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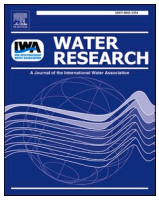

Dilute-and-shoot approach for the high-throughput LC-MS/MS determination of illicit drugs in the field of wastewater-based epidemiology

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ABSTRACT

The determination of illicit drugs in urban influent wastewater (IWW) enables the monitoring of spatial and temporal drug usage trends and assessment of community lifestyle habits. The increasing number of wastewater surveillance studies has emphasized the necessity for the development of rapid, high-throughput methods that maintain high quality data. This work evaluates the use of a dilute-and-shoot methodology, based on direct injection (DI) of centrifuged samples, as an alternative approach to the widely applied sample pre-treatment based on solid-phase extraction, for the liquid chromatography-tandem mass spectrometry determination of seven widely consumed illicit drugs and their metabolites in IWW (amphetamine; cocaine metabolite, benzoylecgonine; ketamine; 3,4-methylenedioxymethamphetamine (MDMA); methamphetamine; cannabis metabolite, 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC—COOH); heroin metabolite, 6-acetylmorphine (6-MAM)). Comparison of both approaches in terms of matrix effects, sensitivity and accuracy, demonstrates the DI method suitability to correctly quantify these analytes in IWW, with a limit of quantification lower than 30 ng L^{-1} for most compounds. After validation of the method and participation in an interlaboratory exercise, the DI method was applied to the analysis of 54 IWW samples collected from different Spanish wastewater treatment plants. Additionally, quality controls were incorporated in each analysis batch to support the DI method applicability and robustness. The use of a 10 μL-DI reduces time-consuming sample preparation, analysis time and measurement uncertainty. Moreover, it supports green chemistry by reducing the consumption of organic solvents and it facilitates logistics by collecting, transporting, and storing less sample volume. The methodology is therefore especially appropriate for monitoring illicit drugs in large wastewater-based epidemiology sampling campaigns or when fast near real-time results are needed.

1. Introduction

Illicit drugs have been monitored in urban influent wastewater (IWW) in the last decade to assess and estimate spatial and temporal consumption trends (González-Mariño et al., 2020; Ort et al., 2014). This approach, which has been termed wastewater-based epidemiology (WBE), is a non-intrusive, anonymous, quick and near real-time tool to estimate drug consumption within communities, providing valuable insights for public health authorities (Castiglioni et al., 2016). The Sewage Analysis CORe Group Europe (SCORE) (2023) annually spearheads a global one-week monitoring program for illicit drugs, promoting collaboration among researchers worldwide and driving forward the field of WBE. Moreover, SCORE has established a comprehensive framework of guidelines, encompassing optimal practices in sampling, sample handling, chemical analysis, back-calculation and data reporting (Castiglioni et al., 2016). To guarantee the precision and dependability of WBE data, the group conducts annual interlaboratory exercises.

Numerous methods can be found in the scientific literature for quantifying illicit drugs (commonly present at the ng L^{-1} level) in complex IWW matrices. Nearly all these methods are based on off-line solid phase extraction (SPE) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Bijlsma et al., 2009; Bones et al., 2007; Castiglioni et al., 2006; Hernández et al., 2018; Christophoridis et al., 2021). HLB (*Hydrophilic and Lipophilic Balanced*) and MCX (*Mixed-mode Cation-eXchange*) are the sorbents most commonly employed. The MCX polymeric sorbent built upon HLB copolymer allows an improved selectivity towards basic analytes (i.e., most illicit drug biomarkers), and may also reduce matrix interferences. However, the inclusion of cannabis in multiresidue methods is of high interest as cannabis remains the most widely used drug worldwide and

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an increased trend towards its legalization for both medicinal and recreational purposes is observed (Bijlsma et al., 2024). Hence, to include non-basic drug biomarkers, such as 11-nor-9-Carboxy-delta-9-tetrahydrocannabinol (THC—COOH) to monitor cannabis, a compromise is often needed where HLB offers a more generic extraction. Moreover, MCX requires an acidification step, which may hamper the stability of biomarkers like THC—COOH (Causanilles et al., 2017). The SPE allows to preconcentrate the analytes, reaching low limits of quantification (LOQ) (Ren et al., 2022; Restrepo-Vieira et al., 2022), and it is also expected to eliminate potential matrix interferences that may affect the analytical method (Prosen et al., 2017). However, SPE is time consuming and requires relatively large sample volumes. High-throughput methods based on fully automated on-line SPE, integrated with the LC-MS/MS system (Heuett et al., 2015; Postigo et al., 2008), as well as on μ-SPE, utilizing 96-well plates (Baz-Lomba et al., 2018; Boogaerts et al., 2023), are good alternative sample preparation approaches to off-line SPE that also reduce sample consumption. Yet a drawback to all SPE is that matrix components coeluting with analytes are also pre-concentrated in the process. Hence, SPE does not ensure the reduction of matrix effects (ME) typically affecting analytes quantification in LC-MS/MS analysis (Simarro-Gimeno et al., 2023), Moreover, SPE may result in analyte losses and contribute to elevate analytical errors linked to the manipulation of samples (Busetti et al., 2012; Poole et al., 2000).

Advances in modern MS/MS instruments have led to notable improvement in sensitivity and have reduced the necessity for sample pre-concentration. One of the current simplest approaches for the determination of organic micropollutants (OMPs) in water is the direct injection (DI) of the samples, even after previous dilution of complex-matrix samples, such as wastewater. This avoids sample handling involved in SPE (e.g. sample loading, drying, eluting, evaporation of eluates), reduces analysis time, costs (e.g., no need for SPE cartridges and less solvent consumption) and ensures high sample throughput and green analytical chemistry. Moreover, it facilitates logistics by collecting, transporting, and storing less sample volume. This is especially beneficial when large sampling campaigns, such as those organized and promoted by SCORE and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA, 2023), are performed. DI offers high reproducibility and minimal sample manipulation (typically filtration and/or centrifugation, and dilution) (Boix et al., 2015). Hence, DI can present a viable alternative to SPE, if the required limits of quantification are achieved (Hernandez et al., 2023).

