Sewage-based epidemiology in monitoring the use of new psychoactive substances: Validation and application of an analytical method using LC-MS/MS

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Abstract

Sewage-based epidemiology (SBE) employs the analysis of sewage to detect and quantify drug use within a community. While SBE has been applied repeatedly for the estimation of classical illicit drugs, only few studies investigated new psychoactive substances (NPS). These compounds mimic effects of illicit drugs by introducing slight modifications to chemical structures of controlled illicit drugs. We describe the optimization, validation and application of an analytical method using liquid chromatography coupled to positive electrospray tandem mass spectrometry (LC–ESI-MS/MS) for the determination of seven NPS in sewage: methoxetamine (MXE), butylone, ethylone, methylone, methiopropamine (MPA), 4-methoxymethamphetamine (PMMA), and 4-methoxyamphetamine (PMA). Sample preparation was performed using solid-phase extraction (SPE) with Oasis MCX cartridges. The LC separation was done with a HILIC (150 x 3 mm, 5 µm) column which ensured good resolution of the analytes with a total run time of 19 min. The lower limit of quantification (LLOQ) was between 0.5 and 5 ng/L for all compounds. The method was validated by evaluating the following parameters: sensitivity, selectivity, linearity, accuracy, precision, recoveries and matrix effects. The method was applied on sewage samples collected from sewage treatment plants in Belgium and Switzerland in which all investigated compounds were detected, except MPA and PMA. Furthermore, a consistent presence of MXE has been observed in most of the sewage samples at levels higher than LLOQ.

Keywords: novel psychoactive substances (NPS), sewage epidemiology, wastewater, LC-MS/MS, methoxetamine

Introduction

Conceptualized in 2001 by Daughton[1], sewage-based epidemiology (SBE) is the analysis of excretion products of illicit drugs in sewage with the purpose of estimating community drug use. Sewage-based epidemiology has been applied since 2005 as an approach complementary to classical investigation methods such as e.g. consumer interviews, medical records, population surveys, and crime statistics for estimating illicit drug use in communities.[2-6] Data obtained from SBE provides information on drug use in a direct, quick and objective way on an international scale.[7,8]

New Psychoactive Substances (NPS) are substances that are not controlled by the 1961 United Nations Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances and that may pose a threat to public health.[9,10] These compounds mimic effects of illicit drugs like cocaine, cannabis and amphetamines – and are produced to evade law enforcement by introducing slight modifications to chemical structures of controlled substances.[11] Currently, there are several classifications of NPS based on 1961 and 1971 (UK Misuse Act).[11] The main families monitored by EU Early warning system (EWS) since 2005 include synthetic cannabinoids, phenethylamines, other substances (derivatives, medicinal products, intermediates and precursors), arylalkylamines, cathinones, opoids, benzodiazepines, tryptamines, aminoindanes, arylcyclohexylamines, piperazines,piperideines and pyrrolidines, and plants and extracts.[12] Often, NPS are easily acquired legally through the internet and smart shops where they are sold under various product labels with misleading information about their effects and safety.[12,13] They are considered a growing problem in many communities and are responsible for numerous fatal intoxications.[14]

The NPS issue is not that recent, it was in the 1960s when some research groups caught on to the idea of drugs with effects similar to an illicit drug being sold legally.[10,15]

However, more recently, the speed at which these compounds appear has tremendously risen. Only since 2009 to 2013, the UNODC have reported the detection of 182 new compounds. Of these compounds, synthetic cathinones, phenylethylamines and phenycylidine-type accounted for 25, 17 and 4% respectively in 2013.[9] However, little is known about the absorption, distribution, metabolism, excretion and toxicity of these substances and we come to learn about most NPS from fatal intoxications when it is too late for intervention.[16,17] Furthermore, data on NPS use and prevalence in different regions of the world is very scarce. With NPS being so transitory and dynamic, SBE has the potential to be usefully applied for their detection and quantification to document their occurrence to appropriate authorities. Being an emerging issue, only a few studies have applied SBE for the analysis of NPS.[18-20] Nonetheless, developed methods should attempt to detect and quantify these compounds at very low concentrations (<10 ng/L) and using a more realistic matrix, like actual sewage, in order to overcome challenges associated with matrix effects. This study confronts factors and challenges associated with application of SBE in analysis of NPS.

In this study, a sensitive analytical method based on solid-phase extraction (SPE) and LC-MS/MS was optimized and validated for the detection of stimulant-type NPS in sewage. This method was applied to sewage samples collected from sewage treatment plants (STPs) within urban areas in Belgium and Switzerland to get an overview of the use of these compounds in the urban areas under investigation, and test if the SBE approach is sensitive enough to pick up the use of these NPS.

