TOTAL POLYPHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY FROM ANTHOCYANIN EXTRACTS

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The aim of this study was to characterize the alcoholic extracts of cranberry, wild blueberry and bluecrop in terms of individual polyphenolic content, total polyphenolic content and antioxidant capacity. Identification and quantification of anthocyanins in alcoholic extracts was performed using MALDI-ToF technique. Antioxidant activity was investigated using radicals DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid). From all the analyzed extracts the wild blueberry extract had the highest antioxidant activity (62.170 µM Trolox equivalents/g fresh weight) while the bluecrop extract had the smallest one (28.043 µM Trolox equivalents/g fresh weight).

Keywords: anthocyanins, polyphenolic content, antioxidant activity, MALDI-ToF

1. Introduction

It is generally known that polyphenols such polyphenolic acids and flavonoids may occur in plant tissues including the fruits, vegetables, soy and tea. Polyphenolic acids are attributed to two large classes of compounds: benzoic acid derivatives and cinnamic acid derivatives. Several studies provide information about the protection induced by polyphenolic acids against some degenerative diseases such us cancer, Alzheimer's illness or cardiovascular diseases. Their beneficial effects on health have been attributed mainly to their antioxidant properties [1-5]. Flavonoids represent the largest group of secondary metabolites usually present as glycosides in plants. They are classified in: flavonols, flavones, flavanols, flavanones, isoflavones, lignans, anthocyanins and proanthocyanins. Flavonoids play an important role in human diet because of their radical scavenging capacity, pharmacological activities and their estrogenic action.

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Belong to the flavonoids family anthocyanins are the most abundant water-soluble plant pigments, responsible for the red, blue and purple color of some fruits and vegetables. Due to the beneficial effects on health and because of their biological and pharmacological properties many scientists studied the occurrence and quantification of anthocyanidins in different categories of foods. The biological properties have been attributed to their antioxidant activity which had an important role in diabetes, cardiovascular and immune diseases and in cancer treatments. Anthocyanins have been studied from many years not only as antioxidants but also as food colorants with nutraceutical benefits [6-11].

Until now was reported more than 500 different anthocyanins but the most important are the six aglycones: dephinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin (Table 1).

According to the literature data there are several methods for identifying and quantifying the antocyanins in plant extracts: HPTLC (high performance thin layer chromatography), HPLC (high performance liquid chromatography), CE (capillary electrophoresis) and LC-MS (liquid chromatography-mass spectrometry) [12-17]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry is a powerful tool used for determination of molecular weight and for structural characterization of anthocyanins [18-21].

The aim of this study was to determine the total polyphenolic content and antioxidant activity in methanolic extracts of cranberry, wild blueberry and bluecrop and to characterize the anthocyanins profile using the MALDI-ToF technique.

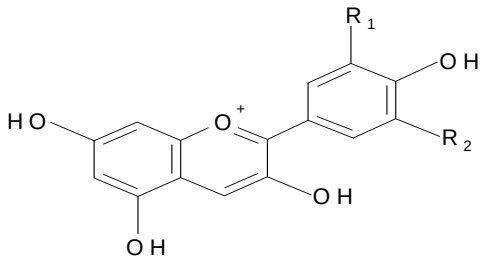

Table1

Chemical structures of anthocyanidins

Compound	**R_1**	**R_2**
Delphinidin	OH	OH
Cyanidin	OH	H
Petunidin	OCH3	OH
Pelargonidin	H	H
Peonidin	OCH3	H
Malvidin	OCH3	OCH3

2. Material and methods

2.1. Plant material

The cranberry, wild blueberry and bluecrop are fruits that belong to the *Vaccinium* genus and were purchased from the Romanian local market. 5 g of each frozen material were added to 10 mL of acidified methanol (0.01%HCl). The samples were kept in the dark at 4^0C for two days and then filtered.

