University of Alberta

Reactions of Anthocyanins and *o*-Quinones in Model Systems and Foods

by

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DEDICATION

To the memory of my friend and teacher

Alex Panasyuk

1971 – 24 June 2010

ABSTRACT

Molecules of anthocyanins and quinones possess distinctive electrophilic character, which is demonstrated by their facile reactions with nucleophiles such as sulfite, thiols, amines and water. In food systems, one of their likely targets would be nucleophilic centers in the side chains of amino acids. Our experiments revealed that on a short-term exposure (1 – 72 h) to free amino acids in solutions with pH < 7 glycosides of cyanidin and quinones of phenolic acids did not yield nucleophilic addition products with most of the amino acids. A notable exception was cysteine, which reacted with oxidized phenolic acids and caused anthocyanin bleaching at elevated temperature. Thermodynamic aspects of the nucleophilic addition reactions were investigated with the aid of computational chemistry.

We have also found that enzymatic browning in apricot puree does not lead to *trans-cis* β-carotene isomerization, contrary to some previous reports. Increased availability of β-carotene for extraction was recorded for browned apple- and pear-apricot purees in comparison with the non-browned purees.

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LIST OF ABBREVIATIONS

BHT - Butylhydroxytoluene

ESI - Electrospray ionization

GC - Gas chromatography

HPLC – High-performance liquid chromatography

m/z - Mass-to-charge ratio

MS - Mass spectrometry

NMR - Nuclear magnetic resonance

PPO - Polyphenol oxidase

TFA - Trifluoroacetic acid

U - Enzyme unit

UV - Ultraviolet

1. INTRODUCTION

Due to the growing demand for functional foods and nutraceuticals, we may see increased application of plant-based extracts in the food industry in the near future (Kwak and Jukes, 2001a, 2001b; Schitler et al., 2003). However, careless application of these extracts may have negative consequences. Nucleophilic groups in the side chains of amino acids which constitute proteins may react with electron-deficient centers in the molecules of anthocyanins and oxidized *o*-diphenols; in some cases, such interactions may result in covalent binding of the phenolic residues to proteins. Adducts with undesirable technological and unpredictable nutritional properties may be formed in these reactions (Papadopoulou and Frazier, 2004). For instance, one of the consequences of the binding of oxidized phenolic compounds to proteins is decreased availability of the essential amino acid lysine (Kroll and Rawel, 2001).

Phenolics can be found in significant quantities in various sources of plant origin – fruits (including berries), vegetables, leaves, stems and processed products based on them. Both anthocyanins and *o*-diphenols are biosynthesized in plants and perform many important functions. For instance, they act as pesticides, antibiotics, and protectors against UV radiation; anthocyanins contribute to coloration of plant tissues; *o*-diphenols participate in separation of the damaged areas of the plant from the healthy part (Shahidi and Naczk, 2004). Simplest plant

phenolics containing the *o*-dihydroxy moiety are derivatives of benzoic and cinnamic acids. Anthocyanins have more complex structures and chemically are glycosides of polyhydroxylated 2-phenylbenzopyrylium (flavylium) cation. They belong to the class of phenolics known as flavonoids.

In the food industry anthocyanins have attracted much interest as safe pigments for coloration of processed foods. However, their color may be susceptible to bleaching in some processing operations and also during storage. Factors that have been connected with color degradation of anthocyanins include pH, temperature, light and presence of preservative or antioxidant agents (Iacobucci and Sweeny, 1983; Castaneda-Ovando et al., 2009; Borkowski et al., 2005).

Anthocyanins may assume different chemical structures depending on pH of the medium. They are particularly unstable in neutral or basic conditions and exist as a mixture of several forms below pH 7. The flavylium cation form ($\mathbf{AH^+}$) predominates around pH 2 and below. It is believed that in water solutions it slowly hydrates yielding colorless chromenols **B-I** and **B-II** (Fig. 1.1). Upon increase of pH, the flavylium cation quickly deprotonates, and the quinoidal form **A** is formed. These three forms exist in equilibrium with each other, and their relative concentrations depend on pH of the medium and structural details of the anthocyanin (Iacobucci and Sweeny, 1983).

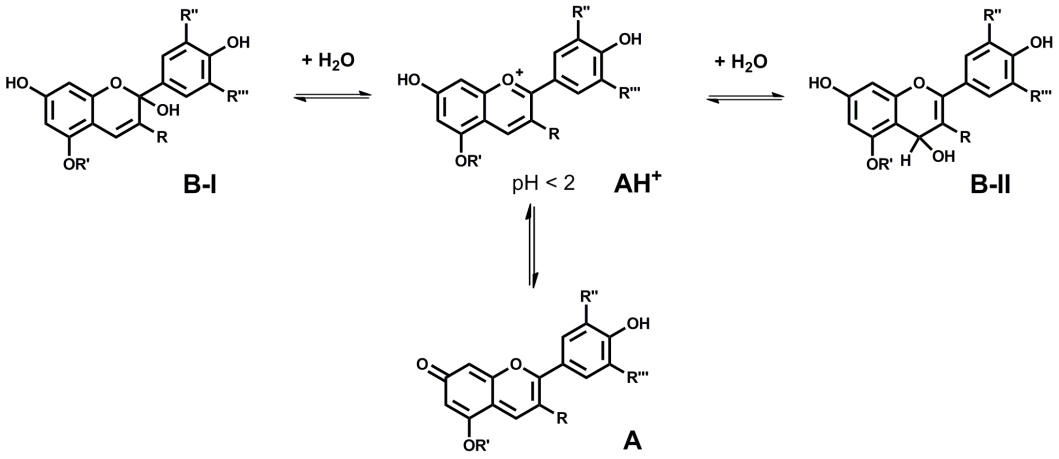


Figure 1.1: Structural transformations of anthocyanins in water.

Phenolics which possess the *o*-dihydroxy moiety are susceptible to enzymatic oxidation catalyzed by a copper-containing enzyme polyphenol oxidase (PPO). The first product in this reaction is the corresponding *o*-quinone. It undergoes further chemical transformations and is converted into dark-colored insoluble polymers (Sanchez-Ferrer et al., 1995). This chain of reactions is known as enzymatic browning. In living plants it is a very important process, because these insoluble polymers create "walls" which separate damaged parts of a plant from the healthy part (Eskin, 1990). The same reaction constitutes a major concern for food processors, because it leads to unwanted changes in chemical, physical, sensory and nutritional properties of foods. However, in some cases enzyme-catalyzed oxidation of phenolics is desirable, as in processing of tobacco, tea, coffee and dates (Mathew and Parpia, 1971; Mayer and Harel, 1979).

PPO activity can be controlled by modifying temperature and pH of food systems as well as by chemical means (Friedman, 1996). Heat treatment applied for a sufficiently long time denaturates the PPO and leads to loss of enzymatic activity; temperatures as high as 80 °C may be necessary to fully denaturate the enzyme in plant tissue (Dijkstra and Walker, 1991; Arslan et al., 1998). The optimal pH range for PPO is between 4.0 and 7.0. Lowering the pH below 4.0 results in retardation of the enzymatic activity (Eskin, 1990).

1.1 Reactions of anthocyanins with *N*-, *O*- and *S*-nucleophiles

The flavylium cation possesses electrophilic character; therefore, in favourable conditions anthocyanins can react with nucleophiles. Many instances of such reactions have been reported in the literature, which are discussed below. In fact, it is because of this susceptibility of natural anthocyanins to reactions with chemical agents employed in food processing that their use as substitutes for artificial colorants can be somewhat limited (Francis and Markakis, 1989; Jackman et al., 1987). Due to peculiarities of the structure of the flavylium cation, most nucleophilic addition products are colorless. Discoloration may occur in foods which contain or were treated with chemicals like sulfites, ascorbic acid, etc. For instance, sulfur dioxide, which is widely employed in the food industry as a preservative, easily reacts with anthocyanins. Many studies have been published on this topic, and several reaction mechanisms have been proposed. The colorless

anthocyanin-bisulfite adduct was identified as a product of direct nucleophilic addition of bisulfite anion to the C4 position of the flavylium ion (Berke et al., 1998). Until their study, it had been thought that the mechanism of the addition reaction involved ring opening of the flavylium cation **1** yielding carbinol **3**, which then reacted with bisulfite, leading to bisulfite-chalcone adduct **4** (Fig. 1.2). Subsequent research demonstrated that the addition product should be either a sulfonate (**5, 6**) or a sulfinate (**7, 8**). Finally, Berke et al., (1998) unambiguously indentified the product of nucleophilic addition of bisulfite to malvidin-3-glucoside as C4-sulfonate. The structure of the adduct was elucidated by a combination of ^{1}H, ^{13}C and ^{33}S NMR spectroscopy.

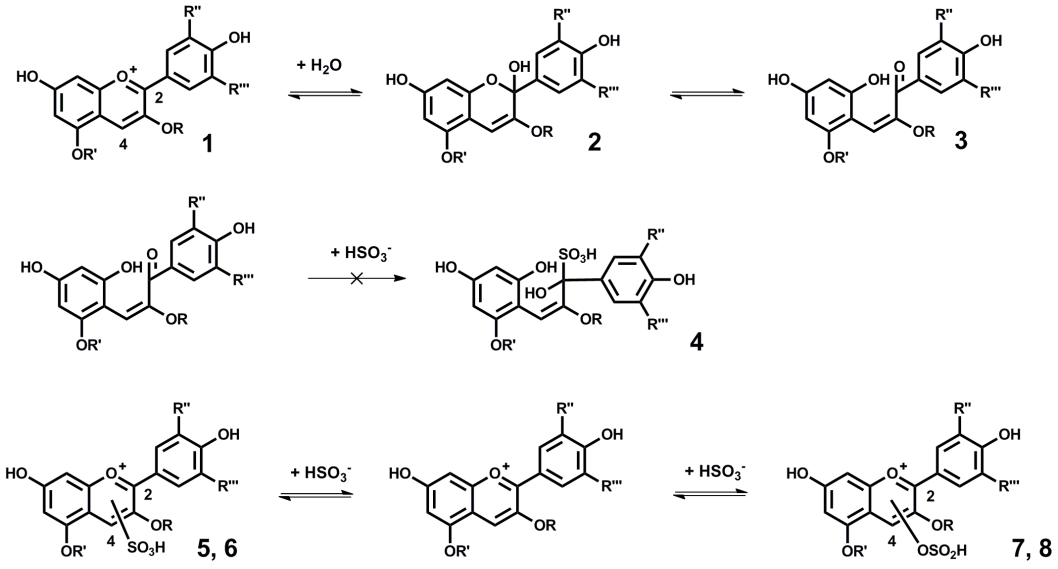
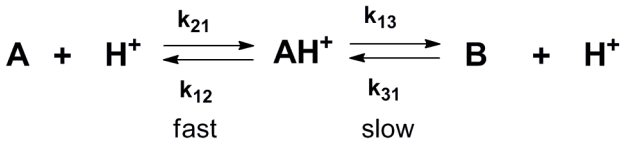


Figure 1.2: Addition of sulfite to the flavylium cation.

The authors pointed out that the equilibrium of the bisulfite addition reaction was quantitatively in favour of the flavylium cation, and that bisulfite and water were in competition with each other for the substrate. The fact that the C4 adduct had been favoured over the C2 was explained on the ground of steric hindrance of the C2 position. Accordingly, the authors put forward a suggestion that chemically modified anthocyanins with bulky substituent introduced in the C4 position would be more resistant towards bisulfite bleaching.

Nucleophilic addition of water to the flavylium cation has been studied in great detail. In acidic water solutions anthocyanins are believed to exist as a mixture of several forms in equilibrium with each other (Fig 1.2). Jurd (1963) proposed that on raising the pH of the solution the flavylium cation form quickly deprotonates into the quinoidal base **A.** On standing it is attacked by water molecules and slowly hydrates into the carbinol **B-II.** Thus, according to Jurd (1963), direct attack of water on the flavylium cation does not take place. Brouillard and Dubois (1977), on the basis of pH jump experiments for malvin (malvidin-3,5-diglucoside), refuted the mechanism put forth by Jurd (1963). Their proposed mechanism



involved direct formation of the carbinol **B** bypassing the quinoidal base **A**. They also estimated rate and equilibrium constants for the transformations of malvin in water at 4 °C: k_{12} = 1.8 (±0.1) × 10^{4} s^{-1}, k_{21} = 1.4 (±0.1) × 10^{8} M^{-1} s^{-1}, k_{13} = 4.7 (±0.2) × 10^{-2} s^{-1}, k_{31} =2.6 (±0.1) M^{-1} s^{-1}, K_{12} = 1.3 (±0.2) × 10^{-4} M, K_{13} = 1.4 (±0.3) × 10^{-2} M. Thus, kinetic product in this reaction is the quinoidal base **A**, whereas thermodynamic product is the carbinol **B**. In further studies, Brouillard extended his kinetic experiments to include malvidin-3-glucoside (Brouillard and Delaporte, 1977). Rate and equilibrium constants for its structural transformations in water were found to be as follows (at 25 °C): k_{12} = 4.7 (±0.4) × 10^{4} s^{-1}, k_{21} = 6.7 (±0.5) × 10^{8} M^{-1} s^{-1}, k_{13} = 8.5 (±1) × 10^{-2} s^{-1}, k_{31} =34 (±3) M^{-1} s^{-1}, K_{12} = 5.7 (±1) × 10^{-5} M, K_{13} = 2.5 (±0.1) × 10^{-3} M; k_{13} (4°C) = 0.95 (±0.1) × 10^{-2} s^{-1}, k_{31} (4°C) =6.2 (±0.3) M^{-1} s^{-1}. Comparison of k_{13} and k_{31} rate constants estimated at 4 °C for malvidin-3,5-diglucoside with the rate constants at the same temperature for malvidin-3-glucoside indicates that glucosylation of the OH group in the C5 position of the anthocyanin increases rate of hydration and slows down degradation of the carbinol **B**. In their studies, Brouillard and Dubois (1977) have demonstrated for both mono- and diglucosides of malvidin that hydration of the flavylium cation $\mathbf{AH}^{+}$ is an endothermic process associated with positive entropy change, and that hydration of the diglucoside occurs faster than hydration of the monoside. These findings were later confirmed by Pina (1998), who revisited thermodynamics and kinetics of the structural transformations of malvidin-3,5-

diglucoside in water and concluded that at low pH hydration of the flavylium cation is indeed endothermic.

Unusual condensation reaction of acetone with cyanidin-3-glucoside and delphinidin-3-glucoside has been recently reported by Lu et al. (2000, 2001). In the process of chromatographic analysis of acetone extracts from blackcurrant seeds, the authors discovered two compounds never before observed in this matrix – glucosides of pyranocyanidin and pyranodelphinidin. While these compounds were present in the acetone extract, alcohol extract did not contain any pyranoanthocyanins. Moreover, the intensity of the pyranoanthocyanin peaks increased with extraction time. Upon further investigation it became evident that these pyranoanthocyanins were in fact formed during extraction of the blackcurrant seed with acetone; they had not been present in the plant material. Nucleophilic addition of acetone took place at the C4 position of the flavylium moiety and was followed by cyclization. The end product was a structure with extended conjugation and increased stability of the chromophore (Fig. 1.3). To confirm formation of these condensation products during extraction, model experiments were performed. Blackcurrant seed anthocyanins were dissolved in acetone, and the solution was monitored for pyranoanthocyanins over the course of three days. Adduct formation was slow at room temperature, but once it was raised to 40 °C the reaction proceeded faster; glucosides of pyranocyanidin and pyranodelphinidin were produced in yields of 20 and 15%, respectively. The

structure of the adducts was elucidated with the aid of ^{1}H and ^{13}C NMR spectroscopy.

Further analysis of the acetone extract from the blackcurrant seed revealed the presence of addition products between acetone and rutinosides of cyanidin and delphinidin (Lu et al., 2002). With the aid of 1D and 2D NMR spectroscopy these colorless compounds were characterized as adducts of acetone to the C2 position of the flavylium moiety of both anthocyanins. Until this publication, there had been no reports where the C2 addition products of anthocyanins were isolated and their structure elucidated.

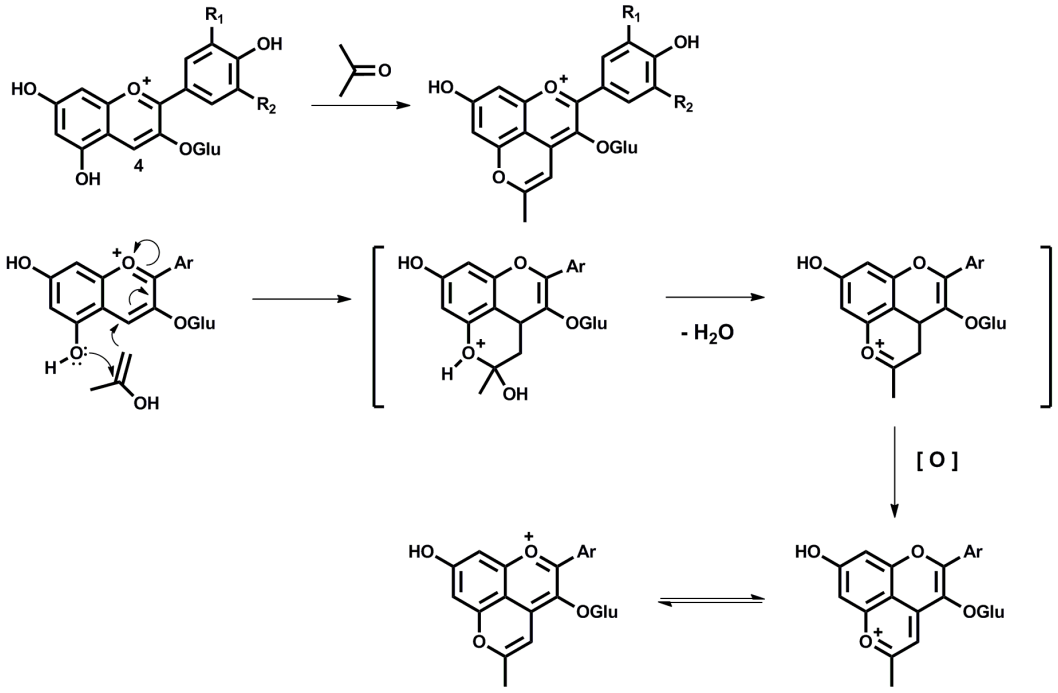


Figure 1.3: Condensation of anthocyanins with acetone (Lu et al., 2001).

Pyranoanthocyanins of similar structure were later synthesized and studied in detail by Olivera et al. (2006). It had been observed that during storage of young red wines anthocyanins, which are largely responsible for their color, are slowly converted into pigments with similar color properties yet different composition (Timberlake and Bridle, 1976; Vivar-Quintana et al., 1999). They were identified as pyranoanthocyanins (Fulcrand et al., 1998; Bakker and Timberlake, 1997). Chemical tests on these compounds revealed increased resistance to hydration and bisulfite bleaching. Olivera with co-workers (2006) synthesized pyranoanthocyanins by reacting isolated anthocyanins with pyruvic acid and performed comprehensive structural and chromatic studies on them. All pyranoanthocyanins that were synthesized were based on the monosides of cyanidin: cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-sophoroside and cyanidin-3-sambubioside. Structural characterization of the adducts was performed by a combination of LC-MS and $^{1}H/^{13}C$ NMR spectroscopy. The proposed mechanism of nucleophilic addition of pyruvic acid to anthocyanins is summarized in Fig. 1.4.

