ANTHOCYANINS, FROM BIOSYNTHESIS IN PLANTS TO HUMAN HEALTH BENEFITS

— review —

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Abstract
Anthocyanins are an important group of highly hydrosoluble pigments in most species of plants. They are produced by secondary metabolism of plants (pentosophosphate, shikimate and flavonoid pathways) and are accumulated in cell vacuoles. In fruits and vegetables anthocyanins are present in the form of glycosides.
The qualitative and quantitative determination of anthocyanins can be performed by classical techniques, such as spectrophotometry, or modern analytical methods, such as HPLC coupled with mass spectrometry detection, liquid chromatography, and thin-layer chromatography. Structural elucidation and conformational analysis can be performed by NMR techniques.
Humans ingest reasonable amounts of anthocyanins through fruits, vegetables and red wines from the diet. Anthocyanins have beneficial role in human health, through their antioxidant properties, being protective against cardiovascular diseases, some types of cancer, diabetes, visual disturbances, liver damage, or UV-B radiation. Supplements containing anthocyanin extracts or new sinergistic blends (OptiBerry) were produced to improve human health.
As they are responsible of plant pigmention, may also offer a natural alternative to food colorings, in particular in soft drinks.

Keywords: anthocyanin, berry, pH differential method, free radicals, biological activity

INTRODUCTION

Apart from primary metabolites, plant cells produce numerous other compounds through secondary metabolism. Generally, this type of metabolism is carried out in specific differentiated cells; the resulted

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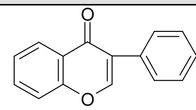
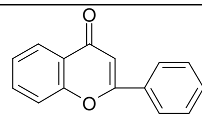
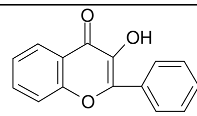
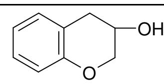
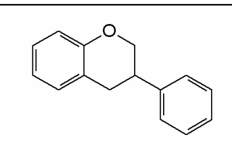
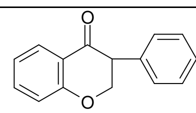
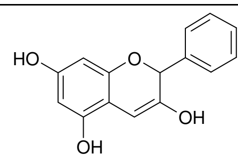
metabolic products are not absolutely necessary to the cell, but they exhibit very important features for the whole plant. Secondary metabolism is induced under certain specific environmental conditions: defense, ultraviolet radiation, saline stress, or nutrient deficits.
The structure of secondary metabolites is more complex than of primary metabolites. Secondary metabolic compounds belong to diverse classes of alkaloids, terpenes and terpenoids, polyisoprene (gutta-percha, balata), phenolic compounds (phenols, phenolic acids, coumarins, naphthoquinones, flavonoids, isoflavonoids, anthocyanins, lignans, lignins), glycosides, tannins, rare amino acids, and amines. Some secondary metabolites are characteristic of a single species, others are common to a group of plants.
Secondary metabolites have different biological functions in plants, as follow (Oancea, 2007):

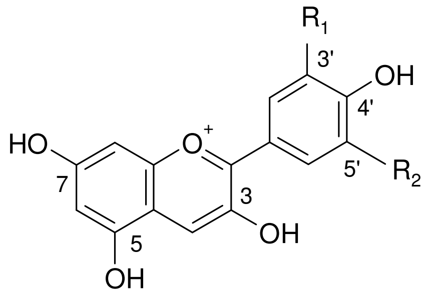
- signal molecules (phytohormones)
- role in plant-animal co-evolution through plant communication with agents of pollination in order to facilitate the reproductive processes (flower pigments)
- defense against pests especially insects (glycosides, alkaloids)
- defense against fungal infections (phytoalexins)
- the inhibition of competitive plant species (allelochemicals)

Over the centuries, the substances produced by secondary metabolism have played an important role in medicine, being used as such or after modification of their chemical structure (synthetic bioactive compounds).
Among the secondary metabolites, anthocyanins represent an important group of phenolic compounds called flavonoids (see Table 1). They function as pigments in plants and are responsible for the bright colors red, purple or blue of the flowers, skin seeds, fruit and some leaves, where they are found in different concentrations and compositions depending on both internal (genetic) or environmental factors (Mohr et al, 1995).
Anthocyanins constitute an important food source of flavonoids (plant products, juices, red wine, grains) (Harbone et al, 1964).

