



Evaluation of methodology for separation and identification of Tetraniliprole in maize (*Zea mays*) using RP-HPLC intended for residual analysis

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ABSTRACT

The aim of this study was to develop and optimize an RP-HPLC technique for the residue determination of tetraniliprole and its metabolite (BCS-CQ 63359). Standardization was carried out by using simplified quick, easy, cheap, effective, rugged and safe (QuEChERS) method in maize (*Zea mays* L.) leaves (fodder) and seeds. The optimized method was validated to fulfill the requirements of SANTE/11813/2017 guidelines including selectivity, linearity, precision and accuracy of detection system. A linearity relationship ($R^2 > 0.99$) between concentration of tetraniliprole/metabolite and peak area over concentration range was observed. Method used reversed phase C₁₈ column (5 μ m, 250 mm $\times$ 4.6 i.d.) along with photodiode array detector with isocratic mobile phase consisting of ACN: H₂O. The flow rate was 0.8 ml/min. and detection gave response at 215 nm. The RSD of peak area ranged from 0.35 to 5.63 % within analytical day and from 1.27 to 4.27 % across analytical days. Limit of detection (LOD) and limit of quantification (LOQ) for both analytes were found to be 0.01 and 0.05 μ g/ml respectively. Overall, the results demonstrate that the proposed method can be effectively implemented for screening and quantization of tetraniliprole and its metabolite as active ingredient taking maize as a matrix.

Key words: Evaluation, Identification, Maize, Methodology, RP-HPLC, Residue analysis, Separation, Tetraniliprole

Maize (*Zea mays* L.) is one of the most important cereals of the world's economy as both food and fodder grown throughout the year. In spite of rising in the area under this crop, the productivity is still low due to numerous reasons. Since the main season for maize cultivation is rainy season, crop is prone to many diseases and pests. Insect pests are one of the major limitations for low yield of maize. The insect pest of maize like fruit borer, corn earworm, leaf miner and stem borer *etc* inflicts serious losses. Several insecticides, formulated as either granules or spray applications, are registered for maize crop. Unsuitable agricultural practices and repeated use of insecticide leads to resistance within the targeted pest population. As an activity of insecticide resistance management, currently available insecticides from diamide group are being sold in some countries as launch progresses. This novel group comprises most advanced insecticides, which are highly efficient due to target specificity and delay of insect resistivity. As these insecticides are ryanodine receptor modulator, they have broad range effectiveness for the control of *Lepidopteran*

pests. They activate muscle ryanodine receptors, which mediate calcium release into the cytoplasm from intracellular stores. As these receptors bind to muscle cell, the calcium floods out which leads to paralysed muscle and death in 72 hr. A new insecticide from phthalic acid diamide group, named as tetraniliprole 480 FS has been developed by Bayer Crop Science. Tetraniliprole is chemically 1-(3-Chloro-2-pyridinyl)-N-(4-cyano-2-methyl-6-((methylamino) carbonyl) phenyl)-3-((5-(trifluoromethyl)-2H-tetrazol-2-yl)methyl)-1H-pyrazole-5 carboxamide. The primary breakdown product of tetraniliprole is BCS-CQ 63359, ISO-common name for which is chinazolinone.

Pesticide sprays can directly influence non-target vegetation, or can drift or volatilize from the affected zone and pollute air, soil and food commodities. Aside from the environmental risk, a high level of pesticide residue can affect the value of the maize grain with its processed products as well and it might eventually reach the consumer leading to health hazards (Handa *et al.* 1988). Therefore, in order to prevent health risks, it is important to monitor the presence of pesticides and regulate their levels. Development of suitable analytical method becomes more interested when the compound under investigation is one of the new insecticides. Nowadays, many sophisticated methods like liquid chromatography (LC) and gas chromatography (GC) coupled with advance detector system has employed for

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accurate and precise quantification of pesticide residue (Donker *et al.* 2015, Ahlawat *et al.* 2017, Lawal *et al.* 2018, Rani *et al.*, 2019). Up to now, there is no publication on extraction methodology and chromatographic analysis of tetraniliprole and its metabolite, the present study aimed at the development of high-performance liquid chromatography (HPLC) method for their determination in maize leaves and seeds. Quick, easy, cheap, effective, rugged and safe (QuEChERS) method introduced by Anastassiades and Lehotay (2003) was developed and validated for extraction. In spite of the fact that MS/MS detector gives higher affectability and selectivity however because of its cost, each quality control research laboratory cannot manage the cost of it. Therefore, an attempt was made to optimize reverse HPLC-DAD method. Also, the proposed method advances the knowledge in the field of detection of tetraniliprole and its metabolite for monitoring programs in developed as well as developing countries.

