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Triterpenoid Content of Berries and Leaves of Bilberry *Vaccinium myrtillus* from Finland and Poland

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Supporting Information

ABSTRACT: Triterpenoid compounds found in free and ester forms in extracts of entire fruits and leaves and in fruit and leaf cuticular waxes of bilberry (*Vaccinium myrtillus* L.) collected in Finland and Poland were identified and quantitated by gas chromatography−mass spectrometry coupled to a flame ionization detector (GC−MS/FID). The main bilberry triterpenoid profile consisted of α- and β-amyrin, α- and β-amyrenone, campesterol, cholesterol, citrostadienol (in berries), cycloartanol, erythrodiol, lupeol, 24-methylenecycloartanol, sitosterol, sitostanol, stigmasterol, stigmasta-3,5-dien-7-one, uvaol, oleanolic and ursolic aldehydes, and oleanolic, ursolic, 2α-hydroxyoleanolic, and 2α-hydroxyursolic acids. Friedelin and D:A-friedooleanan-3β-ol were found only in Finnish plants, whereas D:C-friedours-7-en-3β-ol and taraxasterol were found only in Polish plants. To our knowledge, this is the first thorough description of triterpenoid compounds in this species. The presented results revealed that the triterpenoid profile of bilberry varied considerably between different organs of the plant, regardless of the plant origin, as well as between plant samples obtained from the two geographical locations.

KEYWORDS: *triterpenoids, bilberry (Vaccinium myrtillus L.) fruit and leaf, cuticular waxes, GC−MS, oleanolic acid, ursolic acid, friedelin, taraxasterol*

INTRODUCTION

In recent years, edible berries have attracted significant research and public attention because of their possible beneficial effects on human health.[1,2] Both hydrophilic and lipophilic phytochemicals are found in abundance in these fruits, and thus, the complementary, additive, and/or synergistic effects resulting from the mixture of multiple bioactive compounds are believed to be responsible for the health benefits associated with a diet enriched in berries.[1]

Many wild berries are very popular because of the long tradition of their use as dietary resources with well-known nutritional and medicinal properties. Bilberry (*Vaccinium myrtillus* L.) and lingonberry (also known as cowberry, *Vaccinium vitis-idaea* L.), two of the most abundant noncultivated berries in countries of northern and central Europe and Russia,[3] are readily harvested from the wild[4] and highly valued for their taste and various culinary uses. Despite not being horticultural species thus far, both of these plants are of significant economic importance because of the use of their fruits and sometimes leaves in the production of various foods, pharmaceuticals, cosmetics, and health-care products. Bilberry has been used since the Middle Ages as an antidiabetic, astringent, and antiseptic agent and a treatment for diarrhea.[5] Crude bilberry fruit extracts are now marketed as pharmaceutical preparations for the treatment of ophtalmological diseases and blood vessel disorders.[6] Bilberry is one of the best natural sources of anthocyanins;[7,8] however, other interesting compounds, such as stilbenes[9] and iridoid glycosides,[10] are also found in its berries. The leaves of this plant, traditionally used as a folk medicine treatment for diabetes,[11] have recently been proposed as a potential source of phenolic compounds with many pro-health properties.[12] The less explored organs of bilberry, such as the stems and rhizomes, have also been found to contain phenolics with various biological activities.[13,14] Although the list of phytochemicals identified in bilberry is growing, there is still little available information regarding the triterpenoid content of this plant. These compounds are known to exert numerous biological effects and display various pharmacological activities, and they may contribute to the physiological benefits arising from the consumption of edible berries and other fruits.[15]

Being non-cultivated plants, wild bilberry and lingonberry have not been subjected to agrotechnical methods of selective breeding or plant protection. Because abiotic and biotic environmental stresses significantly modulate the phytochemical profiles of plants, wild berry fruits typically accumulate larger amounts of defensive phytochemicals of more complex chemical composition than their cultivated relatives.[16] Plants of the boreal forest understorey, such as bilberry and lingonberry, are influenced by many abiotic (e.g., light, temperature, growing season length, soil fertility and moisture, winter snow cover, and pollution) and biotic (including canopy composition, interactions with herbivores, neighboring plants, and microbial pathogens or allies) factors. This complex set of external stimuli is regarded as a possible reason for the "chemodiversity" observed among plants of the same species growing in different geoclimates. Indeed, it has been demonstrated that the variation in the content and composition of anthocyanins and other phenolic compounds in bilberry fruits[8,17−20] and leaves[21,22] can

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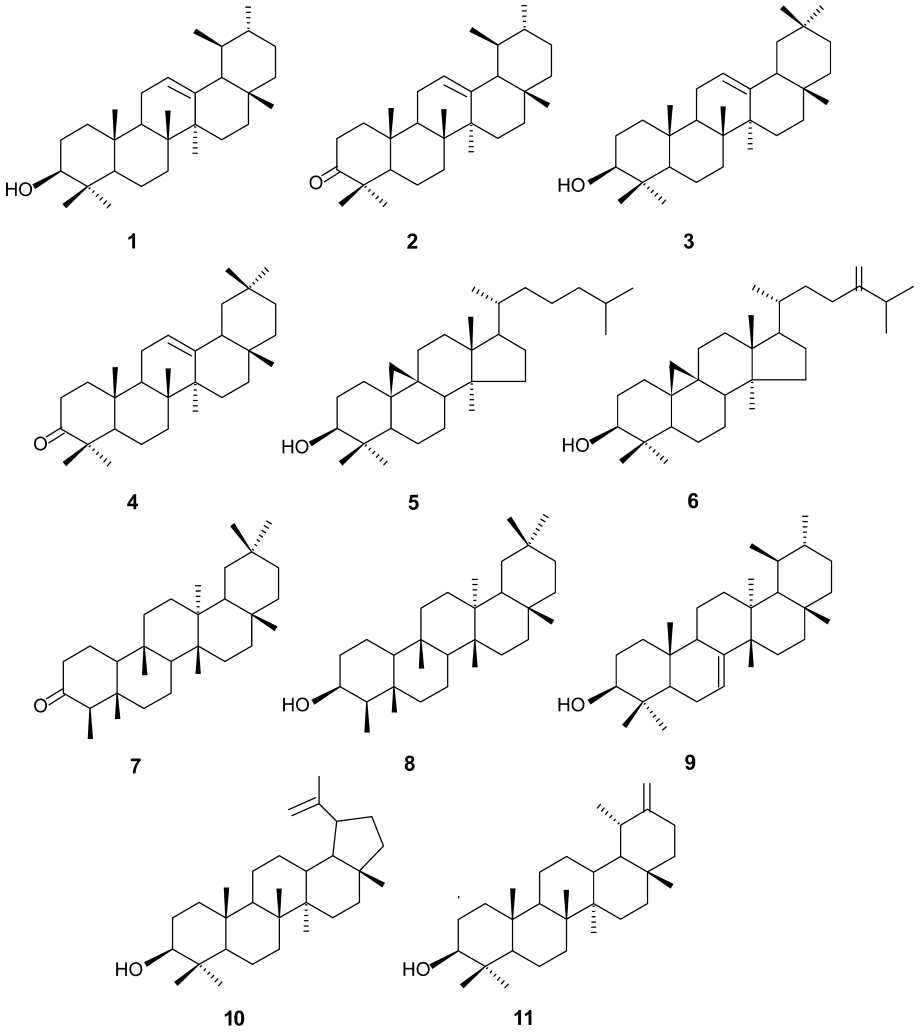


Figure 1. Chemical structures of triterpene monohydroxyalcohols and ketones found in bilberry fruits and leaves: α-amyrin (**1**), α-amyrenone (**2**), β-amyrin (**3**), β-amyrenone (**4**), cycloartanol (**5**), 24-methylenecycloartanol (**6**), friedelin (**7**), D:A-friedooleanan-3β-ol (**8**), D:C-friedours-7-en-3β-ol (**9**), lupeol (**10**), and taraxasterol (**11**).

be very high, thus influencing the antioxidant capacity of the plant material.

