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Off-line ESI-MS/MS profiling of betalains and flavonoid glycosides isolated from (fruit) *Opuntia stricta var. dillenii* and (vegetable) *Atriplex hortensis var. rubra* by countercurrent chromatography

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Abstract:

***Opuntia stricta var. dillenii* and *Atriplex hortensis var. rubra*, two plant species with abundant betalain and flavonoid content, were studied by high performance countercurrent chromatography (HPCCC) coupled with ESI-MS/MS in an off-line detection process. Both CCC runs used *tert.*-butyl-methyl-ether/n-butanol/acetonitrile/water 1% trifluoroacetic acid (v/v/v/v, 2:2:1:5) as a biphasic solvent system within an *elution-extrusion* mode. The resulting fractions were scanned by an off-line sequential injection to ESI-MS/MS approach. The separation of *Opuntia stricta var. dillenii* extract showed a partial isolation of principal betalains (betanin) from their derivatives (betanidin, phyllocactin, feruloyl-betanin, betaxanthin), degraded products (hydroxyl-neobetanin, mono decarboxy-betanins), and flavonoid glycosides (isorhamnetin-rutinoside, kaempferol-rutinoside). In case of *Atriplex hortensis var. rubra*, this study confirmed the coexistence of several flavonoids (quercetin-O-malonyl glucoside, kaempferol-O-malonyl glucoside) and generated few pure pigment fractions (celosianin II) for structural elucidation.**

Keywords: ***Atriplex hortensis var. rubra*, betalains, *countercurrent chromatography* (CCC), ESI-MS/MS, *Opuntia stricta var. dillenii.***

Classification number: **2.2**

Introduction

Red-purple and yellow-orange pigment betalains including betacyanins and betaxanthins are known worldwide as food colorants with positive health properties [1]. This group of pigments abundantly occurs in some families of *Caryophyllales* and their antioxidative effects are numerously reported such as inhibition of tumours (*in-vitro*), reduction of radiation damage [2-6]. Nevertheless, betalains are vulnerable to many exogenic conditions as well as processing techniques such as high temperatures, pH, light, oxygen, and metal ions, etc., making this is a limitation to larger scale betalain recovery and use. The high demand for natural pigments requires innovative research to explore new betalain sources and large-scale purification processes [5-8].

Historically, inorganic silica gel or organic cation-exchangers having high adsorption capacity of plant pigments were used for pigment extraction [9]; however, these resins induce a rapid degradation of labile betalain. Meanwhile, betalain quantification is normally accomplished by UV-Vis spectrophotometry although underestimations and limitations in structural information derived from this method have been reported [10]. The introduction of HPLC-UV-Vis analysis together with LC-MS/MS structural analysis opens a new chapter for betalain investigation [10, 11]. Nevertheless, their unstable nature makes pure betalains (including authentic reference compounds) unavailable and causes problems for comprehensive phytochemical studies [12-16].

All-liquid *countercurrent chromatography* (CCC) is a robust purification technique that uses a variety of biphasic solvent systems in which complex crude extracts are partially distributed between immiscible solvent phase layers and fractionated according to the polarities of the metabolites. The liquid stationary phase layer is kept

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on the coil system due to a strong Archimedean screw force. CCC is a method consisting of multiple continuous liquid-liquid extraction processes and has been widely applied to larger-scale separations of metabolites from various natural product classes. In general, this method of liquid chromatography prevents irreversible sample loss that occurs in solid chromatography; therefore, it is gentle enough for sensitive compounds like the natural pigments betalain and carotenoid [17-19]. Moreover, a variety of available operation modes allow CCC to be flexible for many samples (in term of polarities) and the solvent system has the capability to connect up-to-date monitoring methods such as UV-Vis, ESI-MS/MS, or ACPI-MS/MS. For instance, the combination of CCC with ESI-MS/MS profiling technique has been applied in the discovery many bioactive compounds such as flavonoids, propolis metabolites, and betacyanins from *Impatiens glandulifera Royle* flowers, Saudi-Arabian propolis metabolites, and *Bougainvillea glabra* flowers, respectively [20-22].

