Study of diversity of anthocyanin composition in bilberry *(Vaccinium myrtillus* L.) fruits

Deividas Burdulis, Liudas Ivanauskas[1], Vidmantas Dirsė[1], Saulius Kazlauskas[1], Almantas Ražukas[2]
Department of Pharmaceutical Chemistry and Pharmacognosy,
[1]Department of Analytical and Toxicological Chemistry, Kaunas University of Medicine,
[2]Lithuanian Institute of Agriculture, Vokė branch, Lithuania

Key words: *blueberries; anthocyanins; anthocyanidins; spectrophotometry; high-performance liquid chromatography.*

Summary. *Qualitative and quantitative composition of anthocyanins in bilberry (Vaccinium myrtillus L.) fruits was assayed. The aim of our study was to evaluate total anthocyanin content and their composition in bilberries collected from various regions and at different time. For the quantification of total anthocyanins in frozen fruits, the spectrophotometrical assay was performed. The highest amount of anthocyanins in bilberry fruits, collected in Lithuania, was found in samples from Šilutė (0.399%), the lowest one – from Valkininkai region (0.264%), but higher amounts of anthocyanins were found in the samples collected in Russia (Archangelsk region) and Sweden (Stockholm region). High-performance liquid chromatography was applied for qualitative evaluation of individual anthocyanins in the different material. Quantification of anthocyanidin content was performed after acidic hydrolysis of anthocyanin glycosides. Chromatographic analysis has shown that there are no differences in qualitative composition of anthocyanidins. In all samples, cyanidin was found in the highest quantities (mean amount 0.053 μg/mL). Delphinidin and petunidin was found in quantities 2.5 fold lower than cyanidin, and malvidin and peonidin were found in the smallest quantities. Only in the blueberries collected in Sweden, malvidin was found in the highest amount. It was 1.5 fold higher than amounts of petunidin and delphinidin.*

Introduction

Interest in fruit composition has intensified because of increased awareness of the possible health benefits of some of their nutrients (1). Kalt and Dufour reviewed a number of the beneficial effects ascribed to both wild and domestic blueberries (bilberries), including reduction of coronary heart disease, treatment of urinary tract disorders, and anticarcinogen activity (2). The anthocyanin pigments of the native European blueberry *Vaccinium myrtillus* have long been used for improving visual acuity and treating circulatory disorders (3). There are hundreds of pharmaceutical products derived from *V. myrtillus* (4). Many of these biological properties are believed to be associated with the antioxidant activity of anthocyanin pigments, flavonoids, and other phenolic compounds (1, 5). Prior *et al.* measured total amount of anthocyanins and phenolics and oxygen radical absorbing capacity (ORAC) for four *Vaccinium* species and found a linear relationship between ORAC and anthocyanin and total phenolic content (6). They reported blueberries to be one of the richest sources of antioxidant phytonutrients of fresh fruits and vegetables studied.

Total anthocyanin amount ranges from 300 to 700 mg per 100 g (3, 6). Fourteen anthocyanins have been identified in blueberry fruit, juice, and extract (7–13). These are 3-O-arabinosides, 3-O-glucosides, and 3-O-galactosides of five anthocyanidins: cyanidin, delphinidin, malvidin, petunidin, and peonidin (7, 14).

Bilberry fruits are harvested ripe, usually from July to September. In one study, the highest berry yields came from plants growing in somewhat exposed areas with moderate shade and moderately humid ground (15, 16). It has been determined that as a fruit ripens, the concentration of flavonols and procyanidins decreases while the concentration of anthocyanins increases (8, 17–19).

The aim of our study was to investigate total anthocyanin content, anthocyanin composition, and anthocyanidin content in blueberry fruits collected in different regions of Lithuania, in Russia (Archangelsk region), in Belarus (Grodno region), and Sweden (Stockholm region) and to compare anthocyanin content in fruits collected at different time.

