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Total phenolics and anthocyanin profiles of Romanian wild and cultivated blueberries by direct infusion ESI-IT-MS/MS

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Abstract

Phenolics and anthocyanins were extracted from wild bilberries (Vaccinium myrtillus L.) and cultivated blueberries (Vaccinium corymbosum L.) collected from Breaza/Romania, and analyzed for their content by spectrophotometric techniques. The results have shown a high content of these bioactives in wild bilberry samples compared to the cultivated ones, in accordance with other reported studies. The total phenolics level in blueberry (Bluecrop cultivar), compared to that in bilberry, was found 31 % lower, while the total anthocyanins content of the same cultivar was found up to 45 % lower than of bilberry, depending on the harvest year. The obtained data may become relevant for future estimation studies of anthocyanins daily consumption and for enrichment of the national food composition databases.

Direct-infusion electrospray ionization ion trap tandem mass spectrometry (ESI-IT-MS/MS) was applied to identify individual anthocyanins directly from the crude extracts. The profile of relative abundances of the ions was strongly different. Anthocyanins representing three monoglycosides of five anthocyanidins (cyanidine, delphinidin, peonidin, petunidin, malvidin) were identified in high relative abundance in wild bilberry extracts compared to cultivated blueberry extracts which showed decreased levels (relative abundance of cyanidine and peonidin were <10%). This technique proved to be a valuable method which provides a rapid fingerprint of species-specific anthocyanins.

Key words: *Vaccinium myrtillus* L., *Vaccinium corymbosum* L., phenolics, anthocyanins, pH differential, ESI-MS, MS/MS

Introduction

Vaccinium spp. (blueberries and bilberries) of the *Ericaceae* family are fruits known for their high content of biologically active compounds showing health promoting benefits, in particular protection against chronic diseases, which are supported by epidemiological studies (G. BLOCK & al. [1], W.C. WILLETT [2]). The beneficial effects on human health are due to the high content of phytochemicals. Phytochemicals represent a large class of compounds with high structural variability, such as phenolics, carotenoids, alkaloids, vitamins, nitrogen and organosulfur compounds.

Phenolics are secondary metabolites of plants displaying a wide variety of chemical structures, ranging from simple phenols to highly complex polymeric substances (J.B. HARBORNE [3]). Phenolics have been reported as phytochemicals with high antioxidant, antiulcerative, antimicrobial, anticancer and antiheart disease properties (J. BEATTIE & al. [4], Y. HAMAUZU & al. [5]).

Anthocyanins belong to the class of phenolics, flavonoids respectively. The health positive effects of anthocyanins are based on their free-radical scavenging and antioxidant capacity, which are closely related to their particular chemical structure, mainly to the

position and degree of hydroxylation of both rings of the basic structure (*polyphenolic character*) (H. WANG & al. [6]).

There is a great variability in flavonoid content in plant foods, which depends on genetic background, geographic conditions, climate, farming practices, processing and storage (USDA Database, 2003 http://www.nal.usda.gov/fnic/foodcomp/Data/Flav/Flav02.pdf).

Analysis of phenolics and anthocyanins in fruits and vegetables represents an important task for the estimation of the intake of these biomolecules through diet in particular populations.

Different methodologies for anthocyanin analysis are available, among which UV-Vis spectroscopy and HPLC (regarded as standard methods) coupled to mass spectrometry (HPLC/MS) which proved effective for identification and quantification of anthocyanins, are the main used techniques (J.B. HARBORNE [7], A. BALDI & al. [8]). In particular, in red grapes, MS analysis of anthocyanins has been successfully applied for distinguishing between *Vitis vinifera* and non-*Vitis vinifera* species (J.A. SUGUI & al. [9], M. STOBIECKI [10]). The electrospray ionization-mass spectrometry (ESI-MS) methods are considered versatile ionization techniques, successfully applied for anthocyanin characterization (R. FLAMINI & al. [11], M.M. GIUSTI & al. [12]).

