

Investigating in-sewer transformation products formed from synthetic cathinones and phenethylamines using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry

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Abstract

Recent studies have demonstrated the role of biofilms on the stability of drug residues in wastewater. These factors are pertinent in wastewater-based epidemiology (WBE) when estimating community-level drug use. However, there is scarce information on the biotransformation of drug residues in the presence of biofilms and the potential use of transformation products (TPs) as biomarkers in WBE.

The purpose of this work was to investigate the formation of TPs in sewage reactors in the presence of biofilm mimicking conditions during in-sewer transport. Synthetic cathinones (methylenedioxypyrovalerone, methylone, mephedrone) and phenethylamines (4-methoxy-methamphetamine and 4-methoxyamphetamine) were incubated in individual reactors over a 24 h period. Analysis of parent species and TPs was carried out using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-QToFMS). Identification of TPs was done using suspect and non-target workflows.

In total, 18 TPs were detected and identified with reduction of β -keto group, demethylenation, demethylation, and hydroxylation reactions observed for the synthetic cathinones. For the phenethylamines, N- and O-demethylation reactions were identified. Overall, the experiments showed varying stability for the parent species in wastewater in the presence of biofilms. The newly identified isomeric forms of TPs particularly for methylone and mephedrone can be used as potential

36 target biomarkers for WBE studies due to their specificity and detectability within

37 a 24 h residence time

38

39 **Keywords:** New psychoactive substances; biofilm; biomarkers; stability; LC-

40 QToFMS; wastewater-based epidemiology

Highlights:

- There is a paucity of biomarkers for NPS in wastewater-based epidemiology (WBE)
- Experiments conducted using in-sewer incubations of NPS in the presence of biofilm
- We investigated formation of transformation products (TPs) using LC-QToFMS
- 18 TPs were identified, 5 TPs unique to wastewater matrix interactions
- In-sewer stability and transformations are important considerations in WBE

1. Introduction

The emerging field of wastewater-based epidemiology (WBE) has been instrumental in estimating illicit drug consumption in communities. WBE is now used as a complimentary approach by the European Monitoring Centre for Drug and Drug Addiction (EMCDDA) to monitor spatio-temporal trends in the use of conventional illicit drugs like cocaine, methamphetamine, ecstasy, and amphetamine^{1, 2}. Synthetic cathinones and other phenethylamines have emerged over the last decade as major classes of new psychoactive substances (NPS) in many European countries³. Structurally, synthetic cathinones are ring-substituted phenethylamines with the substitution of a ketone group at the β -carbon position. These substances are often used as alternatives to amphetamine-type stimulants and cocaine because of they possess similar psychoactive effects. Their use has been linked to numerous cases of acute and fatal intoxications^{4, 5}.

Monitoring NPS consumption using WBE has been a growing area of interest in recent years, where few studies⁶⁻¹² have investigated the presence and amount of NPS in wastewater. However, only few NPS have been detected and levels were generally low in sewage. Several factors may contribute to this: firstly, the prevalence of use, when a NPS enters the drug scene its popularity is generally low until it becomes more recognized and thus the concentrations in sewage may be very low^{13, 14}. Secondly, limited information exists about the metabolism and excretion of NPS, and therefore, target biomarkers remain largely unknown.

73 Additionally, the focus on targeted analysis in WBE studies is likely to miss NPS not
74 included in the targeted methods¹⁵. Lastly, their stability and (bio)transformation
75 in wastewater is complex and not (yet) fully understood^{16, 17}.

76 For compounds with unknown or low stability during *in-sewer* transport,
77 (in)stability is considered a major source of uncertainty in the estimation of drug
78 use by WBE and can result in significant under- or overestimation^{18, 19}. Most
79 stability studies related to WBE have focused on *in-sample* stability²³, which
80 involves sample preparation, preservation and storage through the testing of
81 different conditions: filtration of samples, storage at different temperatures, and
82 addition of preservatives²⁰. Few stability studies have considered environmental
83 processes occurring in sewers that may affect the overall fate of target
84 biomarkers^{17, 21} and estimated the possible effect in entire catchments considering
85 known and unknown variables²².

86 Wastewater contains a large number of components and is subject to different
87 environmental processes which are also influenced by the design of the sewer and
88 operation modes^{21, 23}. Presence of biofilms on sewer walls^{21, 24} and processes
89 involving sorption to particulate matter¹⁶, sedimentation, uptake by organisms²³
90 and those causing structural changes of compounds²⁵ should also be considered.
91 Some biomarker stability studies investigated the role of biofilm^{16, 17, 21}, and
92 isolated microbial strains²⁶ on pharmaceuticals and select drugs of abuse.
93 Accounting for aerobic and anaerobic conditions these studies showed that

degradation rates are significantly higher in the presence of biofilm and/or suspended solids. One study modelling *in-sewer* transformation for three catchments of different size showed that in small catchments target biomarkers were mostly affected by biofilm processes. Even though biomarkers in large(r) catchments have on average longer residence times in the sewer, the biomarkers have less contact with biofilm, consequently, their stability is mainly affected by abiotic processes²².

Subsequently, parent compounds can undergo further transformation in the sewage environment, leading to transformation products (TPs) other than those normally observed in biological matrices, such as urine. The ideal WBE biomarker would be one that is stable in wastewater during *in-sewer* transport, specific, and detectable. In many cases, TPs are more stable than the parent compound¹⁶, therefore it would be worthwhile to identify TPs formed during *in-sewer* transport and assess them as potential biomarkers to be used in WBE studies.

Studies conducted to investigate TP formation during wastewater treatment plant (WWTP) processes like ozonation and chlorination were performed by spiking high levels of the compounds of interest individually in reactors²⁷⁻²⁹. Such studies have been instrumental in detecting TPs, which are used for the evaluation of removal efficiencies in WWTPs. Application of individual spike experiments and high-resolution mass-spectrometry (HRMS) techniques would be useful in determining potential biomarkers for NPS to be used in WBE studies.