Correspondence to D. Burdulis, Department of Pharmaceutical Chemistry and Pharmacognosy, Kaunas University of Medicine, A. Mickevičiaus 9, 44307 Kaunas, Lithuania. E-mail: deividas.burdulis@gmail.com

Materials and methods

Determination of total anthocyanins

The total content of anthocyanins was determined by spectrophotometrical method according to the European Pharmacopoeia (20). The 50.0 g of fresh fruits were crushed extemporaneously; 5.0 g of fresh fruits were weighted, and 90.0 mL of methanol was added. The obtained mixture was sonicated for 10 min, put to the thermostat, and left for 30 min at 40°C periodically stirred. The solution was filtered and diluted to 100.0 mL with methanol. A 50-fold dilution of this solution was prepared in a 0.1% (V/V) hydrochloric acid in methanol. The absorbance of the solution was measured at 528 nm (spectrophotometer Unicam "Helios-α") using a 0.1% (V/V) solution of hydrochloric acid in methanol as the compensation liquid. The percentage of anthocyanins was expressed according to cyanidin-3-glucoside chloride. All samples were prepared in triplicate.

Hydrolysis of anthocyanins

To 25.0 mL of methanolic solution, 8.5 mL of concentrated hydrochloric acid was added, and mixture was heated under refluxing condenser for 2 hours. The hydrolyzed solution was filtered through filter (pore size 0.45 μm in diameter) and diluted to 50 mL with methanol.

Chromatographic separation of anthocyanins and anthocyanidins

The chromatographic separation of anthocyanins was carried out on the chromatographic system consisting of Waters 2690 Alliance HPLC separation module equipped with Waters 2487 dual λ absorbance detector (UV/Vis) and Waters 996 photodiode array (PDA) detector (Waters corporation Milford, MA, USA), coupled to personal computer with Waters Millennium 2000® chromatographic manager system (Waters corporation Milford, MA, USA) for data storage and processing. For separation of anthocyanins, the column "Lichrosphere 100" (12.5×4.0 cm, particle size 5 μm) was used. Separation of anthocyanidins was carried out with SUPELCO HYPERSIL ODS (C18) 5U 150.0×4.6 mm column, guarded with a Hypersil ODS (10×4.6 mm in inside diameter; particle size 5 μm) guard column (SUPELCO, Bellefonte USA). The column was kept at ambient temperature. The flow rate was kept constant at 1.0 mL/min for a total run time of 45 min. The gradient elution was used: solvent A – acetonitrile (HPLC grade); solvent B – 4.0% aqueous solution of phosphoric acid. Gradient system: from 7% A at 0 min to 25% A at 45 min. The injection volume for all standard solution and bilberry fruit extracts was 10 μL. The UV/Vis detector was set at 520 nm, PDA 200–800 nm wavelength.

The standards of anthocyanidin chloride salts were purchased from ROTH GmbH. For quantitative determination of anthocyanidins in blueberries, the calibration curves were created for each compound. All samples were run in triplicate.

Results and discussion

Total anthocyanin content in blueberries collected from different regions of Lithuania is presented in Fig. 1.

Anthocyanin content in blueberries was slightly different depending on region (%CV 11.96). The highest content of anthocyanins was found in the samples collected in Šilutė (0.399%), the lowest one –

