Chromatographic fractionation of betacyanins from flowers of Gomphrena globosa

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In this study, a chromatographic fractionation of betacyanin pigments from extract of purple Gomphrena globosa petals was performed by preparative high performance liquid chromatography (prep-HPLC). The particular betacyanins in each collected fractions were tentatively identified by chromatography with optical amd mass spetrometric detection (LC-DAD-ESI-MS).

Betacyanins are natural pigments, which are confirmed to have an antioxidant activity. It was reported that betacyanins can prevent civilization diseases, because of an ability of free radicals scavenging. Betacyanins are present in tissue of plants from Amaranthaceae family, e.g. in petals of Gomphrena globosa. Preparative separation of these particular pigments is difficult, because they are structurally very similar to each other and tend to co-elute. However, in this study, fractions, containing a dominant amount of the principal pigments of Gomphrena globosa inflorescences, were obtained successfully. In the first fractions, two isomeric pigments assigned to gomphrenin I and isogomphrenin I were detected. The fractions of significant amounts of gomphrenin III and isogomphrenin III were obtained separately with sufficient purity. The presence of other, minor gomphrenin-type betacyanins were also confirmed as: gomphrenin II, cis-isomer of gomphrenin II and sinapoyl-gomphrenin I as well as their 15S-diastereomers. Moreover, in other fractions, various unknown pigments were detected

Key words: betacyanins, Gomphrena globosa, plant pigments, LC-MS

Introduction

Betacyanins are water-soluble, natural pigments, which are a subgroup of betalains. Betacyanins are responsible for red-violet colour of various parts of plants: leaves, fruits, roots, flowers as well as mushrooms caps. Their characteristic chemical structures include betalamic acid conjugated with *cyclo*-DOPA or *O*-glycosylated *cyclo*-DOPA. It forms a system of conjugated double bonds, which creates the 1,7-diazaheptamethin chromophore [1-3].

Studies on betacyanins gain importance since their pro-health and antioxidant activities were revealed. Furthermore, it has been reported that they prevent cancer and even have inhibitory effect on the proliferation of tumor cells [3-4].

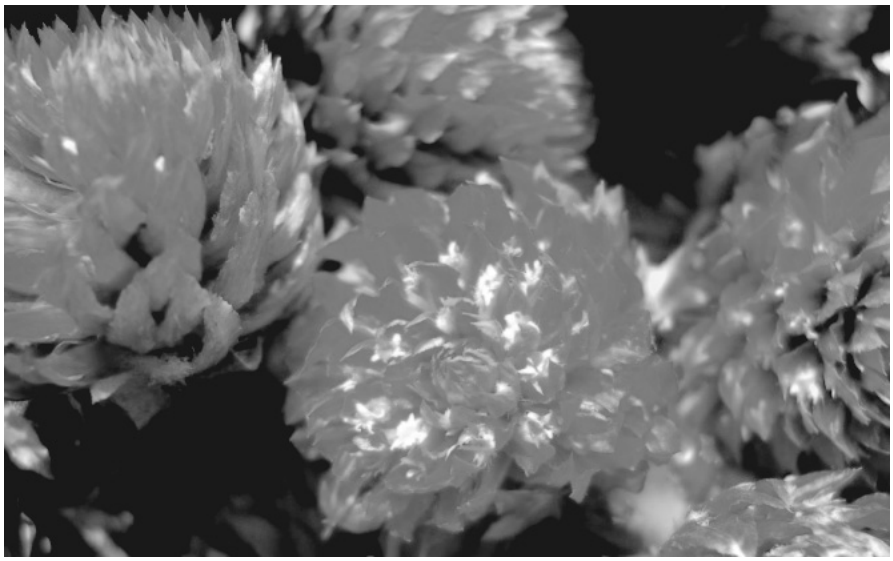

Figure 1. Inflorescences of purple Gomphrena globosa.

