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Estimation of pesticide residues in table grapes by using gas and liquid chromatography coupled with mass spectrometry

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ABSTRACT

The grapes are being exported in increasing quantities from Maharashtra to European countries and a lot of pesticide inputs are being used by the growers. A total number of 578 grape samples collected from Nasik district during December, 2013 to April, 2014 and analyzed for 167 numbers of multi-class pesticide (Organophosphate, Triazine, Pyrimidine, Triazole, Imidazole, Benzimidazole, Nicotinoid, Substituted thiourea, Strobiluron, Dinitroaniline, Morpholine) residue levels using Liquid Chromatography-Mass spectrometry/Mass spectrometry (LC-MS/MS) and Gas Chromatography-Mass spectrometry/Mass spectrometry (GC-MS/MS) by using validated methods. Only four samples were free from pesticide residues and rest were contaminated with 1-13 numbers of pesticides residue. During the study different classes of total 41 number of agro-chemicals had been detected and 116 number of samples were failed with residues of 4-Bromo-2-Chlorophenol, Abamectin, Carbendazim, Chlormequat Chloride, Chloropyriphos, Dinocap, Forchlorfenuron, Hexaconazole, Flusilazole, Profenophos, Spinosad, Thiacloprid, Triazophos, Fipronil and Acephate by exceeding their European Union MRLs.

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INTRODUCTION

Fruits and vegetables are essential components of the human diet since they provide many nutrients which are useful to sustain human body. Fruits and vegetables are commonly used everywhere in world to meet the balance diet and good health (Krol *et al.*, 2000). In India grape (*Vitis vinefera*) is basically a sub-tropical crop and successfully grown in 111.4 thousand ha area with production 1234.9 thousand tons and productivity of 11.1 t/ha (APEDA, Agriexchange). Maharashtra is leading state in production of grapes in the whole country. Nasik and Sangli districts of Maharashtra are at forefront with regard to agricultural land under grape's cultivation.

Like other crops, grapes are also affected by insect-

pests and diseases during production and they reduce the quality and yield. In order to reduce the loss and maintain the quality of fruits during cropping season, pesticides are used together with other pest management techniques to control insect-pests and prevent diseases. The usage of pesticides is increasing because they have rapid action, decrease toxins produced by food infecting organisms and are less labour intensive than other pest control methods. However, the use of pesticides during production often leads to the presence of pesticide residues in fruits and vegetables after harvest.

Several pesticides are noxious substances and can persistent in the environment for a longer period of time. Therefore, it is necessary to control the application of pesticides on crops from health point of view (Freidberg, 2003). On the other hand, different types of new pesticides have been introduced in the market during last few decades to enhance better yield and quality of agricultural products (Manyak and Ajay, 2007 and Srivastava *et al.,* 2001). However, levels of pesticides should be controlled at optimum point due to their relative toxicity to the environment and human health (Jiang *et al.,* 2009).

A few work has been reported on pesticides residue contamination on grapes in India (Hiremath *et al.,* 2010). Therefore, the objective of present work was to assess the pesticides residue concentration levels in table grapes and thus the gathered data would be useful to generate awareness about the lethal effects of these pesticides on human beings with regard to consumers.

MATERIAL AND METHODS

Sample collection:

The samples were collected from farmers' fields of Nasik district and sample size was at least two kilograms. The collected samples were sealed and labeled with a unique sample identity to meet the requirement of APEDA grape RMP guidelines (Annexure 9) and placed in an ice box. All samples were transported to pesticide residues laboratory, National Horticultural Research and Development Foundation (NHRDF), Nasik and were refrigerated at 5ºC. These samples were then extracted and analyzed for pesticide residues within 48 h from the time of their collection.

Chemical and reagents:

Certified reference materials of 167 pesticides were $\geq$ 96% purity and purchased from Dr. Ehrenstorfer GmbH, Ausburg, Germany and Sigma Aldrich, Germany. HPLC grade solvent, methanol was obtained from J.T. Baker, USA. Ethyl acetate for spectroscopy obtained from Merck India Ltd. HPLC grade water was prepared in lab using a Milli-Q water purification system, (Millipore, USA). Ammonium formate, acetic acid, diethelene glycol and sodium sulphate anhydrous were of $\geq$ 97% purity, analytical grade, purchased from Merck India Ltd. Primary secondary amine (PSA) Agilent make was received. The Sodium sulphate anhydrous was heated to 650^0 C for 3 h, cooled in a desiccator and stored in sealed bottle.

