JAP&T
TPA-Timişoara

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Journal of Agroalimentary Processes and Technologies 2010, *16* (1), 36-40

Journal of Agroalimentary Processes and Technologies

Thermal stability of anthocyanins from *Vaccinium myrtillus* L. methanolic extract

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Received: 20 December 2009; Accepted: 02 February 2010

Abstract

The purpose of this study was to investigate the anthocyanins stability of *Vaccinium myrtillus L.* fruits methanolic extract during heating over the temperature range 50-100ºC. The obtained results indicate that the monomeric anthocyanins degradation followed first-order reaction kinetics. The rate constant (k) for anthocyanins degradation, the corresponding half-life values ($t_{1/2}$) and the activation energy (Ea) were also calculated. The anthocyanins stability during storage was determined at -18, 4 and 25 ºC. Antioxidant activities of the extract after thermal exposure (90 and 100ºC) and after 4 months of storage were also evaluated using DPPH radical scavenging assay.

Keywords: anthocyanins, *Vaccinium myrtillus,* thermal degradation, stability, antioxidant activity

1. Introduction

Bilberries (*Vaccinium myrtillus* L.) represent a rich source of phenolic compounds, especially anthocyanins both in quantity and diversity of chemical composition [1-3].

Anthocyanins are the most important group of water-soluble pigments responsible for red, purple, blue and orange color of fruits, vegetables and flower. There is a worldwide interest for use of these pigments as natural food colorants as a consequence of legislative limitations of synthetic dyes. Besides colorant properties, interest for anthocyanins has intensified because their possible health benefits that might arise from their potent antioxidative nature [4-6].

Anthocyanins, like other natural pigments, present a low stability in various environmental conditions. Many factors influence the stability of anthocyanins including pH, temperature, light, oxygen, solvents, the presence of enzymes, ascorbic acid, sugars, copigments, metal ions etc. [7]. Degradation of anthocyanins may occur during extraction, purification, processing or storage.

Temperature is one of the most important factors that influence the stability of the anthocyanins and the rate of degradation, this being the main problem for the applications in food industry. Thermal stability of anthocyanins has been studied for different fruits and vegetables such as blackberries [8], raspberries [9,10], grapes [11], blood orange [12], pomegranate [13], plum [14], red cabbage [15], black carrots [16,17].

In this study, the effect of temperature on the stability of anthocyanins from *Vaccinium myrtillus* L. fruits extract has been studied. Extraction of the pigments was carried out with acidified methanol in ultrasonic conditions. The thermal degradation of anthocyanins was studied during heating and storage of the extract

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at various temperatures. At each temperature and regular time intervals the monomeric anthocyanin content was determined. Also, the degradation kinetic parameters have been evaluated. The anthocyanins degradation could affect the overall antioxidant capacity of the bilberries extract. Therefore, the antioxidant capacity of the bilberries extract was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging method [18].

2. Material and methods

Extraction of anthocyanins.

The ultrasonication method in 99.9% methanol (Sigma-Aldrich) acidified with 0.1% HCl (Merck, 37%), at 25°C, 59 kHz, 60 min. (ultrasonic bath FALC Instruments - Italy) has been used. A stock sample was obtained from 250 g of fresh bilberries (Borlova, Muntele Mic county, harvested in 2009) treated with 500 ml extracting material (solid to solvent ratio 1:2 w/v). After filtration through a Whatman no. 1 filter paper, the methanolic extract has been concentrated in a rotary evaporator at <40°C under vacuum (40-45 mbar) until complete solvent evaporation.

Degradation studies.

The thermal stability of anthocyanins from bilberries was studied at 50, 60, 70, 80, 90 and 100°C. Aliquots of 1 mL bilberries extract were introduced into test tubes well capped to avoid evaporation and placed in a drying chamber with digital adjustment of temperature, preheated to selected temperature. At regular time intervals (10, 40, 70, 100, 130, 160, 190 and 220 min), the tubes with sample were removed from the drying chamber and rapidly cooled into an ice-water bath to stop thermal degradation. Each sample was immediately analyzed for monomeric anthocyanins content. The storage stability of anthocyanins was studied at -18, 4 and 25°C during 122 days. From time to time the stored samples were analyzed for residual anthocyanins content.

Total anthocyanins determination.

Total monomeric anthocyanins content was quantified using the pH differential method described by Giusti and Wrolstad [19]. Samples were diluted in two buffer solutions: potassium chloride buffer 0.025 M (pH 1.0) and sodium acetate buffer 0.4 M (pH 4.5) and then it has been made the measurements of absorbance at 516 nm and 700 nm (to correct for haze). Absorbance readings were made at room temperature against distilled water as blank. A Jasco V 530 UV-Vis spectrophotometer was used for measurements.

