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LC-MS/MS Method, Development and Validation to Determine Three Tetracyclines and Their Epimers in Shrimp Samples

B.SIDDA REDDY[1,2], Y.SUDHAKAR[2], Y.SUBBA RAO[3], P.REDDY PRASAD[1] and N.Y. SREEDHAR*[1]

[1] Department of Chemistry, S. V. University, Tirupati-517 502, A.P. India.
[2]First Source Laboratory Solutions LLP (Analytical), Hyderabad, T.S, India.
[3]DST-PURSE Centre, S. V. University, Tirupati- 517 502, A.P, India.
*Corresponding author Email: sreedhar_ny@rediffmail.com

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ABSTRACT

A modest liquid chromatography/tandem mass spectrometric method was developed and validated to determine three tetracycline antibiotics and their epimers tetracycline, oxytetracycline, chlortetracycline, 4-epi tetracycline, 4-epi oxytetracycline, 4-epi chlortetracycline in shrimp samples respectively. The procedure was involved the extraction of tetracyclines and its epimers from homogenized sample with EDTA-McIlvaine buffer followed by solid phase extraction clean up with Oasis HLB cartridge. The analysis was carried out using Inertsil ODS-3V, 5 micron, 150×4.6 mm column and mobile phase consists of 5 mM Oxalic acid in water (A) and 0.1% formic acid in methanol (B) in gradient mode at a flow rate of 0.8 mL/minutes. The final extracts were analyzed by the sensitive and selective LC/ESI/MS/MS operating in positive ion multiple reaction monitoring (MRM) mode.

Keywords: Tectracylines, Antibiotics, Shrimp, Liquid chromatography, Tandem mass spectrometry.

INTRODUCTION

Tetracyclines (TCs) are a family of broad-spectrum antibiotics that are highly effective against a number of gram-positive and gram-negative bacteria, these antibiotics used worldwide to control bacterial infection and promote aquatic production[1-4]. Therefore, the easy availability and efficiency for the treatment of bacterial infected disease, they were widely used in animal husbandry and aquaculture[5-7]. As with other veterinary antibiotics, when tetracycline is used in food animals, it has the potential to generate drug residues in the animals and animal products which can lead to increases in microbial resistance. Due to the misuse, the antibiotic residues in products of aquatic animal origin (Fish, Shrimp etc.) brought a concern to consumers. The residue of this kind of drugs can be directly toxic or else cause allergic reactions in some hypersensitive individuals[8]. Thus in order to protect human health, the European Union and other regulatory authorities worldwide have established maximum residue limits (MRL) for antibiotic residues in animal products entering the

human food chain. European Union Commission Regulation 37/2010/EC establishes the MRLs for tetracyclines are 100 µg/kg (ppb) in muscle and 300 µg/kg (ppb) in liver for all food producing species[9].

In this respect, it is of great interest to develop analytical procedures capable of determining with good accuracy and sensitivity, the animal tissue concentrations of TCs and to evaluate their presence in edible animal products to protect human health. Accurate monitoring of chemical residue levels in food and agriculture products is essential to assure the safety of the food supply and manage global health risks. The analysis of chemical residues requires techniques sensitive enough to detect and quantify contaminants at or below the maximum residue limit (MRL) of the compound in a given sample matrix. In addition, because of increased food safety regulations and the growing numbers of samples to be analyzed, it is critical that the analytical techniques provide high sample throughput. Several analytical methods have been successfully proposed for the determination of TCs in various matrixes. In particular liquid chromatography in reverse phase mode (RP-HPLC) coupled with different detection systems such as UV [10-15], PDA [16-18], fluorescence is widely used in routine analysis of TCs in different matrixes[19, 20]. On the other hand, during last decade LC-MS/MS methods have been developed and implemented effectively for the determination of TCs mainly due to the known advantages of MS detection over conventional detection methods[21-28]. However based on the author's knowledge no LC-MS/MS method with full validation has been reported for the determination of tetracycline antibiotics along with their epimers in shrimp samples. Therefore the aim of this study is to develop rapid, simple and reliable LC-MS/MS method for the determination of three tetracyclines (chlortetracycline, oxytetracycline and tetracycline) along with their epimers (4-epi chlortetracycline, 4-epi oxytetracycline and 4-epi tetracycline) in shrimp samples.

