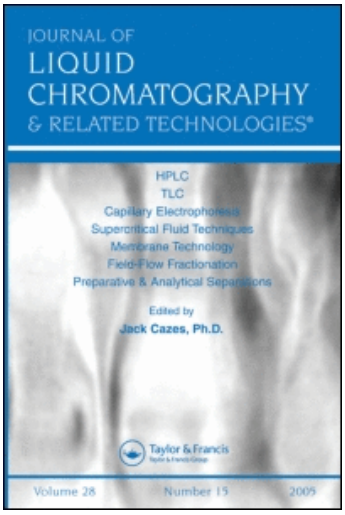

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Combined Florisil, Droplet Counter-Current, and High Performance Liquid Chromatographies for the Preparative Isolation and Purification of Azadirachtin from Neem (*Azadirachta Indica*) Seeds

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COMBINED FLORISIL, DROPLET COUNTER-CURRENT, AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHIES FOR PREPARATIVE ISOLATION AND PURIFICATION OF AZADIRACHTIN FROM NEEM *(AZADIRACHTA INDICA)* SEEDS

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ABSTRACT

An alternate prepurification procedure was developed for the isolation and purification of azadirachtin, a biologically active limonoid, from neem (Azadirachta indica) seeds by the use of Florisil and droplet counter-current chromatographies. The procedure, combined with the use of preparative high performance liquid chromatography, yielded 364.8 mg of >99% pure azadirachtin from 1.5 kg of Indian neem seeds.

INTRODUCTION

Azadirachtin, a biologically active (e.g., insect antifeedant and ecdysis inhibitor) limonoid, has been isolated from the fruit of chinaberry (Melia azedarach L., Meliaceae) (1) and the seeds of neem (Azadirachta indica A. Juss., Meliaceae) (2,3). The isolation and purification of azadirachtin from these plant species are tedious due to the complex chemical nature of the

plant extracts and the similarity-in-structure of azadirachtin to co-occurring compounds (4-6).

A number of chromatographic techniques (e.g., thin layer and open column) (3,7,8) have been used in the isolation and purification of azadirachtin. Recently, high performance liquid chromatography (HPLC) has been employed in a final purification step since a higher degree of purity can apparently be attained with it (9,10). The use of HPLC to purify azadirachtin usually requires sample prepurification in order to avoid column contamination and to increase separation efficiency. We recently reported the use of flash column chromatography as a prepurification step for neem extracts prior to the final purification of azadirachtin by HPLC (11).

In the present paper, we report on an alternate prepurification method for the preparation of samples for HPLC. This method utilizes combined Florisil column displacement chromatography and droplet counter-current chromatography (DCCC).

MATERIALS AND METHODS

Extraction of Azadirachtin from Neem Seeds

Air dried neem seeds (500 g, obtained from India by Vikwood, Ltd., Sheboygan, WI, U.S.A.) were extracted by grinding in methanol (1.0 l) for 5 min with a Waring Blender. The methanolic extract was decanted and the marc was further (x2) extracted with fresh methanol. The methanolic extracts were pooled and concentrated *in vacuo*. The residue was redissolved in aqueous methanol (1.0 l, 1:1, v/v) and partitioned (x3) with equal-volume portions of *n*-hexane, followed by (x3) equal-volume portions of dichloromethane. The dichloromethane extracts were pooled and concentrated *in vacuo*, redissolved in 500 ml of methanol, and back-partitioned (x3) with equal-volume portions of *n*-hexane. After drying, the methanolic layer was subjected to Florisil column chromatography. The entire procedure was repeated three times, each time with 500 g of seeds (with a total of 1.5 kg), to yield an average of 14.79 ± 0.26 g of the methanolic layer.

Florisil Column Chromatography

The methanolic layer described above (14.79 ± 0.26 g) was redissolved in dichloromethane, mixed with Florisil (100 g, 60-100 mesh, Fischer Scientific) and then slowly dried by rotary evaporation. The coated Florisil was placed onto the top of a glass column (115 x 5.0 cm I.D., LGA, Berkeley, CA) dry-packed with Florisil (500 g, 60-100 mesh). The column was eluted first with diethyl ether (1.5 l), and then step-wise with increasing percentages (2.5%, 5.0%, 7.5%, 10.0%, and 20.0%) of methanol in diethyl ether (1.0 l each). The eluates were concentrated *in vacuo* at room temperature.

