https://doi.org/10.31925/farmacia.2026.2.16 **ORIGINAL ARTICLE**

A DUAL-PLATFORM GC-MS/MS AND LC-MS/MS STRATEGY FOR THE TOXICOLOGICAL MONITORING OF SIX EMERGING NEW PSYCHOACTIVE SUBSTANCES (NPS): FIT-FOR-PURPOSE VALIDATION IN HUMAN URINE

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Abstract

Clinical toxicology laboratories require robust and sensitive analytical methods to address the increasing prevalence of new psychoactive substances (NPS). This study developed and validated two complementary analytical methods, gas chromatography-tandem mass spectrometry (GC-MS/MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), for the quantitative analysis of six key NPS in human urine, including α-pyrrolidinovalerophenone (α-PVP), 4-methylethcathinone (4-MEC), 3',4'- methylenedioxypyrovalerone (MDPV), 3,4 – methylenedioxy- alpha- pyrrolidinobutiophenone (MDPB), N-(2-Methoxybenzyl)-4-iodo-2,5-dimethoxyphenethylamine (25-NBOMe) and 4-fluoromethcathinone (4-FMC). Both methods were validated according to established guidelines, assessing parameters such as linearity, sensitivity, precision, and accuracy. Both GC-MS/MS and LC-MS/MS methods demonstrated excellent linearity with coefficients of determination (R^2) above 0.99. However, the LC-MS/MS method proved to be significantly more sensitive and accurate. It achieved a limit of quantification (LOQ) of 1 µg/L, a substantial improvement over the 200 µg/L LOQ of the GC-MS/MS method. Despite the quantitative limitations imposed by higher bias, the GC-MS/MS framework maintains its procedural significance, serving as a vital tool for the confirmatory identification of these emerging NPS. These parallel analytical trajectories act as complementary frameworks, delivering an empirical evaluation of their deployment in clinical practice for thorough toxicological surveillance.

Rezumat

Laboratoarele de toxicologie clinică impun utilizarea unor metode analitice robuste și sensibile pentru a gestiona prevalența tot mai crescută a noilor substanțe psihoactive (NSP). Prezentul studiu a vizat dezvoltarea și validarea a două metode analitice complementare: cromatografia de gaze cuplată cu spectrometria de masă în tandem (GC-MS/MS) și cromatografia lichidă cuplată cu spectrometria de masă în tandem (LC-MS/MS), pentru analiza cantitativă a șase NSP esențiale, din urină, respectiv: α-pirolidinovalerofenona (α-PVP), 4-metiletcatinona (4-MEC), 3',4'-metilendioxi-pirovalerona (MDPV), 3,4-metilendioxi-α-pirolidinobutiofenona (MDPB), N-(2-metoxibenzil)-4-iodo-2,5-dimetoxifenetilamina (25-NBOMe) și 4-fluorometcatinona (4-FMC). Ambele metode au fost validate în conformitate cu ghidurile în vigoare, fiind evaluați parametri precum linearitatea, sensibilitatea, precizia și exactitatea. Atât metoda GC-MS/MS, cât și cea LC-MS/MS au prezentat o linearitate excelentă, cu coeficienți de determinare ($R^2 > 0,99$). Cu toate acestea, metoda LC-MS/MS s-a dovedit a fi semnificativ mai sensibilă și mai exactă, atingând o limită de cuantificare (LOQ) de 1 µg/L, ceea ce reprezintă o îmbunătățire semnificativă față de valoarea LOQ de 200 µg/L obținută prin metoda GC-MS/MS. În pofida limitărilor cantitative, impuse de o eroare sistematică (*bias*) mai ridicată, platforma GC-MS/MS își păstrează relevanța în context clinic, servind drept instrument esențial pentru confirmarea identificării acestor NSP emergente. Aceste metode analitice funcționează complementar, oferind o valoare obiectivă a implementării acestora în practica clinică pentru o supraveghere toxicologică riguroasă.

Keywords: quantification of new psychoactive substance(s), forensic toxicology, GC-MS/MS, LC-MS/MS

Introduction

New Psychoactive Substances (NPS) are chemical compounds that frequently emerge through subtle structural modifications of known psychoactive substances, both licit and illicit. While many are strategically designed to maintain or enhance pharmacological effects whilst evading existing legal frameworks, others represent entirely novel chemical scaffolds or are derived from repurposed pharmaceutical research (1, 2).

The swift spread of new psychoactive substances (NPS), such as synthetic cathinones, synthetic

cannabinoids, synthetic opioids, designer benzodiazepines, phenethylamines, and tryptamines, has become a global public health issue (3). This issue is made worse by the fact that NPS naturally tend to change chemically, which has become a major toxicological priority (4). The dynamic evolution of NPS poses considerable analytical challenges for clinical toxicology laboratories, in terms of timely and reliable detection, identification, and quantification of these constantly evolving compounds (1, 5, 6).

Recent data from the EUDA (European Union Drugs Agency) shows that synthetic cathinones are still an important type of NPS. They are the second largest group of NPS being watched in Europe (7). Although the number of reports varied between 2005 and 2024, this class has been consistently notified, accounting for approximately 18% of all unique NPS identified to date. As indicated in the 2025 European Drug Report, their market dominance is further evidenced by a record-high seizure volume, which reached nearly 48 tonnes in 2024. The clinical profiles of several cathinones, such as α-pyrrolidinovalerophenone (α-PVP), 4-methyl-ethcathinone (4-MEC), 3',4'- methylenedioxypyrovalerone (MDPV), 3,4-methylenedioxy-alpha-pyrrolidinobutiophenone (MDPB), N-(2-Methoxybenzyl)-4-iodo-2,5-dimethoxyphenethylamine (25-NBOMe) and 4-fluoromethcathinone (4-FMC) have necessitated stringent surveillance protocols, as these designer analogs represent a substantial transition from "emerging" to "persistent" status within international toxicovigilance networks. These compounds may constitute a representative series for toxicological analysis, primarily due to their established association with severe, acute toxicological events (8-11).

