



## Research article

# Simultaneous determination of 28 illegal drugs in sewage by high throughput online SPE-ISTD-UHPLC-MS/MS

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## ABSTRACT

This study developed an online solid-phase extraction ultra-high performance liquid chromatography-tandem mass spectrometry (Online-SPE-UHPLC-MS/MS) method for the analysis of 28 illegal drugs in sewage. To achieve this, 28 isotope internal standards (ISTDs) were added to 3 mL sewage samples, the pH was adjusted to 7–8 using hydrochloric acid or 20% ammonia water, followed by centrifugation, filtration, and analysis using UHPLC-MS/MS. The results indicated an excellent linearity of 1–300 ng L<sup>-1</sup>, and cotinine in the concentration range of 20–6000 ng L<sup>-1</sup>, linear correlation coefficient  $R^2 > 0.995$ , with the limit of detection (LOD) of 0.01–6 ng L<sup>-1</sup>, and a limit of quantification (LOQ) of 0.1–20 ng L<sup>-1</sup>. The addition of three concentrates of low (2 ng L<sup>-1</sup>/40 ng L<sup>-1</sup>), medium (20 ng L<sup>-1</sup>/400 ng L<sup>-1</sup>), and high concentration (200 ng L<sup>-1</sup>/4000 ng L<sup>-1</sup>) demonstrated the matrix effect of the target compound between  $\pm 22.0\%$ . The extraction recovery was 70.0–119.4%, and a percent accuracy of 75.7–118.1%. Similarly, the intra- and inter-day precisions were 1.8–20.0% and 1.5–18.9%, respectively. The results cemented the sensitivity, accuracy, reliability, strong specificity, and reproducibility, which can be used to screen 28 illegal drugs in sewage for trace analysis.

## 1. Introduction

According to Article 357 of the Criminal Law of the People's Republic of China (PRC), "Drugs refer to opium, heroin, methamphetamine, morphine, marijuana, cocaine and other narcotic drugs and psychotropic drugs that can make people addicted to drugs," defining drugs as special compounds to be strictly controlled under the stated law. Besides conventional drugs, several new psychoactive substances (NPS) have been developed to escape traditional legal drug control measures or to improve pharmacological activity by modifying the parent drug structure with the objective of anesthesia, excitement, or replacement of similar drugs [1–3]. Drug abuse and NPS have become a global challenge, endangering human health, compromising families' social lives, and threatening social public safety [4]. The Ministry of Public Security, the National Health and Health Commission, and the State Food and Drug

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Administration jointly announced fentanyl-laced to be included in the Supplementary Catalogue of Controlled Varieties of Non-medicinal Narcotic Drugs and Psychotropic Substances from May 1st, 2019, thus classifying dozens of fentanyl-laced as controlled drugs, while NPS such as synthetic cannabinoids, cathinones, and fluoroketamine will be listed as controlled drugs from July 1st, 2021, resulting in controlling 449 types of drugs in China. Therefore, the addition of new drugs brings severe challenges to public safety. In recent years, sewage epidemiology based on sewage analysis has been widely used worldwide to evaluate drug consumption, which can objectively assess the amount of drug abuse in a particular area as an essential means of drug detection [5–7]. Numerous studies have shown that illegal drugs (drugs and NPSs) are discharged into the domestic sewage system in the form of precursors or metabolites after being absorbed by humans, or untreated sewage may be discharged directly into the natural water, making sewage treatment plants release a vital source of illegal drugs contaminating the environment [5,7–10]. Therefore, detecting components and types of illegal drugs in sewage can effectively control drug abuse in cities and contribute to drug control and safety early warning in cities and regions [7].

Different methods, including gas chromatography (GC), liquid chromatography (LC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and solid phase extraction (SPE), have been employed for analyzing illegal drugs in sewage [7,11–13]. The most commonly abused substances included morphine (MOP), methamphetamine (MA), amphetamine (AM), ketamine (KET), norketamine (NK), methylenedioxyamphetamine (MDMA), cocaine (COC), methylenedioxyamphetamine (MDA), and fentanyl (FEN), etc. [7,11,14–16]. Few studies have been reported on popular substances like cathinone (CAT), methcathinone (MC), ephedrine (EPH), pseudoephedrine (PSE), benzylpiperazine (BZP), and norfluoroketamine (NFK) [17–19]. Still, no relevant reports are available on methylenedioxypropylvalerone (MPDV). Furthermore, these NPSs have been observed to rise as reported by public security anti-drug authorities [20,21]. The sewage pretreatment comprises extraction, concentration, and purification but is relatively complicated and time-consuming. Moreover, considering current drug analysis protocols, owing to more new components and abused substances, simply analyzing traditional drugs no longer meets the requirements of analyzing the drug situation, making it imperative to develop new methods and incorporate more kinds of drugs and NPSs in the detection in sewage [22].

Compared to MS or SPE technology, LC-MS/MS offers a wide range of available stationary phases and mobile phases, providing high resolution and efficiency. The technology holds significant potential for scientific research and clinical applications due to its high analytical specificity, broad applicability to analytes with varying molecular weights, and the ability to conduct multi-parameter testing [23,24]. In contrast to GC or GC-MS/MS methods primarily utilized for confirmatory detection (which necessitates analyte polarity and thermal stability), the LC-MS/MS method does not require sample volatilization, this not only reduces sample consumption but also prevents the loss of the analytical target or the generation of new substances during the complex pretreatment process [25,26], thereby minimizing interference with the analysis. Consequently, the use of GC or GC-MS/MS methods for detecting sewage samples is not conducive to analyte recovery. In our research, we selected SPE technology in conjunction with the LC-MS/MS method to analyze common drugs in sewage, considering the advantages of simple operation, reduced consumable costs, shortened processing time, and high recovery rates associated with SPE technology [27–30]. Additionally, the use of the isotope internal standard (ISTD) quantitative method can significantly mitigate the influence of matrix effects on quantitative results, reduce the occurrence of false positives in detection results, and enhance detection sensitivity [31,32]. Therefore, under the conditions of quantitative analysis assisted by the ISTD method, the Online - SPE - UHPLC - MS/MS method was established to quickly, accurately, efficiently, and sensitively analyze illegal drugs in sewage. This method is better suited for the public safety industry to objectively and promptly detect drug abuse and NPSs of abuse, as well as to evaluate different regions.

