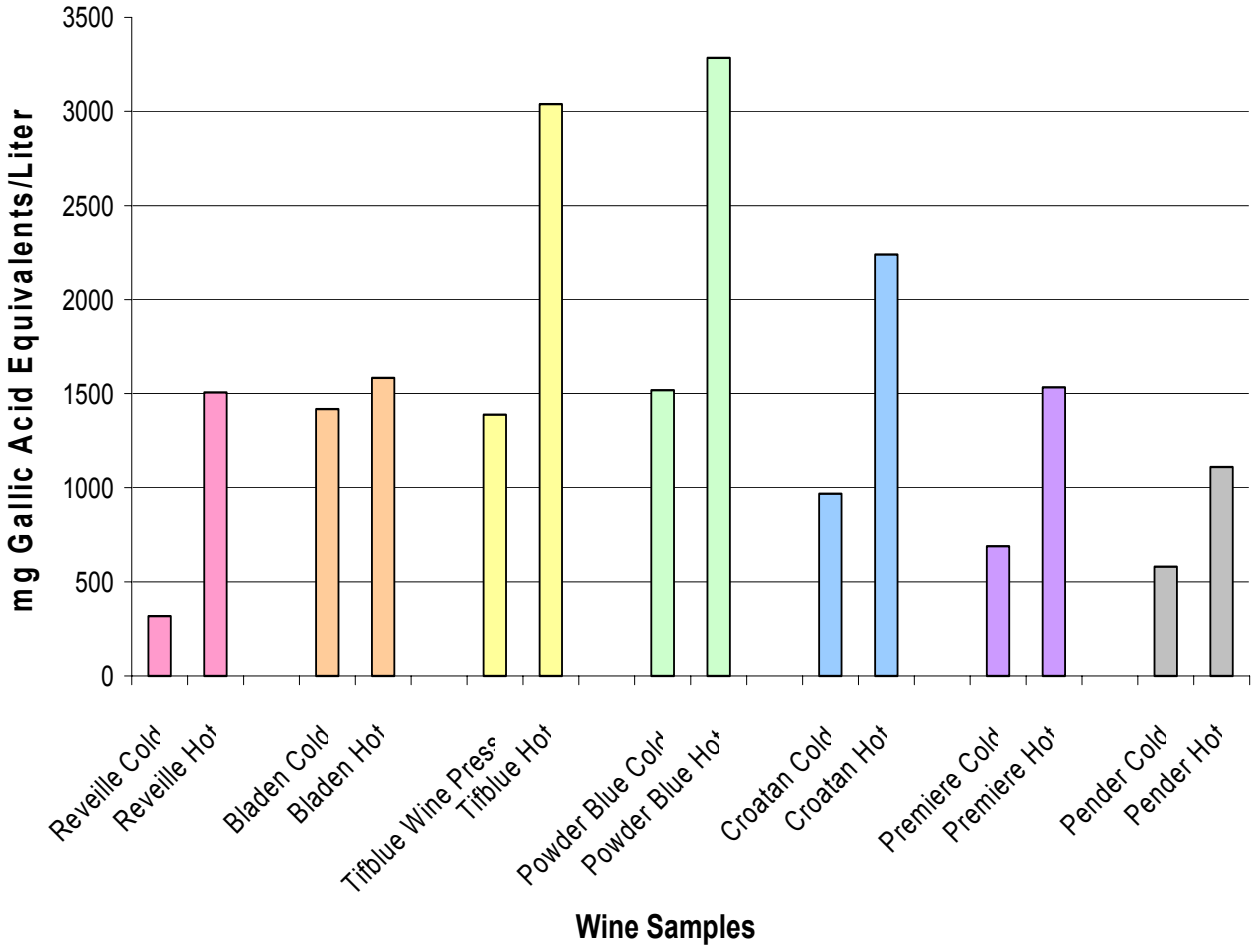


Figure 9. ORAC Values For Cold versus Hot Processed Blueberry Wines.

