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Quantitative Estimation of Fungicide, Pesticide Residues from Pepper (*Piper nigrum L.*) as Obtained in the Selected Area of Kodagu District, Karnataka

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ABSTRACT

Kodagu the one of the major pepper cultivated district in Karnataka state. The present study was carried out in the different villages such as Tuchumaleri, Chikkamandur, Mattur, Nallur, Kunda, Balaji, Kottur, and Mallanakere etc of Ponnempet, Kodagu district. During the pepper cultivation the farmers are used the pesticides in each steps, for high yielding purpose. This study was revealed that to measure the amount of pesticides present in the final product. Aflatoxin is produced by the fungi aspergillusflavus and it having properties of nephrotoxic, teratogenic, carcinogenic, and immunosuppressive. It can be determined by the reliable method like HPLC (column symmetry C-18; 4.6X250 mm) used with detection by florescence (excitation 364 nm, emission 465 nm). Like that the organo phosphors also determined by GC (column summery RTX-5; 30m) the level can be indicated in ppb and ppm respectively.

Keywords: Pepper, Aflotoxin, Organo phosphorous, HPLC, GC.

INTRODUCTION

Pepper (*Piper nigrum L.*) belongs to the family *Piperaceae,* the crop grow well in a temperature range between 25°-40°C and rainfall around 1250 mm. In India, Karnataka, Kerala, Tamilnadu states are play a major role in the production of pepper. As per the 2013-14 report 21,250 tones of black pepper worth Rs. 94,002 lakhs products were exported from India to various countries.

Pepper is one of the important spice used for flavor, and aroma of food and beverages, is produced from the still-green, unripe drupes of the pepper plant. Pepper contains volatile oil so it makes an important contribution to sensory qualities[1]

In Indian ayurveda system the pepper are used as a medicine since 5000 years, as a diet and herbal remedies, it is a source of important drugs such as atropine, codeine, digoxin, morphine, quinine

and vincristine[2].While emphasizing the body, mind and spirit in disease prevention and treatment[3].

Black pepper is a king of spices, used for medicinal agent, preservative, and in perfumery[4]. Its contains major pungent alkaloid Piperine *(1-peperoyl piperidine),* exhibits[5] pharmacological activities like antihypertensive and antiplatelets[6],antioxidant, antitumor**,** anti- asthmatics[7], antipyretic, analgesic, anti-inflammatory, anti-diarrheal, antispasmodic, anxiolytic, antidepressants[8] hepato-protective[9], immuno-modulatory, antibacterial, antifungal, anti-thyroids, anti-metastatic, antimutagenic, etc.

Piperine has been found to enhance the therapeutic efficacy of many drugs, vaccines and nutrients by increasing oral bioavailability by inhibiting various metabolizing enzymes[10].The consumption of black pepper increases the orocecal transit time.[11]and also decreased pressure in arteries, it inhibited high K^+ pre contractions due to blockade Ca^{2+} channel[12], apart from that it having an anti diarrheal property hence in most of the developing countries local peoples, herbal industries, and herbal practioners formulate the peppercorn for diarrhea for all ages[13].

Nerolidol is a natural compound isolated from piper species, having pesticidal activity against various mites. And another compound pipene isolated from volatile oil of peppercorn it is used as an odorant[14].

MATERIALS AND METHODS

Collection of sample

Kodagu is one of the major pepper cultivated district in Karnataka state hence the pepper (*Piper nigrum L*) samples are collected from the different villages of ponnempet. The samples were collected in a zip polythene bags. And preserved in deep freezer.

Survey in farming practices

Questionnaires contained four sections to find out the details like Physical inspection (location, soil type and weather data) general information (type of crop, aware of pesticide effect and recommended dosage) cultivation steps (name of the verity, land preparation, spacing, month of plantation, intercropping, duration and common diseases) pesticide information (name of pesticide used, method of application etc.)

