DIETARY SUPPLEMENTS

New Liquid Chromatography Method with Ultraviolet Detection for Analysis of Anthocyanins and Anthocyanidins in *Vaccinium myrtillus* Fruit Dry Extracts and Commercial Preparations

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The *Vaccinium myrtillus* fruits (bilberry) are a well-known anthocyanins source, and their extracts are widely used in dietary botanicals and pharmaceutical products for the treatment of vascular and vision disorders. Different analytical methods used for standardization of the bilberry extracts and their preparations are available from pharmacopeias and from the literature. However, the methods reported in the literature do not allow the detection of free anthocyanidins, which are markers of poor product quality. A new liquid chromatography method was developed and validated for the identification and quantification of both anthocyanins and anthocyanidins present in bilberry extracts and products. The method shows a good reproducibility and, due to its high specificity, is suitable to identify unequivocally the botanical raw materials used for manufacturing and to evaluate the extract composition, thus ensuring a high degree of product consistency and quality. Forty typical bilberry preparations belonging to 24 different brands were purchased in the marketplace and evaluated for their quality by using the developed method. Results revealed marked differences among the brands despite a common origin and labeling.

Bilberry (*Vaccinium myrtillus* L.) fruits are a well-known source of anthocyanins, and their extracts are widely used in dietary botanicals and pharmaceutical products for the treatment of vascular and vision disorders linked to capillary and venous fragility (1). Bilberry anthocyanins are the main compounds responsible for the therapeutic activity of the extract (2, 3). The term anthocyanin, initially coined to designate the substance responsible for the color of cornflower (from the Greek *anthos,* flower, and *kuanos*, blue) applies to a group of water-soluble pigments responsible for the red, pink, mauve, purple, blue, or violet color of most flowers and fruits. These pigments (the anthocyanins) occur as glycosides, and their aglycones (the anthocyanidins) are derived from the 2-phenylbenzopyrylium cation, more commonly referred to as flavylium cation, a name that emphasizes the fact that these molecules belong to the vast group of flavonoids in the broad sense of the term. The bilberry anthocyanins are C-3 glucosides, galactosides, and arabinosides of cyanidin, peonidin, delphinidin, malvidin, and petunidin. Typical structures of bilberry anthocyanins are shown in Figure 1 along with their anthocyanidins. The majority of the clinical trials have been performed using standardized bilberry extract containing 36% of anthocyanins. This extract was demonstrated to be effective in the therapy of peripheral vascular diseases and venous sensitivity, or to alteration of microcirculation of the retina at a dosage of 320–480 mg/day, corresponding to 100–200 mg/day anthocyanins (1).

Different analytical methods used for standardization of the bilberry extracts and preparations are available from pharmacopeias and from the literature. However, most of them do not satisfy modern analytical requirements and are not convenient for reproducing measurements. The most common analytical methods use UV-visible spectrophotometry that allows the quantification of anthocyanins by detection in the visible region (4–8). In spite of the fact that these methods are very popular, they lack specificity and do not allow the identification of each anthocyanin. As a consequence, these methods are not suitable for the identification of anthocyanins extracts produced with different plant materials (raspberry, blackberry, black currant, elderberry, etc.).

Several thin-layer chromatography methods were developed (6, 7) to qualify and quantify the anthocyanidin content after anthocyanin hydrolysis. A gas chromatography method allows the identification of the quinoline derivatives of 15 anthocyanins (9). Ichiyanagi et al. (10, 11) separated 15 major anthocyanins present in *V. myrtillus* by capillary zone electrophoresis showing the anthocyanin fingerprint of bilberry. Liquid chromatography (LC) seems to be the best technique for standardization of anthocyanin extracts, allowing evaluation of the individual anthocyanins (4, 7, 9, 12–22). Unfortunately, most of these methods do not allow complete separation of all the constituents. Sometimes, in order to simplify the UV-visible and the LC procedures, the materials are submitted to acidic hydrolysis followed by the

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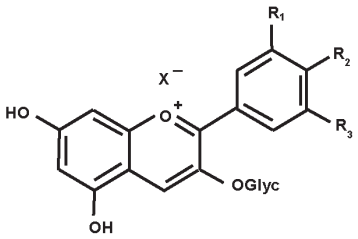


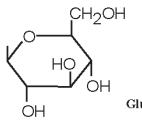
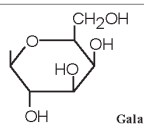
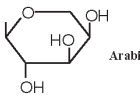
	COMPOUND	R_1	R_2	R_3	Glyc
ANTHOCYANIDIN	Delphinidin	OH	OH	OH	H
	Cyanidin	OH	OH	H	H
	Petunidin	OH	OH	OCH_3	H
	Peonidin	OCH_3	OH	H	H
	Malvidin	OCH_3	OH	OCH_3	H
ANTHOCYANIN	Delphinidin-3-*O*-glucoside	OH	OH	OH	 Glucosyl
	Cyanidin-3-*O*-glucoside	OH	OH	H	
	Petunidin-3-*O*-glucoside	OH	OH	OCH_3	
	Peonidin-3-*O*-glucoside	OCH_3	OH	H	
	Malvidin-3-*O*-glucoside	OCH_3	OH	OCH_3	
	Delphinidin-3-*O*-galactoside	OH	OH	OH	 Galactosyl
	Cyanidin-3-*O*-galactoside	OH	OH	H	
	Petunidin-3-*O*-galactoside	OH	OH	OCH_3	
	Peonidin-3-*O*-galactoside	OCH_3	OH	H	
	Malvidin-3-*O*-galactoside	OCH_3	OH	OCH_3	
	Delphinidin-3-*O*-arabinoside	OH	OH	OH	 Arabinosyl
	Cyanidin-3-*O*-arabinoside	OH	OH	H	
	Petunidin-3-*O*-arabinoside	OH	OH	OCH_3	
	Peonidin-3-*O*-arabinoside	OCH_3	OH	H	
	Malvidin-3-*O*-arabinoside	OCH_3	OH	OCH_3	

