

## Evaluation of Two Different Screening ELISA Assays for Synthetic Cathinones (Mephedrone/Methcathinone and MDPV) with LC-MS Method in Intoxicated Patients

Roda E<sup>1\*</sup>, Lonati D<sup>1</sup>, Buscaglia E<sup>1</sup>, Papa P<sup>2</sup>, Rocchi L<sup>2</sup>, Locatelli CA<sup>1</sup> and Coccini T<sup>1</sup>

<sup>1</sup>Laboratory of Clinical & Experimental Toxicology and Poison Control Centre and National Toxicology Information Centre, Toxicology Unit of the Pavia's Hospital, IRCCS Maugeri Foundation, Pavia, Italy

<sup>2</sup>Laboratory of Analytical Toxicology, Clinical Chemistry Service of Pavia's Hospital, IRCCS Policlinico San Matteo Foundation, Pavia, Italy

\*Corresponding author: Dr. Elisa Roda, IRCCS Maugeri Foundation, Medical Institute of Pavia Laboratory of Clinical & Experimental Toxicology and Poison Control Centre and National Toxicology Information Centre, Via Maugeri, 10 - 27100 Pavia, Italy; Tel: +39/0382/592414; Fax: +39/0382/24605; E-mail: [elisa.roda@fsm.it](mailto:elisa.roda@fsm.it); [elisa.roda@unipv.it](mailto:elisa.roda@unipv.it)

Received date: March 17, 2016; Accepted date: May 3, 2016; Published date: May 10, 2016

Copyright: © 2016 Roda E, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### Abstract

**Context:** Synthetic cathinones are a new trend in the recreational drug market and the paucity of human toxicological data combined with their widespread abuse generated great concern in the international scientific community.

**Objective:** Inside the Italian National Early Warning System (NEWS), clinical urine specimens were collected from patients (n=202) admitted to the Emergency Departments (April 2011-January 2013) for clinically suspected abuse of any kind of unknown new psychoactive substances, to measure synthetic cathinones demonstrating the consistency and reliability of the employed screening assays as useful tools to detect these drugs, with the ultimate objective to advance patients care and management.

**Methods:** Screening analyses were performed using two specific ELISA assays, targeting Mephedrone/methcathinone and MDPV (LOD 0.40 and 20.0 ng/ml, respectively). Data were then compared to determinations gained by LC-MS (LOD 5 ng/ml).

**Results:** (i) Mephedrone/methcathinone: 195/202 samples gave values <7 ng/ml by screening ELISA assay and tested negative by LC-MS. Seven specimens showed concentrations >16 ng/ml (above the upper limit of the standard curve) by screening immunoassay, and only 4 of them resulted positive by LC-MS; (ii) MDPV: 162/167 samples gave values ≤ 60 ng/ml by screening ELISA and tested negative by LC-MS. Five samples showed concentration above the upper limit of the standard curve (>850 ng/ml). Among these, 3/5 samples were confirmed positive by LC-MS (2 for butylone and MDPV, 1 for pentadone and MDPV).

**Discussion and conclusion:** These results emphasize a good overall match between data obtained by the two analytical methods, showing disagreement in few cases concerning positive results; no false negatives were detected by ELISA screening, suggesting the promising usefulness of this reliable tool as first approach in the emergency setting to rapidly detect synthetic cathinones, allowing the clinician to improve differential diagnosis, aiding real-time patient care and management.

**Keywords:** Designer drugs; Bath salts; Cross reactivity; Immunoassay; Emergency departments

### Introduction

The worldwide drug scenario is incessantly transforming in that common drugs of abuse have been joined by “new psychoactive substances” (NPS) that fall outside international drug control conventions. Many of these substances were synthesized and patented in the early 1970s, but only over the last decades they have rapidly emerged in the market supposedly as “legal” alternatives to internationally controlled drugs, thus posing serious risks to human safety. These novel psychoactive substances are marketed as ‘designer drugs’, being this term used, and continuously broadened, to identify and include synthetic substances, mimicking the effects of illicit drugs,

produced by introducing slight modifications to the chemical structure of controlled substances to circumvent drug controls [1].

In 2000s, among NPS, many synthetic cathinones (amphetamine- and cocaine-type stimulants) have received a renewed popularity. These novel compounds, derived from the vegetable cathinone, naturally present in the Khat plant (*Catha edulis*), are marketed as “bath salts” or “plant food” and labeled “not for human consumption” to circumvent the legislation on drugs of abuse [2-4]. The internet market greatly increased the spread of these NPS, sold in specialized shops known as “head” or “smart shops” as well as in online store, being able to respond quickly to changes in the legal status of recreational drugs offering for sale new legal alternatives, thus becoming a matter of threat to public health [5-10].

These “bath salts” are synthetic cathinone powders, distributed under trade names such as ‘Ivory Wave’, ‘White Lightning’ and ‘Vanilla Sky’, typically taken by inhalation (snorting), ingestion, or intravenous/intramuscular injection [6,11]. As with tablets, mephedrone is the most commonly abused in Europe, whereas MDPV and methylone are more prominent in US bath salts [1]. Abuse is documented across population from mid-to-late adolescent to older adults [12].

Like amphetamines, synthetic cathinones exert their stimulant effects via (i) increasing synaptic concentration of catecholamines such as dopamine, serotonin and norepinephrine, and (ii) inhibiting monoamine uptake transporters, with a consequent decreased clearance of the neurotransmitters from the synapse. Furthermore, they may cause release of biogenic amines from intracellular stores [2,13].

