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Simultaneous LC-MS/MS screening for multiple phenethylamine-type conventional drugs and new psychoactive substances in urine

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ABSTRACT

New psychoactive substances are being launched in the drug market at a rapidly growing pace. More than 950 new psychoactive substances have been reported to the United Nations Office on Drugs and Crime. The development of new psychoactive substance abuse has drawn risks on public health and safety. Phenethylamines, along with other stimulants, accounted for the majority of the new psychoactive substances being reported in the past decade. This study presents a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous screening of 74 conventional and artificial phenethylamines in urine samples. The chromatographic analysis was performed by a direct dilute-and-shoot procedure using a Phenomenex Kinetex® Phenyl-Hexyl column (10 cm × 2.1 mm i.d., 1.7 μm) and two mobile phases (A: 0.1% formic acid aqueous solution with 5 mM ammonium acetate, B: 0.1% formic acid methanolic solution). The mass fragments were collected under the multiple reaction monitoring mode. The linearity range located in 1.0–50.0 ng/mL for quantitative analysis. The limit of detection and lower limit of quantification for 74 phenethylamines were 0.5 ng/mL and 1.0 ng/mL, respectively. The method was validated and further applied to analyze authentic urine samples. Twenty samples were tested positive of seven phenethylamines from 67 samples, whereas the contents detected were 9.8 ng/mL to 147.1 μg/mL with dilution factors of 40 to 20,000 folds.

1. Introduction

In the past two decades, new psychoactive substances (NPS) have emerged in drug markets worldwide. Synthetic NPS (also known as designer drugs) including synthetic cannabinoids, synthetic cathinones, and phenethylamines, etc. mimic the properties of substances already scheduled for international control [1,2]. These synthetic compounds are created through substituent replacement or isomerization of the rudimentary structure with a common backbone, leading to a rapid renewal and short shelf time in the drug market [3]. From 2005 to 2019, the number of substances reported to the United Nations Office on Drugs and Crime (UNODC) grew from 166 to more than 950 [2]. The stimulants, such as synthetic cathinones and phenethylamines, accounted for the majority of the reported NPS from 2009 to 2019 [2].

Substituted phenethylamines (or simply phenethylamines, PEAs) are alkaloid-like stimulants functioning as indirect dopamine agonists in central nervous system (CNS) synapses that lead to stimulant effects in human bodies [4]. These stimulants mimic the endogenous catecholamine neurotransmitters of the sympathetic nervous system, such as dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline) [4]. The amphetamine-derived PEAs such as amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxymethamphetamine (MDMA/ecstasy) are widely abused and controlled under the 1971 Convention on Psychotropic Substances of United Nations [5,6].

The PEA-type stimulants are divided into several groups, including conventional PEAs (e.g., 3,4-methylenedioxy-*N*-ethylamphetamine, MDEA; MDMA; *N*-methyl-α-ethyl-3,4-methylenedioxyphenethylamine, MBDB), mescaline-derived compounds (e.g., 3,4,5-trimethoxyamphetamine, TMA isomers), and more recent compounds [7,8]. The more recent PEAs are further classified into various types such as the 2C series (e.g., 4-bromo-2,5-dimethoxyphenethylamine, 2C-B), D series (e.g., 2,5-dimethoxy-4-chloroamphetamine, DOC; 2,5-dimethoxy-4-iodoamphetamine, DOI; 2,5-dimethoxy-4-methylamphetamine, DOM), benzodifurans (e.g., 8-bromo-2,3,6,7-benzo-dihydro-difuran-ethylamine, 2-C-B-Fly), and MDMA analogs (e.g., *p*-methoxymethamphetamine, PMMA) created in the 1960s, had become prevalent worldwide [9–11]. Cases of severe

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intoxication caused by PEA-type substance abuse have been reported. Overdose of PEAs has documented physical and mental effects such as hypertension, hyperthermia, hallucinations, agitation, aggression, dissociation, attention deficit hyperactivity disorder, liver and kidney failure, and even death [1,5,12,13].

Forensic analysis of abusive substances in biological samples could be carried out by immunoassays and chromatographic methods for screening and confirmation in clinical practices [14]. However, immunoassays have drawbacks such as false negatives/positives and the inability to distinguish among the ever-changing NPS [15–17]. Therefore, sensitive and specific methods, including chromatography coupled with mass spectrometry (MS) under ion monitoring mode, are indispensable for further confirmation. The selectivity and sensitivity for detecting target analytes are enhanced by appointing ions of the analytes, whereas the noise from non-target components is reduced [18]. Accordingly, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) have become effective analytical techniques frequently adopted in forensic and clinical toxicology applications for detecting abused substances [11,19].

The chromatographic methods for determining multiple NPSs in bio-samples (e.g. blood and urine) established in recent years (2013–2020) have been reviewed [11,19]. Compared to GC-MS, LC-MS/MS is superior in terms of sensitivity, selectivity, and adaptability, making it a robust tool in forensic analysis [20,21]. However, several deficiencies, including limited coverage (5–34 PEA targets), lack of fatal substances (e.g. *N,N*-DMA, PMA and PMMA), or higher LODs/LOQs (up to 200 ng/mL), were observed from the established LC-MS/MS methods of PEA urinalysis [11,19,21–24]. In addition, given the increasing prevalence of PEAs, there is a need for new analytical methods for detecting more PEAs simultaneously. This study aimed at establishing a screening method to determine 74 PEAs in urine using LC-MS/MS. The method is validated and applied to authentic urine samples collected in Taiwan.

2. Materials and methods

2.1. Reagents

The sources of 74 standards and 10 internal standards (IS) were obtained from five different vendors and the information in detail is listed in Table 1. The standards synthesized by GreenChem were commissioned by Taiwan Food and Drug Administration (TFDA) and have been identically assessed via NMR, HRMS, and FT-IR, whereas the purity for all items are above 95%. LC-MS grade water was purchased from Scharlau (Barcelona, Spain). Artificial urine was purchased from UTAK Laboratories, Inc. (Valencia, CA, USA). A total of 67 authentic urine samples were provided by local law enforcement agencies of Taiwan. The authentic urine samples were stored at −20 °C and acclimated to controlled room temperature before analysis. The sampling of urine specimens in this study followed the regulations of the Ministry of Health and Welfare, Taiwan.

2.2. Instrumentation

The analysis was performed on a Waters Acquity UPLC® system (Waters Assoc., Milford, MA, USA) coupled to an AB SCIEX QTRAP® 5500 mass spectrometer with an electrospray ionization (ESI) source (Applied Biosystems, MDS Sciex, Concord, Ontario, Canada). The analytes were detected by mass spectrometer under the multiple reaction monitoring (MRM) mode. A Phenomenex Kinetex® Phenyl-Hexyl column (10 cm × 2.1 mm i.d., 1.7 µm) was applied in the chromatographic analysis, which was performed at 40 °C with gradient elution at a constant flow rate of 0.3 mL/min using mobile phase A (0.1% formic acid aqueous solution with 5 mM ammonium acetate) and mobile phase B (0.1% formic acid methanolic solution). The gradient elution program was as follows: 0.5–1.0 min 5–30% B, 1.0–1.5 min 30% B, 1.5–2.0 min 30–37% B, 2.0–2.5 min 37% B, 2.5–2.6 min 37–40% B, 2.6–3.0 min 40% B, 3.0–5.0 min 40–46% B, 5.0–5.5 min 46% B, 5.5–8.0 min 46–50% B, 8.0–9.5 min 50–65% B, 9.5–10.5 min 65–100% B. The total run time of chromatographic analysis was 10.5 min. The sample injection volume was 3 µL. Immediately after each sample injection, the needle was rinsed with methanol and water alternately. The ion source of the mass spectrometer was set as follows: ESI in positive mode; ion spray voltage, 5.5 kV; temperature, 550 °C; curtain gas pressure, 30 psi; collision gas pressure, medium level; ion source gas, 50 psi.