In this study, the pre-purified pigment extracts from the cactus fruits of *Opuntia stricta var. dillenii (Opuntia)* and vegetable leaves from *Atriplex hortensis var. rubra* (*Atriplex*) were fractionated by high performance CCC (HPCCC) using the ion-pair solvent system of *tert*.-butyl-methyl-ether/n-butanol/acetonitrile/water,trifluoroacetic acid (TBMe/n-BuOH/ACN/water) (v/v/v/v, 2:2:1:5, 1% TFA). The recovered fractions were selected and monitored by off-line sequential injections to the ESI-MS/MS for the direct identification of eluting betalain pigments and flavonoid glycosides from each cultivar.

Material and method

Samples and chemicals

Samples: Opuntia stricta var. dellini (fruit, Egypt) and *Atriplex hortensis var. rubra* (leaves, Germany) were harvested and stored below -20ºC in dark until analysis.

Chemical: acetonitrile (ACN, LC-MS and HPLC grade, honeywell-Germany), formic acid (LC-MS grade, Sigma-Aldrich-Germany), C18 resin for pigment enrichment (40-63 µm pore, Merck), acetic acid (AcOH ≥ 99.8%, Sigma), ethanol (EtOH, HPLC grade, Sigma). Nanopure water (0.2 µm spore, Wilhelm Werner, Leverkusen). *Tert.*-butyl-methyl-ether and n-butanol (TBMe and n-BuOH, HPLC grade, Honeywell); trifluoroacetic acid (TFA, 99%, Sigma-Aldrich-Germany); propionic acid (LC-MS grade, Sigma-Aldrich-Germany).

Pigment extraction and enrichment

Extraction: each material was treated separately by a similar processing protocol. Briefly, the frozen materials were blended before maceration several times with solvents. Five hundred grams of *Opuntia* was extracted with 1 l ACN:water 0.7% AcOH (1:9, v:v) while 250 g *Atriplex* was extracted with 0.5 l ACN:water (1:1, v:v). The crude extracts were filtered by cotton and paper filters before enrichment by 500 g C18 material in the next step. These steps were done quickly in the dark at room temperature to limit betalain degradation.

Betalain enrichment by C18 adsorption: reversed phase C18 resin was cleaned (three times) by 0.5 l ACN then conditioned (four times) by 0.5 l aqueous AcOH 0.7%. The activated resin was then incubated with each crude extract for 1 h. The pigmented resin was rinsed several times with AcOH 0.7% to remove un-adsorbed sugar, acids, amino acid, etc. The pigment desorption was done by incubating the colored resin with 0.5 l EtOH:AcOH 0.7% (v/v, 2:8) until the purple color was completely released. The colored supernatants were combined, filtered, and immediately freeze dried. The dried powders were stored in the dark at -20ºC to prevent pigment degradation before CCC experiments.

HPCCC operation and offline ESI-MS/MS flow-injection analysis

HPCCC apparatus: the two separations were done on a J-type HPCCC model (Dynamic Extractions, Gwent, UK) that was fabricated with two-connected semi-preparative polytetrafluoroethylene coil columns (total volume of 63+62.5 ml, 1.6 mm internal diameter tube). These columns were mounted on a two-bobbin system. The machine allowed a maximum speed of 1600 rpm under stable temperatures. During operation, the temperature was kept at 30ºC by an external cooling system. The system used a preparative LC pump for solvent delivery.

