DETERMINATION OF POLYPHENOL OXIDASE (PPO) ACTIVITY, ANTHOCYANIN CONTENTS AND THE PHYTONUTRIENT CHANGES IN BLUEBERRY JUICE AS INFLUENCED BY DIFFERENT PROCESSING METHODS

By

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Inhibition of blueberry PPO activity by sodium benzoate, potassium sorbate and potassium metabisulfite and their influence on degradation of individual anthocyanins in an extract was studied. Maceration of blueberries was carried out at 55ºC for 1h with the addition of 0.1% sodium benzoate or with blanching pretreatment at 90ºC for 1min. After maceration pretreatments the extracted juice was processed with traditional hot fill pasteurization, high hydrostatic pressure (HHP) and pulsed electric field (PEF).

Sodium benzoate and potassium metabisulfite were very effective PPO inhibitors in concentrations of 0.1% and 10ppm, respectively. Potassium sorbate was the weakest inhibitor, with 50% PPO remaining.

Degradation of anthocyanins by PPO was dependent on their structure. Tri-phenolic anthocyanins experienced the most degradation, followed by diphenolic and monophenolic compounds, respectively. Sodium benzoate was the most effective at

preventing anthocyanin degradation; potassium metabisulfite did not have any protective effect, while potassium sorbate increased anthocyanin degradation

Blanching of blueberries inactivated native PPO, but also increased the degradation of anthocyanins, especially malvidin glycosides. Addition of 0.1% sodium benzoate decreased PPO activity when compared to frozen blueberries but not in respect to control maceration.

Only 12% of anthocyanins and 33-41% of phenolics were extracted into juice from the frozen fruit. Hot fill pasteurization, high hydrostatic pressure and pulsed electric field did not significantly influence anthocyanins, phenolics and antioxidant activity in blueberry juice.

DEDICATION

I would like to dedicate this research to my late cousin Natasa Vojnovic-Dakovic

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CHAPTER I

INTRODUCTION

Polyphenol oxidase (PPO) is a generic term for a group of enzymes that catalyzes the oxidation of phenolics compounds leading to the development of browning (Kader and others 1997b). The extent to which naturally occurring phenolic substrates contribute to enzymatic browning of individual fruits and vegetables depends on the localization and concentration of the phenols as well as on the color intensity of the macromolecular pigments obtained from the different quinines (Vamos-Vigyazo 1981).

O-quinones, the primary products of the oxidative reaction catalyzed by the enzyme, (a) react with each other to form high molecular weight polymers, (b) form molecular complexes with amino acids or proteins, and anthocyanins, and (c) oxidize compounds that have lower oxidation-reduction potential (Mathew and Parpia 1971). These subsequent reactions may bring about changes in physical, chemical, nutritional and sensory characteristics of food products (Mayer and Harel 1979).

Browning may be prevented not only by inactivating the enzyme, but also by eliminating one of the two substrates necessary for the reaction (Phenols and O_2), or by reacting with the products of enzyme action to inhibit the formation of the colored compounds produced in secondary, non-enzymatic reaction steps (Vamos-Vigyazo 1981).

Many compounds have been identified as possible PPO and browning inhibitors but most of them are not allowed for the use in food. The use of browning inhibitors in food processing is restricted by special requirements such as non-toxicity, wholesomeness, effect on taste, flavor, texture, etc (Vamos-Vigyazo 1981).

Carboxylic acids are shown to be strong inhibitors of PPO (Pifferi and others 1974; Walker and Wilson 1975; Walker and McCallion 1980; Gunata and others 1987; Ferrar and Walker 1996). Benzoic acid and some derivatives of cinnamic acid were found to be mostly competitive inhibitors of PPO (Pifferi and others 1974).

Sulfites are arguably the most versatile food additives available. They act not only as a food preservative but have an important role as inhibitors of enzymatic and non-enzymatic browning as inhibitors of a wide range of enzymes including proteases, oxidases, peroxidases, and are alleged to prevent oxidative spoilage (Wedzicha 1992). Sulfites are highly effective in controlling browning but are subject to regulatory restrictions because of adverse effect on health (Sapers 1993). Sulfites can act as PPO inhibitors and can also react with intermediates to prevent pigment formation (Sayavedra-Soto and Montgomery 1986)

Anthocyanins are the most important water-soluble pigments in plant tissues, and produce blue, red and purple colors. Color of anthocyanins-containing media depends on structure and concentration of the pigment, pH, temperature, presence of copigments, metallic ions, enzymes, oxygen, ascorbic acid, sugars and their degradation products, sulfur dioxide, actual anthocyanins concentration and other factors (Mazza and Miniati 1993). There is experimental evidence that certain anthocyanins and flavonoids have anti-inflammatory properties, and there are reports

that orally administrated anthocyanins are beneficial for treating diabetes, ulcers and may have antiviral and antimicrobial activities and can also provide protection against UV radiation (Mazza and Miniati 1993). Anthocyanins, as a functional food component, can aid in the prevention of obesity and diabetes (Tsuda and others 2003). It is well known that anthocyanins are poor substrates of PPO, and this is probably due to the presences of the sugar moiety causing steric hindrance, since the aglucon forms are often oxidized by PPO (Mathew and Parpia 1971).

Blueberries are a very rich source of anthocyanins and other phenolics. Enzymatic oxidation of phenolics compounds present in blueberries by PPO can occur during processing as soon the berries are damaged. The first reaction step that occurs is oxidation of phenolics substrates to their o-quinones. Quinones are very reactive species which are able, as oxidants, to oxidize other substrates, being reduced in the process to the original phenol, and as electrophiles to react with various nucleophiles (Sarni and others 1995). These reactions of o-quinones, namely oxidation and condensation reactions can lead to discoloration and can involve other phenolics compounds especially anthocyanins. The use of model systems has shown that the enzymatically generated o-quinines play an essential role in the process of anthocyanin degradation (Kader and others 1999a). Degradation of anthocyanins can be inhibited by direct inhibition of PPO and with that formation of o-quinines or by the inhibiting anthocyanins from reacting with already generated o-quinones. Maintaining high antioxidant activity with preserving anthocyanins and other phenolics is of crucial importance for any blueberry processed product. Inhibition of PPO during processing, thus preventing oxidation of phenolics and degradation of

anthocyanins should be a preliminary step in any blueberry processing. Since anthocyanins are heat sensitive use of inhibitors could be the best solution for PPO inhibition without the degradation of anthocyanins.

Health attributes ascribed to blueberries create a need for the development of processed blueberry products that are convenient for the consumer. Blueberries can be pressed into juice and juice concentrate, in order to reach a more wide spread consumption (Rossi and others 2003). Desirable color is a very important sensory characteristic for the consumer of fruits and processed fruit products (Garzon and Wrolstad 2002). Huge economic losses can also be accrued by the company due to the loss of attractive product color. A concord grape juice producer reported about $ 700,000 in losses due to the loss of juice color (Wrolstad and others 1994). The stability of color in blueberries is an important aspect of quality control during processing and storage (Yang and Yang 1987). Not just because they impart color but also because of their possible beneficial effect, particular attention has to be paid to the changes that anthocyanin pigments undergo during processing.

Anthocyanins are not as efficiently extracted in the pressing operation as sugars, acids and other water solubles, which can have a negative impact on juice quality (Skrede and others 2000; Lee and others 2002). The anthocyanins that give color to blueberries exist almost exclusively in the skin, whereas phenolics and other antioxidants are mostly in the flesh (Lee and Wrolstad 2004). Since anthocyanins in blueberries are only found in the skin, skin breaking and maceration are needed to extract color and obtain good quality juice. Lee and others (2002) concluded that heating of berries may have contributed to a breakdown of the skins, increasing

extraction of the color, but still with a substantial amount of anthocyanins left in the press cake. Cold processing of blueberries was not as efficient at transferring antioxidants or phenols to the final juice as hot processing (Carlson 2003).

During juice processing, anthocyanins can be lost due to enzymatic break-down, heat treatment and some are removed with the pulp (Iversen 1999). The primary steps of juice processing (thawing, crushing, depectinization and pressing) contribute to a large loss in total anthocyanins (Skrede and others 2000; Lee and others 2002). The studies listed above reported that substantial losses of anthocyanins and other polyphenolics occurred when blueberries were processed into juice, and in addition different classes of compounds had varying susceptibility to degradation with different processing operations, and the highest losses occurred during milling and depectinization due to the action of native polyphenoloxidase, PPO (Skrede and others 2000).

Processing or any wounding of fruits and vegetables may cause cell disruption that can lead to quinone formation due to the action of PPO leading to product deterioration. Not only the appearance of food and beverages may be affected, but also their taste and nutritional value, often decreasing the quality of the final products (Gandia-Herrero and others 2005). Degradation of anthocyanins occurs when active PPO and its substrate are present in the system. Although anthocyanins are not direct substrates for PPO, anthocyanins with a o-diphenolic B ring were oxidized via enzymatically generated o-quinines of catechol, catechin, chlorogenic acid (Peng and Markakis 1963; Pifferi and Cultrera 1974; Raynal and Moutounet 1989; Wesche-Ebeling and Montgomery 1990b) and caftaric acid (Cheynier and others 1994). It has

been reported that heat inactivation of PPO is unacceptable in anthocyanin-containing juice products, since the high temperature required for PPO inactivation can cause anthocyanin degradation (Siddiq and others 1992).

Processing and storage can also have marked effects on the phenolics' content of fruits that might also affect their health promoting properties (Zafrilla and others 2001). As a general rule, during processing, phenolic biosynthesis is interrupted by the enzyme's destruction and/or cell structure degradation (Tomas-Barberan and Espin 2001a). Processing can enhance phenolic compound degradation (chemically or enzymatically if the oxidative enzymes have not been inactivated) or can produce chemical changes that affect quality characteristics (Tomas-Barberan and Espin 2001a).

Blanching or addition of PPO inhibitors can have a positive effect on juice quality. Blanching of strawberries before juice and concentrate production had a protective effect on anthocyanin pigments, leucoanthocyanins, flavanols, total phenolics and ascorbic acid, and also resulted in improved color stability (Wrolstad and others 1980). Chemical treatments like SO_2, citric acid and $SnCl_2$ had a stabilizing effect on strawberry anthocyanins during processing of strawberry jam (Sistrunk and others 1982). Heat and SO_2 pretreatment before pressing the juice increased recovery of anthocyanins in all processing steps (Lee and others 2002).

There is a demand from consumers and retailers for minimum processed foods that are also safe. Therefore, producers and researchers are searching for alternative food processing methods to gently preserve foods (Houska and others 2006).

Although thermal preservation methods provide safe foods, there is loss of food quality, and nutritional value that is associated with this processing method. Hence, the main objective of low- and non-thermal preservation methods is to minimize the degradation of food quality through limiting heat damage (Senorans and others 2003). Compared with thermal processing, nonthermal methods provide a high retention of flavors and nutrients, giving products a "natural/fresh" taste (Zhong and others 2005).

High voltage pulsed electric fields (PEF) is a promising non thermal processing technology. Treating liquid foods with PEF may inactivate microorganisms and enzymes with only small increases in temperature, simultaneously providing consumers with safe, nutritious, and fresh foods (Aguilar-Rosas and others 2007). Consumer demand for a higher quality, fresh appearing and safe food supply is the ultimate catalyst for the emergence of PEF on a commercial scale (Yeom and others 2002)

Cranberry juice treated by PEF had a similar flavor or aroma profile as the nontreated juice; whereas thermal treatment significantly altered the overall flavor profile of the juice (Jin and Zhang 1999). No differences were observed in the content of anthocyanin pigments between PEF-treated samples and controls. However, thermal treatment significantly reduced their anthocyanin content

High hydrostatic pressure (HHP) is considered a nonthermal technology with the most promising perspective of industrial utilization. There are already known industrial applications in Japan, USA, France and Spain (Houska and others 2006). This technology can enable processors to produce innovative foods with fresh-like, natural-like attributes and natural looking colors which are all aspects that are valued

by consumers (Deliza 2005). In addition information on the benefits that are offered by high pressure technology presented on the juice package had a positive contribution on purchase intention (Deliza 2005). High pressure treatment is expected to be less detrimental than thermal processing to low molecular weight food compounds such as flavoring agents, pigments, vitamins, etc., since covalent bonds are not affected by pressure (Butz and others 2002). The pressure stability of antioxidants is of interest since they play an important role in reducing the risk of free radical-related oxidative damage associated with a number of diseases (Butz and others 2002).

High hydrostatic pressure is a promising alternative to traditional thermal processing techniques of food preservation, but associated changes to a diversity of phytonutrients have not been extensively investigated (Talcott 2003). Making comparisons between HHP and thermal processes is one way to assess its benefits by monitoring destruction of heat labile compounds (Talcott 2003).

The need to preserve valuable phytonutrients in blueberries with minimum impact on the quality and taste in the final product leads to the need for this type of study. The objectives of this study were:

1. To determine inhibition of polyphenol oxidase (PPO) by preservatives and their influence on anthocyanins in a blueberry extract and
2. To determine influence of blueberry mash pretreatment and various processes on anthocyanins, phenolics and antioxidant activity of blueberry juice.

CHAPTER II

LITERATURE REVIEW

Polyphenol oxidase

Polyphenol oxidase (PPO) is a generic term for the group of enzymes that catalyze the oxidation of phenolic compounds that cause browning. Based on substrate specificity there are: Monophenol monoxygenase, cresolase or tyrosinase (EC 1.14.18.1); Diphenol oxidase or catechol oxidase (EC 1.10.3.2); and laccase or p-diphenol oxygen oxidoreductase (EC 1.10.3.1) (Shahidi and Naczk 1995). Numerous reports on enzymes from various sources suggests that there is a copper content of one atom per polypeptide chain or subunit of catechol oxidase (Mayer and Harel 1979). The structure of the active site of the enzyme, in which copper is bound by six or seven histidine residues and a single cysteine residue is highly conserved (Mayer 2006).

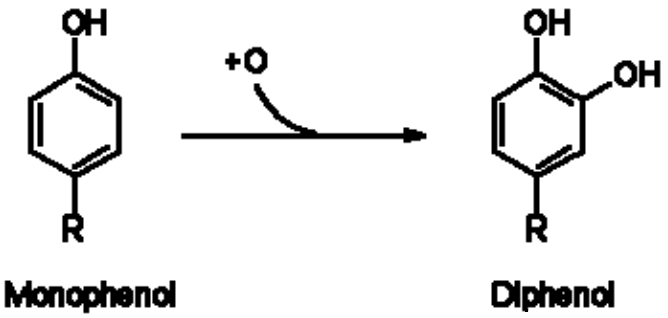
A variety of phenolic compounds car serve as PPO substrates. The compartmentation of the phenolics substrate of the enzyme, both in special cells and within the cell have been reported which results in the separation between the enzyme and the bulk of its phenolics substrates *in situ* (Mayer and Harel 1979).

The most important natural substrates of PPO in fruits and vegetables are catechins, cinnamic acid esters (chlorogenic acid), 3,4-dihydroxy phenylalanine

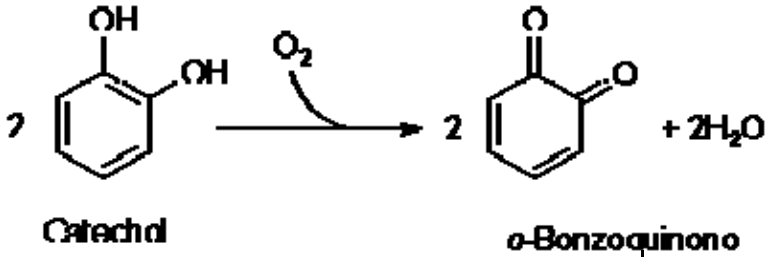
(DOPA), and tyrosine (Vamos-Vigyazo 1981). The sphere of naturally occurring substrates of PPO is limited by the fact that the enzyme does not act on glycosides (Baruah and Swain 1959), and most of the phenolic in plants are present in that form. Negative or positive charges near the phenolic ring, disturb the catalytic process resulting in low activity (Casado-Vela and others 2005). Affinity of the PPO toward substrate is highly dependent on the plant source from which the enzyme is extracted. The crude enzyme extract from concord grapes had activity toward all of the dihydroxyphenols but no activity with monophenols (Cash and others 1976). Although gallic and chlorogenic acid were of dihydroxy configuration, the crude grape enzyme extract did not effectively utilize either of these compounds as its substrate (Cash and others 1976) while the chlorogenic acid was the best substrate for blueberry PPO (Kader and others 1997b). The extent to which naturally occurring phenolic substrates contribute to the enzymatic browning of individual fruits and vegetables depends on the localization and concentration of the phenols as well as on the color intensity of the macromolecular pigments that are obtained from the different quinines (Vamos-Vigyazo 1981).

Polyphenol oxidase catalyzes two basic reactions (Shahidi and Naczk 1995):

1. hydroxylation to the *o*-position adjacent to an existing hydroxyl group of the phenolic substrate (monophenol oxidase activity).



2. and oxidation of diphenol to *o*-benzoquinones (diphenol oxidase activity).



Both reactions utilize molecular oxygen as a co-substrate. Whether a single enzyme system exhibits both mono- and di phenol oxidase activities is still unclear. However, when both monophenol and diphenol oxidases are present in plants, the ratio of monophenol to diphenol oxidase activity is usually 1:10 or as low as 1:40 (Nicolas and Potus 1994).

The primary products of the oxidative reaction catalyzed by the enzyme, the o-quinones, (a) react with each other to form high molecular weight polymers, (b) form molecular complexes with amino acids or proteins, and anthocyanins, and (c) oxidize compounds of lower oxidation-reduction potential (Mathew and Parpia 1971). Non-enzymatic reactions (a) and (b) lead to the formation of brown pigments, the

color which is darker the higher their molecular mass (Vamos-Vigyazo 1981). These subsequent reactions may bring about changes in physical, chemical, nutritional and sensory characteristics of food products (Mayer and Harel 1979). The rate of enzymatic browning reactions depends on the nature and content of phenolic compounds, activity of phenoloxidase present in food, presence of oxygen reducing substances and metal ions, pH and temperature (Shahidi and Naczk 1995).

Products of reaction of type (c) are colorless (Vamos-Vigyazo 1981). In the reaction that belong to group (c), quinines formed by the reaction of PPO, oxidize compounds of lower oxidation-reduction potentials and are again reduced to dihydroxyphenols which provides "fresh" substrate for PPO. This reaction can continue until the enzyme gets inactivated by the reaction products, or compounds of lower oxidation-reduction potential are depleted (Vamos-Vigyazo 1981).

PPO can be found in both soluble and membrane-bound forms in chloroplast, mitochondria, microsomes, peroxisomes and cytoplasm (Tomas-Barberan and Espin 2001b). Serradell and others (2000) concluded that strawberry PPO is associated with the membranes since it was released from the fruit using detergent (Triton X-100) and NaCl. The strength of binding of PPO to the membrane appears to be dependent on the tissue and the stage of development (Mayer and Harel 1979). Solubilization of PPO occurs during ripening or aging of fruits (Mayer and Harel 1979). It was strongly suggested that apple PPO is solubilized and denatured at the later stage of development, but with enough remaining to cause browning (Murata and others 1995). Serradelll and others (2000) reported that PPO is more abundant in immature strawberry fruit than in the ripe one.

One of the oldest suggestions of the physiological role of PPO is that of of o-diphenol synthesis. This suggestion is based on the ability of many catechol-oxidase preparations to oxidase monophenols to the corresponding o-quinones (Mayer and Harel 1979). Changes in the catecholase activity during maturation are related to the formation of anthocyanins (Sanchez-Ferrer and others 1989). PPO has also been reported to be a defensive protein against pathogen attack in various crops (Tomas-Barberan and Espin 2001b).

As with any other enzyme activity, PPO is highly dependent on pH and temperature. The optimum pH of PPO activity varies with the source of the enzyme and with the substrate in a relatively wide range but in most cases is between 4.0 and 7.0 (Vamos-Vigyazo 1981). Optimum activity of the crude enzyme extract occurred at pH 5.9-6.3 while over 50% of its maximum activity was retained at 3.4, the normal pH of the grape juice (Cash and others 1976). Polyphenol oxidase from strawberries showed maximum activity at a pH of 5.3 (Serradell and others 2000).

PPO is not an extremely heat-stable enzyme. Short exposures, in the tissue and solutions, to temperatures of 70 to 90ºC are, in most cases, sufficient for partial or total irreversible destruction of its catalytic function (Vamos-Vigyazo 1981). Most PPO enzymes show optimum activity at temperatures between 30 and 40ºC (Lamikanra and others 1992). Strawberry polyphenol oxidase exhibited maximum activity at 50ºC and than decreased sharply at higher temperatures although it showed considerable activity (15-20%) at extreme temperatures of 0 to 70ºC. These authors also reported that the enzyme retained 71 and 43% of its activity after incubation at 55ºC for 30 and 120 min respectively, while temperatures of 65ºC caused an almost

complete loss of activity even after 30 min. PPO from concord grapes showed a maximum activity between 25 and 30ºC, after which the reaction rate declined very rapidly as temperature increased (Cash and others 1976). Enzyme from Emir grape extract was heat stable at 65ºC, retaining up to 73% of activity after 30 min of heating with a D value of 213 min (Unal and Sener 2006). Activity of apple PPO increased with mild heating and reached a maximum, and as heating progressed, activity decreased, first gradually and then rapidly (Yemenicioglu and others 1997). This increased activity by heat was explained by the activation of latent PPO. The activation of latent PPO can be attributed to protein association and dissociation (Mathew and Parpia 1971).

Blueberry PPO

Kader and others (1997b) used native PAGE to demonstrate that blueberry PPO presents two isoenzymes PPO1 and PPO2. Although they found that caffeic acid as a substrate would provide highest PPO activity, absence of caffeic acid in highbush blueberries would make chlorogenic acid a better substrate for assessing PPO activity in blueberries since it is the major hydroxycinnamic derivative. Oxidation of chlorogenic acid by purified blueberry PPO produced degradation of only 50%, whereas 70% oxidation was obtained when nonpurified PPO was used (Kader and others 1998). These researchers concluded that PPO in the nonpurified extract is probably protected by non-PPO proteins which react with the oxidized polyphenols, thereby sparing the PPO.

The optimum activity of the enzyme was a pH of 4 with the shoulder at pH 5 (Kader and others 1997b). At pH 5, about 80% of the maximum activity remained with the rapid decrease in activity with an increase in pH to 5.5 (Kader and others 1997b). This behavior was explained by the presence of two isoforms. Blueberry PPO was found to be very active at low pH's that are similar to the pH of fruits (3-3.2) (Kader and others 1994).

Inhibition of PPO activity

Browning may be prevented by inactivating the enzyme, by eliminating one of the two substrates necessary for the reaction (Phenols and O_2), or by reacting with the products of enzyme action to inhibit the formation of the colored compounds that are produced in secondary, non-enzymatic reaction steps (Vamos-Vigyazo 1981). In many cases, it is not easy to distinguish the different mechanisms that underlay the action of a browning inhibitor; moreover, some inhibitors act simultaneously on the enzyme and the substrate or products (Vamos-Vigyazo 1981). In general PPO inhibitors can be divided into two groups; those that act primarily on the enzyme and the those that react with the reaction products on the substrate. The use of browning inhibitors in food processing is restricted by special requirements such as non-toxicity, wholesomeness, effect on taste, flavor, texture, etc (Vamos-Vigyazo 1981).

Carboxylic acids are strong inhibitors of PPO (Pifferi and others 1974; Walker and Wilson 1975; Walker and McCallion 1980; Gunata and others 1987; Ferrar and Walker 1996). Benzoic acid (Figure 1) and some derivatives of cinnamic acid (Figure 2) are mostly competitive inhibitors of PPO (Pifferi and others 1974). The

only efficient structural requirement for the inhibitory effect is the association of the carboxyl group with the benzene nucleus or with an unsaturated open chain (Pifferi and others 1974). It was shown that PPO was inhibited by cinnamic and p-coumaric acdis (Walker and Wilson 1975; Walker and McCallion 1980; Gunata and others 1987), benzoic acid (Gunata and others 1987) and to a lesser extent, by ferulic acid (Walker and Wilson 1975; Walker and McCallion 1980).

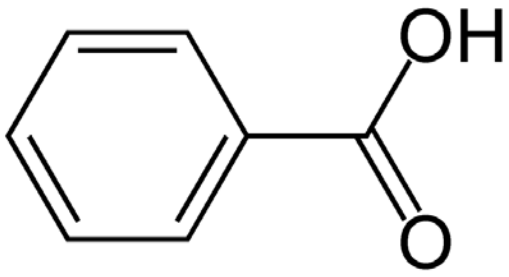


Figure 1. Structure of benzoic acid

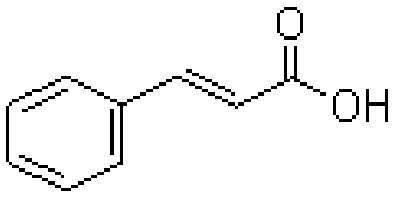


Figure 2. Structure of cinnamic acid.

Hydroxylation, methylation of the benzene ring of these acids and the esterification of the carboxyl group of benzoic and cinnamic acids leads to a considerable decrease in their inhibitory strength (Gunata and others 1987). It has been suggested that for strong inhibition, aromatic acid inhibitors require a free carboxylic group to be substituted directly on the benzene ring (Vamos-Vigyazo 1981). Benzoic acid was found to be a competitive inhibitor when 4-methylcatechol and (+)-catechin were used as substrates and non-competitive with caffeic acid (Gunata and others 1987). Janovitz-Klapp and others (1990) also concluded that benzoic acid is competitive with 4-methyl catechol since it does not affect the apparent Vm and increases the apparent Km. In the system with benzoic acid, phenolic substrate (4-methyl catechol) and oxygen are present, oxygen is the first substrate to be bound to apple PPO, forming an E-O_2 complex to which the benzoic acid as an inhibitor can bind only with enzymatic forms of PPO which are free of other phenolics i.e., E or E-O_2 (Janovitz-Klapp and others 1990b). Some PPO inhibitors require protonation of the acidic group (i.e., low pH) in order to display inhibition (Pifferi and others 1974; Janovitz-Klapp and others 1990a) that should be taken into account when investigating inhibition (Ferrar and Walker 1996). The inhibition of mushroom PPO by benzoic acid and by cyanide, respectively, showed the former to be competitive with catechol (substrate) and non competitive with O_2, while the later inhibitor showed a reversed behavior. This was interpreted as the result of the existence of two distinct substrate binding sites on the enzyme molecule, one of which had a high affinity for aromatic compounds, including phenolic substrates, while the other, which probably contained the enzyme copper, served for

metal-binding agents and oxygen (Duckworth and Coleman 1970). Benzoic acid was found to be a mixed-type inhibitor of gum arabic PPO showing the strongest inhibition among all carboxylic acids tested at a pH of 5.3 (Billaud and others 1996).

The inhibitors of enzymatic browning that react with the reaction products or substrates, can be divided into following groups (Vamos-Vigyazo 1981).

1. Reducing agents acting on the formed quinines by restituting the o-dihydroxy phenols. These compounds are consumed in the process of inhibition, and thus provide only temporary protection against discoloration, unless used in high concentration, in which case reaction inactivation of the enzyme might occur prior to the depletion of reducing agent. Some frequently used representatives of this group are ascorbic acid, SO_2, potassium metabisulfite, 2-mercaptoethanol, and thioglycollate.
2. Quinone couplers forming stable colorless compounds with quinones, thus providing permanent protection as long as they are not entirely consumed. Cysteine, glutathione, benzensulphinic acid, DIECA, Na-ethyl xanthate , among others, are able to perform such reactions.

Sulfites are arguably the most versatile food additives available. They act not only as a food preservative but have an important role as inhibitors of enzymatic and non-enzymatic browning, inhibitors of a wide range of enzymes including proteases, oxidases, peroxidases, and are alleged to prevent oxidative spoilage (Wedzicha 1992). Sulfites are highly effective at controlling browning but are subject to regulatory restrictions because of adverse effect on health (Sapers 1993). The main reason for

the reactivity of sulfites in foods is the high nucleophilicity of the sulfite ion (Wedzicha 1992)

Sulfites have a very complex action on the PPO-polyphenol system(Vamos-Vigyazo 1981). Sulfites can act as PPO inhibitors and can also react with intermediates to prevent pigment formation (Sayavedra-Soto and Montgomery 1986). Sulfites can inhibit browning by combining irreversibly with the quinones to form colorless addition products, at the same time reducing the activity of the enzyme towards both mono-and dihyroxy phenols (Embs and Markakis 1965 and Markakis and Embs 1966). Inhibition of browning involves nucleophilic attack by the sulfite ion on formed quinone by PPO reaction to form sulfonate, where quinone is reduced and sulfonate is unreactive towards PPO (Wedzicha and others 1991). Inhibition of browning is permanent if all the substrate (quinone) is converted to sulfonate (Wedzicha and others 1991). Some authors have reported that sulfite has direct effect on enzyme (Golan-Goldhirsh and Whitaker 1984; Sayavedra-Soto and Montgomery 1986; Valero and others 1992). The product (quinone) accumulation curve in the presence of metabisulfite includes initial an lag period due to the chemical reaction between the quinone products of the enzyme catalysis and metabisulfite and a steady state phase, where all of the inhibitor has been depleted. During the steady state phase the catalytic activity that is expressed is lower than in the absence of metabisulfite, which is probably an indication of the direct effect of metabisulfite on PPO (Valero and others 1992). It is assumed that the inactivation of the oxy form of PPO involves the formation of an enzyme-sulfite complex, which subsequently undergoes an irreversible isomerization reaction that inactivates the enzyme (Valero and others

1992). The main action of sulfite on the enzyme might be sulfitolysis at the vital point for the enzyme activity (Sayavedra-Soto and Montgomery 1986). These researchers also suggested that the formation of PPO-SO_3 complex could have occurred due to the interaction between sulfite and PPO, forming inactive PPO that differed in some properties that was shown by additional bands on electrophoresis and the disappearance of the protein band where the active PPO should have been noticed (Sayavedra-Soto and Montgomery 1986). Presence of the lag phase was also observed and was attributed to the formation of complexes between the o-quinones and sulfite before the onset of browning (Sayavedra-Soto and Montgomery 1986). The inhibitor is gradually consumed in the quinone-coupling precess: thus its action depends on its concentration, as well as on the nature and concentration of the phenols present (Vamos-Vigyazo 1981). In the presence of o-dihydroxy phenols, sulfite may get entirely consumed before complete enzymatic inactivation has occurred (Vamos-Vigyazo 1981). In such a case color formation is delayed and attenuated, but not entirely eliminated (Haisman 1974). In the presence of both mono- and dihydroxy phenols , ascorbic acids interferes (by its reducing action) with the quinone coupling to sulphite, and thus promotes sulphite inhibition of the enzyme (Vamos-Vigyazo 1981).

Sodium metabisulfite was the most potent inhibitor of Emir grape PPO with 5.5, 13.5, 41.1 and 100% inhibition with concentrations of 0.05, 0.1, 0.25 and 0.50 mmol/L respectively (Unal and Sener 2006). It was reported that sodium bisulfite has a direct irreversible inhibition effect on mushroom PPO with an I_{50} value (concentration required to reduce the observed activity by 50%) of 0.20mM (Golan-

Goldhirsh and Whitaker 1984). Presence of sulfites in standardized foods should be declared on the label when the sulfating agents have a functional effect or are present at a detectable level, defined as 10 ppm of more (Title 21, U.S. Code of Federal Regulations 101.100). Use of sulfites to inhibit browning presents some disadvantages: 1) the corrosion of machinery 2) the destruction of nutrients, 3) the production of tissue softening and off-flavors, and 4) adverse health effects (Girelli and others 2004).

