Several important factors, such as pH, exposure to light, oxygen or temperature affect significantly the betalain stability. In particular, the heavy metal ions exert negative effect on stability of betalain colorants and accelerate pigment decomposition. Trace amounts of metal ions may be present in the food products, as well as in food packaging, leading to undesirable colour changes or even discoloration of betalainic foodstuffs. Decarboxylated betalains, such as 2-decarboxy-betanin, generated, e.g. during betalain thermal treatment, may exhibit greater stability than parent pigments. Moreover, obtained derivatives retain attractive color, making them the promising material for study of pigment stability. An effect of Cu²⁺ cations as the most degradative metal ions on 2-decarboxy-betanin stability was investigated in ethanolic and methanolic solutions. The increase of the concentration of copper cations has a negative impact on stability of 2-decarboxy-betanin. The cations of Cu (II) in the presence of organic solvents induce the pigment degradation, while the increase of the concentration of organic solvents enhances the pigment decomposition. The main degradation products of the 2-decarboxy-betanin are compounds possessing absorption maxima at λmax ca. 430 nm. These studies allow obtaining the information needed for proper isolation and treatment of pigments, as well as proper storage of products containing betalains.

**Key words:** betacyanins, 2-decarboxy-betanin, copper ions, betalain stability, Beta vulgaris L.

Structurally, 2-decarboxy-betanin pigment (Fig. 1) belongs to derivatives of red-violet betacyanins which represent a class of nitrogenous betalain plant colorants [1]. They provide colour of many flowers, fruits, as well as leaves, stems, or roots. Betalain occur in some plant families, belonging mainly to the order Caryophyllales and also to higher fungi of genera Basidiomycetes. The most common source of betalain colorant, especially in Europe and North America, is red beet root (Beta vulgaris L.) [2]. Besides of red-violet betacyanins, betalains include yellow-orange betaxanthins. Due to their lower stability, most studies are focused on reddish pigment group. The principal betacyanin in red beet root is betanin (Fig. 1), which is 5-O-glucosides of betanidin (the basic chromophoric aglycone unit). Betanin colourant (E-162) is used in food industry and is produced by vacuum evaporation of red beet juice or spray-drying of betanin concentrate. Moreover, betalains are diet supplements, exhibiting strong antioxidant properties, as well as chemopreventive and anti-inflammatory effect [2].

Decarboxylated betalains, such as 2-decarboxy-betanin, are generated, e.g. during betalain thermal treatment. The studies on thermal degradation of betacyanins from red beet or purple pitaya (Hylocereus polyrhizus), provide information about different decarboxylation and dehydrogenation levels of formed derivatives [1]. The major decarboxylated compounds include 2-decarboxy-betacyanins, 17-decarboxy-betacyanins, 2,17-bidecarboxybetacyanins and 2,15,17-tridecarboxy-betacyanins. They may exhibit greater stability and retain attractive color, making them the promising material for study of pigment stability.

Several important factors, such as pH, exposure to light, oxygen or elevated temperature deteriorate significantly betalain stability. In particular, the heavy metal ions exert negative effect on stability of betalain colorants and accelerate pigment decomposition [3]. Metal ions may be present in the food products, as well as in food packaging, leading to undesirable colour changes or even discoloration of betalainic foodstuffs. Hermetically closed cans with preserved food contain metals such as iron, chromium, tin, copper or aluminum, which may diminish half-lives of betalains. Mainly oxidation of the pigments or their hydrolysis products is cause of their degradation by metal ions [4-6]. It was proved that the Cu²⁺ ions enhanced the oxygen...
uptake by betanin. However, for Zn^{2+} and Al^{3+} ions contradictory findings were obtained [7]. The popular chelating agent, EDTA (ethylenediaminetetraacetic acid), reduces the betanin oxygen uptake. It possesses the ability to prevent metal-catalyzed betanin degradation by its stabilization, as well as complex formation with the metal cations [4-6]. Moreover, trace amounts of different metal can be also present in tested samples upon chromatographic isolations of betalains from plant sources or during their analyses in aqueous-organic solutions, such as methanol, acetonitrile or acetone. These solvents are not applied in food industry, but are frequent used in studies on the pigments in analytical and organic chemistry [3]. Previous studies reported that among metals, Cu^{2+} cations are the most degradative. Furthermore the possibility of a complex formation between betanin and Cu^{2+} as well as Ni^{2+} at pH 7-8 was also stated [3]. The occurrence of complexes at a first stage of the pigment degradation may be helpful in clarification of the betalain oxidation mechanism. Elucidation of this process is crucial due to not fully known antioxidant and chemopreventive activities of betalains.

