Journal of Chromatography B, 878 (2010) 538–550

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Ion-pair high-speed countercurrent chromatography in fractionation of a high-molecular weight variation of acyl-oligosaccharide linked betacyanins from purple bracts of *Bougainvillea glabra*

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ARTICLE INFO

Article history:
Received 26 June 2009
Accepted 21 December 2009
Available online 4 January 2010

Keywords:
Betanin
Betalains
Acyl-oligosaccharide linked betacyanins
Preparative ion-pair countercurrent chromatography
IP–HSCCC
HPLC–DAD–ESI–MS/MS
Trifluoroacetic acid
Perfluorinated carboxylic acids
Bougainvillea glabra
Nyctaginaceae

ABSTRACT

The natural pigment composition of purple bracts of *Bougainvillea glabra* (Nyctaginaceae) consists of a highly complex mixture of betacyanins solely differing by the substitution with a variety of acyl-oligoglycoside units. This study was focused on a two-dimensional chromatography approach, a combination of preparative high-speed countercurrent chromatography (HSCCC) and analytical C18-HPLC with ESI–DAD–MS/MS detection which finally enabled a more detailed view into the pigment profile and elucidated the existence of an overwhelming amount of varying betacyanin structures occurring in *Bougainvillea* bracts. The detected molecular weights of the pigments reached so far unknown high values and ranged up to maximum values of 1653 and 1683 Da for the largest molecules due to oligosaccharide linkage and multiple acyl substitutions. The preparative IP–HSCCC separation yielded 15 complex fractions containing betacyanins of enhanced polarity as well as structures with highly increased lipophilicity. Betacyanin structures extended by large oligosaccharide chains with bigger number of glycoside units and also carrying a reduced number of hydroxycinnamic acid substitutions were characteristic for polar pigments occurring mainly in the early eluting CCC fractions. IP–HSCCC was proven to be extremely effective for fractionating this complex crude betalain pigment extract into more defined '*polarity-windows*'. Structural analysis by analytical LC–ESI–MS/MS in the positive ionization mode detected a total sum of 146 different betacyanin pigments in the CCC fractions of reduced complexity.

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1. Introduction

Bougainvillea glabra (Nyctaginaceae) purple bracts contain a huge spectrum of highly acylated oligoglycosidic linked betacyanins forming an extremely complex pigment profile [1–5]. The betanidin aglycone is the principal chromophore linked to a variety of acylated oligosaccharide units [6,7]. The structural diversity is a chromatographic challenge which prompted us to study this large natural pool of slightly varying pigment structures more intensively. A two-dimensional chromatography approach was required to achieve more structural information and this was perfectly fulfilled by a combination of preparative ion-pair HSCCC combined with a sensitive detection by LC–DAD–ESI–MS/MS.

Recently, betalains have been separated and fractionated by ion-pair assisted high-speed countercurrent chromatography (HSCCC) from plant extracts [8]. One of the important physicochemical properties of betalain plant pigments (Fig. 1) is their significant polarity and ionization in aqueous solutions which in connection with their relatively low stability results in insolubility in any of popular solvents except of water and low-molecular alcohols [6,9]. Because of relatively high hydrophilicity of betalains it was difficult to find appropriate solvent systems capable to separate the pigments, especially the most polar ones. A series of preparative countercurrent chromatography experiments were performed to resolve betanin and isobetanin as an example of polar betacyanins present in cacti fruits from *Hylocereus polyrhizus* at substantial quantities from equally abundant phyllocactin (6′-*O*-malonyl-betanin) as well as hylocerenin (6′-*O*-(3″-hydroxy-3″-methyl-glutaryl)-betanin) [10]. Whereas the separation of betanin from the other pigments was successfully performed, the other polar pigments remained unresolved, however, numerous betacyanins and betaxanthins of higher lipophilicity were relatively easily fractionated for subsequent structural studies [10].

The betacyanins of bracts of *B. glabra* were not completely characterized in spite of several attempts [1–5]. This interesting plant with varieties occurring in very differing color hues contains many oligomeric betacyanin structures extended by several oligosaccharide units and acylation with hydroxycinnamic acids (so far

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doi:10.1016/j.jchromb.2009.12.029

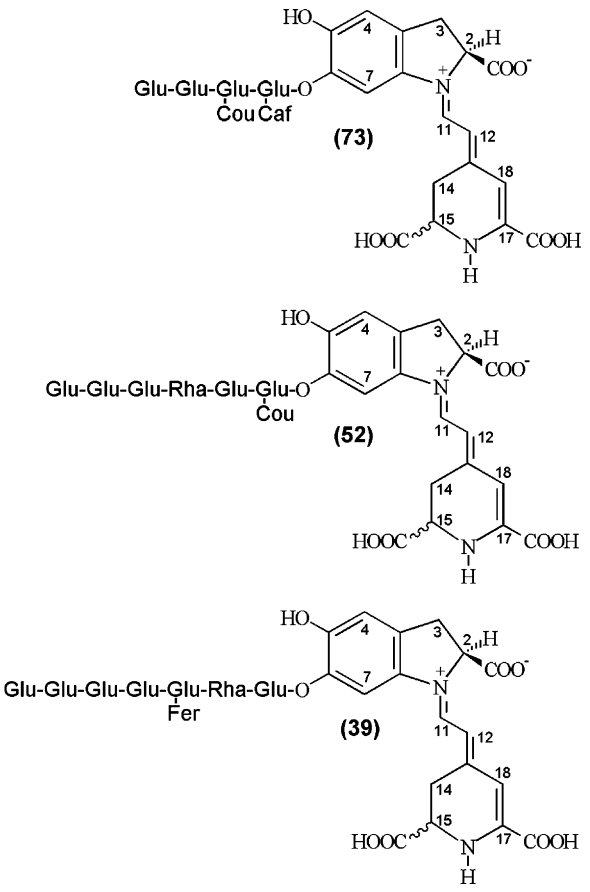

Fig. 1. Chemical structures of chosen betacyanins—the previously identified 4-sugar-ring (acyl-substituted tetroside) betanidin-6-*O*-{(2″-*O*-β-sophorosyl)-[(6′-*O*-*trans*-caffeoyl)-(6″-*O*-*trans*-4-coumaroyl)]}-β-sophoroside (**73**) [4] as well as the tentative structures of the largest pigments detected only in this study (**52** and **39**) (oligoglycoside chain: 6–7-sugars with acyl substitution) present in the HSCCC fractions of *B. glabra* bracts.

identified coumaric, ferulic and caffeic acids). The presence of many isomers overlapping each other in one-dimensional chromatography made the comprehensive analysis of the mixtures not feasible. Only the most abundant pigments were fully characterized and the others tentatively identified [4,5]. Therefore, the separation of betacyanins by preparative ion-pair HSCCC which stands for another dimension in their chromatography – because of a different mode of action [11] – was of our interest. Our first attempt to isolate betacyanins from *Phytolacca americana* berries suggested a complementarity between the modes of their separation in C18-HPLC and IP–HSCCC based on different and rather unexpected elution orders of some groups of the pigments [8]. The phenomenon was much better investigated in this study because of rich diversity of *B. glabra* betacyanins and their isomeric forms.

The principal pigments of purple *B. glabra* bracts exist mostly in the 6-*O*-glycosylated forms of betanidin [1–5]. In the case of orange and yellow pigmented varieties, biosynthesis of betaxanthin-type pigments is dominant [5]. To date, betalain structural characterization (1D- and 2D-NMR) has been greatly hampered by difficulties in isolation of significant amounts of these pigments and their low chemical stability in solutions [6,7,9]. In this contribution, a new chromatographic approach to separation of betacyanins derived from *B. glabra* bracts is presented, based on HSCCC fractionation on a preparative scale of a complex pigment mixture with the application of a solvent system with ion-pair forming trifluoroacetic acid.

2. Experimental

2.1. Reagents

Formic acid, trifluoroacetic acid (TFA), HPLC-grade acetonitrile (ACN), *tert*.-butyl methyl ether (TBME), 1-butanol, methanol and HPLC-grade water were obtained from Merck (Darmstadt, Germany).

2.2. HSCCC and HPLC apparatus

The preparative HSCCC instrument used for the separation of the acyl-oligosaccharide linked betacyanins from the violet bracts of *B. glabra* was a multilayer coil planet J-type centrifuge model CCC 1000 (Pharma-Tech Research, Baltimore, MD, USA). The three preparative coils were connected in series equipped with polytetrafluoroethylene (PTFE) tubing: 165 m × 2.6 mm i.d. with 876 mL theoretical total volume given by manufacturer (850 mL was the measured total volume). The distance (revolution radius = R) of the holder axis of the coils to the central (solar) axis of the instrument was 7.5 cm. The inner β_r-value was measured to be 0.53 at the internal end of the coil and the outer β_r-value was 0.8 (equation: $\beta_r = r/R$; r is defined as the distance from the coil (planetary) axis to the nearest and farthest layer of the PTFE tubes wound on the coil system). The HSCCC system's direction of rotation determined the *head* locations at the periphery of the three coils. The system was operated at 850 rpm in the *head-to-tail elution* mode. For the solvent delivery, a Merck-Hitachi L-6200 A HPLC gradient pump (Tokyo, Japan) was used.

A Gynkotek HPLC system with UVD340U, Gynkotek HPLC Pump Series LPG-3400A and thermostat (Gynkotek Separations, H.I. Ambacht, The Netherlands) was used for the chromatographic analysis. For the data acquisition, the software package Chromeleon 6.0 (Gynkotek Separations) was applied. For the separation of betalains a Luna C18(2) column 250 mm × 3 mm i.d., protected by a guard column (Phenomenex, Torrance, CA, USA) was used (for gradient conditions cf. Section 2.7).

The positive ion electrospray mass spectra were recorded on ThermoFinnigan LCQ Advantage (electrospray voltage 4.5 kV; capillary 250 °C; sheath gas: N_2) coupled to ThermoFinnigan LC Surveyor pump utilizing the HPLC gradient. The MS was controlled and total ion chromatograms and mass spectra were recorded using ThermoFinnigan Xcalibur software (San Jose, CA, USA). The relative collision energies for the CID experiments were set at 40–50% (according to a relative energy scale). Helium was used to improve trapping efficiency and as the collision gas for the CID experiments.

