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Short communication

Qualitative and quantitative determination of sesquiterpenoids in *Achillea* species by reversed-phase high-performance liquid chromatography, mass-spectrometry and thin-layer chromatography

Sabine Glasl, Ulrike Kastner, Johann Jurenitsch*, Wolfgang Kubelka

Institute of Pharmacognosy, University of Vienna, Pharmaziezentrum, Althanstraße 14, A-1090 Vienna, Austria

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Abstract

A reversed-phase high-performance liquid chromatographic method was developed as a universal analysis system in order to determine and quantify antiphlogistic sesquiterpenoids in different *Achillea* species. Identification was performed by HPLC and diode array detection as well as by monitoring the HPLC fractions by TLC and MS. Using santonin as internal standard, HPLC separations were achieved with a methanol–water gradient system using RP 8 LiChrospher 100 (5 μm) as stationary phase. For validation, sample analyses were performed, using the two tetraploid species *A. collina* and *A. pratensis*. The method allows the identification and quantification of the main compounds achillicin, 8α-tigloxy-artabsin, 8α-angeloxy-artabsin, arglanin and santamarin with variation coefficients between 3.4 and 4.7% (total content) using santonin as internal standard. For the different compounds recovery was found between 81 and 107% performing multiple analyses of *A. collina* and *A. pratensis*.

Keywords: *Achillea* species; Sesquiterpenoids

1. Introduction

Yarrow is widely used in folk medicine (7th range of the most popular plants in Austria [1]) against various diseases including internal and external inflammations. Extensive morphological investigations led to a revision of the *Achillea millefolium* group [2–4] providing well defined plant material for isolation and structure elucidation of the main compounds. Extraction of several taxa of the diploid, tetraploid, hexaploid and octoploid level yielded a large number of different, partially labile sesquiterpenoids [5]: the proazulenes on the one hand, which are structurally close to matricin (chamomile), giving a blue colour with heat or acid and on the other hand the non-azulenogenic sesquiterpenes which do not show this reaction.

According to pharmacological studies some of the isolated compounds – proazulenes as well as non-azulenogenic sesquiterpenes – are responsible for the antiphlogistic activity [6,7]. In contrast α-peroxiachifolid and other peroxides show effects against malaria [8], additionally they were shown to trigger allergic contact dermatitis [9,10] due to their α-methylene-γ-lactone structure. This broad spectrum of substances with different effects and properties requires a universal method to check the quality of a sample and to quantify the pharmacologically rel-

*Corresponding author.

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evant substances. Basic approaches were suggested by Rücker et al. [11], using an amperometric detection after HPLC separation, which allows the quantification of oxidizable peroxides only.

The lack of an universal analysis system for quality control and characterization of the genuine sesquiterpene composition of each taxon by its fingerprint required the development of an HPLC method with UV detection on which we report in this paper. Due to the heterogeneity of the plants the use of small drug amounts was basic requirement to perform single plant analyses. The system should allow the separation of sesquiterpenes with differing structures and polarities as well as the absolute quantification of compounds **1–14** (Fig. 1). Determination of the species, of the commercial available drug and of the different remedies (tea, drops, tablets etc.) should be possible. Accuracy and reproducibility were determined by analyzing a series of samples using two tetraploid species, *Achillea collina* (containing proazulenes [12]) and *Achillea pratensis* (containing no proazulenes [13]).

2. Experimental

2.1. Materials

Achillea collina (tetraploid) had been collected at Vösendorf (Austria) in 1995, *Achillea pratensis* (tetraploid) at Laab am Walde (Austria) in 1997. Vouchers of both origins are deposited in the Herbarium of the Institute of Pharmacognosy, University of Vienna (Austria).

The internal standard santonin (99% purity) was purchased from Sigma-Aldrich. All sesquiterpenes used for calibration **1, 2, 3, 4, 5, 7, 8, 9, 10, 13** and **14** have been isolated, purified and structurally elucidated at the Institute of Pharmacognosy, University of Vienna (Austria) [5]. For specification of UV data and R_F values see Table 1.