2.3. Preparation of standard solutions

Standards of the 74 target PEAs and 10 IS were individually dissolved in methanol to reach a concentration of 1.0 mg/mL and 0.1 mg/mL, respectively, to prepare the stock solutions. The working solution consisting of 74 standards and 10 IS was prepared by diluting the stock solutions with 50% methanol aqueous solution to the concentration of 500 ng/mL. All stock and working solutions were stored at −20 °C and acclimated to controlled room temperature before use.

2.4. Pretreatment of urine samples

Urine samples were analyzed using a dilute-and-shoot procedure without any purification. The raw urine sample was centrifuged at 3000*g* for 5 min, and the supernatant was collected. .A mixture of 20 µL supernatant and 20 µL IS working solution (500 ng/mL) was diluted with 50% methanol aqueous solution to the volume of 1 mL. Prior to analysis, the mixed solution was passed through a 0.22 µm PVDF filter. Drug-free urine (DFU) consisted of artificial urine without spiking any target analyte was used as the blank matrix and negative control.

2.5. Validation of the method

To ensure the reliability and feasibility of the present method, validation was carried out following guidelines from “Working Group for Forensic Toxicology Standard Practices (SWGTOX) for Method Validation in Forensic Toxicology” and “Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Specimens” [25,26]. The method was validated in terms of carryover, selectivity, linearity, sensitivity, matrix effects, precision, and accuracy. To evaluate the carryover, DFU was injected (n = 3) immediately after analyzing urine samples spiked with the concentration of the highest calibrator (50 ng/mL) and then calculated the response ratio of residual to LOQ. The acceptable carryover is less than 20% which is regarded ignorable. Selectivity was evaluated by analyzing different DFU samples (n = 20) to ensure that no interfering peaks appeared during the target analysis. Acceptable selectivity was defined by the absence of evident interfering signals from the matrix at retention times near that of characteristic ions for the target.

The linearity was assessed using standard solutions of 74 PEA targets (n = 3) at six concentration levels in series, including 1, 5, 10, 20, 25, and 50 ng/mL (IS of 10 ng/mL included). The peak area ratio of standard/IS was plotted versus the concentration of standard. The least-square method was applied, and a correlation coefficient r higher than 0.995 was considered acceptable. Sample quantification was carried out using the calibration curve based on the IS method in the established linear range. The acceptable ranges for qualitative and quantitative determination were in reference of the criteria set up by TFDA (relative ion ratio > 50%, RSD ± 20%; relative ion ratio 20–50%, RSD ± 25%; relative ion ratio 10–20%, RSD ± 30%; relative

Table 1
MRM parameters for 74 target analytes and 10 IS of phenethylamines.

Item	Analyte	CAS No.	Formula	Molecular weight (g/mol)	Source of drug	Retention time (min)	Ion pairs	DP (V)	CE (eV)	Corresponding internal standard
							Precursor (*m/z*) > Product (*m/z*)			
1	amphetamine	2706-50-5	C9H13N	135.2	a	2.24	136 > 91*	39	21	amphetamine-d8
							136 > 119	39	12	
2	phentermine	1197-21-3	C10H15N	149.2	a	2.61	150 > 65*	38	52	phentermine-d5
							150 > 133	38	14	
3	4-MA (4-methylamphetamine)	41632-56-8	C10H15N	149.2	b	2.94	150 > 105*	42	26	amphetamine-d8
							150 > 133	42	12	
4	methamphetamine	300-42-5	C10H15N	149.2	a	2.39	150 > 119*	40	14.5	methamphetamine-d8
							150 > 65	40	55.5	
5	4-FA (4-fluoroamphetamine)	64609-06-9	C9H12FN	153.2	b	2.43	154 > 109*	40	28	amphetamine-d8
							154 > 137	40	15	
6	N,N-DMA (N,N-dimethylamphetamine)	33286-27-0	C11H17N	163.2	b	2.49	164 > 91*	57	30	MDA-d5
							164 > 119	57	18	
7	N-ethylamphetamine	16105-78-5	C11H17N	163.2	b	2.68	164 > 119*	68	16	MDA-d5
							164 > 65	68	56	
8	5-AEDB (5-(2-aminoethyl)-2,3-dihydrobenzofuran)	796869-33-5	C10H13NO	163.2	d	2.21	164 > 147*	17	15	5-APB-d5
							164 > 91	17	34	
9	PMA (4-methoxyamphetamine)	3706-26-1	C10H15NO	165.2	b	2.46	166 > 121*	39	25	amphetamine-d8
							166 > 91	39	42	
10	4-FMA (4-fluoromethamphetamine)	52063-62-4	C10H14FN	167.2	b	2.59	168 > 109*	50	30	amphetamine-d8
							168 > 83	50	52	
11	FPBA (1-(4-fluorophenyl)butan-2-amine)	23292-09-3	C10H14FN	167.2	b	2.88	168 > 109*	47	28	5-APB-d5
							168 > 83	47	51	
12	4-CA (4-chloroamphetamine)	3706-38-5	C9H12ClN	169.7	b	3.24	170 > 125*	40	31	5-APB-d5
							170 > 153	40	15	
13	5-APB (5-(2-aminopropyl)benzofuran)	286834-80-8	C11H13NO	175.2	d	2.99	176 > 131*	13	27	5-APB-d5
							176 > 159	13	12	
14	5-APDB (5-(2-aminopropyl)-2,3-dihydrobenzofuran)	152623-94-4	C11H15NO	177.2	d	2.57	178 > 161*	13	15	5-APB-d5
							178 > 133	13	27	
15	PMMA (para-methoxymethamphetamine)	3398-68-3	C11H17NO	179.3	b	2.62	180 > 78*	33	55	MDA-d5
							180 > 65	33	62	
16	MDA (3,4-methylenedioxyamphetamine)	6292-91-7	C10H13NO2	179.2	b	2.4	180 > 105*	51	27	amphetamine-d8
							180 > 135	51	25	
17	4-FEA (4-fluoroethamphetamine)	3823-31-2	C11H16FN	181.2	b	2.87	182 > 109*	65	31	MDEA-d5
							182 > 137	65	17	
18	4-MTA (4-methylthioamphetamine)	14116-06-4	C10H15NS	181.3	b	3.36	182 > 137*	44	24	DOB-d5
							182 > 115	44	50	
19	4-CMA (4-chloromethamphetamine)	30572-91-9	C10H14ClN	183.7	b	3.42	184 > 125 *	68	31	DOB-d5
							184 > 153	68	16	
20	5-F-2-MOA (5-fluoro-2-methoxyamphetamine)	–	C10H14FNO	183.2	b	2.91	184 > 139*	40	25	amphetamine-d8
							184 > 109	40	14	
21	3-F-4-MOA (3-fluoro-4-methoxyamphetamine)	–	C10H14FNO	183.2	b	2.52	184 > 139*	36	24	amphetamine-d8
							184 > 167	36	35	
22	fenproporex	16397-28-7	C12H16N2	188.3	b	2.43	189 > 119*	40	15	amphetamine-d8
							189 > 65	40	59	
23	5-MAPB (5-(2-methylaminopropyl)benzofuran)	1823925-53-6	C12H15NO	189.2	d	3.14	190 > 131*	27	26	5-APB-d5
							190 > 159	27	16	
24	5-MAPDB (5-(2-methylaminopropyl)-2,3-dihydrobenzofuran)	–	C12H17NO	191.2	d	2.71	192 > 161*	15	19	5-APB-d5
							192 > 133	15	33	
25	PMEA (para-methoxyethylamphetamine)	93963-24-7	C12H19NO	193.3	b	2.88	194 > 149*	42	18	methamphetamine-d8
							194 > 121	42	27	
26	MDMA (3,4-methylenedioxymethamphetamine)	64057-70-1	C11H15NO2	193.2	e	2.57	194 > 163*	54	16	MDMA-d5
							194 > 105	54	36	

