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Analytical and Bio-Analytical Method Development and Validation of Dichlorvos Pesticide Using RP-HPLC Method

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ABSTRACT

Organophosphorus compounds were synthesised in the 1800s. Later they are used as insecticides in the late 1930s and early 1940s. The German scientist Gerhard Schrader is known for the creation of the basic chemical structure of anticholinesterase organophosphate compounds and development of the first commercialised Organophosperous insecticide. Such chemicals are anticholinesterase insecticide commonly used in agriculture and horticulture. To a lesser extent, they are used for domestic use. Due to the absence of bio persistence in organophosphates, most of the western countries opted to substitute organochlorines with organophosphates. Organophosphate pesticides are commonly used around the world, and contamination by these compounds is a serious public health concern in developing countries. Toxicokinetics and toxicodynamics of OP poisoning not only differ with path or level of exposure. But also the agent's chemical composition. Organophosphates are a group of pesticide that was developed in the 1940s in Germany and soon became an effective defence against agricultural pests. Dichlorvos which is a commonly used group of pesticide is a broad-spectrum organophosphate compound having insecticidal activity. Dichlorvos is a cholinesterase inhibitor exhibiting stomach, contact and systemic mode of action. Therefore, an accurate, fast, cost-effective and straightforward RP-HPLC technique for detecting Dichlorvos was developed. The RP-HPLC method is established by using ACN and Millipore water 50:50 v/v as mobile phase, the Flow rate is maintained at 1.5mL/minute. Detection of Dichlorvos was performed by using a PDA detector at 200nm. By this RP-HPLC procedure, RT of Dichlorvos was identified at 2.9 min.

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INTRODUCTION

Organophosphorus compounds are synthesised in the 1800s, and later they are used as insecticides in the late 1930s and early 1940s (Costa, 2006). The German scientist Gerhard Schrader is known for the creation of the basic chemical structure of anticholinesterase organophosphate compounds and development of the first commercialised Organophosperous insecticide (Costa, 2006). Such chemicals are anticholinesterase insecticide commonly used in agriculture and horticulture (Kwong, 2002). To a lesser extent, they are used for domestic use. Due to the absence of

bio persistence in organophosphates, most of the western countries opted to substitute organochlorines with organophosphates (Rusyniak and Nañagas, 2004). Organophosphate pesticides are commonly used around the world, and contamination by these compounds is a serious public health concern in developing countries (Sukirtha and Usharani, 2013). Toxicokinetics and toxicodynamics of OP poisoning not only differ with path or level of exposure. But also, the agent's chemical composition (Kwong, 2002).

The toxicity mechanism of organophosphates is by suppression of acetylcholinesterase, which results in building up of acetylcholine neurotransmitter and the continues activation of acetylcholine receptors (Jones *et al.*, 1992; Blair *et al.*, 1976; Inoue *et al.*, 2007). The recommended treatment comprises of reactivating blocked acetylcholinesterase with an oxime antidote and suppressing acetylcholine's action on the receptor with atropine (Costa, 2006; Rusyniak and Nañagas, 2004). A patient who received an appropriate diagnosis recover from acute toxicity. Dichlorvos is the active component of many insecticidal formulations (Jones *et al.*, 1992; Blair *et al.*, 1976).

The toxicity of Dichlorvos was reported by FAO/WHO at a joint meeting on pesticide residues (1965, 1967, 1968 and 1970) and a permissible daily intake of 0.004 mg /kg was suggested (Blair *et al.*, 1976).

High concentration exposure over the short-term also up to 50 days of exposure on monkeys and rat to 0.1-0.5mg. The only effect seen was a depression of cholinesterase. Dichlorvos is a cholinesterase inhibitor exhibiting stomach, contact and systemic mode of action (Sharma *et al.*, 1990).

Dichlorvos is a broad-spectrum organophosphate compound having insecticidal activity. In the early days, most organophosperous insecticides were also dangerous to mammals, including humans, i.e. they were not selectively toxic to insects. Chromatographic techniques are commonly used for the chemical isolation, detection and quantification of as many pesticides.

The HPLC, GC or TLC methods were used to determine Dichlorvos, which are having some advantages and disadvantages (Cho *et al.*, 1997; Parrilla *et al.*, 1994). To increase food production, pesticide applications have become vital. India is the world's fifth pesticide consumer. The proposed RP-HPLC method for the detection of Dichlorvos has a short retention time compared to other HPLC methods. Dichlorvos chemical structure is shown in Figure 1.

