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METHOD DEVELOPMENT AND VALIDATION OF RESIDUES OF PESTICIDES IN RICE BY USING LC – MS/MS

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ABSTRACT

A rapid and sensitive method was developed and validated for the determination of around 100 pesticides in rice samples by using HPLC coupled to a triple quadruple detector mass analyser (LC-MS/MS) MS - (Agilent 6400) and LC -(Agilent 1290 infinity 2). The sample preparation step was optimized. Pesticides were extracted from rice samples by using ammonium formation, methanol, formicacid, water. It is a quick easy, cheap, effective, rugged and safe method was used for rice samples. The analytical method was optimized with a total run time of 10minutes with a flow rate of 0.800ml/min. By using Inertsil Column ODS-3V, 150×4.6mm, 5microns. The performance of the method was also evaluated in terms of linearity precision and accuracy and then it was applied to eight commercial rice samples from different suppliers. The results demonstrated the ability of the method to detect all the 20 pesticides with linearity, precision, and accuracy according to the protocols established by the most important organizations and validation guidelines.

KEYWORDS: Tuning, LC-MS, Pesticides, Hexacanazole, Carbendazim, Clothiandin.

INTRODUCTION

Pesticides are agents meant for attracting, seducing, and then destroying any pest. They are a class of biocide These pests include insects, rodents, fungi, weeds, nematodes, algae, etc. In addition to their benefits in agriculture, pesticides are also used to protect public health in controlling the vectors of tropical diseases, such as mosquitoes. But pesticides are also potentially toxic to humans. They may induce adverse health effects comprising cancer, effects on reproduction, immune or nervous systems. Before they can be authorized for use pesticides should be tested for all possible health effects and the results should be analyzed by experts to assess any risks to humans.

MATERIALS AND METHODS

The project work entitled Method development and validation of pesticides in rice by lc-ms/ms was carried out in first source laboratory solution, Hyderabad. The department is equipped with modern sophisticated facilities required for analysis of residues of pesticides in food samples. A new LC-MS/MS method was developed for the determination of residues of pesticides in rice. The LC-MS/MS method was then validated according to ISO17025, FDA and NABL guidelines to indicate that the analytical procedure used is suitable for intended use by using various parameters like selectivity, linearity, precision, accuracy, and system suitability.

Instruments and Specifications

Table 5.1: Instruments Used During Method Development.

S. No.	Name	Make
1	HPLC	Agilent1290 infinity 2
2	Mass Spectrometer	Agilent 6400
3	Micro Pipette-100-1000µL	Lambda Plus
4	Micro Pipette-20-200µL	Lambda Plus
5	Centrifuge	Thermo scientific
6	Ultra Sonicator	Sonica
7	Refrigerator(2-8ºC)	Thermo Fisher Scientific
8	Dual cooling freezer	Thermo lab
9	Weighing Balance	RADWAG (XA .220.3Y)
10	Micro Weighing Balance	Radwag (MYA. 11.3Y)
11	Water Purifier	Milli-Q
12	Vortex	IKA Vortex (VG-3)
13	Multi tube vortexer	Heldolph
14	Evaporator	Biotage

Chemical Specifications

Table 5.2: Chemicals Used During Method Development.

S. No.	Reagents / Materials	Make	Grade	Lot no/batch no
1	Methanol	Honey Well	HPLC	DQ840
2	Acetonitrile	Honey Well	HPLC	WXBC3394V
3	Ammonium formate	Sigma Aldrich	AR	9596266
4	Water	Merck	HPLC	
5	Formic acid	Merck	HPLC	20353053
6	Magnesium sulphate	Sisico	AR	9899075
7	Tri sodium citrate	Merck	AR	QG5Q651372
8	Sodium chloride	Merck	AR	K43957204
9	Sodium hydrogen citrate (Sesquihydrate)	Sigma Aldrich	AR	MKBT2506V

Collection of Reference Standard

Certified reference standards: During method validation, a blank matrix will be spiked with the analyte of interest using solutions of reference standard. It is important that the quality of the reference standard is ensured, as the quality (purity) may affect the outcome of the analysis, and therefore the outcome of the study data. Therefore the reference standards used in this analytical validation and analysis should be obtained from an authentic and traceable source. Suitable reference standards, include certified standards such as compendial standards (sigma Aldrich, Dr.ehrenstorfer), commercial available standards. Suitability of the reference standard should be scientifically justified.They should be stored in frost free refrigerator below -10^{o}c in order to prevent undesirable reactions, such as oxidation, hydrolysis.

