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Fast and Simple Extraction for LCMSMS Analysis of Oxytetracycline Residue in Pork

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Abstract

Pork is very popular as an animal-base protein source of peoples in the Lao PDR. The pig growth promotion, treatment and prevention of diseases frequently relies on the use of antibiotics. In terms of food safety, the analysis of antibiotic residue in pork would be therefore important. Oxytetracycline (OTC) is one of antibiotics used in the pig husbandry. In this study, a fast and simple extraction and analysis method for determining OTC residue in pork was developed. Briefly, 1.0 g of homogenized pork sample in a 50mL-centrifµge tube was extracted with 10 mL of CH_3OH: H_2O mixture (9:1 ratio) assisted with vortex for 10 min, treated with QuEChERS salt and subjected to centrifugation at 5000 rpm. The methanolic extract (1.5 mL) was then cleaned up with 2mL-dSPE tube (containing $MgSO_4$, PSA, and C18), filtered through syringe filter 0.45 micron and analyzed with ESI-LCMS/MS optimized and operated in MRM positive mode. Calibration was prepared with OTC standard solutions (5-100 ng/g). The analytical method was validated with triplicated recovery tests at a spiked level of 50 ng/g. The results unveiled that the method gave very good recovery of 88.7±3.24%. The limits of detection (LOD) and quantification (LOQ) were achieved with 0.36 ± 0.02 and 1.19 ±0.04 ng/g. In consequence, the developed method would be suitable for OTC determination in pork samples.

Keywords: *Antibiotic residue, Oxytetracycline, Pork, LC-MS/MS Analysis.*

1. Introduction

Oxytetracycline (OTC; $C_{22}H_{24}N_2O_9$; Figure 1.) is widely used in veterinary practice as feed additives for growth promotion because of their broad range of activity against bacteria and low cost (Wang et al., 2008), OTC is a broad-spectrum antibiotic that inhibits protein synthesis of susceptible bacteria (Manna et al., 2021). The tetracycline antibiotic class is generally used in veterinary medicine to treat a number of infections, among which oxytetracycline is the most commonly used. Antibiotics/metabolites accumulate in different tissues such as muscle, liver, and kidney due to uncontrolled use of these medications not only for therapy but also for prophylaxis (Verma, 2022). However, OTC residues and degradation products of these substances have many undesirable side effects on the human body, It negatively influences the immune system, affects the human reproductive organs bone and teeth problems in children, gastrointestinal disturbance and hypersensitivity reactions, carcinogenic effects have been found with antibiotics (Frida et al., 2017; Treiber & Beranek-Knauer, 2021). To protect humans from harmful effects of veterinary drug residues in animal-derived food sources, the United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have set standards for maximum residue limits in foods. These limits apply to the parent drug or chemical and its metabolites that may accumulate and be deposited or stored within the cells, tissues or organs following administration of the compound. The acceptable maximum residue limits for tetracycline-based compounds, including chlortetracycline and oxytetracycline, are set at 200 µg/kg, for pig derived muscle, liver and kidney, respectively (Codex Alimentarius, 2018). But unfortunately, it is easily available in Laos and broadly used for livestock and aquaculture (Poupaud et al., 2021). In order to monitor and strict control, the residual level of OTC, sensitive and accurate analytical methods are needed. Capillary electrophoresis, gas chromatography–mass spectrometry (GC-MS), gas chromatography with electron capture detection (GC-ECD), radio immune assay and enzyme immunoassay, microbiological methods, liquid chromatography-tandem mass spectrometry (LC-MS/MS) etc. were used for the screening, confirmatory, and other analytical methods for determining OTC in earlier days (Li et al., 2020). Capillary electrophoresis method is not suitable for routine analysis due to low precision (Colombo & Raffaella, 2019). Methods involved GC-MS, GC-ECD Derivatization of analytes is often required to make analytes to be a volatile derivative when GC is employed to detect veterinary drug residues, GC is not often used for the determination of veterinary drugs because of the requirement of derivatization (Li et al., 2020). Immunoassay method is advanced method for analysis of OTC, but this method is not suitable due to the possibility of obtaining false-positive results arises from matrix interference (Asif Hebbal et al., 2020). Microbiological assays methods are the screening methods employed for qualitative or semi-quantitative detection of antibiotic residues (Asif Hebbal et al., 2020). Liquid chromatography-tandem mass spectrometry methods have been widely used nowadays for analysis of antibiotic residues due to high sensitivity and selectivity. So, the aim of this study was to develop a fast, reliable and user-friendly LC-MS/MS. Coupled with electrospray ionization (ESI) and triplicate mass analysis (QQQ) systems and confirmation methods for the identification and quantification of OTC residues in meat samples. Collected from local markets and supermarkets in Vientiane. With good selectivity, high sensitivity, precision resolution, accuracy and validation to control the safety of food for human consumption.

