

Simultaneous Determination of Four Tetracycline Antibiotics Residues in Chicken by Solid Phase Extraction Coupled with High Performance Liquid Chromatography

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Abstract

Objective: To establish a simple and rapid method for simultaneous determination of Tetracycline, Oxytetracycline, Chlortetracycline and Doxycycline in chicken. **Methods:** After optimized sample pretreatment and chromatographic conditions, tetracycline antibiotics in spiked chicken were extracted by ultrasonic extraction with Na₂EDTA-McIlvaine buffer solution (pH=4.0), purified and enriched by HLB solid phase extraction column, separated by Shim-pack GIST C18 column (4.6×150 mm, 3 μm), methanol-acetonitrile-oxalic acid (1:2:7, V: V: V) as the mobile phase, the flow rate was 0.9 mL/min, the UV detection wavelength was 350 nm, and the peak area was quantified by external standard method. **Results:** The linearity of the four tetracycline antibiotics was good in the range of 0.05-1 mg/kg ($r > 0.997$), and the detection limits were 2.53 μg/kg ~ 5.94 μg/kg according to the S/N ratio (S/N) of 3. The relative standard deviations (RSD, n=6) of precision were 1.51%-4.80%. The recoveries were from 76.81% to 96.34%. **Conclusion:** The method has good selectivity, high accuracy and reproducibility, and is suitable for simultaneous determination of four tetracycline antibiotics in chicken.

Keywords: chicken, tetracycline antibiotics, solid phase extraction, high performance liquid chromatography

1. Introduction

Tetracyclines (TCs) are a kind of natural or semi-synthetic drugs with low price and wide antibacterial spectrum (Bayliss et al., 2019), the chemical structure is shown in Figure 1, which can promote the growth of poultry, control its reproductive cycle and breeding ability (Mesgari Abbasi et al., 2012). As a feed additive, they are widely used for the prevention and control of animal diseases (Sreejith et al., 2020). The common ones are tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC). Some illegal merchants often abuse these drugs for economic benefits, resulting in excessive TCs residue in food. When consumers eat foods with excessive TCs, they may have adverse reactions such as tetracycline teeth, liver damage, digestive dysfunction, antibiotic resistance and so on (Rodriguez et al., 2010; Liu, et al., 2017). Therefore, detection of TCs in animal derived food is of great significance to ensure food hygiene and safety.

At present, the pre-treatment methods of complex matrix for TCs residue include liquid-liquid microextraction (Mohebi et al., 2020), Solid Phase Extraction (SPE) (Sun et al., 2014), molecular imprinting technology (Huang et al., 2019), QuEChERS method (Grande-Martínez et al., 2018), etc., among which Solid Phase Extraction is more commonly used. Bajkacz S et al. (2020) used the SPE method to extract TCs from feces and sludge, and the recovery rate was 45% ~ 85% (Bajkacz et al., 2020). Tolgyesi et al. (2014) used the SPE method to detect TCs in a variety of meat, and the recovery rate in chicken was only 49.4% ~ 69.1%. These solid-phase extraction methods have low recovery rate in extracting and purifying TCs from complex matrix (Tolgyesi et al., 2014).

Currently, the detection methods of TCs include high performance liquid chromatography, electrochemical analysis (Mavritsakis et al., 2019), microbial method (Ntakatsane et al., 2020), ELISA (Chen et al., 2016), colloidal gold immunoassay (Shen et al., 2014), etc., but the most commonly used method for qualitative and quantitative detection of TCs is high performance liquid chromatography analysis. Among them, HPLC connected with photodiode array detector (Sun et al., 2014) is the most commonly used because of its simplicity and low cost. HPLC coupled with fluorescence detector (Kargin et al., 2016) was used for detection, and samples needed pre-column derivatization to be tested on machine, which resulted in cumbersome pre-processing and easy influence on quantitative accuracy. HPLC tandem chemiluminescence detectors (Li et al., 2018) and tandem mass spectrometry (Ren et al., 2016) are expensive and not suitable for routine laboratory testing. China's national standards provide a HPLC method for Tetracycline, Oxytetracycline and Chlortetracycline residues in livestock and poultry meat (Ministry of health of the people's Republic of China, China National Standardization Administration Committee., 2003), and the detection limit is 0.15-0.65 mg/kg. The detection limit of this standard method is too high, and Doxycycline cannot be detected simultaneously.

In this study, SPE and chromatographic conditions were optimized, and a simple and rapid SPE-HPLC-DAD method was established. The method has good selectivity, high recovery, high accuracy, good reproducibility and low detection limit. It was used for simultaneous detection of four TCs in Sample of chicken in Jinan city.