Experimental

Reagents and materials

The reference standards for the investigated compounds (methoxetamine (MXE), butylone, ethylone, methylone, methiopropamine (MPA), 4-methoxymethamphetamine (PMMA), 4-methoxyamphetamine (PMA), see Fig 1 for the chemical structures) and the internal reference standards used (butylone-D_3, ethylone-D_5, methylone-D_3, amphetamine-D_8, methamphetamine-D_8 with purity >98%) were purchased from Cerilliant (Round Rock TX, USA) at concentrations of 1 mg/mL in methanol (MeOH) or acetonitrile (MeCN). Working solutions were prepared for concentrations ranging between 0.005 to 100 ng/µL in MeOH. LC-grade MeCN and MeOH, ammonium hydroxide (NH_4OH), hydrochloric acid (HCl) and ammonium acetate (AmOAc) were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained by purifying demineralized water in an Elga LabWater Purelab Flex system (Veolia Water Solutions & Technologies Belgium, Tienen, Belgium). Oasis HLB (60 mg, 3 mL) and Oasis MCX (60 mg, 3 mL) SPE cartridges were acquired from Waters (New Bedford, MA, USA) and a Supelco Visiprep™ SPE Vacuum Manifold with 24 ports and a self-cleaning dry vacuum system™ Welch 2023 was used for the loading of sewage samples and for the drying of the cartridges.

Sample preparation and extraction

For the method optimization and validation, we used sewage samples collected prior to 2009, in which NPS have not been detected (called further “blank” sewage). These blank samples were pooled and used for the all validation experiments. Prior to extraction, 50 mL sewage were filtered through a 0.7 µm glass filter to remove solid particles. After filtration, the samples were brought to pH 2 using a 6 M HCl solution and spiked with internal standards at a concentration of 100 ng/L. Oasis MCX cartridges were selected for the sample extraction

and were conditioned with 6 mL of methanol, 4 mL Milli-Q water, and 4 mL of Milli-Q water at pH 2 at a rate of 1 mL/min (no vacuum applied). Sewage samples (Table SI-2) were passed through cartridges at a rate of 2.5 mL/min (vacuum applied) and then washed with 3 mL Milli-Q water before drying (30 min). Final extracts were eluted using 2 mL MeOH and 2 mL of 5% NH_4OH in MeOH, collected and evaporated at 30 °C to dryness under a gentle stream of nitrogen, reconstituted in 50 µL MeCN and 50 µL (5 mM AmOAc in water/MeCN, 90/10), vortexed for 60 sec and transferred to an injection vial.

Instrumentation

The liquid chromatographic system was an Agilent 1260 Infinity High Pressure LC (HPLC) fitted with a degasser, a binary high-pressure gradient pump, a thermostated column compartment and an autosampler module. Chromatographic separation was achieved using a Phenomenex Luna HILIC (hydrophilic interaction liquid chromatography) 200 Å (150 x 3 mm, 5 µm) column, with a mobile phase composed of A) 5 mM AmOAc in ultrapure water and B) MeCN, at a flow rate of 0.4 mL/min. The gradient was as follows: 0-0.5 min: 95% B; 0.5-5 min: 95%-90% B; 5-12.5 min: 90%-70% B; 12.6-14.6 min 30% B; 14.7 min 95% B. The total run time including column equilibration was 19 min. The injection volume was optimized based on peak shape and set to 2 µL. The LC system was coupled to an Agilent 6410 triple quadrupole mass spectrometer with an electrospray interface (ESI) for the detection and quantification of compounds. Source parameters were as follows: gas temperature 350 °C, gas flow 10 L/min, nebulizer 40 psi, capillary voltage 4000 V. The mass spectrometer compound dependent parameters were optimized for each compound individually (Table 1).