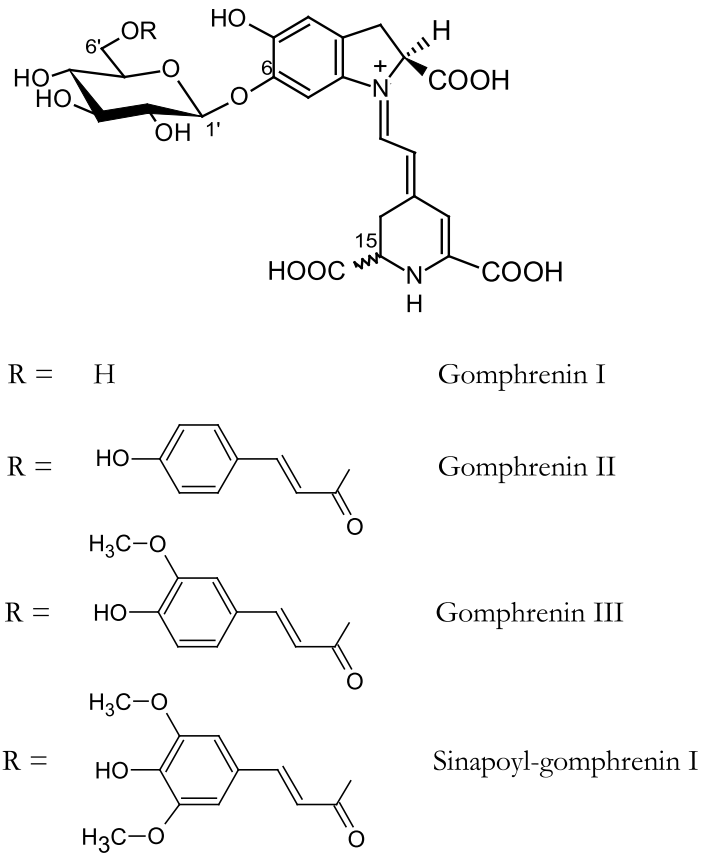

Figure 2. Chemical structures of betacyanins from Gomphrena globosa inflorescences.

Gomphrena globosa is an ornamental plant belonging to *Amaranthaceae* family of *Caryophyllales* order. Within the *Gomphrena* genus, there are several varieties of these plant species. Previous studies proved that the purple petals of their inflorescences contain betacyanin pigments. The first effort in examination of composition of betacyanins in *Gomphrena globosa* inflorescences was conducted in 1967 [5]. The more recent studies proved occurrence of much more varieties of betacyanins. The simplest betacyanin, which was found in the petals of *Gomphrena globosa* is gomphrenin I (betanidin 6-*O*-*β*-glucoside) and its 15*S*-diastereomer (Figure 2). The majority of other *Gomphrena globosa* betacyanins are acylated by hydroxycinnamic acids (ferulic, p-coumaric, sinapic) derivatives of gomphrenin I. [5-7].

The base difficulty of study on these compounds and their afterward exploration is isolation of selected pigments with high purity with efficient purification. For this aim, preparative chromatographic methods are widely explored. However, because of small differences in the structures (Figure 2) and polarities, achievement of appropriate chromatographic peak resolution is troublesome. In general, it requires multistep purification procedures which is uneconomical.

In this study, an attempt to preparatively fractionate betacyanin rich extract of *Gomphrena globosa* petals by chromatography is presented. Obtained results will simplify procedures of the pigments isolation for their further analytical studies.

Materials and Methods

Pigments extraction

Gomphrena globosa inflorescences were ground and subjected to extraction with aqueous solution of 20% acetone (v/v) with addition of ascorbic acid and EDTA. After three-time extraction, the extract was filtered and subsequently concentrated under reduced pressure at 25 °C. Before a step of preparative high performance liquid chromatography (prep-HPLC) fractionation, the concentrated extract was preliminarily purified by low-pressure chromatography on C18 stationary phase. In the next step, the pigment extract was evaporated under reduced pressure at 25 °C in order to concentrate and dispose organic solvents.