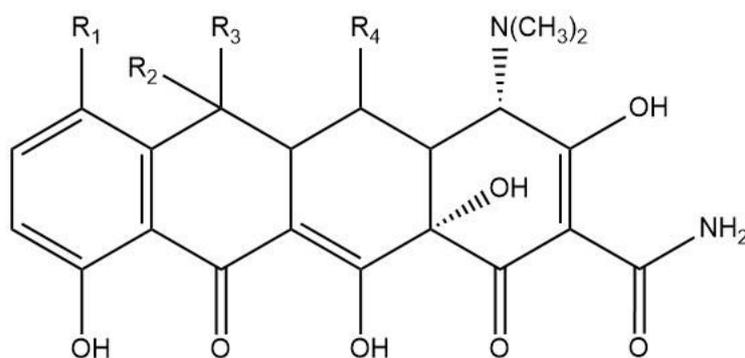


Figure 1. Basic chemical structure of tetracyclines antibiotics

2. Materials and Methods

2.1 Instruments and Reagents

Shimadzu LC-20AT High Performance Liquid chromatograph (including SPD-M20A photodiode array UV visible detector, SIL-20A automatic sampler, LC-20AT solution conveying unit $\times 2$, DGU-20A3R online degassing machine), Shim-pack GIST C18-AQ column (4.6 \times 150 mm, 3 μ m), purchased from Shimadzu Company, Japan; Precellys Evolution Grinder, Purchased from Bertin Technologies; NGSC603-HLB SPE Column (60 mg, 3 mL), purchased from Shanghai Xinhua Experimental Equipment Co., LTD.

Standard products: TC (BWN5365-2016), OTC (BWN5108-2016), CTC (BWN5312-2016), all purchased from Beijing North Weiye Metrology Technology Institute; DC(CN190914-06), Stanford Analytical Chemicals Inc.; Reagents: acetonitrile (chromatographic pure), methanol (chromatographic pure), 88% formic acid (analytical pure), all purchased from Sinopharm Chemical Reagent Co., LTD. Water is ultrapure water purified at the lab.

2.2 Chicken Sample

From March to September 2021, 111 chicken samples (each not less than 700g) were collected from supermarkets and farmers' markets in Jinan urban area. Samples were stored in a freezer at -40 $^{\circ}$ C until extraction.

2.3 Preparation of TCs Mixed Standard Solution

Accurately measured 1 mL of TC, OTC, CTC and DC 100 mg/L standard solutions respectively, added them into a 10 mL volumetric bottle, and diluted with methanol to a final volume of 10 ml to prepare 10 mg/L mixed standard solution, which was used to prepare a series of standard application solutions.

2.4 Preparation of Na₂EDTA-McIlvaine Buffer Solutions with Different pH Values

Mixed 1000 mL of 0.1 mol/L citric acid solution with 625 mL of 0.2 mol/L Na₂HPO₄ solution, weighed 60.49 g Na₂EDTA into the mixture, shook well, until completely dissolved, adjusted pH with hydrochloric acid or NaOH

solution to 3.5, 4.0, 4.5. Three kinds of Na₂EDTA-McIlvaine buffer solutions with different pH values were prepared.

2.5 Optimization of Sample Pre-treatment conditions

2.5.1 Optimization of Extraction Conditions

5 g of chicken samples were accurately weighed and homogenized, then placed in a 50 mL polypropylene centrifugal tube, and extracted with 20 mL, 20 mL and 10 mL of Na₂EDTA-McIlvaine buffer solution in ice water bath three times by ultrasound. In each extraction process, vortex mixing for 1 min, ultrasonic extraction for 10 min, 5000 rpm, 4°C, centrifugation for 5 min. The supernatant was combined, and the volume was fixed to 50 mL. The supernatant was filtered with glass fiber filter paper and then purified.

In order to study the effect of Na₂EDTA-McIlvaine buffer solutions with different pH on TCs recovery, Na₂EDTA-McIlvaine buffer solutions with pH values of 3.5, 4.0 and 4.5 were used to extract TCs from spiked chicken samples respectively.

2.5.2 Purification and Concentration

Activation: In order to study the effects of different activation conditions on TCs recovery, a HLB solid phase extraction column was activated by "6 mL methanol, 6 mL water" and "6 mL methanol, 6 mL water, 6 mL Na₂EDTA-McIlvaine buffer solution".

Sample loading and washing: The sample extract was passed through the column at a rate of 1 mL /min, and the filtrate was discarded. Rinse with 3 ml water, discard the liquid and drain.

