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Botanical Standards

PhytoReport™ #3

Bilberry Identification & Analysis

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PhytoReport™ #3

Bilberry Identification & Analysis

Introduction

Bilberry is the name of the small European blueberry, *Vaccinium myrtillus.* Highly concentrated extracts have been widely used for decades in Europe and the U.S. to treat and prevent ocular, microcirculatory and vascular related disorders. A number of clinical studies support this usage.

Bilberry extracts are generally standardized to their anthocyanins, a group of water-soluble pigments that are responsible for the red, purple, blue and violet colors in many fruits and flowers. Because of the high concentration of the extract (100:1), and the process involved in manufacturing a consistent grade of material, Bilberry is one of the most expensive extracts in regular use for botanical based supplements. But meanwhile because of the high concentration required for Bilberry extracts, the high cost and tight supply of fresh bilberry material, and the process involved in manufacturing a consistent grade of material, Bilberry is one of the most expensive extracts in regular use for botanical based supplements.

For many years a UV test method has been satisfactory to quantify the anthocyanin levels in Bilberry extracts. This method uses Ultra Violet light to detect and quantify the range of color compounds that are present in the bilberry anthocyanins. It is not specific enough however to detect the presence of anthocyanins from other plant sources, or certain synthetic dyes that are added to deceive the UV system.

This PhytoReport™ reviews some of the specific analytical problems deriving from UV analysis, and also the solution found in the use of a practical HPLC method.

Recent Analytical Problems with Bilberry

The term Anthocyanins, initially coined to designate the substance responsible for the color of cornflower, applies to a group of water-soluble pigments responsible for red, pink, mauve, purple, blue, or violet color of most flowers and fruits. In Bilberry, these pigments (the anthocyanins) occur as glycosides (the major form) and their aglycones (the anthocyanidins). In fact, Anthocyanins are read well by the UV method. Until recently therefore the usual method of determining the total anthocyanins in bilberry (*Vaccinium myrtillus*) has been a single-wavelength assay via ultra violet (UV) spectrophotometer. Results reported by different labs expressed as Total Anthocyanins or as Total Anthocyanidins (divided by a factor).

However adulteration has been found in many Bilberry samples approved by the UV method. In these cases, adulterants have been added to either make poor quality bilberry look more potent, or to make fake bilberry look potent and real. The addition of only a small amount of adulterant can make the extract significantly less expensive to manufacture.

A number of these methods of adulteration have been identified:

Addition of dyes:

Azo dyes (synthetic, inorganic chemical compounds) have been used to spike the results given for total anthocyanin content measured by UV in Bilberry extract.

By the addition of dyes, substituting for true anthocyanins, the spectrophotometer is fooled. The European Union views one of the illegal Azo dyes (Sudan I) as both genotoxic and carcinogenic. Mandatory testing for Azo dyes in spices and processed foods has passed into European law [1].

Addition of other plant materials:

Other adulterants used are from plant compounds that are high in anthocyanin levels. These include: mulberry (*Morus nigra),* black bean skins, etc. Bilberry extracts mixed with mulberry or black bean skins have been widely reported in the Japanese market and elsewhere, duping the industry into creating cheaper alternatives and undermining quality.

Because UV does not distinguish between the different forms of anthocyanins (but only determines a total), these spiked materials pass the test for total anthocyanin or total anthocyanidin by UV. In one study, samples were found to have been adulterated by 20-25% addition of other plant materials. A comprehensive review of the adulteration problems and solutions named as Bilberry Extract Adulteration and Laboratory Guidance Document has been published by ABC-AHP-NCNPR Botanical Adulterants Program in 2016 [2].