Recent works have demonstrated the potential of DI for analysis OMPs, such as pharmaceuticals or pesticides, in surface water (Nieto-Juárez et al., 2021) and in more complex matrices, such as IWW and effluent wastewater (EWW) (Botero-Coy et al., 2018; Campos-Mañas et al., 2017; Fabregat-Safont et al., 2023; Simarro-Gimeno et al., 2023; Bade et al., 2023). For the analysis of illicit drugs, some papers using large volume injection (LVI) have also been published (Berset et al., 2010; Chiaia et al., 2008). Recently, Ren et al., 2022 and Restrepo-Vieira et al., 2022, suggested the injection of 30 μL of a previously centrifuged and filtered sample, without any previous dilution, resulting in good method sensitivity although increasing the risk of contamination of the LC-MS/MS instrumentation.

In this work, rapid DI-LC-MS/MS analytical methodology has been developed, establishing a simple procedure for the analysis of seven illicit drugs and metabolites in IWW. This methodology has been validated and compared with a SPE procedure widely applied for illicit drugs determination in wastewater. Special attention has been paid to ME and sensitivity; two relevant parameters closely related to the sample treatment applied. To evaluate the applicability of the developed approach, results of real-world samples and numerous quality control samples analyzed by both methodologies are reported and compared. The use of DI combined with LC-MS/MS analysis using modern/sensitive instruments can be seen as a useful practical alternative approach in WBE, strengthening its utility in the surveillance of illicit drug use at the community level.

2. Experimental

2.1. Chemicals and reagents

Seven biomarkers of illicit drugs have been studied, including parent compounds and human metabolites: amphetamine (AMPH); cocaine metabolite, benzoylecgonine (BE); ketamine (KET); 3,4-methylenedioxymethamphetamine (MDMA); methamphetamine (METH); the main urinary metabolite of cannabis, THC—COOH; and the unique metabolite of heroin, 6-acetylmorphine (6-MAM). Isotopically labelled internal standards (ILIS) used for matrix effect correction and appropriate quantification were: AMPH-d_6, BE-d_3, KET-d_4, MDMA-d_5, METH-d_5, THC—COOH-d_3 and 6-MAM-d_6 (Castiglioni et al., 2016; Hernández et al., 2018). All analytical reference standards were purchased from Merck (Darmstadt, Germany).

Individual 100 mg L^{-1} stock solutions were prepared in methanol (MeOH) and stored in amber glass bottles at −20 ºC. Multi-compound working solutions were prepared at 5 mg L^{-1} by appropriate dilution of the individual stock solutions in MeOH. The mix work solutions containing all analytes were prepared at 500 and 50 μg L^{-1}. The ILIS mix work solution was prepared at 40 μg L^{-1}, except for AMPH-d_6 and THC—COOH-d_3 which were at 400 μg L^{-1} to ensure their measurement.

LC-MS grade MeOH, and formic acid as well as ammonium acetate (>98 %) were supplied by Scharlab (Scharlab, Barcelona, Spain). HPLC-grade water was obtained by purifying demineralized water (ultrapure water) using a Ultramatic Plus GR from Wasserlab (Navarra, Spain). Oasis HLB SPE cartridges (60 mg, 3 cc) were purchased from Waters (Milford, MA, USA).

2.2. Sample collection

IWW samples (24-hour time-proportional every 10 min) were collected from nine wastewater treatment plants (WWTP) located in Spain during 2022 and 2023. All samples were collected, transported to the laboratory, and stored in high-density polyethylene (HDPE) bottles in the dark at −20 °C until analysis. More details on the samples, such as origin (i.e., industrial/rural), sampling dates and population (based on census data of the Spanish National Statistics Institute (INE) (2023)) are included in **Table S1**. Two different types of samples were analyzed: daily samples, corresponding to a single day, and composite samples, corresponding to a pool of several days (i.e., a week).

2.3. Sample treatment

2.3.1. Solid phase extraction (SPE)

The extraction methodology was adapted from a previous study (Bijlsma et al., 2014). Briefly, 25 mL IWW samples were processed by SPE using Oasis HLB cartridges. Prior to loading, samples were centrifuged, 4-fold diluted with ultrapure water and spiked with the ILIS mix solution. Then, 100 mL of the 4-fold diluted samples were percolated by gravity, the cartridges were vacuum dried, and the analytes were eluted with MeOH. The eluates were evaporated to dryness at 40 °C under a gentle stream of nitrogen and reconstituted in 1 mL water:MeOH (90:10, v/v), achieving a preconcentration factor of 25. Finally, 3 μL of the final extract was injected into the UHPLC-MS/MS.

2.3.2. Direct injection (DI)

IWW samples were centrifuged, and two-fold diluted previously to their UHPLC-MS/MS determination. The procedure was as follows: samples were first centrifuged, and 500 μL were taken; subsequently, 50 μL of ILIS mix solution (40 μg L^{-1}) were added, followed by 400 μL of ultrapure water and 50 μL of MeOH, reaching a total volume of 1 mL. Finally, 10 μL of the diluted sample was injected into the UHPLC-MS/MS system.

Figure S1 shows a scheme of the SPE and DI procedures applied.

2.4. Instrumentation

Sample analysis was performed using a Waters Acquity H—Class UPLC system (Waters Corporation, MA, USA) coupled to a triple quadrupole mass spectrometer (Xevo TQS, Waters, Manchester, UK) equipped with an electrospray ionization source (ESI) operated in positive ionization mode. Chromatographic separation was carried out using an Acquity UPLC BEH C_{18} column (50×2.1 mm, 1.7 μm) from Waters, at a flow rate of 0.3 mL/min. Mobile phase consisted on a gradient of A: ultrapure water with 5 mM ammonium acetate and 0.01 % formic acid and B: MeOH (no modifiers added) as follows: 0 min, 10 % B; 3 min, 90 % B; 3.5 min, 90 % B; 3.6 min, 10 % B until 6 min, for re-equilibration of the column. Column temperature was kept at 40 °C and sample manager was kept at 10 °C. All data were acquired and processed using MassLynx v4.1 software (Waters, Manchester, UK). More detailed information on instrument operating conditions and on SPE method validation can be found elsewhere (Bijlsma et al., 2014). Selected transitions, retention times (Rt), cone voltages (CV) and collision energies (CE) are displayed in **Table S2**.

