Ovidius University Annals of Chemistry Volume 21, Number 1, pp.15-19, 2010

Comparative analysis of bilberries alcoholic extracts regarding to anthocyanins content, total phenolics and antioxidant activity

Adina CĂTA*, Mariana N. ŞTEFĂNUŢ, Cristian TĂNASIE and Raluca POP

National Institute of Research-Development for Electrochemistry and Condensed Matter, Str. Dr. A. P. Podeanu 144, 300569 Timişoara, Romania

Abstract The aim of this study was to investigate and compare *Vaccinium myrtillus* L. extracts obtained in ultrasonic condition with different water/methanol and water/ethanol extraction mixture acidified with 0.1% HCl. The extracts were analyzed for monomeric anthocyanins contents, total phenolics content and antioxidant activities. The highest anthocyanins content (3888 mg/L), total phenolics content (6325 mg GAE/L) and the best antioxidant activity were obtained for the bilberries extract with 100% methanol. Also, there is a good correlations between antioxidant activitiy and total phenolics content (R^2 = 0.9763) for water/methanol series extracts.

Keywords: bilberries, anthocyanins, total phenolics, 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 [1-4].

Anthocyanins are the most important group of water-soluble pigments responsible for red, purple, blue and orange colour of fruits, vegetables and flower. Up to date, more than 635 different anthocyanins [5] are reported. They are glycosides of anthocyanidins, mainly cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin [6].

Anthocyanins were incorporated into the human diet many centuries ago. Anthocyanin-rich mixtures and extracts, though did not contain purified compounds, have been used historically to treat various diseases such as hypertension, pyrexia, liver disorders, dysentery and diarrhea, urinary problems including kidney stones and urinary tract infections, common cold, etc [7]. There is an explosive interest for use of anthocyanins as potential nutritional supplements for human diet. Regular consumption of anthocyanins and other polyphenol from fruits, vegetables, juices, wines, jams, and preserves is associated with probable reduced risks of chronic diseases such as cancer, diabet, cardiovascular diseases, neuronal diseases, virus inhibition, Alzheimer's diseases. Anthocyanins and other flavonoids are regarded as important nutraceuticals mainly due to their antioxidant effect, which gives them a potential role in prevention of various diseases associated with oxidative stress [8].

Extraction of phenolics from fruits is, usually, performed by solvent extraction. The most common solvents used for anthocyanins extraction are aqueous mixtures of ethanol, methanol or acetone [9]. For quantitative information regarding the anthocyanins content in crude extracts containing other phenolic compounds, the spectroscopy is the main technique used due to its simplicity and low cost [6].

In this study is presented a comparative analysis regarding the effect of water: alcohol ratios of the extracting system on total monomeric anthocyanins content, total phenolics content and antioxidant activity of *Vaccinium myrtillus* L. fruits extracts. Our study also demonstrates a possible relationship between antioxidant activity and both anthocyanins and phenolics content.

2. Experimental

2.1. A*nthocyanins extraction*

Anthocyanins extraction was carried out with different water/alcohol solutions acidified with 0.1% HCl (Merck, 37%) in ultrasonic conditions for 60 minutes at 25ºC and 59 kHz (ultrasonic bath FALC

ISSN-1223-7221 © 2010 Ovidius University Press

Instruments - Italy).

25 g of bilberries frozen fruits (Borlova, Caraş-Severin County, harvested in 2009) were treated with 100 mL aqueous methanol, and aqueous ethanol respectively, as extracting material (solid to solvent ratio 1:4 w/v). The water : methanol (99.9%, Sigma-Aldrich) and water : ethanol (food grade, 96%) ratios (v/v) were as follows: 100:0, 80:20, 60:40, 40:60, 20:80 and 0:100. After filtration through a Whatman no. 1 filter paper, the extract has been concentrated in a rotary evaporator (Laborota 4000 Efficient, Heidolph, Germany) at <40°C under vacuum (40-45 mbar) until complete solvent evaporation. For an accurate comparative analysis of extracts, they were brought to the same volume (25 mL) with methanol or ethanol, respectively.

2.2. *Determination of total anthocyanins*

Total monomeric anthocyanins content was quantified using a pH differential method described by Giusti and Wrolstad [10]. 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 the absorbance was measured simultaneously at 516 nm and 700 nm (to correct for haze) after 15 minutes of incubation at room temperature. 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). The total monomeric anthocyanins content was expressed 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.

