



A comparative study of SPE- and DLLME-based methods for the determination of opioids and benzodiazepines in urine samples using LC–MS/MS

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ABSTRACT

The determination of drugs of abuse has been gaining increasing attention among the scientific community as drug consumption is considered a major global public health issue. Opioids and benzodiazepines are among the most widely consumed drugs worldwide. Analytical methods for their determination, particularly in urine matrices, primarily rely on chromatographic techniques. However, due to the complexity of the matrix and the low concentration levels at which these drugs are usually present (in the ng/L or µg/L range), efficient sample pre-treatment strategies are essential. This step is of significant importance in the development of environmentally friendly analytical methods. For this reason, in recent years, pre-treatment techniques have been developed based on green analytical chemical principles to address this need.

In this context, we present a comparative study of solid phase extraction (SPE) and dispersive liquid–liquid microextraction (DLLME) for the determination of a group of benzodiazepines and opioids in urine samples using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). The method, based on SPE, was developed using ExtraBond SCX as the sorbent and 7 mL of 5 % of NH₄OH in methanol as the elution solvent, yielding recoveries ranging from 9 to 107 %. The DLLME method used 200 µL of chloroform and 500 µL of ethyl acetate as extractant and dispersant solvents, respectively, achieving recoveries between 14 and 86 %. The DLLME strategy proved to be greener than the SPE method in line with the principles of green analytical chemistry (GAC), as it required lower volumes of solvents, a shorter extraction time, and less energy consumption than the SPE method. Both methods were validated using urine matrices and subsequently applied to 11 urine specimens from women participating in detoxification programmes, confirming the suitability of these methods in toxicological and forensic analyses. The results showed that methadone and diazepam were the most frequently determined compounds, and a trend of polyconsumption was observed in most of the samples.

1. Introduction

The abuse of psychoactive substances remains a global public health concern with significant social health implications. According to the World Drug Report 2023, approximately 1 in 17 individuals between 15 and 64 years of age worldwide had consumed drugs within the preceding 12 months, representing a 23 % increase compared to a decade earlier. Cannabis ranks as the most consumed recreational drug, followed by amphetamines, cocaine, opioids and non-medical uses of pharmaceuticals, such as benzodiazepines. Benzodiazepines and opioids are of particular interest among the range of substances that people abuse, due to their widespread use and their applications in medicine

[1–5].

Opioids, derived from opium, are potent analgesics used for pain relief or as sedatives, but they are also highly addictive. Opioids can be classified as natural (e.g., codeine, morphine, thebaine) or synthetic (e.g., fentanyl, tramadol, buprenorphine, methadone, pethidine). They are abused for their complex psychotropic effects, which give users a feeling of euphoria. However, opioid abuse can lead to severe health complications. Approximately 70 % of overdose-related deaths are attributed to opioids, primarily heroin [1,2]. Compounds such as methadone and buprenorphine have been extensively used in the last few decades in opioid replacement therapy for the treatment of opioid use disorder. These medications are commonly employed in detoxification programmes

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to manage withdrawal symptoms and reduce opioid cravings. Methadone and buprenorphine play a crucial role in stabilizing patients during recovery mitigating opioid dependence, and significantly lowering the risk of relapse [5–7].

Despite historical consumption patterns, recent trends indicate a shift towards the replacement of opioids by other substances, such as benzodiazepines [2]. Benzodiazepines, a class of psychoactive drugs prescribed for anxiety, insomnia, and antiepileptic properties, pose significant health risks when abused. An overdose can produce adverse effects such as blurred vision, dizziness, drowsiness, and in certain cases, coma. Additionally, the consumption of this group of substances is associated with drug-facilitated crimes, where victims are secretly administered these substances, often in alcoholic beverages. Commonly consumed benzodiazepines include diazepam, alprazolam, lorazepam, chlordiazepoxide, flunitrazepam, bromazepam, oxazepam, and nitrazepam, among others [6–8].

Given the serious health problems associated with the consumption of opioids and benzodiazepines, the development of new analytical methods to detect and monitor their consumption has become imperative. Consequently, in recent years, several methods have been developed for the determination of these compounds in various biological matrices such as urine, blood, saliva, and hair [9–16].

Urine analysis has emerged as an important tool for determining and monitoring drug consumption due to the non-invasive sampling method, the minimal need for specialized personnel, and the generation of a large quantity of sample. Furthermore, urine has a wide detection window, ranging from minutes to days or even weeks, making it particularly useful for toxicological investigations. However, drugs are usually present in urine at trace levels ($\mu\text{g/mL}$ or ng/mL), necessitating highly sensitive analytical methods.

As polyconsumption becomes more prevalent, there is a growing need for analytical methods capable of simultaneously identifying several classes of drugs in urine samples. For these reasons, the focus of this study was the development of methods for the determination and quantification of selected benzodiazepines and opioids, representing the two different drug classes.

The determination of these kinds of compounds has generally been done with chromatographic techniques, such as liquid chromatography (LC) or gas chromatography (GC) or with capillary electrophoresis (CE) [12–15,17–20]. Opioids and benzodiazepines are polar compounds; therefore, LC has emerged as the preferred choice, particularly coupled with mass spectrometry (MS) [13,14,19–22]. Given the low concentrations of drugs in urine samples and the complexity of urine as the matrix, sample pre-treatment is required [16,20,23–26].

Pre-treatment techniques developed recently place growing emphasis on the use of environmentally friendly techniques to minimize the ecological footprint of drug detection and determination methods. Solid phase extraction (SPE) is one of the most widely used alternatives, offering selective extraction capabilities with a wide variety of sorbents [21,27–33]. Due to the physicochemical properties and the structure of opioids and benzodiazepines, mixed-mode cation exchange sorbents have been extensively used for selective extraction of these analytes [21,22,30–33].

In recent years, new techniques have been developed to achieve greener analytical methods, in line with the principles of green analytical chemistry (GAC). A method's 'greenness' is often evaluated using instruments like the AGREE-Analytical GREENness Metric Approach. This approach scores methods on a scale from 0 to 1, with a higher score indicating greater greenness [34–36].

Dispersive liquid–liquid microextraction (DLLME) has emerged as a favoured green pre-treatment technique for drug extraction as an alternative to traditional LLE [37–42] because it utilizes reduced volumes of organic solvents (μL range). Traditional DLLME requires a dispersant and an extractant solvent. Common extractants include chlorinated solvents like dichloromethane (DCM), chloroform (TCM) and tetrachloride, and common dispersants include methanol (MeOH),

acetonitrile (ACN), acetone, isopropyl and ethanol [41–47]. This study compares two sample pre-treatment techniques, SPE and DLLME, in combination with LC–MS/MS for the simultaneous determination of a group of opioids and benzodiazepines in urine samples. The main objective of this study is to compare and evaluate the two proposed methods based on SPE and DLLME, for analysing benzodiazepines and opioids in urine samples, with a particular emphasis on method efficiency, accuracy, and environmental impact in line with green chemistry principles. Specifically, we aim to assess the applicability of both methods for analyzing urine samples from women undergoing detoxification treatment. Furthermore, the greenness of each method and its environmental impact were evaluated using the AGREE-approach.

2. Material and methods

2.1. Chemical and reagents

Nine pure analytical drug reference standards, including three benzodiazepines and six opioids (Fig. 1), were acquired from Sigma-Aldrich (St Louis, MO, USA) and LGC Standards (Luckenwalde, Germany). The drugs included morphine (MOR), codeine (COD), tramadol (TRA), heroine (HER), buprenorphine (BUP), bromazepam (BRM), methadone (MET), oxazepam (OXA) and diazepam (DIA).

