A Validated Triple Quadrupole LC/MS/MS Method for Quantitative Analysis of Methylenedioxypyrovalerone (MDPV) and Mephedrone, Common Components of "Bath Salts" in Urine

Application Note

Forensics

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Abstract

Due to the emerging and dangerous popularity of synthetic cathinones – compounds widely marketed as "Bath Salts"– today's forensic laboratories are challenged to screen, confirm, and quantify the controlled forms of those compounds in biological matrices with confidence. This application note describes and evaluates a robust quantitative method for the analysis of two controlled synthetic cathinones, 3, 4-methylenedioxypyrovalerone (MDPV) and 4-methylmethcathione (mephedrone), in urine. The method is validated to demonstrate excellent linearity, lower limit of detection (LOD), reproducibility/precision and lower limit of quantitation (LOQ), with no interferences from structurally similar compounds, and with negligible carryover.

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Introduction

Synthetic cathinones, such as 3, 4-methylenedioxypyrovalerone (MDPV) and 4-methylmethcathione (mephedrone), are emerging substances of abuse in many countries including the US, Europe, and Australia. Synthetic cathinones are of increasing concern in the US, and the number of calls to its Poison Control Centers regarding exposure increased significantly from 304 in 2010 to 6138 in 2011. Of particular concern, several deaths associated with use of these substances have been reported.

Synthetic cathinones are central nervous system (CNS) stimulants, similar in action to methamphetamine and Ecstasy (MDMA), and thought to be highly addictive. Figure 1 shows they are chemically akin in structure to cathinone, an active alkaloid found in the Khat plant of eastern Africa where its fresh leaves are chewed or consumed as tea. When consumed, cathinone causes the brain to release dopamine leading to mild euphoria and excitement, appetite suppression, talkativeness, emotional instability, constipation, manic behavior, and drowsy hallucinations.

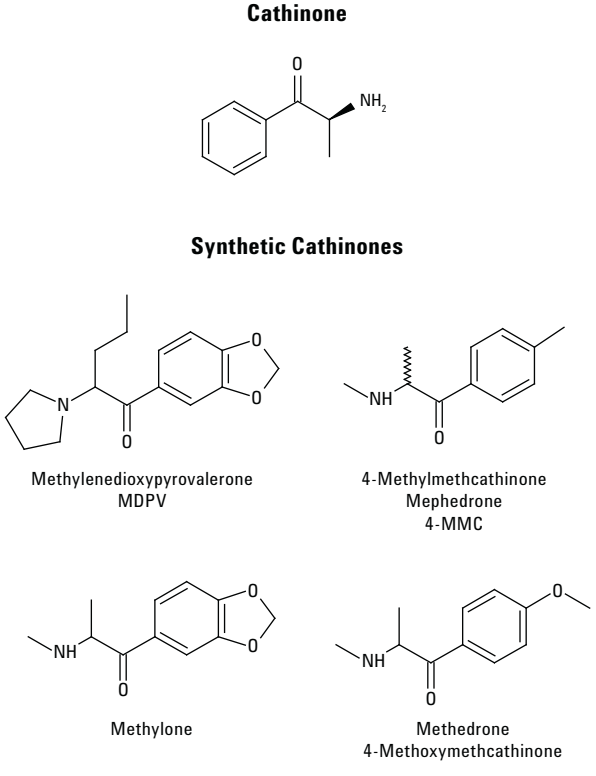

Figure 1. Cathinone and common synthetic cathinones.

In the US, synthetic cathinones are marketed on the Internet, and in convenience and head shops as "Bath Salts" under a variety of brand names such as "Ivory Wave" and "Vanilla Sky." In particular, MDPV and mephedrone use lead to effects similar to that of methamphetamine, cocaine and Ecstasy. Adverse reactions can occur, including dangerously increased heart rate and blood pressure, insomnia, nausea, and vomiting, hallucinations, extreme paranoia and anxiety, seizures, and even death when overdosed or used in combination with other drugs.

MDPV was developed in the 1960s. Though it has no history of a FDA approved medical use, it has been used to treat chronic fatigue and as an anorectic. Currently popular in Europe, the U.K., and Australia, the hydrochloride salt of MDPV is a white to brown powder that is usually "snorted" like cocaine. First synthesized in 1929, Mephedrone is a white crystal or powder that can be formulated as a tablet.

Though MDPV and mephedrone were developed several decades ago, they were not widely known or of concern until the late 1990s and early 2000s. In fact, until 2008, they were primarily abused in Europe. As such, the EU ruled the two drugs illegal in December 2010.

In the US, the first seizure of MDPV and mephedrone was reported in 2008. They are banned in many states including Washington, and effective October 2011, the DEA temporarily placed MDPV and mephedrone, as well as methylone, on schedule 1 of the Controlled Substances Act. It is now illegal to use, possess, sell or manufacture these substances in the US.

Due to these trends, forensics analysis of MDPV and mephedrone, as well as other synthetic cathinones, is expected to increase dramatically. Because these compounds are not detected through existing amphetamine screening immunoassays or confirmatory gas chromatography/mass spectrometry (GC/MS) assays, new methodology is needed. Thus, the objective of this application note is to describe a validated liquid chromatography-triple quadrupole mass spectrometry (LC/MS/MS) method for screening, confirmation, and quantification of MDPV and mephedrone in urine. Developed by Sterling Reference Laboratories and Agilent Technologies, as of the date this application note was published, the method has been used to effectively analyze 561 patient samples. The overall positivity rate was 8% (45 specimens), of which 41 (7.3%) were positive for MDPV, three (0.5%) were positive for mephedrone, and one (0.2%) was positive for both MDPV and mephedrone.

