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Structure−Antioxidant Efficiency Relationships of Phenolic Compounds and Their Contribution to the Antioxidant Activity of Sea Buckthorn Juice

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The phenolic composition of juice derived from fruits of sea buckthorn (*Hippophae rhamnoides*) was investigated by high-performance liquid chromatography (HPLC) with diode array and electrochemical detection. Flavonols were found to be the predominating polyphenols while phenolic acids and catechins represent minor components. Of the seven flavonols identified, isorhamnetin 3-*O*-glycosides were the most important representatives quantitatively. However, because of their structural properties, they were poor radical scavengers as shown by electron spin resonance spectroscopy. Phenolic compounds such as quercetin 3-*O*-glycosides, catechins, and hydroxybenzoic acids with a catechol structure exhibited good antioxidant capacities, but their concentration in sea buckthorn juice was small. These phenolic compounds, determined by HPLC, accounted for less than 5% of the total antioxidant activity of the filtered juice. Ascorbic acid was shown to be the major antioxidant in sea buckthorn juice. Because of its high concentration of 1.22 g/L, it contributes approximately 75% to total antioxidant activity. The remaining difference can be attributed to higher molecular weight flavan-3-ols (proanthocyanidins), which were determined photometrically after acid depolymerization to colored anthocyanidins.

KEYWORDS: Sea buckthorn; *Hippophae rhamnoides*; polyphenols; flavonoids; HPLC; antioxidant activity; ESR; Fremy's salt

INTRODUCTION

Sea buckthorn (*Hippophae rhamnoides*) is a fascinating plant growing widely in various regions of Asia, Europe, and Northern America. In summer, plentiful round yellow orange fruits cover the female plants. The sea buckthorn berries consist of a fairly tough skin and a juice enveloping cellular structures (*1*). Besides juice, further sources of valuable products are the oils of the mesocarp and seed. Sea buckthorn berries belong to the fruits with high contents of natural antioxidants including ascorbic acid (500−14 000 mg/kg), tocopherols (1600 mg/kg), and carotenoids (150−430 mg/kg) (*2*). The complex of polyphenols comprises flavonols (1500−2000 mg/kg), catechins (700−1300 mg/kg), proanthocyanidins (1100−2900 mg/kg), and chlorogenic acids (330−730 mg/kg) (*3*). While the flavonols are reported to consist of glycosides of isorhamnetin, quercetin, and kaempferol (*4*, *5*), detailed structures of phenolic acids and flavan-3-ols are mostly unknown.

Diets rich in fruits and vegetables are reported to protect against certain forms of cancer (*6*). The dietary intake of flavonoids from fruits and vegetables has also been shown to be inversely related to coronary heart disease mortality (*7*, *8*). These atherosclerotic diseases are explained as a consequence of peroxidation of human low-density lipoprotein (LDL) by reactive oxygen species (ROS) (*9*). In many in vitro experiments, phenolic compounds have been shown to inhibit the oxidative damage of LDL (*10*, *11*). Because of their antioxidant characters, flavonoids and phenolic acids are able to reduce free radical formation and to scavenge ROS. Many methods to determine the antioxidant efficiency of polyphenols, which are based on their radical scavenging activities, have been published (*12*−*14*). These methods include the Trolox equivalent antioxidant activity assay (TEAC assay), the oxygen radical absorbance capacity assay (ORAC assay), and the 1,1-diphenyl-2-picrylhydrazyl assay (DPPH radical assay). Frankel and Meyer (*15*) reviewed the methods and concluded that their results were difficult to interpret and compare. Different substrates, system compositions, and analytical methods are employed in the screening tests and result in different orders of antioxidant effectiveness for the same phenolic compounds.

The aim of this work was to identify phenolic acids and flavonoids of sea buckthorn juice. Furthermore, our investigations included the determination of the antioxidant capacity of

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commercially available phenolic compounds and also of flavonols, which have been isolated from sea buckthorn juice. The antioxidant effectiveness obtained by electron spin resonance (ESR) experiments (spin labeling) was related to the structure of the polyphenols. The study also involved the determination and comparison of the contribution to total antioxidant activity (TAA) of filtered sea buckthorn juice supplied by each phenolic compound.

MATERIALS AND METHODS

Sea Buckthorn Juice. Sea buckthorn (*H. rhamnoides* cv. Hergo) fruits were harvested at Fredersdorf near Berlin (Germany) on September 3, 2001. Sea buckthorn juices were prepared at the Institute of Food Technology, Technical University Berlin (Germany). To produce untreated juice (UTJ), the berries were mashed, and the mash was separated immediately into juice and pomace by a Z18-3/401 decanter machine (Flottweg, Vilsbiburg, Germany). An additional procedure was applied for producing enzyme-treated juice (ETJ). Before the juice was separated, the mash was subjected to a treatment with Pectinex 100 L pectolytic enzymes (Novo Nordisk Ferment AG, Dittingen, Switzerland). For pasteurization, UTJ and ETJ were heated at 96 °C for 1.5 min in a plate heat exchanger.

Chemicals. Ascorbic acid was purchased from Merck (Darmstadt, Germany). Chlorogenic acid, protocatechuic acid, isorhamnetin 3-glucoside, isorhamnetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-glucoside, and quercetin 3-rutinoside were from Roth (Karlsruhe, Germany). Caffeic acid, ferulic acid, *p*-hydroxybenzoic acid, (+)-catechin, (−)-epicatechin, isorhamnetin, kaempferol, myricetin, quercetin, and *tert*-butylhydroquinone were obtained from Fluka (Taufkirchen, Germany). Gallic acid was from Serva (Heidelberg, Germany). Vanillic acid, *p*-coumaric acid, quercetin 3-rhamnoside, potassium nitrosodisulfonate (Fremy's salt), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Steinheim, Germany). Reagents and solvents were purchased from Roth or Merck and were of high-performance liquid chromatography (HPLC) or analytical grade quality. HPLC grade water was purified with a deonized water treatment system.