Equipments:

Sophisticated analytical equipments: LC-MS/MS (model: API 4000QTRAP) from ABsciex, GC-MS/MS – Quattro Micro obtained from Waters and other minor apparatus: blender, homogenizer, low volume concentrator, Milli-Q, Centrifuge and Refrigerated micro centrifuge.

Sample extraction and analysis procedure:

2 kg grape berries were blended directly. The blended sample (200 g) further homogenized at 3000 rpm for 1 min. From this, 10 g was transferred in to 50 ml centrifuge tube, extracted with 10 ml of ethyl acetate and followed by adding 10 g of sodium sulphate anhydrous. The mixture was then homogenized at 10000 rpm for 2 minutes followed by centrifugation at 5000 rpm for 5 minutes for phase separation. For GC-MS/MS: 2 ml of extract transferred into eppendorf tube and added 25 mg PSA followed by micro centrifuge at 10000 rpm for 5 minutes, the same has been filter through 0.22 µm ployvinylidene fluoride (PVDF) filter and injected 2 µl in to GC-MS/MS. For LC-MS/MS: Primary secondary amine (PSA, 50 mg) was added to 4 ml of ethyl acetate and it was centrifuged at 10000 rpm. 2 ml of supernatant layer of ethyl acetate collected in to a fresh test tube and evaporated with 200 µl 10% diethelene glycol (DEG) in methanol to dryness under steam of nitrogen gas on low-volume concentrator (Caliper life sciences, Germany) at 35^{0}C. The residues were dissolved and reconstituted (2 ml) with methanol and 0.1% acetic acid (1:1 v/v). This solution was filtered through 0.22 µm ployvinylidene fluoride (PVDF) filter and injected 10 µl in to LC-MS/MS.

LC-MS/MS and GC-MS/MS determination:

Liquid and Gas Chromatography conditions like mobile phase gradient and oven programme of LC and GC were well optimized to detect and quantify the multiclass of pesticides. For each pesticide, mass dependent parameter and source parameters were optimized on both MS/MS equipment methods has been validated (Kaushik *et al*., 2007) and studies at fortification levels of 0.005-0.050 mg kg^{-1} gave mean recoveries from 68 to 106% with relative standard deviation (RSD) ≤16%. Two MS-MS transitions were monitored for each pesticide. The Limit of quantification (LOQ) of all compounds was in compliance to harmonized maximum residue limits (MRLs) of European Union Commission.

RESULTS AND DISCUSSION

Pesticides are a part of majority of chemicals that were applied on grapes growing farms to protect the crop. The present investigation determined the pesticides residues in grapes collected from various grape growing areas of Nasik district and compared them with MRLs set by European Union (http://europa.eu.int). The analytical parameters recovery, limit of quantification (LOQ) and type of equipment of 41 detected pesticides are shown in Table 1.

During the monitoring study a total 578 numbers of grape samples collected for analysis of 167 numbers of agro chemicals (Annexure 9 of RMP-2014) and analyzed on LC-MS/MS and GC-MS/MS by using validated methods, from the results it has been found that only four samples free from pesticide residue and rest were contaminated with 1-13 numbers of pesticide residues. Out of 578 samples, 25 per cent samples were contaminated with five pesticides residue followed by 20 per cent and 14 per cent samples contaminated with four and seven pesticides residue, respectively (Fig. 2).

