Nauka Przyroda Technologie

2018
Tom 12
Zeszyt 1

ISSN 1897-7820 http://www.npt.up-poznan.net http://dx.doi.org/10.17306/J.NPT.00232
Dział: Nauki o Żywności i Żywieniu


KATARZYNA MIKOŁAJCZYK-BATOR[1], JANUSZ CZAPSKI[2]

[1]Department of Natural Science and Quality Assurance
Poznań University of Economics and Business
[2]Institute of Food Technology of Plant Origin
Poznań University of Life Sciences

# CHANGES IN THE CONTENT OF BETALAIN PIGMENTS AND THEIR ANTIOXIDATIVE CAPACITY DURING STORAGE

## ZMIANY ZAWARTOŚCI BARWNIKÓW BETALAINOWYCH I ZDOLNOŚCI ANTYOKSYDACYJNEJ W CZASIE PRZECHOWYWANIA

**Abstract**

**Background.** The red beet is an important source of natural colourants and antioxidants in the diet, such as betalain pigments (betacyanins and betaxanthins). Betalain pigments are commonly used in the food industry for colouring food products.

**Material and methods.** The authors of this study analysed changes in the content of betalain pigments and their antioxidative capacity during the 12-month storage of a betalain preparation (pH 7.0) made from red beet (*Beta vulgaris* L.) in a laboratory. Additionally, the HPLC technique was used to isolate individual betalain pigments from the preparation and to assess their antioxidative activity.

**Results.** During the storage (12 months at 20°C) of the dry preparation (pH 7.0) red pigments decomposed almost completely. The loss of their content amounted to 94%, and their antioxidative capacity decreased only by 31%. The content of yellow pigments, which was measured spectrophotometrically using Nilsson's method, increased over two times. Neobetanin (5-O-β-D--glucopyranosyl neobetanidin) was identified in the mass spectrum of the betalain preparation. Neobetanin is a degradation product of betacyanins. The antioxidative activity of neobetanin was found to be about 2.5 times greater than the antioxidative activity of betanin, which is the most abundant pigment in the red beet. The antioxidative activity of betanidin was also greater (1.9 times per 1 mg) than the antioxidative activity of betanin. Moreover, the antioxidative activity of isobetanin was slightly greater than that of betanin. After 24-hour storage in light and at room temperature neobetanin in an aqueous solution was found to be almost completely degraded.

**Conclusions.** The storage of the dry betalain preparation (pH 7.0) proved that betanin transformed into yellow-orange neobetanin, which exhibited very high antioxidative activity, but, due to the low stability of the pigment during storage, it cannot be used for food colouring.

**Keywords:** red beet, storage, neobetanin, antioxidative capacity

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

## Introduction

Red beet pigments are secondary metabolites synthesised from tyrosine – an amino acid found in plants of the Caryophyllales order, except the Caryophyllaceae and Molluginacea families, and in some higher fungi (Frank et al., 2005). Based on their structure, betalain pigments can be divided into two groups: violet betacyanins e.g. betanin and isobetanin [*beta* (Latin) – beet, *kyanos* (Greek) – blue] and yellow betaxanthins e.g. vulgaxanthin I and II [*beta* (Latin) – beet, *xanthos* (Greek) – yellow] (Wyler et al., 1963).

Betacyanins (e.g. betanin, isobetanin, neobetanin) are water-soluble immonium conjugates of betalamic acid with 3,4-dihydroxyphenylalanine (cyclo-DOPA), which may be glycosylated. Yellow betaxanthins (vulgaxanthin I and vulgaxanthin II) contain different amino acids, e.g. glutamine and glutamic acid.

Betanin, which is a betanidin 5-O-β-D-glucoside, is the most important betacyanin in red beet (*Beta vulgaris* L.) (Fig. 1 a, b). Isobetanin is the C-15 epimer of betanin. Betalains are effective free radical scavengers and they prevent active oxygen-induced and free radical-mediated oxidation of biological molecules (Escribano et al., 1998; Pedreño and Escribano, 2000). For instance, in our previous study (Czapski et al., 2009) the antioxidative capacity of beetroot juice of 11 red beet cultivars ranged from 10.8 to 13.6 μmol of Trolox per 1 ml and it was strongly correlated with the content of red pigments. Moreover, the high antioxidative capacity of these cultivars was caused by their high content of pigments (0.74–1.17 mg/ml and 0.52–0.95 mg/ml for red and yellow pigments, respectively).

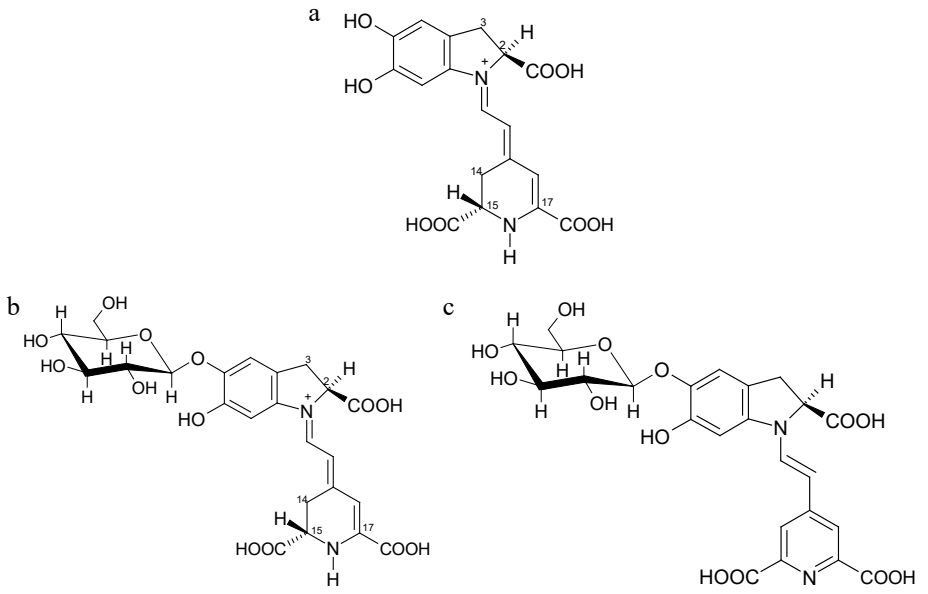


Fig. 1. The chemical structures and atom numbering system of the chromophore system of betanidin (a), betanin (b) and neobetanin (c)

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

Betalains are considered to be unstable compounds. The following factors cause their decomposition: oxidoreductive enzymes; elevated temperature; visible, UV and ionising radiation; transition metal ions and oxygen. The rate of pigment disintegration is also significantly determined by the pH level and water activity (Cai et al., 2003; Herbach et al., 2006). Betacyanin degradation is a widely varied process involving decarboxylation, dehydrogenation, hydrolysis, or deglycosylation. Betanin can be dehydrogenated by C-14,15 (neobetanin – 14,15-dehydrobetanin) (Fig. 1 c). Betacyanins are particularly sensitive to increased temperature (Kanner et al., 2001). Herbach et al. (2004) reported that neobetanin was formed as a result of heating. The presence of large quantities of neobetanin in samples containing betacyanins is the evidence for betanin degradation during processing or preparation.

