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Application Note

As published in "The Handbook of Analytical Methods for Dietary Supplements"

0031 - Bilberry for Anthocyanidins by HPLC

Botanical Name: *Vaccinium myrtillus L.*

Common Names: Whortleberry

Parts of Plant Used: Berries

Uses: To improve visual problems and circulatory disorders.

Modes of Action:

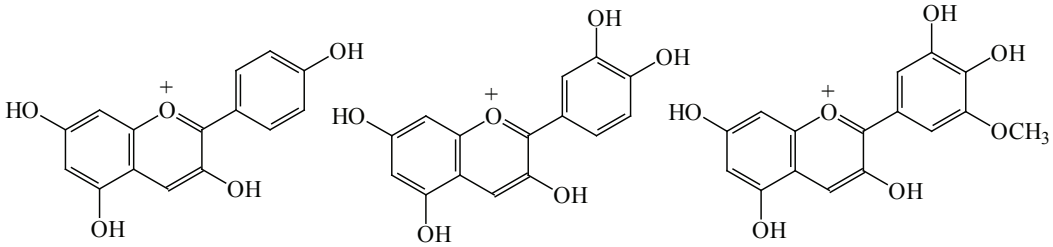
About 20 clinical trials with bilberry have demonstrated positive effects for visual problems and circulatory disorders. The anthocyanins in bilberry are believed to be the bioactive components. The anthocyanins were found to have antioxidant, antiplatelet, anti-inflammatory, wound-healing, anti-ulcer, anti-atherosclerosis, ophthalmic, and vasoprotective activities. The anthocyanins also showed effects on vascular smooth muscle and arteriolar vasomotion. Also, the anthocyanins were found to interact with collagen, phospholipids, and proteoglycans.[1]

Chemistry and Chemical Markers for Quality Control:

The chemical components of bilberry have been studied extensively. Several types of compounds including flavonoids, iridoids, organic acids, sugars, and terpenes have been found in bilberry. The several types of flavonoids [Q: author flavoloids or flavonoids here?] found in bilberry include anthocynins, flavonols, and flavan-3-ols. The ripe fruits were found to contain 5-hydroxylated anthocyanidins (delphinidin, cyanidin, pelargonidin, petunidin, peonidin, and malvidin), each of which is linked with three different sugar units, glucose, galactose, and arabinose. There are at least 15 anthocyanins in bilberry.

Bilberry is also well known to contain proanthocynidins, the amount of which decreases with the progression of ripening.1,2 The volatile components of bilberry were also studied[Q: A—ok as changed?] by GC and GC–MS methods; three compounds, trans-2-hexenal, ethyl 3-methyl butyrate, and ethyl 2-methylbutyrate were found to be responsible for the characteristic aroma of bilberry.1 Currently in the U.S. market, anthocynidins are used as quality control standards for bilberry products.

Major Compounds Identified in Bilberry:



Pelargonidin Cyanidin Petunidin

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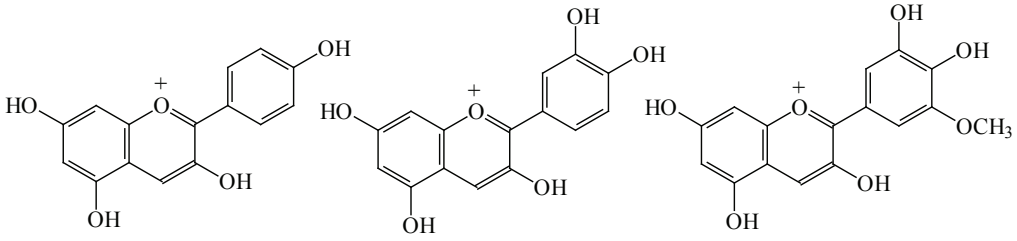

Peonidin Delphinidin Malvidin

Methods of Analysis:

Anthocynidins are usually analyzed by HPLC and colorimetric methods.

Method 1:

The colorimetric method found at www.nsfina.org can be used to determine total anthocyanins in bilberry.
Preparation of buffers: To prepare the pH 1.0 buffer, dissolve 1.49 g of potassium chloride in 100 mL of deionized water, and then mix 25 mL of this solution with 67 mL of 0.2 N hydrochloric acid solution. Adjust the pH to 1.0±0.1 if necessary.

To prepare the pH 4.5 buffer, dissolve 1.64 g of sodium acetate in 100 mL of water and adjust the pH to 4.5±0.1 with hydrochloric acid.