Previously, we examined the triterpenoid profile of the fruits and leaves of lingonberry and found differences in the chemical composition and levels of individual compounds in plants harvested in Finland and Poland.[23] The aim of the present study was to identify the main triterpenoids occurring in bilberry fruits and leaves and to compare the occurrence and abundance of these compounds in plants from distinct geographical sites. Moreover, because triterpenoids in their free and esterified forms are supposed to occur in particularly high concentrations in plant surface cuticles, qualitative and quantitative analyses of these compounds occurring in bilberry fruit and leaf cuticular waxes were also conducted.

MATERIALS AND METHODS

Plant Material. Bilberry fruits and leaves were collected from typical natural forest habitats in northern Ostrobothnia in Finland (65° 066 N, 25° 458 E) and the central Mazovia region in Poland (52° 455 N, 21° 332 E) in early (Poland) and late (Finland) July 2011, i.e., in the middle of the bilberry fruit harvest season in both locations. Because of the difference in latitude, the local geoclimates of the two habitats differ, e.g., in the length of the vegetative season (150 days in Finland and 210 days in Poland), the photoperiod (daylight duration, in June, 22 h and 2 min and 16 h and 47 min; in December, 3 h and 35 min and 7 h and 42 min, in Finland and Poland, respectively), average annual temperature (+2.4 °C in Finland and +7.5 °C in Poland), average annual precipitation (518 mm in Finland and 550 mm in Poland), and snow cover duration (161 days in Finland and 75 days in Poland; however, some winters in this region of Poland happen to be very cold but without snow). The Finnish habitat is a natural *V. myrtillus*-type forest dominated by Scots pine (*Pinus sylvestris*) and some Norway spruce (*Picea abies*), with *V. myrtillus*, *V. vitis-idaea*, and *Rhododendron tomentosum* as the main plants of the understorey. The Polish habitat is a dry forest dominated by Scots pine and some silver birch (*Betula pendula*), with *Juniperus communis*, *V. myrtillus*, *V. vitis-idaea*, and mosses in the undergrowth. Both areas are characterized by nutrient-poor mineral soils. The mean pH values of the forest humus layer vary between 3.9 and 4.1. In both forests, three different collection sites were chosen, placed 250 m apart in an area of about 1 ha. Bilberry forms clonal colonies (genets), being groups of genetically identical individuals (ramets), all originating vegetatively from a single ancestor and connected by rhizomes. Therefore, at every site, 10 ramets were randomly selected within a minimum distance of 10 m of each other to ensure that samples would be collected from different genets and transported to the laboratory, where fresh, green, healthy leaves were randomly detached from the whole plants, mixed, and weighted. Berries were collected directly from the plants in the forest. The replicate 2 g samples of both berries and leaves were prepared from different pooled sample sets of 10−15 g. The Finnish samples

were allowed to dry at room temperature in paper bags and were then sent to the Laboratory of Plant Biochemistry in Warsaw for analysis. Finnish and Polish voucher specimens were deposited, respectively, in the herbaria of the University of Oulu (accession number OULU 10004653) and the University of Warsaw (accession number WA 0000027907).

Chemicals and Standards. All solvents used for extraction and analysis were of analytical grade. Authentic standards were purchased from the following suppliers: α-amyrin and ursolic acid methyl ester from Roth (Karlsruhe, Germany), α-tocopherol from Merck (Darmstadt, Germany), and β-amyrin, lupeol, uvaol, oleanolic acid, campesterol, cholesterol, diosgenin, sitosterol, and stigmasterol from Sigma-Aldrich (Steinheim, Germany). Faradiol, used for total recovery evaluation, was extracted from the flowers of marigold (*Calendula officinalis* L.) in the Laboratory of Plant Biochemistry, University of Warsaw. α-Amyrenone and β-amyrenone were obtained by oxidation of the α-amyrin and β-amyrin standards with chromium trioxide–pyridine in dichloromethane.[24]

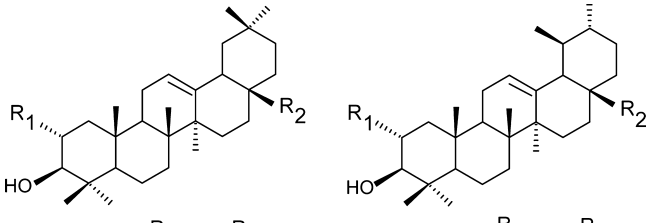
Extraction and Fractionation of Entire Plant Organs. Fruit and leaf samples (2 g, three replicates each) were dried at 60 °C, powdered in a grinding mortar, and extracted with diethyl ether (100 mL) for 8 h in a small Soxhlet apparatus. Extracts were evaporated to dryness at 40 °C under reduced pressure (extract masses were 14–16 and 35–42 mg for fruits and leaves, respectively), and separated by preparative thin-layer chromatography (TLC) on 20 × 20 cm glass plates coated with a 0.25 mm layer of silica gel 60G (Merck) in the solvent system $CHCl_3$/MeOH (97:3, v/v) into three fractions: (i) free (non-esterified) steroids and neutral triterpenes (alcohols, aldehydes, and ketones), (ii) triterpene acids, and (iii) esters. The individual fractions were localized on plates by comparison to standards of oleanolic acid, sitosterol, and α-amyrin and visualized by spraying the relevant part of the plate with 50% H_2SO_4, followed by heating with a hot-air stream. Fractions were eluted from the gel in diethyl ether with at least 10 volumes of the solvent compared to the volume of the scrapped gel. The fractions containing free neutral triterpenes and steroids (R_F of 0.3–0.9) were analyzed directly by gas chromatography–mass spectrometry (GC–MS). The fractions containing triterpene acids (R_F of 0.2–0.3) were first methylated with diazomethane. The fractions containing triterpenoid (triterpene and sterol) esters (R_F of 0.9–1) were subjected to alkaline hydrolysis. The average recovery of α-amyrin, uvaol, stigmasterol, and ursolic acid methyl ester from preparative TLC plates was 98.6, 97.2, 98.9, and 96.1%, respectively.

Extraction and Fractionation of Cuticular Waxes. Fruit and leaf samples (2 g, three replicates each) were extracted by dipping them in 25 mL (fruits) or 40 mL (leaves) of chloroform for 30 s at room temperature. The extracts were decanted and evaporated to dryness under a gentle stream of nitrogen. After weighing, the obtained wax extracts (masses of 1.3–1.6 and 14.3–15.3 mg for cuticular waxes from fruits and leaves, respectively) were separated by preparative TLC and processed as described above for extracts from entire plant organs.

Methylation of Triterpene Acids. Nitrosomethylurea (2.06 g) was added to a mixture of 20 mL of diethyl ether and 6 mL of 25% aqueous KOH. The organic layer was washed with water (3 × 50 mL) and separated from the aqueous layer. Samples containing triterpene acids (not more than 10 mg) were dissolved in 2 mL of the obtained solution of diazomethane in diethyl ether and held at 2 °C for 24 h.

Alkaline Hydrolysis. The ester fraction was subjected to alkaline hydrolysis with 10% NaOH in 80% MeOH at 80 °C for 3 h. Subsequently, 5 volumes of water were added to each hydrolysate. The pH was neutralized with 5% CH_3COOH. The obtained mixtures were extracted with diethyl ether (3 × 10 mL). These extracts were fractionated by preparative TLC as described above. Fractions containing free triterpene alcohols and sterols were directly analyzed by GC–MS, while triterpene acid fractions were methylated prior to this analysis.