Elution: In order to study the effects of the two kinds of eluents on TCs recovery, 10 mL methanol and 0.1% formic acid-methanol were used for column elution at the rate of 1 mL /min to collect the eluents.

Concentration: The eluent was blow-dried with nitrogen at 40 °C, redissolved with 1 mL methanol, filtered through a 0.45 µm microfiltration membrane, and then tested on the machine.

2.6 Optimization of Chromatographic Conditions

Chromatographic column: Shim-pack GIST-C18 (4.6×150 mm, 3 µm); Detector: SPD-M20A photodiode array UV and visible light detector; Injection volume: 20 µL.

Detection wavelengths: The detection wavelengths of 270 nm and 350 nm were used for detection, respectively, to study the influence of different detection wavelengths on chromatographic results;

Mobile phases: acetonitrile-formic acid, acetonitrile-oxalic acid, methanol-acetonitrile-citric acid and methanol-acetonitrile-oxalic acid were used to study the influence of different mobile phases on chromatographic results.

Flow rate: Four flow rates, 0.5mL/min, 0.8mL/min, 0.9mL/min and 1.0mL/min, were used to study the effect of different flow rates on the chromatographic results.

Column temperatures: Three column temperatures, 25°C, 30°C and 35°C, were used to study the influence of different column temperatures on the chromatographic results.

2.7 Methodological Verification

2.7.1 Analytical Parameters

A series of standard application solutions were prepared by mixing standard solution of TCs with a concentration of 10 mg/L, and the standard solution of each concentration was added to the homogenized blank chicken sample. The final concentrations of the samples were 1.0 mg/kg, 0.8 mg/kg, 0.5 mg/kg, 0.2 mg/kg, 0.1 mg/kg and 0.05 mg/kg, respectively. After treatment with the optimized pre-treatment method, the samples were tested on the HPLC. With concentration as abscissa and peak area as ordinate, working curves were drawn and regression equations were fitted.

2.7.2 Standard Recovery Rate

Within the linear range of the determination method, the TCs mixed standard solution was added to the homogenized blank chicken sample, and the final concentration of the sample was 1.0 mg/kg, 0.5 mg/kg, and 0.1 mg/kg, respectively. Three horizontal experiments were conducted for each concentration. After treatment with the optimized post-pretreatment method, the test was conducted on the HPLC. The recoveries of spiked samples with different concentrations were calculated.

2.7.3 Precision

Within the linear range of the determination method, the TCs mixed standard solution was added to the homogenized blank chicken sample, and the final concentration of the sample was 1.0 mg/kg, 0.5 mg/kg, and 0.1 mg/kg, respectively. Six parallel experiments were conducted for each concentration. After treatment with the optimized pre-treatment method, the samples were tested on the HPLC. The precision of the method was obtained by calculating the standard deviation of the spiked samples with different concentrations.

2.7.4 Detection Limit

The blank sample was measured 12 consecutive times, and its standard deviation S was calculated according to the peak area. If the data followed the normal distribution, the response value of the blank sample in a single measurement was 99.7% likely to be within $3S$. Therefore, with a 99.7% confidence limit, the concentration corresponding to $3S$ was the detection limit.

3. Results

3.1 Optimization of Sample Pre-treatment Conditions

3.1.1 Optimization of pH Value of Extraction Solution

In this study, the recovery rates of four TCs were compared when the pH of Na₂EDTA-McIlvaine buffer solution was 3.5, 4.0 and 4.5. As shown in Figure 2, when the pH of the buffer solution was 4.0, the recovery rate of the four TCs was the highest ($P < 0.05$). Therefore, Na₂EDTA-McIlvaine buffer solution with the pH of 4.0 was selected to extract the sample.

