SHIMADZU

Application Report

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Rapid Identification of Isomeric Azadirachtins and Related Triterpenoids in Neem Extract By LC-ESI-MS

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Introduction

The neem, A*zadirachta Indica A. Juss*, occurred mainly in India, is well known for its insecticidal effects. In recent years, there has been a growing interest in neem due to essentially the demand to non-toxic and environmental-friendly pesticides. Neem-based pesticides and hygienic products have been developed and used. Azadirachtin A (Fig. 1) existed mainly in the seeds of neem is a main active component, which functions actually as an effective antifeedant for over 200 pest species. Nevertheless, in the nature plant chemical defense against pests almost never depends on a single compound, but instead on mixtures of compounds. So far, morn 300 compounds have been isolated from neem, and over a hundred of them belong to the class of triterpenoid [1]. Neem-based pesticides and other products are normally made from the extracts of seeds, kernel or leaves of the neem trees by simple formulation. Therefore, the constituents of the products are very complex. There is a demand for rapid analysis of the compositions of neem extract, formulation and products in research and product development work.

The conventional analysis method for neemextracts or formulations is HPLC with UV detection.

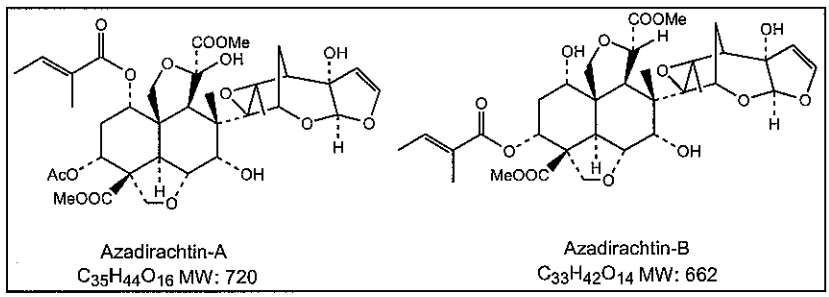

Fig 1 Molecular structures of azadirachtin A & B; ten isomeric azadirachtins have been isolated from neem.

But it has been used mainly in quantitative analysis for a few main components including azadirachtin A, salannin and nimbin etc [2]. For qualitative analysis of neem extracts that normally have complex compositions, a major difficulty faced is lack of standards as many neem triterpenoids not available from a convenient source. For example, it has not a simple method to analyze the isomeric azadirachtins B, D, E, F, G, H, I, K and L, which co-occur with azadirachtin A at low concentrations. The tedious procedure employed in the structure elucidation work of neem compounds, i.e., first isolating each compound via a series separation and purification steps and then carrying out analysis using NMR etc, is obviously not practical for a routine analysis in research and manufacture. Recently, Otmar Schaaf et al [3] used LC-APCI-MS in analysis of neem samples. The obvious advantage of LC/MS is its superior identification capability. This report describes a rapid analysis of neem extract sample using LC-ESI-MS. The mass spectra of the elute peaks obtained under normal and CID (collision induced dissociation) conditions allowed to identify successfully all the major and some minor components without use of any standard compound.

Experimental

The neem extract sample used in this work was obtained in the form of powder from Fortune Biotech Ltd, India. The sample was dissolved in methanol at a rate of 8 mg/25 ml. A LCMS-2010 system (Shimadzu) with collision induced dissociation (CID) capability was used in this work. The CID technique was used to generate fragments by breaking weak chemical bonds of target molecules. The LC and ESI-MS operation conditions are shown in Table 1.

Table 1 LC/MS analytical conditions for neem extract

Column	ODS-VP, 150 mm L x 2.0 mm I.D
Mobile phase	Acetonitrile-water
Liner Gradient	ACN from 35% to 80% in 60 mins
Flow rate	0.2 mL/min
Probe / voltage	ESI-positive mode / -4.5 kV
CDL temp	230 °C
Scan range	m/z 300-800

Results and Discussion

Identification of isomeric azadirachtins

The UV, MS-TIC and MS-MIC chromatograms of the neem extract sample are shown in Fig. 2. It can be seen that the composition of the sample is very complex. Based on the peak intensities, at least 7 major components, 23 minor components as well as some trace components can be detected by both UV and ESI-MS detectors.

