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Characterization of Phenolic Compounds from Lingonberry (*Vaccinium vitis-idaea*)

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Phenolic compounds from the lingonberry (*Vaccinium vitis-idaea*) were identified using LC-TOFMS, LC-MS/MS, and NMR experiments. The compounds were extracted from the plant material using methanol in an ultrasonicator and further isolated and purified using solid-phase extraction and preparative liquid chromatographic techniques. A total of 28 phenolic compounds were at least tentatively identified, including flavonols, anthocyanidins, catechins and their glycosides, and different caffeoyl and ferulic acid conjugates. This is apparently the first report of coumaroyl-hexose-hydroxyphenol, caffeoyl-hexose-hydroxyphenol, coumaroyl-hexose-hydroxyphenol, quercetin-3-*O*-α-arabinofuranoside, kaempferol-pentoside, and kaempferol-deoxyhexoside in the plant, and the flavonol acylglycosides quercetin-3-*O*-[4″-(3-hydroxy-3-methylglutaroyl)]-α-rhamnose and kaempferol-3-*O*-[4″-(3-hydroxy-3-methylglutaroyl)]-α-rhamnose are presented here for the first time ever. In addition, more detailed structure in comparison to earlier reports is described for some compounds previously known to exist in lingonberry.

KEYWORDS: Phenolics; lingonberry; flavonols; anthocyanidins; catechins; proanthocyanidins

INTRODUCTION

Phenolic compounds are a wide group of aromatic compounds that exist naturally in plants and berries, including, for example, flavonoids and aromatic acids produced via shikimate and acetate pathways in plants (*1*). They are connected to a number of biological activities, and their abundance and structural identification from plant-based foodstuffs is continously studied. Lingonberry (*Vaccinium vitis-idaea* L., Ericaceae) is one of the most popular berries in Nordic countries and Russia, and it is used in a number of different forms in the human diet. Furthermore, in the past few years lingonberry products together with another phytochemically similar berry from the *Vaccinium* genus, cranberry, have been increasingly marketed as a natural solution for the treatment of urinary tract infections (*2*−*5*).

In this study, the phenolics in the berry and the aerial parts of the naturally growing lingonberry were studied in a much more detailed manner than earlier, using liquid chromatographic, mass spectrometric, and nuclear magnetic resonance spectroscopic techniques. For most of the compounds, a detailed unambiguous structure was obtained. Many papers concerning the catechins, proanthocyanidins, flavonoids, and other phenolic compounds present in the lingonberry have been published, but only a few of them have concerned the exact structures of the flavonol glycosides and other phenolics and their conjugates (*6*−*10*). Furthermore, many of the studies concerning the flavonoid content of the plants and berries have used hydrolysis in the sample preparation step and, therefore, studied only nonconjugated forms of the flavonoids and phenolics. Here, 28 phenolic compounds were characterized from the berries, leaves, and stems of lingonberry, eight of them being reported for the first time from the plant. To our knowledge, two of these compounds are reported for the very first time.

MATERIALS AND METHODS

Reagents and Materials. The flavonoid standards quercetrin and cyanidin-3-glucoside were purchased from Extrasynthese (Genay, France). HPLC grade acetonitrile and methanol were purchased from Merck (LiChrosolv GG, Darmstadt, Germany). Formic acid, trifluoroacetic acid, and glacial acetic acid were purchased from BDH Laboratory Supplies (Poole, U.K.). Laboratory water was distilled and purified with a Simplicity 185 water purifier (Millipore, Molsheim, France). Deuterated dimethyl sulfoxide, DMSO-d_6, was from Eurisо-Top (Gif sur Yvette, France), whereas deuterated methanol, MeOD-d_4, was from Sigma-Aldrich (Helsinki, Finland).

Sample Preparation and Extraction. The plant material used was collected from the Vaasa region in western Finland during the summer and autumn of 2004 and was identified as lingonberry in the Department of Biology and at the Botanical Gardens at University of Oulu. All plant material was stored in at −18 °C until preparation and analysis. The frozen berries were thawed at room temperature and were dried for 2 h at 40 °C, followed by crushing in a mortar. About 5 g of crushed sample was weighed into 50 mL falcon tubes together with 20 mL of methanol, which was found to be the best extraction solvent in a brief extraction solvent test (methanol, ethanol, acetonitrile, and their various aqueous mixtures were tested; data not shown). The samples were extracted in a GWB Branson 2200 ultrasound sonicator (GWB, Vantaa,

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Finland) at room temperature for 1 h, after which they were centrifuged for 10 min at 3000 rpm with an Eppendorf 5804R centrifuge (Eppendorf, Hamburg, Germany). Before HPLC or LC-MS analyses the samples were diluted 1:2 with ultrapure water to obtain better chromatographic injection conditions. The leaves and the stems of the plant were handled as a single sample, and the samples were dried at room temperature for 7 days, followed by powdering in a mortar. About 100 mg of the powder was weighed into 4 mL sample vials together with 3 mL of methanol. The samples were extracted, centrifuged, and diluted before analysis similarly to berry samples.

Isolation of the Compounds for NMR Experiments. Compounds **11**, **17**, **19**−**24**, and **27** were isolated from the extracts prepared from the leaves and stems of the plant using preparative chromatography. Two 20 mL extracts were prepared as above, followed by pooling and evaporating using a rotary evaporator. The oily suspension obtained was diluted with 1.5 mL of 60% aqueous methanol and was filtered using a 13 mm GHP Acrodisc 0.45 μm syringe filter (Gelman Sciences, Ann Arbor, MI). The compounds were isolated from the sample obtained using preparative liquid chromatoraphy with a Waters Alliance 2690 instrument equipped with a column oven and autosampler, using a 7.8 × 150 mm i.d., 5 μm, XTerra preparative MSC18 column (Waters Corp., Milford, MA) together with a Luna-C18 precolumn (Phenomenex, Torrance, CA). The column temperature used was 30 °C, the eluent flow rate was 1.9 mL/min, and a gradient elution with 0.05% trifluoroacetic acid (A) and methanol (B) was used. A linear gradient elution from 20% B to 42% B in 36 min and to 70% B during the next 8 min (44 min total) was used, followed by column equilibration for 7 min. A Waters 996 photodiode array detector (PDA) was used at a wavelength of 360 nm. The injection volume was 60 μL. The compounds were collected from 10 HPLC runs using manual collection from the flow exiting from the PDA detector. For compounds **11** and **27**, five more additional preparative HPLC runs were carried out with 100 μL injection volumes. The collected samples were dried with a rotavapor and in a nitrogen atmosphere. With some samples, 1 mL of acetone was added to the oily residue to help the drying under the nitrogen flow.

