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Validated HPTLC methods for the determination of salicin in *Salix* sp. and of harpagoside in *Harpogophytum procumbens*

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ABSTRACT

Preparations of *Harpagophytum procumbens* and of *Salix* species are successfully used for the treatment of degenerative rheumatism and painful arthrosis. For the quality control of both drugs, rapid methods of quantification are desirable. Here we report the development of two HPTLC methods enabling the determination of harpagoside in Harpagophyti radix and of salicin in Salicis cortex. We focused on a standardized methodology and thorough validation including two laboratories. The methods allow the analyses of up to 16 samples in parallel demonstrating the proposed methods as very rapid and cost efficient.

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1. Introduction

Phytomedicines are successfully used alone or as adjuvance therapy for the treatment of degenerative rheumatism and painful arthrosis. In this respect roots of *Harpagophytum procumbens* DC and bark of some *Salix* species (*Salix purpurea* L., *Salix daphnoides* Vill., *Salix fragilis* L., et al.) are of special interest. Their efficacy has been proven in clinical studies where strong effects in the short-term treatment of acute episodes of chronic non-specific low back pain was observed [1,2].

The active principle of *H. procumbens* is still unknown, but iridoid glycosides, particularly harpagoside (Fig. 1), are assumed to play a key role in the analgesic and anti-inflammatory effects. Harpagoside is commonly used as a marker substance in quality control of this drug. A minimum content of 1.2% is specified in the European Pharmacopoeia [3]. HPLC methods using UV/VIS [4], MS and NMR [5] detectors to determine the harpagoside content are described in the literature. Also, high-performance thin-layer chromatography (HPTLC) with densitometric evaluation has been reported as analytical tool [6,7].

The pharmacological activity of Salicis cortex is associated in part to the content of phenolic glycosides, such as salicin, salicortin and tremulacin (Fig. 1) [8]. Those are pro-drugs, as they are hydrolysed to salicin and then metabolised to salicyl alcohol and finally to the pharmaceutically active salicylic acid [9]. There are HPLC methods for quantification of the total content of phenolic glycosides after hydrolysis to salicin described in the literature [10,11] and in the European Pharmacopoeia.

For the quality control of both drugs, rapid methods of quantification are desirable. Several different *Salix* species and bastards are acceptable as source for raw material for Salicis cortex [12], even though their content of phenolic glycosides varies widely. For Harpagophyti radix variability is not a problem, but due to limited availability and increasing demand adulterated and inferior plant material is often found on the market.

The aim of our work was the development of two simple and rapid methods of quantification, one for harpagoside in Harpagophyti radix and extracts thereof, and one for salicin from hydrolysis of the phenolic glycosides of Salicis cortex and extracts thereof. We have chosen HPTLC for its simplicity and the ability to analyse multiple samples at comparatively low cost. To address the issue of accuracy and reproducibility we focused on a standardized methodology [13] and thorough validation including two laboratories [14].

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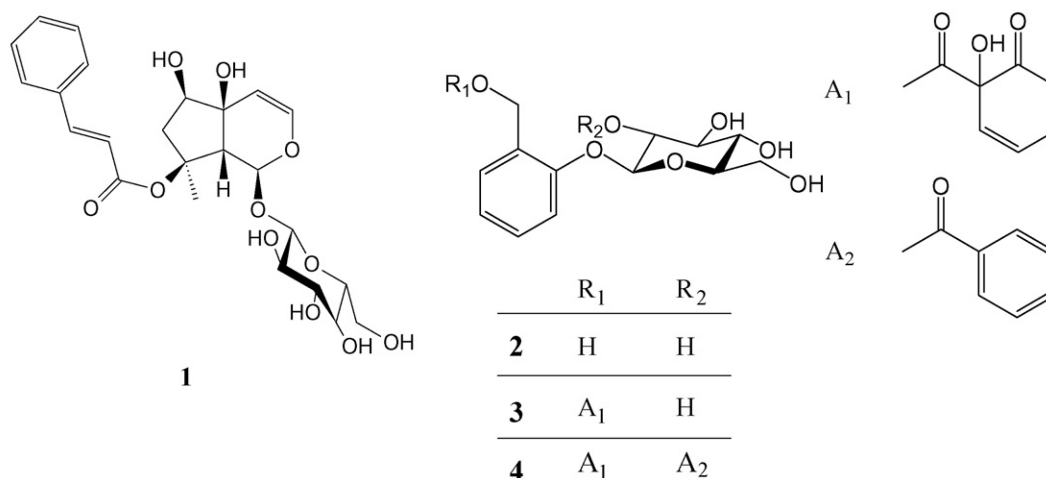

Fig. 1. Chemical structure of harpagoside (**1**), salicin (**2**), salicortin (**3**) and tremulacin (**4**).