2.2. Reagents

Methanol, acetonitrile, Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate, sodium carbonate, trifluoroacetic acid (TFA), delphinidin (Dp), cyanidin (Cy), petunidin (Pt), pelargonidin (Pg), peonidin (Pn) and malvidin (Mv) were purchased from Sigma-Aldrich (Germany). Ultrapure water was Mili Q grade (Milipore). Matrix and the calibration mixture used for MALDI, C104 (Bradykinin, Angiotensin II, Neurotensin, ACTH, Bovine Insulin chain B) were purchased from Bio-Laser.

2.3. Total polyphenolic assay

The total polyphenolic content in sample was determined using Folin-Ciocalteu method [22]. The reagent, 5 mL (diluted 1:10 w/w) was mixed with 1 mL diluted sample and 4 mL of 7.5% sodium carbonate. Samples were kept for 40 min at 50^0C and the absorbance was measured at 765 nm. The content of polyphenols was expressed as gallic acid equivalents per gram fresh weight of fruit (GAE/g FW).

2.4. Free Radical Scavenging Activity on DPPH

The antioxidant activity of the extracts was measured using DPPH free radical scavenging assay [23]. Sample (0.1 mL) was mixed with 1 mL of 25 mM DPPH solution and filled up with methanol to a final volume of 3 mL. The absorbance of the resulting solutions was recorded at 517 nm after 30 minutes of storing at room temperature. For each sample, three replicates were recorded. Inhibition of free radical by DPPH in percent (%) was calculated in the following way:

$$I\ (\%) = 100 \times [(A_{blank} - A_{sample})/A_{blank}]$$

where, A_{blank} is the absorbance of the control reaction mixture excluding the test sample and A_{sample} is the absorbance of the test sample. IC_{50} values which represented the concentration of samples that caused 50% neutralization of DPPH radicals, were calculated from the plot of inhibition percentage of DPPH against concentration.

2.5. Scavenging Activity of ABTS Radical Cation

The mixture containing ABTS stock solution (7 mM) and potassium persulfate (2.45 mM) was incubated at room temperature in the dark for 14-16 hours to obtained ABTS cation radical. Different concentrations of the extracts were added to ABTS solution to give a final volume of 3 mL and the absorbance was measured at 734 nm after 6 minutes of storing at room temperature [24, 25]. A calibration curve was obtained by absorbance reduction of solution in the presence of different concentrations of Trolox: $y = 8756.2x - 0.0248$, $R^2 = 0.9982$. The stable ABTS radical scavenging activity of the extracts was expressed as μM Trolox equivalent antioxidant capacity (TEAC) per gram FW.

2.6. MALDI-ToF equipment

Mass spectra were register on Axima CFR-Plus MALDI-ToF instrument from Shimadzu-Kratos. Desorption/ionization process was made by operating a nitrogen laser at 337 nm. The acceleration voltage was set to 20 with the delay time of 100 ns. Measurements were made in the positive-ion reflectron mode. All spectrums were analyzed using Lunchpad software. A standard mixture C104 (500-3500 Da) was used for the external mass calibration. Matrix used was alpfa-cyano-4-hydroxycinnamic acid (α-CHCA) with a concentration of 5 mg/mL in acetonitrile/ultrapure water/trifluoroacetic acid (50/50/0.1%). The anthocyanidins standards were prepared in methanol and were diluted in TFA 0.1%. All the samples were ten times diluted in triflouroacetic acid 0.1%. The dried droplet technique was applied; matrix and samples were mixed in 1:1 (v/v) ratio and was applied on the MALDI plate. Power laser was set at 52 for MALDI analysis. Tolerance for the recorded masses was ±0.5 Da.

3. Results and discussion

Total polyphenolic content of samples ranged from 5.34 mg GAE/g FW for bluecrop extract to 10.48 mg GAE/g FW for wild blueberry extract (*Table 2*). For cranberry extract, total phenolic content found by Tulio et al. [26] was 3.416 mg GAE/g FW which is much lower than value obtained in our study (9.73 mg GAE/g FW).