EXPERIMENTAL

Chemicals and materials

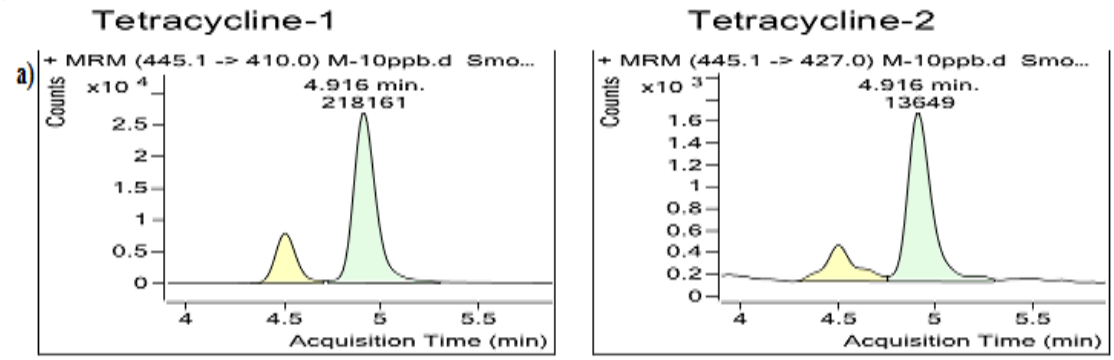
Methanol, acetonitrile and ammonium acetate were purchased from Sigma-Aldrich. Ethylacetate was purchased from Merck & Co. All the other inorganic chemicals and organic solvents were of analytical reagent grade or higher. Standards of tetracycline, chlortetracycline, oxytetracycline, 4-epi chlortetracycline, 4-epi oxytetracycline, 4-epi tetracycline were acquired from Sigma-Aldrich.

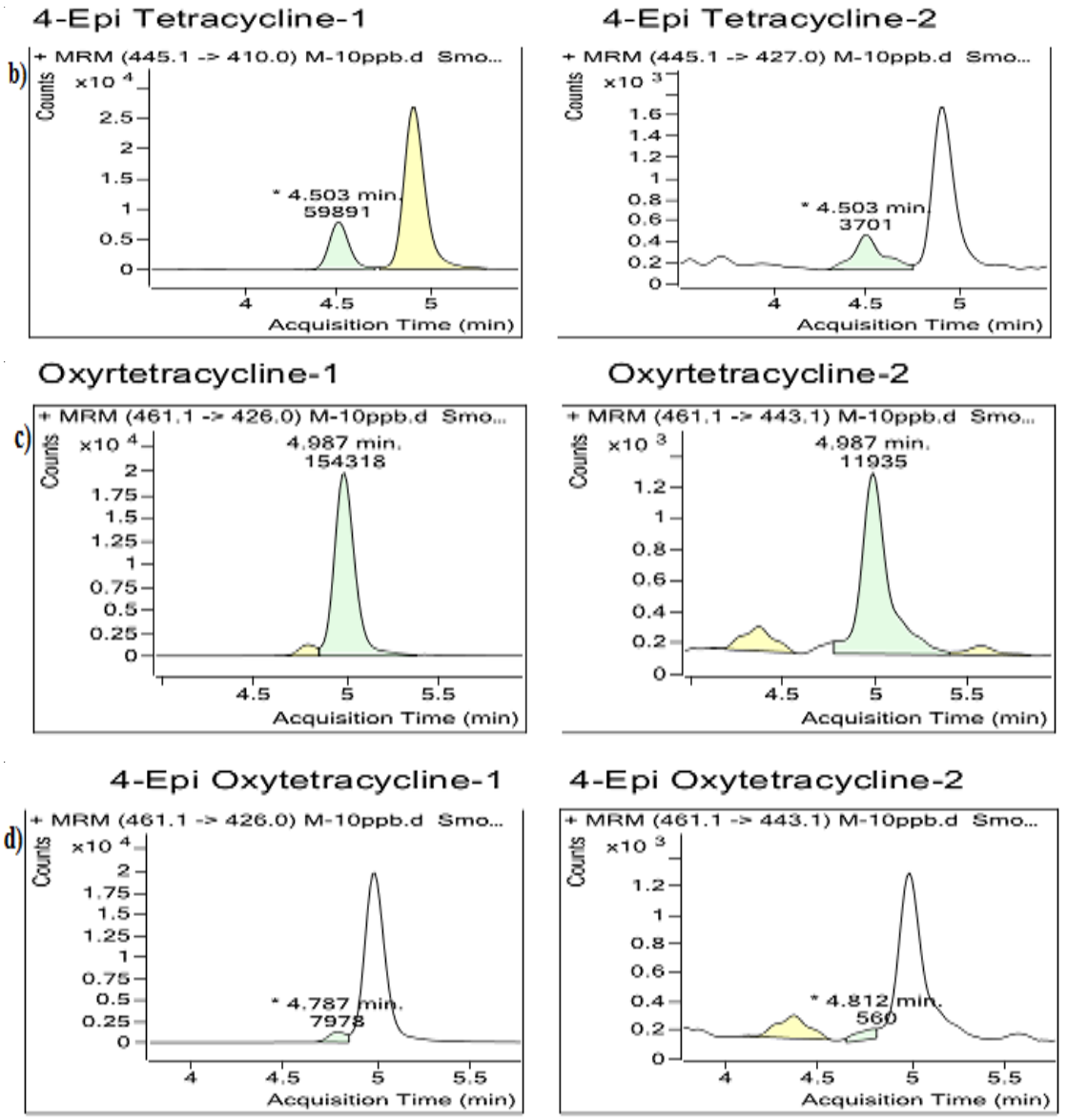
Preparation of stock solution (standard solution)