Method development and validation

Mass spectrometer parameters

All measurements were performed in the positive ionization mode. Optimization of the parameters was carried out by direct injection (without column) of reference standards. Source parameters (gas temperature, gas flow, nebulizer pressure, and capillary voltage) were optimized to acquire an intense protonated molecular species $[M+H]^+$ for each compound. Mass spectrometer compound dependent parameters, fragmentor voltage and collision energy, were optimized to acquire two multiple reaction monitoring (MRM) transitions (qualifier and quantifier) for each compound, and one MRM for the internal standards. Table 1 summarizes the optimized MRM transitions. The most abundant transition in terms of signal to noise ratio (S/N) was chosen as quantifier (Q) and the second most abundant transition as qualifier (q). The Q/q ratio was monitored for variation (RSD < 20%) to provide an additional identification criterion besides the retention time

Liquid chromatography

For LC optimization, a standard mix with all compounds was injected on both HILIC and reversed-phase C_{18} columns, together with the use of different organic phase (MeCN, MeCN with 0.01% formic acid, and MeOH) and buffer (H_2O, 0.1% HCOOH; 5 mM AmOAc in H_2O). The phenethylamine-based compounds (butylone, ethylone, methylone, PMA, PMMA and MPA) are very polar and thus retained well on HILIC columns with MeCN and 5mM AmOAc in H_2O buffer. In addition, different injection volumes (1, 2, 3, or 5 µL) were tested, together with their effect on peak shape.

Solid-phase extraction

The optimization 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

maximum recovery for the compounds of interests, two sorbents (Oasis HLB and MCX) were tested under different pH conditions (2, 7, and 10) using different extraction protocols found in literature.[18,21] In order to estimate the recovery, spiked blank surface water with all compounds at 100 ng/L pre- and post-SPE were compared.[22] The protocol used was adopted with some changes (e.g. solvent volumes) from previous protocols.[21]

Method validation

Method validation was conducted based on the guideline for Bioanalytical Method Validation provided by the European Medicines Agency (EMEA)[23] with some exceptions and was done to evaluate performance features, such as precision, accuracy, selectivity/specificity, linearity, calibration range, recovery, matrix effects, lower limit of quantification (LLOQ), and sensitivity.

Matrix-matched multi-component calibration curves based on internal standards (IS) with seven calibration points ranging from 0.5-200 ng/L (for MXE and methylone) and from 2–200 ng/L (other substances) were prepared by spiking 50 mL blank sewage with analytes of interest before SPE. In addition, a blank sewage sample (processed matrix sample without analyte and without IS), a zero sample (processed matrix with IS) and two QC samples at lower and higher levels in the calibration range (30 and 120 ng/L) were included. Calibration curves (n=3) for quantification were constructed by plotting the ratio between the peak area of the analyte and its corresponding internal standard against the spiked concentration and were 1/x weighted.

Carryover was evaluated by injecting a blank sample fortified with internal standard after the highest concentrated (200 ng/L) calibration standard injection in the instrumental sequence.

The between- and within-run accuracy and precision of the method based on back-calculated concentrations were evaluated by analyzing 11 blank sewage samples (50 mL) spiked with each compound at lower and higher concentration levels in the calibration range (30 and 120 ng/L) over a 3 day validation period. These features were assessed with an acceptance criteria within 85-115% (mean) accuracy and <15% (RSD) precision.[23] LLOQ and limit of detection (LOD) were calculated for S/N 10 and 3, respectively based on five replicates.

Matrix effects were evaluated and quantified during method optimization and validation using blank surface water and blank sewage using the method suggested by Matuszewski et al.[22] This involved the comparison of analyte responses of standards spiked pre-extraction, post-extraction and no extraction (in mobile phase).

Results and Discussion

Method optimization

The SPE recovery experiments were performed in surface water (Table SI-1) to compare performance of the Oasis MCX and HLB sorbents. The experiments proved that the Oasis MCX with the sample brought to pH 2 were the best conditions for the extraction of the investigated NPS with recoveries > 92%. Furthermore, the SPE procedure for NPS spiked in sewage (Table 2) also resulted in similar recoveries. The whole analytical procedure is depicted in Fig SI-1.

In LC optimization, we tested C_{18}-based chromatography since it is the first choice and has general good retention for a broad range of compounds, however, MPA was not well retained. HILIC in combination with AmOAc (A) and MeCN (B) was selected due to its good retention and separation capacity for our NPS of interest. To ensure reproducibility and repeatability of the HILIC during the run, we monitored retention time (t_R) of the matrix-

matched calibrators over 19 injections and RSD was ≤ 1% (Table SI-3). A similar study [24] has demonstrated the stability of chromatographic t_R of HILIC after column conditioning with matrix injections suggesting the stability is affected by matrix and contamination. The injection volume selected (2 µL) gave the best peak shape for the compounds.

