Identification and Quantification of Anthocyanins in Transgenic Purple Tomato

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Abbreviations used: *Del,* Delila; *F3'5'H, flavonoid 3'5'-hydroxylas; PAL, phenylalanine ammonia-lyase;* Ros1, Rosea1.

ABSTRACT: Anthocyanins are natural pigments derived from the phenylpropanoid pathway. Most tomatoes produce little anthocyanins, but the transgenic purple tomato biosynthesizes a high level of anthocyanins due to expression of two transcription factors (*Del* and *Ros1*). This study was to identify and quantify anthocyanins in this transgenic tomato line. Seven anthocyanins, including two new anthocyanins [malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside and malvidin-3-(feruloyl)-rutinoside-5-glucoside], were identified by LC-MS/MS. Petunidin 3-(trans-coumaroyl)-rutinoside-5-glucoside and delphinidin 3-(trans-coumaroyl)-rutinoside-5-glucoside were the most abundant anthocyanins, making up 86% of the total anthocyanins. Compared to undetectable anthocyanins in the wild type, the contents of anthocyanins in the whole fruit, peel, and flesh of the *Del*/*Ros1*-transgenic tomato were 5.2 ± 0.5, 5.1 ± 0.5, and 5.8 ± 0.3 g/kg dry matter, respectively. Anthocyanins were undetectable in the seeds of both wide-type and transgenic tomato lines. Such novel and high levels of anthocyanins obtained in this transgenic tomato may provide unique functional products with potential health benefits.

KEYWORDS: *Anthocyanins, transgenic tomatoes, Delila; Rosea1*

1. Introduction

The natural pigments produced in plants, including chlorophylls, carotenoids, and anthocyanins, are generally synthesized via phenylpropanoid and terpenoid pathways (Gonzali et al., 2009). Anthocyanins (derived from Greek *anthos* (flower) and *kyanos* (dark blue)) are one of the most important water-soluble plant pigments (Delgado-Vargas & Paredes-López, 2003). They are synthesized by the flavonoid branch of the phenylpropanoid pathway through secondary metabolism in higher plants. Among over 600 types of anthocyanins (Xu & Howard,

2012), the majority of anthocyanin aglycones found in nature consist of six anthocyanidins, i.e.,cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin. They share a 2-phenylbenzopyrilium (flavyl-ium) skeleton hydroxylated in 3, 5, and 7 positions, with different substitutions at R1 and R2 (Fig. 1). In comparison with other flavonoids, anthocyanins possess a positive charge on its C-ring, which leads to different colors in response to various pH (Wang & Stoner, 2008).

Anthocyanins present in human foods have received considerable attention due to their possible health-promoting properties such as antioxidant and anti-inflammatory effects (Lim et al, 2013; Bowen-Forbes et al, 2010). Based on food intake data from NHANES 2001-2002, the daily intake of anthocyanins was estimated to be 12.5 mg/day/person in the United States (Xu et al., 2006). The predominant dietary anthocyanins are malvidin, delphinidin, and peonidin glycosides (Bognar et al., 2013), which can be found in many plant foods, including berries, purple sweet potatoes, grapes, and wine.

Tomato (*Solanumlycopersicum L*.) is one of the most important food crops in the world. Its rich red color is due to accumulation of the carotenoid pigments, i.e., lycopene and phytoene, in the peel and flesh (Pannellini et al., 2004; Khachik et al., 2002). However, when compared to anthocyanin-enriched plants, tomatoes generally produce little anthocyanins. Genetic engineering is a powerful approach to induce and enhance biosynthesis of anthocyanins in plants (Schijlen et al., 2004), which has been successfully applied in food crops such as potato and rice (Tanakaand Ohmiya2008; Lukaszewicz et. al., 2004). Several transgenic tomatoes with increased flavonoid levels have also been developed. A transgenic tomato line created by expressing two maize regulatory genes, *Lc* and *C1*, was reported to produce a high level of flavonols rather than anthocyanins (Bovy et al., 2002). Overexpression of the *ANT1*gene encoding a MYB

