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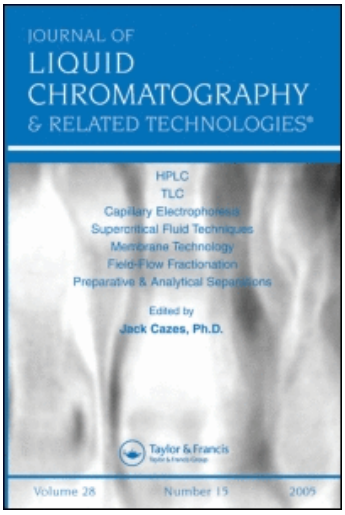


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# ESTIMATION AND ISOLATION OF AZADIRACHTIN-A FROM NEEM [*AZADIRACHTA INDICA A. JUSS*] SEED KERNELS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

P. T. Deota[a]; P. R. Upadhyay[b]; K. B. Patel[b]; K. J. Mehta[b]; B. V. Kamath[a]; M. H. Mehta[b]
[a] Department of Chemistry, Faculty of Science, M. S. University of Baroda, Vadodara, India [b] R & D Centre, GSFC Ltd., Vadodara, India

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# ESTIMATION AND ISOLATION OF AZADIRACHTIN-A FROM NEEM [*AZADIRACHTA INDICA A. JUSS*] SEED KERNELS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

P. T. Deota,[1,*] P. R. Upadhyay,[2] K. B. Patel,[2] K. J. Mehta,[2]
B. V. Kamath,[1] M. H. Mehta[2]

[1]Department of Chemistry
Faculty of Science
M. S. University of Baroda
Vadodara – 390 002, India

[2]GSFC Science Foundation
R & D Centre, GSFC Ltd.
Fertilizernagar, Dist.
Vadodara – 391 750, India

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## ABSTRACT

An improved reverse phase high performance liquid chromatography (HPLC) procedure has been described to estimate azadirachtin-A content in crude methanolic extracts of neem seed kernels using valley-to-valley integration techniques. The present paper highlights a detailed study of extraction, estimation, and purification of azadirachtin-A present in neem seed kernels. We also present, herein, our results of Soxhlet extraction of Aza-A from the defatted neem seed cake as a function of time, which is found to be more efficient than the conventional extraction procedure.



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## INTRODUCTION

The versatility of the neem tree, a species native to India, is growing in tremendous potential for human use.[1] *Azadirachta indica A. Juss* (Neem tree), an indigenous tree of Indo-Pakistan subcontinent, belongs to the family Meliaceae and is widely distributed in Asia, Africa, and other tropical parts of the world.[2] The active compound of the neem seed extracts that has been isolated is a mixture of seven structurally related tetranortriterpenoids known as azadirachtin-A to azadirachtin-G.[3a] Among them, azadirachtin-A (Aza-A) ($C_{35}H_{44}O_{16}$) [Figure 1], which shows promising biological activity, is present in large proportion (~85 %).[3a,4] It has also been shown to be non-mutagenic, biodegradable, and non-toxic to mammals,[5] but sensitive to air, sunlight, heat, and moisture.[6]

In a synthetic endeavor to develop more potential compounds based on Aza-A, we required an efficient method for its isolation from kernels and estimation. A survey through literature, however, revealed that there existed no such method, which would give us exact amounts of purity of Aza-A after extraction and isolation.[7-10] It was, therefore, thought to develop an analytical methodology for our purpose mentioned above.