The aims of this study were: (i) to conduct experiments with real wastewater and biofilm to investigate the formation of TP_s of selected synthetic cathinones (methylenedioxypyrovalerone (MDPV), methylone, mephedrone) and selected phenethylamines (4-methoxy-methamphetamine (PMMA) and 4-methoxy-amphetamine (PMA)), (ii) to identify and characterize the TP_s formed using liquid chromatography quadrupole-time-of-flight mass spectrometry (LC-QToFMS) using suspect and non-target screening approaches, and (iii) to recommend potential biomarkers for these NPS to be used in WBE studies.

2. Materials and methods

2.1 Chemicals and reagents

Chemicals standards for cocaine (COC), mephedrone, MDPV, methylone, PMMA, and PMA were obtained from LGC Standards SARL (Molsheim, France) and Cerilliant (Round Rock, Texas, USA) at the concentration of 1 mg/mL or 100 µg/mL in methanol or acetonitrile. LC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Nanopure water was obtained by purifying demineralized water in an Elga LabWater Purelab Flex system (Veolia Water Solutions & Technologies Belgium, Tienen, Belgium). Formic acid (eluent additive for LC-MS, 98%) was obtained from Sigma-Aldrich (Steinheim, Germany). The internal standards ranitidine-D₆ and fluoxetine-D₅ (with purity > 98%) were purchased from Cerilliant (Round Rock TX, USA) at concentrations of 1 mg/mL in

methanol. Working solutions were prepared for concentrations ranging between 0.005 - 100 ng/μL in methanol.

2.2 Biotransformation reactor setup

The biotransformation experiments were conducted as previously optimised by McCall et al.¹⁷. This protocol was adapted from the OECD international testing guidelines (test 314: Simulation Tests to Assess the Biodegradability of Chemicals Discharged in Wastewater) by additionally including suspended biofilms to the test system. Briefly, the biofilms scraped from Sihl Adliswil manhole in Switzerland were weighed (≈ 23 g) for each reactor (see table SI-1b), homogenized by vigorous mixing with a metal spatula, added to 2 L Erlenmeyer flasks (reactor) and filled up to 0.5 L with wastewater (<1 h old) from a real sewer pumped to feed the Eawag wastewater treatment pilot plant. The wastewater was acclimatized to room temperature (21 ± 1 °C) and all environmental parameters (pH, conductivity, dissolved oxygen content and temperature) were measured. As controls, two reactors out of the seven with the same wastewater were run in parallel: (i) a positive control was spiked with COC, and (ii) a negative control (background) was not spiked. Additionally, a sample (labelled '-15 min') was collected from each reactor prior to the spike, it was useful for confirming that all identified TPs were formed after spike. The batch reactors with suspended biofilms were operated with a biofilm mass that is equivalent to a ratio of intact biofilm surface area to wastewater volume in a real sewer of approx. $33 \text{ m}^2 \text{ m}^{-3}$. Using suspended instead

of intact biofilm showed no substantial differences for transformation of most biomarkers in a previous study, since mass transport limitations seem negligible (see McCall et al.¹⁷ for experimental and theoretical evidence, SI 2.6).

A spiking level of 500 µg/L of each individual NPS (MDPV, methylene, mephedrone, PMMA, and PMA) and COC (the positive control) was applied at time point 0 h. This high concentration spike was necessary to facilitate the detection of minor TPs. Over 24 h, the reactors were shaken in the dark at 45-90 rpm and the environmental process parameters were monitored (Table SI-2). Biomass was measured as total suspended solids (TSS) and volatile suspended solids (VSS) according to European Standard, by the difference in weight of filtered samples, glass fiber filters (GF, 0.75 µm, VWR), before and after heating at 105 °C for 10 h and 550 °C for 2 h. Total solids and volatile solids content of the undiluted biofilm samples was determined similarly (without a filter) using additional biofilm samples.

Samples for evaluation of (bio)transformation and elucidation of TPs were collected before (-15 min) and 2 min after spiking of the NPS (0 h), 2 h, 8 h, and 24 h. For each time point, 5 mL of sample was aliquoted with a 20 mL plastic pipette and filled into 15 mL polypropylene centrifuge tubes. To instantly preserve the sample and stop biological activity, the samples were immediately flash frozen in liquid nitrogen and subsequently stored at -20 °C until analysis on LC-QToFMS.

2.3 Sample preparation

After thawing, 1.5 mL of sample was transferred to a 2 mL Eppendorf tube and centrifuged for 5 min at 8000 rpm. Afterwards, an aliquot of 500 µL was transferred to a 1.5 mL centrifugal filter (0.45 µm), centrifuged for 5 min at 8000 rpm, and transferred to an autosampler vial where 10 µL of a standard mix of fluoxetine-D5 and ranitidine-D6 at 250 µg/L is added before injection into the LC-QToFMS.

2.4 Liquid chromatography quadrupole time-of-flight mass spectrometry

The LC system consisted of an Agilent Infinity 1290 SL binary pump with an integrated two-channel solvent degasser, a thermostated Agilent 1290 HiP-ALS autosampler system (20 µL injection loop) and a 1290 Agilent TCC SL column compartment (Agilent Technologies, Santa Clara, USA). Chromatographic separation was achieved with a Phenomenex Biphenyl (100 mm x 2.1 mm, 2.6 µm) column fitted to a SecurityGuard ULTRA Holder for UHPLC columns (2.1 - 4.6 mm) and maintained at 32 °C. Mobile phase composition consisted of water (A) and of 80:20 acetonitrile:water (B) both with 0.04% of formic acid, with the following gradient: 0 min, 2% B; 2 min, 2% B; 18 min, 40% B; 25 min, 90% B; 29 min, 90% B; 29.5 min, 2% B; 33 min, 2% B. The total run time including column equilibration was 33 min. The injection volume was optimized based on peak shape and set to 2 µL and the flow rate was 0.4 mL/min. The MS system consisted of an Agilent 6530 Accurate-Mass QToF instrument (Agilent Technologies, Santa Clara, USA) operated with jet stream electrospray ion source (Dual AJS ESI source). The method and

instrumental parameters applied for the LC-QToFMS are described in detail in the supplementary information.