LC and LC-MS. The same HPLC system as above was used, together with a 2 × 50 mm i.d., 3 μm, LunaC18 column and a 2.0 × 4.0 mm i.d. Luna-C18 precolumn (Phenomenex). The eluents used were 0.1% formic acid (A) and methanol (B). The initial gradient elution conditions were 6% B, changing linearly to 12% B in 20 min and to 55%B in the next 30 min. The eluent flow rate was 0.3 mL/min, the temperature of the column oven was 30 °C, and the injection volume used was 10−20 μL. The same chromatographic method was used for both berry and leaf/stem samples.

The LC-TOF/MS data in initial screening of the compounds present and accurate mass measurements was acquired using a LCT time-of-flight (TOF) mass spectrometer (Micromass, Altrincham, U.K.), equipped with an ESI Z-Spray ion source. Capillary voltages of 3.5 and −2.7 kV were used in positive and negative ion mode, whereas the cone voltages were set to 24−30 and −40 V, respectively. The HPLC flow was split postcolumn with an Acurate postcolumn stream splitter (LC Packings, Amsterdam, The Netherlands), with a ratio 1/4 to MS and Waters 996 PDA detector, respectively. The desolvation temperature was 350 °C and the source temperature 150 °C. Nitrogen was used as drying gas with a flow rate of 850 L/h. The mass range acquired was m/z 100−1000 using 1.5 s of acquisition time/spectrum. The mass resolution was ca. 5000 in positive ion mode and 4500 in negative ion mode (full width at half-maximum, fwhm). All LC-MS/MS experiments were performed with a Micromass Quattro II triple-quadrupole instrument (Altrincham, U.K.) equipped with a Z-spray ionization source with the same chromatographic method but without postcolumn flow splitting. Capillary voltages of 4.0 and −3.8 kV were used in positive and negative ion mode, respectively. In collision-induced dissociation (CID) of $[M + H]^+$ and $[M - H]^-$ ions the sample cone voltages used were 23 V in positive ion mode and −40 V in negative ion mode, whereas the collision energies varied between 15 and 35 eV. In "pseudo MS^3" experiments in positive ion mode for the 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 of 1.8×10^{-3} mbar. The desolvation temperature used was 350 °C and the source temperature, 150 °C. Nitrogen was used as both drying and nebulizing gas with flow rates of 400 and 20 L/h, respectively. The accurate mass measurements were performed at the end of the study from the samples isolated for NMR measurements, after the samples had been diluted to 1:10 000−1:100 000 with 50% aqueous methanol, depending on their concentration. The Micromass LCT TOF/MS instrument described above was used with negative mode electrospray ionization, using raffinose as a lock mass compound ($[M - H]^- = m/z$ 503.1612). The sample and lock mass compound were both delivered from separate syringe pumps (Harvard Apparatus, Holliston, MA), and the flows were combined using a T-piece before the ESI source. The abundance of the sample and lock mass ions were (300−500 ions/spectrum) adjusted using syringe pump flow rates of 5−20 μL/min.

NMR Spectroscopy. The NMR experiments were carried out at room temperature as gradient enhanced pulse sequences using a Bruker DRX 500 spectrometer at 11.75 T with a 5 mm TXI probe head and a Bruker DPX 400 spectrometer at 9.4 T with a 5 mm BBI probe head equipped with Z-axis gradient coils. Typically, 1−2 s acquisition times and 2−3 s relaxation delays were used for 1D proton spectra with 8−64 scans. For HSQC and HMBC spectra, 200−300 ms acquisition times with 1−2 s relaxation delays were used, with 128 or 256 time increments and 8−192 experiments. For COSY-β spectra (with 30−45° read pulse) and TOCSY spectra, 260−320 ms acquisition times with 1.5−3 s relaxation delays were used with 256 time increments and 1−8 experiments. Mixing time for TOCSY experiments was 60 ms. For DEPT-135 spectra, 400−500 ms acquisition times with 1.8−2.6 s relaxation delays were used. The J_{CH} couplings were optimized for 145 Hz in HSQC and DEPT-135 and for 8 Hz in HMBC experiments. Compounds **17**, **19**−**24**, and **27** were dissolved in DMSO-d_6, and compound **11** was dissolved in MeOD-d_4. Sample volumes used were 450−500 μL. From the compounds dissolved in DMSO-d_6, the coupling constants were determined after the addition of 60−100 μL of D_2O to shift the resonance frequency of residual water in the sample. The Spinworks 2.1 program (*11*) was used for simulating the spectra. Chemical shifts were calibrated using the following solvent signals: DMSO-d_6, ^{1}H 2.5 ppm, ^{13}C 39.51 ppm; MeOD-d_4, ^{1}H 3.31 ppm, ^{13}C 49.15 ppm (in comparison to TMS at 0 ppm). For compounds **21** and **22** the isolated sample amount was not enough for an HMBC experiment in a reasonable time.

RESULTS AND DISCUSSION

In total, 28 compounds were identified from the samples, either unambiguously with exact structure or at least tentatively. **Figure 1** shows LC-TOF/MS chromatograms from the berry and leaf/stem extracts of the plant, acquired with both positive and negative ion mode electrospray. Generally, the weight of sugar units in the glycosides was determined by MS/MS experiments, as well most of the aglycone structures. For more detailed identification of sugar units and their glycosylation sites in the aglycones, and some aglycone structures, ^{1}H NMR spectroscopy together with HSQC, HMBC, COSY, TOCSY, and DEPT experiments were used. For sugar unit identification the ^{1}H chemical shifs and ^{1}H−^{1}H coupling constants were used. As DMSO-d_6 was used as NMR solvent, the residual water signal has a ca. 3.3 ppm resonance frequency, which is within the typical sugar proton chemical shift range. The problem of overlapping signals from analyte protons and water protons was overcome by the addition of a few drops of D_2O into the sample, leading to a change in water signal to higher frequency. In some cases the D_2O addition led to overlapping of water signals with some other analyte signals, when it was moved back toward the lower resonance frequency by increasing the temperature.