At the outset it appeared to us that the analytical methodology for the quantization of Aza-A was still in developmental stage as compared to established synthetic pesticides. Due to its thermal instability and possible adsorption on the stationary phase, a gas chromatographic method is unsuitable for the analysis of Aza-A.[6,7] The preferred method for the estimation of Aza-A is by HPLC-UV.[9-12] A number of analytical and preparative HPLC-UV methods have been reported for isolation and estimation of Aza-A in neem seed kernels.[7-13] However, they involve either a complex procedure[7b, 8,11] or do not report the initial and final purity of the azadirachtin samples[8,10,13] or were not sensitive.[9]

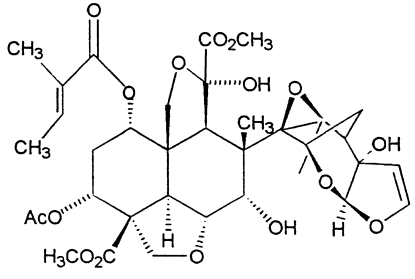


**Figure 1**. Structure of Azadirachtin.

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In this paper, we describe a simplified extraction procedure for Aza-A from neem seed kernels and a quantitative analytical HPLC-UV method for its estimation. Several HPLC-UV determinations showed that Aza-A was always obtained as a distinct peak from other components in methanolic extracts of seed kernels. Hence, we have developed a preparative purification method of Aza-A (up to 91%) from a very complex mixture obtained from plant source.[3b]

## EXPERIMENTAL

### Plant Material

The seeds developed over a period of four months from July to October were collected from Kheda region, Gujarat, India. The soft husk was removed from the seeds using a decoating machine.

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### Solvents

All the solvents (both commercial as well as HPLC grade) were obtained from Qualigens, Glaxo India Ltd. and filtered through a Millipore filter (0.45 μm). Water (18Ω) HPLC grade was purified by Elga Water Purification System Life Science Maxima, and filtered as above. Extractions were carried out at room temperature with an overhead mechanical stirrer for different time intervals unless otherwise mentioned.

### Standard Azadirachtin-A

Analytical grade standard Aza –A was obtained from Sigma Chemical Co. A 1000-ppm stock solution of the standard Aza-A in methanol (0.5 mg/0.5 mL) was prepared and stored between 0 - 4°C. Aliquots of the solutions were diluted with mobile phase prior to use, to give working solutions containing Aza-A in the range of 31.25 to 250 ppm (15.62 –125 μg/0.5 mL), which on injection gave a distinct peak with retention time ($t_R$) of 6.95 minutes (Figure 2A) under the specified conditions.

### Extraction and Enrichment

Extraction of the kernel powder was carried out by the method of Schroeder and Nakanishi[8] with some modifications. The changes made were that extraction with ethanol, vacuum liquid chromatography, and flash chromatography were omitted to make the process simpler and more efficient.