The results from the present study revealed that a total 41 pesticides residue were detected (Table 2), in which high frequency of chlormequat chloride, myclobutanil, azoxystrobin, tetraconazole, mandipropamid, buprofezin and kresoxim methyl (Fig. 1) found in 323, 312, 257, 126, 122 and 83 numbers of samples with higher concentration 0.119, 0.823, 0.278, 0.751, 0.122, and 0.455 mg kg^{-1}, respectively, and other pesticides were also detected in considerable number of samples (Table 2). The analysis test results were compared to EU MRLs for grapes and it has been found that highest concentration (mg/kg) of residues of 4-

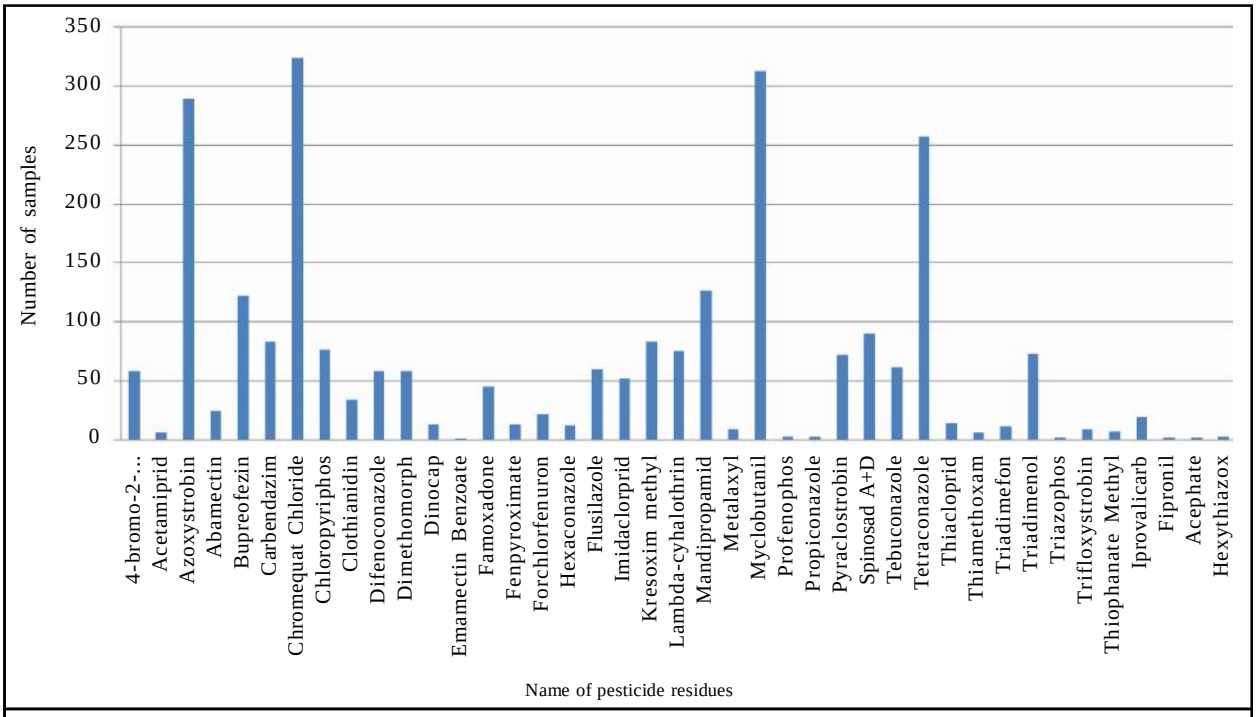

Fig. 1: Abundance of pesticide residues in fresh grape samples

bromo, 2-chloro phenol (0.038); abamectin (0.052); carbendazim (0.564); chlormequat chloride (0.119); chlorpyriphos (0.625); dinocap (0.090); forchlorfenuron (0.099); hexaconazole (0.067); profenophos (0.063); spinosad (0.763); thiacloprid (0.046); triazophos (0.018); fipronil (0.019) and acephate (0.011) residues exceeded

Table 1 : The mean recovery (±RSD) percentage of fortified grape sample for the detected pesticides

