Research on stability of gomphrenin pigments influenced by Cu^{2+} ions

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This paper concerns the results of dehydrogenation of gomphrenin pigments isolated from the purple flowers of *Gomphrena globosa* L. depending on pH and Cu^{2+} activity. Betalains are the plant dyes divided into red-violet betacyanins and yellow-orange betaxanthins. These compounds show pro-health properties proven by numerous studies [1].
Isolation of gomphrenins from the plant material was performed in the following stages: preparative extraction, filtration on a silica layer, preliminary purification and concentration on a bed of strong anion exchanger, preparative chromatographic fractionation and sample liofilization.
The dehydrogenation studies of gomphrenin I and II were carried out in aqueous solutions (pH 3, 5 and 7) catalyzed by Cu^{2+} ions addition. The resulting product mixtures of dehydrogenated and decarboxylated gomphrenin derivatives were monitored by high-performance liquid chromatography with diode-array and mass spectrometric detection (LC-DAD-ESI-MS).
Based on the results, it can be stated that decarboxylated and dehydrogenated gomphrenin II derivatives are more stable than gomphrenin I derivatives, presumably due to acylation of the glucose moiety. The highest levels of gomphrenin I derivatives were observed at pH 7, however, they were mainly monodecarboxylated ones. At pH 3, significant fraction was represented by tridecarboxylated and dehydrogenated derivatives. The lowest yield of the degradation products was indicated at pH 5.

Keywords: Gomphrena globosa L., Betalains, Betacyanins, Gomphrenin, LC-DAD-ESI-MS, Dehydrogenation and decarboxylation

Introduction

Betalains are pigments belonging to the *Caryophyllales* plant order characterized by hydrophilic nature and their greatest stability in slightly acidic pH [1-2].

Due to their chemical structures and biosynthetic pathways, they can be divided into red-violet betacyanins and yellow-orange betaxanthins. Betacyanins are betanidin derivatives – betalamic acid and 3,4-dihydroxyphenylalanine (cyclo-DOPA) adducts, whereas betaxanthins are formed as a result of condensation of α-amino acids or their derivatives with betalamic acid. Betalamic acid is a common building part of all betalains (Figure 1). Their colour intensity is determined by resonating structure with three double bonds creating a chromophoric system – which is called 1,7-diazaheptamethin [2-4]. The main source of betalains, which is used on an industrial scale is beet root (*Beta vulgaris* L.) extract.

Betalains have numerous proven health properties. First of all, they are strong antioxidants, therefore, they scavenge free radicals preventing development of tumours [5]. Moreover, they show anti-lipidemic effects, thereby decrease

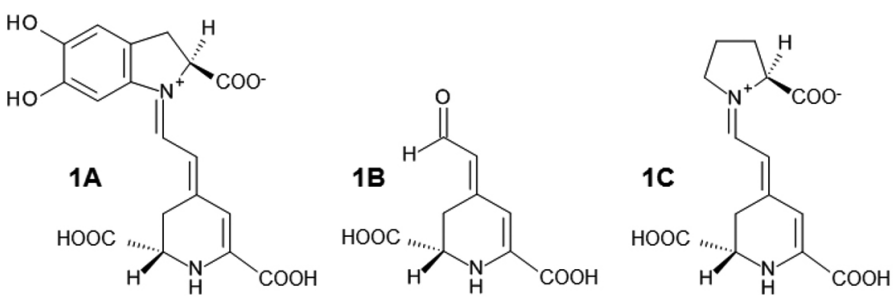

Figure 1. Chemical structures of the simplest betacyanin – betanidin (1A), betaxanthin – indicaxanthin (1C) and their common building part – betalamic acid (1B)

a risk of cardiovascular diseases [6]. Previous preliminary research has shown that they have antimicrobial activity too - betalains have a negative influent on cells functions of the microbes [7].

The main pigments which can be isolated from the purple inflorescences of *Gomphrena globosa* L. are commonly called gomphrenins. Their structures were completely described by nuclear magnetic resonance and mass spectrometry as betanidin-6-*O*-glucoside (gomphrenin I), betanidin -6-*O*-(6'-*O*-coumaroyl)-glucoside (gomphrenin II) and betanidin-6-*O*-(6'-*O*-feruloyl)-glucoside (gomphrenin III). Gomphrenin IV was only tentatively identified as a betanidin-6-*O*-(6'-*O*-sinapoyl)-glucoside, due to its coelution with gomphrenin III [8, 9].

