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Botanical Adulterants Program
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Bilberry Fruit Extract Laboratory Guidance Document

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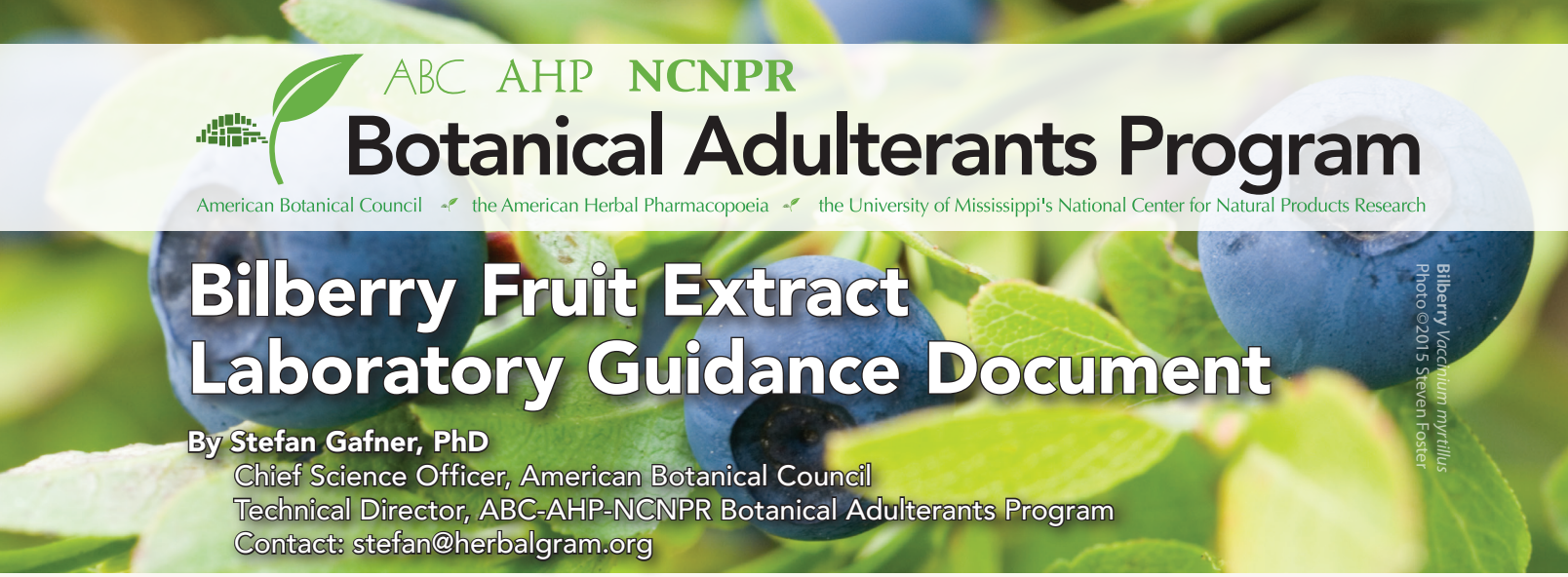

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1. Purpose

Market demand for bilberry (*Vaccinium myrtillus*, Ericaceae) fruit extracts, combined with high prices and falling profit margins have resulted in unscrupulous manufacturers selling various ingredients labeled "bilberry extract." Adulteration predominantly occurs with anthocyanin-rich extracts from other species, e.g., bog bilberry (*V. uliginosum*), lingonberry (*V. vitis-idaea*), European elder (*Sambucus nigra*, Adoxaceae), and Chinese mulberry (*Morus australis*, Moraceae). Additional adulterants reportedly include black soybean (*Glycine max*, Fabaceae) hull or black rice (*Oryza sativa*, Poaceae) extracts, and synthetic colorants like amaranth dye, an azo dye prohibited for use by the United States Food and Drug Administration (FDA) as a suspected carcinogen, and/or charcoal.[1] This Laboratory Guidance Document presents a review of the various analytical technologies and methods used to differentiate between authentic bilberry extracts and potential adulterants.

2. Scope

Previous pharmacopeial test methods for bilberry fruit extract based on UV/Vis absorption of the extract (spectrophotometric methods) are acceptable for quantification of total anthocyanidins, but have proven insufficient to detect adulteration with anthocyanin-rich extracts from other species or synthetic dyes; therefore, other analytical techniques must be used to comply with the legal requirement (for example, according to the Good Manufacturing Practice rule in the United States, and in other countries) to confirm the identity of bilberry fruit extracts. This review is a compilation of published analytical methods for bilberry fruit extracts, and an evaluation of the utility of each method to authenticate bilberry extracts or to detect potential adulterants. This Laboratory Guidance Document *does not cover the analysis of bilberry leaves or bilberry leaf extracts* but may have applications for other anthocyanin-rich berry ingredients, some of which are also known to have quality issues. Analysts can use this review to help guide the appropriate choice of techniques and methods for their specific bilberry materials intended for resale or use in consumer products. A positive assessment of a specific method for testing *V. myrtillus* fruit extracts in their particular matrix in this Laboratory Guidance Document does not remove the responsibility of quality control and laboratory personnel to demonstrate adequate method performance in their own laboratory (and/or in a qualified third-party contract laboratory) using accepted protocols outlined in the Good Manufacturing Practices for dietary supplements in the United States (21 CFR Part 111) and/or by AOAC International, International Organization for Standardization (ISO), the World Health Organization (WHO), and the International Conference on Harmonisation (ICH).

3. Common and Scientific Names

3.1 Common Name: Bilberry[2]

3.2 Other Common Names

English: European blueberry, whortleberry, huckleberry
French: Myrtille, gueule-noire, raisin des bois, vigne des montagnes, ambroche, ambreselle, brimbelle
German: Heidelbeere, Blaubeere, Schwarzbeere, Waldbeere, Bickbeere, Moosbeere
Italian: Mirtillo, ampulette, asaire, bagole, baggiole, cesarelle, giasine, lambrune, murucule
Spanish: Arándano azul, mirtilo
Chinese: *Hei guo yue ju* (黑果越桔)

3.3 Latin Binomial: *Vaccinium myrtillus* L.

3.4 Synonyms: *Vaccinium myrtillus* var. *oreophilum* (Rydb.) Dorn; *Vaccinium myrtillus* subsp. *oreophilum* (Rydb.) Á. Löve, D. Löve & B.M. Kapoor; *Vaccinium oreophilum* Rydb.; *Vaccinium myrtillus* var. *microphyllum* Hook.; *Vaccinium yatabei* Makino[3,4]

3.5 Botanical Family: Ericaceae

Table 1. Known bilberry adulterants of plant origin: Scientific names, family, and common names

Species[a]	Synonym(s)[a]	Family	Standardized common name[b]	Other common names[c-e]
Aronia melanocarpa (Michx.) Elliott	*Aronia arbutifolia* var. *nigra* (Willd.) F.Seym.; *A. nigra* (Willd.) Britton; *Mespilus arbutifolia* var. *nigra* (Willd.) Britton; *Photinia melanocarpa* (Michx.) K.R.Robertson & J.B.Phipps; *Pyrus arbutifolia* var. *nigra* Willd.; *Pyrus melanocarpa* (Michx.) Willd.; *Sorbus melanocarpa* (Michx.) Heynh.	Rosaceae	Not established	Black chokeberry
Glycine max (L.) Merr.	*Dolichos soja* L.; *Glycine angustifolia* Miq.; *G. gracilis* Skvortsov; *G. hispida* (Moench) Maxim.; *Phaseolus max* L.; *Soja japonica* Savi; *S. soja* H.Karst.; *S. viridis* Savi	Fabaceae (Leguminosae)	Soy bean	Sojabean, soya bean, *da dou* (大豆)
Morus australis Poir.	*Morus acidosa* Griff.; *M. bombycis* Koidz.; *M. cavaleriei* H. Lév.; *M. formosensis* Hotta; *M. hastifolia* F.T. Wang & T. Tang ex Z.Y. Cao; *M. inusitata* H. Lév.; *M. longistylus* Diels; *M. nigriformis* (Bureau) Koidz.	Moraceae	Not established	Chinese mulberry, *ji sang* (鸡桑)
Morus nigra L.		Moraceae	Not established	Black mulberry, purple mulberry, *hei sang* (黑桑)
Oryza sativa L.	*Oryza communissima* Lour.; *O. formosana* Masam.& Suzuki; *O. glutinosa* Lour.; *O. montana* Lour.; *O. plena* (Prain) N.P.Chowdhury; *O. praecox* Lour.; *O. rubribarbis* (Desv.) Steud. For a complete list, see references 3 and 6.	Poaceae	Rice	Upland rice, *dao* (稻)
Prunus avium (L.) L.	*Cerasus avium* (L.) Moench; *Druparia avium* (L.) Clairv.	Rosaceae	Sweet cherry	Bird cherry, mazzard cherry, wild cherry

Table 1 continued on the next page.