Quantification and method validation

Selectivity, linearity, LLOQ, precision, accuracy, carry-over, and matrix effects were criteria assessed for the method validation. These requirements were all within the acceptable criteria provided by the EMEA guidelines as illustrated above.[23]

A linear range ($R^2 > 0.99$) from 0.5 ng/L (MXE and methylone) and 2 ng/L (all other compounds) to 200 ng/L was achieved for investigated compounds. The LOD was selected based on the lowest concentration with S/N=3, which was between 0.02- 0.2 ng/L for all compounds. Further, the LLOQ was optimized to achieve the lowest possible concentration that LC-MS/MS instrument could reliably measure and quantify and was selected based on the lowest concentration with S/N=10, which was 2 ng/L for all compounds, except MPA (5 ng/L); MXE and methylone (both 0.5 ng/L). The mean accuracy (%) and precision (% RSD) measured at LLOQ level ranged between 80 - 98% and 1 - 5%, respectively which was within the acceptable criteria of the EMEA guidelines.[23]

Precision and accuracy results at the two spiking levels are presented in Table 2. For all compounds, the inter- and intra-day precision (% RSD) ranged between 1 - 5%. The inter- and intra-day mean accuracy ranged between 98 - 108%.

Oasis MCX with sample at pH 2 gave the highest recovery for all NPS during validation (Table SI-1) where recoveries (done in duplicate) ranged between 92 and 109%. Matrix effects (signal suppression) were between 3 and 7% for all compounds, except MXE which ranged between 14 and 24% (Table 2).

No carryover above 20% of LLOQ of the standard and 5% for the internal standards (IS) was observed in the blank samples which were analyzed after highly concentrated samples in the sequence run, as specified in EMEA guidelines.[23]

Internal standard optimization was necessary for MXE, MPA, PMMA and PMA for which no commercially available labeled IS was available at the time of the experiments. For these compounds, another IS was selected based on chemical similarity and close retention times to the compounds of interest: butylone-D_3 for MXE and amphetamine-D_8 for MPA, PMMA and PMA (Table 1).

Method optimization with sewage

Our study also describes the use of blank sewage for method development, facilitating the characterization and handling of the challenges arising during the analysis of very low concentrations of analytes of low molecular weight (MW < 200). Except for MXE, the matrix effects were low considering that the method was optimized using real sewage water. For MXE, we experienced about 19% signal suppression in sewage (Table 2).

Method optimization for PMA was particularly challenging in sewage, due to an interfering substance that is closely related, with one similar MRM transition (166.1 to 121.0), but a slightly lower retention time (Fig SI-2). For this reason, we narrowed the retention time interval (0.4 min left and right) in the quantification, in order to strictly capture only PMA. We used the 166.1>121.0 MRM transition as the qualifier and finally slightly adjusted the gradient to separate the two peaks. With further experiments, we established that hordenine was the interfering substance present in sewage (Fig SI-2). We optimized parameters for hordenine by injecting a reference standard and found three MRM transitions (166.1 > 121.0; 166.1 > 93.1 and 166.1 > 103.1) and a retention time of 11.2 min.

The method could reliably differentiate the analytes and IS from endogenous components (Figure 2). As in the case of PMA and hordenine, our method can distinguish these two related compounds (Fig SI-2). Hordenine is a phenethylamine that occurs naturally in some plants, including barley which is used to produce beer. It is also used in nutritional supplements and is thus expected to be found in sewage, possibly as an urinary excretion product of beer drinkers.[25]

Sewage samples

Influent 24 h composite sewage samples (SI-Table 2) from six STPs in urban centers – five in Belgium (Dec 2013) and one in Switzerland (Aug 2013) – were used to assess the applicability of the validated method. All tested samples, except two from Belgium, contained MXE at levels between 1.5 and 3.0 ng/L (Table 3). Additional samples were collected over a two-week period in March-April 2014 to monitor trends in MXE loads from three STPs in Belgium (Ninove, Antwerp-Zuid, and Antwerp-Deurne) (Figure 3). Higher loads of MXE from STP Antwerp-Zuid (catchment area of the centre of Antwerp) compared with STP Antwerp-Deurne (catchment area of the suburbs of Antwerp) were observed with a general increase on Sunday and Monday. In STP Ninove (small town), lower loads than those of the two Antwerp STPs were observed, except for the two Friday samples which showed higher loads (Figure 3). MXE is a ketamine analogue and a new recreational drug that is currently not subject to restrictive regulations in most countries. In May 2014, EMCDDA released a report on MXE[26] which indicated that most data gathered on MXE were from intoxications and seizures from 15 European countries not including Switzerland. This study reveals the presence of MXE in sewage from five urban centers within two countries and further monitoring shows the continuous presence of MXE in three different catchments in Belgium over two weeks in 2014.