(*continued on next page*)

Table 1 (*continued*)

Item	Analyte	CAS No.	Formula	Molecular weight (g/mol)	Source of drug	Retention time (min)	Ion pairs Precursor (*m/z*) > Product (*m/z*)	DP (V)	CE (eV)	Corresponding internal standard
27	DMA (2,5-dimethoxyamphetamine)	2801-68-5	C11H17NO2	195.3	b	2.91	196 > 151*	66	23	amphetamine-d8
							196 > 179	66	14	
28	N-hydroxy-MDA (N-hydroxy-3,4- methylenedioxyamphetamine)	74341-83-6	C10H13NO3	195.2	a	2.88	196 > 163*	34	15	amphetamine-d8
							196 > 135	34	28	
29	2C-D (2,5-dimethoxy-4-methylphenethylamine)	25505-65-1	C11H17NO2	195.3	b	3.39	196 > 179*	51	16	amphetamine-d8
							196 > 164	51	27	
30	5-EAPB (5-(2-ethylaminopropyl)benzofuran)	1823776-22-2	C13H17NO	203.3	d	3.46	204 > 131*	34	29	5-APB-d5
							204 > 159	34	9	
31	MBDB (N-methyl-α-ethyl-3,4-methylenedioxyphenethylamine)	128767-12-4	C12H17NO	207.3	d	3.02	208 > 135*	60	25	5-APB-d5
							208 > 51	60	83	
32	MDDMA (N,N-dimethyl-3,4-methylenedioxyamphetamine)	74341-79-0	C12H17NO2	207.2	d	2.67	208 > 163*	21	19	MDMA-d5
							208 > 135	21	29	
33	MDEA (3,4-methylenedioxy-N-ethylamphetamine)	82801-81-8	C12H17NO2	207.3	b	2.84	208 > 163*	57	17	MDEA-d5
							208 > 135	57	30	
34	MMDA (5-methoxy-3,4-methylenedioxyamphetamine)	60676-84-8	C11H16NO3	209.2	b	2.63	210 > 135*	51	27	amphetamine-d8
							210 > 165	51	26	
35	DOM (4-methyl-2,5-dimethoxyamphetamine)	15588-95-1	C12H19NO2	209.3	b	4.42	210 > 165*	63	24	DOB-d5
							210 > 178	63	25	
36	2C-E (2,5-dimethoxy-4-ethylphenethylamine)	923013-67-6	C12H19NO2	209.2	b	3.79	210 > 193*	68	15	MDA-d5
							210 > 178	68	24	
37	mescaline	832-92-8	C11H17NO3	211.3	b	2.18	212 > 165*	40	30	mescaline-d9
							212 > 180	40	24	
38	4-BA (4-bromoamphetamine)	58400-88-7	C9H12BrN	214.1	d	3.56	214 > 169*	57	27	DOB-d5
							214 > 117	57	40	
39	2C-C (4-chloro-2,5-Dimethoxyphenethylamine)	88441-15-0	C10H14ClNO2	215.7	b	3.49	216 > 184*	39	29	DOB-d5
							216 > 199	39	16	
40	DOET (2,5-dimethoxy-4-ethylamphetamine)	22004-32-6	C13H21NO2	223.3	b	4.97	224 > 179*	61	27	DOB-d5
							224 > 192	61	27	
41	lefetamine	24301-90-4	C16H19N	225.3	b	4.1	226 > 103*	61	41	DOB-d5
							226 > 165	61	38	
42	escaline	3166-82-3	C12H19NO3	225.3	b	2.59	226 > 181*	60	22	mescaline-d9
							226 > 91	60	44	
43	TMA-2 (2,4,5-trimethoxyamphetamine)	1083-09-6	C12H19NO3	225.3	b	2.41	226 > 194*	47	28	25I-NBOMe-d3
							226 > 179	47	34	
44	TMA-6 (2,4,6-trimethoxyamphetamine)	23815-74-9	C12H19NO3	225.3	b	2.69	226 > 209*	72	16	25I-NBOMe-d3
							226 > 181	72	28	
45	TMA (3,4,5-trimethoxyamphetamine)	5688-80-2	C12H19NO3	225.3	c	3.58	226 > 209*	64	14	25I-NBOMe-d3
							226 > 181	64	25	
46	6-Cl-MDMA (1-(6-chloro-1,3-benzodioxol-5-yl)-N-methylpropan-2-amine)	–	C11H14ClNO2	227.7	d	3.36	228 > 197*	33	18	DOB-d5
							228 > 169	33	32	
47	DOC (2,5-dimethoxy-4-chloroamphetamine)	42203-77-0	C11H16ClNO2	229.7	d	3.86	230 > 213*	54	17	DOB-d5
							230 > 155	54	34	
48	benzphetamine	5411-22-3	C17H21N	239.4	a	4.62	240 > 91*	70	36	DOB-d5
							240 > 65	70	77	
49	proscaline	61367-69-9	C13H21NO3	239.3	b	3.53	240 > 181*	23	21	DOB-d5
							240 > 121	23	33	
50	2C-T-2 (2,5-dimethoxy-4-ethylthiophenethylamine)	681160-71-4	C12H19NO2S	241.4	b	4.18	242 > 225*	55	15	DOB-d5
							242 > 91	55	59	
51	3C-P (4-propoxy-3,5-dimethoxyamphetamine)	–	C14H23NO3	253.3	b	3.89	254 > 237*	54	12	DOB-d5
							254 > 107	54	38	
52	2C-T-7 (2,5-dimethoxy-4-n-propylthiophenethylamine)	850140-15-7	C13H21NO2S	255.4	b	5.5	256 > 167*	67	35	DOB-d5
							256 > 224	67	24	

(*continued on next page*)

Table 1 (*continued*)