The standard stock solutions were prepared by accurately weighed 10 mg of each standard and is transferred into 10m l volumetric flask and made up to the mark with HPLC grade methanol and stored at -20°C. After proper dilution these stock solutions were used to prepare calibration curve and to fortify the blank shrimp samples to perform the recovery study.

Shrimp samples

Shrimp samples were collected from local market and stored at 20°C before the analysis.





Equipment

Liquid chromatography

The extracts were separated on Inertsil ODS-3V 5 micron, 150×4.6 mm HPLC column using Agilent 1290 HPLC system with a Binary pump, an auto sampler with temperature control, and a column oven kept at 30 °C. Sample aliquots of 5 µL were injected to the HPLC column, mobile phase consists of 5 mM Oxalic acid in Water (A) and 0.1% Formic acid in Methanol (B) in gradient mode with a flow rate of 0.8 ml/minutes. The gradient programme was given in the Table 1. The resultant chromatograms are shown in Figure. 1.

Table. 1: Gradient Program

S.No.	Time (min)	% Composition of A	% Composition of B
1	0	90	10
2	8.0	10	90
3	8.1	90	10
4	10.0	90	10

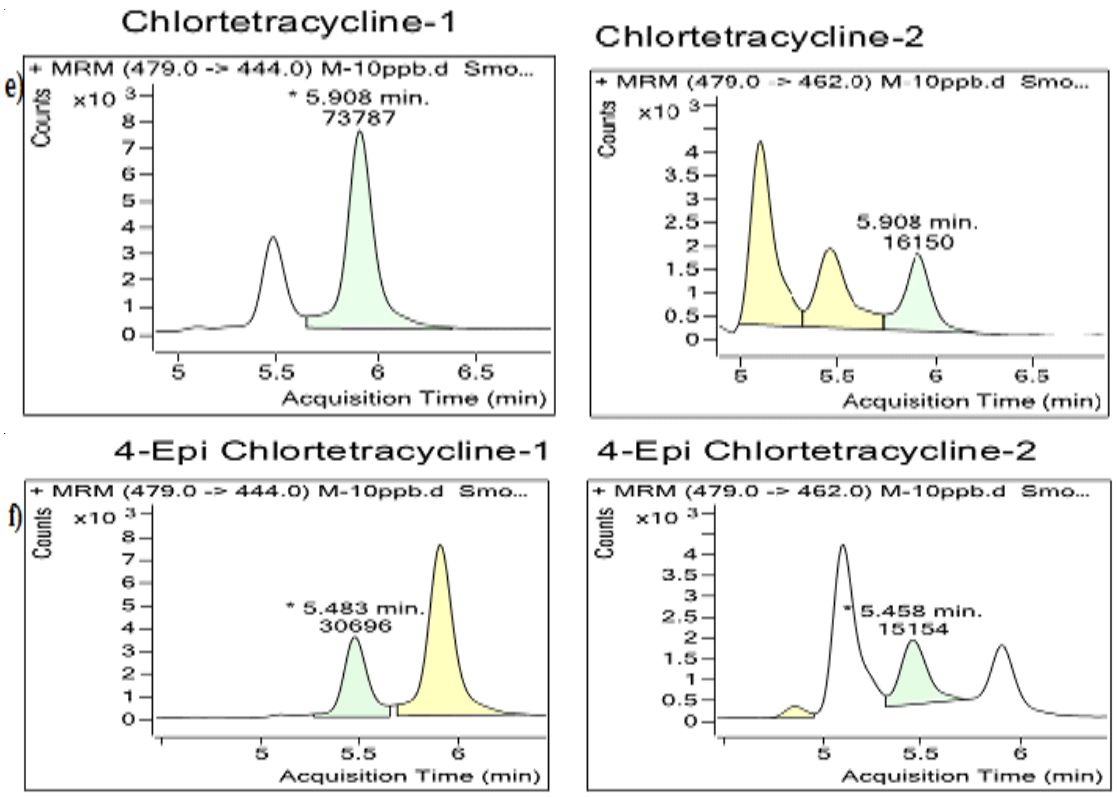


Fig. 1. MRM Chromatograms of TC (a), 4-epiTC (b), OTC(c), 4-epiOTC (d), CTC (e), 4-epi-CTC (f).

Tandem MS analysis

The flow from the LC column was transferred to a triple quadrupole mass spectrometer (Agilent 6470) equipped with an ESI source. The analysis was performed in positive polarity mode for all compounds. Instrument control, data acquisition and evaluation were done with Mass Hunter software, Version B.06.00. Determination was performed by two MRM (multiple reaction monitoring) transitions (one for quantitation and second one for conformation) mode, the MRM dynamics were given in the table 2. The typical source parameters were: Gas Temperature: 350°C, Gas flow: 10 l/min. Nebulizer: 45 psig, Sheath Gas Temperature: 400°C, Sheath Gas flow: 11 L/min. V Cap: 3500 V, Nozzle Voltage: 0V.

