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Isolation of Three Triterpene Saponins, Including Two New Oleanane Derivatives, from *Soldanella alpina* and Hydrophilic Interaction Liquid Chromatography–Evaporative Light Scattering Detection of these Three Saponins in Four *Soldenella* Species

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ABSTRACT:

Introduction – The genus *Soldanella* is one of the few endemic to Europe. Some of its species have relevance in local traditional medicine. Earlier work has indicated the possible presence of saponins in *S. alpina*.

Objective – To investigate *S. alpina* and other related species for the occurrence of saponins.

Methods – Following sequential extraction with *n*-hexane, dichloromethane and ethyl acetate the subsequent methanolic extract of *S. alpina* roots was fractionated after solvent precipitation using fast centrifugal partition chromatography and column chromatography. Structures were elucidated by LC-MS^n, high-resolution MS, hydrolysis experiments and one-dimensional (1D)- and two-dimensional (2D)-NMR. A hydrophilic interaction liquid chromatography method was developed to quantitate saponins in the leaves and roots of four *Soldanella* species.

Results – Three triterpene saponins, two of them new natural products, were isolated from *S. alpina*. Based on an epoxyoleanal aglycone substituted with four sugar units, they were analytically quantitated using a Kinetex 2.6 μm hydrophilic interaction liquid chromatography (HILIC) column together with a mobile phase comprising of ammonium acetate, water and acetonitrile. Method validation confirmed that the assay meets all requirements in respect to linearity, accuracy, sensitivity and precision. All four *Soldanella* species investigated contained the three saponins. The lowest total level of the three saponins (1.09%) was observed in *S. montana* leaves while the highest saponin content (5.14%) was determined in *S. alpina* roots.

Conclusion – The detection of saponins within the genus *Soldanella* is an indication that further phytochemical examination of this genus may reveal more secondary metabolites of interest.

Additional Supporting Information may be found online in the supporting information tab for this article.

Keywords: *Soldanella*; triterpene saponins; hydrophilic interaction liquid chromatography; isolation; quantification

Introduction

The genus *Soldanella* L. (Primulaceae) is one of only 27 genera endemic to Europe and it comprises 16 species found in the Alps and mountainous parts of southern Europe (e.g. Pyrenees, Apennine, Balkan). The rather small (3–20 cm) perennial plants are known as snowbells (English) or Alpenglöckchen (German). They have a basal rosette of simple, orbicular leaves, with flower stalks arising from the centre of the rosette, each stalk bearing one to six white to violet flowers (Zhang and Kadereit, 2002). One of the more widely distributed species is *Soldanella alpina*, a plant sometimes also cultivated for ornamental purposes (it was the flower of the year 2004 in Germany). *Soldanella alpina* has been used as a model organism to study the effects of high temperature or light stress on photoinhibition (Streb *et al.,* 2003a; Laureau *et al.,* 2015), on antioxidative scavenging capacity (Laureau *et al.,* 2011) and the occurrence of metabolites like ascorbate or malate (Streb *et al.,* 2003b). A patent has been filed describing a cosmetic preparation against skin ageing, which contains a *S. alpina* extract as the active ingredient (Dudler and Stangl, 2015). The plant is traditionally used as a sedative drug in some parts of Switzerland (Süßmuth, 2013). However, it should be noted that the term Herba Soldanellae was also used for *Convolvulvus soldanella* (Kosch, 1939) and *Brassica marina* (Frerichs *et al.,* 1949) in former times.

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Previous investigations described the composition of epicuticular waxes of *S. pusilla* (Lütz and Gülz, 1985) and the presence of flavonoids in *S. alpina* (Kroslakova *et al.,* 2016). The occurrence of saponins in *S. alpina* has already been suspected more than 90 years ago (Luft, 1926). Since *S. alpina* has certain relevance in traditional medicine, this present study investigated the occurrence of saponins in this and three other *Soldanella* species.

Experimental

Plant material and chemicals

Three individual samples (i.e. 5–10 entire plants each) of *Soldanella alpina* (SA-1 to SA-3) as well as single samples of *S. pusilla* (SP-1), *S. x transsylvanica* (ST-1) and *S. montana* (SM-1) were analysed. All of them, except SA-3 (Mediplant, Conthey, Switzerland) came from Gärtnerei Eschmann, Emmen, Switzerland. They were harvested in May 2010 (SA-1), June 2011 (SA-2 and SA-3) and May 2012 (SP-1, ST-1 and SM-1). The plant material was authenticated by one of the authors (S. Schwaiger), separated into roots (R) and leaves (L), and dried at ambient temperature. Voucher specimens of all samples are deposited at the Institute of Pharmacy, Pharmacognosy, University of Innsbruck.

All chemicals required for isolation and analysis were of analytical grade (p.a.) quality and were purchased from Merck (Darmstadt, Germany). Deuterated pyridine for NMR experiments came from Euroiso-top (Saint-Aubin Cedex, France). HPLC grade water (18.0 MΩ/cm) was produced by a Satorius Arium 611 water purification system (Göttingen, Germany).

