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Analysis of the sesquiterpenes in *Achillea* species using liquid chromatography-mass spectrometry with positive ion atmospheric pressure chemical ionisation

Katharina Rothwangl-Wiltschnigg, Sabine Glasl, Ingrid Werner, Gottfried Reznicek*

Institute of Pharmacognosy, University of Vienna, PharmaCenter Vienna, Althanstrasse 14, A-1090 Vienna, Austria.

Abstract

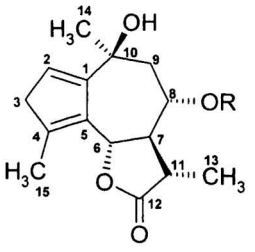
The species of the *Achillea millefolium* group contain different sesquiterpenes which are of chemotaxonomical and pharmacological interest. Therefore the HPLC analysis of these compounds is a fundamental task for the quality control of *Achillea* samples. As difficulties in identification solely on the basis of retention times may arise, the LC was coupled with atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) allowing a selective and sensitive detection and thus the clear identification of the sesquiterpenes.

Keywords

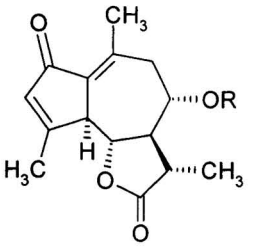
Sesquiterpenoids, *Achillea* species, mass spectrometry, APCI-MS

Introduction

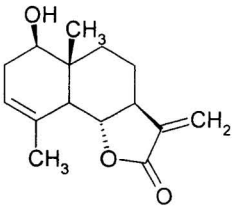
Several species of the polyploid *Achillea millefolium* group are characterized by labile proazulenes and other guaianolides, e.g. 8α-tigloxy- (**1**) resp. 8α-angeloxy-artabsin (**2**) or achillicin (**3**) and stable matricarin-derivatives as matricarin (**4**) or desacetylmatricarin (**5**) as well as eudesmanolides like santamarin (**6**) (Fig. 1), characteristic for the different species which show a typical sesquiterpene pattern [1, 2].



1: 8α-tigloxy-artabsin (R=tigloyl)
2: 8α-angeloxy-artabsin (R=angeloyl)
3: achillicin (R=acetyl)



4: matricarin (R=acetyl)
5: desacetylmatricarin (R=H)



6: santamarin

Fig. 1. Representative sesquiterpenes from different species of the *Achillea millefolium* group

Yarrow is widely used in folk medicine against various diseases including internal and external inflammations and according to pharmacological studies some of these sesquiterpenes are responsible for the anthiphlogistic activity [3, 4]. In contrast some sesquiterpenes show only little or no activity while others even might trigger the undesirable allergic contact dermatitis [5, 6]. Therefore it was an important task to work out a method for the analysis of the sesquiterpenes in *Achillea* species to check the quality of samples. A high performance liquid chromatographic method on reversed phase was developed allowing the quantitative determination of the relevant sesquiterpenoids using UV-detection [7]. It is obvious that the identification of the sesquiterpenes especially in samples with a complex composition or in samples containing compounds of more than one *Achillea* species is difficult on the basis of retention times solely. Thus there was the demand for an alternative detection method allowing the unambiguous identification of these compounds. In this paper the coupling of mass spectrometry

(MS) with high performance liquid chromatography (HPLC) is presented as additional tool for detection of sesquiterpenoids from *Achillea* species.

Experimental

Chemicals

HPLC grade water was prepared by distillation of deionised water on a GFL 4020 distillation instrument, methanol (CHROMASOLV®, HPLC grade) was purchased from Riedel-de Haen, dichloromethane (p. a. grade) from J. T. Baker, ammonium acetate (Ammonium aceticum puriss. cryst.) was obtained from Apoka, Vienna, Austria.

Plant material and reference substances

Achillea species were collected in Austria or they were commercial samples. The plant material was botanically identified at the Institute of Pharmacognosy. Vouchers of the origins are deposited in the Herbarium of the Institute of Pharmacognosy, University of Vienna (Austria).

The sesquiterpenes used for working out and optimising the MS parameter 8α-tigloxy- artabsin (**1**) resp. 8α-angeloxy-artabsin (**2**), desacetylmatricarin (**5**) and santamarin (**6**) have been isolated, purified and structurally elucidated at the Institute of Pharmacognosy, University of Vienna (Austria), purity (HPLC) > 94%.

