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Determination of 10 Antibiotics in Sewage Sludge(SS) by SPE-HPLC-UV Method

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Abstract

A method for the determination of antibiotics in sludge by solid phase extraction, high performance liquid chromatography-ultraviolet detection (SPE-HPLC-UV) was established. The 10 antibiotics were Sulfonamides (SDZ, SM2, SMZ, SMM), Quinolones (PEF, NOR, CIP), Tetracyclines (CTC, DOX) and Chloramphenicol (CAP). The sludge samples were extracted with ultrasonic assisted organic solvent, and the extraction liquid volume and pH of sludge solid phase extraction (SPE) were optimized. The appropriate SPE column and elution liquid volume were selected. The optimum chromatographic parameters and gradient elution mode were determined by the determination of the target antibiotics mixture standard reserve. Sludge sample using methanol: Na2EDTA-Mcllvaine buffer solution was extracted with 1: 1 (v/v). After concentration by solid phase extraction, the target antibiotics were eluted by gradient elution with 0.1% formic acid (A) and methanol (B) in mobile phase at 275 nm.. The linear correlation coefficient R2 of the standard curve was >0.998, the detection limits were 0.04~0.54 μg/kg, and the limits of quantitation were 0.13-1.80 μg/kg. The recoveries of CTC, NOR and CAP were 56.52%~69.88%. The average recoveries of other antibiotics ranged from 82.34% to 107.09%. The relative standard deviation is between 0.58% and 17.74%. This method is simple to operate, has high degree of automation, good repeatability and has practical application prospect.

Keywords

SPE; HPLC; Sewage Sludge; Antibiotic.

1. Introduction

Antibiotics have been widely used in agriculture, medical treatment, livestock breeding and aquaculture through continuous synthesis and development. Antibiotics are mainly used for disease prevention and as growth regulators in poultry and livestock [1]. In particular, in animal husbandry and aquaculture, the use is very large[2] ,up to thousands of tons per year. But most antibiotics can not be absorbed directly, 25% ~ 75% of antibiotics for veterinary drugs are excreted through excretion system [3], and 85% of antibiotics used by human beings are discharged into the environment directly in the form of mother, which brings pollution to soil and water environment[4]. Wang Yao et al. detected water samples from 16 sampling sites in Donghu Lake, Wuhan, and found that 20 antibiotics were detected at different sites[5]. Hu Min detected the antibiotics in typical drinking water in South China. Among 101 antibiotics investigated, 44 antibiotics were detected[6]. Relevant studies have shown that antibiotics with very low residues in soil may also induce soil microorganisms to produce resistance in the case of synergistic action or horizontal gene transfer[7]. These substances are widely

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distributed, durable and potentially harmful to the ecological environment[8]. Antibiotics have antibacterial activity, and their functional groups can produce obvious toxic effects on the growth and development of organisms[9].

As a by-product in the process of sewage treatment, sewage sludge(SS) collects most pollutants in the sewage, which is extremely harmful. The wastewater treatment plants are a major source of antibiotic contamination, and the concentration of antibiotics is mostly ng/L ~ ug/L. Tetracyclines, quinolones, sulfas and other antibiotics are commonly detected. The sewage treatment process can only remove part of antibiotics, and the antibiotics which are not degraded are adsorbed on the sludge by van der Waals force, hydrogen bond, hydrophobic distribution and the like, and the antibiotics can re-enter the soil ecosystem and even groundwater and surface water as the sludge is buried or utilized [10], Accumulatively through food chain and food web, finally causing harm to health, mainly manifested as allergic reaction, organ damage, acute poisoning, increasing carcinogenic rate, deformity rate and mutagenic rate[11], so it is necessary to detect the content of antibiotics in SS.

Solid phase extraction (SPE) is a sample pretreatment technology based on liquid-solid separation and extraction. The solid adsorbent is used to adsorb the sample in solution to separate the sample from the sample matrix and interferer, and then the sample is washed with eluent. Gao Kai et al. establishes a detection method combining solid phase extraction (SPE) and high performance liquid chromatography (HPLC) to simultaneously determine common antibiotics such as sulfanilamides, tetracyclines, quinolones and the like in black and odorous water, and is purified and enriched by an Oasis HLB solid phase extraction column, the recovery rate is good [12]. At present, most of the research on antibiotics is focused on water environment, while the study on the complex solid environment matrix of SS is lacking. In this study, ultrasonic solvent extraction combined with solid phase extraction (SPE) was used to extract antibiotics, and HPLC-UV was used to detect antibiotics in sludge. This method was designed to establish a method with high recovery, low detection limit, high automation and simultaneous detection of multiple antibiotics in sludge.

