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ORIGINAL PAPER

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Identification and quantitation of flavonols in rowanberry (*Sorbus aucuparia* L.) juice

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Abstract Eight flavonols were detected in rowanberry juice, six quercetin and two kaempferol glycosides which were identified as quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, two quercetin 3-*O*-dihexosides, quercetin pentose-hexoside and quercetin hexose-pentoside and two kaempferol dihexosides. The total content of identified flavonols in the rowanberry juice was 291 mg L^{-1}. Quercetin dihexosides contributed 78% to the flavonol content. However, chlorogenic and neochlorogenic acid were the most abundant phenolic compounds in the rowanberry juice (544 and 273 mg L^{-1}, respectively). From a nutritional point of view, rowanberry juice provides 70 mg of flavonols and 196 mg of chlorogenic acids per serving. HPLC-MS^n and HPLC-DAD in combination with solid phase extraction were used to obtain these results.

Keywords Rowanberry juice · Quercetin · Kaempferol · Flavonol glycosides · Mass spectrometry

Introduction

Berries are a traditional part of the Finnish diet (approx. 11 kg per person and year and less than half of this amount is wild berries) [1]. Nowadays, they also gain importance in the European diet thanks to new marketable berry juices. Rowanberry (*Sorbus aucuparia* L.) is a common, yellowish, wild berry that grows in the North of Europe. It is being used to obtain mixture berry juices and rowanberry juice itself. Berries have been described as an important source of flavonoids, particularly flavonols [2, 3]. Flavonoids are supposed to have important biological effects due to their ability to act as antioxidants. Their antioxidant activity affects reactive oxygen species and lipid peroxidation. Kähkönen and coworkers [3] found a high phenolic content and antioxidant activity in several berries. Reactive oxygen species might be involved in pathological diseases such as atherosclerosis, heart diseases, and cancer. The Trolox equivalent antioxidant capacity (TEAC) of quercetin is four-fold higher than that of the antioxidant (pro)vitamins. However, so far no association with cancer mortality has been found in epidemiological studies but flavones seem to be inversely associated with cardiovascular diseases [4].

Flavonols from food are absorbed from the small intestine in humans. For glycosides, the type of sugar and glycosylation site influence the absorption rate [5, 6]. *In vitro* bioavailability assays demonstrated the ability of cells from human small intestine to deglycosylate quercetin 4'-glucoside whereas other quercetin and kaempferol glycosides remained unchanged [7].

Rowanberry is a good source of vitamin C (490 mg kg^{-1}) [2]. Moreover, high quantities of flavonoids and phenolics have been detected in this type of berry, but there are only few reports that give specific identifications and contents. One study found high amounts of ferulic acid [3]. Häkkinen et al. [8] studied the phenolic profile in rowanberries by hydrolysing the phenolic compounds prior to chromatography. Of the compounds detected, 26.9% were quercetin, 0.5% kaempferol, and 0.1% myricetin. In a previous report [9] Häkkinen and Auriola identified four flavonol glycosides in rowanberries as quercetin dihexoside, quercetin deoxyhexose-hexoside, quercetin hexoside, and kaempferol hexose-pentoside by HPLC-ESI-MS. Rechner [10] found high amounts of chlorogenic and neochlorogenic acid in rowanberry juice and detected further peaks with flavonol-typical UV spectra.

This work was initiated to identify these flavonol peaks by LC-MS^n experiments. First of all, we optimised the HPLC gradient method and solid phase extraction procedure for the analysis of flavonoids in rowanberry

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juice and then identified the flavonols occurring in this type of juice by making use of the capabilities of MS^n experiments for sequencing glycosides [11]. The identified flavonols were subjected to quantitative analyses later.

Material and methods

Rowanberry juice sample

Rowanberry juice was obtained from the research center in Geisenheim, Department of Wine Analysis and Beverage Research. 59.4 kg of ground rowanberries and 20 kg of water were macerated for 1 hour and then pressed to yield 69 L of juice and 13 kg of mash.

Sample preparation for qualitative and quantitative analyses

For qualitative analyses using HPLC-DAD-MS, rowanberry juice samples were subjected to solid phase extraction on a polyamide column (J. T. Baker Company, NJ, USA) based on hydrogen bonds formed between the phase material and the phenolic hydroxy groups [11]. 5 mL of sample were applied onto the pre-conditioned column. Then 3 mL of water (Fraction A) were used to remove the sugars. After the wash step, four additive methanol volumes of 5 mL were used to elute phenolic compounds (fractions B, C, D and E). Fractions B, C, D and E were each concentrated to a volume of 2 mL at a rotary evaporator. For quantitative analyses with HPLC-DAD, juice samples were filtered through 0.2 µm polyethersulfone filters and injected without solid-phase extraction.

