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Rapid Identification of Betacyanins from *Amaranthus tricolor*, *Gomphrena globosa*, and *Hylocereus polyrhizus* by Matrix-Assisted Laser Desorption/Ionization Quadrupole Ion Trap Time-of-Flight Mass Spectrometry (MALDI-QIT-TOF MS)

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Natural betacyanins have attracted great attention as food colorants and potential antioxidants. Matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry (MALDI-QIT-TOF MS) is a new and powerful technique for the identification of low molecular weight compounds. This study is the first to employ MALDI-QIT-TOF MS to rapidly identify, within a few minutes, a great number of betacyanins in crude extracts from *Amaranthus tricolor* seedlings, *Gomphrena globosa* flowers, and *Hylocereus polyrhizus* fruits. The fresh crude extract samples without any purification were directly used for MALDI-QIT-TOF MS analysis with 2,5-dihydroxybenzoic acid as a matrix. The MS^2 and MS^3 spectrometric data acquired could provide important characteristic information for structural elucidation of the betacyanins. Fourteen free and acylated betacyanins, belonging to amaranthin-type, betanin-type, and gomphrenin-type betacyanins, respectively, were identified. However, the related isomers should be differentiated with the aid of HPLC.

KEYWORDS: Betacyanins; betalains; MALDI-QIT-TOF MS; *Amaranthus tricolor*; *Gomphrena globosa*; *Hylocereus polyrhizus*; amaranth; pitaya; food colorants; HPLC; low molecular weight compounds

INTRODUCTION

Betalains, water-soluble pigments, can be divided into two large structural groups, red/red-violet betacyanins and yellow betaxanthins (*1*, *2*). So far, approximately 50 betacyanins and 20 betaxanthins have been reported in nature (*3*). Betalains are of great taxonomic significance in higher plants. Betalains and anthocyanins may be found in the angiosperms, but their presence is mutually exclusive. Unlike the more widely distributed anthocyanins, betalains occur only in plants from most families of the order Caryophyllales (*1*, *2*, *4*, *5*). The betalains (mainly betacyanins) from red beet (*Beta vulgaris*), one of the earliest natural colorants developed for use in food systems, are extensively used in the modern food industry (*6*). Betalains are attracting increasing attention because of their use for food coloring and their antioxidant and radical scavenging properties against certain oxidative stress-related disorders (*2*, *5*, *7*−*9*).

Amaranthus tricolor L. is a leafy amaranth from the Amaranthaceae family, and *Hylocereus polyrhizus* (Weber) Britton & Rose belongs to a vine cactus from the Cactaceae family (*9*−*12*). Both are native to the subtropical and tropical regions of Central and South America. *A. tricolor* seedlings or leaves are consumed as a popular vegetable in China and also in other Asian countries and South America. *H. polyrhizus* fruits are known as pitaya or pitahaya (*9*, *10*), which are currently being grown commercially in Taiwan, Vietnam, Malaysia, Israel, Australia, the United States, Mexico, Colombia, and Nicaragua. *Gomphrena globosa* L. (globe amaranth) from the Amaranthaceae family is a popular ornamental plant that is widely cultivated in mild climatic regions. It has been reported that *A. tricolor* seedlings, *H. polyrhizus* fruits, and *G. globosa* flowers are rich in free or acylated betacyanins (*13*−*15*).

Because of the great beneficial effects and taxonomic significance of betalains, many techniques have been used to characterize these compounds. Identification of betacyanins is mainly conducted by comparison of spectroscopic, chromatographic, and electrophoretic properties with authentic standards or literature data and by using traditional and modern analytical techniques (*1*, *2*, *4*, *5*, *16*, *17*), such as paper chromatography, thin-layer chromatography, paper electrophoresis, high-performance liquid chromatography (HPLC), liquid chromatography−mass spectrometry (LC-MS), electrospray ionization tandem mass spectrometry (ESI-MS/MS), nuclear magnetic resonance (NMR), and LC-NMR. The betacyanins isolated from *A. tricolor*, *H. polyrhizus*, and *G. globosa* have been earlier

