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Comparison of chromatographic systems for triterpenoids from Neem (*Azadirachta indica*) seeds

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Abstract

Chromatographic conditions for the isolation and separation of twelve triterpenoids from Neem seeds, including azadirachtin and six closely related compounds are described. The elution order of the compounds using supercritical fluid chromatography and reverse phase HPLC are described. New and corrected NMR spectroscopic data for eleven of these compounds are tabulated.

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

1. Introduction

The Neem tree (*Azadirachta indica*), originally from India and Pakistan, is a fast-growing tree which is now common throughout large areas of the drier tropics. This tree has received much attention in recent years [1–3], thanks to its many valuable properties, but chiefly because of the remarkable insecticidal properties of the substance azadirachtin (Fig. 1) found in its seed kernels [4–6]. In order to assess the value of the crop and to produce an insecticidally active extract, it is important to know the azadirachtin content of seeds or extracts of them. However, the compound is not easily separated from the many compounds of similar structure and polarity, of which over 100 have been isolated [6]. The analysis for azadirachtin is most readily achieved by a chromatographic method using UV detection, since it and its close relatives all have UV chromophores. The problem then is to recognize the azadirachtin peak among the many absorptions seen, and to identify what the other substances are.

The formulation and registering of Neem extracts as commercial insecticide products has necessitated the identification of more of the components present in the mixtures obtained from the seeds. At present there exists no procedure for separating more than a few of these substances, and the descriptions for their isolation are scattered through a number of sources. No single procedure describing the isolation of the major triterpenoids exists. Such information is necessary to obtain the pure reference compounds needed for identification. The Neem triterpenoids can only be identified with certainty with the aid of their NMR spectra, we have therefore tabulated the necessary data from various sources, giving in one place a procedure for isolation, identification and chromatographic analysis for twelve important compounds.

Several groups of authors have described preparative HPLC systems for isolating pure azadirachtin, and some of its derivatives and related compounds [7–11]. The separations are efficient, and give pure

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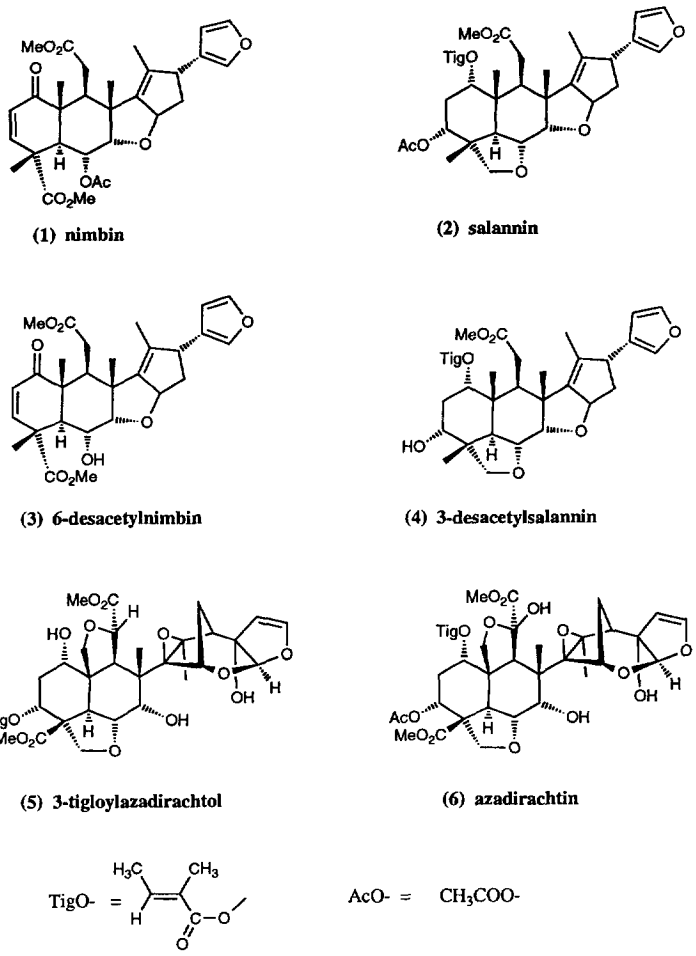
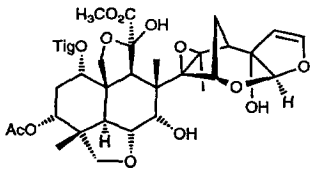


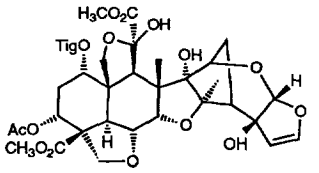
Fig. 1. Structures of compounds.

compounds, (azadirachtin was first obtained in a truly crystalline form in this way [12]), but are costly in their consumption of solvent. The large scale separation of Neem triterpenoids by high-speed counter-current chromatography was reported by Hummel et al. at the 1995 Pittsburgh Conference, held at New Orleans, USA. We have described a new HPLC–NMR method for separating and identifying Neem triterpenoids on an analytical scale [13].

