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Dispersive Liquid-Liquid Microextraction/HPLC Techniques for Determination of Oxytetracycline and Doxycycline Residues in Beef Samples: Method Developments and Statistical Analysis

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Abstract

A rapid, cost-effective and environment-friendly sample pre-treatment method involving dispersive liquid-liquid microextraction (DLLME) and high-performance liquid chromatography (HPLC) was developed and applied for the extraction of oxytetracycline and doxycycline residues in beef samples (liver, kidney and muscle). Several influencing factors associated with the extraction and separation of these antibiotics residues, such as sample size, type and volume of disperser and extraction solvents, centrifugation speed and time, were optimized using Plackett-Burman design and central composite design, while insignificant factors were fixed at values determined using univariate analysis. Figures of merit of the analytical methodology including the limit of detection (LOD), the limit of quantification (LOQ), accuracy (in terms of average recoveries), precision and calibration functions were established according to the European Union commission decision 2002/657/EC. Linearity, in the range of 5–500 μg/kg, was obtained with regression coefficients ranging from 0.9983 – 0.9999. Inter-day repeatability, intra-day precision, LODs and LOQs obtained were 3.81 – 14.90%, 3.80 – 8.70%, 4.21 – 4.69 μg/kg and 14.02 – 15.65 μg/kg respectively. Samples with detectable drug residues have oxytetracycline being the most commonly detected. The developed method was successfully established and the concentration levels of drug residues detected were lower than the European Union set maximum residue level (MRL).

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1. Introduction

Tetracyclines are a group of polyketides used as broad-spectrum antibiotics and are the second most widely used family of antibiotics in human and veterinary medicine due to their high quality and low cost [1]. These drug substances are broken down by animals, and some of the drugs remain in the animal body, while others enter the environment through excreta. The remains of the drug substances in this excreta find their way into the soil and rivers, which would subsequently be absorbed by vegetables, fruits trees and aquatic animals [2]-[5]. Conversely, improper administration of tetracyclines as veterinary drugs has also resulted in the presence of their residues in animal-based food and therefore poses a serious threat including allergic reactions in susceptible users, chronic toxicity, and antimicrobial resistance to consumer health. For protecting human health, the European Union (EU) and the United States Food and Drug Administration (FDA) have regulated the maximum residue limit of this family of antibiotics in animal tissue and milk [1].

Oxytetracycline and doxycycline are among the most extensively used and representative tetracycline in intensively managed feeding operations in animals. They are available over the counter in most African countries, including Nigeria. In most cases, they are used without prescription [6]. In Nigeria, oxytetracycline is the most widely used, and it accounts for over 82.6 %, followed by tylosin at 44.5 %, and gentamycin at 3.8 % [7]. The National Agency for Food and Drug Administration and Control (NAFDAC) and the Pharmacists Council of Nigeria (PCN) are saddled with the responsibilities of establishing guidelines and regulations on the manufacturing, supply and use of antibiotics in Nigeria [8]. Oxytetracycline for example has been found at concentrations of 2.98 mg/kg and 5.17 mg/kg in manures and soils of some farms in countries like China. It is partially absorbed in the gastrointestinal tract of animals with their remains deposited as manure [9]. Meanwhile, doxycycline is more lipid soluble with a higher tendency to persist in animal tissue and, above all, in animal fat, where the concentration can reach the maximum residue limit (MRL) [10,11]. One of the critical difficulties associated with tetracyclines derivatives, such as doxycycline and oxytetracycline, is the treatment of their biological samples. The procedure usually includes: (i) an extraction step with the aid of a suitable solvent and (ii) a clean-up and a pre-concentration stage. The extraction methodologies often adopted involve the use of an acidic buffer (in the range of pH 3–5), a homogenization step and a centrifugation stage. At times, ultrasonication could be utilized to improve extraction efficiency. Recently, various sample preparation methodologies followed by liquid chromatography techniques such as solid phase extraction (SPE), solid phase microextraction (SPME), liquid-liquid microextraction (LLME), DLLME and some tandem methods, such as ultrasound-assisted matrix/SPME, magnetic SPE/DLLME and salt-induced homogenous LLE/DLLME have been applied for the analysis of tetracycline in various complex matrices [10, 12]. The DLLME/HPLC techniques have been preferred due to their simplicity, rapid, cost-effective, environment-friendly and effective detection. However, these methods have not efficiently extracted the drug residues from the sample matrix and in-depth analysis featuring the use of statistical designs to assess and validate the extraction processes has not been extensively investigated.