Figure 1. Structures of anthocyanidins and anthocyanins in bilberry fruits.

measurement of aglycone (anthocyanidin) abundance (9, 13, 18, 19, 22, 23). However, these kinds of analytical methods are far from satisfactory, because if some proanthocyanidins are present in the extracts, they too form anthocyanidins by hydrolysis and provide an overestimation of anthocyanin content. This procedure also does not allow quantification of the real content of anthocyanidins. Indeed, anthocyanidins are degradation products of anthocyanins mainly due to inappropriate storage conditions of the plant material (enzymatic degradation; 24) or unsuitable extraction procedures (18).

In order to overcome these analytical issues, a new LC method has been developed and validated which allows the identification and quantification of all the anthocyanins and anthocyanidins in bilberry extracts and products. The quantification procedure foresees cyanidin-3-*O*-glucoside as an external standard, and the content of each individual anthocyanin is evaluated making use of a molecular weight correction factor. According to the literature (16), there is a direct correlation between molecular weights and responses (absorbance/concentration) between anthocyanins containing similar aglycons (anthocyanidins). However, only cyanidin chloride is easily available on the market at the moment and only cyanidin-3-*O*-glucoside could be produced by synthesis in a suitable amount. Accordingly, molecular weight correction factors with respect to these constituents were introduced for calculation of anthocyanidins and anthocyanins. The method shows good reproducibility and, due to its high specificity, is suitable to identify unequivocally the botanical raw materials used for manufacturing and to evaluate the composition of extracts, providing a high degree of product consistency and quality. A bilberry dry extract was fully characterized by using chromatographic and spectroscopic techniques in order to have a common reference which allows the detection of adulteration with other berries and avoids the use of the expensive standard cyanidin-3-*O*-glucoside for routine analyses. The Italian Pharmacopeia (25) has recently adopted this method described in the bilberry extract monograph. This is the first national monograph that sets the quality of the bilberry extract which has to contain from 32.4 to 39.6% of anthocyanins and not more than 1.0% of anthocyanidins. Additionally, the method described here was used to evaluate the quality of 40 typical bilberry preparations belonging to 24 different brands found in European, American, and Japanese marketplaces.

METHOD

Apparatus

(**a**) *Volumetric flasks.*—20, 25, 50, and 100 mL; class A (Duran®, Hirschmann, Eberstadt, Germany).

(**b**) *Bulb pipets.*—2, 5, and 15 mL; class AS bulb pipets (Hirschmann).

(**c**) *Ultrasonic bath.*—Bransonic 220 (Branson Cleaning Equipment Co., Shelton, CT).

(**d**) *Autosampler vials.*—2 mL Silcote™ CL7 deactivated clear vial with screw-thread cap with a polytetrafluoroethylene (PTFE)/silicone septum (Restek Corp., Bellefonte, PA).

(**e**) *Analytical column.*—Zorbax 5 µm, Extend-C18 (80 Å pore size), 4.6 × 250 mm (Agilent Technologies, Palo Alto, CA).

(**f**) *LC systems.*—Waters Alliance 2690 Separations Module (Milford, MA) equipped with 996 Photodiode Array (PDA) Detector. Waters 600 pump, equipped with 996 PDA Detector and Autosampler Waters 717 plus. Agilent 1100 with diode array detector (DAD) G1315A and Autosampler G1329A with Temperature Controller G1330A. Data analysis was performed with Waters Empower software.

(**g**) *LC/mass spectrometry (MS) system.*—The LC/electrospray ionization (ESI)/MS analyses were performed on a Finnigan MAT LCQ (ThermoFinnigan, San Jose, CA) ion-trap mass spectrometer equipped with a Microsoft® Windows® NT™ data system and an ESI interface. The LC system included a Thermo Separation Product P4000 pump and a Diode Array Thermo Separation Products UV6000LP detector (ThermoFinnigan).

Reagents

(**a**) *Water.*—Deionized and passed trough a carbon filter Milli-Q water purification system (Millipore, Bedford, MA).

(**b**) *Methanol.*—LC grade (J.T. Baker, Phillipsburg, NJ).

(**c**) *Acetonitrile.*—LC grade (J.T. Baker).

(**d**) *Formic acid.*—Analytical grade, 85% (Sigma-Aldrich, St. Louis, MO).

(**e**) *LC mobile phase, solution A.*—Mix well 100 mL formic acid and 900 mL water, and degas before use.

(**f**) *LC mobile phase, solution B.*—Mix well 225 mL methanol, 225 mL acetonitrile, 100 mL formic acid, and 400 mL water, and degas before use.

(**g**) *Hydrochloric acid.*—Analytical grade, 36–38% (J.T. Baker).

(**h**) *Phosphoric acid.*—Analytical grade, 99.999%, 85% solution in water (Sigma-Aldrich).

(**i**) *Dilution solution A.*—Prepare 2% (v/v) hydrochloric solution by adding 20 mL hydrochloric acid to 800 mL methanol. Mix well, transfer to 1000 mL volumetric flask, and dilute to volume with methanol.