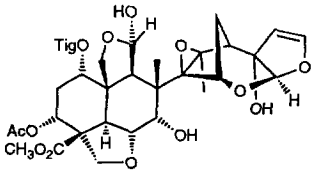
There is some confusion in the literature over the names used for the Neem triterpenoids. Rembold reported that azadirachtin was an impure substance and claimed to separate it into four isomers which he labelled A, B, C and D. He also reported the isolation of three other compounds which he called azadirachtins E, F and G [14,15]. Although structures were proposed for these compounds, no spectroscopic evidence for them has been published. Azadirachtin as isolated by Butterworth and Morgan [4,16] was a single substance. The other compounds reported by Rembold [14] are not isomeric since they have different molecular formulae. The substance called azadirachtin B by Rembold had already been described as 3-tigloylazadirachtol by Kraus's group [17]. Azadirachtins C, E and G remain incompletely described. A sample of azadirachtin F, kindly supplied by H. Kleeberg was found to be identical in retention on supercritical fluid chromatography (SFC) and HPLC and ^{1}H NMR spectrum to azadirachtin H [7]. Since the complete spectral data has been supplied for azadirachtin H but no spectral data was published for azadirachtin F, the name



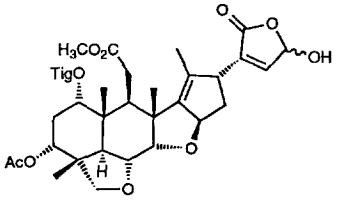
(7) azadirachtin D



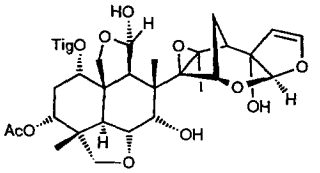
(8) 3-acetyl-1-tigloylazadirachtinin (11*S*)-epimer



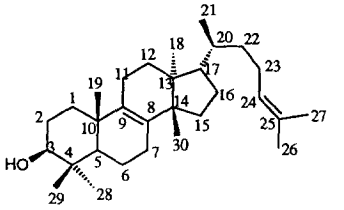
(9) azadirachtin H



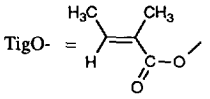
(10) salanninolide



(11) azadirachtin I



(12) tirucallol showing the numbering of this series



TigO- =

AcO- = CH_3COO-

Fig. 1. (*continued*)

azadirachtin H takes precedence for compound **9**, described here.

2. Materials and methods

The light petroleum used throughout was the fraction with b.p. 60–80°C.

2.1. Preparation of triterpenoid extract

Neem seeds (typically 2 kg) of Indian, Ghanaian or Nigerian origin, were de-oiled by grinding in light petroleum (2 dm^3) using a high speed mixer (Linshear Mixers, Stourbridge, UK) and the mixture filtered under vacuum. The resultant meal was re-suspended in light petroleum (2 dm^3) and the grinding and filtering repeated twice more. The combined petroleum extracts were discarded. The meal was suspended in methanol (2 dm^3) and left to soak overnight, then filtered under vacuum, and the meal re-extracted with two further portions of methanol (2 dm^3 each). The combined methanolic filtrate was concentrated to approximately 0.5 dm^3 at 30°C under vacuum.

2.2. Preparative chromatography

A modification of the procedure of Butterworth and Morgan [4] was used. Florex RVM [approx. 200 g, 60–100 mesh, (150–250 μm particle diameter), Whitecourt, London, UK] was added to the above

methanolic extract. The solvent was removed at 30°C under water-pump vacuum in a rotary evaporator. Any residual solvent was removed using an oil pump, leaving the extract coated on the Florex as a free-flowing powder. This powder was placed on the top of a Florex RVM column (ideally 30 g Florex per g Neem extract, typically a 1 kg column) equilibrated in light petroleum.

The column was eluted successively with light petroleum (2.5 dm^3), light petroleum–diethyl ether (1:1, v/v) (5 dm^3), diethyl ether (5 dm^3), diethyl ether–acetone (4:1, v/v) (5 dm^3) and finally with methanol (2.5 dm^3). A single fraction was collected for each solvent composition. These fractions were evaporated to dryness and re-chromatographed on smaller columns to isolate the individual compounds.

