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GC-MS/MS-Based Detection and Quantification of Methamphetamine and Mephedrone in Biological Matrices using Modified dSPE Method

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This research presents a technique combining gas chromatography and tandem mass spectrometry (GC-MS/MS) with a modified dispersive solid phase extraction (D-SPE) method for identifying and quantifying methamphetamine (MTPn) and mephedrone (MPDn) in biological samples. These amphetamine-category new psychoactive substances are increasingly popular among young individuals, making their detection crucial. The method involves a clean-up procedure integrated with GC-MS/MS technology, allowing simultaneous detection of both drugs. Operating the analytical column at 40 ºC with sample concentrations ranging from 5 to 200 ng/mL, the study determined the limit of detection (7.326-20.213 ng/mL) and the limit of quantification (22.2-60.25 ng/mL). The modified dSPE method's efficacy was compared to other extraction techniques, highlighting the sensitivity, accuracy and precision of the GC-MS/MS method. These findings have significant implications for forensic and clinical applications in detecting and quantifying methamphetamine and mephedrone.

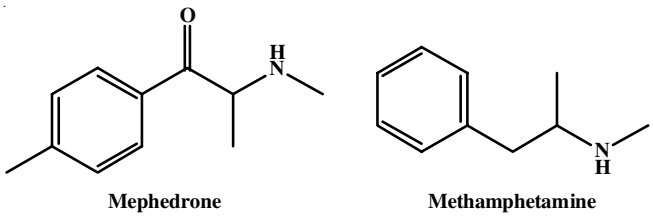
Keywords: Psychoactive substance, Mephedrone, Methamphetamine, Toxicology, Method optimization, Quantitation.

INTRODUCTION

Over the past few years, the illegal drug market has been swamped with an ever-growing variety of new psychoactive substances (NPS) that possess diverse chemical structures. The influx of these substances is staggering, with more than one new substance being introduced every week on average, making it an unprecedented trend in the global illicit drug market [1,2]. Synthetic compounds that mimic traditional drugs of abuse by having psychotropic effects on the body are NPS. These substances are classified under the UN Conventions of 1961 (Single Convention on Narcotic Drugs) and 1971 (Convention on Psychotropic Substances [3]. The minor structural modifications in NPS in comparison to conventional drugs allow them to be legally traded until national restrictions are put in place [4]. NPS includes a large range of stimulants that can be classified into various subgroups, such as synthetic cathinone's, aminoindanes, phenethylamines, piperazines, pipradrols, phencyclidine-type substances and tryptamines [5]. These stimulants typically either enhance or decrease the direct or indirect elevation of neurotransmitters like dopamine, serotonin and/or noradrenaline in the synaptic cleft [6].

Amphetamines are a class of stimulants that are widely abused and known for their potent effects on the central nervous system, including feelings of pleasure and well-being [7]. Amphetamine abuse is still widespread, particularly among young people who need increased energy for physically demanding activities. Illegal sources are used to obtain a new type of synthetic amphetamines with modified ring systems that have become prevalent and popular among young people [8].

The synthetic compound mephedrone (Fig. 1a), also known as 4-methylmethcathinone, is sold online as a research chemical, plant food or legal high labelled "not for human consumption. It is derived from the natural cathinone (Khat) and common street names for mephedrone are 4-MMC, MMCAT and Crab. Mephedrone produces effects similar to other psychostimulants such as MDMA [9,10]. It usually comes in a white, off-white, or yellowish powder or crystals and has a melting point of 170-175 ºC and a boiling point of 259-260 ºC. Mephedrone acts as a central nervous system (CNS) stimulant that produces feelings of euphoria, increased energy, heightened alertness and decreased appetite [11]. Its effects typically last for 2-4 h and it has a rapid onset of action. The substance also possesses the



Mephedrone **Methamphetamine**

Fig. 1. Chemical structure of mephedrone (MPDn) and methamphetamine (MTPn)

ability to be misused and result in addiction. It can lead to a variety of unfavourable outcomes such as restlessness, nervousness, perceptual distortions and heart-related issues [12].

