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Development of Screening Method for Tetracycline Residues in Pork (*Sus scrofa domesticus*) Muscle and Liver Samples

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Antimicrobial residues in pork products are a hazard to public health. They contribute to antimicrobial resistance (AMR) as the exposure of bacteria to trace amounts of these residues can hasten its incidence and emergence. In the Philippines, the quality of meat products is regulated by the National Meat Inspection Service (NMIS) through antimicrobial sensitivity testing (AST) *via* disc diffusion. Analytical methods such as the use of high-performance liquid chromatography (HPLC) may also be used in monitoring antibiotic residues in meat samples. This experimental study aims to develop a screening method for tetracycline residues in pork (*Sus scrofa domesticus*) muscle and liver from public wet markets; and to determine the feasibility of this method based on specified critical parameters such as LOD, LOQ, and linearity. Twenty (20) pork muscle and 14 pork liver samples were collected from stalls in three public wet markets and were subjected to AST through the NMIS and HPLC analysis. The HPLC analysis utilized an isocratic elution with a mobile phase of 85:15 0.1% formic acid in acetonitrile: 0.1% formic acid in water and a flow rate of 1.0 mL/min. The detection of tetracycline was observed at peaks with a retention time of 3.95 ± 0.15 min. The determined retention time was used to identify tetracycline in the pork samples. AST results showed no presence of tetracycline in all samples. HPLC results, however, revealed that two out of 20 pork muscle samples and eight out of 14 pork liver samples contained tetracycline. The results have demonstrated that instrumental methods of analysis such as HPLC are necessary to confirm the absence or presence of the residues and to quantify the concentration to compare it to the established MRL. Further validation studies are recommended to ensure the accuracy and specificity of the optimized method.

Keywords: antibiotic sensitivity testing, antimicrobial residues, HPLC, pork samples, tetracycline

INTRODUCTION

AMR requires action across all government sectors and society. The misuse and overuse of antimicrobial drugs have hastened the process of AMR. Their use in veterinary medicine, as well as in agriculture, has been identified as a significant contributor to the increase in the development of AMR (WHO 2017). Antimicrobials are heavily used by the swine industry for growth promotion and prophylaxis (Teillant 2015). This practice in the swine industry can directly affect Filipinos. It was reported that in 2019, they consume about 14.7 kg *per capita* of pork, more than the world's average pork consumption of 12 kg/capita (OECD 2019).

The World Organization for Animal Health (OIE) has provided the first global and regional analysis of the use of antimicrobial agents in animals in 2018. The 116 participating

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countries – including the Philippines – reported tetracycline, with a proportion of 34.55%, as the most commonly used antimicrobial class in terrestrial food-producing animals. Tetracyclines were also reported as having the largest proportion of quantities (at 31.2%) for antimicrobials used in Asia, the Far East, and Oceania (OIE 2018).

In a review conducted by Schwarz *et al.* (2001), it was reported that tetracyclines account for 65.66% of all antimicrobials used as therapeutics regimens in veterinary medicine – 2294 tons out of 3494 tons. Tetracyclines are applied in veterinary medicine for treatment and prevention of microbial infections. Most widely used within this group are tetracycline, oxytetracycline, and doxycycline.

The Philippine government has come up with an action plan in order to combat antimicrobial resistance in the country. One of its key strategies is to strengthen surveillance and laboratory capacity. They strongly believe that the implementation of an effective monitoring system from the point of slaughter up to the processing of animals, as well as the implementation of surveillance systems focusing on AMR mitigation strategies in animals, is highly essential (DOH 2016).

In accordance with this, different parties led by the NMIS have adapted the Philippine National Standards (PNS) for Veterinary Drug Residues in Food: Maximum Residue Limits (MRLs). This has set the maximum allowable amount of antimicrobial residue safe to consume for humans. According to this, the MRL for tetracycline in pork muscle, liver, and kidney tissues were 200, 600, and 1200 µg/kg, respectively (BPS 2007).