Table 1. General chemical structure and sources of flavonoids.

Class of substances	*Basic chemical structure*	*Biological sources*
FLAVONES		apple, green tea
ISOFLAVONES (daidzein, genistein, glicitein, biochanin A, formononetin)		soybean, sesame, alfalfa
FLAVONOLS		onion, broccoli, tomatoes, apple, cranberries, grapes, green and black tea
FLAVANOLS		onion, tomatoes, red wine, green tea
ISOFLAVANS		soybean, green bean, licorice root
FLAVANONS		citrus peels
ANTHOCYANINS (*aglycon* = *anthocyanidin*)		cherries, raspberries, blackberries, blueberries, oranges, tomatoes, grapes, red wine, red corn, red onion, red cabbage, red-skinned potatoes, eggplant, fennel

Anthocyanins are found in the vacuoles of different plant cells in the form of glycosides. There are about 400 known anthocyanic glycosides (Mazza et al, 1993). Usually, there are 3-glycosides and 3,5-diglycosides, the most common sugar being glucose, but also other carbohydrates (rhamnose, xylose, galactose, arabinose, rutinose). The aglycon (called *anthocyanidin*) is a heterocyclic derivative derived from α-cromen or α-benzopyran. The aglycones are found in very small amounts in fresh plants, as they are quite unstable. The most common aglycones of anthocyanins are pelargonidin, delphinidin, cyanidin, peonidin, petunidin and malvidin. Figure 1 presents the general chemical structure of the aglycon.



R_1 = H, R_2 = H, pelargonidin
R_1 = OH, R_2 = H, cyanidin
R_1 = OH, R_2 = OH, delphinidin
R_1 = OCH_3, R_2 = H, peonidin
R_1 = OCH_3, R_2 = OH, petunidin
R_1 = OCH_3, R_2 = OCH_3, malvidin

Fig. 1. General chemical structure of anthocyanidine.

The aglycones are not only glycosilated but also acylated with different organic acids (caffeic, *p*-coumaric, ferulic and sinapic acids, or acetic, malic, malonic, oxalic and succinic acids) (Eder, 2000).
The most important anthocyanins are the cyanidin glycosides, as they represent 50% of the pigment composition of fruits (Kong et al, 2003).

BIOSYNTHESIS OF ANTHOCYANINS IN PLANTS

The amount of flavonoids in plants varies according to the following factors:

- genetic differences between different plant species
- environmental factors acting during the plant development (light, temperature)
- agronomic factors (methods of cultivation)
- further processings of plant materials

Anthocyanins are abundantly distributed in almost all plant species, and are accumulated in all parts of fruit plants (cherries, raspberries, blackberries, blueberries, currants, oranges, grapes), and vegetables (tomatoes, red corn, red onion, red cabbage, red-skinned potatoes, purple sweet potatoes, eggplant, fennel). In great amounts, they are found in fruits and flowers. Anthocyanins are also found in some products (red wines). The concentration of anthocyanins in fruits is much higher than in vegetables.
The anthocyanins biosynthetic pathway has been completely elucidated, including the identification of the involved enzymes (see Figure 4). The biosynthesis begins with the pentosophosphates pathway, continuous with shikimate pathway, and follows the pathway of flavonoids. Flavonoid biosynthesis in plants starts with the amino acid phenylalanine followed by the elimination of the amino group to produce cinnamic acid, which will be hydroxylated to the *p*-coumaric acid, active in the form of Coenzyme A

thioester. The dihydroflavonols are converted to flavonols (myricetin) which further are glycosylated by glycosyl transferases to the corresponding anthocyanins.