Conventional immunoassays for NPS identification are inherently limited by low analytical specificity and susceptibility to cross-reactivity with structural homologs. This lack of selectivity frequently leads to false-positive results caused by non-target compounds mimicking the chemical profile of the intended analyte and to diagnostic inaccuracies (12). While the gas-chromatography -mass spectrometry (GC-MS) is often employed for the screening of suspected analytes in clinical toxicology, its utility is limited to thermostable compounds and requires extensive sample preparation and derivatization to improve detection (13). Despite advancements in adaptable GC-MS workflows (14-17), refined extraction techniques, and optimized derivatization protocols, technical constraints persist, particularly regarding the underrepresentation of emerging NPS and their metabolites in commercial spectral databases. The establishment of standardized protocols for retention times, derivatization, and fragmentation pathways is therefore necessary for the effective expansion of global spectral libraries (18).

These limitations, along with the absence of comprehensive platforms and standardized protocols for untargeted NPS detection, underscore the pressing necessity for the advancement of rapid, sensitive, and targeted analytical techniques, such as GC-MS/MS. For continuous monitoring, these methods must be used to effectively include NPS detection in regular drug-of-abuse screening (19).

The aim of this study is to develop two robust chromatography-mass spectrometry-based methods, GC-MS/MS and LC-MS/MS, with clinical toxicology utility for the analysis of six high-priority NPS, including: α-PVP, 4-MEC, MDPV, MDPB, 25-NBOMe, and 4-FMC. These NPS were prioritized based on internal data, highlighting their clinical prevalence, significant role in acute intoxications, and the availability of commercial reference standards.

Materials and Methods

Reagents and chemicals

Certified new psychoactive substances reference material: α-PVP, 4-MEC, MDPV, MDPB, 25-NBOMe, and 4-FMC at concentrations of 1 mg/ mL free base in methanol were obtained from Lipomed Inc. (Cambridge, MA, USA). Analytical-grade chloroform, dichloromethane, and dichloroethane (Supelco, Merck) were used as solvents for the extraction phase in GC-MS/MS. For LC-MS/MS, the internal standard (IS) (MDMA-D3 and Methamphetamine-D6 deuterated mixture) (JSM-CL-4005), reagent R1 (JSM-CL-4003), and mobile phases A and B (JSM-CL-4001, JSM-CL-4002) were procured from Jasem® (Istanbul, Turkey) (20).

Preparation of standards and samples

Stock and calibration standards

GC-MS/MS: A primary stock solution containing the six-target new psychoactive substances (α-PVP, 4-MEC, MDPV, MDPB, 25-NBOMe, 4-FMC) was prepared at a concentration of 1000 µg/L by diluting the certified stock standards in methanol. Due to the limited commercial availability of isotopically labeled internal standards for these specific emerging NPS at the time of the study, a rigorous external standard calibration methodology was employed. A series of calibration standards ranging from 200 to 1000 µg/L was then prepared by serial dilution of this stock solution using a micropipette. Calibration curves were constructed daily by plotting the absolute peak area against the analyte concentration. To ensure analytical stability and compensate for the absence of internal standards, quality control (QC) samples at low, medium, and high concentrations were interspersed within each analytical batch to monitor for potential instrument fluctuation or sensitivity changes. Precision standards for method validation were similarly prepared from the same stock.

LC-MS/MS: A stock solution of the target NPS (α-PVP, 4-MEC, MDPV, MDPB, 25-NBOMe, and 4-

FMC) was prepared in methanol at a concentration of 1000 µg/L. After getting the solution, it was mixed with methanol to make it 100 µg/L. Using ultra-pure water as the diluent, we made calibration standards at six different concentrations, from 1 to 100 µg/L, by serial dilution from the 100 µg/L working solution. This aqueous dilution was performed to ensure compatibility with the initial mobile phase composition, thereby preventing solvent strength-induced peak broadening and improving the signal-to-noise ratio during the early stages of the gradient elution**.** The same serial dilution procedure was used to create separate precision standards for method validation. To ensure analytical integrity, all solutions were prepared fresh daily.

Sample preparation

Urine matrices were collected in sterile containers from 10 healthy adult volunteers of both sexes who had pre-screened negative for common drugs of abuse *via* routine immunoassay testing. The study was conducted with the approval of the institutional ethics board, according to Convention No. A - 1893/17.03.2021, and all participant data were rigorously anonymized to ensure confidentiality. These blank matrices were stored at 4°C and utilized for the preparation of fortified samples during method validation. As the primary objective of this study was the analytical development and validation of the GC-MS/MS and LC-MS/MS methods, clinical patient samples were not included in the current scope.