In general, the analytical methods for monitoring antibiotic residues can be classified into two: confirmatory and screening (Cháfer-Pericás *et al.* 2010). The purpose of a screening test is to select samples, which contain one or more residues, to be analyzed with more sophisticated methods (Ferrini *et al.* 2006). The use of HPLC is an efficient separation technique that is fast and reliable with high sensitivity for the analysis of trace residues. The efficiency of analysis varies according to chromatographic conditions and the type of detector used. Ultraviolet detectors have been described to be used for antimicrobial residues analysis (Aman *et al.* 2017).

MATERIALS AND METHODS

Materials

The materials used in this study were pork muscle and liver samples, 0.1 M ethylenediaminetetraacetic acid (EDTA), propylene test tubes, methanol [liquid chromatography – mass spectrometry (LC-MS) grade], formic acid, acetonitrile (LC-MS grade), and distilled water. Tetracycline hydrochloride with 98% purity was purchased from Toronto Research Chemicals. The HPLC instrument used a Shimadzu LC/MS 2020, LabSolutions® software, UCT C18 LC column (column size 4.6 x 150 mm, and pore size of 5 µm) with a photodiode array (PDA) detector system. The materials for the disc diffusion AST were provided by the NMIS.

Sample Collection

Muscle samples were collected from twenty stalls while liver samples were collected from fourteen stalls in three public wet markets in Quezon City, Philippines. The samples were coded accordingly and stored in a cooler immediately after procurement. A 250-g portion of each sample was transported to the NMIS for AST *via* disc diffusion method and another 125-g portion was transported to the UP College of Pharmacy Biochemistry Laboratory for extraction and HPLC analysis.

AST

The entire testing was performed by the NMIS laboratory. The procedures followed the current Laboratory Instruction for the Veterinary Drug Residue Section of NMIS (2010a, b).

HPLC Analysis

Method optimization. The method was optimized by the manipulation of different chromatographic parameters. Both gradient and isocratic elution were assessed, and the ratio of the mobile phases was adjusted. To arrive at the optimized method, the parameters of the width of peak, retention time, tailing factor, presence of interfering peaks, plate height, and plate count were used. Using the optimized method, the retention time for tetracycline was determined by analysis of a commercially available preparation of tetracycline and tetracycline standard solutions.

Linearity, LOD, and LOQ. The linearity, limit of detection (LOD), and limit of quantitation (LOQ) were determined by creating a standard curve from 500.0, 25.0, 10.0, 1.0, 0.5, and 0.2 µg/mL concentrations of tetracycline. The equation of the line and linearity coefficient was determined from the data generated from the standard solutions. Likewise, the LOD and LOQ were determined from the standard deviations of the y-intercept of the linear regression curve produced from each trial reading.

Column efficiency. Column efficiency was determined by gathering data on plate count and tailing factor generated by the instrument and computing for the plate count. The data for plate count and tailing factor were generated using the data analysis capability of the LabSolutions®. Plate

height was computed from the length of the column and plate count. All chromatograms of the standard solutions and samples that contain tetracycline residues were used for the determination of column efficiency.

Instrumentation. The optimized method of HPLC used was a binary mobile phase with an isocratic elution with a run time of 8 min. Mobile phases A and B were composed of filtered distilled water and acetonitrile, respectively. These were acidified with formic acid to a concentration of 0.1%. The ratio of mobile phase A: mobile phase B was 85:15. The mobile phase flow rate was 1 mL/min. HPLC analysis was performed on a C18 column, with a dimension of 4.6 x 150 mm, a pore size of 5 µm, and temperature maintained at 40 °C. The detector used was a PDA detector at a wavelength of 280 nm. The injection volume was 20 µL.

Sample preparation. From a 125-g sample previously thawed and homogenized, a 3-g portion was taken and transferred to a 20-mL polypropylene test tube. A 200-µL 0.1-M aliquot of EDTA was added. Samples were mixed and allowed to stand in the dark for at least 15 min. The tetracycline residue was extracted from the tissues using 15 mL of 70% methanol in distilled water, with 10 min of shaking. The samples were centrifuged at 3800 rpm for 5 min. The supernatant liquid was then transferred into a pre-calibrated plastic petri dish and was concentrated to 1.5 mL through solvent evaporation for 1 h. The concentrated liquid was then filtered through a 0.20-µm pore size syringe nylon filter, and 1 ml of the liquid was transferred into a sample vial. The sample vial was refrigerated and analyzed within 24 h.

