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Chromatographic investigation on acyl migration in betacyanins and their decarboxylated derivatives

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Abstract

Chemopreventive and antioxidant action of betalain pigments can differ in dependence on their stereoselective properties, therefore, it is necessary to use relevant methods for monitoring of their possible stereoisomers. Chromatographic characterisation of a group of new isomers of various 6′-*O*-acylated betacyanins and decarboxylated betacyanins which were generated at low concentration by intramolecular pH-dependent acyl migration was studied in aqueous solutions by HPLC separation with diode-array and mass spectrometric detection. Under alkaline conditions (pH 10.5) the rate of migration was dramatically accelerated, however, always favouring the 6′-*O*-position and it was much less prominent at lower pH (under 7.0). The possible products of the partial rearrangement were tentatively identified as the 3′-*O*- and 4′-*O*-acylated forms and their relative retention times were provided. In malonylated betacyanins and 17-decarboxy-betacyanins the 4′-*O*-forms were characterised in RP-HPLC by higher retention than the 6′-*O* forms, whereas the 3′-*O*-forms were always the most polar. In contrast, the isomerisation of hylocerenin and 17-decarboxy-hylocerenin resulted in different chromatographic profiles of the migration products. In 2-decarboxy- and 2,17-bidecarboxy-betacyanins the 3′-*O*- and 4′-*O*-acylated forms eluted always before the 6′-*O*-acylated betacyanins. The investigations on acyl migration in isolated 4′-*O*-malonyl-betanin confirmed the strong tendency of reverse acyl migration (4′ → 6′) and also partial 4′ → 3′ rearrangement which were leading to the final monoester regioisomeric distribution (%) close to 87:7:6 (6′-*O*-, 4′-*O*-, 3′-*O*-).

Keywords: Acyl migration; HPLC–ESI-MS; HPLC-DAD; Betanin; Phyllocactin; Hylocerenin; Mammillarinin; Apiosyl; Decarboxy-betacyanins

1. Introduction

Betacyanins, water soluble red-violet pigments extensively used as food colorants, can be considered as condensation products of betalamic acid with cyclodopa or its *O*-glycosylated (in most cases 5-*O*-glucosylated) derivatives. Further esterification of the *O*-glycosides with acids such as ferulic, *p*-coumaric or malonic acid is very common [1,2]. Betacyanins exist in two diastereomeric forms differing by the configuration of the C-15 carbon (Fig. 1).

Betanin, the simplest 5-*O*-glucosylated betacyanin (15*S*) and its C-15 isoform (15*R*) are frequently derived from red beet root (*Beta vulgaris* L.). Other edible rich sources of betacyanins are recently investigated species of *Hylocereus* cacti containing betanin, phyllocactin and hylocerenin as the most abundant pigments [3–5]. Growing interest in betacyanins and closely related betaxanthins is stimulated not only by their colorant but also chemopreventive and strong antioxidant properties [6–9].

The most frequent esterification position of the *O*-glucosides is the C-6′ carbon with very few examples of other positions [1,2]. Recently, a new 4′-*O*-malonylated derivative of betanin has been reported in the fruits of *Hylocereus* cacti [5], however, it isomerised to phyllocactin (6′-*O*-malonylated betanin) after isolation due to acyl migration.

The pH-dependent acyl migration between adjacent hydroxy groups on polyhydroxy compounds has been noticed [10–13]. A typical mechanism of acyl migration is based upon a neighbouring group effect with an *ortho* acid ester as an intermediate [10–13] (Fig. 2.). In many instances the proximity in space of the migrating group to the free hydroxyl is sufficient to permit an intermediate cyclic structure [10–13].

The acyl migration was also observed in methylation reactions with the use of silver oxide [10,11,13]. The alkaline conditions played the crucial role in that case and were very

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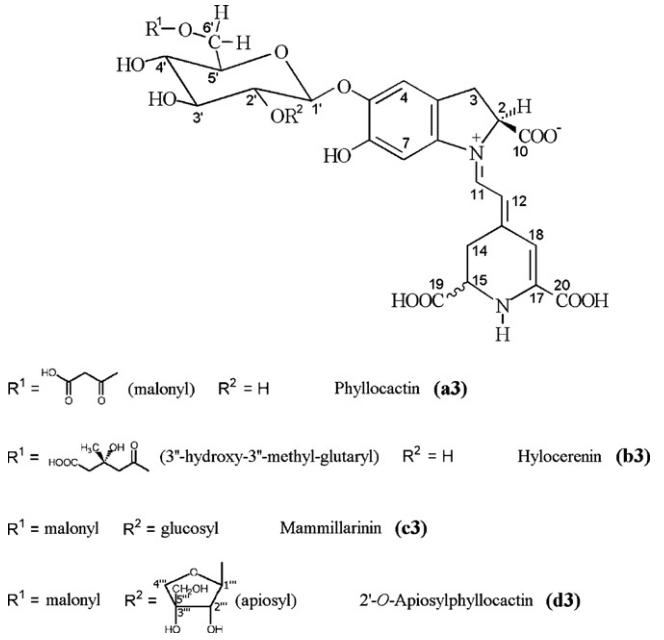

Fig. 1. Chemical structures of 6′-*O*-acylated betacyanins submitted to acyl migration. The phyllocactin and hylocerenin derivatives were obtained by their decarboxylation at carbon no. 2 and/or 17.

often an inducing factor of this phenomenon. However, the acyl migration was also shown to occur in acid solutions [10,11,13].

