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Research Article

Determination of multiple antibiotic residue in eggs using liquid chromatography tandem mass spectrometry (LC-MS/MS)

Nguyen Thi Phuong Mai[1,2*], Le Thanh Huyen[1], Luu Thi Huyen Trang[1], Hoang Thao Ngan[1], Nguyen Thi Hanh[3], Tran Cao Son[1], Nguyen Thi Anh Huong[2]

[1]National Institute for Food Control, Hanoi, Vietnam
[2]Hanoi University of Science, Vietnam National University, Hanoi, Vietnam
[3]Bac Ninh Center for Disease Control, Bac Ninh, Vietnam

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Abstract

Macrolides, quinolones, sulfonamides, and tetracyclines are widely used antibiotic groups in poultry production, and their overuse can lead to residues in eggs, affecting public health. This study developed and validated a method for the simultaneous analysis of 12 antibiotics belonging to the four groups aforementioned in egg samples using LC-MS/MS. Samples were extracted with acetonitrile/0.1% formic acid supplemented with EDTA and cleaned with an Oasis PRiME HLB cartridge before analysis. The method demonstrated good specificity, stable linear range ($R^2 > 0.995$), recovery ranging from 83.8 - 105%, with repeatability (RSD) of 2.50 - 12.4%. The limit of detection and limit of quantification were 15 - 50 µg/kg for the tetracyclines group and 3 - 10 µg/kg for the remaining groups, respectively. When applied to 25 egg samples collected from the Hanoi markets, no antibiotic residues were detected in 21 samples. The remaining four samples showed the presence of oxytetracycline, doxycycline, and sulfamethoxazole, among which one sample contained doxycycline at a notably high concentration of 1360 µg/kg. Due to the limited sample size, these findings should be regarded as preliminary and are insufficient to draw conclusions regarding risk levels at the national scale. Nevertheless, the detection of oxytetracycline, doxycycline, and sulfamethoxazole in egg samples- including one with a relatively high doxycycline concentration- demonstrates the potential applicability of the proposed method and highlights the need for further evaluation through studies with larger sample sizes.

Keywords: *Antibiotic residues in eggs, macrolides, tetracyclines, quinolones, sulfonamides.*

1. INTRODUCTION

Eggs are a food source with high nutritional content and reasonable price. The egg production industry plays an important role in the livestock sector of Vietnam, with output and consumption increasing continuously over the years [1]. To improve productivity and prevent diseases, veterinary drugs, especially antibiotics, are often used in livestock farming. However, the abuse or improper use of antibiotics is still a concern, increasing the risk of antibiotic residues in poultry products, especially eggs [2-4]. A study by Yamaguchi *et al.* (2017) [5] analyzed the residues of 28 antibiotics in 111 egg samples and found that 16 samples detected antibiotics, most of which had concentrations exceeding the limit of quantification (LOQ). Specifically, enrofloxacin was detected in 8 samples with concentrations ranging from 2.4 to 1485 µg/kg, while tilmicosin was detected in 3 samples with concentrations ranging from 49 to 568 µg/kg [5]. In addition, a study conducted in Ho Chi Minh City also noted that 33.1% of 136 chicken egg samples collected from markets contained antibiotic residues [6].

*Corresponding author: Nguyen Thi Phuong Mai (E-mail phuongmainguyen051210@gmail.com)
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Commonly used antibiotic groups in poultry farming include macrolides, quinolones, sulfonamides, and tetracyclines [7–9]. Among them, tetracyclines and some macrolides such as tylosin and erythromycin are used to prevent and treat respiratory diseases as well as necrotizing enteric infections. Fluoroquinolone and quinolone compounds are commonly used in the treatment of gastroenteritis, skin and soft tissue infections, while sulfonamides are used to treat coccidiosis, fowl typhoid, infectious sinusitis and dysentery [4]. The presence of antibiotic residues can lead to many adverse effects on human health, including the development of drug-resistant bacteria, allergic reactions, intestinal microflora disorders, as well as neurotoxicity and immunotoxicity, carcinogenicity and mutagenicity, and effects on the development and metabolism of children with prolonged exposure [4, 9]. According to Circular No. 24/2013/TT-BYT, the maximum allowable residue limits (MRLs) of some antibiotics of the above groups in eggs are presented in **Table 1** [10].

Table 1. *Maximum residue levels of some antibiotics in eggs according to Circular No. 24/2013/TT-BYT [10]*

Antibiotics	Active ingredient	MRL (µg/kg)
Chlortetracycline, Oxytetracycline and Tetracycline	Active ingredient, alone or in combination	400
Tylosin	Tylosin A	300
Erythomycin	Erythromycin A	50