HPLC-MS equipment

The HPLC system consisted of a Perkin Elmer Series 200 autosampler (injection volume 20 µl), Series 200 quaternary pump and LC 235C diode array detector. Separations were carried out using a Hewlett Packard LiChrospher 100 RP 8, 5 µm column (250x4.0 mm) guarded by a Hewlett Packard LiChrospher 100 RP 8, 5 µm guard column (4x4 mm). The binary system employed the eluents methanol and water, 5 mM ammonium acetate was added to the mobile phase. The elution started from 20% methanol to 80% methanol in 90 min (linear; rate=0.66%/min) with a flow of 1.0 ml/min at room temperature.

The HPLC was coupled with a PE-Biosystems API 150 EX mass spectrometer, equipped with the APCI source, the complete HPLC-eluate was introduced into the ion source. The following MS parameters were used: APCI, positive mode, nebulizer gas (NEB) 5, curtain gas (CUR) 12, needle current (NC) 2.5, temperature (TEM) 300°, declustering potential (DP) 10, focusing potential (FP) 200, entrance potential (EP) –10, deflector (DF) –400, channel electron multiplier (CEM) 2700, scan m/z 100 – m/z 400 / sec.

Instrument controlling and data analysis was performed using the PE Biosystems Analyst software, version 1. 1.

Sample preparation

100 mg air dried flowerheads were transferred to a 2 ml reacti vial followed by 1 ml dichloromethane. After ultrasonification for 10 min. at room temperature the solution was filtered into a 10 ml round bottom flask, the drug was washed two times with 1 ml dichloromethane respectively, and also filtered. Altogether this procedure was performed four times, the plant extracts were evaporated entirely and redissolved in 500 µl 80% methanol, 20 µl thereof were analyzed by HPLC-MS.

Results and Discussion

Optimisation of the APCI parameters

Few papers are published concerning API-mass spectrometry of sesquiterpenoids [8-11]. The ideal MS parameters were found using typical sesquiterpenes from *Achillea* like 8α-tigloxy- artabsin (**1**) (Fig. 1) resp. 8α-angeloxy-artabsin (**2**), representing labile proazulenes, desacetylmatricarin (**5**) (Fig. 1), a stable matricarin derivative and santamarin (**6**) (Fig. 1), an eudesmanolide. A constant flow of 80% methanol + 5 mM ammonium acetate with a rate of 1 ml /min was introduced into the mass spectrometer and solutions of the reference compounds (each with 5 µl of 0,001% solution in 80% methanol) were analysed repeatedly by flow injection analysis (FIA) to find out the optimal MS parameters.

Due to the low polarity of the compounds atmospheric pressure chemical ionization (APCI) was employed for ionization whereas electrospray ionization (ESI) did not yield useful mass spectra, neither in the positive, nor in the negative mode.

Influence of buffers

Desacetylmatricarine (**5**), MW 262.5, gave a quasimolecular ion $[MH]^+$ at m/z 263.5 with high intensity in the positive mode (Fig. 2), switching to the negative mode yielded the respective ion at m/z 261.5 $[M-H]^-$ with clearly less intensity thus the further analyses were carried out in the positive mode.

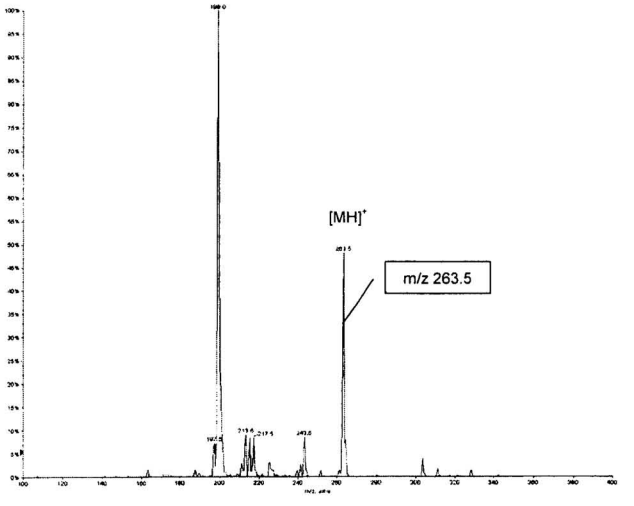


Fig. 2. APCI-mass spectrum of desacetylmatricarin (**5**), MW 262.5.

In contrast the labile proazulenes 8α-tigloxy- artabsin (**1**), MW 346, resp. 8α-angeloxy-artabsin (**2**), showed only some fragments at these conditions and no quasimolecular ions could be found. It is known that the addition of volatile buffers to the mobile phase may enable ionisation and improve the ion abundance. The addition of ammonium acetate resulted in mass spectra of these proazulenes with a prominent peak corresponding to the adduct $[M+NH_4]^+$ beside a quasimolecular ion $[MH]^+$ with low intensity (Fig. 3).

