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Application News

A Simple and Robust Method for Determination of Azadirachtin, Nimbin and Salannin in Neem Oil

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User Benefits

- Efficient and Robust method
- Automation and high throughput

■ Introduction

Neem (*Azadirachta indica*) belongs to the *meliaceas* family and grows in the Indian subcontinent and Southeast Asia. The species have long history as a traditional ingredient for household remedies. Oil extracts from the seeds have been used for soaps and cosmetics and twigs of the plants have been used as tooth-brushes. Farmers also used traditionally various parts of the plants to control insect pests in stored crops and for livestock diseases. Its insecticidal properties and low toxicity to mammals have particularly attracted scientists in chemistry, pharmacology and agriculture and many bioactive compounds have been identified in the plant such as nimbin (anti-inflammatory), nimbidin (antibacterial, anti-ulcer), nimbidol (anti-tubercular, anti-protozoan), gedunin (anti-malaria, anti-fungal), sodium nimbinate (diuretic, anti-arthritic) and salannin[1].

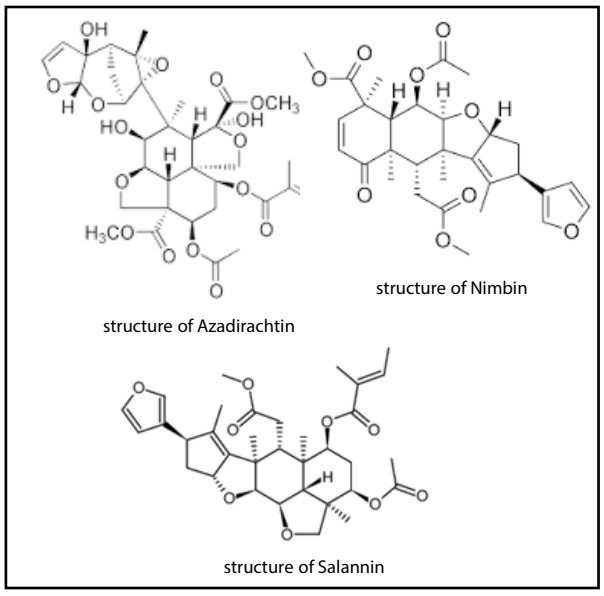


Fig. 1 structures of azadirachtin, nimbin and salannin

Major attention has been paid in recent years to the use of neem-based formulations in the control of pest infestation. The main active ingredient, azadirachtin, has been found to exhibit a variety of properties such as antifeedant and anti- ovicidal effects, disrupting the life cycle of different insects. During the past few years, it has been found that neem oil / extract-based products are highly active against 200 insect species belonging to different orders. Also, azadirachtin has low mammalian toxicity and does not affect most beneficial organisms. Main meliacins in neem oil are azadirachitin, nimbin & salanin (shown in Fig. 1) [2][3].

■ Experimental

A simple and rapid method involving solid phase extraction and liquid chromatography for the determination of azadirachtin-A, azadirachtin-B, nimbin and salannin at nanogram levels in neem oil samples is presented. The chromatographic separations were accomplished using mobile phase consisting of acetonitrile and water. Mobile phase was pumped at a flow rate of 1.0 mL/min at room temperature. Detection was carried out using photodiode array detector at wavelength of 215 nm. LC method was developed using LC-2030C 3D HPLC from Shimadzu Corporation Japan. The method was successfully applied to various neem oil samples collected from different locations in India.

■ Method

The LC conditions are given in Table 1.

Table 1 LC conditions

Column	: Shim-pack™ GIST C18 (250 mm x 4.6 mm,5 µm, P/N : 227-30017-08)
Mobile Phase	: Water
Mobile Phase B	: Acetonitrile
Flow rate	: 1.0 mL/min.
Separation Mode	: Gradient
Gradient Program	: B Conc. 35% (0-14 min) → 85% (14.01-35 min) →85% (35-38 min) → 35% (38.01-42 min) →35% (42- 50 min)
Injection Volume	: 20 µL
Detection	: 215 nm
Run Time	: 50 minutes
Needle Wash	: Water / Acetonitrile = 50 / 50

■ Linearity and quantitation

For quantitative analysis, multipoint calibration curve for all components were prepared and plotted after analysis using condition described in Table 1. Limit of quantitation (LOQ) for azadirachtin A, azadirachtin-B is 0.5 ppm; whereas, for nimbin, salanine it is 0.2 ppm. The S/N, coefficient of regression & %RSD at LOQ level are shown in Table 2. Fig. 2 and Fig. 3 depict the calibration curves for azadirachtin-A, azadirachtin-B, nimbin and salannin.