2.3. Selection of two-phase ion-pairing solvent systems

For the evaluation of suitable solvent systems with ion-pair forming capacity, the previously published solvent system 1-BuOH–ACN–H_2O (0.7% TFA) 5:1:6 (v/v/v) **I** [8,10] was compared to a system TBME–1-BuOH–ACN–H_2O (0.7% TFA) 2:2:1:5 (v/v/v/v) **II**.

The visual partition of pigment components was determined in the first system after shaking of 500 μL 1-butanol, 100 μL ACN and 600 μL acidified (TFA 0.7%) of *B. glabra* crude betacyanin extract in 2 mL vials. In the case of systems **II**, the phase separation followed after shaking of 200 μL TMBE, 200 μL of 1-butanol, 100 μL of ACN and 500 μL *B. glabra* pigment extract in 2 mL vials acidified with 0.7% TFA.

The complexity of the crude extract (known from LC–DAD and LC–ESI–MS analysis) prompted us to do a rough visual estimation of the pigment distribution. For both systems a very good distribution of the colored and obviously more lipophilic pigments between the organic (stationary) and aqueous (mobile) phase (*head-to-tail*

mode) was observed. Finally, we chose system **II** because it was superior by its stationary phase retention based on our results from *H. polyrhizus* betalain separations [10]. Stability of stationary phase is one of the most relevant parameters for a good chromatographic performance during countercurrent chromatography.

2.4. Plant material origin and preparation of crude pigment extract

Plant material of violet-colored *B. glabra* Choisy was collected in April 2007 in Guadalajara-Tonala (Mexico) (approximately ten year old shrub). A herbarium specimen is kept at the Institute of Food Chemistry of the TU-Braunschweig (I.D.: Bg-4-2007-Mex).

The fresh bracts were dried at ambient temperature (25–30 °C) in the shade. For the pigment extraction, the most intensively colored bracts were selected.

For achieving a quantitative yield during the pigment extraction, multiple maceration steps of the dried plant material (∼100 g) were performed with a mixture H2O/ACN/TFA (95:4:1, v/v/v) until no more pigments were visually recognized in the filtrate. The resulting crude pigment solutions were pooled and passed through a large paper filter no. MN 615 1/4 (Macherey & Nagel, Düren, Germany). The filtrate was diluted with nanopure water, frozen and directly lyophilized to prevent pigment degradation. For the preparative IP–HSCCC experiment no further pigment clean-up procedure was performed.

2.5. Lyophilization of resulting IP–HSCCC fractions

The eluted HSCCC fractions (F1–F14 from the *elution* mode and also the complete volume of the *coil system*—F15) – still containing large amounts of solvents – were diluted with nanopure water, then immediately frozen and directly lyophilized (Christ freeze-drier, model Beta 2-8 LD plus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). The fractions of the *elution* mode contained in principle aqueous TFA, with lower amounts of 1-butanol and acetonitrile. More difficult was the resulting high-volume coil-fraction principally consisting of 1-butanol, acetonitrile, *tert*.-butyl methyl ether and TFA. The organic residual solvents had to be condensed in a trap cooled by liquid nitrogen to prevent decomposition of the hybrid vacuum pump (Vacuubrand, Chemistry Hybrid Pump RC 6, Wertheim, Germany). This direct drying of aqueous and organic solvent parts by lyophilization is a crucial step to omit the fast decomposition of pigments and to keep the amount of decarboxylation products as low as possible in the recovered CCC fractions.

Recovered fractions (nos. 1–15) from the IP–HSCCC separation (injection amount 755 mg of *B. glabra* crude pigment extract): F1 (221 mg), F2 (222 mg), F3 (54 mg), F4 (18.5 mg), F5 (6 mg), F6 (5 mg), F7 (6 mg), F8 (4.5 mg), F9 (4.5 mg), F10 (2 mg), F11 (3.5 mg), F12 (4 mg), F13 (2 mg), F14 (5 mg), F15-coil (15.5 mg); sum of recovered yields from HSCCC fractions: 573.5 mg (∼76%).

2.6. IP–HSCCC separation of B. glabra pigments

For the ion-pair assisted HSCCC separation of the freeze-dried pigments of *B. glabra*, the previously studied solvent system TBME–1-BuOH–ACN–H_2O 2:2:1:5 (v/v/v/v) was applied with 0.7% TFA [10].

The multilayer coiled column of the HSCCC was charged by filling the upper organic phase of the solvent system as the stationary phase using a normal HPLC pump (cf. Section 2.2).

The separation was performed in the *head-to-tail* mode with the lower aqueous mobile phase. This procedure is defined as *reversed-phase* mode of CCC in analogy to RP-HPLC.

Subsequently, the freeze-dried crude *B. glabra* pigment sample (755 mg) was dissolved in 10 mL of the solvent system composition consisting of the upper and lower phase. This biphasic sample introduction was suggested by Ito and Conway [11]. The filtered sample solution was introduced into the separation column through a manual low-pressure sample injection valve (Rheodyne, Cotati, CA, USA) and a 25 mL loop without prior column equilibration. The pigment sample was completely soluble in the solvent system, and a precipitate formation was not observed.

The lower aqueous phase was pumped at a flow rate of 3.0 mL/min in the *head-to-tail* direction (*head* located at the periphery of the coil) after the rotation was set to a velocity of 850 rpm.

The elution occurred over 365 min with the mobile phase. The effluent stream from the *tail* outlet of the column was monitored at λ 540 nm using a Knauer K-2501 UV detector (Berlin, Germany) equipped with a preparative cell (0.5 mm path length) and collected into test tubes with a fraction collector (LKB SuperRac 2211, LKB, Bromma, Sweden) at 4 min intervals. The *elution* mode was stopped when the pigment concentration in the eluate had significantly decreased (time ∼365 min or elution-volume ∼1095 mL with a respective K_D value of 1.30).

After the *elution* (fractions F1–F14), the solvent in the coil column potentially containing lipophilic pigments was ejected with nitrogen gas and the stationary phase retention (S_f) was calculated to be 56%. The ejected coil volume was kept as the final and most lipophilic pigment fraction numbered as F15 (yields of CCC fractions, cf. Section 2.5). Analysis of all recovered HSCCC fractions was performed by HPLC–diode array detection (DAD), and HPLC–electrospray ionization (ESI)–MS/MS.

The IP–HSCCC run was performed at ambient temperature with no active temperature control during the separation.

2.7. HPLC–DAD and HPLC–ESI–MS/MS chromatographic system

For the separation of the analytes, the following gradient system was used: 97% A with 3% B at 0 min; gradient to 88% A at 7 min; gradient to 80% A at 35 min; gradient to 64% A at 55 min; (Solvent A—2% aqueous formic acid, Solvent B—acetonitrile). The injection volume was 10 μL, and the flow rate was 0.5 mL/min. The detection of analytes was performed typically at 538, 505, 480, 450 and 310 nm. For the UV–vis spectra aquisition the detection was performed in the DAD mode. The column was thermostated at 35 °C. The same chromatographic conditions were applied for the HPLC–ESI–MS/MS analyses.

3. Results and discussion

3.1. Identification of betacyanins of B. glabra bracts

The complexity of the betacyanin mixtures derived from the *B. glabra* extracts prompted us to study their separation by ion-pair HSCCC technique into simplified mixtures of the pigments for subsequent HPLC analyses. The HSCCC fraction nos. 5–15 contained usually few pigments of higher concentration and fraction nos. 1–4 contained a bulk of various very polar betacyanins at low level, forming in some cases background broadened optical signals during DAD-detection (Fig. 2A–C).

The performed LC–ESI–MS/MS analyses resulted in extraction of the pigment signals even from the background thanks to sufficient clean-up of the HSCCC fractions. Without the help of the HSCCC fortification stage, the analysis of the minor pigments would have been problematic, because of extensive overlapping of compounds of different polarities and substitution patterns.

In Table 1, the mostly tentatively identified pigments were listed in the specified HSCCC fractions where they have been detected.