A Review of Solutions

Because the UV spectrometer has proven unreliable for Bilberry testing, Indena developed a high-performance liquid chromatography (HPLC) method to verify the anthocyanin profile and the concentrations of various Anthocyanins found in bilberry around 2006. According to the company's routine analyses, at least 15 to 20 percent of the bilberry samples they collected on the market were adulterated. Other studies have also addressed both problems and solutions. Two UV approved samples were studied by HPLC method. One extract (adulterated) was found to contain 9% anthocyanins probably not derived from *V. myrtillus*. This adulterant was subsequently identified, using HPLC, mass spectroscopy, and nuclear magnetic resonance, as amaranth, that is, 3-hydroxy-4-[(4-sulfo-1-naphthalenyl)azo]-2,7-naphthalenedisulfonic acid trisodium salts, a synthetic dark red sulfonic acid based naphthylazo dye [3]. The HPLC Chromatograms of these two samples were shown in the Figure 1. These have been great lesson in the value of using higher-precision tests (which equals more expensive tests) to assay raw materials [4].

The Indene HPLC method was later adopted by the European and Italian pharmacopoeias and also by the U.S. Pharmacopeia (USP) with some further modification or improvement. In 2008, an official quality standard monograph for the refined and standardized dry extract of fresh bilberry fruit was added to the European Pharmacopoeia (EP). Also in 2008, the United States Pharmacopeia (USP) published its quality standards monograph for Powdered Bilberry Extract

with one additional control point on the limit of certain Anthocyanidins in the extract compared to EP. Thus, USP does not only specify the list of individual anthocyanosides by HPLC test method and the total % of anthocyanosides (Table 1), but also defines the limit maximum 1.0% of anthocyanidins (Table 2) to ensure the extract is processed from high quality and fresh bilberries. As far as today, USP-HPLC test method is suggested as the most practical to achieve consistent and reliable results when comparing different Bilberry products available on the market today.

Based on ENI internal lab data, both the European (or Indena) HPLC method and the USP-HPLC method give equivalent results on Anthocyanins, while the USP method do not only identify and quantify the 15 anthocyanins (Table 1), but also sets a limit (i.e. <1%) on the 5 small anthocyanidins (Table 2), which may be converted by anthocyanins when the bilberry raw material is not properly stored or stored for a long time. Typical HPLC Chromatograms of a USP Bilberry extract reference standard and a typical lot of ENI bilberry sample were illustrated in Figure 2.

This compendia HPLC test method allows for careful study of the Bilberry anthocyanin HPLC profile, to ensure each anthocyanin can be identified as exclusively from Bilberry. There is still a concern that this method is not sensitive enough to detect potential adulteration by other types of berries. However, as the content of anthocyanins in these berries is usually lower than in bilberry, it might cost more (or about the same) to manufacture an extract with high content of Anthocyanins using other berries vs. bilberry. For this reason, adulteration by using other types of species of berries in manufacturing bilberry extract hasn't become a main concern in the market.

Liquid Chromatography/Mass Spectrometry (LC-MS), a combination of liquid chromatographic separation with mass spectrometric detection, has shown to be another effective way to differentiate a real bilberry extract from an adulterated one. In a real bilberry extract, a total of 15 Anthocyanins can be detected by their specific molecular weight. However, there may be only 2 or 3 peaks identified in an adulterated extract depending on the source and/or extend of the adulteration. Figure 3 shows a comparison of LC-MS chromatogram of a real Bilberry sample and an adulterated one. But similar to EP and USP HPLC method, this more advanced method is not sensitive enough to detect potential adulteration by other types of berries.

Thus, as far as today, the HPLC test methods (EU and USP), and the LC-MS method are suggested as the most practical to achieve consistent and reliable results when comparing different Bilberry products available on the market today.

References:

[1] Daniells, S. (2009). Voices grow louder against bilberry fakes. NUTRAIngredients.com Europe. May 22, 2007. Available at: http://www.nutraingredients.com/news/ng.asp?n=76704&m=1NIE522&c=mumnydmmijxhxvi. Accessed August 29, 2007.

[2] Gafner, S. (2015). Bilberry Extract Adulteration Laboratory Guidance Document. ABC-AHP-NCNPR Botanical Adulterants Program. Retrieved on 8/31/21 from https://www.herbalgram.org/resources/botanical-adulterants-prevention-program/adulterants-bulletins/bilberry-extract-bulletin-april-2016/

[3] Penmanj, K. G., et al. (2006). Bilberry Adulteration Using the Food Dye Amaranth Agric. *Food Chem.* 54, 7378-7382.