2.4.1 Suspect screening data analysis workflow

Suspect lists comprising of name and molecular formulae from previously identified metabolites were created based on *in vitro* and *in vivo* metabolism studies conducted for COC^{30, 31}, mephedrone³², MDPV^{33, 34}, methylone^{35, 36}, PMMA^{31, 37, 38} and PMA^{31, 37}. These lists were then added to the Agilent personal compound database and library (PCDL) manager software (Agilent Technologies, Santa Clara, USA) as separate libraries and further included to the workflow described in our previous work³⁹. The workflow is based on a 'Find by Formula (FbF)' algorithm (Agilent Technologies, Santa Clara, USA) that involves the extraction of accurate masses (calculated based on molecular formula) of expected ions $[M+H]^+/[M-H]^-$ from the data acquired.

2.4.2 Non-target screening data analysis workflow using 'Component Detection' and 'COMPARE LCMS' algorithms

The 'Component Detection Algorithm' (CODA) and 'COMPARE LCMS' algorithm from the ACD/MS Workbook suite were used for a non-target data analysis strategy. CODA which is a molecular feature detection algorithm was useful for peak picking which involved deleting noise and background peaks, recovering mass spectra of pure compounds and separating the co-eluting components within data

sets. The COMPARE LCMS which is a differential analysis algorithm allowed for comparison of two or more data sets with extracted feature candidates and showed the difference between them.

Firstly, we loaded acquired data (0 V full scan segment one) and applied the CODA algorithm which uses parameters set for smoothing, baseline correction, and peak picking parameters (Table SI-2). Secondly, we selected the two chromatograms to compare (e.g. 24 h control and 24 h spiked) and applied the COMPARE LCMS algorithm. Thereafter, a table was generated containing a list of extracted exact masses. The column of interest in the generated result's table labelled 'Uniqueness' was useful for identifying the: 'Unique' (present in one data set), 'Different' (present in both but differ based on set criteria) and 'Similar' (present and same in both) peaks. The 'Unique' peaks were further analysed using target MS/MS to acquire their product ion profiles.

2.4.3 Identification and confirmation of TPs

To communicate confidence of the identifications we applied four of the five levels described by Schymanski et al.⁴⁰. Briefly, the confirmation by injection of a reference standard for determination of retention time (t_R), MS and MS/MS spectra were designated as Level one, whereas for Level two (a or b), a probable structure was proposed based on matching existing (library (a) or literature (b)) spectrum data or using non-reported diagnostic MS/MS product ions evidence. Level 2a confirmations were based on in-house library spectra data available from

previous experiments, and intoxication cases received at our forensic toxicology laboratory (including *in vivo* samples from individual users)^{34, 37, 39, 40}. We considered Level 2a identification as definite but lacking a commercial reference standard to warrant its Level 1 identification. For Level 2b confirmations, we use literature spectra from *in vitro* and *in vivo* metabolism studies on NPS. For level three, a tentative candidate was proposed with a possible structure, however, the exact structure remained unconfirmed due to insufficient information on position of substituents. Lastly, Level four confirmations did not have product ion spectra, as such, were based on matching isotopic information of an extracted exact mass candidate to specific molecular formulae of an extracted exact mass candidate.

2.4.4 Estimating levels of TPs

To aid in estimation of levels of parent compounds and TPs, semi-quantitative calculations were based on peak area and relative response curves were prepared. The relative response for each parent and TP at different time points was calculated as a percentage relative to the peak area of the parent compound at the time of spike (0 h). Based on the classification proposed by McCall et al.²³, we ranked the stability of the parent NPS on the percentage of transformation (low (60-100% transformation), medium (20-60% transformation) or high (0-20% transformation)).

3. Results and Discussion

3.1 *In-sewer* transformation of NPS in the presence of biofilm

The positive control (COC reactor) was used to evaluate the success of the transformation experiments. To this end, the mainly abiotic driven transformation of COC and formation of benzoylecgonine and ecgonine methyl ester were monitored. The levels of COC were only $\approx 10\%$ compared to the initial levels after the 24 h incubation, and in the same reactor an increase in both benzoylecgonine ($\approx 15\%$) and ecgonine methyl ester ($\approx 1\%$) was observed which is consistent with previous transformation studies involving COC^{16, 17, 42}. Vital parameters (pH, dissolved oxygen (DO) and temperature) remained stable through the 24 h incubation period in all reactors (Table SI-1).

