

IDENTIFICATION AND QUANTIFICATION OF ANTHOCYANINS
IN THE TRANSGENIC TOMATO

by

XIAOYU SU

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Approved by:

Major Professor
Dr. Weiqun Wang

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XIAOYU SU

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Abstract

Anthocyanins, a sub-class of flavonoids, are natural pigments derived from phenylpropanoid pathway. Most tomato cultivars found in nature have very low content of anthocyanins, but dark purple tomatoes by ectopic co-expression of two transcription factors *Delila* (*Del*) and *Roseal* (*Ros1*) from snapdragon and *chalcone isomerase* (*CHI*) from onion accumulated high levels of anthocyanins. This study is to identify and quantitate anthocyanins in these transgenic tomato lines. Seven anthocyanins including two new anthocyanins [malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside and malvidin-3-(feruloyl)-rutinoside-5-glucoside] have been identified in transgenic lines by HPLC-MS. The top two anthocyanins are petunidin 3-(trans-coumaroyl)-rutinoside-5-glucoside and delphinidin 3-(trans-coumaroyl)-rutinoside-5-glucoside that contribute for 85% of total anthocyanins in whole fruit. Comparing with undetectable anthocyanins in the wild type, *Del/Ros1*-expressing tomatoes contain total anthocyanins at 4.95 ± 0.42 g/kg dry matter in whole fruit, 5.09 ± 0.62 g/kg dry matter in peel, and 5.56 ± 0.29 g/kg dry matter in flesh, while *CHI* × *Del/Ros1*-coexpressing tomatoes have 9.61 ± 0.71 g/kg dry matter in whole fruit, 29.9 ± 1.64 g/kg dry matter in peel, and 8.65 ± 0.39 g/kg dry matter in flesh. No anthocyanins are detectable in the seeds of each line tested. Enrichment of tomato fruit with new and high anthocyanins may provide potential health-promoting benefits.

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I would like to give my special thanks and express my love and gratitude to my wife and beloved family; for their understanding and endless love, through the duration of my studies.

Dedication

Dedicated to my parents, Yongjia Su and Chunmei Ding, for your endless love, trust and support.

Chapter 1 - Literature review

1.1 Anthocyanins

From a botanic view, the bright, colorful pigments are widely found in many flowers and fruits, which play a major role in both pollination and seed dispersal. The color of flowers and fruits is affected by the presence of different categories of plant pigment belonging to the phenylpropanoid and terpenoid classes; those are chlorophylls, carotenoids and anthocyanins (1). Anthocyanins are one of the most important water soluble natural pigments; over 600 anthocyanins have been reported in nature by now (2). The word “Anthocyanins” original came from two Greek words: anthos and kyanos which represented flowers and dark blue (3).

1.2 Chemical structure

1.2.1 Chemical structure of anthocyanin aglycones

Anthocyanins are a sub-group of flavonoids which are produced by secondary metabolism in higher plants. By comparison with other flavonoids, the anthocyanin takes a positive charge on its C-ring which leads to various colors. The aglycones or anthocyanidins mainly found in nature are cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin. They are sharing the same 2- phenylbenzopyrilium (flavyl-ium) skeleton hydroxylated in 3, 5, and 7 positions, and differ by R1 and R2 substitutes in the B-ring (**Figure 3.5**) (4), and synthesized via the phenylpropanoid pathway (5, 6).

1.2.2 Anthocyanin biosynthesis pathway

Anthocyanins are derived from the phenylpropanoid biochemical pathway of plant secondary metabolism, starting at aromatic amino acid phenylalanine from shikimic acid pathway. Through the catalytic reaction of phenylalanine ammonia-lyase (PAL), this pathway produces an intermediate chalcone-like compound through a polyketide folding mechanism which is commonly found in plants. The chalcone is subsequently isomerized by the enzyme chalcone isomerase (CHI) to the prototype pigment naringenin. Finally, naringenin is oxidized by multiple enzymes such as flavonoid 3' hydroxylase and flavonoid 3' 5'-hydroxylase (F3'5'H) to the final products anthocyanins. (5, 7 and 8).

1.2.3 Anthocyanin biosynthesis pathway in transgenic purple tomaotes

The anthocyanins from transgenic purple tomatoes belong either to the Delphinidin or peonidin and are linked with rutinoside and glucoside (11). In tomato fruits, expression of transcription factors Del and Ros1 causes to activate the transcription of many structural genes involved in the biosynthetic pathway, such as phenylalanine ammonia-lyase (PAL), chalcone isomerase (CHI) and flavonoid 3'5'-hydroxylase (F3'5'H). The activation of PAL provides flavonoid biosynthesis by supporting high levels of flux via the phenylpropanoid metabolism, whereas CHI and F3'5'H are both essential to convert the flux of flavonoid intermediates towards to the anthocyanin products. Meanwhile, CHIxDel/Ros1 transgenic plant, stronger activation of the whole phenylpropanoid pathway, resulted in the significant enhance in both PAL transcription and PAL enzymatic activity and over expression of CHI as indicating higher levels of anthocyanins. (**Figure 3.2**)

1.3 Anthocyanins in human diet

The anthocyanins, contributing to human diet, have a long history, are receiving more attention for their positive health attributes, such as antioxidant, anti-inflammatory, and anti-carcinogenetic effects (9). On the basis of updated food intake data from NHANES 2001–2002, the daily intake of anthocyanins is estimated to be 12.5 mg per day per person in the United States (13).

The food processing leads to extensive decomposition of anthocyanins in canned foods, bread, and baby foods. The reasons are their poor stability and latent destruction during food processing. The chemical structure of anthocyanins includes a positive charge on the C-ring of the aglycone at low pH, but anthocyanins are dysfunctional under neutral or high pH (10). Nowadays, many fruits are seasonal, and therefore frozen storage may be the best way to preserve stability of anthocyanins chemical structure in fruits, which supports consumer's consumption all year (9).

