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Identification of Phenolic Compounds from Lingonberry (*Vaccinium vitis-idaea* L.), Bilberry (*Vaccinium myrtillus* L.) and Hybrid Bilberry (*Vaccinium x intermedium* Ruthe L.) Leaves

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Phenolic compounds from leaves of lingonberry (*Vaccinium vitis-idaea* L.), bilberry (*Vaccinium myrtillus* L.), and the natural hybrid of bilberry and lingonberry (*Vaccinium x intermedium* Ruthe L., hybrid bilberry) were identified using LC/TOF-MS and LC/MS/MS after extraction from the plant material in methanol in an ultrasonicator. The phenolic profiles in the plants were compared using the LC/TOF-MS responses. This is the first thorough report of phenolic compounds in hybrid bilberry. In total, 51 different phenolic compounds were identified, including flavan-3-ols, proanthocyanidins, flavonols and their glycosides, and various phenolic acid conjugates. Of the identified compounds, 35 were detected in bilberry, 36 in lingonberry, and 46 in the hybrid. To our knowledge, seven compounds were previously unreported in *Vaccinium* genus and many of the compounds are reported for the first time from bilberry and lingonberry.

KEYWORDS: Phenolics; lingonberry; *Vaccinium vitis-idaea*; bilberry; *Vaccinium myrtillus*; hybrid bilberry; *Vaccinium x intermedium* Ruthe; flavonols; flavan-3-ols; catechins; proanthocyanidins; phenolic acids

INTRODUCTION

Phenolic compounds are a wide group of secondary metabolites including flavonoids and aromatic acids that are produced via shikimate and acetate pathways in plants (*1*). Various biological activities are associated with plant phenolic compounds, which is the main reason why their abundance and chemical structures are nowadays continuously being studied from plant-based foodstuffs.

Lingonberry and bilberry (*Vaccinium vitis-idaea* L. and *Vaccinium myrtillus* L., Ericaceae) are the characteristic field layer species in boreal forests and among the most significant wild berries in Nordic countries and Russia (*2*). Berries of both species are used in various different forms in the human diet. Hybrid bilberry (*Vaccinium x intermedium* Ruthe) is a rare, natural hybrid of bilberry and lingonberry that was first described in Germany by J.R. Ruthe in 1826 (*3*, *4*). *V. intermedium* displays intermediate characteristics of leaf, stem, and floral morphology of bilberry and lingonberry. As bilberry is deciduous and lingonberry is evergreen, the hybrid drops part of the leaves in the fall, while the other part overwinters similarly to leaves of lingonberry. Some leaves are light green with a number of teeth on the leaf edge as in bilberry leaves, and some are thicker with weakly inrolled blade edge, which resembles lingonberry leaves. The hybrid bilberry rarely develops flowers and berries (*4*).

In this study, phenolics from leaves and stems of lingonberry, bilberry, and hybrid bilberry grown at natural sites or on field were studied using liquid chromatography mass spectrometry (LC/MS). The aim of the study was to compare the phenolic profiles of these closely related species representing different overwintering strategies. Berries of the *Vaccinium* species have been extensively studied in the recent years (*5*−*9*), but less attention has been paid to the chemical composition of leaves and stems as they are not widely used for nutrition. However, due to the high content of phenolic compounds, the leaves and stems of the *Vaccinium* species are potential material for nutraceuticals (*10*). Leaves of bilberry and lowbush blueberry (*Vaccinium angustifolium* Ait.) are recognized as a source of antioxidative (*10*) and antidiabetic compounds, such as chlorogenic acid, quercetin derivatives, proanthocyanidins, and anthocyanins (*11*, *12*). The phenolic profile of the hybrid bilberry has not been reported earlier, but several papers describing catechins, proanthocyanidins, flavonoids, and other phenolic compounds of lingonberry (*5*−*8*, *13*) and bilberry (*14*, *15*) have been published. However, most of the published analyses use hydrolysis in the sample preparation step and, as a result, only nonconjugated forms of the flavonoids and other phenolics are measured. Here, 51 phenolic compounds were identified from leaves and stems of the plants, with seven of them being reported for the first time from *Vaccinium* plants.

MATERIALS AND METHODS

Reagents and Materials. The standard compounds (*trans*-chlorogenic acid, epicatechin, kaempferol, quercetin, quercitrin and rutin) were purchased from Extrasynthese (France). HPLC grade acetonitrile and methanol were purchased from Merck (LiChrosolv GG, Darmstadt,

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Germany). HPLC grade formic acid was purchased from BDH Laboratory Supplies (Poole, England). UP grade (ultrapure, 18.2 MΩ) laboratory water was in-house freshly prepared with Direct-Q (Millipore Oy, Espoo, Finland) purification system.

Sample Preparation and Extraction. The plant material was collected during summer 2005 and autumn 2006 from lingonberry (collected in June) and bilberry (collected in October) plants growing at natural sites in Oulu, Northern Finland. The hybrid plants originate from Pori population in Southern Finland. The plants were propagated by tissue culture as described by Jaakola et al. (*16*) and planted on the test field of the Botanical gardens, University of Oulu in 1998. Leaf and stem samples of the hybrid were collected in May and October, 2005. The leaves collected in May were fully expanded and bilberry leaves collected in October had some signs of senescence, such as loss of chlorophyll, whereas evergreen tissues, such as bilberry stems and lingonberry leaves and stems were similar in appearance regardless of timing of collection. All plant material was collected in the afternoon on a cloudy day, and stored at −20 °C within 1 h until preparation and analysis. Plant leaves and stems were crushed and ground to fine powder using dry ice bath and spatula in glass vial. Crushed plant leaves (100 mg) were exactly weighed into a 4 mL glass vial together with 2 mL of methanol (to obtain extraction concentration of 50 mg of leaves/mL of solvent), which has generally been observed to be the best extraction solvent for plant phenolics in earlier studies (*13*, *17*). The samples were extracted in a GWB Branson 2200 ultrasound-sonicator (GWB, Finland) at room temperature for 1 h, after which they were centrifuged for 10 min at 16100 × *g* at room temperature using Eppendorf 5415D Spin (Eppendorf AG, Hamburg, Germany). Before LC/MS analyses the samples were diluted 1:1 with ultrapure water.

Liquid Chromatography Mass Spectrometry. A Waters Acquity ultraperformance liquid chromatographic (UPLC) system (Waters Corporation, Milford, MA) with autosampler, vacuum degasser, and column oven was used. The analytical column was a 50 × 2.1 mm i.d., 1.8 μm, Waters Acquity HSS T3 (Waters Corporation, Milford). The eluents were 0.1% formic acid (A, pH 2.7) and methanol (B). A linear gradient elution from 10 to 50% B in 12 min was employed, followed by 4 min isocratic elution with 50% B, linear gradient to 90% B in 2 min, 4 min isocratic elution with 90% B, and column equilibration for 2.5 min with initial conditions. The flow rate was 0.5 mL/min and the column oven temperature was 35 °C. Injection volume used was 4 μL. The flow was directed to mass spectrometer (MS) without splitting. UPLC/TOF-MS data was acquired with a Waters LCT Premier XE time-of-flight (TOF) mass spectrometer (Waters Corporation, Milford) using both negative (ESI−) and positive (ESI+) ionization polarities. Leucine enkephalin was used as a lock mass compound ($[M + H]^+ = m/z$ 556.2771 in ESI+ and $[M - H]^- = m/z$ 554.2614 in ESI−). Capillary voltages of 2.8 kV and −2.8 kV were used at ESI+ and ESI−, while the cone voltages were set to 80 V and −40 V, respectively. Aperture 1 voltages of 5 V and 50 V were used in two parallel data acquisition functions, to obtain only molecular ions with the lower voltage and more in-source fragmentation data with the higher voltage. The mass range of m/z 100 − 1100 was acquired. The W-mode ion optics and the dynamic range enhancement (DRE) option were used. The UPLC/MS/MS data was recorded with a Waters Quattro Premier triple quadrupole mass spectrometer. Capillary voltages used were the same as in UPLC/TOF-MS measurements. In CID of $[M + H]^+$ and $[M - H]^-$ ions, the sample cone voltages used were 20 V in positive ion mode and −28 V in negative ion mode, while the collision energies varied between 12 and 45 eV. In "pseudo MS^3" experiments in positive ion mode for identification of aglycones, the $[M + H-\text{glycoside}]^+$ fragments were generated in-source with a cone voltage of 50 V and were further chosen for collision cell CID. In all experiments, the precursor ions were chosen with one unit mass resolution. The collision gas was argon with the CID gas cell pressure 3.6×10^{-3} mbar. In all LC/MS experiments, the desolvation temperature was 350 °C and the source temperature was 150 °C. Nitrogen was used as drying gas with a flow rate of 750 L/h and as nebulizing gas with a full flow rate. The mass spectrometers and UPLC system were operated under MassLynx 4.1 software.