Methanol (Chromasolv, HPLC grade) was from Riedel-de Haën, dichloromethane (analytical-reagent grade) from J.T. Baker.

2.2. Sample preparation

Samples for validation analyses as well as for single plant analyses were obtained by the same procedure. The performance of single plant analyses required smaller amounts than those for validation; these are given in brackets. For validation an amount of 100 g air dried flower heads was separated and homogenized by hand, serving as stock. A 2-g amount (100 mg) air dried flower heads were transferred to a 50-ml flask (2 ml reacti vial) followed by 20 ml (1 ml) dichloromethane and 5 ml (250 μl) internal standard solution (0.1 g santonin in 100 ml methanol). After ultrasonification for 10 min at room temperature the solution was filtered into a 100-ml (10-ml) round-bottom flask, the drug was washed twice with 20 ml (1 ml) dichloromethane and filtered. Altogether this procedure was performed four times, the combined extracts were concentrated in vacuo at less than 40°C to ≈5 ml for the validation analyses. The single plant extracts were evaporated entirely and redissolved in 500 μl either methanol 80% (for qualitative analyses) or dichloromethane (for quantification).

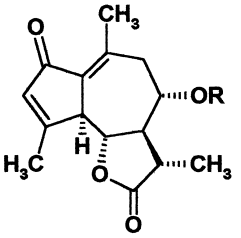
An aliquot of both solutions was transferred into a 1.5-ml vial and 20 μl thereof were analyzed by HPLC.

2.3. HPLC analysis

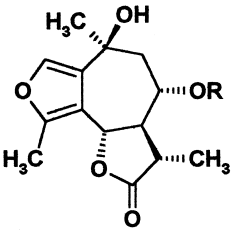
The HPLC system consisted of a Perkin-Elmer system ISS-100 autosampler (injection volume 20 μl), Series 200 pump and LC235C diode array detector (detection at 220 and 255 nm). All computations were performed using the Perkin-Elmer software TURBOCHROM. Separations were carried out using a Hewlett-Packard LiChrospher 100 RP 8 5 μm column (250×4.0 mm) guarded by a Hewlett-Packard LiChrospher 100 RP 8 5 μm guard column (4×4 mm). The binary system employed the eluents methanol and water according to a flat gradient. The elution started from 20% methanol to 80% methanol in 180 min (linear; rate=0.33%/min) with a flow-rate of 1.0 ml/min at room temperature.

The relative retention times of the compounds related to the internal standard santonin are summarized in Table 1.

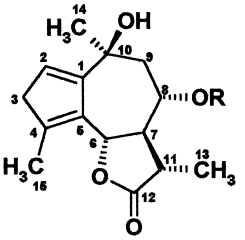
Preparation of HPLC fractions for TLC and MS analyses: The identification of the peaks in the HPLC chromatograms was achieved by the retention times and the UV–Vis spectra (DAD detection) of the respective compounds. As these parameters seemed not sufficient for confirmation, additionally off-line



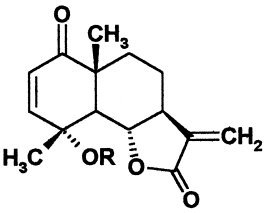
1 8-desacetyl-matricarin (R=H)
2 matricarin (R=acetyl)



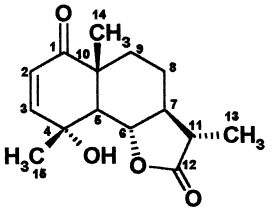
3 3-oxa-achillicin (R=H)
5 8α-tigloxy-3-oxa-artabsin (R=tigloyl)
6 8α-angeloxy-3-oxa-artabsin (R=angeloyl)



