Increases in use of novel synthetic stimulant are not directly linked to decreased use of 3,4-methylenedioxy-N-methylamphetamine (MDMA)

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Abstract

A decline in 3,4-methylenedioxy-N-methylamphetamine (MDMA) use in Adelaide, Australia from 2009 to 2010 was confirmed by us previously. Reports suggested that the shortage in MDMA supply was associated with an increased prevalence of other synthetic stimulants, but quantitative measurements were unavailable. To obtain objective data on the community use of synthetic stimulants, we collected wastewater samples from multiple treatment plants in Adelaide, Australia from 2009 to 2011 and analysed them using solid-phase extraction/liquid chromatography/tandem mass spectrometry (SPE-LC-MS/MS), targeting MDMA and some of the most reported synthetic cathinones and piperazines. Data were temporally compared. MDMA and six other synthetic stimulants were detected and quantified in wastewater samples. While MDMA level decreased markedly from 2009 to 2010 and remained low in 2011, localized increased use of mephedrone, methylone, methylenedioxypyrovalerone (MDPV), benzylpiperazine (BZP), 3-trifluoromethylphenylpiperazine (TFMPP), but not methcathinone, was observed in 2010 and 2011. This suggested that the decline in MDMA use was associated with an increase in the use of a number of other synthetic stimulants. However, the lag time from the decrease in MDMA to the increase in use of a number of these stimulants, together with the highly regionalized use of all synthetic stimulants except methcathinone indicates that there was no direct population wide substitution in response to the reduction in MDMA.

Key words: Wastewater; MDMA; Synthetic stimulant; New psychoactive substance; Temporal comparison; Stability.

Introduction

Drug abuse is a global phenomenon, which causes various health, social and economic problems. Prevalence of drug use in a defined region is crucial information to allow healthcare and policies to be geographically and temporally specialized to improving the cost-efficiency of resources spent on reducing population drug misuse. The information on population drug use is conventionally collected via large-scale surveys, hospital presentations, and customs and police seizures, which provide informative data on drug users' profiles and a snapshot of the drug use situation, but have some limitations with obtaining timely information about fast-changing drug markets. Wastewater analysis directly measures the concentration of drugs of interest (or their metabolites) in municipal wastewater, regardless of their pharmacological properties or street names, and then back-calculates the original disposition or consumption in the contributing population [1]. Thus, it has been used to provide information on drug use in the population with high reliability and timeliness [2,3].

Recently, there has been some attention to the apparently increasing use of novel synthetic cathinones and piperazines. These compounds have similar pharmacological properties to the popular stimulants such as cocaine, amphetamines and 3,4-methylenedioxy-N-methylamphetamine (MDMA) [4-9]. Toxicity [10-14] and some deaths [15-17] have been associated with the recreational use of these drugs. These drugs are sometimes sold online under a variety of names, such as "bath salts" and "plant food" [18], and users often do not know the actual active ingredients when using these drugs [12].

It was reported that MDMA supply experienced a decline in 2009 and 2010 [19,20], which was supported by our previous wastewater analyses [21]. There is now

evidence that this was a temporary phenomenon [22,23]. Meanwhile, reports suggested that the novel synthetic stimulant drugs have increased in popularity since 2006, and particularly since 2009 [20]. Hence, it is possible that the decline of MDMA was associated with increased popularity of the novel synthetic stimulants. It has been suggested that this may have occurred because drug producers may have actively switched to producing these alternative synthetic stimulants or it may have occurred passively because of a shortage in MDMA precursors and stricter laws [20]. However, it is also possible that the two phenomena are unrelated and that the increased popularity of the other synthetic stimulants would have occurred in the absence of any change in MDMA use. An initial step in understanding the change in synthetic stimulant use is to determine whether the amounts of these substances used increased in response to the decrease in MDMA use.

Baker and Kasprzyk-Hordern [24] developed an analytical method for methcathinone, BZP and TFMPP, and detected the latter two in wastewater samples; van Nuijs et al [25] also developed a liquid chromatography-tandem mass spectrometry method for mephedrone and MDPV, but did not find them in wastewater. In this study, we aim to analyse wastewater samples collected in Adelaide, Australia from 2009 to 2011, targeting MDMA and the most reported synthetic cathinones methcathinone, 4-methylmethcathinone (mephedrone), 3,4-methylenedioxy-N-methylcathinone (methylone) and methylenedioxypyrovalerone (MDPV), as well as synthetic piperazines benzylpiperazine (BZP) and 3-trifluoromethylphenylpiperazine (TFMPP) (Figure 1), and temporally compare the data to determine whether the change in MDMA use was associated with an increase in the use of these synthetic cathinones and piperazines. As far as we know, this is also the first reported method for methylone detection in wastewater.

Methods

Chemicals and reagents

MDMA and MDMA-d_5 were purchased from Cerilliant Corp. (Round Rock, TX) as certified solutions at the concentration of 100 mg/L in methanol. The analytical reference substances of the other six synthetic stimulants were provided by Forensic Science South Australia as standard solutions at the concentration of 100 mg/L in methanol. The solutions were diluted to 50 µg/L (MDMA and MDMA-d_5) or 25 µg/L (the other synthetic stimulants) in ethanol and stored at −20 °C. Methanol and formic acid purchased from Merck Pty. Ltd. (Kilsyth, VIC, Australia) as well as distilled water prepared by a water still (Labglass Pty. Ltd., Brisbane, QLD, Australia) were used for liquid chromatography/ tandem mass spectrometry (LC-MS/MS) analyses. Sodium metabisulphite ($Na_2S_2O_5$) was food grade. All other reagents were analytical grade from Chem-Supply Pty. Ltd. (Gillman, SA, Australia). Ammonia (28−30%, w/w) was used at the original concentration or as a 10-time dilution, while hydrochloric acid (1 M), acetic acid (2.5%, v/v) and sodium hydroxide (10%, w/v) were prepared by diluting concentrated reagents with distilled water prior to use.

