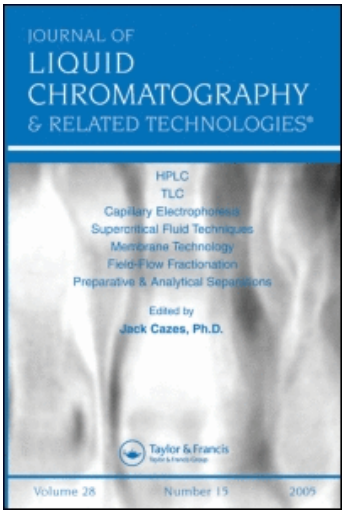

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A DIRECT PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PROCEDURE FOR THE ISOLATION OF MAJOR TRITERPENOIDS AND THEIR QUANTITATIVE DETERMINATION IN NEEM OIL

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ABSTRACT

Isolation of the major triterpenoids from neem oil in pure form by a direct preparative High performance liquid chromatography procedure is presented. As neem oil is an important component of Neem based insecticidal formulations, a simple, isocratic analytical HPLC procedure for quantitation (relative abundance) of these major bioactive triterpenoids has been developed.

INTRODUCTION

Products from the neem tree, *Azadirachta indica* A.Juss. (Meliaceae) have been used in India from ancient times, both in medicine as well as for protection of plants from insect attack. The first crystalline compound nimbin was isolated by Siddiqui from neem oil[1]. But it was only after the isolation by Butterworth and Morgan[2] from neem kernels of azadirachtin, which was shown to be a potent antifeedant for the desert locust *Schistocerca gregaria* at a concentration of 40μg/l, that intense interest was aroused in the chemical investigation of *A. indica*. More than one hundred compounds have been isolated from all parts of the plant, many of them biologically active[3,4,5].

Azadirachtin A has been shown to possess antifeedant activity at concentrations of 10-100 ppm and also ecdysis inhibition activity at concentrations of 1-10 ppm in over two hundred species of insects[6]. Since azadirachtin and its congeners are non-toxic, a number of formulations have been developed for use in agriculture, some based on neem kernel extract and many others on neem oil. Formulations from neem oil contain less of the more polar compounds like azadirachtin A, compared to the kernel extract formulations.

Direct preparative HPLC has been used in this laboratory with great success for the isolation of Azadirachtins A,B,D,H, I and K from neem kernel extract[7,8,9]. During this study the less polar compounds in neem kernel extract such as salannin, nimbin, azadiradione, epoxy azadiradione and 6-deacetylnimbin have also been isolated and identified. In view of the wide use of formulations based on neem oil in India, it was of interest to isolate and identify principal triterpenoid compounds present in neem oil and also estimate their relative abundance. Major triterpenoids like salannin[10], nimbin[1,11], azadiradione, epoxyazadiradione[12], 6-deacetylnimbin[13] have been isolated at different times in different laboratories essentially by conventional column chromatography, using different adsorbents, eluents, and work-up procedures. In a very recent publication[14], HPLC analysis of neem oil using a semi-preparative reverse phase column has been carried out and a few major triterpenoids have been identified by HPLC-MS procedure. In this paper, we report the isolation of the major components of neem oil, in a single experiment by direct preparative HPLC as well as the estimation of the relative abundance of the major components.

MATERIALS AND METHODS

Preparative High Performance Liquid Chromatography was carried out using a Shimadzu LC8A HPLC system linked to CR4A data processor and the peaks detected at 215 nm. Two Shimpack reverse phase (C_{18}) preparative columns (25 cmx 50 mm i.d.) and (25 cm x 20 mm i.d.) were used for preparative runs and Shimpack reverse phase column (C_{18}) (25 cm x 4.6 mm) was used for analysis.

Neem oil, (1 lit) obtained by using a cold mechanical expeller was partitioned between n-hexane and 90% methanol and the methanol extract was concentrated to dryness *in vacuo* at 45° (62.8g). This was subjected to preparative HPLC for the isolation of the triterpenoids.