Regarding the composition of anthocyanins in *Vaccinium* species, it was shown that acylated and non-acylated 3-monoglycosides (glucosides, galactosides, and arabinosides) of cyanidine, delphinidin, peonidin, petunidin and malvidin are the major anthocyanins (G.M. SAPERS & al. [13], A.Z. MERCADANTE & al. [14]). The composition and concentration of these pigments in blueberries are influenced by different conditions, *e.g.* environmental, genetic, and physiological. Anthocyanins are distributed mainly in skin of cultivated blueberries (*Vaccinium corymbosum* L.) and both in skin and flesh of wild bilberries (*Vaccinium myrtillus* L.) also known as European blueberry.

Based on the strong evidence of bioactivities of phenolics and anthocyanins, and the scarcity of data regarding their content in foods from particular regions, this work aimed primarily to determine total phenolics (TP) and total anthocyanins (TA) of wild and cultivated blueberry species coming from the same selected Romanian region (Breaza). Quantitative analysis of TP and TA was performed by UV-Vis spectroscopy. Anthocyanin profiles were investigated by direct-infusion electrospray ionization ion trap tandem mass spectrometry (ESI-IT-MS/MS), technique which may be further used to rapidly distinguish and identify the origin of blueberries, as these fruits are used as raw materials in food and pharmaceutical products, or dietary supplements.

Materials and methods

Materials and reagents

Samples of fresh wild bilberry (*Vaccinium myrtillus* L.) and cultivated highbush blueberry (*Vaccinium corymbosum* L.) Bluecrop cv. were collected from Breaza growing area (Brasov county, Romania). The region of Breaza is situated in the central part of Romania at an altitude of 610 m, has a humid and cold climate (annual average temperatures of 6-8 ^{o}C, annual average rainfall of 700-850 mm), and presents fertile and acidic soils, conditions which are favorable for blueberry growth.

Chemical reagents of analytical grade without further purification were used for preparing the solutions for analysis of TP and TA. Ethanol (> 96 % V/V), methanol (> 99.5 % V/V), hydrochloric acid (37 %) and sodium acetate (trihydrate) were obtained from AdraChim (Bucharest, Romania), potassium chloride was obtained from Chimopar

(Bucharest, Romania), Folin-Ciocalteu reagent was purchased from Merck (Germany), anhydrous sodium carbonate was purchased from Scharlau (Spain), while gallic acid was obtained from Fluka (Germany). Buffer solutions for TA were prepared in distilled water, while solutions for TP analysis were prepared in deionized water.

Extraction and assay of total phenolics (TP)

The TP content in blueberry samples was determined according to the Folin-Ciocalteu spectrophotometric method (V.L. SINGLETON & al. [15]).

Briefly, the blueberry extract in 90 % (V/V) methanol (1 mL) was mixed with distilled deionized water and Folin-Ciocalteu reagent (1 mL) and incubated for 5 min. at room temperature. Then 7 % (m/V) Na_2CO_3 solution was added. After incubation at room temperature for 90 min., the absorbance was measured at 745 nm. The T80 UV/VIS spectrophotometer (PG Instruments Ltd) was used.

Gallic acid was used as standard for the calibration curve. A fivepoint calibration curve of gallic acid in the range of 20-100 mg L^{-1} ($y = 0.0045x - 0.0346$ with R^2 of 0.9995) was constructed. The mean of three readings was used and the TP content was expressed in milligram of gallic acid equivalents/g fresh mass (mg GAE g^{-1} FM).

Extraction and assay of total anthocyanins (TA)

Fruits of selected *Vaccinium* species were homogenized and anthocyanins were extracted in 70 % (V/V) ethanol, at 4 ^oC, for 24 h. The extract was centrifuged at 4 ^oC at 8000 rpm for 10 minutes. The Nűve NF 800R refrigerated centrifuge was used. Extracts were stored at 4 ^oC until analyses.

The TA content in blueberry extracts was determined according to the spectrophotometric pH differential method (R.E. WROLSTAD [16]). Measurements were done in two replicates. The T80 UV/VIS spectrophotometer (PG Instruments Ltd) was used. Total anthocyanins were expressed as cyanidine 3-O-glucoside (Cyn 3-O-G) equivalents (mg $100g^{-1}$ FM).

