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Combination of chromatographic and spectroscopic methods for the isolation and characterization of polar guaianolides from *Achillea asiatica*

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Abstract

Four polar guaianolides, 8α-angeloxy-2α,4α,10β-trihydroxy-6β*H*,7α*H*,11β*H*-1(5)-guaien-12,6α-olide; 8α-angeloxy-1β,2β:4β,5β-diepoxy-10β-hydroxy-6β*H*,7α*H*,11β*H*-12,6α-guaianolide; 8α-angeloxy-4α,10β-dihydroxy-2-oxo-6β*H*, 7α*H*, 11β*H*-1(5)-guaien-12,6α-olide and 8-desacetyl-matricarin, were isolated from *Achillea asiatica* and characterized by TLC, MS, IR, HPLC and diode array detection. Purified extracts were separated by means of flash chromatography. HPLC separations were achieved using different methanol–water gradients as mobile phase and LiChrospher 100-RP8 5 μm or Zorbax SB-C_8 3.5 μm as stationary phases. The chromatographical data are compared to those of the proazulene 8α-tigloxy-artabsin which shows antiinflammatory effects. By means of these characteristics the identification of the guaiaolides with potential antiphlogistic properties is also possible from other sources. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Achillea asiatica*; Plant materials; Guaianolides; Sesquiterpenoids

1. Introduction

Yarrow is a widespread plant used in folk medicine of many Central European and Asian countries because of its various effects. Among the indications are gastric and intestinal disorders as well as inflammation of skin and mucosa which are influenced positively by the antiinflammatory properties of ethanolic and aqueous remedies [1–3]. Diploid and tetraploid taxa of the *Achillea millefolium* group have been shown to contain proazulenes [4] similar to the proazulene matricine in chamomile. These guaianolides are transformed to chamazulene under the influence of acid, light and temperature. The proazulenes and chamazulene exert antiinflammatory activity in the carrageenin edema model [5,6] and in the croton oil ear test [7,8]. Chamazulene has shown inhibition of the leukotriene synthesis and additional antioxidative effects [9], which may contribute to the antiinflammatory activity of chamomile and yarrow. The proazulenes lack any allergenic potential whereas other guaianolides, isolated from hexaploid taxa of the *Achillea millefolium* group, with active sites in the molecule such as α-methylene-γ-lactones or exocyclic methylene groups are known to trigger allergic reactions [10,11]. Therefore, a further proazulene-containing species collected in Mongolia, *Achillea asiatica*, was investigated in order to isolate and characterize new guaianolides as potential active substances without exocyclic methylene group. It

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contains three main proazulenes, 8α-angeloxy-artabsin, 8α-tigloxy-artabsin (**5**) and achillicin (8α-acetoxy-artabsin) which are characteristic for proazulene-containing species [12]. Additionally *Achillea asiatica* is characterized by a remarkably high content of very polar proazulenes and non proazulenes which have been elucidated only recently as 8α-angeloxy-2α,4α,10β-trihydroxy-6β*H*, 7α*H*, 11β*H*-1(5)-guaien-12,6α-olide (**1**), 8α-angeloxy-1β, 2β:4β,5β-diepoxy-10β-hydroxy-6β*H*,7α*H*,11β*H*-12,6α-guaianolide (**2**) and 8α-angeloxy-4α,10β-dihydroxy-2-oxo-6β*H*, 7α*H*, 11β*H*-1(5)-guaien-12,6α-olide (**3**) [13]. The present paper covers the methods to isolate these potential active compounds, including 8-desacetyl-matricarin (**4**) which is well known for other species [4,14] but has not yet been described for *Achillea asiatica*. Additionally their spectroscopic (UV, MS) and chromatographic characteristics (TLC on silica gel, HPLC on RP8) are compared to those of the proazulene 8α-tigloxy-artabsin (**5**) (Fig. 1). By means of these data, identification of the respective compounds in Central European *Achillea* species was also performed.