Betalains are natural antioxidants belonging to the group of cation antioxidants (Kanner et al., 2001). Betanin contains phenolic and cyclic amine groups, which are very good electron donors and exhibit high antioxidative activity (Gliszczyńska-Świgło et al., 2006; Herbach et al., 2004). However, betacyanins are characterised by low bioavailability, which may be a consequence of partial degradation in the digestive system and decreased absorption in the small intestine (Frank et al., 2005; Netzel et al., 2005). Betalains are used exclusively as food colourants (Moreno et al., 2008). Differences in the antioxidative activity between both betalain groups, i.e. betacyanins and betaxanthins, and isolated individual betalain pigments are caused by the chemical structures of these compounds (Zakharova and Petrova, 1998). According to Escribano et al. (1998), betanin exhibits higher antioxidative activity than vulgaxanthin. Gliszczyńska-Świgło et al. (2006) reported that at higher pH betanin became a better hydrogen and electron donor and its free radical-scavenging activity increased.

Betanin (beetroot red – E 162) is approved in the European Union as a food additive for use on the *quantum satis* basis. In the United States the applications of red beet pigments as food additives are specified in the Code of Federal Regulations (Castellar et al., 2003; Moreno et al., 2008). Betalains are not widely distributed in the plant world, but they are used as a source of natural red colour to improve the appearance of numerous food products (Klewicka, 2012; Sawicki et al., 2016). Higher consumer awareness of artificial colourants causes the growing demand for products coloured with natural pigments. Moreover, there is increasing interest in the use of natural food colours due to health benefits such as antioxidative activity. Although betalains have high antioxidative capacity, they are unstable during thermal treatments (Attoe and Von Elbe, 1982), which change their structure and colour.

Unfortunately, so far there has been no essential information about the antioxidative activity of separated betalains because the presence of numerous betalain pigments (approximately 78 structures were reported by Khan and Giridhar, 2015, and Belhadj Slimen et al., 2017) in red beet roots complicates their separation to obtain pure individual compounds.

Red beet pigments are commercially available in the form of concentrates. They are produced by reducing red beet juice to the dry mass content of 40–60%. They are also available in the form of powder concentrates obtained by spray-drying. Powder concentrates exhibit high stability during storage (Sobkowska et al., 1991).

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

In our previous study (Mikołajczyk-Bator and Czapski, 2017) on a betalain preparation solution the most distinct increase in neobetanin was noted at pH 6.5 and 7.0. The increased pH values of the betalain preparation solution were conducive to the accumulation of neobetanin.

In this study the preparation of pigment precipitation was adjusted to pH 7.0 by adding 10% NaOH and it was lyophilised. Part of the lyophilisate was stored for 12 months. Neobetanin, as a pigment with high antioxidative activity, could be used as a potential source of a natural orange pigment for colouring food, e.g. milk-based products (yoghurts, puddings) with pH of about 7.0.

A dry preparation of betalain pigments (pH 7.0) was stored for 12 months at 20°C in order to determine their stability. It was necessary to determine it in the preparation before betalain could be used as a food additive to products with the pH value of 7.0 (e.g. dairy products).

The aim of the study was to determine changes in the content of betalain pigments and their antioxidative capacity during the storage of a dry preparation of red beet (*Beta vulgaris* L.) betalain pigments (pH 7.0) in a laboratory. Additionally, the preparative HPLC technique was used to isolate individual betalain pigments from the preparation and their antioxidative activity was assessed.

## Material and methods

### Betalain pigment preparation procedure

The betalain pigment preparation procedure was set as described in our previous study (Mikołajczyk-Bator and Czapski, 2017) with some modifications. Pigments contained in juice extracted from the 'Chrobry' red beet cultivar were precipitated with 95% ethyl alcohol, where the volumetric ratio between the juice and alcohol amounted to 1 : 7. Next, they were centrifuged at 10,000 × *g* during 15 min. The sediment was the precipitate of betalain pigments. The sediment remaining after centrifugation was dissolved in distilled water and the solution was brought to a pH level of 7.0 by adding 10% NaOH. Then it was transferred in 2 $cm^3$ aliquots into 4 $cm^3$ vials to achieve the same volume in all containers. Next, it was lyophilised.

Prior to assays the lyophilised preparation was dissolved in 1 $cm^3$ of distilled water to obtain the same initial content of betalain pigments in all the samples. The content of betalain pigments was assessed using Nilsson's method (the red pigment content was 1.28 mg/$cm^3$, and the yellow pigment content was 0.32 mg/$cm^3$). Figure 2 shows the flow chart illustrating the experiment.

HPLC was used to separate betalain pigments for analysis. Figure 3 shows the areas of individual peaks generated by pigments contained in the betalain preparation solution. The pigments were identified by comparing the retention times of individual peaks with reference data (Stintzing et al., 2002) and mass spectrometric data.

