Copigmentation reactions and color stability of berry anthocyanins

Maarit Rein

ACADEMIC DISSERTATION

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ABSTRACT

Anthocyanin colors and factors which stabilize and enhance these labile pigments were studied in this thesis. Berry anthocyanin colors were successfully improved by phenolic acid and natural plant extract addition. The results obtained contribute to the understanding of the chemical behavior of anthocyanins.

The color quality of black currant wine was improved by the addition of crowberry juice and grape skin extract. Crowberry juice enhanced the color of black currant wine and grape skin extract stabilized it. The total anthocyanin content decreased in all of the berry wines during storage. However, the decline of color intensity was not as severe as the decline in anthocyanin content, which is an indication of copigmentation reactions taking place during fermentation and storage of the black currant wines.

Strawberry and raspberry juices were enhanced by the addition of plant extracts (black carrot extract, grape skin extract, and rosemary extract). The commercial color enhancers immediately increased the color intensity of these juices. During storage the improved colors were not very stable. The changes in color intensity and λ_{max} of the pure solutions and berry juices and wine were monitored by spectrophotometer.

The effect of phenolic acids on strawberry, raspberry, lingonberry, and cranberry juices was investigated. The phenolic acid enrichment improved and stabilized the juice colors during storage, which was manifested through changes in CIElab parameters and by hyperchromic effect and bathochromic shift detected by spectrophotometer. The simple cinnamic acids, sinapic and ferulic acids enhanced the color of strawberry and raspberry juices the most. The conjugated cinnamic acid, rosmarinic acid, improved and stabilized the color of lingonberry and cranberry juices the most.

Novel anthocyanin derivatives formed between the simple cinnamic acids and juice anthocyanins during storage, which was detected first as new peaks in high-performance liquid chromatography (HPLC) chromatogram, and later identified as pyranoanthocyanins by liquid chromatography mass-spectrometry (LC-MS). The new anthocyanin derivatives were 4-vinylguaiacol and 4-vinylsyringol adducts of pelargonidin and cyanidin. This was the first time pelargonidin 3-glucoside based vinylphenol pyranoanthocyanins and pyranoanthocyanin with more complex glycosyl residues were found. For the first time, pyranoanthocyanins were detected in strawberry and raspberry juices.

Reactions of pure anthocyanins with phenolic acids were studied for 6 months. Immediate intermolecular copigmentation was observed with the monoglucosidic anthocyanins, expressed as hyperchromic effect and bathochromic shift. The strongest color enhancement on the day of preparation was observed with rosmarinic acid and ferulic acid. During storage, ferulic and caffeic acids improved the monoglucosidic anthocyanin colors the most. The color enhanced by rosmarinic acid was not very stabile in the course of time. The trisaccharidic and acylated trisaccharidic cyanidin did not exhibit color enhancement, although they had the best color stability as such during storage.

ACKNOWLEDGEMENTS

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I am greatly indebted to the reviewers Dr. Pirjo Mattila and Prof. Celestino Santos-Buelga for careful and thorough reading of the thesis manuscript and for their constructive criticism and valuable comments.

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Risunen is highly valued for her 100% help always. I would have not made it - or managed the washing machine without her, ever.

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Lausanne, March 2005

Maarit Rein

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I Eiro, M., Hopia, A., Kaukovirta-Norja, A., Lehtinen, P. and Heinonen, M., 2000. Enhancing the Colour of Blackcurrant Wine by Natural Additives. Vitic. Enol. Sci. 55 (1), 3-6.

II Eiro M. J. and Heinonen, M. 2002. Anthocyanin Color Behavior and Stability During Storage: Effect on Intermolecular Copigmentation. J. Agric Food Chem. 50, 7461-7466.

III Rein M. J. and Heinonen, M. 2004. Stability and Enhancement of Berry Juice Color. J. Agric Food Chem. 52, 3106-3114.

IV Rein M. J., Ollilainen V., Vahermo M., Yli-Kauhaluoma, J. and Heinonen, M. 2005. Identification of Novel Pyranoanthocyanins in Berry Juices. Eur. Food Res. Technol. 220:239-244.

Contribution of the author to papers I to IV.

I The author planned the study together with other authors. The experimental study including empirical work and the preparation of the manuscript was carried out by the author. The author wrote the manuscript and Ph.D. Hopia and Prof. Heinonen participated in writing of the manuscript by giving comments and suggestions.

II The author planned the study together with supervisor Prof. Heinonen. The experimental study including empirical work and the preparation of the manuscript was carried out by the author. She was the main author in writing the manuscript.

III The author planned the study together with supervisor Prof. Heinonen. The experimental study including empirical work and the preparation of the manuscript was carried out by the author. The author wrote the manuscript and Prof. Heinonen, participated in writing of the manuscript by giving comments and suggestions.

IV The planning of the study was carried out by the author, Ph.D. Ollilainen, and Prof. Heinonen. The experimental study including empirical work and the preparation of the manuscript was carried out by the author. M.Sc.Vahermo prepared 4-vinylsyringol for the study. Prof. Yli-Kauhaluoma elaborated on the reaction pathway of pyranoanthocyanin formation. Prof. Yli-Kauhaluoma and M.Sc. Vahermo participated in writing the 4-vinylsyringol synthesis in the manuscript. Ph.D. Ollilainen and Prof. Heinonen also participated in writing of the manuscript by giving comments and suggestions.

CONTENTS

1. INTRODUCTION

The color of food has always been a value of quality. In the Inca culture, potatoes with reddish jacket were more valuable than other varieties. Today, the attractive red color of food products, like strawberry jams, blood orange juice, or raspberry jellies is an important quality parameter, which influences consumer behavior. Obtaining a strong and stable color of fruit and berry products, however, is problematic during processing and storage. To better master the behavior of the color of food products, the chemistry of color molecules ought to be in hand. Scientific research on the chemistry of colors, in theoretical and applied level, is essential in order to improve the color of different food products. The need to avoid the use of synthetic colorants and move towards the use of natural food colors has also increased research in this field during the past decades.

Anthocyanins are natural pigments widely distributed in nature. Anthocyanin color molecules are a subclass of flavonoids. They are responsible for the reds, purples, and blues in many flowers, fruits and vegetables. They are found in the petals of petunia, stems of rhubarb, and roots of red radish, for example. Fruits and berries are the most ample sources of anthocyanins in nature. In fruits and berries, anthocyanins are mainly located in the peel, like in apples and grapes, but they are also found in the pulp, as in the case of cherries or blue berries.

Berries and fruits are an important part of the Finnish diet. The consumption of fruits and berries in Finland is around 250g per day per person, of which fresh berries constitute over 50g (Ministry of Agricultural and Forestry, 2003; FINDIET2002). In many researches the positive effect of fruit and berry intake on human health has been reported (Hollman et al., 1996a; Hollman et al., 1996b; Youdim et al., 2002; Knekt et al., 2002; Rissanen et al., 2003). Anthocyanins are considered to contribute to the healthiness of fruits and berries for their antioxidant, anti-carcinogenic, anti-inflammatory, and anti-angiogenic properties for example (Clifford, 2000; Kong et al., 2003; Rossi et al., 2003). Anthocyanins can also improve the nutritional value of processed foods by preventing oxidation of lipids and proteins in the food products (Kähkönen et al., 2001; Kähkönen et al., 2003; Viljanen et al., 2004). However, the stability of anthocyanins becomes most significant also in this case, as in the case of color quality.

Anthocyanins are highly unstable and easily susceptible to degradation. The stability of anthocyanins is affected by pH, storage temperature, presence of enzymes, light, oxygen, structure and concentration of the anthocyanins, and the presence of other compounds such as other flavonoids, proteins, and minerals. The stability of anthocyanin color can be

improved by copigmentation, where the anthocyanin molecule reacts with other natural plant components directly or through weak interactions, resulting in an enhanced and stabilized color (Darias-Martin et al., 2002; Talcott et al., 2003). Anthocyanin copigmentation gives brighter, stronger and more stable colors than what would be expressed by an intact anthocyanin molecule. Copigmentation is known to be responsible for the profuse color variability of bluish flowers and for stable wine colors (Asen et al., 1972; Asen et al., 1975; Brouillard, 1983; Liao et al., 1992; Brouillard and Dangles, 1994; Yabuya et al., 1997; Bloor and Falshaw, 2000), through which the phenomenon was first investigated. Copigmentation of berry products and storage effects on the phenomenon are only faintly studied until now.

2. REVIEW OF LITERATURE

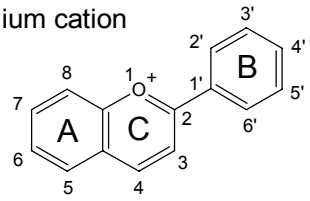
2.1 Berry anthocyanins

2.1.1 Structure of anthocyanins

Anthocyanins belong to the flavonoid group of polyphenols. They have a $C_6C_3C_6$-skeleton typical of flavonoids. Anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium cation, i.e. the flavylium cation (Brouillard, 1982). The main part of anthocyanins is its aglycone, the flavylium cation (table 1), which contains conjugated double bonds responsible for absorption of light around 500 nm causing the pigments to appear red to human eye. The aglycones are called anthocyanidins, which are usually penta- (3,5,7,3′,4′) or hexa-substituted (3,5,7,3′,4′,5′). 22 different anthocyanidins are known today (table 1), but only six of them are significant and most common from food point of view (Francis, 1989). The most important anthocyanidins are pelargonidin, cyanidin, peonidin, delphinidin, malvidin, and petunidin (Figure 1). These aglycones differ in the number of hydroxyl and methoxyl groups in the B-ring of the flavylium cation.

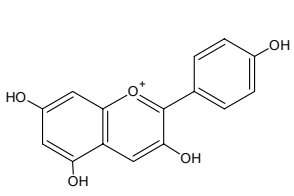
Table 1. The substitution pattern of flavylium cation forming the naturally occuring anthocyanidins known today.

Flavylium cation

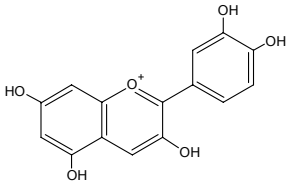


	Substitution pattern							
Anthocyanidin	3	5	6	7	3´	4´	5´	color
Carajurin	H	H	OH	OH	H	OCH_3	OCH_3	-
Arrabidin	H	H	OH	OH	H	OH	OCH_3	-
3´-Hydroxyarrabidin	H	H	OH	OH	OH	OH	OCH_3	-
Apigenin	H	OH	H	OH	H	OH	H	Orange
Luteolin	H	OH	H	OH	OH	OH	H	Orange
Tricetinidin	H	OH	H	OH	OH	OH	OH	Red
Pelargonidin	**OH**	**OH**	**H**	**OH**	**H**	**OH**	**H**	Orange
Aurantinidin	OH	OH	OH	OH	H	OH	H	Orange
Cyanidin	**OH**	**OH**	**H**	**OH**	**OH**	**OH**	**H**	Orange red
5-Methylcyanidin	OH	OCH_3	H	OH	OH	OH	H	Orange red
Peonidin	**OH**	**OH**	**H**	**OH**	**OCH_3**	**OH**	**H**	Red
Rosinidin	OH	OH	H	OCH_3	OCH_3	OH	H	Red
6-Hydroxycyanidin	OH	OH	OH	OH	OH	OH	H	Red
6-Hydroxydelphinidin	OH	OH	OH	OH	OH	OH	OH	Bluish red
Delphinidin	**OH**	**OH**	**H**	**OH**	**OH**	**OH**	**OH**	Bluish red
Petunidin	**OH**	**OH**	**H**	**OH**	**OCH_3**	**OH**	**OH**	Bluish red
Malvidin	**OH**	**OH**	**H**	**OH**	**OCH_3**	**OH**	**OCH_3**	Bluish red
Pulchellidin	OH	OCH_3	H	OH	OH	OH	OH	Bluish red
Eupinidin	OH	OCH_3	H	OH	OCH_3	OH	OH	Bluish red
Capensinidin	OH	OCH_3	H	OH	OCH_3	OH	OCH_3	Bluish red
Hirsutidin	OH	OH	H	OCH_3	OCH_3	OH	OCH_3	Bluish red
Ricciniodin A[a]	OH	H	OH	OH	H	OH	H	-

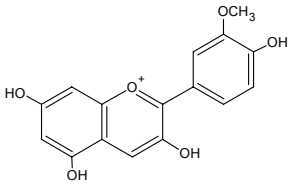
[a]Ring closure on the basis of ether linkage between the 3- and 6´-positions + an additional OH-group at the 2´-position. Addapted from F.J. Fancis (1989), O. Andersen (2002), and Devia et al. (2002). The anthocyanidins in bold are the most important ones regarding foods.



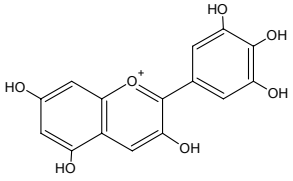
Pelargonidin



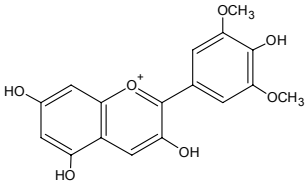
Cyanidin



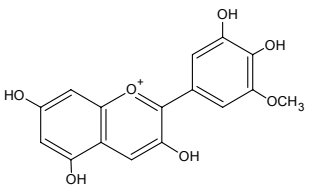
Peonidin



Delphinidin



Malvidin



Petunidin

Figure 1. The most important natural anthocyanidins.

Anthocyanidins are seldom found in nature as such. Anthocyanidins occur in flowers, fruits, and berries mainly in their glycosylated forms (Harborne, 1967), i.e. as anthocyanins. Anthocyanins are much more soluble and stable in water than anthocyanidins, which is due to their glycosylation (Robinson and Leon, 1931; Timberlake and Bridle, 1966a). Anthocyanins are classified by the number of glycosyl units they contain. Monoglycosides comprise of one saccharidic moiety, which is primarily attached to the 3-hydroxyl group of the aglycone (Brouillard 1982). Anthocyanins with glycosylation at 3′- and 4′- positions without C-3 glycosylation have been identified in blue flowers (*Nymphaea caerulea*) (Fossen and Andersen, 1999) and red onion (*Allium cepa* L.) (Fossen et al., 2003). In diglycosides two monosaccharides are attached to 3-hydroxyl and 5-hydroxyl group of the anthocyanidin or seldom to 3-hydroxyl and 7-hydroxyl group, but it is also possible that the two monosaccharides are both attached to C-3. In triglycosides the monosaccharides are attached to the aglycone in such a way, that two of them are in C-3 and one in C-5 or C-7. A trisaccharidic anthocyanin can also have a linear or branched attachment of three monosaccharides at C-3 (Bruneton, 1995). Glycosylations at the position 3′-, 4′-, and 5′- are also possible. The earliest report of cyanidin 4′-glucoside was made in 1968 by Hedin et al. on color of *Hibiscus esculentus* (Hedin et al., 1968). The most common sugars of anthocyanins are monosaccharides in order of frequency: glucose, rhamnose, galactose, arabinose, and xylose. The di- and trisaccharides found most often in anthocyanins are rutinose, sophorose, sambubiose, and glucorutinose, for example (De Ancos et al., 1999a; Kähkönen et al., 2003). Figure 2 shows the most common saccharides found in anthocyanins.

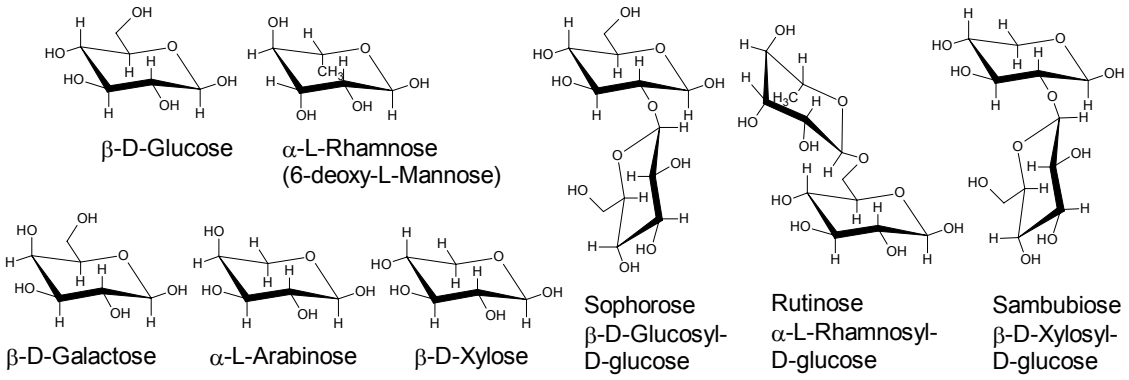


Figure 2. The most common glycosyl units of anthocyanins.

Anthocyanins can also be acylated. Organic acids, which are attached to anthocyanin glycosyl units through an ester bond, are usually either aromatic phenolic acids or aliphatic

dicarboxyl acid or a combination of both. The acids are most commonly linked to the 6-position of the monosaccharide, but anthocyanins with acyl substitution at the 2-, 3-, and 4-positions of the monosaccharide have been elucidated (Cabrita, 1999). The most common phenolic acids in anthocyanins are the derivatives of hydroxycinnamic acids, i.e. *p*-coumaric, ferulic, caffeic, and sinapic acids, and hydroxybenzoic acids, i.e. gallic acid for example. The most common aliphatic acids occurring in anthocyanin molecules are malonic, acetic, malic, succinic, and oxalic acids (Francis, 1989; Bruneton, 1995; Cabrita, 1999). The most common aromatic phenolic acids and aliphatic dicarboxyl acids being part of anthocyanin molecules are shown in figure 3. The complexity of anthocyanins is shown in figure 4, where some differently substituted anthocyanins is presented.

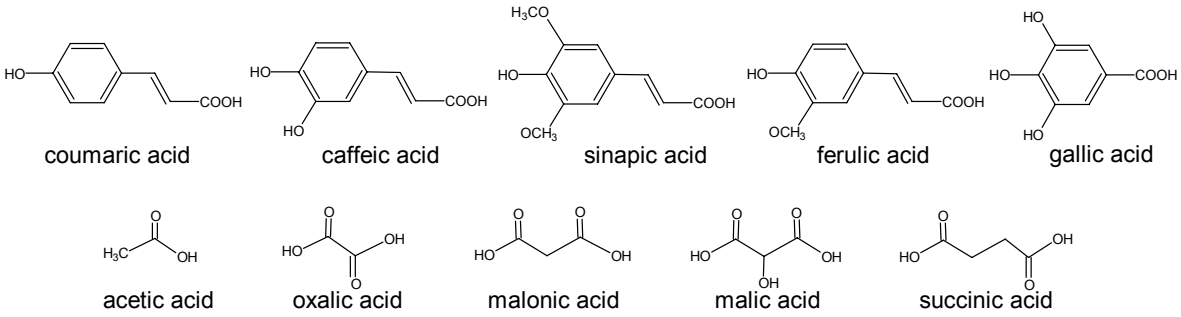


Figure 3. The most common acyl units of anthocyanins.