Table 5.3: List of certified reference standards.

S. No.	Reference standards	Batch No/ Lot. No.	Date of expiry	Storage conditions	Purity
1.	Chlorpyriphos	BCBR6591V	Jan 2021	2-8°c	98.0%
2	Imidacloprid	21030	20/10/2018	20±4°c	99.5%
3	Metalaxyl	SZBD238XV	August-18	20±4°c	99.8%
4	Methamidophos	SZBE202XV	21/07/2017	-20±4°c	99.2%
5	Tricyclazole	21010	`13/12/2016	20±4°c	99.0%
6	Carbendazim	SZBD338XV	DEC-18	20±4°c	99.2%
7	Chlothianidin	30823	AUGUST-17	20±4°c	99.0%
8	Isoprothiolane	31202	FEB-18	4±4°c	97.2%
9	Propiconazole	10607	JUL-17	20±4°c	99.0%
10	Tebuconazole	SZBE029XV	29/01/2019	20±4°c	99.3%
11	Hexaconazole	40613	JUL-18	20±4°c	98.0%

Collection of Samples

Different varieties of rice samples were collected from different places. The rice samples were homogenized, extracted and analyzed as per the sample preparation procedure. On the basis of analysis the following compounds were detected and reported in ppb levels.

Table 5.4: Source of rice samples.

S. No.	Rice Sample Collected From
1.	Tenali
2.	Guntur
3.	Hyderabad
4.	Vijayanagaram
5.	Ongole
6.	Eluru
7.	Kurnool
8.	East Godavari

Method Employed for Sample Preparation

Quechers Method (Quick, Easy, Cheap, Effective, Rugged and Safe)

Extraction

- Homogenized the 250gm of sample and weigh 10g of homogenized sample. Extract the sample in a 50 ml polypropylene centrifuge tube.
- Added 10 ml of water and 10 ml of ACN and vortex for 10 min.

Purification and separation

Add 4gm of magnesium sulphate, 1gm of sodium chloride, 0.5gm of tri sodium citrate dehydrate and 0.5gm of sesquhydrate and vortex for 10 min .Centrifuge at 4000 rpm for 5 minutes.

Evaporation and reconstitution

- Drawn 2mL supernatant in the test tube. Evaporated it to dryness under nitrogen at 35°C.
- Reconstituted with 2 ml of mobile phase-B. Sonicate for 1 min and vortexes for 30 seconds.
- Centrifuged the extract at 4000 rpm for 5 min.
- Inject 1ml of the extract into the LC-MS/MS.

Experimental Work

Tuning

Tuning was carried out in both positive and negative transitions to set Q1 mass and Q3 mass. Tuning of the compounds is done in four steps. They are

1. Full length scanning of the pesticide
2. Product ion scanning.
3. Conformation of the precursor ion scanning.
4. MRM ions scanning.

