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Phenolic profiles differentiate wild bilberry and cultivated blueberry fruit

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ABSTRACT

The dark fruit of European wild-grown blueberry known as common bilberry (*Vaccinium myrtillus* L.) is associated with many health-promoting properties and has a steadily growing global market, especially in the food supplement sector. The adulteration of bilberry products with some less expensive fruits and berries with an intense color is a well-known problem. Phytochemical profiling is a viable option to discern different plant species from each other. In this study, the chemical composition of the fruit of wild bilberry and its close relative, cultivated blueberry (*Vaccinium* sp.), were analyzed and compared. A large number of samples of different geographical origin was collected. The major phenolics, including anthocyanins, chlorogenic acid, and condensed tannins, were determined by liquid chromatography using various detection methods. Significant differences were found in the phenolic profiles between wild-grown bilberry and cultivated blueberry. Principal component analysis indicated that it was possible to distinguish bilberry from cultivated blueberry based solely on their anthocyanin or tannin profiles. However, the geographical origin of the berries could not be identified according to their phenolic compound composition.

1. Introduction

Bilberry, the European blueberry species also known as blue whortleberry (*Vaccinium myrtillus),* grows wild and is native in the northern parts of the world. Its berries are harvested in large quantities in forestry areas in northern Europe, but the species has not been transferred to field cultivation, and neither have any commercial bilberry varieties been released to the market. Bilberry fruit is consumed locally in many countries and has special markets in extract form due to its applications in the pharmaceutical and food industries (Pires, Caleja, Santos-Buelga, Barros, & Ferreira, 2020). The global market for bilberry fruit and products is projected to grow at a CAGR (compound annual growth rate) of 13% in the 2023–2028 forecast period (Expert Market Research, 2022).

The cultivated blueberry (*Vaccinium* sp.) fruit and bilberry fruit resemble each other in appearance and taste to a considerable extent. The production areas of cultivated blueberry have expanded around the world, with a rapidly increasing market size. In 2021, the production area was 163,741 ha, and the production worldwide was 1,113,260.6 t, which were double and almost triple the respective values in 2010 (FAO, 2023). Blueberry fruit is a dominant product in the global market compared with wild bilberry fruit.

The cultivated highbush blueberry varieties belong to several species and species-hybrids. *V. corymbosum* is the dominant ancestor species, and *V. angustifolium* is usually harvested as half-cultivated areas in North America. The so-called half-high varieties have both these species in their genetic background, and *V. australe* and a lowbush species *V. lamarckii* have also been used in variety breeding (Rousi, 1963). The so-called southern blueberry varieties have *V. virgatum* (synonym*V. ashei*) or *V. darrowii* in their genome, and in addition, some other *Vaccinium* species are present in the modern varieties (Edger et al., 2022). The interspecific hybridization and several species included in activities to create blueberry varieties make the blueberry variety group especially divergent in the genetic sense (Campa & Ferreira, 2018). This is likely to be seen as high variability in the chemical composition of the fruit among different varieties.

The blueberry varieties developed or grown in Finland are mainly based on the Northern highbush or lowbush varieties. In addition, *V. uliginosum*, a lowbush blueberry species native to northern regions of the world is included in the genetic background in some varieties

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(Hiirsalmi, 1988). Bilberry belongs to the different sections of the *Vaccinium* genus than cultivated blueberries, and even differs from them with few exceptions by different chromosome number. So far, there are no commercial varieties in the market being hybrids of bilberry and cultivated blueberry (Podwyszynska, Mynett, Markiewicz, Pluta, & Marasek-Ciolakowska, 2021).

Bilberry and blueberry fruit are of special value due to their high content of phenolic compounds with interesting nutraceutical properties. Anthocyanins, proanthocyanidins (condensed tannins, CTs), and chlorogenic acid (5-caffeoylquinic acid, 5-CQA) are the major phenolics in both berries, while other hydroxycinnamic acids and flavonoids have been reported as minor phenolics (Bujor, Le Bourvellec, Volf, Popa, & Dufour, 2016; Colak et al., 2017; Riihinen, Jaakola, Kärenlampi, & Hohtola, 2008; Wang, Fong, Singh, & Vorsa, 2019). Potential health benefits associated with both berries, including antioxidative, prebiotic, neuroprotective, vision enhancing, hypoglycemic, anti-obesity, immunomodulatory and antitumoral activities, improvements in blood circulation, and the potential to reduce the risk of developing cardiovascular disease or diabetes have been comprehensively reviewed in several papers in recent years (Chehri et al., 2022; Miraghajani et al., 2020; Momenyan, Arab, Hasanpour Dehkordi, & Symonds, 2020; Pires et al., 2020; Sharma & Lee, 2022; Silva et al., 2020; Vanekova & Rollinger, 2022). The health-promoting effects are mostly associated with the high polyphenol content of the berries, and particularly with anthocyanins. Bilberry especially is extremely rich in anthocyanins, possessing them throughout the berry, while anthocyanins in blueberry are located mainly in the skin (Burdulis et al., 2009; Riihinen et al., 2008).

