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Structural Investigations of Flavonol Glycosides from Sea Buckthorn (*Hippophaë rhamnoides*) Pomace by NMR Spectroscopy and HPLC-ESI-MS^n

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Four flavonol glycosides were isolated from an extract of sea buckthorn pomace (*Hippophaë rhamnoides*) by Sephadex LH-20 gel chromatography and semipreparative HPLC. Their structures were elucidated by hydrolysis studies, ESI-MS^n, UV, and ^{1}H and ^{13}C NMR spectroscopy. The occurrence of the major flavonol glycoside kaempferol 3-*O*-β-sophoroside-7-*O*-α-rhamnoside in sea buckthorn is described here for the first time. A further 21 flavonol glycosides of Sephadex LH-20 fractions of sea buckthorn pomace were characterized by HPLC-DAD-ESI-MS. The characteristic MS-MS and MS^3 fragmentation pattern of flavonol glycosides previously identified in sea buckthorn juice and of flavonol glycosides identified by NMR spectroscopy gave valuable indications for their identification. The results demonstrate that loss of the sugar moiety from C-7 of the aglycon is more favored than fission of the glycosidic linkage at the C-3 position. Thus, most of the compounds identified were 7-rhamnosides of isorhamnetin, kaempferol, and quercetin, which exhibit different substitution patterns at the C-3 position, mainly glucosides, rutinosides, and sophorosides. In addition, numerous flavonol glycosides were detected lacking a sugar moiety at C-7. Finally, eight flavonol derivatives were identified that are acylated by hydroxybenzoic or hydoxycinnamic acids.

KEYWORDS: Sea buckthorn; *Hippophaë rhamnoides*; polyphenols; flavonol glycosides; kaempferol 3-sophoroside-7-rhamnoside; HPLC; ESI-MS; ^{1}H; ^{13}C; 2D NMR

INTRODUCTION

Epidemiological studies indicate that the dietary intake of flavonoids from fruit and vegetables is inversely related to coronary heart disease mortality (*1*, *2*). The oxidative damage of human low-density lipoprotein (LDL) by reactive oxygen species (ROS) is suggested to be responsible for the development of atherosclerotic diseases (*3*). Inhibition of LDL oxidation by phenolic antioxidants from food has been extensively studied in vitro (*4*, *5*) and has been mainly related to their radical scavenging activity.

Residues such as peels, seeds, pomace, or process waters resulting from food processing may contain substantial amounts of valuable natural antioxidants. The growing interest in the substitution of synthetic food antioxidants by natural ones has led to a considerable number of investigations, which have been reviewed by Moure et al. (*6*).

The fruits of sea buckthorn (*Hippophaë rhamnoides*) are rich sources of flavonols (1500−2000 mg/kg) (*7*), which are reported to consist of glycosides of isorhamnetin, quercetin, and kaempferol (*8*−*11*). Also, other natural antioxidants were found in high concentrations including proanthocyanidins (1100−2900 mg/kg) (*7*), ascorbic acid (500−14000 mg/kg), carotenoids (150−430 mg/kg) (*12*), and tocopherols (50−130 mg/kg) (*13*), which showed a considerable variation within and among the natural populations. Juice extraction from the berries leads to residual press cake, which can be used for seed separation allowing extraction of seed oil. The remaining pulp is a suitable source of "sea buckthorn yellow", which can be extracted by low-percentage alcohol (*14*). This pigment contains mainly flavones and has a potential use as a food coloring material, but also the use of its antioxidant potential would be conceivable.

In our recent work (*15*) we investigated the content of flavonol glycosides, catechins, and phenolic acids in sea buckthorn juice. Furthermore, it has been demonstrated that substitution of hydroxyl groups by sugars or methyl groups had a remarkable influence on the ESR detected antioxidant capacity of flavonols. The aim of this work was the isolation and structure elucidation of major unknown flavonol glycosides of sea buckthorn pomace including the exact determination of the sugar linkages. Further flavonol glycosides that were not isolated in pure state were tentatively identified by high-performance liquid

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chromatography with diode array detection coupled with on-line mass spectroscopy with electrospray ionization source (HPLC-DAD-ESI-MS).

MATERIALS AND METHODS

Reagents and Solvents. Reagents and solvents were purchased from Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany) and were of HPLC or analytical grade quality. HPLC grade water was purified with a deionized water treatment system.

Extraction of Plant Material. Sea buckthorn fruits (*Hippophaë rhamnoides* subsp. *rhamnoides* cv. Hergo) harvested in September 2002 were manufactured into juice as described previously (*15*). The resulting pomace (500 g) was extracted by stirring with 1.2 L of acetone/water (75:25, v/v) at ambient temperature for 1 h. After filtration through a Büchner funnel, acetone was removed using a rotary evaporator, and an aliquot of 175 mL of aqueous solution (corresponding to 250 g of pomace) was defatted with petroleum ether (3 × 200 mL) and freeze-dried.

Isolation and Purification of Flavonol Glycosides. The defatted extract was dissolved in 50 mL of water and applied to a 60 × 4 cm i.d. Sephadex LH-20 column (Pharmacia, Uppsala, Sweden) previously equilibrated with water. After the column had been rinsed with 400 mL of water (discarded), fractions A−M were collected by increasing the methanol content of the eluent from 0 to 100% (v/v) in increments of 10% (400 mL each), followed by 2 × 400 mL fractions of acetone/water (70:30, v/v).

After methanol evaporation of Sephadex LH-20 fraction F (50% methanol), a precipitate developed consisting mainly of flavonol glycoside **10**. Polar impurities were removed by washing the precipitate with water, and after lyophilization, 9.4 mg of flavonol glycoside **10** was obtained.

