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Original Article

Development of QuEChERS/HPLC technique for the determination of veterinary drug residues in beef samples

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Abstract

The application of antibiotics in animals for therapeutic and growth promotion purposes and to meet the need to increase meat production has been reported to cause the development of antibiotic resistance and other health risks due to the consumption of poultry products. Therefore, the need for rapid and accurate analytical methods for the determination of drug residues has become necessary. The Quick, Easy, Cheap, Effective Rugged and Safe (QuEChERS) technique involves liquid-liquid extraction by partitioning with acetonitrile, followed by clean-up with a mixture of magnesium sulphate and a primary secondary amine. The extract obtained was then analyzed using high-performance liquid chromatography (HPLC). The figures of merit of the analytical methodology were determined using optimized parameters and the calibration curve was linear over the tested concentration range. The linearity ranged from 5 to 500 µg/kg for all the drug residues, and the correlation coefficients (r^2) were greater than 0.99. The limit of detection (LOD) ranged within 1.45 – 5.02 µg/kg while the limit of quantification (LOQ) ranged within 4.68 – 6.72 µg/kg. The inter-day and intra-day precisions for beef samples had respective ranges of 1.94 – 19.03 % and 2.78 - 9.04%, and the average recoveries were from 79.48 to 107.48 %. The selectivity was determined by a blank matrix containing external standards and a sample spiked with target analyte and the results indicate a good selectivity with no interferences.

Keywords: antibiotics, HPLC, QuEChERS, sample preparation, veterinary drugs

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1. Introduction

Veterinary drugs are important in the production of livestock for the treatment of infections, for improvement of growth, and to ensure food security (Falowo, & Akimoladun, 2020). The use of veterinary drugs for a long period can cause resistance in animals and lead to water pollution, and they have been used in aquaculture and animal husbandry (Tufa, 2015). Various countries are discouraging the export and the increased use of antibiotics, and the development of new veterinary drugs, to reduce the harmful effects of veterinary drug residues (Hu, & Cowling, 2020).

Oxytetracycline and doxycycline are tetracyclines, which are a group of broad-spectrum antibiotic compounds that have a common basic structure that can be obtained by semi-synthesis or can be directly isolated from several species of *Streptomyces aureofaciens* (Mookantsa, Dude, & Nindi, 2016). Their chemical structure consists of a ring system with different side chains (Patrabansh, Parajuli, & Jha, 2020). Tetracyclines have antibacterial effects on gram-positive and gram-negative bacteria, Chlamydia, rickettsiae, mycoplasma, spirochetes and certain protozoa, with a broad antibacterial spectrum (Mookantsa *et al.*, 2016). Tetracyclines can cause liver damage, exhibit nephrotoxicity, are not conducive to bone and tooth growth, and cause gastrointestinal reactions. Because of these, the Food and Agriculture Organization (FAO) and the EU have established MRLs of these drugs in foods. The acceptable MRLs are set at 100 mg kg^{-1} for muscle, 300 mg kg^{-1} for liver, and 600 mg kg^{-1} for kidney for all food-producing animals (Mookantsa *et al.*, 2016). Tylosin is a macrolide antibiotic containing a mixture of four tylosin derivatives produced by a strain of *Streptomyces fradiae* (European Pharmacopoeia, 2004; Teeter, & Meyerhoff, 2003).

QuEChERS method of extraction involves crushing the sample, using acetonitrile as a solvent for the extraction and separation processes, addition of $MgSO_4$ and other salts to remove water, adding adsorbent to remove impurities, and analysis of the supernatant (using GC-MS and LC-MS). HPLC and SPE principles are similar to the QuEChERS method. Impurity removal is achieved in QuEChERS because it makes use of the interactions between the adsorbent filler and the impurities in the matrix to adsorb impurities. QuEChERS extraction method is widely acceptable for multi-class and multi-residue analysis of different types of veterinary drugs in animal-based foods. (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003). In the present study, a rapid and cost-effective sample preparation method using a quick, easy cheap, effective, rugged and safe method (QuEChERS) was developed for the analysis of drug residues in beef samples by HPLC-UV detector.