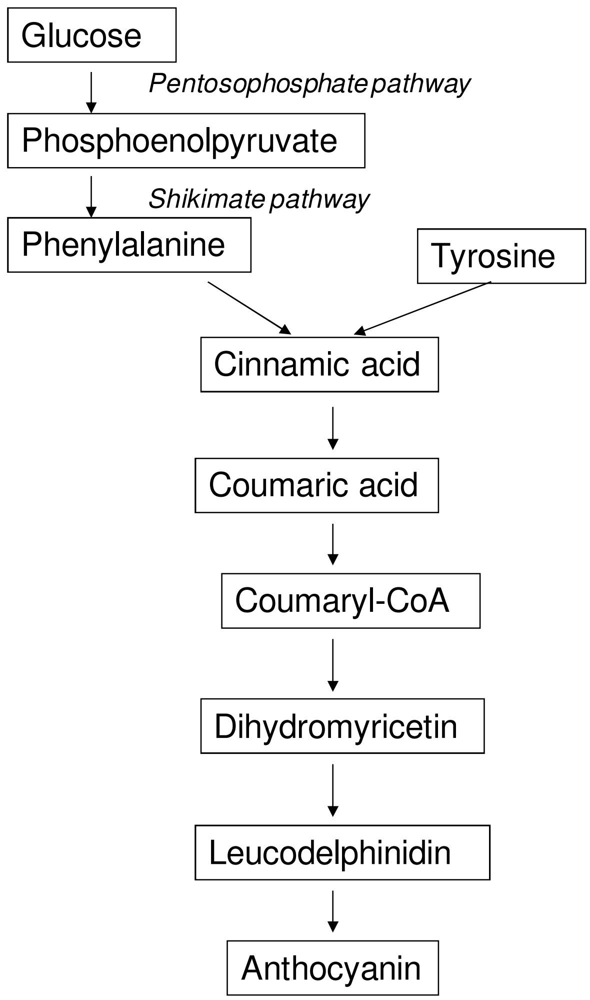


Fig. 4. Biosynthetic pathways of anthocyanins (adapted from *Pascual-Teresa et al, 2008*).

Based on the known biosynthetic pathways of anthocyanins, optimal intervention on the involved enzymes of the secondary metabolic pathways may lead to an increase of anthocyanic content of the plant, as shown by research realised on strawberries (Pascual-Teresa et al, 2008). Also increased anthocyanin content was obtained through genetic improvement in blackcurrants (Brennan, 1996) and raspberries (Graham et al, 2004).

METHODS OF ANALYSIS OF ANTHOCYANINS

Anthocyanins are pigments highly soluble in water and polar solvents. In aqueous solutions, four specific structural forms of anthocyanins coexist in equilibrium, the quinonoidal base (blue, predominantly at pH values between 8 and 10), the flavylium cation (red-orange, predominantly at pH values below 2), the carbinol pseudobase (colorless) and the chalcone C (colorless at pH between 3 and 6) (Brouillard et al, 1993). Percentage of the various specific forms can vary depending on pH and the particular chemical structure of an anthocyanin. These issues are particularly important for selecting the method for analysis of anthocyanins.

Many research studies have shown that anthocyanins are unstable and are oxidized under the action of various factors (pH, temperature, enzymes, UV radiation, SO_2, ascorbic acid, chelating metal ions), resulting in color change and degradation (Rivas-Gonzalo, 2003). Processing and storage at low temperatures can improve the stability of anthocyanins (Delgado-Vargas et al, 2003).

Extraction from plant tissues is an essential step in quantitative analysis of anthocyanins, and solvents used may slightly influence the position of the absorption bands in the spectrophotometric determination.

Qualitative and quantitative determination of anthocyanins in extracts is performed either by classical methods as UV-visible spectroscopy, or by modern techniques, such as HPLC coupled to mass spectrometry or NMR.