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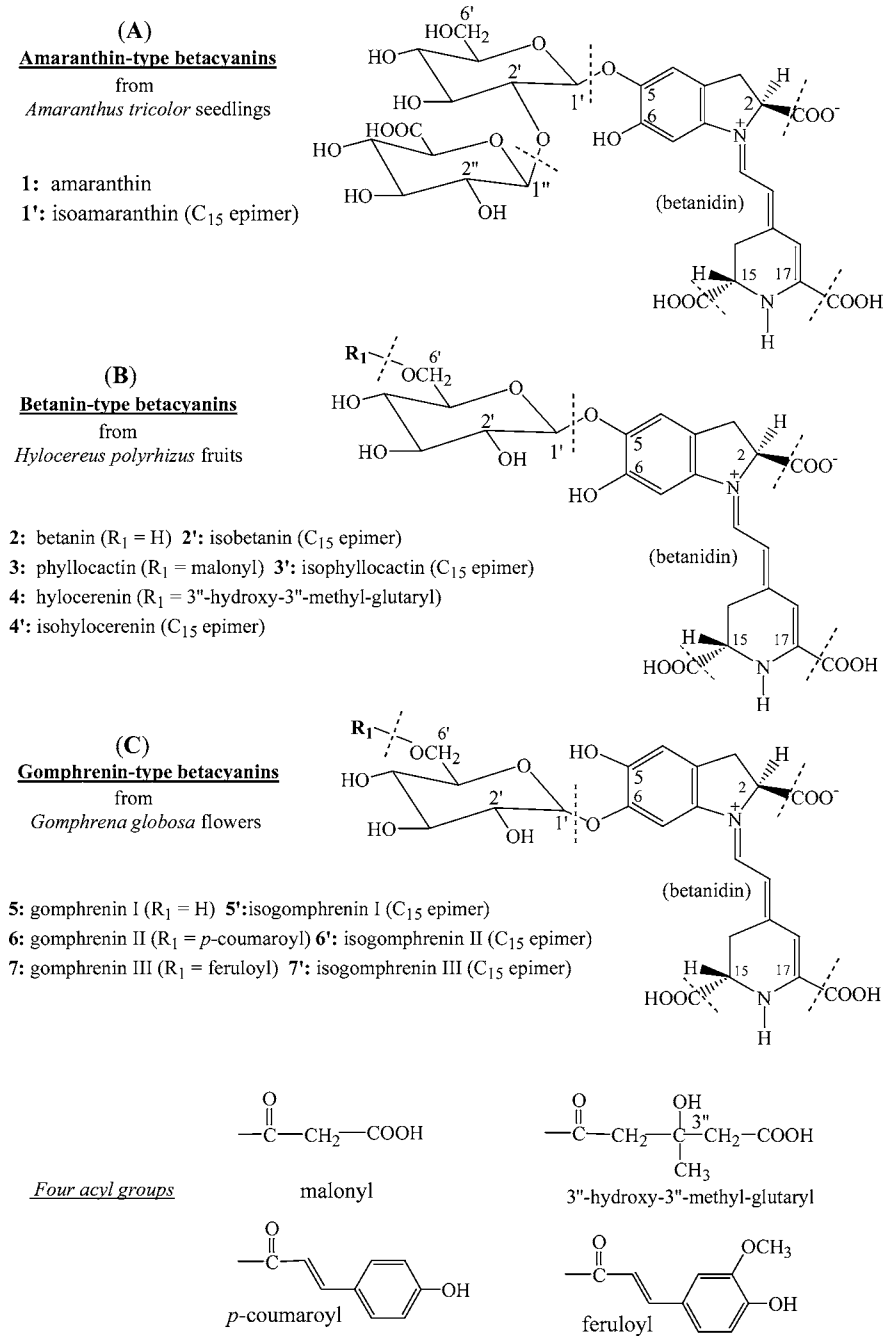

Figure 1. Structures and substitution patterns of free or acylated betacyanins: (**A**) amaranthin-type from *A. tricolor* seedlings; (**B**) betanin-type from *H. polyrhizus* fruits; (**C**) gomphrenin-type from *G. globosa* flowers. Dotted lines represent cleavage positions of fragmentation by MALDI-QIT-TOF MS. Compound numbers correspond to the numbers of the identified betacyanins in **Table 1**.

identified as amaranthin/isoamaranthin, betanin/isobetanin, phyllocactin/isophyllocactin, hylocerenin/isohylocerenin, and gomphrenins/isogomphrenins by HPLC, ESI-MS, and HPLC-ESI-MS (*10*−*14*). Their chemical structures are shown in **Figure 1**.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was originally developed for biomacromolecules (e.g., proteins, lipids, nucleic acids, carbohydrates) (*18*−*21*) and has been recently used for analysis of small biomolecules (e.g., flavonol glycosides, isoflavones, anthocyanins) (*22*−*25*). MALDI-TOF MS possesses many technical advantages, but it is not capable of providing MS^2 and MS^n analyses. A new MALDI MS technique, that is, matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry (MALDI-QIT-TOF MS), has been developed. It requires a hybrid mass spectrometer equipped with a MALDI source and a quadrupole ion trap (QIT), followed by a time-of-flight (TOF) analyzer. This system combines the advantages of conventional MALDI-like rapid analysis time to achieve high-throughput with the ability to perform high-efficiency ion trapping and MS^2 and MS^n analyses and with high sensitivity and resolution by using filtered noise and collision-induced dissociation (CID) methods (*26*−*28*).

So far, MALDI-TOF MS has not been reported for identification of betalains. Recently, we have successfully used MALDI-QIT-TOF MS to identify anthocyanins, flavonols, and hydrolyzable tannins from *Rosa chinensis* flowers (*28*). In the present study, a new MALDI-QIT-TOF MS technique was developed to rapidly and directly identify several different types of betacyanins in crude extracts (without any purification) from *A. tricolor* seedlings, *G. globosa* flowers, and *H. polyrhizus* fruits

within a few minutes. HPLC with a diode array detector (DAD) was also used for differentiation of the isomers of the identified betacyanins.

MATERIALS AND METHODS

Materials and Reagents/Chemicals. Red amaranth seedlings (*A. tricolor*) were obtained from a Hong Kong market. Purple globe amaranth flowers (*G. globosa*) were collected in a Hong Kong garden. Purple pitaya (*H. polyrhizus*) were purchased from a market in Singapore, and the fruits were peeled. Fresh materials of red amaranth seedlings, purple globe amaranth flowers, and purple pitaya pulp were freeze-dried, ground into fine powder, and stored at 4 °C for MALDI-QIT-TOF MS and HPLC analysis. 2,5-Dihydroxybenzoic acid and sodium iodide (NaI) were obtained from Fluka Chemie (Buchs, Switzerland) and HPLC grade organic reagents and formic acid from BDH (Dorset, U.K.).