2. Experimental

2.1. Chemicals and materials

Salicin and harpagoside were purchased from Roth (Karlsruhe, Germany). Dried plant material (Salicis cortex and Harpagophyti radix) were donated by A. Lenherr, Bergapotheke (Zurich, Switzerland). All reagents were of analytical grade and from Roth (Karlsruhe, Germany). Harpagophytum extracts S1858 and S1859 were obtained from Phytolab (Vestenbergsgreuth, Germany).

2.2. Apparatus

HPTLC was performed on equipment made by CAMAG (Muttenz, Switzerand). Instruments included an ATS 4 for sample application, 20 cm × 10 cm Twin Trough Chambers for Chromatogram development, TLC Scanner 3 for densitometric evaluation, immerdion device and plate heater for derivatisation, and WinCATs software version 1.3.3. for instrument control and data evaluation.

2.3. Chromatographic conditions

20 cm × 20 cm HPTLC aluminium sheets with silica gel 60 F_{254} (Merck, Darmstadt, Germany) were cut in 20 × 10 sheets and prewashed with methanol. Samples were applied as bands (6 mm for salicin, 7 mm for harpagoside) using the spray-on technique. Development was performed in saturated chambers (20 min with filter paper) over a distance of 7.5 cm (salicin) or 5.0 cm (harpagoside). After development plates were air-dried for 10 min and then scanned (slit dimension: 5.00 mm × 0.45 mm) in absorbance mode at 272 nm (salicin) or 285 nm (harpagoside). Evaluation was based on peak areas.

2.4. Postchromatographic derivatisation

Postchromatographic derivatisation was necessary for the evaluation of the two dimensional stability TLC. The plate was dipped in anisaldehyde-sulphuric acid solution (anisaldehyde:methanol:acetic acid:sulphuric acid (0.5:85:10:5, v/v)). After air drying for 2 min the plates were heated at 120° C for 5 min.

2.5. Mobile phases

Three mobile phases were evaluated for the chromatography of *Salix* samples—S1 ethyl acetate:methanol:water (77:15:8, v/v) [14], S2 ethyl acetate:methanol:water (77:13:10, v/v) and S3 (ethyl acetate:ethylmethyl ketone:formic acid:water (60:20:2:2, v/v).

Four different mobile phases were evaluated for the determination of harpagoside—H1 ethyl acetate:methanol:water (77:15:8, v/v) [3]; H2 dichloromethane:methanol:acetic acid (79:20:1, v/v); H3 toluene:methanol (76:24, v/v); H4 water:methanol:ethyl acetate:acetic acid (8:15:76:1, v/v).

2.6. Sample preparation

Salicin standard solution: 1.22 mg of salicin was dissolved in 5.0 ml of methanol. Harpagoside standard solution: 1.24 mg of harpagoside was dissolved in 5.0 ml of methanol. 1.0 ml of this solution was diluted with methanol to 25.0 ml. Solutions of *H. procumbens* dried extracts: 35.0 mg of the dried extract were diluted in 100.0 ml methanol.

2.7. Extraction procedures

2.7.1. Salicin procedure

0.5 g of powdered plant material were extracted with 250 ml solvent (methanol, ethanol, water, or ethyl acetate) in a soxhlet-apparatus for 4 h. The solution was evaporated and re-dissolved in 5 ml of methanol. 0.5 ml of sodium carbonate solution (50 g/l) was added for hydrolysis prior to incubation in an ultrasonic bath at 60 °C for 10 min. After cooling, the solution was centrifuged (15,000 × g, 5 min), and filtered. The filter was washed. The filtrate was diluted with methanol to 50.0 ml.