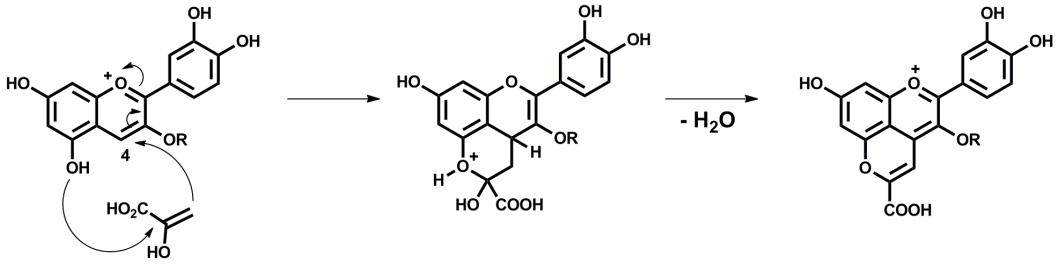


Figure 1.4: Condensation of anthocyanins with pyruvic acid (Olivera et al., 2006).

Chromatic studies in an aqueous solution at pH 2 revealed that the maximum absorption wavelengths of the pyranoanthocyanins were hypsochromically shifted relative to cyanidin-3-glucoside, giving them reddish-orange color. At the same time, raising pH up to neutrality did not have a major effect on the color intensity of the pyranoanthocyanins, whereas cyanidin-3-glucoside was converted into the colorless carbinol. The cyanidin-pyruvic acid adducts also displayed greater resistance to bisulfite beaching than cyanidin-3-glucoside. A small decrease in color intensity of these pigments at bisulfite concentrations of 200 ppm and higher was explained by formation of the nucleophilic addition products at the vacant C2 position of the flavylium moiety.

Bleaching of anthocyanins in the presence of ascorbic acid has long been known in the food industry and has been a source of much controversy. Some authors believe that discoloration of anthocyanins in presence of ascorbic acid is caused by its direct nucleophilic attack on the flavylium cation (Poei-Langston and Wrolstad, 1981; De Rosso and Mercadante, 2007). However, previous studies demonstrated autoxidative nature of this reaction (Sondheimer and Kertesz, 1952). Autoxidation of ascorbic acid in water solutions generates reactive oxygen species ($HOO^{\cdot}$, $HO^{\cdot}$) which can easily attack the flavylium cation, yielding an array of oxidation products (Laroff et al., 1972). For instance, in an argon atmosphere, no trace of reaction between ascorbic acid and apigeninidin was observed. Once air was admitted, bleaching of the anthocyanidin occurred within

a few hours. GC/MS analysis of the reaction mixture revealed the presence of hydroquinone, *p*-hydroxybenzoic acid and phloroglucinol, which had been produced by cleavage of the pyrylium ring of apigeninidin. Moreover, 3-*O*-methyl- and 2-*O*-isovalerylascorbic acids, which cannot undergo autoxidation due to blocked ene-diol function, did not bleach apigeninidin on standing in presence of air (Iacobucci and Sweeny, 1983).

One area of anthocyanin chemistry which has not received enough attention in the literature is nucleophilic addition reactions involving amino acids. Amino acids are found in plant cells both in free and bound form, and some of them possess reactive centers in their side chains which under certain conditions may react with anthocyanins. Therefore, the lack of reports on the reactivity of individual amino acids towards anthocyanins was surprising. Of the relevant studies which have been published to date, neither was explicitly concerned with the amino acid-flavylium cation interactions in conditions characteristic of plant foods. One of these publications is a report by Shriner and Sutton (1963) on reactions of flavylium perchlorate with ethyl esters of α-amino acids. The authors pointed out in the beginning of their paper that since both α-amino acids and anthocyanins occur in the sap of plant cells, it would be of interest to investigate whether these classes of compounds were reactive toward each other. However, their experiments were performed in conditions not characteristic of plant cells. Flavylium perchlorate was incubated with ethyl esters of glycine, alanine, and

phenylalanine in chloroform at room temperature for 8-12 hours. Reaction products were identified as ketimine perchlorates **I** – **III** in Fig. 1.5 by IR spectrometry and chemical tests. The authors also noted that treatment of flavylium perchlorate with free α-amino acids did not result in formation of any addition products.

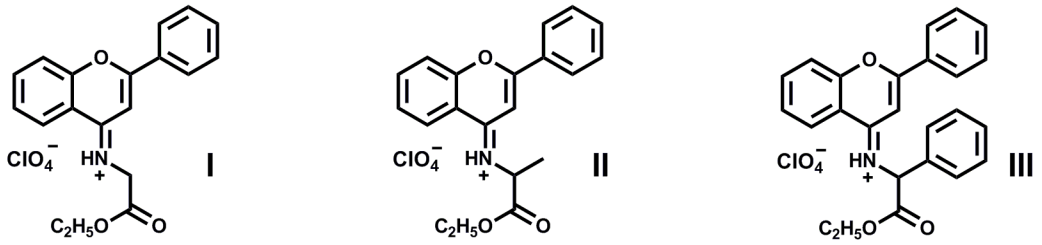


Figure 1.5: Ketimine salts formed in the reaction of flavylium perchlorate with α-amino acids ethyl esters.

Another paper related to amino acid-anthocyanin reactions was published in the proceedings of a conference on food research, which was held by the University of Karachi (Pakistan) in 1996. It was found that fruits of *Carissa carandas* plant grown in Southeast Asia for edible purposes contain "anthocyanin bound protein (ABP)" (Ali and Sayeed, 1996). Dialysis of juice from the fruits yielded a high molecular weight colored fraction, which upon hydrolysis with papain and 2 N HCl produced delphinidin and free serine. This fact led the authors to the conclusion that delphinidin had been bound to serine in the ABP. The protein was found to have molecular weight of 14500 Da, with glycine and

alanine accounting for 90% of the amino acids. The authors were not able to identify the N-terminal amino acid which led them to conclusion that either the N-terminal end of the ABP was derivatized or that it was linked to the anthocyanin. Taking into account all their experimental observations, the authors concluded that the most likely structure for the serine-delphinidin adduct would be one with the amino group of serine inserted into the anthocyanidin moiety in the place of the flavylium oxygen atom. However, no MS or NMR data were available to the authors at the time of writing to confirm or refute their hypothesis. To us, this structure seems to be rather questionable. First of all, the α-amino group in serine is too weak a nucleophile for reaction with flavylium cation in the specified manner. Secondly, the pulp of *Carissa carandas* is highly acidic (pH ~2), which further decreases the reactivity of the α-amino group due to its protonation. For instance, in the above mentioned study of Shriner and Sutton (1963), flavylium perchlorate did not react with free α-amino acids even at pH 7. Thirdly, according to the amino acid composition of the ABP, its lysine and histidine content is three and nine times higher than its serine content. These two amino acids possess nucleophilic centers of greater reactivity than the α-amino group of serine. Therefore, they would be more likely candidates for attachment to delphinidin, both from a physicochemical point of view and basic laws of probability. Unfortunately, the NMR and MS data which the authors intended to publish upon completion of their study have never been published, and the fate of this project is unknown.

1.2 Reactions of oxidized *o*-diphenols with *N*-, *O*- and *S*-nucleophiles

Among monomeric phenolic compounds occurring in foods of plant origin those which possess the *o*-dihydroxy moiety share one common property: their conversion to *o*-quinones upon interaction with polyphenol oxidase in the presence of molecular oxygen. Quinones are highly reactive entities and easily undergo further chemical transformations; for instance, nucleophilic addition reactions. These secondary reactions cause a variety of complex and often undesirable changes in chemical, physical, and nutritional characteristics of foods (Shahidi and Naczk, 2004). Therefore, better characterization of nucleophilic addition reactions involving *o*-quinones is necessary in order to understand all possible implications of such reactions in foods.

Among the nucleophilic substances that have been shown to react with *o*-quinones are some amino acids, glutathione, primary and secondary aliphatic amines, hydroxylamine, alcohols, thiols, etc. For instance, dappled red color of the so-called "Cherry Red" (CR) tobacco is a result of reaction between *o*-diphenols and nornicotine during curing and is an indication of high nornicotine content of tobacco (Penn and Weybrew, 1957). Since tobacco growers are not allowed to produce cultivars with high nornicotine content, CR tobacco has been a persistent problem for them (Bowman and Rawlings, 1985). Wada (1956) was the first to conclude that the red pigment is formed from nornicotine and enzymatically oxidized *o*-diphenols during tobacco curing. Four decades later,

Weeks et al. (1993) unequivocally showed that it is indeed a reaction between *o*-quinones and nornicotine that is responsible for red color formation in the CR tobacco. The authors were not able to determine the structure of the red pigment, since all attempts at isolating the compound failed. Chloroform, hexane, n-butanol, diethyl ether, or polyvinylpyrrolydone could not extract the compound responsible for the color of the CR tobacco. Therefore, the authors had to adopt a different strategy, so they turned to model systems. Reactions in model solutions that contained nornicotine, caffeic/chlorogenic acid and PPO were performed in the temperature interval from 0 to 75 °C. In all cases addition of PPO resulted in almost instant formation of the red color monitored at 570 nm. Ferulic acid, which lacks the *o*-dihydroxy moiety, failed to produce red color in same conditions. Amino acids proline, asparagine, alanine and glutamic acid as well as the alkaloids nicotine and myosmine did not produce red color in the caffeic/chlorogenic acid assay. Unable to isolate caffeic acid quinone, the authors performed experiments with *p*-benzoquinone. The reaction of nornicotine with free *p*-benzoquinone resulted in an instant formation of red color. Upon analysis of the reaction mixture by GC/MS and GC/IR, compounds **I** and **II** were identified as major products of the reaction (Fig. 1.6). Therefore, the authors concluded that reaction of nornicotine with *o*-benzoquinone would give rise to products of similar structure. However, all attempts to separate and identify *o*-quinone adducts from the caffeic/chlorogenic acid assay by HPLC/MS failed.

Figure 1.6: Reaction products of nornicotine with *p*-benzoquinone.

In analytical chemistry, catechols, *o*-aminophenols, and *o*-diamines are often employed as indicator compounds for electrochemical determination of nitrogen and sulfur nucleophiles (White et al., 2001; Nekrassova et al., 2003). Nucleophilic addition reactions of electrochemically derived *o*-quinones with various O-, N- and S-nucleophiles have been studied in great detail. For instance, it has been shown that electrochemical oxidation of catechol derivatives in alcoholic solutions leads to alcoxylation products (Nematollahi and Golabi, 1996; Nematollahi and Golabi, 2001). Catechol and 4-methylcatechol upon oxidation in methanol yielded 4,5-dimethoxy- and 4-methoxy-5-methyl-*o*-benzoquinone, respectively. Similarly, electrochemical oxidation of catechol in ethanol gave 4,5-diethoxy-*o*-benzoquinone. 3,4-Dihydroxybenzaldehyde upon oxidation in methanol gave rise both to alkylation and dimerization products.

Reactions of 3-methylcatechol and 3,4-dihydroxybenzaldehyde with methanol were found to be pH and temperature dependent (Nematollahi and Golabi, 2001). The main product of methoxylation of 3-methyl-*o*-benzoquinone in 0.15M

NaOAc was 3-methoxy-5-methyl-*o*-benzoquinone. Methoxylation significantly decreased susceptibility of the latter quinone towards further nucleophilic reactions, so that no dimethoxylation products were formed. The product of electrochemical oxidation of 2,3-dihydroxybenzaldehyde in 0.15M NaOAc in presence of methanol was 2,3-dihydroxy-6-methoxy-benzaldehyde; polymerization rate was also significant. In a mixture of NaOAc and trichloroacetic acid (15:3, 0.15M NaOAc) dimerization of the quinone was suppressed, and the methoxylation product was formed in much better yield. After increasing temperature of the reaction mixture to 45 °C, the rate of dimethoxylation reaction increased, and part of 2,3-dihydroxy-6-methoxybenzaldehyde was converted into 2,3-dihydroxy-5,6-dimethoxybenzaldehyde. Thus, presence of the –CHO group in the molecule of 2,3-dihydroxybenzaldehyde increased reactivity of its quinone towards nucleophilic attack by methanol, yet also made it more susceptible to polymerization.

Nucleophilic addition of cyanide produced *o*-dihydroxybenzonitriles from electrochemically derived *o*-quinones (Nematollahi et al., 2004). Catechol, 3-methylcatechol, 3-methoxycatechol, 4-*tert*-butylcatechol, 2,3-dihydroxybenzoic and 3,4-dihydroxybenzoic acids when subjected to electrochemical oxidation at pH 7 in presence of NaCN yielded corresponding *o*-dihydroxybenzonitriles. Rates of nucleophilic addition of cyanide were found to be pH-dependent and increased

on increasing pH of the reaction solution. The resultant *o*-dihydroxybenzonitriles were more resistant to oxidation than initial catechols, consequently, nucleophilic addition of more than one cyanide anion was not observed. Derivatives of catechol with electron-donating substituents in the phenolic ring (methoxy, methyl and *tert*-butyl) were less reactive towards cyanide, whereas 2,3- and 3,4-dihydroxybenzoic acids displayed increased reactivity compared to catechol.

Reactions of oxidized catechols with secondary amines yielded corresponding aminobenzoquinones (Kiani et al., 2005; Nematollahi and Hesari, 2005; Raoof et al., 2009). Catechol, when oxidized electrochemically in presence of diethylamine, dibutylamine and dibenzylamine, yielded 4-dialkylamino-*o*-benzoquinones. Reactions of catechol with diethylamine and dibutylamine were studied in pH interval 2-9. At the lower end of this interval no reaction between oxidized catechol and amines was observed; at pH 7 and higher nucleophilic addition products were detected. Similarly to previous observations, nucleophilic addition was limited to one molecule of dialkylamine. Whereas polymerization of *o*-benzoquinone was noticeable in absence and at low concentration of the amines, at higher concentrations the extent of polymerization was negligible. Reactions of 3-methylcatechol and 3-methoxycatechol with dibenzylamine yielded 5-dibenzylamino-3-methyl-12-benzoquinone and 5-dibenzylamino-3-methoxybenzoquinone, respectively. Structures of the dibenzylamino derivatives

were confirmed by UV, IR, ^{1}H NMR, ^{13}C NMR spectroscopy and MS spectrometry.

Sulfite and thiols also readily react with *o*-quinones. Reactions of sulfite with electrochemically oxidized catechol, 4-methylcatechol, 4-*tert*-butylcatechol, 3,4-dihydroxybenzoic acid and 3,4-dihydroxybenzonitrile yielded colorless sulfonate derivatives (Nematollahi et al., 2007). Cyclic voltammetry and controlled-potential coulometry showed that nucleophilic addition of sulfite is faster than other competing reactions involving *o*-quinones (hydration, polymerization, etc.). It was also observed that below pH 3 sulfite failed to react with *o*-quinones, and the rate of the addition reaction increased on increasing pH. Structures of the sulfonates were not elucidated.

Catechol thioethers are main products of (electro)chemical oxidation of catechols in presence of thiols. Catechol, 4-methylcatechol, 4-butylcatechol and 3-methoxycatechol upon oxidation in presence of 2-mercaptobenzoxazole generated corresponding 5-thioethers. Structures of the adducts were confirmed by ^{1}H NMR spectroscopy. Reaction of 1-methyl-1H-imidazole-2-thiol with catechol and 4-*tert*-butylcatechol also yielded 5-thioethers. Their structures were confirmed by ^{1}H NMR spectroscopy and MS spectrometry (Nematollahi and Tammari, 2005; Tammari et al., 2006; Fotouhi et al., 2008; Fotouhi et al., 2009).

The mechanism of nucleophilic addition of thiazolidine-4-carboxylic acid (thiaproline) to *o*-benzoquinone has recently been uncovered (Shahrokhian and

Amiri, 2006). Thiaproline is a component of many pharmaceutical preparations, mainly antitumor and antioxidant agents. Development of a sensitive method for electrochemical determination of thiaproline required better understanding of its reaction with oxidized catechol. Cyclic voltammetry, controlled-potential coulometry, UV-Vis and FT-IR spectroscopy allowed the authors to propose a mechanism which involved the following steps: (A) electrochemical oxidation of catechol to *o*-quinone; (B) addition of thiaproline with its nitrogen atom to the C4 position of the quinone; (C) formation of the oxidized form of the addition product; (D) cyclization into lactone (Fig. 1.7).

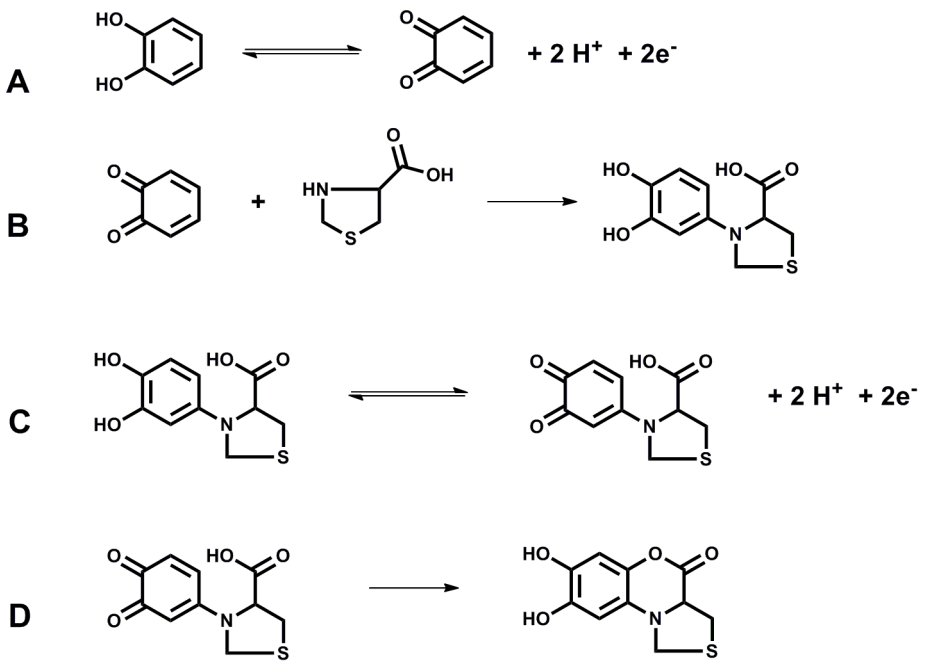


Figure 1.7: Condensation of thiaproline with *o*-benzoquinone (Shahrokhian and Amiri, 2006).

Cysteine reacts with *o*-quinones yielding corresponding cysteinyl- derivatives. Enzymatic oxidation of 4-methylcatechol (MC), chlorogenic acid (CG), catechin (CA) and epicatechin (EC) in presence of excess cysteine gave rise to nucleophilic addition products (Fig. 1.8) – Cys-5-MC (**I),** Cys-2-CG (**II**), Cys-2´-EC (**III**), Cys-5´-EC (**IV**), Cys-2´-CA (**III**), and Cys-5´-CA (**IV**) (Richard et al., 1991).

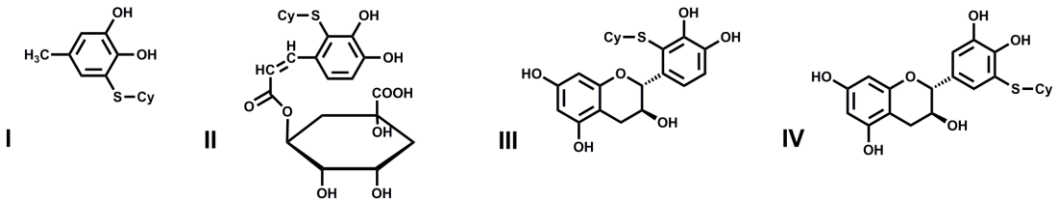


Figure 1.8: Reaction products of oxidized 4-methylcatechol, chlorogenic acid, catechin and epicatechin with cysteine.

In case of chlorogenic acid, formation of small quantities of another adduct was observed. The authors were of opinion that it was either Cys-5-CG or Cys-2,5-CG, since it had been shown that autoxidation of caffeic acid in presence of cysteine yielded a mixture of Cys-2-, Cys-5-, and Cys-2,5- addition products (Cilliers and Singleton, 1990).

