Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 251–259
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Minor betalains in fruits of *Hylocereus* species

Sławomir Wybraniec [a,*], Barbara Nowak-Wydra [b], Katarzyna Mitka [c], Piotr Kowalski [c], Yosef Mizrahi [d]

[a] *Department of Chemical Engineering and Technology, Institute C-1, Faculty of Analytical Chemistry, Cracow University of Technology, ul. Warszawska 24, 31-155 Cracow, Poland*
[b] *Faculty of Chemistry, Adam Mickiewicz University, Grunwaldzka 6, 60-780 Poznań, Poland*
[c] *Department of Chemical Engineering and Technology, Institute C-2, Faculty of Organic Chemistry, Cracow University of Technology, ul. Warszawska 24, 31-155 Cracow, Poland*
[d] *Department of Life Sciences, The Institutes for Applied Research, Ben Gurion University of the Negev, P.O. Box 653, 84105 Beer-Sheva, Israel*

Received 25 June 2006; received in revised form 7 September 2006
Available online 16 November 2006

Abstract

Betacyanins in peel and flesh of fruits of different *Hylocereus* species were identified by means of GC/MS, electrospray MS/MS, HPLC as well as ^{1}H and ^{13}C NMR techniques. As hitherto unknown pigments: betanidin 5-*O*-(2′-*O*-β-D-apiofuranosyl)-β-D-glucopyranoside, betanidin 5-*O*-(4′-*O*-malonyl)-β-D-glucopyranoside and betanidin 5-*O*-[(5″-*O*-*E*-sinapoyl)-2′-*O*-β-D-apiofuranosyl]-β-D-glucopyranoside were elucidated. The sinapoyl moiety attachment position in the structure of betacyanins was established for the first time. The peel contained a more complex pattern of betacyanins with apiofuranosyl moiety. Other recently identified pigments were also present in the samples and their ^{1}H or ^{13}C NMR spectra were recorded. In the case of phyllocactin and its 4′-isomer the migration of the malonyl group was noticed.

Keywords: *Hylocereus polyrhizus*; *Hylocereus ocamponis*; *Hylocereus undatus*; Cactaceae; Acylated betacyanins; Betalains; Betanin; Phyllocactin; Hylocerenin; Sinapic acid; Acyl migration

1. Introduction

Betacyanins and betaxanthins are betalain pigments, which are characteristic for plants of the order Caryophyllales (Strack et al., 2003). Betalains are used in various applications in the food industry due to their colorant properties (Henry, 1996). Some vine cacti species belonging to the subfamily Cactoideae of the tribe Cacteae have been recently investigated and betacyanin pigments were identified in their fruit pulp (Wybraniec et al., 2001; Stintzing et al., 2004). The most frequently investigated was *Hylocereus polyrhizus* [(F.A.C. Weber) Britton & Rose] which is already produced commercially for its glowing deep red–purple flesh fruits (Mizrahi and Nerd, 1999).

Our first structural study on *H. polyrhizus* pigments reported three main betacyanins (betanin, phyllocactin and hylocerenin) as well as their corresponding C-15 diastereoisomers (Wybraniec et al., 2001). Another study provided new ^{1}H and ^{13}C NMR results and reported the possibility of coupling of HPLC to ^{1}H NMR for betacyanin analysis (Stintzing et al., 2004).

Until recent reports (Stintzing et al., 2004; Wybraniec et al., 2005) no ^{13}C NMR data of C14–C15 saturated betacyanins were provided. Because of lability of betalains in highly acidic conditions, the NMR spectra acquisitions were performed in D_2O without acid additions despite signal broadening due to a low protonation degree. D_2O was found to be the most suitable system which afforded the best long-term stability required for gCOSY, gHSQC, and gHMBC experiments (Stintzing et al., 2004).

Several studies reported on betacyanins containing hydroxycinnamic acyl moieties in their structures (Heuer

* Corresponding author. Tel.: +48 12 628 2707; fax: +48 12 633 3374.
E-mail address: swybran@chemia.pk.edu.pl (S. Wybraniec).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.10.002

et al., 1994; Schliemann et al., 1996; Kobayashi et al., 2000). Pigments of pokeberry (*Phytolacca americana*) (Schliemann et al., 1996) and Christmas cactus (*Schlumbergera* × *buckleyi*) (Kobayashi et al., 2000) were shown to contain a rare branched pentose, apiose. Our contribution reports on similar betacyanins which were found in fruit flesh and peel of *Hylocereus* species or hybrids.

2. Results and discussion

HPLC analysis of *Hylocereus ocamponis*, *Hylocereus undatus*, and *Hylocereus purpusii*, as well as hybrids of *Hylocereus costaricensis* × *H. polyrhizus* and *H. undatus* × *H. polyrhizus*, revealed characteristic betacyanin profiles. All fruit flesh samples contained pigments **1**–**7**, while all the peel samples contained pigments **1**–**10**, therefore only a representative HPLC profile of *H. ocamponis* peel was chosen for depiction in Fig. 1. The presence of the most abundant betacyanins (betanin **2**, phyllocactin **4** and hylocerenin **6**) and their 15*R*-isoforms (Fig. 1) was confirmed by their characteristic spectral properties (Table 2) (Wybraniec et al., 2001). Additionally, two betaxanthins were detected (γ-aminobutyric acid-Bx, **I** and indicaxanthin, **II**) which have been already reported in fruits of *Opuntia ficus-indica* and coeluted with the standards prepared according to Stintzing et al. (2002a). The other compounds were characterised for the first time or their new spectral data were generated.

2.1. Betanidin 5-*O*-β-sophoroside

Based on its molecular mass, its sensitivity to β-glucosidase and its retention characteristics, **1** was recently tentatively identified as betanidin 5-*O*-β-sophoroside (Stintzing et al., 2002b), which was found previously in purple bracts of *Bougainvillea glabra* 'Mrs. Butt' (Piattelli and Imperato, 1970; Heuer et al., 1994). It eluted prior to betanin on the reversed phase system due to the presence of a diglucose moiety. The absorption characteristics of **1** (λ_{max} 538 nm) suggested the presence of a 5-*O*-glycosidic bond with betanidin (Piattelli and Imperato, 1970).