## 2. Materials and methods

### 2.1. Instruments, materials and reagents

Two - dimensional - UHPLC - MS/MS (Pisces -I/AB SCIEX QTRAP 6500, SCIEX Company, USA); High-throughput automatic solid phase extractor (Fotector Plus 60, Guangzhou Wilson Tsui Instrument Technology Co., Ltd.); Vortex mixer (MX-S, Dalong Xingchuang Experimental Instrument (Beijing) Co., Ltd.); 0.45  $\mu\text{m}/0.1 \mu\text{m}$  aqueous microporous membrane, PHS-3C<sup>+</sup> acidity meter (Chengdu Century Ark Technology Co., Ltd.). 28 illegal drugs and 28-ISTDs standard stock solutions used were morphine (MOP), MOP-D3, 6-monoacetylmorphine (6-MAM), 6-MAM -D3, codeine (COD), COD-D6, methamphetamine (MA), MA-D5, amphetamine (AM), AM-D5, ketamine (KET), KET-D4, norketamine (NK), NK-D4, 3,4 - methylenedioxyamphetamine (MDMA), MDMA-D5, 3,4 - methylenedioxyamphetamine (MDA), MDA-D5, cocaine (COC), COC-D3, benzoylcegonine (BZE), BZE-D3, methcathinone (MC), MC-D3, fentanyl (FEN), FEN-D5, norfentanyl (NFN), NFN-D5, methadone (MTD), MTD-D3, tramadol, (TRD), TRD-D3, methylenedioxypropylvalerone (MPDV), MPDV-D8, cathinone (CAT), CAT-D5, benzylpiperazine (BZP), BZP-D7, ephedrine (EPH), EPH-D3, pseudoephedrine (PSE), PSE-D3, norfluoroketamine (NFK), NFK-D4, mephedrone (MEP), MEP-D3 (the concentration were all 1.0 mg mL<sup>-1</sup>); fluoroketamine (2-FDCK), 2-FDCK-D4, 4-*p*-methoxymethamphetamine (PMMA), PMMA-D3, noracefentanyl (NAF), NAF-<sup>13</sup>C<sub>6</sub>, and cotinine (COT), COT-D3, with all having concentration of 100  $\mu\text{g mL}^{-1}$ ). The concentrations of 4-anilino-N-phenylethylpiperidine (4-ANNP) and 4-ANNP-D5 were 10  $\mu\text{g mL}^{-1}$  purchased from American Cerilliant Company, and COT (purity >95%) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Other reagents used were methanol, acetonitrile, formic acid, ammonium acetate, ultrapure water, etc.

**Table 1**  
Procedure for online solid phase extraction.

Time (min)	flow rate/(mL.min <sup>-1</sup> )	A (%)	B (%)	C (%)	D (%)
0	2.0	100	0	0	0
0.5	2.0	100	0	0	0
2.0	2.0	0	100	0	0
3.5	2.0	0	0	100	0
4.5	2.0	20	0	0	80
6.0	2.0	20	0	0	80
8.0	2.0	0	100	0	0
10.0	2.0	100	0	0	0

**Table 2**  
Gradient elution conditions of online solid phase extraction.

Time (min)	flow rate (mL.min <sup>-1</sup> )	A (%)	B (%)
0	0.8	90	10
1.0	0.8	90	10
2.5	0.8	75	25
7.5	0.8	5	95
8.5	0.8	5	95
8.6	0.8	90	10
10.0	0.8	90	10

## 2.2. Methods

### 2.2.1. Preparation of standard solution

The stock solutions and working solutions of 28 illegal drugs and 28 ISTDs were prepared with methanol, i.e., MOP, 6-MAM, COD, MA, AM, KET, NK, MDMA, MDA, COC, BZE, MC, FEN, NFN, MTD-D3, TRD, MPDV, CAT, BZP, EPH, PSE, NFK, MEP, 2-FDCK, PMMA, NAF, and 4-ANNP at concentrations of 1  $\mu\text{g mL}^{-1}$ , while COT concentration was 20  $\mu\text{g mL}^{-1}$ . Similarly, 28-ISTD working solutions were prepared i.e., MOP-D3, 6-MAM-D3, COD-D6, MA-D5, AM-D5, KET-D4, NK-D4, MDMA-D5, MDA-D5, COC-D3, BZE-D3, MC-D3, FEN-D5, NFN-D5, MTD-D3, TRD-D3, MPDV-D8, CAT-D5, BZP-D7, EPH-D3, PSE-D3, NFK-D4, MEP-D3, 2-FDCK-D4, PMMA-D3, NAF-<sup>13</sup>C6, and 4-ANNP-D5 at concentration of 25  $\text{ng mL}^{-1}$  in methanol, while COT-D3 concentration was 500  $\text{ng mL}^{-1}$ . All solutions were stored in the dark at  $-4\text{ }^{\circ}\text{C}$ , while 28-ISTDs working solutions were diluted adequately to the required concentration during the test.

### 2.2.2. Working conditions of the instrument

**2.2.2.1. Chromatographic conditions.** The columns used were Phenomenex Kinetex Biphenyl ( $3.0 \times 100\text{ mm}$ ,  $2.6\text{ }\mu\text{m}$ ), and online-SPE column (OASIS HLB Direct Connect HP,  $2.1 \times 30\text{ mm}$ ;  $20\text{ }\mu\text{m}$ ), at column temperature of  $35\text{ }^{\circ}\text{C}$ , with sample injection volume of  $500\text{ }\mu\text{L}$ . The quaternary pump was used for online SPE (equipped with two Oasis HLB), while a binary pump was used for chromatographic separation (provided with Phenomenex Kinetex Biphenyl). The mobile phase A of the quaternary pump was pure water, B was 50% methanol-water (volume fraction, the same as below) solution, C was a mixture of methanol, acetonitrile, and isopropanol (in 1:1:1 ratio), and D was acetonitrile; the online solid phase program is shown in Table 1. Similarly, the mobile phase A for the binary pump was 2 mmol ammonium formate and 0.1% formic acid aqueous solution, and the mobile phase B was acetonitrile. The gradient elution procedure is presented in Table 2.

**2.2.2.2. Mass spectrometry conditions.** The mass spectrometer was equipped with electrospray ionization positive ion mode (ESI<sup>+</sup>), MRM detection method. The spray voltage was 2000 V, with an ion source gas 1 (GS 1) was 55 psi. The ion source temperature (TEM) was  $550\text{ }^{\circ}\text{C}$ , and the curtain gas (CUR) was 35 psi. Similarly, ion source gas 2 (GS 2) was 60 psi; Collision gas (CAD): medium. Quantitative and qualitative ions' collision voltage (CE) and declustering (DP) were optimized. The results are shown in Table 3, and the dwell time was 5 ms.

### 2.2.3. Sample source and pretreatment

Accurately transfer 3 mL of sewage sample, add 100  $\mu\text{L}$  of 28 ISTDs, and adjust the pH to 7–8 using hydrochloric acid or 20% ammonia water. Centrifuge to obtain the supernatant, and filter it through a  $0.45\text{ }\mu\text{m}/0.1\text{ }\mu\text{m}$  water - phase microporous membrane. Finally, extract 500  $\mu\text{L}$  using online SPE for UHPLC - MS/MS analysis. The sewage samples were collected from sewage treatment plants in Guizhou Province, China.