Isolation of triterpene saponins from *S. alpina*

Sequential extraction (5 × 100 mL, 10 min ultrasonic bath for each solvent) of dried and powdered *S. alpina* roots (8.5 g, sample SA-3-R) and subsequent evaporation yielded *n*-hexane (45.8 mg), dichloromethane (39.7 mg), ethyl acetate (18.6 mg) and methanol (2304.4 mg) extracts. The methanolic extract (ca. 2 g) was dissolved in methanol/water (1:1; *v/v*, 4.0 mL) and mixed with acetone (8.0 mL). The resultant precipitate was separated by decantation to yield fraction 1 (17 mg). The remaining solution was mixed with more acetone (4 mL) and kept at room temperature for 24 h. During that time the solution separated into an oily syrup (at the bottom of the beaker) and a light yellow solution that were separated and evaporated to dryness to give fraction 2 (904.0 mg) and fraction 3 (605.6 mg), respectively. Fraction 3 was subsequently dissolved in methanol (8.0 mL) and mixed with diethyl ether (8.0 mL) resulting in a white precipitate (246.5 mg, fraction 3p) and the remaining supernatant, which yielded fraction 3s (383.6 mg) after solvent removal. Fraction 3s was further purified by centrifugal partition chromatography (FCPC A200, Kromaton, Angeres, France) using a solvent system of chloroform/methanol/water/1-propanol (9:12:8:1; *v/v/v/v*). The lower phase was pumped through the system (0.5 mL/min) as mobile phase in descending mode with counter clockwise rotation of 800 rpm. Fractions (2 mL) were collected and checked by HPTLC (LiChrospher silica gel 60 F254 plates, 7 μm, Merck, Darmstadt, Germany) using a developing solution of chloroform/methanol/water/formic acid (6:3.2:0.8:1.2; *v/v/v/v*) and visualised by spraying and heating with anisaldehyde-sulphuric acid reagent. The pooled fractions (fraction 3sA) collected between 160 to 288 mL contained a mixture of two saponins, while the fraction eluting between 362 and 382 mL contained a pure saponin (13.8 mg, **Sap3**; hRf 50.0).

An aliquot (78.6 mg) of fraction 3sA was further separated by silica gel column chromatography (diameter: 2.5 cm, length: 30 cm) using an isocratic elution with chloroform/methanol (1:1, *v/v*). Fractions (3 mL) were monitored using HPTLC yielding **Sap1** (eluting between 180 and 225 mL, 36.3 mg, hRf 54.4) and **Sap2** (eluting between 270 and 360 mL, 17.4 mg, hRf 52.2). Purity and identity of the isolated compounds were verified by HPTLC, LC-MS^3, GC-FID, NMR and HR-ESI-MS (see Supporting Information for LC-MS^n and one-dimensional (1D)- and two-dimensional (2D)-NMR spectra as well as details on hydrolysis/GC-FID experiments).

HR-ESI-MS of isolated compounds

Experiments were performed on a micrOTOF-QII mass spectrometer (Bruker Daltronics, Bremen, Germany) by directly infusing a methanolic solution of each compound (0.25 mg/mL). MS conditions were set to negative electrospray ionisation, a nebuliser pressure of 4.4 psi, a HV capillary voltage of 3.5 kV, 180°C dry temperature, a dry gas flow of 4 L/min and a scan range from *m/z* 100 to 3000, respectively.

Sample preparation

For preparation of the HPLC sample solutions the finely powdered plant material (root or leaf, 50 mg) was extracted with methanol (3 mL) by sonication (15 min). The mixture was centrifuged (5 min, 3500 rpm) and the supernatant placed in a 10 mL volumetric flask. This extraction step was repeated two more times, solutions were combined and the flask filled to volume with methanol. Sample solutions are stable for at least two weeks if stored at 4°C. Prior to HPLC analysis an aliquot of the leaf extract (5.00 mL) or the root extract (10.00 mL) was evaporated to dryness under reduced pressure, the residue was re-dissolved in methanol (1.00 mL) and membrane filtered (GHP Acrodisc 13, 0.45 μm polypropylene membrane, Pall, Port Washington, NY, USA).

Analytical conditions

HPLC experiments were performed on an Agilent 1200 system (Agilent, Waldbronn, Germany) equipped with binary pump, autosampler and column heater, fitted with a Sedex 85 LT ELSD detector from Sedere (Alfortville Cedex, France). The best separation was achieved using a Kinetex HILIC 2.6 μm 100 Å column (150 mm × 4.6 mm, Phenomenex, Torrance, CA, USA), utilising a mobile phase comprising 20 mM ammonium acetate in water (A), and a 9:1 mixture (*v/v*) of acetonitrile and 200 mM ammonium acetate solution (B). Both solutions were adjusted to a pH 4.0 with acetic acid. The linear gradient started at 3% A/97% B to end after 25 min at 8% A/92% B. The column was washed for 5 min with 30% A/70% B before re-equilibration for 20 min. Column temperature, flow rate and sample volume were set to 30°C, 0.8 mL/min and 5 μL, respectively. The evaporative light scattering detection (ELSD) settings were 40°C, a nebuliser pressure of 3.6 bar (nitrogen) and gain 12.