Sampling

Samples were collected as described previously [21,26]. Generally, wastewater samples were obtained from three (named A, B, and C, respectively in this article) independent wastewater treatment plants (WWTP) in Adelaide. All the three WWTP serve suburban areas with populations ranging from 130,000 to 200,000 and similar social-economic status. For practical issues, samples from the WWTP serving the Adelaide CBD area were not collected in this study. 80–100 mL of wastewater sample was taken by autosamplers when every 400,000 L of wastewater passes

through the inlet pipes, which gives a sampling interval of 10–15 min, depending on the wastewater flow. After each 24-h cycle of sample collection, a 1.2 L aliquot was transferred into a polyethylene terephthalate bottle and stored at 4 °C for a maximum of 7 days in the WWTP before transferring to the laboratory.

In each sampling year 6–8 weeks of wastewater samples were collected over the May–July period. Samples were transported from each WWTP to the analytical laboratory at atmospheric temperature within 4 h, and stored at –20 °C until analyses. All samples were extracted using solid-phase extraction (SPE) within one month after arrival at the laboratory.

Sample preparation

All samples were prepared using the method reported previously [21,26]. Briefly, 300 mL samples were thawed, mixed, filtered (GF/A 1.6 μm, Whatman Ltd., Kent, UK), and spiked with 33.3 ng/L MDMA-d_5. The filtered and spiked samples were passed through preconditioned XRDAH506^{TM} solid-phase extraction cartridges (UCT Inc., Bristol, PA). After wash with 6 mL pH 6 buffer, 2 mL 0.1 M acetic acid and 6 mL methanol, the analytes were eluted with 6 mL mixture of 96% dichloromethane: i-propanol (80: 20) / 4% ammonia (28−30%, w/w). The mixture was then evaporated to dryness, and the residue was reconstituted in 20 μL methanol, followed by mixing with 180 μL 0.1% formic acid buffer. The final mixture was stored at 4 °C before instrumental analyses if analysed within one week after preparation, or at –20 °C for long-term storage.

LC-MS/MS

Samples were analysed on a LC-MS/MS system, which consists of an Agilent 1200™ series liquid chromatograph (Santa Clara, CA) and an AB Sciex 4000 Q-Trap™ mass spectrometer (Applied Biosystems Ltd., Toronto, Canada). MDMA-d_5 was used as the internal standard for all analytes including MDMA and the other synthetic stimulants.

The LC-MS/MS methods were identical to those previously reported [21,26,27], except for the compound-dependent parameter settings for the novel synthetic stimulants listed in Table S1. Generally, 10 µL of reconstituted sample was injected into the LC-MS/MS system. The separation was achieved on a Phenomenex Luna™ pentafluorophenyl (PFP[2]) column (3 µm, 100 Å, 50 × 4.6 mm; Phenomenex Inc., Torrance, CA) connected to a PFP(2) guard column (SecurityGuard™; 4 × 2.0 mm; Phenomenex Inc., Torrance, CA). The mobile phase was composed of methanol (solvent A) and 0.1 % formic acid (solvent B) at a flow rate of 0.5 mL/min. The gradient started with 95% B for 1 min and was then decreased to 5% B over the next 14 min and maintained there for 1 min. Then it was brought back to 95% B in 0.1 min and kept there for 2 min. Electrospray ionization source operated in positive mode via multiple-reaction monitoring were used to obtain the mass spectra.

MDMA was quantified using the internal standard MDMA-d_5 and the method of isotopic dilution, as described in previous studies [21,26,27]. The other synthetic stimulants were also quantified using MDMA-d_5 as the internal standard, but with calibration curves generated using the method of standard addition with wastewater samples from the same WWTP. The ranges of the calibration curves were set as 1–50 ng/L for mephedrone, methylone, MDPV and TFMPP, and 1–200 ng/L for

methcathinone, BZP and MDMA. These ranges were based on the linear ranges of the analytes and their concentration ranges in wastewater, as determined in preliminary studies.

Stability and binding tests

Additionally, stability and binding tests were conducted to further validate the method, using methods similar to those in our previous studies [27]. Briefly, stability tests were conducted to evaluate if there is analyte loss during storage [28], and if the stability can be improved under different storage conditions; binding tests investigated if there is analyte loss during the filtration step in sample preparation [29,30] by comparing the recoveries of analytes from filtered and unfiltered samples using liquid-liquid extraction (LLE). All stability and binding tests were conducted in triplicate. The detailed methods and results can be found in the supporting material.