To protect consumers, the tolerance level for the sum of tetracycline drugs in animal muscle has been limited to 2 μg/g by the US Food and Drug Administration, therefore a need for developing an efficient extraction method is required. Accordingly, research into the development of effective methods to detect residues of tetracycline-class drugs have gained attention from researchers to uphold regulations and protect public health [13]. Hence, a rapid, cost-effective and environment-friendly sample pre-treatment method involving DLLME/HPLC was developed and applied for the extraction of oxytetracycline and doxycycline residues in beef samples (liver, kidney and muscle). The development and validation of the procedures were further subjected to statistical analysis using the Plackett-Burman design and central composite design.

2. Materials and Methods

2.1. Materials, Reagents and Instruments

The reagents utilized for this experimental procedure are of analytical grade, unless otherwise specified, and the solvents utilized were of HPLC grade. Acetonitrile, methanol and formic of LC-MS grade products from QREC

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company. Acetone, chloroform, and dichloromethane of HPLC grade obtained from QREC. Sodium hydroxide pellets, sodium chloride, sodium citrate and magnesium sulphate anhydrous are of analytical grade and veterinary drug standards utilized were obtained from Sigma Aldrich. Deionized water was utilized for dissolution and rinsing where required. All glassware including the glass vials was cleaned thoroughly with detergent and a bristle brush and then rinsed with deionized water.

The high-performance liquid chromatography (HPLC) analysis was carried out with a Shimadzu LC -20AT system (Kyoto, Japan) consisting of a degasser, tertiary pump, autosampler, column oven and a fluorescence detector. The chromatographic separation was performed with a C18 150 mm × 4.6 mm Spheris orb 5 ODS-1 (particle size 5m) chromatographic column purchased from Phenomenex (USA).

2.2. Sample Collection

The beef samples were obtained from four different markets (Mandate (8° 28'25.3"N, 4°30'14.4"E), Ipata,(8°49'99.4"N, 4°56'14.8"E) Obo Road (8°48'08.8"N, 4°56'14.2"E) and Oja Tuntun (8°48'83.2" N, 4°53'71.6"E)) in Ilorin metropolis, Kwara State, Nigeria. The samples were collected from each of the markets weekly for 12 weeks between March and June, 2022.

2.3. Preparation of Solutions

Stock standard solutions of 100 μg/mL oxytetracycline and doxycycline were prepared separately in methanol by quantitatively dissolving an amount corresponding to 10–30 mg of the drugs (after correcting for purity, the water of hydration and the fact that some standards were salts) in a 100 mL volumetric flask with methanol. Mixed working standard solutions were then prepared by further diluting 100 μL aliquots of the stock standard solutions (100 μg/mL) in a 100 mL volumetric flask with methanol to obtain a final concentration of 0.1 μg/mL. The standard solutions were stored at a temperature of 2 °C until ready for analysis. Fresh calibration standards were then prepared for every run by fortifying 2.0 g of blank homogenized samples with appropriate volumes of the 0.1 μg/mL mixture of stock standard solutions of oxytetracycline and doxycycline. The spiked samples at five (5) different concentration levels in the range of 5 - 500 μg/kg obtained were taken through the entire DLLME procedure for constructing a matrix-matched calibration curve.

2.4. Sample Treatment

The refrigerated beef samples were removed on the day of analysis, defrost at room temperature and homogenized. The homogenized samples were weighed (2.00 ± 0.005 g) into Falcon tubes and treated with 6 mL of 5:1 (v/v) water/acetonitrile mixture. A 500 mg mixture of salts (magnesium sulfate anhydrous, sodium chloride and trisodium citrate dihydrate (3:1.5:0.5)) was then added to the samples and vortex-mixed for 2 min. The samples were centrifuged (5 °C, 4000 rpm, 5 min) and the supernatant obtained was subjected to the DLLME procedure.

2.5. DLLME Procedure and HPLC Analysis

For the DLLME procedure, the pH of 1 mL aliquots of the supernatants obtained was adjusted to pH 7 using 0.1 M NaOH. 1.5 mL methanol (disperser solvent) and 250 μL chloroform (extracting solvent) were injected rapidly into the sample solutions contained in test tubes. The solutions were quickly taken for centrifugation at 4000 rpm for 5 min. The sedimented phase was withdrawn with a micro-syringe into 2 mL centrifuge tubes, and the solvents were left to evaporate. The residues were then reconstituted in 100 μL of water, and 20 μL were injected into the HPLC. The beef extracts were analyzed isocratically using a 65:25:10 water/methanol/acetonitrile mixture as the mobile phase. The column was kept in a column oven at 30 °*C* at a flow rate of 1.0 ml/min to achieve the optimum resolution of the veterinary drugs. The injection volume was maintained at 10 μL for both the sample and standard solutions.