**Table 3**  
MRM parameters and retention times (RTs) of 28 target compounds and their 28-ISTDs.

Compound	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	CE (eV)	DP (V)	RT (min)
MOP morphine	286.3	201.1 <sup>a</sup> ,165.1	35,52	110	3.33
morphine					
MOP-D3	289.2	201.1 <sup>a</sup> ,165.1	35,52	110	3.33
6-MAM	328.2	165.1 <sup>a</sup> ,211.1	48,35	80	3.35
6-MAM-D3	331.2	165.1 <sup>a</sup> ,211.1	54,36	80	3.35
COD	300.2	165.1 <sup>a</sup> ,199.1	50,40	120	3.26
COD-D6	306.1	165.0 <sup>a</sup> ,202.1	52,42	129	3.26
MA	150.0	119.0 <sup>a</sup> ,91.0	11,20	34	3.29
MA-D5	155.1	124.1 <sup>a</sup> ,96.1	15,21	34	3.29
AM	136.1	91.1 <sup>a</sup> ,119.1	20,11	30	3.29
AM-D5	141.1	96.1 <sup>a</sup> ,124.1	12,8	40	3.29
KET	238.1	125.0 <sup>a</sup> ,179.1	34,19	45	3.52
KET-D4	242.1	129.0 <sup>a</sup> ,183.2	32,24	40	3.52
NK	224.3	125.0 <sup>a</sup> ,179.1	31,20	40	3.45
NK-D4	228.1	129.0 <sup>a</sup> ,183.0	30,21	50	3.45
MDMA	194.5	163.1 <sup>a</sup> ,105.1	16,30	41	3.42
MDMA-D5	199.1	165.1 <sup>a</sup> ,107.0	17,31	40	3.42
MDA	180.3	105.1 <sup>a</sup> ,133.2	27,23	16	3.36
MDA-D5	185.1	110.1 <sup>a</sup> ,138.1	30,25	30	3.45
COC	304.2	182.1 <sup>a</sup> ,150.1	34,32	90	3.72
COC-D3	307.2	185.1 <sup>a</sup> ,153.1	14,25	80	3.72
BZE	290.1	168.1 <sup>a</sup> ,105.0	25,33	80	3.49
BZE-D3	293.1	171.1 <sup>a</sup> ,105.0	16,25	80	3.49
MC	164.4	131.1 <sup>a</sup> ,105.1	26,29	34	3.26
MC-D3	167.1	131.1 <sup>a</sup> ,105.1	26,31	40	3.26
FEN	337.2	188.1 <sup>a</sup> ,105.1	24,34	20	3.91
FEN-D5	342.3	188.1 <sup>a</sup> ,105.1	24,34	20	3.91
NFN	233.2	84.0 <sup>a</sup> ,56.1	21,43	70	3.45
NFN-D5	238.2	84.1 <sup>a</sup> ,56.0	21,43	70	3.45
MTD	310.2	105.1 <sup>a</sup> ,265.2	32,20	43	4.22
MTD-D3	313.2	105 <sup>a</sup> ,268.1	37,20	50	4.22
TRD	264.2	58.2 <sup>a</sup> ,42.0	48,110	40	3.60
TRD-D3	268.4	58.0 <sup>a</sup> ,42.0	48,115	36	3.60
FDCK	222.2	109.1 <sup>a</sup> ,163.1	32,22	30	3.42
FDCK-D4	226.4	113.1 <sup>a</sup> ,167.2	32,21	30	3.42
PMMA	180.1	121.0 <sup>a</sup> ,149.2	20,15	16	3.43
PMMA-D3	183.1	121.0 <sup>a</sup> ,149.1	20,15	16	3.43
NAF	219.2	84.2 <sup>a</sup> ,136.1	21,22	40	3.27
NAF- <sup>13</sup> C6	225.2	84.0 <sup>a</sup> ,142.0	16,18	34	3.27
COT	177.1	80.0 <sup>a</sup> ,98.1	28,27	100	2.84
COT-D3	180.1	80.0 <sup>a</sup> ,101.1	29,27	100	2.84
ANPP	281.2	188.1 <sup>a</sup> ,105.1	24,40	50	3.95
ANPP-D5	286.4	188.2 <sup>a</sup> ,105.1	22,37	50	3.95
MDPV	276.2	126.1 <sup>a</sup> ,175	34,30	100	3.91
MDPV-D8	284.4	134.1 <sup>a</sup> ,175	33,30	100	3.91
CAT	150.0	117.0 <sup>a</sup> ,105.0	29,25	31	2.46
CAT-D5	155.1	137.1 <sup>a</sup> ,122.0	15,30	30	2.46
BZP	177.5	91.1 <sup>a</sup> ,85.0	27,20	60	2.05
BZP-D7	184.2	98.1 <sup>a</sup> ,85.2	27,21	45	2.05
EPH	166.2	148.2 <sup>a</sup> ,133.2	15,27	42	2.50
EPH-D3	169.1	151.1 <sup>a</sup> ,136.1	19,30	40	2.50
PSE	166.2	148.1 <sup>a</sup> ,117.1	16,26	40	2.50
PSE-D3	169.2	151.1 <sup>a</sup> ,117.1	19,30	40	2.50
NFK	208.4	163.3 <sup>a</sup> ,109.0	19,28	27	2.87
NFK-D4	212.3	167.2 <sup>a</sup> ,113.1	9,16	16	2.87
MEP	178.2	160.1 <sup>a</sup> ,145.1	12,18	20	4.03
MEP-D3	181.2	163.2 <sup>a</sup> ,132.1	16,17	21	4.02

<sup>a</sup> represents quantitative ions.