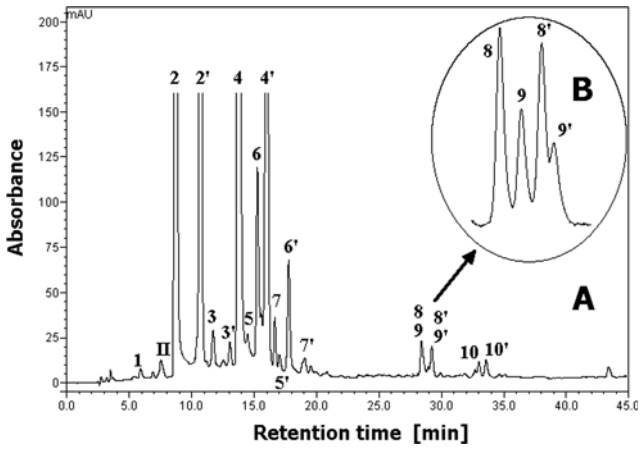

Fig. 1. HPLC elution profile of betacyanins ($\lambda = 538$ nm) from fruit peel of *H. ocamponis* analysed in the gradient System 3 (A) and the improved separation of **8**, **8′**, **9** and **9′** in the gradient System 4 (B).

Our study confirmed these assumptions by the NMR experiments and LC–MS/MS fragmentation of pseudomolecular ion at *m*/*z* 713 to ions at *m*/*z* 551 and 389, suggesting the presence of a second hexose moiety (713 − 551 = 162).

Despite the minute quantities of **1** available for NMR analysis all characteristic signals of betanin confirmed the presence of the aglycone and glucose moieties (Stintzing et al., 2004; Wybraniec et al., 2005) (Table 1). The ^{1}H NMR, 1D TOCSY, gCOSY spectra allowed to distinguish and assign the individual coupled ^{1}H-spin systems of the aglycone (H-2, H3a/b; H-4, H-7; H-11, H-12; H-14a/b, H-15) and of the hexose moieties. The ^{13}C chemical shifts for carbons directly bound to protons were assigned by gHSQC correlations. The β-linkage between the aglycone and glucopyranosyl moiety was indicated by gHMBC correlation between the anomeric proton H-1 and the phenolic carbon C-5 as well as by the three-bond vicinal proton coupling constant $^{3}J_{1'-2'} \sim 7$ Hz. The dihydroindolic system was assigned by gHSQC correlations of H-2, H-3a/b, H-4 and H-7 with their respective carbons. The sugar substitution at C-5 of betanidin was confirmed by the chemical shift difference between H-4 and H-7 of 0.08 ppm. The gHMBC correlation between the second hexose proton H-1‴ and the first hexose carbon C-2′ indicated the second hexose attachment position.

The linkage between the two sugar moieties was definitely established by methylation analysis (Anumula and Taylor, 1992) with subsequent detection of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-glucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol by GC–MS, identified by their characteristic fragmentation pattern (Biermann and McGinnis, 1989). This showed the terminal position of the second glucopyranosyl bound to C-2′ of the first glucopyranosyl moiety.

2.2. 2′-Apiosyl-betanin

Another minor betacyanin **3** (λ_{max} 539 nm) showed a protonated molecular ion at *m*/*z* 683 and its daughter ion fragments at *m*/*z* at 551 and 389 using positive ion mode LC–MS/MS. The mass difference between **3** (*m*/*z* 683) and betanin (*m*/*z* 551) suggested the presence of an additional pentose moiety. From the ratio of the absorbances at 539 nm and 327 nm (1:0.16) the presence of hydroxycinnamoyl residues as acylating moieties in **3** could be excluded (Heuer et al., 1994) and no any other organic acyl residue was identified.

Recent studies on betacyanins from Christmas cactus (*Schlumbergera* × *buckleyi*) confirmed the presence of apiofuranosyl moiety in betacyanin structures. Comparison of retention times between **2** and **3** indicated a decreased overall polarity of **3**. Likewise, an increase of retention time was

Table 1
The ^{1}H and ^{13}C NMR data of pigments **1**, **3** and **7**

No.	Betanidin 5-*O*-β-sophoroside (**1**)		2′-Apiosyl-betanin (**3**)		2′-Apiosyl-phyllocactin (**7**)	
	^{1}H NMR[a]	^{13}C NMR[b,c]	^{1}H NMR[a]	^{13}C NMR[b,c]	^{1}H NMR[a]	^{13}C NMR[b,c]
2	4.87, *dd*, 3.5, 10.5	65.3	4.92, *dd*, 3.4, 10.2	65.1	4.85, *dd* , 3.1, 10.1	65.2
3a/b	3.62, *dd*, 17.2, 10.2 3.14, *dd*, 3.5, 17.1	33.4	3.64, *dd*, 16.6, 9.6 3.17, *dd*, 3.5, 16.9	33.4	3.59, *dd*, 16.8, 10.0 3.33, *dd*, 3.1, 16.8	33.3
4	7.15, *s*	115.9	7.18, *s*	114.2	7.08, *s*	114.4
5		144.8		140.3		144.2
6		146.5		142.0		146.1
7	7.28, *s*	101.6	7.08, *s*	99.5	7.00, *s*	99.6
8		137.9		138.4		138.6
9		127.0		125.5		124.5
10		–[d]		–[d]		–[d]
11	8.18, *bs*	143.7	8.26, *bs*	138.6	8.19, *bs*	143.6
12	5.82, *bs*	105.9	5.88, *bs*	105.8	5.81, *bs*	106.0
13		–[d]		–[d]		–[d]
14a/b	3.18, *bm*	27.2	3.32, *bs* 3.29, *bdd*	27.3	3.27 *bdd* 3.15 *bs*	27.5
15	4.29, *bt*, 7.7	53.9	4.35, *bt*, 7.7	54.0	4.28, *bt*	54.3
17		–[d]		–[d]		–[d]
18	6.24, *bs*	105.1	6.29, *bs*	105.0	6.21, *bs*	104.8
19		–[d]		–[d]		–[d]
20		–[d]		–[d]		–[d]
1′	5.13, *d*, 7.3	101.4	5.10, *d*, 7.0	100.6	5.04, *d*, 7.6	100.8
2′	3.65 (overlap)	–[d]	3.75 (overlap)	80.2	3.69 (overlap)	80.4
3′	3.65 (overlap)	–[d]	3.70 (overlap)	75.5	3.66 (overlap)	75.2
4′	3.65 (overlap)	–[d]	3.58 (overlap)	76.1	3.59 (overlap)	68.7
5′	3.65 (overlap)	–[d]	3.58 (overlap)	69.0	3.73 (overlap)	73.7
6′a/b	3.93, *dd*, 12.1, 2.0 3.67, *dd*, 12.3, 1.6	60.7	3.93, *dd*, 13.2, 1.6 3.78, *dd*, 12.6, 2.0, 4.9	60.5	4.40, *dd*, 12.6, 2.5 4.36, *dd*, 12.7, 4.4	63.1
1″						–[d]
2″a/b					3.29, *s*	37.1
3″						–[d]
1‴	5.02, *d*, 7.3	101.2	5.42, *d*, 3.6	109.8	5.39, *d*, 3.4	110.0
2‴	3.65 (overlap)	–[d]	4.05, *d*, 3.6	76.7	3.99, *d*, 3.4	76.9
3‴	3.65 (overlap)	–[d]		–[d]		–[d]
4‴	3.65 (overlap)	–[d]	4.09, *d*, 10.0 3.87, *s*, 10.3	73.6	4.02, *d*, 10.1 3.80, *d*, 10.1	73.7
5‴	3.65 (overlap)	–[d]	3.63 (a/b)	63.5	3.55 (a/b)	63.4
6‴a/b	3.84, *dd*, 2.2, 12.5 3.73	60.3				