2.6. Method Development

The univariate and multivariate methods were utilized for the method development assessment. For the univariate method, the mass of the sample, extraction time, type and volume of extraction and dispersive solvents were optimized. The extraction solvent and dispersal solvent were carefully selected and the samples for method development were first analyzed to ensure that the target analytes are absent. Meanwhile, for the multivariate method, the Placket-Burman design was employed to determine the significant variables in the DLLME technique. The various factors and levels adopted for the design are presented in Table 1. The significance of factors such as the mass of the sample, the mass of salt mixture, the volume of methanol, the volume of chloroform, centrifugation speed and centrifugation time were estimated, and the design matrices were generated using Minitab® statistical software version 17. The experimental runs were carried out according to the design matrix and the total chromatography peak area (TCPA) was recorded. The Plackett-Burman design screening is in accordance with the documentation of Fang *et al* [14].

The significant factors estimated with the Plackett-Burman design were optimized by the use of a second-order central composite design, utilizing a response-surfaced methodology. The number of points in central composite design (CCD) contains a factorial run of 2 k, axial runs of 2 k and Co centre point runs. Therefore, the total experimental runs (N) of CCD is given by: $N = 2k + 2k + Co$, where Co and k are the number of variables and the number of centre points, respectively [15]. To reduce the effect of uncontrolled variables, the CCD experiments were run in a random manner [16].

Table 1. Factors and level of variables used for P-B design

S/N	Factors	Level	
		Low (-)	High (+)
1	Mass of sample (g)	1	5
2	Mass of salt mixture (g)	0	1.5
3	Volume of methanol (ml)	0.5	2
4	Volume of chloroform (μl)	100	300
5	Centrifugation speed (rpm)	3000	6000
6	Centrifugation time (rpm)	3	9

2.7. Validation of Analytical Methodology and Statistical Analysis

The validation was carried out according to the EU commission decision 2002/657/EC [16]. The method performance was evaluated by the following parameters: matrix effect study, the establishment of matrix-matched calibration, the precision (in terms of intra-day and inter-day relative standard deviation), accuracy in terms of percentage recovery, selectivity and sensitivity, the limit of detection (LOD) and limit of quantification (LOQ). The calibration curve and the graphical representation showing the chromatographic peak area against various parameters were fashioned using Microsoft Excel R software. The Plackett-Burman design matrix (see Table 2) for the six (6) variables was generated and analyzed using Minitab® 17 statistical software package.

In accordance with ICH guidelines, accuracy and precision were assayed using a minimum of nine determinations within a specified range. Three concentration levels were replicated three times for each concentration according to the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH-Topic Q2(R2), 2022) [17]. Accordingly, the accuracy, intra-day and inter-day precision were obtained through spiking of samples at three concentration levels, and on the same day, replicate analyses (3 times) were run for each concentration. The three extractions performed in a single day for estimating the intra-day precision (n = 3), inter-day precision (n = 9) was assessed based on three extractions per day for three days, while accuracy was reported in terms of average recoveries of the spiked sample at various levels of the concentration. A one-way single factor ANOVA was utilized to calculate the variance, as this could give the total sum of squares, between group mean square (BMS) and within group mean square (WMS). BMS was utilized to estimate the variance associated with inter-day

(i.e. between-day variance) and variance related to intra-day variability (i.e. within-day). These two variances were adopted for the determination of repeatability and intermediate precision [18]. More so, the repeatability (intra-day precision) and intermediate precision (inter-day) were calculated according to equations (1) and (2), respectively.

$$\%\ RSD\ (Intra-day) = \frac{\sqrt{\text{WMS}}}{\text{Average Relative Recovery}} \times 100 \quad (1)$$

$$\%\ RSD\ (Inter-day) = \frac{\sqrt{\left(\frac{BMS-WMS}{N}\right) + WMS}}{\text{Average Relative Recovery}} \times 100 \quad (2)$$

where N denotes the number of replicates per day, and the average relative recovery represents the average calculated from daily average recoveries.

3. RESULTS AND DISCUSSION

3.1. Optimization of Factors using Univariate analysis

Optimization of Sample Size, extraction and disperser solvent volume, centrifugation speed and time via univariate analysis. The beef samples optimized by spiking with a standard mixture of the drugs at varied weights (1 - 7 g) were found to be optimum at 2 g for the investigated analytes. The influence of disperser solvent volume was investigated with different volumes of methanol (0.5, 1.0, 1.5 and 2.0 mL) at constant volume of chloroform (250 μL) was optimum at 1.5 mL. Meanwhile, the influence of the extraction solvent volume investigated (at pH 7, 1.5 mL methanol and 100 - 300 μL of chloroform) was optimum at 250 μL. The centrifugation speed varied between 2000 - 6000 rpm at 5 min and was more effective at 4000 rpm, while the centrifugation time varied between 2 -10 min at optimum centrifugation speed was more efficient at 5 min.