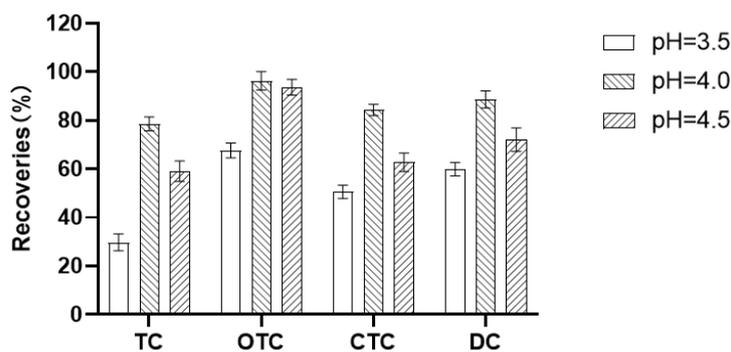


Figure 2. Effect of pH of buffer solution on the recovery of TCs

3.1.2 Optimization of Activation and Elution Conditions of HLB Solid Phase Extraction Column

In this study, the influence of Na₂EDTA-McIlvaine buffer solution on TCs recovery was compared in the activation step of HLB solid phase extraction column. The results showed that, the recovery rate of TCs in the activated Na₂EDTA-McIlvaine buffer solution can be increased by 12.51%-28.14% compared with that without Na₂EDTA-McIlvaine buffer solution. Therefore, “6 mL methanol, 6 mL water, 6 mL Na₂EDTA-McIlvaine buffer solution” was selected to activate the HLB solid phase extraction column with.

The elution effect of methanol was compared with that of methanol containing 0.1% formic acid. The results showed that the elution effect of methanol containing 0.1% formic acid was better, and the recovery rate was from 8.22% to 12.57% higher than that of pure methanol. Therefore, methanol containing 0.1% formic acid was selected as the elution solvent.

3.2 Optimization of Chromatographic Conditions

3.2.1 Optimization of Mobile Phase

In this study, the effects of acetonitrile-formic acid, acetonitrile-oxalic acid, methanol-acetonitrile-citric acid and methanol-acetonitrile-oxalic acid mobile phase systems on the peak type and separation degree of target chromatographic peaks were compared. The results showed that the peak time of the acetonitrile-formic acid mobile phase system was too late and the chromatographic peak shape was too wide. For the acetonitrile-oxalic acid mobile phase system, the peak time is too concentrated, the target peak and impurity peak are difficult to separate completely. In the methanol-acetonitrile-citric acid mobile phase system, TCs were separated from the solvent peak, but the four TCs were not completely separated, and the chromatographic peak front was obvious.

In the methanol - acetonitrile-oxalic acid mobile phase system, the four TCs can be completely separated with a good peak shape and bilateral symmetry. Therefore, the methanol - acetonitrile - oxalic acid mobile phase system was selected. After repeated optimization, when a 0.01 mol/L oxalic acid/methanol/acetonitrile (7:1:2, V: V: V) was used, the separation degree and peak shape of the four TCs were the best. As shown in Figure 3, the retention times of OTC, TC, CTC and DC are 4.179 min, 5.037 min, 10.660 min and 15.946 min, respectively.

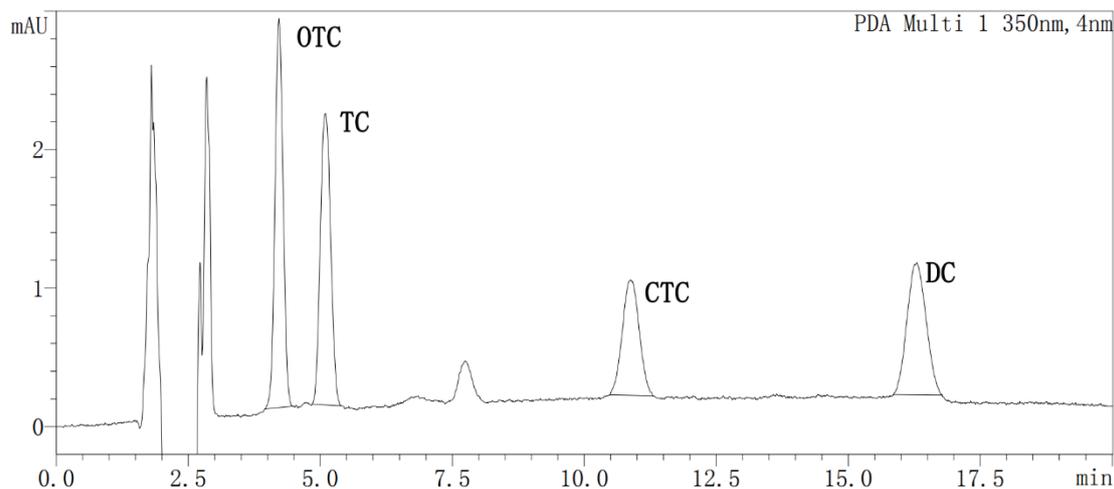


Figure 3. The HPLC chromatogram of TCs-Methanol solution

3.2.2 Optimization of the Detection Wavelength

As shown in Figure 4, the all four TCs veterinary drugs had absorption peaks at about 270 nm and 350 nm, respectively. The spiked recovery chromatograms of the four TCs are shown in Figure 5. Under the condition of 270 nm, the sample chromatograms had more interference from impurity peaks, while under the condition of 350 nm, the chromatograms had a better separation effect, less interference from non-target peaks, high reading precision and a good selectivity. Therefore, 350 nm was selected as the best detection wavelength of TCs.