Table. 2: Multiple Reaction Monitoring transitions (Dynamic MRM)

Analyte(Transition)	Parent ion (Q_1)	Product ion ($Q_{3)}$	Fragmentor (Volts)	CE (Volts)	CAV
4-Epi Chlortetracycline-1	479	444	100	28	7
4-Epi Chlortetracycline-2	479	462	100	18	7
4-Epi Oxytetracycline-1	461.1	426	110	10	7
4-Epi Oxytetracycline-2	461.1	443.1	110	15	7
4-Epi Tetracycline-1	445.1	410	110	10	7
4-Epi Tetracycline-2	445.1	427	110	15	7
Chlortetracycline-1	479	444	100	15	7
Chlortetracycline-2	479	462	100	18	7
Oxyrtetracycline-1	461.1	426	110	10	7
Oxyrtetracycline-2	461.1	443.1	110	15	7
Tetracycline-1	445.1	410	110	10	7
Tetracycline-2	445.1	427	110	15	7

RESULTS AND DISCUSSION

Sample preparation

Collected blank shrimp samples were cleaned thoroughly, cut in to small pieces and homogenized. 5 g of homogenized sample was weighed and taken into 50 mL centrifuge tube, to this 15 mL of Disodium EDTA McIlvaine buffer is added, vortexed for 5 min. separated the aqueous layer by centrifuging the sample with 4500 rpm at 4 °C. The supernatant was transferred into a clean tube and repeated the extraction by adding 15 ml of McIlvaine buffer and combined the aliquots.

SPE Clean UP: The aqueous layer was applied to on preactivated with methanol (6ml), water 93 ml) and Mcvallin buffer (6 ml) HLB 500 mg/ 6cc spe cartridge previously activated with methanol and milli-Q water. After sample loading, the cartridge was given washings with Milli-Q water (6 ml) and dried the cartridge with the help of vacuum. TC antibiotics were eluted with methanol (6 ml) and the eluent was evaporated under a stream of nitrogen at 35°C , and the residue was dissolved in 2 mL 0.1% Formic acid: Methanol (9:1) and analyse on LC-MS/MS.