Catechins and Proanthocyanidins. Catechins were easily detected with both positive and negative electrospray polarities in LC-MS chromatograms. Catechin **3** and epicatechin **6** were

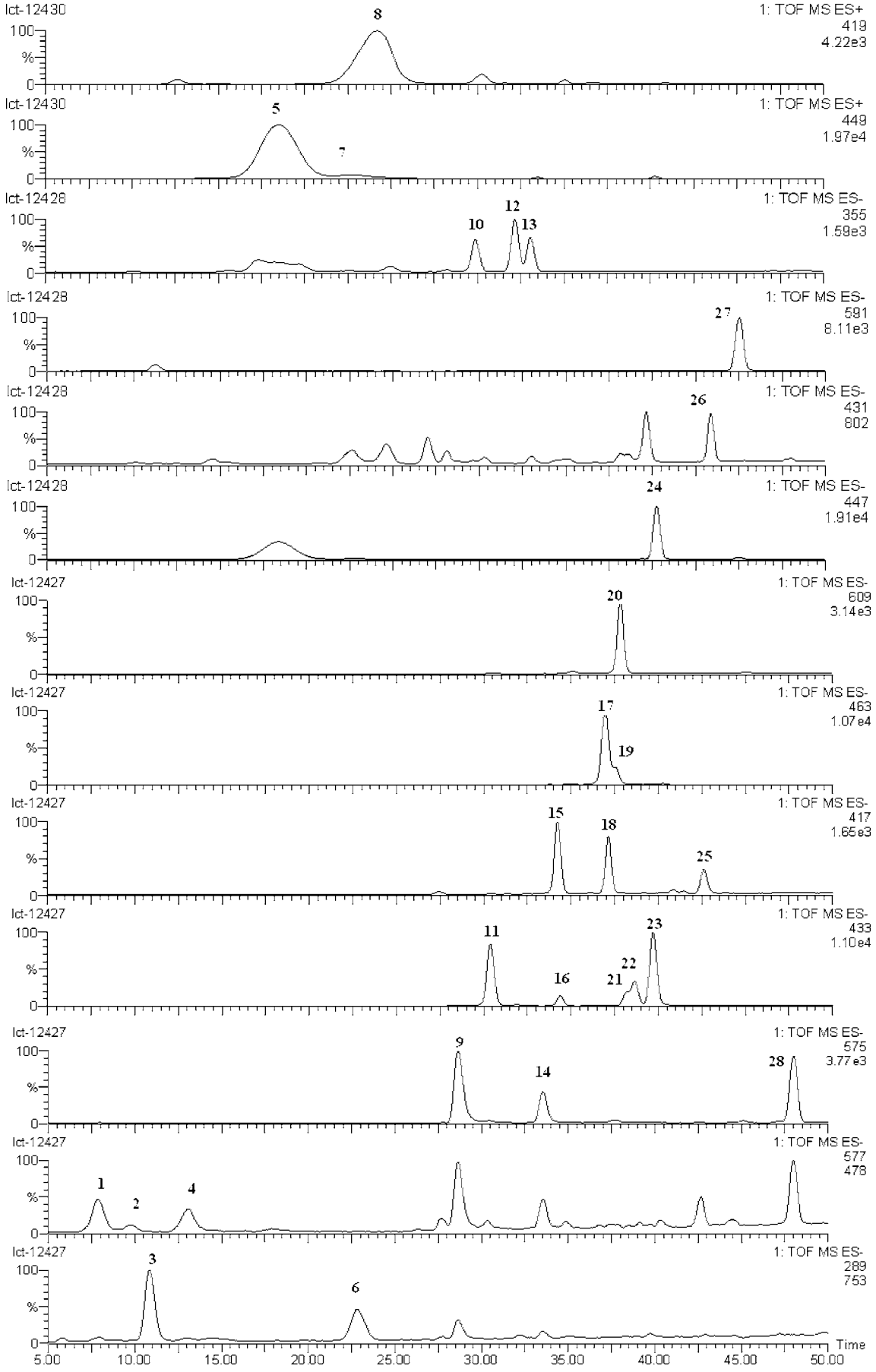

Figure 1. LC-TOF/MS of the methanolic ligonberry extracts.

Table 1. Compounds Identified in Methanolic Extract of Lingonberry and Their LC-MS/MS Data

compd no.	compd name	RT (min)	$[M-H]^-$ (*m*/*z*)	MS/MS (*m*/*z*)	$[M+H]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	MS/MS/MS (*m*/*z*)	berry	leaf
1	proanthocyanidin B	7.0	577		579	427, 409, 291, 289, 247, 139		x	x
2	proanthocyanidin B	8.7	577		579	427, 409, 291, 289, 247, 139		x	x
3	catechin	10.0	289		291	207, 165, 147, 139, 123		x	x
4	proanthocyanidin B	12.2	577		579	427, 409, 291, 289, 247, 139		x	x
5	cyanidin-3-galactoside	18.4	447[b]		449[c]	287		x	
6	epicatechin	22.4	289		291	207, 165, 147, 139, 123		x	x
7	cyanidin-3-glucoside	22.8	447[b]		449[c]	287		x	
8	cyanidin-3-arabinoside	24.2	417[b]		419[c]	287		x	
9	proanthocyanidin A	28.6	575	539, 449, 407, 289, 285	577			x	x
10	ferulic acid–hexoside[a]	29.9	355	193,→ 161, 134, 133				x	
11	2″-caffeoylarbutin	30.4	433	323, 203, 179, 161, 135	435			x	x
12	ferulic acid–hexoside[a]	32.1	355	193,→ 161, 134, 133				x	
13	ferulic acid–hexoside[a]	33.1	355	193,→ 161, 134, 133				x	
14	proanthocyanidin A	33.5	575	539, 449, 407, 289, 285	577			x	x
15	coumaroyl-hexose[a]–hydroxyphenol	34.2	417	307, 187, 163, 145, 119				x	x
16	caffeoyl-hexose[a]–hydroxyphenol	34.4	433	323, 203, 179, 161, 135	435			x	x
17	quercetin-3-*O*-β-galactoside	36.9	463	301, 300, 271, 255	465	303	303, 229, 165, 153, 137	x	x
18	coumaroyl-hexose[a]–hydroxyphenol	37.1	417	307, 187, 163, 145, 119				x	x
19	quercetin-3-*O*-glucoside[a]	37.5	463	301, 300, 271, 255	465	303	303, 229, 165, 153, 137	x	x
20	quercetin-*O*-(hexose–deoxyhexoside)[a]	37.8	609	301, 300	611	465, 303	303, 229, 165, 153, 137	x	x
21	quercetin-3-*O*-β-xyloside	38.1	433	301, 300, 271, 255	435	303	303, 229, 165, 153, 137	x	x
22	quercetin-3-*O*-α-arabinoside	38.6	433	301, 300, 271, 255	435	303	303, 229, 165, 153, 137	x	x
23	quercetin-3-*O*-α-arabinofuranoside	39.7	433	301, 300, 271, 255	435	303	303, 229, 165, 153, 137	x	x
24	quercetin-3-*O*-α-rhamnoside (quercitrin)	40.2	447	301, 300, 271, 255	449	303	303, 229, 165, 153, 137	x	x
25	kaempferol–pentoside[a]	42.6	417	285, 284, 255, 227			287, 213, 165, 153, 121	x	x
26	kaempferol–deoxyhexoside[a]	43.4	431	285, 284, 255, 227			287, 213, 165, 153, 121	x	x
27	quercetin-3-*O*-(4″-HMG)-α-rhamnoside[a]	45.0	591	529, 489, 447, 301, 300	593		303, 229, 165, 153, 137	x	x
28	kaempferol–(HMG) rhamnoside[a]	48.0	575	513, 473, 431, 285, 284	577		285, 213, 165, 153, 121	x	x