2.3. Isolation of nimbin, salannin and 6-desacetylnimbin

The material eluted with light petroleum–diethyl ether (1:1, v/v), was fractionated on a smaller Florex RVM column (100 g), eluting it with light petroleum–diethyl ether (7:3, v/v). Early eluting fractions contained impure nimbin (**1**), which was purified by recrystallization from methanol. Later eluting fractions contained impure salannin (**2**). This was purified by rechromatography using the same solvent system. Later fractions from the Florex column contained impure 6-desacetylnimbin (**3**), which was purified by crystallization from methanol.

2.4. Isolation of 3-desacetylsalannin and 3-tigloylazadirachtol

The material eluted from the large Florex column with diethyl ether (100%), was fractionated on a smaller Florex RVM column, eluting it with light petroleum–diethyl ether (1:4, v/v). Early eluting fractions contained impure 3-desacetylsalannin (**4**), which was purified by recrystallization from ethyl acetate. Later fractions contained impure 3-tigloylazadirachtol (**5**). This was purified by rechromatography using the same solvent system.

2.5. Isolation of azadirachtin and azadirachtin D

The material removed from the large Florex column with diethyl ether–acetone (4:1, v/v) was rechromatographed on a smaller Florex column, eluting with diethyl ether–acetone (49:1, v/v). Early fractions contained pure azadirachtin (**6**). Changing the solvent ratio to diethyl ether–acetone (9:1, v/v) gave impure azadirachtin D (**7**). This was purified by semi-preparative HPLC. Using an isocratic solvent system (acetonitrile–water, 7:13, v/v), azadirachtin D eluted after ~27 min. Other HPLC conditions are described in Section 2.9 below.

2.6. Isolation of salanninolide and 3-acetyl-1-tigloylazadirachtinin

The material removed from the large Florex column with methanol was enriched in compounds eluting both before and after azadirachtin in our SFC system.

Evaporation of the methanol fraction to dryness and recrystallizing the residue from ethyl acetate gave pure 3-acetyl-1-tigloylazadirachtinin (**8**). Then evaporating the mother liquors to dryness and recrystallizing the residue from methanol gave pure salanninolide (**10**).

2.7. Isolation of azadirachtin H, azadirachtin I and SL2

The methanol mother liquors above, after isolating 3-acetyl-1-tigloyl-azadirachtinin and salanninolide were further treated by semi-preparative HPLC. The column was eluted isocratically with acetonitrile–water (3:7, v/v) for 22 min, then with a gradient of acetonitrile–water from 3:7 to 1:1 (v/v) over a 5-min period, then held at 1:1 for 7 min. Two peaks were detected at ~24 and ~26 min. The peak at ~26 min was identified as azadirachtin H (**9**). The peak eluting at ~24 min was separated into two components on SFC. The two were separated by crystallization from methanol, giving azadirachtin I (**11**) as white needles and leaving compound SL2 in solution.

2.8. Supercritical fluid chromatography

The extract from the first Florex column (in methanol, 20 μl) was analysed on a Spherisorb cyanopropyl silica column (150×4.6 mm I.D.) of 5

μm particle size (HPLC Technology, Macclesfield, UK) using an LDC Analytical supercritical fluid chromatograph (Thermo Separation Products, Stone, UK), as described earlier [18]. Separations were carried out using isocratic conditions at a flow-rate of 2 cm^3 min^{-1}, using carbon dioxide–methanol (24:1, v/v, 6.5 mol% methanol) at 3000 p.s.i. (207 bar, 20.6 MPa) and 55°C. Ultraviolet detection was at 217 nm.

2.9. High-performance liquid chromatography

The extract from the first Florex column was analysed on a Sphersorb ODS column (250×4.6 mm I.D.) of 5 μm particle size (HPLC Technology). Separations were carried out using a mobile phase of acetonitrile–water with the following gradient: 0 to 15 min, 32% CH_3CN; 15 to 20 min, 32 to 55% CH_3CN; 20 to 50 min, 55% CH_3CN, at flow-rate of 1 cm^3 min^{-1}, injecting 20 μl of a methanol solution. Detection was at 217 nm using a Unicam UV detector (Pye, Cambridge, UK).

3. Results and discussion

Twelve major polar triterpenoids present in Neem seed kernels have been isolated by column chromatography, with eleven of them identified spectroscopically. The chromatographic behaviour of these compounds has been determined in SFC and HPLC systems.

