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Separation of quercetin, sexangularetin, kaempferol and isorhamnetin for simultaneous HPLC determination of flavonoid aglycones in inflorescences, leaves and fruits of three *Sorbus* species

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous determination of four flavonol aglycones (quercetin, QU; sexangularetin, SX; kaempferol, KA; isorhamnetin, IS) in hydrolyzed extracts from different plant parts of *Sorbus aucuparia* L., *Sorbus aria* (L.) Crantz. and *Sorbus intermedia* (Ehrh.) Pers. Separation of the four compounds was accomplished on a C18 Lichrosphere 100 column (5 μm, 250 mm × 4.6 mm, i.d.) with a methanol gradient elution and recorded at 370 nm. The high resolution of critical bands – SX, KA and IS – was achieved with retention of the last peak (IS) in 19.5 min. The equilibration of the standard mixture by addition of HCl to an acid concentration equal that of hydrolyzed extracts injected was found to be necessary when minimizing calibration error. The correlation coefficients of all the calibration curves showed good linearity ($r > 0.9991$) over the test range. The relative standard deviation of the method was less than 2.8% for intra- and inter-day assays, and the average recoveries were between 95.5 and 102.5%. High sensitivity was demonstrated with detection limits between 0.050 and 0.085 μg/ml. The level of total aglycones was found to be in the range of 687–1515 mg/100 g of dry weight in the inflorescences, 424–1078 mg/100 g in the leaves and 20–60 mg/100 g in the fruits depending on the *Sorbus* species.

1. Introduction

The plant genus *Sorbus* comprises about 100–200 species of deciduous trees or shrubs in the subfamily Maloideae of the family Rosaceae [1,2]. Three of these – *Sorbus aucuparia* L. (rowan tree), *Sorbus aria* (L.) Crantz. (whitebeam) and *Sorbus intermedia* (Ehrh.) Pers. (Swedish mountain ash) – are most common in Polish flora. Fruits of several *Sorbus* species (*S. aucuparia*, *S. aucuparia edulis*, *S. aria*, *S. domestica* and *S. torminalis*) are used as food ingredients [3,4] and also as traditional diuretic, anti-inflammatory, antidiarrhoeal (dried fruits), vasodilatory and vitamin agents [5,6] with high antioxidant activity [4,7–9]. Like the fruits, the inflorescences of *S. aucuparia* are recommended in traditional medicine for similar disorders, i.e., as diuretic and anti-inflammatory agents [3,6]. Recent papers indicated a direct correlation between the antioxidant capacity of *Sorbus* extracts and high content of phenolic compounds [4,9]. So far, various polyphenols have been found in *Sorbus* [10], and among them, flavonoids have been reported as the main bioactive components in *S. aucuparia*, *S. aria* and *S. intermedia* [11–14]. The studies referred to several quercetin (QU) and kaempferol (KA) glycosides in different plant parts, such as inflorescences, leaves and fruits of the three species. Sexangularetin (SX) glucoside has additionally been found in *S. aucuparia* [15], while isorhamnetin (IS) conjugates have been identified in *S. intermedia* [16] and in the related species *S. domestica* [17]. However, no quantitative information is currently available regarding flavonoid content in *Sorbus*, with the exception of detailed studies of *S. aucuparia* fruit (rowanberries) [13,14,18]. The analyses of flavonol aglycones in this material have been accomplished by HPLC, but only QU and KA have been separated, and therefore, the elaborated methods are not suitable for standardization of other *Sorbus* products with more complicated aglycone patterns. Thus, with the wide-ranging use of *Sorbus* in food and traditional medicine, it is necessary to establish a more universal analytical method for quality control and determination of flavonoids.

Plant materials containing flavonoids are often standardized by hydrolysis of the glycosides (which reduces the number of analytes) and subsequent quantification of the released aglycones as prescribed in pharmacopeias [19,20]. Several methods have been used to separate and determine flavonoid aglycones in hydrolyzed media (plant extracts or other biological samples) by HPLC [21–27]. KA and IS have been reported as being especially difficult to

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chromatographically separate, but good resolution has been achieved by several authors so fare [22–24,27–30]. There are, however, only a few reports discussing HPLC separation of SX [31–34]. Even in these cases, the reported resolution of co-occurring bands of SX, KA and IS was not satisfactory [32,33] or the separation required long analysis times [31,34]. Although SX is a relatively rare aglycone, it was found not only in *Sorbus* [15] but also in other rosaceous species like *Crataegus* [35], *Pyrus* [36], *Prunus* and *Dryas* [37], as well as in a number of other plant families, like Crassulaceae [32], Malvaceae [37], Fagaceae, Fabaceae or Cruciferae [38]. Moreover, SX was reported as constituent of numerous bee pollens and honeys with different floral origin [31,34]. The quantitation of SX should therefore be required for many plant materials.