transcription factor was further reported to induce a purple spotting on the epidermis of tomatoes (Mathews et al., 2003). In addition to the *ANT1* gene, combining the *atv* gene with either *Aft* or *Abg* was found to generate anthocyanin petunidin-3-(p-coumaryl)-rutinoside-5-glucoside predominantly in the epidermis of tomatoes up to 0.1% in fresh weight (Mes et al., 2008). Furthermore, expression of two snapdragon (*Antirrhinum majus*) transcription factors, i.e., Delila (*Del*) and Rosea1 (*Ros1*), in the fruit of transgenic tomatoes activated multiple anthocyanin biosynthesis-related genes, including phenylalanine ammonia-lyase (PAL), and flavonoid 3′5′-hydroxylase (F3′5′H) (Butelli et al., 2008). The *Del/Ros1* transgenic tomato grew normally during the green stage and then started to accumulate purple pigments during the ripening stage, exhibiting an intense and uniform purple color both in the peel and flesh (Butelli et al., 2008). According to the reported methods by Butelli et al. (2008), the Park lab has engineered and produced the *Del/Ros1* transgenic tomato line (Lim et al. 2014). Figure 2 shows the whole, cross-section, and freeze dry of ripe wild-type and transgenic *Del/Ros1* fruits.

The objectives of this study were to identify and quantify the anthocyanin profile in this transgenic tomato, and to determine the distribution of anthocyanins in the peel, flesh, and seed of the fruits.

2. Materials and methods

2.1. Materials

Acetonitrile, methanol (MeOH), and formic acid used in this study at either HPLC grade or analytic grade were purchased from Thermal fisher Scientific (Suwanee, GA). Water used was purified through Barnstead E-Pure Deionization System (Dubuque, IA) and filtered by Millpore

0.45 µm membrane (Bedford, MA). A standard of Peonidin-3-glucoside chloride was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Sample preparation and extraction

Wild type (*Solanum lycopersicum* L. cv Rubion) and *Del/Ros1* transgenic purple tomatoes generated in previous studies (Lim et al. 2014) were harvested in the Kansas State University Department of Horticulture greenhouses. For each line, ripe tomatoes were washed with tap water, diced into approximately 0.5 cm cubes, freeze-dried (Labconco, FreeZone 2.5), and ground by a food processor into powder. Prepared powder was then stored at -80℃ until further extraction. For preparation of anthocyanin extracts, 0.05 g of the powder was extracted with 4 mL of acidified MeOH with 1N formic acid at 9:1 (v/v). The flasks containing powder/solvent mixture were wrapped with aluminum foil to avoid light exposure. After a 12-hour extraction, the samples were centrifuged at 2,800 rpm for 30min and then the supernatant was collected and dried by vacuum drier at 25 °C overnight. One mL of the acidified MeOH was added and then the dissolved extract was filtered by Whatman syringe filter (Whatman 0.45um PVDF) for LC-MS/MS analysis.

2.3. Identification and analysis of anthocyanins by LC-MS/MS

LC coupled Electrospray Ionization tandem Mass Spectrometry (LC-MS/MS) was used to carry out anthocyanin identification and quantification. A Shimadzu HPLC system (Kyoto, Japan) was used for chromatographic analysis and separation. This system employed a DGU-20A3 built in degasser, a LC-20AB solvent delivery pump, a SIL-20ACHT auto-sampler, a CTO-20AC column-holding oven, a CBM-20A communicator module, and a SPD-M20A Photodiode Array Detectors. A Waters (Milford, MA) C_{18} reversed phase column (250 mm length, 4.6 mm diameter) was used for anthocyanin separation. Data was analyzed using LC

106 solution software (Kyoto, Japan). Elution was performed with mobile phase A (5%formic acid in
107 de-ionized water) and mobile phase B (5% formic acid in acetonitrile/water 1:1 v: v). An
108 optimum column temperature was set at 25 °C. At a flow rate of 0.8mL/min, the gradient
109 conditions were set as follows: solvent B volume at 5-20% for 35min, 20-50% for 10min, and
110 held at 50% for 10 min before returning to 5% at 60 min. The detector performed a full spectrum
111 scan between 190-800nm, where 520 nm was used for monitoring anthocyanins. Peonidin-3-
112 glucoside was used as an internal standard for quantitation of extraction recovery and the
113 anthocyanin contents were expressed as peonidin 3-glucoside equivalent (PN3GE). Based on a
114 signal-to-noise ratio of 3:1 and the standard deviation of the lowest concentration of PN3G/slope
115 of the calibration line, the detection limit was estimated to be 2 pmol.
116 Mass spectrometric scan was performed on a Bruker Esquire 3000 in positive mode with
117 a scanning interval 500-1200 m/z. Nebulization was conducted at 350 °C aided by concurrent N_2
118 flow at 10 psi; capillary and cone voltages were set at 3.5 kV and 40 V; drying gas flow rate was
119 5 L/min. Mass of precursor ions and reactions of fragments loss were evaluated. Data were
120 analyzed using Bruker Hystar Post Processing software (Bruker Daltonics, GmbH, Billerica,
121 MA). The ESI/MS data was used to confirm the mass of each anthocyanin HPLC peak. The mass
122 spectrometry instrument was controlled by the esquire control 5.3 software (Bruker Daltonics,
123 GmbH, Billerica, MA) and the data were processed with Data analysis 3.3 software (Bruker
124 Daltonics, GmbH, Billerica, MA). Individual identification of each anthocyanin was
125 accomplished by comparison of HPLC retention time, absorbance spectra, and MS spectra with
126 our previously published anthocyanin data (Lim et al. 2013; Xu et al. 2015). The new
127 anthocyanins were identified by matching the mass spectral data with those from the National

