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Short communication

Rapid analysis of anthocyanin and its structural modifications in fresh tomato fruit

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ABSTRACT

Anthocyanin is derived from a flavylium cation structure, and it promotes health in humans and functions in plants as protection against environmental stress. The rapid analysis of anthocyanin structure and content is a critical challenge for improving fruit quality. In this study, the tomato cultivar Indigo Rose, which is a popular purple cultivated tomato used for breeding, was taken as an example for anthocyanin analysis. A rapid analysis method was developed to minimize anthocyanin loss from the fresh fruit. Four new anthocyanins were discovered in the tomato, and the structures of a total of 12 anthocyanins were determined. Among these, petunidin-3-(*trans-p*-coumaroyl)-rutinoside-5-glucoside and malvidin-3-(*trans-p*-coumaroyl)-rutinoside-5-glucoside were the main anthocyanins in Indigo Rose. The structural modifications of these anthocyanins were mainly glycosylation and acylation, and there were also hydroxylation and methylation. Our findings provide new insight into the biosynthesis pathway in tomato fruit.

1. Introduction

Anthocyanin is a health-promoting component (Blando, Berland, Maiorano, Durante, Mazzucato, Picarella, Nicoletti, Gerardi, Mita, & Andersen, 2019; Butelli, Titta, Giorgio, Mock, Matros, Peterek, & Martin, 2008) due to its oxygen radical absorbing capacity (da Silva, Paulo, Barbafina, Eisei, Quina, & Macanita, 2012), which also has potential pharmacological applications (Hyun & Chung, 2004; Seeram, Zhang, & Nair, 2003). In plants, it improves their adaptation to stress (Li, Li, Zhang, Zhang, Jiang, Yu, & Hou, 2017). Therefore, significant interest has been focused on anthocyanins, including their identification, isolation, and structural confirmation (Smidova, Satinsky, Dostalova, & Solich, 2017). The main anthocyanins in plants are based on a flavylium cation structure, including delphinidin, cyanidin, petunidin, peonidin, pelargonidin and malvidin (Alejo-Armijo, Salido, Altarejos, Parola, Gago, Basilio, & Pina, 2016; Pan, Liu, Liu, & Wang, 2019; Xu, Luo, Yu, Jia, Zheng, Bi, & Lei, 2018). Anthocyanins structure analysis is important, however, it is also challenging due to the structural similarities of these compounds.

Anthocyanin is generated through flavonoid biosynthesis from the phenylpropanoid pathway (Sun, Deng, Du, Zhao, Chen, Huang, & Li, 2020), as shown in Fig. 1 (Watkins, Chapman, & Muday, 2017; Zhang, Butelli, Alseekh, Tohge, Rallapalli, Luo, Kawar, Hill, Santino, Fernie, & Martin, 2015). Phenylalanine is catalyzed by phenylalanine ammonia-lyase (PAL) to synthesize cinnamic acid, which is then further converted into coumaric acid by cinnamic acid 4-hydroxylase (CA4H). The 4-coumaroyl CoA is synthesized from coumaric acid by 4-coumarate-CoA ligase (4CL) (Zhang, Butelli, Alseekh, Tohge, Rallapalli, Luo, Kawar, Hill, Santino, Fernie, & Martin, 2015). Then, 4-coumaroyl CoA and malonyl CoA are converted into naringenin chalcone by chalcone synthase (CHS), which is transformed into naringenin by chalcone isomerase (CHI). Afterward, flavanone 3-hydroxylase (F3H) oxidizes naringenin into dihydrokaempferol (Watkins, Chapman, & Muday, 2017). Next, dihydrokaempferol is transformed into myricetin, kaempferol, quercetin or isorhamnetin by flavonol synthase (FLS), flavonoid 3′-hydroxylase (F3′H), flavonoid 3′,5′-hydroxylase (F3′5′H), or flavone 3′-O-methyltransferase 1 (OMT1). Alternatively, dihydrokaempferol is reduced by dihydroflavonol reductase (DFR), and then it is further converted into anthocyanidin by anthocyanidin synthase (ANS). Anthocyanidin is not stable and is modified into a stable form by glycosylation (Zhang, Li, Li, Hu, Yu, Tu, Zhang, Huang, & Chen, 2019). Finally, the anthocyanins are transported into vacuoles.