Similar findings were reported by Sridhar et al. (2001) for reactions of *o*-naphtaloquinone with cysteine and *N*-acetyl-cysteine. Reactions with both amino acids at pH 7 yielded monoaddition products **I** and **II** in Fig. 1.9. Small quantities of quinoneimine **III** were also isolated from the reaction with cysteine. The

authors also studied reactivity of the quinone towards *N*(α)-benzyl-histidine and *N*(α)-acetyl-lysine methyl esters. Reactions with these amino acids were less pronounced than reactions with cysteine; corresponding addition products **IV** and **V** were isolated in yields of about 20% of theoretical. Reaction of *o*-naphtaloquinone with *N*-*t*-Boc-aspartic acid-α-benzyl ester generated a small quantity of dark red unstable adduct **VI**.

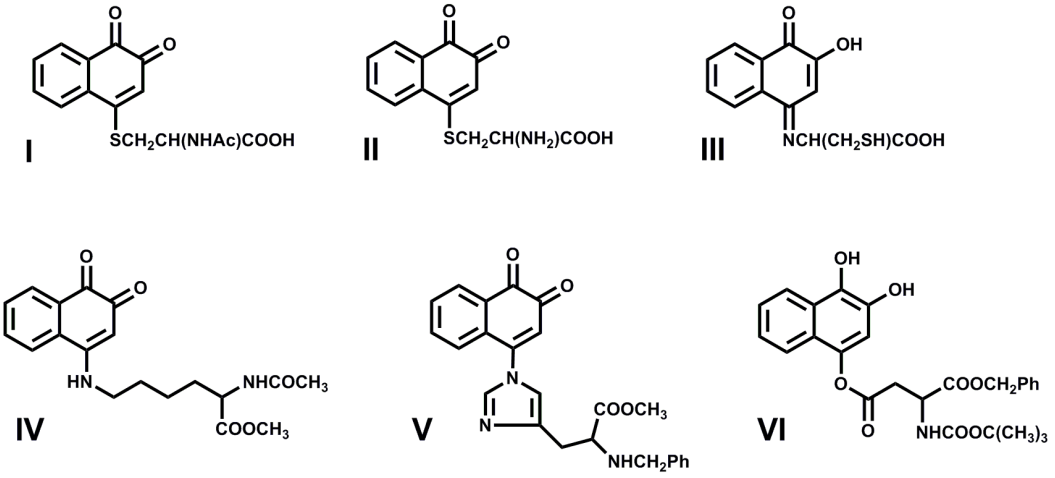


Figure 1.9: Reaction products of derivatized α-amino acids with naphthalene-*o*-quinone.

Covalent addition products were also observed in reactions of enzymatically oxidized chlorogenic acid with *N*-acetyl-L-cysteine and *N*(α)-*t*.-BOC-L-lysine (Schilling et al., 2008). In case of *N*-acetyl-L-cysteine two addition products were detected with *m/z* 514 and 675 in the mass spectrum. These molecular ions correspond to addition products of one and two moieties of *N*-acetyl-L-cysteine to

chlorogenic acid quinone, respectively. Addition products of *N*(α)-*t*.-BOC-L-lysine to monomeric chlorogenic acid quinone were not observed. Instead, two adducts to dimeric chlorogenic acid were detected in very low quantities.

Prigent (2005) reported that incubation of enzymatically generated chlorogenic acid quinone with *N*(α)-*t*.-BOC protected amino acids at pH 7 resulted in formation of adducts with *Lys, Trp, His* and *Tyr*. Free, unprotected amino acids in the same conditions yielded "green color".

Kroll and Rawel (2001) reported formation of covalent addition products between myoglobin and oxidized plant phenols, including *o*- and *p*-hydroxyphenols. Incubation of myoglobin with the phenols at pH 9 resulted in decrease of the amount of free amino groups in the protein. The authors attributed this effect to oxidation of the phenolic substances into quinones in the alkaline solution with subsequent covalent binding of the latter to the protein, for instance, at the amino group of lysine and the heterocyclic nitrogen atom of tryptophan. In fact, it was found that fluorescence properties of tryptophan in the protein treated with the oxidized phenols were different from those in the control myoglobin, which may indicate tryptophan modification by the quinones.

Similar observations were made for lysozyme treated with *o*- and *p*-hydroxyphenols. The amount of free amino groups in the protein treated with the phenols was decreased, with a significant reduction of lysine and tryptophan

residues (Rawel et al., 2001). In reactions of oxidized chlorogenic and caffeic acids with soy glucinin, it was not only lysine and tryptophan that were modified by the covalent addition of the quinones, but also cysteine residues (Rawel et al., 2002).

1.3 Stability of β-carotene during enzymatic browning of fruit purees

Among more than 600 carotenoids known so far, only approximately 50 are active as vitamin A precursors, the most important and most efficient being β-carotene (Schieber and Carle, 2005). Carotenoids mainly occur in their all-*trans*-configuration. Exposure to heat and light, e.g. during food processing, may lead to formation of *cis*-isomers. While light predominantly causes conversion of *trans*-β-carotene to 9-*cis*-β-carotene, thermal impact results in formation of the 13-*cis*-isomer. *Cis*-isomers possess different physicochemical properties compared to their *trans* counterpart such as lower melting points and increased solubility in non-polar solvents. Another consequence of isomerization is a hypsochromic shift in the λ_{max} and smaller extinction coefficients, which leads to decreased color intensity. From the nutritional point of view, *trans*-*cis*-isomerization considerably decreases provitamin A activity of carotenoids. For instance, 13-*cis*-β-carotene and 9-*cis*-β-carotene exhibit only ca. 50% and 35% of the activity of *trans*-β-carotene, respectively. Therefore, knowledge of the isomeric composition of carotenoids in foods including functional foods and nutraceuticals is essential for

an accurate calculation of the vitamin A content. Furthermore, *trans-cis*-isomerization may also lead to changes in bioavailability and antioxidant activity of carotenoids (Schieber and Carle, 2005).

In addition to heat and light, enzymatic browning was reported to cause *trans-cis*-isomerization of β-carotene. To date, only a few investigations on apricot enzymatic browning and associated changes in the chemical composition of apricot tissue have been published. For instance, Dijkstra and Walker (1991) reported levels of phenolic compounds in healthy and rotted apricot tissue; Radi et al. (1997) published a comprehensive study on chemical composition of nine apricot cultivars and their browning susceptibility; De Rigal et al. (2000) studied changes in carotenoid content of apricot puree during browning.

Different opinions exist on the fate of carotenoids and in particular β-carotene, the major carotenoid present in apricots, during enzymatic browning. Radi et al. (1997) reported a 60% decrease in β-carotene content of apricot puree after 30 min browning and ascribed it to enzymatic co-oxidation. In contrast, De Rigal et al. (2000) observed *trans-cis* isomerization of approximately 20% of β-carotene under similar conditions and specifically pointed out that the "total carotenoid amount was not significantly affected by enzymatic browning". It should be noted here that De Rigal et al. (2000) fortified apricot puree in their experiments with the antioxidant BHT to prevent co-oxidation of β-carotene, whereas Radi et al.

(1997) did not add an antioxidant. This fact may explain strikingly different results obtained for β-carotene in their studies.

Therefore, we felt that another study was necessary to clarify whether enzymatic browning in fruit purees containing apricot tissue led to *trans-cis* isomerization of β-carotene.

2. RESEARCH OBJECTIVES

Both anthocyanins and *o*-quinones possess electrophilic properties, and therefore they may react with nucleophilic centers in the side chains of amino acids in food proteins. The main objective of this study was to probe reactivity of selected amino acids at pH < 7 in nucleophilic addition reactions with anthocyanins and *o*-quinones through a combination of experimental and computational methods. To achieve this objective, the following study was proposed:

1. To investigate experimentally whether covalent adducts are formed between anthocyanins and selected amino acids at pH < 7.

2. To investigate whether covalent adducts are formed between enzymatically generated *o*-quinones and the amino acids at pH < 7.

3. To apply molecular modeling to nucleophilic addition reactions of the amino acids with anthocyanins and *o*-quinones in order to computationally estimate the Gibbs energy change in these reactions at room temperature.

Another objective of this study was to examine effects of enzymatic browning on the fate of β-carotene in fruit purees containing apricot tissue and compare the findings with previous reports.

3. MATERIALS AND METHODS

3.1 Experimental methodology

Eight amino acids which possess nucleophilic centers in their side chains and therefore likely to react with electrophilic compounds were selected for this study – *Arg*, *Cys*, *His*, *Lys*, *Pro*, *Ser*, *Thr* and *Trp* (Fig. 3.1).

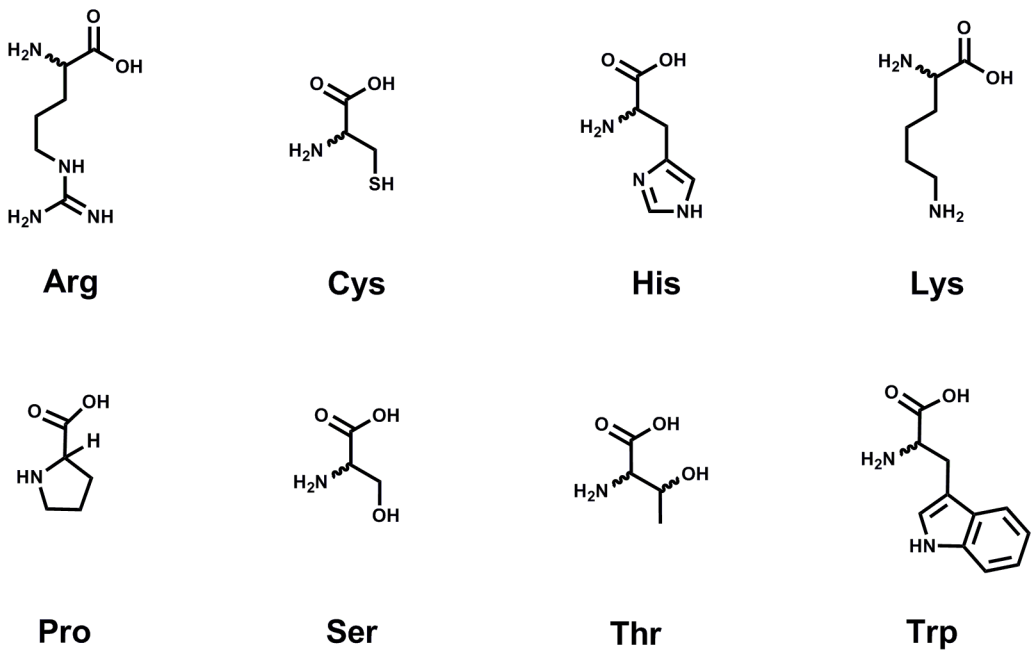


Figure 3.1: Amino acids selected for this study.

Reactivity of these amino acids towards anthocyanins was studied on two derivatives of cyanidin, one of the most widely occurring anthocyanidins: cyanidin-3,5-diglucoside (cyanin) and cyanin-3-rutinoside (keracyanin) (Fig. 3.2).

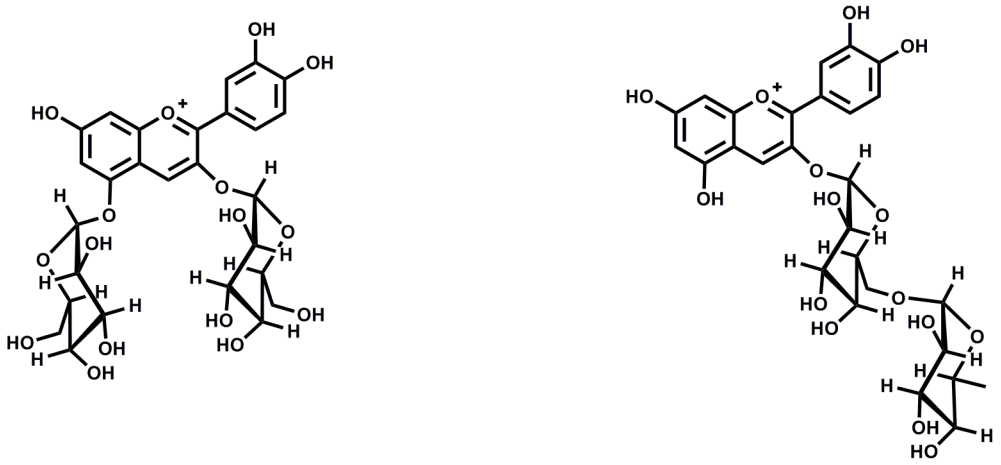


Figure 3.2: Anthocyanins selected for this study.

Reactivity towards quinones was studied on enzymatically generated 3,4-dihydroxybenzoic (protocatechuic) acid quinone and 5-*O*-caffeoylquinic (chlorogenic) acid quinone (Fig. 3.3).

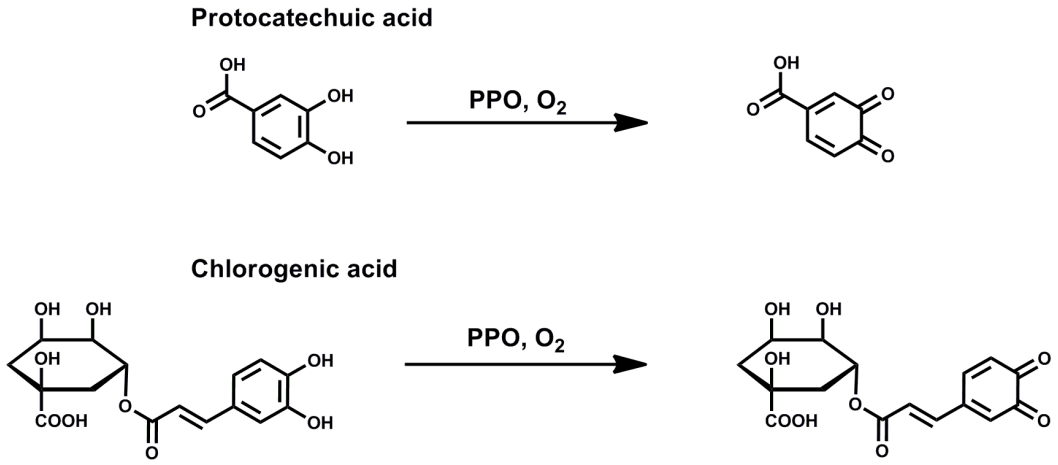


Figure 3.3: Formation of protocatechuic and chlorogenic acid quinones.

Stock solutions of anthocyanins (1.5 μmol in 10 mL) and amino acids (150 μmol in 10 mL) were prepared in two reaction media. Aliquots of 1 mL of the anthocyanin solution (0.15 μmol) were mixed with 1 mL of each amino acid solution (15 μmol) and 3 mL of the corresponding reaction medium. Experiments were performed according to the following scheme:

(a) Reaction medium – water-methanol (1:1) acidified to pH 2 with formic acid, 12 hours reaction time at room temperature;

(b) Reaction medium – water-methanol (1:1) acidified to pH 2 with formic acid, 72 hours reaction time at 37 °C;

(c) Reaction medium – McIlvaine (citrate-phosphate) buffer pH 6.5, 12 hours reaction time at room temperature. This experiment was performed with keracyanin only.

For reactions of the amino acids with quinones, stock solutions of phenolic acids and amino acids (100 μmol in 10 mL) were prepared in the reaction media. Mushroom PPO (1 mg) with activity of ~3500 U/mg was dissolved in 10 mL of water. For each experiment 0.5 mL of the phenolic acid solution (5 μmol) was mixed with 1 mL amino acid solution (10 μmol) and 3 mL of the corresponding medium; then, PPO was added. Experiments were performed according to the following scheme:

(a) Reaction medium – 0.1% formic acid, ~17U PPO, 1 hour reaction time at room temperature;

(b) Reaction medium – 0.1% formic acid, ~17U PPO, 12 hours reaction time at room temperature;

(c) Reaction medium – 0.1% formic acid, ~170U PPO, 1 hour reaction time at 37 °C;

(d) Reaction medium – 0.1% formic acid, ~170U PPO, 12 hours reaction time at room temperature;

(e) Reaction medium – McIlvaine buffer pH 6.5, ~170U PPO, 12 hours reaction time at room temperature.

At the end of incubation time enzymatic activity was inhibited by adding a few drops of 4.5% trifluoroacetic acid solution.

Amino acids, anthocyanins, phenolic acids, solvents and other chemicals were procured from Sigma Chemical Co. (St. Louis, MO).

For experiments on fruit puree browning, organic apricots (*Prunus armeniaca* L.), apples (*Malus domestica* cv. 'Quebec delicious'), and pears (*Pyrus communis* L. cv. 'Bartlett') were purchased from local grocery stores. Fresh, non-bruised fruits were selected. Upon arrival in the laboratory, fruits were quickly peeled (except apricots), cut in small pieces and immediately frozen in liquid nitrogen.

Flash-frozen fruits were transferred into a freeze-dryer and processed for one week. After lyophilization, fruits were homogenized into fine powders using a coffee grinder and stored at -20 °C.

Browning experiments were performed on apricot, apple-apricot, and pear-apricot purees. For each type of puree three experiments were performed each on a different day in order to ensure reproducibility. All experiments were carried out in duplicate. Two media were used for preparation of the purees: distilled water and 1 M NaF. Fluoride solution was used because it inhibits PPO, which is responsible for enzymatic browning. BHT was added to all samples in order to prevent enzymatic co-oxidation of β-carotene. All manipulations were carried out at room temperature under reduced light conditions.

Apricot purees were prepared by mixing 1 g of the apricot powder with 4 mL of each medium supplemented with 0.2 mg BHT in 50 ml plastic centrifuge tubes. Samples were homogenized on a vortex mixer until no dry lumps were visible (ca. 2 min). Apple- and pear-apricot purees (1:1) were prepared in the same way from 1 g of each fruit powder and 6 ml of the corresponding medium. Obtained thick purees were evenly spread over the inner surface of the tubes and left uncovered in the dark for 1 hour. During this time water-based purees gradually changed their color to different shades of brown. The visual intensity of browning increased in the following order: apricot, pear-apricot, apple-apricot puree.

Fluoride-based purees retained their bright orange color, i.e. PPO had been inhibited and no browning had taken place.

Apricot purees were homogenized with 10 ml of acetone and mixtures were left in the dark for 10 min for equilibration. Following the equilibration time the mixtures were centrifuged at 3000 rpm for 4 min, and 4 mL aliquots of the upper acetone layer were transferred into a new set of centrifuge tubes containing 5 mL hexane each. After vigorous mixing followed by 5 min of equilibration, the tubes were topped with distilled water. The upper hexane layer was washed from acetone by careful agitation in water; ~3 mL aliquots of the hexane layer were transferred into a set of amber vials containing 0.5 g anhydrous sodium sulfate each. The same extraction procedure was repeated for apple-apricot and pear-apricot purees except that the volume of acetone used for the initial extraction step was increased to 20 ml, and 10 mL aliquots of the acetone solution were extracted with hexane. Hexane extracts were analyzed by HPLC without further dilution.

3.2 Analytical procedures

The analytical system employed in the study of the reactions between amino acids and *o*-quinones consisted of an Agilent 1200 HPLC-DAD system coupled to an Applied Biosystems 4000 QTrap linear ion trap quadrupole mass spectrometer

equipped with an electrospray interface and controlled by Analyst 1.5 software. Undiluted reaction mixtures (15 μL) were separated on a C18 reversed-phase column (250 × 4.6 mm, 5 μm particle size). For samples containing anthocyanins (20 μL) gradient elution was used: eluent A – 4.5% aqueous formic acid, eluent B – 4.5% aqueous formic acid-acetonitrile (20:80). The elution program was as follows: 0-7 min 15% B, 7-15 min 20% B, 15-16 min 100% B, 16-24 min 0% B. Reaction mixtures containing phenolic acids were separated in the isocratic mode using methanol-water (80:20) acidified with formic acid (4.5%). The diode array detector was set to scan from 200 to 700 nm with 2 nm step width and sampling rate of 2.5 Hz. The mass spectrometer was operated in the following conditions: scanning from 100 to 1000 *m/z*; positive ESI mode for anthocyanins, negative mode for phenolic acids; source temperature 600 °C, collision energy 10 units.