Spectrophotometric analysis of anthocyanins is recorded at 510-540 nm wavelengths depending on the chemical structure (Harborne, 1958), (Giusti et al, 2001). The anthocyanin molecule presents a typical absorption band in the UV region 260-280 nm and two in the visible region 415 nm and 490-540 nm, respectively. By applying the pH differential spectrophotometric method and/or subtractive method based on the structural transformation of anthocyanic chromophore in relation to pH (see Figure 5), the level of total monomeric anthocyanins can be relatively accurate determined (Wrolstad et al, 1995), but without differentiating between various compounds.

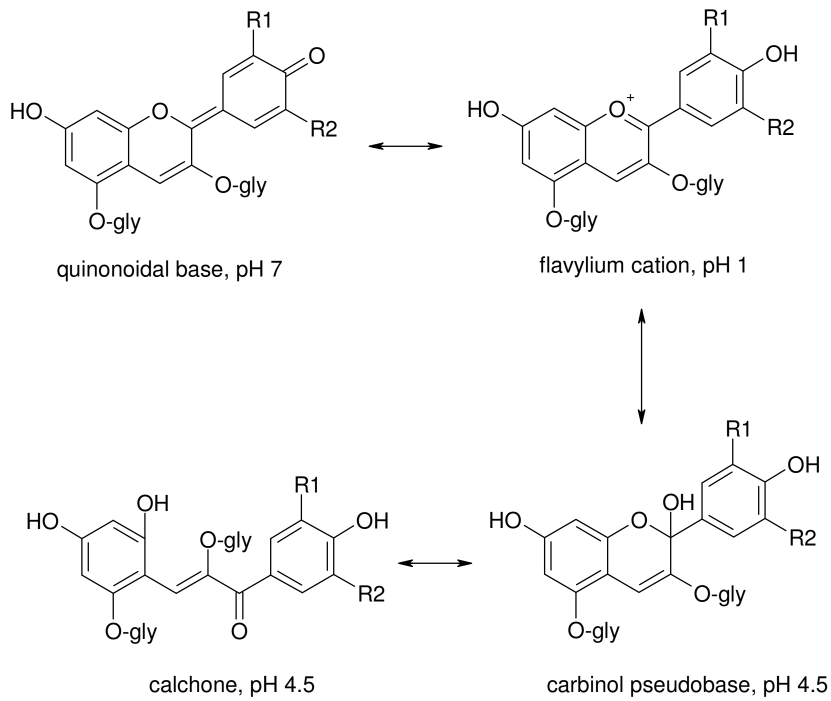


Fig. 5. Structural forms of anthocyanins in relation to pH.

Subtractive spectrophotometric method is based on the absorbance measurement at λ_{max}, followed by a bleaching reaction with sulphite or hydrogen peroxide, and remeasurement of the absorbance for blank (Jackman et al, 1987). This method is applied in order to obtain various degradation indices to assess the quality of foods and beverages colors (Wrolstad et al, 1995).

The spectrophotometric method is a rapid technique that does not require prior hydrolysis of the raw material and was adopted by many research and analysis laboratories worldwide. It has been validated as AOAC official method for determination of monomeric anthocyanins in fruit juices, beverages, natural colorants, and wines with a content of 20-3000 mg/l expressed as cyanidin-3-glucoside equivalents (Lee et al, 2005). The anthocyanin content determined spectrophotometrically is expressed as external standard equivalents, the most commonly used reference material being cyanidin-3-glucoside.

The HPLC method optimized by Adamson et al (Adamson et al, 1999) and Gu et al (Gu et al, 2002), (Gu et al, 2004) was applied to the qualitative and quantitative analysis of anthocyanins, pro-antocyanidins and anthocyanidins. Proantocyanidins are polymers of flavan-3-ol (condensed tannins). In this technique, detection is achieved by diode array detection (DAD) (Gao et al, 1994), mass spectrometry (ESI-MS, FAB-MS, MALDI-MS) (Giusti et al, 1999) or by NMR methods (Mazza et al, 2004). The detection by the electrospray ionization mass spectrometry method (ESI-MS) is particularly useful for determination of anthocyanin metabolites in human plasma or in fruit juices.

