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Analysis of Sesquiterpene Lactones, Lignans, and Flavonoids in Wormwood (*Artemisia absinthium* L.) Using High-Performance Liquid Chromatography (HPLC)−Mass Spectrometry, Reversed Phase HPLC, and HPLC−Solid Phase Extraction−Nuclear Magnetic Resonance

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Today, the medicinal use of wormwood (*Artemisia absinthium*) is enjoying a resurgence of popularity. This study presents a specific and validated high-performance liquid chromatography (HPLC)−diode array detection method for the simultaneous determination and quantification of bioactive compounds in wormwood and commercial preparations thereof. Five sesquiterpene lactones, two lignans, and a polymethoxylated flavonoid were baseline separated on RP-18 material, using a solvent gradient consisting of 0.085% (v/v) *o*-phosphoric acid and acetonitrile. The flow rate was 1.0 mL/min, and chromatograms were recorded at 205 nm. The stability of absinthin was tested exposing samples to light, moisture, and different temperatures. Methanolic and aqueous solutions of absinthin were found to be stable for up to 6 months. This was also the case when the solid compound was kept in the refrigerator at −35 °C. In contrast, the colorless needles, when stored at room temperature, turned yellow. Three degradation compounds (anabsin, anabsinthin, and the new dimer 3′-hydroxyanabsinthin) were identified by HPLC−mass spectrometry and HPLC−solid-phase extraction−nuclear magnetic resonance and quantified by the established HPLC method.

KEYWORDS: *Artemisia absinthium*; wormwood; sesquiterpene lactones; RP-HPLC; HPLC-SPE-NMR; HPLC-MS

INTRODUCTION

Artemisia absinthium L., commonly known as wormwood, is a yellow-flowering, perennial plant distributed throughout various parts of Europe and Siberia and is used for antiparasitic effects and to treat anorexia and indigestion (*1*−*3*). Aerial parts are present in many gastric herbal preparations, in dietary supplements, and in alcoholic beverages, for example, absinthe products, which are enjoying a resurgence of popularity all over the world. The characteristic bitterness of wormwood is caused by sesquiterpene lactones, for example, absinthin (**3**), the main bitter constituent, anabsin (**1**), ketopelenolide b (**2**), and anabsinthin (**6**) (**Figure 1**) (*4*). Besides sesquiterpenes, other constituents show biological activities as well. The lignans epiyangambin (**5**) and sesartemin (**7**) reduce spontaneous locomotor activity and isolation-induced aggression in mice (*5*). Compound **5** possesses antiplatelet aggregation activity in vitro and in vivo (*6*, *7*). The flavonoid artemisetin (**8**) showed marked anti-inflammatory, antitumoral, and antiproliferative activities (*8*−*10*).

Analysis of sesquiterpenes in wormwood using high-performance liquid chromatography (HPLC) (*11*, *12*), high-performance thin-layer chromatography (HPTLC) (*13*), TLC (*14*−*18*), and photometry (*14*, *15*) has been described previously, but as far as we know, there are no reports of analytical methods allowing quantitative determination of the above-mentioned sesquiterpene lactones, lignans, and flavonoids simultaneously. Published HPLC methods focused on the determination of only two sesquiterpene lactones (**1** and **6**) and were not validated at all. These missing and required facts encouraged us to reevaluate and improve the currently available methodology for *A. absinthium*. The new fully validated method can be used for the chemical characterization of wormwood plant material and for quality control of several commercially available herbal preparations.

Another issue addressed in this paper is the stability of analytical markers, which is crucial for quality control. Absinthin (**1**) has been proposed as a marker compound for the assessment of authenticity of absinthe products (*13*). We observed that there is contradictionary knowledge of the stability of absinthin. Older literature describes absinthin as a glucoside, being decomposed by hydrolysis into sugar, a liquid and a resinous compound (*19*). In contrast, Schneider and collaborators (*15*) found the substance to be stable, and the content of absinthin in the stored drug, determined by photometric assay, remained nearly unchanged. To determine the stability of the bitter substance, the degradation behavior of absinthin was investigated under different stress conditions.

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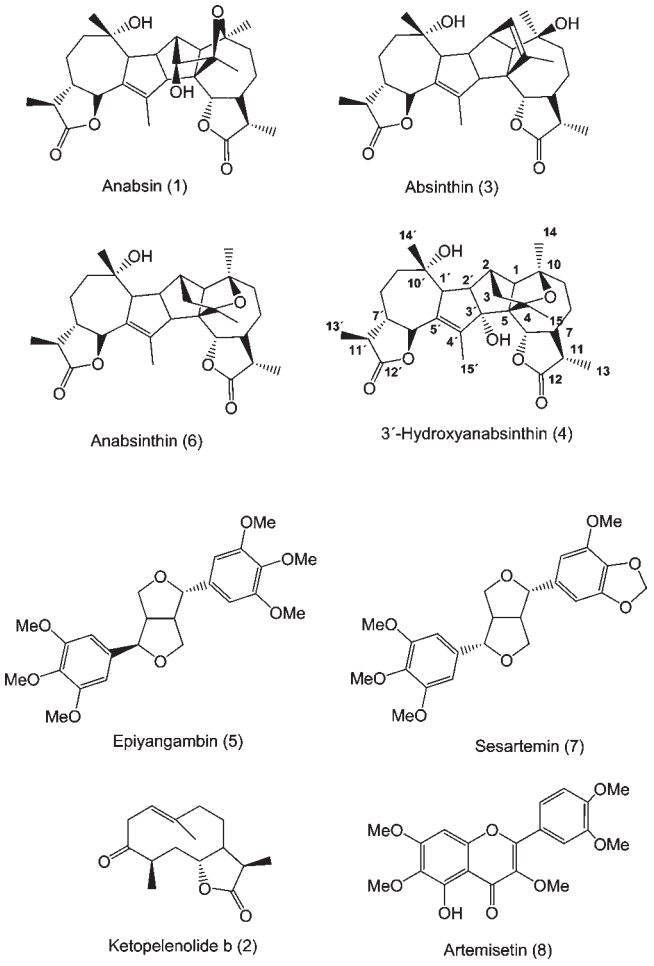

Figure 1. Chemical structures of compounds **1**−**8**.