1.4 Cancer prevention of dietary anthocyanins

1.4.1 Influence of Anthocyanins in cancer cells

Anthocyanins compositions are regarded as an essential factor for inhibiting cancer cell proliferation. A çai fruit was selected due to high monomeric and polymeric anthocyanin content. The chemical composition, anti-proliferative activity, and *in vitro* absorption of monomeric and polymeric anthocyanin fractions from a çai fruit were detected in this experiment. HT-29 human colon adenocarcinoma cells seeded at the density of 2×10^4 cells/well. After 24 hours of incubation, the growth medium was replaced by 500 μ l of media which contained different

concentrations of anthocyanin extracts (from 0.5 to 100 μg cyanidin-3-glucoside/ml). The cell numbers were observed following 48 hours of incubation. In contrast, cell proliferation was inhibited by 50% linear regression analyses by each anthocyanin concentration. In addition, the anti-proliferative activities of anthocyanin monomer, polymer, and mixed fractions were still applied in incubating HT-29 colon tumor cells in different medium fractions. Although monomeric and polymeric anthocyanin fractions and their mixtures significantly reduced the total cancer cell numbers in culture plates, monomeric anthocyanin fractions were found to be more effective in inhibiting cell proliferation when compared to the same concentrations of polymeric fractions (11).

Strawberry fruit includes anthocyanins, which are components not only for fruit pigment but also for health benefits. According to many study results, strawberries have a superior antioxidant capacity among fruits. Human colon cancer cells HT-29 and estrogen-receptor-positive breast cancer cells MCF-7 were incubated 2×10^4 cells in 0.2 ml of medium which were placed in a 96-well microplate for 24 h. Afterward, the medium was replaced by 0.2 ml of strawberry powder extracts medium. Four different concentrations of the extracts were contained in the medium: 0.025%, 0.05%, 0.25%, and 0.5%. The same amount of solvent (50% ethanol) was added for the control wells. After 24 hours of incubation, cells were continually incubated for 1 h for HT-29 cells, and for 3 h for MCF-7 cells after 20 μL of WST-1 were added. As a result, the strawberry extracts significantly suppressed the proliferation on both colon cancer cells (HT-29) and breast cancer cells (MCF-7). Furthermore, the HT-29 cells were inhibited to a higher level than breast cancer cells, and the intensity of inhibition was affiliated with the extract concentration. Particularly for MCF-7 cells, the data showed significant difference in the

inhibition of the proliferation in concentration: 0.5%, 0.25%, and 0.05% compared with the control medium (12).

1.4.2 Influence of Anthocyanins in animal models

Apc^{Min} (Min, multiple intestinal neoplasia) is a point mutation in the murine homolog of the APC gene. Min/+ mice develop multiple intestinal adenomas, as do humans carrying germline mutations in APC. Mice, 4–5 weeks of age, were randomly separated into five different dietary treatments. The five diet groups contained 0, 375, 750, 1500, or 3000 mg of anthocyanin-rich tart cherry extract/kg in powdered diet, and each diet was mixed with 100 mg of sulindac/kg. The diet which contained 100 mg of sulindac/kg was designed according to previous research at MSU (Michigan State University) laboratory because they found some partial inhibition in intestinal tumorigenesis. As a result, the data of small intestinal tumor cells proved that a combination of dietary anthocyanin-rich extract and sulindac were significantly effective in slowing tumorigenesis in the small intestine of APC^{Min} mice than feeding sulindac only. On average, APC^{Min} mice fed anthocyanin-rich extract and sulindac had a 20% smaller tumor area in the small intestine than APC^{Min} mice fed sulindac only. Further, APC^{Min} mice fed a combination of anthocyanin-rich extract and sulindac had a 22% less tumor number in the small intestine than APC^{Min} mice fed sulindac alone (14).

Purple-fleshed sweet potato clone, P40, has enriched anthocyanins which compared with white-fleshed O'Henry and yellow-fleshed NC Japanese. AIN-93M diet was mixed with 20% O'Henry, 20% NC Japanese, 10-30% sweet potato powder in mice diet ingredients. Azoxymethane

(AOM)-induced aberrant crypt foci (ACF) had a close bearing on cancer preventive effect of purple sweet potato diet. AOM injected mice were fed by normal AIN-93M diet, diets containing 10~30% of P40, 20% O' Henry and 20% NC Japanese for 6 weeks. After 6 weeks' AOM injection, all mice were sacrificed. And in the end, ACF were induced in all the animals which injected with 6 weeks' AOM, and ACF were also detected in all the distal portion of the colon. Total number of ACF, whatever large or medium was significantly decreased in colons of mice fed 10-30% P40 diet than mice fed the control diet (15).

Dietary freeze-dried black raspberries were mixed in Fischer 344 rat diet, shown to significant inhibit chemically-induced cancer of the rodent esophagus by 30-60% and even up to 80% for colon cancer. The initiation and progression stages are two essential tumor developments that were inhibited by anthocyanins-enriched dried powder. Berries suppress tumor initiation stage through influencing carcinogen metabolism, leading to reduced degrees of carcinogen-induced DNA damage. Berries inhibit progression stage by slowing the growth rate of premalignant cells, inducing apoptosis, reducing tissue inflammation and inhibiting angiogenesis. The mechanism is that berry components influence multiple signal transduction pathways rely on modification of key regulatory genes such as NFκB, AP-1, P1-3K/Akt, p38/Erk1/2 resulting in effects on downstream genes such as COX-2, VEGF and iNOS (16).

1.5 Potential cancer prevention mechanism

1.5.1 Scavenging reactive oxygen species (ROS)

Anthocyanins, which contain phenolic structure, have higher antioxidant activity because of their electron deficiency (12). Reactive oxygen species (ROS) are natural byproducts via human body metabolism that will constantly accumulate within the body causing oxidative stress.

Oxidative stress, such as high concentration of ROS, have been recognized as incentives in the aging and cancer such as damaging DNA, protein, lipid and cell signaling pathway (17).

Although most of the antioxidant of anthocyanins is contributed by their ability to scavenge ROS, the rest mechanisms still attribute partial antioxidant functions such as: inducing Phase II detoxification enzymes expression, lowering oxidative adducts in DNA conformation, decreasing lipid peroxidation, inhibiting gene mutation through various carcinogens, and decreasing cellular proliferation by regulating signal transduction pathways (4).