Sorbates are considered GRAS. The presence of sorbic acid as an antimicrobial agent had a minimal effect on the browning of avocado puree (Soliva-Fortuny and others 2002). Sorbates are unstable in aqueous solution and suffer and oxidative degradation that depends on pH and the presence of other additives (Campos and Gerschenson 1996; Campos and others 1997). Autoxidative degradation of sorbates proceeds at higher rates at acidic pH values due to the fact that only undissociated molecules are prone to oxidative degradation (Arya 1980). Carbonyl compounds are formed during the degradation of sorbate that can take part in non-enzymatic browning reactions and lead to undesirable changes (Campos and others 1997).

Ascorbic acid is a highly effective inhibitor of enzymatic browning primarily because of its ability to reduce quinones to phenolic compounds before they can undergo further reactions to form pigments (Sapers 1993). However, once added, ascorbic acid has been completely oxidized to DHAA by this reaction, and quinones can accumulate and undergo browning and DHAA can brown non-enzymatically (Sapers 1993).

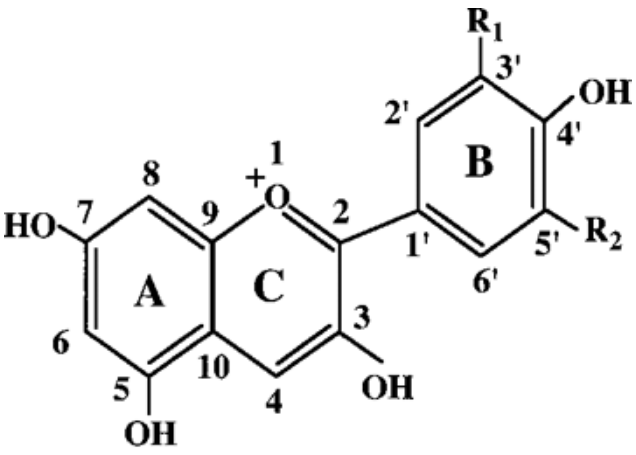
Since PPO is a metalloprotein with copper as the prosthetic group, it can be inhibited by metal chelating agents. Distinct differences were found in the action of this group of inhibitors upon PPO from chloroplasts, mitochondria or the soluble fraction (Vamos-Vigyazo 1981). PPO can also be inhibited by procyanidins, native and oxidized, and by the oxidation products of caffeoylquinic acid and (-)-epicatechin (Le-Bourvellec and others 2004). Some compounds like cyclodextrins have dual effects, activating or inhibiting browning, according to the plant material used (Sojo and others 1999). Proteins, peptides, and amino acids can effect PPO activities by reacting with o-quinones and by chelating the copper at the active site of PPO (Girelli and others 2004). Dipeptides vary widely in their inhibition effect on mushroom PPO ranging from direct, indirect or no inhibition at all (Girelli and others 2004). In recent studies, sodium chlorite emerged as a potential PPO inhibitor with mixed inhibition that is dependent on pH and inhibitor concentration (Lu and others 2006). The search for naturally occurring inhibitors has led to the discovery of a number of active compounds like chalcones and related compounds (Mayer 2006).

Oxalate in spinach leaves and quercetin and leucoanthocyanins in tea leaves have been described as natural inhibitors of catechol oxidase (Mayer 1987). PPO is inhibited by trans rasveratrol with a linear relationship between PPO activity and trans-rasveratrol concentration (Fan and Mattheis 2001). Inhibition of apple PPO by native and oxidized procyanidinas which are oxidation products of caffeoylquininc acid and (-) epicatechin, was reported by Le-Bourvellec and others (2004).

Anthocyanins

Anthocyanins are the most important water-soluble pigments in plant tissue, and impart blue, red and purple colors. The anthocyanins (Greek *anthos*, flower and *kyanos*, blue) are part of the very large and widespread group of plant constituents known collectively as flavonoids. The basic structure of anthocyanins is a flavylium cation with different groups attached to different positions in the molecule. The six most common anthocyanidins or aglucones out of the 17 that occur in nature are: pelargonidin, cyaniding, peonidin, delphinidin, petunidin and malvidin (Figure 3). Differences between the individual anthocyanins are the number of hydroxyl groups in the molecule, the degree of methylation of these hydroxyl groups, the nature and number of sugars attached to the molecule and the position of attachment, and the nature and number of aliphatic or aromatic acids attached to the sugar in the molecule (Mazza and Brouillard 1987b). The sugars most commonly attached to anthocyanidins are glucose, galactose, rhamnose and arabinose (Mazza and Brouillard 1987b). Sugar present in anthocyanin molecule imparts a higher solubility and stability. Sugars, acylated sugars, methoxyl and hydroxyl groups have a marked effect upon the color and reactivity of anthocyanins (Mazza and Brouillard 1987b). Fifteen anthocyanins have been identified in the blueberries cultivated in North America and they are 3-arabinoside, 3-galctoside, and 3-glucoside of peonidin, cyaniding, malvidin, delphinidin and petunidin (Sapers and others 1984; Ballington and others 1987; Mazza and Miniati 1993). The color of anthocyanin-containing media depends on structure and concentration of the pigment, pH, temperature, presence of copigments, metallic ions, enzymes, oxygen, ascorbic acid, sugars and

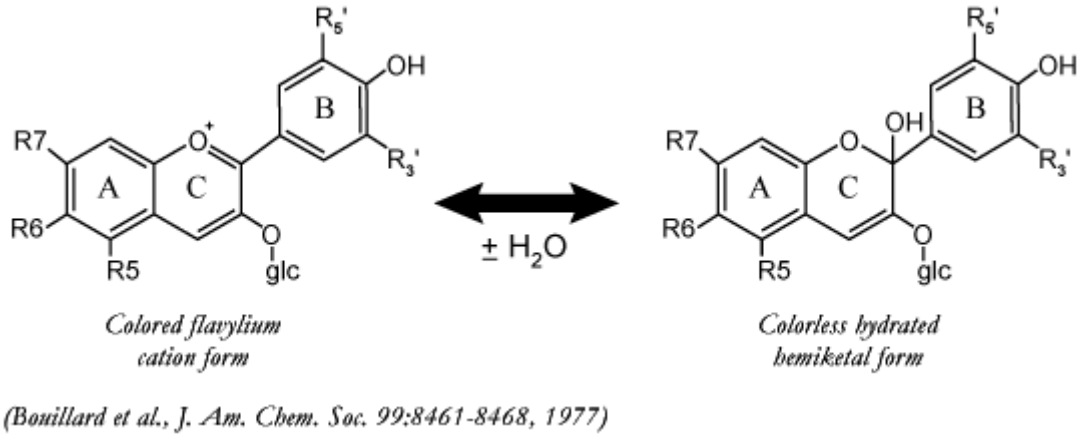
their degradation products, sulfur dioxide, actual anthocyanin concentration and other factors (Mazza and Miniati 1993). Generally as the number of phenolic hydrolysis increases, the color changes from pink to blue. Methoxyl groups replacing hydroxyl reverse the trend (Mazza and Brouillard 1987b). In aqueous media, most of the natural anthocyanins behave like pH indicators. Anthocyanin solutions are red or yellow at low pH's in a form of a flavylium cation, blush at intermediate pH's in the form of a quinoidal base, and colorless at high pH in a form of carbinol pseudobase and chalcone form (Mazza and Brouillard 1987a).



Name	R1	R2
Delphinidin	OH	OH
Petunidin	OCH3	OH
Cyanidin	OH	H
Pelargonidin	H	H
Peonidin	OCH3	H
Malvidin	OCH3	OCH3

Figure 3. Structure of most commonly found anthocyanidins. (http://health.wedar.com/show.asp?id=4701)

It is generally accepted that in acidic solutions anthocyanins exist as equilibrium mixtures of the colored flavylium cation and the colorless carbinol or pseudo base (hemicetal form) (Timberlake and Bridle 1967b) (Figure 4). Thus for example for cyanidin 3-glucoside, at a pH of 3.01, 50 % will be in the colored form and 50% will be colorless (Wrolstad 2004).



(Bouillard et al., J. Am. Chem. Soc. 99:8461-8468, 1977)

Figure 4. Forms of anthocyanins present in acidic solution

The electron deficient anthocyanins molecule is susceptible to nucleophilic attack of compound which may exist naturally in the plant and food materials. Foremost among these nucleophiles is ascorbic acid, which has been shown to accelerate anthocyanin breakdown. Anthocyanins can disappear as monomeric compounds and transform into polymeric forms which result in a color change to a brown pigmentation (Iversen 1999).

The anthocyanins pigment of the native European blueberry or bilberry (*Vaccinium myrtillus*) have long been used for improving visual acuity and treating

circulatory disorders (Mazza and Miniati 1993). There is experimental evidence that certain anthocyanins and flavonoids have anti-inflammatory properties, and there are reports that orally administrated anthocyanins are beneficial for treating diabetes, ulcers and may have antiviral and antimicrobial activities and can also provide protection against UV radiation (Mazza and Miniati 1993). Anthocyanins, as a functional food component, can aid in the prevention of obesity and diabetes (Tsuda and others 2003). The chemical structure (position, number and types of substitution) of an individual anthocyanin molecule has a bearing on the degree to which anthocyanins exert their bioactive properties (Lila 2004). The nature of the sugar conjugated and the aglucone are important determinants of anthocyanin absorption and excretion in both human and rats (McGhie and others 2003). Delphinidin but not malvidin or cyanidin, provided endothelim-dependent vasorelaxation in the rat aorta (Andriambeloson and others 1998).

Degradation of anthocyanins by PPO

It is well known that anthocyanins are poor substrates for PPO, and this is probably due to the presence of the sugar moiety that causes steric hindrance, since the aglucon forms are often oxidized by PPO (Mathew and Parpia 1971). It has been reported that PPO can act on anthocyanins when triphenolic function is present on the B ring of the flavylium structure (delphinidin derivatives) (Sakamura and Obata 1963; Sakamura and others 1965). The loss of about 20% of pigments in a model containing PPO and cyanidin seems to indicate that the enzyme could act directly on this pigment (Wesche-Ebeling and Montgomery 1990b). This could be due to the

presence of cyanidin in the aglucone (no sugar attached) form although the concentration of cyanidin in the aglucone form was not determined (Wesche-Ebeling and Montgomery 1990b). Enzymatic oxidation of phenolic compounds that are present in blueberries by PPO can occur during processing as soon the berries are damaged. The first reaction step that occurs is the oxidation of phenolic substrates to their o-quinones. Quinones are very reactive species which are able, as oxidants, to oxidize other substrates, being reduced in the process to the original phenol, and as electrophiles to react with various nucleophiles (Sarni and others 1995). These o-quinones reactions, namely oxidation and condensation reactions can lead to discoloration and can involve other phenolic compounds, especially anthocyanins. The use of model systems has shown that the enzymatically generated o-quinones play an essential role in the process of anthocyanin degradation (Kader and others 1999a). Although anthocyanins are not direct substrates for PPO, anthocyanins with an o-diphenolic B ring were enzymatically oxidized by generated o-quinines of catechol, catechin, chlorogenic acid (Peng and Markakis 1963; Pifferi and Cultrera 1974; Raynal and Moutounet 1989; Wesche-Ebeling and Montgomery 1990b) and caftaric acid (Cheynier and others 1994). Degradation of anthocyanins occurs when active PPO and its substrate are present in the system. In the model system containing chlorogenic acid, the degradation rate of PPO and anthocyanins was much faster than without chlorogenic acid, and no degradation occurred when the enzymatic extract was inactivated by heating and chlorogenic acid was also not oxidized (Kader and others 1997a). Degradation of anthocyanins is also tied to its structure, which is strictly correlated to the reaction pH (Pifferi and Cultrera 1974).

What ever the PPO substrate used, the degradation of anthocyanins increased as the pH increased with the sharp rise at pH 4.2 (Pifferi and Cultrera 1974). Sarni-Manchado and others (1997) showed that a hemiacetal form of malvidin are more reactive than the flavylium form which is directly dependent on pH. Mechanism and rate of anthocyanin degradation by o-quinones is closely related to their structure, especially their B ring. Sarni and others (1995) concluded that the first reaction step that leads to the discoloration of grape must like model solution depends on the nature of the anthocyanin: o-diphenolic anthocyanins behave mostly as reductants whereas non o-diphenolic anthocyanins act as nucleophiles to yield condensation products. These researchers also concluded that in complex media like grape must, both reactions occur simultaneously but o-diphenolic anthocyanins are degraded faster.

In the presence of chlorogenic acid and enzyme extract, cyanidin-3- rutinoside was rapidly degraded and could not be detected after 20 min of reaction (Raynal and Moutounet 1989). The quinones that are formed from the action of PPO on chlorogenic acid seem to be responsible for the oxidation of anthocyanins because degradation was not evident when quinones were blocked by glutathione (Raynal and Moutounet 1989). In the oxidizing grape must concentration of o-diphenolic anthocyanins like delphinidin cyanidin and petunidin decreased much faster than non o-diphenolic anthocyanins like malvidin and peonidin (Cheynier and others 1994). Degradation of cyanidin- glucoside (o-diphenol) was much faster than the degradation of pelargonidin-3-glucoside and proceeded by a mechanism of coupled oxidation in the presence of the chlorogenic acid o-quinone (Kader and others 2002). The secondary anthocyanin o-quinones generated by coupled oxidation also

proceeded further to condensation products (Sarni and others 1995). Once formed, all condensation products were rapidly degraded to a colorless compound, either by enzymatic oxidation or by reaction with primary or secondary quinines (Sarni and others 1995).

The mechanism of anthocyanin degradation depends not only on the structure of the anthocyanin but also whether the degradation was monitored on one particular anthocyanin or a mixture of anthocyanins. Cheynier and others (1994) reported that non-o-diphenolic anthocyanins may react with caffeoyltartaric o-quinone or secondary quinones that are formed from delphinidin and petunidin-3-glucoside leading to the formation of copolymers. These reactions have also been mentioned by Sarni and others (1995) and can only take place in a mixture of o-diphenolic and non-o-diphenolic anthocyanins.

Degradation of o-diphenolic anthocyanins

Cyanidin glycoside is a diphenolic anthocyanin that is very susceptible to a reaction with o-quinines, secondary products of phenolics oxidation by PPO. Sarni and others (1995) proposed a mechanism of cyaniding-3-glucoside by PPO in the presence of caffeoyltartaric acid o-quinine generated by PPO in the presence of caffeoyltartaric acid as a substrate in a 2 stage process:

1. caffeoyltartaric acid o-quinine + o-diphenolics anthocyanin = caffeoyltartaric acid + anthocyanin o-quinine

Quinines of cyaniding-3-glucoside are extremely unstable and proceed readily to condensation products :

2. anthocyanin o-quinine + anthocyanin = caffeoyltartaric acid-anthocyanin product

The addition of caffeic acid quinine to a cyanidin-3-glucoside solution resulted in the disappearance of the red color and the concomitant formation of a slight orange color (Kader and others 1999a). Kader and others (1999a) proposed identical mechanism of degradation for cyanidin-3-glucoside with caffeic acid quinone as Sarni and others (1995). They did not observed any brown color in the reaction mixture, which means that degradation products of cyanydin-3-glucoside are not involved in the formation of brown polymers. Kader and others (1998) incubated equimolar concentrations of chlorogenic acid and cyaniding-3-glucoside with blueberry PPO and reported that after 10 min of reaction, pigment could not be detected in the reaction mixture and after 20 min, the reaction turned brown. These researchers concluded that cyaniding-3-glucoside is oxidized by a coupled oxidation mechanism involving chlorogenic acid o-quinone that is generated by PPO in the presence of chlorogenic acid which leads to complete discoloration. The ratio of degraded cyaniding-3-glucoside to oxidized chlorogenic acid is relatively constant and equal 2, which means that part of the chlorogenic acid is incorporated into degradation products of cyaniding-3-glucoside (Kader and others 1998)

Degradation of non–diphenolic anthocyanins

The degradation of non-o-diphenolic anthocyanins has been regarded as a two step process involving the enzymatic oxidation of the o-diphenolic substrate into the corresponding o-quinone, followed by the reaction of the enzymatically generated o-

quinone with the anthocyanins (Kader and others 1999b). The latter reaction leads to the formation of adducts which can be oxidized either by enzymatic oxidation or by reaction with the o-quinine with no coupled oxidation occurring as expected by their structure (Sarni and others 1995).

Malvidin glycosides are non o-diphenolic anthocyanins that are less susceptible to the degradation of PPO generated o-quinines. However, Sarni and others (1995) noticed a decrease in malvidin-3-glucoside in the model system in the presence of PPO and caffeoyltartaric acid (PPO substrate) and attributed that decrease to following reaction;

caffeoyltartaric acid o-quinine + anthocyanin = caffeoyltartaric acid –anthocyanin product

There was no degradation of malvidin-3-O-glucoside in the presence of PPO without the substrate (caftaric acid), and no new products appeared in the system with malvidin-3-O-glucoside and caftaric acid as a PPO substrate (Sarni-Manchado and others 1997). This indicated that malvidin-3-O-glucoside is not a direct substrate for PPO and that it does not directly interact with the PPO substrate, caftaric acid in this case (Sarni-Manchado and others 1997). They also concluded that at a pH of 3.4, (where 2 forms of malvidin-3-O-glucoside, flavylium and hemicetal coexist) both reacted with caftaric acid o-quinone that was formed by the action of PPO forming colored flavylium/caftaric acid and hemicetal/caftaric adducts.

Weische-Ebeling and Montgomery (1990) studied the degradation of pelargonidin-3-glycoside by strawberry PPO. A model system containing pelargonidin-3-glycoside + PPO showed a slight loss of pigment (5%), but in the

presence of catechin as a PPO substrate, 50% of pelargonidin-3-glycoside was lost after 24 h. The authors proposed a mechanism of degradation by incorporation of anthocyanins into condensation products of catechin by quinine-phenol reaction. Kader and others (1999) reported that pelargonidin-3-glycoside is not a substrate for PPO since no molecular oxygen was consumed in the mixture of PPO and pelargonidin-3-glycoside. The degradation of pelargonidin-3-glycoside occurred only when both chlorogenic acid and blueberry PPO were present in the mixture. These researchers suggested that chlorogeno-quinone that was generated during the oxidation of chlorogenic acid by PPO plays an important role in the mechanism of pelargonidin-3-glycoside degradation and that the simplest hypothesis to explain this behavior is the concept of a condensation reaction between the chlorogeno-quinone or its degradation products and pelargonidin-3-glycoside. Pelargonidin 3-glucoside reacted with the hydroxycaffeic acid o-quinone formed by the dismutation of caffeic acid in aqueous media which led to the formation of condensation products that contained both caffeic acid and pelargonidin 3-glucoside moieties (Kader and others 2001)

Inhibition of anthocyanins degradation

Degradation of anthocyanins can be inhibited by the direct inhibition of PPO and with that, formation of o-quinines or by the inhibition of reaction of anthocyanins with already generated o-quinones. The quinones formed from the action of PPO on chlorogenic acid seem to be responsible for the oxidation of anthocyanins because the degradation was not evident when quinones were blocked by glutathione (Raynal and

Moutounet 1989). Cheynier and others (1994) also reported that the degradation of anthocyanins was totally inhibited by the addition of glutathione in grape must prior to oxidation. In the conditions when glutathione was added, caffeoyltartaric acid quinones that were by enzymatic oxidation were converted to other products, which prevented them from reacting and degrading the anthocyanins. With larger amounts of the inhibitor glutathione, competition between trapping of the quinones by glutathione and their reduction by anthocyanins was in favor of the former (Cheynier and others 1994)

Addition of ascorbic acid as a reducing agent to the reaction mixture containing pelargonidin-3-glucoside, chlorogenic acid and blueberry PPO induced instantaneous bleaching after 2 min of reaction, (Kader and others 2001). These researchers concluded that in the presence of excess reducing agent, the o-quinones that were formed were reduced to original phenol. On the other hand Sakamura and others (1965) reported that the addition of ascorbic acid retards the loss of pigment as long as ascorbic acid is present in the mixture. Inhibition of PPO activity by phenylthiourea prevented oxidation of chlorogenic acid which in addition prevented the degradation of cyanidin-3-rutinoside (Raynal and Moutounet 1989).

Addition of sulfites was reported both to degrade anthocyanins and to inhibit their degradation by the action of PPO. This bleaching can be reversible or irreversible. The reaction of sulphites with anthocyanins, nicotinamide derivatives, the flavin moiety of flavoenzymes and folate all cause reversible binding of the additive (Wedzicha 1992). The reversible reaction with sulfur dioxide involves reaction with colored flavylium to form colorless chromen-2 (or 4)-sulphonic acid,

which is similar in structure and properties to an anthocyanin carbinol (pseudo) base (Jurd 1963). When sulphur dioxide was added to a diluted blackcurrant juice, the visible color fell rapidly to a minimum value and than rose slowly during the course of several hours (Timberlake and Bridle 1967a). This recovering of the color was attributed to the other substances present in juice (e.g. sugars and dehydroascorbic acid) that were competing with the anthocyanins for SO_2 and were slowly removing it from the initially formed anthocyanin complex (Timberlake and Bridle 1967a).

Sulfur dioxide (8ppm)completely inhibited the degradation of anthocyanin in a model system containing mushroom PPO and catechol as a substrate at pH 6.5 (Goodman and Markakis 1965). In tart cherry juice under similar conditions, but with no catechol added, 30ppm of SO_2 was required for complete inhibition of anthocyanin degradation (Goodman and Markakis 1965). These greater concentration of SO_2 in the juice than in the model system that was necessary for the inhibition of PPO was attributed to the SO_2-binding capacity of carbonyl compounds that are present in the juice.

Influence of the added SO_2 on the content of the colored anthocyanins depends of the content of the SO_2-binding carbonyl compounds present in wine with the same level of total SO_2 that differently influences anthocyanin color in different wines (Dallas and Laureano 1994). Malvidin 3-glucoside is immediately decolorized by an excess of SO_2 (2000mg/l) (Dallas and Laureano 1994). It was found that SO_2 had a strong stabilizing effect on pelargonidin 3-glucoside in canned and bottled strawberries (Adams and Ongley 1973) and in strawberry juice (Bakker and Bridle 1992). Sodium sulphite was found to a have a strong stabilizing effect on

pelargonidin 3-glucoside in canned and bottled strawberries, and it was originally theorized that this was either due to its antioxidant action or to the fact that it forms addition compounds with aldehydes such as furfural and 5-hydroxymethylfurfural (arising from the break down of sugars) which might otherwise condense with the anthocyanin to yield colorless or brown compounds (Adams and Ongley 1973). Incorporation of sulfur dioxide in the extraction medium significantly increased the yield of anthocyanins from apple peel (Timberlake and Bridle 1971).

Blueberry juice

Most of the blueberries in Mississippi are sold on the fresh market (http://www.mdac.state.ms.us/n_library/pub_form/publications/pdf/com_blueberries.pdf.) Mississippi's early season helps growers get top prices before northern blueberries start competing in the market. The perishable nature of blueberries in the fresh form coupled with the decrease in price in southern berries once northern production starts, requires rapid movement to market (Sadfar and Albert 2000) or further processing. There is also a lot of excess fruit that includes unripe, blemished and broken berries that are not suitable for the fresh or frozen markets. Frozen berries are difficult to sell profitably due to supply exceeding demand and high storage costs (Main and others 2001). Use of excess fruit in juice products is especially attractive since machine-harvested fruit can be used (Bakker and others 1998). Health attributes ascribed to blueberries create a need for the development of processed blueberry products that are convenient for the consumer. Of all fruits and vegetables, blueberries are ranked highest in their antioxidant activity (Prior and others 1998).

Both cranberry and blueberry juice contain high molecular weight constituents which selectively inhibit mannose resistant adhesions that are produced by urinary isolates of *Eschericia coli* by binding to the bacterial surface, possibly to the adhesion itself (Ofek and others 1996).

Blueberries are often pressed into juice and juice concentrate, to reach more wide spread consumption (Rossi and others 2003). Recent reports in the beverage industry indicate an increase in the production of functional beverages. Blueberry juice would be a rich source of antioxidants and could be classified as a functional beverage. Blueberries may be the biggest berry beverage breakthrough yet. In Asia and Europe, blueberries are one of the most popular flavors for fruit beverages and juices (http://www.foodprocessing.com/articles/2005/559.html?page=2). In North America, blueberries in beverages are suddenly becoming popular with companies like Izze Beverage Co., extending their line of pure fruit juice sodas with blueberry last year; Leading Brands released TrueBlue blueberry juice cocktail; Island Juice Company produces a line of berry blend juice beverages; Ocean Spray introduced its new cocktail, Organic Cranberry-Blueberry, for a double-shot of health (http://www.foodprocessing.com/articles/2005/559.html?page=2).

Blueberry juice is an expensive product because blueberries are priced higher than other small fruits (Main and others 2001). Blueberry juice can be also mixed with other juices and still produce a blueberry like juice or juice cocktail (Main and others 2001). Berry juices have become widely used in blends with the more traditional juices, such as apple, cranberry, and orange juice (Roberts and others 2004).

The percentage of blueberry juice in the blend and the type of blending juice greatly affects the final product. Concord and Venus grapes blended with blueberry juice resulted in a flavor similar in intensity to pure blueberry juice while apple and cranberry blends produced a juice with diminished blueberry flavor (Main and others 2001).

Blueberry juice processing methods

Juice processing methods affect the quality of the final product. The stability of color in blueberry is an important aspect of controlling quality during processing and storage (Yang and Yang 1987). Anthocyanins exist almost exclusively in the skin, whereas phenolics and antioxidant properties are mostly in the flesh (Lee and Wrolstad 2004). Since blueberries contain anthocyanins that impart the red color in the skin, breaking the skin and maceration of the blueberry mash are necessary steps in order to extract the color and obtain good quality juice. Freezing and heating of the blueberries had a more pronounced effect than pectolytic enzymes on the skin cells, and consequently on the liberation of color pigments. The loss of semipermeability and mechanical injury to the cell membranes appears to determine the total color released (Fuleki and Hope 1964). Crushing of the berries and heating have been used to increase color extraction from the berries and increase yield. Fuleki and Hope (1964) compared three treatments; hot pressing (62ºC for 30 min), cold enzyme pressing and hot enzyme pressing on blueberry juice yield and composition. They reported that the hot enzyme treatment was superior to the other two, although the higher extraction of color was mainly attributed to the increased

temperature and mechanical injury to the skin rather than the action of pectolytic enzymes. Lee and others (2002) also concluded that heating of berries may have contributed towards a breakdown of the skins, which could have increased color extraction, but still with a substantial amount of anthocyanins in the press cake. Reextracting the press cake or crushing the blueberries into fine particles may aid in greater extraction of anthocyanins into the final product (Lee and others 2002). Blueberry puree that was held for 60 min at 60ºC had higher anthocyanin and phenolic concentration than puree held at 25ºC, with anthocyanins being more affected than phenolics (Kalt and others 2000). The increased extraction of anthocyanins was attributed to the increased permeability of membranes in the macerated berries at higher temperatures, and to decreased solubility of oxygen at higher temperatures that decreases oxidative degradation (Kalt and others 2000). Skrede and others (2000) obtained 83% yield of juice with the press cake residue accounting for 10% of the starting material. They also had 7% loss of material in the milling, enzyme maceration and pressing unit operations. Processing operations for the production of juice included thawing (5ºC), milling (6-7mm), depectinization at 43°C for 2h, pressing (0.5bar), pasteurization (90ºC, 1 min), and filtering to obtain single strength juice with 15 Brix and further concentration at 40ºC to 73.5 Brix. Lee and others (2002) compared two pretreatments: addition of 100 ppm SO_2 and heat (95ºC for 2 min) followed by crushing, pectinase treatment, pressing, clarification, pasteurization (90ºC for 90s) and concentration on juice quality. Yield of the juice ranged from 74 to 89% (w/w), with anthocyanins not efficiently extracted, which had a negative influence on juice quality. Pretreatments with heat and SO_2 resulted in

higher recovery of red color pigments with the more intense color (higher chroma) in pasteurized juice compared to control, while the heat treatment samples were darker (smaller L values) than the control and SO_2 treated juice (Lee and others 2002). Rossi and others (2003) reported a juice yield that ranged from 79 to 81% (w/w) when they used a process that consisted of thawing, blanching, milling, depectinization for 1h at room temperature with enzymes, pressing and pasteurization. They concluded that the addition of steam blanching of the fruits should be considered a very important factor when evaluating processed blueberry products for their possible health benefits (Rossi and others 2003). The cold processing of blueberries was not as efficient at transferring antioxidants or phenols to the final juice as methods where heat was used (Carlson 2003). Pasteurization using a steam kettle was the most effective at retaining antioxidant activity as measured by total phenols and ORAC in the final products. One likely reason for this significantly higher level of antioxidant retention is due to exposure to higher heating temperatures resulting in increased permeability of water soluble substances, which then diffused into the liquid stream. The higher heat also degrades enzymes (PPO and glucosidase) that would be harmful to the anthocyanins and lower the oxygen concentration in the final products (Carlson 2003).

PPO in food processing

Polyphenol oxidase (PPO) is a very important enzyme in the food industry. Processing or any wounding of fruits and vegetables may cause cell disruption that can lead to quinone formation due to the action of PPO with subsequent product

deterioration. The appearance of food and beverages may be affected but also the taste and nutritional value, often decreasing the quality of the final products (Gandia-Herrero and others 2005). For the fruit and vegetable processor, the action of PPO is primarily connected to the enzymatic browning of fresh and off-flavor generation in canned or frozen horticultural products, respectively (Vamos-Vigyazo 1981). Both phenomena are of vital importance to the manufacturer as they impart not only the sensory properties and hence, the marketability of a product, but often lower its nutritional value (Vamos-Vigyazo 1981). Enzymatic oxidation of phenolic compounds present in blueberries by PPO can occur during processing as soon the berries are damaged. The first reaction step that occurs is oxidation of phenolics and conversion of substrates to their o-quinones. Quinones are very reactive species which are able, as oxidants, to oxidize other substrates, being reduced in the process to the original phenol, and as electrophiles to react with various nucleophiles (Sarni and others 1995). These reactions of o-quinones, namely oxidation and condensation reactions can lead to discoloration and can involve other phenolic compounds, especially anthocyanins. Degradation of anthocyanins occurs when active PPO and its substrate are present in the system. Although anthocyanins are not direct substrates for PPO, anthocyanins with an o-diphenolic B ring were oxidized via enzymatically generated o-quinines of catechol, catechin, and chlorogenic acid (Peng and Markakis 1963; Pifferi and Cultrera 1974; Raynal and Moutounet 1989; Wesche-Ebeling and Montgomery 1990b) and caftaric acid (Cheynier and others 1994). Thermal treatment and addition of chemical inhibitors are some ways to inhibit the action of PPO.

PPO is not an extremely heat-stable enzyme. Short exposure, in the tissue and solutions, to temperatures of 70 to 90ºC are, in most cases, sufficient for partial or total irreversible destruction of its catalytic function (Vamos-Vigyazo 1981). Blanching (3 min in a steam blanching tunnel) of blueberry fruit was extremely effective at reducing PPO activity, maximizing anthocyanin recovery in the juice (Rossi and others 2003). The PPO in crude extracts of plum, apple, pear and avocado was inactivated at 60-65ºC, whereas for grape PPO, a temperature of 55ºC was enough for inactivation (Weemaes and others 1998). PPO thermal stability can be affected by some substances that are present in the food (Tomas-Barberan and Espin 2001a). Addition of both EDTA and benzoic acid increased the thermal stability of mushroom PPO, whereas gluthatione produced a sensitization to temperature treatments, probably due to an interaction with a disulphide bond of the enzyme (Weemaes and others 1997).