HPLC-DAD-MS system for qualitative analyses

Chromatographic separation was carried out on a Hypersil column (250×4.6 mm, RP-18, 5 µm particle size, Techlab, Erkerode, Germany) 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)] in a gradient program as shown in Table 1. The flow-rate was 1.0 mL/min, and the injection volume was 20 µL.

The HPLC system was equipped with a DAD detector and a mass detector in series. It consisted of a Beckman 126 HPLC pump, a Beckman 507e autosampler and a Beckman 168 photo-diode array detector controlled by Beckman gold nouveau software (Beckman Instruments, Inc., Fullerton, CA, USA). The mass detector was a ThermoQuest/Finnigan MAT LCQ ion-trap mass spectrometer (San José, CA, USA) equipped with an electrospray ionization (ESI) system and controlled by LCQ navigator software. The ionisation parameters were optimised using a constant infusion of chlorogenic acid standard (Serva, Heidelberg, Germany) to the ion source. The heated capillary and voltage were maintained at 280°C and 5 kV, respectively. The full scan mass spectra of the flavonols were measured from *m/z* 120 up to *m/z* 1000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, the collision energy was set at 30%. All mass spectrometry data were acquired in the negative ionisation mode. Total ion chromatograms (TICs) were measured for three alternating scan events: (1) MS (full scan) was used to measure the pseudomolecular ions ($[M–H]^-$), revealing the molecular masses of the components, (2) MS-MS was used to break down the most abundant pseudomolecular ion from MS (full scan), and (3) MS^3 was used to break down the most abundant fragment ion from MS-MS. UV chromatograms were recorded at 280 nm and 320 nm.

Table 1 Gradient program for HPLC analysis

Time (min)	Solvent A[a]	Solvent B[b]
Initial	100%	0%
5	93%	7%
15	88%	12%
50	80%	20%
60	75%	25%
85	50%	50%
90	20%	80%
95	0%	100%

[a] Solvent A=water-acetic acid (99.5:0.5, v/v).
[b] Solvent B=acetonitrile-water-acetic acid (50:49.5:0.5, v/v/v).

HPLC-DAD system for quantitative analyses

These analyses were performed with a Merck-Hitachi gradient liquid chromatograph with a pump model L-6200 and a diode array detector Merck-Hitachi model L-3000. Separations were achieved on a Lichrochart column (Merck, Darmstadt, Germany) (RP-18, 120×4.0 mm, 5 µm particle size), using the mobile phase and gradient program indicated in Table 1. The solvent flow rate was 1 mL min^{-1}. Sample aliquots of 50 µL were injected. Spectra were recorded from 240 to 400 nm every 0.64 s, the UV chromatogram at 320 nm was used for quantitation of flavonol and caffeoylquinic acids. All HPLC analyses were done in triplicate.

Retention times, UV-Vis spectra, and full MS, MS-MS, and MS^3 scans were compared with those of authentic commercial aglycone standards of quercetin (Sigma, Deisenhofen, Germany), kaempferol (Roth, Karlsruhe, Germany), and authentic flavonol glycoside standards that were isolated and identified in previous works. Quercetin 3-*O*-glucoside (isolated from onion) and chlorogenic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were used as standards to obtain quantitative results for the flavonols and chlorogenic acids, respectively.

Results and discussion

Qualitative analysis of flavonols from rowanberry juice

The qualitative analytical method was optimised with regard to the following aspects: a solid-phase extraction method able to enrich and separate the hydroxycinnamic acids and flavonols in different fractions; a mobile phase and eluent program able to separate the phenolic compounds; MS experiments to identify the type of flavonol glycoside occurring in the rowanberry juice.

Polyamide solid-phase extraction (SPE) was applied to enrich and fractionate the phenolic constituents. In fraction B, flavonol peaks 1, 2, 3, 4, 5, and 6 were found whereas in fraction C peaks 7 and 8 were found in significantly higher amounts. In fractions D and E, hy-

Table 2 Composition of polyphenols in polyamide SPE fractions

Fractions	Eluent	Main flavonols and phenolic acids[c]
A	H_2O[a]	Sugars
B	MeOH[b]	Flavonols (peaks 1, 2, 3, 4, 5, 6)
C	MeOH[b]	Flavonols (peaks 1, 2, 7, 8)
D	MeOH[b]	Hydroxycinnamic acids
E	MeOH[b]	Hydroxycinnamic acids

[a] 3 mL of H_2O.
[b] 5 mL of MeOH for every fraction.
[c] Peaks of flavonols are indicated in the Fig. 1 and 2.

