Stability of new psychoactive substances in crude wastewater

Abstract

Those involved in drug testing continue to grapple with the dynamic nature of emerging psychoactive substances (NPS) and their rapid infiltration into society. The challenge extends beyond merely detecting and measuring NPS using analytical tools; it also encompasses the complexities arising from the formation and presence of metabolites and degradation products. This study utilises liquid chromatography time-of-flight mass spectrometry to investigate the stability of new psychoactive substances in wastewater. Seven NPS compounds including 25C-NBOMe, 5F-APINACA 4-hydroxyphenyl, AB-PINACA, APINACA 4-hydroxyphenyl, fentanyl, norfentanyl and MDPV, along with their corresponding internal standard, were examined. Reference material for each NPS compound was introduced into a wastewater sample from a Wessex water treatment plant. The sample was then exposed to four different environments: room temperature, refrigerator temperature, acidification to pH 2, and the introduction of sodium metabisulfite. The findings highlight the critical dependence of storage conditions on target analytes, emphasizing the paramount importance of the time elapsed between collection and analysis for NPS wastewater analysis. Notably, synthetic cannabinoids exhibit limited stability in wastewater whereas cathinone-like substances demonstrate greater stability. Furthermore, metabolites prove to be more stable in wastewater than the parent drug, suggesting that focusing on metabolite detection may be more favourable for future analysis.

Key Words: Wastewater, SPE, LC-ToF-MS, Stability, NPS

1.0 Introduction

New psychoactive substances (NPS) are continuously emerging in the recreational and illicit drug market. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) has been leading the way in monitoring illicit substances and NPS in Europe, including the UK. NPS are mainly synthetic derivatives and analogues of existing controlled drugs, analogues of pharmaceutical products, and naturally occurring compounds such as herbs, fungi, and their extracts to produce a psychoactive effect. In 2022, the EMCDDA published that the current number of NPS being monitored stands at 880 compounds (1). The dynamic nature of NPS makes their detection and identification in the environment difficult compared to the more common illicit substances.

Compared to medicinal drugs, data are scarce on the stability and pharmacokinetics properties of New Psychoactive Substances (NPS). Limited information is available on the chemical stability and metabolism of both new and established NPS analogues. The instability of these compounds, attributed to enzymatic metabolism, hydrolysis, oxidation, and degradation, adds complexity to their detection (2,3).

Most of the analysis conducted in the field of analytical toxicology primarily centres around identifying metabolites or breakdown products rather than the original parent compounds This emphasis on metabolites stems from the intricate metabolic process compounds undergo within the body, coupled with the inherent instability of these compounds (2). While the body's metabolism, driven by enzyme activity, introduces a substantial instability factor, external elements including environmental conditions such as temperature, pH, light, matrix types, and storage conditions can also influence the stability of compounds.

Sewage epidemiology and wastewater testing have emerged as valuable tools for studying drug trends within populations. However, uncertainties surround the stability of target compounds during various stages of the wastewater analysis process. Factors contributing to this uncertainty include the transport of substances from the point of excretion to the sample site at the wastewater treatment plant (WWTP) and the subsequent storage of wastewater samples (4). The stability of these compounds holds significance as it can influence the accuracy of results, leading to potential underestimation or overestimation in the back-calculated data within epidemiological studies (4).

The degradation and formation of drug metabolites in wastewater storage have been examined in studies (5–7). Although the testing approach in these studies varies, a consistent finding emerges: the stability of target compounds can be maintained for several weeks when stored at -20^{o}C.

This study aimed to extend the exploration of the stability of NPS substances within wastewater samples by subjecting NPS to varying temperature conditions and employing different pH levels and additives that could either enhance or deteriorate compound stability. This investigation focused on assessing the stability of the following substances: 25C-NBOMe, 5F-APINACA 4-hydroxypentyl, AB-PINACA, APINACA 4-hydroxypentyl, fentanyl, norfentanyl and methylenedioxypyrovalerone (MDPV) in influent wastewater. The conditions included room temperature, freezer temperature, the addition of sodium metabisulfite, and acidification at pH 2. Samples were analysed on day 4, day 7, day 20 and day 28 to evaluate the changes in compound stability. Click here to enter text.