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

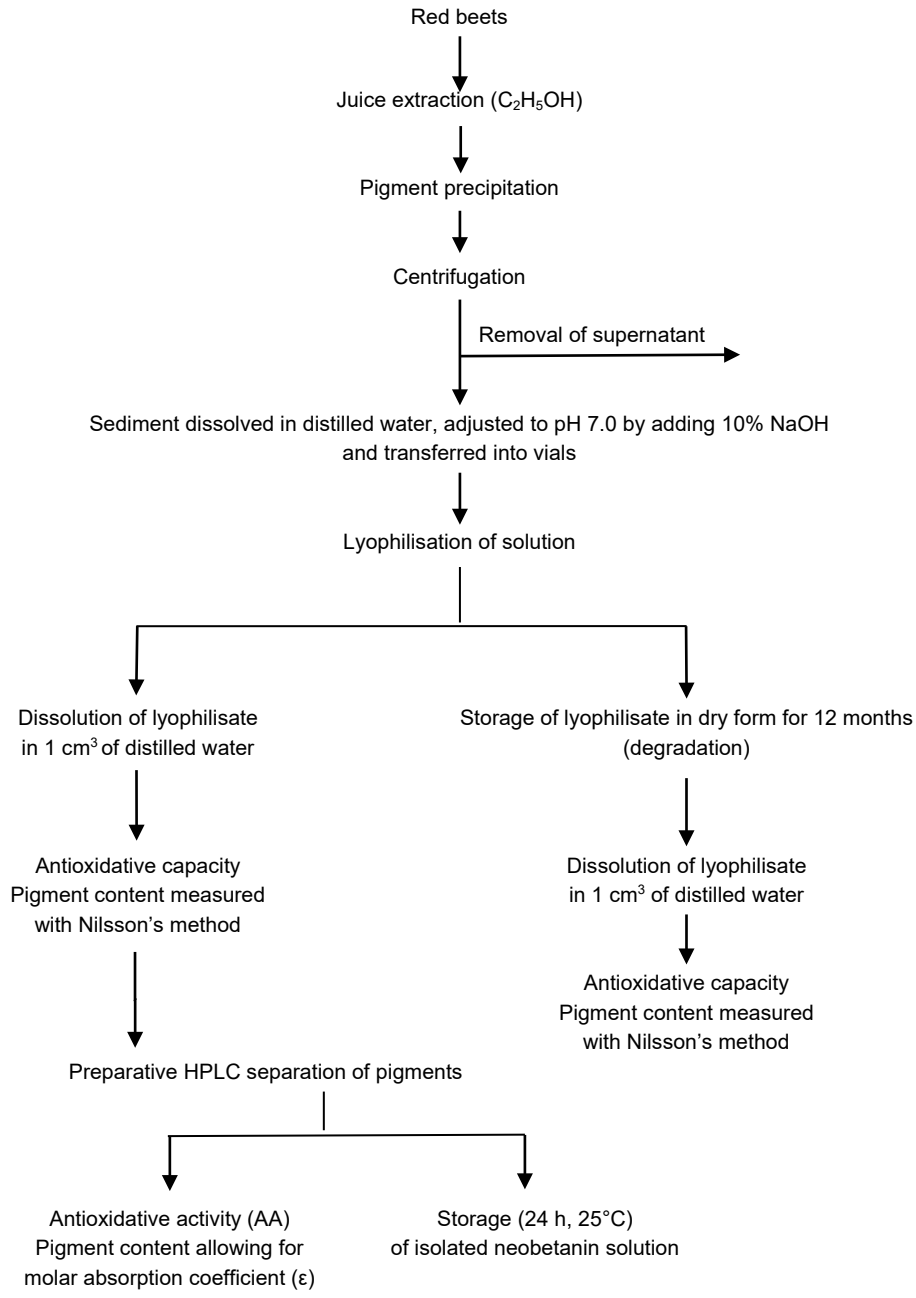


Fig. 2. A flow chart illustrating the experiment

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

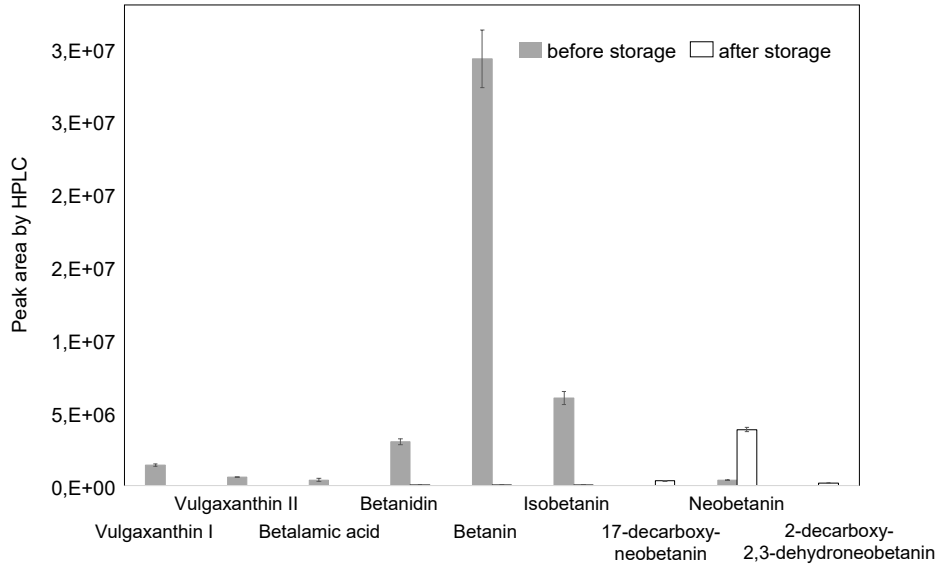


Fig. 3. Peak areas of betalain pigments (λ = 540 nm: betanidin, betanin and isobetanin; λ = 479 nm: 17-decarboxyneobetanin, neobetanin, 2-decarboxy-2,3-dehydroneobetanin; λ = 476 nm: vulgaxanthin I, vulgaxanthin II; λ = 405 nm: betalamic acid) identified by means of HPLC separation in the betalain preparation before storage and in the degraded preparation after storage

## Dry preparation storage

The lyophilised betalain preparation (pH 7.0) was stored at a temperature of 20°C in a glass desiccator filled with silica gel, without access to light. During storage the lyophilised betalain preparation was exposed to oxygen (vials containing lyophilised betalains were filled with air).

After 12 months of storage at room temperature the samples were degraded. Their colour changed from bright violet to orange-brown.

## Antioxidative capacity of betalain preparation solution and pure pigments

The antioxidative capacity of the betalain preparation solution before and after storage was assessed with the ABTS cation radical method developed by Re et al. (1999). A Helios Alfa spectrophotometer with a thermostated compartment (Thermo Electron Corporation) was used for the assessment. The results were expressed as values of the TEAC (Trolox Equivalent Antioxidant Capacity) index – in micromoles of Trolox per 1 $cm^3$.

The antioxidative activity (AA) of pure pigments determined by means of preparative HPLC was converted into a value per 1 mg of pigment and expressed as micromoles of Trolox per 1 mg of pigment.

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

### Measuring content of betalain pigments with spectrophotometry

The content of betalain pigments was assessed with a differencing method developed by Nilsson (1970). The method enabled simultaneous measurement of the content of red betacyanins and yellow betaxanthins. The content of pigments was expressed as milligrams per 1 $cm^3$ of the sample. Absorbance measurements were made at wavelengths of 476, 538 and 600 nm, using a Helios Alfa spectrophotometer (Thermo Electron Corporation).

The content of individual red beet pigments (PC) isolated by means of preparative HPLC from the betalain preparation was calculated with the formula developed by Stintzing et al. (2005):

$$PC = E \times R \times Mm \times 1000/\varepsilon \times l \quad (mg/l)$$

where: E – absorbance of the test sample adjusted to a wavelength of 600 nm, R – multiplication factor of sample dilution, Mm – molecular mass (Da), ε – molar absorption coefficient (l/(mol·cm)), l – thickness of the absorbent layer (cm). The following values of the molar absorption coefficient ε were assumed: 54,000 l/(mol·cm) for betanidin at λ = 538 nm (Kanner et al., 2001), 60,000 l/(mol·cm) for betanin at λ = 538 nm (Herbach et al., 2006), 18,200 l/(mol·cm) at λ = 469 nm for neobetanin (Wyler et al., 1984), 30,000 l/(mol·cm) at λ = 430 nm for betalamic acid (Kimler et al., 1971).

### Analytical HPLC separation

The analytical separation of betalain pigments contained in the betalain pigment preparation was conducted by means of HPLC. The analysis was based on the methodology proposed by Stintzing et al. (2002). The samples were filtered through a Millex--LCR syringe filter unit (Millipore) with the pore size of 0.45 μm, and subjected to chromatographic analysis. HPLC assays were conducted using an HPLC system from Thermo Separation, equipped with a Spectra System UV-300 detector (detector UV-VIS).