MATERIALS AND METHODS

The technical grade analytical standard (certified reference material) of tetraniliprole and its metabolite, *viz.* BCS-CQ 63359 with certified purity >99% were obtained from M/s Bayer Crop Science Ltd, Mumbai, India. HPLC grade acetonitrile (ACN) and water procured from Merck Specialties Pvt Ltd, India. Anhydrous NaCl, anhydrous Na₂SO₄, activated anhydrous MgSO₄ were obtained from Merck, Darmstadt, Germany. Primary secondary amine (PSA) sorbent procured from Sigma-Aldrich, Mumbai, India. The suitability of the solvents and other chemicals was investigated by running reagent blanks before actual analysis.

The high-performance liquid chromatograph (1260 infinity) by Agilent technologies Inc., Santa Clara, CA, USA equipped with a reverse phase (RP) C₁₈ column, a binary pump (Agilent-G1311B) and a photodiode array detector (DAD) (Agilent-G1315D). A ZORBAX® C₁₈ column of 4.6 i.d. and 250 mm length of 5 µm particle size with silica-based packing was used for the separation of tetraniliprole and its metabolite. The samples injected *via* auto sampler. System controller was Agilent Chemstation Software, which regulates solvent gradient, data acquisition and processing.

Different ratios of acetonitrile and water as mobile phases were checked (50: 50 to 100: 0 at the interval of 10). These were practised to obtain separated peak of analytes with best resolution. The favorable conditions were obtained at acetonitrile: water (80:20) in an isocratic mode at flow rate of 0.8 ml/min. Both the solvents used as mobile phases were filtered through a filter of 0.45 µm and well sonicated for nearly 15 min. before being used. Column re-equilibrated under the operating conditions for 30 min. before injecting the samples.

The choice of detection wavelength was based on the scanned absorption spectrum for both the compounds under study. The spectrum was scanned over the range of 200-400 nm at column temperature adopted 30°C. At last, DAD monitoring wavelength was set at 215 nm at an injection

volume of 20 µl.

Stock solutions of both the compounds were prepared individually by dissolving 10 mg standard sample in 10 ml of acetonitrile so that final concentration obtained was 1000 µg/ml. From the stock solution 2, 1, 0.5, 0.25, 0.1, 0.05, 0.01 µg/ml dilutions were prepared by using acetonitrile as diluent.

For sample preparation, QuEChERS method had been performed by taking 15g of homogenized maize leaves out of 500 g representative sample. The maize crop was grown at research area of Entomology Department, CCS Haryana Agricultural University, Hisar (India). Homogenized samples were spiked at different concentration levels under study with three replicates each. Then 30 ml of acetonitrile was added; centrifuged for 3 min. at 1400-1500 rpm. Thereafter, 3 g of anhydrous NaCl was added to the extraction tube and centrifuged for at 3000 rpm (5 min). The organic layer (18 ml) was transferred into test tube containing 9g anhydrous Na₂SO₄ and vortexed. Extracted layer (11 ml) added to sample clean up tubes, which contained 0.9g MgSO₄ and 0.15g of PSA. Clean up tubes were further subjected to vortex and centrifugation. Supernatant extract (6 ml) was transferred to vials and solvent was evaporated under nitrogen stream. Final residues were reconstituted with acetonitrile to make a final volume of 3 ml for further quantification by HPLC.

About 500 g of maize grain samples as a representative were grounded in a homogenizer to form a fine powder. The three replicated samples (10 g each) of maize powder were spiked with tetraniliprole and its metabolite at different fortification levels into 50 ml teflon centrifuge tube. An additional set of three replicates, with no spiking, was set to serve as the control. Double distilled water (20 ml) was added in above powdered samples and mixed on a vortex for 1 min, then added 20 ml acetonitrile in it. Thereafter, the tubes were kept in a deep freezer at -20 °C for 10 min. Using silent crusher, homogenized them at 10000- 12000 rpm to ensure that the dissolvable could have collaborated well with the solvent. Further, 2 g NaCl was added to the sample in the centrifuge tube and extract was centrifuged at 3000 rpm (2 min.). A volume of 10 ml from upper organic layer was moved into another 25 ml test tube containing 10 g anhydrous Na₂SO₄ and then vortexed for 1 min. Subsequently, 6 ml aliquot from it was transferred in cleanup tube containing 0.2 g PSA and 0.6 g anhydrous MgSO₄ and centrifuged for 5 min at 3500 rpm. Afterwards, 4.0 ml of aliquot was concentrated to dryness using vacuum rotavator. The last volume was made 4 ml, which on filtration by utilizing 0.22 µm nylon syringe channel and after that moved into an auto-sampler vial for injection.