The consequences of pH-dependent migration process in acyl glucuronide conjugates of carboxylate drugs are nowadays pharmacologically essential [14]. The phenomenon of acyl migration has not been studied extensively in secondary plant metabolites and especially not in betalains. Because the chemopreventive and antioxidant action of betalains can differ in dependence on their stereoselective properties, it is necessary to use relevant methods for the monitoring of possible stereoisomers. Chromatography is a very useful tool for investigation of acyl migration because of a possibility of monitoring and separation of isomers which are frequently too labile to be isolated for structure elucidation and undergo further acyl rearrangement reaching an equilibrium with other acylated forms [15]. The hyphenated techniques (HPLC-MS and HPLC-NMR) allow new approaches to the structural investigations of the resulting products [16–18]. The only application of HPLC-NMR to analysis of concentrated samples of betacyanins has been performed recently [4]. The use of NMR combined techniques for research on betacyanins is still hindered by their low concentrations in many matrices and their lability in most of solvents required for the experiments [4]. Therefore, in this contribution a chromatographic study on a number of various betacyanins and their decarboxylated derivatives exposed to alkaline environment and detected by means of diode-array detection and mass spectrometry is reported. Our extensive research revealed that pH-depending acyl migration in this group of compounds is a very probable phenomenon which has never been noticed so far.

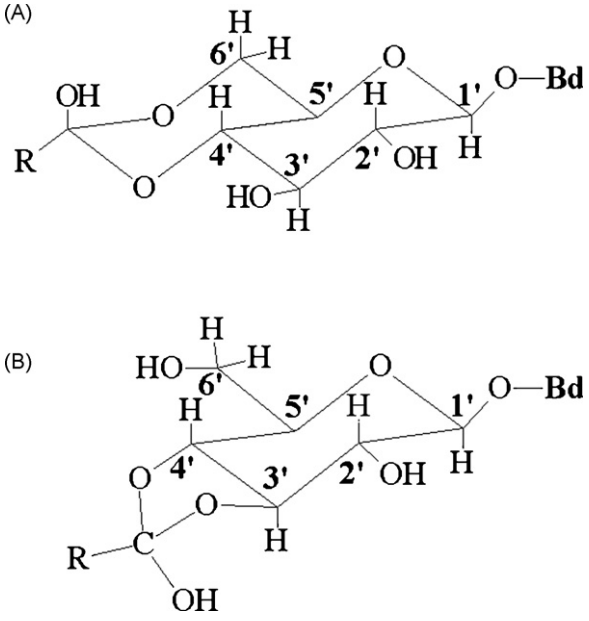

Fig. 2. A presumed intermediate six-membered cyclic structure formed during the acyl migration between the glucosidic *O*-6′ and *O*-4′ oxygens in betacyanins or their decarboxylated derivatives (A) and a five-membered cyclic *ortho* ester formed during the acyl migration between *O*-4′ and *O*-3′ in the pigments (B) [10–13]. (**R**: a fragment of an acyl moiety; **Bd**: the rest of betanidin).

2. Experimental

2.1. Reagents

Sodium hydroxide was obtained from Aldrich (Milwaukee, WI, USA). Formic acid, HPLC-grade acetonitrile and HPLC-grade water were obtained from Merck (Darmstadt, Germany).

2.2. Apparatus

A Gynkotek HPLC system with UVD170S, Gynkotek HPLC Pump Series P580 and thermostat (Gynkotek Separations, H.I. Ambacht, The Netherlands) was used for the chromatographic analysis. For the data acquisition, the software package Chromeleon 4.32 (Gynkotek Separations) was used. For the UV–vis spectra acquisition the detection was performed in the DAD (diode-array detection) mode. For the separation of betacyanins an ONYX monolithic column 100 mm × 4.6 mm i.d. protected by a guard column (Phenomenex, Torrance, CA, USA) was used.

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 applied in the HPLC gradient System 1 or 2. 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.

2.3. Preparation of analyte solutions

Most of betacyanins (phyllocactin, hylocerenin, 2′-apiosylphyllocactin and their C-15 diastereomers) were derived

from extracts of commercially available fruits of *Hylocereus polyrhizus*. 2-Decarboxy-, 17-decarboxy- and 2,17-bidecarboxy-betacyanins were obtained from phyllocactin and hylocerenin as well as their diastereoisomers by preparation procedures described in [19]. Mammillarinin/isomammillarinin were isolated from *Mammillaria* fruits in [20] and were also investigated in this study. The HPLC elution profiles of the pigments was compared with the data from [5,11,20] and was confirmed by mass spectrometric analysis [5,11,20].