The present work attempts to optimize the simultaneous HPLC separation of four flavonol aglycones, QU, SX, KA and IS. The data obtained enabled refinements in HPLC procedures to be made, providing enhanced resolution of the analytes during a relatively short run time. The optimized procedure was then applied to the quantification of flavonoids in hydrolyzed extracts of three Polish *Sorbus* species to evaluate the quality of their inflorescences, leaves and fruits.

2. Experimental

2.1. Plant material

All samples of inflorescences, leaves and fruits of *S. aucuparia* L., *S. intermedia* (Ehrh.) Pers. and *S. aria* (L.) Crantz. were collected in 2006 in the Botanical Garden in Łódź, and were prepared by air-drying in normal conditions and powdering. The inflorescences and leaves were collected in June, whereas the fruits were obtained in October. Voucher specimens (Nos. SAc001-SAc003, SIt001-SIt003, SAr001-SAr003) have been deposited at the Department of Pharmacognosy, Medical University of Łódź, Poland.

2.2. Chemicals, solvents and standards

Analytical-grade hydrochloric acid, methanol (POCh, Poland) and deionized water were used for hydrolysis and extraction procedures, while HPLC-grade methanol (POCh, Poland), water and orthophosphoric acid (Merck, Germany) were used in HPLC analyses. The standards of three aglycones (QU, KA and IS) were from Roth (Germany).

The fourth standard, SX, was isolated from inflorescences of *S. aucuparia*. The dried and powdered plant material (600 g) was pre-extracted with petrol and chloroform in a Soxhlet apparatus, then exhaustively extracted with boiling MeOH. The methanolic extract was evaporated, dissolved in water and re-extracted with diethyl ether (Et_2O). The Et_2O extract (5.5 g) thus obtained was separated by column chromatography on polyamide SC6 (50 g, Macherey-Nagel, Germany) (eluent: C_6H_6–MeOH, 9:1, v/v, isocratic elution) to yield two fractions of free aglycones (30 and 60 mg, respectively), which were separately purified by gel filtration on Sephadex LH-20 (25 g, Sigma–Aldrich, Germany) using MeOH as eluent. Two aglycones were isolated: SX (25 mg) and QU (50 mg). The quality of purification was verified by HPLC analysis and the purity was determined by injecting 20 μl of SX solution (at the concentration of about 0.1 mg/ml) into an analytical HPLC–UV under the conditions described in Section 2.3. As a result, the final purity was above 98% as determined by calculating the peak area percentage. The chemical structure of SX was also confirmed by ^{1}H and ^{13}C NMR studies and by physical properties.

Sexangularetin (8-*methoxykaempferol, herbacetin* 8-*methyl ether*) (*SX*): orange-yellow needles, mp 271–273 °C (MeOH) (Boetius apparatus); ^{1}H NMR δ, ppm: 12.15 (1H, s, OH-5), 8.05 (2H, d, J = 8.6 Hz, H-2′ and H-6′), 6.95 (2H, d, J = 8.6 Hz, H-3′ and H-5′), 6.26 (1H, s, H-6), 3.81 (3H, s, OMe-8). ^{13}C NMR δ, ppm: 176.11 (C-4), 159.29 (C-4′), 156.49 (C-7), 155.42 (C-5), 148.45 (C-9), 146.76 (C-2), 135.71 (C-3), 129.38 (C-2′ and C-6′), 127.46 (C-8), 121.85 (C-1′), 115.60 (C-5′), 115.33 (C-3′), 102.97 (C-10), 98.37 (C-6), 60.97 (8-OMe). The ^{1}H and ^{13}C NMR (125.7 MHz) spectra were recorded on Bruker 500 MHz instrument (in DMSO-d_6, TMS as int. standard). The data agree with those reported in the literature [35].

2.3. Chromatographic instrumentation and conditions

The analyses were carried out on a Waters 600E Multisolvent Delivery System (Waters Co., MA, USA) with a UV–vis dual wavelength absorbance detector (W 2487), a 20 μl sample injector (Rheodyne 7725 i) and a LC workstation equipped with Waters Millenium 32 version 4.0 software for data collection and acquisition. A C18 Lichrosphere 100 column (5 μm, 250 mm × 4.6 mm, i.d.) (Merck, Germany) guarded by a C18 pre-column and maintained at room temperature was used. The detection wavelength was set at 370 nm. The mobile phase consisted of solvent A (0.5%, v/v solution of orthophosphoric acid in water) and solvent B (MeOH) with the elution profile as follows: 0–10 min, 40–60% B (linear gradient, v/v); 10–21 min, 60% B; 21–23 min, 60–40% B (linear gradient); 23–26 min, 40% B; 26–30 min, 40% B (equilibration). The flow rate was 1.0 ml/min.

