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APPLICATION NOTE

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Absence of new psychoactive substances in wastewater from South Wales, UK, revealed by optimised liquid chromatography-time-of-flight analysis

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Abstract

New psychoactive substances are produced and marketed to mimic the effects of their illicit counterparts and to attempt to evade drug tests and prosecution. Here, we present the optimisation, validation and application of an analytical method using liquid chromatography–time-of-flight mass spectrometry to detect and quantify 37 new psychoactive substances and illicit substances in wastewater from South Wales, UK, using a targeted analysis method. Sample preparation was performed using solid-phase extraction with Oasis HLB cartridges. The LC separation was performed using a YMC-Triart Phenyl 450 bar column (12 nm, 5 µm, 100 × 3 mm) which provided good separation and resolution for all targeted analytes with a run time of 9 min. The method was validated using the following parameters: sensitivity, selectivity, linearity, accuracy, precision, recovery and matrix effects. The method was then applied to influent wastewater samples collected from two wastewater treatment plants in Wales, UK.

KEYWORDS
illicit drugs, LC-ToF-MS, new psychoactive substances, solid-phase extraction, wastewater

1 | INTRODUCTION

New psychoactive substances (NPSs) are substances that are not controlled by the United Nations Drug Conventions but pose a similar risk to listed compounds. These compounds mimic the effects of the more common illicit drugs like cocaine, amphetamine and cannabis. They were introduced and marketed to evade drug tests or prosecution by providing slight modifications to the chemical properties and structures of already established compounds.[1] The major NPS groups have been monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) Early Warning System (EWS) since 2005. In 2021, 52 NPSs were first reported in Europe through the EWS with the total NPSs currently being monitored at the EMCDDA reaching 880.[2] Since 2005, 13 NPS categories have now been established by the EMCDDA, and this includes aminoindanes, piperazines, piperidines and pyrrolidines, arylcyclohexylamines, benzodiazepines, tryptamines, arylalkylamines, opioids, phenethylamines, cathinones, synthetic cannabinoids, plants and extracts and other substances (derivatives, medicinal products, intermediates and precursors).[3] The NPS epidemic is not recent; these substances first emerged within society in the 1960s when some research groups identified that drugs can legally be sold that can mimic the effects of illicit drugs.[4]

NPSs and illicit substances have been detected in wastewater using liquid chromatography mass spectrometry (LC–MS) analysis in many countries around the world. A study conducted in multiple European countries in 2016 detected methylenedioxypyrovalerone (MDPV) at 0.65 ng/L and mephedrone at 13.90 ng/L with mephedrone detected again in 2017 at 18.31 ng/L.[5] This study conducted in Australia by Bade et al. explored the quantification of NPSs with method validation parameters covering 32 NPS analytes. The results of this study concluded that only clonazepam, etizolam and alprazolam were detected with concentrations ranging between 0.4–5.8 ng/L.[6]

We present the inaugural study of NPSs within the wastewater of South Wales, UK. The most recent investigation into illicit drugs within the same region transpired in 2009, concentrating on pharmaceuticals and illicit drugs within surface water and wastewater contexts.[7–10] Our liquid chromatography–time-of-flight mass spectrometry (LC-ToF-MS) acquisition method accurately identified and quantified 37 NPSs and illicit substances (35 NPSs plus benzoylecgonine and cannabis) above the instruments and methods' detection limit. Method validation guidance was followed from ISO 17025:2017 and the Scientific Working Group for Forensic Toxicology (SWGTOX). Once the method was validated and within the criteria, the method was applied to wastewater samples collected from two wastewater treatment plants (WWTPs) in South Wales, UK.

2 | MATERIALS AND METHODS

Certified reference standards and deuterated internal standards for the NPS analytes were purchased from either Chiron (Norway) or Merck (Darmstadt, Germany). Dilutions and working standard mixtures with concentrations ranging between 0.02 and 1 ng/mL were further prepared using HPLC-grade methanol (MeOH). HPLC-grade ACN, MeOH and formic acid were purchased from Rathburn Chemicals (Walkerburn, UK). Ammonium acetate was purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained by purifying tap water in an ELIX Millipore water purifier obtained from Millipore (Darmstadt, Germany). Oasis HLB (500 mg, 6 cc) and Oasis MCX (500 mg, 6 cc) SPE cartridges were purchased from Waters (New Bedford, MA, USA).