Method validation

The proposed method was validated for system suitability, specificity, selectivity, sensitivity (limits of detection and limit of quantification), recovery (accuracy), ruggedness, decision limit (CC α) and detection capability (CC β) according to 2002/657/EC decision.

System suitability

System suitability was performed by injecting six replicates of known concentration of standard solution. The results were within the acceptance criteria i.e. % RSD of area was less than 5%. The results have been given in Table 3.

Specificity

Specificity was performed by injecting 20 representative blank samples and sample spiked at MRL, observed that there was no interferences at the retention of analyte. The results have been given in Table 4.

Calibration Curve (Linearity)

Linearity was checked for each compound using six standard solutions with concentrations ranging in concordance with the level of the maximum residue limit (MRL). Three sets Linearity was established using matrix-matched calibration curves. Calibration curves were prepared at levels of matrix blank, 10 ppb, 50 ppb, 100 ppb, 150 ppb and 200 ppb in Shrimp and analyzed with each batch. The results of coefficient of determination greater than 0.99 and the results have been shown in Table 5 and Figure. 2.

Table. 3: System suitability

S. No.	Analyte Name	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6	Mean	SD	%RSD
1	4-epi chlortetracycline	892511	945738	937236	922127	1000941	1004036	950432	44219.1341	4.65
2	4-epi oxytetracycline	397524	377783	387189	371478	359169	373702	377807	13274.4630	3.51
3	4-epi tetracycline	1651314	1684929	1668218	1714973	1770066	1754306	1707301	47687.0313	2.79
4	Chlortetracycline	1922854	1892795	1895596	1892109	1967535	1913028	1913986	29027.5990	1.52
5	Oxytetracycline	3250717	3268198	3346982	3285948	3384171	3258769	3299131	54083.7727	1.64
6	Tetra	37211	38467	38075	38720	39351	38788	38435	73124.	1.90

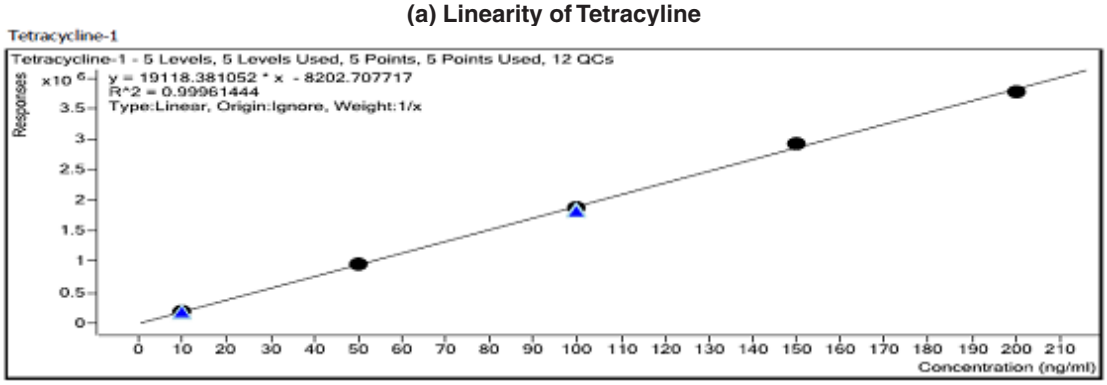
Table. 4: Specificity

S.No.	Analyte Name	Average area of analyte in blank samples	Average of LOQ area
1	4-epi chlortetracycline	444.9	30696
2	4-epi oxytetracycline	1821.4	7978
3	4-epi tetracycline	238.8	59891
4	Chlortetracycline	2220.9	73787
5	Oxytetracycline	1940.8	154318
6	Tetracycline	1973.5	218161