Stock solutions of individual standards were prepared in methanol (MeOH) at a concentration of 1000 mg/L and stored at $-20\text{ }^{\circ}\text{C}$. Working solution of a mixture containing all the compounds were prepared at a concentration of 1 mg/L in MeOH:H₂O (5/95, v/v) and also stored at $-20\text{ }^{\circ}\text{C}$. H₂O and ACN for LC–MS, as well as MeOH, ethyl acetate (EtOAc), acetone (ACE), ethanol (EtOH), TCM and DCM, were supplied by Carlo Erba Reagents (Milano, Italy). Formic acid (purity >98 %) (HCOOH) and ammonium acetate (purity 28–30 %) were supplied by Sigma-Aldrich. Ultrapure water was produced using an Integral Water Purification System (Merck Millipore, Burlington, MA, USA).

Oasis MCX (500 mg) was purchased from Waters (Mildford, MA, USA), and ExtraBond SCX (500 mg) was purchased from Sharlab (Barcelona, Spain). A MiVAc Duo sample concentrator from Genevac (Ipswich, UK) was used to evaporate the solutions.

2.2. Liquid chromatography and MS/MS detection

We used an Agilent 1200 series LC liquid chromatography tandem mass spectrometry coupled with a 6460 QqQ mass spectrometer (MS/MS) equipped with an electrospray ionization (ESI) interface (Agilent Technologies, Santa Clara, CA, USA). The chromatographic column was a Luna Omega Polar C₁₈ (150 mm \times 3.0 mm i.d., 5 μm particle size) supplied by Phenomenex (Torrance, California, USA) with a Security Guard column also from Phenomenex. The mobile phase consisted of A: 0.1 % HCOOH in H₂O and B: 0.1 % HCOOH in ACN in gradient mode.

The gradient starting conditions were 90 % A and 10 % B. After 3 min, the B composition was ramped to 40 % in 5 min. At that point, the B percentage was increased to 100 %, which occurred at minute 16 of the analysis at which point it was returned to the initial conditions, which took 2 min. Once the initial conditions were reached, the composition was maintained for 3 min before the next analysis, resulting in a total chromatographic run time of 24 min. The mobile phase flow rate was 0.4 mL/min, and the column was maintained at 30 $^{\circ}\text{C}$. The injection volume was 5 μL .

In the LC-ESI-QqQ setup, the optimized parameters were as follows: ESI operating under positive mode, source gas temperature of 340 $^{\circ}\text{C}$, nitrogen flow rate of 12 mL/min, nebulizer pressure of 40 psi, capillary voltage of 2500 V, cone voltage of 100 V, and collision energy between 8 and 40 eV. Table 1 shows the selected reaction monitoring (SRM) acquisition parameters for all compounds and the collision energy applied in each transition.

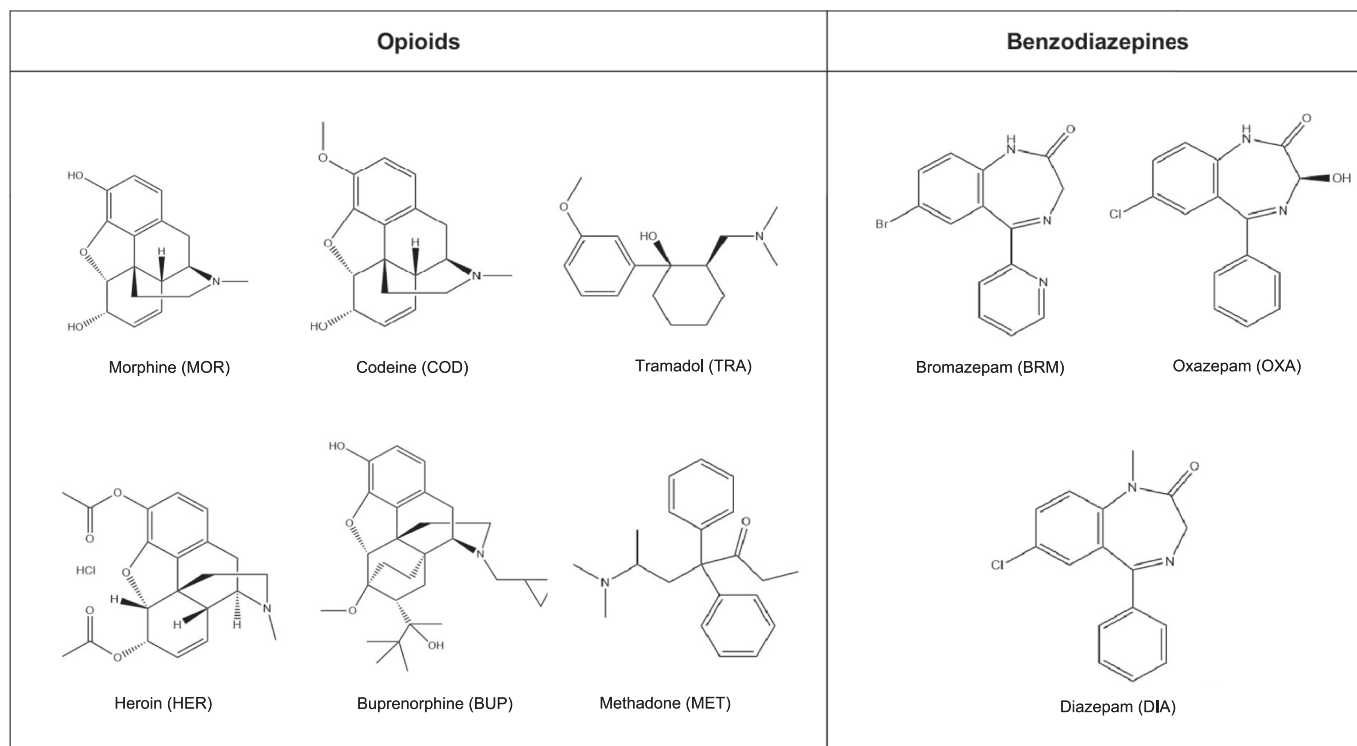


Fig. 1. Chemical structures of the studied benzodiazepines and opioids.

Table 1
Optimized conditions of the MRM acquisition method.

Compound	tr (min)	Precursor ion (m/z)	Cone Voltage (V)	Product ion 1 (m/z) (Q)	Collision energy 1 (eV)	Product ion 2 (m/z) (q)	Collision energy 2 (eV)	Ratio (q/Q) *100
MOR	2.85	296.1	75	165.0	40	153.1	40	66.7
COD	7.17	300.1	125	215.1	40	199.0	40	50.5
TRA	10.52	264.1	75	58.1	30	–	–	–
HER	10.73	370.1	125	268.0	40	211.0	40	72.5
BUP	12.48	468.2	75	396.0	50	55.2	50	17.3
BRM	12.68	317.9	150	209.1	30	182.1	30	64.1
MET	13.64	310.2	125	265.0	20	105.0	30	65.4
OXA	14.03	287.0	50	269.0	20	241.0	20	28.8
DIA	16.05	285.0	125	193.0	30	153.9	30	93.6

2.3. Urine collection

Urine samples were collected from participants at the beginning of their detoxification programs under standardized conditions. The samples were collected in 100 mL sterile polypropylene tubes, and were stored at $-20\text{ }^{\circ}\text{C}$ until analysis. Before their analysis, the samples were thawed and diluted 50:50 (v/v) with ultrapure water adjusted with HCOOH >98 % to achieve the optimal pH for the subsequent sample treatment step, which was pH 3 for the SPE-based method and pH 7 for the DLLME-based method.