2.1 | Sample preparation

Samples for method validation (25 mL of wastewater) were prepared by spiking with a known concentration methanolic standard (50 ng/mL). Because of the physicochemical differences between the compounds, both Oasis HLB and Oasis MCX sorbents were evaluated for extraction.

For Oasis HLB, conditioning of the cartridges was undertaken with 6 mL MeOH and 6 mL of deionised water. The 25 mL samples were loaded under gravity and left to stand in the cartridge for 1 min, the samples were subsequently pulled through under reduced pressure and the remaining sample volume continued loading under reduced pressure until all the samples were pulled through the SPE cartridge. Cartridges were washed with 3 mL of deionised water. After drying under reduced pressure for 5 min, the cartridges were eluted with 8 mL MeOH.

For Oasis MCX, the cartridges were conditioned with 6 mL MeOH, 4 mL deionised water and 4 mL acidified deionised water to pH 2. The 25 mL samples were loaded under gravity to the top and left to stand in the cartridge for 1 min, and the samples were subsequently pulled through under a reduced pressure until all the remaining sample volume was loaded. Cartridges were washed with 3 mL deionised water, 2 mL acidified deionised water (pH 2), followed by 3 mL MeOH. After drying under reduced pressure for 5 min, the cartridges were eluted with 4 mL MeOH followed by 4 mL NH_3:MeOH (5:95 v/v).

Once elution was complete, the eluent was evaporated under a gentle stream of nitrogen at 55°C, and the dried residue was reconstituted in 100 µL of ACN followed by 100 µL of 5 mM ammonium acetate for HLB cartridges and 200 µL 90% MeOH for MCX cartridges. Samples were thoroughly vortexed, then transferred to a 96-deep well plate and injected into the LC-ToF-MS system.

2.2 | Instrumentation

Analysis was carried out using an AB Sciex 5600+ LC-ToF-MS equipped with a binary pump, column oven thermostat and an electrospray ionisation (ESI) source. Chromatographic separation of each drug is performed on a YMC-Triart Phenyl 450 bar column (12 nm, 5 µm, 100 × 3 mm) (Crawford Scientific, UK). The temperature of the column over was set at 50°C, and the sample injection volume was 10 µL. The gradient method was developed over 9 min including equilibrium. The mobile phase consists of (A) 5 mM ammonium acetate and 0.2% formic acid and (B) MeOH. A gradient programme was used, starting at 10% B, 0 min; 2 min; 10% B, 3 min 70% B, 7 min 85% B, 7.5 min 100% B, 8 min 10% B, and hold for 1 min.

An AB Sciex 5600+ time-of-flight mass spectrometer equipped with an electrospray interface was used for the detection and quantification of analytes of interest. The drying gas temperature was 500°C.

Quantitative analysis was performed using Sequential Window Acquisition of all Theoretical fragmentation ion spectra (SWATH®). SWATH® acquisition for this method contains 50 SWATH® windows, 5 Da wide with 1 Da overlap on each side of the window. The first SWATH® window starts at 175 *m/z*, and the final window ends at 505 *m/z* in positive polarity mode. Analyst® software was used for system control, and MultiQuant software was used for quantitative analysis. Peakview was also available, if necessary, for qualitative analysis. The choice of transition ion for each analyte was based on the abundance of the signal, against background noise during the method development. Table S3 gives an overview of the MS parameters and the retention times of all analytes and internal standards.