2. Materials and Methods

2.1 Sample collection

The beef samples were obtained from markets in the Ilorin metropolis. The samples were collected in a polythene bag and were taken to the laboratory and analyzed immediately. The samples that were used for method development, calibration and recovery studies were analyzed to ensure the absence of the target drug residues.

2.2 Chemicals and reagents

Drug residue standards of oxytetracycline, doxycycline and tylosin (which are the most widely used antibiotics in poultry farms) were purchased from Sigma Aldrich. All solvents (methanol, acetonitrile and acetone) used were analytical grade and were purchased from Sigma Aldrich, USA. Anhydrous magnesium sulphate and sodium chloride were purchased from Merck.

2.3 Sample preparation and QuEChERS Procedure

For QuEChERS method development, 200 g of the sample was homogenized by a food processor, and the sample was prepared using the original QuEChERS methods. Briefly, 2 g aliquot of the homogenized sample was weighed and transferred into 15 mL centrifuge tubes, then spiked with 50 µL of drug residue standards at 5, 10, 20 µg/kg and was left to stand for 1 hour at room temperature. This was followed by the addition of 10 mL of a mixture of methanol/acetonitrile/ water (20:25.5:4.5, % v/v) to the mixture and the mixture was shaken manually for 1 minute to ensure that the solvent was mixed thoroughly with the entire sample for complete extraction of the analytes. Anhydrous $MgSO_4$ (2 g) and NaCl (0.5 g) were then added, shaken manually again and the mixture was centrifuged for 5 min at 5,000 rpm (Sirhan, Tan, Al- Shunaq, Abdulra'uf, & Wong, 2014). After centrifugation, about 6 ml of the supernatant was transferred into a 15 mL propylene tube containing pre-weighed 900 mg of PSA and 300 mg of anhydrous Mg_2SO_4. The mixture was centrifuged at 5,000 rpm for 5 min and 20 µL of the supernatant was then injected into HPLC for separation and quantification.

2.4 HPLC analysis

A bulk scientific HPLC (BLC-20 Series) isocratic system with variable wavelength coupled to a fluorescence detector was used for the analysis of extracted tetracyclines and tylosin. The chromatographic separation was carried out on pinnacle DBAQ C-18 Column (250× 4.6 mm i.d, 5 µm, Restek USA). The sample 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 45 ℃ at a flow rate of 1.0 mL/min to achieve the optimum resolution of the drug residues. Three replicate injections were made for each sample and the injection volume was maintained at 20 µL for both the sample and the standard solutions. The calibration curves were constructed from an external standard solution. The average peak area of the compound was plotted against the standard concentration.

2.5 Validation procedure

This was carried out using one-factor-at-a-time (OFAT) and experimental design methods. For the OFAT method, sample size, types and volume of extraction solvent, mass of $MgSO^4$, mass of NaCl and centrifugation speed and time were optimized using spike samples. For the multivariate experiment, the Plackett-Burman design was used to determine the significant variables in the QuEChERS-dSPE technique. The various factors and levels that were used for

the design are presented in Table 1. The significant factors were estimated using the Plackett-Burman design. The design matrix was generated using Minitab® statistical software version 17. Beef samples to be used for method development, matrix-matched calibration, and recovery studies were first analyzed to ensure that the target analytes were absent. The matrix-matched calibration curve was constructed using the external standard calibration method. The calibration of each drug standard was constructed at 8 different concentrations using the working standard solution. The concentrations prepared ranged from 5 to 500µg/kg. The peak area obtained for each drug and external standard was integrated and the calibration curve was constructed by plotting the peak area of the internal standard as a function of concentration. Each concentration point was analyzed in triplicate in three different sample matrices. 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 quantitation (LOQ) were determined using the optimum parameters.