3.2. Optimization via multivariate analysis

The analyzed report for the 12 experimental runs of P-B design for six (6) factors at two levels each was illustrated in the Pareto chart of standardized effects (Figure 1) and normal plot of the standardized effect (Figure 2). It is an illustration of horizontal bars of the screened factors showing a red vertical line across the bars, which indicates the level of significant [19]. Table 2 provides information on the main effects of the factors while all interactions that are present are ignored, using two levels for each factor, with the higher level represented with (+) and the lower with (–) [16]. They were selected based on previous experiments and taking into consideration the limitations of the experimental system. The experiments were designed using a predefined pattern of high and low levels for each number of experiments [15]. The screening indicated that the volume of methanol, volume of chloroform and centrifugation time are significant to extraction efficiency as illustrated by the Pareto chart (Figure 1). The Pareto chart of standardized effects is an illustration of horizontal bars for the screened factors and the red vertical line across the bars indicates the level of significant difference.

The screening experiment obtained indicated that the mass of the sample, centrifugation speed, and mass of the salts mixture ($MgSO_4$, CH_3COONa and NaCl) investigated have no significant effect on the extraction efficiency at $p \leq 0.05$. Therefore, the factors that were not significant (mass of sample, centrifugation speed and mass of salt) were fixed according to the optimal values estimated. However, the volume of methanol, volume of chloroform and centrifugation time (min) that significantly influence ($p \leq 0.05$) extraction efficiency (see Figure 3 and Table 3), were optimized by the second–order CCD. The CCD matrix generated (see Table 4) show that these factors increased the extraction efficiency of the DLLME technique [20].

As can be observed in Figure 3, the optimum volume of methanol, chloroform and centrifugation time were found to be 4.9 mL, 500 μL and 6 min, respectively. The optimum values were subsequently used for the analysis of real samples.