2.3 | Method development and validation

Every compound was quantified using SWATH® acquisition in positive ionisation mode where the mass of the compound was 'searched' within a particular SWATH® window that corresponds to the monoisotopic mass of that compound. An additional confirmation criterion was that the retention time of the compound should not differ more than 2.5% from the calibration or quality control (QC) standards.[11] The source parameters that include collision energy and collision energy spread were optimised to acquire the most intense protonated molecular species $[M + H]^+$ for each compound. The collision energy (CE) for the method was set at 25 eV with a collision energy spread (CES) of 15 eV allowing a CE range from 10 to 40 providing a richer MS spectrum. During the acquisition, the accumulation time is divided by 10 steps, and this is time spent at each point of the CES ramp. Compounds were identified and optimised based on the most intense peak in terms of signal-to-noise (S/N) at MS^1, MS^2 or a combination of both.

LC optimisation was conducted using a standard methanolic mixture containing all 37 analytes injected onto a 5 cm C18 reverse-phase column and a 10 cm phenyl butyl column, together with the use of the same mobile phases ([A] 5 mM ammonium acetate and 0.2% formic acid and [B] MeOH). The compounds' retention time and peak shape were tested to determine the best optimisation.

The optimisation of a suitable SPE cartridge with different sorbent materials plays a crucial role in the attainment of high and reproducible recovery of analytes. To determine the relative recovery over all 37 analytes of interest, two sorbents (Oasis HLB and Oasis MCX) were tested under different extraction methods that were previously trialled in other literature.[12] SPE recovery was assessed by comparing the results of spiked 25 mL samples at a concentration of 50 ng/mL. Relative recovery is presented in Table S4.

Method validation was conducted based on UKAS ISO 17025 and the SWGTOX Standard Practices for Method Validation in Forensic Toxicology (SWGTOX. 2013) with some exceptions. Validation was conducted to evaluate performance features such as accuracy, precision, linearity, recovery and ion suppression.

Calibration working standards were prepared by spiking a 25 mL wastewater sample with a combined methanolic standard containing all 37 analytes before SPE clean-up. The dynamic range was set to contain six calibration points ranging from 0.02 to 1.0 ng/mL. The calibration curve was used for the quantification of crude, influent wastewater samples. As it was not possible to obtain an internal standard for all 37 compounds, 10 internal standards were selected to cover the whole acquisition method (see Table S1). The choice of internal standard was determined by similarities in chemical structure, retention time similarities and monoisotopic mass similarities. An acceptable calibration curve was determined when the R^2 value was >0.99. All target analytes were included in a single calibration curve.

In addition to calibration standards, blank tap water sample (processed sample without IS) and three QC samples at the low, mid and high end of the calibration range (0.08, 0.3 and 0.8 ng/mL). Calibration curves were plotted by the MultiQuant software by plotting the ratio between the peak area of the compound and the corresponding internal standard against the spiked calibration standard concentration.

The intra-day and inter-day accuracy and precision of the method were assessed by analysing spiked samples (25 mL) for all compounds at three concentration levels, situated at the lower end (QC low), mid-range (QC mid) and top-end (QC high) of the linear range. In total, 55 QC samples were analysed over five different days spiked with either low, mid or high QC concentration (0.08, 0.3 and 0.8 ng/mL). Precision and accuracy were assessed with an acceptable criterion within 85% to 115% (mean) accuracy and <15% relative standard deviation (RSD) precision.

Matrix effects were evaluated by ion suppression and quantified during method optimisation and validation. This method was suggested in a previous literature study by Chen et al.[13] that involved a comparison of analyte abundance of standards pre-extraction, post-extraction and with no extraction (neat mobile phase).

Autosampler stability was evaluated by injecting a post-extracted 96-deep well plate containing a calibration (0.02, 0.05, 0.10, 0.25, 0.50 and 1.0 ng/mL), blank and QC material (0.08, 0.30 and 0.80 ng/mL) over 5 days. The 96-deep well plate was kept loaded onto the instrument, in the same compartment. The plate autosampler is kept cooled, and the plate was injected on Days 1, 2, 3 and 5. This was to measure whether the post-extracted, reconstituted samples would remain stable if there was any instrument downtime.

