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ARTICLE

# Flavonol Glycosides of Sea Buckthorn (*Hippophaë rhamnoides* ssp. *sinensis*) and Lingonberry (*Vaccinium vitis-idaea*) Are Bioavailable in Humans and Monoglucuronidated for Excretion

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Glucuronidation and excretion of sea buckthorn and lingonberry flavonols were investigated in a postprandial trial by analyzing the intact forms of flavonol glycosides as well as glucuronides in plasma, urine, and feces. Four study subjects consumed sea buckthorn (study day 1) and lingonberry (study day 2) breakfasts, and blood, urine, and fecal samples were collected for 8, 24, and 48 h, respectively. Both glycosides and glucuronides of the flavonol quercetin as well as kaempferol glucuronides were detected in urine and plasma samples after the consumption of lingonberries; 14% of flavonols in urine were glycosides, and 86% were glucuronidated forms (wt %). After the consumption of sea buckthorn, 5% of flavonols excreted in urine were detected intact, and 95% as the glucuronides (wt %). Solely glucuronides of flavonols isorhamnetin and quercetin were found in plasma after the consumption of sea buckthorn berries. Only glycosides were detected in the feces after each berry trial. Flavonol glycosides and glucuronides remained in blood and urine quite long, and the peak concentrations appeared slightly later than previously described. The berries seemed to serve as a good flavonol supply, providing steady flavonol input for the body for a relatively long time.

**KEYWORDS: Glucuronidation; isorhamnetin; kaempferol; lingonberry; metabolism; postprandial; quercetin; sea buckthorn**

## INTRODUCTION

Sea buckthorn (*Hippophaë rhamnoides* L.) is natively distributed over a wide area of Eurasia, especially in China, where it has traditionally been exploited for soil and water conservation purposes in addition to being an important food and medicinal plant. Recently, the health-related potential of sea buckthorn has raised interest also among Western researchers. The berry has been shown to possess antimicrobial (*1*, *2*) and in vitro radioprotective (*3*) properties as well as chemopreventive (*4*), antineurotoxic (*5*), and antiatherogenic (*6*) properties in animal trials. Sea buckthorn products may also have protective effect against atopic dermatitis (*7*), castric ulcer (*8*), and coronary heart disease (*9*) and inhibit platelet aggregation (*10*) as well as nicotine-induced oxidative stress (*11*). In a recent double-blind placebo-controlled trial in humans, a very modest amount of sea buckthorn (28 g daily) reduced high-sensitivity CRP level in plasma (*12*).

Lingonberry (*Vaccinium vitis-idaea* L.) is a wild, semiwoody chamephyte that keeps its leaves through winter and commonly grows in the northern latitudes. Lingonberry is an abundantly picked wild berry in Scandinavia and Russia. Lingonberry has been shown to possess both chemopreventive (*13*) and antimicrobial (*1*) properties in in vitro trials.

Sea buckthorn and lingonberry contain a wide range of secondary metabolites produced via the shikimate and acetate pathways. The phenolic compounds act as protecting agents, and they are often condensed in the cuticular surface layers of the plant. The phenolics include flavonoids (anthocyanins, flavonols, and catechins), phenolic acids, and stilbenoids, as well as condensed and hydrolyzable tannins. In sea buckthorn, a wide range of flavonol glycosides as well as oligomeric proanthocyanidins have been identified (*14*−*16*). In lingonberry flavonols, anthocyanins, catechins and their glycosides, and different caffeoyl and feruloyl conjugates have been identified (*17*−*19*). The basic structures of flavonols and some flavonol compounds of interest in this study are presented in **Figure 1**.

Phenolics of sea buckthorn have been shown to possess several potential health effects. The flavonoid fraction of sea buckthorn seeds has antihypertensive effect in high-sucrose fed rats (*20*); it inhibits thrombosis in mouse femoral artery and in vitro platelet aggregation (*21*) and enhances wound healing in rats (*22*). Isorhamnetin, a predominant flavonol in sea buckthorn, has been shown to prevent endothelial dysfunction and superoxide

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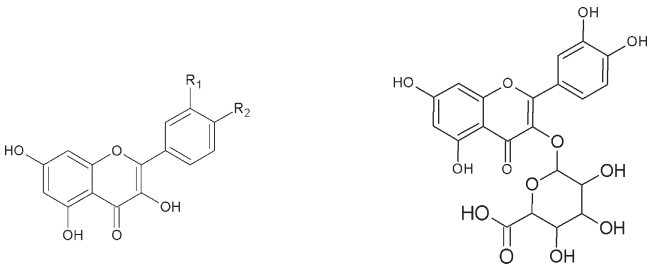


**Figure 1.** Basic structure of a flavonol aglycone, and exemplarily the chemical structures of one flavonol aglycone, quercetin, and one flavonol metabolite of that aglycone, quercetin-3-glucuronide. In the basic structure $R_1$ = OH and $R_2$ = OH for quercetin, $R_1$ = OH and $R_2$ = H for kaempferol, and $R_1$ = $OCH_3$, $R_2$ = OH for isorhamnetin.