2.4. Sample preparation

The sample of plant material was powdered and sieved through a 0.315-mm sieve. An accurately weighed mass (200–400 mg for inflorescences, 300–500 mg for leaves and 1000 mg for fruits) was first defatted by pre-extraction with chloroform to remove waxes (after filtration, the chloroform extract was discarded) and refluxed for 1 h with 30 ml of 95% (v/v) aqueous MeOH and 9 ml of 25% (w/w) hydrochloric acid (7.7 M; 281 g/l). The hydrolysis solution thus obtained consisted of 1.8 M HCl (65 g/l) and 73% aqueous MeOH (v/v). After filtration, the sample was extracted twice with 20 ml of MeOH for 10 min. The combined hydrolysates were diluted with MeOH to 100 ml and filtered through a PTFE syringe filter (13 mm, 2 μm, Whatman, UK). The filtrate was directly injected (20 μl) into the HPLC system. Determinations were performed after three separate extractions of each sample, and each extract was injected in triplicate.

2.5. Optimization of the acid concentration in the standard solution

To evaluate the impact of acid concentration in the standard solution on the quality of separation (resolution and tailing factors), five acidified test solutions were prepared by diluting 1.6 ml of the standard stock solution (see Section 2.6.1.) with five different volumes of 15% (4.4 M; 160.9 g/l) hydrochloric acid (0, 100, 200, 300 and 400 μl, respectively) and MeOH to a final volume of 2 ml. The results are presented in Table 1.

2.6. Method validation

The analytical method was validated by determination of the linearity, precision, accuracy and stability of each analyte.

2.6.1. Standard solutions and calibration curves

The standard stock solution of the four aglycones was prepared in MeOH at the final concentrations of 35.70 μg/ml for QU, 19.15 μg/ml for SX, 20.89 μg/ml for KA and 21.26 μg/ml for IS,

Table 1
Impact of acid concentration on peak tailing and resolution factors

HCl concentration (M)[a]	A_s (symmetry factor, tailing factor)[b]					Resolution factor[c]	
	QU	SX	KA	IS	SX/KA	KA/IS	
0.00	50% h	1.26	1.18	1.18	1.19	1.28	1.33
0.22	50% h	1.20	1.13	1.13	1.12	1.50	1.55
0.44	50% h	1.10	1.05	1.05	1.03	1.77	1.84
0.66[d]	50% h	1.01	1.03	1.03	1.03	2.00	2.05
	5% h	1.07	1.29	1.30	1.28	–	–
0.88	50% h	1.04	1.05	1.05	1.05	2.05	2.10

[a] The concentration of hydrochloric acid in the standard test solution.
[b] Tailing factors calculated for each aglycone peak by the 50 and 5% of the peak height, according to [19b].
[c] Resolution factors for two pairs of adjacent bands calculated using bandwidths at halfhight [19b].
[d] Established optimal HCl concentration.

Table 2
Linearity of standard curves and detection/quantitation limits for the four flavonol aglycones determined

Compound	Linear range (μg/ml)	Calibration equation (linear model)[a]	Correlation factor, r[b]	LOD[c] (μg/ml)	LOQ[c] (μg/ml)
QU	0.714–28.560	$y = 91510x - 35864$	0.9991	0.085	0.283
SX	0.383–15.320	$y = 76276x - 23948$	0.9995	0.055	0.183
KA	0.209–16.712	$y = 78935x - 8179$	0.9999	0.050	0.167
IS	0.425–17.008	$y = 76577x - 19591$	0.9992	0.061	0.203

[a] y = peak area, x = concentration of standards in (μg/ml).
[b] r = correlation coefficient for six or seven (for KA only) data points in the calibration curves ($n = 3$).
[c] LOD: limit of detection, LOQ: limit of quantitation.

respectively. This standard solution was stored in the dark at 4 °C (the HPLC analysis indicated this solution was stable within at least 3 months, which is in agreement with earlier reports [40]). Immediately before calibration the stock solution was diluted with MeOH to 80, 60, 40, 20, 10, 2 and 1% of the starting concentration and adjusted with 15% hydrochloric acid (160.9 g/l; 4.4 M) to an acid concentration of about 2.5% (24.13 g/l; 0.66 M). Briefly, an appropriate volume of the stock solution was mixed with 0.3 ml of 15% acid solution and diluted with MeOH to 2 ml. The acidified standard solutions were stable over at least 14 days when stored in the dark at 4 °C, and over at least 7 days when stored at room temperature. These standard solutions at seven concentrations were injected into the HPLC system to establish calibration curves (with triplicate injections for each concentration). The linearity of analyses for each aglycone were verified by regression analysis and plotted using linear regression of the mean peak area versus concentration (Table 2).

