Research on dehydrogenation of gomphrenin in heated ammonia solutions of organic solvents

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In this report, the influence of ammonia on dehydrogenation of gomphrenin pigment, isolated from purple inflorescences of Gomphrena globosa L., in selected organic solvents (methanol, ethanol, acetone and acetonitrile) at 40°C is presented. Betacyanins are water-soluble, vacuolar plant pigments. Due to their numerous pro-health properties, they can be applied in the pharmaceutical industry and as food colorants. However, as most of natural products, they are less stable than synthetic dyes, therefore, further studies need to be carried out on their stability, but also on determination of their degradation products as well as degradation mechanisms. For the experiments of the ammonia treatment, prolonged isolation of gomphrenin from the plant material including extraction, preliminary purification, preparative HPLC fractionation, concentration and liofilization were performed. The tested pigment dissolved in organic solvents was treated by ammonia and the samples for analysis were taken during and after reaction.
For identification of obtained derivatives of gomphrenin, high-performance liquid chromatography with diode-array and mass spectrometric detection (LC-DAD-ESI-MS) was performed.
As a result of heat processing, the mixture of decarboxylated and dehydrogenated gomphrenin derivatives was detected.
Based on the experimental results, it can be stated that gomphrenin is characterized by the greatest stability in methanolic solutions while it is the most labile in the acetonitrilic ones.

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

Introduction

Betalains are hydrophilic, vacuolar plant pigments occurring in most families of *Caryophyllales* order. Violet betacyanins and yellow betaxanthins are two groups of betalains formed in similar biosynthetic pathways. Betacyanins are *O*-glycoside betanidin derivatives. This substitution can occur at the C-5 or C-6 position. While betacyanins with C-5 substitution are common compounds, betacyanins with C-6 substitution have been discovered only in *Gomphrena globosa*, *Basella rubra* and *Bougainvillea glabra* species [1].

Due to their numerous health benefits (such as anticancer properties, antimicrobial activity, antilipidemic effects), they can be used as food colorants and in the pharmaceutical industry [2-4]. However, as most of natural products, they are less stable than synthetic dyes, therefore, determination of the factors causing their degradation is of great importance. Decarboxylated and dehydrogenated betacyanins are formed as a result of their degradation, e.g., under the influence of thermal treatment and oxidation [5].

Temperature is the most important factor affecting betalains stability during storage and food processing. While heating, betanin decay as a result of isomerisation, decarboxylation as well as hydrolysis, which eventually lead to occurrence of bright brown colour. Betanin can be recreated from its main degradation products while extracts are kept in the low temperature below 10°C. Betanin regeneration concerns the condensation of amine moiety forming a part of cyclo-DOPA-5-*O*-glucoside and an aldehyde moiety of betalamic acid [6]. After prolonged heat processing of betalains solutions, a huge variety of degradation products is generated for example, mono-, bi- and tri-decarboxylated compounds and as a final result, mixtures of dehydrogenated and decarboxylated products are generated. Such complicated profiles were detected in purified extracts obtained from red beet root and violet pitaya [5, 7]. Despite yellow colour, neobetacyanins (14,15-dehydrobetacyanins) structurally are similar to betacyanins, therefore, dehydrogenation of betacyanins and its mechanism is of our interest [5]. In food industry, it is well recognized that products which are not obtained from natural sources are less demanded by the customers, therefore, searching for dyes which are received from natural sources increases. Additionally, studies concerning improvement of their chemical stability are carried out.

Organic solvents are commonly used for extraction of betacyanins from the plants, therefore, it is essential to determine and understand how these solvents influence on betalains stability. In this study, we determined the effect of the solvents and ammonia addition on stability of gomphrenin.

Materials and Methods

Plant material

Dried, violet inflorescences of *Gomphrena globosa* L. were purchased from China market.

Extract preparation and purification

Extract preparation for the experiments included the following stages. In order to obtain gomphrenin pigments, 1 kg of dried, violet *Gomphrena globosa* L. inflorescences was extracted in 1% aqueous solution of formic acid (v/v) and filtered due to high turbidity of the obtained extract. For this purpose, a Buchner funnel with a paper filter and a 3 cm layer of a silica gel was used. Column filled with a bed of strong anion exchanger Sepra™ ZT-SAX with a 30 µm pore size (Phenomenex, USA) was used for further extract purification and concentration. Adsorbed compounds were eluted using 5% formic acid and 50% acetone solution (v/v/v). Obtained eluate was concentrated in a rotary evaporator under reduced pressure.