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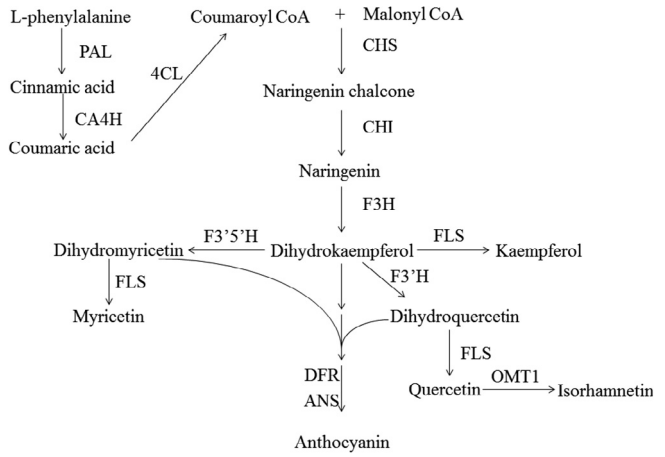

Fig. 1. The biosynthetic pathway for flavonoid is demonstrated with enzymatic steps, which is modified from previously studies (Watkins, Chapman, & Muday, 2017; Zhang, Butelli, Alseekh, Tohge, Rallapalli, Luo, Kawar, Hill, Santino, Fernie, & Martin, 2015). PAL, phenylalanine ammonia-lyase; 4CL, 4-coumarate-CoA ligase; CA4H, cinnamic acid 4-hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; OMT1, flavone 3′-O-methyltransferase 1; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase.

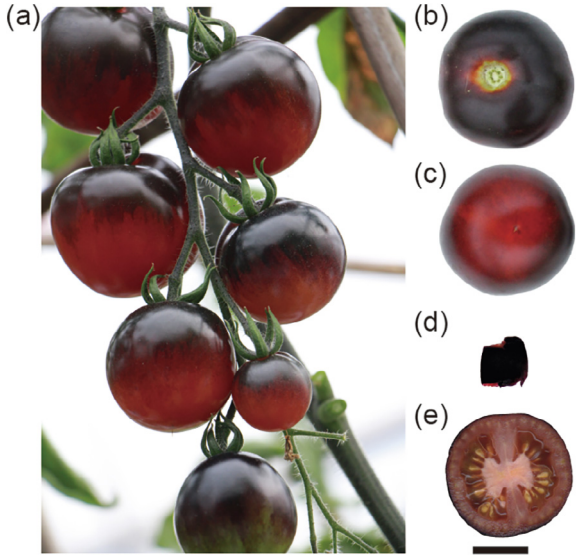

Fig. 2. Photographs of the tomato cultivar Indigo Rose at the red ripe fruit stage. The fruit is shown on the plant (a), in top view (b), in upward view (c), after peeling (d) and in cross section (e). The scale bar is 2 cm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The anthocyanins in tomato (*Solanum lycopersicum*) fruit are biosynthesized in a light-dependent manner (Catola, Castagna, Santin, Calvenzani, Petroni, Mazzucato, & Ranieri, 2017). Nine anthocyanins have been identified with particular structures, including delphinidin-3-(caffeoyl)-rutinoside-5-glucoside, delphinidin-3-(*trans*-coumaroyl)-rutinoside-5-glucoside, delphinidin-3-(feruloyl)-rutinoside-5-glucoside, petunidin-3-(*trans*-coumaroyl)-rutinoside-5-glucoside, petunidin-3-(feruloyl)-rutinoside-5-glucoside, malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside, malvidin-3-(feruloyl)-rutinoside-5-glucoside, petunidin-3-(caffeoyl)-rutinoside-5-glucoside, and delphinidin 3-(caffeoyl)-rutinoside-5-glucoside (Gomez Roldan, Outchkourov, van Houwelingen, Lammers, Romero de la Fuente, Ziklo, & Beekwilder, 2014; Li, Deng, Liu, Young, Zhu, Loewen, & Tsao, 2011; Su, Xu, Rhodes, Shen, Song, Katz, & Wang, 2016; Tohge, Zhang, Peterek, Matros, Rallapalli, Tandron, & Fernie, 2015). Among them, petunidin-3-(*trans-p*-coumaroyl)-rutinoside-5-glucoside is the main anthocyanin (Kiferle, Fantini, Bassolino, Povero, Spelt, Buti, & Gonzali, 2015; Su, Xu, Rhodes, Shen, Song, Katz, & Wang, 2016).