Sr. No.	Name of Pesticides	Class of pesticides	Name of equipment	Limit of quantification (mg/kg)	Fortification level (mg/kg)	Mean recovery (±RSD) (%)
1.	4- bromo-2-chlorophenol	Organophosphates	GC- MS/MS	0.010	0.01	81(±15)
2.	Acetamiprid	Nicotinoids	LC- MS/MS	0.005	0.01	78(±8)
3.	Azoxystrobin	Strobilurin	LC- MS/MS	0.005	0.01	97(±4)
4.	Abamectin	Avermectins	LC- MS/MS	0.010	0.01	76(±11)
5.	Buprofezin	Sulfie ester	LC- MS/MS	0.005	0.01	85(±6)
6.	Carbendazim	Benzimidazole	LC- MS/MS	0.005	0.01	89(±11)
7.	Chlormequat Chloride	Quaternary ammonium	LC- MS/MS	0.005	0.01	94(±7)
8.	Chloropyriphos	Organophosphates	GC- MS/MS	0.005	0.01	83(±16)
9.	Clothianidin	Nicotinoids	LC- MS/MS	0.010	0.01	76(±12)
10.	Difenoconazole	Triazole	LC- MS/MS	0.005	0.01	92(±8)
11.	Dimethomorph	Morpholine	LC- MS/MS	0.005	0.01	86(±9)
12.	Dinocap	Dinitrophenol	LC- MS/MS	0.010	0.01	74(±8)
13.	Emamectin Benzoate	Macrocyclic lactone	LC- MS/MS	0.005	0.01	71(±11)
14.	Famoxadone	Oxazole	LC- MS/MS	0.005	0.01	74(±4)
15.	Fenpyroximate	Pyrazole	LC- MS/MS	0.005	0.01	96(±11)
16.	Forchlorfenuron	Urea derivative	LC- MS/MS	0.005	0.01	102(±5)
17.	Hexaconazole	Triazole	LC- MS/MS	0.010	0.01	87(±10)
18.	Flusilazole	Triazole	LC- MS/MS	0.005	0.01	79(±12)
19.	Imidacloprid	Nicotinoids	LC- MS/MS	0.005	0.01	93(±6)
20.	Kresoxim methyl	Strobilurin	LC- MS/MS	0.005	0.01	80(±9)
21.	Lambda-cyhalothrin	Synthetic Pyrethroids	LC- MS/MS	0.005	0.01	99(±12)
22.	Mandipropamid	Amide	LC- MS/MS	0.005	0.01	85(±15)
23.	Metalaxyl	Acylamino acid funficides	LC- MS/MS	0.005	0.01	91(±8)
24.	Myclobutanil	Triazole	LC- MS/MS	0.005	0.01	95(±11)
25.	Profenophos	Organophosphates	GC- MS/MS	0.005	0.01	84(±6)
26.	Propiconazole	Triazole	LC- MS/MS	0.005	0.01	98(±7)
27.	Pyraclostrobin	Strobilurin	LC- MS/MS	0.005	0.01	94(±9)
28.	Spinosyn A	Macrocyclic lactone	LC- MS/MS	0.005	0.01	106(±4)
28 A.	Spinosyn D	Macrocyclic lactone	LC- MS/MS	0.005	0.01	97(±7)
29.	Tebuconazole	Triazole	LC- MS/MS	0.005	0.01	93(±12)
30.	Tetraconazole	Triazole	LC- MS/MS	0.005	0.01	87(±15)
31.	Thiacloprid	Nicotinoids	LC- MS/MS	0.005	0.01	79(±8)
32.	Thiamethoxam	Nicotinoids	LC- MS/MS	0.005	0.01	84(±11)
33.	Triadimefon	Triazole	LC- MS/MS	0.005	0.01	88(±9)
34.	Triadimenol	Triazole	LC- MS/MS	0.005	0.01	79(±10)
35.	Triazophos	Organophosphates	LC- MS/MS	0.005	0.01	98(±4)
36.	Trifloxystrobin	Strobilurin	LC- MS/MS	0.005	0.01	106(±7)
37.	Thiophanate Methyl	Benzimidazole	LC- MS/MS	0.005	0.01	70(±12)
38.	Iprovalicarb	Carbamates	LC- MS/MS	0.005	0.01	78(±9)
39.	Fipronil	Phenyl pyrazole	GC- MS/MS	0.005	0.005	74(±11)
40.	Acephate	Organophosphates	LC- MS/MS	0.005	0.01	68(±14)
41.	Hexythiazox	Thiazolidine	LC- MS/MS	0.005	0.01	71(±8)

their MRLs among the 48, 25, 3, 6, 2, 2, 2, 12, 16, 3, 1, 7, 2, 2 and 2 numbers of samples, respectively.

