Liquid Chromatography Interface with Tandem- Mass Spectrometry (LCMS/MS) - An Innovative Analytical Technique for Soil Pesticides Residue Analysis

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Abstract: **The multiresidue analysis of pesticides in the soil is a difficult task for the chemist, due to the variety of the different groups of pesticides having a broad range of physico- chemical properties. An innovative analytical technique of LCMS/MS with triple quadrupole on MRM mode which can applicable for monitoring of pesticides residue of different groups existing in 45 soil samples collected from selected vineyards has been developed. The present study highlights on the fundamental principles of LCMS/MS and its employment in the field of agricultural and environmental chemistry.**

Keywords: **Vineyard Soils, Contamination Levels of pesticides, Multiresidue -pesticide analysis, LCMS/MS, Tandem Mass Spectrometry**

I. Introduction

In the 1960's nicotine was used by French farmers to kill lace bugs and in the 1860's arsenic based compounds were used in the United States to control the potato beetle (Ebert *et al.,* 1988). The American Chemical Society has listed more than 15,000 chemical compounds for use as pesticides with more than 35,000 commercial brands. The current world production of formulated pesticides is estimated to be more than 3x 10^9 kg, of which 75% is consumed in the developing countries (McConnell, 1994). Pesticide usage in the developing countries has been on the increase during the past decade. Although only 25% of the annual worldwide production of 3 million tons is used in these countries, 90% of the estimated 3 million yearly poisoning incidents and 99% of the 220,000 reported worldwide pesticide related deaths occurred in the developing countries Pesticide poisoning in these countries is under-diagnosed and under-reported and few agricultural workers have any information on the hazards they are exposed to, either from direct handling of chemicals at work or from exposure to contaminated environments (Santillo*et al*, 1997).

One of the unintended and unfavourable effects of pesticide use is soil contamination. (Lewandowska andWalorczyk , 2010). Pesticides may reach the soil through direct application to the soil surface, incorporation in the top few inches of soil, or during application to crops. Pesticides can also enter ground water resources and surface run-off during rainfall, thereby contributing to the risk of environmental contamination (Dem *et.al.,*2007).

Due to the large number of pesticides on the world market, the development of multi-residue methods is preferred in terms of pesticide residue analysis. (Lacassie*et al.*, 1999). Soil matrices are complex and may be difficult to analyze and require highly selective analytical techniques to determine target compounds and to characterize unknown compounds. The use of Gas Chromatography (GC) and Liquid Chromatography (LC) with Mass Spectrometry (MS) is a well-accepted method for confirmation on the identity of pesticides at very high sensitivity.

Liquid chromatography- with tandem mass spectrometry (LC-MS/Ms) is an innovative approach in the field of chemistry that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used in the various fields, has very high sensitivity and selectivity. Generally its application is oriented towards the general detection and potential identification of chemicals in the presence of other chemicals in a complex mixture. LC-MS-MS is a powerful tool for fast and selective analysis .GC-MS has few limitations. Only compounds with vapor pressures exceeding about 10–10 torr can be analyzed by GC-MS.In contrast, if the substance is dissolved in a mobile phase (liquid) then LC is able to analyze even least volatile or thermally-unstable compounds that are difficult to analyze using GCMS.

Compound dependent parameters which are generally considered in multiresidue pesticide analysis are as follows:

a) Declustering Potential (DP) : The potential difference between the ground and the orifice plate used to minimize solvent cluster ions, which may attach to the sample. The higher the voltage the greater the amount of fragmentation.

b) Collision energy (CE): The amount of energy precursor ion receives as they are accelerated into the collision cell or they accelerate into

c) Entrance Potential (EP): Focuses the ions through the high pressure region.
d) Collision Cell Entrance Potential (CEP): Focuses ions into the collision cell.
e) Collision Cell Exit Potential (CXP).
f) Collision gas (CAD).

II. Experimental

A survey was conducted for analyze the extent of contamination of pesticide residues in the soil of grape growing farms in Nashik district, Maharashtra, India during the year of 2012-13. The details of research design and techniques adopted throughout survey course of are described below.

A. Locality and Weather

Nashik District is located between 18.33 degree and 20.53 degree North Latitude and between 73.16 degree and 75.16 degree East Longitude at Northwest part of the Maharashtra State, at 565 meters above mean sea level.

The climate of Nashik district is characterised, by dryness except in the south-west monsoon season. The year may be divided into four seasons, the winter season from December to February followed by the summer season from March to May and the south-west monsoon season from June to September followed by the post-monsoon season during October and November. The air is very humid during the south-west monsoon season. In the post-monsoon, winter and summer seasons the air is dry. The summer season is the driest part of the year with relative humilities between 20 and 25 per cent only in the afternoons. (nashik.nic.in.)

B. Study area

Niphad, Dindori, and Nasik were selected as study area as these are major grape growing tahasils in the district of Nasik district. Vineyards of five grape cultivators were selected from each talukas for proposed study.

C. Vineyards Classification

Vineyards was classified on the basis of sale market of grapes. Five (5) soil samples from different villages of each taluka were selected as a sample for study by proper soil sampling method in such a way that 2 soil samples from export quality grape growing field and 3soil samples from random field. (Indian sale market).Total 45 representative soil samples were analyzed for its pesticides residues.

D. Soil sampling

Stainless steel auger, spade, khurpi shovel, bucket, plastic sheet, air tight plastic bags were used for collection of soil samples.

Soil samples were collected from the study areas by proper soil sampling methods (Arora and Singh, 2009, NuhMaral, 2010)in three batches to examine pesticides residues in soil throughout the year.

1) August to September 2012 at the time of pruning, before application of pesticides. (Lean Period).
2) November to December 2012 at the time of growth, after application of pesticides. (Peak period).
3) February to March 2013 at the time of harvesting.

Sampling area was cleaned from herb and plant remains .The composite soil samples were drawn from 0-20 cm. depth using appropriate sampling tools from 15 to 20 well distributed spots, moving in a zig-zag manner from each individual sampling site. Soil Samples were collected in between the vineyard rows where most of the vine roots are located. The shovel was dipped up to 20-cm depth. A pit was opened with a shovel. Soil samples were obtained about the 2-cm of thickness, 3–4-cm width and 20-cm length of the part of soil. About 4-5 subsamples per acre from each vineyard were collected. The subsamples were placed into a 16-liter bucket thoroughly mixed on a plastic sheet to ensure that the soil collected was truly representative of each location. Approximately, 1 kg of soil sample was collected. Plant residues and stone pieces were removed by hand. Samples are packed in air tight plastic bags, codes are given as, A to O for 15 samples. A Sampling date, location of the sampling and sampling number were marked on the bags and soil samples were brought to the laboratory for further processing.