2.4. Chromatographic system

For the separation of the analytes the following gradient system was used: 97% A with 3% B at 0 min; gradient to 85% A with 20% B at 35 min (solvent A – 4% HCOOH, solvent B – acetonitrile). The injection volume was 10 μL and the flow rate was 0.5 mL/min. Detection by the UV–vis detector was performed at λ = 538 and 505 nm or in the DAD mode. The column was thermostated at 35 °C.

2.5. Isomerisation experiments

The purified pigments (0.05–2 μmol) (Table 1) dissolved in 300 μL of water were mixed with 600 μL of a buffer (pH 4.0; 4.5; 5.5; 6.5; 7.5; 8.5; 9.5 and 10.5) and incubated for few minutes, hours or days in 2 mL closed glass vials at 4 or 20 °C under nitrogen. After appropriate time interval 10 μL of the samples were analysed by the HPLC system.

3. Results and discussion

The studies on acyl migration in betacyanins were induced by the fact of isomerisation of isolated 4′-*O*-malonyl-betanin from the cacti fruits of *Hylocereus* species to 6′-*O*-malonyl-betanin (phyllocactin) [5]. Phyllocactin is a very common pigment in many cacti species and is present in the *H. polyrhizus* fruits in relatively high concentration. The presence of a small isomeric peak accompanying the peak of phyllocactin has been frequently noticed [5,20,21]. It was also suspected that this compound was another isomer characterised by a different betanidin glycosylation position (O-6 instead of O-5) [5], however, this presumption was not supported because of the lack of slight bathochromic shift to λ_{max} 540–543 nm which was observed for betanidin 6-*O*-β-glucosides [22–24]. Recent study [5] cleared out the identity of this peak as 4′-*O*-malonyl-betanin and indicated the possibility of acyl migration between the glucosidic *O*-4′ and *O*-6′ hydroxyls.

The possibility of acyl migration in betacyanins was considered only once for phyllocactin [22], however, no studies on this phenomenon in betacyanins were performed. The acyl migration is proved to be intramolecular, therefore, mechanisms involved with hydrolysis and re-esterification are not important in the process [25]. Hence, the free carboxyls in the acylating moieties in phyllocactin and hylocerenin as well as their derivatives do not take part in the rearrangement. The formation of the intermediate six-membered strainless cyclic structure (Fig. 2A) between the glucosidic *O*-4′ and *O*-6′ hydroxyls [10–13], which was reported in many cases of migration in acylated β-D-glucosides, could be also responsible for the rearrangement in phyllocactin. The cyclic *ortho* ester has both rings in stable chair forms and can be readily formed.

In order to study the influence of pH on interconversion between 6′-*O*-malonyl-betanin (phyllocactin) and 4′-*O*-malonyl-betanin in aqueous solutions, a series of experiments was performed on the both purified pigments exposed to different pH (range of 4.0–10.5). Afterwards, other betacyanins as well as decarboxy-betacyanins were investigated. In all experiments, the characteristic *m/z* of pseudomolecular ions ($[M+H]^+$) indicated the presence of isomeric forms of the pigments (Table 1) supporting the possibility of acyl migration.

In spite of a certain isomerisation progress, which was frequently noticed in aqueous solutions of purified pigments exposed to room temperature, the starting compositions were far from the equilibrium reached at the end of the experiments. The results of the reversed acyl migration (4′ → 6′) in the hitherto only one isolated 4′-*O*-malonylated derivative (4′-*O*-malonyl-betanin) [5] are presented in Table 2. In Table 3 the retention times as well as the relative retention times of the isomeric forms are provided for the first time and are discussed below.

Table 1
O-acylated betacyanins and decarboxy-betacyanins analysed in this study and their spectral properties

Group no.	Compound	Pigment designation						λ_{max}[a] (nm)	*m/z* $[M+H]^+$
		15*S* forms			15*R* forms (isoforms)				
		3-*O*-	4-*O*-	6-*O*-	3-*O*-	4-*O*-	6-*O*-		
a	Phyllocactin	**a1**	**a2**	**a3**	**a1′**	**a2′**	**a3′**	538	637
b	Hylocerenin	**b1**	**b2**	**b3**	**b1′**	**b2′**	**b3′**	538	695
c	Mammillarinin	**c1**	**c2**	**c3**	**c1′**	**c2′**	**c3′**	538	799
d	2′-Apiosyl-phyllocactin	**d1**	**d2**	**d3**	**d1′**	**d2′**	**d3′**	538	769
e	17-Decarboxy-phyllocactin	**e1**	**e2**	**e3**	**e1′**	**e2′**	**e3′**	505	593
f	17-Decarboxy-hylocerenin	**f1**	**f2**	**f3**	**f1′**	**f2′**	**f3′**	505	651
g	2-Decarboxy-phyllocactin	**g1**	**g2**	**g3**	**g1′**	**g2′**	**g3′**	533	593
h	2-Decarboxy-hylocerenin	**h1**	**h2**	**h3**	**h1′**	**h2′**	**h3′**	533	651
i	2,17-Bidecarboxy-phyllocactin	**i1**	**i2**	**i3**	**i1′**	**i2′**	**i3′**	507	549
j	2,17-Bidecarboxy-hylocerenin	**j1**	**j2**	**j3**	**j1′**	**j2′**	**j3′**	507	607

[a] λ_{max} in the visible range.