The total ionic chromatogram (TIC) of external standards and TIC of positive detected pesticides residue in one of grape sample, respectively, on LC MS/MS (Fig. 3 and 4). The presence of pesticide residues in fruits and vegetables has become a global phenomenon and some of authors have reported the residues of organochlorines (OC), organophosphates (OP) along with fungicide and herbicides in fruit and vegetables from India (Kumari *et al.*, 2002; Shahi *et al*., 2005 and Bhanti and Taneja, 2005) and other countries (Wang *et al.*, 2008 and Quintero *et al*., 2008). None of the grape samples had shown the presence of organochlorine (OC) residues, especially aldrin and DDT residues due to their banned or restricted use. The OP class of pesticides profenophos and chlorpyriphos were widely sprayed by farmers; the same has been confirmed from their spray schedule charts. The outcome of metabolism of profenophos (Dadson *et al.*, 2013), 4-bromo-2-chlorophenol have been detected by exceeding stringent EU MRL (0.01 mg/kg) in 58 samples. Chlorpyriphos detected in 76 samples and exceeds the MRL in two samples only. Abamectin (also called as Avermectin B1) belongs to class Avermectins (insecticidal and antihelmintic compounds derived from soil bacterium *Streptomyces avermitilis*), which was not mentioned in the farmers' spray schedule chart, but a lot of bio-products have been used during the grape cultivation and some these products have abamectin as an active ingredient. The farmers who used bio-products the abamectin residue detected and exceeded MRL in 24 samples. Triazoles class of pesticides hexaconazole and flusilazole fungicides used and detected in 12 and 60 numbers of samples and exceeded the MRL in 12 and 16 samples, respectively. Application of chlormequat chloride (CCC) as plant growth regulator observed in 323 samples and failed to meet the MRL in 6 samples. Nicotinoids class of pesticide thiacloprid residues detected in 14 samples and exceeded in 7 samples. Detection of high levels of pesticides residue in grape samples may be due to injudicious use of pesticides by the farmers without considering proper waiting period.