2.6.2. Limits of detection (LOD) and quantitation (LOQ)

The acidified standard stock solutions were diluted with MeOH to provide serial solutions with their concentrations decreasing to the smallest detectable peaks. This concentration was multiplied to obtain the 3-σ signal-to-noise (S/N) ratio for the LOD of each flavonol, and a value of 10-σ for the limit of quantitation (LOQ, Table 2).

2.6.3. Precision, stability and accuracy studies

The repeatability of the chromatographic method was tested using measurements of the intra- and inter-day variability. The precision was examined using acidified standard solution containing all four flavonoids (in concentrations of 22.85 μg/ml for QU, 12.23 μg/ml for SX, 13.37 μg/ml for KA and 13.61 μg/ml for IS) and real sample of *S. aria* inflorescences. The solutions were injected five times, and the relative standard deviation (R.S.D.) values were calculated for both retention time and integration area and considered as a measure of precision (Table 3). The intra-day variability was determined by analyzing each sample on the same day within 24 h. The inter-day reproducibility was performed on five different days with each sample injected five times on each of 5 days.

To evaluate the accuracy, the recovery tests were carried out in the extract of *S. aria* inflorescences spiked with two different volumes of the standard stock solution (Section 2.6.1). The test extract was prepared using a twice-higher mass of the plant material (800 mg) per the procedure described in Section 2.4, and three portions of 5 ml of the extract were transferred into three volumetric flasks. Two of them were spiked with 0.5 and 1 ml of the standard stock solution. Finally, all three extract samples were diluted with MeOH to 10 ml. Five HPLC analyses were taken for each sample. The accuracy was evaluated by calculating the mean recovery of the four flavonols from the spiked extract solutions versus the non-spiked extract sample (Table 4).

Table 3
Retention parameters and precision data of flavonol aglycones determined

Compound	Retention time RT (min)[a]	k'	Intra-day variability R.S.D. (%)				Inter-day variability R.S.D. (%)			
			Standard solution		Real sample		Standard solution		Real sample	
			RT	Peak area	RT	Peak area	RT	Peak area	RT	Peak area
QU	14.736	6.046	0.44	0.33	1.07	0.87	1.09	0.57	1.85	1.05
SX	17.683	7.440	0.45	0.58	1.55	1.43	1.38	0.88	2.24	1.33
KA	18.464	7.831	0.59	0.31	2.05	1.79	1.53	1.21	2.75	2.12
IS	19.518	8.331	0.59	0.36	1.34	1.09	1.63	1.27	1.98	1.68

[a] Mean values for standard solution tested for inter-day variability ($n = 25$), S.D. = standard deviation.

Table 4
Recoveries of the four flavonol aglycones in the extracts of *Sorbus aria* inflorescences

Compound	Initial mean concentration in the extract[a] (μg/ml)	Amount added (μg)	Concentration after addition[a] (μg/ml)		Recovery (%)	R.S.D.[b] (%)
			Expected	Measured		
QU	10.429	35.70	13.999	13.675	97.69	0.43
		17.85	12.214	12.017	98.38	0.25
SX	2.094	19.15	4.009	3.829	95.51	0.20
		9.57	3.051	2.966	97.20	0.33
KA	1.650	20.89	3.739	3.698	98.91	0.40
		10.45	2.695	2.761	102.45	0.49
IS	11.186	21.26	13.312	12.900	96.90	0.34
		10.63	12.249	11.998	97.95	0.55

[a] Mean concentration of the analyte in the final analytical solution ($n = 5$).
[b] R.S.D. values for $n = 5$.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

The critical step in the quantitation of *Sorbus* flavonoid aglycones is the separation of adjacent bands of SX, KA and IS. As was previously reported [26], the capacity factor (k') of flavonoids varies with the number and position of hydroxyl groups in the flavonoid skeleton. Fig. 1 shows that the mentioned three flavonols possess an identical number and identical positions of free hydroxyl groups, which causes difficulties during separation. The methoxyl groups occurring in the skeletons of SX and IS are too weakly polar to significantly influence the polarity of these molecules versus KA. This is probably the reason that, in spite of some previous work reporting successful separations of KA and IS [22,23,27–29] on short RP-HPLC columns (100–150-mm long), the preliminary tests made in the present study indicated that a longer column should be used in the presence of SX. Therefore, to optimize the resolution and run time, several mobile phases, including MeOH–water, ACN–water and THF–water, were tested on a 250-mm long C18 column (Lichrosphere 100). The MeOH–water gradient system containing 0.5% orthophosphoric acid (v/v) resulted in the best separation at a flow rate of 1 ml/min.