2.3. *Determination of total phenolics*

The total phenolics content of the bilberries extracts were determined by using the Folin-Ciocalteu method [11] and gallic acid as standard. This assay is based on chemical reduction of the Folin-Ciocalteu reagent, a phosphowolframate - phosphomolybdate complex, to blue coloured products by phenolic compounds. The intensity of blue colour is proportional to the concentration of phenolic compunds.

Briefly, 200 µL of each extract (previously diluted 1:10 with double distilled water) or standard solution, 15 mL dd water and 1 mL Folin-Ciocalteu reagent were added to a 20 mL volumetric flask. The contents were mixed and incubated for 5 min at room temperature. Then, 3 mL of 20% (w/v) sodium carbonate solution was added, followed by the addition of dd water to volume and mixing. After incubation for 2 hours at room temperature, the absorbance at 765 nm using a Jasco V 530 UV-Vis spectrophotometer was determined against a blank reagent prepared with dd water.

Gallic acid (GA) was used as standard. The calibration curve was obtained using 10 standard solutions in the range 50-550 mg/L gallic acid. Total phenolics content of the extracts was calculated from the calibration curve (the absorbance at 765 nm vs. gallic acid solution concentration) with the following equation determined by linear regression:

$$A = 1.2335 \cdot 10^{-3} \cdot C - 0.0505\ (R^2 = 0.9972) \qquad (2)$$

Total phenolics content was expressed as mg gallic acid equivalents per litter of fruit extract (mg GAE/L). All samples were analysed in duplicate and the results were expressed as the averages of the two measurements.

2.4. *Determination of antioxidant 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. [12] 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 1:20 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 inhibition of DPPH was followed by monitoring the decrease of absorbance at 515 nm during 4 hours.

3. Results and Discussions

In this paper, an ultrasound-assisted extraction method and different acidified water/alcohol mixtures were used to obtain the bilberries extracts.

The obtained extracts were analyzed to determine the monomeric anthocyanins content, total phenolics content and antioxidant activity.

The changes in total anthocyanins content depending on the water/alcohol ratio are presented in **Fig. 1.**

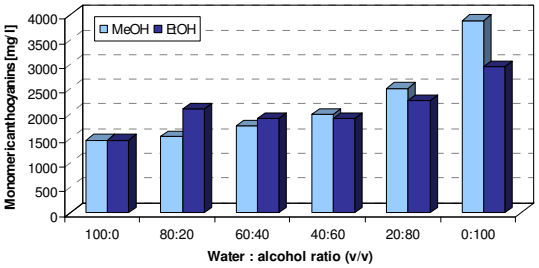

Fig.1. Comparison of anthocyanins content from extracts obtained in water/methanol and water/ethanol systems

The monomeric anthocyanins content increases with increasing the percentage of methanol in the extraction system. This tendency is not observed for water/ethanol extraction, where high values were obtained for 80 % water/20% ethanol extracting system. The amount of monomeric anthocyanins in bilberries extracts ranged from 1462 mg/L to 3888 mg/L for water/methanol extraction, and from 1462 mg/L to 2953 mg/L for water/ethanol extraction.

The results obtained for total phenolics content is shown in **Fig. 2.**

The same increasing trend of total phenolics content with increasing the percentage of alcohol was observed for extracts in water/methanol system. In the case of water/ethanolic extraction, the highest phenolics content were obtained for the extract with 0:100 water to ethanol ratio, followed by the extracts with 20:80 and 80:20 water to ethanol ratio.

The bilberries extracts represent a rich source of phenolic compounds, the total phenolics content varied from 3104 mg GAE/L to 6325 mg GAE/L for water/methanol extraction and from 3104 mg GAE/L to 5226 mg GAE/L for water/ethanol extraction.