Multiresidue pesticide analysis with the help of LCMS/MS used for analysis of soil sample. Multi residue pesticide analysis is a selective and sensitive method of analysis for simultaneous determination of pesticides of different chemical classes.Like a good marriage both liquid chromatography interface with mass spectrometry bring something to their union. LC can split up volatile and semi-volatile compound with great resolution but it cannot recognize them. On the other hand Mass Spectrometry can provide detailed structural information.

E. Instruments

Analytical Balance(Shimadzu),Centrifuge(Thermo), Low volume concentrator (Caliper), GCMS/MS (Perkin Elmer, Clarus500) with auto sampler equipped with an Electron Capture Detector (ECD, 63Ni) with Mass-Lynx software, LCMS /MS (Absciex) with Analyst software, Refrigerator.

F. Glassware / Apparatus

Measuring cylinder 25 ml., micro pipette 0.5 -200 µL, test tube 10 ml., eppendorf tube 2.0 ml., centrifuge tube 50 ml., Beaker 200 ml.- 2lit, 0.2 µm PVDF/nylon membrane filter, 0.22 µm Polytetrafluroethylene(PTFE) membrane filter

G. Reagents

The certified Pesticides standards were purchased from Dr.EhrenstorferGm.bH, Germany. The purity of all pesticide standards were greater than 95%, ethyl acetate (AR / mass grade), anhydrous sodium sulphate ,10% Diethylen glycol (DEG) with methanol (HPLC grade),0.1% acetic acid in water, ammonium formate (AR grade),water (HPLC grade),Primary Secondary Amine (PSA) ,formic Acid, all these were purchased from Merck.

2.8 Preparation of standards

Standards were made up with methanol for LCMS/MS compounds. Stock solution was prepared with concentration around 1000 mg. /litre (ppm) .Working standard (mix standard) was prepared with concentration of 1.0 mg./litre (ppm) .Standard series of suitable concentrations were prepared by the subsequent dilution with respective solvent.

Estimation of pesticides of 65 different groups was carried out using LCMS/MS (Absciex, 4000 Q TRAP)

2.9 Sample Extraction Procedure

Samples were extracted by standard liquid-liquid extraction method. (Zweig *et al.,* 1984; Raikwar*et al.,* 2011)

2.9.1 Extraction procedure for LCMS/MS analysis

For the extraction purpose in 10gm. of soil sample 5 ml. water, 10 ml. ethyl acetate and 10 gm. of anhydrous sodium sulphate were added, then it was homogenized for 2 mins at high speed and centrifuge for 5 minutes at 5,000 rpm. Out of which ,3 ml. of the ethyl acetate phase was taken into a centrifuge tube containing 25mg. primary secondary amine Shake vortex for 1 min. Centrifuge at around 5,000 rpm for five minutes. In 2 ml. cleaned supernatant solution 0.2 ml. 10% diethylene glycol in methanol was added. It is evaporated under gentle stream of nitrogen using low volume concentrator at 35^{0}C. Reconstitute into 1 ml. methanol and 1 ml. 0.1% acetic acid in water. Then centrifuge it at 10,000 rpm for 5 min and filter through 0.2 µm PTFE membrane filter. Inject 10 µl into LCMS/MS as shown in (**Plate 1 to 8)**

Analysis on LCMS/MS

Sequence of injection was as follows

- Inject blank (Methanol).
- Inject standards of suitable concentration within the calibration range.
- Inject samples.

HPLC parameters:

- Two mobile phases:

A : 5 mM ammonium formate dissolved in water: methanol (80:20) (157.7 mg. ammonium formate dissolved in 500 ml. of mobile phase)

B : 5 mM ammonium formate dissolved in methanol: water (90:10) (157.7 mg. ammonium formate dissolved in 500 ml. of mobile phase)

Table 1: HPLC parameters

Step	Total Time (min)	Flow rate (µl/min)	Gradient profile	A (%)	B (%)
0	0.1	600.00	1.0	80.0	20.0
1	1.0	600.00	1.0	90.0	20.0
2	8.0	600.00	1.0	0.0	100.0
3	15.0	600.00	1.0	0.0	100.0
4	16.0	600.00	1.0	80.0	20.0
5	20.0	600.0	1.0	80.0	20 0

- Analytical column: Zorbex (Eclipsed plus-C18)3.5µ, 4.6 x 100 mm (API 4000)

- Flow rate: 0.6 ml./min
- Interface: ESI + ve

Mass parameters

- Source température : 450^0C
- Source Type : Turbo Spray

2.10 Calculation

Concentration of pesticides residues in soil samples were determined by using following formula.

$$\textbf{Concentration} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \frac{\text{Concentration of standard}}{\text{weight of sample}} \times \text{Dilution Factor}$$

For calibration of the instrumentsthe certified Pesticides standards were used. The pesticides were recognizedby comparing with retention indices of the standard solution peaks with those of the samples. The concentrations of analyte were determined by comparing the peak area of the samples and five level calibration curves of the standards. These curves were established by tracing peak areas in accordance with the concentration of analysed pesticide .Calibration was done by linear regression method .The correlation coefficient of calibration curves were ranged from 0.9980 to 0.9990. Results above Limit of Detection (LOD) were taken for calculations and below (LOD) were taken as zero (0) in the calculations.

2.11 Quality control and Safety

All general laboratory safety rules for sample preparation and analysis were followed. All standards were kept in a refrigerator. The method was validated in soil samples by analysis of spiked samples. The identification of the target pesticides were carried out by searching in the appropriate retention time windows (RTWs); the Quantification of the samples was carried out by injecting blank sample extracts spiked with the pesticides at five different concentration levels to perform the calibration curves. Spiked blank soil samples were used as standards. Pesticides were confirmed by their retention times, Recovery studies were performed at 10 and 20 ppb fortification levels of each pesticide. All the recoveries were obtained above 70% with a relative standard deviation between 0.31 and 6.4%.