Identification and Quantification of Triterpenoids by GC–MS/Flame Ionization Detector (FID). An Agilent Technologies 7890A gas chromatograph was used for qualitative (a 5975C mass spectrometric detector) and quantitative (a FID) analyses. Samples dissolved in a 5:1 diethyl ether/methanol mixture were applied (in a volume of 1–4 μL) by split injection 1:10. All samples were analyzed in triplicate. The column was a 30 m × 0.25 mm inner diameter, 0.25 μm, HP-5MS (Agilent Technologies). Helium was used as a carrier gas at a flow rate of 1 mL/min. The following parameters were employed: column temperature, 280 °C; inlet and FID temperature, 290 °C; MS transfer line temperature, 275 °C; quadrupole temperature, 150 °C; ion source temperature, 230 °C; electron impact (EI), 70 eV; m/z range, 33–500; FID gas (H_2) flow, 30 mL/min (hydrogen generator); and air flow, 400 mL/min. Individual compounds (presented in Figures 1–3) were identified by comparing their mass spectra to library data from Wiley 9th ed. and NIST 2008 Library SW (version 2010) or data from the literature and by comparison of their retention times and corresponding mass spectra to those of authentic standards, where available. Quantitation was performed with the use of an external standard method based on calibration curves prepared for typical representatives of each triterpenoid group: α-amyrin, uvaol, oleanolic acid methyl ester, and sitosterol. The total recovery of a known quantity (1 mg) of the triterpene dihydroxyalcohol faradiol (not present in bilberry, with a retention time of 40.0 min), added as an internal standard to the control dried samples of Polish berries prior to their extraction, was 96%.



	R_1	R_2		R_1	R_2
12	H	CH_2OH	**13**	H	CH_2OH
14	H	CHO	**15**	H	CHO
16	H	COOH	**17**	H	COOH
18	OH	COOH	**19**	OH	COOH

Figure 2. Chemical structures of triterpene dihydroxyalcohols, aldehydes, and acids found in bilberry fruits and leaves: erythrodiol (**12**), uvaol (**13**), oleanolic aldehyde (**14**), ursolic aldehyde (**15**), oleanolic acid (**16**), ursolic acid (**17**), 2α-hydroxyoleanolic acid (**18**), and 2α-hydroxyursolic acid (**19**).

Separation by High-Performance Liquid Chromatography (HPLC). Chromatographic analysis of samples containing neutral triterpenes, including the mixtures of α-amyrin and lupeol and α-tocopherol and cholesterol, was performed with a Shimadzu (Japan) chromatographic system equipped with two LC-10AT pumps, a CTO-10AS oven, and a SPD-10A spectrophotometer set at 200 and 254 nm. The column was a 250 × 4.6 mm inner diameter, 4 μm, Synergi MAX-RP 80A (Phenomenex, Torrance, CA). All data were acquired and processed with Shimadzu CLASS-VP (version 5.032) chromatography data system software. The mobile phase (flow rate of 0.6 mL/min) was 100% acetonitrile (isocratic system), and separation was performed at 30 °C.

Statistical Analysis of Data. All data are presented as the mean ± standard deviation of three independent samples of Finnish or Polish plant material analyzed in triplicate. The data were subjected to one-way analysis of variance (ANOVA), and the differences between means were evaluated using the Duncan's multiple-range test. Statistical significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Triterpenoid Profile. Diethyl ether extracts of bilberry fruits and leaves collected in Finland and Poland were separated into three fractions by preparative TLC. Fractions containing

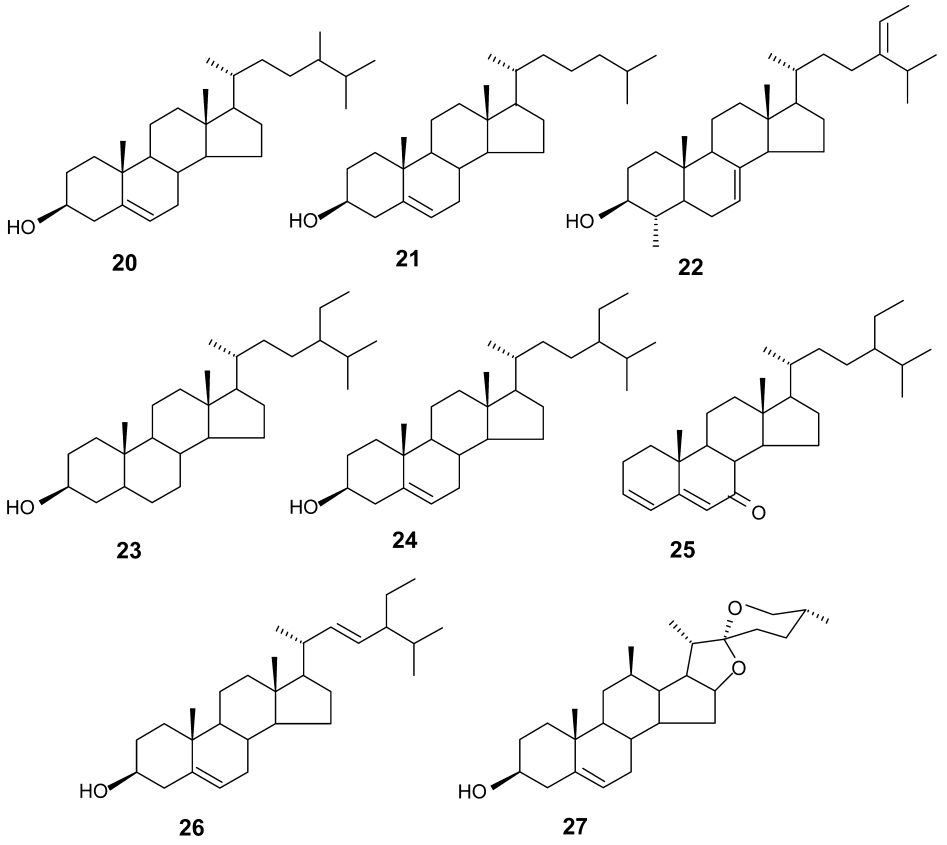


Figure 3. Chemical structures of steroids found in bilberry fruits and leaves: campesterol (**20**), cholesterol (**21**), citrostadienol (**22**), sitostanol (**23**), sitosterol (**24**), stigmasta-3,5-dien-7-one (**25**), and stigmasterol (**26**). Diosgenin (**27**) was identified in cuticular waxes from one sample of Finnish fruits.