Sample Preparation for MALDI-QIT-TOF MS and HPLC Analysis. The freeze-dried sample (20 mg) was extracted in a 1.5 mL vial with 1 mL of 80% methanol at room temperature (~23 °C) for 10 min (continuously vibrating vial) and then filtered by a Whatman syringe filter (cellulose membrane, 0.2 μm) and were immediately used for MALDI-QIT-TOF MS analysis. 2,5-Dihydroxybenzoic acid was used as MALDI-QIT-TOF MS matrix in this study. 2,5-Dihydroxybenzoic acid (100 mM) was prepared in 0.1% TFA methanol. 2,5-Dihydroxybenzoic acid (0.5 μL) and filtered crude extract (0.5 μL) were spotted on a sample plate and then allowed to air-dry at ambient temperature before the sample plate was loaded into the Amixa-QIT instrument. In addition, 3 mM NaI was added in 2,5-dihydroxybenzoic acid solution to observe the sodium adduct of betacyanins. The sample preparation for HPLC analysis was as follows: the freeze-dried sample (1 g) was added to 50 mL of 80% methanol in a conical flask and extracted at ~23 °C for 20 min. The extract was then filtered using a Millipore filter with a 0.2 μm nylon membrane under vacuum at 23 °C. The filtrate was stored at 4 °C until HPLC analysis.

MALDI-QIT-TOF MS. All MS and MS^n spectra were acquired on an Axima MALDI-QIT-TOF MS instrument (Shimadzu Biotech, Kratos, U.K.). This instrument uses a three-dimensional ion trap with a time-of-flight mass measurement stage. Acquisition and data processing were controlled by Launchpad software (Shimadzu Biotech). MALDI was produced using pulsed laser light (337 nm, 3 ns pulse width) generated by a nitrogen laser with a maximum pulse rate of 10 Hz. Each profile resulted from the accumulation of data from two laser shots. A small bias voltage (6−30 V) was applied to the sample plate. The ions were extracted by a negative potential (−10 kV), following ionization. Upon trapping, the ions were cooled using helium. The pressure in the trap was held at 6×10^{-3} Pa. For CID, argon was used as the collision gas. In both the MS and MS^n modes, ions were extracted by applying a potential between the two end-caps and pulsed into the TOF system with an accelerating voltage of 10 kV. The detector was a microchannel plate, and acquisition was made using an eight-bit transient recorder. The instrument was operated in the positive ion mass mode (200−800 Da). Mass spectra from a sum of 200−1000 laser shots were recorded using a laser power of 70 arbitrary units (range of laser power = 70−160). External mass calibration was performed daily using fullerite deposited on the sample plate. The maximum mass error in this work was found to be less than ±0.2 Da in the MS experiment and less than ±0.3 Da in the MS^n ($n \geq 2$) experiment. The typical mass resolution was greater than 6000 in all MS and MS^n experiments.

HPLC-DAD. HPLC analysis was performed using an 1100 series Hewlett-Packard HPLC System (Waldbronn, Germany) consisting of a binary pump and a DAD. A 250 × 4 mm i.d., 5 μm, Nucleosil 100-C18 column with a 4 × 4 mm i.d., 5 μm, Nucleosil 5 C18 guard column (Agilent Technologies, Palo Alto, CA) was used. Linear gradient elution system I was from 10 to 55% solvent B (1.5% H_3PO_4, 20% acetic acid, 25% acetonitrile in H_2O) in solvent A (1.5% H_3PO_4 in H_2O) in 40 min for *A. tricolor* seedlings and *H. polyrhizus* fruits, and gradient system II was from 0 to 100% solvent B in solvent A in 100 min for *G. globosa* flowers. For these two gradient systems, the flow rate was 0.8 mL/min, the injection volume was 20 μL, and detection was at 540 nm. Column temperature was at ~23 °C.

RESULTS AND DISCUSSION

Previous MALDI-TOF MS analyses of both small molecules and large biomolecules usually required the samples to be purified by preparative HPLC and other methods (*20*, *21*, *23*, *24*, *29*). In this study, fresh methanolic crude extracts of *A. tricolor* seedlings, *G. globosa* flowers, and *H. polyrhizus* fruits were not purified and were directly used for MALDI-QIT-TOF MS analysis, hence simplifying the identification procedure. The sample extraction time was only 10 min, and MALDI-QIT-TOF MS determination normally took just 2−6 min per run. **Table 1** shows their MALDI-QIT-TOF MS data, UV−vis absorption maxima (λ_{max}), and retention time (t_R) data. **Figure 2** displays typical MALDI-QIT-TOF MS^2 spectra of $[M + H]^+$ ions of the selected amaranthin-type, gomphrenin-type, and betanin-type betacyanins.

Ionization and Fragmentation of Betacyanins. Ionization of small molecules (e.g., anthocyanins, flavonol glycosides) for MALDI-TOF MS is usually affected by matrices (*29*, *30*). 2,5-Dihydroxybenzoic acid, one of the most common matrices, was examined for desorption and ionization of betacyanins in the present study and could produce high-quality MS, MS^2, and MS^3 spectra (**Figure 2 and 3**). Therefore, all MALDI-QIT-TOF MS spectrometric data and profiles presented were obtained by using 2,5-dihydroxybenzoic acid as matrix. In previous papers (*20*, *28*−*30*), main ion forms of small molecules had protonated $[M + H]^+$ and alkali metal adducts, such as $[M + Na]^+$, $[M + K]^+$, $[M - H + 2Na]^+$, $[M - H + 2K]^+$, and $[M - H + Na + K]^+$. The present study indicated that betacyanins mainly formed a protonated $[M + H]^+$ ion in MALDI-QIT-TOF MS positive mode. The peak signals of alkali metal adducts $[M + Na]^+$ and $[M + K]^+$ were hardly observed. Ishida et al. (*31*) reported that the addition of NaI could significantly increase the relative intensities of the sodium adduct ions of compounds. In the present study, by the addition of an alkali metal (3 mM NaI) in the sample solution, it was also difficult to observe the significant peaks of the sodium adduct of the betacyanins.