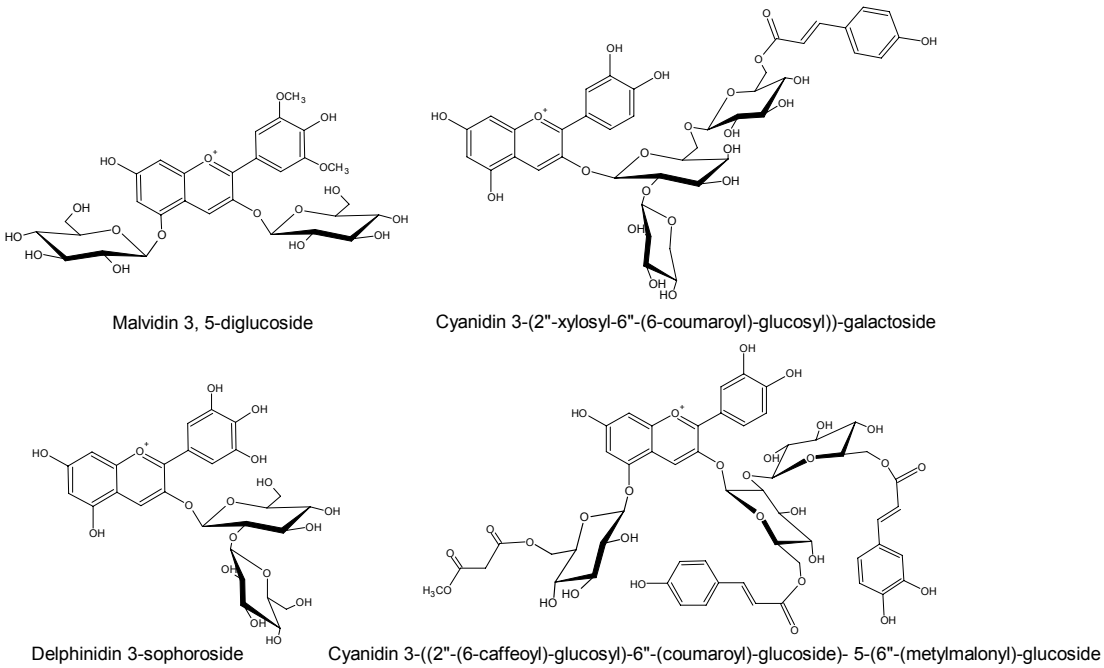


Figure 4. Differently substituted anthocyanins.

2.1.2 Occurrence of anthocyanins in berries

Of the plant kingdom, genus *Vitis*, to which grapes belong, and its species *V. vinifera* of the Vitaceae-family is the most important anthocyanin containing fruit crop in the world (Timberlake and Bridle, 1982). *V. vinifera* is the most cultivated single food plant of which red wine is produced. Other important anthocyanin rich berry families are the Rosaceae- and Ericacea-families. The first includes the genus of strawberry (*Fragaria*) and raspberry (*Rubus*) and the latter of bilberry, cranberry and lingonberry (*Vaccinium*). Besides *Vaccinium*, also *Ribes* is an important genus of the Northern Hemisphere, which includes berry species with high anthocyanin content. Black currant (*Ribes nigrum* L.) is the most cultivated berry crop in Finland, after strawberry, with annual production of over 2000 tons, which is 3% of the domestically consumed berries (Garden Association, 2003; Ministry of Agriculture and Forestry, 2004).

In berries, the anthocyanin concentration correlates well with the darkness of the berry color and hue; the darker blue a berry, the higher its anthocyanin content. The highest anthocyanin content of berries that are consumed in Finland is found in bilberries (*Vaccinium myrtillus*, L.) (300-600 mg/100g fresh weight) (Prior et al., 1998; Kähkönen et al., 2001; Kähkönen et al., 2003) and black currants (80-810 mg/100g) (Toldam-Andersen and Hansen, 1997; Kähkönen et al., 2001; Kähkönen et al., 2003). Other dark berries, such as crowberries (*Empetrum nigrum*, L.) contain anthocyanins 300-560 mg/100g (Linko et al., 1983; Kärppä, 1984; Kähkönen et al., 2001), blueberries (*Vaccinium corymbosum*, L. and other different sub-genera) between 60 mg and 480 mg/100g (Gao and Mazza, 1994; Prior et al., 1998; Heinonen et al., 1998; Prior et al., 2001; Zheng and Wang, 2003) and cranberries (*Vaccinium oxycoccus*, L.) 20-360 mg/100g (Kähkönen et al., 2001; Prior et al., 2001; Wang and Stretch, 2001; Zheng and Wang, 2003). Raspberries (*Rubus ideaeus*, L.) are also a good source of anthocyanins (20-220 mg/100g fresh weight) (Heinonen et al., 1998; De Ancos et al., 1999a; Ochoa et al., 1999; Kalt et al., 1999; Deighton et al., 2000; Kähkönen et al., 2001; Wada and Ou, 2002). Strawberries (*Fragaria ananassa*), having quite a light red hue, contain anthocyanins between 10-80 mg/100g (Heinonen et al., 1998; Kalt et al., 1999; Zabetakis et al., 2000; Kähkönen et al., 2001; Nyman and Kumpulainen, 2001; Cordenunsi et al., 2003; Meyers et al., 2003) and the anthocyanin content of lingonberry (*Vaccinium vitis-idaea*, L.) is in the same range (35-170 mg/ 100g) (Andersen, 1985; Kähkönen et al., 2001; Kähkönen et al., 2003; Zheng and Wang, 2003). The variation of anthocyanin contents in different berries is listed in table 2.

Table 2. Anthocyanin contents of different berry species (mg/100g FW).

BERRY	GENUS	ANTHOCYANINS	REFERENCE
black currant	*Ribes nigrum*, L.	80-810	Toldam-Andersen and Hansen, 1997; Kähkönen et al., 2001; Kähkönen et al., 2003
bilberries	*Vaccinium myrtillus*, L.	300-600	Prior et al., 1998; Kähkönen et al., 2001; Kähkönen et al., 2003
blueberry	*Vaccinium* (different sub-genera)	60-480	Gao and Mazzo, 1994; Prior et al., 1998; Heinonen et al., 1998; Prior et al., 2001; Zheng and Wang, 2003
cranberry	*Vaccinium oxycoccus*, L.	20-360	Kähkönen et al., 2001; Prior et al., 2001; Wang and Stretch, 2001; Zheng and Wang, 2003
lingonberry	*Vaccinium vitis-idaea*, L.	35-170	Andersen, 1985; Kähkönen et al., 2001; Kähkönen et al., 2003, Zheng and Wang, 2003
crowberry	*Empetrum nigrum*, L.	300-560	Linko et al., 1983; Kärppä, 1984; Kähkönen et al., 2001
raspberry	*Rubus ideaeus*, L.	20-220	Heinonen et al.,1998; De Ancos et al.,1999a; Ochoa et al., 1999; Kalt et al.,1999; Deighton et al., 2000; Kähkönen et al., 2001; Wada and Ou, 2002
strawberry	*Fragaria ananassa*	10-80	Heinonen et al., 1998; Kalt et al.,1999; Zabetakis et al., 2000; Kähkönen et al., 2001, Nyman and Kumpulainen, 2001; Cordenunsi et al., 2003; Meyers et al., 2003

The anthocyanin amount in berries varies due to different strains of analyzed berries but also due to different analytical methods used. The total amount of anthocyanins in berries quantified with a single compound, cyanidin 3-glucoside, as done in many studies, differs from the amounts of quantification that is done using individual standard compounds, as shown in the study by Kähkönen et al. (2003). They found the anthocyanin content, when measured as cyanidin 3-glucoside, to be significantly lower than when quantified with several individual anthocyanin standards. Anthocyanin content has also been measured as anthocyanidins in some studies (Nyman and Kumpulainen, 2001, Määttä-Riihinen et al., 2004). Unfortunately, quantification as aglycones looses the valuable information of the naturally occurring glycosylates and their bioactivities.

Different berries have also different amounts of various individual anthocyanins. Strawberry and lingonberry have a simple anthocyanin profile with only one major pigment. The main anthocyanin of strawberry is pelargonidin 3-glucoside (Sondheimer and Kertesz, 1948a; Wrolstad and Putnam, 1969; Goiffon et al., 1991; Lopes-Da-Silva et al., 2002) and the major anthocyanin of lingonberry is cyanidin 3-galactoside (Andersen, 1985; Kähkönen et al., 2003). Black currant contains four major anthocyanins; the 3-glucosides and 3-rutinosides of cyanidin and delphinidin (Chandler and Harper, 1962; Koeppen and Herrmann, 1977; Francis and Andersen, 1984; Slimestad and Solheim, 2002; Kähkönen et al., 2003; Nielsen et al., 2003; Määttä-Riihinen et al., 2004). The six main anthocyanins of cranberry consist of two aglycones, cyanidin and peonidin, which both have three different monosaccharidic substitutes: 3-galactoside, 3-glucoside, and 3-arabinoside (Huopalahti et al., 2000; Zheng and Wang, 2003; Määttä-Riihinen et al., 2004). Other berries like

blueberry, bilberry, and crowberry have much more complex anthocyanin profile. Both bilberry and crowberry contain five main aglycones (delphinidin, cyanidin, petunidin, malvidin, and peonidin) with several different glycosyl substituents (Baj et al., 1983; Karppa et al., 1984; Goiffon et al., 1991; Nyman and Kumpulainen, 2001; Dugo et al., 2001; Määttä-Riihinen et al., 2004). Fifteen different anthocyanins have been identified from bilberry with different combinations of its five aglycones and monosaccharide moieties of 3-galactoside, 3-glucoside, and 3-arabinoside (Dugo et al., 2001; Kähkönen et al., 2003). Crowberry comprises of twelve different anthocyanin monoglucosides, the main anthocyanins being 3-galactosides of cyanidin, delphinidin, and malvidin (Kärppä et al., 1984).

The distribution of different anthocyanins varies between each berry species, but also between different varieties of the same species. De Ancos et al. (1999a) studied the anthocyanins of four cultivates of red raspberries and found the anthocyanin distribution to differ in each raspberry variety. In three of the four varieties cyanidin 3-sophoroside was detected as the main anthocyanin of raspberry. Also in a study of nine red raspberry cultivates cyanidin 3-glucoside and cyanidin 3-sophoroside were identified as the major anthocyanins (Barritt and Torre, 1975). However, in Autumn Bliss-variety the main anthocyanins were cyanidin 3-rutinoside and cyanidin 3-glucoside, and nine different anthocyanins were identified from the variety Ceva (De Ancos et al., 1999a).

Berry anthocyanins, to a large extent, are all non-acylated. However, small quantities of acylated 3-(6´´-coumaroylglucosides) of delphinidin and cyanidin have been identified in black currant (Slimestad and Solheim, 2002). Also blueberry contains some acylated anthocyanins (Gao and Mazza, 1994). The exceptional color properties of grape anthocyanins are postulated to be due to the existence of acylated anthocyanins, which can comprise over 50% of the total anthocyanins in grape (Tamborra et al., 2003). The lack of acylated anthocyanins in northern berry species affects the stability of their color and significantly diminishes the shelf life of food products derived from berries.

2.1.3 Anthocyanin colors in berry foods and wines

Anthocyanin content in food products derived from fruits and berries is much smaller than the original anthocyanin content in the raw material. The manufacturing and processing of berry products lead to deterioration of anthocyanins and the color of the product. During storage the color of berry products degrades even further (Sondheimer and Kertesz, 1948b; Gimenez et al., 2001). The choice of a processing method affects the color quality of the

food products immensely. Blackberry wine produced by Rommel et al. (1992) from HTST-pasteurized, depectinized and fined juice had the best appearance and color stability after storage compared to other processing methods. Juice and pulp treatment with enzymes, like pectinases, increases anthocyanin content and color density (Rommel et al., 1990; Rommel et al., 1992). Addition of anthocyanin containing natural colorants like E163 made of grape skin extract, black currant extract or elderberry extract can be used to improve the color of fruit and berry products. The anthocyanin contents of different berry products are shown in table 3.

Juices and concentrates

The loss of anthocyanins in juice making is quite considerable; the receipt of blueberry anthocyanins during juice pressing was only 50% (Skrede et al., 2000). The anthocyanin content of blueberry juice is somewhat over 300 mg/L (Skrede et al., 2000). Freshly prepared cranberry juice contains only a faint amount of anthocyanins (7.7 mg/100 mL) compared to the amount found in the berries (Huopalahti et al., 2000). Also the anthocyanin content of strawberry juice is nearly half of that of strawberry pulp (Gimenez et al., 2001) and during storage of the juice the anthocyanin content decreases even further (Lundahl et al., 1989). The anthocyanin content of strawberry juices varies between 110 mg/L to 270 mg/L (Gimenez et al., 2001; Garzon and Wrolstad, 2002). However, the anthocyanin half-life of strawberry juice is 30% higher compared to that of strawberry concentrate (Garzon and Wrolstad, 2002). The anthocyanin content of strawberry concentrate is around 130-210 mg/L (Lundahl et al., 1989; Garzon and Wrolstad, 2002). The anthocyanin content of raspberry juice does not diminish as vigorously as in strawberry juice when compared to the intact berries. The anthocyanin content of raspberry juice is 580 mg/L (Rommel et al., 1990). Raspberry juice contains mostly di- and trisaccharidic anthocyanins, which most probably contribute to greater color stability over strawberry or blackberry juices, which contain mainly unstable monosaccharidic anthocyanins (Rommel et al., 1990).

Purees and jams

The color acceptability of berry jams is more dependent on the total anthocyanin content than the percentage of berry puree used in the jam (Spayd and Morris, 1981). Garcia-Viguera et al. (1997) have studied the anthocyanin content and composition of different commercial berry jams, which all had similar range in berry and scurose content. The highest anthocyanin content was found in blackberry jams ranging from 0.2 mg to 27

mg/100g of jam (fresh weight). Raspberry jams contained anthocyanins from traceable amounts to 6 mg/100g of jam. Black currant jam had 0.4 mg of anthocyanins per 100g of jam. Strawberry jams contained small amounts of anthocyanins (0.4 - 2 mg/100g of jam) (Abers and Wrolstad, 1979; Garcia-Viguera et al., 1999), whereas strawberry puree contained anthocyanins 380 mg/L immediately after milling (Bakker and Bridle, 1992). Abers and Wrolstad (1979) proposed that other components of berry products, the so called reactive phenolics (leucoanthocyanins and flavanols), play an important role in the color deterioration of strawberry preserves by forming brown polymeric compounds with anthocyanins. The lacking of protective additives during enzyme treatment of strawberry puree causes likewise anthocyanin degradation (Bakker and Bridle, 1992).

Wines

Anthocyanins are the sole significant and observable difference between red and white grapes and wine. In grapes, 5 to 20 different anthocyanins have been identified, dependent on the *Vitis* genus. Of these anthocyanins, malvidin 3-glucoside (oenin) is the most important one, but also cyanidin and peonidin derivatives are common (Ribéreau-Gayon, 1982). Acylated anthocyanins are typical for red wine, constituting of around 20% of the total anthocyanins (Santos et al., 1991; Burns et al., 2002). The most common acyls being part of wine anthocyanins are *p*-coumaric acid and caffeic acid (Ribéreau-Gayon, 1982). Acylated anthocyanins are thought to contribute to the stability of red wine color during storage. Recently, identification of pyranoanthocyanin derivatives in red table wines and port wines have given elucidation to the color stability and hue changes in the wines during maturation and storage.

The grape varieties used in wine making as well as the vinification and storage conditions during maturation process influence the anthocyanin content of the produced red wine (Pellegrini et al., 2000; Arnous et al., 2001; Sun et al., 2001; Perez-prieto et al., 2003). Some Spanish red wines have been reported to contain between 160-550 mg/L of total anthocyanins after fermentation and 60-260 mg/L after storage (Almela et al., 1996; Perez-prieto et al., 2003). A typical anthocyanin amount in red wines is around 200 mg/L. In young red wines, the typical anthocyanin content is around 500 mg/L (Liao et al., 1992). Wine pressed from red Sangiovese grapes contained 150mg/L anthocyanins after six months of storage (Castellari et al., 2000). Different wines from British Columbia made of Cabernet Franc, Merlot, and Pinot Noir grapes contained anthocyanins from 220 to 280 mg/L after 6-7 months of storage (Mazza et al., 1999).

Table 3. Anthocyanin contents of different berry products (mg/L or mg/100g FW[a,b]).

BERRY	PRODUCT	ANTHOCYANINS	REFERENCE
blueberry	juice	> 300	Skrede et al., 2000
black currant	jam	0.4	Garcia-Vigera et al., 1997
cranberry	juice	0.8	Huopalahti et al., 2000
strawberry	juice	110 - 270	Gimenez et al., 2001; Garzon and Wrolstad, 2002
	concentrate	130 - 210	Lundahl et al.,1989; Garzon and Wrolstad, 2002
	puree	380	Bakker and Bridle, 1992
	jam	0.4 - 2	Abers and Wrolstad, 1979; Garcia-Viguera et al., 1999
raspberry	juice	580 - 670	Rommel et al., 1990
	jam	6	Garcia-Viguera et al., 1997
blackberry	juice	330 - 640	Rommel et al., 1992
	jam	0.2 - 27	Garcia-Viguera et al., 1997
grape	wine	~ 200	Liao et al.,1992; Almela et al., 1996; Mazza et al., 1999; Perez-prieto et al., 2003

[a]Anthocyanin content in juices, concentrate, puree and wine in mg/L
[b]Anthocyanin content in jams mg/100g FW

2.2 Color Stability of anthocyanins

Anthocyanins are highly unstable molecules in food matrix. The color stability of anthocyanins is strongly affected by pH, solvents, temperature, anthocyanin concentration and structure, oxygen, light, enzymes, and other accompanying substances.

2.2.1 Structural effects

The glycosyl units and acyl groups attached to the aglycone, and the site of their bonding, have a significant effect on stability and reactivity of the anthocyanin molecule. Also the substitution pattern of the anthocyanidin, the number and placement of the hydroxyl and methoxyl groups in the aglycone, affect the chemical behavior of the pigment molecule.

The increased hydroxylation of the aglycone stabilizes the anthocyanidin; delphinidin is more stable than cyanidin in acidic methanol (Dao et al., 1998). However, there are discrepancies related to the effect of hydroxylation of the aglycone on molecule stability; in a buffered solution at pH 3.1 cyanidin 3-glucoside was more stable than pelargonidin 3-glucoside but delphinidin 3-glucoside was less stable than cyanidin 3-glucoside. Also petunidin 3-glucoside, with two hydroxyl groups in the B-ring, was less stable than peonidin 3-glucoside, which has one hydroxyl group in the same ring (Cabrita et al., 2000). Increasing methylation of the hydroxyl groups weakens the stability of the anthocyanins. Methoxyl groups at C-4′ and C-7 decrease the stability of a pigment with hydroxyl groups

at these positions (Mazza and Brouillard, 1987a). In a buffered solution cyanidin 3-glucoside was more stable than petunidin 3-glucoside, which differ from the former with an additional 5´-methoxyl. Also peonidin 3-glucoside was more stable than malvidin 3-glucoside, which alike has an extra 5´-methoxyl (Cabrita et al., 2000). Again there is inconsistency related to the effect of methoxylation of the aglycone on pigment stability. In a micellar system malvidin was more stable than pelargonidin, but less stable than cyanidin (Mulinacci et al., 2001), and peonidin 3-glucoside was more stable than pelargonidin 3-glucoside in a buffered solution (Cabrita et al., 2000).

The hydroxyl and methoxyl pattern does not affect only the stability of the anthocyanins but also the color display. The color of anthocyanins changes from pink to blue as the number of hydroxyls increases. Methoxyl groups that replace the hydroxyls reverse the trend (Mazza and Brouillard, 1987a). Also chemical modifications of anthocyanins, such as the synthesis of 3-deoxyanthocyanins, in order to improve stability, have been conducted (Sweeny and Iacobucci, 1977; Sweeny and Iacobucci, 1983; Mazza and Brouillard, 1987b; Dao et al., 1998). However, this produces yellowish anthocyanins, when the much less stable anthocyanins with 3-hydroxyl are dominantly red (Mazza and Brouillard, 1987a).