Hydrolysis of the Juices. Sea buckthorn juices were hydrolyzed to obtain corresponding aglycones with the method described by Häkkinen and Auriola (*16*). For hydrolysis, methanol (25 mL), which contained 2 g/L *tert*-butylhydroquinone, and 6 M HCl (10 mL) were added to 15 mL of juice (final HCl concentration 1.2 M). The mixture was refluxed for 2 h at 85 °C in a 100 mL round-bottomed flask. After the solution was allowed to cool, it was filtered and used for HPLC analysis.

HPLC-Diode Array Detection (DAD)-Electrochemical Detection (ECD). Chromatographic separation of phenolic compounds or filtered sea buckthorn juice was carried out according to Rechner et al. (*17*) on a 250 mm × 4.6 mm i.d., 5 μm Fluofix 120E column (NEOS Company Ltd., Kobe, Japan) connected to a 10 mm × 4.6 mm i.d. guard column of the same material using two solvents [A = water−phosphoric acid (99.5:0.5, v/v); B = acetonitrile−water−phosphoric acid (50:49.5:0.5, v/v/v)]. Gradient elution was performed as follows: 0% B (5 min); 0−25% B in 40 min; 25−80% B in 20 min; 80−100% B in 5 min; 100−0% B in 1 min; 0% B (15 min). The flow rate was 1.0 mL/min, and the injection volume was 20 μL of standard solution or filtered juice. The HPLC system consisted of a Gynkothek model 480 HPLC pump (Dionex, Germering, Germany) and a GINA 50 autosampler (Dionex). The column was maintained at 25 °C by a Haake F3 Thermostat. The detection was performed by a DAD 440 diode array detector (Kontron, Milano, Italy) at 280 and 350 nm (spectroscopic contour plots from 200 to 400 nm) and a L-ECD-6A electrochemical detector (Shimadzu, Kyoto, Japan) at 500 mV in series.

For electrochemical determination of ascorbic acid, solutions of the standard (3−7 mg/L) and of filtered juice (diluted 250-fold) were prepared using metaphosphoric acid (5% in deionized water). The elution was performed isocratically (100% A), and the injection volume was 10 μL. All other conditions were as described above.

Identification of Phenolic Acids. The identity of gallic and protocatechuic acid, which was indicated by HPLC-ECD analysis, was confirmed by HPLC-DAD investigation after extraction. Filtered sea buckthorn juice (10 mL) was extracted three times with *n*-hexane (10 mL) to remove lipophilic substances. The aqueous layer was adjusted to pH 7 with sodium hydroxide solution (0.1 N) and then extracted three times with 10 mL of ethyl acetate to remove flavonoids. The remaining aqueous fraction was adjusted to pH 2 with hydrochloric acid (0.1 N) and extracted three times with 10 mL of ethyl acetate. This solution contains the phenolic acids (*18*). After ethyl acetate was evaporated, the residue was dissolved in 2 mL of methanol−water (50:50, v/v) and used for HPLC-DAD analysis.

Identification of Flavan-3-ols. A flavan-3-ol fraction relatively free of interfering matrix compounds was achieved by fractionating a 2 mL portion of filtered sea buckthorn juice over a Sephadex LH-20 mini coloumn as described by Spanos and Wrolstad (*19*).

Determination of Proanthocyanidins. The proanthocyanidins were determined photometrically after acid depolymerization to the corresponding anthocyanidins as described by Nikfardjam (*20*). In fact, 1 mL of an adequate aqueous dilution of filtered juice was added to 9 mL of a solution of concentrated hydrochloric acid in *n*-butanol (10:90, v/v). The closed vial containing the solution was mixed vigorously and heated for 90 min in a boiling water bath. After the solution was cooled to room temperature, the absorbance at 550 nm was measured using a Novaspec II sectrophotometer (Pharmacia LKB, Uppsala, Sweden). The content of proanthocyanidins (mg cyanidin/L) was calculated by the molar extinction coefficient of cyanidin ($\epsilon = 17\,360$ L mol^{-1} cm^{-1}).

ESR Analysis. Phenolic compounds, ascorbic acid, and Trolox were dissolved in methanol to prepare 0.1 mM solutions. Aliquots (500 μL) were allowed to react with an equal volume of a solution of Fremy's salt (1 mM in phosphate buffer, pH 7.4). The ESR spectrum of Fremy's radical was obtained after 20 min, by which time the reaction was complete. Signal intensity was obtained by integration, and the antioxidant capacity, expressed as moles Fremy's salt reduced by one mole antioxidant, was calculated by comparison with a control reaction with methanol.

For measuring the antioxidant activity of sea buckthorn juice, a 50-fold dilution of filtered juice in water was prepared and reacted as described above. The antioxidant activity, expressed as mmol Fremy's salt reduced by 1 L of filtered juice, was calculated by comparison with a control reaction with distilled water.

Spectra were obtained at 21 °C on a Miniscope MS 100 spectrometer (Magnettech, Berlin, Germany). The microwave power and modulation amplitude were set at 10 dB and 1500 mG, respectively. For the measurement, 50 μL of the reaction mixture was added in a micropipet.

