Research Article

DEVELOPMENT AND VALIDATION OF A NEW METHOD BY UTILIZING HIGH PERFORMANCE THIN LAYER CHORMATOGRAPHY TECHNIQUE FOR ESTIMATION OF ACETAMIPRID IN FORMULATIONS AND EXTRACTS

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ABSTRACT

A determination method for Acetamiprid in mangoes under optimal conditions has been developed. The target compound was identified and quantitatively determined using HPTLC. The matrix-matched calibration curves, used for quantification of the field-incurred analyte, was linear with coefficients of determination (*r*2) exceeding 0.99. The matrix effects ranged from 98.68±0.917 to 101.92±0.479 in the fruits. The limits of detection were found to be 23.70ng and limit of quantitation was found to be71.838ng.The mean recoveries of the analyte ranged between 98.68% and 101.92%. The validated method developed herein was successively applied for the determination of Acetamiprid in field- incurred samples and identified as the main residue in the treated mangoes samples.

INTRODUCTION

Acetamiprid is classified as unlikely to be a human carcinogen. Acetamiprid has a low acute and chronic toxicity in mammals with no evidence of carcinogenicity, neurotoxicity or mutagenicity. It is classified as toxicity category rating II in acute oral studies with rats, toxicity category III in acute dermal and inhalation studies with rats, and toxicity category IV in primary eye and skin irritation studies with rabbits. It is mobile in soil, but degrades rapidly via aerobic soil metabolism, with studies showing its half life between <1 and 8.2 days. The U.S. Environmental Protection Agency (EPA) does not consider it to be environmentally persistent. The EPA considers it "only moderately toxic" to bees; however, some media sources and the recent documentary Vanishing of the Bees have blamed neonicotinoids like acetamiprid for colony collapse disorder. A recent study has implicated acetamiprid as a cause of erectile dysfunction in human males and may be implicated in the problem of declining human fertility, and called into question its safety, particularly where its use may be subject to abuse.

A detailed survey of literature with an objective to find out that the proposed method for the estimation of Acetampride in bulk and fruit extract was already carried out or combination of proposed pesticides reported for other analytical methods.

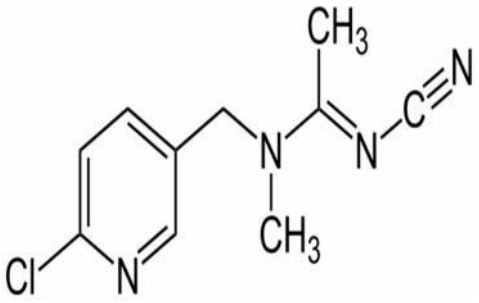


Fig. 1: Molecular structure of Acetamiprid

High performance thin layer Chromatography (HPTLC) Chromatographic system and conditions
The proposed method was performed using following chromatographic conditions as mentioned in Table 1.

Table 1: Chromatographic conditions of HPTLC

Parameters	Specification
HPTLC system	CAMAG HPTLC (Muttenz, Switzerland)
Software	Win CATS HPTLC Software (version 1.3.0)
Applicator	CAMAG Linomat 5 applicator
Syringe (100 µl)	CAMAG Hamilton Micro Syringe
Stationary phase	Silica gel 60 F254 precoated TLC plates (E-Merck, Daemstadt, Germany)
Mobile phase	(n-hexane: ethylacetate) (7:3 v/v)
Chamber saturation (min)	20
Migration distance (mm)	65
Band width (mm)	6
Slit dimensions	6.00 × 0.20 mm, Micro
Scanner	CAMAG TLC Scanner 3
Integrator	Win CATS V 1.3.0
Radiation source	Deuterium (D2)
Scanning wavelength (nm)	254

COLLECTION SAMPLES
Samples of mangoes were collected from various places local markets of Krishna district of Andhra Pradesh and from the collected samples, randomly selected sufficient quantity for current study.