### 3. Results and discussion

#### 3.1. Optimization of chromatographic conditions

Three different columns were used for optimization purposes, i.e., Phenomenex Kinetex Biphenyl column (referred to as "Column 1"), ACQUITY UPLC HSS C18 column (2.1 × 150 mm, 3.5 μm, referred to as "Column 2") and ACE HILIC-N column (2.1 × 100 mm, referred to as "Column 3") with different fillers retaining 28 target compounds. The results showed that "Column 2" was a reverse column, which required starting analysis from a high inorganic phase, making elution time long to separate the targets, making it

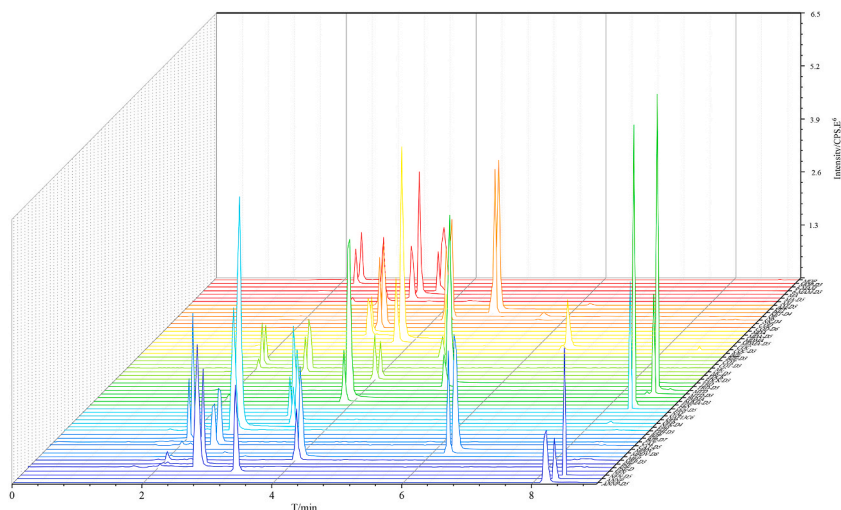


Fig. 1. MRM chromatograms of 28 target compounds and 28-ISTDs.

nonconductive to improve the detection efficiency. The "Column 3" was hydrophilic, used for eluting highly polar substances, but showed poor retention, which was deemed favorable for drugs like AM and its metabolites. The response of MA, AM, MDMA, MDA, EPH, and PSE was improved with "Column 3", but poor elution was observed for BZE, MC, FEN, NFN, MTD-D3, TRD, 2-FDCK, and NFK in terms of retention time. "Column 1" had a core-shell of Biphenyl stationary phase, where surface of core-shell silica gel is modified by the Biphenyl stationary phase with aromatic selectivity, making it selectively hydrophobic, aromatic, and with enhanced polar selectivity, which could achieve good retention and separation ability for 28 target compounds, so "Column 1" was selected. The MRM chromatogram of 28 target compounds and 28-ISTDs obtained by analysis are shown in Fig. 1.

Based on relevant reports, it was discovered that methanol and acetonitrile effectively elute illegal drugs in sewage samples [7,33,34] the organic phase eluent (quaternary pump: B, C, D, binary pump: B). To prevent heat generation from the methanol-water mixture, which can lead to an unstable baseline, we opted for a 1:1 mixed solution of methanol and water as phase B in the quaternary pump. Additionally, we followed previous reports by incorporating a specific amount of ammonium formate as a buffer salt to prevent excessive pressure from pure water in initial conditions and to prolong instrument lifespan. Furthermore, in ESI<sup>+</sup> mode, adding formic acid to the mobile phase proved beneficial in enhancing the resolution and sensitivity of the target [34]. The results (Fig. 1) demonstrate that under these elution conditions, the analysis method achieves satisfactory separation, extraction effectiveness, and stable repeatability for the analysis of 28 target compounds in sewage.

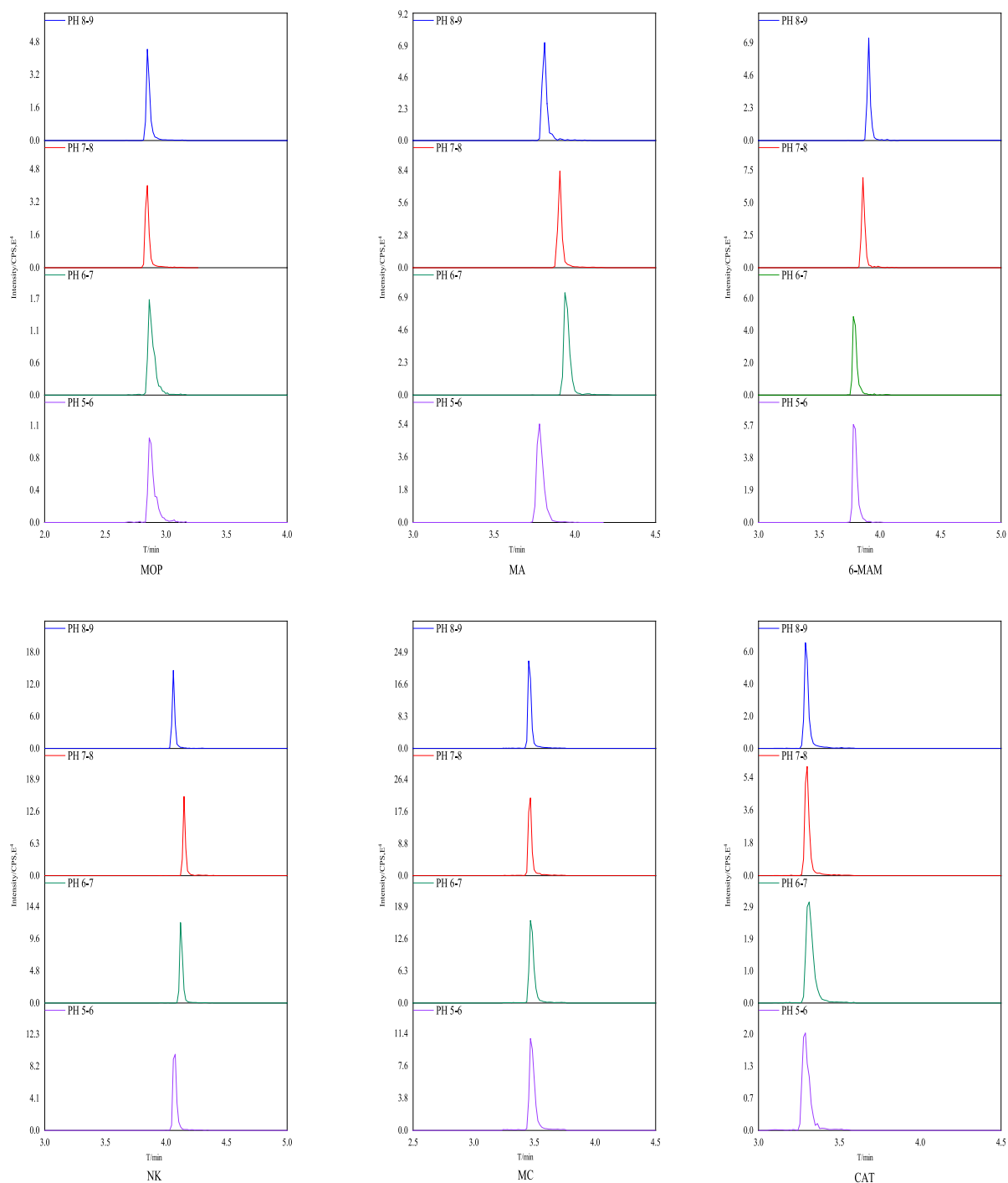
### 3.2. Optimization of sample pretreatment