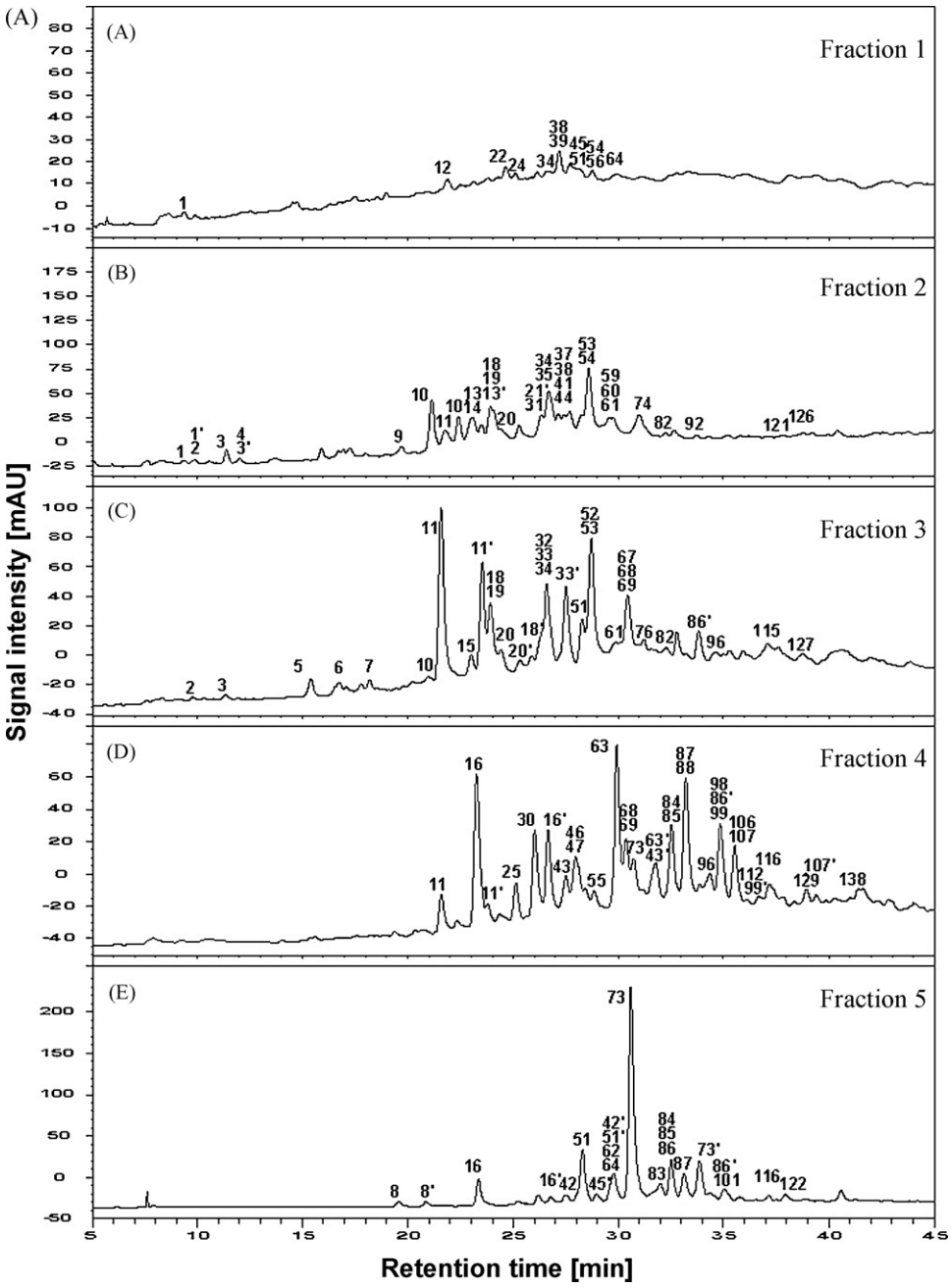

Fig. 2. (A–C) HPLC profiles (A–O) of betacyanins (monitored at λ 538 nm) analyzed in the HSCCC fractions obtained from the extract of *B. glabra* bracts in the HSCCC system. The pigment profile of the extract before the HSCCC run is depicted in the bottom chromatogram (P).

The low optical signal intensities or peak overlappings prevented the analysis of the DAD data for many of the acyl-oligosaccharide substituted betacyanin compounds. Hence, the identification relied solely on the LC–ESI–MS/MS results. The abbreviated structural sequences were deduced primarily from the MS/MS-fragmentation patterns but also from the overall polarity judged by the retention times in analytical C18-HPLC and occurrence in the IP–HSCCC fractions. The polarity of betacyanins is strongly and positively dependent on the number of the sugar units, but it also negatively depends on the increasing number and type of connected acyl-substituents. Previous studies were not reporting betacyanin pigments with higher acylation level than two groups per molecule, therefore, in this study it was also assumed that this maximal level would not be surpassed. However, for pigments with higher number of sugar units, the extensive acylation could not be excluded.

Assuming that the most common hexose-sugar unit in betacyanins was glucose [4], this group was considered as a substituent characterized by a mass cleavage of $\Delta m/z$ 162 Da of the protonated masses detected after the fragmentation in the MS^2 mode. Furthermore, the presence of the rhamnosyl ($\Delta m/z$ 146 Da) moiety was also deduced from the analyses, supported by previous results [2]. Another moiety ($\Delta m/z$ 132 Da) was only tentatively attributed to xylosyl. Interestingly, recent studies based on isolation of only major betacyanins [4] or on LC–MS^n runs of their complicated mixtures [5] were not supporting the presence of these substitutions.

In addition to the sugar units, three acyl moieties were detected: coumaroyl ($\Delta m/z$ 146 Da), caffeoyl ($\Delta m/z$ 162 Da) and feruloyl ($\Delta m/z$ 176 Da), which were the most often identified hydroxycinnamic acid derivatives in the betacyanin structures [4,5]. As

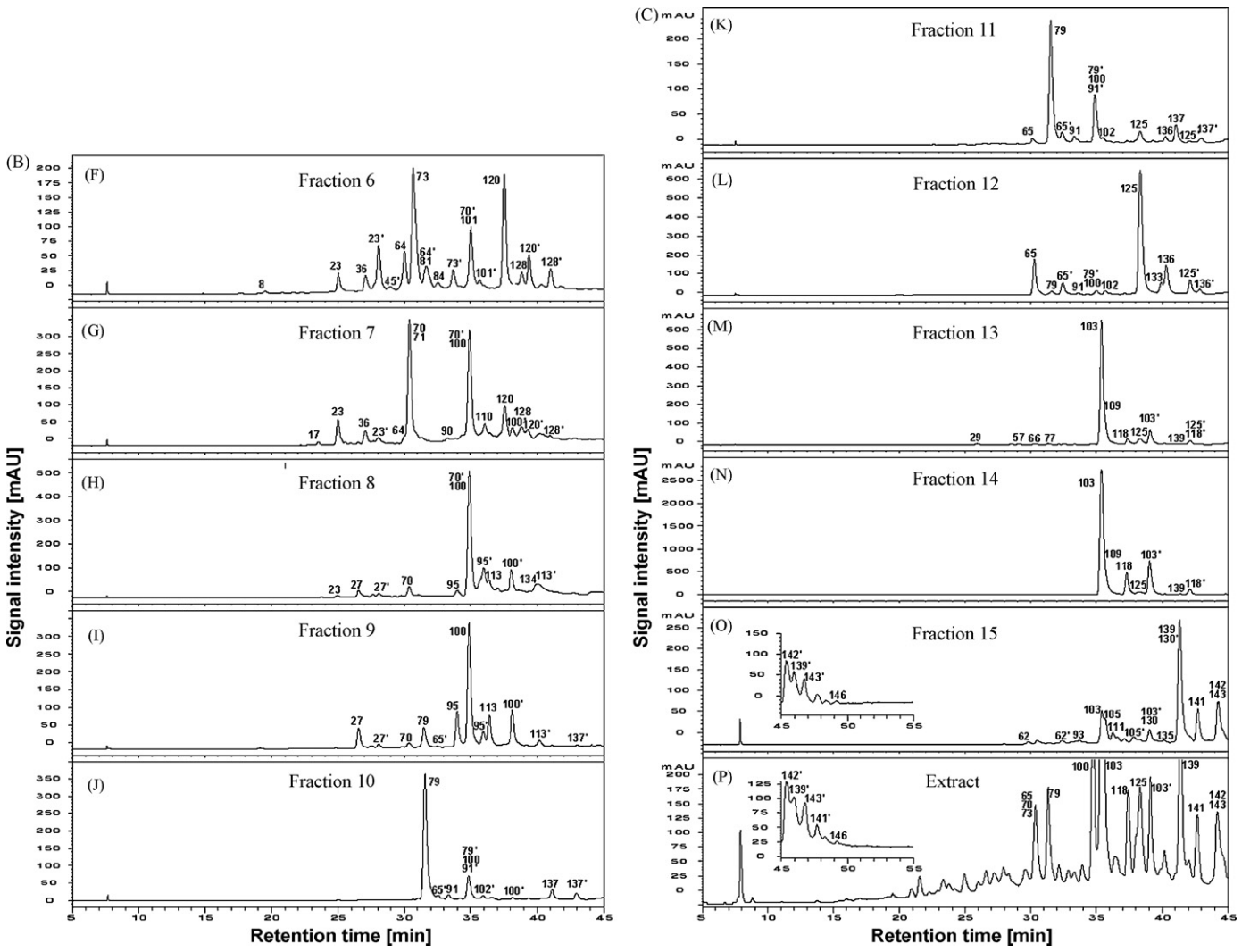

Fig. 2. (*Continue*).

mentioned above, the differentiation between the rhamnosyl- and coumaroyl- as well as glucosyl- and caffeoyl-moieties was based only on the overall polar appearance of the pigments expressed by their chromatographic properties (retention times on C18-HPLC and IP–HSCCC). Unfortunately, the optical data from the DAD detector were too scarce to support the identification of all the pigments. The sinapoyl-groups are a rare form of substitution in betacyanins [8,12] and were also not detected during the MS/MS-fragmentation experiments.

The substitution pattern of the betanidin phenolic groups in most of betacyanins from *Bougainvillea* species was found frequently as 6-*O*-substitution [4], therefore, these structural forms were also assumed in this report, however, the 5-*O*-substitution was not excluded, especially based on the presence of 5-*O*-glucosylated forms in the most polar betacyanins, betanin and glucosylated betanin [5]. The latter pigments were identified by co-elution with authentic standards derived from *Mammillaria* species [13].

The two-dimensional chromatography approach using preparative IP–HSCCC and analytical C18-HPLC (DAD and MS/MS detection) was a very effective combination for pigment analysis and identified 146 different betacyanin structures in the separated CCC fractions. This was an enormous number of pigments surpassing even the number of already known 55 betalains cited recently [14]. Especially the number of all co-eluting pigments in the more polar HSCCC fractions (nos. 1–4) was overwhelming (cf. Tables 1 and 2).

Surprisingly, the presence of a set of oligomeric betacyanins was discovered. The largest oligomeric sugar chains linked to the betanidin-type pigments were counted for 7 sugar units with monoisotopic mass $[M+H]^+$ ion-signals at *m*/*z* 1653 (**19**, **22**, **35**, **38**, **49**, **55** and **59**), then $[M+H]^+$ at *m*/*z* 1683 (**31**, **37**, **39** and **44**), accompanied by the less polar isomeric pigments—but possibly with a shorter oligosaccharide chain of 6 sugars (however, additionally acylated with caffeoyl moiety) with *m*/*z* 1653 (**69**, **78**, **89**, **97**, **104**, **112**, **123** and **127**) and *m*/*z* 1683 (**47**, **92**, **121**, **126**, **131**). The resulting MS/MS-fragmentation data allowed only for the speculative discerning of the isomeric structures, therefore, the chemical structures were completely tentatively proposed, supported by the retention times on C18-HPLC and elution order/fraction number of IP–HSCCC. The presence of the caffeoyl moiety instead of the glucosyl unit was supposed to increase the lipophilicity and hence the retention time of the pigments on IP–HSCCC and particularly stronger on C18-HPLC.