HPCCC operation: the biphasic solvent system of TBMe/1-BuOH/ACN/water/TFA (0.1%) (v/v/v/v, 2:2:1:5) was poured sequentially into a separatory funnel (at 20ºC) before being mixed vigorously until equilibrium was reached. The system was left to form two clear layers (upper layer or organic layer worked as stationary phase while lower layer or aqueous layer used as mobile phase), which were then collected separately and de-gassed shortly before use. In this study of betalains, the '*head to*

tail' and '*elution - extrusion*' modes were applied. Briefly, the stationary phase was firstly pumped to fill the 125-ml coil column. Then, the HPCCC was set to maximum rotation at 1600 rpm. When the temperature reached 30°C, 4 ml/min mobile phase was delivered to the system until only the mobile phase was released (hydrodynamic equilibrium condition). The *stationary phase retention value* S_f was calculated by the ratio of *stationary phase volume* V_S (retained in the system at equilibrium) divided by the *column coil volume* V_C.

Sample preparation and injections: two separations were performed using lyophilized enriched pigment extract (500 mg *Opuntia* and 460 mg *Atriplex*). For each separation, the sample was dissolved in 5 ml of biphasic solvent mixture (2.5 ml aqueous phase and 2.5 ml organic phase), de-gassed, and then filtered (Chromafil Xtra GF-100/25, Macherey & Nagel, Düren, Germany) before delivered into a 5 ml injection loop. When the CCC system was stable at 30ºC, a low-pressure injection port (Reodyne, Cotati, CA, USA) was used to inject the sample into the system to begin the separation. The released fractions were collected at 1 min intervals by a Superfrac (Pharmacia, Uppsala, Sweden) fraction collector. The stationary phase was replaced after the mobile phase passed two coil volumes (approximately 250 ml). The separation was complete when one-coil volume of stationary phase (around 125 ml) was carried through.

ESI-MS/MS profiling by sequential offline injection of collected fractions: for every HPCCC separation, an exact amount of each collected fraction was sequentially injected to the ESI ion trap-MS/MS system (HCT-Ultra ETD II, Bruker Daltonics, Bremen, Germany) through an AS-2000A auto-sampler device (Merck-Hitachi, Japan). For preparation, 300 µl of each fraction was diluted with 1 ml make-up solvent (ACN:water:formic acid (0.1%), v:v, 50:50) and 20 µl propionic acid into a single HPLC vial. Propionic acid was used to eliminate the strong ESI-MS ion signal quenching effect of TFA in the HPCCC fractions. The same volume from each vial (10 µl depending on initial concentration) was injected sequentially into the ESI-MS/MS system within 2 min time intervals to generate a 'mass' selective chromatography profile in the positive ionization mode. Each injection was pumped together with 0.5 ml/min make-up solvent by a binary HPLC-pump (G1312 A, 1100 Series, Agilent Technologies, Waldbronn, Germany). For each ESI-MS/MS injection profile, a base peak ion intensity around 1.0×10^6- to 10^8 was necessary for the relevant ion intensity of a single peak.

ESI-MS/MS set up: ESI detection was set to alternating ionization mode, the *m/z* scan range was 100-2000, the "ultra"-mode and mass scanning rate were 26.000 *m/z* per second, 10.0 l/min nitrogen was used at 320°C for the drying gas, and the nebuliser pressure was set at 60 psi. The high ionization voltage (HV) capillary was -3500 V, HV end plate offset was -500 V, trap drive 79.2, octopole radiofrequency (RF) amplitude was 187.1 V_{pp}, lens 2 60.0 V, Cap Ex 115.0 V, and maximum accumulation time 200 ms. Other notable parameters include averages of five spectra, trap drive level 120%, target mass range *m/z* 500, compound stability 80%, Smart ICC target 100000, ICC charge control "on" and smart parameter setting "active". The most intensive precursor ions (5 to 7 ions) were selected for fragmentation. The remaining MS/MS fragmentation amplitude value was 1 V.

Betalains and flavonoid glycosides identification: the identification of betalains and flavonoid glycosides from each separation were done based on ESI-MS/MS data of the compounds (summarized in Tables 1 and 2) in comparison to that of standard compounds found in literature.