Tomato is a popular fruit, but the levels of its anthocyanins are suboptimal (Sun, Deng, Du, Zhao, Chen, Huang, & Li, 2020). Thus, it is necessary to optimize its anthocyanin content by investigating its anthocyanin regulatory mechanism (Qiu, Wang, Li, Yu, Hui, Yan, & Cao, 2019; Yan, Chen, Huang, Li, Zhi, Yu, & Qiu, 2019). Although many researchers have attempted to improve the anthocyanin content in the epidermis and pericarp of tomato fruit (Anh Thang & Lee, 2019; Sun, Deng, Du, Zhao, Chen, Huang, & Li, 2020), its precise manipulation is still unavailable. One important reason for this is an inadequate interpretation of individual anthocyanins' detailed structures in the anthocyanin biosynthesis pathway. Each anthocyanin has different structural modifications, including hydroxylation, methylation, glycosylation and acylation (Gomez Roldan, Outchkourov, van Houwelingen, Lammers, Romero de la Fuente, Ziklo, & Beekwilder, 2014). Hydroxylation and methylation are structural modifications with hydroxyl and methyl groups, respectively. Anthocyanin glycosylation refers to modification with glucoside on the hydroxyl group of the flavylium cation structure (Zhang, Li, Li, Hu, Yu, Tu, Zhang, Huang, & Chen, 2019). Additional hydroxyl group esterification of the anthocyanin glycosyl results in the acylation, commonly by aliphatic or aromatic acids (Zhao, Yu, Chen, Wen, Wei, Zheng, & Xiao, 2017). These modifications increase the chemical stability of anthocyanins. Notably, the acylation sites and number of acyl groups influence the stability of anthocyanins to different degrees (Luo, Nishiyama, Fuell, Taguchi, Elliott, Hill, & Martin, 2007).

Rapid determination of the anthocyanin structure and its content is significantly influenced by the pretreatment process. In previous studies, lyophilization was applied for pretreatment before extraction (Jeong, Zhao, Jin, Heo, Han, Yoo, & Lee, 2015; Ji, Li, & Li, 2015; Su, Xu, Rhodes, Shen, Song, Katz, & Wang, 2016), which partly damaged the anthocyanin content during the process. To purify the anthocyanins after extraction, column chromatography techniques have been applied using specialized column. Alternatively, solid phase extraction was used with specialized cartridge following the process of activation, equilibration, sample loading, washing and elution (Li, Deng, Liu, Young, Zhu, Loewen, & Tsao, 2011; Mes, Boches, Myers, & Durst, 2008; Minh Anh Thu, 2019; Pan, Liu, Liu, & Wang, 2019). These treatment processes cause the loss of anthocyanins, such as malvidin derivatives. To obtain enough anthocyanin after a long purification process, a large amount of material is needed for the analysis, such as 1 kg tomato peel (Blando, Berland, Maiorano, Durante, Mazzucato, Picarella, Nicoletti, Gerardi, Mita, & Andersen, 2019). In addition, large volumes of extraction solvent are required for adequate extraction (Tohge, Zhang, Peterek, Matros, Rallapalli, Tandron, & Fernie, 2015). Moreover, acid in the extraction buffer could enhance the stability of the anthocyanin compound in the form of a flavylium cation without degradation (Ongkowijoyo, Luna-Vital, & Gonzalez de Mejia, 2018). However, hydrochloric acid might lead to hydrolysis of the glycosidic bond of anthocyanins (Liu, Zhang, Siems, Hill, & Yin, 2015).

In this work, we developed a rapid method to determine anthocyanin content and interpreted the structures of the anthocyanin compounds in tomato. Our process uses a reduced solvent volume, has a low environmental impact, and has high extraction yield. We used a cultivated tomato, Indigo Rose, to analyze its anthocyanins. This cultivar is frequently used in the study of anthocyanin (Ooe, Ogawa, Horiuchi, Tada, Murase, Tsuruma, & Hara, 2016; Sun, Deng, Du, Zhao, Chen, Huang, & Li, 2020). Four new anthocyanins were discovered using our rapid analytical method. Based on their structural modifications, anthocyanins are mainly modified by glycosylation and acylation in tomato. A biosynthetic pathway of anthocyanin was deduced in tomato fruit. Our work provides new insight into the anthocyanin