It has been reported that heat inactivation of PPO is unacceptable in anthocyanin-containing juice products, since the high temperature required for PPO inactivation can cause anthocyanin degradation (Siddiq and others 1992). Carboxylic acid is a strong inhibitor of PPO (Pifferi and others 1974; Walker and Wilson 1975; Walker and McCallion 1980; Gunata and others 1987; Ferrar and Walker 1996). Benzoic acid and some derivatives of cinnamic acid are competitive inhibitors of PPO (Pifferi and others 1974). When 4-methyl catechol is used as a substrate, benzoic acid is a competitive inhibitor of PPO (Gunata and others 1987; Janovitz-Klapp and others 1990b). Benzoic acid at a concentration of 2.5mM (0.03%), inhibited 43% of grape PPO at pH 5 (Gunata and others 1987). The presence of 5mM benzoic acid

lowered enzyme activity by 5%. At higher concentrations of benzoic acid, the enzyme activity was reduced more: 11% at 10mM, 36% at 25mM and 52% at 50mM (Weemaes and others 1997). This decrease in activity is probably due to competition between benzoic acid and catechol for the active site of the enzyme (Weemaes and others 1997).

Acids in blueberries

Sixteen different organic acids were identified in highbush blueberries with the predominant acids being citric, malic, quinic and chlorogenic, averaging 70%, 7%, 4% and 16%, respectively (Markakis and others 1963). In rabbiteye fruit, the percentage contribution by citric, succinic, malic and quinic acid is about 10%, 50%, 34% and 6%, respectively (Kalt and others 1996). Other researchers reported 95% citric acid and 1% to 2% each of quinic and malic acid in ripe Wolcott fruit (Kushman and Ballinger 1968). Succinic and malic acids were the predominant acids in rabbiteye cultivars averaging 50% and 33%, respectively (Ehlenfeldt and others 1994). Among rabbiteye clones, citric acid averaged 10% and was never found to be more than 22%, while quinic acid was consistently present as only a minor constituent, averaging 6% (Ehlenfeldt and others 1994). Quinic acid is found in plants and microorganisms and has a regulation role in the biosynthesis of aromatic compounds in the shikimate pathway (Dewick 1998). The biosynthesis of quinic and shikimic acid are interlinked and both are targets in the search for the new herbicidal, antifugal, antibacterial and antiparasitic agents that may not affect mammals (Pansare and Adsool 2006). High concentrations of quinic acid, between 0.5 and 1.18%, were

reported in juices from different varieties of cranberries and lingenberries but only trace amounts were found in blueberrie's from Germany and Argentina (Jensen and others 2002). Quinic acid is also starting material in the synthesis of viral neuraminidase inhibitors for the treatment of influenza (Kim and others 1997). Quinic and shikimic acids are gaining interest because they can be a starting material in the production of the bird flu medicine, Tamiflu (Bradley 2005). Shikimic acid is also a starting material for the making of Tamiflu, but there is only a limited supply of the acids, with the most abundant source from star anise and the leaves of gingko biloba (Bradley 2005). Tamiflu can also be synthesized from quinic acid found in cinchona bark, but supplies are also limited (Bradley 2005).

Phenolics

Phenolics are present in fruits, vegetables, leaves, nuts, seeds, flowers and barks. Although structurally diverse, phenolics can be classified into two groups – the flavonoids and the nonflavonoids. The flavonoid family includes flavonols (myricetin, querecetin, kaempferol and isorhamnetin), flavan-3-ols (catehin and epcatehin) and anthocyanins. The nonflavonoids encompass galic acid, hydrohycinnamates (p-coumeric acid, caffeic acid, and caftaric) and rasveratol. Anthocyanin flavonoids and hydroxycinnamates are the largest constituents of the total phenolic content of blueberries (Kalt and others 2000). Blueberries are very rich in total phenolics. The total phenolics content of highbush and lowbush blueberries is about 4-fold higher than in strawberries and raspberries (Kalt and others 1999). Reported values of total phenolics in rabbiteye blueberries vary between authors.

Values of 717-961 mg of gallic acid equivalents in 100 g of rabbiteye blueberries (Moyer and others 2002), 230.8 to 457.5 (Prior and others 1998), and 270.02 to 929.62 (Sellappan and others 2002), were reported depending on cultivar. Large differences in total phenolics within the same variety can be attributed to maturity stage of berries, weather conditions during the year, growing season, plant disease, geographic location, extraction method and many other factors (Sellappan and others 2002). Total phenolics play an important role in the plant tissue and the human diet. Many distinctive development features of fleshy fruits, such as loss of astringency and color, are related to changes in the synthesis and accumulation of phenolic compounds. Phenolic compounds are considered nonnutrient biologically active compounds (Sellappan and others 2002). The functionality of these compounds is expressed through their action as an inhibitor or and activator for a large variety of mammalian enzyme systems, and as metal chelators and scavengers of free oxygen radicals (Sellappan and others 2002). Several research groups have suggested the significance of fruit and vegetables phenolics as dietary antioxidants. Their studies suggested that phenolics have substantial antioxidant capacity and may reduce the risks of cardiovascular disease and cancer (Kalt and others 1999).

Changes in anthocyanins during juice processing

Attractive color is a very important sensory characteristic for the consumer of fruits and processed fruit products (Garzon and Wrolstad 2002). Huge economic losses can also be accrued by the company due to the loss of attractive product color. A concord grape juice producer reported an approximate loss of $ 700,000 due to the

loss of juice color (Wrolstad and others 1994). Not just because they impart color, but also because of their possible beneficial effect, particular attention has to be paid to the changes that anthocyanin pigments undergo with processing. Pronounced changes in anthocyanins occurred during all processing steps, with the most dramatic difference between the berry fruit and the initial pressed juice (Skrede and others 2000). Anthocyanins are not as efficiently extracted in the pressing operation as sugars, acids and other water solubles, which can have a negative impact on juice quality (Skrede and others 2000; Lee and others 2002). There was greater than 76% loss of anthocyanins in pasteurized juices when compared to frozen fruit (Lee and others 2002). Anthocyanins as well as other polyphenolics are readily oxidized because of their antioxidant properties and, thus, susceptible to degradative reactions during various unit processing operations (Skrede and others 2000; Rossi and others 2003). The oxidation of anthocyanins may be most significant in fruit juices or beverages, because the aqueous matrix can dissolve large amounts of oxygen during processing, as compared to products with low water content (Kalt and others 2000). The primary steps of processing (thawing, crushing, depectinization, and pressing) contributed a large loss in total anthocyanins (Skrede and others 2000; Lee and others 2002). It was shown that substantial losses of anthocyanins and other polyphenolics occurred when blueberries were processed into juice and that different classes of compounds had varying susceptibility to degradation, with different processing operations with the highest losses occurring during milling and depectinization due to the action of native PPO (Skrede and others 2000).

Stability of anthocyanins in food system is influenced by several factors, including chemical structure of the pigment, total anthocyanin concentration, presence of other phenolics and ascorbic acid, and processing conditions. The relative anthocyanins stability is related to their chemical structure. Diglycosidic substitution is reported to give more stability to the molecule than monoglycosidic substitution ((Mazza and Miniati 1993; Garzon and Wrolstad 2002). The delphinidin glycosides with the greatest lability have 3 ortho phenolics groups in the B ring and the cyanidin and petunidin derivatives, which have the second order of reactivity, have 2 orto phenolics groups (Skrede and others 2000). Peonidin and malvidin glycosides, which have the least reactivity, possess 1 phenolic substituent in the B ring with 1 and 2 adjacent metoxy substituents, respectively (Skrede and others 2000). Anthocyanin stability in products can also be related to total anthocyanin concentration in the products. The higher the total pigment concentration, the higher the stability of the berry juice or products (Garzon and Wrolstad 2002; Rein and Heinonen 2004). Color stability is more dependent on the total anthocyanin content rather than the qualitative anthocyanin composition (Skrede and others 2000). Lower pH (3.0) was found to stabilize the color of anthocyanins in the blueberry puree, because the equilibrium between colored flavylium and colorless pseudobase is shifted toward the flavylium at this pH (Yang and Yang 1987). Phenolics, especially phenolic acids have a protective effect on anthocyanin color (Rein and Heinonen 2004). Total anthocyanins concentration and ascorbic acid are also believed to be major factors that influence the stability of anthocyanins (Garzon and Wrolstad 2002). Fortification of syrups and jams with ascorbic acid caused a decrease in

pigment stability (Garzon and Wrolstad 2002). Not all anthocyanins are affected the same way during processing. Malvidin glycosides comprised 44% of the total anthocyanins in blueberry fruit and increased to 63% in pasteurized juice and concentrate (Skrede and others 2000). Similar results were reported by Lee and others (2002), in which malvidin glycosides in the berry were 51% of total anthocyanins and increased to 60-77% in pasteurized juice and concentrate. Delphinidin glucoside were the most unstable decreasing form 12% in blueberry fruit to 5% in pasteurized juice with only a trace amount present in the initial pressed juice (Skrede and others 2000). Decrease of delphinidin glycosides was also reported by Lee and others (2002) but only for the juice that received no pretreatment prior to pressing.

During juice processing, anthocyanins can be lost due to enzymatic break-down, heat treatment and some are removed with the pulp (Iversen 1999). Activity of PPO is one of the most important factors that influence the color loss of Concord grape juices (Cash and others 1976). After addition of crude PPO extract to juice samples, formation of the brown precipitate was noticed and became greater as reaction time increased. The precipitate appeared to be the end result of enzymatic hydrolysis of anthocyanins to brown degradation products which then became polymerized and settled out of solution (Cash and others 1976). Chlorogenic acid will stimulate PPO destruction of anthocyanins during grape juice processing (Yokotsuka and Singleton 1997). Endogenous enzymes in blueberry fruit can cause pigment degradation in juice processing (Skrede and others 2000). The anthocyanin content of pasteurized single-strength juice was significantly higher than that of initial

pressed juice which was attributed to the action of the endogenous polyphenol oxidase (Skrede and others 2000; Lee and others 2002). Iversen (1999) also reported a higher anthocyanin content in the pasteurized black currant juice than in the raw juice. Kader and others (1997) also reported that endogenous PPO from highbush blueberries caused anthocyanin degradation in crushed fresh berries. The chlorogenic acid present in blueberries is an additional compositional factor which can contribute to the pronounced color degradation in blueberry juice (Skrede and others 2000).

Use of commercially available enzymes is widely used in the juice industry. Although most of the commercial enzyme preparations increase the yield of the juice, they also cause changes in the anthocyanin profiles. These changes include a decrease in anthocyanidin glycosides and formation of unknown components that are attributed to the high galactosidase, glucosidase and arabinosidase activities found in the enzyme preparations (Buchert and others 2005). Wightman and Wrolstad (1995) reported that destruction of the pigment most likely involves two steps. In the first step an enzymatic hydrolysis of the anthocyanin to anthocyanidin and sugar occurs, and than spontaneous transformation of the aglucone pigment causes the formation of colorless juice. As a result of all enzymatic treatments, practically no anthocyanidin galactosides were detected (Buchert and others 2005). Treatment without enzyme addition gave the highest yield of anthocyanins and phenolics (Lee and Wrolstad 2004).

Blanching of strawberries before juice and concentrate production had a protective effect on anthocyanin pigments, leucoanthocyanins, flavanols, total phenolics and ascorbic acid and also resulted in improved color stability (Wrolstad

and others 1980). Heating of the grape must before pressing had the greatest impact on the juice yield, amount of press fraction, pH, TA and color components, and nutraceutical analysis showed higher anthocyanin and phenolic content in the heated must (Threlfall and others 2005). After 2 h mash treatment at 50ºC, 97% of the original anthocyanins content in the black currant berries were intact (Iversen 1999). Blanching of blueberry fruit (steam for 3 min) before milling induced higher anthocyanin retention (23 % instead 12%) when processed into juice with total anthocyanin content of juice from blanched blueberry twice the non blanched one (Rossi and others 2003). They concluded that better retention of anthocyanins for the blanched fruit could be due to the total inactivation of native PPO and increased skin permeability of fruit caused by the heat treatment. Rossi and others (2003) also observed that juice from blanched blueberry fruit before milling was more blue and less red than the juice obtained from the traditional process (without blanching). These color findings were in accordance with the anthocyanin recovery. Percent recovery in the blanching process of delphinidin glycosides and petunidin glycosides (tri-substituted), which are the most intense blue pigments, was higher than that of cyaniding-glycosides and peonidin glycosides (di-substituted).

Chemical treatments like SO_2, citric acid and $SnCl_2$ had a stabilizing effect on strawberry anthocyanins during the processing of strawberry jam (Sistrunk and others 1982). Heat and SO_2 pretreatment before pressing the juice increased recovery of anthocyanins in all processing steps (Lee and others 2002). Less than 22% of the frozen berries' anthocyanins were present in the pressed juice with the control pressed juice having the lowest recovery when compared to the heat and SO_2 treated fruit

(Lee and others 2002). Heated and SO_2 treated pasteurized juices had 1.8 times the anthocyanin content of the control pasteurized juice (Lee and others 2002). Control pasteurized juice had a higher amount of anthocyanins than the initial pressed juice which was mainly attributed to enzymatic losses (Lee and others 2002). The press cake residues contained a substantial amount of anthocyanins (about 43-55% of frozen berry anthocyanins) with the control press cake containing the greatest amounts (Lee and others 2002). Heating of the berries may have contributed toward a breakdown of the skin, which would have increased the extractability of the anthocyanins (Lee and others 2002). Rossi and others (2003) demonstrated the effect of the blanching step prior to the milling of blueberries on the recovery of different anthocyanins is anthocyanin dependent. The anthocyanins that showed the highest benefit from the blanching treatment were, in decreasing order, the glycosides of delphinidin, petunidin , and cyanidin with the exception of cyanidin 3-glucoside that showed the lowest recovery (Rossi and others 2003). Malvidin and petunidin glucoside, having a single hydroxyl group on the phenolics ring were least affected by blanching and PPO inactivation (Rossi and others 2003). The highest percent recovery increase observed for delphinidin-glucoside could also be linked to their higher water solubility, due to the presence of three free phenolics functions (Rossi and others 2003). The proportion of malvidin-glycosides increased with the initial pressing compared to the fresh fruit. Malvidin-glycosides in the berry fruit were 51% of the total anthocyanins and increased to 60 to 77% in pasteurized juice and concentrate (Lee and others 2002). There was a concomitant decrease in delphinidin and petunidin glycosides (Lee and others 2002). Proportion of delphinidin-glycosides

decreased, especially in the control sample with only 8% left, while heat and SO_2 treated had 20 and 23% retained in the pasteurized juice (Lee and others 2002). The relative ratio of delphinidin glycosides versus cyanidin glycosides remained about the same in the juices as compared with the whole berry (Buchert and others 2005).

Changes in phenolics during juice processing

Processing and storage can have a marked effect on the phenolic content of fruits, and might in turn affect their health promoting properties (Zafrilla and others 2001). As a general rule, during processing, phenolic biosynthesis is interrupted by the enzyme's destruction and/or cell structure degradation (Tomas-Barberan and Espin 2001a). Processing can enhance phenolic compound degradation (chemically or enzymatically if the oxidative enzymes have not been inactivated) or can produce chemical changes that affect quality characteristics (Tomas-Barberan and Espin 2001a). Lee and others (2002) reported considerable loss of phenolics during thawing, crushing and pressing with similar results obtained by Skrede and others (2000). Sixty to 65% of blueberry polyphenolics was lost during thawing, crushing and pressing (Lee and others 2002). There were no significant differences in polyphenolics taken after each processing step between the control, heat and SO_2 treated berries (Lee and others 2002). The press cake held 15 to 25% of the frozen berry polyphenolics, which is less than the proportion of anthocyanins left in the press cake (Lee and others 2002). Thirty six to 39% of the polyphenolics in the berries was present in the pasteurized juice. Forty two to 45% of the frozen berry phenolics was lost during juice processing and not accounted in the final pasteurized juices and

press cake (Lee and others 2002). Control pressed juice (no pretreatment) and SO_2 treated pressed juice had a lower value of polyphenolics than their pasteurized juices, but that was not the case for heat treatment. Hot pressed blueberries had higher amounts of total phenolics than cold pressed ones (Lee and others 2002).

Not all phenolic groups are affected the same by processing. Rossi and others (2003) observed a significant recovery of cinammates when blueberry juice was blanched before milling, but lower than total anthocyanins. A significant increase in total cinnamates (phenolic) was observed in the heat treated juice, but lower than that of total anthocyanins (Rossi and others 2003). Extraction of cinnamates from the pulp is less affected by the heat treatment than that of anthocyanins from the fruit skin (Kalt and others 2000). Lee and others (2002) observed no changes in cinnamic acids of juices due to the heat treatment. Skrede and others (2000) reported a considerable loss of flavonol glycosides with processing with the pattern somewhat different than that of anthocyanins and chlorogenic acid. Some flavonol glycosides remained in the press cake residue (7%), but unlike with anthocyanins and chlorogenic acid, initial pressed juice contained higher levels than pasteurized single strength and concentrate which implied that flavonol glycosides were not as susceptible to enzymatic degradation as the anthocyanins and chlorogenic acid. Lee and others (2002) reported that higher levels of flavonol glycosides remained in the press cake (26-35% of the starting material's flavonol-glycosides), with just minor changes after the pressing step. An extensive decrease in procyanidin levels occurred during processing. The levels of procyanidin in initial pressed juice and single strength pasteurized juice was about 40% of the level in the fruit (Skrede and others 2000).

Thermal processing of raspberries into jam did not have much effect on the major phenolic present (Zafrilla and others 2001).

Non-thermal processing

There is the demand of consumers and retailers for minimum processed foods but being safe at the same time. Therefore, producers and researchers are searching for the new food processing methods to gently preserve foods (Houska and others 2006). Even though processed blueberry products may retain a significant portion of antioxidant activity and total phenolic content as compared to fresh and frozen fruit, other forms of bioactivity like antiproliferation power may be compromised. Heat treated products suffered significant losses in antiproliferative activity, even in products in which total phenols and in vitro antioxidant activity were maintained (Schmidt and others 2005). Although thermal preservation methods provide safer foods, there is a loss in food quality that is associated with this processing method. Hence, the main objective of non-thermal preservation methods is to minimize the degradation of food quality through limiting heat damage of food (Senorans and others 2003). Compared with thermal processing, nonthrmal methods provide high retention of flavors and nutrients, giving products a more natural taste (Zhong and others 2005).

Pulsed electric field

Consumer demand for a higher quality, fresh appearing and safe food supply is the ultimate catalyst for the emergence of PEF on a commercial scale (Yeom and

others 2002). Thermal pasteurization is quite efficient in preventing microbial spoilage, but the applied heat may also cause undesirable nutritional and biochemical changes that can affect the overall quality of the final products. High voltage pulsed electric field (PEF) treatment is a promising non thermal processing technique that may radically change liquid food preservation technology. Treating liquid foods with PEF may inactivate microorganisms and enzymes with only a small increase in temperature, simultaneously providing consumers with safe, nutritious, and fresh like quality foods (Aguilar-Rosas and others 2007). PEF treatment might be suitable for the reduction of microbial cell counts in media that contain thermosensitive components like proteins and vitamins, which are difficult to pasteurize by heat processing (Grahl and Markl 1996). Reduction of living cell counts by PEF treatment in media that contain primarily yeast cells, i.e. fruit juices or other acid media, is of great interest, because in this case sufficient lethal effects are already produced by a very low energy output (Grahl and Markl 1996). Application of a pulsed electric field is restricted to food products that can withstand high electric fields, have low electrical conductivity, and do not contain or form bubbles (Senorans and others 2003). The products are minimally affected by the process since damage occurs on a cellular level and flavor and enzyme activity are not significantly diminished (Senorans and others 2003). Products with low pH's (such as apple juice, cranberry juice and orange juice) are more suitable for preservation by the PEF process than the neutral pH products such as milk. It is possible that the high acid environment of the food products may prevent sub-lethally damaged cells from recovery (Wouters and others 2001). The fact that PEF processes that are currently employed can not

inactivate bacterial spores limits the applications of PEF. As a consequence, only high acid conditions that can prevent outgrowth with a limited shelf life are applicable to preserve food products with this technology at present (Wouters and others 2001). PEF can possibly provide a reduction in the amount of energy that is required for fruit juice processing (Toepfl and others 2006).

The results of many researchers indicate that PEF operating with conditions with electric fields between 15 and 80kV/cm, pulse widths between 1 and 100Ms and the frequency of 1-100 pulses were sufficient for inactivation of bacteria and some certain enzymes, while taste and flavors are unaffected (Barbosa-Canovas and others 2001; Wesierska and Trziszka 2007). The initial counts of viable cells in cranberry juice were only around 100 cfu/ml and were not detected after PEF treatment (Jin and Zhang 1999). No differences were observed in the reduction of cell counts between thermal treatment and PEF treatment with an electric field strength of 40 kV/cm for a treatment time of 150μs (Jin and Zhang 1999). PEF treatment of cranberry juice using a bench scale PEF system at 20 kV/cm for 150μs resulted in about a 5 log cycle reduction in aerobic bacteria, yeast and molds, which was as effective as thermal treatment at 90ºC for 90 min (Jin and Zhang 1999).

High intensity pulsed electric field (PEF) processing involves the application of pulses of high voltage (typically 20-80 kV/cm) to food that is placed between 2 electrodes. The PEF system usually consists of a high voltage pulse generator, a treatment chamber and a fluid handling system (Yeom and others 2002). The pulsed generator is capable of converting low voltage electricity into high voltage energy to be stored into capacitors until discharged. Electric pulses are generated when a pair

of high voltage electrodes are charged and discharged in a fraction of a second (Wesierska and Trziszka 2007). To process a product using PEF in a continuous system, the product flows through a series of PEF treatment chambers where it is exposed to the desired electric field strength for a desired amount of time (Yeom and others 2002).

It was observed that PEF-pretreatment caused an increase in anthocyanin concentration in resulting grape juice (Knorr 2003). PEF treatment caused the degradation of cyanindin-3 glycoside in aqueous-methanol solution. As the electric field intensity and the treatment time of PEF increased, degradation of cyanidin-3 glycoside increased significantly (Zhang and others 2007).

Organic acid and volatile aroma compounds of citrus juices did not change with PEF treatment of 28 kV/cm with 50 pulses (Cserhalmi and others 2006). When the most extreme PEF treatment was applied to orange juice, the decrease in the concentration of carotenoids with vitamin A activity was very small (Cortes and others 2006). PEF-treated orange juice retained better color and a higher concentration of vitamin C than heat-pasteurized orange juice through storage but no differences were found in pH, acidity and Brix (Elez-MartÃ-nez and others 2006). Antioxidant activities determined with DPPH method were not different between unprocessed and PEF processed orange juice (Elez-MartÃ-nez and others 2006). For grapes, a juice yield of 87%, similar to that after enzymatic maceration, and an increased content of soluble solids and pigments was reported after cell disintegration by PEF (Toepfl and others 2006). PEF treated cranberry juice had similar flavor and aroma profiles as the controls, and thermal treatment significantly altered the overall

flavor profile of the juice (Jin and Zhang 1999). No significant differences were observed in the content of anthocyanin pigments between PEF-treated samples and controls. However, thermal treatment significantly reduced the anthocyanin pigment content. HTST treatment of apple juice caused a considerable loss of phenols (32.2%) when compared with PEF treatment, which only caused a 14.49% reduction (Aguilar-Rosas and others 2007). The apple juice treated by PEF retained greater amounts of vitamin C and some representative flavor compounds, than the pasteurized juice (94.6ºC for 30s) during storage at 4ºC (Aguilar-Rosas and others 2007). Peroxidase and polyphenol oxidase showed a moderate 30-40% reduction with PEF treatment (Ho and others 1997). PEF treatment reduced 35% of PPO activity which was equivalent to heating the extract for 30 min at 60ºC (Zuckerman and others 2004). Enzyme activity of PPO decreased 38.2% when treated at 33.6kV/cm for 126 μs (Yang and others 2004)

High pressure processing

HPP is considered a technology with the most promising perspective of industrial utilization. There are already known industrial applications, namely in Japan, USA, France and Spain (Houska and others 2006). The most successful products that are treated with high pressure pasteurization process are Golden oysters, orange juice, avocado sauce Guacamole, stewed packed ham, cooked rice and cooked reice mixtures, marinated chicken meat, etc. (Houska and others 2006). One of the main advantages of this process is the almost instantaneous and isostatic pressure transmission to the product, independent of size, shape, and food composition

yielding highly homogenous products (Deliza and others 2005). HPP can enable processors to produce innovative foods with fresh-like, natural-like attributes and natural looking colors which are all aspects valued by consumers (Deliza and others 2005). Information on the benefits offered by high pressure technology presented on the juice package also had a positive contribution on purchase intention (Deliza and others 2005).

High hydrostatic pressure is a promising alternative to traditional thermal processing techniques in food preservation, but associated changes to a diversity of phytonutrients have not been extensively investigated (Talcott and others 2003). Making comparisons between HPP and thermal processes is one way to assess its benefits by monitoring destruction of heat labile compounds (Talcott and others 2003).

High pressure treatment is expected to be less detrimental than thermal processing to low molecular weight food compounds such as flavouring agents, pigments, vitamins, etc., as covalent bondings are not affected by pressure (Butz and others 2002). The pressure stability of antioxidants is of interest since they reduce the risk of free radical-related oxidative damage that is associated with a number of diseases (Butz and others 2002). When used as a pretreatment of juice before processing, High Pressure treatment resulted in a significant increase in anthocyanins (Knorr 2003). In general, HPP was more detrimental to anthocyanins, ascorbic acid, and color characteristic as compared to thermally pasteurized and control juices due to oxidase enzymes that were active during HPP processing of muscadine juice (Talcott and others 2003). Potential mechanisms for destruction include the role of

PPO and/or autoxidative mechanisms, resulting in co-oxidation of anthocyanins and ascorbic acid (Talcott 2003). Without added ascorbic acid, total anthocyanin decreases were equal (3-5%) between treatments, but in the presence of ascorbic acid, anthocyanin losses were influenced by processing and resulted in 12.4% and 18.1% loss during pasteurization and HPP respectively (Talcott and others 2003). Losses ranged from 12 -15% following thermal pasteurization for delphinidin, cyanidin, petunidin and pelargonidin, compared to 15-25% for HPP while peonidin and malvidin were more stable (Talcott and others 2003). For high pressure to be a viable option for fresh muscadine grape juice, issues surrounding removal, inactivation or inhibition of native oxidase are critical for quality retention (Talcott and others 2003). Treatments of 400 MPa of muscadine juice had greater phytonutrients and antioxidant losses than treatment of 550 MPa due to the highly oxidative conditions that resulted from PPO activation during pressurization (Del Pozo-Insfran and others 2007). Anthocyanin degradation was observed at both processing pressures (400 and 550MPa) but was appreciably higher at 400MPa (70% loss) when compared with 550 MPa (46% loss), and these were correlated to antioxidant activity (r=0.86) (Del Pozo-Insfran 2007). After 60 min of 600MPa pressure treatment and extreme temperature processing (95ºC for 60 min), no changes were observed in the total concentration of lucopene of B-carotene when compared to control (Butz an others 2002). Stability of pigments may be explained by a matrix effect: within tissues, the pigments are often compartmentalized and thus protected from adverse influences (Butz and others 2002). Processing pressures of 500 and 800 KPa did not significantly reduced vitamin C and carotenoid content of treated orange juice when compared to

unprocessed orange juice (Fernandez Garcia and others 2001). Water soluble antioxidant capacities of orange and carrot juices can be increased by thermal treatment and maintained by high pressure treatment (Indrawati and others 2004).

CHAPTER III

MATERIALS AND METHODS

EXPERIMENT I

Extraction of anthocyanins and phenolics

Frozen blueberries were homogenized in a commercial blender, weighed (25g), mixed with 25 ml of cold acetone (Fisher Scientific Co., Fair Lawn, NJ) and homogenized using a Brinkmann homogenizer (Polytron, Switzerland) at speed 4 for 2 minutes. An additional 25 ml of 70% acetone was used to wash the homogenizer and for reextraction. The extract was separated from insoluble plant material by filtering the slurry through a Whatman grade No.1 filter paper by vacuum suction using a Buchner funnel. Plant material was reextraced three times with 25 ml of 70% acetone until a clear filtrate was obtained. The volume of the filtrate was recorded and transferred to a separatory funnel where it was mixed with 2 volumes of chloroform (Fisher Scientific Co., Fair Lawn, NJ). The funnel was turned upside a down few times to mix solvents and stored overnight at 4ºC until the clear portion between the two phases was obtained. The aqueous phase (upper portion) that contained the desired extract was transferred to a 500 ml boiling flask. The residual acetone/chloroform was removed in a Brinkman Bushi rotovapor model EL 131 (Brinkman Instruments, Westbury NY) at 40ºC under vacuum.

The remaining aqueous extract was diluted to a known volume (100ml) with acidified deionized distilled water.

Total anthocyanins

Total anthocyanins were determined using the pH differential method. To measure absorbance, the extract was diluted with buffer. The buffers used were pH =1 and pH=4.5. Buffer pH= 1 was made by mixing 125 ml of 0.2N KCl (Fisher Scientific Co., Fair Lawn, NJ) with 385 ml of 0.2N HCl (Fisher Scientific Co.). Buffer pH=4.5 was made by mixing 400ml of 1M sodium acetate (136g/l)(Fisher Scientific Co.) with 240 ml of 1N HCl and 360 ml distilled water. The pH of the buffers was adjusted as required to obtain final pH values of 1 and 4.5. The order of dilution was such that the sample at pH 1 had an absorbance of less than 1, preferably in the range of 0.4 to 0.6. The dilution strength was the same for both pH 1 and pH 4.5 samples. From the obtained extract, in order to obtain an absorbance in this range, the sample had to be diluted 50X, so the dilution factor was 50. The absorbance of the diluted extract was measured by placing the sample in a semimicro disposable cuvette (Fisher Scientific Co.) and reading the absorbance using a Genesys 5 UV/VIS spectrophotometer 336008 Model (Spectronic Analytical Instruments, Leeds, UK) at λ= 510nm and λ= 700nm for both buffers. The maximum absorbance for the anthocyanins was at λ= 510nm. Turbidity or haze was corrected by measuring the absorbance at λ= 700 nm and subtracting this from the absorbance at the wavelength of maximum adsorption, λ= 510. Actual absorbance for the calculation of total anthocyanins was calculated using the following formula:

$A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$

Concentration of total anthocyanins in mg/l was calculated using the following formula:

$C\ (mg/l) = A/\varepsilon L \times MW \times 10^3 \times 50$

where:

A =calculated absorbance

MW = molecular weight of cyanidin–3–glucoside (445,2 g/mol)

E = molar extinction coefficient of cyanidin–3–glucoside (26,900 $M^{-1}\ cm^{-1}$)

L = pathlength (1 cm)

Anthocyanin determination by HPLC-DAD and LC-MS

An Agilent 1100 series HPLC (Agilent Technologies, Santa Clara CA) that is equipped with a quaternary pump, diode array detector, and a Gemini 5µm C18 110A (250 x 4.6 mm) column, fitted with a 4.0 x 3.0 mm inner diameter guard column, from Phenomenex (Torrance, Calif., U.S.A) was used for the separation of anthocyanins. Solvent A was 100% acetonitrile (Fisher Scientific Co.) and solvent B was 10% formic acid (Fisher Scientific Co.) in HPLC grade water (Fisher Scientific Co.). The program used the gradient presented in Table 1 with detection at 520 nm.