RESULTS AND DISCUSSION

Identification of Flavonols. For the separation of the phenolic compounds from sea buckthorn juice, the method of Rechner et al. (*17*) proved to be very suitable. The used reversed phase HPLC column was packed with highly purified silica gel, which is modified by branched fluorocarbon chains (Fluofix), and allowed the determination of phenolic acids and flavonoids within one analytical run. Flavonols of sea buckthorn juice eluting at times between 35 and 65 min were measured at a wavelength of 350 nm (**Figure 1**). The structures of the identified flavonols are shown in **Figure 2**. They have been shown to be the predominating polyphenols in sea buckthorn berries (*21*).

Besides small amounts of the aglycone, isorhamnetin **7**, we identified the 3-*O*-rutinoside **4** and the 3-*O*-glucoside **5** of isorhamnetin by comparison with authentic reference substances. While isorhamnetin **7** has been known to occur in fruits of sea buckthorn since 1944 (*22*), Hörhammer (*23*) first indicated the presence of glycosides **4** and **5** in 1968. Furthermore, we found far smaller concentrations of quercetin 3-*O*-rutinoside **1** and 3-*O*-glucoside **2**. To our knowledge, only the 3-*O*-rutinoside **1** of quercetin is known to occur in fruits of sea buckthorn (*4*, *5*, *24*). The presence of quercetin 3-*O*-glucoside **2** has only been reported for the leaves of this plant (*25*).

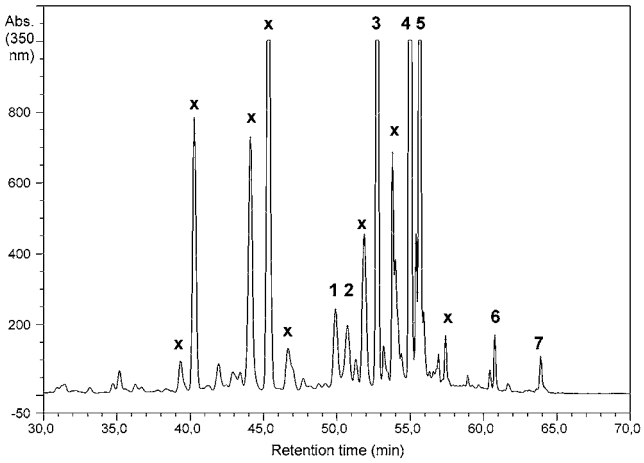
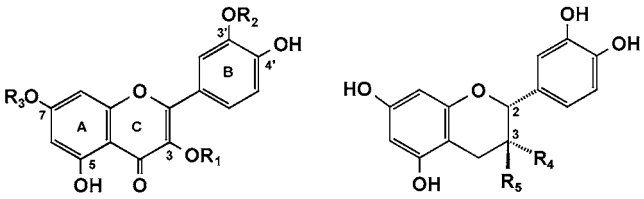


Figure 1. Typical HPLC-DAD (absorbance, 350 nm) plot of filtered sea buckthorn juice. Peaks: (1) quercetin 3-*O*-rutinoside, (2) quercetin 3-*O*-glucoside, (3) isorhamnetin 3-*O*-glucoside-7-*O*-rhamnoside, (4) isorhamnetin 3-*O*-rutinoside, (5) isorhamnetin 3-*O*-glucoside, (6) isorhamnetin 7-*O*-rhamnoside, (7) isorhamnetin, and (x) unidentified flavonol glycosides.



1 R_1=rutinose, R_2=H, R_3=H
2 R_1=glucose, R_2=H, R_3=H
3 R_1=glucose, R_2=CH_3, R_3=rhamnose
4 R_1=rutinose, R_2=CH_3, R_3=H
5 R_1=glucose, R_2=CH_3, R_3=H
6 R_1=H, R_2=CH_3, R_3=rhamnose
7 R_1=H, R_2=CH_3, R_3=H

11 R_4=H, R_5=OH (+)-Catechin
12 R_4=OH, R_5=H (-)-Epicatechin

Figure 2. Structures of flavonols (left) and flavan-3-ols (right) identified in sea buckthorn juice.

In addition, compounds **3** and **6** and peaks marked with x showed a UV spectrum, which is typical for flavonols. Most of these peaks could be attributed to further isorhamnetin glycosides. After hydrolysis of sea buckthorn juices, we found an isorhamnetin concentration of 263 and 240 mg/L for UTJ and ETJ, respectively. In contrast, only 42 mg/L of quercetin and 39 mg/L of kaempferol were detected in UTJ (44 and 50 mg/L in ETJ, respectively). Häkkinen et al. (*26*) investigated the content of quercetin, myricetin, and kaempferol in 25 edible berries and could only detect quercetin (66 mg/kg fresh berries) after hydrolysis of sea buckthorn berries.

Several isorhamnetin glycosides have been isolated from sea buckthorn berries by Lachman et al. (*5*). Their structure was investigated by thin-layer chromatography, UV spectroscopy, and hydrolysis studies. We could confirm the presence of isorhamnetin 3-*O*-glucoside-7-*O*-rhamnoside **3** and isorhamnetin 7-*O*-rhamnoside **6** by NMR and LC-ESI-MS investigations (27).