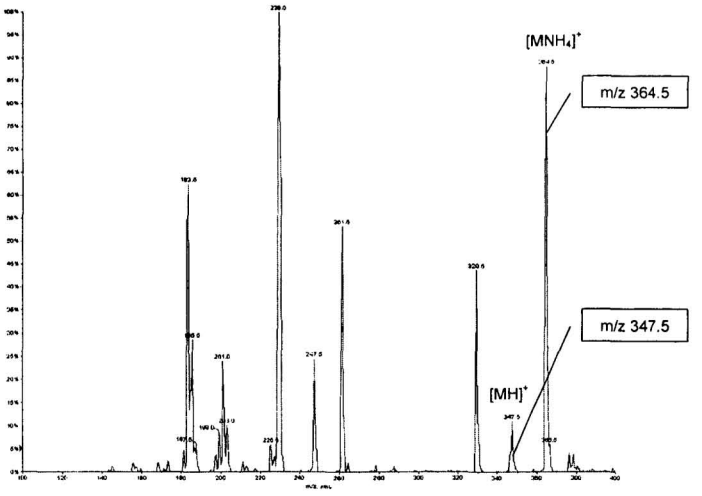


Fig. 3. APCI-mass spectrum of 8α-tigloxy-artabsin (**1**), MW 346.5.

Further investigations showed that a minimum concentration of 2 mM ammonium acetate is necessary to obtain the above described mass spectra. Increasing the concentration to 5 mM buffer leads to moderately increasing peak intensities while the use of higher concentrations up to 10 mM ammonium acetate showed no further advantages. Therefore the addition of 5 mM ammonium acetate to the mobile phase was the best choice.

The same revealed for the eudesmanolide santamarin (**6**), MW 248, also showing ammoniated adducts $[M+NH_4]^+$ besides a quasimolecular ion $[MH]^+$ with low intensity (Fig. 4).

In contrast to the proazulenes and eudesmanolides the matricarin derivative (**5**) did not show any adduct with ammonium acetate, the mass spectrum showed same profile as without addition of ammonium acetate (Fig. 2). This fact was confirmed in all following investigations, therefore two types of sesquiterpenes are found in *Achillea* species: the matricarin type sesquiterpenes giving no adduct with ammonium acetate and showing prominent $[MH]^+$ quasimolecular ions (Fig. 2), on the other hand proazulenes and eudesmanolides are present with $[M+NH_4]^+$ being the base peak and $[MH]^+$ with low abundance (Fig. 3 and 4). The latter compounds

can easily be recognised by the difference of 17 amu between the ions $[M+NH_4]^+$ and $[M+H]^+$.

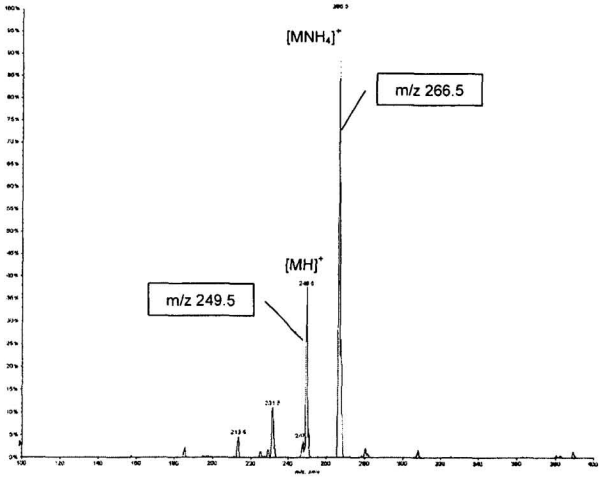


Fig. 4. APCI-mass spectrum of santamarin (**6**), MW 248.5.

Effect of vaporizer temperature and declustering potential

To achieve a reduction of the aerosol droplet size for an improvement of the ionisation efficiency at the given flow rate of the mobile phase of 1 ml/min the vaporizer temperature was increased step by step starting from 200°C. Rising the temperature to 300°C resulted in continous increase of the ion intensities. An increase of the vaporizer temperature to 350°C showed no further effects, whereas temperatures above 350°C produced decreasing ion intensities most likely due to thermal degradation of the compounds. Thus the ideal vaporizer temperature was found to be 300°C.

By applying different potential differences ("declustering potential", DP) in the intermediate pressure region of the mass spectrometer the collision induced dissociation (CID) of compounds may be controlled. A series of investigations with the reference compounds showed that a low declustering potential (DP 1) gives mass spectra where only the quasimolecular ions $[M+H]^+$ resp. $[M+NH_4]^+$ can be

seen. Increasing the potential to DP 10 leads to more and more fragmentation and higher sensitivity of the total ion current (TIC), whereas values above the ideal DP 10 resulted in too much fragmentation so that only very small or no quasimolecular ions were found.