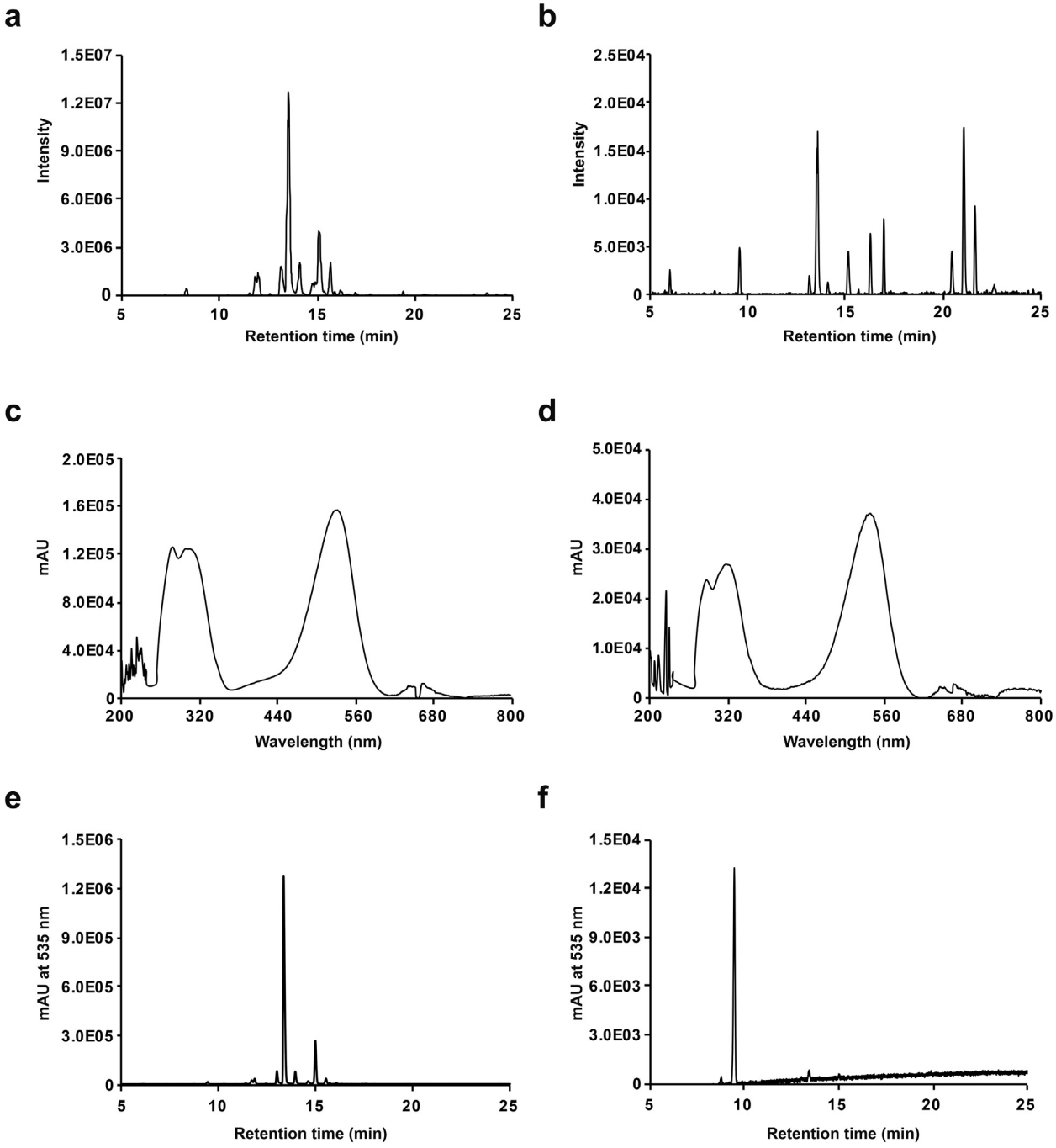

Fig. 3. Liquid chromatograms of quadrupole time-of-flight mass were acquired for anthocyanins in the fruit peel (a) and flesh (b) of the tomato cultivar Indigo Rose. The UV spectra for each anthocyanin were obtained and are shown for anthocyanin at retention time 13.35 min (c) and 14.99 min (d). The corresponding chromatograms of ultraviolet were acquired at 535 nm for fruit peel (e) and flesh (f).

biosynthesis pathway in tomato fruit.

2. Materials and methods

2.1. Plant materials

The seeds of the tomato Indigo Rose were obtained from Johnny's Selected Seeds (http://www.johnnyseeds.com/). The plants were grown in a plastic glasshouse in Guangzhou, Guangdong, in autumn 2018. Each replicate consisted of five red fruits (Fig. 2a–e) from different plants, and three biological replicates were used for each sample.

2.2. Chemicals and reagents

Peonidin-3-glucoside chloride was purchased from Sigma-Aldrich. While methanol, acetonitrile and formic acid were purchased from Fischer Scientific. Water was supplied by a Milli-Q Advantage A10 ultra purification system. Sodium chloride was purchased from Beijing Chemical Works. All chemicals were used as received.