The analytical separation of the samples was conducted in a Waters Spherisorb column sized 4.6 × 250 mm, with octadecylsilane (ODS) stationary phase, filled with 5 μm packing, in the reversed phase (RP) mode.

Separation was carried out at room temperature, at a flow rate of 1 $cm^3$/min, using two mobile phases. Phase A consisted of 0.2% TFA (trifluoroacetic acid) and 10% HCOOH formic acid (65 : 35, v/v). Phase B consisted of 100% ACN (acetonitrile) and 10% HCOOH (80 : 20, v/v). During the initial 15 min isocratic elution with 100% of Phase A was used. Between 15 and 60 min of the analysis a 0–20% gradient of Phase B was applied. HPLC separation was conducted within the visible light range. Readings for the red pigments were made at a wavelength of 540 nm (neobetanin derivatives – 475 nm), for the yellow pigments – at 475 nm, and for betalamic acid – at 405 nm.

### MS analysis

The mass spectrum of the preparation after storage was obtained by electrospray ionisation (ESI) using a high performance liquid chromatography (HPLC) system coupled with a mass spectrometer (HPLC/MS) in a ZQ type chromatograph (Waters). The procedure was based on the methodology described by Stintzing et al. (2002). Detection

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

of the mass range from 200 to 1000 Da was conducted in the negative and positive ion mode.

The conditions of HPLC separation were as follows. For the initial 15 min isocratic elution was used (water/10% HCOOH, 95 : 5, v/v). Between 15 and 75 min of the analysis a 0–20% gradient of water/acetonitrile (20 : 80, v/v) was applied.

### Storage of neobetanin solution

The isolation of neobetanin from the betalain preparation was conducted by means of preparative HPLC. Neobetanin was eluted in 47 min. The conditions of preparative HPLC separation were described in section "Analytical HPLC separation". After the preparative separation the neobetanin solution was stored for 24 h at room temperature (25°C).

### Preparative HPLC separation of red beet pigments

The lyophilised preparation of betalain pigments was dissolved in water. The solution contained 0.016 g of red pigments and 0.004 g of yellow pigments per 1 $cm^3$. The content was measured with Nilsson's method (Nilsson, 1970).

Next, preparative separation of the sample was carried out in a column of the same type as the one used for analytical separation, sized 10 × 250 mm. The separation was conducted at 25°C and a flow rate of 3.5 $cm^3$/min in the RP mode, using two mobile phases with the same compositions as for the analytical separation.

Qualitative identification was based on retention times and analysis of the spectra of HPLC-separated compounds, which were compared with reference data and mass spectrometric data (Stintzing et al., 2002).

### Statistical analysis

All analyses were triplicated and the data were reported as mean ±standard deviation (SD). The results were processed with the Microsoft Excel spreadsheet.

## Results and discussion

### Storage of dry betalain preparation

The HPLC chromatogram of the betalain preparation prior to storage was dominated by a peak generated by betanin. The area of that peak accounted for 72% of the sum total of areas of all peaks generated by other betalain pigments (Fig. 3). HPLC assays were made using an HPLC system equipped with a UV-VIS detector.

In the preparation of betalain pigments degraded by 12-month storage the area of the peak generated by betanin accounted only for about 0.7% of the sum total of all areas of peaks generated by red pigments. Neobetanin represented the highest percentage content in the degraded sample (87%). The sum of the area of all peaks generated by the degraded sample during HPLC separation was assumed as 100%. By contrast, in the unstored sample it amounted only to 0.9% of the total area of peaks generated by the red pigments.

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

Apart from the main peak of neobetanin pigment, other peaks generated by neobetanin derivatives were identified in the betalain preparation after storage. These were yellow-orange pigments such as 17-decarboxyneobetanin and 2-decarboxy-2,3--dehydroneobetanin.

The degradation of the lyophilisate resulted in the formation of neobetanin in the process of dehydrogenation of betanin identified in the mass spectrum (Fig. 4.1).

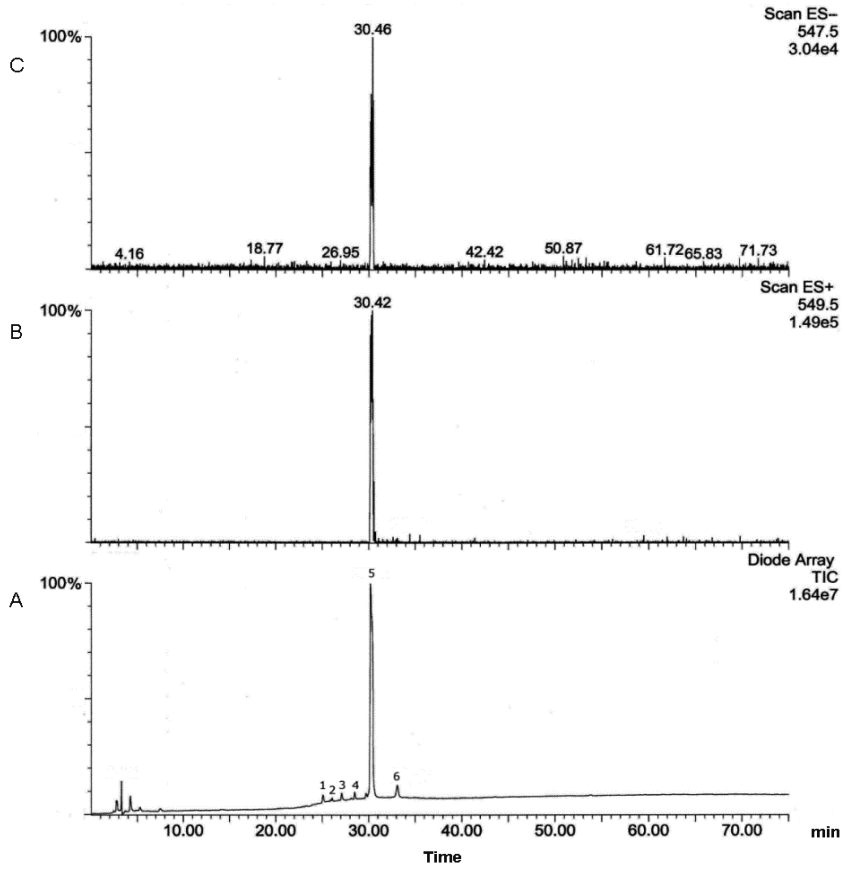


Fig. 4.1. A – the total ion current (TIC) of chromatogram RP-HPLC/ESI-MS** of: 1 – betanidin, 2 – betanin, 3 – isobetanin, 4 – 17-decarboxyneobetanin, 5 – neobetanin, 6 – 2-decarboxy-2,3-dehydroneobetanin, in the stored preparation; neobetanin retention time ($t_R$): 30.23 min; B and C – chromatograms (Extracted Ion Chromatograms) of substances responsible for the ion signal at m/z 549.5 ESI (+) (B) and m/z 547.5 ESI (–) (C)

**A: $H_2O$, B: MeOH; linear gradient: 50% A / 50% B (0 min), 100% B (30 min); flow rate: 0.4 ml/min

Before assessing the content of betalains spectrophotometrically with Nilsson's method (Nilsson, 1970), one ampoule of degraded lyophilisate was dissolved in 1 $cm^3$

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

of distilled water. It resulted in the red pigment content of 0.20 mg/cm$^3$ and yellow pigment content of 1.26 mg/cm$^3$.