Methamphetamine (MTPn, Fig. 1b) is another drug belonging to this category. Its molecular formula is $C_{10}H_{15}N$ and its chemical makeup includes a phenyl ring, a methyl group and an amino group. MTPn is a lipophilic compound with a potent effect on the CNS since it can easily cross the blood-brain barrier [13]. It works by increasing the release of dopamine, norepinephrine and serotonin in the brain, resulting in feelings of euphoria, increased energy and decreased appetite [14]. MTPn has a rapid onset of action and can last for several hours. However, the drug is highly addictive and can result in adverse effects such as anxiety, paranoia, hallucinations and cardiovascular complications [15]. Neurotoxicity and cognitive impairment can also occur with long-term use of MTPn. Furthermore, there has been a rise in seizures by 21% and 122% for NPS such as MTPn and MPDn, respectively [13,16]. It is not uncommon for patients who consume amphetamines to attempt suicide by overdosing on drugs in a life-threatening manner. The instances of reported negative events, including toxicity, fatalities and other harmful effects linked to the abuse of NPSs, are on the rise. Several previous works of literature have documented cases of toxicity and overdose of MPDn and MTPn, ranging from references [17-22]. Identifying NPS and amphetamine type stimulants in biological samples is crucial for forensic and clinical toxicologists. This helps in assessing the prevalence of NPS among the population and diagnosing intoxication and impairment resulting from the use of such substances.

Identifying these compounds in biological samples presents a number of challenges, including the vast number of possible structures and the continuous influx of new compounds, making it a complex task. The literature has documented various methods for analyzing amphetamines in biological samples. The techniques used for analyzing these substances include liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis-mass spectrometry (CE-MS) and gas chromatography-mass spectrometry (GC-MS) [23]. To determine multiple targets of NPS, gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) is still commonly employed. When used in multiple-reaction-monitoring mode (MRM), GC-MS/MS provides high sensitivity and selectivity, with good precision and accuracy over a wide dynamic range, enabling the development of fast analytical methods. In past, several GC-MS/MS applications have been described for determining a variety of NPS in biological matrices such as saliva, blood, urine, oral fluids and hair [24,25].

To employ hyphenated techniques like GC-MS/MS or GC/MS for analyzing highly complex matrices such as saliva and urine, a clean-up step is crucial before instrument analysis. Sample pre-treatment is necessary to obtain an analyzable sample through effective purification that eliminates undesired matrix components, reduces the matrix effect and enhances sensitivity [26]. This was achieved by dispersive solid phase extraction (dSPE) protocol with modification in solvent treatment protocol. Methanol inserted of acetonitrile was utilized in this study to enhance the recovery and lower the matrix interference.

Limited research has been optimized for MPDn and MTPn extraction using a design of experiments and statistical analysis. Yonamine *et al.* [27] first initiated to develop the microextraction solid phase extraction for MPTn using headspace-GC from urine samples and yielded recovery of 75.4-90.5% with LOD and LOQ of 5 ng/mL. A novel fiber coated cyclodextrin derivative also been used by Zhou & Zeng [28] for screening MTPn in human urine samples using GC with recovery rate of 98.2%, although this method devised accurate recovery but still have complexed solvent composition to perform the analysis. In 2018, Zargar *et al.* [29] devised carbon nanomaterial based sorption technique for MTPn from saliva and urine samples with recovery rate of 89.2% in blood and 101% in saliva samples. For MPDn, Alexandridou *et al.* [30] perfomed detection of MPDn from urine and blood samples using GC-MS and obtained accuracy and recoveries in micro levels with matrix effect *i.e.* 118% in blood and 102% in urine sample. Recently, McNeill *et al.* [31] proposed lab on a chip device for detection of MPDn in urine samples using GC-MS, the proposed method was cost effective but still is a qualitative approach.