4. Botanical Description

Botanical descriptions for *V. myrtillus* and its adulterant species are provided in local, national, and international floras and selected publications, e.g., by Ritchie.[5] Identifying and differentiating between *V. myrtillus* and related species requires personnel trained in botany for the assessment of materials with intact botanically characteristic features.

In addition to the species listed in Table 1, synthetic dyes, charcoal, and anthocyanin-rich extracts from other berries (in particular, extracts manufactured in China made from unidentified berries) were also reported as adulterants of bilberry extracts.[11]

Sections 5-8 of this Laboratory Guidance Document discuss macroscopic, microscopic, genetic, and chemical authentication methods for *V. myrtillus*. A comparison among the various approaches is presented in Table 3 at the end of section 8.

5. Identification and Distinction Using Macroanatomical Characteristics

Since bilberries are not cultivated,[11] all commercially available bilberry fruits are wildcrafted; this means that companies are unable to grow their own crop and must rely on a thorough identity testing program to ensure that the correct wild-harvested material is purchased. Macroscopic identification criteria of bilberry fruits can be helpful for companies that purchase the dried fruits to make an extract. Descriptions on macroscopic identification have been published, e.g., in the American Herbal Pharmacopoeia (AHP) monograph by Upton,[12] in the European Pharmacopoeia,[13] and in the book *Herbal Drugs and Phytopharmaceuticals*.[14] The AHP monograph contains a table with criteria to distinguish *V. myrtillus* from *V. uliginosum* and *V. vitis-idaea*. A comparison between *V. myrtillus* and *Aronia melanocarpa* (Rosaceae) is given by Filippini et al.[15] However, a comprehensive macroscopic description of other closely related *Vaccinium* species (e.g., the North

Table 1 continued. Known bilberry adulterants of plant origin: Scientific names, family, and common names

Species[a]	Synonym(s)[a]	Family	Standardized common name[b]	Other common names[c-e]
Ribes nigrum L.	*Botrycarpum nigrum* (L.) Spach; *B. nigrum* (L.) A. Rich.; *Grossularia nigra* (L.) Rupr.; *Ribes cyathiforme* Pojark.; *R. olidum* Moench; *R. pauciflorum* Turcz. ex Ledeb.; *Ribesium nigrum* (L.) Medik.	Grossulariaceae	Black currant	Cassis, European black currant, garden black currant, quinsy berries, squinancy berries, *hei cha biao zi* (黑茶藨子)
Rubus idaeus L.	*Rubus acanthocladus* Borb s; *R. buschii* (Rozanova) Grossh.; *R. chrysoscarpus* Čelak. ex G yer; *R.* × *euroasiaticus* Sinkova; *R. fragrans* Salisb.; *R. frambaesianus* Lam.; *R. obtusifolius* Willd.; *R. sericeus* Gilib. For a complete list, see references 3 and 6.	Rosaceae	Raspberry	Red raspberry, *fu pen zi* (复盆子)
Sambucus nigra L.	*Sambucus graveolens* Willd.	Adoxaceae	European elder	Black elder, black-berried alder, boor tree, bountry, ellanwood, ellhorn
Vaccinium angustifolium Aiton	*Cyanococcus angustifolius* (Aiton) Rydb.	Ericaceae	Blueberry[f]	Lowbush blueberry
Vaccinium corymbosum L.	*Cyanococcus corymbosus* (L.) Rydb.	Ericaceae	Blueberry[f]	Highbush blueberry, giant whortleberry
Vaccinium oxycoccos L.	*Oxycoccus oxycoccos* (L.) MacMill.; *O. palustris* Pers.; *O. quadripetalus* Schinz & Thell.; *O. quadripetalus* Gilib.; *O. vulgaris* Hill; *Schollera oxycoccos* (L.) Roth. For a complete list, see references 3 and 6.	Ericaceae	Cranberry	Small cranberry, *hong mei tai zi* (红莓苔子)
Vaccinium uliginosum L.	*Myrtillus uliginosus* (L.) Drejer; *Vaccinium gaultherioides* Bigelow; *V. occidentale* A. Gray; *V. pedris* Holub; *V. pubescens* Wormsk. ex Hornem.	Ericaceae	Not established	Bog blueberry, bog bilberry, northern bilberry, *du si yue ju* (笃斯越桔)
Vaccinium vitis-idaea L.	*Rhodococcum vitis-idaea* Avrorin; *Vaccinium jesoense* Miq.; *Vitis-idaea punctata* Moench	Ericaceae	Lingonberry	Alpine cranberry, cowberry, foxberry, lingberry, lingenberry, northern mountain cranberry, red bilberry, whortleberry, *yue ju* (越桔)

[a]The Plant List and the Tropicos database.[3,6] A comprehensive list of synonyms can be accessed through both websites.
[b]The American Herbal Products Association's *Herbs of Commerce*, 2nd ed. (2000).[2]
[c]*Herbs of Commerce*, 2nd ed.,[2] the USDA GRIN Database,[4] the USDA PLANTS Database,[7] and the Health Canada website.[8]
[d]*Flora of China*.[9]
[e]*PDR for Herbal Medicines*, 2nd ed.[10]
[f]There are differences in the meaning of "blueberry" and "wild blueberry." In the US dietary supplement trade, the name "blueberry" is restricted to three species, *Vaccinium angustifolium*, *V. corymbosum*, and *V. pallidum*.[2] In Europe, *V. myrtillus* is often called blueberry, though bilberry is the English word which refers to this species in the trade.[2] The hybrid cultivated blueberries from which the majority of the commercial food supply is derived are generally called blueberries. According to Steven Foster, president of Steven Foster Group, Inc., North American wild blueberry, common blueberry, common lowbush blueberry, low sweet blueberry, and lowbush blueberry refer to *V. angustifolium* which is common in the Northeastern United States and is commercially harvested in its habitat. Velvet leaf blueberry (*V. myrtilloides*) is also traded as "wild blueberry," and is mostly wild-harvested in the Canadian maritime provinces. It is safe to assume that "wild blueberry" in a commercial sense refers to both *V. angustifolium* and *V. myrtilloides* (e-mail communication, July 1, 2015).

American blueberry species) is lacking. Macroscopic test methods are obviously inadequate to detect adulteration of bilberry extracts. For correct authentication, additional means of testing (e.g., chemical) should be used.

6. Identification and Distinction Using Microanatomical Characteristics

Detailed microscopic descriptions of *V. myrtillus* are found in several references.[12,13,16,17] The AHP monograph also contains microscopic data on two known adulterants, bog bilberry (*V. uliginosum*) and lingonberry (*V. vitis-idaea*).[12] A paper by Villani et al. compares the micro-anatomical characteristics of bilberry fruit and European elder fruit,[18] but based on the available authoritative resources, there is no reference to find information on *V. myrtillus* and other (i.e., in addition to European elder) known adulterants, e.g., black soybean hull or *Morus australis*.

Comments: While microscopic distinction of a blueberry powder could be helpful to detect adulteration with synthetic dyes, charcoal, or powders from a different berry source, the use in authentication or detection of adulteration of bilberry extracts is limited. (It is conceivable that a synthetic dye or charcoal could be detected as an adulterant of a bilberry extract by microscopy, but no papers in this regard have been located at the time of the publication of this document.) Furthermore, criteria for the identification of a number of adulterant species are lacking. In addition, typical microanatomical features are absent in extracts of bilberry fruit. Therefore, the use of microscopy for the authentication of bilberry extracts and for the detection of its adulterants is generally considered inadequate.

7. Genetic Identification and Distinction

One method described in the literature was evaluated in this review: Jaakola et al.[19]

Comments: High-resolution melting (HRM) of amplicons is a rapid DNA barcoding method that works with samples consisting of fresh and dry material from a single species with intact DNA. The method was able to distinguish bilberries from lingonberry, bog bilberry, blueberry (*V. corymbosum* × *V. angustifolium*), crowberry (*Empetrum nigrum*, Ericaceae), gooseberry (*Ribes uva-crispa*, Grossulariaceae), honeysuckle (*Lonicera caerulea*, Caprifoliaceae), and mountain shadbush (*Amelanchier bartramiana*, Rosaceae). However, the method is not applicable to bilberry extracts since DNA is often damaged (denatured or fragmented) and/or removed via filtration during the extraction process, even though newer DNA methods have shown some success with dried extracts. Also, a genetic assessment is not able to determine the plant part, which is a legal requirement of dietary supplement ingredient identification. Therefore, DNA-based methods are of limited use for bilberry extract authentication or detection of the presence of adulterants.

8. Chemical Identification and Distinction

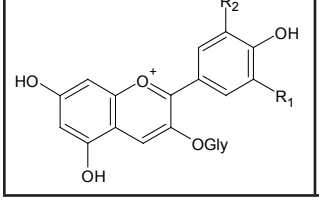
A number of analytical methods have been published for identifying *V. myrtillus* berry extracts, including compendial methods, e.g., by the *European Pharmacopoeia*[13] or the *United States Pharmacopeia*.[20] These methods are cited in the Laboratory Methods section below. Distinction based on the phytochemical profile requires knowledge of the composition of bilberry fruit extracts and its adulterants. The important components in *V. myrtillus* and its adulterating species are listed below with an emphasis on anthocyanins. Obviously, the composition of extracts can vary greatly depending on the manufacturing process.