production in medically induced hypertensive rats' aorta (*23*). Isorhamnetin has also been shown to possess antiproliferative and apoptotic effects in cancer cell cultures in vitro (*24*) as well as to inhibit procarcinogen-activating CYP1 enzyme in vitro (*25*). Although most in vitro research has been done with the aglycone form, in the study of Tribolo et al. (*26*) isorhamnetin-3-glucuronide was shown to modulate the expression of vascular cell adhesion molecule (VCAM). Isorhamnetin-3-glucoside has also shown potential in decreasing the risk of diabetes and its further complications such as cardiovascular diseases by lowering serum glucose concentration and inhibiting aldose reductase action in streptozotocin-induced diabetic rats (*27*). In some in vitro studies, it has been shown that antioxidative activity may be decreased in flavonoid metabolites found in vivo when compared with glycosides or aglycone forms, even though other bioactive effects are less affected by the intestinal and hepatic metabolism of flavonols (*28*−*30*). Lingonberry flavonols have not been investigated as such, although vast research concerning flavonol glycosides known to exist in lingonberries has indicated potential health effects (*31*).

The profiles of the phenolic compounds of sea buckthorn and lingonberry have been already well characterized, but mechanisms of absorption and metabolism of the compounds as conjugates are largely unknown. Some research has been performed both in animals (*32*,*33*) and in human subjects (*34*−*36*) by analyzing aglycone forms of flavonols present in sea buckthorn and lingonberry. However, this approach enables only the detection of absorption, not providing information on metabolites in various tissues. In most studies concerning flavonol metabolism, urine or plasma, but not feces, has been investigated.

The aim of this study was to investigate the absorption and excretion of sea buckthorn and lingonberry flavonols in a postprandial trial by analyzing flavonol glycosides and glucuronidated flavonols in plasma, urine, and feces.

## MATERIALS AND METHODS

**Reagents and Materials.** Methanol and acetonitrile were of HPLC grade and purchased from Sigma-Aldrich (Steinheim, Germany). Trifluoroacectic acid (TFA, ≥98.0%) was obtained from Fluka (Deisenhofen, Germany). Reference compounds (isorhamnetin-3-glucoside, isorhamnetin-3-rutinoside, kaempferol-3-glucoside, kaempferol-3-rutinoside, quercetin-3-rhamnoside, quercetin-3-galactoside, quercetin-3-glucoside, myricetin-3-rhamnoside, and syringetin-3-glucoside) were purchased from Extrasynthese (Genay, France). Flavonol glucuronides (isorhamnetin-3-glucuronide, quercetin-3-glucuronide, and quercetin-7-glucuronide) were obtained from Dr. Paul Needs (Institute of Food Research, Norwich Research Park, U.K.).

Stock solutions of reference compounds (0.5 mg/mL) were prepared in methanol or methanol/dimethyl sulfoxide (9:1, v/v, for isorhamnetin-3-glucoside and isorhamnetin-3-rutinoside) and stored at −70 °C.

Sea buckthorn (*H. rhamnoides* var. Ljubitelskaja) berries were organically cultivated in Finland and purchased from Vinkkilän Luomutuote (Vehmaa, Finland). Lingonberries (*V. vitis-idaea* L.) were of wild Finnish origin and purchased frozen from Pakkasmarja Ltd. (Suonenjoki, Finland).

**Postprandial Study Design.** To investigate the metabolism of sea buckthorn and lingonberry flavonols in humans, a postprandial clinical trial was designed. Four healthy, nonsmoking volunteers (two women and two men) aged on average 26 years (22, 24, 28, and 29 years), BMI 20−25, were recruited. The subjects followed a flavonoid-free diet for 30 h before the study days as well as during the sample collection period of 48 h. Between the study days, there was a wash-out period of 7 days. During the flavonoid-free diet, white bread, white rice, and white pasta were allowed, in addition to all foods of animal origin. After a 10 h fast, the subjects consumed a sea buckthorn breakfast (study day 1, 300 g of frozen sea buckthorn berries and some vanilla yogurt) or lingonberry breakfast (study day 2, 300 g of lingonberries and some vanilla yogurt).

Before the breakfast, a basal sample of blood, urine, and, when possible, feces was collected. Blood samples were drawn at 1, 2, 4, and 8 h after the consumption of berry breakfast, and urine and fecal samples were collected for 24 (0−4, 4−8, 8−12, and 12−24 h) and 48 h (0−12, 12−36, and 36−48 h), respectively, after the consumption of the berry breakfast. The plasma and urine samples collected on study day 1 (sea buckthorn) were frozen and stored at −70 °C immediately. Samples collected on study day 2 (lingonberry) were acidified with 0.1% TFA [0.04:1, v(TFA-$H_2O$)/v(sample)] and stored at −70 °C. Fecal samples were lyophilized for 3−5 days, homogenized, and extracted with acidified methanol (0.1% TFA, 20 mL for 5 g of sample). A 2 mL sample of plasma, a 6 mL sample of urine, and a 5 g sample of lyophilized feces were stored and used for analysis. The total amount of urine and feces excreted was not recorded.

The subjects were provided with a standardized flavonoid-free lunch and an early evening snack on the study day. A flavonoid-free late evening snack was also permitted.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki (2000), and all procedures involving human subjects were approved by the Ethics Committee of the Hospital District of Southwest Finland. Written informed consent was obtained from all subjects. Prior to the study, the subjects were informed about the investigation and their right to discontinue at any time without an explanation, and they had an opportunity to ask questions. The study products used in the study were safe, traditional berry products.