Silica [14,19,20], alumina [4,21] and Florex RVM have all been used as gravity column packing materials for displacement chromatography of Neem extracts. For bulk separation of Neem triterpenoids, we have long recommended the use of Florex RVM [4,13,22], an attapulgite clay, available as a sieved powder suitable for chromatography. It is less retentive than silica or alumina and appears to degrade the Neem triterpenoids less than the other packings. In all cases, the longer the compounds are left in contact with the packing, the greater the loss through degradation. Using the large Florex column, the crude triterpenoid extract was separated into four fractions (Table 1), each fraction was then separated further to give the twelve substances listed.

All of the main triterpenoids present in the seeds, nimbin (**1**), salannin (**2**), 6-desacetylnimbin (**3**), 3-desacetylsalannin (**4**) and azadirachtin (**6**) (Fig. 1) are readily separated using the solvent systems given, but five of the minor triterpenoids are only eluted with methanol. For identification, infrared and mass spectra were used, but identification in this group depends largely upon ^1H NMR spectra, which were recorded at 270 MHz or 500 MHz.

The NMR spectral data of the Neem triterpenoids known up to 1988 are tabulated [23], but the remainder are scattered through the literature, they have therefore been gathered together in Table 2.

Table 1
Substances identified by fractionation of Neem seed triterpenoids on a Florex column

Elution solvent	Material contained	Ref.
Light petroleum	Residual triglyceride oil	–
Light petroleum–diethyl ether (1:1)	Nimbin	[20,21,35,36]
	Salannin	[37–39]
	6-Desacetylnimbin	[20,40]
Diethyl ether	3-Desacetylsalannin	[19]
	3-Tigloylazadirachtol	[12,14,17]
Diethyl ether–acetone (4:1)	Azadirachtin	[4,12,16,41,42]
	Azadirachtin D	[7,14]
Methanol	3-Acetyl-1-tigloylazadirachtinin	[25–27,43]
	Salanninolide	[28,29]
	Azadirachtin H	[8]
	Azadirachtin I	[8]
	SL2 (unidentified,related to azadirachtin)	–

Table 2
^{1}H (at 270 MHz) and ^{13}C (at 62.89 MHz) NMR spectral data for nimbin (**1**), salannin (**2**), 6-desacetylnimbin (**3**), 3-desacetylsalannin (**4**) and azadirachtin (**6**)

