



Stabilization of anthocyanin and skullcap flavone complexes – Investigations with computer simulation and experimental methods

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ABSTRACT

We examined the stabilization of anthocyanins with flavones from the practical and theoretical perspective. The influence of addition of skullcap flavones, heating to 50 °C, and 12 day storage time (in the presence and absence of light) on the stability of anthocyanins in honeysuckle concentrates was investigated experimentally. Theoretical study was conducted with molecular dynamics methods in a model system, preceded by simulated annealing and thermalization. By the methods of the computer simulation of the copigmentation process we determined the sites responsible for the stabilization of a cyanidin quinoidal base–baicalin complex. We revealed both direct and water-mediated hydrogen bondings that keep the lamellar stacking structure of these molecules in the bounded form in water medium. The stacking occurs also due to hydrophobic interactions of the rings of both molecules. The experimental part of the study confirmed the effectiveness of anthocyanins stabilization in a concentrate of honeysuckle with the use of skullcap flavones.

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1. Introduction

There are known over 600 molecular structures of anthocyanins (Konczak & Zhang, 2004) and 23 anthocyanidins, whilst barely six of them are widespread in nature: cyanidin, pelargonidin, delphinidin, peonidin, petunidin, and malvidin (Fig. 1) (Castañeda-Ovando, Pacheco-Hernandez, Paez-Hernandez, Rodriguez, & Galan-Vidal, 2009). The feasibility of attaching two different substituents at C3' and C5' positions of the B-ring and of glycosidation at positions C3, C5, C7, allows for a multitude of potential structural and functional variants of anthocyanins (Barnes & Chung, 2011).

Anthocyanin pigments are claimed to be one of the most important flavonoids owing to their beneficial effect on human health (Konczak & Zhang, 2004). They exhibit a higher antioxidative activity than vitamin C and E (Castañeda-Ovando et al., 2009) and additionally show a high biological activity (Cavalcanti, Santos, & Meireles, 2011). Anthocyanins not only determine the wholesome and nutritional properties of a food product, but also act as a color-modeling agent and determine consumer preferences for product acceptance or rejection.

Unfortunately, anthocyanins belong to unstable compounds. Degradation of anthocyanins proceeds during both processing and storage of products containing them (Cavalcanti et al., 2011).

Likely to occur are, among others, disruption of covalent bonds and oxidation of an anthocyanin. Elucidation of the exact mechanism of these reactions is, however, difficult (Patras, Brunon, O'Donnell, & Tiwari, 2010). One of the reasons behind anthocyanins instability is an insufficient number of electrons in a flavylium ring (Sikorski, 2007). Stabilization may proceed via charge transfer from the Highest Occupied Molecular Orbital (HOMO) to the Lowest Unoccupied Molecular Orbital (LUMO) (Ferreira da Silva et al., 2005).

One of the possibilities to stabilize anthocyanin pigments is their complexation with other phenolic compounds, including: phenolic acids, flavonols, flavanols, flavones and others (Sikorski, 2007; Castañeda-Ovando et al., 2009). The copigmentation constitutes a natural system of pigment protection in fruits, vegetables and flowers (Patras, Brunon, O'Donnell, & Tiwari, 2010). The copigments are rich in π -electrons which are able to associate with electron-poor flavylium cations that determine the typical red color of anthocyanins (Castañeda-Ovando et al., 2009). The efficiency of copigmentation reactions in reducing anthocyanins degradation depends on the type and concentration of compounds taking part in the reaction (Bąkowska, Oszmiański, & Kucharska, 2003a, 2003b). For instance, hydroxyflavones are a perfect electron donor, whilst hydroxybenzoic acids are poor donors of electrons (Ferreira da Silva et al., 2005). The effect of copigmentation is based, principally, on the formation of a π - π complex inducing the bathochromic shift and the hyperchromic effect or through the

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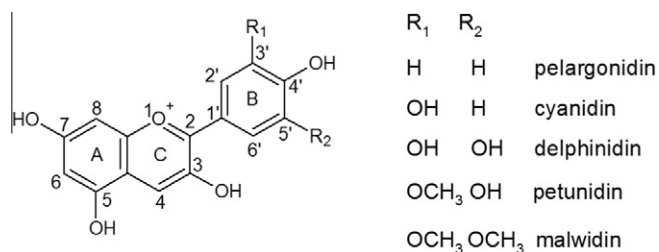


Fig. 1. Aglycones of the most widespread anthocyanins.

stabilization of a flavylum form by a π -complex that shifts the reaction's balance and causes an increment of red color (Castañeda-Ovando et al., 2009).

The formation of a hydrogen bond between a carbonyl group of pigment and hydroxyl groups at aromatic rings of a copigment leads to an increase in the stability and color intensity of anthocyanin pigments. The attachment of a flavonoid to an anthocyanin molecule stabilizes it, thus impeding the nucleophilic attack of water on carbon at the C₂ position by the formation of a stable complex of a lamellar structure (Sikorski, 2007; Santos-Buelga, Escribano-Bailon, & Lattanzio, 2010). Thus far theoretical investigations of anthocyanins simulating their chemical properties have mainly referred to molecular orbitals (Castañeda-Ovando et al., 2009). Studies have also been addressing the stability of flat and non-flat structures of anthocyanins (Sakata, Saito, & Honda, 2006). Compounds energy optimization drives experimental studies into the identification and determination of compounds structure (Barnes & Schung, 2011). It has also been demonstrated that anthocyanin stabilization is feasible via transferring a free electron from copigment onto anthocyanin (Ferreira da Silva et al., 2005). Theoretical studies confirm the possibility of the co-existence of molecular structures differing in their energy by the energy of a hydrogen bond if the molecular structures are in water or water solution (Szymusiak, Oszmiański, & Tyrakowska, 1999). Proton donation by pigment to copigment enables the generation of hydrogen bonds that stabilize the structure. The occurring charge transfer is considered a key factor determining the stability of an anthocyanin-copigment complex (Ferreira da Silva et al., 2005).