free (non-esterified) steroids and neutral triterpenes (alcohols, aldehydes, and ketones), triterpene acids after methylation, and triterpenoids released from their hydrolyzed esters were individually subjected to GC–MS/FID analysis. The fractions containing free steroids and neutral triterpenes, expected to be particularly rich in various triterpenoids as in lingonberry,[23] were analyzed first. Representative GC–FID chromatograms are shown in Figure 4. In the fractions obtained from extracts of Finnish and Polish fruits (chromatograms A and B, respectively), the principal peaks associated with triterpenoid compounds were those with a retention time (t_R) of 17.3 and 18.6 min (compounds **24** and **3**), which had mass spectra of sitosterol and β-amyrin, respectively. As was observed in GC–FID chromatograms obtained previously for lingonberry,[23] a peak with a t_R of 20.2 min (compounds **1** + **10**) was associated with a mixture of two triterpene alcohols, α-amyrin and lupeol, and these compounds could be separated by additional HPLC analysis to permit quantitative analysis based on the relative ratios of peak areas. Smaller peaks were identified as campesterol (**20**), stigmasterol (**26**), sitostanol (**23**), β-amyrenone (**4**), cycloartanol (**5**), α-amyrenone (**2**), stigmasta-3,5-dien-7-one (**25**), 24-methylenecycloartanol (**6**), D:A-friedooleanan-3β-ol (i.e., friedelinol) (**8**) (only in chromatogram A), friedelin (**7**) (only in chromatogram A), D:C-friedours-7-en-3β-ol (**9**) (only in chromatogram B), and taraxasterol (**11**) (only in chromatogram B). The peak with a t_R of 31.0 min (compound **22**) was tentatively identified as citrostadienol by comparison of its retention time and mass spectrum to those available in the literature.[25] The peaks with a t_R of 27.3 and 30.4 min (compounds **16** and **17**) were identified as naturally occurring methyl esters of oleanolic and ursolic acids, respectively. The identification of α- and β-amyrenones was confirmed by a comparison of their retention times and mass spectra to those of compounds obtained by oxidation of the authentic α- and β-amyrin standards. The peak with a t_R of 12.0 min (compound **21**), seen previously in chromatograms of lingonberry fruit extract fractions and unambigouously identified as α-tocopherol,[23] was associated with a mixture of α-tocopherol and cholesterol in chromatograms of bilberry fruit extract fractions. The identification of this peak as a mixture of α-tocopherol and cholesterol was confirmed by GC–MS and HPLC analyses of their authentic standards, examined separately or combined together. However, because of the incomplete separation and different ultraviolet (UV) absorption properties of these compounds, their relative ratio was difficult to establish reliably, and therefore, cholesterol was not included in further quantitative determinations. The well-distinguished peak with a t_R of 40.0 min (IS, chromatogram B) was associated with faradiol, a taraxastane-type tritepene dihydroxyalcohol not occurring in bilberry, that was added to dried Polish berries prior to their extraction and used as an internal standard to determine the recovery. Other peaks that have not been numbered were in most cases identified as aliphatic compounds; for example, the peak with a t_R of 13 min, one of the most significant on the chromatogram of the fraction from Finnish fruits, whereas very small on that from Polish fruits, was associated with long-chain aliphatic diketone, tricosane-2,4-dione.

The three principal peaks detected in chromatograms of leaf extract fractions containing steroids and neutral triterpenes

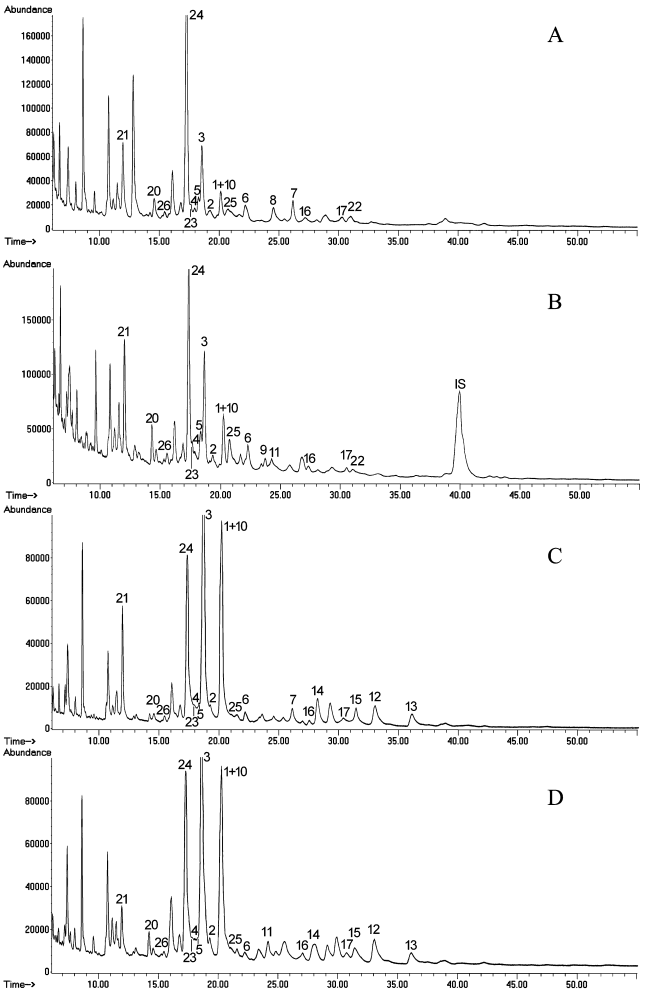


Figure 4. GC−FID chromatograms of the fractions containing sterols and neutral triterpenes (alcohols, aldehydes, and ketones) obtained from diethyl ether extracts of bilberry fruits (A, Finnish; B, Polish) and leaves (C, Finnish; D, Polish). Peaks are numbered according to Figures 1−3. Compound **16** is associated with naturally occurring oleanolic acid methyl ester. Compound **17** is associated with naturally occurring ursolic acid methyl ester. Faradiol (IS) was used as an internal standard.

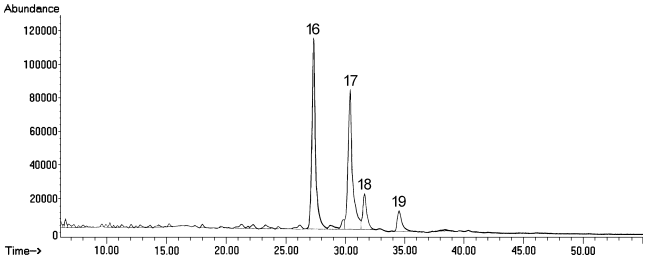


Figure 5. GC−FID chromatogram of the fraction containing methyl esters of triterpene acids from fruits of Polish bilberry. Peaks are numbered according to Figure 2.

(Figure 4, chromatograms C and D for Finnish and Polish samples, respectively) were those of sitosterol (**24**) (t_R of 17.3 min), β-amyrin (**3**) (t_R of 18.6 min), and α-amyrin/lupeol (**1** + **10**) (t_R of 20.3 min). Smaller peaks were associated with several triterpenoids identified in berries, i.e., campesterol (**20**), stigmasterol (**25**), sitostanol (**23**), β-amyrenone (**4**), cycloartanol (**5**), α-amyrenone (**2**), stigmasta-3,5-dien-7-one (**26**), 24-methylenecycloartanol (**6**), friedelin (**7**) (only in chromatogram C), taraxasterol (**11**) (only in chromatogram D), and naturally occurring oleanolic and ursolic acid methyl esters (**16** and **17**), and with some triterpenes not identified in berries, i.e., oleanolic aldehyde (**14**), ursolic aldehyde (**15**), erythrodiol (**12**), and uvaol (**13**). As in chromatograms of fruit extract fractions, the compound **21** (t_R of 12.0 min) was associated with the mixture of α-tocopherol and cholesterol.

Representative GC−FID chromatogram of the fraction containing methyl esters of triterpene acids is shown in Figure 5. Besides oleanolic and ursolic acid methyl esters (**16** and **17**), which, as in lingonberry,[23] were identified in all extracts, another pair of peaks (compounds **18** and **19**), with respective t_R of 31.7 and 34.5 min, were detected, being particularly significant in the extract from Polish leaf samples. These peaks were identified as 2α-hydroxyoleanolic acid (2α,3β-dihydroxyolean-12-en-28-oic acid, **18**), and 2α-hydroxyursolic acid (2α,3β-dihydroxyursan-12-en-28-oic acid, **19**). The major peaks obtained for the hydrolyzed triterpenoid esters were associated with sterols in fruit extracts and amyrins in leaf extracts (chromatograms not shown).