2.4 | Application to wastewater samples

Two WWTPs in Wales, UK, were monitored for 1 month. The samples were collected from Friday to Monday over four weekends. There is a difference between the capacity and population coverage between both WWTPs. WWTP 1 covers an estimated population of 930,624, and WWTP 2 covers an estimated population of 301,443 as provided by the sampling WWTP. During the monitoring campaign, 24 h composite samples were collected from the influent wastewater from both WWTP 1 and WWTP 2. The composite samples were collected using an autosampler located at both WWTPs. The autosampler was programmed to collect a 1 L composite by averaging 10 mL every 15 min. One autosampler (Aquacell P2-Compact, Aquamatic) was installed at each WWTP, and the samples were collected in polypropylene bottles.

Samples were kept frozen until analysis and given 1 day to defrost in a fridge ranging between 3 and 8°C.

All samples were subjected to SPE, and the same procedure was used for all investigated analytes. In detail, all 1 L samples collected were thoroughly shaken and then aliquoted into small samples (25 mL) which in turn were spiked with 100 µL of a mixed internal standard solution at 50 ng/mL. The SPE cartridges were conditioned with 6 mL MeOH and 6 mL deionised water. The samples were loaded onto the cartridges under gravity initially with subsequent reduced pressure applied at a rate of 5 mL/min. Cartridges were then washed with 3 mL of deionised water followed by a reduced pressure drying for 5 min. Elution occurred with 4 mL of MeOH and an additional

4 mL of MeOH. The eluents were dried using a sample concentrator attached to a heating block set at 55°C. Samples were then reconstituted using 100 μL of HPLC-grade acetonitrile followed by 100 μL of 5 mM ammonium acetate. All samples were then transferred to a 96-deep well plate for analysis.

3 | RESULTS AND DISCUSSION

The extraction recovery was measured for both Oasis HLB and Oasis MCX to determine which sorbent was better suited for the extraction of the 37 compounds. Oasis HLB and Oasis MCX recoveries were comparable, both of which provided recoveries of around 50% to 131%. Oasis MCX cartridges required acidification of the samples before extraction and required a more rigorous extraction in comparison with Oasis HLB cartridges. At this stage, the recovery was measured using three different concentration levels (0.08, 0.30 and 0.80 ng/mL) methanolic standards spiked into wastewater. The efficiency of each sorbent was determined by comparing the theoretical concentration of the spiked wastewater compared with the actual concentration of spiked wastewater after extraction. Oasis HLB not only has a simpler, less rigorous extraction method but also provides a slightly greater recovery compared with Oasis MCX. As a result, the extraction method for Oasis HLB was chosen for further validation.

Oasis HLB and Oasis MCX have proven to be popular choices among researchers to cover the wide range of chemical and physical properties of NPSs within their studies.[14–16] MCX has a higher affinity for basic compounds whereas HLB has a higher affinity to a broader range of compounds and is suitable for acidic, basic and neutral compounds.[17–19] Gao et al. utilised the use of MCX cartridges for the detection of MMC, MDPV, BZP, TFMPP and mCPP and determined that the recoveries for these compounds in wastewater are 77.6% to 100.2%.[20] Baz-Lomba et al. performed a comparison study and compared the use of HLB and MCX; the study concluded that even though HLB can potentially provide a lower selectivity for the basic compound in comparison with MCX, HLB is the more sought-after cartridge when targeting a wide range of NPS compounds due to the ability to extract different physicochemical compounds.[21] The HLB cartridges provided a recovery of between 60% and 188% for 51 targeted compounds.

All compounds eluted within 9.0 min, and the total run time, including column re-equilibration, was 9.09 min. The disadvantage of SWATH® acquisition is that the method cannot distinguish between isomer pairs. UR-144 4-hydroxypentyl and UR-144 4-hydroxypentyl cannot be distinguished from each other with both being in the same SWATH® window, fragmentation and retention time. This is also the same for APINACA 4-hydroxypentyl and APINACA 5-hydroxypentyl; therefore, if, for example, UR-144 4-hydroxyphenyl or UR-144 5-hydroxyphenyl is discovered, then both analytes would be reported. No fragmentation ion was discovered for BZP as no second most abundant/unique peak was discovered. Therefore, only the molecular ion was monitored. In this instance, if BZP was discovered, the identification would be qualitative rather than quantitative.