Position	Nimbin (**1**)		Salannin (**2**)		6-Desacetyl-nimbin (**3**)		3-Desacetyl-salannin (**4**)		Azadirachtin (**6**)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	–	–[a]	4.80 (t)	71.39	–	202.17	5.30 (t)	72.82	4.77 (m)	70.58
2	5.58 (d)	125.96	–	27.64	5.86 (d)	126.40	–	30.46	–	29.73
2-Ha	–	–	–[b]	–	–	–	–[b]	–	2.32 (m)	–
2-Hb	–	–	–[b]	–	–	–	–[b]	–	2.26 (m)	–
3	6.34 (d)	147.58	4.97 (t)	71.39	6.42 (d)	148.10	3.88 (m)	70.79	5.51 (br t)	67.00
4	–	47.77	–	42.79	–	47.78	–	44.24	–	52.76
5	3.70 (d)	41.53	2.83 (d)	40.03	3.40 (d)	43.68	2.73 (d)	38.85	3.34 (d)	36.96
6	5.22 (dd)	68.66	4.00 (dd)	72.68	3.93 (dd)	66.10	4.01 (dd)	72.48	4.62 (dd)	73.95
7	4.05 (d)	84.53	4.18 (d)	85.78	4.03 (d)	86.92	4.18 (d)	85.89	4.77 (m)	74.28
8	–	47.08	–	49.15	–	47.33	–	48.99	–	45.56
9	2.87 (m)	38.59	2.76 (dd)	39.50	2.77 (m)	39.06	2.64 (m)	39.51	3.34 (s)	44.69
10	–	47.93	–	40.69	–	47.49	–	40.96	–	50.24
11	–	34.23	–	30.73	–	34.33	–	30.54	–	104.20
11-Ha	2.25 (m)	–	–[b]	–	2.25 (m)	–	–[b]	–	–	–
11-Hb	2.94 (m)	–	–[b]	–	2.93 (m)	–	–[b]	–	–	–
12	–	173.64	–	172.81	–	173.66	–	172.66	–	171.83
13	–	135.08	–	134.89	–	134.83	–	134.89	–	68.70
14	–	146.16	–	146.69	–	146.89	–	146.61	–	70.04
15	5.57 (m)	87.10	5.44 (m)	87.98	5.56 (m)	87.49	5.39 (m)	87.89	4.67 (d)	76.70
16	–	41.59	–	41.50	–	41.47	–	41.32	–	25.07
16-Ha	2.02 (m)	–	–[b]	–	2.03 (m)	–	–[b]	–	–[d]	–
16-Hb	2.19 (m)	–	–[b]	–	2.21 (m)	–	–[b]	–	1.31 (d)	–
17	3.61 (m)	49.47	3.66 (d)	49.52	–[c]	49.62	3.60 (d)	49.42	2.38 (d)	48.63
18	1.69 (d)	12.83	1.67 (d)	13.04	1.69 (s)	12.84	1.66 (d)	13.06	2.01 (s)	18.47
19	1.28 (s)	16.66	0.99 (s)	15.15	1.22 (s)	16.39	0.96 (s)	15.21	–	69.02
19-Ha	–	–	–	–	–	–	–	–	3.63 (d)	–
19-Hb	–	–	–	–	–	–	–	–	4.15 (d)	–
20	–	126.81	–	127.14	–	126.83	–	127.16	–	–[e]
21	7.32 (m)	139.01	7.33 (m)	142.95	7.33 (m)	139.00	7.32 (m)	138.77	5.65 (s)	108.55
22	6.33 (m)	110.48	6.30 (m)	110.65	6.33 (m)	110.46	6.28 (m)	110.69	5.05 (d)	107.56
23	7.23 (m)	143.01	7.26 (m)	138.86	7.24 (m)	143.02	7.25 (m)	142.87	6.46 (d)	146.88
28	–	174.59	–	77.73	–	175.66	–	77.86	–	72.92
28-Ha	–	–	3.72 (m)	–	–	–	4.14 (d)	–	3.75 (d)	–
28-Hb	–	–	3.58 (m)	–	–	–	3.63 (d)	–	4.08 (d)	–
29	1.36 (s)	17.19	1.22 (s)	19.65	1.59 (s)	16.39	1.16 (s)	19.90	–	173.63
30	1.34 (s)	16.70	1.30 (s)	16.95	1.29 (s)	17.13	1.31 (s)	16.90	1.75 (s)	21.40
3-OH	–	–	–	–	–	–	–[b]	–	–	–
6-OH	–	–	–	–	2.19 (m)	–	–	–	–	–
7-OH	–	–	–	–	–	–	–	–	2.83 (br s)	–
11-OH	–	–	–	–	–	–	–	–	5.03 (d)	–
20-OH	–	–	–	–	–	–	–	–	2.89 (br s)	–
12-OCH_3	3.65 (s)	51.66	3.24 (s)	51.48	3.66 (s)	51.60	3.19 (s)	51.38	3.69 (s)	52.76
28-OCH_3	3.73 (s)	53.05	–	–	3.70 (s)	52.93	–	–	–	–
29-OCH_3	–	–	–	–	–	–	–	–	3.80 (s)	53.24
OAc	2.04 (s)	170.58	1.94 (s)	170.33	–	–	–	–	1.95 (s)	169.79
		20.96		20.85						20.80
1′	–	–	–	166.78	–	–	–	166.32	–	166.30
2′	–	–	–	129.17	–	–	–	128.72	–	128.56
3′	–	–	6.96 (m)	137.16	–	–	6.92 (m)	137.90	6.92 (m)	137.89
4′-CH_3	–	–	1.81 (dd)	14.34	–	–	1.84 (dd)	12.19	1.78 (dd)	14.34
5′-CH_3	–	–	1.94 (s)	11.93	–	–	1.92 (s)	14.53	1.86 (s)	11.94

Recorded in C^2HCl_3 with TMS at δ 0.00.
[a] Not recorded in spectrum, should appear at δ 201.73. [b] Signal appears between δ 2.05–2.35. [c] Masked by signal at δ 3.66. [d] Masked by signal at δ 1.75. [e] Masked by C^2HCl_3.

Table 3
^{1}H NMR (at 270 MHz) spectral data of minor triterpenoids found in Neem seeds