RESULTS AND DISCUSSION

In total, 51 compounds were identified unambiguously or at least tentatively from the leaf samples. The **Figure 1** shows LC/TOF-MS chromatograms from leaf extracts of the plants, acquired with negative-ionization mode electrospray. Generally, the weight of sugar units in the glycosides was determined by in-source fragment ion data from LC/TOF-MS experiments, while most of the aglycone structures were identified by MS/MS experiments or by comparison with earlier data from lingonberry phenolics (*13*). The identified compounds and their LC/MS data are shown in the **Table 1**, while relative peak areas for each identified compound in each sample are shown in **Table 2**.

Flavan-3-ols. Catechins were detected with both positive ($[M + H]^+$ and $[M + Na]^+$ ions) and negative electrospray polarities ($[M - H]^-$ ions) in LC/MS chromatograms. Authentic standard was used for identification of epicatechin (compound **14**). Catechin (compound **3**) was identified according to its fragmentation spectrum (**Table 1**) that was identical with epicatechin and with the literature (*13*, *18*, *19*). Gallocatechin and epigallocatechin, compounds **1** and **2**, respectively, were identified by their fragmentation spectra (**Table 1**), where fragment ion typical of catechins with positive ionization mode at m/z 139 (formed via retro Diels−Alder fragmentation, RDA) was dominant. The absence of ion at m/z 123, as observed in MS spectra of catechin and epicatechin, suggests that the additional oxygen atom in compounds **1** and **2** is located in the C-ring (corresponding fragment for compounds **1** and **2** is thus also at m/z 139, overlapping with the RDA-fragment). Fragment ion at m/z 289 corresponds to the loss of water. In negative ionization mode, fragmentation for compounds **1** and **2** was similar to that of compounds **3** and **14**, the only differences observed were a shift of 16 u for fragments at m/z 261 and 219 (fragments at m/z 245 and 203 for compounds **3** and **14**). Their spectrometric characteristics were identical with each other and they were tentatively identified based on their typical retention order published earlier (*20*).

Dimeric and trimeric catechin polymers (trimeric compounds **5**, **9**, **12**, and **20** and dimeric proanthocyanidins **16** and **27**) were also identified according to exact masses and MS fragmentation spectra. Exact masses in positive and negative ionization mode for compounds **16** and **27** suggested a molecular formula $C_{30}H_{24}O_{12}$. Compounds **16** and **27** were identified as A-type proanthocyanidins consisting of catechin (or epicatechin) units based on their fragmentation published earlier in the literature (*13*, *21*). Exact masses for compounds **5**, **9**, and **20** suggested a molecular formula $C_{52}H_{32}O_{13}$ and for compound **12**, a molecular formula $C_{52}H_{34}O_{13}$. Compounds **5**, **9**, and **20** were thus A/B-type proanthocyanidin trimers containing three catechin (or epicatechin) units (one of the (E)C units bonded to central unit with A-type and one with B-type bonding) and compound **12** was a C-type proanthocyanidin trimer. The main fragment ions for compound **12** in negative ionization mode were at m/z 739 (loss of phloroglucinol from extension (E)C subunit), m/z 713 (RDA fragmentation), m/z 695 (RDA-fragmentation + loss of H_2O), m/z 577 (loss of extension (E)C subunit), m/z 575 (loss of terminal (E)C subunit), m/z 451 (loss of extension (E)C unit and loss of phloroglucinol from central (E)C unit), m/z 449 (loss of terminal (E)C and loss of phloroglucinol from extension (E)C unit), m/z 425 (loss of extension (E)C unit and RDA fragmentation), m/z 423 (loss of terminal (E)C unit and RDA fragmentation), m/z 413 (central (E)C unit and phloroglucinol from terminal unit), m/z 289 (terminal (E)C unit), and m/z 287 (terminal (E)C unit), supporting the identification as a C-type proanthocyanidin trimer. Main fragments detected for compound **9** in negative ionization mode were at m/z 711 (RDA fragmentation), m/z 693 (loss of H_2O and RDA-fragmentation), m/z 573 (loss of terminal B-type (E)C unit), m/z 451 (loss of extension A-type (E)C unit and phloroglucinol from central (E)C unit), m/z 411 (extension (E)C unit and phloroglucinol from central (E)C unit), and m/z 289