In MALDI-QIT-TOF MS positive mode, all betacyanins in crude extracts of *A. tricolor* seedlings, *G. globosa* flowers, and *H. polyrhizus* fruits exhibited fragmentation with loss of their carbohydrate residues (C-5 or C-6 position) and the acyl groups at the sugar moiety (C-6′) and with single or multiple decarboxylation (CO_2) at the aglycone (betanidin or isobetanidin) moiety (C-2, C-15, or C-17). The dotted lines in the molecular structures of betacyanins in **Figure 1** represent the cleavage positions fragmented by MALDI-QIT-TOF MS. Fragmentation patterns and ion forms can provide important characteristic information for structural elucidation of the identified compounds. The results of MALDI-QIT-TOF MS analysis showed that the ionization forms and fragmentation patterns of the betacyanins in crude extracts were, to some extent, similar to those of the purified betacyanins observed by electrospray ionization mass spectroscopy (ESI-MS/MS) (*2*, *11*, *14*, *32*−*34*). So far, no-one has reported the cleavage of the carboxyl group (CO_2) at the glucuronyl, malonyl, or 3″-hydroxy-3″-methylglutaryl moiety of betacyanins. Therefore, the cleavage of the carboxyl groups mostly occurs at C-2, C-15, or C-17 positions of the betanidin/isobetanidin moiety. Additionally, we found that the betacyanins fragmented and more easily produced a series of daughter ions with loss of one, two, or three CO_2 at C-2, C-15, or C-17 positions of the betanidin/isobetanidin moiety by MALDI-QIT-TOF MS than by ESI-MS and ESI-MS/MS (*33*−*35*). According to the masses and relative intensities of the observed daughter ions (**Table 1** and **Figure 2**), the first, second, or third decarboxylation of the betacyanins could be assigned.

Table 1. MALDI-QIT-TOF MS and HPLC Analysis of the Betacyanins from *A. tricolor* Seedlings, *H. polyrhizus* Fruits, and *G. globosa* Flowers

no.[a]	betacyanins (trivial/definitive name)	HPLC retention time[b] (min)	HPLC-DAD λ_{max} (nm)	MALDI-QIT-TOF MS (*m/z*) $[M + H]^+$	MALDI-QIT-TOF MS (*m/z*) MS^n experiment (% base peak)
1	amaranthin (betanidin 5-*O*-β-glucuronosylglucoside)	13.4	536	727	MS^2 [727]: 683 (25), 639 (41), 595 (16), 551 (21), 389 (63), 345 (100), 301 (69) MS^3 [727→639]: 595 (21), 389 (74), 345 (100), 301 (61) MS^3 [727→389]: 345 (100), 301 (56), 257 (23)
1′	isoamaranthin (isobetanidin 5-*O*-β-glucuronosylglucoside)	13.7	536		MS^3 [727→345]: 301 (100), 257 (21) MS^3 [727→301]: 257 (100)
2	betanin (betanidin 5-*O*-β-glucoside)	15.3	538	551	MS^2 [551]: 507 (11), 463 (15), 389 (71), 345 (100), 301 (65) MS^3 [551→389]: 345 (100), 301 (69), 257 (22)
2′	isobetanin (isobetanidin 5-*O*-β-glucoside)	16.7	538		MS^3 [551→345]: 301 (100), 257 (18)
3	phyllocactin [betanidin 5-*O*-(6′-*O*-malonyl)-β-glucoside]	20.4	539	637	MS^2 [637]: 593 (15), 551 (12), 549 (10), 505 (15), 389 (77), 345 (100), 301 (67) MS^3 [637→389]: 345 (100), 301 (59), 257 (26)
3′	isophyllocactin [isobetanidin 5-*O*-(6′-*O*-malonyl)-β-glucoside]	24.7	539		MS^3 [637→345]: 301 (100), 257 (19)
4	hylocerenin [betanidin 5-*O*-(6′-*O*-3″-hydroxy-3″-methylglutaryl)-β-glucoside]	23.6	540	695	MS^2 [695]: 651 (18), 607 (13), 563 (17), 551 (13), 389 (71), 345 (100), 301 (62) MS^3 (weak peak signals, no experiment)
4′	isohylocerenin [isobetanidin 5-*O*-(6′-*O*-3″-hydroxy-3″-methylglutaryl)-β-glucoside]	27.3	540		
5	gomphrenin I (betanidin 6-*O*-β-glucoside)	33.3	540	551	MS^2 [551]: 507 (13), 463 (18), 389 (69), 345 (100), 301 (66) MS^3 [551→389]: 345 (100), 301 (71), 257 (23)
5′	isogomphrenin I (isobetanidin 6-*O*-β-glucoside)	35.7	540		MS^3 [551→345]: 301 (100), 257 (19)
6	gomphrenin II [betanidin 6-*O*-(6′-*O*-*E*-*p*-coumaroyl)-β-glucoside]	58.8	310, 552	697	MS^2 [697]: 653 (11), 609 (31), 565 (14), 551 (17), 389 (65), 345 (100), 301 (70) MS^3 [697→389]: 345 (100), 301 (57), 257 (18)
6′	isogomphrenin II [isobetanidin 6-*O*-(6′-*O*-*E*-*p*-coumaroyl)-β-glucoside]	62.0	310, 552		MS^3 [697→345]: 301 (100), 257 (15)
7	gomphrenin III [betanidin 6-*O*-(6′-*O*-*E*-feruloyl)-β-glucoside]	59.6	322, 552	727	MS^2 [727]: 683 (14), 639 (33), 595 (18), 551 (16), 389 (68), 345 (100), 301 (65) MS^3 [697→389]: 345 (100), 301 (58), 257 (17)
7′	isogomphrenin III [betanidin 6-*O*-(6′-*O*-*E*-feruloyl)-β-glucoside]	63.7	324, 552		MS^3 [697→345]: 301 (100), 257 (16)

[a] Compound numbers correspond to the numbers of molecular structures in **Figure 1**. [b] Amaranthin-type betacyanins (**1**/**1′**) and betanin-type betacyanins (**2**/**2′**, **3**/**3′**, and **4**/**4′**) were separated with gradient system I (40 min), and gomphrenin-type betacyanins (**5**/**5′**, **6**/**6′**, and **7**/**7′**) were separated with gradient system II (100 min).