Physical-chemical characterization of extracts

Moisture content of the investigated samples was determined at 105 ^oC using the ML-50 moisture analyzer (A&D Company, Limited). Refractive index (n) and total soluble solids (TSS) of the *Vacciunium spp.* juice obtained by manually pressing was measured by refractometric method using an Abbe AR2008 refractometer (Krüss) at standardised temperature (21 ^oC). Values are expressed as refractometric total soluble substances, ^oBrix. TSS value may be used as indicator of the maturity level reached by given species of plants.

ESI-MS analysis

ESI measurements were performed using the LCQ DECA instrument (ThermoFinnigan, San Jose, CA, USA). Each sample of anthocyanin extract was diluted 1:10 (V/V) in methanol (HPLC grade, Fluka, Germany) for the ESI-MS experiments and directly infused at a flow of 10 μl/min. The ion trap mass spectrometer was operated in positive ion mode. The instrumental conditions were as follow: spray voltage 4.5 kV, 2.5 kV and 0.5 kV, sheath gas (N_2) flow rate 50 (arbitrary units, *a.u.*), entrance capillary temperature 280 °C and entrance capillary voltage 16 V. MS-MS experiments were performed by the selection of the precursor ions and their collision with helium by resonant excitation.

Results and discussion

The physical-chemical characterization of the investigated *Vaccinium spp.* samples is presented in Table 1. Regarding TSS values linked to anthocyanins levels, some studies have found positive correlation between anthocyanins and sugar concentration in fruits (C.H. CRISOSTO & al. [17]) while others did not correlated the soluble solids content to

anthocyanin level, showing a very complex accumulation pattern of TSS and TA in fruits (T. MILOŠEVIĆ & al. [18]).

Among the tested blueberry fruits, results regarding the evaluation of TP and TA contents performed by spectrophotometric method showed higher values in wild blueberries (*Vaccinium myrtillus* L.) compared to the cultivated ones (*Vaccinium corymbosum* L.) from the same growth region. The results are in accordance to other reported studies (J. GUERRERO & al. [19], G. GIOVANELLI & al. [20], A. BUNEA & al. [21]). The TP level in blueberry Bluecrop cv., compared to that in bilberry, was found 31 % lower, while the TA content was found 31- 45 % lower than of bilberry, depending on the harvest year.

Table 1. Physical-chemical characterization of *Vaccinium spp.* samples; TP and TA contents.

Sample	Region	Harvest year	Moisture (g $100g^{-1}$)	TSS (^{o}Brix)	Refractive index (n)	TP (mg GAE $100g^{-1}$ FM)	TA (mg $100g^{-1}$ FM)
Bilberry (*Vaccinium myrtillus* L.)	Breaza (RO)	2010	83.3	13.7	1.3634	nd	215.71
		2011	85.1	9.2	1.3468	355	329.17
Blueberry *(Vaccinium corymbosum* L.) Bluecrop cv.	Breaza (RO)	2010	85.5	16.1	1.3637	nd	96.01
		2011	85.8	10.3	1.3484	110.1	102.72

Note: nd = not determined

In case of wild bilberry, our results showed slightly lower TP and TA contents than other reported values, as follow: 429-670.9 mg GAE $100g^{-1}$ FM (M. JOVANČEVIĆ & al. [22], D. MARINOVA & al. [23]), 360 mg TA $100g^{-1}$ FM as determined by spectrophotometric pH differential method (M. JOVANČEVIĆ & al. [22], A. BUNEA & al. [21]) or 460 mg TA $100g^{-1}$ FM as determined by HPLC method (A. EDER [24]). Differences are due to specific geographic conditions, harvest maturity, and applied analytical methods.