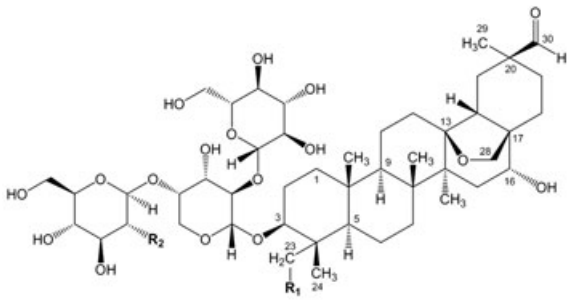
Method validation

A standard stock solution of the three saponins (5 mg each) was prepared with methanol (5.00 mL). Further dilutions were prepared in the ratio of 1:2 with the same solvent. The limits of detection (LOD) and limits of quantitation (LOQ) were visually evaluated, defining concentrations with a peak height of 3- or 10-times the baseline noise, respectively. Accuracy was investigated by spiking sample SA-2-L with different concentrations of the standards (high, medium and low spike). The quantitative results were compared with the theoretically present amount and expressed as recovery rate. Precision was investigated by analysing five individually prepared solutions of sample SP-1-L on day 1. On days 2 and 3 the same procedure was repeated, and the variation within one day (intra-day precision) and within three days (inter-day precision) was calculated based on peak area.

Results and discussion

Structural elucidation of saponins in *S. alpina*

Firstly, the three isolated saponins (Fig. 1) were studied by LC-ESI-MS/MS. The parent ions ($[M-H]^-$) were observed at *m/z* 1059.9 (**Sap1** and **Sap2**) and 1075.9 (**Sap3**). Taking **Sap1** as an example, further fragmentation showed signals at *m/z* 927.6 (MS^2) and 765.1, 603.1 and 471.9 (MS^3). The fragmentation pattern suggested the loss of four sugar units as displayed by observed mass-



	R_1	OR_2
Sap1 (1)	H	α-L-arabinopyranosyl
Sap2 (2)	H	β-D-xylopyranosyl
Sap3 (3)	OH	α-L-arabinopyranosyl

Figure 1. Chemical structure of the main triterpene saponins isolated from *Soldanella alpina*.

differences of 132 and 162. This indicated that the structure of **Sap1** contains two hexoses and two pentoses. **Sap2** and **Sap3** were analysed accordingly (see Supporting Information, Figs S1–S3). Since the mass of the aglycon of **Sap3** differed by 16 to those of **Sap1** and **Sap2** the presence of an additional oxygen atom in the structure was assumed.

The 1D- (^{1}H, ^{13}C) and 2D-NMR spectra (COSY, HSQC, HMBC, NOESY) of the saponins revealed for all three compounds a similar oleanan-type aglycon with an aldehyde function at position 30, a hydroxyl-group at position 16 and an additional ring formation (position 13 to 17) via a -O-CH_2- unit. While **Sap1** and **Sap2** showed no further structural differences, **Sap3** contained an additional hydroxyl-group at position 23, which was differentiated from position 24 by long-range ^{1}H-^{1}H-COSY and NOESY correlations between position 24 and 25. Connectivity and structure of the sugar units were established by ^{1}H-^{1}H-COSY and HMBC correlations, while the α- or β-orientation was deduced from the corresponding coupling constants of anomeric protons ($J <$ 5.1 Hz, α-orientation; $J >$ 6.8 Hz β-orientation). Respective NMR data showed similar chemical shift values except for the terminal aldopentose in **Sap2**. Common features were an initial α-L-arabinose unit which is linked to two β-D-glucose units. The terminal pentose was α-L-arabinose in **Sap1** and **Sap3** while for **Sap2** it was β-D-xylose. The NMR data of all isolated compounds are compiled in Tables 1 (^{1}H-NMR) and Tables 2 (^{13}C-NMR).

