Simultaneous Determination of Sulfonamide, Quinolones, Tetracycline and Macrolide Antibiotics in Soils Using ASE-SPE-HPLC/MS

Ci REN¹, Shuo-xin BAI¹, Yang SONG¹,* and Xue-wen LI¹

¹College of Public Health, Shandong University, Jinan, Shandong 250012, China
*Corresponding author

Keywords: Accelerated solvent extraction, Soil, Antibiotics, High performance liquid chromatography tandem mass spectrometry.

Abstract. This research developed the methods for Simultaneous determination of 4 kinds of antibiotics in soils. Acetonitrile-phosphate was used as extraction solvent, Diatomite(washed by EDTA) was used as dispersing agent. Firstly, soil was extracted by ASE with the parameter conditions: pressure 1500 psi, temperature 70°C, static 10 min, 1 circle, then pre-concentration by SPE, followed by HPLC-MS/MS analysis. Recovery is 72.3%~97.6% for SAs, 63.2%~93.2% for FQs, 60.2%~82.3% for TCs and 67.2%~78.9% for MLs. RSD<10%, r>0.99. Limits of detection is 0.5~0.9µg/kg for SAs, 1.3~1.7 µg/kg for FQs, 0.2~1.1µg/kg for TCs and 0.2~0.3µg/kg for MLs. This method detects 15 kinds of antibiotics in 15 min, the detection accuracy can truly meet the requirements. The Diatomite(washed by EDTA) can increase the recovery of most of the antibiotics. Therefore, this method is worth learning.

Introduction

Antibiotics abuse in breeding industry causes antibiotics residue in manure. Generally, manure is amended to soil without treatment which results in specific soil pollution and serious human health problems [1].

So far, most of the methods reported for residue analysis of antibiotics focus on single type antibiotics, while always gain low recovery. For accelerated solvent extraction (ASE) method has advantages of high extraction efficiency and automatic operation [2], it was identified as a standard method to solid samples extraction by the Environmental Protection Agency [3]. There were some reports on using ASE method to extract antibiotics from soil, but none of them considered the effect of dispersing agent. Therefore, in this study, ASE method was selected to extract multiple antibiotics from soil before analyzed by HPLC-MS/MS. In addition, the roles of different dispersing agents were compared in this process.

Experiment and Material

Instrument, Reagents and Standards

Instrument: HPLC-MS/MS (Agilent, USA); ASE 300 (Dionex, USA); Evaporimeter (Yingdi, China); Oasis hydrophilic-lipophilic balance (HLB) cartridges (200mg, 6ml, Waters, USA); SAX cartridges (500 mg,3 ml, Bonna-Agela Technologies, Tianjin).

Reagents and standards: Sulfapyridine (SPD), Sulfamerazine (SM1), Sulfadimoxine (SDM), Tetracycline (TC), Chlortetracycline (CTC), Oxytetracycline (OTC), Doxycycline (DOX), Erythromycin (ERY), Roxithromycin (ROX), were bought from Fluka company (USA); Methanol and acetonitrile were bought from J.T. Baker company (USA), other reagents were analytical grade; Milli-Q deionized water was used.

Standard Solution and Buffer Solution

Standard solution: stock solutions (100mg/L⁻¹) of each antibiotic were prepared in methanol and stored at -18°C monthly.
Extract buffer solution: 50% methanol and 50% 0.2M citric acid with pH 4.7, 50% phosphate
buffer and 50% acetonitrile with pH=3.0, and1% NH3 and 99% methanol.

**Dispersing Agent**

The sea sand and diatomite were laid inside a muffle furnace in 350°C and dried for 4 hours to
remove organics; Then, 100g sea sand and 50 g diatomite were eluted with 200 ml 0.1M EDTA
solution respectively [4]; Partial drying of the sand and diatomite were completed in vacuum;
Thereafter, sand and diatomite were completely dried in an oven at 100°C.

**Soil Sampling**

Soil was collected in six sites in vegetable area, packaged with aluminum foil immediately. After
transported to lab, the samples will freeze–dried and sieved through a 0.5 mm sieve.