The content of yellow pigments corresponded mainly to neobetanin. The minority corresponded to its derivatives (yellow-orange pigments) in the degraded lyophilisate, which are classified as purple pigments (betacyanins) due to their chemical structure.

During the storage of the preparation the loss of the red pigment amounted to 94%. However, the antioxidative capacity was reduced only by 31%, as compared with the unstored betalain preparation (Table 1). It indicated the high antioxidative capacity of yellow pigments. The HPLC chromatogram and the mass spectrum of the stored betalain preparation indicated the presence of neobetanin as the main peak in the sample (Figs. 4.2, 5).

Table 1. The antioxidative capacity and content of pigment in the unstored betalain preparation solution and after storage at 20°C for 12 months

| Betalain preparation, pH 7.0 | Pigment content assessed with Nilsson's method (mg/cm$^3$) | | TEAC index (μmol of Trolox) | | |
|---|---|---|---|---|---|
| | red | yellow | per 1 cm$^3$ of sample | per 1 mg of red pigments | per 1 mg of yellow pigments |
| Unstored | 3.50 (±0.12) | 0.55 (±0.06) | 38.06 (±0.64) | 10.8 | 69.2 |
| Stored (degraded) | 0.20 (±0.02) | 1.26 (±0.04) | 26.03 (±0.58) | 130.1 | 20.6 |

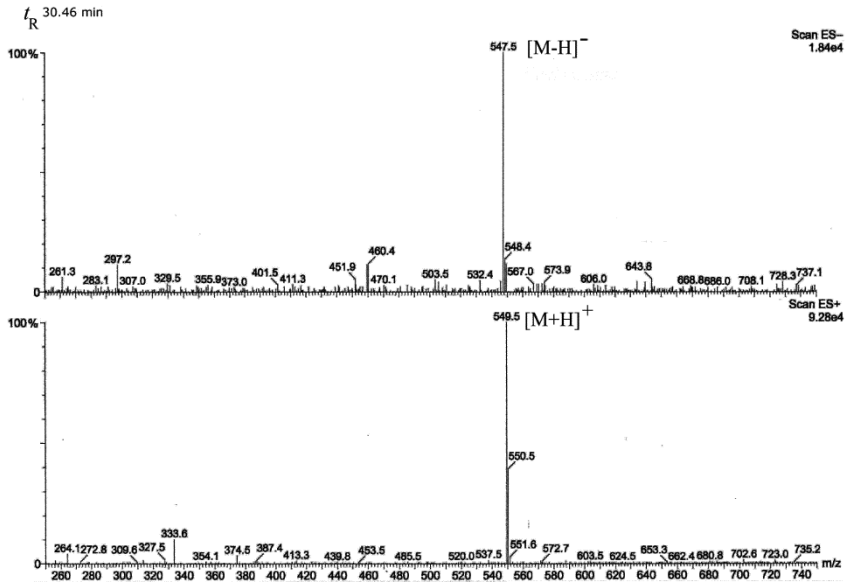


Fig. 4.2. The mass spectrum of the betalain preparation (pH 7.0) stored for 12 months. A neobetanin particle (with the mass of 548.5) gives an ion with the m/z of 547.5 (on negative ions) and an ion with the m/z of 549.5 (on positive ions)

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

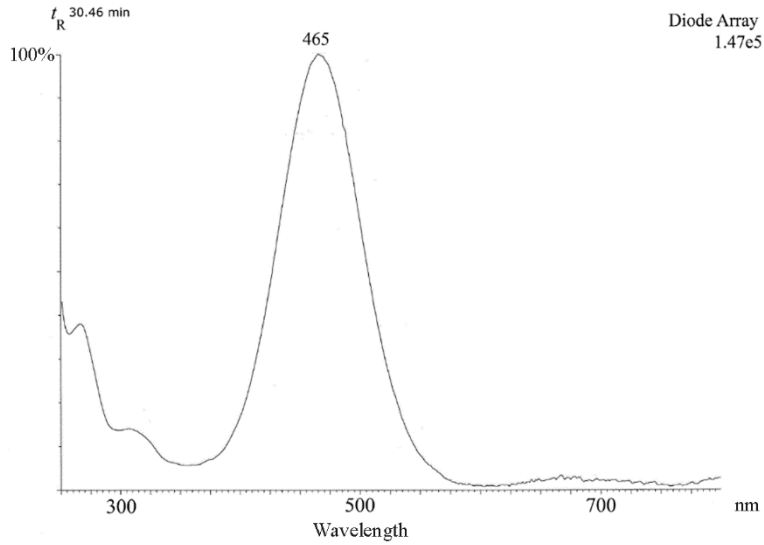


Fig. 5. A UV spectrum of the aqueous solution of neobetanin from the HPLC chromatogram

## Antioxidative activity of isolated betalains

The separation of pigments from the betalain preparation by means of preparative HPLC resulted in the isolation of five pigments: betanidin, betanin, isobetanin, neobetanin and betalamic acid (Table 2).

Table 2. The antioxidative activity and content of pigment in the solution of separated pigments isolated by means of preparative HPLC

| Pigment | Retention time (min) | $\lambda_{max}$ (nm) | Pigment concentration (μg/cm$^3$) | Molar absorption coefficient* | | Molecular mass (Da) | TEAC index (μmol of Trolox per 1 mg of pigment) |
|---|---|---|---|---|---|---|---|
| | | | | ε (l/(mol·cm)) | $\lambda_{max}$ (nm) | | |
| Betanidin | 25.1 | 538 | 3.0 | 54,000 | 538 | 388.14 | 13 (±0.02) |
| Betanin | 26.1 | 538 | 73 | 60,000 | 538 | 550.48 | 6.9 (±0.01) |
| Isobetanin | 27.3 | 538 | 27 | 60,000 | 538 | 550.48 | 8.8 (±0.03) |
| Neobetanin | 30.4 | 474 | 20 | 18,200 | 469 | 548.50 | 17 (±0.03) |
| Betalamic acid | 62.6 | 421 | 8.0 | 30,000 | 430 | 211.17 | 4.5 (±0.02) |

*The molar absorption coefficient specified in the Table is based on reference publications (section "Material and methods").

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

The antioxidative activity (AA) of pure pigments obtained by means of preparative HPLC was converted per 1 mg of pigment and expressed as micromoles of Trolox per 1 mg of pigment.