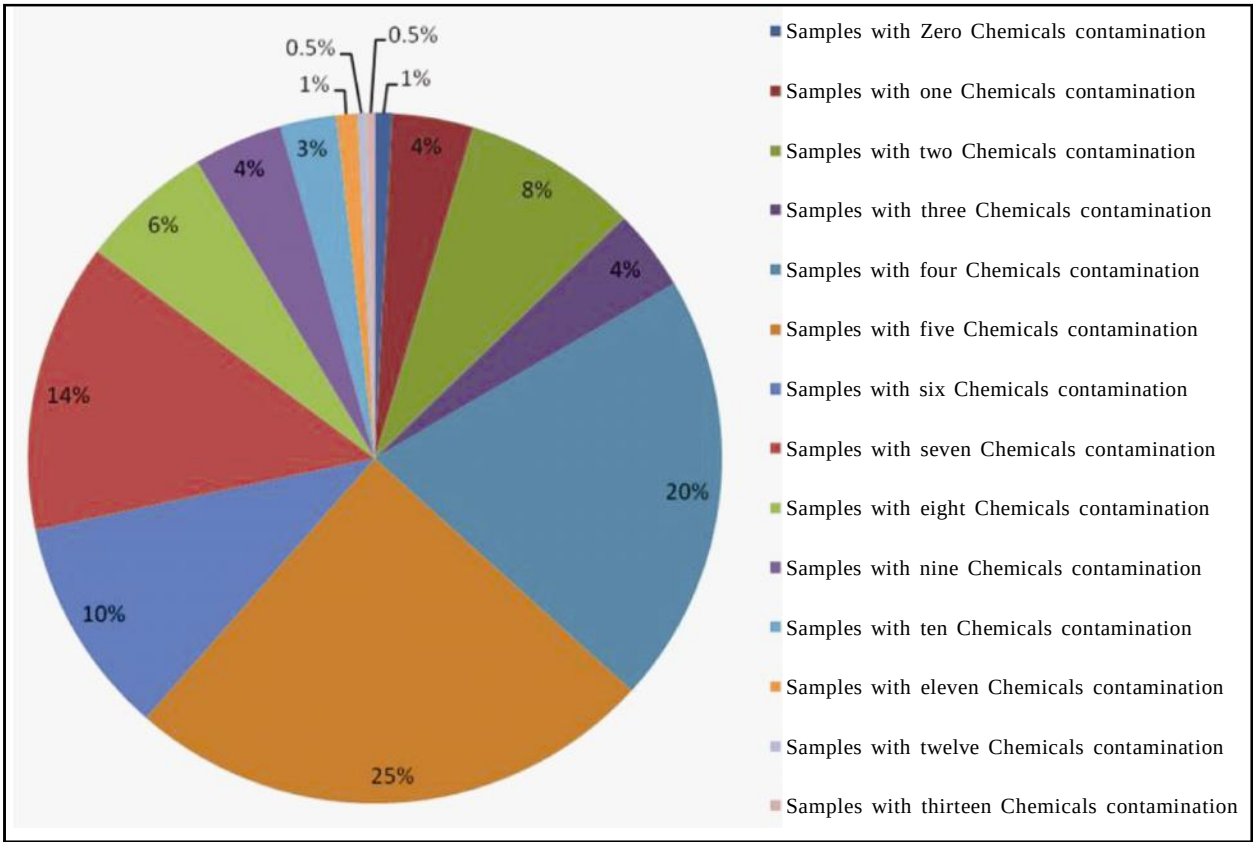

Fig. 2 : Pesticides residue contamination in fresh grape samples

Pesticides mainly OPs and OCs enters and accumulates in to the human body through the consumption of contaminated food commodities (meat, fish, milk, and milk products) and may produce toxic hazards.

A total 116 numbers of grape samples were failed by exceeding EU MRLs with 14 numbers of different classes of agro-chemicals. These may be due to non observance of certain recommended agricultural

