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Rapid and sensitive analysis of azadirachtin and related triterpenoids from Neem (*Azadirachta indica*) by high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

Otmar Schaaf[a], Andrew P. Jarvis[a], S. Andrew van der Esch[b], Germina Giagnacovo[b], Neil J. Oldham[a,*]

[a]*Max-Planck-Institut für Chemische Ökologie, Carl-Zeiss-Promenade 10, D-07745 Jena, Germany*
[b]*E.N.E.A., INN-BIOAG-PRIM, Via Anguillarese km 1.300, I-00060 Rome, Italy*

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Abstract

Based on reversed-phase high-performance liquid chromatography (RP-HPLC) and atmospheric pressure chemical ionization (APCI) mass spectrometry, a HPLC–MS method was developed to permit the rapid qualitative and quantitative analysis of azadirachtin and related tetranortriterpenoids from seeds and tissue cultures of Neem (*Azadirachta indica*). APCI+ standard scanning mass spectra of the major Neem triterpenoids were recorded and utilized to select suitable ions for selected ion monitoring (SIM). Transitions for selective reaction monitoring (SRM) were based on MS–MS experiments. Using SIM, major Neem triterpenoids were detected in callus culture material and seed kernels of *A. indica*. The limit of detection for azadirachtin in extract samples (~1 ng ml^{-1} or 10 pg in SIM mode) was determined to be (with respect to injected absolute amounts) approximately 1000-times lower than values quoted in the literature for existing HPLC methods (~200 ng ml^{-1} or 10 ng). In addition to high sensitivity, the HPLC–MS method is able to tolerate minimal sample preparation and purification, dramatically reducing total analysis time. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Neem; *Azadirachta indica*; Azadirachtin; Triterpenoids

1. Introduction

The Neem tree (*Azadirachta indica* A. Juss, Fam. Meliaceae), originating from India, is one of the richest sources of secondary metabolites in nature. To date more than 300 natural products have been isolated from different parts of the tree, with new compounds added to the list every year [1–3]. Among these compounds, limonoids (tetranortriterpenoids) form the major group (Fig. 1) [4].

The biological activity of Neem had been known for centuries, and preparations have been used in traditional Indian medicine since ancient times. In 1968 Butterworth and Morgan isolated azadirachtin as the main insecticidal component of Neem [5,6]. However, it took 17 years for the correct structure of this highly oxidized triterpenoid to be established [7,8]. Azadirachtin can be isolated in small amounts

*Corresponding author. Tel.: +49-3641-643-628; fax: +49-3641-643-665.
E-mail address: oldham@ice.mpg.de (N.J. Oldham)

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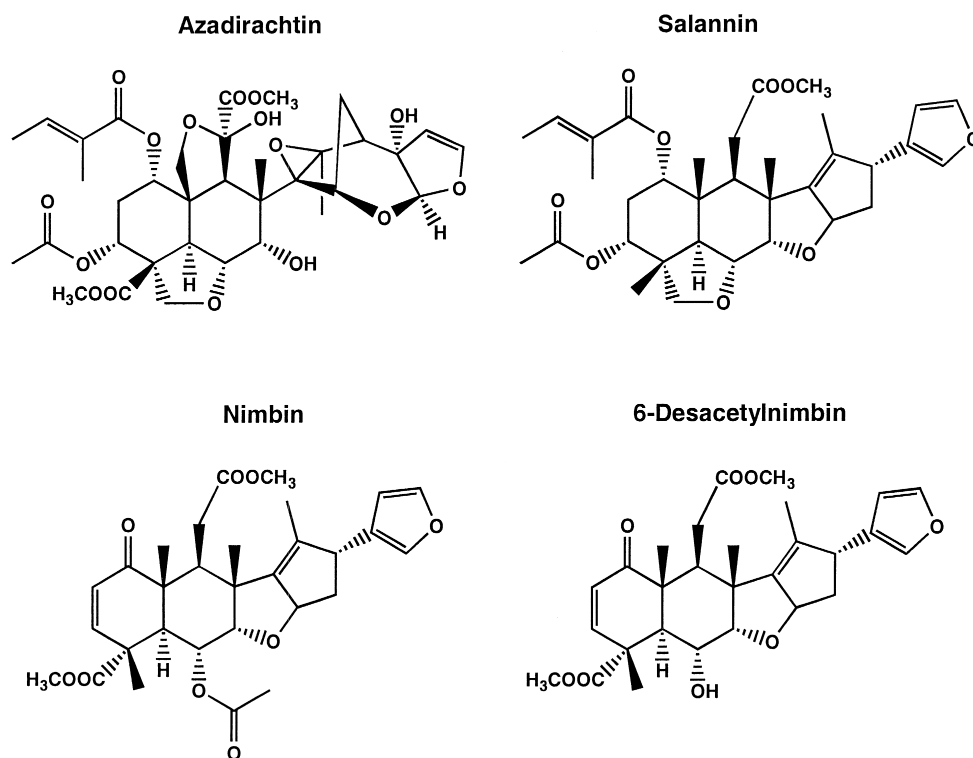