3. Results and Discussion

3.1 Optimization of factors using the OFAT method

The QuEChERS extraction conditions were optimized in order to obtain overall optimum conditions. Sample size, sample/water ratio, the volume of acetonitrile, percentage of acetic acid in acetonitrile, mass of $MgSO_4$, mass of NaCl, centrifuge speed and time were all optimized according to Table 1. The factors were optimized by varying one factor at a time and fixing the other factors.

3.2 Plackets-Burman (P-B) design

A multivariate method was developed for the determination of tetracyclines (oxytetracycline and doxycycline) and tylosin residues in beef. Thus, a P-B design was used for analyzing the most important factors affecting QuEChERS efficiency and recovery. These factors analyzed do not give the exact quantities but help to estimate the significant factors affecting extraction efficiency and also give valuable information on each variable with relatively few and reasonable experimental runs (Abdulra'uf, Sirhan, & Tan, 2015).

The factors and their levels selected are presented in Table 2. PBD was used for the determination of the significant factors that help to predict the behavior of other factors and screen out factors that have little or no effect on extraction efficiency (Stalikas, Fiamegos, Sakka, & Albanis, 2009). The experimental runs were carried out according to the design matrix and the total chromatographic peak area (TCPA) was recorded. The P-B design screening was in accordance with the documentation of Fang *et al.*, (2017) and Kong *et al.*, (2012).

The analyzed report for the 12 experimental runs of P-B design for 8 eight (8) factors at two levels each is illustrated in a Pareto chart (Figure 1) and a normal plot (Figure 2) of standardized effects. This is an illustration with horizontal bars for the screened factors showing a red vertical line across the bars, which indicates the level of significance (Lawal, Wong, Tan, Abdulra'uf, & Alsharif, 2018).

Table 1. Optimization of factors using OFAT method

S/N	Factor	Level range	Optimum level
1	Sample size (g)	1-5	2
2	Sample/water ratio	1:1-2:1	1:1
3	Volume of acetonitrile (ml)	5-20	10
4	Percentage of acetic acid in acetonitrile (%)	0.5-1.5	0
5	Mass of $MgSO_4$(g)	1-5	2
6	Mass of NaCl (g)	0.5-2	0.5
7	Centrifuge speed (rpm)	1000-6000	5000
8	Centrifuge time (min)	3-5	5

Table 2. Factors their levels used in P-B design

S/N	Factor	Levels	
		Low (-)	High (+)
1	Mass of sample (A)	2	10
2	Sample water ratio (B)	1:1	2:1
3	Volume of acetonitrile (C)	5	15
4	Mass of Magnesium sulphate (D)	2	6
5	Mass of NaCl (E)	0	2
6	% of acetic acid in acetonitrile (F)	0	2
7	Centrifugation speed (G)	2000	6000
8	Centrifugation time (H)	2	6