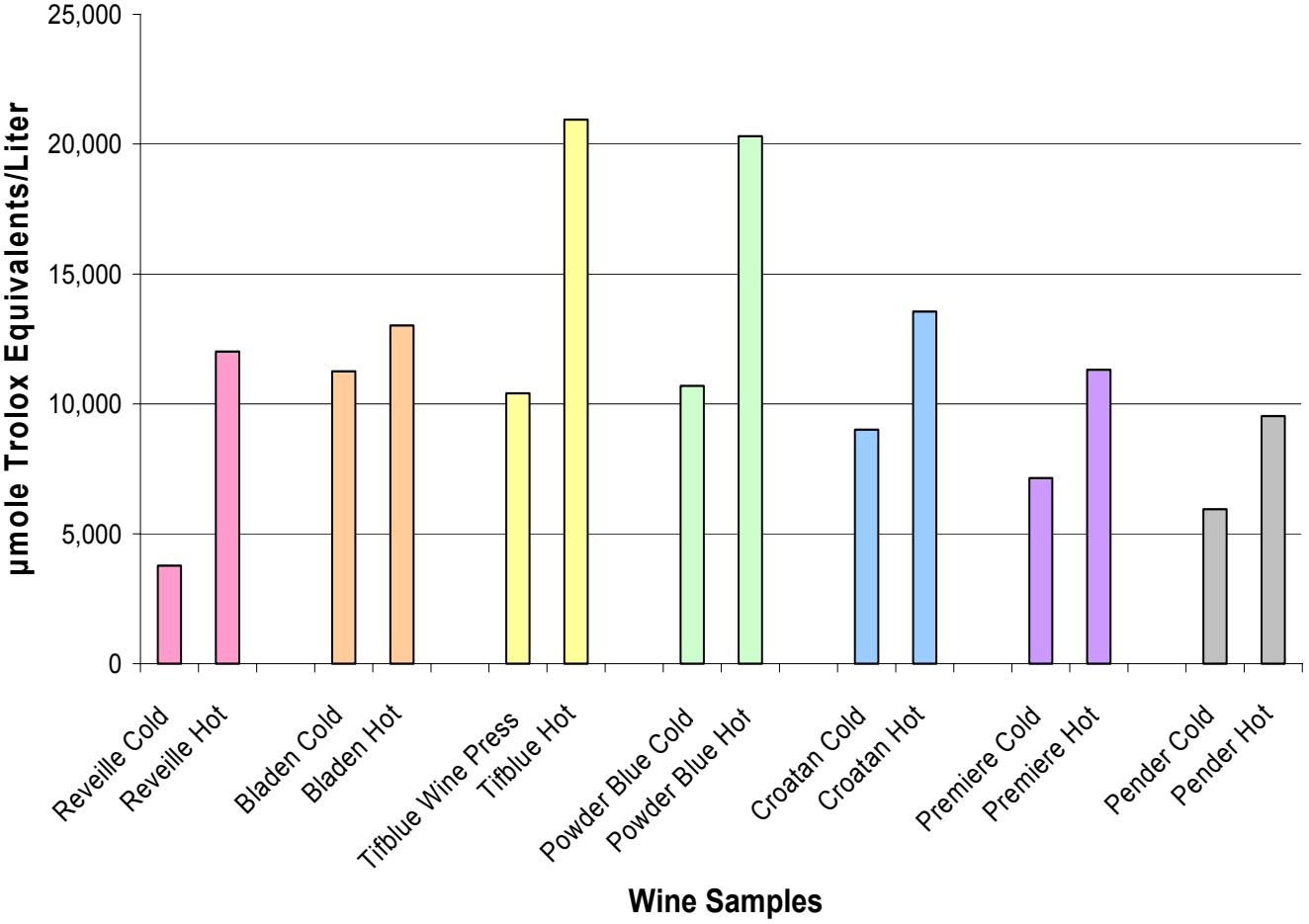


Figure 10. Changes in Total Anthocyanins of Microwaved and Pasteurized Blueberries During Refrigerated Storage.