Fig. 1. Structures of major triterpenoids from the seeds of *Azadirachta indica*.

from all parts of the Neem tree, but is present at highest concentration in the mature seeds [1]. Azadirachtin is particularly well-known for its effectiveness as a defensive compound against insects, acting as a strong anti-feedant and causing growth disruption in many insect species [9,10].

Due to favourable properties such as low toxicity against non-target organisms, low persistence in nature and systemic action [11], azadirachtin and its natural, or synthetic, analogues are highly promising candidates for the development of novel naturally derived insecticides. In fact, a variety of insecticides prepared from Neem extracts are commercially available [12]. However, since these insecticides are based on seed extracts, the concentrations of azadirachtin and related triterpenoids are rather low and may vary due to fluctuating contents in the natural sources, or susceptibility of the compounds to environmental influences (heat, light) [13–15]. Regarding the commercial and industrial relevance of azadirachtin, extensive efforts have been made in recent years to facilitate the in vitro production of azadirachtin. There are now a number of reports of the production of Neem triterpenoids in callus cultures [12,16–20], cell cultures [15,21,22] and hairy roots [12,23] of *A. indica*.

In the past, approaches to the qualitative and quantitative analysis of azadirachtin and other Neem triterpenoids from different sources mostly included reversed-phase high-performance liquid chromatography (RP-HPLC), because of the polarity of the Neem compounds [24–27]. However, the UV detection that is usually utilised for the quantification of azadirachtin is not very sensitive, since the molecule does not carry strong UV absorbing chromophores. The UV signal has to be recorded at low wavelengths (217 nm, ϵ=9200) in order to maximise sensitivity, requiring the use of solvent systems that are UV-transparent in this range [24]. Early attempts at a quantification of azadirachtin in Neem oil, using UV detection, gave the rather low sensitivity of 50 $\mu g\ g^{-1}$ (50 ppm) [25]. Sundaram and Curry developed an improved RP-HPLC method for the analysis of azadirachtin in Neem oil and commercial

formulations (limit of detection 3 ppm), as well as in complex environmental matrices (0.2–0.25 ppm) [24,26]. Supercritical fluid chromatography (SFC) was employed by the Morgan group, in addition to RP-HPLC [28–30]. Recently, Jarvis et al. developed a method for rapid separation of triterpenoids from Neem seed extracts using fractionation with Biotage flash chromatography followed by analysis of the fractions by thin-layer chromatography (TLC), SFC or HPLC [31]. However, all these methods require considerable sample preparation and have rather high limits of detection. A completely different analytical approach was taken by Schütz et al. who developed a highly sensitive enzyme-linked immunosorbent assay (ELISA) for the analysis of azadirachtin within a range of detection from 10 to 1000 ppb [32], though one disadvantage of this method might be its insufficient ability to discriminate between azadirachtin and compounds with closely related structures, e.g. degradation products.

Due to their complexity, the structure elucidation of novel Neem triterpenoids inevitably requires the use of a broad array of modern nuclear magnetic resonance (NMR) techniques (e.g. COSY, NOESY, etc.), which generally means extensive and laborious purification. However, there is a requirement for the rapid and sensitive analysis of known triterpenoids in order to determine their presence in Neem tissue and products. Here we report the suitability of liquid chromatography–mass spectrometry (LC–MS) for the detection of triterpenoids in Neem extracts, using atmospheric pressure chemical ionization (APCI).

