

Article

Identification of Anthocyanin Compounds in Butterfly Pea Flowers (*Clitoria ternatea* L.) by Ultra Performance Liquid Chromatography/Ultraviolet Coupled to Mass Spectrometry

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Abstract: Butterfly pea flower have great sensory attraction, but they have not yet been used widely in Vietnam. Extracts of butterfly pea flowers can be used conveniently as a natural blue colorant for food products. In this study, the identification of anthocyanin compounds in butterfly pea flowers was performed by UPLC coupled with a UV and Mass spectrometer instrument. Positive and negative ion electrospray MS/MS chromatograms and spectra of the anthocyanin compounds were determined. By analyzing the chromatograms and spectra for each ion, five anthocyanins were identified in the butterfly pea flower extract; these were delphinidin-3-(6''-*p*-coumaroyl)-rutinoside, cyanidin 3-(6''-*p*-coumaroyl)-rutinoside, delphinidin-3-(*p*-coumaroyl) glucose in both *cis*- and *trans*- isomers, cyanidin-3-(*p*-coumaroyl)-glucoside) and delphinidin-3-pyranoside. Additionally, based on their intensity, it was determined that cyanidin-3-(*p*-coumaroyl)-glucoside) was the most abundant anthocyanin, followed by cyanidin 3-(6''-*p*-coumaroyl)-rutinoside, delphinidin-3-(*p*-coumaroyl)-glucoside), delphinidin-3-(6''-*p*-coumaroyl)-rutinoside and delphinidin-3-pyranoside. In this study, cyanidin derivatives were discovered in butterfly pea flower extract, where these compounds had not been detected in previous studies.

Keywords: anthocyanin; butterfly pea flowers; cyanidin; delphinidin; UPLC/UV/MS



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1. Introduction

Butterfly pea flowers, known *Clitoria ternatea* L., area plant species belonging to the Fabaceae family [1]. In Vietnam, butterfly pea flowers are usually used as food drinks and as a colorant. Delphinidin is the main anthocyanin responsible for the deep blue to purple color in this flower [2]. The different flower colors are mainly due to the chemical structure of the different anthocyanins or anthocyanidins synthesized in the flower [3]. In the discussion of copigmentation reactions and color stability of berry anthocyanins, it was observed that the color of anthocyanins changes from pink to blue as the number of hydroxyls increases and methoxyl groups replacing the hydroxyls reverse the trend [4]. There is increasing interest in the search and use of natural colorants, of which blue is rare and tends to be sensitive to processing and storage conditions [5]. Butterfly pea flower extract can be used as a natural blue colorant, is convenient to use, and has a longer shelf-life than equivalent plant-based colorants [6]. Therefore, it is very important to identify the main coloring compounds in butterfly pea flowers.

Anthocyanins present in edible fruits, vegetables and flowers have protective effects against diseases, especially cardiovascular disease, certain types of cancer [7], and against several chronic diseases, such as hyperglycemia [8]. Anthocyanins also improve vision [9]. Because of their benefits, anthocyanins are becoming increasingly commercialized and used in foods [10]. Anthocyanins are an important class of water-soluble pigments belonging to the flavonoid family [11]. To date, more than 600 different anthocyanins have been characterized [12]. Structurally, anthocyanins are glycosylated polyhydroxy and polyethoxy derivatives of flavylum salts; they also possess an aglycone form called anthocyanidins. Cyanidin, delphinidin, pelargonidin, peonidin, malvidin, and petunidin are the most common anthocyanidins distributed in the plants [13]. Their different structures depend on the number and position of the hydroxyl and methoxyl groups on the flavylum ring. Normally, anthocyanidins are bound to sugars, giving stability and water solubility to the molecule [14]. Among the sugars, the most common are glucose, galactose, arabinose, rhamnose and xylose, which are usually present as 3-monoglycosides and 3,5-diglycosides. Other sugars such as rutinose (6-O- α -L-rhamnosyl-D-glucoside), sophorose (2-OD-glucosyl-D-glucosides) and sambubiosides (2-O- β -D-xylosyl-D-glucosides) [15,16] are also present.

Anthocyanins are much more soluble and stable in water than anthocyanidins. Therefore, glycosylated forms of anthocyanidins are more common in nature, and aglycones are virtually non-existent in vivo. It has been observed that glycosyl substitution stabilizes anthocyanin molecules [4]. The glycosyl units and acyl groups attached to the aglycone and their binding sites have a significant influence on the stability and reactivity of the anthocyanin molecule [17]. In addition, the number and position of the hydroxyl and methoxyl groups in the aglycone influence the chemical behavior of the pigment molecule. Increased hydroxylation of aglycone stabilizes anthocyanidins, delphinidin is more stable than cyanidin in acidic methanol. However, increasing the methylation of hydroxyl groups will weaken the stability of anthocyanins. Several anthocyanins have been identified with special structures in butterfly pea flowers. The structure of ternatin D1 was identified as delphinidin-3-O-(6-O-malonyl- β -D-glucopyranosyl)-3', 5'-di-O-(6-O-(E-4)-O-(6-O-*Ep*-coumaroyl- β -D-glucopyranosyl)-*p*-coumaroyl)- β -D-glucopyranoside [18]. The structure of deacylternatin was identified as delphinidin-3,3', 5'-tri-O- β -D-glucopyranoside [19]. Terahara et al. [20] also determined the structure of five ternatins (A3, B4, B3, B2 and D2) as delphinidin 3-malonylG with 3'-GCG-5'-GCG, 3'-GCG-5'-GC side chains. 3'-GCGCG-5'-GC, 3'-GCGC-5'-GCG and 3'-GCGC-5'-GC, where G is D-glucose and C is *p*-coumaric acid.

Eight anthocyanins 1 to 8 (ternatins C1, C2, C3, C4, C5, D3 and preternatins A3 and C4) were isolated by Tehara et al. [21]. Structures 1–6 are recognized as delphinidin 3-malonylglucoside with 3'-GCGC-5'-G, 3'-GCGCG5'-G, 3'-GC-5'-G, 3'-GCG-5'-G, 3'-G-5'-G, and 3'-GC-5'-GC, and compounds 7 and 8 as delphinidin 3-glucoside with 3'-GCG-5'-GCG and 3'-GCG-5'-G are the side chains, respectively. Then, Nair et al. [1] went on to identify other delphinidin derivatives. Azima et al. [6] also found only anthocyanidin, delphinidin, but not cyanidin, as in some other ingredients containing anthocyanins. In addition, more recently, Esher et al. [22] identified a simple derivative of delphinidin, delphinidin-3-O-glucoside. The difference between the results obtained is due to different strains of *Clitoria ternatea* with different petal colors and sensitivities of analytical methods. This has been demonstrated by the study of Kazuma et al. [3], where this author identified different anthocyanins in different lines of pea flowers and they are also complex derivatives of delphinidin.

