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Quantitative analysis of drugs of abuse and cognitive enhancers in influent wastewater by means of two chromatographic methods

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Abstract

Sewage-based epidemiology using influent wastewater is used to estimate the consumption trends of (illicit) drugs over a short or long period of time in a subpopulation. The current study aimed to develop two separate methods for the quantitative analysis of selected drugs of abuse (DOA) and cognitive enhancers in influent wastewater using reversed-phase (RP) or hydrophilic interaction liquid chromatography (HILIC) coupled to high-resolution tandem mass spectrometry (LC-HRMS/MS). The performance of RP and HILIC column was evaluated. A simple solid phase extraction was used for sample preparation. Short runtimes of 10 and 15 min on the RP and the HILIC column, respectively, allowed sufficient throughput. A six-point calibration was used for quantification with calibration ranges between 10 and 100 ng/L for all analytes except for benzoylecgonine (BZE, 30–300 ng/L). Method validation was performed according to ICH guideline M10. Analytes such as amphetamine (AMPH), BZE, cocaethylene (CE), cocaine (COC), ethyl sulfate, 4-hydroxy-3-methoxymethamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), methamphetamine, methylphenidate (MPH), and ritalinic acid (RA) were included in method development and validation. Two different column types were necessary for sufficient chromatographic resolution. The analytical setup allowed detection of all other analytes at concentration levels between 1 ng/L for methylphenidate to 10 ng/L for amphetamine. A method for the detection and quantification of DOA, cognitive enhancers, and their biomarkers in wastewater was successfully developed and validated. Moreover, six proof-of-concept samples were analyzed in which AMPH, BZE, COC, MDMA, MPH, and RA were identified and further quantified.

KEYWORDS
cognitive enhancers, drugs of abuse, LC-HRMS/MS, wastewater analysis

1 | INTRODUCTION

Approximately 83 million Europeans aging between 15 and 64 have used illicit drugs in their life. Stimulants such as amphetamines, 3,4-methylenedioxymethamphetamine (MDMA), and cocaine are among the most commonly used drugs of abuse (DOA) in the European union.[1] In addition to DOA, prescription drugs such as methylphenidate, piracetam, and modafinil are abused as cognitive enhancers to improve performances or ease stress.[2]

The extend of current drug abuse and upcoming trends can be monitored by, for example, online surveys, which are easy to perform and cheap. However, one limitation is their highly subjective character, which may lead to under reporting by the participants.[3,4] Hence, complementary and more objective tools, like wastewater-based epidemiology (WBE), are required. This approach allows to monitor drug intake through analyzing compounds of interest after their excretion into wastewater (WW). There is no need of testing individuals,[5] and WBE allows the detection of temporal as well as spatial trends in the abuse of drugs.[6,7]

However, the development of such bioanalytical methods is quite challenging. Hereby, the selection of a suitable biomarker for each drug is crucial. The ideal biomarker is expected to fulfill several criteria. Human specific metabolites should be used to distinguish between human consumption and discarded compounds into WW.[3,5] These metabolites should be excreted in consistent amounts, sufficient for analysis. Furthermore, no sorption to particles, the sewer line itself or filters used for sample (pre-) treatment, should occur. Moreover, biomarkers must be stable under the conditions in WW (in-sewer stability), which includes stability against hydrolysis and stability against biotransformation by microorganisms present in WW.[3,8]

Although reversed-phase (RP) liquid chromatography (LC) is widely used in bioanalysis, separation of highly polar drug metabolites is often not ideal or possible.[9,10] Hydrophilic interaction liquid chromatography (HILIC) as complementary method allows a better separation of polar analytes.[11] The general suitability of HILIC for the separation of hydrophilic DOAs and pharmaceuticals after extracting influent WW using solid phase extraction (SPE) was already demonstrated.[12,13] Particularly very polar pharmaceuticals, which had no retention in classical RP chromatography such as metformin, could be retained using HILIC. Also, the large amount of acetonitrile (ACN) in the mobile phase using HILIC increased the analytical sensitivity.

Aims of this study were first the development of an SPE-based liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS) procedure for identification and quantification of selected DOA, cognitive enhancers, and metabolites in WW. Furthermore, the performance of two analytical LC columns, one RP column and one HILIC column for chromatographic separation of analytes, should be evaluated. Finally, the method should be applied to six proof-of-concept WW grab samples collected over a period of 2 years.