#### 3.2.1. pH of sample solution

In order to enhance extraction and ionization efficiency by investigating the impact of sample pH on the analytes, considering the properties and pKa values of each analyte. 4 separate aliquots of blank sewage samples, each containing 28 target compounds at a concentration of 20 ng L<sup>-1</sup>, were collected. The pH of the samples was adjusted to 5–6, 6–7, 7–8, and 8–9 using hydrochloric acid or 20% ammonia solution, separately. Following the processing of the sewage samples as sections "2.2.3", the chromatographic peak shapes of the target compounds under different pH conditions were depicted in Fig. 2. The results indicated that at a pH of 7–8, the 28 target compounds exhibited higher extraction efficiency, improved chromatographic peak shapes, more symmetrical peaks, increased peak intensities, and reduced peak tailing. Consequently, to ensure optimal chromatographic analysis, the pH of the sewage sample was adjusted if it did not fall within the 7–8 range.

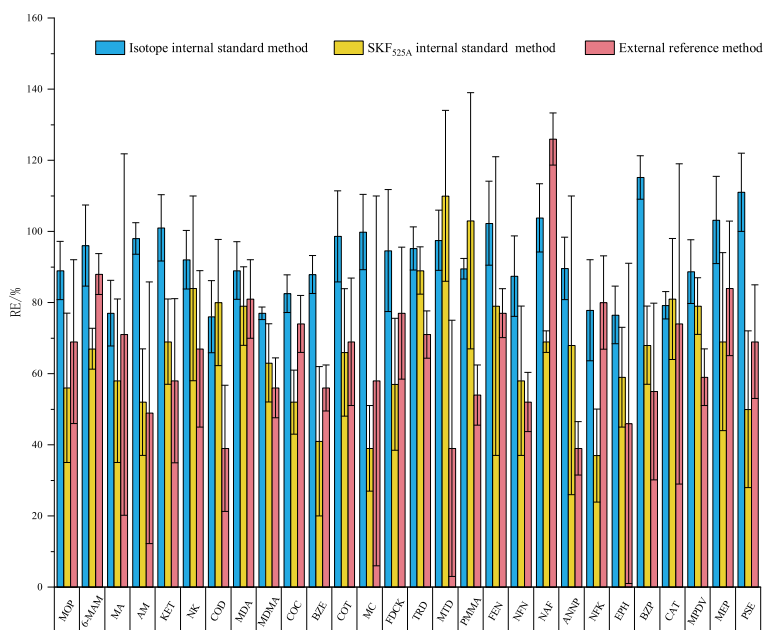
#### 3.2.2. Extraction rate test

The effects of different internal standards on the recovery of domestic sewage target substances were investigated. 28 target compounds were added to 18 blank sewage samples at a concentration of 20 ng L<sup>-1</sup>, and the samples were equally divided into three groups. The order of adding internal standards was the external standard method, SKF<sub>525A</sub> internal standard, and ISTD method, respectively, to investigate the stability of quantitative results and calculate the RSD values of recovery and concentration. The results showed that the recovery of all target compounds was 46.8–126.1% with an RSD of 6.4–50.8% when the external standard method was used for quantification, while 41.7–110.5% with an RSD of 3.1–42.5% in case of conventional internal standard SKF<sub>525A</sub> method. Similarly, the recovery of all target compounds was 76.4–115.2% with an RSD of 3.8–17.1% with ISTD. The results are shown in Fig. 3. These results suggested that recovery of all target compounds was high with good reproducibility when the ISTD method was used. Since all target compounds are controlled and have different properties, using the same method for extraction and analysis may affect some compounds' extraction efficiency and ionization degree. Therefore, the extraction efficiency and ionization degree of all the



**Fig. 2.** Chromatograms of MOP, MA, 6-MAM, NK, MC, and CAT under different pH conditions.

target compounds are uncertain, making recovery of samples low and undesirable precision when using the external standard method or SKF<sub>525A</sub>. The data analysis further showed that the recovery of sample extraction was improved after the isotope (deuterium band) dilution mass spectrometry was used, which could be attributed to the similar properties of ISTD and chemical properties of target compounds except for molecular weight, which could significantly reduce the ME complex, and achieve high detection precision and



**Fig. 3.** Comparison of recoveries of different analysis methods.

sensitivity. Thus, the use of the ISTD method is advocated to be used for analyzing target compounds.

### 3.2.3. Selection of extraction methods

The target compounds were analyzed by manual -, offline -, and online - SPE, where results showed that the manual - and offline - SPE required 50 mL of the sewage sample volume. Similarly, the columns used for manual - and offline - were disposable SPE columns, which made it costly. They also needed nitrogen blowing as pretreatment, which can easily contaminate the samples, leading to false positive results. Moreover, the recovery of all target compounds. The manual - and offline - SPE showed recovery of all targets in 45.2–117.8% and 42.1–123.6%, respectively, which were deemed unsatisfactory, along with a complicated process and longer analysis time. In contrast, the online - SPE for automatic solid-phase extraction required only 3 mL of the sewage sample, which could be injected directly for analysis without any pretreatment, ensuring a 100% utilization rate of the sample. Moreover, the SPE column can be reused, effectively reducing the analysis time and cost of consumables. The efficiency of online SPE was found to increase even in the case of a large number of samples. It showed recovery of all target compounds in the range of 88.0–110.7%, effectively reducing sample loss with an increased recovery rate of target compounds. Therefore, online - SPE for automatic solid-phase extraction is advocated for further analysis.

Additionally, the adsorption and extraction efficiency of three different solid phase extraction cartridges with different stationary phases for all target compounds were investigated, i.e., Oasis HLB (2.1 × 30 mm direct connect HP), XBridge C18 (2.1 × 30 mm direct connect HP), and XBridge C8 (2.1 × 30 mm direct connect HP). The overall results showed that some targets, including MOP, COT, KET, NFN, 4-ANNP, MPDV, BZP, and PSE, had poor retention efficiency on the XBridge C18 extraction column, with COT recovery of only 32.5%. The XBridge C8, solid-phase extraction column, showed unsatisfactory retention efficiency and recovery for MOP, COT, AM, BZE, NAF, 4-ANNP, MPDV, BZP, and MEP, which was in the range of 42.9–61.8%, which may translate into an increased probability of false negative results of the above compounds at low concentrations. Similarly, the Oasis HLB solid-phase extraction column showed recoveries of MOP and COT up to 71.2% and 72.1%, respectively, while the percent recovery of the other 26 target compounds was 79.6–109.8%, indicating all target compounds were well retained on the Oasis HLB solid-phase extraction column, thus ensuring the extraction recovery at the pretreatment end of the sample. These results advocated using the Oasis HLB solid phase extraction column to adsorb the target compounds.