[a] Hexose, deoxyhexose, and pentose sugar conjugates. [b] Possibly a deprotonated molecule of cyanidin quinoidal form. [c] M^+ ion; → MS/MS of *m*/*z* 193 ion; HMG = 3-hydroxy-3-methylglutaroyl.

identified according to their MS/MS spectra (**Table 1**), which were identical with the literature (*12*). As their spectra were identical with each other, the identification was made according to their typical retention order, according to which catechin elutes before epicatechin (*13*). The catechin polymers, proanthocyanidins **1**, **2**, **4**, **9**, and **14**, were also identified on the basis of their MS/MS spectra, which were similar to those presented earlier (*14*). All of their fragment ions may be difficult to interpret as the multiple losses of water molecules may form several dissociation pathways and all of the cleavages may occur in both monomeric units in the dimeric structure. The spectra of compounds **1**, **2**, and **4** were identical with each other, as well the spectra of compounds **9** and **14**. The main fragment ions at *m*/*z* 427, 409, 291, 289, and 271 are typical in MS/MS of $[M+H]^+$ ions for B-type proanthocyanidins, whereas ions at *m*/*z* 449, 407, 289, and 285 correspond to the MS/MS fragment ions of $[M-H]^-$ ions for A-type proanthocyanidins. However, due to the lack of standard compounds it was not possible to elucidate the structure of the proanthocyanidins in more detail. Also, the compounds were not isolated for NMR measurements due to their low abundance in the samples. Catechins and proanthocyanidins are very typical constituents of *Vaccinium* berries and have been reported in a number of publications (*8*−*10*).

Anthocyanins. Three anthocyanin,s **5**, **7**, and **8**, were detected from the samples and were identified as cyanidin-3-galactoside (**5**), cyanidin-3-glucoside (**7**), and cyanidin-3-arabinoside (**8**) by comparison of the LC-MS data with the ealier literature (*8*, *9*, *15*). Compounds **5** and **7** were distinguished on the basis of their retention order and much higher abundance of compound **5**. Also, the broad chromagraphic peak shape at the pH 2.7 used is typical for anthocyanins. The peak shape was improved very much by using a pH of 1.5 for chromatography, due to the more stable flavylium cation form (data not shown) (*16*).

Flavonols. Compounds **17** and **19**−**28** were identified as conjugates of quercetin and kaempferol. The abundance of kaemferol glycosides was much lower than that of quercetin glycosides, which is in accordance with the earlier literature (*17*). In all of these compounds the flavonol was identified with LC-MS/MS measurement of the aglycone 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 (**Table 1**). For quercetin, the positive ion mode fragment ions after collision of the *m*/*z* 303 were the ions at *m*/*z* values 229, 165, 153, and 137, whereas for kaempferol the corresponding ions after CID of *m*/*z* 287 were seen at *m*/*z* 213, 165, 153, and 121, both being in accordance with the known literature data (*18*). Also, the sugar moieties were identified as hexose, deoxyhexose, or pentose sugars according to the losses of −162, −146, or −132 amu from the molecular ions, respectively.

For compounds **19** and **20** their abundances were so low that the isolated sample amounts were not enough for NMR measurements, and their sugar units were therefore not identified further with spectroscopic methods. Also, their glycosylation sites in the quercetin aglycone could not be identified, but **19** was identified as quercetin-3-*O*-glucoside as it has been reported from the berry in earlier studies (*8*, *9*), and also the *O*-diglycoside **20** (quercetin-hexose-deoxyhexoside) has been identified from the lingonberry (*6*). The accurate mass measured for the $[M-H]^-$ ion of **19** was *m*/*z* 463.0883, whereas the calculated value for $C_{21}H_{19}O_{12}$ is *m*/*z* 463.0877, supporting the identification, and for **20** the *m*/*z* 609.1437 was obtained in comparison to the calculated value of *m*/*z* 609.1456 for $C_{27}H_{29}O_{16}$. In addition, in the CID of the $[M-H]^-$ ion, the intensities of fragment $[Y0-H]^{\bullet-}$ ions at *m*/*z* 300 (homolytic cleavage) in comparison to $Y0^-$ ions (heterolytic cleavage) at *m*/*z* 301 were about 180−200% for both **19** and **20**. These ratios are closely similar to those obtained for other quercetin-3-*O*-