2.3. Pretreatment of fresh tomato fruit for anthocyanin analysis

The pretreatment process was modified from previous studies (Fibigr, Šatínský, & Solich, 2017; Su, Xu, Rhodes, Shen, Song, Katz, &

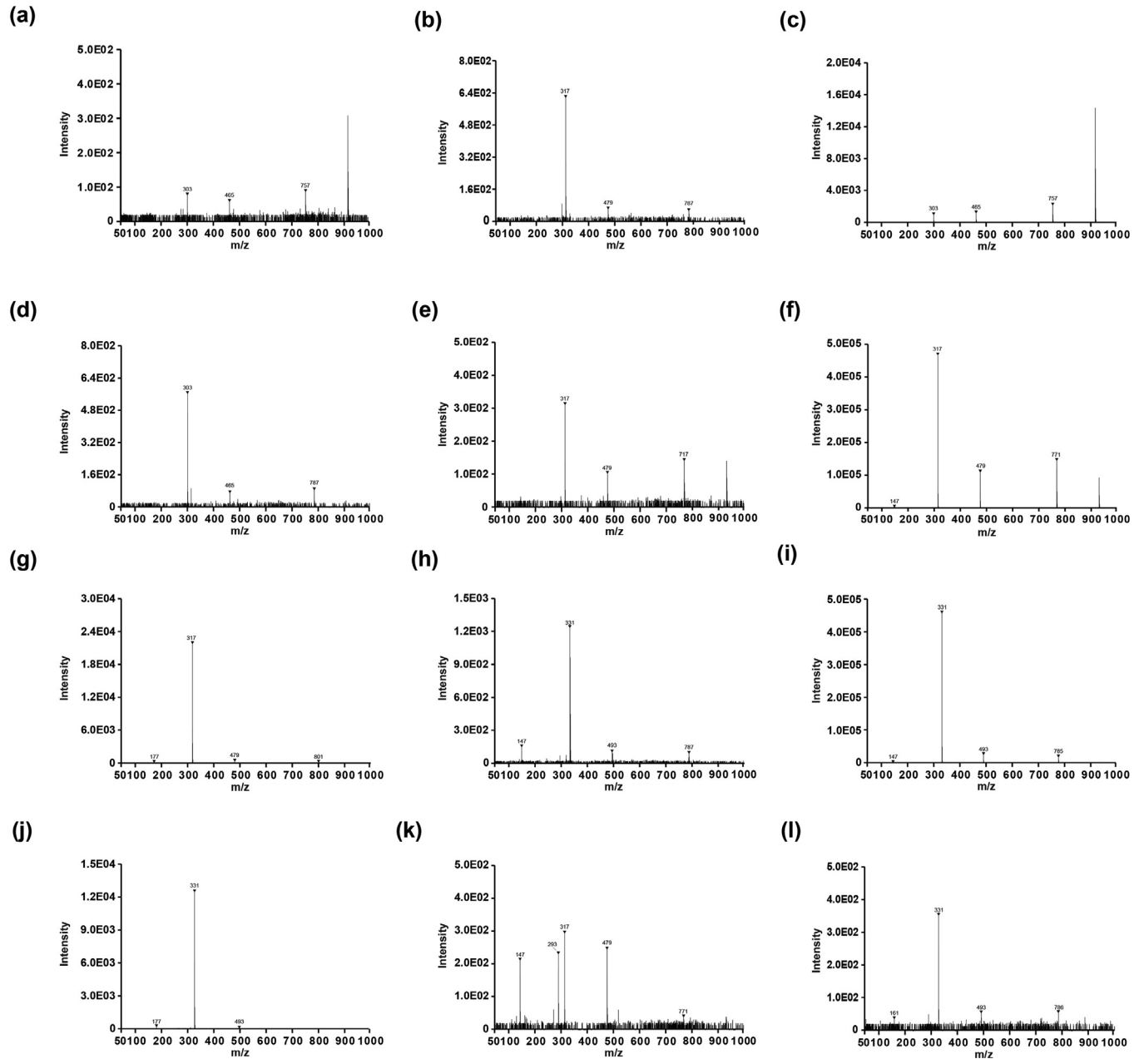

Fig. 4. The MS^2 fragments of the 12 anthocyanins (from a to i).

Wang, 2016). In detail, the fruits were washed with water after sampling and then peeled. The peel and flesh were manually ground with liquid nitrogen into powder in a mortar immediately after harvest. Afterward, 0.4 g sample was extracted with 4 ml methanol/formic acid (9:1, v/v) in a brown glass tube to avoid light exposure. At the same time, 15 μg peonidin-3-glucoside chloride was added to the sample as an internal standard for quantitation of the anthocyanin content, which were expressed as its equivalent. The sample was extracted for 12 h at 4 °C after homogeneous agitation. Then, the sample was centrifuged at 3900 rpm for 20 min, and 2 ml of the supernatant was transferred into another brown glass tube. The extraction solution was evaporated under nitrogen gas flow, and 1 ml methanol/formic acid (9:1, v/v) was added to the dried extract to dissolve the target component. The dissolved solution was filtered through a 0.22 μm nylon membrane for further analysis.