Anthocyanins are more stable and soluble in aqueous solutions than anthocyanidins (Andersen, 2002). Therefore the glycosylated forms of anthocyanidins are more common in nature and the aglycones hardly exist as such *in vivo*. It has been shown that glycosyl substitution stabilizes the anthocyanin molecule (Timberlake and Bridle, 1966a; Timberlake and Bridle, 1966b). The color stability of cyanidin is higher than that of malvidin, but lower than that of malvidin 3-glucoside (Mazza and Brouillard, 1987b). The place of the glycosyl attachment influences the stability of the anthocyanin. In a study of isomeric β-glycosides of pelargonidin, glycosylation at C-3 produced the most stable anthocyanin over 5-, 7-, and 4´-glucosides (Leon et al., 1931). Also the type of glycosyl units influences the anthocyanin stability; cranberry anthocyanins with galactose were more stable than with arabinose during storage (Starr and Francis, 1968). The half-life of cyanidin 3-rutinoside is 65 days in buffer solution of pH 2.8 in room temperature, where as the half-life of the corresponding anthocyanidin, cyanidin, is only 12 hours (Iacobucci and Sweeny, 1983). Bronnum-Hansen and Flink (1985) reported that the anthocyanins containing the disaccharide, sambubiose, were more stable than glucose containing monosaccharidic anthocyanins. The slow hydrolysis of the 3-glycosyl moiety of an anthocyanin in acidic conditions is proposed to be behind the better color stability of these pigments (Mazza and Brouillard, 1987a). The sugar substitutions also affect the perceived color of the anthocyanin pigments. Anthocyanin 3-glucosides have been found to be more

colored than the corresponding 3,5- and 5-glucosides (Mazza and Brouillard, 1987a). The increasing number of glucose units gives rise to more yellow pigments (Giusti et al., 1999).

Acylation further increases the stability of anthocyanins (Bassa and Francis, 1987). The exceptional hues and stable colors of flowers are due to acylated anthocyanins. The anthocyanin color of flower tradescantia (*Tradescantia pallida*, v.) was much more stable than anthocyanins from concord grapes and red cabbage, presumably due to its diverse acylation pattern (Baublis et al., 1994). In monoacylated anthocyanins, the acyl group occurs mainly at the 3-C monosaccharide and at the 6-position of the monosaccharide (Harborne, 1964). Polyacylated anthocyanins are more stable than monoacylated anthocyanins and they posses high color stability throughout the entire pH range from acidic to alkali (Asen, 1976). Red raddish extracts with diacylated anthocyanins are more stable than red-fleshed potato extracts with monoacylated anthocyanins (Rodriguez-Saona et al., 1999). The type of an attached acyl affects the anthocyanin stability. Anthocyanins with aromatic acyl substituents are more stable than the ones with aliphatic acyls (Stintzing and Carle, 2004). Within the aromatic acid group the type of acid affects the stability alike. Caffeic acid induces more stable color properties than *p*-coumaric acid for example (Francis, 1989). Acylation of anthocyanins results in more intense color, especially with diacylation, and also in a changed hue. *P*-coumaric acid induces more yellowish hue to pelargonidin 3-sophoroside-5-glucoside and ferulic acid more bluish (Giusti et al., 1999). Anthocyanins with B-ring acylation produce stable and more intense coloration at pH values of 4-5.5 (Francis, 1992; Baublis et al., 1994).

2.2.2 Concentration effects

Increased anthocyanin concentration promotes higher color stability (Giusti and Wrolstad, 2003). Color stability of strawberry syrup was greatly improved by increasing the concentration of anthocyanins. It was observed that the total anthocyanin concentration was more important in relation to color stability than the different types of individual anthocyanins (Skrede et al., 1992). Increased anthocyanin concentrations also increases color intensity by multifold. Change of cyanin concentrations from 10^{-4} to 10^{-2} produced a 300-fold increment of the color intensity (Asen et al., 1972). Increasing the content of anthocyanins improves their stability through self-association (Brouillard, 1982; Dao et al., 1998).

2.2.3 pH

Anthocyanins are more stable in acidic media at low pH values than in alkaline solutions with high pH values. However, anthocyanins are known to display a huge variety of color variations in the pH range from 1-14. The ionic nature of anthocyanins enables the changes of the molecule structure according to the prevailing pH, resulting in different colors and hues at different pH values (Brouillard, 1982; von Elbe and Schwartz, 1996).

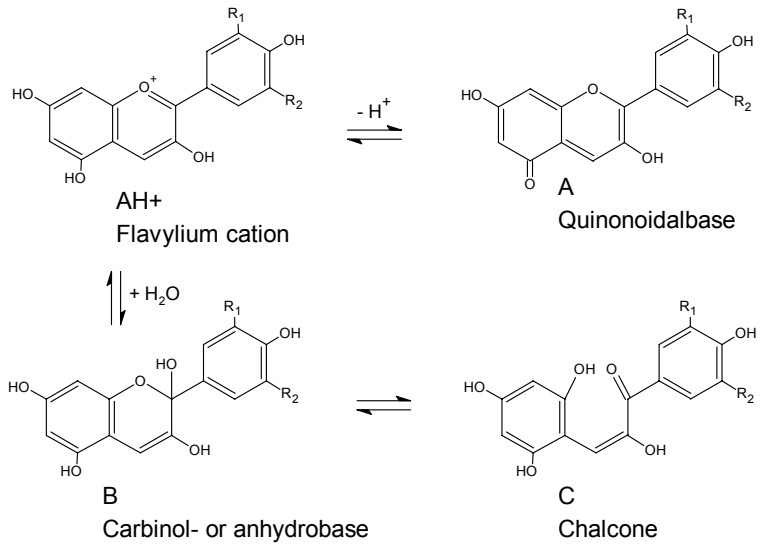


Figure 5. The main four equilibrium forms of anthocyanin existing in aqueous media.

In acidic aqueous solutions anthocyanins exist as four main equilibrium species: the quinonoidal base A, the flavylium cation AH+, the carbinol or pseudobase B, and the chalcone C (figure 5). In a very acidic media (pH 0.5) the red flavylium cation is the only predominating equilibrium species. Increasing the pH inflicts in decrease of both the color intensity and the concentration of the flavylium cation, as it is hydrated by nucleophilic attack of water, to the colorless carbinol form. The carbinol form has lost its conjugated double bond between the A- and B-ring and therefore does not absorb visible light (Brouillard, 1982). Also a rapid proton loss of the flavylium cation takes place as the pH shifts higher and the colored quinonoidal form rises. When pH increases further, the carbinol form yields, through ring opening, the colorless chalcone. The relative amounts of each equilibrium form vary with both the pH value and the structure of a certain anthocyanin (Mazza and Brouillard, 1987a; Jackman et al., 1987). In figure 6 is presented the distribution of the four main equilibrium species of malvidin 3-glucoside. In pH values between 4-5.5 very little color is left since the colorless carbinol and yellowish chalcone

species dominate. The quinonoidal form is the only colored species existing above pH value 5 but it exists in a neglectable percentage and does not affect the overall color of solution noticeably (Jackman et al., 1987). In studies on anthocyanin stability at a wide pH range in aqueos solutions, it was noticed that some anthocyanins showed improved color stability in alkaline region around pH 8-9, although the color intensities were modest (Fossen et al., 1998; Cabrita et al., 2000). The intensity of anthocyanins in alkaline solutions has been observed to increase near pH 10 but not to reach as high intensities as in acidic media, and also to have different color characterisitic concerning hue and λ_{max}, than in same solutions around pH 1. (Torskangerpoll and Andersen, 2005).

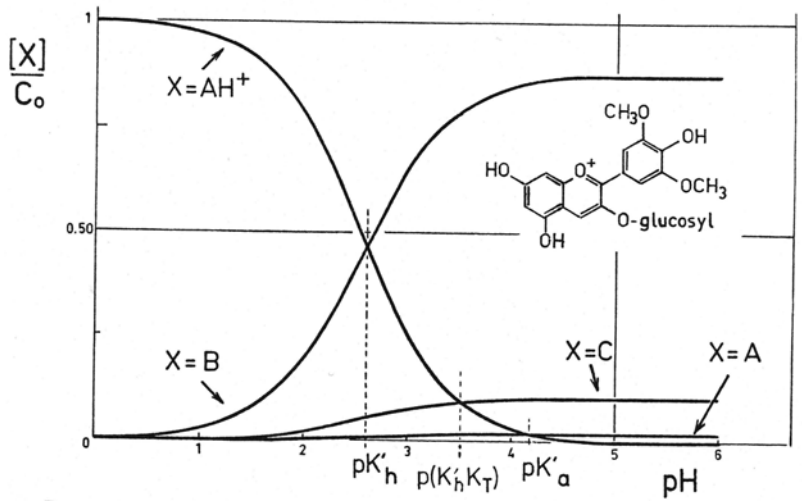


Figure 6. The distribution of the different Malvidin 3-glucoside equilibrium forms according to pH. AH+) Flavylium cation; A) Quinonoidalbase; B) Carbinol or anhydrobase; C) Chalcone (Brouillard, 1982).

The loss of anthocyanin color, as we understand it, is dependent on pH by the equilibrium of the four forms of anthocyanins, of which AH+, the flavylium cation, is the most stable and the most colored species. The flavylium cation exists, as demonstrated, in acidic media, and the equilibrium species dominating in alkali media degrade further easily and fast (Belitz and Grosch, 1999). Figure 6 demonstrates the significance of pH on anthocyanin color well: the color intensity is multifold at low pH value compared to higher pH value. If the pH does not favor the flavylium cation form, the color is lost. There are several ways to affect the color stability of the flavylium form *in vivo* or in food products at pH values close to neutral. The color of an alkali solution can also be reverted by changing the pH value back to acidic. In this case the anthocyanin equilibrium forms shift back to the equilibrium where the red colored flavylium form predominates. However, if the pH value is too high and unstable ionic chalcones have already been formed, this revitalising of color can not be achieved anymore (Brouillard, 1982).

2.2.4 Temperature

Anthocyanin stability is affected by temperature. The degradation rate of anthocyanins increases during processing and storage as the temperature rises (Palamidis and Markakis, 1978; Maccarone et al., 1985). Temperature rise in pH values 2-4 induces the loss of the glycosyl moieties of the anthocyanins, by hydrolysis of the glycosidic bond (Adams, 1973). This leads to further loss of anthocyanin color, since the aglycones are much less stable than their glycosidic forms. It is postulated that the formation of a chalcone is the first step in thermal degradation of anthocyanins (Markakis et al., 1957; Adams, 1973). Eventually thermal degradation leads to brown products, especially in the presence of oxygen (Markakis et al., 1957). Thermal degradation of anthocyanins follows first order kinetics (Markakis et al., 1957; Keith and Powers, 1965; Rhim, 2002; Ahmed et al., 2004). High temperature together with high pH caused degradation of cherry anthocyanins resulting in three different benzoic acid derivatives (Seeram et al., 2001) also a trihydrobezaldehyde has been identified as an end product of thermal degradation of anthocyanins (Furtado et al., 1993). Coumarin 3,5-diglycosides are also common thermal degradation products of anthocyanin 3,5-diglycosides (von Elbe and Schwartz, 1996). Figure 5 shows the proposed thermal anthocyanin degradation pathways.

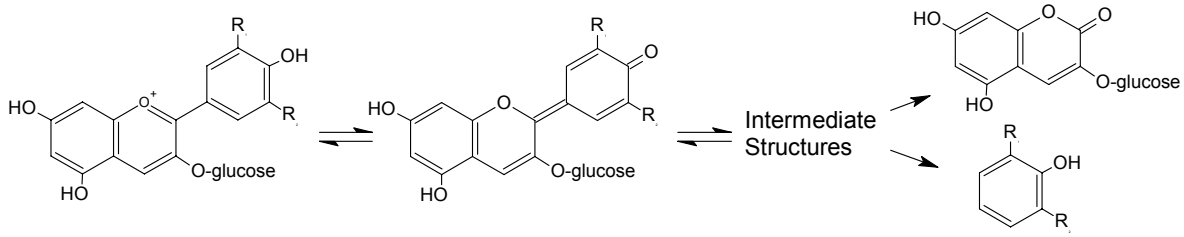


Figure 5. Degradation of anthocyanin monoglucoside at pH 3.7 accelerated by heat.

In general, the same structural factors, which enhance anthocyanin pH stability, also increase their thermal stability (von Elbe and Schwartz, 1996). Of three anthocyanin glucosides tested, pelargonidin 3-glucoside was more stable at 100°C than petunidin 3-glucoside, which was more stable than malvidin 3-glucoside (Keith and Powers, 1965). Arabinoses of cyanidin and peonidin were more stable to heat treatment than the corresponding galactoses (Attoe and von Elbe, 1981). In a thermal degradation study of four different anthocyanin extracts from different plant sources, red cabbage anthocyanins were the most stable ones, following black currant anthocyanins, grape skin anthocyanins, and the least stable were the anthocyanins of elderberry. The complex sugar residues of red

cabbage anthocyanins were proposed to be protective against thermal degradation (Dyrby et al., 2001). By decreasing pH value thermal degradation can be hindered. Also removal of oxygen protects anthocyanins from thermal degradation, and it cannot be overlooked that many other factors affect, together with heat, the stability of anthocyanins (Markakis et al., 1957, Daravingas and Cain, 1968).

Temperature can also have a positive effect on anthocyanins. Temperature is important for anthocyanin formation during storage of fresh berries for example. The anthocyanin content in strawberries and raspberries stored fresh for eight days was increased in storage temperatures over 0°C (Kalt et al., 1999). Also the anthocyanin content of different cranberry varieties increased during three months storage at 15°C (Wang and Stretch, 2001).

2.2.5 Oxygen

Oxygen amplifies the impact of other anthocyanin degradation processes. As previously mentioned the removal of oxygen protects against thermal degradation. The presence of oxygen, together with elevated temperature, was the most detrimental combinations of many factors tested against color deterioration of different berry juices and isolated anthocyanins (Nebesky et al., 1949). Oxygen together with ascorbic acid was also found damaging to the anthocyanin stability of cranberry juice. Contrary to thermal degradation, the galactosides were more stable than the arabinosides of cranberry anthocyanins against degradation induced by oxygen and ascorbic acid (Starr and Francis, 1968). Oxygen induced anthocyanin instability is affected by pH; the higher the pH, the more forcefull anthocyanin degradation in the presence of oxygen (Markakis, 1982). Light induced degradation of anthocyanins is dependent on molecular oxygen (Attoe and von Elbe, 1981). The deleterious effect of oxygen on anthocyanins can take place through direct oxidative mechanism and/or through indirect oxidation, where the oxidized components of the media further react with anthocyanins giving rise to colorless or brown products (Jackman et al., 1987). Anthocyanins react also with oxygen radicals, i.e. peroxyradicals. In such reactions anthocyanins act as antioxidants, and this feature of anthocyanins is considered to attribute to the healthiness of anthocyanin pigments against cardiovascular diseases for example (Matsufuji et al., 2003; Garcia-Alonso et al., 2004; Rossetto et al., 2004).

2.2.6 Light

Light affects anthocyanins in two different ways. Light is essential for the biosynthesis of anthocyanins, but it also accelerates their degradation (Markakis, 1982). Anthocyanins preserve their color much better when kept in the dark; the difference was seen already after 24 hours when anthocyanins were stored in light and for comparison in the dark at room temperature in pH 2.3 (Kearsley and Rodriguez, 1981). The color of carbonated grape anthocyanin beverages was lost only by 30% when kept in the dark, and in window light exposure 50% of the pigments were lost in otherwise same storage conditions. The most vigorous anthocyanin loss (70%) was observed under fluorescent light with slightly elevated storage temperature (Palamidis and Markakis, 1978). Furtado et al. (1993) found the end products of light induced degradation of anthocyanins to be the same as in thermal degradation, however, the kinetic pathway of the degradation reaction was different involving the excitation of the flavylium cation.

2.2.7 Enzymes

Inactivation of enzymes improves anthocyanin stability (Garcia-Palazon et al., 2004). The most common anthocyanin degrading enzymes are glycosidases, which break the covalent bond between the glycosyl residue and the aglycone of an anthocyanin, resulting in the degradation of the highly unstable anthocyanidin (Huang, 1955; Huang, 1956). Peroxidases and phenolases, such as phenol oxidases and polyphenol oxidases, which both are found naturally in fruits and berries themselves, are also common anthocyanin degradation enzymes (Pifferi and Cultrera, 1974; Kader et al., 1997).

Phenolases can react directly with anthocyanins, but the destruction of anthocyanins is much more efficient when other phenolic compounds, such as catechol and caftaric acid are present (Peng and Markakis, 1963; Yokotsuka and Singleton, 1997). However, Sarni-Manchado et al. (1997) stated that no malvidin 3-glucoside degradation was observed by polyphenol oxidase alone, but only in the presence of caftaric acid.

In enzymatic degradation of anthocyanins, quinones play an important role (Sakamura et al., 1966). Enzymes first oxidize other phenolic compounds in the media to their corresponding quinones, which then react with anthocyanins resulting in anthocyanin degradation, and leading to brown condensation products. This has been observed in many studies with pure anthocyanins, like pelargonidin 3-glucoside (Kader et al., 2001) and cyanidin 3-glucoside (Kader et al., 1999), with wine model solutions (Sarni et al., 1995),

and with berry products (Yokotsuka and Singleton, 1997; Skrede et al., 2000; Kader et al., 2002). Of the anthocyanin forms, Pifferi et al. (1974) noticed the anhydrobase to be the most susceptible to enzymatic oxidation. The structure of anthocyanins affects their stability against enzyme activity (Yokotsuka and Singleton, 1997). Malvidin glycosides were more stable than delphinidin glycosides against polyphenol oxidase (Skrede et al., 2000). Also cyanidin was observed to react directly with polyphenol oxidase, but pelargonidin did not react at all (Wesche-Ebeling and Montgomery, 1990).

2.2.8 Ascorbic acid

Fortification of fruit and berry juices with ascorbic acid is a common method to protect against oxidation and to increase the nutritional value of a food product. Ascorbic acid is thought to have several different roles in anthocyanin color stability. Anthocyanin decomposition is accelerated by the presence of ascorbic acid (Meschter, 1953; Starr and Francis, 1968; Poei-Langston and Wrolstad, 1981; Marti et al., 2002). Ascorbic acid enhances polymer pigment formation and bleaches anthocyanin pigments (Poei-Langston and Wrolstad, 1981). Direct condensation between anthocyanins and ascorbic acid has been postulated as a mechanism for anthocyanin degradation (Poei-Langston and Wrolstad, 1981). Also the formation of hydrogen peroxide from ascorbic acid oxidation can influence anthocyanin stability (Meschter, 1953; Markakis, 1982; Talcott et al., 2003). However, the stability of acylated anthocyanins has been observed to increase in the presence of ascorbic acid (Del Pozo-Insfran et al., 2004). Anthocyanins are also considered to be protected by ascorbic acid against enzymatic degradation (Talcott et al., 2003).

2.2.9 Sugars

Sugars are naturally present in fruits and berries, and during food production processes they are often added to different berry and fruit products, such as juices and preserves. Sugars, as well as their degradation products, are known to decrease anthocyanin stability (Meschter, 1953; Thakur and Arya, 1989). In a study by Daravingas and Cain (1968) all the tested sugars (sucrose, fructose, glucose, and xylose) increased anthocyanin degradation, behaving all in the same way. Of the typical sugar degradation products, furfural accelerated anthocyanin pigment deterioration more prominently than hydroxy-methylfurfural (Meschter, 1953). The reactions of anthocyanins with both the degradation products of sugars and ascorbic acid, yield in the formation of brown pigment polymers. (Krifi et al., 2000).