Semipreparative HPLC fractionation

In order to pigment isolation from the obtained extract, a semipreparative HPLC system with UV-Vis detector was used. The column filled with a Bischoff C18 stationary phase (Bischoff Chromatography, Germany), 250 mm x 30 mm, 10 µm, with a 10 mm x 10 mm guard column (Phenomenex, USA) was used. The following gradient system was used: 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 of signal was carried out at 505 nm.

Purified gomphrenin I pigment was obtained using preparative HPLC system: 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.

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

LC-DAD-ESI-MS analysis

The collected fractions after semipreparative separations as well as after thermal treatment experiments in organic solvents, were analyzed by an LCMS-8030 mass spectrometric system (Shimadzu, Japan) coupled to a liquid chromatograph composed of autosampler (SIL-20ACXR), two pumps (LC-20ADXR) and detector with photo-diode array model (SPD-M20A). The MS system equipped with an electrospray ion source was working in positive ion mode (ESI+) at an electrospray voltage 4.5 kV. The column was a Kinetex C18 150 mm x 4,6 mm, 5 µm, and the sample solutions were separated in 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 20 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.

Experiments on dehydrogenation of gomphrenin

Gomphrenin samples were dissolved in methanolic, ethanolic, acetonic and acetonitrilic aqueous solutions (90:10, v/v). To each sample, 50 µl of ammonia was added in order to increase pH value. The samples were heated for 5, 10 and 30 min at 40 ^{0}C, respectively. Immediately after sampling of the mixtures, the formic acid was added to neutralize the ammonia in the taken samples. All of the samples were concentrated to 50-100 µl and diluted with water to 500 µl. Afterwards, they were analyzed by LC-DAD-ESI-MS.

Results and Discussion

As a result of performed experiments, a mixture of decarboxylated and dehydrogenated gomphrenin derivatives was obtained. Their chemical structures are shown in Figure 1.

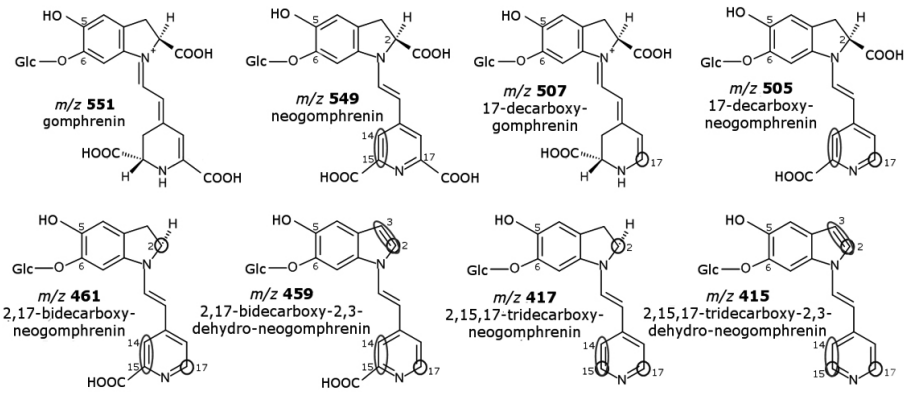

Figure 1. Chemical structures of gomphrenin and decarboxylated and dehydrogenated gomphrenin derivatives

According to previous studies, dehydrogenation can occur at carbons C-14,15 which results in formation of pyridinic system but also C-2,3 creating additional dehydrogenated derivatives, while decarboxylation can take place starting from carbons C-2,17 [8].

Thermal degradation of gomphrenin pigment in methanolic (MeOH) ammonia solution

In Table 1, normalized concentrations of gomphrenin derivatives were shown which were formed as a result of gomphrenin heating in ammonia solution of methanol.

The absolute signal values for generated derivatives during the thermal treatment are presented in Figure 2. Based on the results, it can be observed that the highest signal intensities were obtained for gomphrenin derivatives after 10 min of heating (Table 1). Among all of formed dehydrogenated and decarboxylated compounds, 17-decarboxy-gomphrenin (*m/z* 507) is observed in the largest amounts in each sample. However, its content in the samples plummets with the increase of heating time. After 30 min of thermal treatment, only a half content of 17-decarboxy-gomphrenin remains in relation to its content obtained after 5 min of heating. In each sample, the lowest quantities of 17-decarboxy-neogomphrenin (*m/z* 505) are formed. Neogomphrenin (*m/z* 549) is present at low levels as well, however, its content grows slightly during the heating.

Worth noting is that the prolonged heating causes formation of derivatives, which had been tetradehydrogenated, i.e., dehydrogenated neogomphrenin (*m/z* 415) as well as 2,17-bidecarboxy-2,3-dehydro-neogomphrenin (*m/z* 459).

Thermal degradation of gomphrenin pigment in ethanolic (EtOH) ammonia solution

The normalized concentrations of gomphrenin derivatives, which were formed as a result of gomphrenin heating in ethanolic ammonia solution were shown in Table 2.