2.4. UPLC-QTOF-MS conditions for anthocyanin analysis

UPLC-QTOF-MS was used for anthocyanin determination in this study, with a method modified from previous studies (Fibigr, Šatínský, & Solich, 2017; Thu, 2019). A Waters ACQUITY UPLC I-Class-Xevo G2-XS QTOF/PDA eLambda Detector was used. An ACQUITY UPLC CSH C18 (2.1 mm × 100 mm i.d., 1.7 μm) column was used for analysis. Acetonitrile with 5% formic acid was mobile phase A and water with 5% formic acid was mobile phase B. The run was at a flow rate of 0.15 ml/min with initial 2.5% A. Solvent A changed from 2.5% to 10% in 5 min, then from 10% to 25% in 15 min and held at 25% for 5 min, returning from 25% to 2.5% in 5 min. The column and FTN temperatures were 25 °C and 20 °C, respectively. The photo-diode array (PDA) wavelength range was from 190 nm to 800 nm. The injection volume was 1 μl for all samples. Mass was set in positive ion mode for anthocyanin identification. The ESI source parameters were set as follows: eletrospray capillary voltage, 2.0 kV; source temperature, 100 °C;

Table 1
The identified anthocyanins in liquid chromatograms and detected mass fragments in the tomato cultivar Indigo Rose.

No.	Retention time for UV (min)	Retention time for MS (min)	Putative identification	$[M + H]^+$ (*m/z*)	MS^2 fragments
A	11.41	11.55	Delphinidin-3-(*cis*-*p*-coumaroyl)-rutinoside-5-glucoside	919	757 (Dpd + 3*cis*-pCouR), 465 (Dpd + 5Glc), 303 (Dpd)
B	11.71	11.83	Petunidin-3-(caffeoyl)-rutinoside-5-glucoside	949	787 ([Ptd + 3CaR), 479 (Ptd + 5Glc), 317 (Ptd)
C	11.86	11.97	Delphinidin-3-(*trans*-*p*-coumaroyl)-rutinoside-5-glucoside	919	757 (Dpd + 3*trans*-pCouR), 465(Dpd + 5Glc), 303 (Dpd)
D	12.45	12.58	Delphinidin-3-(feruloyl)-rutinoside-5-glucoside	949	787 (Dpd + 3FerR), 465 (Dpd + 5Glc), 303 (Dpd)
E	**13.01**	**13.16**	**Petunidin-3-(*cis*-*p*-coumaroyl)-rutinoside-5-glucoside**	**933**	**771 (Ptd + 3*cis*-pCouR), 479 (Ptd + 5Glc), 317 (Ptd)**
F	13.35	13.52	Petunidin-3-(*trans*-*p*-coumaroyl)-rutinoside-5-glucoside	933	771 (Ptd + 3*trans*-pCouR), 479 (Ptd + 5Glc), 317 (Ptd), 147 (*trans*-*p*-coumaroyl)
G	13.95	14.11	Petunidin-3-(feruloyl)-rutinoside-5-glucoside	963	801 (Ptd + 3FerR), 479 (Ptd + 5Glc), 317 (Ptd), 177 (feruloyl)
H	**14.61**	**14.77**	**Malvidin-3-(*cis*-*p*-coumaroyl)-rutinoside-5-glucoside**	**947**	**787 (Mv + 3*cis*-pCouR), 493 (Mv + 5Glc), 331 (Mv), 147 (*cis*-*p*-coumaroyl)**
I	14.99	15.08	Malvidin-3-(*trans*-*p*-coumaroyl)-rutinoside-5-glucoside	947	785 (Mv + 3*trans*-pCouR), 493 (Mv + 5Glc), 331 (Mv), 147 (*trans*-*p*-coumaroyl)
G	15.33	15.68	Malvidin-3-(feruloyl)-rutinoside-5-glucoside	977	493 (Mv + 5Glc), 331 (Mv), 177 (feruloyl)
K	**15.73**	**15.90**	**Petunidin-3-(*trans*-*p*-coumaroyl-rhamonside)-glucoside-5-glucoside**	**933**	**771 (Ptd-3-Glc-pCouRha), 479 (Ptd + 5Glc), 317 (Ptd), 293 (*trans*-*p*-coumaroyl-Rha), 147 (*trans*-*p*-coumaroyl)**
L	**16.07**	**16.18**	**Malvidin-3-(*p*-methoxy-*trans*-coumaroyl)-rutinoside-5-glucoside**	**961**	**786 (Mv + 3*trans*-pCouR), 493 (Mv + 5Glc), 331 (Mv), 161 (*p*-methoxy-*trans*-coumaroyl)**

*Dpd, delphinidin; Ptd, petunidin; Mv, malvidin; Glc, glucoside; Rha, rhamonside; pCouR, (*p*-coumaroyl)-rutinoside; CafR, (caffeoyl)-rutinoside; FerR, (feruloyl)-rutinoside.

desolvation temperature, 300 °C; desolvation gas flow, 800 L/h; cone gas flow, 50 L/h. The mass acquisition was in Fast DDA for 30 min. The analyzer was in resolution mode. The precursor molecular ion mass was acquired over the range from 900 m/z to 1000 m/z in a continuum mode. The MS/MS for fragments were over the range from 50 m/z to 1000 m/z in a continuum mode. A maximum of 5 MS/MS ions were acquired for identification. The collision ramp was used to obtain the fragments. The ramp range was from 6 V to 80 V.