The harvesting and marketing chains of bilberry, with considerable export quantities, are well developed in Northern European countries. However, the complex global marketing networks, with actors at several levels and in different parts of world, are vulnerable to fraud. The adulteration of berries and berry-based food products is typically caused by intentional and unintentional mislabeling, when valuable and high-quality berries are replaced by berries of lower value and quality or of a different geographical origin (Lee, 2016). It has already been reported that wild bilberries have been replaced with cultivated blueberries (Lee, 2016). Bilberry extracts have been substituted with lower-cost extracts from other anthocyanin-rich plants, including different blueberry species (*V. angustifolium, V. corymbosum, V. floribundum*), wild cherry (*Prunus avium*), black chokeberry (*Aronia melanocarpa*), black soybean (*Glycine max*) hull, black rice (*Oryza sativa*), and mulberry (*Morus australis, M. nigra*) (Gafner et al., 2023).

Although blueberry and bilberry fruits can be distinguished by an experienced quality controller, based on their morphological features, products derived from these berries, such as jams, juices, or powders, pose a challenge in terms of visual differentiation. While DNA analysis stands out as a highly accurate method for verifying authenticity, its application is labor-intensive and requires specialized knowledge and skills that may not be available in all laboratory settings.

The composition differences of flavonoids and phenolic acids in the single genotypes of bilberry and cultivated blueberry plants have previously been compared (Riihinen et al., 2008). Bornsek et al. (2012) have shown that the fruit of cultivated blueberry varieties are lower than bilberry fruit in their content of some phenolic compounds, especially anthocyanins. We hypothesized that the difference in the chemical composition of cultivated blueberry and wild bilberry fruit could be used as a reliable tool to distinguish them. Our goal was to identify distinct phenolic compounds that could serve as key markers, enabling the differentiation of bilberry fruit from cultivated blueberries. We explored an especially large set of blueberry varieties, either grown in or imported to Finland, and compared them with a large number of wild bilberry populations over a wide geographical area in Finland. To the best of our knowledge, this is the first time the phenolic profiles of bilberry and cultivated blueberry fruit have been compared to this extent.

2. Material and methods

2.1. Berry samples

A total of 35 samples of ripe, fresh bilberries, 43 samples of blueberries cultivated in Finland, and 38 samples of cultivated blueberries imported to Finland were acquired in 2021 and 2022. The bilberry fruit was purchased in local markets in the Oulu region or directly from pickers during the typical bilberry harvest period between July and August. The geographical location of the bilberry collection area ranged between the latitudes 63° 31′–66°18′ N and the longitudes 26° 15' – 29° 10′ E. The samples of Finnish cultivated blueberry fruit were either harvested on Luke's Piikkiö Experimental Site in Kaarina in South-West Finland (60° 23′ N, 22° 33′ E), consisting of 38 varieties, or purchased from Finnish berry farmers in Central Finland (62° 53' – 64° 07′ N, 25° 22' – 27° 40′ E), consisting of five varieties.

The cultivated blueberry fruit imported to Finland was purchased in different shops in the Oulu region. The fruit originated in Southern Europe (Spain), Central Europe (Germany, Poland), Eastern Europe (Ukraine), North Africa (Morocco), Southern Africa (Republic of South Africa, Zimbabwe), and South America (Argentina, Peru). In only a few batches of imported blueberries was the name of the variety indicated, such as "Star," "Bluecrop," "Cargo," and "Biloxi."

The fresh samples were pretreated at Luke in Oulu, Finland. Each sample batch was about 1.0–1.5 kg, of which approximately 30 g of intact berries were randomly selected for measurements and frozen at −20 °C. The frozen samples were weighed and crushed before freeze-drying (Freezone 4.5 Plus, Labconco Corporation, Kansas City, MO, US.) for three to four days. The dry matter percentage of the samples was then determined. The samples were crushed into a fine powder and sent to the Luke chemistry laboratory at Jokioinen, Finland, for chemical analyses.

2.2. Chemical analysis

2.2.1. Anthocyanins and chlorogenic acid

60–90 mg of a freeze-dried berry sample was weighed into a 1.5 mL Eppendorf tube. 1 mL of extraction solvent (65% methanol (aq.) with 4% acetic acid) was added, and the sample solution was vigorously mixed with a vortex mixer. The sample was placed in an ultrasonic bath (400 W) for 20 min and centrifuged, after which the extract was poured into a 5 mL volume flask. The extraction procedure was repeated three times, and the final volume was adjusted to 5 mL. The extract was filtered and analyzed using Agilent 1100 high performance liquid chromatography (HPLC), equipped with diode array detection (DAD). Separation was done on a Phenomenex Kinetex EVO C18 column (250 × 4.6 mm, 5 μm) using a gradient elution designed for bilberry anthocyanins and described in European Pharmacopeia 8.0 (EDQM, 2013). The UV spectra of the peaks were recorded by DAD, and wavelengths of 535 nm and 329 nm were used to determine anthocyanins and chlorogenic acid (5-caffeoylquinic acid, 5-CQA) respectively. Authentic chlorogenic acid (MilliporeSigma, Munich, Germany) was used as an external standard for the quantification of 5-CQA, while anthocyanins were quantified as cyanidin-3-glucoside (Extrasynthese, Lyon, France) equivalents.