Currently, there have been many studies in the world focusing on the simultaneous determination of antibiotic residues in eggs, in which liquid chromatography-tandem mass spectrometry (LC-MS/MS) is widely applied, with the main difference being the extraction solvent and the matrix cleanup step [11-15]. For example, Heller *et al.* (2006) successfully developed a method for the simultaneous analysis of 29 antibiotics from the sulfonamides, tetracyclines, fluoroquinolones and beta-lactam groups in egg samples, using sodium succinate extraction buffer and matrix cleanup using an Oasis HLB cartridge [11]. Next, Piatkowska, Jedziniak and Zmudzki (2015) used a mixture of acetonitrile:water (8:2, v/v) containing 0.1% formic acid and EDTA for extraction and cleanup using a HybridSPE solid-phase extraction column [12]. In another study, Wang (2017) chose a simple extraction procedure using acetonitrile:water mixture (90:10, v/v) with Na_2EDTA, without applying a matrix cleanup step but still analyzed effectively on a C18 column with a water/acetonitrile mobile phase containing 0.1% formic acid [13]. Similarly, Ma *et al.* extracted with acetonitrile supplemented with 0.02 mol/L EDTA, cleaned with a PRiME HLB cartridge and separated on a C18 column with a methanol/water mobile phase containing 0.1% formic acid [14]. In addition, Yamaguchi *et al.* (2017) used acetonitrile for extraction, matrix cleanup by d-SPE (C18); while Li (2018) applied the QuEChERS procedure with $MgSO_4$ /NaCl salt combined with d-SPE (PSA/C18/$MgSO_4$) [5, 15]. Thus, despite differences in procedural details, most studies are based on the general framework of using acetonitrile - often with added acid and/or EDTA - as the extraction solvent, combined with a specialized matrix cleanup step (SPE, d-SPE or QuEChERS).

However, in Vietnam, there is still no standardized procedure that allows simultaneous analysis of multiple antibiotic groups in eggs. Therefore, in this study, the LC-MS/MS method was used to develop a procedure for simultaneous detection and quantification of 12 antibiotic active ingredients belonging to 4 groups: macrolide, fluoroquinolone, sulfonamide and tetracycline in egg samples.

2. MATERIALS AND METHODS

2.1. Analysis objects and samples

This study focused on 12 antibiotics including: 3 macrolide antibiotics (tylosin, tilmicosin, erythromycin); 2 fluoroquinolone antibiotics (enrofloxacin, ofloxacin); 3 sulfonamide antibiotics (sulfamethoxazole, sulfadimidine, Sulfisomidine) and 4 tetracycline antibiotics (tetracycline, oxytetracycline, chlortetracycline, doxycycline).

The sample subjects were poultry egg samples randomly purchased from markets in Hanoi during the period from September to October 2025. Of which, a total of 25 samples were collected, including: 16 chicken egg samples, 7 duck egg samples and 2 quail egg samples in the areas of Cau Giay, Long Bien, Gia Lam, Tay Ho, Ba Dinh.

2.2. Standards and chemical reagents

All chemical reagents used in this study were of analytical grade for LC-MS/MS. The chemicals included acetonitrile (C_2H_3N), formic acid (HCOOH), ammonium formate (NH_4HCO_2), $Na_2EDTA.2H_2O$ from Merck, Germany; anhydrous magnesium sulfate ($MgSO_4$), sodium chloride (NaCl) from Xilong, China; C18 adsorbent with a particle size of 40 µm from Agilent, USA; deionized water obtained from a Milli-Q ultrapure water purifier. Details of the 12 antibiotic standards and co-agents used in the study are presented in **Table 2**.

***Table 2**. Information of the standards used*

No.	Name of standard	Code	Lot	Purity/Concentration
1	Tylosin tartrate	DRE-C17895600	1432912	91.2%
2	Tilmicosin	DRE-C17582000	G1490214	84.8%
3	Erythromycin	DRE-C13203490	1493103	97.2%
4	Enrofloxacin	DRE-C13170000	H1583380	99.8%
5	Ofloxacin	DRE-C15717000	G1527754	95.9%
6	Sulfonamides Mixture (including sulfadimidine, sulfisomidine, sulfamethoxazole)	DRE-GA09000590AL	2-H533315AL	100µg/mL
7	Tetracycline hydrochloride	DRE-C17396150	G1357741	95.1%
8	Oxytetracycline dihydrate	DRE-C15819990	1505270	95.1%
9	Chlortetracycline hydrochloride	DRE-C11509100	1566477	91.5%
10	Doxycycline hyclate	DRE-C13084280	1550014	95.8%
11	Enrofloxacin Hydrochloride-d5	DRE-C13170100	G1481114	97.0%
12	Demeclocycline hydrochloride	DRE-C12128000	G1487965	92.9%

Individual stock solutions were prepared by dissolving each standard in methanol at 1000 mg/L, and the standard solutions were diluted to the appropriate concentration. The Sulfonamides mixture is ready to use. The standard solutions were all stored at 2 - 4°C prior to use.

2.3. Equipment and instruments

The main equipment used in the study was a SCIEX Exion LC 20AD liquid chromatography system LC-MS/MS (USA) coupled with an AB SCIEX Triple Quad 6500+ mass spectrometer detector, an Xbridge Premier C18 reversed-phase chromatography column (2.1 x 150 mm, 3.5 µm) and corresponding pre-column. Other equipment included: Sample homogenizer (Phillips); analytical balance accurate to 0.01 mg (Mettler Toledo); technical balance accurate to 0.01 g (Mettler Toledo), centrifuge (Mikro 200R, Hettich) and vortex shaker (Vortex 3, IKA). In addition, the study used some common laboratory equipment and tools such as micropipettes with adjustable volumes from 10 - 100 µL and from 100 - 1000 µL (Eppendorf), various volumetric flasks, centrifuge tubes, and measuring cylinders.

2.4. Research methods

2.4.1. Analytical methods

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to simultaneously analyze 12 antibiotic substances in eggs. The mass spectrometry system used an electrospray ionization source in positive ion mode. Detection and quantification were performed in multiple reaction monitoring (MRM) mode, with each compound monitored via a parent ion and two characteristic daughter ions. The daughter ion with the higher signal intensity was selected for quantification, while the remaining ion was used to confirm the selectivity and specificity of the method.