MATERIALS AND METHODS

Instrumentation and Chromatographic conditions

For the current study, high- pressure liquid chromatography (HPLC) LC-20AD with PDA detector is used. The separation was attained by using a Phenomenex Luna C18 column (250 mm X 4.60 mm 5μ). The run time was set to 10min. Acetonitrile (ACN) and Millipore water (50:50 v / v) at a flow rate of 1.5 ml/min are used as the mobile phase. The temperature of the column was set at 40°C. The wavelength of detection was set at 200 nm. PHENEX PTFE0.02μm syringe sensor is used for filtration purposes.

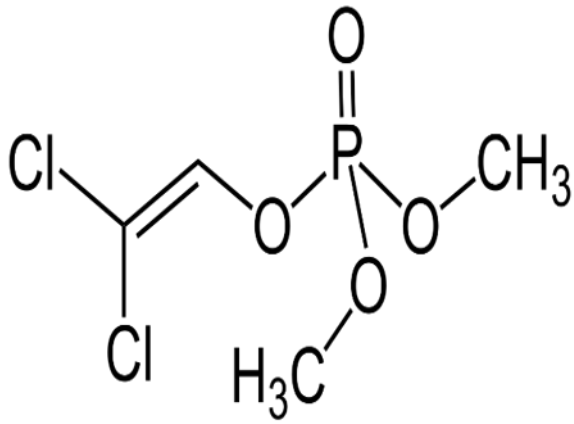

Figure 1: Chemical structure of Dichlorvos

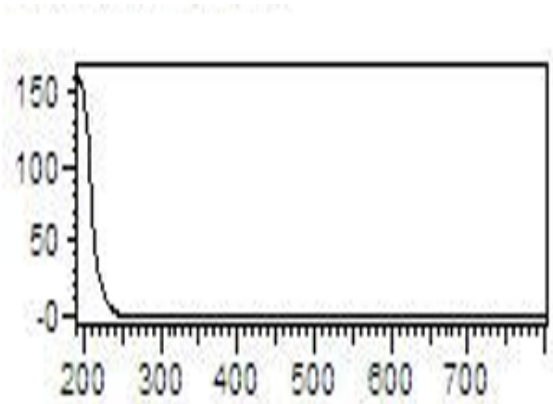

Figure 2: Uv spectrum

Chemicals and reagents

Dichlorvos standard was procured from Sigma Aldrich, Bengaluru. Action-3, which is a Dichlorvos marketed formulation manufactured by Jayakrishna pesticides private limited., was procured from

Table 1: Optimized Chromatographic conditions

Column	Phenomenex luna C18 column (250 mm X 4.60 mm 5μ)
Wavelength	200nm
Flow rate	1.5ml/min
Detector	PDA
Injection volume	10μl
Mobile phase	ACN and Millipore water 50:50 (v/v)
Retention time	2.9 min

Table 2: System suitability results

Parameters	Acceptance criteria	Results
Tailing factor	NMT 2.0	1.270
Theoretical plates	NLT 2000.0	8652.304

Table 3: Concentration andpeak area for calibration curve

Concentration	Area
10	549627
20	937318
30	1271496
40	1670206
50	2147712

Table 4: Method precision intraday studies

Concentration	Peak area	Concentration	Peak area	Concentration	Peak area
10	545384	30	1237318	50	2170206
10	546254	30	1229528	50	2194037
10	541658	30	1241167	50	2138502
10	536916	30	1206536	50	2097184
10	534522	30	1283667	50	2198853
10	544521	30	1231128	50	2095633
average	541542.5		1238224		2149069
STD deviation	4407.041		23110.79		42044.54
%RSD	0.813794		1.866447		1.956407

Acceptance criteria: The RSD calculated on 8 determinations must be $\leq$ 2.0%

Table 5: Method precision interday studies

Concentration	Peak area	Concentration	Peak area	Concentration	Peak area
10	535624	30	1218965	50	2193468
10	546985	30	1204563	50	2185524
10	546685	30	1208265	50	2099835
10	539863	30	1218167	50	2122558
10	548467	30	1205837	50	2192538
10	546997	30	1218462	50	2184935
average	544103.5		1212377		2163143
Std deviation	4695.094		6254.223		37448.89
%Rsd	0.862904		0.515865		1.731226

Acceptance criteria: The RSD calculated must be $\leq$ 2.0%.