Thus, according to GC−MS analysis, the main triterpenoid profile of bilberry has the following composition: (i) four triterpene acids, oleanolic, ursolic, 2α-hydroxyoleanolic, and 2α-hydroxyursolic, (ii) several triterpene monohydroxyalcohols, α-amyrin, β-amyrin, cycloartanol, 24-methylenecycloartanol, and lupeol, occurring in plants from both locations, D:A-friedooleanan-3β-ol found only in Finnish berries, D:C-friedours-7-en-3β-ol found only in Polish berries, and taraxasterol found only in Polish berries and leaves, (iii) two triterpene dihydroxyalcohols, erythrodiol and uvaol, found only in leaves, (iv) two triterpene aldehydes, oleanolic and ursolic, also found only in leaves, (v) three triterpene ketones, α-amyrenone and β-amyrenone found in plants from both locations and friedelin found only in Finnish plants, and (vi) several steroids, campesterol, cholesterol, sitosterol, sitostanol, stigmasterol, stigmasta-3,5-dien-7-one, and citrostadienol, with the latter found only in berries.

The chemical structures of these compounds are shown in Figures 1−3. To our knowledge, this is the first thorough description of the triterpenoid profile of bilberry.

Comparison of the triterpenoid profiles of berries and leaves of bilberry revealed significant differences. The two basic oleanane and ursane monohydroxyalcohols, α- and β-amyrin, and the ketones, α- and β-amyrenone, were found in both berries and leaves, whereas some other consecutive derivatives, namely, the dihydroxyalcohols, erythrodiol and uvaol, and the aldehydes, oleanolic and ursolic, were detected only in leaves. The mono- and dihydroxyacids were found in both berries and leaves. Citrostadienol, detected previously in bilberry seeds,[25] was found exclusively in berries. These data revealed that the triterpenoid profile of bilberry varies considerably in different organs of the plant. Moreover, considerable variation was apparent in the triterpenoid profile of plant samples obtained from the two geographical locations. D:A-friedooleanan-3β-ol was detected exclusively in Finnish berries, and friedelin was detected in both Finnish berries and leaves. D:C-friedours-7-en-3β-ol was found only in Polish berries, and taraxasterol was found in both Polish berries and leaves.

Triterpenoid Content of Bilberry Fruits. The results of quantitative determination of individual triterpenoids identified in Finnish and Polish bilberry fruits are presented in Table 1. The total triterpenoid content was similar in fruits originating

Table 1. Contents of Triterpenoids in Fruits of Bilberry Collected in Finland and Poland in July 2011[a]

compound	Finnish berries (μg/g of dry weight ± SD)	Polish berries (μg/g of dry weight ± SD)
	Free	
α-amyrin (**1**)[b]	64.7 ± 3.4 a	81.1 ± 4.1 b
α-amyrenone (**2**)	13.4 ± 0.9 a	12.6 ± 0.6 a
β-amyrin (**3**)	133.1 ± 5.5 a	122.5 ± 7.3 a
β-amyrenone (**4**)	30.9 ± 2.6 a	28.9 ± 1.7 a
cycloartanol (**5**)	38.6 ± 2.2 a	32.8 ± 1.6 b
24-methylenecycloartanol (**6**)	35.8 ± 2.1 a	45.5 ± 2.7 b
friedelin (**7**)	66.1 ± 2.8	nd
friedelinol (**8**)	29.2 ± 1.6	nd
friedoursenol (**9**)	nd	59.4 ± 4.1
lupeol (**10**)[b]	2.2 ± 0.1 a	10.1 ± 0.5 b
taraxasterol (**11**)	nd	20.7 ± 1.2
sum of neutral triterpenes	414.0	413.6
oleanolic acid (**16**)	1679.2 ± 67.9 a	2029.6 ± 101.2 b
ursolic acid (**17**)	1353.3 ± 49.7 a	1420.3 ± 85.2 a
hydroxyoleanolic acid (**18**)	21.9 ± 1.3 a	36.4 ± 2.1 b
hydroxyursolic acid (**19**)	15.8 ± 0.7 a	25.1 ± 1.2 b
sum of triterpene acids	3070.2	3511.4
campesterol (**20**)	58.3 ± 4.1 a	42.1 ± 2.1 b
citrostadienol (**22**)[c]	25.2 ± 1.4 a	10.6 ± 0.4 b
sitostanol (**23**)	62.7 ± 3.6 a	20.9 ± 1.0 b
sitosterol (**24**)	882.3 ± 46.8 a	755.7 ± 37.8 b
stigmastadienone (**25**)	30.1 ± 1.4 a	31.5 ± 1.8 a
stigmasterol (**26**)	14.2 ± 0.7 a	19.1 ± 0.9 b
sum of steroids	1072.8	879.9
	Esters	
α-amyrin	5.6 ± 0.4 a	1.7 ± 0.1 b
β-amyrin	7.8 ± 0.5 a	2.4 ± 0.1 b
24-methylenecycloartanol	11.6 ± 0.6 a	9.5 ± 0.4 b
oleanolic acid	18.3 ± 1.1 a	26.9 ± 1.6 b
ursolic acid	12.8 ± 0.7 a	16.2 ± 0.8 b
campesterol	3.8 ± 0.2	nd
sitosterol	61.1 ± 4.1 a	10.8 ± 0.7 b
stigmasterol	10.9 ± 0.6 a	1.1 ± 0.1 b
sum of esters	131.9	68.6
total	4688.9	4873.5

[a]Results are referenced to fruit dry weight and expressed as the mean ± SD of three independent samples analyzed in triplicate. Results in rows not sharing a common letter are significantly different ($p < 0.05$). [b]Calculation based on the relative ratios of HPLC peak areas. [c]Tentative identification.

from both countries (differing by 4%) and accounted for approximately 4.7 and 4.9 mg/g of dry fruit weight of Finnish and Polish berries, respectively. As in lingonberry fruits,[23] the two isomeric acids, oleanolic and ursolic, were the most abundant compounds, constituting 65 and 70% of all triterpenoids in Finnish and Polish berries, respectively. Moreover, considerable amounts of another pair of isomers, 2α-hydroxyoleanolic and 2α-hydroxyursolic acids, were found in bilberry fruits from both locations. The total level of triterpene acids was higher (by 13%) in Polish berries than in Finnish berries, with respective oleanolic/ursolic acid ratios of 1:0.7 and 1:0.8. Oleanolic acid was the principal triterpene compound in bilberry fruit, which makes it unique among other edible berries of the genus *Vaccinium*. Previous studies have shown that ursolic acid was the predominant isomer in lingonberry *V. vitis-idaea* and also *Vaccinium macrocarpon*, *Vaccinium oxycoccus*, and *Vaccinium corymbosum*.[23,26,27]

The total content of the neutral triterpenes was strikingly similar in Finnish and Polish berries, with β-amyrin being the dominant compound. However, regardless of this quantitative similarity, significant qualitative differences were observed in the detailed profiles of this fraction. Friedelin and D:A-friedooleanan-3β-ol, together comprising 23% of neutral triterpenes, were found only in Finnish fruits, whereas taraxasterol and D:C-friedours-7-en-3β-ol (19% of the neutral triterpenes) were detected exclusively in Polish berries.

The total content of steroids was markedly higher (by 18%) in Finnish berries than in Polish berries. Sitosterol was the dominant compound among phytosterols (82 and 86% of the steroid fraction in Finnish and Polish fruits, respectively). Triterpenoid esters were present in Finnish berries in a total amount almost twice those found in Polish berries. The levels of sitosterol and stigmasterol esters were considerably higher (almost 6 and 10 times, respectively) in Finnish fruits than in Polish fruits, whereas esters of oleanolic and ursolic acids were more abundant (almost twice) in the latter.