Table 1. Linear gradient program used for the separation of anthocyanins

Time (min)	Solvent %	
	Acetonitrile	10% formic acid
0	7	93
17	15	85
22	30	70
24	100	0
34	100	0

The injection volume was 25µl for pure extract of anthocyanin identification and 100 µl for diluted extract for anthocyanins degradation by PPO. The column was at room temperature and samples were filtered with 0.45 µm syringe filter (Fisher Scientific Co) before HPLC injection.

Anthocyanins were analyzed with LC-MS using Bruker Esquire(Bruker Daltonics, Bremen, Germany) in positive mode scanning from 100-500 m/z. located at Mississippi State Chemical Laboratory. The software employed was Esquire LC NT version 3.1 (Bruker Daltonics, Bremen, Germany). The program used same liner gradient like for HPLC separation (Table 1) except that solvent B was 0.1% formic acid in water.

Extraction of Polyphenoloxidase (PPO)

Ten grams of frozen blueberries was mixed with 20 ml cold (~4ºC) 200 mM phosphate buffer pH =7 containing 0.1% Triton 100-X (Sigma Chemical Co, St.Louis, MO) and 2% PVPP (Sigma Chemical Co.). The mixture was centrifuged at 10000 g for 15 min at 4ºC using a Sorvall® RC 5B plus fixed angle centrifuge (Sorvall Products, L.P. Newton, CT). Triton 100-X was used to reverse or inhibit formation of tannin-protein complexes (Wesche-Ebeling and Montgomery 1990c). Blueberries contain a high level of phenolic compounds that may interfere in the extraction of the enzyme, or its enzymatic assay. Therefore PVPP, a not-specific phenolic absorbent, was included in the extraction buffer (Serradell and others 2000). The pellet was discarded and the supernatant was used for all further experiments as an enzyme source.

Determination of total protein

Total protein was determined using the Micro-Bradford assay by placing 1 ml of 25 times diluted protein extract with 1 ml of Bradford reagent (Sigma Chemical Co.) directly in the cuvette. After 15 min of incubation, absorbance was recorded at 595 nm using a Genesys 5 UV/VIS spectrophotometer Model 336008 (Spectronic Analytical Instruments, Leeds, UK). Calculations of the total protein were made according to the standard curve (Figure 5). Standard curve was constructed by placing 1ml of a known concentration of BSA protein (Sigma Chemical Co.) with 1 ml of Bradford reagent and recording the absorbance at 595nm after 15 min of incubation.

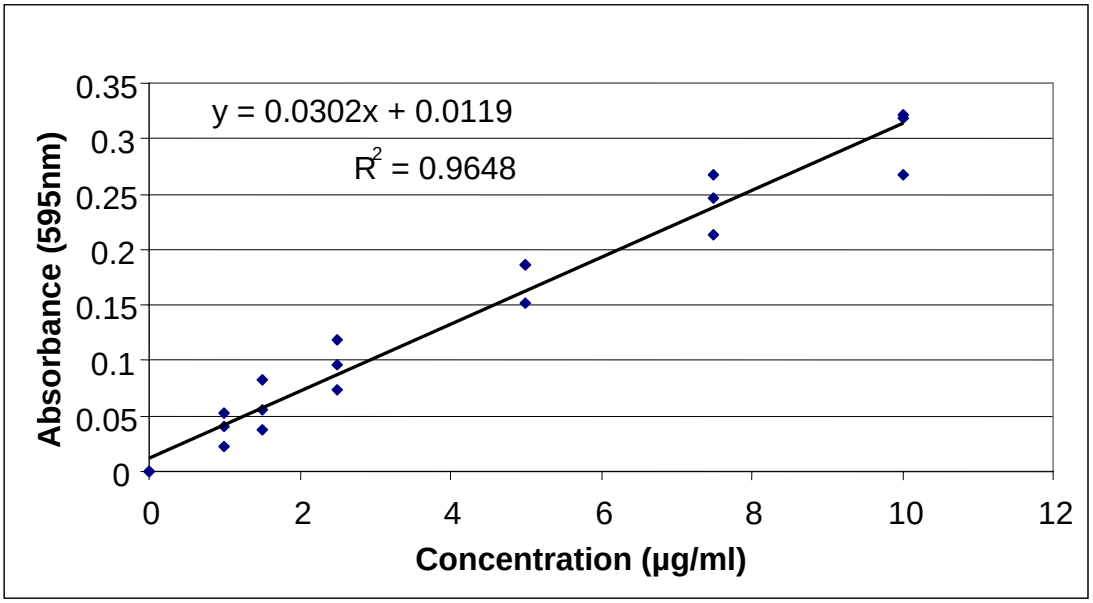


Figure 5. Standard curve for determination of total protein with BSA as a standard

PPO assay

Polyphenol oxidase activity was measured polarographically using a digital controller Model 10 equipped with a 7 ml Pyrex Rank inverted O_2 electrode (Rank Brothers, Cambridge, England) (Figure 6), and oxygen consumption was recorded using an ADC16 data logger (Pico Technology, St. Neots, UK). The instrument was standardized using air saturated water, assuming that 100% oxygen was equivalent to 233 µmol dissolved oxygen per liter (the solubility of O_2 in water equilibrated with air at 30ºC) (Wrolstad 2001). To determine the electrode response and set zero oxygen concentration, few crystals of sodium diothionite (Sigma Chemical Co.) were placed in an electrode chamber that contained distilled water prior to performing the assay. In order to optimize the working concentration of the enzyme to ensure that assay gives a true measure of the initial rate of reaction, and that the relationship between enzyme concentration and rate lies within the liner range, the range finding was performed using different concentrations (amounts) of enzyme in the assay (Wrolstad 2001). The total assay volume was 3 ml containing 0.5ml (100mM) 4-methylcatechol (Sigma Chemical Co.), 0.2 ml enzyme extract and Mcillvane buffer pH=3.6. The temperature in the electrode incubation chamber was kept at 30ºC by circulating water heated in the Isotemp 202 water bath (Fisher Scientific Co.) to 30ºC with a pump (Manostat, New York, NY). The assay was performed for 180 s and the rate of reaction was calculated from the initial linear portion of the obtained curve. Results were expressed in µmol O_2/min/100g of berries.

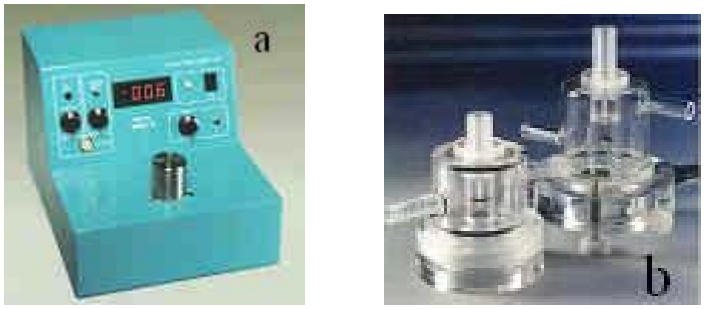


Figure 6. Digital controller model 10 (a) and Rank inverted oxygen electrode (b)

Inhibition study

The polarographic method can be very useful in inhibition studies since it can indicate whether compounds were actually inhibiting PPO per se or merely affecting the formation of colored products (Ferrar and Walker 1996).

Food grade sodium benzoate, potassium sorbate (Xena International, St. Charles, IL), potassium metabisulfite (Basf Corporation, Florham Park, NJ) and a combination of sodium benzoate and potassium metabisulfite were used for the PPO inhibition study. Concentrations of inhibitors that were used are determined according to the standards currently used in the food industry (Table 2). Concentrations of 0.1% sodium benzoate and potassium sorbate are the maximum allowable concentrations (Title 21, U.S. Code of Federal Regulations) for the use in food, while a concentration of ≤10 ppm of sulfite is not necessary for declaration on the label (Title 21, U.S. Code of Federal Regulations 101.100).

The effect of an inhibitor on PPO activity was determined by placing the designated amount of inhibitor solution in the electrode chamber together with 0.5ml (100mM) 4-methylcatechol (Sigma Chemical Co.), 0.2ml enzyme extract and Mcillvane

buffer pH=3.6 to a total volume of 3 ml. Final concentrations of the inhibitors that were used are presented in Table 2. The temperature in the electrode incubation chamber was kept at 30ºC by circulating water heated in the Isotemp 202 water bath (Fisher Scientific Co.) to 30ºC with a pump (Manostat, New York, NY). The assay was performed for 180 s and the rate of reaction was calculated from the linear portion of the obtained curve. Activity of the enzyme was calculated in μmol O_2/min/100g of berries and expressed as a percentage compared to the enzyme activity without the inhibitor that was designated as 100%. Linear regression lines for each inhibitor were obtained by transforming percent activity to log percent activity and plotting that against inhibitor concentrations.

Table 2. Concentration of inhibitors used in the PPO inhibition study.

	Inhibitor levels				
Inhibitors	**0**	**1**	**2**	**3**	**4**
benzoate (%)	0	0.025	0.05	0.075	0.1
sorbate (%)	0	0.025	0.05	0.075	0.1
sulfite (ppm)	0	4	6	8	10
benzoate(%)/sulfite(ppm)	0	0.025/8	0.05/8	0.075/8	0.1/8

Degradation of anthocyanins by blueberry PPO in the model system

The model system contained 400 μl of anthocyanin extract (205.59±13.05 mg/100g of blueberries); 200 μl of crude enzyme extract (31.14 ±0.67 mg/100g of blueberries of total protein and PPO activity of 397±38 μmol O_2/min/100g of berries) and 500 μl of 100mM 4-methyl catechol as a PPO substrate. Different volumes of inhibitors were added to make up final concentrations of 0.05 and 0.1 % of sodium benzoate and potassium sorbate, 4 and 10 ppm of potassium metabisulfite and 0.05 %/ 8ppm and 0.1%

/ 8ppm sodium benzoate/potassium metabisulfite. Total volume was adjusted to 3ml with Mcviline buffer pH 3.6. Total reaction time was 10 min at room temperature. After the reaction samples were filtered through a 0.45 micro filter (Fisher Scientific Co.) and analyzed with a HPLC utilizing the same linear gradient used for the separation and identification of anthocyanins (Table 1). Peak areas that were obtained with incubating just 400 µl of anthocyanin extract in a total volume of 3 ml in buffer was designated as 100 %. Anthocyanin extract was incubated separately for 10 min with the enzyme extract and 4-methyl catechol and no degradation of anthocyanin was observed.

Statistical analysis

For the analysis of the inhibitor effect on the PPO activity, each inhibitor was treated as a separate experiment. Completely randomized designs with inhibitor concentrations as treatments with three replications were used to determine the effect of inhibitor concentration on PPO activity. The least significant difference test was used to separate treatments means ($p < 0.05$). Slopes from the obtained regression lines for each inhibitor were treated as separate treatment and were analyzed in CR design and means were separated with LSD.

A split plot design was utilized for the analysis of anthocyanins destruction by PPO with and without the inhibitors. Inhibitor was designated as a main plot and the concentration of inhibitor as a split plot. Since the interaction between main and split plot was significant, mean separation was conducted on treatment combinations (inhibitor concentration) using SNK.

EXPERIMENT II

Maceration pretreatment of blueberry mash

Frozen blueberries were obtained from a local blueberry packer and were kept frozen (-15ºC) for about 10 months prior to processing. Blueberries were processed into juice at the Mississippi State University Dept. of Food Science Nutrition and Health Promotion Ammerman-Hearnsberger pilot plant. Frozen berries were heated in a steam kettle and constantly stirred during maceration. There were three maceration treatments (Figure 7) prior to pressing blueberries into juice: Control (no treatment), initial heat treatment and sodium benzoate treatment. Control fruit were thawed macerated at 55ºC for 1h, pressed, placed into half gallon plastic jugs and frozen for further analysis. The heat treatment procedure differed only in that the fruit was heated to 90ºC for 1 min prior to the maceration treatment. Sodium benzoate treatment differed from the control in that 0.1% of sodium benzoate (Xena International, St. Charles, IL) was added during the maceration step. All three treatments were replicated three times. Approximately 41 kg of frozen berries were used for each replication. After maceration, berries were pressed using a vertical press, and the juice was passed through a few layers of cheese cloth to remove seeds and pieces of skin. The juice was weighed to calculate the yield of the juice and then frozen for further treatment.

Blueberry juice filtration

Before pasteurization, blueberry juice samples were thawed overnight in the refrigerator and first filtered through polypropylene string wound with a 100 micron

cartridge (FCPSF1100) and then through 20 micron (FCPSF1020) filters (Siemens Water Technologies, Lowel, MA) to remove particles. The resulting juice was frozen at -20ºC until needed.

Juice treatments

Frozen, filtered juice was defrosted overnight in a refrigerator prior to treatments. For heat pasteurization, thawed blueberry juice was processed in a steam jacketed kettle for 1 min at 90ºC. Hot juice was immediately filled into 6 oz glass bottles and kept refrigerated (~3 d) at 4ºC prior to analysis.

For Pulsed Electric treatment (PEF) thawed, blueberry juice samples, were transported to Louisiana State University in an ice chest (~ 5h) with ice and immediately processed. Pulsed Electric Field equipment was located at Louisiana State University in the Department of Dairy and Animal Science. Pulsed Electric field was carried out in a continuous flow bench scale system (OSU-4K, The Ohio State University, Columbus, OH) using square wave pulses (Figure 8). The flow rate was 60 ml/min and was controlled by a pump. The PEF processing conditions were 25 kV/cm electrical field applied in bipolar mode, 3 μs pulse width, 10,000 μs pulse period making a total of 600 μs of pulse in 1 second (1000000 s x 6 μs/ 10000 s) and 200 pulses per second (600 μs/3 μs). Processed juice was filled in autoclaved 6 oz glass bottles. A portion of the filled bottled juice was frozen for further analysis.

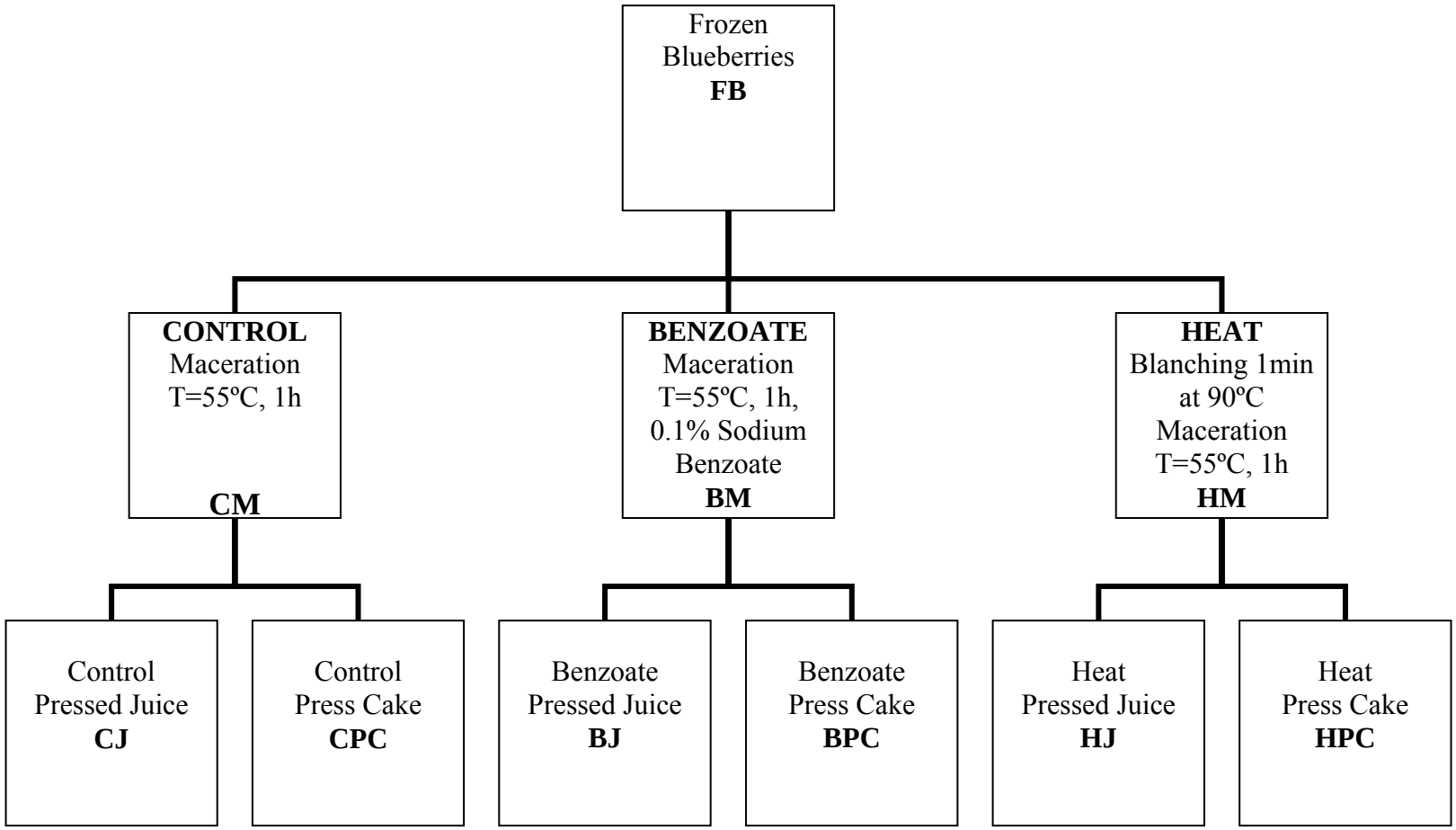


Figure 7. Schematic representation of blueberry mash maceration treatments.

For High Pressure Processing (HPP), thawed blueberry juice (125ml) was vacuum packed in double bags and processed at 3 different pressures: 200, 400 and 600 MPa using a Quintus Food Press QFP 35-L600 with 7XS-6000 Intensifier Pump. These experiments were performed at Virginia Polytechnic Institute (Blacksburg, Virginia). The control sample was also vacuum packaged but was not subject to high pressure treatment. All samples were ran with the water starting at room temperature (about 20ºC) and with a holding time of 5 min. Pressures of 200, 400 and 600 MPa reached maximum vessel temperatures of 21, 27 and 32ºC, respectively. Each pressure treatment was replicated three times.

Figure 8. OSU-4K Pulsed Electric Field Equipment.

Statistical analysis

Completely randomized designs with three replications were utilized to analyze data obtained from maceration and juice processing treatments The least significant difference test was used to separate treatments means ($p < 0.05$).

Analysis

Extraction of anthocyanins and phenolics

Frozen blueberry mash was homogenized in a commercial blender, weighed (25g) and processed as described in experiment I.

Anthocyanin determination by HPLC-DAD

Anthocyanins in the blueberry mash, pomace and juice were determined as described in experiment I. The only difference was that the concentration of anthocyanins was determined according to cyanindin-3-glucoside standard curve (Figure 9). An external standard was prepared by dissolving different amounts on cyanidin-3 glucoside in acidified HPLC grade water.

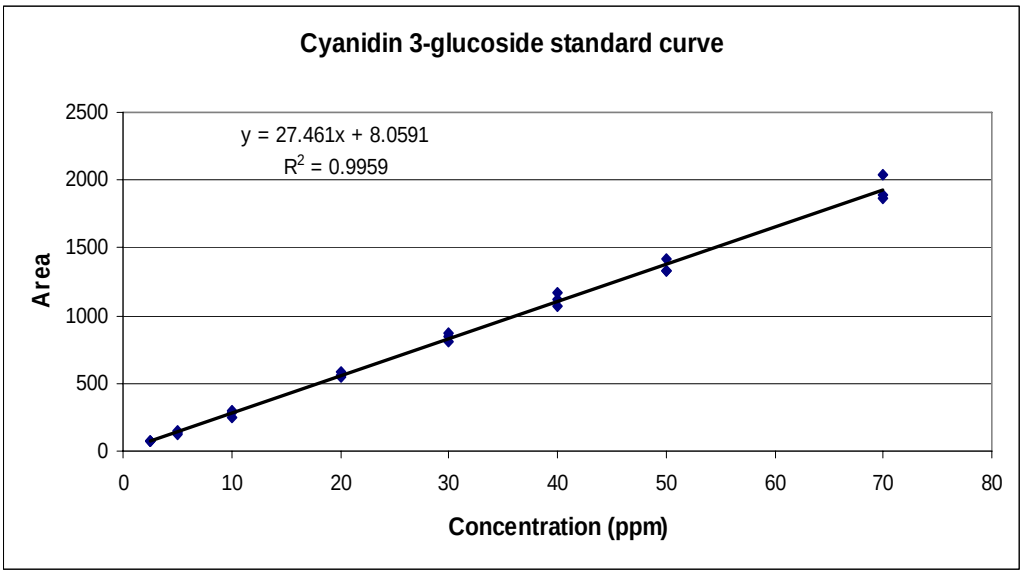


Figure 9. Cyanidin 3-glucoside standard curve

Extraction and assay of Polyphenoloxidase (PPO)

Ten grams of frozen blueberry mash was used to determine PPO activity as described in experiment I.

Total phenolics

Total phenolics in the blueberry extracts were determined with the Folin-Ciocalteu reagent by the method of Singleton and Rossi using gallic acid as the standard (Singleton and Rossi 1965). Twenty microliters of sample were mixed directly in the cuvette with 1.58 ml of water and 100 µl of Folin-Ciocalteu reagent. The cuvette was incubated between 1 and 8 min and 300 µl of 20% sodium carbonate was added. Samples were incubated at room temperature for 2 h and the absorbance was recorded at 765 nm using a Genesys 5 UV/VIS spectrophotometer Model 336008 (Spectronic

Analytical Instruments, Leeds, UK). Results were obtained using the gallic acid standard curve (Figure 10) and expressed as mg gallic acid equivalents in 100 g of blueberries.

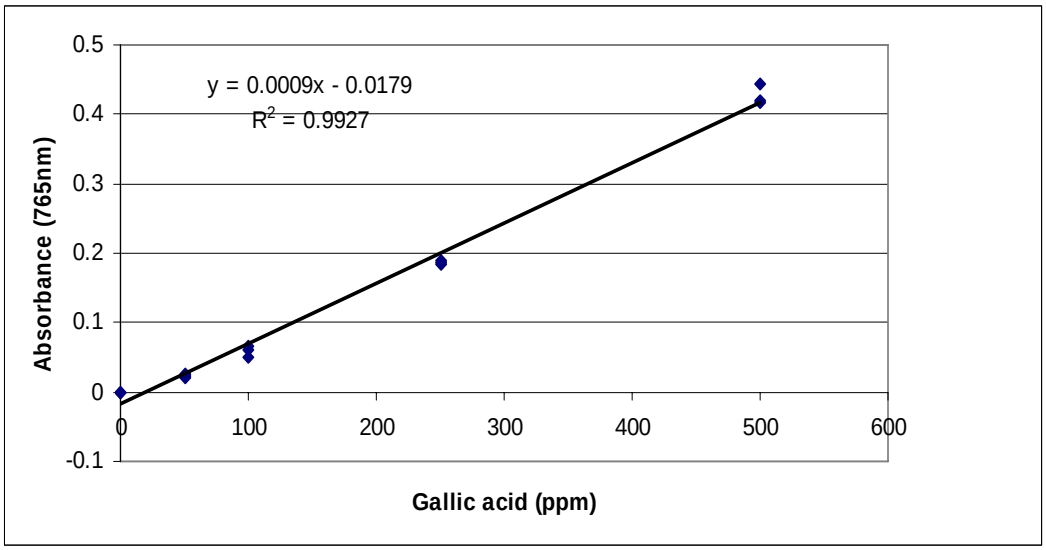


Figure 10. Gallic acid standard curve.

Polymeric color

Polymeric color was determined using the method described by Wrolstad (1976). The same extract that was used for the determination of anthocyanins and phenolics was diluted 50 times with distilled water. Two cuvettes were used for each sample. In each cuvette 2.8 ml of diluted sample were placed. In the first cuvette, 0.2 ml of freshly made 20% potassium metabisulfate (Fisher Scientific Co.) was added, and 0.2 ml of distilled water was placed in the second cuvette. Samples were left to equilibrate at room temperature (21°C) for 15 min. Absorbance for all samples was measured at λ=420nm, 510nm and 700nm (to correct for haze) against the blank cell filled with distilled water, using a Genesys 5 UV/VIS spectrophotometer Model 336008 (Spectronic Analytical

Instruments, Leeds, UK). Color density of the control sample (treated with water) was calculated using the formula;

$CD = [(A_{420} - A_{700}) - (A_{510} - A_{700})] \times 50$

Polymeric color of the bisulfite bleached sample was calculated as follows:

$PC = [A= (A_{510} - A_{700}) + (A_{510} - A_{700})] \times 50$

Percent polymeric color was calculated as:

% PC = (Polymeric color/ color density) x 100

Antioxidant activity

The antioxidant activities of the extracts were determined using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma Chemical Co, St.Louis, MO) as a free radical, (Brand-Williams and others 1995). In its radical form, DPPH absorbs at 515 nm, but upon reduction by an antioxidant or radical species, the absorption disappears. Aliquots of 0.075 ml 5x diluted extract were added to 1.9 ml of 0.025g/l DPPH solution in methanol. The decrease in absorbance was determined at 515 nm at zero minutes, and every minute for a period of 20 min using a Genesys 5 UV/VIS spectrophotometer Model 336008 (Spectronic Analytical Instruments, Leeds, UK). The concentration of DPPH in the reaction medium was calculated from the calibration curve (Figure 11). The percentage of remaining DPPH ($\%DPPH_{REM}$) at the steady state, was determined as follows:

$$\%DPPH\cdot_{REM} = [DPPH\cdot]_T / [DPPH\cdot]_{T=0}$$

Results were expressed graphically by plotting time against the percentage of DPPH remaining after a given time.

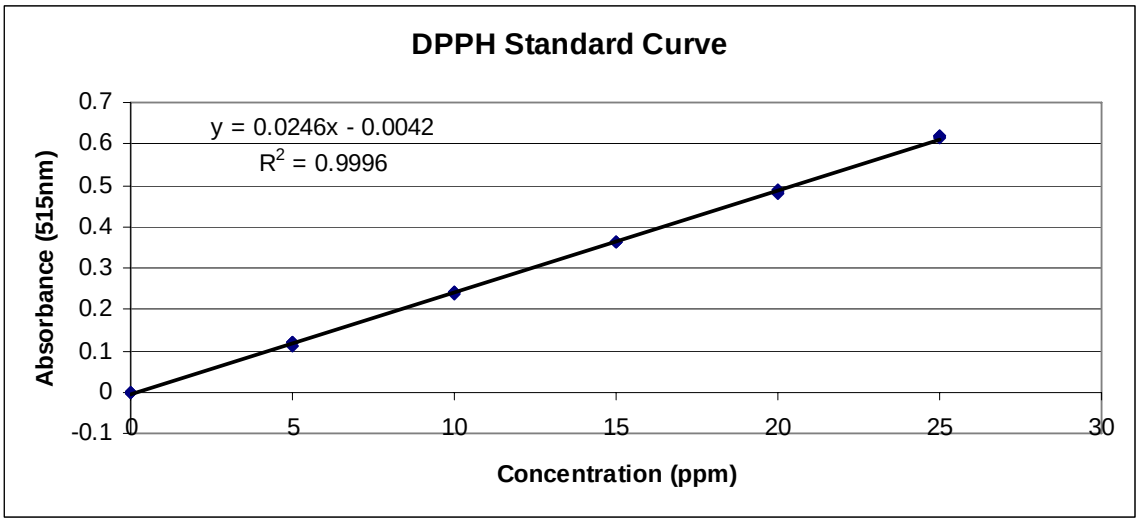


Figure 11. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) standard curve

Acids

An Agilent 1100 series HPLC (Agilent Technologies, Santa Clara CA) equipped with quaternary pump, diode array detector and a Gemini 5µm C18 110A (250 x 4.6 mm) column, fitted with a 4.0 x 3.0 mm inner dia guard column, from Phenomenex (Torrance, Calif., U.S.A) was used for the separation of acids.

An isocratic elution with 0.01M H_2SO_4 as the mobile phase, with the flow rate of 0.5 ml/min, column temperature of 35ºC and UV detection at 214nm was used. Standard curves of quinic (Figure 12), malic (Figure 13), citric (Figure 14) and shikimic acid (Figure 15) were used to calculate the concentration of these acids in the sample.