[a] ^{1}H NMR δ [ppm], mult, J [Hz].
[b] ^{1}H NMR δ [ppm].
[c] ^{13}C chemical shifts were derived from gHSQC or gHMBC.
[d] Chemical shifts were not observable.

observed recently for another apiose containing betacyanin, 2′-apiosyl-phyllocactin, in comparison to phyllocactin (Kobayashi et al., 2000).

Therefore, comparison of **3** with an authentic sample of apiin containing terminal apiose by methylation analysis (Anumula and Taylor, 1992; Kobayashi et al., 2000) confirmed unambiguously the identity of the pentose residue, as well as the linkage between the two sugar moieties. The detection of 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylapitol by GC–MS, identifed by its characteristic fragmentation pattern (Wagner and Demuth, 1972), clearly showed the terminal position of this pentose, which is bound to C-2′ of the glucose as indicated by the detection of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-glucitol.

The characteristic signals in ^{1}H and ^{13}C NMR of the aglycone and glucopyranosyl systems were similar to the signals of **1** (Table 1). In addition, the ^{1}H NMR, 1D TOCSY, gCOSY spectra indicated a furanose moiety by the presence of another anomeric proton with a small coupling constant (δ 5.42, 1H, *d*, $J = 3.6$ Hz, H-1‴) as well as by the presence of AB quartet methylene signals (δ 3.80 and 4.02, 1H each, *d*, $J = 10.1$ Hz each, H-4‴a and H-4‴b, respectively) and a singlet (δ 3.55, 2H, *s*, H-5‴). The presence of the apiofuranosyl moiety was further supported by gHSQC indicating two methylenes (C-4‴ and C-5‴), however, no signal corresponding to a quaternary carbon could be identified. Unfortunately, the attachment position of the apiofuranose at the glucopyranosyl moiety

could not be detected in the HMBC spectrum because of too small amounts of the analytical sample. Recent study on apiose-derived betacyanin (Kobayashi et al., 2000) suggested a β-configuration of the apiofuranosyl anomeric proton based on the coupling magnitude $^3J_{1'''-2'''}$ (3.9 Hz) (Schliemann et al., 1996; Schwind et al., 1990). The magnitude of $^3J_{1'''-2'''}$ found in our study (3.4 Hz) also suggested the β-configuration of the glycosidic linkage completing the structure elucidation of **3** as betanidin 5-*O*-(2′-*O*-β-D-apiofuranosyl)-β-D-glucopyranoside (Fig. 2).

2.3. 4′-Malonyl-betanin

Compound **5** was identified as isomeric to already known phyllocactin **4** (Figs. 1 and 2) (Minale et al., 1966). The LC–MS spectra of **5** were similar to the spectra of phyllocactin (Kobayashi et al., 2000; Wybraniec et al., 2001). The protonated molecular ion ($[M + H]^+$) for compound **5** was found at *m/z* 637 [550 (betanin)+86 (malonyl)+H]$^+$ suggesting the presence of the malonyl moiety and the daughter ions at *m/z* 551 $[\text{betanin} + H]^+$ and 389

