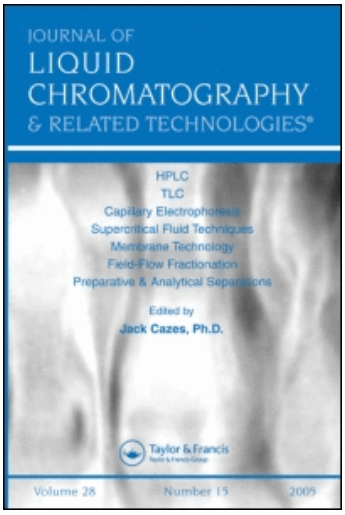

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ESTIMATION OF AZADIRACHTIN CONTENT IN NEEM EXTRACTS AND FORMULATIONS

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ABSTRACT

A high performance liquid chromatographic reversed-phase procedure has been developed whereby azadirachtin content can be estimated in crude extracts of neem and in dust formulations of neem. An estimation of the azadirachtin content is achieved through the use of an external azadirachtin standard and valley-to-valley integration. Since azadirachtin seems to be the most potent insect feeding deterrent in these extracts and formulations, its content is a measurement of potency and represents an attempt at standardization.

INTRODUCTION

Azadirachtin ($C_{35}H_{44}O_{16}$) is a tetranortriterpenoid (1,2) (Fig. 1) present in neem kernels [*Azadirachta indica* A. Juss. (*Melia azadirachta* L., *M. indica* Brandis., Margosa tree or Indian lilac)] and the chinaberry tree (*M. azedarach* L., Persian lilac). It is a highly active feeding deterrent and growth regulator for insects (3).

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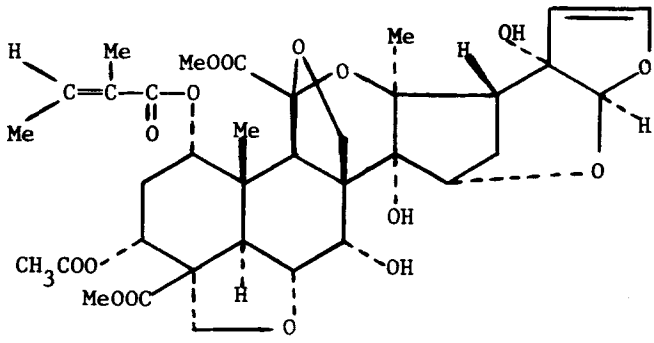

FIGURE 1 Structure of azadirachtin ($C_{35}H_{44}O_{16}$) as proposed by Zanno et al. (2).

Azadirachtin has not yet become a generally used insect feeding deterrent for several reasons: 1) it is difficult to isolate and the yield is low (4), 2) formulations have not yet been stabilized (5), and 3) its effectiveness on any one particular insect pest in the field has not been totally studied, etc. (6-10).

In order to study the effectiveness of crude extracts or formulations containing neem, we developed a high performance liquid chromatographic (HPLC) technique for estimating the azadirachtin content of these samples. The technique is partially based on some of our prior work dealing with the preparative isolation of azadirachtin (4) and the effects of sunlight on azadirachtin (5).

MATERIALS AND METHODS

Apparatus

A Waters Associates Model ALC-100 Liquid Chromatograph equipped with a Model 720 System Controller, a Model 730 Data Module, two Model 6000A Pumps, a U6K Injector, and a Model 440 Absorbance Detector with an Extended Wavelength Module at 214 nm was used for all HPLC. The column for HPLC, in a Z-Module™ Radial

Compression Separation System, was a 10 μ Radial-Pak™ μBondapak® C_{18}.

A Comitrol Model MG, Urschel Cutter, with a 0.15 cm cutting head; a Hamilton Kettle, style A 227 L, double motion Teflon scraper agitator; a Sparkler Filter with E-5 filter paper; a Precision Scientific, 3 L (D-1) laboratory, vacuum, glass evaporator; and a Stokes Vacuum Dryer were used for the large scale extraction of neem seed kernels.

A Brinkmann Centrifugal Grinding Mill ZM-1 was used to decrease the particle size of hexane extracted neem kernel powder needed for kaolin (hydrated aluminum silicate) formulations.

Ethanol (95%) was obtained from Publicker Industries, Inc. Solvents (methanol, methylene chloride, acetone, and ether) were HPLC grade and obtained from Fisher Scientific Co. Hexane (laboratory grade) was also obtained from Fisher Scientific Co. Water was distilled. Kaolin (USP) was obtained from Mallinckrodt Inc.