Method validation

Both the SPE-LC-MS/MS and LLE-LC-MS/MS methods used in this study were validated. Limits of quantification (LOQ) and linear ranges were determined in a creek water sample mixed with 0.01 % (w/v) laundry powder and 0.1 % (v/v) urine from a volunteer who has not history of drug use to simulate wastewater, since drug-free wastewater was unobtainable. This "artificial wastewater" was prepared to represent drug-free wastewater for the determination of LOQ and linear range based on the fact that the environmental matrix, human waste and household chemicals are the major sources of wastewater matrix. An analyte concentration in the "artificial wastewater" sample that gave a signal to noise ratio of 10 was set as the LOQ. Linear range was the concentration range of the analytes that gave linear instrumental responses. Absolute recovery, relative recovery (i.e. precision) and reproducibility (n

= 7, inter-day RSD) of all methods were tested on a 1:1:1 (v/v/v) mixture of real wastewater samples obtained from the three WWTP.

Data analyses

Data conversion

Measured concentrations of MDMA and synthetic drugs were converted into disposed amount of each drug *per* day *per* 1,000 of the population, using the method described by Zuccato et al [1] and taking into consideration of the daily wastewater volume and the contributing population. Since the level of drugs in a wastewater sample may vary by the sampling year, sampling location and sampling day of the week, all converted data were grouped and compared in different ways as below. GraphPad Prism™ 5 (GraphPad Software Inc., La Jolla, CA) was used for all statistics.

Temporal comparison

Weekly disposition of each drug was calculated by adding the daily values over 7 days and expressed as disposition *per* week *per* 1,000 of the population. Means and standard error of the mean of estimated weekly disposition were calculated for each WWTP in each year.

Two-way analysis of variance was used, with the sampling year and WWTP as the two factors. Tukey's multiple comparisons were applied to determine which year showed significant ($p < 0.05$) higher or lower values.

Weekly use pattern

For the year and WWTP that shows the highest level of each drug, daily disposition data were categorized and averaged according to their sampling day of the week to show the weekly use pattern of the drug.

Results

Validation

Table S2 and Table S3 summarise the validation data for the analytical methods used in this study, namely SPE-LC-MS/MS and LLE-LC-MS/MS, respectively. Both methods showed satisfactory sensitivity, linear range, recovery, accuracy and precision.

Stability and binding tests

In untreated wastewater, 15 % of MDPV degraded in 3 days. Approximately 20 % of mephedrone and TFMPP were lost after one week. Methylone and BZP levels dropped 20 % after the 2-week storage (Figure S1). All drugs were stable for up to two weeks if samples were stored at 4 °C or −20 °C, acidified to pH 2 or preserved by 2 g/L $Na_2S_2O_5$ (data not shown). Furthermore, no noticeable change of analyte concentration was observed in reconstituted sample extract stored at 4 °C or −20 °C (data not shown).

No significant loss of analytes was observed after filtration (data not shown), indicating that the drugs analysed in this study have little affinity to particulate matters or filter papers.

Temporal comparison

The disposition of each drug was grouped by WWTP and temporally compared (Figure 2). MDMA level declined markedly in 2010 as previously reported [21] and remained low in 2011. Meanwhile, methcathinone disposition was consistent from 2009 to 2011 and there was a similar consumption level across WWTP. The remaining synthetic stimulants showed very different patterns of use. Firstly, their use was geographically localized, occurring predominantly (although not exclusively) in one WWTP. While the use of all these stimulants increased after 2009, only mephedrone experienced an increase in 2010; for all the other synthetic stimulants the increase occurred in 2011.

Weekly use pattern

MDMA shows a strong pattern of excretion on Sunday compared to other days of the week, reflecting a predominant pattern of weekend (particularly Saturday night) use (Figure 3). Mephedrone, methylone and BZP also showed higher disposition on weekends, whereas the levels of methcathinone, MDPV and TFMPP were more consistent over the week.

Discussion

In this study MDMA and six other synthetic stimulants were detected and quantified in wastewater samples. The response to the pronounced decrease in MDMA level [21] differed across the various synthetic stimulants. Methcathinone use, which was the highest of the stimulants in 2009, did not change with the decrease in MDMA use. Use of mephedrone increased in 2010, while use of the remaining compounds did not increase until 2011. In addition, while methcathinone use was similar across different

regions, the use of the remaining synthetic stimulants was predominantly in one region. This suggested that the decline in MDMA was associated with increased use of novel synthetic stimulants other than methcathinone. However, for most of these stimulants there was a lag between the decline in MDMA use and their increased use, and hence the relation is not a direct one. It can therefore be concluded that the novel synthetic stimulants did not directly replace MDMA across the population.

The weekly disposition patterns of the synthetic stimulants were found to be inconsistent in this study. While MDMA, mephedrone, methylone and BZP levels peaked on the weekend, methcathinone, MDPV and TFMPP dispositions were relatively consistent throughout the week. This could possibly be due to longer elimination half-lives for methcathinone, MDPV and TFMPP, which might smooth out the weekly trend. However, data on their pharmacokinetic properties in human subjects are required to support this hypothesis. Alternatively, there may be differences in the patterns of use as there are between other stimulants.

Rust et al [31] analysed 325 hair samples from 2009 and 2010 that originally tested positive for amphetamines or MDMA, and found mephedrone in 11 cases and methylone in 1 case. BZP, methcathinone, MDPV and TFMPP were also targeted but not found. This suggested some limited overlap of MDMA users and users of the other synthetic stimulants. A survey carried out in London nightclubs in July 2011 also revealed that although overall 65.8% reported previous use of the novel synthetic stimulants, frequent use was rare (except for mephedrone) [32]. Both studies support our conclusion that the novel synthetic stimulants only partially replaced MDMA in the market.