A 50 mL urine aliquot was combined with 2 mL of a phosphate buffer (sodium dihydrogen phosphate/dipotassium phosphate, 14.7 g/7.3 g/1000 mL H_2O, pH 7). This solution was subjected to a liquid-liquid extraction (LLE) using 6 mL of a pre-mixed organic solvent mixture (chloroform/dichloromethane/dichloroethane, 1:1:1, v/v/v). The solution was stirred for 15 min with a digital magnetic stirrer and centrifuged at room temperature for 10 min at 3500 RPM using an EBA 200 Hettich centrifuge. The organic layer was collected, and the solvent was evaporated to dryness in a *Memmert* UN30 oven at a constant temperature of 40º C. The remaining residue was reconstituted in 1 mL of extraction solvent for later GC-MS/MS analysis. The external calibration standards were treated with the same evaporation and reconstitution procedure as the samples in order to correct for possible analyte losses during the preparation procedures. A reconstitution volume of 1 mL was employed to ensure complete analyte solubility and to minimize 'wall effects' or surface adsorption. While a lower volume would increase the concentration factor, the current approach provides the necessary sensitivity for clinical detection limits while ensuring sufficient extract for precise, replicate injections.

Urine matrices for LC-MS/MS analysis were collected and stored in the same manner. An aliquot of 50 µL of urine was mixed with 25 µL of internal standard. After an initial 10-second vortex, 175 µL of reagent R1 was added. The sample was vortexed again for 10 sec and centrifuged at 13.000 RPM for 5 min. Next, 150 µL of the supernatant was transferred to a vial insert for injection into the LC-MS/MS system.

Instrumentation and analytical conditions

GC-MS/MS Method

Chromatographic separation was carried out on an Agilent 8890 GC System (Agilent Technologies, Houston, TX, USA) coupled to a 7010B triple quadrupole mass spectrometer (Agilent Technologies, Houston, TX, USA) and a 7693A Autosampler (Agilent Technologies, Houston, TX, USA) with a DB-5ms Ultra Inert column (30 m x 0.25 mm ID x 0.25 µm df). Helium was used as the carrier gas at a constant flow of 1 mL/min. The GC oven temperature program was: 120°C (0.5 min hold), ramped at 15°C/min to 300°C (2.5 min hold). The front inlet was operated in pulsed splitless mode at 260°C (25 psi pulse until 0.5 min, then 50 mL/min purge flow to split vent at 0.75 min). At 15.00 min, a post-column backflush was started with an oven temperature of 300°C. The autosampler was programmed to inject 1 µL (10 µL syringe size) and heated at 50°C for vial mixing (2 cycles, 10 sec, 1000 rpm).

The Agilent Mass Selective Detector (MSD) was used for detection with Electron Ionization (EI) at 70 eV. The temperatures of the ion source and the transfer line of the MSD were 230°C and 280°C, respectively. Data were acquired in Multiple Reaction Monitoring (MRM) mode.

LC-MS/MS Method

Chromatographic separation was performed on an Agilent 1290 Infinity II LC System with Autosampler (Agilent Technologies, Houston, TX, USA) utilizing an Agilent Zorbax RRHD Eclipse Plus C18 column (2.1 x 50 mm, 1.8 µm). The LC system was coupled to a 6475 LC/TQ triple quadrupole mass spectrometer (Agilent Technologies, Houston, TX, USA) using gradient elution with Solvent A (100% water) and Solvent B (100% acetonitrile) at a constant flow rate of 0.7 mL/min. The gradient started at 10% B and increased to 80% B at 3.15 min, 95% B at 7.15 min, and returned to 10% B at 9.15 min. The total running time was 12.15 min. The column zone temperature was maintained at 40°C while the multisampler was at 15°C.

Detection was carried out using an Agilent Triple Quadrupole (TQ) mass spectrometer in positive polarity with an Agilent Jet Stream Electrospray Ionization (AJS ESI) source. Data were collected in Dynamic Multiple Reaction Monitoring (dMRM) mode with a time filter window of 0.07 min. Source parameters: capillary voltage 2500 V, drying gas flow 10.0 L/min, nebulizer pressure 40.0 psi, sheath gas flow 11.0 L/min.

Statistical analysis

All statistical analyses were performed in Microsoft Excel Version 16.92. The mean, standard deviation

(SD), relative standard deviation (RSD), and bias were calculated for the validation injections.

The analysis was performed using a linear regression (weight:1/x) calibration model based on the analyte concentration vs. peak area relation. The calibration curve was prepared from five concentration levels within the analytical range. In the GC-MS/MS method, the instrument response was taken as the peak area of the analyte (without internal standard), and in the LC-MS/MS method, the response was expressed as the peak area ratio of the analyte to the internal standard.

Calibration standards were run in duplicate at each concentration level, and linearity was evaluated over the concentration ranges indicated for each analytical method. R^2 gave an initial indication of linearity, but other statistical parameters, such as slope and intercept of the regression model, and standard error of the estimate, were evaluated to ascertain the adequacy of the calibration. In addition, analysis of variance (ANOVA) was applied to test the statistical significance of the linear regression (F-test for regression) and to confirm that the model adequately describes the relationship between analyte concentration and instrument response ($p < 0.05$ at a 95% confidence level).

Analytical validation protocol

The methodology was validated according to ANSI/ASB Standard 036 (2019) guidelines.

The overall method accuracy was determined by evaluating both the extraction recovery and matrix effects of spiked analytes from a negative urine matrix. This assessment, which covered the entire analytical workflow from sample preparation to final instrumental analysis, accounted for potential sources of error throughout the complete process. Recovery was determined by comparing the peak areas of samples spiked before extraction with those spiked after extraction, following the completion of the full extraction procedure. The recovery percentage was calculated using the following formula:

$$\text{Recovery (\%)} = (\text{Area}_{\text{spiked pre- extraction}}/\text{Area}_{\text{spiked post extraction}}) \times 100,$$

Area pre-extraction spiked = Peak area of the sample spiked before extraction; Area post-extraction spike = Area of the peak of the sample spiked after extraction.