Sample Preparation:

Weigh about 75 mg of powdered bilberry extract into a 100-mL volumetric flask and add about 80 mL of distilled water. Sonicate for 15 minutes or until dissolved. Cool to room temperature and dilute to volume with water. If the solution is not clear, centrifuge a portion to remove particulates. Dilute 1 mL of the bilberry solution to 25 mL with the pH 1.0 buffer and mix. And then dilute another 1 mL of the bilberry solution to 25 mL with the pH 4.5 buffer.

Testing:

Zero the spectrophotometer with distilled water. Measure the absorbance of the pH 1.0 and 4.5 sample preparations at 510 and 700 nm, respectively.

Calculations:

Calculate the difference in absorbance between the two samples using the following equation:

$$\text{Absorbance} = (A_{510nm\ pH\ 1.0} - A_{700nm\ pH\ 1.0}) - (A_{510nm\ pH\ 4.5} - A_{700nm\ pH\ 4.5})$$

Calculate the %wt/wt of total anthocyanins in the sample using:

$$\%wt/wt = \text{Absorbance} \times MW \times DF \times V \times 100/(\varepsilon \times L \times wt)$$

Where:
A = Absorbance
ε = Cyd-3-glu molar absorbance (26,900)
MW = anthocyanin molecular weight (449.2)
DF = dilution factor
V = final volume (mL)
wt = sample weight (mg)
L = cell pathlength (usually 1 cm)

Method 2:

The method of Nyman and Kumpulainen[3] can be used to analyze the hydrolyzed aglycones of bilberry anthocyanins.

Sample Preparation:

Weigh about 0.5 g of dried sample into a 70-mL screw-cap test tube, add 18 to 60 mL of 2 M hydrochloric acid in methanol, and hydrolyze in a 90°C water bath for 50 minutes. Cool to room temperature and dilute to volume (25 to 100 mL) using methanol.

Chromatography:

Column: C18 Inertsil ODS(3), 3 µm, 150 x 4.0 mm with a C18 guard column.
Mobile phase: Solvent A = water–formic acid (90:10), solvent B = acetonitrile.
Gradient:

Time (minutes)	%A	%B
0	96	4
8	85	15
23	85	15
24	20	80
27	20	80
28	96	4
34	96	4

Flow rate: 0.8 mL/minute
Injection volume: 10 µL
Detection wavelength: 530 nm for delphinidin, cyanidin, petunidin, peonidin, and malvidin and 510 nm for pelargonidin.
Column temperature: 35°C

Validation Data:

Not available

Method 3:

The method of Chandra et al.[4] can be used to identify the anthocyanins in bilberry.

Sample Preparation:

Not specified

Chromatography:

Column: Agilent Hypersil ODS HPLC column, 5 µm, 125 x 4.0 mm.
Mobile phase: Solvent A = water (0.5% phosphoric acid), solvent B = water–acetonitrile–acetic acid–phosphoric acid (50:48.5:1.0:0.5 vol/vol/vol/vol).
Gradient: 20% B to 60% B in 26 minutes, then to 20% B in 4 minutes, and keep at 20% B for 5 minutes.
Flow rate: 0.8 mL/minute
Injection volume: 10 µL
Column temperature: 30°C
Detection wavelength: 520 nm

Validation Data:
Not available

Method 4:

The method of Dugo et al.[5] was used.

Sample Preparation:
Pound about 200 g of fruit in a mortar to obtain the extract; centrifuge a 5-mL aliquot of extract at 6000 rpm for 20 minutes.

Chromatography:
Column: Restek Pinnacle ODS, 5 µm, 250 × 4.6 mm.
Mobile phase: Solvent A = water–formic acid (9:1), solvent B = water–formic acid–acetonitrile (4:1:5).
Gradient: Hold at 12%B for 1 minute, then linear to 30% B in 25 minutes, and then to 100% B in 9 minutes.
Flow rate: 1 mL/minute
Injection volume: 20 µL
Detection wavelength: 518 nm

Validation Data:
Not available

References:

1. Morazzoni P, Bombardelli E. Vaccinium myrtillus L. Fitoterapia. 1996;66(1):3–29.
2. Azar M, Verette E, Brun S. Identification of some phenolic compounds in bilberry juice Vaccinium myrtillus. J Food Sci. 1987;52(5):1255–7.
3. Nyman NA, Kumpulainen JT. Determination of anthocyanidins in berries and red wine by high-performance liquid chromatography. J Agric Food Chem. 2001;49(9):4183–7.
4. Chandra A, Rana J, Li Y. Separation, identification, quantification, and method validation of anthocyanins in botanical supplement raw materials by HPLC and HPLC-MS. J Agric Food Chem. 2001;49(8):3515–21.
5. Dugo P, Mondello L, Errante G, et al. Identification of anthocyanins in berries by narrow-bore high-performance liquid chromatography with electrospray ionization detection. J Agric Food Chem. 2001;49(8):3987–92.

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