(**j**) *Dilution solution B.*—Prepare diluted phosphoric solution by adding 100 mL phosphoric acid to 800 mL water. Mix well, transfer to 1000 mL volumetric flask, and dilute to volume with water.

(**k**) *Reference standards.*—Cyanidin chloride and cyanidin-3-*O*-glucoside chloride were obtained from Indena SpA R&D Laboratories (Settala, Italy) based on the Robertson and Robinson syntheses (26–29). Cyanidin chloride and cyanidin-3-*O*-glucoside reference standards were chemically characterized by LC/PDA, nuclear magnetic resonance (NMR), Fourier transform-infrared, and MS analyses (purity of 98.8 and 90.8%, respectively). *V. myrtillus* dry extract reference standard was produced and standardized by LC/PDA, NMR, and LC/MS analysis at Indena R&D Laboratories (cyanidin-3-*O*-glucoside chloride content 3.6%, total anthocyanins 36.2%).

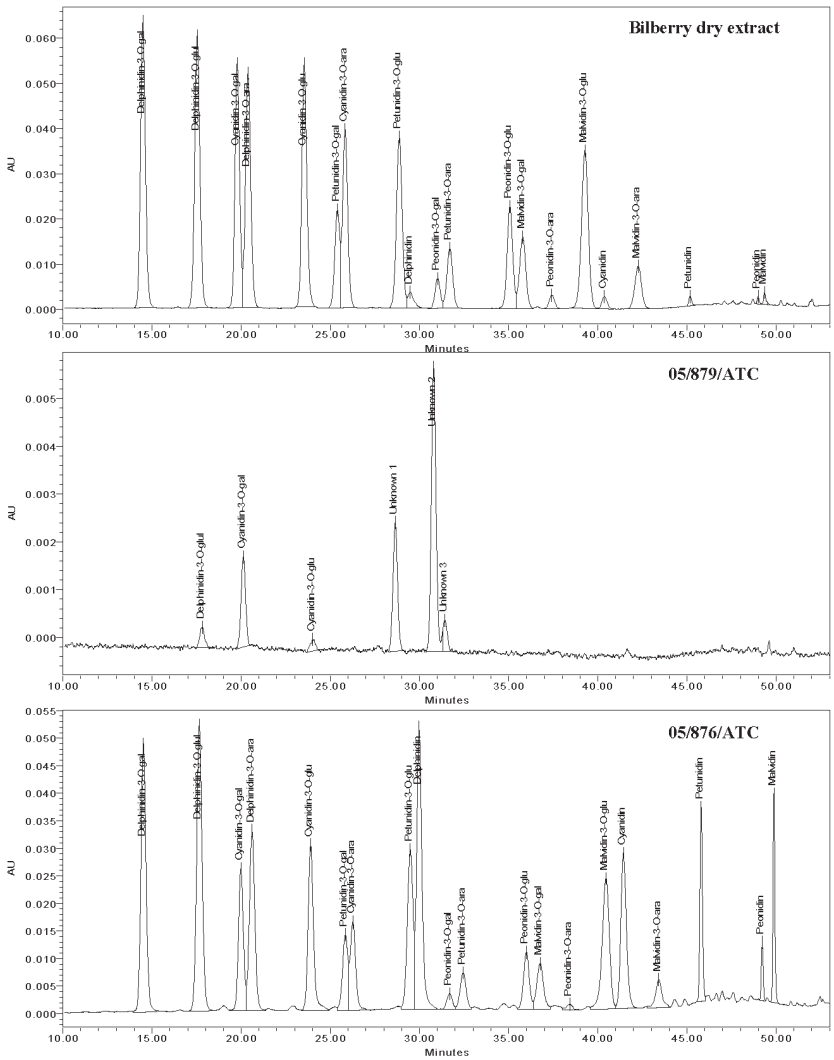


Figure 2. Comparisons between the typical profile of bilberry dry extract and chromatograms belonging to 2 commercial preparations.

Table 1. Peak identification with the retention time (RT) relative to the cyanidin-3-*O*-glucoside and the corresponding molecular weight (MW)

Peak	Components	RT	Relative RT	MW_r
1	Delphinidin-3-*O*-galactoside	11.882	0.61	500.84
2	Delphinidin-3-*O*-glucoside	14.238	0.73	500.84
3	Cyanidin-3-*O*-galactoside	16.287	0.84	484.84
4	Delphinidin-3-*O*-arabinoside	16.726	0.86	470.81
5	Cyanidin-3-*O*-glucoside	19.468	1.00	484.84
6	Petunidin-3-*O*-galactoside	21.059	1.08	514.88
7	Cyanidin-3-*O*-arabinoside	21.625	1.11	454.81
8	Petunidin-3-*O*-glucoside	24.208	1.24	514.86
9	Delphinidin	25.004	1.29	338.70
10	Peonidin-3-*O*-galactoside	26.387	1.36	498.86
11	Petunidin-3-*O*-arabinoside	26.951	1.38	484.85
12	Peonidin-3-*O*-glucoside	30.193	1.55	498.86
13	Malvidin-3-*O*-glucoside	30.816	1.58	528.89
14	Peonidin-3-*O*-arabinoside	32.474	1.67	468.84
15	Malvidin-3-*O*-galactoside	34.171	1.76	528.89
16	Cyanidin	35.436	1.82	322.70
17	Malvidin-3-*O*-arabinoside	37.087	1.91	498.86
18	Petunidin	40.478	2.02	352.72
19	Peonidin	44.141	2.18	336.72
20	Malvidin	44.788	2.21	366.75

Solutions

(**a**) *Blank solution.*—Pipet 5 mL dilution solution A into a 20 mL volumetric flask and dilute to volume with dilution solution B.