Likewise, the isomeric betacyanins with oligosaccharide chains of 5 and 6 connected sugar units with the monoisotopic ion-signals $[M+H]^+$ at *m*/*z* 1491 and *m*/*z* 1521 were also divided into 2 subgroups differing by the lack of the caffeoyl moiety in 6-ring structures **12**, **24**, **25**, **34**, **41**, **46**, **52**, **58** and **67** (*m*/*z* 1491) as well as in **40**, **50**, **53**, **54**, **60**, **74** and **80** (*m*/*z* 1521) or the

Table 1
Chromatographic, spectrophotometric and mass spectrometric data (the nominal monoisotopic *m*/*z* values) of the analyzed pigments found in *Bougainvillea glabra* bracts HSCCC fractions. Most of compounds were tentatively identified, unless inversely specified.[c].

No.	Compound	R_t [min]	Fraction no.	λ_{max}[a] [nm] I	λ_{max}[b] [nm] II	Abs. ratio II:I	*m*/*z* $[M+H]^+$	Monoisotopic *m*/*z* from MS/MS of $[M+H]^+$
1	-Glu Glu[c,e]	9.3	1–2	n.d.[d]	537	n.d.	**713**	551; 389
1′	-Glu Glu[c]	9.9	1–2	n.d.	536	n.d.	**713**	551; 389
2	-Glu[c]	9.7	2–3	n.d.	535	n.d.	**551**	389
2′	-Glu[c]	10.5	2–3	n.d.	n.d.	n.d.	**551**	389
3	-Glu Glu[c]	11.3	2–3	n.d.	539	n.d.	**713**	551; 389
3′	-Glu Glu[c]	12.0	2–3	n.d.	539	n.d.	**713**	551; 389
4	-Glu[c]	11.9	2	n.d.	539	n.d.	**551**	389
5	-Glu(Cou) Glu	15.3	3	n.d.	546	n.d.	**859**	713; 697; 551; 389
6	-Glu(Cou) Glu	16.7	3	n.d.	n.d.	n.d.	**859**	713; 697; 551; 389
7	-Glu(Cou) Glu	18.2	3	n.d.	544	n.d.	**859**	713; 697; 551; 389
8	-Glu Glu(Caf)	19.6	5–6	n.d.	n.d.	n.d.	**875**	713; 551; 389
9	Unknown	19.6	2	n.d.	n.d.	n.d.	**1227**	No fragmentation
9′	Unknown	20.2	2	n.d.	n.d.	n.d.	**1227**	No fragmentation
8′	-Glu Glu(Caf)	20.8	5	n.d.	n.d.	n.d.	**875**	713; 551; 389
10	-Glu Glu(Cou) Glu Glu	21.1	2–3	n.d.	543	n.d.	**1183**	1021; 859; 713; 551; 389
11	-Glu Glu(Cou) Glu	21.6	2–4	314	542	1:0.44	**1021**	859; 713; 551; 389
12	-Glu(Cou) Glu Rha Glu Glu Glu	21.9	1	n.d.	n.d.	n.d.	**1491**	1329; 1167; 1021; 1005; 859; 697; 551
10′	-Glu(Cou) Glu Glu Glu	22.3	2	n.d.	543	n.d.	**1183**	1021; 859; 697; 551; 389
13	-Glu Glu Glu(Cou) Glu Glu	22.8	2	n.d.	n.d.	n.d.	**1345**	1183; 1021; 875; 713; 551; 389
14	-Glu Glu(Fer) Rha Glu Xyl	23.0	2	n.d.	n.d.	n.d.	**1329**	1197; 1035; 889; 859; 713; 551; 389
15	Unknown	23.0	3	n.d.	n.d.	n.d.	**1037**	No fragmentation
16	- Glu(Caf) Glu[c]	23.3	4–5	n.d.	544	n.d.	**875**	713; 551; 389
17	-Glu(Mal) Glu Rha	23.5	7	n.d.	n.d.	n.d.	**975**	829; 667; 389
11′	-Glu Glu(Cou) Glu	23.6	2–4	314	542	1:0.48	**1021**	859; 713; 551; 389
18	-Glu(Cou) Glu Rha Glu Glu	23.8	2–3	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
19	-Glu(Cou) Glu Glu Rha Glu Glu Glu	23.8	2–3	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1021; 859; 697; 551
13′	-Glu Glu Glu(Cou) Glu Glu	24.0	2	n.d.	n.d.	n.d.	**1345**	1183; 1021; 875; 713; 551; 389
20	-Glu Glu(Fer) Glu	24.4	2–3	n.d.	n.d.	n.d.	**1051**	889; 713; 551; 389
21	-Glu(Cou) Glu Glu	24.5	2	n.d.	n.d.	n.d.	**1021**	859; 697; 551; 389
22	-Glu(Cou) Glu Rha Glu Glu Glu Glu	24.6	1	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1005; 859; 697; 551
23	-Glu Glu(Cou)[c]	25.0	6–8	n.d.	541	n.d.	**859**	713; 551; 389
24	-Glu(Cou) Glu Rha Glu Glu Glu	25.1	1	n.d.	n.d.	n.d.	**1491**	1329; 1167; 1021; 1005; 859; 697; 551
25	-Glu(Cou) Glu Glu Rha Glu Glu	25.1	4	n.d.	n.d.	n.d.	**1491**	1329; 1167; 1021; 1005; 859; 697; 551
20′	-Glu Glu(Fer) Glu	25.2	2–3	n.d.	n.d.	n.d.	**1051**	889; 713; 551; 389
26	-Glu(Mal) Glu Rha	25.5	7	n.d.	n.d.	n.d.	**975**	829; 667; 389
28	Unknown	25.8	6–7	n.d.	n.d.	n.d.	**859**	No fragmentation
29	Glu(Caf)	26.0	13	n.d.	546	n.d.	**713**	551; 389
30	-Glu Glu Glu Glu Glu(Caf)	26.1	4	n.d.	n.d.	n.d.	**1361**	1199; 1037; 875; 713; 551; 389
31	-Glu Rha Glu(Fer) Glu Glu Glu Glu	26.2	2	n.d.	n.d.	n.d.	**1683**	1521; 1359; 1197; 1021; 697
18′	-Glu(Cou) Glu Rha Glu Glu	26.2	3–4	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
21′	-Glu Glu(Cou) Glu	26.2	2	n.d.	n.d.	n.d.	**1021**	859; 713; 551; 389
32	-Glu(Cou) Glu Glu	26.5	3	n.d.	n.d.	n.d.	**1021**	859; 697; 551; 389
27	-Glu Glu Glu Glu(Caf)	26.6	8–9	n.d.	553	n.d.	**1199**	1037; 875; 713; 551; 389
33	-Glu Glu Glu(Cou) Glu Glu	26.6	3	n.d.	n.d.	n.d.	**1345**	1183; 1021; 875; 713; 551; 389
34	-Glu(Cou) Glu Rha Glu Glu Glu	26.6	1–3	n.d.	n.d.	n.d.	**1491**	1329; 1167; 1021; 1005; 859; 697; 551
16′	-Glu(Caf) Glu[c]	26.7	4–5	n.d.	n.d.	n.d.	**875**	713; 551; 389
35	-Glu(Cou) Glu Glu Rha Glu Glu Glu	26.9	2	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1021; 859; 697; 551
36	Unknown	27.1	6–7	n.d.	n.d.	n.d.	**859**	No fragmentation
37	-Glu Rha Glu(Fer) Glu Glu Glu Glu	27.1	2	n.d.	n.d.	n.d.	**1683**	1521; 1359; 1197; 1021
38	-Glu(Cou) Glu Rha Glu Glu Glu Glu	27.2	1–3	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1005; 859; 697; 551
39	-Glu Rha Glu(Fer) Glu Glu Glu Glu	27.2	1	n.d.	n.d.	n.d.	**1683**	1521; 1359; 1197; 1021; 859
40	-Glu Rha Glu(Fer) Glu Glu Glu	27.2	3–4	n.d.	n.d.	n.d.	**1521**	1359; 1197; 1035; 1021; 859; 697; 389
29′	-Glu(Caf)	27.3	13	n.d.	n.d.	n.d.	**713**	551; 389
41	-Glu(Cou) Glu Rha Glu Glu Glu	27.4	2	n.d.	n.d.	n.d.	**1491**	1329; 1167; 1021; 1005; 859; 697; 551

Table 1 (*Continued*)