3. Results and discussion

3.1. Rapid analysis of anthocyanins

To simplify the pretreatment process and avoid lyophilization (Jeong, Zhao, Jin, Heo, Han, Yoo, & Lee, 2015; Ji, Li, & Li, 2015; Su, Xu, Rhodes, Shen, Song, Katz, & Wang, 2016), tomato fruit tissues were frozen in liquid nitrogen immediately after harvest, to keep their original state and to quench all biosynthetic processes. An internal standard was added to the extraction solvent to evaluate the extraction recovery and to improve quantitative accuracy while using lower amounts of extraction solvent (Tohge, Zhang, Peterek, Matros, Rallapalli, Tandron, & Fernie, 2015). Formic acid was chosen for pretreatment and UPLC- QTOF/PDA analysis to enhance the anthocyanin stability and to avoid hydrolysis of the glycosidic bond (Liu, Zhang, Siems, Hill, & Yin, 2015; Ongkowijoyo, Luna-Vital, & Gonzalez de Mejia, 2018). After extraction, the purification was finished with centrifugation and filtration with a 0.22 μm nylon membrane, instead of expensive column chromatography or solid phase extraction (Li, Deng, Liu, Young, Zhu, Loewen, & Tsao, 2011; Mes, Boches, Myers, & Durst, 2008; Thu, 2019; Pan, Liu, Liu, & Wang, 2019). The precursor molecular ion mass was acquired over a range from 900 m/z to 1000 m/z to minimize the background, which was adjustable according to different crops.

We analyzed the anthocyanins in the fruit peel and flesh of Indigo Rose, one of the cultivated tomatoes with anthocyanins enrichment in its peel (Fig. 2a). The mass chromatograms for peel and flesh were acquired (Fig. 3a and b). To ensure efficiency and accuracy, UV and mass were synchronously used for anthocyanin analysis in our work. The main anthocyanins were at the retention times 13.52 min and 15.08 min in mass chromatogram straight after ultraviolet (UV) absorption, whose spectra were gathered at 13.35 min and 14.99 min in the UV chromatogram (Fig. 3c and d). Both of them had maximum UV absorption at 535 nm. Therefore, the determinative wavelength was 535 nm for all anthocyanins. The UV chromatograms for peel and flesh at 535 nm were acquired (Fig. 3e and f). After analyzing the mass and UV chromatogram, a total of 12 anthocyanins were found in Indigo Rose. When using our simplified analytical method, a 0.4 g fresh sample of tomato fruits will be enough for analysis. Thus, a simplified method has been developed, which is suitable for tomato anthocyanins analysis and can be completed in one day.

3.2. Anthocyanin structural modifications of tomato fruit

To obtain the detailed structures of the detected anthocyanins, the MS^2 fragments of the 12 anthocyanins were further analyzed (Fig. 4 and Table 1). Based on their structure, we found that petunidin-3-(*trans*-*p*-coumaroyl)-rutinoside-5-glucoside is the main anthocyanin in the peel of the Indigo Rose fruits, which is consistent with previous studies (Blando, Berland, Maiorano, Durante, Mazzucato, Picarella, Nicoletti, Gerardi, Mita, & Andersen, 2019; Su, Xu, Rhodes, Shen, Song, Katz, & Wang, 2016).

Various anthocyanin fragments analyses have previously been reported in tomato (Mes, Boches, Myers, & Durst, 2008). The modification sites and the numbers of modification groups were further analyzed, along with mass technology development. In this study, the detected 12 anthocyanins in the tomato fruits were found to be

Table 2
The individual anthocyanin contents (mg/kg FW) in the fruit flesh and peel of the tomato cultivar Indigo Rose.