While reported studies only focus on the sample pretreatment processes, which is a method miniaturization and results in either higher matrix effect or poor recovery, present approach retained the clean-up step based on the original method due to the complex matrix nature of the biological samples. The clean-up step was used to eliminate any significant matrix effect, resulting in clearer extracts and protecting the GC column from potential source contamination in the long term. This method displayed excellent recovery of MPDn and MTPn in ppb levels from urine and saliva, surpassing prior GC-MS/MS research.

This work aims to develop a comprehensive workflow system that encompasses simple sample preparation to reliable quantitative performance. It focuses on the development and validation of a modified-dSPE technique combined with GC-MS/MS analysis of two NPS drugs of abuse, specifically saliva and urine samples. The goal of the workflow system is to provide forensic laboratories with a practical, sensitive, fast and robust approach for routine toxicological analysis.

EXPERIMENTAL

Analytical grade solvents, namely acetonitrile, methanol and ethyl acetate were procured from Sigma-Aldrich (USA). whereas hydrochloric acid and *N*-methyl-*bis*-trifluoroacetamide was sourced from Thermo-Fischer Scientific (USA). To carry out the extraction and clean-up, prepacked Q-sep™ QuEChERS extraction salts (containing 6 g $MgSO_4$ and 1.5 g NaOAc; AOAC

2007.01 method) and Q-sep™ QuEChERS dispersive-SPE (dSPE) tubes (containing 150 mg $MgSO_4$ and 50 mg C_{18}; AOAC 2007.01 method) were purchased from Merck (Germany).

Stock solution and internal standards: To prepare the stock standard solutions, methanol was used to dissolve the substances at a concentration of 1 mg/mL. The working solutions were then created by diluting the stock solution in distilled water at various concentrations, including 5, 10, 20, 50, 100 and 200 ng/mL. Additionally, a mixed internal standard (IS) solution was generated for all analytes, with a concentration of 1 µg/mL in CAN.

Method validation: The workflow system for toxicological analysis was developed and validated by assessing relevant quantitative method parameters, including selectivity, limits of detection (LODs), limits of quantification (LOQs), linearity, precision, reproducibility, process efficiency, sample stability and carry-over. Selectivity was confirmed by examining blank samples to ensure no peaks were present that could interfere with the substances of interest. LOD and LOQ were determined by analyzing saliva and urine samples at decreasing concentrations, with LOD established at a concentration giving an S/N > 3 for at least three ions for each substance. The linearity of the method was verified by spiking samples with the standard mixture to obtain various concentrations. Reproducibility was assessed by analyzing three replicates at two concentration levels and accuracy was evaluated by comparing the calculated value with the theoretical sample concentration. Process efficiency was determined through the recovery of analytes from the sample matrix, while carry-over effects were evaluated by analyzing a blank sample after injection of the highest calibrator.

The procurement of chemicals and equipment for extraction and clean-up was conducted according to standard protocols. The required chemicals were obtained from a reputable supplier and were of analytical grade. The equipment used for extraction and clean-up was validated and maintained regularly to ensure optimal performance.

Stock and working solutions for analysis were prepared according to established protocols. Stock solutions were prepared by dissolving the appropriate amount of each substance in a suitable solvent at a concentration of 1 mg/mL. Working solutions were prepared by diluting the stock solutions to the required concentrations using the appropriate solvent. All solutions were stored in a cool, dry place and were protected from light to ensure stability. Prior to analysis, solutions were vortexed and filtered through a 0.22 µm membrane filter to remove any particulate matter.

dSPE extraction protocol: The optimization process for dSPE extraction involved the implementation of two techniques. Firstly, a uniform artificial sample of saliva and urine spiked with MPDn and MTPn at various concentrations ranging from 5 to 200 ng/mL was mixed with a diluent comprising of 10 mL of methanol and 10 mL of Milli Q water. The mixture was homogenized for 10 min on a wrist action shaker, followed by the addition of an EN D-SPE salt pouch. The mixture was vortexed for 1 min before being centrifuged at 6000 rpm for 10 min at 2-8 ºC. From the supernatant layer obtained, 6 mL was transferred to a 15 mL centrifuge tube. In the second step, the mixture was subjected to a cleanup process using 500 mg of magnesium sulfate and 250 mg of primary and secondary amine (PSA) to simplify the compound. The sample was vortexed for 2 min, centrifuged at 6000 rpm for 6 min at 2-8 ºC and then poured into a tube containing 150 mg of $MgSO_4$. The resulting extract was clean and 200 µL was transferred to a different vial, with 10 uL from each sample directly injected into the GC-MS/MS apparatus.