ETHANOLIC EXTRACTION OF MANGO POWDER

- Fresh mangoes were randomly collected from various locations and cut into small pieces and then dried under shade drying.
- After drying of pieces were pulvizerised in cutter mill then powder was collected.
- ***In first step*** accurately 500gm of mango powder was taken and transferred into cleaned Round bottom flask (RBF).
- 500ml of ethanol was transferred into flask and closed with lid (stopper).This flask was kept aside for 3days for extraction process.
- After 3 days of extraction process extract was filtered through whatmann filter paper and filtrate was collected.
- The above collected filtrate was taken into beaker and it was kept in heating mantle at 50°C. Alcohol was evaporated and residue remains in the beaker in the form of paste. The heat process was stopped and the extract was dried and taken into closed vessel under aseptic conditions.
- ***In second step*** remain volume of flask was made up with 50ml of ethanol and flask was kept aside 3days for extraction process.
- After extraction process the extract was filtered through whatmann filter paper and filtrate was collected. The collected filtrate was taken into beaker and it was kept in heating mantle at 50°C. Alcohol was evaporated and residue remains in the beaker in the form of paste. The heat process was stopped and the extract was dried and taken into closed vessel under aseptic conditions.

PURIFICATION OF PESTICIDES FROM FORMULATION
The 1g of powder material was taken in round bottom flask containing 50ml of hexane and stirred for 2 hrs. The content was filtered and the filtrate was discarded. The residue was taken in 100ml ethyl acetate and refluxed for 3hrs, cooled to room temperature and filtered. The filtrate contains ethyl acetate layer was evaporated and the resultant solid was dried under vacuum. The isolated compound was analyzed for its purity by TLC and Mass spectrum. The result was presented in Fig. 2.
The purified compound was used for further analysis and identification in the vegetable/fruits/plant extracts.

OPTIMIZATION OF METHOD

Optimization and detection of UV wavelength

The sensitivity of HPTLC method that uses UV/VIS detection depends upon the proper selection of detection wavelength. An ideal wavelength is one that gives good response for the drug that is to be analyzed. In the present study, by appropriate dilution of each stock solution, various concentrations of Acetamiprid were prepared. Each solution was scanned in the spectrum mode and their spectra were observed. The wavelength selected for the analysis was 254 nm at which Acetamiprid showed significance absorbance.

Preparation of stock solutions

Standard Acetamiprid 10 mg was weighed and transferred to a 10 ml volumetric flask. 5 ml of methanol was added to dissolve the drug. The flask was shaken and volume was made up to the mark with methanol to give a solution containing concentration of 1000 µg/ml (stock solution A). From this stock solution, pipette out 1 ml and place it in 10 ml volumetric flask. The volume was made up to mark with methanol to give a solution containing concentration of 100 µg/ml (stock solution B).

Preparation of sample solutions

10 mg of Ethanol extract of Mango was accurately weighed and transferred to 10 ml volumetric flask containing 5 ml methanol. The flask was shaken and volume was made up to the mark with methanol. The solution was filtered through Whatmann filter paper (No. 41) to give a solution of concentration 1000 µg/ml. From the above solution pipette out 1 ml and make up the volume to 10 ml with methanol to give a solution containing 100 µg/ml. From this solution, appropriate volume was injected to the TLC plate.

HPTLC analysis

Analyses were performed on 10 × 10 cm aluminum backed silica gel F254 HPTLC plates (E-Merck, Darmstadt, Germany). Before using, the plates were pre-developed with methanol and then dried in an oven at 50°C for 5 min. Standard solution and sample solution were applied to the plate as 6 mm bands by means of a Camag Linomat-V (Muttenz, Switzerland) sample applicator equipped with 100 µl syringe (Hamilton, Reno, Nevada, USA); the distance between the bands was 11.6 mm with n- hexane: ethyl acetate (7:3 v/v) as mobile phase in a Camag twin trough chamber previously saturated for 20 min. The average development time is 20 min. After development, the plate was dried at 110°C in an oven for 10 min. Densitometric scanning at 254 nm was then performed with a Camag TLC Scanner equipped with Win-Cat software, version 1.3.0 using a Deuterium light source. The slit dimensions were 6.00 mm × 0.20 mm.

VALIDATION OF THE HPTLC METHOD

Linearity

Standard solution equivalent to 300, 600, 900, 1200, 1500 ng per band of Acetamiprid was applied to a pre-developed HPTLC plate. The plate was developed, dried and scanned as described above. The chromatograms were obtained and peak area was determined for each concentrations of drug solution (Table 2). A calibration plot was constructed by plotting peak area against amount of Acetamiprid (ng) (Fig. 3). The linearity of response for Acetamiprid was assessed in the concentration ranges 300- 1500 ng per band; the slope, intercept, and correlation coefficient were also determined (Table 3). 3D graph of the linearity studies is given in Fig. 4.