Usually, HPLC analysis of anthocyanins is performed on reverse-phase columns, using binary elution systems (aqueous acidified solvents). In the RP-HPLC, elution proceeds in the following order: polar phenolic acids, diglycosilated anthocyanins, monoglycosilated anthocyanins, aglycones, and finally acylated anthocyanins. In direct HPLC, because of the complex chromatograms (great number of peaks), prior acid hydrolysis of the extract is realised, and consequently specific anthocyanidin composition and concentration can be determined.

HPLC method is able to separate and quantify all pro-anthocyanidins from mono- to decamers, and polymers with a degree of polymerization greater than ten (data refer to the sum of all forms of a proanthocyanidin for a certain oligomeric fraction). Gu et al. have validated this method to demonstrate the efficiency of extraction and specificity of separation and quantification. Reverse phase HPLC can not be applied to the separation of pro-antocyanidins with a degree of polymerization greater than three (Adamson et al, 1999). The most common pro-anthocyanidins in foods are pro-cyanidins (epicatechin polymers), pro-delphinidin (gallate polymers) and pro-pelargonidin (derived from afzelechin) (Gu et al, 2004). Unfortunately, analytical technology has not reached the stage where these compounds may be quantified separately, but using mass spectrometry it became possible to identify those peaks corresponding to oligomers with different degrees of polymerization.

Literature also reports identification, separation and quantification of anthocyanins and anthocyanidins by *other techniques*, such as thin layer chromatography (TLC) (Harborne, 1998), liquid chromatography (LC) coupled with mass spectrometry (MS) (Glassgen et al, 1992) or capillary electrophoresis (CE) (da Costa et al, 1998). Structural elucidation and three-dimensional conformation of anthocyanins was done by using mass spectrometry and NMR techniques, in both one and two dimensions (Giusti et al, 1998).

HEALTH EFFECTS AND APPLICATIONS OF ANTHOCYANINS

More recent studies in this field have focused on the beneficial effects of anthocyanins on human health (Weisel, 2006). Humans ingest anthocyanins through fruits, vegetables and red wine daily intake.

The most important role of anthocyanins is the antioxidant activity, and protection against DNA damage, these compounds being able to capture dangerous free radicals as superoxide radical O_2^{-}, hydroxyl radical $HO^{\cdot}$, hydrogen peroxide, H_2O_2, and singlet oxygen $^1O_2$ – chemical species that lead to lipid peroxidation of cell membranes. Antioxidant activity was closely related to the chemical structure of anthocyanins, in particular to the position and degree of hydroxylation of both rings of the basic structure (polyphenolic character) (Rice-Evans et al, 1995).

As result of the antioxidant properties of anthocyanins, it was shown that a diet rich in these bioactive compounds may prevent hyperlipidemia (Xia et al, 2007) and cardiovascular diseases (Garcia-Alonso et al, 2004). Research in the field is based largely on the results of *in vitro* and in some cases *in vivo* investigations of anthocyanins extracts (elderberry, blueberry, strawberry, red wine, wild bilberry, cranberry, raspberry seed). *in vivo* studies of the antioxidant activity are useful for the determination of total antioxidant activity in plasma after a diet rich in anthocyanins.

The anti-inflammatory and protective effects of anthocyanins are explained by the neutralization of the enzymes that destroy the connective tissues of veins, antioxidant capacity against free radical oxygens and repair of the damaged proteins in the blood vessel walls.

