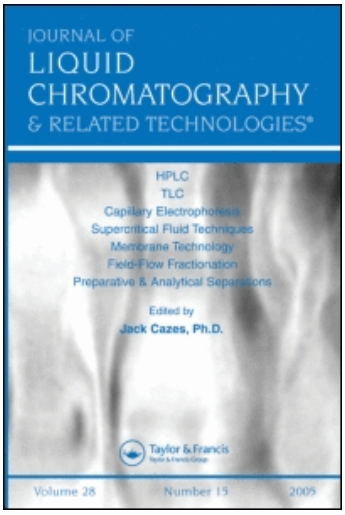

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Method Development for Determination of Anthocyanidin Content in Bilberry (*Vaccinium myrtillus* L) Fruits

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Abstract: The optimal conditions for blueberry fruits extraction were chosen for better sample preparation. The biggest amount of anthocyanins was found in methanolic extracts. Ultrasonic extraction was performed for comparison with the maceration method. Ten minutes of ultrasonic extraction has shown the best recovery of anthocyanins in all analysed samples. The optimization of acid hydrolysis conditions was determined as well. At various times and concentrations of HCl, the hydrolysis of anthocyanins was conducted. Five major anthocyanidins (delphinidin, cyanidin, petunidin, peonidin, and malvidin) were estimated by high performance liquid chromatography (HPLC) in bilberry fruit extracts. A simple HPLC method has been developed for the analysis of anthocyanidins in bilberry extracts. The main steps of HPLC method validation were estimated, respectively.

Keywords: Anthocyanins, Bilberries, HPLC analysis, Extraction, Hydrolysis

INTRODUCTION

Bilberry (Vaccinium myrtillus L) is a small sub-shrub widely known and found throughout central and northern Europe, northern Asia, and North America.

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Bilberry has been used as a food for centuries due to its nutritive properties.[1–3] Nowadays, fresh, frozen, or dried bilberry fruits, as medical crude drugs, are described in various pharmacopoeias.[4–6] Bilberries are rich in water (up to 90%) sugars, pectins, organic (citric, malic, lactic) and phenolic (vanillic, caffeic, chlorogenic) acids, tannins (mostly catechol), vitamins, and anthocyanins.

Anthocyanins of bilberry fruits, exists as C-3 *O*-glucosides, *O*-galactosides, and *O*-arabinosides of cyanidin, delphinidin, petunidin, peonidin, and malvidin.[1–3,5–7] The anthocyanins constitute the major flavonoid group that is found in fruits, flowers, and vegetables. These, natural colorants, consist of an aglycone (anthocyanidine) and sugar(s). The anthocyanins are derived from the 2-phenylbenzopyrylium cation, more commonly known as flavylium cation (Figure 1).[6,8]

The pharmacological properties of bilberry fruits are attributed to the presence of anthocyanins. Recent scientific researches with human cells and rats demonstrated that water soluble pigments may have a positive influence on many health conditions. The antioxidant properties of anthocyanins have been demonstrated by various experiments.[9–13] Antioxidant activities may contribute to the prevention of heart disease, cancer, and inflammatory disease.[9,14–16] Two aglycone anthocyanins (delphinidin and cyanidin) were used to examine their effects on cell cycle progression and on induction of apoptosis in human cancer cells (uterine carcinoma and colon adenocarcinoma cells) and in normal human fibroblasts.[14] Recently, it was determined that bilberry extract induced apoptotic cell bodies and nucleosomal DNA fragmentation in HL60 cells.[15] Scientific studies revealed the bioactivities of anthocyanins in bilberries and assessed their potential antiproliferation and apoptosis induction effects using two colon cancer cell lines, HT-29 and Caco-2.[16] It has been reported that anthocyanins rich supplementation could prevent impaired object recognition memory and elevated levels of the oxidative stress responsive protein, nuclear factor kappa B in aged rats. New experiments indicate that it may be possible to overcome genetic predispositions to Alzheimer disease through an anthocyanins rich diet.[17,18] In vitro