In order to determine the stability of 2-decarboxy-betanin, affected by different concentrations of copper ions, a set of degradation experiments was performed, including the influence of pH (3-8) and organic solvents, such as ethanol and methanol.

**Material and methods**

The spectrophotometric measurements of tested solutions of 2-decarboxy-betanin were monitored by registration of UV-Vis spectra in a microplate reader Inifnite M200 (TECAN, Austria). Experiments were carried out in 96-well polystyrene microplates, consisting of eight rows (A to H) and 12 columns (1 through 12).

On the first stage of the study, an effect of Cu^{2+} cations at different concentrations on the stability of 2-dBt in ethanolic and methanolic solutions was studied.

Each time, 20 μl of pigment, 20 μl of buffer and appropriate volumes of water and organic solvent were added to obtain the desired concentrations. Moreover, in columns 1 through 6, the 10 μl of copper sulphate solution (6.5 μM) were sequentially introduced into the wells of the microplate. Additionally, in columns 7 through 10, the 10 μl of Cu^{2+} (650 μM) was added. In rows A to H, concentrations of organic solvents differed from 0 to 80% (v/v) (0, 15, 25, 40, 50, 60, 70, 80% (v/v)). In the experiment, acetonitrile (3-5.5) and phosphate (5.5-8) buffers were used.

In each case, the microplates were inserted to the microplate reader for 24-hour spectrophotometric monitoring of the UV-Vis spectra at 25 °C in a wide range of the visible spectrum (350-550 nm). After each experiment, 200 μl samples were collected and submitted to solvent evaporation in a lyophilizer and then were freeze-dried and dissolved in 200 μl of demineralized water for HPLC-DAD analysis. HPLC analysis was performed with a Gynkotek HPLC system with UVD340U, Gynkotek HPLC pump Series P580 and thermostat (Gynkotek Separations, H.I. Ambacht, the Netherlands). The analytical column was a Luna C-18(2) 250x3 mm I.D., 5 μm (Phenomenex, Torrance, CA, USA). The following gradient system was used: 3% A in B at 0 min, 16% A in B at 17 min and a gradient to 50% A in B at 30 min (A, acetonitrile; B, 2% formic acid in water). The injection volume was applied 10 μL at the flow rate of 0.5 mL/min. Detection was carry out with a DAD (diode array detection) system at 538, 505, 480 and 310 nm, respectively. The column was thermostated at 35 °C.

**Results and Discussion**

Spectrophotometric analyses enable determination of 2-decarboxy-betanin stability in different tested solutions. The experiment was carried out at broad pH range 3-8 in selected aqueous-organic (methanol and ethanolic) solvents at differentiated copper cations concentrations. Betacyanin derivative, such as 2-dBt possess absorption maximum at λ_{max} 533 nm. Obtained data allowed to observation of changes during reaction of pigment with copper, based on arising of new or shifted absorption bands. Recent studies reported that oxidation of betacyanins leads to formation of decarboxylated and dehydrogenated betacyanins [3]. Metal ion-catalysed decomposition of betacyanins, leading to their oxidation, also results in a formation of such derivatives. Therefore, the presence of new absorption bands indicates the creation of betacyanin derivatives in their visible spectra in the range of 390-500 nm.

**Comparison of the spectra profiles obtained for the methanolic and ethanolic solutions without copper cations**

Spectra profiles obtained without copper cations addition for each of the organic solvent were compared to determine the stability of 2-dBt in the solutions. The results obtained after 2, 5 and 20 hours of the experiment were compared (Fig. 2).