The monomeric anthocyanin pigment concentration was calculated according to the following equation:

$$C(mg/l) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times l} \qquad (1)$$

where: $A=(A_{516}-A_{700})_{pH\ 1.0}-(A_{516}-A_{700})_{pH\ 4.5}$, MW is the molecular weight, DF is the dilution factor, ε is the molar absorbance and l is the pathlength (1 cm). Pigment content is calculate as cyaniding-3-glucoside equivalents (MW=449.2 and ε=26900). Each sample was analyzed in duplicate and the results were expressed as the averages of the two measurements.

Free radical scavenging activity.

The free radical scavenging activity of the bilberries extracts was perform by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay according to the procedure described by Brand-Williams et al. [18] with some modifications. This assay is based on the spectrophotometric measurements of the loss of DPPH colour caused by consumption of DPPH radical by antioxidant species present in the sample.

In order to evaluate antioxidant activities, each sample has been diluted 1:25 v/v with methanol, so that concentration differences between samples were maintained. Antioxidant solution in methanol (0.1 ml) was added to 2.9 ml of a solution $6 \cdot 10^{-5}$ mol/l DPPH in methanol.

The reduction of DPPH was followed by monitoring the decrease of absorbance at 515 nm during 6 hours. A Jasco V 530 UV-Visible spectrophotometer was used for measurements.

3. Results and discussion

Anthocyanins degradation during heating.

Variation of the monomeric anthocyanins concentration in time at 50, 60, 70, 80, 90 and 100°C is shown in Figure 1. The thermal degradation of monomeric anthocyanins followed first-order reaction kinetics. In all cases the r^2 values were higher than 0.90 indicating a good data fit.

Our results are in agreement with previous studies reporting first-order reaction kinetics for the thermal degradation of anthocyanins from different sources [8,12,16].

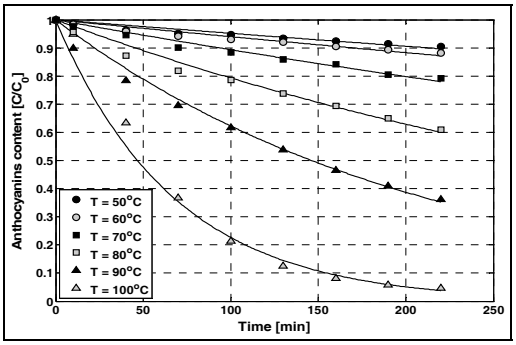

Figure 1. Degradation of anthocyanins in bilberries extract during heating

The first-order reaction rate constant (k) at each temperature was calculated by the following equation:

$$C_t = C_0 \cdot \exp(-k \cdot t) \quad (2)$$

where C_0 is the initial monomeric anthocyanins concentration and C_t is the anthocyanins concentration after time (t) of heating.

The half-lives (time necessary for 50% degradation of anthocyanins) were calculated using the following equation:

$$t_{1/2} = -\ln 0.5 / k \quad (3)$$

Kinetic parameters for thermal degradation of anthocyanins are given in Table 1. As expected, the degradation rate of anthocyanins increased with increasing heating temperature and time. The total anthocyanins content significantly decreases during heating at 90°C and 100°C.

Table 1. The values of rate constants and half-lives for anthocyanins degradation at different temperatures

Temperature [°C]	$k \cdot 10^3$ [min^{-1}]	$t_{1/2}$ [h]
50	0.405 (0.908)*	28.6
60	0.622 (0.933)	18.6
70	1.128 (0.974)	10.2
80	2.319 (0.982)	5.0
90	4.742 (0.992)	2.4
100	14.843 (0.990)	0.8

* Numbers in parentheses are the determination coefficients

The dependence of the degradation rate constant on temperature was represented by the Arrhenius equation (4):

$$k = k_0 \cdot e^{-E_a / RT} \quad (4)$$

where: k_0 is the frequency factor (min^{-1}), E_a is the activation energy (kJ/mol), R is the universal gas constant (8.314 J/mol/K) and T is the absolute temperature (K). The effect of the temperature on the anthocyanins degradation rate constant is shown in Figure 2. The calculated value of the activation energy was 70.56 kJ/mol ($r^2 = 0.963$).

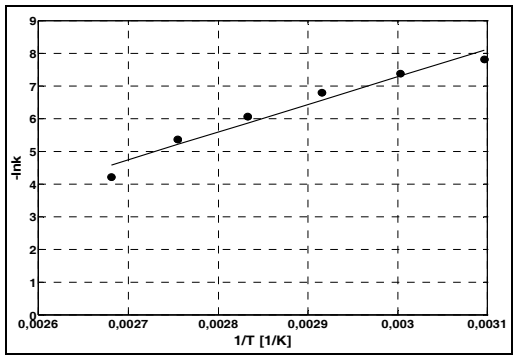

Figure 2. The Arrhenius plots for degradation of anthocyanins in bilberries extract during heating

Anthocyanins degradation during storage.

The degradation of anthocyanins from bilberries extract was also studied during long-term storage at -18, 4 and 25°C (Figure 3).