[4]. Liva, R. (2007). New FDA cGMPs for Supplements: Smoke or Substance? *Integrative Medicine*. 6 (5), 28~32.

Attachments:

[1] Figure 1: HPLC Chromatogram of a typical Bilberry sample and an adulterated sample.

[2] Figure 2: HPLC Chromatogram of USP powdered bilberry extract reference standard and a typical ENI Bilberry sample.

[3] Figure 3: Comparison of LC-MS Chromatogram of a typical ENI Bilberry sample and an adulterated sample.

Table 1: Fifteen (15) Bilberry Anthocyanins by USP37 monograph of Powdered Bilberry Extract (USP spec 36%):

Analyte	Relative Retention Time
Delphinidin-3-*O*-galactoside chloride	0.61
Delphinidin-3-*O*-glucoside chloride	0.73
Cyanidin-3-*O*-galactoside chloride	0.84
Delphinidin-3-*O*-arabinoside chloride	0.86
Cyanidin-3-*O*-glucoside chloride	1.00
Petunidin-3-*O*-galactoside chloride	1.08
Cyanidin-3-*O*-arabinoside chloride	1.11
Petunidin-3-*O*-glucoside chloride	1.24
Peonidin-3-*O*-galactoside chloride	1.36
Petunidin-3-*O*-arabinoside chloride	1.39
Peonidin-3-*O*-glucoside chloride	1.55
Malvidin-3-*O*-galactoside chloride	1.58
Peonidin-3-*O*-arabinoside chloride	1.67
Malvidin-3-*O*-glucoside chloride	1.76
Malvidin-3-*O*-arabinoside chloride	1.91

Table 2: Five (5) Bilberry Anthocyanidins by USP37 monograph of Powdered Bilberry Extract (USP limit < 1%):

Analyte	Relative Retention Time
Delphinidin chloride	1.28
Cyanidin chloride	1.82
Petunidin chloride	2.08
Peonidin chloride	2.27
Malvidin chloride	2.30