### 3.3. Linearity, LOD, and LOQ

The linearity, LOD, and LOQ were determined by mixing blank sewage samples with different volumes of standard solutions in concentrations of 1, 2, 5, 10, 20, 50, 100, 200, and 300 ng L<sup>-1</sup>, while the COT concentration was 20, 40, 100, 200, 400, 1000, 2000, 4000, and 6000 ng L<sup>-1</sup>. They were analyzed as per the procedure outlined in section “2.2.3”. Each concentration was analyzed in pentaplicates and plotted concentration (X) vs. average peak area (Y) to get a linear regression equation. Similarly, the LOD and LOQ

**Table 4**  
Linearity, LOD and LOQ parameters of 28 target compounds.

Compound	Linear range (ng.L <sup>-1</sup> )	Regression equation	Coefficient (R <sup>2</sup> )	LOD (ng.L <sup>-1</sup> )	LOQ (ng.L <sup>-1</sup> )
MOP	1–300	y = 0.0149x+0.00316	0.9992	0.3	1
6-MAM	1–300	y = 0.0167x+0.00668	0.9996	0.3	1
COD	1–300	y = 0.0236x+0.00306	0.9991	0.3	1
MA	1–300	y = 0.0177x-0.00381	0.9992	0.3	1
AM	1–300	y = 0.051x-0.035	0.9993	0.1	1
KET	1–300	y = 0.016x-0.00725	0.9990	0.01	0.1
NK	1–300	y = 0.0179x-0.00359	0.9995	0.3	1
MDMA	1–300	y = 0.00868x-0.00204	0.9992	0.1	1
MDA	1–300	y = 0.0149x+0.024	0.9991	0.3	1
COC	1–300	y = 0.101x-0.0234	0.9991	0.1	1
BZE	1–300	y = 0.118x-0.0295	0.9996	0.1	1
MC	1–300	y = 0.037x-0.00756	0.9993	0.3	1
FEN	1–300	y = 0.0175x-0.00145	0.9991	0.1	1
NFN	1–300	y = 0.0307x-0.0123	0.9991	0.1	1
MTD	1–300	y = 0.0194x-0.00388	0.9994	0.1	1
TRD	1–300	y = 0.022x-0.000424	0.9995	0.1	1
FDCK	1–300	y = 0.0248x-0.00639	0.9991	0.3	1
PMMA	1–300	y = 0.0313x-0.00621	0.9994	0.3	1
NAF	1–300	y = 0.0339x-0.0167	0.9992	0.1	1
COT	20–6000	y = 0.00111x+0.000162	0.9998	6	20
ANPP	1–300	y = 0.0196x-0.0047	0.9994	0.3	1
MDPV	1–300	y = 0.2175x -0.00988	0.9976	0.05	0.1
CAT	1–300	y = 0.1292x + 0.0933	0.9993	0.2	0.5
BZP	1–300	y = 0.107x + 0.00638	0.9991	0.1	0.4
EPH	1–300	y = 0.016x - 0.00439	0.9993	0.1	0.5
PSE	1–300	y = 0.0825x - 0.00581	0.9987	0.1	0.5
NFK	1–300	y = 0.0133x + 0.0044	0.9997	0.1	0.2
MEP	1–300	y = 0.0698x + 0.00125	0.9996	0.6	1

were determined by calculating 3 times signal-to-noise ratio (3 S/N) and 10 times signal-to-noise ratio (10 S/N), respectively. Data is presented in Table 4, which shows that the linear relationship of COT was good in the range of 20–6000 ng L<sup>-1</sup>, while the linear relationship of the other 27 target compounds was good in the range of 1–300 ng L<sup>-1</sup>. The correlation coefficients of all target compounds were found to be R<sup>2</sup> > 0.995, with LOD of 0.01–6 ng L<sup>-1</sup> and LOQ of 0.1–20 ng L<sup>-1</sup>.

### 3.4. Matrix effect and extraction recovery

The ME is a crucial LC-MS analysis phenomenon characterized by ion enhancement or inhibition. The ME and extraction recovery (RE) were investigated simultaneously. Six blank sewage samples from different sources were mixed with varying volumes of standard solutions described in section “2.2.1” to prepare quality control samples with low, medium, and high concentrations. The concentrations of COT were 40, 400, and 4000 ng L<sup>-1</sup>, while the remaining 27 had concentrations of 2, 20, and 200 ng L<sup>-1</sup>. All prepared samples were pretreated as outlined in section “2.2.3”. All samples and pure standard solutions with low, medium, and high concentrations were analyzed using UHPLC - MS/MS, respectively. The peak areas were labeled as S<sub>sample</sub> and S<sub>standard</sub>. Similarly, the blank sewage samples from different sources were also pretreated as per the details outlined in section “2.2.3”, followed by the addition of standard solution with the same mass concentration after evaporation, where the peak area was labeled as S. All the samples were analyzed in six replicates, and results averaged. The percent ME and RE were calculated using the following relations.