The HPLC system for analysis of β-carotene stereoisomers was from Shimadzu and consisted of SIL-10 ADvp autoinjector, SCL-10 Avp system controller, LC10-ATvp solvent module, and SPD-M10Avp diode array detector. Carotenoids were separated on a YMC C_{30} reversed-phase column (250 mm × 4.6 mm i.d., particle size 5 μm) with gradient elution from 100% eluent A to 56% B at the flow rate of 1 ml/min. Eluent A consisted of methanol-*tert.*-butyl methyl ether-water (81:15:4, v:v), eluent B was pure *tert.*-butyl methyl ether. The diode array detector was set to scan in the range 300-500 nm.

HPLC-grade standards of all-*trans*-β-carotene, 13-*cis*-β-carotene, and 9-*cis*-β-carotene were supplied by Carotenature (Lupsingen, Switzerland).

Peak identification was based on retention times and shape of UV-Vis absorption spectra. Quantification was performed by the external standard method. Purity of the standards had been checked before use. It had been found that the all-*trans* standard contained 20% *cis*-isomers. 9-*Cis* and 13-*cis* isomer standards contained only ~87% and ~65% of the corresponding *cis*-isomer. These numbers were taken into account in the calculations. Calibration curves covered the concentration range of all samples. All manipulations involving standard solutions and sample extracts were carried out avoiding exposure to light and thermal impact.

3.3 Computational approach

With the aid of computational physical chemistry it is possible to analyze driving forces which prompt spontaneous chemical changes and factors which determine rates of chemical reactions. Thermodynamics is the basis for examination of the tendency toward chemical reaction. The Gibbs free energy determines whether a chemical reaction can occur spontaneously and can be calculated as

$$\Delta_r G = \Delta_r H - T\Delta_r S \,,$$

where $\Delta_r H$ is the enthalpy change in the course of a reaction, $\Delta_r S$ is the entropy change, and T is the absolute temperature. Enthalpy is the heat energy change in the course of a reaction; entropy can be defined as a measure of dispersal of the energy. Heat energy is liberated during exothermic reactions and consumed in endothermic reactions. Entropy increases during processes involving dissociation and decreases during associative processes. It is the combination of enthalpy and entropy contributions (the Gibbs function) that correctly describes driving forces behind chemical reactions. In fact, a chemical reaction can only occur spontaneously when the associated Gibbs energy change is negative. This does not mean that reactions for which $\Delta_r G$ is positive do not occur at all. In such case, equilibrium will be shifted towards reactants and the extent of this shift will depend on the magnitude of $\Delta_r G$. For chemical reactions associated with a large, positive change in $\Delta_r G$ the equilibrium will be almost fully shifted towards reactants (Isaacs, 1987).

The Gibbs energy change during a chemical reaction can be either measured experimentally or calculated within statistical thermodynamics. Experimental measurements require specialized equipment and are not always possible. However, due to the advancements in high-performance computing, changes in thermodynamic functions associated with chemical reactions can be estimated using mathematical models of the reactions. So-called *ab initio* molecular orbital theory can be used to predict useful properties of chemical systems by applying

various approximations and mathematical transformations to the equations of quantum mechanics. The fundamental equation of quantum mechanics is the Schrödinger equation. Various properties of atomic and molecular systems can be obtained by solving this equation. In practice, it is impossible to find exact analytical solutions of the Schrödinger equation for all but the smallest systems. In order to obtain reasonably accurate solutions of the quantum-mechanical equations for chemical systems of practical interest, an elaborate system of approximations and mathematical treatments has been constructed and programmed into software packages, which as of lately have become increasingly available to the general chemical community. Due to these advancements, modern computational chemistry allows scientists whose main area is not theoretical chemistry to make useful predictions concerning chemical systems of their interest (Bachrach, 2007).

In this study, computational chemistry was used to investigate reactions of model flavylium cation and *o*-benzoquinone with specific amino acids in order to assess thermodynamic stability of the expected adducts and to provide mechanistic insight into the experimentally observed behavior. Molecular geometries of the amino acids, flavylium cation, *o*-benzoquinone and the adducts were first modeled in water solution to obtain equilibrium structural parameters. Once the relaxed molecular structures were located, vibrational frequencies and thermochemical parametrs were computed for them. For all computations

'B3LYP/6-31G(d)' approximation to the Schrödinger equation was used as implemented in the *Gaussian 03* system of programs (Frisch et al., 2004). Polarized continuum model (PCM) was employed to take into account solvent effects. In this method, solute is placed into a cavity of solvent characterized by its dielectric constant. The 'B3LYP/6-31G(d)' approximation to the Schrödinger equation was tested on many types of organic reactions and was shown to perform well in reproducing experimentally measured thermochemical parameters (Foresman and Frisch, 1994). Hundreds of research papers have been published that successfully employed this model in uncovering mechanistic aspects of organic reactions, including recent theoretical studies on anthocyanidins and *o*-quinones (Estevez and Mosquera, 2008; Woodford, 2005; Hatzipanayioti et al., 2006). Therefore, we felt that this method was well suited for modeling our systems of interest.

4. RESULTS AND DISCUSSION

4.1 Reactions of the anthocyanins with amino acids

Incubation of the anthocyanins with the amino acids in 1% formic acid solution did not result in visible color degradation. When HPLC-DAD-MS chromatograms of the reaction mixtures were recorded after 12 hours at room temperature, in the DAD trace no evidence of the adduct formation was found either. In the reaction mixtures which contained cyanin, its peak was detected at ~7.8 min with UV-Vis absorption maxima at 242, 278 and 514 nm and ions in the mass spectrum at *m/z* 611.1 (molecular ion), 449.1 (loss of one glucose), and 247.1 (loss of two glucose moieties). Keracyanin eluted at ~8.7 min giving UV-Vis absorption at 240, 280, and 520 nm and MS signal at *m/z* 595 (molecular ion). In those reactions which contained tryptophan, keracyanin coeluted with it due to similar retention characteristics on the employed HPLC column.

Results of the HPLC-DAD-MS runs are summarized in Tables 4.1 and 4.2. In the TIC chromatograms of the keracyanin-arginine and keracyanin-lysine reactions ions were detected at *m/z* ratios close to those of the expected adducts, yet their intensities were so low that no corresponding peaks were recorded in the DAD trace. The fact that these ions were observed for the amino acids most likely to react with the anthocyanins – arginine and lysine – makes it quite possible that the observed ions were indeed the molecular ions of the covalent adducts between

these amino acids and keracyanin. However, their low intensity suggests that at low pH and ambient temperature amino acids do not cause anthocyanin bleaching similar to that brought about by sulfite. Therefore, from this point of view nucleophilic addition reactions of amino acids to anthocyanins are unlikely to be a concern for the food industry.

Increasing temperature from ambient to 37 °C and incubation time from 12 to 72 hours did not result in increased formation of the addition products (see Tables 4.3 and 4.4). This observation allowed us to exclude the possibility that it had been high activation barrier of the addition reactions that had caused them to proceed at a very slow rate. If it had been the case, then increased temperature would have imparted enough energy to some reactant molecules to overcome the activation barrier and yield detectable amounts of nucleophilic addition products. This did not happen. However, increased temperature resulted in higher degradation rate of the anthocyanins, which was especially noticeable in reactions with cysteine. In fact, reaction of keracyanin with cysteine at increased temperature resulted in almost complete disappearance of the keracyanin peak from the DAD trace and precipitation of white solid from the solution. A large number of peaks of unidentified nature were recorded in the chromatogram of the supernatant. It seems likely that this reaction is similar to what is published for reaction of anthocyanins with ascorbic acid (Iacobucci and Sweeny, 1983). Ascorbic acid causes autooxidation of the flavylium cation, giving rise to a wide

array of degradation products. It may be that atmospheric oxygen triggered similar reaction involving cysteine which has an easily oxidizable sulfhydryl group. Cysteine was converted into less soluble cystine (which precipitated), and peroxide generated in this reaction attacked keracyanin, giving a complex mixture of oxidation products. Nucleophilic addition products between cysteine and the anthocyanins were not found in the DAD and TIC chromatograms of the reaction mixtures; therefore, this reaction was not pursued further.

One may argue that in 1% formic acid nucleophilic centers in the side chains of amino acids will not be reactive enough to attack anthocyanins. Therefore, reactions with keracyanin were also performed at pH 6.5 and ambient temperature. No difference was found regarding nucleophilic addition products in comparison with 1% formic acid (see Table 4.5).

Table 4.1: Summary of cyanin-amino acid experiments in 1% formic acid for 12 h at ambient temperature.

Reaction	**Compound**	**$[M+H]^+$ (*m/z*, Da)** ***calcd / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
	Cyanin (STD)	611.16 / 611.03 (7.822)	7.86 (112)	242, 278, 514
Cyanin –	Cyanin	611.16 / 611.13 (7.924)	7.87 (128)	242, 278, 514
Arginine	Cyanin-Arg	785.27 / not detected	not observed	-
Cyanin –	Cyanin	611.16 / 611.13 (8.028)	7.89 (109)	240, 278, 514
Cysteine	Cyanin-Cys	732.18 / not detected	not observed	-
Cyanin –	Cyanin	611.16 / 611.19 (7.943)	7.87 (121)	240, 278, 514
Histidine	Cyanin-His	766.23 / not detected	not observed	-
Cyanin –	Cyanin	611.16 / 611.20 (7.999)	7.87 (122)	242, 278, 514
Lysine	Cyanin-Lys	757.27 / not detected	not observed	-
Cyanin –	Cyanin	611.16 / 611.16 (8.012)	7.87	240, 278, 514
Proline	Cyanin-Pro	726.22 / not detected	not observed	-
Cyanin –	Cyanin	611.16 / 611.15 (8.021)	7.87 (130)	242, 278, 514
Serine	Cyanin-Ser	716.20 / not detected	not observed	-
Cyanin –	Cyanin	611.16 / 611.14 (7.892)	7.87 (123)	242, 278, 514
Threonine	Cyanin-Thr	730.22 / not detected	not observed	-
Cyanin –	Cyanin	611.16 / 611.09 (8.008)	7.87 (163)	240, 278, 514
Tryptophan	Cyanin-Trp	815.25 / not detected	not observed	-

Table 4.2: Summary of keracyanin-amino acid experiments in 1% formic acid for 12 h at ambient temperature.

Reaction	**Compound**	**$[M+H]^+$ (*m/z*, Da)** ***calcd / detected (t, min)***	**R_t, min, (int., mAU)**	**λ_{max} (nm)**
	Keracyanin (STD)	595.17 / 594.93 (8.785)	8.72 (131)	240, 280, 520
Keracyanin – Arginine	Keracyanin	595.17 / 595.14 (8.832)	8.73 (137)	240, 280, 520
	Keracyanin-Arg	769.28 / 769.51 (26.108)	not observed	-
Keracyanin – Cysteine	Keracyanin	595.17 / 595.15 (8.817)	8.73 (136)	240, 280, 520
	Keracyanin-Cys	716.19 / not detected	not observed	-
Keracyanin – Histidine	Keracyanin	595.17 / 595.08 (8.836)	8.73 (136)	240, 280, 518
	Keracyanin-His	750.24 / not detected	not observed	-
Keracyanin – Lysine	Keracyanin	595.17 / 595.13 (8.829)	8.73 (135)	240, 280, 518
	Keracyanin-Lys	741.27 / 741.47 (11.481)	not observed	-
Keracyanin – Proline	Keracyanin	595.17 / 595.12 (8.800)	8.73 (141)	240, 280, 520
	Keracyanin-Pro	710.23 / not detected	not observed	-
Keracyanin – Serine	Keracyanin	595.17 / 595.12 (8.803)	8.73 (142)	240, 280, 520
	Keracyanin-Ser	700.21 / not detected	not observed	-
Keracyanin – Threonine	Keracyanin	595.17 / 595.18 (8.833)	8.74 (133)	240, 280, 520
	Keracyanin-Thr	714.22 / not detected	not observed	-
Keracyanin – Tryptophan	Keracyanin	595.17 / 594.75 (8.771)	8.76 (2539)[1]	244, 272, 380, 518
	Keracyanin-Trp	799.26 / not detected	not observed	-
	Tryptophan	205.10 / 205.09 (8.941)	8.76 (2539)	244, 272, 380, 518

[1] Keracyanin coeluted with tryptophan

Table 4.3: Summary of cyanin-amino acid experiments in 1% formic acid for 72 h at 37 °C.

Reaction	**Compound**	**$[M+H]^+$ (*m/z*, Da)** ***calcd / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
	Cyanin (STD)	611.16 / 611.17 (7.847)	7.81 (98)	240, 278, 512
Cyanin –	Cyanin	611.16 / 611.17 (7.933)	7.83 (126)	240, 278, 514
Arginine	Cyanin-Arg	785.27 / not detected	not observed	-
Cyanin –	Cyanin	611.16 / 611.10 (7.941)	7.82 (68)	240, 278, 514
Cysteine	Cyanin-Cys	732.18 / not detected	not observed	-
Cyanin –	Cyanin	611.16 / 611.08 (7.868)	7.83 (89)	242, 278, 514
Histidine	Cyanin-His	766.23 / 766.62 (37.167)	not observed	-
Cyanin –	Cyanin	611.16 / 611.17 (7.948)	7.83 (108)	242, 278, 514
Lysine	Cyanin-Lys	757.27 / not detected	not observed	
Cyanin –	Cyanin	611.16 / 611.07 (7.906)	7.82 (117)	242, 278, 514
Proline	Cyanin-Pro	726.22 / 726.58 (36.172)	not observed	-
Cyanin –	Cyanin	611.16 / 611.11 (7.913)	7.83 (128)	242, 278, 514
Serine	Cyanin-Ser	716.20 / 716.30 (24.37)	not observed	-
Cyanin –	Cyanin	611.16 / 611.12 (7.856)	7.82 (109)	240, 278, 514
Threonine	Cyanin-Thr	730.22 / not detected	not observed	-
Cyanin –	Cyanin	611.16 / 611.13 (7.890)	7.81 (109)	242, 278, 514
Tryptophan	Cyanin-Trp	815.25 / not detected	not observed	-

Table 4.4: Summary of keracyanin-amino acid experiments in 1% formic acid for 72 h at 37 °C.

Reaction	**Compound**	**$[M+H]^+$ (*m/z*, Da)** ***calcd / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
	Keracyanin (STD)	595.17 / 595.17 (8.873)	8.74 (324)	240, 280, 518
Keracyanin – Arginine	Keracyanin	595.17 / 595.11 (8.881)	8.74 (295)	240, 280, 520
	Keracyanin-Arg	769.28 / not detected	not observed	-
Keracyanin – Cysteine	Keracyanin	595.17 / 595.06 (8.756)	8.76 (15)	240, 280, 520
	Keracyanin-Cys	716.19 / not detected	not observed	-
Keracyanin – Histidine	Keracyanin	595.17 / 595.06 (8.728)	8.75 (297)	240, 280, 520
	Keracyanin-His	750.24 / not detected	not observed	-
Keracyanin – Lysine	Keracyanin	595.17 / 595.11 (8.696)	8.74 (296)	240, 280, 520
	Keracyanin-Lys	741.27 / 741.39 (11.473)	not observed	-
Keracyanin – Proline	Keracyanin	595.17 / 595.19 (8.752)	8.74 (286)	240, 280, 518
	Keracyanin-Pro	710.23 / not detected	not observed	-
Keracyanin – Serine	Keracyanin	595.17 / 595.04 (8.709)	8.74 (323)	240, 280, 518
	Keracyanin-Ser	700.21 / not detected	not observed	-
Keracyanin – Threonine	Keracyanin	595.17 / 595.30 (8.874)	8.74 (301)	240, 280, 520
	Keracyanin-Thr	714.22 / not detected	not observed	-
Keracyanin – Tryptophan	Keracyanin	595.17 / 595.16 (8.728)	8.78 (2540)	240, 272, 380, 520
	Keracyanin-Trp	799.26 / not detected	not observed	-
	Tryptophan	205.10 / 205.03 (8.941)	8.78 (2540)	240, 272, 380, 520

Table 4.5: Summary of keracyanin-amino acid experiments in McIlvaine buffer pH 6.5 for 12 h at ambient temperature.

Reaction	**Compound**	**$[M+H]^+$ (*m/z*, Da)** ***calcd / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
	Keracyanin (STD)	595.17 / 595.10 (8.892)	8.78 (44)	240, 280, 520
Keracyanin –	Keracyanin	595.17 / 595.10 (8.763)	8.77 (34)	240, 280, 520
Arginine	Keracyanin-Arg	769.28 / not detected	not observed	-
Keracyanin –	Keracyanin	595.17 / 595.15 (8.854)	8.78 (39)	240, 282, 520
Cysteine	Keracyanin-Cys	716.19 / not detected	not observed	-
Keracyanin –	Keracyanin	595.17 / 595.11 (8.866)	8.78 (35)	240, 280, 520
Histidine	Keracyanin-His	750.24 / not detected	not observed	-
Keracyanin –	Keracyanin	595.17 / 595.12 (9.028)	8.78 (34)	240, 280, 518
Lysine	Keracyanin-Lys	741.27 / not detected	not observed	-
Keracyanin –	Keracyanin	595.17 / 595.16 (8.854)	8.77 (39)	240, 280, 520
Proline	Keracyanin-Pro	710.23 / not detected	not observed	-
Keracyanin –	Keracyanin	595.17 / 595.10 (8.942)	8.79 (36)	240, 280, 518
Serine	Keracyanin-Ser	700.21 / not detected	not observed	-
Keracyanin –	Keracyanin	595.17 / 595.08 (8.818)	8.77 (37)	240, 282, 520
Threonine	Keracyanin-Thr	714.22 / not detected	not observed	-
Keracyanin –	Keracyanin	595.17 / 595.12 (8.955)	8.77 (1736)	242, 270, 380, 520
Tryptophan	Keracyanin-Trp	799.26 / not detected	not observed	-
	Tryptophan	205.10 / 205.05 (8.928)	8.77 (1736)	242, 270, 380, 520

4.2 Theoretical findings - anthocyanins

In order to gain deeper understanding of the experimentally observed behavior of the anthocyanins towards the amino acids, thermodynamics of nucleophilic addition reactions with a model flavylium cation has been evaluated using techniques of computational chemistry. As a first step, equilibrium structures of the anthocyanidin, amino acids and nucleophilic addition products were located via energy minimization in a computational model of water solution. Then, vibration frequency calculations were performed, which are necessary in order to compute thermodynamic functions such as enthalpy (*H*), entropy (*S*) and the Gibbs function (*G*) using standard equations of statistical thermodynamics. Knowing the values of these thermodynamic functions for reactants and products, changes in *H, S,* and *G* were calculated for each reaction as a difference between the sum of the values of the corresponding function for products and the sum of the values for reactants. Reactions that were considered in this study are summarized in Figs. 4.1 – 4.4 along with the associated Gibbs energy change ($\Delta_r G$).