Table 2 Summary of calibration curves

Limonoids	r^2	LOQ		
		Conc. In ppm	%RSD (n=6)	S/N
Azadirachtin-A	0.9995	0.50	1.5	64
Azadirachitin-B	0.999	0.50	1.2	70
Nimbin	0.9997	0.20	0.8	52
Salannin	0.9995	0.20	0.95	77

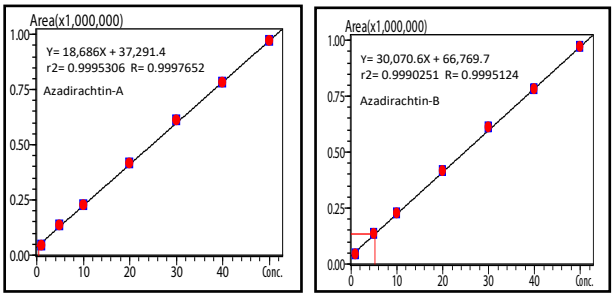


Fig.2 Calibration curves for azadirachtin-A and azadirachtin-B

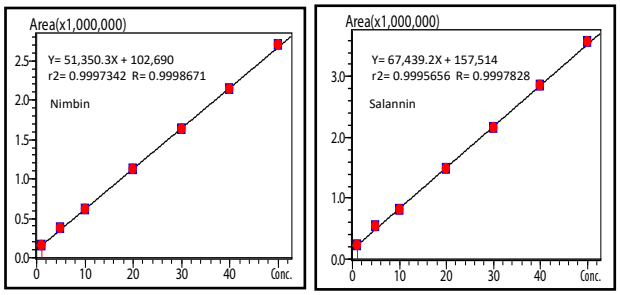


Fig.3 Calibration curve for nimbin and salannin

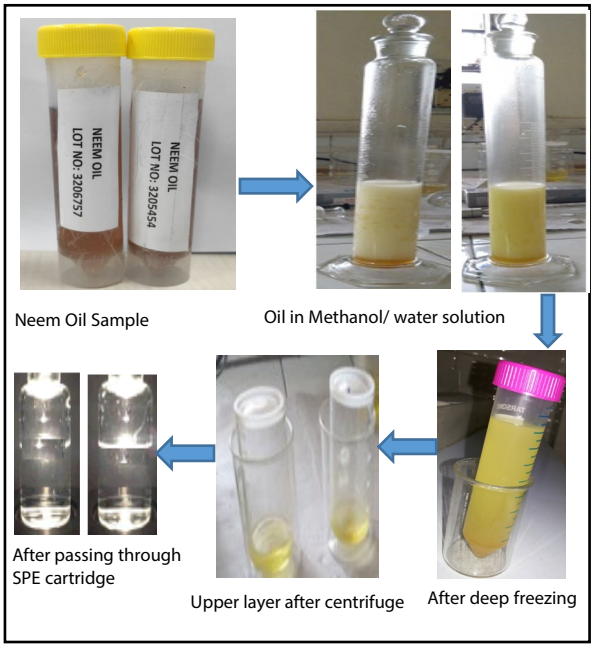
■ Sample preparation

I. Weigh Accurately (approx. 5 g) sample quantity so as to contain 2 mg of azadirachtin in 50 ml volumetric flask and dissolve in a mixture of methanol and water (90:10). Make up to mark and shake well for five minutes as shown in below images of sample preparation.

II. Sonicate the sample solution for approx. 15 minutes. again, shake vigorously for approx. 5 minutes and sonicate for 15 minutes then place in deep freezer for 15 minutes.

III. Take supernatant in centrifuge tube and close the lid. Centrifuge for 30 minutes at 5000 RPM in order to separate the layers.