Zuba and Byrska [33] analysed 449 seized ‘legal high’ samples from October 2008 to June 2011 in Poland, and found that most of the pills were mixtures of two or more ingredients, including but not limited to MDPV, BZP and TFMPP. However, mephedrone was often the sole product component. This finding together with others reports [31, 32] that suggested higher prevalence of mephedrone over the other synthetic stimulants indicated that mephedrone might be produced and distributed in a different way from the other synthetic stimulants. This hypothesis is supported by our data, which showed that the increase in mephedrone use occurred in the year before the increase in use of methylone, MDPV, BZP and TFMPP.

In this present study, we used the parent drugs of the synthetic stimulants as the analytical targets, mainly due to the fact that the analytical standards of their metabolites were not available at the time when this study was carried out. Limited metabolic studies [34-39] suggested that the parent forms exist in the urine of rats and humans after intake of these novel synthetic stimulants, but the excretion ratios from human subjects, which are essential to back-calculate the consumption [1], are largely unknown. If more metabolic data for these stimulants become available in the future, it might be possible to back-calculate the consumption of these novel synthetic stimulants in the population based on the disposition data in this study.

In conclusion, the decline in MDMA use was associated with some increases in the use of some other synthetic stimulants in a localized manner. This demonstrates that there was no population wide substitution of novel synthetic stimulants for MDMA.

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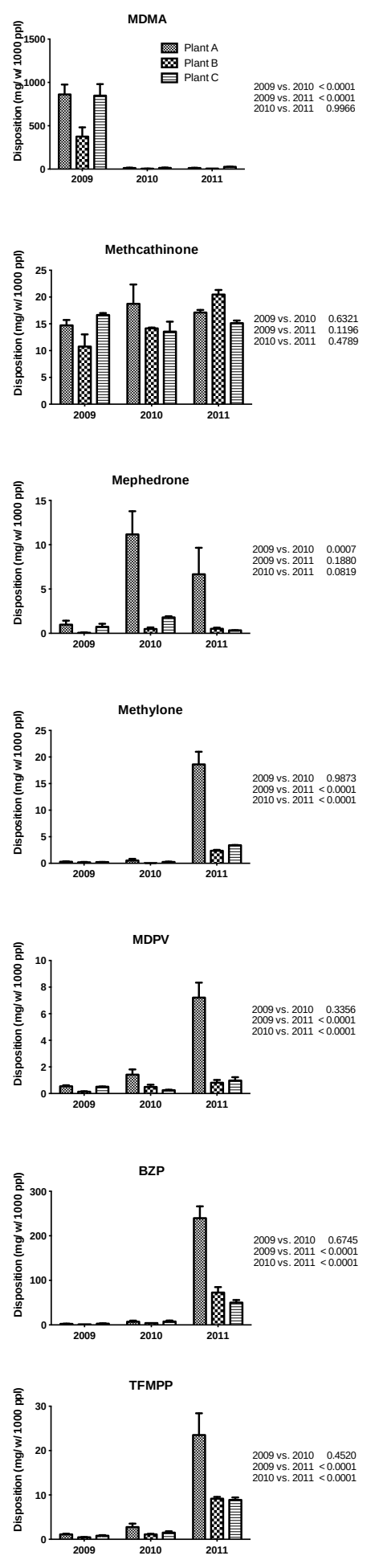
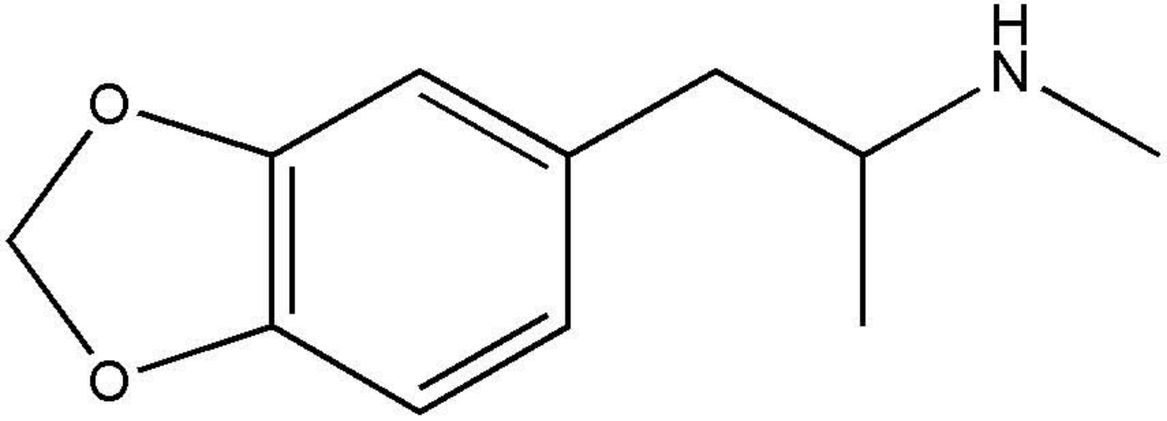
Captions for figures

Figure 1. Chemical structures of synthetic drugs analysed in this study. MDMA: 3,4-methylenedioxy-N-methylamphetamine; mephedrone: 4-methylmethcathinone; methylone: 3,4-methylenedioxy-N-methylcathinone; MDPV: methylenedioxypyrovalerone; BZP: benzylpiperazine; TFMPP: 3-trifluoromethylphenylpiperazine.