Table 2. NMR Data of the Isolated Compounds **11**, **17**, and **27**

11	δ_H	J_{HH} (Hz)	δ_C	**17**	δ_H	J_{HH} (Hz)	δ_C	**27**	δ_H	J_{HH} (Hz)	δ_C
p-OH-phenol				aglycone				aglycone			
1			152.1	2			156	2			157.4
2 (×2)	6.86 dd	$J_{(23)} = 8.9$ $J_{(22)} = 3$	119.3	3			133.2	3			133.9
3 (×2)	6.66 dd	$J_{(32)} = 8.9$ $J_{(33)} = 3$	116.5	4				4			177.5
4			153.9	5 (OH)			161	5 (OH)	12.61		161
glucose				6	6.2 d	$J_{(68)} = 2$	98.3	6	6.21 d	$J_{(68)} = 2$	98.5
1″	4.95 d	$J_{(1''2'')} = 8.1$	102.1	7 (OH)			163.9	7 (OH)			163.9
2″	5.04 dd	$J_{(2''3'')} = 9.5$	75	8	6.4 d	$J_{(86)} = 2$	93.3	8	6.4 d	$J_{(86)} = 2$	93.5
3″	3.67 dd	$J_{(3''4'')} = 9$	76	9			156	9			156.4
4″	3.49 dd	$J_{(4''5'')} = 10$	71.3	10			103.8	10			104
5″	3.46 m	$J_{(5''6a'')} = 2$ $J_{(5''6b'')} = 5.2$	78.1	1′			120.9	1′			120.5
6a″	3.93 dd	$J_{(6a''6b'')} = 12.1$	62.3	2′	7.52 d	$J_{(2'6')} = 2.2$	115.7	2′	7.3 d	$J_{(2'6')} = 2.1$	115.3*[a]
6b″	3.74 dd			3′ (OH)			144.7	3′ (OH)			145
caffeoyl acid				4′ (OH)			148.2	4′ (OH)			148.3
1′			168.2	5′	6.81 d	$J_{(5'6')} = 8.4$	114.9	5′	6.88 d	$J_{(5'6')} = 8.3$	115.2*
2′	6.33 d	$J_{(2'3')} = 15.9$	114.8	6′	7.66 dd	$J_{(6'5')} = 8.4$ $J_{(6'2')} = 2.2$	121.8	6′	7.24 dd	$J_{(6'5')} = 8.3$ $J_{(6'2')} = 2.1$	120.7
3′	7.61 d	$J_{(3'2')} = 15.9$	147.3	galactose				rhamnose			
4′			127.5	1″	5.37 d	$J_{(1''2'')} = 7.8$	101.5	1″	5.2 d	$J_{(1''2'')} = 1.5$	101.4
5′	7.05 d	$J_{(5'9')} = 2$	115	2″	3.54[b] dd	$J_{(2''3'')} = 9.5$	71	2″	4.02 dd	$J_{(2''3'')} = 3$	69.7
6′			146.7	3″	3.36[b] dd	$J_{(3''4'')} = 3.4$	73	3″	3.74 dd	$J_{(3''4'')} = 9.8$	67.7
7′			149.5	4″	3.65[b] dd	$J_{(4''5'')} = 1$	67.8	4″	4.74 dd	$J_{(4''5'')} = 9.9$	72.7
8′	6.78 d	$J_{(8'9')} = 8.2$	116.4	5″	3.32[b] m	$J_{(5''6a'')} = 6$ $J_{(5''6b'')} = 6.1$	75.6	5″	3.47 m	$J_{(5''6'')} = 6.2$	67.8
9′	6.96 dd	$J_{(9'5')} = 2$ $J_{(9'8')} = 8.2$	122.9	6_a″	3.43[b] dd	$J_{(6a''6b'')} = 11.0$	60	6″	0.75d	$J_{(6''5'')} = 6.2$	16.9
								glutaric acid			
								I			170
								IIa	2.62 d	$J_{(IIaIIb)} = -14.5$	45.3*
								IIb	2.57 d		
								III			68.8
								IVa	2.54 d	$J_{(IVaIVb)} = -15.3$	45.2*
								IVb	2.49 d		
								V			172.2
								VI	1.28 s		27.3

[a] Identifications marked with an asterisk may be vice versa. [b] Chemical shift after D_2O addition into DMSO.

glycosides with the same instrument and same CID parameters, suggesting that the glycosylation sites in both of these compounds were in the 3-position of the quercetin aglycone (data not shown) (*19*). These ratios have been reported to be dependent on the glycosylation site, if instrument type and parameters are carefully adjusted (*19*−*21*).

As compounds **17**, **21**−**24**, and **27** were known to be quercetin glycosides, NMR measurements were mainly used to identify the sugar unit and its glycosylation site in the aglycone (**Tables 2** and **3**). Compound **17** was identified as quercetin-3-*O*-*β*-galactoside (hyperin). The HMBC spectrum showed a correlation between the anomeric galactose proton and quercetin carbon at the 3-position, giving the attachment site of the galactose on quercetin. This flavonoid has also been reported earlier in lingonberry (*8*, *9*). The accurate mass measured for the $[M - H]^-$ ion of **17** was *m*/*z* 463.0885, whereas the calculated value for $C_{21}H_{19}O_{12}$ is *m*/*z* 463.0877.

Compounds **21**−**23** had identical MS/MS spectra, suggesting that they were quercetin−pentose sugar conjugates. The chemical shifts and J_{HH} coupling constants from NMR experiments enabled the sugar units to be identified as *β*-xylose in **21**, α-arabinose in **22**, and α-arabinofuranose in **23**. Due to the low sample amounts obtained after isolation, it was not possible to measure the HMBC spectra of **21** and **22**, and the glycosylation sites of quercetin are not known for sure. However, **21** can be identified as quercetin-3-*O*-*β*-xyloside, which has been reported from the plant earlier (*9*). In addition, also compound **21** had a similar homolytic/heterolytic fragment ion ratio $[Y0 - H]^{\bullet -}$/$Y0^-$ in the CID of the $[M - H]^-$ ion as quercetin-3-*O*-glycosides ($[Y0 - H]^{\bullet -}$ about 350% of $Y0^-$), supporting the identification of the glycosylation site at the 3-position of quercetin (*19*, *21*). The ^1H and ^{13}C chemical shifts of **22** were, however, similar to the ealier data reported for quercetin-3-*O*-α-arabinoside (quaijaverin), enabling the identification (*22*). The accurate mass measurements for **21** and **22** gave *m*/*z* values of 433.0762 and 433.0770, respectively, whereas the calculated value for $C_{20}H_{17}O_{11}$ is *m*/*z* 433.0771. The glycosylation site of arabinofuranose on the quercetin aglycone in **23** was identified with the HMBC correlation between the anomeric sugar proton and the quercetin carbon in the 3-position, enabling the exact identification of the compound as quercetin-3-*O*-α-arabinofuranose (avicularin). The correlation is shown in the HMBC spectrum of **23** in **Figure 4**, as an example of the identification of the glycosylation site. The sugar identification as the furanose form was supported also by the TOCSY correlation between the anomeric proton at the 1″-position and the other sugar proton at the 4″-position. The NMR data of **23** were identical with the earlier literature data (*23*). The accurate mass measured for the $[M - H]^-$ ion of **23** was *m*/*z* 433.0772, whereas the calculated value was the same as for **21** and **22**. Compounds **21** and **22** were reported earlier in the plant (*8*, *9*), but **23** with arabinose in five-membered cyclic form has not been identied earlier to our knowledge.