One serious problem in the separation of flavonoids is peak tailing, which has been connected with dissociation of the hydroxyl groups. The presence of acid in a mobile phase can prevent this effect by changing the pH, thereby improving peak symmetry of analytes [22,39]. However, the routine addition of 0.5% orthophosphoric acid to the mobile phase was only sufficient to reduce tailing in real *Sorbus* extracts. Regrettably, for the standard solution it was inadequate. Therefore, the influence of different acid concentrations in the mobile phase (0.5–2.5% orthophosphoric acid, v/v) and in the standard solution was tested (Table 1). In the standard solution, hydrochloric acid was used for tests to obtain conditions similar to plant hydrolyzates. Better results were observed with increased acid concentration in the standard solution than when the acid concentration in the mobile phase was elevated. With the limit of high peak symmetry ($1.07 \leq A_s$ (symmetry factor) ≤ 1.30, at 5% of the peak height), excellent resolution could be obtained when the HCl concentration in the standard mixture was not less than 24 g/l (0.66 M), and this concentration was used in the further studies as the most similar to that in *Sorbus* real samples (25.30 g/l, 0.70 M, in the final dilution). Stability tests of the acidified standard solution proved it to be stable for at least 14 days at 4 °C.

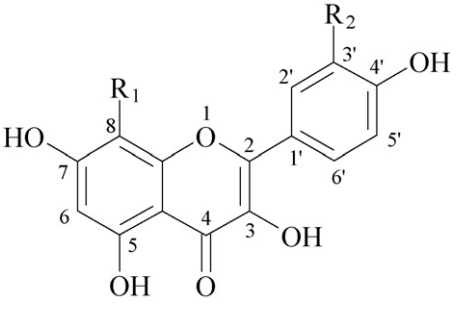

Fig. 1. Structures of four flavonol aglycones found in the *Sorbus* extracts.

	R_1	R_2
Quercetin (QU)	H	OH
Sexangularetin (SX)	OCH_3	H
Kaempferol (KA)	H	H
Isorhamnetin (IS)	H	OCH_3

As reported [22], tailing problems are mainly a function of the stationary phase, and many popular C18 materials (including Lichrosphere 100) are described as causing excessive peak tailing, especially during separation of free flavonoids [23]. The addition of HCl to the standard aglycones tested in the current study markedly reduced peak tailing, improved peak shape and enabled better separation on the Lichrosphere 100 stationary phase.

The acid concentration has moreover a great impact on detection of the flavonoid aglycones studied. As was shown in Fig. 2, with the increasing HCl concentration in the standard solution, the significant increase of peak heights with little changes of their surface areas were observed. The procedure described above matched the analytical conditions for the standard mixture to those of the real hydrolyzed samples, thereby reducing potential calibration error. Matching of the acid concentration in standard and sample solutions has been previously suggested for HPLC analyses of flavonoid aglycones by Hertog et al. [40] and by Justesen et al. [24], but without wider discussion.

As reported in Table 1, the optimized chromatographic conditions resulted in high separation quality. The resolution factor R_s calculated basing on peak widths at half-height [19b] was 2.00 and 2.05 for the SX/KA and KA/IS peak pairs, respectively, which suggested baseline separation. True baseline resolution, however, was only observed for low concentrations of the aglycones due to peak tailing and the non-Gaussian peak shape. Alternative R_s values were estimated based on the height of the valley between two adjacent bands [40]. The calculated height of the valley relative to the height of the smaller band was between 0.9 and 1.6% for both of the peak pairs. The R_s values were therefore not less than 1.46 for a 1/1 band-size ratio, and not less than 1.50 for a 2/1 band-size ratio [41]. The selectivity factor α, calculated from the equation $\alpha = k'_2/k'_1$ for three pairs of adjacent bands, was 1.053 for $\alpha_{SX/KA}$, 1.064 for $\alpha_{KA/IS}$ and 1.120 for $\alpha_{SX/IS}$. The retention parameters (retention times and k' values) obtained in the optimized conditions are listed in Table 3.