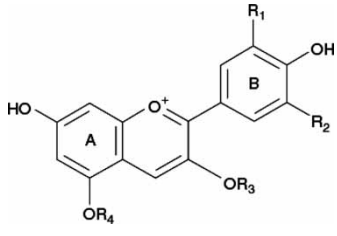

Figure 1. Structural formula of anthocyanins. Cyanidin: R1 = OH; R2, R3, R4 = H. Delphinidin: R1, R2 = OH; R3, R4 = H. Peonidin: R1 = OCH3; R2, R3, R4 = H. Petunidin: R1 = OCH3; R2 = OH; R3, R4 = H. Malvidin: R1, R2 = OCH3; R3, R4 = H. Pelargonidin: R1, R2, R3, R4 = H.

experiments proved that anthocyanins reduce retinal pigment epithelium cell damage as well.[19]

Numerous techniques are used for separation and quantification of anthocyanins. The most studied methods are high performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE).[20–25] Moreover, HPLC has been a method of choice for the analysis of anthocyanins in most fruits and their products. However, few studies deal with the method development and validation of anthocyanins in fruits extracts. Thus, the aim of the present study was to develop an HPLC method for the analysis of anthocyanidins in bilberry extracts and to select the optimal conditions for extraction and acid mediated hydrolysis.

EXPERIMENTAL

Raw Materials

Wild bilberry (*Vaccinium myrtillus* L) fruit samples were collected randomly in natural sampling sites in the territory of Lithuania in 2006. Fresh, ripe fruit samples were frozen and stored in the freezer at $-19 \pm 1^{\circ}$C until use. Loss on drying was carried out for the freshly crushed sample by drying it in the oven at 100–105°C as described in the European Pharmacopoeia. All results were recalculated for the absolutely dried material.

Chemicals

All solvents, reagents, and standards used in this study were of analytical grade. Acetonitrile (HPLC grade) and orthophosphoric acid (85% purity, HPLC grade) were purchased from Sigma–Aldrich GmbH, (Buchs, Switzerland). Methanol was provided by Roth (Karlsruhe, Germany), ethanol by Stumbras AB (Kaunas, Lithuania). Hydrochloric acid (35%) was purchased from Lachema (Neratovice, Czeck Republik). Anthocyanidin (cyanidin chloride, delphinidin chloride, petunidin chloride, peonidin chloride, and malvidin chloride) standards were of HPLC purity grade and purchased from Roth (Karlsruhe, Germany) and Chromadex (Santa Ana, USA). Water was deionized and filtered through a Millipore filter system (Millipore, USA) before use.

Extraction Procedures

Approximately 50 g of frozen berries were crushed in a pounder to produce a thick puree. A 5 g (accurate eight) aliquot was placed into a 100 mL flat bottom flask and various extractants (95 mL) were added to determine the

influence of extracting solvents. Pure water, methanol (concentration varied in the range from 30 to 100% (v/v)), and ethanol (concentration varied in the range from 30 to 96% (v/v)), were tested on the recovery of anthocyanins by the spectrophotometric method. According to previous data, two different extraction methods (maceration and ultrasonic agitation) were assayed, respectively. Selected extractant, in the optimal concentration ratio, was used for further analysis. Maceration and sonication extractions were performed at different time intervals. Maceration studies were carried out on the laboratory shaker, type 358S (Lubawa, Poland). Shaking speed was 150 ± 5 c.p.m. An ultrasonic bath BioSonic UC 100 (Coltene/Whaledent, Mahwah, NJ, USA) was used for sonication with occasional swirling. Three replicates of samples were prepared between all analysts.