The DPPH and ABTS methods were used to measure the antioxidant activity of the extracts based on their ability to reduce the radical cation. The IC_{50} values calculated by DPPH method was between 0.071 mg/g FW for cranberry extract and 0.053 mg/g FW for bluecrop extract. The ABTS reducing power in bluecrop extract had the lowest value while the extract of wild blueberry had the highest value (*Table 2*).

Table 2

Total polyphenolic content and antioxidant activity found in sample extracts

Sample extracts	Total phenolics mg GAE/g FW	IC_{50} mg/g FW	TEAC µM/FW
Cranberry	9.73	0.071	41.395
Wild blueberry	10.48	0.057	62.170
Bluecrop	5.34	0.053	28.043

Correlation coefficient (r) was calculated in order to determine the relationship between polyphenolic content and antioxidant capacity. As illustrated in Fig. 1 a good linear correlation was found between the total polyphenolic content and DPPH radical scavenging (r=0.946) and ABTS reducing activity (r=0.928). A positive relationship between total polyphenolic content and antioxidant activity was found in all the analyzed extracts.

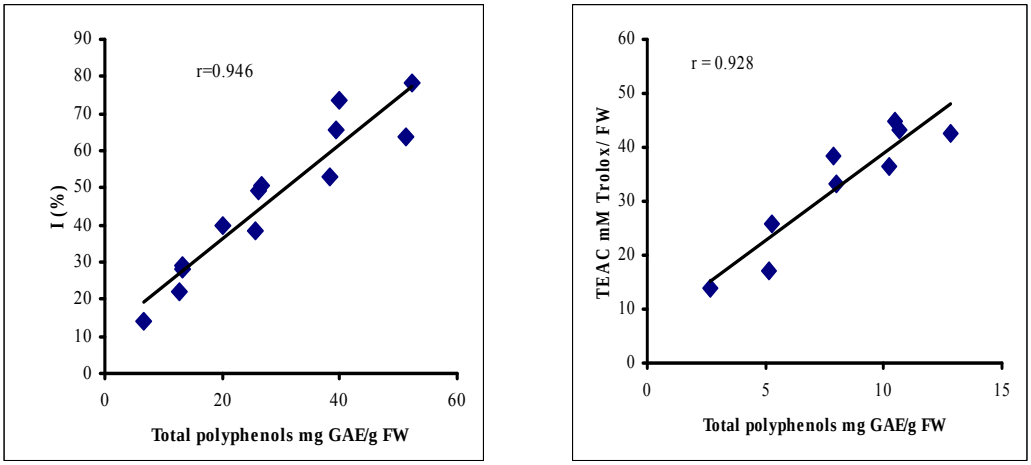

Fig. 1. Correlation between total polyphenolic content and antioxidant activity

The samples were analysed using MALDI-ToF and identification of compounds was made by comparing with analysis of the standards. Also the MS/MS fragmentation of the ions 449, 463, 493 and 595 confirmed the presence of anthocyanins. The main anthocyanidins and anthocyanins found was: cyanidin,

peonidin, delphinidin, petunidin, malvidin, pelargonidin-3-O-glucoside (Pg-3-glc), cyanidin-3-O-glucoside (Cy-3-glc), peonidin-3-O-glucoside (Pn-3-glc), delphinidin-3-O-glucoside (Dp-3-glc), petunidin-3-O-glucoside (Pt-3-glc), malvidin-3-O-glucoside (Mv-3-glc) and cyaniding-3-O-p-coumaric (Cy-3-p-coum). The MS/MS process requires a greater laser power (59) and a higher number of profiles. The molecular mass and relevant fragments are presented in *Table 3*.

Table 3

Masses and relevant MALDI fragments found in extracts

Compound	Mass Weight [Da]	$[M+H]^+$	MS/MS
Cy	287	288	
Pn	301	302	
Dp	303	304	
Pt	317	318	
Mv	331	332	
Pg-3-glc	433	434	
Cy-3-glc	449	450	316;286
Pn-3-glc	463	464	331;303
Dp-3-glc	465	466	
Pt-3-glc	479	480	
Mv-3-glc	493	494	331
Cy-3-p-coum	595	596	331

The results showed that the highest amount of anthocyanidins and anthocyanins was found in wild blueberry extract. In all samples the predominant constituent was Dp with a content ranged between 22 and 28%. The amount of Pn-3-O-glc and Cy-3-p-coum was rather low in wild blueberry extract while in cranberry and in bluecrop extracts was not found (Table 4).