Unfortunately, as most natural products, betalains are relatively labile compounds. Factors like pH, oxygen, light, metals and higher temperature cause their degradation [10]. Especially, heavy metal ions cause decomposition of betalains by their catalytic activity [11].

During purification, isolation and storage, these compounds are vulnerable to an influence of different metals. Due to the fact that gomphrenins can be potentially used in pharmaceutical industry, investigation of their degradation pathways and determination of conditions of their increased stability is demanded. Therefore, determination of pH influence on gomphrenins stability under the catalytic impact of copper ions was the main purpose of this study.

Materials and Methods

Plant material

Dried, violet inflorescences of *Gomphrena globosa* L. were purchased from China in October 2016.

Extract preparation

Extraction of 1 kg of plant material was carried out with 1% aqueous solution of formic acid (v/v). For preliminary purification of the obtained extract, a silica gel column was applied. Afterwards, for further preconcentration, a column filled with a bed of strong anion exchanger Sepra™ ZT-SAX with a 30 µm pore size (Phenomenex, USA) was used. The betacyanins fraction was eluted using 5% formic acid and 50% acetone solution (v/v/v) and was concentrated in a rotary evaporator under reduced pressure.

Semipreparative HPLC fractionation

For isolation of gomphrenin pigments from the extract of *Gomphrena globosa* L., a semipreparative HPLC system with UV-Vis detector was used. The column was a Bischoff C18 (Bischoff Chromatography, Germany), 250 mm x 10 mm, 10µm, with a 10 mm x 10 mm guard column (Phenomenex, USA) under chromatographic system: 80% A in B at 0 min; gradient to 75% A in B at 20 min, gradient to 70% A in B at 30 min, gradient to 65% A in B at 40 min (A – 1% HCOOH in H2O (v/v), B – acetone).The injection volume was 15 ml. The detection was carried out at 505 nm.

In order to obtain a purified gomphrenin I, the following system was used: 92% A in B at 0 min, gradient to 90% A in B at 20 min, gradient to 88% A in B at 30 min, gradient to 86% A in B at 40 min (A – 1% HCOOH in H2O (v/v), B – acetone). The injection volume was 25 ml.

Purified gomphrenin II was obtained using preparative HPLC system under isocratic system: 75% A in B (A – 1% HCOOH in H2O (v/v), B – acetone). The injection volume was 20 ml.

Obtained fractions containing the pigments were concentrated under reduced pressure and subjected to freeze-drying.

LC-DAD-ESI-MS analysis

In order to analyze the collected fractions after semipreparative separations as well as for analysis of the samples after dehydrogenation experiments, high-performance liquid chromatography with diode-array and mass spectrometric detection was used. An LCMS-8030 mass spectrometric system (Shimadzu, Japan) coupled to chromatographic apparatus containing autosampler (SIL-20ACXR), two pumps (LC-20ADXR) and detector with photo diode array model (SPD-M20A) was applied. The MS system equipped with an electrospray ion source was working in positive ion mode (ESI+) at electrospray voltage 4.5 kV. The HPLC column was a 150 mm x 4,6 mm, 5 µm, Kinetex C18 and the samples solutions were pumped through the column under the following gradient system: 95% A in B at 0 min; gradient to 70% A in B at 5 min, gradient to 55% A in B at 12 min, gradient to 5% A in B at 15 min (A – 2% HCOOH in H2O (v/v), B – pure methanol).The column was thermostated at 40 ^{0}C. The injection volume was in the range of 20-50 µl. The detection was performed in the full PDA range and at selected wavelengths (540, 505, 480, 440 and 400 nm). The LC-MS system was controlled with LabSolutions software (Shimadzu), which recorded total ion chromatograms and mass spectra.

Dehydrogenation experiments of gomphrenin pigments

As a source of copper ions, 10 mM $CuSO_4$ aqueous solution was used. Dehydrogenation studies of gomphrenin pigments catalyzed by copper ions was performed in aqueous solutions buffered at pH 3, 5 and 7. Each time, 150 µl aqueous solution of gomphrenin pigments, 20 µl of buffers and 30 µl of a copper sulfate solution were introduced into microplate wells and analyzed by LC-DAD-ESI-MS.