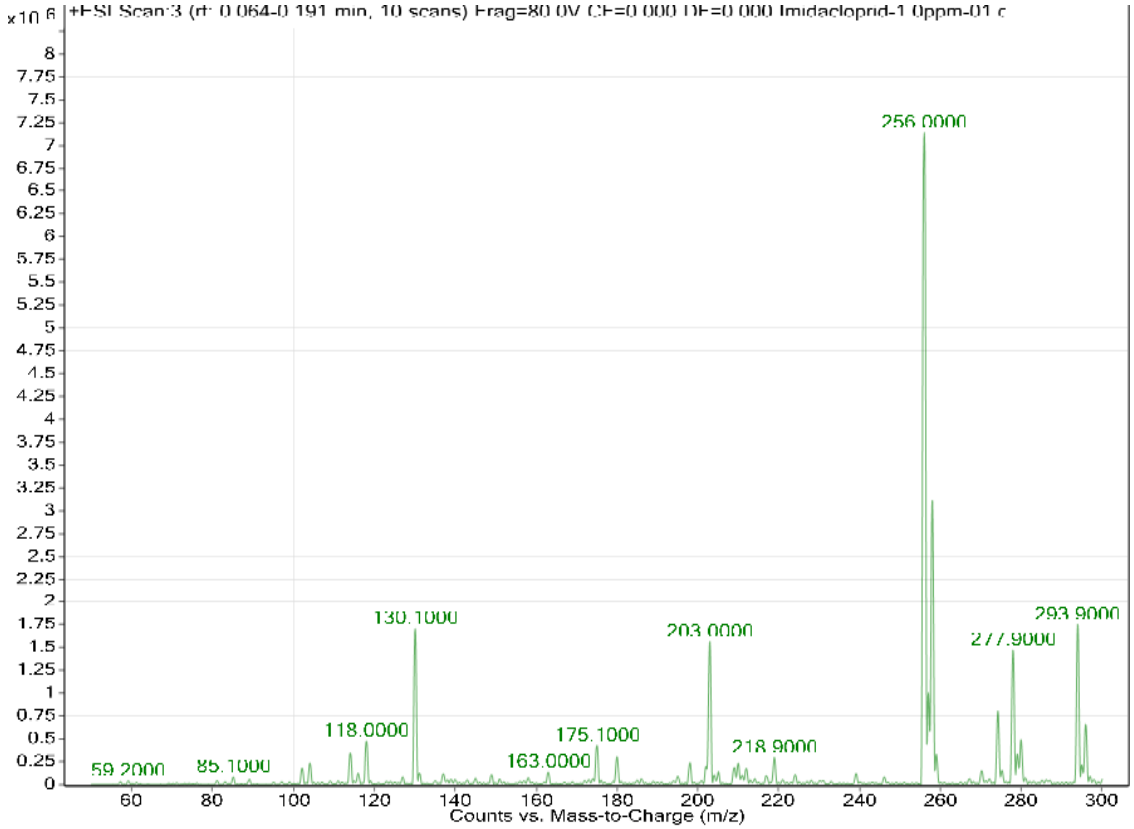


Fig. 6.1: Full Scanning of Imidacloprid.