Calibration curve was designed for tetraniliprole and its metabolite. Linearity was drawn between range 0.01-2 µg/ml. These working standards were also subjected for analysis of inter-day and intra-day precision in chromatographic responses. The calibration curve was further utilized for obtaining regression characteristics including slope, correlation coefficient and standard deviation. For the

method proposed accuracy, precision, specificity, robustness, ruggedness etc. were measured as discussed below.

The accuracy of the method is the closeness of the measured value to the true value of the sample (Al-Rimawi 2014). For determination of accuracy of the method under study, four different spiking level of tetraniliprole and its metabolite were checked for determining recovery values. Precision of the method calculated as % RSD (Relative Standard Deviation) was confirmed by analyzing the intra-day and inter-day results. In other words, repeatability was studied by running three replicates of same sample with same spiking concentration and reproducibility was evaluated by running three replicates of same sample with same spiking concentration across three different days. Results obtained in % RSD were calculated by using following formula:

$$\%RSD = \frac{SD}{Mean} \times 100$$

SD= Standard Deviation of analyte concentration, Mean= Average of analyte concentration.

Specificity of HPLC method was investigated to study the interfering effects of inert material with the desired peak. However, no overlapping peaks at retention times corresponding to the analytes were found. Current method was also optimized for good resolution of desired peaks in presence of other pesticides in matrix. The limit of detection (LOD) is usually defined as the lowest quantity or concentration of a component that can be reliably detected with a given analytical method (Armbruster and Pry 2008). By serial dilution, LOD was acquired in signal/noise ratio of 3:1 (Nakagawa *et al.* 2006). Moreover, it was calculated by using:

$$LOD = \frac{10 \times \sigma}{S}$$

where " σ " standard deviation of analyte and " S " is the slope of calibrative curve. The limit of quantification (LOQ) of an analyte is its lowest amount in a sample that can be determined quantitatively with precision. LOQ was obtained by analysis signal/noise ratio 10:1. Likewise LOD, respective

formula will be:

$$LOQ = \frac{10 \times \sigma}{S}$$

Robustness was performed by estimating change in standardized method by small but purposeful variation in detector wavelength 215 ± 2 nm, mobile phase ACN: Water (60:40) ratio ± 2 and flow rate 0.8 ± 2 ml/min. The ruggedness was tested by using two separate columns of same specification. While using different column, rest of the HPLC conditions like wavelength of detector, flow rate and mobile phase composition were kept steady. Roughness was equally estimated by repeatability and reproducibility acquired when in same research facility, same example, same instrument is dealt with by various administrators on various days. Triplicates of each recovery sample were used for statistical analysis and resulting values are expressed as mean $\pm$ S.E. Two level factorials of design (2^2) for analysis of variance and F-test were analysed using the computer program SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA) to assess any significant differences between the means ($p < 0.05$).

RESULTS AND DISCUSSION

The present investigation was carried out to develop a reversed phase HPLC method. Similar attempt for method validation of a novel diamide pesticide (chlorantraniliprole) was made using HPLC by Badaway (2018). Preliminary experiments were conducted with the purpose of getting best instrumental conditions that would allow better separation with high sensitivity and unambiguous identity. Under the chosen conditions, tetraniliprole and its metabolite showed individual isolated peak at 3.77 ± 0.5 min. and 4.92 ± 0.5 min. respectively, with an average of five injections and offer suitable chromatograms in real samples of maize. No considerable peaks were observed in non-spiked control samples.