According to the computational model, nucleophilic addition reactions between amino acids and the flavylium cation in water are all associated with a significant positive change in the Gibbs function. This change becomes even more dramatic when the fact that some of the amino acids are very likely to exist in the protonated state in the reaction conditions is taken into account. Such a marked

positive change in $\Delta_r G$ for the model reactions is an indication of low stability of the addition products in acidic conditions. Nucleophilic addition reactions with the amino acids were found to be significantly endothermic ($\Delta_r H$ of ca. +40 kcal/mol) and associated with a decrease in entropy ($T\Delta_r S$ of ca. -15 kcal/mol), i.e. unfavorable from both enthalpy and entropy points of view. On the other hand, in presence of an excess of free protons in form of H_3O^+, protonation of the nucleophilic groups in the side chains of *Arg*, *His*, *Lys*, *Pro* and *Trp* is favored by a significant decrease in enthalpy ($\Delta_r H$ ranging from -10 to -40 kcal/mol, i.e. the reactions are exothermic) and associated with only slightly unfavorable change in $T\Delta_r S$ (ca. -1 kcal/mol). Combination of these two factors makes protonation of these five amino acids a facile process associated with a large equilibrium constant, which further decreases their initially low reactivity towards flavylium cations. From the thermodynamic data it is also clear that in acidic conditions covalent adducts between the amino acids and flavylium cation could be easily converted into the initial flavylium cation and protonated amino acids. Even though –OH groups of *Ser* and *Trp* and –SH group of *Cys* are unlikely to be protonated in mildly acidic conditions, these amino acids displayed low reactivity towards the anthocyanins in the experimental study and low affinity for the model flavylium cation in the computational model ($\Delta_r G$ of ca. 50 kcal/mol, $\rightarrow K << 1$).

By extrapolating the findings for the model anthocyanidin onto the anthocyanins from the experimental study, one could conclude that equilibrium of

the addition reactions would be to a very large extent shifted towards the reactants; formation of significant quantities of the covalent adducts in these experimental conditions would be an unlikely process.

According to the computational findings, addition of sulfite to the flavylium cation is an exothermic reaction with $\Delta_r H$ of ca. -3 kcal/mol and due to somewhat unfavorable entropy change (entropy tends to decrease during associative processes) would be a reversible, enthalpy-driven process. Also, the C4 addition product is favored over the C2 addition product. These theoretical predictions are fully supported by the experimental findings (Berke et al., 1998), and this fact confirms the ability of the chosen computational model to give qualitatively correct description of the reactivity of anthocyanins in nucleophilic addition reactions.

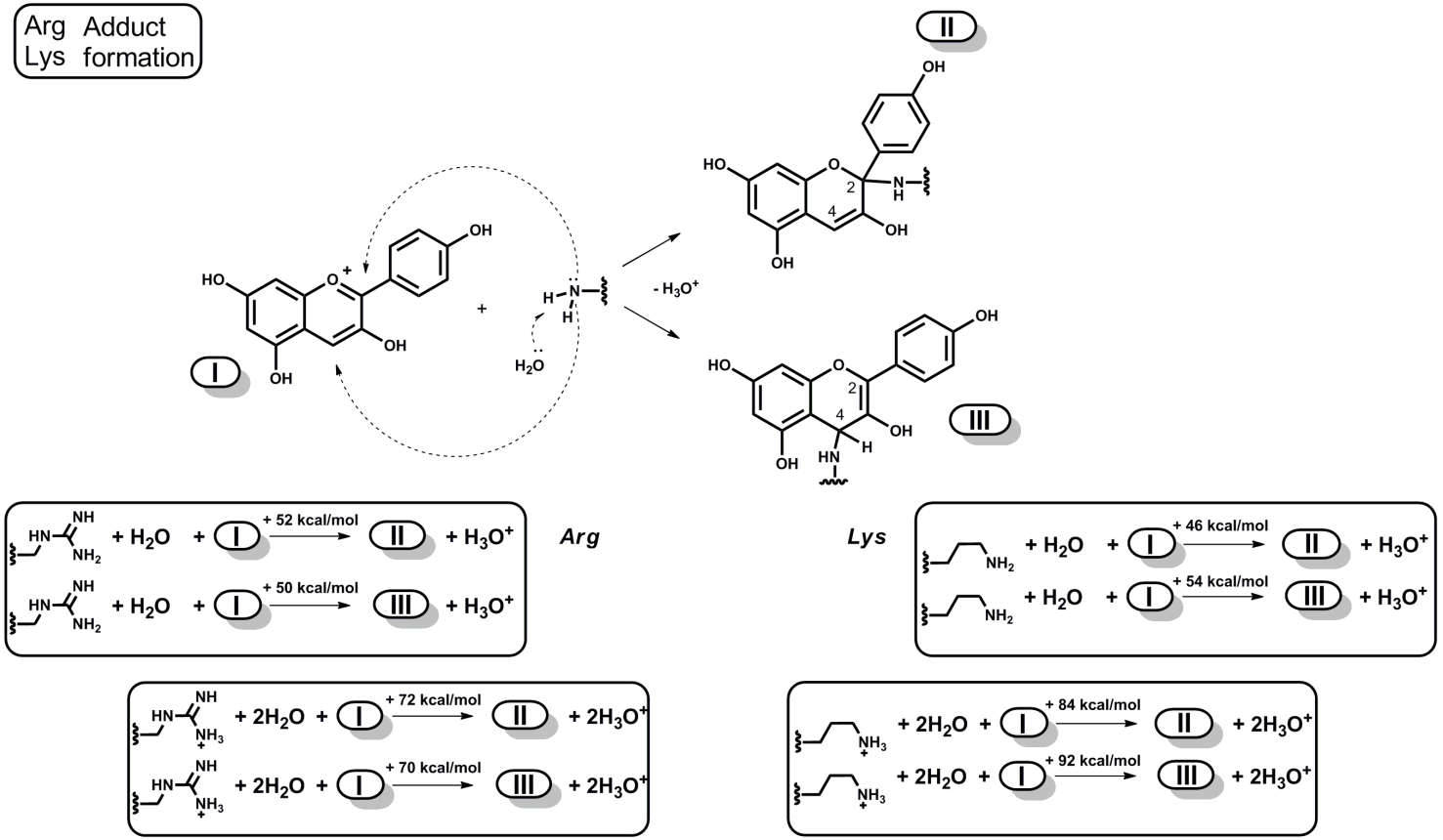


Figure 4.1: Nucleophilic addition of arginine and lysine to the model anthocyanidin and the associated $\Delta_r G$ changes.

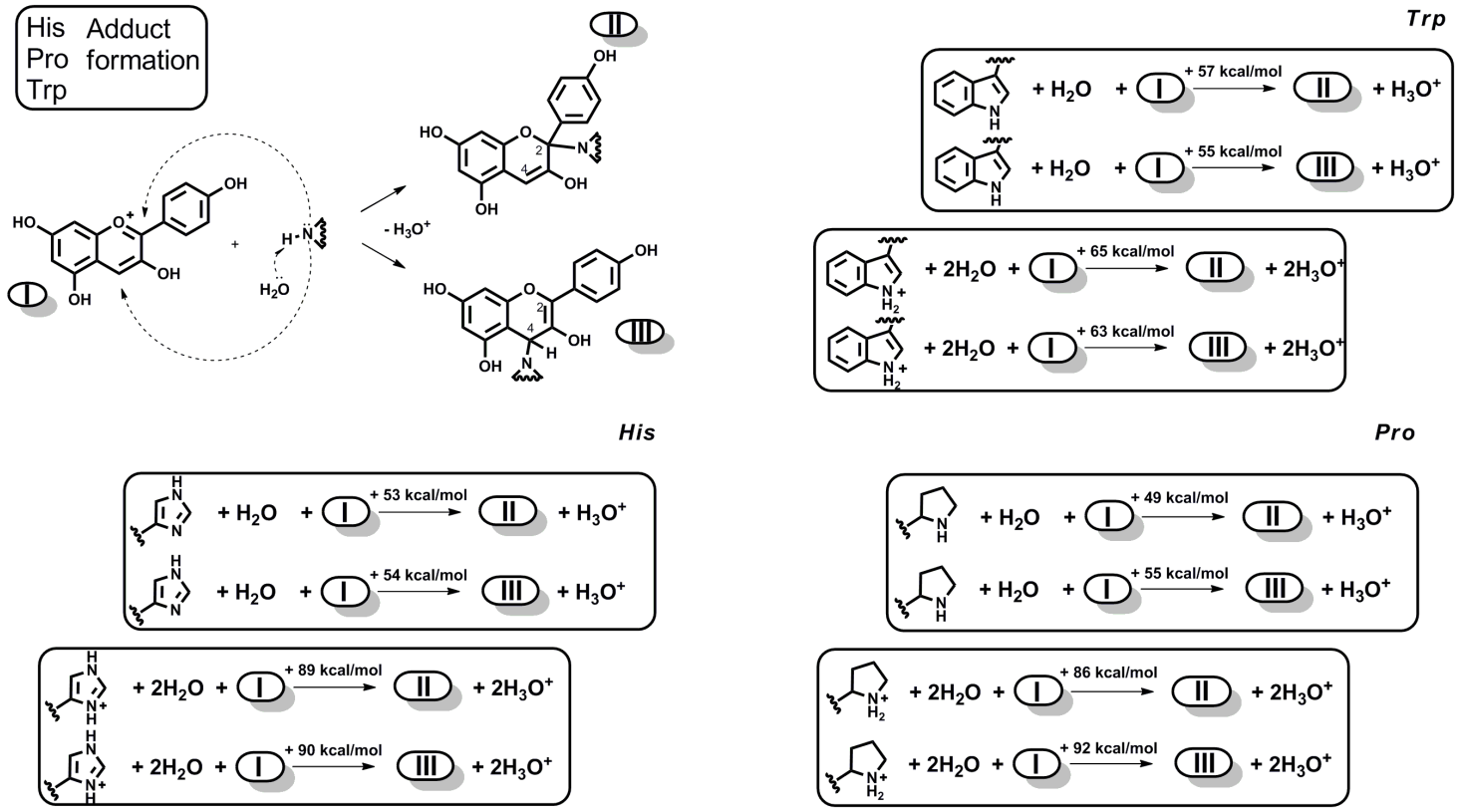


Figure 4.2: Nucleophilic addition of histidine, proline and tryptophan to the model anthocyanidin and the associated $\Delta_r G$ changes.

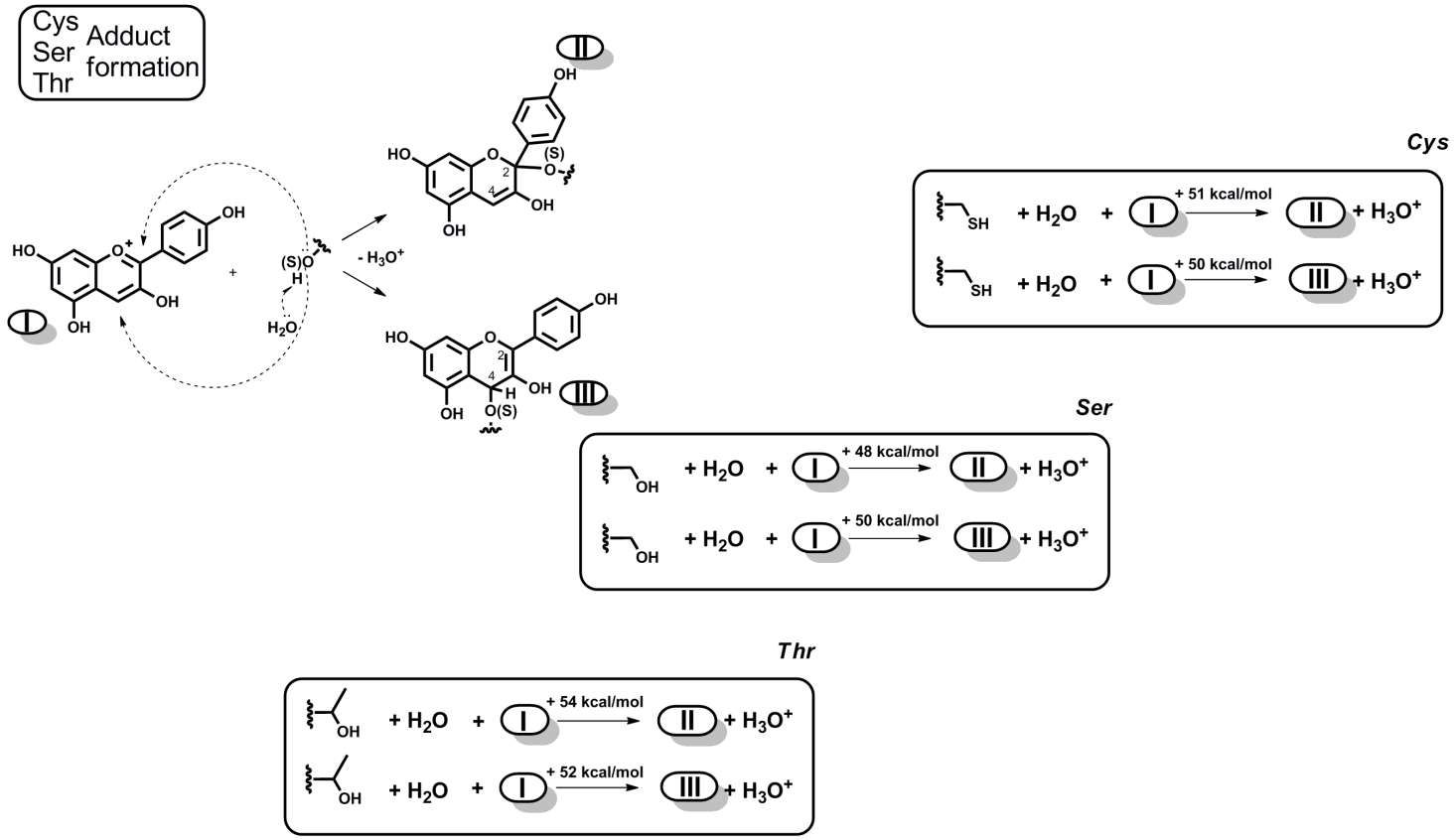


Figure 4.3: Nucleophilic addition of cysteine, serine and threonine to the model anthocyanidin and the associated $\Delta_r G$ changes.

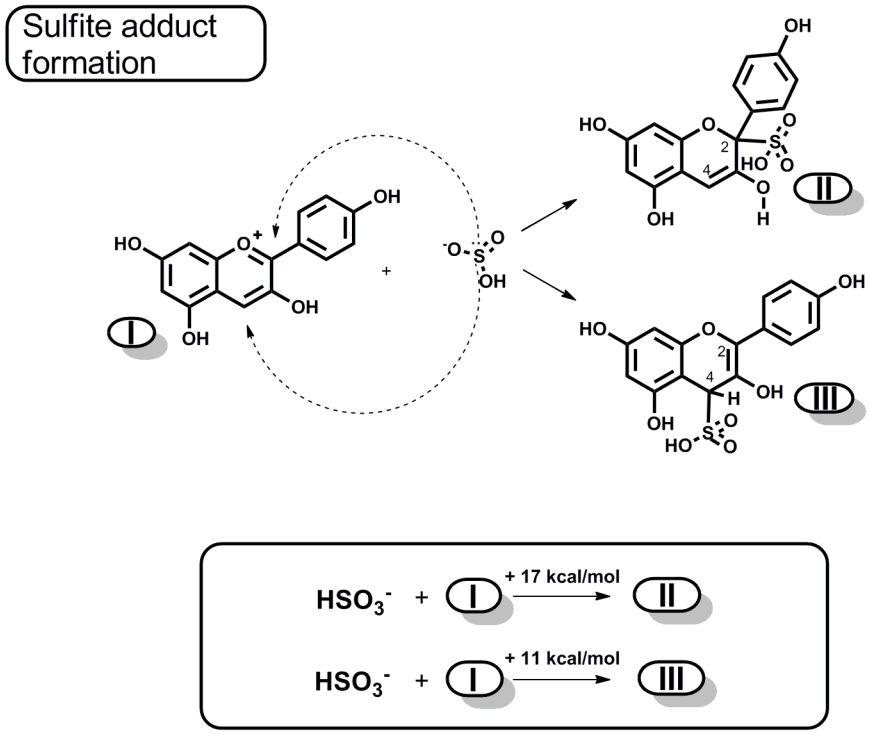


Figure 4.4: Nucleophilic addition of sulfite to the model anthocyanidin and the associated $\Delta_r G$ changes.

4.3 Reactions of the quinones with amino acids

Incubation of the phenolic compounds with amino acids in presence of PPO in 0.1% formic acid solution at ambient temperature resulted in a gradual development of yellow color characteristic of the quinones. After about 1 hour reaction time the color started to darken, and at this point aliquots were withdrawn for HPLC analysis; enzymatic activity in the aliquots was stopped with a drop of 4.5% trifluoroacetic acid. Remaining reaction mixtures were left for additional 11 hours at room temperature. At the end of this time trifluoroacetic acid was added, and another set of aliquots taken for HPLC analysis. Tables 4.6, 4.7, 4.8 and 4.9 contain summary of the HPLC-DAD-MS analysis of the two sets of aliquots taken in this experiment. In the set of samples taken after 1 hour of incubation no significant degradation of the phenolic acids was observed. Protocatechuic acid eluted at ~5.2 min giving a signal with *m/z* 153 in the mass spectrum and UV absorption maxima at 260 and 294 nm. Chlorogenic acid eluted at ~7.3 min giving a signal with *m/z* 353 in the mass spectrum and UV absorption maxima at 246 and 326 nm. Nucleophilic addition products were detected in significant quantities only for reactions with cysteine. In case of protocatechuic acid, the ratio of its peak height in the DAD trace to the adduct peak height was ~10:1 after 1 hour incubation, whereas for chlorogenic acid this ratio was ~2:1. Also, in the sample from the reaction of protocatechuic acid with tryptophan a very small

amount of a substance was detected which gave signal in the mass spectrum at *m/z* close to that of the expected adduct.

In the second set of samples taken after 12 hours of reaction time effects of the enzymatic activity were more evident. In some of the samples which contained chlorogenic acid its hydrolysis products were detected (caffeic and quinic acids) as well as formation of 3- and 4-caffeoylquinic acids which were identified by their *m/z* signals and characteristic elution pattern relative to the peak of 5-caffeoylquinic acid (Schilling et al., 2008). Adducts of cysteine with both phenolic compounds were found in increased quantities relative to the data recorded after 1 hour incubation. Peak height ratio of the phenolic acid to cysteine adduct changed from ~10:1 to ~1:8 for protocatechuic acid and from ~2:1 to ~1:1 for chlorogenic acid. Small quantities of histidine adducts were found for both phenolics and also lysine adduct for protocatechuic acid. However, the quantities of these adducts were negligible in comparison with the quantities of cysteine adducts formed over the same length of time.

Incubation of the phenolics with the amino acids and tenfold increased amount of the enzyme at 37 °C for 1 hour did not have a major effect on formation of the adducts except for cysteine (Tables 4.10 and 4.11). However, more pronounced browning (i.e. quinone polymerization) was evident. Peak height ratio of the phenolic acid to cysteine adduct was found to be roughly 1:2 for both phenolics, i.e. increased temperature and enzyme concentration

intensified adduct formation. Formation of the histidine adduct was recorded for chlorogenic acid yet not for protocatechuic acid. However, traces of a compound which might be the tryptophan adduct were detected in the TIC chromatogram for the latter phenolic acid. Almost complete disappearance of the chlorogenic acid peak was observed in the reactions with arginine, histidine and lysine due to its conversion into degradation products and dimers.

Performing the reactions with tenfold increased amount of the enzyme at room temperature for 12 hours resulted in formation of cysteine, histidine and traces of tryptophan adducts with both phenolics (Tables 4.12 and 4.13). Adducts of the phenolics with tryptophan as well as histidine adduct with chlorogenic acid were only detected in trace amounts in the TIC chromatograms.

