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# Standardization of Method for Estimation of Neonicotinoids using HPLC

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**Abstract—***Chemical or natural pesticides/ Insecticides are used to control various pests and disease carriers, control weeds, insect infestation and diseases on the agriculture crops. There are many different types and classes of pesticides/ Insecticides which are effective against specific or on wide range of pests / insects. The perfect insecticide should have the characteristics such as efficacy, economic viability and safety. The agriculture crops with pesticides are later used by humans as their food or for feeding their cattle population. Over the years of their use, now insecticide/ pesticide resistance has been observed in many cases. That means those pesticides/ Insecticides are not able to control pest or insect growth in crop fields. This is called as Insecticide resistance. It is a major driving force behind the need for development of new insecticides.*

*Neonicotinoids are a class of neuro-active insecticides chemically similar to nicotine. They represent a unique model, which would act as poison for a neurotransmitter receptor. Today, Neonicotinoids are registered globally in more than 120 countries and found to be effective against sucking pests such as aphids, leafhoppers, planthoppers, thrips, whiteflies, etc and accounted for a worldwide turnover of approx. 1.7 billion US $ (Ralf Nauen and peter Jeschke 2008) .*

*All neonicotinoids act on the insect central nervous system as the agonists of the postsynaptic nicotinic acetylcholine receptors (Bai et al., 1991). The first neonicotinoid insecticide introduced to the market was imidacloprid in 1991. The present investigation was done to standardize a method for the simultaneous quantification of some of neonicotinoids namely acetamiprid, thiocloprid & imidacloprid and to use the method developed to examine these neonicotinoids in the food samples.Cabbage was taken for the present study The investigation was successful as these pesticides got separated at HPLC conditions with detection at 254 nm and flow rate of 1.0 ml/min and in cabbage sample Imidacloprid and Acetamiprid were detected.*

**Keywords***: HPLC- High Pressure Liquid Chromatography, Neonicotinoids, Thiocloprid, Imidacloprid and Acetamiprid.*

## 1. INTRODUCTION

Chemicals have been used to manage insects & pests for many decades. Initially crude inorganic compounds (ashes, sulphur) were used which used to function primarily as cuticle poisons. DDT transform insect control around the world. Its low cost, broad spectrum of contact action, selective toxicity between insects and mammals, persistence and ease of formulation, made it the most widely used insecticide ever manufactured. In 1941 parathion was discovered. The third major class is carbamates, which was discovered in early 1950's. In 1970's synthetic pyrethroids were introduced; they kill the insect by inhibition of axonic transmission of nervous system. Insecticide resistance is a major driving force behind the need for development of new insecticides and search of herbal and organic insecticides.

Neonicotinoids are a class of neuro-active insecticides chemically similar to nicotine. In the 1980s Shell and in the 1990s Bayer started work on their development (Kollmeyer, Willy D. et al, 1999). Neonicotinoids includes acetamiprid, clothianidin, imidacloprid, nitenpyram, thiocloprid and thiamethoxam. The first neonicotinoid insecticide introduced to the world market was imidacloprid in 1991 and it is the most widely used one too. Compared to organophosphate and carbamate insecticides, neonicotinoids cause less toxicity in birds and mammals than insects. Some breakdown products are also toxic to insects ( *Tomizawa M, Casida JE, 2005).*

Neonicotinoids represent a class of insect-selective ligands of nicotinic acetylcholine receptors. Today, Neonicotinoids are registered globally in more than 120 countries and found to be effective against sucking pests such as aphids, leafhoppers, planthoppers, thrips, whiteflies, etc and accounted for a worldwide turnover of approx. 1.7 billion US $ (Ralf Nauen and peter Jeschke 2008). The well-known member of this group Nicotine (as crushed tobacco leaves), a naturally occurring plant origin compound has been used as an insecticide for over 200 years. At present nicotine cannot compete with the broad spectrum insecticides. High mammalian toxicity coupled with narrow insecticidal spectrum and low persistence under field conditions lead to its replacement by safer and more effective synthetic insecticides. Since it acts on a different target in the nervous system of insects, development of cross-resistance between anticholinesterase insecticides and nicotinoids is unlikely. Therefore, nicotinoids represent a unique model for new

generation of synthetic nicotinoids, which would act as poison for a neurotransmitter receptor.

Studies have revealed nicotinic acetylcholine receptors (nAChR) to be the molecular targets of neonicotinoids. They act antagonistically on the nAChR at the synapses in the insect central nervous system, first stimulating the post-synaptic membranes, and then paralyzing nerve conduction. As a result, there is no cross- resistance thus fits well into resistance management strategies. These compounds have low mammalian toxicity. This selectivity is due to insensitivity of mammalian nicotinic acetylcholine receptors and high sensitivity of analogous receptors in insects (Kumar R. and Dikshit A.K., 2001). All neonicotinoids have a mode of action that binds at a specific site in the central nervous system of insects. This causes excitation of the nerves and eventual paralysis, which leads to death. There is no cross-resistance to conventional insecticide classes such as carbamates, organophosphates, and pyrethroids (Yamamoto *et al.*, 1996).