Table 6: Accuracy

Level of recovery	Amount of formulation	Amount of Pure drug	The total amount of drug	Peak area	Difference	% Recovery	Mean
50	20	10	30	36085012	35535385	98.47686	
50	20	10	30	36084635	35535008	98.47684	98.47684
50	20	10	30	36081465	35531838	98.47671	
100	20	20	40	78090259	77152941	98.7997	
100	20	20	40	71885632	70948314	98.6961	98.6961
100	20	20	40	71852784	70915466	98.6955	
150	20	30	50	82284526	81013030	98.45476	
150	20	30	50	82276485	81004989	98.45461	98.45461
150	20	30	50	82276352	81004856	98.4546	

Acceptance criteria: Mean % recovery and individual at each level should be between 102.0% and 98.0%
% Recovery= (Amount of drug recovered/ Amount of drug added)*100

Table 7: LOD and LOQ

Average of SD			
101937			
102339	102339	LOD	0.046μg/ml
102659			
Average of slope			
12302			
12301	12301	LOQ	0.139μg/ml
12377			

Table 8: Robustness

Parameters	Change in units	Acceptance criteria	Results
Wavelength	205 $\pm$3	%RSD $\leq$ 2	1.329
Flow rate	1ml/min $\pm$0.1	%RSD $\leq$ 2	1.256
Column temperature	40°c $\pm$5°c	%RSD $\leq$ 2	0.546
Mobile phase ratio	Acetonitrile: orthophosporic acid 0.1% 50:50 (v/v) $\pm$2	%RSD $\leq$ 2	0.217

Table 9: Ruggedness

Concentration	Trial 1	Trial 2	Mean	SD	%RSD
by changing the analyst					
0	0	0	0	0	0
10	542356	5354867	2948612	3402959	0.925648
20	931234	931025	931129.5	147.7853	0.765426
30	1215482	12256718	6736100	7807333	0.896253
40	1610250	16203652	8906951	10319094	0.925625
By changing the instrument					
0	0	0	0	0	0
10	539524	545264	542394	4058.793	0.748311
20	935246	945698	940472	7390.68	0.785848
30	12854691	1226524	7040608	8222356	0.862546
40	1621534	1660214	1640874	27350.89	0.965246

Table 10: Calibration data of Dichlorvos

Sl.no	Conc. (μg/ml)	Peak area
1	0	0
2	10	312932
3	20	615335
4	30	907831
5	40	1095066
6	50	1513468

Table 11: Results showing precision for Dichlorvos (within run)

Concentration	Mean peak area	Mean concentration	SD	%CV
10 (LLOQ)	305015.4	9.9026	0.8183	8.2643
20 (LQC)	585185.0	19.962	0.8725	4.3708
30 (MQC)	861875.4	29.897	1.0632	3.5563
40(HQC)	1136209.2	39.748	1.9101	4.8055

Table 12: Results showing precision for Dichlorvos (between run)

Concentration	Mean peak area	Mean concentration	SD	%CV
10 (LLOQ)	307135.8	9.97877	0.7763	7.7795
20 (LQC)	581728.6	19.8809	0.8446	4.2483
30 (MQC)	862136.0	29.9069	1.0649	3.5609
40(HQC)	1134797.2	39.6973	1.8912	4.7640

Table 13: Results showing recovery for Dichlorvos

Standards	Concentration	Analytical Peak Area	Bioanalytical Peak area	% Recovery
LLOQ	10	549627	312932	56.93533978
LQC	20	937318	615335	65.64847789
MQC	30	1271496	907831	71.39865167
HQC	40	1670206	1095066	65.56472675
UQC	50	2147712	1513468	70.46885243

Table 14: Result showing stability for Dichlorvos

Stability	Standards	Concentration μg/mL	Mean recovered concentration μg/mL)	SD	%CV
Bench-top	LQC	20	19.9169	0.5020	2.5205
	HQC	40	39.2690	0.7317	1.8633
Freeze and	LQC	20	19.9370	0.2522	1.2650
thaw	HQC	40	39.7585	0.7294	1.8347
Long term	LQC	20	19.9120	0.2621	1.3166
stability	HQC	40	38.3938	0.0700	0.1827