Similarly, the matrix effect was calculated as:

$$\text{ME (\%)} = (\text{Area}_{\text{post-extraction spiked}}/\text{Area}_{\text{neat solution}}) \times 100$$

Area post-extraction spiked = Peak area of a blank matrix sample that has undergone the extraction process with the analyte added to the final extract.

Neat solution area = Peak area from a standard prepared directly in neat solvent without any extraction steps.

For method validation, 50 mL aliquots of urine (GC-MS/MS) and 1 mL aliquots (LC-MS/MS) were fortified in triplicate with the target NPS mixture at 1000 µg/L and 25 µg/L, respectively, for recovery studies, and at 100 µg/L for matrix effect (ME) evaluation in LC-MS/MS.

The limit of detection (LOD) for GC-MS/MS was calculated statistically as LOD = (3.3 * SD)/S, where SD is the standard deviation of the response, and S is the slope of the calibration curve. The LOD for LC-MS/MS was empirically determined at the lowest level of calibrator (1 µg/L), where the signal-to-noise (S/N) ratios were approximately 3.

Selectivity was evaluated using blank urine from six different sources, and carry-over was monitored *via* solvent blanks injected after every five samples.

Results and Discussion

Extraction method recovery and matrix effect

The extraction recovery and matrix effect results for the target NPS are summarized in Table I. Recovery rates for the GC-MS/MS method ranged from 61.23% (MDPV) to 90.08% (4-MEC), whereas the LC-MS/MS method demonstrated higher recoveries ranging from 97.77% to 99.74%. Matrix effect evaluations (Table I) showed minimal interference for the majority of compounds, though significant ion suppression was observed for MDPB in the LC-MS/MS method (51.30%). Due to low recovery and insufficient reproducibility in the GC-MS/MS trials, 4-FMC was excluded from further analysis within that platform.

Analytical method validation

The two distinct chromatographic methods, GC-MS/MS and LC-MS/MS, were developed and validated for the sensitive and specific quantification of NPS in human urine. The validation process, originally based on SWGTOX guidelines, was subsequently updated to align with ANSI/ASB Standard 036 (2019) and included a rigorous assessment of several key parameters: selectivity, carry-over, calibration curve, lower limit of quantification (LLOQ), accuracy and preci-sion, recovery, matrix effect, dilution integrity, and stability (21, 22). While statistical precision was evaluated at one concentration level, the method's suitability for clinical implementation is fully discussed as a current limitation. The triple quadrupole MS/MS conditions were optimized to provide the best sensitivity and specificity for each target NPS. Table II presents the optimized parameters for the quantification of the five-target NPS by GC-MS/MS and LC-MS/MS. The tables show the actual Multiple Reaction Monitoring (MRM) transitions (m/z) used for each compound and their retention times (tR). The table shows the fragmentation patterns employed in the GC-MS/MS method to obtain high selectivity and sensitivity. Similarly, for the LC-MS/MS method, it displays the precursor and product ion pairs that were selected for strong and reliable detection. The data in these tables are valuable for the identification and quantification of these compounds in the analysed samples

Table I

Recovery and matrix effect of GC-MS/MS and LC-MS/MS Methods for NPS in urine

	GC-MS/MS		LC-MS/MS	
	Extraction recovery (%)	Matrix effect (%)	Extraction recovery (%)	Matrix effect (%)
25-NBOMe	88.70	98.82	97.77	120.20
4-MEC	90.08	94.14	99.01	96.72
α-PVP	77.69	112.19	99.74	90.44
MDPB	78.94	96.95	99.50	51.30
MDPV	61.23	111.05	98.66	94.87
4-FMC	-	-	98.38	97.75

Table II

Optimized GC-MS/MS and LC-MS/MS parameters for NPS quantification

Parameters GC-MS/MS	**25-NBOMe**	**4-FMC**	**4-MEC**	**α-PVP**	**MDPB**	**MDPV**
Transition 1 (quantifier)	150→91	-	72→ 44	126→96	112→55	126→97
CE(V) Transition 1	20	-	20	30	20	20
Dwell (ms) Transition1	25	-	100	25	25	25
Transition 2 (qualifier)	121→91	-	119→91	126→84	112→70	126→84
CE(V) Transition 2	20	-	20	20	20	20
Dwell (ms) Transition 2	25	-	25	25	25	25
Retention times (t_R)	13.45	-	6.57	8.42	10.49	10.90
Electron Energy (eV)	70 eV					
Injection volume	1 µL					
Parameters LC-MS/MS	**25-NBOMe**	**4-FMC**	**4-MEC**	**α-PVP**	**MDPB**	**MDPV**
Transition 1 (quantifier)	336→91.1	182.1→164.1	192.1→174.1	232.2→ 91.1	262.1→112.1	276.2→126.1
CE(V) Transition 1	50	10	10	20	24	28
Transition 2 (qualifier)	336→121	182.1→149.1	192.1→144.1	232.2→77.1	262.1→161.1	276.2→175
CE(V) Transition 2	18	20	32	56	20	20
Fragmentor (V)	105	295	75	93	108	80
Retention times (t_R)	5.49	4.35	4.78	4.93	4.66	4.96
Polarity	+	+	+	+	+	+
Injection volume	5 µL					

Selectivity and carry-over

No endogenous interferences were observed at the specific retention times of the target NPS in any of the blank urine sources tested. Additionally, the absence of detectable peaks in the interspersed solvent blanks confirmed that the workflow is free from carry-over.