(**b**) *System suitability solution.*—Use a working *V. myrtillus* dry extract system suitability test.

(**c**) *Cyanidin reference standard solutions.*—Prepare a solution with a concentration of about 0.01 mg/mL. For instance, in duplicate, accurately weigh 10 mg cyanidin reference standard into a 20 mL volumetric flask. Add about 15 mL dilution solution A, and dissolve. Dilute to volume with the same solvent, and mix. Pipet 2.0 mL of this solution into a 100 mL volumetric flask, and dilute to volume with dilution solution B.

(**d**) *Cyanidin-3-O-glucoside reference standard solution.*—Prepare a solution with a concentration of about 0.08 mg/mL. For instance, in duplicate, accurately weigh 10 mg cyanidin-3-*O*-glucoside reference standard into a 25 mL volumetric flask. Add about 15 mL dilution solution A, and dissolve. Dilute to volume with the same solvent, and mix well. Pipet 2.0 mL of this solution into a 10 mL volumetric flask, and dilute to volume with dilution solution B.

(**e**) *Working V. myrtillus dry extract reference standard solution.*—In duplicate, accurately weigh 125 mg *V. myrtillus* dry extract reference standard into a 25 mL volumetric flask. Add about 15 mL dilution solution A, and dissolve. Dilute to volume with the same solvent and mix well. Pipet 5.0 mL of this solution into a 20 mL volumetric flask and dilute to volume with dilution solution B to obtain a final concentration of 1.25 mg/mL.

(**f**) *V. myrtillus dry extract.*—Accurately weigh 125 mg *V. myrtillus* dry extract to be examined into a 25 mL volumetric flask. Add about 15 mL dilution solution A and dissolve. Dilute to volume with the same solvent, and mix. Pipet 5.0 mL of this solution into a 20 mL volumetric flask and dilute to volume with dilution solution B to obtain a final concentration of 1.25 mg/mL.

(**g**) *Capsules containing V. myrtillus dry extract (single or multiple components).*—In duplicate, open and empty 3–5 capsules into a 50 mL flask, adding shells. Add about 60% of the needed volume of methanol–hydrochloric acid (9 + 2, v/v) mixture (total volume: 50 mL of solvent for about 120 mg bilberry extract); sonicate for 10 min, shaking manually at least 5 times. Transfer the solution into a suitable volumetric flask, wash the flask and the capsules 3 times with a mixture of methanol–hydrochloric acid (98 + 2, v/v), and dilute to the final volume (50 mL for about 120 mg bilberry extract) with the same solvent. Transfer 10 mL of this solution into a 20 mL volumetric flask and dilute to volume with dilution solution B. If the previous preparation does not allow completely removing the bilberry extract from the capsule shell, proceed as follows: In duplicate, open and empty the capsules into a 50 mL round-bottom flask, adding shells. Add about 60% of the needed volume of methanol (total volume: 50 mL for about 120 mg bilberry extract) and heat under reflux for 20 min in a water bath, shaking manually several times. Let the solution cool and carefully add, with stirring, an amount of hydrochloric acid to obtain a final acid concentration of 2%, v/v. Transfer the solution into a suitable volumetric flask, wash the flask and the capsules 3 times with a mixture of methanol–hydrochloric acid (98 + 2, v/v), and dilute to the final volume (50 mL for about 120 mg bilberry extract) with the same solvent. Transfer 10 mL of this solution into a 20 mL volumetric flask and dilute to volume with diluted phosphoric acid (10 mL phosphoric acid diluted to 100 mL with water).

(**h**) *Tablets containing V. myrtillus dry extract (single or multiple component).*—Weigh 20 tablets and calculate the average tablets weight. Grind at least 10 tablets with a mortar and pestle to create a powder consistency capable of passing through a No. 60 sieve. Accurately weigh 125 mg powder and proceed as described for (**g**) above.

LC Procedure

Set the column oven temperature at 30°C. Set the temperature oven of the autosampler at 4°C. Set the UV detector at 535 nm. Equilibrate the column by passing about 100 mL degassed initial conditions mobile phase at a flow rate of 1.0 mL/min or until a steady baseline is obtained. Use a step

gradient of LC mobile phase, solution A (A) and LC mobile phase, solution B (B), according to the following profile, with flow rate set at 1.0 mL/min: 0–35 min, 93–75% A, 7–25% B; 35–45 min, 75–35% A, 25–65% B; 45–46 min, 35–0% A, 65–100% B; 46–50 min, isocratic 0% A, 100% B; 50–51 min, 0–93% A, 100–7% B; 51–60 min, isocratic 93% A, 7% B. A typical chromatogram is shown in Figure 2. The relative retention times for each anthocyanidin and anthocyanin, along with their molecular weights (MW), are listed in Table 1.