MPA and PMA were not detected in any of the samples at a LOD of 0.7 and 0.2 ng/L, respectively. Butylone, ethylone and PMMA were detected; however the concentrations were below LLOQ (Table 3). Methylone was detected and quantified in only two samples from Switzerland at levels slightly higher than LLOQ (Table 3, Fig SI-3). The results from these samples demonstrate the importance of SBE in revealing the occurrence of NPS within catchment areas of urban centers and the need to develop very sensitive analytical methods. The compounds that were not detected could be absent in the sewage or present in the form of metabolites which were not targeted in the present study. Further studies on the biotransformation of NPS need thus to be carried out to provide SBE with information regarding additional biomarkers of NPS parent drugs.

Future of SBE in NPS analysis

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 depending on the area served by the STP and on the prevalence of its use.[27] Therefore, pooled urine analysis[25,28] would be useful in detecting the occurrence of NPS before dilution into sewage. However, SBE has the advantage of representing entire populations served by the corresponding STPs. Reid et al.[19] measured the presence of N-5-hydroxypentyl JWH-018, a metabolite of the synthetic cannabinoid JWH-018, in two STPs in Norway using SBE. Kankaanpää et al.[19] also detected MDPV in two regions in Finland using SBE. Subsequently, it was imperative to optimize the LLOQ for each compound and develop a sensitive analytical method to detect and quantify low concentrations. It would be a valuable approach to combine pooled urine analysis and SBE in tackling the issue of NPS in communities. SBE requires a specific, reliable, stable biomarker for the NPS of interest. However, studies on NPS are fairly recent and much information is unknown about them.

With several emerging studies on *in vivo* and *in vitro* metabolism[29,30], biomarkers can be identified for their detection in sewage. In addition, the studies on stability of biomarkers under different conditions would contain pertinent information in determining the most reliable biomarker.

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Supplementary information is available

The supplementary information contains three figures and three tables.

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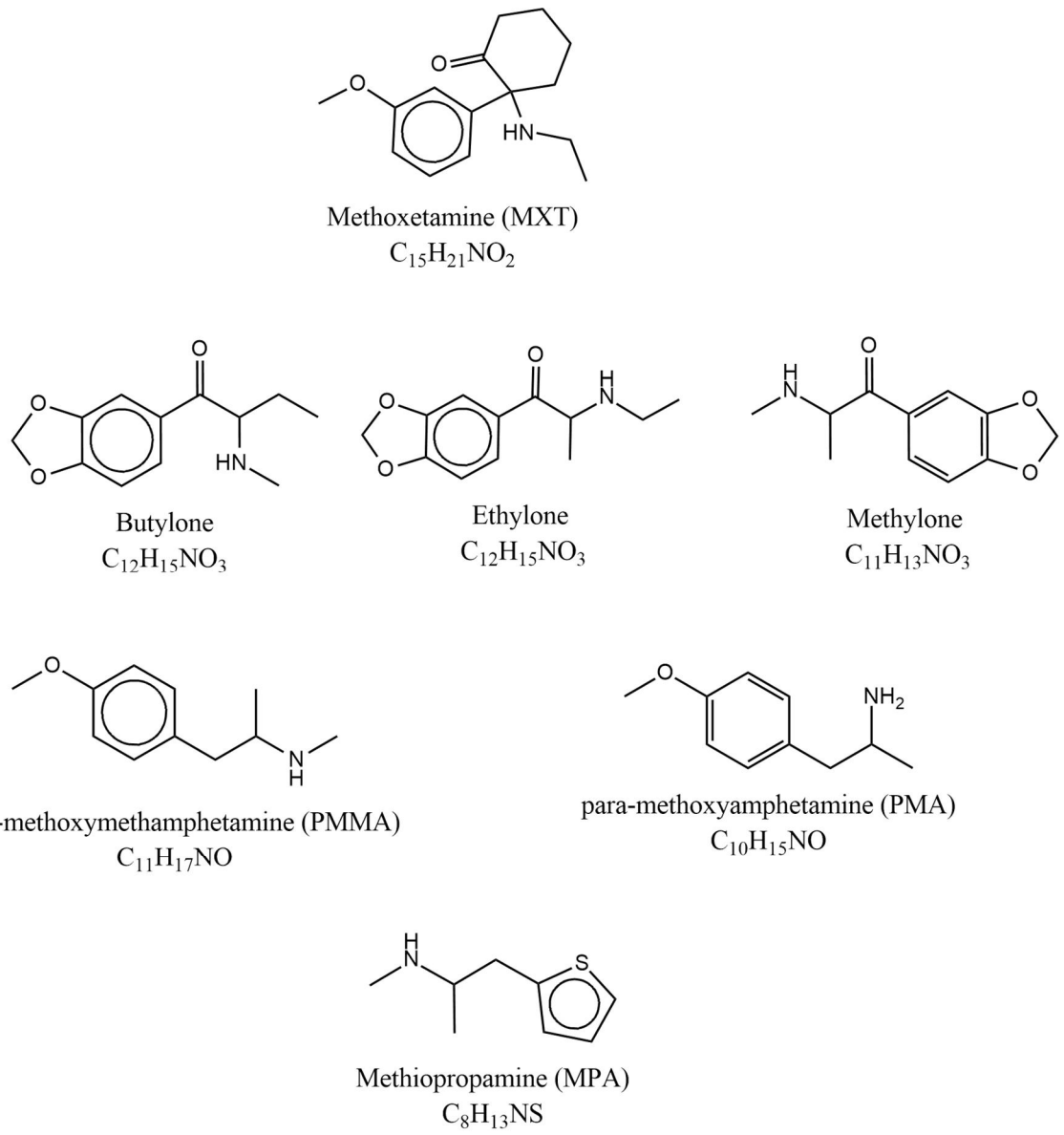