Chemicals / materials used

Acetone, petroleum ether, Dichloro methane. Diethyl ether, Sodiumsulphate, Florosil, sodium chloride, tween 20, Methanol, KBr, conc. Nitric acid, acetonitril, butter paper, flutted filter paper, micro fiber filter paper, IAC(immune affinity column) and HPLC water.

Stock and working standard

Stock and working standard of organo phosphorous pesticides of appropriate concentration in acetone (*standard from EPA of equivalent*). Store the standards under refrigeration. (Shelf life for stock standard is one year and that for working standard is six months)

Analysis of Aflatoxin

- Weigh the sample into a blender jar.
- Add NaCl into blender jar.
- Add 100 ml of 80:20 methanol & HPLC water mix.
- Cap blender jar and seal with Parafilm.
- Blend at high speed for 1 minute
- Then filter it into a beaker through fluted filter

Table 1: Aflatoxin Standard

Peak	Name	Ret. Time	Area	Area%	Conc.
1	G2	7.067	264859	11.573	20.000
2	G1	8.114	660603	28.866	80.000
3	B2	8.916	445142	19.451	20.000
4	B1	10.402	917926	40.110	80.000
Total			2288527	100.000	

paper.

- Pipette 10 ml of filtrate into 50 ml graduated cylinder.
- If the sample is nutmeg, oregano, or black pepper, add 40 mL of 20%Tween-20 solution to the graduated cylinder. If the sample is not one of the products mentioned, add 40 ml of DI Distilled water to the cylinder. Mix thoroughly.
- Filter the contents of the graduated cylinder through a glass fiber filter into a 250 ml beaker. This filtrate will be used for the Aflatest column.

Immuno-Affinity Column Clean-Up

- Attach an Aflatest-P column to the pump stand.
- Pipette 10 ml of filtrate to the syringe and pass through the IAC column
- Once the entire sample has passed through the column, rinse the column with 10 ml of HPLC water. Repeat HPLC water rinse.
- Place 20 ml stoppered test tube under the tip of the column and add 1 ml of methanol to the column.
- Collect all the methanol eluent in the test tube. And transfer the sample to a sample vial. The sample is now ready for injection to the HPLC.[15]

Analysis of Organo Phosphorous

- Rinse thoroughly all the glass wares first with acetone and then with petroleum ether before starting the analysis
- Always do a reagent blank along with sample
- Sample preparation: whole spice:-The entire sample is reduced in six by mixing and quartering and a subsample of 100g taken for crushing/blending. A representative sample of 20g (in duplicate) is taken for analysis.
- Crushed/ground spices: - The entire sample is reduced in size by mixing and quartering and a subsample of 100g is taken. From this a representative sample of 20g is taken for analysis.

Table 2: Organo phosphorous Standard

Peak	Ret. Time	Name	Area	Height	Conc.	Units
1	2.267	Dichlorovos	9179844	1568984	2000.00	pG
2	3.235	Acephate	1816787	389424	4000.00	pG
3	4.772	Omethoate	3891511	756206	4000.00	pG
4	5.519	Monocrotofos	141960	44365	4000.00	pG
5	5.670	Phorate	10316861	1888548	2000.00	pG
6	6.310	Dimethoate	2394844	611262	2000.00	pG
7	6.870	Diazinon	5129908	1414095	2000.00	pG
8	7.083	Disulfoton	3150731	900220	2000.00	pG
9	7.253	Etrimphos	2821356	850826	2000.00	pG
10	8.133	Parathion methyl	525406	1626396	2000.00	pG
11	8.720	Pirimiphos methyl	9816919	1781049	2000.00	pG
12	8.882	Malalthion	2870442	938102	2000.00	pG
13	10.128	Quinolphos	5806111	2071751	2000.00	pG
14	11.096	Profenophos	3184702	1170602	2000.00	pG
15	12.077	Ethion	749567	284785	1000.00	pG
16	12.397	Triazophos	4486193	1655339	2000.00	pG
17	13.804	Phosmet	1026677	389577	2000.00	pG
18	14.524	Phosalone	3009899	1017151	2000.00	pG
19	15.313	Azinphos ethyl	2254325	691752	2000.00	pG
Total			77302702	20050434		