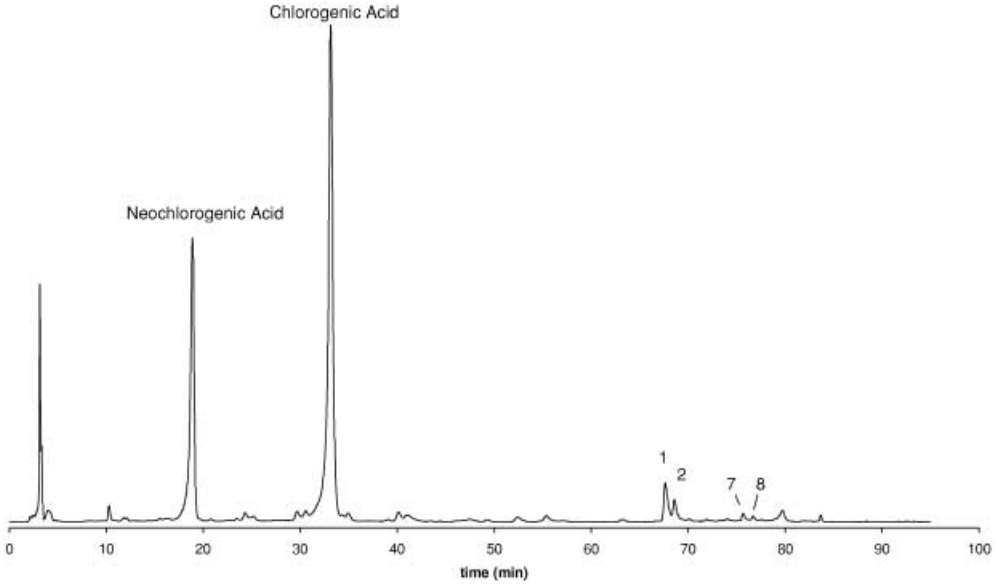

Fig. 1 Chromatogram of rowanberry juice at 320 nm without previous extraction

droxycinnamic acids were found (Table 2, the peaks are numbered according to Fig. 2).

Two gradient systems with methanol and acetonitrile as organic solvents were tested: (1) Solvent A=water-acetic acid (99:1, v/v), solvent B=methanol-water-acetic acid (50:49.5:0.5, v/v/v) and (2) solvent A=water-acetic acid (99.5:0.5, v/v), solvent B=acetonitrile-water-acetic acid (50:49.5:0.5, v/v/v). It was finally found that the second mobile phase containing acetonitrile separated the phenolic constituents in the rowanberry juice using the gradient program described in Table 1 better, although peaks 4 and 6 were not resolved properly (Fig. 2). UV detection was fixed at 280 and 320 nm, as all of the compounds showed appreciable absorption at these wavelengths.

HPLC-DAD analyses were performed in order to provide the characteristic UV spectra for every peak and to identify the compounds by their retention times and by standard addition. HPLC-ESI-MS^n was found to be a useful technique to determine the compound mass, to confirm the type of flavonol aglycone by its specific fragmentation pattern, and the possible type of sugar bound to the aglycone. Since mass spectroscopic techniques do not differentiate between isomeric sugars, they will not provide information regarding the exact glycosidic substitution other than the number of carbons or the presence of deoxy sugars.

The two highest peaks of the chromatogram (Fig. 1) belonged to neochlorogenic acid [retention time (RT) 19.1 min] and chlorogenic acid (RT 33.7 min). These peaks have been identified in rowanberry by Rechner in a previous report [10]. Ion masses in the negative ionisation mode and the specific fragmentation of both isomeric phenolic acids matched with those of standards (*m/z* $[M–H]^–$=353; MS-MS fragments of *m/z* 353=191; 179; 173; 135).