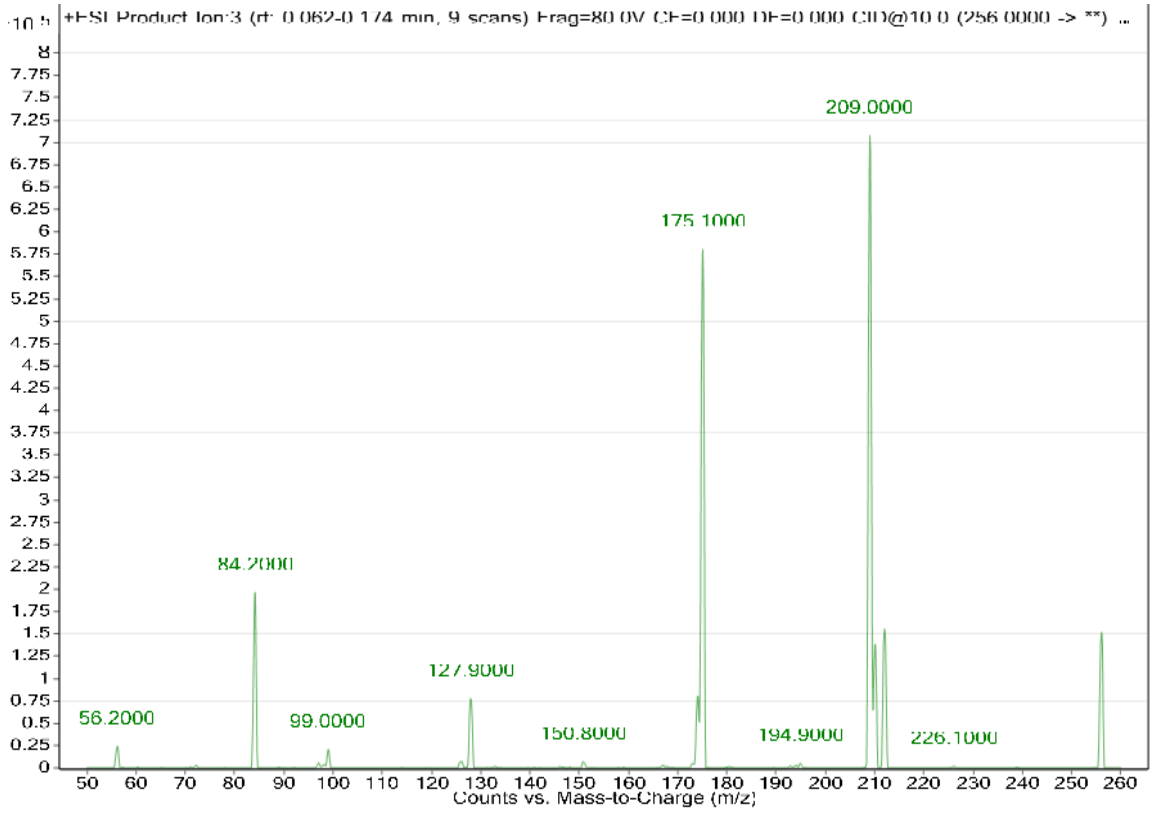


Fig. 6.2: Product Ion Scanning of Imidacloprid.

