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Quantitative Estimation of Residues of Neem Compounds (NfC and NC) and Dimilin (IGR) in Treated *Callosobruchus analis* F. and Contact Media (Filter Paper and Glass) by HPLC

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Abstract: Employing high performance liquid chromatography, quantitative estimation of neem compounds, NfC and NC, was carried out in treated insects, *Callosobruchus analis*, and in contact media, in comparison with an IGR, Diflubenzuron (Dimilin). Adult specimens of *Callosobruchus analis* were treated with impregnated filter paper mimicking an absorbent surface and treated glass mimicking a non-absorbent surface of grain storing containers.

In the case of filter paper impregnation, the recovery of NfC was 4.57 µg/10 µl and 1.28 µg/10 µl in treated insect samples, and 0.71 µg/10 µl and 0.50 µg/10 µl in treated filter paper samples after 24 and 144 h of treatment, respectively. Recovery of NC was 0.71 µg/10 µl after 24 h and no trace was found after 144 h in treated insect samples, whereas treated filter paper samples showed 0.21 µg/10 µl and 0.07 µg/10 µl recovery after 24 and 144 h of treatment, respectively. The recovery of Dimilin was 0.14 µg/10 µl and 0.08 µg/10 µl in treated insect samples, and 0.36 µg/10 µl and 0.14 µg/10 µl in treated filter paper samples after 24 and 144 h of treatment, respectively.

In the case of the glass film method, NC treated insects showed 1.42 µg/10 µl and 0.71 µg/10 µl recovery, while the glass rinsed samples showed 0.71 µg/10 µl and 0.07 µg/10 µl recovery after 24 and 144 h of treatment, respectively. Dimilin treated *C. analis* showed 0.84 µg/10 µl and 0.33 µg/10 µl recovery after 24 and 144 h of treatment, while the glass rinsed samples showed 0.12 µg/10 µl recovery after 24 h and no trace was found after 144 h of treatment. NfC extract showed no significant effect by this method and therefore samples were not analysed.

Key Words: Residues, neem compounds, dimilin (IGR), *Callosobruchus analis*, HPLC

Introduction

Natural products of plants are a vast source of bioactive substances, but have been exploited only to a limited extent as models in the synthesis of practical insecticides, acaricides and phagodeterrents.

The search for alternative pesticides for environmental safety and low human toxicity with an ability to control insects is growing. Neem plays an important role in this context. Naqvi (1996) and Khan and Ahmed (2000) have reviewed neem as a grain protectant. However, in order to recommend neem as a grain protectant, it is necessary to evaluate its persistence on treated surfaces as well as its efficacy.

In the present study, the persistency of neem compounds NfC and NC was determined in comparison with Dimilin, diflubenzuron, employing high performance liquid chromatography (HPLC).

Materials and Methods

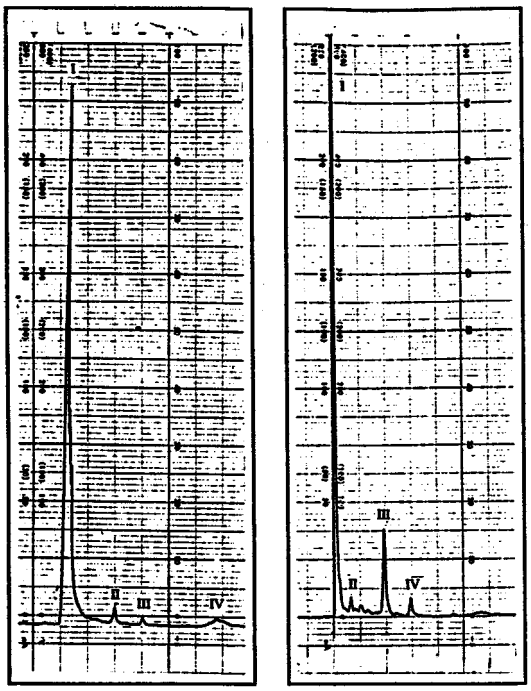
Adults of *Callosobruchus analis* F. were obtained from the GSRI, PARC, Insect Rearing Laboratory and had been reared in the Department of Zoology, University of Karachi, for the previous 5 years at 28 ± 2 °C and 65 ± 5% R.H. HPLC was used to determine the residues of crude neem extract (NfC) and isolated compounds NC, i.e. nimolicine and Dimilin, in treated insects and contact