Droplet Counter-Current Chromatography (DCCC)

Chromatography was carried out on an Eyela Model D.C.C.-300-G3 Droplet Counter-Current Chromatograph (Tokyo Rikakikai, Tokyo, Japan) equipped with 300 standard glass tubes (40 x 0.2 cm I.D.) interconnected with teflon tubing (0.5 mm I.D.). The DCCC solvent system used was chloroform-toluene-methanol-water (5:5:7:2, v/v/v/v). The top and bottom layers of the solvent mixture were used as the stationary and mobile phases, respectively. Two of the Florisil column eluates (7.5% and 10.0% methanol in diethyl ether eluates, referred to as "b_1" and "b_2", respectively) were dissolved in a 1:1 (v/v) mixture (10 ml) of both mobile and stationary phases and each eluate was aspirated separately into the DCCC sample chamber. The flow rate was set at 5.0 ml/h and the effluent was collected in 5.0 ml fractions with a Pharmacia Frac-100 fraction collector. Fractions containing azadirachtin were pooled and concentrated *in vacuo* to yield averages (for 3 DCCC separations) of 440.1 ± 34.3 mg for b_1 and of 291.1 ± 14.6 mg for b_2.

Thin Layer Chromatography (TLC)

TLC was performed either on 40 x 8 mm Polygram SILG/UV_{254}, (0.25 mm, Macherey-Nagel) or on 20 x 20 cm prescored silica gel GHLF (0.25 mm, Analtech) plates. The plates were developed with the bottom layer of the DCCC solvent mixture and visualized by

spraying with a vanillin-sulfuric acid-ethanol (3 g:1.5 ml:100 ml) spray reagent followed by heating.

High Performance Liquid Chromatography (HPLC)

Normal and reversed phase preparative and reversed phase analytical HPLC procedures used were slight modifications of the methods of Yamasaki et al. (11). Normal and reversed phase preparative HPLC steps were carried out with a Hewlett-Packard Model 1081B liquid chromatograph and a Micromeritics 750 solvent delivery system, respectively. Both systems were equipped with a Negretti and Zambra injector with a fixed loop size. Effluents were detected using either a Micromeritics 787 variable-wavelength UV-Visible detector set at 218 nm or a Pharmacia single path monitor UV-1/214 with a 214 nm filter. Detected peaks and retention times were recorded using a Hewlett-Packard 3388A integrator.

The normal phase preparative HPLC step was performed with a Phenomenex Maxsil 5 silica column (particle size 5 μm, 25 x 2.0 cm I.D.) eluted isocratically with isopropanol-n-hexane (1:3, v/v) at a flow rate of 5.0 ml/min and an average pressure of 1100 p.s.i. The reversed phase preparative HPLC step was performed with a Phenomenex Ultrex 5 phenyl column (particle size 5 μm, 25 x 2.25 cm I.D.) eluted isocratically with acetonitrile-water (3:7, v/v) at a flow rate of 4.0 ml/min and an average pressure of 2100 p.s.i. Both columns were protected with an Alltech stainless-steel guard column (5.0 x 0.46 cm I.D.) packed with the corresponding Alltech pellicular packing material.

Reversed phase analytical HPLC was carried out using the same HPLC system described above for the reversed phase preparative step. Analytical HPLC was performed on a Phenomenex Ultrex phenyl stainless-steel column (particle size 5 μm, 25 x 0.46 cm I.D.) eluted isocratically with acetonitrile-water (3:7, v/v) at a flow rate of 1.0 ml/min and an average pressure of 1000 p.s.i. The percentage of azadirachtin in each purification step was determined by analytical HPLC from a standard curve generated by

using authentic azadirachtin as an external standard (peak height vs. amount of azadirachtin).

Identification of Azadirachtin and Azadiradione

Azadirachtin isolated from neem seeds was identified by spectral comparison (IR, PMR) and cochromatography (TLC, HPLC) with an authentic sample. Azadiradione was identified by comparison of IR, PMR, and MS (FAB+, CI, and EI) spectra with literature data (12).

RESULTS AND DISCUSSION

A prepurification method for the isolation of azadirachtin from extracted and partitioned neem seeds was developed by combining Florisil column displacement chromatography and DCCC, followed by the final purification with preparative HPLC. The method gave 364.8 mg of >99% pure (as indicated on analytical HPLC) azadirachtin from 1.5 kg of Indian neem seeds. Dry weights, percent purity and percent yield of azadirachtin from sequential purification steps are shown in Table 1.

