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Research Article

Determination of some Tetracycline antibiotics and their epimers residues in meat and meat products by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

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Abstract

The study was conducted with the aim of developing and validating a method for determining the content of some tetracycline group substances including tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), metacycline (MC), doxycycline (DC) and epimers: 4-epi oxytetracycline (4-epi OTC), 4-epi tetracycline (4-epi TC), 4-epi chlortetracycline (4-epi CTC) in meat and meat products by liquid chromatography tandem mass spectrometry (LC-MS/MS). The sample processing procedure was quick and simple: the sample was extracted with McIlvaine-EDTA buffer, and the protein was precipitated with trichloroacetic acid. Then, the extract was cleaned on a HLB solid-phase extraction column. The separation was performed on a reversed-phase X-bridge C18 column (150 mm x 2.1 mm id; 3.5 µm), mobile phase channel A was 0.1% formic acid in H_2O, channel B was ACN; flow rate 0.5 mL/min, injection volume 10 µL. The validated method showed good specificity, the standard curve of the analytes was linear in the concentration range of 15 – 150 µg/kg. The limit of detection (LOD) and limit of quantification (LOQ) were achieved with the corresponding analytes of 5 µg/kg and 15 µg/kg. The accuracy was assessed by recovery in the range of 84.23 – 108.5%, the precision was assessed by repeatability and internal reproducibility met the requirements according to AOAC. The method was applied to analyze the content of tetracycline antibiotics and epimers in 10 food samples (chicken, pork, ham, sausage) collected from the market.

Keywords: *TC, OTC, CTC, 4-epi OTC, 4-epi TC, 4-epi CTC, meat, meat products, LC/MS-MS*

1. INTRODUCTION

In the typical diet of Vietnamese families, meat presents the primary source of animal-derived food consumption. Otherwise, it is also a traditional food that is widely accepted in most cultures and countries around the world, including Vietnam. Therefore, unsafe meat is one of the risks influencing community health. During breeding, antibiotics are commonly used to prevent and treat diseases. However, the overuse of antibiotics can lead to their accumulation in animal meat and milk. Consumption of such food, which residues antibiotics, may induce the resistance of antibiotics or allergy [1].

Among antibiotic groups, the tetracyclines group (TCs), one of the broad-spectrum antibiotics, is generally used to effectively treat the infection caused by both negative and positive bacteria in humans and animals. Besides, TCs are also used as growth stimulants in livestock and aquaculture. Similar to the drug, the residue of TCs in animals and products from animals. Additionally, TCs are utilized as growth promoters in livestock and aquaculture due to their side effect on the enhancement of developmental processes [2]. However, similar to other veterinary drugs, the use of TCs in livestock farming induces the TCs residues in animals and animal-

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derived products. This residual presence of TCs raises significant concern about antibiotic resistance, complicating the treatment of bacterial infections in animals and humans. A particularly alarming issue is the potential transmission of antibiotic-resistant microorganisms from animal-derived products to humans [3]. Furthermore, the consumption of foods containing TCs can lead to chronic toxicity and long-term health risks such as impaired fetal development, digestive disorders, inflammation, cytotoxicity, and diseases related to the immune system [4].

To mitigate potential adverse effects, the Ministry of Health has issued Circular No. 24/2013/TT-BYT named "Regulations on the maximum limit of veterinary drug residues in food" [5], in which the maximum residue limit (MRL) of CTC, OTC, TC in pork and poultry, was established at 200 µg/kg. Additionally, national regulatory agencies and international organizations, such as Codex Alimentarius, the European Commission (EC), and the FDA have implemented stringent regulations regarding TC residues [6]. The European Commission (EC) has set the MRL of each TC at 100 µg/kg, 300 µg/kg, and 600 µg/kg for the muscles, liver, and kidneys of all animals, respectively. For isomers of TCs, the allowable level was calculated based on the concentration of parent antibiotics (TC, CTC, OTC) and their main metabolites are 4-epi TC, 4-epi CTC, and 4-epi OTC [7].