Preparative fractionation

A preparative HPLC system with a PUMP 64 (Knauer, Germany) was applied for the fractionation with a gradient: 18% A in B at 0 min; gradient to 19% A in B at 40 min (A, acetone; B, 1% formic acid in water). A semi-preparative column was Bischoff C18 (Bischoff Chromatography, Germany) 250 mm x 30 mm i.d., 10 µm particle i.d., with a 10 mm x 10 mm i.d. guard column with the same mate-

rial. The injection volume was 60 mL and the flow rate 3 mL/min. The detection of signal was performed at 505 nm by a UV-Vis detector (Knauer, Germany). The temperature during chromatographic isolation was ambient. As a result, 14 fractions were collected and analysed by LC-DAD-ESI-MS.

LC-DAD-ESI-MS analysis

The pigments in each fraction were identified and recorded on a Shimadzu LCMS-8030 (Japan) mass spectrometry system by a positive ion electrospray interface. The electrospray voltage was 4.5 kV; temperature of capillary was 250°C and sheath gas was N_2. A Kinetex monolithic column 100 x 4.6 mm i.d. with a guard column (Phenomenex, Torrance, CA, USA) thermostated at 40 °C was used. The injection volume was 40 *m*L and the flow rate was 0.5 mL/min. An applied gradient system was: 15% A in B at 0 min; gradient to 40% B in A at 12 min (A, acetonitrile, B, 2% (v/v) formic acid). The controlling software, LabSolutions LCMS Ver. 5.6 Shimadzu (Japan), was used for mass spectra recording and elaboration.

Results and discussion

The obtained chromatographic fractions contained dominant particular pigments. Therefore, the isolation of a sin-

Table 1. LC-MS data of characteristic ions of pigments from inflorescences of Gomphrena globosa.

No.	Compound[a]	t_R (min)	λ_{max} (nm)	*m/z* $[M+H]^+$
1	betanidin-6-*O*-*β*-glucoside [gomphrenin I]	6.6	539	551
1′	isobetanidin-6-*O*-*β*-glucoside [isogomphrenin I]	7.2	539	551
2	unknown	12.1	-	713
3	betanidin-6-*O*-(6′-*O*-*cis*-4-coumaroyl)-*β*-glucoside [*cis*-isomer of gomphrenin II]	12.8	543	697
3′	isobetanidin-6-*O*-(6′-*O*-*cis*-4-coumaroyl)-*β*-glucoside [*cis*-isomer of isogomphrenin II]	13.3	543	697
4	betanidin-6-*O*-(6′-*O*-*cis*-4-feruloyl)-*β*-glucoside [*cis*-isomer of gomphrenin III]	12.9	544	727
4′	isobetanidin-6-*O*-(6′-*O*-*cis*-4-feruloyl)-*β*-glucoside [*cis*-isomer of isogomphrenin III]	13.3	544	727
5	sinapoyl-gomphrenin I	13.7	-	757
5′	sinapoyl-isogomphrenin I	15.2	-	757
6	betanidin-6-*O*-(6′-*O*-*trans*-4-feruloyl)-*β*-glucoside [gomphrenin III]	14.0	544	727
6′	isobetanidin-6-*O*-(6′-*O*-*trans*-4-feruloyl)-*β*-glucoside [isogomphrenin III]	15.2	544	727
7	betanidin-6-*O*-(6′-*O*-*trans*-4-coumaroyl)-*β*-glucoside [gomphrenin II]	14.1	544	697
7′	isobetanidin-6-*O*-(6′-*O*-*trans*-4-coumaroyl)-*β*-glucoside [isogomphrenin II]	15.0	544	697

[a] tentatively identified based on LC-MS data and [5-7]

Table 2. Distribution of betacyanins in the obtained chromatographic fractions from inflorescences of Gomphrena globosa.