Limit of detection, precision, accuracy and ion suppression were the criteria assessed for the method validation. This criterion was assessed against the SWGTOX requirements as stated above.[22] A linear range (R^2 > 0.99) from 0.02 to 1.0 ng/mL was achieved for all compounds investigated in this study. LOD was determined by assessing 20 blank samples, which were between 0.004 and 0.020 ng/mL for all analytes. LOQ was deemed to be the lowest calibrator level used within this study which was 0.02 ng/mL.

Precision and accuracy results at three control levels are illustrated in Table S4. For all analytes, the inter-day and intra-day mean accuracy was between 77% and 100% which is within the acceptance criteria for UKAS ISO 17025 at 33% for analytes less than 10 ng/mL. The intra-day precision ranged between 8% and 20% whereas the inter-day precision ranged between 7% and 30%. The intra-day precision is within the ISO 17025 acceptance criteria, but for some analytes (5F-APINACA and benzoylecgonine), the inter-day precision is high even though within the 33% criteria. This could be because the levels that this study is investigating are at the detection limit of the instrument; therefore, any slight degradation in the mobile phase, column or standard material causing the slightest variation will cause a dramatic change in the precision of the compound.

Ion suppression was calculated by comparing the response of each QC concentration (0.08, 0.30 and 0.80 ng/mL) spiked into 25 mL samples, pre-extraction, post-extraction and with no extraction (neat mobile phase mixture). The matrix ion suppression averaged at 50% between all analytes for QC concentration at 0.08 ng/mL, averaged at 53% at QC concentration at 0.3 ng/mL and averaged at 46% at QC concentration of 0.8 ng/mL.

The internal standard was tested for recovery. Ten internal standard compounds were chosen for this study as not all compounds had commercially available internal standards. For the analytes without internal standards, the 10 chosen internal standards were distributed between all 37 analytes and chosen based on chemical similarity and close comparable retention times.

Autosampler stability was measured to ensure that if instrument downtime occurred while samples were loaded on the instrument, the samples would remain stable. The 96-deep well plate remained loaded onto the instrument for 5 days. Autosampler stability was measured by injecting an extracted Calibration 6 and repeating the injection every day for 5 days. The results provided evidence that the samples can remain stable across 5 days if the 96-well plate is to remain on the instrument. As noted in Section 2, the autosampler on the AB Sciex Exion LC is chilled which may provide a better environment for stability in comparison with autosamplers that are not chilled.

3.1 | Application to WWTP samples

Influent 24 h composite sewage samples from two WWTPs in Wales were used to determine whether any NPSs were present within the local cities and towns that feed into the WWTP. All samples were tested against this validated method. Benzoylecgonine and 11-Nor-9-carboxy-THC were added as target analytes because it is well

TABLE 1 Results of South Wales crude wastewater samples for benzoylecgonine and alprazolam in mg/day/1000 inhabitants.

Analyte	Sample number and date								
	1	2	3	4	5	6	7	8	9
	17 October	18 October	23 October	24 October	24 October	25 October	30 October	30 October	31 October
	Wastewater treatment plant								
	1	1	1	1	2	1	1	2	1
Benzoylecgonine	3.4	3.8	2.7	2.1	1.4	2.0	7.4	2.4	5.7
Alprazolam	-	-	-	-	-	1.7	-	-	-

Note: No NPS was detected among all samples analysed.

TABLE 1 (Continued)

Analyte	Sample number and date								
	10	11	12	13	14	15	16	17	18
	31 October	01 November	06 November	06 November	07 November	07 November	08 November	13 November	14 November
	Wastewater treatment plant								
	2	1	1	2	1	2	1	2	2
Benzoylecgonine	1.8	4.1	1.0	2.6	1.6	2.1	8.5	1.0	2.5
Alprazolam	-	-	-	-	-	-	-	-	-

Note: No NPS was detected among all samples analysed.

documented that benzoylecgonine is often detected in wastewater. The detection of benzoylecgonine in wastewater samples serves as a useful indicator of method performance; 24 h composite samples were collected over a total of 4 weeks, Friday to Monday. The results of the wastewater samples can be seen in Table 1.