Since synthetic cathinones are a pandemic trend, the paucity of human toxicological data combined with the numerous cases of abuse, dependence, severe intoxication and drug-related deaths, signalled in several Countries, has generated great concern in the scientific community. Thus, International agencies and national institutions have issued extensive reports to monitor this emerging trend of abuse, as well as to schedule and banned some NPS [1,14-20]. In Italy, as a result of acute intoxications including deaths, mephedrone was placed under the regulatory control in June 2010, as reported in the Presidential Decree 309/90 (as amended) on the “regulation of narcotic drugs and psychotropic substances, prevention, treatment and rehabilitation of drug addiction”. Subsequently, new decrees entered into force placing under control synthetic cathinone 3,4-methylenedioxypropylone (MDPV) and structure analogues, derived from 2-amino-1-phenyl-1-propanone, for one or more substitutions on the aromatic ring and/or on the nitrogen and/or on the terminal carbon [18,21].

Currently, the information available about the human pharmacokinetics and pharmacodynamics of cathinone derivatives as well as the short and long-term toxicological effects of these NPS are very limited, thus their potential consequences are not well defined and generally poorly known.

In Italy the dimension of this problem is still indefinite; ambiguous signs/symptoms often characterize the clinical presentation of the patients admitted to the National Emergency Departments (EDs), contributing to underestimate or misjudge this phenomenon, potentially reverberating on the patient management. The identification of intoxication cases consistently related to synthetic cathinones abuse on the Italian EDs may allow the national regulatory agencies to engage actions designed to prevent and control this growing misuse.

So far, even though a number of national and international bans have been enacted, [15,16,18,19,22] the abuse of these designer drugs still continues and exponentially grows. For this reason it is difficult to validate and maintain comprehensive analytical methods for accurate detection of these compounds in biological specimens. Commonly, screening methods, such as immunoassay, are employed by toxicology laboratories for the first, presumptive identification of drugs of abuse, followed by confirmatory analysis, such as gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometry (MS). As a consequence, in recent years, a critical need emerged in the field of toxicology to (i) study the activities of these NPS in screening assays, as well as to (ii) identify and validate reliable screening tests for multiple designer drugs, to be successfully applied other than classical analytical methods in human specimens.

With this purpose, inside the Italian National Early Warning System (NEWS) Project - Department for Antidrug Policies-Presidency of the Italian Council of Ministers (Rome), in which the Pavia Poison Control Centre (IRCCS Salvatore Maugeri Foundation) is the National Coordinating Centre for clinico-toxicological aspects, our Laboratory of Clinical Toxicology (IRCCS Salvatore Maugeri Foundation) performed screening analysis in urine samples of poisoned patients, admitted to EDs for a clinically suspected abuse of any kind of unknown NPS, followed by confirmatory analyses carried out by the Laboratory of Analytical Toxicology of the IRCCS Foundation Policlinico San Matteo. The investigation aimed at measuring synthetic cathinones to demonstrate the consistency and reliability of the employed screening assays as useful tools to rapidly detect these NPS, with the final goal to improve patient care and management.

## Materials and Methods

### Human urine specimens

The present study design was reviewed and approved by the Ethic Committee of our Hospital (1099 CE/2015).

The study was conducted on a total of 202 clinical urine specimens collected from severely intoxicated patients admitted to Italian emergency departments (EDs) inside the NEWS Project, from April 2011 to January 2013, for a clinically suspected abuse of a broad range of unknown NPS. Patients were informed of their biological specimens use for this research project and signed the consent forms, following the regulation established by Department for Antidrug Policies-Presidency of the Italian Council of Ministers (Rome). The urine samples sent to our lab were collected in plastic containers (not adsorbing drugs), divided into two aliquots (one for the first-step screening ELISA analysis and the other one for the following confirmatory determination, respectively) and immediately stored at -20°C until examinations.

In addition, twenty-five healthy, non-smoker, non-drug abusers volunteers were recruited and their urines underwent contextual screening analysis to determine synthetic cathinones (i.e. both Mephedrone/Methcathinone and MDPV) background levels.

### Reagents and consumables

Mephedrone/Methcathinone (Bath Salt I) and MDPV (Bath Salt II) determination was achieved using two specific enzyme-linked immunosorbent (ELISA) commercial kits, purchased from RANDOX Laboratories Ltd (Crumlin, Co. Antrim, UK).

For confirmatory analyses, all chemical reagents were obtained from Sigma Aldrich (Sigma, Milan, Italia), and the certified reference standards from LGC (LGC Standards, Teddington, Middlesex, UK).

### Immunoassay screening procedure

The ELISA assays allow to semi-quantitatively measure, with high specificity, the level of several Mephedrone/Methcathinone and MDPV parent compounds and derivatives (see Table 1). Standard curves for Mephedrone/Methcathinone and MDPV kits ranged between 0 to 0.16 ng/ml and 0 to 850 ng/ml, respectively. LOD: 0.40 and 20.0 ng/ml for Mephedrone/methcathinone (Bath Salt I) and MDPV (Bath Salt II), respectively.

According to the manufacturer's instructions, urines were centrifuged at 13000 rpm for 60 sec and then diluted 1:4 with sample diluent. Subsequently, 25 microL of standard solution or urine samples and 100 microL of conjugate diluent were added to each appropriate well. All calibrators and samples were performed in duplicate. After incubation at room temperature for 1 h in the dark, the plates were washed six times with diluted wash buffer.