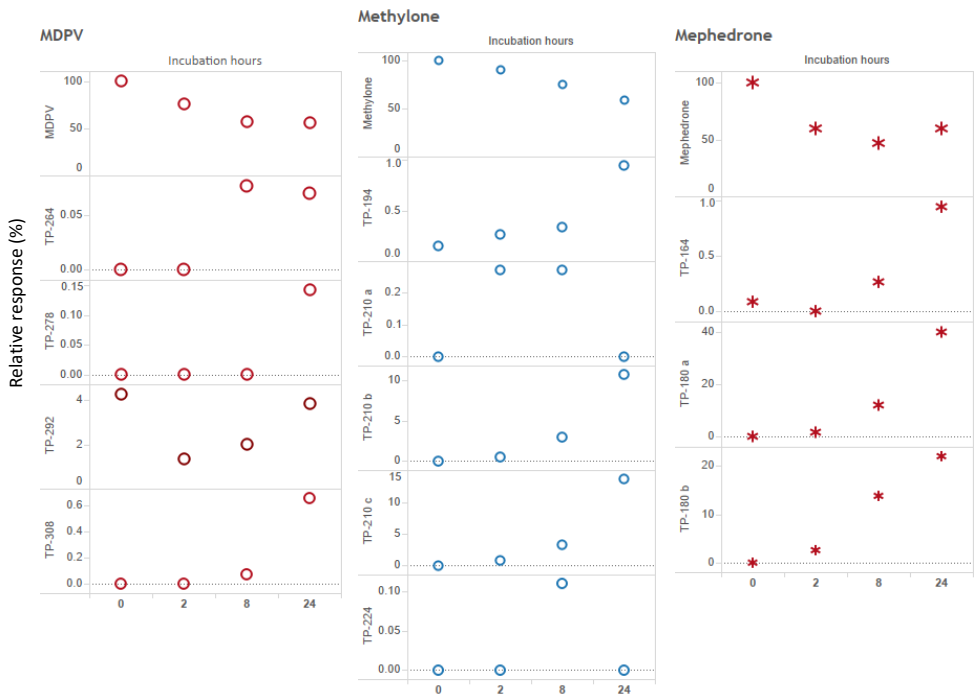


Figure 1. Relative response of MDPV, methylone, mephedrone and their TPs over 24 h incubations in sewage containing biofilm

3.2 Synthetic cathinones (MDPV, methyldone and mephedrone)

3.2.1 MDPV

MDPV was observed to be of medium stability as its levels decreased to $\approx 55\%$ over 24 h (Table SI-3, Figure 1). Six TPs originating from MDPV were detected and identified (Table 1). A similar study involving MDPV incubation in wastewater in the presence of biofilm by McCall et al.¹⁷ showed a consistent pattern for aerobic biotransformation of MDPV. However, Mardal et al.³³ did not observe as significant a decrease of MDPV over a 24 h biotic incubation. This discrepancy could be attributed to the differences in experimental setup, particularly the airflow into the reactors affecting the dissolved oxygen in the two systems. Additionally, Mardal et al.³³ reported a total of 12 TPs for a 10-day incubation of MDPV, whereas, we detected and identified six TPs formed within a 24 h residence time.

TP-264 (m/z 264.1579) was detected at the 8 h time point and was likely formed between 2-8 h of incubation at very low levels ($< 0.5\%$ relative response). Due to its low abundance, no MS/MS spectrum could be acquired, however, we confirmed its identity using the t_R from an in-house MDPV *in vitro* metabolism extract³⁴ (Figure SI-1) present in our in-house library. Consequently, we assigned the Level 2a confidence. We proposed TP-264 as being formed through the loss of the methylene group (Figure 2). This TP has been previously identified as a major biotransformation product in *in vitro*^{34, 43}, *in vivo*⁴³ and *in-sewer*³³ studies.

We detected two TPs with m/z 308.1481 and 308.1467 (TP-308a and TP-308b) at t_R 10.12 and 11.31 min, respectively (Table 1). TP-308a was detected at the 2 h time point at low levels 0.9% and diminished to < 0.2% after 24 h incubation (Figure 1). TP-308b was detected at the 8 h time point at low levels < 0.1% and increased to 0.6% after 24 h of incubation. A MDPV biotransformation product with this exact mass has been previously identified in an *in vitro*³⁴ metabolism study as a metabolite formed through a dihydroxylation of MDPV without cleavage of the pyrrolidine ring. However, in an *in-sewer* study by Mardal et al.³³ the dihydroxylation of MDPV was proposed through hydroxylation of the alkyl group, followed by *N*-dealkylation and further oxidation of the cleaved pyrrolidine ring. TP-308a was not present at a high abundance and no MS/MS spectrum could be acquired, and therefore only a Level 4 confirmation could be assigned. We confirmed TP-308b as the dihydroxylated structure proposed by Negreira et al.³⁴ (without cleavage on the pyrrolidine ring) based on the fragmentation pattern (Figure SI-2) and the t_R information included in the in-house database and therefore assigned Level 2a identification (Figure SI-1).

Additionally, two TPs with m/z 278.1746 and 278.1646 (TP-278a and TP-278b) were detected at t_R 9.18 and 11.73 min respectively (Table 1). In previous studies^{33, 34, 43, 44} MDPV biotransformation products with this same exact mass have been identified. Meyer et al.⁴³ proposed the demethylenyl-methyl form (methylcatechol-pyrovalerone) of MDPV and detected it *in vitro* and *in vivo*. Negreira et al.³⁴ proposed

the dihydro-MDPV resulting from the reduction of the β -keto group based on the fragmentation pattern.

Table 1. Transformation products identified for MDPV over 24 h *in-sewer* incubations in the presence of biofilm

Compound	^a t _R	Measured <i>m/z</i>	^b Δm	Diagnostic product	Chemical Formula	^c Level
	(min)	[M+H] ⁺	(ppm)	ions		
MDPV	11.66	276.1590	-1.45	135.044, 149.0224, 175.0736, 205.0846, 233.1052	[C ₁₆ H ₂₂ NO ₃] ⁺	1
TP-264	7.51	264.1579	-7.57	not found	[C ₁₅ H ₂₂ NO ₃] ⁺	4
TP-278a	9.18	278.1746	-1.80	not found	[C ₁₆ H ₂₄ NO ₃] ⁺	4
TP-308a	10.12	308.1481	-3.57	not found	[C ₁₆ H ₂₂ NO ₅] ⁺	4
TP-308b	11.31	308.1467	-8.11	135.0448, 177.0901, 260.1254	[C ₁₆ H ₂₂ NO ₅] ⁺	3
TP-278b	11.73	278.1646	-37.75	not found	[C ₁₆ H ₂₄ NO ₃] ⁺	2a
TP-292	13.75	292.1540	-1.03	126.1301, 149.013, 205.106	[C ₁₆ H ₂₂ NO ₄] ⁺	3

Retention time (min); ^b m/z accurate mass measurement error; ^c Identification level according to Schymanski et. al⁴⁰

Paul et al.⁴⁴ detected three forms with this exact mass in human urine and proposed the methylcatechol-pyrovalerone isomers, and dihydro-MPDV⁴⁴. In addition, Mardal et al.³³ studied the microbial biotransformation of MDPV in wastewater and detected

one TP with the same exact mass and tentatively identified it as the methylcatechol-pyrovalerone³³. TP-278a and TP-278b were detected solely in the 24 h extract at low levels of < 0.5%, and thus were formed between 8 h and 24 h time points (Table SI-3). We tentatively identified the TP-278b as dihydro-MDPV based on the comparison of the t_R with a previously injected *in vitro* metabolism extract from a previous study (Figure SI-1). However, we could not acquire MS/MS spectra for the two TPs since they were present at a low abundance.