2 | MATERIALS AND METHODS

2.1 | Reagents and materials

Amphetamine (AMPH) sulfate, AMPH-d_5, benzoylecgonine (BZE), BZE-d_3, cocaethylene (CE), CE-d_8, cocaine (COC) hydrochloride (HCl), COC-d_3, ethyl sulfate (EtS) sodium salt, EtS-d_5, 5-hydroxy-indolacetic acid (5-HIAA), 5-HIAA-d_5, 4-hydroxy-3-methoxymethamphetamine (HMMA), MDMA HCl, MDMA-d_5, methamphetamine (METH) HCl, METH-d_5, methylphenidate (MPH) HCl, MPH-d_9, modafinil, modafinilic acid, piracetam, ritalinic acid (RA), and RA-d_{10} were obtained from LGC (Wesel, Germany). All other chemicals were purchased from VWR (Darmstadt, Germany). Water was purified with a Millipore (Merck, Darmstadt, Germany) filtration unit, which purifies water to a resistance of 18.2 $\Omega \times$ cm.

Stock solutions of each compound were prepared at concentrations of 100 µg/L in ACN, calculated for the active compound. The internal standard (IS) solution contained 150 µg/L BZE-d_3, 1 mg/L EtS-d_5, and all other isotopically labeled compounds at concentrations of 50 mg/L in ACN. Calibrator (Cal) and quality control (QC) working solutions were separately prepared in ACN, and final concentrations in surface water (rainwater) as surrogate blank matrix are shown in Table S1.

2.2 | Sample preparation

Sample preparation was performed according to a previously published procedure[6] with minor modifications. WW samples (10 mL) were fortified with IS solution (final concentration 50 ng/L, except BZE-d_3 150 ng/L and EtS-d_5 1 µg/L) and then filtered via Phenex-PTFE 25-mm syringe filters 0.2 mm (Phenomenex, Aschaffenburg, Germany) to remove particles. The SPE was performed using Isolute 200 mg/10 mL (3-mL XL) HCX cartridges (Biotage, Uppsala, Sweden). Cartridges were primed with 1-mL methanol (MeOH) and 1-mL purified water. Cartridges were then loaded with 10 mL of WW and subsequently washed with purified water, 0.1 M hydrochloric acid, and MeOH (1 mL each). Targeted compounds were eluted using a mixture of MeOH and NH_3 (35%, 98/2, *v/v*, 1.25 mL). Eluates were then partitioned into two equal aliquots and evaporated to dryness under a gentle stream of nitrogen at 40°C. The residues were reconstituted using a mixture of ACN and formic acid (50 µL, 99/1, *v/v*, HILIC samples) or a mixture of water and formic acid (50 µL, 99/1, *v/v*, RP C_{18} (C_{18}) samples).

2.3 | Instrumental settings

Samples were analyzed using a Thermo Fisher Scientific (TF, Dreieich, Germany) Dionex UltiMate 3000 consisting of a degasser, a quaternary pump, a DL W2 wash system, and an HCT PAL autosampler (CTC Analytics AG, Zwinger, Switzerland). The system was coupled to a TF Q-Exactive orbitrap mass spectrometer, equipped with a heated electrospray ionization II source (HESI-II). Calibration was done prior to analysis according to the manufacturer's recommendations using external mass calibration. The final conditions of the LC-system

using the C_{18} column were as follows: Waters AQUITY UPLC BEH C_{18} column (100 × 2.1 mm, 1.7 µm; Massachusetts, USA); gradient elution was done with 2 mM ammonium formate solution containing 0.1% (*v*/*v*) formic acid (eluent A) and 2 mM ammonium formate solution in ACN/MeOH (50/50, *v*/*v*) containing 0.1% (*v*/*v*) formic acid and 1% (*v*/*v*) water (eluent B). The flow rate was at 0.500 mL/min with the following gradient settings: 0–1 min 85% A, 1–3 min to 40% A, 3–6 min to 30% A, 6–8 min 1% A, 8–9.2 min hold 1% A, 9.2–9.21 min to 85% A, 9.21–10 min hold 85% A.

The final conditions of the LC-system with the HILIC column were as follows: Merck SeQuant ZIC-HILIC column (150 × 2.1 mm, 3 µm; Merck, Darmstadt, Germany); gradient elution with 200 mM aqueous ammonium acetate solution (eluent C) and ACN containing formic acid 0.1% (*v*/*v*) (eluent D). The flow rate was at 0.500 mL/min with the following gradient settings: 0–1 min hold 1% C, 1–1.8 min to 10% C, 1.8–9 min hold 10% C, 9–9.5 min to 60% C, 9.5–10.5 min hold 60% C, 10.5–10.6 min to 1% C, 10.6–15 min hold 1% C. Chromatography on both columns was performed at 40°C.