Experimental

Method Overview

Spiked synthetic urine samples were prepared and then extracted using cation exchange solid phase extraction (SPE) columns. SPE was used rather than simple sample dilution and direct injection ("dilute and shoot") because the SPE method introduces much cleaner samples into the mass spectrometer. As a result, ion suppression and ion source cleaning tasks are minimized, and sensitivity is enhanced. Dilute and shoot methods introduce dirtier samples – raw diluted urine – into the mass spectrometer. However, laboratories performing only a small number of bath salts analyses and that do not mind frequent source cleaning may prefer to "dilute and shoot."

The extracted samples were injected into a LC/MS/MS system equipped with electrospray ionization. Two multiple reaction monitoring (MRM) transitions were monitored for each analyte and internal standard. Retention time and the ratios of the selected ions, relative to the internal standards, were used for detection and quantification. Calibration curve development and quantitative analysis were performed using MassHunter data analysis software. Quantitative analysis was performed through interpolation of the analyte response against the calibration curves. The method was evaluated and validated based on the following criteria: linearity, lower LOD and LOQ, reproducibility, interferences, and carryover.

Synthetic Urine, Calibrator, Quality Control, and Internal Standard Solutions

Synthetic urine (solution to be spiked and the negative control or blank) was prepared by dissolving the following in 800 mL deionized water. Deionized water was then added to bring the final volume to 4,000 mL.

20.0 g	NaCl (ACS reagent grade)
2.0 g	creatinine (Sigma Cat. No. C4255-100G)
40.0 g	urea (ACS reagent grade)
38.6 g	monosodium phosphate monohydrate (ACS reagent grade)
32.3 g	disodium phosphate heptahydrate (ACS reagent grade)
4.0 g	sodium azide (ACS reagent grade)
3 to 5 drops	yellow food coloring (food grade, McCormick)

The pH of the synthetic urine was then adjusted to 6.5 using 1% HCl.

Calibrator solutions were made with MDPV (Cayman, Cat. No.10684) and mephedrone (Cerilliant Cat. No. M-138) stock solutions dissolved in methanol. To construct calibration curves for MDPV and mephedrone over the range of 1 to 5000 ng/mL, calibrator compound was spiked in synthetic urine at the 1, 5, 10, 25, 50, 100, 500, 1,000, and 5,000 ng/mL level.

Quality Control (QC) specimens. Three QC specimens were prepared in synthetic urine: negative, 40% of positive cutoff and +25% of positive cutoff. The positive cutoff was administratively set at 25 ng/mL, thus the corresponding 40% and +25% QC specimens were nominally set at 10 ng/mL and 31 ng/mL, respectively.

Deuterium-labeled internal standard solutions of MDPV-D8 (Cayman, Cat. No. 10679) and mephedrone-D3 (Cerilliant, Cat. No. M-139) in HPLC-grade methanol were prepared at 500 ng/mL in deionized water.

Sample Preparation

Figure 2 shows an overview of the cation exchange SPE sample preparation procedure. The procedure is designed to enable preparation of over 300 samples per 8-hour shift with ease.

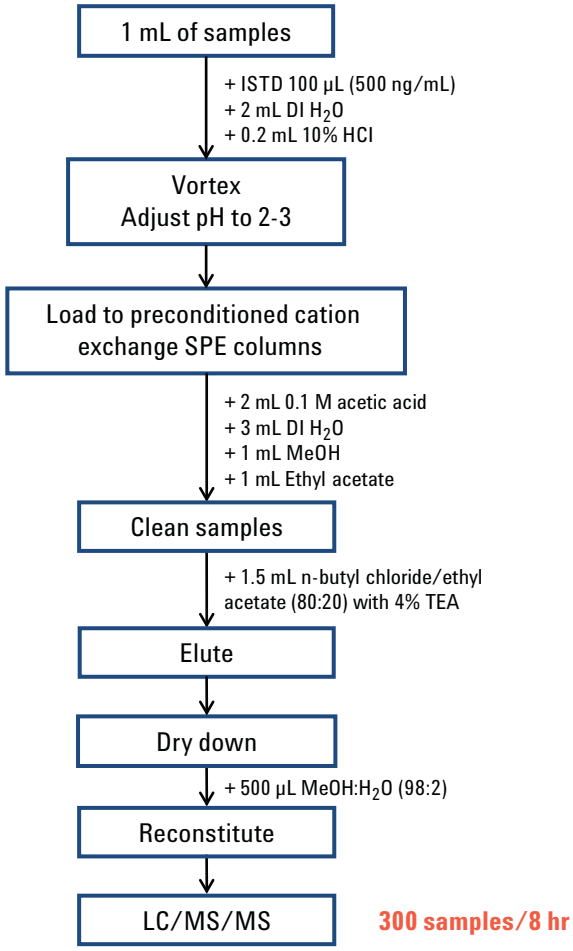

Figure 2. Sample preparation overview.

Using a calibrated micropipette, 1 mL of the calibrator, 40% QC, +25% QC, and negative control urine solutions were transferred to individual 16×100 mm labeled culture tubes. Next, using a calibrated repeating pipettor with calibrated pipette dispensers, 100 µL of the 500 ng/mL internal standard solution was transferred to the samples in the culture tubes. Deionized water (2 mL) was likewise added to each of the culture tubes. The pH was adjusted to 2–3, with the addition of 0.2 mL of 10% HCl to each of the culture tubes.