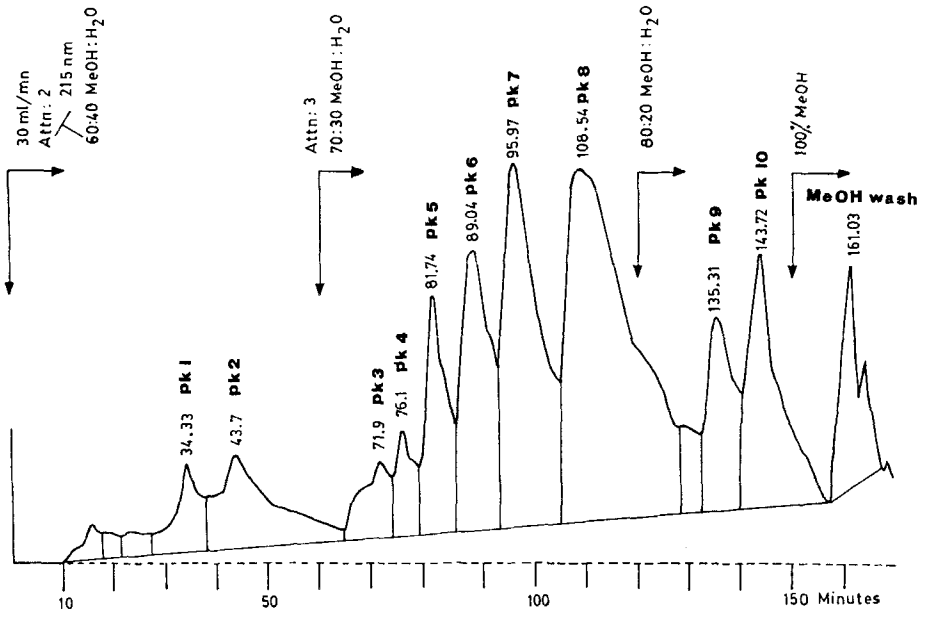

Fig.1 Preparative High performance liquid chromatogram of Neem oil triterpenoids

RESULTS AND DISCUSSION

For each preparative run 5 g of the above residue from methanol extract was dissolved in 20 ml of methanol, filtered through a Millipore filter (0.25μm) and then injected into the preparative column (25 cm x 50 mm i.d). The eluent flow rate was 30 ml/min throughout the run. During the first sixty minutes 60:40 MeOH: H_2O was used and the more polar compounds like azadirachtins were eluted. From 60 minutes the eluent was changed to 70:30 methanol:water and atleast six major peaks eluted out (Fig. 1).

Pk-1(rt 34.3 min.) and Pk-2 (rt 43.7 min.) were found to contain azadirachtins A, B, D, H and I in different proportions. Pk-3(rt 71.9 min.) and Pk-4 (rt 76.1 min.) gave only trace quantities of a material which showed the presence of a number of closely eluting compounds by analytical HPLC .Pk-5 (rt 81.7 min.) was found to be pure deacetyl nimbin (175 mg),while Pk-6 (rt 89 min.)(200 mg), Pk-7 (rt 95.9 min.)(448 mg) and Pk-8 (rt108.5 min.)(880mg), on evaporation and crystallisation in methanol:water, gave pure azadiradione, nimbin and salannin respectively.