Extraction

- Transfer the above weighed sample to a 500 ml stoppered conical flask
- Add freshly prepared acetone-water mixture
- Keep on the laboratory shaker for 1hour with occasional shaking
- Filter through whatman No.1 filter paper in a bucher funnel, using vacuum pump
- Shake well and transfer 100 ml of filtrate to the 250ml separating funnel
- To this add 50ml petroleum ether first and then 50ml methylene dichloride
- Shake vigorously for about 2 minutes and then allow to stand for the separation of layer
- After clear separation layer, transfer the lower layer to another 250 ml separating funnel.
- To this add 100 ml MDC. Shake and allow to stand.
- Collect the upper layer of step-7 to a beaker after passing through a funnel containing anhydrous sodium sulphate which is supported by glass wool pre wetted with petroleum ether
- After the separation of layer, collect the lower layer of the 2nd separating funnel to the same beaker by passing through the same funnel containing sodium sulphate
- Transfer this solution quantitatively to rotary evaporator flask/water bath and concentrate to minimum volume. This is ready for clean up.

Florosil Cleanup for Organo phosphorous

- Place activated florosil (about 4") in the column; add about 0.5" sodium sulphate pre wet column with 50 ml petroleum ether.

Table 3: Amount of Pesticides present in sample no. 3

S.no	Compound Name	Results in ppm	EU Limits in ppm
1.	Diazinon	0.01	0.1
2	Malathion	0.01	0.02
3	Quinophos	0.01	0.1
4	Profenophos	0.02	0.07
5	Ethion	0.02	5