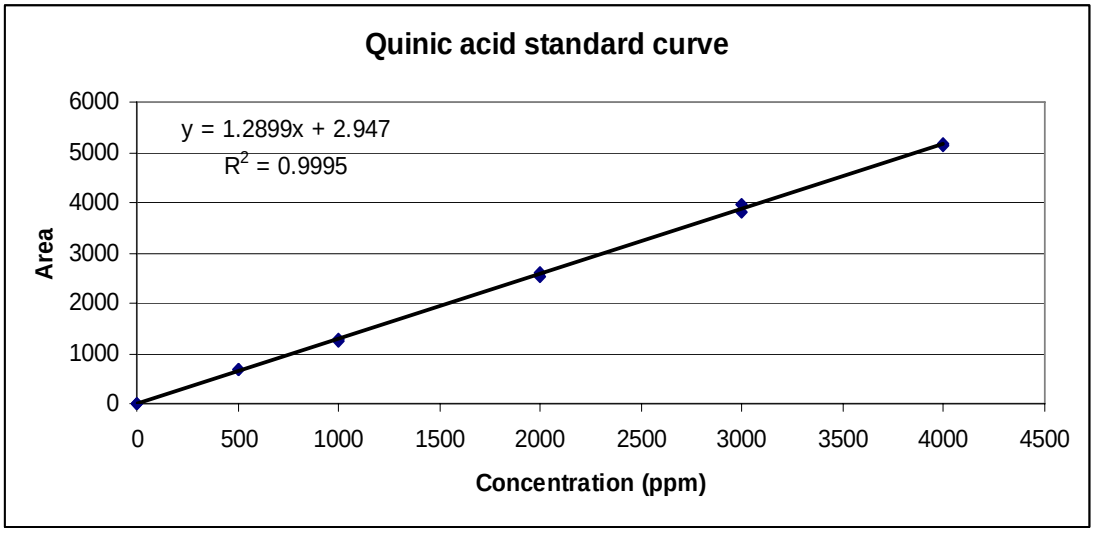


Figure 12. Quinic acid standard curve

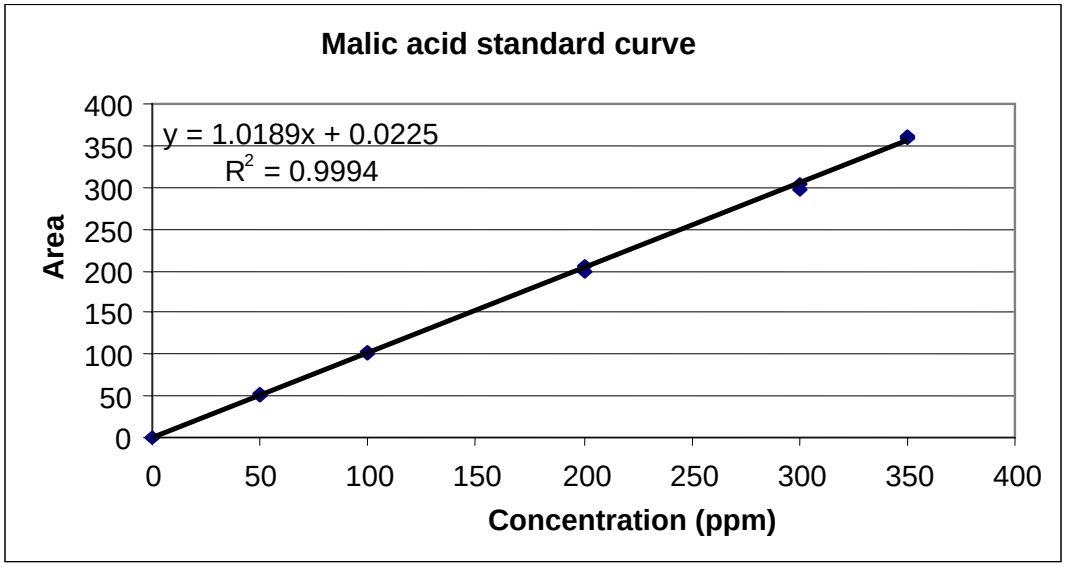


Figure 13. Malic acid standard curve

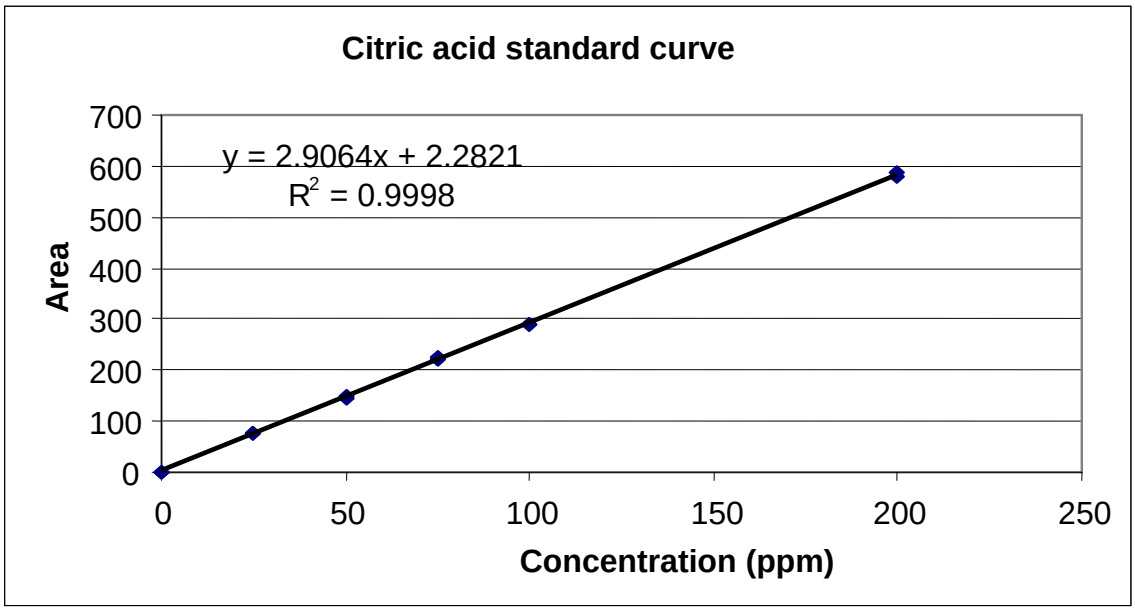


Figure 14. Citric acid standard curve

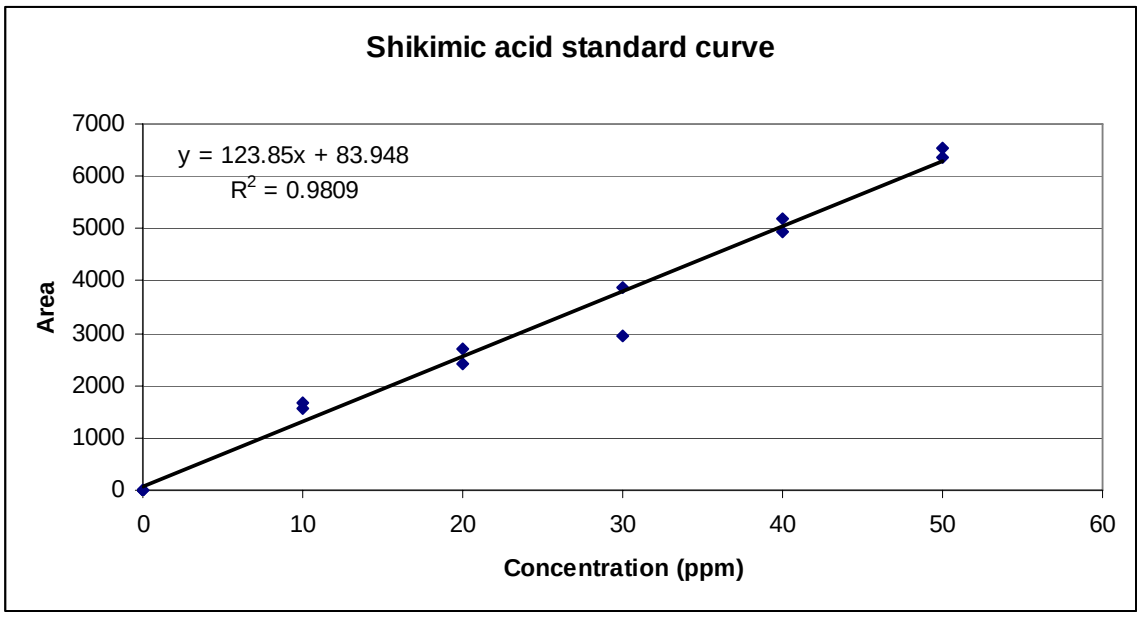


Figure 15. Shikimic acid standard curve

CHAPTER IV

RESULTS AND DISCUSSION

EXPERIMENT I

Enzyme activity and inhibition

Significant differences were found between treatments (concentrations) for all inhibitors used ($p<0.01$). Addition of sulfite and combination of sulfite and benzoate had significantly higher regression slopes that benzoate alone and sorbate indicating better inhibition effect (Table 3).

Increasing the percentage of benzoate from 0 to 0.025 to 0.05 significantly decreased PPO activity (Figure 16). Utilization of 0.1 % sodium benzoate, which is the maximum that is allowed for use in food products (21CFR 184.1733), decreased PPO activity to only 5.05% in comparison to the activity without inhibitor (Figure 16). This PPO activity was not significantly different ($p>0.05$) from the activity of 8.78% when 0.075 % of benzoate was used.

When 4-methyl catechol is used as a substrate, benzoic acid is found to be a competitive inhibitor of PPO (Gunata and others 1987; Janovitz-Klapp and others 1990b). Benzoic acid at 2.5mM (0.03%) inhibited 43% of grape PPO at pH 5 (Gunata and others 1987) while in the present study a 76 % inhibition (23.98 % activity) was observed with 0.025 % at pH 3.6, using 4-methy catechol as a substrate. The addition of

0.025% sodium benzoate reduced PPO activity to 23.98 % (Figure 16) while the same concentration of potassium sorbate reduced it to only 81.97 %, clearly indicating that the sodium benzoate is a better inhibitor. Benzoic and sorbic acid had a similar inhibition effect on PPO from banana, mushroom and apple, while sodium metabisulfite had the highest inhibition effect at 3300 μM, the same concentration of (Ferrar and Walker 1996). The similarity in inhibition effect between benzoate and sorbate can be explained by the high concentrations used, 4.03% and 3.7% respectively, in comparison to 0.1% as the highest concentration used in our experiments. Potassium sorbate also significantly decreased PPO activity but with almost 50% of activity remaining when the maximum allowable concentration of 0.1% was used. Benzoic acid is a better inhibitor of gum arabic PPO compared to sorbic acid (Billaud and others 1996). Piffere and others (1974) also concluded that inhibitors containing the benzene nucleus showed a greater effectiveness than aliphatic compounds, with benzoic acid being a better inhibitor than sorbic acid. Of all the inhibitors used, potassium sorbate was the weakest PPO inhibitor with a significantly lower regression slope (Table 3). The presence of sorbic acid as an antimicrobial agent had a minimal effect on the browning of avocado puree (Soliva-Fortuny and others 2002).

Potassium metabisulfite in concentration as low as 4 ppm inhibited almost 70% of PPO activity (Figure 17). There was no significant difference ($p>0.05$) in PPO activity between 8 and 10 ppm sulfite (Figure 17), which is very important from the practical point of view since amounts of less than 10 ppm of sulfites do not have to declared on the product label (Title 21, U.S. Code of Federal Regulations 101.100). Inhibition of PPO in the presence of sodium metabisulfite using an O_2 –electrode indicated that the enzyme

was directly inhibited rather than by inhibition through secondary o-quinones. Ferrar and others (1996) concluded that sodium metabisulfite inhibition effect is probably due to the formation of colorless sulpho-quinones, rather than PPO inhibition per se, although the O_2 –electrode assay also showed inhibition, suggesting that enzyme inactivation may also occur. Direct and indirect effects of sulfites were reported by various authors and the mode of action depends on the inhibitor concentration, substrate and the complexity of the system (presence of other compounds).

Sodium metabisulfite was the most potent inhibitor of Emir grape PPO with 5.5, 13.5, 41.1 and 100% inhibition with concentrations of 0.05, 0.1, 0.25 and 0.50 mmol/L respectively (Unal and Sener 2006). Inhibition of PPO by sulfite in concentration of 0.04 mg/ml was instantaneous below pH 4 (Sayavedra-Soto and Montgomery 1986). Potassium metabisulfite at a concentration of 1mM inhibited 74% of strawberry PPO (Wesche-Ebeling and Montgomery 1990a). Metabisulfite at concentrations of 10µM, 0.1mM 0.4mM and 1mM inhibited 0, 31, 100 and 100% PPO activity from grapes respectively (Nunez-Delicado and others 2005). $Na_2S_2O_5$ inhibited 90, 98 and 100 % PPO activity from concord grapes in concentrations of 0.05, 0.5 and 5mM respectively (Cash and others 1976).

A combination of 0.05% of sodium benzoate with 8ppm of sulfite inhibited almost all PPO activity (Figure 18). There were significant differences between regression slopes between benzoate and the benzoate/sulfite combination, indicating that addition of 8ppm of sulfite had a significant effect on PPO inhibition (Table 3). From the present study it appears that the combination of benzoic acid and potassium metabisulfite (reducing agent) has a greater inhibition affect on blueberry PPO than either inhibitor by

itself. Similar results were obtained with the combination of benzoic acid and ascorbic acid (reducing agent) where browning of the apple was inhibited to a greater extent when a combination of inhibitors were used rather than either treatment alone. This demonstrates that the inhibition effect appears to be synergistic rather than additive (Sapers and others 1989). Ferrar and Walker (1996) also concluded that the majority of strong inhibitors were either aromatic or sulfhydryl compounds while aliphatic compounds exhibited weaker inhibition effect. This is in accordance to conclusions presented in this work since sodium benzoate and potassium metabisulfite were better inhibitors than potassium sorbate as demonstrated by the slopes of the inhibition (Table 3). Mechanism of inhibition may depend on the substrate and enzyme source (Ferrar and Walker 1996) which may explain some differences between results obtained in the current study and previous studies.

Identification of anthocyanins

Anthocyanins were identified at 520 nm according to previous studies (Kader and others 1996; Lee and others 2002; Kahkonen and others 2003; Nakajima and others 2004; Buchert and others 2005). Peak identification was confirmed with HPLC/ESI-MS (Figure 20). Anthocyanins (mostly in the oxonium form at low pH) generate positive ions that can be detected by MS. An anthocyanin's single positive charge allows the mass-to-charge ratio (m/z) to correspond directly to the molecular weight of the anthocyanins (Lee and Wrolstad 2004). Fifteen different anthocyanins were identified in the rabbiteye blueberry extract (Table 4) and the clear peaks can been seen on the extracted ion chromatogram (Figure 20). Fourteen anthocyanins were separated using

HPLC and had visible peaks at 520nm (Figure 19). Peonidin 3- arabinoside coeluted together with malvidin 3-glucoside (peak #13) and identification was only possible using mass spectroscopy. Lee and Wrolstad (2004) also found a low concentration of peonidin 3-arabinoside in highbush 'Rubel' blueberries which made its identification difficult. Coelution of the two can be clearly seen on the extracted ion chromatogram of anthocyanins, peaks 13 and 13a (Figure 20). In the same figure it can be seen that malvidin 3-galactoside and peonidin 3-glucoside also coelute, although their separation was possible using HPLC (Figure 19), possibly due to the use of 10% formic acid as opposed to only 0.1% formic acid used in HPLC-MS. Coelution of peonidin 3-glucoside with malvidin 3-galactoside (peaks 11 and 12) and peonidin 3-arabinoside and malvidin 3-glucoside (peaks 13 and 13a) was previously reported (Prior and others 2001; Lee and Wrolstad 2004; Nakajima and others 2004). Identical m/z and ion chromatograms of glycosides and galactosides of delphinidin (Figures 22 and 23), cyanidin (Figure 25 and 26), petunidin (Figures 31 and 32) peonidin (Figures 34 and 35) and malvidin (Figures 38 and 39) can be noted although they have different retention times. Their identification was possible due to the clear differences in retention times and the fact that galactosides elute before glycosides in the C18 column (Figures 21, 24, 30, 33 and 37). Extracted ion chromatograms and ion chromatograms of all 15 anthocyanins are presented in figures 21-41. Similar anthocyanin profiles to this study were reported for highbush blueberries grown in Europe (Kader and others 1996) and highbush "Rubel" blueberries grown in Oregon (Lee and Wrolstad 2004).

Derivatives of malvidin were the most abundant anthocyanins (42.1%), followed by delphinidin (19.86 %), cyanidin (11.58%), petunidin (16%) and peonidin (6.6 %).

Malvidin 3-galactoside was the most abundant individual anthocyanin, with 18.9%. Anthocyanins with the galactose in the molecule were the most abundant (44.68%) followed by the ones with the glucose (32.53%) and arabinose (18.93%).

Derivatives of malvidin and delphinidin accounted for 37 and 31% of the total anthocyanins, respectively, while 3-monogalactoside derivatives constituted about 41% (Kader and others 1996). Malvidin and delphinidin derivatives were the major anthocyanins in highbush blueberries (77.2%) (Vaccinium corymbosum L.cv. Rubel) (Lee and others 2002). Malvidin 3-galactoside was a major anthocyanin in highbush 'Rubel' blueberries grown in Oregon with malvidin glycosides being most abundant accounting for 54.7% of all anthocyanins followed by delphinidin (25.7%), petunidin (14.7%), cyanidin (3.9%) and peonidin (1%) based on the peak area percentage (Lee and Wrolstad 2004). Malvidin 3-galactoside predominates in lowbush and 'Tifblue' blueberries (Prior and others 2001), in highbush 'Rubel' blueberries (Lee and others 2002) and rabbiteye blueberries grown in Japan (Nakajima and others 2004). Malvidin derivatives were most abundant in highbush blueberries, comprising 44% of the total anthocyanin in blueberries (Skrede and others 2000).

Degradation of anthocyanins

In the model system, anthocyanins were degraded by the action of quinones formed by the action of blueberry PPO on 4-methyl catechol. Blueberry PPO degraded 36% of the total anthocyanins present in the model system (Figure 42). Degradation of anthocyanins was closely related to their structure. Triphenolic anthocyanins suffered the highest percentage of degradation (delphinidin 77 %), followed by diphenolic (petunidin

48% and cyanidin 24%) and monomeric (malvidin 19% and peonidin 16%) (Figure 43). All individual derivatives of these anthocyanins (glycosides, galactosides and arabinosides) followed a similar pattern of degradation (Figures 45-62).

The relative anthocyanin stability is related to their chemical structure. The delphinidin glycosides with the greatest liability have 3 ortho phenolic groups in the B ring and the cyanidin and petunidin derivative, which have the second order of reactivity, have 2 ortho phenolic groups. Peonidin and malvidin glycosides, which have the least reactivity, possess one phenolic substituent in the B ring with one and two adjacent methoxyl substituents, respectively (Skrede and others 2000). The high degradation of delphinidin anthocyanins can also be explained by the possible direct action of PPO. It was previously shown that PPO can act on anthocyanins when triphenolic function is present on the B ring of the flavylium structure as in the case of delphinidin (Sakamura and Obata 1963).

Fifty three percent of nasunin (delphinidin 3-(p-coumaroylrutinoside)-5-glucoside) was destroyed by PPO and the destruction increased to 98 % with the addition of chlorogenic acid (Sakamura and Obata 1963). This suggests that high degradation of delphinidin derivatives may be the result of direct action by PPO and indirect action by PPO generated quinones. Delphinidin glycosides were the most unstable during processing of the blueberry juice which mostly contributed to the action of blueberry PPO (Skrede and others 2000). Delphinidin-glycosides (based on its structure, delphinidin is most labile) were degraded the most, and malvidin-glycosides appear to have degraded the least by PPO, especially when the starting material was whole berries (Lee and Wrolstad 2004), which implies that the native enzymes present in the blueberry

destroyed anthocyanins (Skrede and others 2000). In a study of the Oxygen Radical Absorbance Capacity (ORAC) of different anthocyanins, it was reported that delphinidin and cyanidin derivatives had higher Trolox equivalents than malvidin and peonidin (Wang and others 1997), which is similar to results that were observed in this study. Wesche-Ebeling and Montgomery (1990) reported increased degradation of o-dipehnolic anthocyanins in comparison to non o-diphenolic in a model system containing PPO and catechin as the PPO substrate. After 24 h, 60% of cyanidin anthocyanins was destroyed when compared to 50% pelargonidin anthocyanins (Wesche-Ebeling and Montgomery 1990b). In the presence of chlorogenic acid and enzyme extract, cyanidin-3- rutinoside was rapidly degraded and could not be detected after 20 min of reaction (Raynal and Moutounet 1989).

Malvidin derivatives should not be oxidized by the enzyme or by coupled oxidation (Sarni and others 1995). Degradation may occur due to the incorporation of anthocyanins into condensation products by quinone-phenol reactions as postulated by Wesche-Ebeling and Montgomery (1990). Sarni and others (1995) also demonstrated the degradation of both cyanidin and malvidin derivatives, with cyanidin degrading more rapidly than malvidin. The faster degradation of cyanidin-3-glucoside was due to its particular capacity (related to the o-diphenolic moiety) to be invoked in coupled oxidation, although the presence of methoxyl groups like in malvidin may also impede nucleophilic addition into quinone (Sarni and others 1995). Competition between cyanidin and malvidin 3-glucoside for the PPO generated o-quinine resulted in favor of the o-diphenol (cyanidin) (Sarni and others 1995), that can also explain more degradation of cyanidin in this study when compared to malvidin. Anthocyanins with arabinose in

their molecule seem to degrade more than the anthocyanins with glucose and galactose (Figure 44).

Addition of 0.05 and 0.1% sodium benzoate significantly decreased the percent of anthocyanins that were degraded by the PPO from 35% to 25% when 0.05% was used, and to 18 with 0.1% benzoate (Figure 42). With the addition of 0.1% sodium benzoate, almost 50% of degraded anthocyanins were protected. Protection of anthocyanin degradation by benzoate was not in the same order for all anthocyanins. The more the anthocyanins are susceptible to degradation the more they were protected by benzoate. At 0.1 %, benzoate protected approximately 30% of delphinidin (Figures 45-48), 25% of petunidin (Figures 49-52), 12% of cyanidin (Figures 53-56), 13% of malvidin (Figures 57-60) and 8% of peonidin (Figures 61-63). This is especially important since delphinidin and petunidin anthocyanins have the highest antioxidant activity and give the characteristic blue color to the blueberries.

Anthocyanin extracts contained additional compounds including acids and other phenolics. These compounds did not interfere with the inhibition of blueberry PPO by sodium benzoate. Even with an excess PPO substrate present, the generation of quinones that degrade anthocyanins is very fast, and benzoate was able to protect anthocyanins.

Addition of 8 ppm of potassium metabisulfite to the different concentrations of sodium benzoate (0.05% and 0.1%) did not significantly affect anthocyanin degradation compared to the addition of benzoate alone. Potassium metabisulfite and potassium sorbate did not have any protective effect on total and individual anthocyanin degradation by PPO. Potassium sorbate in the concentration of 0.1% significantly increased degradation of delphinidin (Figures 45-48) and petunidin (Figures 49-52) anthocyanins.

It is well known that sulfites can bleach anthocyanins, but since the order of degradation with sulfites was the same as without them, it can be concluded that the degradation of anthocyanins in the system with added sulfites was not due to the bleaching of anthocyanins but due to the action of PPO.

Although sulfites inhibited PPO activity (Figure 17), they did not protect anthocyanins that were present in the model solution. Concentration of sulfites (strong reducing agents) in our model system, might not be enough to reduce 4-methyl catechol quinones back to 4-methyl catechol which would protect anthocyanins. In the presence of excess reducing agent, o-quinines that were formed from the chlorogenic acid were reduced to o-diphenol (Kader and others 1999). Sulfur dioxide (8ppm) completely inhibited the degradation of anthocyanin in a model system containing mushroom PPO and catechol as a substrate at pH 6.5 (Goodman and Markakis 1965). In tart cherry juice, under similar conditions, but with no catechol added, 30ppm of SO_2 were required for a complete inhibition of anthocyanin degradation (Goodman and Markakis 1965). This greater concentration of SO_2 in the juice than in the model system necessary for the inhibition of PPO was attributed to the SO_2-binding capacity of carbonyl compounds present in the juice. Influence of the added SO_2 on the content of the colored anthocyanins depends on the content of the SO_2-binding carbonyl compounds present in wine with the same level of total SO_2 influencing differently anthocyanins color in different wines (Dallas and Laureano 1994). It was found that the amount of anthocyanins decreased less rapidly with the high concentration of SO_2 (Ribereau-Gayon and others 1983). Anthocyanin extract used in our model system contained other phenolic compounds and acids from the blueberries that might react with SO_2 before it

was able to inhibit PPO activity and formation of o-quinones that are responsible for anthocyanin destruction. Higher concentrations of potassium metabisulfite might be needed for the inhibition of anthocyanin degradation.

One of the reasons that sulfite did not inhibit the degradation of anthocyanins is that it may be in the bound form. Bound sulfite usually represents the portion of the additive which is present as hydroxysulphonate adducts that are formed by reactions between carbonyl groups and HSO_3-. (Wedzicha 1992). Such adducts are decomposed slowly when sulfite treated foods are acidified or during titration with iodine, but rapidly if the pH of samples is raised to pH >10 or when acidified solutions are boiled (Wedzicha 1992). A well known observation of the browning of sulfite treated foods, while there is still residual sulfite present, indicates that in such situations the concentration of free sulphite may be depleted to an extend that it becomes kinetically limiting (Wedzicha 1992). The inhibition of enzymatic browning catalyzed by polyphenol oxidase is potentially capable of leading to irreversible binding of sulphites (Wedzicha 1992). In the case of 4-methyl catechol as a PPO substrate, sulfite can react with the formed o-quinone by forming 4-sulphocatechol which is neither oxidized by an enzyme nor does inhibit the enzyme (Wedzicha 1992).

Failure of potassium metabisulfite to protect against the degradation of anthocyanins could also be due to the fact that some of the sulfite was tight up in the enzyme sulfite complex after the enzyme had enough time to react with 4-methyl catechol and form quinones that reacted with anthocyanins.

Table 3. Slopes of the inhibitor regression lines.

Treatment	slope
Benzoate	0.307 b
Sorbate	0.078 c
Sulfite	0.54 a
Benzoate/sulfite	0.528 a
LSD	0.0785
CV	11.45

Means within the column followed by the same letter (abc) are not significantly Different ($p < 0.05$)

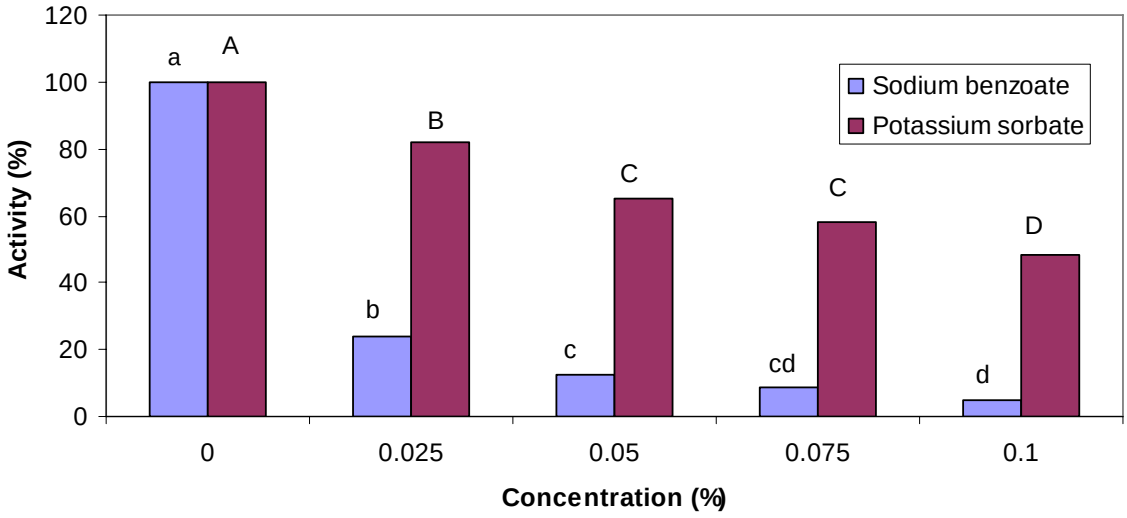


Figure 16. Effect of sodium benzoate and potassium sorbate on blueberry PPO activity

Means followed by the same letter (abcd and ABCD) are not significantly different ($p<0.05$).

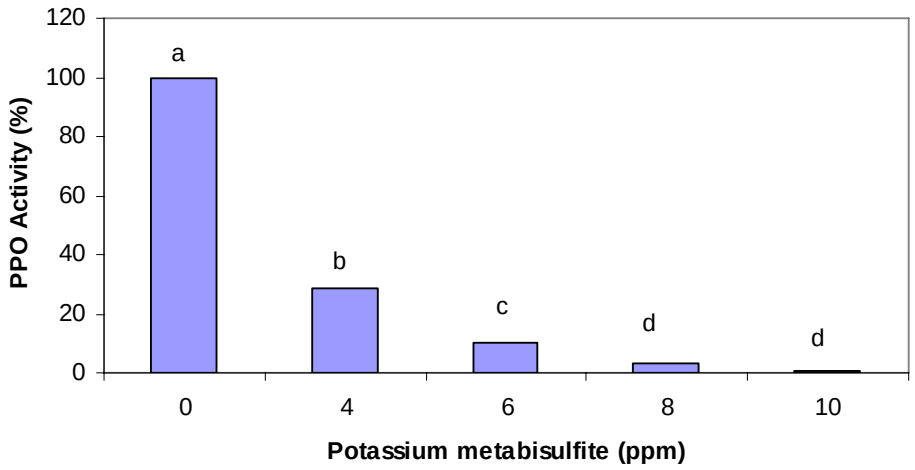


Figure 17. Effect of potassium metabisulfite on blueberry PPO activity

Means followed by the same letter (abcd) are not significantly different ($p<0.05$)

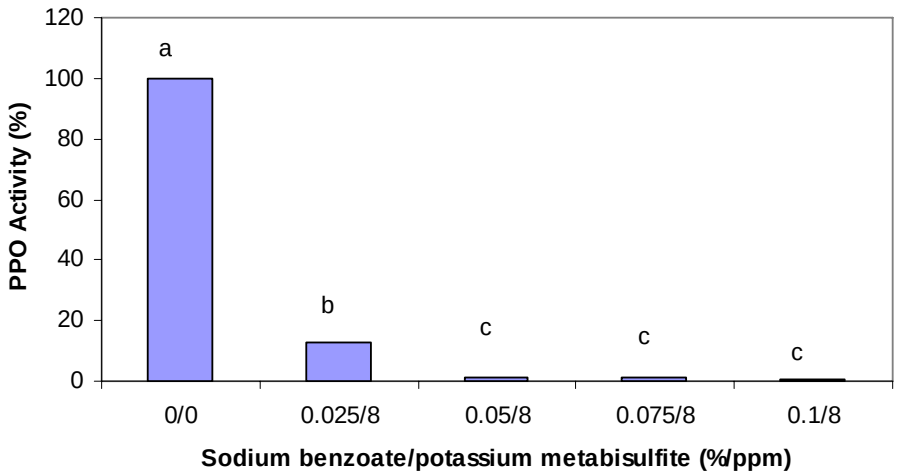


Figure 18. Effect of combination of sodium benzoate and potassium metabisulfite on blueberry PPO activity

Means followed by the same letter (abcd) are not significantly different ($p<0.05$).