The linearity of standard curve forms the plots of integrated peak area verses concentration of the standard and expressed in terms of correlation co-efficient (R^2). Fig 1 shows calibration curve for tetraniliprole and its metabolite

Table 1 Recovery studies of fortified maize leaves with tetraniliprole and its metabolite

Fortification levels ($\mu\text{g/ml}$)	Tetraniliprole		BCS-CQ 63359	
	Recovery mean ($\mu\text{g/ml}$) [% Recovery $\pm$ S.D.]	% RSD	Recovery mean ($\mu\text{g/ml}$) [% Recovery $\pm$ S.D.]	% RSD
0.05	0.0425 [85 $\pm$ 3.72]	4.37	0.045 [90 $\pm$ 0.32]	0.35
0.1	0.860 [86 $\pm$ 1.50]	1.74	0.091 [91 $\pm$ 4.9]	5.38
0.25	0.227 [91 $\pm$ 2.52]	2.76	0.255 [102 $\pm$ 2.30]	2.25
0.50	0.465 [93 $\pm$ 4.31]	4.63	0.540 [108 $\pm$ 4.31]	3.99

Table 2 Recovery studies of fortified maize grain with tetraniliprole and its metabolite

Fortification levels ($\mu\text{g/ml}$)	Tetraniliprole		BCS-CQ 63359	
	Recovery mean ($\mu\text{g/ml}$) [% Recovery $\pm$ S.D.]	% RSD	Recovery mean ($\mu\text{g/ml}$) [% Recovery $\pm$ S.D.]	% RSD
0.05	0.050 [100 $\pm$ 3.43]	3.43	0.048 [97 $\pm$ 3.65]	3.76
0.1	0.106 [106 $\pm$ 1.70]	1.60	0.102 [102 $\pm$ 5.09]	4.99
0.25	0.272 [109 $\pm$ 4.70]	4.31	0.255 [102 $\pm$ 4.50]	4.41
0.50	0.550 [110 $\pm$ 3.51]	3.19	0.525 [105 $\pm$ 1.61]	1.53

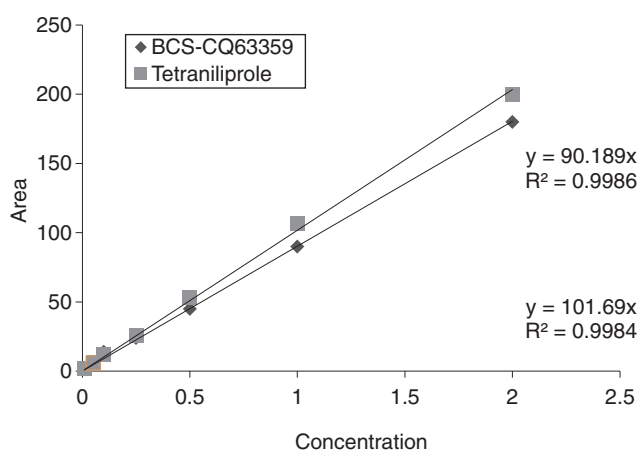


Fig 1 Calibration curve of tetraniliprole and its metabolite.

with R^2 value greater than 0.999. Recovery obtained at all concentrations and conditions investigated were more than 82% in all the samples under study. The precision values found were in agreement with those obtained in previous related studies (Liang *et al.* 2006). Accuracy was measured by 12 determinations (for each analyte) over a minimum of 4 concentration levels covering the specified range (0.5, 0.25, 0.1, 0.05 $\mu\text{g/ml}$) in case of maize leaves as well as seeds (Table 1-2). At each level of studies, % RSD values of replicates provided the precision for leaves as well as grains in terms of repeatability (RSD_r) and reproducibility (RSD_R) (Table 3-6). As per the results demonstrated in given data, % RSD values ranges from 0.35 to 5.63 % within analytical days and from 1.27 to 4.27 % across analytical days. Two-factor ANOVA findings at 5% level of significance, signified

Table 3 Recovery and RSD values of spiked samples of maize leaves with tetraniliprole

Day ↓	% recovery				Repeatability (% RSD_r)				Reproducibility (% RSD_R)			
	0.05	0.1	0.25	0.50	0.05	0.1	0.25	0.50	0.05	0.1	0.25	0.50
Fortification level ($\mu\text{g/ml}$)→												
1	85.33	86.33	91.00	93.00	4.37	1.74	2.76	4.63	2.30	3.68	2.84	3.17
2	89.00	85.00	96.00	99.00	4.68	2.07	3.10	4.89				
3	87.33	91.00	92.00	97.00	2.57	2.65	1.88	3.84				
<i>Factors</i>	<i>CD</i>		<i>SE (m)</i>		<i>CD</i>		<i>SE (m)</i>					
Days	0.92		0.312		0.026		0.009					
Fortification level	1.06		0.360		0.030		0.010					
Days $\times$ Fortification level	1.83		0.624		0.052		0.018					

Table 4 Recovery and RSD values of spiked samples of maize grain with tetraniliprole