Total Anthocyanins Measurement

Total anthocyanin contents of berry extracts were measured using the spectrophotometric method. The samples were determined using a model Unicam Helios ALPHA spectrophotometer (Unicam, Cambridge, UK). The obtained extract was filtrated through a paper filter into a 100.0 volumetric flask and diluted with appropriate extractant to 100.0 mL. A 50-fold dilution of this solution in a 0.1% v/v of hydrochloric acid in the extractant was prepared. The absorbance of the solution was measured at 528 nm wavelength, using a 0.1% v/v solution of hydrochloric acid in the appropriate extractant. Results are expressed as percentage of cyanidin 3-glucoside equivalents.[4]

Acid Mediated Hydrolysis of Anthocyanins

After acid hydrolysis the anthocyanin content was converted to five major anthocyanidins. Various amounts (4, 6, 8.5 mL) of 35% hydrochloric acid were added to 25 mL of prepared extract, and heated in a water bath with a refluxing condenser. At various times, the hydrolysis of anthocyanins with the selected amount of 35% hydrochloric acid was conducted for the method optimization. Hydrolyzed samples were passed through 0.22 μm pore size membrane filters (Carl Roth GmbH, Karlsruhe, Germany) prior to HPLC injection.

Chromatographic Analysis

Commercially available anthocyanidin standards were dissolved together in methanol to form a standard mixture of cyanidin chloride, delphinidin

chloride, petunidin chloride, peonidin chloride, and malvidin chloride. The standard mixture was diluted in methanol to 4/5, 3/5, 2/5, 1/5 of the initial concentrations. These standard solutions and the stock solution were injected into the HPLC system to generate a five point calibration curve for five anthocyanidin standard compounds separately. All standard solutions were measured in triplicate. Peak areas of the anthocyanidins in fruit extracts were within the linear range of the calibration curves. These standards were also used to further confirm the identities of anthocyanidins in the fruit extracts.

Experiments were performed with Waters 2690 Alliance HPLC system, equipped with Waters 2487 Dual λ Absorbance Detector (UV/Vis), and Waters 996 Photodiode Array (PDA) Detector (Waters corporation Milford, MA, USA), coupled to a personal computer with Waters Millennium 2000® chromatographic manager system (Waters corporation Milford, MA, USA) for data storage and processing. Separation of anthocyanidins was carried out using a Supelco Hypersil ODS (C_{18}) column (100 × 3 mm i.d.; particle size, 3 μm), guarded with a Hypersil ODS (10 × 4.6 mm i.d.; 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. Mobile phases were A, acetonitrile and B 4% aqueous ortophosphoric acid (V/V). The system was run with a gradient program: 0–37 min, 7–25% A, 37–40 min, 25–7% A and 40–45 min 7% A. There was a 5 min post run at initial conditions for the column equilibration. The injection volume for all standard solutions and bilberry extracts was 10 μL. The UV/Vis detector was set at 520 nm, PDA 200-800 nm wavelength. Identification of anthocyanidins was achieved by comparing their retention times and UV-Vis spectra, obtained with a PDA detector, with those of the standards.

Statistical Data Analysis

All of the assays were performed in $n = 3$ assays. The mean values, standard deviations were obtained using the SPSS (Chicago, JAV) computer package.

RESULTS AND DISCUSSION

Extraction Method Optimization

Anthocyanins are soluble in water and alcohols, but insoluble in apolar organic solvents. Usual extractants for extraction of anthocyanins are ethanolic or methanolic solutions. A small amount of hydrochloric, acetic, citric, formic, tartaric, or trifluoroacetic acid may be added to improve stability of the anthocyanins.[6,23–27] In the present study, the obtained

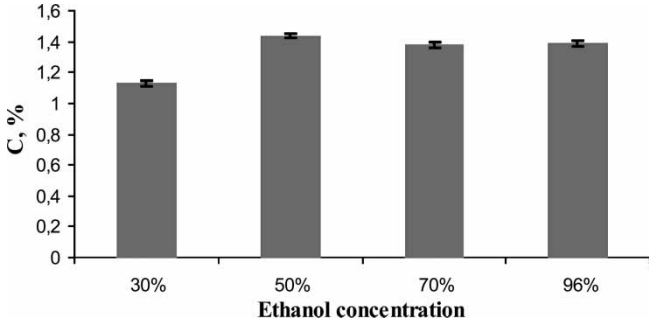

Figure 2. Extraction yields for the total amount of anthocyanins in bilberry extracts, using different concentration of ethanol.