Extraction was performed on a Multi-prep SPE workstation (Biochemical Diagnostics). The cation exchange SPE columns (Biochemical Diagnostics, Cat. No. 1410082-0, GV-65), were conditioned with 1 mL of methanol followed by 1 mL of 5% sodium bisulfite which were allowed to flow through the system by gravity.

To extract the prepared samples, each was poured into the corresponding labeled SPE column. Next 2 mL of 0.1 M acetic acid was pipetted into each SPE column, followed by 3 mL of deionized water, 1 mL of methanol and finally, 1 mL of ethyl acetate. Between each solution addition, the liquid was allowed to flow by gravity until there was no liquid observed above the column bed.

Sample elution was performed outside of the vacuum box into elution tubes. Elution solvent (1.5 mL), n-butyl chloride/ethyl acetate 80:20 with 4% TEA, was pipetted into each column and allowed to flow by gravity until there was no liquid above the column bed. The elution tubes were placed in aluminum dry down blocks and evaporated to dryness under a gentle stream of nitrogen at 34–40 °C.

The samples were reconstituted with 0.5 mL of methanol:deionized water (2:98) and let to sit for 20 minutes at ambient temperature before transferring to autosampler vials. Once the samples were reconstituted, they were ready for LC/MS/MS analysis.

LC/MS/MS Analyses

LC/MS/MS analyses were performed using an Agilent 1200 Series LC System coupled to an Agilent 6460 Series Triple Quadrupole Mass Spectrometer. The total run time is 4.2 minutes per sample. The LC System was equipped with an autosampler, degasser, binary pump, and thermosttated column compartment. Separation was performed on an Agilent Polaris C18 column. The LC operating parameters are shown in Table 1.

Table 1. LC Operating Parameters

Column	Agilent Polaris C18, 50 × 2.0 mm, 5 µm			
Injection volume	1 µL			
LC gradient	Time	Flow	Mobile Phase B	Mobile Phase A
	0	0.8	2%	98%
	2	0.8	30%	70%
	2.5	0.8	90%	10%
	3.5	0.8	90%	10%
	3.6	0.8	2%	98%
Mobile phase A:	100% deionized water containing 0.1% formic acid			
Mobile phase B:	100% methanol containing 0.1% formic acid			

The Agilent 6460 Series Triple Quadrupole LC/MS/MS System was equipped with an electrospray ionization (ESI) source operated in positive ion mode. The MS operating parameters are shown in Table 2.

Table 2. MS Operating Parameters

Nebulizing gas	Nitrogen, (ultra high purity), 99.999%	
Collision cell gas	Nitrogen, (ultra high purity), 99.999%	
Ion source parameters	Gas temperature	300°C
	Gas flow	10 L/min
	Nebulizer	20 psi
	Sheath gas heater	350°C
	Sheath gas flow	8 L/min
	Capillary	4,000 V
	Nozzle voltage	0 V
Detector parameters	EMV	200

Two MRM transitions were monitored for each analyte and internal standard. The MRM transitions and MS/MS specific parameters for the mephedrone and MDPV compounds monitored are shown in Table 3.

Table 3. MRM Transitions and MS/MS Parameters

Compound	Precursor ion	Product ions monitored	Dwell time (ms)	Fragmentation voltage	Collision energy voltage
Mephedrone	178.1	160.1/145.0	50	95	8/20
Mephedrone-D3	181.1	163.1/148.1	50	90	8/20
MDPV	276.2	135.0/126.1	50	130	24/24
MDPV-D8	284.2	134.5/149.0	50	130	28/32

Calibration Curve Construction

In order to construct calibration curves for MDPV and mephedrone over the range of 1 to 5 000 ng/mL, five replicates (one injection of five extractions, n = 5) were made at each level (1, 5, 10, 25, 50, 100, 500, 1,000, and 5,000 ng/mL). Calibration curves were constructed by Agilent MassHunter Software using least-squares linear regression of the ratio of the quantitation ion abundance of the analyte/internal standard versus the concentration of the calibrators.

Results and Discussion

The total ion chromatogram (TIC) and MRM chromatograms for the MDPV and mephedrone, deuterated and nondeuterated forms in synthetic urine at 25 ng/mL, are shown in Figure 3. Due to the selectivity of the LC/MS/MS technique, chemical noise was negligible and response was strong. Ideally-shaped Gaussian peaks for quantitation were also observed.