Item	Analyte	CAS No.	Formula	Molecular weight (g/mol)	Source of drug	Retention time (min)	Ion pairs Precursor (*m/z*) > Product (*m/z*)	DP (V)	CE (eV)	Corresponding internal standard
53	clobenzorex	5843-53-8	C16H18ClN	259.8	c	5.69	260 > 91*	79	34	DOB-d5
							260 > 125	79	37	
54	2C-B (4-bromo-2,5-dimethoxyphenethylamine)	56281-37-9	C10H14BrNO2	260.1	b	3.81	260 > 213*	32	44	DOB-d5
							260 > 91	32	58	
55	6-Br-MDMA (1-(6-bromo-1,3-benzodioxol-5-yl)-N-methylpropan-2-amine)	–	C11H14BrNO2	272.1	d	3.66	272 > 241*	16	19	DOB-d5
							272 > 213	16	32	
56	DOB (4-bromo-2,5-dimethoxyamphetamine)	29705-96-2	C11H16BrNO2	274.1	b	4.24	274 > 178*	58	28	DOB-d5
							274 > 199	58	38	
57	4-EA-NBOMe (4-ethyl-N-(o-methoxybenzyl)amphetamine)	–	C19H25NO	283.4	d	9.07	284 > 121*	44	24	25I-NBOMe-d3
							284 > 91	44	59	
58	25H-NBOMe (2-(2,5-dimethoxyphenyl)-N-(2-methoxybenzyl) ethanamine)	1566571-52-5	C18H23NO3	301.4	d	6.03	302 > 121*	33	23	DOB-d5
							302 > 91	33	51	
59	2C-I (2,5-dimethoxy-4-iodophenethylamine)	64584-32-3	C10H14INO2	307.1	b	4.46	308 > 291*	48	18	DOB-d5
							308 > 276	48	31	
60	25D-NBOMe (2-(2,5-dimethoxy-4-methylphenyl)-N-(2-methoxybenzyl)ethanamine)	1539266-35-7	C19H25NO3	315.4	d	4.7	316 > 121*	51	25	DOB-d5
							316 > 91	51	54	
61	3,4-DMA-NBOMe (N-(o-methoxybenzyl)-3,4-dimethoxyamphetamine)	–	C19H25NO3	315.4	d	7.87	316 > 121*	17	24	25I-NBOMe-d3
							316 > 91	17	55	
62	DOI (2,5-dimethoxy-4-iodoamphetamine)	42203-78-1	C11H16INO2	321.2	b	4.97	322 > 178*	54	29	DOB-d5
							322 > 135	54	41	
63	25C-NBF (4-chloro-N-[(2-fluorophenyl)methyl]-2,5-dimethoxybenzeneethanamine)	1539266-21-1	C17H19ClFNO2	323.8	d	6.83	324 > 199*	61	27	DOB-d5
							324 > 184	61	39	
64	25G-NBOMe (2,5-dimethoxy-N-[(2-methoxyphenyl)methyl] -3,4-dimethylbenzeneethanamine)	1797132-54-7	C20H27NO3	329.4	d	9.32	330 > 121*	43	24	25I-NBOMe-d3
							330 > 91	43	60	
65	25C-NB3OMe (4-chloro-2,5-dimethoxy-N-[(3-methoxyphenyl) methyl] benzeneethanamine)	1566571-57-0	C18H22ClNO3	335.8	d	7.68	336 > 121*	44	27	25I-NBOMe-d3
							336 > 199	44	25	
66	25P-NBOMe (2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-4-propyl-benzeneethanamine)	1539266-43-7	C21H29NO3	343.5	d	10.48	344 > 121*	24	25	25I-NBOMe-d3
							344 > 91	24	66	
67	25N-NBOMe (2-(2,5-dimethoxy-4-nitrophenyl)-N-(2-methoxybenzyl) ethanamine)	1566571-65-0	C18H22N2O5	346.4	d	6.14	347 > 121*	30	23	DOB-d5
							347 > 91	30	59	
68	25T2-NBOMe (4-(ethylthio)-2,5-dimethoxy-N-[(2-methoxy-phenyl)methyl]benzeneethanamine)	1539266-51-7	C20H27NO3S	361.5	d	9.36	362 > 121*	23	27	25I-NBOMe-d3
							362 > 91	23	65	
69	25B-NBF (4-bromo-N-[(2-fluorophenyl)methyl]-2,5-dimethoxybenzeneethanamine)	1539266-17-5	C17H19BrFNO2	368.2	d	7.51	368 > 243*	28	28	25I-NBOMe-d3
							368 > 228	28	37	
70	25T7-NBOMe (2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-4-(propylthio)benzeneethanamine)	1539266-55-1	C21H29NO3S	375.5	d	10.33	376 > 121*	26	29	25I-NBOMe-d3
							376 > 91	26	64	
71	25T4-NBOMe (2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-4-[(1-methylethyl)thio]benzeneethanamine)	1566571-73-0	C21H29NO3S	375.5	d	10.09	376 > 121*	48	27	25I-NBOMe-d3
							376 > 91	48	64	
72	25B-NBOMe (N-(2-methoxybenzyl)-4-bromo-2,5-dimethoxyphenethylamine)	1539266-15-3	C18H22BrNO3	380.2	b	8.76	380 > 65*	72	105	25I-NBOMe-d3
							380 > 93	72	44	

(*continued on next page*)

Table 1 (*continued*)

Item	Analyte	CAS No.	Formula	Molecular weight (g/mol)	Source of drug	Retention time (min)	Ion pairs	DP (V)	CE (eV)	Corresponding internal standard
							Precursor (*m/z*) > Product (*m/z*)			
73	25I-NBF (N-(2-fluorobenzyl)-4-iodo-2,5-dimethoxyphenethylamine)	1539266-13-1	C17H19FINO2	415.2	d	8.74	416 > 291*	47	25	25I-NBOMe-d3
							416 > 276	47	39	
74	25I-NBOMe (N-(2-methoxybenzyl)-4-iodo-2,5-dimethoxyphenethylamine)	1043868-97-8	C18H22INO3	427.3	b	9.72	428 > 65*	117	113	25I-NBOMe-d3
							428 > 272	117	23	
75	amphetamine-d8	145225-00-9	C9H5D8N	143.3	e	2.2	144 > 97	50	24	–
76	phentermine-d5	1330236-21-9	C10D5H10N·HCl	190.7	e	2.57	155 > 96	42	32	–
77	methamphetamine-d8	136765-40-7	C10H7D8N	157.3	e	2.36	158 > 93	36	33	–
78	5-APB-d5 (5-(2-aminopropyl)benzofuran-d5)	–	C11H8D5NO·HCl	216.7	e	2.96	181 > 164	27	12	–
79	MDA-d5 (3,4-methylenedioxyamphetamine-d5)	136765-42-9	C10H8D5NO2	184.3	e	2.39	185 > 168	58	14	–
80	MDMA-d5 (3,4-methylenedioxymethamphetamine-d5)	136765-43-0	C11H10D5NO2	198.3	e	2.55	199 > 165	22	19	–
81	MDEA-d5 (3,4-methylenedioxy-N-ethylamphetamine-d5)	160227-43-0	C12H12D5NO2	212.3	e	2.82	213 > 163	61	18	–
82	mescaline-d9	–	C15H18ND9	230.44	e	2.16	221 > 204	59	16	–
83	DOB-d5 (4-bromo-2,5-dimethoxyamphetamine-d5)	–	C11H11D5BrNO2·HCl	315.7	e	4.22	279 > 262	67	16	–
84	25I-NBOMe-d3 (4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl] benzeneethanamine-d3)	–	C18H19D3INO3·HCl	466.8	e	9.69	431 > 124	26	26	–

a. Sigma-Aldrich Corporation (St. Louis, MO, USA).
b. GreenChem Corporation (Taichung, Taiwan).
c. LGC Limited (Teddington, UK).
d. Cayman Chemical (Ann Arbor, MI, USA).
e. Cerilliant Corporation (Austin, TX, USA), methanolic solutions (1 mg/mL).
* quantifier.

ion ratio ≦ 10%, RSD ± 50%). The acceptable retention time deviation of target was ±0.2 min.