2.4. SPE method

The selected sorbent in this study was the mixed-mode strong cation-exchange silica sorbent Extra Bond SCX (500 mg). The SPE protocol began with a conditioning step using 10 mL of MeOH followed by 10 mL of ultrapure water adjusted to pH 3 with HCOOH. After conditioning, 10 mL of a mixture of urine:H₂O (50:50, v/v) at pH 3 was loaded onto the sorbent. Subsequently, a washing step with 1 mL of MeOH was performed, and finally, the analytes were eluted from the sorbent using 7 mL of 5 % NH₄OH in MeOH. Following elution, 100 μL of 1 % chloridric acid (HCl) in MeOH was added to the extracts, which were then

evaporated to dryness using a miVAC Duo Concentrator. The dried extracts were reconstituted with 0.5 mL of the initial mobile phase and filtered through a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter before being transferred to a vial for analysis.

2.5. DLLME method

For the DLLME method, 5 mL of urine:H₂O mixture at pH 7 adjusted with HCOOH (50:50, v/v) was placed in a 10 mL vial. Then, 500 μL of ethyl acetate (as the dispersant solvent), and 200 μL of TCM (as the extractant solvent) were rapidly added simultaneously to ensure proper dispersion. The vials were vortexed for 60 s to form a cloudy solution and then centrifuged for 5 min at 4000 rpm to separate the solvents. The organic layer was then transferred to a vial and evaporated to dryness using a gentle stream of N₂. The residue was reconstituted with 0.5 mL of the initial mobile phase, filtered through a 0.45 μm PTFE syringe filter, and transferred to a vial for analysis.

2.6. Validation

The validation was performed following the guidelines for workplace drug testing in urine [48]. The parameters evaluated include linearity,

instrumental detection and quantification limits (LOD and LOQ), method detection and quantification limits (MDL and MQL), recovery (R %), repeatability (intra-day) and reproducibility (inter-day).

Instrumental linearity was assessed using standards ranging between 0.05 to 250 ng/mL considering the determination coefficient (r^2). The LOQ was defined as the lowest concentration value on the calibration curve with a signal-to-noise (S/N) ratio ≥ 10 , while the LOD was defined as the lowest detectable concentration with a S/N ratio ≥ 3 .

For method validation, linearity was evaluated using a matrix-matched calibration curve for each compound, with which the coefficient r^2 was assessed. Linearity for both SPE and DLLME methods was studied in the ng/mL range. MQL was the lowest value on the calibration curve, and MDL was defined as the lowest concentration at which compounds could be detected after the extraction method with S/N ratio ≥ 3 .

Repeatability and reproducibility were evaluated using urine spiked at three different concentration levels (5, 25, and 60 ng/mL), selected to represent low, medium and high concentrations within the linearity range of the calibration curves. These parameters were evaluated in terms of relative standard deviation (%RSD) with five replicates ($n = 5$) for each concentration.

Recovery was calculated as the ratio between the signal obtained after the extraction process and the signal of a standard at the same concentration and was also evaluated at three different concentration levels (5, 25, 60 ng/mL).

2.7. Analysis of real samples

Eleven urine samples, provided by the Centre Catal  de la Solidaritat (CECAS) in Tarragona, Spain, from anonymous women starting a drug abuse detoxification, were analysed. All samples were collected in 100 mL sterilised polypropylene tubes upon the patients' admission to the centre and frozen at -20°C until analysis. Although the samples were not collected on the same day, they were all analysed on the same day, with each sample analysed in triplicate ($n = 3$).

3. Results and discussion

3.1. LC-MS/MS optimization

For the LC separation, the stationary phase and the mobile phase were selected based on previous studies conducted by our research group, wherein several drugs, including the compounds under study, were determined in urine matrices [21,49]. These previous studies covered a high number of drugs of different families, including some benzodiazepines and opioids. However, to optimize the separation for the specific compounds in this study, we made modifications to the method. Specifically, adjustments were made. These changes resulted in a shorter analysis time (now 24 min), and improved chromatographic separation, with enhanced peak resolution. Fig. 2 shows the satisfactory optimal separation obtained.

All MS/MS parameters were optimized by injecting individual standard solutions of each compound at a concentration of 1 mg/L in a MeOH:H₂O (50:50, v/v) solution. Both positive and negative ESI modes were evaluated, and the positive mode was selected as it provided better ionization efficiency and higher signal-to-noise ratios for all analytes. The acquisition mode used in this study was SRM mode, wherein a precursor ion, specifically $[M+H]^+$, and two product ions were selected for each analyte. The exception was TRA, for which only one product ion was selected due to the absence of additional characteristic fragments. This approach aligns with findings from other studies, which also reported the selection of a single product ion for TRA [50–52].

The cone voltage was optimized at a range of 50 to 175 V, and the optimal voltage values for each analyte are detailed in Table 1. As the table shows, for most analytes, the optimal voltage was 75 or 125 V, except for OXA, for which the optimal voltage was 50 V, and for BUP, whose optimal voltage was 150 V. For the optimization of the collision energy, a range of energies between 0 and 50 eV (in steps of 10 eV) was evaluated initially, and then the range was narrowed down to identify the optimal collision energy. For example, in the case of MET, the initial assessment indicated an optimal collision energy of between 20 and 40 eV. We then evaluated a more precise evaluation within this range, using 5 eV increments, resulting in optimal energies for product ion 1 (20 eV) and product ion 2 (30 eV). All these parameters are summarized in Table 1.

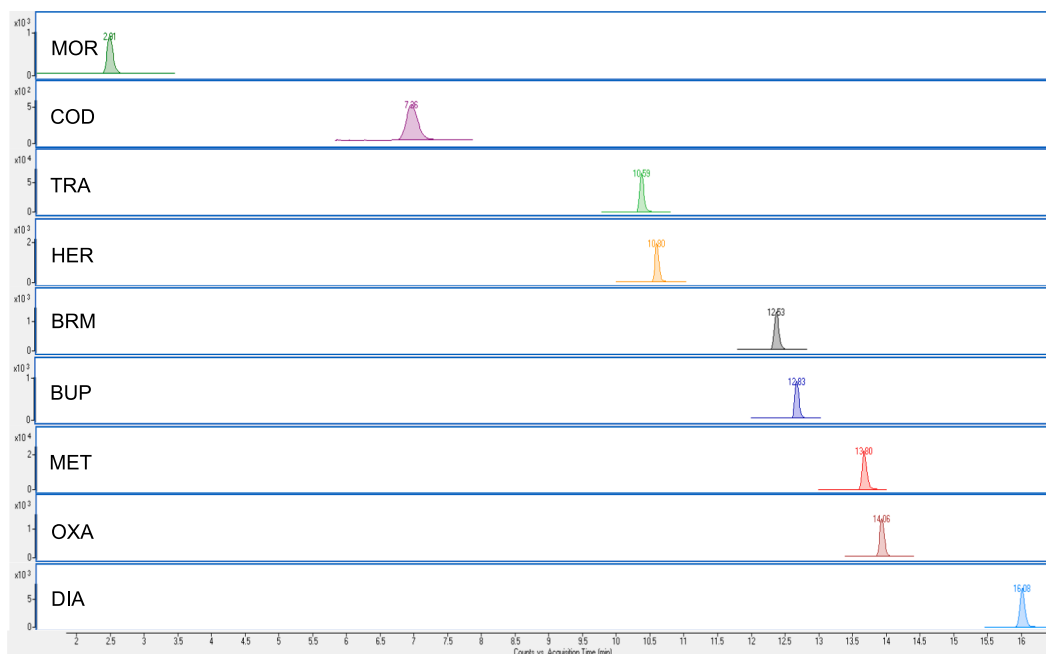


Fig. 2. Chromatogram of a urine spiked at 25 ng/mL analysed using the optimized LC-MS/MS conditions.