No NPSs were discovered within the samples from both WWTP. One sample contained 1.2 ng/mL of alprazolam. As expected, benzoylecgonine was discovered in all samples collected between both WWTPs. The concentration of benzoylecgonine varied and was dependent on the day; Sundays seemed to produce the highest values of benzoylecgonine compared with other days in the week (Figures 1 and 2). WWTP 1 shows a higher concentration overall of benzoylecgonine compared with WWTP 2. WWTP 1 covers a larger area and has a greater collection due to a higher population. As benzoylecgonine was discovered, it has proven that there are no issues with the method in its entirety, but there were little to no targeted analytes present within this collection area. This may be because NPS has evolved greatly from the targeted analytes investigated within this method or individuals are not consuming as much NPSs in Wales post-2016 legislation implementation and are sticking to the more

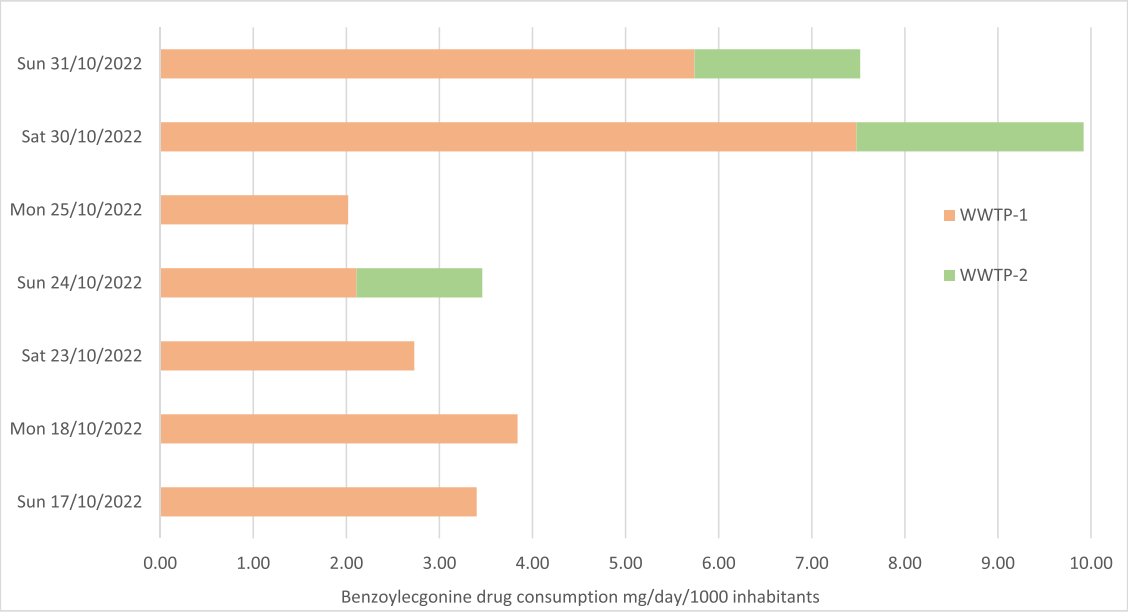

FIGURE 1 Consumption of benzoylecgonine in mg/day/1000 inhabitants in South Wales Sunday 17 October–Sunday 31 October.