Identification of Phenolic Acids. The use of electrochemical detection allowed the determination of very low amounts of phenolic compounds with a catechol or pyrogallol structure. We could identify gallic acid (3,4,5-trihydroxybenzoic acid) **9** and protocatechuic acid (3,4-dihydroxybenzoic acid) **10** in sea buckthorn juice by comparison with the retention times of standards (**Figure 3**). Because the amount of these acids was too small for detection by UV, an extraction with ethyl acetate at different pH values was necessary (*18*). Thus, a large amount

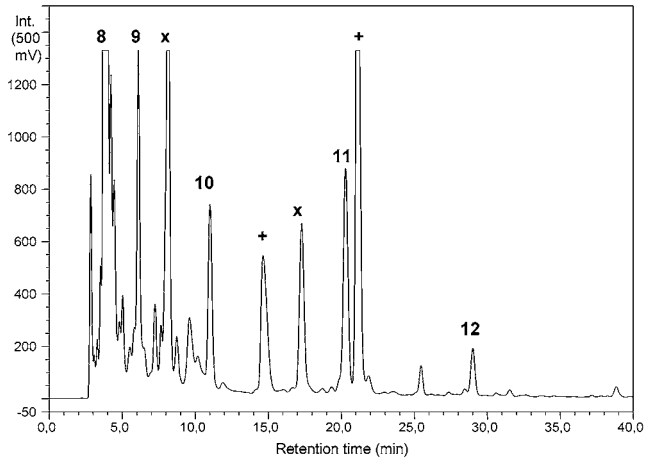


Figure 3. Typical HPLC-ECD (intensity, 500 mV) plot of filtered sea buckthorn juice. Peaks: (8) ascorbic acid, (9) gallic acid, (10) protocatechuic acid, (11) (+)-catechin, (12) (−)-epicatechin, (x) unidentified flavan-3-ols, and (+) unidentified compounds.

of interfering matrix was removed and the spectra of the sample peaks corresponded with those of the standards. While protocatechuic acid **10** is known to occur in dried fruits of sea buckthorn (*28*), gallic acid **9** was previously found only in the leaves of this plant (*29*).

Identification of Flavan-3-ols. The HPLC-ECD chromatogram of sea buckthorn juice showed also the occurrence of (+)-catechin **11** and (−)-epicatechin **12** (**Figure 3**). The identity of these flavan-3-ols (**Figure 2**) was confirmed by comparison of the UV spectra after the juice was cleaned up over a small column filled with Sephadex LH-20 (*19*). Catechins are known to occur in sea buckthorn berries (*4*), but no detailed structures are reported. Peaks marked with x (**Figure 3**) showed UV spectra similar to catechins with a maximum at 270 nm, which indicates the presence of gallocatechins.

Sea buckthorn fruits are reported to contain also flavan-3-ols of higher molecular weight (*4*). In general, these proanthocyanidins can be determined by reversed-phase HPLC methods up to a degree of polymerization of four. Because of the lack of standard substances, the content of proanthocyanidins was determined photometrically after acid depolymerization to anthocyanidins (*20*). HPLC investigation of the hydrolysate obtained suggested that the predominating subunits of sea buckthorn proanthocyanidins are gallocatechins (data not shown). Further work will be required to identify these prodelphinidins.

Antioxidant Capacity of Phenolic Substances. Free radical scavenging is the generally accepted mechanism for antioxidants to inhibit lipid oxidation. Free radicals can be measured by using ESR spectroscopy. The efficiency of an antioxidant compound is expressed as its ability to reduce a synthetic free radical species, Fremy's salt (*30*). By using this spin labeling method, we tested several phenolic compounds detected in sea buckthorn juice. To confirm possible structure−antioxidant efficiency relationships, we also involved some polyphenols in the study, which do not occur in sea buckthorn berries. The antioxidant capacity indicates the quantity of Fremy's salt (mol), which is reduced by 1 mol of antioxidant compound (**Table 1**).

In general, the antioxidant capacity of compounds possessing an *o*-diphenolic arrangement (catechol structure) was higher than in monophenols (**Table 1**) due to their ability to form *o*-quinones when reacting with free radicals. We could demonstrate this relationship by comparing the values of *p*-hydroxybenzoic acid (0.6 mol Fremy's salt/mol) and protocatechuic acid **10** (5.8 mol Fremy's salt/mol). Replacing the 3-hydroxyl group of proto-

Table 1. Antioxidant Capacity (mol Fremy's Salt/mol Antioxidant; Mean ± SD of Duplicate Assays) of Phenolic Comounds, Ascorbic Acid, and Trolox

compound	OH substitution	other substitution	antioxidant capacity (mol Fremy's salt/mol)
	hydroxybenzoic acids		
gallic acid	3,4,5		8.1 ± 0.2
protocatechuic acid	3,4		5.8 ± 0.3
p-hydroxybenzoic acid	4		0.6 ± 0.2
vanillic acid	4	3-OMe	0.2 ± 0.2
	hydroxycinnamic acids		
caffeic acid	3,4		7.0 ± 0.4
chlorogenic acid[a]	3,4		6.7 ± 0.0
ferulic acid	4	3-OMe	1.5 ± 0.2
p-coumaric acid	4		0.6 ± 0.2
	flavan-3-ols		
epicatechin	3,5,7,3′,4′		6.7 ± 0.2
catechin	3,5,7,3′,4′		6.1 ± 0.3
	flavonols		
myricetin	3,5,7,3′,4′,5′		8.2 ± 0.4
quercetin	3,5,7,3′,4′		8.0 ± 0.1
isorhamnetin	3,5,7,4′	3′-OMe	2.7 ± 0.1
kaempferol	3,5,7,4′		2.5 ± 0.1
	flavonolglycosides		
quercetin 3-*O*-rhamnoside	5,7,3′,4′	3-Orha	6.2 ± 0.0
quercetin 3-*O*-glucoside	5,7,3′,4′	3-Oglu	7.1 ± 0.1
quercetin 3-*O*-galactoside	5,7,3′,4′	3-Ogal	7.8 ± 0.2
quercetin 3-*O*-rutinoside	5,7,3′,4′	3-Orut	6.4 ± 0.0
isorhamnetin 7-*O*-rhamnoside	3,5,4′	3′-OMe, 7-Orha	2.6 ± 0.2
isorhamnetin 3-*O*-glucoside-7-*O*-rhamnoside	5,4′	3-Oglu, 3′-OMe 7-Orha, 3′-OMe	1.2 ± 0.4
isorhamnetin 3-*O*-glucoside	5,7,4′	3-Oglu, 3′-OMe	0.4 ± 0.2
isorhamnetin 3-*O*-rutinoside	5,7,4′	3-Orut, 3′-OMe	0.5 ± 0.1
ascorbic acid	3,4		4.0 ± 0.3
Trolox[b]	6		4.2 ± 0.0