Instrumentation condition: The GC-MS/MS analysis was conducted using an Agilent Technologies 7890B-7000D instrument in Santa Clara, CA, U.S.A. The Agilent J&W HP-5MS ultra inert column (15 m × 0.25 mm, i.d., film thickness 0.25 µm; Agilent Technologies) was used as the column. The GC conditions consisted of high-purity helium as the carrier gas with a flow rate of 2.25 mL/min. The initial temperature was held at 80 ºC for 1.5 min followed by a ramp up to 280 ºC at a rate of 25 ºC/min and a final hold for 15 min, resulting in a total run time of 23.7 min. The solvent delay time was 7.5 min, with a forward inlet temperature of 250 ºC and a split ratio of 5:1. The MS/MS conditions included an ion source temperature of 230 °C, quadrupole temperature of 150 ºC, positive electron ionization voltage at 70 eV in single stage GC-MS mode and molecular ion as a precursor ion using collision energies at an optimal 25 eV for product ion spectra.

RESULTS AND DISCUSSION

Method optimization: The effectiveness of dSPE method depends on various factors and its selectivity can be modified. Hence, it is crucial to evaluate specific parameters, such as the sample/solvent ratio, extraction solvent, pH, type and quantity of partition salts, type of agitation and cleaning sorbents. Such studies allow for changes to traditional methods and an improvement in the extraction process [32,33]. The dSPE method was assessed in two stages, with a design experiment used to determine the optimal extraction conditions. The preliminary assessment involved comparing the amplitude and statistical significance of various parameters on the response using normal plots generated for each amphetamine. Fig. 2 shows calibration plots for MPDn and MTPn in saliva and urine samples, indicating a linear relationship between the response and concentration. The goodness of fit parameter (R^2) was between 0 and 1, indicating that the regression predictions fit the data well.

Implementing GC-MS/MS condition: In this study, the optimal conditions for chromatography and spectrometry were established using the GC-MS/MS system by directly diffusing pure standard solutions. To identify each sample, the system detected precursor ions and employed different collision energy voltages to identify two distinct product ions. The quantifier ion and the peak with the second-highest intensity served as the qualifier ion among the transitions. Multiple reactions monitoring (MRM) transitions and dwell periods were used to automatically adjust the parameters. The composition of the mobile phase has a significant impact on quantification specificity, including buffer solution concentration and pH and MS conditions. Addition of 5 mM ammonium acetate to the mobile phase reduces peak width and produces a more symmetrical