Position	3-Tigloyl-azadirachtol (**5**)	Azadirachtin D (**7**)	Salanninolide (**10**)	Azadirachtin H (**9**)	Azadirachtin I (**11**)	3-Acetyl-1-tigloyazadirachtinnin (**8**)
1-H	3.53 (m)	4.93 (m)	4.90 (t)[b]	5.38 (t)[b]	4.97 (t)	4.74 (t)
2-Ha	3.33 (m)	–[d]	–[f]	–[g]	2.21 (m)	–
2-Hb	2.08 (m)	–[d]	–[f]	–[g]	2.23 (m)	–[j]
3-H	5.55 (t)	5.11 (m)	4.99 (t)[b]	5.54 (t)[b]	5.36 (m)	5.51 (t)
5-H	3.33 (m)	3.13 (d)	2.73 (d)	3.37 (d)	3.12 (d)	3.18 (d)
6-H	4.54 (m)	4.70 (m)	3.98 (dd)	4.47 (dd)	4.07 (dd)	4.43 (dd)
7-H	4.74 (d)	4.70 (m)	4.26 (d)	4.66 (m)	4.62 (m)	4.45 (d)
9-H	3.19 (m)	3.35 (s)	2.53 (dd)	2.65 (s)	2.64 (s)	3.55 (s)
11-H	4.48 (br s)	–	–[f]	5.42 (m)	5.36 (m)	–
15-H	4.58 (d)	4.68 (m)	5.43 (m)	4.59 (br d)	4.58 (m)	4.18 (br s)
16-Ha	–[a]	–[e]	–[f]	1.70 (m)	1.70 (m)	–[k]
16-Hb	–[a]	1.34 (d)	–[f]	–[a]	1.30 (m)	–[k]
17-H	2.36 (d)	2.39 (d)	3.61 (d)	–[g]	2.35 (d)	–[j]
18-CH_3	2.05 (s)	2.07 (s)	1.84 (s)	1.99 (s)	1.97 (s)	1.55 (s)
19-CH_3	–	–	0.94 (s)	–	–	–
19-Ha	3.48 (m)	3.84 (d)	–	3.76 (d)	–[i]	–[l]
19-Hb	3.95 (m)	4.21 (d)	–	4.10 (d)	–[i]	4.27 (d)
21-H	5.67 (s)	5.71 (s)	–	5.67 (s)	5.69 (s)	5.67 (s)
22-H	5.03 (d)	5.04 (d)	6.76 (s)	5.06 (d)	5.04 (d)	4.88 (d)
23-H	6.43 (d)	6.45 (d)	5.96 (d)	6.46 (d)	6.44 (d)	6.38 (d)
28-Ha	3.85 (d)[b]	3.80 (d)[b]	3.49 (d)[b]	–[h]	–[i]	–[l]
28-Hb	4.04 (d)[b]	4.16 (d)[b]	3.73 (d)[b]	–[h]	–[i]	4.05 (d)
29-CH_3	–	1.06 (s)	1.21 (s)	–	1.07 (s)	–
30-CH_3	1.46 (s)	1.75 (s)	1.30 (s)	1.33 (s)	1.33 (s)	1.70 (s)
1-OH	3.41 (br s)	–	–	–	–	–
7-OH	–[c]	2.99 (br s)	–	2.71 (br s)	2.90 (br s)	–
11-OH	–	5.11 (s)	–	3.10 (br d)	3.24 (br d)	–[m]
14-OH	–	–	–	–	–	–[n]
20-OH	2.88 (br s)	3.37 (br s)	–	2.95 (br s)	3.06 (br s)	6.11 (s)
23-OH	–	–	5.31 (d)	–	–	
12-CO_2Me	3.76 (s)	3.70 (s)	3.45 (s)	–	–	3.70 (s)
29-CO_2Me	3.76 (s)	–	–	3.80 (s)	–	3.77 (s)
3-OAc	–	1.94 (s)	2.00 (s)	1.86 (s)	1.88 (s)	2.00 (s)
3′-H	6.96 (m)	6.90 (m)	6.98 (m)	6.96 (m)	6.95 (m)	6.91 (m)
4′-CH_3	1.79 (dd)	1.78 (dd)	1.84 (br d)	1.78 (dd)	1.77 (dd)	1.82 (m)
5′-CH_3	1.85 (d)	1.85 (s)	1.91 (br s)	1.85 (m)	1.84 (m)	1.87 (d)

Recorded in C^2HCl_3 with TMS at δ 0.00.
[a] Masked by impurity at δ 1.26.
[b] These assignments may be reversed.
[c] Masked by signal at δ 3.33.
[d] Identification uncertain, should occur between δ 2.10 and δ 2.35.
[e] Masked by signal at δ 1.75.
[f] Identification uncertain, should occur between δ 2.05 and δ 2.45.
[g] Identification uncertain, should occur between δ 2.36 and δ 2.40.
[h] Masked by singlet at δ 3.80.
[i] Identification uncertain, should occur between δ 3.65 and δ 3.75.
[j] Identification uncertain, should occur between δ 2.10 and δ 2.30.
[k] Identification uncertain, should occur between δ 1.80 and δ 1.90.
[l] Masked by signal at δ 3.70.
[m] The 11-OH was not determined.
[n] Masked by signal at δ 4.43.