Sugars have also been found to protect anthocyanins and the color of berry products. Sucrose protected anthocyanins from degrading during frozen storage, and also prevented browning, and the formation of polymeric pigments, which is probably due to the inhibition of enzymatic reactions or the hindering of different condensation reactions by sucrose (Wrolstad et al., 1990). Also the lowering of water activity by sugars can be protective against anthocyanin degradation (De Ancos et al., 1999b).

2.3 Copigmentation

The color of anthocyanins can be stabilized and enhanced by copigmentation reactions. The copigmentation phenomenon was observed already in 1916 by Willstätter and Zollinger, who noticed the color of a grape pigment, oenin (malvidin 3-glucoside), to change its hue to bluer red by the addition of tannin or gallic acid. Copigmentation was first studied with flower colors. Robinson and Robinson (1931) observed the same anthocyanin to produce different color hues in different flower petals, and different anthocyanins to produce the same colors in different parts of plants. In plant vacuoles, the free anthocyanins ought to be colorless in the prevailing weakly acidic pH conditions. Since anthocyanins are nevertheless colorful in nature, their colored forms must be strongly stabilized by other natural components, the so-called copigments, existing in the cells of flowers, fruits and berries (Brouillard, 1982).

Copigmentation can be a valuable, natural tool for improving the color of anthocyanin rich food products, the color of which can be stabilized and enhanced by the addition of different plant extracts rich in copigments. It has been observed already that copigmentation is more intense in berry juices than with the purified anthocyanin molecules of these juices (Wilska-Jeszka and Korzuchowska, 1996). This indicates that several other components of the juice material play a role in the copigmentation phenomenon than just one added copigment molecule.

Copigmentation can take place through several interactions (figure 7). The most important mechanisms of copigmentation are the intermolecular and the intramolecular complex formations. Self-association and metal complexation are also possible means through which copigmentation occurs.

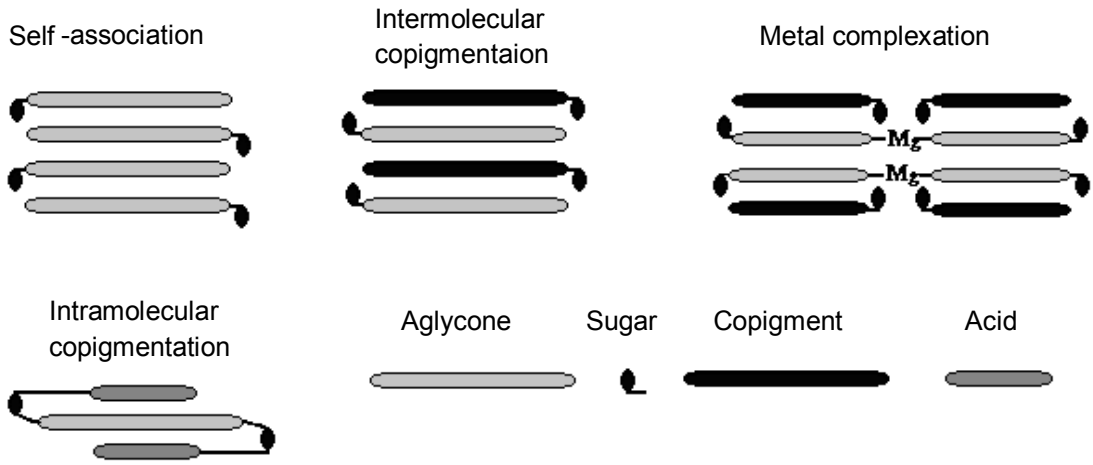


Figure 7. Anthocyanin interactions.

Copigmentation phenomena is observed as a bathochromic shift ($\Delta\lambda_{max}$), i.e. the shift of the maximum absorption wavelength (λ_{max}), in the visible range towards higher wavelength, which is also called the bluing effect, since the color of an anthocyanin changes from red to more blue through copigmentation (Asen et al., 1972), or as a hyperchromic effect (ΔA), in which the intensity of an anthocyanin color (A) is fortified by copigmentation. Both the flavylium cation and the quinonoidal base play a role in the bathochromic shift, but the hyperchromic effect is achieved only by the stabilization of the latter (Asen et al., 1972). Figure 8 demonstrates the change in absorption maximum wavelength (bathochromic shift) and in the color intensity (hyperchromic effect) for cyanidin 3-glucoside copigmented with rosmarinic acid.

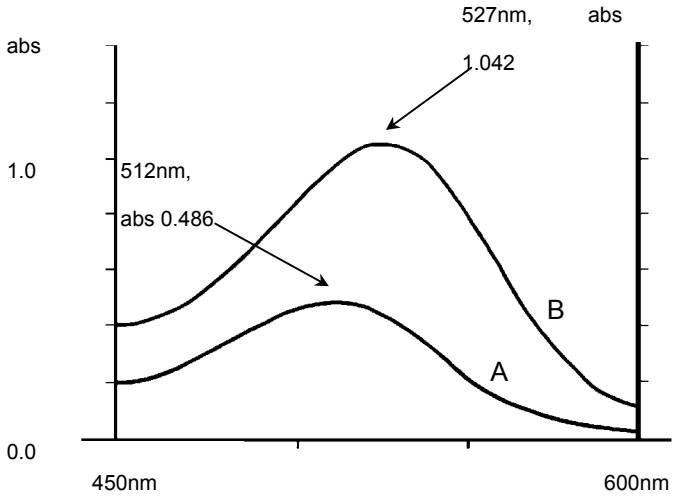


Figure 8. Copigmentation as hyperchromic effect and bathochromic shift.
A) Cyanidin 3-glucoside, B) Cyanidin 3-glucoside + rosmarinic acid

Like all the reactions of anthocyanins, also copigmentation reactions are strongly affected by pH (Williams and Hrazdina, 1979; Wilska-Jeszka and Korzuchowska, 1996), temperature (Bakowska et al., 2003), concentrations (Scheffeldt and Hrazdina, 1978; Bakowska et al., 2003), solvents (Brouillard et al., 1989; Brouillard et al., 1991), and molecule structures. According to Asen (1976), the anthocyanin concentration needs to be above 3.5 x 10^{-5} M before copigmentation reactions are possible, and also the concentration of the copigment is critical. It has been observed that the color intensity of strawberry and chokeberry juices increased linearly with the increasing copigment addition (Wilska-Jeszka and Korzuchowska, 1996). The effect of concentration ratios has also been clearly demonstrated; the higher the copigment concentration over the anthocyanin concentration, the more efficient copigmentation effect was observed (Asen et al., 1972; Scheffeldt and Hrazdina, 1978). The increment of temperature hinders the copigmentation phenomenon (Cai et al., 1990; Wilska-Jeszka and Korzuchowska, 1996). At very low pH values, where the flavylium cation dominates, copigmentation reactions are much weaker than at pH values between 2 and 5 where also the quinonoidal equilibrium form exists (Williams and Hrazdina, 1979). Also the solvent system affects the copigmentation phenomenon. The structure of water, in liquid state, governs the molecular association between an anthocyanin and its copigment (Mazza and Brouillard, 1990). The stronger the network of hydrogen bonded tetrahedral water molecules is, the stronger copigmentation phenomenon can be achieved (Brouillard et al., 1991). Therefore, the addition of methanol, formamide, or salts to the solvent reduces the copigmentation effect (Brouillard et al., 1989).

In wines, instability and reactivity of anthocyanins, together with copigmentation reactions, are thought to be responsible for the changing color of aging wines (Boulton, 2001; Darias-Martin et al., 2002; Gutierrez, 2003; Boselli et al., 2004). In fruit and berry products, by copigmentation the color of juices (Wilska-Jeszka and Korzuchowska, 1996; Dufour and Sauvaitre, 2000), purees, jams and syrups could be enhanced and stabilized, improving consumer acceptance and prolonging product shelf-life. However, this has not yet been well established in food products and more research is needed in the field of copigmentation of anthocyanin rich foods.

2.3.1 Copigments

Copigments are colorless or only very slightly, mainly yellowishly, colored molecules occurring naturally in plant kingdom in cells alongside anthocyanins. A wide range of different molecules has been found to act as copigments. The most common structurally unrelated copigment compounds are flavonoids, and other polyphenols, alkaloids, amino

acids, and organic acids (Brouillard et al., 1989). The most studied group of copigments is the flavonoids, of which flavones, flavonols, flavanones, and flavanols have been under profound examination (Asen et al., 1972; Chen and Hrazdina, 1981; Baranac et al., 1997a; Baranac et al., 1997c). Also phenolic acids, i.e. hydroxycinnamic acids and hydroxybenzoic acids, have been of interest for copigmentation researchers, and have shown a prominent effect on the enhancement and stabilization of anthocyanin (Markovic et al., 2000). However, phenolic acids have not been studied as extensively as flavonoids.

Of the flavonols, rutin is one of the most efficient copigment, and also quercetin has been found to produce strong copigmentation (Scheffeldt and Hrazdina, 1978; Williams and Hrazdina, 1979; Chen and Hrazdina, 1981; Ballington et al., 1987; Baranac et al., 1997b; Gonnet, 1999; Bakowska et al., 2003,). Rutin induced a bathochromic shift of 30 nm and quercetin of 28 nm to malvidin 3,5-diglucoside at pH 3.2 (Chen and Hrazdina, 1981). However, at very low pH values (< 2.0) intermolecular copigmentation induced by rutin resulted only in bathochromic shift and not in increase of color intensity (Williams and Hrazdina, 1979).

Of the phenolic acids, sinapic acid has been found to be one of the most efficient copigment but also ferulic acid has exhibited strong color enhancement and bathochromic shift (Asen et al., 1972; Markovic et al., 2000). Benzoic acids are on the contrary quite week copigments (Asen et al., 1972). Other copigments such as tannins (Robinson and Robinson, 1931; Cai et al., 1990), caffeine (Brouillard et al., 1991), and the conjugated hydroxycinnamic acid, chlorogenic acid, (Brouillard et al., 1989, Brouillard et al., 1991) have also been investigated. Purines were observed to be efficient copigments (Brouillard et al., 1991) with the synthetic anthocyanin 7-hydroxy-3,4´-dimethoxyflavylium (Wigand et al., 1992). Also amino acids have shown color enhancement, especially proline and arginine, but no bathochromic shift was observed (Asen et al., 1972).

Fruits and berries as such do not contain free phenolic acids in high amounts. However, the free phenolics existing in plant materials can be regarded as potential copigments (Gao and Mazza, 1994). Chlorogenic acid is the main free phenolic acid of blue berries existing in considerable amounts (200-1000 mg/kg FW) (Gao and Mazza, 1994; Skrede et al. 2000). Modest amounts of ferulic and coumaric acid have been detected likewise in blue berries (Mattila et al. 2004). In raspberries and strawberries only chlorogenic acid has been detected to exist in non-bound form (Mattila and Kumpulainen, 2002).

2.3.2 Intermolecular copigmentation

Intermolecular interaction is a more prominent means of copigmentation in fruits and berries, and products derived from them, than intramolecular copigmentation, due to the lack of acylated anthocyanins in fruits and berries. The mechanism for intermolecular copigmentation was first thought to be a weak complex formation (Robinson and Robinson, 1931). Later, intermolecular copigmentation was defined as the interactions between a colored anthocyanin and a colorless copigment, which is not bound covalently to the anthocyanin molecule (Brouillard, 1983). Hydrogen bonding and hydrophobic interactions have been suggested as the main mechanistic driving forces for intermolecular copigmentation, resulting in 1:1 complex formation (Asen et al., 1972; Williams and Hrazdina, 1979; Chen and Hrazdina, 1981; Brouillard et al., 1989; Cai et al., 1990). Also ionic (electrostatic) interactions have been speculated as possible means for intermolecular copigmentation (Chen and Hrazdina, 1981). However, Brouillard et al. (1991) stated that the coulombic repulsion or attraction between anthocyanin and its copigment are not the driving forces for copigmentation.

Intermolecular interactions can occur with both the flavylium cation and the quinonoidal base forms of the anthocyanin (Asen et al., 1972; Williams and Hrazdina, 1979; Chen and Hrazdina, 1981). Since both these colored equilibrium forms of anthocyanins are almost planar, with efficiently delocalized π-electrons, the interactions with copigments, having the same structural features, make the interactions between the flavylium cation or quinonoidal base and copigment much more easier and probable. This results in an overlapping arrangement of the two molecules (figure 9A), preventing nucleophilic attack of water on the anthocyanin molecule. The formation of hydrogen bonds between the keto group of the quinoidal base form of an anthocyanin and a flavonol copigment has been suggested as a possible means of complex formation (figure 9B) (Williams and Hrazdina, 1979). In such a case, the keto group in the 7- or 4′-position of the anthocyanin would hydrogen bond with the 7-, 3′-, or 4′-hydroxyl group of a flavonol (Williams and Hrazdina, 1979).

Unlike metal complexation, intermolecular copigmentation occurs with all the main anthocyanidins (Chen and Hrazdina, 1981). The structure of anthocyanins, as well as of copigments, affects the magnitude of copigmentation. For 3-monoglucosides, maximum complex formation and color enhancement was observed to occur at pH 3.5-4.2, for 3,5-diglucosides and 3-acylglucoside-5-glucosides at pH 3.1, and at little higher pH values, approximately 4.0-4.5 for 3-acylglucosides (Williams and Hrazdina, 1979; Wilska-Jeszka and Korzuchowska, 1996). The typical pH values of berry juices are around 3.5, which

settle well between the observed pH values for maximum copigmentation of differently substituted anthocyanins. It has been stated that the more hydroxyl groups are present in the flavonoid copigment, the stronger the copigmentation and complex formation, and that the most important hydroxyl group for the complex formation is the one situated at C-7 (Chen and Hrazdina, 1981). Methoxyl groups of the copigments have been observed to decrease the copigmentation effect (Chen and Hrazdina, 1981).

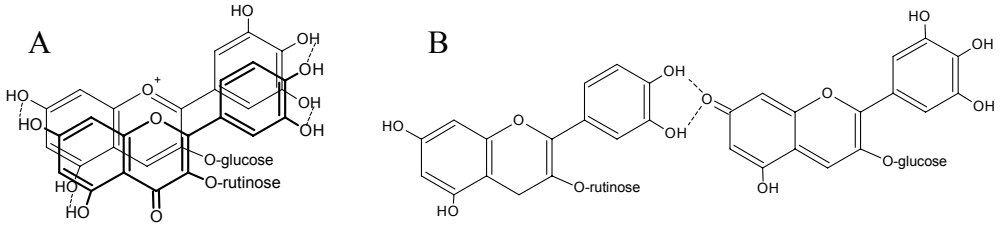


Figure 9. Two complex formations through intermolecular interactions between delphinidin 3-glucoside and rutin. A) Interlocked complex and B) Parallel complex (adapted from Williams and Hrazdina, 1979 and Osawa, 1982).

2.3.3 Intramolecular copigmentation

Intramolecular copigmentation is loosely defined as such that the copigment is part of the anthocyanin molecule (Brouillard, 1983). The specified definition is such that covalent acylation of the anthocyanin molecule stabilizes the pigment (Francis, 1989; Dangles et al., 1993). This acylation is always attached to a glycosyl moiety of an anthocyanin. Intramolecular copigmentation is thought to be stronger and more effective in stabilizing the anthocyanin color than intermolecular copigmentation probably due to strengths of the bonds (Brouillard, 1982).

The exceptional color stability of flower colors near neutral pH values was associated with intramolecular copigmentation, since anthocyanins of flowers are often acylated. This applies also to black carrot extracts and other edible plant materials which contain acylated anthocyanins in high amounts (Giusti and Wrolstad, 2003). The aromatic residues of acyl groups of an anthocyanin interact with the positively charged flavylium cation so that the reactivity of the carbon C-2 and C-4 with nucleophilic reactants, e.g. water molecules, is hindered (Brouillard, 1981; Brouillard, 1983). Brouillard (1981) was the first to suggest this protective stacking to take place on both sides of the anthocyanin aglycone and called it a sandwich type of stacking. He also stated that it is hydrophobic interactions between the flavylium aglycone and the aromatic residues of the acyl groups that are the driving forces for this stacking conformation.

The number of acyl groups, their structure, and the position of attachment to glycosyl residues, as well as the structure and number of saccharides affect the intramolecular copigmentation (Brouillard, 1983; Dangles et al., 1993). Copigmentation effect of monoacylated anthocyanins is not as efficient as that of polyacylated anthocyanins. It is believed that in the case of a monoacylated anthocyanin, one side of the chromophore is unprotected and water is capable of attacking the unprotected side of the aglycone, and only a partial stabilization is established (Brouillard, 1983). The glycosyl residues are considered as spacers, which enable the folding of the acyl group on the aglycone to take place. Interaction of an acyl group attached to a glycosyl residue, which is at any of 3-, 5-, 7-, 3´- or 5´-positions of the anthocyanin chromophore, with the aglycone should be possible (Brouillard, 1983). Acylated substituents at C-7 and C-3´ have been observed to completely protect the anthocyanin from hydration, resulting in a stability of the anthocyanin also at slightly acidic or neutral milieu (Figueiredo et al., 1999). However, caffeic acid residue esterified to a glycosyl unit at 3´-position of delphinidin produced a copigmentation effect, but when it was esterified to a glycosyl unit at 5-position of the same anthocyanidin no significant copigmentation effect was observed (Yoshida et al., 1992). In the case of pelargonidin 3-(caffeyl) sophorosyl-5-glucoside, the glycosyl spacer was considered too short to allow a close enough contact of the acyl moiety to adequately protect the chromophore (Dangles et al., 1993).

2.3.4 Self-association

Asen et al. (1972) noticed that the increment of anthocyanin concentration from 10^{-4} to 10^{-2} M caused a change of absorbance maximum from 507 to 502 nm, and a 300-fold increase in absorbance of color intensity, and reasoned this to be due to anthocyanin self-association. The mechanism of self-association has been discussed to be analogous to the stacking-like interactions (Hoshino et al., 1980). Self-associations of anthocyanins have been observed to take place during wine aging and it is assumed that they may partially contribute to the color of aged wines (Ribéreau-Gayon, 1982).

2.3.5 Metal complexation

Metal complexation is only of little interest to the food industry due to undesired contamination of food products by metals, and therefore it shall be only briefly mentioned here. The many variations of flower colors were originally explained to be due to complex formation between blue metal chelates with red flavylium salts (Asen et al., 1972). The

most common metals in anthocyanin complexes are tin (Sn), copper (Cu), iron (Fe), aluminum (Al), magnesium (Mg), and potassium (K) (Starr and Francis, 1974; Markakis, 1982). Only cyanidin, delphinidin and petunidin based anthocyanins, which have more than one free hydroxyl group in the B-ring, are capable of metal chelation (Osawa, 1982). Metals have been shown to stabilize the color of different berry products, such as strawberry puree (Wrolstad and Erlandson, 1973), cranberry juice cocktail (Starr and Francis, 1974), and crowberry juice (Kallio et al., 1986).

2.4. Pyranoanthocyanins

Anthocyanin derived molecules, formed through direct reactions between anthocyanins and small molecules, resulting in a new ring formation are called pyranoanthocyanins. Pyranoanthocyanins exhibit very stable and strong color even at higher pH values (Sarni-Manchado et al., 1996; Fulcrand et al., 1996; Bakker and Timberlake, 1997; Fulcrand et al., 1998). They are also resistant to SO_2 discoloration (Sarni-Manchado et al., 1996; Bakker and Timberlake, 1997; Francia-Aricha et al., 1997). Pyranoanthocyanins have become known only recently within the last decade. In the course of wine aging, the formation of new anthocyanin derived pigments contribute to the changing color, and it was through wine pigmentation studies that pyranoanthocyanins were first found (Sarni-Manchado et al., 1996; Fulcrand et al., 1996). In the beginning, pyranoanthocyanins were studied in model wine solutions (Sarni-Manchado et al., 1996; Romero and Bakker, 1999; Salas et al., 2003), and now the same pigments have been identified also in authentic wines (Mateus and de Freitas, 2001; Mateus et al., 2002a; Hayasaka and Asenstorfer, 2002; Alcalde-Eon et al., 2004). In black carrot juice (Schwarz et al., 2004) and blood orange juice (Hillebrand et al., 2004) pyranoanthocyanins have been found only in very small portions. In berry products, such as juices or concentrates, pyranoanthocyanins have not yet been identified. Pyranoanthocyanins could be very promising pigments contributing to the color of different berry products as stable and intense color molecules.