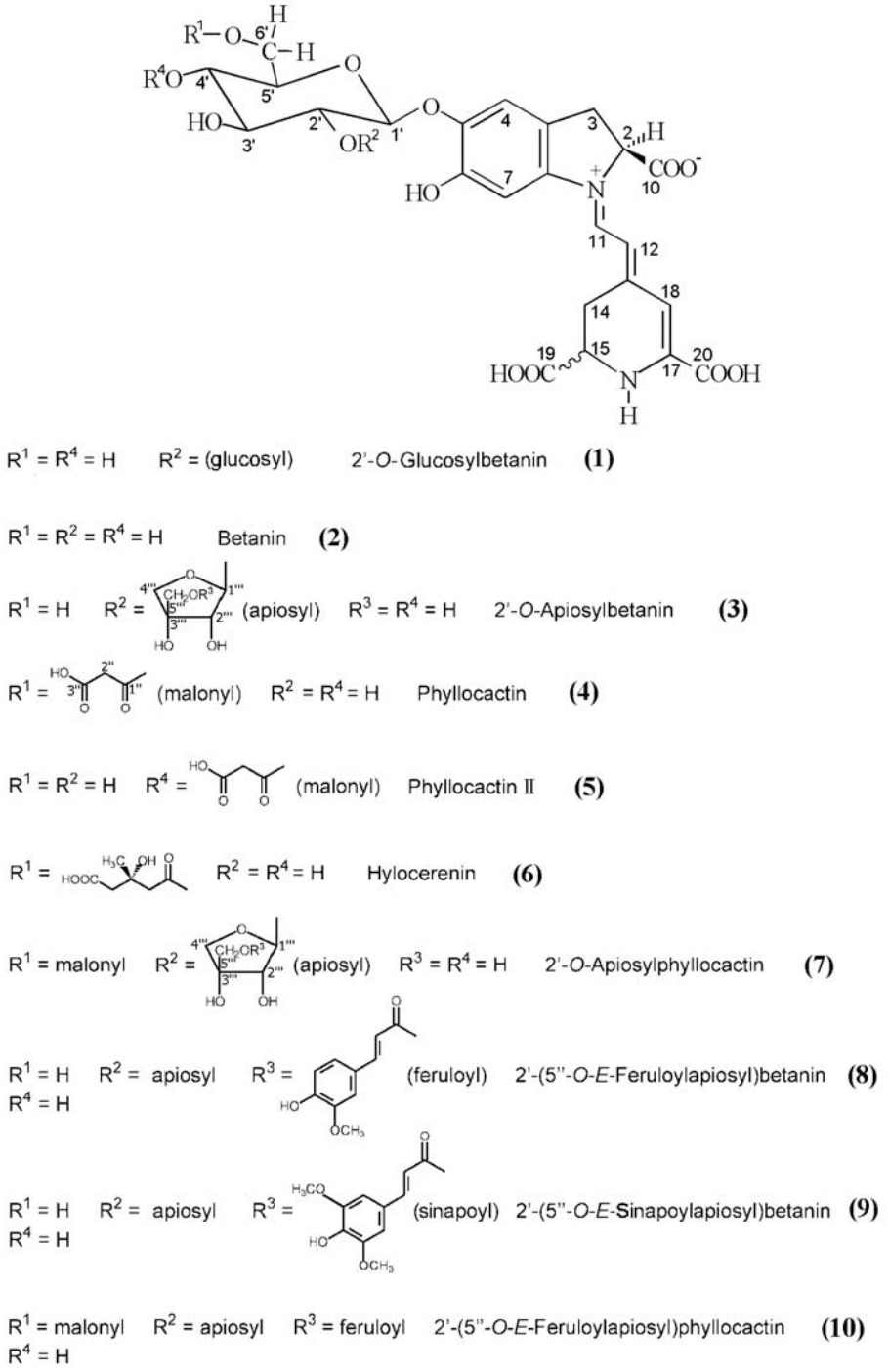

Fig. 2. Chemical structures of betacyanins found in *Hylocereus* cacti.

Table 2
Chromatographic (HPLC gradient System 3), spectroscopic and mass spectrometric data of the analysed pigments found in fruit peel of *H. ocamponis*

No.	Compound	R_t (min)	λ_{max}[a] (nm) I:	λ_{max}[b] (nm) II:	Abs. Ratio II:I	m/z $[M+H]^+$	m/z from MS/MS of $[M+H]^+$
1	Betanidin 5-*O*-β-sophoroside	5.9	–	539	–[c]	713	551; 389
I	γ-Aminobutyric acid-betaxanthin	6.9	–	461	–	297	–
II	Indicaxanthin	7.5	–	475	–	309	–
2	Betanin	8.7	–	538	–	551	389
2′	Isobetanin	10.7	–	538	–	551	389
3	2′-Apiosyl-betanin	12.0	–	539	–	683	551; 389
3′	2′-Apiosyl-isobetanin	13.0	–	539	–	683	551; 389
4	Phyllocactin	13.9	–	538	–	637	619; 593; 551; 389
5	4′-Malonyl-betanin	14.5	–	538	–	637	619; 593; 551; 389
4′	Isophyllocactin	15.2	–	539	–	637	619; 593; 551; 389
6	Hylocerenin	16.0	–	539	–	695	677; 651; 633; 551; 389
7	2′-Apiosyl-phyllocactin	16.6	–	540	–	769	683; 551; 389
5′	4′-Malonyl-isobetanin	17.1	–	538	–	637	619; 593; 551; 389
6′	Isohylocerenin	17.8	–	538	–	695	551; 389
7′	2′-Apiosyl-isophyllocactin	19.1	–	540	–	769	683; 551; 389
8	5″-*O*-*E*-Feruloyl-2′-apiosyl-betanin[d]	28.4	329	551	1:0.49	859	683; 551; 389
9	5″-*O*-*E*-Sinapoyl-2′-apiosyl-betanin[d]	28.4	330	550	1:0.50	889	683; 551; 389
8′	5″-*O*-*E*-Feruloyl-2′-apiosyl-isobetanin[d]	29.2	329	550	1:0.47	859	683; 551; 389
9′	5″-*O*-*E*-Sinapoyl-2′-apiosyl-isobetanin[d]	29.2	329	549	1:0.51	889	683; 551; 389
10	5″-*O*-*E*-Feruloyl-2′-apiosyl-phyllocactin	33.0	328	551	1:0.49	945	769; 683; 551; 389
10′	5″-*O*-*E*-Feruloyl-2′-apiosyl-isophyllocactin	33.6	331	550	1:0.53	945	769; 683; 551; 389

[a] λ_{max} of hydroxycinnamoyl moiety (HCA/I), –, no absorbance band.
[b] λ_{max} of betaxanthins or betacyanins in the visible range (II).
[c] Ratio of absorbance at λ_{max} (Vis) and at 320 nm is *ca.* 1:0.1 (−).
[d] The data were generated using System 4 as well as for purified compounds.

$[\text{betanidin} + H]^+$. Additional ions at m/z 619 and 593 were assigned to losses of H_2O and CO_2 (Table 2). However, the different retention times of **4** and **4′** from **5** suggested, that **5** was not a phyllocactin, but an isomeric compound differing from phyllocactin by the position of the acyl moiety attached to the glucosyl unit or an acylated betanidin 6-*O*-β-glucoside (Minale et al., 1967). In a recent LC–MS study the structure of this compound was tentatively proposed as betanidin 5-*O*-(6′-*O*-3-hydroxy-butyryl)-β-D-glucopyranoside (Stintzing et al., 2002b), however in this study the GC-FID analysis of methylated hydrolysate of **5** resulted in identification of dimethyl malonate ester.