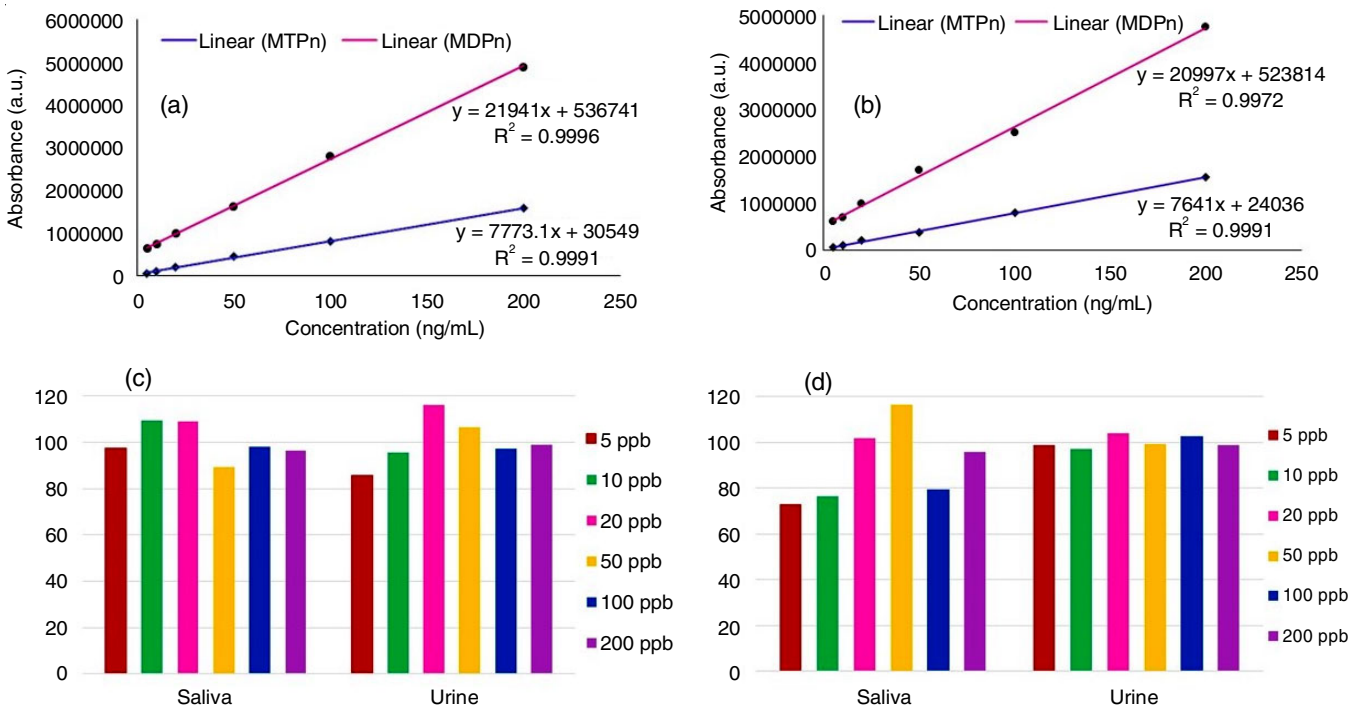


Fig. 2. Linearity plots of MTPn and MDPn in (a) saliva and (b) urine; (c) recovery percentage plot of MTPn and (d) recovery percentage plot of MDPn

peak than water. Formic acid (0.1%) improved the signal responses of MPDn and MTPn.

The GC conditions for MPDn and MTPn in saliva and urine samples were optimized and are presented in Table-1, including retention times. The MS parameters were optimized using a 100 ng/mL tuning solution in both positive and negative ionization modes. The results indicated that both drugs were more sensitive and had minimal background noise in positive ionization mode. The precursor/product ion mass transitions for MTPn were measured at 262→280 *m/z*, while those for MPDn were 148→163 *m/z*. The response and retention time of both drugs in artificial saliva and urine samples at concentrations ranging from 5 ng/mL to 200 ng/mL are illustrated in Figs. 3 and 4, as determined by GC-MS/MS.

Limit of detection and quantification: To ensure the method's linearity, calibration curves were created using six concentration levels and peak area to standard internal ratios of analytes. Statistical software such as IBM-SPSS and GraphPad were utilized to analyze the data. The range of concentration was designed to cover therapeutic levels for all drugs, with a working range of 10 ng/mL. The LOD and LOQ were determined using signal-to-noise ratios of 3:1 and 10:1, respectively and the results are summarized in Table-2. The analysis showed that the calibration range was suitable for a linear curve with $1/x^2$ weighting for most NPS. The linear regression coefficient $R^2 > 0.9999$ indicated a reliable level of model adjustment fit.