Table 2
Time-dependent monoester regioisomeric distribution of acyl migration products after exposition of purified 4′-*O*-malonyl-betanin to different pH conditions

Time (min)	Regioisomeric distribution (%)														
	pH 6.5			pH 7.5			pH 8.5			pH 9.5			pH 10.5		
	3-*O*-	4-*O*-	6-*O*-	3-*O*-	4-*O*-	6-*O*-	3-*O*-	4-*O*-	6-*O*-	3-*O*-	4-*O*-	6-*O*-	3-*O*-	4-*O*-	6-*O*-
Temp. 4 °C															
0	0	93	7	0	93	7	0	93	7	0	93	7	0	93	7
1	1	92	7	1	68	31	1	52	47	2	43	55	2	34	65
25	2	89	9	2	58	40	2	37	61	3	28	69	3	10	87
60	3	76	22	3	50	47	3	28	69	4	18	79	4	7	90
180	3	64	32	4	39	58	4	18	78	4	11	85	4	7	89
360	4	56	40	5	30	65	5	13	82	6	9	86	6	7	88
720	5	47	48	5	19	76	5	9	85	6	7	87	6	7	87
1440	5	39	56	5	12	83	6	8	86	6	7	88	6	7	87
Temp. 20 °C															
0	0	93	7	0	93	7	0	93	7	0	93	7	0	93	7
1	3	60	37	3	47	50	5	10	85	6	7	87	6	7	87
25	5	40	56	5	29	66	6	7	87	6	7	87	6	7	87
60	5	26	68	5	19	76	6	7	87	6	7	87	6	7	87
180	5	20	75	5	8	86	6	7	87	6	7	87	6	7	87
360	6	12	82	6	8	87	6	7	87	6	7	87	6	7	87
720	6	10	84	6	7	87	6	7	87	6	7	87	6	7	87
1440	6	9	85	6	7	87	6	7	87	6	7	87	6	7	87

3.1. Acyl migration in phyllocactin (6′-O-malonyl-betanin)

The intramolecular migration in phyllocactin and other 6′-*O*-acylated pigments could be observed only under alkaline conditions. Below pH 7.0 practically no changes in ratio between 6′-*O*- and 4′-*O*-acylated forms were observed, except of the degradation of the pigments completing after few days. Therefore, further experiments performed in order to observe the final products of acyl migration of phyllocactin and other betacyanins as well as their decarboxylated derivatives were conducted at pH 10.5 (Table 3). In each case the two C-15 diastereoisomeric forms were studied separately in order to avoid misinterpreta-

Table 3
Retention times of betacyanin and decarboxy-betacyanin isomers obtained after chromatographic analysis (HPLC gradient System 1) of purified 6′-*O*-forms submitted to acyl migration at pH 10.5

Compound	Group no.	Retention time (min)					
		Isomer no. (acylation position)					
		1 (**3′-*O*-**)	**2** (**4′-*O*-**)	**3** (**6′-*O*-**)	**1′** (**3′-*O*-**)	**2′** (**4′-*O*-**)	**3′** (**6′-*O*-**)
Phyllocactin	**a**	15.1	17.4	16.1	17.3	20.0	18.3
Hylocerenin	**b**	21.8	22.8	20.9	24.3	25.1	23.3
Mammillarinin	**c**	11.4	14.5	12.5	13.3	15.8	14.2
2′-*O*-Apiosyl-phyllocactin	**d**	20.8	23.4	22.2	23.2	25.6	24.4
17-Decarboxy-phyllocactin	**e**	18.0	21.4	20.0	21.8	24.2	22.7
17-Decarboxy-hylocerenin	**f**	20.3	23.7	22.8	24.2	27.2	25.4
2-Decarboxy-phyllocactin	**g**	24.2	24.6	28.1	23.8	24.8	26.7
2-Decarboxy-hylocerenin	**h**	27.7	28.4	30.6	27.4	28.2	29.0
2,17-Bidecarboxy-phyllocactin	**i**	26.5	27.5	30.5	26.3	27.9	29.3
2,17-Bidecarboxy-hylocerenin	**j**	31.2	32.7	33.3	30.8	31.7	32.1
Relative retention time[a]							
Phyllocactin	**a**	0.93	1.09	1.00	1.09	1.28	1.16
Hylocerenin	**b**	1.05	1.10	1.00	1.18	1.22	1.13
Mammillarinin	**c**	0.89	1.19	1.00	1.08	1.32	1.17
2′-*O*-Apiosyl-phyllocactin	**d**	0.93	1.06	1.00	1.05	1.17	1.11
17-Decarboxy-phyllocactin	**e**	0.89	1.08	1.00	1.10	1.24	1.15
17-Decarboxy-hylocerenin	**f**	0.88	1.04	1.00	1.07	1.21	1.13
2-Decarboxy-phyllocactin	**g**	0.85	0.86	1.00	0.83	0.87	0.95
2-Decarboxy-hylocerenin	**h**	0.90	0.92	1.00	0.89	0.92	0.94
2,17-Bidecarboxy-phyllocactin	**i**	0.86	0.89	1.00	0.85	0.91	0.96
2,17-Bidecarboxy-hylocerenin	**j**	0.93	0.98	1.00	0.92	0.95	0.96