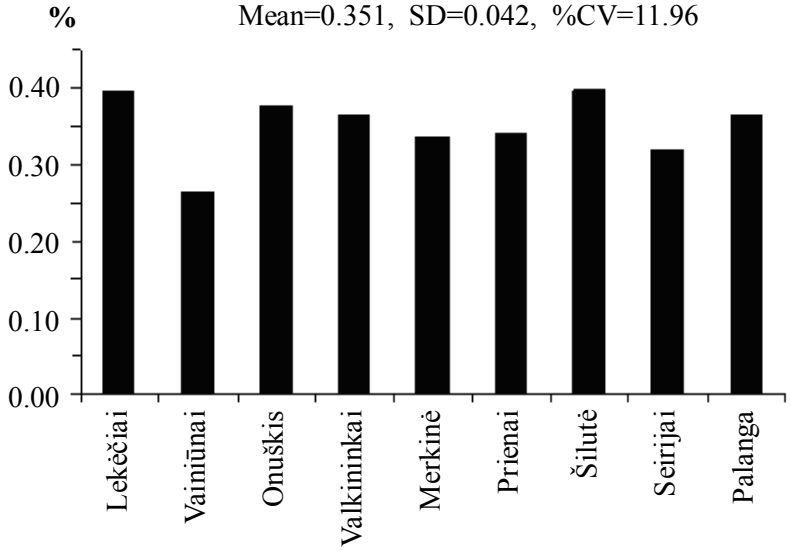

Fig 1. **Total anthocyanin content in blueberries from different Lithuanian regions**
SD – standard deviation, CV – coefficient of variation.

in Valkininkai region (0.264%). All results, except that from Valkininkai, agree with literature data (Mazza and Miniati (3), Prior *et al.* (6), and others). The low content of anthocyanins in the berries from Valkininkai may be related to early collection of fruits (20 July).

The anthocyanin content from different countries is represented in Fig. 2. The highest content of anthocyanins was found in berries collected in Russia (Archangelsk region) and Sweden (Stockholm region). These results may be related to the late collection time of berries (the end of August, the beginning of September) and more northern places of collection. Berries from these regions were most ripened, and this has the influence on anthocyanin content. As mentioned by Brenneisen and Steineger (1981a), Moeck (1994), Morazzoni and Bombardelli (1996), Upton *et al.* (2001), the amount of anthocyanin in the berries increases when they ripen. The amount of anthocyanins was similar in the samples collected in Belarus and Lithuania (5% difference).

Chromatographic separation of anthocyanins

The chromatogram of anthocyanin separation in blueberries is represented in Fig. 3. The identification of anthocyanins was performed according to the

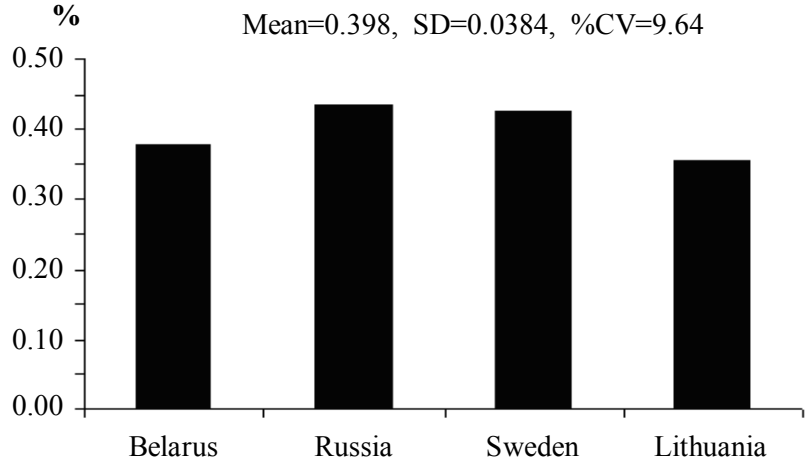

Fig. 2. **Total anthocyanin content in blueberries from different countries**
SD – standard deviation; CV – coefficient of variation.