The results of the study showed that neobetanin, the orange pigment formed by betanin dehydrogenation, exhibited very high antioxidative activity (AA): 17 µmol of Trolox per 1 mg of pigment. By contrast, the antioxidative activity of 1 mg of betanin was about 2.5 times smaller, i.e. 6.9 µmol of Trolox. The antioxidative activity of betanidin expressed per 1 mg of pigment was 1.9 times greater (13 µmol of Trolox) than that of betanin. Moreover, the antioxidative activity of isobetanin was also slightly greater than that of betanin, i.e. 8.8 µmol of Trolox per 1 mg of pigment (Table 2). Betalamic acid exhibited the lowest antioxidative activity, i.e. 4.5 µmol of Trolox.

The spectral analysis of neobetanin made immediately after preparative separation and after 24 h of storage of the solution at room temperature (25ºC) as well as after exposure to oxygen and light showed that the pigment had almost entirely degraded (Fig. 6). Thus, the experiment proved neobetanin to be a rather unstable pigment, whose decomposition was precipitated by the aqueous environment.

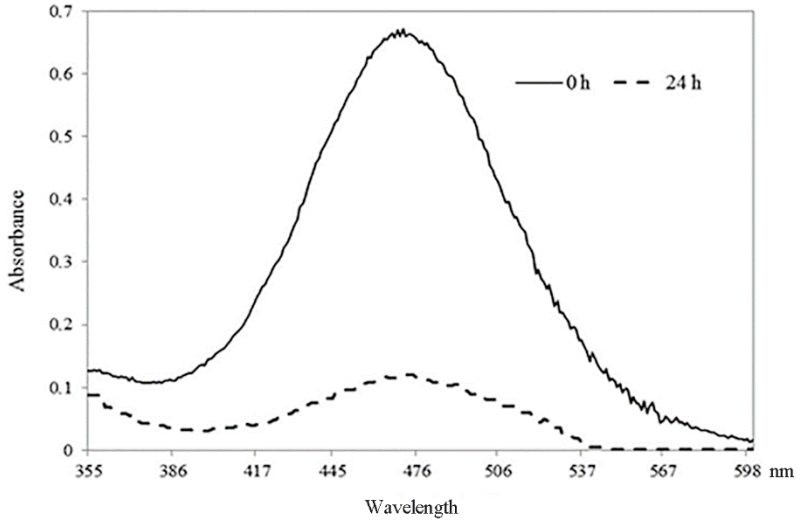


Fig. 6. The absorption spectrum of neobetanin recorded immediately after preparative HPLC separation (0 h) and after 24 h of storage (24 h) at room temperature (25°C)

Unfortunately, the large number of betalain pigments (so far there have been 78 structurally different betalain pigments identified (Belhadj Slimen et al., 2017; Khan and Giridhar, 2015) and their unstable nature complicates their separation so as to obtain pure individual compounds. The isolation of betalains from plant materials is a notably complicated and time-consuming task. Therefore, there are no comparisons of the antioxidative activity of individual betalains. Our results are in agreement with the results of the study by Gliszczyńska-Świgło and Szymusiak (2007), who found that the radical

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

scavenging activity of betanidin within the pH range of 2–4 was about 5.4 to 1.4 times greater than the radical scavenging activity of betanin. The authors indicated that the relatively high antiradical activity of betanidin, at physiologically relevant acidic pH, where cationic and mono-deprotonated forms dominated, may have been caused by the presence of a catechol moiety in the betanidin molecule (Gliszczyńska-Świgło and Szymusiak, 2007).

The experiment showed that neobetanin was responsible for the increase in the content of yellow pigments in the betalain preparation (pH 7.0) degraded during storage, as assessed with Nilsson's method. At the same time, the decomposition of betaxanthins (characteristic yellow pigments found in red beets) was observed. Orange-coloured neobetanin ($\lambda_{max}$ = 474 nm) is formed from betanin, which is a red pigment (Herbach et al., 2004).

Neobetanin (5-O-β-D-glucopyranosyl neobetanidin or 14,15-dehydrobetanin) was identified as a new class of orange pigments belonging to the betalain group (Mabry et al., 1967) in multiple plants of the Caryophyllales order. As early as 1967, Mabry et al. (1967) found that neobetanin was formed from betanin and betanidin was probably formed by oxygenation in the air, which caused deprotonation of the basic coupled system, e.g. in the presence of diazomethane, pyridine and acetic anhydride, amines or sodium acetate.

In 2004 Herbach et al. (2004) were the first to report that the pigment was a product of thermal degradation of betanin during the heating of red beet juice. In spite of its orange colour ($\lambda_{max}$ = 465 nm or $\lambda_{max}$ = 470 nm according to Stintzing and Carle, 2004; $\lambda_{max}$ = 474 nm in our study), neobetanin is structurally classified as one of betacyanins (red pigments). The presence of a large quantity of neobetanin in samples containing betacyanins is the evidence for betanin degradation during processing or preparation (Herbach et al., 2004). Mixtures of mono-, bi-, and tridecarboxylated betacyanins together with their corresponding neobetacyanins were obtained from *Beta vulgaris* root juice as heating degradation products of betacyanins (Wybraniec, 2005).

According to reports, the stability of both pigment groups varies according to the chemical structure of these compounds. The process of condensation of betalamic acid with amino acids or amines for betaxanthins, or cyclo-DOPA glucoside for betanidin, causes variation in the stability of chemical structures of pigments (Cai et al., 1998; Kobayashi et al., 2001; Pedreño and Escribano, 2000).

The antioxidative capacity of betalains has been attributed to the molecular structure of these pigments, which depends on their ability to donate hydrogen to reactive species. Glycosylation reduced the antioxidative activity of betacyanins, whereas acylation increased it (Stintzing and Carle, 2004). The antioxidative activity of betalains may increase according to the number and position of hydroxyl groups in the molecule. The C-5 position of the hydroxyl group in the aglycone is responsible for the increase in their antioxidative activity (Azeredo, 2009; Cai et al., 2005). Betanidin is more reactive than betanin and neobetanin due to the presence of the 5,6-dihydroxyl moiety, which results in its high antioxidative activity (Wybraniec et al., 2011). This study confirmed that betanidin exhibited higher antioxidative activity than betanin.

The results of the experiment indicate that an increase in the sample pH to 7.0 may alter the antioxidative activity of neobetanin and betanin.

The orange-coloured solution of neobetanin isolated by means of preparative HPLC became discoloured after 24 h of storage at room temperature (25ºC), and the absorption of light within the visible range practically dropped to zero. However, the absorption of light within the UV range increased and the maximum was recorded at a wavelength of 243 nm (the absorbance level rose from 0.728 to 1.400). The decomposition of neobetanin probably resulted in cyclo-DOPA glucoside, which is more stable in an acidic rather than alkaline environment (Herbach et al., 2004). The pH value of the sample in the experiment was 1.5. The maximum light absorption by cyclo-DOPA glucoside depends on the environmental pH and ranges from 217 nm at pH 0.5 to 311 nm at pH 12.5 (Wyler et al., 1984). Herbach et al. (2004) detected cyclo-DOPA glucoside as a product of thermal degradation of betanin after 1 h of heating at 85°C. However, the compound was markedly degraded after heating juice at 85°C for 8 h.

