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AI-Assisted SPE-HPLC-UV Method for Multi-Class Antibiotic Detection in Sewage Sludge

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Abstract: A rapid and sensitive analytical method was established for the simultaneous determination of ten antibiotics in sewage sludge (SS) using solid-phase extraction combined with high-performance liquid chromatography and ultraviolet detection (SPE-HPLC-UV). The targeted antibiotics spanned four major classes, including sulfonamides, quinolones, tetracyclines, and chloramphenicol. Key parameters such as extraction solvent composition, pH, and eluent volume were systematically optimized to improve recovery and reduce matrix interference. Under optimal conditions, the method achieved excellent linearity ($R^2 > 0.998$), low detection limits (0.04–0.54 µg/kg), and satisfactory recovery rates (56.52%–107.09%). Furthermore, this study highlights the potential of integrating artificial intelligence (AI) into the analytical workflow. By leveraging machine learning algorithms, future studies may enable rapid prediction of recovery trends, automated optimization of extraction conditions, and intelligent screening of complex sludge matrices. The combination of traditional analytical chemistry with AI-driven optimization offers promising avenues for efficient, reproducible, and scalable monitoring of trace antibiotics in environmental samples.

Keywords: Solid-Phase Extraction (SPE); High-Performance Liquid Chromatography (HPLC-UV); Sewage Sludge; Antibiotics; Recovery Efficiency; AI-Assisted Analysis

1. Introduction

Antibiotics have been extensively used in agriculture, medicine, livestock, and aquaculture due to their broad-spectrum antimicrobial activity and effectiveness in disease control and growth promotion in animals [1,2]. However, most antibiotics are not fully metabolized in organisms; approximately 25% − 75% of veterinary antibiotics are excreted unchanged, and up to 85% of antibiotics consumed by humans are directly released into the environment [3,4]. Consequently, antibiotic residues have been detected in various aquatic and terrestrial environments. Studies have reported the presence of multiple antibiotic classes in surface water bodies and even in drinking water supplies across different regions, highlighting the widespread and persistent nature of these contaminants [5,6].

These antibiotic residues, even at trace levels, pose a serious threat to ecosystems. They may promote antibiotic resistance through horizontal gene transfer and selective pressure on soil microbes [7], and cause toxic effects to organisms due to their stable chemical structures and persistent biological activity [8,9]. As a concentrated byproduct of municipal wastewater treatment, sewage sludge (SS) accumulates residual antibiotics via adsorption mechanisms such as van der Waals forces and hydrophobic interactions [10]. Improper disposal of sludge may lead to re-entry of antibiotics into the soil and water systems, posing long-term risks to ecological and human health through food chain accumulation [11].

While several studies have explored antibiotic contamination in aqueous environments [12], research on the detection and quantification of antibiotics in solid matrices such as SS remains limited due to its complex composition. Solid-phase extraction (SPE) has emerged as a preferred sample preparation technique, offering high selectivity and enrichment capability [13,14]. In this context, high-performance liquid chromatography coupled with ultraviolet detection (HPLC-UV) provides a robust and sensitive analytical platform for multi-residue detection of antibiotics in complex matrices.

In recent years, artificial intelligence (AI) has demonstrated significant potential in enhancing environmental monitoring workflows. For instance, AI can be applied to predict the recovery efficiency of antibiotics under various extraction conditions, identify optimal elution strategies through pattern recognition, and automate peak identification and quantification in chromatographic data. Additionally, machine learning algorithms can model the nonlinear relationship between sludge matrix properties (e.g., organic matter, pH, ionic strength) and antibiotic behavior, enabling more precise pretreatment adjustments and detection calibration.

Therefore, this study aims not only to establish a sensitive and reproducible SPE-HPLC-UV method for the detection of 10 antibiotics in SS, but also to explore the prospects of integrating AI-assisted modeling and optimization into analytical protocols for complex environmental matrices.