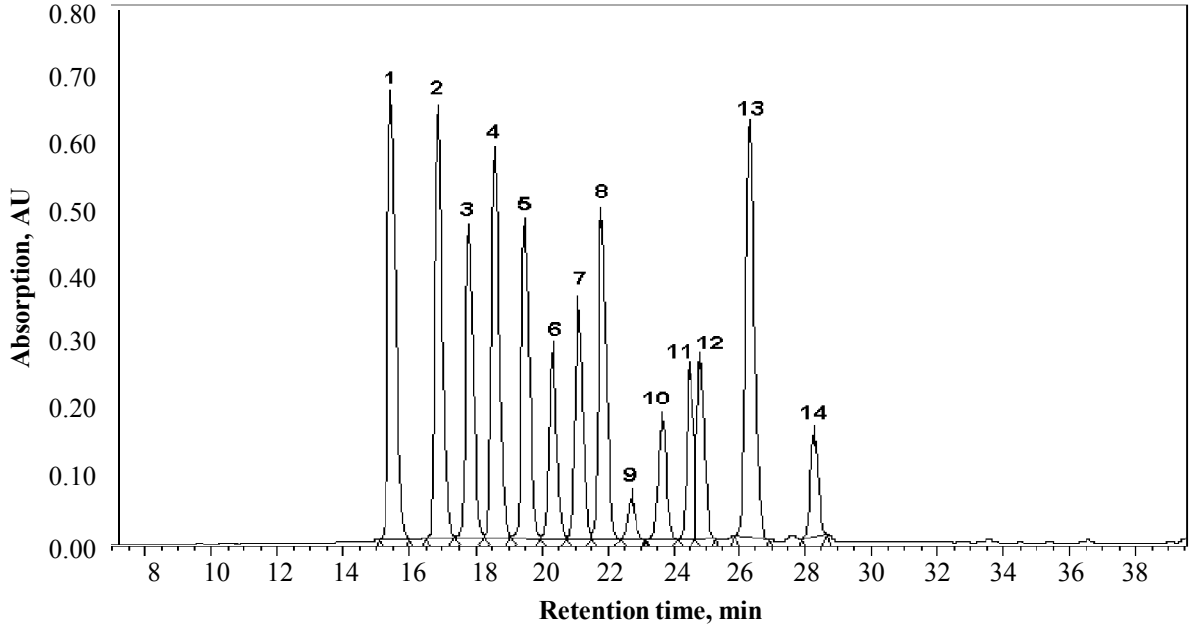

Fig. 3. **Representative chromatogram of blueberry anthocyanins**
Peaks: 1 – delphinidin 3-galactoside; 2 – delphinidin 3-glucoside; 3 – cyanidin 3-galactoside; 4 – delphinidin 3-arabinoside; 5 – cyanidin 3-glucoside; 6 – petunidin 3-galactoside; 7 – cyanidin 3-arabinoside; 8 – petunidin 3-glucoside; 9 – peonidin 3-galactoside; 10 – petunidin 3-arabinoside; 11 – peonidin 3-glucoside; 12 – malvidin 3-galactoside; 13 – malvidin 3-glucoside; 14 – malvidin 3-arabinoside.

literature data (21, 22). The most researchers investigating anthocyanin composition in blueberries have reported mainly 14 anthocyanins. In our study, we also found 14 anthocyanins. The chromatograms were identical in all samples, and no differences were observed. For confirmation of the presence of anthocyanins, spectra of each peak were registered (Fig. 4). Acidic hydrolysis was chosen because this method greatly simplifies quantification of anthocyanidin content. Hydrolyzed anthocyanidin aglycones can be separated, identified, and assayed easily.

Analysis of anthocyanidins

The chromatogram of anthocyanidins is presented in Fig. 5. Anthocyanidin composition of blueberries, collected in Lithuania, is given in Table 1.

Chromatographic analysis has shown that there are no differences in qualitative composition of anthocyanidins. In all samples, cyanidin was found in the highest quantities (mean amount 0.053 μg/mL). Delphinidin and petunidin were found in quantities 2.5 fold lower than cyanidin, and malvidin and peonidin were found in the smallest quantities.

The analysis of anthocyanidin composition in blueberries from different countries is represented in Table 2.

From the data represented in Table 2, it can be stated that there are no differences in qualitative composition of blueberries, collected in different countries. In all samples, cyanidin was found in the highest quantities, then delphinidin and petunidin; peonidin and malvidin. Only in the blueberries collected in Sweden, malvidin was found in the highest quantity (1.5 fold) than petunidin and delphinidin.

We have investigated the content and quantities of anthocyanidins in the blueberries collected at different time. The data are presented in Table 3.