Sample analysis. The solutions were injected into the HPLC instrument in three replicates. A solvent blank was used to correct for possible solvent responses. The resulting area under the curve and retention time corresponding to tetracycline (tR = 3.95 ± 0.15 min) for each solution injected was recorded. The concentration of tetracycline was expressed in mean values with a %RSD. The concentrations of tetracycline in all pork muscle and liver samples were compared with the MRL of tetracycline in pork muscle and liver, respectively, as specified by the PNS for the Veterinary Drug Residues in Food.

RESULTS

AST

Out of the 20 muscle samples and 14 liver samples submitted to the NMIS, no zone of inhibition was observed in all samples. It was reported that there were no detectable amounts of tetracycline residues in all samples submitted for AST *via* disc diffusion.

HPLC Analysis

Method optimization. The wavelength of maximum absorbance was determined to be 280 nm. It was observed that the peaks of tetracycline eluted at the retention time of 3.95 ± 0.04 min. The peaks corresponding to tetracycline at the determined retention time was observed to increase as the concentration of the standard increase (Figure 1; Table 1).

Linearity, LOD, and LOQ. The standard curve of tetracycline was constructed (Figure 2). The determined %RSD for all concentrations were within the range of 0.66–9.64%. The equation of the line was computed at y = 56746x – 160750 with an R^2 value of 0.9995.

Column efficiency. Column efficiency was observed based on plate height, plate count, and tailing factor (Table 2). It can be observed that the standard has a higher column efficiency than the sample.

Sample analysis. Two out of 20 muscle samples (Figure 3; Table 3) and eight out of 14 liver samples (Figure 4; Table 4) contained tetracycline based on retention time. This was observed in all three public wet markets, where most were detected on liver samples.

DISCUSSION

AST

The reports from the NMIS showed the absence of tetracycline from all the samples. The minimum zone of inhibition for tetracycline is 14 mm for samples to test positive. However, a study by Shahbazi *et al.* (2015), where a zone of inhibition of ≥ 2 mm was considered positive, revealed that a certain concentration of the residue can already be quantified at this measurement. Inhibition zones in microbial diffusion tests can be influenced by other parameters such as agar depth, length of incubation and of pre-incubation, batch preparation, and the length of storage. In addition, inhibition zones were basically dependent on the nature of the inhibitor or the antimicrobial present and on its concentration (Ferrini *et al.* 2007). It was possible that low concentrations of antibiotics (*i.e.* at MRL levels) in tissues were not detected by the AST. This was one of the problems associated with microbial diffusion tests – their lack of sensitivity, which usually results in a false positive or a false negative report (Cháfer-Pericás *et al.* 2010). Thus, a confirmatory assay such as liquid chromatography is required for confirmation and quantification of screening test results (Shahbazi *et al.* 2015).