media. Standard samples of Dimilin, NC and NfC were prepared and standard chromatograms were obtained to use for comparison with the chromatograms obtained from the samples of treated insects and treated filter paper of glass rinsed samples.

The cleaning technique, extraction of fat, Soxhlet extraction, sorption and alumina and silica column techniques for the separation of fat from pesticides were adopted from Holden and Marsden (1969).

For the detection of pesticides, HPLC was carried out on Shimadzu LC-3A using a packed column, Zorbax™ NH_2 Shimpack GLC-ODS. It is a polar bounded phase with particle size of about 7 µm in diameter. n-Hexane was used as mobile phase for Dimilin while methanol was used as mobile phase for NfC and NC, with a flow rate of 0.5 ml/min at pressure 200 kg/cm^2. A UV detector was used at a wavelength of 250 nm and absorbance 0.32 with chart speed as 2.5 mm/min.

NfC (methanolic fraction of neem seed extract, i.e. mixture of tetranortriterpenoids) and NC (nimolicine, i.e. isolated compound from the fraction) were obtained from Dr. Beena S. Siddiqui of H.E.J. Research Institute of Chemistry, University of Karachi. Dimilin was obtained from Dr. A.C. Grosscurt of Holland.



Chromatograph 1: Chromatograph showing the amount of standard NfC samples after the injection of 1 µg/ 10 µl. I = Solvent peak, II = NfC, III and IV = Other components.

Chromatograph 2: Chromatograph showing the amount of NfC in adult of *C. analis* by HPLC after 24 hours of treatment by filter paper impregnation method. I = Solvent peak, II and IV = Other components, III = NfC.

Results

Quantitative analysis of pesticides was carried out by HPLC in treated *C. analis* and contact media with neem compounds (NfC and NC) and Dimilin by filter paper impregnation and glass film method after 24 and 144 h of post-treatment. The retention time of each peak of treated samples was calculated and then compared with the peak of the respective standard pesticides used for the treatment. Samples of 10 µl were injected for the analysis.

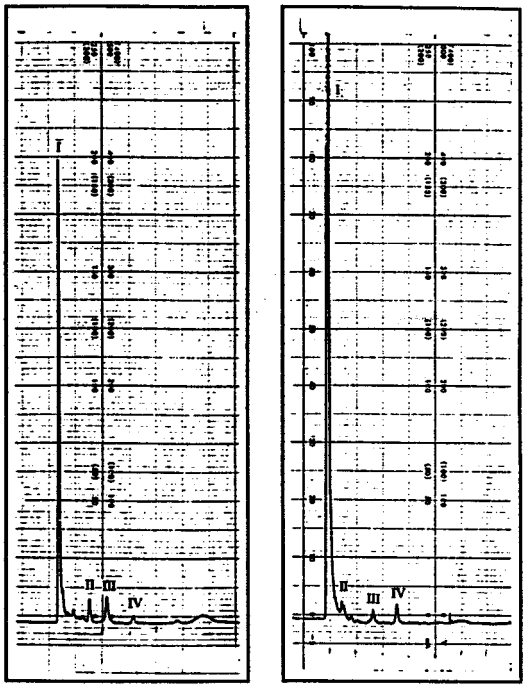
FILTER PAPER IMPREGNATION METHOD:

NfC Treatment:

For the analysis of the standard NfC, 1 µg/10 µl was injected. Four peaks were obtained on the chromatogram. The solvent peak appeared after 360 s while the peak of standard NfC appeared after 800 s along with 2 small peaks of other components of NfC (chromatogram-1). HPLC analysis was carried out at 24 and 144 h of post treatment. In the case of C. analis, insects were treated with 39.28 µg/cm^2 for 24 h exposure to NfC. Recovery of the compound was 4.57 µg/10 µl. Two small peaks also appeared after 504 and 1176 s as other components of NfC (chromatogram-2). In the case of 144 h of exposure of *C. analis* adults of NfC, the recovery of compound was 1.28 µg/10 µl. In the case of treated surface (39.28 µg/cm^2) after 24 h, recovery of NfC was 0.71 µg/10 µl (chromatogram-3). After 144 h, recovery was 0.50 µg/10 µl.

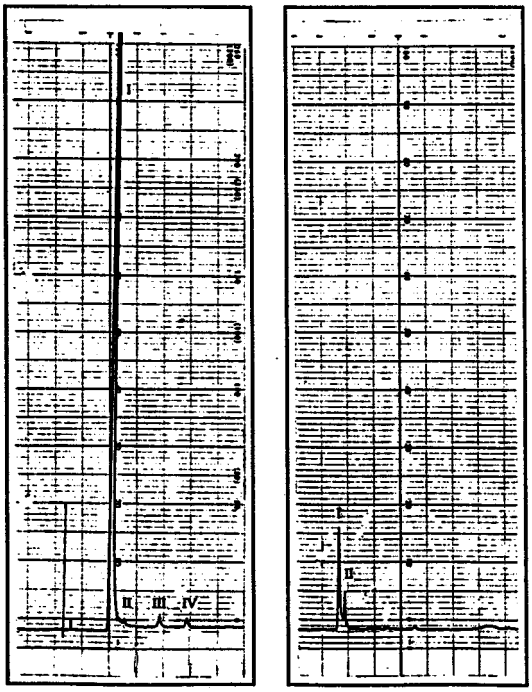
NC Treatment:

A sample (1 µg/10 µl) of NC was injected. After 360 s, the solvent peak appeared and, after 482 s, the standard compound peak appeared (chromatogram-4). In the case of insects treated with NC (7.16 µg/cm^2), the standard peak appeared after 504 s and the other 2 peaks may be of other components or metabolites



Chromatograph 3: Chromatograph showing the amount of NfC in adult of *C. analis* by HPLC after 144 hours of treatment by filter paper impregnation method. I = Solvent peak, II and IV = Other components, III = NfC.

Chromatograph 4: Chromatograph showing the amount of NfC in media treated samples after 24 hours of treatment by filter paper impregnation method. I = Solvent peak, II and IV = Other components, III = NfC.



Chromatograph 5: Chromatograph showing the amount of NfC in media treated samples after 144 hours of treatment by filter paper impregnation method. I = Solvent peak, II and IV = Other components, III = NfC.

Chromatograph 6: Chromatograph showing the amount of NC standard aftre the injection of 1 µg/10 µl. I = Solvent peak, II = NC.

(chromatogram-5). Recovery of the compound was 0.71 µg/10 µl (24 h of exposure to NC). No compound peak appeared after 144 h of exposure but the solvent peak appeared after 360 s. In the case of treated surface, recovery of the compound was 0.21 µg/10 µl after 508 s following exposure for 24 h (chromatogram-6). Two other small peaks also appeared, which may be of the other components. After 144 h of NC treatment, recovery of the compound was 0.07 µg/10 µl. The standard peak appeared after 528 s and the other 2 small peaks may be of the other components. However, the recovery of NC was poor.