Our results revealed that during ripening process, there is an increase in the quantity of anthocyanidins. The highest quantities of anthocyanidins were found in the berries collected in September (approximately 1.5 fold higher as compared to July). Only the amount of malvidin was almost the same during all collection period (0.016–0.017 μg/mL).

Conclusions

From the study results the following conclusions can be drawn:

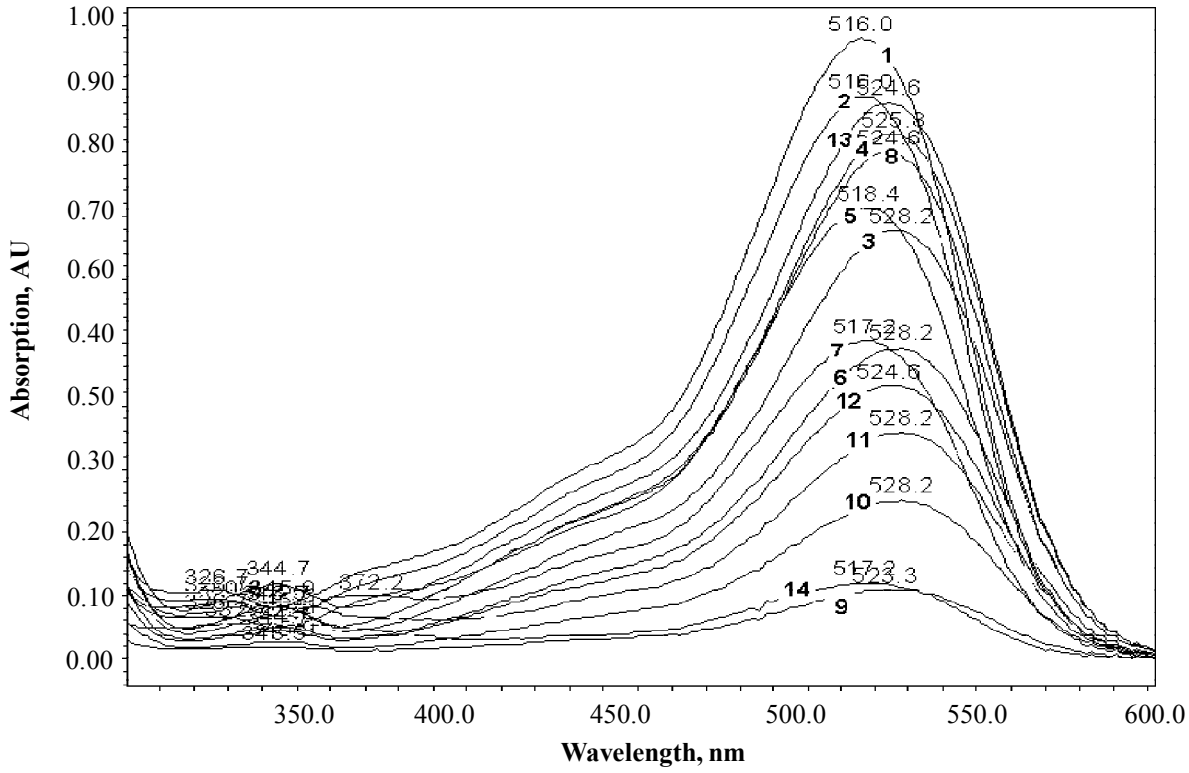

Fig. 4. **The spectra of anthocyanins**

Spectra of anthocyanins: 1 – delphinidin 3-galactoside; 2 – delphinidin 3-glucoside; 3 – cyanidin 3-galactoside; 4 – delphinidin 3-arabinoside; 5 – cyanidin 3-glucoside; 6 – petunidin 3-galactoside; 7 – cyanidin 3-arabinoside; 8 – petunidin 3-glucoside; 9 – peonidin 3-galactoside; 10 – petunidin 3-arabinoside; 11 – peonidin 3-glucoside; 12 – malvidin 3-galactoside; 13 – malvidin 3-glucoside; 14 – malvidin 3-arabinoside.