Despite many studies regarding anthocyanins in berries [23,24], studies on anthocyanins in butterfly pea flowers are still limited. In this study, anthocyanin composition in butterfly pea flowers (grown in Can Tho, Vietnam) was determined by UPLC/UV/MS for the first time.

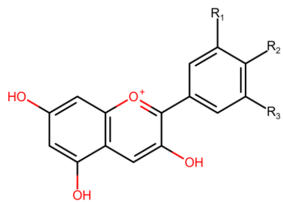
More recently, faster methods have been proposed for the quantification of polyphenols using more powerful chromatographic systems such as UHPLC in combination with a UV detector [25,26] or mass analyzer [27]. The aim of this study was to develop a UPLC

method using a UV detector and an ESI-triple quadrupole (QQQ) mass analyzer for the simultaneous determination of the major anthocyanin compounds of butterfly pea flowers. This study will provide insight into the composition of anthocyanin in butterfly pea flowers so that the extract can be applied more effectively.

2. Results and Discussion

The identification and assignment of anthocyanin peaks was mainly performed based on the comparison between their retention times (RT) and mass spectrometry data with standards, references [28] as shown in Table 1. The chemical structures and molecular weights of six common anthocyanidins and the most common sugars and acylation groups are shown on the basis of preferences. The study of anthocyanin composition in butterfly pea flowers was started by using the basic anthocyanin structure as a reference, taking into account previous literature data on anthocyanin structure [29–33].

Table 1. Chemical structures and molecular weight of six common anthocyanidins.

Six Common Anthocyanidins:	Anthocyanidin	R ₁	R ₂	R ₃	MW
	Pelargonidin	H	OH	H	271
	Cyanidin	OH	OH	H	287
	Delphinidin	OH	OH	OH	303
	Peonidin	OMe	OH	H	301
	Petunidin	OMe	OH	OH	317

UPLC chromatograms (Figure 1) for total ions in butterfly pea flowers were collected. It was observed that the acquisition times of anthocyanin peaks ranged from 5.5 to 7.0 min, approximately, in mass chromatogram straight after UV absorption. Unlike other groups of flavonoids or polyphenols, anthocyanins exist in the cationic form, so a positive ion mode has often been used to analyze them. Due to the positive charge and phenolic groups of anthocyanins, these compounds can readily donate protons to free radicals. However, it is difficult to distinguish anthocyanins from flavonol glycosides in active ionization mode using MS, for example between delphinidin glycoside and quercetin glycoside, or between cyanidin glycoside and kaempferol glycoside. These flavonoids may have the same molecular ions and mass fragmentation patterns as the corresponding anthocyanins.

Meanwhile, unlike the MS data collected in the positive ionization mode, the mass spectrometry data obtained with negative ionization provide a wide range of ions that are specific for anthocyanins [34,35]. Therefore, in this work, mass chromatograms of anthocyanins in both positive ion mode and negative ion mode were investigated.

The results in Figure 1a,b also show that in the negative ion mode, the total ion chromatogram for anthocyanins is simpler than in the positive mode. Additionally, the results also show that all ions exhibited the maximum UV absorption at 535 nm, which is consistent with the previous study by Wang et al. [36] on anthocyanins in the black tomato variety Indigo Rose. Therefore, the determination wavelength was 535 nm for all anthocyanins and the UV chromatogram for butterfly pea flowers extract was at 535 nm (Figure 1c).

To ensure efficiency and accuracy, a combination of spectrophotometric methods, ultraviolet/visible (UV/Vis) and mass spectrometry (MS) methods were performed for anthocyanin identification. Therefore, after UV chromatographic analysis, mass spectrometry data for total ions in both positive and negative modes were also acquired (Figure 2). To obtain the detailed structures of the detected anthocyanins, the MS/MS fragments of anthocyanins were further analyzed. The literature was also used concurrently to focus on and narrow down specific compounds.