Compound no.	Relative content of pigment in prep-HPLC fraction analysed by LC-DAD-MS													Total peak area
	1	2	3	4	5	6	7	8	9	10	11	12	13	
1	0,92	0,08	-	-	-	-	-	-	-	-	-	-	-	380
1′	0,78	0,22	-	-	-	-	-	-	-	-	-	-	-	410
2	-	-	-	-	-	0,57	0,43	-	-	-	-	-	-	35
3	-	-	-	-	0,17	0,26	0,43	0,13	-	-	-	-	-	230
3′	-	-	-	-	-	-	0,33	0,67	-	-	-	-	-	120
4	-	-	-	0,60	0,40	-	-	-	-	-	-	-	-	100
4′	-	-	-	0,15	0,09	0,59	0,17	-	-	-	-	-	-	230
5	-	-	-	0,16	0,07	0,22	0,52	0,03	-	-	-	-	-	290
5′	-	-	-	-	-	-	0,13	0,26	0,61	-	-	-	-	115
6	-	-	-	-	-	-	0,08	0,74	0,12	0,02	0,01	-	0,02	2025
6′	-	-	-	-	-	-	-	0,02	-	0,66	0,18	0,11	0,03	1420
7	-	-	-	-	-	0,04	-	-	0,75	0,15	0,06	-	-	335
7′	-	-	-	-	-	-	-	-	-	-	0,43	0,43	0,13	230

gular betacyanin pigment was simplified. Table 1 includes the chromatographic and mass spectrometric data of detected pigments. The betacyanins were tentatively identified by a comparison of their detected *m/z* values of protonated molecular ion $[M+H]^+$, retention times and absorption maxima with data from literature. Table 2 presents contents of the pigments in particular fractions based on LC-MS data and chromatograms (Figures 3-4).

In the first fraction, two isomeric pigments of protonated molecular ion $[M+H]^+$ *m/z* 551 were detected, con-

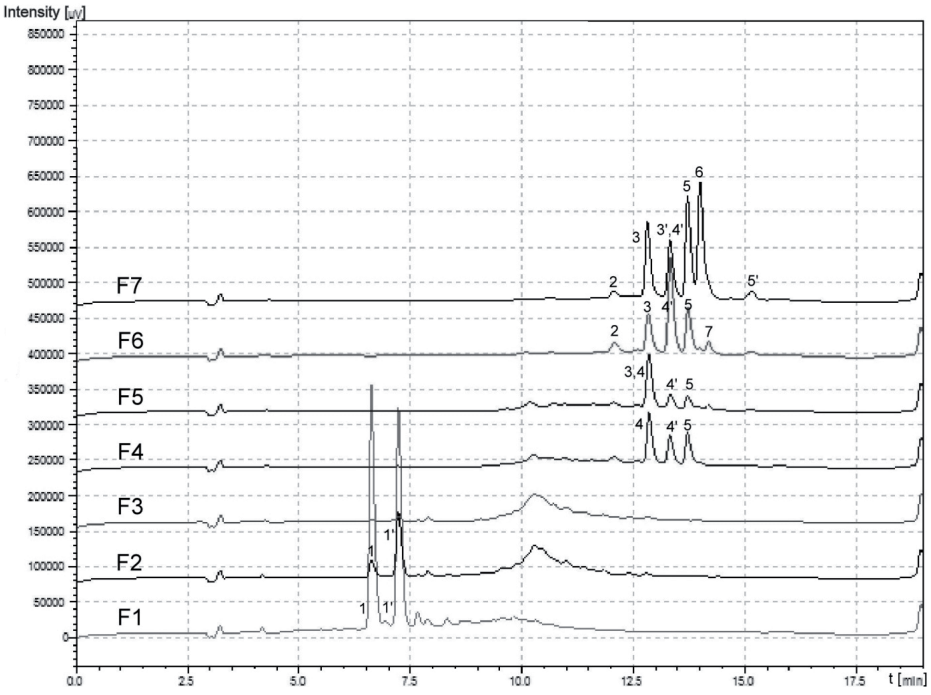

Figure 3. Chromatogram of LC-MS assay of the first 7 fractions from inflorescences of Gomphrena globosa obtained by preparative chromatography