Currently, various methods have been employed to individually or simultaneously analyze the TCs in meat and meat products. In Vietnam, the standard method using liquid chromatography detector UV-Vis has been issued for the analysis of tetracycline antibiotic residues in meat and meat products [8]. A number of other methods are also used to analyze the antibiotic content of TCs such as the spectral method [9], electrochemical method [10, 11], liquid chromatography using PDA detectors, and MS [12-14]. However, previous studies have primarily focused on the TCs, without effectively separating their isomers while the TCs are easily degraded by light, reducing oxidation conditions, and pH. Specifically, 4-epi TC, 4-epi CTC, and 4-epi OTC isomers are generated under acidic environmental conditions (pH 2–6) and can be reverted to their active form in alkaline conditions or in the presence of metals [15]. Therefore, optimizing conditions and selecting appropriate chromatographic columns play a vital role in separating isomers from each other. In this study, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was selected for the simultaneous analysis of eight antibiotics of TCs and their isomers in meat and meat products for the development of the analysis process and ensure the safety of food supplies and protect the health of consumers.

2. MATERIALS AND METHODS

2.1. Study subjects and samples

Eight antibiotics of the TCs group including TC, CTC, OTC, MC, DC and isomers of TCs including 4-epi TC, 4-epi CTC and 4-epi OTC were selected as the subjects of analysis in this study.

The selected sample subjects are 10 samples of meat and meat products randomly collected from markets in Hanoi city in July 2024. The samples included: 05 samples of chicken, 02 samples of pork, 02 samples of beef and 01 sample of spring rolls.

2.2. Chemicals and standards

Standard substances: tetracycline (LGC, UK), 97.94% purity, lot number G1127754; oxytetracycline (LGC, UK), 97.5% purity, lot number G125763; chlortetracycline (Sigma, Germany), purity 91.95%, lot number BCCH2550; metacycline (LGC, UK), purity 95.61%, lot number G1482386; doxycycline (LGC, UK), 98.00% purity, lot number 1116543; 4-epi oxytetracycline (LGC, UK), 79.7% purity, lot number 1380563; 4-epi tetracycline (HPC, Japan), 94.24% purity, lot number 823709; 4-epi chlortetracycline (HPC, Japan), purity 87.42%, lot number 820195; demeclocycline companion standard of LGC (UK), purity 92.85%, lot number G1487965.

Chemicals: acid citric monohydrat ($C_6H_8O_7$); acid formic (HCOOH); acid trichloracetic (TCA); natri biphosphat (Na_2HPO_4); natri hydroxit (NaOH); $Na_2EDTA.2H_2O$; acid phosphoric, acetonitrile (ACN); methanol (MeOH), water.

McIlvaine pH 4.0 buffer: Accurately weigh 28.4 g of anhydrous Na_2HPO_4, dissolve with deionized water in a 1 L volumetric flask, then fulfill to 1 L, shake the mixture well. Accurately weigh 21.0 g of citric acid

monohydrate, dissolve with deionized water into a 1 L volumetric flask, then fullfil to 1 L. Mix 1 L of the citric acid solution with 625 mL of Na_2HPO_4 solution. Adjust pH to 4 ± 0.05 by adding 0.1 M HCl or 0.1 M NaOH.

McIlvaine-EDTA buffer: Accurately weigh 60.0g of $Na_2EDTA.2H_2O$, add 1.625 L of McIlvaine buffer and mix until it dissolves.

2.3. Equipments

The primary analytical instrument utilized in this study was the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system, which comprised a Shimadzu HPLC 20AD system coupled with an AB SCIEX Triple Quad 6500+ mass spectrometer. The chromatographic separation was performed using an X-Bridge C18 reversed-phase column (150 mm × 2.1 mm, 3.5 µm) along with a corresponding precolumn. Additionally, sample preparation involved the use of a Waters Oasis HLB solid-phase extraction (SPE) cartridge (60 mg, 3 cc) to enhance analyte purification and concentration. Additionally, the study utilizes various standard laboratory equipment and instruments.

2.4. Research methods

2.4.1. Analytical methods

The LC-MS/MS method with many advantages such as high selectivity, accuracy and sensitivity is suitable for research purposes for simultaneous analysis of eight antibiotics of TCs and TCs isomers in meat and meat products. The analytical conditions for the LC-MS/MS system were adapted from previous studies [6] with mobile phase consist of channel A (0.1% formic acid) and channel B (ACN). The flow rate was set at 0.5 mL/min, and the injection volume was 10 µL. A gradient elution program was investigated to optimize the separation efficiency eight tetracycline and isomeric antibiotics.