Sr. No.	Name of Chemical	RT (min)	Q	Q1	Q2	CE (V)	CXP (V)	DP (V)	CE (V)	CXP (V)	LOD (ng/g)	LOQ
1	Acetamiprid	5.8	223	126	56	27	7	60	35	3	0.6	2.0
2	Atrazine	8.9	216	174	104	26	14	54	45	6	0.3	1.0
3	Azoxystrobin	9.2	404	372	344	22	4	53	32	2	0.3	1.0
4	Benalaxyl	10.6	326.2	148.3	208.3	30	10	65	23	10	0.3	1.0
5	Buprofezin	12.39	306	201	116	20	9	32	24	7	0.3	1.0
6	Carbandazim	6.86	192	160	132	30	7	33	43	6	0.3	1.0
7	Carbaryl	8.3	202	145	127	13	6	53	40	6	0.3	1.0
8	Carbofuran	7.9	222	165	123	20	8	55	28	6	0.3	1.0
9	Clothianidin	5.4	250	132	169	29	6	50	20	5	0.6	2.0
10	Cymoxanil	6.4	199	128	111	22	6	48	31	5	1.0	2.5
11	Dimethoate	6.0	230	125	199	29	4	50	18	2	0.5	1.5
12	Dimethomorph	9.77	388.2	301	165.2	29	7	95	45	13	0.6	2.0
13	Fenamidone	9.4	312	92	236	35	3	53	21	5	0.5	1.5
14	Flusilazole	10.38	316	165	247	37	8	13	28	2	0.5	1.5
15	Hexaconazole	10.9	314	70	159	38	2	52	38	6	0.5	1.5
16	Imidacloprid	5.15	256	175	209	29	8	55	21	11	0.6	2.0
17	Iprobenphos	10.4	289	205	91	15	10	46	37	6	0.6	2.0
18	Iprovalicarb	10 .0	321.3	119.3	203.1	33	10	61	13	16	0.3	0.1
19	Kresim Methyl	10.66	314.2	222.1	116.1	19	16	51	19	8	0.3	1.0
20	Metalaxyl	8.94	280	220	192	20	8	58	26	8	0.3	1.0
21	Myclobutanil	10.54	289	70	125	50	2	67	29	5	0.3	1.0
22	Penconazole	10.6	284	159	70	36	8	56	45	2	0.6	2.0
23	Phosalone	11.04	368	182	111	30	9	68	60	4	0.5	1.5
24	Propiconazole	10.6	342	159	69	33	8	30	40	2	0.6	2.0
25	Pyraclostrobin	10.88	388	194	296	18	10	20	18	3	0.3	1.0
26	Simazine	8 .0	202	124	132	27	6	60	27	6	0.5	1.5
27	Spinosad A	13.86	732	142	98.1	43	10	111	103	10	0.3	1.0
28	Tebuconazole	10.6	308	70	125	55	4	61	59	8	0.6	2.0
29	Tetraconazole	10.13	372.2	159.1	70.1	49	14	76	53	12	0.5	1.5
30	Thiamethoxam	4.1	292	132	211	31	6	52	18	10	1.0	2.5
31	Tridemefon	9.8	294	197.1	225	23	14	66	19	8	0.3	1.0
32	Triazophos	9.8	314	162	119	25	7	29	49	5	0.3	1.0

(**RT**: retention time; **Q**: protonated parent ion; **Q1**: quantifier ion; **Q2**: qualifier ion; **DP**: declustering potential; **CE**: Collision energy; **CXP**: collision cell exit potential; **LOD**: limit of detection; **LOQ**: limit of quantification.)

Table 2: Liquid chromatography tandem mass spectrometer (LC-MS/MS) specific parameters for target pesticides

❖ **Stepwise experimental part during research work**

Plate 1: Standard solution of pesticides

Plate 2 : Extraction of soil samples for its pesticides residues.

Plate 3: Centrifuge at10,000r.p.m.

Plate 4: Evaporation under gentle stream of nitrogen using low Volume concentrator

Plate 5: Filtration through 0.2 µm PTFE membrane filter in a vial.

Plate 6: Extracted soil samples with code A to O

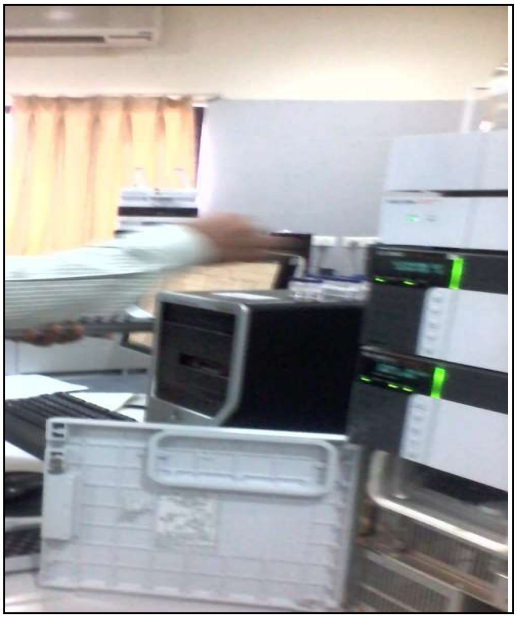

Plate 7: Vials are placed in a tray Plate 8:Setting of LCMS parameters.

III. Results and Discussion

The concentration of pesticide residues detected in the soil samples collected at the time of harvesting are reported in **table 3**.All collected soil samples were found to be contaminated with seventeen pesticides residues differ widely in contamination levels. Some pesticides such as imidacloprid, dimethomorph shows large variation with relatively high concentration values for different soil samples. While other pesticides were found to be detected with low concentration which reduces the possibility of biomagnifications of pesticides in grapes and avoid contamination in grapes.