2.4.2. Sample preparation method

Based on reference [14], the sample processing procedure for the simultaneous determination of 12 antibiotics in egg samples was performed as follows: Weigh 2.0 g of homogenized egg sample into a 50 mL centrifuge tube, then add 2 mL of 0.02 mol/L EDTA solution. The sample was vortexed for 1 min before adding 8 mL of extraction solvent; three solvent systems including: acetonitrile (ACN), ACN containing 0.1% formic acid and ACN containing 1% formic acid were selected for investigation. The mixture was then left at 4°C for 30 min to enhance protein separation, then centrifuged at 5000 rpm for 5 min. The supernatant was collected and loaded directly onto an Oasis PRiME HLB solid-phase extraction column. The entire eluate was collected and used for analysis by LC-MS/MS system. In addition, enrofloxacin-d5 was used as an internal standard for the quinolone group; while demeclocycline was used for the tetracycline group.

In addition to the PRiME HLB cartridge, two other common matrix treatment and cleanup methods, QuEChERS and solid-liquid extraction (SLE), were also investigated and compared to select a suitable procedure. For the QuEChERS procedure, referring to the literature [15], 2g of homogenized egg sample was weighed into a 50 mL centrifuge tube, then extracted with 10 mL of acetonitrile and added with a salt mixture of 4 g of $MgSO_4$ and 1 g of NaCl. The resulting organic phase was further cleaned with C18 adsorbent, then filtered and analyzed by LC-MS/MS. For the SLE method [13], 2g of sample was extracted with 7.5 mL of acetonitrile with the addition of 0.5 mL of 0.1 M Na_2 EDTA solution; the mixture was vortexed and sonicated for 15 min, then left at 4°C for 30 min. The supernatant after centrifugation was filtered through a 0.2 µm filter and collected in a vial for LC-MS/MS analysis.

The blank samples were selected from egg samples that had been tested by LC-MS/MS and confirmed to be free of analytes.

2.4.3. Method validation

The analytical method was validated and evaluate according to AOAC Guidelines for Standard Method Performance Requirements [16]. The validation criteria include: specificity, limit of detection, limit of quantification, calibration curve and linearity, accuracy (assessed through recovery efficiency), and precision (assessed through repeatability, expressed as relative standard deviation - RSD%). The analyte was quantified based on a matrix-matched calibration curve.

2.4.4. Application

The method was applied to simultaneously detect and quantify 12 antibiotics belonging to four groups: macrolide, fluoroquinolone, sulfonamide and tetracycline in 25 egg samples. Those egg samples were collected, including 11 samples of free-range chicken eggs, 5 samples of industrial chicken eggs, 7 samples of duck eggs, and 2 samples of quail eggs. They were randomly collected from markets in Hanoi as part of a pilot sampling study.

2.4.5. Data processing method

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

The content of the analytes was determined based on the calibration curve. The calibration curve was constructed from the signal obtained when analyzing the standard solution or blank sample supplemented with standards at different concentrations. In particular, for the fluoroquinolone and tetracycline groups, the signal was represented by the ratio between the peak area of the analyte and the corresponding internal standard, while for the remaining compounds, the signal was represented by the peak area of the analyte.

In the case where the calibration curve was established using the standard solution in the solvent, the content of antibiotic active ingredients in the egg sample was calculated according to the formula:

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

In which, X is the analyte content in the sample (µg/kg); V is the volume of extract (mL), C is the analyte concentration calculated according to the standard curve (µg/L); k is the dilution factor and m is the sample mass (g).

3. RESULTS AND DISCUSSION

3.1. Mass spectrum conditions

Based on reference [14] and the structural characteristics of the analytes, fragmentation conditions were automatically set on the mass spectrometer system. The parent ion fragments, daughter ion fragments and corresponding MS/MS parameters for each compound are presented in **Table 3**.

Table 3. *Parameters for multiple reaction monitoring (MRM)*

Analytes	ESI	Molecule weight (g/mol)	Parents ion (m/z)	Daughter ions (m/z)	Collision Energy (V)	Cell exit potential (V)	Application
Tylosin	(+)	916.1	916.0	174.3	47	4	Quantitative
				772.2	40	4	Qualitative
Tilmicosin	(+)	869.1	869.6	174.1	47	4	Quantitative
				696.5	47	4	Qualitative
Erythromycin	(+)	733.9	734.4	158.4	10	4	Quantitative
				576.4	10	4	Qualitative
Enrofloxacin	(+)	359.4	360.3	316.0	47	4	Quantitative
				342.0	47	4	Qualitative
Ofloxacin	(+)	361.4	362.5	318.2	47	4	Quantitative
				261.3	47	4	Qualitative
Sulfamethoxazole	(+)	253.3	254.1	156.2	47	4	Quantitative
				188.0	47	4	Qualitative
Sulfadimidine	(+)	278.3	279.0	124.0	47	4	Quantitative
				92.0	47	4	Qualitative
Sulfisomidine	(+)	278.3	279.0	124.1	29	4	Quantitative
				186.0	23	4	Qualitative
Tetracycline	(+)	444.4	445.0	410.0	29	4	Quantitative
				427.0	25	4	Qualitative
Oxytetracycline	(+)	460.4	461.0	426.0	30	4	Quantitative
				443.0	17	4	Qualitative
Chlortetracycline	(+)	478.9	479.0	260.1	77	4	Quantitative
				444.1	31	4	Qualitative
Doxycycline	(+)	444.4	445.2	321.0	35	4	Quantitative
				154.1	37	4	Qualitative
Enrofloxacin-d5	(+)	364.4	365.2	321.0	23	10	Internal standard for the quinolones
Demeclocycline	(+)	464.9	465.1	448.2	25	4	Internal standard for the tetracyclines