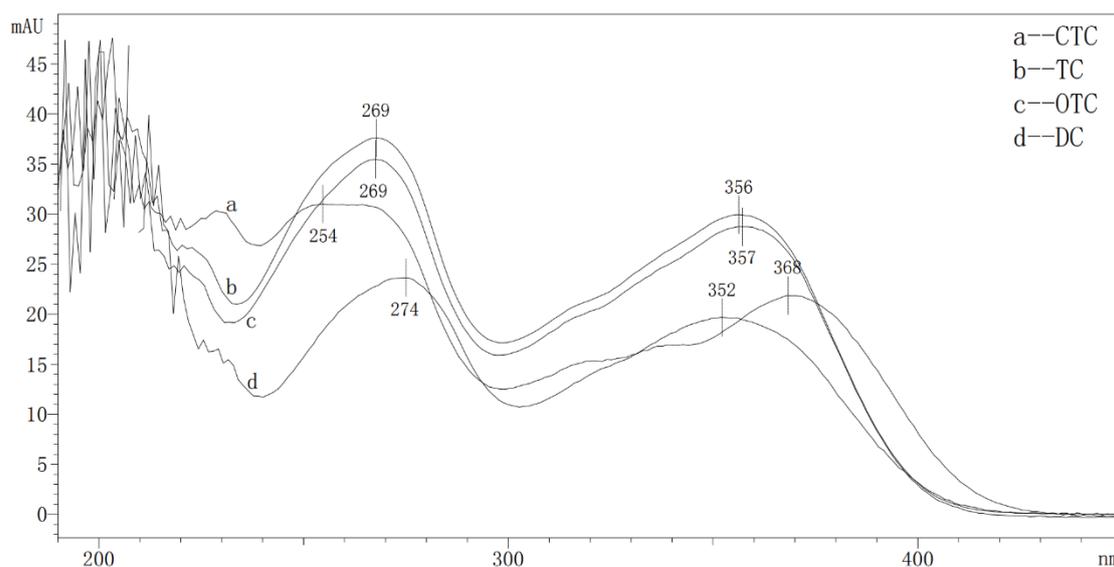


Figure 4. The UV absorption curve of four TCs

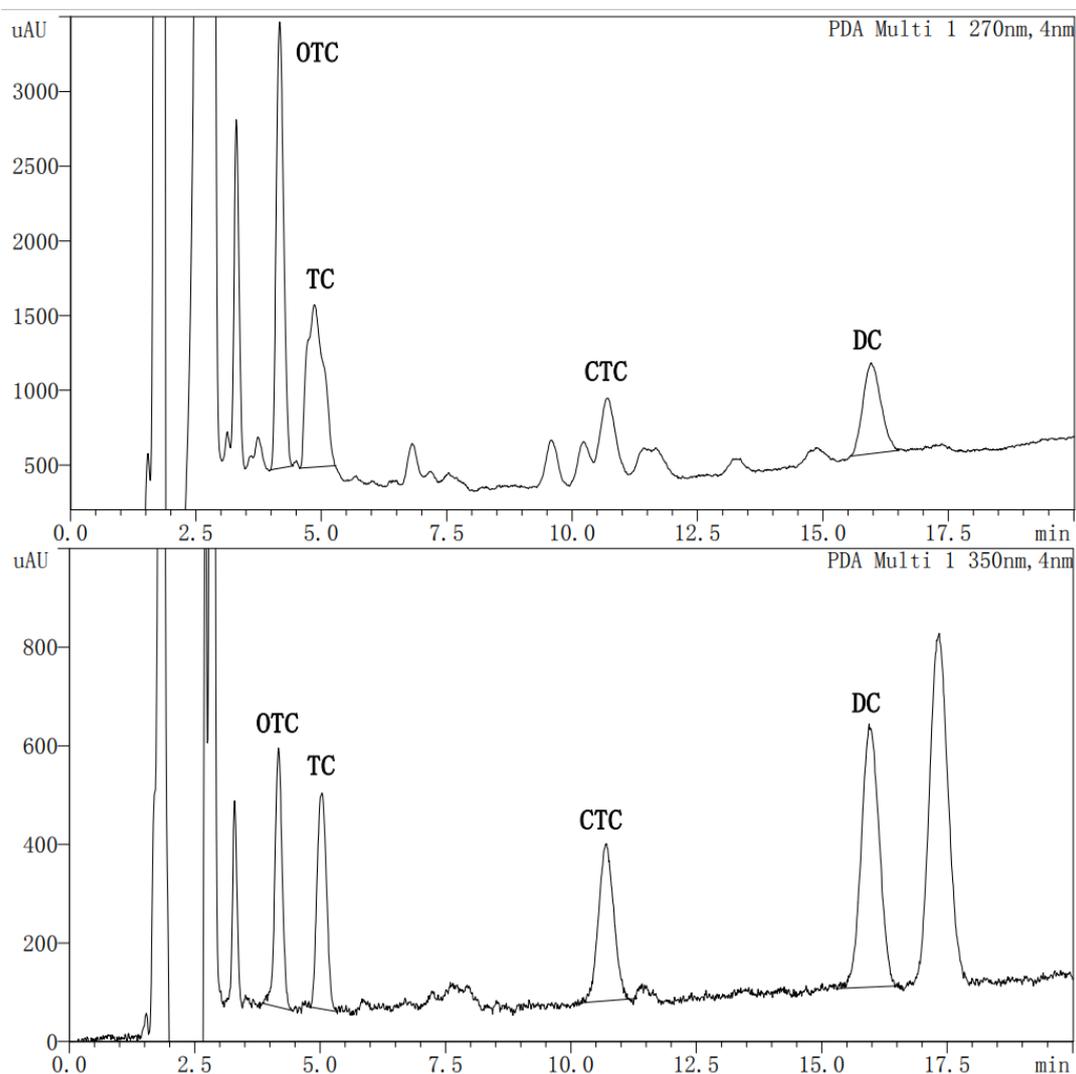


Figure 5. Chromatograms of four TCs spiked samples with detection wavelengths of 270nm and 350nm

3.2.3 Optimization of Flow Rate and Column Temperature

In this study, the effects of four flow rates of 0.5 mL/min, 0.8 mL/min, 0.9 mL/min and 1.0 mL/min on chromatographic results were compared. The results showed that the retention time of the four TCs was too long when the flow rate was 0.5 mL/min. When the flow rate was 1.0 mL/min, the column pressure was higher than the safe level, which affected the column efficiency. When the flow rate was 0.8 mL/min and 0.9 mL/min, the retention time was shorter and the column pressure was at a reasonable level. Compared with the two flow rates, the flow rate of 0.9 mL/min could reach the peak faster within 20 min, so 0.9 mL/min was selected.