Sensitivity was evaluated in terms of the LOD and LLOQ (lower limit of qualification), defined by the estimated signal-to-noise (S/N) ratios of 3 and 10, respectively. Six replicates (n = 6) were used for each analyte.

The matrix effect was assessed by comparing the calibration curves for the standard solution and the urine matrix. All analytes were prepared at serial concentrations of 1.0–50.0 ng/mL (including 10 ng/mL IS) in either DFU or 50% methanol aqueous solution. The matrix effect was evaluated with three replicates (n = 3) and calculated using the following formula:

$$\text{Matrix effect} = \frac{S_m - S_s}{S_s} \times 100\%$$

where S_m is the slope of the calibration curve in the DFU matrix, and S_s is that in 50% methanol aqueous solution.

To evaluate the precision and accuracy, quality control (QC) was introduced for analyte-spiked urine samples. The intra-day and inter-day precision (% CV) and accuracy (%) were assessed at various concentration levels from low to high (5.0, 25.0, and 50.0 ng/mL) in triplicate over five different runs. The acceptable rage of precision and accuracy are ± 20% and 80–120%, respectively. The procedure was in reference of the criteria for QC in chemical analysis by TFDA (for 1.0–10.0 ng/mL, recovery 60–125%, RSD 30%; for 10.0–100.0 ng/mL, recovery 70–120%, RSD 20%).

3. Results and discussion

3.1. Method development

From the pre-test of the ESI source, the positive mode (i.e. monitoring protonated molecules $[M+H]^+$) for PEA targets demonstrated a more robust response than in negative mode. Results were consistent with previous findings; hence, the ESI^+ mode was selected to discriminate multiple PEA targets [27,28]. The compound-specific MRM parameters, including declustering and collision potentials, were optimized. The monitoring and quantitative ions were collected, and the respective higher relative intensities were obtained. The MRM parameters and reference IS for each analyte are shown in Table 1, whereas the extract ion chromatogram (XIC) in urine spiked with all analytes of interest at 25 ng/mL is presented in Fig. 1. Separation was achieved in 11 min of a single run, with the first analyte 5-AEDB eluting at 2.21 min and the last analyte 25P-NBOMe at 10.48 min. It could be seen that over two third of the targets (57 items, 1–51, 54–56, 59, 60, and 62) distributed in the interval of 2.0–5.0 min. The similarity of PEA-type substances especially NPS has drawn difficulties for identification. The MRM mode has become a powerful tool for analyzing multiple PEA analogs through monitoring transitions under specific detection windows with designated retention times.

A couple of PEAs in this study were analogs possessing similar chemical structures. These PEAs may display the same mass fragments with similar retention times, leading to possible misinterpretation of results. The targets with the same mass fragments are grouped and listed as follows: phentermine, 4-MA, and methamphetamine (150); *N*,*N*-DMA, *N*-ethylamphetamine, and 5-AEDB (164); 4-FMA and FPBA (168); MDA and PMMA (180); 4-FEA and 4-MTA (182); 4-CMA, 5-F-2-MOA, and 3-F-4-MOA (184); PMEA and MDMA (194); DMA, *N*-hydroxy-MDA, and 2C-D (196); MBDB, MDDMA, and MDEA (208); MMDA, DOM, and 2C-E (210); lefetamine, escaline, TMA-2, TMA-6, and TMA (226); benzphetamine and proscaline (240); clobenzorex and 2C-B (260); 25D-NBOMe and 3,4-DMA-NBOMe (316); and 25T7-NBOMe and 25T4-NBOMe (376). To prevent misinterpretation, the retention time of each analyte should be inspected. The cross contributions were further examined by subsequent method validation to ensure limited impact on the analysis to guarantee reasonable specificity for quantifying analytes with the same mass pattern.

3.2. Method validation

To ensure the reliability and credibility of the present method for forensic analysis, validation was performed in terms of carryover and selectivity. The carryover was assessed to prevent the erroneous identification on analyzing targets. The result was shown in Table 2 which the carryovers for 66 targets were within 20%, regarded ignorable interference on target determination of subsequent sample. Worth noticing, the carryovers of eight 25-series PEAs (item 64–67, 69, 70, 72, and 74 in Table 2) ranged in 25.9–71.3%, indicating the residue appeared in the subsequent blank. As a result, attention should be paid in qualitative analysis to avoid the false-positive resulted from the residue of preceding sample, i.e. sufficient eluting should be done till no residue was observed in the blank. To assess the selectivity, the interfering peaks, IS traces, and cross-interference among analytes were inspected. As previously mentioned, PEA targets with the same mass fragments were also examined, and cross contributions were eliminated. The result indicated that the present method is selective for all analytes.

Linearity was assessed in the concentration range of 1.0–50.0 ng/mL based on the correlation coefficient, r. All analytes had r values above 0.995, corresponding to the IS selected for the respective target during qualification. The LOD and LLOQ determined for all analytes were 0.5 and 1.0 ng/mL, respectively. Comparing the results with previously reported methods, the present method demonstrated a broader coverage of target analytes (74 items) while ensuring good sensitivity (LOD of 0.5 ng/mL and LLOQ of 1.0 ng/mL), which is a requisite for screening multiple PEA-type NPS [21–24]. The present method offers good performance in qualitative and quantitative analysis of PEA-type NPS at a low limit from sensitivity validation.

For the analysis of forensic specimens, the matrix effect was assessed to determine whether sample pretreatment or purification was necessary. The matrix effect might affect the sensitivity, precision, accuracy, and reproducibility of the analytical method and the quantification of target analytes. In this study, urinalysis was implemented using a direct dilute-and-shoot procedure without sample pretreatment, and hence it is necessary to evaluate the matrix effect. The measured matrix effect ranged from −18.3% to 19.0% for all analytes, which satisfies the criterion of less than ±20%. The assay's precision and accuracy were evaluated by performing intra-day and inter-day experiments with triplicate samples at three concentration levels. The ion ratios of all analytes were higher than 50% with precision ranged from 0% to 20%, whereas the accuracy was 80%—120%. These values are satisfactory considering the respective criteria of within ±20% and 80%—120%, respectively. The results of the matrix effect, precision, and accuracy are presented in Table 3.

3.3. Application

Authentic urine samples collected by local law enforcement agencies were analyzed using the present method. A total of 67 samples were analyzed, and the targets detected at levels above their LODs are listed in Table 3. These samples were tested beforehand by immunoassays or chromatographic methods and identified positive of conventional PEAs, such as amphetamine, methamphetamine, and MDA. Afterwards, the samples were further analyzed applying the present method to see if additional PEAs presented. Twenty samples were tested positive of seven PEAs from 67 samples, whereas the contents detected were 9.8 ng/mL to 147.1 μg/mL with dilution factors of 40–20,000 folds. Of note, when the contents of targets within one sample vary tremendously, the dilution integrity