Numerous *in vitro* studies have investigated the role of various bioactive constituents, mainly phenolics, in the well-known pharmacological effects of bilberry. However, the isolated compounds often failed to exhibit the same activities as whole fruit extracts.[5,6] These findings support the hypothesis of synergism of different phytochemicals and suggest that, at

Table 2. Contents of Triterpenoids in Leaves of Bilberry Collected in Finland and Poland in July 2011[a]

compound	Finnish plants (μg/g of dry weight ± SD)	Polish plants (μg/g of dry weight ± SD)
	Free	
α-amyrin (**1**)[b]	711.9 ± 35.5 a	631.4 ± 37.9 b
α-amyrenone (**2**)	11.7 ± 0.4 a	22.2 ± 1.1 b
β-amyrin (**3**)	919.1 ± 39.7 a	987.2 ± 49.4 a
β-amyrenone (**4**)	15.4 ± 0.7 a	34.7 ± 1.7 b
cycloartanol (**5**)	20.3 ± 1.1 a	22.8 ± 0.9 a
24-methylenecycloartanol (**6**)	12.1 ± 0.6 a	12.7 ± 0.6 a
erythrodiol (**12**)	46.7 ± 2.6 a	54.7 ± 3.3 b
friedelin (**7**)	23.3 ± 1.2	nd
lupeol (**10**)[b]	24.6 ± 0.9 a	63.4 ± 2.5 b
oleanolic aldehyde (**14**)	75.2 ± 3.1 a	51.9 ± 2.6 b
taraxasterol (**11**)	nd	37.9 ± 2.2
ursolic aldehyde (**15**)	46.1 ± 2.8 a	33.2 ± 1.3 b
uvaol (**13**)	28.6 ± 1.5 a	36.8 ± 2.1 b
sum of neutral triterpenes	1935.0	1988.9
oleanolic acid (**16**)	853.2 ± 51.2 a	873.6 ± 48.4 a
ursolic acid (**17**)	747.7 ± 44.8 a	776.2 ± 47.5 a
hydroxyoleanolic acid (**18**)	50.1 ± 2.5 a	164.7 ± 107.9 b
hydroxyursolic acid (**19**)	33.9 ± 2.0 a	115.8 ± 68.9 b
sum of triterpene acids	1684.9	1930.3
campesterol (**20**)	11.1 ± 0.4 a	16.7 ± 0.8 b
sitostanol (**23**)	6.9 ± 0.3 a	8.2 ± 0.5 b
sitosterol (**24**)	610.9 ± 36.7 a	671.4 ± 40.3 a
stigmastadienone (**25**)	38.3 ± 2.2 a	20.8 ± 1.2 b
stigmasterol (**26**)	5.4 ± 0.3 a	5.2 ± 0.3 a
sum of steroids	672.6	722.3
	Esters	
α-amyrin	30.2 ± 1.8 a	18.3 ± 1.1 b
β-amyrin	44.3 ± 1.7 a	20.5 ± 1.0 b
cycloartanol	2.5 ± 0.1 a	0.8 ± 0.1 b
24-methylenecycloartanol	19.1 ± 0.7 a	4.9 ± 0.2 b
oleanolic acid	5.8 ± 0.2 a	9.7 ± 0.5 b
ursolic acid	4.9 ± 0.2 a	8.1 ± 0.5 b
sitosterol	6.2 ± 0.3 a	9.8 ± 0.4 b
stigmasterol	3.1 ± 0.2 a	5.8 ± 0.3 b
sum of esters	116.1	77.9
total	4408.6	4719.4

[a]Results are referenced to leaf dry weight and expressed as the mean ± SD of three independent samples analyzed in triplicate. Results in rows not sharing a common letter are significantly different ($p < 0.05$). [b]Calculation based on the relative ratios of HPLC peak areas.

least in some cases, triterpenoids could be the missing link in the chain of various compounds exerting specific activities, which produce the total effect of complex extracts obtained from entire fruits.

The possible synergism was suggested for phenolics and triterpenoids from cranberry *V. macrocarpon*,[28] and it might be likely in the case of other berries possessing such a combination of bioactive phytochemicals, including other members of the genus *Vaccinium* (family Ericaceae), i.e., highbush blueberry *V. corymbosum*[29] and rabbiteye blueberry *Vaccinium ashei*,[30] as well as some representatives of other plant families, e.g., sea buckthorn *Hippophae rhamnoides* of Elaeagnaceae.[31] In cranberries, the main triterpenoid was ursolic acid found in the free form as well as *cis*- and *trans*-*p*-hydroxycinnamate esters, and the inhibition of the growth of several types of tumor cells (including breast, colon, prostate, lung, cervical epidermoid, and leukemia cell lines) was demonstrated for these compounds.[26−28,32] The occurrence of ursolic acid, α- and β-amyrin, erythrodiol, uvaol, lupeol, betulin, β-sitosterol, and β-sitosterol 3-*O*-β-glucopyranoside in rabbiteye blueberry extracts exerting significant cytotoxic activity against human lung and colon cancer cells was described.[30] Ursolic acid accompanied by its 19-hydroxy derivative, pomolic acid (3β,19α-dihydroxy-urs-12-en-28-oic acid), and β-sitosterol 3-*O*-β-glucopyranoside was found in highbush blueberry extracts reported to inhibit proliferation of the leukemia cell line.[29]

Triterpenoid Content of Bilberry Leaves. The results of quantitative determination of individual triterpenoids identified in bilberry leaves collected in Finland and Poland are presented in Table 2. The total content of triterpenoids in Finnish and Polish samples accounted for approximately 4.4 and 4.7 mg/g of dry leaf weight, which was slightly lower (by 6 and 4%) than the total triterpenoid content of the respective berries. Different from what was observed in berries, the neutral triterpenes (and not the triterpene acids) were the predominant fraction, constituing 44 and 42% of all triterpenoids in leaves of Finnish and Polish plants, respectively, with β-amyrin as the most abundant compound. Oleanane- and ursane-type triterpenes were found in considerable amounts in leaves of plants from both locations. Generally, within each class of triterpene

Table 3. Contents of Triterpenoids in Cuticular Waxes of Bilberry Fruits Collected in Finland and Poland in July 2011[a]