In order to verify the composition of the individual sugar chains, hydrolysis and GC-experiments were performed. Each saponin was subjected to acidic hydrolysis, the reaction product was derivatised and analysed by GC in comparison to standard compounds (see Supporting Information for experimental details). For **Sap1** and **Sap3** only signals corresponding to L-(+)-arabinose and D-(+)-glucose were found, while **Sap2** revealed the presence of L-(+)-arabinose, D-(+)-xylose and D-(+)-glucose (Figs S4–S6). Accordingly, **Sap1** was elucidated as 3β,16α-dihydroxy-13β,28-epoxyolean-30-al 3-*O*-[(*O*-β-D-glucopyranosyl-(1→2)-*O*-[*O*-α-L-arabinopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranoside)], **Sap2** as 3β,16α-dihydroxy-13β,28-epoxyolean-30-al 3-*O*-[(*O*-β-D-glucopyranosyl-(1→2)-*O*-[*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranoside)], and **Sap3** as 3β,16α,23-trihydroxy-13β,28-epoxyolean-30-al 3-*O*-[(*O*-β-D-glucopyranosyl-(1→2)-*O*-[*O*-α-L-arabinopyranosyl-(1→2)-β-D-glucopyranosyl (1→4)]-α-L-arabinopyranoside)]. The deduced molecular formulae were confirmed by HR-ESI-MS revealing for **Sap1** and **Sap2** an *m/z* value of 1059.5413 (calculated $[M-H]^-$ 1059.5381, mass error 3.2 ppm) and for **Sap3** *m/z* 1075.5366 (calculated $[M-H]^-$ 1075.5331, mass error 3.5 ppm). **Sap2** has already been isolated as deglucocyclamin from *Cyclamen hederifolium* (Altunkeyik *et al.,* 2012) and as ardisiacrispin A from several sources including *Ardisia crispa* (Jansakul *et al.,* 1987), *Lysimachia foenum-graecum* (Dai *et al.,* 2017) and *Labisia pumila* (Avula *et al.,* 2011). The latter publication also reports on a quantitative HPLC-ELSD method. **Sap1** and **Sap3** have not previously been described.

Table 1. ^{1}H-NMR data of isolated compounds (in pyridine-d_5; 600.19 MHz; σ in ppm; J in parentheses)

Position	Sap1	Sap2	Sap3
1	H_a 1.66 d (12.2)	H_a 1.67 d (11.4)	H_a 1.73[a]
	H_b 0.89 d (12.5)	H_b 0.89 d (13.0)	H_b 1.00[a]
2	H_a 1.83 dd (12.8, 25.4)	H_a 2.03 br d (12.6)	H_a 2.12[a]
	H_b 2.02 d (12.5)	H_b 1.85 q (12.5)	H_b 1.97[a]
3	3.18[a], 1H	3.19[a], 1H	4.11[a], 1H
4	—	—	—
5	0.70 d (11.6), 1H	0.70 d (11.4), 1H	1.54 d (12.7), 1H
6	H_a 1.44[a]	H_a 1.45[a]	H_a 1.69[a]
	H_b 1.40 d (15.0)	H_b 1.40[a]	H_b 1.45[a]
7	H_a 1.55[a]	H_a 1.50[a]	H_a 1.69[a]
	H_b 1.22[a]	H_b 1.22[a]	H_b 1.20 d (12.2)
8	—	—	—
9	1.28 d (12.2), 1H	1.28[a], 1H	1.40[a], 1H
10	—	—	—
11	H_a 1.75 dq (4.5, 13.6, 13.9)	H_a 1.75 m	H_a 1.78 dd (2.8, 12.7)
	H_b 1.44[a]	H_b 1.45[a]	H_b 1.46[a]
12	H_a 2.12[a]	H_a 2.11[a]	H_a 2.09[a]

(*Continues*)

