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Study of anthocyanins from *Vaccinium Myrtillus* L. frozen fruits

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Abstract

The paper proposes the anthocyanins extraction from *Vaccinium Myrtillus* L. frozen fruits. Experiments were performed in solvents like methanol, ethanol using two different extraction methods. The extracts have been analyzed by UV-VIS technique, FT-IR and high-performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 apparatus equipped with photodiode array detector for characterized the anthocyanins. It has been identified a number of fifteen anthocyanins in bilberries extracts. Qualitative composition of anthocyanidins was performed after acidic hydrolysis of extracts.

Keywords: anthocyanins, *Vaccinium Myrtillus* L., fruits, extraction, FT-IR, UV-Vis, HPLC

1. Introduction

Anthocyanins represent one of the most widely distributed classes of flavonoids in plants. They are water-soluble pigments responsible for red, purple, blue and orange color of fruits, vegetables and flower. Interest for anthocyanins has intensified because their possible health benefits, one of the important properties of flavonoids being their strong antioxidant activity in metabolic reaction [1,2].

The physiological functions of *Vaccinium Myrtillus* L. fruits may be attributed to their abundance of phenolics, including anthocyanins. Phenolic compounds possess a wide spectrum of biochemical activities, such as antioxidant, antimutagenic, anticargionogenic effects, as well as ability to modify gene expression. Also, phenolics in the human diet may exert the protection against coronary heart disease, diabetic retinopathy, oxidative stress, hepatotoxicity and atherosclerosis [1,2,3].

The aim of this study is to investigate the total anthocyanins content and anthocyanins composition in *Vaccinium Myrtillus* L. fruits collected in 2008 from Cluj County (Romania).

2. Material and methods

Extraction of Anthocyanins.

Two different extraction methods were tested: the maceration in two steps at 4°C (50 ml fresh extracting solvent for 48 hours, plus 50 ml fresh extracting solvent for 24 hours) and ultrasonication at 25°C, 59 kHz, 60 min. (ultrasonic bath FALC Instruments - Italy). The methanol acidified with 0.1% HCl and ethanol acidified with 0.1% HCl have been used. The following notations for the obtained extracts have been used: A – MeOH macerated sample, B – EtOH macerated sample, C – MeOH sonicated sample and D – EtOH sonicated sample.

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25 g of *Vaccinium Myrtillus* L. frozen fruits were treated with 100 ml extracting material for each experiment (solid to solvent ratio 1:4 w/v). After filtration, the extracts were evaporated under vacuum (40-45 mbar) at 40°C until complete solvent evaporation.

Hydrolysis of anthocyanins.

To 5 g extract, 10 ml CH_3OH, 2.5 ml HCl 37% (Merck) and 1 ml H_2O ultra pure water were added. Mixture was heated under reflux and under stirring for 3 hours, then centrifuged and filtered.

Total Anthocyanins Determination.

Total anthocyanins were quantified using the pH differential method described by Giusti and Wrolstad [4]. This method, based on reversible structural transformations of anthocyanin pigments in different pH solutions [5], permits a rapid and accurate measurement of the total anthocyanins even in the presence of polymerized degraded pigments and other interfering compounds.

A Jasco V 530 UV-Vis spectrophotometer was used for measurements. Samples were diluted in buffer solutions of pH 1.0 (potassium chloride buffer) and pH 4.5 (sodium acetate buffer) and then it has been made the measurements of absorbance for each solution at 514 nm and 700 nm (to correct for haze). The absorbance (A) was calculated using the following relation:

$$A = (A_{515} - A_{700})_{pH\,1.0} - (A_{515} - A_{700})_{pH\,4.5} \quad (1)$$

The monomeric anthocyanin pigment concentration in the original sample was calculated using the following formula:

$$C\,(mg/l) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times l} \quad (2)$$

where MW is the molecular weight , DF is the dilution factor, ε is the molar absorbance (26900) and l is the pathlength (1 cm). Each sample was analyzed in duplicate and the results were expressed as the averages of the two measurements.

HPLC Analysis of Anthocyanins and Anthocyanidins.

Analysis of anthocyanins was performed by applying a previously developed method [6]. Chromatographic system consists of Dionex Ultimate 3000 apparatus (Dionex Corporation, USA) equipped with a photodiode array (PDA) detector, a 20 µl injection loop and a 4.6 x 150 mm, 5 µm C-18 Acclaim® 120 Silica-Based reversed-phase column (Dionex Corporation). Chromatograms were recorded and processed with Chromeleon software. The column was maintained at 40°C using a thermostatted column compartment Dionex TCC-3000.

For HPLC analysis of anthocyanins, fruits extract was dissolved (1:25 v/v) in 0.5% trifluoroacetic acid (TFA). The mobile phase consists in an aqueous solution of 20% methanol containing 0.5% TFA. The program run in isocratic conditions at a flow rate of 1.5 ml/min.

