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Analysis of azadirachtin by supercritical-fluid chromatography

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ABSTRACT

The quantitative determination of the natural insecticide azadirachtin in crude extract of Neem seeds can be conveniently carried out by packed column supercritical-fluid chromatography, with detection by UV absorption at 210–220 nm and using carbon dioxide–methanol as mobile phase. The least detectable amount of azadirachtin was 10 ng at 212 nm.

INTRODUCTION

Much attention has been focused in recent years on the use of naturally occurring pesticides, and among these, the most promising for commercial development is azadirachtin (Fig. 1A) found in the seeds of Neem tree (*Azadirachta indica* A.

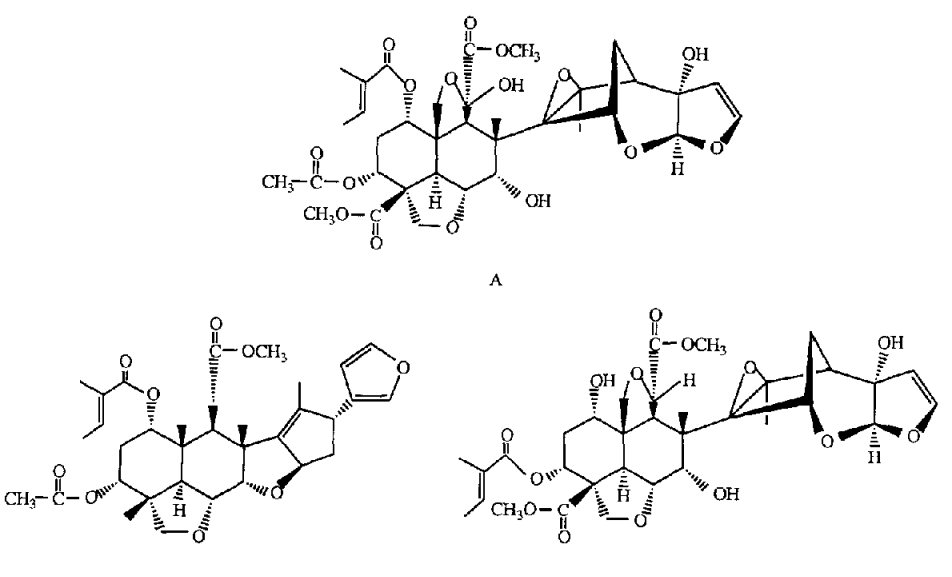

Fig. 1. Structures of A, azadirachtin; B, salannin; C, 11-desoxy-3-desacetylazadirachtin.

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Juss). Azadirachtin [1–3] is a highly oxidized triterpenoid substance, it is an antifeedant substance for some insects [4] and a potent inhibitor of growth and development for many pest species [5]. It is, however, not the only triterpenoid present in Neem seeds possessing these insecticidal properties, and it is highly probable that commercial products will be more or less pure mixtures of these triterpenoids, among which, azadirachtin will be the most important and the most active compound. A rapid and reliable method for determining azadirachtin is therefore required, in order to determine the quality and potency of such extracts.

Azadirachtin is non-volatile and highly polar. It is unsuited therefore for gas chromatography. It has an UV absorption, due to the α,β-unsaturated carbonyl chromophore in the tiglate ester and the vinyl ether, but the absorption maximum is at very short wavelength (λ_{max} 212 nm, falling to 6% of this value at 254 nm). Liquid chromatography is not the best system for quantifying this compound because most solvents absorb strongly at the short wavelength required for sensitive detection. Two high-performance liquid chromatographic (HPLC) methods have been described, using reversed-phase columns, one with methanol–water and detection at 214 nm [6], and the other using acetonitrile–water with detection at 218 nm [7]. Retention times in these systems are 11.5 and 10.3 min respectively. The rapid equilibration and shorter retention times of supercritical-fluid chromatography (SFC) and the excellent transparency of supercritical carbon dioxide at short UV wavelengths compelled us to investigate the analysis of azadirachtin by SFC. We describe a simple and rapid method for the quantitative determination of azadirachtin in crude extracts of Neem by SFC.