2.5. Matrix effect evaluation

IWW samples of distinct origins (urban, rural, and industrial) were collected from 5 WWTPs to evaluate both SPE and DI methodologies in terms of ME, and the possible variations among samples. Additionally, in two of these WWTPs, samples were collected at different time intervals to investigate variations in ME within a single WWTP. A total of 7 different IWW samples were therefore studied. A common difficulty in this field is the lack of representative genuine blank samples, as nearly all samples contain some of the illicit drugs investigated, especially BE and THC—COOH. Therefore, ILIS compounds were used instead for ME assessment. These compounds, absent in IWW samples, are theoretically affected by ME in the same way as the natural analytes. Hence, the signal of ILIS in wastewater (2 μg L^{-1} concentration in the final sample extract) was compared with ILIS standards of the same concentration prepared in solvent (see **Figure S2**). ME was calculated using Eq. (1). ME are considered significant if they exceed ±20 % (SANTE, 2021).

$$ME\ (\%) = \frac{signal\ ILIS\ in\ sample - signal\ ILIS\ in\ solvent}{signal\ ILIS\ in\ solvent}\ x\ 100 \qquad (1)$$

2.6. DI method validation

The performance of the DI method was evaluated in terms of linearity, selectivity, accuracy, precision, limit of detection (LOD) and LOQ, following the proposal for validation of chromatographic methods for OMPs determination in water samples (Hernández et al., 2023). To this aim, five IWW samples of different origin were used to cope with the high variability of wastewater samples in terms of matrix composition and analyte concentration and illustrate the robustness of the methodology.

Linearity was evaluated by analyzing standard solutions in ultrapure water:MeOH (90:10) at nine concentrations from 0.01 to 10 μg L^{-1} in triplicate. Satisfactory linearity was assumed when regression coefficient (R^2) was >0.99 with residuals lower than 20 %.

Accuracy and *precision* were evaluated by the analysis of each IWW sample ($n = 5$) at four concentration levels: 0.1, 0.5, 2.5 and 10 μg L^{-1}. Mean recoveries in the range 70 – 120 % were considered satisfactory. The precision, expressed as RSD, reflected the reproducibility of the method for analysis of five different samples. It was considered as satisfactory when it was ≤30 %.

The LOD was estimated as follows: a) when the analyte was not detected in the "blank" samples (i.e., AMPH, KET, MDMA, METH and 6-MAM) the LOD was calculated from the IWW samples spiked at 100 ng L^{-1} (500 ng L^{-1} for AMPH) as the concentration corresponding to a signal-to-noise ratio (S/N) = 3, using the quantification transition (Q); b) when the compound was present in the "blank" samples and it could be quantified (BE and THC—COOH), the LOD was calculated from the "blank" sample as the concentration corresponding to a $S/N = 3$.

The LOQ was calculated similarly to LOD but applying a $S/N = 10$. In addition, at least one of the confirmation transitions needed to show an S/N ratio of 3.

2.7. Quality control (QC) analysis

In this work, special attention was paid to the quality of the analysis to ensure the reliability, accuracy and consistency of the results reported. This is especially important in long-term studies or when analyzing samples with different characteristics (e.g., IWW from different locations). QC samples at different concentration levels were included in each batch of samples: 100, 400 and 800 ng/L for SPE; and 100, 400, 2500 and 10,000 ng/L for DI. Individual QCs recoveries ranging from 60 to 140 % were considered satisfactory (Hernández et al., 2023).

3. Results and discussion

3.1. Dilution factor optimization

IWW samples are prone to strong ME; therefore, a dilution of these complex-matrix samples appears as a simple way to minimize it. In this study, a IWW sample was fortified with the seven compounds under study at two concentration levels, 0.5 and 2.5 μg L^{-1} (by triplicate), and analyzed without dilution, and two- and five-fold diluted. When analyte-ILIS correction was used, no significant differences were observed in recoveries between non-diluted and diluted samples (**Table S3**). Recovery could not be calculated for BE at 0.5 μg L^{-1} due to the high concentration present in the "blank" sample. A two-fold dilution was selected to reach a compromise between ME reduction (evaluated in the next section) and the sensitivity of the methodology. Moreover, it must be considered that diluting the samples implies introducing less matrix than injecting raw IWW samples, which is always beneficial for the overall instrument performance over-time (Celma et al., 2019).

3.2. Matrix effect evaluation

The results obtained from the SPE and DI methods (2-fold dilution) are shown in **Tables S4** and **S5** respectively, and in Fig. 1.

SPE revealed consistent ME for most of the compounds in all the samples (**Table S4**). AMPH and METH showed an ion enhancement of around 50 %, while BE, KET, and THC—COOH had substantial ion suppression effects. In contrast, no robust behavior was observed for MDMA and 6-MAM which presented high ME variability between the samples. These disparities are presumed to stem from variations in the sample matrix, attributed to the intricate and variable nature of the composition of IWW. This complexity hinders the assessment of certain parameters, such as ME, and impedes the establishment of a definitive and robust value for them (Asimakopoulos et al., 2017). Our data illustrate that SPE methodology is strongly affected by ME and that the composition IWW can notably vary between different locations, and also reflects inter-day variations within the same WWTP.

Table S5 shows the ME for the same samples analyzed by DI. As it can be seen, the results are considerably different from those obtained using the SPE method. In general, DI presented ME lower than SPE. This was the case of AMPH (16 %), BE (−10 %), KET (6 %) and THC—COOH (−20 %), with in general negligible matrix effects (ME < ±20 %). On the contrary, MDMA (+44 %) and 6-MAM (−50 %) were affected by higher ME in DI compared to SPE (+9 and +12 %, respectively). METH showed similar ME (around 50 %) by both methods.