Table 1. *(Continued)*

Position	Sap1	Sap2	Sap3
	H_b 1.44[a]	H_b 1.44[a]	H_b 1.44[a]
13	—	—	—
14	—	—	—
15	H_a 2.21 dd (4.5, 14.6)	H_a 2.21 dd (3.7, 14.1)	H_a 2.19 dd (4.3, 14.0)
	H_b 1.50 d (14.9)	H_b 1.51[a]	H_b 1.42[a]
16	4.22[a], 1H	4.23[a], 1H	4.19[a], 1H
17	—	—	—
18	1.40 d (15.0), 1H	1.41[a], 1H	1.39[a], 1H
19	H_a 2.87 t (13.4)	H_a 2.87 t (13.3)	H_a 2.84 t (13.4)
	H_b 2.13 m	H_b 2.14[a]	H_b 2.10[a]
20	—	—	—
21	H_a 2.57 dt (4.2, 13.1, 13.5)	H_a 2.57 dd (4.7, 13.5)	H_a 2.54 dt (3.7, 12.7, 13.1)
	H_b 2.10 m	H_b 2.11 m	H_b 2.08[a]
22	H_a 1.98 dd (3.0, 13.9)	H_a 1.98 dd (4.2, 13.3)	H_a 1.95[a]
	H_b 1.60 dd (4.4, 13.5)	H_b 1.60 dd (4.7, 13.6)	H_b 1.60 dd (5.2, 13.7)
23	1.24 s, 3H	1.24 s, 3H	H_a 4.28[a]
			H_b 3.74[a]
24	1.10 s, 3H	1.11 s, 3H	1.09 s, 3H
25	0.85 s, 3H	0.86 s, 3H	0.95 s, 3H
26	1.31 s, 3H	1.31 s, 3H	1.33 s, 3H
27	1.56 s, 3H	1.57 s, 3H	1.49 s, 3H
28	H_a 3.56 d (7.2)	H_a 3.56 d (6.9)	H_a 3.55 d (7.4)
	H_b 3.19 m	H_b 3.18 m	H_b 3.17 d (7.4)
29	1.04 s, 3H	1.04 s, 3H	1.00 s, 3H
30	9.66 s, 1H	9.65 s, 1H	9.63 s, 1H
α-L-Arabinose unit at position 3			
1′	4.81 d (4.5), 1H	4.82 d (5.1), 1H	5.04 d (4.5)[a], 1H
2′	4.57[a], 1H	4.63[a], 1H	4.61[a], 1H
3′	4.30[a], 1H	4.30[a], 1H	4.21[a], 1H
4′	4.27[a], 1H	4.30[a], 1H	4.22[a], 1H
5′	H_a 4.64 dd (3.1, 7.3)	H_a 4.58 d (12.9)	H_a 4.58[a]
	H_b 3.69 d (11.7)	H_b 3.69 d (11.7)	H_b 3.60 d (11.9)
β-D-Glucose unit at position 2′			
1″	5.50 d (6.8), 1H	5.52 d (7.2), 1H	5.51 d (7.6), 1H
2″	4.10 t (7.5), 1H	4.09 t (7.6), 1H	4.13[a], 1H
3″	4.27[a], 1H	4.25 d (9.9), 1H	4.27[a], 1H
4″	4.24[a], 1H	4.22[a], 1H	4.26[a], 1H
5″	4.04[a], 1H	4.06[a], 1H	4.05[a], 1H
6″	H_a 4.57[a]	H_a 4.56[a]	H_a 4.58[a]
	H_b 4.43[a]	H_b 4.44[a]	H_b 4.44[a]
β-D-Glucose unit at position 4′			
1‴	5.02 d (7.4), 1H	5.08 d (7.9), 1H	5.04 d (8.2)[a], 1H
2‴	3.94 t (6.9), 1H	4.01 dd (17.6, 24.7), 1H	3.99 m, 1H
3‴	4.22[a], 1H	4.22[a], 1H	4.23[a], 1H
4‴	4.22[a], 1H	4.22[a], 1H	4.24[a], 1H
5‴	3.81 t (7.0), 1H	3.80 br s, 1H	3.79 m, 1H
6‴	H_a 4.45[a];	H_a 4.44[a]	H_a 4.40[a]
	H_b 4.32[a]	H_b 4.32[a]	H_b 4.32[a]
Terminal pentose unit at position 2‴			
1⁗	4.93 d (3.2), 1H	5.10 d (7.9), 1H	5.00 br s[a], 1H
2⁗	4.04[a], 1H	4.54[a], 1H	4.05[a], 1H
3⁗	4.15 dd (8.9, 12.7), 1H	4.16 dd (17.6, 23.0), 1H	4.17[a], 1H
4⁗	4.22[a], 1H	4.32[a], 1H	4.23[a], 1H
5⁗	H_a 4.57[a]	H_a 4.66 d (12.4)	H_a 4.59[a]
	H_b 3.73 t (10.9)	H_b 3.89 d (12.0)	H_b 3.73[a]

[a]Signals overlapping.

Table 2. ^{13}C-NMR data of isolated compounds (in pyridine-d_5; 150.91 MHz; in ppm)

Position	Sap1	Sap2	Sap3
1	39.65 t	39.65 t	39.62 t
2	27.05 t	27.05 t	26.44 t
3	89.52 d	89.50 d	82.86 d
4	40.20 s	40.18 s	44.13 s
5	56.17 s	56.15 s	48.33 s
6	18.41 t	18.38 t	18.11 t
7	34.82 t	34.79 t	34.48 t
8	43.01 s	42.98 s	43.00 s
9	50.89 d	50.87 d	50.96 d
10	37.28 s	37.26 s	37.26 s
11	19.62 t	19.60 t	19.64 t
12	34.14 t	33.12 t	33.14 t
13	86.82 s	86.79 s	86.79 s
14	45.07 s	45.07 s	45.04 s
15	37.35 t	37.33 t	37.30 t
16	77.37 d	77.35 d	77.33 d
17	44.50 s	44.48 s	44.45 s
18	53.80 d	53.78 d	53.80 d
19	33.85 t	33.83 t	33.79 t
20	48.79 s	48.77 s	48.74 s
21	30.96 t	30.93 t	30.89 t
22	32.82 t	32.80 t	32.77 t
23	28.56 q	28.53 q	65.41 t
24	17.12 q	17.07 q	13.71 q
25	16.86 q	16.85 q	17.41 q
26	19.00 q	18.98 q	18.99 q
27	20.25 q	20.22 q	20.15 q
28	78.28 t	78.11 t	78.08 t
29	24.60 q	24.57 q	24.49 q
30	208.03 d	207.99 d	207.96 d
α-L-Arabinose unit at position 3			
1′	105.15 d	105.19 d	104.11 d
2′	80.22 d	80.55 d	80.75 d
3′	73.74 d	73.78 d	73.81 d
4′	79.08 d	78.39 d	78.56 d
5′	64.68 t	64.82 t	64.54 t
β-D-Glucose unit at position 2′			
1″	105.38 d	105.42 d	105.60 d
2″	76.72 d	76.83 d	76.69 d
3″	78.67 d	78.60 d	78.76 d
4″	72.31 d	72.34 d	71.88 d
5″	76.59 d	78.63 d	76.61 d
6″	63.48 t	63.82 t	63.14 t
β-D-Glucose unit at position 4′			
1‴	104.67 d	104.51 d	104.49 d
2‴	85.89 d	84.63 d	85.89 d
3‴	78.05 d	78.60 d	78.28 d
4‴	71.57 d	71.67 d	71.52 d
5‴	78.83 d	78.81 d	78.82 d
6‴	62.80 t	62.82 t	62.75 t
Terminal pentose unit at position 2‴			
1⁗	108.15 d	107.37 d	108.14 d
2⁗	78.45 d	74.02 d	78.28 d
3⁗	71.18 d	74.59 d	71.17 d
4⁗	78.13 d	69.28 d	78.10 d
5⁗	67.95 t	67.44 t	67.96 t