Similarly to what was stated above, compound **24** was identified as quercetin-3-*O*-α-rhamnose (quercitrin) after elucidating the aglycone as quercetin by MS/MS, identifying the

Table 3. NMR Data of the Isolated Compounds **21**–**24**

21	δ_H	J_{HH} (Hz)	δ_C	**22**	δ_H	J_{HH} (Hz)	δ_C	**23**	δ_H	J_{HH} (Hz)	δ_C	**24**	δ_H	J_{HH} (Hz)	δ_C
aglycone				aglycone				aglycone				aglycone			
2				2				2			156.9	2			157.3
3				3				3			133.4	3			134
4				4				4				4			
5 (OH)	12.61			5 (OH)	12.65			5 (OH)	12.65		160.8	5 (OH)	12.66		161
6	6.2 d	$J_{(68)} = 2$	98.5	6	6.2 d	$J_{(68)} = 2$	98.4	6	6.2 d	$J_{(68)} = 2$	98.4	6	6.2 d	$J_{(68)} = 2.1$	98.5
7 (OH)	10.89			7 (OH)	10.89			7 (OH)	10.89		163.8	7 (OH)	10.9		163.9
8	6.4 d	$J_{(86)} = 2$	93.4	8	6.4 d	$J_{(86)} = 2$	93.1	8	6.41 d	$J_{(86)} = 2$	93.3	8	6.39 d	$J_{(86)} = 2.1$	93.5
9				9				9			156.4	9			156.3
10				10				10			103.9	10			104
1′				1'				1′			120.9	1′			120.7
2′	7.57 d	$J_{(2'6')} = 2$	115.9	2′	7.5 d	$J_{(2'6')} = 2.2$	115.5	2′	7.47 d	$J_{(2'6')} = 2.2$	115.3	2′	7.29 d	$J_{(2'6')} = 2.1$	115.4
3′ (OH)	9.77*[a]			3′ (OH)	9.77*			3′ (OH)	9.74*		144.8	3′ (OH)			144.8
4′ (OH)	9.3*			4′ (OH)	9.21*			4′ (OH)	9.28*		148.1	4′ (OH)			148.1
5′	6.85 d	$J_{(5'6')} = 8.4$	115	5′	6.84 d	$J_{(5'6')} = 8.4$	115.1	5′	6.85 d	$J_{(5'6')}$ 8.4	115.3	5′	6.86 d	$J_{(5'6')} = 8.3$	115.2
6′	7.54 dd	$J_{(6'5')} = 8.4$ $J_{(6'2')} = 2$	121.2	6′	7.66 dd	$J_{(6'5')} = 8.4$ $J_{(6'2')} = 2.2$	121.9	6′	7.55 dd	$J_{(6'5')} = 8.4$ $J_{(6'2')} = 2.2$	121.5	6′	7.25 dd	$J_{(6'5')} = 8.3$ $J_{(6'2')} = 2.1$	120.9
xylose				arabino-pyranose				arabino-furanose				rhamnose			
1″	5.34 d	$J_{(1''2'')} = 7.3$	101.4	1″	5.27 d	$J_{(1''2'')} = 5.2$	101.1	1″	5.58 d	$J_{(1''2'')} = 1.3$	107.6	1″	5.25 d	$J_{(1''2'')} = 1.6$	101.6
2″	3.3[b] dd	$J_{(2''3'')} = 8.7$	69.1[b]	2″	3.75 dd	$J_{(2''3'')} = 7.1$	70.5	2″	4.15 dd	$J_{(2''3'')} = 3.8$	81.9	2″	3.97 dd	$J_{(2''3'')} = 3.3$	69.9
3″	3.19[b] dd	$J_{(3''4'')} = 8.5$	75.9	3″	3.51 dd	$J_{(3''4'')} = 3.3$	71.4	3″	3.71 dd	$J_{(3''4'')} = 6.2$	76.6	3″	3.5 dd	$J_{(3''4'')} = 9.3$	70.2
4″	3.31[b] m	$J_{(4''5a'')} = 9.4$ $J_{(4''5e'')} = 5.3$	73.4[b]	4″	3.64 m	$J_{(4''5a'')} = 5.4$ $J_{(4''5b'')} = 2.7$	65.8	4″	3.54 m	$J_{(4''5a'')} = 3.7$ $J_{(4''5b'')} = 5.2$	85.6	4″	3.14 dd	$J_{(4''5'')} = 9.4$	71
$5_e''$	3.63 dd	$J_{(5a''5e'')} = 11.4$	65.8	$5_a''$	3.59 dd	$J_{(5a''5b'')} = 11.5$	64	$5_a''$	3.32 dd	$J_{(5a''5b'')} = -11.9$	60.4	5″	3.2 m	$J_{(5''6'')} = 6.1$	70.5
$5_a''$	2.96 dd			$5_b''$	3.21dd			$5_b''$	3.27 dd			6″	0.81 d	$J_{(6''5'')} = 6.1$	17.3

[a] Identifications marked with an asterisk may be vice versa. [b] Chemical shift after D_2O addition into DMSO.

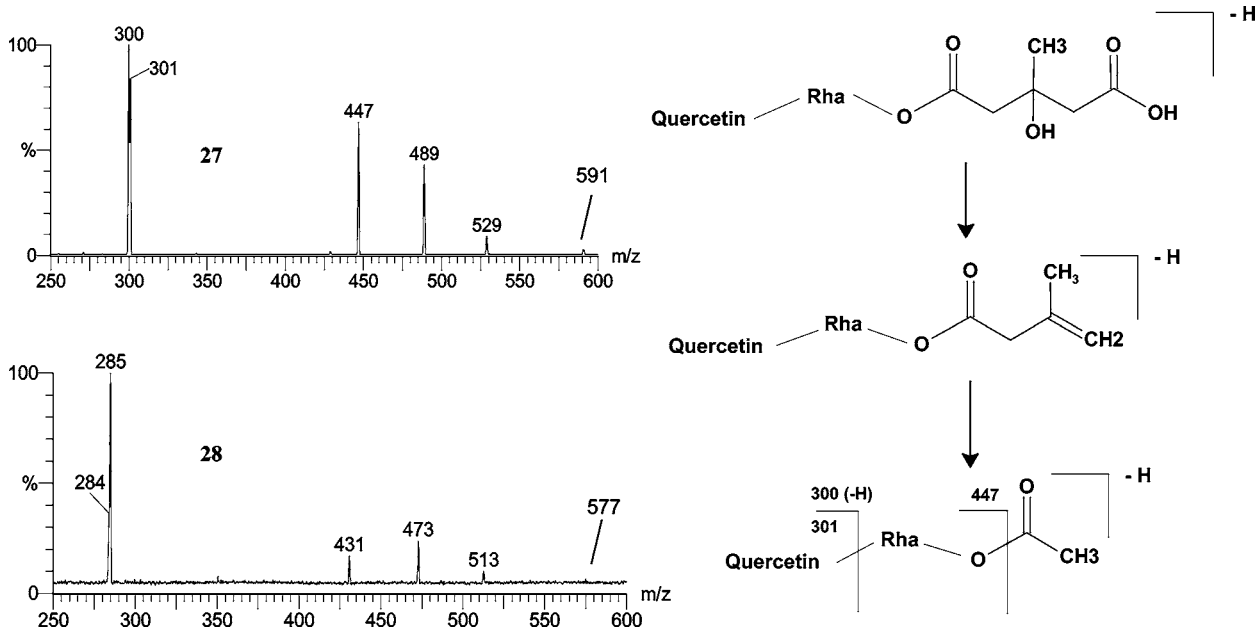

Figure 2. ESI$^-$ MS/MS spectra of compounds **27** (**A**) and **28** (**B**) and the suggested fragmentation pathway for compound **27**.