extracts were hydrolysed immediately. Therefore, acidic solutions were not applied for the extraction procedure. The measurement of total anthocyanins was performed by a spectrophotometric method. Two different extraction methods (maceration and ultrasonic agitation) were tested in the initial stages of this study. The maceration method was used to determine the influence of the extractant polarity on the recovery of anthocyanins. We investigated the extraction yields with various ratios of methanol (concentration varied in the range from 30 to 100% (v/v)), ethanol (concentration varied in the range from 30 to 96% (v/v)), and water. Quantitative results obtained for the total amount of anthocyanins in bilberry extracts are shown in Figures 2 and 3. As can be seen from these figures, the optimal extractant concentration is 100% methanol. Pure water gave the least extraction yield; only 0.71% of total anthocyanins were estimated in aqueous extracts. On the basis of obtained data, the extraction of bilberry fruits was performed by maceration and sonication methods, using 100% methanol as extractant. Twenty-four hours of maceration gave the best results among all samples tested at different time intervals (Figure 4). However, a 10 min period of ultrasonic agitation indicated a higher extraction yield. Total antocyanin recoveries, obtained with different periods of sonication, are presented in Figure 5. This graphical chart shows that longer sonication periods decreased extraction yield in all estimated samples. Thus, the 10 min period of sonication was selected for further research.

Hydrolysis Method Optimization

Acid hydrolysis of more than 15 anthocyanins, present in bilberry extracts, yields 5 major anthocyanidins. The hydrolysis method was chosen because acid hydrolysis greatly simplifies quantification of the anthocyanidins

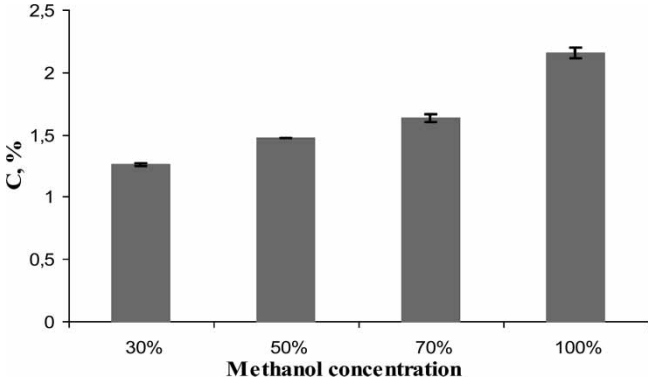

Figure 3. Extraction yields for the total amount of anthocyanins in bilberry extracts, using different concentration of methanol.

content. Hydrolysed anthocyanidin aglycones can be completely separated, identified and assayed.

The HPLC analysis of anthocyanins is complicated due to the reference compounds unavailability in the market. Mass spectrometry revealed that complete separation of all anthocyanins in a single HPLC run is very difficult, and this may influence the accuracy of the total anthocyanin calculations.[23] The hydrolysis of anthocyanins was conducted at various time intervals and concentrations of hydrochloric acid. Acid mediated hydrolysis was carried out and five major anthocyanidins (delphinidin, cyanidin, petunidin, peonidin, and malvidin) were estimated by HPLC in bilberry fruit extracts. The preliminary hydrolysis studies with different amounts of 35% hydrochloric acid were performed for 2 hours (Table 1), and revealed that 8.5 mL of hydrochloric acid gave optimal percentage of the total antocyanidins. Malvidin was an exception; the malvidin amount decreased slightly (3.3%) with an increased amount of hydrochloric acid. The hydrolysis rate of anthocyanins and the formation of aglycons were