Soil Sample codes	Carbandazim	Azoxystorbin	Imidacloprid	Flusilazole	Dimethomorph	Thiamethoxam	Fenamidone	Pyraclostorbin	Clothianidin	Iprovalicarb	Hexaconazole	Tridemefon	Penconazole	Spinosada A	Metalaxyl 1	Tetraconazole 1	Myclobutanil 1	Total
A	4.5	22.5	623.00	4.3	30.3	8.8	0.20	2.5	136.0	0	5.2	0	0	0	0	0	3	840.3
B	71.5	23.7	8.5	0.5	48.7	5.3	0	7.9	0	0	0	0	0	0	0	0	13.7	179.8
C	0.6	1.9	2.9	2.4	73.1	0	4.8	0	0	1.8	0	0	1.1	0	0	0	1.2	89.8
D	0	0	0.9	0.3	1.3	0.2	0	0	2	0	0	0	0	0	0	0	0	4.7
E	0	0	1.2	0	2.7	0	0	0	0	0	0	0	0	0	0	0	0	3.9
F	1.6	0	38.3	16.7	105	0	2.3	7.6	1.2	0	1.9	0	0	0	0	0	0	174.6
G	1.9	0	17.1	2	21.1	2.1	0	0	6.4	0	0	0	0	0	0	0	0	50.6
H	9	9.7	67	8	123	0	0	7.3	4.1	0	1.1	0	0	0	2	0	1.4	232.6
I	3.7	118	45.6	5.7	176	10.9	47.1	48.4	1.3	118	6.6	67.7	0	3.7	0	5.5	48.9	714.8
J	8.4	3.5	0	0	23	0	0	0	0	0	0	0	0	0	0	0	0	34.9
K	3.1	151	66.5	0	539	11.5	0	15.7	14.5	13.9	2.3	0	0	0	0	0	7.4	826.6
L	15.7	51.9	10	0	545	0	25	0	0	0	5.5	0	0	0	0	0	0	655.1
M	6.4	34.6	114	0	105	1.6	0	8.1	0	0	0	0	0	0	0	0	2.9	273.1
N	3.5	4	5.8	0	52.2	0.2	0.4	0.8	0	0	0	0	0	0	0	0	0	66.9
O	1.5	8.2	84.6	11.8	73	0.4	0	1.9	19.9	3.3	7.8	0	0	0	0	0	0	212.4
Mean	8.76	28.60	72.36	3.44	127.89	2.73	5.32	6.68	12.36	9.13	2.02	4.51	0.07	0.24	0.13	0.366	5.23	290.67
STD	17.85	45.98	156.45	5.10	174.85	4.24	13.22	12.44	34.71	30.33	2.80	17.48	0.28	0.95	0.51	1.42	12.65	306.48
Lower bound	-0.46	5.0	-8.42	0.81	37.61	0.54	-1.50	0.25	12.36	-6.53	AA0.57	-4.51	-0.073	0.0003	-0.13	-0.36	-1.30	33.6173
Upper bound	17.98	52.34	153.14	6.08	218.17	4.922	12.14	13.10	30.28	24.79	3.47	13.5	0.21	0.74	0.39	1.09	11.76	565.932

Table3: Contamination of Pesticide Residues in soil samples collected at the time of harvesting

❖ **Chromatograms, mass spectrum, linear regression curves of pesticides detected**

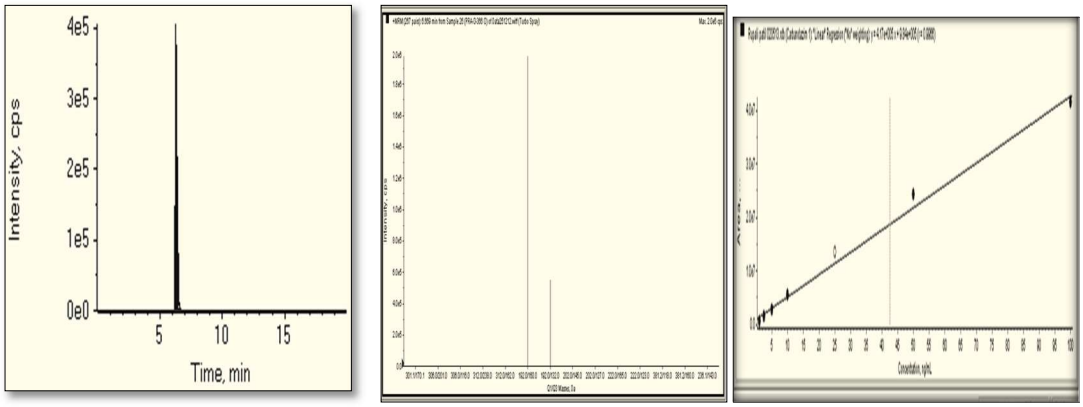

Figure.1: Representative Chromatogram, mass spectrum, linear regression Curve of Carbendazim

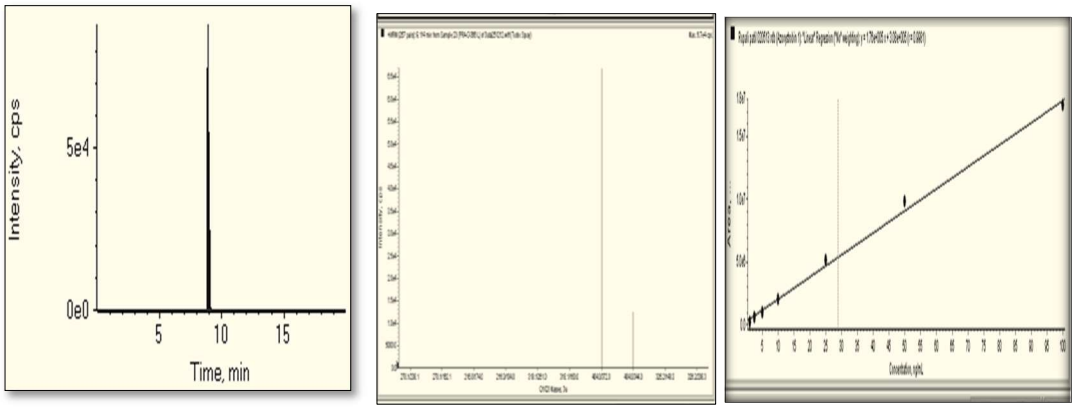

Figure 2 : Representative Chromatogram mass spectrum, linear regression curve of Azoxystorbin