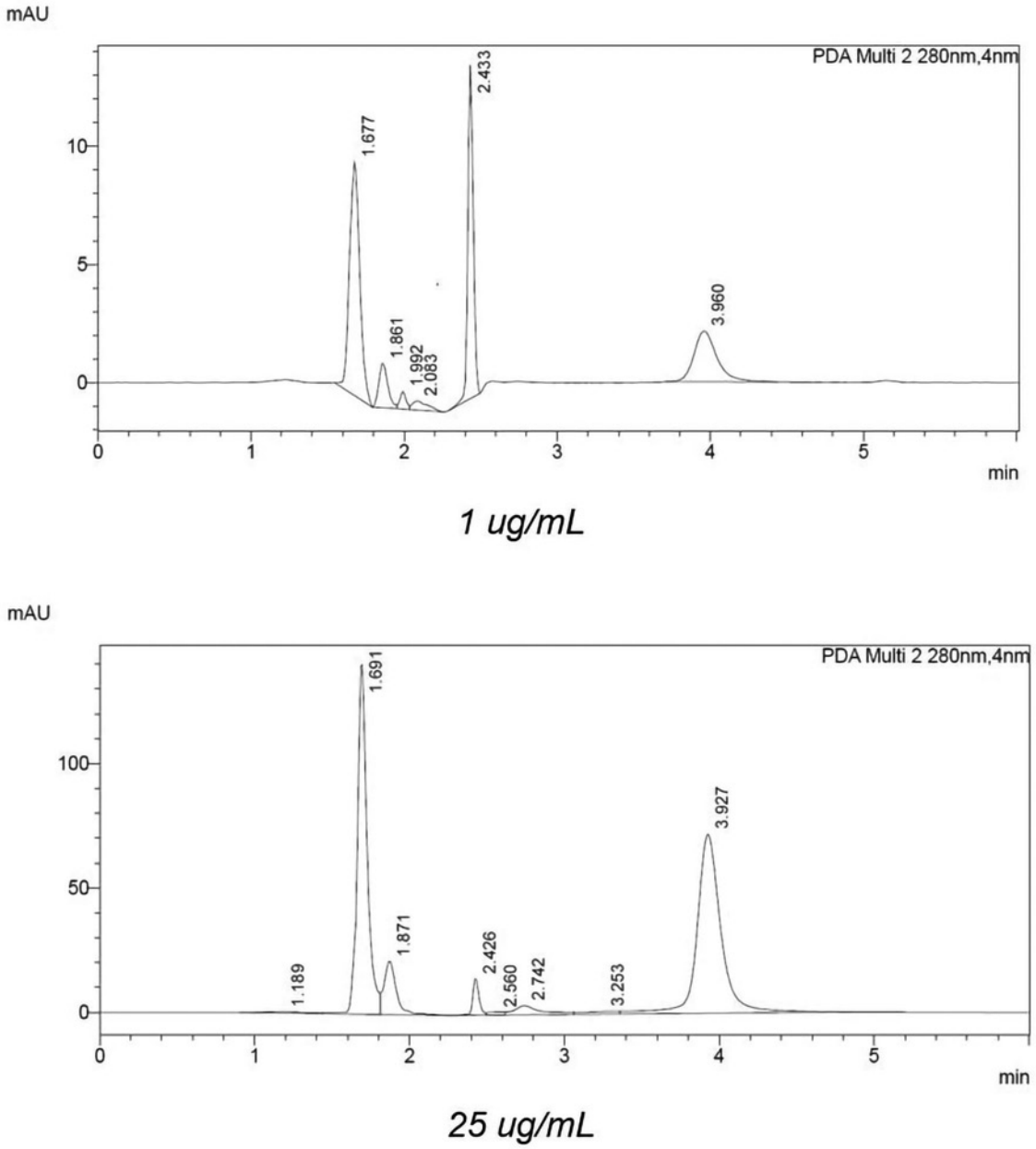

Figure 1. HPLC chromatograms of tetracycline standard solutions observed at 280 nm (tR = 3.95 ± 0.15 min).

Table 1. HPLC responses of the tetracycline standard solutions determined at 280 nm.

Concentration (µg/mL)	Area under the curve (mAU, mean ± % RSD)	Retention time (min, mean ± %RSD)
500.0	2.8238 x 10^7 ± 0.9800	3.851 ± 0.2100
25.0	7.8584 x 10^5 1± 1.7200	3.938 ± 0.0820
10.0	2.7145 x 10^5 ± 6.9400	3.964 ± 0.1530
1.0	2.1969 x 10^4 ± 1.7700	3.950 ± 0.2350
0.5	8.7190 x10^3 ± 0.6600	3.949 ± 0.2270
0.2	4.2560 x 10^3 ± 9.6400	3.920 ± 0.2870

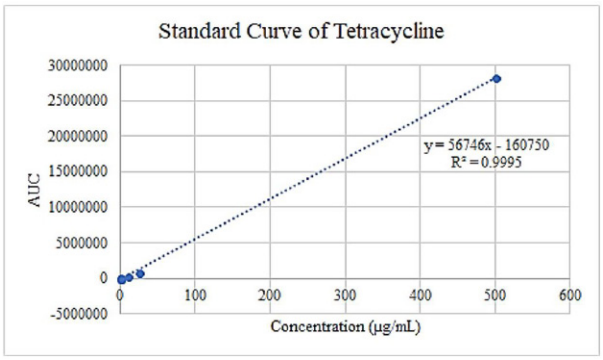

Figure 2. Linear regression curve of tetracycline standard at concentrations 0.20, 0.50, 1.00, 10.00, 25.00, and 500.00 µg/mL.