Betacyanins from *A. tricolor* Seedlings. Earlier and recent reviews (*1*, *2*, *8*) have shown that betacyanins include four main groups, that is, amaranthin, betanin, gomphrenin, and descarboxybetanin. The materials tested in this study, *A. tricolor* seedlings, *H. polyrhizus* fruits, and *G. globosa* flowers, are rich in amaranthin-type, betanin-type, and gomphrenin-type betacyanins, respectively. From **Table 1**, compounds **1**/**1′** were primarily assigned as amaranthin/isoamaranthin in the crude extracts of *A. tricolor* seedlings by UV−vis λ_{max} and t_R data and by comparison with our previous literature data including ESI-MS data (*14*). This assignment was easily confirmed by MALDI-QIT-TOF MS, giving the expected protonated parent molecular ion $[M + H]^+$ (*m/z* 727) and typical daughter ions. MALDI-QIT-TOF MS could further provide MS^n data for the structural elucidation of betacyanins. Further evidence was the formation of characteristic fragments in the MS^2 and MS^3 experiments. **Figure 2A** shows that amaranthin/isoamaranthin fragmented and produced two typical daughter ions, that is, $[M + H - 176]^+$ at *m/z* 551 with loss of the glucuronosyl moiety (176 Da) and $[M + H - 338]^+$ at *m/z* 389 ($[\text{betanidin} + H]^+$) with loss of the glucuronosylglucose moiety (338 Da). This was similar to the result observed by ESI-MS in our previous study (*14*). The tested amaranthin/isoamaranthin easily produced a series of daughter ions with loss of one, two, or three CO_2 (44 Da) at the C-2, C-15, or C-17 position of the betanidin moiety. In the MS^2 experiment for amaranthin/isoamaranthin, fragments at *m/z* 683 ($727 - CO_2$), *m/z* 639 ($727 - 2CO_2$), *m/z* 595 ($727 - 3CO_2$), *m/z* 345 ($727 - 338 - CO_2$), and *m/z* 301 ($727 - 338 - 2CO_2$) were obtained, in addition to two typical daughter ions *m/z* 551 ($727 - 176$) and *m/z* 389 ($727 - 338$) (**Table 1** and **Figure 2A**). In the MS^3 experiment, the daughter ion *m/z* 639 further produced characteristic fragments at *m/z* 595, 389, 345, and 301. At the same time the daughter ion *m/z* 389 further produced fragments at *m/z* 345, 301, and 257 ($727 - 338 - 3CO_2$), the daughter ion *m/z* 345 also further produced fragments at *m/z* 301 and 257, and the daughter ion *m/z* 301 further produced a main fragment at *m/z* 257 (**Table 1**). **Figure 3** shows detailed MS^3 spectra of these daughter ions.

Betacyanins from *H. polyrhizus* Fruits. Consistent with previous findings (*11*), compounds **2**/**2′**, **3**/**3′**, and **4**/**4′** were preliminarily identified as betanin, phyllocactin, and hylocerenin and their C-15 epimers, respectively, in the crude extracts of *H. polyrhizus* fruits by their relative retention times and absorption maxima and by comparison with the related literature data. MALDI-QIT-TOF MS data confirmed the identity of these compounds, displaying the expected protonated parent molecular ions $[M + H]^+$ (*m/z* 551, 637, and 695) and many typical daughter ions (**Table 1**). The MS^2 and MS^3 experiments of these betacaynins from *H. polyrhizus* fruits provided more information for their structural elucidation. In the MS^2 experiment of the parent ion $[M + H]^+$ (*m/z* 551) for betanin/isobetanin (**Figure 2B**), we obtained the fragments at *m/z* 507 ($551 - CO_2$), *m/z* 463 ($551 - 2CO_2$), *m/z* 389 ($551 - 162$ [glucose]), *m/z* 345 ($551 - \text{glucose} - CO_2$), and *m/z* 301 ($551 - \text{glucose} - 2CO_2$). The characteristic ion *m/z* 389 was the most important daughter ion $[\text{betanidin} + H]^+$. Betanidin was the basic structural unit (aglycone) of all betacyains (**Figure 1**). In the MS^2 experiment of the parent ion $[M + H]^+$ (*m/z* 637) for phyllocactin/isophyllocactin (**Figure 2C**), we observed the fragments at *m/z* 593 ($637 - CO_2$), *m/z* 551 ($637 - 86$ [malonyl group]), *m/z* 549 ($637 - 2CO_2$), *m/z* 505 ($637 - 3CO_2$), *m/z* 389 ($637 - 86 - 162$ [glucose]), *m/z* 345 ($637 - 86 - 162 - CO_2$), and *m/z* 301 ($637 - 86 - 162 - 2CO_2$). In the MS^2 experiment of the parent ion $[M + H]^+$ (*m/z* 695) for hylocerenin/isohylocerenin, the fragments at *m/z* 651 ($695 - CO_2$), *m/z* 607 ($695 - 2CO_2$), *m/z* 563 ($695 - 3CO_2$), *m/z* 551 ($695 - 144$ [3″-