No.	Compound	R_t [min]	Fraction no.	λ_{max}[a] [nm] I	λ_{max}[b] [nm] II	Abs. ratio II:I	m/z $[M+H]^+$	Monoisotopic m/z from MS/MS of $[M+H]^+$
42	-Glu(Cou) Glu Rha Glu	27.5	5	n.d.	n.d.	n.d.	**1167**	1005; 859; 697; 389
30′	-Glu Glu Glu Glu Glu(Caf)	27.5	4	n.d.	n.d.	n.d.	**1361**	1199; 1037; 875; 713; 551; 389
43	-Glu(Cou) Glu Rha Glu Glu	27.5	2–3	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
33′	-Glu Glu Glu(Cou) Glu Glu	27.5	3	n.d.	n.d.	n.d.	**1345**	1183; 1021; 875; 713; 551; 389
44	-Glu Rha Glu(Fer) Glu Glu Glu Glu	27.5	1	n.d.	n.d.	n.d.	**1683**	1521; 1359; 1197; 1021; 859
45	-Glu Glu(Fer)	27.9	4–6	n.d.	n.d.	n.d.	**889**	713; 551; 389
46	-Glu(Cou) Glu Glu Rha Glu Glu	27.9	4	n.d.	n.d.	n.d.	**1491**	1329; 1167; 1021; 1005; 859; 697; 551
23′	-Glu Glu(Cou)[c]	28.0	6–7	n.d.	543	n.d.	**859**	713; 551; 389
27′	-Glu Glu Glu Glu(Caf)	28.1	8–9	n.d.	n.d.	n.d.	**1199**	1037; 875; 713; 551; 389
47	-Glu Rha Glu(Fer) Glu Glu Glu Caf	28.2	2	n.d.	n.d.	n.d.	**1683**	1521; 1359; 1197; 1021; 889
48	-Glu(Fer) Glu Glu	28.2	2	n.d.	n.d.	n.d.	**1051**	889; 727; 551; 389
49	-Glu(Cou) Glu Glu Rha Glu Glu Glu	28.2	2	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1021; 859; 697; 551
50	-Glu Rha(Fer) Glu Glu Glu Glu	28.2	1–4	n.d.	n.d.	n.d.	**1521**	1359; 1197; 1035; 1021; 859; 697; 389
51	-Glu(Cou) Glu Rha Glu Glu	28.2	3–5	n.d.	551	n.d.	**1329**	1167; 1005; 859; 697; 389
52	-Glu(Cou) Glu Rha Glu Glu Glu	28.6	2–3	318	548	1:0.57	**1491**	1329; 1167; 1021; 1005; 859; 697; 551
53	-Glu Rha(Fer) Glu Glu Glu Glu	28.7	1–2	n.d.	n.d.	n.d.	**1521**	1359; 1197; 1035; 1021; 859; 697; 389
54	-Glu Rha Glu(Fer) Glu Glu Glu	28.7	4	n.d.	n.d.	n.d.	**1521**	1359; 1197; 1035; 1021; 859; 697; 389
55	-Glu(Cou) Glu Rha Glu Glu Glu Glu	28.8	1	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1021; 1005; 859; 697; 551
45′	-Glu Glu(Fer)	29.0	5–6	n.d.	n.d.	n.d.	**889**	713; 551; 389
56	-Glu(Caf)	29.1	13	n.d.	546	n.d.	**713**	551; 389
57	-Glu Rha Glu(Fer) Glu Glu	29.2	2	n.d.	n.d.	n.d.	**1329**	1197; 1035; 889; 859; 697; 551; 389
58	-Glu(Cou) Glu Rha Glu Glu Glu	29.5	2	n.d.	n.d.	n.d.	**1491**	1329; 1167; 1021; 1005; 859; 697; 551
59	-Glu(Cou) Glu Glu Rha Glu Glu Glu	29.6	2	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1021; 859; 697; 551
60	-Glu Rha Glu(Fer) Glu Glu Glu	29.8	2–3	n.d.	n.d.	n.d.	**1521**	1359; 1197; 1035; 1021; 859; 697; 389
61	-Glu(Cou)	29.8	15	n.d.	n.d.	n.d.	**697**	551; 389
42′	-Glu(Cou) Glu Rha Glu	29.8	5	n.d.	n.d.	n.d.	**1167**	1005; 859; 697; 389
51′	-Glu(Cou) Glu Rha Glu Glu	29.8	4–5	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
62	-Glu(Cou) Glu Rha Glu Glu	29.9	4–5	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
63	-Glu Glu(Fer) Rha Glu Glu	29.9	4	n.d.	n.d.	n.d.	**1359**	1197; 1035; 889; 859; 713; 551; 389
64	-Glu(Cou) Glu(Caf) Glu Glu	30.0	5–7	312	544	1:0.45	**1345**	1183; 1021; 875; 859; 697; 551; 389
65	-Glu(Cou) Glu(Caf) Glu	30.1	11-12	316	549	1:0.92	**1183**	1021; 875; 859; 697; 551; 389
66	-Glu(Caf)	30.2	13	n.d.	n.d.	n.d.	**713**	551; 389
67	-Glu(Cou) Glu Rha Glu Glu Glu	30.3	2–3	n.d.	n.d.	n.d.	**1491**	1329; 1167; 1021; 1005; 859; 697; 551
68	-Glu(Cou) Glu Glu	30.3	3–4	n.d.	n.d.	n.d.	**1021**	859; 697; 551; 389
69	-Glu(Cou) Glu Rha Glu Glu Glu Caf	30.3	3–4	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1021; 1005; 859; 697; 551
70	-Glu(Cou) Glu[c]	30.3	7–9	312	544	1:0.42	**859**	697; 551; 389
71	-Glu(Mal) Glu Rha	30.3	7	n.d.	n.d.	n.d.	**975**	829; 667; 389
72	-Glu(Cou) Glu(Caf) Glu	30.5	5	n.d.	n.d.	n.d.	**1183**	1021; 875; 859; 697; 551; 389
73	-Glu(Caf) Glu(Cou) Glu Glu[c]	30.7	4–6	312	546	1:0.85	**1345**	1183; 1021; 875; 859; 713; 551; 389
74	-Glu Rha Glu(Fer) Glu Glu Glu	31.0	2–3	n.d.	n.d.	n.d.	**1521**	1359; 1197; 1035; 1021; 859; 697; 389
75	-Glu(Cou) Glu Rha Glu Glu Caf	31.1	4	n.d.	n.d.	n.d.	**1491**	1329; 1167; 1021; 1005; 859; 697; 551
71′	-Glu(Mal) Glu Rha	31.1	7	n.d.	n.d.	n.d.	**975**	829; 667; 389
76	-Glu(Cou) Glu Rha Glu Glu	31.2	3	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
77	-Glu(Caf)	31.3	13	n.d.	n.d.	n.d.	**713**	551; 389

Table 1 (*Continued*)

No.	Compound	R_t [min]	Fraction no.	λ_{max}[a] [nm] I	λ_{max}[b] [nm] II	Abs. ratio II:I	m/z $[M+H]^+$	Monoisotopic m/z from MS/MS of $[M+H]^+$
78	-Glu(Cou) Glu Rha Glu Glu Glu Caf	31.3	2	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1021; 1005; 859; 697; 551
79	-Glu(Caf) Glu(Cou) Glu[c]	31.5	9–12	318	548	1:0.89	**1183**	1021; 875; 859; 713; 551; 389
80	-Glu Rha(Fer) Glu Glu Glu Glu	31.5	2–3	n.d.	n.d.	n.d.	**1521**	1359; 1197; 1035; 1021; 859; 697; 389
64′	-Glu(Cou) Glu(Caf) Glu Glu	31.6	6	n.d.	548	n.d.	**1345**	1183; 1021; 875; 859; 697; 551; 389
81	-Glu(Cou) Glu Glu Rha	31.7	6	n.d.	n.d.	n.d.	**1167**	1021; 859; 697; 389
62′	-Glu(Cou) Glu Rha Glu Glu	31.8	4–5	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
63′	-Glu Glu(Fer) Rha Glu Glu	31.8	4	n.d.	n.d.	n.d.	**1359**	1197; 1035; 889; 859; 713; 551; 389
82	-Glu(Fer) Glu Glu	32.1	2–3	n.d.	n.d.	n.d.	**1051**	889; 727; 551; 389
83	-Glu(Cou) Glu Rha Glu	32.1	5	n.d.	n.d.	n.d.	**1167**	1005; 859; 697; 389
61′	-Glu(Cou)	32.4	15	n.d.	n.d.	n.d.	**697**	551; 389
84	-Glu(Fer) Glu	32.5	4–6	n.d.	546	n.d.	**889**	727; 713; 551; 389
85	-Glu(Cou) Glu Glu Rha Glu Caf	32.5	4–5	n.d.	n.d.	n.d.	**1491**	1329; 1167; 1021; 1005; 859; 697; 551
86	-Glu(Caf) Glu(Fer) Glu Glu	32.5	5	n.d.	n.d.	n.d.	**1375**	1213; 1051; 889; 875; 713; 551; 389
65′	-Glu(Cou) Glu(Caf) Glu	32.5	9-12	312	547	1:0.86	**1183**	1021; 875; 859; 697; 551; 389
87	-Glu(Caf) Glu(Fer) Glu Glu	33.1	4–5	n.d.	553	n.d.	**1375**	1213; 1051; 889; 875; 713; 551; 389
88	-Glu Rha(Fer) Glu Glu Glu Caf	33.1	4	n.d.	n.d.	n.d.	**1521**	1359; 1197; 1035; 1021; 859; 697; 389
89	-Glu(Cou) Glu Glu Rha Glu Glu Caf	33.2	3	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1021; 859; 697; 551
90	-Glu Rha Xyl Cou	33.3	7	n.d.	n.d.	n.d.	**975**	829; 697; 551; 507; 389
91	-Glu(Fer) Glu(Caf) Glu	33.3	10–11	n.d.	542	n.d.	**1213**	1051; 889; 875; 727; 551; 389
92	-Glu Rha Glu(Fer) Glu Glu Glu Caf	33.8	2	n.d.	n.d.	n.d.	**1683**	1521; 1359; 1197; 1021
93	-Glu(Cou)	33.8	15	n.d.	n.d.	n.d.	**697**	551; 389
73′	-Glu(Caf) Glu(Cou) Glu Glu[c]	33.8	5–6	n.d.	548	n.d.	**1345**	1183; 1021; 875; 859; 713; 551; 389
76′	-Glu(Cou) Glu Rha Glu Glu	33.8	3	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
94	-Glu Rha Glu(Fer) Glu Glu Caf	33.9	4	n.d.	n.d.	n.d.	**1521**	1359; 1197; 1035; 1021; 859; 697; 389
90′	-Glu Rha Xyl Cou	33.9	7	n.d.	n.d.	n.d.	**975**	829; 697; 551; 507; 389
95	-Glu(Caf) Glu(Fer) Glu	34.0	8–9	n.d.	552	n.d.	**1213**	1051; 889; 875; 713; 551; 389
96	-Glu(Cou) Glu Glu	34.3	3–4	n.d.	n.d.	n.d.	**1021**	859; 697; 551; 389
97	-Glu(Cou) Glu Rha Glu Glu Glu Caf	34.3	2	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1005; 859; 697; 551
98	-Glu(Cou) Glu Glu Rha Glu Caf	34.8	4	n.d.	n.d.	n.d.	**1491**	1329; 1167; 1021; 1005; 859; 697; 551
99	-Glu(Fer) Glu Rha Glu	34.8	4	n.d.	n.d.	n.d.	**1197**	1035; 1021; 1005; 889; 859; 727; 697; 389
86′	-Glu(Caf) Glu(Fer) Glu Glu	34.8	4–5	n.d.	n.d.	n.d.	**1375**	1213; 1051; 889; 875; 713; 551; 389
79′	-Glu(Caf) Glu(Cou) Glu[c]	34.9	10–12	322	549	1:0.91	**1183**	1021; 875; 859; 713; 551; 389
100	-Glu(Cou) Glu(Cou) Glu Glu	34.9	7–12	315	548	1:0.87	**1329**	1167; 1005; 859; 697; 389
70′	-Glu(Cou) Glu[c]	35.0	6–8	314	544	1:0.47	**859**	697; 551; 389
101	-Glu(Cou) Glu Glu Rha	35.1	5–6	n.d.	n.d.	n.d.	**1167**	1021; 859; 697; 389
91′	-Glu(Fer) Glu(Caf) Glu	35.1	10–11	n.d.	n.d.	n.d.	**1213**	1051; 889; 875; 727; 551; 389
102	-Glu(Cou) Glu Rha Glu Glu	35.2	11–12	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
103	-Glu(Cou) Glu(Cou) Glu[c]	35.4	13–15	314	541	1:0.95	**1167**	1005; 859; 697; 389
104	-Glu(Cou) Glu Rha Glu Glu Glu Caf	35.4	3	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1021; 1005; 859; 697; 551
105	-Glu(Cou) Glu(Caf)	35.5	15	n.d.	551	n.d.	**1021**	875; 859; 713; 697; 389
106	-Glu Rha(Fer) Glu Glu Glu Caf	35.5	3–4	n.d.	n.d.	n.d.	**1521**	1359; 1197; 1035; 1021; 859; 697; 389
107	-Glu(Cou) Glu Glu Rha	35.5	4	n.d.	n.d.	n.d.	**1169**	1021; 859; 697; 389
108	-Glu(Cou) Glu(Cou) Glu Glu Oxa	35.5	8	n.d.	n.d.	n.d.	**1401**	1357; 1329; 1167; 1005; 859; 697; 551
93′	-Glu(Cou)	35.5	15	n.d.	n.d.	n.d.	**697**	551; 389
101′	-Glu(Cou) Glu Glu Rha	35.7	6	n.d.	n.d.	n.d.	**1167**	1021; 859; 697; 389