2. Materials and Methods

2.1 Chemicals and reagents

Certified standard, that is, Oxytetracycline hydrochloride (OTC, $C_{22}H_{24}N_2O_9$. ClH) purchased from Sisco Research Laboratories Pvt. Ltd, India, Ethylene Diamine Tetra Acetic Acid Disodium salt (EDTA di-sodium salt) purchased from Ajax Finechem, Australia, Magnesium Sulfate ($MgSO_4$), Sodium Chloride (NaCl), Trisoduium citrate dehydrate, Disodium hydrogen citrate sesquihydrate purchased from Restek, USA, Formic acid, LC-MS/MS grade acetonitrile (ACN) and LC-MS/MS grade methanol were purchased from Fisher Scientific, Korea , C18 and Primary secondary amine (PSA) was purchased from Restek, USA, De-ionised water (18.2 MΩcm)

2.2 LC-MSMS system and conditions

Analysis was performed using liquid chromatography–mass Spectrometer (LC-MS/MS, Model Shimadzu-8045) coupled with ESI interface and QQQ mass analyzer. Ultra-fast liquid chromatography (LC; Model- Shimadzu Prominence) system contained degassing unit (DGU-405), solvent delivery module (LC-40DXS), auto sampler (SIL-40DXS)), column oven (CTO-40C), N_2 gas (drying & nebulizing gas), air (heating gas), and Ar gas (collision gas) were used for sample analysis.

Chromatographic separation of OTC was achieved using Kintex 2.6u XB-C18 100A reversed-phase column (100 mm L × 2.1 mm i.d) operated at column oven temperature of 40℃. Mobile phases: A (H_2O + 0.1% formic acid) and B: ACN were used. The flow rate of the mobile phase was 0.5 ml/min. The injection volume was 5 µl. A mobile phase operated in gradient elution with program start 0-1 min 0% of mobile phase B, 2-4 min 25% of mobile phase B, 5- 8 min 100% of mobile phase B, 8.50-10 min 0% of mobile phase B was performed.

The ESI interface was used and operated in positive mode. The flow rate of nebulizing gas 3 L/min, drying gas 10 L/min, heating 10 L/min, respectively. The temperature of interface, desolvation line, and heat block was 300℃, 250℃, and 400℃, respectively. The optimization to find the optimum energies for the multiple reaction monitoring (MRM) mode for quantification for OTC was performed.

2.3 OTC standard preparation

OTC stock solution of 1000 mg/L was prepared by taking 0.01 g OTC standard in 10 ml volumetric flask followed by making up to the mark with deionized water (H_2O), and then OTC primary standard solution of 1 mg/L was prepared consequently. Finally, a series of OTC working standard solution with concentrations in the range of 5–100 ng/L was prepared. To study the effect of matric, a mixture of CH_3OH: H_2O at a 9:1 v/v ratio was used to prepare the matrix solvent for standard preparation (Matrix-Matched calibration solution).