The dichloromethane-soluble fraction was chromatographed on an open column of Florisil (2,3,8). We chose coarse Florisil since it resulted in a 5-fold enrichment of azadirachtin from the crude dichloromethane fraction (Table 1) in a time frame shorter than that possible with fine Florisil. In addition, we were able to load a larger sample (14.79 g) onto the column of Florisil (500 g), with minimal column overloading, than was possible with direct loading of a solution of the sample, by first precoating the sample onto 100 g of Florisil. Thus, each 14.79 ± 0.26 g of sample containing $1.7 \pm 0.2\%$ of azadirachtin loaded onto the Florisil column afforded in less than 1 h, 2 fractions which combined yielded 2.96 ± 0.39 g containing 8.4-9.5% of azadirachtin (Table 1).

Azadirachtin was eluted predominantly in 2 fractions from the Florisil column; in 7.5% methanol in diethyl ether (b_1) and in 10.0% methanol in diethyl ether (b_2). The other fractions

TABLE 1.

Percentage of Azadirachtin Found in Sequential Purification Steps and the Percent Yield of Azadirachtin.

Purification	X ± S.D.		% Yield of Azadirachtin
	Dry wt. (mg) ± S.D.	% Azadirachtin ± S.D.*	
a. Dichloromethane partition	14790±260	1.7±0.2	>95
b_1. Florisil column eluate 7.5% methanol in diethyl ether	1980±180	8.4±2.4	42
b_2. Florisil column eluate 10.0% methanol in diethyl ether	980±210	9.50±1.3	24
c_1. DCCC of b_1	440.1±34.3	28.0±7.2	74
c_2. DCCC of b_2	291.1±14.6	34.7±5.0	>99
d. Normal phase prep. HPLC	252.9±68.8	55.4±10.7	62
e. Reversed phase prep. HPLC	121.6±35.8	99.9	87

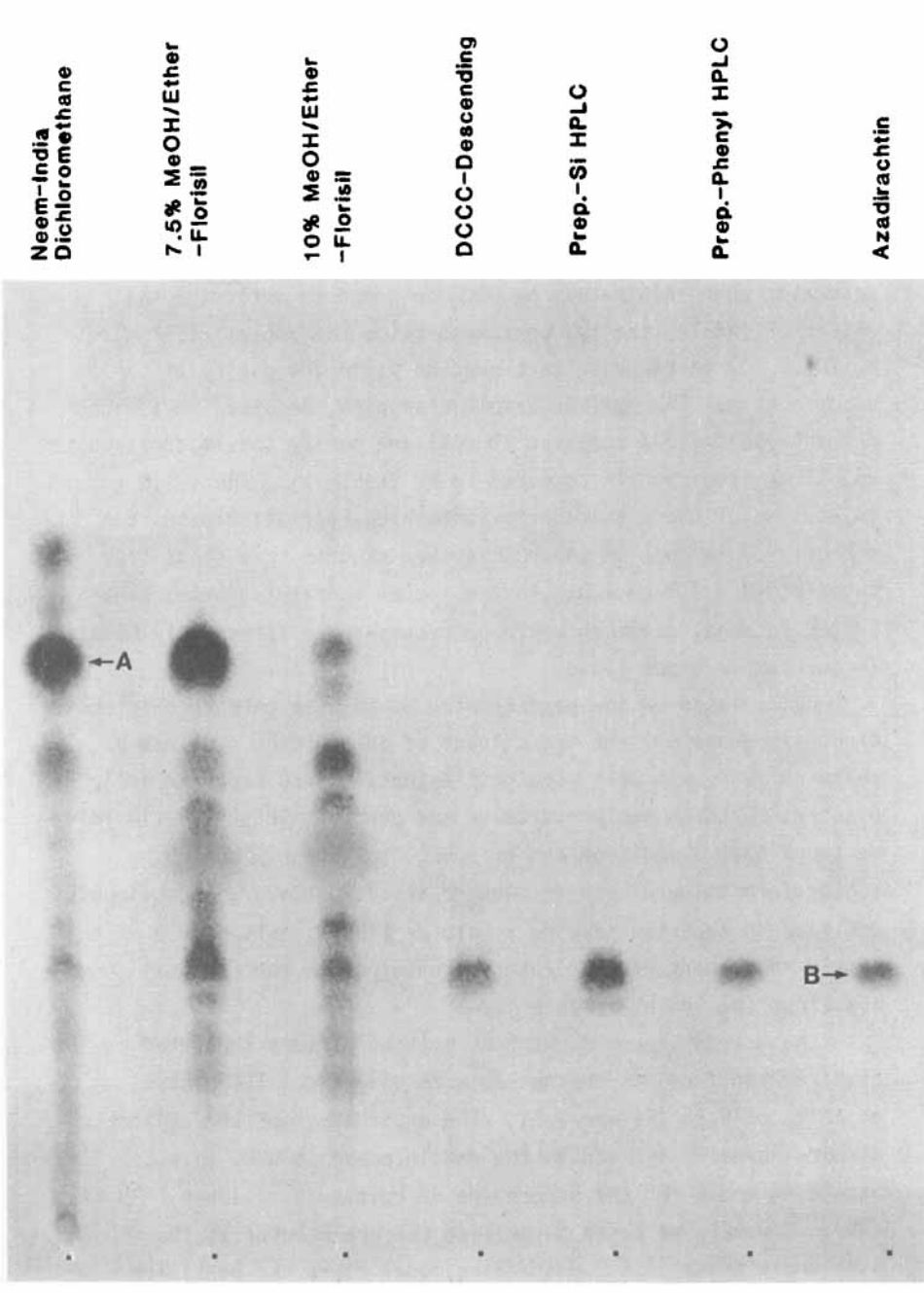
*Analyzed by analytical phenyl HPLC; the concentration of azadirachtin was quantified using external standard (peak height vs. amount of azadirachtin)

