

# Rapid preconcentration method for the determination of azadirachtin-A and -B, nimbin and salannin in neem oil samples by using graphitised carbon solid phase extraction

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A simple and rapid method involving solid phase extraction and liquid chromatography for the determination of azadirachtin-A and -B, nimbin and salannin at nanogram levels in neem oil samples is presented. The neem oil samples are defatted and the compounds of interest extracted by mixing the sample with hexane and passing the hexane solution through a graphitised carbon black column. After washing the column with 2 ml of hexane, azadirachtin-A and -B, nimbin and salannin are eluted with 5 ml of acetonitrile and quantified using HPLC with UV detection. The recoveries of azadirachtin-A and -B, nimbin and salannin in fortified oil samples were 97.4–104.7%. The upper limit of quantification is up to  $100 \mu\text{g ml}^{-1}$  without any additional clean-up and with little interference from lipids during the analysis by HPLC. The method was successfully applied to various neem oil samples collected from different locations in India.

## Introduction

Major attention has been paid in recent years to the use of neem based formulations (*Azadirachtica indica*, A.Juss) in the control of pest infestation.<sup>1</sup> The main active ingredient, azadirachtin, has been found to exhibit a variety of properties such as anti-feedant and anti-ovicidal effects, disrupting the life cycle of different insects.<sup>2–4</sup> 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.<sup>5,6</sup> Unlike synthetic chemical insecticides, which are mostly contact neurotoxins, azadirachtin is a selective compound affecting the endocrine system of insects in addition to being an anti-feedant.<sup>7</sup> Because of this selectivity and its rapid degradation,<sup>2,8</sup> azadirachtin is considered to be less damaging than synthetic insecticides to the environment and to pose a much smaller threat to non-target organisms, including humans *via* food residues, surface and ground water contamination or accidental exposure.<sup>9–11</sup> Further, it is readily biodegradable and hence is perceived to be environmentally safe and ecologically acceptable.<sup>12–14</sup> In general, neem oil contains azadirachtin at a concentration of approximately 0.3% (but this may vary depending on the locality), along with other components such as nimbin, salannin and meliantriol. Most of the formulations currently used are based on the content of azadirachtin,<sup>15–17</sup> although other active ingredients of neem oil have also been found to exhibit potent insecticidal activity. There is currently an increasing demand for the ability to monitor azadirachtin at lower and lower concentrations because standardisation and commercialisation of all the neem products are solely based on azadirachtin content. In recent years, the use of graphitised carbon black (GCB) solid phase extraction (SPE) methods have been described for the analysis of environment samples for a variety of pesticides and herbicides.<sup>18,19</sup> In continuation of our experiments<sup>20</sup> with graphitised carbon black as a solid phase extraction material for the concentration of pyrethroids in oil samples, we report a rapid and simple method for the determination of active ingredients azadirachtin-A and -B, nimbin and salannin in neem oil samples. The method

involves a simple clean-up procedure for the removal of interferences.

## Experimental

Reference standards of azadirachtin-A and -B, nimbin and salannin were obtained from Trifolio-M, (Lahnau 2, Germany). Trace analysis grade acetonitrile was supplied by Merck, (Darmstadt, Germany). All other chemicals were of analytical reagent grade. The high performance liquid chromatographic (HPLC) system, supplied by Shimadzu (Kyoto, Japan) consisted of a model LC-10 AT pump and SPD-10A UV-VIS detector interfaced to a Winacds data station supplied by Aimil (Bangalore, India). A Supelcosil LC-18 stainless steel reversed phase column ( $15.0 \text{ cm} \times 4.6 \text{ mm id}$ ) was used for HPLC separation. An isocratic solvent system consisting of methanol, acetonitrile and water (35 + 15 + 50) was prepared and used as the mobile phase at a flow rate  $1.0 \text{ ml min}^{-1}$ .

## Standard solutions

Individual stock standard solutions of azadirachtin-A and -B, nimbin and salannin were prepared in trace analysis grade acetonitrile by dissolving 1 mg of each compound in 10 ml of acetonitrile and storing at  $-4^\circ\text{C}$ . Working standard solutions were prepared by diluting the stock standard solutions to obtain final concentrations of  $10 \mu\text{g ml}^{-1}$  of each compound. These standard solutions were used for the preparation of calibration solutions and for the preparation of fortified samples.