3.2. SPE optimization

In this study, two commercial mixed-mode strong cationic exchange sorbents were evaluated for their ability to extract opioids and benzodiazepines from urine samples. These sorbents were chosen based on the physicochemical properties of the compounds. These sorbents can retain these compounds by cationic interactions and/or reverse phase interactions. Most of the compounds in this study have pKa values of higher than 8, so at pH values lower than pH 8, these compounds predominantly exist in their cationic form, enabling interactions with the sulfonic acid groups ($-\text{SO}_3^-$) present on the sorbent structure. Conversely, OXA and DIA, with pKa values of 1.5 and 3.3 respectively, only remain in their cationic form under highly acidic conditions. Nevertheless, all the compounds have aromatic parts in their structure (Fig. 1), allowing them to be retained by reverse phase interactions. Additionally, the choice of mixed-mode strong cationic exchange sorbents for the extraction of these compounds is supported by previous studies [21,22,31,32,53], which have demonstrated the effectiveness of such sorbents in selectively extracting various drug groups, including some of the studied compounds.

The sorbents compared in the present study were Extra Bond SCX (500 mg) and Oasis MCX (500 mg). Both sorbents have sulfonic groups on their structure but differ in their base composition. Oasis MCX is polymer-based, while Extra Bond SCX is silica-based. The polymeric structure of Oasis MCX, obtained from polyvinylpyrrolidone-divinylbenzene, generally provides a higher retention capacity than silica, allowing more compounds to be retained on its structure [33].

Both sorbents were evaluated simultaneously, using recovery values as the key factor for the comparison, with optimized parameters including loading conditions (pH and sample volume), clean-up conditions (solvent volume and type) and elution conditions (solvent volume and type).

Initially, the sample loading pH was optimized using 5 mL of ultra-pure water spiked with all analytes at a concentration of 2.5 ng/mL. Based on existing literature, elution was performed with 5 mL of 5 % of NH_4OH in MeOH, followed by the addition of 0.1 mL of 1 % HCl solution before evaporation to dryness using a miVAC Duo Concentrator. The addition of HCl was necessary because acidified methanol helps to stabilize certain analytes by reducing their volatility and preventing degradation at high temperatures, ensuring more accurate quantification after the drying step. Finally, the extracts were reconstituted with 0.5 mL of the initial mobile phase for LC-MS/MS analysis [21,33].

The sample pH was evaluated to optimize compound retention. Given the pKa values of most of the compounds, which are higher than 8, they exist in their cationic form at pH values below 8. Thus, pH values below or equal to 7 (3, 4 and 7) were tested. Lower pH values were not considered to prevent potential damage to the silica structure of Extra Bond SCX [33].

Our study showed higher recoveries at pH 3 for both sorbents, ranging from 50 % to 100 %. At pH 7, recoveries decreased, with Oasis MCX showing slightly higher values than the silica sorbent, indicating potential reverse phase interactions. pH 4 also yielded lower recoveries compared to pH 3. These findings confirmed the suitability of pH 3 for our selected conditions. Other authors, such as Musile et al. [31] also defined pH 3 as optimal for the extraction of a broad range of compounds, including MOR, COD and MET using an in-house cationic mixed-mode sorbent. Similarly, Pascual-Caro et al. [21] evaluated different pH values ranging from 2 to 6 and found that pH 4 was the optimal for extracting a large group of illicit drugs (40 compounds), including most of the compounds in this study, except for TRA and OXA. The authors selected the best conditions for the most compounds; in contrast the present study, it focussed on a more specific group of compounds and the pH conditions changed to achieve more optimal extraction. The pH 3 conditions were also evaluated using a mixture of urine:H₂O (50:50, v/v), and the recoveries achieved were at the same order as those presented previously.

A sample mixture of urine:H₂O (50:50, v/v) was evaluated to optimize the clean-up, loading and elution conditions. Urine samples were diluted 50:50 to minimize the matrix interferences and to ensure more accurate pH control. The elution solvent, a mixture of NH_4OH :MeOH (5:95, v/v), was selected based on its demonstrated effectiveness in disrupting ionic interactions between analytes and the sorbent [21,33]. To ensure complete elution, three different volumes of solvent (5 mL, 7 mL and 10 mL) were evaluated. Both sorbents achieved higher recoveries with 7 mL and 10 mL compared to 5 mL, indicating incomplete elution with the lower volume. No significant differences were found between 7 mL and 10 mL; therefore, 7 mL was selected for its lower solvent requirement and shorter evaporation time, enhancing method sustainability.

A clean-up step using MeOH was incorporated to remove interferences and increase extraction selectivity [21,33]. Different MeOH volumes (1, 2 and 5 mL) were tested. Using 5 mL caused elution of OXA and DIA during the washing step, indicating that at the working pH they were not in their cationic form and were only retained by reverse phase interactions, while no losses were observed for the other compounds. To minimize losses of DIA and OXA, the volume of MeOH was reduced to 1 mL, resulting in partial elution of these compounds during the washing step.

Fig. 3 shows the recoveries achieved with both sorbents. As shown in the figure, Extra Bond SCX generally yielded higher recoveries than Oasis MCX for most compounds, except for OXA and DIA (20 points lower), which was more strongly retained in the polymeric structure than the silica structure by reverse phase interactions. Therefore, Extra Bond SCX was selected as the optimal sorbent for the extraction of the studied compounds.

The final optimization step involved determining the loading volume. Both 5 mL and 10 mL of a mixture of urine:H₂O (50:50, v/v) were tested and achieved comparable results. Therefore, 10 mL was chosen to make it possible to detect lower concentration values. Higher volumes of sample were not studied to avoid increased extraction time, energy consumption and waste generation, which would make the method less environmentally friendly. Moreover, the 10 mL volume was sufficient to achieve the expected lower concentrations of the compounds (levels of $\mu\text{g/L}$) in urine samples.

After optimization, the optimal SPE extraction conditions with Extra Bond SCX sorbent involved loading 10 mL of a mixture of urine:H₂O (50:50, v/v) at pH 3, followed by clean-up with 1 mL of MeOH, and elution with 7 mL of NH_4OH :MeOH (5:95, v/v). Subsequently, 100 μL of 1 % HCl in MeOH was added before evaporating to dryness using the MiVac. The dried extracts were reconstituted with 500 μL of the initial mobile phase, filtered through a 0.45 μm PTFE syringe filter, and transferred to injection vials. This method achieved recoveries exceeding 50 % for all compounds except OXA and DIA, with the highest recoveries for MOR, COD, TRA and MET, all over 85 %. Overall, the recovery values in this study were higher than those reported in previous studies. For instance, Pascual-Caro et al. [21], using ExtraBond SCX sorbent with 5 mL of sample at pH 4 for the extraction of a group of 40 drugs, reported recoveries lower than those found in the present study; for example, the recoveries of COD and MET were between 15 and 30 points lower. Similarly, Yang et al. [32], using the ACCUBOND EVIDEX sorbent, a silica bifunctional (C_8/SA) sorbent for the determination of 10 drugs of abuse, including MOR and COD, also obtained lower recoveries compared to this study. For example, their method yielded 82 % recovery for COD and 71 % for MOR, while this study achieved 88 % recovery for both compounds. In another study Fernández et al. [54] used Oasis MCX sorbent with an automated SPE system to extract 23 opioids and metabolites. While their recovery values were comparable to those in the present study for most compounds, their elution solvent was a mixture of DCM and isopropanol ammonia (80:20, v/v) presenting a disadvantage in terms of environmental sustainability compared to the present study, which used basified MeOH as the elution solvent.