Owing to their instability, it is recommended to determine color changes and stability of anthocyanins under conditions of basic pH (Cabrita, Fossen, & Andersen, 2000; Castañeda-Ovando et al., 2009), therefore a quinoidal base was selected for theoretical computations. Changes in the color of these compounds are more significant in the alkaline region due to their instability (Cabrita et al., 2000). The equilibrium constant of the hydration reaction Kh is significantly higher than that of the proton transfer reaction Ka, which reduces stability of the flavylum cation (Sikorski, 2007).

Derivatives of cyanidin are widespread in many raw materials, including black currant, elder, chokeberry, cherries, strawberries, and blackberries. In fruits and vegetables, the most commonly occurring (as much as 50%) are cyanidin derivatives especially in the form of cyanidin-3-glucoside (Castañeda-Ovando et al., 2009; Kong, Chia, Goh, Chia, & Brouillard, 2003). In turn, baicalin is one of the flavones occurring jointly with scutellarin, wogonin and its glycoside in roots of skullcap (*Scutellaria baicalensis*). As demonstrated by experimental research, skullcap flavones enable achieving a high stability of anthocyanins (Bąkowska et al., 2003a, 2003b; Kalisz, Kalisz, & Oszmiański, 2004; Oszmiański, Kalisz, & Kalisz, 2001).

Structures of compounds participating in the copigmentation process and complexes formed with, among others anthocyanins, are in the focus of interest of many experimentalists. The complexes formed by some anthocyanins with flavonoids have, so

far, been investigated as linearly-flat and cyclic systems (Russo, Toscano, & Ucella, 2000; Szymusiak, Oszmiański, & Tyrakowska, 1999). In the case of anthocyanins copigmentation with other phenolic compounds, it has been suggested that the interaction is elapsing due to a lack of chemical bonds (Castañeda-Ovando et al., 2009). The description of the lamellar structure of complexes of anthocyanin pigments with flavonoids has so far been inexplicit.

2. Materials and methods

2.1. Molecular dynamics computations

The experimental system was constituted by a molecule of anthocyanin and a molecule of flavone participating in the copigmentation reactions. For optimization of geometry and quantum-mechanical computations, use was made of aglycone occurring most frequently in food of plant origin, i.e. cyanidin – in the form of a quinoidal base possessing two hydroxyl groups in the A ring and a ketone group in the B ring. The second reagent was baicalin 7-O-glucuronide occurring in the root of skullcap (*Radix Scutellariae baicalensis*). They served to produce a preparation to be used in the experimental part of the study.

Investigations with methods of molecular modeling were carried out applying the software package Sybyl, ver. 6.5 (Tripos Inc., St. Louis, USA). Using its Build module, preliminary molecular structures of molecules were built and were assigned electric charges on atoms according to a standard method by Gasteiger – Hückel. Next, the structures were subjected to energy minimization according to a BFGS algorithm. In numerical optimization, the Broyden–Fletcher–Goldfarb–Shanno (BFGS) method is a method for solving nonlinear optimization problems (which lack constraints) (Fletcher, 1987). The prepared molecules were elaborated with the semi-empirical PM3 method implemented into Mopac software, being part of the Sybyl package. MOPAC (Molecular Orbital PACKage) is a computer program designed to implement semi-empirical quantum chemistry algorithms (Young, 2001). PM3 (Parameterized Model number 3) is a semi-empirical method for the quantum calculation of a molecular electronic structure. It is based on simplifying assumptions that atomic orbitals of different atoms do not overlap and that some electrons are forming cores of atoms. Such simplifications need introducing empirical parameters into equations of quantum chemistry (Young, 2001; Stewart, 1989). Potential derived electric charges were determined for the molecular geometry of the lowest energy and these charges were ascribed to atoms. Successive investigations were conducted with methods of molecular dynamics in Tripos forcefield using parameterization of electric interactions with the potential derived atomic charges determined with the PM3 method. A forcefield is a recipe how to compute energy of a molecule or a system of molecules when atoms change their mutual positions. If bonds' lengths, planar angles between bonds and dihedrals (angles between 2 intersecting planes formed by 2 triples of atoms) change from their equilibrium values, then some excess of energy occurs. Also nonbonding interactions like electrostatic (and also H-bonding) and Van der Waals interactions are dependent on the mutual position of atoms (Clark, Cramer, & Van Opdenbosch, 1989; White, 1977).

Molecules of baicalin and a quinoidal base of cyanidin were tentatively arranged in a structure of a hypothetical complex of "sandwich" type, and the system's volume was determined. The own preliminary study on molecular modeling as well as investigations conducted by other experimentalists (Szymusiak, Oszmiański, & Tyrakowska, 1999) demonstrated that in the environment deprived of water, the system was unstable and was disintegrating, therefore the structure was fixed in an aqueous environment being typical of

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