MATERIALS AND METHODS

General Procedures. Column chromatography (CC) was performed using silica gel 60 (40−63 μm, 230−400 mesh; Merck, Darmstadt, Germany) and Sephadex LH-20 (Sigma-Aldrich, Vienna, Austria). Semipreparative reversed phase HPLC was performed on a Dionex P 580 pump. The column used was a 250 mm × 10.0 mm i.d., 10 μm, Synergi MAX-RP column (Phenomenex, Torrance, CA). TLC was performed with silica gel 60 F_{254} plates (0.25 mm; Merck). Optical rotation was measured with a Perkin-Elmer 341 polarimeter. The melting point was determined on a Kofler melting point apparatus microscope (Reichert, Austria). The Fourier transform infrared (FTIR) spectrum was recorded on a Bruker IFS 25 FTIR spectrometer in transmission mode within the range of 4000 to 600 cm^{-1}. The sample was applied to a ZnSe disk of 2 mm thickness. NMR spectra were recorded on a Bruker Avance 300 spectrometer at 300 MHz and a Bruker Avance AV 600 spectrometer at 600 MHz. High-resolution mass spectra were acquired on a micrOTOF-Q II mass spectrometer (Bruker-Daltonics, Bremen, Germany). Ionization was performed in positive electrospray ionization (ESI) mode. The nebulizer, dry gas, and probe temperature of the mass spectrometer were set to 10 psi (nitrogen), 4 L/min (nitrogen), and 180 °C, respectively; the mass scan range was set in the range of *m*/*z* 100−1000.

Materials. *A. absinthium* plant material for the isolation of compounds **2**, **3**, **5**, **7**, and **8** was purchased from Caesar & Loretz (Hilden, Germany). Samples AA-1 to AA-11 (*A. absinthium* herb) were obtained from different vendors in Germany and Austria. Samples AA-1, AA-4, and AA-6 to AA-8 were a gift of Bionorica AG (Neumarkt, Germany). Samples AA-2 and AA-3 were supplied by Alfred Galke (Gittelde, Germany), sample AA-5 was supplied by Kräuter Mix (Abtswind, Germany), and sample AA-9 was supplied by Abtswinder Naturheilmittel (Abtswind, Germany). Sample AA-10 was obtained from Leopold Bichler (Innsbruck, Austria), and sample AA-11 was obtained from Köhle Heilmittel (Velden, Austria).

Samples AA-12 to AA-18 were commercially available products provided by different companies. Further information about the samples can be obtained from the authors. Samples AA-12 and AA-18 were a capsule form of wormwood. Samples AA-13 to AA-17 were liquid preparations containing 20−70% of ethanol, prepared by maceration or percolation using methods given in the homeopathic and Ph. Eur. monographs. Reference specimens of all samples are deposited at the Institute of Pharmacy, University of Innsbruck, in Innsbruck, Austria.

Chemicals. Acetonitrile (gradient grade), formic acid, and *o*-phosphoric acid (all p.a. grade) were obtained from Merck. Methanol, ethyl acetate, dichloromethane, and acetone (all p.a. grade) were purchased from VWR (Vienna, Austria). Ethanol was obtained from Kögl Pharm (Innsbruck, Austria). Nitrogen (99.995%) for mass spectrometry experiments was produced by a nitrogen generator (Peak Scientific Instruments Ltd., Fountain Crescent, United Kingdom). Deuterated acetonitrile (99.8%) was purchased from Cambridge Isotope Laboratories (Andover, MA). All solvents for preparative separations were distilled prior to use, and water was produced by reverse osmosis followed by distillation.

Isolation Procedures. The dried and powdered plant material (ca. 420 g) was macerated with 3.5 L of dichloromethane for 5 days. The resulting solution was dried in vacuo to yield 20.4 g of crude extract. The extract was chromatographed on silica gel employing a gradient of dichloromethane and methanol (from 98:2 to 40:60, v/v), giving four fractions (A 1−4). Fraction A 3 (4.1 g) was subjected to Sephadex LH-20 CC using CH_2Cl_2 and acetone (85:15, v/v) as the mobile phase resulting in five fractions (B 1−5). Fraction B 2 (1.2 g) was further subjected to Sephadex LH-20 CC using acetone, yielding five fractions (C 1−5). Compound **8** (24.5 mg) was obtained by crystallization from acetone as yellow needles. Fraction C 3 (100.0 mg) was dissolved in MeOH and separated by semipreparative HPLC in 15 min using a simple isocratic run with a mixture of water and acetonitrile (40:60, v/v). The flow rate was adjusted to 3.5 mL/min, and the detection wavelength set to 205 nm. All separations were performed at 40 °C. This procedure allowed the isolation of substance **2** [22.1 mg; retention time (RT), 7.4 min], **3** (25.0 mg; RT, 7.9 min), **5** (13.7 mg; RT, 9.2 min), and **7** (11.6 mg; RT, 10.3 min). The isolation process was monitored by TLC, HPLC−diode array detection (DAD), and HPLC-DAD-MS. TLC was performed on silica gel plates, with CH_2Cl_2 and MeOH (98:2, v/v) or CH_2Cl_2 and acetone (9:1, v/v) as the mobile phase and vanillin/sulfuric acid as the detection reagent.