**Method Development.** For sample pretreatment, a method previously reported by us for anthocyanin analysis in urine (*37*) was used. Ultrahigh-performance liquid chromatographic (uHPLC) analysis of flavonols was developed by testing solvents defined in the literature (*38*, *39*) at different concentrations and combinations. The gradient was optimized to enable a sufficient chromatographic separation of analytes in about 5 min. For identification of the different isomers of flavonol glucuronides, also another inlet method of a longer run time of 14.5 min was developed. Tandem mass spectrometric (MS/MS) analysis was performed in the multiple reaction mode (MRM). Ionization energies, collision energies, and daughter ions were carefully optimized for reference compounds.

**Method Validation.** Detection and quantification limits of the analytical method were determined by analyzing different concentrations of reference compounds in TFA/$H_2O$/MeOH (0.1:39.9:60, by volume). In accordance with Bioanalytical Method Validation Guidance for Industry (2003), the detection limit was considered to be the concentration that gave a signal-to-noise ratio (S/N) > 3, and the quantification limit was considered to be the concentration that gave S/N > 10. The lowest concentration of reference compounds analyzed was 0.2 ng/mL.

Repeatability was tested by spiking reference compounds (a concentration of 100 ng/mL of each) into urine that did not contain flavonols and by analyzing samples as several parallel samples within one day (five parallel samples) and in three consecutive days (three parallel samples each day). The yield of the extraction step was tested by spiking blank urine samples before the extraction and the extract after the extraction at a concentration

of 100 ng/mL. Freeze-and-thaw stability was tested by analyzing one urine sample before and after two freeze-and-thaw cycles.

The applicability of validation results for other matrices analyzed was tested by comparing the matrix effects of all three matrices. Reference compounds were spiked (a concentration of 100 ng/mL of each) into blank plasma, urine, and fecal samples pretreated as described below and analyzed by uHPLC-MS/MS to compare the retention times and responses.

**Quantification of Sea Buckthorn and Lingonberry Flavonols.** Half a gram of frozen berries was weighed, thawed, homogenized, and extracted once with 4 mL of 0.1% TFA/$H_2O$ and twice with 1 mL of 0.1% TFA/MeOH (1:1, v/v) by shaking the berries for 20 s, sonicating for 5 min, and shaking in a vortex shaker for 20 s and subsequently centrifuging for 5 min at 3400*g*. Supernatants were combined and diluted with 8 mL of 0.1% TFA/$H_2O$. Extracts were applied in Supelco (C18, 500 mg) solid phase extraction tubes preconditioned with 2 mL of methanol and 2 mL of 0.1% TFA/$H_2O$. Tubes were washed with 2 mL of 0.1% TFA/$H_2O$, and analytes were eluted with 1 mL of TFA/$H_2O$/MeOH (0.1:39.9:60, by volume). The samples were analyzed as such with the uHPLC-MS/MS method described below and quantified with the QuanLynx program with an internal standard method.

**Physiological Sample Preparation.** Sample pretreatment from a method previously reported by us for anthocyanin analysis from urine (*37*) was used for flavonol analysis. Briefly, acidified plasma, urine, or fecal samples were purified with solid phase extraction with 96-well plates with 2 mg of Oasis packing material (Waters, Milford, MA). Samples were injected directly from the collection plate wells into the uHPLC system.

**uHPLC-MS/MS Method.** All MS analyses were carried out on uHPLC-MS/MS equipment consisting of an Acquity UPLC system with a 50 mm × 2.1 mm, 1.7 $\mu$m, Acquity UPLC BEH $C_{18}$ column and a Quattro Premier tandem quadrupole mass spectrometer (Waters). Elution of the berry and physiological samples was performed using 1% acetic acid in water as solvent A and acetonitrile as solvent B. The gradient was optimized to achieve baseline separation of all the critical pairs of the flavonol glycoside reference compounds. The flow rate was set at 0.45 mL/min. Initial solvent composition for 0.45 min was 90% A and 10% B. Subsequently, the compounds were eluted with a gradient from 0.45 to 2 min, resulting in 82.5% A and 17.5% B. After that, the proportion of B was increased to 70% for column wash and the initial conditions were stabilized at 4.8−5.3 min. The total run time was 5.3 min.

For the identification of the different isomers of flavonol glucuronides synthesized, glucuronide reference compounds as well as some physiological samples were analyzed by another uHPLC method. Elution was performed using 0.33% formic acid in water as solvent A and methanol as solvent B. The gradient was optimized to achieve baseline separation of analytes. The flow rate was set at 0.3 mL/min. Initial solvent composition was 85% A and 15% B. Subsequently, compounds were eluted with a gradient from 0 to 12 min, resulting in 30% A and 70% B. After that, the proportion of B was increased to 95% for column wash. The total run time was 14.5 min.