2. Experimental

2.1. Tissue culture

The *Azadirachta indica* callus cultures were initiated, in 1990, from sterile seedlings, grown from seeds obtained from Togo in West Africa. The culture medium was optimised with the callus cell lines, and the most effective medium was called Vm. This medium consists of Murashige and Skoog basal salts with minimum organics (MS basal medium; No. 5519; Sigma, Deisenhofen, Germany) supplemented with sucrose (30 g l^{-1}), indolebutyric acid (IBA, 19.6 μM; 4 mg l^{-1}), 6-benzylaminopurine (BAP, 8.87 μM; 2 mg l^{-1}), casein hydrolasate (1 g l^{-1}), agar (1%, w/v). All reagents were purchased from Sigma.

The callus cultures are maintained in culture tubes (150×20 mm) containing 8 ml of agarised medium. Incubation conditions are at 28°C either in the dark or with a light/dark cycle of 16/8 h with the light at 2500 Lux. The cultures are regularly subcultured (every 16–21 days).

2.2. Extraction

Freeze–dried callus tissue (5 g) was ground in liquid nitrogen and extracted with methanol (3×100 ml). After addition of 150 ml water the solution was partitioned against 3×50 ml CH_2Cl_2. The methanol–water layer (upper) was discarded, the dichloromethane layers combined and dried over $MgSO_4$. The extract was evaporated to dryness in a rotatory evaporator (400 mbar, room temperature) and re-dissolved in 5 ml methanol prior to analysis by HPLC–MS.

Dried Neem seeds, originating from India, were supplied by Professor E.D. Morgan, University of Keele, Keele, UK. The husks of 5 g of seeds were removed and the kernels deoiled by grinding in light petroleum (3×30 ml for 20 min). After filtration, the filtrate (containing the oil) was discarded and the dry meal ground in methanol (3×30 ml for 20 min). The combined, filtered extracts were concentrated to 20 ml in a rotatory evaporator and water (20 ml) was added. This solution was partitioned against CH_2Cl_2 (2×40 ml) and the combined CH_2Cl_2 layers evaporated to dryness (400 mbar, room temperature). The residue was re-dissolved in 5 ml methanol and analysed by HPLC–MS.

2.3. Instrumentation

A Hewlett-Packard Series 1100 HPLC system, equipped with a UV detector, was connected to a Quattro II quadrupole-hexapole-quadrupole tandem mass spectrometer (Micromass, Altrincham, UK). Analyses were performed with an APCI interface

(Z-spray source). Data were recorded and analysed with MassLynx 3.1 software (Micromass).

2.4. HPLC conditions

A Supelcosil LC-18 RP-HPLC column (10 cm$\times$4.6 mm I.D., 5 μm pore diameter, column temperature 30°C; Supelco, Deisenhofen, Germany) served as the stationary phase, while a gradient of water and acetonitrile (ACN)+0.1% trifluoroacetic acid (TFA) was employed as the mobile phase. The gradient used in APCI mode (constant flow-rate 0.5 ml min^{-1}) started with 20% ACN+TFA (5 min isocratic), and over a period of 10 min the percentage of ACN+TFA was increased to 65% (10 min isocratic) and subsequently decreased again to 20% ACN+TFA (2 min isocratic). UV signals were recorded at 217 nm with an attenuation of 1000 and a response of 2000 ms.

2.5. MS conditions

Samples were analysed in APCI positive mode with a probe temperature of 400°C and a source block temperature of 150°C. Nitrogen (300 l h^{-1}) was used as the APCI sheath and drying gas, and argon (2$\times$10^{-3} mbar) as the collision gas for selective reaction monitoring (SRM) experiments. The source was operated with a corona pin voltage of 3.50 kV, and a cone voltage of 25 V. Suitable reactions and conditions for SRM experiments were established for azadirachtin, nimbin and salannin by a series of product ion scans (cone voltage 30 V, collision energy 15 eV).

2.6. Analysis and quantification

In order to compare the sensitivity and selectivity of different MS methods for the analysis of azadirachtin, standard as well as extract samples were analysed in full scan, selective ion monitoring (SIM) and SRM modes using the APCI interface. The dwell time for each ion was 1.00 s. For the extract samples, selected ion scans for each compound were restricted to specific retention time windows (cortisol: 0.0 to 15.0 min, azadirachtin: 15.0 to 18.0 min, 6-desacetylnimbin: 18.0 to 20.6 min, nimbin: 20.6 to 22.5 min, salannin: 21.5 to 32.0 min).