Imprecision, accuracy and recovery: The ANOVA analysis was utilized to assess imprecision and Table-3 presents the values of slope, intercept and standard errors. The results for imprecision and recovery are consistent with earlier studies and all samples met the imprecision acceptance criteria of not surpassing a 20% variation. The recovery rate for MPDn and MTPn ranged from 98.6% to 103.85% for MPDn in saliva samples, 92.91% to 103.8% for MPDn in urine samples, 85.8%

TABLE-1
RETENTION TIME AND MASS DATA BY CHARGE RATIO OF METHAMPHETAMINE AND MEPHEDRONE DRUG

Compound	Retention time (min)	Quantifier		Qualifier	
		m/z	eV	*m/z*	eV
Methamphetamine	7.452	262	67	280	14
Mephedrone	7.609	148	29	163	17

TABLE-2
ANALYTICAL AND CALIBRATION DATA OF MEPHEDRONE (MPDn) AND METHAMPHETAMINE (MTPn) IN BIOLOGICAL MATRICES

Drugs	Matrices	Slope	Intercept	RT	R^2	LOD	LOQ	RSD
Methamphetamine	Saliva	7773.1	30549	4.439	0.99	11.528	33.535	1.164
	Urine	7641	24036	4.439	0.99	11.187	33.902	1.149
Mephedrone	Saliva	21941	536741	4.733	0.99	7.326	22.2	1.179
	Urine	20997	523814	4.733	0.99	20.213	60.251	1.162