2.2.2. Proanthocyanidins

Proanthocyanidins (condensed tannins) were determined by ultra-high performance liquid chromatography (UHPLC) according to Korkalo et al. (2020). Briefly, a dry sample was weighed (20–30 mg) into a 1.5 mL Eppendorf tube and mixed with 1 mL of depolymerization reagent (3 g cysteamine, 56 mL methanol, 4 mL 13 M HCl). The samples were incubated at 65 °C for 60 min, after which they were placed in an ice bath to stop the reaction. The degradation products, i.e., free flavan-3-ols (terminal units) and their cysteaminyl derivatives (extension units) were determined by UHPLC equipped with DAD and fluorescence detection (FLD). Catechin, epicatechin, gallocatechin, and

epigallocatechin were purchased from MilliporeSigma (Munich, Germany), and procyanidin B2 from Extrasynthese (Lyon, France).

2.2.3. Liquid chromatography—mass spectrometry

UHPLC separation followed by quadrupole time-of-flight mass spectrometry (QTOF-MS) was performed for selected samples. The compounds were separated on a Waters Acquity BEH C18 (1.7 μm, 2.1 mm × 150 mm) column using a gradient described elsewhere (Hellström, Granato, & Mattila, 2020). Electrospray ionization (ESI) was used in positive mode for anthocyanins and chlorogenic acid and in negative mode for thiolyzed proanthocyanidins, with capillary voltages of +0.5 kV and −1 kV respectively. The sampling cone was set to 35 V, and the extraction cone to 4 V. MS/MS analyses were conducted by data independent acquisition (MS^E) centroid data mode in a full scan m/z 50–1500 with a 0.2 s scan time. In the MS^E function, the precursor ions from the low-collision energy MS-mode were fragmented using high collision energy ramped up from 25 to 45 V.

2.3. Statistical analyses

The differences in the levels of individual 21 parameters between the three groups (bilberry, cultivated domestic blueberry, cultivated imported blueberry) were evaluated with one-way ANOVA, and Tukey's HSD test was used in pairwise comparisons. The data set consisted of 116 observations and 21 parameters for ANOVA. The exploratory data analysis was conducted using unsupervised classification procedure principal component analysis (PCA). All the statistical analyses were performed using R Statistical Software (v4.2.0; R Core Team (2022)).

3. Results

3.1. Anthocyanins and chlorogenic acid

The anthocyanins in both cultivated blueberries and bilberries were based on five anthocyanidins, namely delphinidin, cyanidin, petunidin, peonidin, and malvidin, as identified according to UV spectra, retention order, and MS data. Galactoside, glucoside, and arabinoside derivatives of all five anthocyanidins were found in all bilberries and in most of the cultivated blueberries. Typical HPLC chromatograms of bilberry and blueberry anthocyanins can be found in supplementary data (Figs. S1–S2). Peonidin-3-arabinoside could not be determined in six blueberry samples and peonidin-3-galactoside and peonidin-3-glucoside were not determined in one blueberry sample. In general, galactose was the dominant sugar attached to anthocyanins in cultivated blueberries, while in bilberries, sugars were more evenly distributed, and the most common was glucose (Table 1). Anthocyanins acylated with acetic acid were frequently found. The total content of anthocyanins varied greatly, being 4.8–33 g/kg DW in cultivated blueberries and 29–65 g/kg DW in bilberries. Generally, the content was significantly higher in bilberries (48 ± 8.9 g/kg, mean ± sd) than in blueberries (17 ± 7.1 g/kg).

Acylated anthocyanins were detected in all bilberry samples, while 10 cultivated blueberry samples lacked them. However, the content of acylated forms was always quite moderate in bilberries (0.38 ± 0.07 g/kg DW, mean ± sd), and their average content was much higher in blueberries (1.4 ± 2.5 g/kg DW). In general, the major anthocyanidin in blueberries was malvidin, followed by delphinidin, while in bilberries, delphinidin was the major aglycon, followed by cyanidin (Table 1). Chlorogenic acid was detected and determined in every sample, and the content was generally higher in cultivated blueberries (4.41 ± 1.96 g/kg DW, mean ± sd) than in bilberries (1.59 ± 0.34 g/kg DW).

3.2. Proanthocyanidins

The average proanthocyanin content was 11 ± 5.0 g/kg DW (mean ± sd) and 14 ± 1.4 g/kg in cultivated blueberries and bilberries respectively (Table 1). Proanthocyanidins were based on (epi)catechin and (epi)gallocatechin structural unit, i.e., they were mixtures of procyanidins (PCs) and prodelphinidins (PDs). PC was always the dominant form, and its proportion varied between 66 and 100% and 69 and 80% in blueberries and bilberries respectively. Prodelphinidins could not be detected in five blueberry samples. Terminal units were essentially (epi) catechins. The proportions of terminal units differed significantly between blueberries and bilberries, i.e., the major terminal unit was always catechin in blueberries and epicatechin in bilberries. In general,

Table 1
Major phenolic compounds in cultivated blueberries (*Vaccinium* sp.) and wild bilberries (*V. myrtillus*). Contents given as mean ± standard deviation in dry weight (DW); in bilberries n = 35, in domestic blueberries n = 43, and in imported blueberries n = 38. Interpretation of superscripted letters a, b, c: not sharing any letter are significantly different by the Tukey-test at the 5% level of significance. Values for Blueberry, mean (n = 81) are calculated from all the results of domestic and imported blueberries.