1.5.2 Anti-cell proliferation

Making through cell culture trials, high concentration of anthocyanins extracts from dark fruits and vegetables have potential anti-proliferative activity towards cancer cell *in vitro* (18). The various anthocyanins inhibited cell proliferation through restricting regulator proteins on cell cycle (e.g., p53, p21, p27, cyclin D1, cyclin A, etc.) (18). Among six anthocyanins tested, ortho-dihydroxyphenyl structure on the B-ring highly inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cell transformation and activator protein-1 transactivation. Delphinidin suppressed the phosphorylation of protein kinases in the extracellular signal-regulated protein kinase (ERK) pathway at early times and the c-Jun N-terminal kinase (JNK) signaling pathway at later time. Two mitogen-activated protein kinase (MAPK) specific inhibitors could specifically block the pathway of both JNK and ERK. In the end, the evidences prove that anthocyanidins may contribute the cancer prevention by suppressing MAPK pathway (19).

1.5.3 Induction of apoptosis

Apoptosis, an automatic pathway of cell self-destruction that is regulated by nuclear DNA, is activated either a vivo or vitro stimulus. It is a physiological processing to regulate nuclear DNA-damage such as uncontrolled cell growth and tumor cells. Anthocyanin-rich

extracts from berries and grapes involve different types of pure anthocyanins and anthocyanidins, have potential pro-apoptotic effects in multiple cell types *in vitro* (20, 21, and 22). In the intrinsic pathway, anthocyanin induces cytochrome *c* release in cancer cells membrane and modifies caspase-dependent anti- and pro-apoptotic proteins. In the extrinsic pathway, the express of Fas and FASL (FAS ligand) are operated by anthocyanins which causing cancer cells apoptosis (23).

1.5.4 Induction of differentiation

Induction of cellular differentiation can prevent and treat cancer which forwards a less toxic chemotherapy pathway. *In vitro*, leukemic cells with anthocyanins (25–200 µg/ml) culture medium induces cancer cell differentiation through four evidences: a) led to reduction of nitroblue tetrazolium (NBT), a marker for granulocyte/monocyte differentiation; b) induced differentiation of leukemic cells into a monocyte/macrophage-like phenotype; c) increase naphthol AS-D chloroacetate activity, a marker for granulocytic differentiation; and, d) led to increase the number of α -naphthyl acetate esterase positive cells which forwarding differentiation to the monocytic/macrophagic lineage (24). Inducing leukemic cell differentiation was linked with reduced cell proliferation and down-regulation of c-myc. Anthocyanins also led differentiation in melanoma cells through increasing dendritic outgrowth which accompanied by rearranging the microtubular network (16). This will apparently increase the expression of “brain specific” cytoskeletal components (NF-160 and NF-200 neurofilament proteins) in the cells (16).

In this part of study, three varieties of tomatoes, Wild, Del/Ros1, and CHIXDel/Ros, were provided by Dr. Park in Horticulture of Kansas State University. To identify novel characteristics of transgenic tomatoes, we identified and quantitated anthocyanins in edible CHIXDel/Ros and Del/Ros1 transgenic tomato from extracted tomatoes solution by HPLC-MS and compared them to control, while Wild type.

1.6 Reference

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Chapter 2 - Experiment

2.1 Abstract

Anthocyanins, a sub-class of flavonoids, are natural pigments derived from phenylpropanoid pathway. Most tomato cultivars found in nature have very low content of anthocyanins, but dark purple tomatoes by ectopic co-expression of two transcription factors *Delila* (*Del*) and *Roseal* (*Ros1*) from snapdragon and *chalcone isomerase* (*CHI*) from onion accumulated high levels of anthocyanins. This study is to identify and quantitate anthocyanins in these transgenic tomato lines. Seven anthocyanins including two new anthocyanins [malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside and malvidin-3-(feruloyl)-rutinoside-5-glucoside] have been identified in transgenic lines by HPLC-MS. The top two anthocyanins are petunidin 3-(trans-coumaroyl)-rutinoside-5-glucoside and delphinidin 3-(trans-coumaroyl)-rutinoside-5-glucoside, that contribute for 85% of total anthocyanins in whole fruit. Comparing with undetectable anthocyanins in the wild type, *Del/Ros1*-expressing tomatoes contain total anthocyanins at 4.95 ± 0.42 g/kg dry matter in whole fruit, 5.09 ± 0.62 g/kg dry matter in peel, and 5.56 ± 0.29 g/kg dry matter in flesh, while *CHI* × *Del/Ros1*-coexpressing tomatoes have 9.61 ± 0.71 g/kg dry matter in whole fruit, 29.9 ± 1.64 g/kg dry matter in peel, and 8.65 ± 0.39 g/kg dry matter in flesh. No anthocyanins are detectable in the seeds of each line tested. Enrichment of tomato fruit with new and high anthocyanins may provide potential health-promoting benefits.

2.2 Introduction

The bright-colorful are widely found in many flowers and fruits which play a major role in both pollination and seed dispersal (1). The color of flowers and fruits is affected by the presence of different categories of pigment belonging to the phenylpropanoid and terpenoid

classes, whose are chlorophylls, carotenoids and anthocyanins and so on (2). As one of the most important water soluble natural pigment, over 600 anthocyanins have been reported in nature by now (3). The word "Anthocyanins" originated on two Greek words: anthos and kyanos which represented flowers and dark blue (4). As a sub-class of flavonoids, anthocyanins are natural pigments derived from phenylpropanoid pathway through secondary metabolism in higher plants. The aglycones or anthocyanidins mainly found in nature are cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin. They are sharing the same 2- phenylbenzopyrilium (flavylium) skeleton hydroxylated in 3, 5, and 7 positions, and differ by R1 and R2 substitutes in the B-ring (**Figure 3.5**) and synthesized via the phenylpropanoid pathway. By comparison with other flavonoids, the anthocyanin takes a positive charge on its C-ring which leads to various colors (5). Many recent cell culture studies, animal studies and epidemiological studies have demonstrated potential association between anthocyanin intake and clinical disease prevention (6, 15 and 16).