Compound	% Cross Reactivity
<b>Mephedrone/Methcathinone (Bath Salt I) kit</b>	
Mephedrone HCl (4-MMC)	100
Methylone	63
Flephedrone HCl (4-FMC)	45
R(+)-Methcathinone HCl	44
Methcathinone	43
3-FluoroMethcathinone (3-FMC)	16
4-Methylethcathinone (4-MEC)	10
Ethylone HCl	7
N-ethylcathinone HCl	4
Buphedrone HCl	<1
S(-)-Methcathinone	<1
S(-)-cathinone	<1
R(+)-cathinone	<1
Bupropion HCl	<1
Beta-ethyl Methcathinone	<1
<b>MDPV (Bath Salt II) kit</b>	
3,4-Methylenedioxypropylvalerone (MDPV) HCl	100
3'-4'-Methylenedioxy-alpha-pyrrolidinobutiophenone (MDPBP) HCl	96
Naphyrone HCl	27
Pyrovalerone HCl	17
4'- Methyl-alpha-pyrrolidinohexaphenone (4'-Me-a-PHP) HCl	15
4' Methyl-alpha-pyrrolidinobutiophenone (MPBP) HCl	13
Pentylone HCl	9
3',4'-Methylenedioxy-alpha-pyrrolidinopropiophenone (MDPPP) HCl	4
Butylone HCl	4
Desmethyl pyrovalerone (alpha-PVP) HCl salt	2
Pentadone HCl	<1

**Table 1:** Summarized specificity of the ELISA kits employed for the screening analyses, based on the manufacturer's indications.

Next, 125 microL of one shot substrate solution was added and the plates were placed in the dark at room temperature for 20 min. The reaction was stopped by adding 100 microL of stop solution and the absorbance was read at 450 nm, also using a reference 630 nm filter. Finally, to interpret the results and to calculate the urine concentrations, a 4 parameter curve fit method was used to generate a standard log10 curve; the mean absorbance of controls and samples was calculated and then plotted against the standard curve.

### Confirmatory urine samples study: Liquid chromatography-tandem mass spectrometry (LC/MS) analysis

Presumptive positive and negative urine specimens, based on the values obtained by screening assay, were confirmed with a liquid chromatography-tandem mass spectrometry (LC-MS) method, modified from Chimalakonda et al. and Dresen et al. [23,24]. Specifically, for the LC-MS analysis, urine samples (1 ml) were additivated with 100 ng of dosulepin as internal standard, 100 microL of NaOH 1N (pH 14) and extracted with a mixture of exane:ethylacetate (3:1 v/v) by vortex mixing for 2 min. The organic layer was transferred to a new glass tube and evaporated to dryness under nitrogen stream.

The samples were then reconstituted in 10 microL of methanol and 100 microL of mobile phase A. 20 microL of the reconstituted sample was injected into a Waters LC-MS apparatus, constituted by an Alliance liquid chromatographic system coupled with a Waters Quattro Micro triple quadrupole equipped with a Z-spray electrospray source. The chromatographic separation was performed using a Waters Xbridge C18 column operating in gradient mode at 30°C. The mobile phases were: (A) 5 mM ammonium formate in water, pH 3 with formic acid, (B) 0.1% formic acid in acetonitrile. The gradient started from 95% A, 5% B, got up to 20% A, 80% B in 12 min and returned to the original condition in 6 min. Flow rate was 0.2 ml/min. Mass spectrometer analysis was performed in positive ionization (ESI) and the acquisition was made in Multiple Reaction Monitoring mode (MRM) with two transitions for each analyte.

### Results

Presumptive negative and positive urine specimens, based on ELISA screening determinations, were analysed with a LC-MS confirmatory method for 15 parent cathinones and derivates. Limits of detection (LOD) was 5 ng/ml for all the screened cathinones, based on the availability of certified reference standards.

Specifically, the followings were determined: Mephedrone or 4-methylmethcathinone (4-MMC), 3,4-Dimethylmethcathinone (3,4-DMMC), Flephedrone or 4-FluoroMethcathinone (4-FMC), 4-Methylethcathinone (4-MEC), Ethylone ( $\beta$ k-MDEA), Buphedrone or  $\alpha$ -methylamino-butyrophenone (MABP), 3,4-Methylenedioxypropylvalerone (MDPV), Naphyrone or naphthylpyrovalerone (O-2482), Pentylone ( $\beta$ k-MBDP), Butylone ( $\beta$ k-MBDB), Pentadone or  $\alpha$ -methylamino-valerophenone, N,N-DimethylCathinone or metamfepramone, Ethcathinone or ethylpropion (ETH-CAT), Methedrone or 4-methoxymethcathinone ( $\beta$ k-PMMA), Methylone or 3,4-methylenedioxy-N-methylcathinone (MDMC or bk-MDMA).

We identified the analyzed urine samples as following: (i) true positive specimens, those screened and confirmed positive, (ii) true negative specimens, those negative in both assays; (iii) false (i.e. mismatching) positive specimens, those screened positive, but not

confirmed by LC-MS for synthetic cathinones; (iv) false negatives, sample screened negative but confirmed positive for one or more synthetic cathinones.

Table 2 details which drug of abuse screened by ELISA assay, as specified by the manufacturer, was determined by LC-MS, based on certified reference standards supply.