2. Experimental Part

2.1 Main Instruments and Reagents

The sewage sludge (SS) samples used in this study were collected over seven consecutive days from a municipal wastewater treatment plant to ensure representativeness. After being frozen, dried, and sieved, the samples were stored at room temperature for analysis. Antibiotics were extracted using ultrasonic-assisted solvent extraction with a mixture of methanol and Na_2 EDTA–McIlvaine buffer (1:1, v/v). The supernatants from three extraction rounds were combined and diluted to 350 mL with ultrapure water to control the methanol content, ensuring compatibility with the subsequent solid-phase extraction (SPE) process. Antibiotics were enriched using Oasis HLB cartridges under optimized pH and flow conditions, followed by methanol elution and nitrogen drying prior to HPLC-UV analysis.

To address the variability inherent in complex sludge matrices, anhydrous sodium sulfate was used as a simulated sludge to optimize pretreatment conditions. Among three tested extraction solvents, methanol–Na_2 EDTA–McIlvaine buffer showed the highest recovery efficiency across most antibiotics, with more than 80% recovery for the majority of compounds. This system was thus selected as the optimal extraction solvent. The effect of extraction pH was also investigated, and pH 3 provided the best overall performance, particularly for compounds like NOR, CTC, and DOX. In terms of elution volume, 6 mL of methanol was sufficient for most antibiotics, although slightly higher recoveries were observed for NOR when using larger volumes. Considering both efficiency and economy, 6 mL was used as the final elution volume.

As the complexity of sludge composition and the diversity of antibiotic physicochemical properties increase, manual optimization of extraction conditions becomes labor-intensive. In future work, artificial intelligence (AI) techniques such as machine learning regression and optimization algorithms can be employed to model the relationship between matrix conditions and recovery efficiency. AI-assisted workflows may automatically recommend solvent systems, extraction pH, and eluent volumes based on sample characteristics, thus improving throughput and reproducibility. Moreover, pattern recognition tools could help identify outliers and anomalies in experimental data, further enhancing the robustness of sludge antibiotic analysis.

2.2 Sample Pretreatment

2.2.1 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.

2.2.2 Ultrasonic Centrifugal Extraction

Weighing 1.000 ± 0.001 g of sieving sludge sample, adding 10 mL of extract liquid (Methanol- Na2EDTA-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).

2.2.3 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: Na2EDTA-Mcllvaine buffer solution = 1:1(v/v); extract 2: Methanol: acetonitrile: Na2EDTA-Mcllvaine buffer solution = 1:1:2(v/v/v); extract 3: acetonitrile: Na2EDTA-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 extraction efficiency. Therefore, extraction solution 1: methanol: Na2EDTA-Mcllvaine buffer solution = 1:1(v/v) was used as the final extraction solvent in this study.

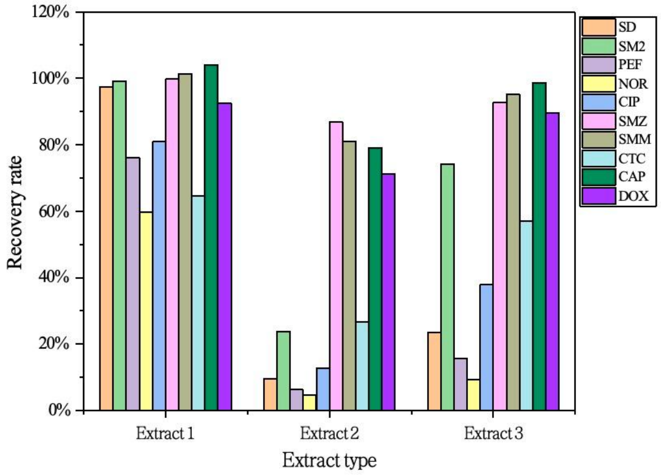

Fig 1. Effects of different extracts on antibiotic recovery

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.