[a] 5-Caffeoylquinic acid. [b] Commonly used as reference substance for comparison of antioxidant activity.

catechuic acid **10** by a methoxy group as in vanillic acid had a suppressive influence on the antioxidant capacity (0.2 mol Fremy's salt/mol). The same ranking order as for these hydroxybenzoic acids was also found for cinnamic acid derivatives (**Table 1**). We observed maximum antioxidant capacities for caffeic and chlorogenic acid (7.0 and 6.7 mol Fremy's salt/mol) exhibiting a catechol structure.

The structural advantage of an additional hydroxyl group confers on gallic acid **9** (8.1 mol Fremy's salt/mol) an enhancement of antioxidant capacity as compared to protocatechuic acid **10** (**Table 1**). Because of its pyrogallol structure, it shows a greater oxidizability and the quinone formed can be stabilized by resonance structures. The importance of a pyrogallol structure for maximum antioxidant activity of hydroxybenzoic acid derivatives was also described by Rice-Evans et al. (*12*) for the TEAC assay and by Cao et al. (*13*) for the ORAC assay.

Comparing the flavonol aglycones, we could demonstrate the importance of a catechol structure in the B-ring for the antioxidant properties (**Table 1**). For quercetin (8.0 mol Fremy's salt/mol), we could observe a significantly higher antioxidant capacity than for kaempferol (2.5 mol Fremy's salt/mol). However, radical scavenging properties were hardly affected by an additional hydroxyl group in the B-ring, as for myricetin, which reduced 8.2 mol Fremy's salt/mol. A possible explanation for the fact that myricetin possessed a lower antioxidant capacity than expected was given by Burda and Oleszek (*31*). These

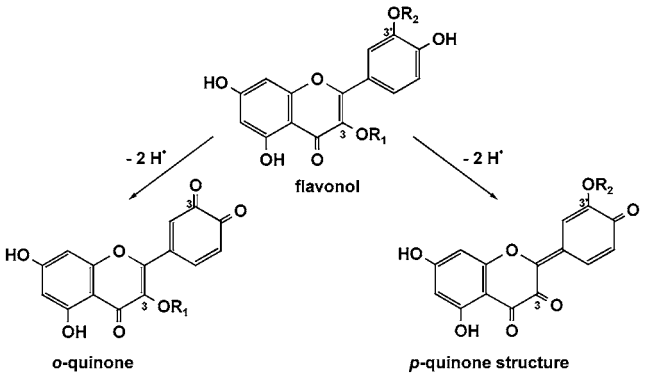


Figure 4. Influence of the flavonol substitution pattern (R_1, R_2) on the ability to form quinone structures by oxidation with Fremy's salt (left pathway for R_1 = H or sugar and R_2 = H; right pathway for R_1 = H, R_2 = H, or CH_3).

authors found quercetin to be more effective than myricetin in scavenging DPPH• and inhibiting oxidation in a β-carotene−linoleic acid model system. This could be attributed to the high oxidation sensitivity of myricetin, which caused its rapid decomposition during measurement. While the same relationship in antioxidant effectiveness was observed in the TEAC assay (*12*), an opposite result was found by measuring inhibition of LDL oxidation (*11*). Gardner et al. (*30*) observed a higher antioxidant potential for myricetin than for quercetin by ESR. However, the values were even smaller (1.3 mol Fremy's salt/mol for quercetin and 2.2 mol Fremy's salt/mol for myricetin) as compared to our results, which could be attributed to the use of different solvent systems. The reduction potentials of various hydroxyl groups bound to the flavan nucleus may change in different solvent systems depending on their state of protonation or deprotonation (*32*).

Substitution of the 3-hydroxyl group of quercetin with a glycosyl group caused an 11−26% decrease in antioxidant capacity (**Table 1**), which is in accordance with the results of Gardner et al. (*30*). However, the values ranging from 5.9 to 7.1 mol Fremy's salt/mol for the individual quercetin 3-*O*-glycosides proved them to be excellent radical scavengers. These results suggested that the *o*-diphenolic structure in the B-ring is important for effective radical scavenging of flavonols. Even if the 3-hydroxyl group is substituted by a sugar (**Figure 4**; flavonol with R_1 = sugar, R_2 = H), it could react with free radicals to form an *o*-quinone. Investigations with DPPH• showed that glycosidation of the 3-hydroxyl group of quercetin did not alter the antiradical activity of quercetin (*31*). While the LDL oxidation method showed similar results (*11*), other findings proved quercetin to be more antioxidant than its glycoside, quercetin 3-*O*-rutinoside **1** (*12*−*13*).

The important role of the catechol structure in the B-ring of a flavonol was demonstrated in the case of isorhamnetin **7** (**Figure 4**; flavonol with R_1 = H, R_2 = CH_3). This quercetin 3′-*O*-methyl ether and also its 7-*O*-rhamnoside **6** are able to quench Fremy's radical and are thereby oxidized to *p*-quinone structures. They exhibited antioxidant capacities of 2.7 and 2.6 mol Fremy's salt/mol, which are significantly lower than for quercetin and its 3-*O*-glycosides (**Table 1**).