contained only traces (<3%) or undetectable amounts of azadirachtin. The high yield of the 5% methanol in diethyl ether fraction (3.78 $\pm$ 0.43 g) was in large part due to the presence of another limonoid, azadiradione, which represented approximately 37% of the fraction (Figure 1,A).

Although fractions b_1 and b_2 each contained 8.4-9.5% azadirachtin and could have been combined, they were chromatographed separately by DCCC in order to determine the effect of sample size (b_1 contained twice the amount (1.98 g) of b_2 (0.98 g)) on the resultant percent yield and purity of azadirachtin. The smaller sample size of b_2 resulted in a higher percent yield (>99% compared to 74%) and purity (34.7% compared to 28.0%) of azadirachtin compared to b_1 (Table 1). Thus, 1.0 g injections of the azadirachtin-containing Florisil eluates can effectively be made on the DCCC system used in this study (300 tubes of 40 x 0.2 cm I.D.), but a system containing tubes of a larger internal diameter would be necessary to effectively handle larger sample sizes (13).

DCCC, based on the partitioning of solutes between droplets of mobile phase solvent and columns of surrounding stationary phase solvent, has been used most extensively to separate polar plant extracts on semipreparative and preparative scales (14-18). We found that a modification of a DCCC solvent mixture (chloroform:toluene:methanol:water, 5:5:7:2, v/v/v/v), previously utilized to separate peptide alkaloids (19), resulted in a 3- to 4-fold enrichment of azadirachtin contained in the Florisil fractions (b_1 and b_2) (Table 1).

The bottom layer of the DCCC solvent mixture separated azadirachtin from its contaminants on silica gel TLC plates at an R_f of 0.25 (Figure 1,B). The upper layer of the solvent mixture normally is used as the mobile phase in DCCC (i.e., ascending mode) for the separation of compounds of low R_f (<0.4) (20). However, we chose to utilize the upper layer as the stationary phase (i.e., descending mode) since the partition coefficient value (K) of azadirachtin was 0.52, while the K value



of azadirachtin for the ascending mode was 1.92 (unpublished data). Thus, the use of the descending mode resulted in a 3-fold purification of azadirachtin in the much shorter time period of 44-62 h (azadirachtin was still retained in the stationary phase even after 96 h when the DCCC was operated in the ascending mode).

The advantages of DCCC (as compared to the use of solid support chromatography) are lower consumption of solvent and quantitative recovery of samples since no solid support, which might cause irreversible adsorption, is involved. Due to the conservative pooling of the DCCC eluates from b_1 (larger sample size), only 74% of the azadirachtin (c_1) was recovered. However, complete recovery of the azadirachtin (c_2) was made from the DCCC eluates from b_2 (smaller sample size).

Final purification of azadirachtin was accomplished with HPLC. The normal and reversed phase preparative HPLC steps were modifications of our previously reported method (11). Even though silica gel preparative HPLC did not appear to significantly concentrate azadirachtin further (Figures 1 and 2), we included it in our isolation scheme in order to eliminate persistent yellow colored impurities and other impurities not readily separated from azadirachtin by the use of other chromatographic methods (including reversed phase HPLC). The difficulty of separating these impurities from azadirachtin is reflected in the low percent yield of azadirachtin in the normal phase step (62%, Table 1). The subsequent phenyl preparative HPLC step resulted in azadirachtin of single peak purity, as monitored by analytical phenyl HPLC (Figures 1 and 2). The approximate t_R's for

FIGURE 1

Thin layer chromatographic analysis of azadirachtin (compound B) from the sequential purification of neem seeds. The TLC plate was developed once with the bottom layer of chloroform:toluene: methanol:water (5:5:7:2 v/v/v/v)) and visualized by spraying with vanillin-sulfuric acid-ethanol (3g:1.5 ml:100 ml) followed by heating.