Since pyranoanthocyanins cause a more stable color and a changed hue, could their effect on color not be classified as copigmentation? They are formed from intact anthocyanins and for example phenolic acids, which are considered as efficient copigments. Through the interpretation of the loose definition of intramolecular copigmentation, a covalent bonding between an anthocyanin and a copigment, which results in an enhanced color, can be regarded as copigmentation. Earlier, when copigmentation was defined by the phenomena on the color and there was no adequate means of identification of the anthocyanin

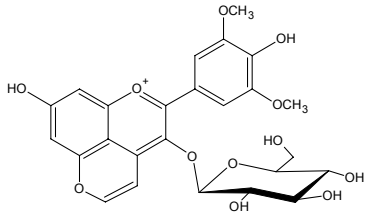
complexes, which in the case of intermolecular copigmentation is insufficient even today, misinterpretations on copigmentation were possible. Therefore it is incomplete to concentrate on the phenomena of copigmentation, without discovering the reasons behind the changed color hue and color stability. Nowadays, with the state of the art analytical equipment, it is indeed essential and possible to identify the molecules behind copigmentation phenomena and not only report the observable facts.

2.4.1 Formation and structures

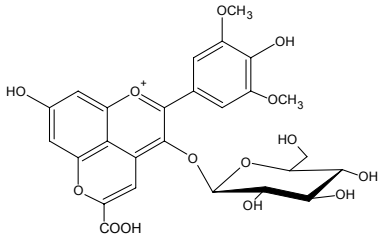
The first pyranoanthocyanins found were malvidin 3-glucoside and malvidin 3-(6-coumaroyl)glucoside derivatives of 4-vinylphenol (Fulcrand et al., 1996). The smallest pyranoanthocyanin is the vitisin B, malvidin 3-glucoside-4-vinyl (figure 10), which consists of malvidin 3-glucoside to which CH=CH is bind at C-4 and C-5 positions (Bakker and Timberlake, 1997; Asenstorfer et al., 2001; Morata et al., 2003). More complex pyranoanthocyanins have also been identified, of which a malvidin 3-glucoside derivative with two catechin units attached through a D-ring of the pyranoanthocyanin, is a good example (Francia-Aricha et al., 1997). Also pyranoanthocyanins with other anthocyanin aglycones and anthocyanins with different glycosyl and acyl residues are known (Asenstorfer et al., 2001, Lu et al., 2002). The most common anthocyanin aglycone identified in pyranoanthocyanins is malvidin, since it represents the major anthocyanins of wines, in which pyranoanthocyanins have been mostly studied. Pyranoanthocyanin derivatives of peonidin, petunidin, delphinidin, and cyanidin have also been identified to a lesser extent (Hayasaka and Asenstorfer, 2002; Alcalde-Eon et al., 2004; Pozo-Bayon et al., 2004). Some of the different pyranoanthocyanins known today are shown in figure 10.

The formation of the new wine pigments was first observed to take place through condensation reactions between anthocyanins and flavanols (Liao et al., 1992; Rivas-Gonzalo et al., 1995; Dallas et al., 1996; Francia-Aricha et al., 1997), mediated through acetaldehyde (Bakker et al., 1993; Rivas-Gonzalo et al., 1995; Bakker and Timberlake, 1997; Es-Safi et al., 1999) and puryvic acid environments (Fulcrand et al., 1998; Romero and Bakker, 1999). Earlier, pyranoanthocyanins were considered to form from direct covalent reactions of anthocyanins with vinylphenols (Fulcrand et al., 1996), the latter being wine fermentation products, resulting from enzymatic decarboxylation of phenolic acids (Grando et al., 1993; Coghe et al., 2004). Fulcrand et al. (1996) suggested two possible pathways for the formation of pyranoanthocyanins; a cycloaddition, or an electrophilic addition of a vinylphenol double bond to an anthocyanin nucleus, followed by an oxidation step. The electrophilic addition mechanism was favored, because of the

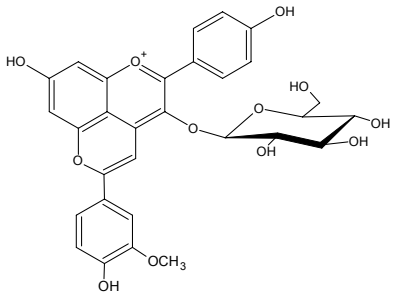
electrophilic site at C-4 and the nucleophilic site at C-5 hydroxy group of the anthocyanin. The substitution at C-4 of anthocyanins, as is in the case of pyranoanthocyanin formation, stabilizes the pigment molecule because it prevents the hydration of the pyranoanthocyanin to a colorless carbinol base (Fulcrand et al., 1996). The reaction mechanism of pyranoanthocyanin formation is not clear yet and several different pathways could exist. Recently, anthocyanin reactions with intact simple cinnamic acids have been put forward (Schwarz and Winterhalter, 2003). Pyranoanthocyanin formations were considered to be quite fast in model solutions (Sarni-Manchado et al., 1996; Fulcrand et al., 1996; Schwarz and Winterhalter, 2003).



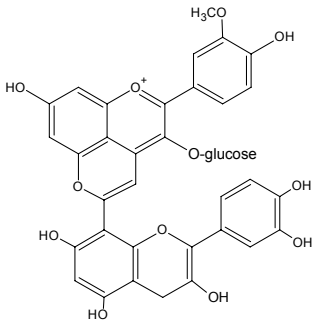
Vitisin B



Malvidin 3-glucoside pyruvic adduct



Pelargonidin 3-glucoside, 4-vinylguaiacol



Peonidin 3-glucoside, 4-vinylcatechin

Figure 10. Structures of four different pyranoanthocyanins.

2.4.2 Occurrence and manifestation in wines and berries

Pyranoanthocyanins exhibit more orange-red tint than their corresponding intact anthocyanins and are therefore considered to be in part responsible for the color of some aged wines (Sarni-Manchado et al., 1996; Francia-Aricha et al., 1997; Mateus et al., 2002b; Alcalde-Eon et al., 2004). A hypsochromic shifts of the maximum absorbance wavelength (λ_{max}) was observed with malvidin 3-glucoside derivatives of around 18-19 nm for vitisin A and 36-39 nm for vitisin B, depending on the solvent (Bakker and Timberlake, 1997). The color of pyranoanthocyanins is more enduring during storage than that of plain anthocyanins, and can be considered more constant with regards to colorimetric parameters

(Sarni-Manchado et al., 1996). Also stable pyranoanthocyanins with a unique violet hue have been synthesized (Schwarz and Winterhalter, 2003). Unusually intense bluish colored pyranoanthocyanins have been synthesized from malvidin based pyruvic acid adducts and 8-vinylcatechin (Mateus et al., 2004) and isolated form wines (Mateus et al., 2003b). Stable color and a different hue of pyranoanthocyanins is most probably due to delocalization by resonance of the positive charge of the C ring of the flavylium cation, which is partly shared by the oxygen atom of the new D ring (Bakker and Timberlake, 1997). Of course the color change is also due to the increment of double bond conjugation. Near neutrality the formation of stable quinonoidal bases is responsible for the bluer hues of pyranoanthocyanins.

Identification of pyranoanthocyanin pigments in red table wines and sparkling red wines have been reported recently (Dangles and Elhajji, 1994; Hayasaka and Asenstorfer, 2002; Vivar-Quintana et al., 2002; Schwarz and Winterhalter, 2003; Schwarz et al., 2003; Alcalde-Eon et al., 2004; Pozo-Bayon et al., 2004; Schwarz et al., 2004). Also port wine has proven to contain a considerable amount of pyranoanthocyanins (Mateus and de Freitas, 2001; Mateus et al., 2001; Romero and Bakker, 2001; Mateus et al., 2002a; Mateus et al., 2002b; Mateus et al., 2003a; Mateus et al., 2003b). Most of the known pyranoanthocyanins today have been found in wines, and only few of such have been identified elsewhere. Bakker and Timberlake (1997) have detected pyranoanthocyanins in frozen grapes after storage, but not in the skins of fresh grapes. Pyranoanthocyanins have been identified in grape skin extracts (Revilla et al., 1999), grape pomace (Amico et al., 2004), black carrot juice (Schwarz et al., 2004), strawberries (Andersen et al., 2004), red onion (Fossen and Andersen, 2003), and black currant seeds (Lu et al., 2000; Lu et al., 2002). In red onion, two pyranoanthocyanins were identified; 5-carboxypyrano-anthocyanidin 3-glucoside and 5-carboxypyranoanthocyanidin 3-(6´´-malonyl)glucoside. The pyranoanthocyanin derivatives found in black currant seeds consisted of cyanidin and delphinidin aglycones with either 3-glucoside or 3-rutinoside glycosyl unit, with methylpyranoside D-ring or 4-vinylphenol adduct. In strawberry, 5-carboxypyrano-pelargonidin 3-glucoside was found in very small quantities.

Identification of pyranoanthocyanins in berry products has been non-existent. For one, because they have not been examined, two because in berries pyranoanthocyanins present only extremely small quantities of the total anthocyanins, and three because berry juices and other products lack the fermentation process of wines, through which pyranoanthocyanins are formed in much more considerable quantities. The lack of fermentation in berry juices prevents the formation of these new stable color molecules that are found in reactions mediated, for example, through acetaldehyde in wines.

3. AIMS OF THE STUDY

The overall aim of this thesis was to study different anthocyanin colors, and factors that enhance and stabilize them. It was of interest to examine effects of physico-chemical factors and storage on anthocyanin pigments and their stability, and on copigmentation phenomena. The aim was to gain knowledge of anthocyanin chemical behavior during storage and to better understand the anthocyanin color display in berry juices and wine.

The main objectives were:

- To evaluate the addition of natural color enhancers, such as plant extracts and anthocyanin rich berry juice, on the color enhancement and stability of berry wine (**I**) and berry juices (**III**).

- To study the copigmentation effects of phenolic acids on pure anthocyanins (**II**) and on berry juices (**III**).

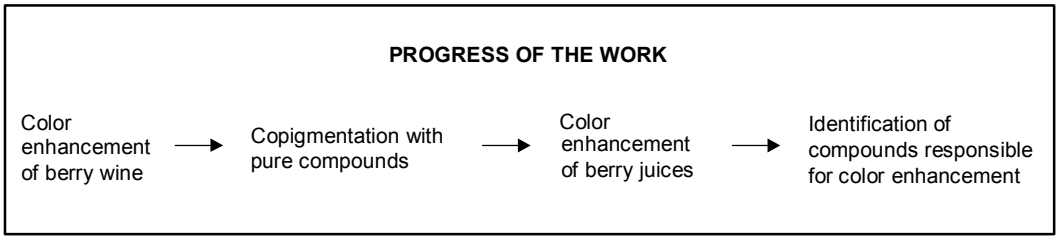
- To investigate the stability of the anthocyanin colors during storage (**I**, **II**, **III**).

- To identify the novel berry juice anthocyanins responsible for the enhanced juice color (**IV**).

4. MATERIALS AND METHODS

The evolution of the work is presented in Scheme 1. and experimental design of the work is presented in Scheme 2. More detailed materials and methods are described in the original papers **I-IV**.

Scheme 1. Evolution of the thesis work.



Scheme 2. Experimental design of the work.

STUDY	PIGMENT MEDIUM	COLOR ENHANCERS	METHODS
I	**Black currant wine**	**Color enhancers** Grape Skin Extract (GSE) Crowberry	**HPLC** Changes in anthocyanin, flavonoid and phenolic acid content **Spectrophotometry** Hyperchromic effect bathochromic shift
II	**Pure anthocyanins** Pelargonidin 3-glucoside Cyanidin 3-glucoside Malvidin 3-glucoside Cyanidin 3-(xylosyl-glucosyl)-galactoside Cyanidin 3-(xylosyl-(coumaroyl-glucosyl))-galactoside	**Pure phenolic acids** Ferulic acid Caffeic acid Chlorogenic acid Rosmarinic acid Gallic acid	**Spectrophotometry** Hyperchromic effect bathochromic shift
III	**Berry juices** Strawberry juice Raspberry juice Lingonberry juice Cranberry juice	**Pure phenolic acids +** **Color enhancers** Ferulic acid Sinapic acid Rosmarinic acid Black Carrot Extract (BCE) Grape Skin Extract (GSE)	**HPLC** Changes in anthocyanin content **Spectrophotometry** Hyperchromic effect bathochromic shift **CIELab** Changes in color parameters
IV	**Berry juices** Strawberry juice Raspberry juice **Pure anthocyanin** Pelargonidin 3-glucoside	**Pure phenolic acids** Ferulic acid Sinapic acid 4-Vinylsyringol	**HPLC** Changes in anthocyanin content **LC-MS** Identification of novel pyrano-anthocyanins

4.1 Pure compounds

4.1.1 Anthocyanins

The anthocyanins used were, pelargonidin 3-glucoside (callistephin), cyaniding 3-glucoside (kuromanine), malvidin 3-glucoside (oenin), cyanidin 3-*O*-(2″-*O*-*β*-xylopyranosyl-6″-*O*-*β*-glucopyranosyl)-*β*-galactoside, and cyanidin 3-*O*-(2″-*O*-*β*-xylopyranosyl-6″-*O*-(6-*O*-(*E*-coumaroyl)-*β*-glucopyranosyl))-*β*-galactoside. The chemical structures of these anthocyanins are shown in figure 11.

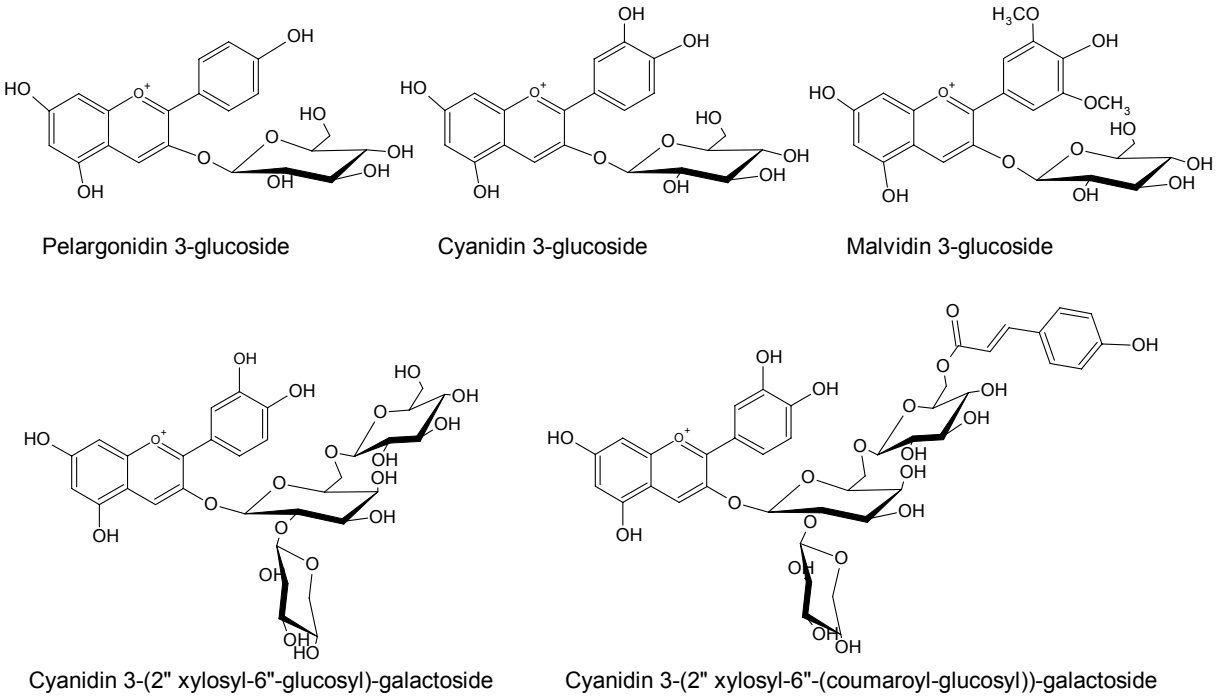


Figure 11. The sturctures of anthocyanins used in the studies.

4.1.2 Copigments

The copigments used were ferulic acid, sinapic acid (**II, III, IV),** rosmarinic acid (**II, III)**, caffeic acid (**II)**, chlorogenic acid (**II)**, gallic acid (**II**), and 4-vinylsyringol (**IV)**. The chemical structures of these copigments are shown in figure 12.

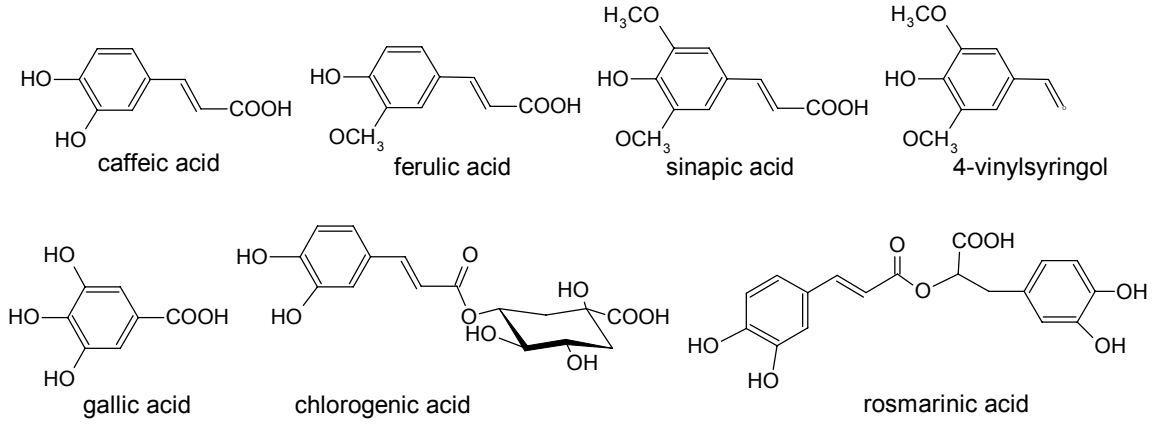


Figure 12. The sturctures of different copigments used in the studies.

4.2 Juices and wine

4.2.1 Berries

Black currants (*Ribes nigrum* L.) were used for berry wine making (**I**). Berry juice was obtained from frozen lingonberries (*Vaccinium vitis-idaea*, L.) and cranberries (*Vaccinium oxycoccus*, L.) from the previous summer crop, while strawberry (*Fragaria ananassa*) and raspberry (*Rubus ideaeus*, L.) juices were brought from local supermarket (**III**). Also frozen strawberries and red raspberries were used as juice material (**IV**).

4.2.2 Color enhancers

All the berry juices and wine were enhanced with natural color enhancers (**I**, **III**). Crowberry (*Empetrum nigrum* L.) juice and Grape skin extract (E 163), in the concentration of 10 g/L, were used as wine color enhancers (**I**). Grape skin extract (Enocyanin), Black carrot extract, and Color'Plus®, a non-anthocyanin rosemary extract, were used as color enhancers in the amount of 2 g/L of berry juice (**III**).