No.	Component name	Fruit peel (mg/kg)	Fruit flesh (mg/kg)
A	Delphinidin-3-(*cis-p*-coumaroyl)-rutinoside-5-glucoside	14.86 ± 1.20	7.24 ± 0.93
B	Petunidin-3-(caffeoyl)-rutinoside-5-glucoside	47.84 ± 8.41	7.24 ± 0.93
C	Delphinidin-3-(*trans-p*-coumaroyl)-rutinoside-5-glucoside	67.59 ± 7.48	7.24 ± 0.90
D	Delphinidin-3-(feruloyl)-rutinoside-5-glucoside	13.86 ± 0.92	7.25 ± 0.91
E	Petunidin-3-(*cis-p*-coumaroyl)-rutinoside-5-glucoside	176.87 ± 12.54	7.26 ± 0.94
F	Petunidin-3-(*trans-p*-coumaroyl)-rutinoside-5-glucoside	2733.25 ± 246.79	8.40 ± 0.93
G	Petunidin-3-(feruloyl)-rutinoside-5-glucoside	189.48 ± 25.96	7.23 ± 0.91
H	Malvidin-3-(*cis-p*-coumaroyl)-rutinoside-5-glucoside	51.07 ± 3.17	7.24 ± 0.92
I	Malvidin-3-(*trans*-p-coumaroyl)-rutinoside-5-glucoside	566.21 ± 37.58	7.57 ± 0.83
G	Malvidin-3-(feruloyl)-rutinoside-5-glucoside	88.76 ± 7.83	7.29 ± 0.95
K	Petunidin-3-(*trans-p*-coumaroyl-rhamonside)-glucoside-5-glucoside	13.97 ± 0.93	7.24 ± 0.93
L	Malvidin-3-(*p*-methoxy-*trans*-coumaroyl)-rutinoside-5-glucoside	14.16 ± 3.17	7.24 ± 0.93

*The result is indicated as the mean ± SD (n = 3).

modified by hydroxylation, methylation, glycosylation and acylation. Four of the anthocyanins have not been reported before (Table 1), including petunidin-3-(*cis-p*-coumaroyl)-rutinoside-5-glucoside, malvidin-3-(*cis-p*-coumaroyl)-rutinoside-5-glucoside, petunidin-3-(*trans-p*-coumaroyl-rhamonside)-glucoside-5-glucoside and malvidin-3-(*p*-methoxy-*trans*-coumaroyl)-rutinoside-5-glucoside. Two of these had *cis*-hydroxycinnamic acyl groups, which are less stable than the anthocyanin substituent of *trans*-hydroxycinnamic acyl groups (George, Figueiredo, Toki, Tatsuzawa, Saito, & Brouillard, 2001; Yoshida, Okuno, Kameda, & Kondo, 2002).

To visually classify the anthocyanins, we showed all of their structures (Fig. S1 from 1 to 12). Based on their structural modifications and reports from prior studies, the biosynthetic pathway of anthocyanins in tomato fruit was proposed (Fig. S2). Straight after flavonoid biosynthesis pathway, delphinidin is methylated to form petunidin and malvidin. Then, they are modified by glycosylation and acylation to generate other kinds of anthocyanins. The pathway has four branches to form delphinidin derivatives, petunidin derivatives and malvidin derivatives. The acylation shelters anthocyanin from nucleophilic attack with a parallel folding, which leads to a higher stability than anthocyanins only modified by glycosylation (Fig. S3). There are also hydroxylation and methylation, depending on composition of the enzymatic clusters during biosynthesis (Fig. S2).

3.3. Anthocyanin content in tomato fruit

We further determined the contents of the different anthocyanins in the fruit peel and flesh of Indigo Rose (Table 2). The total contents of anthocyanins reached 3977.93 mg/kg in the fruit peel, which was much higher than in the flesh (88.44 mg/kg). These results indicate that anthocyanins are enriched in the Indigo Rose peel. The petunidin-3-(*trans-p*-coumaroyl)-rutinoside-5-glucoside and malvidin-3-(*trans*-p-coumaroyl)-rutinoside-5-glucoside were the two main anthocyanins in the peel, composing approximately 68.7% and 14.2% of the total anthocyanins content, respectively. The content of *cis*-hydroxycinnamic anthocyanins was much lower than the *trans*-hydroxycinnamic anthocyanins in the peel, due to their different stabilities (Table 2). In the future, tomato could be developed into a pharmacological source of antioxidant compounds by improvement of the anthocyanin, in addition to being one of the most popular fruits in the world.

4. Conclusion

In conclusion, a rapid analytical method for anthocyanin determination was developed in this study, with a shortened pretreatment process and high efficiency. The detailed structures of the isolated anthocyanins were obtained. Four new anthocyanins were discovered, out of a total of 12 anthocyanins found in the tomato fruit. According to our proposed anthocyanin biosynthetic pathway in tomato fruit, the modifications of anthocyanin in tomato fruits are mainly glycosylation and acylation, and there are also hydroxylation and methylation.

CRediT authorship contribution statement

Haijing Wang: Conceptualization, Writing - original draft. **Shuai Sun:** Data curation, Investigation. **Zhen Zhou:** Formal analysis. **Zhengkun Qiu:** Resources. **Xia Cui:** Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127439.

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