Calculations

To determine the total content of anthocyanins (T%), calculate the content of each anthocyanin (T_i%) by means of the following equation:

$$T_i\% = \frac{area_i \times C_{std} \times Q}{area_{std} \times C_{sample}} \times \frac{MW_i}{MW_{std}} \times 100$$

where $area_i$ is the peak area of the ith anthocyanin in the sample solution; $area_{std}$, the mean peak area of cyanidin-3-*O*-glucoside chloride for the reference solutions; C_{sample}, the sample concentration in the sample solution; C_{std}, the reference concentration in the reference solution; Q, the purity of cyanidin-3-*O*-glucoside chloride reference standard; and MW_i and MW_{std}, the molecular weight of the ith anthocyanin and cyanidin-3-*O*-glucoside chloride, respectively. Calculate the total content of anthocyanins (T%) by means of the following equation:

$$T\% = \sum T_i\%$$

For determination of total content of anthocyanidins (T%), calculate the content of each anthocyanidin (T_i%) by means of the following equation:

$$T_j\% = \frac{area_j \times C_{std} \times Q}{area_{std} \times C_{sample}} \times \frac{MW_j}{MW_{std}} \times 100$$

where $area_j$ is the peak area of the jth anthocyanidin in the sample solution; $area_{std}$, the mean peak area of cyanidin chloride for the reference solutions; C_{sample}, the sample concentration in the sample solution; C_{std}, the reference concentration in the reference solution; Q, the purity of cyanidin chloride reference standard; and MW_j and MW_{std}, the molecular weight of the jth anthocyanidin and cyanidin chloride, respectively. Calculate the total content of anthocyanidins (T%) by means of the following equation:

$$T\% = \sum T_j\%$$

Identification and Peak Purity

The specificity of the method and the identification of anthocyanidins and anthocyanins in *V. myrtillus* dry extract were assessed by LC/MS and LC/PDA analyses. The LC conditions are described above. Mass spectrometer conditions were optimized in order to achieve maximum sensitivity. ESI conditions: source voltage, 5.0 kV; sheath gas, nitrogen; flow rate, 60 arbitrary units; source current, 80 μA; capillary voltage, 10 V; and capillary temperature, 250°C. Full scan spectra from *m/z* 150–2000 in the positive-ion mode were obtained at 1 scan/s. The acquisition of the ion trap conditions were set in automatic gain control with a max-inject time of 200 ms. Identification of anthocyanins was based on the $[M]^+$ and $[M\text{-sugar unit}]^+$ ions observed in the spectrum of each peak; anthocyanidins were identified by the $[M]^+$. Specificity using LC/PDA analyses is assessed by determination of elution peak integrity and library matching for anthocyanins and anthocyanidins with PDA analyses. Evaluation of elution peaks integrity and library matching are performed by spectral contrast technique.

Linearity

Seven levels of concentration were prepared for cyanidin chloride and cyanidin-3-*O*-glucoside chloride, the reference compounds used for the quantification of anthocyanidins and anthocyanins, respectively. The studied concentration ranges were 5.0–200.0 μg/mL for cyanidin-3-*O*-glucoside chloride and 0.075–15.0 μg/mL for cyanidin chloride. The method showed linear responses in the studied concentration ranges for both cyanidin-3-*O*-glucoside chloride and cyanidin chloride, with correlation coefficients of 0.99994 and 0.99965, respectively.

Stability

The stability of cyanidin chloride and cyanidin-3-*O*-glucoside chloride in the reference solutions and in each quantified component of bilberry extract in the test solution was evaluated. The analyses were performed by injecting the stability solutions kept at 4°C every 4 h over 48 h. The regression lines of the stability studies were calculated for every constituent using the area responses versus time intervals. The responses do not change more than 5% making reference to time zero.

Cyanidin-3-*O*-glucoside chloride and each constituent of *V. myrtillus* dry extract are stable in test and reference solutions at 4°C up to 48 h. Cyanidin chloride is stable in reference solution at 4°C up to 36 h.

The stability of the anthocyanins during capsules and tablets extraction procedures was evaluated by submitting the *V. myrtillus* dry extract reference standard to the same procedures and evaluating the anthocyanins recovery.

Chromatographic Precision, Repeatability, and Intralaboratory Precision

The chromatographic precision was determined by evaluating the relative standard deviation (RSD) of the responses for each anthocyanin and anthocyanidin peak area for 10 replicate injections of the *V. myrtillus* dry extract reference standard solutions. The RSD of the responses was <1.0 and 1.5% for anthocyanins and anthocyanidins, respectively. Chromatographic precision results are reported in Table 2.

To evaluate the repeatability of the method, 3 solutions at 70, 100, and 130% of the working concentration of

Table 2. Chromatographic precision and repeatability results

	Chromatographic precision results[a]	Repeatability results[b]	
	Peak area RSD, %	Content, %	RSD, %
Cyanidin 3-*O*-arabinoside	0.14	2.46	0.95
Cyanidin 3-*O*-galactoside	0.64	3.33	0.53
Cyanidin 3-*O*-glucoside	0.05	3.58	0.49
Delphinidin 3-*O*-arabinoside	0.53	3.77	0.49
Delphinidin 3-*O*-galactoside	0.06	4.42	0.50
Delphinidin 3-*O*-glucoside	0.08	4.54	0.40
Malvidin 3-*O*-arabinoside	0.26	0.88	0.45
Malvidin 3-*O*-galactoside	0.70	1.39	0.64
Malvidin 3-*O*-glucoside	0.27	3.47	0.87
Peonidin 3-*O*-arabinoside	0.97	0.20	0.96
Peonidin 3-*O*-galactoside	0.38	0.44	0.61
Peonidin 3-*O*-glucoside	0.58	1.66	0.30
Petunidin 3-*O*-arabinoside	0.18	1.03	0.47
Petunidin 3-*O*-galactoside	0.21	1.60	0.54
Petunidin 3-*O*-glucoside	0.06	3.13	1.03
Total		35.90	0.23
Cyanidin	1.50	0.046	1.25
Delphinidin	0.71	0.088	1.14
Malvidin	0.79	0.021	1.36
Peonidin	1.13	0.009	1.74
Petunidin	0.73	0.020	1.64

[a] Relative standard deviation (RSD, %) of the responses for each anthocyanin and anthocyanidin peaks area for 10 replicate injections of the *V. myrtillus* dry extract reference standard solutions.