Table 1 (*Continued*)

No.	Compound	R_t [min]	Fraction no.	λ_{max}[a] [nm] I	λ_{max}[b] [nm] II	Abs. ratio II:I	m/z $[M+H]^+$	Monoisotopic m/z from MS/MS of $[M+H]^+$
109	-Glu(Cou) Glu(Cou) Glu	35.8	13–14	n.d.	n.d.	n.d.	**1167**	1005; 859; 697; 389
87′	-Glu(Caf) Glu(Fer) Glu Glu	35.8	5	n.d.	n.d.	n.d.	**1375**	1213; 1051; 889; 875; 713; 551; 389
95′	-Glu(Caf) Glu(Fer) Glu	35.9	8–9	n.d.	552	n.d.	**1213**	1051; 889; 875; 713; 551; 389
102′	-Glu(Cou) Glu(Cou) Glu Glu	36.0	10–11	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
110	-Glu(Cou) Glu(Cou) Glu (Mal) Glu	36.1	7	n.d.	n.d.	n.d.	**1445**	1283; 1167; 1005; 859; 697; 551
111	-Glu(Cou) Glu(Caf)	36.1	15	n.d.	n.d.	n.d.	**1021**	875; 859; 713; 697; 389
112	-Glu(Cou) Glu Rha Glu Glu Glu Caf	36.2	3–4	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1021; 1005; 859; 697; 551
113	-Glu(Cou) Glu(Cou) Glu Glu	36.4	8–9	n.d.	547	1:0.92	**1329**	1167; 1005; 859; 697; 389
114	-Glu(Cou) Glu(Caf)	36.4	15	n.d.	551	n.d.	**1021**	875; 859; 713; 697; 389
99′	-Glu(Fer) Glu Rha Glu	36.7	4	n.d.	n.d.	n.d.	**1197**	1035; 1021; 1005; 889; 859; 727; 697; 389
115	-Glu(Fer) Glu Glu	37.0	3	n.d.	n.d.	n.d.	**1051**	889; 727; 713; 389
116	-Glu(Fer) Glu	37.2	4–5	n.d.	n.d.	n.d.	**889**	727; 713; 551; 389
117	-Glu(Cou) Glu(Cou) Glu (Mal) Glu	37.2	7	n.d.	n.d.	n.d.	**1445**	1283; 1167; 1005; 859; 697; 551
118	-Glu(Cou) Glu(Cou) Glu	37.3	13–14	312	546	1:0.88	**1167**	1005; 859; 697; 389
119	-Glu(Cou) Glu Rha Glu Glu	37.5	3	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
120	-Glu(Cou) Glu(Fer) Glu Glu	37.6	6–7	318	549	1:0.92	**1359**	1197; 1035; 889; 859; 727; 697; 389
105′	-Glu(Cou) Glu(Caf)	37.7	15	n.d.	551	n.d.	**1021**	875; 859; 713; 697; 389
121	-Glu Rha Glu(Fer) Glu Glu Glu Caf	37.8	2	n.d.	n.d.	n.d.	**1683**	1521; 1359; 1197; 859
122	-Glu(Cou) Glu Glu Rha Glu Caf	37.9	5	n.d.	n.d.	n.d.	**1491**	1329; 1167; 1021; 1005; 859; 697; 551
123	-Glu(Cou) Glu Rha Glu Glu Glu Caf	38.0	3	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1021; 1005; 859; 697; 551
100′	-Glu(Cou) Glu(Cou) Glu Glu	38.1	7–9	315	547	1:0.93	**1329**	1167; 1005; 859; 697; 389
124	-Glu Rha(Fer) Glu Glu Glu Caf	38.2	4	n.d.	n.d.	n.d.	**1521**	1359; 1197; 1035; 1021; 859; 697; 389
125	-Glu(Cou) Glu(Fer) Glu	38.2	11–14	316	548	1:0.89	**1197**	1035; 889; 859; 727; 697; 389
126	-Glu Rha Glu(Fer) Glu Glu Glu Caf	38.8	2	n.d.	n.d.	n.d.	**1683**	1521; 1359; 1197; 1035; 889;
127	-Glu(Cou) Glu Rha Glu Glu Glu Caf	38.9	3	n.d.	n.d.	n.d.	**1653**	1491; 1329; 1167; 1005; 859; 697; 551
128	-Glu(Cou) Glu(Fer) Glu Glu	38.9	6–7	n.d.	547	n.d.	**1359**	1197; 1035; 889; 859; 727; 697; 389
129	-Glu(Cou) Glu Rha Glu Glu	38.9	4	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
130	-Glu(Caf) Glu(Fer)	39.1	15	n.d.	553	n.d.	**1051**	889; 875; 713; 551; 389
103′	-Glu(Cou) Glu(Cou) Glu[c]	39.1	13–15	314	546	1:0.87	**1167**	1005; 859; 697; 389
131	-Glu Rha Glu(Fer) Glu Glu Glu Caf	39.2	2	n.d.	n.d.	n.d.	**1683**	1521; 1359; 1197
132	-Glu(Cou) Glu(Caf)	39.3	15	n.d.	551	n.d.	**1021**	875; 859; 713; 697; 389
120′	-Glu(Cou) Glu(Fer) Glu Glu	39.3	6–7	322	549	1:0.94	**1359**	1197; 1035; 889; 859; 727; 697; 389
107′	-Glu(Cou) Glu Glu Rha	39.4	4	n.d.	n.d.	n.d.	**1169**	1021; 859; 697; 389
133	-Glu(Cou) Glu(Fer) Glu	39.6	12	319	547	1:0.84	**1197**	1035; 889; 859; 727; 697; 389
134	-Glu(Cou) Glu(Cou) Glu Glu Oxa	39.9	8	n.d.	552	n.d.	**1401**	1357; 1329; 1167; 1005; 859; 697; 551
135	-Glu(Cou) Glu(Caf)	40.0	15	n.d.	551	n.d.	**1021**	875; 859; 713; 697; 389
136	-Glu(Cou) Glu(Fer) Glu	40.1	11–13	318	548	1:0.98	**1197**	1035; 889; 859; 727; 697; 389
113′	-Glu(Cou) Glu(Cou) Glu Glu	40.2	8–9	n.d.	n.d.	n.d.	**1329**	1167; 1005; 859; 697; 389
128′	-Glu(Cou) Glu(Fer) Glu Glu	41.0	6–7	n.d.	547	n.d.	**1359**	1197; 1035; 889; 859; 727; 697; 389
137	-Glu(Fer) Glu(Fer) Glu	41.1	9–11	n.d.	550	n.d.	**1227**	1065; 889; 727; 551; 389
138	-Glu Rha(Fer) Glu Glu Glu Caf	41.2	4	n.d.	n.d.	n.d.	**1521**	1359; 1197; 1035; 1021; 859; 697; 389
139	-Glu(Cou) Glu(Cou)[c]	41.2	13–15	n.d.	546	n.d.	**1005**	859; 697; 551; 389
130′	-Glu(Caf) Glu(Fer)	41.2	15	n.d.	552	n.d.	**1051**	889; 875; 713; 551; 389
140	-Glu(Cou) Glu(Cou)	41.5	15	n.d.	n.d.	n.d.	**1005**	859; 697; 551; 389
125′	-Glu(Cou) Glu(Fer) Glu	42.0	11–13	321	547	1:0.83	**1197**	1035; 889; 859; 727; 697; 389
118′	-Glu(Cou) Glu(Cou) Glu	42.1	13–14	318	546	1:0.70	**1167**	1005; 859; 697; 389
141	-Glu(Cou) Glu(Cou)	42.5	15	n.d.	548	n.d.	**1005**	859; 697; 551; 389

Table 1 (*Continued*)