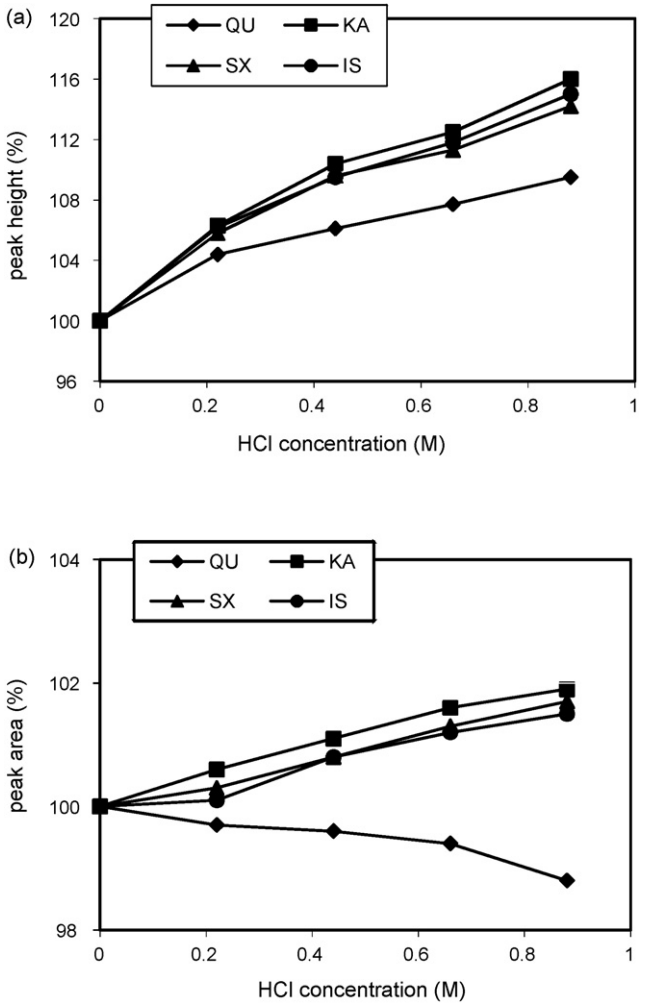

Fig. 2. Effect of HCl concentration in the standard solution on the peak heights (a) and peak areas (b) of flavonoid aglycones. All graph points are the mean of a triplicate analysis.

The separation of four flavonols on a 250-mm long C18 column in less than 20 min with high resolution and simple elution procedure enabled refinements in HPLC procedures to be made earlier [31–34]. The previous works have indeed not discussed the resolution, but the enclosed HPLC chromatograms allow examination of the separation quality obtained. The shortest analysis time has been reported by Ferreres et al. [33] with elution of IS in about 10 min, but the resolution of adjacent SX, KA and IS bands was inadequate. Soler et al. [31] achieved successful separation of KA and IS, however the elution time for IS increased to about 34 min, and the resolution of SX and KA remained unsatisfactory. Stevens et al. [32] reported retention times relative to rutin. The calculated α values for band pairs SX/KA, SX/IS and KA/IS (1.041, 1.010 and 1.053, respectively) were also lower than those obtained in the present study. The best results were published by Martos et al. [34]. The following α values were obtained: $\alpha_{SX/KA} = 1.04$, $\alpha_{KA/IS} = 1.16$ and $\alpha_{SX/IS} = 1.21$, but the retention of IS required 30.5 min. In all studies cited [31–34] short conventional C18 columns (100 or 125 mm) were used for HPLC separations, which could partially explain the relatively low resolution obtained for SX, KA and IS bands.

3.2. Linearity, precision, accuracy and stability

The four calibration curves exhibited linear regressions of at least $r > 0.9991$ (Table 2). The high sensitivity of the method