[b] Relative standard deviation (RSD, %) of the content of each anthocyanin; anthocyanidin of triplicate sample preparations at 3 levels of concentrations of the *V. myrtillus* dry extract solutions.

V. myrtillus dry extract were prepared. For each level, 3 different solutions were prepared, and each solution was injected 3 times. The RSD for each anthocyanin and total anthocyanidins content was calculated to estimate the method precision. The RSD of the content for each anthocyanin, for total anthocyanins, and for total anthocyanidins was <2.0% in all experiments. Repeatability results are reported in Table 2.

To establish the effects of random events on repeatability of the analytical procedure, the repeatability trials were performed with 2 different instrumentation systems, by 2 analysts on different days. The overall RSD for each anthocyanin and total anthocyanidins content was calculated in order to evaluate the intralaboratory precision of the method.

The RSD of the content of total anthocyanins was <1.5%; the RSD of the content of total anthocyanidins was <18.0%.

Limit of Quantitation

The limit of quantitation (LOQ) for the cyanidin chloride, the reference substance used in the anthocyanidin quantitation, was evaluated on the basis of the signal-to-noise (S/N) ratio.

Determination of the S/N ratio is performed by comparing measured signals from samples with known low concentrations of cyanidin chloride with those of blank samples, and by establishing the minimum concentration at which the analyte can be reliably quantified by measuring the RSD of peak area for 6 replicate injections of cyanidin chloride that should be not more than 10.0%. A typical S/N ratio is 10:1.

The cyanidin chloride LOQ is 0.075 μg/mL (8.3% RSD).

Recovery Studies

The method accuracy was determined at 3 levels of concentration by calculating the percent recovery of cyanidin-3-*O*-glucoside chloride and total anthocyanins by fortifying *V. myrtillus* dry extract test with *V. myrtillus* reference standard. Three enrichment solutions were added to a suitable amount of *V. myrtillus* dry extract test in order to reach, respectively, 70, 100, and 130% of the working concentration.

Table 3. Anthocyanosides testing results of the products under exam, with the identification number, label claim, marketplace, and daily dosage

Identification No.	Declared composition	Market-place	Anthocyanins, assay, %	Anthocyanidins, assay, %	Anthocyanins, mg/dosage form	Daily dosage	Daily dosage, mg/day of anthocyanins
05/913/ATC	Bilberry ex., 160 mg	Italy	35.69	0.18	57.11	2 hard-gel caps	114.2
05/914/ATC	Bilberry ex., 160 mg	Italy	33.68	0.21	53.89	2 hard-gel caps	107.8
05/801/ATC	Anthocyanins 25%, bilberry ex. 250 mg, 3 caps	Japan	40.2	0.27	33.5	2–3 soft-gel caps	100.5
05/800/ATC	Anthocyanins 25%, bilberry ex. 250 mg, 3 caps	Japan	37.54	0.34	31.28	2–3 soft-gel caps	93.8
05/681/ATC	Blueberry ex., 240 mg, 4 caps	Japan	34.58	0.28	20.75	2–4 soft-gel caps	83.0
05/680/ATC	Blueberry ex., 240 mg, 4 caps	Japan	34.16	0.23	20.5	2–4 soft-gel caps	82.0
05/909/ATC	Bilberry ex., 160 mg	USA	37.01	0.26	59.21	1 hard-gel cap	59.2
05/753/ATC	Bilberry anthocyanins 36%, 160 mg	Japan	36.23	0.52	57.97	1 soft-gel cap	58.0
05/757/ATC	Bilberry anthocyanins 36%, 160 mg	Japan	34.7	0.96	55.52	1 soft-gel cap	55.5
05/678/ATC	Bilberry anthocyanins 36%, 85 mg	Japan	32.57	1.2	27.69	2 soft-gel caps	55.4
05/679/ATC	Bilberry anthocyanins 36%, 85 mg	Japan	32.54	0.88	27.66	2 soft-gel caps	55.3
05/752/ATC	Bilberry anthocyanins 36%, 160 mg	Japan	34.45	0.52	55.11	1 soft-gel cap	55.1
05/756/ATC	Bilberry anthocyanins 36%, 160 mg	Japan	33.63	0.98	53.81	1 soft-gel cap	53.8
05/683/ATC	Bilberry anthocyanins 25%, 120 mg	Japan	19.9	0.09	11.94	2–4 soft-gel caps	47.8
05/802/ATC	Bilberry ex., 140 mg, 4 caps	Japan	30.99	0.75	10.85	4 soft-gel caps	43.4
05/803/ATC	Bilberry ex., 140 mg, 4 caps	Japan	30.87	0.55	10.81	4 soft-gel caps	43.2
05/755/ATC	Blueberry ex., 60 mg	Japan	36.01	0.16	21.61	2 soft-gel caps	43.2
05/754/ATC	Blueberry ex., 60 mg	Japan	33.48	0.27	20.09	2 soft-gel caps	40.2
05/831/ATC	Anthocyanins 25%, bilberry ex., 120 mg, 2 caps	Japan	33.2	0.3	19.92	2 soft-gel caps	39.8
05/830/ATC	Anthocyanins 25%, bilberry ex., 120 mg, 2 caps	Japan	33.08	0.32	19.85	2 soft-gel caps	39.7
05/682/ATC	Bilberry anthocyanins 25%, 120 mg	Japan	15.7	0.06	9.42	2–4 soft-gel caps	37.7
05/875/ATC	Bilberry ex., 80 mg	USA	38.96	0.93	31.16	1 hard-gel cap	31.2
05/828/ATC	Anthocyanins 25%, bilberry ex. 160 mg, 4 caps	Japan	18.68	0.1	7.47	4 soft-gel caps	29.9
05/829/ATC	Anthocyanins 25% bilberry ex. 160 mg, 4 caps	Japan	18.52	0.09	7.41	4 soft-gel caps	29.6
05/878/ATC	Bilberry ex., 30 mg	USA	44.51	1.67	13.35	2 hard-gel caps	26.7
05/877/ATC	Bilberry ex., 30 mg	USA	41.08	0.36	12.32	2 hard-gel caps	24.6
05/881/ATC	Bilberry ex., 60 mg	USA	37.87	0.45	22.72	1 hard-gel cap	22.7
05/876/ATC[a]	Bilberry ex., 80 mg	USA	27.67	4.99	22.14	1 hard-gel cap	22.1
05/862/ATC[a]	Anthocyanins 25%, bilberry ex., 80 mg	USA	21.31	2.24	17.04	1 hard-gel cap	17.0
05/912/ATC	Bilberry ex., 40 mg	USA	40.55	0.39	16.22	1 hard-gel cap	16.2
05/911/ATC	Bilberry ex., 40 mg	USA	37.26	0.21	14.91	1 hard-gel cap	14.9