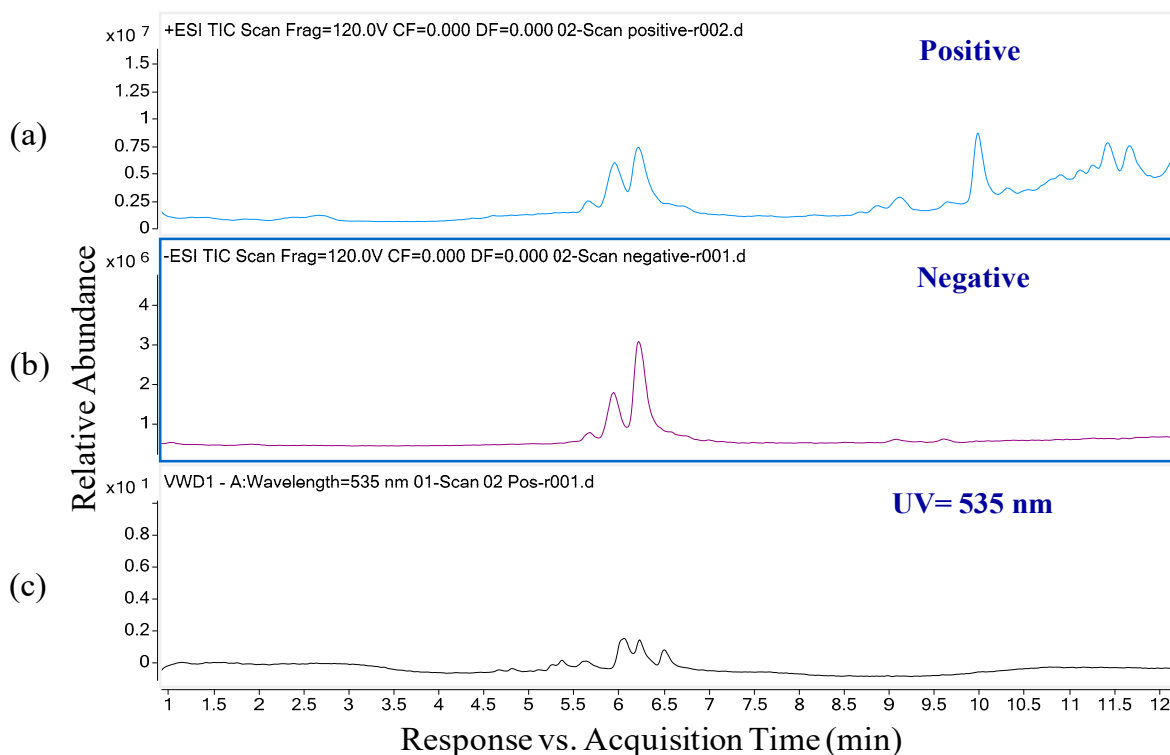


Figure 1. Total ion chromatograms in positive mode (a) and negative mode (b) and the corresponding chromatogram of UV at 535 nm (c).

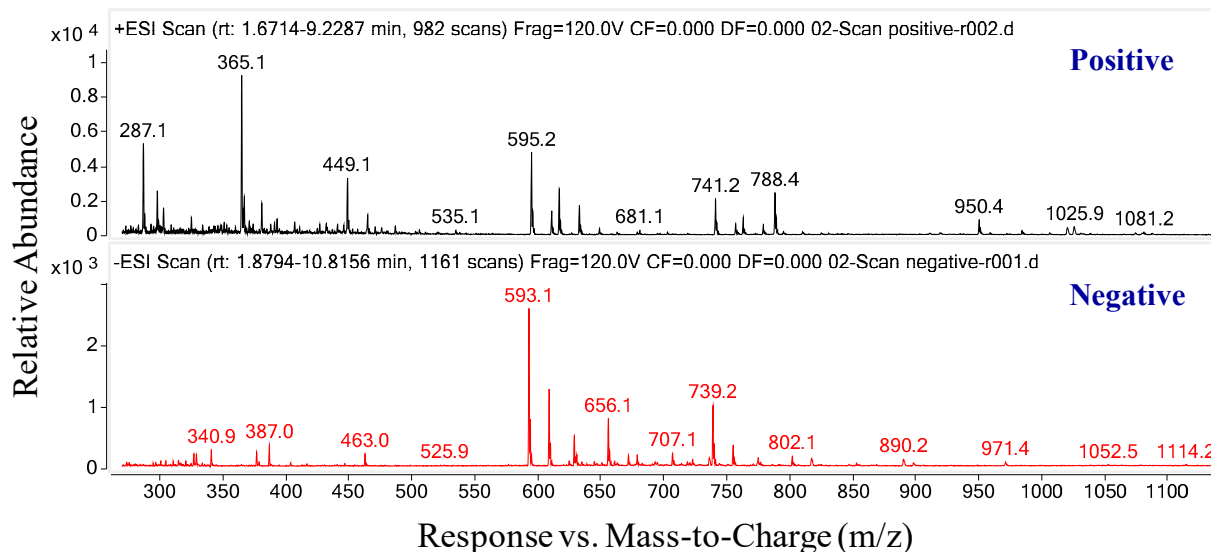


Figure 2. Total ion spectra in positive and negative modes.

The structure of anthocyanins was determined by the anthocyanin molecular weight ion $[M + H]^+$ and the backbone anthocyanidin molecular weight ion (MS/MS). The m/z ratios and UPLC-MS/MS ion graphs of each parent ion and their daughter fragments are shown in Figures 3–7 and Table 2. Five anthocyanins were identified, including three delphinidin derivatives and two cyanidin derivatives, responsible for the blue color of butterfly pea flowers. The ion chromatograms of the MS/MS product in positive and negative mode and the spectrum of the first anthocyanin eluent component shown in Figure 3 are similar.