2. Experimental

2.1. Materials

Achillea asiatica was collected in Central Mongolia (Töv aimag) near the river Shavart at the time of full blooming in 1998. Voucher specimens were deposited at the Institute of Biology and Biotechnology, Mongolian Academy of Sciences, Ulaanbaatar. *Achillea ceretanica* Sennen (tetraploid) was propagated in vitro at the Institute of Pharmacognosy [15], *Achillea collina* (tetraploid) was collected at Vösendorf, Lower Austria, *Achillea asplenifolia* (diploid) at Rust, Burgenland, and *Achillea roseoalba* (diploid) at Parrendorf, Carinthia. Voucher specimens of the Middle European taxa were deposited in the Herbarium of the Institute of Pharmacognosy, University of Vienna.

Solvents for extraction and flash chromatography were of distilled quality, for further purification and TLC of analytical-reagent grade (dichloromethane: Merck; acetone: J.T. Baker; methanol: Riedel-de Haën) and for HPLC of Chromasolv grade (methanol: Riedel-de Haën).

2.1.1. Extraction, purification and isolation

Herb of *Achillea asiatica* was dried over a period of 4 weeks at room temperature protected from direct sunlight. Dry tissue (1 kg) was ground and extracted three times in portions with dichloromethane (total volume 10 l) by ultrasonification for 10 min at room temperature. The combined extracts were concentrated to dryness in a rotary vacuum evaporator at 40°C maximum to prevent degradation of the labile proazulenes. The resulting oily green residue (29.0 g) was redissolved in 500 ml dichloromethane and 500 ml of 50% aqueous methanol were added. The round bottom flask containing the resulting two layers was transferred to the rotary vacuum evaporator and treated in vacuo at 40°C. This procedure represented a soft extraction, as the more volatile dichloromethane evaporated by bubbling through the upper aqueous methanol layer. The sesquiterpenes remained dissolved in this methanolic phase, the very apolar compounds precipitated and were removed by filtration. The aqueous methanolic phase was kept until unification with following ones, the residue was redissolved in dichloromethane and treated again with aqueous methanol 50%. All together this procedure was carried out for four times. A final TLC check of the residue did not show any more sesquiterpenes, the combined aqueous methanolic layers were extracted with dichloromethane and yielded 3.9 g of a yellow, aromatic smelling oil. Further separation into ten fractions corresponding to increasing polarity of the eluent mixtures (v/v, volume: 1 l each) was performed by flash chromatography on ICN Silica TSC, 60A (5× 20 cm, 20 ml/min): CH_2Cl_2, CH_2Cl_2–acetone 9:1, 8:2 (compounds **2**, **3**, **4**), 7:3, 6:4, 1:1 (compound **1**), CH_2Cl_2–methanol 9:1, 8:2, 7:3 and 6:4. Further purification was performed by repeated column chromatography of the respective fractions on silica gel 60 (1×20 cm, 5 ml/min) starting with pure CH_2Cl_2 and continuing with a more fine gradient of CH_2Cl_2–acetone mixtures (v/v, volume: 500 ml each): CH_2Cl_2–acetone 95:5, 9:1, 85:15, 8:2, 1:1. Compounds **1**–**4** were isolated by HPLC (LiChrospher 100-RP8) employing either an isocratic system