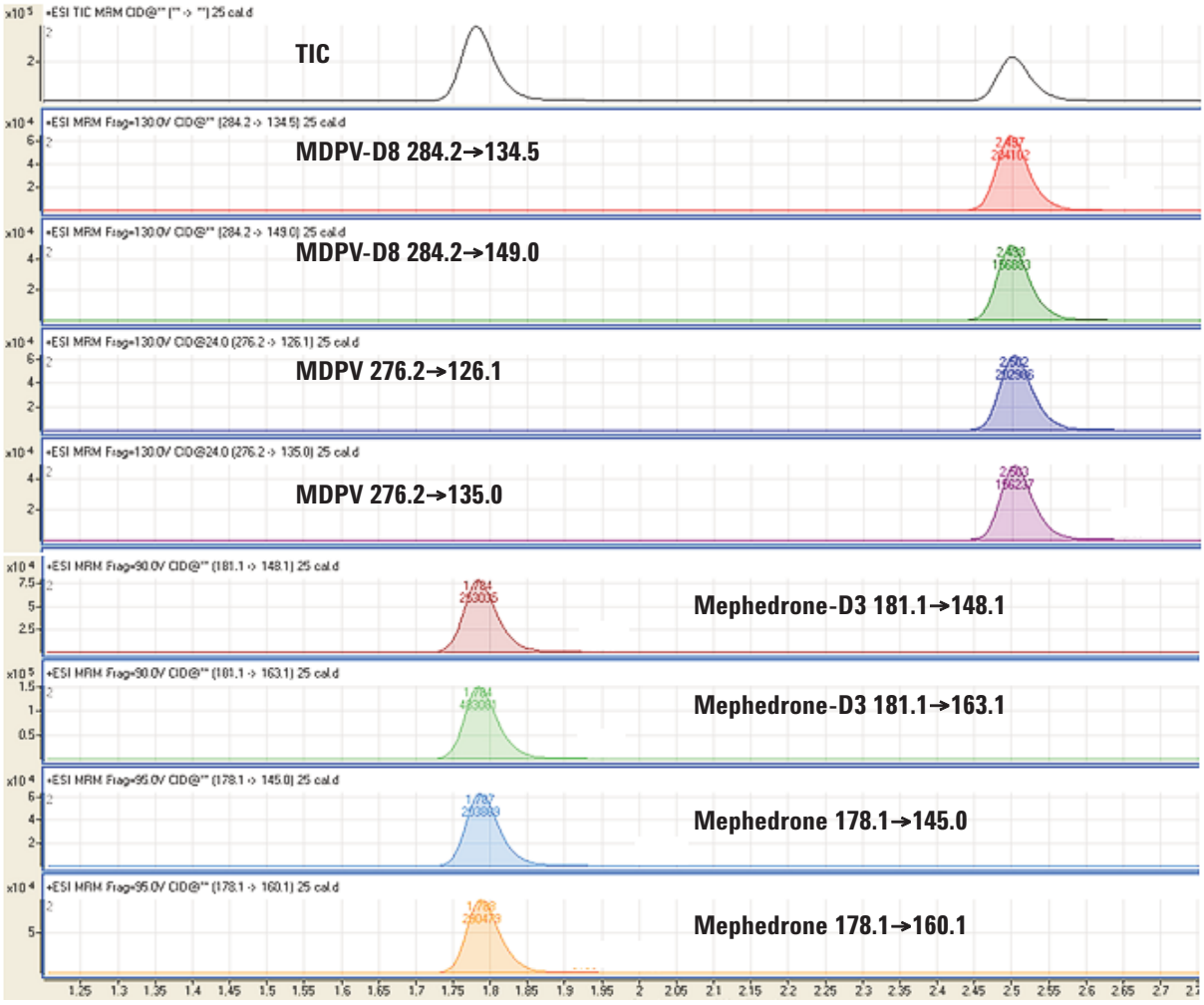

Figure 3. Total ion chromatogram (TIC) and MRM chromatograms for the MDVP and mephedrone deuterated and non-deuterated forms, in synthetic urine. Response for all compounds at 25 ng/mL was strong.

Calibration curves for MDPV and mephedrone spiked in synthetic urine over the range of 1 to 5,000 ng/mL are shown in Figure 4. Method linearity was excellent over the entire range of concentrations, including at the low end of the calibration curve, with an average correlation coefficient (R^2) greater than 0.999.

Based on the five replicates, an average signal-to-noise ratio (S/N) of 19 ± 6 and 24 ± 9 was obtained at 1 ng/mL for MDPV and mephedrone, respectively. Theoretically, a lower limit of detection (LOD) could be reached, but 1 ng/mL was determined to be practical for most routine analyses. Figures 5 and 6 show the method response for MDPV and mephedrone, respectively, at 1 ng/mL.

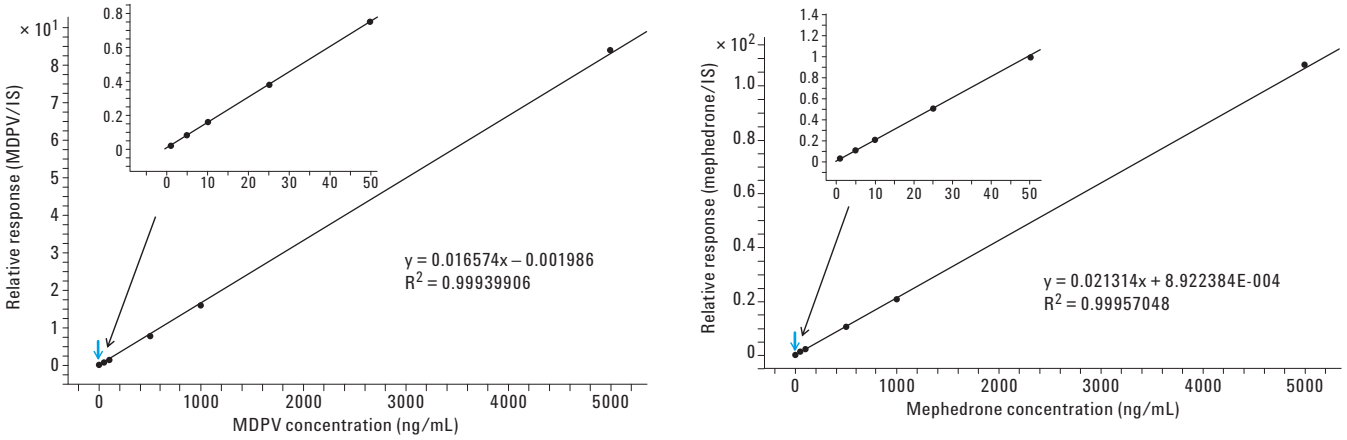

Figure 4. MDPV and mephedrone calibration curves demonstrated the excellent linearity of the method, even at low analyte concentration (insets).