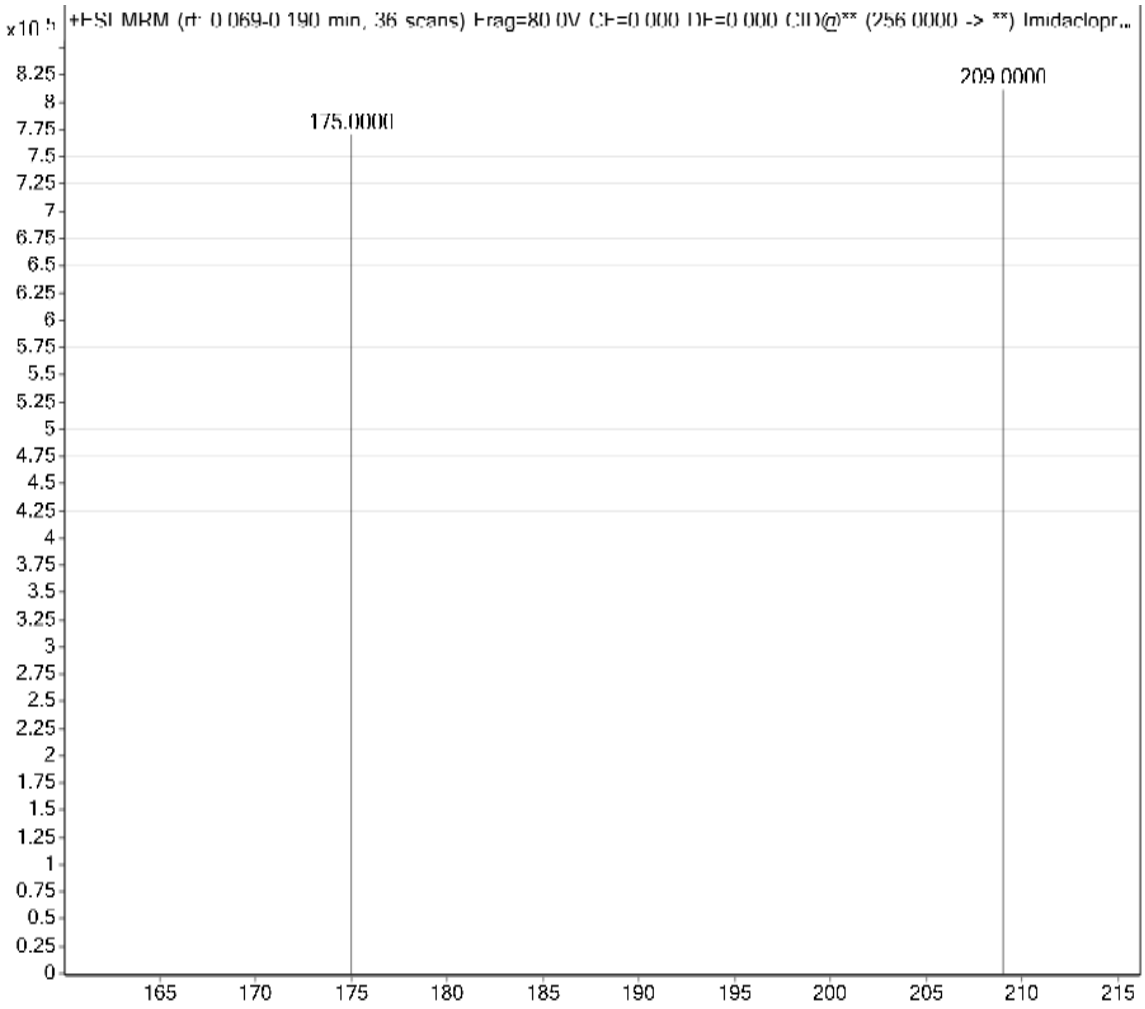


Fig. 6.3: MRM Ions of Imidacloprid.

In this way scanning is done for all the following 11 pesticides

1. Chloropyriphos
2. Metalaxyl
3. Methamidophos
4. Tricyclazole
5. Carbendazim
6. Chlothianidin
7. Isoprothiolane
8. Propiconazole
9. Tebuconazole
10. Hexaconazole.
11. Imidacloprid

MRM Parameters (Multi Residual Monitoring)

From tuning of the above pesticides yields to precursor ion, product ion, collision energies, fragmentation energies, retention time & polarity**.** The responses and energies of the pesticides are tabulated below.

Table 6.1: MRM ions of pesticides.

Compound name	Precursor ion ms1 response	Product ion Ms2 response	Frag (v)	CE (v)	Ret time	Polarity
Methamidophos	141.8	124.9	62	10	3.43	Positive
		93.9		14		
Imidacloprid	256	209	73	10	5.3	Positive
		175		10		
Metalaxyl	280.2	220	78	9	10.18	Positive
		160		21		
Chloropyriphos	350	198	65	10	15.02	Positive
		96.8		38		
Tricyclazole	190	163	120	25	7.7	Positive
		136		30		
Carbendazim	192.2	159.9	93	18	4.63	Positive
		132		30		
Chlothianidin	250.1	169	52	6	5.56	Positive
		131.9		10		
Isoprothiolane	291.1	231	80	8	11.43	Positive
		188.8		20		
Propiconazole	342.1	158.9	111	33	12.59	Positive
		69.1		17		
Tebuconazole	308	125	106	40	12.36	Positive
		70		21		
Hexaconazole	314.1	158.9	99	34	12.72	Positive