Table 2. Column efficiency data expressed as plate height, plate count, and tailing factor on standard and sample solutions.

Parameter	Standard	Sample
Plant height (mm)	0.0409	0.8129
Plate count	3891.0000	2057.0000
Tailing factor	1.3048	1.1416

interfering peaks. A commercially available dosage form of tetracycline was analyzed, and the retention time was determined to be at 3.95 min. This corresponds to the peak detected in both the standard solutions and the dosage form that was used for testing.

Linearity, LOD, and LOQ. Critical parameters of linearity, LOD, and LOQ were determined. The linearity of the chromatographic response was checked using the standard solutions of tetracycline using five concentrations from 0.2–500.0 µg/mL. The linear regression curve showed good linearity with an R^2 value of 0.995 over the range of 0.2–500.0 µg/mL. The computation for the LOD and LOQ were taken from the ICH Q2 (2005), which made use of the standard deviation of the y-intercepts of the regression lines. The computed Instrumental detection limits for LOD and LOQ were 23.9 µg/kg and 72.3 µg/kg, respectively. According to the Bureau of Product Standards (2007), the MRL of tetracycline was 200 µg/kg in pork muscle and 600 µg/kg in pork liver. The MRLs in both pork muscle and liver were within the LOD and LOQ, which demonstrated that the method could

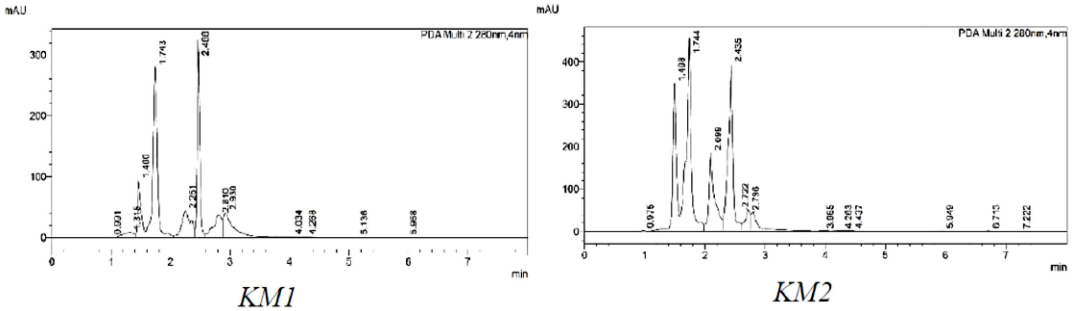

Figure 3. HPLC chromatograms of pork muscle samples observed at 280 nm (tR =3.95 ± 0.15 min).

Table 3. Retention time and concentration of tetracycline in pork muscle samples (n = 3).

Sample	Retention time (min, mean ± %RSD)	Area under the curve (mAU, mean ± % RSD)	Concentration (µg/kg, mean ± %RSD)
Standard	3.9500 ± 0.2350	2.1969 x 10^4 ± 1.7700	1.0000 (ug/mL)
KM1	4.0440 ± 0.3320	1.4312 x 10^4± 95.8400	1542.51 ± 8.8400
KM2	3.9620 ± 0.0760	3.9970 x 10^3± 1.0100	1451.61 ± 0.0200

HPLC Analysis

Method optimization. The determined wavelength of maximum absorbance for tetracycline was 280 nm. This was also the wavelength of detection used in the assay for tetracycline (USP 2012). The optimization of the chromatographic method was aimed to maximize sensitivity and minimize runtime. This resulted in an isocratic elution using a binary mobile phase. The short retention time lead to a peak, which was free from quantitatively determine the low levels of compounds in the sample matrices.