8.1 Chemistry of *Vaccinium myrtillus* and the Potential Adulterants

***Vaccinium myrtillus*:** Dry bilberry fruit contains up to 10% of catechin-type tannins (min. 1% according to PhEur),[13] proanthocyanidins, and anthocyanins. The anthocyanins are mainly glucosides, galactosides, or arabinosides of delphinidin, cyanidin, and – to a lesser extent – malvidin, peonidin, and petunidin (Figure 1).[21] However, there are considerable differences in the quantitative composition of anthocyanins, e.g., glucosides are almost completely absent in some samples from Eastern Finland.[22] Flavonols include quercetin- and kaempferol-glycosides. The fruits also contain other phenolic compounds (e.g., chlorogenic acid, caffeic acid, *o*-, *m*-, and *p*-coumaric acids, and ferulic acid), citric and malic acids, and volatile compounds.[12,14] Marker compounds that can be used to detect adulteration with other berry or fruit extracts are indicated in Table 2.

Aronia melanocarpa: Black chokeberry fruit contains up to 5.2% proanthocyanidins and up to 2% anthocyanins,[23] mainly cyanidin-3-*O*-galactoside, with lesser amounts of cyanidin-3-*O*-arabinoside, -xyloside, and -glucoside. [24,25] Other flavonoids include glycosides of quercetin.[26] The fruit also contains other phenolic compounds (chlorogenic acid and neochlorogenic acid), malic and citric acids, and volatile compounds.[23] The much simpler and very different anthocyanin pattern of chokeberry fruit can be used to distinguish it from bilberry fruit. In addition, the presence of high amounts of neochlorogenic acid or quercetin-3-*O*-rhamnosyl-(1→6)-galactoside is indicative of adulteration with *Aronia* spp. or berries from other species.

Glycine max: Dry hydroalcoholic extracts of soybean contain mainly sugars (58-65%), proteins (5-7%), lipids (4-7%), minerals (7-10%) and saponins (6-10%). The content of the characteristic isoflavones in crude extracts is between 0.8 and 2%,[27] with 6''-*O*-malonylgenistin, 6''-*O*-malonyldaidzin, genistin, and daidzin as the quantitatively most important.[28,29] A study comparing soybean with variously colored seed coats determined that only black



Cyanidin-3-*O*-glycoside: R_1 = H, R_2 = OH
Delphinidin-3-*O*-glycoside: R_1 = OH, R_2 = OH
Malvidin-3-*O*-glycoside: R_1 = OCH_3, R_2 = OCH_3
Peonidin-3-*O*-glycoside: R_1 = H, R_2 = OCH_3
Petunidin-3-*O*-glycoside: R_1 = OCH_3, R_2 = OH

Gly: glucose, galactose, or arabinose

Figure 1: Anthocyanins occurring in bilberry fruits

seed coat soybeans contain cyanidin-3-*O*-glucoside. [30] A specific extract of the black soybean hull was shown to contain 39.7% proanthocyanidins, 9.2% cyanidin-3-*O*-glucoside, and 6.2% catechin.[31] Besides cyanidin-3-*O*-glucoside, which makes up to 80% of anthocyanins in black soybean, delphinidin-3-*O*-glucoside (ca. 13%) and petunidin-3-*O*-glucoside (3-4%) were quantitatively next, while six other anthocyanins were found in very low amounts.[28] Analysis of a black soybean hull market sample confirmed cyanidin-3-*O*-glucoside as the major anthocyanin and peonidin-3-*O*-glucoside as a minor component.[32] The presence of isoflavones and the different anthocyanin pattern, dominated by cyanidin-3-*O*-glucoside but lacking the anthocyanin-arabinosides,[33] allow a distinction between black soybean hull and bilberry extracts.

Morus australis: The fruits of *Morus australis* are rich in anthocyanins, predominantly cyanidin-3-*O*-rutinoside, but also cyanidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, the alkaloid 1-deoxynojirimycin, and the flavonoid rutin.[34,35] The anthocyanidin-rutinosides and 1-deoxynojirimycin are absent in *Vaccinium* berries and can be used as markers to detect adulteration. Rutinosides of cyanidin and pelargonidin are reportedly good markers for adulteration since they occur in many *Morus* species (e.g., *M. atropurpurea*, *M. alba*, and *M. nigra*).[21]

Morus nigra: Black mulberry is a good source of organic acids and phenolics. The fruit contains 3.5-19.9% malic acid and 0.6-2.3% citric acid.[36] Chlorogenic acid (0.05-0.14%) was found to be the prominent phenolic acid, while rutin (0.07-0.21%) is the major flavonoid. The main anthocyanins are cyanidin-3-*O*-glucoside (0.01-0.70%) and cyanidin-3-*O*-rutinoside (0.005-0.57%). The concentrations of pelargonidin-3-*O*-glucoside and pelargonidin-3-*O*-rutinoside are below 0.03% for both fresh and dry mulberry.[37-39] In addition, the berry contains the alkaloid 1-deoxynojirimycin, which was found in juices of eight different mulberry species (at concentrations between 30 and 80 mg/mL), including *M. nigra*.[35] This alkaloid, or cyanidin-3-*O*-rutinoside and pelargonidin-3-*O*-rutinoside, can be used as markers for adulteration with this and other mulberry species.

***Oryza sativa*:** The composition of various rice parts (endosperm, bran, and hull) has been the subject of a number of reviews.[40,41] Rice hulls consist mainly of lignin, hemicellulose, cellulose, and hydrated silica.[42] Cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside are the main anthocyanins of black rice extract.[32] Anthocyanin content in rice bran strongly depends on color, with black rice bran having the most, followed by purple, red, and brown rice bran. The major anthocyanin of rice bran is cyanidin-3-*O*-glucoside, accounting for 51-84% of the total, followed by peonidin-3-*O*-glucoside (6-16%), cyanidin-3-*O*-rutinoside (3-5%), and cyanidin-3-*O*-galactoside (1-2%).[41] The major flavones in bran are tricin, luteolin, and apigenin, with tricin found in unpigmented rice hulls. No flavones have been reported from pigmented hulls. Other compounds in bran and hulls are phenolic acids (e.g., ferulic acid, *p*-coumaric acid, sinapic acid) and tocopherols.[41] An indication of bilberry extract adulteration with pigmented rice could be the presence of a large amount of cyanidin-3-*O*-glucoside, although other extracts (e.g., made from soybean hulls or European elder berries) would lead to a similar outcome. Tricin (5,7,3'-trihydroxy-2',4'-dimethoxyflavone), cyanidin-3-*O*-rutinoside, or large amounts of cyanidin-3-*O*-glucoside can be used to detect adulteration with rice bran extracts.

Prunus avium: Sweet cherry fruit contains high levels of sugars and sugar alcohols, with up to 8.9 g, 7.6 g, and 6.8 g/100 g fresh fruit for glucose, fructose, and sorbitol, respectively.[43] Other important constituents are the polyphenols, especially anthocyanins, phenolic acid derivatives (predominantly neochlorogenic acid, with lower amounts of chlorogenic acid and caffeoylquinic acid), catechin, epicatechin, and rutin.[43-45] The main anthocyanin is cyanidin-3-*O*-rutinoside with 5.7-128.9 mg/100 g fresh weight (fw), followed by cyanidin-3-*O*-glucoside (0.4-34.8 mg/100 g fw) and peonidin-3-*O*-rutinoside (0.01-8.4 mg/100 g fw). Other anthocyanins reported from wild cherries are peonidin-3-*O*-glucoside and peonidin-3-*O*-rutinoside.[43-45] The presence of cyanidin-3-*O*-rutinoside and pelargonidin-3-*O*-rutinoside, while not exclusive for sweet cherry, is an indication of bilberry extract adulteration with other materials.