Switching the reaction medium from 0.1% formic acid to McIlvaine buffer with pH 6.5 had a drastic effect on stability of the phenolic compounds. After 12 hours at room temperature, chlorogenic acid peak disappeared from all chromatograms. Protocatechuic acid peak was also significantly diminished in all samples yet still visible in the DAD trace (Tables 4.14 and 4.15). The only amino acid which yielded stable adducts in these conditions was cysteine. It is worth mentioning that in all previous experiments both phenolics yielded monoadducts with cysteine; however, according to the MS data, at pH 6.5 protocatechuic acid was converted into the diadduct, whereas chlorogenic acid still yielded the monoadduct.

Table 4.6: Summary of protocatechuic acid-amino acid experiments in 0.1% formic acid. Low PPO (17U), 1 h reaction time at ambient temperature.

Reaction	**Compound**	**$[M]^-$ (*m/z*, Da)** ***calcd / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
	PCA (STD)	153.02, 151.01 / 153.05 (5.359)	5.23 (334)	260, 294
PCA – Arginine	PCA	153.02, 151.01 / 153.06 (5.237)	5.19 (364)	260, 294
	PCA-Arg	325.11, 323.10 / not detected	not observed	-
	PCA-2Arg	497.21, 495.19 / not detected	not observed	-
PCA – Cysteine	PCA	153.02, 151.01 / 153.02 (5.424)	5.25 (329)	260, 294
	PCA-Cys	272.02, 270.01 / 272.06 (3.358)	3.17 (22)	244, 300
	PCA-2Cys	391.03, 389.01 / not detected	not observed	-
PCA – Histidine	PCA	153.02, 151.01 / 153.04 (5.309)	5.25 (349)	260, 294
	PCA-His	306.07, 304.06 / not detected	not observed	-
	PCA-2His	459.13, 457.11 / not detected	not observed	-
PCA – Lysine	PCA	153.02, 151.01 / 153.03 (5.322)	5.25 (350)	260, 294
	PCA-Lys	297.11, 295.10 / not detected	not observed	-
	PCA-2Lys	441.20, 439.18 / not detected	not observed	-
PCA – Proline	PCA	153.02, 151.01 / 153.02 (5.462)	5.24 (372)	260, 294
	PCA-Pro	266.07, 264.06 / not detected	not observed	-
	PCA-2Pro	379.11, 377.09 / not detected	not observed	-
PCA – Serine	PCA	153.02, 151.01 / 153.04 (5.454)	5.26 (337)	260, 294
	PCA-Ser	256.05, 254.04 / not detected	not observed	-
	PCA-2Ser	359.07, 357.05 / not detected	not observed	-
PCA – Threonine	PCA	153.02, 151.01 / 153.03 (5.316)	5.24 (348)	260, 294
	PCA-Thr	270.06, 268.05 / not detected	not observed	-
	PCA-2Thr	387.10, 385.08 / not detected	not observed	-
PCA – Tryptophan	PCA	153.02, 151.01 / 153.03 (5.289)	5.25 (340)	260, 294
	PCA-Trp	355.09, 353.08 / not detected	not observed	-
	PCA-2Trp	557.17, 555.15 / not detected	not observed	-

Table 4.7: Summary of chlorogenic acid-amino acid experiments in 0.1% formic acid. Low PPO (17U), 1 h reaction time at ambient temperature.

Reaction	**Compound**	**$[M]^-$ (*m/z*, Da)** ***calcd / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
	CGA (STD)	353.09, 351.08 / 353.24 (7.458)	7.34 (449)	246, 326
CGA –	CGA	353.09, 351.08 / 353.20 (7.455)	7.29 (779)	246, 326
Arginine	CGA-Arg	525.18, 523.17 / not detected	not observed	-
	CGA-2Arg	697.28, 695.26 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.24 (7.354)	7.30 (627)	246, 326
Cysteine	CGA-Cys	472.09, 470.08 / 472.22 (4.547)	4.55 (299)	250, 326
	CGA-2Cys	591.10, 589.09 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.17 (7.845)	7.29 (505)	246, 326
Histidine	CGA-His	506.14, 504.13 / not detected	not observed	-
	CGA-2His	659.19, 657.17 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.26 (7.379)	7.33 (764)	246, 326
Lysine	CGA-Lys	497.18, 495.17 / not detected	not observed	-
	CGA-2Lys	641.27, 639.25 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.19 (7.448)	7.31 (372)	246, 326
Proline	CGA-Pro	466.13, 464.12 / not detected	not observed	-
	CGA-2Pro	579.18, 577.16 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.10 (7.495)	7.30 (337)	246, 326
Serine	CGA-Ser	456.11, 454.10 / not detected	not observed	-
	CGA-2Ser	559.14, 557.12 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.17 (7.493)	7.29 (368)	246, 326
Threonine	CGA-Thr	470.13, 468.12 / not detected	not observed	-
	CGA-2Thr	587.17 , 585.15 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.21 (7.398)	7.36 (398)	246, 326
Tryptophan	CGA Trp	555.16, 553.15 / 555.08 (11.367)	11.23 (<1)	-
	CGA-2Trp	757.24, 755.22 / not detected	not observed	-

Table 4.8: Summary of protocatechuic acid-amino acid experiments in 0.1% formic acid. Low PPO (17U), 12 h reaction time at ambient temperature.

Reaction	**Compound**	**$[M]^-$ (m/z, Da)** ***calcd / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
PCA – Arginine	PCA	153.02, 151.01 / 153.05 (5.328)	5.24 (19)	260, 294
	PCA-Arg	325.11, 323.10 / not detected	not observed	-
	PCA-2Arg	497.21, 495.19 / not detected	not observed	-
PCA – Cysteine	PCA	153.02, 151.01 / 153.04 (5.394)	5.32 (14)	260, 294
	PCA-Cys	272.02, 270.01 / 272.04 (3.398)	3.21 (104)	246, 300
	PCA-2Cys	391.03, 389.01 / not detected	not observed	-
PCA – Histidine	PCA	153.02, 151.01 / 153.04 (5.346)	5.23 (166)	260, 294
	PCA-His	306.07, 304.06 / 306.11 (2.441)	2.39 (19)	262, 300
	PCA-2His	459.13, 457.11 / 459.60 (17.828)	not observed	-
PCA – Lysine	PCA	153.02, 151.01 / 153.04 (5.409)	5.23 (303)	260, 294
	PCA-Lys	297.11, 295.10 / 297.06 (24.175)	not observed	-
	PCA-2Lys	441.20, 439.18 / 441.30 (33.265)	not observed	-
PCA – Proline	PCA	153.02, 151.01 / 152.98 (5.451)	5.23 (311)	260, 294
	PCA-Pro	266.07, 264.06 / not detected	not observed	-
	PCA-2Pro	379.11, 377.09 / not detected	not observed	-
PCA – Serine	PCA	153.02, 151.01 / 153.04 (5.334)	5.23 (326)	260, 294
	PCA-Ser	256.05, 254.04 / not detected	not observed	-
	PCA-2Ser	359.07, 357.05 / not detected	not observed	-
PCA – Threonine	PCA	153.02, 151.01 / 153.03 (5.384)	5.23 (311)	260, 294
	PCA-Thr	270.06, 268.05 / not detected	not observed	-
	PCA-2Thr	387.10, 385.08 / not detected	not observed	-
PCA – Tryptophan	PCA	153.02, 151.01 / 153.04 (5.422)	5.30 (307)	260, 294
	PCA-Trp	355.09, 353.08 / not detected	not observed	-
	PCA-2Trp	557.17, 555.15 / not detected	not observed	-

Table 4.9: Summary of chlorogenic acid-amino acid experiments in 0.1% formic acid. Low PPO (17U), 12 h reaction time at ambient temperature.

Reaction	**Compound**	**$[M]^-$ (*m/z*, Da)** ***calcd / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
CGA – Arginine	CGA	353.09, 351.08 / 353.21 (7.628)	7.57 (252)	246, 326
	CGA-Arg	525.18, 523.17 / not detected	not observed	-
	CGA-2Arg	697.28, 695.26 / not detected	not observed	-
CGA – Cysteine	CGA	353.09, 351.08 / 353.26 (7.647)	7.53 (567)	246, 326
	CGA-Cys	472.09, 470.08 / 472.20 (4.716)	4.68 (532)	250, 326
	CGA-2Cys	591.10, 589.08 / not detected	not observed	-
CGA – Histidine	CGA	353.09, 351.08 / 353.19 (7.714)	7.56 (94)	246, 326
	CGA-His	506.14, 504.13 / 506.18 (2.142)	not observed	-
	CGA-2His	659.19, 657.17 / not detected	not observed	-
CGA – Lysine	CGA	353.09, 351.08 / 353.18 (7.663)	7.54 (262)	246, 326
	CGA-Lys	497.18, 495.17 / not detected	not observed	-
	CGA-2Lys	641.27, 639.25 / not detected	not observed	-
CGA – Proline	CGA	353.09, 351.08 / 353.21 (7.505)	7.56 (281)	246, 326
	CGA-Pro	466.13, 464.12 / not detected	not observed	-
	CGA-2Pro	579.18, 577.16 / not detected	not observed	-
CGA – Serine	CGA	353.09, 351.08 / 353.16 (7.748)	7.54 (121)	246, 326
	CGA-Ser	456.11, 454.10 / not detected	not observed	-
	CGA-2Ser	559.14, 557.12 / not detected	not observed	-
CGA – Threonine	CGA	353.09, 351.08 / 353.20 (7.689)	7.55 (329)	246, 326
	CGA-Thr	470.13, 468.12 / not detected	not observed	-
	CGA-2Thr	587.17, 585.15 / not detected	not observed	-
CGA – Tryptophan	CGA	353.09, 351.08 / 353.20 (7.09)	7.55 (365)	246, 326
	CGA Trp	555.16, 553.15 / not detected	not observed	-
	CGA-2Trp	757.24, 755.22 / not detected	not observed	-

Table 4.10: Summary of protocatechuic acid-amino acid experiments in 0.1% formic acid. High PPO (170U), 1 h reaction time at 37 °C.

Reaction	**Compound**	**$[M]^-$ (*m/z*, Da)** ***calcd / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
	PCA (STD)	153.02, 151.01 / 153.06 (5.398)	5.37 (332)	260, 294
PCA –	PCA	153.02, 151.01 / 153.04 (5.418)	5.35 (294)	260, 294
Arginine	PCA-Arg	325.11, 323.10 / not detected	not observed	-
	PCA-2Arg	497.21, 495.19 / not detected	not observed	-
PCA –	PCA	153.02, 151.01 / 153.05 (5.391)	5.36 (154)	260, 294
Cysteine	PCA-Cys	272.02, 271.01 / 272.03 (3.384)	3.20 (292)	246, 300
	PCA-2Cys	391.03, 389.01 / 390.91 (7.323)	not observed	-
PCA –	PCA	153.02, 151.01 / 153.02 (5.376)	5.37 (74)	260, 294
Histidine	PCA-His	306.07, 304.06 / not detected	not observed	-
	PCA-2His	459.13, 457.11 / not detected	not observed	-
PCA –	PCA	153.02, 151.01 / 153.04 (5.554)	5.36 (312)	260, 294
Lysine	PCA-Lys	297.11, 295.10 / not detected	not observed	-
	PCA-2Lys	441.20, 439.18 / not detected	not observed	-
PCA –	PCA	153.02, 151.01 / 153.04 (5.370)	5.35 (321)	260, 294
Proline	PCA-Pro	266.07, 264.06 / not detected	not observed	-
	PCA-2Pro	379.11, 377.09 / not detected	not observed	-
PCA –	PCA	153.02, 151.01 / 153.04 (5.390)	5.36 (298)	260, 294
Serine	PCA-Ser	256.05, 254.04 / not detected	not observed	-
	PCA-2Ser	359.07, 357.05 / not detected	not observed	-
PCA –	PCA	153.02, 151.01 / 153.05 (5.403)	5.36 (312)	260, 294
Threonine	PCA-Thr	270.06, 268.05 / not detected	not observed	-
	PCA-2Thr	387.10, 385.08 / not detected	not observed	-
PCA –	PCA	153.02, 151.01 / 153.03 (5.345)	5.36 (318)	260, 294
Tryptophan	PCA-Trp	355.09, 353.08 / 355.15 (12.365)	not observed	-
	PCA-2Trp	557.17, 555.15 / not detected	not observed	-

Table 4.11: Summary of chlorogenic acid-amino acid experiments in 0.1% formic acid. High PPO (170U), 1 h reaction time at 37 °C.

Reaction	**Compound**	**$[M]^-$ (*m/z*, Da)** ***calcd / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
	CGA (STD)	353.09, 351.08 / 353.27 (7.660)	7.60 (451)	246, 326
CGA –	CGA	353.09, 351.08 / 353.14 (7.651)	7.57 (8)	246, 326
Arginine	CGA-Arg	525.18, 523.17 / not detected	not observed	-
	CGA-2Arg	697.28, 695.26 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.25 (7.649)	7.58 (430)	246, 326
Cysteine	CGA-Cys	472.09, 470.08 / 472.19 (4.673)	4.66 (884)	250, 326
	CGA-2Cys	591.10, 589.08 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.10 (7.610)	7.58 (5)	244, 324
Histidine	CGA-His	506.14, 504.13 / 506.12 (2.393)	2.38 (23)	246, 290
	CGA-2His	659.19, 657.17 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.14 (7.659)	7.59 (7)	246, 326
Lysine	CGA-Lys	497.18, 495.17 / not detected	not observed	-
	CGA-2Lys	641.27, 639.25 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.22 (7.712)	7.59 (447)	246, 326
Proline	CGA-Pro	466.13, 464.12 / not detected	not observed	-
	CGA-2Pro	579.18, 577.16 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.23 (7.669)	7.59 (445)	246, 326
Serine	CGA-Ser	456.11, 454.10 / not detected	not observed	-
	CGA-2Ser	559.14, 557.12 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.22 (7.652)	7.59 (450)	246, 326
Threonine	CGA-Thr	470.13, 468.12 / not detected	not observed	-
	CGA-2Thr	587.17, 585.15 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.24 (7.652)	7.59 (493)	246, 326
Tryptophan	CGA Trp	555.16, 553.15 / not detected	not observed	-
	CGA-2Trp	757.24, 755.22 / not detected	not observed	-

Table 4.12: Summary of protocatechuic acid-amino acid experiments in 0.1% formic acid. High PPO (170U), 12 h reaction time at ambient temperature.

Reaction	**Compound**	**$[M]^-$ (m/z, Da)** *calcd / detected (t, min)*	**R_t, min,** *(int., mAU)*	**λ_{max} (nm)**
	PCA (STD)	153.02, 151.01 / 153.04 (5.427)	5.36 (330)	260, 294
PCA – Arginine	PCA	153.02, 151.01 / 153.04 (5.453)	5.36 (311)	260, 294
	PCA-Arg	325.11, 323.10 / not detected	not observed	-
	PCA-2Arg	497.21, 495.19 / not detected	not observed	-
PCA – Cysteine	PCA	153.02, 151.01 / 153.03 (5.376)	5.35 (82)	260, 294
	PCA-Cys	272.02, 270.01 / 272.03 (3.368)	3.20 (362)	246, 300
	PCA-2Cys	391.03, 389.01 / 391.02 (2.906)	not observed	-
PCA – Histidine	PCA	153.02, 151.01 / 153.02 (5.399)	5.36 (130)	260, 294
	PCA-His	306.07, 304.06 / 306.05 (2.376)	2.25 (17)	244, 294
	PCA-2His	459.13, 457.11 / not detected	not observed	-
PCA – Lysine	PCA	153.02, 151.01 / 153.04 (5.407)	5.36 (299)	260, 294
	PCA-Lys	297.11, 295.10 / 279.05 (15.54)	not observed	-
	PCA-2Lys	441.20, 439.18 / not detected	not observed	-
PCA – Proline	PCA	153.02, 151.01 / 153.04 (5.397)	5.36 (324)	260, 294
	PCA-Pro	266.07, 264.06 / not detected	not observed	-
	PCA-2Pro	379.11, 377.09 / not detected	not observed	-
PCA – Serine	PCA	153.02, 151.01 / 153.04 (5.363)	5.35 (331)	260, 294
	PCA-Ser	256.05, 254.04 / not detected	not observed	-
	PCA-2Ser	359.07, 357.05 / not detected	not observed	-
PCA – Threonine	PCA	153.02, 151.01 / 153.04 (5.424)	5.36 (329)	260, 294
	PCA-Thr	270.06, 268.05 / not detected	not observed	-
	PCA-2Thr	387.10, 385.08 / not detected	not observed	-
PCA – Tryptophan	PCA	153.02, 151.01 / 153.04 (5.386)	5.36 (321)	260, 294
	PCA-Trp	355.09, 353.08 / 355.14 (12.708)	not observed	-
	PCA-2Trp	557.17, 555.15 / 557.63 (24.277)	not observed	-

Table 4.13: Summary of chlorogenic acid-amino acid experiments in 0.1% formic acid. High PPO (170U), 12 h reaction time at ambient temperature.

Reaction	**Compound**	**$[M]^-$ (*m/z*, Da)** ***calcld / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
	CGA (STD)	353.09, 351.08 / 353.24 (7.508)	7.55 (1171)	246, 326
CGA –	CGA	353.09, 351.08 / 353.18 (7.595)	7.49 (95)	244, 326
Arginine	CGA-Arg	525.18, 523.17 / not detected	not observed	-
	CGA-2Arg	697.28, 695.26 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.23 (7.540)	7.55 (354)	246, 326
Cysteine	CGA-Cys	472.09, 470.08 / 472.21 (4.795)	4.63 (892)	250, 326
	CGA-2Cys	591.10, 589.08 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.08 (7.516)	7.55 (9)	246, 326
Histidine	CGA-His	506.14, 504.13 / 506.25 (2.456)	not observed	-
	CGA-2His	659.19, 657.17 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.14 (7.534)	7.55 (41)	246, 326
Lysine	CGA-Lys	497.18, 495.17 / not detected	not observed	-
	CGA-2Lys	641.27, 639.25 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.18 (7.641)	7.49 (178)	246, 326
Proline	CGA-Pro	466.13, 464.12 / not detected	not observed	-
	CGA-2Pro	579.18, 577.16 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.20 (7.644)	7.49 (156)	246, 326
Serine	CGA-Ser	456.11, 454.10 / not detected	not observed	-
	CGA-2Ser	559.14, 557.12 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.18 (7.649)	7.50 (244)	246, 326
Threonine	CGA-Thr	470.13, 468.12 / not detected	not observed	-
	CGA-2Thr	587.17, 585.15 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / 353.19 (7.449)	7.49 (199)	246, 326
Tryptophan	CGA-Trp	555.16, 553.15 / 555.03 (9.129)	not observed	-
	CGA-2Trp	757.24, 755.22 / not detected	not observed	-

Table 4.14: Summary of protocatechuic acid-amino acid experiments in McIlvaine buffer pH 6.5. High PPO (170U), 12 h reaction time at ambient temperature.