## Conclusions

The storage of the dry preparation (pH 7.0) of red beet pigments for 12 months at room temperature was found to induce a very large loss of red pigments and yellow betalain pigments and a prominent increase in the amount of yellow pigments other than betaxanthins. The loss of betacyanin pigments over this period was much greater than the decrease in the antioxidative capacity, totalling 94% and 31%, respectively.

The analysis of the mass spectrum of the stored preparation revealed the presence of the main peak generated by neobetanin in the degraded preparation of betacyanins both in positive ions $[M+H]^+$, where the m/z amounted to 549.5, and negative ions $[M-H]^-$, where the m/z amounted to 547.5. Neobetanin has poor stability. After storing the solution at 25°C for 24 h the pigment was nearly completely decomposed.

To sum up, the storage of the betalain preparation (pH 7.0) proved that betanin transformed into yellow-orange neobetanin. The colour of stored food products, with the content of betalain preparation may change from red-violet to yellow as a result of neobetanin formation. As neobetanin is not stable during storage at room temperature, it should not be used for the colouring of food that requires storage.

## References

Attoe, E. L., Von Elbe, J. H. (1982). Degradation kinetics of betanin in solutions as influenced by oxygen. J. Agric. Food Chem., 30, 4, 708–712. http://dx.doi.org/10.1021/jf00112a021

Azeredo, H. M. C. (2009). Betalains: properties, sources, applications, and stability – a review. Int. J. Food Sci. Technol., 44, 12, 2365–2376. https://dx.doi.org/10.1111/j.1365-2621.2007.01668.x

Belhadj Slimen, I., Najar, T., Abderrabba, M. (2017). Chemical and antioxidant properties of betalains. J. Agric. Food Chem., 65, 4, 675–689. http://dx.doi.org/10.1021/acs.jafc.6b04208

Cai, Y., Sun, M., Corke, H. (1998). Colorant properties and stability of *Amaranthus* betacyanin pigments. J. Agric. Food Chem., 46, 11, 4491–4495. http://dx.doi.org/10.1021/jf980457g

Cai, Y., Sun, M., Corke, H. (2003). Antioxidant activity of betalains from plants of the Amaranthaceae. J. Agric. Food Chem., 51, 8, 2288–2294. http://dx.doi.org/10.1021/jf030045u

Cai, Y., Sun, M., Corke, H. (2005). HPLC characterization of betalains from plants in the Amaranthaceae. J. Chromatogr. Sci., 43, 9, 454–460. https://dx.doi.org/10.1093/chromsci/43.9.454

Castellar, R., Obón, J. M., Alacid, M., Fernández-López, J. A. (2003). Color properties and stability of betacyanins from *Opuntia* fruits. J. Agric. Food Chem., 51, 9, 2772–2776. http://dx.doi.org/10.1021/jf021045h

Czapski, J., Mikołajczyk, K., Kaczmarek, M. (2009). Relationship between antioxidant capacity of red beet juice and contents of its betalain pigments. Pol. J. Food Nutr. Sci., 59, 2, 119–122.

Escribano, J., Pedreño, M. A., García-Carmona, F., Muñoz, R. (1998). Characterization of the antiradical activity of betalains from *Beta vulgaris* L. roots. Phytochem. Anal., 9, 3, 124–127. https://dx.doi.org/10.1002/(SICI)1099-1565(199805/06)9:3<124::AID-PCA401>3.0.CO;2-0

Frank, Th., Stintzing, F. C., Carle, R., Bitsch, I., Quaas, D., Straß, G., Bitsch, R., Netzel, M. (2005). Urinary pharmacokinetics of betalains following consumption of red beet juice in healthy humans. Pharmacol. Res., 52, 4, 290–297. http://dx.doi.org/10.1016/j.phrs.2005.04.005

Gliszczyńska-Świgło, A., Szymusiak, H. (2007). Molecular insight in the pH-dependent radical--scavenging activity of betanidin. Pol. J. Nat. Sci. Suppl., 4, 17–24.

Gliszczyńska-Świgło, A., Szymusiak, H., Malinowska, P. (2006). Betanin, the main pigment of red beet: molecular origin of its exceptionally high free radical-scavenging activity. Food Additiv. Contam., 23, 11, 1079–1087. https://dx.doi.org/10.1080/02652030600986032

Herbach, K. M., Stintzing, F. C., Carle, R. (2004). Impact of thermal treatment on color and pigment pattern of red beet (*Beta vulgaris* L.) preparations. J. Food Sci., 69, 6, C491–C498. https://dx.doi.org/10.1111/j.1365-2621.2004.tb10994.x

Herbach, K. M., Stintzing, F. C., Carle, R. (2006). Betalain stability and degradation – structural and chromatic aspects. J. Food Sci., 71, 4, R41–R50. https://dx.doi.org/10.1111/j.1750-3841.2006.00022.x

Kanner, J., Harel, S., Granit, R. (2001). Betalains – a new class of dietary cationized antioxidants. J. Agric. Food Chem., 49, 11, 5178–5185. http://dx.doi.org/10.1021/jf010456f

Khan, M. I., Giridhar, P. (2015). Plant betalains: chemistry and biochemistry. Phytochemistry, 117, 267–295. https://dx.doi.org/10.1016/j.phytochem.2015.06.008

Kimler, L., Larson, R. A., Messenger, L., Moore, J. B., Mabry, T. J. (1971). Betalamic acid, a new naturally occurring pigment. J. Chem. Soc. D Chem. Commun., 21, 1329–1330. http://dx.doi.org/10.1039/C29710001329

Klewicka, E. (2012). Betacyjaniny – biodostępność i biologiczna aktywność. Żywn. Nauka Technol. Jakość, 81, 2, 5–21.

Kobayashi, N., Schmidt, J., Wray, V., Schliemann, W. (2001). Formation and occurrence of dopamine-derived betacyanins. Phytochemistry, 56, 5, 429–436. http://dx.doi.org/10.1016/S0031-9422(00)00383-6

Mabry, T. J., Wyler, H., Parkih, I., Dreiding, A. S. (1967). The conversion of betanidin and betanin to neobetanidin derivatives. Tetrahedron, 23, 7, 3111–3127. https://dx.doi.org/10.1016/S0040-4020(01)83368-8

Mikołajczyk-Bator, K., Czapski, J. (2017). Effect of pH changes on antioxidant capacity and the content of betalain pigments during the heating of a solution of red beet betalains. Pol. J. Food Nutr. Sci., 67, 2, 123–128. https://dx.doi.org/10.1515/pjfns-2016-0012

Moreno, D. A., García-Viguera, C., Gil, J. I., Gil-Izquierdo, A. (2008). Betalains in the era of global agri-food science, technology and nutritional health. Phytochem. Rev., 7, 2, 261–280. http://dx.doi.org/10.1007/s11101-007-9084-y

Netzel, M., Stintzing, F. C., Quaas, D., Straß, G., Carle, R., Bitsch, R., Bitsch, I., Frank, Th. (2005). Renal excretion of antioxidative constituents from red beet in humans. Food Res. Int., 38, 8–9, 1051–1058. http://dx.doi.org/10.1016/j.foodres.2005.03.016

Nilsson, T. (1970). Studies into the pigments in beetroot (*Beta vulgaris* L. ssp. *vulgaris* var. *rubra* L.). Lantbrukshögsk. Ann., 36, 179–219.