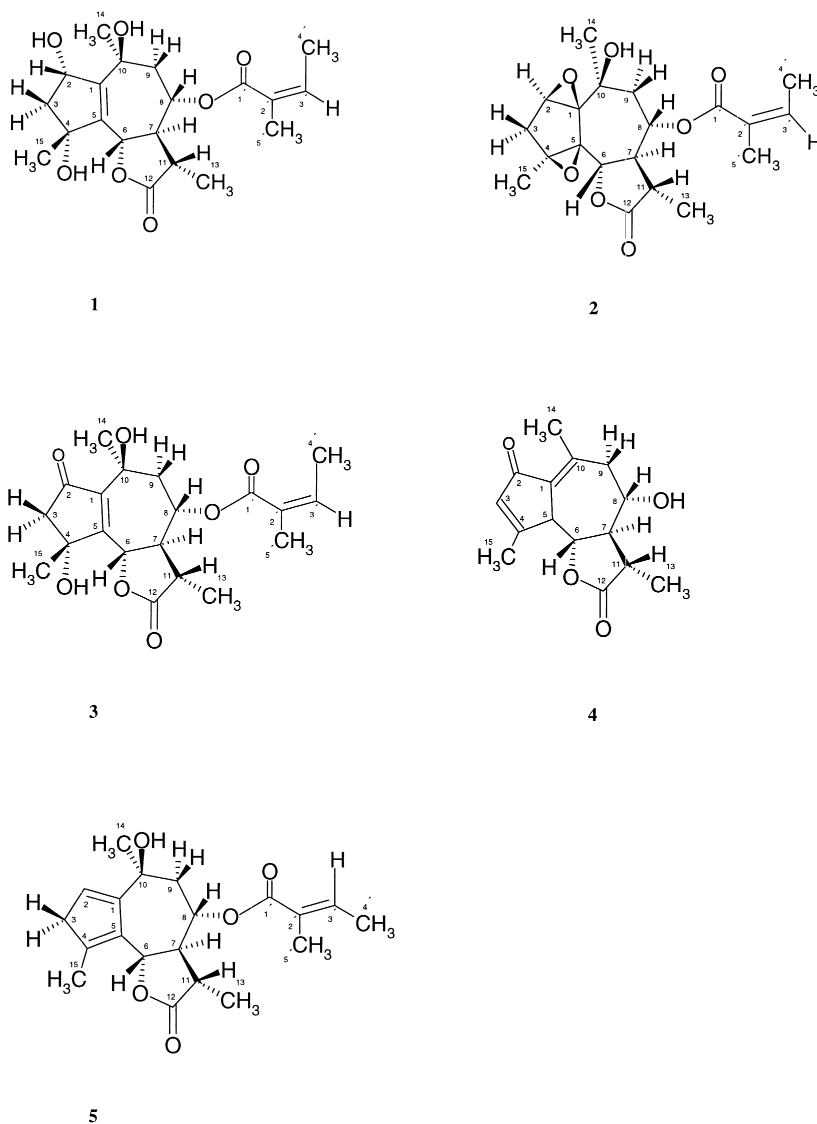


Fig. 1. Structures of the sesquiterpenes **1**–**5**.

(compounds **2**–**4**, 55% methanol, see Fig. 2) or a gradient system (compound **1**, rate: 0.6%/min). The corresponding fractions were collected, extracted with CH_2Cl_2, purified over a small silica gel cartridge and measured by NMR: **1** (14 mg), **2** (1.6 mg), **3** (3.0 mg), **4** (2.0 mg). The yields of these labile compounds do not represent their real concentrations in the plant.

2.1.2. Sample preparation for identification analyses

To identify the guaianolides in the Middle European *Achillea* species, flower heads were exclusively used for extraction. This tissue lacks chlorophyll and is characterized by a higher content of proazulenes compared to the leaves. First, 2 g flower heads were extracted one time with 20 ml dichloromethane by