Analysis of Aflatoxin by HPLC

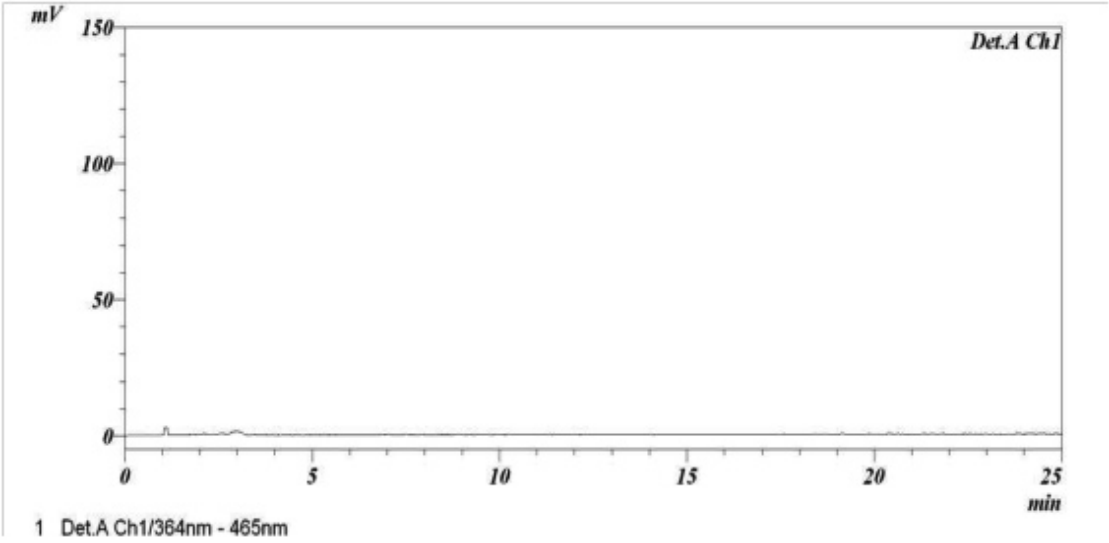

Fig.1: Aflatoxin blank

- Transfer the concentrate to sintered column using 8ml HPLC grade acetone, and rinse walls of chromatographic tube with additional small portion of acetone. Drain with 100 ml of petroleum ether and collect and collect it in a 500 ml beaker.
- Elute column at about 5ml/minute with 200ml diethyl ether-petroleum ether (50:50). Collect the eluant in the beaker containing petroleum ether in step-2
- This elute is ready for concentration step for organo phosphorous pesticides by roatary evaporator/water bath.
- Concentrate the above extract to minimum volume.
- Concentrate the extract using a water bath/ K. D Evaporator/ Rotary evaporator
- Rinse the content with acetone and transfer to a graduated stopped test tube
- Make up to 4ml with acetone. This is ready for GC analysis[16].

Calculation

Aflatoxin was quantified using the following formula

$$\frac{\text{Conc.}}{20\ \mu l} \times \frac{100 \times 1\text{ml}}{10\text{ml}} \times \frac{50\text{ml}}{10\text{ml}} \times \frac{100\text{ml}}{25 \times 10^3 (\text{mg})}$$

Organo phosphorous was quantified using the following formula

$$\text{OP (conc.)} = \frac{\text{PG (conc.)}}{2\ \mu l} \times 1000 \times \frac{6.0\ \mu l}{100\ \text{ml}} \times \frac{350\ \text{ml}}{20\ \mu g} \times \frac{1}{10^6}$$

$$= \text{PG (conc.)} \times \frac{0.525}{10^3}$$

RESULTS AND DISCUSSION

Kodagu, one of the major pepper cultivated district of the state Karnataka. A total of ten farmers having plantations with pepper crop from different villages of ponnempet, kodagu district were interviewed. They were majorly cultivating pepper plants. Distance of 10-15 feet of panniru verity with a support of forest trees or gliricidia plant, and coffee is an intercropping in sand mixed and black soil. Most of the farmers not aware of pesticide effect on human health. Some of them were engaged in