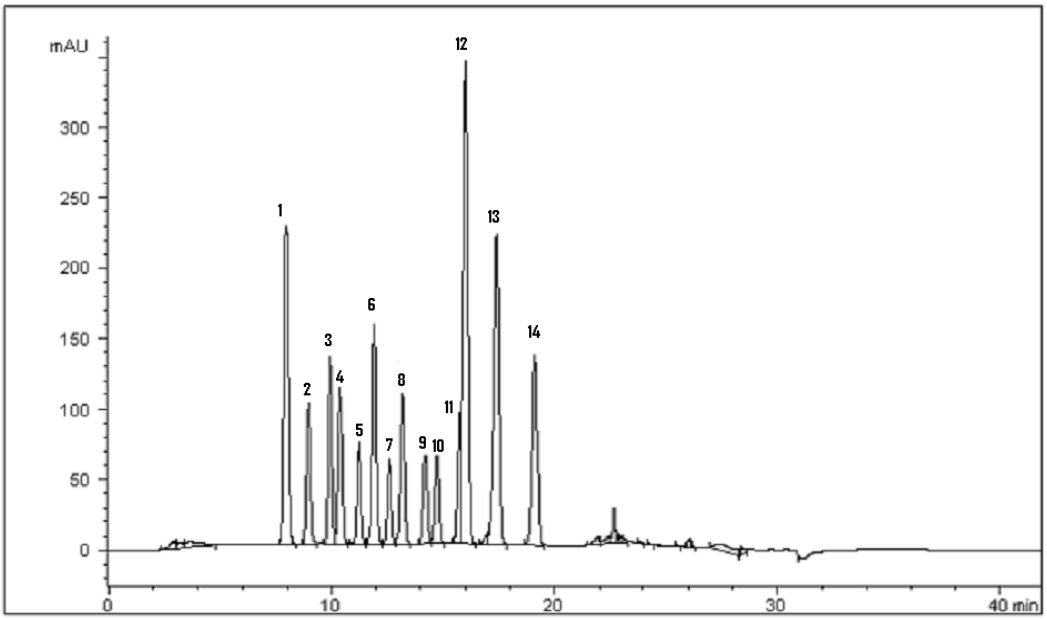


Figure 19. HPLC chromatogram of 14 anthocyanins detected at 520 nm using DAD

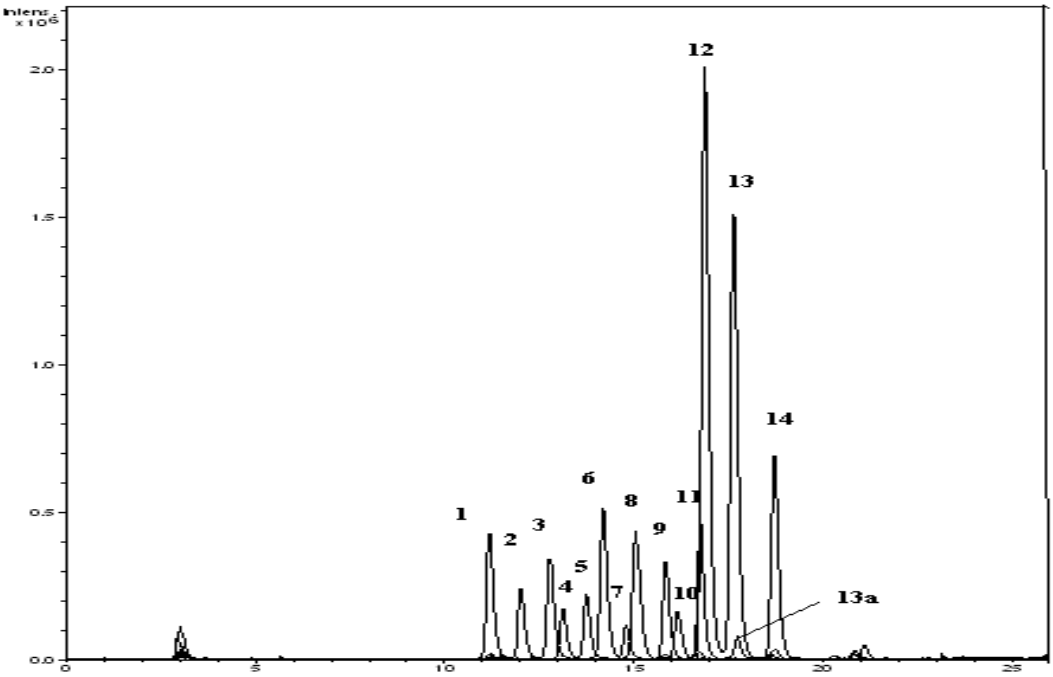


Figure 20. Extracted ion chromatogram of 15 identified anthocyanins

Table 4. Retention times, % area and m/z of total and aglucone of identified anthocyanins in blueberry extract using High Pressure Liquid Chromatography

peak #	Name	Abbreviation	RT	% area	m/z total	m/z aglucone
1	Delphinidin-3-galactoside	Dp-3-gal	7.3	10	465	303
2	Delphinidin-3-glucoside	Dp-3-glc	8.24	4.83	465	303
3	Cyanidin-3-galactoside	Cy-3-gal	9.2	5.68	449	287
4	Delphinidin-3-arabinoside	Dp-3-ara	9.62	5.03	435	303
5	Cyanidin-3-glucoside	Cy-3-glc	10.5	3.3	449	287
6	Petunidin-3-galactoside	Pt-3-gal	11.2	7.3	479	317
7	Cyanidin-3-arabinoside	Cy-3-ara	11.83	2.6	419	287
8	Petunidin-3-glucoside	Pt-3-glc	12.4	5.6	479	317
9	Peonidin-3-galactoside	Pn-3-gal	13.5	2.8	463	301
10	Petunidin-3-arabinoside	Pt-3--ara	14.02	3.1	449	317
11	Peonidin-3-glucoside	Pn-3-glc	15.03	3.8	463	301
12	Malvidin-3-galactoside	Mv-3-gal	15.33	18.9	493	331
13	Malvidin-3-glucoside	Mv-3-glc	16.7	15	493	331
13a*	Peonidin-3-arabinoside*	Pn-3-ara	16.7		433	301
14	Malvidin-3-arabinoside	Mv-3-ara	18.4	8.2	463	331

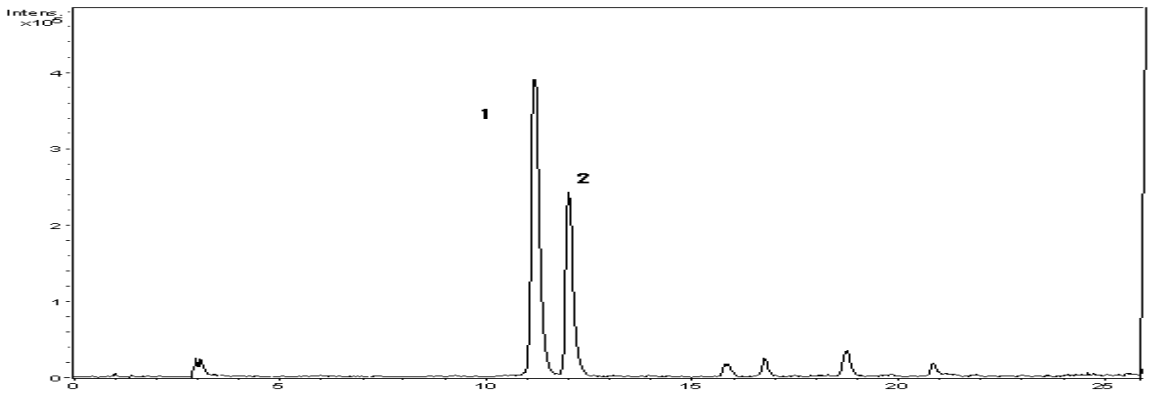


Figure 21. Extracted chromatogram of delphinidin 3-galactoside and delphinidin 3-glucoside m/z 465

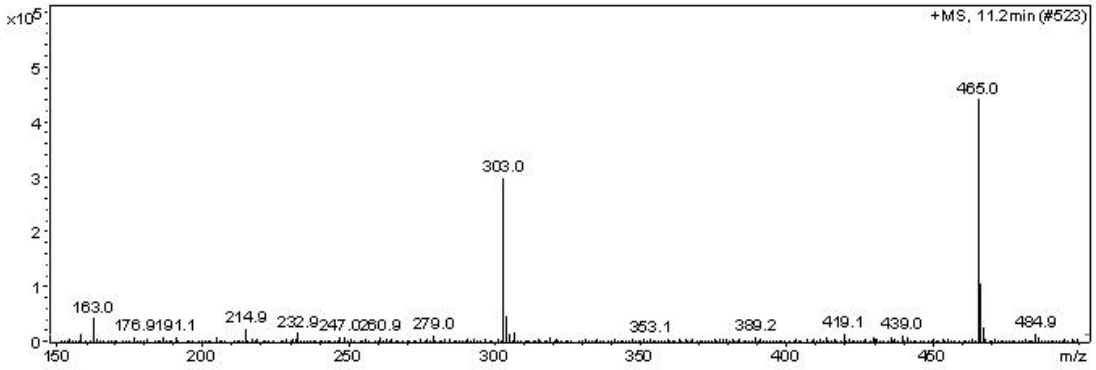


Figure 22. Ion chromatogram of delphinidin 3-galactoside (peak # 1)

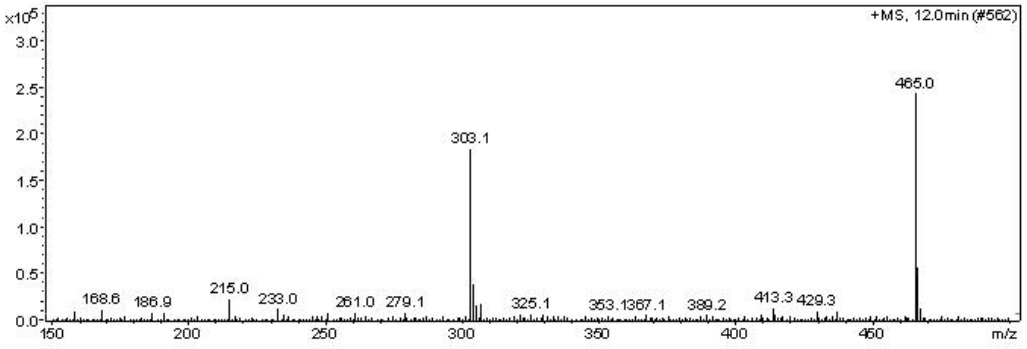


Figure 23. Ion chromatogram of delphinidin 3-glucoside (peak # 2)

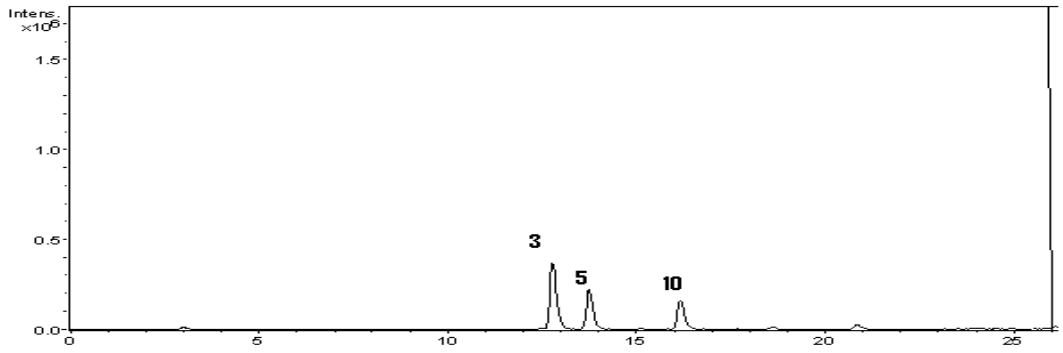


Figure 24. Extracted chromatogram of cyanidin 3-galactoside, cyanidin 3-glucoside and petunidin 3-arabinoside m/z 449

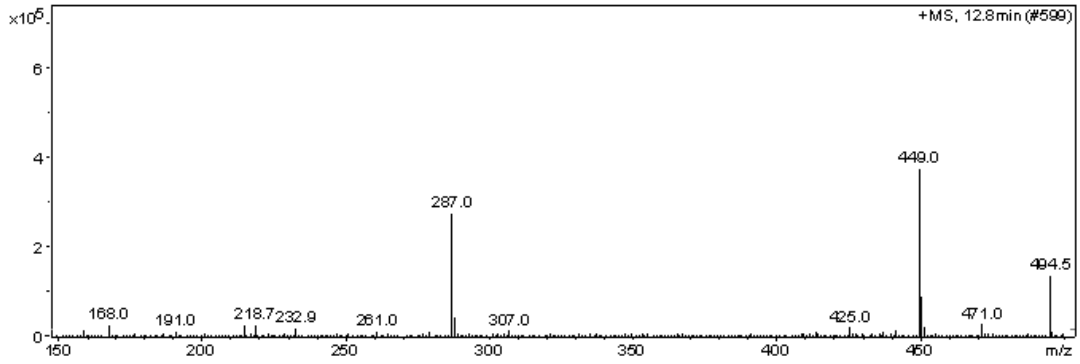


Figure 25. Ion chromatogram of cyanidin 3-galactoside (peak # 3)

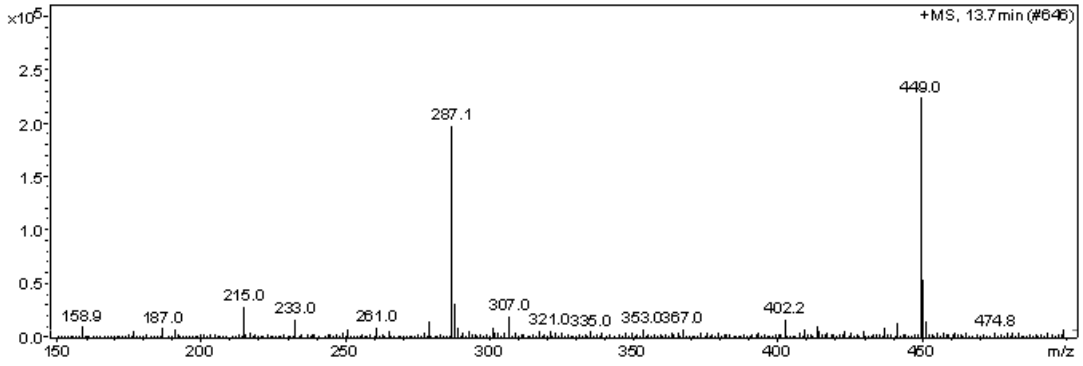


Figure 26. Ion chromatogram of cyanidin 3-glucoside (peak # 5)

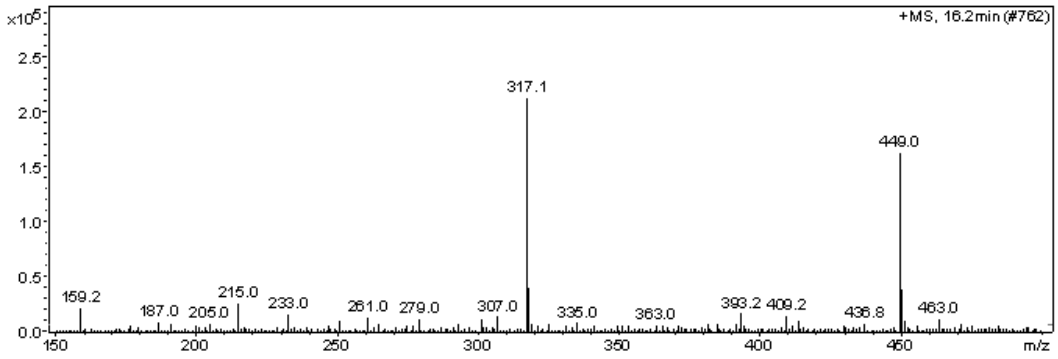


Figure 27 . Ion chromatogram of petunidin 3-arabinoside (peak # 10).

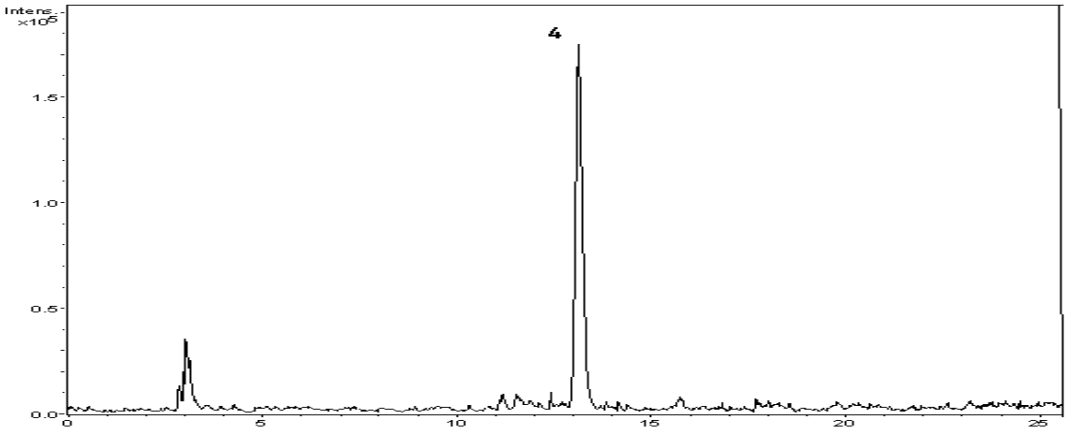


Figure 28. Extracted chromatogram of delphinidin 3- arabinoside m/z 435

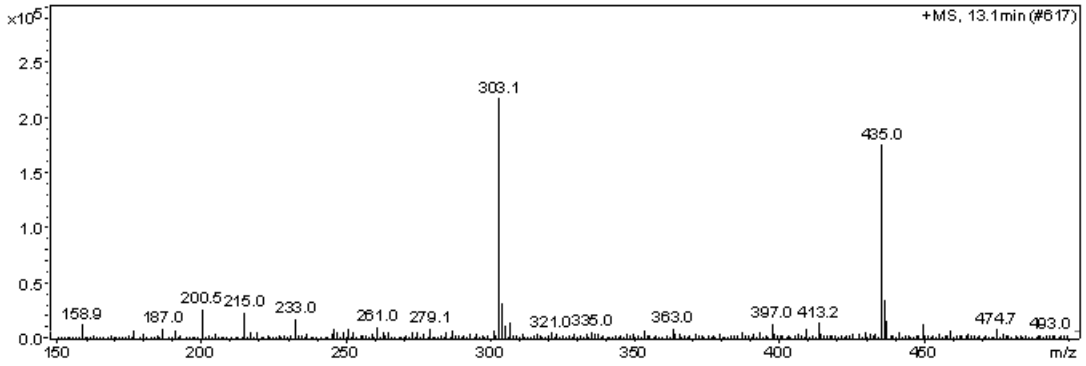


Figure 29. Ion chromatogram of delphinidin 3- arabinoside (peak # 4)

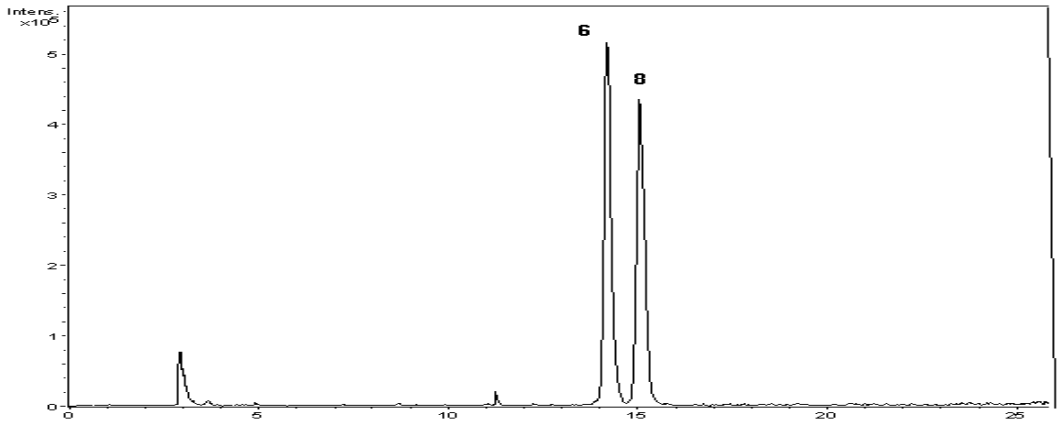


Figure 30. Extracted chromatogram of petunidin 3-galactoside and petunidin 3-glucoside m/z 479.

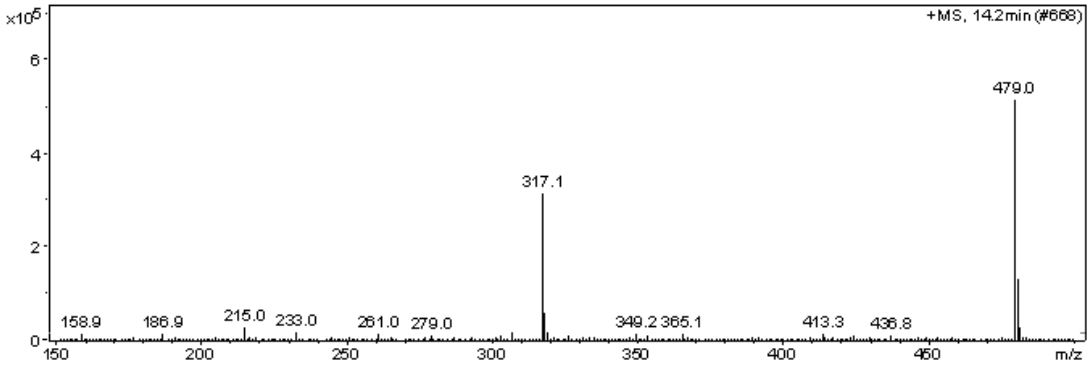


Figure 31. Ion chromatogram of petunidin 3-galactoside (peak # 6)

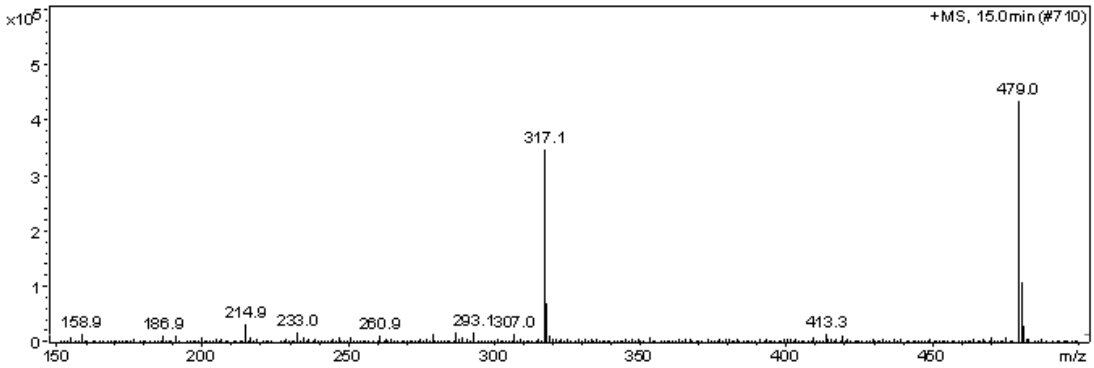


Figure 32. Ion chromatogram of petunidin 3-glucoside (peak # 8)

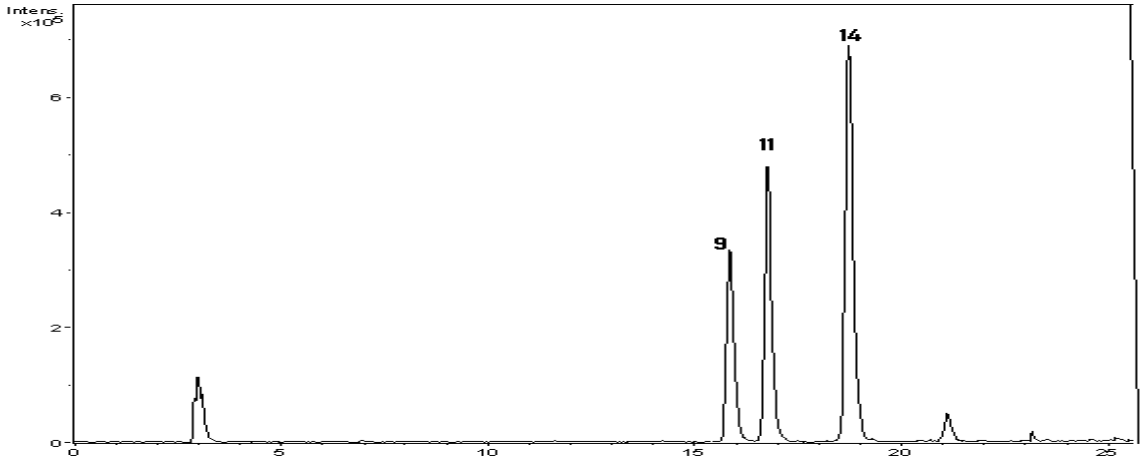


Figure 33. Extracted chromatogram of peonidin 3-galactoside, peonidin 3-glucoside and malvidin 3-arabinoside m/z 463

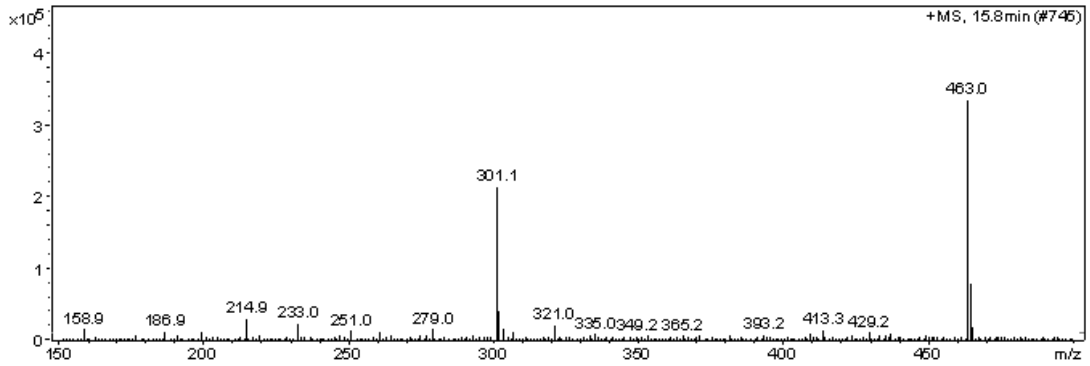


Figure 34. Ion chromatogram of peonidin 3-galactoside (peak # 9)

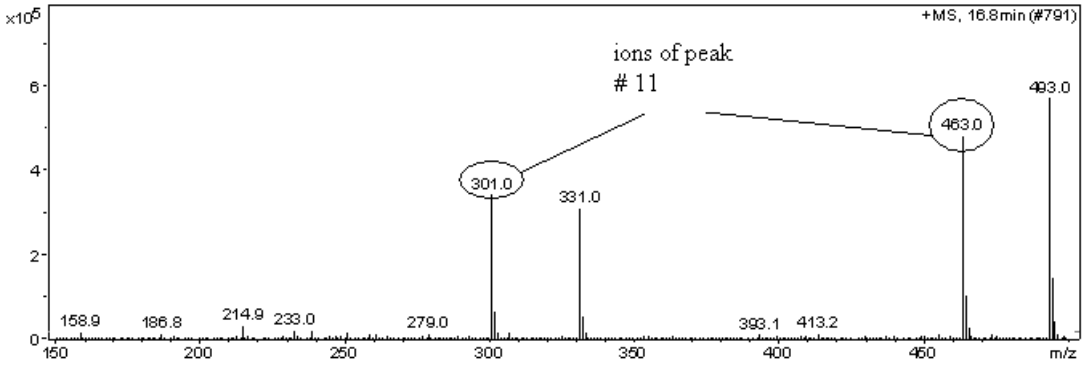


Figure 35. Ion chromatogram of peonidin 3-glucoside (peak # 11)

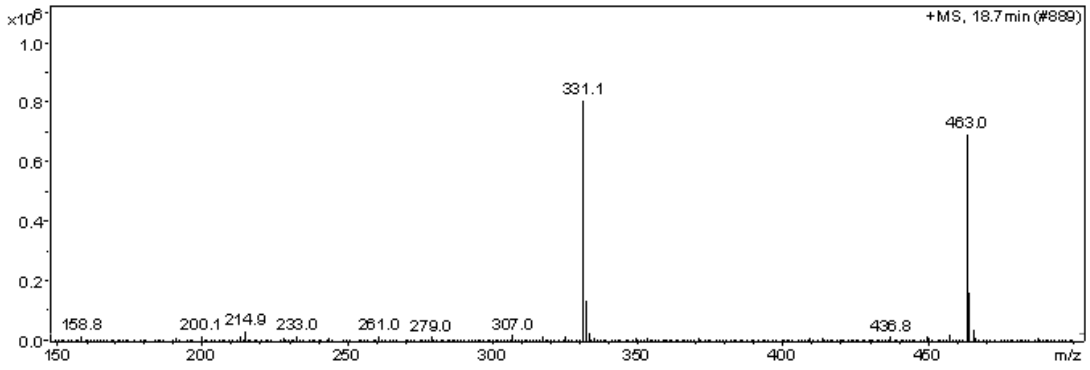


Figure 36. Ion chromatogram of malvidin 3-arabinoside (peak # 14)

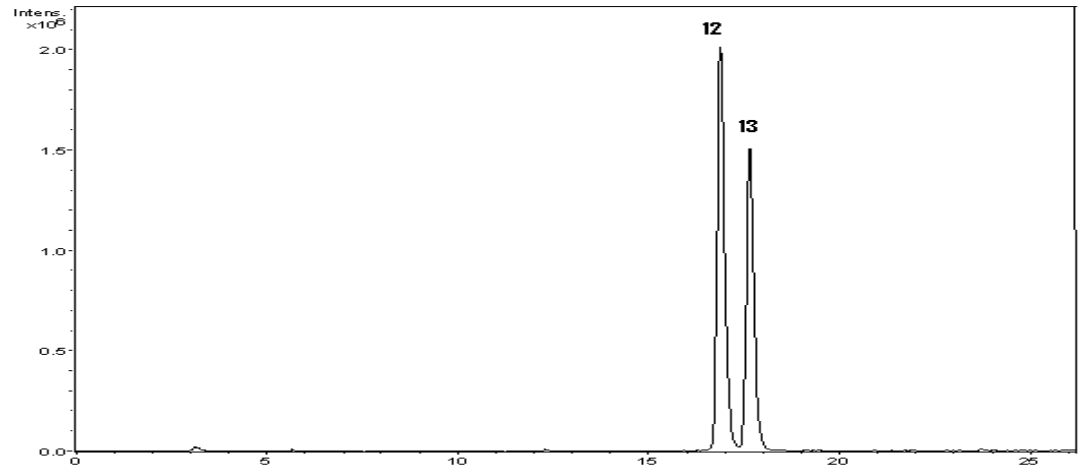


Figure 37. Extracted chromatograms of malvidin 3-galactoside and malvidin 3-glucoside m/z 493

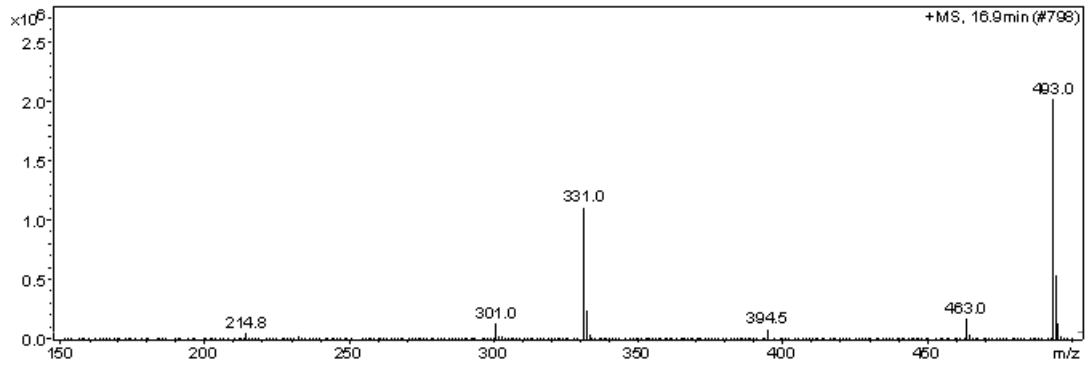


Figure 38. Ion chromatogram of malvidin 3-galactoside (peak #12)

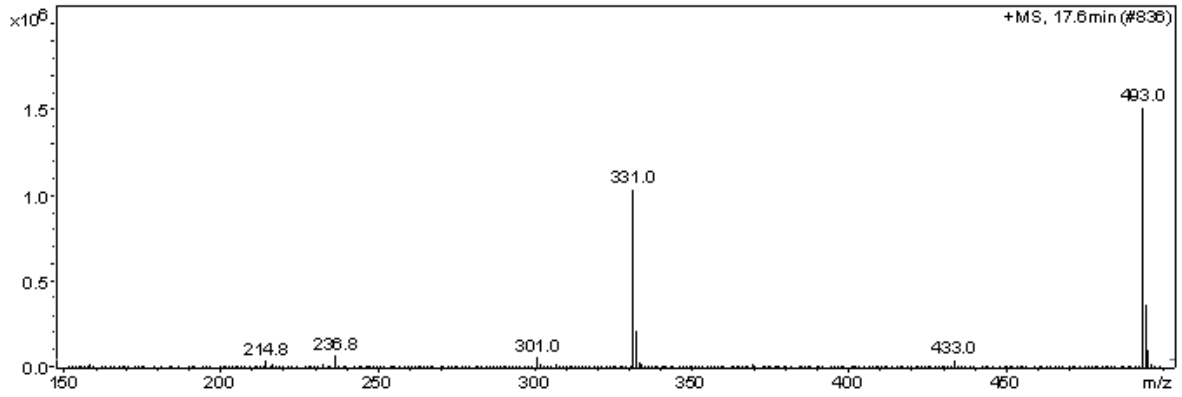


Figure 39. Ion chromatogram of malvidin 3-glucoside (peak #13)