For enzymatic hydrolysis a solution of flavonol glycoside **10** (9.0 mg) in 0.1 M acetate buffer (pH 5) was incubated with 10 mg of β-glucosidase (7.1 units/mg) (Boehringer, Ingelheim, Germany) at 37 °C. The reaction was monitored by HPLC-DAD as previously described (*15*), and further 10 mg portions of β-glucosidase were added until it was complete. The solution was applied to a 10 × 1 cm i.d. Sephadex LH-20 column and fractionated with 20 mL of water (discarded) and 20 mL of methanol. After evaporation of methanol, 8.9 mg of impure solid were cleaned up by semipreparative HPLC on a 250 × 10 mm i.d., 5 μm, Fluofix 120E column (NEOS Co., Ltd., Kobe, Japan) connected to a 10 × 10 mm i.d., 5 μm, Hypersil BDS C8 guard column. Using two solvents [A, water/acetic acid (99.5:0.5, v/v); B, acetonitrile/water/acetic acid (50:49.5:0.5, v/v/v)], the sample was fractionated by isocratic elution (70% A). After chromatographic separation was performed at a flow rate of 5 mL/min using a Gynkothek model 480 HPLC pump (Dionex, Germering, Germany), the flow was split into 0.1 mL/min for detection at 280 nm using a variable-wavelength UV−vis detector (Linear Instruments, Reno, NV) and 4.9 mL/min for fraction collection. After solvent evaporation, 3.2 mg (48%) of flavonol glycoside **1** was obtained.

For isolation of compounds **15** and **16** Sephadex LH-20 fraction B (10% methanol) was evaporated to yield 200 mg of solid and further fractionated by semipreparative HPLC (90% A). A final purification step was necessary (92% A) to yield pure compounds **15** (10.4 mg) and **16** (8.3 mg).

Acid Hydrolysis. After dissolving 1−2 mg in 1 mL of 1 N HCl (flavonol glycosides **10**, **15**, and **16** and lyophilized Sephadex LH-20 fractions C−H) or 1 N methanolic HCl (flavonol glycoside **1**), flavonol glycosides were hydrolyzed by heating to 80 °C for 4 h in a pressure-stable vial. After lyophilization, the residue was dissolved in methanol and the aglycons were identified by HPLC-DAD as previously described (*15*). For sugar analysis aliquots of the methanolic solutions were applied to a silica gel 60, 20 × 10 cm HPTLC plate (Merck, Darmstadt, Germany) and developed with chloroform/methanol/water (65:30:5, v/v/v). Sugar spots were visualized by aniline/diphenylamine/phosphoric acid (*16*), and sugars were identified by comparison with R_f values and coloring of reference substances.

UV−Vis Spectroscopy. UV−vis spectra of flavonol glycosides **1**, **10**, **15**, and **16** and bathochromic shifts after addition of diagnostic reagents were recorded with an Uvikon 930 spectrometer (Kontron, Milano, Italy) according to the method of Mabry et al. (*17*).

NMR Spectroscopy. ^{13}C, $^{1}H-^{1}H$-COSY, and HMQC spectra of flavonol glycosides **10**, **15**, and **16** dissolved in DMSO-d_6 were recorded using a DPX 300 MHz spectrometer (Bruker, Rheinstetten, Germany). ^{1}H and ROESY spectra of flavonol glycosides **1**, **10**, **15**, and **16** were measured with an AMX 600 MHz spectrometer (Bruker).

ESI-MS. ESI mass spectra (negative ionization) of purified flavonol glycosides **1**, **10**, **15**, and **16** were recorded using an Agilent 1100 series LC/MSD trap controlled by LCMSD software (version 4.1). Methanolic solutions of flavonol glycosides (10 mg/L) were injected using a syringe pump (5 mL/min). Nitrogen was used as dry gas (5 L/min, 300 °C) and nebulizer gas (15 psi). The capillary, end plate, and capillary exit voltages were set at 3500, −500, and −120 V, respectively. The full-scan mass spectra were measured from *m*/*z* 100 to *m*/*z* 1000. MS-MS and MS^n spectra were recorded by isolation and fragmentation of the pseudomolecular ion of interest.

High-Resolution Mass Spectrometry (HRMS). HRMS (positive or negative electrospray ionization) was applied on a LCQ-MS (Finnigan MAT, Bremen, Germany).

*Isorhamnetin 7-O-α-L-rhamnoside (**1**)* was obtained as a yellow amorphous solid: UV λ_{max} (nm, MeOH) 254, 269 sh, 289 sh, 372; +NaOMe 247, 266, 323 sh, 435 (slow decomposition); +NaOAc 255, 294 sh, 375; +NaOAc+H_3BO_3 255, 300 sh, 375; ESI-MS, *m*/*z* (relative intensity) 497 $[M + Cl]^-$ (17), 461 $[M - H]^-$ (100); MS-MS of 461, 446 $[M - H - CH_3]^{2-}$ (7), 315 $[M - H - rha]^-$ (100), 300 $[M - H - rha - CH_3]^{2-}$ (6); HRMS (negative ionization), *m*/*z* 461.1072 found, 461.1078 calcd for $C_{22}H_{21}O_{11}$; ^{1}H NMR data are shown in **Table 2**.

*Isorhamnetin 3-O-β-D-glucoside-7-O-α-L-rhamnoside (**10**):* was obtained as a yellow amorphous solid: UV λ_{max} (nm, MeOH) 254, 267 sh, 356; +NaOMe 247 sh, 265, 400; +NaOAc 255, 266 sh, 360, 426 sh; +NaOAc+H_3BO_3 255, 266 sh, 350; ESI-MS, *m*/*z* (relative intensity) 659 $[M + Cl]^-$ (29), 623 $[M - H]^-$ (100); MS-MS of 623, 477 $[M - H - rha]^-$ (100), 461 $[M - H - glu]^-$ (26), 315 $[M - H - rha - glu]^-$ (31); HRMS (positive ionization), *m*/*z* 647.1582 found, 647.1583 calcd for $C_{28}H_{32}O_{16}Na$; ^{13}C and ^{1}H NMR data are shown in **Tables 1** and **2**.

*Isorhamnetin 3-O-β-D-sophoroside-7-O-α-L-rhamnoside (**15**)* was obtained as a yellow amorphous solid: UV λ_{max} (nm, MeOH) 254, 266 sh, 356; +NaOMe 248 sh, 266, 397; +NaOAc 257, 266 sh, 347, 413 sh; +NaOAc+H_3BO_3 255, 266 sh, 359; ESI-MS, *m*/*z* (relative intensity) 821 $[M + Cl]^-$ (100), 785 $[M - H]^-$ (28); MS-MS of 785, 639 $[M - H - rha]^-$ (100); MS^3 of 785 → 639, 477 $[M - H - rha - glu]^-$ (3), 459 $[M - H - rha - glu - H_2O]^-$ (21), 315 $[M - H - rha - glu - glu]^-$ (100); HRMS (positive ionization), *m*/*z* 809.2108 found, 809.2111 calcd for $C_{34}H_{42}O_{21}Na$; ^{13}C and ^{1}H NMR data are shown in **Tables 1** and **2**.