Table 3. (*continued*)

Identification No.	Declared composition	Market-place	Anthocyanins, assay, %	Anthocyanidins, assay, %	Anthocyanins, mg/dosage form	Daily dosage	Daily dosage, mg/day of anthocyanins
05/910/ATC[a]	Bilberry ex., 90 mg	USA	11.76	0.75	10.59	1 soft-gel cap	10.6
05/851/ATC[a]	Bilberry ex., 40 mg	USA	25.83	6.5	10.32	1 hard-gel cap	10.3
05/863/ATC[a]	Bilberry ex., 37.5 mg	USA	2.2	0	0.82	1 hard-gel cap	0.8
05/879/ATC[a]	Bilberry ex., 60 mg, 4 caps	USA	1.65	0	0.25	1 hard-gel cap	0.3
05/880/ATC[a]	Bilberry ex., 60 mg, 4 caps	USA	0.7	0	0.1	1 hard-gel cap	0.1
05/864/ATC[a]	Bilberry ex., 40 mg	USA	0	0	0	1 hard-gel cap	0.0
05/915/ATC[a]	Bilberry ex., 50 mg	Italy	0	0	0	4 soft-gel caps	0.0
05/916/ATC[a]	Bilberry ex., 50 mg	Italy	0	0	0	4 soft-gel caps	0.0
05/852/ATC[a]	Blueberry ex., 120 mg	Malaysia	0	0	0	1–2 Tablets	0.0

[a] LC profile not comparable with that of the typical *V. myrtillus* LC profile.

Accuracy for cyanidin chloride is determined at 3 levels of concentration by calculating the percent recovery for cyanidin chloride by adding cyanidin chloride reference to *V. myrtillus* dry extract test. Three enrichment solutions were added to a suitable amount of *V. myrtillus* dry extract test in order to reach, respectively, 50, 100, and 150% of the working concentration.

The obtained mean percent recoveries were found to be 101.1% for cyanidin-3-*O*-glucoside chloride, 104.6% for total anthocyanins, and 102.9% for cyanidin chloride.

Results and Discussion

The present analytical method was developed to obtain good specificity and reproducibility and to generate accurate quantitative data for the determination of both anthocyanins and anthocyanidins in bilberry extract and relative typical formulations.

The LC-UV chromatogram exhibited the presence of 15 anthocyanins and 5 anthocyanidins. The LC/MS and LC/DAD analyses allowed the peak attribution reported in Table 1 and showed that the method is specific for the quantification of the identified peaks.

The method showed linear response in the concentration ranges of 5–200 μg/mL for cyanidin-3-*O*-glucoside chloride and 0.075–15 μg/mL for cyanidin chloride, with a coefficient of correlation >0.999 for both compounds.

The stability test showed that cyanidin-3-*O*-glucoside chloride and each constituent of *V. myrtillus* dry extract are stable in test and reference solutions at 4°C up to 48 h, and cyanidin chloride is stable in reference solution at 4°C up to 36 h. Furthermore, anthocyanins have been shown to be stable during capsule and tablet extraction procedures.

Repeatability studies were performed in the range of 70–130% of the nominal working concentration. The RSD values (precision) for all the quantified constituents were <2.0%.

The intralaboratory precision was assessed by means of intralaboratory trials with 2 different analysts on different days, using 2 different LC systems. The RSD of the anthocyanins contents was <3.0% and for the anthocyanidins was <18.0%.

The recovery results were 101.1% (2.2% RSD) for cyanidin-3-*O*-glucoside chloride, 104.6% (1.5% RSD) for total anthocyanins, and 102.9% (5.0% RSD) for cyanidin chloride. The above results lie within 95 and 105%, showing that the method performed well in terms of accuracy.

The analysis of the products found in the marketplace revealed marked differences among them despite their common origin, as reported in Table 3. The preliminary evaluation of the producer-declared composition showed 3 different preparation types: those containing extracts with a declared value of 36% of anthocyanins, those containing extracts with a declared value of 25% of anthocyanins, and products which contain a generic "bilberry extract" without any declared content of anthocyanins. On the basis of the obtained LC results, the products can be divided into 4 main categories, taking into consideration the advised daily dosage

of anthocyanins ["Standardized extracts of bilberry fruit containing 36% of anthocyanins: 320–408 mg/day," equivalent to 115–173 mg/day of anthocyanins (1)]; products with a daily dosage between 80 and 120 mg/day (15%); products with a daily dosage between 20 and 60 mg/day (52%); products with a daily dosage <20 mg/day (23%); and products which lack anthocyanins (10%). Furthermore, 25% of the analyzed products exhibited an LC profile different from the typical LC profile of bilberry extract, as shown in Figure 2. Among these, 5 products showed a higher content of anthocyanidins (>1%); outlying partial degradation of anthocyanins occurred during the manufacturing process or storage, while in 2 cases the use of berries different from *V. myrtillus* was evident.