Anthocyanidins analysis has been performed using hydrolyzed extract (1:5 v/v in 0.5% TFA). In this case, the mobile phase consists in 90% solvent A – aqueous solution of 20% methanol containing 0.5% TFA and 10% solvent B – methanol. The flow rate was kept constant at 2 ml/min using isocratic conditions.

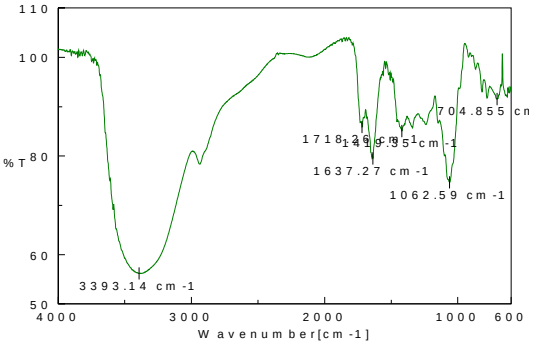
Chromatographic profiles were recorded at 520 nm for both anthocyanins and anthocyanidins evaluation. All reagents were HPLC grade and were obtained from Sigma-Aldrich, Germany. Ultra pure water was obtained by an EASYpure® RoDi system for water purification.

3. Results and discussion

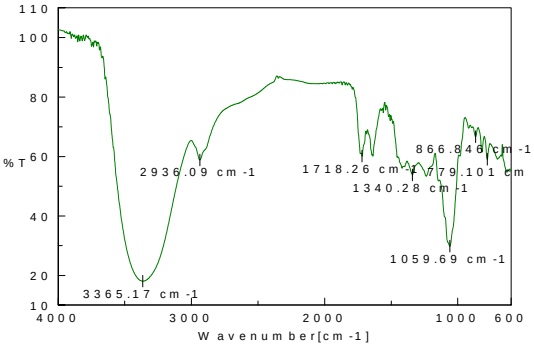
The obtained extracts have been characterized by FT-IR spectra, UV-VIS technique, and high-performance liquid chromatography (HPLC). FT-IR spectra were made by Jasco FTIR 430 apparatus (Figure 1).

It can be seen that there are intensive bands in the 1059-1062 cm^{-1} span that is characteristic of glycoside bond. The

intensive bands 3365-3393 cm^{-1} are attributed ν_{OH} of associated –OH (ν_{OH} sugar vibration band expected frequency about 3400 cm^{-1}). At 2830-2936 cm^{-1} there are the bands of ν_{CH} vibrations of $-CH_2$ and δ_{CH} at 1450-1475 m. The intensive band at 1718 and 1637-1650 cm^{-1} is most probably the result of $\nu_{C=O}$ vibrations of C=O group of aglicon-pyranoside (galactoside, glucoside, arabinoside, etc.) or some vitamins, while the ν_{C-O} vibration occurs at 1240-1300 cm^{-1}. In this regard, the FT-IR technique was not enough to diagnostic the differences between these extracts.



a.



b.

Figure 1. IR spectrum of extract A (a) and extract B (b)

The anthocyanins content in frozen fruits of *Vaccinium Myrtillus* L. were determined by a pH differential method. Anthocyanins present a maximum absorbance at a wavelength of around 520 nm at pH 1.0. At pH 1.0, the flavylium cation is the predominant species and has a red color, in aqueous media at pH 4.5 hydration reaction generates the colorless carbinol pseudo-base (Figure 2). The color change of anthocyanins with pH is exemplified by visible spectra of fruits extract A in buffer solution with pH 1.0 and 4.5 (Figure 3).

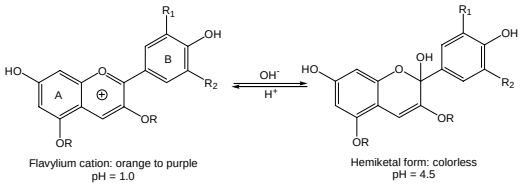


Figure 2. Structural forms of anthocyanins at pH 1.0 and pH 4.5

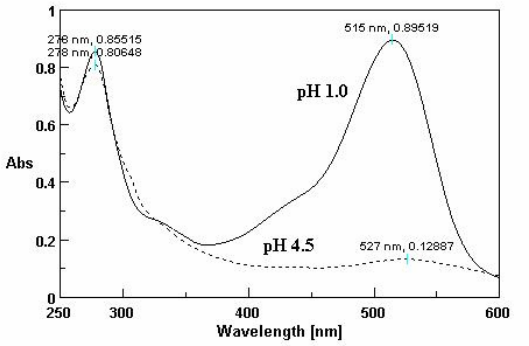


Figure 3. Spectral characteristics of *Vaccinium Myrtillus* L. fruits extract A in pH 1.0 and pH 4.5 buffers

The anthocyanins concentration in *Vaccinium Myrtillus* L. extracts obtained by the two different extraction methods is presented in Table 1. Pigment content is calculate as cyaniding-3-glucoside equivalents (MW=449.2 and ε=26900).