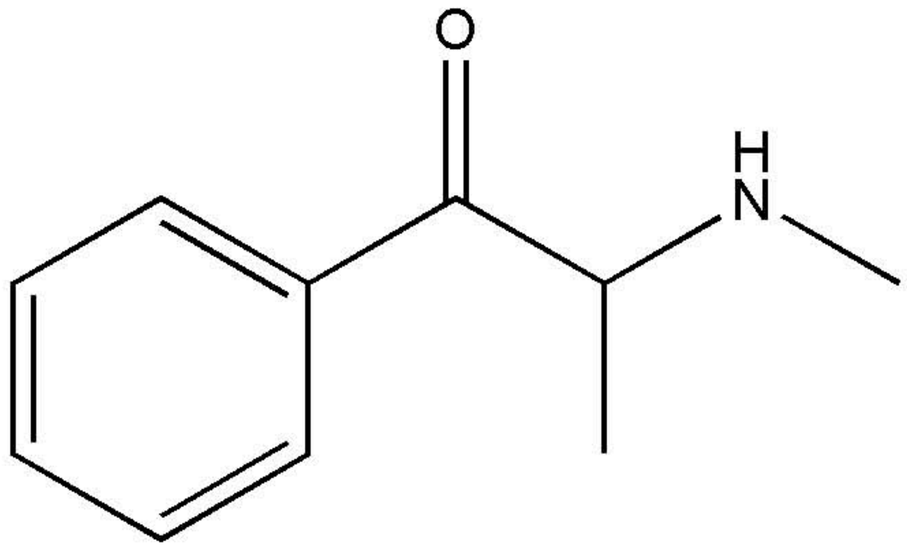
Figure 2. Weekly drug disposition (mg/ week/ 1,000 people) in each year and WWTP served area. MDMA: 3,4-methylenedioxy-N-methylamphetamine; mephedrone: 4-methylmethcathinone; methylone: 3,4-methylenedioxy-N-methylcathinone; MDPV: methylenedioxypyrovalerone; BZP: benzylpiperazine; TFMPP: 3-trifluoromethylphenylpiperazine. Two-way analysis of variance with Tukey's multiple comparisons. *p* values of multiple comparisons are displayed to the left of each figure.

Figure 3: Disposition of analysed drugs on each day of the week. 2009 data of WWTP A was displayed for MDMA; 2011 data of WWTP B for methcathinone; 2010 data of WWTP A for 4-methylmethcathinone (mephedrone); and 2011 data of WWTP A for 3,4-methylenedioxy-N-methylcathinone (methylone), methylenedioxypyrovalerone (MDPV), benzylpiperazine (BZP) and 3-trifluoromethylphenylpiperazine (TFMPP).

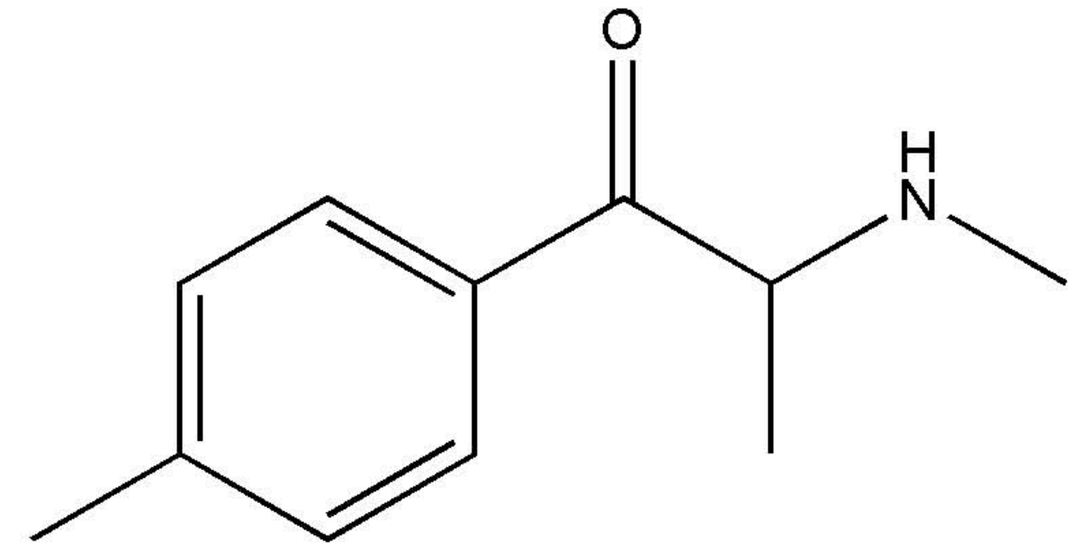

MDMA
Plant A
Plant B
Plant C
Disposition (mg/ w/ 1000 ppl)
1500
1000
500
0
2009
2010
2011
2009 vs. 2010 < 0.0001
2009 vs. 2011 < 0.0001
2010 vs. 2011 0.9966
Methcathinone
Disposition (mg/ w/ 1000 ppl)
25
20
15
10
5
0
2009
2010
2011
2009 vs. 2010 0.6321
2009 vs. 2011 0.1196
2010 vs. 2011 0.4789
Mephedrone
Disposition (mg/ w/ 1000 ppl)
15
10
5
0
2009
2010
2011
2009 vs. 2010 0.0007
2009 vs. 2011 0.1880
2010 vs. 2011 0.0819
Methylone
Disposition (mg/ w/ 1000 ppl)
25
20
15
10
5
0
2009
2010
2011
2009 vs. 2010 0.9873
2009 vs. 2011 < 0.0001
2010 vs. 2011 < 0.0001
MDPV
Disposition (mg/ w/ 1000 ppl)
10
8
6
4
2
0
2009
2010
2011
2009 vs. 2010 0.3356
2009 vs. 2011 < 0.0001
2010 vs. 2011 < 0.0001
BZP
Disposition (mg/ w/ 1000 ppl)
300
200
100
0
2009
2010
2011
2009 vs. 2010 0.6745
2009 vs. 2011 < 0.0001
2010 vs. 2011 < 0.0001
TFMPP
Disposition (mg/ w/ 1000 ppl)
30
20
10
0
2009
2010
2011
2009 vs. 2010 0.4520
2009 vs. 2011 < 0.0001
2010 vs. 2011 < 0.0001