Glycosidation of the 3-hydroxyl group of isorhamnetin **7** or isorhamnetin 7-*O*-rhamnoside **6** further diminished the antioxidant capacity. The values for isorhamnetin 3-*O*-glycosides **3**−**5** ranged from 0.4 to 1.2 mol Fremy's salt/mol (**Table 1**). The poor radical scavenging activities of isorhamnetin 3-*O*-glycosides **3**−**5** could be explained by the substitution of the 3- and

Table 2. Concentration of Ascorbic Acid and Phenolic Compounds of Sea Buckthorn Juices (mg/L; Mean ± SD of Duplicate Assays) and Their Contribution to TAA (mmol Fremy's Salt/L and %)

	UTJ		ETJ	
	concentration (mg/L)	contribution to TAA (mmol Fremy's salt/L)	concentration (mg/L)	contribution to TAA (mmol Fremy's salt/L)
ascorbic acid **8**	1220 ± 64	28.1 (82.1%)	1220 ± 85	28.1 (71.6%)
phenolic acids				
gallic acid **9**	1.5 ± 0.1	0.07 (0.2%)	2.6 ± 0.0	0.12 (0.3%)
protocatechuic acid **10**	2.1 ± 0.2	0.08 (0.2%)	2.9 ± 0.3	0.10 (0.3%)
flavan-3-ols				
catechin **11**	19 ± 1	0.40 (1.2%)	26 ± 2	0.55 (1.4%)
epicatechin **12**	2.8 ± 0.1	0.06 (0.2%)	5.2 ± 0.3	0.12 (0.3%)
proanthocyanidins[a]	351 ± 16	7.4[b] (21.2%)	573 ± 6	12.1[b] (30.8%)
flavonols				
quercetin 3-*O*-rutinoside **1**	14.5 ± 0.4	0.15 (0.4%)	11.4 ± 0.6	0.12 (0.3%)
quercetin 3-*O*-glucoside **2**	9.0 ± 0.4	0.13 (0.4%)	7.7 ± 0.1	0.11 (0.3%)
isorhamnetin 3-*O*-glucoside-7-*O*-rhamnoside **3**	75 ± 4[c]	0.14 (0.4%)	66 ± 3[c]	0.13 (0.3%)
isorhamnetin 3-*O*-rutinoside **4**	181 ± 9	0.13 (0.4%)	182 ± 9	0.13 (0.3%)
isorhamnetin 3-*O*-glucoside **5**	75 ± 4	0.07 (0.2%)	87 ± 4	0.08 (0.2%)
isorhamnetin 7-*O*-rhamnoside **6**	1.3 ± 0.2[d]	0.01 (<0.1%)	1.3 ± 0.0[d]	0.01 (<0.1%)
isorhamnetin **7**	1.4 ± 0.3	0.01 (<0.1%)	0.4 ± 0.0	<0.01 (<0.1%)

[a] Concentration determined as cyanidin after acid depolymerization according to Nikfardjam (*20*). [b] Value calculated from the antioxidant capacity of the monomer catechin. [c] Concentration determined as isorhamnetin 3-*O*-glucoside. [d] Concentration determined as isorhamnetin.

3′-hydroxyl group by the sugar and methoxy groups (**Figure 4**; flavonol with R_1 = sugar, $R_2 = CH_3$). Thus, these compounds can be oxidized neither to *o*- nor to *p*-quinones.

The flavan-3-ols, catechin **11** and epicatechin **12**, studied were excellent radical scavengers. Their antioxidant capacity of 6.1 and 6.7 mol Fremy's salt/mol (**Table 1**) was approximately the same as measured for the quercetin 3-*O*-glycosides. However, they were less effective in scavenging Fremy's salt than the flavonol aglycone quercetin. Because of the lack of the 2,3-double bond and the 4-oxo function, flavan-3-ols are unable to support electron delocalization between the A- and the B-rings, stabilizing the aryloxyl radical after hydrogen donation (*12*). While the TEAC assay and ORAC assay confirmed quercetin to be a better antioxidant than the catechins (*12, 13*), the flavan-3-ols were more effective in inhibiting the copper catalyzed oxidation of human LDL in vitro (*11*).

Our findings on the relationship between the radical scavenging activity and the chemical structure of phenolic compounds showed great similarities with the hierachy of antioxidant effectivness described by Rice-Evans et al. (*12*). We can confirm the structural criteria for maximum antioxidant activity of flavonoids: the *o*-dihydroxy structure in the B-ring, the 3-hydroxyl group in the C-ring, and the 2,3-double bond in conjugation with an oxo function in the C-ring.

Contribution of Identified Constituents to TAA of Filtered Sea Buckthorn Juice. The concentrations of ascorbic acid and phenolic compounds in sea buckthorn juices are shown in **Table 2**. Our investigations comprised two juices, which were produced under different conditions. In contrast to UTJ, the mash of juice termed ETJ was treated with pectolytic enzymes before separation and pasteurization. Thus, we could observe differences in the content of antioxidant compounds in the two sea buckthorn juices. While in both cases the concentration of ascorbic acid and flavonols were quite similar, the juice produced by adding pectolytic enzymes (ETJ) showed higher values for phenolic acids, flavan-3-ols, and proanthocyanidins, which correlated with the detected total phenol content (data not shown).