2.4.2. Sample preparation

Based on the reference document [18], the solid phase extraction method is used to purify sample. In detail, weigh approximately 2 g ± 0.10 g of the homogenized sample into a 50 mL centrifuge tube. Add 60 µL of 1 µg/mL demeclocycline surrogate standard and the mixture was allowed to stand for 10 minutes. Subsequently, add 10 mL of pH 4.0 buffer (selected based on buffer optimization studies), followed by vortexing for 2 minutes. The sample was then centrifuged at 6000 rpm for 5 minutes. The supernatant was transferred to a second centrifuge tube, while the remaining residue is extracted for the second time with 10 mL of pH 4.0 buffer solution, then centrifuged at 6000 rpm for 5 minutes. To precipitate proteins, TCA was added (based on optimized TCA volume). The mixture was shaken for about 30 seconds before centrifuged at 6000 rpm for 5 minutes. The resulting extract was filtered through filter paper into a 50 mL centrifuge tube and purified by SPE Oasis HLB column. Firstly, the column is activated with 3 mL of MeOH, then 3 mL of distilled water. The sample was loaded into column at a flow rate of no more than 2 mL/min. Afterward, 3 mL of distilled water is added to remove impurities. The column was dried under vacuum for 1 minute to eliminate residual solvents. Finally, the sample is eluted with 4 mL of MeOH solution. The eluate was filtered through a 0.2 µm membrane into a 2 mL vial and analyzed on an LC-MS/MS system.

2.4.3. Method validation

Method validation was evaluated based on the specificity, linear range and calibration curve, limit of detection (LOD), limit of quantification (LOQ), precision (intra-day repeatability and inter-day reproducibility), and accuracy assessed through recovery studies. The results are calculated based on the peak area ratio of the analyzer and the surrogate standard, as displayed on the LC-MS/MS software. During the analysis, blank samples, spiked blank samples, and standard samples were used for method validation.

Blank sample: chicken sample does not contain 8 analytes. The sample is processed according to the surveyed and selected process.

Spike sample: 300 µL of a mixed 8-analyte standards mixed in MeOH at a concentration of 1 µg/mL is added to 2 g of blank samples. The calibration curve was constructed using a blank matrix with five calibration points at concentrations of 15, 30, 50, 100, and 150 µg/kg.

During the investigation of sample preparation conditions and method validation, chicken meat was selected as a representative matrix for meat and meat products.

2.4.4. Data processing methods

The results were automatically calculated using Analysis version 1.7 (AB SCIEX, USA) software and processed by Microsoft Excel 2019 software.

The results are calculated based on the cabibration curve of the ratio of the standard peak/surrogate standard area to the standard concentration.

The analyte content (ng/g) is calculated according to the following formula:

$$X = \frac{V\ x\ C_m\ x\ k}{m}$$

In which: V is the volume of the final extract (mL), C_m is the concentration of antibiotics in the sample extract calculated according to the cabilbration curve (ng/mL), m is the weight of the analysis sample (g), k is the dilution factor, X is the antibiotic content in the sample (ng/g).

3. RESULTS AND DISCUSSION

3.1. Optimization the analytical condition on LC-MS/MS Instrument

3.1.1. Selection of analytical conditions on the mass spectrum

Based on references [13-15], the simultaneous analysis of TCs and isomers was conducted by LC-MS/MS with electron spray ionization in positive mode (ESI (+)). The mass spectrometry conditions were tuned by instrument software, where a standard solution of each TCs at a concentration of 50.0 ng/mL was injected directly into aerosol source. The results of the precursor ions, daughter ions and the corresponding optimal parameters for each TCs are presented in **Table 1**.

Table 1. *Mass spectrometry of TCs*

Analyte	Retention time (min)	Precursor ions (m/z)	Daughter ion (m/z)	CE (eV)
4-epi TC	5.38	445	410	27
			427	25
4-epi OTC	5.45	461	426	30
			443	17
OTC	5.79	461	426	16
			443	18
TC	6.41	445	410	25
			427	19
4-epi CTC	7.96	479	444	31
			462	25
CTC	9.25	479	444	16
			462	16
MC	9.76	443	426	26
			201	48
DC	10.13	445	321	35
			154	37
Demeclocycline (IS)	7.69	445	430	15

3.1.2. Optimization of mobile phase gradient elution program

Based on the study by Anna Gajda *et al.* [16], The mobile phase consisting of channel A (0.1% formic acid) and channel B (ACN) along with a C18 column was selected for investigation the simultaneous analysis of TCs and their isomers on LC-MS/MS. The results indicated that acid formic and ACN combination provided the best signal of the analytes with symmetrical peak shape. Therefore, the optimized analytical conditions for the LC-MS/MS method were established as follows: mobile phase composition (channel A: 0.1% formic acid, channel B: ACN), a flow rate of 0.5 mL/min, an injection volume of 10 µL, and chromatographic separation performed on an X-Bridge C18 reversed-phase column (150 mm × 2.1 mm, 3.5 µm) with a corresponding guard column.