4.3 Analytical methods

4.3.1 Pure solutions (II)

Anthocyanins were dissolved in 0.5% TFA at the concentration of 0.2 mM. The copigments were dissolved into 10% DMSO in 0.02 M ammonium acetate at the concentration of 0.02 M. The pure solutions of anthocyanins and copigments were diluted 1:1 together, resulting in the final concentrations of 0.1 mM for anthocyanin and 0.01 M for copigment, and molar ratio of 1:100. This molar ratio was used in storage studies. Molar ratios of 1:50 and 1:10 were also established, keeping the anthocyanin concentration constant. Reference solutions of the anthocyanins were prepared by dissolving them 1:1 with 10% DMSO in 0.02 M ammonium acetate. Right after dissolving and mixing the pure compounds, the pH of the solution was set to 3.4 with 25% ammonia or 10M HCl. The chosen pH value is the natural pH of strawberry juice.

4.3.2 Wine making (I)

Blackcurrant juice was diluted with water in the ratio 1:2. Three different batches were made, one contained blackcurrant juice, one 10% crowberry juice with blackcurrant juice, and one 10% grape skin extract with blackcurrant juice. 0.5 ml Lalvin V1116 *S. cerevisiae* yeast suspense (0.1 g/ml), 5 ml Vitamon Combi wine nutrient (12 mg/ml), and 20 g sucrose was dissolved into the juices. The incubation time of the juices was two weeks in a shaker (40 rpm, +19°C). After incubation, the wines were sterile filtrated and stored in dark in +17°C for six months. During fermentation and storage, pH and absorbance of the wines was determined. Also anthocyanin content of the wines was measured by high performance liquid chromatography (HPLC).

4.3.3 Juice preparation (III, IV)

Frozen lingonberries, cranberries, strawberries and raspberries were thawn and crushed before centrifuging to gain juice. The juices were clarified with a tenfold pile of GF/A fiberglass filters and then they were further filtered through a 0.8 μm membrane filter. The commercial strawberry and raspberry juices were processed as above. The commercial strawberry and raspberry juices consisted of 35 % berries, added sugar and water (**III**). Lingonberry and cranberry juices were diluted with water to 35 % juice (**III**). Juices made from strawberries and red raspberries in study **IV** were diluted to 25 %. The anthocyanin content of the juices was determined by HPLC.

The copigments were added individually into the juices in tenfold molar amounts compared to the anthocyanin molar amount. This equals to 125-605 μg/ml of ferulic acid, 145-700 μg/ml of sinapic acid, and 230-1120 μg/ml of rosmarinic acid depending on the juice. The copigments were dissolved into the berry juices as such**.** The natural color enhancers, Grape skin extract, Black carrot extract, and Color'Plus®, were dissolved into the commercial strawberry and raspberry juices in the amount of 2 g/L of juice. The juices were put in ultrasonic bath for 10 min in order to ease the copigment dissolvement. Strawberry and raspberry juices were ultrasonicated for 45 min to brake down microbial cell walls for sterilization. The juices were kept for storage at room temperature in daylight, in sealed, but not vacuum-sealed, tubes in order to avoid contamination and evaporation. pH of the juices was measured in the beginning of storage. In study **III**, sugar content was measured with a refractometer and total phenolic content was calculated according to the Folin-Ciocalteu method (1992) and expressed as gallic acid equivalents (GAE), milligrams per liter of juice.

4.3.4 Spectrophotometric measurements of color intensity (I-III).

Absorption spectra of pure anthocyanin solutions and berry juices were recorded using a UV-visible spectrophotometer, scanning the visible range from 450 to 600 nm. The change in the maximum absorbance (A_{max}) at varying wavelengths (λ_{max}) presented the change in the color intensity, and revealed a possible hyperchromic effect (ΔA_{max}) and bathochromic shift ($\Delta\lambda_{max}$), resulting from a copigmentation reaction.

4.3.5 CIELAB analysis of color changes of the juices (III)

CIELAB parameters were determined with Minolta Chroma Meter CR-210 colorimeter using the illuminant D_{65} diffused illumination. The measured parameters were L* for lightness, a* for redness, and b* for yellowness. With these calculations of C* for chroma, h for hue angle, and ΔE for total color change were made with the following equations:

(1) $C^* = [(a^*)^2 + (b^*)^2]^{1/2}$

(2) $h = \arctan(b^*/a^*)$

(3) $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$

4.3.6 HPLC-analysis

Wine flavonoids and phenolic acids (I)

From black currant wine (**I**), anthocyanins and other flavonoids were determined by HPLC method developed by Lamuela-Raventós and Waterhouse (1994) (Lamuela-Raventos and Waterhouse 1994), where a C-18 column (Nova-Pak, Waters) was used with a modified gradient elution program. A Spherisorp S5 ODS2 precolumn, 3 Waters 501-series pumps and a Waters diodearray UV-visible detector were used. Wavelengths 280 nm for catechins, 320 nm for phenolic acids, 365 nm for flavonols, and 520 nm for anthocyanins were used in the detection. Column temperature was +40°C.

Anthocyanins of juices and pure solutions (III-IV)

The anthocyanin content of juices and pure solutions (**III-IV**) was analyzed with a HPLC system consisting of a 2690 separation module, a PDA996 diodearray detector, and a Millenium 2020C/S software data module. A Zorbax SB C18 column (150mm x 4.6 mm, 5

μm; Agilent, USA) with a C18 guard column was used for separation. The mobile phase consisted of 10% formic acid (solvent A) and 100% CH_3CN (solvent B). The elution conditions are described in more detail in study **III**. The flow rate was 1.0 ml/min and injection volumes 30-100 μl. Wavelengths of 280 nm and 520 nm were used for detection, and the spectra from 200-600 nm were recorded. Column temperature was +40°C. The anthocyanin content was calculated using external standards. The standards used were pelargonidin 3-glucoside, 3-glucoside, 3-rutinoside, 3-arabinoside, and 3-galactoside of cyanidin, and 3-rutinoside and 3-glucoside of dephinidine. The formation of new anthocyanin derived products was monitored with HPLC as described above and the formed novel peaks in the chromatograms were fractionated for MS analysis (**IV**).

4.3.7 Mass-spectrometric analysis (IV)

Nano-ESI-MS was applied to the analysis of the pure compound solutions and the juice fractions obtained from HPLC runs. Esquire-LC quadrupole ion trap (QIT) mass spectrometer was equipped with a nano-electrospray interface and DataAnalysis 3.0 software (Bruker Daltonics, Bremen, Germany). The instrument was operated in positive ion scan mode under the following conditions optimized for smaller molecular weight ions/larger molecular weight ions: needle voltage 650 V; dry temperature 30 °C; skimmer 1 potential 30/57; capillary exit offset 25/24 V; trap drive value 55/74. Nitrogen was used as nebulizer gas (0.2 psi) and tandem mass spectrometry (MS/MS) was performed using helium (99.996%) as collision gas. Samples were diluted with 0.1 M ammonium formate in the ratio of 10:1 in order to help the ionization of the copigment solutions and the juice fractions. The berry juices were also run through LC system equipped with an HP 1100 solvent delivery system, autosampler, DAD, elution splitter unit (1:50) (Acurate, LC Packings, CA, U.S.A.), and ChemStation Plus A.09.01 software Agilent (Agilent Technologies, CA, U.S.A.) with the same column and elution conditions as was used with the HPLC system. The LC was connected to ESI-MS operated on positive ion mode under the following conditions: needle voltage 2800 V; dry temperature 300 °C; skimmer 1 potential 45; capillary exit offset 15 V; trap drive value 65. This was conducted to confirm the peak identification based on the juice fractions.

4.3.8 Statistical analysis

Statistical analysis of variance (ANOVA) was conducted using Statgraphics plus, version 3.2. Significant ($P < 0.05$) differences between means of four (colorimetric measurements)

or three (HPLC and juice composition measurements) replicates were identified using Tukey's procedure (**II**, **III**).

5. RESULTS

5.1 Enhancement of berry wine and juices with natural color enhancers (I, III)

5.1.1 Berry wine

Natural color enhancers improved the color of black currant wine immediately after addition and during storage. The most intense color in the beginning of fermentation was gained with blackcurrant juice enhanced with crowberry juice, the color of which was 35% more intense than that of the plain black currant juice. The second strongest color was obtained with blackcurrant juice enriched with grape skin extract, which was 10% stronger than the color of the non-enhanced black currant juice.

Color stability

During storage, the color intensity decreased in all the wines. The color intensity of blackcurrant wine enriched with crowberry juice decreased 60% during storage, remaining nonetheless the strongest after six months. The color intensity of plain blackcurrant wine decreased by 57%, and the color of blackcurrant wine enhanced with grape skin extract decreased only 55% by the end of storage (**I**; figure 1).

The wavelength of the maximum absorbance (λ_{max}) of the wines shifted during the study, which was detected as bathochromic shift and denotes of copigmentation. In the beginning of fermentation, the maximum absorbance was detected at 516 nm in all the black currant juices. The greatest bathochromic shift in the wines was detected after 15 weeks of storage. Black currant wine enhanced with crowberry juice had the highest wavelength of 523 nm. The plain non-enhanced black currant wine and black currant wine enhanced with grape skin extract both had the highest λ_{max} at 520 nm. By the end of storage the wavelengths were closer to the values of the beginning (520-517 nm).

Changes of anthocyanin content

In the beginning of fermentation, the highest anthocyanin content was in black currant juice enriched with crowberry juice (520 mg/L) and the lowest in plain black currant juice (360 mg/L). The anthocyanin contents decreased rapidly during fermentation, by nearly halving, and in storage it diminished even further. The highest anthocyanin content after storage was in black currant wine enhanced with grape skin extract (6.0 mg/L, 1.7%). Plain black currant wine contained 4.9 mg/L (1.4 %) of anthocyanins and black currant wine enhanced with crowberry juice 4.7 mg/L, (0.9 %), after 110 days (**I**; figure 2).

5.1.2 Berry juices

The color of strawberry and raspberry juices was improved by the commercial color enhancers immediately after their addition and during 100 days of storage. Grape skin extract (E163) instantly increased the color intensity of strawberry juice by 190% and black carrot extract by 166%. The immediate enhancement was modest with Color'Plus® (53%) in strawberry juice. After 75 days of storage, the color intensity of strawberry juice enhanced with black carrot extract decreased only by 20 %. However, the color of strawberry juice enriched with grape skin extract became brownish during storage and λ_{max} was not measurable by spectrophotometer in the scanned range after 51 days. This was the case also with Color'Plus® (**III**; figure 2A).

In raspberry juice it was likewise grape skin extract that induced the strongest color. The color of raspberry juice immediately increased by 70 % after grape skin extract addition. The addition of black carrot extract increased raspberry juice color immediately by 58 % and Color'Plus® by 19 %, respectively. After 75 days of storage, the color intensity of raspberry juice decreased only 56 % when enhanced with grape skin extract and 77 % with black carrot extract. The color intensity of raspberry juice enhanced with Color'Plus® was not more stable than the plain non-enhanced juice (**III**; figure 2B).

Changes of color parameters

The color hue of all the juices changed in the course of time. The hue angle of strawberry and raspberry juices enriched with color enhancers expanded during storage. This resulted in yellowing of the juices. Black carrot extract induced the least yellowing to strawberry juice, and grape skin extract to raspberry juice. The addition of the extracts affected the lightness of the initial berry juice color significantly, making the juices darker than the

original non-enhanced juices, which is obvious due to the increment of the initial anthocyanin molecules. The color of all the juices lightened during storage. Also the chroma, i.e. the tinctorial characteristic of the juices, was affected. The chromas of raspberry juice enhanced with the commercial extracts, in the end of storage, were lower than the chroma of the original plain raspberry juice. With strawberry juice it was the opposite; the chromas of the juice enhanced with the plant extracts were higher in the end of storage than the original plain juice before storage. The total color change during storage (ΔE) was greater with grape skin extract in both the juices than with black carrot extract.

Changes of anthocyanin content

The total anthocyanin content of all the juices decreased during storage. In the beginning of the study, the anthocyanin content of the plain raspberry juice was 81 mg/L and of the plain strawberry juice 39 mg/L. Black carrot extract increased the anthocyanin content of the juices by 20 mg/L, grape skin extract with less than 10 mg/L, and since Color'Plus® does not contain anthocyanins, it did not affect the anthocyanin content of the juices. After 100 days of storage, the plain non-enhanced raspberry and strawberry juices contained only 1 % of the original anthocyanins. The highest anthocyanin content in the end of storage was observed in strawberry juice enhanced with black carrot extract, retaining 35% (20 mg/L) of the initial content. In the end of storage, the anthocyanin content of strawberry juice enhanced with grape skin extract was only 5% of the original, and Color'Plus® sustained 4% of the initial anthocyanins. Raspberry juice enhanced with grape skin extract had 5% of the original anthocyanins left, in the end of storage, and less than 2% with the other color enhancers.

5.2 Enhancement of berry juices by phenolic acids (III)

The color of strawberry, raspberry, lingonberry, and cranberry juices was improved by phenolic acid addition. An increase in color intensity was observed in the juices immediately after copigment addition. The enhancement of the color during storage was more intensive in strawberry and raspberry juices than in lingonberry and cranberry juices.

Strawberry juice color was enhanced by all the three phenolic acids, ferulic acid, sinapic acid, and rosmarinic acid, during storage. This enhancement was most efficient with sinapic acid, which was little over 100% by the end of storage (**III**; figure 1A). The best color enhancers in raspberry juice were ferulic and sinapic acids. After 100 days, sinapic acid had induced around 30% and ferulic acid around 35% more intense color in raspberry juice

compared to the plain non-enhanced juice. The enhancement of raspberry juice color by rosmarinic acid was not stable during storage even though it started as the strongest (**III**; figure 1B).

The color of cranberry and lingonberry juices enhanced differently than the color of strawberry and raspberry juices. The color of lingonberry and cranberry juices was strongest through out the storage period when enriched with rosmarinic acid. Rosmarinic acid intensified cranberry juice color over 110% and lingonberry juice color almost by 50% more than the color of the plain non-enhanced juice, in the end of storage. Sinapic acid increased cranberry juice color by 70% and lingonberry juice color by 30%, by the end of storage compared to the plain non-enhanced juices at the same time point. Ferulic acid was the most modest color enhancer of the phenolic acids with cranberry and lingonberry juices, increasing the juice colors by 30 and 10%, respectively.

Effect on color stability

The color stability of all the four berry juices improved by the addition of phenolic acids. The plain strawberry juice lost its redness quickly during storage, but when sinapic acid was added, the color intensity of strawberry juice in the end of storage was 104% of the original intensity of the non-enhanced juice. The color of strawberry juice enriched with ferulic acid was 98% and with rosmarinic acid 84% of the original intensity of the non-enhanced juice color in the end of storage. With raspberry juice the color stabilizing effect was more modest and observed with ferulic and sinapic acids. Ferulic acid maintained 35% and sinapic acid 33% of the original color intensity of raspberry juice after 103 days. The color intensity of the plain non-enhanced cranberry juice decreased during storage by 80% and lingonberry juice by 67%. By the end of storage cranberry juice copigmented with rosmarinic acid had 42% of the original color left, with sinapic acid 33% and with ferulic acid 25%, respectively. Lingonberry juice enhanced with rosmarinic acid had 48% of the original color left, with sinapic acid 42% and with ferulic acid 37%.

Changes of color parameters

The tone of color of all the juices changed in the course of time. Phenolic acid addition inhibited the expansion of the hue angle in all the juices, both immediately after supplementation and during storage. Sinapic acid was most efficient in this inhibition, especially in strawberry juice. In raspberry juice, sinapic and ferulic acids reduced the increment of the hue angle, and rosmarinic acid did not have any effect. In cranberry and

lingonberry juices rosmarinic and sinapic acids were more effective in this inhibition than ferulic acid (**III**; table 1).

Lightness of the berry juices was affected by the phenolic acid addition somewhat during storage. The lightness of the juices was affected in the course of time and not so much immediately after addition (**III**; table 2).

Chroma of the original plain raspberry, lingonberry and cranberry juices decreased during storage. The addition of rosmarinic acid reduced the decrement of chroma in these juices. In strawberry juice the addition of ferulic and sinapic acids increased the value of chroma after 100 days, indicating of a more vivid juice color development during storage (**III**; table 1).

The total color difference (ΔE) was greatest in cranberry juice and smallest in strawberry juice over the storage period. In lingonberry and cranberry juices, the ΔE was the smallest in the juices enhanced with rosmarinic acid, and for that reason rosmarinic acid evidently stabilized the color of these juices more than the other phenolic acids. Ferulic and sinapic acids induced a similar stabilizing effect to raspberry juice. In strawberry juice ΔE did not reveal any statistically significant stabilization of the color, which is surprising since the changes of chroma and hue angle clearly indicate of efficient color enhancement and stabilization by the phenolic acids (**III**; table 1).

Changes of anthocyanin content

The total anthocyanin content of all the juices decreased during storage. The plain lingonberry juice had the highest anthocyanin content (0.16 g/L), cranberry juice the second highest (0.14 g/L), raspberry juice the second lowest (0.08 g/L), and strawberry juice the lowest (0.04 g/L), in the beginning of the study. Lingonberry juice sustained the highest anthocyanin content even after storage, which was 13% of the original amount. 90% of cranberry juice anthocyanins were lost during storage. Raspberry and strawberry juices lost almost all, 99%, of their anthocyanins during storage.

The anthocyanin content of lingonberry juice diminished after 100 days by 72% when rosmarinic acid was added. With sinapic acid it decreased 78% and with ferulic acid 84%. Also in cranberry juice, rosmarinic acid sustained the anthocyanin amount most efficiently during storage. The anthocyanin content of cranberry juice enhanced with rosmarinic acid decreased by 75%, with sinapic acid 83%, and with ferulic acid 87%. The total amount of the anthocyanin content in strawberry juice diminished by 74% with sinapic acid addition,

with ferulic acid enrichment by 84%, and 81% with rosmarinic acid addition. In raspberry juice the anthocyanin content decreased more than 90% with sinapic acid and ferulic acid addition. Rosmarinic acid did not sustain the anthocyanin content of raspberry juice at all after 100 days of storage. Figure 13 shows the changes of anthocyanin contents of the enhanced juices during storage.

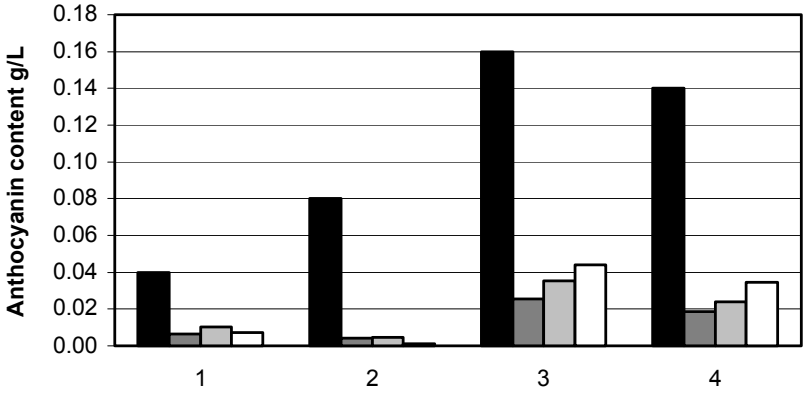


Figure 13. Changes in anthocyanin contents of enhanced juices during storage. 1) Strawberry juice, 2) Raspberry juice, 3) Lingonberry juice, and 4) Cranberry juice. The black bars present the initial anthocyanin concentration of each juice. Dark gray bars the anthocyanin content of the juices enhanced with ferulic acid after 75 days; Light gray bars juice+sinapic acid; White bars juice+rosmarinic acid.