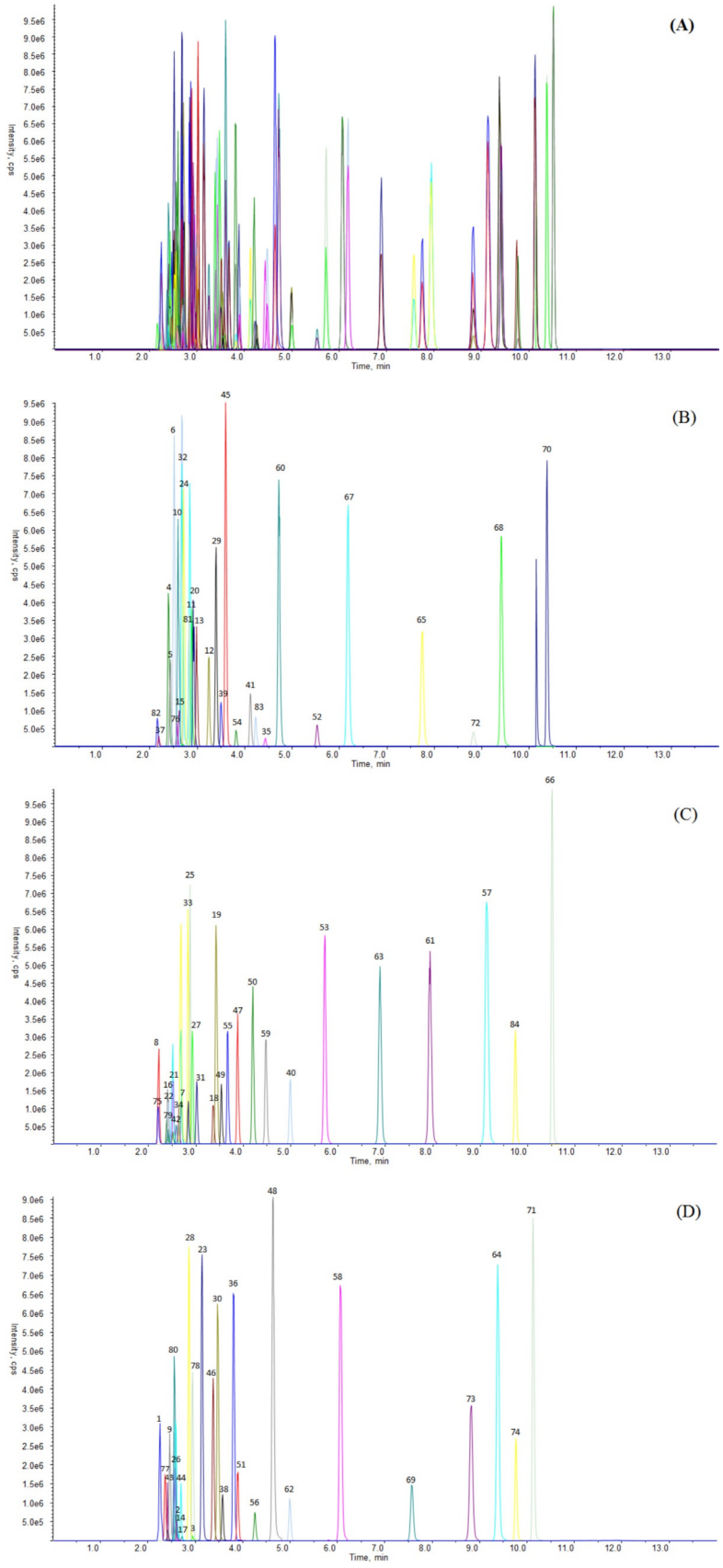

Fig. 1. Extract ion chromatograms (XIC) of 74 targets and 10 IS of PEAs at 25 ng/mL in urine, arranged by item number of analytes from Table 1: (A) Overall XIC; (B), (C), and (D) separate XICs.

Table 2
Carryover, matrix effect, precision, and accuracy for 74 phenethylamines.

Item	Analyte	Carryover (%)	Matrix effect (%)	Spiked concentration (ng/mL)	Intra-day		Inter-day	
					Precision (% CV)	Accuracy (%)	Precision (% CV)	Accuracy (%)
1	amphetamine	0.0	−18.1	5	4	86	13	92
				25	8	112	10	108
				50	9	92	9	95
2	phentermine	0.0	15.3	5	12	103	17	115
				25	8	112	16	113
				50	10	94	13	96
3	4-MA	3.7	6.6	5	13	117	12	110
				25	13	116	12	111
				50	3	104	8	97
4	methamphetamine	3.2	−9.4	5	13	116	20	102
				25	7	104	17	92
				50	15	113	17	90
5	4-FA	3.7	−5.8	5	7	108	20	110
				25	9	92	10	103
				50	20	98	15	103
6	*N*,*N*-DMA	3.5	17.3	5	6	98	16	81
				25	5	92	16	84
				50	12	94	19	82
7	*N*-ethylamphetamine	2.8	−7.8	5	20	82	19	117
				25	10	89	18	112
				50	13	118	13	103
8	5-AEDB	7.0	−8.5	5	7	103	19	115
				25	14	103	17	112
				50	14	101	17	99
9	PMA	2.5	14.6	5	17	90	19	112
				25	12	104	14	113
				50	18	98	17	106
10	4-FMA	6.3	−18.3	5	1	88	13	95
				25	2	111	7	110
				50	2	99	7	93
11	FPBA	4.7	−16.4	5	9	105	15	112
				25	8	112	8	115
				50	10	97	7	95
12	4-CA	7.5	7.1	5	12	103	20	116
				25	14	114	18	120
				50	12	105	15	109
13	5-APB	9.7	4.2	5	5	87	14	94
				25	6	93	10	97
				50	4	84	14	83
14	5-APDB	0.0	−4.5	5	7	82	13	111
				25	3	109	18	108
				50	8	107	17	98
15	PMMA	4.5	−13.2	5	9	104	18	106
				25	4	117	16	109
				50	9	96	15	83
16	MDA	0.0	−3.0	5	8	89	16	84
				25	18	87	15	117
				50	18	81	19	114
17	4-FEA	3.1	−16.6	5	8	114	13	105
				25	8	112	8	109
				50	9	106	14	94
18	4-MTA	10.7	−5.9	5	16	102	13	104
				25	8	97	16	96
				50	7	83	7	82
19	4-CMA	8.1	−1.6	5	20	105	15	112
				25	3	110	8	109
				50	11	91	9	90
20	5-F-2-MOA	4.3	0.2	5	17	95	17	113
				25	7	104	9	108
				50	17	98	15	97
21	3-F-4-MOA	10.8	6.7	5	9	88	18	106
				25	7	96	18	94
				50	6	110	12	113

(*continued on next page*)

Table 2 (*continued*)