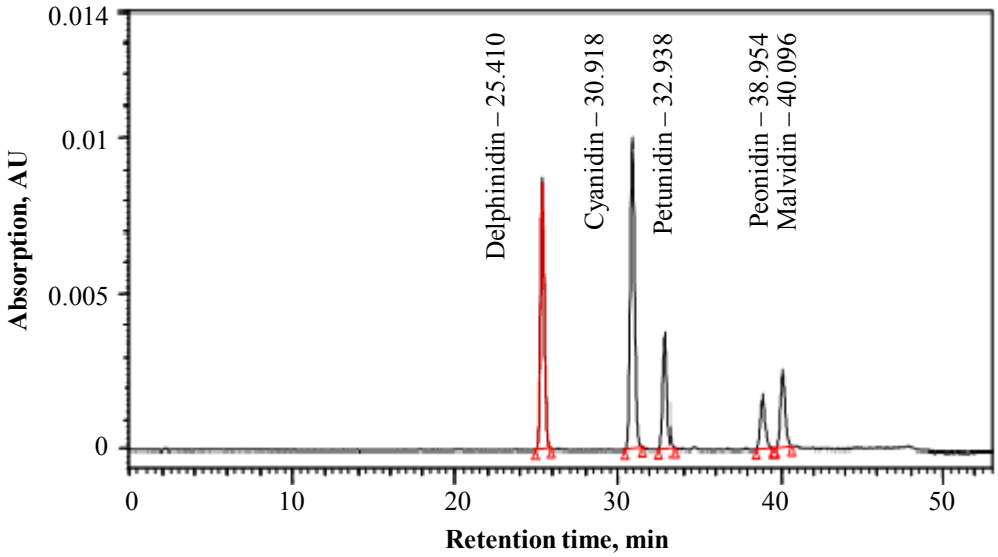

Fig 5. **Representative chromatogram of anthocyanidins**

Table 1. **Anthocyanidin contents in the blueberries from different regions of Lithuania**

Region	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin	Units
Vainiūnai	0.006	0.021	0.007	0.004	0.003	µg/mL
Palanga	0.013	0.040	0.015	0.009	0.008	µg/mL
Šilutė	0.021	0.060	0.023	0.016	0.014	µg/mL
Merkinė	0.016	0.051	0.017	0.011	0.008	µg/mL
Onuškis	0.027	0.077	0.028	0.018	0.016	µg/mL
Seirijai	0.023	0.056	0.023	0.012	0.012	µg/mL
Valkininkai	0.021	0.063	0.022	0.014	0.012	µg/mL
Prienai	0.022	0.059	0.023	0.014	0.012	µg/mL
Mean	0.019	0.053	0.020	0.012	0.011	µg/mL
SD	0.0067	0.0166	0.0064	0.0044	0.0041	
% CV	35.26	31.32	32.00	36.66	37.27	

SD – standard deviation, %CV – coefficient of variation.

Table 2. **Anthocyanidins in the blueberries collected in different countries**

Country	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin	Units
Lithuania	0.019	0.053	0.020	0.012	0.011	µg/mL
Russia	0.023	0.061	0.023	0.013	0.012	µg/mL
Belarus	0.022	0.059	0.022	0.012	0.018	µg/mL
Sweden	0.021	0.050	0.020	0.009	0.029	µg/mL
Mean	0.021	0.056	0.021	0.011	0.017	µg/mL
SD	0.002	0.005	0.002	0.002	0.008	
% CV	0.93	0.88	0.78	1.52	4.66	

SD – standard deviation; %CV – coefficient of variation.

***Table 3*. Anthocyanidin content in blueberries collected at different time**

Month	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin	Units
July	0.021	0.054	0.022	0.012	0.017	μg/mL
August	0.022	0.058	0.021	0.016	0.016	μg/mL
September	0.028	0.077	0.028	0.018	0.016	μg/mL
Mean	0.024	0.063	0.024	0.015	0.016	μg/mL
SD	0.004	0.012	0.004	0.003	0.001	
% CV	16.00	19.51	16.00	19.92	3.53	

SD – standard deviation; % CV – coefficient of variation.