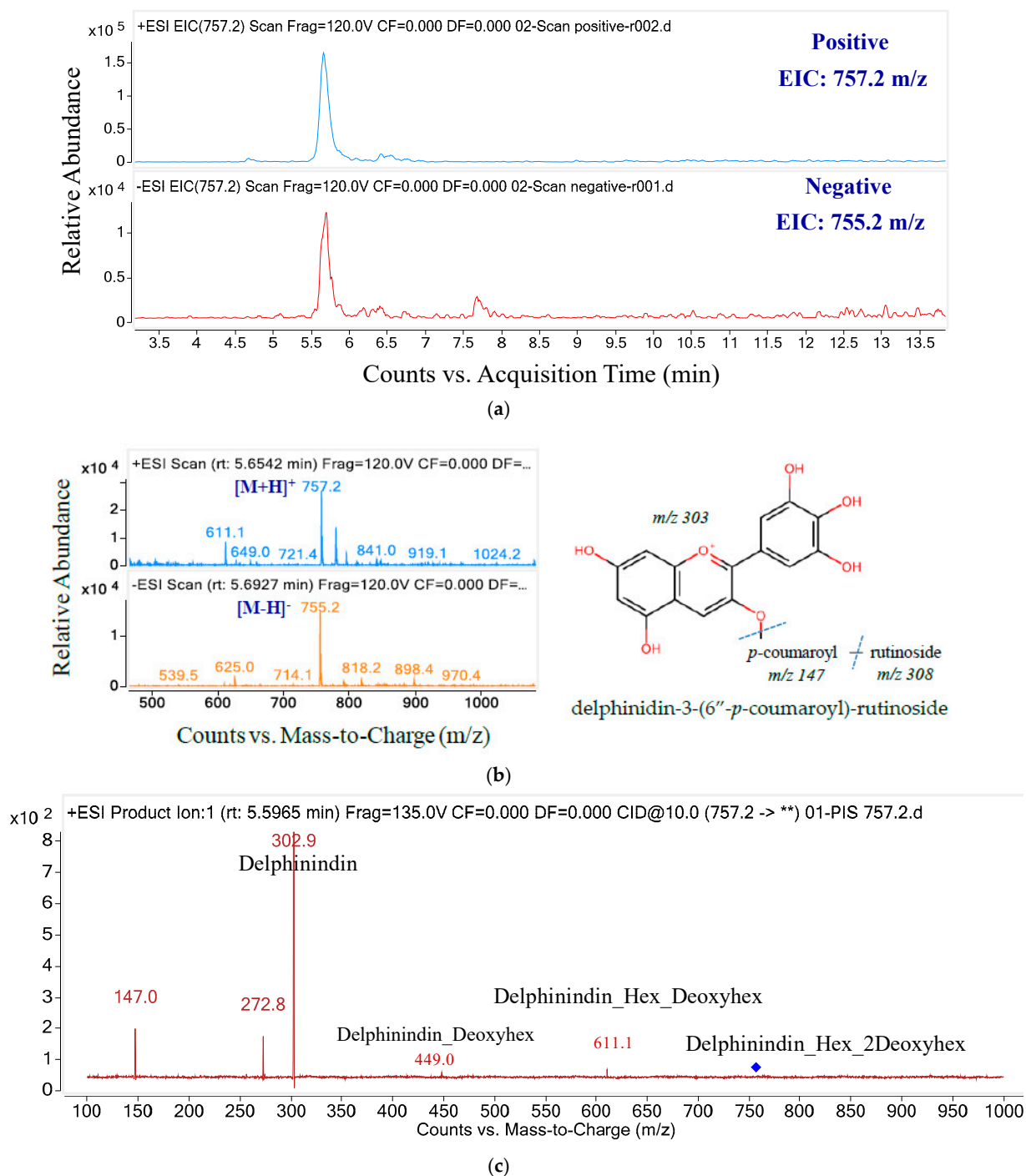


Figure 3. The ion chromatograms (a) and ion spectra (b) and MS/MS fragments (***) of parent mass 757.2 (c).

Compound 1 at RT 5.62 with $[M + H]^+$ m/z 757.2 and $[M + H]^-$ m/z 755.2 yielded MS2 fragments at m/z 611.1, 449, 302.9, 272.8 and 147. The fragmentation of m/z 611.1 can be transiently explained from the parent ion due to the loss of the pentose group, while m/z 449 can be explained by the loss of 162 Da from the precursor ion m/z 611, possibly due to the loss of a hexose residue. The rest of the structure (m/z fragment 147), obtained from the parent ion m/z 752.7, is shown in Figure 3b.

The m/z transition from 757.2 to 302.9 and 147 confirms that the aglycone is delphinidin (m/z 303), and that the compound has *p*-coumaroyl (m/z 147). In addition, a fragment with m/z 611 showed that rutinose (m/z 308) was linked to delphinidin. This evidence suggests that compound 1 is delphinidin-3-(6''-*p*-coumaroyl)-rutinoside [37].

Showing a similar signal to the positive ion analysis, the negative ion electrospray tandem mass chromatogram and the spectrum of the second anthocyanin are shown in Figure 4. Compound 2 exhibited a molecular ion $[M + H]^+$ with m/z 741.1 and produced a major fragment with m/z 286.9 that may correspond to cyanidin (m/z 287). In addition, the m/z transition from 594.9 to 286.9 implied the loss of rutinose (m/z 308). Thus, with a *p*-coumaroyl fragment (m/z 147), this compound could be tentatively identified as cyanidin 3-(6''-*p*-coumaroyl)-rutinoside [37].