Limit of detection (LOD) and Limit of quantitation (LOQ)

The limit of detection (LOD) and limit of quantitation of Acetamiprid were determined by using standard deviation of the response and slope approach as defined as in International Conference on Harmonization (ICH) guidelines.

Precision

Precision was evaluated by using standard solutions containing Acetamiprid at concentration covering the 300, 600 and 900 ng per band. The precision of the method in terms of intra-day precision (% R.S.D) was determined by analyzing Acetamiprid standard solution in the range (100-300 ng per band) three times on the same day. The inter-day precision (% R.S.D) was assessed by analyzing these solutions (300-900 ng

per band) on three different days over a period of one week. The results of the precision studies are shown in Table 4.

Accuracy

Accuracy is the closeness of the test results obtained by the method to the true value. To study the Accuracy, the formulations were weighed and also analysis of the same was carried out. Recovery studies were carried out using standard addition method by adding known amount of standard drug solution (50%, 100% and 150%) to the sample solution. % Recovery was calculated and reported in the Table 4.

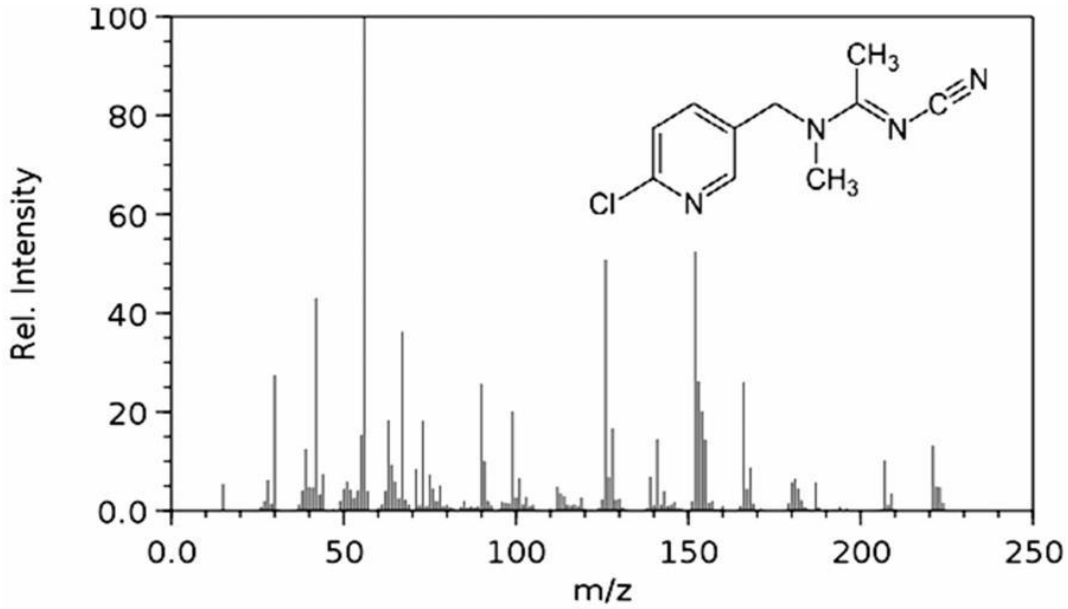
5. ETHANOLIC EXTRACTION

500gm of mango powder was taken and mixed with 500ml of ethanol solvent and extraction was carried out by traditional method and 10 %w/w yield of dried residue.

6.1 PURIFICATION OF ACETAMIPRID FROM ITS FORMULATION

The isolated compound was analyzed for its purity by TLC and Mass spectrum. In TLC plate appearance of single spot when stain iodine crystals.

Fig. 2: Mass spectrum of Acetamiprid



From the mass spectrum, the molecular ionic peak of the Acetamiprid was found to be 222.2 m/z and which was matched with standard Acetamiprid**.**

6.2. HPTLC ANALYSIS

Table 2: Characteristic parameters of Acetamiprid for the proposed HPTLC method

Parameters	HPTLC
Detection wavelength (nm)	254
Calibration range (ng per band)	300-1500
Mobile phase (n-hexane: ethylacetate)	(7:3 v/v)
Regression equation (Y*)	y = 3.9348 x - 9.4429
Slope (m)	3.9348
Intercept (c)	-9.4429
Correlation coefficient (r2)	0.9997
Limit of detection (ng per band)	23.70
Limit of quantification (ng per band)	71.838