1. The total quantities of anthocyanins depend on place and time of collection.
2. The content of anthocyanin in blueberries was slightly different depending on region (% CV 11. 96).
3. The fruits of blueberries contain five anthocyanidins. Cyanidin makes up almost half (about 46%) of total anthocyanidin amount found in all investigated samples.

Acknowledgements

Authors thank the Foundation of Science of Kaunas University of Medicine for supporting this work.

Antocianų sudėties įvairovės tyrimai mėlynės (*Vaccinium myrtillus* L.) vaisiuose

Deividas Burdulis, Liudas Ivanauskas[1], Vidmantas Dirsė[1], Saulius Kazlauskas[1], Almantas Ražukas[2]

Kauno medicinos universiteto Farmacinės chemijos ir farmakognozijos katedra,

[1]Analizinės ir toksikologinės chemijos katedra, [2]Lietuvos žemdirbystės instituto Vokės skyrius

Raktažodžiai: mėlynės antocianai, antocianidinai, spektrofotometrija, efektyvioji skysčių chromatografija.

Santrauka. Tirta mėlynės (*Vaccinium myrtillus* L.) vaisių biologiškai aktyvių junginių (antocianų) kiekinė ir kokybinė sudėtis.

Tyrimo tikslas. Ištirti antocianų kiekį mėlynėse, surinktose skirtinguose augimo regionuose, bei kiekinės ir kokybinės sudėties įvairavimų priklausomumą nuo vaistinės augalinės žaliavos paruošų laiko. Bendras antocianų kiekis šaldytose uogose nustatytas spektrofotometriniu metodu. Didžiausias suminis antocianų kiekis Lietuvos teritorijoje rinktuose ėminiuose nustatytas Šilutės (0,399 proc.), o mažiausias Valkininkų (0,264 proc.) regionuose, tačiau didesni antocianų kiekiai rasti ėminiuose, rinktuose Rusijoje (Archangelsko regione) ir Švedijoje (Stokholmo regione). Kiekinė antocianų sudėtis mėlynės vaisiuose nustatyta efektyviosios skysčių chromatografijos metodu. Antocianų aglikonų kiekinė sudėtis nustatyta atlikus antocianų glikozidų rūgštinę hidrolizę. Atlikus tyrimus efektyviosios skysčių chromatografijos metodu, nustatyta, kad antocianidinų kokybinė sudėtis tirtuose ėminiuose yra identiška. Analizuotuose ėminiuose rasti didžiausi kiekiai cianidino (vidutiniškai – 0,053 μg/ml). Delfinidino ir petunidino kiekiai nustatyti 2,5 karto mažesni nei cianidino, o peonidino ir malvidino rasti mažiausi kiekiai. Švedijoje rinktuose ėminiuose aptiktas didžiausias santykinis malvidino kiekis. Jis didesnis už delfinidino ir petunidino kiekį.

Adresas susirašinėti: D. Burdulis, KMU Farmacinės chemijos ir farmakognozijos katedra, A. Mickevičiaus 9, 44307 Kaunas. El. paštas: deividas.burdulis@gmail.com

References

1. Rice-Evans CA, Miller NJ, Pagana G. Structure – antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 1996;20:933-56.
2. Kalt W, Dufour D. Healthy functionality of blueberries. Hort Technology 1997;7:216-21.
3. Mazza G, Miniati E. Anthocyanins in fruits, vegetables and grains. Boca Raton, Fla.: CRC Press Inc.; 1993. p. 362.
4. Kalt W, McDonald JE. Chemical composition of lowbush blueberry cultivars. J Amer Soc Hort Sci 1996;121:142-6.
5. Wang II, Cao G, Prior RL. Oxygen radical absorbing capacity of anthocyanins. J Agric Food Chem 1997;45:304-9.
6. Prior RL, Cao G, Martin A, Sofic E, McEwen J, O'Brien C, Lischner N, Ehlenfeldt M, Kalt W, Krewer G, Maunland CM.

Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. J Agric Food Chem 1998;46:2686-93.
7. Baj A, Bombardelli E, Gabetta B, Martinelli EM. Qualitative and quantitative evaluation of *Vaccinium myrtillus* anthocyanins by high-resolution gas chromatography and high-performance liquid chromatography. J Chromatography A 1983; 279:365-72.
8. Brenneisen VR, Steinegger E. Quantitativer Vergleich der Polyphenole in Fruchten von *Vaccinium myrtillus* L. unter Schiedlichen Reifegrades. Pharm Acta Helv 1981a:56:180-5.
9. Brenneisen VR, Steinegger E. Zur Analytik der Polyphenole der Fruchte von *Vaccinium myrtillus* L. (Ericaceae). (Analysis of polyphenols in *Vaccinium myrtillus* L. (Ericaceae) fruits.) Pharm Acta Helv 1981b;56:180-5.
10. Goiffon JP, Brun M, Bourrier MJ. High-performance liquid chromatography of red fruit anthocyanins. J Chromatography 1991;537:101-21.
11. Krawczyk U, Petri G. Application of RP-HPLC and spectrophotometry in standardization of bilberry anthocyanin extract. Arch Pharm 1992;325:147-9.
12. Petri G, Krawczyk U, Kery A. Spectrophotometric and chromatographic investigation of bilberry anthocyanins for qualification purposes. Acta Pharm Hung 1994;65:117-22.
13. Petri G, Krawczyk U, Kery A. Spectrophotometric and chromatographic investigation of bilberry anthocyanins for qualification purposes. Microchem Journal 1997;55:12-23.
14. Martinelli EM, Baj A, Bombardelli E. Computer-aided evaluation of liquid-chromatographic profiles for anthocyanins in *Vaccinium myrtillus* fruits. Anal Chim Acta 1986;191: 275-81.
15. Gozin AA. Effect of ecological factors on the level of biologically active agents in *Vaccinium myrtillus* berries. Restit Resur 1972;8:245.
16. Vanhaelen M, Lejoly J, Hanocq M, Molle L. Climatic and geographical aspects of medicinal plant constituents. In: Wijesecera ROB, editor. The medicinal plant industry. Boca Raton: CRC; 1991. p. 59-75.
17. Burdulis D, Ivanauskas L, Jakštas V, Janulis V. Mėlynės (*Vaccinium myrtillus* L.) vaisių vaistinės augalinės žaliavos antocianų tyrimas efektyviosios skysčių chromatografijos metodu. (Analysis of anthocyanin content in bilberry (*Vaccinium myrtillus* L.) fruit crude drugs by high-performance liquid chromatography method.) Medicina (Kaunas) 2007;43(2): 568-74.
18. Moeck S. Vaccinium. In: Hansel R, Keller K, Rimpler H, Schneider G, editors. Hagers Handbuch der Pharmazeutischen Praxis. (Hagers handbook of pharmaceutical practice.) Vol. 6 (P-Z). Berlin: Springer; 1994. p. 1051-67.
19. Morazzoni P, Bombardelli E. *Vaccinium myrtillus* L. Fitoterapia 1996;67(1):3-29.
20. European Pharmacopoeia. Strasburg: Council of Europe; 2005. p. 1099-100.
21. Skrede G, Wrolstad RE, Durst RW. Changes in Anthocyanins and polyphenolics during juice processing of highbush blueberries (*Vaccinium corymbosum* L.). J Food Sci 2000;65(2): 357-64.
22. Wu X, Prior RL. Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: fruits and berries. J Agric Food Chem 2005, 53:2589-99.

Received 30 October 2007, accepted 20 November 2007
Straipsnis gautas 2007 10 30, priimtas 2007 11 20