Peaks 1 and 2 displayed identical UV spectra with the same shape and ipsochromic shift compared to the quercetin aglycone as with quercetin 3-*O*-glucoside (Table 3). This suggests a similar *O*-glycosylation in the position 3 of the aglycone ring (compare Mabry et al. [12]). In addition, sugar bonds in the position 3 have been described as being more labile than other C-positions in the aglycone ring [13]. Full mass (MS) spectra of the two peaks showed pseudomolecular ions $[M–H]^–$ at *m/z*=625 (Fig. 2, D). Fragment ions at *m/z*=300 and 301 ($[quercetin–H]^–$) were found in the MS-MS-scan of this ion (Table 3). The highest relative abundance belonged to *m/z*=300, which showed an MS^3 fragmentation to *m/z*=271 and 255 corresponding to the fragmentation of several quercetin glycoside standards (Table 3). The occurrence of peaks at *m/z*=463, 445 and 179 ($[M–H–hexose]^–$, $[M–H–hexose–H_2O]^–$ and $[hexose–H]^–$) indicated that the quercetin was diglycosylated with two hexose moieties (Table 3). Therefore, peaks 1 and 2 were considered to be quercetin 3-*O*-hexose-hexosides. Häkkinen and Auriola [9] identified a quercetin hexose-hexoside with the same ion mass (*m/z*=627 in the positive ionisation mode) in rowanberry in which both sugar moieties were glucose.

The peaks 3 and 4 at RT=71.3 and 73.5 min had UV spectra similar to those of peaks 1 and 2 (Table 3). However, the maxima and shoulders were a bit different. The $[M–H]^–$ ions at *m/z*=595 (Fig. 2,A), their MS-MS fragmentation to *m/z*=300 with highest relative abundance and *m/z*=301 and the MS^3 fragmentation of *m/z* 300 confirmed that the aglycone was quercetin (Table 3). A fragment at *m/z*=463 in the MS-MS scan of the peak 3 $[M–H]^–$ ion (Table 3) showed that the terminal sugar was a pentose and the sugar bound to the aglycone ring a hexose. However, the MS-MS scan of the peak 4 $[M–H]^–$ ion showed a characteristic fragment at *m/z*=433 (Table 3). Therefore, the terminal sugar was, in this case, a hexose and the sugar bound to the aglycone a pentose.

At RT=72.9 and 73.6 min (peaks 5 and 6) eluted two compounds with kaempferol-like UV spectra and with

Table 3 UV and ESI mass spectral data (negative mode) of rowanberry juice flavonols

Peak	Retention time (min)	UV absorption (nm)	$[M–H]^–$ in MS[c]	$[M–H]^–$ in MS-MS[c]	$[M–H]^–$ in MS^3 of $X^–$[c]	Structure assignment
1	66.9	256m[a] 283sh[b] 354 m	625.3 (100)	505.1 (22) 463.1 (18) 445.3 (43) 301.2 (42) 300.2 (100) $X^–$ 179.1 (2)	271.4 (100) 255.5 (40)	Q 3-*O*-hexose-hexoside[d]
2	68.0	256 m 283 sh 354 m	625.3 (100)	505.2 (15) 463.2 (16) 445.2 (67) 301.2 (28) 300.2 (100) $X^–$ 179.1 (5)	271.3 (100) 255.4 (50)	Q 3-*O*-hexose-hexoside
3	71.3	266 m 309 sh 350 m	595.2 (100)	475.2 (12) 463.2 (10) 445.2 (23) 301.2 (20) 300.2 (100) $X^–$	271.3 (100) 255.4 (65)	Q hexose-pentoside
4	73.5	266 m 309 sh 350 m	595.6 (100)	433.2 (10) 415.2 (30) 301.3 (20) 300.2 (100) $X^–$	271.3 (100) 255.4 (70)	Q pentose-hexoside
5	72.9	263 m 289 sh 349 m	609.5 (100)	447.3 (6) 429.2 (47) 285.2 (100) $X^–$ 284.2 (43)	257.3 (100)	K hexose-hexoside[e]
6	73.6	263 m 289 sh 352 m	609.7 (100)	447.3 (14) 429.2 (100) 285.2 (78) $X^–$ 284.1 (75)	257.2 (100)	K hexose-hexoside
7	75.1	256 m 283 sh 354 m	463.6 (100)	301.2 (100) $X^–$ 300.2 (67)	273.2 (9) 257.3 (10) 179.1 (100) 151.2 (30)	Q 3-*O*-galactoside
8	76.1	256 m 283 sh 356 m	463.7 (100)	301.1 (100) $X^–$ 300.2 (68)	273.2 (19) 257.4 (18) 179.1 (100) 151.2 (40)	Q 3-*O*-glucoside
Std[f]	75.7	255 m 282 sh 355 m	463.1 (100)	301.1 (100) 300.2 (73) $X^–$	271.3 (100) 255.4 (45)	Q 3-*O*-galactoside
Std	76.5	256 m 283 sh 356 m	463.2 (100)	301.2 (100) $X^–$ 300.3 (40)	273.2 (12) 257.3 (12) 179.2 (100) 151.2 (44)	Q 3-*O*-glucoside