The absolute signal values for generated derivatives during the thermal treatment were presented in Figure 3. As a result of gomphrenin heating in ethanolic solution, the greatest amounts of degradation products were obtained after 5 min (Table 2). In this case, the lowest content of 17-decarboxy-gomphrenin (*m/z* 507) is observed only after 5 min of heating. During the thermal treatment, its content declines significantly and after 30 min of heating,

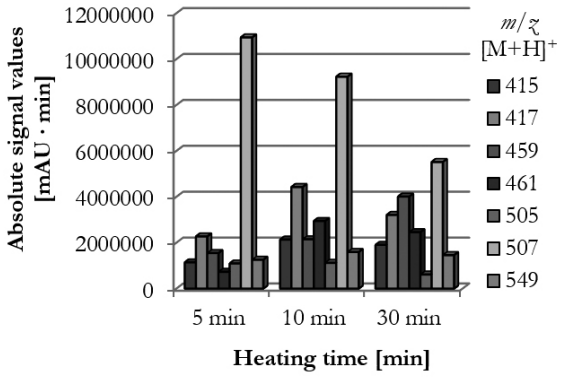

Figure 2. Betacyanins profiles in the samples submitted to heating at 40°C in MeOH

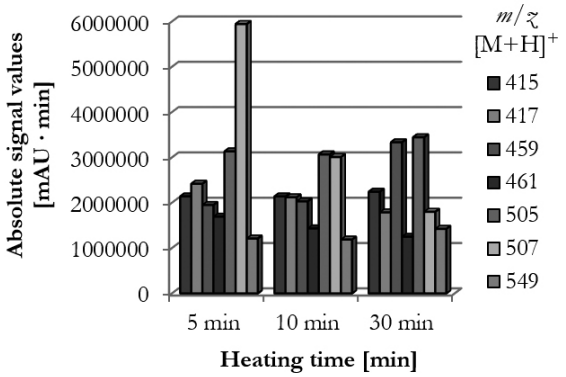

Figure 3. Betacyanins profiles in the samples submitted to heating at 40°C in EtOH

Table 1. Normalized concentration [%] and the total peak area of gomphrenin derivatives in the samples after heating experiments in methanol

m/z $[M+H]^+$	Normalized concentration [%]		
	5 min	10 min	30 min
415	6.0	9.0	9.9
417	12.0	18.8	16.7
459	8.2	9.1	20.9
461	3.8	12.5	12.8
505	5.8	4.8	3.2
507	57.6	39.1	28.8
549	6.6	6.7	7.6
Total HPLC peak area [AU·min·10^6]	19.0	23.6	19.2

Table 2. Normalized concentration [%] and the total peak area of gomphrenin derivatives in the samples after heating experiments in ethanol

m/z $[M+H]^+$	Normalized concentration [%]		
	5 min	10 min	30 min
415	11.6	14.3	14.7
417	13.1	14.1	11.7
459	10.5	13.5	21.8
461	9.2	9.6	8.2
505	16.9	20.5	22.5
507	32.1	20.1	11.8
549	6.5	7.9	9.3
Total HPLC peak area [AU·min·10^6]	18.0	15.3	13.9

merely about one third of 17-decarboxy-gomphrenin remains in relation to its content obtained after 5 min of heating. Based on Table 2, it can be noticed that neogomphrenin (*m/z* 549) arises slowly. Interestingly, normalized concentrations of decarboxylated gomphrenin derivatives (*m/z* 461 and *m/z* 417) reach the highest levels after 10 min of heating, but concentrations of their corresponding dehydrogenated derivatives (*m/z* 459 and *m/z* 415, respectively) increase constantly during the heating time.

In reference to foregoing observation it can be stated that gomphrenin shows lower stability in ammonia solution of ethanol than in the methanol solution.

Thermal degradation of gomphrenin pigment in acetonic (Ac) ammonia solution

In Table 3, normalized concentrations of gomphrenin derivatives were depicted, which were formed as a result of gomphrenin heating in acetonic ammonia solution.

The absolute signal values for generated derivatives during the thermal treatment were presented in Figure 4. Performed analysis proved that after 5 min heating of gomphrenin in the acetonic solutions the highest levels of derivatives are obtained, which is very similar to the results obtained for ethanol. Surprisingly, 2,15,17-tridecarboxy-neogomphrenin (*m/z* 417) is observed in the largest amounts, reaching the highest level after 10 min of heating (Table 3). Another derivative present in the large amounts is 17-decarboxy-neogomphrenin (*m/z* 505). In each sample, neogomphrenin (*m/z* 549) levels remain steady during the increase of heating time. Worth noting is that derivative concentrations after 10 and 30 min are very similar for most generated compounds. The only exception is observed for 2,15-bidecarboxy-neogomphrenin (*m/z* 461) whose content rises slowly during the heating.