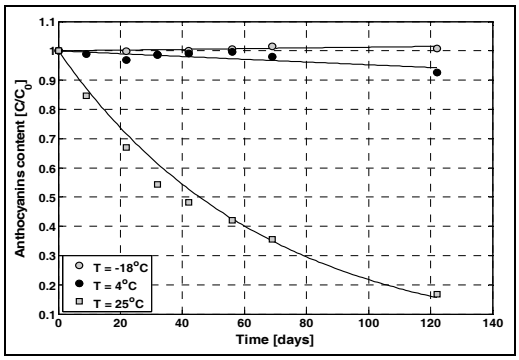

Figure 3. Degradation of anthocyanins in bilberries extract during storage

Storage temperature had a strong influence on the degradation of anthocyanins. Total monomeric anthocyanins content decreases much faster at 25°C compared to refrigerated storage at 4°C. The degradation of anthocyanins during storage at 25°C was also fitted to a first-order reaction kinetic. The following kinetic parameters were obtained: k = 0.0152 day^{-1} ($r^2 = 0.984$) and $t_{1/2}$ = 45.5 days. After 4 months of storage, the anthocyanins losses from

bilberries extract were about 7% at 4°C and 83% at 25°C. There was no anthocyanins degradation during storage at -18°C.

Free radical scavenging activity.

The antioxidant activity of the extract was estimated by the ability to scavenging the DPPH radical. The DPPH concentration in the reaction medium was calculated from the calibration curve with the following equation determined by linear regression ($r^2 = 0.99987$) [20]:

$$A_{515} = 11048 \cdot c_{DPPH} + 3.6828 \cdot 10^{-3} \quad (5)$$

For each sample, the amount of the remaining DPPH expressed as a percentage was calculated as:

$$Remaining\ DPPH\ [\%] = \frac{(c_{DPPH})_t}{(c_{DPPH})_{t=0}} \cdot 100 \quad (6)$$

where $(c_{DDPH})_t$ is the value of DPPH concentration in the presence of extract at time *t*.

The percentage of remaining DPPH concentration against reaction time for the extract at three selected temperatures is illustrated in Figure 4. In Table 2 is presented the percentage of remaining DPPH concentration after 6 hours. The lower this value, the higher is antiradical efficiency.

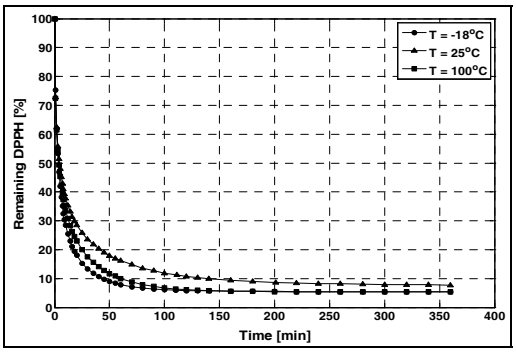

Figure 4. DPPH scavenging activity of anthocyanins in bilberries extract

It was observed minor differences of antioxidant activity after 122 days between the extract stored at -18°C and those stored at 4°C. Even if the anthocyanins content exhibits a major decrease during storage at 25°C, the antioxidant activity was not significantly modified.

Most interestingly, after 220 minutes heating at 90°C and 100°C, the antioxidant activity of extract was almost unchanged compared to the extract stored at -18°C. This behavior can be explained by cleavage of covalent bonds or enhanced oxidation reactions due to thermal processing.

The resulting polyphenolic degradation products may also possess antioxidant properties [21].

Table 2. The percentage of remaining DPPH concentration after 6 hours

Nr. Crt.	Temperature [°C]	Remaining DPPH at the time t = 6 h [%]
1.	- 18°C, after 122 days	5.37
2.	4 °C, after 122 days	5.72
3.	25 °C, after 122 days	7.72
4.	90°C, after 220 min. heating	5.51
5.	100°C, after 220 min. heating	5.31

4. Conclusions

The present study revealed that in general high temperatures and long storage periods affect the anthocyanins content in crude (unpurified) bilberries extracts.

As expected, a very fast degradation occurred with temperature increasing: 70-100°C. The obtained results indicate that the monomeric anthocyanins degradation followed first-order reaction kinetics.

During storage at -18°C and 4°C, anthocyanins showed a high stability. A fast degradation of bilberries anthocyanins occurs in the sample stored at 25°C.

The antioxidant activity is not correlated with the decrease of monomeric anthocyanins content. High DPPH radical scavenging activities of bilberries extract was obtained after thermal processing; this may by possible if formed degradation products have also antioxidant properties.

Acknowledgments

This work is part of the project no. 52145 / 2008 - "Hypoglycemic and antioxidant dietary supplements with anthocyanidinic structure - SAHASA", carried out under the 4th Programme-Partnerships in priority areas of The National Plan for Research-Development and Innovation 2007-2013 (Romania).

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