Confirmatory LC-MS analyses	Screening Analyses % Cross Reactivity	ELISA kits
Mephedrone (4-MMC)	100	(Mephedrone/Methcathinone)
Methylone	63	(Mephedrone/Methcathinone)
4-FluoroMethcathinone (4-FMC)	45	(Mephedrone/Methcathinone)
4-Methylethcathinone (4-MEC)	10	(Mephedrone/Methcathinone)
Ethylone (βk-MDEA)	7	(Mephedrone/Methcathinone)
Buphedrone (MABP)	1	(Mephedrone/Methcathinone)
MDPV	100	(MDPV)
Naphyrone (O-2482)	27	(MDPV)
Pentylone (βk-MBDP)	9	(MDPV)
Butylone (βk-MBDB)	4	(MDPV)
Pentedrone	1	(MDPV)
N,N-DimethylCathinone	not detected	---
3,4-Dimethylmethcathinone (3,4-DMMC)	not detected	---
Ethcathinone (ETH-CAT)	not detected	---
Methedrone (βk-PMMA)	not detected	---

**Table 2:** Drug of abuse screened by ELISA, as reported in given manufacturer's indications, was determined by LC-MS, based on certified reference standards supply.

Regarding the n=25 samples from healthy volunteers (considered as controls), tested to determine the cathinones background levels, screening analysis measured urinary values ranging from 0.0 to 0.8 ng/ml and 0.0 to 57.2 ng/ml, for Mephedrone/Methcathinones (Bath Salt I) and MDPV (Bath Salt II), respectively.

**Mephedrone/methcathinone (Bath Salt I) determination:** on a total of 202 biological specimens, 195 samples gave values <7 ng/ml by screening ELISA assay, thus being considering as presumptive negatives; the same samples subsequently were tested as negative by LC-MS analysis, with value <5 ng/ml, thus demonstrating that all 195 samples were true negatives.

Seven urine samples showed concentrations >16 ng/ml (above the upper limit of the standard curve) by screening immunoassay, and only 4 of them resulted true positive when dosed by LC-MS (Table 3A). Regarding the n=3 false (i.e. mismatching) positive specimens (Table 3A), it has to be pointed out that 2 samples belonged to subjects screened and confirmed as polydrug abusers; specifically, one urine

was positives for delta-9-tetrahydrocannabinol (Δ-9-THC), Amphetamine (AMP) and 3,4-methylenedioxy-methamphetamine (MDMA) and the second sample for benzoylecgonine (i.e. cocaine) (COC) and MDMA, respectively, while the remaining one was tested positive for Methoxetamine (MXE) by LC-MS. Notably, no false negatives were detected.

**MDPV (Bath Salt II) determination:** On a total of 167 specimens analyzed, 162 samples gave values ≤ 60 ng/ml by screening ELISA assay and considered as presumptive negatives. When tested by LC-MS, the same samples were confirmed as true negatives. 5 urine samples were screened as presumptive positive; among these, 3 specimens, showing concentrations above the upper limit of the standard curve (>850 ng/ml) in screening, were confirmed as true positive by LC-MS analysis (Table 3B). Concerning the false (i.e. mismatching) positive specimens (n=2) not confirmed by LC-MS (Table 3B), n=1 urine was screened positive for COC and the other sample for Ketamine (KET), as further confirmed by the following LC-MS analyses. Importantly, once more, no false negatives were determined.

A: Mephedrone/Methcathinones				
Total biological samples	True Confirmed Negatives	False negatives	True Confirmed positives	FALSE (i.e. mismatching) positives
n=202	n=195	0	n=4	n=3 (*)
	<7 ng/ml (Screening analyses)		>16 ng/ml (Screening analyses)	
	<5 ng/ml (LC-MS analyses)		>5 ng/ml (LC-MS analyses)	
B: MDPV				
Total biological samples	True Confirmed Negatives	False negatives	True Confirmed positives	FALSE (i.e. mismatching) positives
n=167	n=162	0	n=3	n=2 (*)
	≤ 60 ng/ml (Screening analyses)		>850 ng/ml (Screening analyses)	
	<5 ng/ml (LC-MS analyses)		>5 ng/ml (LC-MS analyses)	

**Table 3:** Comparison between screening results (ELISA assay) and LC-MS data. (\*) Discrepancy between ELISA results for Mephedrone/Methcathinones and MDPV (>16 ng/ml and >850 ng/ml, respectively) and LC-MS data (negative).

Notably, Table 4 details the true positives samples, n=4. The urine specimens screened as presumptive positive for both Mephedrone/methcathinone and MDPV, were the same, except for one sample screened as presumptive negative for MDPV only.

Among the four urines screened positives for Mephedrone/methcathinone (Bath Salt I), n=1 specimen was positive for 4-MEC and n=1 for mephedrone, fully matching with the declared specificity of the ELISA assay package insert (see Table 1, Bath Salt I).

Regarding the other two presumptive positives in screening, they were both LC-MS confirmed positive for butylone, which is known to

crossreact (about 50%) with Bath Salt I, targeting Mephedrone/methcathinone, although butylone is exclusively included in the specificity stated by the manufacturer for MDPV kit (Bath Salt II) [25].

Concerning the n=3 samples screened as positives for MDPV, LC-MS confirmatory analyses demonstrated n=2 sample were positive for

both butylone and MDPV, and the other one for pentedrone and MDPV, accordingly to the specificities enumerated in the MDPV ELISA kit package insert (see Table 1, Bath Salt II).