Cortisol (0.01 mg ml^{-1}) was employed as an internal standard for the quantification of azadirachtin in extract samples. The following concentrations of azadirachtin (mg ml^{-1} methanol) were used for calibration: 0.0005, 0.005, 0.02, 0.04, 0.06, 0.08, 0.1, 0.15, 0.20. Each injection (10 μl) of standard or extract samples was repeated three times using an autosampler.

2.7. Chemicals

All solvents used were of HPLC grade. Triterpenoids in Neem extracts were identified by comparison of their retention times and APCI+ mass spectra with the corresponding standards. Azadirachtin, nimbin and salannin isolated from Indian Neem seeds were kindly supplied by Professor E.D. Morgan and Dr. G.R. Jones, University of Keele. 6-Desacetylnimbin was prepared by saponification of nimbin (10 mg) in a saturated solution of Na_2CO_3 in methanol (1 ml). After stirring for 16 h at room temperature, the methanol was evaporated and the residue dissolved into water. The pH was adjusted to 5 with HCl and the solution partitioned against ether. The ether was removed and diazomethane in ether (1 ml) was added to the residue. After 16 h at 4°C, the solution was evaporated to dryness giving an approximately 50:50 mixture of nimbin and 6-desacetylnimbin (by HPLC–APCI+ MS).

3. Results and discussion

3.1. APCI+ mass spectra of Neem triterpenoids

The APCI+ mass spectrum of azadirachtin (Fig. 2a) showed only a weak protonated molecule $[M+H]^+$ at m/z 721, but a relatively intense sodium adduct ion $[M+Na]^+$ at m/z 743 caused by traces of Na^+ in the HPLC solvent. The base peak (m/z 703) was formed by elimination of water $[M+H-H_2O]^+$. Though every effort was made to standardise the conditions, changes in the relative intensities of ions