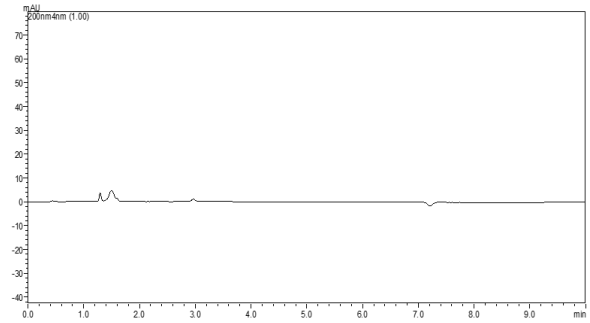

Figure 3: Blank chromatogram

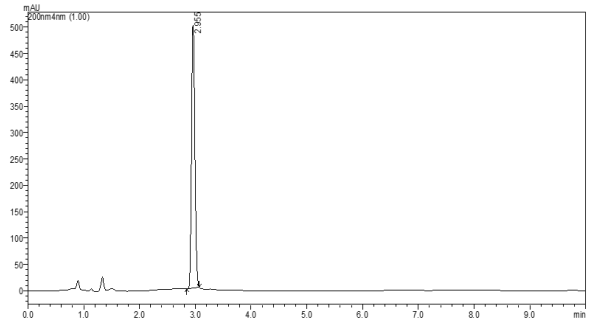

Figure 4: Standard chromatogram of Dichlorvos at 100μg/ml concentration showing RT at 2.9 min

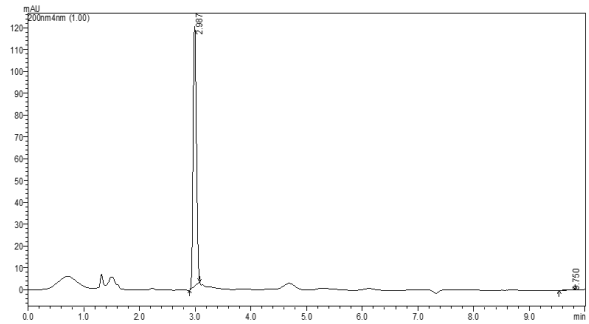

Figure 5: Sample chromatogram of Dichlorvos at 20μg/ml concentration showing RT at 2.9 min

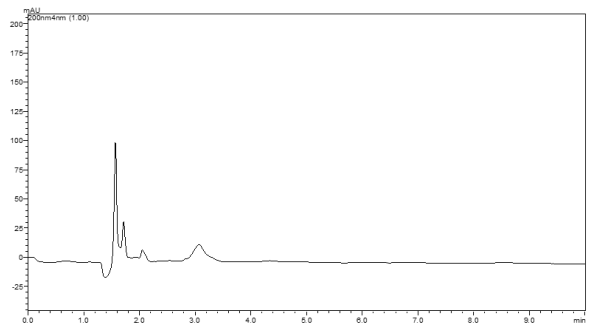

Figure 6: Chromatogram of blank serum

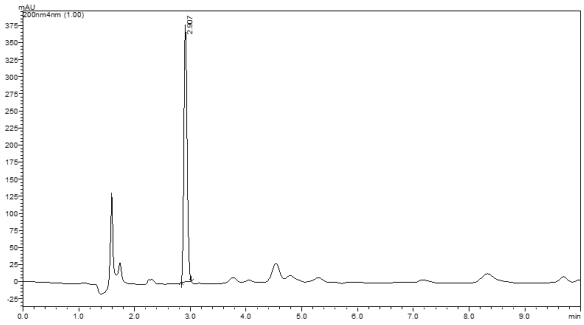

Figure 7: Standard chromatogram of Dichlorvos

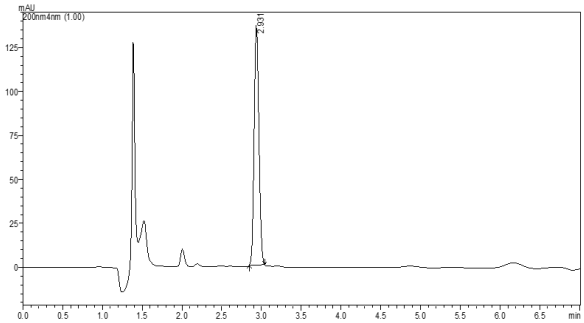

Figure 8: Sample chromatogram of Dichlorvos

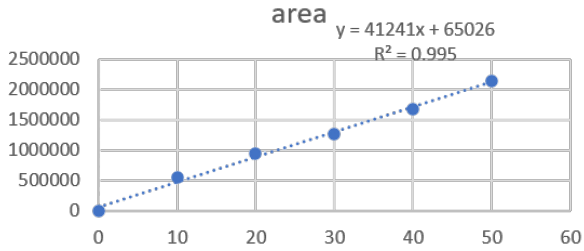

Figure 9: Calibration curve forDichlorvos

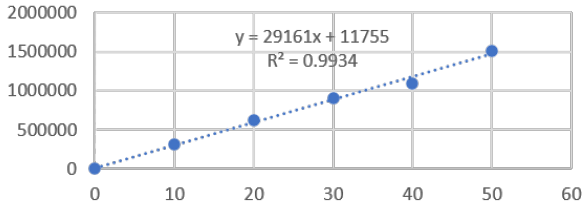

Figure 10: Linearity graph of Dichlorvos

a local market. All chemicals used were analytical grade purchased from Merck pharmaceuticals. HPLC grade ACN and Millipore water is used as mobile phase. HPLC grade ACN was used as the diluent for preparation of the solutions.