The anthocyanins, contributing to human diet, have a long history, are receiving more attention for their positive health attributes, such as antioxidant, anti-inflammatory, and anti-carcinogenetic effects (7). On the basis of updated food intake data from NHANES 2001–2002, the daily intake of anthocyanins is estimated to be 12.5 mg/day/person in the United States (NHANES 2001–2002) (7). As a result, in recent years, numbers of different genome approaches have been adopted to active the synthesis pathway of flavonoids in plants (8) such as crops, potato and rice (9, 10). Tomato, one of the most famous fruit containing lycopene as a dietary carotenoids, has been demonstrated to be associated with a decreased risk of chronic diseases, such as prostate cancer and cardiovascular disease (11, 12), however it has never been reported to contain anthocyanin in nature. Bovy et al reported (13) two regulatory genes Lc and C1 which

control anthocyanin biosynthesis in maize were encoded in tomatoes resulted in fruit containing higher levels of flavonols, but still no anthocyanin synthesis was found. Subsequently, co-expression two selected transcription factors Delila(Del) and Roseal(Ros) from snapdragon (*Antirrhinum majus* L) activated anthocyanin synthesis pathway and lead to high levels of anthocyanins throughout the fruit flesh and peel, as a result, purple color accumulated (2). Del/Ros1 and CHI×Del/Ros1 developed normally during green stage and slight purple pigmentation emerged in transgenic fruit close to the end of the fruit mature green stage. The purple and blue pigmentation rapidly occupied mature green fruits by in the past few days after its start (14). At maturity, three different lines of tomato were sent to lab that waiting for identification. Lines showed a range of phenotypes, with wild control for red color, medium purple for Del/Ros1 and dark purple for CHI×Del/Ros1.

Transgenic purple tomatoes (*Ipomoea batatas* L.) exhibit an intense blue or purple color in both skin and flesh of the fruits is derived from a high level of anthocyanins accumulation. The color of fruits is shown in **Figure 3.1**.The anthocyanins from transgenic purple tomatoes belong either to the Delphinidin or peonidin and are linked with rutinoside and glucoside (14). In tomato fruits, expression of transcription factors Del and Ros1 causes to active the transcription of many structural genes involved in the biosynthetic pathway, such as *phenylalanine ammonia-lyase* (PAL), *chalcone isomerase* (CHI) and *flavonoid 3'5'-hydroxylase* (F3'5'H). The activation of PAL provides flavonoid biosynthesis by supporting high levels of flux via the phenylpropanoid metabolism, whereas CHI and F3'5'H are both essential to convert the flux of flavonoid intermediates towards to the anthocyanin products. Meanwhile, CHI×Del/Ros1 transgenic plant, stronger activation of the whole phenylpropanoid pathway, resulted in the significant enhance in both PAL transcription and PAL enzymatic activity and over expression

of CHI as indicating higher levels of anthocyanins. (**Figure 3.2**)

In this part of study, three varieties of tomatoes, Wild, Del/Ros1, and CHI×Del/Ros1, were provided by Dr. Park in Horticulture of Kansas State University. To identify novel characteristics of transgenic tomatoes, we identified and quantitated anthocyanins in edible CHI×Del/Ros1 and Del/Ros1 transgenic tomato from extracted tomatoes solution by HPLC-MS and compared them to control, while Wild type.

2.3 Material and methods

2.3.1 Reagent

1. All organic solvents (methanol, acetonitrile, formic acid) were HPLC grade, and purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA).
2. Internal standard, Peonidin-3-glucoside chloride was obtained from Sigma-aldrich (St. Louis, MO, USA).
3. Transgenic Purple tomatoes were provided from the Dr. Sunghun Park in Horticulture of Kansas State University, Kansas.

2.3.2 Sample preparation and extraction

a. Whole Fruits

Purple tomato samples were randomly taken from each line, cleaned with paper towel, diced into 0.5cm cubes, freeze-dried (Labconco, FreeZone 2.5) and ground by pestle and mortar into powder. Prepared powder was store at -80°C until use.

b. Peel, Flesh, seed

Purple tomato samples were randomly taken from each line, cleaned with paper towel. Then, the purple tomato was slightly kneaded until the texture of tomatoes became soft. The peel and flesh of fruits were separated by tweezers, diced flesh into 0.5 cubes, collected all the seeds, freeze-dried and ground by pestle and mortar into powder. Prepared powder was stored at -80 °C until use.

For preparation of anthocyanin extracts, 0.05g of powder was extracted with 4ml of acidified MeOH (1N formic acid, 9:1) to obtain a sample to solvent ratio 1:80. The flasks containing powder/solvent mixture were sealed with aluminum foil to avoid exposure to light. After 12 hours extraction, the mixture was centrifuged (2,800rpm, 30min) and the supernatant was kept. The supernatant was dried by vacuum drier at 25 °C overnight. 1ml acidified MeOH (1N Formic acid, 9:1) was added to re-dissolve the dried powder. Transfer 1ml constant volume sample to HPLC vial through Whatman syringe filter (Whatman 0.45µm PVDF).

HPLC-MS/ESI analysis of anthocyanins

The quantification of anthocyanins in transgenic purple tomato extracts was based on previous methods. (35, 36). Extract analysis was implemented on a high phase liquid chromatography, HPLC (Shimadzu), and Esquire 3000+ electrospray ion-trap mass spectrometer ESI/MS (Agilent). A WATERS C18 stationary phase column (4.6mm x 250mm i.e., 5µm) was applied for sample separation. An optimum column temperature of 25 °C was set. The mobile phase A consisted of 5% formic acid in double deionized water (ddiH₂O), and the mobile phase B consisted of 95:5 mixtures of acetonitrile and formic acid. At a flow rate of 0.8mL/min the gradient conditions were as follows: solvent B: 0min, 5%; 35min, 20%; 45min, 35%; 55min, 50%; 60min, 5%. For HPLC-MS analysis, peaks were spiked with peonidin-3-glucoside as an

internal standard and detected by monitoring absorbance upon 520nm wavelength for trial anthocyanins. The quantification measurement of each anthocyanin was based on comparison of relative retention time (RT), percentage peak area, and spectral data with anthocyanin internal standard, peonidin 3-glucoside (0-100 μ M). Right after the HPLC analysis, the Agilent mass spectrometry was applied in confirming the mass of the each anthocyanin HPLC peaks.