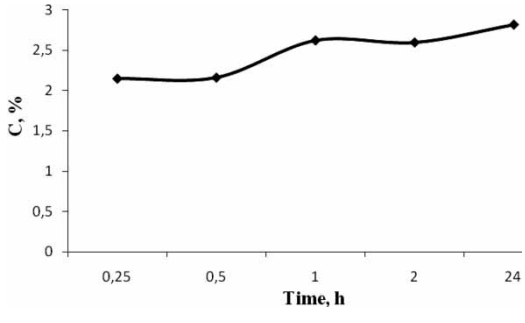

Figure 4. Effect of maceration time on anthocyanidin yields.

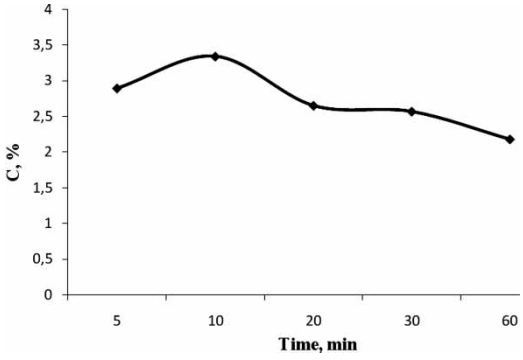

Figure 5. Effect of sonication time on anthocyanidin yields.

determined by the change in the peak pattern in the HPLC chromatogram (Figure 6). The hydrolysis reaction with 4 mL of acid converted most anthocyanins to aglycons, but small peaks of anthocyanins with retention times of 5–14 were still detected (Figure 6a). After hydrolysis with 8.5 mL of hydrochloric acid, anthocyanin peaks completely disappeared (Figure 6b). Six mL and 8.5 mL of hydrochloric acid gave similar amounts of individual anthocyanidns, only amounts of delphinidin and malvidin remained unchangeable,

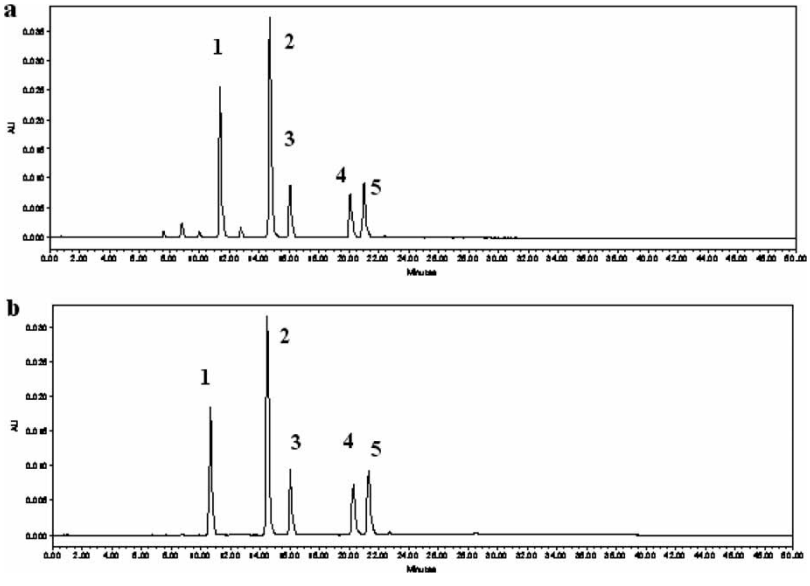

Figure 6. HPLC chromatograms of bilberry extracts after 4 mL hydrochloric acid hydrolysis (a) and 8.5 mL hydrochloric acid hydrolysis (b).