[a] m, peak maxima; [b] sh, shoulder; [c] Relative abundance given in brackets; [d] Q, quercetin; [e] K, kaempferol; [f] Std, standard

$[M–H]^–$ ions at *m/z*=609 (Fig. 2, C and Table 3). It is important to underline that peaks 4 and 6 were not properly resolved in the UV chromatogram but are easily held apart if single ion chromatograms are plotted (Fig. 2, A and C). Fragmentation of the pseudomolecular ions for the two peaks and further fragmentation of the main fragment at *m/z*=285 resulted in a fragment ion at *m/z*=257, a characteristic fragment of the kaempferol aglycone. Fragments at *m/z*=429 and 447 corresponded to $[M–H–hexose]^–$ and $[M–H–hexose–H_2O]^–$ (Table 3). Therefore, peaks 5 and 6 were identified as kaempferol hexose-hexosides. We did not find kaempferol hexose-pentosides as has been described in a previous report [9].

The UV spectra of the peaks 7 and 8 at RT=75.7 and 76.7 min corresponded to that of quercetin glucoside (Table 3). In the ESI-MS analyses, a signal at *m/z*=463 was found as the $[M–H]^–$ ion in both peaks (Fig. 2, B). The MS-MS experiments showed signals at *m/z*=301 (main signal) and 300 for both peaks. Further fragmentation (MS^3) of the fragment at *m/z* 301 yielded the same characteristic peaks as the fragmentation of the quercetin aglycone. It might be important to point out that the characteristic signals found in the MS-MS of the quercetin aglycone standard ($[M–H]^–$ at *m/z*=301) appeared at *m/z*=273 and 257 while the fragments obtained from the peak at *m/z*=300, the signal of highest relative abundance

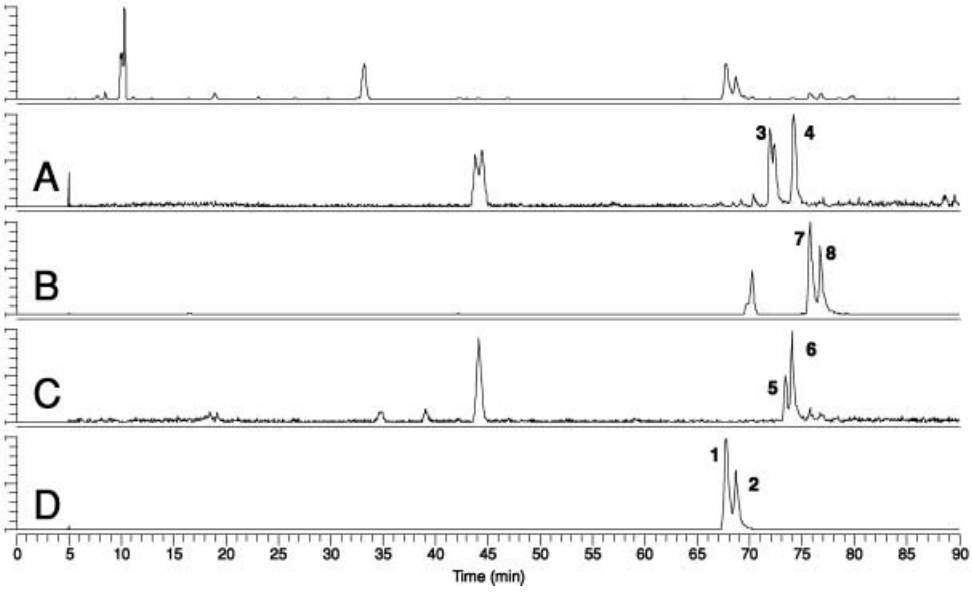

Fig. 2 Base peak chromatogram of rowanberry juice and single mass chromatograms at *m/z*=595.5 (A), *m/z*=463.5 (B), *m/z*=609.5 (C), and *m/z*=625.5 (D)

in the diglycoside MS-MS experiments, appeared at *m/z*=271 and 255. Taking into account all the information provided by diode array detection and ESI-MS, we concluded that peaks 7 and 8 were two quercetin 3-*O*-hexosides. Injections of 20 µL of rowanberry juice sample with added standard quercetin 3-*O*-galactoside or quercetin 3-*O*-glucoside (isolated in our laboratory) confirmed the identity of the two quercetin glycosides. Quercetin 3-*O*-galactoside eluted at RT=75.1 min and quercetin 3-*O*-glucoside at 76.1 min. This is in agreement with previous studies that described quercetin 3-glucoside in rowanberry [9, 14].