Since TCs and their isomers share the same molecular mass and fragmentation pattern, selecting an appropriate gradient program is essential for achieving effective separation. To enhance the resolution between isomers, the optimization of mobile phase was investigated by gradually decreasing the concentration of channel A. The finalized gradient program is presented in **Table 2**.

Table 2. *Mobile phase gradient*

Time (min)	Concentration	
	Channel A (%)	Channel B (%)
0.0	95	5.0
0.5	95	5.0
17.0	60	40
17.1	5.0	95
19.0	5.0	95
25.0	95	5.0
30.0	95	5.0

Upon implementing this gradient program, the results show that the chromatographic peaks of TC and 4-epi TC, CTC and 4-epi CTC, OTC and 4-epi OTC have been separated as the chromatograms shown in **Figures 1**.

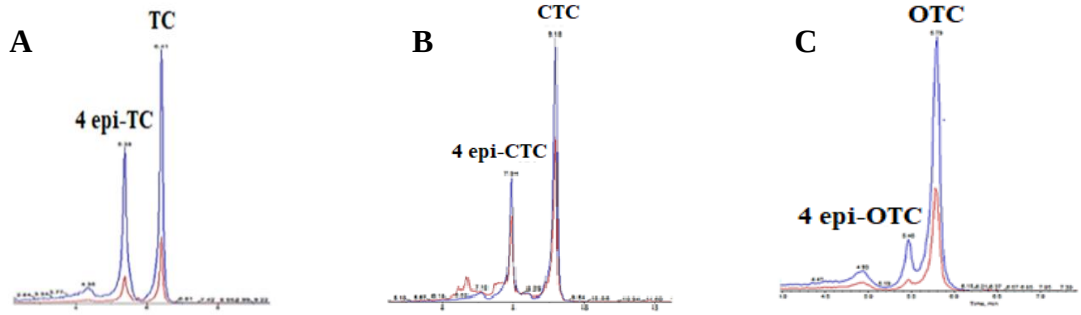

Figure 1*. Chromatogram of TC and 4-epi TC (A); CTC and 4-epi CTC (B); OTC and 4-epi OTC (C)*

3.2. Optimize sample preparation

3.2.1. Survey of sampling conditions

Based on a review of the literature, most of the samples were prepared by a similar methodology in which variations extraction solvents was used. Consequently, three extraction processes were selected for investigation, each processes utilizing a different extraction solvent: Process 1 using 0.02 M oxalic acid buffer [16], process 2 using KH_2PO_4 buffer [17] and process 3 using EDTA-McIlvaine [18]. All solvents were adjusted to pH of 4.0. The results comparing the recovery of TCs antibiotics using different extraction processes are shown in **Figure 2**.