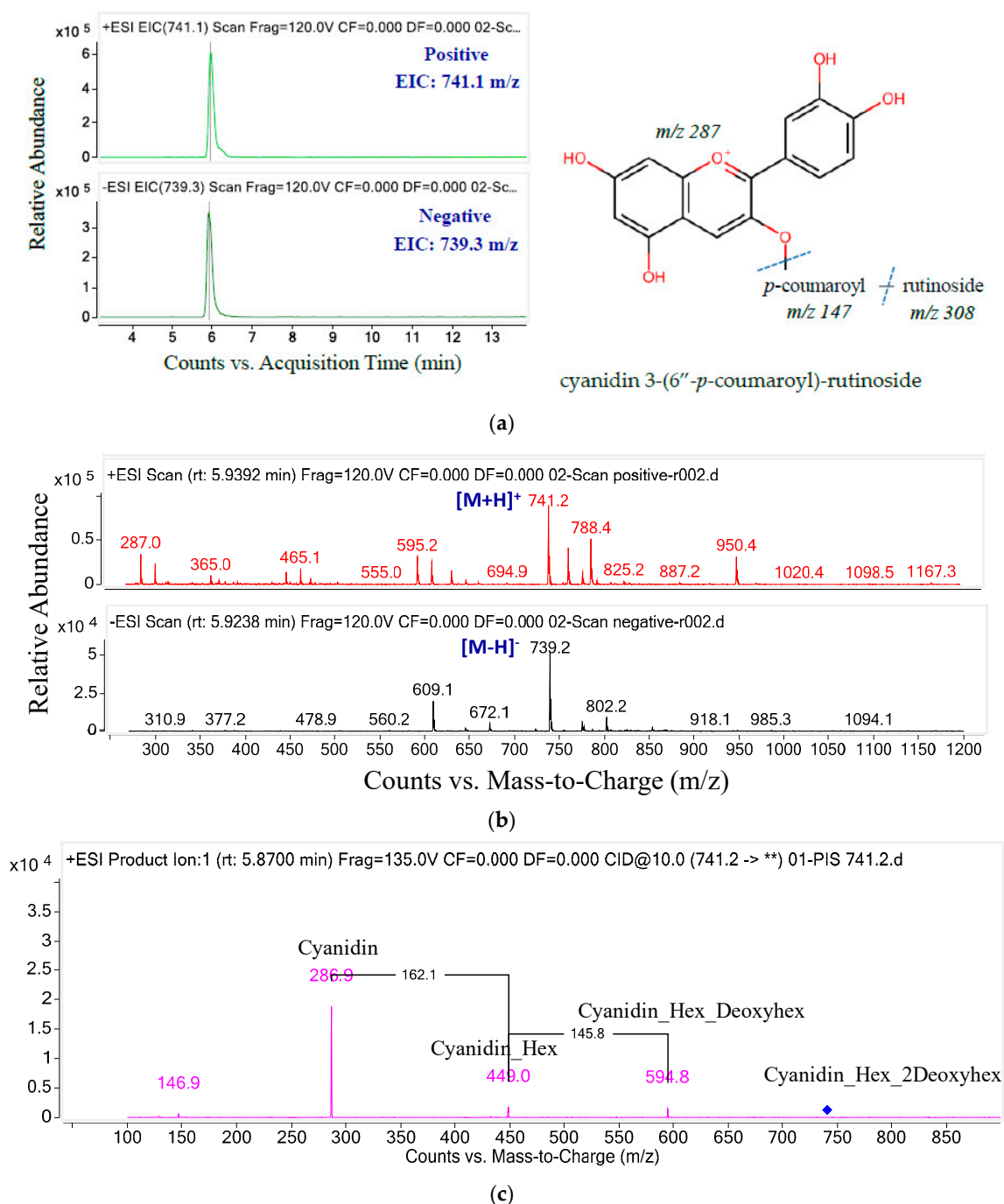
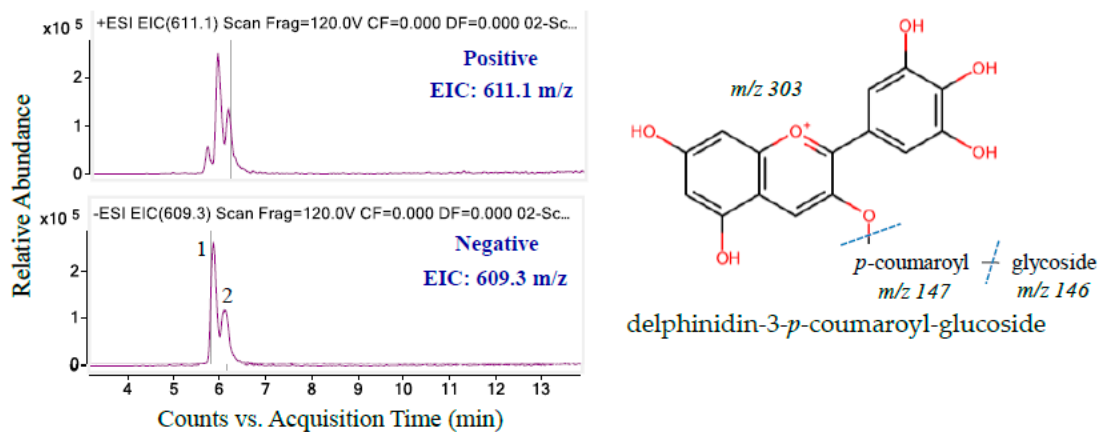
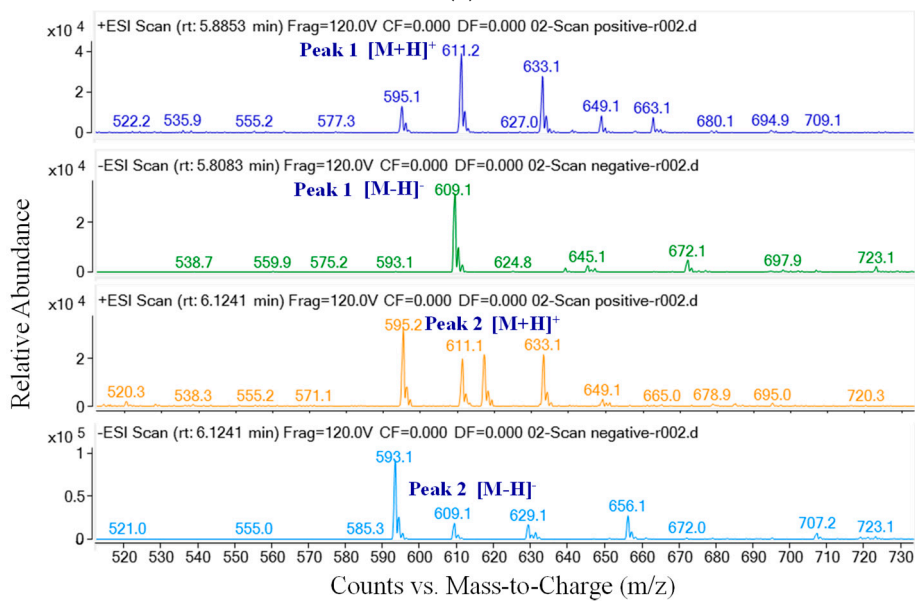


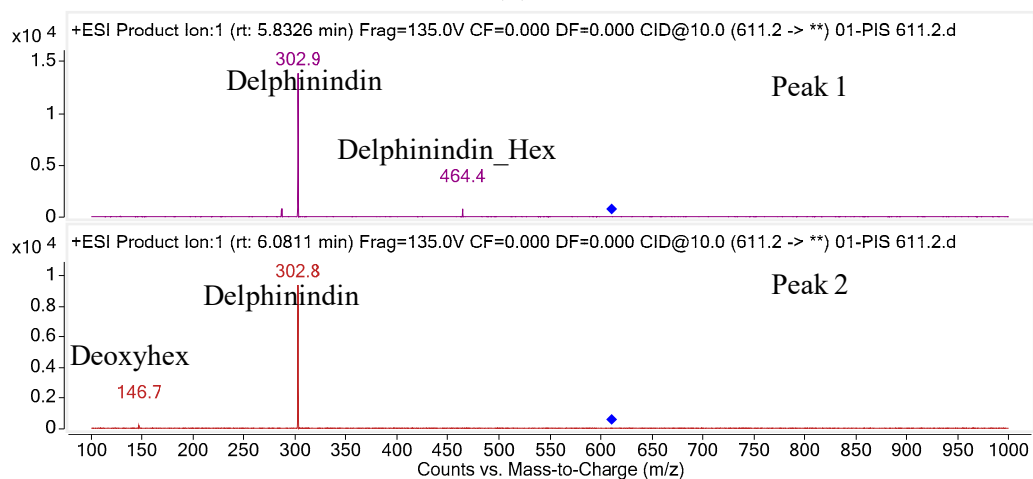
Figure 4. The ion chromatograms (a) and ion spectra (b) and MS/MS fragments (**) of parent mass 741.2 (c).



(a)



(b)



(c)

Figure 5. The ion chromatograms (a) and ion spectra (b) and MS/MS fragments (**) of parent mass 611.2 (c).