The final HESI-II source and MS conditions were as follows: ionization mode: positive and negative; sheath gas, 60 arbitrary units (AU); auxiliary gas flow rate, 10 AU; spray voltage, 4.00 kV; auxiliary gas heater temperature, 320°C, ion capillary temperature, 320°C; and S-lens RF level, 50.0. Mass spectrometry experiments after C_{18} column separation were performed using parallel reaction monitoring (PRM) in positive mode with a scheduled inclusion list containing the precursor masses of interest, adjusted normalized collision energies (NCEs). The settings for PRM experiments were as follows: resolution 17,500; automatic gain control (AGC) target 2e5; maximum injection time (IT) 250 ms; isolation window, 1 *m*/*z*; high-energy collisional dissociation (HCD) with NCE 30, 40 eV. Mass spectrometry experiments after HILIC column separation were performed using PRM in positive and negative mode in a single analytical run. Settings in positive mode were the same as described for the C_{18} column; settings for negative mode were as follows: resolution 17,500; AGC target 2e5; maximum IT 250 ms; isolation window, 1 *m*/*z*; HCD with NCE 10 eV. TF Xcalibur Qual Browser version 4.1.31.9 was used for data handling. Masses of the precursor ions (*m*/*z*) used for the inclusion list, polarity, and adjusted NCE are listed in Table S2.

2.4 | Method validation

The method was validated according to the ICH guideline M10 on bioanalytical method validation and study sample analysis.[14] For identification, MS^2 spectra were compared to a database,[15] and for quantification, peak area ratios of the quantifier ions of analytes and IS in MS^2 (see Table S2) were used.

2.4.1 | Calibration curve and lower limits of quantitation

The calibration curve consisted of six calibration standards (Cal 1–6), prepared by spiking aged surface water, as blank matrix, with different calibrator solutions and the IS mix. QC lower limit of quantification (LLOQ), QC low, QC medium (mid), and QC high were prepared by spiking blank matrix with different QC spike solutions, prepared separately from the calibration standards (final concentrations in are listed in Table S1) and IS. Calibrators and QC samples were extracted as described above (Section 2.2). Each analytical run consisted of Cal 1–6, QC LLOQ, QC low, QC mid, QC high, a blank matrix sample, and a zero sample (blank matrix spiked with IS). Back calculated concentrations of the calibration standards should be within ±15% of the nominal value (±20% for QC LLOQ), and at least 75% of the calibrators must fulfill this criterion. The limit of detection was set to be equal to the LLOQ, and the LLOQ was accepted in case the back calculated concentrations were within ±20%.

2.4.2 | Selectivity and carry-over

Selectivity was tested by extraction (see Section 2.2) of six different sources of surface water (blank matrix), which were analyzed individually on both columns as described in Section 2.3. Acceptance criteria (AC) are met if the analyte response is less than 20% of the LLOQ or 5% of the IS. Carry-over was tested by injecting blank extracts after QC high samples ($n = 3$) on both columns; no carry-over greater than 20% of the LLOQ for the analytes or 5% for the IS should be observed to fulfill AC.

2.4.3 | Dilution integrity

Dilution integrity was determined by spiking blank matrix with analyte concentrations 10 times the QC high and diluting these 1:10 with blank matrix ($n = 5$). Accuracy and precision of all analytes should be within ±15%.

2.4.4 | Matrix factors (MFs), recovery, and studies on co-eluting analytes

MFs and recovery were determined using blank matrix from six different sources. Three sets of samples at QC low and QC high were prepared ($n = 6$): set 1 consisted of pure solutions of analytes and IS, set 2 consisted of blank matrix spiked with analytes and IS after the extraction, and set 3 consisted of blank matrix spiked before the extraction. Pure solutions of analytes and IS were prepared in ACN and formic acid (99/1, *v*/*v*, HILIC samples) or a mixture of water and formic acid (99/1, *v*/*v*, C_{18} samples). Sample preparation of set 2 was performed as described in Section 2.2 without IS fortification prior to SPE. Sample preparation of set 3 was performed as described in Section 2.2.

For each analyte and IS, the MF was calculated, according to the guideline of the European Medicines Agency,[16] using the ratio of set 1 and set 2, and the IS-normalized MF of each analyte was calculated by dividing the analytes MF by the MF of the corresponding IS. The IS-normalized MF of HMMA was calculated by using MDMA-d_5 as IS, because no isotopically labeled analog of HMMA was available to be included in this study.

Additionally, the recovery (RE) according to Matuszewski et al.[17] was tested. RE was calculated via the peak area ratio of set 3 to set

2 for each analyte and IS. Since for every analyte, except HMMA, a corresponding isotopically labeled analog was available, the IS-normalized RE was calculated additionally, as described for the IS-normalized MF, CVs should not exceed 15%.

Ion suppression or enhancement was tested according to Remane et al. at concentrations of 10 µg/L for all co-eluting analytes and IS,[18] CVs for all co-eluting analytes and IS should be within 15%.