Ribes nigrum: Black currant fruit contains high levels of polyphenols, especially anthocyanins, phenolic acid derivatives (both hydroxybenzoic and hydroxycinnamic acids), flavonols (glycosides of myricetin, quercetin, kaempferol, and isorhamnetin), and proanthocyanidins (between 120 and 166 mg/100 g fresh berries).[46-47] The main anthocyanins are delphinidin-3-*O*-rutinoside and cyanidin-3-*O*-rutinoside, but delphinidin- and cyanidin-3-*O*-glucoside are also found.[47-49] The best markers for the presence of extracts made from berries of *R. nigrum* are delphinidin-, cyanidin-, and myricetin-3-*O*-rutinoside.[21]

***Rubus* spp.**: Due to the large number of distinct species and hybrids, it is beyond the scope of this Laboratory Guidance Document to provide a comprehensive phytochemical review of all *Rubus* spp. The conclusions regarding the composition of *Rubus* spp. in this paragraph are based on review articles by Lee et al.[50] and Kaume et al.[51] Fresh blackberry (*Rubus* spp. according to Kaume et al.)[51] fruit contains over 88% water, 5.3% total fiber, 4.9% total sugar (mainly glucose and fructose), 1.4% protein, and 0.5% total lipids. Total anthocyanins reportedly vary between 38-326 mg/100 g fw in blackberry samples.[51] Phenolic acids (free and conjugated forms of hydroxycinnamic and hydroxybenzoic acids), catechin, epicatechin, and flavonol-glycosides (quercetin- and kaempferol-glycosides) make up the phenolic monomers that have been reported in *Rubus* fruits. Typically, these compounds are less abundant than the phenolic polymers (ellagitannins) or anthocyanins.[50] Anthocyanins from *Rubus* fruits are mainly derivatives of cyanidin with non-acylated glycosyl moieties; however, anthocyanins containing acylated sugars such as cyanidin-3-*O*-malo-

nylglucoside and cyanidin-3-*O*-dioxalylglucoside can be found occasionally, e.g. in blackberries, at low concentrations. Cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside appear to be common to all *Rubus* spp., but vary with regard to the relative amounts. Cyanidin-3-*O*-rutinoside, also occurring in mulberry and cherry species (Table 2), is not found in bilberry fruit, and can be used as a marker compound for adulteration. In black raspberries, cyanidin-3-*O*-xylosylrutinoside is the predominant anthocyanin.[50] The presence of this anthocyanin is indicative for either black raspberry or red currant (*Ribes rubrum*, Grossulariaceae) fruit (Table 2).

***Sambucus nigra*:** European elder berries are also rich in polyphenolic compounds. The anthocyanin content is dominated by cyanidin-3-*O*-glucoside and cyanidin-3-*O*-sambubioside, with lesser amounts of cyanidin-3-*O*-sambubioside-5-*O*-glucoside and cyanidin-3,5-*O*-diglucoside.[24,47,52] Other phenolic compounds occurring in European elder berries are chlorogenic acid, rutin, and smaller amounts of isoquercitrin.[52] The proanthocyanidin content was established in one publication as 23 mg/100 g fresh black elder berries.[47] Black elder berries can be distinguished from bilberry by the presence of cyanidin-3-*O*-sambubioside and cyanidin-3-*O*-sambubioside-5-*O*-glucoside.

Vaccinium angustifolium: The qualitative composition of lowbush blueberries is quite similar to that of bilberry. According to Primetta,[21] the content of chlorogenic acid is higher in lowbush and highbush blueberries when compared to bilberry. The total anthocyanin content is lower in blueberries than in bilberries. However, blueberry (highbush and lowbush) has a higher relative malvidin content.[21] Kalt reported the presence of anthocyanins with acetylated sugar moieties, with malvidin-3-*O*-acetylgalactoside and malvidin-3-*O*-acetylglucoside being most abundant, in lowbush and velvet leaf (*V. myrtilloides*) blueberries.[53] The occurrence of eight different anthocyanin-acetylglycosides in lowbush blueberry was reported by Wu and Prior.[54] Therefore, these acetylated anthocyanins can be used as marker compounds to detect the presence of lowbush blueberry extracts.

Vaccinium corymbosum: Highbush blueberries also have a chemical composition that is very similar to bilberry. The variability in the anthocyanin pattern among highbush blueberries cultivated in various geographic locations and those collected in the wild, as outlined by Kalt,[53] makes a distinction based on chemical markers particularly difficult. Highbush blueberries reportedly contain higher amounts of chlorogenic acid,[21] but this compound alone is not a suitable marker for adulteration. The presence of acetylated anthocyanins, which can be used as markers for adulteration with lowbush and velvet leaf blueberry species, has been reported by several authors,[53,55] but appears to be inconsistent.[53] However, the relative amount of malvidin-3-*O*-glucoside and malvidin-3-*O*-galactoside – which are among the major anthocyanins in blueberries but are less abundant in bilberry fruit, where delphinidin- and cyanidin-glycosides are predominant – can be used as a criterion to indicate substitution or admixture of blueberry (highbush and lowbush) material.[11,53,56]

Vaccinium oxycoccos: Compared to bilberry fruit, cranberry contains relative high amounts of flavonols, mainly galactosides and other glycosides of quercetin and myricetin, but low amounts of phenolic acids (caffeic acid, ferulic acid, and *p*-hydroxybenzoic acid).[57,58] The contents of organic acids in freeze-dried cranberry from Poland was 14.7%, 7.5%, and 5.8% for citric, malic, and quinic acids, respectively,[59] and has been found to be higher in cranberry juice compared to bilberry and blueberry juices.[60] Cranberry from Western Canada contained five major anthocyanins, with cyanidin-3-*O*-glucoside, cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-galactoside, peonidin-3-*O*-galactoside, and peonidin-3-*O*-arabinoside in order of decreasing quantities.[61] The most abundant anthocyanins in cranberries from Finland were cyanidin-3-*O*-arabinoside (23.1% of total anthocyanins), peonidin-3-*O*-galactoside (21.5%), cyanidin-3-*O*-galactoside (19.2%), and peonidin-3-*O*-arabinoside (14.1%).[62] Based on the available data on anthocyanins, cranberry extracts can be distinguished from bilberry extracts by the absence of delphinidin- and pelargonidin-glycosides and the presence of relatively high amounts of peonidin-3-*O*-galactoside and peonidin-3-*O*-arabinoside.

Vaccinium uliginosum: The qualitative composition of bog blueberries is quite similar to that of bilberry, but there are some quantitative differences that can be used to detect adulteration. The berries of *V. uliginosum* are among the richest sources of flavonols – for example, myricetin, quercetin, and rutin (30-100 mg/100 g fw) compared to the berries of *V. myrtillus* (1-11 mg/100 g fw).[63-65] In bog blueberries, delphinidin- and malvidin-glycosides predominate; however, the anthocyanin composition reportedly varies depending on the geographical origin of the material. The main anthocyanin in bog blueberries from Norway and wildcrafted material from China is malvidin-3-*O*-glucoside, while cultivated Chinese material contains cyanidin-3-*O*-glucoside as the major anthocyanin.[21,66-68] The range of the relative proportions of cyanidin and malvidin (calculated either as glycosides or as aglycones after hydrolysis) is different in the berries of *V. myrtillus* (26-40% and 9-15%, respectively) compared to the berries of *V. uliginosum* (4-10% and 28-49%, respectively).[21]

Vaccinium vitis-idaea: Phytochemical research on lingonberry has mainly focused on the phenolic composition (anthocyanins, flavonols, phenolic acids, and proanthocyanidins). The anthocyanin pattern in lingonberries is rather simple and consists mainly of cyanidin-glycosides (predominantly cyanidin-3-*O*-galactoside).[64,69,70] The flavonol composition reportedly consists of quercetin-glycosides (quercitrin, hyperoside, and quercetin 4"-(3-hydroxy-3-methylglutaroyl)rhamnoside [HMG-rhamnoside]) and kaempferol derivatives.[21,71] The presence of quercetin-HMG-rhamnoside, or the different anthocyanin composition, has been reported to be useful in the detection of adulteration of bilberry fruit extract with that made from lingonberry.[21]

Table 2: Phenolic acid, anthocyanin, and flavonol marker compounds in berries and fruit other than bilberry and related *Vaccinium* spp.*