Subsequent linkage analysis between the sugar moiety and the acyl substituent was performed by mild methylation analysis (Prehm, 1980) followed by the procedure of partially methylated alditol acetate preparation (Anumula and Taylor, 1992). The detection of the prevailing 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol by GC–MS, identifed by its retention time and characteristic fragmentation pattern (Biermann and McGinnis, 1989), indicated the position of the malonyl moiety which was bound to C-4′ carbon of the glucosyl unit. Under the mild conditions of the Prehm derivatization procedure the acyl linkage is generally not lost, however some 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol was detected, indicating partial destruction of the acyl linkage during the methylation. Parallel methylation analysis of **5** in basic conditions (Anumula and Taylor, 1992), under which all acyl residues are lost, resulted in detection of solely 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, confirming the presence of the glucosyl moiety in the structure of **5**.

Because of compound **5** instability under ^1H and ^{13}C NMR spectra aquisition or in acidic conditions, no NMR spectra could be acquired. After prolonged time of exposure to room temperature compound **5** partially isomerised to phyllocactin, which was observed in chromatograms and LC–MS/MS spectra. In addition, the presence of malonylated betanidin 6-*O*-β-glucoside could rather be excluded on the basis of the lack of slight bathochromic shift to λ_{max} 540–543 nm which was observed for betanidin 6-*O*-β-glucoside (Minale et al., 1967; Heuer et al., 1992a). Alkaline deacylation of **5** and subsequent acidification of the resulting mixture with HCl resulted in the liberation of a mixture of **1/1′** confirming the 5-*O*-β-glucosidation in **5**. Therefore, a possibility of acyl migration was considered. The pH-dependent acyl migration between adjacent hydroxy groups on polyhydroxy compounds has been frequently noticed (Fischer, 1920; Haines, 1976) and this possibility was also considered for phyllocactin (Minale et al., 1966), however no studies on this phenomenon occurring

in betacyanins were performed. Our further studies carried out at different pHs confirmed the malonyl migration (from the C-6′ to C-4′ carbon or vice versa, depending on the isomer taken for the reaction) being the fastest at pH 10–11 in reaching the interconversion equilibrium (Wybraniec, S., unpublished). Therefore, in connection to the carbohydrate linkage analysis results, compound **5** was identified as betanidin 5-*O*-(4′-*O*-malonyl-β-D-glucopyranoside).

2.4. 2′-Apiosyl-phyllocactin

Another minor betacyanin **7** showed a protonated molecular ion at *m*/*z* 769 and its daughter ion fragments at *m*/*z* at 683, 551 and 389 using positive ion mode LC–MS/MS. The mass and the fragmentation pattern suggested the presence of 2′-apiosyl-phyllocactin recently found in Christmas cactus (*Schlumbergera* × *buckleyi*) (Kobayashi et al., 2000). The difference between **7** (*m*/*z* 769) and betanin (*m*/*z* 551) suggested the presence of an additional malonyl and pentose moiety. From the ratio of the absorbances at 540 nm and 329 nm (1:0.17) the presence of hydroxycinnamoyl residues as acylating moieties in **7** could be excluded (Heuer et al., 1994). Furthermore, no any other acylated moieties were found.

The linkage analysis performed after methylation (Anumula and Taylor, 1992) of **7** confirmed the identity of the glucose and pentose residues, as well as their linkage. As in the case of **3**, the detection of 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylapitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylglucitol by GC–MS, showed the terminal position of apiose, bound to the C-2′ of the glucose unit. The malonyl residue was lost under the basic conditions of the derivatization procedure. The aliphatic acid analysis after esterification (Donner et al., 1997) revealed malonyl moiety bound to the C-6′ of the glucose unit as indicated by the Prehm methylation (Prehm, 1980) followed by the procedure of partially methylated alditol acetate preparation (Anumula and Taylor, 1992) and the detection of 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-glucitol by GC–MS.

The identity of the carbohydrate system was confirmed by the alkaline deacylation of **7** and subsequent acidification of the resulting mixture with HCl. The liberation of a mixture **3**/**3**′ indicated the presence of the 2′-*O*-β-D-apiofuranosyl-β-D-glucopyranosyl system in the structure of **7**.

The ^{1}H and ^{13}C NMR spectra of **7** were virtually the same as of **3** (Table 1) except of the signals confirming the presence of the malonyl moiety bound to the C-6′ of the glucose moiety as indicated by the downfield shift of the glucopyranosyl proton signals H-6′a/b in ^{1}H NMR and for the C-6′ in gHSQC and gHMBC in comparison to **2** or **3** derived structures. Thus, the compound **7** was identified as betanidin 5-*O*-(2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl)-β-D-glucopyranoside. The coelution of **7** with the already identified compound isolated from *Schlumbergera* flowers confirmed the identification. As in the case of **3**, an increased retention time of **7** in comparison to **4** (differing from **7** by the lack of the apiofuranosyl moiety) was observed, confirming, that the presence of the apiofuranosyl moiety decreased the overall polarity of the molecule.

2.5. Hydroxycinnamoyl apiofuranosyl derivatives

Inspection of chromatograms of fruit peels (for example *H. ocamponis*, Fig. 1) revealed additional pigments **8**–**10** and their isoforms. All of them exhibited the ratio of the absorbances at ca. 550 nm and 330 nm (1:0.5), indicating the presence of one hydroxycinnamoyl acylating moiety in each compound.

During the mass spectrometric analyses the protonated molecular ions ($[M + H]^+$) for compounds **8**, **9** and **10** were found at *m*/*z* 859, 889 and 945, respectively. The masses and the fragmentation patterns indicated the presence of apiofuranosyl moiety in each case, because the fragments at *m*/*z* 683, 551 and 389 were found. The mass differences between **8** (*m*/*z* 859), **9** (*m*/*z* 889) and **3** (*m*/*z* 683) suggested the presence of feruloyl ($859 - 683 = 176$) and sinapoyl ($889 - 683 = 206$) moieties in **8** and **9**, respectively. The sinapoyl moiety had been only tentatively detected in betacyanins of *Iresine herbstii* (Minale et al., 1966) and *Gomphrena globosa* flowers (Heuer et al., 1992a,b).

The identity of the carbohydrate system was initially indicated by the alkaline deacylation of **8**–**10** and subsequent acidification of the resulting mixtures with HCl. In each case the liberation of a mixture of **3**/**3**′ suggested the presence of the 2′-*O*-β-D-apiofuranosyl-β-D-glucopyranosyl system in the structures of **8**–**10**.