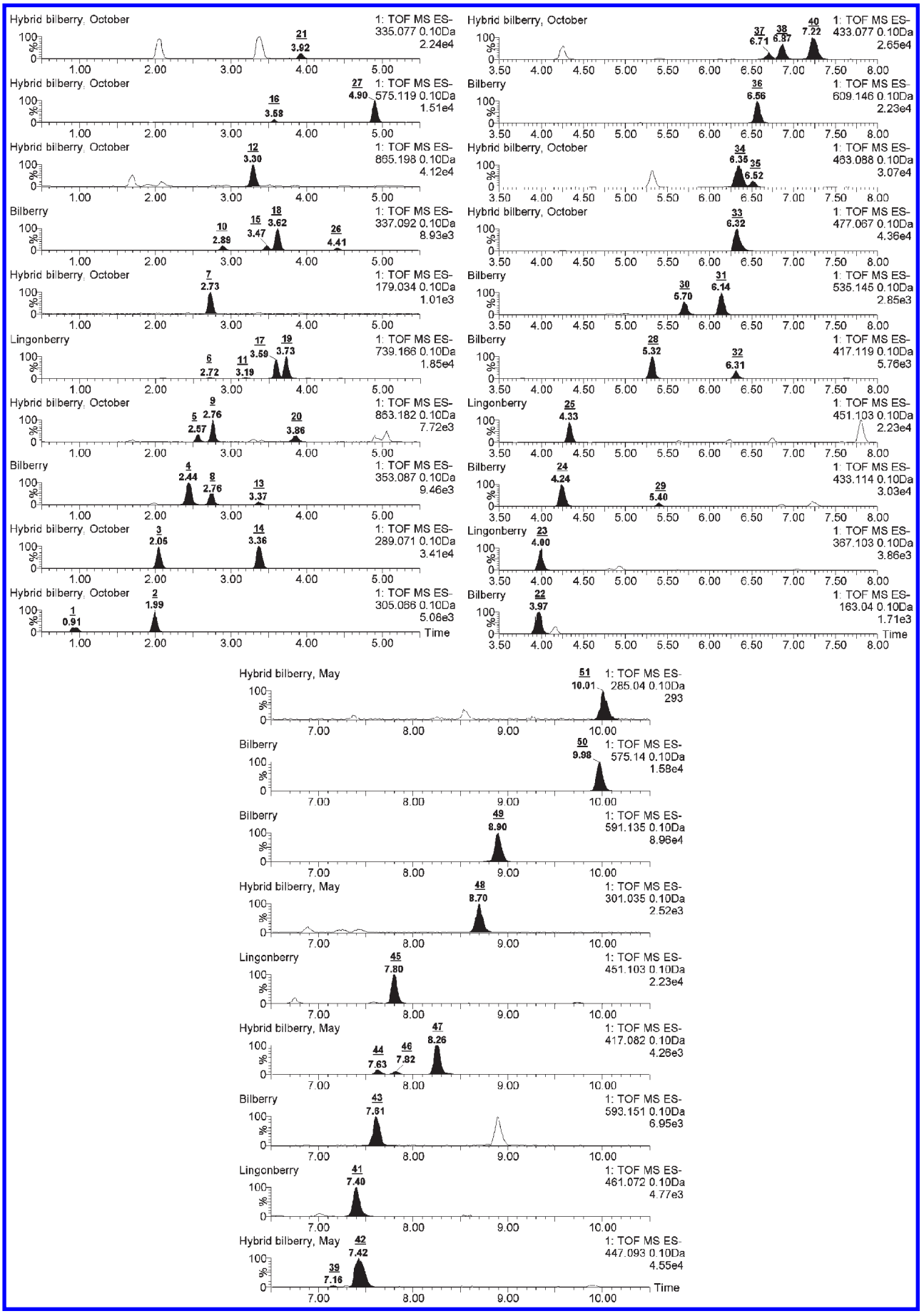

Figure 1. LC/TOFMS ion chromatograms for characterized compounds, acquired from the methanol extracted leaf samples. Peak numbers refer to **Tables 1** and **2**.

Table 1. Compounds Identified from Methanolic Extracts of Bilberry, Lingonberry, and Hybrid Bilberry and their LC/MS Data: Peak Numbers Refer to **Figure 1**

									m/z of the main fragment ions	
no.	compound name	t_R [min]	$[M-H]^-$ (*m/z*)	$[M-H]^-$ (*m/z*, calcd)	$[M+H]^+$ (*m/z*)	$[M+H]^+$ (*m/z*, calcd)	$[M+Na]^+$ (*m/z*)	$[M+Na]^+$ (*m/z*, calcd)	$[M-H]^-$ in-source fragments	$[M+H]^+$ in-source fragments
1	gallocatechin	0.94	305.0658	305.0661	307.0779	307.0818	329.0648	329.0637	261, 219, 179, 165, 139, 137	289, 169, 139
2	epigallocatechin	1.99	305.0671	305.0661	307.0808	307.0818	329.0613	329.0637	261, 219, 179, 165, 139, 137	289, 169, 139
3	catechin	2.04	289.0701	289.0712	291.0879	291.0869	313.0654	313.0688	245, 203, 179, 165, 139, 137, 125	273, 239, 207, 165, 147, 139, 123
4	caffeoyl quinic acid isomer 1 (*trans*-chlorogenic acid)	2.43	353.0885	353.0873	355.1029	355.1029			191, 179	195, 163, 135
5	proanthocyanidin trimer type A/B 1	2.56	863.1843	863.1823	865.1957	865.1980			711, 693, 573	713, 695, 575, 533
6	cinchonain IIx isomer 1[c]	2.72	739.1664	739.1663	741.1796	741.1819			629, 587, 569, 449, 435, 339, 289	643, 571, 451
7	caffeic acid	2.73	179.0322	179.0344					135	
8	caffeoyl quinic acid isomer 2	2.76	353.0901	353.0873	355.1001	355.1029			191, 179, 173, 135	195, 163, 135
9	proanthocyanidin trimer type A/B 2	2.77	863.1850	863.1823	865.2017	865.1980			711, 693, 573, 451, 411, 289	713, 695, 575, 533, 287
10	coumaroyl quinic acid isomer 1	2.89	337.0905	337.0923			361.0905	361.0899	173, 163, 119	
11	cinchonain IIx isomer 2[c]	3.18	739.1667	739.1663	741.1824	741.1819			629, 587	643, 571, 451
12	proanthocyanidin trimer type B	3.29	865.2008	865.1980	867.2107	867.2136	889.1947	889.1956	739, 713, 695, 577, 575, 533, 451, 449, 425, 423, 413, 289, 287	769, 753, 715, 697, 579, 577, 427, 425, 409, 407, 289, 247
13	caffeoyl quinic acid isomer 3 (*cis*-chlorogenic acid)	3.37	353.0869	353.0873	355.1043	355.1029	377.0859	377.0849	191, 179	195, 163, 135
14	epicatechin	3.37	289.0713	289.0712	291.0866	291.0869	313.0683	313.0688	245, 203, 179, 165, 139, 137, 125	273, 239, 207, 165, 147, 139, 123
15	coumaroyl quinic acid isomer 2	3.46	337.0922	337.0923	339.1040	339.1080			191, 173, 163	179, 147, (119)
16	proanthocyanidin dimer type A 1	3.56	575.1224	575.1190	577.1369	577.1346			539, 449, 407, 289, 285	
17	cinchonain IIx isomer 3[c]	3.58	739.1661	739.1663					629, 587, 569, 449, 339, 289	643, 589, 571, 451, 437, 405, 289
18	coumaroyl quinic acid isomer 3	3.60	337.0952	337.0923	339.1090	339.1080			173, 163, 119	179, 147, 119
19	cinchonain IIx isomer 4[c]	3.72	739.1684	739.1663					629, 587, 569, 449,339, 289	643, 589, 571, 451, 437, 405, 289
20	proanthocyanidin trimer type A/B 3	3.86	863.1846	863.1823	865.1964	865.1980			711, 693, 575, 289, 287	713, 695, 575, 533, 289, 287
21	caffeoyl shikimic acid	3.93	335.0753	335.0767	337.0908	337.0923	359.0753	359.0743	179, 161, 135	
22	*p*-coumaric acid	3.98	163.0370	163.0395					119	
23	feruloyl quinic acid isomer 1	4.00	367.1013	367.1029	369.1143	369.1186			193, 191, 173	177
24	2-*O*-caffeoylarbutin	4.25	433.1125	433.1135	435.1275	435.1291	457.1115	457.1111	323, 203, 179, 161, 135	
25	cinchonain Ix isomer 1[c]	4.35	451.1011	451.1029	453.1212	453.1186			341, 321, 289, 231, 217, 189, 177	343, 325, 301, 259, 191
26	coumaroyl quinic acid isomer 4	4.41	337.0929	337.0923	339.1091	339.1080	361.0875	361.0899	191, 163, 145	179, 147
27	proanthocyanidin dimer type A 2	4.90	575.1196	575.1190	577.1328	577.1346			539, 449, 407, 289, 285	437, 425, 299, 287
28	cumaroyl-hexose hydroxyphenol	5.32	417.1213	417.1186	419.1339	419.1342	441.1166	441.1162	307, 187, 163, 145, 119	
29	caffeoyl-hexose-hydroxyphenol	5.40	433.1129	433.1135			457.1088	457.1111	323, 203, 179, 161, 135	
30	coumaroyl iridoid isomer 1	5.71	535.1454	535.1452			559.1407	559.1428	491, 373, 371, 329, 311, 209, 191, 163	427, 379, 357, 339, 193, 179, 175, 165, 147
31	coumaroyl iridoid isomer 2	6.14	535.1450	535.1452			559.1442	559.1428	491, 373, 371, 329, 311, 209, 191, 163	427, 379, 357, 339, 193, 179, 175, 165, 147
32	cumaroyl-hexose hydroxyphenol	6.31	417.1179	417.1186			441.1154	441.1162	307, 187, 163, 145, 119	
33	quercetin-3-*O*-glucuronide	6.32	477.0689	477.0669	479.0853	479.0826			301, 175	303[a]
34	quercetin-3-*O*-β-galactoside	6.33	463.0869	463.0877	465.1011	465.1033			301, 300	303[a], 185
35	quercetin-3-*O*-glucoside	6.51	463.0869	463.0877			487.0858	487.0852	301, 300	303[a], 185
36	quercetin-3-*O*-rutinoside (rutin)	6.57	609.1446	609.1456	611.1610	611.1612			301, 300	465, 303[a]