Fig. 1 illustrates the disparity in the ME between the two methods. DI

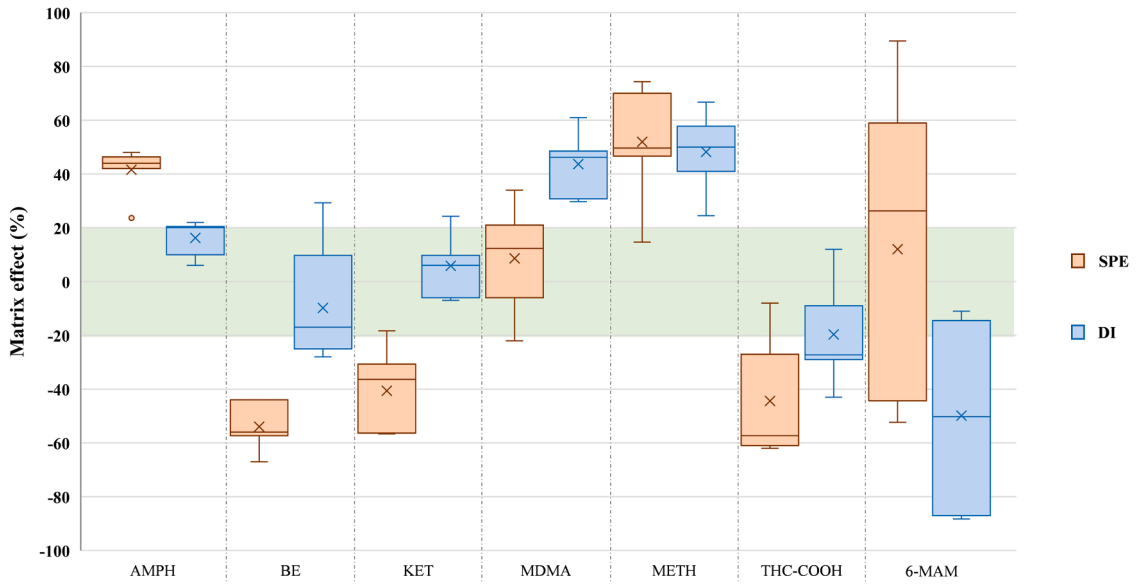

Fig. 1. Matrix effect for SPE and DI procedures in IWW, evaluated using ILIS compounds. Mean value represented with 'X' and median with the dash.

presented lower ME for all compounds, except for MDMA. Besides, narrower data spread (smaller boxes) was in general obtained for DI too. The only exception was BE, although its ME average and median remained within −20 % to 20 %, which is considered as negligible ME. Large boxes were observed for 6-MAM, indicating the wide variation in ME, complicating the evaluation and comparison of ME by both methods.

Although lower matrix impact would be expected after SPE (SPE also serves as a clean-up), our data suggest that this step was not beneficial from the point of view of matrix effects in comparison with the direct injection of 2-fold diluted samples (lower ME and narrower data spread observed in DI). Thus, although cartridges concentrate the analytes, it seems that coeluting matrix interferences are concentrated too (Bisceglia et al., 2010).

3.3. SPE recovery

Despite the SPE procedure applied in this work has been widely applied for monitoring illicit drugs in wastewater, we performed an evaluation of the SPE cartridge recovery (**Figure S2**). In these experiments were also performed using ILIS instead of the natural analytes to overcome the problem associated to the presence of these drugs in the "blank" wastewater samples used in the experiments. The results (**Table S6**) showed satisfactory recoveries for all compounds, except for AMPH (average recovery 39 %) and METH (55 %), possibly due to the absence of an additional clean up step as included by Bijlsma et al. (2014). Obviously, an analytical SPE procedure using ILIS as surrogates would allow to correct not only for ME but also for SPE losses. Therefore, the addition of ILIS to the samples before being subjected to SPE is highly recommended.

3.4. Direct injection method validation

Table 1 summarizes the results obtained in the validation of the DI methodology. Satisfactory linearity was observed from 0.01 to 10 μg L^{-1} for most of the compounds. The lower sensitivity for AMPH and THC—COOH forced to increase the lowest calibration point up to 0.02 (AMPH) or to 0.05 μg L^{-1} (THC—COOH). Regarding BE, the high sensitivity of the transition initially selected for quantification (290 > 168) limited linearity to 0.01–2.5 μg L^{-1} i.e., at concentrations higher than 2.5 μg L^{-1} the detector got saturated. This issue was solved using a less sensitive q1 transition (290 > 82) for quantification. The corresponding ILIS transition (293 > 85) was also selected to ensure correct quantification.

All compounds showed excellent recovery (as no sample treatment is applied, the term accuracy can be better used in the present study), with average values in the range of 80–114 % and RSD ≤ 22 % at the four concentration levels tested. Validation at the lowest level (0.1 μg L^{-1})

Table 1
Direct injection method validation. Mean accuracy (5 different IWW samples, $n = 1$ each) and RSD, in brackets (both in%).

Compound	Linear Range (μg·L^{-1})	"Blank" sample conc. range (ng·L^{-1})	Accuracy,% (RSD,%)				LOD (ng·L^{-1})	LOQ (ng·L^{-1})	**SPE LOQ (ng·L^{-1})
			0.1 (μg·L^{-1})	0.5 (μg·L^{-1})	2.5 (μg·L^{-1})	10 (μg·L^{-1})			
AMPH	0.02–10	0	nd	107 (11)	98 (6)	91 (6)	30	110	100
BE[a]	0.01–2.5	1711 - 4502	*	114 (14)	102 (5)	96 (1)	3	10	2
KET	0.01–10	0 - 52	83 (13)	95 (3)	101 (3)	97 (1)	6	20	20
MDMA	0.01–10	50 – 89	90 (4)	92 (2)	96 (2)	95 (1)	6	20	30
METH	0.01–10	0	80 (6)	92 (3)	98 (4)	96 (4)	6	20	82
THC—COOH	0.05–10	d – 265	93 (22)	91 (6)	95 (3)	91 (5)	30	100	60
6-MAM	0.01–10	0	86 (11)	92 (6)	97 (3)	93 (3)	10	30	37

[a] For the highest calibration level of BE, the signal of the Q transition was saturated. It was possible to increase the linearity up to 10 μg L^{-1} using q-transition responses (correcting with the corresponding ILIS transition).

nd: not detected.

* Recovery could not be calculated, due to the high concentration observed in "blank" sample.

** (Bijlsma et al., 2014).

was not possible for two compounds: BE, due to the high analyte concentrations present in the "blank" samples, and AMPH, due to the low sensitivity for this compound.

LOQs were estimated between 10 and 30 ng L^{-1} for all compounds, except for THC—COOH (100 ng L^{-1}) and AMPH (110 ng L^{-1}). LODs ranged from 3 to 30 ng L^{-1}.