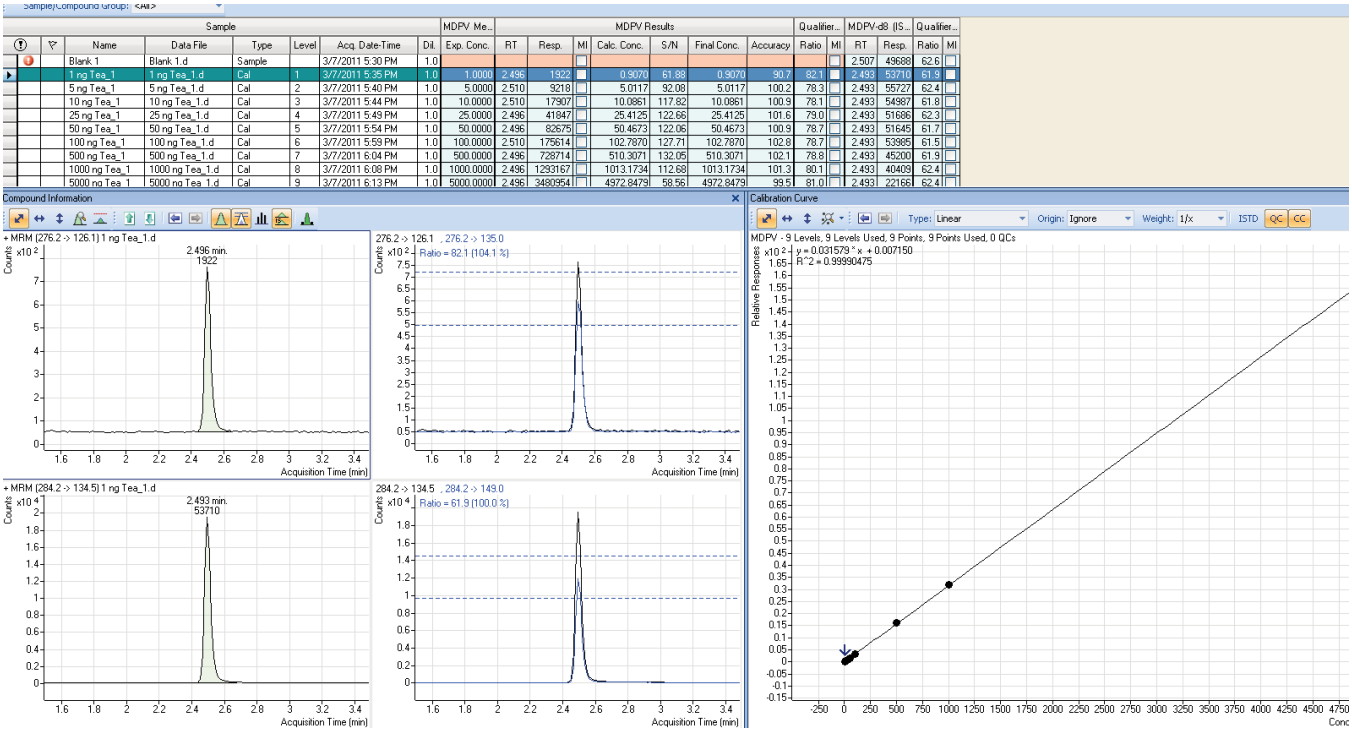

Figure 5. Method response for MDPV at 1 ng/mL, the LOD.