2.7.2. Harpagoside procedure 1 (according to Ph. Eur.)

0.5 g of powdered plant material were extracted with 50 ml solvent (water, methanol, or ethanol) under stirring for 1 h and subsequently filtered. The filter (with the residue) was extracted with 50 ml of the same solvent under reflux for 1 h and subsequently filtered. Both filtrates were combined, evaporated and dissolved in 25.0 ml of methanol. 1.0 ml of this solution was diluted to 25.0 ml with methanol.

2.7.3. Harpagoside procedure 2

0.5 g of powdered plant material was extracted with 250 ml solvent (water, methanol, or ethanol) by using a soxhlet-apparatus for 4 h. The solution was evaporated and redissolved in 25.0 ml of methanol. 1.0 ml of this solution was diluted to 25.0 ml with methanol.

3. Results and discussion

3.1. Development of a method for the quantification of salicin in bark of Salix sp.

3.1.1. Evaluation of the optimal extraction solvent

Methanol, ethanol, water and ethyl acetate were evaluated as extraction solvents for Salicis cortex (from *S. purpurea*). A simplified procedure was selected for comparison: 0.5 g of powdered plant material was extracted for 10 min with 5.0 ml of solvent in an ultrasonic bath at 50 °C. The extracts were hydrolysed as described in "Salicin procedure". The salicin amount was compared by densitometry (272 nm) after chromatography with mobile phase S1. The methanol extract showed a two times higher amount of salicin, whereas water and ethyl acetate extracted only marginal amounts. Consequently, methanol was used as extraction solvent.

3.1.2. Mobile phase

All tested mobile phases gave a good resolution, but the mobile phase S2 produced the most suitable R_f value ($R_f = 0.38$) for the target compound and the smoothest base line for quantification (Fig. 2).

3.2. Development of a method for the quantification of harpagoside in roots of H. procumbens

3.2.1. Evaluation of the optimal extraction solvent

As described for Salix bark water, methanol, and ethanol were evaluated as extraction solvent. The harpagoside amounts were compared by densitometry at 285 nm after chromatography with mobile phase H2. Methanol and ethanol extracted similar amounts, while water was a poor solvent. Similar results were obtained for extraction procedures 1 and 2.

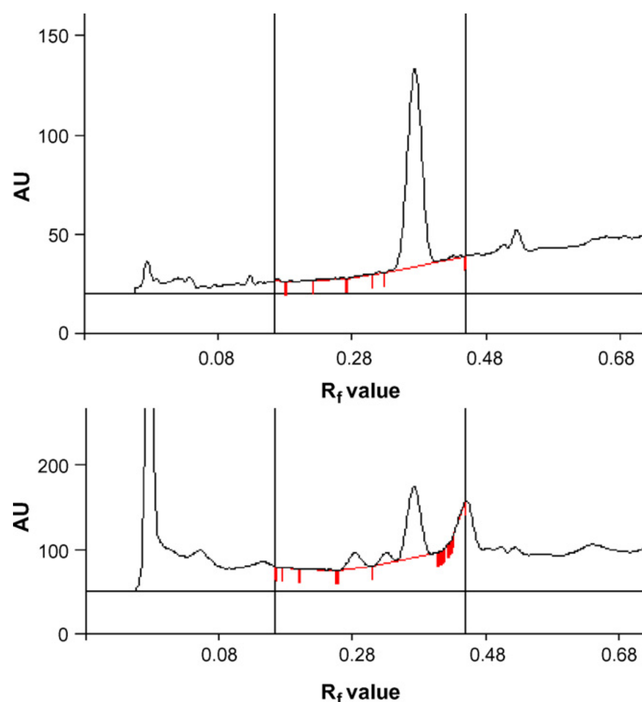

Fig. 2. HPTLC chromatograms at 278 nm of salicin ($R_f = 0.38$) as standard (top) and in *Salix purpurea* bark (bottom).