2.4 Extraction

A representative portion of the defrosted sample (20 g) was weighed and mixed with 25 mg of EDTA per gram sample. The sample and the EDTA were homogenized for 1 min using a blender,(Frida et al., 2017), Homogenized samples 1g with were extracted by using quick, easy, cheap, effective, rugged, and safe (QuEChERS) method with some modification. Briefly, 1.0 g of homogenized pork sample in a 50mL-centrifµge tube was extracted with 10 mL of CH_3OH: H_2O mixture (9:1 ratio) assisted with vortex for 10 min, treated with QuEChERS salt and subjected to centrifugation at 5000 rpm. The methanolic extract (1.5 mL) was then cleaned up with 2mL-dSPE tube (containing $MgSO_4$, PSA, and C18), filtered through syringe filter 0.45 micron and analyzed with ESI-LCMS/MS optimized and operated in MRM positive mode (Figure2.).

2.5 Analytical method validation

The method was validated by following the EU Commission Decision, 2002/657/EC (COMMISSION DECISION, 2002).

Specificity was confirmed by injecting control samples extract and selectivity was evaluated by analyzing standard OTC, blank matrices, and sample matrices spiked with OTC simultaneously and monitoring retention time.

Unwanted components interfering with analytes were analyzed by comparing the chromatogram of the standard OTC, blank matrices, and matrices spiked with OTC.

Limit of detection (LOD) and Limit of Quantification (LOQ) was estimated by statistical method (Gunawan, 2018).

Linearity was carried out at five concentrations ranging from 5 to 100 μg/kg. A calibration curve was constructed by plotting the peak area versus concentration.

The accuracy was evaluated in terms of percentage recoveries of each sample which calculated from matrix-matched calibration curve and matrix effect was calculated by comparing with that of calibration curve of standard OTC with mobile phase and matrix-matched. For recovery experiment, meat sample (2 g) was taken in Teflon tube. Samples were spiked with OTC standard solutions at 50 μg/kg levels for repeatability (intra-day) and reproducibility (inter-day), and the sample was allowed to stand for 1 hr. to let the OTC to be absorbed into the samples. The precision of the method was estimated by determining the relative standard deviation (RSD).

Matrix effect (%) was calculated with reference to peak area of matrix of control sample and peak area of standard solvent. To evaluate matrix effect, matrix-matched calibration was used

3. Results

3.1 Mass spectrometric Detection and Optimization

At first, in this analysis precursor ion (461) was identified using flow injection method in Q3 scan mode without using any collision energy (Figure3.). The parent and product ions were first optimized by using an OTC standard solution of 250 μg/kg in both positive and negative polarity mode. Due to deprotonation of OTC, the intensity of precursor ion was much higher in positive mode. After that, optimization of the MS/MS parameters was performed (Table 1). Three characteristic fragmentations of the product ions (426, 443, and 444) were monitored applying collision energy using MRM event optimization method (Figure 3). Later separations were performed by passing sample through LC column where retention time of OTC was 2.79 min (Figure 3B).

3.2 Chromatographic Separation

Most of the previous LC methods of Oxytetracycline analysis studied used C18 silica particles as the stationary phase. A C8 stationary phase was reported in very few papers(Ewelina & Kwiatek, 2021; Kardani et al., 2023). Thus, the influence of stationary phase type (C18 and C8 silica particles) on penicillin separation was evaluated. In this study chromatographic columns of Kintex 2.6u XB-C18 100A reversed-phase column (100 mm L × 2.1 mm i.d) were evaluated for the separation of Oxytetracycline from a mixture of standard solutions. gradient elution conditions were evaluated with columns. The parameters of width of the peak, resolution and retention factors were used to select the best separation procedure. Thus, the Kintex 2.6u XB-C18 100A column was selected for LC–MS/MS studies. Under these conditions, the run time for the separation of the Oxytetracycline obtained in LC–MS/MS was approximately 10 min.