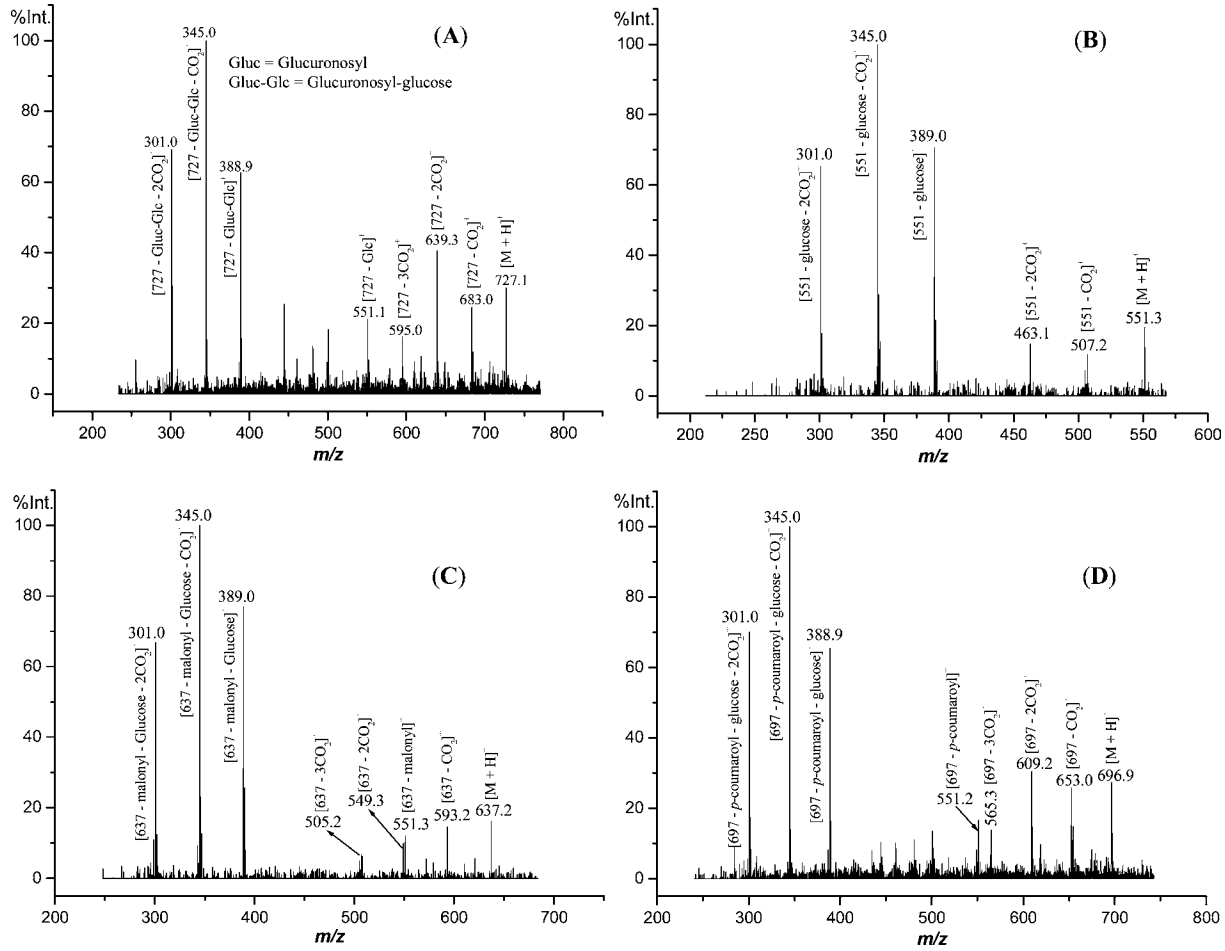

Figure 2. MALDI-QIT-TOF MS $[M + H]^+$ ion spectra (MS^2) of (**A**) amaranthin/isoamaranthin (*m*/*z* 727) from *A. tricolor* seedlings, (**B**) betanin/isobetanin (*m*/*z* 551) and (**C**) phyllocactin/isophyllocactin (*m*/*z* 637) from *H. polyrhizus* fruits, and (**D**) gomphrenin II/isogomphrenin II (*m*/*z* 697) from *G. globosa* flowers.

hydroxy-3″-methylglutaryl group]), *m*/*z* 389 (695 − 144 − 162 [glucose]), *m*/*z* 345 (695 − 144 − 162 − CO_2), and *m*/*z* 301 (695 − 144 − 162 − $2CO_2$) were observed (**Table 1**). Characteristic ions at *m*/*z* 551 and 389 indicated that both phyllocactin/isophyllocactin and hylocerenin/isohylocerenin were derivatives of betanin and betanidin. The mass differences of 86 (*m*/*z* 637−551) and 144 (*m*/*z* 695−551) suggested that the aliphatic acyl groups (i.e., malonyl and 3″-hydroxy-3″-methylglutaryl) connected to the C-6′ at the glucose of betanin/isobetanin (**Figure 1**). This was in agreement with previous ESI-MS and NMR results (*11*, *12*, *16*, *17*, *32*). In addition, in the MS^3 experiment of the prominent daughter ions (*m*/*z* 389 and 345) from betanin/isobetanin and phyllocactin/isophyllocactin, we could further observe their fragments at *m*/*z* 345 (389 − CO_2), *m*/*z* 301 (389 − $2CO_2$), and *m*/*z* 257 (389 − $3CO_2$) and *m*/*z* 301 (345 − CO_2) and *m*/*z* 257 (345 − $2CO_2$), respectively (**Table 1**).