**Sampling Preparation**

ASE procedure: 3.0 g soil was mixed with 13 g diatomite in a mortar and then put into a 34mL
extraction cell. Dionex glass fiber filters were placed at the bottom and top of it to avoid the block
of the end caps by the particles. The analytes were extracted with methanol-citric acid (pH=4.7) at
70 °C and 1.500 psi for 10 min of static time, in one cycle, at 60% of flush volume, purged for 60 s
with nitrogen.

Solid-phase extraction (SPE) procedure: Firstly, the ASE extracts (containing methanol) were
evaporated to a half volume, then diluted with water to the methanol concentration less than 5%. SAX-HLB tandem cartridges were pre-conditioned with 5mL methanol and 5mL water. The ASE
extracts passed through tandem cartridges at a speed of ca 3 ml/min. Subsequently, the HLB was
washed with 10ml water to flush impurity, freeze–dried for 15 min. Finally, HLB cartridge was
eluted with 6 ml methanol. Eluant evaporated with nitrogen at 40°C and redisolved with 1ml
methanol–water (50:50, v/v), passed through 0.22 um organic membrane filter before analysis.

**HPLC-MS/MS Analysis**

XDB-C18 column (4.6×50 mm, 1.8 µm) was used to separate target antibiotics, 10ul redisolved
solution was injected into the chromatographic system. The mobile phase consisted of 0.1% formic
acid (A) and acetonitrile (B), and ramped at a flow rate of 0.4mL/min from 10% B to 40% in 6 min
and 40% to 95% in 2 min, kept for 2 min, then ramped to 10% in 5 min.

Mass spectrometric analysis equipped with an electrospray ionization source that operated in the
positive ionization mode. The nebulizer pressure was set to 30psi, drying gas was N2 .The flow rate
and temperature of drying gas was 10 L/min and 350°C. The capillary voltages were 3000 V. Sample acquisition was performed in the multiple reaction monitoring mode. The optimal
conditions are summarized in Table 1.

| Antibiotics          | Quality (min) | Quantifier (m/z) | FVc (v) | CEd (v) |
|----------------------|---------------|------------------|---------|---------|
| Sulfapyridine(SPD)   | 250.0/155.8   | 250.0/183.8      | 120     | 15/10   |
| Sulfamerazine(SM1)   | 265.0/155.9   | 265.0/171.9      | 120     | 15/15   |
| Sulfadimoxine(SDM)   | 310.9/107.9   | 310.9/155.8      | 100     | 20/25   |
| Ciprofloxacin(CIP)   | 332.0/288.0   | 332.0/314.0      | 120     | 15/15   |
| Norfloxacin(NOR)     | 320.0/233.0   | 320.0/301.9      | 100     | 20/20   |
| Enrofloxacin(ENR)    | 360.0/315.9   | 360.0/342.0      | 150     | 22/15   |
| Lomefloxacin(LOM)    | 352.2/265.1   | 352.2/334.0      | 120     | 25/20   |
| Fleroxacin(FLE)      | 370.2/269.0   | 370.2/326.0      | 120     | 15/25   |
| Ofloxacin(OFL)       | 362.2/261.0   | 362.2/318.1      | 120     | 15/25   |

continued
Table 1. Optimal condition for analytes in MRM mode (continued).

| Antibiotics          | Quality (min) | Quantifier (m/z) | FVc (v) | CEd (v) |
|----------------------|---------------|------------------|--------|--------|
| Tetracycline(TC)     | 445.1/410.0   | 445.1/427.0      | 120    | 10/15  |
| Oxytetracycline(OTC) | 461.2/425.9   | 461.2/427.9      | 120    | 10/15  |
| Chlorotetracycline(CTC) | 479.0/443.0 | 479.1/461.9      | 120    | 10/20  |
| Doxycycline(DOX)     | 445.2/153.9   | 445.2/427.9      | 120    | 15/30  |
| Erythromycin(ERY)    | 734.4/158.0   | 734.4/576.2      | 150    | 15/25  |
| Roxithromycin(ROX)   | 837.5/558.2   | 837.5/679.3      | 150    | 20/20  |

Results and Discussions

Optimization of the ASE Procedure

The research optimizes the ASE procedure from two aspects: instrumented and un-instrumented parameter.

Extraction solvent: Deionized water, methanol-1%NH3, methanol-citric acid (pH=4.7) and acetonitrile-phosphate (pH=3.0) were used as extraction solvents. Sea sand was used as dispersing agent. The spiking soil (200µg /kg) was extracted at room temperature. Finally acetonitrile-phosphate was selected as the extraction agent. Because recoveries of some antibiotics cannot be accepted for quantitative analysis, the method needed further optimization.