Marker compound not found in bilberries	Source plant(s): Common name (Latin name)
Phenolic acids	
Caffeoyltartaric acid (syn: caftaric acid)	Grape (*Vitis vinifera*)
Coumaroyltartaric acid	Grape
Feruloyltartaric acid	Grape
Anthocyanins	
Delphinidin-3,5-*O*-diglucoside	Pomegranate (*Punica granatum*); muscadine grape (*Vitis rotundifolia*)
Delphinidin-3-*O*-rutinoside	Black currant (*Ribes nigrum*); European elder (*Sambucus nigra*)
Cyanidin-3-*O*-2G-glucosylrutinoside	Raspberry (*Rubus idaeus*); *Rubus* hybrids; red currant (*Ribes rubrum*)
Cyanidin-3-*O*-sophoroside-5-*O*-rhamnoside	Raspberry
Cyanidin-3-*O*-sambubioside-5-*O*-glucoside	American elder (*Sambucus nigra* ssp. *canadensis*); European elder
Cyanidin-3-*O*-2G-xylosylrutinoside	Red currant; black raspberry (*Rubus occidentalis*)
Cyanidin-3,5,-*O*-diglucoside	Pomegranate; raspberry; American elder; European elder; fox grape (*Vitis labrusca*); muscadine grape
Cyanidin-3-*O*-sophoroside	Black mulberry (*Morus nigra*); raspberry; *Rubus* hybrids; red currant
Cyanidin-3-*O*-rutinoside	Black mulberry and other *Morus* spp.; sweet cherry (*Prunus avium*); sour cherry (*Prunus cerasus*); European gooseberry (*Ribes uva-crispa*); black currant; red currant; red-flower currant (*Ribes sanguineum*); Andes berry (*Rubus glaucus*); *Rubus* hybrids; raspberry; boysenberry (*Rubus loganobaccus*)
Cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)-sambubioside-5-*O*-glucoside	American elder
Cyanidin-3-*O*-(6″-*O*-dioxalyl)-glucoside	Raspberry; *Rubus* hybrids
Petunidin-3,5-*O*-diglucoside	Muscadine grape
Petunidin-3-*O*-rutinoside	Black currant
Peonidin-3,5-*O*-diglucoside	Muscadine grape
Peonidin-3-*O*-rutinoside	European gooseberry; black currant
Pelargonidin-3,5-*O*-diglucoside	Pomegranate; muscadine grape
Pelargonidin 3-*O*-rutinoside	Strawberry (*Fragaria vesca*); black mulberry and other *Morus* spp.; sweet cherry; Nanking cherry (*Prunus tomentosa*); Andes berry; raspberry
Pelargonidin 3-*O*-glucoside	Strawberry; black mulberry; raspberry
Pelargonidin 3-*O*-(6″-*O*-malonyl)-glucoside	Strawberry
Flavonol glycosides	
Myricetin 3-*O*-rutinoside	Black currant
Myricetin 3-*O*-(6″-*O*-malonyl)-glucoside	Black currant
Quercetin-3-*O*-rhamnosyl-(1→6)-galactoside	Black chokeberry (*Aronia melanocarpa*)
Quercetin-3-*O*-arabinosyl-(1→6)-glucoside	Black chokeberry
Quercetin 3-*O*-glucosyl-(1→6)-xyloside	*Rubus* hybrids
Quercetin 3-*O*-xylosyl-(1→6)-glucuronide	*Rubus* hybrids
Quercetin 3-*O*-(6″-*O*-malonyl)-glucoside	Strawberry; black currant
Isorhamnetin 3-*O*-rutinoside	Black currant; European elder; American elder
Kaempferol 3-*O*-rutinoside	Black currant; European elder; American elder
Kaempferol 3-*O*-(6″-*O*-malonyl)-glucoside	Strawberry; black currant

*Modified from reference 21.

8.2 Laboratory Methods

Note: Unless otherwise noted, all methods summarized below are based on using only the fruit of bilberry and its known adulterants.

8.2.1 HPTLC

Methods from the following sources were evaluated in this review: Upton,[12] the PhEur 8.4 monograph for bilberry extract,[13] the *USP 38-NF 33* Powdered Bilberry Extract monograph,[20] the CAMAG application note,[72] Wagner and Bladt,[73] the PhEur 8.4 monograph for dry bilberry fruit,[74] and the *USP Dietary Supplements Compendium*.[75]

Comments: HPTLC fingerprints are a good means to authenticate bilberry fruit extracts and detect adulteration, although there are obvious differences among the various published methods. The sample preparation generally consists in dissolving the bilberry extract (or powdered dry fruit in references 72 and 74) in methanol by shaking for 10-15 min, and subsequent filtration or centrifugation – allowing for the preparation of a test sample using a low amount of solvent in less than 30 min. The *n*-butanol-acetic acid-water (5:1:2) solvent system on silica gel plates leads to anthocyanin tailing, which does not allow for a clear distinction of the anthocyanin pattern.[73] However, the same system with cellulose plates provides better separation and peak shapes. The chromatographic conditions described in references 12, 72, 74, and 75 use silica gel plates and a single mobile phase consisting of formic acid, water, and *n*-butanol (although the solvent proportions in reference 74 are different than those described in 12, 72, and 75) and provide suitable conditions for bilberry authentication. With this method, adulteration with amaranth dye at concentrations as low as 0.25% can be detected.[72] An advantage of references 12, 72, and 75 (compared to the compendial methods in 13, 20, and 74) is the inclusion of color photographs enabling a comparison with commercially available extracts.

The *USP 38-NF 33*[20] and PhEur 8.4[13] monographs for bilberry extract use the same stationary and mobile phase in their HPTLC methods for bilberry extract analysis, but different standard compounds: either a reference bilberry extract[20] or cyanidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside[13] are used. Note that in PhEur 8.6, the entire suite of bilberry monographs will be harmonized to include only HPTLC on silica gel plates with anhydrous formic acid, water, and *n*-butanol as detailed in reference 74 (Eike Reich, e-mail communication, May 29, 2015). The chromatographic systems described in references 12, 13, 20, 72, 74, and 75 are expected to adequately distinguish bilberry extracts from extracts of other fruit species. However, the monographs for bilberry extract[13,20] require two consecutive developing steps before visualization and use cellulose as the stationary phase without having a better resolution of the anthocyanin bands than when using the conditions of 12, 72 and 75, or 74. For species discrimination, the derivatization with anisaldehyde reagent (Figures 3 and 4) is most suitable. With derivatization, the sugar composition of bilberry fruit extract becomes visible, which can provide additional information about the type of extract and whether or not sugar was added to the extract.

8.2.2 HPLC and UHPLC

Methods described in the following literature were evaluated in this review: the PhEur 8.4,[13] the *USP 38-NF 33*,[20] Lätti et al.,[22] Chandra et al.,[24] Govindaraghavan,[32] Kalt et al.,[53] Može et al.,[65] Cassinese et al.,[76] Penman et al.,[77] Buchert et al.,[78] Burdulis et al.,[79] Díaz-García et al.,[80] Fanali et al.,[81] Gardana et al.,[82] Ichiyanagi et al.,[83] Jovančević et al.,[84] Müller et al.,[85] Nakajima et al.,[86] Obón et al.,[87] Yamamoto et al.,[88] and Zhang et al.[89] Specific comments on strengths and weaknesses of each of the methods are listed in Appendix 1, Table 4.

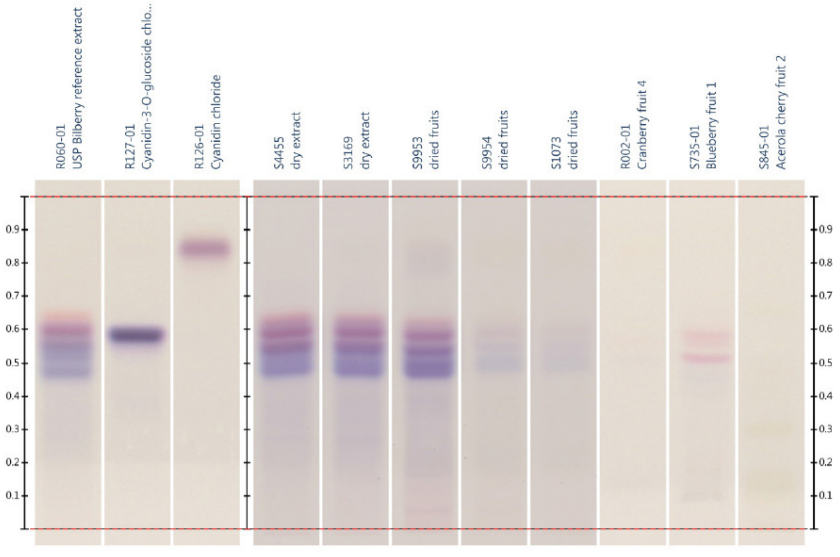


Figure 2: HPTLC analysis of bilberry fruit extract, bilberry fruit, cranberry fruit, blueberry fruit, and acerola cherry (*Malpighia* sp., Malpighiaceae) fruit according to reference 74;

Detection: visible light. Lane 2: cyanidin-3-*O*-glucoside chloride; lane 3: cyanidin chloride. Image provided by CAMAG (Muttenz, Switzerland)

Comments: Adulteration of bilberry extracts with natural or synthetic dyes, or anthocyanin-containing extracts, can be detected with HPLC fingerprinting methods. For routine quality control, quick and easy sample preparation methods are provided in the *European Pharmacopoeia* and the *USP 38-NF 33*.[13,20] The solvent of choice is usually 2% hydrochloric acid in methanol with extracts dissolved under sonication. Note that the anthocyanin stability is limited using this solvent. Based on the run time, separation quality, and thorough validation, the HPLC-

UV methods presented in references 13, 20, 32, and 76 appear to be the optimal choices.

The USP monograph has additional features to authenticate bilberry: delphinidin-3-*O*-galactoside and delphinidin-3-*O*-glucoside should be the largest peaks; the cyanidin-3-*O*-galactoside, delphinidin-3-*O*-arabinoside, and cyanidin-3-*O*-glucoside peaks should be of similar size; and the size of each of the remaining anthocyanin peaks in the chromatogram should be smaller than the cyanidin-3-*O*-glucoside peak. This could be problematic since some authentic bilberry fruit samples (although authentication methods were not detailed) were found to contain more malvidin-3-*O*-glucoside than cyanidin-3-*O*-glucoside. In this situation, some authentic bilberry fruit extracts could be rejected if the specifications outlined in the USP monograph were to be followed.[32] If run time is critical, the conditions described by Yamamoto et al.[88] are a good choice since they provide a similar separation efficiency in a 20-min run as the HPLC methods do in 50 min.[13,20,32,76] It should be noted that this requires UHPLC instrumentation, which operates under higher pressure than standard HPLC equipment. Validation and system suitability parameters are lacking for the published UHPLC method.