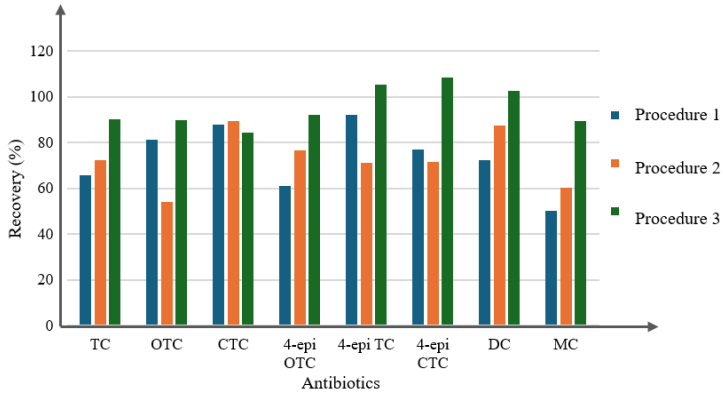

Figure 2. *Survey results of 03 processes using different extraction solvents*

As shown in **Figure 2**, process 1 observed the highest recovery for 4-epi TC (92.05%) but yielded low recoveries for TC, 4-epi OTC and MC, ranging from 50.15% to 65.79%. Process 2 exhibited optimal performance for DC (89.58%) but relatively lower recoveries for OTC, MC. Process 3 demonstrated consistently high and stable recoveries of all analytes ranging from 84.23% to 108.5%. Given that tetracycline antibiotics and their isomers are highly sensitive to light and acidic conditions [19], the selection of extraction solvent is crucial. Process 3 uses an acidic extraction solution, which also contains EDTA - McIlvaine buffer - pH 4.0 for the highest overall performance therefore chosen for this study.

3.2.2. Investigation of Trichloracetic acid (TCA) concentraition

TCA is a weak acid that induced the precipitation of proteins [20], allowing the effectively remove protein from the sample matrix.To optimize the extraction process, the amount of TCA must be determined to maximize protein precipitation, and enhance sample cleanliness, and minimize protein interference in analyte detection. In detail, the TCA amounts of 120, 150, 200, 300, 500 µL of 1.0 g/mL TCA was evaluated. The recovery and results of the TCA optimization were shown in **Figure 3**.

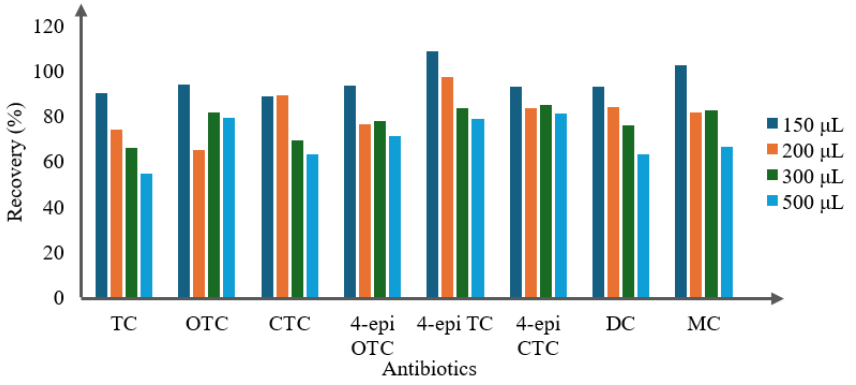

***Figure 3**. Results of TCA selection survey*

When 120 µL of TCA was added to the sample, the protein was not completely precipitated which led to the extraction solution being stuck inside the SPE Oasis HLB column. Therefore, the assay was evaluated TCA volumes of 150, 200, 300, and 500 µL.

As shown in **Figure 3** a significant drop of recoveries of TC, OTC, and 4-epi OTC, all falling below 80%, was recored when the increasing the TCA volume to 200, 300, and 500 µL. In contrast, 150 µL of TCA yielded high recoveries with overall more than 85%. The observed decline in recovery with increasing TCA volume can be attributed to the acidification of the extraction solution, as TCA is weak acid. Given that tetracycline antibiotics are highly sensitive to acidic conditions, excessive TCA addition negatively impacts recovery efficiency. Therefore, the appropriate amount of TCA to remove the maximum amount of protein in the selected sample was 150 µL.

3.3. Method validation

3.3.1. Specificity

Specificity was determined by analyzing 3 samples: blank sample, a standard sample and a spike sample. The results indicated that the retention time in the spiked sample closely matched that of the standard sample in the solvent matrix, with a deviation of no more than 1%, thereby meeting the AOAC requirement [21].

In addition, im mass spectrometry, the identification points (IP) is an important value for evaluating specificity, the IP score is calculated as follows: 1 point for LC separation, 1 point for each precursor ion and 1.5 points for each daughter ion. As shown in **Table 1**, each analyte contains 1 precursor ion and 2 daughter ions, all substances have an IP score of ≥ 5, which meets the IP score requirement (IP ≥ 5) [21].

Thus, the method meets the requirements of specificity, suitable for applying the analysis of TCs and isomers (TC, OTC, CTC, MC, DC, 4-epi OTC, 4-epi TC, 4-epi CTC).

