HPLC-Based Quantitative Detection of Tylosin in Soil

Kang Wang¹, Shaopeng Yu², Jia Zhou¹*

¹College of Geographical Science Key Laboratory of geographical environment detection and spatial information service in cold region of Heilongjiang Province, Harbin Normal University, Harbin 150025, China
²School of Geography and Tourism, Harbin Institute of Technology, Harbin 150086, China
* E-mail: harbin_zhoujia@hrbnu.edu.cn

Abstract. The sample was extracted under the ultrasonic assistance and purified through the solid-phase extraction, and then the residual tylosin in soil was detected via the high-performance liquid chromatography (HPLC). Acetonitrile-aqueous solution was used as the extracting agent in the ultrasound-assisted extraction. The HLB solid-phase extraction column was activated using 5 mL of methanol and 5 mL of ultrapure water, leached with 5 mL of water and 5 mL of 2% ammonium hydroxide + 5% methanol solution, and eluted with 5 mL of methanol in the end. The determination was implemented using the HPLC method, where the detected wavelength \( \lambda \) value was 290 nm, the sample size was 20 \( \mu \)L, and the column temperature was 30 \( ^{\circ} \)C. Acetonitrile-0.01 mol/L monopotassium phosphate solution (pH=2.5) = 60:40 (V: V) was taken as the mobile phase in the detection of response degree to tylosin in soil. The detection results were as follows: LOD was 0.019mg/kg, LOQ was 0.062mg/kg, the coefficient of variation of intra-day precision was smaller than 10%, and that of inter-day precision was smaller than 15%.

1. Introduction

Tylosin is one of macrolide antibiotics [1]. The antibiotic-induced environmental pollution problems have become increasingly severe and urgent, but a large quantity of tylosin dregs remains to be properly disposed in China [2-5]. The enormous antibiotic residues in soil will lead to potential environmental risks, harm human health, and bring about microbial drug resistance-triggered risks [6]. The antibiotic detection in soil will be of great research values and application potentials for guaranteeing the safety of microbial drug residues, providing the resource base to the greatest extent, and realizing the development of low-carbon economy and circular economy in China.

This study aims to detect the tylosin residues in the selected soil. To be specific, the tylosin residues were pretreated using 105\(^{\circ}\)C oven-drying method, physiochemical analysis method [10], etc., followed by the detection through ultrasound-assisted extraction, solid-phase extraction-based purification and high-performance liquid chromatography (HPLC) [11]. The experiment was standardized by reference to Environmental Protection Law of the People’s Republic of China [12] and Law of the People’s Republic of China on the Prevention and Control of Environmental Pollution by Solid Waste [12].

2. Materials and Methods

2.1. Reagents and instruments
High-performance liquid chromatograph (UV detector), HLB solid-phase extraction column (200 mg, 3 mL), vortex oscillator, centrifuge, rotary evaporator, PH meter, vacuum pump, Agilent TC-C18 chromatographic column (4.6 mm×250 mm, 5μm), and polypropylene centrifugal tube. Tylosin standard (purity=99.9%, Hubei Yuancheng Saichuang Technology Co., Ltd), ammonium dihydrogen phosphate, phosphoric acid, Na2EDTA-McIlvaine (analytically pure), hydrochloric acid, sodium acetate, methanol, acetonitrile (HPLC level), ammonium hydroxide (analytically pure), and ultrapure water.

2.2. Soil sample
In this project, the soil sample was collected from Yinchuan Helan County Biotech Park, which was located at downstream and downwind part of the underground water in the environmental protection zone. A proper amount of tylosin standard was accurately weighed, diluted with mobile phase into 100 mg/L standard stock solution, and then preserved away from light in a refrigerator at 4°C. Its physiochemical properties are seen in the following Table 1, 2 and 3.