Chapter 3 - Result and discussion

3.1 HPLC-MS/ESI analysis of anthocyanins from transgenic purple tomatoes

Through HPLC analysis, anthocyanins profiling was detected; retention time changed from 23min to 38min under 520 nm with flow rate 0.8ml/min. A total of 7 anthocyanins have been detected in both transgenic lines by HPLC, by contrast, anthocyanins are undetectable in wild and all fruit seeds (**Figure 3.3**). The identification of each anthocyanin compound in samples was confirmed by the using Esquire 3000+ electrospray ion-trap mass spectrometer ESI/MS. Table 3.1 shows MS spectrum of each individual anthocyanin standard in positive ESI mode. The mass spectra fragmentation patterns in transgenic tomatoes demonstrated delphinidin and peonidin derivatives were same as previously reported to be found in both transgenic tomatoes (14), but no reliable report was found about the presence of malvidin derivatives in CHI×Del/Ros and Del/Ros1 transgenic purple tomatoes. By matching fragmental patterns to literatures, two unique new anthocyanins, (malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside and malvidin-3-(feruloyl) -rutinoside-5-glucoside) were detected in our samples which never reported before. (**Figure 3.4 a,b**)

Peonidin-3 glucoside chloride (2μM), as an internal standard, was added in the extracts, and was also used as a standard for quantification. The Anthocyanin content of purple tomatoes was measured by peonidin 3-glucoside chloride equivalent (PN3GE) value. Total anthocyanin content of CHI×Del/Ros1 was 9.61g PN3GE/kg dry weight, and it was significantly higher than Del/Ros1 which was 4.95g PN3GE/kg dry weight ($p < 0.001$).(**Table2**)

Through data analysis, petunidin-3-(trans-coumaroyl)-rutinoside-5-glucoside and delphinidin-3-(trans-coumaroyl)-rutinoside-5-glucoside occupied top two anthocyanins content, that also contribute for 85% of total anthocyanins in whole fruit, by contrast, two new

anthocyanins, malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside and malvidin-3-(feruloyl)-rutinoside-5-glucoside, only contributed 6% of total anthocyanins in transgenic tomatoes. (**Table 3.3**)

Discussion

The HPLC profiling from transgenic tomatoes Del/Ros1 and CHI \times Del/Ros1 are similar as shown by the HPLC chromatograms (**Figure 3.3**). Differences only found in peak area or anthocyanin content of each spike, which was converted to PN3GE value. According to comparison of relative retention time (RT), percentage peak area, and spectral data, the first 5 anthocyanin peaks of HPLC chromatograms were matching with previous studies (14), which only contributed petunidin, delphinidin as anthocyanidins or anthocyanin precursors. Moreover, in our study, two unique new anthocyanins [malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside and malvidin-3-(feruloyl)-rutinoside-5-glucoside] have been identified in transgenic lines by HPLC-MS with retention time 36 and 38min, which contribute to a new anthocyanidin. Total anthocyanin content of CHI \times Del/Ros1 was 9.61g PN3GE/kg dw, and it was significantly higher than Del/Ros1 which was 4.95g PN3GE/kg dw ($p < 0.001$).

In view of new anthocyanins detected in both breeds of transgenic tomatoes, two possible reasons may be accompanied with new anthocyanidin found in both transgenic tomatoes. Extract and HPLC method are considered as the primary reasons, causing distinct peak separation than previous detection. The differences between the two compared methods are shown below. (**Table.4**)

In our method, 10 percent of formic acid was added in methanol before extraction, acidic mixture extract provides a dominant low pH condition for stability characteristics of

anthocyanins. Then I retarded flow rate and solvent B ratio during gradient elution in order to get good separation.

Secondly, anthocyanins often accumulated at specific developmental stages and may be induced by numerous exterior and interior environmental factors including visible and UVB radiation, fertilizer, cold temperatures, insect and water stress. The subsequent production and localization of anthocyanins in root, stem, flower, and especially fruit tissues may allow the plant to develop resistance environmental stresses (17). Such environmentally induced anthocyanins lifting may also help new anthocyanin arising from the interplay of genetic and environmental factors.

CHI×Del/Ros1 transgenic tomato, as a genetic engineering product, has elevated total anthocyanin content even close to 2 times than Del/Ros1(P<0.001). Similarly, the flesh of CHI and Del/Ros1 contained 1.6-fold higher anthocyanin content than transgenic tomatoes only treated with Del/Ros1 (P<0.001). The most striking feature of this ectopic co-expression of two transcription factors is a very high anthocyanin content in fruit peel (29.89g/kg DW). Although peel accounts for only about 5% of fruit mass, co-expression of CHI and *Del/Ros1* gene, significantly increases peel anthocyanin content up to 5.8-fold than Del/Ros1 peel (P<0.001).

"French Paradox" is an obviously paradoxical pathological phenomenon that French people have a relatively low incidence of coronary heart disease (CHD) although they intake of a high-fat diet, have been attributed to the higher consumption of red wine containing high levels of polyphenolic compounds (18). Malvidin, a new anthocyanidin found in our sample, occupies a dominant position in the polyphenols of red wine and grape together with other anthocyanidins, phenolic acids and flavonoids (19, 20 and 21). These new discoveries are helpful to make direction of further CHD research. Recent findings also indicate a potential preventive role of

dietary Malvidin against proliferation of human cancer cell lines (22, 23) and chronic inflammatory diseases such as diabetes and hypertension (16, 24).

In conclusion, seven anthocyanins have been identified in both CHI×Del/Ros1 and Del/Ros1 transgenic tomatoes, including 2 new anthocyanins (malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside and malvidin-3-(feruloyl)-rutinoside-5-glucoside). Total anthocyanin contents are 9.6 and 5.0 g/kg dry matter in CHI×Del/Ros1 and Del/Ros1, respectively. Enrichment of tomato fruit with such new and high anthocyanin contents may provide a potential health-promoting benefit. For the future, studies performed using in vivo and in vitro (animal and cellular) experimental model systems are critical components of the effort to identify anti-proliferation and anti-inflammatory effects. Experimental studies permit the evaluation of possible chance of elongating lifespan and inhibiting cancer cell growth under experimental group and control group. The new transgenic purple tomatoes, CHI×Del/Ros1, may contribute to functional food as an excellent therapeutic agent for preventing cancer in human.