8.2.3 UV/Vis Spectrophotometry

Methods described in the following literature were evaluated in this review: Upton,[12] the Institute for Nutraceutical Advancement,[90] the PhEur 6.0,[91] and AOAC International.[92]

Comments: While all these methods have the advantage of being simple and quick, and can be performed with relatively affordable instrumentation, their use to detect adulteration of bilberry extracts is limited. The methods described in references 12 and 91 are basically the same (minor differences exist in the sample preparation) and may allow the detection of adulteration with charcoal, but other adulterants will absorb at the test wavelength (528 nm) and may lead to erroneously high values for anthocyanin content. The INA method[90] and the official AOAC method[92] use pH-dependent differences in absorption (anthocyanins exist as the intensely colored oxonium or flavylium ions at pH 1.0, whereas at pH 4.5, they occur as colorless carbinols) at 520 nm to calculate anthocyanin contents. This method has been shown to detect adulteration with synthetic dyes, but it is not capable of identifying anthocyanin-based adulterants from other natural sources. Therefore, UV/Vis spectrometry is inadequate as a means to detect adulteration of bilberry extracts.

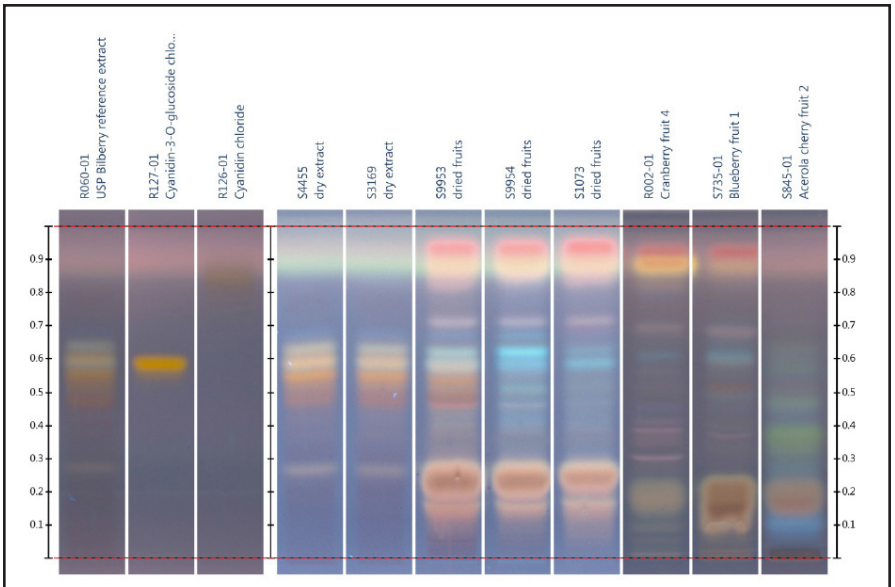


Figure 3: HPTLC analysis of bilberry fruit extract, bilberry fruit, cranberry fruit, blueberry fruit, and acerola cherry fruit using the stationary and mobile phase specified in reference 74; Detection: anisaldehyde reagent, viewed under UV light at 366 nm. Lane 2: cyanidin-3-*O*-glucoside chloride; lane 3: cyanidin chloride. Image provided by CAMAG (Muttenz, Switzerland)

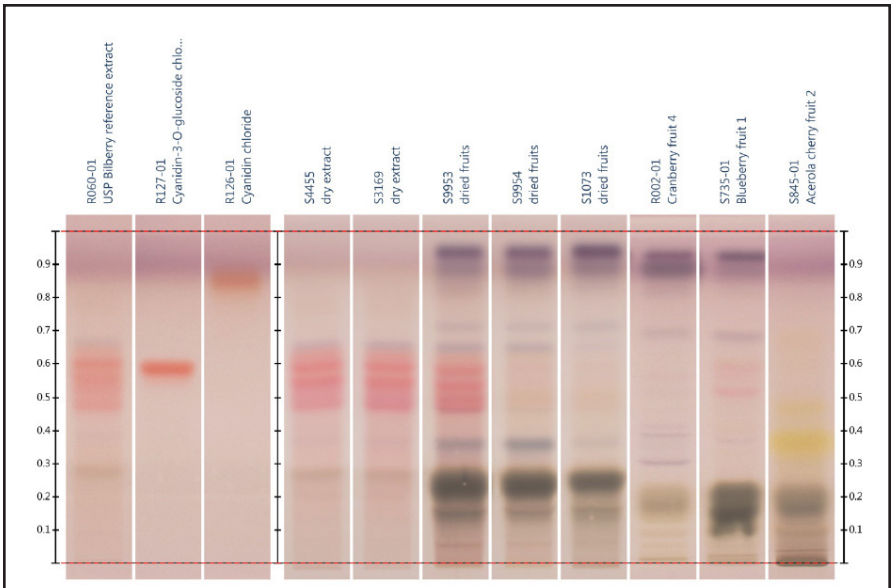


Figure 4: HPTLC analysis of bilberry fruit extract, bilberry fruit, cranberry fruit, blueberry fruit, and acerola cherry fruit using the stationary and mobile phase specified in reference 74; Detection: anisaldehyde reagent. Lane 2: cyanidin-3-*O*-glucoside chloride; lane 3: cyanidin chloride. Image provided by CAMAG (Muttenz, Switzerland)

9. Conclusion

Based on an evaluation of published methods, the most effective approach to detect adulteration of bilberry fruit extracts may be based on the evaluation of a phytochemical fingerprint. Several published HPTLC methods have shown their ability to distinguish bilberry fruit extract and its major adulterants.[12,13,20,72,74,75] The HPTLC methods of choice for detection of bilberry fruit extract substitution are described in the *European Pharmacopoeia*[74] and in references 12, 72, and 75 using the anisaldehyde reagent for detection. Admixtures of other anthocyanin-containing extracts can also be detected in many instances using HPTLC, a possible exception being if bilberry and blueberry extracts are mixed.

HPLC has the added benefit that peak size can be easily evaluated, which is a helpful tool for the detection of bilberry and adulterant extract mixtures. Several authors and compendia propose comparable methods,[13,20,32,76] which can be recommended based on ease of use, extensive validation, and proven ability to detect a wide array of adulterants. Care should be taken when using the additional criteria established in the USP for authentication, since the natural variability in bilberry may possibly lead to rejection of extracts made from authentic material from certain geographical locations.[32]

Note: A number of identity tests for bilberry extracts are offered by third-party analytical laboratories. According to input from five contract laboratories, the main testing methods are HPTLC and HPLC-UV. Additional testing methods (FT-NIR and HPLC-MS) are offered by some laboratories, or can be developed upon request.

10. References

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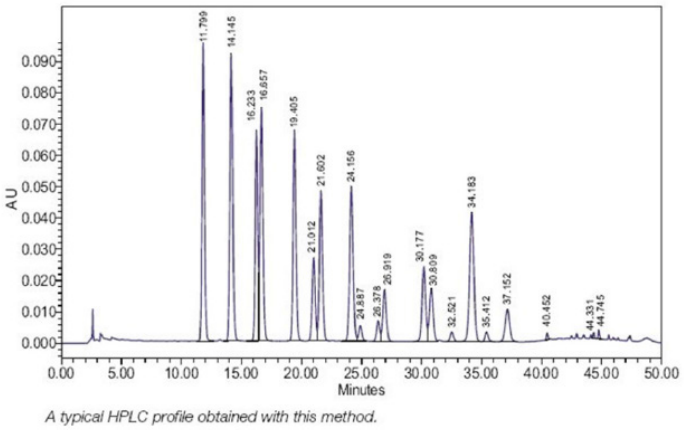

COMPOUND	RETENTION TIME
Delphinidin-3-O-galactoside	11.8
Delphinidin-3-O-glucoside	14.15
Cyanidin-3-O-galactoside	16.23
Delphinidin-3-O-arabinoside	16.66
Cyanidin-3-O-glucoside	19.41
Petunidin-3-O-galactoside	21.01
Cyanidin-3-O-arabinoside	21.6
Petunidin-3-O-glucoside	24.16
Delphinidin	24.89
Peonidin-3-O-galactoside	26.38
Petunidin-3-O-arabinoside	26.92
Peonidin-3-O-glucoside	30.18
Malvidin-3-O-glucoside	30.81
Peonidin-3-O-arabinoside	32.52
Malvidin-3-O-galactoside	34.18
Cyanidin	35.41
Malvidin-3-O-arabinoside	37.15
Petunidin	40.45
Peonidin	44.33
Malvidin	44.75

Figure 5. HPLC-UV chromatogram of an authentic bilberry extract analyzed according to the conditions outlined in the *European Pharmacopoeia*;[13] Image provided by Indena S.p.A. (Milan, Italy).