Table 1. Continued

no.	compound name	t_R [min]	$[M-H]^-$ (*m/z*)	$[M-H]^-$ (*m/z*, calcd)	$[M+H]^+$ (*m/z*)	$[M+H]^+$ (*m/z*, calcd)	$[M+Na]^+$ (*m/z*)	$[M+Na]^+$ (*m/z*, calcd)	*m/z* of the main fragment ions	
									$[M-H]^-$ in-source fragments	$[M+H]^+$ in-source fragments
37	quercetin-3-*O*-β-xyloside	6.71	433.0779	433.0771	435.0940	435.0927			301, 300, 271, 255	303[a]
38	quercetin-3-*O*-α-arabinoside	6.87	433.0770	433.0771	435.0920	435.0927			301, 300, 271, 255	303[a]
39	kaempferol-*O*-hexoside	7.15	447.0949	447.0927			471.0914	471.0903	285, 284	287[b]
40	quercetin-3-*O*-α-arabinofuranoside	7.22	433.0758	433.0771	435.0934	435.0927			301, 300, 271, 255	303[a]
41	kaempferol-3-*O*-glucuronide	7.37	461.0711	461.0720	463.0860	463.0877			285, 175	287[b]
42	quercetin-3-*O*-α-rhamnoside (quercitrin)	7.43	447.0921	447.0927	449.1059	449.1084			301, 300, 271, 255	303[a]
43	kaempferol-*O*-(hexose-deoxyhexoside)	7.61	593.1486	593.1506	595.1617	595.1663			417, 285, 284	503, 331, 287[b]
44	kaempferol-*O*-pentoside	7.63	417.0822	417.0822	419.0995	419.0978			285, 284	287
45	cinchonain Ix isomer 2[c]	7.80	451.1033	451.1029	453.1138	453.1186			341, 321, 289, 231, 217, 189, 177	
46	kaempferol-*O*-pentoside	7.83	417.0811	417.0822			441.0795	441.0798	285, 284, 255, 227	287[b]
47	kaempferol-*O*-pentoside	8.26	417.0811	417.0822			441.0780	441.0798	285, 284, 255, 227	287[b]
48	quercetin	8.71	301.0373	301.0348	303.0464	303.0505				257, 229, 165, 153, 137[d]
49	quercetin-3-*O*-(4″-HMG[e])-α-rhamnoside	8.90	591.1354	591.1350	593.1485	593.1506	615.1293	615.1326	529, 489, 447, 301, 300	303[a]
50	kaempferol-(HMG[e])-rhamnoside	9.97	575.1388	575.1401	577.1536	577.1557			513, 473, 431, 285, 284	287[b]
51	kaempferol	10.01	285.0401	285.0399	287.0562	287.0556				241, 213, 165, 153, 121[d]

[a] Aglycone identified as quercetin based on the similar fragmentation in MS/MS compared to authentic standard. [b] Aglycone identified as kaempferol based on the similar fragmentation in MS/MS compared to authentic standard. [c] x = a, b, c, or d. [d] Fragments from MS/MS measurements. [e] HMG = 3-hydroxy-3-methylglutaryl.

(terminal (E)C unit). A similar fragmentation pattern was obtained for compound **5**, but due to low abundance, only fragment ions at *m/z* 711, 693, 575, and 289 were detected. This suggests that the terminal (E)C unit in compounds **5** and **9** was B-type and extension (E)C unit was A-type. The structural difference between compounds **5** and **9** is in stereochemistry of subunits (epicatechin/catechin), and cannot be distinguished by means of MS. For **20**, the detected fragment ions were at *m/z* 711, 693, 575, 289, and 287 (loss of extension (E)C units and formation of quinone-methide functionality). Presence of ion at *m/z* 287 suggests that the terminal (E)C unit was A-type and extension (E)C unit was B-type. Catechins and proanthocyanidins have been reported earlier from *Vaccinium* plants in many publications (*7*, *8*, *13*).

For the compounds **6**, **11**, **17**, and **19** and compounds **25** and **45**, a unique fragmentation behavior in comparison to other phenolic compounds was observed. Compounds **6**, **11**, **17**, and **19** showed molecular ions with negative ionization mode at *m/z* 739.1661−739.1684 (calcd for $C_{39}H_{31}O_{15}$ = 739.1663), while compounds **25** and **45** had molecular ions at *m/z* 451.1011−451.1033 (calcd for $C_{24}H_{19}O_9$ = 451.1029). Fragment ions detected for compounds **6**, **17**, and **19** were at *m/z* values 629, 587, 569, 449, 435, 339, and 289 in negative-ion mode. For compound **11**, only two abundant fragments were detected (due to low abundance of the compound) at *m/z* 629 and *m/z* 587. Fragment ions detected for compounds **25** and **45** were at *m/z* 341, *m/z* 321, *m/z* 289, *m/z* 231, *m/z* 217, *m/z* 189, and *m/z* 177 in negative-ion mode and at *m/z* 343, *m/z* 325, *m/z* 301, *m/z* 259, and *m/z* 191 in positive-ion mode. Compounds **25** and **45** were identified as cinchonain Ix (*x* = a, b, c, or d) and compounds **6**, **11**, **17**, and **19** were identified as cinchonain IIx (*x* = a, b, c, or d). Structures for these compounds together with proposed fragmentation pathways are presented in **Figures 2** and **3**. Apparently, this the first report describing the MS-fragmentation of cinchonains. A more detailed identification of compounds **6**, **11**, **17**, **19**, **25**, and **45** was not possible by means of MS. Cinchonains have earlier been detected for example from *Cinchona succirubra* and *Eriobotrya japonica* (*22*, *23*), but not from *Vaccinium* plants.

Flavonols. The compounds **48** and **51** were identified as quercetin and kaempferol flavonols using authentic standards. The compounds **33**−**44**, **46**, **47**, **49**, and **50** were identified as glycosides of quercetin and kaempferol. Abundance of kaemferol glycosides was clearly lower than that of quercetin glycosides, which is in agreement with earlier reports on lingonberry (*13*, *24*). For all these compounds, the flavonol aglycone was identified with LC/MS/MS measurement with a triple quadrupole mass spectrometer after cleavage of the conjugate sugar from the molecular ion by using in-source MS/MS with high cone voltage (except for compound **44**). For quercetin and kaempferol, the fragmentation was in accordance with the known literature data (*13*, *25*). Identification of sugar moieties was done by classifying them as hexose, deoxyhexose, or pentose sugars according to the in-source fragment ions (neutral losses of −162, −146, or −132 amu from the molecular ions, respectively). For more detailed identification of the sugar units of quercetin glycosides, the retention times were compared with data published by Ek et al. (*13*), and authentic standards were used for identification of quercitrin (quercetin-3-*O*-α-rhamnoside, compound **42**) and rutin (quercetin-3-*O*-rutinoside, compound **36**). Most of the kaempferol glycosides were only tentatively identified as comparable data concerning the retention behavior of kaempferol glycosides in analytical conditions similar to this study was not available. It is likely that the glycosylation site in kaempferol glycosides is also at the 3-*O*-position. MS/MS