Anthocyanins (mg/100g, DW)				
Compound	Bilberry	Blueberry, Domestic	Blueberry, Imported	Blueberry, Mean
Dl-3-Gal	^{b}650 ± 150	^{a}270 ± 110	^{a}220 ± 150	240 ± 130
Dl-3-Glc	^{a}600 ± 130	^{b}150 ± 87	^{c}35 ± 43	97 ± 91
Dl-3-Ara	^{a}590 ± 130	^{b}190 ± 71	^{c}140 ± 64	170 ± 70
Cn-3-Gal	^{a}490 ± 88	^{b}70 ± 34	^{c}28 ± 19	50 ± 35
Cn-3-Glc	^{a}480 ± 84	^{b}45 ± 25	^{c}7.7 ± 12	27 ± 26
Cn-3-Ara	^{b}480 ± 71	^{a}26 ± 19	^{a}11 ± 6.1	19 ± 16
Pt-3-Gal	^{a}150 ± 59	^{a}160 ± 65	^{a}130 ± 84	150 ± 80
Pt-3-Glc	^{a}340 ± 130	^{b}120 ± 68	^{c}29 ± 33	78 ± 71
Pt-3-Ara	^{b}140 ± 29	^{a}86 ± 34	^{a}72 ± 26	80 ± 31
Po-3-Gal	^{a}51 ± 9.8	^{b}22 ± 12	^{c}11 ± 5.8	17 ± 11
Po-3-Glc	^{a}190 ± 38	^{b}35 ± 22	^{c}8.4 ± 7.4	23 ± 21
Po-3-Ara	^{a}28 ± 5.6	^{b}17 ± 8.5	^{c}8.7 ± 6.5	13 ± 8.7
Mv-3-Gal	^{b}180 ± 51	^{a}290 ± 120	^{a}300 ± 190	290 ± 150
Mv-3-Glc	^{a}330 ± 150	^{b}230 ± 130	^{c}50 ± 52	150 ± 140
Mv-3Ara	^{b}82 ± 43	^{a}180 ± 60	^{a}200 ± 78	190 ± 70
Acylated	^{b}38 ± 7.4	^{a}240 ± 305	^{b}34 ± 44	140 ± 250
Total	^{a}4810 ± 890	^{b}2130 ± 580	^{b}1280 ± 530	1730 ± 700

Proanthocyanidins				
Parameter	Bilberry	Blueberry Domestic	Blueberry Imported	Blueberry, Mean
Content (mg/100g)	^{a}1430 ± 140	^{a}1380 ± 320	^{b}800 ± 490	1110 ± 500
PC/PD	^{a}74/26	^{a}71/29	^{b}82/18	76/24
DP	^{a}14 ± 2	^{b}40 ± 16	^{c}24 ± 10	33 ± 16
Terminal cat/epicat	^{a}21/79	^{b}69/31	^{c}76/24	72/28

Phenolic acids (mg/100g, DW)				
Compound	Bilberry	Blueberry Domestic	Blueberry Imported	Blueberry, Mean
5-CQA	^{a}160 ± 34	^{b}510 ± 180	^{c}390 ± 220	440 ± 200

Dry Matter				
	Bilberry	Blueberry Domestic	Blueberry Imported	Blueberry, Mean
Dry Matter (g/100g)	^{a}14.5 ± 1.8	^{a}14.7 ± 2.1	^{a}14.9 ± 1.2	14.6 ± 2.0

Anthocyanins: Dl = delphinidin, Cn = cyanidin, Pt = petunidin, Po = peonidin, Mv = malvidin, Gal = galactoside, Glc = glucoside, Ara = arabinoside.
Proanthocyanidins: PC/PD = average proportions of procyanidins/prodelphinidins in proanthocyanidins, DP = average degree of polymerization, Terminal cat/epicat = average proportions of proanthocyanidins terminal units (catechin/epicatechin).
Phenolic acids: 5-CQA = 5-caffeoylquinic acid (chlorogenic acid).

the average degree of polymerization (DP) in proanthocyanidins was significantly higher in cultivated blueberry (33 ± 16, mean ± sd) than in bilberry (14 ± 2), but the variation of DP was also very high, especially in blueberry samples.

3.3. Liquid chromatography—mass spectrometry

While the identification of anthocyanins was mostly based on retention order, the literature, and UV spectra, some samples were further analyzed by UHPLC-MS to obtain additional information about the compounds. Although the chromatographic conditions were not optimized for anthocyanins, all major glycosides could be detected (Table 2). Acetic acid appeared to be the organic acid attached to the acylated anthocyanins.

The UHPLC-MS analyses of thiolyzed samples confirmed that the structural units of both cultivated blueberry and bilberry proanthocyanins were catechin, epicatechin, and epigallocatechin, and the last appeared only as an extension unit (Table 2).

3.4. Principal component analysis (PCA)

Of the 21 parameters, 12 showed significant differences between all three berry groups, and one parameter, i.e., the content of petunidin-3-galactoside (Pt-3-Gal), had no differences between any groups (Table 1). Five parameters had no difference between domestic blueberry and imported blueberry but had differences between the other two comparisons (domestic blueberry versus bilberry and imported blueberry versus bilberry), while two parameters had no difference between domestic blueberry and bilberry but had differences between the other two comparisons (imported blueberry versus domestic blueberry and imported blueberry versus bilberry).