Table 1. Influence of hydrochloric acid on hydrolysis effect

Amount of HCl (ml)	Delphinidin (%)	Cyanidin (%)	Petunidin (%)	Peonidin (%)	Malvidin (%)	Total (%)
4	0.38 ± 0.02	1.13 ± 0.01	0.32 ± 0.01	0.34 ± 0.02	0.30 ± 0.01	2.48 ± 0.03
6	0.33 ± 0.02	1.19 ± 0.01	0.38 ± 0.02	0.34 ± 0.02	0.29 ± 0.01	2.53 ± 0.02
8.5	0.33 ± 0.02	1.21 ± 0.02	0.39 ± 0.01	0.35 ± 0.01	0.29 ± 0.01	2.57 ± 0.04
10	0.24 ± 0.02	1.18 ± 0.02	0.36 ± 0.03	0.30 ± 0.01	0.26 ± 0.01	2.34 ± 0.06

Values are means ± S.D of free aliquots.

but amounts of other anthocyanidins increased slightly. The increment of hydrochloric acid gave less percentage of individual anthocyanidins. Thus, 8.5 mL of hydrochloric acid was used for further studies. Later hydrolysis analysis at various times (Table 2) indicated that a 2 hour period afforded the highest total and individual amounts of anthocyanidins. Only delphinidin was an exception. The amount of delphinidin decreased with 2 hours of hydrolysis. This was in agreement with data previously published in literature, that delphinidin is not stable under hydrolysis conditions.[24] When the time was increased up to 2.5 hours, the percentage of total anthocyanidins decreased. Therefore, 2 hours of hydrolysis was used for prospective investigations.

Chromatographic Separation Optimization

The parameters for HPLC method development and validation have been defined and discussed in literature. In the present study, the main steps of HPLC method validation were estimated, respectively. This developed method was validated using bilberry (*Vaccinium myrtillus* L) fruit extract, but can be successfully adapted to many anthocyanin containing raw materials.

Solid phase extraction (SPE) is a very popular procedure for cleaning up a botanical matrix, prior to HPLC injection. In our case, all hydrolyzed anthocyanidns may be clearly determined at detection wavelength (520 nm); meanwhile, other compounds and impurities are not seen. SPE is quite complicated in this occasion due to the complexity of the botanical sample. Purification of extracts may cause loss of anthocyanins as well. Previous studies with bilberry extracts demonstrated that SPE did not give any significant difference between using extraction, and those not using any cleanup.[25,27] Therefore, the SPE cleanup step was rejected in our study.

The usual choice for organic modifier in RP-HPLC analysis is between acetonitrile and methanol. Acetonitrile/water mixtures show approximately 2.5 times lower viscosity than equivalent methanol/water eluents; this may afford 2.5 faster flow rates with acetonitrile as organic modifier.[28] Because of aforementioned beneficial properties acetonitrile was selected for further method development. Nowadays a majority of the HPLC methods use various amounts of acids in mobile phase, in order to maintain the acidic environment to ensure the stability of anthocyanins in the form of flavylium cation. Our method was optimized using 4% aqueous orthophosphoric acid. Initial analysis with the standards mixture was performed to attain optimal chromatographic separation. Furthermore, the same conditions were applied to bilberry fruit extracts. Resolution (Rs) is a degree of chromatographic separation of two adjacent peaks.[29] Desirable resolution value (Rs >2) was achieved for both standards and extracts aliquots. Calibration curves of standard compounds afforded good

Table 2. Effect of hydrolysis time on anthocyanidin yields

Time of hydrolysis (hours)	Delphinidin (%)	Cyanidin (%)	Petunidin (%)	Peonidin (%)	Malvidin (%)	Total (%)
1	0.27 ± 0.06	0.88 ± 0.19	0.29 ± 0.06	0.24 ± 0.05	0.30 ± 0.05	1.89 ± 0.41
1.5	0.34 ± 0.01	1.12 ± 0.02	0.36 ± 0.01	0.30 ± 0.01	0.26 ± 0.01	2.39 ± 0.04
2	0.33 ± 0.02	1.21 ± 0.02	0.39 ± 0.01	0.35 ± 0.01	0.29 ± 0.01	2.57 ± 0.05
2,5	0.29 ± 0.01	1.20 ± 0.01	0.37 ± 0.01	0.35 ± 0.01	0.29 ± 0.01	2.49 ± 0.02

Values are means ± S.D of free aliquots.