Institute of Standards and Technology Mass Spectra Library data (NIST08, National Institute of Standards and Technology, Gaithersburg, MD, USA).

2.4. Statistical analysis

Data were analyzed using SAS statistical software, version 9.3 (SAS Institute, Cary, NC, USA). Results were evaluated by one-way ANOVA using a general linear model procedure followed by Tukey's post-hoc test. The results were presented as means ± SD, and a probability at $p \leq 0.05$ was considered significant.

3. Results and discussion

3.1. Chromatographic separation

The objectives of this study were focused on characterizing the anthocyanin profile in a transgenic purple tomato and quantifying the anthocyanin content in each part of the tomato using HPLC-MS/MS. The profile of the anthocyanin peaks from transgenic tomato *Del/Ros1* was shown by HPLC chromatogram in Fig. 3. While no anthocyanin peaks were detectable in the wild type, a total of seven peaks were eluted at the retention times between 23 and 38 min in the transgenic *Del/Ros1* fruits. Of these, peaks 2 and 4 were the major anthocyanins and their peak areas appeared to be more than half of the total anthocyanin peak areas.

3.2. Mass spectrometric identification

Following HPLC separation, LC-MS/MS data were characterized by monitoring the molecular ion characteristics for each peak. The m/z ratio of each intact anthocyanin and its daughter fragments are listed in Table 1. As shown in Table 1, delphinidin (Dpd m/z 302), petunidin (Ptd m/z 316), and malvidin (Mv m/z 331) were the three anthocyanidin aglycones detected in the transgenic tomato line. Five of the seven anthocyanins including delphinidins

(peaks 1-3) and petunidins (peaks 4-5) were reported previously by Bultelli et al. (2008). However, two new malvidins (peaks 6-7) were found in the transgenic tomato line for the first time. As shown in Figure 4a, the ions of peak 6, i.e., malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside (m/z 947), produced three fragments of m/z 785, 493, and 331. Transition 947 to 785 and 947 to 493 implied the loss of glucose (m/z 162) and p-coumaroyl (m/z 454), respectively. Transition 947 to 331 produced malvidin aglycone (m/z 331) caused by the loss of glucose and p-coumaroyl. In Figure 4b, malvidin-3-(feruloyl)-rutinoside-5-glucoside (peak 7) produced transitions of 977 to 815, 493, and 331 m/z. Transition 977 to 815 and 947 to 493 indicated the loss of glucose (m/z 162) and feruloyl (m/z 484), respectively, while transition 947 to 331 produced malvidin aglycone (m/z 331).

When compared with other glycosylated anthocyanidins, malvidins have been found to have stronger inhibitory effects on nitric oxide production in LPS/IFN-γ -activated RAW 264.7 mouse macrophage cells due to better absorption and better free radical scavenging activity (Wang and Mazza, 2002). In addition, possible health benefits of dietary malvidins have been reported because of anti-proliferative (Seeram & Zhang, 2003; Hyun & Chung, 2004) and anti-inflammatory activities (Jing et al. 2008; Wedick et al. 2012).

The reason why two new anthocyanins could be detected in the transgenic line may relate to the modified extraction and HPLC method that allowed for a distinct peak separation. In this study, 10% of formic acid was added to the methanol before extraction, creating a low pH environment for anthocyanin stabilization. The decrease of flow rate and solvent B ratio during the gradient elution might also provide better peak separation. Lastly, with a C18 stationary phase column, more polar solvent A and less polar solvent B mobile phase may carry out a better gradient elution.