Betacyanins from *G. globosa* Flowers. Compounds **5**/**5′**, **6**/**6′**, and **7**/**7′** in **Table 1** were preliminarily assigned as gomphrenins I, II, and III and their C-15 epimers, respectively, in the crude extracts of *G. globosa* flowers by their absorption maxima and relative retention times and by comparison with our previous data including ESI-MS data (*14*). These betacyanins were readily identified by MALDI-QIT-TOF MS, providing the expected protonated parent molecular ions $[M + H]^+$ (*m*/*z* 551, 697, and 727) and many typical daughter ions (**Table 1**). In the MS^2 experiment of the parent ion $[M + H]^+$ (*m*/*z* 551) for gomphrenin I/isogomphrenin I, the fragments at *m*/*z* 507 (551 − CO_2), *m*/*z* 463 (551 − $2CO_2$), *m*/*z* 389 (551 − 162 [glucose]), *m*/*z* 345 (551 − glucose − CO_2), and *m*/*z* 301 (551 − glucose − $2CO_2$) were observed (**Table 1**). In the MS^2 experiment of the parent ion $[M + H]^+$ (*m*/*z* 697) for gomphrenin II/isogomphrenin II (**Figure 2D**), we observed the fragments at *m*/*z* 653 (697 − CO_2), *m*/*z* 609 (697 − $2CO_2$), *m*/*z* 565 (697 − $3CO_2$), *m*/*z* 551 (697 − 146 [*p*-coumaroyl group]), *m*/*z* 389 (697 − 146 − 162 [glucose]), *m*/*z* 345 (697 − 146 − 162 − CO_2), and *m*/*z* 301 (697 − 146 − 162 − $2CO_2$). In the MS^2 experiment of the parent ion $[M + H]^+$ (*m*/*z* 727) for gomphrenin III/isogomphrenin III, the fragments at *m*/*z* 683 (727 − CO_2), *m*/*z* 639 (727 − $2CO_2$), *m*/*z* 595 (727 − $3CO_2$), *m*/*z* 551 (727 − 176 [feruloyl group]), *m*/*z* 389 (727 − 176 − 162 [glucose]), *m*/*z* 345 (727 − 176 − 162 − CO_2), and *m*/*z* 301 (727 − 176 − 162 − $2CO_2$) were observed (**Table 1**). According to Heuer et al. (*13*) and our earlier study (*14*), three kinds of gomphrenins/isogomphrenins belong to the gomphrenin-type betacyanins (substituted at C-6 of betanidin/isobetanidin), differing from betanin-type betacyanins (substituted at C-5 of betanidin/isobetanidin) (**Figure 1**). The mass differences of 146 (*m*/*z* 697−551) and 176 (*m*/*z* 727−551) indicated the presence of aromatic acyl groups (i.e., coumaroyl and feruloyl) at the C-6′ of glucose in gomphrenins/isogomphrenins (**Figure 1**). Gomphrenin I/isogomphrenin I had the same molecular mass as betanin/isobetanin, and they produced similar parent ion and daughter ions. However, the relative intensities (percent) of the ions observed in the MS^2 and MS^3 spectra, UV−vis λ_{max}, and HPLC retention times were different (**Table 1**). Furthermore, in the MS^3 experiment of the prominent daughter ions (*m*/*z* 389 and 345) from all gomphrenins/isogomphrenins, we could