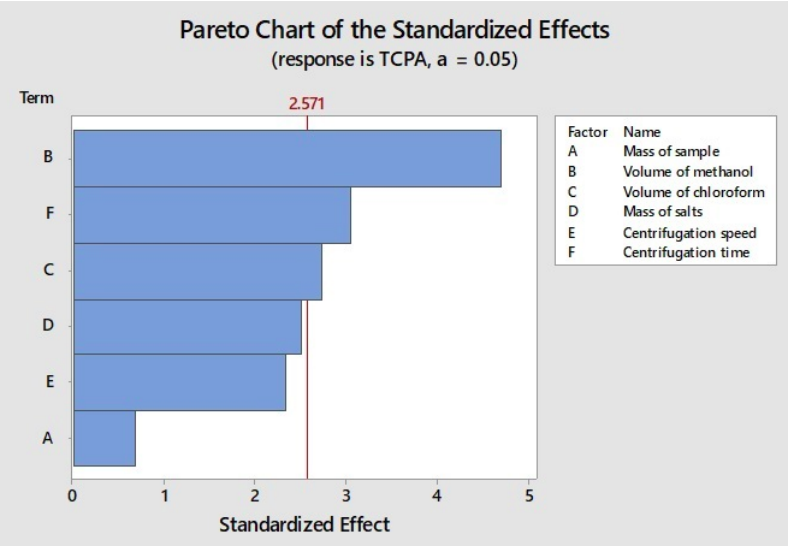

Figure 1. Pareto chart of standardized effects of Plackett-Burman runs

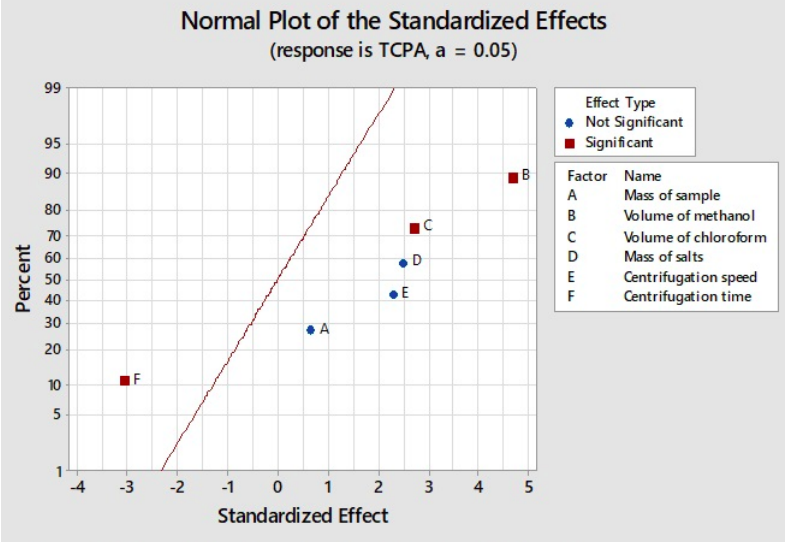

Figure 2. Normal plot of standardized effects of Plackett-Burman runs

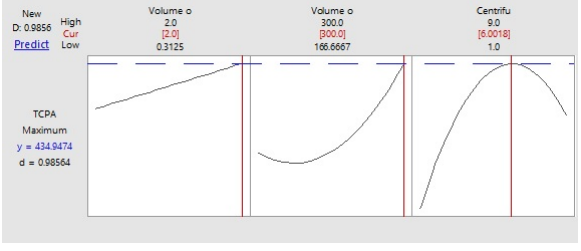

Figure 3. Optimization curve of significant factors for DLLME

Table 2. Design Matrix for Plackett - Burman Design (PBD)*

StdOrder	RunOrder	A	B	C	D	E	F	Observed	Predicted
2	1	5	2	100	1.5	3000	3	290.93	279.81
10	2	5	0.5	100	0	6000	9	223.72	222.79
11	3	1	2	100	0	3000	9	234.62	235.09
8	4	1	0.5	300	1.5	6000	3	290.04	277.55
1	5	5	0.5	300	0	3000	3	239.4	247.53
12	6	1	0.5	100	0	3000	3	227.73	223.09
4	7	5	0.5	300	1.5	3000	9	238.91	243.77
6	8	5	2	300	0	6000	9	288.17	276.27
5	9	5	2	100	1.5	6000	3	285.36	296.55
7	10	1	2	300	1.5	3000	9	270.27	272.85
9	11	1	0.5	100	1.5	6000	9	230.78	236.07
3	12	1	2	300	0	6000	3	284.17	293.31

N.B.: *Design generated using Minitab® Statistical Software Version 17;
A, mass of sample; B, volume of methanol; C, volume of chloroform;
D, mass of salt; E, centrifugation speed; F, centrifugation time.

Table 3. Significant factors of DLLME

S/N	Factors	Level	
		Low	High
A	Volume of methanol (mL)	0.5	2
B	Volume of chloroform (μL)	100	300
C	Centrifugation time (min)	3	9

3.3. Method validation

3.3.1. Linearity

The linearity assessment conducted using a standard solution of the target analyte in the concentration range of 5 – 500 μg/kg with a matrix-matched external standard calibration curve was found to be linear with a regression coefficient > 0.99 as indicated in Table 5.

3.3.2. LOQ and LOD

Estimation of LOD and LOQ was processed via the signal/noise ratio method, and the LOD and LOQ were calculated using the signal/noise ratio of 3:1 (i.e. 3σ/S) and 10:1 (i.e. 10σ/S) respectively. σ and S are the standard deviation of the response and slope of the calibration curve respectively. The LOD obtained for oxytetracycline and doxycycline were 4.69 and 4.21 μg/kg, respectively, while their LOQ were 15.65 and 14.02 respectively. The obtained figures of merit were comparable with those reported on the analysis of veterinary drug residues using various methods of extraction [21, 22].

3.3.3. Precision (Intra-day and Inter-day)

Table 6 shows the intra-day and inter-day precision of the target analytes in beef samples. The liver sample was estimated to range from 3.89 – 4.44% (intra-day) and 4.14 – 10.30% (inter-day). The kidney sample ranged from 6.29 – 6.46% (intra-day) and 7.75 – 13.48% (inter-day), while the intra-day and inter-day precision in muscle samples ranged from 7.07 – 7.08% and 7.32 – 14.90%, respectively. This result agreed with those reported by Moema et al. [23] in which a % RSD between the range 4.0 – 7.0% was obtained for fluoroquinolone using dispersive liquid-liquid microextraction.

Table 4. Design Matrix for Central Composite Design (CCD)

StdOrder	RunOrder	PtType	Blocks	A	B	C	Observed	Predicted
15	1	0	1	1.25	200	6	436.40	386.45
13	2	-1	1	1.25	200	1	335.58	341.66
7	3	1	1	0.5	300	9	351.93	357.28
19	4	0	1	1.25	200	6	435.06	386.45
18	5	0	1	1.25	200	6	359.10	386.45
6	6	1	1	2	167	9	341.60	353.87
20	7	0	1	1.25	200	6	358.37	386.45
3	8	1	1	0.5	300	3	432.26	414.65
16	9	0	1	1.25	200	6	428.04	386.45
4	10	1	1	2	300	3	405.41	407.11
5	11	1	1	0.5	167	9	346.17	343.90
8	12	1	1	2	300	9	426.26	407.53
10	13	-1	1	2	200	6	363.66	381.85
2	14	1	1	2	167	3	379.65	366.43
9	15	-1	1	0.312	200	6	393.28	392.70
11	16	-1	1	1.25	233	6	362.55	387.31
12	17	-1	1	1.25	300	6	399.25	424.40
17	18	0	1	1.25	200	6	384.66	386.45
1	19	1	1	0.5	167	3	398.64	414.25
14	20	-1	1	1.25	200	6	360.21	386.45