Dispersing agent: According to the reports, due to the chelation between EDTA and metal ion [4], EDTA washed dispersing agents could improve extraction efficiency. Compared with common diatomite, EDTA washed diatomite has a better extraction result.

The sea sand was recommended as dispersing agent[5], there are no report about using diatomite as dispersing agent. This research got some satisfying results with diatomite used, especially for the TCs.

Optimization of circle times: On the basis of the result, 1 circle is selected as the optimal cycle time.

Optimization of extraction temperature: Used 40℃, 55℃, 70℃ and 85℃ as extraction temperatures. Overall consideration, 70℃ was elected as the optimal extraction temperature.

As is said above, the extraction agent is acetonitrile-phosphate buffer, the dispersing agent is EDTA- diatomite, the extraction temperature is 70℃, the circulation is 1 times, the pressure is 1500 psi, the purging volume is 60%, the nitrogen-blow is 60s, the static extraction is 10 min.

Linear Range, Limit of Detection and Recovery

The linearity of the analytical methods was demonstrated building the calibration curves (5, 25, 50, 100, 200 and 500µg/kg of each target antibiotics). The calibration curves shown good linearity between 5 and 500µg/kg, r>0.99. The limit of detection (LOD) was estimated at a S/N of 3. Recovery was 72.3%~97.6% for SAs, 63.2%~93.2% for FQs, 60.2%~82.3% for TCs, 67.2%~78.9% for MLs. LOD was 0.5~0.9µg/kg for SAs, 1.3~1.7µg/kg for FQs, 0.2~1.1µg/kg for TCs, 0.2~0.3µg/kg for MLs. For each recovery of the 15 kinds of antibiotics please refer to table 2.

Table 2. Recoveries and LOD of 15 kinds of antibiotics(n=3).

| Antibiotics          | LOD (µg /kg) | 200 Recovery(%)RSD(%) | 50 Recovery(%)RSD(%) |
|----------------------|--------------|-----------------------|---------------------|
| Sulfapyridine(SPD)   | 0.5          | 81.6/9.7              | 72.3/7.7            |
| Sulfamerazine(SM1)   | 0.9          | 82.3/7.1              | 97.6/4.6            |
| Sulfadimoxine(SDM)   | 0.7          | 93.6/4.8              | 88.7/4.4            |
| Ciprofloxacin(CIP)   | 1.6          | 81.3/3.2              | 67.4/6.6            |

continued
Table 2. Recoveries and LOD of 15 kinds of antibiotics (n=3) (continued).

| Antibiotics     | LOD (µg /kg) | 200                  | 50                  |
|-----------------|--------------|----------------------|---------------------|
|                 |              | Spiking Levels(µg /kg)| Recovery(%)/RSD(%)  | Recovery(%)/RSD(%) |
| Norfloxacin(NOR)| 1.6          | 79.4                 | 4.3                 | 90.2               | 4.7                |
| Enrofloxacin(ENR)| 1.7          | 78.6                 | 8.3                 | 73.4               | 3.8                |
| Lomefloxacin(LOM)| 1.3          | 86.8                 | 5.6                 | 63.2               | 4.3                |
| Fleroxacin(FLE)  | 1.6          | 68.9                 | 8.9                 | 72.4               | 5.4                |
| Ofloxacin(OFL)   | 1.3          | 77.4                 | 7.6                 | 64.5               | 5.2                |
| Tetracycline(TC) | 0.2          | 78.9                 | 6.3                 | 82.3               | 3.8                |
| Oxytetracycline(OTC)| 0.2      | 67.8                 | 4.2                 | 71.2               | 2.9                |
| Chlortetracycline(CTC)| 0.3  | 75.3                 | 7.7                 | 60.2               | 2.1                |
| Doxycycline(DOX) | 1.1          | 85.4                 | 5.1                 | 77.6               | 6.3                |
| Erythromycin(ERY)| 0.3          | 68.5                 | 4.3                 | 77.4               | 2.7                |
| Roxithromycin(ROX)| 0.2         | 78.9                 | 3.5                 | 67.2               | 7.6                |

Determination of Soil Samples

Analyzing the 6 samples that were gathered in different place on the basis of methods mentioned above, the results were in table 3. There were varying degrees of antibiotics in different samples. The concentration of SAs were ND~27.9 µg/kg, TCs were ND~47.7 µg/kg, FQs were ND~80.2 µg/kg, MLs were ND~19.23µg/kg.