2.5.1. Feruloylated and sinapoylated apiosyl-betanin

In the HPLC gradient System 3 applied, the overlapping pairs **8**–**9** and **8**′–**9**′ could not be resolved (Fig. 1A) and only the mass spectrometric monitoring definitely indicated the different compounds. For adequate separation of **8**, **9** and their isoforms in analytical (System 4, Fig. 1B) or semi-preparative (System 2) HPLC 14–20% formic acid was used as eluent component. In highly concentrated acid conditions after few steps of isolation the pigments were separated enough from each other and any other impurities monitored in DAD-HPLC for subsequent spectroscopic identification. The purified fractions of **9** or **9**′ did not contain any traces of **8**/**8**′. The order of elution indicated the presence of a less polar **9**/**9**′ than **8**/**8**′. However, the quantities and purity (decreasing because of the compound lability) of the isolated pigments were not sufficient for the NMR structure elucidation.

The coelution of **8** and **8**′ with the same compound isolated from *Schlumbergera* flowers suggested the presence of betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl]-β-D-glucopyranoside, previously found in *P. americana* (Schliemann et al., 1996) and *Schlumbergera* × *buckleyi* (Kobayashi et al., 2000). In addition, the possibility that **9** was identical to a feruloylated diglucoside, for which a mass spectrometric protonated molecular ion ($[M + H]^+$)

was also found at *m/z* 889 (Vogt et al., 1999; Kugler et al., 2004), was readily excluded after co-chromatography (HPLC System 3) with later eluting betanidin monoferuloyl-5-*O*-β-D-diglucoside isolated previously from the Swiss Chard (Kugler et al., 2004).

Alkaline hydrolysis of isolated **8**, **8′**, **9** and **9′** with subsequent HPLC analysis (System 3) of the hydrolysates confirmed the presence of ferulic acid (from **8** and **8′**) and sinapic acid (from **9** and **9′**), both eluting later than **8**, **8′**, **9** and **9′**.

For the carbohydrate linkage as well as for the acyl attachment position analysis in **8** and **9**, the same procedures as in the previous cases were performed, i.e. the methylation by the Prehm method for partially methylated alditol acetate preparation by Anumula method.

The detection of 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylapitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-glucitol by GC–MS, again showed the terminal position of apiose, bound to the C-2′ of the glucose unit. Furthermore, the analysis of partially methylated alditol acetates prepared by the Prehm methylation followed by the procedure of Anumula resulted in detection of the prevailing 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylapitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-glucitol, indicating the position of the acyl moiety attachment to the C-5‴ atom of the apiofuranosyl unit. Some 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylapitol was also detected indicating partial cleavage of the acyl linkage during the methylation.

Therefore, the structures of **8** and **9** were identified as betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl]-β-D-glucopyranoside (**8**) and betanidin 5-*O*-[(5″-*O*-*E*-sinapoyl)-2′-*O*-β-D-apiofuranosyl]-β-D-glucopyranoside (**9**). This is the first case, where the sinapic acid is found as acylating substituent in the apiofuranosyl betacyanin structure.

2.5.2. Feruloylated apiosyl-phyllocactin

The pseudomolecular ions of **10** and **10′** at *m/z* 945 and the MS/MS fragmentation ions at *m/z* 859, 769, 683, 551 and 389, indicated the presence of malonyl (the mass difference of $945 - 859 = 86$) and feruloyl ($945 - 769 = 176$) in addition to apiofuranosyl moiety. This was readily supported by the coelution of **10** and **10′** with the pigments isolated from *Schlumbergera* × *buckleyi* flowers. The quantities of the isolated pigment were too scarce for the NMR structure elucidation, therefore only carbohydrate linkage analysis was performed. The detection of 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylapitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-glucitol by GC–MS, showed the typical terminal position of apiose, bound to the C-2′ of the glucose unit. The quantities of **10** or **10′** were not sufficient to confirm the position of the malonyl and feruloyl moieties by the Prehm method, however the detected carbohydrate linkage together with the pigment coelution results allowed the identification of **10** as betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl]-β-D-glucopyranoside.

2.6. Summary

The presence of new apiofuranosyl betacyanins in fruit flesh and peel of *Hylocereus* species was shown. For some compounds the ^{1}H, 1D TOCSY, gCOSY, gHSQC, and gHMBC NMR spectra were recorded for the first time in spite of minute quantities of the isolated pigments available. Interestingly, the peel samples contained hydroxycinnamoyl apiofuranosyl betacyanins and especially sinapoyl moiety was found for the first time in their structure. A new pigment structure isomeric to phyllocactin was identified with the malonyl moiety bound to C-4′ of glucopyranosyl unit instead of C-6′ as a result of possible acyl migration.

3. Experimental

3.1. Plant material

Fruits of *H. ocamponis*, *H. undatus*, and *H. purpusii*, as well as hybrids of *H. costaricensis* × *H. polyrhizus* and *H. undatus* × *H. polyrhizus* were selected for this study. The origin of the species and hybrids was described by Tel-Zur et al. (2004). The clones were introduced as cuttings to Israel from the Huntington Botanical Garden in California or from other cacti gardens from Israel and elsewhere. The hybrids were made in Beer Sheva (Israel) at BGU (Tel-Zur et al., 2004). For preparation of reference substances, Christmas cactus and Swiss chard was purchased from a local supplier.

3.2. Pigment extraction

For pigment isolation typically 500 g of freeze-dried fruit flesh or peel powder was three times extracted with 1200 ml 80% aq. MeOH and filtered through a 0.2 μm i.d. pore size filter (Millipore, Bedford, MA) and finally through a layer of 0.040 mm silica gel (J.T. Baker, Deventer, Holland) to obtain clear solution. The extract was rotovaporated for removing part of MeOH under reduced pressure at 25 °C and diluted with water before being freeze-dried. The flowers of Christmas cactus and Swiss chard petioles were processed by a similar procedure.