		70		18		

RESULTS AND DISCUSSION

Batch Analysis

Batch analysis was performed by collecting rice samples from different places and they are analyzed by using the **Quechers** method and the results are tabulated below. The results are expressed in ppb level i.e.; (µg/kg)**.**

Table 7.1: Batch analysis of the rice samples.

Compound Name	Sample-1 Tenali Rice	Sample-2 Guntur Rice	Sample-3 Kurnool Rice	Sample-4 Hyderabad Rice	Sample-5 Ongole Rice	Sample-6 East. Godavari Rice	Sample-7 Vijayanagaram Rice
Carbendazim	BLQ	5	68	10	BLQ	6	BLQ
Chlorpyriphos	11	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Chlothianidin	BLQ	3	17	4	BLQ	16	BLQ
Hexaconazole	BLQ	BLQ	BLQ	BLQ	6	BLQ	BLQ
Imidacloprid	67	BLQ	BLQ	BLQ	7	BLQ	BLQ
Isoprothiolane	BLQ	BLQ	78	BLQ	3	175	BLQ
Metalaxyl	16	46	16	15	31	52	28
Methamidophos	5	BLQ	3	BLQ	BLQ	BLQ	BLQ
Propiconazole	BLQ	BLQ	18	2	8	BLQ	13
Tebuconazole	BLQ	15	BLQ	3	BLQ	BLQ	BLQ
Tricyclazole	41	8	25	46	17	BLQ	BLQ

Maximum residual limits (MRL) for following pesticides

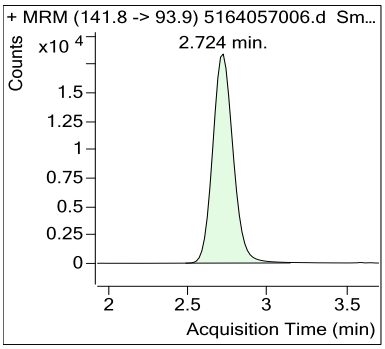
Food Safety and Standards Act, 2006, the Food Safety and Standards Authority of India proposes to make Food Safety and Standards Regulations in so far as they relates to Food Safety and Standards (Contaminants, Toxins and Residues) Regulations, 2011.