Plant Material and Formulations

1) Neem extracts on a small scale were prepared by grinding 50 g of neem kernels in a Waring Blendor with 100 ml 95% ethanol or an appropriate solvent for 30 sec. The mixture was then filtered through Whatman #1 filter paper. When neem was extracted with methylene chloride or ether, the solvent was removed in vacuo and replaced with 100 mL 95% ethanol for HPLC analysis.

2) Neem extract on a large scale (Fig. 2) was prepared by first grinding 114 kg of neem seed kernels into a coarse powder with a Comitrol Model MG, Urschel cutter. The first 23 kg was extracted with 95 L 95% ethanol for 2 hr at room temperature in a Hamilton Kettle. The mixture was allowed to settle and the supernatant was clarified by filtration with a Sparkler Filter. The filtrate was concentrated at a temperature generally below 40°C in a Precision Scientific Evaporator. The yield of extract was 4 L. The extraction was repeated twice with yields of 2.5 L and 0.25 L, respectively. The neem seed kernel marc was dried in

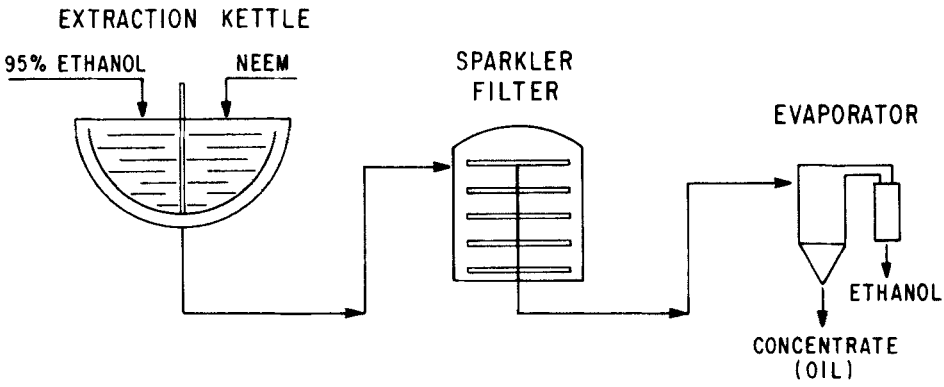

FIGURE 2 Pilot plant extraction process.

a Stokes Vacuum Dryer to 2.2% volatiles. The remaining 91 kg of ground neem seed kernels was extracted with 76 L 95% ethanol for 2 hrs, allowed to settle overnight, and filtered. Eight similar extractions of this large batch gave a total yield of 19.5 L after concentration. A 1 g sample from each of the extracts was prepared for analysis by adding 10 mL 95% ethanol.

3) Neem-kaolin formulations were prepared by first grinding 454 g of neem seed kernels with 1 L hexane in a Waring Blendor for 60 sec. The mixture was filtered through Whatman #1 filter paper and the procedure repeated. The hexane-extracted powder was air dried and then ground in the Brinkman Mill with a screen of 0.5 mm pore size. The powder was then mixed with kaolin on a weight to weight basis. The neem-kaolin formulations (1 g) and 10 mL 95% ethanol were mixed for the analytical estimation of azadirachtin content.

Sample Preparation

The neem-kaolin formulations (1.0 g) were mixed with 2-10 mL 95% ethanol and sonicated for 10 min. The mixtures were then centrifuged at low speed for 5 min and the supernatant was used for the analytical estimation of azadirachtin content.