Additionally, Musile et al. [31], in their determination of a group of

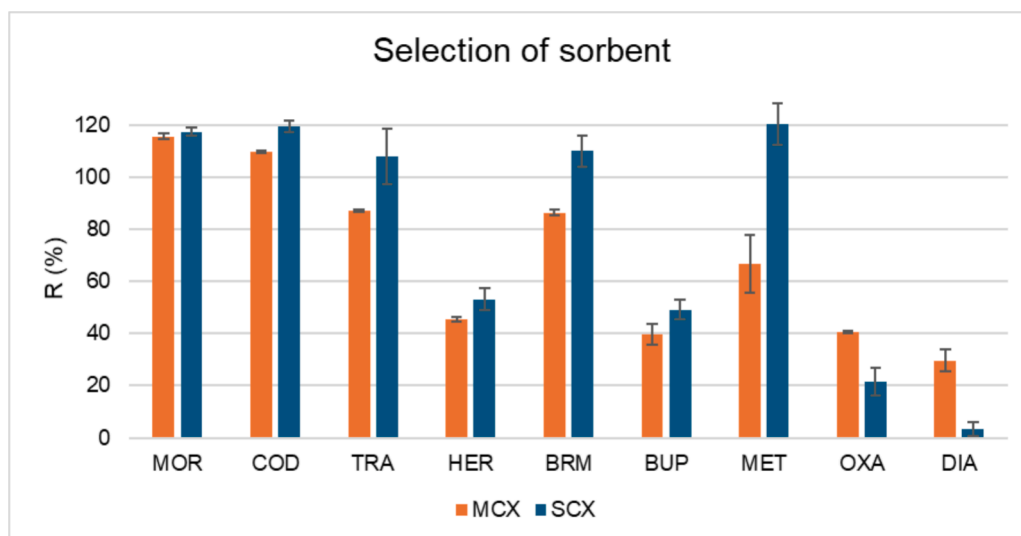


Fig. 3. Comparison of recoveries achieved with sorbents Oasis MCX and ExtraBond SCX in the optimization of SPE-based method.

drugs, including MOR, COD and MET, used an in-house mixed mode sorbent, and the achieved recoveries were in the same range as those in this study. However, their method required a higher elution volume (9 mL), which is a drawback in terms of greenness when compared to the lower solvent volume used in present study.

Thus, the specificity of the present method, focused on two families of drugs, had a direct impact on achieving more efficient extraction compared to previously reported methods, enhancing the analytical performance and reliability of the results for the determination of opioids and benzodiazepines than in previous research.

3.3. DLLME optimization

The DLLME procedure involves the rapid addition of low volumes of an extractant solvent along with a dispersant solvent to an aqueous sample. In this procedure, both solvents are added simultaneously to promote efficient dispersion and extraction. The extractant solvent, typically an organic solvent, must be immiscible with the aqueous sample and denser than water. Conversely, the dispersant solvent should be miscible in both phases, facilitating the interaction of analytes with the extraction solvent [43,47,55,56]. Selecting the most appropriate solvents is critical for achieving efficient extraction. Other important parameters, such as sample pH and the type and volume of solvents, also require optimization. For the optimization, the recovery values were used as the key response factor.

Before optimizing the solvents, the sample pH was optimized, as pH significantly influences extraction efficiency. For effective extraction, the compounds must be in their neutral form. To optimize this parameter, 200 μ L of chloroform (TCM) was used as the extractant, and 500 μ L of MeOH as the dispersant. The choice of these solvents was based on previous literature [37,41], including studies by Ranjbari et al. [41] and Fischella et al. [37], where TCM and MeOH were successfully used as extractant and dispersant, respectively, to extract similar compounds.

Two pH values, 7 and 10, were evaluated according to the physicochemical properties of the compounds under study. A working solution of 5 mL of urine:H₂O (50:50, v/v), spiked with all analytes at a concentration of 2.5 ng/mL, was adjusted to the corresponding pH using NH₄OH. As for SPE, urine samples were previously diluted 50:50 to reduce matrix interferences and to allow for more precise and effective pH adjustment.

The recoveries achieved for most compounds, except MOR, COD and TRA, were higher than 30%. The low recoveries of these three drugs at both pH values may be attributed to their polar characteristics, due to

the presence of alcohols and/or phenolic groups in their structures (log P values lower than 1.35) (Fig. 1), resulting in reduced affinity with the organic solvent and consequently, lower extraction efficiency. For all compounds, except BUP, higher recoveries were achieved at pH 7. Therefore, pH 7 was selected for extraction.

The selection of dispersant and extractant solvents is crucial in the development of a DLLME method. Different combinations of extractant and dispersant solvents were evaluated, including TCM and DCM as extractants, and five dispersant solvents: MeOH, EtOH, ACE, ACN and EtOAc. The extraction efficiency of the different combinations of solvents was evaluated using a mixture of 5 mL of urine:H₂O (50:50, v/v) at pH 7, spiked with all the analytes at a concentration of 2.5 ng/mL. Generally, the volume of extractant is lower than the dispersant volume [38–41]; thus, the volume of dispersant was fixed at 500 μ L and the extractant at 200 μ L.

Table 2 shows the recoveries obtained with the different solvent combinations. MOR, COD and TRA consistently exhibited the lowest recoveries across most solvent combinations, aligning with their polar characteristics.

Regarding the extractant, for most of the compounds, recoveries achieved with TCM were generally higher than those with DCM, except when combined with EtOH, where DCM yielded slightly better results (most of the compounds were 6 points higher). Consequently, TCM was selected as the extractant solvent.

Comparing the results achieved with TCM and different dispersants, ACE and EtOAc provided the highest recoveries for most compounds. Recoveries with EtOAc were consistently higher for all compounds, except for HER, BRM and MET, making it the optimal dispersant solvent.

Next, the extractant volume was optimized while keeping the dispersant volume at 500 μ L of EtOAc. Different volumes of TCM (150 μ L, 200 μ L and 250 μ L) were tested. Compound recoveries achieved with 150 μ L were lower, while those with 200 μ L and 250 μ L were comparable. To minimize solvent usage and maintain efficient green extraction, 200 μ L of TCM was selected as the optimal extractant volume, balancing efficiency and environmental sustainability.

In the next optimization step, we evaluated different volumes of dispersant solvent (200 μ L, 500 μ L, 1000 μ L and 1500 μ L). Recent trends in DLLME have demonstrated that eliminating the dispersant solvent can yield satisfactory recoveries. For example, Zhu et al. [57], developed a method for the determination of piperazines without using a dispersant solvent, achieving recoveries exceeding 70%. Similarly, Mercieca et al. [58], developed a dispersant-free method for the extraction of cannabinoids with recoveries higher than 50%. Considering these positive

Table 2Comparison of recoveries (%R) achieved with different dispersants (500 μ L) and extractant (200 μ L) solvents in the DLLME-based method.