2.0 Materials and Methods

2.1 Chemicals and reagents

Certified reference standards and deuterated internal standards for the NPS analytes were purchased from either Chiron (Norway) or Merck (Darmstadt, Germany) at concentrations of 1 mg/mL or 100 ug/mL in methanol (MeOH) or acetonitrile (AcN). Dilutions and working standards with concentrations ranging between 0.02 and 1 ng/mL were further prepared using HPLC grade MeOH. HPLC grade AcN, MeOH and formic acid were purchased from Rathburn Chemicals (Walkerburn, UK) whereas the 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 (500mg, 6cc) and Oasis MCX (500mg, 6cc) SPE cartridges were purchased from Waters (Milford, MA, USA).

2.2 Instrumentation

The stability of the 7 NPS compounds was investigated using an AB Sciex 5600+ liquid chromatography time-of-flight mass spectrometry (LC-ToF-MS) coupled with an electrospray ionisation (ESI) source. Chromatographic separation was performed on a YMC-Triart Phenyl 450 bar Column (12nm, 5µm, 100 x 3mm) (Crawford Scientific, UK). The column oven temperature was set at 50^{o}C with a sample injection volume of

10µL. A 9.02 min gradient method was developed including equilibrium time. The mobile phase consisted of (A) 5mM ammonium acetate and 0.2% formic acid and (B) Methanol. The gradient programme started 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.

Quantitative analysis was performed using Sequential Window Acquisition of all THeoretical fragmentation ion spectra (SWATH®). SWATH® acquisition for this method contained 50 SWATH® windows, 5 Da wide with 1 Da overlap on each side of the window. The first SWATH® window started at 175 m/z and the final window ended at 505 m/z in positive mode. The choice of fragmentation ion for each analyte was based on the abundance of the signal, against background noise during the method development phase using tap water. Table 1 provides an overview of the MS parameters the retention times of all analytes and corresponding internal standards.

Table 1 Retention Time, SWATH windows, precursor, and fragment ions for each compound

This method was validated in concordance with UKAS ISO 17025. Recovery was evaluated by introducing a methanolic standard with a concentration of 50 ng/mL into wastewater. Solid phase extraction (SPE) was performed using Oasis HLB cartridges, which demonstrated an average recovery of 46% for all analytes. Linearity was established through a six-point calibration at concentrations 20, 50, 100, 250, 500 and 1000 ng/L. A satisfactory 6-point calibration (R2 >0.99) was achieved for all analytes. The limit of detection (LOD) ranged from 4 to 20 ng/L within wastewater, with the limit of quantification (LOQ) set at the lowest calibrator level of 20 ng/L. The method's intra- and inter-day accuracy and precision were evaluated using three concentration levels within the linear range: lower (80 ng/L), mid-range (300 ng/L), and upper (800 ng/L) in spiked wastewater. The mean accuracy for intra- and inter-day assessments ranged between 77% and 100%. Intra-day precision fell within the range of 8% to 20%, while inter-day precision ranged from 7% to 30%, within ANOVA employed as the statistical tool.

3.0 Stability Study

3.1 Sample Preparation

To investigate the stability of the target compounds in influent wastewater, a 28-day experiment was executed. Wessex Water collected an influent wastewater sample via a spot collection at a WWTP in the South of England. The wastewater sample was tested to ensure there was no target analyte presence before the experiment started.

After confirmation that the sample was negative for target analytes, 500mL of wastewater sample was spiked with 56uL of combined methanolic standard (10 μg/mL) and mixed thoroughly. The spiked sample was subsequently aliquoted into 16 samples, 4 samples for room temperature analysis, 4 samples for refrigerator temperature analysis, 4 samples with sodium metabisulfite and 4 samples acidified to pH 2. Subsequent samples were brought to pH 2 with HCL and stored at refrigerator temperature (3 – 6^{o}C) until analysis followed by aliquots with the addition of sodium metabisulfite spiked at 2 g/L and stored at refrigerator temperature until analysis.

Each sample was analysed with a set of calibrations (0.02, 0.05, 0.1, 0.25, 0.5, 1 ng/mL), three quality control materials (0.08, 0.30, 0.80 ng/mL) and blanks to ensure a quantitative result could be obtained. The temperature of the room and refrigerator where the stability aliquots were kept were monitored daily and the temperature remained stable throughout the study at 20^{o}C. One set of samples was analysed directly after spiking with the combined methanolic standard and was regarded as the initial concentration (day = 0).