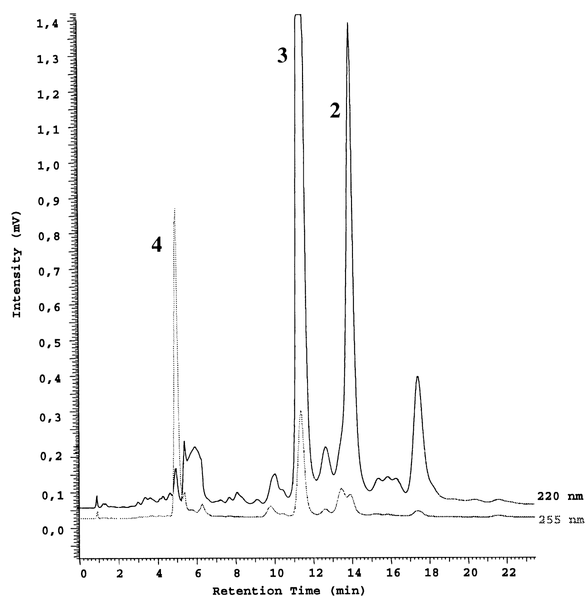


Fig. 2. HPLC chromatogram of a fraction containing compounds **2**, **3** and **4** Stationary phase: LiChrospher 100-RP8 5 μm; mobile phase: methanol–water (55:45, v/v) isocratic; detection: 220 and 255 nm.

ultrasonification for 10 min at room temperature. The resulting residue (0.1 g) was fractionated over a cartridge (I.D. 0.5 mm) filled with 1 g silica gel using CH_2Cl_2 and CH_2Cl_2–acetone mixtures (v/v, volume: 20 ml each) as mobile phase: CH_2Cl_2, CH_2Cl_2–acetone 9:1 (compounds **5**, **4**), 8:2 (compounds **1**, **2**, **3**), 7:3 (compounds **1**, **3**), 1:1. The respective fractions were concentrated to dryness in a rotary vacuum evaporator at 40°C maximum and redissolved in 200 μl CH_2Cl_2. Then, 50 μl thereof were analyzed by HPLC with diode array detection (DAD), running a gradient system with a rate of 0.6%/min.

2.2. HPLC

All semipreparative and analytical separations were performed on a Merck Hitachi liquid chromatograph consisting of a Rheodyne injection unit, a L-7100 pump, a L-7450 diode array detector (monitoring wavelength 220 nm) and a D-7000 interface. All computations were performed using the Merck D-7000 HSM data system. Separations were carried out on the stationary phases Hewlett-Packard LiChrospher 100 (Merck)-RP8 5 μm column (250×4.0 mm) and Hewlett-Packard Zorbax (DuPont) SB-C_8 3.5 μm column (75×4.6 mm), both guarded by a Hewlett-Packard LiChrospher 100-RP8 5 μm guard column (4×4 mm). The mobile phase consisted of varying methanol–water mixtures (v/v), flow-rate was 1 ml/min at room temperature. System 1: start at 20% methanol to 80% methanol in 90 min (linear gradient; rate=0.66%/min); system 2: start at 20% methanol to 80% methanol in 180 min (linear gradient; rate=0.33%/min); system 3: 55% methanol (isocratic system). The retention times of the compounds in the respective systems are summarized in Table 1.

2.3. TLC

Silica gel 60 Merck plates (0.25 mm) were used with CH_2Cl_2–acetone (9:1, v/v) or CH_2Cl_2–methanol (9:1, v/v) as mobile phases. Chromatograms

Table 1
Retention times t_R and retention factors k of **1**–**5** in different HPLC systems

Compound	HPLC LiChrospher 100-RP8 55% isocratic, t_0=3 min[b]		HPLC LiChrospher 100-RP8 0.66%/min, t_0=3 min[b]		HPLC Zorbax SB-C_8 55% isocratic, t_0=1 min[b]		HPLC Zorbax SB-C_8 0.66%/min, t_0=1 min[b]		HPLC Zorbax SB-C_8 0.33%/min, t_0=1 min[b]	
	t_R (min)	k[a]	t_R (min)	k[a]	t_R (min)	k[a]	t_R (min)	k[a]	t_R (min)	k[a]
1	6	1.0	37	11.3	2	1.0	26	25.0	37	36.0
2	14	3.7	54	17.0	4	3.0	39	38.0	62	61.0
3	11	2.7	55	17.3	3	2.0	33	32.0	50	49.0
4	5	0.7	26	7.7	2	1.0	19	18.0	24	23.0
5	57	18.0	75	24.0	24	23.0	67	66.0	118	117.0

[a] Calculated: $k = \frac{t_R - t_0}{t_0}$.