The instrument parameters are automatically optimized after the parent fragments and daughter ions for the analytes have been selected, including: curtain gas (CUR) 35 psi, collision gas (CAD) 8 psi, ionspray voltage (IS) 4500V, temperature 450°C, ion source gas 50 psi and entrance potential 10 V.

3.2. Mobile phase investigation

The mobile phase composition was investigated with two different systems, including a mobile phase system supplemented with 0.1% formic acid and a mobile phase system containing a mixture of 0.1% formic acid with 10 mM ammonium formate. The choice of mobile phase system was based on the shape and symmetry of the chromatographic peaks. The results of the investigation are presented in **Figure 1**.

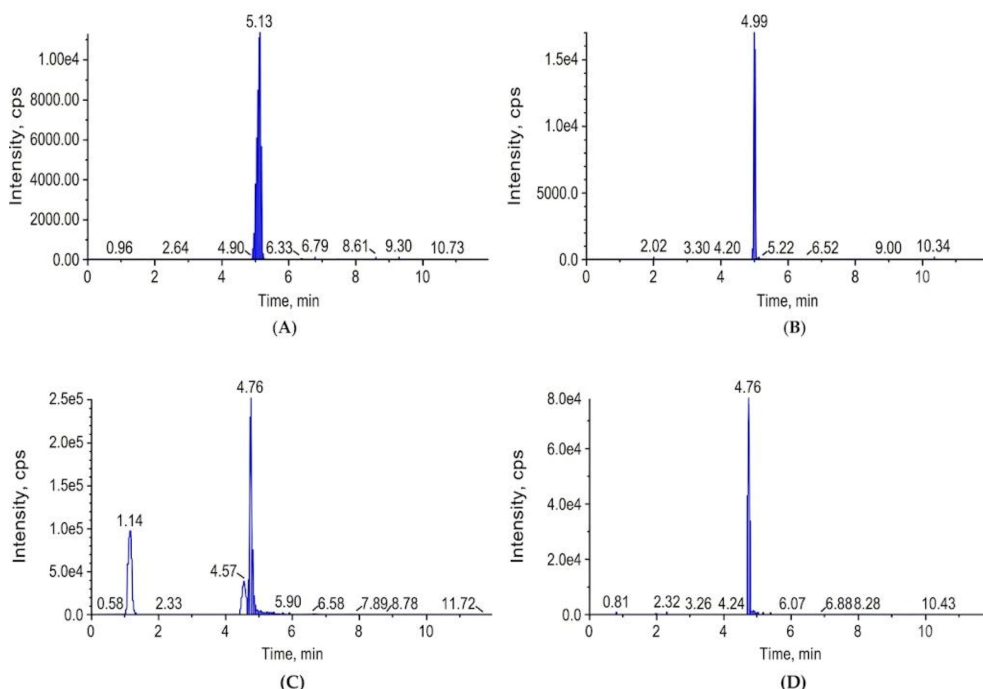

Figure 1. *Chromatograms of erythromycin with two mobile phase systems: (A) 0.1% formic acid + 10 mM NH_4HCO_2/ACN, (B) 0.1% formic acid/ACN; and of enrofloxacin with two mobile phase systems: (C) 0.1% formic acid + 10 mM NH_4HCO_2/ACN, (D) 0.1% formic acid/ACN*

By comparing the chromatographic characteristics between the two mobile phase systems, and the chromatograms of erythromycin and enrofloxacin in **Figure 1**, the results show that the mobile phase system containing 0.1% formic acid produces relatively sharp chromatographic peaks with good symmetry and narrower widths on the C18 column, indicating that the separation process is stable and the interaction between the analyte and the stationary phase is relatively consistent. On the contrary, when using the mobile phase system supplemented with 10 mM ammonium formate, the peak shape is less stable, as shown by the phenomenon of "peak tail" broadening, reduced symmetry and a tendency to reduce resolution between compounds with close retention times. On that basis, the mobile phase system containing 0.1% formic acid was selected. This result is consistent with the general principle in LC–MS/MS and the report of Freitas *et al.* [17], in which acidic mobile phases promote positive ionization of most antibiotics in this study, thereby improving ionic strength and detection ability [13, 17].

From the results of the mobile phase survey, a gradient program was set up with phase A being a solution containing 0.1% formic acid and phase B being acetonitrile, at a flow rate of 0.4 mL/min. The mobile phase composition was kept at 100% A for the first 2 min, then gradually changed to 100% B until 9.5 min and back to 100% A at 12 min to equilibrate the column before the next injection.

3.3. Optimize sample preparation conditions

3.3.1. Investigation of sample treatment procedures

The results of the background cleaning procedures are presented in **Figure 2.** The blank sample was spiked with a standard mixture of 12 antibiotics at 50 µg/kg. The procedure was developed based on reference [14] with acetonitrile as the extraction solvent.