The effects of column temperatures of 25 °C, 30 °C and 35 °C on the separation degree and retention time of target chromatographic peaks were compared. The results show that the separation degree of TC and OTC was not good when the column temperature was 35 °C. When the column temperature was 25°C and 30°C, the separation degree of the four TCs was good, but the retention time was shorter at 30°C, so the column temperature was chosen to be 30°C.

3.3 Methodological Verification

3.3.1 Linear Relationship and Detection Limit

It can be seen from Table 1 and Figure 6 that in the range from 0.05 mg/kg to 1.0 mg/kg, the four TCs had a good linear relationship with correlation coefficients greater than 0.997. The limits of detection for TC, OTC, CTC and DC were 3.67 µg/kg, 2.53 µg/kg, 5.94 µg/kg and 3.90 µg/kg, respectively.

Table 1. Linear relationship and detection limit of four TCs in spiked sample

Compound	Regression equation ¹	Correlation coefficient	LOD($\mu\text{g}/\text{kg}$)
TC	$Y=21365X-33.635$	0.9998	3.67
OTC	$Y=30955X+5.4582$	0.9974	2.53
CTC	$Y=13210X+127.12$	0.9996	5.94
DC	$Y=20119X+158.44$	0.9994	3.90

Note. ¹ X is tetracycline content, $\mu\text{g}/\text{kg}$; Y is peak area, $\text{mAU}\cdot\text{min}$.