TP-292 (m/z 292.1540) was formed immediately after spiking of MDPV in the reactor at 0 h. Its levels were much higher at the beginning of the experiment and diminished after 2 h and then steadily increased again to levels > 3% after 24 h of incubation. TP-292 corresponds to a hydroxylation of MDPV, this is consistent with the forms of m/z 292.1543 which have been proposed from *in vitro*^{34, 43}, *in vivo*⁴³ and *in-sewer*³³ studies. Based on the fragmentation pattern (Figure SI-3), two possible structures were proposed with the intact pyrrolidine ring which are unique compared to previous MDPV biotransformation studies^{33, 34, 43}. This was attributed to the two fragments: at m/z 126.1277 which suggests the presence of the pyrrolidine ring, and at m/z 86.0600 which has two possible structures that could be formed from either the hydroxyl aryl or hydroxyl pyrrolidine forms of the TP-292. As such, Level 3 confirmation for this TP could be assigned.

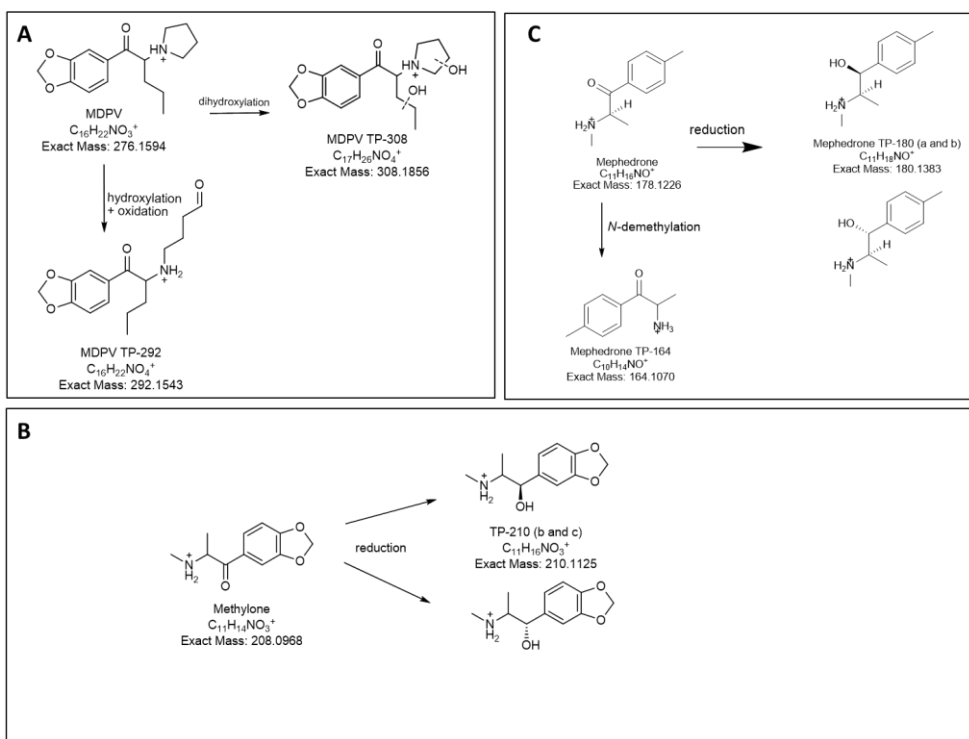


Figure 2. Proposed *in-sewer* biotransformation pathway of MDPV (A), methylone (B), and mephedrone (C).

Overall, over the 24 h incubation period of MDPV, the TPs formed at significant levels were TP-308b and TP-292. Based on their detectability after the 24 h residence time these TPs could be good biomarkers to include in suspect screening list when performing WBE studies. In contrast, Mardal et al.³³ suggested a TP with *m/z* 278.1751 as the best biomarker for WBE, which we detected at low levels.

3.2.2 Methylone

Methylone in the presence of biofilm showed medium stability where its levels decreased to approximately 60 % after 24 h of incubation (Table SI-3, Figure 1).

Five TPs were detected and tentatively identified from the *in-sewer* incubation experiments.

TP-224 (*m/z* 224.0909), which corresponds to the hydroxylation of methylone, was detected at low levels (< 0.2%) at the 8 h time point (Figure 1). This TP has been previously identified in *in vitro* metabolism studies as *N*-hydroxyl-methylone⁴⁵. MS/MS spectra were not acquired for this TP, since the levels were lower than the parameters set.

Table 2. Transformation products identified for methylone over 24 h *in-sewer* incubations in the presence of biofilm

Compound	^a t _R	Measured	^b Δ <i>m</i> (ppm)	Diagnostic	Chemical	^c Level
		<i>m/z</i> [M+H] ⁺		product ions	Formula	
Methylone	6.86	208.0961	-3.36	132.0821, 160.0741, 190.0810	[C ₁₁ H ₁₄ NO ₃] ⁺	1
TP-224	1.97	224.0909	-3.57	not found	[C ₁₁ H ₁₄ NO ₄] ⁺	4
TP-210a	2.23	210.1112	-6.19	not found	[C ₁₁ H ₁₆ NO ₃] ⁺	4
TP-210b	5.83	210.1118	-3.33	103.0556, 177.0797, 192.1010	[C ₁₁ H ₁₆ NO ₃] ⁺	2a
TP-194	5.93	194.0804	-4.12	not found	[C ₁₀ H ₁₂ NO ₃] ⁺	4
TP-210c	6.25	210.1114	-5.24	103.0550, 177.0810, 192.1029	[C ₁₁ H ₁₆ NO ₃] ⁺	2a

