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# Note

## Simple method for the isolation of azadirachtin by preparative high-performance liquid chromatography

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Azadirachtin, first isolated by Butterworth and Morgan[1] from neem (*Azadirachta indica* A. Juss.) kernels, has been studied intensively during the past 15 years by organic chemists probing and finally solving[2–5] its intricate molecular architecture and by entomologists on account of its powerful antifeedant and hormonal activity towards many species of insects[6]. Although azadirachtin is present to the extent of 0.2–0.6% in neem kernels, its isolation[1,3,7–12] in a pure state is tedious and time consuming, involving repeated partitioning between solvents and elaborate chromatography under a variety of conditions. It is neither necessary nor practical to use azadirachtin as such for insect control. Only small amounts are required for biological studies and as an analytical standard.

In the course of numerous high-performance liquid chromatographic (HPLC) determinations of the azadirachtin content of ethonolic extracts of neem kernels, it was observed that azadirachtin could always be obtained as a peak distinct from those of most other components. This suggested the possibility of developing a simple procedure for the isolation of azadirachtin by direct preparative HPLC, using an ODS column and methanol–water (60:40) as eluent. The azadirachtin obtained by the direct preparative HPLC method was identical in all respects with a sample prepared by the procedure of Schroeder and Nakanishi[12], which was modified after the vacuum liquid chromatographic stage as we did not succeed in improving the azadirachtin content by carbon tetrachloride crystallisation. The azadirachtin-rich fraction from vacuum liquid chromatography was further purified by medium-pressure liquid chromatography on silica gel with a final purification by preparative HPLC on an ODS column. The specific rotation and $^1H$ and $^{13}C$ NMR data were in excellent agreement with data reported by Kraus *et al.*[5] and Butterworth *et al.*[7].

### EXPERIMENTAL AND RESULTS

HPLC studies were carried out on a Shimadzu Model LC-8A system linked to a C-R4A data processor. The peaks were detected with a Shimadzu SPD-6AV detector at 215 nm. A Shimpack analytical ODS column (15 cm × 4.6 mm I.D.) was used for

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analytical studies and a Shimpack preparative ODS column (25 cm × 20 mm I.D.) for preparative work.

Neem kernel powder (15 g) was defatted with hexane in a Soxhlet apparatus for 2 h. The dry, defatted powder was extracted with four 15-ml portions of boiling 95% ethanol on a hot water-bath for 10 min, each time decanting the extract through a filter-paper and diluting to 100 ml with 95% ethanol.

A 20-$\mu$l portion was removed for determination of the azadirachtin content by analytical HPLC with methanol–water (60:40) as eluent. Valley-to-valley integration of the azadirachtin peak and calculation of the azadirachtin content from a calibration graph of peak area (ordinate) *versus* azadiractin content in nanograms (abscissa) showed a total content of 0.4%.

The alcohol was removed *in vacuo* at 45°C and the residue was dried to constant weight (1.5 g). A weighed portion of the residue was also analysed for azadirachtin content and the result was in agreement with the earlier analysis.

The hexane extract was repeatedly shaken with 90% methanol. The hexane layer on evaporation yielded an oil (5.1 g). The residue from the methanol layer (0.5 g) contained a detectable amount of azadirachtin as shown by HPLC. Apparently, the oil carries with it a small amount of azadirachtin and other compounds. Similar fractions from a number of runs can be pooled and processed for recovery of azadirachtin. It is essential, however, to defat the kernels with hexane, as they contain 30% of oil and the azadirachtin content is *ca.* 0.3%. It is not desirable to load the reversed-phase column with an excess of non-polar material.

*Isolation of pure azadirachtin by preparative HPLC*

A 500-mg amount of the ethanol extract residue was dissolved in 1 ml of methanol, centrifuged at 15 500 *g* to remove insoluble matter, filtered through a Millipore filter (0.25 $\mu$m) and then injected into the preparative column and eluted with methanol–water (60:40). The flow-rate was adjusted at 10 ml/min. for the first 25 min. Under these conditions, components of high polarity were eluted in the first 10 min, followed by a small peak. The azadirachtin peak appeared next and was centred at 14.4 min during the preparative run. As the left-hand segment of the peak ascended from the baseline, the eluent was collected until the peak had attained two thirds of the maximum height (fraction A). Collection of the azadirachtin fraction was begun when this two thirds of peak height had been reached and was ended when the peak descended to the baseline again (fraction B). Fraction A contained, in approximately equal amounts, azadirachtin with a retention time of 5.3 min on an analytical ODS column and a compound with a slightly shorter retention time of 5.1 min. Fraction B yielded azadirachtin, identical with an authentic sample as shown by HPLC. At 18.5 min, another peak was detected which was also collected for further investigation. After 25 min, the flow-rate was increased to 50 ml/min. to remove all of the less polar components from the column, this process taking about 45 min. At least six distinct peaks were collected for further investigation. The less polar compounds could be removed better by eluting the column with pure methanol for 20 min at a flow-rate of 25 ml/min. Restabilization of the column with methanol–water (60:40) for another 20 min at a flow-rate of 25 ml/min renders it ready for the next run.

*Preparation of the standard sample*

Neem kernels (1 kg) were extracted with three 1-l portions of methanol over a period of 3 days. The methanol was removed *in vacuo* at 45°C and the residue partitioned between aqueous methanol–hexane and aqueous methanol–ethyl acetate as reported by Schroeder and Nakanishi[12]. The residue of the ethyl acetate extract (34.5 g) was subjected to vacuum liquid chromatography on silica gel (Acme, 250–400 mesh, 500 g) using hexane–ethyl acetate (25:75). The azadirachtin-containing fraction yielded a residue (11.4 g) which was subjected to medium-pressure liquid chromatography on a Büchi column (450 mm × 49 mm I.D.) loaded with silica gel (Acme, 250–400 mesh, 500 g). The column was eluted with hexane–ethyl acetate (25:75) and fifteen 250-ml fractions were collected. The azadirachtin-enriched residue of fraction 9 (2.8 g) was subjected to preparative HPLC in portions with methanol–water (60:40). From 500 mg of this enriched fraction 170 mg of azadirachtin were isolated. It had a retention time of 5.3 min when analysed on an analytical ODS column eluted with methanol–water (60:40) at a flow-rate of 0.8 ml/min. $[\alpha]_D^{28}$ − 53°. $^1H$ and $^{13}C$ NMR data were in excellent agreement with earlier data[5,7].

## DISCUSSION

Using an ODS column of I.D. 20 mm, the optimum batch size was 500 mg of the residue from the ethanolic extract (the total residue from 15 g of kernels was 1.5 g). Using the procedure described under Experimental and Results, each run could be completed in about 1 h, yielding 10–15 mg of azadirachtin and several runs can be effected in succession. Using larger preparative columns (I.D. 50 mm and above), which are commercially available, the batch size can be increased to 5 g.

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