*Kaempferol 3-O-β-D-sophoroside-7-O-α-L-rhamnoside (**16**)* was obtained as a yellow amorphous solid: UV λ_{max} (nm, MeOH) 266, 318 sh, 350; +NaOMe 243, 270, 300 sh, 341 sh, 384; +NaOAc 266, 354, 408sh; +NaOAc+H_3BO_3 266, 318sh, 351; ESI-MS, *m*/*z* (relative intensity) 791 $[M + Cl]^-$ (100), 755 $[M - H]^-$ (89); MS-MS of 755, 609 $[M - H - rha]^-$ (100); MS^3 of 755 → 609, 447 $[M - H - rha - glu]^-$ (10), 429 $[M - H - rha - glu - H_2O]^-$ (71), 285 $[M - H - rha - glu - glu]^-$ (100); HRMS (positive ionization), *m*/*z* 779.2006 found, 779.2005 calcd for $C_{33}H_{40}O_{20}Na$; ^{13}C and ^{1}H NMR data are shown in **Tables 1** and **2**.

HPLC-DAD-ESI-MS. The Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) consisted of a binary HPLC pump, an autosampler, a degasser, a thermostat, and a photodiode array detector and was controlled by ChemStation software. Chromatographic separation of membrane-filtered (0.45 μm PTFE) (Roth, Karlsruhe, Germany) Sephadex LH-20 fractions C−H of pomace extract was carried out on a 250 × 4.6 mm i.d., 5 μm, Fluofix 120E column (NEOS Co. Ltd.) connected to a 10 × 4.6 mm i.d. guard column of the same material using two solvents [A, water/acetic acid (99.5:0.5, v/v); B, acetonitrile]. Gradient elution was performed as follows: 5−35% B in 30 min; 35−50% B in 5 min; 50% B (5 min); 50−5% B in 1 min; 5% B (15 min) at a flow rate of 1.0 mL/min and a column temperature of 30 °C. After detection by DAD at 280 and 350 nm (spectroscopic contour plots from 200 to 400 nm), the flow was split,

Table 1. ^{13}C NMR Spectroscopic Data of **10**, **15**, and **16** in DMSO-d_6 (300 MHz)[a]

carbon	10	15	16
aglycon			
2	156.9	156.5	156.0
3	133.3	133.3	133.3
4	177.6	177.7	177.7
5	161.0	161.0	161.0
6	99.4	99.4	99.4
7	161.6	161.7	161.7
8	94.7	94.7	94.4
9	156.0	156.0	156.2
10	105.7	105.7	105.6
1′	121.0	121.0	120.8
2′	113.5	113.1	131.2
3′	149.7	149.7	115.4
4′	147.0	147.2	160.2
5′	115.3	115.5	115.4
6′	122.4	123.1	131.2
OMe	55.8	55.9	
sugar at C-3			
1″	100.8	98.3	97.9
2″	74.4	82.2	82.4
3″	76.5	76.7	76.7
4″	70.1	69.6	69.6
5″	77.6	76.6	76.6
6″	60.6	60.6	60.5
1‴		103.6	104.1
2‴		74.4	74.4
3‴		77.0	77.0
4‴		70.2	70.1
5‴		77.6	77.6
6‴		60.9	60.8
sugar at C-7			
1‴	98.4		
2‴	70.1		
3‴	70.3		
4‴	71.7		
5‴	69.9		
6‴	18.0		
1⁗		98.4	98.4
2⁗		69.9	69.8
3⁗		70.3	70.2
4⁗		71.7	71.6
5⁗		69.8	69.5
6⁗		18.1	18.1

[a] Chemical shifts (δ) are in ppm. Assignments were confirmed by HMQC, but carbons having almost the same chemical shifts may be reversed. ^{13}C NMR of **1** was not measured because of limited amounts.

and 0.35 mL/min was subjected to ESI-MS analysis. ESI-MS experiments were performed as described above with the exception of the conditions of dry and nebulizer gas, which were set at 10 L/min (350 °C) and 40 psi, respectively. MS-MS and MS^3 experiments were performed by isolation and fragmentation of the most abundant pseudomolecular ion.

RESULTS AND DISCUSSION

Sea buckthorn pomace extract was fractionated on a Sephadex LH-20 column using a water/methanol gradient (*18*). Flavonol glycosides of Sephadex LH-20 fractions C−H (20−70% methanol) of sea buckthorn pomace were separated by HPLC and detected at 350 nm (**Figure 1**). Three major flavonol glycosides (**10**, **15**, and **16**) were isolated and further purified by semipreparative HPLC on a Fluofix stationary phase. The structures of these compounds were elucidated by NMR spectroscopy, and other components were identified by HPLC-DAD-ESI-MS investigations of the Sephadex LH-20 fractions.

Structure Elucidation of Flavonol Glycosides. Acid hydrolysis of flavonol glycoside **10** yielded the aglycon isorhamnetin, which was identified by HPLC-DAD (*15*). HPTLC analysis (*16*) of the sugars released showed the presence of β-D-glucose and α-L-rhamnose, in the ratio 1:1. The ESI mass spectrum (negative ionization) of compound **10** showed a quasi molecular ion peak at *m*/*z* 623 $[M - H]^-$ and the adduct *m*/*z* 659 $[M + Cl]^-$. MS-MS fragmentation of *m*/*z* 623 produced ions at *m*/*z* 477 $[M - H - 146]^-$, *m*/*z* 461 $[M - H - 162]^-$, and *m*/*z* 315 $[M - H - 146 - 162]^-$, which proved compound **10** to be an isorhamnetin-glucorhamnoside. The UV spectroscopic analysis of **10** using customary shift reagents indicated the presence of a 3,7-diglycoside (*17*). Final identification of **10** was performed by ^1H and ^{13}C NMR spectroscopy. The ^1H NMR spectrum (**Table 2**) allowed us to assign all flavonol aglycon protons, the rhamnose methyl protons, and the β-D-glucose and α-L-rhamnose anomeric protons (*19*). The positions of sugar linkages were derived from the ^{13}C spectrum by comparison with data from the literature (*20*, *21*). The chemical shift of δ 133.3 observed for the aglycon C-3 of **10** was consistent with the shifts of isorhamnetin 3-*O*-glycosides. Ito et al. (*21*) observed a remarkable upfield shift ($\Delta\delta$ 2.7) for the C-7 of an isorhamnetin 3,7-diglycoside (δ 161.8) compared to an isorhamnetin 3-glycoside (δ 164.5). Our findings showed a chemical shift of δ 161.6 for C-7 (**Table 1**); thus, flavonol