6.3 PREPARATION OF STANDARD CURVE AND ANALYSIS OFEXTRACT

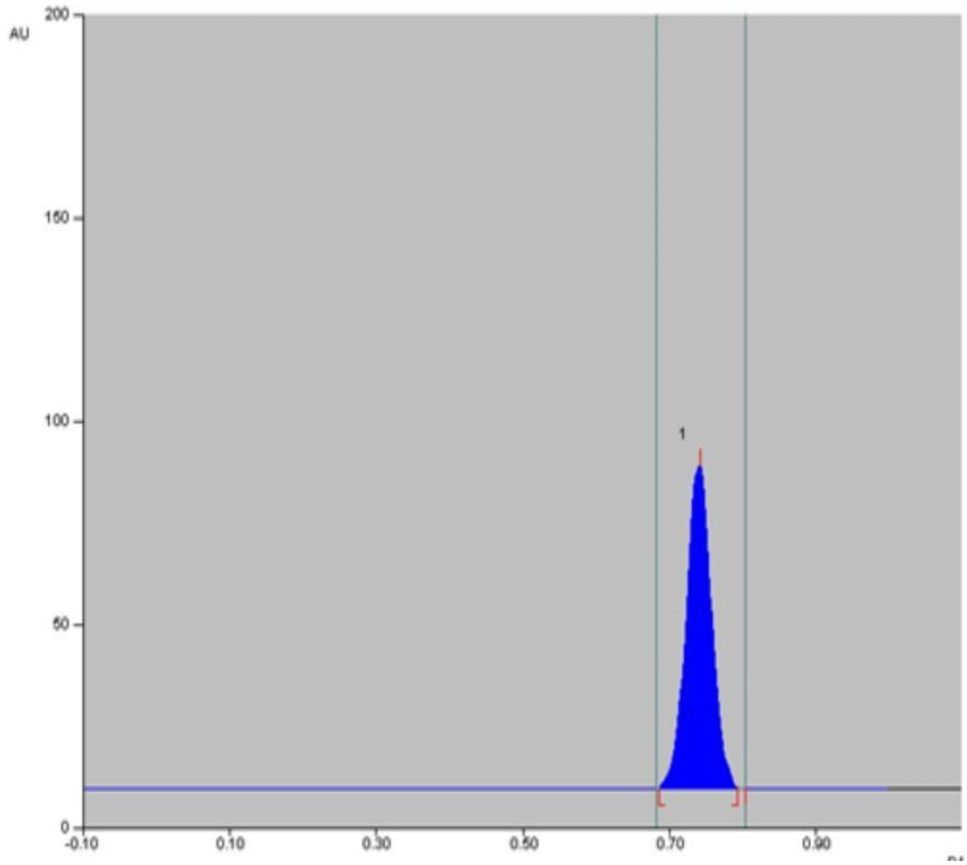


Fig. 3: Chromatogram of 600 ng of standard solution of Acetamiprid by HPTLC method

From the graph, it was clear that the calibration curve was linear in the concentration range of 300-1500 ng. The equation for isolated compound was found to be y = 3.9348 x - 9.4429 with correlation coefficient of 0.9997, where 'X' is the response in peak area, 'Y' is the concentration in ng.

The chromatograms obtained from isolated compound and the ethanol extract are shown in Figure 3. To ascertain the purity of peak in test samples, it's *in situ* reflectance spectrum was compared with that of standard isolated compound. The clear superimposibility confirms the purity of peak. The isolated compound content of the extract was found to be **10.13% w/w** of fruit of mango.