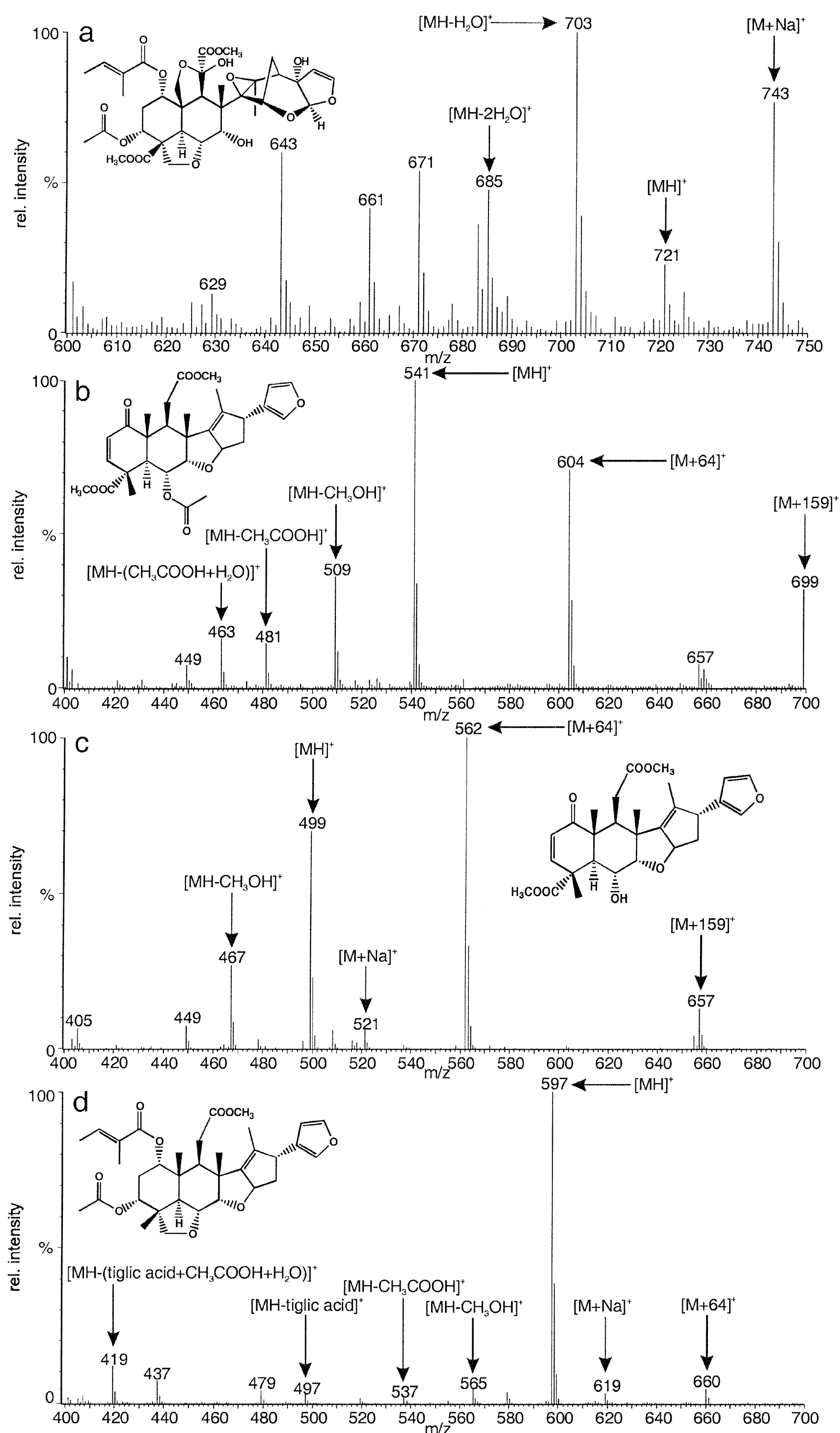

Fig. 2. APCI+ mass spectra of the triterpenoids studied; (a) azadirachtin, (b) nimbin, (c) 6-desacetylnimbin, (d) salannin; experimental conditions see text.

were observed over time. Therefore, in the SIM mode, three ions (including the sodium adduct ion) were chosen to allow accurate quantification of azadirachtin. Hence, *m/z* 743, 703 and 685 (resulting from cleavage of two water molecules from $[M+H]^+$) were selected for SIM.

In contrast to azadirachtin, the protonated molecules $[M+H]^+$ in the spectra of nimbin (*m/z* 541, Fig. 2b) and salannin (*m/z* 597, Fig. 2d) formed the base peaks. Major fragments were mostly derived from cleavage of ester bonds by successive elimination of methanol $[M+H-CH_3OH]^+$ and/or acetate and tiglate groups. The spectra of 6-desacetylnimbin and salannin exhibited very strong $[M+H]^+$ ions and only little fragmentation. Formation of sodium adduct ions was constantly weak (*m/z* 619 for salannin) or absent (6-desacetylnimbin). For that reason, only the $[M+H]^+$ ions were selected for SIM experiments.

Two conspicuous ions at $[M+64]^+$ (base peak for 6-desacetylnimbin) and $[M+159]^+$ appeared in the spectra of nimbin and 6-desacetylnimbin. The presence of these adduct ions was not only observed in the spectra of extract samples, but also in the corresponding standards. The $[M+64]^+$ ion might be due to adduct formation with Na^+ [+23] and acetonitrile (CH_3CN) [+41], while [M+159] might be formed by addition of Na^+ and ($CF3COO^-$ Na^+) [+136]. These hypotheses are supported by the results of MS–MS experiments with nimbin, showing an emergence of *m/z* 563 as the only significant product ion for both *m/z* 699 $[M+159]^+$ and 604 $[M+64]^+$. The ion in question can be formed from *m/z* 699 by loss of sodium trifluoroacetate (−136) and from *m/z* 604 by loss of acetonitrile (−41). A parent ion scan for *m/z* 563 confirmed its origin from both *m/z* 604 and 699.

Although the spectrum of salannin also exhibited an [M+64] ion, it was of considerably lower intensity than for the nimbin skeleton. The highest stability of response for nimbin in SIM mode was obtained using the combination of ions *m/z* 699, 604, 541 $[M+H]^+$ and 509 $[M+H-CH_3OH]^+$.

Based on the MS–MS experiments (product ion scans), we chose the following fragmentations in order to perform SRM analyses: *m/z* 703→685 (azadirachtin), *m/z* 541→509 (nimbin) and *m/z* 597→419 (salannin).

3.2. Analysis of Neem extracts with HPLC–APCI+ MS

The limits of detection for azadirachtin in standard and Neem seed extract samples (see Section 3.3) were determined by injecting serial tenfold dilutions in methanol, using different MS methods in the APCI+ mode. For standards, SRM was the least sensitive (but most selective) method, with a detection limit of 140 $\mu g\ ml^{-1}$ (1.4 μg), followed by standard scanning MS (*m/z* 600 to 750), which gave a detection limit of 14 $\mu g\ ml^{-1}$ (0.14 μg). In SIM mode the lowest detectable amount of azadirachtin was 1.4 pg (140 $pg\ ml^{-1}$). Thus, SIM, as the most sensitive method, was selected for the analysis of Neem extracts.