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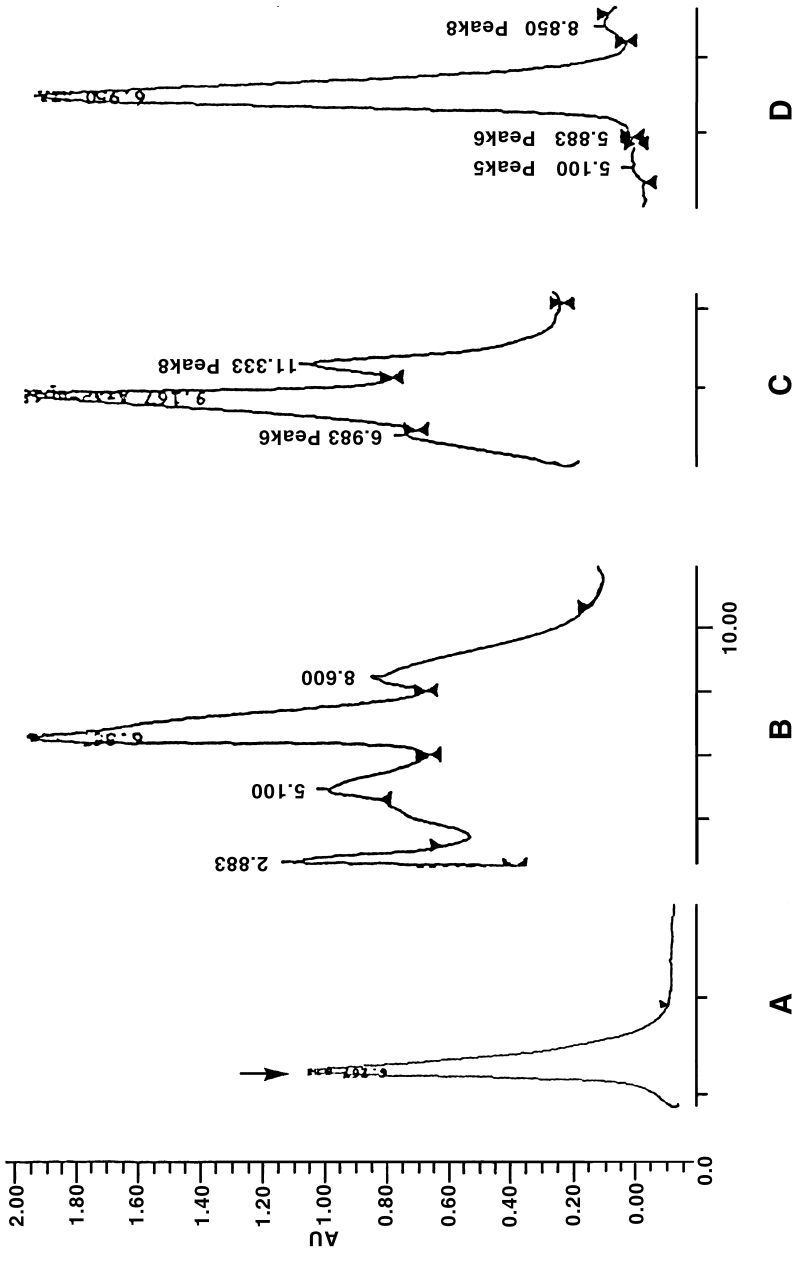


**Figure 2.** Chromatograms of Azadirachtin; (a) Standard; (b) Crude powder from methanol extracts; (c) In preparative mode; (d) Fractions A and B.

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Finely ground powder of neem seed kernel (500 g) was extracted with hexane (1.0 l ) for 30 minutes (Figure 3). The hexane extract was filtered and the process repeated with fresh hexane three more times. The pooled hexane extracts were concentrated under reduced pressure in a rotary evaporator between 45-50°C, which yielded fatty oil (A) (40-45%) having 0.0235% Aza-A (w/w of oil) as determined by following the procedure of Sundaram *et al.*[7] The dry defatted cake (B, 250 g) was then extracted with methanol (6 × 500 mL).

The six methanolic extracts were analyzed for Aza-A content by analytical HPLC-UV method (*vide infra)*. These combined extracts were then concen-