MATERIALS AND METHODS

Apparatus

The SFC equipment used in this work was essentially supplied by LDC Analytical (Stone, U.K.), except as otherwise indicated.

The cylinder of CO_2 was fitted with a dip-tube delivering liquified gas from the bottom of the cylinder to a CO_2 pump (CP 3000) fitted with a clamp-on heat exchanger, through which refrigerated water–methanol was circulated from a cooler (Haake C, F.R.G.). Another pump (Constametric 3000) was used to deliver the modifier liquid, methanol. The two pumps were controlled by a GC-4000 gradient controller which adjusted flow-rate and modifier concentration and could be used to produce solvent programming. The reciprocating pumps gave almost pulseless transfer of mobile phase at high pressure to a dynamic mixer. The mobile phase then passed through a pressure pulse dampener (Negretti, Southampton, U.K.) for smoother baseline operation at high sensitivity and then into the column oven (Model SpH 99). The column oven contained an injection valve (Rheodyne 7125, Anachem, Luton, U.K.) built into the oven wall and fitted with a 20-μl loop for injection of the sample. The mobile phase then passed to a guard column packed with 5-μm amino-propyl silica and then to the analytical column and then through a 2-μm filter. The eluate flowed then from the oven to the detector, an SM 3100X UV detector fitted with an 8-μl high pressure, stainless-steel UV flow cell, designed to stand operation up to 560 bar and 100°C. The absorbance at a selected wavelength was reported by a computing integrator (CI 4000, LDC). The eluate then flowed to a pressure regulator

(Model 26-1721-24-084, Tescon, Elk River, MN, U.S.A.), fitted in the column oven. The depressurized mixture of carbon dioxide–methanol was bubbled through water in a flask.

Columns

Eight columns were used in this work. Six columns were packed with 5-μm particles. These were an aminopropyl silica column (250 × 4.6 mm I.D., LDC), a cyanopropyl silica column (150 × 4.6 mm I.D., LDC), two octadecyl silica columns (150 × 4.6 mm I.D. and 250 × 4.6 mm I.D., both LDC), a ADExII Diol column (250 × 4.6 mm I.D., Jones Chromatography, Hengoed, Mid-Glamorgan, U.K.) and a methyl-coated column (150 × 4.6 mm I.D., Jones Chromatography). Another aminopropyl column (150 × 4.6 mm I.D., Hichrom, Worthing, U.K.) was packed with 7.5-μm particles. A silica column (100 × 4.6 mm I.D. HPLC Technology, Macclesfield, U.K.) was packed with 3-μm particles.

Reagents

A standard solution was prepared from azadirachtin (1 μg μl^{-1} in methanol) isolated from the seeds of Neem by a modification of the method of Butterworth and Morgan [4]. Ten-fold dilutions in methanol were made of this solution down to 10 pg μl^{-1}, a calibration curve was prepared by injecting 10 μl of these standard solutions in the range of 10^{-3} to 10 μg using an absorbance range from 0.1 to 0.002 units. These solutions were normally kept in the refrigerator.

Extracts of Neem seeds from India and West Africa, containing concentrates of azadirachtin were examined under the same conditions, diluting the crude solution as required in methanol. For each determination 10 μl diluted extract was injected.

Operation

To eliminate some of the variables, a constant pressure of 3000 p.s.i. was chosen, and since temperature did not greatly affect separations, and to avoid any possibility of decomposition at higher temperatures, all work was carried out at 55°C. The UV detector was set at 212 nm. The delivery system was operated over a range from 1.5 to 3.0 ml min^{-1} and from 1 to 20% methanol. From this range, a CO_2–methanol ratio of 19:1 and a flow-rate of 2.0 ml min^{-1} were chosen for routine operation.

RESULTS AND DISCUSSION

The UV absorption spectrum of azadirachtin in methanol was first determined on a solution containing 50 μg ml^{-1} over the range 350 to 190 nm. The absorption maximum was found to be at 212 nm. Absorption fell to 6% of the maximum value at 254 nm. It was therefore considered practical to measure absorption in SFC using carbon dioxide–methanol (UV cut off at 203 nm) as modifier.