2. Experimental Part

2.1 Main Instruments and Reagents

Agilent 1260infinity high performance liquid chromatography (Agilent Technology Co., Ltd., China); HGC numerical control automatic solid phase extraction instrument (Shanghai Hegong Scientific Instrument Co., Ltd.); KQ2200DE numerical control ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd.); TTL-DC nitrogen blowing instrument (Beijing Tongtailian Technology Development Co., Ltd.); KQ2200DE numerical control ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd.); TD5G desktop low speed centrifuge (Hunan Kaida Scientific Instrument Co., Ltd.); XH-2000-1 Scroll Mixer (Tianjin TEST Instrument Co., Ltd.).

10 antibiotic standards: Sulfadiazine (SDZ), Sulfadimethoxazole (SMZ), Sulfamethazine (SM2), Sulfamethoxine (SMM), Norfloxacin (NOR), Aureomycin (CTC). Doxycycline (DOX), Chloramphenicol (CAP) (98%, Aladdin), Pefloxacin (PEF) (99%, McLean), Ciprofloxacin (CIP) (98%, Rohn's reagent), The mass concentration of the standard sample is 100 mg/L, stored in a refrigerator at -20 °C in the dark, mixed with the standard solution, used and prepared each time. methanol, ethyl acetate (chromatographic grade, purity > 99.9%); formic acid, acetic acid, hydrochloric acid (all analytically pure); The experiment water is ultrapure water.

2.2 Liquid Chromatography Condition

The column was EclipseXDB-C18 (5 um,4.6 mmx250mm, Agilent). Mobile phase A was 0.1% formic acid-aqueous solution, and mobile phase B was pure methanol. Gradient elution conditions such as B; 20~35 min,50%~90%:0~15 min,10%~25%B; 15-20 min,25%~50%B; 35~36 min,90%~10%B. Column temperature 30 °C; Flow rate: 0.8 mL/ min; sample size: 10 uL; Uv detection wavelength: 275 nm.

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2.3 Sample Pretreatment

2.3.1 Sample Collection and Preservation

The SS samples used in this study were mixed samples taken continuously for 7 days from the same sewage treatment plant. Put an ice pack in the incubator, put the frozen fresh sludge in the incubator and express it back to the laboratory (1-2 days). After the sludge is frozen, dried, ground and screened (<80 mesh), it is placed in a drying oven at room temperature for airtight storage to be measured.

2.3.2 Ultrasonic Centrifugal Extraction

Weighing 1.000 ± 0.001 g of sieving sludge sample, adding 10 mL of extract liquid (Methanol-Na_2EDTA-Mcllvaine buffer solution, 1: 1, v/v) oscillating and vortex mixing, ultrasonicating for 10 min, centrifuging for 15 min by a low-speed centrifuge at 4500 r/min to separate supernatant fluid. extracting twice again according to the above steps, combining the three extracting solutions, diluting the three extracting solutions with ultrapure water to a constant volume of 350 mL, and ensuring the methanol content in the solution to be lower than 5% ((To prevent the high content of organic matter in the solution from causing the HLB column detachment during solid phase extraction, and the target antibiotics cannot be retained on the HLB).

2.3.3 Solid Phase Extraction(SPE)

Antibiotics were extracted using a solid phase extraction unit combined with Oasis HLB column. Activate the column with methanol and ultrapure water 6 mL each. and staying on the column for 4-6 minutes. It was then allowed to flow out at a flow rate of 0.5 mL/min. Do not allow the column to dry up during this process, so as not to affect the activity of the column. The diluted extract was adjusted to pH = 3 with diluted hydrochloric acid, and then enriched by HLB solid phase extraction column at a flow rate of 2.5 mL/min. After the loading of the solution, the column was rinsed with 10 mL ultrapure water to remove impurities, and then drained for 10~15 min to remove moisture. Elution of target antibiotics with 6 mL of methanol at a flow rate of 0.5 mL/min (Ensure that the eluate stays on the column for about 5 min during elution).