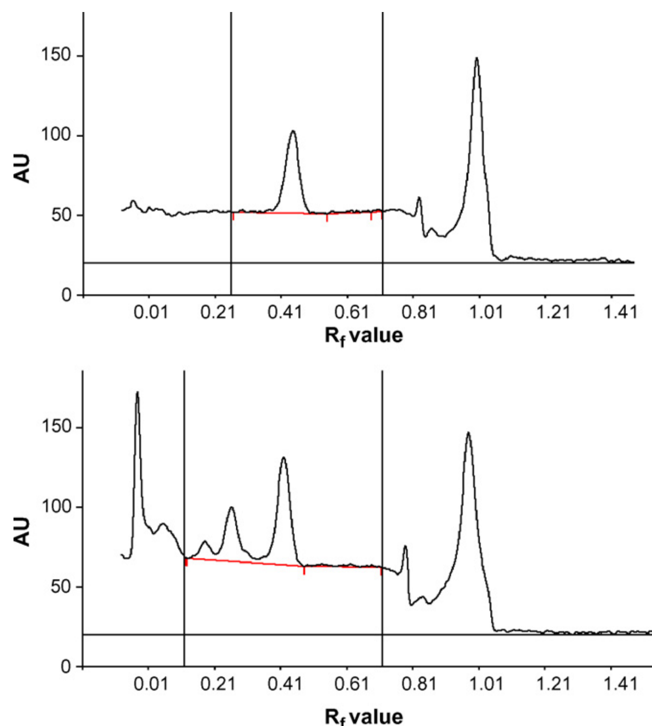

Fig. 3. HPTLC chromatograms at 285 nm of harpagoside ($R_f = 0.42$) as standard (top) and in *Harpagophytum procumbens* root (bottom).

3.2.2. Mobile phase

With the mobile phase H1 and H4 the harpagoside peak showed significant tailing. Mobile phases H2 and H3 gave adequate resolutions. H2 gave the smoothest baseline and the best peak shape (Fig. 3) and was therefore selected for all further experiments.

3.3. Validation

3.3.1. Stability

The stability of both analytes during chromatography was established by two-dimensional chromatography with the selected mobile phases S1 and H2, respectively, on a 10 cm × 10 cm HPTLC-plate. Neither evaluation of fluorescence quenching at 254 nm nor inspection under white light after postchromatographic derivatisation with anisaldehyde-sulphuric acid solution showed any artefact.

Both methods were validated for quantitative analysis with regard to specificity, correlation, precision, accuracy and precision of extraction procedure.

3.3.2. Specificity

The identity of the salicin- and harpagoside-bands in the corresponding samples was demonstrated by comparing the R_f value and the UV spectra with those of standard solutions (data not shown). Absorption maxima of 272 nm for salicin and of 285 nm for harpagoside were verified. The purity of the salicin- and of the harpagoside-band in the corresponding sample was confirmed by comparing the UV spectrum at the start, maximum and end of the peak. All UV spectra were identical (correlation coefficient $r > 0.999$; data not shown). The methods are specific for salicin and harpagoside, respectively.

3.3.3. Working range

For salicin, a regression curve based on a second-order polynomial function was generated by applying 12 volumes between

Table 1
Validation data for three salicin standard solutions, *Salix pentandra* extract (Spen), and *S. purpurea* extract (SPur)

	Mass (μg)	S.D. of within-day precision		S.D. of reproducibility		S.D. of reproducibility between different laboratories		Accuracy: deviation from calculated value (%)
		μg	%	μg	%	%	μg	
Standard 1	0.48	0.013–0.036	2.86–7.83	0.03	3.24	0.019	4.04	97.89–103.59
Standard 2	1.20	0.027–0.059	2.34–4.96	0.01	1.37	0.025	2.13	99.88–102.64
Standard 3	2.40	0.091–0.116	3.83–4.90	0.01	1.06	0.066	2.66	102.63–104.75
SPen	1.97	0.017–0.096	0.89–4.98	0.06	3.07	0.03	1.53	–
SPur	1.78	0.035–0.082	2.01–4.66	0.07	4.19	0.01	1.55	–

Amounts correspond to 5 μl of sample.

1 and 10 μl of the salicin standard solution on a plate resulting in 0.244 μg/band to 2.44 μg/band. The lowest and the highest amount of the regression curve were applied three times. The equation

$$A = -6.9 \times 10^{-5}\,\mathrm{m}^2 + 0.947\ \mathrm{x\ m} + 20.61 \qquad (R^2 = 0.99945)$$

with the applied salicin mass *m* and the absorption *A* was obtained.