As it is shown in Table 3, the antibiotics in soil belong to different kinds and are in low concentration. It is necessary to develop its sensitivity and stability.

Table 3. Antibiotics concentration in soil samples (µg /kg).

| Antibiotics     | soil samples |
|-----------------|--------------|
|                 | 1  | 2  | 3  | 4  | 5  | 6  |
| Sulfapyridine(SPD) | 2.9 | 5.9 | 2.4 | ND | 4.5 | 3.5 |
| Sulfamerazine(SM1)  | ND | ND | 5.3 | 3.7 | 8.5 | ND |
| Sulfadimoxine(SDM)  | 27.1 | 27.9 | ND | ND | ND | ND |
| Ciprofloxacin(CIP)  | 35.5 | 28.2 | 35.9 | ND | ND | ND |
| Norfloxacin(NOR)   | 39.3 | ND | 30.0 | ND | 15.9 | ND |
| Enrofloxacin(ENR)  | 6.4 | 64.5 | ND | 10.5 | ND | ND |
| Lomefloxacin(LOM)  | 7.5 | ND | 27.2 | ND | 12.63 | 46.3 |
| Fleroxacin(FLE)    | ND | ND | ND | 10.0 | ND | ND |
| Ofloxacin(OFL)     | 34.8 | 56.7 | 80.2 | 54.8 | ND | 31.84 |
| Tetracycline(TC)   | ND | ND | ND | ND | 7.8 | ND |
| Oxytetracycline(OTC)| 2.3 | 2.6 | ND | 4.7 | ND | 3.3 |
| Chlortetracycline(CTC) | ND | 2.7 | ND | 4.8 | ND | 2.7 |
| Doxycycline(DOX)   | 47.7 | 28.2 | ND | 25.4 | 8.8 | ND |
| Erythromycin(ERY)  | ND | 2.5 | ND | 11.4 | ND | 9.9 |
| Roxithromycin(ROX) | ND | ND | 8.48 | ND | 19.23 | ND |

Comment “ND” shows Non-detected

Summary

ASE-SPE-HPLC-MS/MS method has been developed and optimized for Simultaneous determination of sulfonamide, quinolones, tetracycline and macrolide antibiotics in soils. Recovery was 72.3%~97.6% for SAs, 63.2%~93.2% for FQs, 60.2%~82.3% for TCs and 67.2%~78.9% for MLs. RSD<10%, r>0.99. Limits of detection was 0.5~0.9 µg /kg for SAs, 1.3~1.7 µg/kg for FQs, 0.2~1.1 µg /kg for TCs and 0.2~0.3 µg /kg for MLs. This method determined 15 kinds of antibiotics in 15 min, the detection accuracy can meet the actual sample requirements. This method can be applied to analyzing the antibiotics residues in soil.
References

[1] H.S. Zeng, Y.L. Yan. The present situation of the antibiotic abuse and coping strategies, J. Chinese Health Service Management. 5(2010):341-343.

[2] Carabias-Martínez R, Rodríguez-Gonzalo E, Revilla-Ruiz P. et al. Pressurized liquid extraction in the analysis of food and biological samples[J]. Journal of Chromatography A, 2005, 1089(1): 1-17.

[3] Sanchez-Brunete C, Miguel E, Tadeo J L. Analysis of 27 polycyclic aromatic hydrocarbons by matrix solid-phase dispersion and isotope dilution gas chromatography-mass spectrometry in sewage sludge from the Spanish area of Madrid[J]. Journal of Chromatography A, 2007, 1148(2): 219-227.

[4] Carretero V, Blasco C, Pico Y (2008) J Chromatogr A 1209:162-173.

[5] Vicente A, Vazquez-Roig P, Blasco C, Yolanda Picó. Determination of tetracycline residues in soil by pressurized liquid extraction and liquid chromatography tandem mass spectrometry [J]. Anal Bioanal Chem, 2009, 394:1329–1339.