Table 2. Relative LC/TOF-MS Peak Areas of Each Detected Compound in Each Plant: Peak Numbers Refer to **Figure 1** and **Table 1**

		relative share of each compound from the total combined peak area of all detected compounds in each plant, %			
no.	compound name	bilberry	lingonberry	hybridMay[a]	hybridOct[b]
1	gallocatechin	0.02	ND[c]	ND	0.32
2	epigallocatechin	0.07	ND	ND	0.88
3	catechin	0.20	0.02	0.96	6.40
14	epicatechin	1.75	ND	0.83	6.54
Total Catechins		2.0	0.0	1.8	14.1
6	cinchonain IIx isomer 1	0.13	ND	0.20	ND
11	cinchonain IIx isomer 2	0.16	ND	0.19	ND
17	cinchonain IIx isomer 3	3.65	ND	0.38	ND
19	cinchonain IIx isomer 4	4.23	ND	0.43	0.57
25	cinchonain Ix isomer 1	4.66	ND	0.90	0.80
45	cinchonain Ix isomer 2	5.64	ND	1.31	0.99
Total Cinchonains		18.5	0.0	3.4	2.4
5	proanthocyanidin trimer type A/B 1	0.03	0.12	0.49	0.54
9	proanthocyanidin trimer type A/B 2	0.39	ND	0.72	0.99
12	proanthocyanidin trimer type B	1.38	ND	0.40	2.91
16	proanthocyanidin dimer type A 1	ND	1.20	0.60	0.64
20	proanthocyanidin trimer type A/B 3	ND	ND	0.11	0.58
27	proanthocyanidin dimer type A 2	ND	0.09	2.61	4.67
Total Proanthocyanidins		1.8	1.4	4.9	10.3
4	caffeoyl quinic acid isomer 1 (*trans*-chlorogenic acid)	17.08	3.55	8.21	12.34
7	caffeic acid	0.16	0.61	1.98	0.20
8	caffeoyl quinic acid isomer 2	ND	1.66	ND	ND
10	coumaroyl quinic acid isomer 1	0.03	0.54	0.03	0.01
13	caffeoyl quinic acid isomer 3 (*cis*-chlorogenic acid)	10.52	0.32	1.93	7.28
15	coumaroyl quinic acid isomer 2	1.69	0.56	1.01	3.11
18	coumaroyl quinic acid isomer 3	0.16	2.71	0.39	0.20
21	caffeoyl shikimic acid	0.48	0.18	0.30	1.08
22	*p*-coumaric acid	0.02	0.64	0.42	0.02
23	feruloyl quinic acid isomer 1	0.83	0.04	0.08	0.12
24	2-*O*-caffeoylarbutin	ND	10.38	3.22	3.12
26	coumaroyl quinic acid isomer 4	1.56	0.28	0.63	ND
28	cumaroyl-hexose hydroxyphenol	ND	1.31	1.24	1.12
29	caffeoyl-hexose-hydroxyphenol	ND	1.03	0.27	0.27
32	cumaroyl-hexose hydroxyphenol	ND	0.54	0.10	0.09
Total Phenolic Acids		32.5	24.4	19.8	29.0
30	coumaroyl iridoid 1	0.62	1.50	0.34	0.40
31	coumaroyl iridoid 2	0.36	5.85	2.13	1.61
Total Iridoids		1.0	7.4	2.5	2.0
33	quercetin-3-glucuronide	30.31	ND	18.15	10.2
34	quercetin-3-*O*-β-galactoside	4.06	6.30	10.07	8.31
35	quercetin-3-*O*-glucoside	0.99	3.87	3.46	1.74
36	quercetin-3-*O*-rutinoside (rutin)	ND	7.59	ND	ND
37	quercetin-3-*O*-β-xyloside	ND	0.25	3.15	1.54
38	quercetin-3-*O*-α-arabinoside	2.92	0.78	7.11	3.98
39	kaempferol-hexoside	0.28	0.09	0.36	0.18
40	quercetin-3-*O*-α-arabinofuranoside (avicularin)	ND	2.34	11.43	7.29
41	kaempferol-3-glucuronide	1.34	ND	ND	0.28
42	quercetin-3-*O*-α-rhamnoside (quercitrin)	0.73	5.37	12.35	8.37
43	kaempferol-*O*-(hexose-deoxyhexoside)	ND	2.48	ND	ND
44	kaempferol-*O*-pentoside	0.03	0.04	0.10	0.04
46	kaempferol-*O*-pentoside	ND	0.06	0.82	0.22
47	kaempferol-*O*-pentoside	ND	0.01	0.07	0.03
48	quercetin	0.03	0.17	0.46	ND
49	quercetin-3-*O*-(4′′-HMG)-α-rhamnoside	3.48	32.17	ND	ND
50	kaempferol-(HMG)-rhamnoside	ND	5.35	ND	ND
51	kaempferol	ND	ND	0.05	ND
Total Flavonols		44.2	66.9	67.6	42.2

[a] HybridMay = hybrid bilberry collected in May. [b] HybridOct = hybrid bilberry collected in October. [c] ND = compound not detected.

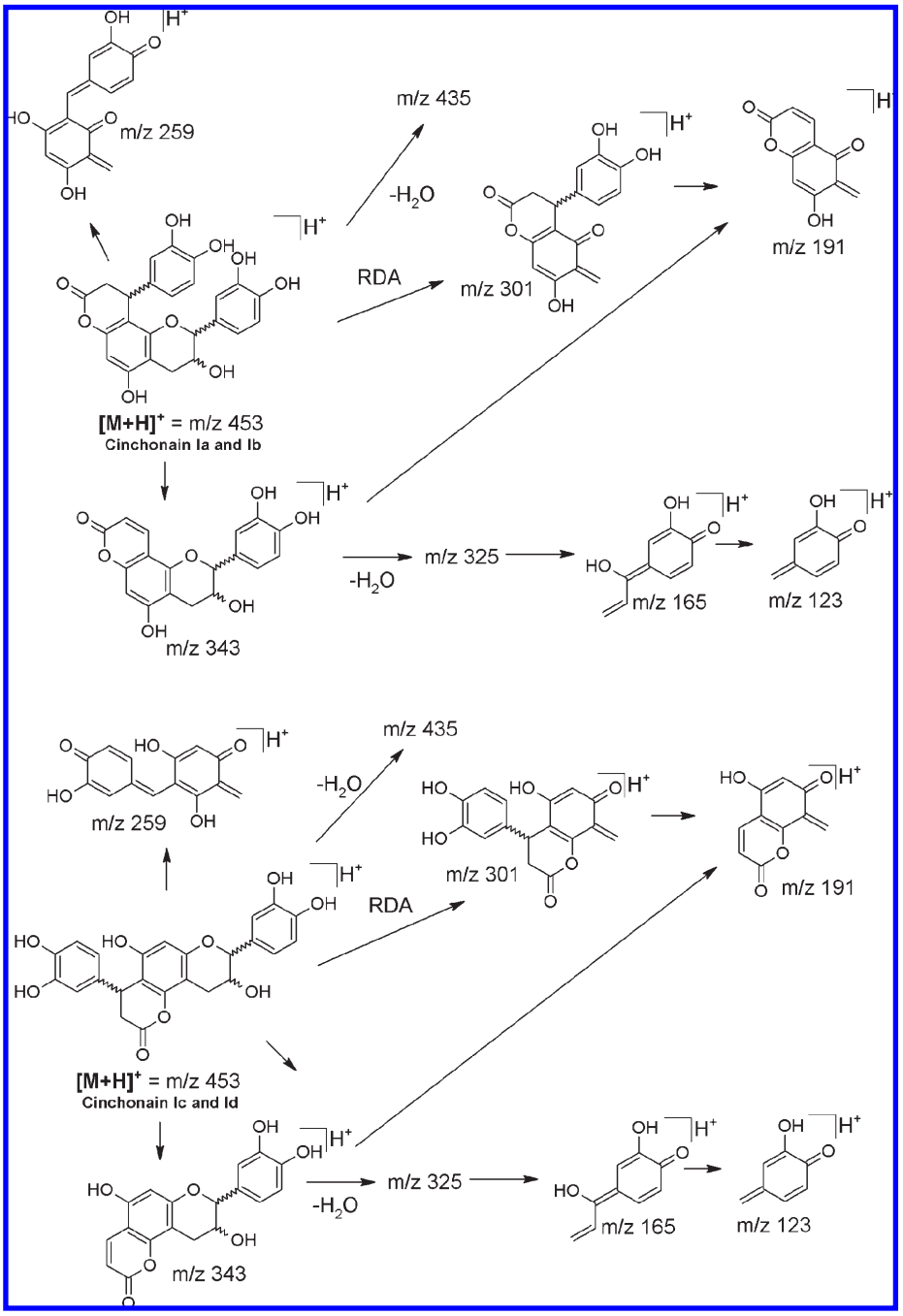

Figure 2. Suggested fragmentation pathway of compounds **25** and **45** in positive ionization mode (ESI+).