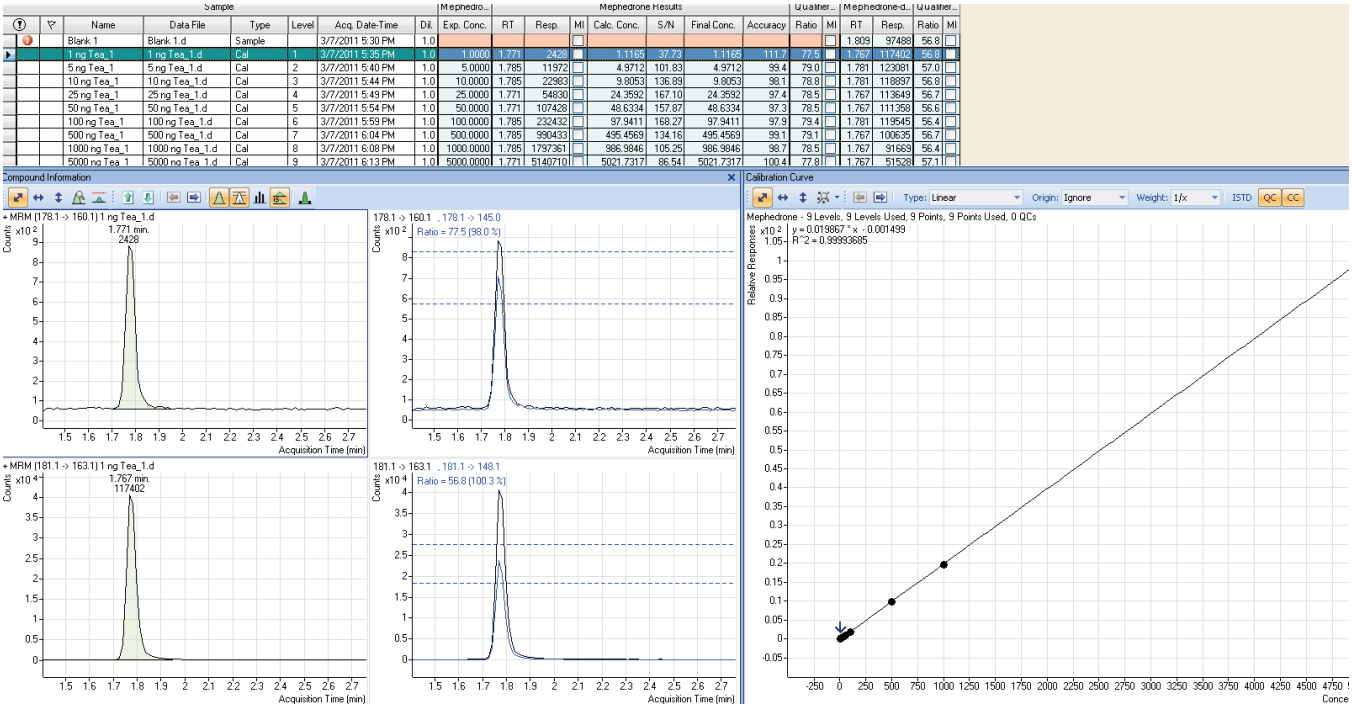

Figure 6. Method response for mephedrone at 1 ng/mL, the LOD.

The excellent reproducibility (precision at n = 5) of the method for the nine calibrators spiked in synthetic urine is shown in Table 4. Imprecision was within 5% relative standard deviation (RSD). At 5 ng/mL, the level of the second to the lowest calibrator, at least 80% accuracy was obtained when developing the calibration curves. Thus, 5 ng/mL was chosen as the reasonable limit of quantitation (LOQ) for the method.

Table 4. Method Precision (n=5) for the Calibrator Compounds in Synthetic Urine

	Average conc. ng/mL	
Expected conc ng/mL	**MDPV (RSD%)**	**Mephedrone (RSD%)**
1	1.33 (3.83%)	1.21 (4.91%)
5	4.98 (3.98%)	4.96 (3.14%)
10	9.53 (2.49%)	9.69 (1.72%)
25	22.61 (1.84%)	23.94 (1.81%)
50	45.65 (2.64%)	47.17 (0.82%)
100	93.81 (3.12%)	96.81 (1.32%)
500	479.46 (0.92%)	485.71 (1.79%)
1000	991.13 (1.40%)	975.75 (0.98%)
5000	5042.34 (0.33%)	5045.73 (0.39%)

Though no regulatory agencies have set cut-off values for MDPV and mephedrone, 25 ng/mL seems a suitable choice based on the results described here.

To test for possible interferences, six compounds similar in structure to MDPV and mephedrone were spiked in the blank urine to reach the relatively high concentrations of 1×10^6, 1×10^6, 1×10^6, 5×10^4, 5×10^3, and 5×10^3 ng/mL for phenylpropanolamine (PPA), ephedrine, pseudoephedrine, phentermine, amphetamine and methamphetamine, respectively. Figure 7 shows the structures and molecular weights of these compounds. Because their molecular weights are different from both MDPV and mephedrone, no interferences were expected to be observed. The samples were prepared, extracted and run through the LC/MS/MS system as described earlier.

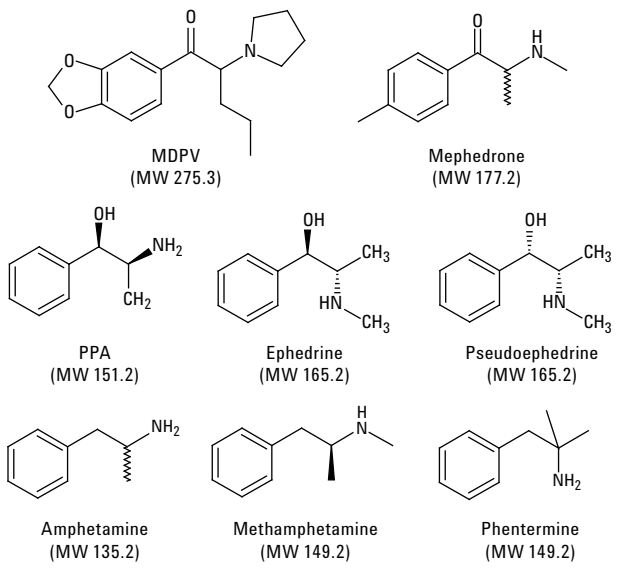

Figure 7. Six compounds similar in structure to MDPV and mephedrone.

As expected, no interferences were found. Figure 8 shows the MRM chromatograms for the blank urine sample spiked with ephedrine at $1x10^6$ ng/mL, a high concentration in comparison to that expected for the target synthetic cathinones. The four peaks shown are of the MDPV-D8 and mephedrone-D3 transitions.

To check for carryover, the negative control urine was analyzed after five injections of 10,000 ng/mL MDPV and mephedrone. Small peaks resulting from carryover were observed. Figure 9 and Figure 10 show the calculated concentration of MDPV and mephedrone in the blank sample was 3.18 ng/mL and 1.53 ng/mL, respectively. Because both values are well below the chosen cutoff, 25 ng/mL, carryover was considered negligible.