Column efficiency. The US Food and Drug Administration (2015) has set values for analytical method performance requirements: tailing factor limit should be equal to or less than 2, and the plate count should be more than 2000. Column performance needs to be sensitive, reproducible, and robust. The tailing factor affects the accuracy of the quantitation. The tailing factor from the determination

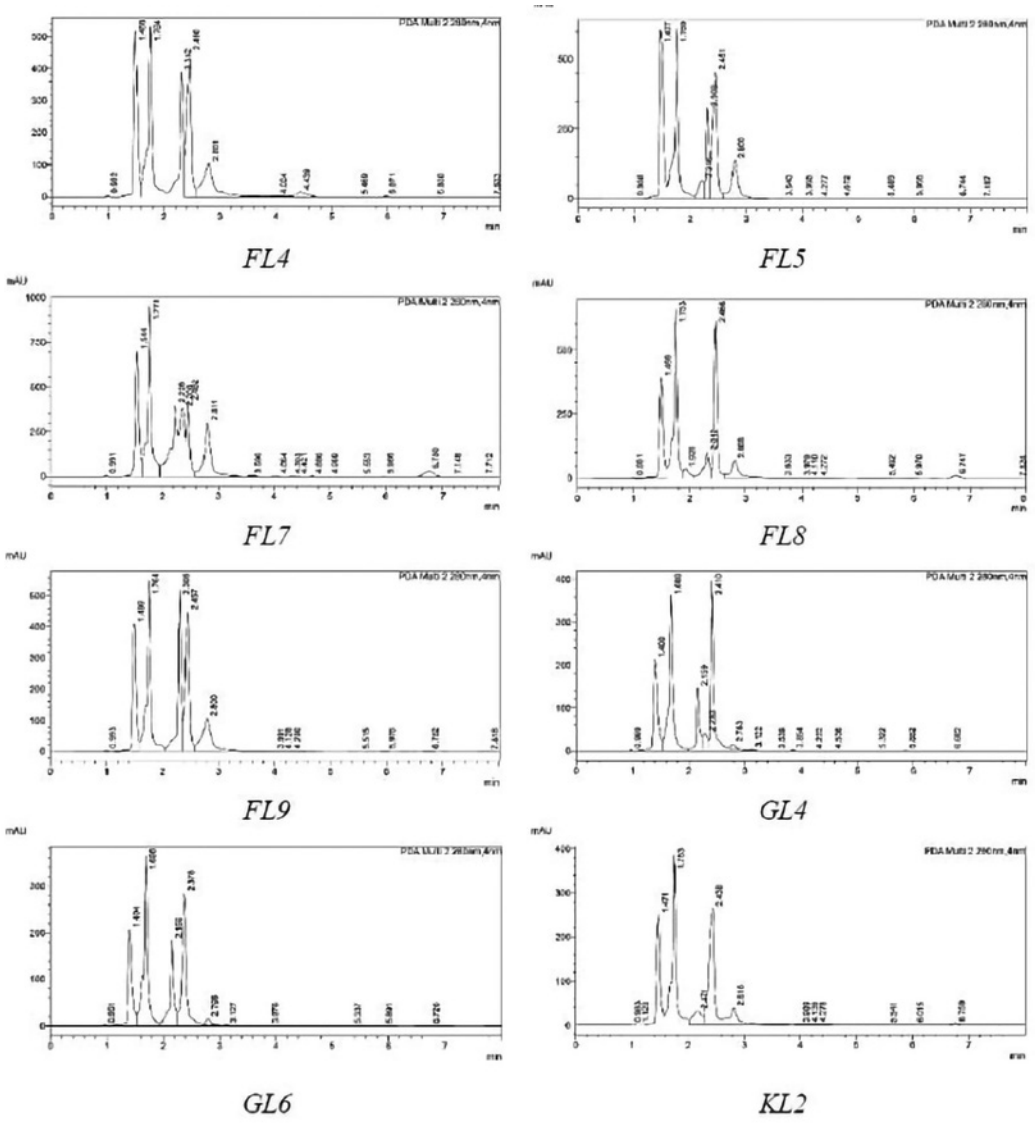

Figure 4. HPLC chromatograms of pork liver samples observed at 280 nm (tR =3.95 ± 0.15 min).

Table 4. Retention time and concentration of tetracycline in pork liver samples (n = 3).