6.4 LINEARITY VALUES

Table 3: Calibration data of Acetamiprid by HPTLC method

S. No.	Concentration (ng per band)	Peak Area
1	0	0
2	300	1202.8
3	600	2316.7
4	900	3517.1
5	1200	4672.0
6	1500	5941.4

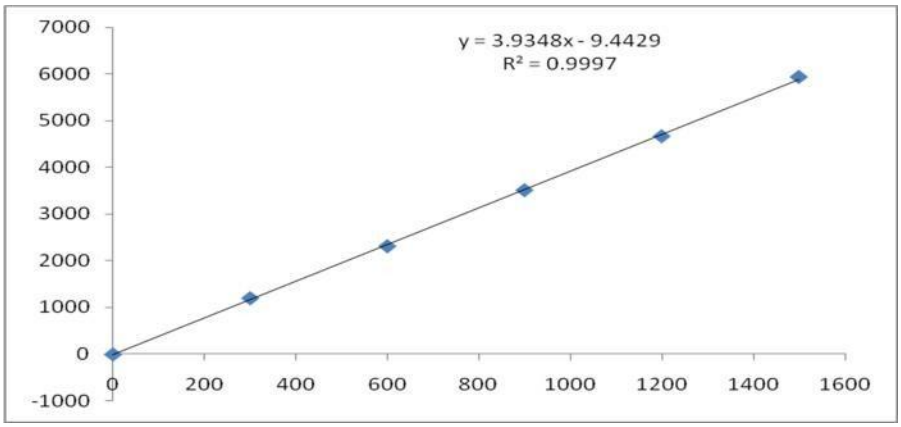


Fig. 4: Calibration curve of Acetamiprid by HPTLC method

Calibration plots of peak area against concentration were linear in the range of 300 to 1500 ng of isolated compound. The calibration line was represented by linear equation y = 3.9348 x - 9.4429, with a correlation coefficient of 0.9997, where X is response in peak area and Y is concentration (Fig. 4).

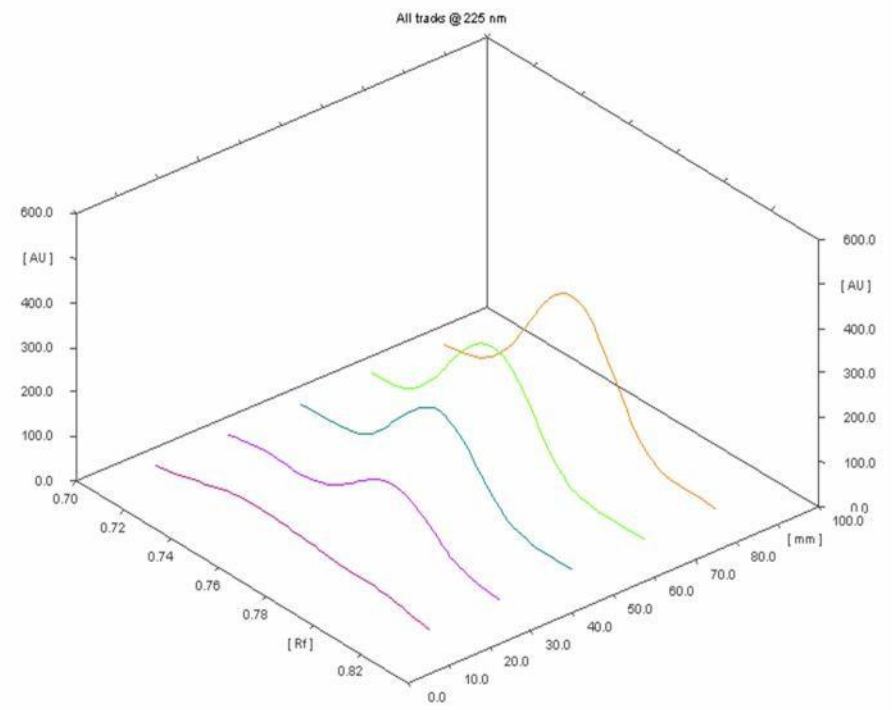


Fig. 5: 3D view of calibration curve of Acetamiprid by HPTLC method

6.5 LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ)

The limit of quantification (LOQ) and limit of detection (LOD) were calculated by using equations, LOD=3×N/B and LOQ=10×N/B, where N is the standard deviation of the peak area of the standard (n=3), taken as a measure of the noise and B is the shape of the corresponding calibration curve. The LOQ was found to be 71.838 ng and LOD as 23.70 ng.

6.6 PRECISION AND ACCURACY

The precision of the method in terms of intra-day precision (% R.S.D) and the inter- day precision (% R.S.D) studies shows that it's were in the range of acceptance criteria

Table 4: Precision results of Acetamiprid by HPTLC method

Concentration (ng per band)	Intra-day precision Mean ± S.D**	% R.S.D	Inter-day Precision Mean ± S.D**	% R.S.D
600	943.3 ± 15.42	0.90	945.26 ± 0.66	1.56
900	1618.6 ± 9.77	1.00	1658 ± 17.74	1.28
1200	2199.3 ± 24.5	0.64	2215.4 ± 40.61	0.80

**Average of three determinations

6.7 RECOVERY & REPRODUCIBILITY STUDIES

The recovery values obtained were in the range of 98.68 ± 0.917 to 101.92 ± 0.479 showing the reliability and reproducibility of the method.