[a] Generated using Minitab Statistical Software version 17

N.B.: A, volume of methanol; B, volume of chloroform; C, centrifugation time

Table 5. Linearity range (μg/kg) of the developed DLLME method

Drug Residue	R^2	Linearity (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)
Oxytetracycline	0.9999	10 – 500	4.69	15.65
Doxycycline	0.9998	5 – 500	4.21	14.02

3.3.4. Recovery and selectivity

The precisions and accuracies (in terms of relative recoveries) of the developed method in beef are presented in Table 6. Recoveries in beef samples ranged from 95.33% – 109.04%, 81.94% – 104.22% and 98.85% – 106.67% in liver, kidney and muscle samples respectively These are considered acceptable according to the SANCO guideline [24], which put method performance criteria of mean recoveries within 70 – 120% with precisions ≤ 20%.

Selectivity describes the extent to which a method can be utilized to assess a particular analyte in mixtures/or matrices without interferences from other species. It also revealed an insight into the capability of an analytical instrument to produce signals that represent the target specie(s) and not interfering components [25]. In this work, selectivity assessment was carried out by extracting a blank matrix containing the external standard, and a sample spiked with the analyte of interest. The chromatogram obtained (see Figure 4) indicated a good selectivity with no interferences.

Table 6. Accuracy, Inter-day and Intra-day Precision of the Drug residues in Beef Samples

Analytes	Added (μg/kg)	Liver			Kidney			Muscle		
		Recovery (%)	Inter (%)	Intra (%)	Recovery (%)	Inter (%)	Intra (%)	Recovery (%)	Inter (%)	Intra (%)
Oxytetracycline		103.24	4.14	3.89	85.34	7.75	6.29	101.48	14.90	7.08
Deoxycycline		95.33	10.30	4.44	104.22	13.48	6.46	106.67	7.32	7.07