[b] For the determination of t_0 thiourea was used.

were developed at room temperature. Samples were redissolved in CH_2Cl_2 (amounts variable, depending on the amount of the respective fraction), as reference served a CH_2Cl_2 extract of chamomile (reference substance: matricine, R_F=0.40). Developed TLCs were examined under $UV_{255\ nm}$. Additionally modified acetic acid–phosphoric acid reagent [16] served as selective and very sensitive detection reagent for the proazulenes giving blue-green coloured spots after heating at 140°C. The treatment of the sprayed plate with steam causes a change of the colour into bright blue as well as a remarkably intensification of the stains so that proazulenes of even very low concentrations become visible.

2.4. Mass spectrometry

For mass spectrometry electron impact ionization (EI), chemical ionization (CI) (both Shimadzu QP-1000 EX MSPAC 200) and atmospheric pressure chemical ionization (APCI) (PE Sciex API 150EX) with direct inlet were employed as ionization modi. EI-mode: ion source: 250°C, 70 eV; vacuum: 4×10^{-6} Torr (1 Torr=133.322 Pa); scan: 40–500/2 s; heating rate of sample vial: 80°C/min; CI-mode: ion source: 180°C, 200 eV; reactant gas: CH_4–N_2O (77:23) 4.8 and isobutane 4.0, pre-pressure: 1 bar; vacuum: 5×10^{-5} Torr; scan: 40–500/2 s; heating rate of sample vial: 80°C/min; APCI-positive mode: injection volume: 10 μl of a 0.01% solution; mobile phase: methanol–water (10 mmolar ammonium acetate) 40:60; step size: 0.5 amu; dwell time: 7.0 ms; scan: 200–400/2.81 s; gases: nebulizer gas: 10, curtain gas: 12; controls: needle current: 2.20, temperature: 325.0, orifice: 20.0, rng: 129.5, Q0: −5.10, IQ1: −7.00, stubby focusing rod: −10.00, Q1 rod offset (Q1 ion energy): −6.0, CEM deflector plate: −400.0, channel electron multiplier: 2700.0.

3. Results and discussion

In proazulene-containing species, the derivatives of artabsin (tigloxy- (**5**), angeloxy- and acetoxy-artabsin) represent the main sesquiterpenes, their content in *Achillea collina* comes to 0.3% [17]. As esters of unsaturated C-5 carbonic acids, they are rather apolar with only one free hydroxyl group and elute from silica gel with CH_2Cl_2–acetone (9:1). Usually this mobile phase is used for TLC fingerprints of different *Achillea* samples. In contrast **1**–**4** are present in only very low concentrations as can be estimated semiquantitatively by the size of the spots and the intensity of colour with EP reagent. Additionally they are of higher polarity, especially compound **1**, which shows three free hydroxyl groups and requires the addition of methanol instead of acetone to the mobile phase; otherwise, no migration takes place. The R_F of 8-desacetyl-matricarin (**4**, R_F=0.25) in CH_2Cl_2–acetone (9:1) hints a higher polarity than that of **5** (R_F=0.50) as acetic acid lacks. Compounds **1**–**3** would be expected in the same polarity range as 8α-tigloxy-artabsin due to their esterization with angelic acid. Nevertheless, their polarity corresponds to that of non esterized 8-desacetylmatricarin (**4**) rather than of 8α-tigloxy-artabsin (**5**) considering the chromatographical behaviour on silical gel and RP8. Obviously the polarity of the substances is not only determined by whether or not an ester is present, but depends as well on the additional oxidation of positions 2 and 4.