compound	Finnish berries (μg/mg of wax extract)	Polish berries (μg/mg of wax extract)
	Free	
α-amyrin (**1**)[b]	5.5 ± 0.2 a	9.1 ± 0.8 b
α-amyrenone (**2**)	2.6 ± 0.1 a	2.2 ± 0.1 b
β-amyrin (**3**)	10.4 ± 1.2 a	9.0 ± 0.8 a
β-amyrenone (**4**)	3.8 ± 0.1 a	3.6 ± 0.2 a
cycloartanol (**5**)	5.6 ± 0.1 a	3.8 ± 0.2 b
24-methylene-cycloartanol (**6**)	4.2 ± 0.2 a	4.0 ± 0.2 a
friedelin (**7**)	4.1 ± 0.2	nd
friedelinol (**8**)	3.1 ± 0.2	nd
friedours-7-en-3-ol (**9**)	nd	4.8 ± 0.8
lupeol (**10**)[b]	0.3 ± 0.1 a	0.5 ± 0.1 a
taraxasterol (**11**)	nd	4.0 ± 0.3
sum of neutral triterpenes	39.6	41.0
oleanolic acid (**16**)	88.9 ± 3.6 a	109.1 ± 7.5 b
ursolic acid (**17**)	68.8 ± 5.2 a	84.7 ± 5.5 b
hydroxyoleanolic acid (**18**)	1.2 ± 0.1 a	2.2 ± 0.2 b
hydroxyursolic acid (**19**)	0.7 ± 0.1 a	1.7 ± 0.1 b
sum of triterpene acids	159.6	197.7
campesterol (**20**)	6.2 ± 0.2 a	4.1 ± 0.2 b
sitostanol (**23**)	4.8 ± 0.1 a	3.6 ± 0.2 b
sitosterol (**24**)	53.1 ± 2.3 a	42.9 ± 2.7 b
stigmasta-3,5-dien-7-one (**25**)	15.5 ± 1.3 a	9.2 ± 1.0 b
stigmasterol (**26**)	3.9 ± 0.2 a	2.7 ± 0.1 b
sum of steroids	83.5	62.5
	Esters	
α-amyrin	0.5 ± 0.1 a	0.2 ± 0.1 b
β-amyrin	0.8 ± 0.1 a	0.4 ± 0.1 b
24-methylene-cycloartanol	1.2 ± 0.2 a	0.8 ± 0.2 a
oleanolic acid	1.6 ± 0.2 a	1.8 ± 0.2 a
ursolic acid	1.2 ± 0.2 a	1.5 ± 0.1 a
sitosterol	6.2 ± 0.4 a	2.0 ± 0.2 b
stigmasterol	1.1 ± 0.1	tr
sum of esters	12.6	6.7
total	295.3	307.9

[a]Results are referenced to fruit wax extract mass and expressed as the mean ± SD of three independent samples analyzed in triplicate. Results in rows not sharing a common letter are significantly different ($p < 0.05$). [b]Calculation based on the relative ratios of HPLC peak areas.

Table 4. Contents of Triterpenoids in Cuticular Waxes of Bilberry Leaves Collected in Finland and Poland in July 2011[a]

compound	Finnish plants (μg/mg of wax extract)	Polish plants (μg/mg of wax extract)
	Free	
α-amyrin (**1**)[b]	23.9 ± 1.9 a	21.2 ± 1.4 a
α-amyrenone (**2**)	0.4 ± 0.1 a	0.5 ± 0.1 a
β-amyrin (**3**)	39.3 ± 2.8 a	44.9 ± 4.3 a
β-amyrenone (**4**)	0.6 ± 0.1 a	1.0 ± 0.1 b
cycloartanol (**5**)	1.1 ± 0.1 a	1.3 ± 0.1 a
24-methylene-cycloartanol (**6**)	0.5 ± 0.1 a	0.6 ± 0.1 a
erythrodiol (**12**)	2.4 ± 0.3 a	2.9 ± 0.2 a
friedelin (**7**)	1.4 ± 0.1	nd
lupeol (**10**)[b]	0.8 ± 0.1 a	1.4 ± 0.2 b
oleanolic aldehyde (**14**)	5.1 ± 0.3 a	3.9 ± 0.3 b
taraxasterol (**11**)	nd	2.3 ± 0.2
ursolic aldehyde (**15**)	3.8 ± 0.2 a	3.0 ± 0.2 b
uvaol (**13**)	1.7 ± 0.2 a	1.9 ± 0.2 a
sum of neutral triterpenes	81.0	84.9
oleanolic acid (**16**)	31.6 ± 5.7 a	34.1 ± 1.8 a
ursolic acid (**17**)	27.9 ± 4.4 a	26.2 ± 2.9 a
hydroxyoleanolic acid (**18**)	1.8 ± 0.2 a	6.4 ± 2.1 b
hydroxyursolic acid (**19**)	1.3 ± 0.2 a	4.6 ± 1.9 b
sum of triterpene acids	62.6	71.3
campesterol (**20**)	0.9 ± 0.1 a	1.7 ± 0.2 b
sitostanol (**23**)	0.6 ± 0.1 a	0.7 ± 0.1 a
sitosterol (**24**)	8.3 ± 0.6 a	8.9 ± 0.7 a
stigmasta-3,5-dien-7-one (**25**)	2.2 ± 0.2 a	2.1 ± 0.2 a
stigmasterol (**26**)	0.6 ± 0.1 a	0.5 ± 0.1 a
sum of steroids	12.6	13.9
	Esters	
α-amyrin	1.0 ± 0.1 a	0.6 ± 0.1 b
β-amyrin	1.4 ± 0.2 a	0.8 ± 0.1 b
cycloartanol	0.4 ± 0.1 a	0.2 ± 0.1 a
24-methylene-cycloartanol	3.2 ± 0.2 a	1.1 ± 0.2 b
oleanolic acid	2.9 ± 0.1 a	4.1 ± 0.2 b
ursolic acid	1.7 ± 0.1 a	2.7 ± 0.2 b
sitosterol	1.1 ± 0.1 a	2.0 ± 0.2 b
stigmasterol	0.4 ± 0.1 a	1.1 ± 0.1 b
sum of esters	12.1	12.6
total	168.3	182.7

[a]Results are referenced to leaf wax extract mass and expressed as the mean ± SD of three independent samples analyzed in triplicate. Results in rows not sharing a common letter are significantly different ($p < 0.05$). [b]Calculation based on the relative ratios of HPLC peak areas.

derivatives (monohydroxyalcohols, ketones, dihydroxyalcohols, and aldehydes), compounds based on the oleanane skeleton were predominant, with β-amyrin and β-amyrenone more abundant than α-amyrin and α-amyrenone and erythrodiol and oleanolic aldehyde found at higher levels than uvaol and ursolic aldehyde. The lupane-type triterpene, lupeol, as well as two tetracyclic triterpene monohydroxyalcohols, cycloartanol and 24-methylenecycloartanol, were also detected in leaves of plants from both countries. Cycloartanol and 24-methylenecycloartanol were present in similar amounts, whereas lupeol was much more abundant in Polish leaves. The triterpene ketone, friedelin (comprising 1% of the neutral triterpene fraction), was found exclusively in the leaves of Finnish plants, whereas taraxasterol (2% of the fraction) was detected only in Polish leaves.

The content of triterpene acids in leaves was significantly lower than in berries (by 45% in both Finnish and Polish leaves), and oleanolic acid was again the predominant isomer, with an oleanolic/ursolic acid ratio of approximately 1:0.9 in leaves of plants from both locations. The contents of oleanolic and ursolic acids were comparable in Finnish and Polish leaves, but levels of 2α-hydroxyoleanolic and 2α-hydroxyursolic acids

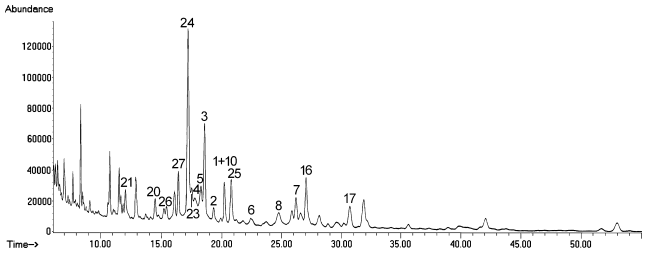


Figure 6. GC−FID chromatogram of the fraction containing sterols and neutral triterpenes (alcohols, aldehydes, and ketones) obtained from chloroform-soluble fruit cuticular waxes from Finnish bilberry. Peaks are numbered according to Figures 1−3.

were more than 3-fold higher in the latter. However, the contents of both hydroxyoleanolic and hydroxyursolic acids seemed to be highly variable, ranging in Polish samples from traces to 276 and 182 μg/g of dry weight for hydroxyoleanolic and hydroxyursolic acids, respectively. In comparison, the majority of the other quantitated compounds showed minor variability, not exceeding 5−6%. The sterol fraction was also less abundant in bilberry leaves than in berries (by 37% in Finnish plants and 15% in Polish plants), again with sitosterol as the main compound, which accounted for more than 90% of all steroids in leaves of plants of both countries. The total content of triterpenoid esters was higher in Finnish leaves than in Polish leaves, although the esters of oleanolic and ursolic acids as well as sitosterol and stigmasterol were more abundant in Polish samples.