Reaction	**Compound**	**$[M]^-$ (*m/z*, Da)** ***calcd / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
	PCA (STD)	153.02, 151.01 / 152.98 (6.174)	5.91 (24)	260, 294
PCA –	PCA	153.02, 151.01 / 153.00 (6.069)	5.89 (3)	258, 292
Arginine	PCA-Arg	325.11, 323.10 / not detected	not observed	-
	PCA-2Arg	497.21, 495.19 / not detected	not observed	-
PCA –	PCA	153.02, 151.01 / not detected	not observed	260, 294
Cysteine	PCA-Cys	272.02, 270.01 / not detected	not observed	-
	PCA-2Cys	391.03, 389.01 / 391.06 (3.146)	3.09 (354)	238, 300
PCA –	PCA	153.02, 151.01 / 153.03 (5.978)	5.91 (10)	260, 294
Histidine	PCA-His	306.07, 304.06 / not detected	not observed	-
	PCA-2His	459.13, 457.11 / not detected	not observed	-
PCA –	PCA	153.02, 151.01 / 153.02 (6.122)	5.99 (3)	260, 294
Lysine	PCA-Lys	297.11, 295.10 / not detected	not observed	-
	PCA-2Lys	441.20, 439.18 / not detected	not observed	-
PCA –	PCA	153.02, 151.01 / 153.00 (5.989)	5.89 (25)	260, 294
Proline	PCA-Pro	266.07, 264.06 / not detected	not observed	-
	PCA-2Pro	379.11, 377.09 / not detected	not observed	-
PCA –	PCA	153.02, 151.01 / 153.02 (5.976)	5.90 (14)	260, 294
Serine	PCA-Ser	256.05, 254.04 / not detected	not observed	-
	PCA-2Ser	359.07, 357.05 / not detected	not observed	-
PCA –	PCA	153.02, 151.01 / 152.96 (5.939)	5.90 (15)	260, 294
Threonine	PCA-Thr	270.06, 268.05 / not detected	not observed	-
	PCA-2Thr	387.10, 385.08 / not detected	not observed	-
PCA –	PCA	153.02, 151.01 / 153.01 (6.091)	5.98 (1)	260, 294
Tryptophan	PCA-Trp	355.09, 353.08 / not detected	not observed	-
	PCA-2Trp	557.17, 555.15 / not detected	not observed	-

Table 4.15: Summary of chlorogenic acid-amino acid experiments in McIlvaine buffer pH 6.5. High PPO (170U), 12 h reaction time at ambient temperature.

Reaction	**Compound**	**$[M]^-$ (m/z, Da)** ***calcd / detected (t, min)***	**R_t, min,** ***(int., mAU)***	**λ_{max} (nm)**
	CGA (STD)	353.09, 351.08 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / not detected	not observed	-
Arginine	CGA-Arg	525.18, 523.17 / not detected	not observed	-
	CGA-2Arg	697.28, 695.26 / 697.23 (10.752)	not observed	-
CGA –	CGA	353.09, 351.08 / 353.06 (4.491)	not observed	-
Cysteine	CGA-Cys	472.09, 470.08 / 472.21 (6.040)	5.87 (612)	250, 326
	CGA-2Cys	591.10, 589.08 / 591.09 (4.211)	not observed	-
CGA –	CGA	353.09, 351.08 / not detected	not observed	-
Histidine	CGA-His	506.14, 504.13 / not detected	not observed	-
	CGA-2His	659.19, 657.17 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / not detected	not observed	-
Lysine	CGA-Lys	497.18, 495.17 / not detected	not observed	-
	CGA-2Lys	641.27, 639.25 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / not detected	not observed	-
Proline	CGA-Pro	466.13, 464.12 / not detected	not observed	-
	CGA-2Pro	579.18, 577.16 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / not detected	not observed	-
Serine	CGA-Ser	456.11, 454.10 / not detected	not observed	-
	CGA-2Ser	559.14, 557.12 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / not detected	not observed	-
Threonine	CGA-Thr	470.13, 468.12 / not detected	not observed	-
	CGA-2Thr	587.17 , 585.15 / not detected	not observed	-
CGA –	CGA	353.09, 351.08 / not detected	not observed	-
Tryptophan	CGA Trp	555.16, 553.15 / not detected	not observed	-
	CGA-2Trp	757.24, 755.22 / not detected	not observed	-

4.4 Theoretical findings - quinones

In the same way as it had been done for anthocyanins, molecular modeling was applied to nucleophilic addition reactions between the amino acids and model *o*-benzoquinone. Protocatechuic acid quinone was chosen a model substrate, and thermodynamics of nucleophilic addition reactions was evaluated for the three most likely pathways (Fig. 4.5 - 4.7). Again, as a first step, equilibrium structures of the quinone, amino acids and nucleophilic addition products were located in water solution via energy minimization procedure. Then, vibration frequency analysis was performed in order to evaluate enthalpy (*H*), entropy (*S*) and the Gibbs function (*G*) for each molecular structure. Having obtained the values of these thermodynamic functions for reactants and products, $\Delta_r G$ could be calculated for each reaction as a difference between the sum of the Gibbs energies of products and the sum of the Gibbs energies of reactants.

For all amino acids considered in this study, $\Delta_r G$ change in the addition reactions with the model quinone was in the range of 1-11 kcal/mol. This indicates that in favorable conditions nucleophilic addition of the amino acids to *o*-quinones can take place. However, accumulation of significant quantities of the addition products in the reaction mixtures will be subject to certain conditions, including the following:

(a) sufficient stability of the nucleophilic addition products towards further chemical and enzymatic transformations;

(b) rate of the nucleophilic addition reaction should be close to or higher than rates of any competing reactions involving *o*-quinone;

(c) availability of the reactive form of amino acid in the reaction conditions.

For instance, if we consider nucleophilic addition reactions between cysteine and the model quinone, they are characterized by the lowest $\Delta_r G$ among the amino acids in this study, with the pathway leading to the 2-Cys derivative being the most favorable (only +1 kcal/mol). Incidentally, it has been shown by ^{1}H NMR that addition of cysteine to chlorogenic acid quinone led to the 2-Cys conjugate as the major product (Richard et al., 1991). Sulfhydryl group of cysteine is not susceptible to protonation in mildly acidic conditions, which makes it available for nucleophilic attack on the quinone. Electron density around the sulfur atom is much more diffuse than the compact electron pairs of the oxygen and nitrogen atoms. This fact may have a lowering effect on the activation barrier of the reactions involving sulfur nucleophiles. In addition, it has been shown that cysteinyl phenolic acids are not substrates for PPO (Sanada et al., 1976); hence, once they are formed, they do not undergo further enzymatic transformations, which is how cysteine blocks enzymatic browning. Therefore, in case of cysteine there is a combination of several favorable factors which make its nucleophilic addition to *o*-quinones a highly facile process.

Amino acids that conjugate with *o*-quinones via the nitrogen atom do not satisfy some of the abovementioned conditions. Firstly, *Arg*, *His*, *Lys* and *Pro* are

susceptible to protonation in acidic conditions (reactions are highly exothermic, with $\Delta_r G$ of ca. -30 kcal/mol). Tryptophan, however, is less susceptible to protonation ($\Delta_r G$ of ca. -8 kcal mol). From Fig. 4.5 and 4.6 it is obvious that protonation effectively deactivates nucleophilic centers of the amino acids in the reactions with the quinone: whereas $\Delta_r G$ of the reactions between free amino acids and the quinone is between 5 and 11 kcal/mol, same reactions involving protonated amino acids have $\Delta_r G$ of 30-50 kcal/mol (except for *Trp*). Also, it is not known whether *o*-dihydroxyaminophenols can be a substrate for PPO and enter the cycle of enzymatically induced transformations, yet it is common knowledge that hydroxyaminophenols and quinone imines are quite unstable in presence of molecular oxygen and readily undergo chemical reactions leading to high molecular weight compounds. Therefore, at least two unfavorable factors work against accumulation of significant quantities of *o*-quinone adducts with *Arg*, *His*, *Lys*, *Pro* and *Trp*: (a) protonation of the nucleophilic centers of the amino acids in acidic conditions; and (b) susceptibility of the adducts towards further transformations.

As for serine and threonine, they are not easily protonated in mildly acidic solutions, and the changes in $\Delta_r G$ for the reactions with the model *o*-quinone are also quite favorable, especially for serine. However, for reasons that cannot be identified with the available data, these amino acids did not yield significant quantities of the addition products in the reactions with oxidized *o*-diphenols.

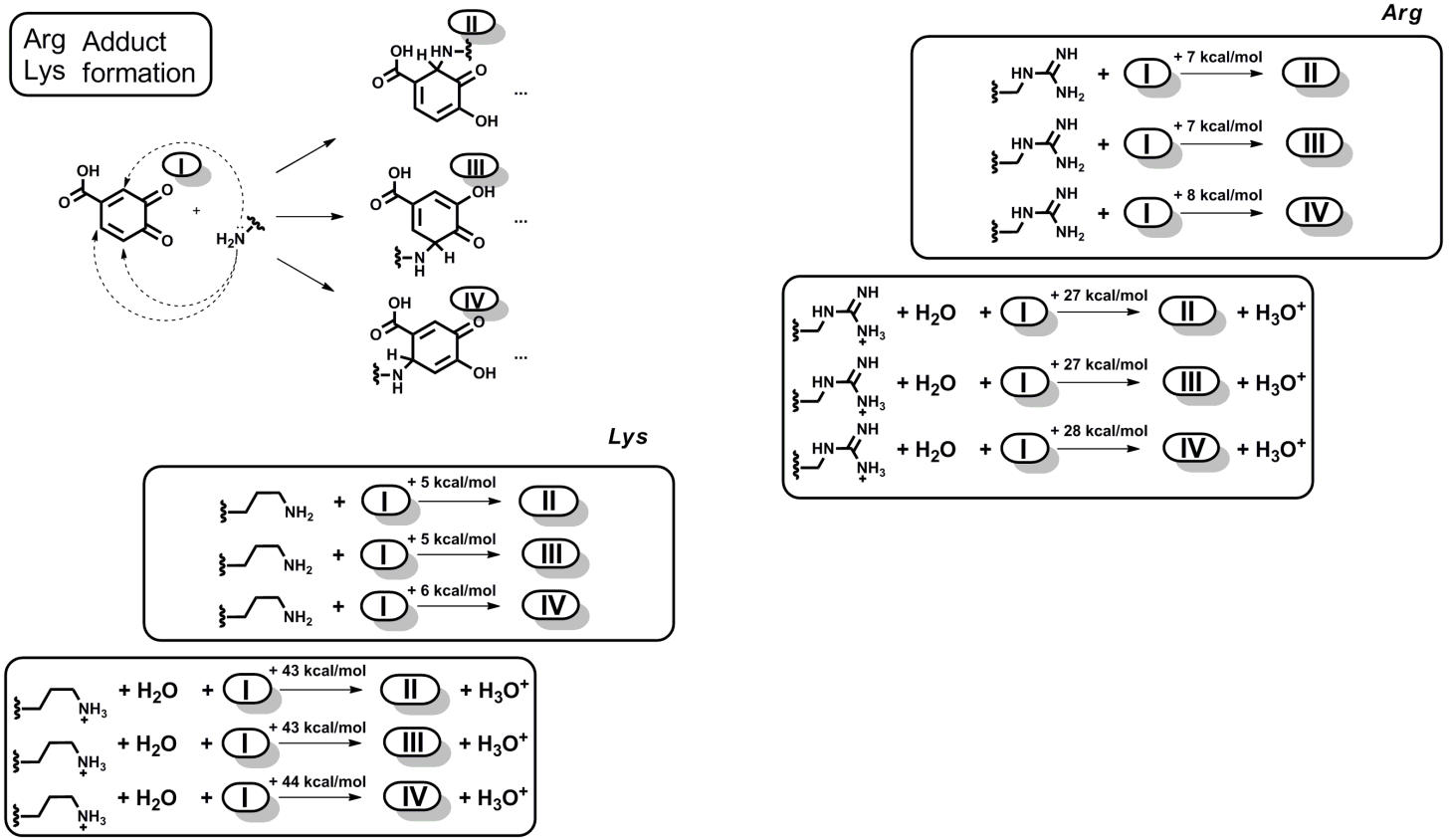


Figure 4.5: Nucleophilic addition of arginine and lysine to protocatechuic acid quinone and the associated $\Delta_r G$ changes.

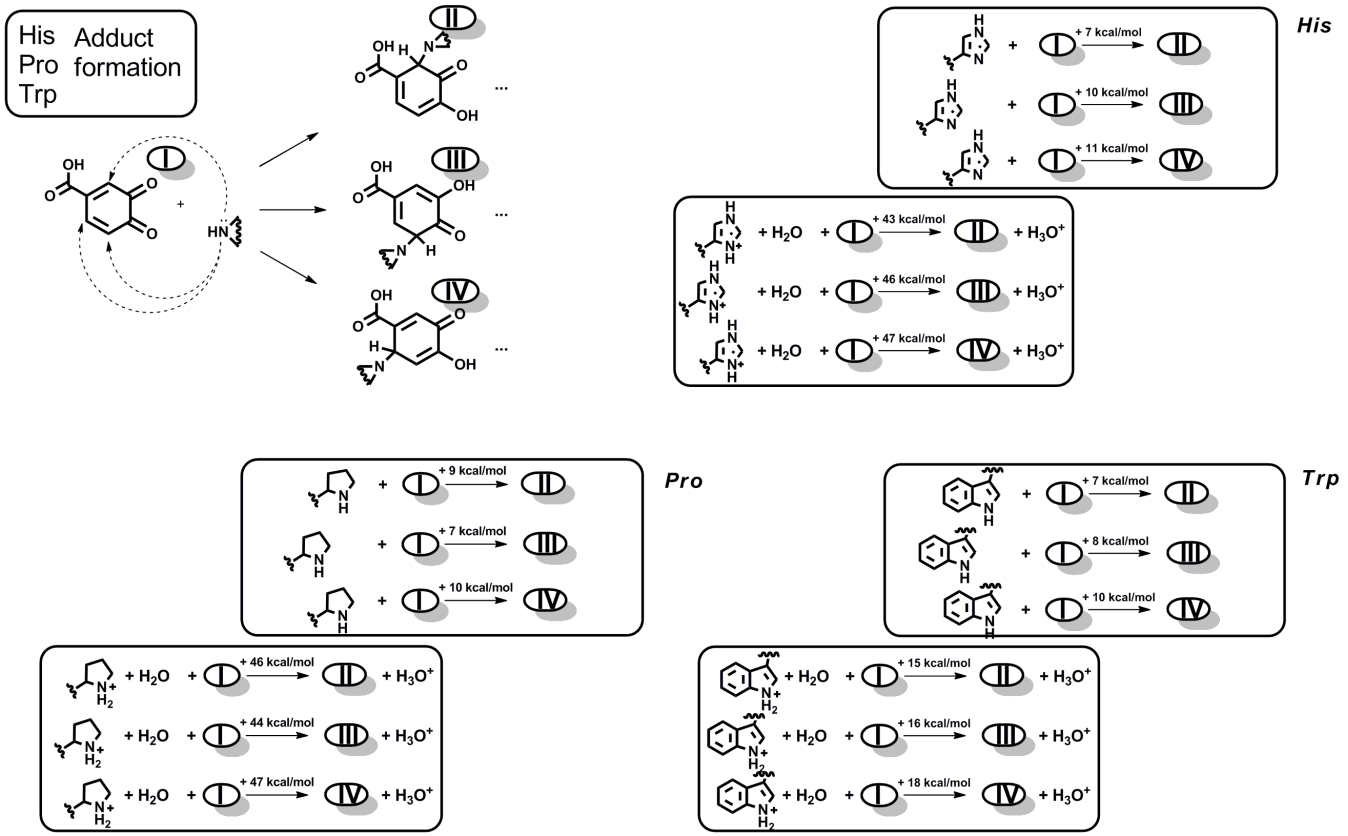


Figure 4.6: Nucleophilic addition of histidine, proline and tryptophan to protocatechuic acid quinone and the associated $\Delta_r G$ changes.

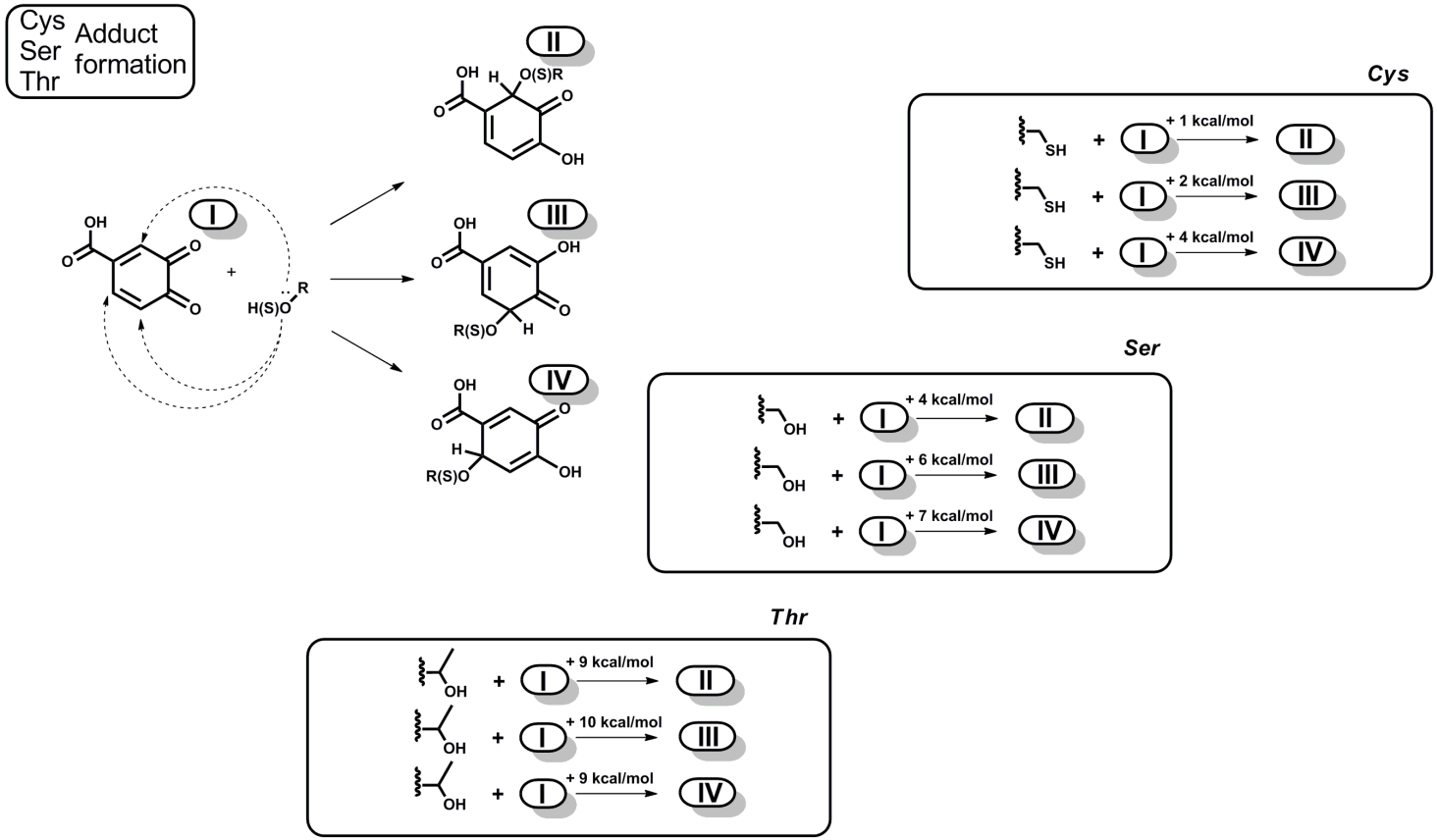


Figure 4.7: Nucleophilic addition of cysteine, serine and threonine to protocatechuic acid quinone and the associated $\Delta_r G$ changes.

4.3 Stability of β–carotene during enzymatic browning of fruit purees

A chromatogram of the β-carotene standard containing three stereoisomers is shown in Fig. 4.8.