Day ↓	% recovery				Repeatability (% RSD_r)				Reproducibility (% RSD_R)			
	0.05	0.1	0.25	0.50	0.05	0.1	0.25	0.50	0.05	0.1	0.25	0.50
Fortification level ($\mu\text{g/ml}$)→												
1	100.0	106.0	109.0	110.0	3.43	1.60	4.31	3.19	2.00	2.77	2.87	4.27
2	102.0	106.0	103.0	101.3	3.64	2.99	5.33	4.21				
3	98.0	101.0	107.0	106.0	3.92	3.92	4.64	3.77				
<i>Factors</i>	<i>CD</i>		<i>SE (m)</i>		<i>CD</i>		<i>SE (m)</i>					
Days	0.85		0.86		0.026		0.009					
Fortification level	0.99		0.99		0.030		0.010					
Days $\times$ Fortification levels	1.72		1.72		0.052		0.018					

Table 5 Recovery and RSD values of spiked samples of maize leaves with metabolite (BCS-CQ 63359)

Day ↓	% recovery				Repeatability (% RSD_r)				Reproducibility (% RSD_R)			
	0.05	0.1	0.25	0.50	0.05	0.1	0.25	0.50	0.05	0.1	0.25	0.50
Fortification level ($\mu\text{g/ml}$)→												
1	90.0	91.0	102.0	108.0	0.35	5.38	2.25	3.99	1.27	2.88	1.71	3.37
2	90.3	95.0	99.0	101.0	1.70	2.72	4.36	1.37				
3	92.0	90.0	102.0	104.0	2.75	3.93	2.72	3.71				
<i>Factors</i>	<i>CD</i>		<i>SE (m)</i>		<i>CD</i>		<i>SE (m)</i>					
Days	0.824		0.281		0.008		0.003					
Fortification level	0.951		0.324		0.010		0.003					
Days $\times$ Fortification level	1.647		0.561		0.017		0.006					

Table 6 Recovery and RSD values of spiked samples of maize grain with metabolite (BCS-CQ 63359)

Day ↓ Fortification level (µg/ml)→	% recovery				Repeatability (% RSD _r)				Reproducibility (% RSD _R)			
	0.05	0.1	0.25	0.50	0.05	0.1	0.25	0.50	0.05	0.1	0.25	0.50
1	97.0	102.0	102.0	105.0	3.76	4.99	4.41	1.53	3.09	3.19	3.01	1.47
2	94.0	103.0	98.0	102.0	2.57	2.82	2.42	2.55				
3	101.0	97.0	104.0	104.0	2.34	3.59	5.63	4.79				
Factors	CD		SE (m)		CD		SE (m)					
Days	0.85		0.29		0.01		0.003					
Fortification level	0.98		0.33		0.01		0.003					
Days × Fortification level	1.70		0.58		0.02		0.006					

the difference between mean values obtained for various levels of fortification. This variation is acceptable because of % recovery range as per SANTE/11813/2017 guidelines which is 80-120 % up to fortification level of 1 µg/ml. Similarly, % RSD values are also acceptable because their range lies below 20% (Chauhan *et al.* 2018).

Matrix match studies for tetraniliprole and its metabolite at different spiking levels showed no interfering effects with desired peaks. The absence of interference peak indicates that method can be used for routine residual analysis of samples. Likewise, the effects of inert materials were not discovered clashing to the chromatographic responses, which accounted for the specificity of the optimized method.

Using the linearity data and applying the formula aforementioned, LOD and LOQ of the proposed method was found to be 0.01 and 0.05 µg/ml for tetraniliprole as well as its metabolite. Minute modification in mobile phase combination, flow rate and in wavelength conferred negligible effect on analytical results of developed method. By and large, the developed method was found to be robust and rugged which showed similarity with previously presented work (Akoijam *et al.* 2015).

Conclusion

The significance of the method validation for a novel pesticide is not only to develop a method carefully for the accurate interpretation of the toxicological findings in routine residual investigations but also to demonstrate its applicability, especially about quality management and accreditation. The newly developed HPLC-DAD method has satisfactory validation characteristics like linearity, accuracy, precision, robustness, specificity *etc.* for determination of tetraniliprole and its metabolite. Overall results showed that the validated method could be successfully implemented for routine analysis of tetraniliprole along with its metabolite using QuEChERS technique. Moreover, the presented procedure can be applied for monitoring programs at low cost. Further field studies are necessary to contribute for the establishment of the maximum residue limits.

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