$$ME (\%) = \frac{S - S_{standard}}{S_{standard}} \times 100\% \quad (1)$$

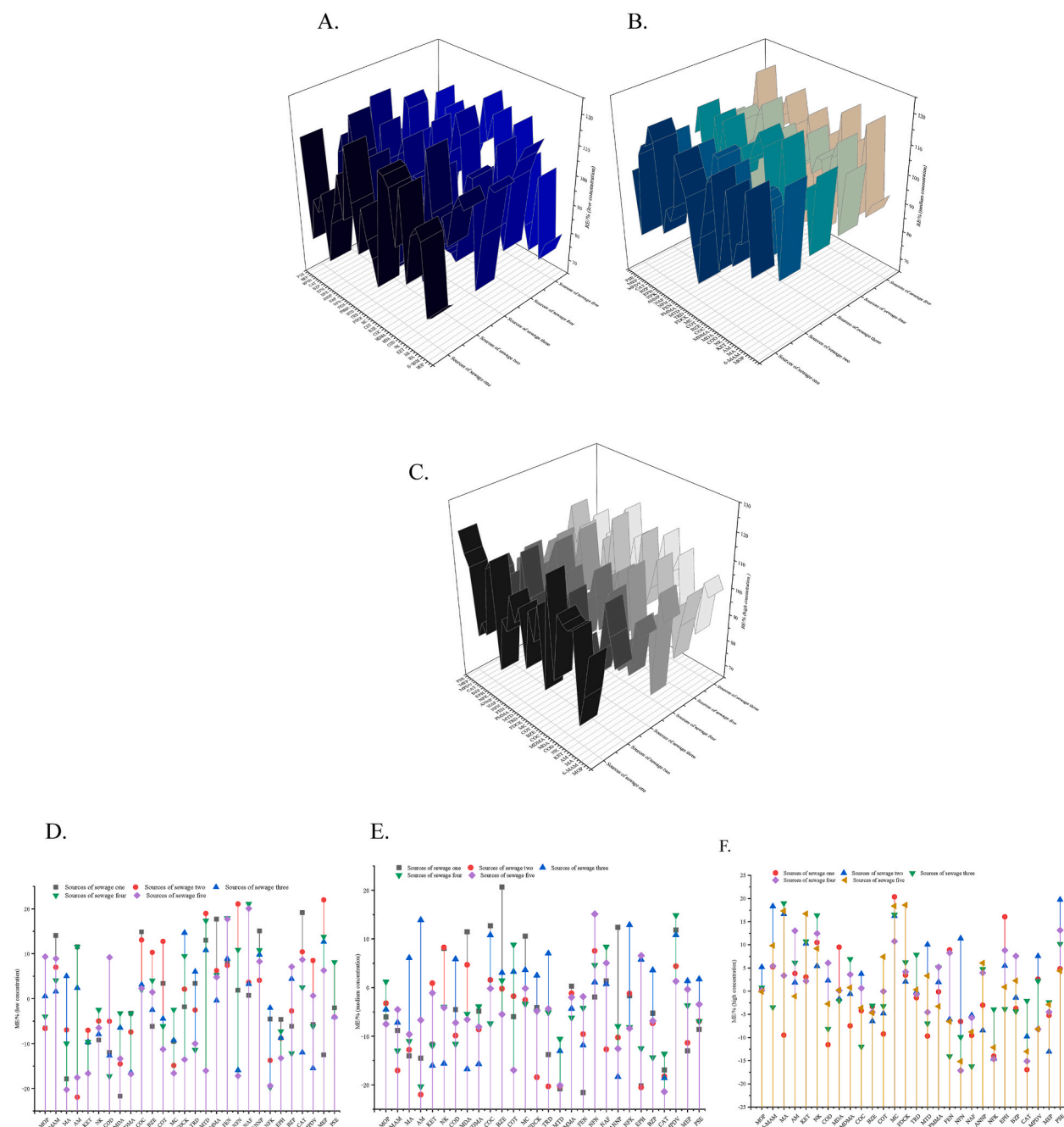
$$RE (\%) = \frac{S_{sample}}{S} \times 100\% \quad (2)$$

The results are shown in Fig. 4, which demonstrates the percent REs of 70.0–117.6%, 72–117.4%, and 76.2–119.4% for low, medium, and high mass concentrations, respectively. Similarly, the percent MEs for three mass concentrations were -21.9 ~ +21.9%, -21.5 ~ +20.6%, and -16.9 ~ +20.3%, respectively. As shown in Fig. 3, since the sewage matrix was relatively complex with ionization effects [7], some target compounds easily degraded in the matrix. Consequently, it was observed that 28 illegal drugs, including MA, MDA, AM, KET, and NFK, were inhibited, while FMMA and CAT were enhanced by the matrix. Nevertheless, they all met the methodological requirements, and the other 21 target compounds had no apparent matrix influence, cementing that the quantitative results of the method are accurate and reliable.

### 3.5. Precision and accuracy

Fifteen blank sewage samples from the same source were mixed with different volumes of standard solutions (detailed in section





**Fig. 4.** REs (A, B, C) and MEs (D, E, F) of 28 target compounds in different blank sewage samples ( $n = 6$ ).

“2.2.1”) to prepare quality control samples with low, medium, and high mass concentrations of COT (40, 400, 4000  $\text{ng L}^{-1}$ ), and other target compounds (2, 20, and 200  $\text{ng L}^{-1}$ ), and analyzed five times for recovery measurement. The intra-day precision (intra-RSD) can be obtained by analyzing the same concentration within one day, and inter-day precision (Inter-RSD) can be obtained by measuring different concentrations and different time points. Accuracy is expressed by bias, with the added mass concentration as the reference value and the average value of continuous analysis for six days as the measured value; the accuracy of the method was calculated from the percentage difference between the measured average and reference values. The results are shown in Figs. 5 and 6, where the accuracy (low, medium, and high) for the target compounds was found to be 75.7–117.3%, 82.0–118.1%, and 82.7–113.3%, with corresponding intra-RSD of 1.8–19.9%, 2.8–20.0%, and 2.1–12.9%, respectively. The relative standard deviation of the peak area of the three mass concentration points of inter-RSD were 4.2–18.9%, 2.3–18.8%, and 1.5–17.9%, respectively. These results showed that the analysis method can meet the requirements of trace analysis of 28 target compounds in sewage.

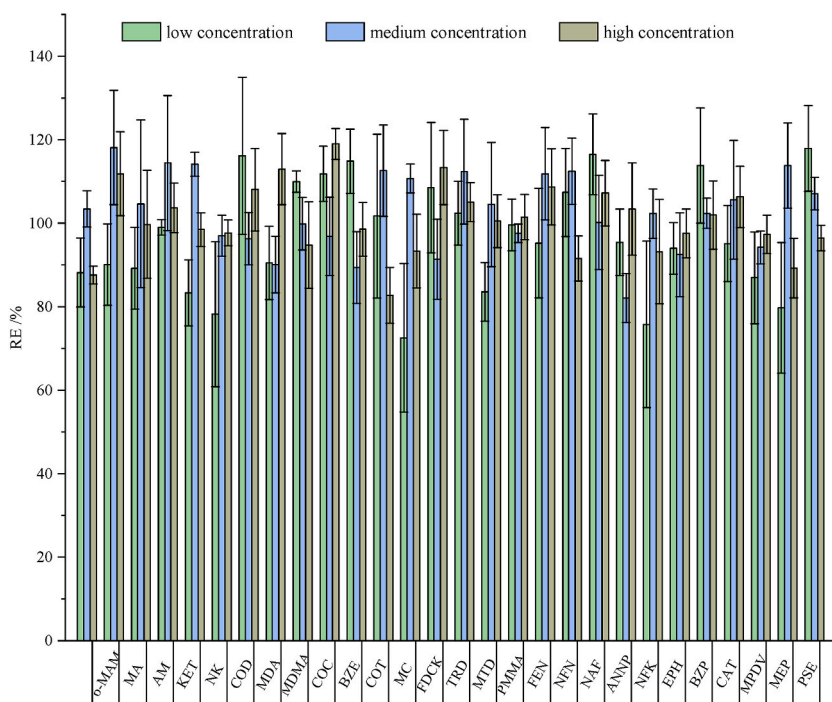


Fig. 5. Recoveries and intra-RSDs of 28 target compounds.