It is known that oxidative stress is involved in more than one hundred disease conditions in animals and humans, so that other biological effects of anthocyanins in relation to their antioxidant activity were reported, as follow:

(*a*) Increase of visual acuity; *Vaccinium myrtillus* extracts were shown to improve the night vision in human subjects; in particular, anthocyanins are beneficial in patients with diabetic retinopathy, through the influence on the permeability of retinal vessels;

(*b*) Decrease of the permeability of the blood-brain barrier to toxic substances; acylated anthocyanins from red cabbage were shown to prevent toxins (Paraquat)-induced oxidative stress (Igarashi et al, 2000);

(*c*) *In vitro* inhibition of platelet aggregation (cardioprotectant agents) (Istudor, 1996), (Tamas et al, 2000);

(*d*) Antitumor activity, inhition of angiogenesis (Liu et al, 2005); anthocyanins of fruit extracts (blueberry, bilberry, cranberry, strawberry,

raspberry) were shown to inhibit either the initiation of carcinogenesis or the formation of tumors (esophageal, colon, liver); in particular cyanidin and delphinidin inhibited the growth of certain tumor cells by inhibition of the epidermal growth-factor receptor, while the corresponding glycosides were inactive, and malvidin from grapes less active; structural moiety was found responsible for the high effect of berry anthocyanins on the vascular endothelial growth-factor expression and release (antiangiogenetic effect);
(*e*) Antimutagenic activity (Hope Smith et al, 2004);
(*f*) Stimulation of insulin in pancreatic cells (Jayaprakasam et al, 2005);
(*g*) Protective effects against liver damage (Wang et al, 2000);
(*h*) Beneficial effects on neurodegenerative processes in Parkinson's or Alzheimer's disease; this is probably due to the ability of anthocyanins to reduce inflammation and oxidative stress in brain (Joseph et al, 2003), (Lau et al, 2007). It was shown that berries improve dopamine release in the brain resulting in a better communication between brain cells. High intake of anthocyanins through diets rich in fruits and vegetables could inhibit or reverse age-related changes in brain and behavior;
(*i*) Antiulcer properties (Cristoni et al, 1987);
(*j*) Protection against UV-B radiation (290-320 nm); in particular cyanidin acts as a skin photoprotective agent (Sharma, 2001).

As a result of their pharmacological properties, anthocyanins have for long been used for therapeutic purposes. Thus, crude extracts of *Vaccinium myrtillus* administered orally, intravenously or intramuscularly are able to reduce the capillary permeability and fragility.

OptiBerry is a health-promoting product in the form of a sinergistic combination of six selected extracts from wild blueberry and bilberry (*Vaccinium myrtillus* and *Vaccinium corymbosum*), cranberries, elderberries, raspberries and strawberries. This product has shown excellent antioxidant and antiangiogenetic activities, and also antiatherosclerosis, anticarcinogenic and antibacterial properties (effective against *Helicobacter pylori* pathogen responsible for the occurrence of gastrointestinal disorders including gastric ulcer and cancer) (Zafra-Stone et al, 2007). The results indicate that OptiBerry has low cytotoxicity, being a safe food and dietary supplement.

In the form of powders, anthocyanins (grapes, tomatoes, red cabbage) are used as natural food additives in confectionery and soft drinks. Anthocyanins from red cabbage are stable over a broader pH, so that are used as natural alternatives to synthetic blue colorings for foods with neutral pH (Bridle et al, 1997). Anthocyanins of *Hibiscus* are used both in soft drinks and medical herbs.

CONCLUSIONS

Anthocyanins are important pigments and antioxidants commonly found in plant foods. These compounds which are composed of an aglycon (anthocyanidine) and sugar molecules, are produced in plants by pentosophosphate, shikimate and flavonoid pathways.
Classical and modern techniques are available for the analysis of anthocyanins extracts (spectrophotometry, HPLC, TLC, LC, MS, NMR).
Anthocyanins exhibit antioxidant properties which made them good protective agents against age-related diseases (cardiovascular diseases, neurological diseases, inflammation processes). Anthocyanins have also other beneficial health effects, such as improvement of visual acuity, anticancer and antimutagenic properties, antidiabetic effects, antimicrobial potencies and skin protection against UV-B radiation.
As pigments, anthocyanins may be used as natural alternatives to synthetic food colorings.

Acknowledgements

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