5.3 Copigmentation of pure anthocyanins (II)

5.3.1 Immediate intermolecular copigmentation effect

The strongest immediate copigmentation was observed in the 1:100 molar ratio with malvidin 3-glucoside with all the five phenolic acids (gallic, ferulic, caffeic, rosmarinic and chlorogenic acid). Intermolecular copigmentation in terms of hyperchromic effect was relatively strong also in cyanidin 3-glucoside and pelargonidin 3-glucoside solutions. The acylated anthocyanin, cyanidin 3-(2″-xylosyl-6″-(coumaroyl-glucosyl))-galactoside, possessed the strongest color intensity compared to the other anthocyanins. On the day of preparation, however, it did not show any statistically significant intermolecular copigmentation and neither did the trisaccharidic anthocyanin, cyanidin 3-(2″-xylosyl-6″-glucosyl)-galactoside (**II**; figure 2).

Of the copigments, rosmarinic acid and ferulic acid were the best color enhancers on the day of preparation. They both increased the color intensity of malvidin 3-glucoside by

160%. Caffeic acid and chlorogenic acid were relatively good copigments in the beginning of the study. The intermolecular copigmentation effect of caffeic acid and chlorogenic acid was of the same magnitude with all the anthocyanins, including the trisaccharidic forms. Caffeic acid increased the color of the monoglucosidic anthocyanins by 30–110% and chlorogenic acid by 40–110%, respectively. Gallic acid produced a moderate color enhancement on the day of preparation, increasing the absorbance of the anthocyanins at λ_{max} by 9–45% (**II**; figure 2).

The biggest bathochromic shift was induced by rosmarinic acid with malvidin 3-glucoside ($\Delta\lambda_{max}$ 18.6 nm). The second highest shift emerged with ferulic acid, ranging from $\Delta\lambda_{max}$ 17.6 nm with malvidin 3-glucoside to $\Delta\lambda_{max}$ 5.5 nm with cyanidin 3-(2″-xylosyl-6″-(coumaroyl-glucosyl))-galactoside (**II**; table 1).

5.3.2 Copigmentation and color stability during storage

During the six months storage period, cyanidin 3-(2″-xylosyl-6″-(coumaroyl-glucosyl))-galactoside had the best color stability of the studied anthocyanins. Its color diminished only by 30% of the original (**II**; figure 4C). Malvidin 3-glucoside lost its color quickly; its color was imperceptible after 55 days (**II**; figure 4D). Pelargonidin 3-glucoside and cyanidin 3-glucoside retained 20% and 25% of their color (**II**; figures 4A and 4B), and cyanidin 3-(2″-xylosyl-6″-glucosyl)-galactoside had 40% of its color left in the end of the study.

Copigment addition increased anthocyanin color stability in general during storage (**II**; figure 4). Ferulic acid and caffeic acid addition increased, to a great extent, the color of pelargonidin 3-glucoside throughout the storage period, at the end of storage being 220% and 190% of the original color intensity. Caffeic acid also enhanced cyanidin 3-glucoside color throughout the storage period, and ferulic acid the color of malvidin 3-glucoside. Although rosmarinic acid enhanced anthocyanin colors strongly on the day of preparation, it maintained the colors poorly in storage. Chlorogenic acid stabilized the anthocyanin colors somewhat. Gallic acid was the weakest copigment with all the anthocyanins inducing a yellowish tint to the anthocyanin solutions. Copigmentation reactions of the acylated anthocyanin and the trisaccharidic anthocyanin were not significant during storage. The color stability of the acylated anthocyanin was diminished by gallic, ferulic and caffeic acids during storage. In figure 14 is shown the increase in color intensity of monoglucosidic anthocyanins induced by the simple cinnamic acids.

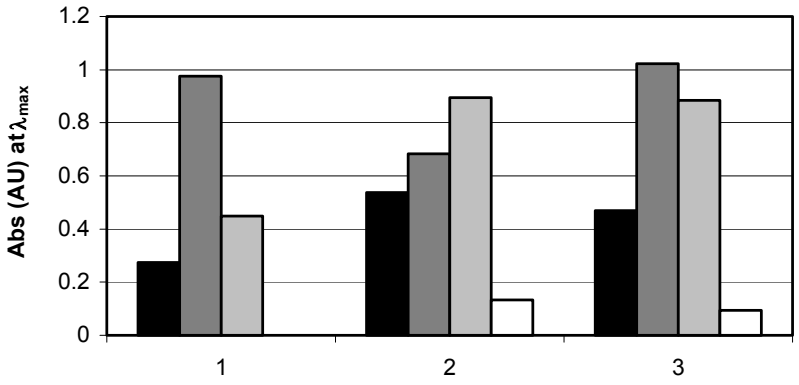


Figure 14. Changes in color absorbance ($\Delta\lambda_{max}$) of anthocyanin monoglucosides copigmented with simple cinnamic acids. 1) Malvidin 3-glucoside, 2) Cyanidin 3-glucoside, 3) Pelargonidin 3-glucoside. Black bars present the initial absorbance of non-enhanced anthocyanins. Dark gray bars present the absorbance of monoglucosides enhanced with ferulic acid after six months storage; Light gray bars monoglucosides+caffeic acid; White bars the final absorbance of non-enhanced anthocyanins.

5.4 Identification of novel pyranoanthocyanins (III, IV)

The berry juices enriched with phenolic acids formed novel peaks to the end of their HPLC chromatogram measured at 520nm, indicating the formation of new anthocyanin compounds during storage (**III**; figure 4 and 5). The number of the novel compounds was in relation to the number of original individual anthocyanin compounds in the juices. The greatest number of novel compounds was discovered in cranberry juice. With strawberry juice one major novel compound emerged. With strawberry juice the area of these novel peaks grew with time, indicating an increase in the amount of the new pigment formation during storage. This increment was not observed with the other juices, but neither did any other juice sustain its color as efficaciously when enhanced with phenolic acids than strawberry juice (**III**; figure 1 A).

Each phenolic acid induced these novel compounds differently with the four berry juices. Sinapic acid formed the most abundant peaks in strawberry juice. With raspberry juice, it was ferulic acid that induced the most pronounced peaks. The reactions in lingonberry and cranberry juices differed from the two former juices. Sinapic and ferulic acids produced only modest peaks with lingonberry and cranberry juices, but rosmarinic acid stabilized the anthocyanins of lingonberry and cranberry juice but did not induce new peaks into their chromatograms.

The new major anthocyanin derived pigment found in strawberry juice, after the addition of ferulic acid, had a *m/z* value of 579, which corresponds to the mass of a pyranoanthocyanin, pelargonidin 3-glucoside – vinylguaiacol adduct. The major anthocyanin derived pigment obtained from strawberry juice enhanced with sinapic acid was a [M+] ion with *m/z* 609, which corresponds to the mass of pelargonidin 3-glucoside – vinylsyringol adduct, another pyranoanthocyanin.

The new anthocyanin derived pigments in raspberry juice, after the addition of ferulic acid had *m/z* values of 903, 757, 741, and 595 corresponding to the cyanidin 3-glycosylrutinoside –, cyanidin 3-sophoroside –, cyanidin 3-rutinoside –, and cyanidin 3-glucoside – vinylguaiacol pyranoanthocyanin adducts, respectively. Figure 15 shows the mass spectra of the new pyranoanthocyanin molecules detected in raspberry juice after ferulic acid addition. The addition of sinapic acid induced the formation of cyanidin 3-glycosylrutinoside –, cyanidin 3-sophoroside –, cyanidin 3-rutinoside –, and cyanidin 3-glucoside – vinylsyringol pyranoanthocyanin adducts with corresponding mass units (*m/z*) of 933, 787, 771, and 625, respectively.

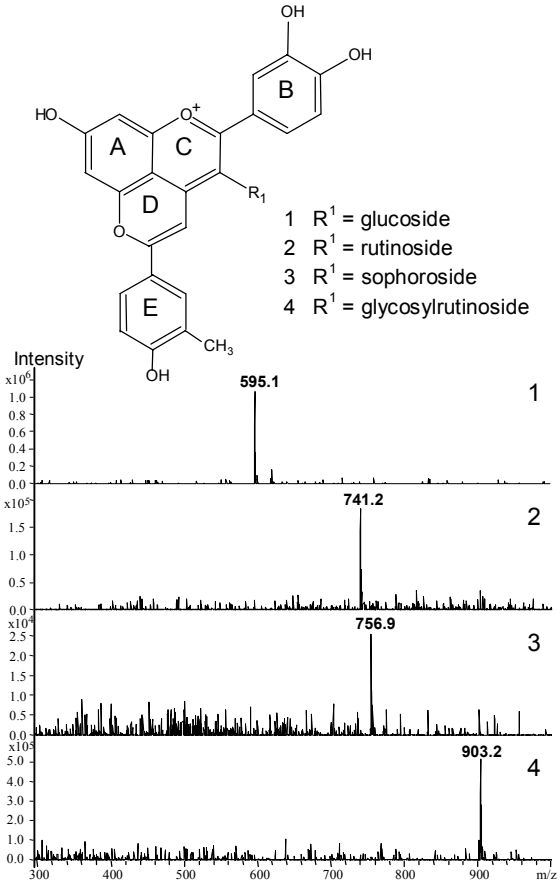
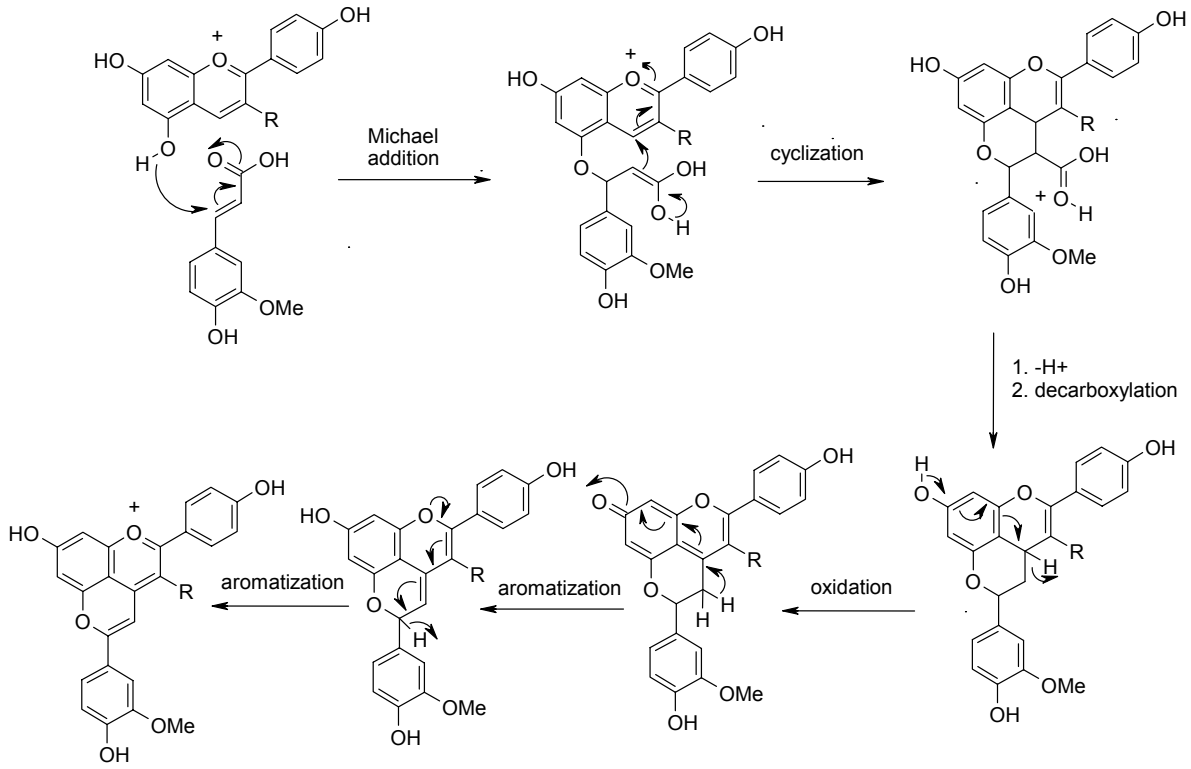


Figure 15. Full scan mass spectra and molecular structures of cyanidin based pyranoanthocyanin adducts detected in raspberry juice enhanced with ferulic acid.

The proposed mechanism for the pyranoanthocyanin formation is shown in Scheme 3. It was confirmed that the reaction proceeds through the formation of vinylphenols from the cinnamic acids. Synthesized 4-vinylsyringol reacted in strawberry juice with pelargonidin 3-glucoside, resulting in the same pyranoanthocyanin adduct as was obtained with the addition of sinapic acid in strawberry juice, and in the reaction of pure pelargonidin 3-glucoside with sinapic acid.

Scheme 3. Proposed reaction mechanism of the formation of new pyranoanthocyanin from pelargonidin 3-glucoside and ferulic acid. R = glucoside residue.



6. DISCUSSION

6.1 Enhancement of berry wine and juices with natural color enhancers (I, III)

6.1.1 Berry wine

The use of natural color enhancers intensified the color of black currant wine. Grape skin extract is a widely used natural food colorant, which has a food additive number E163 in EC, and is manufactured as wine industry side product from grape pomace. Grape skin extract is rich in anthocyanins, containing them in average 0.3 to 4 g/100g (Mazza, 1995). The small dark blue berry of crowberry has one of the richest anthocyanin content of wild berries, containing in average 0.4 g/100g FW of anthocyanins (Kärppä et al., 1984; Kähkönen et al., 2001), and thus is a promising color enhancer. Also other substances of these natural color enhancers, such as other flavonoids and phenolic acids, can contribute to the enhanced color of black currant wine but also the introduction of other anthocyanins non-typical for black currant in high concentrations can induce enhanced color to the berry wine.

Crowberry juice was the most efficient enrichment in enhancing the color of black currant wine, in which the anthocyanin content was greatest both in the beginning and after fermentation. Best color stability was obtained by using grape skin extract as a color enhancer, as the color intensity of black currant wine enhanced with grape skin extract decreased the least during storage. However, the overall color stability was not greatly improved by the use of color enhancers, as the color intensities decreased in all the wines during fermentation and storage.

The anthocyanin content in all the wines declined a great deal already during fermentation and this regression continued throughout the storage period. However, the decrement of anthocyanin content did not take place in the same ratio with the decline of color intensity. This was due to copigmentation reactions where anthocyanins condensed with each other, and with other organic molecules existing in the berries to form new pigments. Therefore the anthocyanin contents diminished but the color intensities did not decline as rigorously. These copigmentation reactions were similar to the ones known to take place in grape wines. The shift of the maximum absorbance (λ_{max}) towards higher wavelength during the study, i.e. the bathochromic shift, also supports the assumption of copigmentation reactions

taking place. The observed bathochromic shift can be also due to formation of new anthocyanin derivative pigments having bluer hues. It is well known that acetaldehyde present in wines can react with catechin and the formed adduct can condense with anthocyanins giving rise to violet pigments (Rivas-Gonzalo et al., 1995; Escribano-Bailón et al., 2001).

The anthocyanin content of blackcurrant wine, especially when enhanced with crowberry juice and grape skin extract, which both increase the amount of anthocyanin, is comparable with that of grape wines in the beginning of fermentation. In grape wines the anthocyanin content in the beginning of fermentation varies between 80 – 400 mg/L (Almela et al., 1996) and after fermentation between 100 - 800 mg/L (Ribéreau-Gayon, 1982; Liao et al., 1992). Even after fermentation the anthocyanin content of blackcurrant wine can be compared to that of an average grape wine, but the decline in anthocyanin content during storage is much more severe in blackcurrant wine than in grape wine. In grape wine, after one-year storage time, the anthocyanin content has decreased by 50% (Ribéreau-Gayon, 1982). Of berry wines, raspberry wine anthocyanins have been shown to be more stable than that of blackberry wines. Blackberry wine lost 85-100% of its anthocyanins during processing and storage (Rommel et al., 1992) and raspberry wine around 50% (Rommel et al., 1990). In blackcurrant wine, the anthocyanin content diminished to nearly one-tenth already after four months. This shows that the stability of anthocyanins in berry wines is greatly lower than in ordinary red table wines.

6.1.2 Berry juices

The non-enhanced raspberry and strawberry juices lost their anthocyanin pigments and redness quickly during storage. Forceful decrement of anthocyanin content of different berry products during storage have been reported before for strawberry preserves (Abers and Wrolstad, 1979), strawberry and black currant syrups (Skrede et al., 1992), strawberry juice (Garzon and Wrolstad, 2002), red radish and red-fleshed potato juices (Rodriguez-Saona et al., 1999), beverages containing sweet potato anthocyanins (Bassa and Francis, 1987) and grape anthocyanins (Palamidis and Markakis, 1978), blood orange juice concentrate (Kirca and Cemeroglu, 2003), raspberry pulp (Ochoa et al., 1999), and raspberry juice concentrate (Withy et al., 1993), as well as for cranberry juice cocktail (Starr and Francis, 1974). It is well known that strawberry products are one of the most labile in color wise of all berry and fruit products. The stability of the color of berry products has been investigated concentrating on the effects of processing methods (Rommel et al., 1990; Jiang et al., 1990; Rommel et al., 1992; Zabetakis et al., 2000;

Skrede et al., 2000; Talcott et al., 2003), packaging material and added preservatives (Thakur and Arya 1989), and the addition of sugar (Wrolstad et al., 1990) and ascorbic acid (Skrede et al., 1992), but no studies focussed on color enhancement by the addition of natural anthocyanin extracts have been conducted before.

The effects on color stabilization and color enhancement of the commercial natural plant extracts on the berry juices were not as significant as the corresponding of the phenolic acids. Although the initial intensifying of the berry juice color by the enhancers was strong, the stability of the color was fairly poor, except with black carrot extract in strawberry juice. The introduction of anthocyanin pigments, which do not naturally belong to the juices and the increment of the anthocyanin amount itself stabilized the juice colors somewhat in the case of black carrot and grape skin extracts. This is in agreement with previous works, in which the color stability was found to increase as the total anthocyanin content increased (Skrede et al., 1992; Garzon and Wrolstad, 2002).

6.2 Enhancement of berry juices by phenolic acids (III)

The addition of phenolic acids to the four different berry juices improved the juice color by enhancing the anthocyanin color intensity and stabilizing the color during storage. Anthocyanin content of all the juices diminished during storage, most pronouncedly in strawberry and raspberry juices. The effects of flavonol addition on anthocyanin color in model systems (Shrikhande and Francis, 1974) and in blood orange juice (Maccarone et al., 1985) has also been studied before. In these studies flavonols, i.e. rutin and quercetin, but also tartaric and caffeic acids reduced the loss of anthocyanins. These results support our findings and hypothesis of a protective effect of phenols, especially phenolic acids, on anthocyanin color. Enhancement and stabilization of the color of berry juices by phenolic acids during storage had not been studied previously and hence novel information on anthocyanin enhancement and copigmentation in food models was provided in study **III**.

The color enhancement by different phenols has been previously reported concerning mostly only red wine (Mateus et al., 2001; Darias-Martin et al., 2002). The assumption has been that most copigmentation reactions in wine involve fermentation. Here it was shown that anthocyanin copigmentation reactions do not require fermentation and take place also in non-fermented 45 min sonicated berry juices. In study (**II**) with pure compounds, copigmentation occurred more intensively with pelargonidin 3-glucoside, the main anthocyanin of strawberry, than with cyanidin 3-glucoside, which is one of the main anthocyanins of raspberry. These same observations were made here with the juices

substantiating that copigmentation reactions, which were seen with pure compounds, can be accomplished also in food matrix.