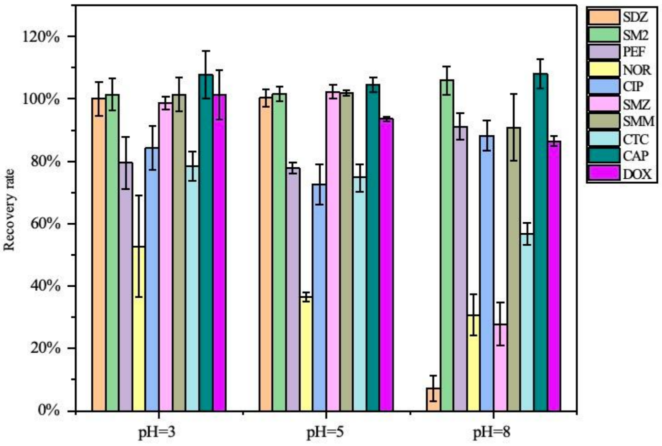

Fig 2. The recovery rate of antibiotics at different pH

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.

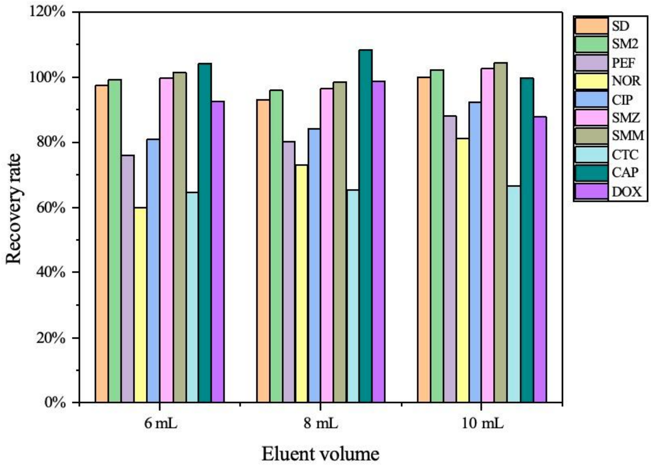

Fig 3. Effect of different eluent volume on antibiotic recovery

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:

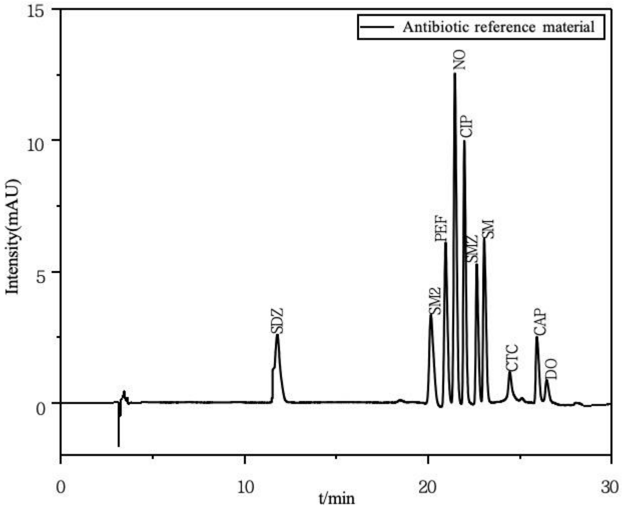

Fig 4. Liquid chromatography of antibiotics

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 R2 >=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(R2)	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	0.2	0.65
CAP	y=0.02*x-0.73	0.998	0.54	1.8

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%

4. Conclusion

In this study, a robust analytical method was established for the simultaneous detection of 10 antibiotics from four major classes in sewage sludge using solid-phase extraction (SPE) combined with high-performance liquid chromatography and ultraviolet detection (HPLC-UV). Under optimized experimental conditions, the method demonstrated excellent linearity ($R^2 \geq 0.998$), low detection limits (LOD: 0.04–0.54 μg/kg), and quantification limits (LOQ: 0.13–1.80 μg/kg). Recovery rates for the target antibiotics ranged from 56.52% to 107.09%, indicating good method accuracy and reliability.

Compared with traditional approaches, this method offers higher automation, sensitivity, and reproducibility, making it well-suited for trace-level antibiotic monitoring in complex sludge matrices. Furthermore, the study underscores the potential of integrating artificial intelligence into analytical workflows. AI-assisted modeling and optimization tools could further enhance the efficiency of parameter selection, improve prediction of recovery trends, and support intelligent quality control across varying sample conditions. This fusion of classical analytical chemistry with data-driven techniques represents a promising direction for advancing the environmental detection of emerging contaminants.

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