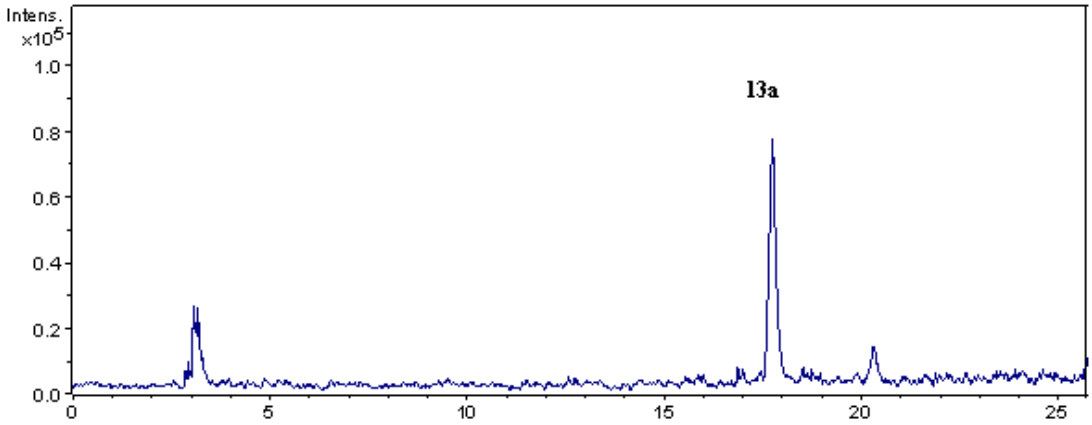


Figure 40. Extracted chromatogram of peonidin 3-arabinoside m/z 433

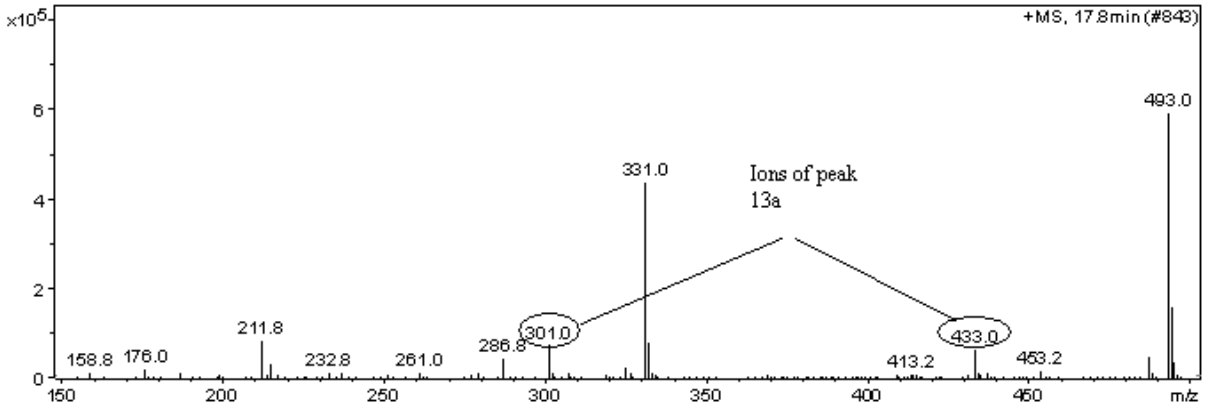


Figure 41. Ion chromatogram of peonidin 3-arabinoside

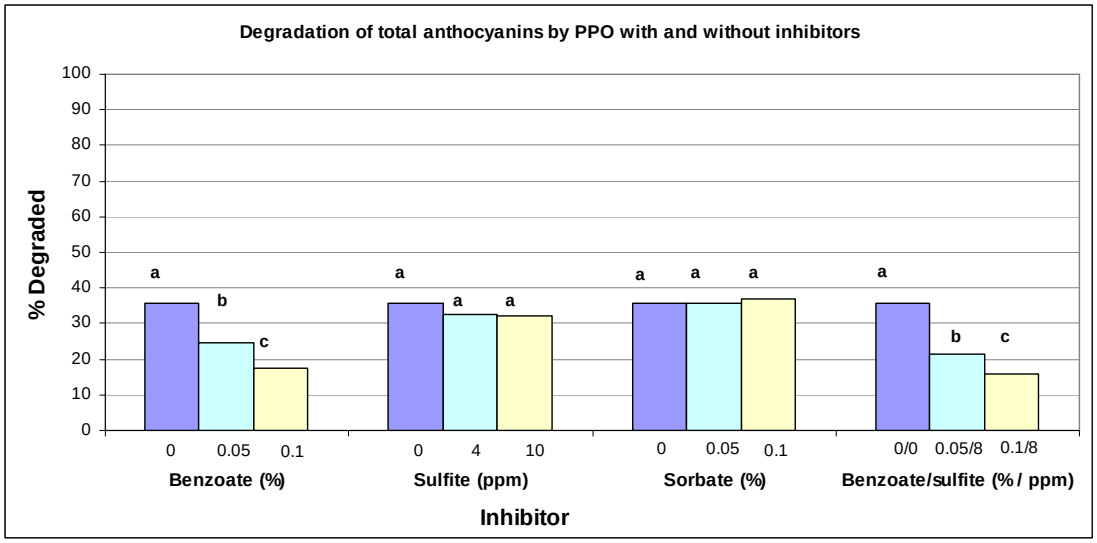


Figure 42. Degradation of total anthocyanins by blueberry PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

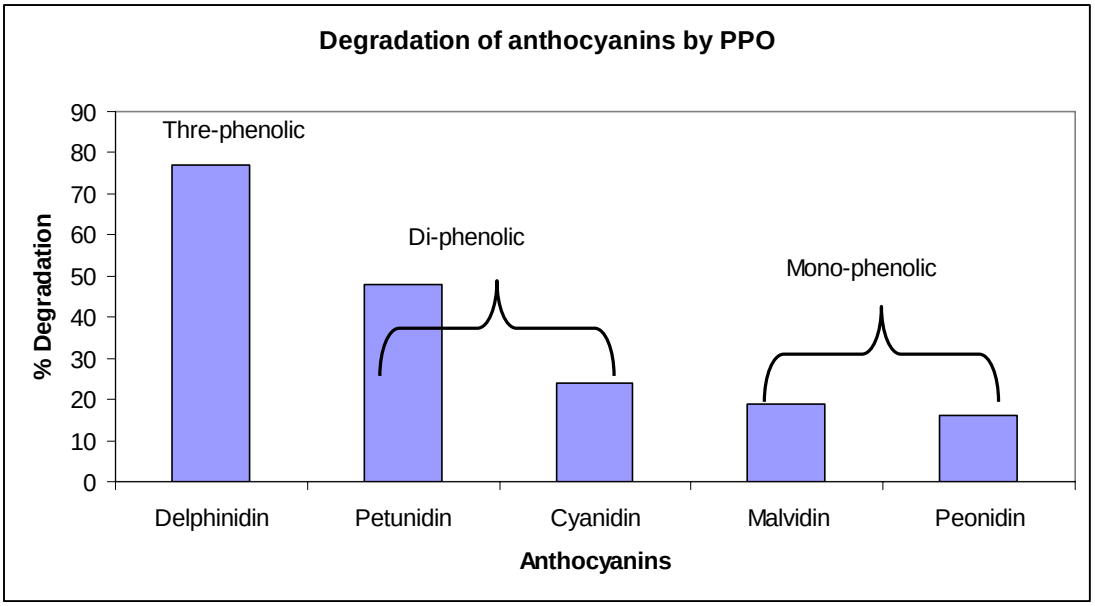


Figure 43. Degradation of anthocyanins by blueberry PPO

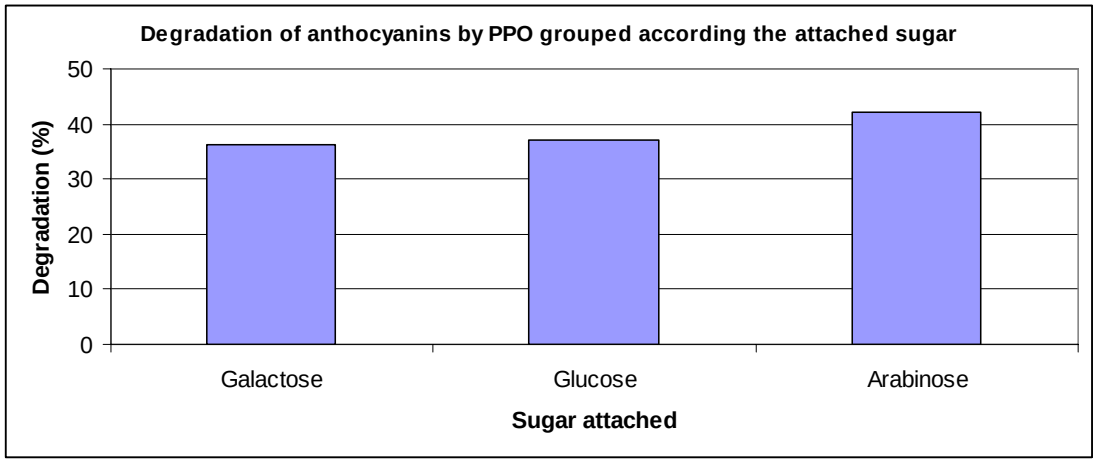


Figure 44. Degradation of anthocyanins by PPO grouped according to the sugar in the molecule

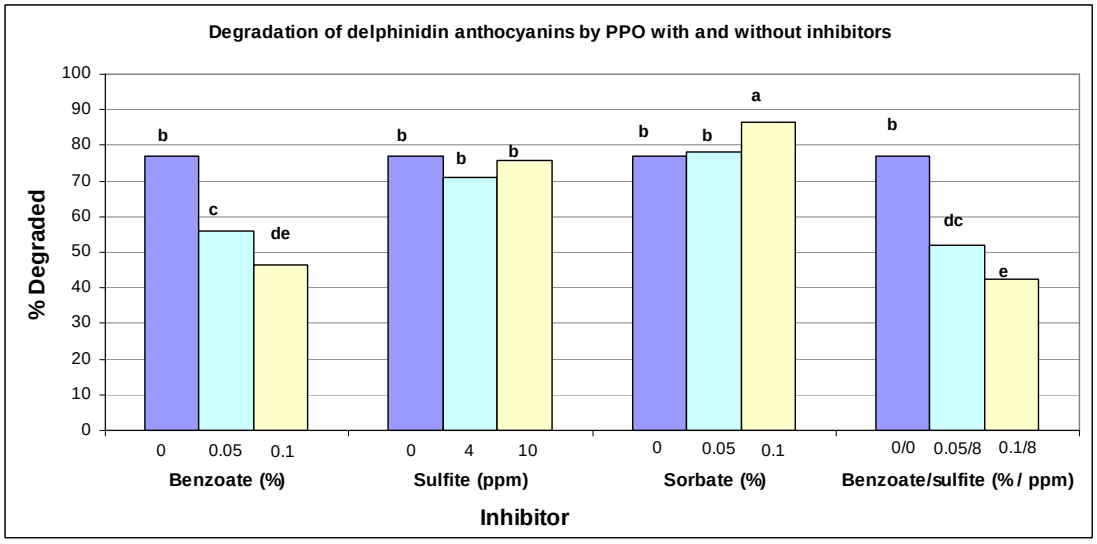


Figure 45. Degradation of delphinidin anthocyanins by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

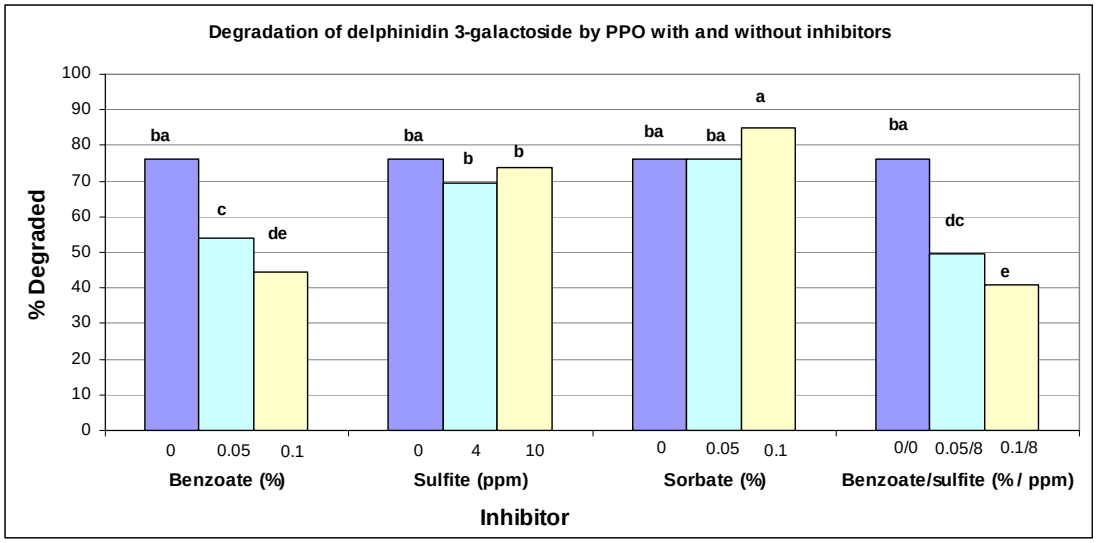


Figure 46. Degradation of delphinidin 3-galactoside by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

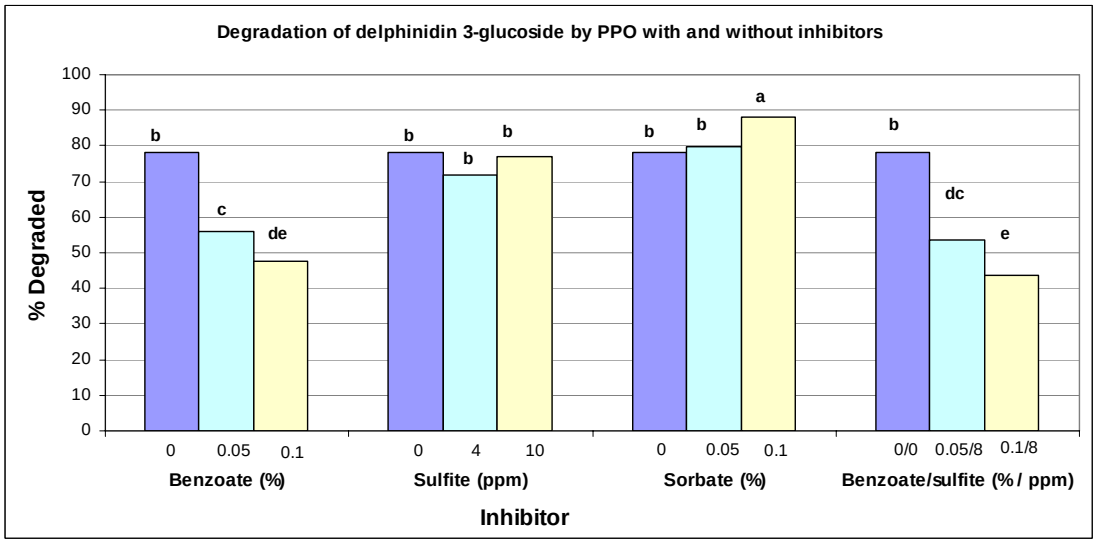


Figure 47. Degradation of delphinidin 3-glucosde by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

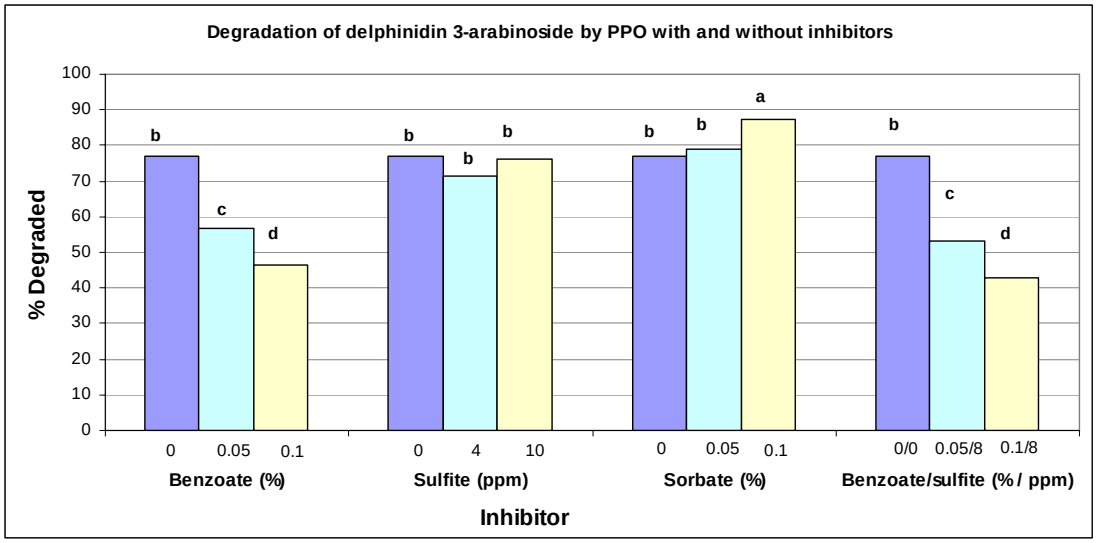


Figure 48. Degradation of delphinidin 3-arabinosede by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

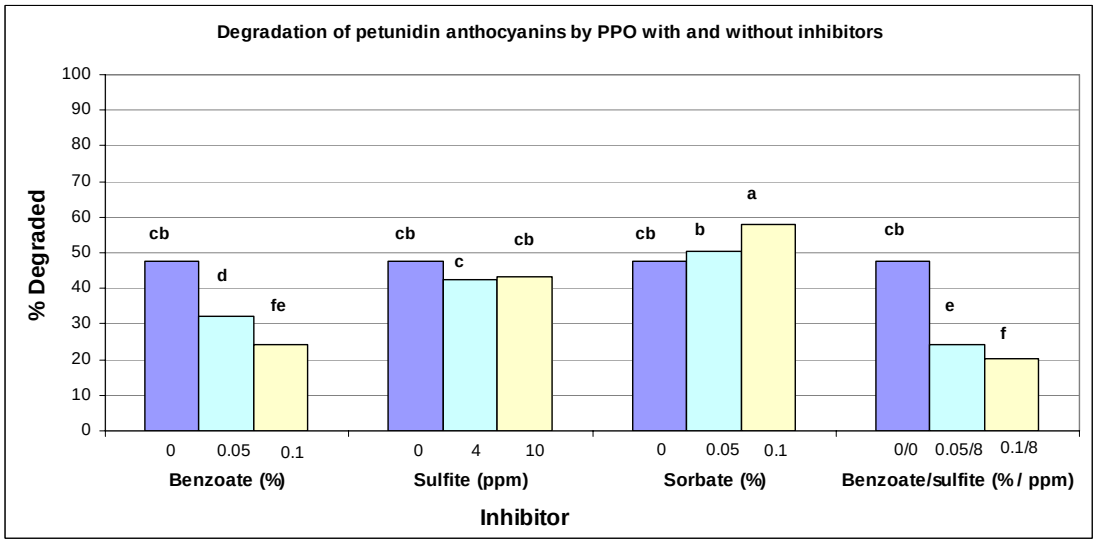


Figure 49. Degradation of petunidin anthocyanins by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

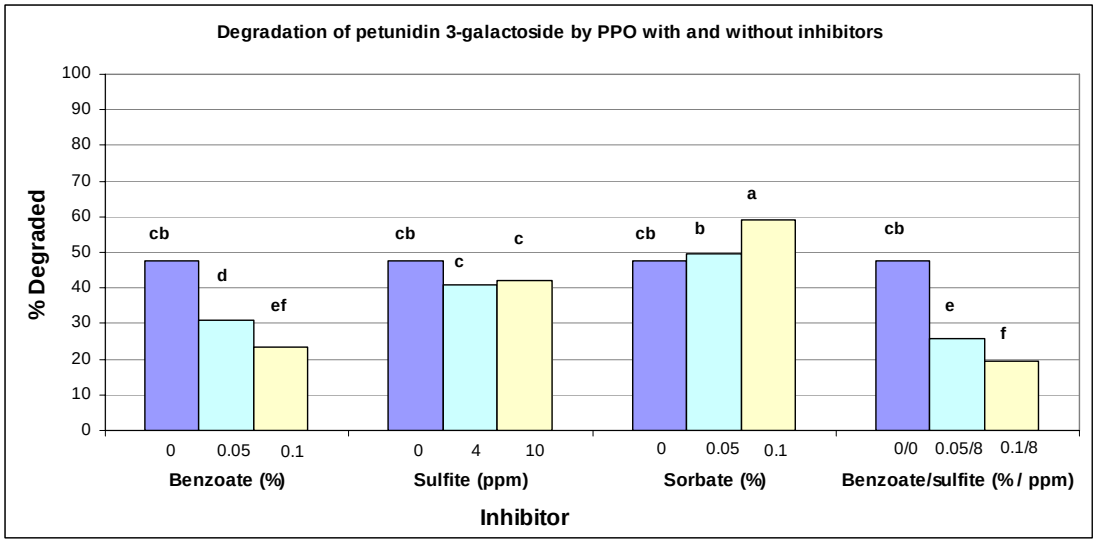


Figure 50. Degradation of petunidin 3-galactoside by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

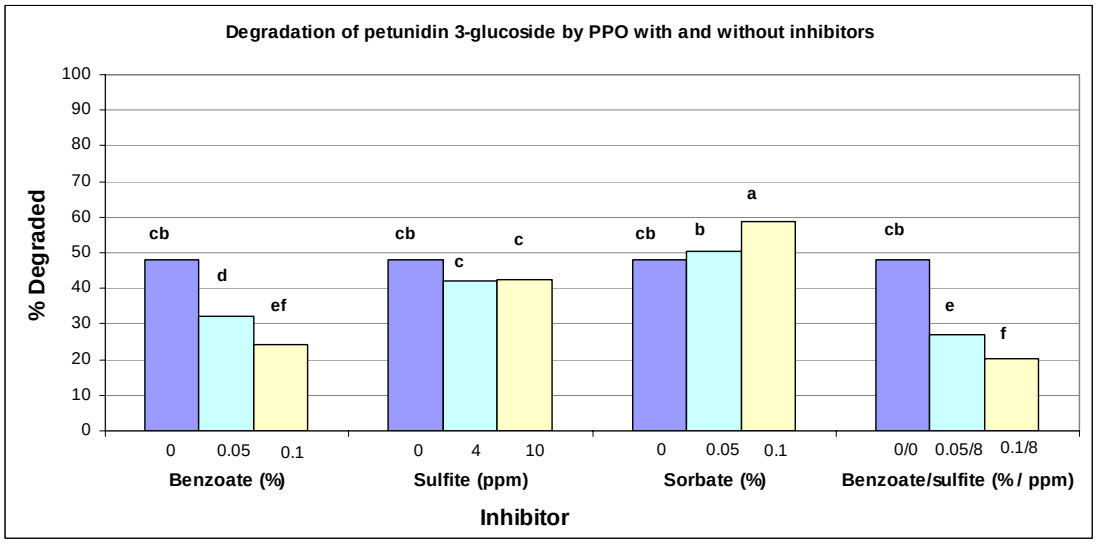


Figure 51. Degradation of petunidin 3-glucoside by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

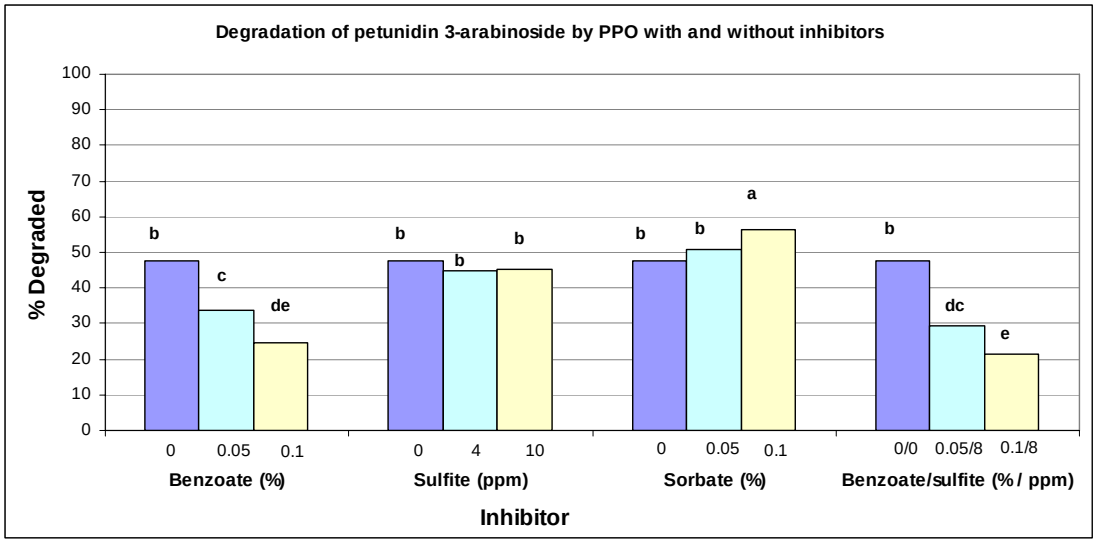


Figure 52. Degradation of petunidin 3-arabinoside by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

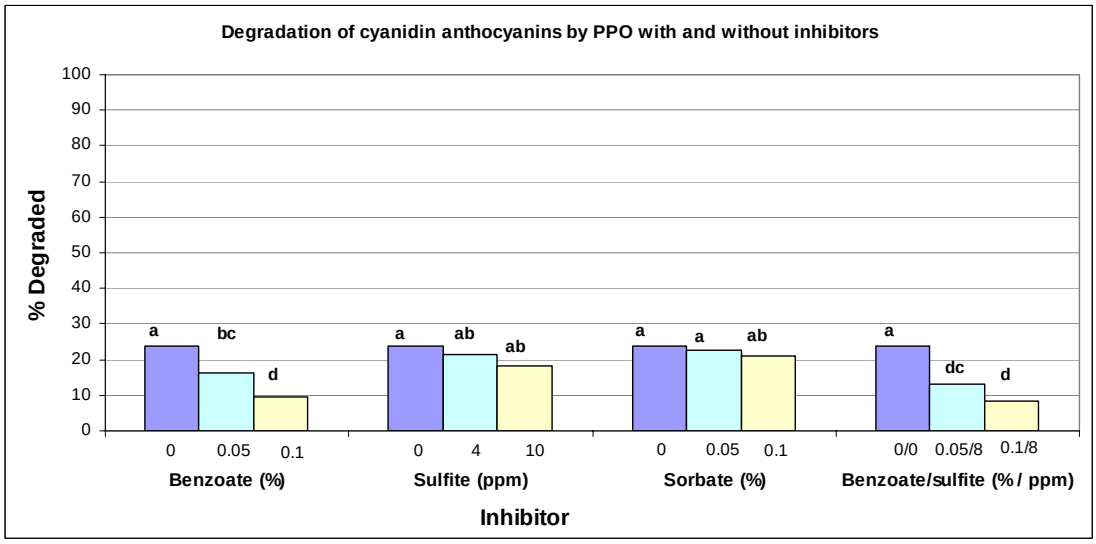


Figure 53. Degradation of cyanidin anthocyanins by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

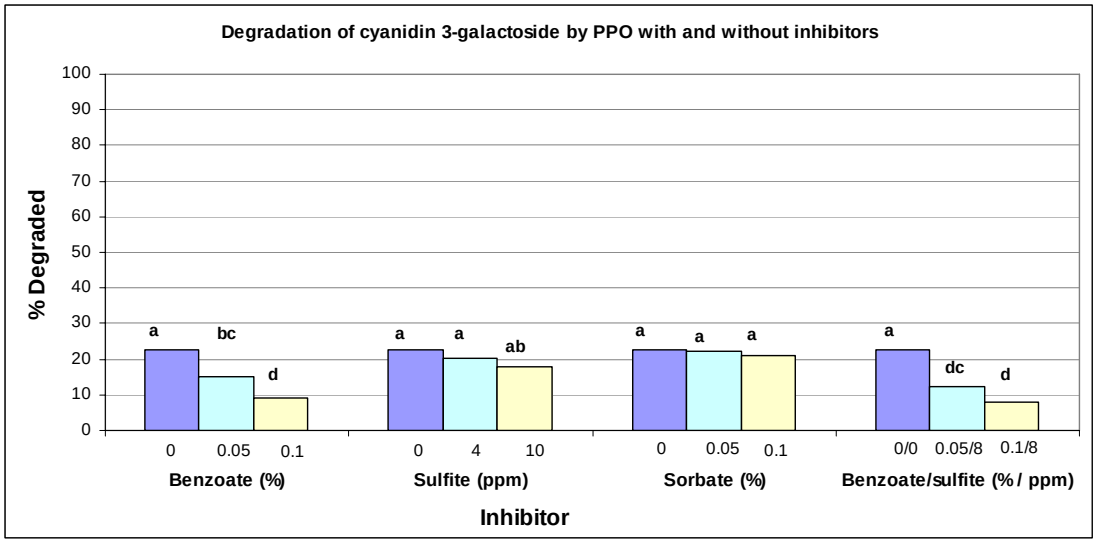


Figure 54. Degradation of cyanidin 3-galactoside by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

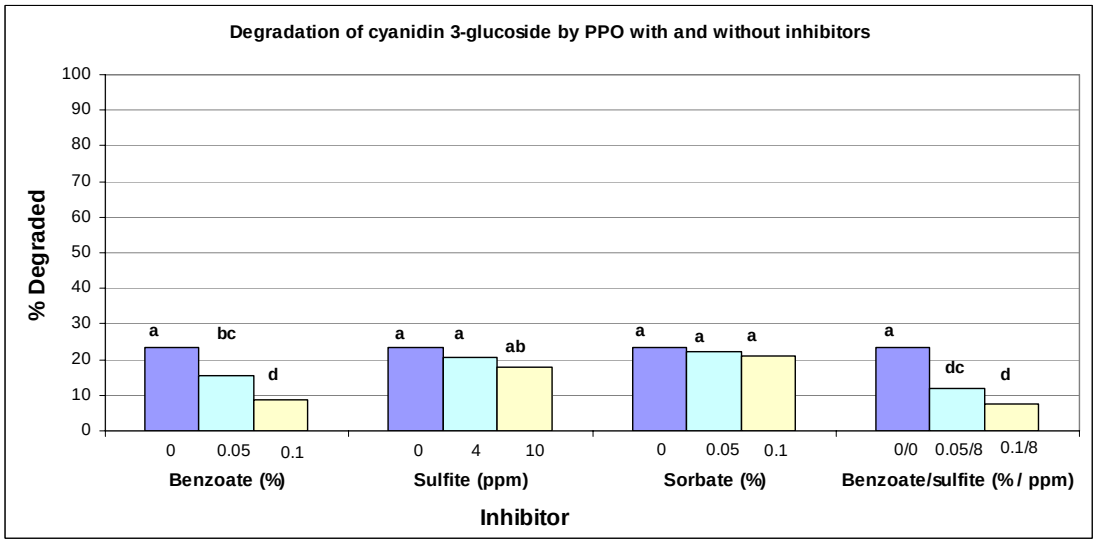


Figure 55. Degradation of cyanidin 3-glucoside by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