The ^{1}H NMR spectra of the minor triterpenoids are given in Table 3. In some cases the spectral data differ from that previously recorded. The original data for azadirachtin H [8] has recently been corrected [24]. The numbering of these compounds is based on that of tirucallol (**12**) their presumed biosynthetic progenitor.

Compound **8**, the rearranged product of azadirachtin, has been isolated from a plant source for the first time. This type of structure (actually the 11-methoxy derivative) was first described by Kraus as methyl 1-tigloyl-11-hydroxy-meliatinin-4-carboxylate [25] and then as 3-acetyl-1-tigloyl-azadirachtinin [26], which name was used by Ley et al. in 1988 [27], who first prepared compound **8** from azadirachtin under acidic conditions, through opening of the epoxide ring. Compound **8** is readily epimerized at C_{11}, and so a mixture of epimers is often encountered. In later papers Ley refers to these as (11*R*)- and (11*S*)-azadirachtinin. Compound **10** has a γ-hydroxybutenolide ring in place of the furan ring of salannin. This compound has also been isolated from *Melia dubia* seeds [28], Neem seeds [29] and formed as a photo-oxidation product of salannin ([28,30]; Johnson et al. in preparation). The compound has been variously called compositolide [28], photosalannin [28] and salannolide [29], but to be consistent with the system of trivial names given to other compounds containing the hydroxybutenolide structure, we refer to this compound as salanninolide.

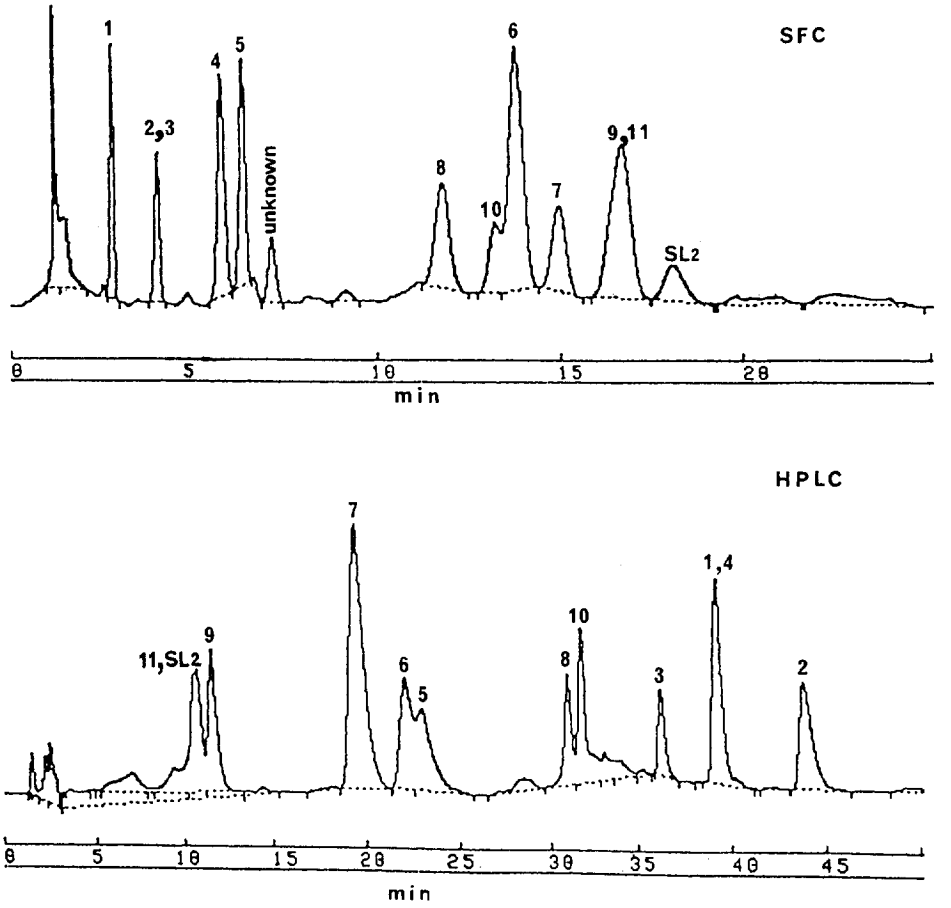


Fig. 2. Chromatograms of triterpenoids found in Neem seeds and discussed here. Above, SFC and below HPLC. The numbers of peaks correspond to the numbering in Fig. 1, the structure of the compound SL2 has not yet been determined. The peak areas do not represent their natural abundance in seeds, as the minor components have been enriched to make them more easily visible.