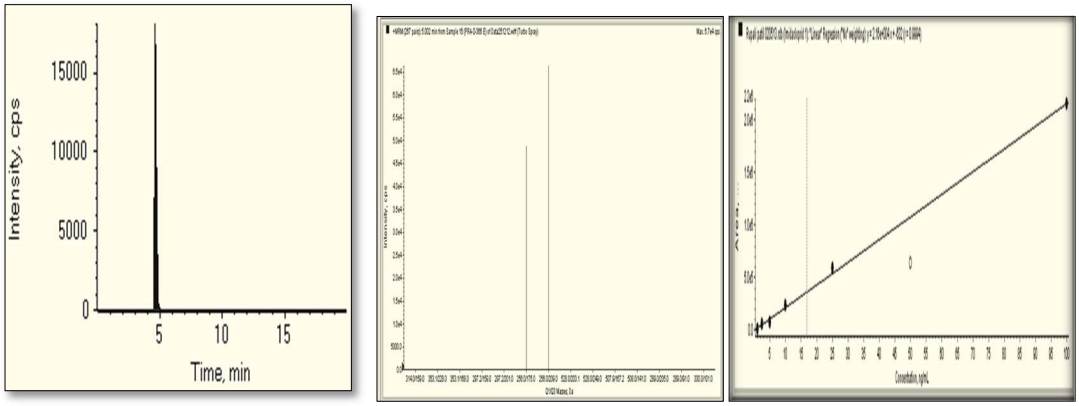

Figure 3 : Representative Chromatogram mass spectrum, linear regression curve of Imidacloprid

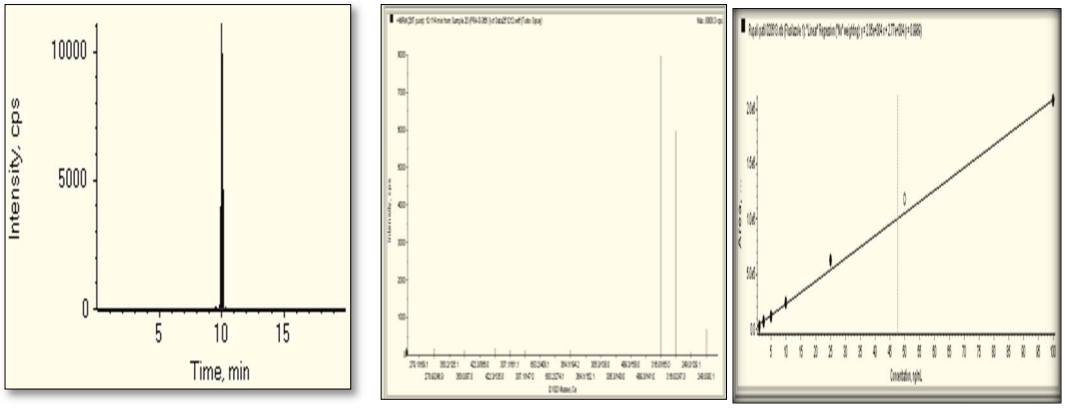

Figure 4 : Representative Chromatogram mass spectrum, linear regression curve of Flusilazole

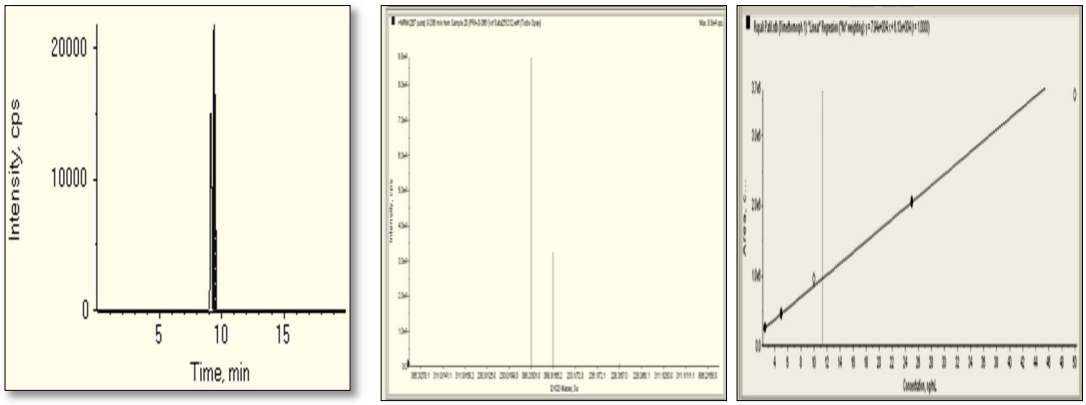

Figure 5 : Representative Chromatogram mass spectrum, linear regression curve of Dimethomorph

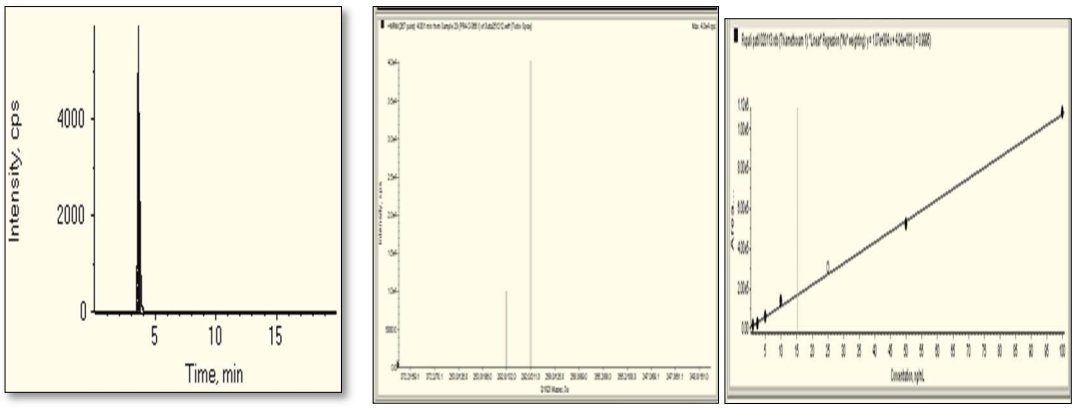

Figure 6 : Representative Chromatogram mass spectrum, linear regression curve of Thiamethoxam