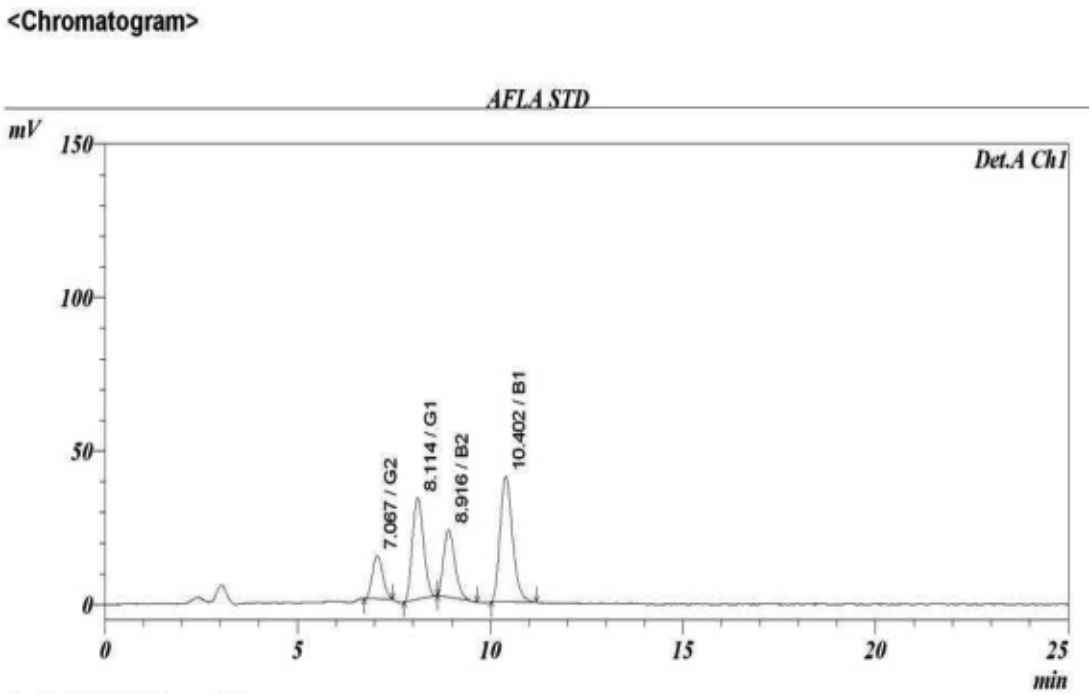

Fig. 2: Aflatoxin standard

the application of pesticides for the high yielding purpose and revealed via interview, the generally used pesticides in this district were copper sulphate, bodomixture, trichoderma, copper oxichloride etc. applied through power/hand spraying for controlling black and yellow wilt diseases.[17]. This study shows the quantitative estimation of fungicide, pesticide residual content in the collected pepper samples