Analytical method development

Selection of wavelength

The λ max of Dichlorvos was determined by using UV-visible spectrophotometer 1800. Uv spectrum for Dichlorvos is shown in Figure 2.

Mobile phase selection and preparation

Dichlorvos being less polar, different mobile phase combinations of various ratios were tried for the selection of mobile phase. The standard Dichlorvos drug was injected with various combination of mobile phase at different ratios and flow rate for the peak optimisation. The procedure was continued until obtaining a sharp peak. The sharp peak was obtained at 50:50(v/v) of ACN and Millipore water.

Preparation of standard stock solution

The standard stock of Dichlorvos was prepared by dissolving 10mg of the standard drug in 10ml of HPLC grade acetonitrile to obtain 1mg/ml concentration. From the stock solution, the standard stock solutions of 10, 20, 30, 40, and 50μg/ml were prepared. All dilutions were made up by using HPLC grade acetonitrile.

Sample preparation

0.131ml of marketed formulation (Action-3) containing 76% of Dichlorvos was diluted to 100ml by using HPLC grade acetonitrile to form 1mg/ml solution. From the above sample solution, pipette out 0.2ml and make-up to 10ml by using HPLC grade acetonitrile to get 20μg/ml solution. The above resolution was passed through 0.20μm syringe filter and injected to RP-HPLC.

Optimization of the method for Dichlorvos

Study of the effect of various parameters in developing method was carried out. Initially, the solubility of Dichlorvos in multiple solvents was tested. Then a suitable column for separation is selected for the proposed method. To achieve a proper separation of eluted compounds in HPLC, the chromatographic conditions were optimised. Initially, different diluent was tested to elute the drug.

Flow rate and mobile phase choice are determined based on peak parameters like tailing factor or asymmetry, run time, resolution. Acetonitrile and Millipore water in ratio 50:50 (v/v) was used as mobile phase at a flow rate of 1.5ml/min. The blank chromatogram was shown in Figure 3.

The standard and sample chromatogram of Dichlorvos at 2.9 min were shown in Figure 4 and Figure 5 respectively. Chromatographic conditions used for the method is shown in Table 1.

Bio-analytical method development

Preparation of standard stock

Accurately weigh 10mg of pure Dichlorvos into 10ml volumetric flask dissolve and make-up the volume by using HPLC grade acetonitrile to get 1mg/ml concentration.

From the above solution prepare 75, 150, 225, 300 and 375μg/ml so that after diluting it with serum and ACN final concentration will be 10, 20, 30, 40 and 50μg/ml.

Optimized extraction procedure

After trying with a different combination of serum and drug volume for protein precipitation method by using acetonitrile, the following method is finalized.

In this procedure, acetonitrile act as a precipitating protein agent. To the Eppendorf tube add 100μl of human serum and 100μl of a drug, Vortex the above mixture in a vortex meter for 20 seconds. To the mixture add acetonitrile and make-up the volume to 1.5ml then centrifuge it at 9500 RPM for 10 minutes at 4°c. The supernatant is filtered through 0.20μm syringe filter and injected to RP-UFLC. The blank chromatogram was shown in Figure 6. The standard and sample chromatogram of Dichlorvos at 2.9 min were shown in Figure 7 and Figure 8 respectively. Chromatographic conditions used for the method is shown in Table 1.

RESULTS AND DISCUSSION

The precise, quick and easy RP-HPLC technique for the identification of Dichlorvos has been developed. The proposed technique was evaluated following the ICH Q2(R1) guidelines. The RT of Dichlorvos was found to be 2.9min from the chromatogram. And the coefficient of correlation was found to be 0.995 for analytical and 0.9934 for bio-analytical method. The quantification limit (LO Q) was found to be 0.139μg / ml, and the detection limit (LOD) was found to be 0.461μg / ml. The inter-day and intra-day precision value (RS D percentage) were identified to be less than two.