3.5. Testing the methods with fortified ultrapure water and interlaboratory samples

The accuracy of both procedures was also assessed by testing ultrapure water fortified at four different concentration levels 0.1, 0.4, 0.8 and 2.5 μg L^{-1}. In the case of SPE, ILIS were added as surrogates (i.e. added to the samples before SPE). Although ultrapure water does not represent the complexity of wastewater, it was chosen as preliminary experiment to check the accuracy for some analytes at the lowest levels due to the difficulties in finding genuine "blank" IWW samples free of illicit drugs. Table 2 shows the differences (calculated as percentage) between measured and theoretical concentrations for each compound. No major differences between DI and SPE were observed.

The participation of our research group in the SCORE network implies that we must take part in the annual interlaboratory exercises organized to ensure accurate quantification and reporting of the data (van Nuijs et al., 2018). This interlaboratory test involves assessing three tap water samples fortified with varying levels of illicit drugs. For each sample, a minimum of three individual values must be reported. The reported mean, along with the mean of means of the other participating groups, is used to calculate the Z-score. Participants pass the interlaboratory test successfully when their Z-score is within the −2 to 2 range. Taking the opportunity during the yearly method review, we also conducted DI analysis on these interlaboratory samples.

Table 3 shows the results obtained, including the concentrations obtained for both the DI and SPE methods, the group means, standard deviations, nominal spikes, and corresponding Z-score for each method. The SPE method successfully passed the interlaboratory exercise, for all compounds yielding Z-scores within of −2 to 2. Similarly, the DI method demonstrated good performance, with the only exception of BE in the sample M2, which showed a value slightly above −2. It is not easy to understand the reason of this apparent discrepancy, as sample M2 had the highest spiked concentration (350 ng L^{-1}), and the QCs analyzed during the interlaboratory study presented excellent recoveries for BE (around 100 %). It must be noticed that the interlaboratory nominal spike for BE does not represent the actual concentration found in most IWW samples, which normally is at the ppb levels (i.e. above 1000 ng L^{-1}).The interlaboratory exercise provided a valuable means of re-evaluation of the efficacy and accuracy of the DI method, which may be used as an alternative approach to SPE for the seven drug biomarkers studied in this work.

Table 2
Difference (calculated as percentage) between expected and measured concentrations for SPE and DI methods in ultrapure water spiked at four concentration levels.

Compounds	Difference (%) between expected and measured concentrations							
	0.1 μg·L^{-1}		0.4 μg·L^{-1}		0.8 μg·L^{-1}		2.5 μg·L^{-1}	
	SPE	DI	SPE	DI	SPE	DI	SPE	DI
AMPH	−6	−22	3	9	2	−3	−1	11
BE	4	7	8	−1	7	5	3	7
KET	−10	25	−4	7	−1	4	−4	6
MDMA	−18	−16	−7	1	−9	4	2	4
METH	5	−16	2	−8	5	8	9	7
THC-COOH	0	−9	8	1	22	−10	20	−8
6-MAM	−6	−6	−5	−4	−1	−7	12	1

3.6. SPE and DI quality controls

Results from QCs analyzed together with wastewater samples along this study (both for SPE and DI) are shown in Table 4. The average recoveries were highly satisfactory for DI, with most values between 80 and 110 %, and RSDs (reproducibility) were mostly lower than 20 %. When using SPE, the recoveries were also satisfactory, except for AMPH and METH, which were around 50 %. It is noteworthy that the original SPE methodology with HLB cartridges (Bijlsma et al., 2014) employed an additional clean-up with Oasis MCX SPE and dispersive primary secondary amine to incorporate AMPH and METH in the analysis of IWW. The absence of clean-up in the generic SPE procedure applied in the present work led to dirtier sample extracts, where the ILIS correction might be less efficient. AMPH and METH ILIS contained 6 and 5 deuterium atoms, respectively, a fact that might affect their ionization efficiency differently than their corresponding parent compound, especially in the more concentrated SPE extracts.

DI recoveries could not be obtained for BE at the lowest spiked levels, 0.1 and 0.4 μg/L, due to the high concentration of this compound in the "blank" samples used to prepare QCs. In contrast, the highest QCs (above 1 μg L^{-1}), presented average recovery of 90 % and very low variability (RSD < 15 %), ensuring a reliable quantification. The same situation occurred in the SPE procedure, where only the highest level (0.8 μg/L) could be evaluated for BE. It is worth highlighting the higher RSDs of SPE compared to DI, surely because of the sample treatment applied, which introduced higher variability in the results. For a correct interpretation of data, it must be considered that these results correspond to the average of individual QCs recoveries from different campaigns analyzed over a year, so greater variations are expected (i.e. reproducibility versus repeatability).

3.7. Application to the analysis of IWW samples

In this work, 54 samples were analyzed using both methods, following the sample treatment outlined in Figure S1. The fact that different samples from several WWTPs were analyzed implies high variability in sample composition and in the results obtained, but also offers a realistic picture of the subject treated. Table 5 presents the results obtained and the percentage differences. The DI method was unable to quantify KET or MDMA in several samples, due to their low concentration levels, which raise questions about the significance of reporting such minute values versus the practicality of real-time results. Furthermore, some major differences for BE may be due to the signal saturation observed in the SPE method for this compound for both the quantification and the first confirmation (q1) transitions in many samples. In those cases where q1 was saturated, quantification was performed with the second confirmation (q2) transition, but using the ILIS signal corresponding to q1 transition, as the equivalent to q2 was not initially included in the MS method (only the quantification and first confirmation transitions were acquired for the ILIS). This surely affected the quantification, as ME correction might not have been appropriate, and introduces some doubts about the concentration reported by the SPE procedure in samples with high BE concentrations. This fact, together with the excellent QC recoveries obtained in the DI method, reinforce the applicability of this procedure in wastewater samples.

Among all compounds found in the wastewater samples, BE was the most frequently quantified (detection frequency of 100 %), with maximum concentration of up to 5 μg L^{-1}. Despite the objective of this work was not to estimate the drug use in the populations under study (it would require considering other factors, as the daily mass load taking into account the WWTP flow), no consume pattern of cocaine was observed along the week on the basis of the BE concentrations found. Similarly, THC—COOH was detected in all samples (except one) without weekend pattern. KET, MDMA and METH were the drugs with the lowest frequency of detection, with maximum concentrations of 123, 246 and 32 ng L^{-1}, respectively.