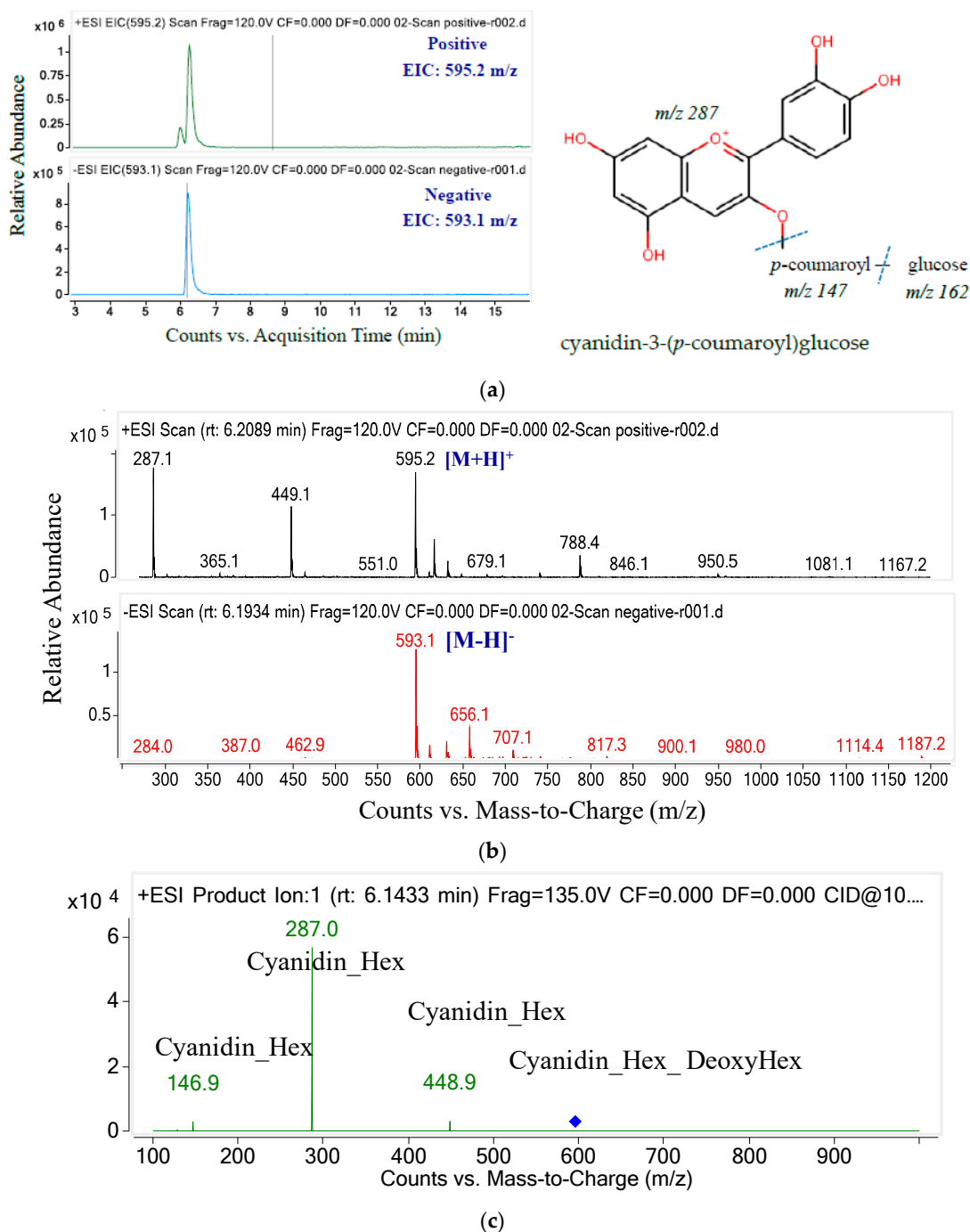


Figure 6. The ion chromatograms (a) and ion spectra (b) and MS/MS fragments of parent mass 595.2 (c).

Three peaks can be observed in the chromatogram in the positive ion mode, but only two in the negative ion mode (Figure 5). That means that the third anthocyanin has two isomers. Peaks 1 and 2 have the same daughter fragment of delphinidin aglycone (m/z 302.9 and 302.8). In peak 1, the m/z transition from 464.4 to 302.9 indicates a loss of glucose. Meanwhile, peak 2 produces the main fragment, m/z 146.7, corresponding to *p*-coumaroyl. In addition, because the *cis-p*-coumaroyl derivative has a higher polarity, it elutes earlier than its *trans* configuration [38]. Therefore, peak 1 was tentatively identified as delphinidin-3-(*cis-p*-coumaroyl-glucoside) and peak 2 was provisionally identified as delphinidin-3-(*trans-p*-coumaroyl-glucoside) [39].