Hydrophilic interaction liquid chromatography (HILIC)–evaporative light scattering detection (ELSD) analysis

Since the saponins possessed basically no UV absorption, even close to 200 nm, ELSD was investigated under reversed phase HPLC conditions using C-8 (Zorbax Eclipse XDB-C8, 5 μm particle size), C-12 (Synergi Max-RP, 4 μm), C-18 (Synergi Fusion-RP, 4 μm) or pentafluorophenyl (Kinetex PFP, 2.6 μm) stationary phases. However, on all these materials the compounds co-eluted. With an amino column (Supelcosil LC-NH_2, 3 μm) operated in normal phase mode at least some peak splitting was noticed, yet the baseline separation of all three saponins was only possible on hydrophilic interaction liquid chromatography (HILIC) material (Fig. 2). After modifying HPLC parameters (see later) the required analysis time was less than 13 min and signals with excellent peak shape and resolution were obtained.

HILIC phases are polar and most commonly based on silica material or they show zwitterionic characteristics (Zuo *et al.*, 2014). Both modes were tested and an unbonded silica core-shell material (Kinetex HILIC 2.6 μm 100 Å) yielded better results with respect to resolution and repeatability than the zwitterionic mode (ZIC-HILIC 3.5 μm 100 Å column). Since HILIC is mainly based on liquid-liquid chromatography, columns generally require longer equilibration periods (e.g. 20 min in the current case) compared to reversed phase (RP)-HPLC. A mobile phase typical for HILIC containing acetonitrile and aqueous ammonium acetate was best suited. However, pH and buffer molarity needed to be finely tuned to achieve the desired separation, because both parameters have a significant influence on the activity of the silanol groups of the stationary phase. Adjusting the mobile phase to pH 5.0 resulted in prolonged analysis time, at pH 3.0 the saponin signals partially overlapped; thus a pH of 4.0 was selected. A higher buffer molarity resulted in a better separation but also broader signals, by utilising a solvent gradient peak symmetry could be improved again. The influence of column temperature was as expected (i.e. prolonged retention at lower temperatures); however, an unexpected observation was made when varying the ELSD nebuliser temperature. At 40°C peak height and symmetry were much more enhanced than at 70°C. The saponins are obviously thermo-labile to a certain extent, so that the unique low temperature technique of the utilised ELSD was favourable to facilitate a more sensitive detection. Selected chromatograms indicating the impact of

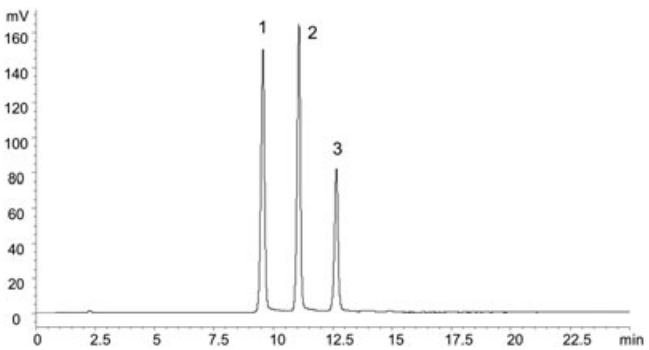


Figure 2. Separation of the three standard compounds **Sap1** (**1**), **Sap2** (**2**) and **Sap3** (**3**) under optimised conditions (column: Kinetex HILIC 2.6 μm 100 Å, 150 mm × 4.6 mm; mobile phase: 20 mM ammonium acetate in water with pH 4.0 (A), 9:1 mixture of acetonitrile and 200 mM ammonium acetate with pH 4.0 (B); gradient: 3A/97B in 25 min to 8A/92B; temperature: 30°C; flow rate. 0.8 mL/min; sample volume: 5 μL; evaporative light scattering detection (ELSD): 40°C, 3.6 bar (nitrogen), gain 12).

individual separation parameters are shown in the Supporting Information.