Furthermore, **Table 2** shows the contribution of each phenolic compound and ascorbic acid to TAA of the filtered juices. This was calculated on the basis of concentration, antioxidant capacity (**Table 1**), and molecular mass of the compound. The calculated value for proanthocyanidins is based on the antioxidant capacity of the monomer catechin. Lu and Foo (*33*) showed that proanthocyanidins up to trimers are as efficient radical scavengers as the monomeric subunits. The percentage of antioxidant activity, which is due to the individual constituents, was calculated by comparison of its antioxidant activity with TAA of the juices determined by spin labeling using Fremy's salt. It should be noted that influence of lipophyllic antioxidants such as tocopherols and carotenoids on the antioxidant activity of the juices was minimized by removing the oil phase (pulp oil) by filtration.

The predominating antioxidant compound of filtered sea buckthorn juice was ascorbic acid **8** (**Table 2**). Because of its high concentration and its antioxidant capacity of 4.0 mol/mol Fremy's salt, its contribution to TAA of both juices was 28.1 mmol Fremy's salt/L (corresponding to 82.1% for UTJ and 71.6% for ETJ). Catechin **11**, epicatechin **12**, protocatechuic acid **10**, and gallic acid **9** showed higher antioxidant capacities than ascorbic acid (**Table 1**). However, because of their low concentration, all quantified catechins and phenolic acids contributed only 1.8% to TAA of UTJ and 2.3% to TAA of ETJ (**Table 2**). For example, the concentration of gallic acid **9** in UTJ was 1.5 mg/L. Its calculated contribution to TAA of the juice was only 0.07 mmol Fremy's salt/L. If the concentration of gallic acid **9** was as high as the value of ascorbic acid **8**, it would reduce 57 mmol Fremy's salt/L.

As compared to other flavonol glycosides, the potent radical scavengers quercetin 3-*O*-glucoside **1**, quercetin 3-*O*-rutinoside **2**, isorhamnetin **7**, and isorhamnetin 7-*O*-rhamnoside **6** were found in rather small quantities in sea buckthorn juice (**Table 2**). Thus, the total amount of these compounds contributed only 0.9% to TAA of UTJ (ETJ, 0.7%). Although isorhamnetin 3-*O*-glycosides **3**−**5** exhibited higher concentrations, their contribution to TAA of the juices was also very low (UTJ, 1.0%; ETJ, 0.8%) according to their poor radical scavenging properties. It should be noted that the list of the determined phenolic compounds could not be complete because of the lack of reference substances. With exception of compounds **1**, **2**, **6**, and

7, we could not detect any significant peak in the HPLC-ECD chromatogram that would correspond to a flavonol. According to the potential of the ECD working electrode, substances showing poor antioxidant properties such as isorhamnetin 3-*O*-glycosides **3**−**5**, gave no response by ECD detection. Thus, in the case of the flavonols, the influence of further unknown compounds on TAA of sea buckthorn juice could be excluded.

Furthermore, the results presented in **Table 2** demonstrate that proanthocyanidins were the main phenolic antioxidants in sea buckthorn juice. These so-called condensed tannins showed a concentration that is comparable to the flavonol glycosides. Provided that they show similar radical scavenging properties as their monomeric units, they explain 21.7 and 30.8% of TAA of UTJ and ETJ, respectively.

Summation of antioxidant activities contributed by the quantified constituents led to values of 36.7 mmol Fremy's salt/L for UTJ and 41.6 mmol Fremy's salt/mol for ETJ. These values were close to the measured TAA, which was 34.1 and 39.1 mmol Fremy's salt/L for UTJ and ETJ, respectively. Provided that no interactive effects occurred, this model for the antioxidant compounds in filtered sea buckthorn juice exhibits a good approach toward the true antioxidant activity of the juices.

In conclusion, this study indicates that ascorbic acid and proanthocyanidins are the major hydrophyllic antioxidants of sea buckthorn juice. Besides proanthocyanidins, further quanitatively important phenolic compounds of sea buckthorn juice are isorhamnetin 3-*O*-glycosides. Their contribution to TAA of the juice is negligible because they only show limited radical scavenging properties. The physiological significance of dietary antioxidants depends on their mechanism of absorption and biotransformation. In the case of the flavonol glycosides, it is generally considered that they are first hydrolyzed by the digestive microflora before being absorbed (*4*). However, investigations of Hollman et al. (*34*) indicate that quercetin glycosides from onions are more readily absorbed by the human small intestine than the aglycone form. To evaluate a possible physiological significance of the isorhamnetin 3-*O*-glycosides, more data about their bioavailability are necessary.

LITERATURE CITED

(1) Beveridge, T.; Li, T. S. C.; Oomah, D.; Smith A. Sea buckthorn Products: Manufacture and composition. *J. Agric. Food Chem.* **1999**, *47*, 3480−3488.

(2) Dr. Duke's Phytochemical and Ethnobotanical Databases (http://www.ars-grin.gov/duke/plants.html).

(3) Shapiro, D. K.; Garanovich, I. M.; Anikhimovskaya, L. V.; Narizhnaya, T. I. Biochemical and morphological characteristics of promising forms of common sea buckthorn. *Rastit. Resur.* **1978**, *14*, 560−564.

(4) Kühnau, J. The flavonoids. A class of semi-essential food components: Their role in human nutrition. *World Rev. Nutr. Diet.* **1976**, *24*, 117−191.

(5) Lachman, J.; Pivec, V.; Hubacek, J.; Rehakova, V. Flavonoid substances in the fruits of sea buckthorn (*Hippophae rhamnoides*). *Sci. Agric. Bohemoslov.* **1985**, *3*, 169−182.

(6) Block, G. A. A role for antioxidants in reducing cancer risk. *Nutr. Rev.* **1992**, *50*, 207−213.