PCA of all 21 parameters showed a clear separation between the samples originating from blueberry and bilberry, and the first two principal components explained 69.1% of the total variance (Supplementary data, Table S1). There was no clear separation between domestic and imported blueberry (Fig. 1a). The three different subsets of the parameters described below also gave the same separation result as all 21 parameters (Fig. 1b–d, Tables S2–S4). The first subset had only tannin parameters, i.e., total content, average degree of polymerization, the proportion of prodelphinidins vs. procyanidins, and the proportion of terminal units (catechin vs. epicatechin). The second subset included anthocyanins in their native forms (15 anthocyanidin glycosides and their acylated derivatives), and in the third subset, anthocyanins were regrouped according to their aglycon forms, i.e., the total content of delphinidin derivatives, cyanidin derivatives, petunidin derivatives, peonidin derivatives, and malvidin derivatives. Respectively, in the three subset analyses, two principal components explained 82.9%, 80.0%, and 97.4% of the total variance.

Table 2
TOF-MS/MS^E data of phenolic compounds (anthocyanins, thiolyzed proanthocyanidins & 5-caffeoylquinic acid) of bilberries and cultivated blueberries.

Anthocyanins

Anthocyanin (Rt, min)	Molecular formula	Monoisotopic mass	Detected mass (*m/z*)	Mass difference (ppm)	Characteristic aglycon fragment
Delphinidin 3-galactoside (5.57)	$C_{21}H_{21}O_{12}$	465.1033	465.1021–465.1035	−2.6–0.4	303.0496–303.0505
Delphinidin 3-glucoside (5.85)	$C_{21}H_{21}O_{12}$	465.1033	465.1018–465.1038	−3.2–1.1	303.0490–303.0511
Cyanidin 3-galactoside (5.92)	$C_{21}H_{21}O_{11}$	449.1084	449.1075–449.1086	−2.0–0.4	287.0548–287.0567
Delphinidin 3-arabinoside (6.08)	$C_{20}H_{19}O_{11}$	435.0927	449.0914–449.0930	−3.0–0.70	303.0493–303.0505
Cyanidin 3-glucoside (6.20)	$C_{21}H_{21}O_{11}$	449.1084	449.1081–449.1090	−0.7–1.3	287.0549–287.0566
Petunidin 3-galactoside (6.30)	$C_{22}H_{23}O_{12}$	479.1190	479.1178–479.1200	−2.5–2.1	317.0649–317.0664
Cyanidin 3-arabinoside (6.53)	$C_{20}H_{19}O_{10}$	419.0978	419.0971–419.0994	−1.7–3.8	287.0548–287.0566
Petunidin 3-glucoside (6.63)	$C_{22}H_{23}O_{12}$	479.1190	479.1178–479.1194	−2.5–0.8	317.0651–317.0662
Petunidin 3-arabinoside (6.84)	$C_{21}H_{21}O_{11}$	449.1084	449.1071–449.1089	−2.9–1.1	317.0649–317.0663
Peonidin 3-galactoside (6.90)	$C_{22}H_{23}O_{11}$	463.1240	463.1235–463.1247	−1.1–1.5	301.0707–301.0714
Peonidin 3-glucoside (6.96)	$C_{22}H_{23}O_{11}$	463.1240	463.1227–463.1247	−2.8–1.5	301.0706–301.0713
Malvidin 3-galactoside (6.98)	$C_{23}H_{25}O_{12}$	493.1346	493.1338–493.1356	−1.6–2.0	331.0801–331.0818
Acetylated delphinidin 3-galactoside (7.10)	$C_{23}H_{23}O_{13}$	507.1139	507.1128–507.1143	−2.2–0.8	303.0501–303.0508
Peonidin 3-arabinoside (7.15)	$C_{21}H_{21}O_{10}$	443.1135	443.1126–443.1149	−2.1–3.2	301.0704–301.0721
Malvidin 3-glucoside (7.20)	$C_{23}H_{25}O_{12}$	493.1346	493.1332–493.1346	−2.8–0.0	331.0813–331.0818
Malvidin 3-arabinoside (7.35)	$C_{22}H_{23}O_{11}$	463.1240	463.1228–463.1245	−2.6–1.1	331.0806–331.0815
Acetylated delphinidin 3-glucoside (7.54)	$C_{23}H_{23}O_{13}$	507.1139	507.1126–507.1168	−2.6–5.7	303.0497–303.0506
Acetylated cyanidin 3-galactoside (7.57)	$C_{23}H_{23}O_{12}$	491.1190	491.1184–491.1201	−1.2–2.2	287.0555–287.0557
Acetylated petunidin 3-galactoside (7.73)	$C_{24}H_{25}O_{13}$	521.1295	521.1288–521.1295	−1.3–0.0	317.0655–317.0659
Acetylated cyanidin 3-glucoside (8.15)	$C_{23}H_{23}O_{12}$	491.1190	491.1179–491.1193	−2.2–0.6	287.0551–287.0557
Acetylated petunidin 3-glucoside (8.32)	$C_{24}H_{25}O_{13}$	521.1295	521.1281–521.1295	−2.7–0.0	317.0653–317.0670
Acetylated peonidin 3-galactoside (8.32)	$C_{24}H_{25}O_{12}$	505.1346	505.1331–505.1346	−3.0–0.0	301.0710–301.0712
Acetylated malvidin 3-galactoside (8.42)	$C_{25}H_{27}O_{13}$	535.1452	535.1443–535.1458	−1.7–1.1	331.0806–331.0829
Acetylated peonidin 3-glucoside (8.80)	$C_{24}H_{25}O_{12}$	505.1346	505.1331–505.1354	−3.0–1.6	301.0709–301.0718
Acetylated malvidin 3-glucoside (8.82)	$C_{25}H_{27}O_{13}$	535.1452	535.1435–535.1469	−4.5–3.2	331.0803–331.0833