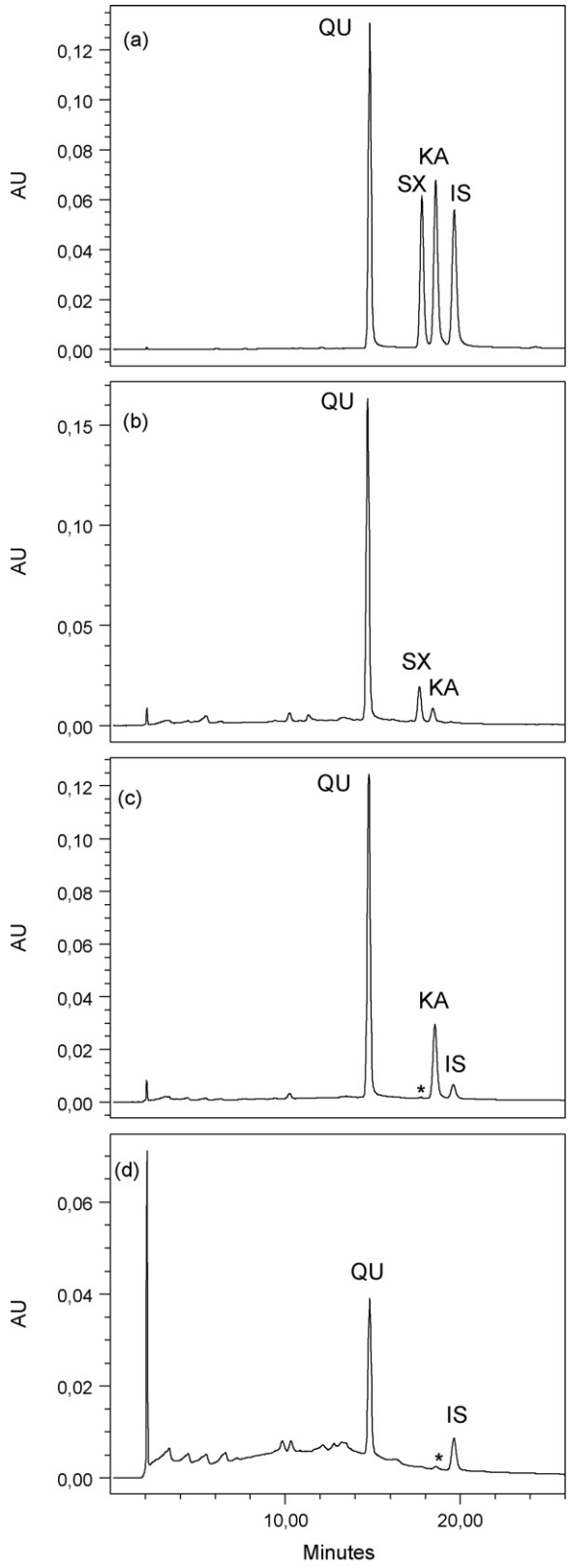

Fig. 3. Representative HPLC chromatograms of (a) standard solution at a high concentration (QU: 22.85 μg/ml, SX: 12.23 μg/ml, KA: 13.37 μg/ml, IS: 13.61 μg/ml); (b) real sample of *S. aucuparia* inflorescences; (c) real sample of *S. aria* leaves; (d) real sample of *S. intermedia* fruits. The analytes detected in traces were marked with an asterisk.

Table 5
Content of four flavonol aglycones in the inflorescences, leaves and fruits of three *Sorbus* species[a]

Sample	QU		SX		KA		IS		Total (mg/100 g)[b]	
	mg/100 g	R.S.D. (%)	mg/100 g	R.S.D. (%)	mg/100 g	R.S.D. (%)	mg/100 g	R.S.D. (%)	Aglycones	Glycosides
Sorbus aucuparia										
Inflorescences	1110.7	1.66	158.2	2.08	75.2	0.85	nd	nd	1344.1	2067.4
Leaves	881.1	2.41	nd	nd	196.9	2.23	nd	nd	1078.0	1666.0
Fruits	53.8	1.27	nd	nd	6.4	1.36	nd	nd	60.2	92.9
Sorbus intermedia										
Inflorescences	1053.4	1.25	117.3	0.51	29.3	1.59	314.8	1.28	1514.8	2320.7
Leaves	303.6	1.75	<LOQ	–	52.0	2.61	68.5	2.50	424.1	652.6
Fruits	32.5	2.04	nd	nd	2.4	3.87	9.5	3.00	44.4	68.2
Sorbus aria										
Inflorescences	291.6	2.15	52.6	1.16	43.7	1.52	299.3	2.04	687.2	1049.0
Leaves	518.9	3.13	14.8	3.50	254.6	2.04	99.8	3.03	888.1	1371.9
Fruits	9.4	2.45	nd	nd	2.4	3.00	8.5	2.71	20.3	31.1

[a] Mean aglycone content in mg per 100 g of dry weight of the plant material. R.S.D. for $n = 9$ (3 extractions and 3 injections for each extraction); nd: peak not detected.
[b] The conversion factors for calculation of total glycosides ($f_1 = 1.54$ for the aglycone QU (MW 302.24), $f_2 = 1.57$ for KA (MW 286.24) and $f_3 = 1.51$ for SX and IS (MW 316.27)) were determined with the molecular weight of glucosides: isoquercitrin (QU 3-*O*-β-D-glucopyranoside, MW 464.38), astragalin (KA 3-*O*-β-D-glucopyranoside, MW 448.38), SX and IS 3-*O*-β-D-glucopyranosides (MW 478.41)). Calculation: total glycosides = (f_1 × total QU) + (f_2 × total KA) + (f_3 × (total SX + total IS)).

was demonstrated with the low LOD (0.050–0.085 μg/ml) and LOQ (0.167–0.283 μg/ml). The highest LOD and LOQ were calculated for QU. These values were lower than those reported by other authors, working with conventional RP-C18 columns but without acid addition to the standards [23,25,26]. Similar values (LOD = 0.050–0.010 μg/ml) have been found for QU and KA by Repolles et al. [21], who worked with a monolithic C18 column, and by Justesen et al. [24], who worked with an acidified standard solution.