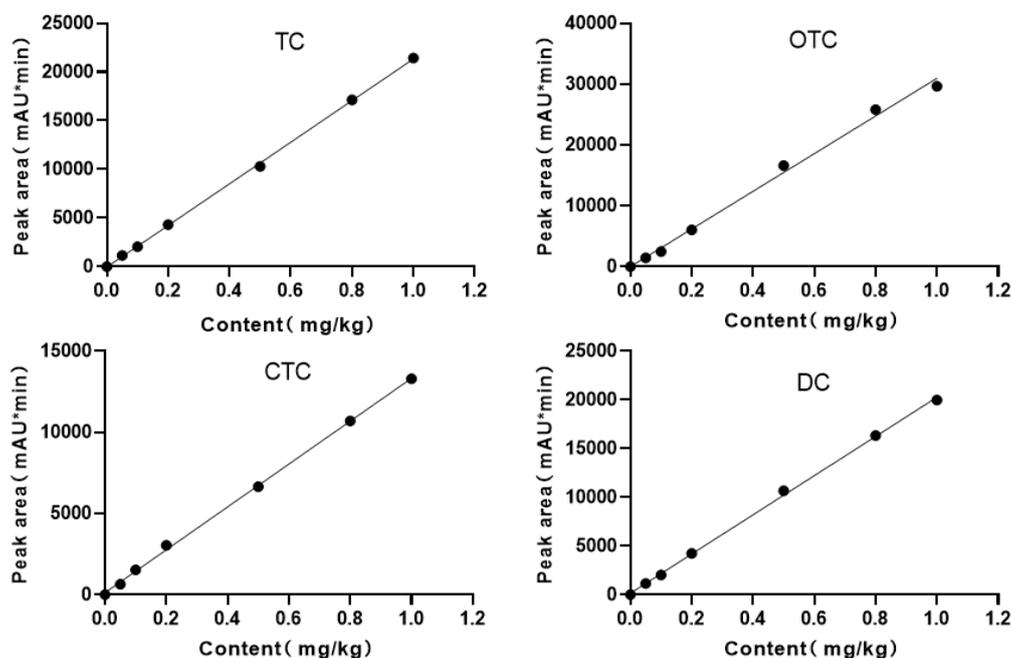


Figure 6. Working curves of four TCs in spiked samples

3.3.2 Spiked Recovery and Precision

It can be seen from Table 2 that the spiked recoveries of the four TCs in chicken ranged from 76.81% to 96.34% with RSD from 1.51% to 4.80%, which can meet the requirements of trace detection of TCs in chicken.

Table 2. Recovery and repeatability for four TCs in spiked chicken

Compound	Concentration (mg/kg)	Recovery($\bar{X}\pm\text{SD}$, n=3)	RSD (%)
TC	0.1	81.87 \pm 1.25	1.53
	0.5	76.81 \pm 2.36	3.07
	1	78.61 \pm 2.11	2.69
OTC	0.1	96.17 \pm 1.71	1.77
	0.5	95.37 \pm 1.76	1.84
	1	96.34 \pm 1.72	1.78
CTC	0.1	87.54 \pm 2.09	2.38
	0.5	84.29 \pm 1.28	1.51
	1	86.31 \pm 1.91	2.22
DC	0.1	86.79 \pm 1.99	2.30
	0.5	88.65 \pm 3.37	3.81
	1	87.11 \pm 4.18	4.80

3.3.3 Inspection Status of Chicken Samples

Table 3 shows the detection of four TCs residues in 111 chicken samples. The suitability of the extraction and HPLC methods was evaluated by analyzing the real sample.

Table 3. TCs residue detection in 111 chicken samples

Compound	Detection number	Detection rate (%)	Detection quantity ($\bar{X} \pm SD$)	Repeatability (RDS %)
TC	14	12.61	39.28 \pm 12.74	2.56
OTC	15	13.51	34.07 \pm 20.07	1.94
CTC	29	26.13	52.38 \pm 17.08	2.15
DC	23	20.72	79.30 \pm 47.60	3.28

4. Discussion

4.1 Optimization of Sample Pre-treatment Conditions

Due to the strong polarity of TCs, it is easy for them to form conjugated compounds with proteins in chicken (Li et al., 2021) and chelates with metal ions (Qu et al., 2009), resulting in difficult extraction of TCs. In this study, a Na₂EDTA-McIlvaine buffer solution was used to extract TCs from chicken. The McIlvaine buffer solution can reduce the bonding action between TCs and protein. EDTA as a competitive chelating agent can eliminate the interference of metal ions, and then improve the recovery rate. In addition, TCs are easy to decompose when exposed to light and heated, so a low temperature is required during the ultrasonic extraction and light is avoided. In this study, an ice water bath was used for ultrasonic extraction and light was avoided at the same time to ensure recovery and lipid removal.