Table 3. Characteristics of quantitative analysis

Anthocyanidin	Regression equation	Correlation coefficient (R^2)	LOD (μg/mL)	LOQ (μg/mL)
Delpinidin	$Y = 5.33 \cdot 10^6 \times + 7.70 \cdot 10^3$	0.9993	0.034	0.080
Cyanidin	$Y = 2.54 \cdot 10^6 \times + 3.82 \cdot 10^3$	0.9997	0.076	0.018
Petunidin	$Y = 2.45 \cdot 10^6 \times - 1.24 \cdot 10^3$	0.9991	0.085	0.190
Peonidin	$Y = 2.39 \cdot 10^6 \times + 1.37 \cdot 10^3$	0.9929	0.080	0.186
Malvidin	$Y = 3.35 \cdot 10^6 \times + 4.82 \cdot 10^3$	0.9945	0.055	0.127

correlation for all anthocyanidins. Correlation coefficient (R^2) values were higher than 0.99 for all analytes and confirmed the linearity of the proposed method. The linear regression equations, linear correlation coefficients, and determination limits are presented in Table 3. Limit of detection (LOD) and limit of quantification (LOQ) were determined for compounds by the signal-to-noise ratio. These parameters of limit tests are important for qualitative and quantitative analysis of investigating analytes. LOD values ranged from 0.034 to 0.085 μg/mL and LOQ accordingly from 0.018 to 0.190 μg/mL. To confirm the precision of the developed method, repeatability of the results was examined. Five replicate injections of methanolic bilberry extracts were carried out. As can be seen from Table 4, repeatability and reproducibility for retention times and peak areas were agreeable. They didn't exceed 3.4% for retention time and 4.0% for peak area.

The developed HPLC method is very suitable for quantification of anthocyanidins in bilberry raw materials. This method was applied to 6 samples, collected from different natural sampling sites, for quantification of

Table 4. Repeatability of HPLC method

	RSD (%)					
	Retention time			Integrated area		
Anthocyanidin	Run-to-run	Day-to-day	Extraction-to-extraction	Run-to-run	Day-to-day	Extraction-to-extraction
Delpinidin	0.53	3.39	1.33	1.64	0.32	3.96
Cyanidin	0.34	3.15	1.05	1.89	1.27	2.65
Petunidin	0.36	2.12	0.93	1.75	0.85	2.77
Peonidin	0.36	1.55	0.80	1.83	1.09	1.91
Malvidin	0.28	1.32	0.72	1.40	3.07	2.77

Table 5. Distribution of anthocyanidins in bilberry fruit extracts

Sampling sites	Anthocyanidins (%)					
	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin	Total
Kurtuvėnų park	0.27	0.97	0.33	0.23	0.15	1.96
Skliausčių forest	0.30	0.98	0.34	0.38	0.23	2.21
Darbėnų forest	0.28	0.77	0.34	0.24	0.21	1.85
Menciškės forest	0.27	0.71	0.29	0.18	0.20	1.65
Onuškio forest	0.26	0.77	0.30	0.23	0.25	1.81
Medvalakio forest	0.27	0.71	0.30	0.27	0.19	1.74
M	0.27	0.87	0.32	0.26	0.21	—
Min	0.26	0.71	0.29	0.18	0.15	—
Max	0.30	0.98	0.34	0.38	0.25	—
$S_{\bar{x}}$	0.01	0.05	0.01	0.03	0.01	—
CV (%)	3.99	13.83	7.20	23.03	14.97	—

anthocyanidins. Obtained results and their statistical assessments are presented in Table 5. Patterns of anthocyanidins in all investigated samples were similar in distribution. From our data we found that cyanidin is a dominant anthocyanidin in all crude drug samples.

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