From the results obtained, the conventional QuEChERS procedure using unbuffered acetonitrile with $MgSO_4$/NaCl salt mixture showed very low extraction efficiency for tetracycline and fluoroquinolone antibiotics in egg matrix. The low efficiency of these two groups of substances can be explained based on the physicochemical properties and matrix interactions specific to each group of substances. Tetracycline has a strong affinity for Ca^{2+} and Mg^{2+}, which are present at high concentrations in egg samples and are supplemented by $MgSO_4$. In the absence of complexing agents such as EDTA, tetracycline easily forms chelate complexes

and is not transferred to the acetonitrile phase after extraction. At the same time, the silica matrix of the C18 sorbent contains silanol groups, which can bind to tetracycline, leading to significant loss during d-SPE/SPE cleanup. For quinolones, at neutral pH, quinolones exist mainly in the zwitterion form with relative polarity, which reduces the solubility in ACN. On the other hand, quinolones in this form can also form complexes with metal ions or interact with proteins and phospholipids in eggs. The SLE procedure showed low recoveries for erythromycin and oxytetracycline (below 50%).

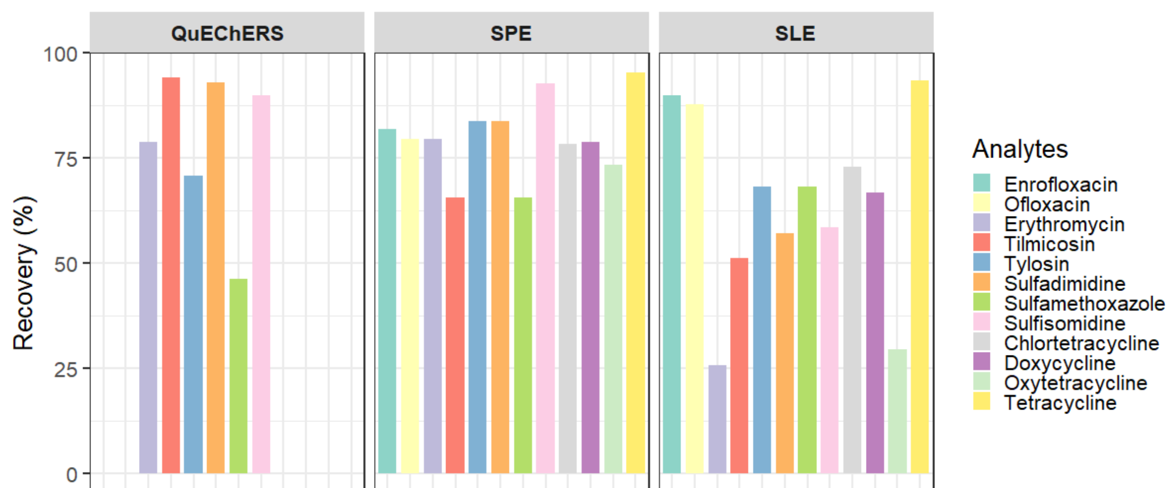

Figure 2. *Results of the investigation on the cleaning process of egg sample matrix*

In addition, the recoveries of most analytes were significantly lower than those obtained using the Oasis PRiME HLB SPE procedure. This observation is consistent with the findings of Xia *et al.* (2016) [18], who reported that the absence of a matrix cleanup step resulted in reduced recovery due to the high lecithin content in egg matrices. These phospholipids both increase the background signal and cause ion suppression at the ESI source through the mechanism of affecting the solvent removal process of the downstream mobile phase, slowing down droplet breakup and reducing the number of released ions [19]. Xia *et al.* also demonstrated that the Oasis PRiME HLB cartridge effectively removed phospholipids, thereby reducing the background noise, limiting ion suppression and significantly improving the recovery and sensitivity of the analysis for antibiotics in egg matrices [18, 19]. The results of the survey showed that the SPE procedure using the Oasis PRiME HLB cartridge is suitable for the simultaneous analysis of antibiotics in egg matrices. This choice is also consistent with previously published results and at the same time provides a basis for explaining the design of some studies [11, 14, 20, 21], in which the Oasis PRiME HLB cartridge is also preferred.

3.3.2. Investigation of extraction solvent

The extraction solvent study was conducted on a blank sample supplemented with a standard mixture of 12 antibiotics at a concentration of 50 µg/kg. Three solvent systems were evaluated including ACN, ACN containing 0.1% formic acid and ACN containing 1% formic acid. The recovery results of the analytes with each solvent system are presented in **Figure 3**.

Based on the acid-base properties and ionization states of the antibiotic groups, ACN supplemented with 0.1% formic acid was determined to be a suitable extraction solvent in this study. Sulfadimidine has two pKa constants (HB^+ = 2.4; HA = 7.4), so it mainly exists in the uncharged form in the pH range 3 - 6. In contrast, sulfamethoxazole (HA = 5.6) and Sulfisomidine (pKa = 7.6) exhibit only one pKa constant related to the dissociation of the proton at the sulfonamide group and mainly exist in the unionized form at pH < 4. The formation of the unionized form under the extraction conditions increases the hydrophobicity of the compounds, thereby increasing the extraction efficiency into the acetonitrile phase. Quinolones simultaneously carry a carboxylic acid group and a piperazinyl base. At acidic pH, the piperazinyl group is protonated while the carboxyl group is predominantly in the undissociated form, leaving enrofloxacin (HA = 6.0; HB^+ = 8.8) and ofloxacin (HA = 5.97; HB^+ = 9.28) to exist predominantly in the cationic form, significantly reducing their ability to form complexes with metal ions. The tetracycline group and its derivatives have three to four pKa constants related to the phenolic, enolic-dicarbonyl, and dimethylamino groups. Specifically, tetracycline has