Table 3
2023 Interlaboratory test data for DI and SPE methods. Information on nominal spike, means of means and standard deviation provided by SCORE.

Compound	Sample	DI mean conc. ($ng{\cdot}L^{-1}$)	SPE mean conc. ($ng{\cdot}L^{-1}$)	DI Z-SCORE	SPE Z-SCORE	Nominal spike ($ng{\cdot}L^{-1}$)	Mean of means ($ng{\cdot}L^{-1}$)	Standard deviation ($ng{\cdot}L^{-1}$)
AMP	M1	71	81	−1.30	−1.08	125	116	32
	M2	50	61	−1.40	−0.96	80	78	17
	M3	115	133	−0.70	−0.46	185	157	58
BE	M1	72	96	−1.90	−1.21	180	132	29
	M2	171	240	***−2.10***	−1.02	350	300	44
	M3	67	93	−1.10	−0.47	150	115	29
KET	M1	123	134	−1.10	−1.24	170	166	25
	M2	83	91	−1.40	−1.06	110	108	16
	M3	63	71	−1.10	−0.75	85	83	15
MDMA	M1	98	100	−0.90	−0.90	140	131	32
	M2	81	91	−1.20	−0.83	115	113	26
	M3	225	235	−1.10	−0.93	300	294	62
METH	M1	36	37	−1.30	−1.27	60	55	13
	M2	150	148	−0.90	−0.95	200	194	45
	M3	98	103	−1.20	−1.10	145	141	25
THC-COOH	M1	52	78	−1.20	−0.27	160	85	27
	M2	165	194	−0.80	−0.55	375	246	103
	M3	244	291	−0.70	−0.34	525	321	108
6-MAM	M1	53	76	−1.00	−0.69	200	120	63
	M2	849	898	−0.10	0.08	140	948*	448*
	M3	81	102	−1.00	−0.75	210	146	60

* SCORE is still investigating the discrepancy between means of means and nominal spike for 6-MAM in sample M2.

Table 4
QCs recoveries for the SPE and DI procedures.

Compound	Quality Controls average Recoveries and RSDs (in brackets), all expressed in%						
	DI				SPE		
	0.1 $\mu g{\cdot}L^{-1}$	0.4 $\mu g{\cdot}L^{-1}$	2.5 $\mu g{\cdot}L^{-1}$	10 $\mu g{\cdot}L^{-1}$	0.1 $\mu g{\cdot}L^{-1}$	0.4 $\mu g{\cdot}L^{-1}$	0.8 $\mu g{\cdot}L^{-1}$
AMPH	nd	117 (29)	110 (8)	99 (8)	67 (11)	**56** (34)	**53** (28)
BE	*	*	95 (9)	85 (15)	*	*	116 (27)
KET	101 (14)	101 (3)	96 (7)	90 (8)	105 (10)	102 (9)	108 (16)
MDMA	92 (16)	91 (10)	86 (7)	85 (13)	81 (27)	78 (31)	84 (29)
METH	94 (16)	92 (13)	82 (7)	79 (9)	**49** (39)	**46** (36)	**51** (33)
THC—COOH	*	104 (26)	99 (7)	97 (7)	85 (35)	92 (21)	98 (21)
6-MAM	97 (22)	113 (19)	104 (10)	99 (10)	109 (18)	104 (18)	106 (18)

nd: not detected.
* Concentration of the sample was similar or higher than the spiked level, and recovery could not be calculated.

Data obtained show that the DI-LC-MS/MS can be used for analysis of seven illicit drugs/metabolites, allowing their quantification at low concentrations without any preconcentration step. The average LOQs for DI analysis were rather similar to those for SPE, except for THC—COOH which exhibits a slightly higher LOQ by DI. Although DI is a viable alternative for the rapid analysis of IWW samples, very low analyte concentrations (in general, below 10 ng/L) may lead to false negative results, that however could be quantified by using SPE. This may occur for those drugs that are commonly present at lower concentrations in wastewater (e.g. METH, MDMA or AMPH in samples from this study). In this context, the question about how significant the reporting of low analyte concentrations is in terms of use/consumption in population arises. Finding a compromise between providing data on very low illicit drugs concentrations in wastewater and the utility of such data, versus using longer sample treatments potentially subjected to higher errors, seems a reasonable way to face this issue.

3.8. Overview from the WBE perspective

SCORE conducts a global multi-city study every year, which involves the analysis of IWW samples during a week to estimate community consumption (Sewage Analysis CORe Group Europe (SCORE), 2023). Population normalized mass loads (PNML) from all European cities is presented by the EMCDDA every year. The anonymized data from 2022 is shown in Table S7.

We made a simulation to estimate how many cities could be monitored using the DI method presented in this paper. Five virtual cities of different size were simulated including IWW flow data based on average flow data reported by cities of similar sizes. Based on the LOQs reported for the DI method, PNML (mg/1000inhabitants/day) of each virtual city were calculated, obtaining the minimum value that could be reported by DI (Table 6). These average values were compared with the minimum PNML reported by the EMCDDA, and subsequently the number of cities that could be monitored without reporting false negative results were obtained. Table 7 shows that nearly all cities could be efficiently monitored for BE and THC—COOH by the DI method, and around 70 % for AMPH, MDMA and METH. The lowest percentage of applicability for the DI method was for KET with only 44 % of cities that could be monitored. However, only 16 cities reported data for this drug; so, there is not enough data to reach robust conclusions in comparison with the rest of drugs included in EMCDDA data.

This simulation indicates that the DI method is sensitive enough and that most of the cities participating in SCORE could be monitored using this approach. For the remaining cities the question of a valid cut-off value may raise. In other words, if the concentration of a certain drug in wastewater is very low surely there is not a relevant problem for that substance in the monitored city.

Table 5
Concentrations obtained in the analysis of IWW samples by DI and SPE LC-MS/MS. Difference (%) between both methods, considering the value obtained by SPE as the reference.