No.	Compound	R_t [min]	Fraction no.	λ_{max}[a] [nm] I	λ_{max}[b] [nm] II	Abs. ratio II:I	m/z $[M+H]^+$	Monoisotopic m/z from MS/MS of $[M+H]^+$
136′	-Glu(Cou) Glu(Fer) Glu	42.8	11–12	n.d.	548	n.d.	**1197**	1035; 889; 859; 727; 697; 389
137′	-Glu(Fer) Glu(Fer) Glu	43.0	9–11	n.d.	552	n.d.	**1227**	1065; 889; 727; 551; 389
142	-Glu(Fer) Glu(Cou)	44.2	15	n.d.	548	n.d.	**1035**	889; 859; 727; 697; 551; 389
143	-Glu(Fer) Glu(Cou)	44.6	15	n.d.	548	n.d.	**1035**	889; 859; 727; 697; 551; 389
142′	-Glu(Fer) Glu(Cou)	45.4	15	n.d.	546	n.d.	**1035**	889; 859; 727; 697; 551; 389
139′	-Glu(Cou) Glu(Cou)	45.9	15	n.d.	546	n.d.	**1005**	859; 697; 551; 389
143′	-Glu(Fer) Glu(Cou)	46.7	15	n.d.	546	n.d.	**1035**	889; 859; 727; 697; 551; 389
144	-Glu(Fer) Glu(Fer)	47.2	15	n.d.	n.d.	n.d.	**1065**	889; 727; 551; 389
141′	-Glu(Cou) Glu(Cou)	47.7	15	n.d.	n.d.	n.d.	**1005**	859; 697; 551; 389
145	-Glu(Fer) Glu(Cou)	48.3	15	n.d.	546	n.d.	**1035**	889; 859; 727; 697; 551; 389
146	-Glu(Fer) Glu(Fer)	49.2	15	n.d.	n.d.	n.d.	**1065**	889; 727; 551; 389

[a] λ_{max} of hydroxycinnamoyl moiety (HCA/I).
[b] λ_{max} of betaxanthins or betacyanins in the visible range (II).
[c] Previously identified betacyanins/isobetacyanins [4]:

1/1′—Betanidin-5-*O*-β-sophoroside [bougainvillein-r-I];
2/2′—Betanidin-5-*O*-β-glucoside [betanin];
3/3′—Betanidin-6-*O*-β-sophoroside [bougainvillein-v];
4/4′—Betanidin–6-*O*-β-glucoside [gomphrenin I];
16/16′—Betanidin-6-*O*-(6′-*O*-*trans*-caffeoyl)-β-sophoroside;
23/23′—Betanidin-6-*O*-(6″-*O*-*trans*-4-coumaroyl)-β-sophoroside;
70/70′—Betanidin-6-*O*-(6′-*O*-*trans*-4-coumaroyl)-β-sophoroside;
73/73′—Betanidin-6-*O*-{(2″-*O*-*β*-sophorosyl)-[(6′-*O*-*trans*-caffeoyl)-(6″-*O*-*trans*-4-coumaroyl)]}-β-sophoroside;
79/79′—Betanidin-6-*O*-{(2″-*O*-β-glucosyl)-[(6′-*O*-*trans*-caffeoyl)-(6″-*O*-*trans*-4-coumaroyl)]}-β-sophoroside;
103/103′—Betanidin-6-*O*-[(2″-*O*-β-glucosyl)-(6′,6″-di-*O*-*trans*-4-coumaroyl)]-β-sophoroside;
139/139′—Betanidin-6-*O*-(6′,6″-di-*O*-*trans*-4-coumaroyl)-β-sophoroside.

[d] No absorbance band detectable due to low signal intensity or peak co-elution.
[e] Glu = glucosyl; Rha = rhamnosyl; Xyl = xylosyl; Cou = coumaroyl; Fer = feruloyl; Caf = caffeoyl; Oxa = oxalyl; Mal = malyl.

presence of caffeoyl moiety in 5-ring structures **75**, **85**, **98** and **122** (m/z 1491) as well as in **88**, **94**, **106**, **124** and **138** (m/z 1521).

The speculative proposals might explain the big range of polarities of the oligomeric isomers experienced in HPLC columns. However, all of these pigments were possibly too polar to be separated in the IP–HSCCC solvent system, therefore, they were eluted in the first 3–4 polar fractions.

In some cases, the possibility of multi-branched sugar structures couldn't be excluded solely by using the information from the MS/MS experiments—showing identical fragment ions of cleaved glucosyl moieties being detached in either case.

Table 2
The most abundant betacyanin (only the 15*S* forms) distribution in the recovered fractions obtained from *Bougainvillea glabra* bracts by IP–HSCCC. The highest pigment values in the fractions are written in bold letters.

No.	R_t	m/z	Relative content of pigment in HSCCC fractions analyzed by HPLC–DAD–MS														Total HPLC peak area
	[min]	$[M+H]^+$	Betacyanin fraction no.														
			2	3	4	5	6	7	8	9	10	11	12	13	14	15[a]	
11	21.6	1021	9.1	**79.2**	11.7												7700
23	25.0	859					22.9	**68.8**	8.3								10,900
16	23.3	875			**69.0**	31.0											11,600
52	28.6	1491	**52.4**	47.6													2100
65	30.1	1183										7.7	**87.5**	4.8			10,400
70	30.3	859					3.9	**78.9**	13.3	3.9							38,000
73	30.7	1345			4.4	**71.2**	24.4										9000
79	31.5	1183								6.5	**49.1**	22.2	21.0	1.2			42,800
87	33.1	1375			**62.9**	37.1											3500
91	33.3	1213									**52.6**	47.4					1900
95	34.0	1213							**49.4**	**50.6**							2470
100	35.0	1329						22.9	**40.7**	30.3	2.7	1.7	1.0	0.7			29,700
103	35.4	1167												22.6	**73.6**	3.8	106,000
125	38.2	1197										5.0	**82.4**	5.4	7.2		27,900
128	38.9	1359					**62.5**	37.5									9600
137	41.1	1227								4.9	**65.6**	29.5					6100
139	41.2	1005												3.0	8.3	**88.7**	16,900
142	44.0	1035														100.0	5000

[a] Fraction no. 15 was recovered from the complete solvent volume of the coil system from the CCC-apparatus.

An elevated HPLC peak for which the λ_{max} 548 nm and λ_{HCA} 318 nm were obtained from the DAD was detected for **52**. The ratio of absorbances λ_{max}: λ_{HCA} was close to the value 0.5, thus confirming the attachment of one acyl moiety to the sugar chain.

The analyses of the HPLC profile (Fig. 2C) of the *B. glabra* crude pigment extract confirmed the presence of the known structures from the previous studies based on NMR, LC–MS/MS and inter-sugar linkage analysis [4,5], tracing the non-acylated betacyanins (Table 1): **3** (*m*/*z* 713) and **4** (*m*/*z* 551) as well as the acylated betacyanins: **16** (*m*/*z* 875), **23** (*m*/*z* 859), **70** (*m*/*z* 859), **73** (*m*/*z* 1345), **79** (*m*/*z* 1183), **103** (*m*/*z* 1167) and **139** (*m*/*z* 1005) (cf. Table 1). These pigments gave high signal intensities during the HPLC chromatographic analyses, therefore, they were readily identified and were used as the guiding reference points for the further speculations of the other unknown and complex substituted pigment structures. Pigment **73** (betanidin-6-*O*-{(2″-*O*-β-sophorosyl)-[(6′-*O*-*trans*-caffeoyl)-(6″-*O*-*trans*-4-coumaroyl)]}-β-sophoroside) had been regarded as the longest-chain of acyl-substituted oligomeric sugars linked to a betacyanin found in the nature, so far. Interestingly, for each of the reference acylated pigment, more isomeric structures of similar or different linkages were detected (e.g. a selection of pigments with $[M+H]^+$ ion-signals at *m*/*z* 1167, but different retention times on C18-HPLC). In addition, all the coumaroylated pigments which we had named reference betacyanins had their corresponding analogues characterized by higher HPLC retention times and acylation with ferulic acid instead of coumaric acid: **45** (*m*/*z* 889), **84** (*m*/*z* 889), **86** or **87** (*m*/*z* 1375), **91** or **95** (*m*/*z* 1213), **125** (*m*/*z* 1197) and **142** (*m*/*z* 1035). Two exchanges of the coumaroyls into feruloyls in **103** (*m*/*z* 1167) and **139** (*m*/*z* 1005) resulted in the appearance of their bi-feruloylated analogues: **137** (*m*/*z* 1227) and **144** (*m*/*z* 1065), respectively. For most of these pigments, it was possible to tentatively determine their diastereomeric forms, taking into account the HPLC retention time differences between the forms.

Some unknown pigments **17**, **26, 71** and **90** occurred with ion-signals $[M+H]^+$ at *m*/*z* 975. Interestingly, they all were eluted in CCC fraction 7 but were characterized by very different elution times on C18-HPLC. From the observed MS/MS-fragmentation pattern ($\Delta m/z$ 278) for **17**, **26** and **71** we assume the complete cleavage of a glucosyl-malyl subunit ($\Delta m/z$ 162 + 116) directly attached to the betanidin aglycone. The bifunctionality caused by the two carboxyl groups of malic acid may result in different malyl-esters with potentially different retention times on C18-HPLC. The fragmentation pattern of **90** was different and resulted in another structure deduction based on $\Delta m/z$ 146 (probably coumaroyl), $\Delta m/z$ 132 (xylosyl) and $\Delta m/z$ 146 (rhamnosyl). The postulated single esterification with coumaric acid was supported by a low retention time in comparison with the doubly esterified most lipophilic pigments (e.g. **139**). The presence of xylosyl was also tentatively assumed for the structure of **14**.

Surprisingly, the presence of possibly triply acylated oligomeric betacyanins was concluded for **108** and **134** (*m*/*z* 1401) as well as for **110** and **117** (*m*/*z* 1445) which would stand for the completely unusual acylation pattern in betacyanins based on oxalyl (in **108** and **134**) and malyl (in **110** and **117**) in addition to two coumaroyl moieties (cf. Table 1).

3.2. HSCCC separation of betacyanins from violet bracts of *B. glabra*

For the separation of the violet-colored betacyanin pigments from the *B. glabra* bracts a solvent system containing TFA which was previously applied for the study of betacyanins from the cacti fruits of *H. polyrhizus* [10] was used. The lipophilicity of the pigments of *B. glabra* was supposed to be much higher. Micro-scale partitioning assays of the pigment crude extract for CCC solvent evaluation [11] enabled a good partitioning and distribution of betacyanins between the two solvent phases which was the prerequisite of their good separation. Nevertheless, our first tests revealed a polar pigment group of betacyanins eluted in the first HSCCC fractions.