3.2 Reference

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Table 3.1 MS spectrum of each individual anthocyanin

Peak identification	[M+H] ⁺ (<i>m/z</i>)	Detected fragments
1. Delphinidin-3-(caffeoyl)-rutinoside-5-glucoside	935	772.57 (M+Glc), 464.52 (dpd+Glc), 302.19 (dpd)
2. Delphinidin-3-(trans-coumaroyl)-rutinoside-5-glucoside	919	757.44(M+Glc), 464.59 (dpd+Glc), 302.31 (dpd)
3. Delphinidin-3-(feruloyl)-rutinoside-5-glucoside	949	786.58 (M+Glc), 463.65 (dpd+Glc), 303.06 (dpd)
4. Petunidin-3-(trans-coumaroyl)-rutinoside-5-glucoside	933	771.51 (M+Glc), 478.64 (ptd+Glc), 316.31(ptd)
5. Petunidin-3-(feruloyl)-rutinoside-5-glucoside	963	799.59 (M+Glc), 477.90 (ptd+Glc), 316.29(ptd)
6. Malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside	947	784.59 (M+Glc), 492.65 (ptd+Glc), 330.72 (Mv)
7. Malvidin-3-(feruloyl)-rutinoside-5-glucoside	976	814.55 (M+Glc), 492.62 (ptd+Glc), 330.70 (Mv)

Characteristics of the Anthocyanins Found in transgenic purple tomatoes by mass spectrometry. The purified compounds were analyzed by HPLC /MS. Glc, glucose; dpd, delphinidin; ptd, petunidin; Mv, Malvidin.

Table 3.2 Total anthocyanin content of *CHI*×*Del/Ros1* and *Del/Ros1*.

	Whole Fruit	Peel	Flesh	Seed
Wild	UD	UD	UD	UD
<i>Del/Ros1</i>	4.95 ±0.42	5.09 ±0.62	5.56 ±0.29	UD
<i>CHI</i> × <i>Del/Ros1</i>	9.61 ±0.71	29.89 ±1.64	8.65v ±0.39	UD

Total anthocyanin content of *CHI*×*Del/Ros1* was 9.61g PN3GE/kg dw, and it was significantly higher than *Del/Ros1* which was 4.95g PN3GE/kg dw ($p < 0.001$).

Table 3.3 Individual anthocyanin content from each part of transgenic tomatoes

<i>mg/kg dry matter</i>	<i>Del/Ros1</i>			<i>CHI × Del/Ros1</i>		
	Whole	Peel	Flesh	Whole	Peel	Flesh
Delphinidin3-(caffeoyl)-rutinoside-5-glucoside	133.03±5.76	84.80±8.64	151.32±10.38	212.02±10.82	220.75±13.74	258.41±11.38
Delphinidin3-(trans-coumaroyl)-rutinoside-5-glucoside	1380.18±144.01	1147.38±131.99	1596.35±99.77	2311.38±139.92	5001.07±360.60	2424.46±90.62
Delphinidin3-(feruloyl)-rutinoside-5-glucoside	51.88±6.93	26.77±2.02	63.19±2.88	68.18±5.15	46.64±5.94	89.79±12.22
Petunidin3-(trans-coumaroyl)-rutinoside-5-glucoside	2717.12±178.85	3322.41±430.90	2961.57±124.25	6146.79±531.64	22688.15±1214.85	4884.84±233.97
Petunidin 3-(feruloyl)-rutinoside-5-glucoside	433.47±86.20	233.33±14.48	542.67±23.79	404.49±21.82	345.86±40.38	603.45±38.47
malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside	234.46±13.45	270.88±28.15	252.59±29.95	466.02±34.32	1586.37±56.32	386.52±18.84
malvidin-3-(feruloyl) -rutinoside-5-glucoside	46.06±3.40	24.44±1.32	51.55±2.88	65.68±9.68	43.77±3.47	75.83±8.20

Table 3.4 Anthocyanins extraction methods

<i>Current method</i>	<i>Butelli's Method</i>
1. Frozen powdered fruit was extracted with 9:1 methanol/ Formic acid.	1. powdered plant material was extracted with 50% methanol and then with 100% methanol
2. 95% water,5% formic acid (solvent A) , 95% acetonitrile, 5% formic acid (solvent B)	2. 87% water, 3% acetonitrile (CAN) and 10% acetic acid (solvent A) or 40% water, 50% ACN and 10% acetic acid (solvent B)
3. flow rate of 0.8 ml/min	3.flow rate of 1 ml/min
4. solvent B gradient elution: 0min, 5%; 35min, 20%; 45min, 35%; 55min, 50%; 60min, 5%.	4. solvent B gradient elution: 0min, 6%; 20min, 20%; 35min, 40%; 40min, 60%; 45min, 90%; 60min, 6%

FIGURE LEGENDS

FIGURE 3.1. Phenotypes of wild-type (left column), Del/Ros1C (middle column) and Del/Ros1N (right column) tomato fruit harvested at the ripening stages.

FIGURE 3.2. Anthocyanin biosynthesis in tomato plants. PAL, phenylalanine ammonia lyase; CHI, chalcone isomerase; F3'5'H, flavonoid-3'5'-hydroxylase

FIGURE 3.3. HPLC chromatography of Anthocyanins.

FIGURE 3.4. Mass-spectrum of selected two new anthocyanins: peaks 6 and 7.

FIGURE 3.5. Structures of common anthocyanidins and anthocyanins



Figure 3.1 Phenotypes of wild-type (left column), Del/Ros1C (middle column) and Del/Ros1N (right column) tomato fruit harvested at the ripening stages.