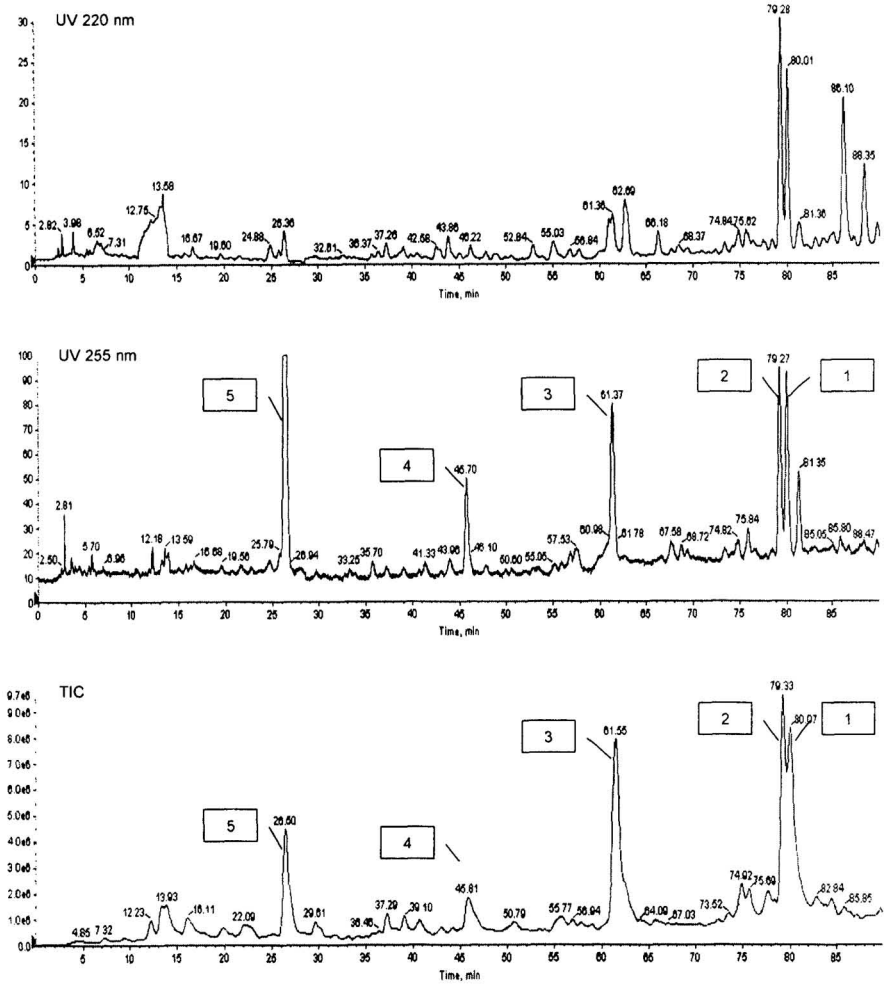


Fig. 5. HPLC analysis of *Achillea collina*, detection with UV at 220 nm (top) resp. 255 nm (middle) and TIC of APCI mass spectrometry (below); 8α-tigloxy-artabsin (**1**), 8α-angeloxy-artabsin (**2**), achillicin (**3**), matricarin (**4**), desacetylmatricarin (**5**).

All other parameters (nebulizer gas, curtain gas, needle current, focusing potential, entrance potential and deflector) were investigated in the same manner, but only minor effects on the mass spectra could be observed.

LC-MS detection of sesquiterpenoids from Achillea

Fig. 5 shows the TIC as well as the UV traces from an analysis of *Achillea collina*. Using LC- APCI MS the sesquiterpenoids can selectively be detected.

The detection limit for these compounds was investigated by injection of various amounts of the reference sesquiterpenes 8α-tigloxy- (**1**) resp. 8α-angeloxy-artabsin (**2**), desacetylmatricarin (**5**) and santamarin (**6**). These series of analyses demonstrated that this method is effective for the detection of the sesquiterpenes down to levels of 1 ng in the scan mode (m/z 100 – m/z 400 / sec.) and showed that these compounds are detected in the same intensities. Thus, LC-MS offers a much better sensitivity than UV-detection.

The introduced HPLC-APCI MS method allows the selective and sensitive detection of the sesquiterpenes from different species of the *Achillea millefolium* group. A clear identification of the compounds is possible by the use of the mass spectra even if a complex composition of the sesquiterpenes or a mixture of different species is present. This is an indispensable requirement for the analysis and quality control of medicinally used *Achillea* samples or extracts.

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