Sample ID	ELISA Screening		LC-MS analyses					
	Mephedrone/Methcathinone	MDPV	Mephedrone	Butylone	Other Cathinones	4-MEC	Pentedrone	MDPV
1	+	+	-	+	-	-	-	+
2	+	-	-	-	-	+	-	-
3	+	+	-	+	-	-	-	+
4	+	+	+	-	-	-	+	+

**Table 4:** True positive samples. +: positive; -: negative. Full colours (i.e. green and yellow) indicate accordance between LC-MS results and screening data obtained with the specific ELISA kit. Merged colours (green+yellow in LC-MS analyses) indicate accordance between LC-MS results (specifically for butylone) and screening data obtained with both ELISA kit. Weak yellow is just to indicate the specimen screened and confirmed as true negative by both methods.

Moreover, the LC-MS analyses showed that the only specimen determined as presumptive negative for MDPV (but also presumptive positive for Mephedrone/methcathinone) in screening, tested as true negative for Butylone, Pentedrone and MDPV, resulting as true positive for the 4-MEC only, which is included among the compounds detected by the Mephedrone/methcathinones kit (Bath Salt I).

Sample ID	LC-MS determination	ELISA Screening analyses	
	MXE	Mephedrone/Methcathinone (*)	MDPV (*)
	+	0.1	2.4
#1	+	0.2	5.7
#2	+	0	3.1
#3	+	0	7.5
#4	+	0.4	14.7
#5	+	0.7	8.8
#6	+	0.2	2.6
#7	+	0.3	15.9

**Table 5:** Comparison between urinary specimens screened and confirmed negative for both Mephedrone/Methcathinones and MDPV,

and confirmed positive for MXE by LC-MS. (\*) Urinary values determined by both ELISA assays fell within the background range levels (0.0-0.8 ng/ml for Mephedrone/Methcathinones and 0.0-57.2 ng/ml for MDPV).

Based on the clinical history and health conditions at admission to EDs, n=7 subjects were evaluated for suspected MXE assumption, and confirmed positive by LC-MS. The same patients were screened with both Mephedrone/Methcathinone (Bath Salt I) and MDPV (Bath Salt II) assays resulting negatives, as further confirmed by LC-MS (Table 5).

## Discussion

Even though (i) several confirmatory LC-MS/MS and GC-MS methods have been previously published for the determination of synthetic cathinones in biological samples, [26-33] and (ii) an increasing number of labs are using MS-based technology, regrettably rather few hospital-based clinical laboratories possess the capability to perform these tests and to give a robust clinical toxicological consultation. Thus, screening methods such as ELISA immunoassays continue to be widely employed in hospital-based laboratories, representing a rapid and cost-efficient approach to gain basic information about the drug content of a biological specimen [34-36].

Few specific immunoassays are available for new designer drugs, including the newest compounds such as the synthetic cathinones and their derivatives [25,36,37]. Noteworthy, it has to be remarked that the

recently developed ELISA assays, presently applied in our study, were specifically designed to detect synthetic cathinones.

Altogether, our results highlighted a good global correspondence between results obtained with the Mephedrone/Methcathinone and MDPV kits and data gained by LC-MS, employed to confirm cathinones (and their derivatives) presence, showing the complete absence of false negative data, and evidencing a mismatch only in very few cases related to positive results. Thus, the total lack of false negatives further support the reliability of the screening test approach.

Specifically, with regard to Mephedrone/Methcathinone (Bath Salt I), in accordance to our results, previous literature data demonstrated that the Randox Mephedrone/Methcathinone kit was specific for several cathinone derivatives (i.e. mephedrone, methcathinone, methylone, 4-MEC, and 4-FMC) [36]. The same authors also reported that the Mephedrone/Methcathinone kit did neither demonstrate cross-reactivity towards MDPV (indicating the possibility that the nitrogen-containing ring system on MDPV hindered the antibodies activity) nor towards other phenethylamines. Regarding the n=3 false (i.e. mismatching) positive specimens, not confirmed by LC-MS, it has to be reported that two samples belonged to subjects screened and confirmed as polydrug abusers, positives for  $\Delta$ -9-THC, AMP, MDMA and for COC and MDMA, respectively, while the remaining one was tested positive for MXE by LC-MS. We can exclude the occurrence of potential interference/crossreactivities of these substances with the antibodies/enzyme employed for the ELISA assay, based on (i) the manufacturer's indications, clearly reporting that even urinary concentrations as high as 7 - 10 microg/ml of  $\Delta$ -9-THC, AMP, MDMA, and COC elicited a negative response when tested with Mephedrone/Methcathinone kit, (ii) our lab experience testing THC-, AMP-, MDMA- and COC-true positive specimens (other than those included in these study), demonstrating the lack of any reactivity using Mephedrone/Methcathinone assay (data not shown), as well as (iii) recent experimental literature data from Swortwood et al., [36] assessing the crossreactivities of designer drugs, e.g. cathinones, with several types of immunoassay. Moreover, it has to be mentioned that Ellefsen et al. [25] using Randox Drugs of Abuse (DOA-V) biochip array technology assay, assessed crossreactivities of some cathinones, included in those determined by MDPV (Bath Salt II) kit, with Bath Salt I targeting Mephedrone/Methcathinone. In particular, crossreactivities of about 50% and 1% were reported for methedrone/butylone and for 4-MPBP/MDPPP, respectively.

Concerning the MDPV analysis, accordingly to our data, previous experimental investigation demonstrated that the Randox MDPV kit was extremely selective, particularly for butylone [36]. With respect to the false (i.e. mismatching) positives (n=2), not confirmed by LC-MS, these two urine specimens were screened and confirmed positive for different substances (i.e. COC and KET), belonging to subjects recognized as polydrug abusers. Similarly to what previously hypothesised for Mephedrone/Methcathinone test, once more, for MDPV kit, we can rule out the occurrence of non-specific crossreactivities of the two mentioned substances (i.e. COC and KET) based on our previous lab dosages showing that COC- and KET-true positive samples resulted negative when tested with MDPV assay (data not shown), in accordance to the manufacturer's data (MDPV-negative response even at COC and KET urinary concentrations as high as 10 microg/ml).