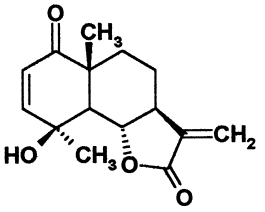
4 8α-acetoxy-artabsin (=achillicin, R=H)
7 8α-tigloxy-artabsin (R=tigloyl)
8 8α-angeloxy-artabsin (R=angeloyl)



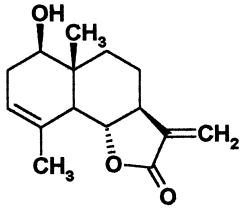
10 arglanin (R=H)
11 4-hydroperoxy-arglanin (R=OH)



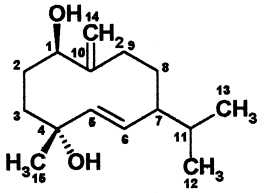
9 tauremisin



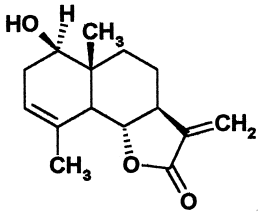
12 4-epi-arglanin



13 santamarin



14 5E,10(14)-germacradien-1β,4α-diol



santonin (internal standard)

Fig. 1. Structures of the quantified sesquiterpenes **1**–**14** and of the internal standard santonin.

TLC–MS was performed by sampling the HPLC fractions corresponding to each compound repeatedly. The fractions were extracted with dichloromethane, the organic layer was removed under reduced pressure, the residue was redissolved in ≈50 μl dichloromethane and used for TLC confirmation and

Table 1
Relative retentions times, λ_{max}, R_F, characteristics under $UV_{255\ nm}$, reaction with spray reagents and correction factors referred to the internal standard santonin of the compounds **1**–**14**

Compound	Molecular mass	Relative t_R (min)[a]	λ_{max}[b] (nm)	R_F	$UV_{255\ nm}$[c]	EP reagent[d]	AS reagent[e]	Correction factor[g]	Equation of the calibration curve[f]
8-Desacetyl-matricarin (**1**)[#]	262	0.58±0.02	262	0.40	+++	−	Violet	0.78	$y = 1.2939x - 0.002$
Matricarin (**2**)[#]	304	1.17±0,02	260	0.80	+++	−	Violet	1.28	$y = 0.6979x + 0.0308$
3-Oxa-achillicin (**3**)[#]	308	1.49±0.08	211	0.60	−	Pink	Violet	3.02	$y = 0.3339x - 0.0006$
Achillicin (**4**)	306	1.66±0.07	247	0.65	++	Blue-green	Brown-green	4.78	$y = 0.2204x - 0.0034$
8α-Tigloxy-3-oxa-artabsin (**5**)[#]	348	2.33±0.17	216	0.65	−	Pink	Violet	0.97	$y = 0.9757x - 0.0018$
8α-Angeloxy-3-oxa-artabsin (**6**)[#]	348	2.33±0,17	216	0.75	−	Pink	Violet	0.97	
8α-Tigloxy-artabsin (**7**)	346	2.52±0.20	220	0.70	++	Blue-green	Brown-green	1.65	$y = 0.6727x + 0.0081$
8α-Angeloxy-artabsin (**8**)	346	2.55±0.20	220	0.80	++	Blue-green	Brown-green	1.65	$y = 0.5373x - 0.0045$
Tauremisin (**9**)	264	0.54±0.02	216	0.50	−	−	−	1.18	$y = 0.8217x + 0.0018$
Arglanin (**10**)	262	0.58±0.02	213	0.50	+	Yellowish	Green	0.95	$y = 0.8666x + 0.0698$
4-Hydroperoxy-arglanin (**11**)	278	0.63±0.01	219	0.60	+	Yellow	Orange	0.95	
4-Epi-arglanin (**12**)[#]	262	0.73±0.01	211	0.55	+	Yellowish	Green	0.95	
Santamarin (**13**)	248	1.30±0.02	202	0.70	−	−	Blue-violet	1.61	$y = 0.609x + 0.0044$
5*E*,10(14)-Germacradien-1β,4α-diol (**14**)	238	1.84±0.03	201	0.40	−	−	Blue-violet	6.82	$y = 0.1433x + 0.001$