Classification of the juices

The four juices can be classified into two different groups by their composition, but also by their copigmentation reactions and color behavior during storage. The difference in anthocyanin profiles of the juices is one of the main reasons for the different copigmentation and color enhancement reactions. Strawberry and raspberry have similar anthocyanin profiles, and correspondingly so do lingonberry and cranberry (De Ancos et al., 1999a; Prior et al., 2001; Lopes-Da-Silva et al., 2002; Kaehkoenen et al., 2003), which is also evident from their HPLC-data.
The copigmentation reactions were similar within strawberry and raspberry juices, the reactions of which differed from the reactions perceived in lingonberry and cranberry juices, which again were similar to one another. Strawberry juice color was enhanced the most by the phenolic acid addition. Raspberry juice was comparatively receptive to color enhancement. The color development observed in lingonberry and cranberry juices was more modest.

Garzon and Wrolstad (2002) came to the conclusion when studying the stability of strawberry juice and concentrate that the higher the total pigment concentration, the more stable the berry juice or product, which is in an agreement with results reported here on overall anthocyanin stability. Skrede et al. (1992) stated that color stability is more dependent on the total anthocyanin content rather than the qualitative anthocyanin composition. This is true for overall color stability, but when it comes to color enhancement, especially through copigmentation reactions, the results are very much dependent on the individual anthocyanin composition. Obviously, the structural characteristics of each anthocyanin, i.e. the level of methoxylation and hydroxylation, glycosyl and acyl substitutions, contribute to the manifestation of copigmentation. Thus, the individual anthocyanin composition affects the stability and enhancement of each studied berry juice.

Also the composition of other molecules of each berry variety and the chemical and physical characteristics of the berry juices affect the observed reactions in the juices. Strawberry and raspberry juices contained less phenolics and anthocyanins than cranberry and lingonberry juices, the amounts of which are in accordance with previous studies on berry phenolics (Kähkönen et al., 2001; Prior et al., 2001). The pH and sugar content was significantly higher in the two former juices compared to the latter ones.

Classification of the copigments

The phenolic acids, which were used as copigments, can also be classified into two groups by their chemical structure for one, but also by their manifestation of copigmentation in the juices. Sinapic and ferulic acids induced the strongest hyperchromic effects to strawberry and raspberry juices. They also formed new pigment molecules with strawberry and raspberry anthocyanins, which were observed as novel peaks in the HPLC-chromatograms. In strawberry juice, sinapic acid, the dimethoxylated cinnamic acid, was more efficient color enhancer than monomethoxylated ferulic acid. Asen et al. (1972) noticed also that of all the cinnamic and benzoic acids examined in their copigmentation studies, sinapic acid produced the strongest copigmentation effect. Thus it can be concluded that the increasing methoxylation of the simple cinnamic acids lubricates both the color enhancement and the formation of anthocyanin derivatives in juices containing mainly monoglycosidic anthocyanins.

The enhancement by sinapic and ferulic acids was not as strong with lingonberry and cranberry anthocyanins. Instead, it was perceived that rosmarinic acid affected the color stability of these juices most efficiently. Rosmarinic acid obviously stabilized lingonberry and cranberry anthocyanins via intermolecular copigmentation reactions since their color was stabilized and the diminishing of anthocyanins during storage was reduced. Rosmarinic acid did not induce new anthocyanin compounds in the juices, since no such were detected with HPLC.

The different pKas of the phenolic acids can affect the stabilization of the berry juice colors. The pKas of ferulic and sinapic acids are around 4.6. The pKa of rosmarinic acid is 2.8, which indicates that in the pH of lingonberry and cranberry juices most of rosmarinic acid is dissociated, unlike sinapic or ferulic acids. Obviously the negatively charged rosmarinic acid protects anthocyanins more effectively in the acidic juices. This difference in chemical behavior between rosmarinic acid, and ferulic and sinapic acids, with anthocyanins, was also shown in the study with pure compounds (**II**).

Changes in color parameters

When it comes to berry juice color, color enhancement, and stability, the picture is incomplete if only looking at the λ_{max} of the absorption spectrum of a juice. Therefore it was important to monitor the total color change and different aspects effecting the manifestation of berry juice color with the CIELAB color space system, which enables an

approach to the changes of juice colors where the changes in hue, chromatic saturation, and overall lightness are taken into account.

The total color changes (ΔE) of the juices, with and without color enhancers, showed clearly that the changes during storage were visible also to the naked eye. All the juices yellowed during storage, which was indicated by the increment of hue angle. This is in accordance with previous studies where strawberry juice (Garzon and Wrolstad, 2002), preserves (Abers and Wrolstad, 1979), and blackcurrant syrups (Skrede et al., 1992) obtained higher hue angles and became more yellow during storage. The hue angle of strawberry juice enhanced with ferulic acid in the end of storage was significantly greater than the one of the plain non-enhanced juice in the end of storage, and yet the a*-value, standing for the redness, was almost the same. Therefore the a*-value is not sufficient enough for interpretation of the stability of red berry juice color. For example, the juice color of strawberry juice enhanced with ferulic acid can be considered red, but it has yellowed during storage, since the b*-value had increased significantly, and also the hue angle had increased.

Bathochromic shift of a juice color does not enlighten the change of tint sufficiently enough either. Since the bathochromic shift marks only the change of the absorption maximum (λ_{max}) of a spectrum, it disregards the effects of other wavelengths which are also important in the definition of the overall berry juice color. Here the benefit of measuring the CIELAB coordinates is shown most clearly in the case of raspberry juice, the color of which was undeterminable as λ_{max} in the red region after storage time of 51 days, and yet the hue values on the CIELAB scale show that raspberry juices with and without additions have reddish color after storage time of 103 days.

6.3 Copigmentation of pure anthocyanins

6.3.1 Immediate intermolecular copigmentation effect

The strongest immediate copigmentation effect was observed with the monoglucosidic anthocyanins over the trisaccharidic and acylated anthocyanins. As Dangles et al. (1993), have pointed out, two or more cinnamic acid esters present in an anthocyanin induce strong enough intramolecular copigmentation, which prevents intermolecular copigmentation from taking place. Our results indicate that one acyl group is enough for adequate intramolecular copigmentation and three monosaccharidic moieties, to prevent intermolecular copigmentation. Hoshino et al. (1980), showed that the acylated anthocyanin, delphinidin

3-coumaroyl glucoside-5-glucoside (awobanin), exhibited stronger intermolecular copigmentation than its unacylated form, delphinidin 3,5-diglucoside (delphin), but their study was conducted with a flavone and not with phenolic acids.

Of the copigments, rosmarinic and ferulic acids were the best color enhancers on the day of preparation. Likewise, caffeic and chlorogenic acids were relatively good copigments on the day of preparation. In a study conducted by Davies and Mazza (1993) caffeic acid exhibited 40–50% greater copigmentation effect than chlorogenic acid with a diacylated anthocyanin, pelargonidin 3-coumaroyl glucoside-5-malonoyl glucoside (monardein). However, caffeic and chlorogenic acids had similar effects on pelargonidin 3-glucoside, as reported also in our results.

Although the monoglucosidic anthocyanin, malvidin 3-glucoside, itself had weak color stability compared to the trisaccharidic anthocyanins for example, it enabled strong copigmentation to take place. This is in accordance with earlier studies in which malvidin-based anthocyanins exhibited greater copigmentation effects than other anthocyanins (Mazza and Brouillard, 1990; Davies and Mazza, 1993). According to Mazza and Brouillard (1990), the copigmentation effect increases with the degree of methoxylation and glycosylation of the anthocyanin chromophore. This was the case with methoxylation but not with glycosylation; malvidin 3-glucoside exhibited stronger color enhancement than cyanidin 3-glucoside, but the trisaccharidic form of cyanidin exhibited weaker copigmentation than its monoglucosidic analogue. Therefore it seems to be likely that it is the number of positions of glycosyl attachments in the anthocyanin chromophore, rather than the actual number of the monosaccharidic moieties that increase the copigmentation effect. Obviously also the degree of hydroxylation affects the copigmentation reaction, hence pelargonidin 3-glucoside produced stable enhanced color with one free hydroxyl group in the B-ring, but the copigmentation effect was not as strong with cyanidin 3-glucoside possessing two free hydroxyl groups in the B-ring. These findings of structural importance on the outcome of anthocyanin color enhancement stability are in agreement with previous studies (Davies and Mazza, 1993; Fossen et al., 1998; Cabrita et al., 2000).

6.3.2 Copigmentation and color stability during storage

Copigment addition increased anthocyanin color stability in general during storage, which is in agreement with other studies (Yabuya et al., 2000; Darias-Martin et al., 2001; Malien-Aubert et al., 2001). The color enhancement reactions were efficient with the monoglucosidic anthocyanins. Surprisingly, the addition of caffeic acid and ferulic acid

greatly increased the color of pelargonidin 3-glucoside throughout the entire storage period. The other acids used in the study where not as good in the color enhancement and stabilization of the monoglucosidic anthocyanins as the simple cinnamic acids.

The acylated anthocyanin, cyanidin 3-(2″-xylosyl-6″-(coumaroyl-glucosyl))-galactoside, had the best color stability during storage. Also the trisaccharidic anthocyanin, cyanidin 3-(2″-xylosyl-6″-glucosyl)-galactoside, possessed more stable color than the monoglucosidic anthocyanins. However, copigmentation reactions of the acylated and the trisaccharidic anthocyanins during storage were not significant, resulting most probably from their sterically compact structure, which protects and stabilizes them, and prevents the copigments from intervening with the anthocyanin chromophore to form any kind of inter- or intramolecular complexes. Gallic, ferulic, and caffeic acids decreased the color stability of the acylated anthocyanin during storage, which suggests that these phenolic acids interfere with, and diminish the protective intramolecular mechanism of the acylated anthocyanin.

To our knowledge there are no previous reports on the stability of intermolecular copigmentation complexes of anthocyanins with phenolic acids during storage. The most interesting findings with the pure compounds were the reactions of ferulic and caffeic acids with the monoglucosidic anthocyanins. These copigments increased the color intensity of the monoglucosidic anthocyanins throughout the storage period. It can be speculated that pyranoanthocyanin derivatives, similar to the ones that were formed in berry juices, occurred also in the course of time in these solutions of pure anthocyanins and simple cinnamic acids. Unfortunately, HPLC follow-up was not done to these solutions, and no supporting evidence is available.

Classification of the copigments

On the basis of these results, the copigments in study (**II**) can be classified into three categories. The behavior and association of caffeic and ferulic acids with the anthocyanins differed from those of chlorogenic and rosmarinic acids, and gallic acid differed further from all the other copigments. This classification is also supported by their chemical structures. Ferulic and caffeic acids, which are simple cinnamic acids, with and without a methoxyl group, were the best copigments. Chlorogenic and rosmarinic acids, which are conjugated cinnamic acid derivatives, can be considered as moderate copigments, and gallic acid, which is a simple benzoic acid, was the poorest copigment. The different stoichiometric and equilibrium constants for chlorogenic and caffeic acids indicate their

difference of associations with anthocyanins and support their different classification as copigments (Davies and Mazza, 1993).

6.3.3 Effect of anthocyanin and copigment concentration

The results of the addition of copigments at three concentration levels showed that the outcome of copigmentation is dependent on molar ratio, which is in agreement with previous works (Asen et al., 1972; Davies and Mazza, 1993). The highest anthocyanin/copigment molar ratio of 1:100 resulted in the strongest copigmentation effect. It can be stated that the higher the copigment excess the more pronounced the copigmentation effect. However, when the copigmentation concentration exceeds a certain level, no further changes in absorbance can be observed, therefore the molar ratio cannot be raised to an unlimited extent (Hoshino et al., 1980).

6.4 Identification of novel pyranoanthocyanins (III, IV)

Copigmentation, i.e. color intensification and stability, which was observed first with pure anthocyanins and phenolic acids, was then applied successfully to berry juices. The paling of strawberry and raspberry juices was hindered with the copigment addition and the formation of stable red-orange juice color was observed in the course of time. Behind the changed color of strawberry and raspberry juices were new anthocyanin derivatives, pyranoanthocyanins. From the HPLC-chromatogram of the enhanced juices, the development of the novel anthocyanin compounds was observed during storage. The formation of new color molecules was observed both in sterile and non-sterile juices. The formation of these anthocyanin derivatives took place more rapidly in the non-sterile juice than in the sterile. The newly found compounds were identified to comprise either of a 4-vinylguaiacol or a 4-vinylsyringol adduct, depending on the used cinnamic acid, linked to 4- carbon and 5-hydroxyl positions of the anthocyanin.

Anthocyanin monoglucosides are known to form adducts to their 4- carbon and 5-hydroxyl positions, the smallest pyranoanthocyanins being vitisin B (Bakker and Timberlake, 1997) and other pyruvic acid derivatives (Fulcrand et al., 1998; Mateus et al., 2001; Vivar-Quintana et al., 2002). Intact cinnamic acids have also been observed to form pyranoanthocyanins with monoglucosides. These have been perceived mainly with malvidin 3-glucoside (Schwarz et al., 2003; Hakansson et al., 2003). Peonidin, petunidin, delphinidin, and cyanidin (Hayasaka and Asenstorfer, 2002; Alcalde-Eon et al., 2004; Pozo-Bayon et al., 2004) based adducts have been identified to a lesser extent. This is the

first time that pelargonidin 3-glucoside based vinylphenol pyranoanthocyanins are found. Most recently, only a vinyl pyruvic adduct of pelargonidin 3-glucoside has been identified as a minor pigment molecule in strawberry (Andersen et al., 2004). The glycosyl residues detected in pyranoanthocyanins have been mainly 3-monoglucosides, and to a lesser degree 3-rutinosides have been reported (Lu et al., 2002). This is the first time pyranoanthocyanins are described with more complex sugar residues. To our knowledge pyranoanthocyanins have not been detected in strawberry and raspberry juices before.

The same retention times on the HPLC chromatogram, UV-vis spectra, and m/z values, as the here found strawberry juice pyranoanthocyanins, were obtained with the model solution of pure pelargonidin 3-glucoside, enhanced with either ferulic acid or sinapic acid. This confirms the anthocyanin aglycone to be pelargonidin and the reactions to take place with pelargonidin 3-glucoside and the studied cinnamic acids. The raspberry variety used in the study had four major anthocyanins, and the same number of novel compounds was detected after cinnamic acid addition (figure 13). Also in strawberry juice, which contained one major anthocyanin in the beginning, one major new anthocyanin adduct was detected after storage.

The proposed mechanism for the reaction between an anthocyanin and a cinnamic acid is shown in Scheme 3. It differs from previous reaction mechanisms proposed (Schwarz et al., 2003). It was confirmed that the reaction involves the formation of vinylphenol form from the cinnamic acids. Synthesized 4-vinylsyringol reacted in strawberry juice with pelargonidin 3-glucoside resulting in the same pyranoanthocyanin adduct as was obtained with the addition of sinapic acid in strawberry juice, and pure pelargonidin 3-glucoside with sinapic acid. Previously pyranoanthocyanins were considered to form from direct covalent reactions of anthocyanins with vinylphenols (Fulcrand et al., 1996), the latter being wine fermentation products resulting from enzymatic decarboxylation (Grando et al., 1993; Coghe et al., 2004). Now, however, other reaction pathways have been put forward, and Schwarz et al. (2003) suggested of a reaction of wine anthocyanins with intact simple cinnamic acids. They found the reaction to be quite fast in a model solution as did Fulcrand et al. (1996), and Sarni-Manchado et al. (1996).

In study (**II**) on color stability with pure compounds, in model solution of pelargonidin 3-glucoside, it was noticed that the color intensity reached its highest level after 50 days of storage. In study (**IV**) on development of novel anthocyanin derivatives in strawberry and raspberry juices, the formation of new compounds was well ahead already after 11 days of storage. This is slow compared to reactions in wine model solutions but fast compared to reactions in authentic wines. It was also noticed that in the non-sterile juices, the enhanced

color development was faster than in the 45-minute ultrasonicated juices. In the non-fermented, sterile but not pasteurized, berry juices, it is possible for enzymatic activity to occur and speed up the formation of pyranoanthocyanins. This is a probable explanation for the difference in reaction times between pure compounds in model solutions and anthocyanins with cinnamic acids in juice media.

7. CONCLUSIONS

Attractive color is one of the most important sensory characteristics of fruit and berry products. However, the color of red berry products is unstable and susceptible to degradation. Anthocyanin colors can be enhanced and stabilized by the addition of different plant extracts and phenolic acids. This enhancement was established both with pure anthocyanins in model solutions and in food product applications, i.e. black currant wine and four different berry juices.

In wine and juice making the anthocyanin content and color quality of the product depends mostly on raw materials and processing methods used. Maintaining a strong and stable color in berry wines and juices is problematic during processing and storage. In this study it was shown that the color of black currant wine could be enhanced by grape skin extract or by crowberry juice. Crowberry induced stronger color and grape skin extract more stable color to the wine. Natural plant extracts, black carrot, grape skin, and rosemary extracts, enhanced strawberry and raspberry juices. This enhancement is most probably due to the overall increment of anthocyanin content in the berry juices and wine, in the case of black carrot and grape skin extracts, but also other substances of the extracts, like other flavonoids and phenolic acids, may take part in the color enhancement reactions.

Berry juice colors were successfully enhanced by the addition of phenolic acids alike. The color of berry juices faded quickly during storage. The addition of cinnamic acids improved the color of the juices by stabilizing and enhancing their color. The reactions observed with the four berry juices and added natural phenolic acids differed significantly by their mechanisms and manifestations. Intermolecular copigmentation reactions are most likely responsible for the color enhancement by the conjugated cinnamic acid, rosmarinic acid, which protected lingonberry and cranberry juice anthocyanins. In raspberry and strawberry juices the simple cinnamic acids, sinapic and ferulic acids, formed new anthocyanin derived pigment molecules, pyranoanthocyanins, which possessed more stable and stronger colors compared to the color of the intack berry juices.

The color of pure anthocyanin molecules was successfully affected by the addition of different copigments. The immediate copigmentation effect, i.e. the color enhancement perceived on the day of preparation, differed from the phenomena observed during storage. At first rosmarinic acid induced the strongest color enhancement. During storage the most efficient copigmentation was observed with ferulic and caffeic acids. The data obtained from the study with pure compounds revealed adequately the effects of molar ratio and the structures of copigments and anthocyanins, on copigmentation phenomena. Most probably the same new pigments, which were detected in berry juices, were generated in the course of time in the model solutions of pure compounds, but since they were not monitored, no distinct elucidation is to support this assumption.

Until now, the similar color enhancement reactions, as observed in this study, have been thought to take place in grape wines through fermentation, and with the help of fermentation side products and during wine ageing. The newly found anthocyanin vinylphenol adducts detected in berry juices were identified to comprise either of 4-vinylguaiacol or 4-vinylsyringol, depending on the used cinnamic acid, linked to 4- carbon and 5-hydroxyl positions of the anthocyanin. This is the first time color enhancement and the formation of new anthocyanin vinylphenol adducts were observed in non-fermented juices. This was also the first study where pyranoanthocyanins were identified with a pelargonidin 3-glucoside entity and also the first time when more complex glycosyl residues were detected in pyranoanthocyanins.

The enhancement of berry juices with cinnamic acids can be regarded as a promising mean of improving unstable juice color. The most popular way of attchieving attractive juice color should not be in the development of new food additives, but rather throught optimising the berry phenolic content in the raw materials, in balancing between the anthocyanin content to that of the copigments. Plant breeding of the raw materials could also be a potential mean in increasing and optimising the phenolic contents.

The current results may be of use in improving the color quality of berry products and in the development of foods with anthocyanin-rich ingredients. To fully benefit from the findings of this research in the development of berry food products, these physico-chemical characteristics should be supplemented with other qualitative and sensory aspects.

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