Table 5: Recovery study data of Acetamiprid by HPTLC method

Level of recovery (%)	Amount of drug added (mg)	Amount of drug recovered (mg)*	% Recovery ± S.D*
50	5	5.05	101.92 ± 0.479
100	10	9.95	99.55 ± 0.286
150	15	14.80	98.68 ± 0.917

*Average of three determinations

7 .1 SUMMARY OF THE PRESENT WORK

- Among the modern Analytical tools HPTLC is a powerful analytical method equally suitable for qualitative and quantitative analytical tasks. HPTLC is playing an important role in today analytical world.
- India ranks first among world's mango producing countries accounting for about 50% of the world's mango production, particularly Andhra Pradesh is a major producing state in India. In view of increase the yield of quality products and to prevent from insect pests mostly mealy bug, hopper, inflorescence midge, fruit fly and scale insects, using of pesticides by growers tremendously increased, in order to control these diseases, without seeking advice and professional assistance to prevent/control diseases and disorders in the crop and spraying of inappropriate chemicals in excess to control pest particularly Acetamiprid. Various researchers reported the presence of such chemicals found in the fruits are prone to highly health risks by elevated plasma ALP in consumed of fruits might be due to acute hepatocellular damage and destruction of epithelial cells in gastrointestinal tracts and carcinogenic as well.
- Our attempts to develop and validate a simple, economic and accurate method for Acetamiprid and its quantitative determination in fruit extract using HPTLC technique.
- Mango fruit was collected randomly and extracted with ethanol and the excess solvent was distilled under vacuum. The dried ethanol extract was used for further uses.
- Acetamiprid formulation brought from market and isolated pure compound from formulation by using hexane and the content was filtered and the filtrate was discarded. The residue was treated with ethyl acetate and refluxed for 3hrs, cooled to room temperature and filtered; the resultant solid was dried under vacuum. The isolated compound was confirmed by mass spectrum and the molecular ionic peak of the acetamiprid was found to be 222.2 m/z and which was matched with standard acetamiprid. The purity acetamiprid was confirmed by TLC.

- From the mass spectrum, the molecular ionic peak of the acetamiprid was found to be 222.2 m/z and which was matched with standard acetamiprid**.**
- The sensitivity of HPTLC method that uses UV/VIS detection depends upon the proper selection of detection wavelength. An ideal wavelength is one that gives good response for the drug that is to be analyzed; the wavelength selected for the analysis was 254 nm at which Acetamiprid showed significance absorbance.
- In HPTLC method Linear least square regression analysis showed there was a good linear relationship (r^2 > 0.9997) between peak area and concentration in the range 300-1500 ng per zone; the regression equation of the calibration plots was y = 3.9348 x - 9.4429. Acetamiprid was resolved by using n- hexane: ethylacetate (7:3 v/v) as mobile phase.
- To ascertain the purity of peak in test samples, it's *in situ* reflectance spectrum was compared with that of standard isolated compound. The clear superimposibility confirms the purity of peak. The isolated compound content of the extract was found to be **10.13% w/w** of fruit of mango.

7.2 CONCLUSION

The developed analytical method for the simultaneous estimation of Acetamiprid utilising HPTLC techniques was found to be simple, precise, specific, accurate quick, reliable and reproducible. The method was completely validated exhibits satisfactory data for the parameters tested. The out-come results from this described method indicate that it can be used for quantitative analysis of the compound. A HPLC screening method was successfully developed and employed to determine a minute quantity of target pesticide in mangoes. The potential of HPTLC for such residue application has been shown to be important as a technique complementary to traditional analysis, as a result shows good sensitivity and recoveries and allows for rapid sample analysis, moreover it requires only small volumes of solvent per sample and does not use any chlorinated solvents. It covers a wide range of fruits and vegetables, and is ideally suited for use in a regulatory laboratory.

From the results of our findings it was concluded that extracts of fruit contains residue of pesticide more than permissible quantity. Various research works reported toxicity of acetamiprid causes acute hepatocellular damage and destruction of epithelial cells in gastrointestinal tracts and carcinogenic as well, this may due to over-usage of pesticide by growers. In order reduce the toxicity of pesticide; the growers need have sought advice and professional assistance to prevent/control diseases and disorders in the crop.

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