Conclusions

An LC method was developed and validated for the analysis of *V. myrtillus* extracts, allowing the identification and quantification of 15 anthocyanins and 5 anthocyanidins. The method was found to be specific and suitable for routine analysis because of its reproducibility and accuracy. The results of the analyses of the preparations found in the marketplace show that only 15% of the products analyzed allow the administration of a quantity of anthocyanins that was demonstrated to be effective in the clinical trials. Furthermore, all the products exhibit labels which are not clear and often not true. Hence, there is an urgent need for common standards of quality for bilberry preparations in order to ensure their efficacy and safety. The application of the present method together with clear information on the labels is desirable in order to enhance the security and confidence of the consumers.

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References

(1) European Scientific Cooperative on Phytotherapy (ESCOP) Monographs (2003) 2nd Ed., *Myrtilli fructus*, Thieme Publishers, New York, NY, pp 345–350

(2) Prior, R.L., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., Lischner, N., Ehlenfeldt, M., Kalt, W., Krewer, G., & Mainland, C.M. (1998) *J. Agric. Food Chem.* **46**, 2686–2693

(3) Wightman, J.L.D. (2004) *American Chemical Society (ACS) Symposium Series* 871 (Nutraceutical Beverages), 123–132

(4) Krawczyk, U., & Petri, G. (1992) *Arch. Pharm.* **325**, 147–149

(5) Melegari, M., & Albasini, A. (1988) *Atti Soc. Nat. Mat. Modena* **119**, 31–41

(6) Petri, G., Krawczyk, U., & Es Kery, A. (1994) *Acta Pharm. Hung.* **64**, 117–122

(7) Petri, G., Krawczyk, U., & Kery, A. (1997) *Microchem. J.* **55**, 12–23

(8) Simard, R.E., Bourzeix, M., & Heredia, N. (1980) *Can. Inst. Food Sci. Technol. J.* **13**, 115–117

(9) Baj, A., Bombardelli, E., Gabetta, B., & Martinelli, E.M. (1983) *J. Chromatogr.* **279**, 365–372

(10) Ichiyanagi, T., Tateyama, C., Oikawa, K., & Konishi, T. (2000) *Biol. Pharm. Bull.* **23**, 492–497

(11) Ichiyanagi, T., Kashiwada, Y., Ikeshiro, Y., Hatano, Y., Shida, Y., Horie, M., Matsugo, S., & Konishi, T. (2004) *Chem. Pharm. Bull.* **52**, 226–229

(12) Ichiyanagi, T., Hatano, Y., Matsugo, S., & Konishi, T. (2004) *Chem. Pharm. Bull.* **52**, 628–630

(13) Zhang, Z., Kou, X., Fugal, K., & McLaughlin, J. (2004) *J. Agric. Food Chem.* **52**, 688–691

(14) Ekker, K.I., Pimenova, V.V., Levin, L.G., & Kiseleva, M.G. (2003) *Vopr. Pitan.* **72**, 28–35

(15) Dugo, P., Mondello, L., Errante, G., Zappia, G., & Dugo, G. (2001) *J. Agric. Food Chem.* **49**, 3987–3992

(16) Chandra, A., Rana, J., & Li, Y. (2001) *J. Agric. Food Chem.* **49**, 3515–3521

(17) Huopalahti, R., Jarvenpaa, E.P., & Katina, K. (2000) *J. Liq. Chromatogr. Rel. Technol.* **23**, 2695–2701

(18) Martinelli, E.M., Scilingo, A., & Pifferi, G. (1992) *Anal. Chim. Acta* **259**, 109–113

(19) Goiffon, J.P., Brun, M., & Bourrier, M.J. (1991) *J. Chromatogr.* **537**, 101–121

(20) Hong, V., & Wrolstad, R.E. (1990) *J. Agric. Food Chem.* **38**, 698–708

(21) Ballington, J.R., Ballinger, W.E., Maness, E.P., & Luby, J.J. (1988) *Can. J. Plant Sci.* **68**, 241–246

(22) Martinelli, E.M., Baj, A., & Bombardelli, E. (1986) *Anal. Chim. Acta* **191**, 275–281

(23) Nyman, N.A., & Kumpulainen, J.T. (2001) *J. Agric. Food Chem.* **49**, 4183–4187

(24) Francis, F.J. (1989) *Crit. Rev. Food Sci. Nutr.* **28**, 273–314

(25) *Farmacopea Ufficiale della Repubblica Italiana* (2005) XI Edizione I Supplemento, Istituto Poligrafico e Zecca dello Stato, Rome, Italy, pp 1469–1472

(26) Robertson, A., & Robinson, R. (1927) *J. Chem. Soc.*, 242–247

(27) Robertson, A., & Robinson, R. (1927) *J. Chem. Soc.*, 1710–1717

(28) Robertson, A., & Robinson, R. (1927) *J. Chem. Soc.*, 2196–2206

(29) Robertson, A., & Robinson, R. (1928) *J. Chem. Soc.*, 1460–1472