Table4

The amount of anthocyanin found in samples (n=3)

Compound	Cranberry extract (% amount)	Wild blueberry extract (% amount)	Bluecrop extract (% amount)
Cy	16.16 ±0.2	13.23±0.16	2.99 ±0.12
Pn	5.53 ±0.18	2.43 ±0.24	2.63 ±0.32
Dp	28.23 ±0.21	28.26 ±0.26	22.34 ±0.25
Pt	9.63 ±0.13	6.2 ±0.40	8.45 ±0.34
Mv	7.97 ±0.15	6.204 ±0.12	17.75 ±0.28
Pg-3-glc	0	0.81 ±0.17	0
Cy-3-glc	10.85 ±0.14	10.91 ±0.28	6.43 ±0.19

Pn-3-glc	2.84 ±0.2	2.99 ±0.18	9.83 ±0.14
Dp-3-glc	8.97 ±0.15	16.22 ±0.24	9.39 ±0.22
Pt-3-glc	3.62 ±0.26	2.98 ±0.16	6.3 ±0.58
Mv-3-glc	6.2 ±0.32	6.13 ±0.1	13.9 ±0.49
Cy-3-p-coum	0	0.62 ±0.23	0

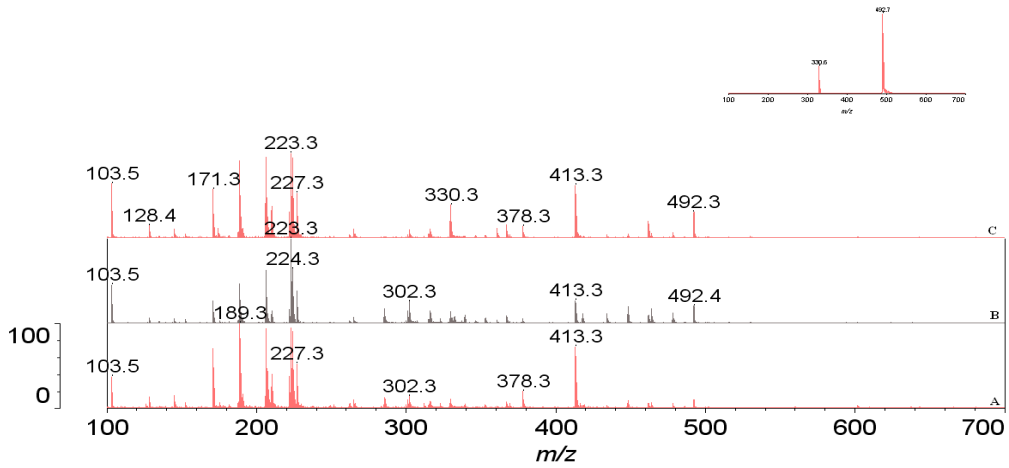

Fig 2. MALDI-TOF mass spectrum in positive reflectron mode of: **A**-cranberry extract; **B**-wild blueberry extract; **C**-bluecrop extract. Inset is MS/MS spectrum for Mv-3-O-glc (493 *m/z*)

4. Conclusion

The results of this study revealed that, among the examined extracts, wild blueberry extract presented the highest values for polyphenolic content and antioxidant activity while the bluecrop extract presented the lowest one. Our results are similar to other Romanian reported studies [27].

We demonstrated for the first time the applicability of matrix-assisted laser desorption/ionization mass spectrometry for the rapid analysis of anthocyanins and anthocyanidins in different fruits samples from Romanian market (wild and cultured). The amounts of anthocyanins varied from 0.62% to 28.26% and were significant greater in wild assortments of fruits. The obtained results confirm the value of studied fruits for human consumption due to their antioxidant properties which made them suitable for maintaining/improving human health.

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