A number of columns and conditions, were examined to find suitable conditions for the elution of azadirachtin. The retention and column characteristics for azadirachtin under a constant set of conditions of temperature, flow-rate and mobile phase composition are given in Table I for the columns used. Relatively short retention times and good peak shape were easily obtained. The aminopropyl and cyanopropyl columns seemed most suitable for azadirachtin. The effect of flow-rate and methanol

TABLE I

EFFECT OF STATIONARY PHASE ON RETENTION AND SEPARATION EFFICIENCY (k', n, R_s) OF AZADIRACHTIN UNDER SFC

Temperature: 55°C; mobile phase: 5% methanol in carbon dioxide; flow-rate: 3.0 ml min^{-1}; and pressure: 3000 p.s.i.

Column	Manufacturer	t_R	k'	n	R_s
5 μm Cyanopropyl 150 × 4.6 mm I.D.	LDC Analytical	6.80	5.52	8213	28.8
5 μm Aminopropyl 250 × 4.6 mm I.D.	LDC Analytical	10.23	4.32	2616	15.11
5 μm ODS 150 × 4.6 mm I.D.	LDC Analytical	2.00	1.20	711	7.16
5 μm ODS 250 × 4.6 mm I.D.	LDC Analytical	9.86	4.62	1080	0.94
5 μm C-1 150 × 4.6 mm I.D.	Jones Chrom.	3.57	0.63	189	2.0
5 μm Diol 250 × 4.6 mm I.D.	Jones Chrom.	9.21	3.40	2770	15.8
7.5 μm Aminopropyl 150 × 4.1 mm I.D.	Hichrom	9.53	8.62	9082	28.5
3 μm Silica 100 × 4.6 mm I.D.	HPLC Tech.	4.54	1.93	3664	14.95

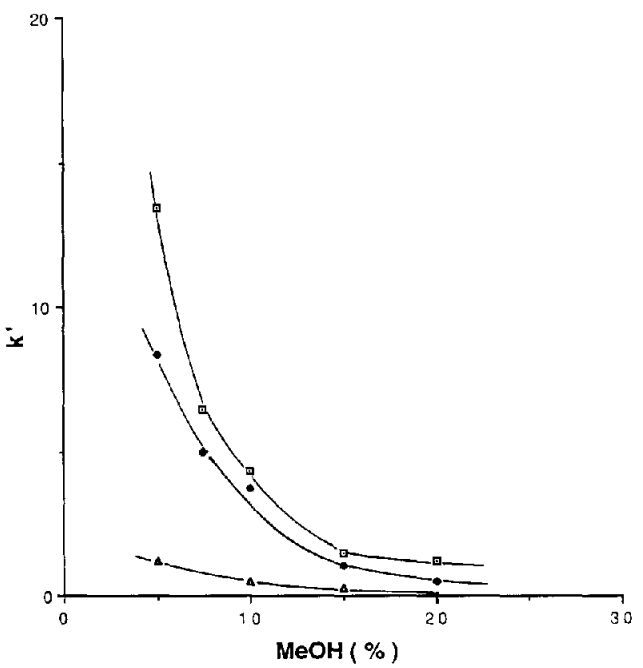

Fig. 2. Plot of capacity factor, k', for azadirachtin against percentage of methanol in the mobile phase at 55°C and a flow-rate of 3.0 ml min^{-1} on three different columns. □ = 7.5-μm aminopropyl, 150 mm; ◆ = 5-μm aminopropyl, 250 mm; △ = 5-μm C-1, 150 mm.