Fig. 3 shows the HPLC chromatogram from analysis of a crude extract of freeze–dried *A. indica* callus tissue culture material. The data are from a single analysis, with Fig. 3a showing the UV detector response (217 nm) and Fig. 3b–f the SIM responses of cortisol (internal standard), azadirachtin, 6-desacetylnimbin, nimbin and salannin. No purification of the methanolic extract had been undertaken apart from addition of water and partitioning against dichloromethane. The triterpenoids were resolved to clear and distinct peaks in the total ion current (TIC) chromatograms, but were undetectable in the UV chromatogram. Azadirachtin, the most polar compound analysed, eluted first, followed by 6-desacetylnimbin, nimbin and salannin, a result in accordance with the findings of Johnson and Morgan using a Sphersorb ODS semipreparative HPLC column [29].

As expected, the deoiled methanolic Neem seed extract contained triterpenoids in amounts that were orders of magnitude higher than in the callus extract. Hence, triterpenoid peaks were detected in the UV trace. However, the UV chromatogram did not afford satisfactory selectivity, resulting in overlapping and unresolved peaks of coeluting compounds. As within the callus culture sample, SIM detection gave clear, resolved peaks.

3.3. Quantification of azadirachtin in Neem callus and seed extracts

Using SIM, with cortisol as an internal standard,

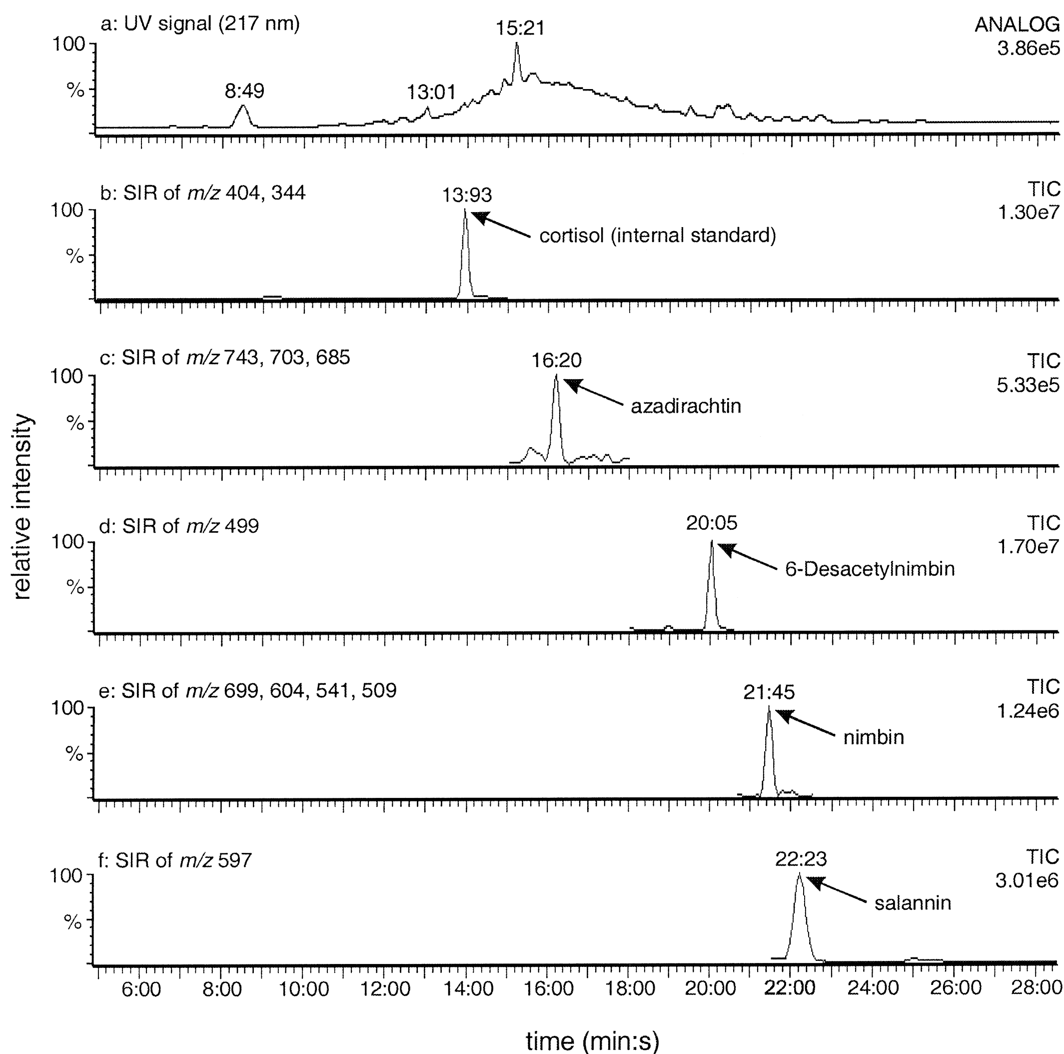

Fig. 3. HPLC chromatograms from a single analysis of *Azadirachta indica* callus tissue. (a) UV detector output (217 nm), (b–f) SIM of cortisol (internal standard; b), azadirachtin (c), 6-desacetylnimbin (d), nimbin (e), salannin (f); TIC=total ion current.

azadirachtin gave a linear response within the range 5 ng to 2 μg (total number of data points=27). The resulting linear calibration curve $y=ax+b$ had a slope of $a=33.3366$ (± 1.0414) and an intercept of $b=0.0396$ (± 0.1009). Correlation coefficients were calculated as $r=0.99$ (SD=0.35) and $r^2=0.98$.

The concentrations of azadirachtin in the callus and seed extracts were calculated from the calibration curve using the mean response values of three sequential injections. The callus extract contained 0.5 μg of azadirachtin per ml, which corresponds to 0.5 μg g^{-1} freeze–dried callus material (0.00005%). The concentration of azadirachtin in the seed extract was considerably higher and amounted to 867 μg ml^{-1} or 867 μg g^{-1} dried seeds (0.0867%).