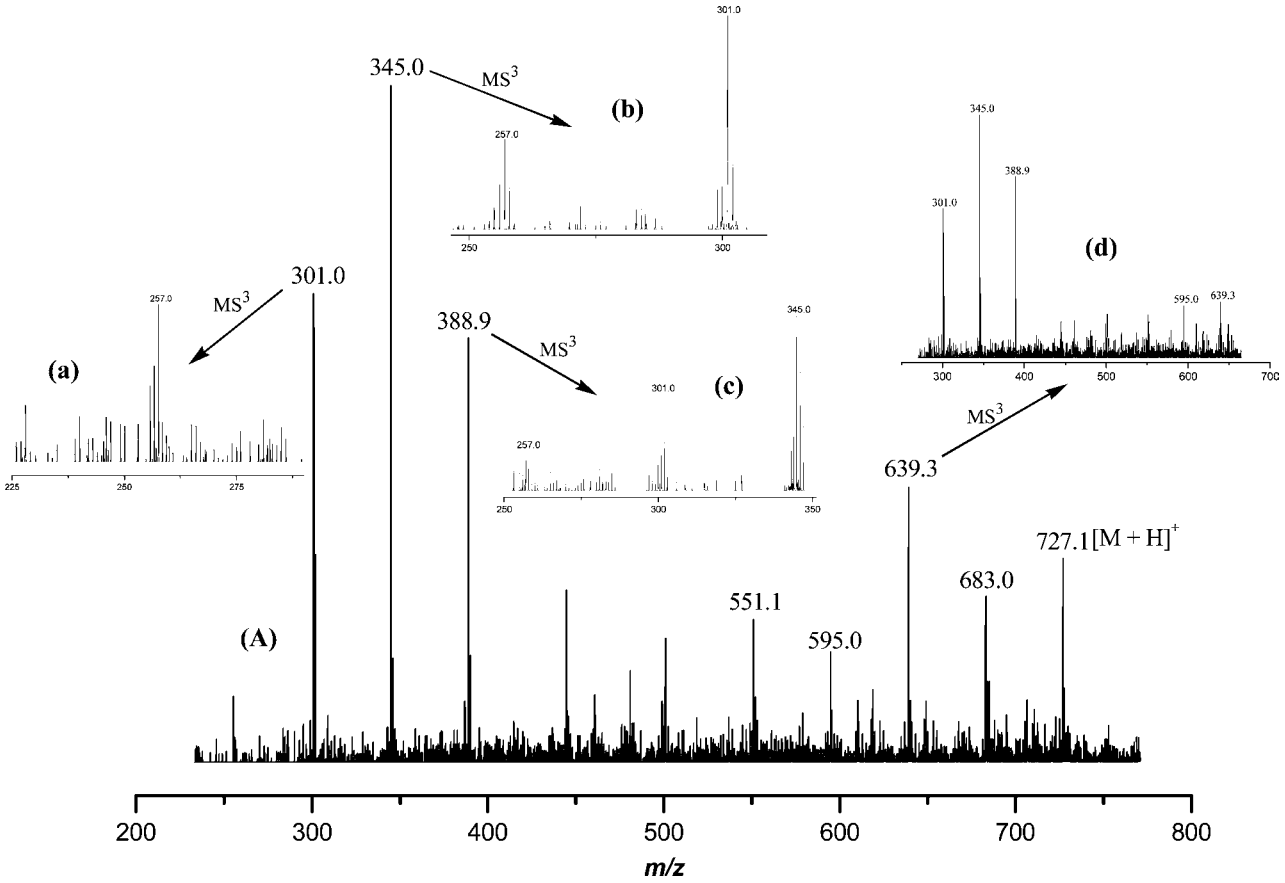

Figure 3. Examples for MS^2 (**A**) and MS^3 (**a**–**d**) spectra of MALDI-QIT-TOF MS $[M + H]^+$ ions of amaranthin/isoamaranthin (*m*/*z* 727) from *A. tricolor* seedlings.

observe their fragments at *m*/*z* 345 (389 − CO_2), *m*/*z* 301 (389 − $2CO_2$), and *m*/*z* 257 (389 − $3CO_2$) and *m*/*z* 301 (345 − CO_2) and *m*/*z* 257 (345 − $2CO_2$), respectively (**Table 1**).

Advantage and Disadvantage of MALDI-QIT-TOF MS. MALDI MS was originally and widely used for large biomolecules, such as proteins, lipids, oligo- or polysaccharides, and tannins (*18*−*21*), and recently has been also used for small biomolecules (low molecular weight compounds), such as anthocyanins and flavonol glycosides (*22*−*24*). In the present study, the crude samples (without any purification) of the betacyanins extracted in 80% methanol for 10 min were dropped into the sample plate with 2,5-dihydroxybenzoic acid as a matrix for direct analysis. MALDI-QIT-TOF MS was used to rapidly and simultaneously identify various kinds of betacyanins in the crude extracts of three plant materials within a few minutes. The current study and previous papers (*26*−*28*) have suggested that MALDI-QIT-TOF MS not only possesses the advantages of conventional MALDI MS, such as ease of sample preparation, rapid generation of spectra, wide applicability combined with a good tolerance toward contaminants, and the ability for the simultaneous determination of masses in complex samples, but also can produce MS^2 and MS^n spectra for structural elucidation of compounds because it is equipped with a MALDI source and a quadrupole ion trap time-of-flight analyzer. Therefore, the biggest advantage of MALDI-QIT-TOF MS is its ability to rapidly analyze a great number of crude extract samples of betacyanins within a short time.

In contrast to MALDI-QIT-TOF MS, conventional ESI-MS or ESI-MS/MS analysis via the direct injection mode normally requires purified samples, whereas LC-MS analysis needs longer times for chromatographic separation and purification of betacyanins (usually 40−100 min). However, MALDI-QIT-TOF MS has its own disadvantages. It gives masses of only molecular parent and daughter ions and could not distinguish isomers of the identified compounds. The isomers have the same molecular masses and similar UV−vis spectra, but possess different retention times. In the present study, although MALDI-QIT-TOF MS could not distinguish the isomers of the betacyanins, common reversed-phase HPLC was used to easily identify various betacyanins and their isomers by comparison with literature data (relative values of retention times) (*11*−*14*). **Table 1** shows that seven pairs of betacyanins/isobetacyanins (**1**/**1′**, **2**/**2′**, **3**/**3′**, **4**/**4′**, **5**/**5′**, **5**/**5′**, **6**/**6′**, and **7**/**7′**) had significantly different retention times.

Additionally, betanin (isobetanin) and gomphrenin (isogomphrenin I) possess the same formula ($C_{24}H_{26}N_2O_{13}$) and the same mass weight (550), but have structural differences at C-5 and C-6 (**Figure 1**). Because the relative intensities (percent) of the ions observed in the MS^2 and MS^3 spectra for these two types of betacyanins were a little different (**Table 1**), it was possible for MALDI-QIT-TOF MS to differentiate them. By comparison, HPLC could readily distinguish them, because their retention times were significantly different and their UV−vis spectra were also a little different (**Table 1**).

In conclusion, MALDI-QIT-TOF MS is a valuable and feasible technique to rapidly and simultaneously identify various kinds of betacyanins in the crude extracts from *A. tricolor* seedlings, *G. globosa* flowers, and *H. polyrhizus* fruits within a few minutes. This is the first report on the betacyanins identified by MALDI-QIT-TOF MS. In the positive scan mode, all identified betacyanins exhibited fragmentation with loss of their carbohydrate residues and the acyl groups at the sugar moiety and with single or multiple decarboxylations at the betanidin/isobetanidin moiety. The MS^2 and MS^3 spectrometric data

acquired could provide important information for structural elucidation of the betacyanins. However, the MALDI-QIT-TOF MS technique could not distinguish the isomers of the betacyanins, which should be done with the aid of conventional HPLC or LC-MS.

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