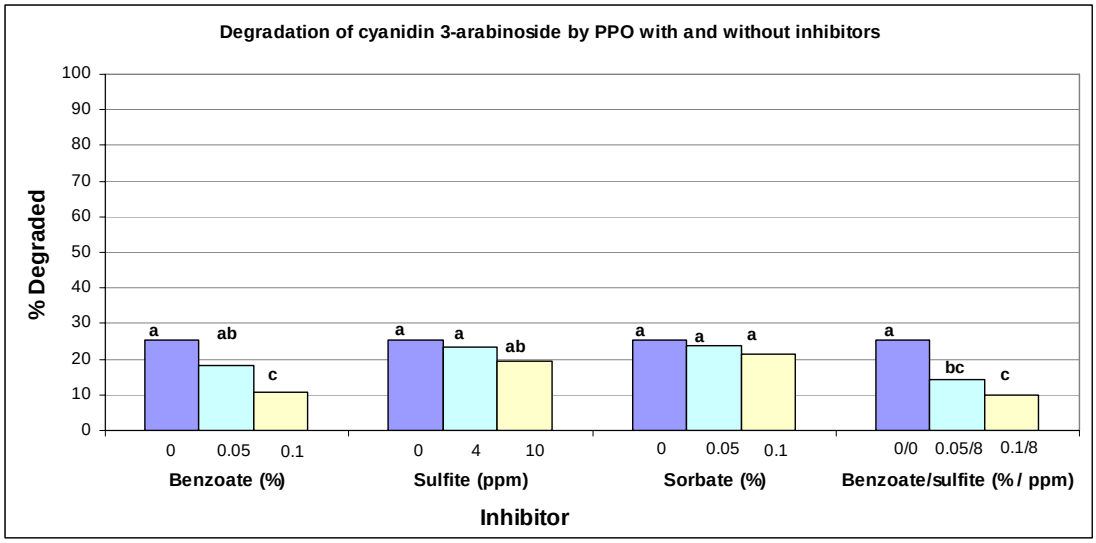


Figure 56. Degradation of cyanidin 3-arabinoside by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

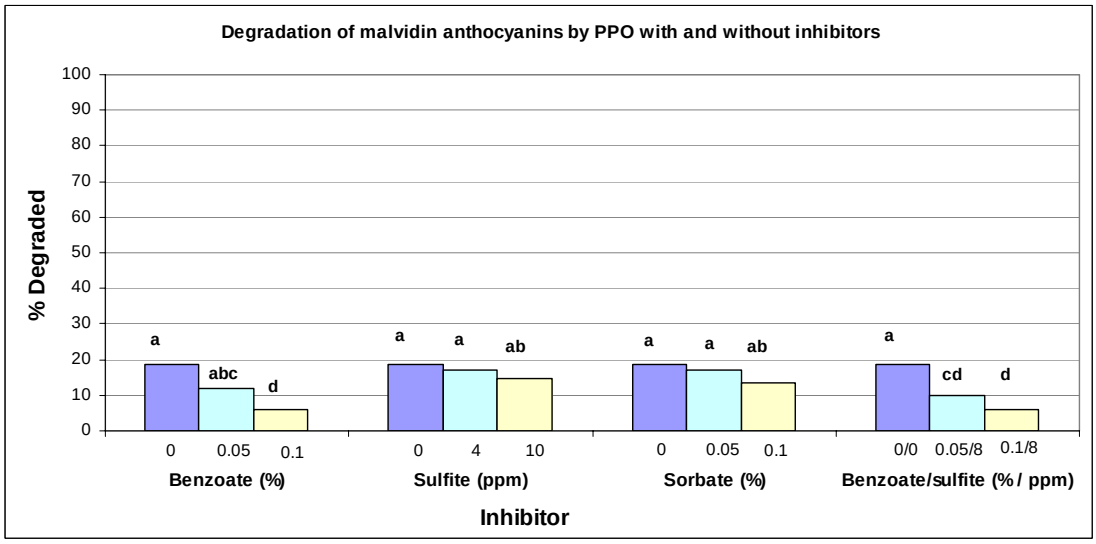


Figure 57. Degradation of malvidin anthocyanins by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

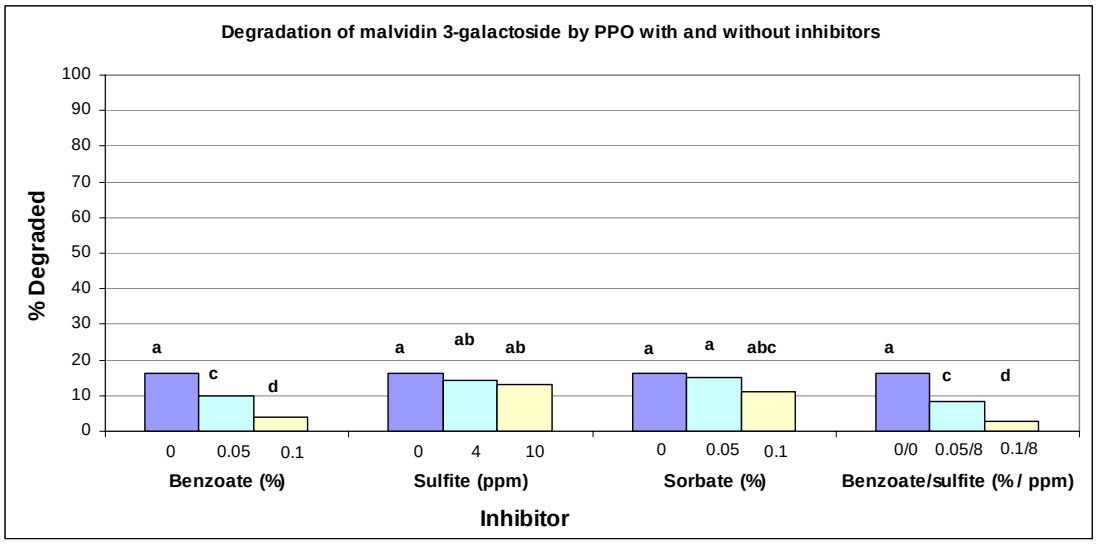


Figure 58. Degradation of malvidin 3-galactoside by PPO with and without Inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

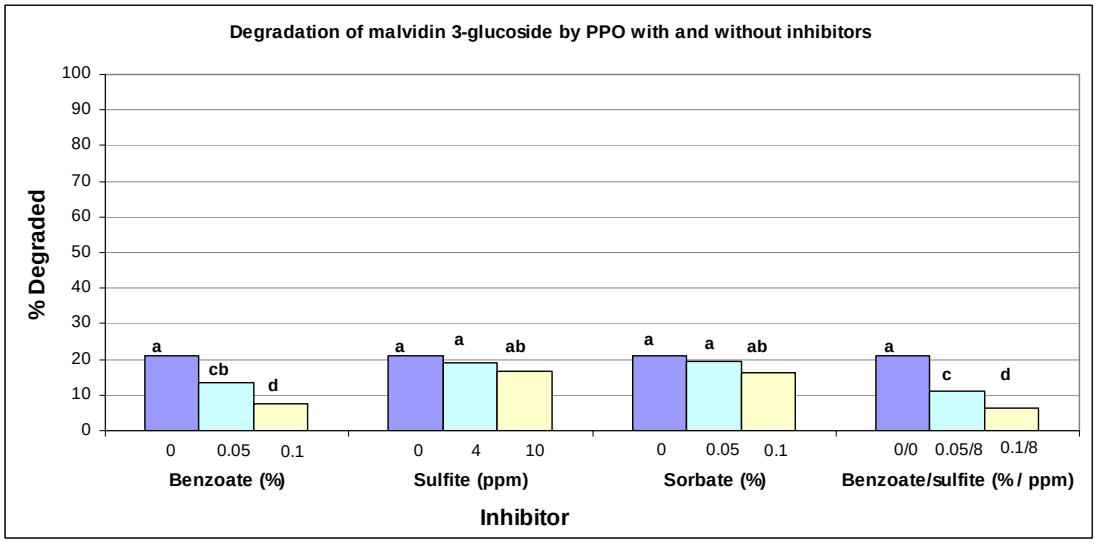


Figure 59. Degradation of malvidin 3-glucoside by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

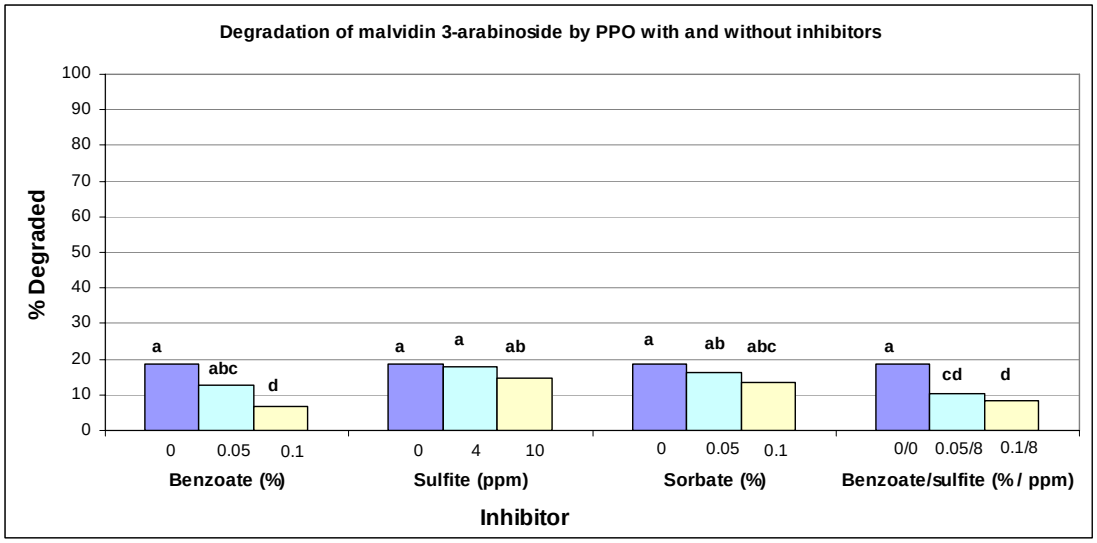


Figure 60. Degradation of malvidin 3-arabinoside by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

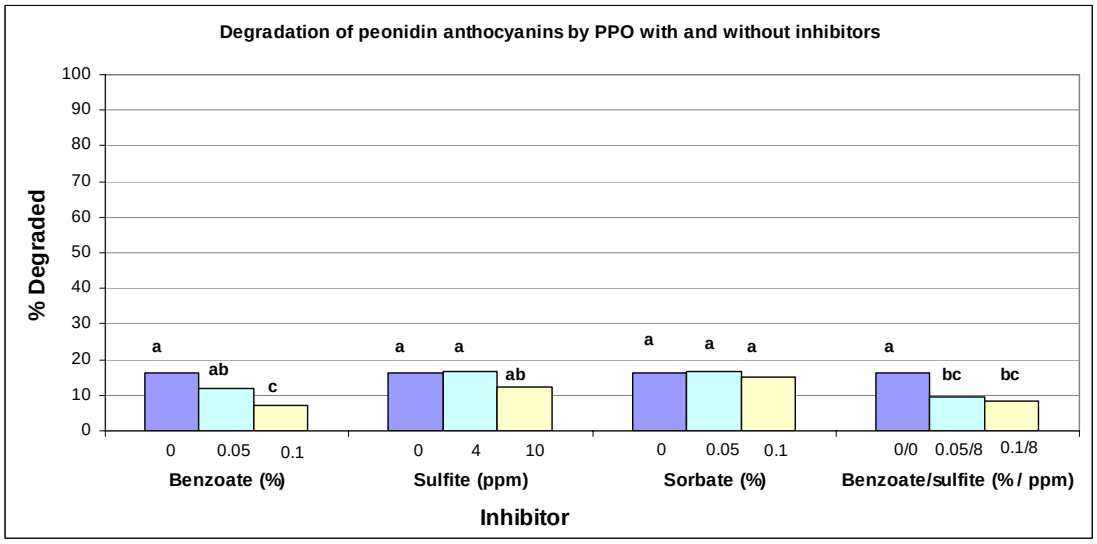


Figure 61. Degradation of peonidin anthocyanins by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

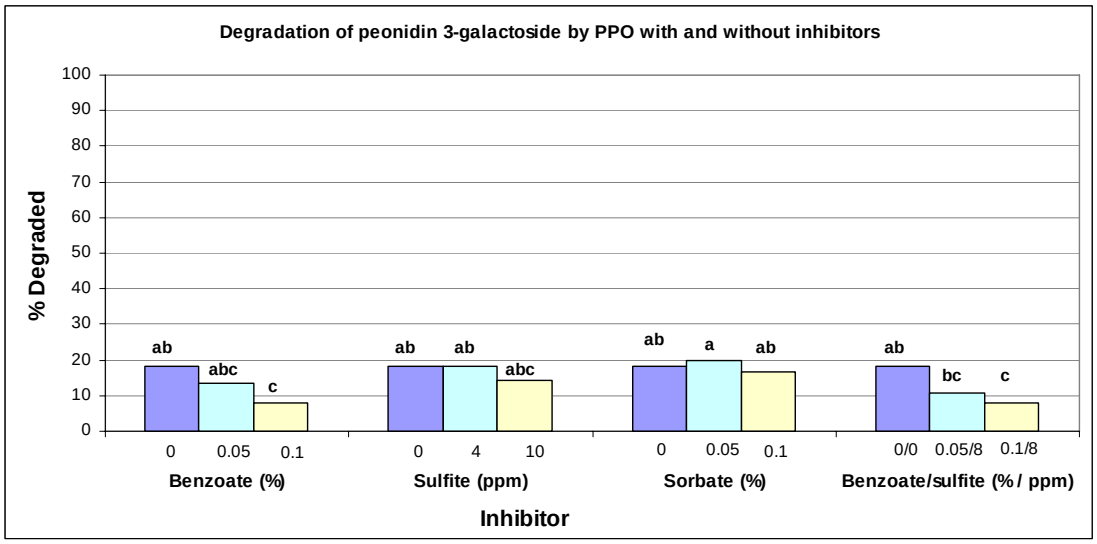


Figure 62. Degradation of peonidin 3-galactoside by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

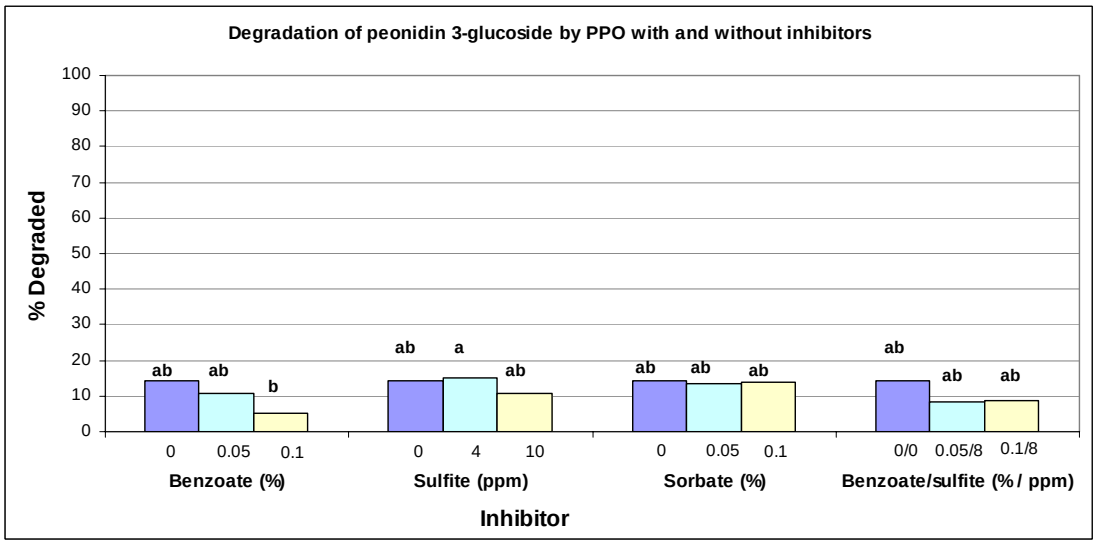


Figure 63. Degradation of peonidin 3-glucoside by PPO with and without inhibitors

Means followed by the same letter are not significantly different ($p<0.05$)

EXPERIMENT II

Identification and quantification of anthocyanins

Fourteen different anthocyanins were identified in the frozen rabbitey blueberry extract (Table 4) and clear peak separations can been seen on the HPLC chromatogram detected at 520 nm (Figure 19). Separation of Peonidin 3- glucoside and malvidin 3-galactoside, peaks number 11 and 12 (Figure 19) was variable. In most of the analysis for different treatments, the two coeluted and no clear pattern of separation could be found. Because of that, it was impossible to analyze data for peonidin 3- glucoside, so the data was omitted in all analysis. Determination of total anthocyanins by HPLC method was a summation of individual anthocyanin peaks detected at 520 nm. Individual anthocyanins were quantified by comparisons with an external standard of cyanidin-3-glucoside.

Concentration of total anthocyanins in frozen blueberries was 328 mg/100 g. These value fall within the range of 25 to 495mg/100g for highbush blueberries (Mazza and Miniati 1993). In the frozen berries, malvidin glucoside was the most abundant (50.43%) followed by delphinidin (19.3), petunidin (15.64), cyanidin (10.68%) and peonidin (4.1%). Concentrations of individual anthocyanins expressed as mg/L of cyanidin 3–glucoside are presented in table 3. Malvidin and delphinidin derivatives were the major anthocyanins in highbush blueberries (77.2%) (*Vaccinium corymbosum* L.cv. Rubel) (Lee and others 2002). Malvidin 3-galactoside was a major anthocyanin in highbush 'Rubel' blueberries grown in Oregon with malvidin glycosides being most abundant accounting for 54.7% of all anthocyanins followed by delphinidin (25.7%),

petunidin (14.7%), cyanidin (3.9%) and peonidin (1%) based on the peak area percentage (Lee and Wrolstad 2004). Malvidin 3-galactoside predominates in lowbush (*V. angustifolium)* and 'Tifblue' (*V. ashei*) blueberries (Prior and others 2001), in Highbush 'Rubel' blueberries (Lee and others 2002) and rabbiteye (*V. ashei*) blueberries grown in Japan (Nakajima and others 2004). Malvidin derivatives were most abundant in highbush blueberries, comprising 44% of the total anthocyanins in blueberries (Skrede and others 2000).

Identification and quantification of acids

Separation of acid standards (Figure 64) and acids in the blueberry extract (Figure 65) was carried out at 214 nm. The acids that were identified in frozen blueberry extract were quinic, malic, citric and shikimic. The most abundant acid identified in the blueberry extract was quinic 941.6 mg/100g, followed by malic 89.4 mg/100g, citric 29.4 mg/100g and shikimic 8.9mg/100g (Table 6). Conflicting reports about acid concentrations were found in the literature. In rabbiteye fruit, the percentage contribution of citric, succinic, malic and quinic acid is about 10%, 50%, 34% and 6%, respectively (Kalt and others 1996). In the present research, blueberry extract was also screened for succinic acid but it was not detected. Other researchers reported a average of 95% citric acid and 1% to 2% each of quinic and malic acid in ripe "Wolcott" fruit (Kushman and Ballinger 1968). Succinic and malic acids were the predominant acids in rabbiteye cultivars averaging 50% and 33%, respectively (Ehlenfeldt and others 1994). Among rabbiteye clones, citric acid averaged 10% and was never found to be more than 22%,

while quinic acid was consistently present only as a minor constituent, averaging 6% (Ehlenfeldt and others 1994).

PPO activity in blueberry mash

Maceration treatment affected ($p<0.05$) PPO activity (Figure 70). Maceration temperature at 55ºC reduced ($p<0.05$) PPO activity from 233 to 52.3 μmolO_2/min/100g. Although there were no significant differences between the control treatment (60 min maceration at 55ºC) and heat treatment, no PPO activity was detected in blueberry mash that was heat treated at 90C and then macerated for 60 min at 55ºC. This is to be expected since PPO is not an extremely heat-stable enzyme. Short exposures, in the tissue and solutions, to temperatures of 70 to 90ºC are, in most cases, sufficient for partial or total irreversible destruction of its catalytic function (Vamos-Vigyazo 1981). Blanching blueberry fruit for 3 min in a steam tunnel totally inactivated PPO (Rossi and others 2003). Inactivation of PPO did not begin to occur until the grape juice was heated above 60ºC and heating above 87ºC was necessary to completely inactivate the enzyme (Montgomery and others 1982). Activity of the PPO was reduced 100 times when the temperature was raised to 88-99ºC, to 4 and 3 μmoles O_2/min/2.5ml from 445 μmoles O_2/min/2.5ml, while at 60ºC PPO activity decreased 10 times to 44 μmoles O2/min/2.5ml (Montgomery and others 1982). The PPO in crude extracts of plum, apple, pear and avocado was inactivated at 60-65ºC, whereas in grape extracts, 55ºC was enough for inactivation (Weemaes and others 1998).

Addition of 0.1% of sodium benzoate to blueberry mash significantly reduced PPO activity compared to frozen blueberries but was also unexpectedly significantly

higher than a control sample without added inhibitor (Figure 70). It could be possible that the addition of sodium benzoate increased thermal stability of PPO and that the added amounts were not enough to inhibit enzyme. Thermal stability of PPO can be affected by some substances present in the food (Tomas-Barberan and Espin 2001a). Addition of both EDTA and benzoic acid increased the thermal stability of mushroom PPO, whereas gluthatione produced a sensitization to temperature treatments, probably due to an interaction with a disulphide bond in the enzyme (Weemaes and others 1997). Benzoic acid at 50 mM protected PPO from temperature treatment, thus increasing the thermal stability of the enzyme (Weemaes and others 1997). It could also be possible that some other phenolic compounds that are abundant in blueberries are bound to PPO without inhibiting activity but preventing sodium benzoate to bind to an enzyme and inhibit its activity. There are two distinct substrate binding sites on the enzyme molecule, one of which had a high affinity for aromatic compounds, including phenolic substrates, while the other, which probably contained the enzyme copper, served for metal-binding agents and oxygen (Duckworth and Coleman 1970). In the system with benzoic acid, phenolic substrate (4-methyl catechol) and oxygen are present, oxygen is the first substrate to be bound to the apple PPO forming $E\text{-}O_2$ complex to which benzoic acid as an inhibitor can bind only with enzymatic forms of PPO which are free of other phenolics i.e., E or $E\text{-}O_2$ (Janovitz-Klapp and others 1990b).

Degradation of anthocyanins, acids and phenolics in blueberry mash

Since blueberries contain anthocyanins that impart their color only in the skin, breaking the skin and maceration of the mash are necessary to extract the color and

obtain good quality juice. Freezing and heating of the blueberries had a more pronounced effect than pectolytic enzymes on the skin cells, and consequently on the liberation of color pigments. The loss of semipermeability and mechanical injury to the cell membranes appears to determine the total color released (Fuleki and Hope 1964). Significant differences were found between maceration treatments in respect to concentration of total anthocyanins (Figure 66). No significant differences were found between frozen blueberries and control mash treatment (Figure 66), proving that 55ºC was not high enough to affect anthocyanins but on the other hand was high enough to decrease PPO activity almost 4 times (Figure 70), thus protecting anthocyanins and preserving the red color.

Blueberry puree that was held for 60 min at 60ºC had higher concentrations of anthocyanins and phenolics than the one held at 25ºC, with anthocyanins being more affected than phenolics (Kalt and others 2000). The increased extraction of anthocyanins was attributed to the increased permeability of membranes in the macerated berries at higher temperatures and to decreased solubility of oxygen at higher temperatures that can prevent oxidative degradation (Kalt and others 2000). After 2h mash treatment at 50ºC, 97% of the original anthocyanin content in black currant berries were intact (Iversen 1999).

Maceration treatments with added sodium benzoate and heat had a significantly lower concentration of total anthocyanins (Figure 66). Heat treatment at 90ºC inactivated PPO, but degraded approximately 30% of anthocyanins. The kinetics of thermal degradation is generally first order up to 110˚C and is influenced by temperature, plant species, pH, presence of oxygen and sugar content. A logarithmic relationship between

temperature and pigment degradation has been described (Francis 1989). Although PPO activity can degrade anthocyanins, degradation of anthocyanins in the heat treated (blanched) blueberry mash can be the result of the heat treatment rather than the action of PPO since PPO activity was barely detectible in the heat treated mash (Figure 70).

The benzoate treatment also significantly reduced total anthocyanins, but possibly due to the action of PPO rather than heat, since 55ºC was not enough to degrade anthocyanins (Figure 66). Addition of sodium benzoate decreased PPO activity only by 42% from 233 to 137 $\mu molO_2/min/100g$ (Figure 70) leaving enough active enzyme to degrade anthocyanins. It was shown that substantial losses of anthocyanins and other polyphenolics occurred when blueberries were processed into juice and that different classes of compounds had varying susceptibility to degradation with different processing operations with the highest losses occurring during milling and depectinization due to the action of native PPO (Skrede and others 2000).

Not all anthocyanin aglucones were effected the same by the treatments. No significant differences were found between frozen blueberries and control mash in respect to all anthocyanin aglucones (Figure 67). All anthocyanin aglucones except cyanidin aglucones were significantly decreased by heat treatment (Figure 67) when compared to frozen berries. Heat treatment had the highest negative influence on malvidin anthocyanins (~34 % degraded), followed by delphinidin and petunidin (28% degraded), and cyanidin (20% degraded), in comparison to the frozen berries (Figure 67). The three most representative blueberry anthocyanins (i.e.malvidin glycosides) showed a minor recovery increase with blanching (Rossi and others 2003). Sodium benzoate significantly decreased all anthocyanin aglucones due to the action of PPO that was still

active (Figure 67). Treatment with benzoate showed a similar trend of anthocyanin degradation except that malvidin glycosides were less degraded than with the heat treatment, with only 25% degradation. These results suggest that malvidin glycosides are more susceptible to heat degradation than to the action of PPO. Malvidin glycosides are non o-diphenolic anthocyanins which are less susceptible to degradation of PPO generated o-quinines. Nevertheless, Sarni and others (1995) noticed a decrease in malvidin-3-glucoside in a model system in the presence of PPO and caffeoyltartaric acid (PPO substrate).

Individual anthocyanins were affected by maceration treatment similarly to their aglucones (Table 5). Maceration treatments did not have a significant influence on polymeric color (Figure 69). This indicates that the association of anthocyanins with each other and with other phenolics occurred within the blueberries and was not affected in any way by treatments.

No significant differences were found between maceration treatments in respect to total phenolic concentration (Figure 68). Total phenolic concentration was retained during jam processing of some berries (Amakura and others 2000; Zafrilla and others 2001). Thermal processing of raspberries into jam had a minimal effect on the major phenolics present (Zafrilla and others 2001).

Significant differences were found between blueberry mash maceration treatments in respect to the concentration of identified acids (Table 6). Concentrations of quinic acid and citric acids were significantly higher in heat treated mash than in the control maceration treatment (Table 6). Heat treatment probably helped increase the extraction of these acids from the blueberry skin and seeds.

Juice yield

Yield of blueberry juice was around 52% and there were no significant differences between maceration treatments (Figure 75). Higher yields for blueberry juice were reported in previous studies:79-81 % (Rossi and others 2003), 83% (Roberts and others 2004), 83%(Skrede and others 2000), and 78-82% for both cold and hot pressed juice (Carlson 2003). Higher yields may be due to the use of added pectolytic enzymes, smaller scale production and use of more sophisticated pressing equipment.

Anthocyanins, phenolics and acids in pressed juice and press cake

After pressing, anthocyanins are released into juice either in soluble form or in small suspended fruit particles (Iversen 1999). No significant differences in anthocyanins were found between different maceration treatments in extracted juice (Figure 71). Although, heat treated blueberry mash had a significantly lower concentration of total anthocyanins after 60 min of maceration when compared to the control (Figure 66), no significant differences in anthocyanins in the extracted juice could be the consequence of better extraction of anthocyanins from the heat-broken skin. Recovery of anthocyanins was 12 % from the control and benzoate treated mash, and 17% from the heat treated juice. Anthocyanins are not as efficiently extracted in the pressing operation as are sugars, acids and other water solubles, which can have a negative impact on juice quality (Skrede and others 2000; Lee and others 2002). It was shown that substantial losses of anthocyanins and other polyphenolics occurred when blueberries were processed into juice and that different classes of compounds had varying susceptibility to degradation

with different processing operations, with the highest losses occurring during milling and depectinization due to the action of native PPO (Skrede and others 2000).

Pretreatments with heat and SO_2 resulted in higher recovery of red color pigments with a more intense color (higher chroma) in pasteurized juice when compared to the control, while the heat treated samples were darker (smaller L values) than the control and SO_2 treated juice (Lee and others 2002). Only 12% of total blueberry anthocyanins were extracted in juice, increasing to 23% when the juice was blanched (Rossi and others 2003). Rossi and others (2003) reported that the total anthocyanin content of juice from blanched blueberry fruit was twice that of non blanched fruit. These researchers concluded that this was the result of PPO inactivation and the greater extraction yield was linked to the increase of fruit skin permeability caused by the heat treatment.

No significant differences in anthocyanin aglucones were found between control extracted, benzoate and heat treated juices (Figure 72). Heat extracted juice had significantly higher amounts of delphinidin, cyanidin and petunidin anthocyanins compared to benzoate treated juice (Figure 72). Malvidin glycosides were the most readily extracted anthocyanins for all treatments, contributing approximately 57-65% of the total anthocyanins that were present in the juice, while their contribution in the frozen blueberry fruit was aproximatelly 50%. Malvidin glycosides comprised 44% of the total anthocyanins in blueberry fruit and increased to 63% in pasteurized juice and concentrate (Skrede and others 2000). Similar results were reported by Lee and others (2002) where malvidin glycosides in the berry were 51% of total anthocyanins and increased to 60-77% in pasteurized juice and concentrate. Contribution of delphinidin and petunidin anthocyanins to total anthocyanins decreased in extracted juice compared to their

contribution in frozen berries, while cyanidin and peonidin ratios remained the same. Delphinidin anthocyanins decreased the most of all anthocyanins, decreasing from 19% contribution of total anthocyananins present in the frozen berries to 11% in the pressed juice. Delphinidin glucoside were the most unstable decreasing from 12% in blueberry fruit to 5% in pasteurized juice with only trace amounts in the initial pressed juice (Skrede and others 2000). Decreases in delphinidin glycosides was also reported by Lee and others (2002) but only for the juice that received no pretreatment before pressing. There was a concomitant decrease in delphinidin and petunidin glycosides (Lee and others 2002). The proportion of delphinidin-glycosides decreased especially in the control sample with only 8% left, while heat and SO_2 treated samples had 20 and 23% retained in the pasteurized juice (Lee and others 2002). The relative ratio of delphinidin glycosides versus cyanidin glycosides remained about the same in the juices as compared with the whole berry (Buchert and others 2005). Rossi and others (2003) obtained very different results with the higher percent recovery increase of delphinidin glycosides and petunidin –glycosides which have a more intense blue than that of cyanidin-glycosides and peonidin-glycosides, which are orange in color. The highest percentage recovery increase observed for delphinidin-glycosides could also be linked to their higher water-solubility, due to the presence of three free phenolic functions (Rossi and others 2003).

The anthocyanins that showed the highest benefits from the blanching treatment were, in decreasing order, the glycosides of delphinidin, petunidin , and cyanidin with the exception of cyanindin-3-glucoside that showed the lowest recovery (Rossi and others 2003). The three most representative blueberry anthocyanins (i.e.malvidin glycosides) showed a minor recovery increase with blanching (Rossi and others 2003). In the present