Method validation

The developed HILIC method was validated for linearity, LOD and LOQ, selectivity, accuracy and precision (Table 3). For ELSD the observed peak area (A) is related to the quantity of analyte on-column (m) through the relationship $A = am^x$, where (x) is the slope of the response line and (a) is a response factor (Ganzera and Stuppner, 2005). Thus, the logarithmic values for A and m will show a linear trend ($\log A = a + x\log m$). Considering these facts resulted in correlation coefficients for all three saponins higher than 0.998, within a linear range from at least 1000 to 37 μg/mL. The determined LOD (≤ 6.7 μg/mL) and LOQ (≤ 20.3 μg/mL) values are higher than those usually achievable by UV-vis (if compounds show absorbance) or MS, but they are typical for this detection technique. Selectivity of the method was assured by two facts. First, all relevant signals in the samples were symmetrical without any shoulders. Second, as an ELSD does not provide any

Table 3. Validation data of the developed hydrophilic interaction liquid chromatography (HILIC) method.

Parameter/compound	Sap1	Sap2	Sap3
Regression equation[a]	$y = 1.562x - 1.543$	$y = 1.508x - 1.396$	$y = 1.483x - 1.550$
Correlation coefficient	0.9994	0.9986	0.9991
Range[b]	1050–12.9 (5250–64.5)	1080 – 13.3 (5400 - 66.5)	1000 – 37.0 (5000 - 185.0)
Limit of detection (LOD)[b]	3.9 (19.5)	4.0 (20.0)	6.7 (33.5)
Limit of quantification (LOQ)[b]	12.9 (64.5)	13.3 (66.5)	20.3 (101.5)
Accuracy (high spike)[c]	103.5	102.0	100.9
Accuracy (medium spike)[c]	98.1	98.7	99.2
Accuracy (low spike)[c]	97.3	97.4	100.4
Precision (intra-day)[d]	6.38	5.73	5.38
Precision (inter-day)[e]	2.83	4.16	3.82

[a]For evaporative light scattering detection (ELSD): y = log (peak area), x = log (concentration in μg/mL).
[b]In μg/mL (absolute amounts in ng based on 5 μL injection volume in parenthesis).
[c]Expressed as recovery rates in percentage.
[d]Maximum deviation within one day based on peak area in percentage (n = 4).
[e]Deviation within three days based on peak area in percentage.

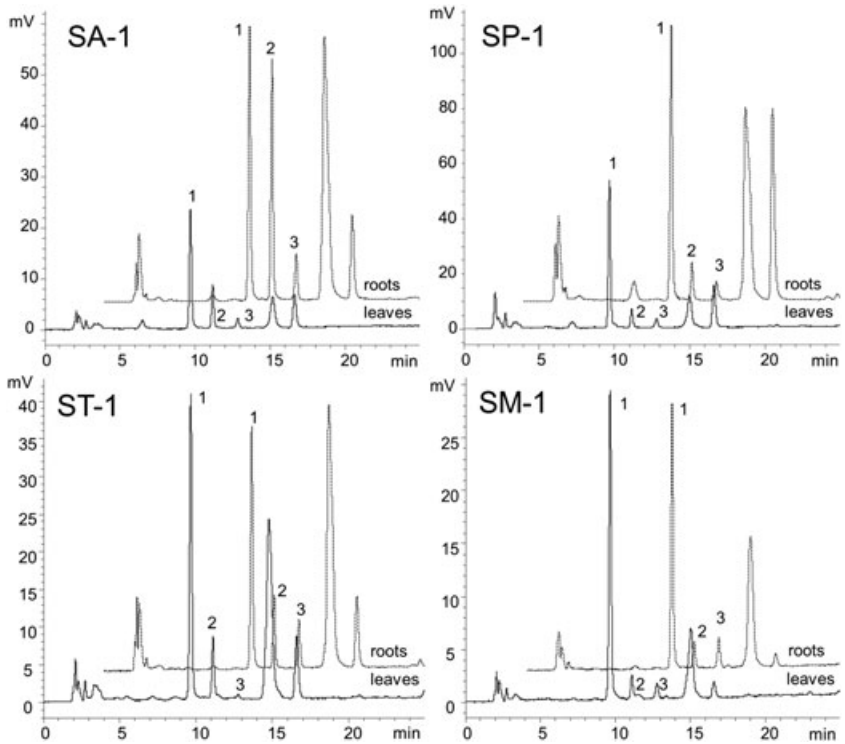


Figure 3. Comparison of root (R) and leaf (L) extracts of *Soldanella alpina* (sample SA-1), *S. pusilla* (SP-1), *S. x transsylvatica* (ST-1) and *S. montana* (SM-1) analysed by hydrophilic interaction liquid chromatography (HILIC)–evaporative light scattering detection (ELSD); separation conditions as for Fig. 2.

spectroscopic data to show peak purity based on an UV-spectra, LC-MS experiments were performed (see Supporting Information for conditions). The HILIC method comprised only volatile chemicals, thus it could readily be hyphenated with an iontrap MS without any modifications. Respective results showed no indications for co-eluting compounds (data not shown in detail). Even if detection of the saponins would have been possible with MS as well, in this study we selected the more economic option (ELSD) because it might easier be available in many laboratories and it equally was suitable for the analysis of saponins in *Soldanella* samples.