2.4.5 | Stability

Stability of the stock solution was tested over 6 weeks; samples were stored at −22°C, and aliquots were analyzed once a week ($n = 3$). As the extracts for both columns were prepared using the same stock solution, stability was only determined using the HILIC column. Furthermore, stability in the autosampler was tested for processed QC low and QC high samples ($n = 3$, 24 h at 10°C), as well as freeze thaw stability (3 cycles, 24 h per cycle at −22°C) and long-term stability over 106 days at −22°C for unprocessed samples. Concentrations should be within ±15% of nominal values for both QC levels when analyzed directly after the sample preparation and after the applied storage conditions via a freshly prepared calibration. Short-term stability and benchtop stability were not applicable for this study as the samples were directly frozen after sampling and hence not tested.

2.4.6 | Accuracy and precision

Accuracy and precision were evaluated for QC LLOQ, QC low, QC mid, and QC high samples. Within-run accuracy and precision experiments consisted of five sets of QC samples, measured within a single analytical run. Between-run accuracy and precision were determined with three different runs on two different days. QC concentrations were back calculated via calibration curves, and AC for accuracy experiments are met if mean concentrations are within ±15% of the nominal values (±20% for QC LLOQ). For precision experiments, the coefficient of variation (CV) should be within ±15% for all QC samples (±20% for QC LLOQ) to meet the AC.

2.5 | WW sample collection, treatment, and application of the method

Proof of concept WW grab samples ($n = 6$ in total) were obtained between June 2021 and March 2023, at two different sampling points. Samples were acidified with acetic acid (0.1% *v*/*v*) and stored at −22°C until the final sample preparation.

3 | RESULTS AND DISCUSSION

3.1 | Samples preparation

As described in Section 2.2, sample preparation was performed according to Meyer et al. with minor modifications.[6] We were able to use the same sample preparation (the same extract) prior to analysis by C18 and HILIC. This resulted in lower costs and higher sample throughput in contrast to individual sample preparation prior to C18 and HILIC. Best peak shapes were obtained on the C_{18} column if the analytes were reconstituted in a mixture of water and formic acid (50 µL, 99/1, *v*/*v*), and on the HILIC column with a reconstitution mixture containing ACN and formic acid (50 µL, 99/1, *v*/*v*). Therefore, two different extracts were necessary for the analysis using two different columns (see Figures 1 and 2). A volume of 50-mL reconstitution solvent was used to increase the concentration factor up to 100. The extraction and/or separation of 5-HIAA, modafinil, modafinilic acid, and piracetam was not reproducible, and these analytes were thus not included in the method validation.

3.2 | Method validation

Aged rainwater was used as blank matrix although it does not necessarily reflect the complexity of influent WW. However, influent WW cannot be used as blank matrix for validation as it contains the analytes of interest per se. Thus, a surrogate matrix must be used, and aged rainwater seems to be one reasonable alternative although there are still some limitations.

3.2.1 | Selectivity and carry-over

Analysis of six blank samples showed no interfering signals in PRM runs with either the C_{18} or HILIC column. Furthermore, on both columns, no carry-over of analytes in blank runs after the QC high ($n = 3$) could be observed. Additionally, blank samples should be injected between study samples and after Cal 6, as potential carry-over was only tested up to concentrations of QC high.

3.2.2 | Dilution integrity

The selection of the concentration range was based on previously studies and expected concentrations. Higher concentrations during validation might have increased the risk of carry-over and were thus avoided. Instead, the dilution integrity was tested to also allow the reliable quantification of higher concentrations after dilution. All analytes met the AC for the 1:10 dilution after analysis on both columns except for HMMA and EtS (Table S3). Relative mean concentration and CV of HMMA were >15% (55%, CV 22%), and for EtS, the IS could not be detected.

3.2.3 | MFs, recovery, and studies on co-eluting analytes

IS-normalized MF, RE, and IS-normalized RE are listed in Tables 1 and 2. After C_{18} separation, CVs obtained for IS-normalized MF of QC low