Figure 1: Chemical structures of the investigated new psychoactive substances.

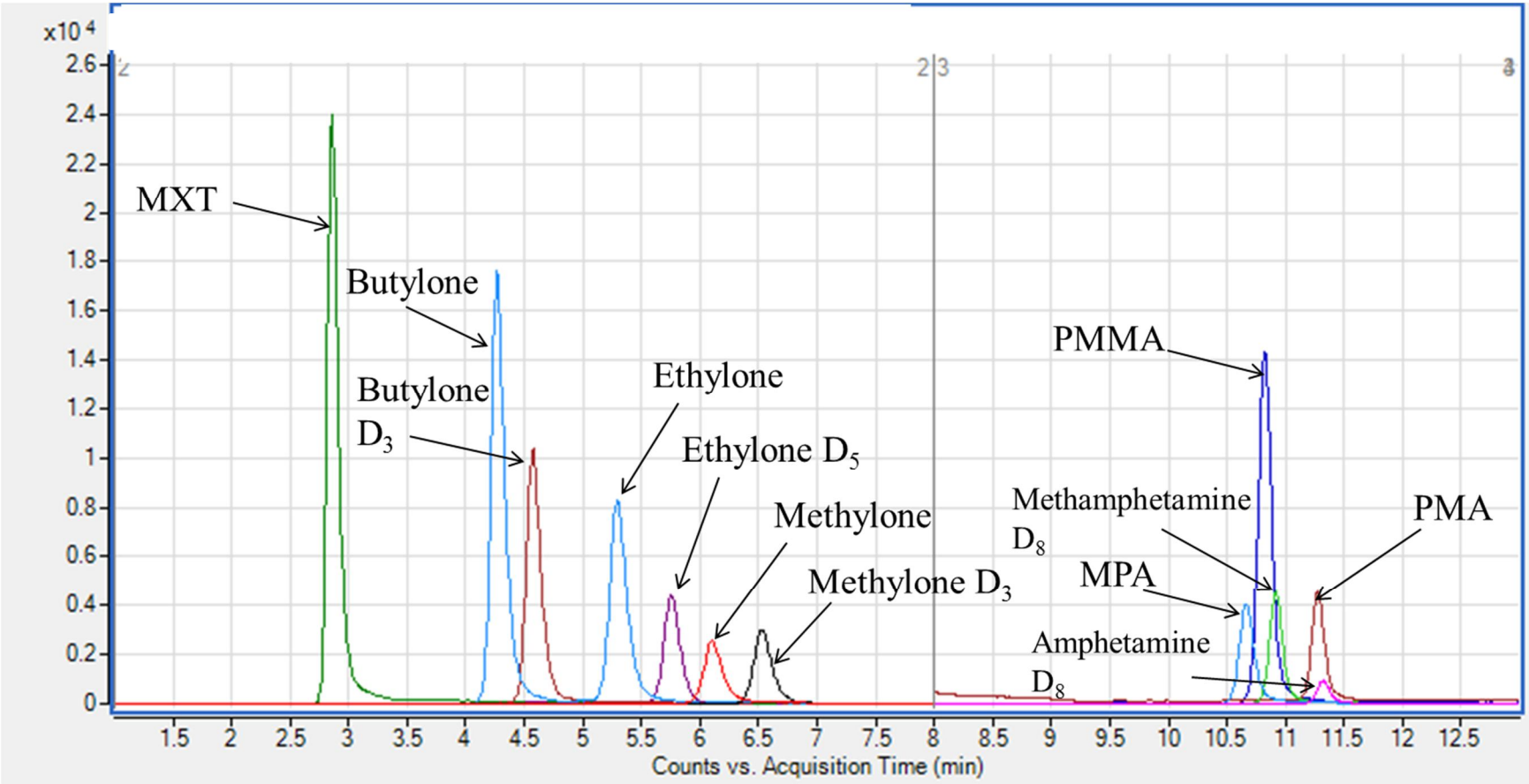

Figure 2: Chromatogram of the quantifier transitions in blank wastewater spiked at QC high concentration.

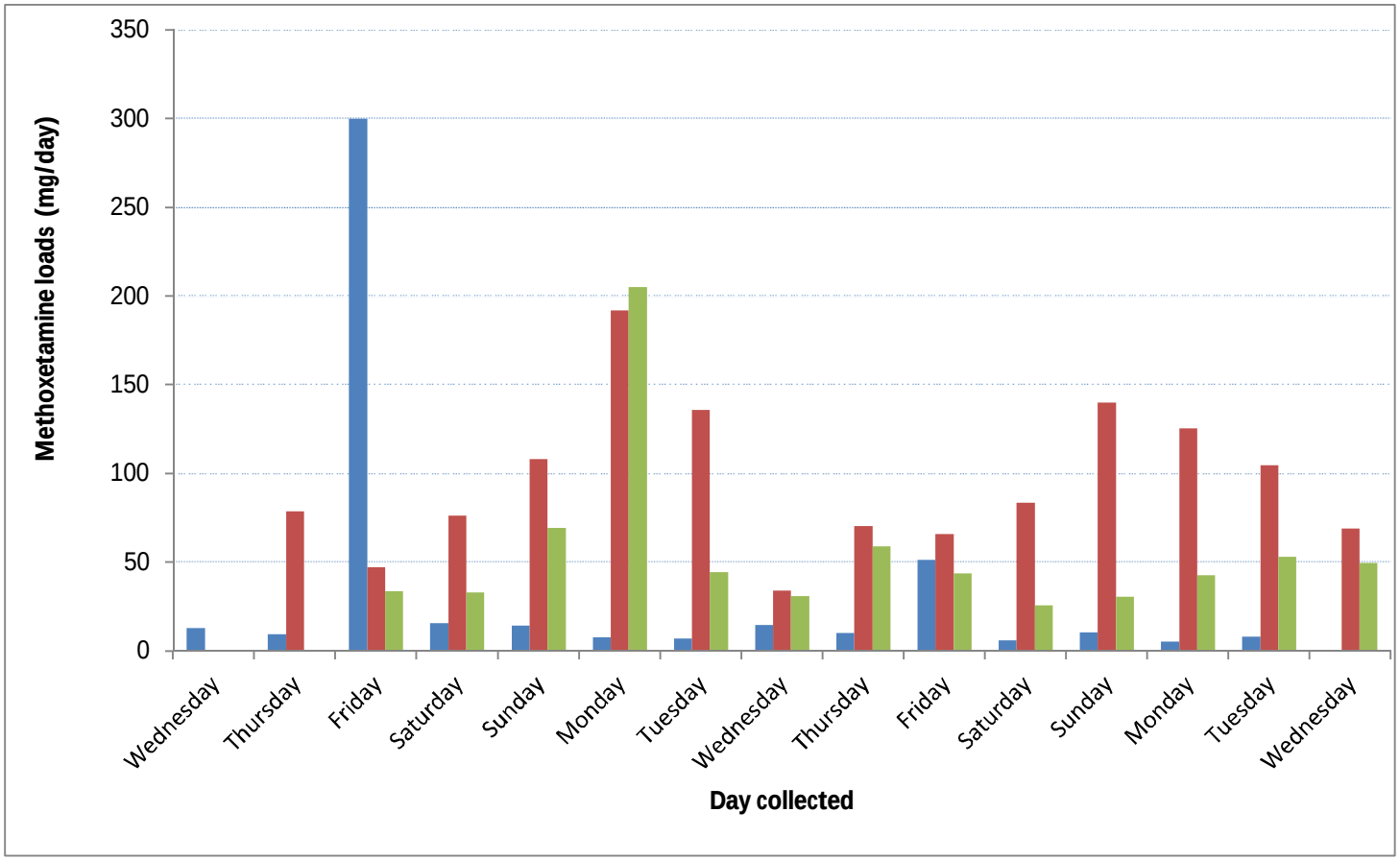

Figure 3: Daily variation in mass loads (mg/day) of MXE for Ninove-Belgium (19 March - April 1, 2014), Antwerp-Zuid (April 3 – April 16th 2014), and Antwerp- Deurne (April 4 – April 18th, 2014)

Table 1: Optimized MRM transitions and retention times for selected compounds and the deuterated internal standards.