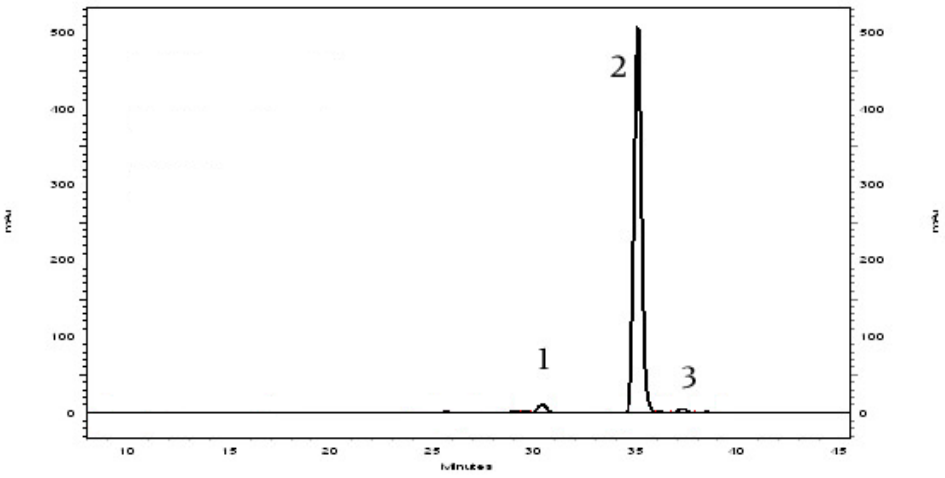


Figure 4.8: Separation of β-carotene stereoisomers by HPLC. From left to right: 1 - 13-*cis*-β-carotene; 2 - all-*trans*-β-carotene; 3 - 9-*cis*-β-carotene.

Here, good baseline separation was obtained for the three stereoisomers of interest. The elution pattern of these three compounds was in agreement with the results obtained in previous studies (Marx et al., 2000). Confirmation of the identity of the individual peaks was also done by analyzing their UV-VIS spectra and comparing them with those of the standards. Fig. 4.9 shows the spectra of the three β-carotene stereoisomers.

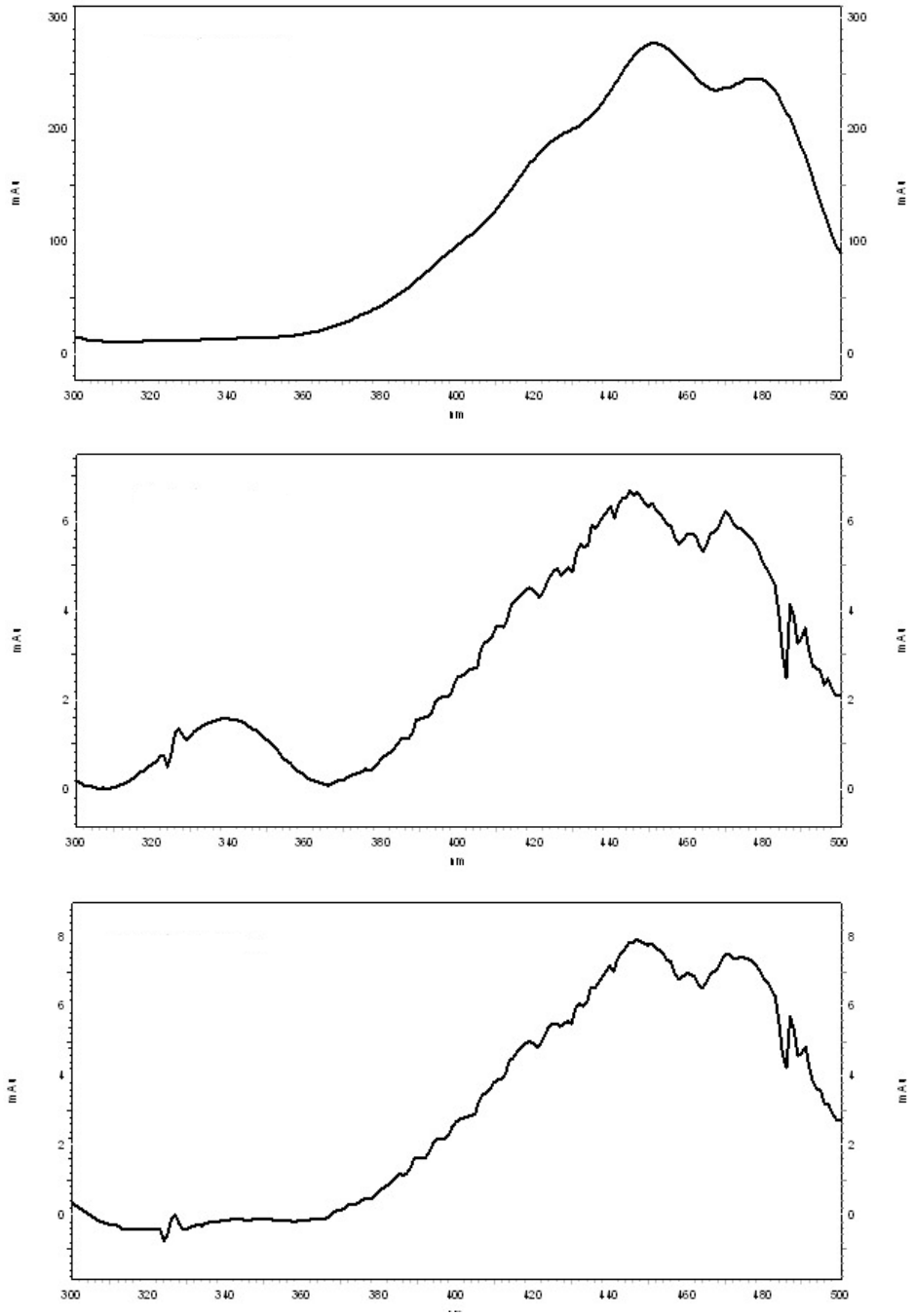


Figure 4.9: UV-VIS spectra of β-carotene stereoisomer standards. From top to bottom: all-*trans*-β-carotene, 13-*cis*-β-carotene, 9-*cis*-β-carotene.

The isomers of β-carotene can be distinguished from one another by the size of the so-called *cis* peak in the region between 300 and 360 nm. The closer the *cis* double bond to the centre of the molecule, the greater the size of the *cis*-peak. In Fig. 4.9, 13-*cis*-β-carotene has the greatest *cis*-peak, followed by smaller *cis*-peak for 9-*cis*-β-carotene, and no *cis*-peak in the spectrum of all-*trans*-β-carotene.

Results of our experiments on apricot puree browning (summarized in Fig. 4.10) do not confirm *trans*-*cis* β-carotene isomerization reported by De Rigal et al. (2000) yet are in line with the observations of Radi et al. (1997) for *trans*-β-carotene. Concentrations of the three isomers of β-carotene in the hexane extracts from the browned apricot purees were generally found to be lower than concentrations of the same isomers in the extracts from non-browned purees. Since both browned and non-browned purees were prepared, incubated, and processed under the same conditions, the observed behavior of β-carotene isomers may be attributed to their degradation during enzymatic browning.

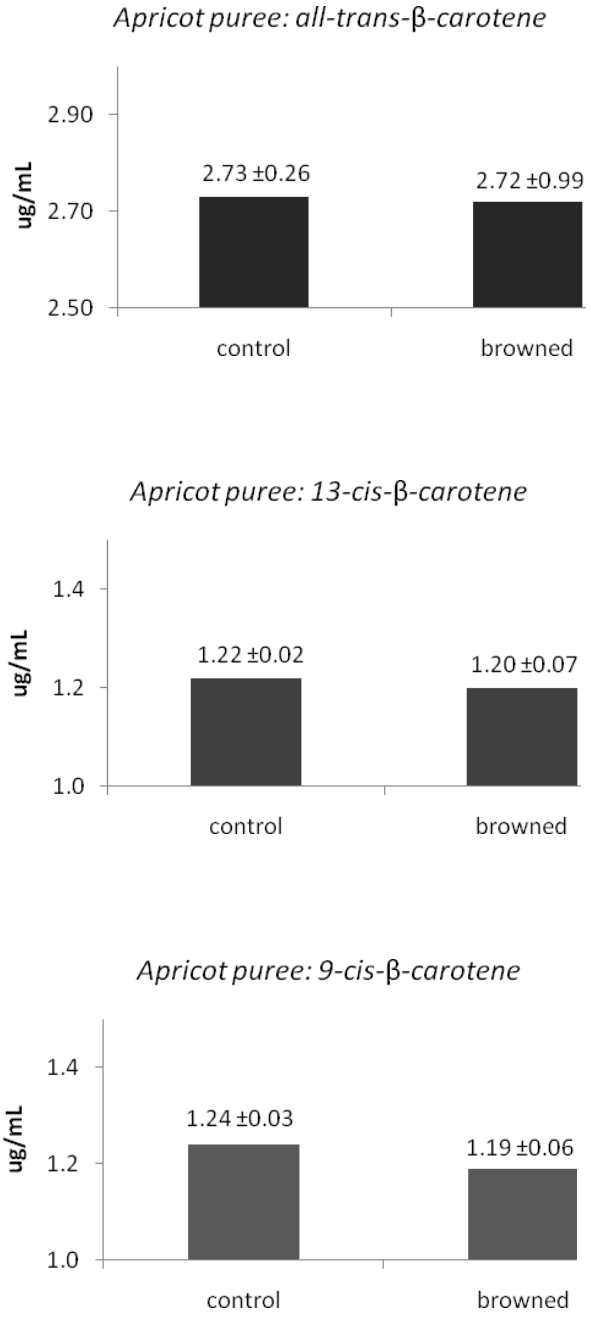


Figure 4.10: Effect of apricot puree browning on β-carotene stereoisomers. Concentration on the vertical axis is that of the hexane extract (see experimental section). Standard deviation was determined using data from four experiments.

Apple and pear tissue contain both phenolic compounds and PPO yet do not contain β-carotene. If there were a simple relationship between the degree of enzymatic browning and degradation of β-carotene, addition of apple or pear tissue to apricot puree would result in even more pronounced browning and increased rate of β-carotene degradation. However, results obtained in apple-apricot and pear-apricot browning experiments did not confirm this (Fig. 4.11 and 4.12).

Even though enzymatic browning visually was more intensive in apple- and pear-apricot purees than in pure apricot puree, concentrations of all three isomers of β-carotene in the hexane extracts from browned apple- and pear-apricot purees were always found to be higher than concentrations in the extracts from non-browned purees. Since browning experiments exhibited good reproducibility both in case of apple-apricot and pear-apricot puree, these results, albeit somewhat controversial, could not be dismissed on the ground of statistical insignificance. It might be that as a result of intensive browning in the apple- and pear-apricot purees β-carotene in the apricot tissue became more readily available for extraction with organic solvents than in pure apricot puree. In such case degradation of β-carotene caused by enzymatic browning could have been masked by extraction of additional β-carotene which was less accessible to organic solvents in pure apricot puree.

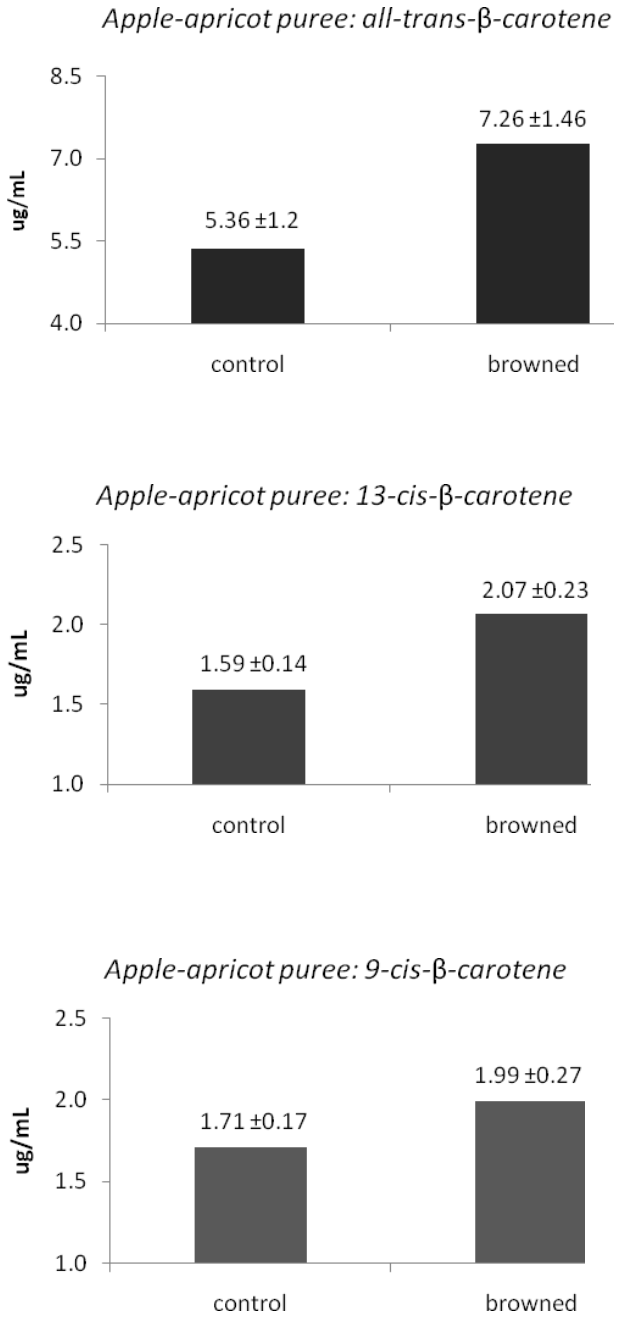


Figure 4.11: Effect of apple-apricot browning on β-carotene stereoisomers. Concentration on the vertical axis is that of the hexane extract (see experimental section). Standard deviation was determined using data from six experiments.

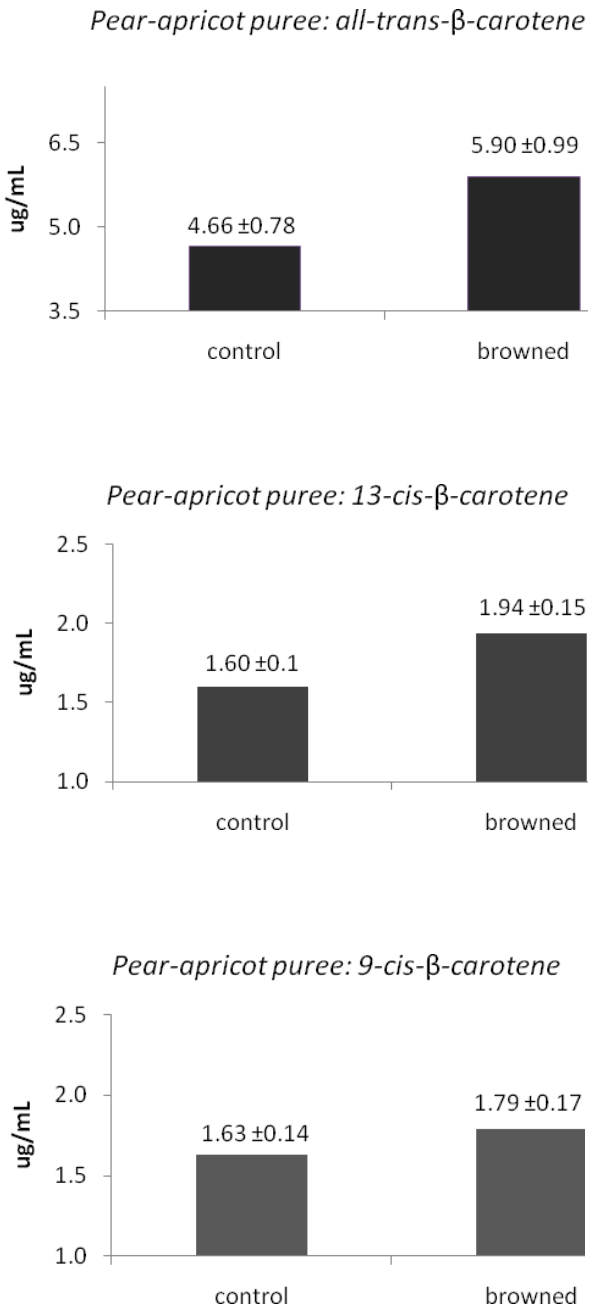


Figure 4.12: Effect of pear-apricot puree browning on β-carotene stereoisomers. Concentration on the vertical axis is that of the hexane extract (see experimental section). Standard deviation was determined using data from six experiments.

Conclusions

In this work we have investigated reactivity of eight amino acids in nucleophilic addition reactions with anthocyanins and oxidized *o*-diphenols. Despite the fact that flavylium cation form of anthocyanins possesses electrophilic properties and is known to react with nucleophiles such as sulfite and water, incubation of mono- and diglucosides of cyanidin with the amino acids did not result in formation of measurable quantities of the addition products. By applying molecular modeling to nucleophilic addition reactions between the amino acids and a model flavylium cation, we have estimated changes in the Gibbs function associated with these reactions ($\Delta_r G$). It was found that all of the considered reactions with amino acids are characterized by large, positive $\Delta_r G$ meaning that equilibrium will be shifted to a high extent towards reactants. Also, nucleophilic addition products with *Arg, His, Lys, Pro* and *Trp* are predicted to be unstable in acidic conditions due to the fact that their dissociation into the reactants is facilitated by a significant negative $\Delta_r G$ of the amino acid protonation reactions. Therefore, in our opinion, at pH < 7 nucleophilic addition reactions between anthocyanins and side chains of the amino acids are unlikely to cause anthocyanin bleaching similar to that induced by sulfite. Judging by the low reactivity of the amino acids towards anthocyanins, it can also be concluded that on a short-term contact (12 – 72 h) at ambient temperature formation of significant quantities of

anthocyanin-protein adducts via nucleophilic addition is unlikely. However, at elevated temperatures cysteine may induce anthocyanin bleaching most likely caused by peroxide generated during its autoxidation in presence of the molecular oxygen, similarly to what is documented for ascorbic acid.

Of the eight amino acids considered in this study, only cysteine readily reacted with enzymatically oxidized protocatechuic and chlorogenic acids in mildly acidic conditions. Small amounts of nucleophilic adducts were also formed in reactions with histidine. Other amino acids only generated trace amounts of the adducts detected by mass spectrometry, or none at all. Increasing pH of the reaction medium to 6.5 did not result in increased formation of nucleophilic addition products, yet caused almost complete degradation of the initial phenolic compounds after 12 hours incubation time. Application of molecular modeling to nucleophilic addition reactions between the amino acids and protocatechuic acid quinone revealed that the reactions are characterized by only slightly positive $\Delta_r G$, which indicates that in favorable circumstances these reactions can take place. In fact, cysteine, which is the only amino acid that yielded significant quantities of the adducts, is predicted by the model to be the most reactive of the amino acids towards *o*-quinones, which is confirmed by previous experimental reports. Accumulation of significant quantities of nucleophilic adducts in reactions of the remaining seven amino acids with *o*-quinones is likely to be disadvantaged by several unfavorable factors. Among these factors the most

obvious are deactivation of the amino acids by protonation, high activation barriers of the addition reactions, existence of competing reactions involving *o*-quinones and susceptibility of the adducts towards further chemical and enzymatic transformations. Thus, according to the model, formation of protein-polyphenol conjugates through the sulfhydryl group of cysteine is a likely process; this conclusion is in perfect agreement with previous experimental reports. However, involvement of other amino acids in these reactions will be dependent on the physicochemical properties and chemical composition of the reaction medium among other factors.

We have also studied effects of enzymatic browning in apricot, apple-apricot, and pear-apricot purees on their β-carotene content and stereoisomeric profile. *Trans-cis* isomerization of β-carotene during enzymatic browning of apricot puree as documented by De Rigal et al. (2000) was not confirmed in our experiments. Instead, our results indicate that β-carotene degradation took place. This observation is in line with previous results of Radi et al. (1997) for *trans*-β-carotene. We have also found that not only *trans*-β-carotene, but also its *cis*-isomers degraded during enzymatic browning of apricot puree.

Browning of apple-apricot and pear-apricot purees while visually more intensive due to additional phenolic compounds and PPO introduced with apple and pear tissue yielded unexpected results. Increased amounts of all three β-carotene isomers were extracted from browned mixed fruit purees in comparison

with the non-browned ones. Additional investigations are needed to fully understand the nature of this effect.

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