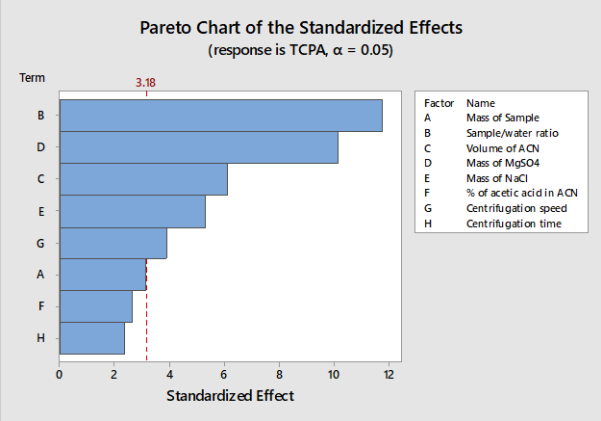


Figure 1. Pareto chart of the standardized effects

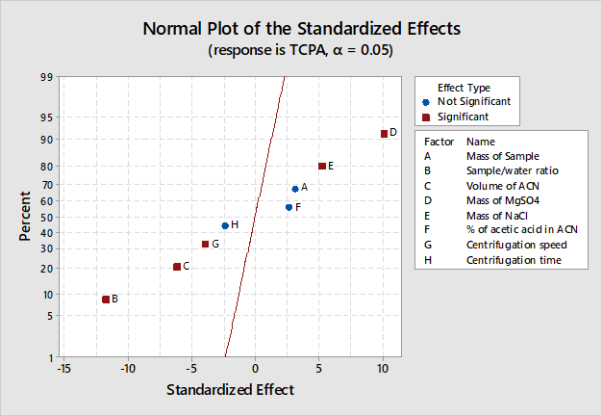


Figure 2. Normal plot of the standardized effects

3.3. Central composite design

It can be observed from Figures 1 and 2 that the mass of the sample, percentage of acetic acid in acetonitrile and centrifuge time did not significantly affect extraction efficiency. Therefore, they were fixed according to the optimal value estimated using the OFAT approach. The volume of acetonitrile, sample/water ratio, mass of $MgSO_4$, mass of NaCl and centrifugation speed, which were found to significantly affect extraction efficiency, were further optimized by the second-order central composite design (CCD), utilizing response surface methodology (RSM). These factors (Table 3), increased the extraction and clean-up efficiencies of the QuEChERS technique (Curbelo, Asensio-Ramos, Herrera-Herrera, & Harnandez-Borges, 2012).

Table 3. Significant factors used for CCD matrix

S/N	Significant factor	Levels	
		Low	High
1	Volume of Acetonitrile (A)	5	20
2	Mass of $MgSO_4$ (B)	2	6
3	Mass of NaCl Ratio (C)	0	2
4	Centrifugation Speed (D)	3000	6000
5	Sample/Water (E)	1:1	1:2

3.4 Figures of merit of analytical methodology

Validation procedures were carried out in order to verify whether the analytical procedure used is suitable. This is very essential in ensuring the optimal utilization of analytical resources (Chan, 2011).

To determine the linearity, 5 concentration levels of each veterinary drug were analyzed and the calibration curve was constructed (Abdulrauf and Tan, 2014). A set of calibration curves were prepared with concentrations ranging from 5 to 500 µg/kg using an external standard calibration method. The calibration curve was linear over the tested concentration range. The linearity ranged over 10 – 500 µg/kg for oxytetracycline, and from 5 to 500 µg/kg for doxycycline and tylosin. The correlation coefficients (r^2) were greater than 0.99 for all the tested drug residues (Table 5).

As presented in Table 5, the inter-day and intra-day precisions of target analytes ranged in 2.31 – 7.41% and 2.78 – 7.13% respectively for the liver sample, in 1.94 – 9.63% and 4.74 – 8.64% for the kidney sample, and in 7.53 – 19.03 % and 7.27 – 9.04 % for the muscle samples. The results obtained in this study agree with the results obtained by Mookantsa *et al.* (2016), who obtained RSD of 5.9 – 6.4% for doxycycline and 7.7 – 9.4% for oxytetracycline using dispersive liquid-liquid microextraction.

Table 5 shows the accuracy (relative recoveries) with the developed method for different samples. Recoveries ranged in 98.07-106.53% for the liver, 89.19-107.78% for the kidney and 79.48-103.77% for the muscle samples. These are all acceptable according to the SANCO guidelines (SANCO, 2013), which state that the method performance criteria require that mean recoveries should be within the range of 70 – 120% with precisions less than or equal to 20%. The average recoveries obtained in this study align with the report of Mookantsa *et al.* (2011), who obtained average recoveries ranging from 83 – 99%, but as a result of the fact that the design of experiments was employed to optimize factors, the optimized factors gave improved results.