Linearity was shown for harpagoside between 9.9 and 99.2 ng/band by spraying 9 different volumes (1–10 μl) of the harpagoside standard on a plate. The highest (99.2 ng/band) and the lowest amount (9.92 ng/band) were applied three times. The equation

$$A = 19.69 \times m + 43.14 \qquad (R^2 = 0.99933)$$

with the applied harpagoside mass m and the absorption *A* was obtained (Fig. 4).

For both substances a working range was chosen starting with the lowest quantifiable concentration to avoid or minimize non-linearity regression curves. Furthermore, residual plots were generated. No trends were observed indicating that the model represents the data correctly.

3.3.4. Precision

Precision data were subdivided into intra-day precision, inter-day precision and inter-laboratory precision. Intra-day precision was obtained by analysing the same sample three times within 1 day. This was repeated on two different days by different analysts using freshly prepared samples and mobile phases. The results of the three experiments were used to calculate the inter-day precision. For inter-laboratory precision, an intra-day precision was carried out in a collaborating laboratory.

Three different amounts (2, 5 and 10 μl) of standard solution were applied for each experiment resulting in about 0.48, 1.20 and 2.4 μg salicin per band or about 20.0, 50.0 and 100 ng harpagoside per band, respectively. Furthermore, 5 μl of different extracts were applied: extracts of *Salix pentandra* and *S. purpurea* or an extract of Harpagophyti radix 1 as well as of solutions of S1658 and S1659, respectively. Results are shown in Tables 1 and 2.

3.3.5. Accuracy

Extracts were spiked with standard solutions. One millilitre *Salix pentandra* extract was spiked with 1 ml standard solution. The calculated amount (0.32 mg/ml) was compared with the experimentally determined amount (0.31 ± 0.01 mg/ml, *n* = 3) and a deviation of −0.72% was calculated. A spiked *H. procumbens* extract (1 ml extract and 1 ml harpagoside standard solution) was used to show accuracy of the harpagoside method. The experimentally determined amount (12.4 ± 0.2 μg/ml, *n* = 3) showed a deviation of

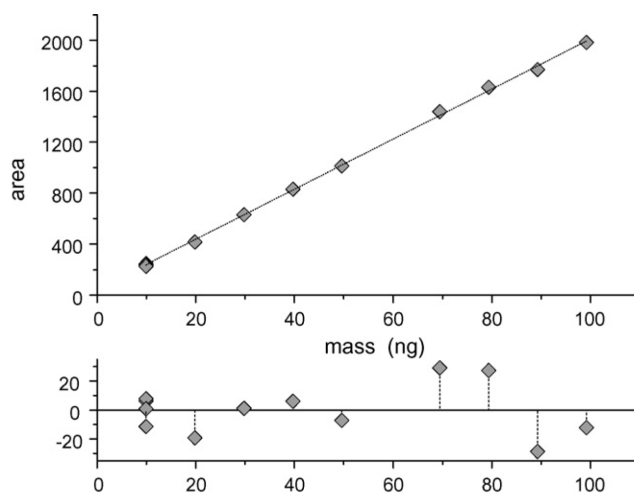

Fig. 4. Regression curve and residual plot of harpagoside between 9.9 and 99 ng/spot.

Table 2
Validation data of three harpagoside standard solutions and extracts of *Harpagophytum procumbens*

Sample	Mass (μg)	S.D. of within-day precision		S.D. of reproducibility		S.D. of reproducibility between different laboratories		Accuracy: deviation from calculated value (%)
		μg	%	μg	%	%	μg	
Standard 1	20	0.54–1.00	3.08–4.77	0.52	2.37	0.78	3.81	101.95–106.76
Standard 2	50	1.96–2.25	4.44–4.75	1.79	3.53	0.22	0.45	96.28–102.73
Standard 3	100	0.51–3.99	0.63–4.10	1.45	1.52	0.65	0.70	95.65–98.23
Harpag. radix 1								
Extraction 1	69.8	0.19–1.89	0.28–2.64	2.49	3.62	2.78	3.98	–
Extraction 2	65.1	–	–	–	–	2.74	4.29	–
S1858	42.1	0.22–1.90	0.53–3.19	1.23	2.88	–	–	–
S1859	40.3	1.26–1.38	2.96–3.63	1.59	4.02	–	–	–

Harpagophyti radix 1 was extracted according to Harpagophytum extraction 1. S1858 and S1859 were dried extracts re-dissolved in methanol. Amounts correspond to 5 μl of sample.