Compound	TCM (extractant)					DCM (extractant)				
	Dispersants					Dispersants				
	MeOH	ACE	ACN	EtOH	EtOAc	MeOH	ACE	ACN	EtOH	EtOAc
MOR	8.5	3.8	12.8	10.0	12.8	4.2	11.8	10.6	9.7	14.2
COD	22.2	11.0	29.4	23.4	17.7	7.0	14.6	22.8	17.8	29.6
TRA	23.1	12.4	45.7	25.1	26.8	8.0	17.1	36.7	21.1	28.7
HER	30.0	120.0	64.5	38.6	62.8	26.4	120.1	50.0	45.4	72.5
BRM	65.2	105.5	65.0	35.3	81.1	20.4	100.4	52.1	41.2	123.0
BUP	94.5	80.2	39.6	21.4	84.8	41.9	54.1	31.4	27.0	51.2
MET	48.1	80.6	78.7	41.0	78.4	27.2	86.7	60.8	51.7	66.0
OXA	47.4	63.9	59.4	31.5	82.0	25.3	53.5	48.8	37.3	74.8
DIA	74.0	70.0	57.2	31.7	92.7	28.4	52.8	39.5	38.7	72.1

results, we investigated the feasibility of omitting the dispersant solvent in our study focusing on the extraction of opioids and benzodiazepines.

Fig. 4 shows the results obtained for the optimization of the volume of dispersant for all of the compounds except for MOR, COD and TRA, which exhibited consistently low recoveries across all tested volumes, evidencing their distinct behaviour. For the remaining analytes, our results clearly demonstrate the crucial role of the dispersant solvent in the DLLME process. When no dispersant solvent (0 μ L EtOAc) was used, recoveries fell below 50 %. This indicates that for our target analytes, the dispersant solvent played a crucial role in enhancing extraction efficiency. When we increased the volume of EtOAc from 200 μ L to 500 μ L, we observed a clear improvement in recoveries. This trend highlights the fact that the dispersant solvent facilitates the distribution of the extraction solvent through the aqueous sample, enhancing contact with the analytes and thus improving extraction efficiency. However, when the dispersant volume was increased beyond 500 μ L, recoveries did not significantly improve. Therefore, 500 μ L was defined as the optimal volume of solvent, as this volume allows effective analyte recovery while providing a method which aligns with green chemistry principles, ensuring a sustainable extraction protocol.

The optimal DLLME extraction conditions were established as follows: extraction of a 5 mL of a mixture of urine:H₂O (50:50, v/v) at pH 7 using 200 μ L of TCM as the extractant solvent and 500 μ L of EtOAc as the dispersant solvent. Under these conditions, the recoveries achieved for most compounds exceeded 20 %, except for MOR and COD. DIA, BUP and BRM had the highest recoveries, both exceeding 80 %.

In comparison with other similar studies, the recoveries achieved in our study are significantly higher demonstrating the effectiveness of our method. For instance, Fisichella et al. [37], used 100 μ L of TCM as the

extractant and 250 μ L of MeOH as the dispersant solvent for the extraction of a group of benzodiazepines including DIA, OXA and BRM. Their reported recoveries for these compounds were 65 % for DIA and 38 % for OXA and BRM, whereas our study achieved recoveries exceeding 80 % for these analytes. This substantial improvement can be attributed to various factors, including the choice of dispersant solvent. In our case, we used EtOAc instead of MeOH, and our results showed that EtOAc was more efficient in promoting the dispersion of the extractant solvent within the aqueous phase, enhancing the interaction of the analytes with the extractant solvent. Another factor that could contribute in the increase of the recoveries in the present study was the volume of TCM used as extractant, which was 200 μ L, double the volume used by Fisichella et al. [37].

Similarly, Ghambarian et al. [39], developed a method for the determination of a group of benzodiazepines, including DIA. In their study, the authors used acetone (1 mL) as the dispersant and a mixture of dodecane/butylacetate (150 μ L) as the extractant. They reported recoveries around 60 %, significantly lower than those achieved in our study. This highlights the critical role solvent selection plays in extraction efficiency. In another study, Ranjbari et al. [41] reported higher recoveries (around 100 %) for MET using 250 μ L of TCM as the extractant and 2.5 mL of MeOH as the dispersant, but their method required significantly larger solvent volumes, particularly for the dispersant. This presents a drawback in terms of environmentally sustainability. Additionally, the method of Ranjbari et al. [41], focused on MET, which limits its applicability in cases of polyconsumption, which is common in forensic and clinical toxicology where multiple substances often need to be determined simultaneously [39,41].

The versatility of our method allows for the simultaneous extraction

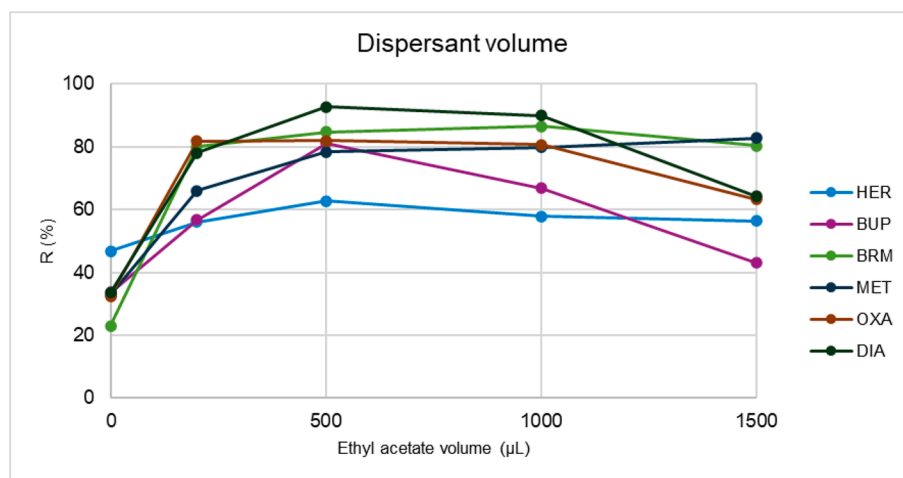


Fig. 4. Optimization of the volume of dispersant solvent for the DLLME-based method. Comparison of the recoveries achieved with 0 μ L, 200 μ L, 500 μ L, 1000 μ L and 1500 μ L of EtOAc.

of compounds from different drug families, benzodiazepines and opioids. This capability is particularly valuable in forensic and clinical toxicology, where a wide range of substances frequently need to be determined from complex matrices.

3.4. Methods validation

The instrumental linearity of the methods in the present study ranged from 0.05 to 5 to 100–250 ng/mL. LODs achieved ranged from 0.05 ng/mL to 0.1 ng/mL, while LOQs ranged between 0.05 and 5 ng/mL. Precision, evaluated in terms of the reproducibility (inter-day) and repeatability (intra-day) of the method using %RSD ($n = 5$), yielded satisfactory results with values of less than 4 % for all compounds.

The following subsections present and discuss the validation results of the SPE-based method (Table 3) and the DLLME-based method (Table 4). For both methods, matrix-matched calibrations were performed, and recoveries and precision were evaluated at three concentration levels in urine: low (5 ng/mL), medium (25 ng/mL) and high (60 ng/mL).