Sample concentration: The collected eluate was blown to dryness by blowing nitrogen, redissolved with methanol to 1 mL, vortexed for about 3 min, filtered into a 1.5 mL brown sample bottle using 0.22 μm filter, and stored at -20 °C, to be measured by the machine.

3. Results and Discussion

3.1 Optimization of Sample Pretreatment Conditions

Due to the complex matrix of sludge samples, it would be relatively difficult to select actual sludge samples to optimize sample pretreatment conditions, so anhydrous sodium sulfate was selected as the simulated sludge in this study. Weigh 1 g anhydrous sodium sulfate, add 1 mL of standard antibiotic mixed solution with concentration of 1 mg/L, mix well and store in refrigerator overnight to simulate sewage plant sludge samples.

3.1.1 Selection of Extraction Solvent

The extraction efficiency of antibiotics in sludge was closely related to the types of extraction solvents. This study mainly investigated the extraction efficiency of the following three extraction solvents, including extraction solution 1: methanol: Na_2EDTA-Mcllvaine buffer solution = 1:1(v/v); extract 2: Methanol: acetonitrile: Na_2EDTA-Mcllvaine buffer solution = 1:1:2(v/v/v); extract 3: acetonitrile: Na_2EDTA-Mcllvaine buffer solution = 1:1(v/v). The above three extracts were separately added to 1g simulated sludge. According to the same pretreatment method, extraction, enrichment, analysis and calculation were carried out. After detection, analysis and calculation, the recovery rates of 10 targeted antibiotics were shown in Figure 1. Extract 1 has a good effect on the extraction of target antibiotics. Except NOR and CTC, the recovery rate of other antibiotics could reach more than 80%. In addition to the recovery rates of SMZ, SMM, CAP and DOX above 80% in extract 2 and extract 3, the recovery rates of the other antibiotics were all below 40%, which could not reach the ideal

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extraction efficiency. Therefore, extraction solution 1: methanol: Na_2EDTA-Mcllvaine buffer solution = 1:1(v/v) was used as the final extraction solvent in this study.

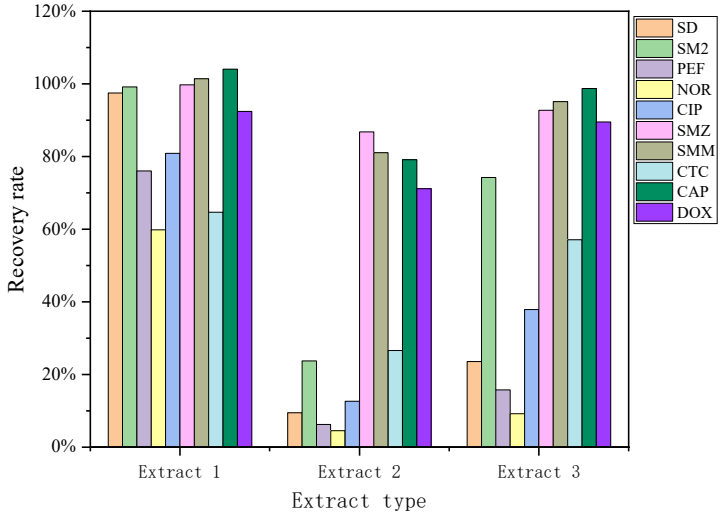

Fig 1. Effects of different extracts on antibiotic recovery

3.1.2 Optimization of pH of Extraction Solution

The simulated sludge sample extract obtained in advance was diluted to 350 mL with ultra-pure water, and the pH of the added solution after dilution was adjusted to 3, 5, 8. Under different pH conditions, the extraction efficiency of Oasis HLB solid phase extraction column on target antibiotics was investigated, so as to determine the optimal pH value of sample extract. Oasis HLB solid phase extraction column was activated with 6 mL methanol and 6 mL water. After sample loading, the solid phase extraction column was washed with 10 mL ultra-pure water and finally 6 mL. The target substance was eluted with methanol, 6 mL of methanol eluting liquid nitrogen was blown to near-dry, redissolved to 1 mL and filtered for detection.