Result and discussion

HPCCC-off-line ESI-MS/MS profile of Opuntia pigment extract

There were many studies to compare the bioactivities of the most common prickly pear metabolites, which indicate that pigments and flavonoid glycosides from *Opuntia stricta* show the highest antioxidant activities, especially that of *Opuntia dillenii* [23, 24]. Therefore, this prompted us to investigate the pigment and polyphenolic metabolites of this cultivar for a potential process for natural pigment. The hyphenation of preparative HPCCC with an off-line ESI-MS/MS as the molecular-weight-visualization method enabled the fractionation and identification of around 500 mg of pre-cleaned pigment extract. In general, the colored betalains were nicely fractionated from non-colored flavonoid glycosides. From there, a few minor pigments and degradation products were significantly concentrated into a few fractions although they were not detected in the crude extract by LC-ESI-MS (data not shown). Table 1 summarizes the principle phytochemistry from *Opuntia fruits* pigment

extract and their MS data elucidated from this study. Fig. 1 illustrates the dried sequential injected vials (upper) and the generated injection ESI-MS/MS profile (lower) of this HPCCC run with selected single ion traces.

Table 1. ESI-MS/MS data of betalains in *Opuntia stricta var. dillenii* fruits (positive ionization mode).

No	*m/z*	MS^2	Compounds	References
1	551	389	15*S*/15*R* betanin 15*S*/15*R* gomphrenin	[11-16]
2	637	551, 389	15*S*/15*R* phyllocactin	[11-16]
3	568	549, 387	Hydroxyl-neobetanin	[25-27]
4	309	263	Indicaxanthin	[14]
5	689	645, 603	Unknown	
6	389	345	15*S*/15*R* betanidin	[11-16]
7	625	317, 257, 479	(Isorhamnetin-O-rutinoside	[26]
8	595	449, 287	Kaempferol-O-rutinoside	[26]
9	506	268	Unknown	
10	620	602, 342	Unknown	
11	563	-	Unknown	
-	727	389	15*S*/15*R* feruloyl betanin	[26]
-	507	345	Degraded betacyanins (mono decarboxy-betanins)	[11-16, 25]
-	358	196, 150	Degraded betacyanins (cyclo-dopa 5-O-β-glucoside)	[11-16, 25]

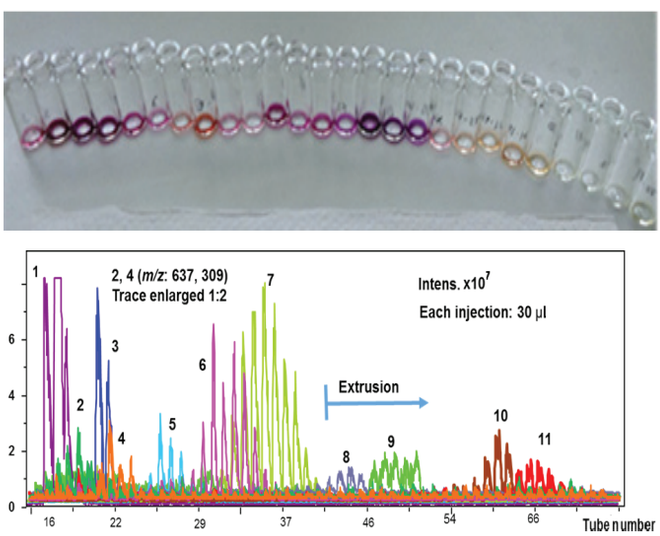

Fig. 1. The dried sequential injected vials and sequential ESI-MS/MS off-line injection profile of C18 purified *Opuntia* pigment extract fractionated by preparative HPCCC (pos. ionization mode) with visualized selected single target ion traces (*m/z* 100-1500).