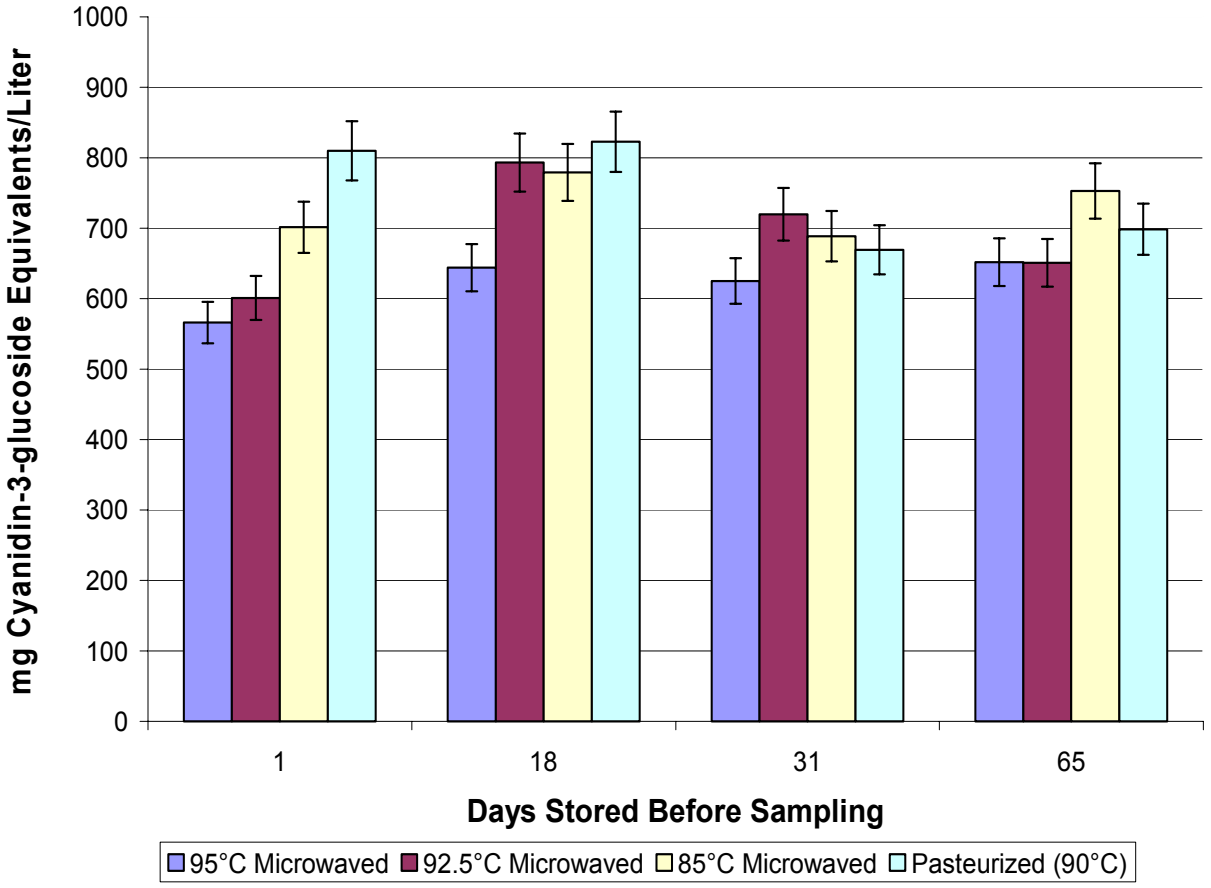


Figure 11. ORAC Changes in Microwaved and Pasteurized Blueberry Juices Over Time

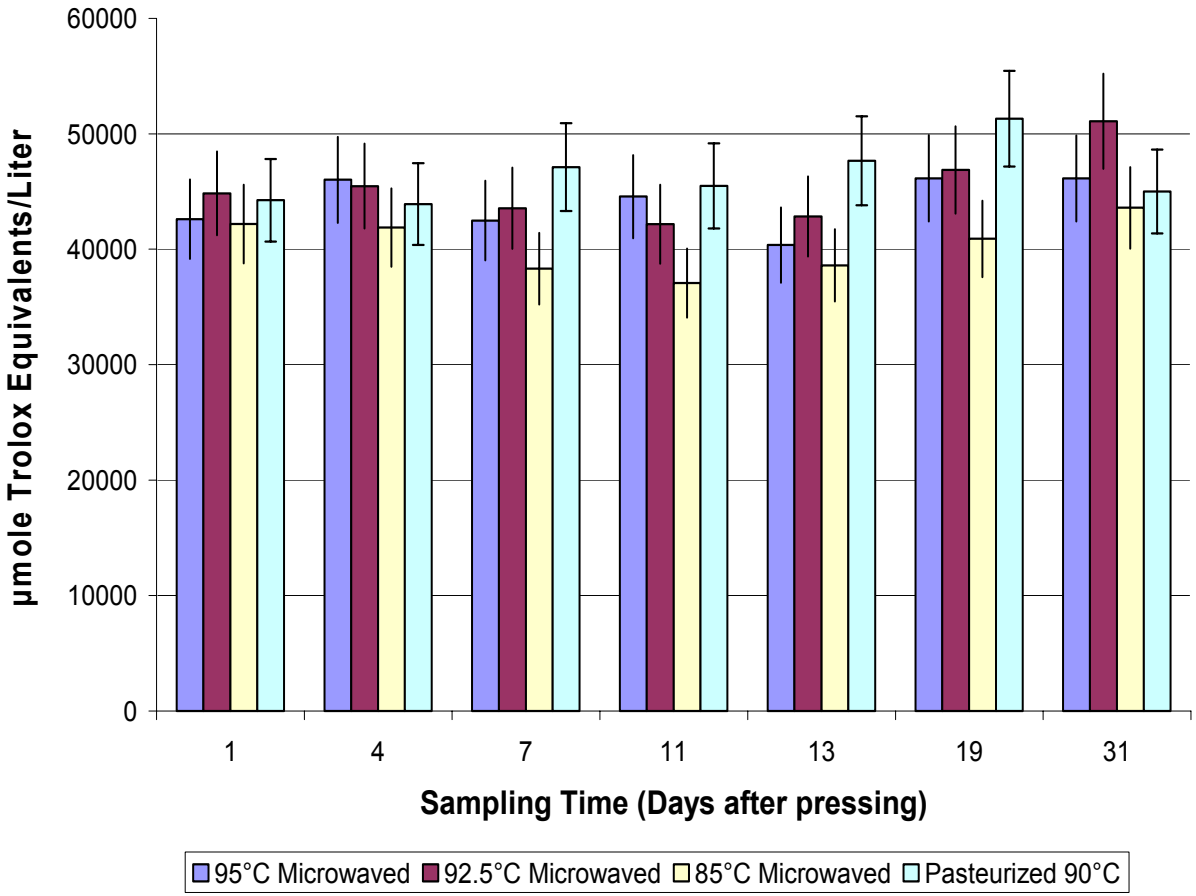


Figure 12. Total Phenol Changes in Microwaved and Pasteurized Blueberry Juices Over Time.