So far, not avoidable degradations of the genuine pigments by decarboxylation and dehydrogenation of betacyanins induced by perfluoroacidic solvent systems used during HSCCC has been observed in earlier studies [8,10]. This phenomenon was noticed in many thermally degraded betalainic products [15–18] and can be also expressed at room temperature, especially in organic solvents [18]. However, the glycosylation position of the phenolic groups at C-5 or C-6 carbons in the betanidin (aglycone) unit has a deciding effect on the pigment chemical stability. In general, it was regarded, that the stability of the 6-*O*-glucosylated forms was much higher thanks to intramolecular interactions [19] and this fact was confirmed indirectly in our study assuming that most of the pigments found in *B. glabra* bracts were the 6-*O*-glucosylated betacyanins. There were no artefacts of the pigments found in all the HSCCC fractions which was a marvelous result if compared to our recent IP–HSCCC separations of betanin [8] and acylated betanin with aliphatic acids [10]. Pigment stabilization in solution is well known for highly acylated anthocyanin structures. In analogy, highly acylated betacyanins may be protected by intra- and/or inter-molecular stabilization [20]. Aromatic systems such as the betacyanin aglycone with its *cyclo*-Dopa ring system and hydroxycinnamic acid groups could stack and build up π–π interactions preventing chemical alteration.

In Fig. 2C, the HPLC chromatogram of the crude *B. glabra* pigment extract monitored at λ 540 nm was depicted. The applied conditions in the IP–HSCCC allowed for a good separation of most of the acylated principal reference pigments of *B. glabra* from each other as well as from the bulk of the polar betacyanins. Thus, the principal compounds were eluted mainly in fraction nos. 4–5 (**16**, *m*/*z* 875), 6–8 (**23**, *m*/*z* 859), 7–8 (**70**, *m*/*z* 859), 5–6 (**73**, *m*/*z* 1345), 10–12 (**79**,

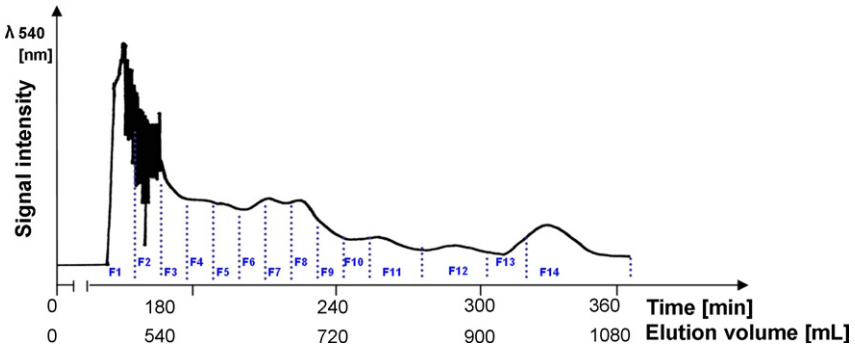

Fig. 3. IP–HSCCC chromatogram of directly recovered crude pigment extracts (755 mg) of violet *B. glabra* bracts in the solvent system TBME-1–BuOH–ACN–H_2O 2:2:1:5 (v/v/v/v) using 0.7% TFA. HSCCC conditions: flow rate: 3.0 mL/min; CCC-operation: *head-to-tail* mode; velocity: 850 rpm and detection wavelength λ 540 nm.

m/*z* 1183), 13–14 (**103**, *m*/*z* 1167), and in the coil-fraction 15 (**139**, *m*/*z* 1005). However, these pigments were accompanied by other new betacyanins tentatively identified in this study from which the most prominent were: **100** (*m*/*z* 1329, mainly in the fractions 7–9), **125** (*m*/*z* 1197, fraction 12) and **120** (*m*/*z* 1359, fraction 6).

An interesting observation of the IP–HSCCC separation of some analogs was also noticed. Recent study on *P. americana* pigments indicated the reversed elution order of feruloylated and sinapoylated betacyanins (the analogs differing only by the presence of the methoxy group) in C18-HPLC and IP–HSCCC [8]. Moreover, it concerned the pairs of the diastereomeric forms which resulted in separation of the analog pairs between each other in HSCCC in contrast to HPLC providing a valuable way for their preliminary separation. In this study, the best example of such inversion of elution orders could be observed for the most prominent coumaroylated betacyanins in comparison to their feruloylated analogs (Table 2). Starting with the most concentrated compound **103** (*m*/*z* 1167) as well as its diastereomer **103**′, eluted in the fractions 13–14, it was striking that the mono-feruloylated analog pair **125**/**125**′ (*m*/*z* 1197) was eluted mainly in the earlier fraction (12) in spite of its higher retention time in HPLC. Moreover, the bi-feruloylated analog pair **137**/**137**′ (*m*/*z* 1227) was eluted even in the much earlier fractions (10–11) than the previous ones. Other good examples of this phenomenon with the coumaroyl-feruloyl analogues (Table 2) were observed for the pairs of **23**/**23**′ (fractions 6–8; *m*/*z* 859) and **45**/**45**′ (4–6; *m*/*z* 889); **70**/**70**′ (6–9; *m*/*z* 859) and **84**/**84**′ (4–6; *m*/*z* 889); **73**/**73**′ (4–6; *m*/*z* 1345) and **87**/**87**′ (4–5; *m*/*z* 1375); **65**/**65**′ (11–13; *m*/*z* 1183) and **91**/**91**′ (10–11; *m*/*z* 1213) as well as **79**/**79**′ (9–12; *m*/*z* 1183) and **95**/**95**′ (8–9; *m*/*z* 1213). These results show the complementary nature of both techniques, allowing for the effective separations of the pigments in sequencial use of IP–HSCCC and C18-HPLC.

Unfortunately, this effect was not possible to trace for the most lipophilic betacyanins: coumaroyl-feruloyl-bi-feruloyl analogues of the pairs of **139**/**139**′ (*m*/*z* 1005), **142**/**142**′ (*m*/*z* 1035) and **144**/**144**′ (*m*/*z* 1065), respectively, which were eluted in the coil-fraction 15 and recovered by evaporation from the completely retained stationary organic phase. This proved the highest lipophilic character of the betacyanins in the analyzed mixture. The *elution–extrusion* (EE) approach of Berthod et al. [21–23] should enable further fractionation of these acyl-loaded pigments.

In the HSCCC chromatogram (Fig. 3) some of the prominent pigments formed well separated peaks, however, the polar fractions consisted of a mixture of many betacyanins, especially in fractions 2–4, which was also visible in the chromatogram (Fig. 3) where the peak area of the polar fraction was higher than the area of the following peaks.

The betacyanins containing long oligomeric saccharide chains of 7-, 6- and 5-sugar linked units and solely a mono-acylated position were grouped mostly in the polar fractions 1–4 and were practically not resolved during IP–HSCCC. The acidic concentration of 0.7% TFA in the biphasic CCC-system was not strong enough for improving the partition ratios K_D of the polar pigments and ideal selectivity factors α proving a better separation were not achieved. Likewise, many other polar pigments of lower abundance were present in these fractions forming a group of elevated background optical absorbance. It would be, therefore, worth separating them in another solvent system having stronger ion-pairing properties. For such a solvent system, heptafluorobutyric acid (HFBA) could be used, which already has been applied in one of our parallel studies on separation of highly polar betacyanins of *H. polyrhizus* fruits [10].

The presence of the most polar and low-molecular betacyanins was also acknowledged in the first three IP–HSCCC fractions, namely, the 6-*O*-glycosylated **3**/**3**′ (*m*/*z* 713) and **4** (*m*/*z* 551) as well as the 5-*O*-glycosylated **1**/**1**′ (*m*/*z* 713) and **2**/**2**′ (*m*/*z* 551) (betanin/isobetanin). The most prominent was **3**/**3**′, however, all of the pigments were found at low levels, possibly as a results of a degradation at the highly acidic environment. The possibility of degradation of **2**/**2**′ as well as fast decomposition of **1**/**1**′ in the solvent systems used for HSCCC was recently noticed [10]. Especially, the presence of the acidified organic solvents accelerates the decarboxylation and dehydrogenation of the pigments [18]. Even non-acylated 6-*O*-glycosylated betacyanins are susceptible to partial decomposition, because they are potentially less protected by the intramolecular stabilization [19]. However, because of minute quantities of the most polar pigments, their degradation products were not detected.

4. Conclusions

This contribution proved that the development of an appropriate solvent system using ion-pair forming perfluoroacidic reagents such as TFA for the IP–HSCCC separation of betacyanins is a preparative chromatographic '*tool*' of high potential and versatility. Separations of polar, medium-polar and lipophilic pigments into more defined '*polarity-windows*' can effectively be done by HSCCC applying ion-pair reagents. The two-dimensional chromatographic approach using preparative IP–HSCCC in combination with sensitive LC–ESI–MS/MS analysis enabled the detection of 146 different betacyanins of rather high-molecular weights based on differing acyl-oligoscacharide linkages in a wide polarity range present in purple bracts of *B. glabra*.

Nevertheless, the very polar and high-molecular weight pigments of *B. glabra* (early eluting fractions) still await the application of a further improved solvent system. Increasing the partition ratios K_D at higher extent by enhancing the affinity of the pigments to the stationary organic CCC-phase will require stronger ion-pair reagents.

These research efforts, in addition to understanding of the behavior of the pigments in IP–RP–HPLC chromatography, will also allow for anticipating the nature of emerging ion pairs in the studies of their distribution in HSCCC systems of both the normal and reversed phases. Despite many chromatographic similarities during the separation of the pigments in HPLC and HSCCC, in some cases, there were also significant differences noticed, such as inversion of elution for feruloyl- and coumaroyl-substituted betacyanins.

The ion-pair '*tool*' using different perfluorinated carboxylic acids is giving many options for constructing applications to be taylored specifically for crude pigment CCC separations. In addition, the *EE* approach suggested by Berthod et al. [21–23] is another optional variation and can be performed to secure a complete pigment recovery. Generally, the IP–HSCCC methodology can be widely used for separation of charged natural product analytes, e.g. such as alkaloids and anthocyanins.

Further studies will be necessary to isolate pure compounds to elucidate complete interglycosidic linkages and points of acylation by modern 1D/2D-NMR techniques.

Acknowledgements

The authors are grateful to Mr. José Ramos-Cortés (Guadalajara, Mexico) for the supply of *Bougainvillea glabra* bracts. This research was financed in part by Polish Ministry of Science and Higher Education for years 2007–2010 (Project No. N205 2991 33).

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