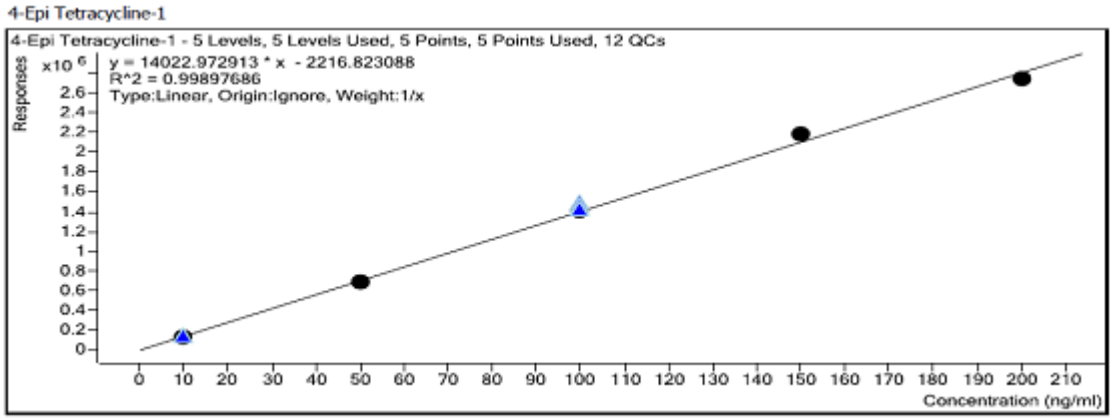
Table. 5: Linearity Results

S.No.	Compound	Slope (m)	Coefficient of determination (R^2)
1	4-epi chlortetracycline	4472.684	0.998901
2	4-epi oxytetracycline	1986.989	0.99912
3	4-epi tetracycline	9320.932	0.998678
4	Chlortetracycline	8843.196	0.998558
5	Oxytetracycline	15773.01	0.998562
6	Tetracycline	20477.41	0.998768