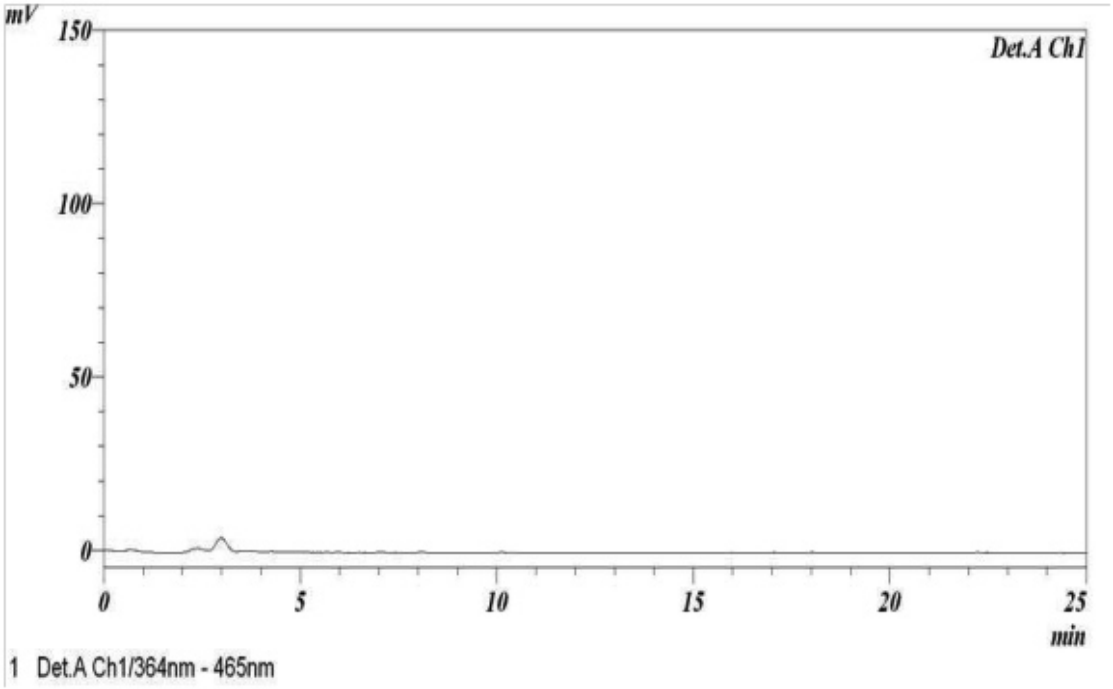

Fig. 3: Aflatoxin sample

Analysis of Organo Phosphorous by GC

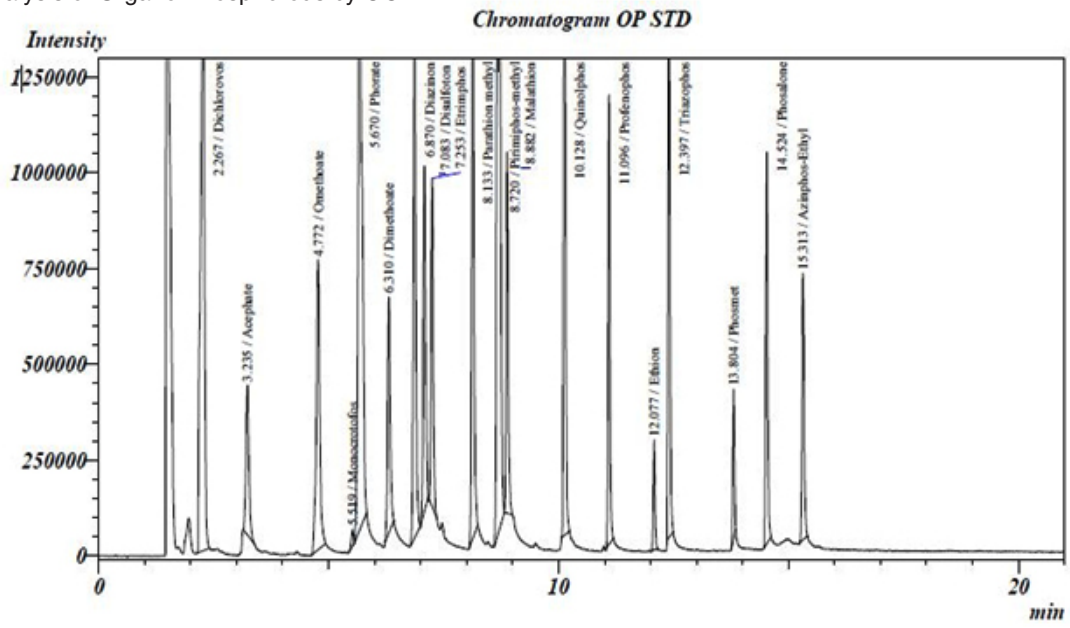

Fig. 4: Organo phosphorous Standard

by ASTA method using HPLC and GC. The analysis of ten samples the result shows the absence of aflotoxins (G2 G1 and B2, B1). In the pesticides (Organo phosphorous) totally analyzed nineteen chemical compounds from each collected sample in that Diazinon, Malathion, Quinophos, Profenophos