In case of cultivated blueberries (Bluecrop cv.), the TP content was found lower than other reported values, such as 306.9 mg GAE $100g^{-1}$ FM (K. SKUPIEN [25]), while the TA content was similar to values reported by other authors, 94.6-164.5 mg $100g^{-1}$ FM as determined by spectrophotometric pH differential method (I. ŚCIBISZ & al. [26]) or 143 mg $100g^{-1}$ FM as determined by HPLC method (M.J. CHO & al. [27]).

Our results indicated lower TP and TA contents of Romanian cultivated blueberries (Bluecrop cv.) than those reported by BUNEA & al. [21], differences due to the diverse geographical origin of samples (central part of Romania *vs.* North-West part of Romania), probably different harvest years/maturity levels (not specified), different extraction technologies starting with the used solvents (70 % ethanol *vs.* acidified methanol), time and temperature of extraction, storing conditions before analysis (fresh *vs.* freezing). Freezing storage has been shown to increase the level of anthocyanins in several fruits (B. GONCALVES & al. [28], S. OANCEA & al. [29]). We have used ethanol as safer extraction solvent considering some future food applications of the obtained crude extracts.

The results suggest a wide genetic (different *Vaccinium* species) and environmental variability in blueberries, with respect to TP and TA contents. Different growth conditions such as day length, light intensity, temperature, greatly influence the phytochemical based quality of blueberries, as shown by I. MARTINUSSEN & al. [30]. All these considerations advocate for standardized rules developed for anthocyanins extraction and analysis.

We have further investigated the anthocyanins profile in the selected wild and cultivated *Vaccinium spp.* by using ESI-MS technique. As such studies are based on a

previous HPLC-DAD analysis of anthocyanins, here we have determined the anthocyanins profiling by direct infusion ESI-IT-MS/MS analysis of the crude bioextracts in order to have an immediate profile which may rapidly be used to the establishment of the origin of blueberries in various fruit derived products.

The ESI-MS profiles of wild bilberry anthocyanin extract obtained by using different capillary voltages (*i.e.* in presence of different electrostatic field) are reported in Fig. 1. The electrospray phenomena depend on geometric parameters of the ion source and on chemical-physical characteristics of the solvent. The voltage necessary to activate the electrospray phenomenon has been calculated by SMITH (J.N. SMITH [31]) as shown in Eq. /1/.

$$V_{on} = 2x10^5(\gamma r_c)^{1/2}\ln(4d/r_c) \quad /1/$$

where V_{on} is the potential required for the onset of electrospray, γ is the surface tension of the solvent, and r_c the radius of the capillary.

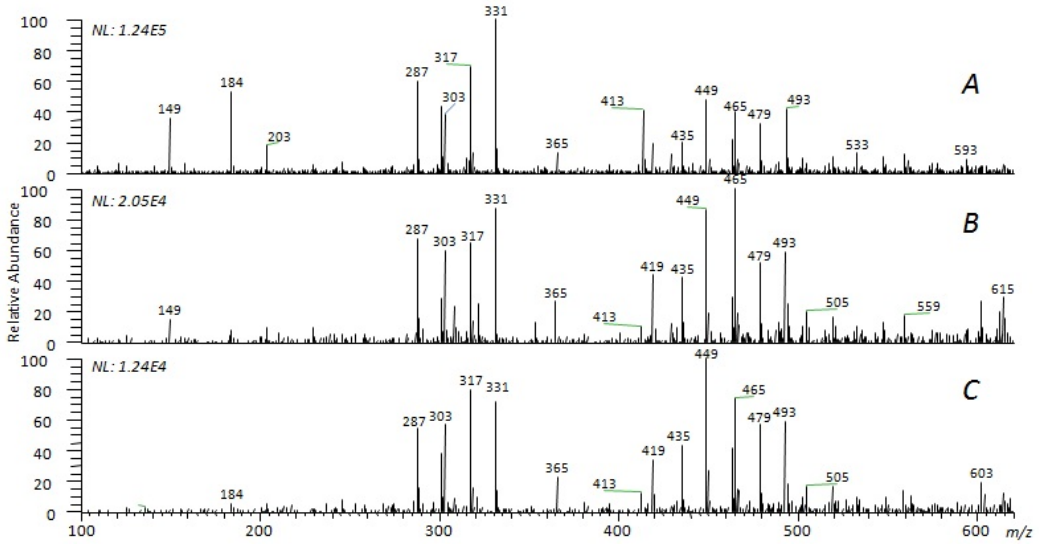

Figure 1. ESI-MS profiles of the anthocyanin extract of wild bilberry (*Vaccinium myrtillus* L.) from Breaza Romanian region, as resulted by applying different source voltages: **A)** 4.5 kV; **B)** 2.5 kV; **C)** 0.5 kV.