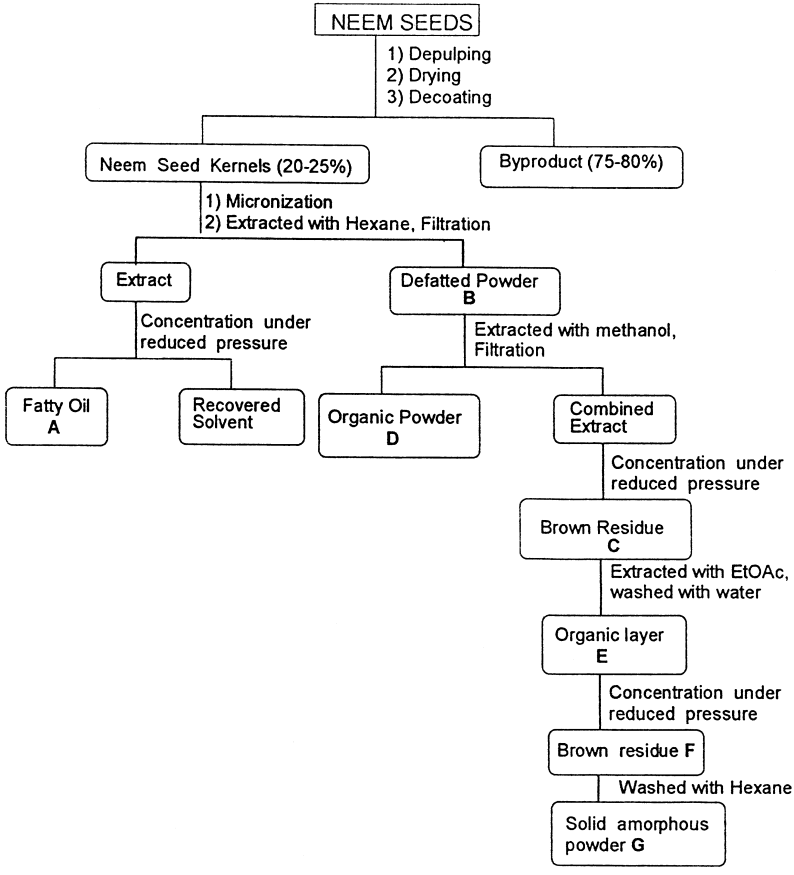


**Figure 3**. Flow chart of Neem Seed Extraction Procedure.

trated under vacuum, after filtration, to give a brown residue (C, 200 mL) and organic powder (D). The brown concentrated residue containing Aza-A was further extracted with ethyl acetate (300 mL) for 30 minutes to give (E). This was then washed with distilled water (150 mL) to remove water-soluble compounds such as carbohydrates, proteins, and minerals followed by passing it through a column of anhydrous sodium sulfate (17mm id × 45cm). The column was rinsed with ethyl acetate (25mL) which was added to (E).

It was then concentrated under reduced pressure to furnish a brown residue (F), which was washed with hexane (4 × 15 mL) to give a dark brown amorphous solid (G, 6.5 g). A weighed portion of this solid was dissolved in methanol for the estimation of Aza-A content in it using analytical HPLC-UV method (*vide infra*). It should be mentioned here that the extraction procedure reported, herein, is much shorter and economical than the one reported earlier.[7b]

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**Soxhlet Extraction**

Finely ground powder of neem seed kernels (500 g) was extracted with hexane (1.0 L). The defatted solid cake (B, 230 g) obtained by following the above procedure was subjected to Soxhlet extractions for Aza-A enrichment using methanol (500 mL). These experiments were carried out for 24 and 72 hours independently and their Aza-A contents were analyzed by HPLC-UV method (*vide infra*) at the interval of 4 h.

**High Performance Liquid Chromatography**

Analytical as well as preparative HPLC-UV studies were carried out on a Waters LC-4000 system linked with chromatography software Millennium 2010. The HPLC system was equipped with a Flow controller (Waters model No: 600), a tunable absorbance UV Detector (190-600 nm, Waters model No: 486), a guard column, Rheodyne injector 7010, and an automatic fraction collector. The instrument employed an automatic degassing system and dual pump heads with common drive flows. The Millennium 2010 provided the chromatograph, calibration curve, peak area, area %, and retention time ($t_R$) etc.

Analytical HPLC-UV was carried out on a reverse phase $C_{18}$ Bondapak column (3.9mm id × 300 mm) with 10 μm particle size and 125Å pore size. The solvent system consisting of methanol: water (60:40 v/v) was run isocratically at a flow rate of 1 mL/min and an average pressure of 1500-2000 psi for an injection volume of 20 μL. The peak corresponding to Aza-A was detected at 217 nm. Detected peaks were integrated (valley-to-valley) and retention time was recorded using Millennium 2010, set at peak width of 30.00, retention window 5%, and threshold 25. The chromatographic run time was 30 minutes.