Fig. 2 shows the recovery of the target antibiotics at different pH conditions by solid phase extraction columns, NOR was relatively poor compared to other antibiotics at these three pH conditions. However, the highest recovery of NOR was 57.58% at pH 3. SDZ, SM2, SMZ and SMM can get better recoveries under acid condition. When the pH of the solution was 8, the recovery of SDZ and SMZ was very low to 30% or less. The pH value of CAP is less affected, and the recovery rate of CAP is about 100% under three pH conditions. The recoveries of CTC and DOX decreased with the increase of pH, and the highest recoveries were 76.38% and 103.55% respectively at pH 3. Therefore, when the pH value of the extract is adjusted to 3, the target antibiotic can achieve a better recovery rate, and when the pH value is lower, the interference caused by some substances with high pKa value in the solution can be reduced. Therefore, pH = 3 is the optimum pH for the extract in this study.

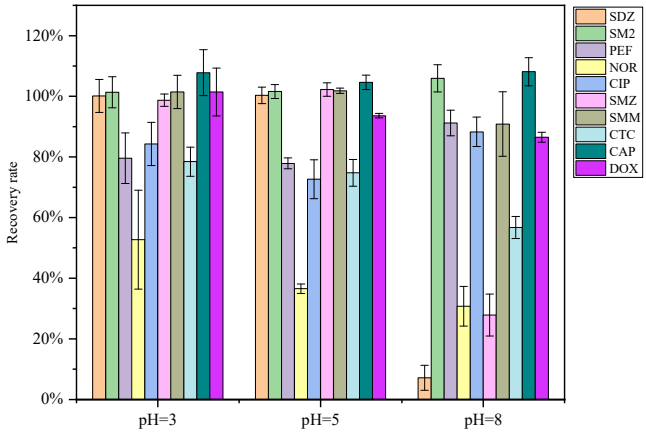

Fig 2. The recovery rate of antibiotics at different pH

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3.1.3 Optimization of Eluent Volume

In this study, under the same conditions, the effects of 6 mL, 8 mL and 10 mL of methanol on the recovery of each antibiotic were investigated. The recovery results of each antibiotic were shown in Fig. 3. The recoveries of NOR and CTC are relatively low, increasing the usage of eluent, increasing the recovery of NOR from 60% to 80%, and almost keeping the recovery of CTC at about 65%. The recoveries of sulfa antibiotics and CAP were the highest and kept at about 100%. When the amount of elution solvent was 6 mL, they were almost completely eluted. The elution efficiency of the other antibiotics was not significantly improved with the increase of the dosage. Therefore, the optimal amount of elution solvent used was 6 mL.

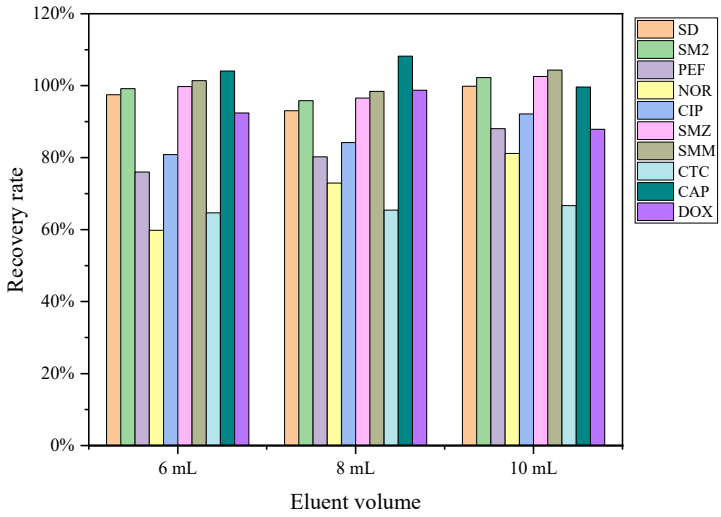

Fig 3. Effect of different eluent volume on antibiotic recovery

3.1.4 Liquid Chromatography Condition Optimization

The selection of mobile phase A and B is the key to the retention and separation of organic matter in the chromatography column. Wang Fan et al. select 0.1% formic acid-water solution and acetonitrile to carry out gradient elution separation on 15 antibiotics in sewage and sludge of SS treatment plant[13]. Liu Siguang et al. separated antibiotics from the sediments by selecting 0.1% by volume of formic acid-water solution (A) and 0.1% by volume of acetonitrile (B) [14]. Thus adding a certain proportion of acid into the mobile phase is helpful to achieve the ideal separation effect. Therefore, 0.1% volume fraction of formic acid aqueous solution (A) and methanol (B) are selected as mobile phases and separated by gradient elution mode. The liquid phase separation chromatogram of the 10 antibiotics is shown in Figure 4:

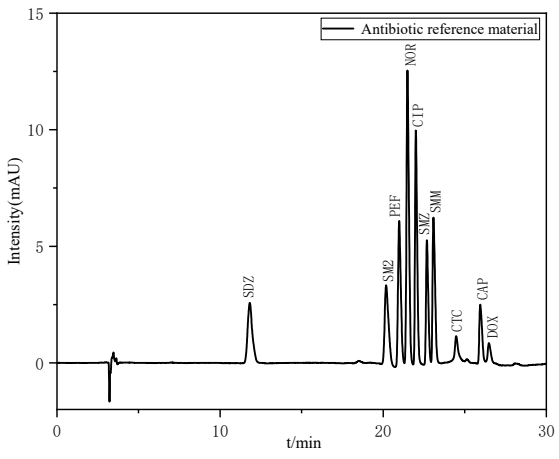

Fig 4. Liquid chromatography of antibiotics

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3.2 Linear Range and Detection Limit

Mixed standard solutions with concentration gradients of 1, 5, 10, 50, 100, 200, 500, 1000, 2000 μg/L were prepared and analyzed in sequence. The results showed that the linear relationship of 10 SAs antibiotics in the range of 1~2000 μg/ L was good, and the correlation coefficient R^2 >=0.998. In practice, 3 times of the signal to noise ratio (S/N =3) is often taken as the detection limit (LOD), and 10 times of the signal to noise ratio (S/N =10) is taken as the limit of quantitation (LOQ), see Table 1.

Table 1. Linear equation, correlation coefficient, detection limit and quantification limit of 10 antibiotics

Antibiotic	Linear recovery equation	Correlation coefficient(R^2)	LOD(μg/kg)	LOQ(μg/kg)
SDZ	y=0.05*x+0.42	0.999	0.19	0.63
SM2	y=0.04*x+0.18	0.999	0.15	0.48
PEF	y=0.08*x-0.27	0.999	0.08	0.26
NOR	y=0.11*x-3.08	0.999	0.04	0.13
CIP	y=0.10*x+0.17	0.999	0.05	0.16
SMZ	y=0.04*x+0.62	0.999	0.09	0.31
SMM	y=0.06*x+0.29	0.999	0.08	0.26
CTC	y=0.02*x+0.62	0.998	0.43	1.44
DOX	y=0.03*x+0.03	0.999	0.20	0.65
CAP	y=0.02*x-0.73	0.998	0.54	1.80

3.3 Standard Recovery and Precision

Adding 1 mL of antibiotic mixed solution with concentration of 1 ppm into 1 g simulated sludge was carried out three parallel experiments to calculate the recovery rate and RSD of the added substances, see Table 2.

Table 2. Recovery rate and RSD of antibiotic

Antibiotic	Recovery rate 1	Recovery rate 2	Recovery rate 3	Average recovery rate	RSD
SDZ	102.79%	102.30%	101.61%	102.24%	0.58%
SM2	96.69%	97.81%	97.54%	97.35%	0.60%
PEF	86.12%	85.24%	75.66%	82.34%	7.05%
NOR	70.53%	76.87%	62.25%	69.88%	10.49%
CIP	92.74%	94.38%	84.50%	90.54%	5.85%
SMZ	97.04%	99.03%	97.22%	97.76%	1.12%
SMM	107.29%	108.45%	105.54%	107.09%	1.37%
CTC	62.64%	50.11%	56.82%	56.52%	11.09%
DOX	89.39%	89.94%	120.35%	99.89%	17.74%
CAP	66.27%	57.59%	69.01%	64.29%	9.28%

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4. Conclusion

In this study, a method for the analysis of 4 classes and 10 kinds of antibiotics in sludge was established based on SPE and HPLC-UV technology. Under the optimized experimental conditions, the 10 target antibiotics had a good linear relationship with the correlation coefficient $R^2 \geq 0.998$, the LOD and LOQ were 0.04-0.54 μg/ kg and 0.13-1.80 μg/ kg. The recoveries ranged from 56.52% to 107.09%. Compared with the traditional method, this method is simple, automatic, sensitive and reproducible, and can meet the requirements of trace antibiotics analysis in sludge.

Acknowledgments

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