In case of methanol solution, V_{on} has been calculated to be of the order of 2.2 kV: under this value no electrospray takes place. In Fig. 1 A, the spectrum obtained in ESI conditions ($V_{capillary}$= 4.5 kV) is reported. Many ions are detectable due to either protonated molecules of species present as neutrals in the solution or ions originally present in the solution. The spectra obtained by setting 2.5 kV and 0.5 kV on the spray in capillary, are reported in Figs. 1 B and 1 C, respectively. As observed, some species disappear and changes in relative abundance become detectable. In particular, the ionic species in the *m/z* range 400-500 become more abundant. These results can be explained by the activation of ion mobility phenomena in presence of high electrical field. With V= 4.5 kV, the ion of smaller dimensions exhibit a speed higher than those corresponding to larger ions and consequently their intensities become higher. In presence of small electrical field [Fig. 1 C, V= 0.5 kV] these ion mobility phenomena are strongly reduced and the discrimination between high and low mass ions is no more present. Consequently, the spectrum of Fig. 1 C can be considered the real mapping of the charged species present in the original solution directly injected into the source operating in pneumatic spraying conditions. These conditions seem to be the most

suitable ones to highlight possible differences in anthocyanins profile of wild and cultivated blueberry fruits.

The spectra obtained by pneumatic spraying of wild and cultivated blueberry anthocyanin extracts are reported in Fig. 2. As noticed, wide differences are present, most probably due to different species (*Vaccinium myrtillus* L. and *Vaccinium corymbosum* L.) as well as different growing conditions (wild and in field). Actually, the abundance of the different ions due to anthocyanin compounds is strongly differentiated

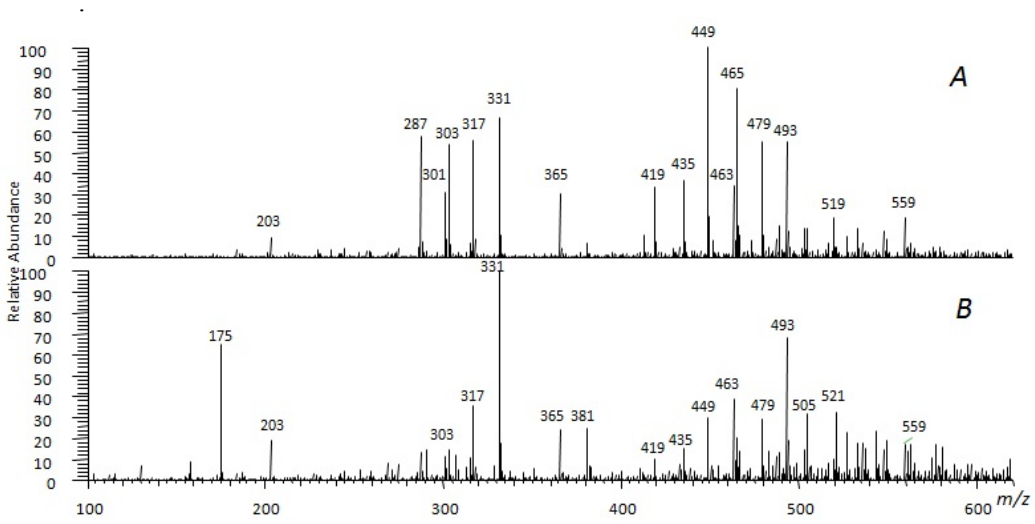

Figure 2. Comparative ESI-MS anthocyanin profiles of extracts of wild bilberry *Vaccinium myrtillus* L. (**A**), and cultivated blueberry *Vaccinium corymbosum* L. (**B**) fruits from Breaza Romanian region.