The brown amorphous solid (1.0 g, 9.14% purity) obtained after concentration of organic layer (F), was dissolved in methanol and filtered through a Nylon 66 filter (0.45 μm removal rating and 0.13 mm diameter). This solution (1 mL) was then injected into a preparative reverse phase $C_{18}$ Bondapak (19 mm id · 300mm) column, with 15-20 μm particle size and 125 Å pore size, and eluted isocratically with methanol: water (60:40 v/v). The peak was detected at 217 nm under 400-500 psi column pressure at a flow rate of 15 mL/min. Under these conditions Aza –A peak appeared at 9.16 min during the preparative run. When the Aza-A peak ascended from the baseline, the eluent was collected until the peak was at maximum (Fraction 'A') and the peak descended to the baseline again (Fraction 'B'). The presence of the Aza-A in fractions 'A' and 'B' was confirmed by the analytical HPLC-UV (*vide supra*). The less polar compounds could be removed by eluting the column with pure methanol for 10 to15 min at a flow rate of 20 mL/min. Restabilization of the column was done with methanol: water (60:40 v/v) for another 20 - 25 min.[10]

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## RESULTS AND DISCUSSION

Enrichment by solvent extraction and partition was carried out to prepare neem fractions rich in azadirachtin (Figure 3). The extraction of Aza-A with methanol was found to be more efficient, when the ground neem seeds were defatted with hexane. Hexane extracts removed neem oil having Aza-A content of 0.0235% (w/w of oil).[19]

For complete recovery of Aza-A, the defatted powder (B) was extracted six times with methanol at room temperature.[13] Methanol extractions removed the traces of oil left with kernels along with Aza-A, soluble sugars, amino acids and proteins. To ensure near-complete extraction of Aza-A from defatted neem cake, we applied the present HPLC-UV method, which proved to be very useful. Our results showed negligible quantities of Aza-A (< 0.0041%) left in the defatted neem seed cake after six extractions.

The Aza-A content in the six methanolic extracts ranged from 0.21 to 0.0041%. An analytical HPLC-UV method was used to estimate Aza-A content in crude powder. For reverse phase HPLC-UV method, aqueous methanol (60:40 v/v) was suitable for determination and separation of Aza-A giving sharp peaks and good resolution without much baseline drift. The retention time ($t_R$) of crude Aza-A in our system was 6.9 min (Figure 2B). The concentration of Aza-A in the crude methanol extracts and in the solid powder (G) was determined from the calibration curve obtained by plotting of peak area versus Aza-A concentration, by injecting 20 μL of standard Aza-A solutions in triplet, ranging in concentration from 31.25 to 250 ppm. From the HPLC-UV analysis and calculations, the percentage purity of Aza-A in crude powder was found to be 9.14%, the same in seed kernels was found to be 0.11% and in fruits 0.029%. Our results are in close agreement with those reported in literature.[14,19]

The preparative HPLC-UV method used in this study was chosen on the basis of the results obtained from analytical HPLC-UV. Each 1mL injection of methanolic solution containing ~9.14% Aza-A content in the residue and each run was completed in 60 min yielding 5-10 mg of 88 to 90% pure Aza-A. Seed kernels (500 g) gave 6.5 g of crude powder containing 9.14% Aza-A. From 1g of this powder, 90 mg of the 88 to 90% pure Aza-A was isolated. The retention time ($t_R$) of Aza-A in our system was 9.16 min (Figure 2C). Fractions 'A' and 'B' (*vide supra*) were found to contain approximately equal amounts Aza-A (Figure 2D). IR, UV, and PMR spectra of the sample of Aza-A purified by this method were found to be in good agreement with those reported in the literature.[15-18]

In Soxhlet extraction experiments, Aza-A content was determined by analytical HPLC-UV method by taking samples of extracted solutions at 4 h interval for total period of 24 and 72 h, independently. The HPLC-UV results showed that the enrichment of the Aza-A in the extracted solutions by Soxhlet method (0.449%) was higher than the normal extraction method (0.386%, after