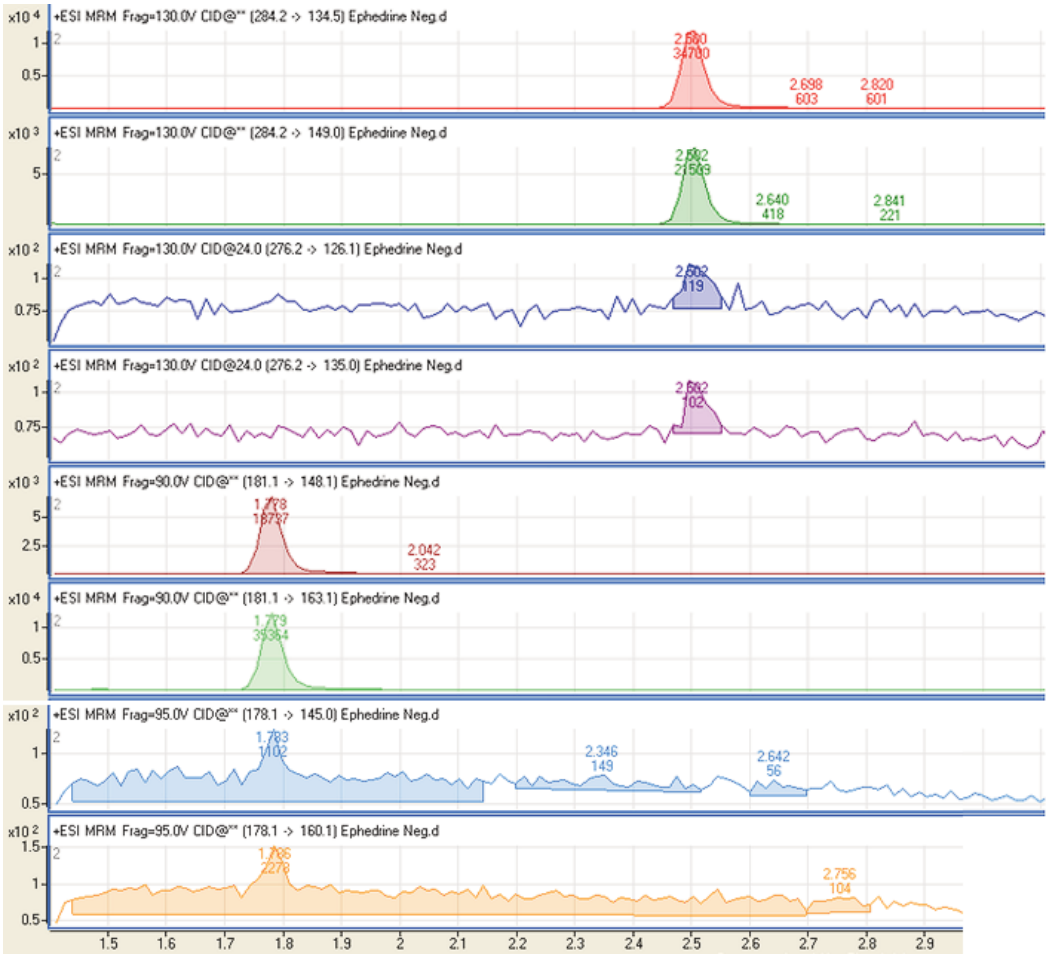

Figure 8. MRM chromatograms of blank urine spiked with ephedrine at $1x10^6$ ng/mL. No interferences were observed.