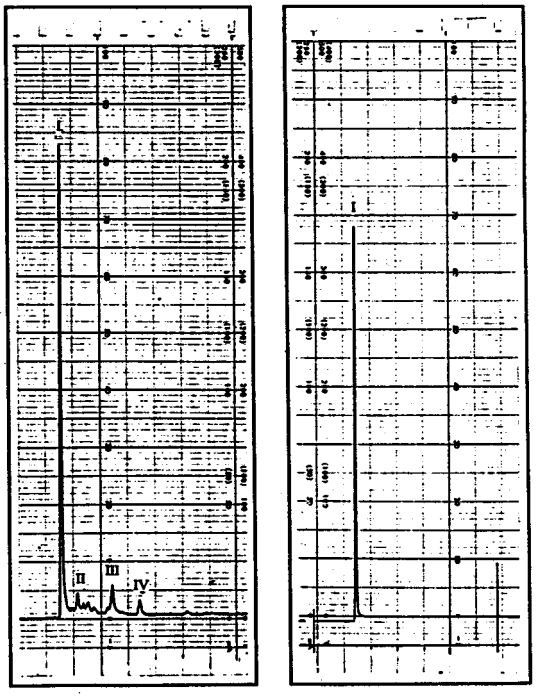
Dimilin Treatment:

In the case of Dimilin, the standard peak appeared after 456 s (chromatogram-7). In the insect body after 24 h of treatment with 13.0 µg/cm^2 dose the compound peak appeared after 456 s, while the other peaks may be other components. Recovery of the compound was 0.14 µg/10 µl (chromatogram-8). After 144 h of treatment the compound peak appeared after 432 s and the other peaks that also appeared may be of the other components. Compound recovery was 0.08 µg/10 µl. The chromatogram showed that after 24 h the insects have more Dimilin than after 144 h. In media treated samples the standard peak appeared after 456 s (chromatogram-9) and recovery of the compound was 0.36 µg/10 µl. After 24 h of treatment the standard compound peak appeared after 480 s and recovery of the compound was 0.14 µg/10 µl, and the other small peaks may be of the other components.

GLASS FILM METHOD:

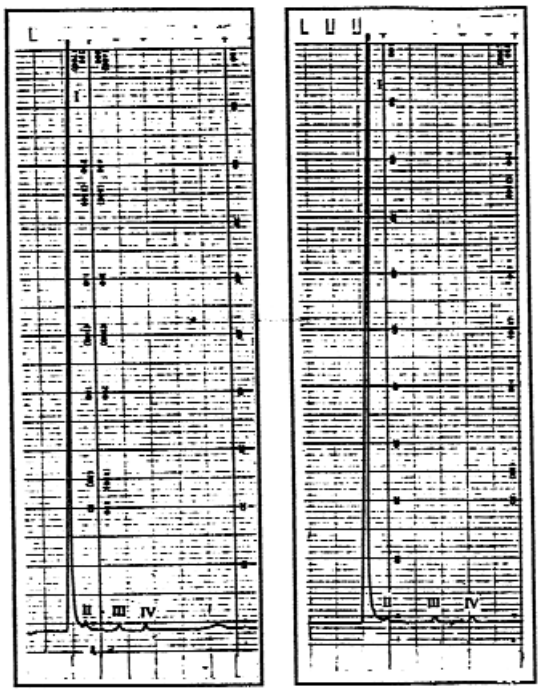
NC Treatment:

In the case of NC treatment, 10.0 µg/10 µl was applied and the standard peak of the compound appeared after 504 s along with 3 peaks. Recovery of NC was 1.42



Chromatograph 7: Chromatograph showing the amount of NC in adult *C. analis* after 24 hours of treatment by filter paper impregnation method. I = Solvent peak, II = NfC, III and IV = Other components.

Chromatograph 8: Chromatograph showing the amount of NC in adult *C. analis* after 144 hours of treatment by filter paper impregnation method. I = Solvent peak.



Chromatograph 9: Chromatograph showing the amount of NC in media treated samples after 24 hours of treatment by filter paper impregnation method. I = Solvent peak, II = NfC, III and IV = Other components.

Chromatograph 10: Chromatograph showing the amount of NC in media treated samples after 144 hours of treatment by filter paper impregnation method. I = Solvent peak, II = NfC, III and IV = Other components.