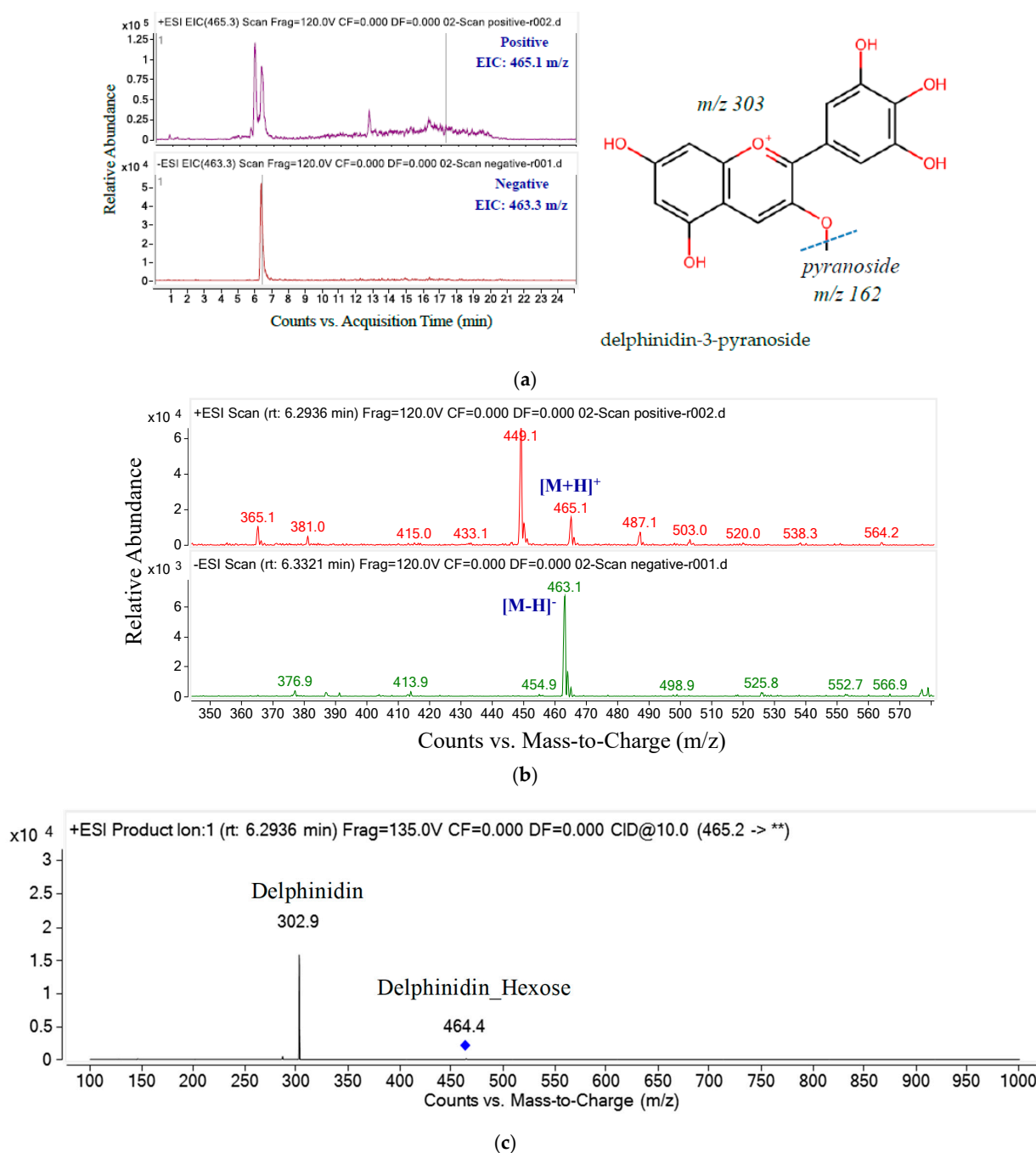


Figure 7. The ion chromatograms (a) and ion spectra (b) and MS/MS fragments (**) of parent mass 465.2 (c).

The chromatograms and MS/MS negative and positive ion electrospray of the final anthocyanin are shown in Figure 6. Two peaks appeared in the mass chromatogram in positive ion mode, but only one peak appeared in negative ion mode. Compound 4 showed molecular ion $[M + H]^+$ with m/z 595.2 and produced two major fragments with m/z 287.0 (cyanidin) and m/z 146.9 (*p*-coumaroyl). Together with the m/z transition from 448.9 to 287.0, indicating glucose loss (m/z 162), peak 4 was identified as cyanidin-3-(*p*-coumaroyl)-glucose [39].

Similarly, there is a difference between the ion chromatograms in positive and negative ion modes for the final anthocyanins with three and two peaks, respectively (Figure 7). The molecular ion $[M + H]^+$ of compound 5 produced a major fragment with m/z 302.9, corresponding to delphinidin aglycone (m/z 303). The m/z transition from 464.4 to 302.9

indicated a loss of pyranose (m/z 162). Therefore, peak 5 was expected to be recognized as delphinidin-3-pyranoside (delphinidin-3-glucoside or delphinidin-3-galactoside) [39].

Table 2. Anthocyanin compounds identified in butterfly pea flower.

Peak	Molecular Ion [M + H] ⁺ (m/z)	Detected Fragments (MS/MS)	Anthocyanins
1	757.2	611.1; 449.0; 302.9; 272.8; 147.0	Delphinidin-3-(6''- <i>p</i> -coumaroyl)-rutinoside
2	741.1	594.8; 449.0; 286.9; 146.9	Cyanidin 3-(6''- <i>p</i> -coumaroyl)-rutinoside
3	611.2	302.8; 146.7	Delphinidin-3-(<i>cis-p</i> -coumaroyl)-glucoside)
3'	611.1	464.4; 302.9	Delphinidin-3-(<i>trans-p</i> -coumaroyl)-glucoside)
4	595.2	448.9; 287.0; 146.9	Cyanidin-3-(<i>p</i> -coumaroyl)glucose
5	465.1	302.9	Delphinidin-3-pyranoside

In summary, after analyzing the chromatograms and spectra of each ion, a total of five anthocyanins were found in the butterfly pea flowers. These were delphinidin-3-(6''-*p*-coumaroyl)-rutinoside, cyanidin 3-(6''-*p*-coumaroyl)-rutinoside, delphinidin-3-(*p*-coumaroyl) glucose in both *cis* and *trans* isomers, cyanidin-3-(*p*-coumaroyl)-glucoside) and delphinidin-3-pyranoside. The modifications of these anthocyanins were mainly glycosylation and acylation. In other studies, some delphinidin derivatives were also found in butterfly pea flowers but these compounds had larger sizes, as evidenced by the larger m/z values of the [M + H]⁺ ion, for example 950, 1296, 1534 [1] or 830.21, 1551.42, 903.22 [22]. In particular, cyanidin derivatives have not been detected/discovered in previous studies. In addition, based on their intensity, we found that cyanidin-3-(*p*-coumaroyl)-glucoside) was the most abundant anthocyanin, followed by cyanidin-3-(6''-*p*-coumaroyl)-rutinoside, delphinidin-3-(*p*-coumaroyl)-glucoside), delphinidin-3-(6''-*p*-coumaroyl)-rutinoside, and finally delphinidin-3-pyranoside (Figure 8).