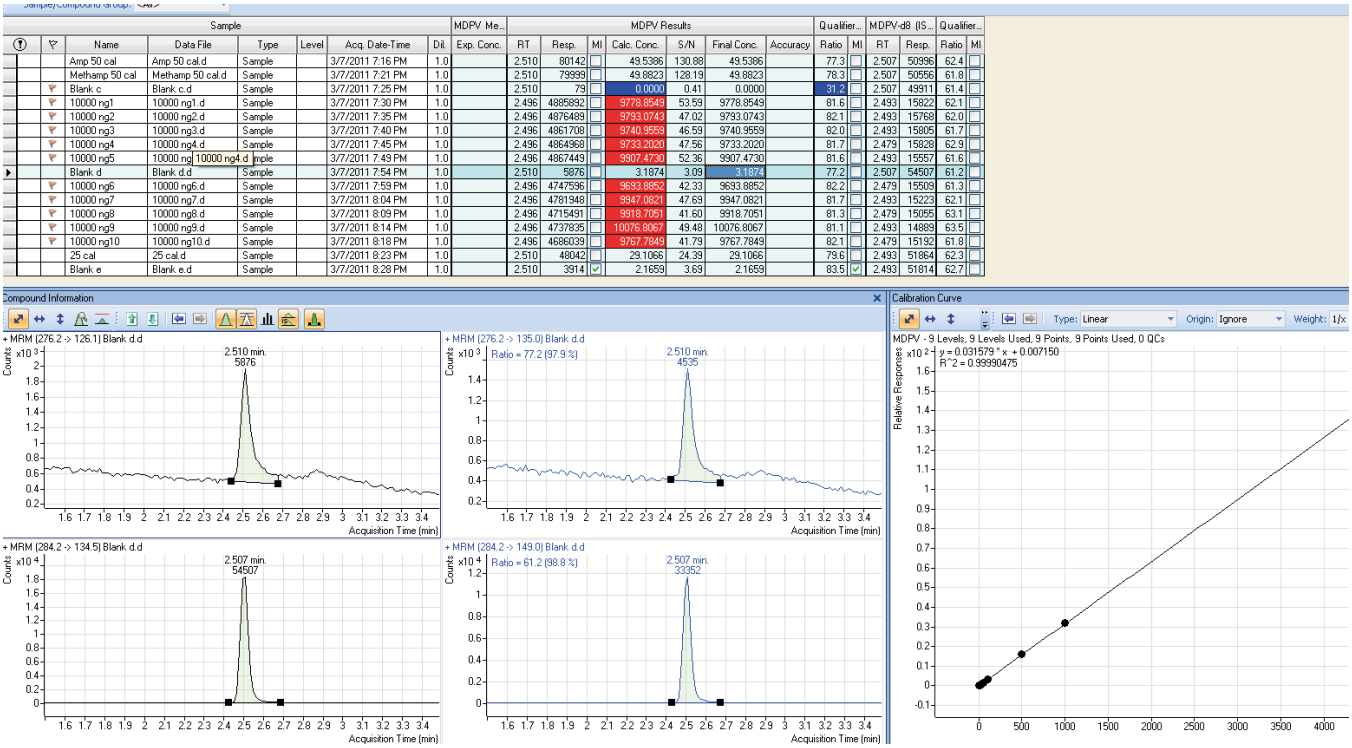

Figure 9. MDPV carryover is negligible at 3.18 ng/mL and below the cutoff value of 25 ng/mL.

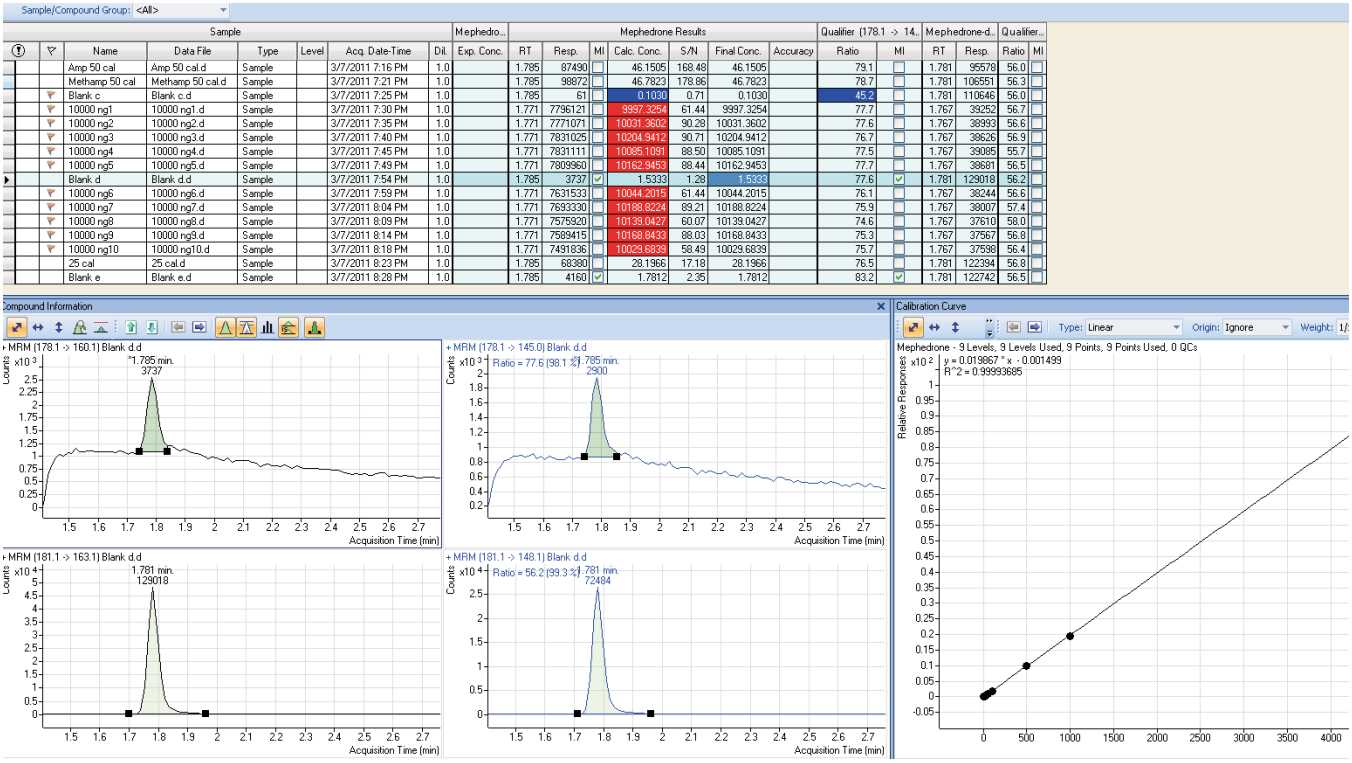

Figure 10. Mephedrone carryover is negligible at 1.53 ng/mL and below the cutoff value of 25 ng/mL.

Conclusion

Due to its selectivity and sensitivity, LC/MS/MS is a powerful technique for screening, confirmation and quantification of abused synthetic cathinones, such as MDVP and mephedrone, in complex biological matrices such as urine. The LC/MS/MS method described here offers forensics laboratories an easy and robust approach that demonstrates excellent linearity, LOD, reproducibility and LOQ, with no interferences from structurally similar compounds, and with negligible carryover. The method can also be easily modified to include the analysis of other synthetic cathinones as the need arises.

Further Reading

1. American Association of Poison Control Centers. http://www.aapcc/org.
2. DEA Drug Fact Sheet, Bath Salts or Designer Cathinones (Synthetic Stimulants). http://www.justice.gov/dea/pubs/abuse/drug_data_sheets/Bath_Salts.pdf.
3. Federal Register Vol. 76, No. 174. September 8, 2011.
4. Spiller, H.A. et al. Clinical experience with and analytical confirmation of "bath salts" and "legal highs" (synthetic cathinones) in the United States. Clin. Toxicol. 2011; Jul;49(6): 499-505.

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