The development of neonicotinoid insecticides has provided growers with invaluable new tools for managing some of the world's most destructive crop pests, primarily those of the order Hemiptera (aphids, whiteflies, and plant hoppers) and Coleoptera (beetles), including species with a long history of resistance to earlier-used products. To date, neonicotinoids have proved relatively resilient to the development of resistance, especially when considering aphids such as *Myzus persicae* and *Phorodon humuli*.

New and new pesticides are entering into the pesticide market neonicotinoids are one of them. Since these are quite new pesticide and not much work has been done detect and quantify these pesticides in food samples so, the present investigation was taken up with the objectives to standardize a method for the simultaneous quantification of some of neonicotinoids namely Acetamiprid, Thiocloprid & Imidacloprid and to use the developed method to examine neonicotinoids namely Acetamiprid, Thiocloprid & Imidacloprid in the food sample in the year 2006.

## 2. MATERIALS AND METHOD

**Equipments used:** HPLC Instrument, Orbital Shaking Incubator, Vacuum Evaporator, Vacuum pump.

**Chemicals used:** HPLC grade reagents of CDH, Polypharm, Nice, Sigma, qualigens brands were used in the study.

**Raw Material:** Cabbage samples were taken for the study.

## 3. PROCEDURE

### 3.1 For standard:

**3.1.1. Stock solutions** were prepared by dissolving the standard in acetonitrile. Standard solutions are prepared as acetamiprid; 617 ppm, thiocloprid; 560 ppm & imidacloprid; 1540 ppm. Stock solutions so prepared were stored at $4^0$C.

**3.1.2. Working standards** were prepared by diluting stock solution to 100 times so as to obtain following acetamiprid; 6.17 ppm, thiocloprid; 5.60 ppm, imidacloprid; 15.40 ppm. 1 ppm of each pesticide is also prepared. A mix is also prepared by mixing 1 ml each of acetamiprid; 6.17 ppm, thiocloprid; 5.60 ppm & imidacloprid; 15.40 ppm. Working standards were stored at $4^0$C.

**3.1.3. Mobile phase** was obtained by combining and mixing 25ml acetonitrile (HPLC grade) & 75ml deionized water (HPLC grade) well & then by deaerating.

3.1.4. Following HPLC conditions were used:

| | |
|---|---|
| Column | Supelco C-8 RP |
| Flow rate | 1ml/min |
| Injection Volume | 20 µl |
| Temperature | Ambient temperature. |
| Detection | Absorbance detector, wavelength 254nm |

**3.1.5 Identification of pesticide:** Peak of imidacloprid, acetamiprid and thiocloprid were determined by identifying retention time.

### 3.2 For sample:

3.2.1**.** Samples were procured from local Hisar (Haryana, India) market.

3.2.2. For sample preparation, samples were ground so as to pass a 40-mess sieve and sodium sulphate is added into it so as to absorb moisture. Samples are stored at or below $4^o$C and at dark.

3.2.3 Pesticide extraction and quantification is done by following procedure :

1. **Sampling:** About 10-25g ground homogenous sample of food was taken.
2. **Spicking:** 1ml of acetamiprid, thiocloprid, imidacloprid & mix of each pesticide was separately added to sample's so that four test sample's can be made and replicates were also made. One control is also made with no added pesticide.
3. **Extraction:** For extraction, sample is taken in a stoppered conical flask and 75 ml of extracting solvent acetone: water (4:1) is added. The sample is soaked overnight in 50 ml of extracting solvent in an orbital shaker; the supernatant liquid is collected by decanting. Then 25 ml of extracting solvent is added to the sample again and then conical flask is again kept in orbital shaker for half an hour. The supernatant liquid is again collected by decanting and then these both are mixed.
4. **Drying:** The above extract is then dried in a vacuum flash evaporator to near dryness or till about 1-2 ml is left in the flask.

5. **Partitioning:** Liquid-Liquid partitioning was done in a separating funnel after adding saturated sodium chloride solution and then 100, 50, 30, 20 ml Dichloromethane (DCM) was added. Lower layer was collected in a flask by putting cotton and sodium sulphate in a funnel.
6. **Concentration:** Above extract was concentrated upto 2-3 ml in vacuum flask evaporator.
7. Add acetonitrile (or add the mobile phase taken for estimating standards) to above concentrate and transfer quantitatively by adding 50, 30, 20 ml of acetonitrile.
8. Transfer quantitatively elute obtained in step 6 to column by giving 3-4 wash.
9. **Column chromatography:** Column was made with florisil + charcoal (3g & 0.1g) respectively.
10. **Prewetting:** After prewetting column with hexane (AR grade) & the column is ready for elution.
11. Collect the elute obtained after passing and discarding hexane. Then pass the concentrate (obtained in step 5) dissolved in acetonitrile through the column and elute is then collected.
12. The above elute obtained in step 10 was concentrated to 1-2ml in a vacuum evaporator. Now this was ready for injecting in HPLC at the same conditions that are standardized for standard.