Compounds **1**, **4**, and **6** were isolated from a mixture of absinthin and degradation products and identified by HPLC−solid-phase extraction (SPE)−NMR experiments. In a second step, semipreparative HPLC was used to obtain higher amounts of the degradation products. Forty milligrams of the mixture was dissolved in MeOH and separated by semipreparative HPLC as described above, yielding 2 mg of **1** (RT, 6.2 min), 1 mg of **4** (RT, 8.1 min), and 15 mg of **6** (RT, 10.3 min).

Stability Study. Stability tests were carried out with a methanolic and an aqueous ethanol extract of wormwood and the powdered plant material, respectively. The methanolic extract was prepared by mixing 1.0 g of ground wormwood with 2.0 g of diatomaceous earth and extracting it using accelerated solvent extraction (ASE 110; Dionex, Idstein, Germany) with 10 mL cells. The extraction was performed under the following conditions: pressure, 1500 psi; room temperature; 10 min static extraction with MeOH; rinsed with the same solvent; and purged using pressurized nitrogen gas at 1500 psi. The cycle was repeated three times. The combined extracts were evaporated to dryness and redissolved in 20.0 mL of MeOH.

The aqueous ethanol sample solution (20% v/v ethanol) was prepared by maceration at room temperature for 3 days. The ground wormwood (1.0 g) was extracted with 30.0 mL of 60% (v/v) ethanol, adding water gradually to obtain 100.0 mL of an aqueous solution with a final alcohol content of 20% (v/v). The powdered plant material (5.0 g) was stored in brown glass, and 1.0 g was extracted after 1, 3, and 6 months with MeOH using ASE (ASE 110; Dionex) as described above.

All of the samples were stored in climatic chambers under the following conditions: Long-term stability studies were conducted by storage at 25 °C and 60% relative humidity (RH). Accelerated stability testing was conducted by storage at 40 °C and 75% RH. Furthermore, samples were stored in a cool, dark place under refrigerated conditions (refrigerator at +4 °C). Samples were withdrawn at day 0 and months 1, 3 and 6 and analyzed for absinthin contents by HPLC.

To investigate the stability of the pure substance, 20.0 mg of absinthin was stored in an exsiccator at room temperature and exposed to light, and 5.0 mg was kept in a cool, dark place under frozen conditions (freezer at −35 °C). After 1, 3, and 6 months, the absinthin contents were determined by HPLC.

HPLC-SPE-NMR. Chromatography was performed using an Agilent 1200 Liquid Chromatograph (Agilent, Waldbronn, Germany) equipped with quaternary pump, autosampler, column oven, and diode array detector. A mixture of absinthin and its degradation compounds (in total 20.0 mg) was dissolved in 1.5 mL of acetonitrile. Separations were performed on a 150 mm × 4.6 mm i.d., 5 μm, Zorbax Eclipse XDB-C18 column (Agilent), with a 4 mm × 4 mm i.d., 5 μm, LiChrospher 100 RP-18 guard column (Merck). The flow rate was adjusted to 0.8 mL/min, 10 × 20 μL of sample was injected, and the temperature was 30 °C. Gradient elution was performed using water (A) and acetonitrile (B) with the following gradient combination: 0 min, 98A/2B; 10 min, 85A/15B; 40 min, 30A/70B; 45 min, 5A/95B; and 50 min, 5A/95B. The Bruker/Spark Prospekt 2 SPE unit (Bruker BioSpin, Rheinstetten, Germany, and Spark, Emmen, Holland) was used to automatically trap the chromatographic peaks on HySphere C18 cartridges (10 mm × 2 mm; Spark). Every peak was trapped three times. After chromatographic separation, water was added to the eluent of the column with a Knauer K120 HPLC pump (Knauer, Berlin, Germany) at a flow rate of 1.3 mL/min (makeup pump), to provide proper retention of the peaks under study on the sorbing material of the cartridges. The trapped peaks were dried with nitrogen gas to remove residual solvents and eluted with 153 μL of deuterated acetonitrile to a Bruker Avance AV 600 MHz spectrometer (Bruker Biospin).

Furthermore, in the second step, the absinthin peak (injection volume, 20 μL) was trapped three times on six different cartridges. Trapped peaks were dried with nitrogen gas to remove residual solvents and stored under acid-free conditions. To investigate the degradation behavior of absinthin, NMR measurements were done over a period of 6 months. Every month, one analyte peak was eluted with deuterated acetonitrile and transferred to the spectrometer via the peak sampling unit.

Spectroscopic Data. *Anabsin* (***1***). ^{1}H NMR (acetonitrile-d_3): δ 2.56 (1H, s, H-1), 2.19 (1H, s, H-2), 3.66 (1H, d, J = 7.9 Hz, H-3), 4.27 (1H, d, J = 10.5 Hz, H-6), 1.96 (1H, H-7), 1.67 (2H, H-8), 1.66 (2H, H-9), 2.32 (1H, H-11), 1.16 (3H, d, J = 7.2 Hz, H-13), 1.44 (3H, s, H-14), 1.19 (3H, s, H-15), 2.71 (1H, s, H-1′), 2.75 (1H, m, H-2′), 3.43 (1H, d, J = 9.4 Hz, H-3′), 4.73 (1H, d, J = 10.9 Hz, H-6′), 2.04 (1H, H-7′), 1.66 (2H, H-8′), 1.74 (2H, H-9′), 2.32 (1H, H-11′), 1.18 (3H, d, J = 6.4 Hz, H-13′), 1.25 (3H, s, H-14′), 1.90 (3H, s, H-15′). ^{13}C NMR (acetonitrile-d_3): δ 64.1 (C-1), 50.1 (C-2), 73.3 (C-3), 90.5 (C-4), 63.1 (C-5), 83.3 (C-6), 49.3 (C-7), 25.5 (C-8), 39.0 (C-9), 78.8 (C-10), 43.2 (C-11), 178.2 (C-12), 12.6 (C-13), 27.4 (C-14), 15.0 (C-15), 57.2 (C-1′), 43.8 (C-2′), 54.0 (C-3′), 134.8 (C-4′), 145.2 (C-5′), 81.9 (C-6′), 49.8 (C-7′), 22.9 (C-8′), 42.7 (C-9′), 74.4 (C-10′), 43.2 (C-11′), 178.9 (C-12′), 12.6 (C-13′), 29.8 (C-14′), 17.0 (C-15′).