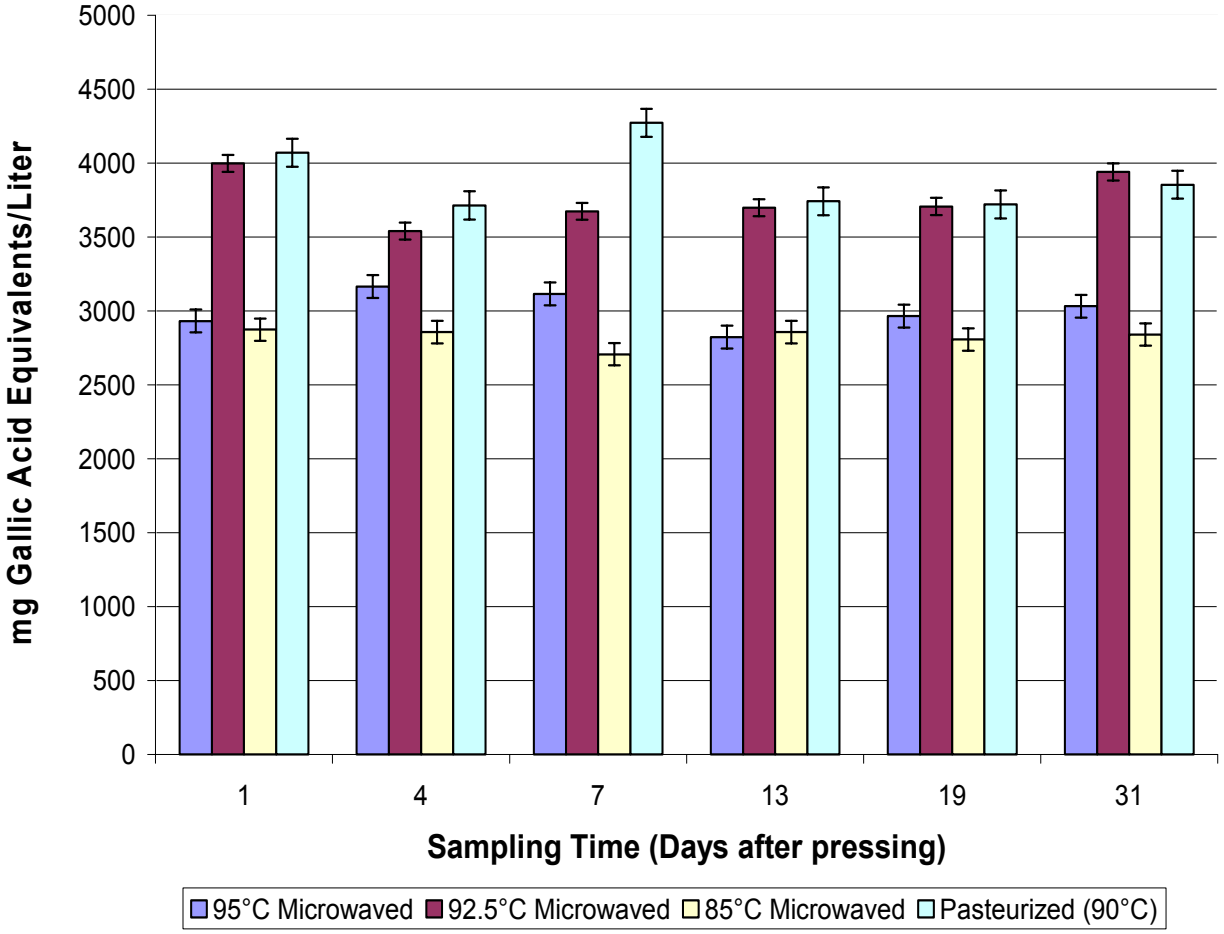


Figure 13. ORAC Values for Different Processing Methods of Blueberry Juices

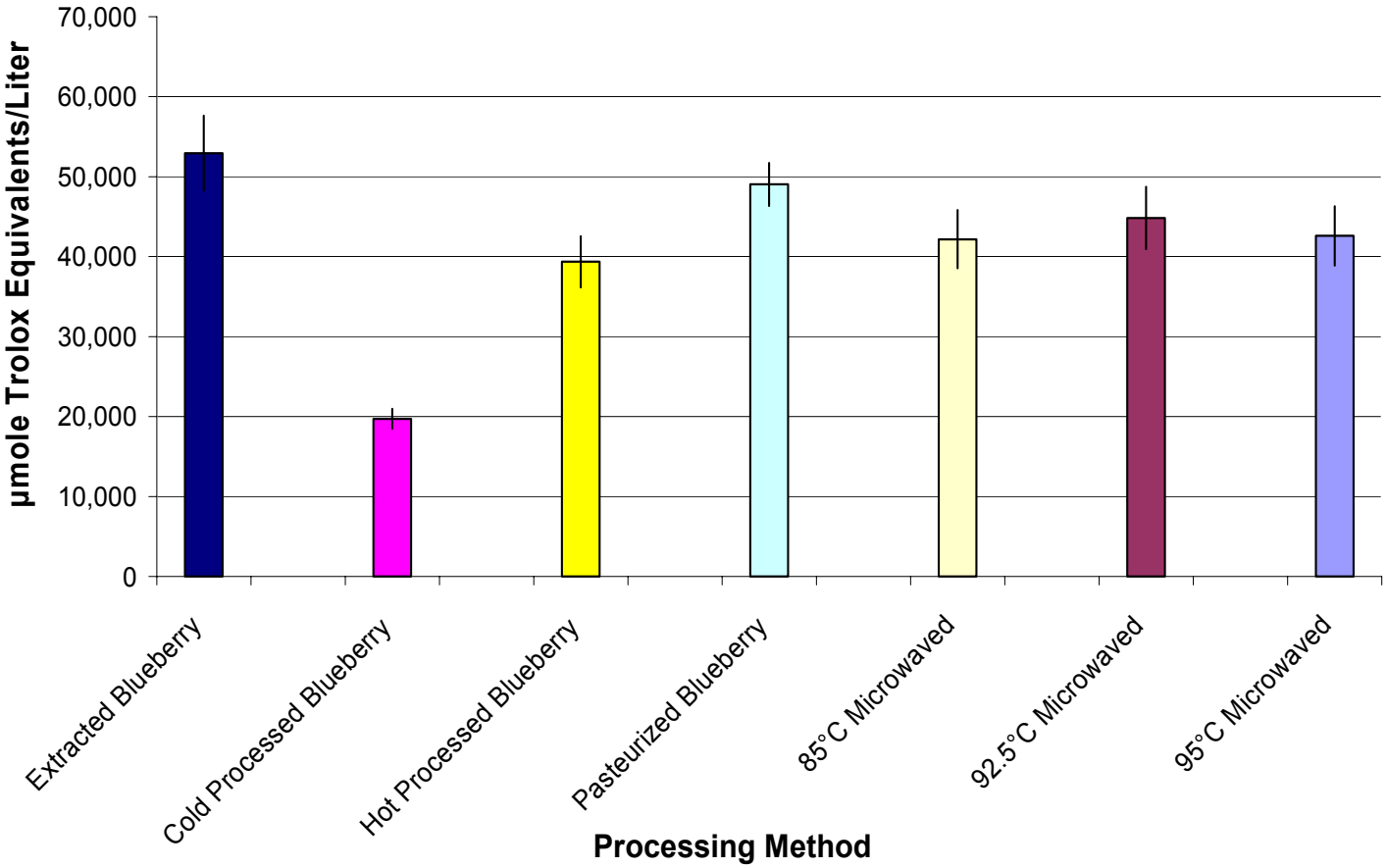
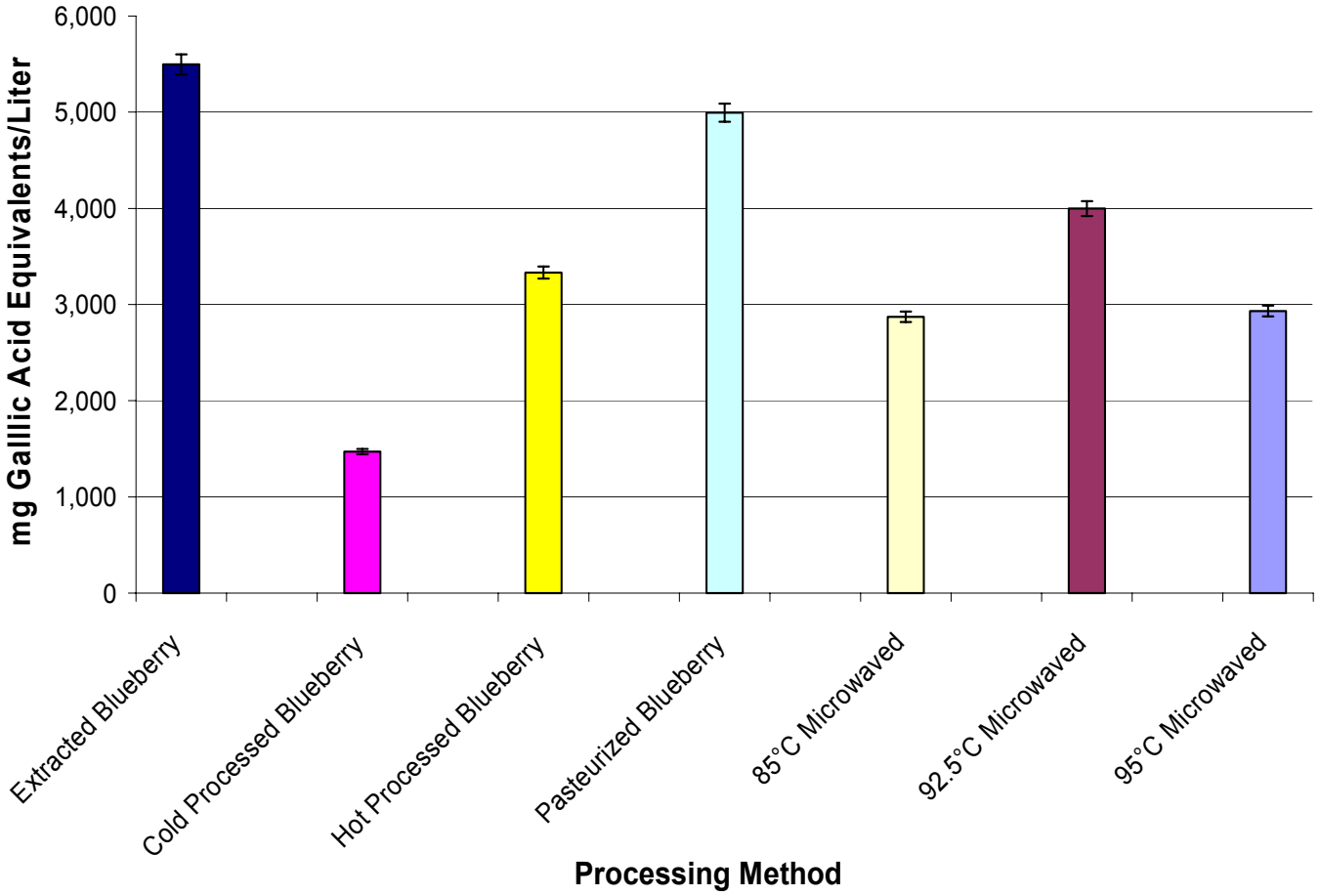


Figure 14. Total Phenol Values for Different Processing Methods of Blueberry Juices.



