

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

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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.

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 μ m 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 μ m) 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 μ m) 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 μ L. 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 –

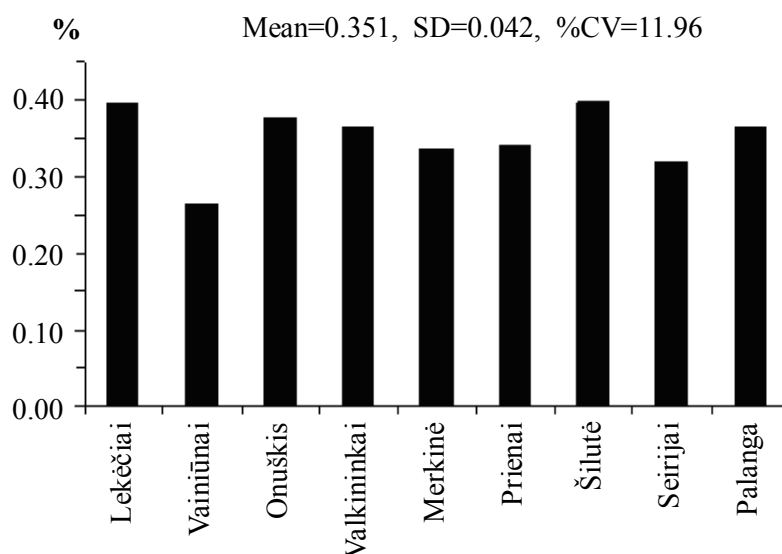


Fig 1. Total anthocyanin content in blueberries from different Lithuanian regions
SD – standard deviation, CV – coefficient of variation.

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 (Archangel'sk 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. Ber-

ries 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

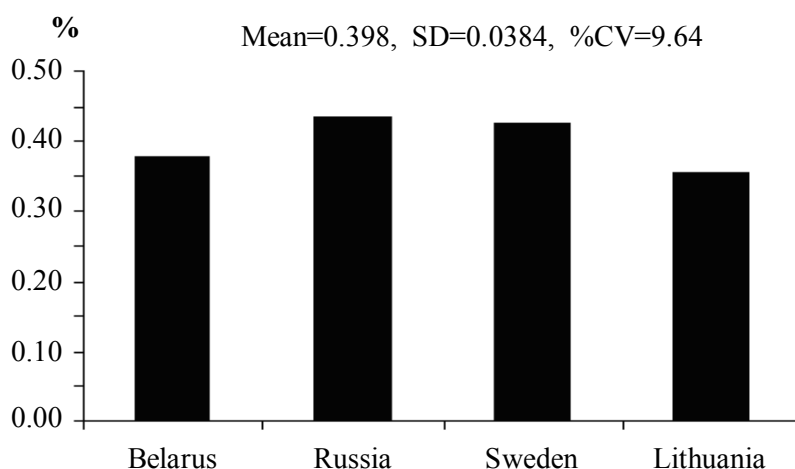


Fig. 2. Total anthocyanin content in blueberries from different countries

SD – standard deviation; CV – coefficient of variation.

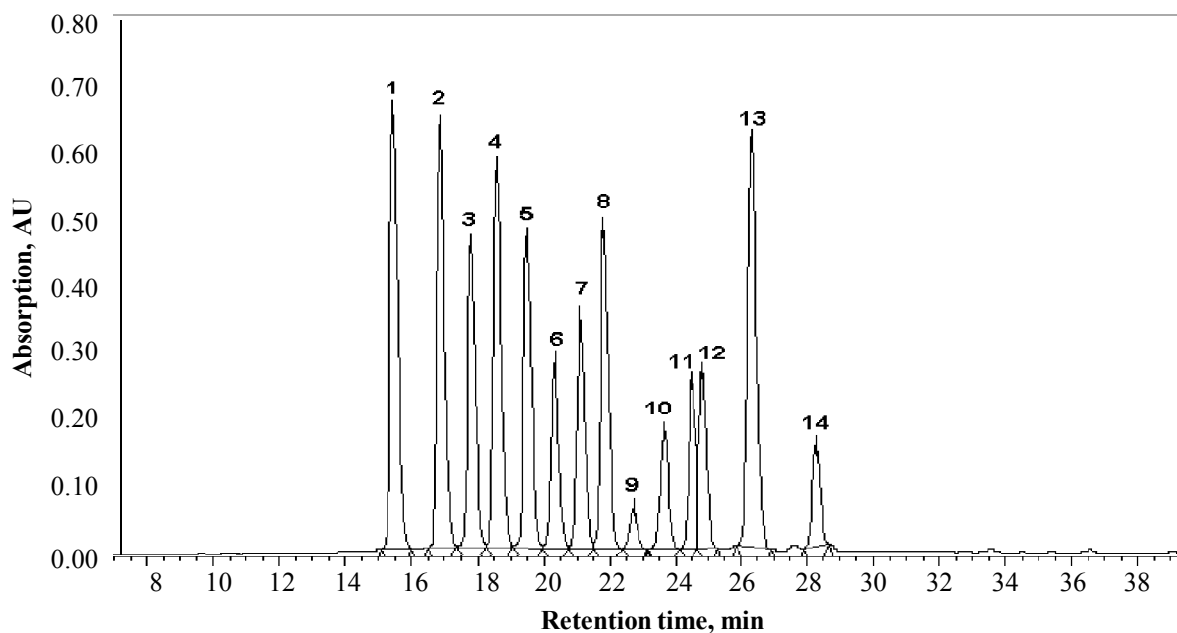


Fig. 3. Representative chromatogram of blueberry anthocyanins

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 $\mu\text{g}/\text{mL}$). 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 $\mu\text{g}/\text{mL}$).