In order to obtain the structural assignment of the most abundant species detected in pneumatic spray conditions, a series of MS-MS experiments have been performed and the obtained collisional spectra have been compared to those reported in literature (E.E. NICOUÉ & al. [32], L.R. HOWARD & al. [33]). The structural assignment has been performed on the basis of molecular mass and of the characteristic fragments obtained by collision of the molecular species. The results are reported in Table 2.

Table 2. Comparison of relative abundances of the ions present in the anthocyanin profiles identified from ESI-MS analysis in wild bilberry *Vaccinium myrtillus* L. (sample **A**) and cultivated blueberry *Vaccinium corymbosum* L. (sample **B**) fruits from Breaza Romanian region.

$[M]^+$ (*m/z*)	MS/MS fragments (*m/z*)	Relative abundance in samples		Anthocyanidine/Anthocyanin
		A	B	
203	185, 157	9.17	18.60	
287	213, 231, 241, 259, 269	59.66	<10	cyanidine
301	286	31.01	<10	peonidin
303	257	54.07	13.71	delphinidin
317	302	56.69	33.14	petunidin
331	270, 287, 299, 316	66.73	100	malvidin
365	203, 184	32.47	25.69	203 + 162
381	201, 219		24.59	219 + 162
419	287	35.06		cyanidine 3-arabinoside

435	303	36.32		delphinidin 3-arabinoside
449	317, 287	100	30.87	cyanidine 3-glucoside/ cyanidine 3-galactoside / petunidin pentoside
463	301, 331	33.38	39.26	peonidin 3-galactoside/ peonidin 3-glucoside/ malvidin 3-arabinoside
465	303	81.54		delphinidin 3-glucoside/ delphinidin 3-galactoside
479	317	56.25	29.65	petunidin 3-glucoside/ petunidin 3-galactoside
493	331	57.07	67.98	malvidin 3-galactoside/ malvidin 3-glucoside/ delphinidin 3-acetyl-glucoside
505	401, 313, 301		29.31	peonidin 3-acetyl-glucoside
519	501, 487	19.04		petunidin 3-acetyl-rhamnoside
521	503, 417, 329, 291, 317		31.14	petunidin 3-acetyl-glucoside
543	481, 439		24.04	162 + 381
549	517		21.73	
559	497, 455, 379, 329	19.16	16.81	
563	531		16.29	

The ionic species in the *m/z* range 287-331 reported in Table 2 correspond to the most common anthocyanidins which are the aglycones of the anthocyanins: cyanidine, peonidin, delphinidin, petunidin and malvidin. All the five anthocyanidins were identified in the investigated wild bilberry sample, but pelargonidin was not found, which is in agreement with other reported results (E.E. NICOUÉ & al. [32]). Levels of cyanidine and peonidin in cultivated blueberry sample were found of low relative abundance (< 10 %). The structure of the ion with *m/z* 365, as suggested by MS-MS experiments (loss of neutral species of 162 Da), seems to contain a sugar linked to a structure with molecular mass of 203 Da. This ion is detectable in both spectra of wild and cultivated *Vaccinium spp.* This molecule is not necessarily ascribable to the class of the flavonols. The same may be supposed for the ion with *m/z* 381 found only in cultivated blueberry sample, where collisional experiments showed again a loss of 162 Da that indicates the presence of an hexose in its structure. The ion at *m/z* 449 is present in both samples, however with different relative abundances. The collisional experiments showed two product ions of 449 in wild bilberry sample as shown in Fig. 3: at *m/z* 287 and 317 respectively, that may be interpreted as products of cyanidine 3-glucoside/cyanidine 3-galactoside or petunidin 3-arabinoside, both generated from the parent ion at *m/z* 449.