^a Retention time (min); ^b *m/z* accurate mass measurement error; ^c Identification level according to Schymanski et. al⁴⁰

Three TPs with m/z 210.1112, 210.1118, and 210.1114 (TP-210a, TP-210b and TP210c) were detected at t_R 2.23, 5.8 and 6.2 min respectively (Table 2). TP-210a was detected at the 2 and 8 h time points at low levels \approx 0.3% (Figure 1). TP-210b and c appeared at higher levels (>0.5 %) at the 2 h time point, and their levels steadily increased over the 24 h incubation to 10.6 and 13.7%, respectively. TP-210b and TP210c are probably structural isomers formed through the reduction of the β -keto group on methylone, with fragments at m/z 192.1019 and 177.0784 which are indicative of an intact methylenedioxy group (Figure SI-4). Only one isomer (dihydromethylone) has been previously detected *in vitro* and *in vivo*^{35, 45-47} metabolism studies. TP-210a was proposed to be one form of *O*-demethylated methylone (Figure SI-5), however, it could not be confirmed in this study since the MS/MS spectra were not acquired due to its low abundance, as such, we assigned it a Level 4 confirmation (Table 2).

Methylone's TP-210b and TP-210c are likely good biomarkers that can be used as targets and /or suspects in analytical methods applied in WBE studies, particularly because they are formed at higher levels during the normal residence time of 24 h.

3.2.3 Mephedrone

Mephedrone in the presence of biofilm had the lowest stability where its levels decreased to $< 50\%$ after 24 h of incubation (Table SI-3, Figure 1). This stability pattern was consistent with several other aerobic biotransformation studies^{17, 16, 48, 49}. Two studies^{16, 17} further showed that mephedrone was subject to chemical

transformation under abiotic conditions (autoclaved wastewater, tap and mineral water). Three TPs were detected and identified from our *in-sewer* incubation experiments.

Two TPs with m/z 180.1383 (TP-180a and TP-180b) were detected at t_R 7.16 and 7.50 min, respectively (Table 3). TP-180a and TP-180b appeared to be formed between 0 and 2 h of incubation, and they both increased over 24 h to > 35% for TP-180a and > 20% for TP-180b (Figure 1). TP-180a and TP-180b corresponded to the reduction of the β -keto group of mephedrone (Figure 2). In addition, based on their similar product ions at m/z 162.1277 and m/z 147.1037 they are likely to be structural isomers (Figure SI-6). A study involving mephedrone metabolism identified one metabolite with m/z 180.1383 as 4-methylephedrine (dihydromephedrone) which was also identified in human urine, and their analysis showed a similar fragmentation pattern as in our study³². We confirmed the identity of TP-180b as (\pm)4-methylephedrine (dihydromephedrone) using an analytical standard.

TP-164 with m/z 164.107 at t_R 7.2 min was formed immediately after spike at very low levels and only increased to \approx 1% after 24 h incubation. Based on its accurate mass, MS/MS spectra and t_R , we confirmed the identity as nor-mephedrone which has previously been identified *in vitro* and in human urine formed through the *N*-demethylation of mephedrone^{32, 46}.

Table 3. Transformation products identified for mephedrone over 24 h *in-sewer* incubations in the presence of biofilm

Compound	^a t _R	Measured m/z [M+H] ⁺	^b Δm (ppm)	Diagnostic product ions	Chemical Formula	^c Level
Mephedrone	8.1	178.1250	13.47	91.0543, 119.0856, 145.0884, 160.1117	[C ₁₁ H ₁₆ NO] ⁺	1
TP-180a	7.16	180.1385	1.11	147.1037, 162.1268	[C ₁₁ H ₁₈ NO] ⁺	2a
TP-164	7.2	164.1061	-5.48	91.0558, 146.0956	[C ₁₀ H ₁₄ NO] ⁺	1
TP-180b	7.5	180.1381	-1.11	91.0536, 147.1058, 162.127	[C ₁₁ H ₁₈ NO] ⁺	1

^a Retention time (min); ^b m/z accurate mass measurement error; ^c Identification level according to Schymanski et. al.⁴⁰

In general, TP-180a and TP-180b would appear to be suitable biomarkers for mephedrone based on their abundance over the 24 h incubation and considering that mephedrone is highly unstable.

3.3 Phenylethylamine compounds (PMMA and PMA)

3.3.1 PMMA

PMMA showed the highest stability, its levels decreased by approximately 15% after 24 h incubation (Table SI-4, Figure 3). Three TPs were detected for PMMA

(Table 4) from the *in-sewer* incubation experiments. TP-152_{PMMA} with m/z 152.1078 at t_R 4.99 min was detected at low abundance and as such MS/MS spectra were not acquired for this TP. PMMA *in vitro* and *in vivo* metabolism has been well studied^{37, 50} and a metabolite with m/z 152.1070 has been reported as *p*-OH-amphetamine, however, the t_R for TP-152_{PMMA} (4.99 min) did not match the *p*-OH-amphetamine t_R in our in-house database which indicates it is a unique TP.

Two TPs with m/z 166.1226 (TP-166a and TP-166b) at t_R 3.49 and 6.89 min respectively were also detected. Their identities were confirmed based on the accurate mass, t_R and MS/MS spectra. TP-166a and its product ions at m/z 77.0379, 91.0559, 107.0496 and 135.0804 formed through *O*-demethylation of PMMA matched *p*-OH-methamphetamine identified in previous studies³⁷ (Figure 4). TP-166a was formed after 2 h of incubation increased to \approx 46% after 24 h of incubation, this makes it a good biomarker candidate. TP-166b at 6.83 min with the product ion m/z 149.094 formed through the *N*-demethylation of PMMA and was a match for PMA previously identified in a previous study^{31, 37, 51}; it was formed immediately after spike and increased slightly over 24 h to \approx 3%.

TP-166a would be an ideal biomarker for PMMA given its high abundance over the 24 h period, however, it has also been identified as a metabolite of methamphetamine³¹, as such, it is not specific and cannot be used. In any case, PMMA is highly stable and should be detectable if present.