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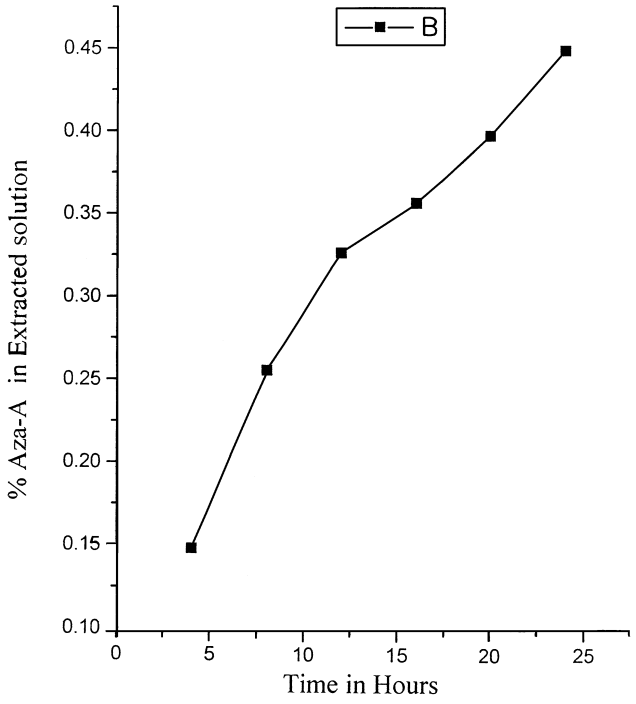


**Figure 4**. Plot of % Aza-A extracted vs. time (24 hrs) by Soxhlet method.

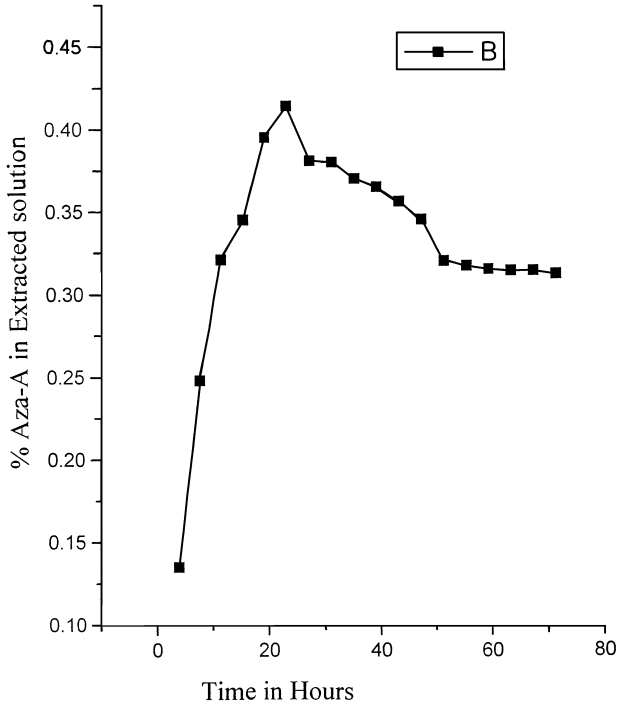


**Figure 5**. Plot of % Aza-A extracted vs. time (72 hrs) by Soxhlet method.

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six extractions with methanol) thus showing the former method to be more efficient. The HPLC-UV results and Figures 4 and 5 showed that in Soxhlet extraction, the amount of Aza-A in the extracted solutions increased up to 24 h initially and decreased gradually, threafter, perhaps due to decomposition of Aza-A.

## CONCLUSIONS

The present study describes an improved reverse phase HPLC method, for the quantification of Aza-A in neem seed kernels and its purification of single peak purity using the preparative HPLC-UV method. In addition, a short and economical extraction procedure of Aza-A from kernels is presented. The HPLC-UV results show that the Soxhlet method, which gives a higher percentage of extracted Aza-A, is preferable over a conventional one. Other applications of this procedure could include the determination of Aza-A in degraded neem formulations, which is the subject of our future study.

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