Table 2. ^1H NMR Spectroscopic Data of **1**, **10**, **15**, and **16** in DMSO-d_6 (600 MHz)[a]

proton	1	10	15	16
aglycon				
6	6.42 d (1.7)	6.44 d (2.0)	6.43 d (2.1)	6.42 d (2.1)
8	6.87 d (1.7)	6.83 d (2.0)	6.85 d (2.1)	6.82 d (2.1)
2′	7.78 br d	7.94 d (1.9)	7.81 d (2.1)	8.07 d (8.9)
3				6.91 d (8.9)
5′	6.93 d (8.5)	6.93 d (8.5)	6.92 d (8.4)	6.91 d (8.9)
6′	7.76 br dd	7.55 dd (8.5, 1.9)	7.68 dd (8.4, 2.1)	8.07 d (8.9)
OMe	3.84 s	3.83 s	3.85 s	
sugar at C-3				
1″		5.57 d (6.7)	5.74 d (7.2)	5.69 d (7.3)
2″		3.2 m[b]	3.52 m[b]	3.45 m[b]
3″		3.2 m	3.52 m[b]	3.45 m
4″		3.1 m	3.32 m[b]	3.1–3.3 m
5″		3.1 m	3.12 m	3.1–3.3 m
6a″		3.57 d (11.9)	3.52 m	3.45 m
6b″		3.45 m	3.32 m	3.29 m
1‴			4.61 d (7.8)	4.61 d (7.8)
2‴			3.04 m[b]	3.08 m[b]
3‴			3.4 m[b]	3.1–3.3 m
4‴			3.3 m[b]	3.1–3.3 m
5‴			3.12 m	3.1–3.3 m
6a‴			3.52 m	3.56 m
6b‴			3.43 m	3.45 m
sugar at C-7				
1″	5.56 d (1.4)			
2″	3.83 br s			
3″	3.63 br d			
4″	3.29 m			
5″	3.44 m			
6″	1.11 d (6.2)			
1‴		5.55 br s		
2‴		3.83 br s[b]		
3‴		3.63 dd (9.2, 3.2)[b]		
4‴		3.3 m[b]		
5‴		3.4 m[b]		
6‴		1.10 d (6.1)		
1⁗			5.56 d (1.3)	
2⁗			3.83 m[b]	5.54 d (1.4)
3⁗			3.62 dd (9.3, 3.1)[b]	3.83 dd (3.1, 1.8)[b]
4⁗			3.32 m[b]	3.62 dd (9.3, 3.3)[b]
5⁗			3.43 m[b]	3.25 m[b]
6⁗			1.11 d (6.2)	3.45 m[b]

[a] Chemical shifts (δ) are in ppm, and coupling constants (*J*) in Hz are given in parentheses. [b] Signals were assigned by ^1H–^1H-COSY.

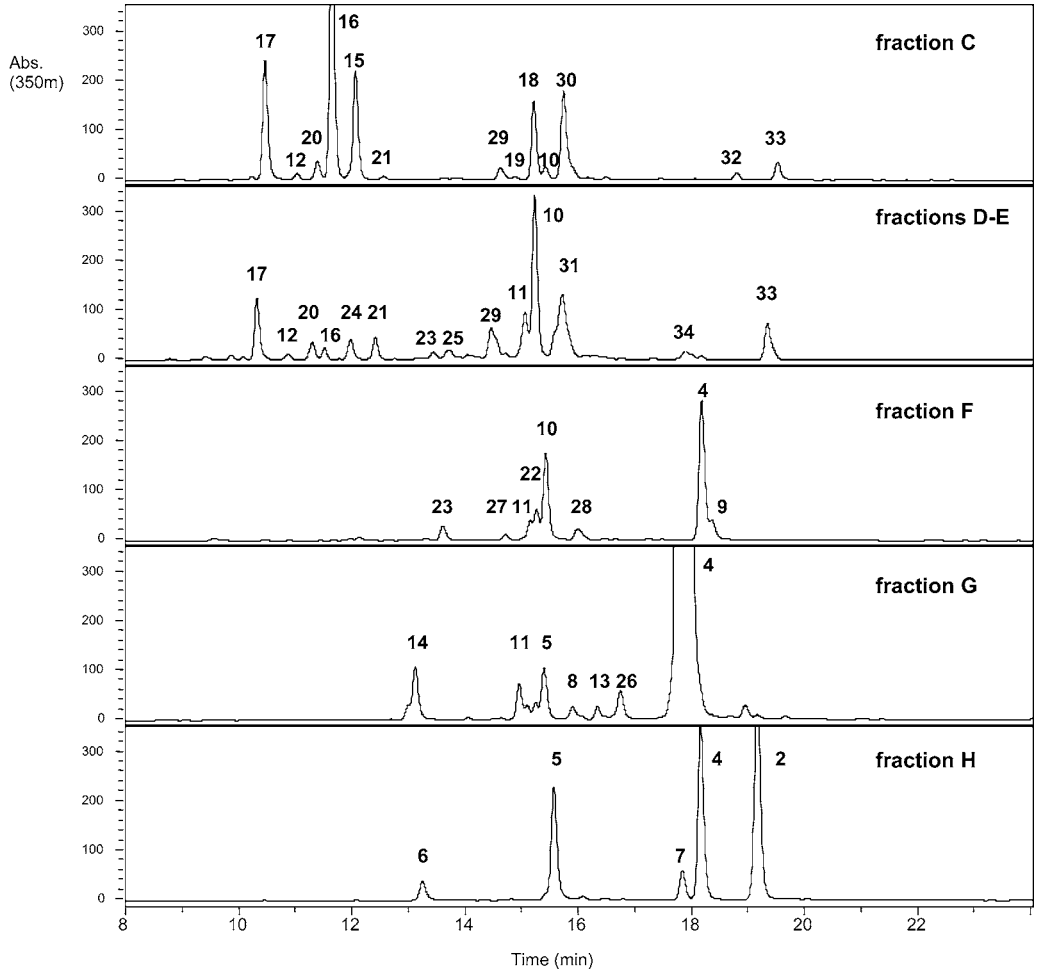
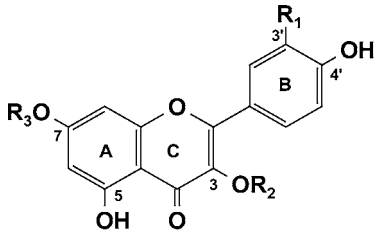


Figure 1. HPLC-DAD (350 nm) plots of Sephadex LH-20 fractions C–H of sea buckthorn pomace extract. For peak identification see **Table 3**.