WWTP	Date	Concentration (ng·L^{-1})														
		BE			KET			MDMA			METH			THC—COOH		
		SPE	DI	*Diff*	SPE	DI	*Diff*	SPE	DI	*Diff*	SPE	DI	*Diff*	SPE	DI	*Diff*
A	T-7/4/2022	3974	3137	*−21*	–	–	–	30	29	*−2*	–	–	*0*	383	524	*37*
	F-8/4/2022	5816	4727	*−19*	–	–	–	37	33	*−11*	–	–	*0*	346	365	*5*
	S-9/4/2022	6260	4430	*−29*	–	–	–	41	29	*−27*	–	–	*0*	322	371	*15*
	S-10/4/2022	5442	4743	*−13*	–	–	–	61	55	*−10*	–	–	*0*	241	306	*27*
	M-11/4/2022	4727	4376	*−7*	–	–	–	70	64	*−8*	–	–	*0*	271	330	*22*
	T-12/4/2022	4326	3613	*−16*	–	–	–	43	39	*−10*	–	–	*0*	341	441	*30*
	W-13/4/2022	4080	5332	*31*	–	–	–	72	87	*21*	–	–	*0*	242	248	*2*
B	T-7/4/2022	1794	1623	*−10*	57	54	*−5*	<LOQ	–	*	–	–	*0*	164	141	*−14*
	F-8/4/2022	2190	2065	*−6*	70	57	*−18*	<LOQ	–	*	–	–	*0*	189	179	*−5*
	S-9/4/2022	2081	1776	*−15*	59	44	*−26*	<LOQ	–	*	–	–	*0*	187	172	*−8*
	S-10/4/2022	2806	2351	*−16*	42	27	*−37*	<LOQ	–	*	–	–	*0*	186	189	*1*
	M-11/4/2022	4068	3488	*−14*	49	37	*−25*	<LOQ	–	*	–	–	*0*	166	169	*2*
	T-12/4/2022	2185	1907	*−13*	48	40	*−17*	<LOQ	–	*	–	–	*0*	170	211	*24*
	W-13/4/2022	1920	1709	*−11*	45	33	*−25*	<LOQ	–	*	–	–	*0*	182	249	*37*
C	T-7/4/2022	2133	1645	*−23*	<LOQ	–	*	<LOQ	20	*	–	–	*0*	181	170	*−6*
	F-8/4/2022	2110	1790	*−15*	–	–	–	30	23	*−23*	–	–	*0*	226	208	*−8*
	S-9/4/2022	2414	2082	*−14*	<LOQ	–	*	47	45	*−4*	–	–	*0*	259	224	*−13*
	S-10/4/2022	2957	2287	*−23*	<LOQ	–	*	<LOQ	20	*	–	–	*0*	216	208	*−4*
	M-11/4/2022	2793	2383	*−15*	<LOQ	–	*	55	49	*−12*	–	–	*0*	206	209	*2*
	T-12/4/2022	2552	2213	*−13*	<LOQ	–	*	61	49	*−21*	–	–	*0*	299	255	*−15*
	W-13/4/2022	2084	1737	*−17*	<LOQ	–	*	43	39	*−10*	–	–	*0*	251	238	*−5*
I	W-23/3/2022	2685	2613	*−3*	108	91	*−16*	73	86	*17*	<LOQ	32	*	329	345	*5*
	F-25/3/2022	2733	2571	*−6*	98	81	*−17*	61	58	*−5*	<LOQ	27	*	277	367	*32*
	S-26/3/2022	4068	3268	*−20*	112	81	*−28*	97	82	*−15*	<LOQ	27	*	290	396	*37*
	S-27/3/2022	3557	3118	*−12*	96	71	*−27*	138	141	*2*	<LOQ	21	*	318	383	*20*
	M-28/3/2022	4904	3389	*−31*	135	109	*−19*	246	246	*0*	<LOQ	21	*	343	404	*18*
	T-29/3/2022	3344	2875	*−14*	175	123	*−30*	187	166	*−11*	<LOQ	–	*	346	453	*31*
	W-30/3/2022	2598	2403	*−8*	94	71	*−24*	95	82	*−13*	<LOQ	20	*	282	339	*20*
D	T-26/4/2022	1889	1885	*0*	<LOQ	–	*	<LOQ	24	*	–	–	*0*	166	214	*28*
	W-27/4/2022	1702	1539	*−10*	–	–	–	<LOQ	20	*	–	–	*0*	185	251	*36*
	T-28/4/2022	2052	1810	*−12*	–	–	–	<LOQ	20	*	–	–	*0*	190	258	*36*
	F-29/4/2022	1921	1757	*−9*	–	–	–	<LOQ	20	*	–	–	*0*	197	240	*22*
	S-30/4/2022	2699	2627	*−3*	<LOQ	–	*	30	27	*−11*	–	–	*0*	202	202	*0*
	S-1/5/2022	5116	3190	*−38*	<LOQ	–	*	56	55	*−2*	–	–	*0*	226	234	*3*
	L-2/5/2022	5116	3415	*−33*	<LOQ	–	*	67	67	*0*	–	–	*0*	268	311	*16*
E	22/06/22 - 06/07/22	2845	3125	*10*	<LOQ	–	*	<LOQ	–	*	<LOQ	–	*	307	253	*−18*
F	22/06/22 - 06/07/22	1442	1185	*−18*	<LOQ	–	*	<LOQ	–	*	–	–	*0*	115	100	*−13*
G	22/06/22 - 06/07/22	3334	3489	*5*	12	–	*	<LOQ	–	*	–	–	*0*	124	152	*23*
H	22/06/22 - 06/07/22	2474	2102	*−15*	–	–	–	<LOQ	–	*	–	–	*0*	275	320	*16*
E	24/08/22 - 07/09/22	3802	3079	*−19*	–	–	–	<LOQ	36	*	–	–	*0*	315	326	*3*
F	24/08/22 - 07/09/22	2151	2060	*−4*	–	–	–	<LOQ	<LOQ	*0*	–	–	*0*	145	159	*10*
G	24/08/22 - 07/09/22	4939	4892	*−1*	16	–	*	<LOQ	20	*	–	–	*0*	80	–	*
H	24/08/22 - 07/09/22	3790	3055	*−19*	–	–	–	56	53	*−5*	–	–	*0*	335	326	*−3*
C	T-20/10/2022	2126	1613	*−24*	10	<LOQ	*	<LOQ	<LOQ	*	–	–	*0*	279	273	*−2*
	F-21/10/2022	2554	2043	*−20*	10	<LOQ	*	<LOQ	<LOQ	*	–	–	*0*	358	423	*18*
	S-22/10/2022	3388	2562	*−24*	27	20	*−25*	<LOQ	20	*	–	–	*0*	359	461	*29*
	S-23/10/2022	3575	2791	*−22*	11	<LOQ	*	50	66	*31*	–	–	*0*	336	499	*48*
	M-24/10/2022	3977	3329	*−16*	15	<LOQ	*	52	62	*18*	–	–	*0*	300	348	*16*
	T-25/10/2022	3042	2263	*−26*	11	<LOQ	*	30	31	*4*	–	–	*0*	402	272	*−32*
	W-26/10/2022	2473	1978	*−20*	12	<LOQ	*	<LOQ	21	*	–	–	*0*	433	499	*15*
E	18/01/23 - 01/02/23	6353	3819	*−40*	–	–	–	<LOQ	25	*	–	–	*0*	287	243	*−15*
F	18/01/23 - 01/02/23	2632	2282	*−13*	–	–	–	45	46	*2*	–	–	*0*	241	215	*−11*
G	18/01/23 - 01/02/23	4378	3889	*−11*	–	–	–	<LOQ	22	*	–	–	*0*	236	271	*15*
H	18/01/23 - 01/02/23	3922	3303	*−16*	–	–	–	<LOQ	20	*	–	–	*0*	400	354	*−12*