Results and Discussion

Figure 2 presents betacyanins which were detected in the extract before the chromatographic fractionation. Betacyanins were identified based on their chromatographic and spectrometric properties compared with literature data [12,

Table 1. Major betacyanins detected in *Gomphrena globosa* L. extract

No.	Compound (trivial name)	Rt [min]	*m/z* [M+H]+
1	betanidin-6-*O*-*β*-glucoside (gomphrenin I)	7.3	551
1'	isobetanidin 6-*O*-*β*-glucoside (isogomphrenin I)	7.7	551
2	betanidin-6-*O*-(6'-*O*-*cis*-4-coumaroyl)-*β*-glucoside (*cis*-isomer of gomphrenin II)	11.0	697
2'	isobetanidin-6-*O*-(6'-*O*-*cis*-4-coumaroyl)-*β*-glucoside (*cis*-isomer of isogomphrenin II)	11.5	697
3	betanidin 6-*O*-(6'-*O*-*cis*--feruloyl)-*β*-glucoside (cis-isomer of gomphrenin III)	11.0	727
3'	betanidin 6-*O*-(6'-*O*-*cis*--feruloyl)-*β*-glucoside (cis-isomer of isogomphrenin III)	11.5	727
4	sinapoyl-gomphrenin I	11.9	757
4'	sinapoyl-isogomphrenin I	13.2	757
5	betanidin-6-*O*-(6'-*O*-4-coumaroyl)-*β*-glucoside (gomphrenin II)	12.2	697
5'	betanidin 6-*O*-(6'-*O*-feruloyl)-*β*-glucoside (gomphrenin III)	13.1	727
6	isobetanidin-6-*O*-(6'-*O*-4-coumaroyl)-*β*-glucoside (isogomphrenin II)	12.2	697
6'	isobetanidin 6-*O*-(6'-*O*-feruloyl)-*β*-glucoside (isogomphrenin III)	13.3	727

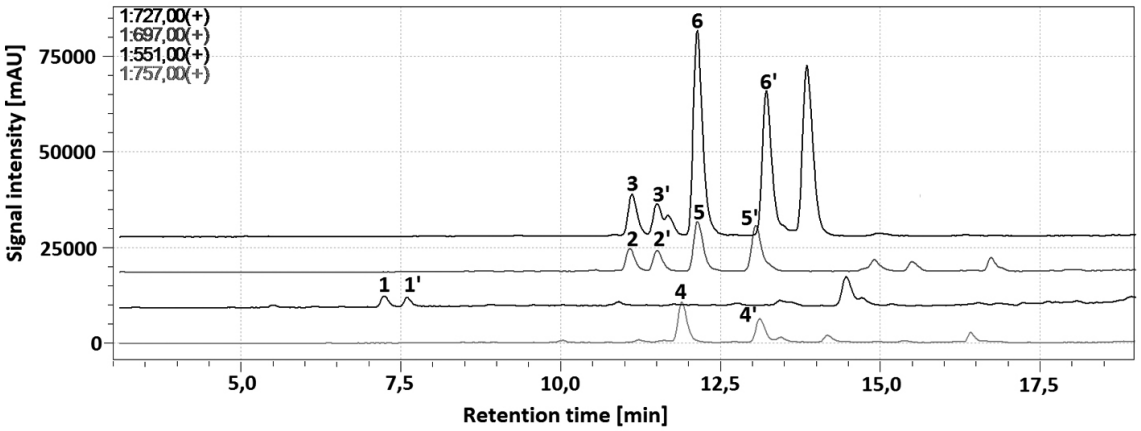

Figure 2. Chromatogram of LC-MS assay (in selected ion monitoring mode) of the *Gomphrena globosa* L. extract