3.3 Extraction and Method validation

3.3.1 Linearity

The linearity of the chosen method was evaluated by using blank pork samples spiked with standard solutions to construct calibration curves, which were populated with the area under the curve versus the concentration at five levels. The calibration curves were generated for each oxytetracycline in both pork samples, and they showed excellent linearity within the concentration ranges that are the linearity was checked for standard solutions containing total OTC in the range from 5 to 100 μg/kg. The square of the correlation coefficient (R^2) for OTC was 0.9992 and 0.9998 for the matrix-matched and standard calibration curve, respectively. LOD and LOQ values 0.36 and 1.19 μg/Kg, respectively.

3.3.2 Accuracy and precision

The recoveries of the OTC ranged between 88.73±3.24%. The precision of the method was estimated by determining the RSD calculated from results generated under the intra-day (n = 3) and inter-day (n = 9), and RSD was found in the range between 1.67% and 3.91% (Table 2). And MRM chromatograms was performed (Figure4.)

3.3.3 Matrix effect

For the OTC, the matrix enhancement effects were mild in the 17.85 % From this result, it was concluded that samples matrix interfered with the detection of OTC. So, matrix-matched calibration curves were used for quantitative analysis. was performed (Figure5.)

4. Discussion

The development of techniques comfort-table for routine analysis has been rendered feasible by OTC evaluation in pig samples. because the analytical method was validated with triplicated recovery tests at a spiked level of 50 μg/kg. The results revealed that the method gave an excellent recovery, Considering that very low limits of detection (LOD) and limits of quantitation (LOQ) were reached and the matrix enhancing effects were not severe, to compared with other studies looking at residues, previous studies found that, such as Developing a Highly Validated and Sensitive HPLC Method for Simultaneous Estimation of Oxytetracycline in Their Dosage Forms, the limits of detection were reported to be 0.07 μg/ml for oxytetracycline, and 99.96 ± 0.98 was found recovery (Sebaiy et al., 2019), Oxytetracycline residue levels in beef in Dodoma region, Tanzania, The detection and quantification limit were 18.2 and 54.6 ng/g, respectively(Frida et al., 2017), Determination of some antibiotic residues (tetracycline, oxytetracycline and penicillin-G) in beef sold for public consumption at Dukem and Bishoftu (Debre Zeyit) towns, central Ethiopia by LC/MS/MS, The average recovery was very satisfactory, ranging from 97.52 % to 111.34 % (Uma & Ashenef, 2023), Determination of tetracycline, oxytetracycline and chlortetracycline residues in seafood products of Saudi Arabia using high performance liquid chromatography–Photo diode array detection the RSD was less than 2%, while the recovery was 95–105% (Alanazi et al., 2021), The People's Republic of China determined OTC in milk recovery was 84.2% and the limits of detection (LOD) was 5 g/kg (Shi et al., 2020). The literature doesn’t have a report on the determination of OTC in Laos, but this developed method can be applied to the determination of OTC in Laos PDR because it is sufficiently sensitive and reproducible in the routine analysis of OTC in pork within a short period of time.

5. Conclusion

A mixture of methanol-water at a ratio of 9:1 provided efficient extraction of OTC from the pork sample. Selecting a suitable extracting solvent depends on the solubility of the analyte in the solvent. The small amount of water presented in the solvent mixture could be ignored as it was removed during addition of QuEChERS salts and dSPE clean-up. LCMSMS with MRM mode provide very sensitive and selective detection of OTC. This leads to acceptable and repeatable recovery results which are in the range of 70-120%. The results obtained in this study indicated that the developed method is sufficiently sensitive and reproducible in the routine analysis of OTC in pork within a short analysis time. It is expected that this method could be applicable to analyze OTC in other food matrices.