[a] In relation to the corresponding 6′-*O*-isomer of the pigment 15*S* form, calculated from the reduced retention times.

tion of the resulting data but in each chromatogram a small peak of the opposite form always appeared, however, it was usually possible to recognise it by its retention time. Such isomerisation between the 15*S* and 15*R* forms had been frequently noticed [2].

Interestingly, except of the small peak of isophyllocactin (due to epimerisation at the C-15 carbon), not only the major isomeric peak of 4′-malonyl-betanin (**a2**) was formed but also another minor peak (**a1**) preceding the huge peak of phyllocactin (Fig. 3B). The experiments were repeated also for isophyllocactin and resulted in a similar peak profile (Fig. 3C). The detection of phyllocactin derivatives by LC–MS/MS confirmed that the additional peak was another isomer of phyllocactin and it was very probable that another migration process took place leading to a formation of 3′-malonyl-betanin or 2′-malonyl-betanin. The proximity of the two hydroxyls at the C-4′ and C-3′ carbon favoured the first rearrangement product, therefore, the structure of the minor product of the acyl migration was tentatively identified as 3′-malonyl-betanin. In this case the possible intermediate can be formed as a five-membered cyclic *ortho* ester (Fig. 2B) between adjacent hydroxyl groups having an equatorial–equatorial disposition without major bond distortion [10–13]. The results of the experiments suggested that the intramolecular migration started from *O*-6′ and proceeded to *O*-3′ through *O*-4′ until reaching the equilibrium at a given pH still favouring the *O*-6′ position. In addition, the similarity of the chromatographic profiles (Fig. 3D and F) of mammillarinin (phyllocactin derivative—see results below) rearrangement products also suggested the 4′ → 3′ migration in phyllocactin because in mammillarinin the 4′ → 2′ process was not possible (no hydroxyl is present at the C-2′ carbon).

3.2. Acyl migration in 4′-*O*-malonyl-betanin

Further experiments were performed with isolated 4′-*O*-malonyl-betanin which appeared as a very labile compound, easily isomerising to phyllocactin, but also, in certain extent, to another isomer, presumably 3′-*O*-malonyl-betanin (Fig. 4). Preparative isolation of the 4′-*O*-malonyl-betanin from the natural source [5] was possible because of the high concentration of phyllocactin in the sample with which sufficiently concentrated 4′-*O*-malonyl-betanin existed in the equilibrium.

The reversed acyl migration (4′ → 6′) proceeded almost immediately (within less than 1 min) when a high pH (10.5) was applied to the pigment solution (Table 2). Further increase of pH (beyond 11.4) would result in the fast hydrolysis of the immonium conjugates and partial degradation of the pigments. Decreasing pH of the solutions resulted in a slower isomerisation rate of 4′-*O*-malonyl-betanin and at pH 6–7 it took several hours for reaching the equilibrium at 20 °C or few days at 4 °C. However, after few days a progressing degradation of the pigments was also detectable.

The results of the experiments performed on 4′-*O*-malonyl-betanin (Table 2) confirmed, that the most favoured position of the malonyl moiety was the C-6′ carbon and the intramolecular migrations 4′ → 3′ and 4′ → 6′ proceeded until reaching