REFERENCES

de Ancos, B. Gonzalez, E.M. and Cano, M.P. (2000). "Ellagic acid, vitamin C, and total phenolic contents and radical scavenging capacity affected by freezing and frozen storage in raspberry fruit." J. Agricultural and Food Chemistry, Vol. 48, No. 10, 4565-4570

de Ancos, B. Cano, M.P. Hernandez, A. and Monreal, M. (1999). "Effects of microwave heating on pigment composition and colour of fruit purees," J. of Science of Food and Agriculture, Vol. 70, 663-670

Andriambeloson, E. Magnier, C. Haan-Archipoff, G. Lobstein, A. Anton, R. Beretz, A. Stoclet, J.C. and Andriantsitohaina, R. (1998). "Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta." J. of Nutrition, Vol. 128, No. 12, 2324-2333

Adriambeloson, E. Stoclet, J.C. and Andriantsitohaina, R. (1996). "Effects vasculaires d'extracts vegetaux contenant des derives polyphnoliques." In Polyphenolics Communications. Vercauteren, J. Cheze, C. Dumon, M.C. and Wber, J.F. 421-422. Bordeaux, France

Ballinger, W.E. and Kushman, L.J. (1970). "Relationship of Stage of Ripeness to Composition and Keeping Quality of Highbush Blueberries." J. Amer. Soc. Hort. Sci., Vol. 95, 239-242

Baublis, A. Spomer, A. and Berber-Jimenez, M.D. (1994). "Anthocyanin Pigments: Comparison of Extract Stability," J. of Food Science, Vol. 59, No. 6, 1219-1222

Boniface, R. Miskulin, M. Robert, L. and Robert, A.M. (1986). "Pharmacological properties of Myrtillus anthocyanosides: correlation with results of treatment of diabetic microangiopahty." in Flavonoids and Bioflavonoids. Farkas, L. Gabor, M. and Kallay, F. editors, Amsterdam, Elsevier, pg 293-301

Bridle, P. Garcia-Viguera, C. and Tomas-Barberan, A. (1996). "Analysis of anthocyanins by capillary zone electrophoresis." J. Liquid. Chrom. and Rel. Technol., Vol. 19, No. 4, 537-545

Brouillard, R. (1982). in Anthocyanins as Food Colors. Markakis, P. editor, New York.

Cignarella, A.M. Nastasi, E. Cavalli, E. and Puglisi, L. (1996). "Novel lipid-lowering properties of Vaccinium myrtillus L leaves, a traditional antidiabetic treatment in several models of rat dyslipidaemia: a comparison with ciprofibrate." Thrombosis Res. Vol 84, No. 5, 311-322

Combs, G.F. Jr. (1998). The Vitamins; Fundamental Aspects in Nutrition and Health Second Edition, Academic Press Inc. U.S.

Connor, A.M. Luby, J.J. Hancock, J.F. Berkheimer, S. and Hanson, E.J. (2002). "Changes in Fruit Antioxidant Activity among Blueberry Cultivars during Cold-Temperature Storage," J. of Agricultural and Food Chemistry, Vol. 50, 893-898

Cristoni, A. and Magistretti, M.J. (1987). "Antiulcer and healing activity of Vaccinium myrtillus anthocyanosides." Farmaco. Vol. 42, No. 2, 29-43

Cross, C.E. van der Vliet, A. Louie, S. Theile, J.J. and Halliwell, B. (1998). "Oxidative stress and antioxidants at biosurfaces: plants, skin and respiratory tract surfaces." Environmental Health and Perspect., Vol. 106, 1241-1251

Daub, M.E. and Briggs, S.P. (1983). "Changes in tobacco cell membrane composition and structure caused by the fungal toxin cercosporin." J. of Plant Physiology, Vol. 71, 763-766

Davies, K.J. Sevanian, A. Muakkassah-Kelly, S.F. and Hochstein, P. (1986). "Uric acid-iron ion complexes. A new aspect of the antioxidant functions of uric acid." J. of Biochemistry, Vol. 235, No. 3, 747-754

Delledonne, M. Xia, Y. Dixon, R.A. and Lamb, C. (1998). "Nitric oxide functions as a signal in plant disease resistance." Nature, Vol. 394, 585-588

Demrow, H.S. Slane, P.R. and Folts, J.D. (1995). "Administration of wine and grape juice inhibits in vivo platelet activity and thrombosis in stenosed canine coronary arteries." Circulation, Vol. 91, No. 4, 1182-1188

Diplock, A.T. (1985). Fat soluble Vitamins. Pg 319, Heinemann, London

Durkee AB, Jones JD. (1969). "A mild procedure for extraction and fractionation of anthocyanin, proanthocyanin and other polyphenols of apple peel", Phytochem, Vol. 8, 909–911

Durst, R.W. Lee, J. and Wrolstad, R.W. (2002). "Impact of Juice Processing on Blueberry Anthocyanins and Polyphenolics: Comparison of Two Pretreatments." J. of Food Science. Vol. 67, No. 5

Eck, P. (1988). Blueberry Science. Rutgers Press, Brunswick, N.J.