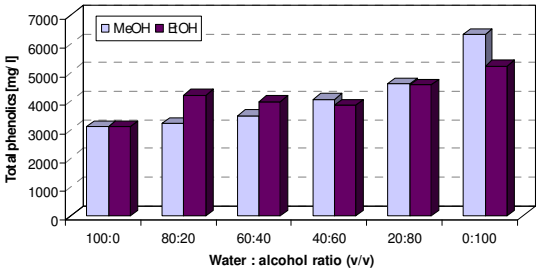

Fig.2. Comparison of total phenolics content from extracts obtained in water/methanol and water/ethanol systems

The antioxidant activity of the extracts 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 ($R^2 = 0.99987$):

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

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

$$\text{Re}maining\ DPPH\ [\%] = \frac{(c_{DPPH})_t}{(c_{DPPH})_{t=0}} \cdot 100 \quad (4)$$

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

The scavenging of the free radical by the studied extracts show similar pattern curves of remaining DPPH versus time. The reaction occurs rapidly in the first minutes and then slowed. The lower step can be due to the antioxidant properties of the slow reacting components originally present in the sample and/or due to the reaction products formed during rapid phase [13]. The percentage of remaining DPPH concentration against reaction time is exemplified in **Fig. 3** for the extracts obtained in water/methanol system.

In **Table 1** is presented the percentage of remaining DPPH concentration after 4 hours of reaction between the extracts and DPPH radical for the two studied cases. The lower this value, the higher is antiradical efficiency.

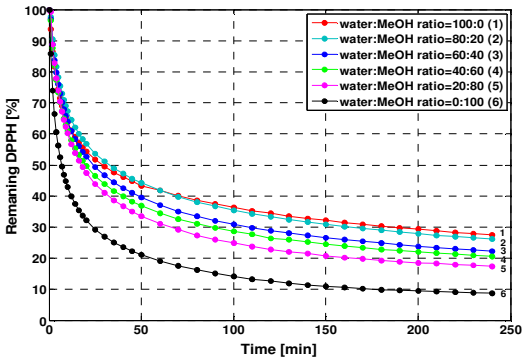

Fig.3. DPPH scavenging kinetic curves for bilberries extracts obtained in water/methanol system

Table 1. The percentage of remaining DPPH concentration after 4 hours

Water:alcohol ratio (v/v)	Remaining DPPH [%]	
	Water/MeOH system	Water/EtOH system
100:0	27.52	27.52
80:20	26.14	17.72
60:40	22.26	18.85
40:60	20.63	17.11
20:80	17.45	14.38
0:100	8.75	15.14

In the methanolic extracts series, the antioxidant activity of bilberries extracts increases with increasing the percentage of methanol in the extraction system. In the ethanolic extracts series, the higher antioxidant activity was obtained for 20 % water/80% ethanol extracting system, even if this extract does not present the highest content of anthocyanins or total phenolics. Comparing antioxidant activities of the bilberries extracts in the two cases, it is observed higher antioxidant activities in the ethanol series, except the extracts with 100% alcohol.

The correlations between antioxidant activitiy and monomeric anthocyanins content for the two extraction systems are presented in **Fig. 4.**

The following equations determined by linear regression regarding the relationship between antioxidant activity and anthocyanins content was obtained, Eq.(5) for methanol series and Eq.(6) for ethanol series. There is good correlation between antioxidant activity and anthocyanins content for the bilberries extracts from methanol series.

$$y = -0.0073 \cdot x + 36.507 \qquad (R^2 = 0.9645) \qquad (5)$$

$$y = -0.0074 \cdot x + 33.881 \qquad (R^2 = 0.5946) \qquad (6)$$

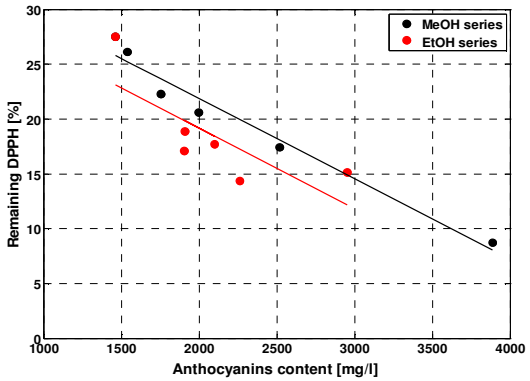

Fig.4. Correlation between anthocyanins content and antioxidant activity

In **Fig. 5** are shown the correlations between antioxidant activitiy and total phenolics content for the two studied extraction systems.