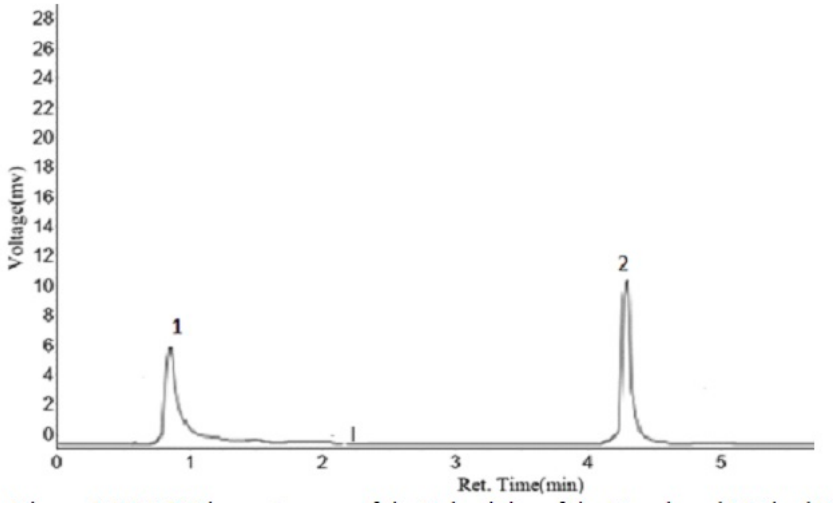

Figure 4. HPLC Chromatogram of the Selectivity of the Developed Method Peak

Table 7. Concentration (μg/kg) of target analyte in beef liver samples

Week	Ipata (n = 12) (mean±SD)		Oja Tuntun (n = 12) (mean±SD)		Obo road (n = 12) (mean±SD)		Mandate (n = 12) (mean±SD)	
	OT	DT	OT	DT	OT	DT	OT	DT
1	572.49±27.20	561.94±23.62	502.00±70.28	587.34±31.81	509.85±24.24	463.69±23.86	560.90±26.66	509.91±26.25
2	468.23±28.29	456.73±19.19	515.32±31.12	502.26±25.85	417.02±25.21	376.89±19.39	458.92±27.73	414.53±21.33
3	563.19±4.65	586.95±24.68	542.23±38..76	578.08±33.25	501.95±4.15	484.02±24.93	551.99±4.56	532.83±27.42
4	160.65±6.76	157.73±6.62	176.99±7.44	173.14±8.90	143.01±6.01	129.93±6.68	157.56±6.62	142.90±7.35
5	579.58±10.38	440.00±87.71	450.91±93.84	595.01±34.44	516.54±9.25	501.53±25.83	568.11±10.17	551.97±28.42
6	178.49±13.85	168.82±7.08	196.51±15.24	185.55±9.53	159.01±12.34	138.98±7.15	174.94±13.58	152.96±7.86
7	520.32±48.80	594.92±25.00	553.12±62.17	432.22±33.68	538.93±22.00	490.92±25.26	592.90±24.08	539.85±27.79
8	552.33±25.04	538.45±22.63	564.44±34.81	592.05±30.49	491.98±22.31	444.01±22.86	541.11±24.54	488.49±25.15
9	497.92±19,60	491.11±20.64	547.36±21.56	540.28±27.81	443.72±17.47	405.01±20.86	487.93±19.21	445.52±22.94
10	555.08±26.04	540.11±22.70	489.67±70.80	593.97±30.56	494.72±23.20	445.58±22.93	543.95±25.52	489.99±25.23
11	578.91±14.69	581.69±24.45	510.00±65.16	588.98±32.93	515.86±13.09	479.70±24.70	567.11±14.39	527.84±27.17
12	481.07±20.21	471.36±19.81	528.92±22.23	518.45±26.68	428.70±18.01	388.56±20.01	471.01±19.81	427.70±22.01
MRL	600	600	600	600	600	600	600	600

N.B: OT (Oxytetracycline) and DT (Doxycycline)

3.3.5. DLLME application on real meat samples

The proposed extraction method was adequately applied for the determination of various veterinary drugs in meat samples. The samples were found to contain oxytetracycline and doxycycline, as these drugs' residues were detected probably as a result of their frequent usage but their withdrawal periods were not observed. The concentration levels of the drug residues detected in beef are reported in Tables 7-9. For the samples collected weekly for 12 weeks, the mean ± SD showed that the levels are lower than the European Union set maximum residue level (MRL), hence, the meat is fit for human consumption. For beef liver samples, Mandate market had the highest residue of mean ± SD of 592.90 ± 24.08 μg/kg of oxytetracycline in week 7, while Oja Tuntun had the highest residue level with mean ± SD 595.01 ± 34.44 μg/kg of doxycycline in week 5. For the beef kidney sample, Oja Tuntun market had the highest drug residue of 665.50 ± 27.03 μg/kg of oxytetracycline in week 7, 669.17 ± 34.44 μg/kg was the highest residue level of doxycycline in Oja Tuntun market in week 5. In beef muscle samples, Oja Tuntun had the highest residue level with mean ± SD 196.41 ± 20.65 μg/kg of oxytetracycline in week 6, Oja Tuntun also had the highest level of residue in doxycycline with mean ± SD of 185.35 ± 9.53 μg/kg in week 6.

The data in Tables 7-9 were analyzed using single-factor ANOVA to determine whether the antibiotic content

Table 8. Concentration (μg/kg) of target analyte in beef kidney samples

Week	Ipata (n = 12) (mean±SD)		Oja Tuntun (n = 12) (mean±SD)		Obo road (n = 12) (mean±SD)		Mandate (n = 12) (mean±SD)	
	OT	DT	OT	DT	OT	DT	OT	DT
1	572.00±27.20	561.23±23.62	629.49±29.92	617.98±31.82	509.74±24.24	463.59±23.86	561.20±26.66	510.11±26.25
2	467.93±28.29	456.23±19.19	515.22±31.12	502.16±25.85	417.53±25.21	376.69±19.39	459.12±27.73	414.53±21.33
3	562.99±4.65	586.32±24.68	619.37±5.12	645.51±33.25	501.95±4.15	484.18±24.93	551.99±4.56	532.78±27.43
4	160.65±6.76	157.53±6.61	176.69±7.43	172.94±8.90	143.28±6.02	129.73±6.68	157.46±6.62	142.90±7.35
5	579.88±10.38	608.00±25.57	637.80±11.42	669.17±34.44	516.44±9.25	502.03±25.83	567.95±10.17	551.77±28.42
6	178.19±13.85	168.72±7.08	196.31±15.24	185.55±9.53	158.97±12.34	139.28±7.15	174.74±13.58	152.76±7.86
7	604.02±24.58	594.32±24.99	665.50±27.03	654.16±33.68	538.83±21.89	490.72±25.26	593.20±24.09	539.95±27.79
8	552.00±25.04	538.00±22.63	607.21±27.55	592.05±30.49	492.28±22.31	444.11±22.86	541.35±24.54	488.69±25.15
9	497.52±19.60	491.01±20.64	547.56±21.56	539.98±27.81	443.22±17.46	405.04±20.86	487.83±19.21	445.82±22.94
10	555.00±26.04	539.91±22.70	610.45±28.64	593.67±30.58	494.82±23.20	445.28±22.93	543.95±25.52	490.09±25.23
11	578.91±14.69	581.69±24.44	636.45±16.15	639.80±32.93	515.96±13.09	480.00±24.70	567.10±14.39	527.64±27.17
12	480.47±20.21	471.01±19.81	529.12±22.23	518.55±26.68	428.60±18.01	388.96±20.01	471.01±19.81	427.50±22.01
/MRL	1200	1200	1200	1200	1200	1200	1200	1200