The diastereomeric pair *15S/15R* betanin (**1**) in Fig. 1 eluted initially at low retention volumes (fraction 15) after the so-called *break-through* of solvents until fraction 14. Subsequently, they appeared as the most intensive ion signal with $[M+H]^+$ at *m/z* 551 in the initial fractions 16 to 18 together with cyclo-dopa-5-O-β-glucoside (*m/z* 358), which is one precursor molecular of betanin. On the other hand, traces of betalamic acid ($[M+H]^+$ at *m/z* 212), the other betanin precursor, appeared later at larger elution volumes and apart in tube 46 to 50 (data not shown in Fig. 1). Similarly, the three most abundant degradation products of betanin, including neobetanin (or dehydro betanin, $[M+H]^+$ at *m/z* 549), mono decarboxy betanin ($[M+H]^+$ at *m/z* 507), and decarboxy neobetanin (*m/z* 505), were also found in traces within these initial tubes fractions. These degraded products of betanin mixed together made a distinct dark red color for fraction 14. Interestingly, these red compounds or betanin artifacts were not detected in the *Opuntia dillenii* crude extract scanned by LC-ESI-MS experiment before (data not shown). The presence of betanin artifacts in these HPCCC fractions suggest that the separation process could provoke pigment degradation. These degradative effects were seen in previous works on betalain purification using the CCC technique as an usual result of processing steps by impacts of solvents, oxygen contact, and separation time [25, 27].

The ESI-MS/MS data of the following fractions from tube 18-21 showed high concentrations of phyllocactin epimeric mixture (**2**, *15S/15R*). These epimers were partly fractionated from the betanin epimers. The higher elution time, and therefore higher affinity to the liquid stationary phase, could be related to the slightly decreased polarity of phyllocactin by the malonyl-substitution at the glucose unit. The strong ion signal of **3** (*m/z* 568), indicating a neobetanin derivative, was found close to **2** (tube 21 and 22). This compound was reported before as hydroxy-neobetanin, which is a neobetanin structured with an additional hydroxyl group bound to the C14-C15 double bond (Herbach, et al. (2005) [25]). This red derivative was well separated from betanins although it mixed with phyllocactins. Betanidin epimers (**6,** *m/z* 389), the aglycone of betanin, were either eluted at much longer elution times or much later than betanins (from tube 31 to 37) in this HPCCC run. This could be related to the neutral loss Δm/z 162, which was identical to the cut-off of one glucose unit from betanin structure. Thus, the missing hydrophilic glucose group implies that betanidin

is more lipophilic and remained longer in the organic stationary phase. These later fractions also carried more polar epimers of *15S/15R* feruloyl-betanin (*m/z* 727, MS^2 389), which could be identified as lampranthin II or gomphrenin III based on a neutral loss cleavage of the feruloyl unit (Δm/z 176). These betacyanin derivatives have been seen in *O. ficus indica* before [26]. Surprisingly, Jerz, et al. (2013) [26] detected very polar betanidin 5-O-β-sophoroside epimers (*15S/15R, m/z* 713, MS/MS 389) from their IPHSCCC purification of *O. ficus* pigments while these compounds were not found in this study of *O. stricta*. Moreover, *15S/15R* hylocerenins (*m/z* 695, MS/MS 389) are typical cactus betacyanins but were not recognized in these separations, which is in agreement with the report of Jerz, et al. (2013) [26] on *O. ficus* pigments using CCC technique.

Beside betacyanins, the only betaxanthin found after the C18 cleaning step was indicaxanthin (**4**, *m/z* 309). This yellow pigment was isolated completely from the purple betanin although it was partly mixed with a neobetanin derivative (**3**). Meanwhile, isorhamnetin-rutinoside and kaempferol-O-rutinoside (**7**, *m/z* 625, MS/MS 317 and **8**, *m/z* 595, MS/MS 287, respectively) were seen as the two major concentrated flavonoid glycosides from the extract and they eluted apart from each other. However, **7** partly mixed with betanidin (**6**). Interestingly, there were several novel minor concentrated compounds, namely, **5**, **9**, **10,** and **11,** which were detected by higher ion intensities in the extrusion mode (from fraction 42 to the end). These structures could be metabolites from the fruits that need further CCC studies to verify. In general, this HPCCC off-line ESI-MS/MS approach could partly purify the pigment betanin from its derivatives and flavonoid glycosides, resulting in some fractions of a few pure *15S/15R*-betanin and isorhamnetin-rutinoside for further NMR experiments (NMR data not shown).