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Table 3. Comparison among the different approaches to authenticate bilberry.

Method	Applicable to	Pro	Contra
Macroscopic	- Unprocessed plant parts	Quick Inexpensive No solvents required	No automation/statistics Outcome relies on analyst's expertise Challenging for cut and sifted material
Microscopic	- Unprocessed plant parts	Quick Inexpensive	No automation/statistics Outcome relies on analyst's expertise Difficult or impossible to distinguish closely related species
Genetic	- Unprocessed plant parts - Cut and sifted - Powdered	Able to distinguish closely related species	Labor-intensive sample preparation and analysis Expensive equipment Unable to differentiate plant parts Cannot detect dyed or pre-extracted materials May not be applicable to highly processed materials[a]
HPTLC	- Cut and sifted - Powdered - Extracts	Quick Basic systems affordable for smaller labs	No statistics High-end equipment expensive Detection of adulteration challenging when related *Vaccinium* species are mixed Need for standard compounds
HPLC-UV	- Cut and sifted - Powdered - Extracts	Standard equipment in many laboratories Ideal for compounds with strong chromophore (e.g., phenolic acids)	Equipment expensive Mostly quantitative (less specific than HPLC-UV/MS) Detection of adulteration challenging when related *Vaccinium* species are mixed Need for standard compounds
HPLC-UV/MS	- Cut and sifted - Powdered - Extracts	Qualitative and quantitative State-of-the-art statistical evaluation possible	Equipment expensive Detection of adulteration challenging when related *Vaccinium* species are mixed
UV/Vis	- Cut and sifted - Powdered - Extracts	Quick Inexpensive Method based on absorption at different pHs is able to detect adulteration with synthetic dyes	Unable to detect adulteration in most cases No statistics
FT-NIR	- Powdered - Extracts	Quick Inexpensive State-of-the-art statistical evaluation Result does not rely on analyst's expertise	Need to build-up reference library No data on the ability to detect adulteration (except blueberry[93])

[a]Heat, UV light, radiation, and mutagenic chemicals (e.g., polyaromatic hydrocarbons) can damage DNA. Extracts made using lipophilic solvents will not contain DNA.

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Appendix 1

Table 4: Comments on the published HPLC methods to authenticate bilberry extracts and detect adulteration.

Reference	Comments
EP 8.4,[13] USP 38-NF 33,[20] Govindaraghavan,[32] Cassinese[76]	The validated HPLC-UV fingerprinting methods detailed by the USP[20] and the *European Pharmacopoeia*[13] have only minor differences (the difference being the concentration of formic acid in the mobile phase). Both provide excellent separations with good peak shapes over the 50-min run time.[a] Data from industry[11] have shown that these methods are capable of detecting adulteration with a variety of anthocyanidin-containing extracts and amaranth dye.
Lätti[22]	This fingerprint method shows a good separation of anthocyanins and acceptable peak shapes. The run time is long[a] (65 min) but provides good conditions for flavonol-glycoside analysis and allows separation of some of the anthocyanin pentosides from known adulterants (e.g., the berries of *Vaccinium uliginosum*). Based on differences in anthocyanin patterns, bilberry samples from various origins and closely related *Vaccinium* species can be distinguished. The gradient conditions between 20 and 38 min are not given in the paper, but consist of 10% solvent A - 90% solvent B, before dropping to 89% solvent B at 38 min (Anja Primetta, e-mail communication, August 13, 2015). The column temperature is not indicated. The method has been validated but parameters for system suitability are lacking.
Chandra[24]	This is a validated method that can be used to distinguish bilberry from other anthocyanin-containing ingredients like tart cherry, European elder, and black chokeberry. The run time is short, but the chromatogram likely contains a number of unresolved peaks, e.g., delphinidin-3-*O*-arabinoside and peonidin-3-*O*-glucoside. There are no peaks eluting after 16 min, so the run time[a] can be shortened. The sample preparation is quick and easy. The use of MS in addition to the UV detection provides additional information on peak identity. System suitability parameters are not available.
Kalt[53]	This fingerprint method has shown the ability to distinguish bilberry fruit extracts from extracts of closely related North American *Vaccinium* species (*V. angustifolium*, *V. corymbosum*, and *V. myrtilloides*). Despite the long run time, not all of the peaks are well separated. The column temperature is not specified. The high injection volume (50 µL) of the sample solution carries the risk of peak broadening (although the peak shape looks acceptable) and precipitation of certain components at the injection step. The method has not been validated and parameters for system suitability are lacking.
Može, anthocyanins[65]	This method has a 40-min run time[a] and has been at least partly validated (the extent of the validation is not detailed). The method is able to distinguish bilberry from highbush blueberries (*V. corymbosum*), but it is not clear if admixtures of the known adulterants charcoal or amaranth dye would be picked up using the MS detector. Due to the lack of images, the separation and peak shape cannot be assessed. The use of an MS as a detection device requires more expensive instrumentation. Parameters for system suitability are not indicated.
Može, flavonols, phenolic acids, and stilbenes[65]	This method has been at least partly validated (the extent of the validation is not detailed). The run time[a] is 60 min but the method can distinguish bilberry from highbush blueberries (*V. corymbosum*), although the amounts of most flavonols, phenolic acids, and stilbenes is rather low in the samples analyzed. It is not clear how well charcoal or amaranth dye ionizes in negative ion mode and if admixtures of such adulterants could be detected. Due to the lack of images, the separation and peak shape cannot be assessed. The use of an MS as a detection device requires more expensive instrumentation. Parameters for system suitability are not indicated.
Penman[77]	The conditions provide a good separation of bilberry anthocyanins and can be used as a fingerprinting method for anthocyanin-containing materials. Sample preparation is quick and easy. The run time[a] (42 min) could be shortened, since no peak is eluting after 26 min. The column temperature is not specified. The method has not been validated and parameters for system suitability are lacking.
Buchert[78]	The conditions provide an acceptable separation of bilberry anthocyanins and can be used as a fingerprinting method for anthocyanin-containing materials. The sample preparation using an enzymatic digestion is not applicable to routine QC. The run time[a] is rather long (70 min). The method has not been validated and parameters for system suitability are lacking.

Table 4 Continued: Comments on the published HPLC methods to authenticate bilberry extracts and detect adulteration.

Reference	Comments
Burdulis[79]	The conditions provide a good separation of bilberry anthocyanins and can be used as a fingerprinting method for anthocyanin-containing materials. The sample preparation described applies to fresh fruit only. The run time[a] (45 min) could be shortened, since no peak is eluting after 30 min. The authors specify two different columns for the separation of anthocyanins, so the actual stationary phase is unclear. The method has not been validated and parameters for system suitability are lacking.
Díaz-García[80]	This method provides acceptable separation in 25 min.[a] Some of the anthocyanins are barely separated (e.g., cyanidin-3-*O*-glucoside and petunidin-3-*O*-galactoside). The method can be used to analyze anthocyanins, flavonols, hydroxycinnamic acids, hydroxybenzoic acids, flavan-3-ols, and stilbenes in one run. Bilberry fruit can be distinguished from other anthocyanin-containing fruits (e.g., strawberry, sour cherry, cranberry, black grape). A UHPLC system is prerequisite. The method has not been validated and parameters for system suitability are lacking.
Fanali, HPLC-UV[81]	This is a validated method, although the validation was done using blueberry juice (no scientific name was given, but the composition of the juice was very similar to bilberry). The run time is 56 min,[a] but could be shortened, since the last peak elutes just before 40 min. Some peaks are barely separated and the peak shapes of some of the later eluting peaks (e.g., peonidin-3-*O*-glactoside) are less than perfect. Various berry juices can be distinguished based on anthocyanin profile. System suitability parameters are not indicated.
Fanali, Nano-LC-ESI-IT-MS[81]	This method has been validated, although using blueberry juice (see above). The run time[a] is fairly short and many peaks are overlapping, but the use of an MS allows separating the co-eluting anthocyanins based on the different molecular weights. Various berries can be distinguished based on anthocyanin profile. The nano-column is not commercially available and has to be hand-made. The validation data are inferior compared to the HPLC-UV analysis developed by the same authors. Savings in time and solvents are offset by the increased costs for the equipment and time used to fabricate the nano-column. System suitability parameters are not indicated.
Gardana[82]	This UHPLC-UV/MS method provides good separation, with only petunidin-3-*O*-glucoside and malvidin-3-*O*-galactoside overlapping, although the run time[a] of 53 min is on the longer side for a UHPLC method. The stability of anthocyanins in MeOH-H_2O (1:9), which is used for sample preparation of extracts, needs be established. The sample injection volume of 50 µL is high and carries the risk of peak broadening (although the peak shape looks acceptable) and precipitation of certain components at a flow rate of 0.5 mL/min. The method is able to detect adulteration with black mulberry, chokeberry, and blackberry. The use of an MS as a detection device requires more expensive instrumentation, and the method has not been validated and parameters for system suitability are lacking.
Ichiyanagi[83]	The method provides a good separation with a run time[a] of 43 min. Due to the isocratic conditions, the later eluting peaks are very broad, which may affect quantitative data. The sample preparation is very quick and easy. There are no data on other anthocyanin-containing materials, including known bilberry adulterants. The absence of a washout step using isocratic conditions (20% aqueous methanol) carries the risk of appearance of ghost peaks in subsequent chromatograms and that the more lipophilic bilberry components get stuck on the column and thus may shorten its life span. The method has not been validated and parameters for system suitability are lacking.
Jovančević[84]	The method analyzes bilberry anthocyanidins after hydrolysis in 2N HCl at 100°C for 1 hr. The separation is good, but the exact HPLC conditions are unknown (the method description ends after 22 min, but in the image of the chromatogram, the peaks for peonidin and malvidin elute after ca. 29 and 31 min, respectively). There are no data on other anthocyanin-containing materials, including known bilberry adulterants. The stability of anthocyanins and anthocyanidins in boiling 2N HCl is not known. The method has not been validated and parameters for system suitability are lacking.
Müller[85]	The separation in this partially validated fingerprint method is excellent, but the method is long[a] (65 min) and has an additional 45 min for washout and re-equilibration. The internal standard elutes during the washout period, which is not ideal. The method can distinguish bilberry from highbush blueberries (*V. corymbosum*). A quantification using standard compounds for all 15 bilberry anthocyanins is not feasible in practice, since this is very expensive and some of the standards are not commercially available. Parameters for system suitability are not indicated.