| Sample                  | C (%) | N (%) | C/N | pH  | Water content % |
|-------------------------|-------|-------|-----|-----|-----------------|
| Fresh slag of tylosin   | 46.93 | 6.36  | 7.38 | 8.37 | 12.7            |
| Process 1#              | 46.87 | 6.56  | 7.14 | 8.09 | 7.6             |
| Process 2#              | 38.76 | 5.48  | 7.07 | 7.75 | 8.5             |

| Sample                  | P2O5 (mg/kg) | K2O (mg/kg) | Protein (g/100g) | Tylenol residues (μg/g) |
|-------------------------|--------------|--------------|------------------|------------------------|
| Fresh slag of tylosin   | 128.92       | 11354        | 39.75            | 204.5                  |
| Process 1#              | 74.86        | 10636.4      | 41               | 142.87                 |
| Process 2#              | 615.42       | 5275.4       | 34.25            | 17.71                  |

| Sample | As(mg/L) | Cd(mg/L) | Cr(mg/L) | Pb(mg/L) | Hg(mg/L) |
|--------|----------|----------|----------|----------|----------|
| Fresh slag of tylosin   | 0.8      | 0.3      | 7.2      | 7.1      | not detected |
| Process 1#               | 2.7      | 0.8      | 8        | 8.2      | not detected |
| Process 2#               | 0.8      | 0.6      | 14.5     | 3.1      | not detected |

2.3. Sample pretreatment
1 g (accurate to 0.01) of the sample was prepared in advance, 50 mL of acetonitrile-water=9:1 (V:V) was added into a 50 mL polypropylene centrifugal tube, the pH value was regulated to 4 using phosphoric acids in acetonitrile solution, the mixture was vortexed for 1 min, the ultrasound-assisted extraction lasted 30 min and then the centrifugation lasted 10 min at rate of 4,000 rpm, and the supernatant was removed finally. The rotary evaporation of the extracting solution was conducted through 40°C water bath until it was nearly dried, 0.5 mL of methanol solution was added into a 10 mL centrifugal tube, 12.30 g of sodium acetate was weighed and added together with 900 mL of water until it was dissolved, the pH value was regulated to 5.5 using hydrochloric acids (1 mol/L), and the solution was buffered with sodium acetate (0.15 mol/L). The solution was radically vortexed for 1 min until all residues were dissolved to become solution, which was then transferred to the 10 mL centrifugal tube. 5 mL of methanol and 5 mL of ultrapure water were transferred using a transfer pipette in order to activate HLB solid-phase extraction column, the pretreated sample was then added and made to pass this column at speed of 1 mL/min. The sample solution flowing out was rinsed using 5 mL of water and 5 mL of 2% ammonium hydroxide and then eluted using 5% aqueous methanol, all liquids were filtered and dried, and final analyte was shaken and detected via 0.45 μm filter membrane.

2.4. HPLC chromatographic detection conditions
Chromatographic condition: The chromatographic column was Agilent TC-C18 column (4.6 mm×250
mm, 5 μm), mobile phase: acetonitrile-0.01 mol/L monopotassium phosphate solution (pH=2.5) =60:40 (V: V), flow velocity: 1 mL/min, detection wavelength: 290 nm, sample size: 20 μL, and column temperature: 30℃.

3. Discussion

3.1. Condition optimization for mobile phases

3.1.1. Selection of organic mobile phase and inorganic mobile phase
According to the tylosin properties, tylosin is dissolvable in organic solvents such as methanol solution and acetonitrile solution, and moreover, it can be easily dissolved in weakly acidic solution. From related literatures, acetonitrile should even more be used as organic mobile phase in comparison with methanol, and its advantage lies in the peak symmetry in the chromatograph. Therefore, acetonitrile was finally chosen as the organic mobile phase for the sake of easier observation and data acquisition and calculation. The commonly used three solutions were chosen as the inorganic mobile phases, namely, monopotassium phosphate, sodium perchlorate and ammonium dihydrogen phosphate solutions.

The three inorganic mobile phases were tested in the experiment to obtain their respective chromatographic peaks, when they were compared in the aspects of response signal intensity and peak pattern, it was found that when sodium perchlorate and monopotassium phosphate were used as the inorganic mobile phases, the chromatographic peaks were wide and the degree of separation was unsatisfactory. Therefore, the experiment was the optimal with acetonitrile as organic mobile phase and 0.01 mol/L ammonium dihydrogen phosphate as the inorganic mobile phase.