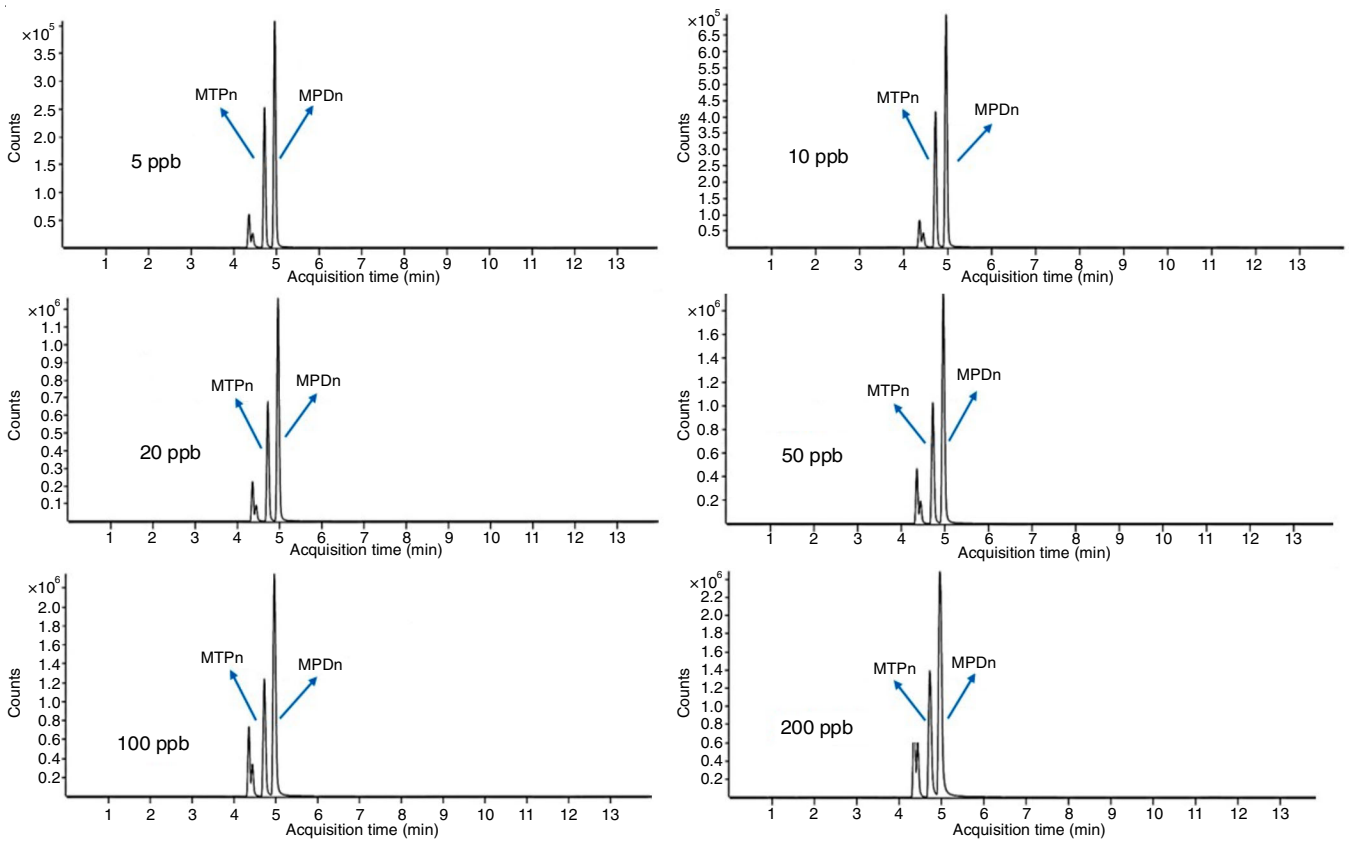


Fig. 3. Chromatograms of mephedrone (MPDn) and methamphetamine (MTPn) in simulated saliva at different concentrations of 5, 10, 20, 50, 100 and 200 ppb