FSSAI (Food safety and standard authority of India) has established the maximum residual limits for different pesticides in rice. The following are the tolerance limits for the pesticides that are detected in the rice samples.

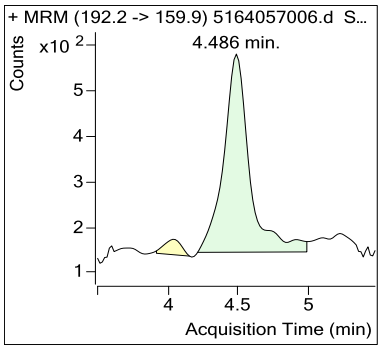
Table 7.2: MRL of pesticides.

S. No.	Compounds	Food	Tolerance Limit (µg/Kg, ppb)
1	Carbendazim	Rice	500
2	Chlorpyriphos	Rice	50
3	Chlothianidin	Rice	10
4	Hexaconazole	Rice	20
5	Imidacloprid	Rice	50
6	Isoprothiolane	Rice	100
7	Metalaxyl	Rice	50
8	Methamidophos	Rice	100
9	Propiconazole	Rice	50
10	Tebuconazole	Rice	50
11	Tricyclazole	Rice	20

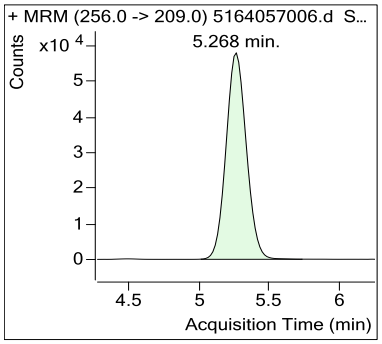
Batch Analysis Chromatograms



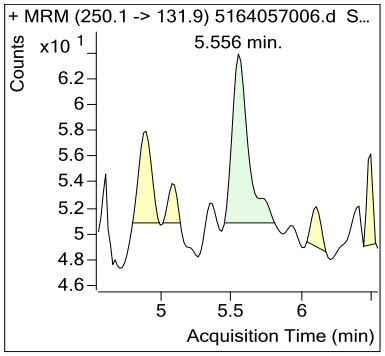
Methamidophos



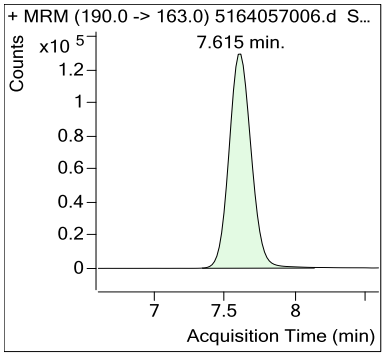
Carbendazim



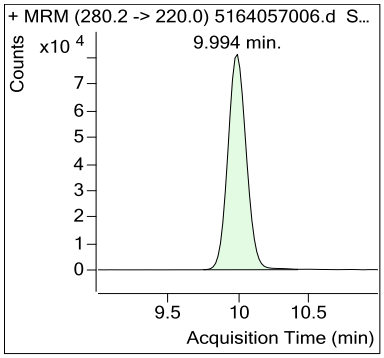
Imidacloprid



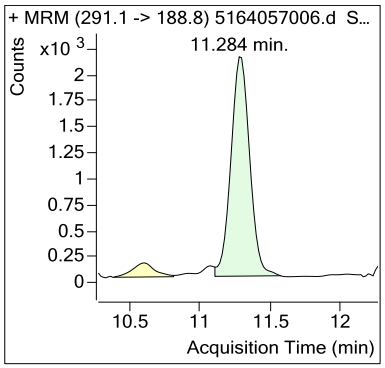
Clothianidin



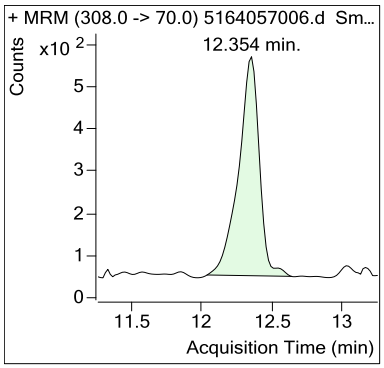
Tricyclazole

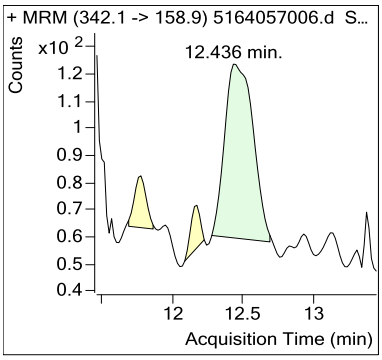


Metalaxyl



Isoprothiolane





Tebuconazole

Linearity

Linearity was established using matrix-matched calibration curves. Five calibration curves were prepared at levels of Matrix blank, 10ppb, 20ppb, 50ppb, 75ppb and 100ppb, in rice.

These curves were prepared on two different days and plot a graph through software against the concentration of the each Analyte and its response.

Acceptance criteria

The Correlation Coefficient (R^2) ≥ 0.99.

Table 7.5: Linearity.

Compound Name	R^2
Carbendazim	0.995
Chlorpyriphos	0.995
Chlothianidin	0.995
Hexaconazole	0.996
Imidacloprid	0.995
Isoprothiolane	0.994
Metalaxyl	0.995
Methamidophos	0.996
Propiconazole	0.995
Tebuconazole	0.995
Tricyclazole	0.995

Precision

The precision of the analytical method explains the closeness of repeated individual measures of analyte. Precision is expressed as the coefficient of variation (CV). The statistical method for estimation of the precision should be predefined and calculated according standard practice. Method precision was performed by injecting six spiked samples at 10 ppb, 50 ppb and 100 ppb.

Acceptance criteria: % RSD of area ≤20% and % Accuracy (Recovery) was injecting the spiked sample at 10ppb, 50ppb and 100ppb levels. Acceptance criteria in between 70-120%.

Formula: Precision=standard deviation /mean volume* 100.

Table 7.6: Precision At 10PPB.

Compound Name	RUN-01	RUN-02	RUN-03	RUN-04	RUN-05	RUN-06	Mean	Stdev	%CV
Carbendazim	591844	586020	581571	579314	565480	581707	580989.33	8801.49	1.51
Chlorpyriphos	23044	21451	21998	21264	20174	22195	21687.67	971.60	4.48
Chlothianidin	59982	59431	58041	57761	58415	59087	58786.17	857.94	1.46
Hexaconazole	99909	96609	94246	90887	96632	92926	95201.50	3190.41	3.35
Imidacloprid	95119	92807	93345	91868	91988	92508	92939.17	1197.65	1.29
Isoprothiolane	496157	486053	485460	474503	482158	491610	485990.17	7508.30	1.54
Metalaxyl	464929	459826	446516	446697	448685	454350	453500.50	7610.57	1.68
Methamidophos	282943	280021	277480	274271	275382	278172	278044.83	3148.49	1.13
Propiconazole	71604	66943	64539	65112	70945	69942	68180.83	3055.27	4.48
Tebuconazole	152593	150208	144691	143111	144451	153128	148030.33	4465.62	3.02
Tricyclazole	360292	355703	351618	350418	348459	357223	353952.17	4518.20	1.28

Table 7.7: Precisionat 50PPB.