spectrum of compound **44** could not be obtained due to low abundance, thus, the identification of the aglycone was done only based on the exact mass (in-source fragment ion in positive ionization mode at *m*/*z* 287.0528, calcd for $C_{15}H_{11}O_6$ = 287.0556). The kaempferol diglycoside, compound **43**, showed similar fragmentation compared to compound **36** with a loss of 146 u corresponding to the loss of deoxyhexose sugar, and a loss of 308 u (146 u + 162 u) due to the loss of hexose and deoxyhexose sugars. This indicates that the hexose sugar was directly bonded to aglycone and that the deoxyhexose was bonded to hexose sugar in both compounds, suggesting that compound **43** might be kaempferol-3-*O*-rutinoside. Quercetin-*O*-(hexose-deoxyhexoside) has been reported from lingonberry earlier (*5*, *13*). The glucuronide conjugates of quercetin and kaempferol were identified based on the neutral loss of 176 amu from the molecular ion and by the presence of a fragment ion at *m*/*z* 175 in negative ionization mode (loss of 18 amu (H_2O) from glucuronic acid residue). The quercetin-3-*O*-glucuronide has also been reported as the main flavonoid in bilberry leaves (about 1% of total dry weight) (*26*).

For quercetin glycosides, the high relative intensity of $[Y_0 - H]^{\bullet -}$ fragment ion (> 100%) at *m*/*z* 300 (homolytic cleavage) in comparison to Y_0^- ions (heterolytic cleavage) at *m*/*z* 301 suggested that the glycosylation site for all detected quercetin glycosides was in the 3-position (*27*). This observation was in good harmony with earlier reports on identification of lingonberry quercetin glycosides (*7*, *13*).

The compounds **49** and **50** were identified as quercetin-3-*O*-[4″-(3-hydroxy-3-methylglutaryl)]-α-rhamnose and kaempferol-3-*O*-[4″-(3-hydroxy-3-methylglutaryl)]-α-rhamnose, respectively. Their in-source fragmentation spectra were identical to those reported for lingonberry earlier (*13*).

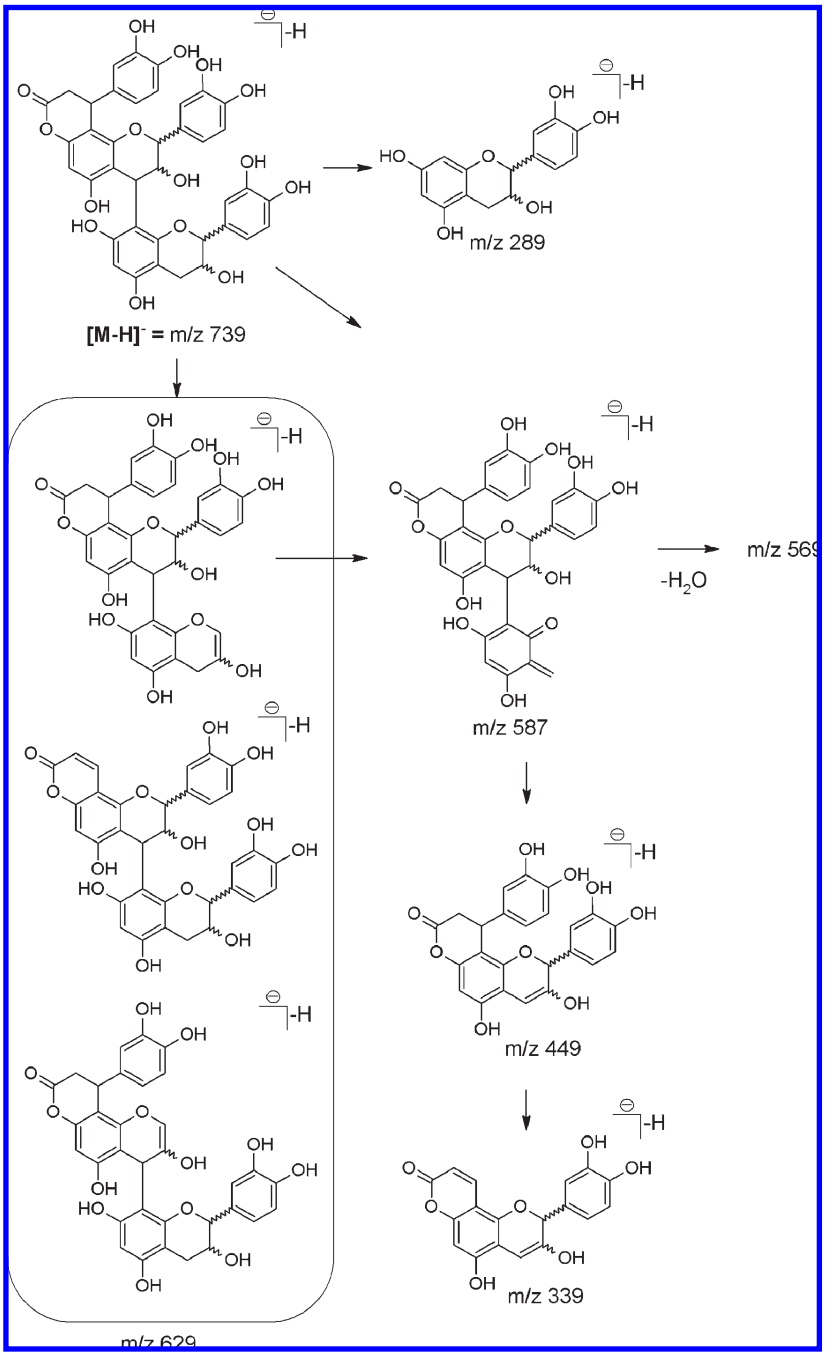

Figure 3. Suggested fragmentation pathway of compounds **6**, **11**, **17**, and **19** in negative ionization mode (ESI−).

Other Phenolic Compounds. Compounds **7** and **22** were identified as caffeic acid and *p*-coumaric acid, based on their exact masses and fragmentation in negative ionization mode. Compounds **4**, **8**, and **13** were identified as caffeoyl quinic acids. The fragmentation of caffeoyl quinic acid isomers was not completely similar. The main fragment ion for compounds **4** and **13** was at *m*/*z* 191 (quinic acid moiety), and the minor fragment ion was detected at *m*/*z* 179 (caffeic acid moiety). Compound **4** was identified as *trans*-chlorogenic acid using an authentic standard and compound **13** was identified as *cis*-chlorogenic acid according to earlier literature data on their similar fragmentation (*28*). When pure *trans*-chlorogenic acid was kept in an ultrasonic bath in methanol (mimicking the extraction of leaves), a small amount of *cis*-isomer was formed from *trans*-chlorogenic acid. Such isomerization of cinnamic acid esters has earlier been observed during irradiation of *trans*-cinnamic acid ester solutions with UV-light (*28*). This suggests that at least part of the detected *cis*-isomer was formed during the extraction. Compound **8** showed major fragment ions at *m*/*z* 191, 179, 173, and 135 (further loss of CO_2 from caffeic acid moiety), most intense fragment ion being at *m*/*z* 173 (loss of H_2O from quinic acid moiety), suggesting that compound **8** was cryptochlorogenic acid or its steroisomer (*cis*-isomer of caffeic acid or different conformational form of quinic acid) as proposed by Fang et al. (*29*). Compounds **10**, **15**, **18**, and **26** were tentatively identified as coumaroyl quinic acid isomers according to their exact masses and fragmentation. All of them showed molecular ion at *m*/*z* 337 in negative ionization mode and fragment ion at *m*/*z* 163, corresponding to coumaroyl moiety. Compounds **15** and **26** showed major fragment ion at *m*/*z* 191, corresponding to quinic acid moiety. Compounds **10** and **18** showed major fragment ion at *m*/*z* 173, corresponding to the loss of H_2O from quinic acid