3′-Hydroxyanabsinthin (***4***). Colorless amorphous solid; mp 257−258 °C ($CHCl_3$); $[\alpha]_D^{25}$ +92.0 ($CHCl_3$, c 1.7). UV (MeOH) λ_{max}: 205 nm (HPLC online). FTIR ν_{max}: 3413 (br), 2962, 2922, 2852, 2351, 2290, 1772, 1590, 1455, 1381, 1321, 1238, 1175, 1145, 1098, 1038, 1011, 970, 926, 880, 832, 627 cm^{-1}. MS (ESI) m/z (rel. int. %) 513 $[M + H]^+$ (12), 495 $[M - H_2O + H]^+$ (100), 477 $[M - 2H_2O + H]^+$ (92). HR-MS (ESI) m/z 535.2649 $[M + Na]^+$ (calcd for $C_{30}H_{40}O_7$ + Na, 535.6226).

Sample Preparation for Quantification Purposes. The finely powdered plant material (1.0 g) was mixed with 2.0 g of diatomaceous earth, placed in 10 mL stainless steel cells, and extracted with methanol using ASE (ASE 110; Dionex). The cell was filled with MeOH and pressurized (1500 psi). Three static cycles of 10 min and ambient temperature were used. After each extraction step, the pressure was released, and the extract was collected in a 60 mL glass vial. The cell was rinsed with fresh solvent and purged using pressurized nitrogen gas at 1500 psi. The extracts were combined; each sample solution was assayed in triplicate. Liquid samples of wormwood preparations were injected directly without any sample preparation.

HPLC Conditions for Stability Studies. Stability testing analyses were performed on an Agilent 1200 series HPLC instrument, equipped with binary pump, autosampler, column oven, and photodiode array detector (Agilent). The stationary phase was a 150 mm × 4.6 mm i.d., 5 μm, Zorbax Eclipse XDB-C18 column (Agilent), with a 4 mm × 4 mm i.d., 5 μm, LiChrospher 100 RP-18 guard column (Merck). The mobile phase was comprised of 0.085% (v/v) *o*-phosphoric acid in water (A) and acetonitrile (B). Elution was performed using the following gradient: 0 min, 99A/1B; 40 min, 5A/95B; 45 min, 5A/95B; 45.5 min, 99A/1B; and 55 min, 99A/1B (total runtime, 45 min; equilibration period, 10 min). The flow rate, temperature, and injected sample volume were adjusted to 1.0 mL/min, 30 °C, and 10 μL, respectively. Detection was performed at 205 nm.

Quantitative HPLC and HPLC-MS Conditions. Quantification studies were performed on a LaChrom Elite HPLC system (Merck-Hitachi, Tokyo, Japan), equipped with a L-2200 autosampler, L-2100 quaternary pump, L-2300 column oven, and L-2400 UV-detector. An optimum separation of the compounds **1**−**8** was achieved on a 150 mm × 4.6 mm i.d., 5 μm, Fortis C18 column (HPLC Service, Breitenfurt, Austria), with a 4 mm × 4 mm i.d., 5 μm, LiChrospher 100 RP-18 guard column (Merck). The mobile phase was comprised of 0.085% (v/v) *o*-phosphoric acid in water (A) and acetonitrile (B). Separations were performed by gradient elution: 0 min, 70A/30B; 10 min, 50A/50B; 22 min, 30A/70B; 27 min, 2A/98B; 35 min, 2A/98B; 35.5 min, 70A/30B; and 47 min, 70A/30B (total runtime, 35 min; equilibration period, 12 min). The flow rate, temperature, and injected sample volume were adjusted to 1.0 mL/min, 30 °C, and 10 μL, respectively. Detection was performed at 205 nm.

HPLC-MS experiments were performed on an Esquire 3000 iontrap mass spectrometer (Bruker-Daltonics), which was coupled to the HPLC apparatus (split ratio 1:3). Eluent A was changed and was comprised of a mixture of water and formic acid in the ratio of 99.9:0.1 (v/v); otherwise, the same separation conditions as described above for quantitative analysis were used. For optimum MS results, ionization was performed in positive ESI mode. The nebulizer, dry gas, and probe temperature of the mass spectrometer were set to 30 psi (nitrogen), 10 L/min (nitrogen), and 350 °C, respectively; the mass scan range was set in the range of m/z 100−1000.

Calibration. A standard stock solution was prepared by dissolving seven standard compounds in 5.00 mL of methanol (1.00 mg of **1** and **6** and 2.00 mg of **2**, **3**, **5**, **7**, and **8**). Six additional calibration levels were prepared by diluting this solution 1:2 with methanol. The standard solutions were stable for at least 1 month if stored at +4 °C (confirmed by reassaying). Compound **4** was quantified based on the calibration data of the structurally similar **6**.