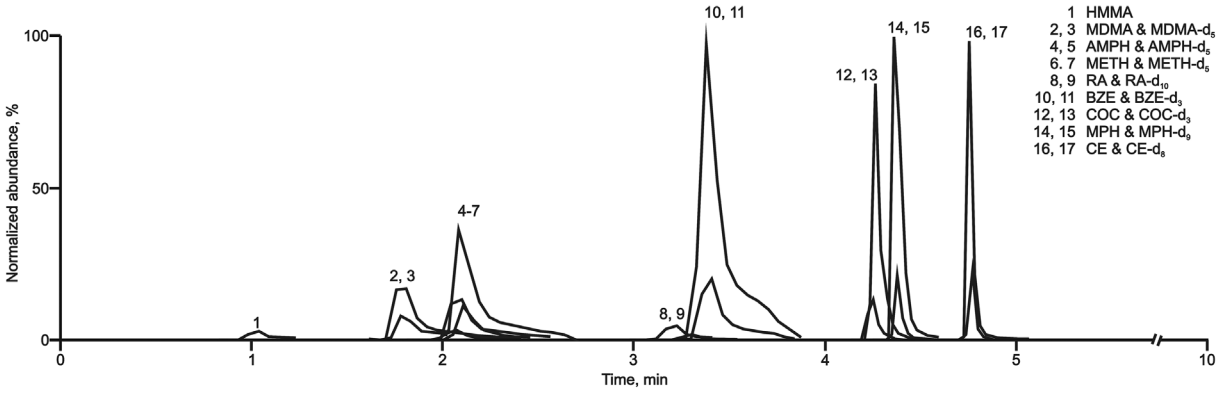

FIGURE 1 Extracted quantifier ion chromatogram of an extracted lower limit of quantification (LLOQ) sample after separation using the C_{18} column; AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; RA, ritalinic acid.

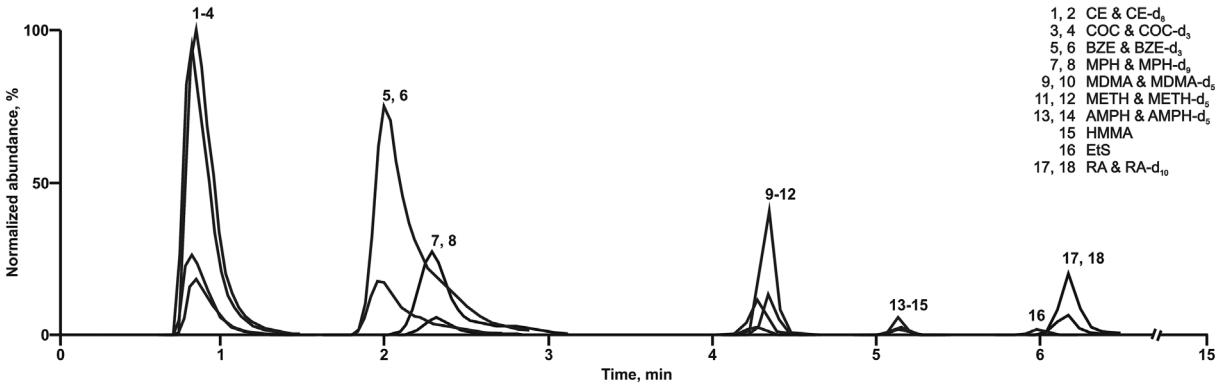

FIGURE 2 Extracted quantifier ion chromatogram of an extracted lower limit of quantification (LLOQ) sample after separation using the hydrophilic interaction liquid chromatography column; AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; EtS, ethyl sulfate; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; RA, ritalinic acid.

TABLE 1 Internal standard (IS)-normalized matrix factor (MF), recovery (RE), IS-normalized RE, and their coefficients of variation (CV) calculated for quality control (QC) low and QC high ($n = 6$), analyzed on the C_{18} column.

	QC low			QC high		
Analyte	IS-normalized MF, % (CV, %)	RE, % (CV, %)	IS-normalized RE, % (CV, %)	IS-normalized MF, % (CV, %)	RE, % (CV, %)	IS-normalized RE, % (CV, %)
AMPH	94 (5)	73 (33)	112 (8)	101 (4)	80 (9)	100 (8)
BZE	101 (3)	48 (30)	97 (13)	98 (3)	54 (16)	105 (7)
CE	102 (8)	15 (26)	148 (7)	105 (10)	14 (28)	87 (13)
COC	100 (8)	19 (31)	103 (13)	98 (7)	26 (30)	108 (9)
EtS	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
HMMA	29 (14)	105 (51)	203 (13)	28 (13)	135 (11)	369 (25)
MDMA	99 (2)	55 (40)	111 (9)	96 (12)	63 (16)	111 (6)
METH	125 (10)	57 (35)	148 (27)	102 (13)	61 (16)	169 (23)
MPH	100 (3)	43 (40)	105 (13)	93 (11)	46 (18)	104 (7)
RA	100 (2)	69 (20)	119 (11)	96 (5)	62 (15)	105 (6)

Abbreviations: AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; EtS, ethyl sulfate; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; n. a., not available; QC: quality control; RA, ritalinic acid.

TABLE 2 Internal standard (IS)-normalized matrix factor (MF), recovery (RE), IS-normalized RE, and their coefficients of variation (CV) calculated for quality control (QC) low and QC high ($n = 6$), analyzed on the hydrophilic interaction liquid chromatography column.