Item	Analyte	Carryover (%)	Matrix effect (%)	Spiked concentration (ng/mL)	Intra-day		Inter-day	
					Precision (% CV)	Accuracy (%)	Precision (% CV)	Accuracy (%)
22	fenproporex	2.9	−10.6	5	19	116	17	117
				25	12	99	10	116
				50	16	85	9	90
23	5-MAPB	7.7	5.0	5	13	111	19	118
				25	9	107	12	111
				50	9	110	18	99
24	5-MAPDB	4.1	2.1	5	18	91	16	100
				25	14	105	14	103
				50	11	100	15	83
25	PMEA	4.1	−0.2	5	12	110	9	102
				25	18	103	20	116
				50	10	97	11	89
26	MDMA	6.7	1.5	5	10	96	18	99
				25	8	101	16	110
				50	13	94	11	99
27	DMA	5.9	−13.4	5	13	86	13	93
				25	3	100	7	109
				50	9	103	10	102
28	*N*-hydroxy-MDA	0.0	−11.9	5	12	89	8	89
				25	10	93	20	107
				50	12	107	16	99
29	2C-D	4.4	−13.4	5	6	91	9	106
				25	4	117	9	119
				50	3	97	6	98
30	5-EAPB	7.0	−2.4	5	8	83	15	94
				25	7	97	9	99
				50	2	90	18	92
31	MBDB	5.0	6.7	5	10	106	17	113
				25	17	102	14	103
				50	13	89	13	82
32	MDDMA	6.0	1.8	5	11	109	19	117
				25	6	81	14	90
				50	7	89	15	107
33	MDEA	3.6	−3.0	5	15	90	17	101
				25	9	98	14	95
				50	7	98	20	82
34	MMDA	0.0	18.2	5	5	99	19	118
				25	4	98	16	111
				50	13	96	13	97
35	DOM	17.8	18.4	5	2	103	5	106
				25	12	108	8	107
				50	7	93	5	93
36	2C-E	5.8	6.6	5	9	113	12	98
				25	13	105	15	113
				50	5	105	9	95
37	mescaline	0.0	−12.1	5	10	93	13	98
				25	8	95	13	108
				50	9	92	11	100
38	4-BA	6.0	16.2	5	12	104	16	112
				25	13	105	12	108
				50	10	90	15	94
39	2C-C	9.7	−2.6	5	19	94	13	102
				25	8	111	13	113
				50	14	100	15	93
40	DOET	6.0	−6.1	5	8	109	8	102
				25	7	112	7	108
				50	7	90	7	95
41	lefetamine	7.6	−6.9	5	6	109	9	99
				25	11	114	12	104
				50	13	89	9	93
42	escaline	0.0	−14.5	5	2	111	17	86
				25	5	109	13	80
				50	5	92	18	94

(*continued on next page*)

Table 2 (*continued*)

Item	Analyte	Carryover (%)	Matrix effect (%)	Spiked concentration (ng/mL)	Intra-day		Inter-day	
					Precision (% CV)	Accuracy (%)	Precision (% CV)	Accuracy (%)
43	TMA-2	6.8	13.3	5	10	96	18	84
				25	10	114	9	109
				50	11	113	11	104
44	TMA-6	5.3	2.1	5	6	97	5	103
				25	7	113	11	105
				50	13	96	15	91
45	TMA	0.0	−4.8	5	12	116	16	113
				25	18	103	14	113
				50	12	100	16	98
46	6-Cl-MDMA	18.1	17.0	5	8	88	13	97
				25	8	90	13	97
				50	5	88	8	84
47	DOC	0.0	16.4	5	5	96	6	103
				25	1	103	4	103
				50	8	89	7	86
48	benzphetamine	10.1	17.8	5	7	98	11	103
				25	10	105	12	98
				50	9	81	9	82
49	proscaline	4.6	−8.2	5	2	104	15	105
				25	15	100	9	105
				50	11	85	12	94
50	2C-T-2	13.8	12.8	5	8	101	8	109
				25	7	100	8	100
				50	6	85	5	86
51	3C-P	0.0	18.5	5	9	91	8	95
				25	9	108	7	107
				50	10	97	13	90
52	2C-T-7	0.0	−3.3	5	8	113	8	107
				25	9	111	7	110
				50	7	88	8	96
53	clobenzorex	12.9	−13.7	5	2	85	7	95
				25	4	106	6	106
				50	3	89	4	89
54	2C-B	13.9	17.7	5	9	109	10	111
				25	8	113	9	111
				50	2	93	8	92
55	6-Br-MDMA	20.0	10.9	5	13	108	13	98
				25	9	105	14	107
				50	13	82	12	90
56	DOB	0.2	−4.9	5	13	108	12	101
				25	6	116	9	115
				50	8	95	8	101
57	4-EA-NBOMe	3.9	−11.3	5	9	104	8	97
				25	8	108	8	108
				50	10	85	7	91
58	25H-NBOMe	4.2	3.9	5	9	107	7	102
				25	12	106	10	107
				50	8	93	9	87
59	2C-I	0.1	7.6	5	6	107	9	98
				25	16	108	12	111
				50	13	101	13	93
60	25D-NBOMe	1.6	11.2	5	16	89	9	88
				25	3	103	7	101
				50	8	92	10	84
61	3,4-DMA-NBOMe	4.5	−8.3	5	4	97	7	89
				25	6	100	4	102
				50	0	86	4	91
62	DOI	17.6	10.4	5	1	100	5	105
				25	4	107	6	107
				50	1	91	3	92
63	25C-NBF	4.6	3.8	5	9	106	7	102
				25	11	103	7	102
				50	5	83	8	90

(*continued on next page*)

Table 2 (*continued*)

Item	Analyte	Carryover (%)	Matrix effect (%)	Spiked concentration (ng/mL)	Intra-day		Inter-day	
					Precision (% CV)	Accuracy (%)	Precision (% CV)	Accuracy (%)
64	25G-NBOMe	30.2	8.5	5	3	90	8	90
				25	5	109	8	113
				50	1	93	4	95
65	25C-NB3OMe	67.3	−9.0	5	4	107	7	113
				25	3	102	6	104
				50	1	94	4	94
66	25P-NBOMe	37.2	−4.5	5	4	85	7	95
				25	4	110	6	114
				50	2	91	3	92
67	25N-NBOMe	56.6	11.9	5	3	93	6	100
				25	4	95	9	111
				50	2	100	4	97
68	25T2-NBOMe	4.8	−13.2	5	3	94	3	94
				25	4	115	6	108
				50	8	98	5	96
69	25B-NBF	25.9	−7.9	5	3	92	3	93
				25	2	114	7	105
				50	2	99	3	96
70	25T7-NBOMe	63.8	14.5	5	6	105	7	109
				25	6	110	6	111
				50	3	94	4	94
71	25T4-NBOMe	4.0	−2.3	5	8	106	6	104
				25	10	111	7	112
				50	7	83	9	92
72	25B-NBOMe	51.6	15.0	5	2	104	3	105
				25	3	109	4	108
				50	1	95	2	96
73	25I-NBF	6.2	−17.5	5	1	93	3	96
				25	2	113	6	105
				50	4	97	3	95
74	25I-NBOMe	71.3	19.0	5	3	104	5	106
				25	2	103	6	105
				50	1	93	4	93

Table 3
Targets detected above LOD from authentic urine samples.

Sample No.	Target detected	Content (μg/mL)	RSD (%)
2	*N,N*-DMA	0.6	2.8
	PMA	7.3	2.2
	PMMA	0.2	2.1
3	amphetamine	2.0	0.9
4	4-CA	5.6	0.8
	N,N-DMA	7.1	1.0
7	methamphetamine	0.7	1.5
12	methamphetamine	15.6	3.5
19	methamphetamine	9.8 ng/mL	2.6
22	methamphetamine	9.2	1.4
23	methamphetamine	12.3	0.4
26	amphetamine	4.6	0.5
	N,N-DMA	3.0	1.9
	PMA	0.4	0.8
28	*N,N*-DMA	1.3	3.5
29	*N,N*-DMA	4.6	1.9
32	*N,N*-DMA	1.7	1.1
33	methamphetamine	17.2	4.1
34	methamphetamine	2.0	1.8
	PMA	0.4	0.3
	MDA	13.8	0.4
36	methamphetamine	3.2	0.5
43	methamphetamine	6.7	0.6
44	methamphetamine	9.3	0.6
52	methamphetamine	80.4	1.7
57	methamphetamine	147.1	0.2
60	methamphetamine	7.1	2.3