3.2 Solid-phase extraction

Filtering before SPE did not occur as part of this method. The universal, polymeric reverse-phase SPE cartridges Waters HLB (Milford, MA, USA) were used for this method. In detail, samples (25 mL) were spiked with 100 µL of a mixed internal standard solution at 50 ng/mL. The SPE cartridges were conditioned with 6 mL methanol and 6 mL deionised water. The samples were loaded onto the SPE cartridges and allowed to pass through the cartridges by gravity, subsequent sample volume was then passed through the cartridges under vacuum at a 5 mL/min rate. The cartridges were then washed with 3 mL of deionised water followed by a vacuum drying for 5 minutes. Elution occurred with 4 mL of methanol and an additional 4 mL of methanol. The eluents were dried using a sample concentrator attached to a heating block set at 55^{o}C. Samples were reconstituted using 100 µL of HPLC grade acetonitrile followed by 100 µL of 5mM ammonium acetate. All samples were then transferred to a 96-deep well plate for analysis.

4.0 Results

The stability results in this study have been presented using a standardised metric originally published by McCall et al., which categorises stability into low (60-100%), medium (20-60%), and high (0-20%) based on the reduction from the original

concentration. While McCall et al. utilised this metric across 24 hours (unless otherwise stated in the paper), this study utilised the McCall et al, principles for degradation classification, but coupled with our own experimental conditions and over 28 days.

AB-PINACA

AB-PINACA displayed remarkably low stability under various storage conditions, exhibiting a concentration depletion ranging from 60 to 100% over 28 days. The refrigerator and sodium metabisulfite conditions showed the most significant concentration depletion, reaching a 100% reduction by day 28. AB-PINACA's instability was evident early on, with a minimum of 90% depletion by day 4 across all four storage conditions. The result from each storage condition strongly suggests that this synthetic cannabinoid lacks stability within the wastewater. Consequently, the chances of detecting AB-PINACA in wastewater are greatly diminished due to the compound's inherent instability.

5F-APINACA 4-Hydroxypentyl

5F-APINACA 4-hydroxypentyl, the main metabolite of 5F-APINACA or 5F-AKB48 was more stable in wastewater in comparison to AB-PINACA but still exhibited low stability (60-100%) across all four storage conditions over 28 days. The stability of 5F-APINACA 4-hydroxypentyl performed better under sodium metabisulfite and room temperature than AB-PINACA with a 70% concentration reduction by day 4. At room temperature, by day 7 stability of 5F-APINACA 4-hydroxypentyl had completely diminished, but with sodium metabisulfite, the depletion was more gradual until day 28 when the concentration depleted by 95%. At the refrigerator and pH 2.01 conditions, the stability of 5F-APINACA 4-hydroxypentyl had diminished by day 4 therefore, similarly to AB-PINACA the likelihood of detection is very small after 4 days in wastewater.

APINACA 4-Hydroxypentyl

The stability profile of the APINACA 4-hydroxypentyl metabolite mirrors that of other synthetic cannabinoids analysed in this study across diverse storage conditions over 28 days. APINACA 4-hydroxypentyl demonstrates low stability (60-100%). In wastewater treated with sodium metabisulfite, wastewater stored at room temperature, or under refrigeration the compound exhibits exceptionally low stability, with a concentration depletion exceeding 90% by day 4. Even when the wastewater is acidified to pH 2 using HCL, APINACA 4-hydroxypentyl maintains a low stability (60-

100%), but with a more gradual depletion observed until day 20, contrasting with the rapid depletion seen in other storage conditions.

Fentanyl

Under refrigerator storage conditions, fentanyl demonstrates the fourth-highest stability among the seven different compounds, despite still exhibiting low stability (60-100%) over 28 days. Refrigerator conditions emerged as the most favourable storage condition, while acidification proved to be the least suitable, resulting in nearly maximum concentration depletion by day 4. This implied that fentanyl is unsuitable for acidic conditions due to its nature as a weak base, causing it to ionise and acquire a positive charge in acidic environments. All other storage conditions exhibited comparable levels of stability for fentanyl, all indicating low stability, with a concentration depletion exceeding 80% by day 4.

Methylenedioxypyrovalerone (MDPV)

MDPV's highest stability was observed when the wastewater was stored in the refrigerator (over 28 days) even though the stability was still deemed low (60-100%) by day 4. MDPV under refrigerator conditions did not completely deplete and stabilised in concentration by day 4 which continued by day 28. This similar trend can be seen across room temperature conditions and acidification to pH 2. The stability of MDPV was compromised when stored with sodium metabisulfite, resulting in a concentration depletion exceeding 99% by the fourth day.