	QC low			QC high		
Analyte	IS-normalized MF, % (CV, %)	RE, % (CV, %)	IS-normalized RE, % (CV, %)	IS-normalized MF, % (CV, %)	RE, % (CV, %)	IS-normalized RE, % (CV, %)
AMPH	102 (11)	147 (75)	107 (14)	99 (3)	393 (48)	99 (2)
BZE	121 (25)	75 (44)	109 (46)	100 (2)	148 (46)	107 (4)
CE	94 (12)	98 (24)	121 (10)	100(1)	139 (32)	107 (5)
COC	97 (8)	99 (25)	122 (15)	100 (1)	143 (34)	107 (4)
EtS	81 (45)	92 (217)	IS n.d.	100 (4)	1 (18)	IS n.d.
HMMA	15 (61)	196 (94)	150 (103)	134 (10)	415 (48)	434 (48)
MDMA	100 (5)	108 (110)	110 (9)	101 (5)	123 (40)	112 (4)
METH	102 (6)	185 (122)	110 (14)	103 (6)	116 (40)	110 (7)
MPH	96 (6)	104 (41)	116 (9)	99 (2)	146 (34)	106 (5)
RA	97 (2)	930 (208)	109 (10)	97 (2)	129 (16)	107 (5)

Abbreviations: AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; EtS, ethyl sulfate; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; QC, quality control; RA, ritalinic acid.

and QC high were below 15%, except for HMMA (see Table 1). After HILIC separation, HMMA, BZE, and EtS did not meet the AC given by the ICH.[14]

CVs of the IS-normalized RE were within 15% for most analytes. METH (C_{18} column), BZE, EtS (HILIC column), and HMMA (both columns) did not meet the AC. In comparison, CVs of RE calculated according to Matuszewski et al. were above 15% for all analytes, which could be due to variations in the extractions or fluctuations during the measurements. Because there was a corresponding IS for each analyte except for HMMA, it was expected that the IS-normalized RE would show lower CVs than the RE. Differences in meeting the AC for RE between both columns might be explained with varying column performances during method validation. Furthermore, it could be influenced by the different reconstitution solvents used for both columns.

Ion suppression or enhancement was tested according to Remane et al.[18]; CVs for all co-eluting analytes and IS were below 15% (Table S4). The co-eluting analytes can be identified in Figures 1 and 2.

3.2.4 | Stability

Analysis of stability samples was performed after HILIC separation. Stability of the stock solution was given over a 6-week interval at −22°C. Values of the stability in the autosampler are listed in Table S5. All analytes (except EtS and HMMA) passed the criteria for the stability in the autosampler. Relative mean concentrations and CVs of the freeze and thaw stability are listed in Table S6. HMMA, METH, and EtS did not meet the AC set by the ICH; METH only fulfilled the ICH criteria for the first two freeze and thaw cycles and failed the third cycle in QC low samples.[14] Values for the long-term stability are given in Table S7. HMMA did not meet the AC set by the ICH guidelines.[14] Furthermore, MDMA failed the requirements for long-term stability as well as EtS and HMMA.

3.2.5 | Accuracy and precision

A linear calibration model was used for all analytes. Within- and between-run accuracy and precision results are shown in Tables 3 and 4. All analytes, except HMMA, showed relative mean concentrations within ±15% of the nominal concentration for QC low, QC mid, QC high, and ±20% for QC LLOQ, as well as CVs < 15% after C_{18} separation. AMPH, EtS, and HMMA did not meet the AC after HILIC separation most likely due to poor peak shape at low concentrations and lack of a labeled standard of HMMA. The poor peak shape can lead to issues with reproducible determination of peak areas. The remaining analytes fulfilled the AC set by the ICH.[14]

HMMA did not pass any of the validation experiments, with either column. A possible explanation might be that no corresponding isotope labeled IS was available for this analyte. Although MDMA-d_5 is the deuterated analog of HMMAs parent compound, it could not compensate for variations during extractions or analysis. Furthermore, EtS-d_5 could not be detected in any of the validation experiments, even though it could be detected in the spike solution used for the whole validation process. This was likely due to a poor extraction performance, as EtS-d_5 could be detected in stock stability samples.

TABLE 3 Within- and between-day accuracy (A) and precision (P), of quality control (QC) lower limit of quantification (LLOQ), QC low, QC mid, and QC high ($n = 5$), analyzed via the C_{18} column. EtS was not included in the C_{18} method.