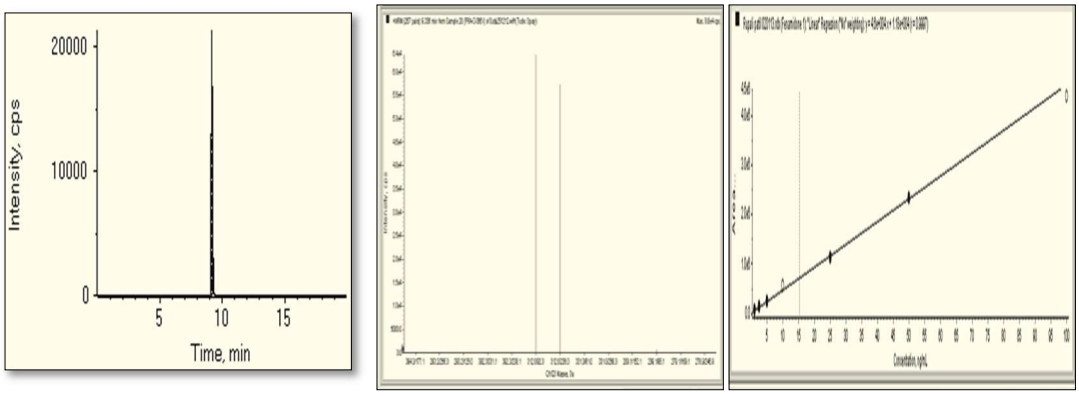

Figure 7 : Representative Chromatogram mass spectrum, linear regression curve of Fenamidone

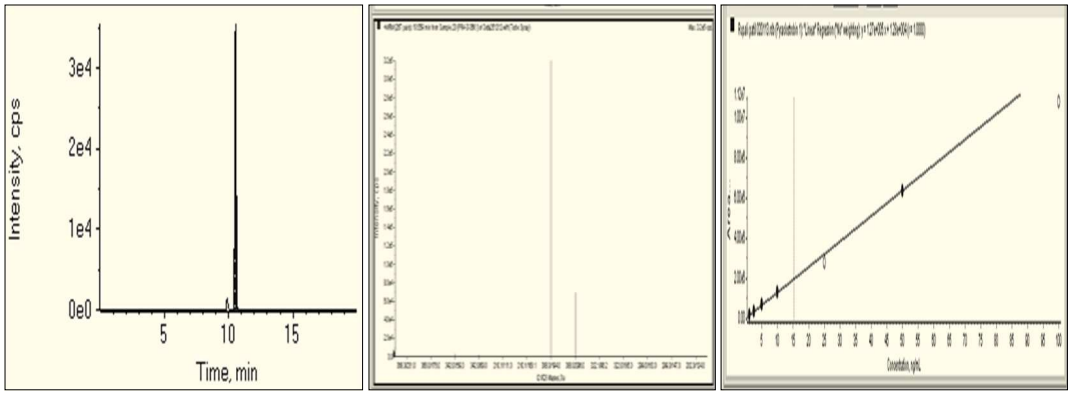

Figure 8 : Representative Chromatogram mass spectrum, linear regression curve of Pyraclostorbin

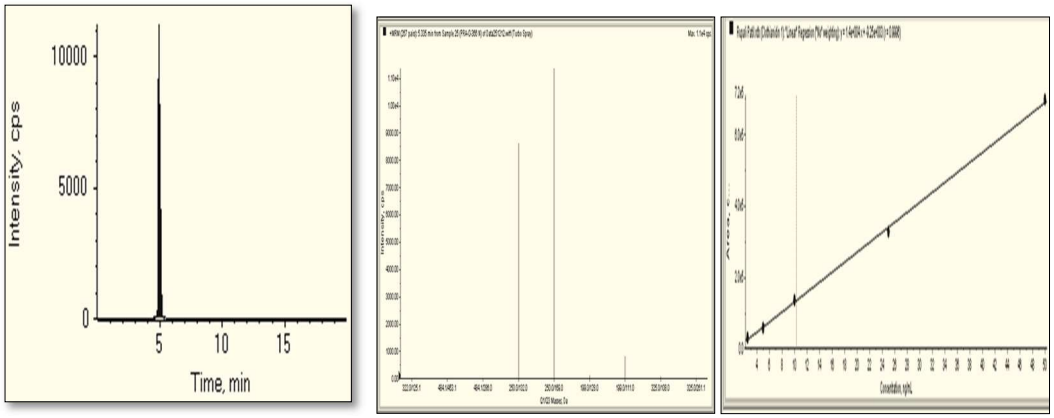

Figure 9 : Representative Chromatogram mass spectrum, linear regression curve of Clothianidin

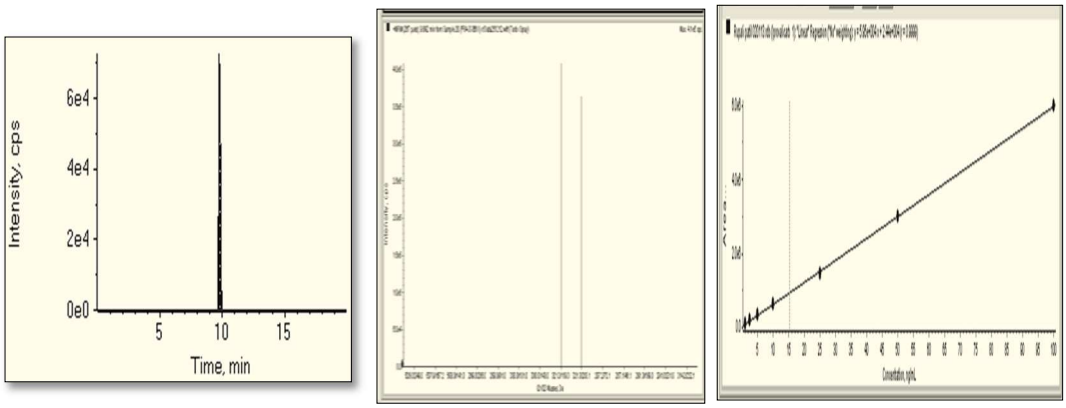

Figure 10 : Representative Chromatogram mass spectrum, linear regression curve of Iprovalicarb