For the HPLC separations with methanol–water as

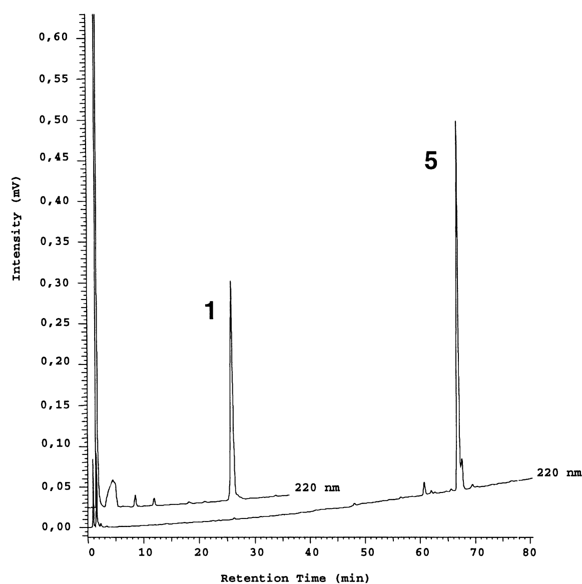
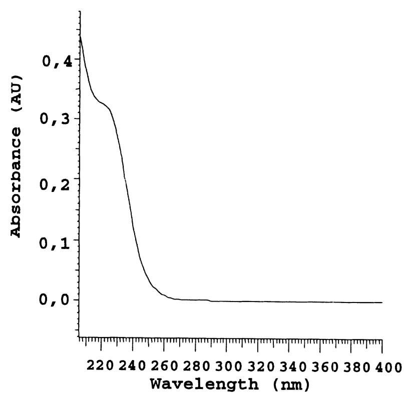
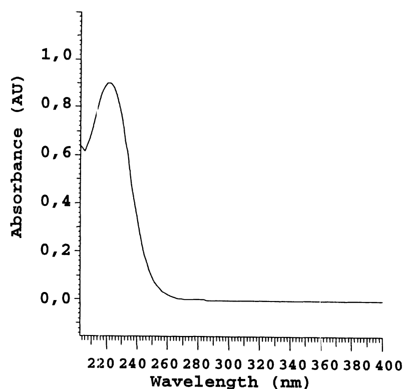


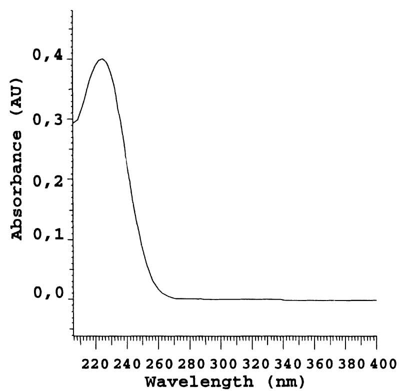
Fig. 3. HPLC chromatograms of the pure compounds **1** and **5**; stationary phase: Zorbax SB-C_8 3.5 μm; mobile phase: methanol–water gradient system, start at 20% (v/v) up to 80% in 90 min, rate: 0.66%/min; detection: 220 nm.



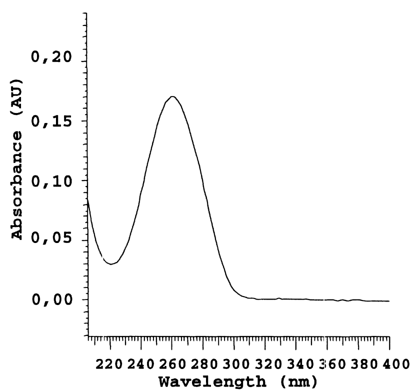
Compound **1**



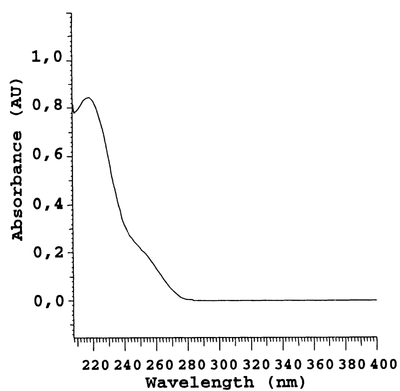
Compound **2**



Compound **3**



Compound **4**



Compound **5**

Fig. 4. UV-spectra of the sesquiterpenes **1**–**5** recorded on line in methanol–water by DAD during HPLC run (stationary phase: LiChrospher 100-RP8 5 μm; mobile phase: methanol–water gradient system, start at 20% (v/v) up to 80% in 90 min, rate: 0.66%/min).

mobile phase, three different systems were employed. The retention times of **1**–**5** were determined in two gradient systems (rate: 0.33 and 0.66%/min) and one isocratic system (55%) using two different stationary phases. Due to the small particle size (3.5 μm) and the reduced length of the column, the retention times on Zorbax SB-C_8 were shorter (Table 1) at comparable or even better separation efficiency (Fig. 3).