## Solid phase extraction

Graphitised carbon black has been shown to be a valuable sorbent material for SPE for a variety of pollutants in water<sup>21–24</sup>. Graphitised carbon black (500 mg) from Indo-National, (Chennai, India), was placed in a 1 cm stainless steel-glass cartridge between two Teflon frits. The cartridge was attached

**Table 1** Mean recoveries ( $n = 6$ ) of azadirachtin-A and -B, nimbin and salannin

Amount fortified/ $\mu\text{g ml}^{-1}$	Azadirachtin-A		Azadirachtin-B		Nimbin		Salannin	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
100	104.7	2.14	102.0	1.95	101.3	1.97	102.0	1.86
50	103.8	3.13	100.5	3.18	101.9	3.18	99.1	2.19
30	100.8	2.27	99.2	2.42	100.4	2.64	100.5	1.34
20	102.4	2.41	99.3	1.61	99.2	1.97	99.3	1.47
10	99.6	2.04	99.4	1.77	98.2	1.57	97.4	1.70

to a solvent recovery flask connected to a vacuum pump and was conditioned by rinsing with 10 ml of hexane.

### Preconcentration of azadirachtin-A and -B, nimbin and salannin on solid phase extraction cartridges

A 1.0 ml volume of oil sample was taken in a test-tube and 0.2–0.5 ml of 10.0, 20.0, 30.0, 50.0 and 100  $\mu\text{g ml}^{-1}$  standard solutions of azadirachtin-A and -B, nimbin and salannin were added, mixed thoroughly and allowed to stand for 5 min. A 10 ml volume of hexane was added and each tube was shaken vigorously for 3 min. The sample was transferred to the SPE column reservoir and allowed to percolate for 5 min, then a vacuum was applied to drain the oil out completely. After ensuring that the oil had drained completely, the column was slowly washed with 2 ml of hexane. Azadirachtin and other active ingredients were slowly eluted with 5 ml of acetonitrile. The acetonitrile layer was collected, filtered and analyzed by HPLC.

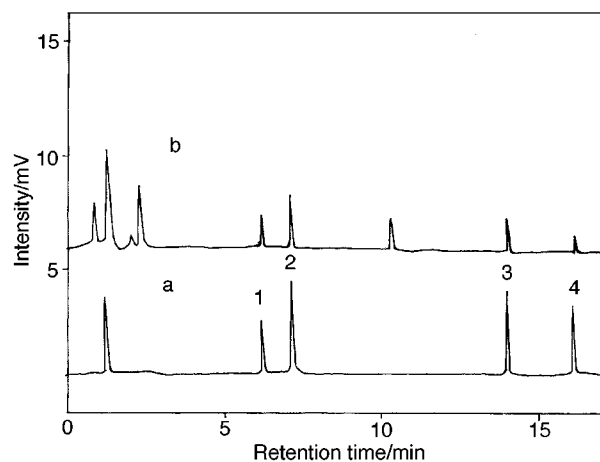
### Recoveries of azadirachtin in fortified neem oil samples

Neem oil samples purified after passage through the GCB cartridge column were fortified with known standards of azadirachtin-A and -B, nimbin and salannin and processed as described earlier. The recovery details are presented in Table 1. Azadirachtin-A and -B showed 99.2–104.7% recoveries at fortification levels of 10–100  $\mu\text{g ml}^{-1}$  with relative standard deviations (RSDs) of 1.61–3.18%, whereas nimbin and salannin showed 97.4–102.0% recoveries with RSDs in the range 1.57–3.18%.

## Results and discussion

The proposed procedure consists in the SPE extraction of azadirachtin in place of the usual liquid–liquid partition step followed by column clean-up. Relatively large volumes of oil sample can be passed through the cartridge and pesticides in small amounts can be concentrated on the surface of the sorbent.