6. Conflict of Interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

7. Acknowledgments

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8. References

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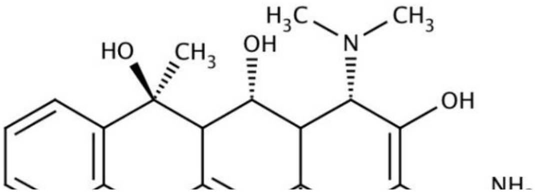
Table 1Multiple reaction monitoring (MRM) method for product ions and voltage parameters of detection of oxytetracycline

Name	**Retention time (min)**	**MRM method (*m/z*)**		**Voltage (*V*)**		
		Precursor ion $[M-H]^-$	**Product ion**	**Q1 Pre Bias**	**CE**	**Q3 Pre Bias**
Oxytetracycline	2.79	461	426	-21	-20	-28
			443	-21	-14	-29
			444	-21	-16	-29

Table 2 Intraday and Interday recovery of oxytetracycline in pork samples

Sample	Spiking level (ng/g)	Intraday-1 (*n* = 3)		Intraday-2 (*n* = 3)		Intraday-3 (*n* = 3)		Interday (*n* = 9)	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Pork	50	87.12	3.91	87.99	2.85	91.07	1.67	88.73	3.24

Figure1. Chemical structure of Oxytetracycline (OTC)



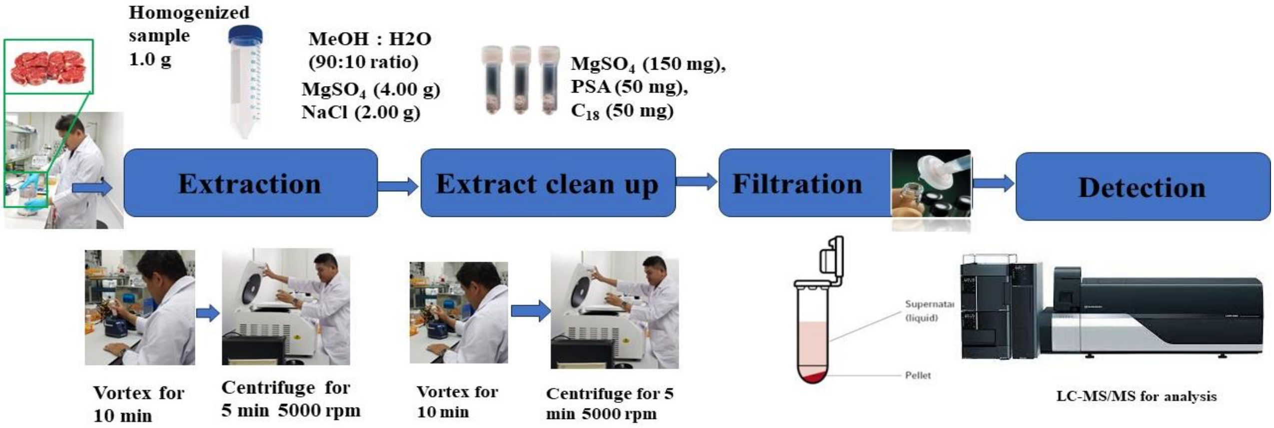


Figure 2. Schematic Representation of OTC analysis, Starting from Extraction, Extract Clean up, Filtration to Detection