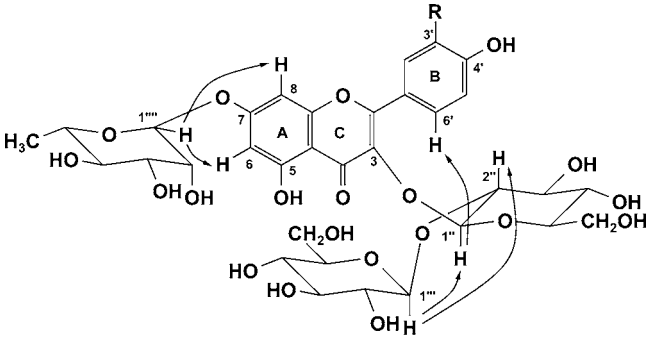
1 R_1=OCH_3, R_2=H, R_3=*α-L*-rhamnose
10 R_1=OCH_3, R_2=*β-D*-glucose, R_3=*α-L*-rhamnose
15 R_1=OCH_3, R_2=*β-D*-sophorose, R_3=*α-L*-rhamnose
16 R_1=H, R_2=*β-D*-sophorose, R_3=*α-L*-rhamnose

Figure 2. Structures of flavonol glycosides **1**, **10**, **15**, and **16** identified in sea buckthorn pomace.

glycoside **10** has to be an isorhamnetin 3,7-diglycoside. The ^{1}H NMR chemical shifts (**Table 2**) of the anomeric protons of *β*-D-glucose (δ 5.57, d, J = 6.7 Hz, H-1″) and α-L-rhamnose (δ 5.55 ppm, br s, H-1‴) indicated that glucose is linked to the C-3 and rhamnose to the C-7 of isorhamnetin (*19*). An opposite assignment would require a signal between δ 4.90 and 5.05 for *β*-D-glucose and a signal between δ 4.96 and 5.36 for α-L-rhamnose. Therefore the structure isorhamnetin 3-*O*-*β*-D-glucoside-7-*O*-α-L-rhamnoside was assigned to **10** (**Figure 2**).

This assignment could be confirmed after enzymatic hydrolysis of **10** using *β*-glucosidase (*21*), which produced flavonol glycoside **1**. ESI-MS analysis of **1** showed a base peak at *m/z* 461 $[M - H]^-$ and the adduct *m/z* 497 $[M + Cl]^-$. MS-MS of *m/z* 461 yielded *m/z* 446 $[M - H - 15]^{2-}$, *m/z* 315 $[M - H - 146]^-$, and *m/z* 300 $[M - H - 146 - 15]^{2-}$, whereas acid hydrolysis led to isorhamnetin and α-L-rhamnose. As expected, α-L-rhamnose was still attached to isorhamnetin in flavonol glycoside **1**. The linkage of the rhamnose moiety to the C-7 of isorhamnetin was confirmed by UV spectroscopy (*17*). ^{1}H NMR analysis of **1** showed a signal at δ 5.56 (d, J = 1.4 Hz, H-1″), which is characteristic for an anomeric proton of α-L-rhamnose linked to the aglycon C-7 (**Table 2**). This assignment was confirmed by a ROESY experiment. Strong negative ROEs were observed to the protons in the A-ring of isorhamnetin (δ 6.42, d, J = 1.7 Hz, H-6; and δ 6.87, d, J = 1.7 Hz, H-8) by irradiation of the anomeric proton of α-L-rhamnose (H-1″), which was in accordance to the results of Nielsen and Norbaek (*22*, *23*). Consequently, **1** was identified as isorhamnetin 7-*O*-α-L-rhamnoside (**Figure 2**), which resulted from enzymatic hydrolysis of flavonol glycoside **10**. Flavonol glycoside **1** was also detected in fraction J obtained from Sephadex LH-20 fractionation of sea buckthorn pomace by comparison with the retention time of the isolated compound using HPLC-DAD as described previously (*15*).

UV shifts and ^{13}C and ^{1}H NMR spectroscopic data of the aglycon part of flavonol glycoside **15** were similar to the results obtained for **10** (**Tables 1** and **2**). Thus, it was suggested that **15** is a further isorhamnetin 3,7-derivative, which released *β*-D-glucose and α-L-rhamnose (ratio 2:1) on acid hydrolysis. The ESI mass spectrum (negative ionization) of **15** showed a quasi molecular ion peak at *m/z* 785 $[M - H]^-$ and the adduct at *m/z* 821 $[M + Cl]^-$. The MS-MS fragment *m/z* 639 $[M - H - 146]^-$ indicated a loss of an α-L-rhamnose unit. Further fragmentation of *m/z* 785 → 639 (MS^3) produced ions at *m/z*



15 R=OCH_3
16 R=H

Figure 3. ROESY correlations (arrows) between sugar and aglycon part of flavonol glycosides **15** and **16** (only the important correlations are described).