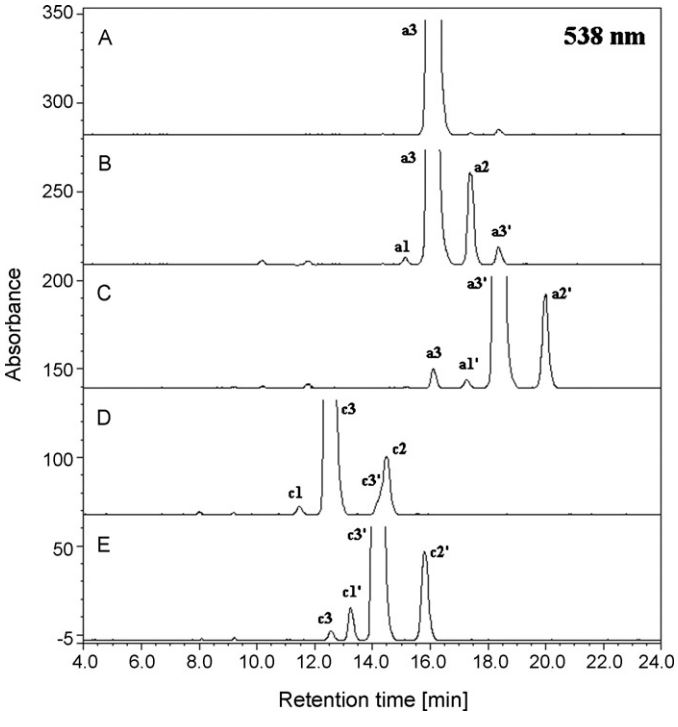

Fig. 3. Chromatographic profiles (HPLC gradient System 1) of purified phyllocactin (A), phyllocactin/isophyllocactin submitted to acyl migration (B and C) and mammillarinin/isomammillarinin submitted to acyl migration (D and E). The analysis started after 10 min of reaction in aqueous solution (pH 10.5) at 20 °C.

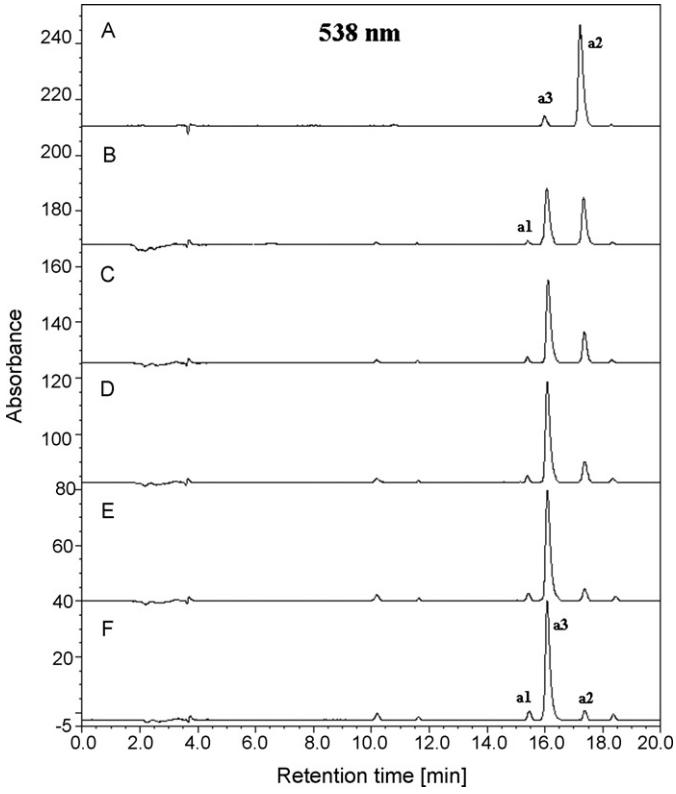

Fig. 4. Malonyl migration in 4′-*O*-malonyl-betanin monitored after 0 min (A), 1 min (B) 25 min (C), 60 min (D), 180 min (E) and 720 min (F) of reaction in aqueous solution (pH 9.5) at 4 °C (HPLC gradient System 1).

the equilibrium at a given pH with the monoester regioisomeric distribution (%) close to 87:7:6 (**a3**:**a2**:**a1**).

3.3. Acyl migration in other betacyanins

Very recent studies revealed a new malonylated betacyanin, mammillarinin (**c3**), which was identified as betanidin 6′-*O*-malonyl-5-*O*-*β*-sophoroside [20] and can be regarded as glucosylated phyllocactin. In analogy to the migration in phyllocactin, two other betacyanins (**c2/c2′**) eluting after their corresponding isomers, mammillarinin/isomammillarinin (**c3/c3′**), were tentatively identified as betanin/isobetanin 4′-*O*-malonyl-5-*O*-*β*-sophoroside, the acyl migration products [20]. Therefore, in this study a migration of the malonyl positioned at the first glucose unit in mammillarinin for the comparison to the migration in phyllocactin was also investigated.

The experiments confirmed that **c2/c2′** could be a result of alkalic isomerisation (6′ → 4′) of mammillarinin/isomammillarinin (Fig. 3D and E), however, for each diastereomer the additional minor peak (**c1/c1′**) of isomer observed in the chromatograms suggested the presence of another acyl migration product, presumably betanin/isobetanin 3′-*O*-malonyl-5-*O*-*β*-sophoroside (Table 3).

The betanin 4′-*O*-malonyl-5-*O*-*β*-sophoroside (**c2**) coeluted with isomammillarinin (**c3′**) in the gradient System 1, but increasing the concentration of formic acid in eluent A to 8% allowed for their separation (results not shown).

Another malonylated betacyanin, 2′-*O*-apiosyl-phyllocactin (**d3**), which posses a similar structure to mammillarinin except of the second sugar moiety (apiosyl), was isolated from *Hylocereus* cacti [5] as well as from other sources [26,27]. Because of the similarity of the structures it was possible to compare the isomerisation results between 2′-*O*-apiosyl-phyllocactin, phyllocactin and mammillarinin. Arising peaks (**d1/d1′** and **d2/d2′**), presumably due to the acyl migration, together with the peaks of the starting substrates (**d3/d3′**) formed similar chromatographic profiles as in the case of phyllocactin and mammillarinin (Table 3, Fig. 5C and D). Because no hydroxyl is present at the C-2′ carbon, the presence of 2′-*O*-forms was excluded. This confirmed the possible assignation of the peaks as 3′-*O*- (**d1/d1′**) and 4′-*O*-forms (**d2/d2′**).