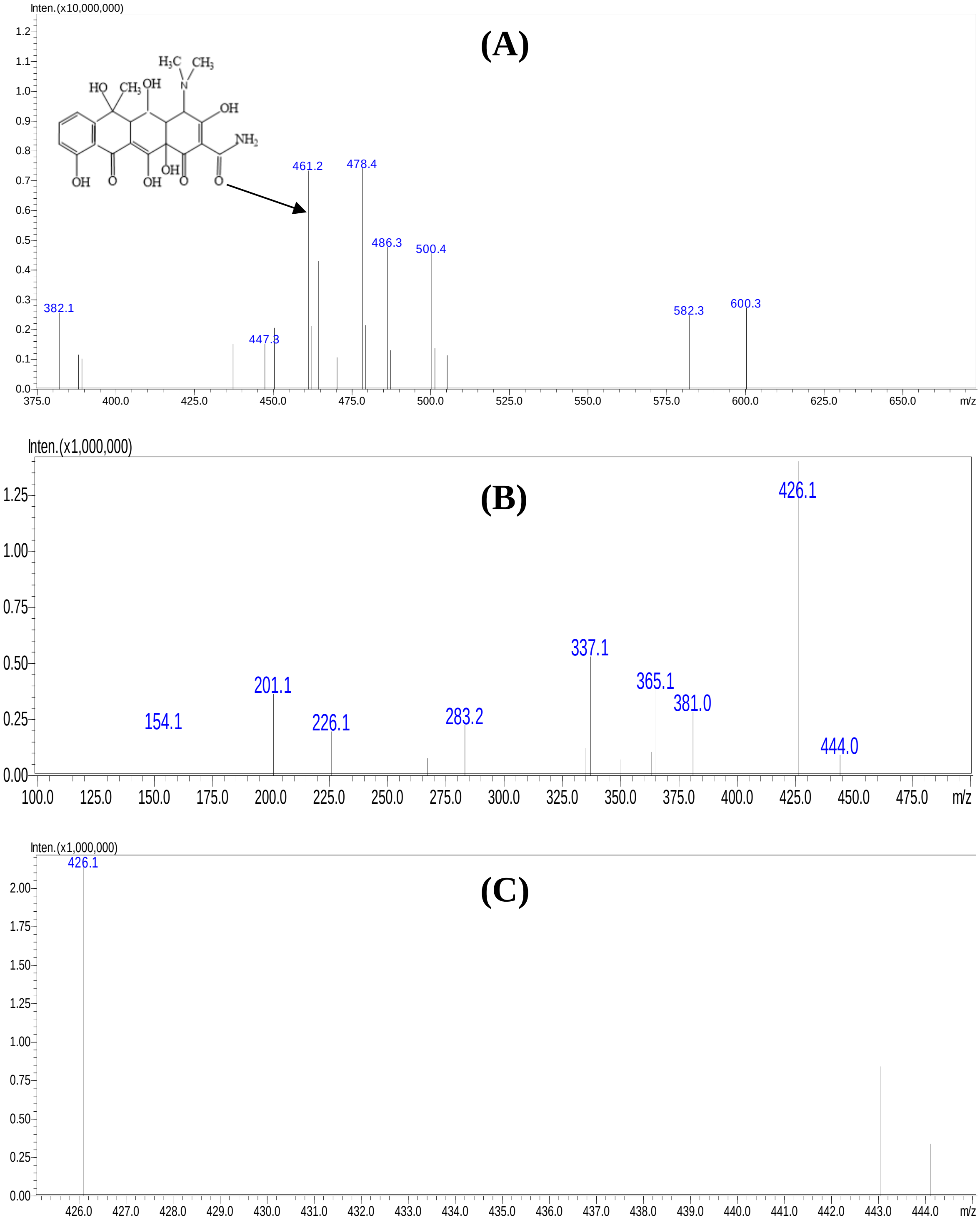


Figure 3. LC-MS/MS spectrums of (A) Full Scan mass spectrum of Oxytetracycline, (B) Product Ion Scan mass spectrum of Oxytetracycline (C) product ions after optimization