	Relative mean concentration (A), % (CV (P), %							
	Within-run				Between-run			
Analyte	QC LLOQ	QC low	QC mid	QC high	QC LLOQ	QC low	QC mid	QC high
AMPH	112 (9)	100 (11)	101 (9)	106 (7)	102 (12)	100 (4)	101 (3)	108 (4)
BZE	111 (2)	109 (2)	103 (3)	109 (2)	104 (1)	102 (4)	99 (5)	109 (1)
CE	91 (6)	104 (6)	95 (9)	108 (8)	95 (9)	99 (6)	96 (1)	108 (2)
COC	93 (10)	101 (3)	101 (5)	107 (2)	92 (9)	100 (3)	100 (3)	107 (6)
EtS	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
HMMA	51 (4)	73 (11)	83 (18)	156 (22)	75 (31)	105 (13)	123 (15)	152 (4)
MDMA	93 (2)	103 (3)	108 (4)	111 (2)	92 (9)	98 (10)	102 (10)	112 (6)
METH	88 (3)	96 (7)	111 (4)	97 (5)	96 (9)	97 (11)	103 (8)	102 (3)
MPH	118 (3)	100 (7)	107 (4)	112 (2)	97 (5)	97 (2)	102 (5)	109 (7)
RA	105 (3)	105 (6)	93 (10)	108 (2)	101 (8)	95 (13)	95 (4)	110 (5)

Abbreviations: AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; CV, coefficient of variation; EtS, ethyl sulfate; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; mid, medium; MPH, methylphenidate; n. a., not available; QC, quality control; RA, ritalinic acid.

TABLE 4 Within- and between-day accuracy (A) and precision (P), of quality control (QC) lower limit of quantification (LLOQ), QC low, QC mid, and QC high ($n = 5$), analyzed via the HILIC column.

	Relative mean concentration (A), % (CV (P), %							
	Within-run				Between-run			
Analyte	QC LLOQ	QC low	QC mid	QC high	QC LLOQ	QC low	QC mid	QC high
AMPH	n.d.	90 (4)	100 (8)	106 (10)	n.d.	96 (36)	100 (19)	106 (12)
BZE	108 (2)	107 (2)	102 (5)	107 (5)	106 (2)	101 (5)	100 (5)	111 (4)
CE	98 (1)	106 (2)	99 (6)	110 (1)	95 (3)	100 (4)	98 (5)	112 (2)
COC	102 (9)	106 (2)	100 (6)	110 (3)	97 (4)	100 (5)	98 (5)	111 (2)
EtS	IS n. d.	IS n. d.	IS n. d.	IS n. d.	IS n. d.	IS n. d.	IS n. d.	IS n. d.
HMMA	84 (10)	93 (10)	112 (26)	100 (11)	166 (38)	116 (133)	148 (129)	137 (133)
MDMA	83 (2)	102 (3)	109 (3)	109 (3)	90 (14)	96 (9)	102 (10)	111 (5)
METH	99 (5)	101 (7)	103 (7)	111 (1)	105 (3)	96 (4)	102 (6)	112 (6)
MPH	94 (1)	106 (2)	99 (8)	109 (1)	93 (7)	100 (4)	99 (6)	108 (6)
RA	104 (4)	103 (3)	96 (10)	111 (3)	102 (5)	94 (9)	96 (4)	113 (4)

Abbreviations: AMPH, amphetamine; BZE: benzoylecgonine; CE, cocaethylene; COC, cocaine; CV, coefficient of variation; EtS, ethyl sulfate; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; mid, medium; MPH, methylphenidate; n.d., not detected; RA, ritalinic acid; QC, quality control.

3.3 | Application of the method to analyze WW samples for proof-of-concept

Tables 5 and 6 show the results of the analysis of six WW grab samples obtained from two different sites (one and two). Analysis of the samples allowed the identification and quantification of the DOA and the cognitive enhancers included in the presented method. AMPH, METH, MDMA, COC, and its metabolite BZE were mainly detected in the samples originating from site two. Detecting only little concentrations of METH compared with AMPH is in accordance with previously published data by Ort et al.[7] describing the differences in AMPH and METH distribution in Europe. In samples from both sites, MPH and its metabolite RA could be identified, which could originate from patients with ADHD, as MPH is the most commonly prescribed medication for ADHD in Germany.[19] However, MPH also is misused as a cognitive enhancer.[20]

TABLE 5 Concentrations of drugs of abuse and cognitive enhancers in wastewater grab samples, analyzed using the C_{18} column.

		Analyte concentration, ng/L									
Sampling month	Sampling site	AMPH	BZE	CE	COC	EtS	HMMA	MDMA	METH	MPH	RA
JUN 21	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	13	18
DEC 21	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	23
	two	88	<30	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	14
APR 22	one	n. d.	<30	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	<10	14
	two	>100	<30	n. d.	22	n. d.	n. d.	>100	19	<10	>100
AUG 22	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	<10	n. d.	n. d.	n. d.
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
NOV 22	one	n. d.	120	n. d.	17	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	two	>100	56	n. d.	<10	n. d.	n. d.	n. d.	n. d.	n. d.	>100
MAR 23	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	44	n. d.	15	>100
	two	56	n. d.	n. d.	14	n. d.	n. d.	<10	n. d.	n. d.	n. d.