The mass spectra of peaks 12 and 14 are shown in Fig. 3. Peaks 12 and 14 can be assigned to azadirachtin A and B, respectively, although their protonated molecule peaks (i.e., $[M+H]^+$ at m/z 721 and m/z 663) are very small. The mass spectrum of azadirachtin A (peak 12) is in association with that reported by Otmar Schaaf et al [3]. The base peaks at m/z 703 for azadirachtin A and m/z 645 for azadirachtin B correspond to $[MH-H_2O]^+$, formed via elimination of a molecule of water from their protonated molecules. In fact, there are 3 hydroxyl groups that can be eliminated as water on the molecule skeletons (Fig. 1).

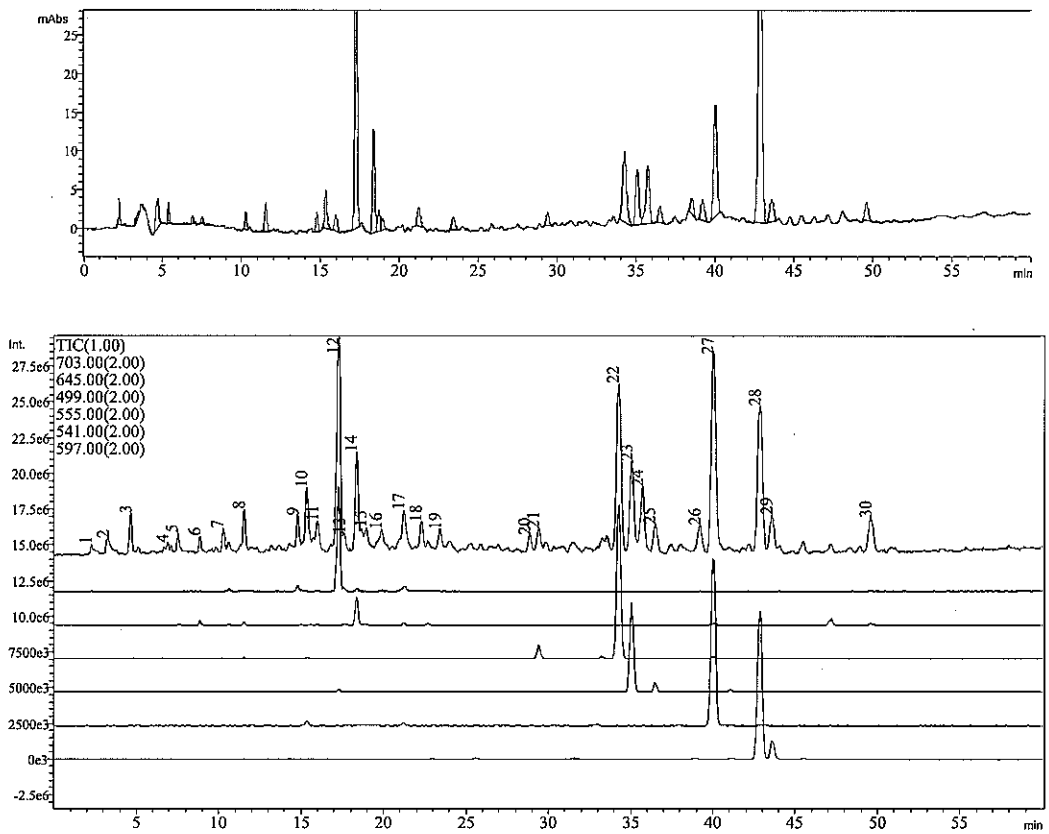

Fig. 2 UV chromatogram at 217 nm (a), MS-TIC and MS-MIC chromatograms (b) of neem extract sample.