Calibration curve

The linearity of the method was established by analyzing five NPS: α-PVP, 4-MEC, MDPV, MDPB, and 25-NBOMe, at five concentration levels (200 - 1000 µg/L) in duplicate analytical runs using GC-MS/MS, with each replicate subjected to a single instrumental reading (n = 2 total injections *per* level). The statistical summary of the regression analysis is presented in Table III. The models were characterized by good linearity and high coefficients of determination (R^2) ranging from 0.9908 (MDPV) to 0.9982 (MDPB). The high adjusted R^2 values also supported the fact that the models fit the data well.

All 5 regression models were statistically significant, and p-values were well below the significance level of 0.05. This is further supported by the high F-values of the ANOVA, ranging from 323.58 for MDPV to 1655.03 for MDPB. The slopes of the calibration curves were different, with the lowest for 25-NBOMe (23.53) and the highest for 4-MEC (128.91). This difference suggests that 4-MEC produced the largest change in signal for a given change in concentration, while 25-NBOMe produced the smallest.

Table III

Statistical analysis of calibration models for NPS by GC-MS/MS

	R-Square (R^2)	*Std. Error*	*Intercept*	*p-Value*	*Slope*	*p-Value*	*F (ANOVA)*	*Significance F*
25-NBOMe	0.9978	405.23	-4482.40	1.82E-03	23.53	4.44E-05	1349.09	4.44E-05
4-MEC	0.9968	2671.95	-5244.70	1.58E-01	128.91	7.73E-05	931.11	7.73E-05
α-PVP	0.9919	2637.33	-1783.70	5.65E-01	80.08	3.08E-04	368.81	3.08E-04
MDPB	0.9982	647.81	-350.70	6.41E-01	41.67	3.27E-05	1655.03	3.27E-05

	R-Square (R^2)	*Std. Error*	*Intercept*	*p-Value*	*Slope*	*p-Value*	*F (ANOVA)*	*Significance F*
MDPV	0.9908	1758.85	-2182.50	3.22E-01	50.03	3.75E-04	323.58	3.75E-04

For the LC-MS/MS method, linearity was validated by analyzing six NPS (α-PVP, 4-FMC, 4-MEC, MDPV, MDPB, and 25-NBOMe) in duplicate at six concentration levels, ranging from 1 to 100 µg/L. The statistical summary of the regression analysis is detailed in Table IV. The linearity of the models for all six compounds was excellent, with coefficients of determination (R^2) ranging from 0.9946 for 25-NBOMe to 0.9999 for 4-FMC. The slopes of the calibration curves were different; 25-NBOMe showed the lowest slope (12290.34), and 4-MEC the highest (16549.83). All regression models were statistically significant with p-values ranging from 1.09E-05 to 4.07E-07, suggesting a strong correlation between analyte concentration and instrument response. The high F-values of the ANOVA also indicated the significance of the models.

Table IV

Statistical analysis of calibration models for NPS by LC-MS/MS

	R-Square (R^2)	*Std. Error*	*Intercept*	*p-Value*	*Slope*	*p-Value*	*F (ANOVA)*	*Significance F*
25-NBOMe	0.9946	38253.45	-17481.43	4.57E-01	12290.34	1.09E-05	740.21	1.09E-05
4-FMC	0.9999	18864.79	-9281.56	4.25E-01	14366.03	3.46E-07	4158.53	3.46E-07
4-MEC	0.9994	17353.79	-10146.06	3.52E-01	16549.83	1.41E-07	6521.81	1.41E-07
a-PVP	0.9995	9686.96	-5292.83	3.81E-01	10271.96	9.22E-08	8063.08	9.22E-08
MDPB	0.9993	7800.07	-4934.62	3.18E-01	7065.59	1.73E-07	5883.96	1.73E-07
MDPV	0.9990	10500.98	-6607.94	3.20E-01	7679.31	4.07E-07	3834.91	4.07E-07

The performance of both methods, GC-MS/MS and LC-MS/MS, was tested at the top of their calibration ranges, respectively. For GC-MS/MS, a standard concentration of 1000 µg/L was measured in the range between 918.86 µg/L (4-MEC) and 1091.07 µg/L (α-PVP). For the LC-MS/MS method, the upper limit was set at a concentration of 100 µg/L. Mean measured values ranged from 83.44 µg/L (4-FMC) to 120.25 µg/L (25-NBOMe), exhibiting different levels of accuracy. The relative standard deviation (RSD) values were low (0.89 - 4.58%), indicating good precision. The calculated bias for these samples ranged from -16.56% (4-FMC) to 20.25% (25-NBOMe).

LOD

The LOD values for the two analytical platforms showed different sensitivity profiles *per* compound. LOD values for the GC-MS/MS method were statistically calculated from the regression parameters obtained in the validation protocol. The limits obtained were 56.82 µg/L for 25-NBOMe, 68.40 µg/L for 4-MEC, 108.68 µg/L for α-PVP, 51.30 µg/L for MDPB, and 116.02 µg/L for MDPV. These variations are attributed to the specific ionization efficiencies and fragmentation patterns of the NPS subclasses under electron ionization (EI) conditions. On the other hand, the LC-MS/MS method provided a dramatic increase in sensitivity with a functional LOD of 1 µg/L for all the target analytes. At this concentration, signal-to-noise (S/N) requirements for both detection and quantification were always fulfilled, indicating the high efficiency of the electrospray ionization (ESI) source and the dynamic MRM approach for these compounds in urine matrices. While the theoretical LOD could be lower based on S/N ratios, 1 µg/L was selected as the functional limit to ensure maximum reliability at the lowest point of the calibration curve.