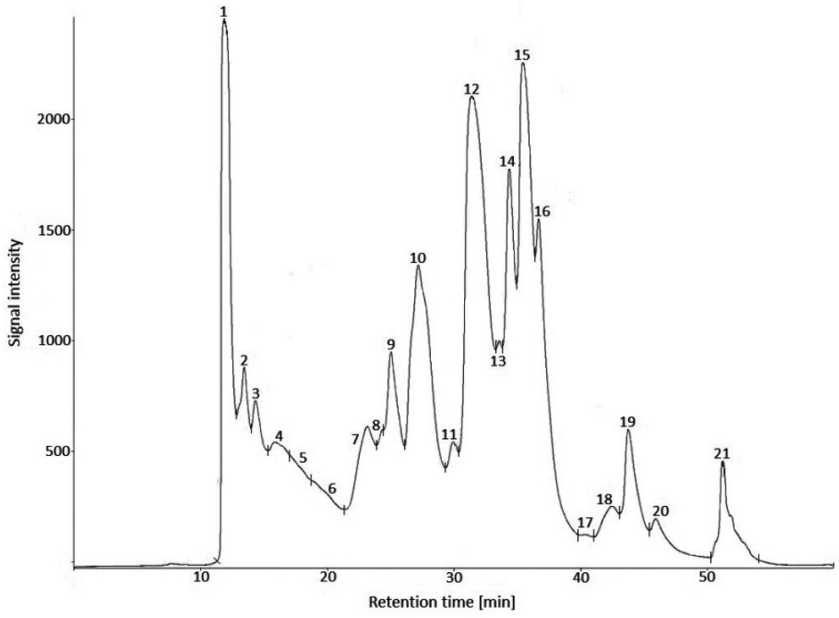

Figure 3. Chromatogram obtained after semipreparative HPLC separation of gomphrenins from the flowers of *Gomphrena globosa* L.

13]. A violet extract of *Gomphrena globosa* L. inflorescences is a rich source of acylated gomphrenins. The significant quantities of gomphrenin III (**6**) and isogomphrenin III (**6'**) with pseudomolecular ions at *m/z* 727 were observed. The first two chromatographic peaks correspond to gomphrenin I (**1**) and isogomphrenin I (**1'**) with *m/z* 551. These compounds are the most hydrophilic, therefore, they are eluted as first (in reversed phase system). Gomphrenin II (**5**) and isogomphrenin II (**5'**) with *m/z* 697 as well as sinapoyl-gomphrenin I (**4**) and sinapoyl-isogomphrenin I (**4'**) with *m/z* 757 were detected in the extract sample, too.

In Figure 3, the chromatogram obtained as a result of semipreparative HPLC is shown. After this separation, 21 fractions were collected. Gomphrenin I with its isomer were present in the first two fractions, gomphrenin II/ isogomphrenin II occurred in the largest amounts in fractions 14/16, respectively and gomphrenin III/ isogomphrenin III were detected mainly in fractions 12/15, respectively.

Gomphrenin I dehydrogenation

As a result of performed experiments, a mixture of decarboxylated and dehydrogenated gomphrenin I derivatives was formed. Gomphrenin derivatives were identified based on their chromatographic and spectrometric properties compared with previous oxidation gomphrenin studies [14]. Due to relatively small signal intensities, it was not possible to define absorption maxima, thus obtained data are only identified tentatively.

Table 2. Gomphrenin I oxidized derivatives

m/z $[M+H]^+$	Compound[a]
549	Neogomphrenin
507	17-decarboxy-gomphrenin
505	17-decarboxy-neogomphrenin
461	2,17-bidecarboxy-neogomphrenin
459	2,17-bidecarboxy-2,3-dehydro-neogomphrenin
417	2,15,17-tridecarboxy-neogomphrenin
415	2,15,17-tridecarboxy-2,3-dehydro-neogomphrenin

[a] tentatively identified

Table 3. Normalized concentration of gomphrenin I derivatives depending on pH formed under copper ions assisted oxidation of gomphrenin I

m/z $[M+H]^+$	Normalized concentration [%]		
	pH 3	pH 5	pH 7
415	3.7	4.6	3.1
417	10.1	9.1	2.7
459	11.0	13.1	7.6
461	4.7	5.5	4.6
505	11.1	14.6	11.9
507	59.5	53.1	51.2
549	0.0	0.0	18.8
Total HPLC peak area $[AU \cdot min \cdot 10^6]$	16.9	13.7	18.3

The main dehydrogenated gomphrenin derivative is neogomphrenin, which results from oxidation of carbons C-14,15 and forming the pyridinic system (Figure 4). Decarboxylated derivatives arise as a result of a division of the bond at carbons C-2,15,17. Moreover, additional dehydrogenation at carbon C-2,3 is possible.