Sample	Retention time (min, mean ± %RSD)	Area under the curve (mAU, mean ± % RSD)	Concentration (µg/kg, mean ± %RSD)
Standard	3.9500 ± 0.2350	2.1969 x 10^4 ± 1.7700	1.0000 (ug/mL)
FL4	4.0290 ± 0.1250	1.5560 x 10^3 ± 4.6900	1430.1001 ± 0.0400
FL5	3.9980 ± 0.1440	6.6370 x 10^3 ± 11.5900	1474.8800 ± 0.4600
FL7	4.0610 ± 0.1030	1.4001 x 10^3 ± 2.7800	1539.7600 ± 0.2200
FL8	3.9720 ± 0.1600	3.8350 x 10^3 ± 4.4100	1447.5300 ± 0.1000
FL9	3.9940 ± 0.1090	1.9940 x 10^3 ± 3.1100	1433.9700 ± 0.0400
GL4	3.8450 ± 0.4660	1.1924 x 10^4 ± 0.8900	1521.4700 ± 0.0600
GL6	3.8720 ± 0.1160	5.1070 x 10^3 ± 2.6400	1461.4000 ± 0.0800
KL2	3.9750 ± 0.0120	2.1710 x 10^3 ± 4.8600	1435.5300 ± 0.0600

of columns efficiency for both the standard solutions and the sample solutions were found to be less than 2, which is an acceptable value. The plate count, however, determines how many peaks can be located per unit runtime. The computed plate count was more than 2000, which is acceptable.

Sample analysis. Two out of 20 muscle samples were found to contain tetracycline residues, detected at levels ranging from 1452–1542 µg/kg. Consequently, eight out of 14 liver samples contained tetracycline residues from 1430–1539 µg/kg. The identification of tetracycline residues was done through the retention time. All positive samples exceeded the tetracycline MRL of 200 and 600 µg/kg for pork muscle and liver, respectively. Tetracycline residues were found more on liver samples than in muscles, which can be attributed to the accumulation of tetracycline in excretory organs such as the liver. This raises concern towards the public to consume pork liver more carefully (Shahbazi *et al.* 2015). A study done by Ramatla *et al.* (2017) observed that although antibiotic residues were detected from all organs, the liver stood out as the organ with the highest detection level at 30%. This was interpreted to be the result of the liver's role in metabolism and detoxification processes.

The detection of tetracycline in pork was not surprising since, according to the OIE (2018), one of the largest proportions of all reported antimicrobial classes used in food-producing animals in Asia and the Pacific was tetracycline. In the Philippines, tetracycline was also being constantly monitored by the NMIS, among five other antibiotic classes. These suggest that tetracycline may have been extensively used in pig farms in the country. The results of the AST, however, were not sensitive enough to provide a more accurate approach to the monitoring of tetracycline use. In this study, the HPLC analysis was able to detect 11 false-negative results obtained from AST, which is equal to a frequency of 29.41%. This high false-negative rate yielded by the AST suggests the use of more sensitive and quantitative analyses such as HPLC, among others, is needed. However, despite the advantages of the HPLC, a validated method should be in place for the proper identification and quantification of residues.

CONCLUSION

Results obtained from the HPLC analysis compared to the AST *via* disc diffusion have demonstrated that instrumental methods are necessary to detect trace amounts of drug residues in pork (*Sus scrofa domesticus*) muscle and liver samples.

This study has demonstrated that an instrumental method of screening tetracycline residues in pork muscle and liver may prove to be a useful adjunct to currently available methods of determining tetracycline residues. Results of the AST *via* disc diffusion showed that no samples contained detectable amounts of tetracycline based on the observation of the zone of inhibition. However, HPLC analysis revealed that two out of 20 pork muscle samples and eight out of 14 pork liver samples contained tetracycline residues. The MRL was exceeded in all 10 samples.

The developed method could establish better performance characteristics with further validation. The developed HPLC analysis requires further optimization and validation studies to determine the extent of its accuracy and specificity in determining the presence of tetracycline residues in pork muscle and liver. Recovery studies can be performed to further validate the specificity and sensitivity of the method. Column efficiency could further be improved by lessening the tailing factor and improving plate count – this could be done by varying the proportion and flow rate of the mobile phase.

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