LOQ

The Limit of Quantification (LOQ) was validated at nominal concentrations of 200 µg/L for the GC-MS/MS method and 1 µg/L for the LC-MS/MS method. These functional limits were validated based on a Coefficient of Variation (CV) below 20%, and a Signal-to-Noise ratio (S/N) ≥ 10. For the LC-MS/MS method, the LOD and LOQ approach is at 1 µg/L, as this concentration represents the lowest level validated with the precision and accuracy necessary for clinical applications. The experimentally determined LOQ values for the six types of NPS are comprehensively presented in Table V. The established concentrations fall well within the linearity range of the analytical method, which confirms that analyte quantitation is performed with demonstrated precision and accuracy.

Table V

Assessment of limits of quantification for the six types of NPS by GC-MS/MS and LC-MS/MS

Compound	**GC-MS/MS (200 µg/L)**			**LC-MS/MS (1 µg/L)**		
	LOQ, µg/L	SD, µg/L	RSD, %	LOQ, µg/L	SD, µg/L	RSD, %
25-NBOMe	257.21	16.91	6.78	1.16	0.11	9.18
4-MEC	201.09	9.02	4.50	1.30	0.13	10.33

Compound	GC-MS/MS (200 µg/L)			LC-MS/MS (1 µg/L)		
	LOQ, µg/L	SD, µg/L	RSD, %	LOQ, µg/L	SD, µg/L	RSD, %
α-PVP	210.90	13.60	6.59	1.32	0.10	7.95
MDPB	163.38	10.00	6.13	1.32	0.24	16.24
MDPV	177.64	5.30	2.93	1.24	0.08	6.76
4-FMC	-	-	-	1.02	0.05	5.29

Note: LOQ = limit of quantification; SD = standard deviation; RSD = relative standard deviation. "–" indicates not determined

LC-MS/MS consistently gave lower LOQs, indicating better sensitivity. The RSD values of most analytes were within the recommended ranges, which indicated the reliability of the two methods. The higher RSD obtained for MDPB by LC-MS/MS may be due to compound-specific analytical difficulties. Significantly, 4-FMC was detected only by LC-MS/MS, which showed high sensitivity and precision. In conclusion, the obtained results confirm the applicability of both methods for the quantification of new psychoactive substances, with LC-MS/MS being more sensitive.

Precision and accuracy

Intra-day precision (repeatability) and accuracy were determined on six replicates of fortified urine samples prepared and analyzed on the same day. Each replicate was analyzed by a single instrumental reading (n = 6 total injections *per* level). At concentration levels of 1000 µg/L (for GC-MS/MS) and 100 µg/L (for LC-MS/MS), both methods demonstrated high repeatability with low coefficients of variation (CV) and acceptable bias (Table VI).

Table VI

Analytical precision and accuracy for NPS methods

Compound	GC-MS/MS				LC-MS/MS			
	Mean, µg/L	SD, µg/L	CV, %	BIAS, %	Mean, µg/L	SD, µg/L	CV, %	BIAS, %
25-NBOMe	1004.07	6.00	0.60	0.40	120.25	1.07	0.89	20.25
4-MEC	918.86	3.04	0.33	-8.17	97.95	2.11	2.15	-2.05
α-PVP	1091.07	4.58	0.42	9.20	105.74	4.84	4.58	5.74
MDPB	960.36	9.50	0.99	-3.95	112.09	3.72	3.31	12.09
MDPV	1081.13	8.02	0.74	8.17	109.08	2.84	2.60	9.08
4-FMC	-	-	-	-	83.44	2.09	2.51	-16.55

Note: SD = standard deviation; CV = coefficient of variation; BIAS = percent deviation from the true or expected value. "–" indicates not determined

The intermediate precision (reproducibility) of the method was evaluated by analyzing three independent replicates over 3 consecutive days by 2 different analysts. To ensure method reliability, analysts prepared fresh solutions and mobile phases independently each day, with each replicate subjected to a single instrumental reading (totaling n = 18 readings *per* concentration level across the study). For this assessment, concentration levels of 500 µg/L (for GC-MS/MS) and 25 µg/L (for LC-MS/MS) were utilized (Table VII).

Table VII

Analytical precision and accuracy for NPS methods

Compound	GC-MS/MS				LC-MS/MS			
	Mean, µg/L	SD, µg/L	CV, %	BIAS, %	Mean, µg/L	SD, µg/L	CV, %	BIAS, %
25-NBOMe	648.39	7.83	1.21	29.68	24.48	0.60	2.46	-2.23
4-MEC	445.64	3.40	0.76	-10.87	25.49	0.28	1.11	0.22
α-PVP	444.46	6.56	1.47	-11.11	27.63	0.35	1.28	7.60
MDPB	354.14	1.60	0.45	-29.17	29.74	0.34	1.13	16.32
MDPV	351.71	6.61	1.88	-29.66	28.19	0.31	1.11	10.57
4-FMC	-	-	-	-	22.47	0.42	1.86	-11.25

Note: SD = standard deviation; CV = coefficient of variation; BIAS = percent deviation from the true or expected value. "–" indicates not determined

Both GC-MS/MS and LC-MS/MS methods consistently delivered high precision, with coefficients of variation (CV) well below 2.5%, indicating the reliability of the techniques for routine applications.