3.3. Anthocyanin quantification in transgenic tomato

While anthocyanins were undetectable in the wild-type, the content of anthocyanins in the *Del/Ros1* transgenic tomato is equally distributed, with 5.1 ± 0.5 g/kg DW in the peel and 5.8 ± 0.3 g/kg DW in the flesh. Total anthocyanin contents in *Del/Ros1* are near 5.2 ± 0.5g PN3GE/kg DW, or 0.5% of dry weight, which is higher than some of the anthocyanin-enriched foods such as red raspberry (3.9 g/kg DW by Wang & Lin 2000), strawberry (3.2 g/kg DW by Wang & Lin 2000), and mulberry (2.1 g/kg DW by Bae & Suh, 2007). Anthocyanins were undetectable in the seeds of both wide-type and transgenic tomato lines.

Table 2 lists the content profile of individual anthocyanin in the whole, peel, and flesh of the transgenic tomato line. The predominant anthocyanins were delphinidin-3-(trans-coumaroyl)-rutinoside-5-glucoside and petunidin-3-(trans-coumaroyl)-rutinoside-5-glucoside, which contributed to nearly 86% of the total anthocyanins. The reason why they are the highest among all the anthocyanins is not clear, but it may be due to the transgenic *Del/Ros1*-induced overexpression of the genes that relate to the specific anthocyanin biosynthesis pathway in which delphinidin-3-(trans-coumaroyl)-rutinoside-5-glucoside is an immediate precursor for petunidin-3-(trans-coumaroyl)-rutinoside-5-glucoside (Holton & Cornish 1995). Two new anthocyanins, malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside and malvidin-3-(feruloyl)-rutinoside-5-glucoside made up 6% of the total anthocyanins.

In conclusion, seven anthocyanins, including 2 new anthocyanins, have been identified in the *Del/Ros1* transgenic tomato. Compared to undetectable anthocyanins in the wild type, the *Del/Ros1* transgenic tomato produced a high level of anthocyanins that may provide unique functional products with potential health benefits.

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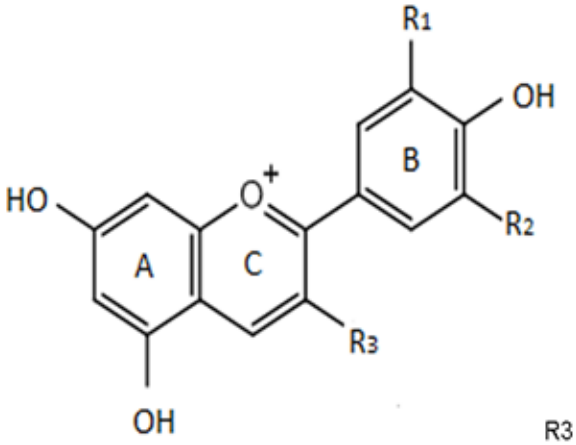
Figure Legends

Figure 1. Structures of common anthocyanidins and anthocyanins

Figure 2. Representative images of the whole, cross-section, and freeze dry of the ripe wild-type (left column) vs. the transgenic *Del/Ros1* tomato fruit (right column).

Figure 3. Representative HPLC chromatograms of anthocyanins in the wild-type and the transgenic *Del/Ros1* tomatoes (the peak number corresponding to anthocyanin name is shown in Table 1).

Figure 4. Mass spectrometric data of two new malvidins detected in the transgenic *Del/Ros1* purple tomato: a) Malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside; b) malvidin-3- (feruloyl) -rutinoside-5-glucoside (Mv, Malvidin; Glc, glucose; 3FR, 3-(feruloyl)-rutinoside; 3PR, 3-(p-coumaroyl)-rutinoside).



	R1	R2
Cyanidin	OH	H
Pelargonidin	H	H
Delphinidin	OH	OH
Petunidin	OCH3	OH
Peonidin	OCH3	H
Malvidin	OCH3	OCH3