[a] 32 HPLC analyses performed over a period of 2 months (mean±standard deviation).
[b] Recorded on line in methanol–water by DAD detection during HPLC run.
[c] Number of '+' indicates the intensity of fluorescence quenching zones.
[d] Modified acetic acid–phosphoric acid reagent [14] (0.25 g dimethylaminobenzaldehyde, 50 g acetic acid, 5 g phosphoric acid 85%, 20 ml water).
[e] Anisaldehyde–sulphuric acid reagent [15] (17 g ethanol, 2 g sulphuric acid conc., 1 g anisaldehyde).
[f] x = Concentration $_{sample}$ (mg/ml); y = concentration $_{santonin}$ (mg/ml)*area $_{sample}$/area $_{santonin}$ calibration analyses were performed within the range of 0.03–1.4 mg/ml; in the drug the main compounds are within this range, the others (signed by [#]) were outside of this calibration range (lower concentration, see Table 2).
[g] Calculated: $\frac{\text{weight}^{*}_{\text{substance i}}\text{area}_{\text{internal standard}}}{\text{weight}^{*}_{\text{internal standard}}\text{area}_{\text{substance i}}}$

off-line MS confirmation (molecular masses, R_F values on silica-gel as well as the characteristic colours with the detection reagents see Table 1).

2.4. Thin-layer chromatography

Silica gel 60 Merck plates (0.25 mm) were used with dichloromethane–acetone (9:1, v/v) as mobile phase and a dichloromethane extract of chamomile as reference (matricin: $R_F = 0–050$). After detection with modified acetic acid–phosphoric acid-reagent [14] and anisaldehyde–sulphuric acid-reagent [15] under heat (140°C) different coloured stains resulted (Table 1).

2.5. Mass spectrometry

A Shimadzu QP-1000 EX MSPAC 200 with direct inlet and two possible ionisation modi (EI-MS, CI-MS) was used for all of the sesquiterpenes. EI-mode: ion source: 250°C, 70 eV; vacuum: $4 \cdot 10^{-6}$ torr; scan: 40–500/2 s; heating rate of sample vial: 80°C/min; CI-mode: ion source: 180°C, 200 eV; reactant gas: ammonia 2.6, pre-pressure: 1 bar; vacuum: $5 \cdot 10^{-5}$ torr; scan: 40–500/2 s; heating rate of sample vial: 80°C/min.

2.6. Calibration

The HPLC method was calibrated for all substances except 4-epi-arglanin (**12**), 4-hydroperoxy-arglanin (**11**) and 8α-angeloxy-3-oxa-artabsin (**6**) by preparing methanolic standard solutions. Aliquots of varying amounts (0.03 mg/ml to 1.4 mg/ml) occasionally occurring in the plants were mixed with the internal standard santonin (0.5 mg/ml), evaporated under nitrogen and diluted in 500 μl methanol 80%, so that the concentrations accorded to those of the sample solutions. The correction factors were determined in relation to the internal standard santonin (Table 1). The internal standard santonin was detected at 255 nm ($\lambda_{max} = 245$nm), whereas the peak

areas of the respective compounds were determined at either 220 or 255 nm, depending on their UV characteristics (Table 1). Over the selected concentration range from 0.03 to 1.4 mg/ml the calibration curves showed linearity. Depending on the available amount of each compound at least three and maximal six concentration levels were analyzed. The substances **6, 11** and **12** were not available for calibration. For **11** and **12** a very similar absorption coefficient to arglanin (**10**) could be assumed, as all of the compounds were characterized by the same chromophore as well as by a nearly equal molecular mass. Therefore, the correction factor of arglanin was used for the quantification of both compounds. The same was applied to 8α-angeloxy-3-oxa-artabsin (**6**) which was calculated by the correction factor of its tigloxy derivative (**5**).