µg/10 µl after 24 h (chromatogram-10). After 144 h of treatment the standard peak appeared after 504 s. The other 3 peaks may be of other components, and recovery of the compound was 0.71 µg/10 µl. In media treatment the standard peak appeared after 490 s and the other 2 peaks may be metabolites or other components (chromatogram-11) and recovery was 0.71 µg/10 µl after 24 h. After 144 h of treatment the compound peak appeared after 508 s and the other 2 small peaks may be of the other components. The recovery of the compound was 0.07 µg/10 µl.

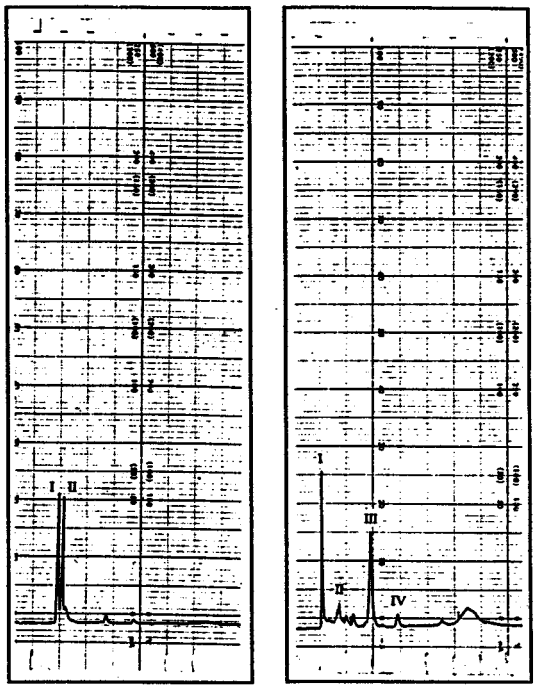
Dimilin Treatment:

Dimilin treated insect samples (3.2 µg/cm^2) were injected in HPLC after 24 and 144 h of insect treatment. After 24 h of exposure, the standard compound peak appeared after 460 s and another 5 peaks also appeared, which may be of metabolites or other components (chromatogram-12). Recovery of the compound was 0.84 µg/10 µl. After 144 h of exposure, the standard peak appeared after 460 s; subsequently, 2 peaks were obtained. Recovery of the compound was 0.33 µg/10 µl. In treated surface samples, the standard peak appeared after 480 s and other peaks also appeared after the standard compound (chromatogram-13), which may be of the other components. Recovery of the compound was 0.12 µg/10 µl after 24 h of treatment. There was no trace of compound in the 144 h treated surface sample.

Discussion

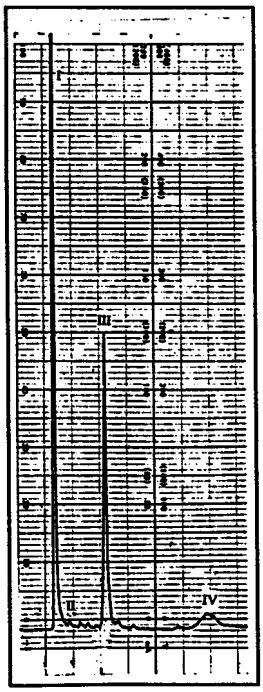
NfC and NC:

Neem components have so far been the most interesting plant origin constituents from neem seeds (Butterworth and Morgan, 1968) because they are very potent insect antifeedants and insect growth regulators (Kubo and Klocke, 1982; Sieber and Rembold, 1983;



Chromatograph 11: Chromatograph showing the amount of dimilin in standard samples after the injection of 1 µg/10 µl. I = Solvent peak, II = Dimilin.

Chromatograph 12: Chromatograph showing amount of dimilin in adult *C. analis* after 24 hours of treatment by filter paper impregnation method. I = Solvent peak, II = Dimilin, III, IV and V = Other components.



Chromatograph 13: Chromatograph showing the amount of dimilin in adult *C. analis* after 144 hours of treatment by filter paper impregnation method. I = Solvent peak, II = Dimilin, III and IV = Other components.