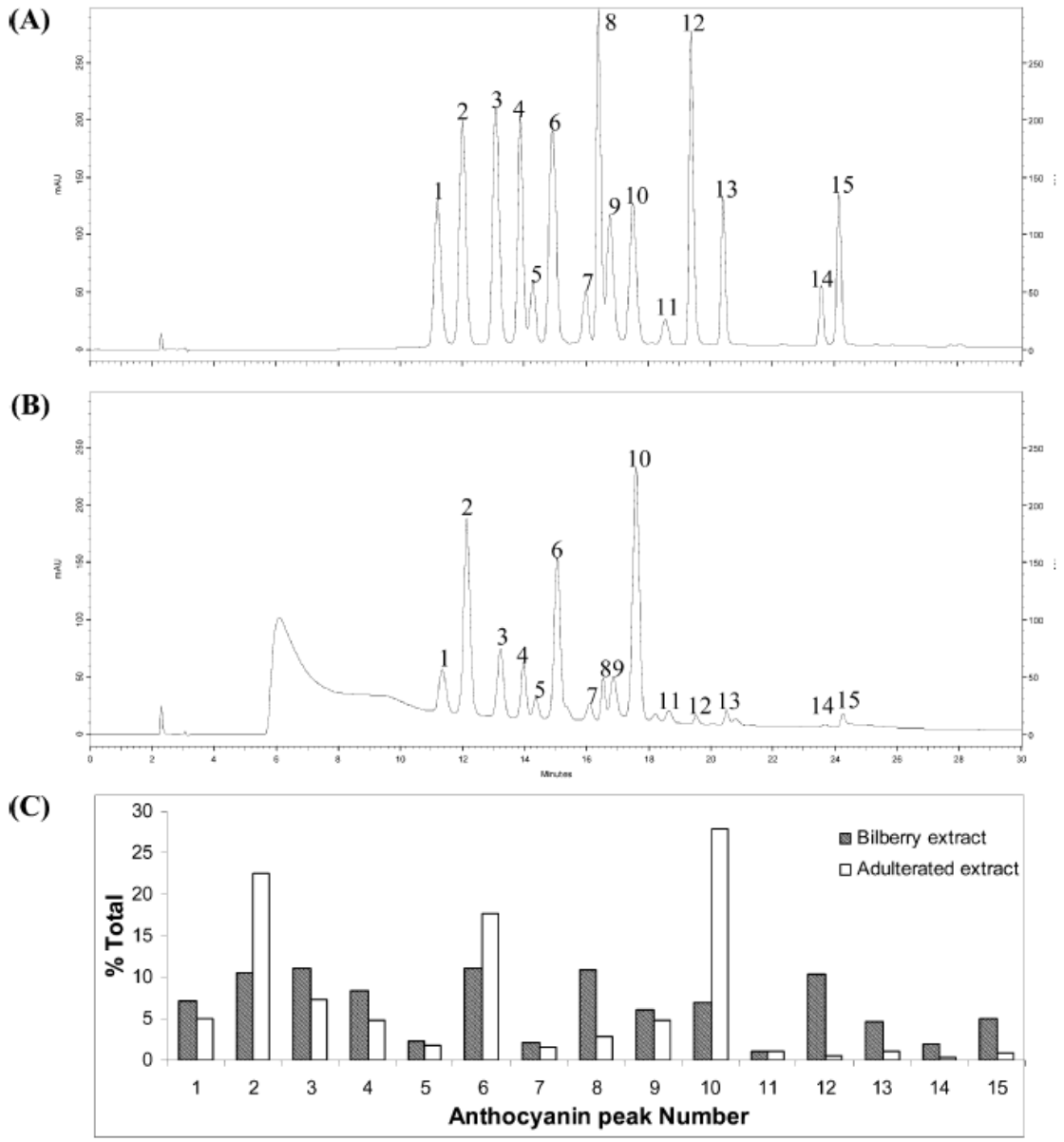

Figure 1. HPLC traces at 540 nm for two purported bilberry extracts: (**A**) bilberry extract; (**B**) adulterated extract; (**C**) comparison of percent of total for each anthocyanin present in both extracts calculated from peak areas.
Peaks: 1, delphinidin 3-O-galactoside; 2, delphinidin 3-O-glucoside; 3, cyaniding 3-O-galactoside; 4, delphinidin 3-O-arabinoside; 5, cyanidin 3-O-glucoside; 6, petunidin 3-O-galactoside; 7, cyanidin 3-O-arabinoside; 8, petunidin 3-Oglucoside; 9, peonidin 3-O-galactoside; 10, petunidin 3-O-arabinoside; 11, peonidin 3-O-glucoside; 12, malvidin 3-O-galactoside; 13, peonidin 3-Oarabinoside; 14, malvidin 3-O-glucoside; 15, malvidin 3-**O**-arabinoside (Source from reference [3]).

Figure 2(A)