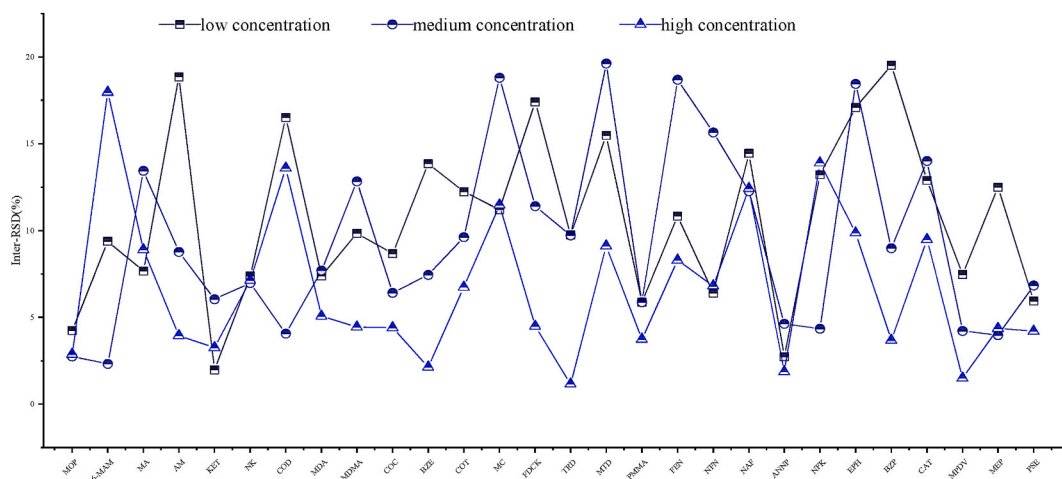


Fig. 6. The inter-RSD of 28 targets compounds.

### 3.6. Long-term stability

For long-term stability of target compounds in sewage, 15 samples containing COT ( $400 \text{ ng L}^{-1}$ ) and 27 other target compounds ( $20 \text{ ng L}^{-1}$ ) were prepared from blank sewage, stored at  $4 \text{ }^{\circ}\text{C}$ , and analyzed at different times of day 1, 2, 4, 8, 16, 20th. The results showed that the existence of target compounds for a longer duration suffered a significant loss, where on day 16, the mass concentrations of COT, COC, 6-MAM, and BZE significantly reduced with a recovery of 35.2–48.6%, while the recovery of the other 24 target compounds was 51.5–71.3%. Therefore, sewage samples should be analyzed immediately or not stored for more than 16 days to detect all target compounds.

### 3.7. Applications

The online - SPE method established in this study was applied to analyze 2653 sewage samples collected from 18 sewage treatment

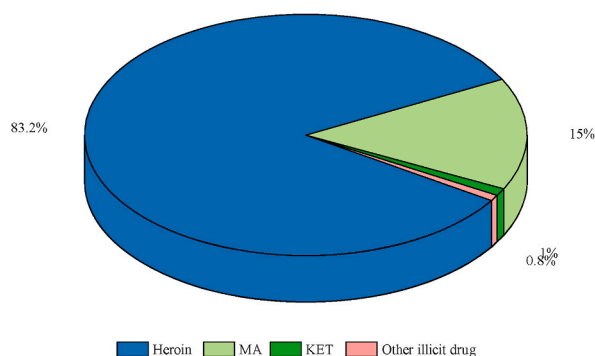


Fig. 7. Distribution of illegal drugs in an area of Guizhou Province in 2020.

**Table 5**

Analysis of the content of illegal drugs (Quantitative results in the linear range of sewage samples).

Compound	Minimum level (ng.L <sup>-1</sup> )	Maximum level (ng.L <sup>-1</sup> )
MA	1.2	205.4
KET	1.1	163.5
MOP	1.2	296.5
COD	2.7	105.2
PSE	25.7	268.2
MTD	1.5	278.3
TRD	6.8	169.2
CAT	1.7	147.5

plants in the first quarter of 2020 in a particular area of Guizhou province, China. The results showed that the main components of illegal drugs in this batch of sewage samples were heroin, COD, MA, KET, MC, CAT, PSE, 2-FDCK, MTD, and TRD (Fig. 7). At the same time, the content of the positive sewage samples was analyzed; the results are shown in Table 5. According to Fig. 7, heroin (calculated by 6-MAM) accounted for 83.2%, MA for 15%, KET for 1%, and other abuse substances for 0.8%. It was observed that the abuse of illegal drugs in that area was mainly traditional drugs. According to Table 5, the detected MOP concentration in this area was relatively high, which could be due to the wastewater containing high concentrations of MOP in some hospitals being directly discharged into the municipal pipeline network to enter the sewage treatment plant without strict treatment, or some restaurants might be using additives such as illegal use of poppy shells, which will also lead to high concentration of MOP entering sewage. In addition, MOP, COD, compound licorice tablets, and other drugs used by residents may also be entering the sewage after metabolism, increasing MOP concentration in the sewage. The detected PSE and TRD were mainly from the commonly used pharmaceutical ingredients in medical treatment, and people may have caused the abnormally high concentration of their respective samples during the medication process or the wastewater around the hospital being discharged into the sewage pipeline without strict treatment. Similarly, MTD is mainly used for treating the withdrawal syndrome of drug addicts, which might be released into the sewage pipeline around the drug rehabilitation center when there is a high situation analysis. In contrast, most MA, COD, COT, and KET conditions might be caused by abuse, illegal processing, and illegal use in people's lives.

#### 4. Conclusion

Based on online SPE - UHPLC - MS/MS and ISTD method, an analytical method for rapid analysis of 28 illegal drugs in sewage samples by direct injection was established. The technique is simple, fast, and requires less samples. The sample must only pass through a water - phase filter membrane before analysis, significantly reducing the analysis time. Moreover, it also provides an early warning for analyzing sewage water, which is not easily stored for a long time. The whole analysis can be done quickly, significantly improving the detection efficiency and extending to the screening and quantitative analysis of multi-target illegal drugs in sewage.

#### CRedit authorship contribution statement

**Shunqin Chen:** Writing – original draft, Software. **Han Yang:** Writing – original draft, Data curation. **Shan Zhang:** Writing – original draft, Software, Data curation. **Faze Zhu:** Software, Resources. **Shan Liu:** Visualization, Validation, Data curation. **Gao Han:** Investigation. **Qing Diao:** Validation, Software, Investigation. **Wenbo Ding:** Resources, Methodology. **Yuemeng Chen:** Writing – review & editing, Conceptualization. **Peng Luo:** Writing – review & editing, Validation, Funding acquisition, Conceptualization. **Yubo Liu:** Writing – review & editing, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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