HPCCC off-line ESI-MS/MS profile of Atriplex pigments extract

Atriplex hortensis var. rubra is known for its high content of celosianin II and flavonoid glycosides (particularly sulphated flavonoids), which gives the plant its typical purple color and shows a strong antioxidant capacity [28]. The HPCCC run of this enriched *Atriplex* pigment and polyphenol extract was performed using the same procedure as the *Opuntia* pigment above. As a result, the pigments (betalains) and non-pigmented compounds (flavonoids) were effectively fractionated. Table 2 summarizes the principle compounds elucidated from this separation and their MS/MS data. Fig. 2 indicates the adjusted ESI-MS/MS profile of recovered fractions eluting from this HPCCC run, which is displayed by selected single ion traces using the positive ionization mode.

Table 2. ESI-MS/MS data of betalains and flavonoids from *Atriplex* leaves (positive ionization mode).

No	*m/z*	MS^2	Compounds	References
1	727	551, 389	*15S/15R* amaranthin	[11-17, 29]
2	551	389	*15S/15R* betanin	[11-17, 29]
3	903	551, 389	*15S/15R* celosianin II ((2"-O-E-feruloyl)-amaranthine)	[28, 29]
4	551	302.7	Quercetin-O-malonyl glucoside	[22, 28, 29]
5	535	287	Kaempferol-O-malonyl glucoside	[22, 28, 29]
6	419	287	Kaempferol-O-pentoside	[22, 28, 29]
7	499	367, 287	Kaempferol-3-O-sulphate-7-O α-arabinopyranoside	[22, 28, 29]
-	265	248, 177	Unknown	
-	611	455, 302.7	Quercetin-rhamnose-glucose	[22, 28, 29]
-	667	535, 287	Kaempferol-arabino-glucose-malonyl	[22, 28, 29]
-	595	449, 287	Kaempferol-rhamnose-glucose	[22, 28, 29]

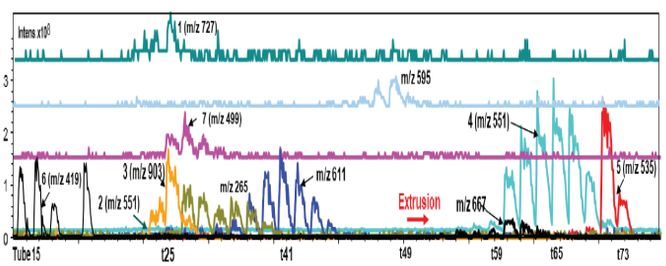

Fig. 2. Adjusted off-line ESI-MS/MS profile of *Atriplex* pigment extract eluted from HPCCC (possitive ionization mode).

The high content of the epimeric mixture of celosianin II (*15S/15R-(2"-O-E-feruloyl)-amaranthine*, (**3**), was represented by a strong molecular ion signal $[M+H]^+$ at *m/z* 903. In contrast, celosianin I, a typical betacyanin from this cultivar, was not found. Meanwhile, signals from amaranthin (*15S/15R*, **1**, *m/z* 727) and betanin (*15S/15R*, **2**, *m/z* 551) were found only in trace amounts. Similarly, these betacyanins were identified by the abundant fragmented ion signals at *m/z* 551 (betanin) and *m/z* 389 (betanidin). As can be seen from the profile, amaranthin (**1**) started eluting early, followed by betanin (**2**), then celosianin II (**3**) in which (**3**) was apart from (**2**) but (**3**) mixed completely with (**1**). Because celosianin II was recovered in some specific fraction, a few fractions were selected for NMR structural elucidation (data not shown). Notably, the degraded betacyanins, together with the unidentified pigment (traces, *m/z* 889), have

been found before within the HSCCC run of *Atriplex* extract but they were not detected in this HPCCC run [29]. This indicates the lighter effect of this new HPCCC approach to vulnerable betalains as compared to the HSCCC approach.