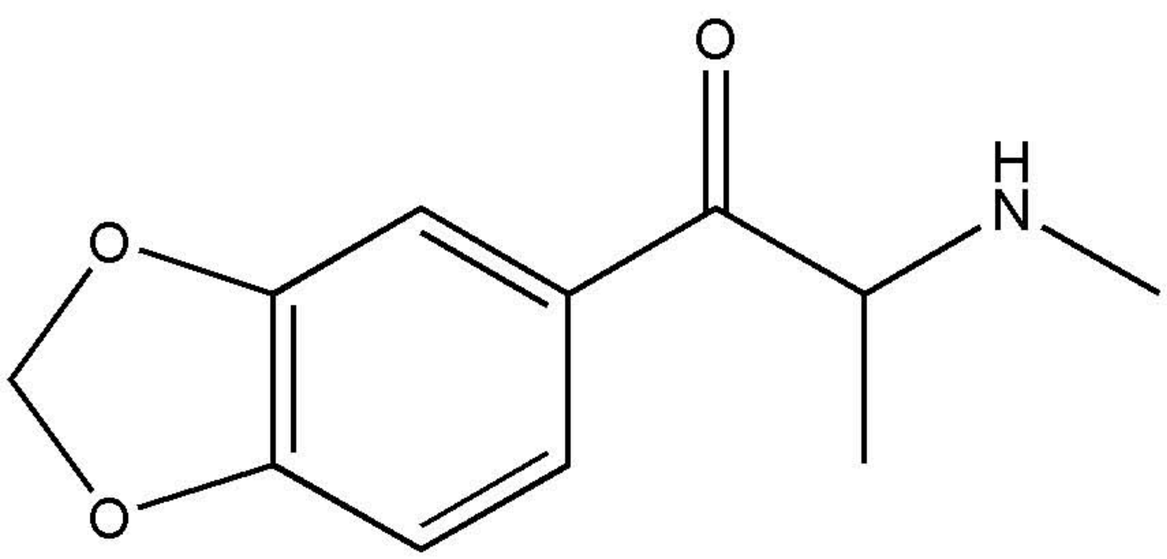
MDMA



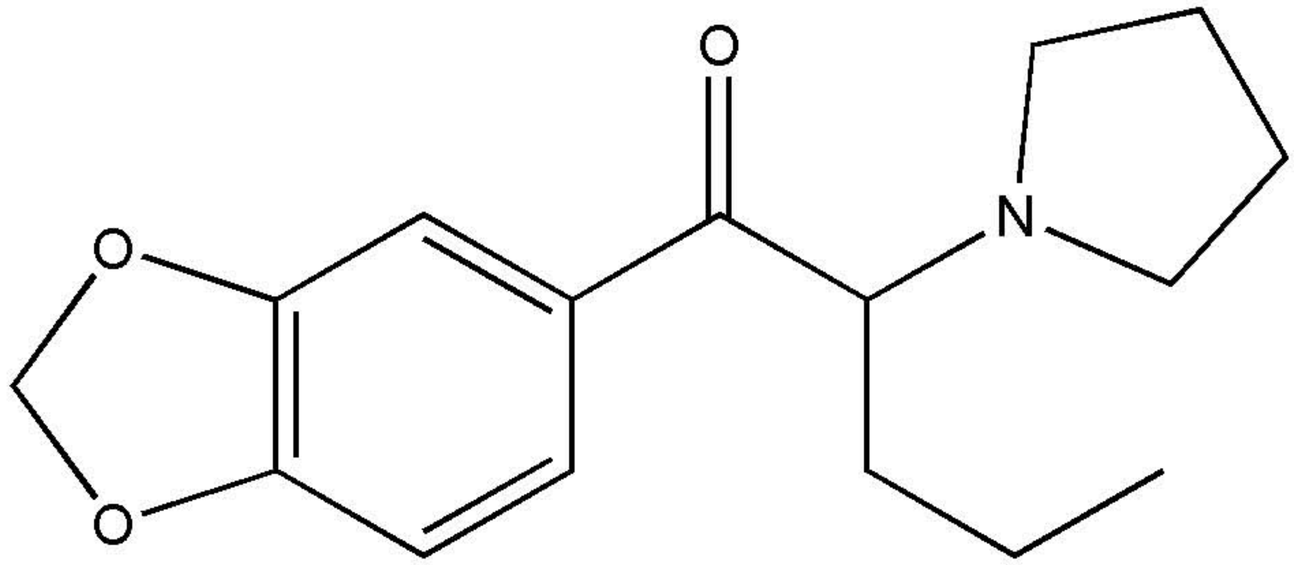
Methcathinone



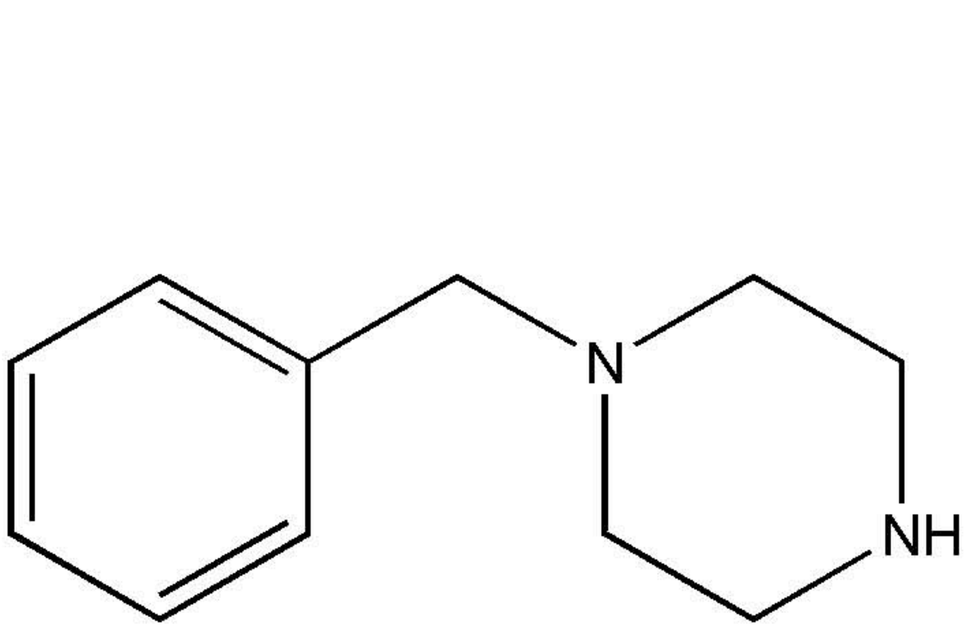
Mephedrone



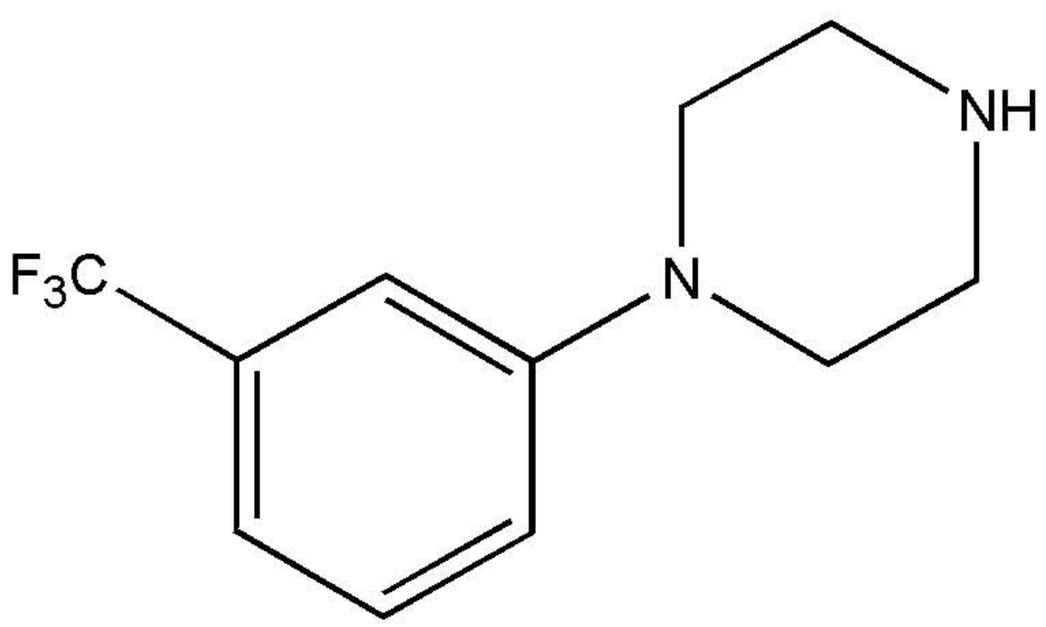
Methylone



MDPV



BZP



TFMPP

Methods for stability and binding tests

Stability tests were carried out in fresh wastewater samples that were stored in different conditions, including: no treatment, storage at 4 °C, −20 °C, acidification, preservative-addition and filtration. Concentration of analytes was measured on the starting day (day 0) and on days 1, 2, 3, 7 and 14 thereafter to show whether there was significant change. A change of more than 15% was considered significant formation (> 115%) or degradation (< 85%). Stability of analytes in reconstituted extract was evaluated by comparing the analytes' peak areas with a standard solution prepared in ethanol and stored at −20 °C after storage at either 4 °C or −20 °C or 1, 3, 7 and 14 days.

For binding tests, 100 mL sample was spiked with reference standards to ensure that the concentrations of these analytes were above the LOQ of the analytical method. After 2 h, half of the mixed sample was filtered under vacuum using 1.6 µm GF/A glass microfiber filters (Whatman Ltd., Kent, UK), while the other 50 mL remained unfiltered. Analytes in filtered and unfiltered samples were then extracted using liquid-liquid extraction (LLE) and ~~analyzed~~ analysed by LC–MS/MS [~~25~~27]. Data were then compared by paired two-tailed t test using GraphPad Prism™ 5 (GraphPad Software Inc., La Jolla, CA) to assess whether there was significant analyte loss after filtration.