3.3. Pigment purification

For isolation the pigment extract was chromatographically concentrated by solid phase extraction on C18 cartridges (Merck, Darmstadt, Germany) according to the procedure of Stintzing et al. (2002b). For preparative isolation of the pigments subsequent cleanup was performed on DEAE Sephadex A-25 gel (Aldrich, Milwaukee, WI). Typically, the extract was dissolved in 100 ml of distilled water and run through a short column filled with the gel (3 cm × 1 cm i.d.). After rinsing with water and acetonitrile the betacyanin fraction was immediately eluted with acidified

methanol (methanol/TFA acidified water at pH 2, 95:5, v/v). The eluates were pooled and rotovaporated for removing part of methanol under reduced pressure at 25 °C and diluted with water before being freeze-dried. The freeze-dried residue was submitted to semipreparative HPLC for isolation of betacyanins.

3.4. Semipreparative HPLC

For the semipreparative isolation of betacyanins from the purified extracts a Gynkotek HPLC system with UVD170S, Gynkotek HPLC pump Series P580 and thermostat (Gynkotek Separations, H.I. Ambacht, The Netherlands) was used. The semipreparative column used was a 250 × 10 mm i.d., 10 μm Luna C18(2) (Phenomenex, Torrance, CA, USA) under the following gradient system (System 1): 6% A in B at 0 min; gradient to 10% A in B at 30 min. (A, acetonitrile; B, 2% HCOOH in H_2O). In each case the injection volume was 100 μl and the flow rate was 3 ml min^{-1}. Detection was generally performed at 538, 505, 480 and 310 nm with a DAD UV–vis detector. The columns were thermostated at 30 °C. For the isolation of **8**, **8′**, **9** and **9′** the chromatographic system (System 2) was the same except of the composition of B (14–20% HCOOH in H_2O). The 14% HCOOH solution was sufficient for the first step of **8**, **8′**, **9** and **9′** fractionation and the HCOOH concentration was differently set in subsequent steps of the pigment purification for the adequate resolution of the adjacent peaks. All obtained fractions (diluted with water after isolation in the System 2) were submitted to freeze-drying and analysis or another fractionation step.

3.5. Carbohydrate linkage analysis

The linkage between the carbohydrate moieties was established by GC–MS analysis of partially methylated alditol acetates prepared by permethylation of betacyanins in basic conditions (dispersed NaOH in DMSO) using methyl iodide with subsequent hydrolysis, reduction and peracetylation as described by Anumula and Taylor (1992). Any existing acyl-linked organic residues are lost under these conditions. For analysis of betacyanins containing apiofuranosyl moiety, apiin [apigenin 7-*O*-(2′-*O*-β-D-apiofuranosyl)-β-D-glucopyranoside from Alchem (Toruń, Poland) was used as standard.

The GC–MS analyses were performed on Finnigan gas chromatograph equipped with a 30-m Rtx-5 capillary column (Restek Co., Bellefonte, PA) connected to a Finnigan GCQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA) running in the 70 eV electron-impact mode. The gas chromatographic program used was 80 °C (1 min)–10 °C/min–300 °C. The analytes were identified by their retention time and characteristic fragmentation pattern (Biermann and McGinnis, 1989).

The position of acylation of the carbohydrate moieties was established by the above procedure with changed first step (permethylation of betacyanins) which was performed according to the standard procedure of Prehm (1980) using methyl trifluoromethanesulfonate in trimethyl phosphate. Under these mild conditions the prevailing final products are derived mainly from permethylated carbohydrate units with preserved acyl linkage, however a partial loss of acyl units is also observed. For analysis of **8** and **9** containing acylated apiofuranosyl moieties 5″-*O*-*E*-feruloyl-2′-apiosyl-betanin was isolated from the flowers of Christmas cactus (Kobayashi et al., 2000) and used as standard.

3.6. Deacylation of betacyanins

For the determination of the type of aliphatic acyl groups attached to the carbohydrate moieties, acid hydrolysis of betacyanins was performed according to Donner et al. (1997) in a mixture of 2 N HCl and MeOH under N_2 atmosphere for 1 h in boiling water. The acids were determined as methyl esters by GC-FID after their extraction with chloroform and drying with sodium sulphate. The analyses were performed on a GC 6000 Vega Series 2 gas chromatograph (Carlo Erba Instruments, Milan, Italy) equipped with a DB-FFAP or DB-1701 capillary column (J&W Scientific, Folsom, CA). As standards, dicarboxylic acids were subjected to the same methylation procedure and run by GC.

Hydroxycinnamic acyl moieties were identified as liberated acids by HPLC (under the gradient System 3, see below) after alkaline hydrolysis of betacyanins in deoxygenated 0.1 N NaOH for 10 min at 60 °C, according to Schliemann et al. (1996). Subsequent acidification of the resulting mixture with 0.1 M HCl resulted in restoration of epimerised mixture of deacylated betacyanins (Minale et al., 1966).

3.7. Analytical HPLC

The analytical HPLC system was the same as in the semipreparative mode except of the analytical column (Synergi Hydro-RP 250 × 3 mm i.d., 4 μm (Phenomenex, Torrance, CA, USA)), the gradient system (System 3) (from 7% A in B at 0 min; to 13% A in B at 40 min), an injection volume of 10 μl and a flow rate of 0.5 ml min^{-1}. For the separation of **8**, **8′**, **9** and **9′** a higher formic acid concentration in B was used (14%) (System 4).

3.8. LC–ESI–MS/MS analysis

The positive ion electrospray mass spectra were recorded on a ThermoFinnigan LCQ Advantage mass spectrometer (electrospray voltage 4.5 kV; capillary 250 °C; sheath gas: N_2) coupled to ThermoFinnigan LC Surveyor pump applied in the HPLC gradient System 3 or 4. The MS was controlled and total ion chromatograms and mass spectra were recorded using ThermoFinnigan Xcalibur software (San Jose, CA, USA). Helium was used to improve trapping efficiency and as the collision gas for CID experiments. The relative collision energies for MS/MS analyses were set at 30% (according to a relative energy scale).

3.9. NMR experiments

The NMR spectra of **1** (1 mg), **3** (1 mg) and **7** (1.5 mg) were recorded on a Bruker Avance 600 MHz instrument in not acidified D_2O at 300 K. The reference for the ^1H chemical shifts was the residual solvent signal at $\delta = 4.70$ ppm (D_2O) relative to TMS. All 1D (1H, 1D TOCSY) and 2D NMR (gCOSY, gHSQC, gHMBC, g = gradient enhanced) measurements were performed using standard Bruker pulse sequences.