477 [M − H − 146 − 162]$^-$, *m*/*z* 459 [M − H − 146 − 162 − H_2O]$^-$, and *m*/*z* 315 [M − H − 146 − 162 − 162]$^-$. As in flavonol glycoside **1**, the signal of the anomeric proton of the α-L-rhamnose appeared at δ 5.56 (d, J = 1.3 Hz, H-1′′′′) (**Table 2**). Furthermore, this proton exhibited a ROE correlation with H-6 (δ 6.43, d, J = 2.1 Hz) and H-8 (δ 6.85, d, J = 2.1 Hz), indicating that the rhamnosyl moiety is attached to the isorhamnetin C-7 (**Figure 3**). By irradiation of the anomeric proton at δ 5.74 (d, J = 7.2 Hz, H-1′′) a negative ROE was observed on H-6′ (δ 7.68, dd, J = 8.4, 2.1 Hz), which is consistent with a C-3-linked β-D-glucose (*22*, *23*) (**Figure 3**). The linkage position to the second β-D-glucose was deduced from a significant downfield shift of $\Delta\delta$ 7.8 for the C-2′′ (δ 82.2) of **15** compared to the shift of C-2′′ (δ 74.4) in flavonol glycoside **10** (**Table 1**). Moreover, the anomeric proton at δ 4.61 (d, 7.8 Hz, H-1′′′) showed a negative ROE correlation to H-1′′ and H-2′′ (δ 3.52, m) (**Figure 3**), indicating that the second glucosyl unit is linked to C-2′′ (*23*, *24*). Thus, **15** was identified as isorhamnetin 3-*O*-(2-*O*-β-D-glucosyl)-β-D-glucoside-7-*O*-α-L-rhamnoside (isorhamnetin 3-*O*-β-D-sophoroside-7-*O*-α-rhamnoside) (**Figure 2**).

Besides β-D-glucose and α-L-rhamnose (ratio 2:1), acid hydrolysis of flavonol glycoside **16** yielded kaempferol, which was confirmed by UV, ^{13}C, and ^{1}H NMR spectroscopy. ESI-MS analysis showed a quasi molecular ion peak at *m*/*z* 755 [M − H]$^-$ and MS-MS and MS^3 fragmentation patterns that were quite similar to those of flavonol glycoside **15**, with the exception of a mass difference of 30 Da. Furthermore, we observed similar ^{13}C and ^{1}H NMR spectroscopic data for the sugar parts as well as for the A- and C-rings of the aglycons. Finally, the same ROE correlations were found for flavonol glycoside **16** as for compound **15** (**Figure 3**), proving its structure as kaempferol 3-*O*-(2-*O*-β-D-glucosyl)-β-D-glucoside-7-*O*-α-L-rhamnoside (kaempferol 3-*O*-β-D-sophoroside-7-*O*-α-rhamnoside) (**Figure 2**).

Although the occurrence of flavonol glycosides **1**, **10**, and **15** in sea buckthorn fruits was assumed (*10*, *11*), their structures were not confirmed by ^{13}C and ^{1}H NMR spectroscopic data. The presence of flavonol glycoside **16** has been described for the leaves of *Shepherdia argentea* Nutt. (*25*) and *Ravensara anisata* (*26*). To our knowledge, the occurrence of **16** in sea buckthorn is described here for the first time.

Identification of Flavonol Glycosides by HPLC-DAD-ESI-MS. The flavonol glycosides detected within the fractions resulting from Sephadex LH-20 fractionation (**Figure 1**) were grouped into monoglycosides, rutinosides, 3,7-diglycosides, 3-sophoroside-7-rhamnosides, 3-rutinoside-7-rhamnosides, further 3-diglycoside-7-rhamnosides, further di- and triglycosides, and acylated glycosides (**Table 3**). Spectroscopic contour plots (200−400 nm) in combination with MS^n fragmentation pattern were used for their identification. The MS^n fragmentation behavior of flavonol glycosides previously identified in sea buckthorn juice (*15*) and of isolated compounds **1**, **10**, **15**, and **16** gave valuable indications for their structural characterization.

After acid hydrolysis fractions C−H produced glucose and rhamnose, but no other sugars were detected by HPTLC. Although the occurrence of minor amounts of other sugars could not be excluded, the flavonol glycosides were designated using glucose and rhamnose.

Besides **1** we found further flavonol monoglycosides **2** (isorhamnetin 3-glucoside) and **3** (quercetin 3-glucoside) fractions by comparison with authentic reference substances (**Table 3**).

Furthermore, we detected isorhamnetin 3-rutinoside **4**, which has been shown to be the major flavonol glycoside in sea buckthorn juice (*15*). MS-MS fragmentation of **4** ([M − H]$^-$ at *m*/*z* 623) produced almost exclusively the aglycon pseudomolecular ion [M − H − 146 − 162]$^-$ at *m*/*z* 315 (**Table 3**). A similar fragmentation pattern was observed for the previously identified quercetin 3-rutinoside **5** (*15*), which allowed us to identify further rutinosides. We also observed a complete loss of the disaccharide rutinose without formation of a fragment [M − H − 146]$^-$, which would correspond to a loss of rhamnose in **6** (myricetin rutinoside) and **7** (kaempferol rutinoside). The most abundant daughter ions resulting from MS-MS fragmentation of flavonol rutinoside **8** showed a mass-to-charge ratio of *m*/*z* 331, indicating its structure as myricetin methyl ether rutinoside. Compound **9**, which yielded *m*/*z* 345 on MS-MS, was tentatively identified as myricetin dimethyl ether rutinoside.

In contrast to **4**, the MS-MS spectrum of **10** ([M − H]$^-$ at *m*/*z* 623) showed the most abundant daughter ion at *m*/*z* 477 [M − H − 146]$^-$, corresponding to a loss of rhamnose from the C-7 of isorhamnetin (**Table 3**). The daughter ion at *m*/*z* 461 [M − H − 162]$^-$ resulting from the loss of the C-3-bonded glucose showed a lower intensity. The higher sensitivity of the glycosidic linkage at the C-7 position toward collision-induced fragmentation was also described by Llorach et al. (*27*). Due to these findings we identified **11** (kaempferol 3-glucoside-7-rhamnoside), **12** (isorhamnetin 3,7-diglucoside), and **13** (isorhamnetin 3-pentoside-7-rhamnoside). The most abundant ion on MS-MS fragmentation of **14** ([M − H]$^-$ at *m*/*z* 609) was *m*/*z* 301 [M − H − 146 − 162]$^-$. The rhamnose ([M − H − 146]$^-$ at *m*/*z* 463) and the glucose fission product ([M − H − 162]$^-$ at *m*/*z* 447) were less abundant and showed almost the same intensities (**Table 3**). Due to the preferred linkage position of the rhamnose moiety **14** was tentatively identified as quercetin 3-glucoside-7-rhamnoside, but an opposite assignment of the sugar moieties would also be conceivable.