Otherwise, it has to be considered for the mismatching data obtained from either screening ELISA assay that these not confirmed positive specimens were tested by LC-MS only for certified reference

standards available in our labs (see Materials and Methods section and Table 2 for details). Therefore, the discrepancy between screening and confirmatory data could be ascribable to the presence of substance/metabolites not yet included in LC-MS analyses, but still identified by ELISA assay. In fact, further recent literature data seem to support this hypothesis demonstrating that even 4-MPBP and MDPPP cross-react with Bath Salt II (targeting MDPV/MDPBP) [25].

Moreover, even though the patient samples were collected within 12 hours from the admission to EDs, and properly stored at -20°C until the analyses, we cannot exclude that the instability of synthetic cathinones in urines (over the interval between screening and confirmatory determination) could have also contributed to the false positive screening rate [30].

Approaching another issue, grounding on previous literature demonstrating that some cathinones, including MDPV and butylone, produced false positive results in Phencyclidine (PCP) immunoassays, [8,38-40] we reason about the possibility that an inverse mechanism may occur.

Thus, with the aim to clarify this hypothesis, we evaluated the potential cross-reactivity of NPS, structural analogues of KET and PCP, i.e. MXE, [41-44] with the employed ELISA screening assays. These data plainly demonstrated that no interferences/crossreactivities occur when testing MXE-positive urine specimens for cathinones presence with the two ELISA kits.

Current literature data on the metabolism of the available bath salts are still limited, and predicting their half life time as well as detection window is complex.

Furthermore the required "effective" dose for these synthetic stimulants is much lower than their cocaine/ecstasy/amphetamine counterparts (usual dose of about 100 mg), thus resulting in lower excreted metabolite levels, even though accompanied by higher psychoactive potency. To date, MDPV is known to produce psychoactive effects with intake doses as low as 3 to 5 mg, depending on its route of administration, with reported average dose of approximately 5-20 mg, [45] while mephedrone has typical doses ranging between 25 and 75 mg, with 90 mg being considered an elevated dose [46].

The pharmacokinetics of bath salts (i.e. MDPV) has not yet been rigorously studied; Ross and co-authors [45] produced preliminary data, based on a single case report study, of a rapid clearance from the blood with a half-life of 1.88 hours. A recent epidemiological investigation demonstrated that drug effect timing depends on the route of administration, possibly starting within minutes after with nasal insufflation. Furthermore, the drug's "rush" may peak at 90 minutes, with duration of action of 3-4 hours, subsequently followed by about 1 hour come-down, for a total experience typically in the range of 6-8 hours [47].

Marinetti and Antonides [31] reported the occurrence of desired effects within 30-45 min (after intake), enduring from 1 to 3 hours, with the counterpart undesirable side outcomes lasting from hours to days. Winder et al. [46] provided detailed information on both MDPV and Mephedrone pharmacokinetics, summarizing the available literature data. Specifically, typical intake doses of 5-25 and 25-75 mg were reported for MDPV and Mephedrone, respectively. The drug onset ranged from 60 to 90 minutes for MDPV, with an estimated duration effect of 2.5 hours. Regarding the Mephedrone, the peak effect was expected within 30 minutes, followed by a rapid withdrawal.

Based on (i) the available pharmacokinetic and pharmacodynamic study, and (ii) the comparison with the pharmacokinetic of classical stimulant drugs, a predicted detection window of 48-72 hours can be hypothesised in urine specimens [45,46,48-52].

Regarding to bath salt concentrations detected in biological fluids, despite MDPV and mephedrone have been directly implicated in a number of case reports/series/fatalities relating to individuals presenting to healthcare facilities with acute toxicity, [53-56] there is no unanimous consensus on what constitutes toxic or lethal levels.

Our screening data reporting MDPV and Mephedrone/methcathinone urinary levels of about 850 and 16 ng/ml, respectively, in positive samples, further corroborated by the confirmatory analyses by LC-MS, seem to be in line with the available literature data.

In a retrospective case series of 236 patients reported by two USA poison centres with exposures to bath salts, [57] MDPV was detected in 13 of 17 live patients, with blood serum levels ranging from 24 to 241 ng/ml (mean 58 ng/ml). GC-MS determination in urine samples demonstrated MDPV values ranging between 34 and 1386 ng/ml, with a mean level of 856 ng/ml. Furthermore, quantitative analysis performed on postmortem samples detected MDPV in blood at 170 ng/ml and in urine at 1400 ng/ml.

Thornton and colleagues [34] described a case report of psychosis after reportedly insufflating a "bath salt" product; LC-TOF/MS testing revealed both MDPV and flephedrone. MDPV concentrations in serum and urine were 186 and 136 ng/ml, respectively, while flephedrone levels of 346 and 257 ng/ml were determined in the serum and urine, respectively. Ojanperä et al. [29] evaluating urine specimens from patients with a history of stimulant misuse, reported a range of MDPV of 40 to 3800 ng/ml, with a median value of 160 ng/ml. In a case report documenting hyperthermia and multiorgan failure after abuse of bath salts, patient urine, from the day of admission, tested positive for MDPV showing a concentration of 140 ng/ml by LC/MS [58]. Marinetti and Antonides [31] describing several intoxication cases, reported MDPV blood concentration of 6 - 368 ng/ml, with an average of 100 ng/ml. Moreover, in a recent case of confirmed MDPV related death, the serum and urine concentrations were 82 ng/ml and 670 ng/ml respectively [9,55].