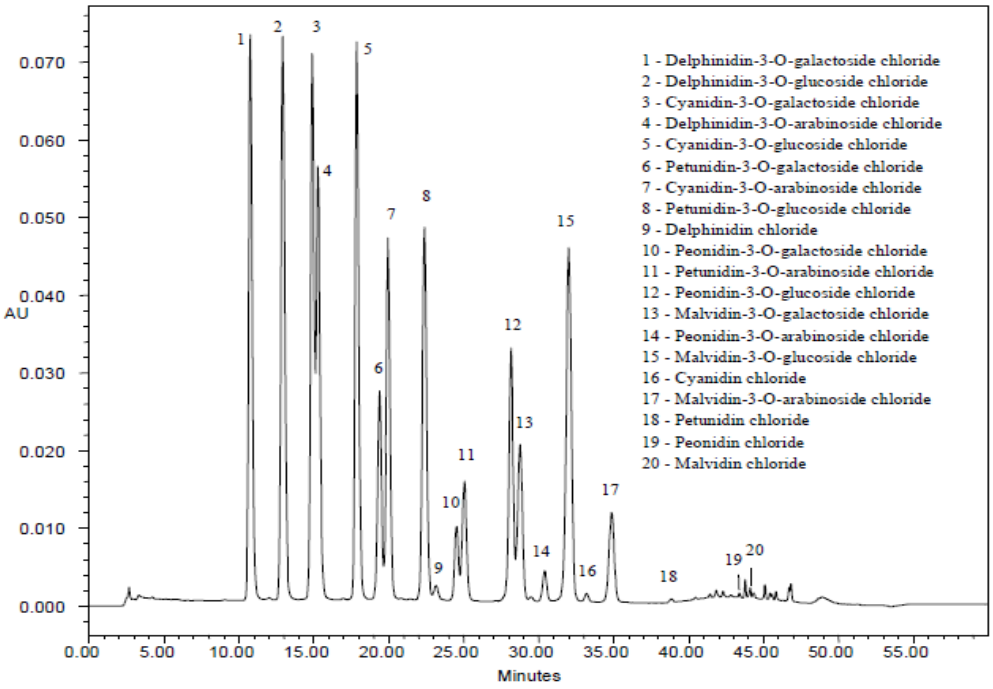

Figure 2(B)

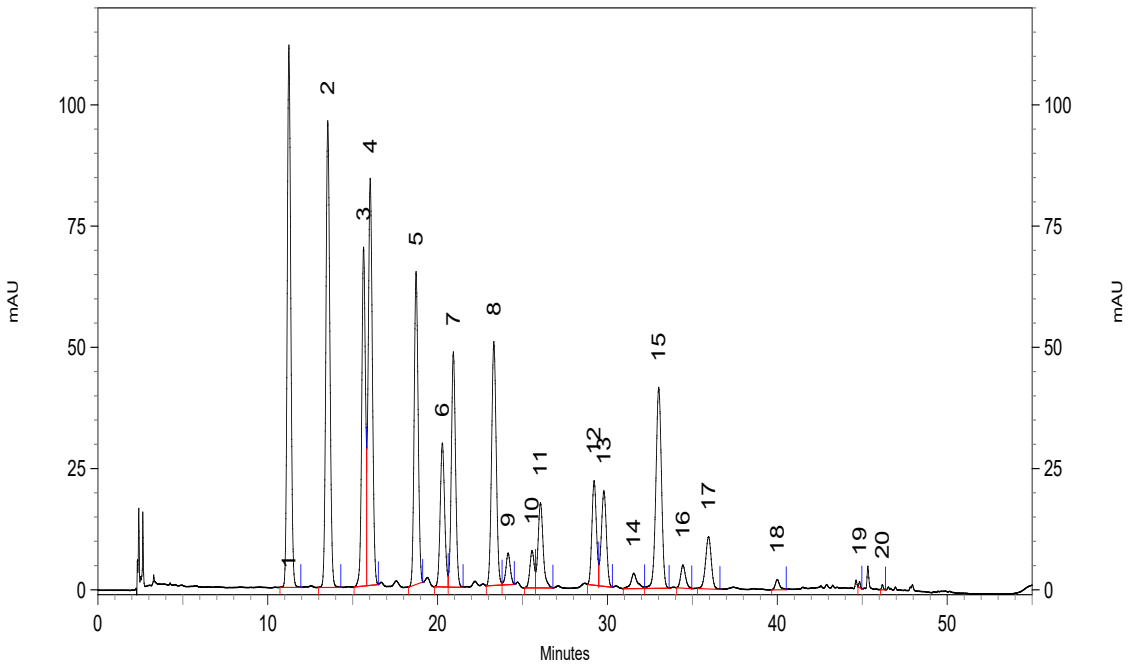

Figure 2(A) - HPLC Chromatogram of a USP powdered bilberry extract (Cat No. 1071268, Lot F0H286); 2(B) - HPLC Chromatogram of a typical lot of ENI Bilberry extract.