HA_1 = 3.3, HA_2 = 7.7, and HB^+ = 9.7; chlortetracycline has HA_1 = 3.3, HA_2 = 7.4, and HB^+ = 9.3; doxycycline has HA_1 = 3.2, HA_2 = 7.6, HB^+ = 8.9, and HA_3 = 11.5; Oxytetracycline has HA_1 = 3.3, HA_2 = 7.3 and HB^+ = 9.1. At pH 4, they can appear in the zwitterion form; while maintaining a certain degree of hydrophobicity due to the polycyclic backbone, this form is able to form complexes with Ca^{2+}/Mg^{2+}. When the pH is reduced below the pKa_1, the phenolic and enolic groups are almost completely protonated, greatly reducing the presence of the zwitterion form and converting tetracycline to a less complexing cationic form that is easily released into the ACN phase. Macrolides are weak bases (pKa of the HB^+ form 7.7 - 9.6) and tend to be unstable in strongly acidic media. However, under the 0.1% formic acid conditions used for the extraction, these compounds remained stable over the operating period and existed mainly in the protonated form. Although mainly present in the cationic form, macrolides maintain their ability to distribute into the ACN phase due to a certain degree of hydrophobicity of the molecule, which is indirectly reflected by logKow values such as 3.80 (tilmicosin), 3.06 (erythromycin) and 1.63 (tylosin).

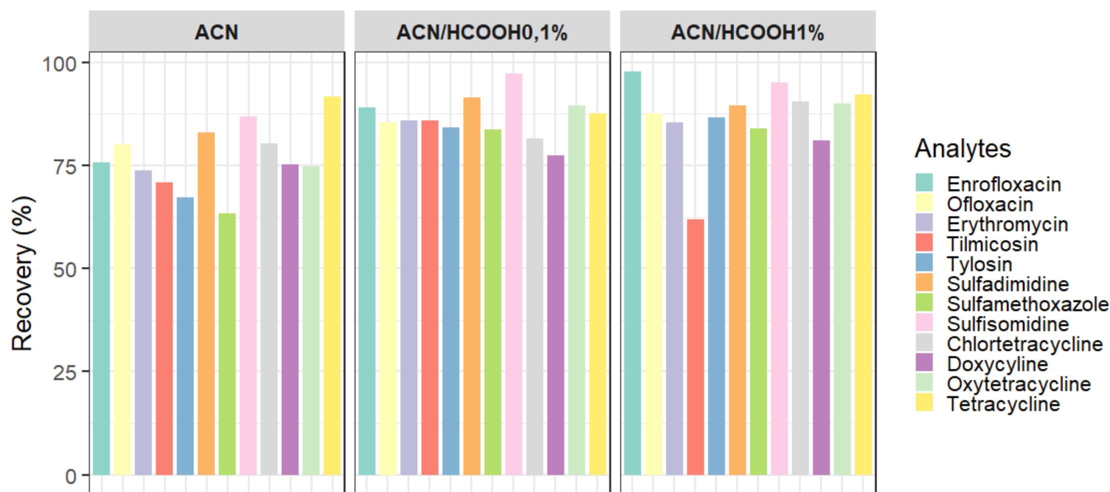

Figure 3. *Results of investigating extraction solvent*

The results (**Figure 3**) show that the recovery of tilmicosin is significantly reduced when using ACN containing 1% formic acid, while most antibiotics also give low recoveries when using pure ACN. These results clearly reflect the role of pH in the extraction process: too acidic environments reduce the stability of macrolides, while unadjusted pH environments cause sulfonamides, tetracyclines and quinolones to exist in an ionized state that is not favorable for the release process from the sample matrix. These results are consistent with previous studies [22], in which sulfonamides achieved the highest performance in the pH range of 5 - 6, while macrolides were more stable and extracted more effectively at neutral or weakly acidic pH. Overall, the ACN solvent system containing 0.1% formic acid created a suitable pH environment, helping to maintain a favorable ionization state for the release of antibiotic compounds from the sample matrix, while ensuring the chemical stability of the compounds in the study. In addition, tetracycline tends to form stable complexes with Ca^{2+} and Mg^{2+} ions as well as interact with proteins and silanol groups; therefore, the addition of a complexing agent such as EDTA or Na_2EDTA is necessary to prevent this complexation and improve the extraction efficiency [22].

3.4. Method validation

3.4.1. Selectivity

In LC-MS/MS, identification points (IP) are an important parameter used to evaluate the specificity of the analysis. According to the scoring rule, the LC separation technique is converted into 1 point, each parent ion contributes 1 point and each product ion contributes 1.5 points [23]. Based on the results presented in **Table 3,** each analyte uses 1 parent ion and 2 product ions, corresponding to a total of 5 IP points; therefore, the method meets the specificity requirement for LC-MS/MS (IP $\geq$ 5).