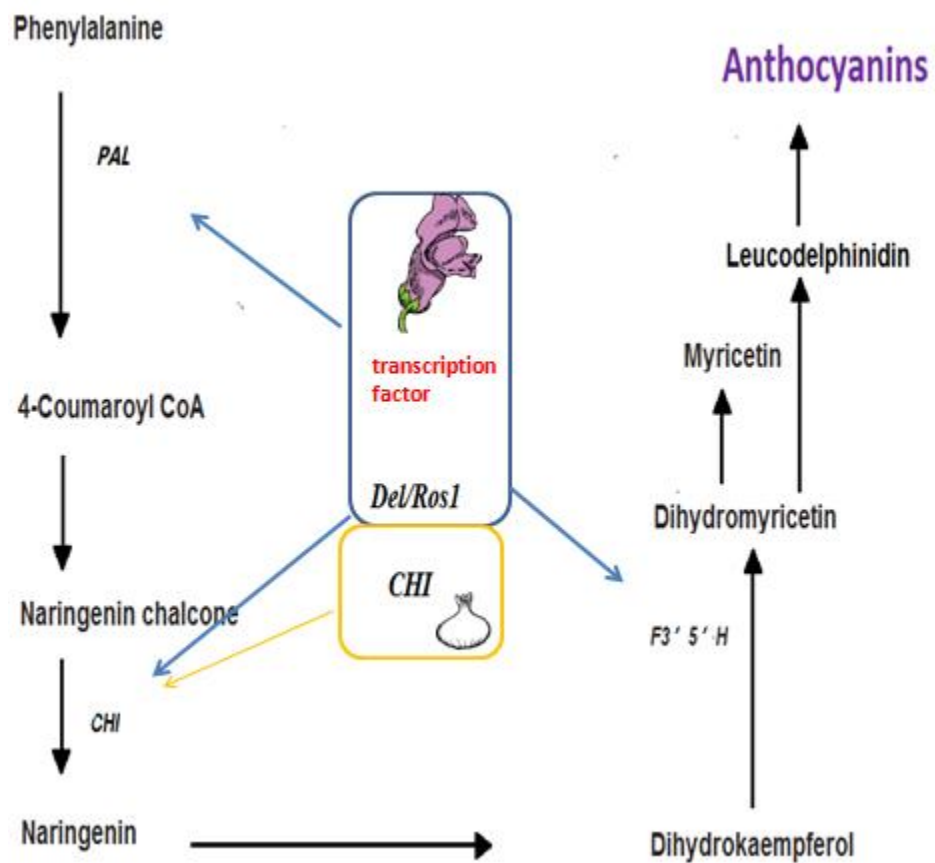


Figure 3.2 Anthocyanin biosynthesis in tomato plants. PAL, phenylalanine ammonia lyase; CHI, chalcone isomerase; F3'5'H, flavonoid-3'5'-hydroxylase.

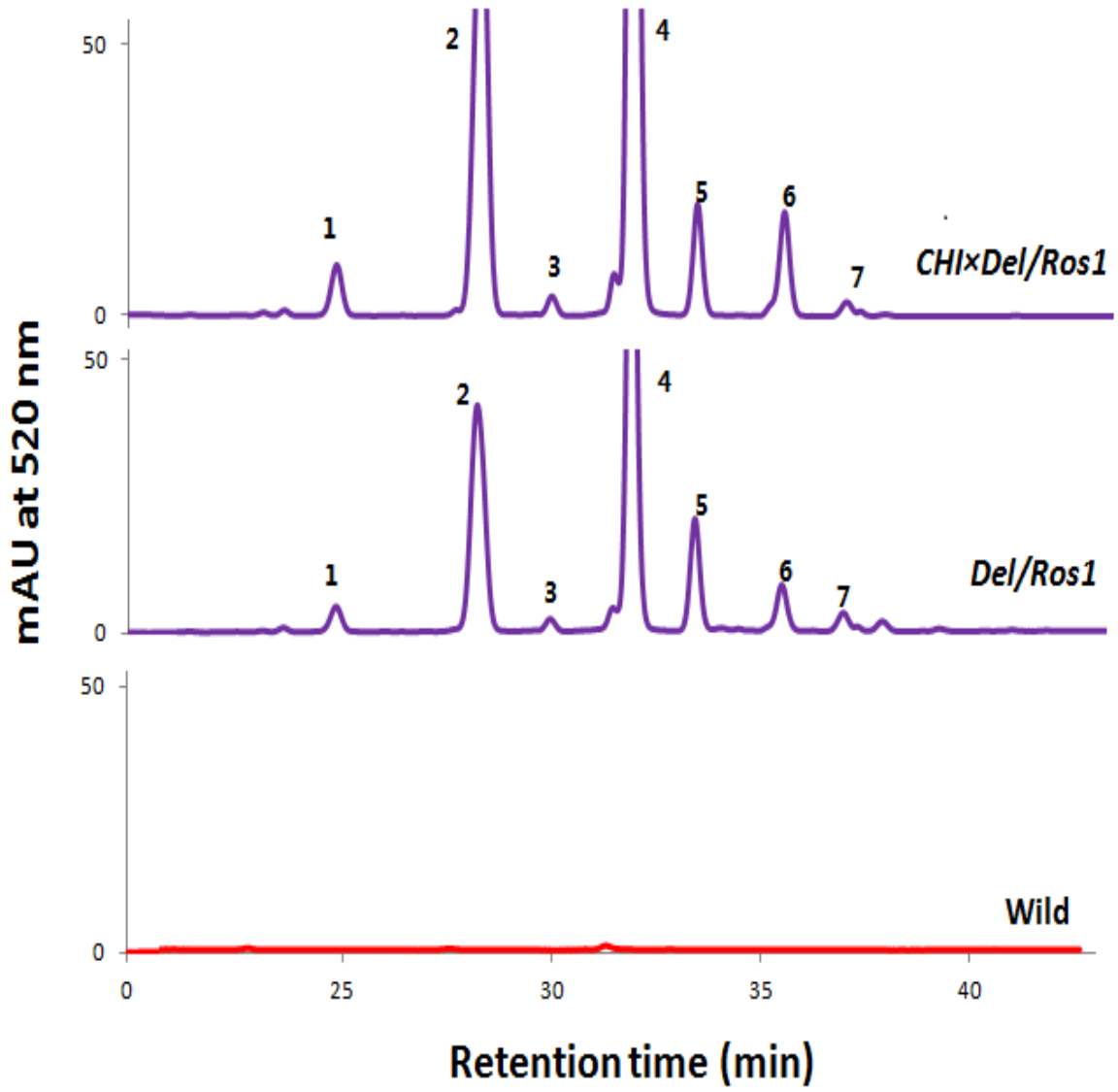
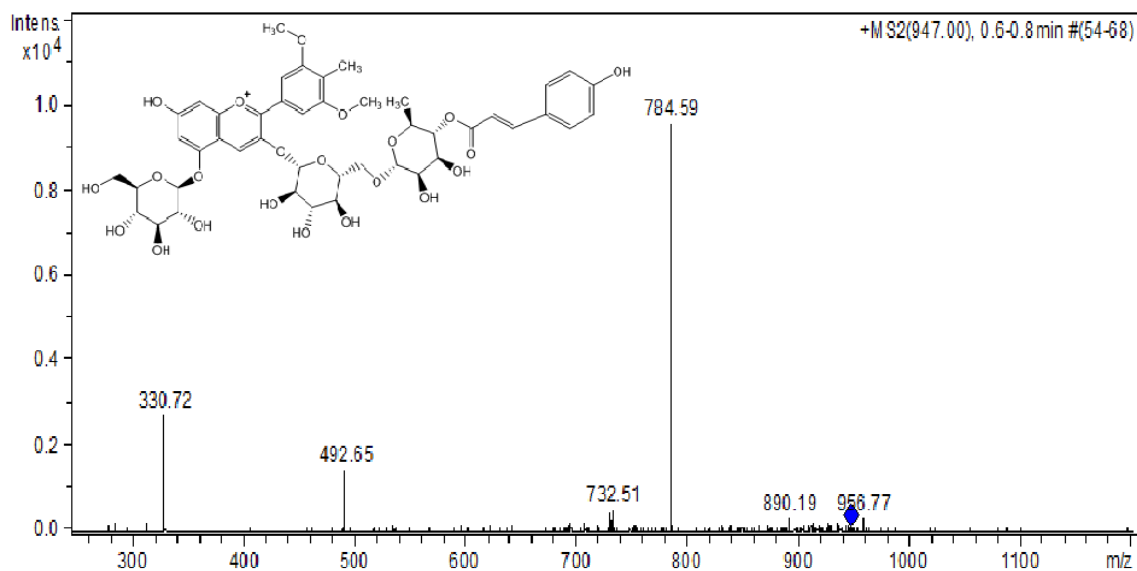