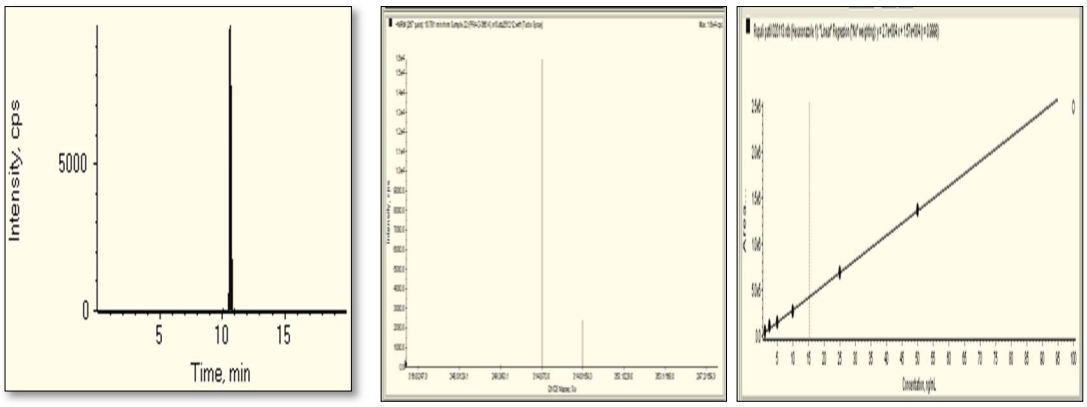

Figure 11 : Representative Chromatogram mass spectrum, linear regression curve of Hexaconazole

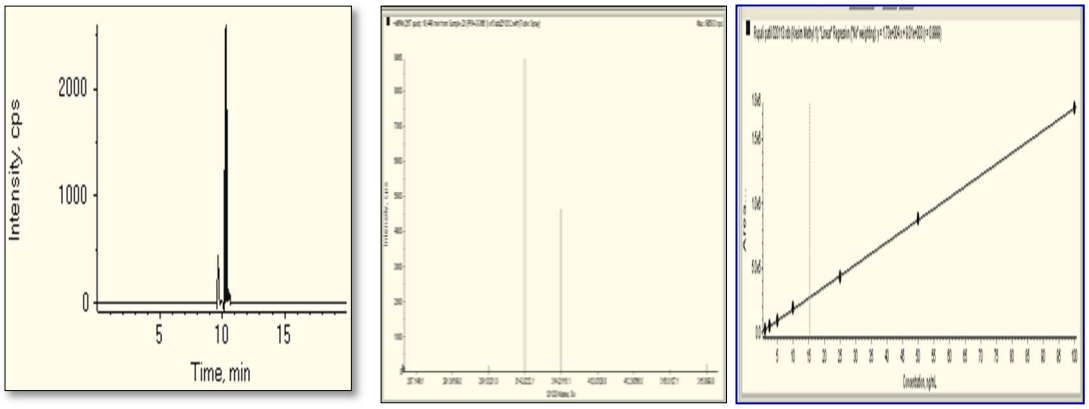

Figure 12 : Representative Chromatogram mass spectrum, linear regression curve of Tridemefon

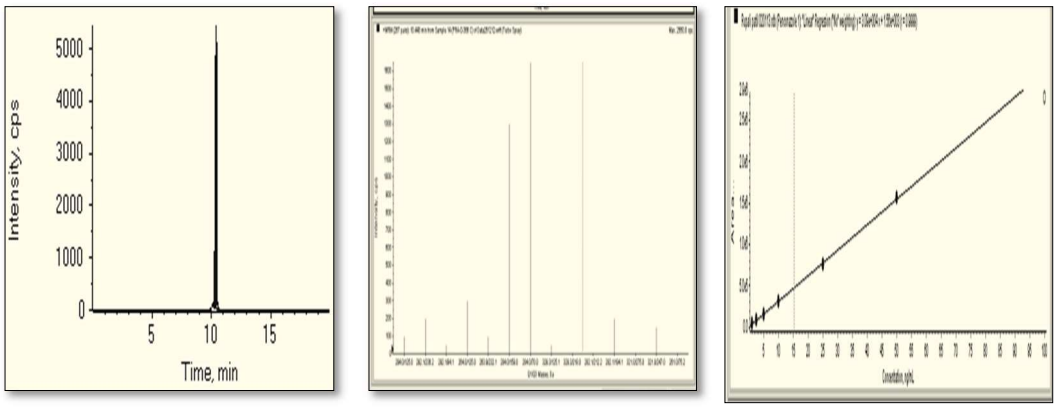

Figure 13: Representative Chromatogram mass spectrum, linear regression curve of Penconazole

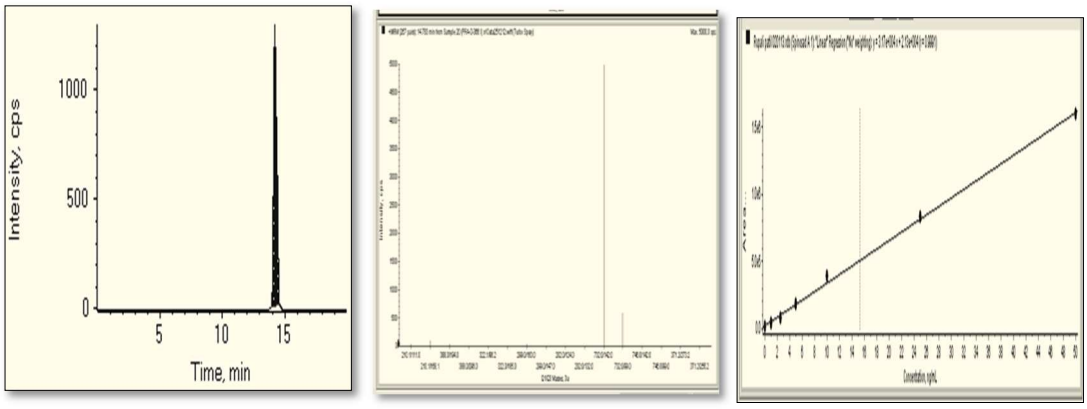

Figure 14: Representative Chromatogram mass spectrum, linear regression curve of Spinosad A

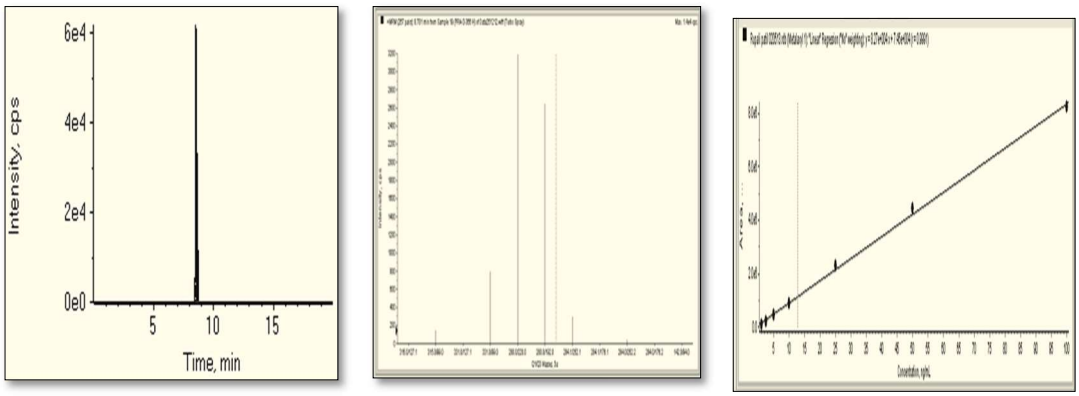

Figure 15: Representative Chromatogram mass spectrum, linear regression curve of Metalaxyl