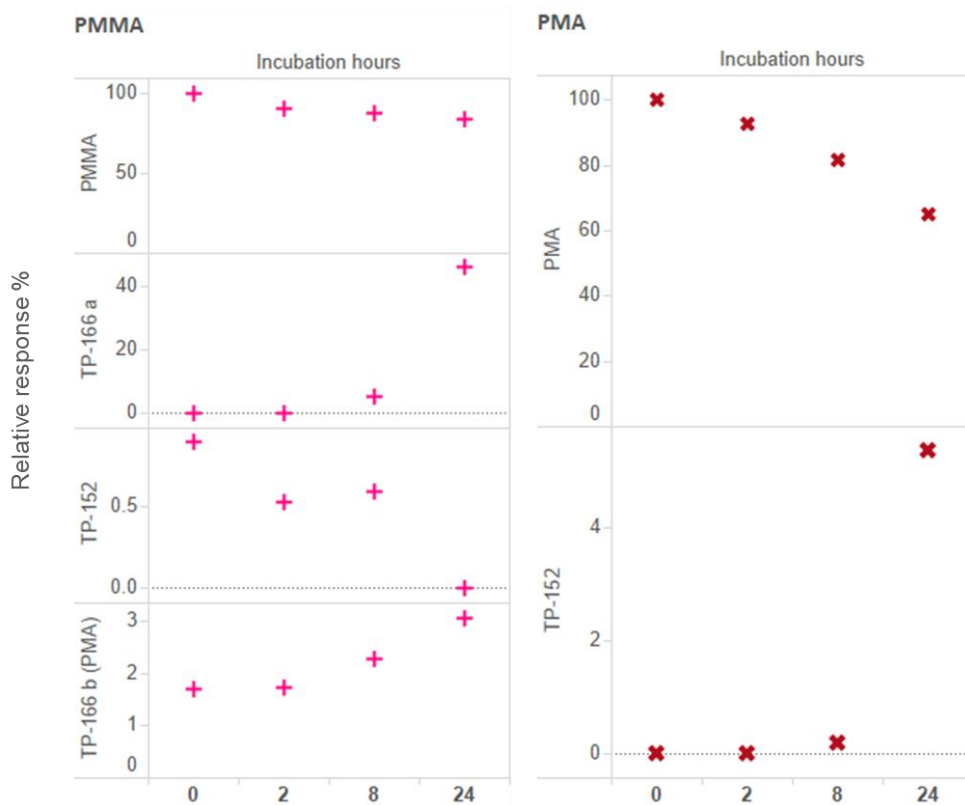


Figure 3. Relative response of PMMA, PMA, and their TPs over 24h incubations in wastewater containing biofilm in sewage containing biofilm

Table 4. Transformation products identified for PMMA and PMA over 24 h *in-sewer* incubations in the presence of biofilm

Compound	^a t _R	Measured <i>m/z</i> [M+H] ⁺	^b Δ <i>m</i> (ppm)	Diagnostic product ions	Chemical Formula	^c Level
PMMA and TPs						
PMMA	7.93	180.1385	1.11	77.0393, 91.0559, 107.0855, 121.067, 149.0954	[C ₁₁ H ₁₈ NO] ⁺	1
TP-166a	3.49	166.1226	0	77.0379, 91.0533, 107.0496, 135.0804	[C ₁₀ H ₁₆ NO] ⁺	2a
TP-152 _{PMMA}	4.99	152.1078	5.26	not found	[C ₉ H ₁₄ NO] ⁺	4
TP-166b (PMA)	6.83	166.1214	-7.22	149.094	[C ₁₀ H ₁₆ NO] ⁺	1
PMA and TPs						
PMA	6.87	166.122	-3.61	91.0557, 121.0646, 149.0955	[C ₁₀ H ₁₆ NO] ⁺	1
TP-152 _{PMA}	2.2	152.1083	8.55	107.0496, 135.0806	[C ₉ H ₁₃ NO] ⁺	2a

^a Retention time (min); ^b *m/z* accurate mass measurement error; ^c Identification level according to Schymanski et. al⁴⁰

3.3.2 PMA

PMA had medium stability, its levels decreased by approximately 35% after 24 h incubation (Table SI-4, Figure 3). One TP of PMA with *m/z* 152.1070 at t_R 2.20 min and with product ions at *m/z* 107.0496, and 135.0806 (Table 4) was detected and confirmed to be the *O*-demethylated product (Figure 4). Its identity was consistent with the PMA metabolite *p*-OH-amphetamine and identified in a previous study³⁷. However, since *p*-OH-amphetamine is also a metabolite of amphetamine³¹, it would not be the ideal biomarker for PMA in WBE studies. Interestingly, PMA detected in wastewater could be a result of *in*-

sewer transformation of PMMA, PMMA and/or PMA and/or mebeverine⁵² consumption. This makes PMA a non-specific biomarker.