3.1.2. Selection of volume ratio for mobile phases
The matching ratio of organic and inorganic mobile phases was related to their polarity and would directly decide whether the substance separation effect was the optimal at the elution rate. The proportion of mobile phases should be selected after the mobile phases were chosen, and four different acetonitrile-ammonium dihydrogen phosphate proportions were selected: V/V=65:35; V/V=60:40; gradient elution 1; gradient elution 2. The detailed volume ratios of gradient eluting solutions are seen in Table 4.

Based on an analysis, isocratic elution might generate the peak spreading phenomenon, while the gradient elution would contribute to high signal intensity, symmetric peak pattern and good separation effect without peak broadening phenomenon. To sum up, acetonitrile-ammonium dihydrogen phosphate of gradient elution 1 should be chosen for the mobile phases.

Table 4. Volume ratio of solution in gradient elution procedure

| Type              | 0-9min(V:V) | 10-19min(V:V) | 20-25min(V:V) |
|-------------------|-------------|---------------|---------------|
| Gradient elution 1| 25:75       | 50:50         | 75:25         |
| Gradient elution 2| 30:70       | 35:75         | 30:70         |

3.2. Condition optimization for extracting agent and purifying flushing fluid

3.2.1. Determination of extracting agent
Though being slightly soluble in water, tylosin is freely soluble in organic solutions, so the used extracting agent must be of very good adaptation to tylosin. Given this, four extracting solutions were available, namely, methanol, acetonitrile, acetonitrile/water (V/V=9: 1) and Na2EDTA-McIlvaine. The response value and extraction recovery of the liquid-phase chromatograph were used as the evaluation indexes to select the optimal extracting agent.

In the chromatographs of Na2EDTA-McIlvaine and phosphate solutions, high peaks of solvent impurities appeared, but no peaks of target substances were displayed. If the methanol solution was
used as the extracting agent, this would give rise to broad peak and peak asymmetry, uneven base line and impurities disturbance. However, if the acetonitrile solution was chosen, the target substances would present excellent peak pattern, and the degree of separation would conform to the test standard to a great extent.

3.2.2. Selection of volume ratio for extracting agent
Different acetonitrile-water volume ratios were used. Figure 1 shows the values of extraction recovery under the matching conditions corresponding to volume ratios of 1:1, 7:3 and 9:1. With permeability and degreasing capacity, acetonitrile can dissolve most drugs and remove proteins. Therefore, acetonitrile-water (V/V=9:1) was taken as the extracting agent, and its pH value was regulated to 4 as the optimal pH value.

![Fig 1. Extraction recovery of each concentration](image)

3.3. Optimization of purification conditions
The base line stability and resolution of target substances in the chromatograph should be improved by purifying the impurities contained in the sample, so as to improve the detection quality of target substances, protect the chromatographic system from pollution risk, and purify the sample through as many as chromatographic columns of multiple impurities. According to the rinsing conditions in Table 5, the optimal purifying flushing solutions should be 2% ammonium hydroxide and 5% aqueous methanol, which can not only purify the impurities of solid-phase extraction column as much as possible but also elute the target substances.

![Table 5. SPE optimization of different purification conditions](image)

4. Conclusion
20 μL of the sample filtrate was injected, the peak area was recorded, and the retention time was about 5.76 min.

The attempt was made in this experimental study to extract tylosin in soil through ultrasound-assisted extraction and solid-phase extraction, and quantitatively determine it via HPLC. According to the experimental results, its LOD was 0.019 mg/kg, LOQ was 0.062 mg/kg, coefficient of variation of intra-day precision was smaller than 10%, and that of inter-day precision was smaller than 15%. The proposed method is verified to be a fast, stable and accurate soil tylosin detection.
method, which can satisfy the requirements for the sample detection by virtue of favorable objectivity and precision.

Fig 2. Determination of tylosin in soil by HPLC chromatogram

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