In terms of accuracy, some GC-MS/MS analyzed compounds showed bias values within generally acceptable ranges, with several analytes (α-PVP, 4-MEC) with bias < 20%. The higher bias for some compounds (MDPB and MDPV) in the intermediate precision study is most likely due to compound-specific thermal stability problems during GC injection. However, this does not detract from the method's general applicability to qualitative and quantitative NPS screening. The coefficients of variation and bias values were within the acceptance criteria as *per* the LC-MS/MS guideline. This indicates that the

LC-MS/MS method can provide accurate and precise measurements for most of the target compounds. In conclusion, these outcomes show that both approaches are reliable and accurate methods for the quantification of these novel psychoactive substances within established bioanalytical guidelines for the majority of results.

Accuracy

The accuracy of the analytical methods was characterized by assessing the measurement bias, defined as the systematic deviation between the observed mean concentration and the analyte's certified reference value. While acceptable performance generally mandates that the calculated bias remains within the ± 20% limits specified by specialized guidelines.

The results demonstrated that the LC-MS method exhibited significantly better compliance with the established quality requirements compared to the GC-MS/MS method (Table VI and Table VII), particularly for the cathinone subclass.

Robustness

The robustness of the analytical methods was evaluated by the analysis of the analytical response under varying operational conditions, specifically through the assessment of intermediate precision (multi-day and multi-analyst trials). These replicated trials systematically evaluated clinical parameters, including external conditions such as sample homogenization, accurate pipetting, and mixing with the extraction solvent. The extraction procedure for LC-MS was far more robust than the one for GC-MS (Table VI and Table VII).

System suitability and Analyte stability

The system suitability was evaluated by the analysis of the variability of the retention times (tR) and the response reproducibility of the prepared calibration standards. The retention times recorded across multiple analytical days were highly stable, exhibiting a maximum difference of 0.05 minutes, which is safely within the ± 2% limits specified by the guidelines. The stability of the analytes was also assessed by analyzing processed extracts and fortified matrix samples under different storage conditions (benchtop and autosampler stability). The results confirmed the linearity of the method and the analyte concentrations, indicating no significant degradation of the target NPS analytes during the analytical timeframe.

Comparative performance analysis of GC-MS/MS and LC-MS/MS methods for NPS detection

The strong performance features discussed in the previous sections illustrate the practical utility of these methods for routine high-throughput screening. The ever-increasing influx of NPS increasingly challenges the development of rapid and comprehensive analytical methods in clinical toxicology laboratories.

Gas chromatography-tandem mass spectrometry (GC-MS/MS) has been used for synthetic cathinones (SCs), but its application is often hindered by extensive sample preparation and frequent need for analyte derivatization. Such procedures are often necessary to achieve the analytical sensitivity and selectivity required, as exemplified by the work of Shih P-H *et. al.* for phenethylamines (23).

Alexandridou *et al.* also report the same approach, who, using acetylated and silylated derivatization agents, managed to achieve reliable and fast quantification of five NPS in blood and detection of six NPS in urine. The method was tested for robustness by means of an intra-laboratory comparison (24). However, the development of a validated GC-MS/MS method in addition to LC-MS/MS is essential for analytical redundancy and adds to the overall versatility of the laboratory.

GC-MS/MS remains the workhorse in many clinical settings, due to lower operational costs and wider availability (25). The presence of a validated, derivatization-free GC protocol thus offers a readily available alternative for qualitative screening and confirmation in laboratories without high-end LC-MS/MS instrumentation. The GC-MS/MS method developed in this study complies with the validation protocol proposed by Denia *et al.,* who recommend implementing a GC method for the determination of NPS by removing the derivatization step. The study was evaluated for other complex biological matrices, such as oral fluids, and provides a simplified workflow that maintains analytical integrity and optimizes operational speed for rapid toxicological assessment (26).

Although historically derivatization was the method of choice to convert polar, non-volatile amines into volatile derivatives suitable for GC (27) and remains an optional technique recommended by the UNODC (28) to mitigate challenging chromatographic behavior such as peak broadening, the present method avoids these laborious steps while maintaining analytical integrity. Most of these limitations have been overcome in recent years with the introduction of LC-MS/MS, allowing simpler sample preparation methods (liquid-liquid extraction or direct injection without aqueous phase removal) (17). Unlike the "dilute and shoot" approaches, the LLE protocol used in this study was able to effectively separate the NPS analytes from endogenous urinary compounds, such as inorganic salts and highly polar urea, which usually compete for charge during Electrospray Ionization (ESI). This is in line with the approach of Dresen *et. al.* (29), who also used LLE to enhance the detection sensitivity for low concentration levels, but whose screening procedure is still considerably more time-consuming due to mandatory evaporation and reconstitution steps.

Furthermore, our method is more sensitive than the work of Chen *et. al.,* who performed similar LLE extraction from urine samples. The protocol they used reported a LOQ range of 1 to 50 ng/mL. The present method achieved a much narrower and refined LOQ

value of 1 ng/mL, which ensures high precision for trace-level detection (30).