After 2 h, the tested pigment features high stability in the whole pH range, except of methanol solutions at pH 3, where slight decrease of absorbance is noticed. After 10 h, the increase of organic solvents concentration induces 2-dBt degradation, especially at pH 3 and 8. In 45% (v/v) and 60% (v/v) solutions, increased pigment decomposition at above mentioned pH is also observed. The hypochromic shift of absorption maxima λ_{max} at these conditions to ca. 450 nm is noticed, indicating a generation of 2-dBt derivatives. A similar trend is observed for the final stage of the analysis. After 20 h of the experiment, the presence of new absorption bands at λ_{max} ca. 450 nm at pH 3 in 60% (v/v) methanol is detected (Fig. 2).
Fig. 2. Spectra of 2-decarboxy-betanin in aqueous-organic solutions (methanol, ethanol) after 20 h of incubation
Determination of Cu (II) concentration influence on 2-dBt degradation in methanolic and ethanolic solutions

The next step of presented study was determination of the effect of different concentrations of Cu (II) ions on degradation of 2-decarboxy-betanin in methanolic and ethanolic solutions. The results, obtained after 20 hours of reaction in both solvents are shown in Figs. 3-4.

Higher concentration of copper ions (650 μM) results in a rapid degradation of 2-dBt. However, at low concentration (6,5 μM) the pigment is relatively stable – a slight decrease of absorbance at pH 3 for all concentrations of the solvents is visible. For 650 μM Cu²⁺, the significant changes in the spectra, even at 15% (v/v) concentrations of organic solvents and considerable absorbance decrease is noticed. Absorption maxima are shifted to 450 nm at pH 7-8 and the largest shift occurs for 45 and 60% (v/v) methanol concentration. In these cases, formation of a new absorption band at λ max ca. 430 nm is noticed which is presumably

Fig. 3. Spectra of 2-decarboxy-betanin in aqueous methanolic solutions after 20 h of incubation, with 6.5 μM Cu²⁺ and 650 μM Cu²⁺ at 25 °C
a result of new derivatives generation by decarboxylation and dehydrogenation of the pigment. After 10 h of the experiment, at the lower concentration of copper, an absorption band at $\lambda_{\text{max}}$ ca. 460 nm occurs in 45% and 60% (v/v) methanol. At higher concentrations, the pigment is almost completely degraded at any pH value. Prolonged experiment results in similar tendency to pigment degradation as well as the generation of new absorption band.

Analysis of absorption spectra in ethanolic solutions for all copper concentrations exhibits similar degradative results. For 650 μM Cu$^{2+}$, the 2-decarboxy-betanin degradation rate is higher than for lower concentrations of copper in all tested organic solvents. After 2 hours of reaction, the hypsochromic shifts of absorption maxima $\lambda_{\text{max}}$ to ca. 430 nm is formed in 45% and 60% (v/v) ethanolic solutions. Prolonged experiment leads to almost complete degrada-
Effect of Cu (II) Cations on 2-Decarboxy-betanin Stability in Aqueous-Organic Solutions

Effect of Cu (II) Cations on 2-Decarboxy-betanin Stability in Aqueous-Organic Solutions

New absorption bands arise at 430 nm in 45% and 60% (v/v) ethanol.

Betacyanin stability may be improved by elimination or reduction of disadvantageous conditions such as light and oxygen. The tested samples were not encapsulated, thus oxygen in atmospheric air could enhance decomposition of betacyanins. Additionally, decomposition of 2-dBt under influence of copper cations leads to the pigment oxidation. The scientific interest is in conduct of study in an argon or a nitrogen atmosphere, the latter was reported to improve betanin stability [8-9]. New decarboxylated and dehydrogenated derivatives of 2-decarboxy-betanin as well as metal-pigment complexes are generated as a result of the tested reactions.

Conclusions

The increase of the concentration of copper cations has a negative effect on stability of 2-decarboxy-betanin. Decline of the pigment stability is noticed with the occurrence of the absorption maximum shifts or generation of new absorption bands as a result of a reaction catalyzed by Cu (II) ions. The cations of Cu (II) in the presence of organic solvents induce an increasing degradation, while the increase of the concentration of organic solvents enhances further the pigment decomposition. The main degradation products of the 2-decarboxy-betanin in all solvents are compounds possessing absorption maxima at \( \lambda_{\text{max}} \) ca. 430 nm.

Due to the extensive use of betalain pigment as food additives, it is important to determine factors influencing their decomposition, which may be associated with a decrease of the intensity of their color. These studies allow obtaining the information needed for proper isolation and treatment of pigments, as well as proper storage of products containing betalains.

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