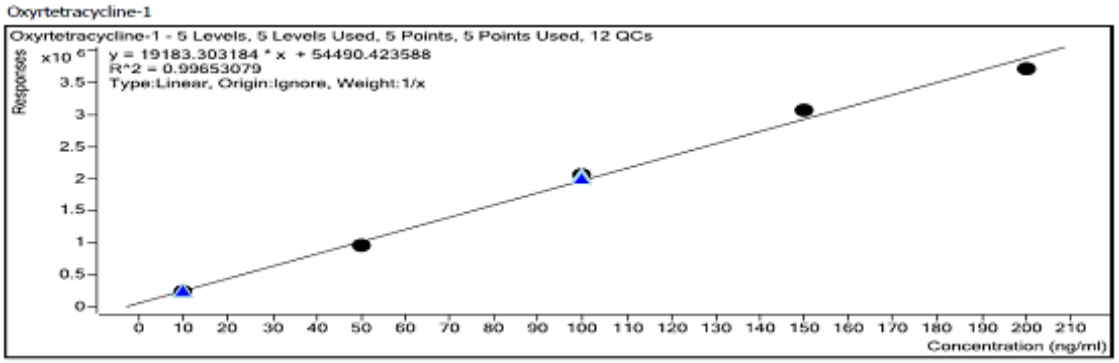
(a) Linearity of Tetracyline



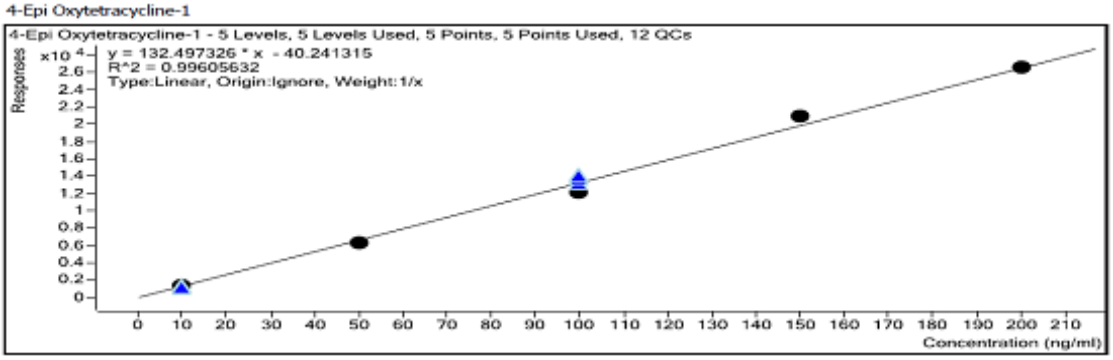
(d) Linearity of 4-epi-Oxy Tetracycline



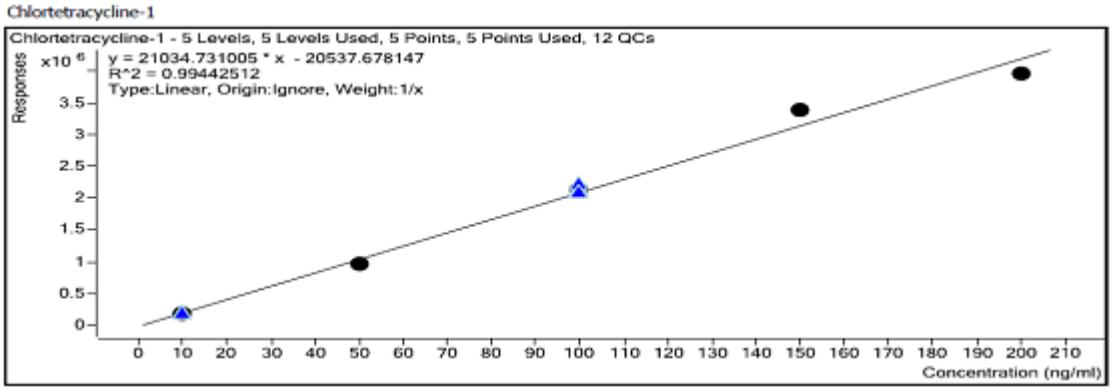
(c)Linearity of Oxy Tetracycline



(d) Linearity of 4-epi-Oxy Tetracycline



(e)Linearity of Chlorotetracycline



(f) Linearity of 4-epi-Chlorotetracycline

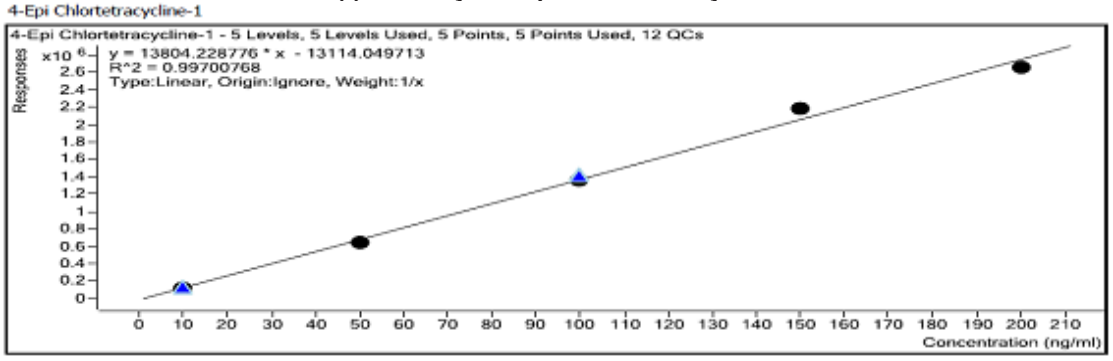


Fig. 2. Linearity diagram of TC (a), 4-epi TC (b), OTC(c), 4-epi OTC (d), CTC (e), 4-epi-CTC (f).

Table. 6: Limit of Detection and Limit of Quantification

S.No.	Compound	SD (STEYX)	Slope	LOD	LOQ
1	4-epi chlortetracycline	24310.06	4472.684	17.93625	54.35229
2	4-epi oxytetracycline	9663.506	1986.989	16.04919	48.63392
3	4-epi tetracycline	55580.3	9320.932	19.67775	59.62955
4	Chlortetracycline	55064.58	8843.196	20.54835	62.26774
5	Oxytetracycline	98082.35	15773.01	20.52061	62.18366
6	Tetracycline	117829.7	20477.41	18.98863	57.54131

Limit of Detection and Limit of Quantification

Calculated the Limit of Detection (LOD) and Limit of Quantification (LOQ) by using standard deviation (STEYX) of the response and the slope of the linearity and the obtained LOD and LOQ values are given in the Table 6.