should be considered to ensure the valid qualification of each target. Chromatograms of urinalysis from three authentic samples are shown as Fig. 2. The result indicated the present method possessed a good performance in discriminating multiple PEAs while the background noise and matrix interferences are eliminated and retained a good specificity and sensitivity in qualitative and quantitative analysis. The seven identified targets are four scheduled substances (amphetamine, methamphetamine, MDA, and PMA) and three NPS (4-CA, *N,N*-DMA, and PMMA). Amphetamine and methamphetamine were the two PEAs most commonly detected in the surveyed samples. In 2019, amphetamine and methamphetamine were the second-largest group of illicit substances (38.5% of the total) seized in Taiwan, whereas methamphetamine was the most frequently detected illicit substance in urine drug tests (37,617 among 231,947 cases) [29]. Furthermore, four of the samples we tested (No. 2, 4, 26, and 34) contained 2 or 3 PEAs simultaneously. Up to December 2020, Taiwan's early warning system of drug abuse ("Analytic Laboratory Urine and Drug Abuse Report System", UDARS) received reports of 34 PEAs in total, which takes the third place among all types of NPS [30]. The top five PEA-type NPS reported most often in Taiwan are methoxymethamphetamines (including PMMA), MDDMA, chloroamphetamines (including 4-CA), *N,N*-DMA, and 2C-I. Three of the monitored PEAs were detected from the surveyed samples in this study. Therefore, the results of urinalysis show the trend consistent with those observed in Taiwan in PEA-type NPS. Besides NPS, four controlled substances were also detected. Based on the results above, the present method is a potential tool for screening multiple PEAs in preliminary or confirmatory analyses.

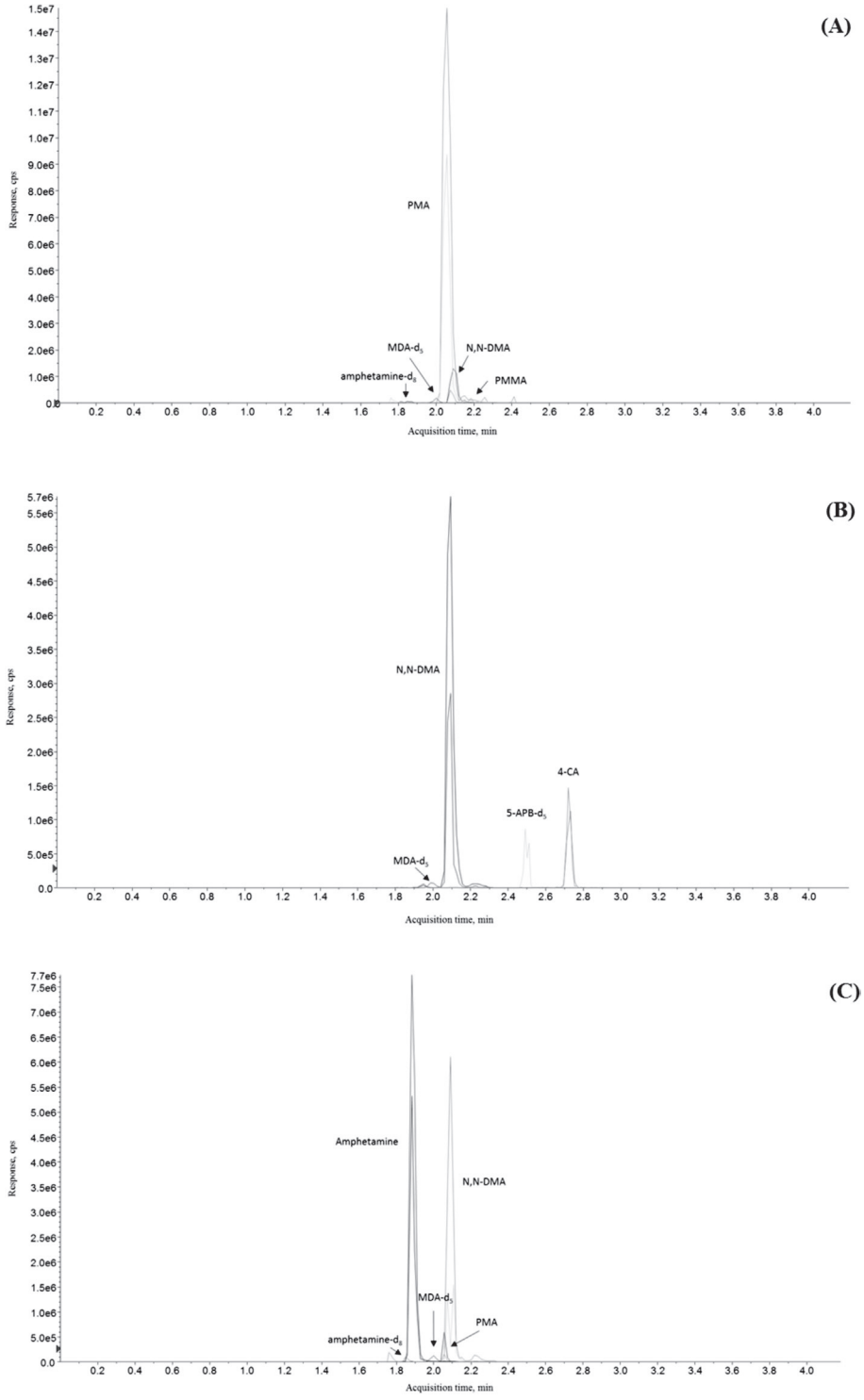

Fig. 2. Chromatograms of urinalysis from three authentic samples detected positive of target PEAs: (A) *N,N*-DMA, PMA and PMMA (sample No. 2); (B) 4-CA and *N,N*-DMA (sample No. 4); (C) amphetamine, *N,N*-DMA and PMA (sample No. 26).

4. Conclusions

This study presents a direct dilute-and-shoot LC-MS/MS method. The method was applied for screening a total of 74 PEA targets in urine and was further validated and applied to urinalysis of authentic samples. Out of 67 samples, 20 were detected positive, with a total of seven identified targets. These results suggest the present method as a potential tool for preliminary or confirmatory analysis of PEAs among both controlled substances and NPS, with possible application for forensic purposes.

CRediT authorship contribution statement

Shu-Yu Fan: Conception and design of study, Acquisition of data, Analysis and/or interpretation of data, Approval of the version of the manuscript to be published. **Chi-Zong Zang:** Conception and design of study, Acquisition of data, Analysis and/or interpretation of data, Drafting the manuscript, Approval of the version of the manuscript to be published. **Po-Han Shih:** Acquisition of data, Analysis and/or interpretation of data, Approval of the version of the manuscript to be published. **Ya-Chun Ko:** Conception and design of study, Revising the manuscript critically for important intellectual content, Approval of the version of the manuscript to be published. **Ya-Hui Hsu:** Conception and design of study, Revising the manuscript critically for important intellectual content, Approval of the version of the manuscript to be published. **Mei-Chih Lin:** Conception and design of study, Revising the manuscript critically for important intellectual content, Approval of the version of the manuscript to be published. **Su-Hsiang Tseng:** Revising the manuscript critically for important intellectual content, Approval of the version of the manuscript to be published. **Der-Yuan Wang:** Revising the manuscript critically for important intellectual content, Approval of the version of the manuscript to be published.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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