(7) Hertog, M. G. L.; Feskens, E. J. M.; Hollman, P. C. H.; Katan, M. B.; Kromhut, D. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet* **1993**, *342*, 1007−1011.

(8) Hertog, M. G. L.; Kromhut, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; et al. Flavonoid intake and long-term risk of coronary heart disease and cancer in the Seven Countries Study. *Arch. Intern. Med.* **1995**, *153*, 381−386.

(9) Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **1989**, *320*, 915−924.

(10) Frankel, E. N.; Kanner, J.; German, J. B.; Parks, E.; Kinsella, J. E. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **1993**, *341*, 454−457.

(11) Teissedre, P. L.; Frankel, E. N.; Waterhouse, A. L.; Peleg, H.; German, J. B. Inhibition of in vitro human LDL oxidation by phenolic antioxidants from grapes and wines. *J. Sci. Food Agric.* **1996**, *70*, 55−61.

(12) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.

(13) Cao, G.; Sofic, E.; Prior, R. L. Antioxidant and prooxidant behavior of flavonoids: Structure−activity relationship. *Free Radical Biol. Med.* **1997**, *22*, 749−760.

(14) Sanchez-Moreno, C.; Larrauri, J. A.; Saura-Calixto, F. A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food Chem.* **1998**, *76*, 270−276.

(15) Frankel, E. N.; Meyer, A. S. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric.* **2000**, *80*, 1925−1941.

(16) Häkkinen, S.; Auriola S. High-performance liquid chromatography with electrospray ionization mass spectrometry and diode array ultraviolet detection in the identification of flavonol aglycons and glycosides in berries. *J. Chromatogr. A* **1998**, *829*, 91−100.

(17) Rechner, A.; Patz, C.-D; Dietrich, H. Polyphenolanalytik von Fruchtsäften und Weinen mittels HPLC/UV/ECD an einer fluorierten RP−Phase. *Dtsch. Lebensm. Rundsch.* **1998**, *94*, 363−365.

(18) Delage, E.; Bohoun, G.; Baron, A.; Drilleau, J.-F. High-performance liquid chomatography of the phenolic compounds in the juice of some French cider apple varieties. *J. Chromatogr.* **1991**, *555*, 125−136.

(19) Spanos, G. A.; Wrolstad, R. E. Influence of processing and storage on the phenolic composition of Thompson seedless grape juice. *J. Agric. Food Chem.* **1990**, *38*, 1565−1571.

(20) Nikfardjam, M. S. P. Polyphenole in Weissweinen und Traubensäften und ihre Veränderung im Verlauf der Herstellung. Ph.D. Thesis. Justus Liebig University Giessen, Germany, 2001.

(21) Häkkinen, S.; Heinonen, M.; Kärenlampi, S.; Mykkänen, H.; Ruuskanen, J.; Törrönen, R. Screening of selected flavonoids and phenolic acids in 19 berries. *Food Res. Int.* **1999**, *32*, 345−353.

(22) Bielig, H. J. Die Farbstoffe der Sanddornbeere. *Ber. Dtsch. Chem. Ges.* **1944**, *77*, 748−753.

(23) Hörhammer, L.; Wagner, H.; Khalil, E. Über die Flavonolglykoside des Sanddorns. *Lloydia* **1966**, *29*, 225−229.

(24) Purve, O.; et al. Flavonoids of *Hippophae rhamnoides* growing in Mongolia. *Chem. Nat. Compd.* **1978**, 341.

(25) Mukhamed'yarova, M. M.; Chumbalov, T. K. Polyphenols of the leaves of *Hippophae rhamnoides* II. *Chem. Nat. Compd.* **1977**, 243.

(26) Häkkinen, S. H.; Kärenlampi, S. O.; Heinonen, I. M.; Mykkänen, H. M.; Törrönen, A. R. Content of the flavonols quercetin, myricetin and kaempferol in 25 edible berries. *J. Agric. Food Chem.* **1999**, *47*, 7, 2274−2279.

(27) Rösch, D.; Mügge, C.; Kroh, L. W. Unpublished results.

(28) Yoshida, T.; Tanaka, K.; Chen, X. M.; Okuda, T. Tannins from *Hippophae rhamnoides*. *Phytochemistry* **1991**, *30*, 663−666.

(29) Chumbalov, T. K.; Mukhamed'yarova, M. M.; Polyakov, V. V. Polyphenols of the leaves of *Hippophae rhamnoides*. *Chem. Nat. Compd.* **1976**, 597.

(30) Gardner, P. T.; McPhail, D. B.; Crozier, A.; Duthie, G. G. Electron spin resonance (ESR) spectroscopic assessment of the contribution of quercetin and other flavonols of the antioxidant capacity of red wines. *J. Sci. Food Agric.* **1999**, *79*, 1011−1014.
(31) Burda, S.; Oleszek, W. Antioxidant and antiradical activities of flavonoids. *J. Agric. Food Chem.* **2001**, *49*, 2774−2779.
(32) Gardner, P. T.; McPhail, D.; Duthie, G. G. Electron spin resonance spectroscopic assessment of the antioxidant potential of teas in aqueous and organic media. *J. Sci. Food Agric.* **1998**, *76*, 257−262.
(33) Lu, Y.; Foo, L. Y. Antioxidant and radical scavenging activity of polyphenols from apple pomace. *Food Chem.* **2000**, *68*, 81−85.
(34) Hollmann, P. C. H.; de Vries, J. H. M.; Leeuwen, S. D.; Mengelers, M. J. B.; Katan, M. B. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr.* **1995**, *62*, 1276−1282.

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