sugar unit as α-rhamnose with J_{HH} coupling constants and chemical shifts, and finally localizing the glycosylation site by correlation between rhamnose anomeric proton and quercetin 3-carbon in HMBC. The identification was also confirmed by using authentic quercitrin standard. Quercitrin is also a compound known to exist in lingonberry (*8*, *9*). The accurate mass measured for the [M − H]$^-$ ion of **24** was *m*/*z* 447.0919, whereas the calculated value for $C_{21}H_{19}O_{11}$ is *m*/*z* 447.0927.

Compounds **25** and **26** were identified as pentose and deoxyhexose sugar conjugates of kaempferol, respectively. Kaempferol is known to be a very minor component of lingonberry flavonols, and only kaempferol-3-glucoside has been reported from the berry, but not these two compounds (*8*, *9*).

Compounds **27** and **28** were identified as quercetin-3-*O*-[4″-(3-hydroxy-3-methylglutaroyl)]-α-rhamnose and kaempferol-3-*O*-[4″-(3-hydroxy-3-methylglutaroyl)]-α-rhamnose, respectively. Their ESI$^-$/MS/MS spectra are shown in **Figure 2**, together with the suggested fragmentation pathway using compound **27** as an example. Both compounds show similar fragmentation patterns, except that all of the peaks in the spectrum of compound **28** have *m*/*z* ratios that are 16 units lower. The spectra showed the losses of the conjugates from the flavonol aglycones as both heterolytic and homolytic cleavages, leading to fragment ions at *m*/*z* 301 and 300 for **27** and at *m*/*z* 284 and 285 for **28**. The ions at *m*/*z* 447 and *m*/*z* 431 are due to the losses of 3-hydroxy-3-methylglutaroyl units from the rhamnose sugar, and the rest of the fragment ions are due to the fragment ions cleaving from this 3-hydroxy-3-methylglutaroyl unit. The identification of the sugar groups and the 3-hydroxy-3-methylglutaroyl units was confirmed using NMR experiments for compound **27**. The sugar moiety was identified as a rhamnose, and the structure of the 3-hydroxy-3-methylglutaroyl unit was confirmed using NMR experiments (TOCSY, HSQC, HMBC, DEPT). The HMBC spectrum showed a correlation between

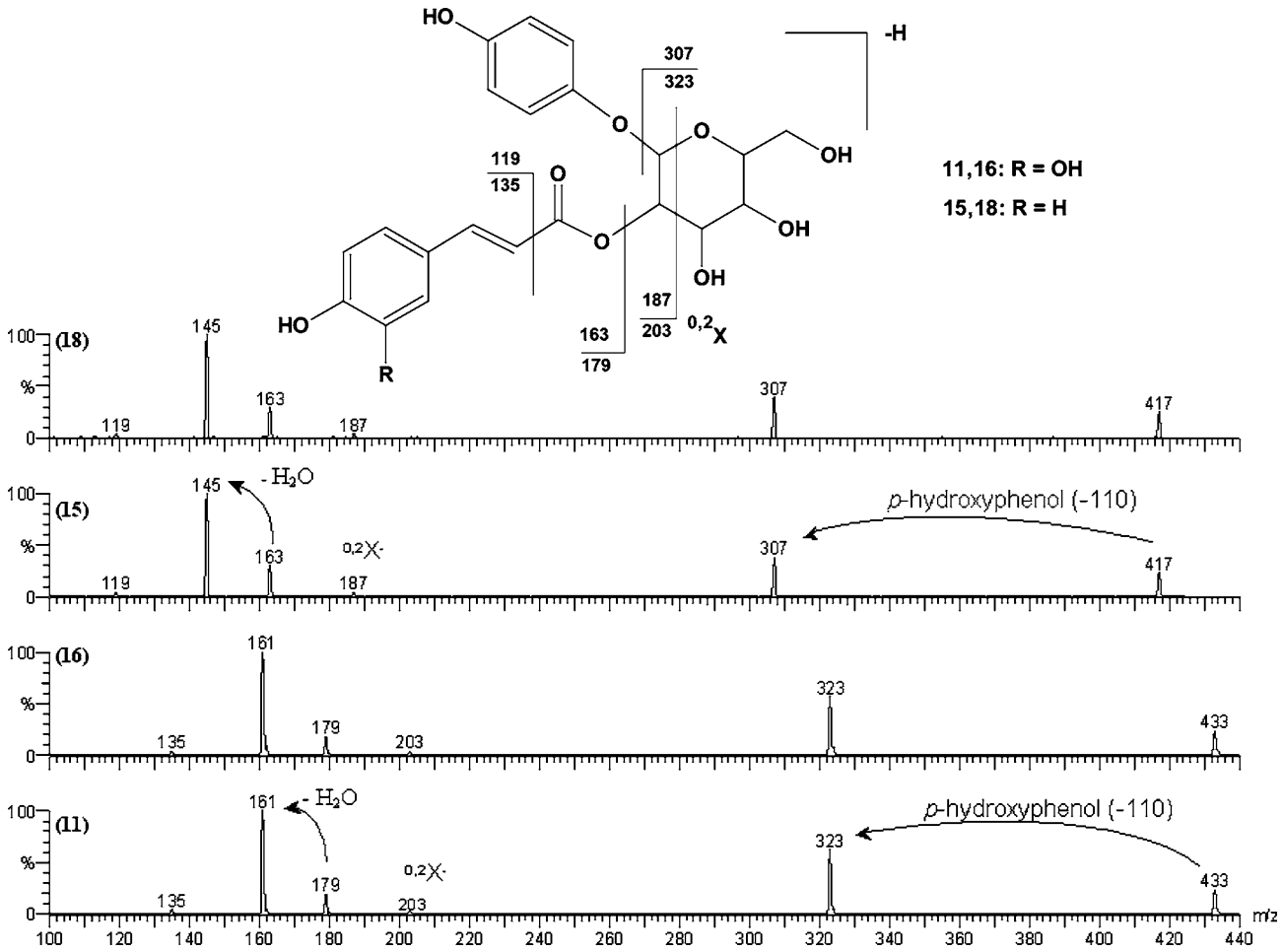

Figure 3. ESI$^-$ MS /MS spectra of compounds **11**, **15**, **16**, and **18**, together with the suggested fragmentation pathway.