**Identification of Flavonol Glycosides and Their Metabolites in Physiological Samples.** Identification of flavonols in physiological samples was performed by spiking blank urine with reference compounds, when available, and comparing their retention times and parent and product ions in MS/MS. Identification of some flavonol metabolites was based on comparison of the retention time and respective *m*/*z* values of the precursor and product ion information to that found in the literature (*15*, *18*, *19*, *36*, *40*), because all of the reference compounds could not be obtained. As indicated in **Table 1**, isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside, quercetin-3-glucoside, quercetin-3-galactoside, kaempferol-3-glucoside, kaempferol-3-rutinoside, quercetin-3-rhamnoside, myricetin-3-rhamnoside, quercetin-3-glucuronide, quercetin-7-glucuronide, and isorhamnetin-7-glucuronide were used for identification. Reference compounds were not obtained for kaempferol glucuronides, quercetin-3-xyloside, and -arabinoside. Detection was carried out by using electrospray ionization in positive ion mode with the desolvation gas flow set to 598 L/h, capillary voltage to 3.2 kV, cone voltage to 13−15 V, and collision energy to 10−22 eV (optimized separately for each reference compound). MS/MS data were collected in the MRM mode by monitoring the transition of precursor and product ions specific for each compound.

All compounds were quantified by uHPLC-MS/MS analysis with one internal standard, syringetin-3-glucoside (*m*/*z* 509.4/347.0). To eliminate the error from differences in response, a standard curve was made by spiking flavonol-free blank urine with 0, 40, 200, 1000, and 5000 ng/mL of all the reference compounds used. For quantification of compounds for which the reference compounds could not be obtained, a standard curve of the most similar compound (another glycoside of the same aglycone or the same glycoside part with different aglycone) was utilized. Glucuronides were quantified as a sum because of coelution, and identification was performed by comparing retention times obtained with another inlet method.

**Glucuronide Synthesis.** To tentatively identify the substitution positions of glucuronides detected in plasma and urine samples, quercetin, kaempferol, and isorhamnetin aglycones were derivatized into corresponding glucuronides by UGT1A1 enzyme. Briefly, 65 $\mu$L of UDP-glucuronic acid, 6.25 $\mu$L of aglycone (10 mM in MeOH), 379 $\mu$L of potassium phosphate (20 mM, pH 7.0), and 25 $\mu$L of UGT1A1 enzyme (12 mg/mL) were mixed and incubated at 37 °C for 6 h. Reaction was stopped by adding 1.5 mL of acetone. The reaction mixture was centrifuged for 10 min at 16000*g*, and acetone was evaporated from the supernatant with nitrogen. The sample was analyzed by uHPLC-MS/MS both with the 5.3 min method used for physiological samples and with the other method developed to separate different isomers.

## RESULTS

**Method Validation.** The yield of the SPE extraction was 29−58% for different flavonol conjugates, and the repeatability (CV) of the method was 4.4−28.4%. The variance explained in a regression analysis, $R^2$, of the standard curve of quercetin-3 -galactoside was 0.9946. Detection and quantification limits for the reference compounds were 0.75 and 1.5−3.1 ng/mL in standard dilutions, respectively. Freeze-and-thaw stability was 75%, and freeze-and-thaw cycles were thus minimized in analyses. All samples were analyzed after a single freeze-and-thaw cycle. Intensity differences of the peaks of reference compounds as well as retention time shifts were modest in the matrices analyzed; thus, validation with urine was applicable to all sample matrices. The uHPLC-MS/MS run of 5.3 min utilizing small solvent volumes together with small 96-well plate solid phase extraction columns made the method economical and environmentally responsible.

**Flavonols in Berries.** As presented in **Table 1**, isorhamnetin-3-rutinoside was the major flavonol in sea buckthorn berries [54% (wt %)] and quercetin-3-rhamnoside in lingonberries [33% (wt %)]. Isorhamnetin-3-glucoside, isorhamnetin-3-galactoside, and quercetin-3-glucoside were also detected, accounting for 22, 8, and 13% (wt %, respectively) of the total flavonols in sea buckthorn. Quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-xyloside/-arabinoside were detected in lingonberries, accounting for 29, 4, 6, 5, and 19% (wt %, respectively) of the total flavonols. Isorhamnetin-3-glucoside, isorhamnetin-3-rutinoside, quercetin-3-glucoside, and quercetin-3-galactoside were identified by comparing the retention times and the mother and daughter ions in the uHPLC-MS/MS analysis with those of the reference compounds, and isorhamnetin-3-galactoside and quercetin-3-xyloside/-arabinoside were tentatively identified with the help of literature data on *m*/*z* values and retention time. On the basis of the berry analysis, the quantity of flavonols that the human subjects consumed in the postprandial trial was 12.1 mg on day 1 (sea buckthorn, SB) and 18.1 mg on day 2 (lingonberry, LB).

**Identification of Flavonol Derivatives from Physiological Samples.** Flavonol glycosides in their intact form prevailed in feces, whereas glucuronides were the most abundant analytes in urine. Both glycosidic and glucuronidic forms were detected in plasma after the lingonberry meal, whereas after the sea buckthorn meal