				Quantifier		Qualifiers	
Compound name	**Retention time (min)**	**Precursor ion (m/z)**	**Fragmentor voltage (V)**	**Product ion (m/z)**	**Collision energy (V)**	**Product ions (m/z)**	**Collision energy (V)**
Methoxetamine	2.9	248.3	90	203.2	5	121.0	25
Butylone	4.6	222.1	90	146.1	25	131.0	35
Butylone-D_3	4.9	225.1	90	177.1	15		
Ethylone	5.8	222.2	80	174.1	15	146.1	25
Ethylone-D_5	6.2	227.1	100	179.1	15		
Methylone	6.6	208.1	100	190.1	5	160.1	15
Methylone-D_3	6.8	211.1	95	163.1	15		
Methiopropamine	9.8	156.1	80	125.0	5	97.1	15
Amphetamine-D_8	10.3	144.0	80	127.0	5		
PMMA	10.9	180.1	90	149.1	5	121.0	15
Methamphetamine-D_8	9.9	158.1	80	93.1	15		
PMA	11.4	166.1	60	149.1	3	121.0	7

Table 2: Method validation criteria: solid-phase extraction recovery, accuracy and precision (intraday and interday) at two quality control (QC) concentrations (low=30 ng/L; high=120 ng/L).

Compounds	**Linear range (ng/L)**	**SPE Recovery (n=3) (% ± SD)**	**Matrix Effects (n=4) (% ± SD)**	**Accuracy (%)**				**Precision (RSD %)**			
				Intra-day		**Interday**		**Intra-day**		**Interday**	
				QC low (n=5)	**QC high (n=5)**	**QC low (n=11)**	**QC high (n=11)**	**QC low (n=5)**	**QC high (n=5)**	**QC low (n=11)**	**QC high (n=11)**
Methoxetamine	0.5 - 200	99 ± 3	19± 5	101	96	101	98	1	4	1	2
Butylone	2 - 200	96 ± 2	3 ± 2	108	100	104	100	3	1	4	1
Ethylone	2 - 200	95 ± 10	5 ± 1	99	100	100	101	1	1	1	2
Methylone	0.5 - 200	105 ± 1	7 ± 2	100	100	100	100	1	1	1	1
Methiopropamine	5 - 200	96 ± 3	4 ± 1	102	106	101	103	4	2	4	3
PMA	2 - 200	94 ± 7	5 ± 1	99	99	98	99	3	2	3	1
PMMA	2 - 200	97 ± 3	4 ± 7	100	100	100	100	1	1	1	1

Table 3: Concentrations (ng/L) of the substances under investigation in influent sewage from sewage treatment plants (STPs) in Belgium and Switzerland. D= above LOD, ND= below LOD.

STP	Date	MXE	Butylone	Ethylone	Methylone	MPA	PMMA	PMA
Antwerp-Noord (BE)	03/12/13	1.8	ND	ND	ND	ND	ND	ND
Antwerp-Zuid (BE)	02/12/13	3.1	ND	D	ND	ND	ND	ND
Antwerp-Zuid (BE)	3/4 - 15/4/2014	0.7-3.4	ND	ND	ND	ND	ND	ND
Antwerp-Deurne (BE)	4/4 - 18/4/2014	0.7 – 2.7	ND	ND	ND	ND	ND	ND
Boechout (BE)	02/12/13	1.9	D	ND	ND	ND	ND	ND
Boechout (BE)	03/12/13	1.7	ND	ND	ND	ND	ND	ND
Ninove (BE)	19/3 - 27/3/2014	0.8-12.5	ND	ND	ND	ND	ND	ND
Ruisbroek (BE)	11/12/13	ND	ND	ND	ND	ND	ND	ND
Zele (BE)	13/12/13	ND	ND	ND	ND	ND	ND	ND
Zurich (CH)	9/8/13	2.5	ND	D	2.5	ND	D	ND
Zurich* (CH)	10/8/13	1.8	D	D	0.6	ND	D	ND
Zurich* (CH)	11/8/13	1.5	ND	ND	ND	ND	ND	ND

*: Saturday Street Parade took place in Zurich (CH) during this period