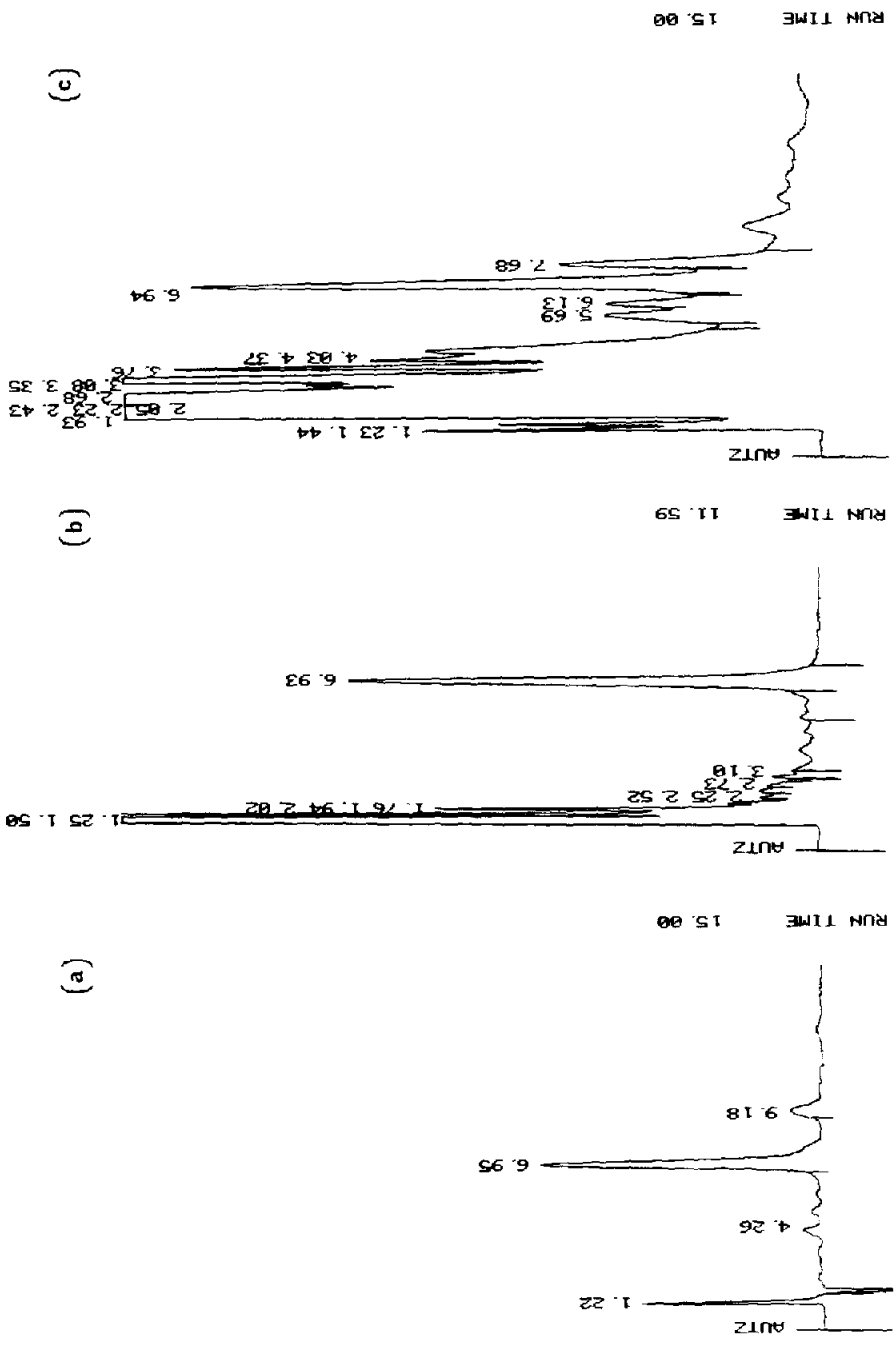

Fig. 3. Chromatograms obtained of (a) almost pure azadirachtin; (b) a partially purified solid concentrate obtained from Neem seeds containing 30% azadirachtin; and (c) a liquid extract of Neem seeds containing 4% of azadirachtin. Column, 7.5-μm aminopropyl silica, at 55°C and 2.0 ml min^{-1}, 7.5% methanol in CO_2. Retention times in min. Azadirachtin at 6.9 min.