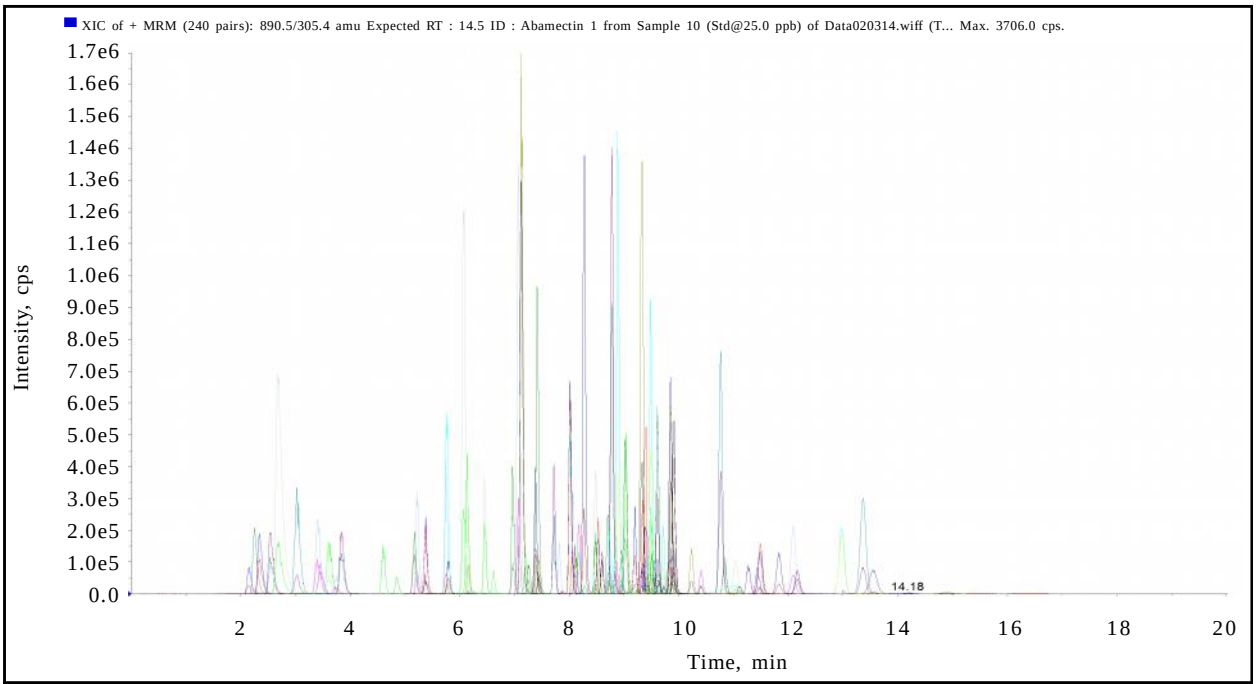

Fig. 3 : TIC of Standards of multi residue pesticides on LC MS/MS

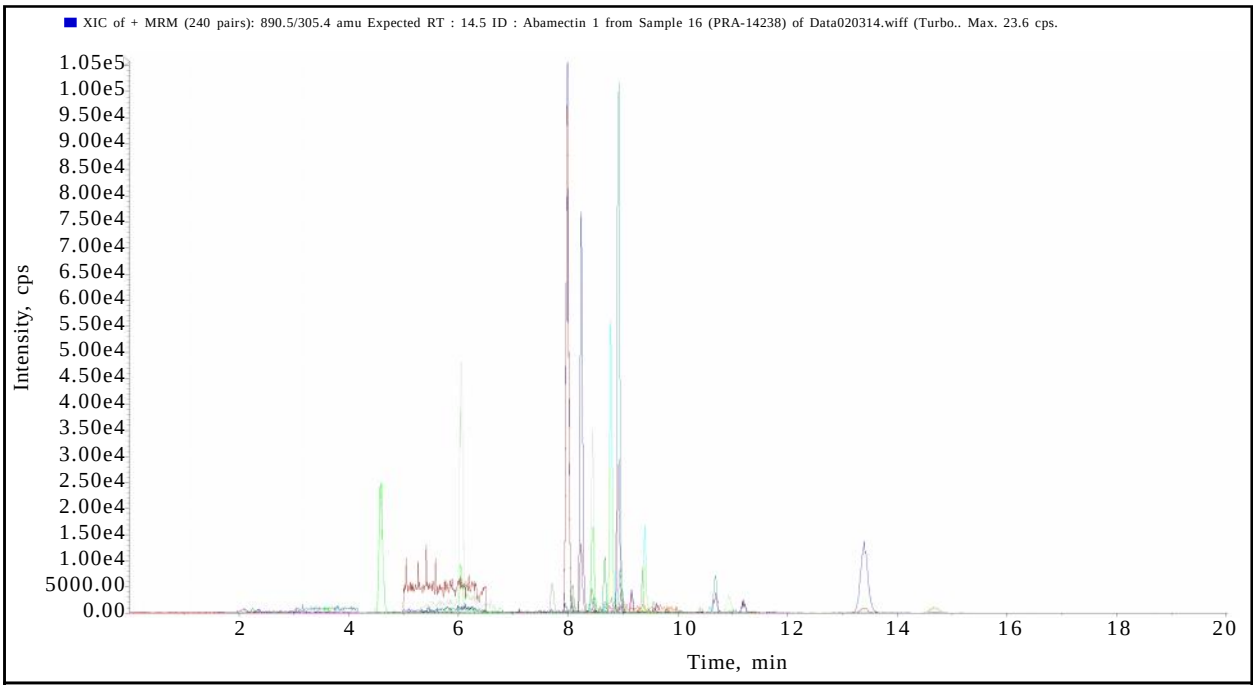

Fig. 4 : TIC of one of grape sample of multi residue pesticides on LC MS/MS

practices like respecting the safety interval between the last pesticide application and the harvest of the crop or higher dose of pesticides sprays than recommended or early crop harvesting by ignoring their Pre-Harvest Interval (PHI) as a result so many numbers of pesticides residue detected in grape samples. In order to avoid

Table 2 : Pesticide residue levels in grape samples of Nasik district, Maharashtra