For determination of the methods accuracy recovery experiments were performed. Individually weighed portions of sample SA-2-L were spiked with three concentrations of the saponins (high spike: 120 μg/mL per compound, medium spike: 80 μg/mL, low spike: 40 μg/mL). After extraction and analysis, maximum deviations between theoretical and observed values of 97.3% (**Sap1**, low spike) and 103.5% (**Sap1**, high spike) were found. Finally, intermediate precision was assured by analysing individually prepared solutions of SP-1-L over a three-day period. Within one day the results varied by no more than 6.4% (**Sap1**, $n = 5$), inter-day precision was even below 4.2% (**Sap2**). Accordingly, all of the required validation criteria were fulfilled, so that the analytical procedure could be used for a reliable quantification of saponins in diverse *Soldanella* samples.

Quantification of samples

Three snowbell species and one hybrid, including three samples of *S. alpina*, were available for quantitative studies. To test the extraction efficiency, two samples (SA-1-R and SA-1-L) were treated as described under "sample preparation" and then re-extracted once more with methanol. Comparison of the two sequential extracts showed no quantifiable amounts of the three target analytes in these extracts.

The HILIC–ELSD chromatograms of the methanolic extracts of *S. alpina*, *S. pusilla*, *S. x transsylvanica* and *S. montana* (Fig. 3) were quantitated against the calibration curves (Fig. 4). Each sample solution was assayed in triplicate and a maximum relative standard deviation of 4.1% (**Sap3** in SM-1-L) was observed. All samples investigated, regardless if roots or leaves, contained the three saponins, with **Sap1** always being the dominant compound (from 0.58% in SA-2-R to 2.52% in SA-3-R). In all *S. alpina* specimens **Sap2** (up to 0.67% in leaves and 1.81% in roots) was the second most abundant saponin. However, in the other species, particularly in root material, **Sap3** (up to 0.47% in *S. pusilla* roots) was taking this position. In most samples the total saponin content was higher in leaves compared to roots (e.g. in SA-2: 2.43% vs. 1.44%, in ST-1: 2.76% vs. 1.60%), except in two *S. alpina* samples which contained a higher percentage of saponins in the roots. In this respect it should be noted that the major compounds eluting right after **Sap3** are saccharides. Saccharose (Rt = 14.9 min) was unambiguously identified by NMR after isolation but the amount of the second substance (Rt = 16.5 min) was not sufficient for an exact structural determination. Preliminary NMR and MS-data indicate the trisaccharide kestose. As it was not relevant for this study, neither the differentiation of 1- or 6-kestose nor the quantification of these two sugars was considered. The highest overall saponin content (5.14%) was found in one of the *S. alpina* samples. However, the content seems rather variable in this species as another root sample also harvested in June 2011 only contained 1.44% total saponins.

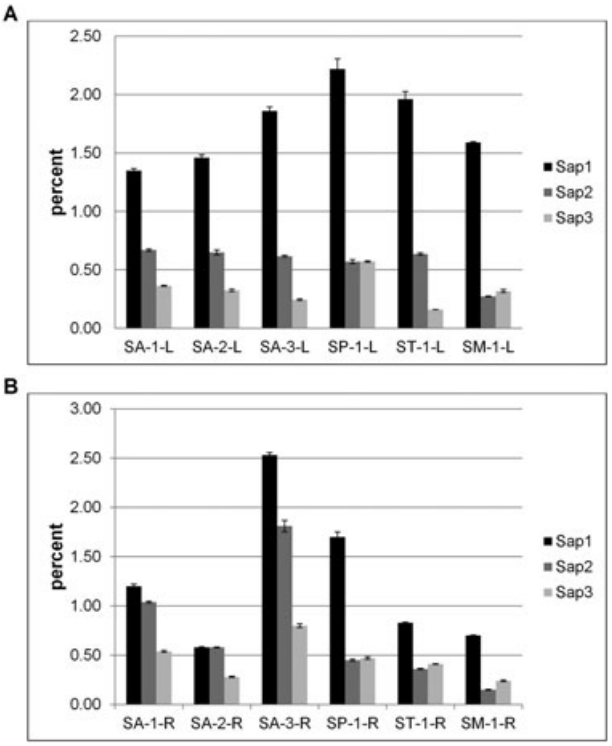


Figure 4. Content of saponins (in percentage by weight) in *Soldanella alpina* (SA), *S. pusilla* (SP), *S. x transsylvanica* (ST) and *S. montana* (SM) samples: (A) leaves and (B) roots. Relative standard deviations ($n = 3$) are indicated with bars.

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Declaration of interest statement

The authors have declared no conflict of interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.