3.4.1. SPE-based method validation

Table 3 summarizes all validation parameters for the SPE method developed in this study. The linearity range, obtained by matrix-matched calibration curves using 10 calibration points, ranged from the MQL to 75 ng/mL for all compounds. The MQL ranged from 1.25 to 5 ng/mL, and the MDLs ranged from 0.1 to 5 ng/mL. These values were significantly lower than the acceptance criteria established by the guidelines for workplace drug testing in urine (200 ng/mL) [48], demonstrating the method's high sensitivity. The method exhibited good precision, with reproducibility (intra-day) and repeatability (inter-day) expressed as %RSD ($n = 5$) at three concentration levels with values of less than 30 % in all cases. Compared to other studies, such as that of Pascual-Caro et al. [21], which also developed a method based on SPE-LC-MS/MS for determining 40 drugs of abuse including some of our target analytes, the MDLs and MQLs were in the same range of concentrations and were significantly lower than the expected concentration ranges in urine samples. Fernández et al. [54], developed a method for the determination of 23 opioids and metabolites, with a linear range bigger than the present study, but the MDLs and MQLs were higher than in the present study. Similarly, Musile et al. [31] developed a method for the determination of 16 basic drugs using an in-house sorbent, achieving higher limits, in comparison with this study. The present method demonstrated reliable performance for simultaneously quantifying a group of benzodiazepines and opioids.

3.4.2. DLLME-based method validation

The validation of the DLLME method was performed with a matrix-

matched calibration, using 10 calibration points, the results of which are summarized in Table 4. The calibration range extended from the MQL and 80 ng/mL. MDLs achieved ranged from 0.1 ng/mL to 2.5 ng/mL, and MQL ranged from 1 ng/mL to 2.5 ng/mL. These limits were considerably lower than the acceptance criteria established by the guidelines by workplace drug testing in urine (200 ng/mL) [48]. Also, these limits were lower than those reported in other studies, such as Meng et al. [40], who used DLLME combined with capillary electrophoresis for the determination of a group of drugs of abuse, including MOR and COD. [39].

Precision, evaluated as intra-day and inter-day %RSD ($n = 5$) at three levels of concentration, exhibited values of less than 30 % in all cases. This method demonstrated good performance in simultaneously quantifying a group of benzodiazepines and opioids at the typical levels that these compounds can be found in urine samples.

3.5. Application to real cases

After the development and validation of both methods, they were applied to the analysis of 11 urine samples from women initiating a detoxification programme at CECAS. Table 5 presents the results for the compounds detected in each specimen. For samples 8 and 9, the concentration of MET exceeded the validated linearity range. To ensure accurate quantification, the extracts obtained after SPE or DLLME method were diluted prior to their analysis to bring their concentrations within the validated linear range of the methods. To evaluate the potential variation in recoveries when analyte concentration exceeded the linear calibration range, urine samples were spiked at high concentrations (1000 ng/mL and 2000 ng/mL) and analysed using both methods, with the extracts diluted prior to analysis. The results confirmed that the recoveries remained consistent and were not affected by the dilution process, validating the application of this approach.

At least one compound was detected in each specimen, with a predominant trend of drug polyconsumption observed in 9 out of the 11 analysed samples, where more than one compound could be determined. This observation is consistent with previous findings from several recent studies, which reported an increasing trend of polyconsumption [2,17,21,59].

MET was detected in the highest number of specimens (10 out of 11), although quantification was not possible in all cases. Using the SPE method, MET could be quantified in 40 % of the specimens at concentrations ranging from 2.5 to 1980 ng/mL. Conversely, using the DLLME method, we were only able to quantify MET in two specimens (8 and 9) with concentrations of 172 and 1205 ng/mL, respectively.

HER was detected in 36 % of the samples and could only be quantified in a sample with the DLLME method, specifically in specimen 11, at a concentration of 1.6 ng/mL. The SPE method could detect but not

Table 3
Validation results of SPE-based method.

Compound	MDL (ng/mL)	MQL (ng/mL)	Lineal range (ng/mL)	5 ng/mL			25 ng/mL			60 ng/mL		
				R%	Intra-day precision RSD (%) (n = 5)	Inter-day precision RSD (%) (n = 5)	R%	Intra-day precision RSD (%) (n = 5)	Inter-day precision RSD (%) (n = 5)	R%	Intra-day precision RSD (%) (n = 5)	Inter-day precision RSD (%) (n = 5)
MOR	0.25	1.25	1.25 – 75	83.2	4.1	10.9	88.4	3.5	24.9	107.9	5.4	2.8
COD	0.25	2.5	2.5 – 75	78.1	9.3	14.5	99.3	13.1	12.2	86.3	5.9	4.1
TRA	0.1	1.25	1.25 – 75	88.1	7.5	2.3	116.7	10.9	9.3	84.8	8.2	6.7
HER	1.5	5	5 – 75	42.6	25.3	28.7	53.2	27.1	24.9	47.5	28.6	29.0
BRM	0.5	2.5	2.5 – 75	61.9	15.8	17.9	69.7	18.4	16.4	42.5	13.1	10.6
BUP	1.25	2.5	2.5 – 75	31.5	15.7	10.4	40.6	20.1	24.6	44.4	17.3	9.4
MET	1.25	2.5	2.5 – 75	80.4	3.5	9.7	91.2	26.8	23.4	70.9	8.9	7.7
OXA	0.25	1.25	1.25 – 75	15.0	4.9	6.1	19.9	17.1	18.1	14.1	7.6	9.5
DIA	1.25	2.5	2.5 – 75	11.2	25.7	25.3	8.7	22.8	25.1	9.6	20.2	26.2

Table 4

Validation results of DLLME-based method.

Compound	MDL (ng/mL)	MQL (ng/mL)	Lineal range (ng/mL)	5 ng/mL			25 ng/mL			60 ng/mL		
				R%	Intra-day precision RSD (%) (n = 5)	Inter-day precision RSD (%) (n = 5)	R%	Intra-day precision RSD (%) (n = 5)	Inter-day precision RSD (%) (n = 5)	R%	Intra-day precision RSD (%) (n = 5)	Inter-day precision RSD (%) (n = 5)
MOR	0.5	1	1 – 70	9.9	10.1	6.7	9.9	29.0	32.3	14.3	12.0	8.3
COD	0.5	1	1 – 70	17.0	14.3	12.9	17.1	24.3	39.0	25.0	14.6	13.2
TRA	0.1	1	1 – 70	19.3	16.8	12.0	24.0	26.0	31.1	29.7	12.8	14.6
HER	1	1	1 – 70	40.9	12.3	7.0	58.5	16.0	12.5	45.8	8.8	16.2
BRM	0.1	1	1 – 80	78.1	20.6	5.0	80.5	22.9	19.5	70.1	8.0	11.0
BUP	2.5	2.5	2.5 – 80	63.2	16.1	10.2	80.5	19.6	14.9	71.2	16.0	18.0
MET	0.25	1	1 – 70	73.2	14.3	12.0	72.7	14.5	19.0	68.9	11.1	14.1
OXA	0.1	1	1 – 80	64.2	12.0	4.7	77.3	11.9	16.6	56.5	17.6	16.1
DIA	0.1	1	1 – 80	73.0	18.2	9.0	86.8	18.3	13.4	71.7	18.4	22.7

Table 5

Compounds detected with both methods in urine samples from women starting a detoxification programme (n = 3).