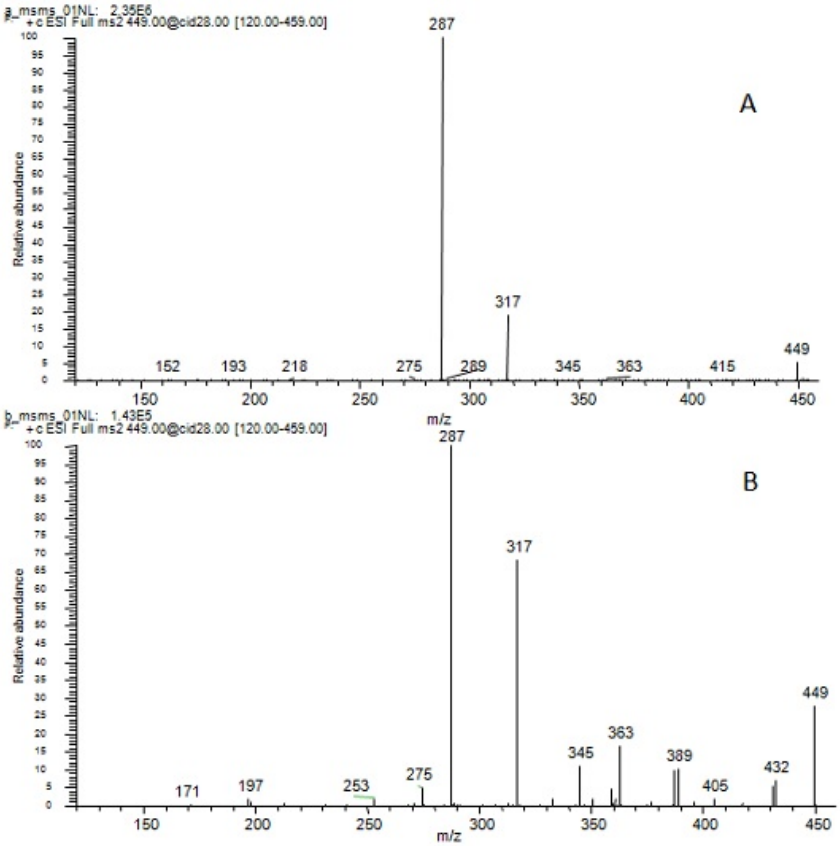

Figure 3. MS-MS spectra of the ion at *m/z* 449 of anthocyanin extracts of wild bilberry *Vaccinium myrtillus* L. (**A**), and cultivated blueberry *Vaccinium corymbosum* L. (**B**) fruits from Breaza Romanian region.

In fact, in case of cultivated blueberry sample, the MS-MS spectrum showed the fragment at *m/z* 317 four times less abundant compared to that at *m/z* 287, due to minor content of petunidin 3-arabinoside in the anthocyanin extract. The same difference in abundance of product ions can be observed for the ion at *m/z* 463, that may indicate the presence of either peonidin glycosides or malvidin pentosides in the two investigated samples. For the ions at *m/z* 465, 479 and 493 respectively, similar indications can be concluded: delphinidin hexosides, petunidin hexosides and malvidin hexosides, respectively. Some acetyl derivatives of glycosilated compounds were also found, as shown in Table 2. Furthermore, one of the product ions of the ion at *m/z* 543 is 381, originating from a loss of a neutral moiety of 162 Da, suggesting a second glycosilation of the species detected at *m/z* 219.

Fig. 4 summarizes the relative abundances of the ionic species due to anthocyanin compounds found in the wild and cultivated *Vaccinium spp*. anthocyanin extracts. The differences are highly evident: wild bilberry sample (*Vaccinium myrtillus* L.) exhibited abundance in ions at *m/z* value < 493 with the most abundant specie resulted at *m/z* 449, while cultivated blueberry sample (*Vaccinium corymbosum L.* L.) showed more glycosilated compounds.