Table 3
Content of salicin in dried bark of five different *Salix* species

Species	Content (%)
Salix eleagnos Scop.	3.87 ± 0.11 (3.86 ± 0.01, 3.77 ± 0.06, 3.99 ± 0.06)[a]
Salix fragilis L.	2.40 ± 0.03
Salix daphnoides Vill.	3.30 ± 0.03
Salix pentandra L.	3.96 ± 0.11
Salix purpurea L.	2.03 ± 0.01

[a] Mean value and standard deviation of three different extracts. The values for each single extract are present in parenthesis.

Table 4
Content of harpagoside in dried roots and dried extracts

Extract	Content (%); $n = 3$
Harpagophyti radix 1[a]	1.71 ± 0.07 (1.74 ± 0.07, 1.63 ± 0.03, 1.77 ± 0.09)[a]
Harpagophyti radix 2	1.20 ± 0.01
S1858	2.41 ± 0.03
S1859	2.26 ± 0.04

[a] Mean value and standard deviation of three different extracts. The values for each single extract are present in parenthesis.

4.2% to the calculated amount (11.9 μg/ml). At each time 5 μl of the extracts and the spiked extracts were used.

Furthermore, mean values of the intra-day precision at the different concentrations were compared with the values calculated by inserting the actual concentration into the calibration curve. Accuracy was expressed as percentage of the mean value related to the calculated value (=100%, Tables 1 and 2).

3.3.6. Precision of the extraction procedures

The *Salix* extraction procedure was performed with bark of *Salix eleagnos* and harpagoside extraction procedure 1 was carried out with Harpagophyti radix 1. Both extractions were repeated three times on different days and the content of salicin and harpagoside was determined using 5 μl of the extracts, respectively. The three salicin and harpagoside contents are given in parenthesis in the first row of Tables 3 and 4, respectively. The determined contents of salicin showed a relative standard deviation of 2.8%. A standard deviation of 4.09% was calculated for the determined contents of harpagoside.

3.4. Content in different species, plants or dried extracts

To show the suitability of the salicin-method for different *Salix* species, extraction and determination of salicin content were performed for five species (Table 3). No substances interfered with the salicin peak.

The applicability of the validated harpagoside-method for different plant material and dried extracts was investigated (Table 4). No substance interfered with the peak of harpagoside.

4. Conclusion

The proposed method for quantification of salicin in willow bark is the first validated HPTLC method to the best of our knowledge. The method allows the determination of the total content of phenolic glycosides. For all investigated *Salix*-species hydrolysis results in a well-separated salicin peak without any co-eluting substances. The determined amounts of salicin in various bark samples are within the pharmacopoeial limits. The proposed HPTLC method for determination of harpagoside results in a symmetrical, well-resolved peak for the target compound. The content of harpagoside in the investigated plant samples was within the pharmacopoeial limits whereas one of the investigated extracts did not meet the minimum level of 1.2%. This underlines the importance of quality controls for this drug.

For the determination of harpagoside in *H. procumbens* HPTLC methods have already been developed [6,7]. They show similar statistical values as the method developed here. However, in our study inter-laboratory precision was shown for a HPTLC method of harpagoside for the first time. Due to the "open system" TLC environmental and human effects have strong influences on the results of HPTLC methods. Especially, quantitative methods of HPTLC can result in different contents if they are performed in different laboratories. The determined inter-laboratory precision showed similar deviations as the intra-day precision in one laboratory. This could also be shown for the HPTLC method of Salicin demonstrating the universal validity of the developed HPTLC methods.

Because of the possibility of analysing up to 16 samples in parallel the proposed methods are very rapid and cost efficient. About 4 min and 2 ml of mobile phase are required per sample. In contrast, the current HPLC-methods of the two drugs used in European Pharmacopoeia [3,12] take more than 30 min and needs 30 ml of the mobile phase per sample. The achieved precision and accuracy is sufficient for general quality control.

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