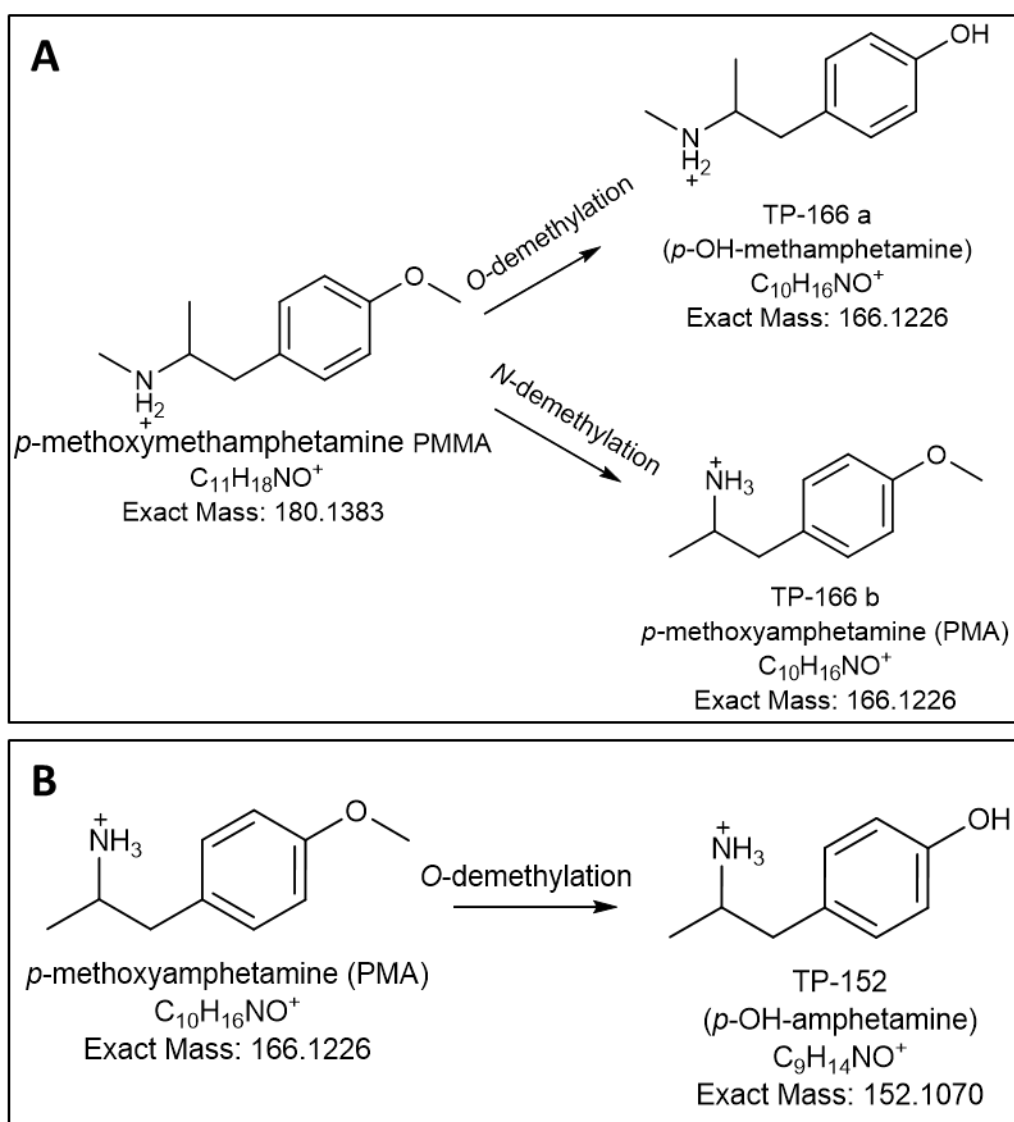


Figure 4. Proposed *in-sewer* biotransformation pathway of PMMA (A) and PMA (B)

4. Conclusions

Overall, the experiments led to the identification of 18 TP3 for five NPS (MDPV, methylone, mephedrone, PMMA, and PMA), nine of which were confirmed using accurate mass, t_R and fragment ions (Level 1 and 2a). The identification of many TP3 was limited to Level 2-4 due to the unavailability of commercial reference standards. The *in-sewer* experiments demonstrated the importance of biofilm which affects stability and (bio)transformation, consequently, introducing five unique TP3

formed through matrix interactions as was observed for MDPV, methylone, mephedrone and PMMA. In addition, we observed comparable *in-sewer* stability for the synthetic cathinones (MDPV, methylone, and mephedrone) and similar biotransformation reactions including reduction of carbonyl group for the three. The *in-sewer* stability of the two phenylethylamines (PMMA and PMA) was different, however, their biotransformation reactions were similar (*O*-demethylation). Additionally, the residence time of 24 h was useful in determining the possible biomarkers of interest. In a previous study, mean hydraulic residence times (HRT) were collected from relatively large cities, ranging from 1-12 h with an average of 3.8 h (20 cities in 2012) and 4.3 h (30 cities in 2013) {Ort, 2014 #750}. Therefore, the TPs formed after 8 h at very low levels are likely to be undetectable in influent wastewater due to dilution and HRT.

The *in-sewer* experiments led to the formation of 13 TPs previously identified as human metabolites during *in vitro* metabolism studies^{32, 34, 35, 37}. This shows that *in-sewer* transformations contribute to the levels of drug target residues measured in influent samples for WBE monitoring. The correction factor applied to WBE's back-calculation model relies on the human excretion pharmacokinetic data to generate the molar ratio (parent: metabolite), however, it does not account for levels from *in-sewer* transformation⁵³. Therefore, it is important to identify TPs formed *in-sewer* as they may influence accuracy of the correction factor in WBE calculations. Exploring the impact of TPs on correction factors would be worthy in future studies.

Analysis of identified TPs has limitations, many of them do not have commercially available reference standards which limits their targeted quantitation, and our understanding of their LC-MS attributes like matrix effects. Therefore, future *in-sewer* stability and/or biotransformation studies should consider replicate reactors to aid in estimation and accounting for analytical variability.

Recently, NPS monitoring using WBE is shifting from targeted to suspect screening so as to 'catch up' with the dynamic changes in NPS markets^{1, 15}. The application of wide-scope screening techniques

485 (qualitative) based on HRMS for WBE purposes casts a wider net and reduces the likelihood of missing
486 compounds as is the case in targeted analysis. Furthermore, qualitative techniques offer the
487 opportunity for retrospective data analysis. The identification of TPs is particularly useful for database
488 development and imperative to suspect screening which is reliant on a comprehensive
489 library/database. Information on identified TPs will be useful for reporting detection frequencies of
490 circulating NPS. The newly identified TPs in this study can be used as potential biomarkers in targeted
491 and suspect analysis.

492 In conclusion, the study showed the importance of understanding the fate of compounds during *in-*
493 *sewer* transport for WBE purposes, and the potential for such experiments in the identification of
494 biomarkers for the monitoring of NPS.

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