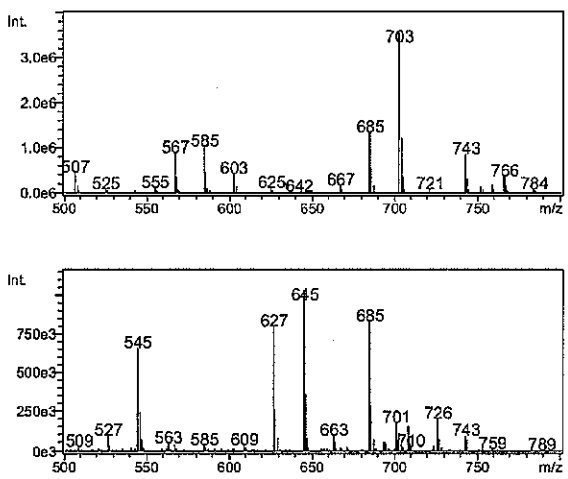

Fig. 3 Mass spectra of elute peaks 12 (a) and 14 (b).

The mass peaks of m/z 685, 667 for azadirachtin A and m/z 627, 609 for azadirachtin B are due to the protonated molecules of losing 2 and 3 water molecules, respectively. The mass peaks at much lower range, i.e., m/z 585 567 for azadirachtin A and m/z 545, 527 for azadirachtin B are resulted from further elimination of tiglic acid to form $[MH-H_2O-C_5H_8O_2]^+$ and $[MH-2H_2O-C_5H_8O_2]^+$, respectively. The mass peaks appeared at higher mass range, m/z 743 for azadirachtin A and m/z 685 for azadirachtin B, are due to sodium adduct ion $[M+Na]^+$ [3].

The mass spectra of peaks 7~18 (except peak 9 & 17) were found to have a common pattern as summarized in Table 2. First, there are three mass peaks with mass spans of 40 and 18 from the base mass peak (middle, m/z = P). For azadirachtin A, these three characteristic mass peaks are m/z 703(P), 743(P+40) and 685 (P-18); and m/z 645 (P), 685 (P+40) and 627 (P-18) for azadirachtin B. Second, the mass peak of protonated molecules of both compounds, which intensity were very low, appeared at m/z = P+18. In other words, the base peak, P, is actually due to the protonated molecule after eliminating a water molecule in the ionization process. The mass peak of "P-18" is due to eliminating a second water molecule and the "P+40" due to the adduct ions with Na^+, i.e., $[M+Na]^+$. It seems that azadirachtin molecules have a strong tendency to form sodium adduct ions [3]. Third, two mass peaks at low mass range were observed. They are due to further elimination of a TgOH molecule (molar mass = 100) from either P or P-18 ions.

To date, 10 isomeric azadirachtins, namely, azadirachtin A, B, D, E, F, G, H, I, K and L, have been isolated from neem and their structures have been established [1]. It is worth to note that each of the azadirachtins has 2 to 3 hydroxyl groups and a tigloyl group on the molecular ring-skeletons and these groups are eliminable. The common pattern of the mass spectra of peaks 7~18 (except peaks 9 & 17) suggests that they correspond to different isomeric azadirachtins or the analogues. This finding enables us to find six azadirachtins, i.e., type A, B, I, D, L and F in the sample as shown in Table 2. In addition, peak 8 is believed to correspond to either azadirachtin G or H, which share a same MW (662).

Table 2 Identification of isomeric azadirachtins in neem extract sample

Peak No	Characteristic Mass Peaks							Azadirachtin Found	
	P-136	P-118	P-100	P-60	P-18	P	P+40	Type	MW
7	-	483	501	-	583	601	641	I	618
8	-	527	545	-	627	645	685	G or H	662
10	523	541	559	-	641	659	699	D	676
12	567	585	-	-	685	703	743	A	720
14	-	527	545	-	627	645	685	B	662
15	-	-	-	627	669	687	727	L	704
16	-	529	547	-	629	647	687	F	664
Ions Assigned	$[MH-3H_2O-TgOH]^+$	$[MH-2H_2O-TgOH]^+$	$[MH-H_2O-TgOH]^+$	$[MNa-TgOH]^+$	$[MH-2H_2O]^+$	$[MH-H_2O]^+$	$[MNa]^+$		