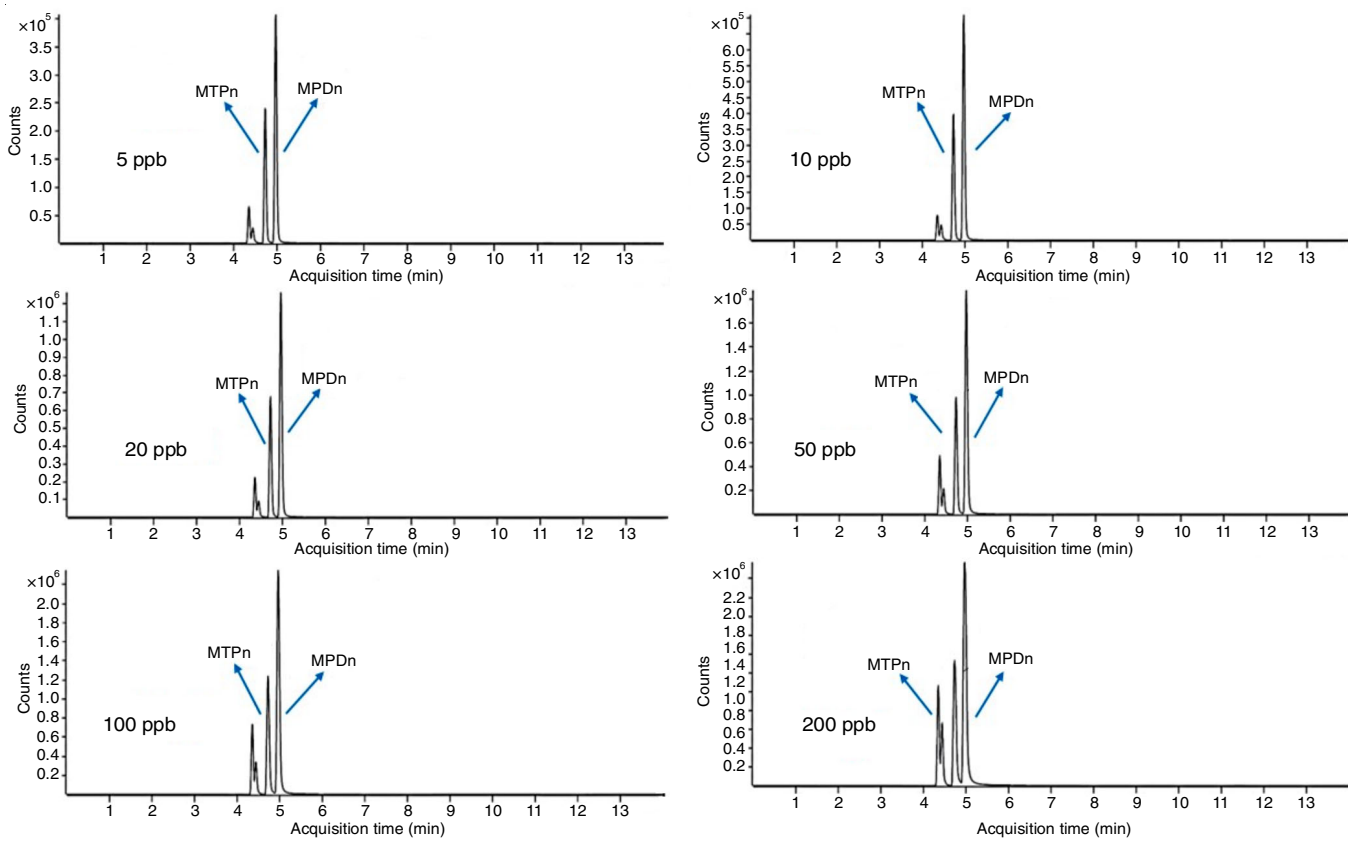


Fig. 4. Chromatograms of mephedrone (MPDn) and methamphetamine (MTPn) in simulated urine at different concentrations of 5, 10, 20, 50, 100 and 200 ppb

TABLE-3
RECOVERY PERCENTAGE OF MEPHEDRONE (MPDn) AND METHAMPHETAMINE (MTPn) IN BIOLOGICAL MATRICES

Spiked concentration	Extracted concentration from saliva (ng/mL)		Recovery (%)		Extracted concentration from urine (ng/ML)		Recovery (%)	
	MTPn	MDPn	MTPn	MDPn	MTPn	MDPn	MTPn	MDPn
5	4.8744	3.654	97.488	73.08	4.2895	4.9318	85.79	98.636
10	10.9587	7.6454	109.587	76.454	9.5626	9.7205	95.626	97.205
20	21.8306	20.3088	109.153	101.544	23.2091	20.7727	116.0455	103.8635
50	44.6606	58.1489	89.3212	116.2978	53.3231	49.5264	106.6462	99.0528
100	98.1676	79.3043	98.1676	79.3043	97.1695	102.4378	97.1695	102.4378
200	193.1708	191.0459	96.5854	95.52295	197.4462	197.6108	98.7231	98.8054

to 116% for MTPn in saliva and 95.6% to 111% for MTPn in urine samples. For the lowest concentration (5 ppb), the method had excellent recovery for both amphetamines, with a range of 85.8% to 98.6% for both matrices. However, for 20 ppb injection volume, the method exhibited high matrix effects, resulting in a recovery percentage range of 103.8% to 116% as tabulated in Table-3.

Matrix interference and carryover: We observed matrix effects for all analytes at two concentration levels, namely the lower quality control (LQC) of 5 ng/L and higher quality control (HQC) of 200 ng/L. These effects showed variations of less than 20% at both levels, which is considered acceptable data and meets validation standards. The interference test confirmed the method's selectivity for both amphetamine drugs, as no common compound in artificial urine and saliva samples was detected. However, we detected carryover at the highest quantities from previous injections of the target drug.

Conclusion

New psychoactive substances (NPS) have become a global concern due to their potent toxicity and the occurrence of mortality cases associated with their use. They pose a significant threat to human lives and are often used as drugs of abuse. Analyzing analytes in complex specimens encountered in clinical and forensic settings, such as biological samples, can be challenging, requiring a highly sensitive and selective method. This study presents a novel approach to detect and quantify methamphetamine (MTPn) and mephedrone (MPDn) in forensically important biological matrices using GC-MS/MS analysis. The developed method demonstrated good linearity, accuracy and precision and successfully applied to both synthetic and real samples, with a recovery rate of 85-116% for MTPn and 73-116% for MPDn. This study provides a reliable method for detecting and quantifying these drugs in forensic and clinical settings, but further research is needed to validate the method on a larger sample size and explore its applicability to different matrices and drug combinations.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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