Conclusions

From the study results the following conclusions can be drawn:

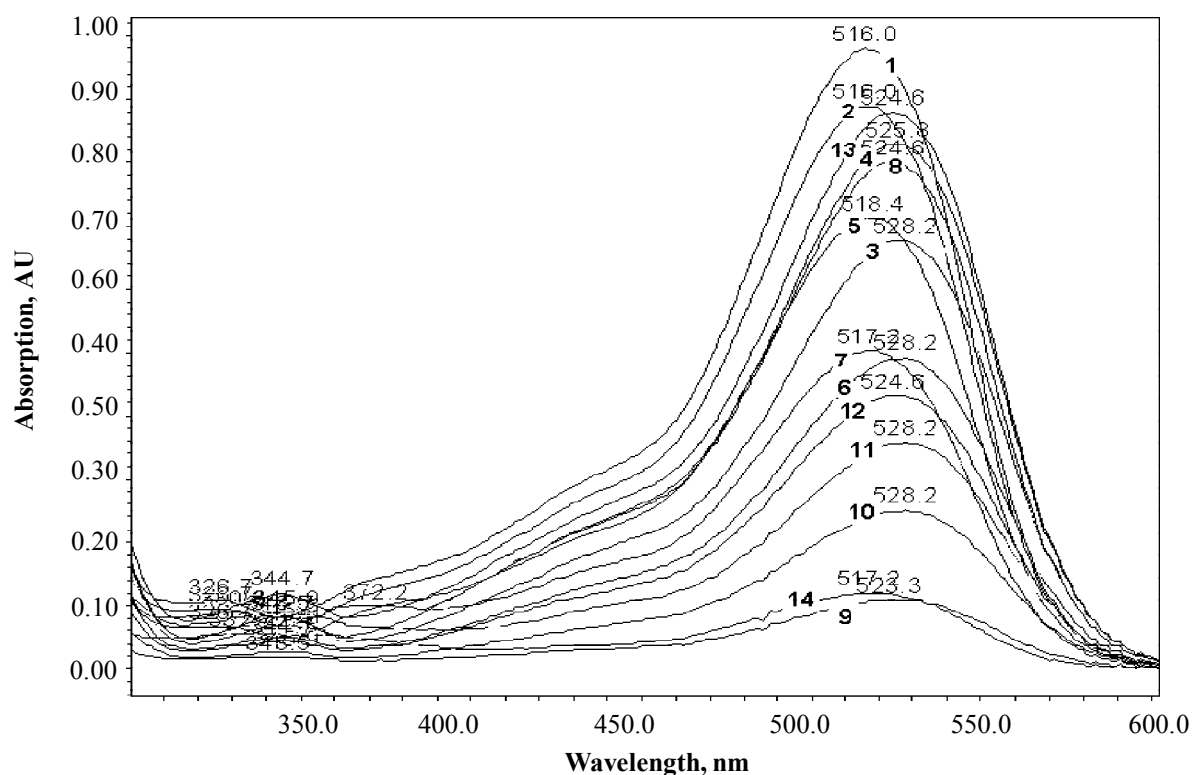


Fig. 4. The spectra of anthocyanins

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.

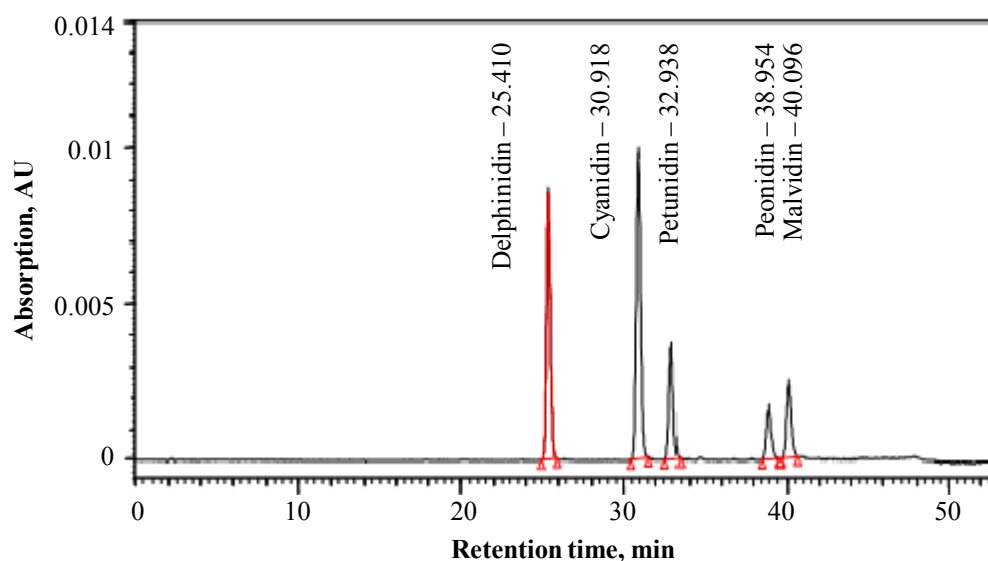


Fig 5. Representative chromatogram of anthocyanidins

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.

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

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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.

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