No major interference from lipids was observed during the process. In addition, SPE columns can be used repeatedly at least four times by simply washing with 10 ml of acetone followed by 10 ml of water each time. The elution of the oil takes place efficiently and also more quickly after mixing the oil with 5 ml of hexane. Absolute recoveries were determined by using external calibrations. Additionally there were no interferences during the analysis of oil samples by HPLC for azadirachtin-A and -B, nimbin and salannin. Use of a graphitised carbon black SPE method facilitated the preconcentration of azadirachtin-A and -B, nimbin and salannin and the removal of all impurities associated with oil samples to a major extent, because there are no silanol group interactions with graphitised carbon black, and the adsorption of the compound is solely on carbon. As a result of the factors noted above, this method is an

**Fig. 1** HPLC of (a) standards and (b) sample. Peaks: 1, azadirachtin-B; 2, azadirachtin-A; 3, nimbin; and 4, salannin.**Table 2** Effect of temperature on the stability of azadirachtin-A and -B, nimbin and salannin (30  $\mu\text{g g}^{-1}$  fortification level) when stored in a graphitised carbon black cartridge column at different temperatures

Active ingredient	Recovery (%)					
	At 6 h			At 72 h		
	4 °C	20 °C	30 °C	4 °C	20 °C	30 °C
Azadirachtin-A	102.6	101.8	99.8	102.4	91.2	81.2
Azadirachtin-B	100.8	100.4	98.8	100.8	88.3	78.4
Nimbin	99.6	99.2	98.7	99.6	89.9	74.1
Salannin	99.4	98.9	98.3	99.6	86.2	75.2

improvement on other techniques reported for these compounds.<sup>13,17,27</sup> The chromatogram presented in Fig. 1 shows the clear, sequential separation of azadirachtin-B and -A, nimbin and salannin at retention times of 6.0, 7.0, 14.2 and 16.4 min, respectively.

### Effect of storage

All the cartridges containing azadirachtin and other active ingredients were stored at three different temperatures, 4, 20 and 30 °C, for 72 h to determine the effect of storage conditions on stability. Cartridge samples were stored in a temperature controlled oven/refrigerator. After 72 h, the cartridges were removed from the oven/refrigerator and allowed to come to room temperature. Azadirachtin and other active ingredients were eluted using 5 ml of acetonitrile and analysed by HPLC (Table 2). The results show that samples stored at 4 °C for 72 h retain the initial recovery levels. At 20 °C, on average 10–15% lower recoveries were observed and at 30 °C the recoveries fell by 18–27%.

## Applications to real samples

The method was successfully applied to real samples. Neem seeds collected from three locations in India (Padappai, Chennai; Rajendra Nagar, Hyderabad; and Bangalore City) were crushed and the oil extracted. A 1–2 g amount of oil sample was weighed and processed as described earlier. Analysis of the results showed the maximum concentration of nimbin (3.24–3.53%) in the neem kernels collected from Hyderabad, Andhrapradesh, whereas kernels from Karnataka (Bangalore) and Tamilnadu (Chennai) showed almost equal concentrations (0.70–0.80%) of nimbin. Small amounts of salannin (0.06–0.07%) were also observed in the sample collected from Andhrapradesh, whereas the samples from Karnataka and Tamilnadu did not contain detectable levels of salannin. Samples from Andhrapradesh contained the highest levels (0.24–0.31%) of azadirachtin-A and -B whereas the Karnataka and Tamilnadu samples contained less azadirachtin (0.17–0.21% and 0.26–0.33%, respectively). In general, azadirachtin is very labile when exposed to air, moisture and sunlight. This may be due to the presence of C–C  $\pi$ -bonds.<sup>25,26</sup> The strained molecular structure consisting of epoxide rings and ester groups may make azadirachtin prone to undergo addition, ring cleavage, etc. Further, its instability to UV radiation may also affect the percentage of azadirachtin present in neem seed kernels.

## Conclusions

A sensitive and rapid method for the sequential determination of azadirachtin-A and -B, nimbin and salannin in neem oil samples has been developed. The method shows no interferences during the HPLC analysis of the neem oil samples after preconcentration using graphitised carbon black. Further, the recoveries of azadirachtin-A and -B, nimbin and salannin were higher in comparison with other methods. Major interferences associated with fats/lipids of neem oil samples were simply removed without affecting the active components. As there is no other simple and rapid method for the determination of azadirachtin-A and -B, nimbin and salannin in neem oil samples, the proposed method should be of value. Further, the utility of graphitised carbon black was established once again in the SPE and preconcentration of pesticides. Similar results were observed for some organophosphorus and organochlorine pesticides when tested under the same conditions. Further studies are in progress and the results will be published elsewhere.

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