Table 1. Total anthocyanins content in *Vaccinium Myrtillus* L. fruits extract

Extract	A	B	C	D
Total anthocyanins C [mg/l]	26259	25700	14687	9779

Qualitative composition of anthocyanins in bilberries extracts was evaluated by HPLC analysis. For all extracts, the HPLC chromatograms obtained at 520 nm revealed 15 peaks. In Figure 4 is presented the chromatogram for the extract A. Identification of detected components, presented in Table 2, was performed according to the literature data [6].

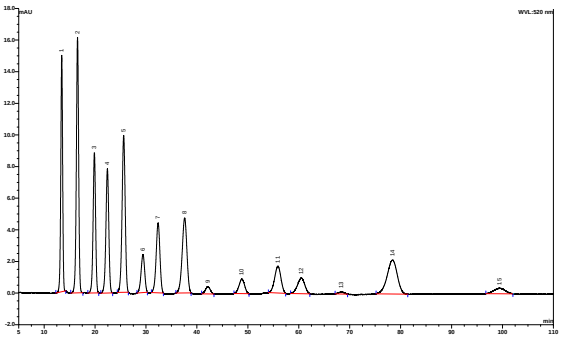


Figure 4. HPLC chromatogram at 520 nm for extract A

Table 2. Chromatographic separation of anthocyanins

Peak No.	Compound	Retention time, min.
1	delphinidin-3-galactoside	13.454
2	delphinidin-3-glucoside	16.557
3	cyanidin-3-galactoside	19.863
4	delphinidin-3-arabinoside	22.426
5	cyanidin-3-glucoside	25.616
6	petunidin-3-galactoside	29.398
7	cyanidin-3-arabinoside	32.388
8	petunidin-3-glucoside	37.596
9	peonidin-3-galactoside	42.185
10	petunidin-3-arabinoside	48.821
11	peonidin-3-glucoside	55.933
12	malvidin-3-galactoside	60.508
13	peonidin-3-arabinoside	68.391
14	malvidin-3-glucoside	78.429
15	malvidin-3-arabinoside	99.431

The first method applied in HPLC analysis of anthocyanidins was the same used in anthocyanins separation. The disadvantage of this method was the long separation time (only three anthocyanidins were recorded in three hours). In order to improve the analysis conditions, the method was modified by increasing the methanol content in mobile phase, so the time of analysis was reduced to less then 80 min.

The chromatogram of anthocyanidins is presented in Figure 5. Five major peaks were separated (Table 3).

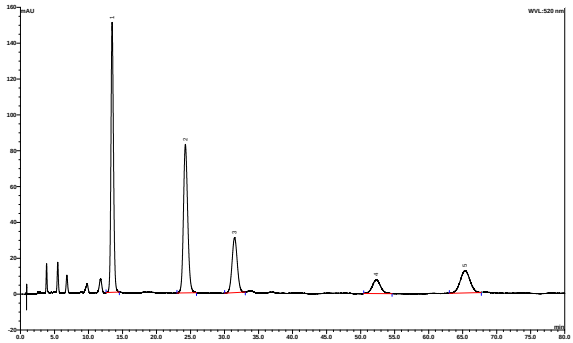


Figure 5. HPLC chromatogram at 520 nm for hydrolyzed extract A

Table 3. Chromatographic separation of anthocyanidins

Peak No.	Compound	Retention time, min.
1	Delphinidin	13.471
2	Cyanidin	24.241
3	Petunidin	31.478
4	Peonidin	52.313
5	Malvidin	65.364

Along these some small quantities of unhydrolyzed anthocyanins have been recorded. Identification of delphinidin, cyanidin and malvidin was performed by comparison with chloride standards (Fluka), peonidin and petunidin were assigned according to literature data [7]. In all samples, delphinidin was found in the highest quantities, followed by cyanidin, petunidin, malvidin and peonidin.

4. Conclusions

The anthocyanins content in *Vaccinium Myrtillus* L. fruits depend on type of solvent and applied extraction method. The best results were obtained with acidified methanol and ethanol in maceration

technique. The ultrasonic method is also appropriate due to reduced processing time and simplicity.

It has been identified a number of fifteen major compounds in bilberries extracts and five anthocyanins aglycons obtained by acidic hydrolysis, confirming the literature data.

This work is part of the national project "Hypoglycemic and antioxidant dietary supplements with anthocyanidinic structure" carried out under the 4th Programme-Partnership in priority areas of the National Plan for Research-Development and Innovation 2007-2013. The further objectives regard: separation, identification and quantitative determination of anthocyanins and anthocyanidins from other fruits like black currant, red currant, raspberry, strawberry, mulberry, grapes and blackberry, determination of their "in vitro" antioxidant activity and "in vivo" testing of extracts on rats with experimentally induced diabetic disease.

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