Validation. The HPLC method was validated for linearity, limit of detection and quantification, peak purity, accuracy, precision, repeatability, and ruggedness. The limit of detection (S/N ratio of 3) and limit of quantification (S/N ratio of 10) were determined by serial dilution of standard solutions containing the relevant compounds. Peak purity and identity were confirmed by UV, HPLC-MS, and NMR spectroscopy. Accuracy was determined by spiking sample AA-1 with three concentrations of standard compounds (low, medium, and high spike). For this purpose, known amounts of **1**−**8** were added to the dry, powdered plant material, which was then extracted and assayed as described before. The actually found amounts in relation to the theoretically present ones were expressed as percent of recovery.

Precision (intra- and interday) of the method was verified by analyzing sample AA-1 5-fold on three consecutive days. By comparing variations within the same days, intraday precision was determined by observing differences; within the 3 days, interday precision was deduced.

Repeatability was confirmed by evaluating consistency of RTs and standard deviations (maximum relative standard deviation of 4.9%). Finally, ruggedness of the developed method can be concluded by the fact that the same results were obtained on two different HPLC instruments (LaChrom Elite and HP 1200).

RESULTS AND DISCUSSION

Isolation and Structure Elucidation of the Compounds. Chromatographic separation of the dichloromethane extract of wormwood resulted in the isolation of compounds **2**, **3**, **5**, **7**, and **8**. Identity and purity of the isolated compounds were confirmed by chromatographic (TLC and HPLC) and spectroscopic (1D and 2D NMR and HPLC-MS) methods in reference to literature values (*12*, *20*−*23*). The purity of all isolated compounds was ≥95% (determined by HPLC).

Compounds **1**, **4**, and **6** were isolated from a mixture of absinthin and degradation products and identified by HPLC-SPE-NMR. NMR assignments of **6** are in accordance with published data (*12*). Compound **1** was identified as anabsin. Its structure was already established for the first time in 1979 (*24*), but NMR data for this diguaianolide reported in literature were found to be fragmentary. Thus, in this paper, the complete assignment of proton and carbon NMR signals is shown.

Table 1. NMR Signal Assignment of 3′-Hydroxyanabsinthin (**4**) and Anabsinthin (**6**)[a]

	3′-hydroxyanabsinthin (**4**)		anabsinthin (**6**)	
position	^{13}C NMR[b]	^{1}H NMR	^{13}C NMR[b]	^{1}H NMR
1	62.7	2.80, 1H, s	63.8	2.48, 1H, s
2	40.6	2.40, 1H, m	42.5	2.23, 1H, s
3	34.6	1.66, 1.27, 2H	35.9	1.55, 1.67, 2H
4	87.9		88.0	
5	65.7		62.3	
6	82.7	4.31, 1H, d, J = 10.2 Hz	83.5	4.23, 1H, d, J = 10.5 Hz
7	48.0	2.03, 1H	49.5	1.95, 1H
8	29.5	1.43, 1.90, 2H	25.0	1.64, 1.84, 2H
9	39.5	1.59, 1.78, 2H	35.9	1.56, 1.76, 2H
10	78.1		77.7	
11	42.4	2.20, 1H	43.2	2.29, 1H
12	178.3		178.9	
13	11.8	1.19, 3H, d, J = 7.2 Hz	12.7	1.16, 3H, d, J = 6.8 Hz
14	27.5	1.27, 3H, s	27.6	1.25, 3H, s
15	16.9	1.15, 3H, s	17.4	1.15, 3H, s
1′	54.9	2.49, 1H, m	57.5	2.57, 1H, s
2′	51.1	2.85, 1H, m	44.6	2.75, 1H, d, J = 10.5 Hz
3′	104.5		53.7	3.41, 1H, d, J = 10.2 Hz
4′	140.7		145.9	
5′	116.6		133.7	
6′	82.2	4.79, 1H, d, J = 11.3 Hz	82.2	4.74, 1H, d, J = 11.3 Hz
7′	49.4	2.04, 1H	50.1	1.98, 1H
8′	25.0	1.63, 1.81, 2H	24.0	1.64, 1.84, 2H
9′	44.1	1.57, 1.86, 2H	42.1	1.63, 1.78, 2H
10′	74.0		73.8	
11′	42.5	2.34, 1H	43.5	2.31, 1H
12′	178.3		178.9	
13′	13.8	1.19, 3H, d, J = 6.8 Hz	12.9	1.18, 3H, d, J = 6.8 Hz
14′	30.8	1.31, 3H, s	30.0	1.22, 3H, s
15′	15.1	1.98, 3H, s	17.1	1.89, 3H, s

[a] Measured in CD_3CN at 600 MHz; spectra are referenced to solvent residual and solvent peaks of acetonitrile-d_3 at δ_H = 1.94 ppm and δ_C = 1.24 ppm, respectively. [b] Values deduced from HMBC and HSQC data.

Compound **4**, a new dimer, has not been described in the literature before and is named 3′-hydroxyanabsinthin. The molecular formula of **4** was established as $C_{30}H_{40}O_7$ (MW = 512) by HRESIMS. Further ESI-MS data indicated the quasi-molecular ion peak at m/z 513 $[M + H]^+$ and fragment ions at m/z 495 $[M - H_2O + H]^+$ and 477 $[M - 2H_2O + H]^+$. NMR spectra revealed the presence of six methyl, five methylene, ten methine, nine quaternary carbons, and two hydroxyl groups. A combination of ^{1}H, heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), and correlation spectroscopy (COSY) experiments suggested that **4** was a sesquiterpene lactone with a diguaiane skeleton. In the FTIR spectrum, a broad valence vibration between 3600 and 3200 cm^{-1} indicated the presence of hydroxyl groups in the molecule. A strong absorption band at 1772 cm^{-1} due to the C=O stretching of the γ-lactone rings was detectable. ^{1}H and the ^{13}C NMR data for **4** were very similar to those of anabsinthin (**Table 1**), but in contrast to anabsinthin, HSQC and HMBC spectra did not show the proton signal at C-3′. Instead of the C-3′ signal at δ_C 53.7 ppm, the HMBC spectrum showed a resonance at δ_C 104.5 ppm, assignable to a hydroxyl-bearing quaternary carbon. The downfield shift of this signal can be explained with the formation of a strong intramolecular hydrogen bond between the hydroxyl group and the oxygen at C-6 and the high ring strain. As this oxygen atom is α-oriented in all absinthin derivatives, the respective hydroxyl group at C-3′ has to be α-orientated as well. This is also corroborated by the other remaining NMR signals, which were comparable to those of anabsinthin, except for the resonances of H-1, H-1′, and H-2′ (Figure 1 of the Supporting Information). A β-substitution of the hydroxyl group in position C-3′ would result in a conformation change of the whole molecule and for sure lead to significant shift changes in comparison to anabsinthin.