In the light of the obtained results, it can be stated that the solvent causes gomphrenin degradation in higher extent resulting in generation of large amounts of tridecarboxy-derivatives at the beginning of the heating experiment.

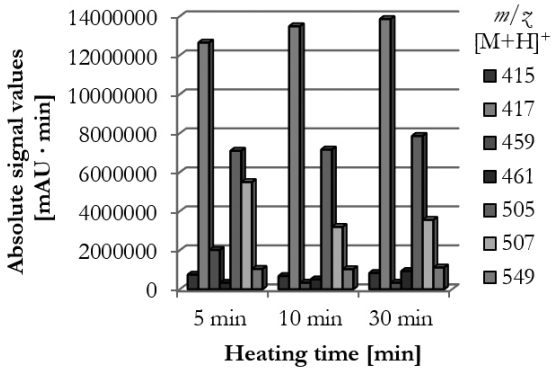

Figure 4. Betacyanins profiles in the samples submitted to heating at 40°C in Ac

Table 3. Normalized concentration [%] and the total peak area of gomphrenin derivatives in the samples after heating experiments in acetone

m/z [M+H]+	Normalized concentration [%]		
	5 min	10 min	30 min
415	2.5	2.6	2.9
417	43.1	51.1	48.6
459	6.8	1.3	1.2
461	1.1	1.9	3.3
505	24.2	27.2	27.6
507	18.7	12.1	12.5
549	3.5	3.9	3.9
Total HPLC peak area [AU·min·10^6]	29.4	26.4	28.5

Thermal degradation of gomphrenin pigment in acetonitrilic (ACN) ammonia solution

In Table 4, the normalized concentrations of gomphrenin derivatives were shown, which were obtained as a result of gomphrenin heating in acetonitrilic ammonia solution.

The absolute signal values for generated derivatives during the thermal treatment were presented in Figure 5. Heating of acetonitrilic solutions resulted in formation of only four gomphrenin derivatives which are mono- and

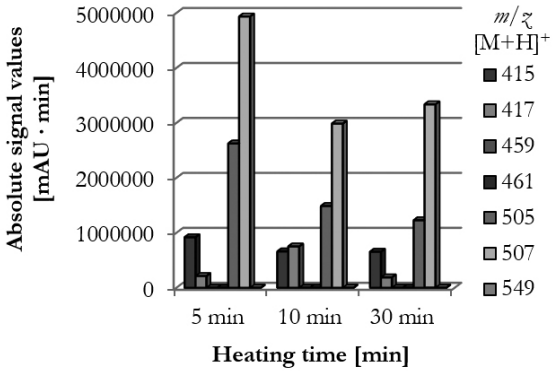

Figure 5. Betacyanins profiles in the samples submitted to heating at 40°C in ACN

Table 4. Normalized concentration [%] and the total peak area of gomphrenin derivatives in the samples after heating experiments in acetonitrile

m/z [M+H]+	Normalized concentration [%]		
	5 min	10 min	30 min
415	10.6	11.2	12.1
417	2.5	12.7	3.5
505	30.2	25.3	22.7
507	56.8	50.8	61.7
Total HPLC peak area [AU·min·10^6]	8.7	5.9	5.4

tridecarboxylated compounds but no bidecarboxylated compounds were observed. The compound detected in the largest amount was 17-decarboxy-gomphrenin (*m/z* 507) (Table 4). At the beginning of the heating test, significant concentrations of monodecarboxylated gomphrenin derivatives (*m/z* 507) with corresponding dehydrogenated compound (*m/z* 505) were observed. During the prolonged heating, the concentration of tridecarboxylated compounds (*m/z* 415 and 417) increases slightly and for compound of *m/z* 417 reaches the peak after 10 min of the test and then declines fast.

It can be concluded, that among all used solvents, acetonitrile degrades gomphrenin at the highest extent. The least variety of formed derivatives is caused presumably by quick conversion to the betacyanins final degradation product – betalamic acid.

Conclusions

In all the tested solvents, the yields of generated 2,17-bidecarboxy-gomphrenin (*m/z* 461) and 2,15,17-tridecarboxy-gomphrenin (*m/z* 417) as well as their corresponding dehydrogenated derivatives are increased during the heating of the ammonia solutions. At the same time, the concentration levels of preliminarily formed monodecarboxylated derivatives are decreasing due to their further reactions. The highest and lowest dehydrogenation rates were observed for acetonitrilic and methanolic solutions, respectively.

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