In addition, the specificity of the method is evaluated through the ratio between the quantitative ion and the qualitative ion of the analytes, in which the ion ratio of the standard mixture solution is compared with the corresponding ratio in the spiked blank sample at equivalent concentrations. The results showed that the relative

deviation of the ion ratio did not exceed ± 40%, meeting the requirements for analyte confirmation. In addition, when analyzing the blank sample, the standard mixture solution and the spiked blank sample with at least 6 repetitions [23], the results showed that the blank sample did not show any signal of the analytes; at the same time, the retention time of 12 antibiotics between the standard mixture solution and the spiked blank sample did not differ significantly (< 1%), confirming the stability and good discrimination ability of the method.

The matrix effect was evaluated using a standard solution and a spiked sample at a concentration of 100 µg/kg. The results demonstrated a significant difference between the analytical signals of the spiked sample and the standard solution (> ± 20%), indicating a pronounced matrix effect that may influence the analytical performance. Therefore, matrix-matched calibrations were employed to minimize matrix interferences during analyte quantification.

3.4.2. Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) of the method were determined based on the signal/noise ratio (S/N) [23]. The LOD was established by analyzing a blank sample spiked with a standard mixture of 12 analytes at decreasing concentrations until reaching a S/N ratio of 3 [23]. The LOQ was determined by multiplying the S/N ratio by a factor of 10. For four substances belonging to the tetracycline group, the LOD and LOQ values were 15 and 50 µg/kg, respectively; for the remaining eight substances, the LOD and LOQ were 3 and 10 µg/kg, respectively. Compared with the limits specified in Circular No 24/2013/TT-BYT, the method showed suitable sensitivity for detecting and quantifying analytes for food safety control. The LOD and LOQ of each analyte were indicated in **Table 4**.

3.4.3. Calibration curve and linearity

Matrix-matched calibration curves for the determination of 12 antibiotics by LC-MS/MS were constructed at concentration levels of 50, 80, 100, 300, and 500 µg/kg for tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), and doxycycline, and at 10, 20, 50, 80, and 100 µg/kg for enrofloxacin, ofloxacin, sulfadimidine, sulfisomidine, sulfamethoxazole, erythromycin, tilmicosin, and tylosin. Calibration curve equations together with the correlation coefficients presented in **Table 4**. In which, x is the concentration of the analyte, and y is the chromatographic signal, expressed as the peak area of the analyte for groups without using the internal standard, or as the ratio between the peak area of the analyte and the internal standard for the fluoroquinolone and tetracycline groups.

Table 4. *Calibration curve equation and correlation coefficient*

Analytes	LOD (µg/kg)	LOQ (µg/kg)	Standard curve equation	Correlation coefficient (R^2)
Enrofloxacin	3.0	10.0	y = 0.00641x + 0.018	0.9996
Ofloxacin	3.0	10.0	y = 0.00461x – 0.0116	0.9990
Erythromycin	3.0	10.0	y = 508x + 3030	0.9996
Tilmicosin	3.0	10.0	y = 1830x - 9050	0.9978
Tylosin	3.0	10.0	y = 4970x + 75900	0.9958
Sulfadimidine	3.0	10.0	y = 10400x + 175000	0.9988
Sulfamethoxazole	3.0	10.0	y = 7910x + 172000	0.9996
Sulfisomidine	3.0	10.0	y = 13400x + 284000	0.9986
Chlortetracycline	15.0	50.0	y = 0.00953x + 0.255	0.9946
Doxycycline	15.0	50.0	y = 0.000922x + 0.0206	0.9996
Oxytetracycline	15.0	50.0	y = 0.00577x + 0.00311	0.9998
Tetracycline	15.0	50.0	y = 0.0119x + 0.0131	0.9986

The results in **Table 4** show that the standard curve equations of the 12 substances all have correlation coefficients $R^2 > 0.990$. Therefore, in the investigated concentration range, there is a linear relationship between the chromatographic signal (represented by the peak area of the analyte or the ratio between the peak area of the analyte and the internal standard for the two tetracycline and fluoroquinolone groups) and the corresponding

analyte concentration. The deviation at all concentration points does not exceed ± 15%, meeting the AOAC requirements [16].

3.4.4. Precision and accuracy of the method

The trueness (assessed by recovery, H%) and precision (assessed by repeatability (within-day), expressed as relative standard deviation - RSD%) were determined by analyzing spike samples at three concentration levels: 50, 100 and 500 µg/kg for the tetracycline group; 10, 20 and 100 µg/kg for the remaining eight substances. Each concentration level was analyzed in six replicates using optimal sample preparation procedure. The trueness and precision results are presented in **Table 5.**

Table 5. *Precision and accuracy results on egg sample matrix*

	Average recovery (%) ± RSD (%)		
Analytes	**10 µg/kg**	**50 µg/kg**	**100 µg/kg**
Erythromycin	89.6 ± 4.7	94.1 ± 6.0	98.7 ± 2.7
Tilmicosin	102.8 ± 8.5	95.9 ± 12.4	90.5 ± 7.7
Tylosin	91.6 ± 6.0	95.7 ± 6.0	100.7 ± 2.5
Sulfamethoxazole	99.9 ± 8.4	102.0 ± 5.1	96.7 ± 5.3
Sulfisomidine	90.3 ± 9.4	104.9 ± 4.2	97.0 ± 3.1
Sulfadimidine	94.1 ± 11.9	99.6 ± 6.1	97.3 ± 4.8
Enrofloxacin	91.7 ± 8.6	94.8 ± 11.3	98.7 ± 3.4
Ofloxacin	103.1 ± 7.0	85.6 ± 5.2	97.3 ± 5.9
	Recovery ± RSD		
Analytes	**50 µg/kg**	**100 µg/kg**	**500 µg/kg**
Oxytetracycline	96.5 ± 10.2	95.5 ± 8.2	91.5 ± 9.3
Chlortetracycline	83.8 ± 3.5	97.5 ± 4.3	93.1 ± 8.5
Tetracycline	95.5 ± 6.8	100.1 ± 6.7	86.8 ± 6.2
Doxycycline	94.5 ± 9.1	96.3 ± 5.9	98.7 ± 7.6