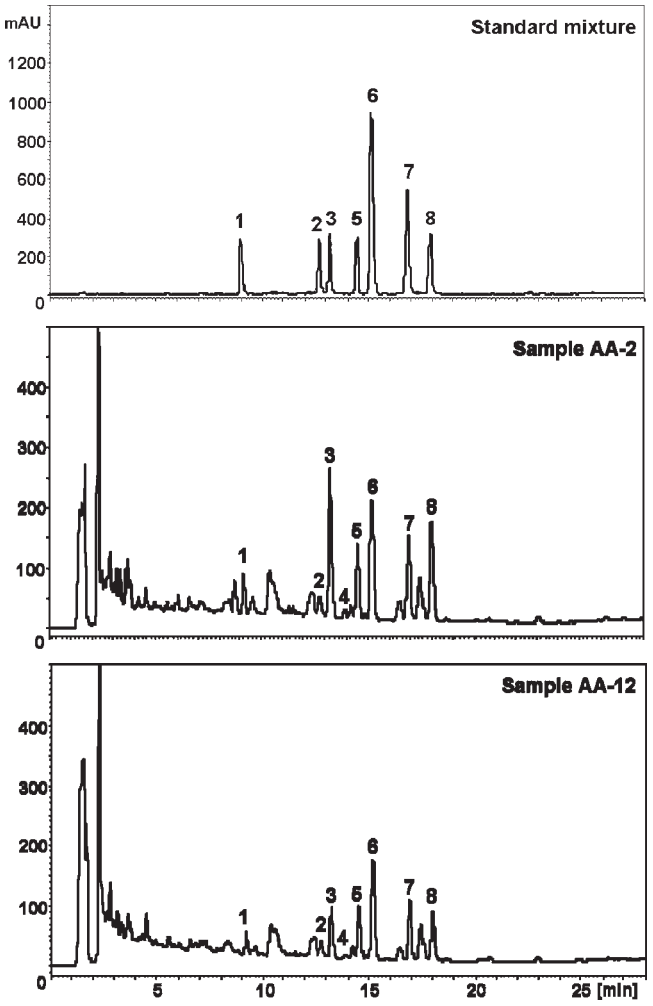

Figure 2. Separation of a standard mixture of compounds **1**−**8** and HPLC analysis of samples AA-2 and AA-12 extracted with methanol obtained under optimized HPLC conditions. Peak assignments: 1, anabsin; 2, ketopelenolide b; 3, absinthin; 4, 3′-hydroxyanabsinthin; 5, epiyangambin; 6, anabsinthin; 7, sesartemin; and 8, artemisetin.

Method Development and Validation. All analytical methods reported on wormwood are limited to single compounds, for example, absinthin (**1**) and anabsinthin (**6**), or a group of constituents (sesquiterpene lactones). They are not suitable for the qualitative and quantitative determination of various pharmacologically relevant groups of compounds. Because of the structural diversity of the analytes in wormwood (sesquiterpenes, lignans, and flavonoids), the HPLC method had to be optimized to achieve a satisfactory separation of the compounds.

For a good separation, the mobile phase had to be acidic (0.085% v/v *o*-phosphoric acid added to solvent A). No separation of **2** and **3** was observed when methanol was used as the organic solvent; good separation was achieved with acetonitrile. Concerning the stationary phase, out of a number of different columns tested (C-8, C-12, C-18, phenyl-hexyl, and cyano column), best results were obtained with a Fortis C18. Different particle sizes (3−5 μm), pore sizes (80−300 Å), and additives to the mobile phase (e.g., buffers or THF) did not improve the results either. Setting the wavelength to 205 nm and applying a column temperature of 30 °C resulted in baseline separation of **1**−**8** with an analysis time of less than 30 min (**Figure 2**). For HPLC-MS experiments, only the mobile phase

Table 2. Stability of Absinthin in Solution and in the Crude Plant Material[a]

storage conditions	1 month	3 months	6 months
		methanolic extract[b]	
long-term	100.5 (0.6)	98.4 (0.1)	99.6 (0.1)
accelerated	99.6 (1.7)	100.6 (0.1)	99.4 (1.0)
refrigerated	101.4 (1.7)	101.3 (0.03)	99.9 (0.01)
		20% v/v ethanol extract[c]	
long-term	98.0 (0.1)	98.3 (1.5)	99.1 (0.6)
accelerated	100.7 (0.3)	98.9 (0.7)	99.3 (1.0)
refrigerated	98.9 (1.1)	100.3 (1.0)	100.1 (0.7)
		powdered herb[d]	
long-term	99.8 (1.4)	80.9 (1.9)	79.4 (0.1)
accelerated	100.2 (0.2)	82.9 (0.1)	74.0 (0.6)
refrigerated	100.1 (0.3)	92.4 (0.04)	92.0 (0.7)

[a] Results are based on recovery rates in percent, and relative standard deviations are given in parentheses ($n = 2$). [b] Initial concentration (day 0): 45.8 μg/mL. [c] Initial concentration (day 0): 7.7 μg/mL. [d] Initial concentration (day 0): 48.0 μg/mL.