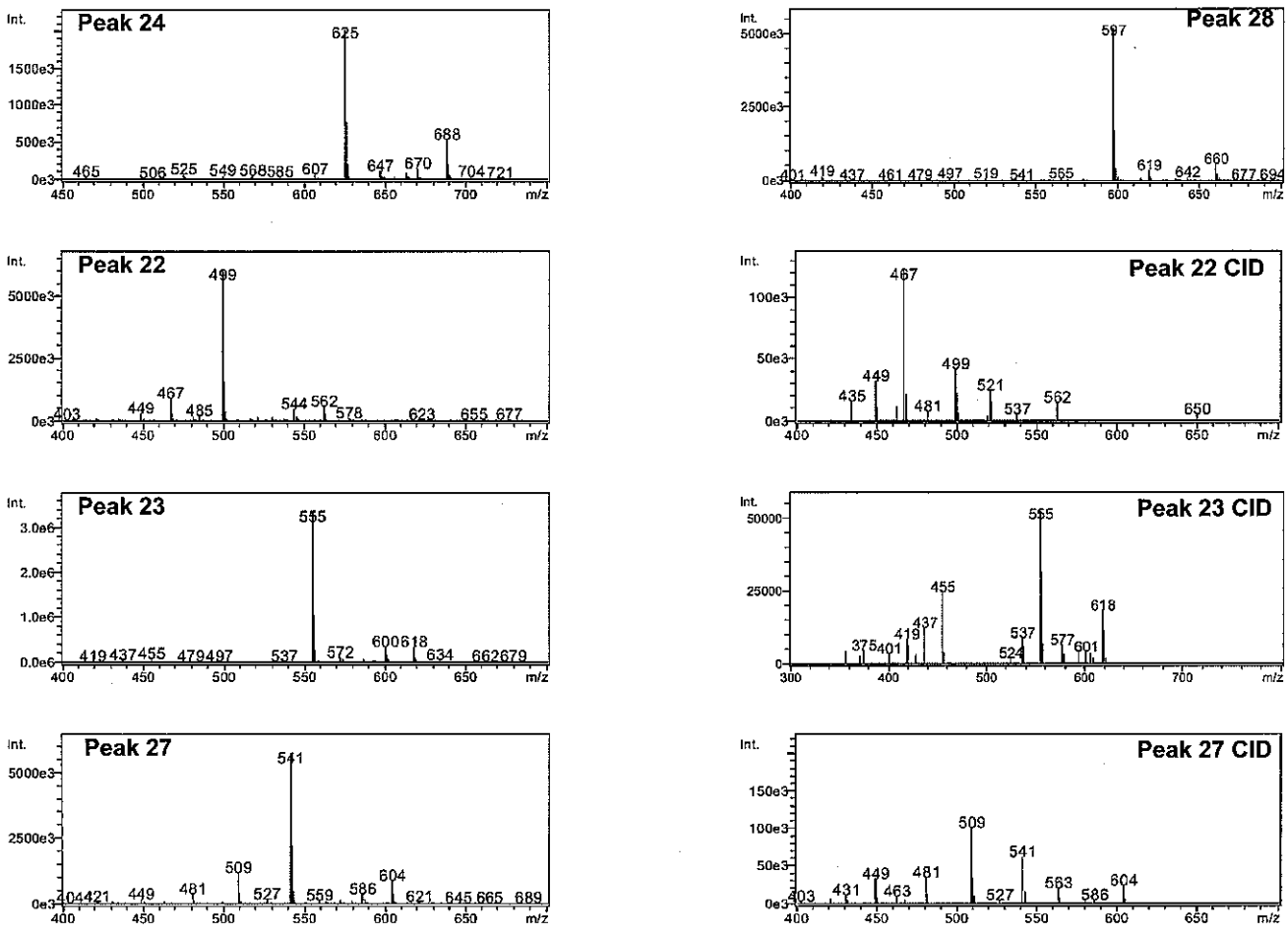

Fig. 4 Mass spectra of major elute peaks under normal and CID conditions.

Identification of other triterpenoids

Figure 4 shows the mass spectra of other 5 main peaks. The identification of these major components seems to be quite straightforward from their "simple" mass spectra, each with only a base mass peak corresponding to the protonated molecule. We tabulated all the triterpenoids isolated from neem according to their molecular weight (MW) [1]. It has been found that that for protonated molecule with m/z 625 there is only one candidate, i.e., ohichinolide-B with a MW of 624; and for m/z 597 also only one candidate, i.e., salannin with a MW of 596. Therefore, peak 24 and peak 28 are identified as ohichinolide-B and salannin, respectively (Table 3). However, as can be seen in Table 4, there are four candidates for m/z 499, three for m/z 555 and three for m/z 541. The sharing of identical MW by several compounds makes difficult in their identification.