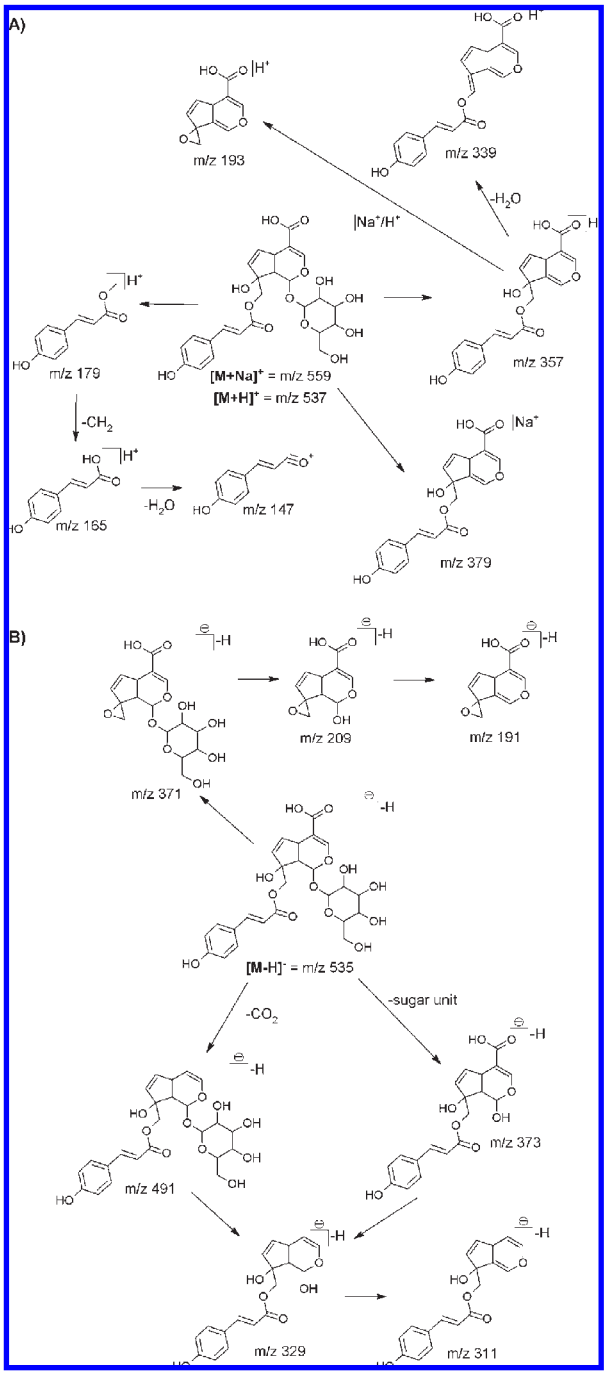

Figure 4. Suggested fragmentation pathway of compounds **30** and **31** in (A) positive ionization mode (ESI+) and (B) negative ionization mode (ESI−).

moiety. Compounds **10**, **15**, **18**, and **26** could not be identified in more detail based on the data acquired, but structural difference between compounds **10**, **15**, **18**, and **26** is probably the position of esterification (esterification to positions 1, 3, 4, and 5 of quinic acid) or *cis*/*trans*-isomerism. Compound **23** was identified as feruloyl quinic acid, based on its exact mass in positive and negative-ion mode and fragmentation in negative-ion mode, giving fragment ions at *m*/*z* 193.0496 (calcd for $C_{10}H_9O_4$ = 193.0501, corresponding to ferulic acid), 191.0535, and 173.0431 (calcd for $C_7H_{11}O_6$ = 191.0556 and for $C_{11}H_9O_5$ = 173.0450, corresponding to quinic acid and dehydrated quinic acid, respectively).

According to exact mass, fragmentation data and retention behavior, the compound **24** was identified as 2″-caffeoylarbutin, which has previously been reported from lingonberry (*13*). Compound **29** showed exactly similar mass spectrometric data to **24** and was therefore identified as its isomer caffeoyl-hexose-hydroxyphenol. The compounds **28** and **32** were identified as isomeric forms of coumaroyl-hexose-hydroxyphenol based on their fragmentation in negative ionization mode that was identical with the literature (*13*). Structural differences between compounds **24** and **29**, as well as between **28** and **32**, are probably in the sugar unit (glucose/galactose) or *cis*/*trans*-isomerism of caffeoyl- and coumaroyl acid units. Also, compounds **28**, **29**, and **32** have been previously reported in lingonberry (*13*).

Accurate mass measured for $[M - H]^-$ and $[M + Na]^+$ ions (**Table 1**) of compounds **30** and **31** corresponded to molecular formula $C_{25}H_{28}O_{13}$. Accurate masses for fragment ions detected in the in-source fragment ion spectra were used to obtain more structural information. The in-source fragmentation spectra of compounds **30** and **31** were identical. Presence of ion at *m*/*z* 163.0372 (calcd *m*/*z* for $C_9H_7O_3$ = 163.0395) in negative-ion mode and presence of ion at *m*/*z* 147.0423 (calcd for $C_9H_7O_2$ = 147.0446) in positive-ion mode suggested a coumaroyl unit to exist in the structure. In negative-ion mode, a fragment ion corresponding to a loss of 44 amu was detected (measured *m*/*z* 491.1555, calcd for $C_{24}H_{27}O_{11}$ = 491.1553), suggesting the presence of carboxylic acid in the structure. Loss of coumaroyl unit from parent ion gave a rise to a fragment ion at *m*/*z* 371.0971 (calcd for $C_{16}H_{19}O_{10}$ = 371.0978). Ion at *m*/*z* 209.0434 (calcd for $C_{10}H_9O_5$ = 209.0450) is caused by the loss of 162 amu from ion at *m*/*z* 371, suggesting the presence of glucose or galactose sugar in the structure. Ion at *m*/*z* 373.0927 (calcd for $C_{19}H_{17}O_8$ = 373.0923) corresponds to the loss of sugar unit directly from the parent ion, suggesting that the sugar unit and the coumaroyl unit are not linked, but directly attached to the aglycone part of the molecule. Ion at *m*/*z* 329.1023 (calcd for $C_{18}H_{17}O_6$ = 329.1025) corresponds to the loss of CO_2 from ion at *m*/*z* 373 (or a loss of sugar unit from ion at *m*/*z* 491, which gives the same structure). Ion at *m*/*z* 311.0929 (calcd for $C_{18}H_{15}O_5$ = 311.0919) is formed by subsequent loss of H_2O from ion at *m*/*z* 329. Ion at *m*/*z* 191.0354 (calcd for $C_{10}H_7O_4$ = 191.0344) is caused by subsequent loss of H_2O from ion at *m*/*z* 209. The structure of the aglycone part is impossible to exactly identify by the MS data only. Two different iridoid glycosides were detected by Jensen et al. (*6*) from cranberry (*Vaccinium macrocarpon*, L.), lingonberry, and bilberry juices and they were identified as monotropein and 6,7-dihydromonotropein. Also coumaroyl iridoids have been reported in cranberry juice (*30*). Presence of monotropein in the lingonberry and bilberry juice suggests, together with the MS data obtained and interpreted here, that structures of the compounds **30** and **31** might be 10-*p*-*trans*- and 10-*p*-*cis*-coumaroyl-1*S*-monotropein. This conclusion is also supported by the fact that the coumaroyl iridoids reported from cranberry (which is phytochemically very closely related to lingonberry), were identified as 10-*p*-*trans*- and 10-*p*-*cis*-coumaroyl-1*S*-dihydromonotropein (*30*). 10-*p*-*trans*-coumaroyl-1*S*-monotropein (vaccinoside) has been reported from *Vaccinium bracteatum* (*31*) and 10-*p*-*cis*-coumaroyl-1*S*-monotropein (andromedoside) has been reported from *Andromeda polifolia* (*32*), but not from bilberry or lingonberry. To verify the identification of compounds **30** and **31** would require NMR analysis of the compounds. The suggested fragmentation pathway of 10-*p*-coumaroyl-1*S*-monotropein in both positive and negative ionization mode is presented in **Figure 4**.