* Not calculated due to the absence of quantitative data from the DI and/or the SPE procedure.

Table 6
Population normalized mass loads (mg/day/1000inh) of virtual cities considering the LOQs of the DI method (in ng·L^{-1} in brackets).

Virtual city	Population	Flow data (m^3/day)	AMPH (100)	BE (10)	KET (20)	MDMA (20)	METH (20)	THC—COOH (100)	6-MAM (30)
A	50,000	8000	17.6	1.6	3.2	3.2	3.2	16.0	4.8
B	100,000	20,000	22.0	2.0	4.0	4.0	4.0	20.0	6.0
C	200,000	38,000	20.9	1.9	3.8	3.8	3.8	19.0	5.7
D	500,000	80,000	17.6	1.6	3.2	3.2	3.2	16.0	4.8
E	1,000,000	250,000	27.5	2.5	5.0	5.0	5.0	25.0	7.5
		Average	21.1	1.9	3.8	3.8	3.8	19.2	5.8

Table 7
Population normalized mass loads (mg/day/1000inh) from EMCDDA data. Estimation of cities that could be efficiently monitored by using the DI method.

	AMPH	BE	KET	MDMA	METH	THC—COOH
Average	82.6	257.3	19.2	17.4	47.6	75.3
Median	49.4	121.1	2.6	8.5	7.5	62.9
Maximum	873.4	2381.4	203.6	182.3	922.3	180.8
Minimum	1.7	0.1	0.2	0.6	0.3	21.4
Total cities reported	101	109	16	105	95	58
Cities that could be monitored by DI	72	103	7	83	65	58
Percentage monitored by DI (%)	71	94	44	79	68	100

No data reported for 6-MAM.

3.9. Benefits and future application

Direct injection analysis presents several benefits and holds promising future applications for analyzing illicit drugs in IWW. Firstly, it aligns with the principles of green chemistry by minimizing the need for sample preparation, thus reducing solvent consumption and the generation of waste and plastics. This approach contributes to environmental sustainability, but also streamlines the analytical process and involves fewer errors in sample treatment (e.g. the evaporation step can cause losses of some compounds (Baker and Kasprzyk-Hordern, 2011)), allowing high-throughput analyses, which is very valuable when large monitoring events are involved. Also, as other drugs become of interest and consumption increases, they can easily be incorporated into this method. This could be more challenging with the SPE method, as the choice of a selective sorbent to suit a wide range of chemical classes may be complicated. The main advantage lies in the rapid analysis and the capability to process larger sample campaigns. The reduction of sample size (i.e., from approximately 100 mL to only 1 mL) needed for analysis, facilitates sample collection and transport, but also results in the reduction in sample storage space and duration. Samples can even be promptly thawed and analyzed, upon arrival at the laboratory, if UHPLC-MS/MS equipment is accessible. Thus preventing compound degradation and the potential adsorption of compounds such as THC—COOH to suspended particles matter during extended storage periods (McCall et al., 2016). The main drawback would be the lower LOQs attainable as there is not a pre-concentration step as occurs in SPE. However, this work shows that most of the conventional illicit drugs could be efficiency monitored in a given population with relatively low drug consumption.

With the improvement of sensitivity of analytical instruments and its quick and efficient workflow, it is expected that DI methods will become increasingly important in the field of wastewater-based epidemiology ensuring timely monitoring of illicit drug trends, and enabling early interventions to emerging public health issues.

4. Conclusions

This study presents a rapid and sensitive method based on direct injection LC-MS/MS for the quantification of 7 illicit drugs in urban IWW samples, reaching detection capability of around 30 ng L^{-1} without the need for a preconcentration step. A detailed comparison has been made with the most applied SPE method, focusing on the evaluation of matrix effects, sensitivity and suitability. The DI approach has demonstrated minimal matrix effects, while the SPE based method was affected by higher matrix effects in general, and led to partial analyte losses, particularly affecting AMPH and METH. Validation of the DI method in different wastewater samples showed acceptable results for most analytes, except for the lowest levels of AMPH. The interlaboratory exercise confirmed that DI-LC-MS/MS might be used as an alternative to SPE-LC-MS/MS. In addition, 54 IWW samples from 9 WWTPs in Spain were analyzed by both methodologies, together with the required quality controls, showing the robustness of DI method, supported by excellent accuracy data along the time. The data presented in this paper highlights that DI represents a fast, cost-effective, and efficient alternative, significantly reducing time consumption in comparison to the conventional SPE for the analysis of illicit drugs in IWW by LC-MS/MS.

CRediT authorship contribution statement

Elisa Gracia-Marín: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Félix Hernández:** Writing – review & editing, Visualization, Resources. **María Ibáñez:** Writing – review & editing, Visualization, Supervision, Conceptualization. **Lubertus Bijlsma:** Writing – review & editing, Supervision, Resources, 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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121864.

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