**Table 1.** Retention Times, Responses, MRM Shifts, and Identification Data for Each Compound Analyzed; Concentrations of Analytes in the Berries Consumed as a Study Meal as well as in Urine, Plasma, and Feces of Study Subjects

| | | | | | | | | | occurrence ($C_{max}$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | retention time | | | | identification | | | in berries (mg/100 g) | | in physiological samples (ng/mL for plasma and urine, ng/g for feces) | | | | | |
| no. | compd | short method | glucuronide method | response[a] | MRM shift | ref compd | synthesized ref compd | literature data | SB | LB | plasma (SB) | urine (SB) | feces (SB) | plasma (LB) | urine (LB) | feces (LB) |
| 1 | syringetin-3-glucoside | 2.72 | | | 509.4 > 347.0 | x | | | IS | IS | IS | IS | IS | IS | IS | IS |
| 2 | isorhamnetin-3-glucoside | 2.74 | | 1 | 479.4 > 316.9 | x | | | 0.88 | ND[b] | ND | 19.4 | 153.1 | NA[c] | NA | NA |
| 3 | isorhamnetin-3-galactoside | 2.50 | | | 479.4 > 316.9 | | | x | 0.22 | ND | ND | ND | ND | NA | NA | NA |
| 4 | isorhamnetin-3-rutinoside | 2.50 | | 0.39 | 625.5 > 316.9 | x | | | 2.2 | ND | ND | ND | 495.9 | NA | NA | NA |
| 5 | isorhamnetin-3-glucuronide[d] | 3.60 | 6.69 | | 493.0 > 317.0 | x | x | | NA | NA | sum | sum | ND | NA | NA | NA |
| 6 | isorhamnetin-7-glucuronide[d] | 2.90 | 6.18 | | 493.0 > 317.0 | | x | | NA | NA | 910[e] | 510[e] | ND | NA | NA | NA |
| 7 | isorhamnetin-4′-glucuronide[d] | 3.60 | 6.85 | | 493.0 > 317.0 | | x | | NA | NA | | | ND | NA | NA | NA |
| 8 | kaempferol-3-glucoside | 2.60 | | 0.83 | 449.4 > 286.9 | x | | | 0.01 | 0.04 | ND | ND | ND | ND | ND | ND |
| 9 | kaempferol-3-rutinoside | 2.37 | | 1.16 | 595.5 > 287.0 | x | | | 0.08 | 0.01 | ND | ND | 29.5 | ND | ND | ND |
| 10 | kaempferol-*x*-rutinoside | 1.60 | | | 595.5 > 287.0 | | | x | ND | ND | ND | ND | ND | ND | ND | ND |
| 11 | kaempferol-7-glucuronide | 2.60 | 6.00 | | 463.0 > 287.0 | | | | NA | NA | 81.2[e] | ND | ND | 6.4 | 59 | ND |
| 12 | kaempferol-3-glucuronide | 2.94 | 6.43 | | 463.0 > 287.0 | | | x | NA | NA | ND | ND | ND | ND | ND | ND |
| 13 | quercetin-3-glucoside | 1.96 | | 0.57 | 465.0 > 303.3 | x | | | 0.55 | 0.23 | ND | 7.7 | ND | ND | ND | ND |
| 14 | quercetin-3-galactoside | 1.87 | | 0.63 | 465.0 > 303.3 | x | | | 0.04 | 1.70 | ND | ND | 45.0 | ND | ND | 430 |
| 15 | quercetin-3-rhamnoside | 2.60 | | 0.64 | 449.4 > 303.0 | x | | | ND | 2.00 | ND | ND | ND | 5.1 | 19. | 620 |
| 16 | quercetin-3-arabinoside | 2.32[c], 2.47[d] | | | 435.0 > 303.0 | | | x | ND | 0.3[f]; 1.1[g] | NA | NA | NA | ND | ND | ND |
| 17 | quercetin-3-xyloside | 2.19 | | | 435.0 > 303.0 | | | x | ND | 0.34 | NA | NA | NA | ND | 7.3 | 690 |
| 18 | quercetin-3-glucuronide | 1.80 | 5.44 | | 479.0 > 303.0 | x | x | | ND | ND | ND | 62[e] | ND | 4.4 | 80 | ND |
| 19 | quercetin-7-glucuronide | 1.80 | 4.79 | | 479.0 > 303.0 | x | x | | ND | ND | ND | ND | ND | ND | ND | ND |
| 20 | quercetin-4′-glucuronide | 1.80 | 6.27 | | 479.0 > 303.0 | | x | | ND | ND | ND | ND | ND | ND | ND | ND |
| 21 | quercetin-3′-glucuronide | 1.80 | 7.04 | | 479.0 > 303.0 | | x | | ND | ND | ND | ND | ND | ND | ND | ND |
| 22 | myricetin-3-rhamnoside | 1.79 | | 0.86 | 465.4 > 319.1 | x | | | ND | ND | ND | ND | ND | ND | ND | ND |

[a] Determined from the physiological samples analysis method. [b] ND, not detected. [c] NA, not analyzed. [d] Quantitated as a sum because of coelution, only identification was made with longer inlet method. [e] Average of three study subjects, because one subjects samples could not be analysed to glucuronides after SB consumption. [f] Furanose form. [g] Pyranose form.