Normalized concentrations of gomphrenin I derivatives in the samples depending on pH are presented in Table 3 and absolute signal values are depicted in Figure 5.

Based on the results, it can be stated that neogomphrenin was formed only at pH 7. In all the samples, the highest concentration was observed for 17-decarboxy-gomphrenin (*m/z* 507) and the lowest one for 2,15,17-tridecarboxy-2,3-dehydro-neogomphrenin (*m/z* 415). The concentration of gomphrenin degradation products at different pH value solutions decrease with the increase of their decarboxylation level. The highest levels of gomphrenin I derivatives can be observed at pH 7 and they are mainly monodecarboxylated and dehydrogenated. The lowest amounts of tridecarboxylated betacyanins and corresponding dehydrogenated ones were also observed. In general, the lowest concentrations of derivatives were detected in the sample at pH 5, which confirms previous research pointing to the greatest betacyanins stability in the weak acidic pH [15].

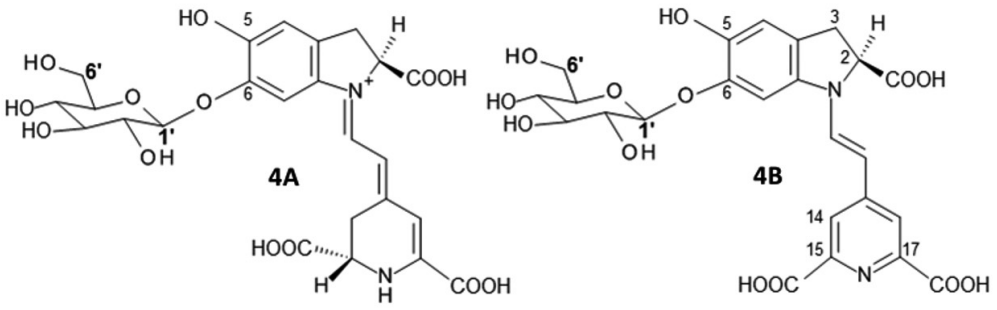

Figure 4. Chemical structures of gomphrenin I (4A) and neogomphrenin (4B)

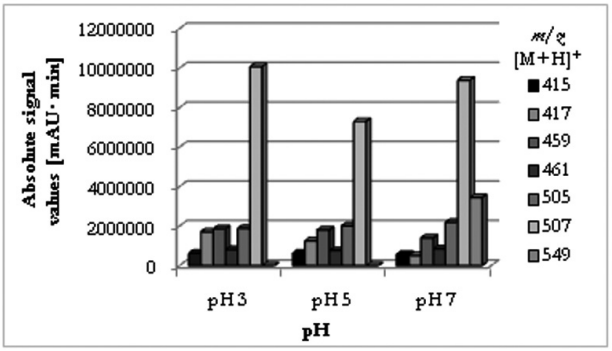

Figure 5. Chromatographic MS signal values of gomphrenin I derivatives formed under copper ions assisted oxidation of gomphrenin I

Table 4. Gomphrenin II oxidized derivatives

m/z $[M+H]^+$	Compound
695	Neogomphrenin II
653	17-decarboxy-gomphrenin II
651	17-decarboxy-neogomphrenin II
605	2,17-bidecarboxy-2,3-dehydro-neogomphrenin II

Table 5. Normalized concentration of gomphrenin II derivatives depending on pH formed under catalytic oxidation of gomphrenin II by copper ions

m/z $[M+H]^+$	Normalized concentration [%]		
	pH 3	pH 5	pH 7
605	7.4	16.7	0.0
651	20.5	29.1	10.6
653	72.1	54.2	18.1
695	0.0	0.0	71.3
Total HPLC peak area [AU·min·10^6]	4.1	1.8	15.3

Gomphrenin II dehydrogenation

Gomphrenin II derivatives generated as a result of oxidation are presented in Table 4. Gomphrenin II is a hydroxycinnamic acid derivative (coumaroyl) as a result of acylation of the glucose moiety in gomphrenin I. Its dehydrogenation presumably occurs in the same way as for gomphrenin I (Figure 6).