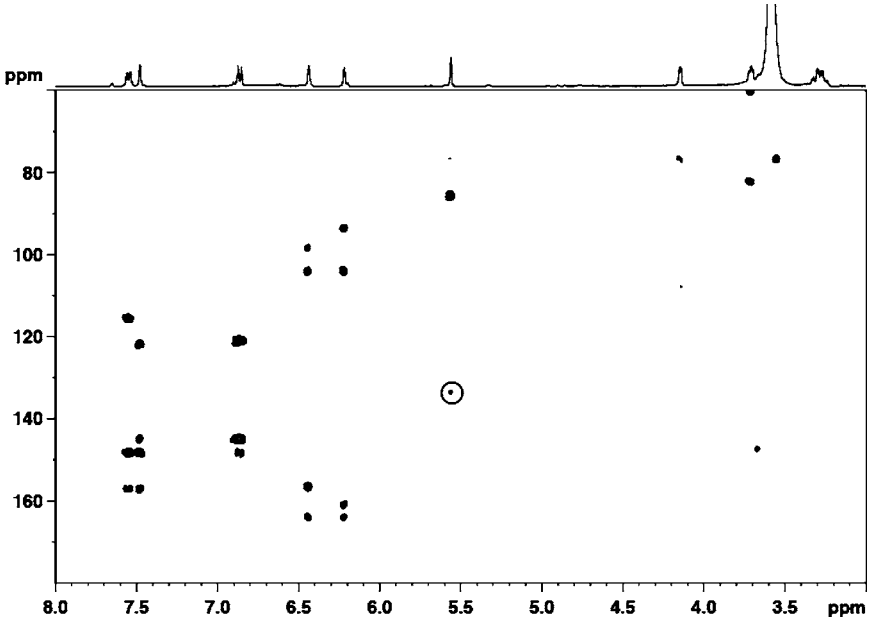

Figure 4. HMBC spectrum of compound **23**. The correlation used for elucidating the glycosylation site is marked.

the rhamnose 4″-proton and the carbonyl group of the 3-hydroxy-3-methylglutaroyl unit, localizing the attachment site on the rhamnose moiety. The same HMBC spectrum showed also a correlation between the rhamnose 1′-proton and the quercetin carbon at position 3, localizing also the attachment site of the rhamnose on flavonol aglycone. The accurate mass measured for [M − H]$^-$ ion of **27** was *m*/*z* 591.1346, whereas the calculated value for $C_{27}H_{27}O_{15}$ is *m*/*z* 591.1350. To our knowledge, these two flavonol acylglucosides have not been reported earlier, but the closely similar quercetin-3-*O*-(6″-3-hydroxy-3-methylglutaroyl)-*β*-galactoside has been reported from blueberry (*24*, *25*).

Other Phenolics. Compound **11** was identified as 2″-caffeoylarbutin according to its MS and NMR data. The pair

of doublet of doublets with integral of two protons at ^{1}H chemical shifts 6.66 and 6.86 ppm and mutual coupling constants of 8.9 and 3 Hz were identified as *p*-hydroxyphenol protons. The caffeoyl group was easily identified from COSY, HSQC, and HMBC spectra, the data being identical with the literature (*26*). The double-bond configuration was shown to be trans according to the 15.9 Hz vicinal coupling constant between the double-bond protons. The sugar moiety was identified as glucose by MS data and using J_{HH} coupling constants and chemical shifts. The HMBC spectrum showed a correlation between the anomeric glucose proton and *p*-hydroxyphenol carbon and a correlation between the glucose 2″-proton and the caffeoyl group carbonyl carbon, supporting the identification. The accurate mass measured for the $[M - H]^-$ ion of **11** was *m*/*z* 433.1138, whereas the calculated value for $C_{21}H_{21}O_{10}$ is *m*/*z* 433.1135. The compound has been reported in the lingonberry earlier (*27*). Compound **16** showed similar mass spectrometric data as **11** and was therefore identified as its isomer caffeoyl-hexose(sugar)-hydroxyphenol. However, as the compound was not isolated for NMR studies, it was not possible to elucidate its exact structure from the data. Compounds **15** and **18** also showed similar mass spectrometric fragmentation in comparison to **11** and **16**, the only difference being a 16 amu lower molecular weight and fragment ions. On this basis, compounds **15** and **18** were identified as isomeric forms of coumaroyl-hexose (sugar)-hydroxyphenol. **Figure 3** shows the MS/MS spectra and suggested fragmentation for these compounds. As the structure and stereochemistry of compound **11** are exactly known from the NMR experiments, compound **16** may be its cis isomer, or alternatively the glucose is replaced by galactose. Corresponding structural difference (cis/trans or glucose/galactose) between compounds **15** and **18** may also be speculated. To our knowledge, compounds **15**, **16**, and **18** have not been reported earlier from the plant, even though a number of coumaroyl conjugates are known to exist, similar to number of ferulic acid conjugates (*8*, *9*). Compounds **10**, **12**, and **13**, which were detected only in the berry samples, were identified as hexose sugar conjugates of ferulic acid according to their MS/MS spectra of the $[M - H]^-$ ions at *m*/*z* 355 that showed loss of hexose sugar (−162 amu) and further MS/MS spectra of the aglycone fragment ion *at m*/*z* 193, which resulted in fragment ions at *m*/*z* 161 and 133 due to neutral losses of methanol and acetic acid. The three isomeric forms may have different hexose moieties, or they may differ in cis/trans configuration of the double bond.

Twenty-eight phenolic compounds were characterized from the berries and leaves/stems of lingonberry using LC-MS/MS and NMR methods, most structures being identified unambiguously. Six of these compounds were detected only in the berries, whereas the rest were detected in both sample types. Eight of the compounds were reported for the first time from the lingonberry, and two of the compounds, flavonol acylglycosides quercetin-3-*O*-[4″-(3-hydroxy-3-methylglutaroyl)]-α-rhamnose and kaempferol-3-*O*-[4″-(3-hydroxy-3-methylglutaroyl)]-α-rhamnose, were reported entirely for the first time.

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