Analytical method validation

System suitability

The standard stock of Dichlorvos was injected six times for testing system suitability parameters, The results were shown in Table 2.

Linearity

It is the ability to obtain experimental results equal to the analyte content in the specimen. The calibration curve was attained by using five different concentrations in triplicate 10, 20, 30, 40, and 50μg/ml and the linearity was established by applying linearity expression y=mx+c, and the slope was calculated. The calibration curve for Dichlorvos is shown in Figure 9. The concentration and peak area was established in Table 3.

Precision

The repeatability of the method was validated by using different concentration of the drug 10, 30 and 50 μg/ml. The above solutions were prepared from the stock solution and used to inject in interday and intraday for the evaluation of precision. The concentrations were prepared at three different times in a day for intraday studies. The results for accuracy was shown in Table 4 and Table 5.

Accuracy

It is the closeness of the obtained value to the true value of the sample, to check the accuracy of the method, the formulation was spiked with 50%, 100% and 150% of Dichlorvos standard drug. The results were analysed to find the % recovery of the Dichlorvos. The result for accuracy was given in Table 6.

Limit of detection and limit of quantification

The LOD and LOQ for the HPLC method were determined by using a calibration standard. LOD can be calculated as per the ICH guidelines by using the formula LO D=3.3$\times$N$\div$S, N is the standard deviation and S is the slope. LOQ can be calculated by the formula LOQ=10$\times$N$\div$S where N is the standard deviation and S is the slope. The results for LOD and LOQ was shown in Table 7.

Robustness

A method can stay unchanged when small differences in parameters are applied. The robustness of the suggested technique was verified by increasing and decreasing the wavelength, flow rate, column temperature and 30μg / mL concentration were injected. The result of the robustness was shown in Table 8.

Ruggedness

An experimental procedure's ruggedness is its ability to remain unaffected by minor or intentional changes in system parameters. The ruggedness of the proposed procedure is validated by changing analyst and instrument. The result was shown in Table 9.

Bioanalytical method validation

Calibration curve

It consisted of a matrix sample processed without analyte and matrix sample with calibration standards. It is showing good linearity over the range of 10 to 50μg/ml with a coefficient of correlation 0.9934. The calibration curve for Dichlorvos is shown in Figure 10. The concentration and peak area was shown in Table 10.

Specificity/selectivity

An analytical technique can differentiate and quantify the analyte in the presence of other components in the sample. For selectivity blank plasma of two different lots were taken and analysed. Selectivity was assessed by comparing the extracted blank plasma response with extracted LLOQ. At the RT of the drug, No significant interference from the blank plasma was observed.

Sensitivity

This parameter was evaluated by injecting six different aliquots of extracted LLOQ concentration. Percentage deviation from the nominal concentration and percentage CV were calculated. The developed method was found to be sensitive to %CV.

Accuracy and precision

Within run and between run accuracy were performed by five replicates of LLOQ, LQC, MQC and HQC. Between run, accuracy was assessed by analysing sample on different days.

The accuracy and precision for all batches at LLOQ, LQC, MQC and HQC levels were calculated. Mean percentage nominal concentration and CV for all the batches were found to be within the acceptance limit. The results for precision is shown in Table 11 and Table 12.

Recovery

After spiking the extracted QC samples were analysed and percentage recovery at each level was calculated by comparing the peak area of low, medium and high QC levels.

Mean recovery across all the QC levels is found to be 66.0%. Results for recovery is shown in Table 13.

Stock solution stability

Both main stock and spiking stock of Dichlorvos was found to be stable at 2-10°c for 20 days (long term) and 8 hours at room temperature.

Bench top stability

Low and high QC was prepared and kept at the benchtop at room temperature for a minimum of 4 hrs (stability samples).

Then analysed the response is compared with the freshly prepared calibration standard responses. Mean percentage change was calculated

Freeze and thaw stability

The samples were exposed to three freeze-thaw cycles. The peak area response is then compared with standard calibration responses.

Mean percentage change was calculated and verified against acceptance criteria.

Long term stability

The stability of the sample is evaluated by keeping it for an extended period in freeze state and extracted then analysed. The response is compared with a fresh calibration standard response. Results for stability is shown in Table 14.

CONCLUSION

The developed RP-HPLC approach has been validated in terms of device suitability, linearity, precision, accuracy, LOD and LOQ, robustness and ruggedness in compliance with the ICH guidelines. It was inferred from the above finding that the system developed was reliable, accurate and unique for the detection of Dichlorvos.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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