2.7. Validation

To investigate the performance of the method seven analyses of *Achillea collina* and six analyses of *Achillea pratensis* were repeated over a period of 3 days. For recovery three repeated analyses of 10–70% addition of individual compounds (**1**, **4**, **7**, **8**, **9**, **10**, **13**) were performed on 3 different days. The standards were added to the drug before extraction. The data of these validation experiments are shown in Table 2.

3. Results and discussion

The aim of this project was to establish an universal analysis method, applicable to different achillea taxa. Therefore two species were chosen for development which contain sesquiterpenes of very different polarities: *Achillea collina* (containing apolar, labile proazulenes) and *Achillea pratensis* (containing polar eudesmanolides). The extraction was performed with several solvents (methanol, methanol–water, dichloromethane) and examined semiquantitatively by TLC. The highest yield could be obtained using dichloromethane which additionally offers the advantage of evaporation at low temperature (40°C maximum). Thus, a careful treatment of the unstable proazulenes is guaranteed. Using dichloromethane, an 98% extraction yield of the guaianolides could be reached and demonstrated by HPLC analyses after extracting twice, whereas the yield of eudesmanolides in the same range required four extractions. The evaporated combined extracts for qualitative analyses were resuspended in methanol–water (80:20, v/v), representing a further purification step, as lipophilic oily ballast is not soluble.

For quantitative analyses dichloromethane was used, since only this solvent guaranteed complete solubility of the compounds to be quantified. Since the use of pulverized drug did not yield higher amounts of sesquiterpenes but gave solutions with higher concentrations of impurities (intensive yellow colour), unground flower heads were applied.

Besides extraction, the eudesmanolides also required the optimization of the HPLC system to allow the separation of compounds **9**, **10** and **11** (see Fig. 2). Variations of the mobile phase (methanol, acetonitrile, isocratic and gradient systems) as well as different stationary phases (RP 2, RP 8 and RP 18) were tested. Acetonitrile as modifier did not give a sufficient separation of the eudesmanolides **9**, **10** and **11**, using LiChrospher RP 2, RP 8 and RP 18 as stationary phases. The same results were found when LiChrospher RP 2 and RP 18 columns in combinations with methanol–water were used. Finally acceptable separations were obtained using LiChrospher RP 8 material (5 μm) and a flat methanol–water gradient (0.33%/min). The flat gradient system offers the advantage to record compounds with different polarities within one single run but requires a run time of more than 2 h. As Fig. 2 shows, the elution of the less polar proazulenes **7** and **8** of *A. collina* takes 148 min, whereas the eudesmanolides **9**–**13** of *A. pratensis* elute within 85 min due to their high polarity.

The requirements for the internal standard were commercial availability, high purity, identical chemical behaviour as the sesquiterpenes, chemical stability and a suitable position in the chromatograms. Among matricin, parthenolid (both labile) and santonin, the latter complied with all requirements. Quantifications as well as relative retention times were calculated according to peak areas and retention times, respectively.

The lability of some compounds required gentle processing in general, as, for example, extraction of the respective HPLC fractions with dichloromethane