Abbreviations: AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; EtS, ethyl sulfate; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; n. d., not detected; one, sampling site one; RA, ritalinic acid; two, sampling site two.

TABLE 6 Concentrations of DOA and cognitive enhancers in wastewater grab samples, analyzed using the hydrophilic interaction liquid chromatography column.

		Analyte concentration, ng/L									
Sampling month	Sampling site	AMPH	BZE	CE	COC	EtS	HMMA	MDMA	METH	MPH	RA
JUN 21	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	18
DEC 21	one	n. d.	<30	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	<10	24
	two	>100	40	n. d.	17	n. d.	n. d.	n. d.	n. d.	n. d.	17
APR 22	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	<10	14
	two	>100	<30	n. d.	22	n. d.	n. d.	>100	n. d.	n. d.	>100
AUG 22	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	<10	n. d.	n. d.	23
	two	27	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	11
NOV 22	one	n. d.	121	n. d.	17	n. d.	n. d.	n. d.	n. d.	n. d.	36
	two	n. d.	57	n. d.	<10	n. d.	n. d.	n. d.	n. d.	n. d.	>100
MAR 23	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	44	n. d.	15	>100
	two	56	n. d.	n. d.	12	n. d.	n. d.	<10	n. d.	n. d.	28

Abbreviations: AMPH, amphetamine; AW, sampling site one; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; DOA, drugs of abuse; EtS, ethyl sulfate; HKW, sampling site two; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; n. d., not detected; one, sampling site one; RA, ritalinic acid; two, sampling site two.

3.4 | LC-HRMS/MS analysis and impact of the different LC columns

For both columns, sufficient chromatographic separation should be achieved within short runtimes. Using the C_{18} column, an analytical run was performed within 10 min, including a washout phase. Furthermore, only three pairs of coeluting analytes were present for the C_{18} compared with the HILIC. Separation of analytes was possible within the first 5 min of a run. For the HILIC column, separation of analytes was performed within 15 min, also including a washout phase. However, baseline separation of analytes could not be achieved as there were five clusters of coeluting analytes. Figures 1 and 2 show the chromatographic separation of all analytes and IS included in the method validation at the LLOQ.

Different MS settings were tested during method development, whereas the PRM mode with adjusted collision energies and time windows was able to provide highest sensitivity with the lowest LOIs (see Table S2).

Although most analytes fulfilled all validation criteria across both columns, differences were observed. In terms of analysis time, the C_{18}

column allowed shorter runtimes compared with the HILIC. Furthermore, three analytes did not fulfill all validation criteria using the C_{18} column (METH, MDMA, and HMMA), whereas five analytes did not fulfill at least one criterium after HILIC separation (AMPH, BZE, HMMA, MDMA, and EtS). Regarding the peak shape, the HILIC column showed better peak shapes compared with the C_{18} column. Because HILIC columns are better suited for analysis of polar compounds, it might be expected that the performance separation of metabolites would be better compared with the C_{18} column. When considering the accuracy and precision results, relative mean concentrations after analysis across both columns showed no major differences. Only for AMPH, the results of the QC LLOQ after HILIC separation deviated from the C_{18} column. Comparing the concentrations determined in the proof-of concept samples (see Tables 5 and 6), differences between the values obtained with both columns for the same samples were observed. Concentrations for AMPH and RA were in general higher when quantified after HILIC separation compared with C_{18} separation. MPH and COC concentrations were slightly higher when analyzed after C_{18} separation.

4 | CONCLUSIONS

Two methods for qualitative and quantitative analysis of four DOA, one cognitive enhancer, and three of their biomarkers in WW was successfully developed and validated using RP-LC and HILIC. One common simple sample preparation via SPE was used, and analysis via LC-HRMS/MS was possible within short run times of 10 (C_{18} column) or 15 min (HILIC column). Validation results showed advantages of the C_{18} over the HILIC column, as only METH and HMMA did not fulfill all validation parameters via the C_{18}, whereas AMPH, BZE, EtS, and HMMA did not fulfill the criteria set by the ICH using the HILIC. Thus, only HMMA could not by reliably quantified. The used SPE did not allow the extraction of EtS, but its analysis should be possible by dilute and shoot as shown by Mastroianni et al.[21] or Rodríguez-Álvarez et al.[22] A two-column setup is necessary for the analysis of all analytes included in this method, because on neither column, all analytes met the criteria set by the ICH (see Table S8). Evaluation of proof-of-concept samples allowed the quantification of several analytes in the WW grab samples. The presented method is thus ready to be used for monitoring trends in the consumption of DOA and cognitive enhancer in WW samples.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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