The MS-MS spectra of **15** ([M − H]$^-$ at *m*/*z* 785) and **16** ([M − H]$^-$ at *m*/*z* 755), which showed ions only at *m*/*z* 639 and 609 [M − H − 146]$^-$, clearly indicated that removal of the rhamnosyl moiety from the hydroxyl group of C-7 is much more favored than loss of sophorose or glucose from C-3 (**Table 3**). MS^3 fragmentation of *m*/*z* 785 → 639 (compound **15**) yielded ions at *m*/*z* 459 [M − H − 146 − 162 − H_2O]$^-$ and *m*/*z* 315 [M − H − 146 − 162 − 162]$^-$, which was in contrast to rutinosides that showed a complete loss of the disaccharide. A fragmentation pattern similar to that of **15** and **16** was also observed for compound **17** (quercetin 3-sophoroside-7-rhamnoside).

MS-MS fragmentation of **18** (isorhamnetin 3-rutinoside-7-rhamnoside, ([M − H]$^-$ at *m*/*z* 769) yielded a fragment at *m*/*z*

Table 3. HPLC-DAD-ESI-MS Data of Flavonol Glycosides from Sea Buckthorn Pomace

peak	t_R (min)	fraction	UV (nm)	$[M-H]^-$ (*m/z*)	MS-MS[d] (*m/z*)	MS^3 (*m/z*)	structure assignment[e]
monoglycosides							
1[b]		J[c]					I 7-rha
2[a]	19.1	H–I[c]	255, 266sh, 354	477	314,* 315 (44)	300 (45), 285 (100)	I 3-glu
3[a]		I[c]					Q 3-glu
rutinosides							
4[a]	18.1	F–H	266, 266sh, 355	623	315,* 300 (25)	300 (100)	I 3-rut
5[a]	15.5	G–H	255, 266sh, 354	609	301,* 271 (24)	271 (100)	Q 3-rut
6	13.2	H	265, 355	625	316,* 317 (70)	287 (34) 271 (100)	M-rut
7	17.6	H	266, 348	593	285*	255 (100)	K-rut
8	15.9	G	230, 270, 360sh	639	331,* 316 (43)	316 (100)	M-Me-rut
9	18.4	F	255, 266 sh, 358	653	345,* 330 (20), 315 (12)	330 (100), 315 (29), 301 (17)	M-$(Me)_2$-rut
3,7-diglycosides							
10[b]	15.2	D–F	255, 267sh, 353	623	477,* 461 (38), 315 (19)	314 (100)	I 3-glu-7-rha
11	15.0	D–G	266, 347	593	447*, 431 (59), 285 (38)	285 (31), 284 (100)	K 3-glu-7-rha
12	11.0	C–E	255, 266sh, 352	639	477,* 315 (17)	315 (32), 314 (100)	I 3,7-diglu
13	16.3	G	255, 268sh, 354	593	461,* 447 (36), 315 (67)	315 (100)	I 3-pen-7-rha
14	13.1	G	255, 267sh, 352	609	463 (87), 447 (98), 301*	271 (100)	Q 3-glu-7-rha
3-sophorosides-7-rhamnosides							
15[b]	12.2	B–C	256, 267sh, 354	785	639*	459 (37), 315 (100)	I 3-soph-7-rha
16[b]	11.5	B–E	266, 348	755	609*	429 (83), 285 (100), 284 (83), 255 (31)	K 3-soph-7-rha
17	10.5	B–E	256, 266sh, 354	771	625*	463 (30), 445 (18), 301 (100), 300 (96)	Q 3-soph-7-rha
3-rutinosides-7-rhamnosides							
18	15.2	B–C	255, 266sh, 354	769	623*	315 (100), 300 (25)	I 3-rut-7-rha
19	14.8	B–C	266, 344	739	593*	285 (100)	K 3-rut-7-rha
further 3-diglycosides-7-rhamnosides							
20	11.3	C–E	266, 344	755	609*	429 (14), 285 (100), 284 (43)	K 3-glu-glu-7-rha
21	12.6	C–E	266, 344	739	593*	429 (33), 284 (100), 255 (37)	K 3-glu-rha-7-rha
further di- and triglycosides							
22	15.2	F	265, 348	739	593 (4), 575 (34), 285 (34), 284*	255 (100)	K-glu-rha-rha
23	13.5	D–F	255, 266sh, 354	755	609 (21), 591 (18), 300*	271 (100)	Q-glu-rha-rha
24	12.0	D–E	255, 266sh, 351	771	625 (13), 609 (9), 591 (21), 300*	271 (100)	Q-glu-glu-rha
25	13.7	D–E	266, 349	755	575 (45), 285*	255 (100)	K-glu-glu-rha
26	16.7	G	255, 268sh, 355	623	314*	300 (100)	I-glu-rha
acylated glycosides							
27	14.7	F	264sh, 270, 320	917	771,* 625 (51)	625 (100), 445 (7), 301 (7)	Q 3-cou-glu-glu-7-rha
28	15.9	F	260sh, 270, 310, 360sh	931	785*	639 (100), 459 (11), 315 (28)	I 3-cou-glu-glu-7-rha
29	14.5	C–E	230, 268sh, 334	977	831,* 771 (59), 625 (52)	639 (30), 625 (100), 445 (12), 315 (7), 300 (9)	I 3-OHfer-glu-glu-7-rha
30	15.7	B–E	245, 270sh, 334	991	845*	653 (100), 639 (66), 459 (8), 329 (40), 314 (20)	Q-$(Me)_2$-3-OHfer-glu-glu-7-rha
31	15.7	D–E	236, 254, 268sh, 330	961	815*	653 (100), 639 (54), 609 (25), 329 (17), 315 (42), 300 (15)	Q-$(Me)_2$-3-caf-glu-glu-7-rha
32	18.8	B–C	266, 348	921	775*	609 (100), 591 (43), 429 (42), 285 (28), 284 (25)	K 3-OHvan-glu-glu-7-rha
33	19.5	B–E	256, 270sh, 356	869	723*	639 (20), 621 (21), 459 (37), 315 (100), 314 (74)	I 3-acyl-glu-glu-7-rha
34	17.8	D–E	255, 270sh, 355	855	709,* 625 (16), 607 (10), 446 (14), 301 (13)	625 (75), 607 (47), 463 (13), 445 (14), 300 (100)	Q 3-acyl-glu-glu-rha

[a] Compounds were identified by comparison with reference substances using HPLC-DAD as described previously (*15*). [b] Compounds were isolated and identified by NMR spectroscopy. [c] Fractions I and J were not investigated by HPLC-DAD-ESI-MS. [d] An asterisk (*) indicates the most abundant ion after MS-MS fragmentation, which was used for MS^3. [e] I, isorhamnetin; K, kaempferol; M, myricetin; Q, quercetin; Me, methyl; acyl, unidentified acyl with *m/z* 84; caf, caffeoyl; cou, coumaroyl; OHvan, hydroxyvanilloyl; OHfer, hydroxyferuloyl; glu, glucoside; pen, pentoside; rha, rhamnoside; rut, rutinoside; sop, sophoroside. Relative intensities are given in parentheses.