Eck, P. (1970). Influence of Ethrel Upon Highbush Bluberry Fruit Ripening." Hort. Science, Vol. 5, 23-25

Edenharder, R. Kurz, P. John, K. Burgard, S. and Seeger, K. (1994). "In vitro effect of vegetable and fruit juices on the nutagenicity of 2-amino-3-methylimidazo [4,5-fquinoline, 2-amino-3,4-dimethylimidazo [4,5-fquinoline and 2-amino-3,8-dimethylimidazo [4,5-fquinozaline." Food Chem. Toxicol. Vol. 32, No. 5, 443-459

Ehlenfeldt, M.K. and Prior, R.L. (2001) "Oxygen Radical Absorbance Capacity (ORAC) and Phenolic and Anthocyanin Concentrations in Fruit and Leaf Tissues of Highbush Blueberry," J. of Agricultural and Food Chemistry, Vol. 49, 2222-2227

Ernster, L. and Nelson, B.D. (1981). in Biomedical and Clinical Aspects of Coenzyme Q, Folkers, K, and Yamamura, Y editors., pg 159-168, Elsevier, Amsterdam

Eskilsson, C.S. and Bjorklund, E. (2000). "Analytical-scale microwave-assisted extraction," J. of Chromatography, Vol. 902, 227-250

Folin O, Denis W. (1912). "On phosphotungstic-phosphomolybdic compounds and colour reagents." J Biol Chem, Vol. 12, 239-43

Food and Nutrition Board. (1998). "Proposed definition of dietary antioxidants." http://www.2nas.edu/fnb/21a2.html

Foyer, C.H. and Halliwell, B. (1976)."The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism." Planta Vol. 133, 21-25

Francis, F.J. Harborne, J.B. and Baker, W.G. (1966). "Anthocyanins in the lowbush blueberry, Vaccinium angustifolium." J. of Food Science, Vol. 31, 583-587

Ganzler, K. Salgo, A. and Valko, K. (1986). "Microwave Extraction: A novel Sample Preparation Method for Chromatography," Journal of Chromatography, Vol. 371, 299-306

Gao, L. and Mazza, G. (1996). "Extraction of Anthocyanin Pigments from Purple Sunflower Hulls," J. of Food Science, Vol. 61, No. 3, 600-603

Gao, L. and Mazza, G. (1994). "Rapid Method for Complete Chemical Characterization of Simple and Acylated Anthocyanins by High-Performance Liquid Chromatography and Capillary Gas-Liquid Chromatography," J. of Agricultural and Food Chemistry, Vol. 42, 118-125

Garcia-Viguera, C. Zafrilla, P. Romero, R. Abellan, P. Artes, F. and Tomas-Barberan, F.A. (1999). "Color Stability of Strawberry Jam as Affected by Cultivar and Storage Temperature." J. of Food Science, Vol. 64, No. 2

Garcia-Viguera, C. Zafrilla, P. and Tomas-barberan. (1998) "The use of Acetone as an Extraction Solvent for Anthocyanins from Strawberry Fruit." Phytochem. Anal. Vol. 9, 274-277

Gimenez, J. Kajda, P. Margomenou, L. Piggott, J.R. and Zabetakis, I. (2001). "A study on the colour and sensory attributes of high-hydrostatic-pressure jams as compared with traditional jams," ," J. of Science of Food and Agriculture, Vol. 81, 1228-1234

Goto, T. (1987). "Structure, stability and color variation of natural anthocyanins", Progress Chem Org. Nat. Prod. Vol. 52, 113-158
Gutteridge, J.M.C. and Halliwell, B. (1994). Antioxidants in Nutrition, Health, and Disease. Oxford University Press, Oxford

Halliwell, B. and Gutteridge, J.M.C. (1989). Free radicals in biology and medicine. 2nd Edition, Clarendon Press, Oxford.

Haslam, E. (1989). Plant Polyphenols; Vegetable Tannins Revisited, Cambridge University Press, Melbourne Australia

Hazen, R.A. Bushway, A.A., Bussell, B.L. and Davis-Dentici, K. (2003). http://ift.confex.com/ift/2000/techprogarm/paper_2250.htm

Heagle, S.A. (1989). "Ozone and Crop Yield." Annu. Rev. Phytopathology, Vol. 27, 397-423

Hertog, M.G.L. Hollman, P.C.H. Katan, M.B. and Kromhout, D. (1993). "Dietary antioxidant flavonoid intake and risk of coronary heart disease: the Zutphen Elderly Study." Lancet Vol. 342, 1007-1111

Hong, V. and Wrolstad, R.E. (1990). "Characterization of anthocyanin-containing colorants and fruit juices by HPLC/phododiode array detection," J. of Agricultural and Food Chemistry, Vol. 38, No. 3, 698-708

Hong, N. Yaylayan, V.A., Raghavan, G.S. Pare, J.R. and Belanger, J.M. (2001). "Microwave-assisted extraction of phenolic compounds from grape seed," Nat. Prod. Left, Vol. 15, No. 3, 197-204

Howell, A.B. Der Marderosian, A. and Foo, L.Y. (1998). "Inhibition of the adherence of P-fimbriated *Escherichia coli* to uroepithelial-cell surfaces by proanthocyanidin extracts from cranberries," New England J. of Medicine, Vol. 339, No. 15, 1085-1086

Huang, D. Ou, B. Hampsch-Woodill, M. Flanagan, J.A. and Deemer, E.K. (2002). "Development and Validation of Oxygen Radical Absorbance Capacity Assay for Lipophilic Antioxidants Using Randomly Methylated beta-Cyclodextrin as the Solubility Enhancer," J. of Agricultural and Food Chemistry, Vol. 50, 1815-1821

Ichiyanagi, T. Oikawa, K. Tateyama, C. and Konishi, T. (2001). "Acid Mediated Hydrolysis of Blueberry Anthocyanins," J. of Chem. Pharm. And Bull. Vol. 49, No. 1, 114-117

Inze, D. and Montagu, M.V. (2002). Oxidative Stress in Plants. TJ International Ltd. Padstow Cornwall England

Jabs, T. Dietrich, R.A. and Dangl, J.L. (1996). "Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide." Science, Vol. 273, 1853-1856

Jayle, G.E. Aubry, M. Gavini, H. Graccini, G. and de la Baume, C. (1965). "Etude concernant l'action sur la vision nocturne." Ann. Oculistique, Vol. 198, No. 6, 556-562
Jimenez, M. and Garcia-Carmona, F. (1999). "Oxidation of the Flavonol Quercetin by Polyphenol Oxidase," J. of Agricultural and Food Chemistry, Vol. 47, No. 1, 56-60

Kader, F. Irmouli, M. Zitouni, N. Nicolas, J.P. and Methce, M. (1999). "Degradation of Cyanidin 3-Glucoside by Caffeic Acid o-Quinone. Determination of the Stoichiometry and Characterization of the Degradation Products," J. of Agricultural and Food Chemistry, Vol. 47, 4625-4630

Kader, F. Haluk, J.P. Nicolas, J.P. and Metche, M. (1998). "Degradation of Cyanidin 3-Glucoside by Blueberry Polyphenol Oxidase: Kinetic Studies of Mechanisms," J. of Agricultural and Food Chemistry, Vol. 46, No. 8, 3060-3065