N.B: OT (Oxytetracycline) and DT (Doxycycline)

Table 9. Concentration (μg/kg) of target analyte in beef muscle samples

Week	Ipata (n = 12) (mean±SD)		Oja Tuntun (n = 12) (mean±SD)		Obo road (n = 12) (mean±SD)		Mandate (n = 12) (mean±SD)	
	OT	DT	OT	DT	OT	DT	OT	DT
1	149.01±23.23	149.01±27.56	151.67±20.63	162.32±3.82	151.67±10.62	139.08±3.86	152.11±25.98	176.40±6.25
2	138.22±17.27	138.22±10.16	139.08±10.38	154.23±5.85	145.67±17.95	141.15±9.39	143.17±10.14	168.34±2.33
3	141.10±20.90	141.10±24.14	176.89±21.15	165.54±3.25	148.23±16.66	137.77±24.93	178.32±16.69	178.30±2.43
4	139.23±12.54	139.23±11.13	176.99±15.24	173.34±8.90	176.89±18.04	130.03±6.68	157.36±6.62	142.80±7.35
5	133.32±23.22	133.32±28.61	167.90±23.73	154.32±4.44	141.13±17.73	141.13±5.83	165.55±21.36	151.67±8.42
6	178.79±13.85	168.72±7.08	196.41±20.65	185.35±9.53	159.37±12.34	139.38±7.15	174.84±13.58	152.86±7.86
7	139.08±24.07	152.11±22.52	141.13±16.58	152.11±3.68	136.51±22.30	136.51±5.26	182.20±26.61	148.23±7.79
8	141.15±26.86	143.17±18.68	149.01±20.65	143.17±3.49	149.01±12.78	149.01±2.86	176.17±13.98	176.89±5.15
9	137.77±10.84	178.32±17.55	176.40±16.58	178.32±7.81	176.40±14.50	138.22±2.85	145.55±12.01	154.45±2.94
10	135.98±20.97	155.34±13.47	168.34±22.76	152.33±3.58	168.34±14.59	141.10±2.93	190.23±18.54	159.11±5.23
11	141.13±29.13	165.55±29.20	178.30±29.54	165.55±3.93	178.30±19.04	139.23±4.70	187.90±10.04	162.56±7.17
12	144.23±19.33	162.23±15.58	142.50±14.28	164.45±6.68	142.50±13.93	133.32±2.01	157.54±17.93	178.43±2.01
MRL	200	200	200	200	200	200	200	200

N.B: OT (Oxytetracycline) and DT (Doxycycline)

varies significantly at $p \leq 0.05$. There was no significant difference in the concentration of oxytetracycline in beef liver samples obtained from different markets, while a significant difference was observed in the concentration of oxytetracycline in beef kidney and muscle samples obtained from various markets where samples were taken. A similar trend was observed in the concentration levels of doxytetracycline in beef liver, kidney and muscle samples obtained in different markets.

4. Conclusion

A rapid, simple and cost-effective method (DLLME) was successfully utilized for the analysis of oxytetracycline and doxycycline in beef using HPLC to determine their prevalence below and or above MRLs. This method was developed, optimized and validated for the determination and extraction of these drugs residue in the samples bought from four different markets in the Ilorin metropolis, Nigeria. Methanol and chloroform were used as the extraction and disperser solvents, respectively. Under the optimal conditions, validation parameters such as recoveries (95 to 109%), LODs (4.21 to 4.69 μg/kg), LOQs (14.02 to 15.65 μg/kg) and linearity (10 – 500 and 5 – 500 μg/kg) for oxytetracycline and doxycycline, respectively, were established indicating the applicability of the method to real samples as an extraction method. All the samples analyzed contain oxytetracycline and doxycycline at a level below the EU set MRLs of 600, 1200 and 200 μg/kg of oxytetracycline and doxycycline for beef's liver, kidney and muscle

respectively. This observation indicates that the beef samples analyzed were compliant with the EU limit, and therefore fit for human consumption.

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