Quantitative analyses of flavonols and phenolic acids in rowanberry juice

Few studies describe the flavonol content of rowanberry [2, 3, 15] and none that of rowanberry juice. However, the phenolic content of other berries like strawberry, raspberry, or black and red currant have been studied more intensely [2, 3, 15, 16, 17, 18]. We quantified the amount of phenolic compounds in the studied rowanberry juice (Table 4, data are calculated in mg of quercetin equivalent) to assess its dietary benefit. The total flavonol content was 291 mg L^{-1}, corresponding to 70 mg per serving (one serving is estimated as 240 mL of juice according to FDA [19]). The two quercetin 3-*O*-hexose-hexosides [QHH(1) and QHH(2)] showed the highest concentration (78% of the total flavonol content). Q3Gal and Q3Glu contributed slightly more to the total flavonol content than QHP, QPH, KHH(1), and KHH(2) (Table 4). In 1996, the quercetin, kaempferol and flavonoid intakes in Denmark were 12.4, 2.4, and 26 mg day^{-1}, respectively [16]. Only one serving of rowanberry juice provides amounts of 34 mg quercetin and 2 mg kaempferol. Therefore, the consumption of this type of juice could be recommended to increase the intake of antioxidative species.

Table 4 Flavonol content in rowanberry juice (calculated as quercetin equivalents)

Peak	Flavonol[a]	Content in rowanberry juice in mg L^{-1}
1	QHH (1)	114.9±4.8
2	QHH (2)	116.5±3.8
3	QHP	6.0±0.3
4+6	QPH+KHH (2)	15.2±2.3
5	KHH (1)	9.1±0.4
7	Q3Gal	16.3±1.4
8	Q3Glu	13.3±1.3
	Total	291.3±13.3

[a] QHH, quercetin hexose-hexoside; QHP, quercetin hexose-pentoside; QPH, quercetin pentose-hexoside; KHH, kaempferol hexose-hexoside; Q3Gal, quercetin 3-*O*-galactoside; Q3Glu, quercetin 3-*O*-glucoside.

Chlorogenic and neochlorogenic acid were also quantified. Their contents in the rowanberry juice were 544 mg L^{-1} and 273 mg L^{-1}, respectively. The phenolic acid content was approximately 3 times higher than the total flavonol content.

Conclusion

Negative ion electrospray MS provides a potent analytical tool for the identification of plant phenolics, especially for minor compounds. In combination with HPLC-DAD, this technique permitted the detection of eight flavonols: six quercetin glycosides (quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, two quercetin 3-*O*-hexose-hexosides, a quercetin pentose-hexoside and a quercetin hexose-pentoside) and two kaempferol hexose-hexosides. From an analytical point of view, it was striking that the collision-induced fragmentation of glycosylated quercetin led to a fragment ion at *m/z*=300 in addi-

tion to the pseudomolecular ion of quercetin at *m/z*=301. In the fragmentation spectra of the diglycosides this ion was the main signal.

These results might be useful for the characterisation of rowanberry juices and the detection of adulterations with other types of berry juices. Quercetin glycosides, kaempferol glycosides, and isorhamnetin glycosides have already been used as characteristic compounds of fruit juices permitting detection of the presence of other juices in mixtures [20]. However, further specific techniques should be applied to identify the sugars in the six flavonol diglycosides that have been detected.

The total content of identified flavonols in the rowanberry juice was 291 mg L^{-1} (calculated in mg of quercetin equivalents). Quercetin 3-*O*-hexose-hexosides contributed 78% to the flavonol content. Chlorogenic and neochlorogenic acid were the most abundant phenolic compounds (544 and 273 mg L^{-1}, respectively). From a nutritional point of view, rowanberry juice provides 70 mg of flavonols and 196 mg of chlorogenic acids per serving. The juice could be an agreeable means to increase the intake of phenolic compounds in specific diets. However, since the bioavailability of flavonoids and phenolics from berries as well as from other sources is poorly known, their ultimate role in the health effects of berries remains to be investigated [1].

Statistics

Quantitative results and standard deviation are calculated as media of three values.

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