Thiolyzed proanthocyanidins

Compound (Rt, min)	Deprotonated molecular formula	Monoisotopic mass	Detected mass (*m/z*)	Mass difference (ppm)	Characteristic flavan-3-ol QM - fragment
Epigallocatechin-cysteaminylthioether (4.84)	$C_{17}H_{18}NO_7S$	380.0804	380.0788–380.0796	−4.2–−2.1	303.0479–303.0488
Catechin-cysteaminylthioether (4.95)	$C_{17}H_{18}NO_6S$	364.0855	364.0837–364.0843	−4.9–−3.3	287.0527–287.0536
Epicatechin-cysteaminylthioether (5.65)	$C_{17}H_{18}NO_6S$	364.0855	364.0836–364.0847	−5.2–−2.2	287.0536–287.0548
Catechin (5.76)	$C_{15}H_{13}O_6$	289.0712	289.0692–289.0707	−6.9–−1.7	–
Epicatechin (6.81)	$C_{15}H_{13}O_6$	289.0712	289.0694–289.0711	−6.2–−0.3	–

Phenolic acids

Compound (Rt, min)	Protonated molecular formula	Monoisotopic mass	Detected mass (*m/z*)	Mass difference (ppm)	Characteristic caffeic acid fragment
5-Caffeoylquinic acid (5.85)	$C_{16}H_{19}O_9$	355.1029	355.1017–355.1037	−3.4–2.3	181.0493–181.0510

Rt = retention time.
QM – fragment = quinone methide fission cleavage product.

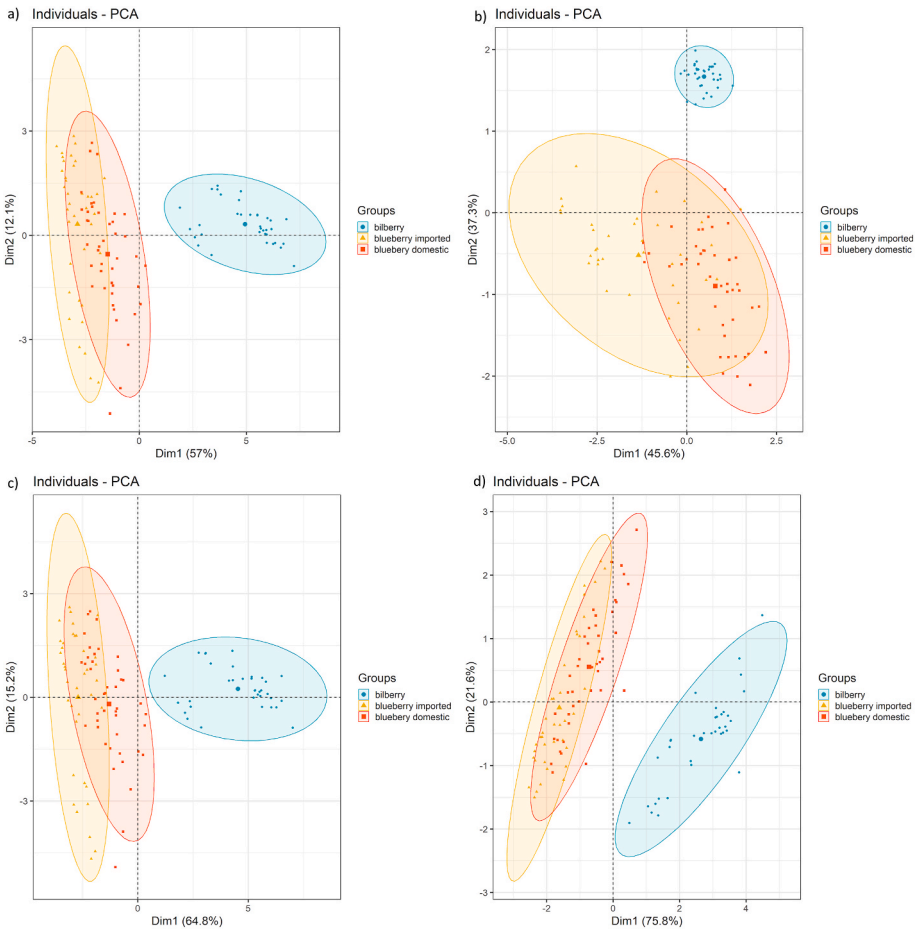

Fig. 1. Principal component analysis (PCA) score plot of bilberries, domestic blueberries and imported blueberries: a) all 21 parameters, b) tannin parameters, c) anthocyanins, and d) anthocyanidins included in the analyses.

The PCA loadings of all parameters (Fig. 2a) indicate that group separation is mostly influenced by the content of three anthocyanins (Mv-3-Gal, Pt-3-Gal, Mv-3-Ara) and the proportion of terminal units in proanthocyanidins (TUcat), as well as by the content of chlorogenic acid (5-CQA). When only proanthocyanidins are considered (Fig. 2b), the proportion of terminal units and the average degree of polymerization are the most indicative parameters. Three anthocyanins (Mv-3-Gal, Pt-3-Gal, Mv-3-Ara) are the main distinguishing factors among native anthocyanins (Fig. 2c) and malvidin when anthocyanin aglycons are considered (Fig. 2d).