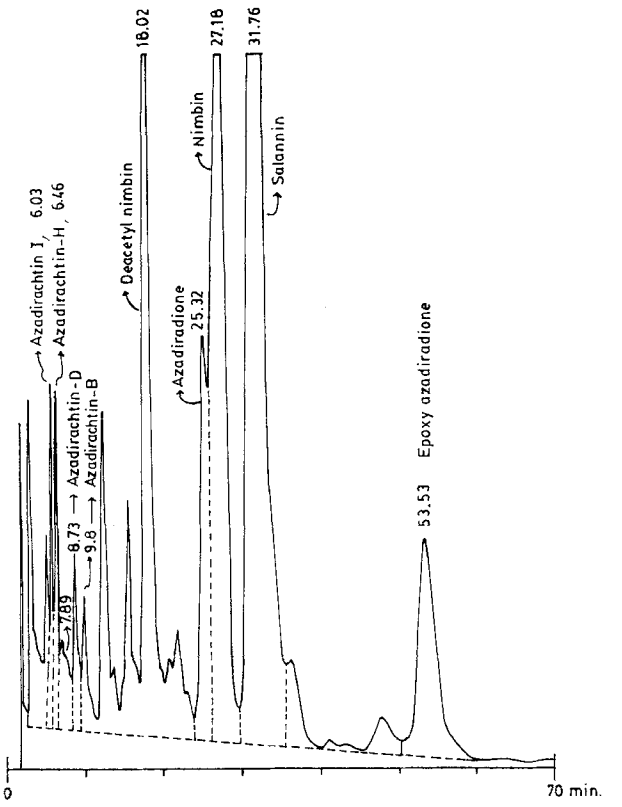

Fig.2 Analytical High performance liquid chromatogram of Neem oil triterpenoids

On changing the solvent to 80:20 methanol:water at 130 minutes, two major peaks were eluted with retention times 135.3 minutes and 143.7 minutes respectively. Both the peaks were collected and concentrated to dryness. The residue from the peak at 135.3 minutes was a complex mixture with a number of peaks in analytical HPLC and was not further investigated.

The peak eluting at 145 minutes (175 mg) was subsequently subjected to one more preparative HPLC using 25 cm x 20 mm i.d. column with 50:50 CH_3CN : H_2O as eluent and 15 ml/min flow rate. Peak eluting out at 45 min was collected and concentrated *in vacuo* to yield 110 mg of pure epoxy azadiradione.

Table 1. Quantitative estimation of major triterpenoids in neem seed oil

Compound	Retention time (min)	*Area/μg of pure compd.	mg. of compound/lit. of neem oil (in %)	
			R1	R2
Azadirachtin I	6.03	2117.40	1015.87 (0.109)	974.12 (0.097)
Azadirachtin H	6.46	1037.20	2521.40 (0.252)	2352.50 (0.235)
Azadirachtin A	7.89	2128.90	283.56 (0.028)	220.96 (0.022)
Azadirachtin D	8.73	1875.02	1141.85 (0.114)	1011.29 (0.101)
Azadirachtin B	9.8	1018.68	2107.2 (0.210)	1741.28 (0.174)
6-deacetyl nimbin	18.02	3658.76	4844.42 (0.48)	4971.46 (0.5)
Azadiradione	25.32	4694.86	2091.56 (0.21)	2102.76 (0.21)
Nimbin	27.18	4502.42	4956.9 (0.496)	4949.08 (0.496)
Salannin	31.76	4494.40	14010.6 (1.4)	14438.2 (1.44)
Epoxy azadiradione	53.53	7809.80	1313.4 (0.13)	1486.8 (0.144)

*based on average of 3 replicates and 4 concentrations

The identity and purity of the isolated triterpenoids were further confirmed by spectral data and comparison with standard samples by analytical HPLC[9].

For quantitation of the isolated triterpenoids, pure samples of Azadirachtins A, B,D,H, I, deacetylnimbin, azadiradione, nimbin, salannin, and epoxy azadiradione were accurately weighed (about 1 mg), dissolved in methanol and made up to 1 ml. Then three aliquotes of 20 μl, 15 μl, 10 μl and 5μl of each of the compounds were analysed on

a RP_{18} column (Shimpack ODS, 25 cm x 4.6 mm, detection at 215 nm, CH_3CN : H_2O 50:50 as the eluent at 1 ml/minute). Areas of peaks (valley to valley) of each concentration and compound were measured and average area per μg of each compound was calculated. The compounds eluted in the following order: Retention times are indicated in parantheses. Azadirachtin I (6.03 min.), Azadirachtin H (6.46 min.), Azadirachtin A (7.89 min.), Azadirachtin D (8.73 min.), Azadirachtin B (9.8), 6-deacetylnimbin (18.02 min.), Azadiradione (25.32 min.), nimbin (27.18 min.), salannin (31.76 min.) and epoxyazadiradione (53.53 min.). For quantitative analyses of these compounds in neem oil, a standard solution (32mg/10 ml) of the methanol extract residue (see above) was made and analysed with the same column and experimental conditions as for the pure compounds, injecting 10 μl of the solution. A representative HPLC chromatogram is given in Fig.2. Compounds are identified by retention times and area measurements (valley to valley) were used to calculate the amounts present.

Table 1 presents data on the relative abundance of the major triterpenoids in neem oil from two different sources.

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