(eluent A) had to be changed slightly to ionize and detect all compounds of interest in one analytical run.

Validation of the established method was performed according to ICH guidelines (*25*). Data presented in Table 1 of the Supporting Information indicate linearity within the tested range (0.6−430 μg/mL), with a correlation coefficient of 0.997 and higher. The determined limits of detection (S/N ratio of 3, based on a 10 μL injection) and limits of quantitation (S/N ratio of 10) were found to be below 67 and 200 ng/mL and demonstrated the methods sensitivity. Online acquisition of UV and MS spectra of single peaks showed that the method is specific and selective, and there is no interference or overlap of coeluting impurities.

Accuracy was determined by means of recovery experiments spiking plant material with three different concentrations of standard compounds and ranged from 96.4 to 102.4% for all compounds (Table 2 of the Supporting Information). Relative standard deviations below 4.9% and very stable RTs over the whole study period (approximately 300 injections) indicated the methods repeatability. Finally, over a period of 3 days, precision of the assay was determined. Intraday and interday precision are observed as indicated by the relative standard deviation values ranging from 0.4 to 4.9% and from 0.5 to 3.9%, respectively (Table 3 of the Supporting Information).

Stability Studies. The stability of analytes in samples is a prerequisite for the reliable quantification. Therefore, full validation of a method must include stability studies to confirm stability of the analytes during the whole analytical procedure including storage prior to analysis and sample preparation.

According to the ICH guidelines, plant material was stored under different stress conditions for 6 months. The results of long-term, accelerated, and refrigerated stability studies conducted on wormwood are shown in **Table 2**. Extracts of wormwood were found to be stable for up to 6 months. The pure methanolic extracts by ASE and the 20% v/v ethanol solutions prepared by maceration showed more than 98% absinthin content. Both extracts stored at 40 °C were opalescent at the end of 6 months, while the extracts kept under refrigerated conditions showed some precipitation with no significant loss of absinthin. Extracts stored under room temperature exhibited no visible opalescent effect or precipitates.

In contrast, powdered plant material revealed a considerable decrease of absinthin when stored for 6 months under accelerated, room temperature, and refrigerated conditions. Recovery rates for absinthin in the plant material ranged from 74.0 to 92.0%. When kept at 40 °C, a 20% reduction in absinthin content was observed. Almost the same loss of absinthin was observed, when storing the plant material at room temperature. Low temperature conditions did not stop decomposition of absinthin, but a slow-down of the degradation process took place (recovery rate, 92.0%).

Absinthin, isolated as an amorphous solid, was stable during frozen storage conditions at −35 °C. In contrast, the colorless compound, when stored in an exsiccator at room temperature and exposed to light, turned yellow. HPLC analysis of the yellowish powder stored for 3 months at 25 °C showed three additional peaks of unidentified degradation products with UV spectra similar to that observed for absinthin. These compounds were isolated by HPLC-SPE-NMR and identified as dimeric sesquiterpene lactones. The major degradation product was anabsinthin (**6**), followed by anabsin (*1*) and the new dimer 3′-hydroxyanabsinthin (**4**). It is known that in weak acidic medium the diguaianolide absinthin is transformed to the isomeric anabsinthin; the hydroxyl group at C-10 binds to the carbon−carbon double bond in position 3,4 resulting in an intramolecular rearrangement and formation of the tetrahydrofuran ring (**1**). Degradation of absinthin was also observed by 1D ^{1}H NMR experiments. The compound was trapped on C18 cartridges and stored under nitrogen atmosphere, and changes were studied over a period of 6 months. Inspection of the ^{1}H NMR spectrum of the eluted compounds revealed the presence of additional signals due to the presence of anabsinthin, for example, a doublet assignable to H-6 (δ_H = 4.23 ppm, J = 10.5 Hz).

Analysis of Samples. Prior to sample analysis, optimum extraction conditions were determined. Different solvents (methanol, water, and mixtures thereof) and extraction procedures (sonication, shaking, ASE, or refluxing) were evaluated. ASE with methanol was shown to be most efficient and rapid (three cycles of extraction for 10 min each). Following this procedure assures an exhaustive extraction.

Typical sample chromatograms are shown in **Figure 2**. All of the quantified compounds were separated well and could be assigned by comparing their RTs and UV spectra with those of the previously isolated compounds. Compound **4** was identified by comparison of its UV spectra with that of the structurally closely related **6** and by HPLC-MS studies. The MS signals of each compound were assignable as $[M + H]^+$ ions or the $[M - H_2O + H]^+$ ion fragment, and the deduced molecular masses are in good agreement to reported literature values (Figure 2 of the Supporting Information).

Eleven samples of wormwood and seven commercially available herbal preparations were analyzed by the developed HPLC method. Samples AA-1 to AA-3 were different batches obtained from one source; this was also the case with samples AA-4 and AA-5. Nine samples of wormwood plant material came from different vendors. Samples AA-12 and AA-18 were solid preparations in the form of capsules; five of the commercial products (AA-13 to AA-17) were liquid preparations. All of the marketed formulations were labeled to contain *A. absinthium* herb. Whether a product contained wormwood or not could be determined in all investigated samples unambiguously. Quantitative results are presented in **Table 3** and confirmed the correct labeling of five commercial products (samples AA-12 to AA-16). Samples AA-17 and AA-18 contained no wormwood but other herbal extracts. UV and MS spectra of the two samples revealed the absence of compounds **1**−**8**.