4. Discussion

Most of the fruit studied in our research was acquired in 2022. Some blueberry varieties were included in both the 2021 and 2022 studies, and some varieties were collected from different regions. They were all considered as separate samples because it is known that environmental factors such as climate and other growing conditions may affect the polyphenol content of bilberry and blueberry fruit (Mikulic-Petkovsek, M., Schmitzer, V., Slatnar, A., Stampar, F., & Veberic, R., 2015; Spinardi, A., Cola, G., Gardana, C. S., & Mignani, I. 2019; Cerezo et al., 2020). Differences in varieties, harvest locations, and times were all beneficial to covering the highest possible variation in fruit quality.

The anthocyanins in cultivated blueberries and bilberries were galactosides, glucosides, and arabinosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin, and acylation with acetic acid was common, in line with previous studies (Bujor et al., 2016; Colak et al., 2017; Grace, Xiong, Esposito, Ehlenfeldt, & Lila, 2019; Lätti, Riihinen, & Kainulainen, 2008; Wang et al., 2019; Zoratti, Jaakola, Häggman, & Giongo, 2015). While, Wendelin, Korntheuer, Baumann, & Brandes (2018) previously reported that bilberries exclusively contained non-acylated anthocyanins, our study found low but consistent levels of acylated anthocyanins in all bilberry samples. Yet the average content of acylated forms was much higher in cultivated blueberries than in bilberries. Ienascu, Balcu, Segneanu, Cata, and Damian (2009) and Zoratti et al. (2015) have already reported the presence of acylated anthocyanins in bilberry. Significant variability in anthocyanin and chlorogenic acid content was observed, particularly in cultivated blueberries. Previous reports have highlighted substantial differences in anthocyanin content among various cultivated blueberry samples, linked to cultivar distinctions, genotypic variations, and environmental factors (Yang et al., 2022). Similar variations have been noted among different bilberry populations, influenced by both environmental factors and the genetic background of the populations (Lätti et al., 2008; Åkerström, Jaakola, Bång, & Jäderlund, 2010). Bilberry had a significantly higher average anthocyanin content and lower chlorogenic acid content than cultivated blueberries, and the determined levels were generally consistent with previous studies (Aksic et al., 2019; Bujor et al., 2016; Burdulis et al., 2009; Colak et al., 2017; Grégrová et al., 2021; Lätti et al., 2008; Pires et al., 2020; Spinardi, Cola, Gardana, & Mignani, 2019).

The condensed tannins were mixtures of procyanidins and prodelphinidins in most of the samples. Some previous studies have found only procyanidins in cultivated blueberries, namely, *V. corymbosum* (Gu et al., 2004; Wang et al., 2019), and in the current study, some blueberry cultivars also lacked prodelphinidins. However, the tannins in bilberry samples were always mixtures of procyanidins and prodelphinidins, agreeing with previous studies (Hellström, Törrönen, & Mattila, 2009; Suvanto, Karppinen, Riihinen, Jaakola, & Salminen, 2020). A similar

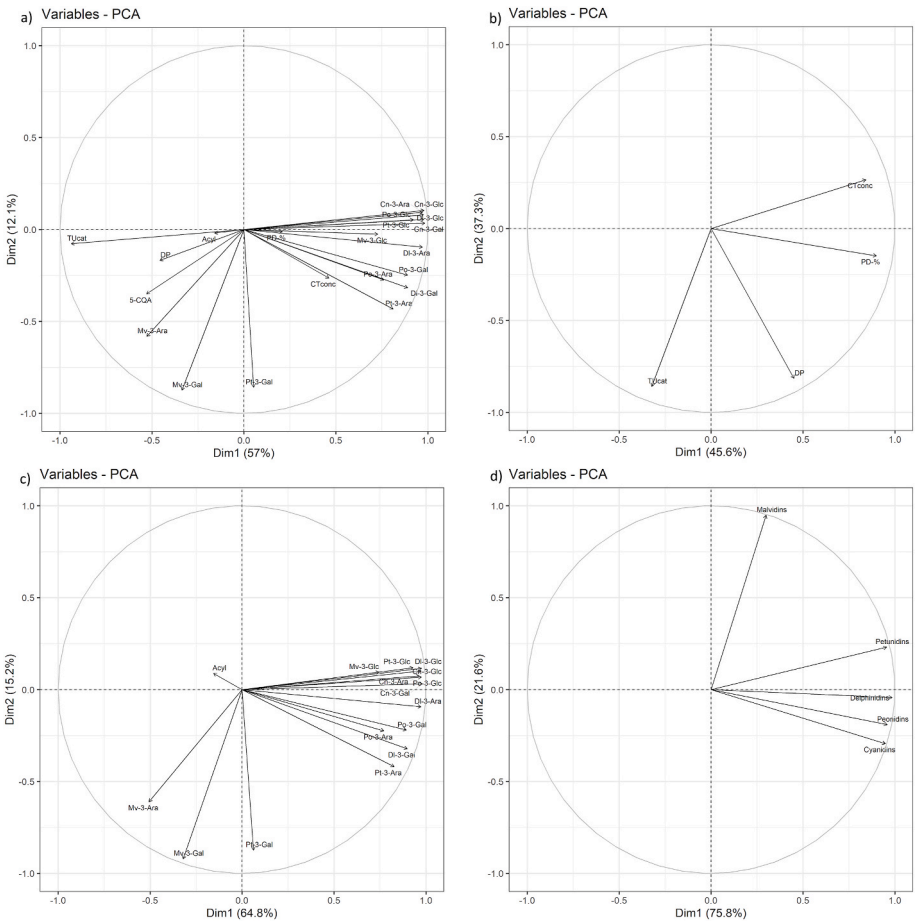

Fig. 2. Principal component analysis (PCA) loading plot of bilberries, domestic blueberries and imported blueberries: a) all 21 parameters, b) tannin parameters, c) anthocyanins, and d) anthocyanidins included in the analyses.