Parallely, several cases of fatal poisoning with mephedrone have been reported in the last years, [59-61] as also described by Advisory Council on the Misuse of Drugs from the UK. [22]. Some of these cases involved combined use with other drugs, such as cannabis or heroin, and tested mephedrone levels in blood and urine as high as 500 and 198000 ng/ml, respectively. In a published series of 4 fatalities in Scotland measured mephedrone concentrations in blood ranged from 1200 to 22000 ng/ml.

On the other hand, Lusthof et al. [53] describe a case of extreme agitation and death after the use of mephedrone in The Netherlands; toxicological analyses by GC-MS in post-mortem samples demonstrated mephedrone levels of 5100 and 186000 ng/ml in blood and urine, respectively.

A recent work evaluating clinical features in analytically confirmed cases of 3-Methylmethcathinone (3-MMC, a structural analogue of mephedrone) exposure among patients presenting to hospitals in Sweden, established serum concentrations ranging between 2 to 1490 ng/ml (median 91 ng/ml), while urinary levels were 7-290000 ng/ml (median 3050 ng/ml) [62].

From a clinical point of view, patients coming to medical attention with bath salt intoxication can display agitation, combative behavior, psychosis, tachycardia, and hyperthermia [57,58,63,64]. Health care workers should be cognizant that patients presenting with this constellation of symptoms may have taken bath salts, other than cocaine, ecstasy or amphetamine, thus needing a primarily supportive treatment with benzodiazepines for agitation and excessive sympathetic stimulation, as well as aggressive cooling for severe hyperthermia [2,45,57,65].

In this clinical context, a pivotal support to improve differential diagnosis as well as to aid real-time patient care and management may come from the employment of both Mephedrone/Methcathinone and MDPV screening assay as useful tools in emergency setting to rapidly detect these NPS, also allowing the clinician to address a focused and quick treatment.

## Conclusions

In summary, our data highlight a good overall match between results obtained with the two analytical methods, evidencing an incongruity only in very few cases related to positive results.

This discrepancy may be due to: (i) non-specific interference/crossreactivities of substances, other than cathinones, with the ELISA kit, (ii) presence of substance/metabolites not yet included in LC-MS evaluated standards, but still identified by ELISA assay or (iii) instability of synthetic cathinones in urines, over the interval between screening and confirmatory determination.

Importantly, no false negatives were detected by screening analysis, thus suggesting the promising usefulness of this rapid and reliable tool as first approach in the emergency setting, followed by confirmatory LC-MS determination, even though the current availability of reference standards is limited, due to the continuous turnover of new synthetic substances in the drug market.

## Acknowledgements

This publication arises from the activities of the Italian National Early Warning System (NEWS) Alert Project from the Department for Antidrug Policies-Presidency of the Italian Council of Ministers, Rome, Italy. We wish to thank our technical staff for the analytical determinations.

## Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## References

1. German CL, Fleckenstein AE, Hanson GR (2014) Bath salts and synthetic cathinones: an emerging designer drug phenomenon. *Life Sci* 97: 2-8.
2. Baumann MH, Partilla JS, Lehner KR (2013) Psychoactive "bath salts": not so soothing. *Eur J Pharmacol* 698: 1-5.
3. Zawilska JB, Wojcieszak J (2013) Designer cathinones--an emerging class of novel recreational drugs. *Forensic Sci Int* 231: 42-53.
4. Hill SL, Thomas SH (2011) Clinical toxicology of newer recreational drugs. *Clin Toxicol (Phila)* 49: 705-719.
5. Davies S, Wood DM, Smith G, Button J, Ramsey J, et al. (2010) Purchasing 'legal highs' on the internet-is there consistency in what you get? *QJM* 103: 489-493.