Table 4 Continued: Comments on the published HPLC methods to authenticate bilberry extracts and detect adulteration.

Reference	Comments
Nakajima[86]	The fingerprint method has good separation (the two pairs of overlapping peaks in UV trace can be separated using extracted ion chromatograms from MS detection), acceptable peak shapes, and easily distinguishes bilberry, black currant, chokeberry, and elder berry. The separation time[a] is 60 min. The sample preparation is time-consuming, in part due to a purification step using an Amberlite® XAD-7 resin. The injection volume is missing, and the stability of the anthocyanin-rich fractions in water needs to be evaluated. The method has not been validated and parameters for system suitability are lacking.
Obón, anthocyanins[87]	This reasonably short fingerprint method shows good separation (only peonidin-3-*O*-arabinoside is missing) and peak shapes, and easily distinguishes bilberry from seven other anthocyanin-containing fruits and purple carrots (*Daucus carota*, Apiaceae). In addition, the method proved its ability to detect adulteration with seven commercial synthetic and natural red pigments. The sample preparation is short and simple. The method has not been validated and parameters for system suitability are lacking.
Obón, hydroxycinnamic and hydroxybenzoic acids[87]	This is a short fingerprint method; however, the optimization for anthocyanins comes at the price of an insufficient separation for the phenolic acids, in particular the early eluting hydroxybenzoic acid peaks. Detection using UV at 260 nm or 320 nm is standard in many laboratories, but the more selective fluorescence detector may have to be added. Based on the various fingerprints, the authors state that "it is not possible to use phenolic acid and catechin profiles for the fingerprinting of a fruit or vegetable juice." The method has not been validated and parameters for system suitability are lacking.
Yamamoto, HPLC[88]	The fingerprint method is almost identical to the official methods described by the USP[20] and PhEur[13]. It has good separation (only one pair of overlapping peaks in UV trace) and acceptable peak shapes. No comparison between bilberry and other anthocyanin-containing extracts is given, but the method has proven its ability to detect adulteration (with black currant, in this case). The sample preparation is quick and easy. The method has not been validated and parameters for system suitability are lacking; however, since the only difference with the USP is the fact that this method starts with a 91% aqueous phase (rather than 93% as in the USP), the argument can be made that a full validation is not necessary, and the system suitability parameters can be adopted from the official method.
Yamamoto, UHPLC[88]	The separation of this fingerprint method is comparable to the HPLC method published by the same authors described above,[88] but with the added advantage of a short run time[a] of 20 min. The test samples and sample preparation steps are the same as for the HPLC method. For laboratories equipped with a UHPLC system, this is a good method for authentication and to detect adulteration. However, the method has not been validated and parameters for system suitability are lacking.
Zhang, fingerprint[89]	This fingerprinting method has a short run time[a] of 35 min, although it could be even shorter since the last anthocyanin of interest elutes before 15 min, and the last peak before 25 min. The short chromatography time comes at the expense of the separation, since a number of bilberry constituents are co-eluting. The sample preparation is quick and easy. The ability to detect adulteration has not been evaluated, but based on other fingerprinting methods, it should be adequate for the purpose. Once again, the method has not been validated and parameters for system suitability are lacking.
Zhang, hydrolysis[89]	This method has been developed for quantitative analysis of anthocyanins in bilberry extract. The anthocyanidins are separated under isocratic conditions after hydrolysis. The separation is good, although the isocratic conditions lead to an obvious broadening of the later-eluting peaks. The sample preparation is more time-consuming due to the hydrolysis step. The fact that only anthocyanidins are measured impacts the ability of this method to distinguish bilberry extracts from other anthocyanidin-containing extracts, and as such, it is not adequate as a means to detect adulteration. The method has not been validated and parameters for system suitability are lacking.

[a]The run times do not include the time used to return to initial conditions and equilibrate, since this information is not always provided in the publications.

Note: The term "validated" is used when a method has been validated for quantitative analysis, but not in terms of qualitative identification according to LaBudde and Harnly.[94]

Table 5. Comparison of different published HPLC methods for *V. myrtillus*. Sample preparation steps and times are indicated only for dry bilberry extracts, not for fresh fruit or fruit juice.

Reference	Number of samples[a]	Origin of samples	Sample preparation: handling[b] / duration [min][c]	Column Type	Run time [min][d]	Detection wavelength (UV) or ion mode (MS)
EP 8.4,[13] USP 38-NF 33,[20] Govindaraghavan,[32] Cassinese[76]	4[32]	USP powdered bilberry extract RS (1), commercial bilberry extracts (3)[32]	4/45[20] 5/50[13,32,76]	C18	50	UV: 535
Lätti[22]	179	Harvested by authors	n/a	C18	65	UV: 520
Chandra[24]	1	Commercial raw material	4/30	C18	26	UV: 520 MS (positive)
Kalt[53]	1	Commercial fresh fruit	n/a	C18	65	UV: 280, 520
Može, anthocyanins[65]	7	Harvested by authors	n/a	C18	40	MS (positive)
Može, flavonols, phenolic acids, and stilbenes[65]	7	Harvested by authors	n/a	C18	60	MS (negative)
Penman[77]	2	Commercial raw material	4/45	C18	42	UV: 540
Buchert[78]	1	Commercial fresh fruit	n/a	C18	70	UV: 520
Burdulis[79]	11	Harvested by authors	n/a	C18	45	UV: 520
Díaz-García[80]	1	Commercial fruit juice	n/a	C18	38.8	UV: 520
Fanali, HPLC-UV[81]	-	Commercial fruit juice	n/a	C18	56	UV: 518
Fanali, Nano-LC-ESI-IT-MS[81]	-	Commercial fruit juice	n/a	C18	33	MS (positive)
Gardana[82]	45	Commercial frozen fruit (19), extracts (14), capsules (6), and juices (6)	17/55 (extracts)	C18	53	UV: 520 MS (positive)
Ichiyanagi[83]	1	Commercial product	3/15	C18	43	UV: 520
Jovančević[84]	11	Harvested by authors	n/a	C18	unclear	UV: 520
Müller[85]	13	Commercial fruit and fruit juice	n/a	C18	65	UV: 520
Nakajima[86]	1	Commercial frozen fruit	n/a	C18	60	UV: 500-550 MS (positive)
Obón, anthocyanins[87]	1	Commercial fruit juice	n/a	C18	38	UV: 520
Obón, hydroxycin-namic and hydroxylbenzoic acids[87]	1	Commercial fruit juice	n/a	C18	38	UV: 260, 320; Fluorescence
Yamamoto, HPLC[88]	14	Commercial bilberry extracts (11) and bilberry combination products (3)	4/45	C18	50	UV: 535
Yamamoto, UHPLC[88]	14	Commercial bilberry extracts (11) and bilberry combination products (3)	4/45	C18	20	UV: 535
Zhang, fingerprint[89]	2	Commercial bilberry extracts	4/35	C18	35	UV: 525
Zhang, hydrolysis[89]	2	Commercial bilberry extracts	6/125	C18	30	UV: 530

[a]Number of bilberry samples analyzed
[b]Number of sample preparation steps involved (see Appendix 1)
[c]Estimated based on description provided in the reference (see Appendix 1)
[d]Not including the time used to return to initial conditions and equilibrate
n/a: not applicable