Kalt, W. Ryan, D.A.J. Duy, J.C. Prior, R.L. Ehlenfeldt, M.K., and Vander Kloet, S.P. (2001). "Interspecific Variation in Anthocyanins, Phenolics, and Antioxidant Capacity among Genotypes of Highbush and Lowbush Blueberries (*Vaccinium* Section *cyanococcus* ssp.)," J. of Agricultural and Food Chemisty, Vol. 49, 4761-4767

Kalt, W. and Dufour, D. (1997). "Health functionality of blueberries." Hort. Technol. Vol. 7, No. 3, 216-222

Kalt, W. Forney, C.F., Martin, A. and Prior, R.L. (1999). "Antioxidant Capacity, Vitamin C, Phenolics, and Anthocyanins after Fresh Storage of Small Fruits," J. of Agricultural and Food Chemistry, Vol. 47, 4638-4644

Kauss, H. Jeblick, W. Ziegler, J. and Krabler, W. (1994). "Pretreatment of parsley (*Petroselinum crispum*) suspension cultures with methyl jasmonate enhanced elicitation of activated oxygen species." Plant Physiology, Vol. 105, 89-94

Kermasha, S., Bisakowski, B., Ramaswamy, H.S. and van de Voort, F.R. (1993) "Comparison of microwave, conventional and combination heat treatments on wheat germ lipase activity," International Journal of Food Science and Technology, Vol. 28, 617-623

Kimura, K. Ida, M. Yosida, Y. Ohiki, K. Fukumoto, T. and Sakui, N. (1994). "Comparison of Keeping Quality between Pressure-processed Jam and Heat-processed Jam: Changes in Flavor Components, Hue, and Nutrients during Storage," J. of Bioscience, Biotechnology, and Biochemistry, Vol. 58, No. 8, 1386-1391

Krause, G.H. (1994). "The role of oxygen in photoinhibition of photosynthesis" in Causes of Photooxidative Stress and Amelioraton of Defense Systems in Plants. CRC Press, Boca Raton, pg 43-76

Landbo, A.K. and Meyer, A.S. (2001). "Enzyme-assisted extraction of antioxidative phenols from black currant juice press residues (Ribes nigrum)",J Agric Food Chem. Vol. 49, No. 7, 3169-3177

Laplaud, P.M. Lelubre, A. and Chapman, M.J. (1997). "Antioxidant action of Vaccinium myrtillus extract on human low density lipoproteins in vitro: initial observations." Fundamental Clinical Pharmacol. Vol. 11, No. 1, 35-40

Lee, J. Durst, R.W., and Wrolstad, R.E. (2002). "Impact of Juice Processing on Blueberry Anthocyanins and Polyphenolics: Comparison of Two Pretreatments," J. of Food Science, Vol. 67 No. 5, 1660-1667

Makus, D.J. and Ballinger, W.E. (1973). "Characterization of Anthocyanins During Ripening of Fruit of Vaccinium corymbosum L." J. Amer. Soc. Hort. Sci., Vol. 98, 99-101

Markakis, P. (1982). "Anthocyanins as food additives," Anthocyanins as Food Colors. Academic Press, Inc., New York, 245-253

Mazza, G. and Oomah, B.D. (2000) Functional Foods & Nutraceuticals Seris: Herbs, Botanicals & Teas," Technomic Publishing Company, Inc. Lancaster

Mazza, G. (1997). "Anthocyanins in edible plant parts: a qualitative and quantitative assessment." in Antioxidant Methodology: In vivo and In Vitro Concepts. AOCS Champaign, IL. Chapter 8, 119-140

Mazza, G. and Miniati, E. (1993). "Anthocyanins in Fruits, Vegetables, and Grains." CRC Press Inc. Boca Ratan, FL. Pgs 85-130

McCord, J.M. and Fridovich, I. (1969). "Superoxide Dismutase: an Enzymic Function for Erythrocuprein (Hemocuprein)." J. Biol. Chem. Vol. 244, 6049-6055

Mehlhorn, H. (1990). "Ethylene-promoted ascorbate peroxidase activity protects plants against hydrogen peroxide, ozone, and paraqua." Plant Cell Environ., Vol. 13, 971-976

Moore, J. N. (1994). "The blueberry industry of North America," Horticulture Technology, Vol. 4, No. 2, 96-102

Neubeck, W. and Seidel, D. (1975). "Direct method for measuring lipoprotein-X in serum." Clin. Chem. Vol. 21, No. 7, 853-856

Ofek, I. Goldhar, and Sharon, N. (1996). "Anti-Eschericia coli adhesion activity of cranberry and blueberry juices." In Toward Anti-Adhesion Therapy for Microbial Diseases. Kahane and Ofek editors. Plenum Press, N.Y. pg 179-183

Ofek, I. Goldhar, D. Zafriri D. Lis, H. Adar, R. and Sharon, N. (1991). "Anti-*Escherichia coli* adhesion activity of cranberry and blueberry juices," New England J. of Medicine, Vol. 324, No. 22, 1599

Ou, B. Hampsch-Woodill, M. and Prior, R.L. (2001). "Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe," J. of Agricultural and Food Chemistry, Vol. 49, 4619-4626

Petri, G. Krawczyk, U. and Kery, A. (1997). "Spectrophotometric and chromatographic investigation of bilberry anthocyanins for quantification purposes." Microchem. J. Vol. 55, No. 1, 12-23

Price, A.H. and Hendry, G.A.F. (1991). "Iron-catalysed oxygen radical formation and its possible contribution to drought damage in nine native grasses and three cereals." Plant Cell Environ. Vol. 14, 477-484

Prieur, C. Rigaud, J. Cheynier, V. and Moutounet, M. (1994)."Oligomeric and polymeric procyanidins from grape seeds", J. Phytochemistry, Vol. 36, No. 3, 781-784

Prior, R.L. Cao, G. Martin, A. Sofic, E. McEwen, J. O'Brien, C. Lischner, N. Ehlenfeldt, M. Kalt, W. Krewer, G. and Mainland, M.C. (1998). "Antioxidant Capacity As Influenced by Total Phenolic and Anthocyanin Content, Maturity, and Variety of Caccinium Species," J. of Agricultural and Food Chemistry, Vol. 46, 2686-2693

Renaud, S. and de Lorgeril, M. (1992) "Wine, alcohol, platelets, and the French paradox for coronary heart disease", Lancet. Vol. 20, No. 339, 1523-1526.