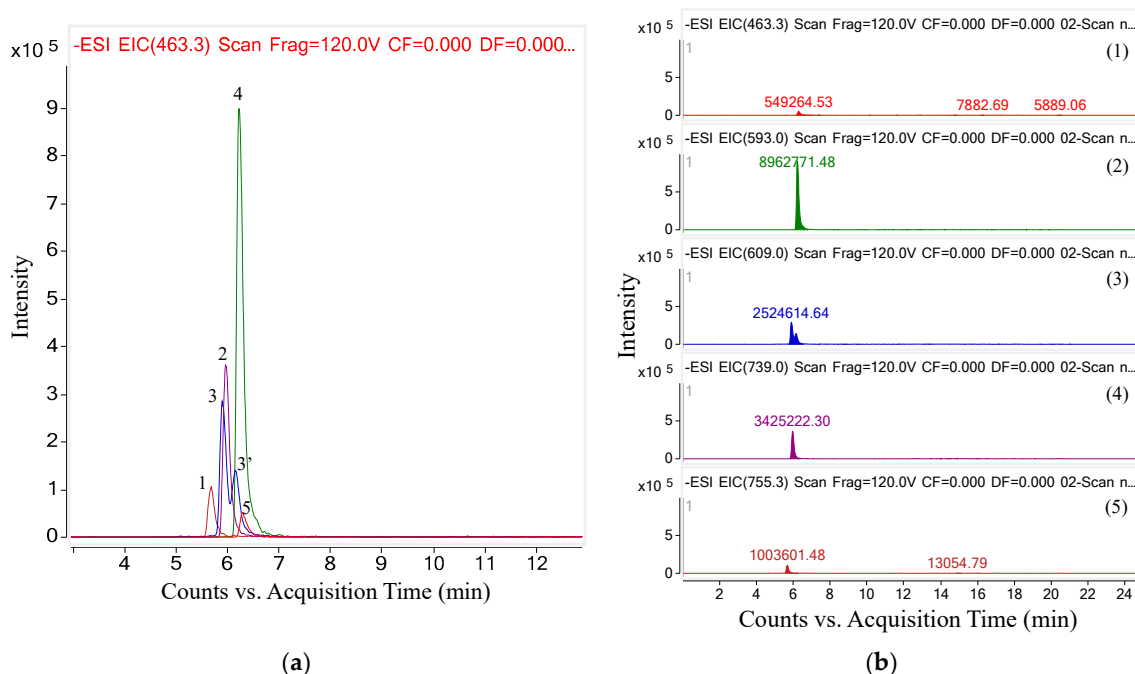


Figure 8. UPLC-MS/MS profile of five anthocyanins found in butterfly pea flower (a) and the comparison of their intensity (b). (1) Delphinidin-3-(6''-*p*-coumaroyl)-rutinoside, (2) Cyanidin 3-(6''-*p*-coumaroyl)-rutinoside, (3) Delphinidin-3-(*cis-p*-coumaroyl)-glucoside, (3') Delphinidin-3-(*trans-p*-coumaroyl)-glucoside, (4) Cyanidin-3-(*p*-coumaroyl)glucose and (5) Delphinidin-3-pyranoside.

3. Materials and Methods

3.1. Chemicals and Materials

HPLC methanol, acetonitrile, formic acid, and acetic acid were purchased from Merck KGaA, Darmstadt, Germany. All chemicals were analytical grade. Milli-Q water (Milli-Q IQ 7003/7005, Merck, NJ, USA) was used.

Butterfly pea flowers were grown at the College of Agriculture, Can Tho University, Vietnam. Butterfly pea flowers were freeze-dried in an Alpha 2–4 DL freeze dryer (Martin Christ, Germany) at $-80\text{ }^{\circ}\text{C}$ and 0.001 mbar to 3–5% moisture content and finely ground before extraction.

3.2. Ultra Performance Liquid Chromatography/Ultraviolet/Mass Spectrometry (UPLC/UV/MS) Analysis

3.2.1. Extraction

An amount of 5g lyophilized butterfly pea powder was extracted with 50 mL methanol: water (60:40, *v/v*) using sonication (WUC-A10H, Daihan, Korea) for 60 min at room temperature ($23\text{--}25\text{ }^{\circ}\text{C}$). The slurry mixture was centrifuged at $16,000\times g$ for 30 min (Z323K, Hermle, Germany).

3.2.2. Purification Procedure

The extraction solution (100 μL) prepared above was put into a centrifuge tube and 700 μL of ice ethanol was added. The tubes were vortexed for 15 s and kept at $-80\text{ }^{\circ}\text{C}$ for 60 min. The tubes were then centrifuged at $21,000\times g$ for 30 min. The supernatant was filtered through a 17 mm (0.2 mm) PVDF syringe filter (VWR Scientific, Seattle, WA, USA) and dried at $40\text{ }^{\circ}\text{C}$ under vacuum.

The residue was activated with 6 mL of methanol 100%. This mixture was passed through 200 mg C18 solid-phase extraction cartridge (Water, MA, USA) and washed with water (6 mL), then eluted with methanol (6 mL). The methanol fraction containing the parent anthocyanins was concentrated to dryness, and dissolved before HPLC analysis.

3.2.3. The UPLC/UV/MS Conditions

Anthocyanins and its derivatives were determined using LC-ESI-QQQ (6460 Triple Quadrupole System, Agilent, CA, USA) in combination with a UV detector (1260 Infinity, Agilent, CA, USA). Both positive and negative ion electrode mass spectra and tandem mass spectra were recorded. The injection volume was 3.0 μL . Separations of anthocyanins and anthocyanidin aglycones were performed on analytical column Zorbax Eclipse C18 ($2.1\times 50.0\text{ mm}$, 1.8 μm , Agilent, CA, USA). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) each containing 0.1% formic acid. The flow rate was 0.3 mL/min and the gradients between the time points were as follows: 0–5 min, 0–30%B; 5–8 min, 30–75%B; 8–25 min, 75–100%B. UV-Visible absorption spectra of anthocyanins were recorded at 535 nm. The MS conditions were as follow: gas temperature, $275\text{ }^{\circ}\text{C}$; gas flow, 8 L/min; Nebulizer, 45 psi; sheath gas temperature, $350\text{ }^{\circ}\text{C}$; sheath gas flow: 12 L/min; capillary: 3500 V (positive), 3500V (negative); Nozzle voltage: 500 V (positive), 500V (negative); and scan mass: 270–1000 (*m/z*) (positive).

4. Conclusions

The mass spectrometry behaviors of anthocyanins in positive and negative ionic modes were studied and were demonstrated to be a valuable tool for identifying anthocyanins from butterfly pea flowers. A new strategy was developed based on observation to distinguish anthocyanin compounds in this flower. Data were generated from UPLC/MS using a developed method that is able to provide rapid and reliable anthocyanin determination with the use of a UV detector. The obtained results show the potential of butterfly pea flowers in anthocyanin extraction and further use as safe colorant in food processing.

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