Fig 3. (A)

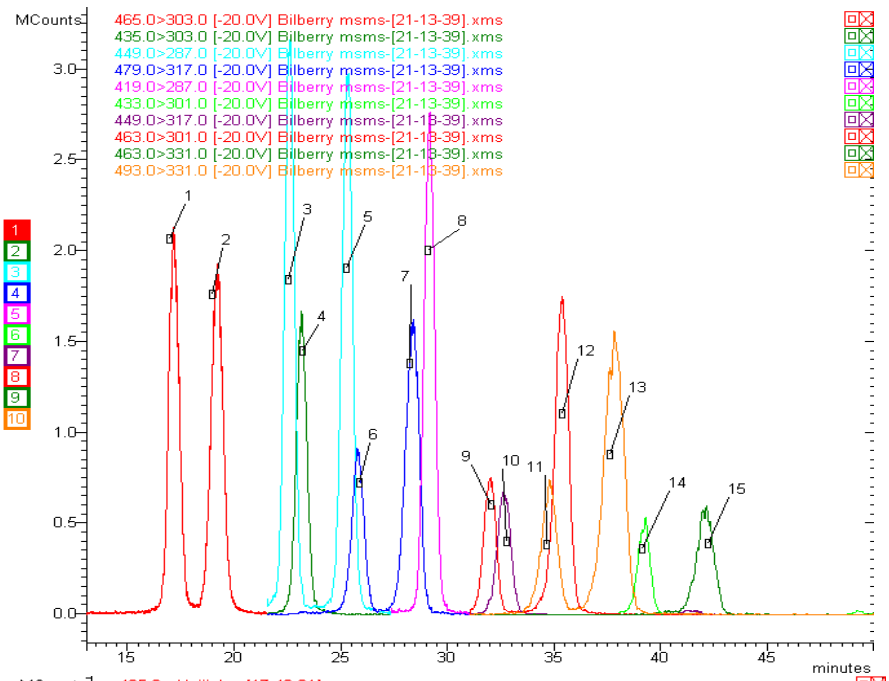

Fig 3. (B)

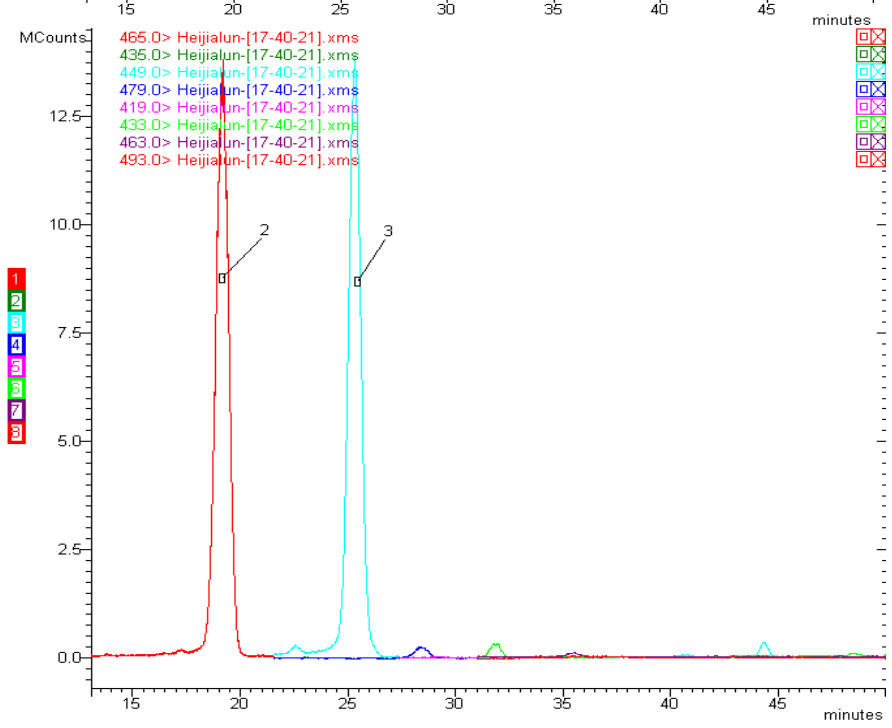

Figure 3: Comparison of LC-MSMS Chromatograms (A) Bilberry extract supplied by ENI, in which 15 bilberry Anthocyanins are clearly identified; (B) An adulterated extract, only two peaks of Bilberry Anthocyanins can be identified by the LC-MS machine. of 2 and 3.