Repeatability and Within Laboratory Reproducibility

Repeatability was performed by injecting seven spiked samples at 50 ppb, 100 ppb and 150 ppb and calculated the mean, standard deviation and % RSD. The results were within the acceptance criteria i.e. % RSD of area ≤20. For within Laboratory reproducibility the above procedure was performed and calculated the mean, standard deviation and %RSD for all the analyses at 50 ppb (0.5 MRL), 100 ppb (MRL) and 150 ppb (1.5 MRL).

Recovery/Accuracy (Trueness)

Recovery was performed by injecting seven spiked samples at 50 ppb, 100 ppb and 150 ppb and calculated the mean, the standard deviation and the coefficient of variance for these concentrations, calculate the recovery as accuracy (trueness) by dividing the detected mean concentration by the fortified value and multiply by 100 and the results are given in Table 7.

Ruggedness

Ruggedness was performed by injecting seven spiked samples with two different analysts and two different lots of extraction solvents at permitted level. Calculated the Mean, standard deviation and % RSD. The results were within the acceptance criteria i.e. % RSD of area ≤7 and the results were given in Table-8.

Decision Limit (CC α)

Decision limit was performed by injecting seven spiked samples at 50 ppb, 100 ppb and 150 ppb in three batches and calculated the y-intercept and slope. The decision limit (at á = 5 %) was calculated at permitted limit for all the compounds, the concentration at permitted limit (MRL) plus 1.64 times the standard deviation of the within-laboratory reproducibility. The results were given in table 9.

Table. 7: Recovery/Accuracy

S.No.	Compound	Mean (Concentration in ppb)			% Recovery/Accuracy (Concentration in ppb)		
		Batch -1	Batch -2	Batch -3	Batch -1	Batch -2	Batch -3
1	4-epi chlortetracycline	43.2018	44.8266	42.5916	86.40	89.65	85.18
2	4-epi oxytetracycline	42.7798	42.7275	46.8627	85.56	85.45	93.73
3	4-epi tetracycline	41.7066	41.5344	41.5838	83.41	83.07	83.17
4	Chlortetracycline	54.7178	54.5396	52.6245	109.44	109.08	105.25
5	Oxytetracycline	53.2315	51.2158	50.6047	106.46	102.43	101.21
6	Tetracycline	57.7920	55.6192	55.2160	115.58	111.24	110.43

Detection Capability (CC β)

Detection capability (β = 5 %) was calculated, the concentration at decision limit plus 1.64 times the standard deviation of the 20 samples fortified at decision limit. The results were shown in table 9 and the recoveries of the 20 samples are meeting the criteria.

Table. 8: Ruggedness

S.No.	Compound	Mean	SD	%RSD
1	4-epi chlortetracycline	43.54	1.67	3.84
2	4-epi oxytetracycline	44.12	2.89	6.55
3	4-epi tetracycline	41.61	0.91	2.18
4	Chlortetracycline	53.96	2.10	3.89
5	Oxytetracycline	51.68	1.76	3.40
6	Tetracycline	56.21	2.19	3.90

Table. 9 : Decision limit (CC α) and detection capability (CC β)

S.No.	Compound	Decision limit (CC α)	Detection capability (CC β)
1	4-epi chlortetracycline	114.4879	128.9759
2	4-epi oxytetracycline	111.8852	123.7703
3	4-epi tetracycline	108.6466	117.2932
4	Chlortetracycline	114.8092	129.6185
5	Oxytetracycline	109.4829	118.9658
6	Tetracycline	108.2438	116.4877

CONCLUSIONS

A simple positive ion LC-MS/MS method was developed and validated for the determination of tetracyline antibiotic residues in raw shrimp meat. Based on the results obtained, acceptance criteria for all validation parameters such as system suitability, specificity, calibration curve (Linearity), repeatability, within laboratory reproducibility, recovery, decision limit (CC α) and detection capability (CC β) have been met for the method used to estimate the tetracylines in shrimp samples as per protocol and as well as EU guideline council Directive 2002/657/EC. The method requires only a small sample volume, needs minimal manual sample preparation and reasonable run time. For this reason, the method could be useful for determination of tetracylines in shrimp samples. Henceforth considered the method is validated and can be used for further intended purpose.

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