25C-NBOMe

25C-NBOMe showed strong stability in wastewater regardless of storage conditions with high stability (0-20%) observed across refrigerator, room temperature and acidification over 28 days. Under refrigerator conditions, 25C-NBOMe exhibited high stability until day 28 when the concentration decreased to medium stability. Under acidification, high stability can be seen until day 28 when the concentration has depleted to medium stability but the concentration depletion by day 28 was only a reduction of 23%. 25C-NBOMe under sodium metabisulfite storage conditions exhibits the least stability, with low stability observed by day 4 but the concentration does plateau from this point with a maximum concentration depletion of 81% by day 28.

Norfentanyl

Overall, norfentanyl stability performed better than its parent substance fentanyl in all storage conditions besides sodium metabisulfite over 28 days. Even though norfentanyl still exhibited low stability like fentanyl, the concentration depletion is more gradual and less dramatic than the parent drug. The stability of norfentanyl was greatest under refrigerator storage conditions with a less gradual decline to a maximum of 71% concentration depletion by day 28. Norfentanyl under sodium metabisulfite conditions exhibited the worst stability with a dramatic fall in concentration by day 4 and a total concentration depletion of 95% by day 28. Under the sodium metabisulfite condition, fentanyl stability outperformed norfentanyl.

Figure 1 Graphs illustrating each storage condition for each target compound.

5.0 Discussion

The synthetic cannabinoids under scrutiny in this study, namely AB-PINACA, APINACA 4-hydroxypentyl and 5F-APINACA 5-hydroxypentyl, undergo significant metabolism within the liver, leading to the excretion of metabolites primarily in urine, while the parent synthetic cannabinoids are infrequently detected in wastewater. The hepatic cytochrome P450 enzyme system oxidises synthetic cannabinoids, resulting in glucuronide-conjugated analogues (9). Previous publications indicated that synthetic cannabinoids are stable for 20 days at -20^{o}C or at 4^{o}C with the addition of a preservative (10). Contrary to expectations, within 7 days under each storage condition, the concentration of each synthetic cannabinoid decreased to approximately 6-7% of its original concentration for room temperature, pH 2, sodium metabisulfite, and 1% for refrigerator temperature. Analytical challenges associated with detecting synthetic cannabinoids in wastewater may arise from their lipophilic properties and high metabolic rate, resulting in minimal excretion of the parent compound (10). Given the lipophilic nature of synthetic cannabinoids, the true concentration of these substances may be present in the particulate fraction of wastewater, owing to their excretion in faecal waste. A study by Pandopulos et al. investigated the stability of cannabis and synthetic cannabinoids within biosolids (11). The results concluded that all analytes were stable within 2 hours of sludge collection at room temperature, however, within 24 hours, 36% of the analysis did not meet the proposed +/- 20% criteria, indicating instability, whereas AB-FUBINACA, AB-PINACA, MMB2201 and AB-CHMINACA metabolite all fell below 50% (11). The study also found that the mass load of cannabis markers in primary sludge exceeded that detected in influent wastewater in Australia, suggesting that lipophilic properties result in an accumulation within the particulate fraction of wastewater, presenting an

alternative for cannabinoid monitoring. Given the extensive metabolism of synthetic cannabinoids, such as AB-PINACA converting to AB-PINACA carboxylic acid, future studies may find it more beneficial to focus target analysis on the most abundant metabolites (12,13).

The stimulant compounds within this study including MDPV and 25C-NBOMe are highly metabolised, like the SCRAs in the liver. 25C-NBOMe Is predominantly metabolised by O-demethylation, followed by O-di-methylation and hydroxylation (14). Phase 1 metabolites are extensively conjugated in human urine with glucuronic acid. (14). A study conducted by Bade et al in 2017 investigated the stability of 25C-NBOMe and MDPV in wastewater by spiking a known concentration reference material into wastewater, acidifying the samples to pH 2 and leaving samples at refrigerator temperature (4^{o}C), room temperature (20^{o}C) and freezer temperature (-20^{o}C) for 7 days (15). The results of this study mirror the results presented by Bade et al., where the results concluded that 25C-NBOMe exhibited high stability after 7 days under all conditions therefore the degradation of 25C-NBOMe, maintained above 80% (15). The current study investigated 4 different storage scenarios across a longer time frame but the results at 7 days for 25C-NBOMe matched at pH 2.01 but were high stability (60-100%) except for the sample with sodium metabisulfite which exhibited low stability (0-20%). Bade et al concluded that MDPV exhibited medium stability (20-60%) across room temperature and refrigerator at pH 2 (15), this coincides with this study as MDPV also displayed medium stability at refrigerator and pH 2.01 but did exhibit low stability (0-20%) at room temperature and sodium metabisulfite.