**3.3 For Determination:**

1. HPLC system was switched on & was allowed to warm up and equilibrate for a minimum of 30 minutes with mobile phase flowing. The flow rate should be 1.0 ml/min. for all three pesticides
2. Standard was injected by adjusting detectors sensitivity to give peak heights of 50-90% of full scale. Repeat injection until peak height is reproducible.
3. Sample solution was injected: intersperse with standard solution injection after every 9 samples to ensure accurate quantification.
4. The investigation was carried out to standardize HPLC condition to simultaneously detect some of the neonicotinoids namely acetamiprid, thiocloprid & imidacloprid. A number of HPLC conditions were tried. Injection volume in HPLC, type of column, flow rate were also changed to select the optimum concentration for extraction.

**3.4 For Identification:** Peak of imidacloprid, acetamiprid and thiocloprid are identified by comparing the retention time with standard in the same condition.

**3.5 Calculation:**

Formulae is used for the calculations which is given as picture 1.

## 4. RESULTS AND DISCUSSION

A number of HPLC conditions were tried. Out of the columns tried, Reverse phase (RP) Supelco C-8 column was found best in separating the pesticides. Different concentrations of Acetonitrile: Water and Methanol: Water were tried but separation was better with Acetonitrile: Water (25:75). Sometimes injection volume in HPLC, flow rate were changed. Finally a condition was reached where these neonicotinoids got separated with a sharp peak and at different retention time.

The investigation was successful as these pesticides got separated at following HPLC conditions:

- Detection : 254 nm
- Mobile phase : Acetonitrile:Water (25:75)
- Flow rate : 1.0ml/min
- Column : Supelco C-8
- Injected volume : 20µl

Pesticide standards and sample extract were injected in HPLC and peaks were obtained at the conditions standardized. The result of each graph so obtained from HPLC provided details of Retention time (Rt), Height, Area, Height%, Area% and AR/HT of the peak. By using the formula given in point 3.5, amount of pesticides were calculated and presented in Table 1.

The results obtained are discussed below and tabulated in table 1:

1. When Acetamiprid (standard of 6.17 ppm concentration) was injected in the HPLC at the standardized conditions. A single sharp peak was obtained at Rt of 5.62 minutes and area of that peak was 369115.

$$\text{Amount of pesticide (mg/l)} = \frac{\text{Peak area of sample} \times \text{Concentration of standard} \times \text{Volume of standard injected} \times \text{Volume make up}}{\text{Peak area of standard} \times \text{Weight of sample} \times \text{Volume of sample injected}}$$

**Picture 1: Formula used for calculation**

**Table 1: Results of Investigation**

| S. No. | Sample | Amount of Imidacloprid | Amount of Acetamiprid | Amount of Thiocloprid |
|---|---|---|---|---|
| 1 | CABBAGE | 0.9387 ppm | 2.065 ppm | Not detected |

2. When Imidacloprid (standard of 15.4 ppm concentration) was injected in the HPLC at the conditions standardized. A single sharp peak was obtained at Rt of 4.70 minutes and area of that peak was 426925.
3. When Thiocloprid (standard of 5.6 ppm concentration) was injected in the HPLC at the conditions standardized. A single sharp peak was obtained at Rt of 7.93 minutes and area of that peak was 2670594.

4. When Cabbage (sample) extract was injected in the HPLC at the conditions standardized. Then two different peaks were obtained at retention time of 4.71 minutes and 5.68 minutes and these values are quite close to Imidacloprid (4.70 minutes) and Acetamiprid (5.62 minutes) peaks obtained when standard was injected.

## 5. CONCLUSION

The above investigation was carried out to standardize HPLC condition to simultaneously detect some of the neonicotinoids namely Acetamiprid, Thiocloprid & Imidacloprid. Imidacloprid was the first to come out of the column i.e. it has a minimum retention time in the column followed by Acetamiprid and the Thiocloprid with a retention time of 4.70 min, 5.62 min, 7.93 min respectively. Thus in the samples a retention time closer to the standard was taken for calculations. In cabbage sample Imidacloprid & Acetamiprid were detected, with a retention time of 4.71 min & 5.68 min respectively. The difference between the retention time of standard and sample may be because of manual error in pressing the run button (integrator) and HPLC nobe. There are number of factors which may contribute to this difference as mentioned below:

- There may be degraded metabolites of neonicotinoids, which are interfering in the detection.
- The food mix is made up of complex system consisting of protein, carbohydrate, fat, vitamins& minerals.

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