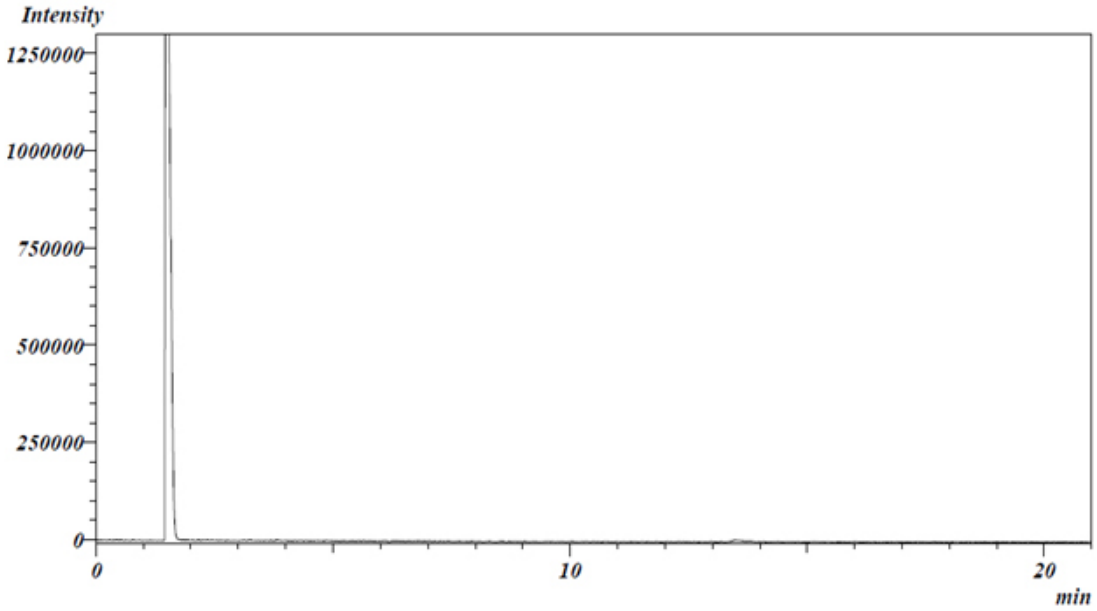

Fig. 5: Organo phosphorous Blank

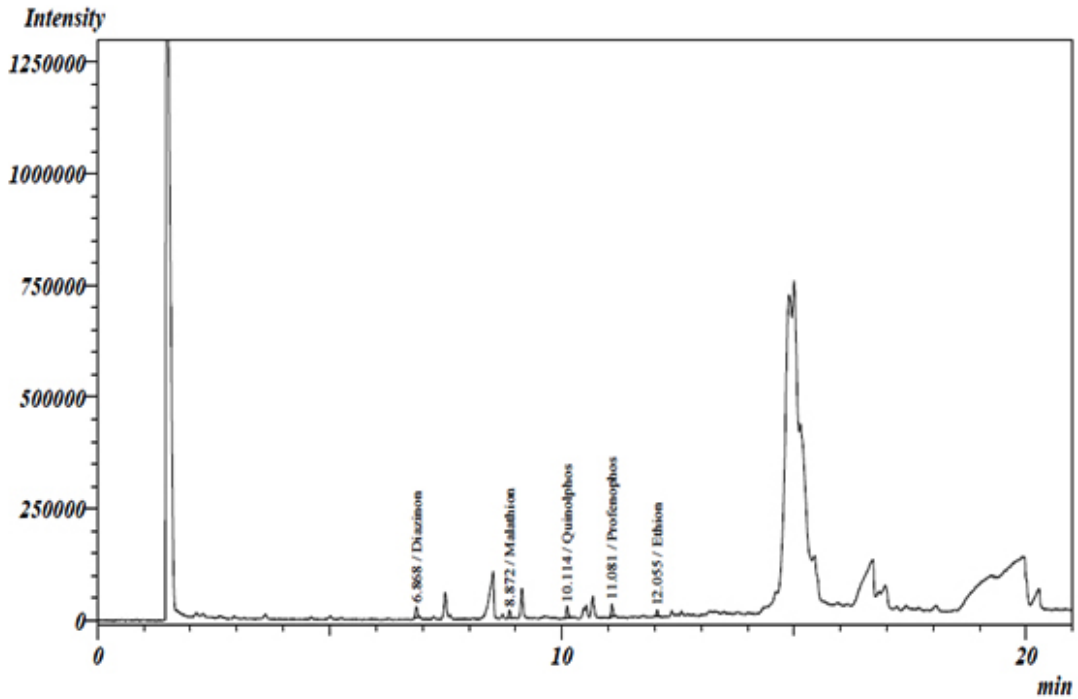

Fig. 6: Organo phosphorous Sample-3

Fig. A

Fig. B

Fig. C

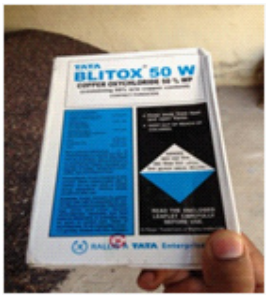

Fig. D

Fig. E

Fig A and B are disease effected leaf, Fig C and D are chemicals used during the cultivation of pepper plant and Fig.E Gliricidia (Supporting Plant)

and ethion are present in sample number three that was collected from the mattur village. But it also in acceptable range (*within EU limits*) 1.e. 0.01, 0.01, 0.01, 0.02 and 0.02ppm respectively.

CONCLUSION

As per the survey and quantitative estimation of fungicide(Aflotoxin), pesticide(Organo phosphorous) analysis results shows the samples collected from the different villages of Ponnempet of Kodagu district Karnataka was within the *EU* limits hence it was free from fungicides, pesticides contents.

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