In addition to the pigments, these initial HPCCC fractions also contained the hydrophilic flavonoid glycosides kaempferol 3-O-sulphate-7-O α-arabinopyranoside (**7**, *m/z* 499) and kaempferol-O-pentoside (**6**, *m/z* 419). These two kaempferol derivatives were identified based on their MS/MS fragmentation signal at *m/z* 287 and their sulphated linkage, which makes the structure more polar and better bound to the mobile phase. While compound **6** appeared far away from amaranthin and celosianin II, flavonoid **7** was partly co-eluted with both of these major pigments. On the other hand, some flavonoid-O-glycosides appeared to be slightly more lipophilic and concentrated in the stationary phase within the *extrusion-mode*. For instance, there was effective fractionation of quercetin-O-malonyl-glucoside (**4**, *m/z* 551) and kaempferol-O-malonyl glucoside (**5**, *m/z* 535) from the rest of compounds and from each other. This is because they were the two flavonoid glycosides released at the end of the separation, which indicates their low polarities. Besides, the 'unknown' structures $[M+H]^+$ at *m/z* 265 and *m/z* 611 were also partly fractionated from each other and from the pigments since they came in the middle of the MS profile. However, their quantities were not sufficient for further investigation by NMR experiments. Therefore, their structural identification was tentatively accomplished by comparisons with the flavonoid MS/MS fragmentation data available in literature [22, 28, 29]. Similarly, kaempferol-O-rhamnosyl-O-glucoside (*m/z* 595) was well isolated and concentrated in several center fractions of the HPCCC while kaempferol-O-arabinosyl-O-malonyl-glucoside (*m/z* 667) was completely co-eluted with part of major compound **4** in several early fractions within *extrusion-mode* (the recovered amount was limited). These end fractions of *extrusion* also contained traces of other unknown ion signals at *m/z* 565, 435, etc. (data not shown), which were supposed to be flavonoid glycosides based on MS/MS fragmentation and indicative of neutral loss differences, namely, Δ*m/z* 86, Δ*m/z* 80, Δ*m/z* 132, and Δ*m/z* 146 representing malonyl, sulphate, pentose, and desoxy-hexose, respectively.

Recently, the similar application of LC-MS/MS coupled with IPHPCCC to investigate the whole betalain metabolome of Vietnamese red dragon fruit (*Hylocereus polyrhizus*) was reported [30]. While this ESI - MS/MS profiling and IPHPCCC method was used to study the polar metabolites from of *Opuntia stricta var. dillenii* in detail, analytical and semi-preparative IPHPCCC separations were done to compare and evaluate the re-productivity of the method [31].

Conclusions

This report contributes to the basic phytochemical knowledge of betalains and flavonoid constituents of *Atriplex hortensis var. Rubra* and *Opuntia stricta var. dillenii* by application of the all-liquid chromatography technique HPCCC. The off-line hypenation of semi-preparative HPCCC separations with the sensitive ESI-MS/MS profiling was able to fractionate principal pigments from its derivatives and from most of the flavonoids glycosides. This approach supplies molecular weight data and fragmentation data of all ionizable molecules available in every single injected HPCCC fraction. From then, the compounds of interest can be directly pooled for further analysis. These results are in agreement with previous studies about the applications of CCC on betalain and flavonoid purification from these two plants. This work also demonstrates the potential for larger scale recoveries of bioactive compounds from plant materials of *Atriplex hortensis* and *Opuntia stricta* using CCC all-liquid separation methodology.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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