The results presented in **Table 5** show that the recoveries of the tetracycline group compounds ranged from 83.8 - 100%, with RSD values ranging from 3.50 - 10.2% on egg matrix. For the remaining eight compounds, the recoveries were 85.6 - 105% and the RSDs ranged from 2.50 - 12.4%. According to AOAC [16], the concentration level of 10 - 50 µg/kg required recoveries in the range of 60 - 115%, while the level of 100 - 500 µg/kg required recoveries of 80 - 110% and repeatability with RSDs less than 32%. The results showed that all the analytes fully met the criteria for accuracy and precision according to AOAC [16]. Therefore, the analytical method demonstrated acceptable reliability, meeting the requirements in determining residues of 12 antibiotics belonging to the fluoroquinolone, tetracycline, sulfonamide and macrolide groups in egg samples. Measurement uncertainty was not evaluated and is therefore acknowledged as a limitation of the study.

3.5. Analysis of real sample

The validated method was applied to screen and quantify 12 antibiotics in 25 egg samples collected in Hanoi. The results showed that 21/25 samples did not detect antibiotic residues. The recoveries were evaluated using spiked samples at a concentration of 100 µg/kg. The recoveries obtained for the spiked samples ranged from 85.5 to 108% for the 12 investigated antibiotics, which met the AOAC requirements (acceptable recovery range of 80 - 110% at this concentration level).

In the remaining four samples, oxytetracycline was detected in two samples but both were below the limit of quantification; the remaining two samples recorded the presence of doxycycline and sulfamethoxazole with concentrations of 1360 and 30 µg/kg, respectively (illustrative chromatograms are shown in **Figure 4**). Although doxycycline is not authorized for use in laying hens; therefore, any detectable residue in eggs may warrant attention regarding its use. In addition, when compared with the Vietnamese maximum residue level (MRL) of 400 µg/kg for tetracycline, oxytetracycline, and chlortetracycline in eggs, the detected doxycycline concentration of 1360 µg/kg appears relatively high despite the absence of an official MRL.

These preliminary findings suggest the possible presence of antibiotic residues at specific sampling points and indicate the need for further studies focusing on exposure assessment and risk evaluation.

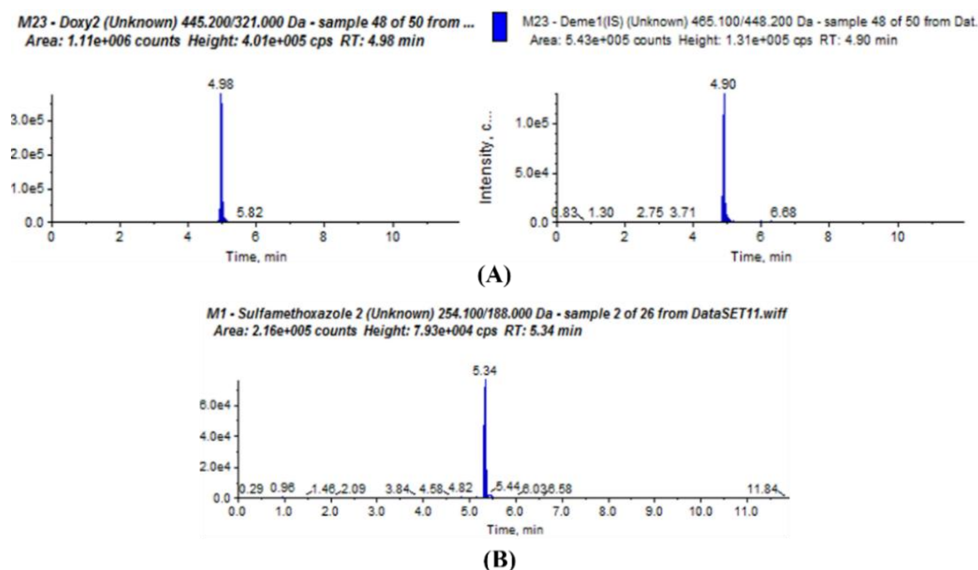

***Figure 4**. Chromatograms of two samples for doxycycline (A) and sulfamethoxazole (B) detection*

4. CONCLUSION

The study successfully developed a procedure for simultaneous analysis of 12 antibiotics belonging to four groups: tetracycline, sulfonamide, fluoroquinolone and macrolide in egg matrix, meeting the validation requirements according to AOAC guidelines. As a preliminary investigation, the method was applied to 25 randomly collected egg samples from local markets; antibiotic residues were not detected in 21 samples. The remaining four samples showed the presence of oxytetracycline, doxycycline, and sulfamethoxazole, including one sample with a doxycycline concentration of 1360 µg/kg. Eggs are a highly consumed food and play an important role in the diet of Vietnamese people. These findings highlight the need for further in-depth studies on exposure and risk assessment to support enhanced domestic food safety management.

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