Hylocerenin was the only non-malonylated betacyanin analysed in this study (Table 3, Fig. 5A and B). This pigment, found solely in the fruits of epiphytic cacti, was structurally elucidated during the past few years [3,4] and its decarboxylated derivatives were studied as well [16,28]. From Fig. 5A and B it could be concluded that the chromatographic profiles of hylocerenin/isohylocerenin alkaline isomerisation products were different from the profiles of phyllocactin/isophyllocactin, however, still two pairs of isomers (**b2/b2′**) were formed. Because the 4′-*O*-form of phyllocactin isomers (**a2**) eluted later than the 3′-*O*-form (**a1**) and because in the case of other pigments one prevailing isomer (presumably the 4′-*O*-form) was always arising at distinctly higher concentration and eluting later than the minor isomer (presumably the 3′-*O*-form), it was in analogy tentatively assumed that the later eluting hylocerenin isomer (**b2**) was the 4′-*O*-form. Therefore, it was likely that the acyl migration products were 3′-*O*-(3″-hydroxy-3″-methyl-glutaryl)-betanin (**b1**) and 4′-*O*-(3″-hydroxy-3″-methyl-glutaryl)-betanin (**b2**) as well as their isoforms (**b1′** and **b2′**), respectively.

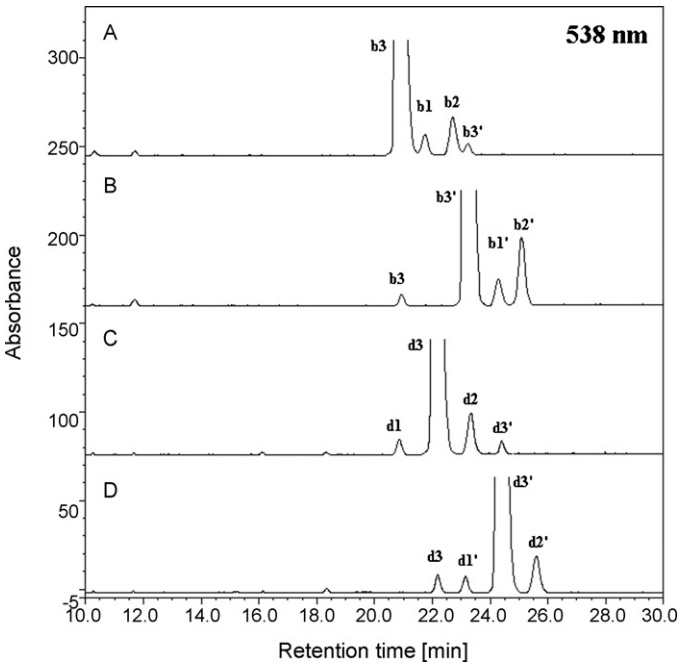

Fig. 5. Chromatographic profiles (HPLC gradient System 1) of hylocerenin/isohylocerenin (A and B) and 2′-*O*-apiosyl-phyllocactin/-isophyllocactin (C and D) submitted to acyl migration. The analysis started after 10 min of reaction in aqueous solution (pH 10.5) at 20 °C.

3.4. Acyl migration in decarboxylated betacyanins

Upon elevated temperature, in aqueous or ethanolic solutions betacyanins form 2-decarboxylated or 17-decarboxylated derivatives [16,28,29], respectively. Further heating results in formation of 2,17-bi- and 2,15,17-tridecarboxy-betacyanins in the both solutions [16,29]. Therefore, some of these compounds are always present in heated solutions or industrial formulations containing betacyanins. The 2-decarboxylated betacyanins were also reported in plants [30]. During our recent studies, the derivatives of betanin, phyllocactin and hylocerenin were frequently isolated for structure elucidation [16,28] and chromatographic studies [31], therefore, these compounds could be chosen for subsequent experiments. Because the acylated derivatives (decarboxylated phyllocactin and hylocerenin) differ from their precursors only by the lack of the carboxyls at betanidin part, the acyl migration in these pigments was very probable. It was also interesting to observe the elution profiles of the isomeric compounds. A parallel study [32] proved the reversed elution order of diastereomers of 2-decarboxy- and 2,17-bidecarboxy-betacyanins (15*R* before the 15*S* form) in comparison to betacyanins and 17-decarboxy-betacyanins. Therefore, some meaningful differences of the elution profiles between each group of the isomers were expected.