Figure 3.3 HPLC chromatography of Anthocyanins.

a) Peak 6: malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside



b) Peak 7: malvidin-3-(feruloyl)-rutinoside-5-glucoside

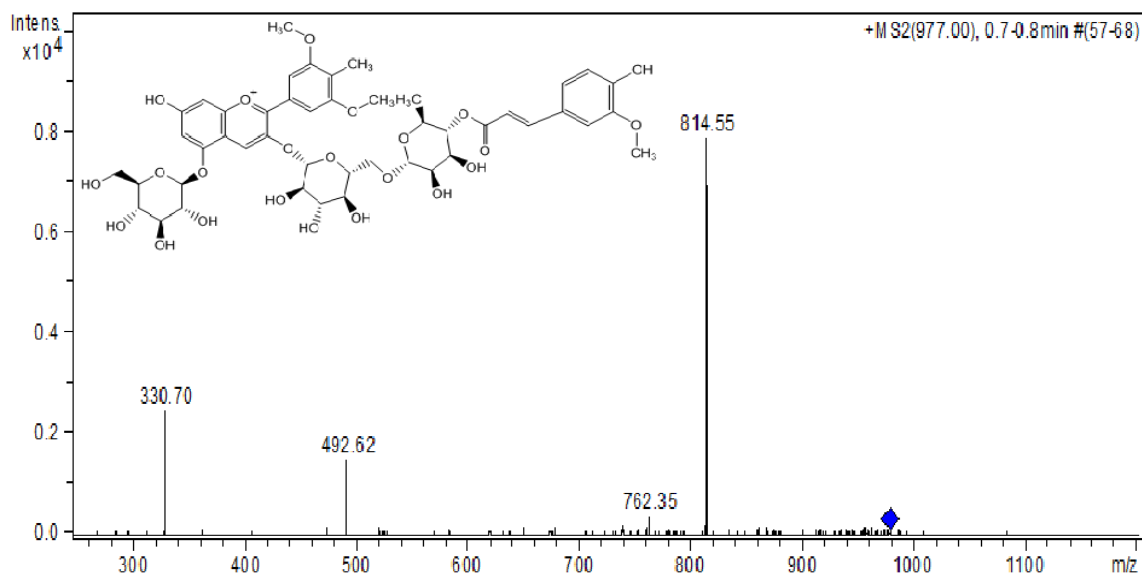
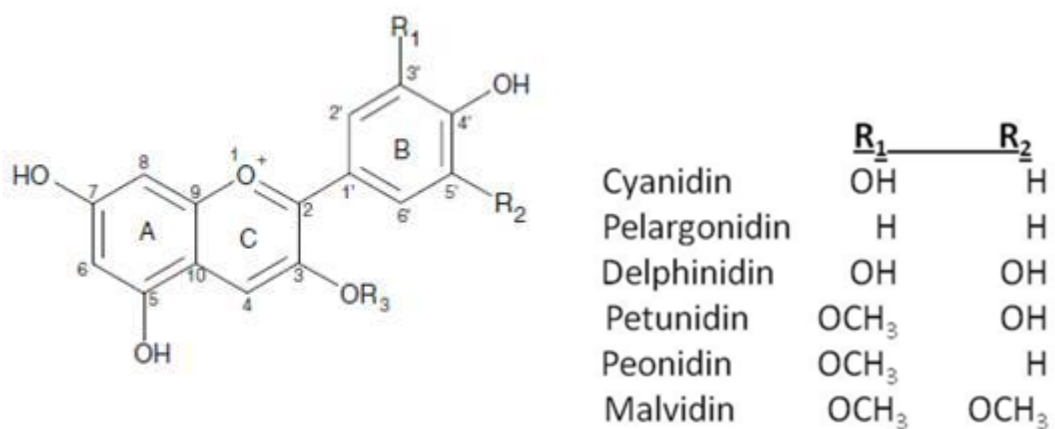


Figure 3.4 Mass-spectrum of selected two new anthocyanins: peaks 6 and 7.



R₃ = Glucose, galactose, rhamnose, xylose, or arabinose

Figure 3.5 Structures of common anthocyanidins and anthocyanins.