Compound Name	RUN-01	RUN-02	RUN-03	RUN-04	RUN-05	RUN-06	Mean	Stdev	%CV
Carbendazim	2396619	2418757	2429211	2528312	2417923	2550298	2456853.33	65111.32	2.65
Chlorpyriphos	85784	83988	82052	85264	82373	90320	84963.50	3019.76	3.55
Chlothianidin	235712	234698	237645	244150	233862	240754	237803.50	3963.90	1.67
Hexaconazole	374344	395844	391490	404564	381714	401540	391582.67	11665.28	2.98
Imidacloprid	374062	379049	373695	389300	373691	390305	380017.00	7853.69	2.07
Isoprothiolane	2006474	2014629	2000639	2080032	1957414	2119729	2029819.50	59118.25	2.91
Metalaxyl	1836893	1829937	1829095	1881759	1812629	1909314	1849937.83	37241.62	2.01
Methamidophos	1113766	1129767	1128919	1183385	1142582	1199490	1149651.50	34014.12	2.96
Propiconazole	286025	279940	288350	293253	278899	299885	287725.33	8002.62	2.78
Tebuconazole	598980	590098	596581	617616	600710	623028	604502.17	12886.26	2.13
Tricyclazole	1482462	1474960	1489620	1549333	1464419	1582830	1507270.67	47509.40	3.15

Table 7.8: Precision AT 100PPB.

Compound Name	RUN-01	RUN-02	RUN-03	RUN-04	RUN-05	RUN-06	Mean	Stdev	%CV
Carbendazim	5484904	5781523	5267666	5225827	5202704	5156901	5353254.17	238968.12	4.46
Chlorpyriphos	180190	193131	184824	182175	178704	173403	182071.17	6632.08	3.64
Chlothianidin	507230	527025	493020	488098	479994	473462	494804.83	19573.29	3.96
Hexaconazole	820789	854943	779287	761997	765288	752811	789185.83	40135.32	5.09
Imidacloprid	805678	844898	779595	784049	779270	768764	793709.00	27881.28	3.51
Isoprothiolane	4353330	4597122	4211574	4255758	4170830	4120202	4284802.67	172353.44	4.02
Metalaxyl	4015986	4236276	3858113	3825501	3840005	3817557	3932239.67	166131.75	4.22
Methamidophos	2484743	2640776	2406301	2409422	2394156	2397640	2455506.33	96785.27	3.94
Propiconazole	591749	639055	568638	565411	564448	568174	582912.50	29319.92	5.03
Tebuconazole	1221989	1276626	1172612	1169157	1182934	1165075	1198065.50	43692.11	3.65
Tricyclazole	3358960	3532984	3248539	3224846	3202209	3169510	3289508.00	135635.11	4.12

DISCUSSION

On the basis of batch analysis, I conclude that some pesticides are beyond the limit in rice collected from different places. In Kurnool and Eluru, clothianidin, tricyclazole & isoprothiolane pesticides were detected and they are beyond the limit. In Tenali and Hyderabad, imidacloprid and tricyclazole pesticides were detected and they are beyond the limit. In Guntur, ongole, vijayanagaram areas pesticides usage is below the limit so no toxic effects were seen with that rice.

Linearity was performed and the R^2 values of the calibration curve are within the acceptance criteria.7.

Precision and accuracy were performed and the values of %RSD of area and recoveries are within the acceptance criteria.

CONCLUSION

The method was developed and validated for the estimation of residues of pesticides in rice by lc-ms/ms. Rice samples were collected from different places and were analyzed. On the basis of analysis I conclude that some pesticides are beyond the limit in rice collected from different places.

In Kurnool and Eluru, clothianidin, tricyclazole&isoprothiolane pesticides were detected and they are beyond the limit. In Tenali and Hyderabad, imidacloprid and tricyclazole pesticides were detected and they are beyond the limit. In Guntur, ongole, vijayanagaram areas pesticides usage is below the limit so no toxic effects were seen with that rice.

Not only in rice, are Pesticides used in different food commodities to control the pests, insects, rodents, fungi, weeds, nematodes, algae etc.

Pesticides in rice yields to severe acute and long term health effects like abdominal pain, dizziness, headaches, nausea, vomiting, skinproblems, eyeproblems, cancer, leukemia, lymphoma, brain, kidney, breast, prostate, pancreas, liver, lung, and skin cancers.

Pesticide exposure leads to birth defects, fetal and altered fetal growth, and reduced fertility in males, dementia, and neurological problems. Not only health problems, it effects the environment also l air, soil, water, marine organisms like fishes and fish eating birds.

When we consume the rice that contains more amount of pesticide it leads to acute and chronic health problems. So pesticides usage should be reduced.

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