Revilla, I. Gonzalez-SanJose, M.L. (2002). "Multivariate evaluation of changes induced in red wine characteristics by the use of extracting agents." J Agric Food Chem, Vol. 50, No. 16, 4525-30

Rice-Evans, C.A. and Burdon, R.H. (1994). Free Radical Damage and its Control. Elsevier, New York U.S.

Rimm, E.B. Stampfer, M.J. Ascherio, A. et al. (1993). "Vitamin E consumption and the risk of coronary heart disease in men." New England J. of Medicine, Vol. 328, 1450-1456

Rodriguez-Lopez, J.N. Fenoll, L.G. Tudela, J. Devece, C. Sanches-Hernandez, D. de los Reyes, E. and Garcia-Canovas, F. (1999). "Thermal Inactivation of Mushroom Polyphenoloxidase Employing 2450 MHz Microwave Radiation," J. of Agricultural and Food Chemistry, Vol. 47, 3028-3035

Rodriguez-Saona, L.E. and Wrolstad, R.E. (2001). Current Protocols in Food Analytical Chemistry: Extraction, Isolation, and Purification of Anthocyanins. John Wiley and Sons, U.S.

Sapers, G.M. Burgher, A.M. Phillips, J.G. Jones, S.B. and Stone, E.G. (1984). "Color and Composition of Highbush Blueberry Cultivars." J. Amer. Soc. Hort. Sci., Vol. 109, 105-111

Sapers, G.M. Jones, S.B. and Phillips, J.G. (1985). "Leakage of anthocyanins from skin of thawed, frozen highbush bluberres (*Vaccinium corymbosum L.*)," J. of Food Science, Vol. 50, 432-436

Sarni-Manchado, P. Cheynier, V. and Moutounet, M. (1997). "Reactions of Polyphenoloxidase Generated Caftaric Acid o-Quinone with Malvidin 3-o-Glucoside," J. of Phytochemistry, Vol. 45, No. 7, 1635-1369

Sato, M. Suzuki, Y. Yanai, T. Ikoma, G. Takamatsu, H. and Hanamure, K. (2001) "Composition of Anthocyanins and the Physiological Effects of Ingestion on Blueberry Wines," Am. J. of Enol. Vitic. Vol. 52, No. 1, 58-59

Satue-Garcia, M.T. Heinonen, M. and Frankel, E.N. (1997). "Anthocyanins as antioxidants on human low-density lipoprotein and lecithin-liposome systems." J. of Agricultural and Food Chemistry, Vol. 45, No. 9, 3362-3367

Scalbert, A. and Williamson, G. (2000). "Dietary intake and bioavailability of polyphenols." J. of Nutrition, Vol. 130 (8 Supplemental), 2073S-2085S

Seeram, N.P. Bourquin, L.D. and Nair, M.G. (2001). "Degradation Products of Cyanidin Glycosides from Tart Cherries and Their Bioactivities," J. of Agricultural and Food Chemistry, Vol 49, 4324-4929

Seib, P.A. and Tolbert, B.M. (1982). Advances in Chemistry Series Vol. 200, pg 366, American Chemical Society, Washington D.C.

Siddiq, M. Arnold, J.F. Dinha, N.K. and Cash, J.N. (1994). "Effect of Polyphenol Oxidase and its Inhibitors on Anthocyanin Changes in Plum Juice," J. of Food Processing and Preservation, Vol. 18, 75-84

Singleton, V.L. and Rossi, J.A. (1965). "Colormetry of total phenolics with phosphomolybdicphosphotungstic acid reagents." Am. J. of Enol. Vitic. Vol. 16, 144-158

Stampfer, M.J. Hennekins, C.H. Manson, J.E. et al. (1993). "Vitamine E consumption and the risk of coronary disease in women." New England J. of Medicine, Vol. 328, 1444-1449

Tanchev, S.S. and Yoncheva, N. (1974). Kinetics of Thermal Degradation of the Anthocyanins Delphinidin-3-rutinoside and Malvidin-3-glucoside," Die Nahrung, Vol. 18, No. 8, 747-752

Tanchev, S. and Ioncheva, N. (1974). "Products of Thermal Degradtion of the anthocyanins Cyanidin-3-glucoside, Cyanidin-3-rutinoside, and Cyanidin-3-sophoroside'" Die Nahrung, Vol. 20, No. 10, 889-893

Teramura, A.H. and Sullivan, J.H. (1994). "Effects of UV-B radiation on photosynthesis and growth of terrestrial plants." Photosynthesis Res., Vol. 39, 463-473

Varma, S.D. (1986). "Inhibition of aldose reductase by flavonoids: possible attenuation of diabetic complications." in Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure-Activity Relationships. Alan R. Liss, Inc. pg. 343-358

Villatta, M. (1998). "Cultivated blueberries: a true-blue baking ingredient." Cereal Foods World, Vol. 43, No. 3, 128-130

Wang, S.Y. Zheng, W. and Galletta, G.J. (2002). "Cultural System Affects Fruit Quality and Antioxidant Capacity in Strawberries," J. of Agricultural and Food Chemistry, Vol. 50, 6534-6542

Watt, B.K. and Merrill, A.L. (1963). Composition of Foods. U.S. Department of Agriculture Handbook, Vol. 8.

Wesche Ebeling, P. and Montgomery, M.W. (1990). "Strawberry polyphenoloxidase: its role in anthocyanin degradation." Journal of Food Science, Vol. 55, No. 3, 731-734, 745

Wise, R.R. and Naylor, A.W. (1987). "Chilling enhanced peroxidation: evidence for the role of singlet oxygen and superoxide in the breakdown of pigments and endogenous antioxidants." Plant Physiology, Vol. 83, 278-282

Wrolstad, R.E. (2000). "Anthocyanins", Chp.11, pp 237-252, In, Natural Food Colorants. F. J. Francis and G. J. Lauro editors,. Marcel Dekker, Inc., NY.

Yokotsuka, K. and Singleton, V.L. (1997). "Disappearance of anthocyanins as grapejuice is prepared and oxidized with PPO and PPO substrates", Am. J. of Enol. Vitic Vol. 48, 13-25

Zabetakis, I. Leclerc, D. and Kajda, P. (2000). "The Effect of High Hydrostatic Pressure on the Strawberry Anthocyanins," J. of Agricultural and Food Chemistry, Vol. 48, 2749-2754

Zhang, H.L. Bushway, A. Work, T. Camire, M.E. and Work, R. (1997). "Prevention of anthocyanin leakage of individually quick frozen (IQF) lowbush blueberries in blueberry muffins," Acta Horticulture, Vol. 446, 211-217