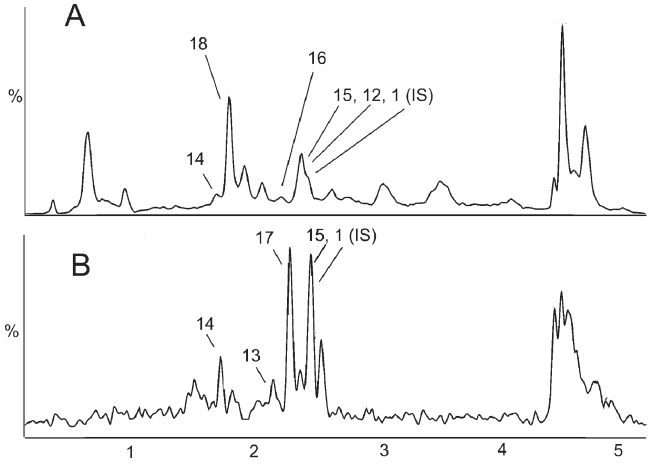


**Figure 2.** Total ion chromatograms of the HPLC-MS/MS analysis of urine (**A**) and feces (**B**) samples of one subject taken at 4−8 h and at 12−24 h after the consumption of lingonberries, respectively. Identification of peaks indicated is presented in **Table 1**.

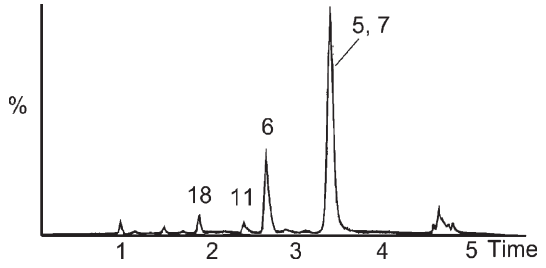


**Figure 3.** Total ion chromatogram of flavonol glucuronide analysis of the urine sample of one subject taken at 4−8 h after the consumption of sea buckthorn. Identification of peaks indicated is presented in **Table 1**.

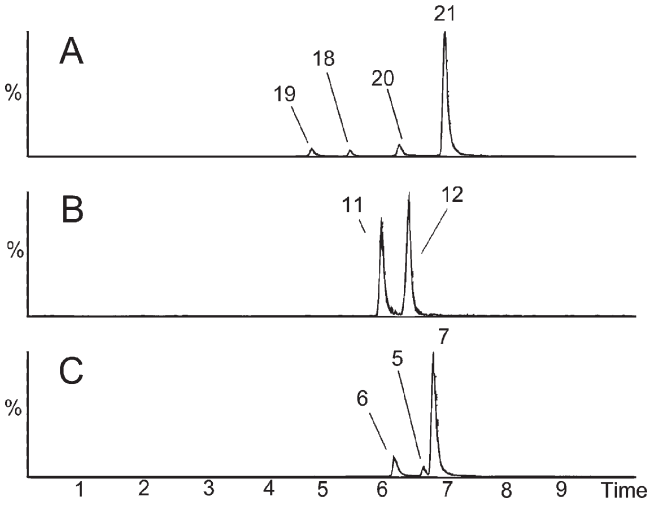


**Figure 4.** Total ion chromatograms of flavonol glucuronides synthesized from quercetin (**A**), kaempferol (**B**), and isorhamnetin (**C**) aglycones. Identification of synthesis products is based on the retention times and MS data of reference compounds quercetin-3-glucuronide, quercetin-7-glucuronide, and isorhamnetin-3-glucuronide as well as retention order information found in the literature. Identification of the peaks indicated is presented in **Table 1**.

only glucuronides were detected. **Figure 2** shows total ion chromatograms of a simultaneous flavonol glycoside and flavonol glucuronide analysis of urine and fecal sample of one subject

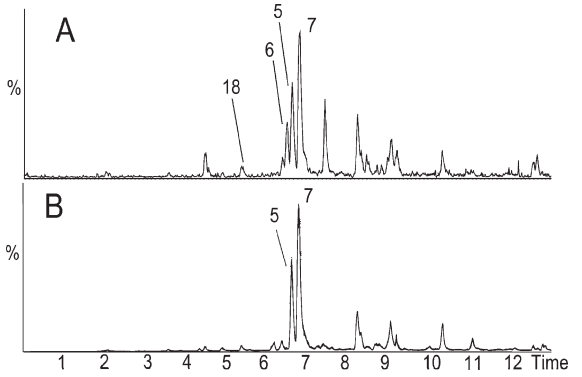


**Figure 5.** Total ion chromatograms of flavonol glucuronide analysis of plasma (**A**) and urine (**B**) samples after the consumption of sea buckthorn. Identification of peaks indicated is presented in **Table 1**.

taken at 4−8 h and at 12−24 h after the consumption of lingonberries, respectively. **Figure 3** shows an ion chromatogram of flavonol glucuronide analysis of the urine sample of one subject taken at 4−8 h after the consumption of sea buckthorn.

Isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside, quercetin-3-glucoside, quercetin-3-galactoside, and quercetin-3-rhamnoside were identified by comparison with reference compounds based on the retention times and detection of parent and product ions of MS analysis as indicated in **Table 1**. Quercetin-3-xyloside and -arabinoside were analyzed at $m/z$ values of 435.00/303.00, but reference compounds for these glycosides were not available. Three peaks were detected in this mass window in the lingonberry sample, whereas only one (**Figure 2**, peak 17) was present in physiological samples, that is, in urine. On the basis of the retention properties found in the literature (*17*, *19*), this was presumably quercetin-3-xyloside.