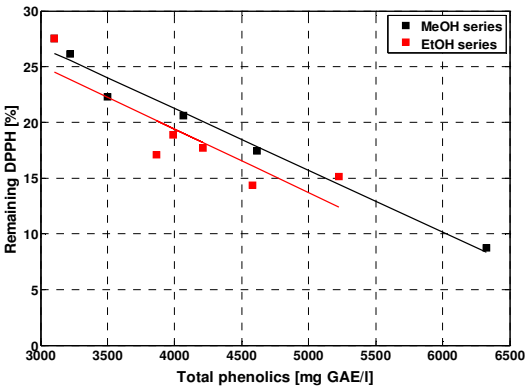

Fig.5. Correlation between total phenolics content and antioxidant activity

Linear relationships between antioxidant activity and total phenolics content are given by Eq.(7) for methanol series and Eq.(8) for ethanol series extracts.

$$y = -0.0056 \cdot x + 43.440 \qquad (R^2 = 0.9763) \qquad (7)$$

$$y = -0.0057 \cdot x + 42.201 \qquad (R^2 = 0.7386) \qquad (8)$$

Also, notice a good correlation between antioxidant activity and total phenolics content for methanol series extracts. The values of the determination coefficients are acceptable, indicating a suitable correlation by linear regressions between antioxidant activities and both anthocyanins and total phenolics content for bilberries extracts obtained in water/methanol systems. Lower values for determination coefficients were obtained in the of ethanol series extracts. This can be explained by the possible presence of other nonphenolics antioxidant compounds such as carotenoids, vitamins, etc. which can contribute to overall antioxidant activity of the extracts.

4. Conclusions

The performed studies indicate that bilberries extracts are a rich source of anthocyanins and phenolic compounds, and also possess a significant antioxidant activity. The best results regarding monomeric anthocyanins content, total phenolics content and antioxidant activity were obtained at extraction with 100% methanol. However, for food industry, the extractions with ethanolic solution are more convenient. The correlations antioxidant activity-anthocyanins content, and antioxidant activity-total phenolics content depend on the extraction solvent, the best determination coefficients was found for bilberries extracts obtained in water/methanol systems.

5. 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).

6. References

* E-mail address: adina.cata@yahoo.com

[1]. R.L. Prior, G. Cao, A. Martin, E. Sofic, J. McEwen, C. O'Brien, N. Lischner, M. Ehlenfeldt, W. Kalt, G. Krewer and C.M. Mainland, Journal of Agricultural and Food Chemistry **46**, 2686-2693 (1998).

[2]. D. Burdulis, L. Ivanauskas, V. Dirsė, S. Kazlauskas and A. Ražukas, Medicina (Kaunas) **43**, 971-977 (2007).

[3]. T. Ichiyanagi, Y. Hatano, S. Matsugo and T. Konishi, Chemical & Pharmaceutical Bulletin **52**, 628-630 (2004).

[4]. I. David, M.N. Ştefănuţ, A. Căta, I. Ienaşcu, R. Pop, C. Tănasie and I. Balcu, Journal of Agroalimentary Processes and Technologies **15**, 348-352 (2009).

[5]. J. He and M.M. Giusti, Annual Review of Food Science and Technology **1**, 163-187 (2010).

[6]. A. Castañeda-Ovando, Ma. de Lourdes Pacheco-Hernández, Ma. E. Páez-Hernández, J.A. Rodríguez and C.A. Galán-Vidal, Food Chemistry **113** (4), 859-871 (2009).

[7]. I. Konczak and W. Zhang, Journal of Biomedicine and Biotechnology **2004** (5), 239-240 (2004).

[8]. Ø.M. Andersen and M. Jordheim, The anthocyanins. In Ø.M. Andersen and K.R. Markham (Eds), *Flavonoids: Chemistry, biochemistry and applications*, CRC Taylor & Francis: Boca Raton, 2006, p. 472-537.

[9]. M.P. Kähkönen, A.I. Hopia and M. Heinonen, Journal of Agricultural and Food Chemistry **49**, 4076-4082 (2001)

[10]. M.M. Giusti and R.E. Wrolstad, Unit F1.2. *Anthocyanins. Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy, Current Protocols in Food Analytical Chemistry*, 2001, F1.2.1-F1.2.13., John Wiley & Sons, Inc.

[11]. A.L. Waterhouse, Unit I1.1. *Polyphenolics. Determination of Total Phenolics, Current Protocols in Food Analytical Chemistry*, I1.1.1-I1.1.8., 2002, John Wiley & Sons, Inc.

[12]. W. Brand-Williams, M.E. Cuvelier and C. Berset, Lebensmittel-Wissenschaft Und-Technologie **28**, 25-30 (1995).

[13]. D.I. Tsimogiannis and V. Oreopoulou, Innovative Food Science and Emerging Technologies **7**, 140-146 (2006).