Table 3 Main components found in neem sample

Peak No	m/z (+)	Compounds	Formula	M/W
12	685	Azadirachtin A	$C_{35}H_{44}O_{16}$	720
14	645	Azadirachtin B	$C_{33}H_{42}O_{14}$	662
22	499	6-Desacetyl nimbin	$C_{28}H_{34}O_8$	498
23	555	3-Desacetyl salannin or 2,3-dehydrosalannol	$C_{32}H_{42}O_8$	554
24	625	Ohichinolide-B	$C_{35}H_{44}O_{10}$	624
27	541	Nimbin	$C_{30}H_{36}O_9$	540
28	597	Salannin	$C_{34}H_{44}O_9$	596

s – suspected

Table 4 Neem compounds with identical MW

MW	Compound & Formula			
498	6-Desacetyl nimbin $C_{28}H_{34}O_8$	6-O-Acetylnimbadiol $C_{28}H_{34}O_8$	Nimocin $C_{33}H_{38}O_4$	1-α-Methoxy-1,2-dihydronimbinin $C_{29}H_{38}O_7$
540	Nimbin $C_{30}H_{36}O_9$	4-Epinimbin $C_{30}H_{36}O_9$	Isonimolicinolide $C_{30}H_{36}O_9$	-
554	3-Desacetyl salannin $C_{32}H_{42}O_8$	2,3-dehydrosalannol $C_{32}H_{42}O_8$	Vilasinin triacetate $C_{32}H_{42}O_8$	-

The mass spectra obtained under CID condition (Fig. 4) provide a solution to this difficulty. For peak 22, the fragments under CID were found at m/z 481, 467, 449 and 435. First, the fragment of m/z 481 and 467 can be attributed to $[MH-H_2O]^+$ and $[MH-CH_3OH]^+$, respectively. They were resulted from elimination of H_2O and CH_3OH from the molecule. Second, the fragments of m/z 449 & 435 can be attributed to $[MH-CH_3OH-H_2O]^+$ and $[MH-2CH_3OH]^+$, respectively. In the four possible structures, only the first two can lose water molecule, and only the first one can lose two molecules of CH_3OH to give m/z435 ion. Thus, peak 22 is believed to correspond to 6-desacetyl nimbin.

For peak 23, the fragments observed under CID included ions of m/z 537, 455, and 437. The fragment of m/z 455 can be attributed to $[MH-TgOH]^+$. Vilasinin has not a TgO- group, thus, its presence can be excluded. The mass peak at m/z 537 is due to $[MH-H_2O]^+$ ion. However, we cannot further distinguish the two structures since their similarity in structure and function groups.

For peak 27, the fragments observed under CID condition include m/z 509, 481and 449. They can be assigned to $[MH-CH_3OH]^+$, $[MH-CH_3COOH]^+$ and $[MH-CH_3OH-CH_3COOH]^+$, respectively. These fragments formed via elimination to lose CH_3OH (-32), CH_3COOH (-60) or both (-92) from their parent molecule. Both nimbin and 4-epinimbin are possible to generate these fragments, but isonimolicinolide can generate only $[MH-CH_3COOH]^+$. Hence, the presence of isonimolicinolide is excluded. In fact, nimbin and 4-epinimbin are stereoisomers with the -COOMe group pointing to above or below the molecule plane. They cannot be separated by a reversed-phase column and identified by UV and/or MS detectors. However, the natural occurrence of 4-epinimbin in the neem tree is only at trace level. It is believed that peak 27 is contributed mainly by nimbin.

Conclusions

The results show that LC-ESI-MS provides a rapid method for detection and identification of neem compounds in neem extracts. All the major components, isomeric azadirachtins and some minor components (not present) were identified from the mass spectra of the elute peaks without use of any standard. By using the CID, structure information of some compounds were obtained and used to distinguish the compounds with identical MW. The mass spectra of azadirachtins were found to have a common pattern. This characteristic pattern was used successfully in identification of the isomeric azadirachtins.

References

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