Acknowledgement

This study was financed by Foundation for Supporting of Polish Pharmacy and Medicine Development at POLPHARMA S.A. Pharmaceutical Plant in the frame of the Research Project No. 015/2002.

References

Anumula, K.R., Taylor, P.B., 1992. A comprehensive procedure for preparation of partially methylated alditol acetates from glycoprotein carbohydrates. Anal. Biochem. 203, 101–108.

Biermann, C.J., McGinnis, G.D., 1989. Analysis of Carbohydrates by GLC and MS. CRC, Boca Raton.

Donner, H., Gao, L., Mazza, G., 1997. Separation and characterization of simple and malonylated anthocyanins in red onions, *Allium cepa* L. Food Res. Int. 30, 637–643.

Fischer, E., 1920. Migration of the acyl in glycerides. Chem. Ber. 53, 1621–1633.

Haines, A.H., 1976. Relative reactivities of hydroxyl groups in carbohydrates. Adv. Carbohydr. Chem. Biochem. 33, 11–109.

Henry, B.S., 1996. Natural food colours. In: Hendry, G.A.F., Houghton, J.D. (Eds.), Natural Food Colorants. Blackie Chapman & Hall, London, pp. 40–79.

Heuer, S., Wray, V., Metzger, J.W., Strack, D., 1992a. Betacyanins from flowers of *Gomphrena globosa*. Phytochemistry 31, 1801–1807.

Heuer, S., Wray, V., Metzger, J.W., Strack, D., 1992b. Betacyanins from flowers of *Gomphrena globosa*. Phytochemistry 33, 1553–1554.

Heuer, S., Richter, S., Metzger, J.W., Wray, V., Nimtz, M., Strack, D., 1994. Betacyanins from bracts of *Bougainvillea glabra*. Phytochemistry 37, 761–767.

Kobayashi, N., Schmidt, J., Nimtz, M., Wray, V., Schliemann, W., 2000. Betalains from Christmas cactus. Phytochemistry 54, 419–426.

Kugler, F., Stintzing, F.C., Carle, R., 2004. Identification of betalains from petioles of differently colored Swiss chard (*Beta vulgaris* L. ssp. *cicla* [L.] Alef. Cv. Bright lights) by high-performance liquid chromatography–electrospray ionization mass spectrometry. J. Agric. Food Chem. 52, 2975–2981.

Minale, L., Piattelli, S., De Stefano, S., Nicolaus, R.A., 1966. Pigments of Centrospermae VI. Acylated betacyanins. Phytochemistry 5, 1037–1052.

Minale, L., Piattelli, S., De Stefano, S., 1967. Pigments of Centrospermae VII. Betacyanins from *Gomphrena globosa* L. Phytochemistry 6, 703–709.

Mizrahi, Y., Nerd, A., 1999. Climbing and columnar cacti: new arid land fruit crops. In: Janick, J. (Ed.), Perspectives on New Crops and New Uses. American Society for Horticultural Sciences Press, Alexandria, pp. 358–366.

Piattelli, M., Imperato, F., 1970. Betacyanins from Bougainvillea. Phytochemistry 9, 455–458.

Prehm, P., 1980. Methylation of carbohydrates by methyl trifluoromethanesulfonate in trimethyl phosphate. Carbohydr. Res. 78, 372–374.

Schliemann, W., Joy IV, R.W., Komamine, A., Metzger, J.W., Wray, V., Strack, D., 1996. Betacyanins from plants and cell cultures of *Phytolacca americana*. Phytochemistry 42, 1039–1046.

Schwind, P., Wray, V., Nahrstedt, A., 1990. Structure elucidation of an acylated cyanogenic triglycoside, and further cyanogenic constituents from *Xeranthemum cylindraceum*. Phytochemistry 29, 1903–1911.

Stintzing, F.C., Schieber, A., Carle, R., 2002a. Identification of betalains from yellow beet (*Beta vulgaris* L.) and cactus pear [*Opuntia ficus-indica* (L.) Mill.] by high-performance liquid chromatography–electrospray ionization mass spectrometry. J. Agric. Food Chem. 50, 2302–2307.

Stintzing, F.C., Schieber, A., Carle, R., 2002b. Betacyanins in fruits from red–purple pitaya, *Hylocereus polyrhizus* (Weber) Britton & Rose. Food Chem. 77, 101–106, 517.

Stintzing, F.C., Conrad, J., Klaiber, I., Beifuss, U., Carle, R., 2004. Structural investigations on betacyanin pigments by LC NMR and 2D NMR spectroscopy. Phytochemistry 65, 415–422.

Strack, D., Vogt, T., Schliemann, W., 2003. Recent advances in betalain research. Phytochemistry 62, 247–269.

Tel-Zur, N., Abbo, S., Bar-Zvi, D., Mizrahi, Y., 2004. Clone identification and genetic relationship among vine cacti from the genera *Hylocereus* and *Selenicereus* based on RAPD analysis. Sci. Hort. 100, 279–289.

Vogt, T., Ibdah, M., Schmidt, J., Wray, V., Nimtz, M., Strack, D., 1999. Light-induced betacyanin and flavonol accumulation in bladder cells of *Mesembryanthemum crystallinum*. Phytochemistry 52, 583–592.

Wagner, H., Demuth, G., 1972. 6-*O*-(D-Apiofuranosyl)-1,6,8-trihydroxy-3-methyl-anthrachinon, ein neues Glykosid (Frangulin B) aus der Rinde von *Rhamnus frangula* L. Tetrahedron Lett. 49, 5013–5014.

Wybraniec, S., Platzner, I., Geresh, S., Gottlieb, H.E., Haimberg, M., Mogilnitzki, M., Mizrahi, Y., 2001. Betacyanins from vine cactus *Hylocereus polyrhizus*. Phytochemistry 58, 1209–1212.

Wybraniec, S., Nowak-Wydra, B., Mizrahi, Y., 2005. ^1H and ^{13}C NMR spectroscopic structural elucidation of new decarboxylated betacyanins. Tetrahedron Lett. 47, 1725–1728.