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Caption for Figure S1.

Figure S1. Analyte stability in untreated wastewater in 14 days. Data are expressed as average value of remaining percentages of Day 0, n = 3. Mephedrone~~:~~, 4-methylmethcathinone; methylone~~:~~, 3,4-methylenedioxy-N-methylcathinone; MDPV~~:~~,

methylenedioxypyrovalerone; BZP~~:~~, benzylpiperazine; TFMPP~~:~~, 3-trifluoromethylphenylpiperazine. Initial concentration was 25 ng/L for all the drugs.#

Table S1. Selected mass spectrometric parameters used in the analysis of 3,4-methylenedioxymethamphetamine (MDMA), methcathinone, 4-methylmethcathinone (mephedrone), 3,4-methylenedioxy-N-methylcathinone (methylone), methylenedioxypyrovalerone (MDPV), benzylpiperazine (BZP) and 3-trifluoromethylphenylpiperazine (TFMPP) for Applied Biosystems 4000 Q-Trap™.

Transition	**Q_1 m/z**	**Q_3 m/z**	**Dwell time (ms)**	**DP[a] (V)**	**EP[b] (V)**	**CE[c] (V)**	**CXP[d] (V)**
MDMA 1#	194	163	60	50	10	20	30
MDMA 2	194	105	40	50	10	30	30
MDMA 3	194	135	40	50	10	35	30
Methcathinone 1#	164	146	80	50	10	20	10
Methcathinone 2	164	130	40	50	10	42	10
Methcathinone 3	164	105	40	50	10	28	10
Mephedrone 1#	178	160	80	50	10	20	10
Mephedrone 2	178	144	40	50	10	45	10
Mephedrone 3	178	119	40	50	10	32	10
Methylone 1#	208	160	80	50	10	25	10
Methylone 2	208	190	40	50	10	20	10
Methylone 3	208	58	40	50	10	45	10
MDPV 1#	276	126	80	70	10	40	10
MDPV 2	276	135	40	70	10	40	10
MDPV 3	276	175	40	70	10	30	10
BZP 1#	177	91	80	50	10	35	10
BZP 2	177	65	40	50	10	65	10
BZP 3	177	85	40	50	10	25	10
TFMPP 1#	231	188	80	50	10	35	10
TFMPP 2	231	119	40	50	10	45	10
TFMPP 3	231	168	40	50	10	40	10
MDMA-d_5*#	199	165	60	50	10	20	30

[a] Declustering potential.
[b] Entrance potential.
[c] Collision energy.
[d] Collision cell exit potential.
Transitions used for quantification.
* Internal standard.

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Table S2. Validation data of the SPE–LC–MS/MS method used in this study. MDMA: 3,4-methylenedioxymethamphetamine; Mephedrone: 4-methylmethcathinone; methylone: 3,4-methylenedioxy-N-methylcathinone; MDPV: methylenedioxypyrovalerone; BZP: benzylpiperazine; TFMPP: 3-trifluoromethylphenylpiperazine.

	LOQ (ng/L)	Linear range (ng/L)	R^2	Absolute recovery (%; mean ± 95% CI)	Relative recovery (%; mean ± 95% CI)	RSD (%)
MDMA	1	1−1000	0.995	89.3 ± 2.8	100.0 ± 2.1	3.66
Methcathinone	1	1−1000	0.994	98.15 ± 11.42	117.49 ± 9.89	10.76
Mephedrone	1	1−3000	0.993	98.60 ± 3.86	104.27 ± 4.24	4.87
Methylone	1	1−2000	0.995	99.45 ± 1.81	102.38 ± 1.93	2.39
MDPV	1	1−2000	0.992	98.68 ± 1.09	100.04 ± 1.11	1.30
BZP	1	1−2000	0.994	104.60 ± 5.45	92.39 ± 4.84	6.36
TFMPP	1	1−2000	0.998	101.15 ± 1.24	100.78 ± 1.19	1.39

Note: LOQ and linear ranges were determined on “artificial wastewater”.

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Table S3. Validation data of the LLE–LC–MS/MS method used in the binding tests. MDMA: 3,4-methylenedioxymethamphetamine; Mephedrone: 4-methylmethcathinone; methylone: 3,4-methylenedioxy-N-methylcathinone; MDPV: methylenedioxypyrovalerone; BZP: benzylpiperazine; TFMPP: 3-trifluoromethylphenylpiperazine.

	LOQ (ng/L)	Linear range (ng/L)	R^2	Absolute recovery (%; mean ± 95% CI)	Relative recovery (%; mean ± 95% CI)	RSD (%)
MDMA	2	2–1000	0.995	70.74 ± 1.72	91.33 ± 2.21	2.09
Methcathinone	2	2–500	0.990	53.55 ± 6.06	101.04 ± 7.44	14.42
Mephedrone	2	2–2000	0.994	66.83 ± 2.32	99.54 ± 3.66	4.68
Methylone	2	2–1000	0.994	72.07 ± 1.95	97.31 ± 2.76	3.66
MDPV	2	2–1000	0.997	53.11 ± 5.31	111.22 ± 11.19	13.41
BZP	2	2–1000	0.994	35.09 ± 3.45	112.86 ± 10.29	13.01
TFMPP	2	2–1000	1.000	65.28 ± 3.55	101.54 ± 5.80	7.21

Note: LOQ and linear ranges were determined on “artificial wastewater”.

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