The use of MS/MS or multiple reaction monitoring (MRM) provides characteristic fragment ions, and the enhanced chromatographic separation provides a substantial increase in specificity, making LC-MS/MS a powerful tool for the analysis of difficult isomers or co-eluting compounds (13, 31, 32). Such a parameter might be required for the purpose of establishing the validated quantitative framework, as suggested by Ambach *et. al.,* especially when a limited analyte panel is required or for specific high-priority cases (33).

A limitation of the current study is the lack of internal standards for the GC-MS/MS framework, which is mainly due to the limited commercial availability of isotopically labeled standards for these particular emerging NPS. However, such limitations are often encountered in NPS screening, and methodological integrity was preserved through the use of a strict external calibration protocol as described in the methodology (27).

This dual-platform approach was deliberately designed to exploit the complementary nature of the two techniques, an approach also performed in previous studies to maximize compound coverage and cross-platform verification (34, 35). GC-MS/MS offers a different fragmentation pattern and retention behavior as an independent confirmatory tool to provide higher forensic and clinical confidence of the results, whereas LC-MS/MS offers better sensitivity for trace analysis. This dual-approach avoids the risk of false negatives, as demonstrated in earlier single-platform studies (18), by offering a robust secondary confirmation if an analyte (4-FMC) is thermally unstable for GC. Moreover, the LC-MS/MS method showed excellent robustness for all critical analytical parameters determined in this study. Moreover, the increased sensitivity (LOQ of 1 µg/L for all NPS analyzed, compared to LOQ of 200 µg/L for the GC-MS/MS method) is a clear advantage. This lower LOQ is required to detect the trace concentrations of NPS typically found in biological samples, which exceeds several traditional screening methods that lack sensitivity to detect high-potency molecules such as 25-NBOMe. Although the LOQ for the GC-MS/MS is higher than that of the LC-MS/MS, it is still of clinical relevance. For acute intoxications from synthetic cathinones, urine concentrations are often found within the range that our GC method can detect (36), but the LC-MS/MS platform is sensitive enough to be used in chronic use or trace level screening.

The LC-MS/MS method was more robust and precise than GC-MS/MS for routine analysis, in agreement with previous reports (37). The latter's significant bias for compounds such as 25-NBOMe and MDPV suggests a relative lack of accuracy documented in other high-potency synthetic drug assays. Moreover, the low recovery of 25-NBOMe is consistent with previous data (25, 37) and confirms that its detection still poses a major analytical challenge, even in the transition between different biological matrices.

Clinical implications and future perspectives

The present study, in which urine was used as the main matrix, improves the practical applicability of these validated methods in cases of acute intoxication. Although blood analysis is the gold standard for correlating drug concentrations with immediate clinical impairment, urine provides a critical diagnostic advantage due to its extended detection window and the usually higher concentrations of NPS metabolites, as also reported by Pardo Marin *et. al.* (38). Biotransformation is an important mechanism in the elimination of NPS, however identification of parent compounds in urine remains a major approach in forensic and clinical toxicology. This study highlights the persistence of these parent compounds that allows a definitive toxicological confirmation even if patients arrive several hours after ingestion.

Similar to other clinical trends, routine toxicological screening was only performed in 15% of emergency presentations with acute drug toxicity in the Euro-DEN Plus project data (2013 to present), which is representative of the standard practice in European hospitals (39). In these real-world conditions, NPS detection was only possible by mass spectrometry (MS). Immunoassays did not detect these substances even in the case of self-reported use. The incorporation of these specific NPS: α-PVP, 4-MEC, MDPV, MDPB, 25-NBOMe, and 4-FMC in our current analytical scope directly addresses these diagnostic gaps and highlights the clinical importance of the method.

Also, although symptomatic measures are generally sufficient for adequate patient management, particular compounds have a great importance for early detection from a public health perspective. This is particularly the case when new substances emerging on the market lead to localized clusters of intoxications, where rapid analytical confirmation is critical to inform emergency response. Such cases have been reported by Grafinger *et. al.,* who reviewed the clinical implications of NPS (40).

This study is the first in the regional context of Romania to present a validated dual platform framework as a practical tool for the clinician to bridge the gap between clinical suspicion and analytical proof. This 'fit-for-purpose' approach, however, guarantees that the procedures are primed for seamless integration in routine diagnostic workflows where rapid turnaround is prioritized. Forensic samples must be rigorously validated for evidentiary purposes, while clinical toxicology is focused on the rapid identification of analytes to guide patient care (37, 41).

Conclusions

The present study successfully developed two complementary analytical platforms for the detection of

α-PVP, 4-MEC, MDPV, MDPB, 25-NBOMe, and 4-FMC in urine, each optimized to its own instrumental strengths. Although the LC-MS/MS method showed enhanced quantitative rigor with higher sensitivity and less bias, the GC-MS/MS platform offers a useful alternative analytical route. The use of these different but complementary modalities allows laboratories to have complete diagnostic coverage and methodological integrity. In the end, the combination of both platforms offers a powerful, scalable solution that can satisfy the changing needs of clinical toxicological casework.

One limitation of this study is the lack of authentic clinical specimens, as no patients suspected of having consumed these specific NPS were encountered during the period of analytical development. However, the rigorous validation in fortified matrices according to ANSI/ASB Standard 036 confirms that this methodological framework is ready for the next step in research: the prospective application to real clinical cases.

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Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki. This study was conducted in strict adherence to ethical guidelines, with approval from the institutional review board, and all patient data were rigorously anonymized to ensure confidentiality. Informed consent was obtained from all subjects involved in the study.

AI use disclosure

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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