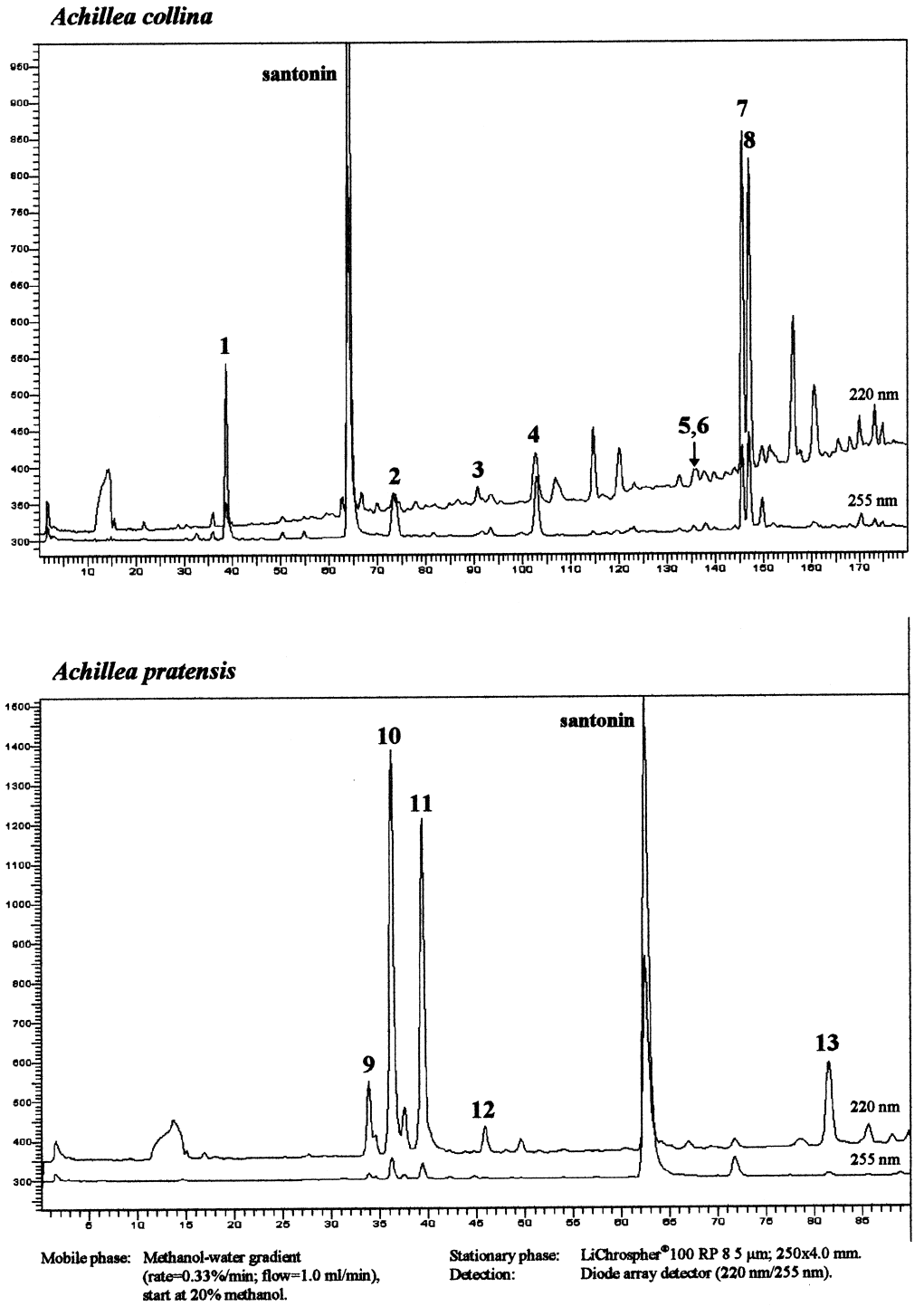


Fig. 2. HPLC chromatograms of a sample of *Achillea collina* and *Achillea pratensis* recorded at 220 and 255 nm.

and evaporation at 40°C maximum. Due to this fact the fresh preparation of a sample solution was necessary to get correct quantitative results. The content of proazulenes decreased with increasing age of the solution.