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

Figure 1

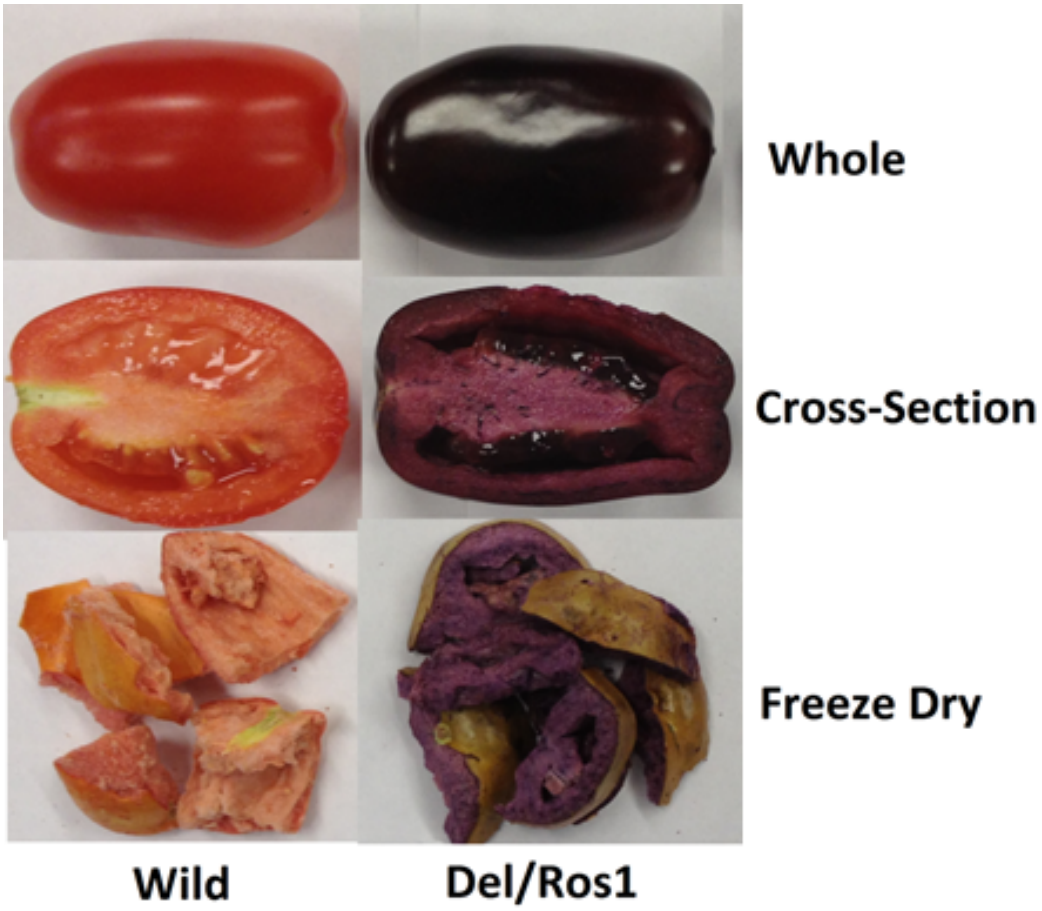


Figure 2

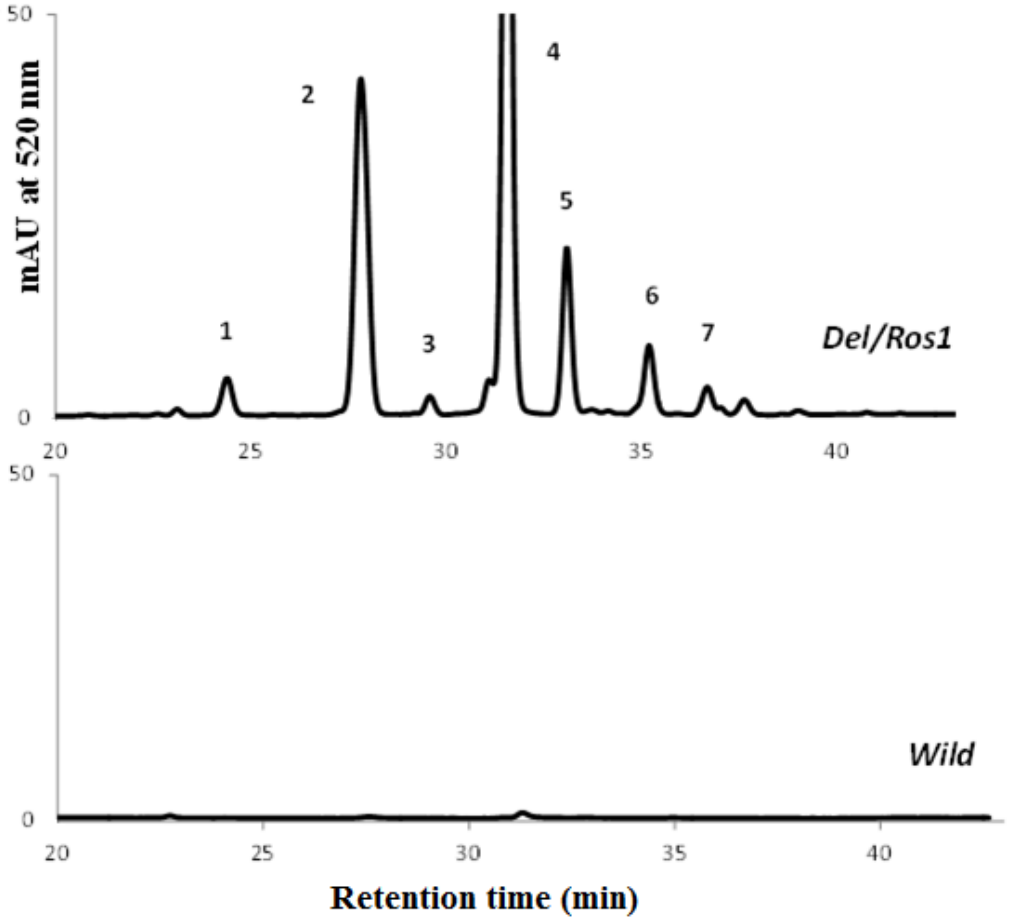