Fentanyl and Norfentanyl are the final compounds under investigation in this study. While norfentanyl is the primary metabolite for fentanyl, it can also be present independently. Following human consumption, fentanyl undergoes extensive metabolism in the liver, primarily through the hepatic pathway (16–18). D. R Baker et al., explored the stability of fentanyl and norfentanyl in wastewater by spiking raw wastewater and allowing the samples to remain at neutral pH at either refrigerator temperature (2^{o}C) or room temperature (19^{o}C) (19). The samples were re-tested at 12 hours, 24 hours, and 72 hours, using the same measurement technique as published by Bade et al in 2017 (15). The findings indicated that fentanyl exhibits high stability in wastewater within the first 24 hours under both temperature conditions. It maintains high stability at 72 hours in the refrigerator by experiencing a substantial drop to low stability at 72 hours at room temperature. On the other hand, norfentanyl demonstrates significantly greater stability than fentanyl. It maintains high stability (0-20% reduction)

within the initial 72 hours at refrigerator temperature and remains high until 72 hours at room temperature, after which the concentration drops to medium stability (20-60%) at room temperature. In contrast to the stability study conducted by D. R. Baker (19), although the storage durations differed between that study and this study, norfentanyl once again exhibited markedly higher stability than fentanyl by the fourth day under both refrigeration and room temperature conditions.

Figure 2 Graph representing the stability differences between parent drug and metabolite fentanyl and norfentanyl per storage condition.

Understanding the stability of NPS in wastewater is crucial for several reasons. NPS are bioactive and are therefore susceptible to degradation and metabolism by the bacteria present in the wastewater or by other transformation reactions. Detecting NPS in wastewater collected several hours before analysis may lead to significantly elevated or decreased values compared to the actual concentration initially present in the sample. In 2006, the first stability investigation in wastewater was conducted, focusing solely on drugs of abuse at the time (20). This study explored one storage condition, where samples were stored in dark bottles for three days at 4ºC, resulting in a decrease in cocaine, norcocaine, and cocaethylene by 36%, 15% and 13% respectively. Subsequent stability investigations expanded on the 2006 study, exploring different storage conditions by varying storage length, adjusting pH, and employing a range of temperatures (4,21,22). 2013 saw the first stability investigation specifically focused on emerging New Psychoactive Substances (2), though it was conducted in blood and plasma rather than wastewater. Another stability study in 2020 investigating synthetic cannabinoids in wastewater was concluded at room temperature, 24 of 30 cannabinoids were not stable in untreated wastewater for up to 14 days but keeping untreated samples at 4ºC improved stability for one week. Adding the preservative sodium metabisulfite stabilised 14 / 30 synthetic cannabinoids for 7 days. Acidification improved 25 / 30 synthetic cannabinoids for 7 days at room temperature (10). Cathinones stability was investigated during a study in 2017 using wastewater. The wastewater samples were at natural pH and acidified to pH 2 and kept at 3 different temperatures (20 ºC, 4 ºC and -20 ºC) for 24 h, 48 h, 7 days, and 30 days (15).

Conclusion

The results presented in this study underscore that some new psychoactive substances (NPS) exhibit limited stability in wastewater over extended periods (a month), emphasising the critical importance of the time elapsed between collection

and analysis. Furthermore, it becomes apparent that various categories of NPS demonstrate distinct stability profiles under diverse conditions, posing challenges for methods that investigate different NPS categories. The likelihood of detecting cathinone-like substances appears higher than that of synthetic cannabinoids. However, if wastewater treatment is employed to enhance synthetic cannabinoid stability, it may inadvertently eliminate fentanyl-like substances. This issue holds significant implications for wastewater analysis, as the degradation of compounds or the formation of metabolic residues can lead to the underestimation or overestimation of back-calculated drug use. This study represents another stride toward comprehending the dynamic nature of NPS and their stability in biological matrices.

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