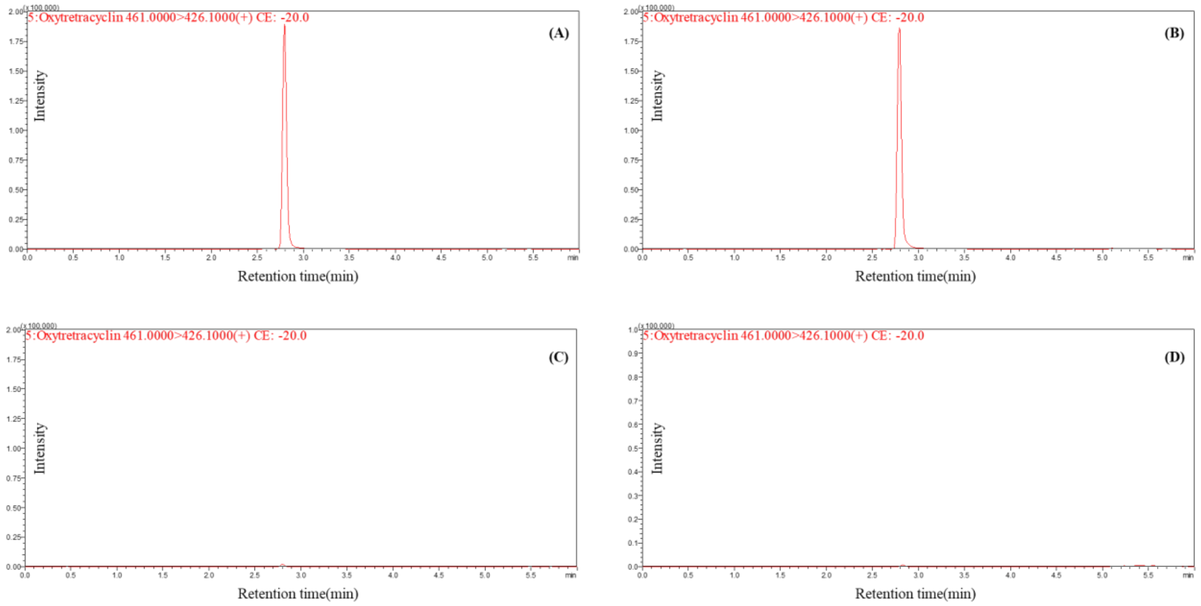


Figure 4. MRM chromatograms of (A) OTC Matrix-Matched 50 ng/g, (B) OTC spike sample 50 ng/g, (C) Pork sample, (D) Blank

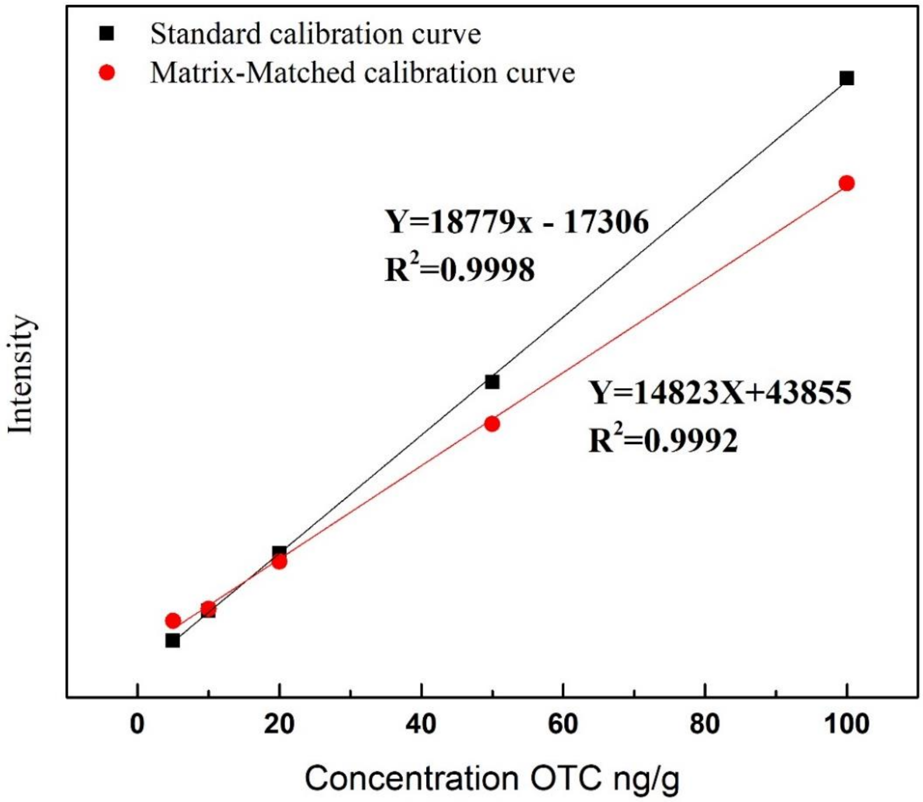


Figure 5. Standard calibration curve and Matrix-Matched calibration curve