mAU at 520 nm
50
0
20
25
30
35
40
1
2
3
4
5
6
7
Del/Ros1
Wild
Retention time (min)

Figure 3

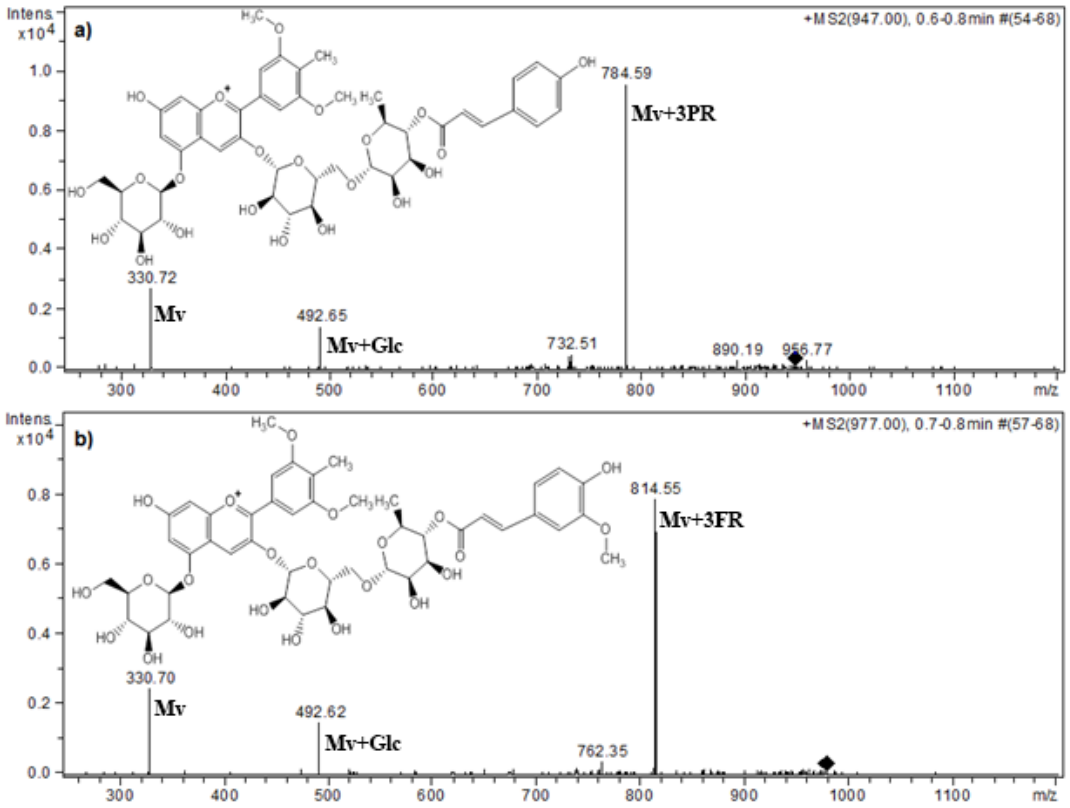


Figure 4