Specimen	Founded analytes	Concentration (ng/mL)	
		SPE	DLLME
1	MOR	<MQL	n.d.
	TRA	1.7 ± 1.1	n.d.
	HER	<MQL	<MQL
	BRM	n.d.	<MQL
	MET	<MQL	<MQL
	DIA	n.d.	<MQL
2	MET	2.5 ± 1.6	n.d.
	OXA	<MQL	2.8 ± 0.1
	DIA	<MQL	3.5 ± 0.1
3	BRM	<MQL	<MQL
	MET	6.2 ± 0.2	4.75 ± 0.2
4	MET	<MQL	<MQL
5	MET	<MQL	<MQL
	DIA	n.d.	<MQL
6	TRA	1.6 ± 0.1	<MQL
	MET	<MQL	<MQL
	DIA	n.d.	<MQL
7	MET	<MQL	<MQL
8	HER	<MQL	<MQL
	MET	1980 ± 10	1205 ± 20
9	MET	199 ± 10	172.5 ± 1.2
	DIA	n.d.	1.2 ± 0.2
10	HER	<MQL	<MQL
	OXA	n.d.	1.4 ± 0.1
	DIA	n.d.	1.2 ± 0.2
11	HER	<MQL	1.6 ± 0.1
	MET	<MQL	<MQL

quantify HER due to a higher MQL.

DIA only could not be quantified using the SPE-based method due to the low recoveries and lower concentration values, but it was detected in 6 out of 11 specimens with the DLLME method. Additionally, other compounds detected in fewer specimens with the DLLME method included OXA (2 specimens), TRA and BRM (2 specimens).

TRA could only be quantified using the SPE method, at concentrations of 1.6 and 1.7 ng/mL in specimens 6 and 1, respectively. Meanwhile, OXA could only be quantified using the DLLME method, with concentrations ranging from 1.4 to 2.8 ng/mL and the highest concentration detected in specimen 2.

The results presented in Table 5 indicate that benzodiazepines are more commonly consumed than opioids. This is likely due to their general use in medicine for the treatment of anxiety and insomnia. Moreover, MET was the most frequently detected opioid, which aligns with the experimental context in which samples were obtained from women following a detoxification programme. MET has been extensively used in recent decades for treating drug disorders [41].

3.6. Method comparison

In this study, two methods were developed, validated and compared. The comparison of validation methods (Tables 3 and 4) revealed higher recoveries for HER, BRM, BUP, OXA and DIA with the DLLME-method, with recoveries exceeding 60 %. For MET the recovery was over 75 %, which was significantly higher than that achieved with the SPE method. Conversely, lower recoveries were observed for MOR, COD and TRA, likely due to their highly polar nature, making efficient extraction challenging with non-polar organic solvents like TCM. On the other hand, for MOR, COD, TRA, and MET, the SPE method yielded the highest recoveries, surpassing 85 %. Additionally, HER and BRM exhibited recoveries higher than 50 %, whereas DIA and OXA achieved the lowest recoveries (below 25 %). The difference in recoveries between the DLLME and SPE methods is primarily due to the distinct physicochemical properties of benzodiazepines and opioids as well as their charge states during the extraction.

MDLs achieved with both methods were within the same concentration range, with no significant differences observed. However, consistent with the extraction efficiency results, the DLLME method achieved lower MDL values for benzodiazepines and HER. MET had lower MDL values with DLLME while the lowest MDL for BUT was achieved with the SPE method. Other opioids also had the lowest MDL values with the SPE method.

One of the primary objectives of this study was to develop the most environmentally friendly method feasible. To accomplish this, the methods were evaluated using the AGREE-Analytical GREENness Metric Approach, with which the environmental impact of each step of the developed methods was evaluated on a scale from 0 to 1, with 1 indicating the highest level of environmental friendliness. Fig. 5 illustrates the classification of both methods (SPE-based and DLLME-based) according to their environmental scores, with the DLLME-LC method receiving a higher score of 0.54 compared to the SPE-LC method with a score of 0.49. The scores encompass 12 different points of evaluation [36,60]. Although there was not a great difference between the scores of the two methods, the DLLME-LC method achieved a higher score,

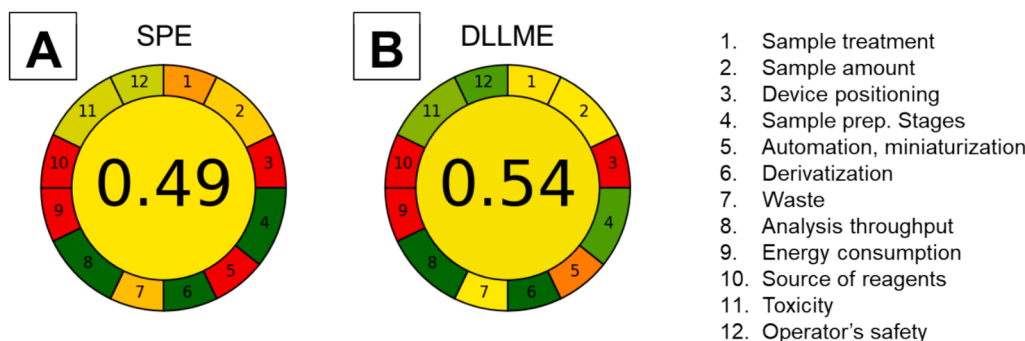


Fig. 5. Method evaluation according to the AGREE-Analytical GREENess Metric Approach: Evaluation of SPE method (A) and DLLME method (B).

making it the more environmentally friendly method.

Several factors contributed to the higher environmental score of the DLLME-based method. Specifically, the SPE-based method scored lower in points 2 and 7 compared to the DLLME method, as it required larger volumes of solvent and sample, resulting in the increased generation of waste. However, scores for other points were similar for both methods, which is why the colours shown for each point in Fig. 5 are similar. Points 3, 9 and 10 are the most critical points in both methods, represented in red in both graphics in Fig. 5. Point 9 evaluated the energy consumption of equipment, related in both cases to the higher energy consumption of the LC-MS/MS equipment. Point 10 assessed the origin of the reagents, and as both methods made use of non-bioreagents, both achieved lower scores. Point 3 evaluated the positioning of the analytical device (in-line, off-line, on-line, at-line), with off-line being the least green option, used in both methods.

In conclusion, based on the AGREE method, it is evident that the DLLME method is slightly greener than the SPE method.

4. Conclusions

In this study, two methods based on SPE and DLLME followed by LC-MS/MS were successfully developed and validated for the determination of a group of opioids and benzodiazepines in urine samples. For the optimization of the SPE method, two SPE sorbents were evaluated and compared, and ultimately Extra Bond SCX was selected as the optimal choice, as it achieved recoveries above 50 % for most of the compounds. The optimization of the DLLME method involved testing two extractant solvents and five dispersant solvents, with TCM (extractant) and EtOAc (dispersant) selected as the optimal solvents as they yielded recoveries exceeding 30 % for most of the compounds under study. Both methods were successfully validated. To demonstrate the practical applicability of the methods, twelve urine samples from women undergoing a detoxification process were analysed. A trend of polyconsumption was observed in most samples (10 out of 11), with MET being the most frequently detected and quantified compound at the highest concentration using both methods. Finally, according to the AGREE, the DLLME method (0.54) demonstrated greater environmental sustainability than the SPE method (0.49), highlighting its potential as an eco-friendlier approach in this context.

Overall, our findings contribute to the advancement of analytical methodologies for drug analyses in urine samples and emphasize the importance of considering the environmental impact when developing analytical methods.

Compliance with ethical standards

Ethical approval: All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals per-

formed by any of the authors.

Informed consent: Informed consent was obtained from all individual participants included in the study.

CRedit authorship contribution statement

Pol Clivillé-Cabré: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Luana M. Rosendo:** Validation, Methodology, Investigation, Conceptualization. **Francesc Borrull:** Project administration, Funding acquisition, Conceptualization. **Carme Aguilar:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Marta Calull:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, 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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Data availability

No data was used for the research described in the article.

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