To define the site of glucuronidation, reference compounds of isorhamnetin-3-glucuronide, quercetin-3-glucuronide, and quercetin-7-glucuronide were obtained and other glucuronides were synthesized. A total ion chromatogram of the synthesized glucuronides is shown in **Figure 4**. Synthesized glucuronides were identified on the basis of the reference compounds obtained and known retention order (*41*). In synthesized glucuronides, peaks appearing at $m/z$ values 493/317, 463/287, and 479/303 indicated the presence of isorhamnetin monoglucuronide, kaempferol monoglucuronide, and quercetin monoglucuronide, respectively, due to the cleavage of the characteristic fragments of $m/z$ 176. Because some glucuronides coeluted in our 5.3 min inlet method, also another HPLC method was utilized to obtain baseline separation of isomers.

A subset of plasma and urine samples was analyzed by uHPLC-MS/MS with the longer inlet method to obtain information on the substitution sites of the glucuronides in the physiological samples. Chromatograms of flavonol glucuronide analysis of plasma and urine samples after the consumption of sea buckthorn are presented in **Figure 5**. The glucuronide isomers that could be identified (quercetin-3- and isorhamnetin-3-glucuronide, peaks 18 and 5, respectively) or tentatively identified (isorhamnetin-7- and -4′-glucuronides, peaks 6 and 7, respectively) on the basis of reference and synthesized glucuronides, respectively, are indicated.

Kaempferol glucuronides were tentatively identified on the basis of the $m/z$ values of precursor and parent ions, and isomerism was assumed on the basis of the retention order of different quercetin glucuronide isomers (*41*), because a reference compound could not be obtained.

After the consumption of sea buckthorn, isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside, quercetin-3-glucoside,

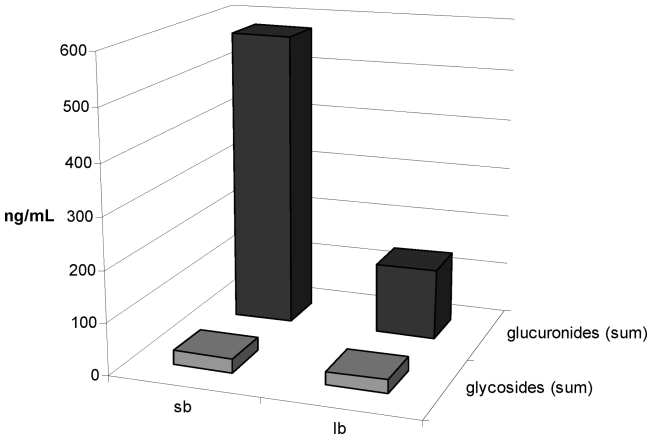


**Figure 6.** Maximum concentrations of summed flavonol glucuronides and summed flavonol glycosides in urine during the 24 h sample collection period after the sea buckthorn (sb) or lingonberry (lb) breakfast.

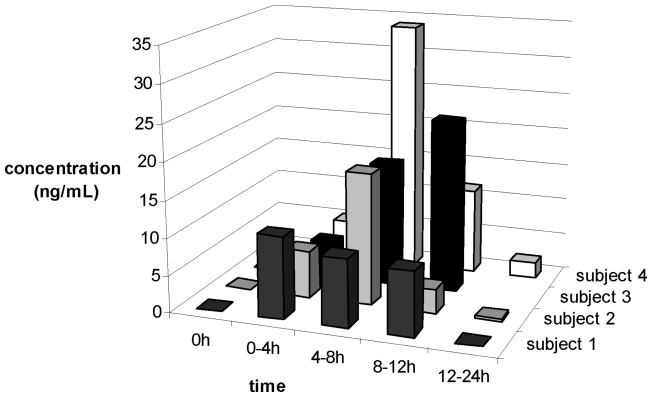


**Figure 7.** Concentrations of isorhamnetin-3-glucoside in urine samples of the four study subjects after the ingestion of sea buckthorn.

and kaempferol-3-rutinoside were detected in feces, isorhamnetin-3-glucoside, quercetin-3-glucoside, isorhamnetin glucuronides, and quercetin-3-glucuronide in urine, and isorhamnetin glucuronides and kaempferol-7-glucuronide in plasma. After consumption of lingonberries, quercetin-3-galactoside, quercetin-3-rhamnoside, and quercetin-3-xyloside were detected in feces, quercetin-3-xyloside/-arabinoside, quercetin-3-glucuronide, and quercetin-3-rhamnoside in urine, and quercetin-3-rhamnoside, quercetin-3-glucuronide, and kaempferol-7-glucuronide in plasma. These compounds were not detectable before the consumption of the berries, and the concentrations decreased in plasma and urine almost to baseline values during the collection period. For feces, the 48 h collection period was rather short as the concentrations of the flavonol glycosides did not reach baseline values at the 48 h time point. The flavonol-free diet of 32 h, however, was sufficient, as only minor amounts of analytes were detectable at baseline.

**Quantities of Flavonol Derivatives in Physiological Samples.** Flavonol glycosides ingested in this study as berry components were excreted steadily and efficiently glucuronidated, although large interindividual differences in the excretion rates and profiles could be observed. All of the compounds detected in the physiological samples taken before and during the 48 h sample collection period after the sea buckthorn or lingonberry breakfasts, and their quantities, are presented in **Table 1**.