In this study, a HLB solid phase extraction column was used for purification and enrichment. The solid phase extraction column is usually activated by methanol and water, but TCs are more stable in a weak acidic environment. In this study, a Na₂EDTA-McIlvaine buffer solution was used to provide a weak acidic environment at the end of extraction column activation, and the results showed that the spiked recovery rate was significantly improved. In the loading process, the pH value of Na₂EDTA-McIlvaine buffer solution will affect the surface potential of packing in SPE column and the existence form of TCs (Guo et al., 2020), and then affect the absorption performance of SPE column for TCs. Therefore, this study compared three Na₂EDTA-McIlvaine buffer solutions with different pH values, and found that when the pH=4.0, all the four TCs had high recovery rates, which may be because the four TCs mainly existed in the form of neutral molecules at pH=4.0. The interaction between TCs molecules without charge and the packing of HLB SPE column is stronger (Seifrtov áet al., 2009).

4.2 Optimization of Chromatographic Conditions

4.2.1 Optimization of Detector and Detection Wavelength

TCs have ultraviolet absorption and fluorescence properties. TCs show strong fluorescence properties only when they form chelates with metal ions (Kargin et al., 2016). In addition, a fluorescence detector can only be used for detection after a derivatization of the samples to be tested. The derivatization operation is cumbersome and easy to affect the accuracy of quantification, so photodiode array ultraviolet and visible light detector is used in this study. TCs have two chromophores. After a spectral scanning, it is found that the four TCs have a strong UV absorption near 270 nm and 350 nm. In this study, it is found that at 270 nm and 350 nm, the four TCs have a great response value, but at 350 nm, the chromatogram interference signal is less.

4.2.2 Selection of Chromatographic Columns

The phenolic hydroxyl and enol hydroxyl groups contained in the TCs molecules have a certain polarity. If the chromatographic column with a high polarity of the normal phase bonding is selected, the strong interaction between TCs and the column will easily lead to an elution difficulty and a long retention time. Therefore, the reverse bonding phase Shim-Pack GIST-C18 column was selected as the detection column of TCs in this study.

4.2.3 Optimization of the Mobile Phase

TCs are easy to combine with metal ions to form chelates that can be absorbed on the silicon hydroxyl groups in the reversed-phase bonded phase chromatographic column, resulting in chromatographic peak tailing and broadening. In this study, the influence of different flows on the detection results was compared, and repeated experiments confirmed that 0.01 mol/L oxalic acid/methanol/acetonitrile (7:1:2, V: V: V) was finally determined as the mobile phase. The four TCs had a good peak shape, left-right symmetry and good separation effect, which may be because when oxalic acid was used as the mobile phase, oxalic acid, as a strong chelating agent, can combine with metal ions to avoid trailing chromatographic peaks and broaden them (Zhou et al., 2012).

4.2.4 Optimization of the Column Temperature and Flow Rate

Flow rate and column temperature affect the retention time and separation effect of the four TCs. The lower the flow rate is and the longer the TCs retention time is, the shorter the TCs retention time is. But too fast a flow rate will lead to too high column pressure, decreased column efficiency, and damage to the column. The temperature increase obviously accelerates the peak time of TCs, improves the peak shape and adjusts the column efficiency, but the high temperature can lead to the decrease of TCs separation degree. It was verified by repeated experiments that the separation of the four TCs was good and the retention time was short when the final flow rate was 0.9 mL/min and the column temperature was 30 °C. The detection could be completed within 20 min, and the column pressure was also at a safe level.

4.3 Methodological Verification

Under the optimized conditions of this study, the linear range of the four TCs was wide, and the linear relationship between 0.05 and 1.0 µg/mL was good ($r > 0.997$), and the sensitivity was high. The recovery rate ranged from 76.81% to 96.34% with high accuracy. The relative standard deviations were all less than 4.80%, with high precision and good stability. Low detection limit. It can meet the requirements of quantitative detection of TCs in chicken. Compared with the national standard of China (Ministry of health of the people's Republic of China, China National Standardization Administration Committee., 2003), our new method has better detection limit, better sensitivity, and can detect doxycycline simultaneously

5. Conclusion

In this study, SPE pretreatment and chromatographic conditions were optimized, and an SPE-HPLC-DAD method was established for the simultaneous detection of TC, OTC, CTC and DC in chicken samples. The method has the advantages of sensitivity, efficiency, simplicity, low cost and batch detection, and is suitable for the simultaneous detection of four TCs residues in large quantities of chicken. The establishment of the detection method greatly improves detection efficiency, shortens the detection cycle, reduces the detection cost, has a wide application prospect, has significant economic and social benefits.

6. Ethics Declarations

6.1 Conflict of Interest

The authors declare that they have no conflict of interest.

6.2 Ethical Approval

This article does not contain any studies with human or animal subjects.

6.3 Informed Consent

Informed consent was obtained from all individual participants included in the study.

7. Funding

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8. Data Availability Statements

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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