heterogenous tannin structure was also more common among cultivated blueberries. It should be noted that all the domestic blueberries studied were hybrid cultivars, while the cultivars of imported blueberries remained generally unknown, although many of them were likely hybrid cultivars too. Prodelphinidins have been detected in cultivated blueberry in the previous studies of Aksic et al. (2019), Kimura, Ogawa, Akihiro, and Yokota (2011), and Riihinen et al. (2008). The total content of CTs varied greatly in cultivated blueberries but was generally in good accordance with the literature (Gu et al., 2004; Wang et al., 2019). Bilberries had less variation in the CT content. The terminal unit was predominantly epicatechin in bilberry CTs, consistent with Bujor et al. (2016) while in blueberry CTs it was catechin, in line with Gu et al. (2003).

The results showed that cultivated blueberries and bilberries had characteristic differences in their phenolic profiles. This outcome aligns with expectations, considering that the biosynthesis of phenolics is genetically regulated, and thus differences in levels are expected between different species. PCA separated blueberries from bilberries, whether all the phenolic compounds were included or just either of the major groups, i.e., tannins or anthocyanins. Furthermore, the anthocyanin data could be compressed to aglycon level, and a distinctive separation between blueberries and bilberries was still achieved. From an analytical perspective, this could be an advantage, as the very complicated anthocyanin profiles of cultivated blueberries and bilberries could be simplified by applying acid hydrolysis to the extracts, resulting in only five anthocyanidins to be determined. In wild bilberries, the major anthocyanidins were delphinidin and cyanidin, while in cultivated blueberries, malvidin dominated, followed by delphinidin, in line with the recent study of Wang et al. (2022). Yet particular care should be taken if identification is based on aglycons, as some other common anthocyanin sources such as grape may have overlapping aglycon profiles with bilberries or blueberries (Filip, Vlassa, Copaciu, & Coman, 2012). No differences could be identified between the phenolics in domestic and imported blueberries.

Various analytical chemistry techniques have been developed to assess the authenticity of berries and berry-based foods, including DNA-based methods, electronic sensors, chromatography, and various spectroscopy methods (Ongkowijoyo, Luna-Vital, & Gonzalez de Mejia, 2018; Salo, Nguyen, Alakärppä, & Klavins, 2021; Traksele & Snitka, 2022). Liu, Hu, and Yan (2020) developed the HPLC fingerprint method to distinguish bilberry samples from other berries, including cultivated blueberries. They used similarity evaluation software to compare the anthocyanin profiles of different samples and could demonstrate significant differences between the species. Similarly, Grégrová et al. (2021) found differences in anthocyanin profiles between cultivated blueberries and wild bilberries, with bilberries having higher proportions of anthocyanidin-glucosides than blueberries. In the current study, the average proportion of anthocyanidin-glucosides was also significantly higher in bilberries than in cultivated blueberries. However, Zoratti et al. (2015) observed that the proportions of sugar moieties in bilberry anthocyanins depended on the growing site, and the proportion of glucosides in their study ranged between 30 and >60%, depending on the habitat.

The geographical origin of bilberries and blueberries has been successfully identified by Raman spectroscopy (Traksele & Snitka, 2022) and by analyzing the stable isotopic ratios of mineral elements (Klavins et al., 2021). The results from the current study indicate that HPLC-based chemical profiling according to phenolic compounds does

not necessarily provide sufficient information to identify the berries' geographical origin.

5. Conclusions

Cultivated blueberries have a highly diverse genetic background that induces variation in their chemical composition. It was found that imported blueberries could not be separated from domestic Finnish blueberries according to their phenolic profile, i.e., neither group had sufficiently distinctive features to indicate the geographical origin. The genetic background of wild bilberries is more uniform, and they show less variation in their chemical composition. It was evident that wild bilberries could be differentiated from cultivated blueberries based on their phenolic profiles. Distinguishable features were found in both major phenolic groups, anthocyanins and proanthocyanidins. The results showed that phenolic profiling could be a feasible way to detect bilberry adulterations. This approach proves particularly valuable in processed products, in which the original visual characteristics of the fruits may be absent. In a broader context, the chemical characterization of secondary metabolites appears to be a quite straightforward method for verifying the authenticity of plant products, and there is potential for its wider application in the future.

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CRediT authorship contribution statement

Jarkko Hellström: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Conceptualization. **Saila Karhu:** Writing – review & editing, Writing – original draft, Supervision, Resources. **Jouni Karhu:** Writing – review & editing, Software, Methodology, Formal analysis. **Eila Järvenpää:** Writing – review & editing, Resources, Conceptualization. **Anna-Liisa Välimaa:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116080.

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