For the validation analyses homogenous plant material had to be provided. As single plants with a weighed portion of 100 mg flower heads showed too high variability, a stock of 100 g dried flower heads homogenized by hand was prepared, serving as a source for 2 g deposits per analysis. This gave acceptable results (see Table 2) for the total contents of guaianolides (RSD 4.7%) and eudesmanolides (RSD 3.4%) analyzed on different days. The RSD values of the single compounds varied from 4.4 to 25.1% depending on the concentration of the respective substance. The relatively high variances of the values of compounds **2**, **3**, **5**, **6** and **12** (10.0–25.1%) are due to their occurrence in traces. This fact also implies the combined quantification of the two 3-oxa-derivatives **5** and **6**. The concentrations of **1**, **2**, **3**, **5**, **6** and **12** were determined although their concentrations were not within the calibration range. It has to be emphasized that all determinations were related to reference substances isolated at our institute (Section 2.1). The recoveries of the seven added substances were in the range of 80–107%, lowest values were found for the proazulenes **4**, **7** and **8** owing to their lability.

Single analyses were performed, as the RSDs of several samples analyzed in duplicate was $<2\%$.

4. Conclusions

The presented method for extraction and analysis by HPLC in combination with TLC and MS allows the qualitative and quantitative determination of different *Achillea* species. It offers the possibility of qualitative single plant analyses, as small amounts of flower heads give characteristic patterns depending on the respective taxon. Additionally, the main proazulenes and eudesmanolides can be quantified sensitively using more sample. Thus a method is provided to examine the commercially available drug, the variability of the proazulene content within

Table 2
Content (g/100 g dried weight) of the compounds of seven samples of *Achillea collina* and six samples of *Achillea pratensis*, mean values, standard deviation, relative standard deviation and recovery as 10–70% addition of seven components

Species	Compound	Sample							Mean value	SD[a]	RSD (%)[b]	Recovery (%)[c]
		A	B	C	D	E	F	G				
A. collina	8-Desacetyl-matricarin (**1**)	0.030	0.030	0.026	0.031	0.032	0.031	0.033	0.031	0.002	7.0	93
	Matricarin (**2**)	0.031	0.027	0.034	0.031	0.025	0.028	0.027	0.029	0.003	10.7	
	3-Oxa-achillicin (**3**)	0.023	0.019	0.016	0.018	0.018	0.020	0.012	0.018	0.004	19.8	
	Achillicin (**4**)	0.103	0.104	0.121	0.125	0.100	0.098	0.105	0.108	0.011	9.8	86
	8α-Tigloxy-/8α-angeloxy -3-oxa-artabsin (**5**,**6**)	0.008	0.011	0.013	0.007	0.008	0.006	0.009	0.009	0.002	25.1	
	8α-Tigloxy-artabsin (**7**)	0.141	0.158	0.163	0.163	0.153	0.149	0.153	0.154	0.008	5.1	80
	8α-Angeloxy-artabsin (**8**)	0.155	0.137	0.164	0.146	0.142	0.165	0.146	0.151	0.011	7.4	81
	Total content	0.491	0.485	0.537	0.523	0.476	0.501	0.476	0.498	0.024	4.7	
A. pratensis	Tauremisin (**9**)	0.038	0.043	0.037	0.047	0.043	0.038		0.041	0.004	9.2	107
	Arglanin (**10**)	0.209	0.204	0.218	0.207	0.201	0.191		0.205	0.009	4.4	102
	4-Hydroperoxy-arglanin (**11**)	0.173	0.178	0.150	0.143	0.175	0.162		0.163	0.015	9.0	
	4-epi-Arglanin (**12**)	0.016	0.015	0.015	0.015	0.013	0.012		0.015	0.002	10.0	
	Santamarin (**13**)	0.103	0.107	0.096	0.100	0.095	0.096		0.100	0.005	4.8	94
	Total content	0.539	0.547	0.516	0.511	0.528	0.499		0.523	0.018	3.4	

[a] Standard deviation.
[b] Relative standard deviation.
[c] Performed as 10–70% addition of the average contents of the individual components. The addition was performed in triplicate on 3 different days.

different species, as well as to investigate the rate of transfer into aqueous remedies.

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