The chromatographic data of 17-decarboxy-phyllocactin/-isophyllocactin (**e3/e3′**) (Fig. 6A and B) and 17-decarboxy-

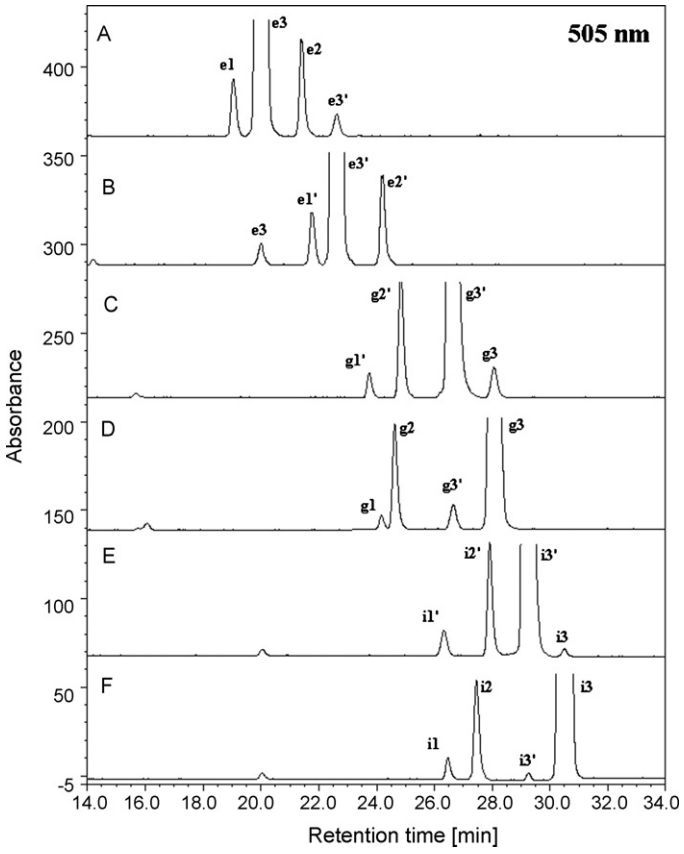

Fig. 6. Chromatographic profiles (HPLC gradient System 1) of 17-decarboxy-phyllocactin/-isophyllocactin (A and B), 2-decarboxy-phyllocactin/-isophyllocactin (C and D) and 2,17-bidecarboxy-phyllocactin/-isophyllocactin (E and F) submitted to acyl migration. The analysis started after 10 min of reaction in aqueous solution (pH 10.5) at 20 °C.

hylocerenin/-isohylocerenin (**f3/f3′**) isomerisation are summarised in Table 3. In the case of 17-decarboxy-phyllocactin there were two peaks of isomers arising which presumably appeared as a result of acyl migration. The chromatographic profiles were similar to the profile of betacyanin isomers, therefore it was tentatively possible to assign the peaks as 4′-*O*-malonyl-17-decarboxy-betanin/-isobetanin (**e2/e2′**) and 3′-*O*-malonyl-17-decarboxy-betanin/-isobetanin (**e1/e1′**).

As a result of hylocerenin/-isohylocerenin derivative (**f3/f3′**) isomerisation only one peak arising from each pigment could be detected (Table 3) which was tentatively assigned as 4′-*O*-(3″-hydroxy-3″-methyl-glutaryl)-17-decarboxy-betanin/-isobetanin (**f2/f2′**). However, taking into account the chromatographic profiles of hylocerenin isomers (**b1-b3** as well as **b1′-b3′**), it could be possible that the peaks of 3′-*O*-acylated isomers (**f1/f1′**) coeluted with the huge peaks of 6′-*O*-acylated forms (**f3/f3′**).

In the case of 2-decarboxylated and 2,17-decarboxylated derivatives of phyllocactin (Fig. 6C–F) and hylocerenin, the resulting chromatograms were similar to each other but significantly different from the chromatograms of betacyanins (Table 3). The major products of acyl migration (**g2/g2′**, **h2/h2′**, **i2/i2′**, **j2/j2′**), presumably 4′-*O*-acylated isomers, always eluted before the substrates but after the minor products (**g1/g1′**, **h1/h1′**, **i1/i1′**, **j1/j1′**), presumably 3′-*O*-acylated isomers. As it was expected before, the different elution profiles in this group of pigments corresponded well to the reversion of the elution order of the substrate diastereomers (Fig. 6C–F) [32]. Therefore, in general, on the basis of the performed experiments in the current and in previous study [32], it can be concluded that the loss of the carboxyl at the C-2 carbon is always determining these interesting chromatographic phenomena in the group of betalains.

4. Conclusions

The study confirmed the possibility of chromatographic separation of the analysed stereoisomers of betacyanins and decarboxylated betacyanins with characteristic chromatographic patterns in different groups of compounds. The acyl migration in the pigments is a strongly pH-dependent phenomenon and is responsible for the presence of detectable quantities of the 4′-*O*-forms of betacyanins in plant tissue which exist in equilibrium with the prevailing 6′-*O*-forms. Further studies on acyl migration in other rarely occurring acylated betacyanins present as the main forms in plants (2′-*O*- and 3′-*O*-acylated pigments instead of the 6′-*O*-forms) shall complement the results of this report.

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