Govindachari et al. describe a further compound which they call azadirachtin K [31], which we have not isolated, and 11-acetylazadirachtin H, known as marrangin, from *Azadirachta excelsa* [32] has not been found in Neem extracts.

4. Conclusions

There have been a number of reports on HPLC systems for analysis of azadirachtin (quoted in Refs. [12,33]). For such complex mixtures as the neem triterpenoids, the use of both SFC and HPLC techniques has advantages as shown in Fig. 2. The cyanopropyl silica phase used for SFC generally behaves as a normal-phase absorbent. This is indicated by the similarity of elution order of the five main triterpenoids in the SFC system (Fig. 2) compared with the Florex RVM chromatography column (Table 4). Small but useful differences are seen in the two systems, in particular, 3-acetyl-1-tigloylazadirachtinin (**8**) and salanninolide (**10**) are strongly retained on Florex RVM, but both elute before azadirachtin on SFC. The reversed-phase HPLC system has a general reversal of order (Table 4) but small changes in order of elution indicate that factors other than just polarity affect retention. Azadirachtins H and I, unseparated in the SFC system are separated in RP-HPLC on an ODS column, while azadirachtin I and the unknown compound SL2 are unresolved. The unidentified analog of azadirachtin, SL2, is the most polar, on the SFC system, of the series of compounds isolated from seeds. However, in RP-HPLC it elutes second, after azadirachtin I. Nimbin and 3-desacetylsalannin are unresolved and azadirachtin and 3-tigloylazadirachtol are barely separated on the HPLC system (Fig. 2), but are resolved by over 8 min in SFC. An ethanol–water HPLC solvent system has also been suggested for separating this latter pair of compounds [34]. The limitations of SFC on the column chosen is shown in the case of salannin and 6-desacetylnimbin which were initially separated by only 0.2 min, and as the column aged, resolution decreased.

Earlier we reported that azadirachtin (**6**) and 3-tigloylazadirachtol (**5**) [17] eluted close together in SFC [18]. This was an error, more careful identification has shown that 3-tigloyazadirachtol (**5**) elutes well before azadirachtin itself. The mis-identified peak eluting before azadirachtin is 3-acetyl-1-tigloyl-azadirachtinin (**8**). Within the azadirachtin group, an hydroxyl at C_{11} is important in increasing polarity. This can be seen with azadirachtin eluting over 8 min later than 3-tigloylazadirachtol (**5**) on SFC. On the other hand a C_{28} carbomethoxy group appears to reduce polarity compared with a C_{28} methyl group. Azadirachtin D (**7**), (with a C_{28} methyl group) elutes after azadirachtin, and azadirachtin I (**11**) (C_{28} methyl) elutes after azadirachtin H (**9**) in SFC. The

Table 4
Elution order of the isolated triterpenoids using Florex RVM gravity column chromatography, SFC and reversed-phase HPLC

Elution order	Florex RVM gravity column	SFC	Reversed-phase HPLC
a	Nimbin	Nimbin	Azadirachtin I, SL2
b	Salannin	Salannin[b]	Azadirachtin H
c	6-Desacetylnimbin	6-Desacetylnimbin[b]	Azadirachtin D
d	3-Desacetylsalannin	3-Deacetylsalannin	Azadirachtin
e	3-Tigloylazadirachtol	3-Tigloylazadirachtol	3-Tigloylazadirachtol
f	Azadirachtin	3-Acetyl-1-tigloyl- azadirachtinin	3-Acetyl-1-tigloyl-azadirachtinin
g	Azadirachtin D, 3-acetyl-1-tigloyl-azadirachtinin[a], salanninolide[a], azadirachtin H[a], azadirachtin I[a], SL2[a]	Salanninolide	Salanninolide
h		Azadirachtin	6-Desacetylnimbin
i		Azadirachtin D	Nimbin, 3-desacetylsalannin
j		Azadirachtin H, azadirachtin I	Salannin
k		SL2	

[a] Eluted with methanol.
[b] Co-elute on older SFC column.

order is largely reversed in HPLC. Also, the lack of a methoxycarbonyl group at C_{11} causes azadirachtins H and I to elute after azadirachtin and azadirachtin D in SFC and before them in HPLC.

The chief differences between SFC and HPLC for this work is the much longer time required for an HPLC separation (43.6 min) and the much larger volume of solvent required. The SFC separation time is shorter (18.1 min) and the greater part of the mobile phase is provided by the relatively cheap, non-toxic and non-flammable carbon dioxide.

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