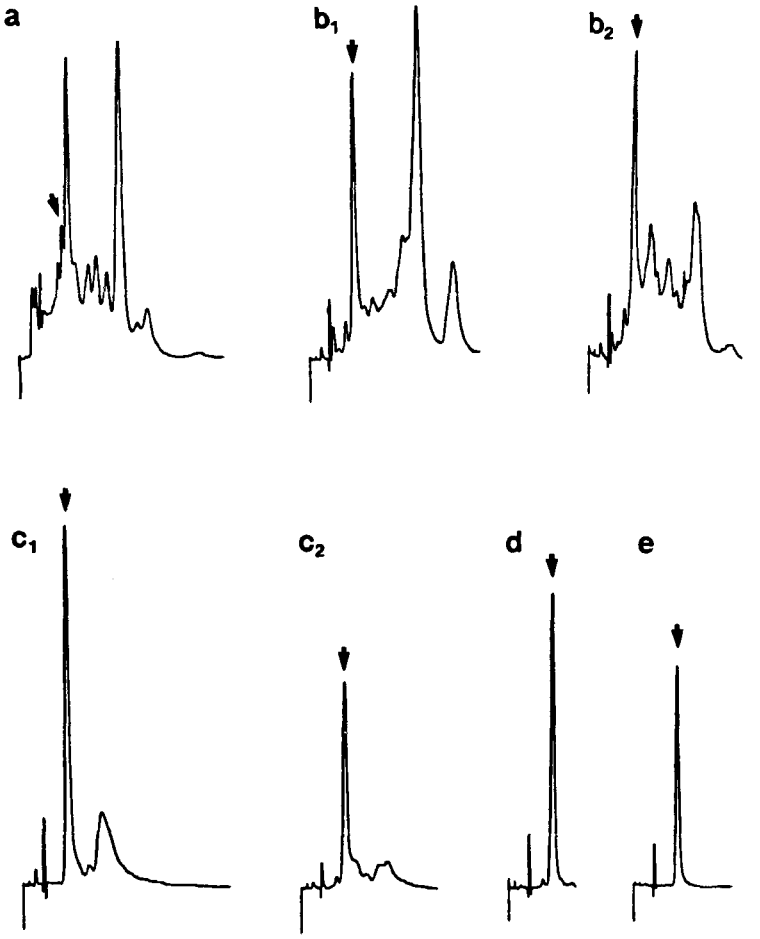


FIGURE 2.

Analytical high performance liquid chromatograms of azadirachtin (arrows) recovered from: (a) dichloromethane partition; (b_1) 7.5% methanol in diethyl ether eluate; (b_2) 10.0% methanol in diethyl ether eluate; (c_1) DCCC of b_1; (c_2) DCCC of b_2; (d) preparative silica gel HPLC;[1] (e) preparative phenyl HPLC.[2] Analytical HPLC was performed with a phenyl column (25 x 0.46 cm I.D., 5 μM) eluted isocratically with acetonitrile-water (3:7 v/v) at 1.0 m/min.

azadirachtin in the normal and reversed phase preparative systems were 35 and 60 min, respectively.

Preparative HPLC, when coupled with appropriate prepurification steps, is the best available method, in our experience, for the purification of azadirachtin to single peak purity as monitored by analytical HPLC. Combined Florisil column displacement chromatography and DCCC were found to adequately prepurify azadirachtin from neem for final purification with HPLC.

Although our procedure, which involves a combination of complementary methods, may seem tedious and time-consuming, in our hands the various methods were necessary to attain azadirachtin of single peak purity from the chemically-complex neem seed extracts. However, in those situations where absolutely pure azadirachtin is not needed (e.g., in certain applications for insect control), certain of the steps in our procedure can be deleted. For example, Florisil, which has long been used successfully in the isolation of azadirachtin (2,3,8), can sometimes suffice as the sole chromatographic method in the purification of azadirachtin from extracted and partitioned neem seeds. DCCC, which can further purify (at least 3-fold) the azadirachtin-containing Florisil fractions, might also suffice as the sole chromatographic method in the purification of azadirachtin, especially if the number and internal diameter of the tubes used are maximized. Both Florisil column chromatography and DCCC can easily be scaled up to handle even greater sample sizes than reported here. Thus, depending on the degree of purity required by the individual investigator, some or all of the methods in our procedure can effectively be utilized in the purification of azadirachtin from neem seeds.

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