3.3.2. Limit of detection (LOD) and Limit of quantitative (LOQ)

The LOD and LOQ of the method were evaluated by analyzing standard samples of a mixture of analytes with decreasing concentrations until a detectable signal was observed. Chromatograms were recored and S/N ratio was also determined. LOD and LOQ were denified as the concentrations where the S/N ratio was 3-folds and 10-folds, respectively. The results showed that the LOD of the antibiotics was 5 µg/kg and the LOQ was 15 µg/kg.

3.3.3. Calibration curve

The calibration curve was established using blank samples spiked with analyte concentrations of 15 µg/kg, 30 µg/kg, 50 µg/kg, 100 µg/kg, and 150 µg/kg. The relationship between the analyte-to-internal standard peak area ratio and the analyte concentration was examined. The results, presented in **Table 3**, indicate a strong correlation, with a coefficient (R^2) ranging from 0.995 to 1. Additionally, the deviation of data points from the calibration curve remained within ± 15% [21]. These findings confirm that the calibration curve demonstrates a reliable correlation between signal intensity and analyte concentration with satisfactory linearity.

***Table 3**. Standard line results – Repeatability – Retracement - Repeatability*

Analytical substances	Standard line	R^2	R (%)	RSD (%)	RSDr (%)
TC	y = 0.250x - 0.345	0.9986	87.4 - 99.6	0.7 - 4.33	4.63
CTC	y = 0.020x - 0.038	0.9999	90.1 - 98.9	1.14 - 3.53	2.28
OTC	y = 0.195x - 0.396	0.9985	88.0 - 103	0.84 - 3.14	1.73
MC	y = 0.0215x + 0.176	0.9965	85.8 - 104	2.70 - 6.20	4.87
DC	y = 0.0192x + 0.112	0.9971	85.5 - 110	4.80 - 7.50	6.35
4-epi OTC	y = 0.109x - 0.491	0.9998	85.8 - 110	3.15 - 8.24	6.91
4-epi TC	y = 0.103x - 0.689	0.9999	85.9 - 109	4.80 - 7.04	4.52
4-epi CTC	y = 0.0688x - 0.498	0.9999	86.1 - 110	3.50 - 7.00	3.81

3.3.4. Repeatability and reproducibility

Repeatability (RSD) was measured at three levels of 15 µg/kg, 30 µg/kg and 150 µg/kg of spike sample. The results are shown in **Table 3**. Reproducibility (RSDr) was evaluated through analyses conducted by two independent testers. Each tester performed six replicate analyses at a concentration of 30 µg/kg, and internal reproducibility was calculated using the pooled standard deviation method. The results obtained in **Table 3** show that repeatability was in the range of 0.7 - 8.24%, and reproducibility is in the range of 1.73 - 6.91%. According to AOAC 2016 regulations [21], at concentrations of 10 - 100 ppb, the required repeatability is less than 15%, and the reproducibility is less than 22%. Therefore, the analytical method meets the requirements for repeatability and repeatability of AOAC.

3.3.5. Correctness

The correctness was assessed by analyzing the spike sample at 3 concentration level sequivalent to 1 x LOQ, 2 x LOQ, and 10 x LOQ level. The results shown in **Table 3** show that the recovery was > 80% at all levels. According to AOAC regulations, for concentrations of 10 - 100 ppb, the required recovery is 80 - 110%. Therefore, the recovery results of the method were satisfactory according to the AOAC [21].

3.4. Analysis of the content of some TCs and isomers in actual samples

After validation, the method was applied to analyze the concentrations of selected tetracyclines (TCs) and their isomers in 10 randomly collected food samples from the market. The results showed that 9 out of 10 samples did not detect the analytes. CTC and 4-epi CTC were detected in 01 pork sample (100% lean meat) with the content of 121 µg/kg and 46.5 µg/kg, respectively. The chromatographic results of these two TCs were shown in **Figure 4** and **Figure 5**.