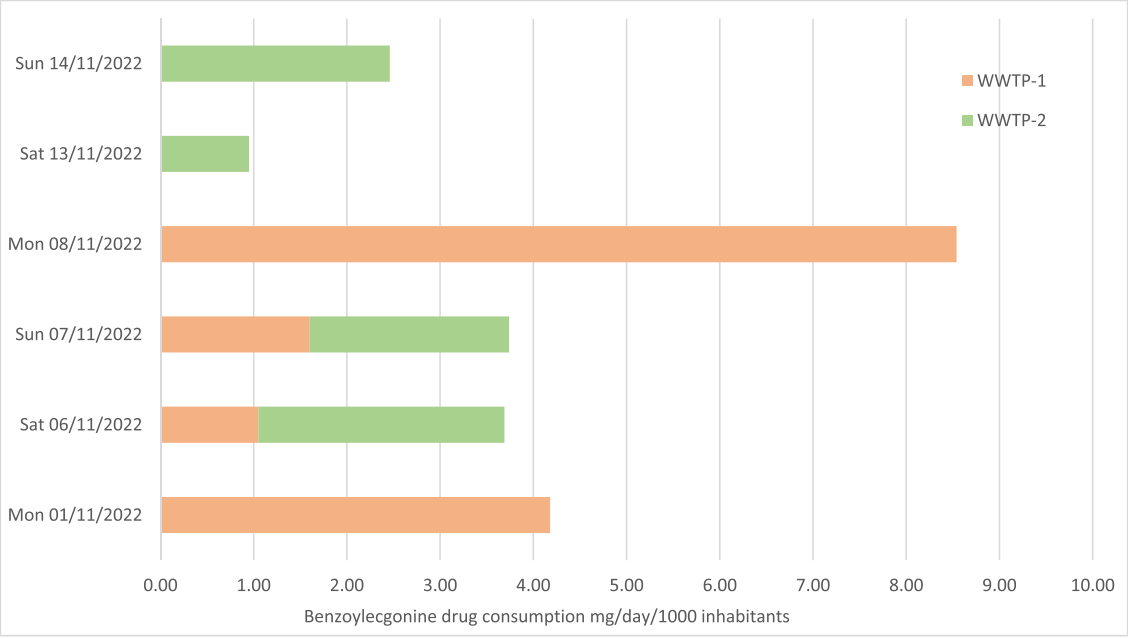

FIGURE 2 Consumption of benzoylecgonine in mg/day/1000 inhabitants in South Wales Monday 1 November–Sunday 14 November.

common illicit drugs. Plus, this could indicate that NPS is being used but not in sufficient amounts to be detectable. The results from these samples demonstrate the importance of wastewater testing in revealing that it is still possible to detect illicit drugs within populated areas. The other compounds within this study that were not discovered could be an indication that the consumption of NPSs has evolved to other NPS types during the development stages of this method. It is well documented that NPS is a dynamic arena that is constantly evolving to other substances to evade legislation. These results could lend support to the importance of untargeted analysis in comparison with targeted because of the dynamic nature of NPSs within a population setting.

When a new NPS emerges in a population area, its initial popularity is low until it gains recognition, resulting in low wastewater concentrations.[5] Some NPSs fade quickly, while others become popular.[23] Various NPS testing approaches are possible. Pooled urine analysis is more concentrated but limited to specific populations, unlike wastewater testing, which offers broader insights.[24] This study focused on targeting NPS analysis with cocaine and cannabis, suitable for well-established substances but less ideal for dynamic NPSs.[25] Non-targeted wastewater analysis has potential, especially for new substances, although it may struggle to detect metabolites.[25] Biomarkers in vivo and in vitro studies[26–28] can aid in rapid NPS identification, improving certified reference material production.[29]

4 | CONCLUSION

The combination of LC-ToF-MS instrumentation with an efficient solid-phase extraction procedure produced a sensitive and robust method for the evaluation of NPSs and traditional illicit substances in wastewater. The analytical method's validation of ISO 17025:2017 and SWGTOX standards confirmed its reliability for the extraction and analysis of this wide array of structurally different NPSs and illicit substances within an adequate concentration interval.

The application of the developed method to WWTP samples in Wales is, to our knowledge, the first study performed in Wales, UK, to assess the use of NPSs by wastewater-based epidemiology. No NPSs were detected in the wastewater, but the detection of benzoylecgonine within all samples demonstrates a widespread use of cocaine within the region. However, further research is needed to evaluate the dynamic tendencies of NPSs in Wales, UK, region and determine the average time taken for the use of certain NPS compounds to evolve by collaborating with services such as WEDINOS. This will help determine whether targeted compounds within a method are still fit for population surveying.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest within this study.

DATA AVAILABILITY STATEMENT

The data underlying this article will be shared on reasonable request to the corresponding author.

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SUPPORTING INFORMATION

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

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