Bilberry leaves sampled from either country contained considerably smaller amounts of triterpenoids than lingonberry leaves.[23] Moreover, some significant compositional differences were identified between these two *Vaccinium* species. Besides typical phytosterols and steroids, the triterpenoids of bilberry leaves are predominantly intermediates of subsequent transformations of oleanane and ursane triterpenes, including alcohols, aldehydes, ketones, and acids. In contrast, lingonberry leaves have been shown to contain considerable amounts of triterpenoids based on other types of skeletons, in addition to high amounts of ursolic and oleanolic acids. Thus, the obtained results might suggest that the triterpenoid content of leaves reflect the differences in the adaptation to climate conditions and the survival strategies employed by deciduous *V. myrtillus* and evergreen *V. vitis-idaea*. Triterpenoids, with their various biological activities, might protect the leaves against the pressure of biotic agents, such as insect herbivores and fungal pathogens, with the latter being a serious threat under snow cover.[33] Thus, the high levels of fernenol, particularly in Finnish lingonberry leaves, that are completely covered by snow for many months, could be related to the supposed antifungal properties of this compound.[34]

The results presented in this study show some qualitative and quantitative differences in the triterpenoid content between bilberry samples collected in only one (chosen as the most typical) location in each country; therefore, they cannot be assumed as an general pattern for all natural populations occurring in Finland or Poland. However, as in the case of lingonberry,[23] this comparison is a good example of chemodiversity of plant material, which can be used for functional foods, dietary supplements, cosmetics, or any other plant-derived products. Thus, our research again supports the strong need for the standardization of plant material, particularly that obtained from wild plant species, and the evaluation of its quality and composition before usage for specific applications.

Wild habitats vary in many environmental attributes, including climatic conditions and soil fertility; moreover, abiotic factors usually interfere with complex biotic influences. Because of the complexity involved, it is difficult to predict the impact of an individual environmental factor and to provide one general explanation of the observed effects. To avoid a risk of unpredictable fluctuations in plant composition as well as to facilitate the harvest, a big effort is currently made for domestication of lingonberry and bilberry.[35] This solution could also be advantageous to prevent the reduction in natural plant resources, overexploitation, and destruction of wild habitats. However, the lower level of bioactive phytochemicals might be expected in cultivated plants, and this feature could decrease the value of commercially obtained material for the nutraceutical, pharmaceutical, or cosmetic industries. Thus, the new agricultural technologies with possible application of controlled environmental stress in the optimization of high-yield cultivation should be developed for wild plant domestication.[36]

Triterpenoid Content of Bilberry Fruit and Leaf Cuticular Waxes. Extracts from fruit and leaf cuticular waxes were obtained from the respective plant organs by dipping them into chloroform and gently mixing for 30 s. This method has been shown to extract only between 30 and 73% of cuticular waxes, depending upon the plant species,[37] but despite this possible incomplete recovery, it is the preferred method because it avoids the risk of solvent penetration across the cuticle and extraction of compounds present in deeper tissues. Other methods of studying compounds in cuticular waxes, incuding longer extractions of mechanically or enzymatically isolated cuticle are difficult to apply to small berries, and the extracts may be contaminated by triterpenoids originating from epidermis, collenchyma, and sometimes even parenchyma.[15]

A GC−MS/FID chromatogram of cuticular wax extract obtained from Finnish berries is presented in Figure 6. Generally, the same triterpenoids identified in extracts of the entire organs were detected. It is noteworthy that the identification of individual neutral triterpenes and steroids was easier and more precise in the fractions of wax extracts than in the respective fractions from entire organs, because of the lack of contamination with various low-polarity phenolics and products of chlorophyll degradation. Besides triterpenoids, mainly hydrocarbons were present in the wax extracts, and these formed distinct and clearly separated peaks.

Surprisingly, in the fraction of neutral triterpenes and steroids obtained from one of the Finnish fruit samples, a considerable peak with a t_R of 16.5 min was unambiguously identified as diosgenin (compound **27** in Figure 6). The identification of this compound was confirmed by a comparison of its retention time and mass spectrum to those of an authentic diosgenin standard. Diosgenin is a steroid aglycone (sapogenin), often occurring in the form of glycosides called saponins, that is present in a variety of plants, although to our knowledge, it has never previously been found in any *Vaccinium* species. The occurrence of diosgenin in bilberry requires further investigation, because it was detected in no other sample obtained from either Finland or Poland. This finding revealed that, in some bilberry populations or even individual plants, the unexpected and unpredictable presence of various triterpenoids can disturb the general, rather uniform, and stable patterns of these compounds. Such exceptional individual plants that

produce, for example, very low amounts of anthocyanins have previously been identified in the Finnish bilberry population.[17]

The results of quantitative determination of individual triterpenoids identified in fruit and leaf cuticular waxes extracted from bilberry plants collected in Finland and Poland are presented in Tables 3 and 4. The relative ratios of individual compounds and whole fractions were generally similar to those observed in entire organs, indicating that a significant proportion of detected triterpenoids accumulates in the surface cuticle of bilberry fruits and leaves. The only exception was the level of triterpene acids that were lower than expected from the relative ratios of these compounds to neutral triterpenes and steroids found in the entire organs.

Our results suggest that GC−MS profiling of cuticular waxes might be a convenient method for preliminary investigation of free and ester forms of triterpenoids in entire plant organs when searching for triterpenoid-rich sources. The benefits of this procedure are (i) the rapid extraction (30 s instead of 8 h), (ii) the small amount of obtained extracts (1.3−1.6 and 14.3−15.3 mg of fruit and leaf cuticular wax extracts compared to 14−16 and 35−42 mg of extracts from the entire organs) which facilitates separation by preparative TLC (or any other kind of chromatography) or can even permit direct GC−MS analysis after derivatization, and (iii) the easier and more precise identification of compounds because of lower levels of contaminants. These features make cuticular wax profiling an interesting alternative to the analysis of triterpenoids in total organ extracts. However, this method is suitable only as an initial screen because it cannot provide data on the total triterpenoid content of a plant, and some compounds may not be detected because of their localization in other tissues (e.g., citrostadienol is probably present mainly in the seeds and not the cuticular wax of bilberry fruit). Cuticular wax profiling provides data mainly on triterpenoids present in free and ester forms and not on those in the form of saponins, although some sterol monoglycosides have also been detected in waxes of fruits of various plants.[15] Thus, wax profiling may be adequate for Ericaceae species (as shown for *V. myrtillus*, *V. vitis-idaea*, and *Calluna vulgaris*[38]), which synthesize and accumulate large amounts of free tritepenoids, but it might be insufficient for plants producing triterpenoids mainly in the form of saponins, accumulated in other tissues. Further investigation and more comparative data are required to fully evaluate the usefulness of cuticular wax profiling for triterpenoid screening.

■ ASSOCIATED CONTENT

Supporting Information

GC−MS data of triterpenoids and steroids identified in bilberry fruits and leaves (Table 1S), calibration data for quantitative determination of identified triterpenoids (Table 2S), and HPLC chromatogram of the fraction of neutral triterpenes and steroids from fruits of Polish bilberry compared to HPLC chromatogram of the mixture of the authentic standards of α-amyrin and lupeol (Figure 1S). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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