623 $[M-H-146]^-$, which indicated a loss of rhamnose from C-7 (**Table 3**). MS^3 fragmentation of *m/z* 769 → 623 led to *m/z* 315 $[M-H-146-146-162]^-$, which corresponded to a complete loss of the disaccharide. Compound **19**, exhibiting a fragmentation pattern similar to that of **18**, was identified as kaempferol 3-rutinoside-7-rhamnoside.

Compounds **20** ($[M-H]^-$ at *m/z* 755) and **21** ($[M-H]^-$ at *m/z* 739) also lost rhamnose on MS-MS fragmentation, yielding ions at *m/z* 609 and 593 $[M-H-146]^-$. MS^3 of *m/z* 755 → 609 (compound **20**) led to *m/z* 429 $[M-H-146-162-H_2O]^-$ and *m/z* 285 $[M-H-146-162-162]^-$, whereas MS^3 of *m/z* 739 → 593 (compound **21**) yielded *m/z* 429 $[M-H-146-146-H_2O]^-$ and *m/z* 284 $[M-H-146-146-162-H]^{2-}$. Consequently, the structures of **20** and **21** were kaempferol 3-glucosyl-glucoside-7-rhamnoside and kaempferol 3-rhamnosyl-glucoside-7-rhamnoside, respectively.

A further kaempferol glycoside **22** ($[M-H]^-$ at *m/z* 739) was found to yield *m/z* 284 $[M-H-146-146-162-H]^{2-}$ with the highest abundance on MS-MS. Thus, the structure of **22** was concluded as kaempferol dirhamnosylglucoside (**Table 3**). As in the case of **22** the fragmentation behavior of **23** (quercetin diglucosylrhamnoside), **24** (quercetin dirhamnosylglucoside), **25** (kaempferol diglucosylrhamnoside), and **26** (isorhamnetin rhamnosylglucoside) allowed no accurate assignment of the glycosyl moiety.

The UV spectra of compounds **27**−**31** revealed a very broad band I shifted to a wavelength between 310 and 334 nm, whereas all other flavonols exhibited a maximum of band I between 344 and 360 nm (**Table 3**). This observation indicated that flavonols **27**−**31** were acylated with hydroxycinnamic acid derivatives (*27*). As with all other identified acyl derivatives, compound **27** ($[M-H]^-$ at *m/z* 917) released rhamnose on MS-MS fragmentation, yielding *m/z* 771 $[M-H-146]^-$ as the most intense ion. MS^3 fragmentation of *m/z* 917 → 771 led to *m/z* 625 $[M-H-146-146]^-$, *m/z* 445 $[M-H-146-146-162-H_2O]^-$, and *m/z* 301 $[M-H-146-146-$

162 − 162]$^-$, which corresponded to a loss of coumaric acid (*m*/*z* 146) and subsequent loss of two glucose molecules. Consequently, we identified **27** as quercetin 3-coumaroylglucosylglucoside-7-rhamnoside and compound **28**, exhibiting a similar fragmentation pattern, as isorhamnetin 3-coumaroylglucosylglucoside-7-rhamnoside. Compound **29** showed ions at *m*/*z* 639 [M − H − 146 − 192]$^-$, *m*/*z* 625 [M − H − 146 − 192 − 14]$^-$, and *m*/*z* 315 [M − H − 146 − 192 − 162 − 162]$^-$ on MS^3 of *m*/*z* 977 → 831. The acyl moiety of **29** (isorhamnetin 3-hydroxyferuloyl-glucosyl-glucoside-7-rhamnoside) was suggested to be hydroxyferulic acid (*m*/*z* 192). Similar results were obtained for **30** (quercetin dimethyl ether 3-hydroxyferuloylglucosylglucoside-7-rhamnoside). Compound **31**, which was suggested to consist of caffeic acid (*m*/*z* 162) as the cinnamoyl constituent, was identified as quercetin dimethyl ether 3-caffeoylglucosylglucoside-7-rhamnoside.

In contrast to the hydroxycinnamic acid derivatives the UV spectra of compounds **32**−**34** showed similar properties to nonacylated flavonol glycosides (**Table 3**). Although **32** (kaempferol 3-hydroxyvanilloylglucosylglucoside-7-rhamnoside) was suggested to consist of hydroxyvanillic acid (*m*/*z* 166), the acyl moiety of compounds **33** and **34** was unknown.

To our knowledge the occurrence of acylated flavonol derivatives in sea buckthorn fruits, juice, or pomace has never been described before. However, Yoshida et al. (*28*) isolated kaempferol 3-*O*-(6-*O*-*p*-coumaroyl)-*β*-D-glucoside (tiliroside) from the leaves of this plant.

In conclusion, the present study demonstrates the structural elucidation of major flavonol glycosides from sea buckthorn by hydrolysis studies, ESI-MS^n, UV, and ^1H and ^{13}C NMR spectroscopy. These findings and the results of our previous study (*15*) allowed us to characterize further flavonol glycosides by their UV spectra and their characteristic MS-MS and MS^3 fragmentation behavior. Our results indicated that most of the flavonols from sea buckthorn are either 3-glycosides or 3,7-diglycosides. With regard to the antioxidant properties of flavonols, glycosidation at the C-3 position plays an important role. In fact, 3-glycosides exhibit lower antioxidant activities than their aglycons (*29*). Isorhamnetin 3-glycosides especially are very poor radical scavengers due to their inability to form quinonic structures by oxidation (*15*). However, whether structure−antioxidant activity relationships determined by in vitro methods are of physiological significance remains to be demonstrated.

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