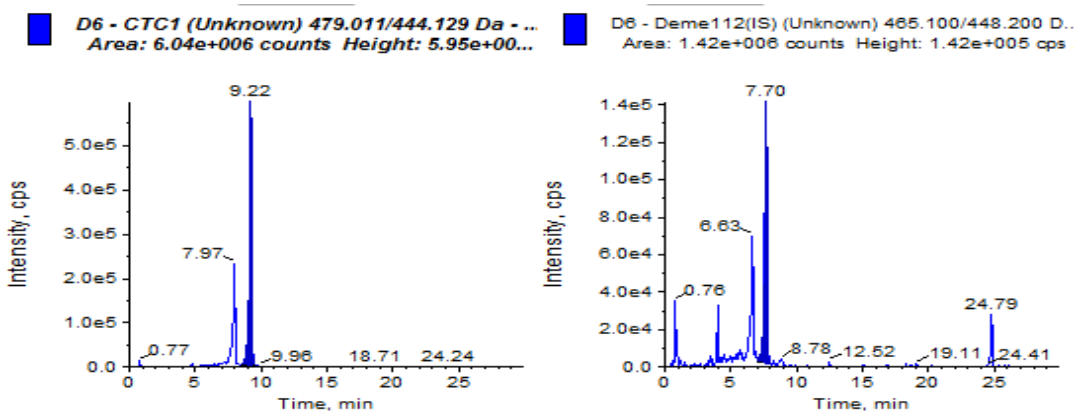

***Figure 4**. Chromatography analysis of pork samples showed positive results for CTC*

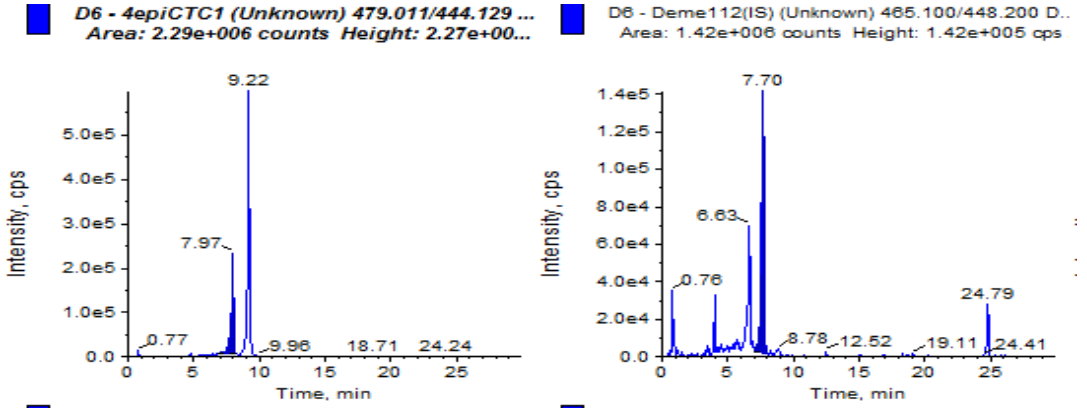

***Figure 5.** Chromatographic analysis of pork samples showed positive results for 4-epi CTC*

The results presented in **Figures 4** and **Figures 5** indicate that CTC and 4-epi were detected in the same pork sample. However, the content of these TCs in the sample is lower than the permissible limitation as specified in Circular No. 24/2013/TT-BYT [5]. This study provides a preliminary assessment of the level of TCs antibiotic residues in some meat and meat products on the market today.

A study by Fulya Tasci *et al.* [22] have been simultaneously identified OTC, 4-epi OTC, TC, 4-epi TC, CTC, 4-epi CTC and DC in milk. The researchers analyzed antibiotic residues in 130 samples, including 10 samples of colostrum, 31 samples of processed milk (cows and goats), 68 samples of raw cow's milk, and 21 samples of raw goat's milk collected at random in Burdur Province, Turkey. The results showed that 6/21 samples of raw goat's milk and 6/68 samples of raw cow's milk were detected of TC and 4-epi TC esidues exceeding the maximum allowable limits. Therefore, future research will focus on developing and expanding studies on milk samples in Vietnam to conduct an initial risk assessment of TCs and their isomers in dairy products.

4. CONCLUSION

The study successfully developed a method for simultaneous determination of the content of tetracycline antibiotics including TC, OTC, CTC, MC, DC and isomers 4-epi OTC, 4-epi TC, 4-epi CTC in meat and meat products by LC-MS/MS. The method was validated and demonstrated the specificity, standard line, precision, correctness and detection limit and quantitative limit, which all met the requirements of the AOAC. The method was applied to analyze the content of TCs and isomers in 10 food samples collected on the market. The results revealed that one sample contained antibiotic residues from the TC group; however, the detected levels were below the permissible limits set by the Ministry of Health. Moving forward, this method will continue to be used for the analysis of meat and meat products and can be further extended to other food matrices to ensure quality control and protect consumer rights.

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