Pedreño, M. A., Escribano, J. (2000). Studying the oxidation and the antiradical activity of betalain from beetroot. J. Biol. Educ., 35, 1, 49–51. https://dx.doi.org/10.1080/00219266.2000.9655736

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med., 26, 9–10, 1231–1237. https://dx.doi.org/10.1016/S0891-5849(98)00315-3

Sawicki, T., Bączek, N., Wiczkowski, W. (2016). Betalain profile, content and antioxidant capacity of red beetroot dependent on the genotype and root part. J. Funct. Foods, 27, 249–261. https://dx.doi.org/10.1016/j.jff.2016.09.004

Sobkowska, E., Czapski, J., Kaczmarek, R. (1991). Red table beet pigment as food colorant. Int. Food Ingred., 3, 24–28.

Stintzing, F. C., Carle, R. (2004). Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. Trends Food Sci. Technol., 15, 1, 19–38. https://dx.doi.org/10.1016/j.tifs.2003.07.004

Stintzing, F. C., Herbach, K. M., Mosshammer, M. R., Carle, R., Yi, W., Sellappan, S., Akoh, C. C., Bunch, R., Felker, P. (2005). Color, betalain pattern, and antioxidant properties of cactus pear (*Opuntia* spp.) clones. J. Agric. Food Chem., 53, 2, 442–451. http://dx.doi.org/10.1021/jf048751y

Stintzing, F. C., Schieber, A., Carle, R. (2002). Identification of betalains from yellow beet (*Beta vulgaris* L.) and cactus pear [*Opuntia ficus-indica* (L.) Mill.] by High-Performance Liquid Chromatography – Electrospray Ionization Mass Spectrometry. J. Agric. Food Chem., 50, 8, 2302–2307. http://dx.doi.org/10.1021/jf011305f

Wybraniec, S. (2005). Formation of decarboxylated betacyanins in heated purified betacyanin fractions from red beet root (*Beta vulgaris* L.) monitored by LC–MS/MS. J. Agric. Food Chem., 53, 9, 3483–3487. http://dx.doi.org/10.1021/jf048088d

Wybraniec, S., Stalica, P., Spórna, A., Nemzer, B., Pietrzkowski, Z., Michałowski, T. (2011). Antioxidant activity of betanidin: electrochemical study in aqueous media. J. Agric. Food Chem., 59, 22, 12163–12170. http://dx.doi.org/10.1021/jf2024769

Wyler, H., Mabry, T. J., Dreiding, A. S. (1963). Zur Struktur des Betanidins. 6. (vorläufige) Mitteilung. Über die Konstitution des Randenfarbstoffes Betanin. Helv. Chim. Acta, 46, 5, 1745–1748. https://dx.doi.org/10.1002/hlca.19630460529

Wyler, H., Meuer, U., Bauer, J., Stravs-Mombelli, L. (1984). Cyclodopa glucoside (= (2*S*)-5-(β-D--glucopyranosyloxy)-6-hydroxyindoline-2-carboxylic acid) and its occurrence in red beet (*Beta vulgaris* var. *rubra* L.). Helv. Chim. Acta, 67, 5, 1348–1355. https://dx.doi.org/10.1002/hlca.19840670520

Zakharova, N. S., Petrova, T. A. (1998). Relationship between the structure and antioxidant activity of various betalains. Prikl. Biokhim. Mikrobiol., 34, 2, 199–202.

## ZMIANY ZAWARTOŚCI BARWNIKÓW BETALAINOWYCH I ZDOLNOŚCI ANTYOKSYDACYJNEJ W CZASIE PRZECHOWYWANIA

**Abstrakt**

**Wstęp.** Buraki ćwikłowe są ważnym źródłem naturalnych barwników i przeciwutleniaczy w diecie, takich jak betalainy (betacyjaniny i betaksantyny). Barwniki betalainowe są powszechnie stosowane w przemyśle spożywczym.

**Materiał i metody.** Badano preparat barwników betalainowych o zwiększonej wartości pH (7,0) uzyskanych laboratoryjnie z buraka ćwikłowego (*Beta vulgaris* L.). Stosując preparatywną chromatografię HPLC, wyizolowano z preparatu poszczególne barwniki i określono ich aktywność antyoksydacyjną, a także oceniono zmiany ich zawartości w preparacie podczas 12-miesięcznego przechowywania.

Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232

**Wyniki.** W czasie 12-miesięcznego przechowywania suchego preparatu o pH 7,0 w temperaturze 20°C nastąpił niemal całkowity rozkład barwników czerwonych. Straty ich zawartości wyniosły 94%, z jednoczesnym spadkiem zdolności antyoksydacyjnych tylko o 31%. Zawartość barwników żółtych badana spektrofotometrycznie metodą Nilssona wzrosła ponad dwukrotnie. Stosując spektrometrię mas, zidentyfikowano na widmie masowym przechowywanego preparatu neobetaninę (5-O-β-D-glukopiranozyloneobetanidynę), która jest produktem degradacji betacyjanin. Wykazuje ona około 2,5 razy większą aktywność antyoksydacyjną niż betanina, występująca w burakach ćwikłowych w największych ilościach. Również aktywność antyoksydacyjna betanidyny jest większa (1,9 razy na 1 mg) niż aktywność antyoksydacyjna betaniny. Także aktywność antyoksydacyjna izobetaniny jest nieco większa niż betaniny. Przechowywanie neobetaniny przez 24 h w roztworze wodnym w temperaturze pokojowej z dostępem światła spowodowało niemal całkowitą degradację tego barwnika.
**Wnioski.** Stwierdzono, że przechowywanie suchego preparatu betalain o pH 7,0 powoduje przekształcenie betaniny w żółto-pomarańczową neobetaninę, która wykazuje bardzo dużą aktywność przeciwutleniającą, jednak mała stabilność tego barwnika w czasie przechowywania wyklucza jego stosowanie do barwienia żywności.

**Słowa kluczowe:** burak ćwikłowy, przechowywanie, neobetanina, zdolność antyoksydacyjna

*Corresponding address – Adres do korespondencji:*
*Katarzyna Mikołajczyk-Bator, Katedra Przyrodniczych Podstaw Jakości, Uniwersytet Ekonomiczny w Poznaniu, al. Niepodległości 10, 61-875 Poznań, Poland, e-mail: Katarzyna.Mikolajczyk-Bator@ue.poznan.pl*

*Accepted for publication – Zaakceptowano do opublikowania:*
*30.03.2018*

*For citation – Do cytowania:*
*Mikołajczyk-Bator, K., Czapski, J. (2018). Changes in the content of betalain pigments and their antioxidative capacity during storage. Nauka Przyr. Technol., 12, 1, 113–129. http://dx.doi.org/10.17306/J.NPT.00232*