As with standard samples, the sensitivity of SIM and SRM was compared by the analysis of azadirachtin in a Neem seed extract, using serial tenfold dilutions of the quantified seed extract sample (no usable chromatogram was obtained by standard scanning MS due to overlapping peaks and poor

signal-to-noise ratio). The limit of detection for azadirachtin in SIM mode was determined to be 8.67 pg (corresponding to a concentration of 0.867 ng ml^{-1}). Thus, SIM analysis of azadirachtin in crude seed extracts is slightly less sensitive than the analysis of standard samples (limit of detection 1.4 pg). However, compared to the best published values for conventional HPLC analysis of azadirachtin in extracts (10 ng injected or 200 ng ml^{-1} [26]), this means an improvement of sensitivity approximately by a factor of 1000 with respect to injected absolute amounts.

Consistent with the standards, again a substantial loss of sensitivity was observed for SRM (limit of detection 86.7 ng or 8.67 μg ml^{-1}) compared to SIM. This result was unexpected, since SRM is commonly regarded as the most sensitive method for crude samples, because of its selectivity and superior signal-to-noise ratio (S/N). The relatively poor performance of SRM in our experiments may, at least in part, be explained by the mode of construction of the mass spectrometer employed, which is equipped with two detectors. The intermediate detector, located after the first quadrupole analyser, gives improved sensitivity in the standard MS mode (either full scan or SIM) by dramatically reducing ion path length. Another consideration is the already high S/N observed in SIM mode, caused by a relatively low background, which reduced the impact of SRM on S/N.

4. Conclusions

Atmospheric pressure chemical ionization HPLC–MS provides a very powerful tool for the monitoring and analysis of the polar triterpenoids in Neem tissues and preparations, since it combines the resolving power of RP–HPLC with the sensitivity of MS. Additionally, high selectivity is supplied by the possibility to perform SIM or SRM experiments. In terms of sensitivity and selectivity, the benefits of HPLC–MS techniques – with a limit of detection in the picogram range in SIM mode – for the analysis of Neem extracts are evident, when compared to conventional HPLC methods. After a simple extraction procedure, HPLC–SIM-MS permits very rapid determination of the presence of azadirachtin and related limonoids in crude extracts, without extensive and time-consuming purification. It allows down-scaling of experimental designs and should also be of interest for routine analyses of commercial Neem preparations.

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