Based on the degradation results of gomphrenin II, significantly lower amounts of decarboxylated and dehydrogenated derivatives are generated. Interestingly, no tridecarboxylated derivatives were noticed at any applied pH. Similarly to neogomphrenin I neogomphrenin II (*m/z* 695) formed only in the sample at pH 7 but in the largest amount.

The 2,17-bidecarboxy-2,3-dehydro-neogomphrenin II (*m/z* 605) was formed but at low concentration levels only in the samples at pH 3 and 5 (Table 5). In that case, the sample at pH 5 is characterized by the lowest concentration of betacyanins, too. As in the case of gomphrenin I, most

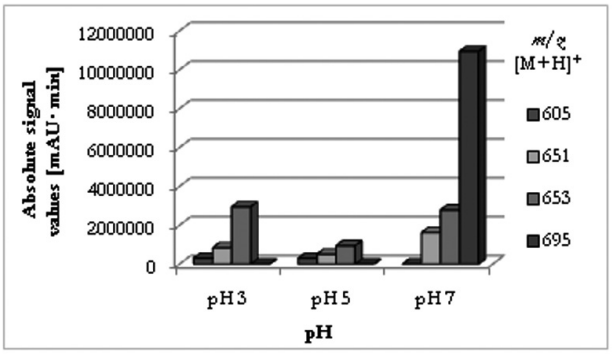

Figure 7. Chromatographic MS signal values of gomphrenin II derivatives formed under catalytic oxidation of gomphrenin II by copper ions

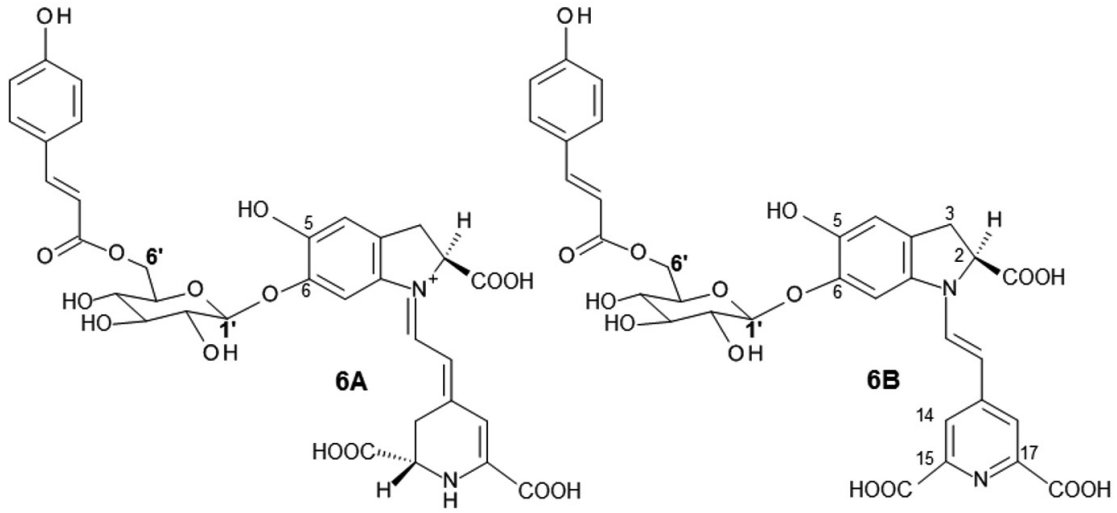

Figure 6. Structural patterns of gomphrenin II (6A) and neogomphrenin II (6B)

of betacyanins were formed with dominating neogomphrenin II at pH 7. In contrast, at pH 3 and 5, the monodecarboxylated derivatives dominate.

Conclusions

Gomphrenin pigments show the greatest stability against catalytic oxidation at pH 5. Both decreasing and increasing the pH causes the formation of larger amounts of decarboxylated and dehydrogenated derivatives. At pH 3, a significant fraction of gomphrenin I derivatives with higher decarboxylation and dehydrogenation levels was detected, while at pH 7, there is the highest amount of the mono-derivatives. Acylated gomphrenins are characterized by greater stability against catalytic oxidizing than gomphrenin I, therefore, they seem to be better suited for the use in the food industry.

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