Despite differences observed in the content of single constituents of the five wormwood preparations, there are some common characteristics. All of the samples (except sample AA-7) showed a common pattern in which **3** and **5** were the major compounds.

Table 3. Quantitative Results for Plant Material (AA-1 to AA-11) and Commercial Products (AA-12 to AA-18)[a]

sample	1	2	3	4	5	6	7	8
AA-1[a]	0.06 (0.4)	0.02 (0.8)	0.3 (0.7)	0.0003 (0.3)	0.5 (0.6)	0.05 (0.8)	0.05 (1.0)	0.2 (0.8)
AA-2[a]	0.1 (1.4)	0.06 (4.9)	0.5 (0.4)	0.0002 (4.9)	0.9 (0.5)	0.4 (0.9)	0.6 (1.1)	0.2 (0.8)
AA-3[a]	0.02 (1.7)	0.04 (2.6)	0.2 (0.7)	0.0002 (2.8)	0.5 (0.8)	0.03 (0.9)	0.04 (0.6)	0.08 (0.4)
AA-4[b]	0.02 (2.5)	0.02 (0.3)	0.1 (0.6)	0.0005 (0.03)	0.5 (1.1)	0.04 (1.0)	0.04 (1.0)	0.08 (1.3)
AA-5[b]	0.01 (2.6)	0.06 (1.6)	0.1 (1.1)	0.0005 (3.0)	1.2 (0.9)	0.07 (0.9)	0.08 (1.5)	0.1 (0.9)
AA-6	0.02 (1.9)	0.02 (1.2)	0.1 (0.3)	0.0001 (0.2)	0.5 (0.4)	0.04 (0.3)	0.04 (0.4)	0.09 (0.2)
AA-7	0.01 (3.6)	0.07 (3.2)	0.05 (1.3)	0.0004 (0.2)	0.6 (0.6)	0.04 (1.6)	0.06 (1.1)	0.1 (1.1)
AA-8	0.06 (1.2)	0.07 (1.5)	0.2 (1.0)	0.0001 (0.8)	0.3 (1.3)	0.02 (1.2)	0.04 (1.8)	0.09 (0.9)
AA-9	0.03 (0.6)	0.07 (2.0)	0.2 (0.4)	NQ	0.6 (0.4)	0.02 (1.8)	0.04 (0.7)	0.09 (0.9)
AA-10	0.04 (0.8)	0.01 (1.3)	0.1 (0.3)	0.0001 (0.4)	0.3 (0.9)	0.02 (1.0)	0.03 (0.8)	0.09 (0.9)
AA-11	0.03 (0.9)	NQ	0.2 (1.1)	NQ	0.3 (0.8)	0.01 (1.0)	0.02 (1.5)	0.03 (0.6)
AA-12	0.07 (1.2)	0.05 (1.5)	0.2 (0.1)	0.0002 (0.1)	0.6 (1.1)	0.03 (0.3)	0.04 (0.2)	0.08 (0.3)
AA-13	25.2 (2.2)	6.9 (4.8)	267.1 (0.5)	NQ	1176.0 (0.9)	49.9 (0.7)	98.0 (0.6)	58.7 (0.5)
AA-14	6.7 (0.8)	1.3 (0.8)	171.5 (0.3)	NQ	508.2 (0.2)	15.0 (0.2)	19.7 (0.1)	1.5 (1.6)
AA-15	NQ	1.3 (0.4)	59.5 (0.8)	NQ	127.0 (0.01)	2.8 (0.1)	6.5 (0.1)	12.5 (0.1)
AA-16	10.9 (0.1)	18.2 (4.2)	300.1 (0.1)	NQ	1064.2 (0.3)	30.9 (0.1)	76.2 (0.03)	43.8 (0.2)
AA-17	ND	ND	ND	ND	ND	ND	ND	ND
AA-18	ND	ND	ND	ND	ND	ND	ND	ND

[a] Values for compounds **1**−**8** in percent for samples AA-1 to AA-12 and in μg/mL for AA-13 to AA-16; relative standard deviations are given in parentheses ($n = 3$). Superscript letters a and b refer to the same supplier but different batches; NQ, not quantified; ND, not detected.

Compound **5** was present in an amount of up to 1.2% (1176.0 μg/mL in the liquids), and the highest content of **3** was 0.5% (300.1 μg/mL in the liquids). In the herbal material, compounds **5** and **7** exhibited the highest variation of all compounds, differing from 0.3 to 1.2% and from 0.02 to 0.6%, respectively. In contrary, the occurrence of **2** (0.01−0.07%) and **4** (0.0001−0.0005%) was more consistent.

Most of the samples analyzed in this study (except AA-17 and AA-18) contained all eight compounds. Compound **1** was detected but not quantified in sample AA-15 because of the limit of quantification. This was also the case with compound **2** in sample AA-11. Compound **4** was present in all samples, but the content was below 0.0005% and therefore only partially quantifiable. At present, it is unclear whether **4** is a natural compound or only a degradation product of absinthin. Further experiments are mandatory to determine the occurrence of the substance in fresh plant material.

The here presented HPLC method enables for the first time the simultaneous determination of several bioactive compounds in *A. absinthium*. The method is fully validated and applicable to commercial products. The marker substance absinthin is stable in solution for up to 6 months; this is also the case when the solid compound is kept in the refrigerator. Three decomposition compounds were detected by HPLC-MS and HPLC-SPE-NMR and identified as dimeric sesquiterpene lactones. The major degradation product of absinthin was anabsinthin followed by anabsin and a new dimer, the hydroxylated form of anabsinthin.

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Supporting Information Available: Additional information regarding data validation, HPLC-MS analysis, and NMR structure elucidation. This material is available free of charge via the Internet at http://pubs.acs.org.

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