After consumption of sea buckthorn, 5 wt % of flavonols excreted in urine were detected intact and 95 wt % as the glucuronidated form. The lingonberry meal resulted in somewhat different proportions of intact and glucuronidated forms as 14 wt % of flavonols analyzed from urine were glycosides and 86 wt % were glucuronidated forms. Profiles of the derivatives in urine are illustrated in **Figure 6**.

## DISCUSSION

Quantities of flavonol glycosides in feces were about 100 times higher than in urine. However, after the consumption of sea buckthorn, the quantities of glucuronides in urine were of the same magnitude as the quantities of glycosides in feces. Because the total volume of urine or mass of feces was not measured, the total excreted amounts of flavonols are not known. However, it can be concluded on the basis of this study that glucuronidation and excretion of isorhamnetin glycosides of sea buckthorn in urine are approximately equally important excretion routes to the excretion in feces without. It must be taken into account that some of the isorhamnetin glucuronides detected might be methylated and glucuronidated from quercetin glycosides also present in sea buckthorn. In our study design, monoglucuronidation of isorhamnetin and methylation and monoglucuronidation of quecetin could not be distinguished.

Interindividual differences in both absorption extent and time as well as relative proportions of different metabolites were high. **Figure 7** shows the concentrations of isorhamnetin-3-glucoside in the urine of individual study subjects.

Also in previous studies, large interindividual differences between study subjects in highest and lowest excreted concentrations have been reported (*42*, *43*). In a study by Bonetti et al., excreted concentrations of kaempferol was correlated with the body mass index of the seven subjects participating in the study (*42*). However, excretion rates of flavonols have been remarkably similar in all study subjects (*42*, *43*). The effect of genotype on the ability to metabolize bioactive compounds such as flavonols should be investigated in the future.

The maximum concentration of flavonol derivatives in plasma after the ingestion of lingonberries with yoghurt appeared at 2−4 h time points. This is slightly later than previously reported by Mullen et al. (*44*) after the ingestion of onions. However, Moon et al. (*45*) found the peak concentration of quercetin aglycone and its metabolites to appear even later, at 3−4 h after the ingestion of quercetin aglycone. Differences observed might be due to different food matrices, resulting in potential flavonoid−protein interactions with milk proteins in yogurt, or due to different sugar moieties attached to flavonol aglycone. The highest concentrations of flavonols in urine were found at 8−12 h of the sample collection period. Bonetti et al. (*42*) have reported the peak concentration of kaempferol in urine at 2−8 h after the consumption of beans. Kaempferol, however, has been reported to be excreted more efficiently than quercetin (*46*), indicating that the results might not be comparable. After the ingestion of onions, Mullen et al. (*47*) found the highest level of quercetin metabolites in urine during the 0−4 h collection period. Onions in this study were cooked, which might contribute to faster absorption than in the present study, in which the study subjects consumed uncooked berries. Berries thus seem to serve as foods or food components providing a relatively slow and steady flavonol supply to the body during several hours after ingestion.

Compared with the lingonberry meal, peak concentrations of flavonol monoglucuronides in plasma and urine samples analyzed in the present study were higher after the consumption of sea buckthorn, whereas the concentrations of flavonol glycosides in feces were higher after the lingonberry meal. This indicates that sea buckthorn and lingonberry flavonols are quite differently

metabolized. Also, compared with isorhamnetin, quercetin was excreted more in its intact glycosidic forms.

The human ability to absorb isorhamnetin-3-rutinoside has been indicated in an in vitro trial showing transportation through the Caco-2 cell layer by MRP-transporter proteins (*48*). Human studies concerning isorhamnetin metabolism, however, have not been performed prior to the present study. Bioavailability of isorhamnetin has been previously indicated (*34*, *35*). After the consumption of onions, both intact quercetin glycosides (*49*) and glucuronidated and sulfonated forms have been detected (*44*, *50*). Proportions of the intact and metabolized quercetin species in plasma or urine are known to vary widely (*51*, *52*), and in some studies, only glucosides (*49*, *50*) have been found. In this study, quantifiable amounts of both flavonol glycosides and glucuronides were detected after the ingestion of both berries in urine and after lingonberry consumption in plasma. However, after the sea buckthorn meal, glucuronides were present in urine at 10−50 times higher concentrations than glycosides, and in plasma samples glycosides were below the detection limits, whereas after the lingonberry meal, differences between glycoside and glucuronide concentrations in urine and plasma were more modest. This indicates that flavonols present in lingonberries are less prone to monoglucuronidation than the main flavonol in sea buckthorn, isorhamnetin, and are possibly directed toward other metabolic routes such as sulfonation and methylation. According to the present study, isorhamnetin, which exists as mono- and diglycoside derivatives in the sea buckthorn berries, is efficiently monoglucuronidated in vivo and is readily bioavailable.

In summary, this paper presents a rapid approach for flavonol analysis from various physiological samples as well as from berries and application of the method to the postprandial study of sea buckthorn and lingonberry flavonols. Our results indicate that isorhamnetin glucosides are absorbed and glucuronidated by routes similar to those of other flavonol glycosides investigated to date. Berries seem to serve as a good flavonol supply, providing a steady flavonol input to the body for a relatively long time. By consuming three berry meals daily, flavonol metabolite concentrations in blood would stay relatively high throughout the day, which would potentially have positive effects on health status.

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