concentration on retention were studied further. Convenient flow-rates were found between 2 and 4 ml min^{-1}. The capacity factor, k', was not greatly affected by changing the polarity of solvent with the methyl coated (C-1) column (Fig. 2). On two of the aminopropyl columns, however, decreasing methanol proportion gave large increase in separating efficiency, as is commonly found in SFC operation. This change in retention is valuable when it is necessary to separate the azadirachtin from large amounts of less polar contaminants in crude seed extracts. Optimum operation on the 7.5-μm aminopropyl silica column was obtained at flow-rate 2.0 ml min^{-1} and 7.5% methanol as shown in Fig. 3a. At these conditions, resolution (R_s) of 28.62 and plate number (n) of 12 365 were obtained. By comparison with Table I, these conditions were obviously an improvement on the standard set of conditions used to compare column performance. A plot of log concentration against log absorbance over four orders of magnitude is shown in Fig. 4. Good linearity was obtained over different absorbance settings. The least detectable amount was found by successive dilution to be 10 ng injected, with a signal-to-noise ratio of 5.

A chromatogram of almost pure azadirachtin is shown in Fig. 3a. A solid extract containing more than 30% of azadirachtin is shown in Fig. 3b and a crude Neem extract containing a wide range of ethanol-soluble triterpenoids, including azadirachtin (4%, w/v) is shown in Fig. 3c.

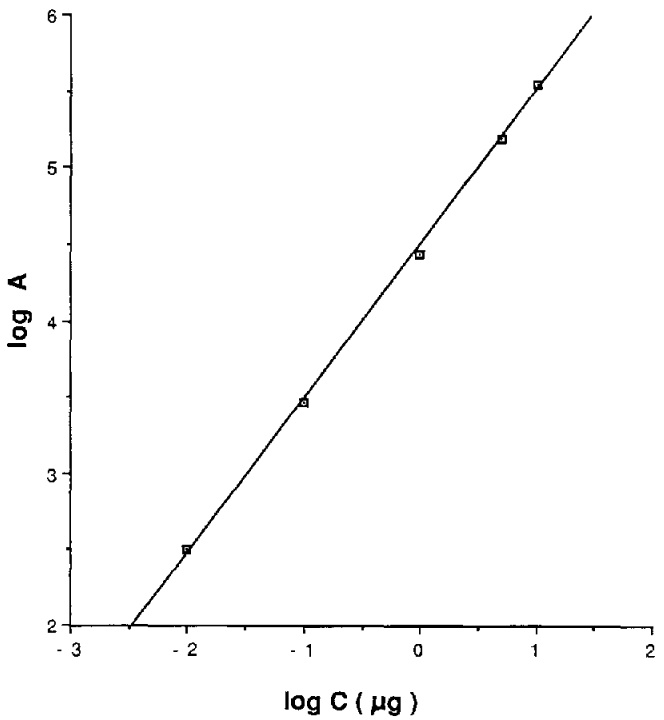

Fig. 4. Plot of absorbance against concentration of azadirachtin over the range 10 μg to 10 ng, using the absorbance range from 0.10 to 0.002 units, on an aminopropyl column with a flow-rate of 1.8 ml min^{-1}. Absorbance measured at 212 nm.

In most Neem extracts, the next most abundant triterpenoid product is salannin (Fig. 1B) which also has a very short wavelength UV absorption, and possesses some insect repellent [8] and growth-disrupting properties. Its retention time is relatively sort (0.20 of that of azadirachtin on the 7.5-μm aminopropyl column), where there can be a lot of other interfering compounds in crude extracts. Desoxydesacetylazadirachtin [9] (Fig. 1C), a compound closely related to azadirachtin, is usually present in small quantities and elutes just before azadirachtin (at 6.13 min in Fig. 3c).

SFC is a suitable system for determining azadirachtin in crude extracts of Neem. Almost all the accompanying substances are less polar than azadirachtin and are eluded quickly. On reversed-phase HPLC they elute very slowly or must be removed by some pre-treatment. The UV absorbing peak for azadirachtin can be readily separated from the other substances present in a relatively short time and accurate quantification made. The method could be valuable in the analysis of commercial Neem exracts.

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