Sr. No.	Name of Pesticides	Number of samples contaminated	Average residue level (mg/kg)	Minimum residue level detected (mg/kg)	Maximum residue level detected (mg/kg)	Number of samples Exceeds EU MRL	EU MRL (mg/kg)
1.	4- bromo-2-chlorophenol	58	0.022	0.010	0.038	58	0.01
2.	Acetamiprid	6	0.090	0.020	0.297	0	0.50
3.	Azoxystrobin	289	0.125	0.010	0.503	0	2.00
4.	Abamectin	24	0.019	0.010	0.052	24	0.01
5.	Buprofezin	122	0.137	0.010	0.903	0	1.00
6.	Carbendazim	83	0.099	0.010	0.565	3	0.30
7.	Chlormequat Chloride	323	0.180	0.010	0.119	6	0.05
8.	Chloropyriphos	76	0.107	0.010	0.625	2	0.50
9.	Clothianidin	34	0.338	0.010	0.121	0	0.70
10.	Difenoconazole	58	0.048	0.010	0.129	0	0.50
11.	Dimethomorph	58	0.067	0.010	0.426	0	3.00
12.	Dinocap	13	0.028	0.010	0.09	2	0.05
13.	Emamectin Benzoate	1	0.012	0.012	0.012	0	0.05
14.	Famoxadone	45	0.066	0.010	0.23	0	2.00
15.	Fenpyroximate	13	0.073	0.010	0.0254	0	0.30
16.	Forchlorfenuron	22	0.029	0.010	0.099	2	0.05
17.	Hexaconazole	12	0.037	0.011	0.067	12	0.01
18.	Flusilazole	60	0.047	0.010	0.142	16	0.05
19.	Imidacloprid	52	0.051	0.010	0.232	0	1.00
20.	Kresoxim methyl	83	0.078	0.010	0.455	0	1.00
21.	Lambda-cyhalothrin	75	0.051	0.010	0.115	0	0.20
22	Mandipropamid	126	0.148	0.010	0.751	0	2.00
23.	Metalaxyl	9	0.054	0.012	0.119	0	2.00
24.	Myclobutanil	312	0.043	0.010	0.823	0	1.00
25.	Profenophos	3	0.032	0.010	0.063	3	0.010
26.	Propiconazole	3	0.083	0.018	0.2	0	0.30
27.	Pyraclostrobin	72	0.060	0.010	0.121	0	1.00
28.	Spinosad A+D	90	0.083	0.011	0.763	1	0.50
29.	Tebuconazole	61	0.083	0.010	0.234	0	2.00
30.	Tetraconazole	257	0.058	0.010	0.278	0	0.50
31.	Thiacloprid	14	0.035	0.010	0.046	7	0.020
32.	Thiamethoxam	6	0.047	0.010	0.103	0	0.90
33.	Tridimefon	11	0.043	0.010	0.25	0	2.00
34.	Triadimenol	73	0.049	0.010	0.181	0	2.00
35.	Triazophos	2	0.007	0.014	0.018	2	0.010
36.	Trifloxystrobin	9	0.042	0.010	0.135	0	5.00
37.	Thiophanate Methyl	7	0.030	0.011	0.035	0	0.10
38.	Iprovalicarb	19	0.102	0.010	0.658	0	2.00
39.	Fipronil	2	0.012	0.005	0.019	2	0.005
40.	Acephate	2	0.010	0.010	0.011	2	0.010
41.	Hexythiazox	3	0.030	0.011	0.025	0	1.00

such occurrences, the producers should adhere to the recommended, authorized and correct ways of using pesticides to control insect-pests and diseases in their crops. These chemicals are toxic by nature, but when used on the appropriate and safe manner as specified on the labels, they should not be harmful to the users, consumers and environment.

Conclusion:

The present study indicates that existence of wide range of pesticides residue in grape samples and those concentration levels exceeding the EU MRLs. To avoid adverse effects of residues of pesticides on public health, it is a necessity to set up control measures so as to make sure that each pesticide should be below MRL in the grapes or other fruits and vegetables to be marketed. Regular evaluation of pesticides residue should be recommended for formulation of standards and quality control of pesticides.

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