3.5 Analysis of real sample

Samples were collected weekly for 12 weeks and the mean ± SD showed that the veterinary drug contents of the samples were all below the maximum residue level (MRL).

In liver samples, 357.96 ± 91.34µg/kg was found to be the highest for oxytetracycline (Oja Tuntun market) on week 11, and 170.19 ± 51.00µg/kg was observed to be the highest for doxycycline (Ipata market) on week 5, and 80.34 ± 14.24µg/kg was the highest for tylosin (Ipata market) on week 8. In kidney samples of beef, 340.80 ± 81.93µg/kg was the highest for oxytetracycline which was found in the Ipata market on week 5, 306.16 ± 72.19µg/kg was also found in the Ipata market to be the highest of the doxycycline on week 11, while 80.24 ± 24.92µg/kg was observed to be the highest of tylosin which was found in Ipata market as well on week 8.

Table 4. Linearity ranges (µg/kg) of the developed QuEChERS method

Drug residue	Linear equation	R^2	Linear range (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
Oxytetracycline	y = 8046.4x – 12296	0.9999	10 – 500	4.79	15.96
Doxycycline	y = 9823.7x – 35391	0.9998	5 – 500	5.02	16.72
Tylosin	y = 10257x – 53695	0.9983	5 – 500	1.45	4.68

Table 5. Accuracy, intra-day and inter-day precisions of the drug residues in beef samples

Analyte	Liver				Kidney			Muscle		
	Added (µg/kg)	Rec. (%)	Inter (%)	Intra (%)	Rec. (%)	Inter (%)	Intra (%)	Rec. (%)	Inter (%)	Intra (%)
Oxytetracycline	100	98.07	7.41	7.13	103.33	1.94	4.74	79.48	19.03	9.04
Deoxycycline	100	106.53	2.82	3.93	107.78	5.19	5.10	103.77	7.53	7.27
Tylosin	100	100.08	2.31	2.78	89.19	9.63	8.64	98.85	10.24	8.38

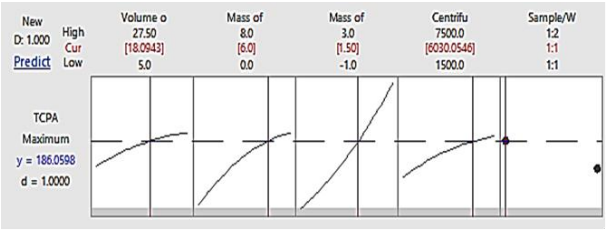


Figure 3. Desirability optimization plot

For muscle beef samples, 102.07 ± 39.52 µg/kg was the highest, which was recorded in the Ipata market on week 9, 104.60 ± 87.23µg/kg was the highest which was also recorded at the Ipata market on week 12 and 78.45 ± 21.10 µg/kg was the highest recorded also at Ipata market on week 12.

The data were further analyzed by comparing the drug residues based on markets. The mean value for oxytetracycline in liver, kidney and muscle varies significantly because the calculated F-value is greater than the critical F-value. Also, the mean values for doxycycline vary significantly for the liver and kidney but do not vary significantly for muscle. More so, the mean value for tylosin does not vary significantly for the liver but varies significantly for the kidney and muscle at $p \leq 0.05$.

4. Conclusions

The importance of frequent monitoring of drug residues in animal-based foods has brought about the development of various sample preparation methods. The QuEChERS method has proven to be fast, accurate and efficient for qualitative and quantitative analysis of drug residues in beef (cow). The advantages of the QuEChERS method over other traditional methods are its low cost, rapid sampling, simple operational methods, being environmentally friendly, and high recovery and enrichment factor. QuEChERS sample can be easily quantified with HPLC or/and GC because the extraction step is the same irrespective of the chromatographic instrument. Food analysis is very important in quality control and assurance. Therefore, the QuEChERS technique described in this study has been shown to be very efficient, effective, versatile and rapid for the analysis of drug residues and contaminants from beef (cow) and other food samples.

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