The results shown in Tables 3 and 4 indicate that the developed analytical method was reproducible with good recovery and stability. The intra- and inter-day variations for all analytes were less than 1.63 and 2.75%, respectively. The higher values were observed for the real samples, probably because of matrix error, and for retention times due to the lack of a column thermostat. Validation of the method was achieved by determination of the recovery during standard addition. The average recoveries were satisfactory for all four flavonoids, with values ranging from 95.51 to 102.45%, thereby confirming the robustness of the analytical method.

3.3. Optimization of the hydrolysis process

The most effective hydrolysis conditions for *Sorbus* extracts were determined in the preliminary screenings based on previous optimization studies by Hertog et al. [40]. The highest level of aglycones could be obtained with a hydrolysis solution of 1.8 M HCl (65 g/l) in 73% aqueous MeOH (v/v) and a reflux time of 60 min. As the analyzed *Sorbus* samples contain tannin-type proanthocyanidins and easy hydrolysable flavonol glycosides, complete hydrolysis needed relatively high HCl concentration and a short reaction period, but the data are still in agreement with earlier findings [40]. Interestingly, the most effective concentration of MeOH in the reaction medium is higher than that reported by Hertog et al. [40], probably due to the significant presence of relatively weak polar flavonols (SX, IS and KA) in *Sorbus* extracts.

3.4. Sample analysis

The sample solutions of the hydrolyzed *Sorbus* extracts were injected directly and separated under the optimized conditions mentioned above. The typical HPLC–UV profiles are illustrated in Fig. 3. The content of each flavonol in nine different *Sorbus* samples was determined by the corresponding regression equation and results were summarized in Table 5. It shows that the total content of flavonol aglycones varied strongly among plant material types from 687 to 1515 mg/100 g of dry weight in inflorescences, 424–1078 mg/100 g in leaves to 20–60 mg/100 g in fruits. For *S. aucuparia* and *S. intermedia* the highest amount of flavonoids was observed in inflorescences, whereas for *S. aria* the highest concentrations were present in leaves. Among the four analyzed aglycones, QU was the dominant component in all samples. The levels of the other three flavonols were significantly lower with the exception of the IS level in *S. aria* inflorescences and fruits, which was similar to the QU level. Interestingly, all four studied flavonols were simultaneously detected only in *S. intermedia* and *S. aria* (both species classified taxonomically in the same *Sorbus* subgenus *Aria* [1,2]), while in *S. aucuparia* (a member of the subgenus *Aucuparia*) IS was absent, which may be important from a chemotaxonomic point of view. SX, the second methoxyflavonol detected in all species, has often been reported as a constituent of bee pollen [31,34,35,37,38] and as being present in flowers. However, in the current study low or trace levels of SX were also detected in the leaves of *S. intermedia* (<LOQ) and *S. aria* (14.8 mg/100 g). This was probably due to strong tomentose leaves of both species and, therefore, to strong pollen adsorption on the leaf surface.

Flavonoid content was determined in the present work for the first time for all tested *Sorbus* samples, with the exception of *S. aucuparia* fruits. Wild rowanberries have been analyzed by Häkkinen et al. [18], finding flavonoid aglycones on the total level of 63 mg/kg of fresh weight. In addition, Kähkönen et al. [8] found flavonoid glycosides at a level of 165 mg/100 g of dry weight, calculated as rutin (approximately 82 mg/100 g calc. as QU). These results appeared to be in good agreement with those obtained in the present study, which found that the total content of aglycones in Polish rowanberries amounted 60 mg/100 g of dry weight.

4. Conclusion

The RP-HPLC method reported here represents a simple, accurate and rapid technique for simultaneous determination of four flavonol aglycones, including the methoxylated flavonols SX and IS. The assay is reproducible, sensitive, fully validated and was successfully applied to hydrolyzed extracts of different plant parts of three *Sorbus* species. For eight of the nine *Sorbus* samples tested, this is the first report regarding flavonoid content. Furthermore, the method can be used for quality control of flavonoids in other plant materials with similar aglycone patterns. Considering

the known bioactivity of flavonoids and the high levels found in *Sorbus* samples, use of these plant resources appears to be very promising.

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