IV. Take 2 ml of upper layer with the help of pipette into SPE cartridge. Elute it into 10 ml volumetric flask with repeated washing with a mixture of methanol and water (90:10). Make up to the mark, shake well.



■ Results and Discussion

A sensitive and rapid method for the sequential determination of azadirachtin-A and azadirachtin-B, nimbin and salannin in neem oil samples have been developed. The method shows no interferences during the HPLC analysis of the neem oil samples, after cleaning of sample using SPE cartridge. Analysis chromatogram of mixed standards is shown in Fig. 4. Analysis chromatogram of neem oil sample is shown in Fig. 5.

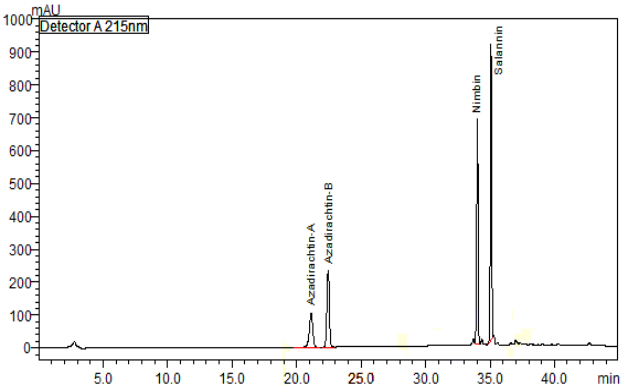


Fig. 4 Mixed standards (Azadirachtin, nimbin & salanin) chromatogram

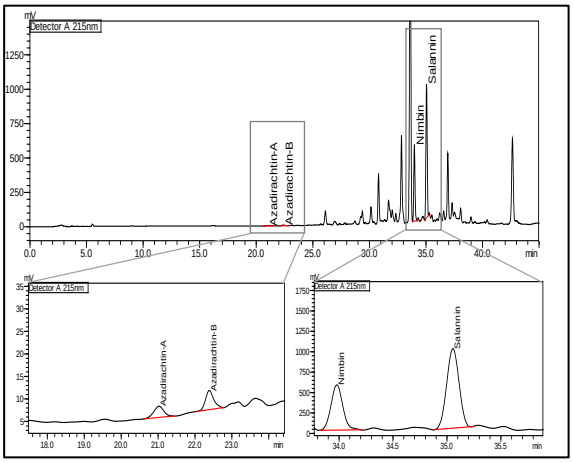


Fig.5 Chromatogram of Neem oil analysis along with zoomed chromatograms for showing peaks azadirachtin- A & B and peaks of Nimbin & salanin in sample chromatogram

■ Conclusion

A sensitive and rapid method for the sequential determination of azadirachtin A and azadirachtin B, nimbin and salannin in neem oil samples has been developed. Method was checked for linearity, ruggedness , robustness and specificity. The method shows no interferences during the HPLC analysis of the neem oil samples after cleaning of sample using SPE cartridge and co-elution in case close eluting peaks were checked using PDA peak purity function. Different neem oil samples, used for coating of urea were analysed with developed method. Average amount of total azadirachitine observed in different samples were between 200 mg/ kg to 1000 mg/kg. Average nimbin and salanin determined from different sample were in range of 2500- 5000 mg/Kg and 3500- 6000 mg/Kg. Actual amount of azadirachtin, nimbin and salannin is calculated using linearity curve and using dilution factors used in sample preparation. Concentration of azadirachtin, nimbin and salannin in neem oil can also be calculated using custom calculations of LabSolutions software. During method development and studies of neem oil samples, if neem oil samples kept at ambient temperature for 1-2 months, then there observed degradation of neem oil contents. In degradation of neem oil either azadirachtin A converted to azadirachtin-B or degradation in total azadirachtin observed.

■ References

1. Hisashi Kato-Noguchi et al, Molecules **2014**, 19, 6930-6940.
2. Andrew P. Jarvis et al, *Journal of Chemical Ecology* ***1997***, *23, 2841-2860.*
3. Atmakuru Ramesh et al, *The Analyst* , **1999**, 124,19-21.

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