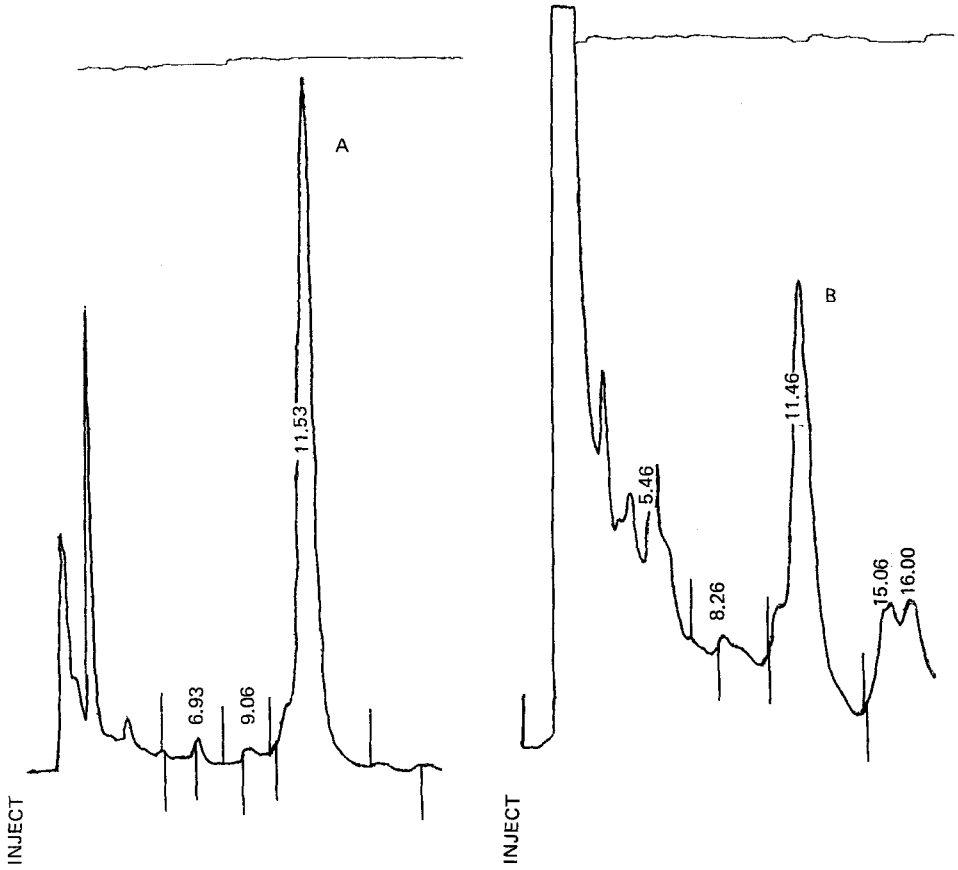

FIGURE 3 A typical chromatogram of (A) the standard, azadirachtin, and (B) a crude neem sample.

Chromatographic Procedure

A standard azadirachtin sample (1.0 µg/10 µL) was injected into a 10 µ Radial-Pak µBondapak C_{18} in a Z-Module Radical Compression Separation System with a flow rate of 2 mL/min (50/50 methanol/water) and detection at 214 nm.

The Model 730 Data Module was set with a peak width of 40, noise rejection of 6, and valley-to-valley integration. At 214 nm and 0.05 attenuation, 1.0 µg of azadirachtin gave a 1/2 scale peak

which was entered in the calibration table as an external standard.

Solutions prepared from crude neem extracts or neem-kaolin samples were injected and the peak corresponding to the azadirachtin standard was analyzed by the Data Module at the same attenuations. A typical chromatogram of the standard, azadirachtin, and a crude neem sample is shown in Fig. 3.

When crude neem extracts were analyzed, it was necessary to flush the column after each run with a 10 min flush of 100% methanol. This was accomplished by the System Controller with a 10 min linear program from 50/50 methanol/water to 100% methanol, a hold for 10 min at 100% methanol, and then another 10 min linear program from 100% methanol to 50/50 methanol/water. This procedure was not necessary with the analyses of the neem-kaolin samples because the neem kernels in these formulations had been pre-extracted with hexane.

RESULTS AND DISCUSSION

1) The efficiency of azadirachtin extraction from neem kernels was determined by a series of small scale extractions. Of the solvents tried, 95% ethanol was the most effective for the removal of azadirachtin from the kernels (Table 1).

2) The utilization of this estimation technique for monitoring the completeness of azadirachtin removal by extracting neem kernels in 95% ethanol has proven to be very useful. The amount of azadirachtin/g of extract for the 3 successive extracts from the 23 kg batch revealed that azadirachtin was effectively removed (1 - 1.36 µg/g, 2 - 2.72 µg/g, 3 - 0.22 µg/g). The amount of azadirachtin/g of extract for each of the 8 successive extracts from the 91 kg batch also revealed the completeness of azadirachtin extraction.

3) The stability of the kaolin-neem powder formulations was demonstrated by this estimation technique also. It was found that the 1:4 neem:kaolin formulation contained 474 µg azadirachtin/g of extract and 2 months later contained 465 µg/g. Other neem-kaolin

TABLE 1
Extraction of Neem Kernels

Solvent	µg Azadirachtin/10 µL Solvent
95% Ethanol	2.80
Methanol:Water 85:15	2.60
Methanol	2.19
Methylene Chloride	1.73
Ether	1.28
Acetone	0.74

formulations of 10%, 5%, 2%, and 1% were also analyzed and found to be stable with time and room temperature.

This high performance liquid chromatographic technique has been extremely valuable in estimating azadirachtin content. Other applications of this technique could include the determination of azadirachtin yield from different batches of neem kernels; determination of azadirachtin degradation in other neem extracts; determination of azadirachtin content in extracts of other Melia species; and possibly the determination of azadirachtin content in the environment (insects, food, clothing, animals, soil, etc.).

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Trade names are used in this publication solely for the purpose of providing specific information. Mention of a commercial or a proprietary product does not constitute a recommendation, guarantee, or warranty of the product by the U.S. Department of Agriculture or an endorsement by the Department over the products mentioned.

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