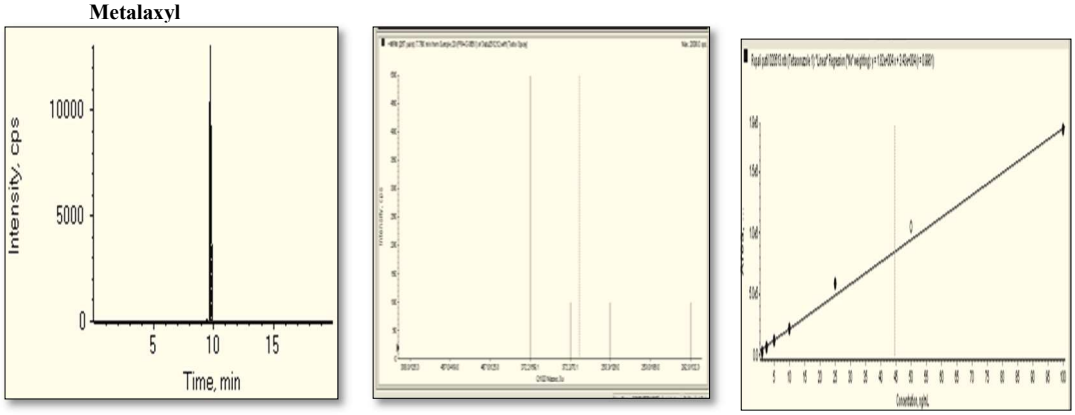

Figure 16: Representative Chromatogram mass spectrum, linear regression curve of Tetraconazole

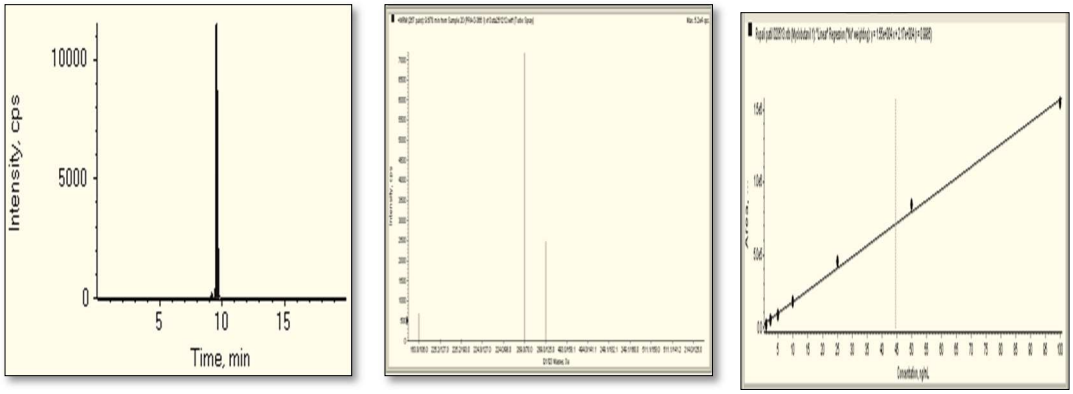

Figure 17: Representative Chromatogram mass spectrum, linear regression curve of Myclobutanol

Out of seventeen pesticides detected dimethomorph, imidacloprid and carbendazim were the most often detected pesticides found to be contamination levels relatively in high concentration with highest percent contamination of 100.0%, 86.66 %, 86.66% respectively .This indicates that use of these pesticides in grape growing farms was on higher side throughout the year.

Azoxystorbin was investigated in eleven soil samples with percent contamination of 73.33%. followed by pyraclostorbin, thiamethoxam and flusilazole with 60.0 % while clothianidin with 53.33% .Pesticide residues of hexaconazole and myclobutanil detected in seven soil samples with percent contamination of 46.66% followed by fenamidone with 40%, iprovalicarb contaminates four soil sample with percent contamination of 26.66% remaining five pesticides such as, tridemefon, penconazole, metalaxyl, tetraconazole, and spinosad A were detected in only one soil sample with low percent contamination of 6.66%.

The mean of ∑ pesticides was found to be 290.67µg kg^{-1} at 95% confidence level. Soil sample A shows highest contamination with concentration of 840.30 µg kg^{-1} followed by 826.60 µg kg^{-1} in K, 714.8 µg kg^{-1} in I, 655.10 µg kg^{-1} in L,273.10 µg kg^{-1} in M, 232.60 µg kg^{-1} in H, 212.40µg kg^{-1} in O, 179.80 µg kg^{-1} in B, 174.60 µg kg^{-1} in F, 89.80 µg kg^{-1} in C, 66.90 µg kg^{-1} in N, 50.60 µg kg^{-1} in G, 34.90 µg kg^{-1} in J, 4.70 µg kg^{-1} in D, 3.9 µg kg^{-1} in E respectively.

The contamination levels of most of the soil samples were noticed onhigher level on account of the overall effect of frequent use of pesticides throughout the year .These pesticides have high persistence in soil and degrade according to their half-

life. Contamination of pesticides of triazole group such as, flusilazole, penconazole, tetraconazole, hexaconazole, myclobutanil, tridemefon were found on higher side probably due to their slow degradation having half-life of fourteen to four twenty (14-420) days(Kookana et.al, (1998)

IV. CONCLUSIONS

The LCMS/MS method described in this work is rapid and allows the simultaneous determination of higher number of compounds in a single run .It is the most authentic, less laborious technique for multiresidue pesticide analysis of soil samples as, all pesticide residues were detected at a time. It is prescribed for the monitoring of pesticides with a broad range of physico-chemical properties.

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