Compound **21** was identified as caffeoyl shikimic acid based on its fragmentation in negative ionization mode. Fragment ion at *m*/*z* 179.0318 (calcd for $C_9H_7O_4$ = 179.0344) suggested the presence of caffeic acid. Fragment ion at *m*/*z* 201.0163 (calcd for $C_9H_6O_4Na$ = 201.0164) was identified as sodium adduct of

two times deprotonated caffeic acid moiety (strong sodium adduct $[M - 2H + Na]^-$ of the parent ion was detected also in negative ionization mode). Fragment ions at m/z 161 and 135 are further dissociation products of caffeic acid moiety, caused by subsequent losses of H_2O and CO_2 from ion at m/z 179, respectively. Caffeoyl shikimic acid has previously been reported from yerba mate (*Ilex paraguariensis*, L.) and green tea (*Camelia sinensis*, L.) (*33*), but to our knowledge not from *Vaccinium* plants.

Comparison of Phenolic Profiles of Lingonberry, Bilberry, and their Hybrid. As quantification was not performed, the absolute amounts of individual compounds cannot be presented. However, by comparing the LC/TOF-MS peak areas of different compounds, clear differences between species can be observed. For relative quantification, $[M - H]^-$ ions were used for all phenolic acids, catechins, proanthocyanidins, and flavonol glycosides and $[M + H]^+$ ions were used for cinchonains. Relative peak areas of each component in each species are presented in **Table 2**. It is, however, worth stressing that these percentual values are formed assuming similar LC/MS-response between all compounds, which may not be the case.

Main phenolic compounds in bilberry were **33** (quercetin-3-*O*-glucuronide), **4** (*trans*-chlorogenic acid), **13** (*cis*-chlorogenic acid), and **45** (cinchonain Ia, Ib, Ic, or Id), having 30, 17, 11, and 6% share, respectively, of the combined LC/TOF-MS peak area of all detected phenolic compounds in bilberry. This is generally in good agreement with earlier literature, as the chlorogenic acids and quercetin-3-glucuronide have been reported as the main phenolic compounds in bilberry leaves (*26*). Main components in lingonberry leaves were compounds **49** (quercetin-3-*O*-(4″-3-hydroxy-3-methylglutaryl)-α-rhamnoside), **24** (2-*O*-caffeoylarbutin), **36** (quercetin-3-*O*-rutinoside), **34** (quercetin-3-*O*-β-galactoside), **31** (10-*p*-*trans*-coumaroyl-1*S*-monotropein), **42** (quercetin-3-*O*-α-rhamnoside, quercitrin), and **50** (kaempferol-HMG-rhamnoside), having 32, 10, 8, 6, 6, 5, and 5% share, respectively, of the combined LC/TOF-MS peak area of all detected phenolic compounds in lingonberry. Compound **49** has been reported from lingonberry only once before (*13*) and, interestingly, based on the LC/MS response, it seems to be the main phenolic compound in lingonberry leaves. 2-*O*-Caffeoylarbutin together with different quercetin- and kaempferol-glycosides have been reported from lingonberry berries and leaves earlier (*13*). Main components in hybrid bilberry leaves collected in May (hybridMay) were compounds **33**, **42**, **40** (quercetin-3-*O*-α-arabinofuranoside, avicularin), **34**, **4**, and **38** (quercetin-3-*O*-α-arabinoside, guaijaverin), having 18, 12, 11, 10, 8, and 7% share, respectively, of the combined LC/TOF-MS peak area of all detected phenolic compounds in hybridMay. Main components in hybrid bilberry leaves collected in October (hybridOct) were compounds **4**, **33**, **42**, **34**, **40**, **13**, **14** (epicatechin), and **3** (catechin), having 12, 10, 8, 8, 7, 7, 7, and 6% share, respectively, of the combined LC/TOF-MS peak area of all detected phenolic compounds in hybridOct.

Seasonal variation in the phenolic composition was thus clear when comparing the leaves of hybridMay and hybridOct. Flavonols were the main group of phenolic compounds in all plants. Combined relative peak areas of all flavonols (glycosides and aglycones) in hybridMay, hybridOct, bilberry, and lingonberry was 68, 42, 44, and 67%, respectively, of the combined peak area of all detected phenolic compounds. Relative amount of all phenolic acids and phenolic acid conjugates (excluding coumaroyl iridoids) in hybridMay, hybridOct, bilberry, and lingonberry was 20, 29, 33, and 24%, respectively. Combined relative peak areas of cinchonains in hybridMay, hybridOct, and bilberry was 3, 2, and 18%, respectively, and they were not detected at all in lingonberry. Relative peak area of simple flavan-3-ols (catechin, epicatechin, gallocatechin, and epigallocatechin) was clearly highest in hybridOct, being 14% of the combined peak area of all detected phenolics. Relative amount of simple flavan-3-ols in hybridMay, bilberry, and lingonberry was only 2, 2, and < 1%, respectively. Relative amount of proanthocyanidins (excluding cinchonains) in hybridMay, hybridOct, bilberry, and lingonberry was 5, 10, 2, and 1%, respectively. It is worth stressing that the amount of proanthocyanidins is not very comparable as the extraction method used for sample preparation only extracts the readily soluble (oligomeric) proanthocyanidins and the mass spectrometric detection was adjusted so that all proanthocyanidins with the degree of polymerization (DP) > 4 are not detected. Likely, the degree of polymerization of proanthocyanidins is different between plants and, thus, the amount of detected proanthocyanidins does not reflect the total content of proanthocyanidins. A relative amount of coumaroyl iridoids (compounds **30** and **31**) in hybridMay, hybridOct, bilberry, and lingonberry was 2, 2, 1, and 7%, respectively.

There are clear differences in hybridMay and hybridOct when compared with bilberry or lingonberry. Neither of hybrid bilberry samples shows clear correlation to bilberry or lingonberry. Two compounds (**51**, kaempferol aglycone; **20**, trimeric proanthocyanidin type A/B) that were not detected in bilberry or lingonberry, were detected in hybrid bilberry, even though their quantities were low. There was also one compound (**49**, quercetin-3-*O*-(4″-HMG)-α-rhamnoside) detected in bilberry and lingonberry but not in hybrid bilberry. The differences in phenolic composition of different samples are at least partially caused by seasonal variation as it has been reported earlier that the phenolic composition of *Vaccinium* plants varies within growth season (*17*, *34*).

A total of 51 phenolic compounds were identified from leaves of lingonberry, bilberry, and hybrid bilberry using LC/TOFMS and LC/MSMS methods. Of these compounds, 35 were detected in bilberry, 36 of them were detected in lingonberry, and 46 were detected in hybrid bilberry (collected in May or in October). To our knowledge, seven of the compounds were reported for the first time from *Vaccinium* plants. All the main phenolic components detected in *V. x intermedium* Ruthe were detected also in *V. vitis-idaea* or in *V. myrtillus* or both.

Many of the compounds detected in the leaves of the three *Vaccinium* plants, for example, proanthocyanidins and cinchonains, have previously been reported to have antiviral (*35*), antimicrobial (*35*), antidiabetic (*23*), and antioxidant (*36*) activities. Leaves of bilberry have traditionally been used for treating diabetes and according to our results, they have high content of cinchonains. Qa'dana et al. (*23*) reported that the cinchonain Ib isolated from *Eriobotrya japonica* induced insulin secretion both in vitro and in vivo in rats. Therefore, it is possible that the cinchonains are responsible for the antidiabetic (blood glucose lowering) effects of bilberry leaves. As the leaves of *Vaccinium* species are a rich source of phenolic compounds (*10*), they can in the future serve as a commercial source of specific compounds or fractions for pharmaceutics, cosmetics and natural product markets.

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