All compounds show fluorescence quenching spots at $UV_{255\ nm}$. Only **1** and **5** represent proazulenes showing a bright blue colour after spraying with modified acetic acid–phosphoric acid reagent [16], heating at 140°C and following steam treatment. **2** gives an olive-green colour, **3** an orange colour and **4** no colouring with this reagent. 8-desacetylmatricarin (**4**) is characterized by two double bonds (1,10; 3,4) in conjugation to a ketone function in position 2 of the molecule and therefore shows a λ_{max} at 260 nm. Due to its characteristic UV spectrum (Fig. 4) **4** can be identified reliably under $UV_{255\ nm}$ as strong fluorescence quenching spot on TLC as well as in HPLC at 255 nm wavelength. In contrast, **3** shows only one double bond conjugated with a ketone function in position 2 resulting in a λ_{max} of 226 nm. **1** and **2** showed their maximum at 222 nm, 8α-tigloxy-artabsin (**5**) was characterized by a λ_{max} of 218 nm together with a shoulder at 255 nm (Fig. 4).

Due to the low concentrations compared to the main proazulenes **1**–**4** were enriched after extraction by separation over a silica gel cartridge. For identification of **1**–**4** in the Middle European *Achillea* species the resulting fractions CH_2Cl_2–acetone (8:2) and (7:3) were analyzed by HPLC. The fractions of repeated runs corresponding to the respective compounds were collected and extracted with CH_2Cl_2, the organic layer was removed under reduced pressure, the residue was redissolved again in CH_2Cl_2 and used for TLC confirmation and off-line MS confirmation (molecular masses, R_F values on silica gel, colours with detection reagent see Table 2).

4. Conclusions

The combination of chromatographic and spectroscopic data of the compounds **1**–**5** allow their identification in extracts of *Achillea* flower heads. The high efficiency of TLC methods in combination with selective detection and HPLC in combination with spectroscopic measurements are shown for the example of very polar guaianolides. Consequently, an adaption of the previous described method [17] for quality control and quantification is possible now providing the basis for further research with high relevance for medicinal chemistry. Compounds of similar structure but less polarity show antiphlogistic activity in the croton oil ear test, which might also apply to **1**–**5**. The respective pharmacological studies are presently carried out. Due to the high polarity of **1**–**4**, a considerable rate of transfer into aqueous remedies which are mainly applied in folk medicin can be expected. Comparative quantitative analyses of dichloromethane, ethanolic and aqueous extracts are performed presently.

Table 2
Sum formulas, molecular masses, λ_{max}, characteristics under $UV_{255\ nm}$, R_F and reaction with EP reagent

Compound	Sum formula	Molecular mass	λ_{max} (nm)	$UV_{255\ nm}$ [a]	R_F		EP reagent [b]
					CH_2Cl_2–acetone (9:1, v/v)	CH_2Cl_2–methanol (9:1, v/v)	
1	$C_{20}H_{28}O_7$	380	222	+	0	0.54	Blue
2	$C_{20}H_{26}O_7$	378	222	+	0.19	0.79	Blue
3	$C_{20}H_{26}O_7$	378	226	+	0.23	0.81	Brown
4	$C_{15}H_{18}O_4$	262	260	+ +	0.25	0.68	No colour
5	$C_{20}H_{26}O_5$	346	218/255	+	0.50	0.90	Blue

[a] Number of + indicates the intensity of fluorescence quenching zones.
[b] Modified acetic acid–phosphoric acid reagent [16].

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