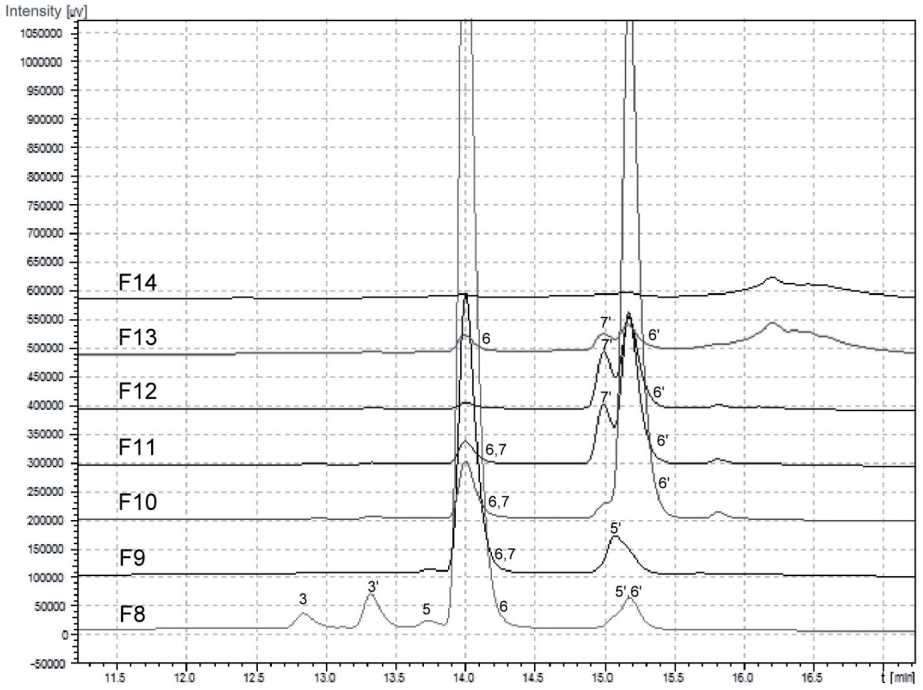

Figure 4. Chromatogram of LC-MS assay of the last 7 fractions from inflorescences of Gomphrena globosa obtained by preparative chromatography

firming the presence of gomphrenin I (**1**) and isogomphrenin I (**1'**). The latter isomer dominates in the 2nd fraction. The subsequent fractions (4-7) are mixtures of several pigments. Due to similarity in the structures of gomphrenin II, gomphrenin III and sinapoyl-gomphrenin I (Figure 2) the separation is not a trivial problem, however, the fractions which contain prevailing amount of one pair of the pigment diastereomers are obtained. For instance, in fraction 4, compounds with $[M+H]^+$ *m/z* 727 are assigned to *cis*-isomer of gomphrenin III (**4**) and *cis*-isomer of isogomphrenin III (**4'**). Moreover, fraction 6 is rich in **4'**, whose peak intensity is as twice higher as the other compounds in the sample.

Fraction 8 is important, because it comprises almost only gomphrenin III (**6**), with negligible contamination by its 15*S*-diastereomer (**6'**) as well as *cis*-isomer of gomphrenin II (**3**), sinapoyl-gomphrenin I (**5**) and their isomers **3'**, **5'** respectively. The fraction 9 is devoid of compounds: **3**, **3'**, **5**. In this fraction a high amount of gomphrenin III (**6**) is found as well, but the pigment is co-eluted with gomphrenin II (**7**), which is undesired. However, in this fraction, the co-elution of compounds **5'** and **6'** is not present, therefore, an isolation of **5'** could be possibly simplified in the further preparative step. Fraction 10 is also relevant, because it is characterised by the highest amount of isogomphrenin III (**6'**) with a minor amount of gomphrenin III (**6**) and gomphrenin II (**7**). In fractions 11-13, beside compounds: **6**, **6'** and **7**, isogomphrenin II (**7'**) is detected.

Interestingly, the results show that separation of *cis*-isomers (F4-F5) from *trans*-isomers (F9-F13) was carried out effortless.

Conclusions

1. The fractionation of *Gomphrena globosa* inflorescence extract was performed successfully. The several fractions containing significant amounts of one dominant betacyanin were obtained. Moreover, the *cis*- and *trans*-isomers were separated.
2. It is worth mentioning that the sample preparation involved only one step of purification and then the extract was directly subjected to separation. Because of similarity of these compound structures, for obtaining higher amounts of the pigments (preparative scale) they usually required multistep purification by means of chromatographic methods which results in high consumption of chromatographic eluents, energy and time. In this study, the preparative chromatography separation was performed only one time.

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