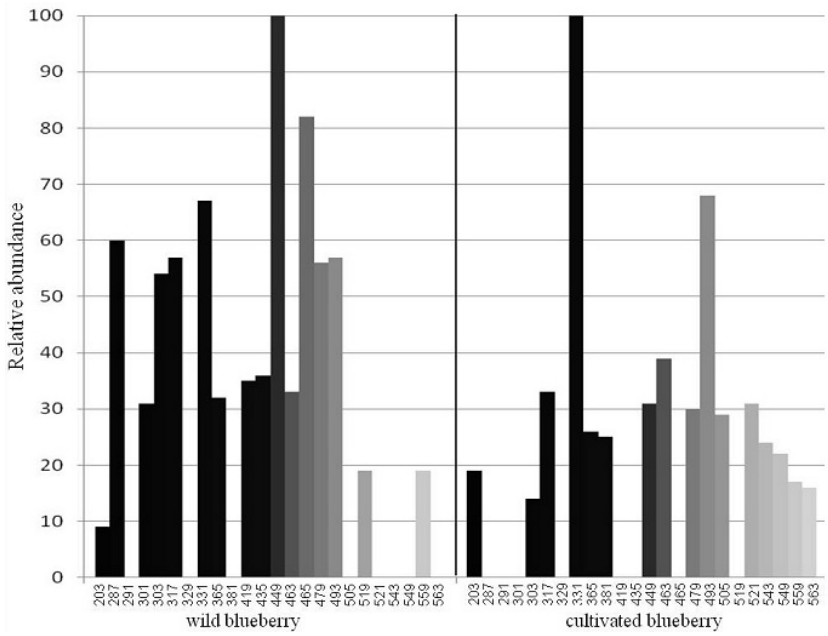

Figure 4. Fingerprint of relative abundances of the ions representing anthocyanins in wild bilberry (*Vaccinium myrtillus* L.) and cultivated blueberry (*Vaccinium corymbosum* L.) fruits from Breaza Romanian region, as obtained from ESI-MS experiments.

These results are important through the achievement of a fast anthocyanins fingerprint by ESI-MS of crude extracts of blueberry fruits of different origin, so distinguishing the starting material of *Vaccinium spp.* used in different food products (*e.g.* juices and jams) and supplements. ESI-MS proved to be a valuable and rapid technique for the identification of a group of anthocyanins with different masses, though it can not differentiate anthocyanin isomers (R. FLAMINI & al. [11]).

Moreover, results regarding the evaluation of TP and TA contents in wild and cultivated blueberries from the selected region are important for future epidemiological studies regarding the estimation of anthocyanins intake from diet in Romanian population.

Conclusions

The results from the analysis of total phenolics and anthocyanins in bilberry and blueberry fruits collected from Breaza Romanian growing area have shown an increased content in wild blueberries (*Vaccinium myrtillus* L.) compared to cultivated ones (*Vaccinium corymbosum* L.) as determined by UV-Vis spectroscopy, in accordance with other reported studies. The TP level in blueberry Bluecrop cv., compared to that in bilberry, was found 31 % lower, while the TA content was found up to 45 % lower than of bilberry, depending on the harvest year.

Qualitative analysis of anthocyanins in crude extracts was carried out by direct-infusion ESI-MS/MS experiments. The abundance of the different ions due to anthocyanins was strongly different. Anthocyanins representing three monoglycosides of five anthocyanidins (cyanidine, delphinidin, peonidin, petunidin, malvidin) were identified in high relative abundance in wild bilberry extracts compared to cultivated ones, the last showing decreased levels (relative abundance of cyanidine and peonidin were < 10 %). Samples from

wild bilberries exhibited abundance in ions at *m/z* value < 493 with the most abundant specie at *m/z* 449, while cultivated blueberries showed more glycosilated compounds.

As overall conclusion, the obtained data on TP and TA contents in *Vaccinium spp.* of great commercial interest may become significant for further studies on estimation of the anthocyanins daily consumption and for enrichment of national databases regarding foods composition. The development of public health programs on nutrition issues requires information on phenolics and anthocyanins composition in plant foods. Also, the applied ESI-MS/MS technique in order to obtain a fast identification of individual anthocyanins may be used as a potential tool to rapidly differentiate species-specific anthocyanins (*anthocyanins profiling by MS*) in crude extracts.

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