Schmutterer, 1987). Neem compounds are isolated from neem seeds by column chromatography followed by HPLC (with methanol as solvent) and identified by NMR (Rembold et al., 1980). Govindachari et al. (1990) reported the isolation of the neem compound azadirachtin by HPLC using an ODS column and methanol:water (60:40) as solvent. Nizam (1993) studied NFB and nimocinol, the allied compound of NfC and NC, on HPLC. In the present case, the neem extracts NfC (mixture) and nimolicine (NC) were studied on HPLC from treated *C. analis* and contact media, i.e. impregnated filter paper and treated glass after 24 and 144 h of treatment. For standard NfC and NC 1 µg/10 µl compounds were injected in HPLC. In the case of NfC treated *C. analis* (filter paper impregnation method) 5.47 µg and 1.28 µg and in media treated 0.71 µg and 0.5 µg recovery of compounds was observed after 24 and 144 h, respectively. In the case of NC treated *C. analis* (filter paper impregnation method) 0.71 µg after 24 h and after 144 h no trace of the parent compound was found, while media treated samples showed 0.21 µg and 0.07 µg recovery after 24 and 144 h, respectively.

In *C. analis* treated with NC by glass film method 1.42 µg and 0.71 mg recovery after 24 and 144 h was noted, whereas media treated samples showed 0.71 µg and 0.07 µg recovery. Nizam (1993) reported 60% and 36% recovery of NfB (neem fraction B) and nimocinol. In the present case, recovery of NfC was greater than that of NC. This may be due to the fact that NfC and NfB are crude forms while nimocinol and NC (nimolicine) are isolated compounds. In both cases, recovery of the crude form is higher than that of isolated compounds possibly because a greater quantity of crude form was applied. When we compare the recovery of compounds obtained by 2 different methods (filter paper impregnation and glass film) it was higher in the glass film method than in filter paper impregnation method, possibly because most of the compound was absorbed by the filter paper.

Dimilin:

Dimilin appears to inhibit a number of enzyme systems in different insects and its effect in some cases on

chitin synthesis may be secondary. Chang and Stokes (1979) and Chang and Woods (1979) reported the metabolic pathway of Diflubenzuron (Dimilin) in the boll weevil and penfluron in the housefly by radio chromatograms. Chang and Stokes (1979) reported 57.3% of compound (Dimilin) recovery in the weevil after 4 days post-treatment by injection method. Chang and Woods (1979) reported 95.2% recovery of penfluron in flies treated by injection method after 1-week treatment. In the present case, the amount of Dimilin (filter paper impregnation) recovered from treated *C. analis* was 0.14 µg and 0.08 µg, whereas the media treated samples showed 0.36 µg and 0.14 µg after 24 and 144 h of treatment, respectively. In the present case, recovery in media samples was 36% and 4% after 24 and 144 h of treatment. When the present results are compared with a previous study of Dimilin (Chang, 1978), which indicated the metabolic pathway of penfluron and Diflubenzuron in houseflies, the recovery is lower in the present case. This may be due to the indirect method of application.

In the glass film method, *C. analis* treated with Dimilin showed 0.84 µg and 0.33 µg of the compound, while in media treated sample 0.12 µg and no trace of the parent compound were observed after 24 and 144 h, respectively. A similar observation was reported by Nizam (1993), namely no trace of the parent compound Dimilin was detected after 24 h by HPLC in housefly samples treated with Dimilin. Furthermore, in the present case, 85% and 33% recovery in treated insects after 24 and 144 h, and 12% recovery in media treated samples after 24 h and no trace after 144 h were found. The difference in the results may be due to the different method of treatment. Spates and Wright (1980) reported that only 11.25% of Dimilin was recovered by paper chromatography from *Stomoxys calcitrans* when treated topically. Metabolites of Dimilin were also recorded in the present case in the form of peaks. In the same case, chromatograms of metabolites of Dimilin were also observed but these were not identified because the standards of these were not available due to limited time and funds.

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