6. Addiction (EMCDDA) (2011) Report on the risk assessment of mephedrone in the framework of the council decision on new psychoactive substances. Luxembourg. The Publications Office of the European Union; 2011193.
7. Winstock AR, Mitcheson LR, Deluca P, Davey Z, Corazza O, et al. (2011) Mephedrone, new kid for the chop? *Addiction* 106: 154-161.
8. Kasick DP, McKnight CA, Klisovic E (2012) "Bath salt" ingestion leading to severe intoxication delirium: two cases and a brief review of the emergence of mephedrone use. *Am J Drug Alcohol Abuse* 38: 176-180.
9. Coppola M, Mondola R (2012) Synthetic cathinones: chemistry, pharmacology and toxicology of a new class of designer drugs of abuse marketed as "bath salts" or "plant food". *Toxicol Lett* 211: 144-149.
10. DAWN (drug abuse warning network) Report (2013) Highlights of the 2011 Drug Abuse Warning Network (DAWN) Findings on Drug-Related Emergency Department.
11. Kavanagh P, O'Brien J, Power JD, Talbot B, McDermott SD (2013) 'Smoking' mephedrone: the identification of the pyrolysis products of 4-methylmethcathinone hydrochloride. *Drug Test Anal* 5: 291-305.
12. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (2012) Drug profiles: synthetic cathinones. EMCDDA, Lisbon, Portugal.
13. Cozzi NV, Sievert MK, Shulgin AT, Jacob P 3rd, Ruoho AE (1999) Inhibition of plasma membrane monoamine transporters by beta-ketoamphetamines. *Eur J Pharmacol* 381: 63-69.
14. Council Decision (2010) Council decision of 2 December 2010 on submitting 4-methylmethcathinone (mephedrone) to control measures (2010/759/EU).
15. Drug Enforcement Administration (DEA) (2011) Schedules of controlled substances: temporary placement of three synthetic cathinones in Schedule I. *Fed Regist* 76: 65371-65375.
16. American Association of Poison Control Centers (AAPCC) (2013) Bath salts data.
17. EMCDDA-Europol Joint Report (2010) Joint report on a new psychoactive substance: 4-methylmethcathinone (mephedrone).
18. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (2015). New psychoactive substances in Europe. An update from the EU Early Warning System. EMCDDA, Publications Office of the European Union, Luxembourg.
19. Dargan PI, Sedefov R, Gallegos A, Wood DM (2011) The pharmacology and toxicology of the synthetic cathinone mephedrone (4-methylmethcathinone). *Drug Test Anal* 3: 454-463.
20. Schifano F, Albanese A, Fergus S, Stair JL, Deluca P, et al. (2011) Mephedrone (4-methylmethcathinone; 'meow meow'): chemical, pharmacological and clinical issues. *Psychopharmacology (Berl)* 214: 593-602.
21. National Early Warning System (NEWS), <http://www.en.allertadroga.it>
22. Advisory Council on the Misuse of Drugs (ACMD) (2010). Consideration of the cathinones.
23. Chimalakonda KC, Hailey C, Black R, Beekman A, Carlisle R, et al. (2010) Development and validation of an LC-MS/MS method for determination of phencyclidine in human serum and its application to human drug abuse cases. *Anal Methods* 2: 1249-1254.
24. Dresen S, Kempf J, Weinmann W (2006) Electrospray-ionization MS/MS library of drugs as database for method development and drug identification. *Forensic Sci Int* 161: 86-91.
25. Ellefsen KN, Anizan S, Castaneto MS, Desrosiers NA, Martin TM, et al. (2014) Validation of the only commercially available immunoassay for synthetic cathinones in urine: Randox Drugs of Abuse V Biochip Array Technology. *Drug Test Anal* 6: 728-738.
26. Meyer MR, Wilhelm J, Peters FT, Maurer HH (2010a) Beta-keto amphetamines: Studies on the metabolism of the designer drug mephedrone and toxicological detection of mephedrone, butylone, and methylone in urine using gas chromatography-mass spectrometry. *Anal Bioanal Chem* 397: 1225-1233.
27. Meyer MR, Du P, Schuster F, Maurer HH (2010b) Studies on the metabolism of the a-pyrrolidinophenone designer drug methylenedioxypropylvalerone (MDPV) in rat and human urine and human liver microsomes using GC-MS and LC-high-resolution MS and its detectability in urine by GC-MS. *J Mass Spectrom* 45: 1426-1442.
28. Strano-Rossi S, Cadwallader AB, de la Torre X, Botre F (2010) Toxicological determination and in vitro metabolism of the designer drug methylenedioxypropylvalerone (MDPV) by gas chromatography/mass spectrometry and liquid chromatography/quadrupole time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 24: 2706-2714.
29. Ojanperä IA, Heikman PK, Rasanen JJ (2011) Urine analysis of 3,4-methylenedioxypropylvalerone in opioid-dependent patients by gas chromatography-mass spectrometry. *Ther Drug Monit* 33: 257-263.
30. Concheiro M, Anizan S, Ellefsen K, Huestis MA (2013) Simultaneous quantification of 28 synthetic cathinones and metabolites in urine by liquid chromatography-high resolution mass spectrometry. *Anal Bioanal Chem* 405: 9437-9448.
31. Marinetti LJ, Antonides HM (2013) Analysis of synthetic cathinones commonly found in bath salts in human performance and postmortem toxicology: method development, drug distribution and interpretation of results. *J Anal Toxicol* 37: 135-146.
32. De Castro A, Lendoiro E, Fernández-Vega H, Steinmeyer S, López-Rivadulla M, et al. (2014) Liquid chromatography tandem mass spectrometry determination of selected synthetic cathinones and two piperazines in oral fluid. Cross reactivity study with an on-site immunoassay device. *J Chromatogr A* 1374: 93-101.
33. Namera A, Kawamura M, Nakamoto A, Saito T, Nagao M (2015) Comprehensive review of the detection methods for synthetic cannabinoids and cathinones. *Forensic Toxicol* 33: 175-194.
34. Thornton SL, Gerona RR, Tomaszewski CA (2012) Psychosis from a bath salt product containing flephedrone and MDPV with serum, urine, and product quantification. *J Med Toxicol* 8: 310-313.
35. Wohlfarth A, Weinmann W, Dresen S (2010) LC-MS/MS screening method for designer amphetamines, tryptamines, and piperazines in serum. *Anal Bioanalysis Chem* 396: 2403-2414.
36. Swortwood MJ, Hearn WL, Decaprio AP (2014) Cross-reactivity of designer drugs, including cathinone derivatives, in commercial enzyme-linked immunosorbent assays. *Drug Test Anal* 6: 716-727.
37. Petrie M, Lynch KL, Ekins S, Chang JS, Goetz RJ, et al. (2013) Cross-reactivity studies and predictive modelling of "Bath Salts" and other amphetamine-type stimulants with amphetamine screening immunoassays. *Clin Toxicol* 51: 83-91.
38. Bell C, George C, Kicman AT, Traynor A (2011) Development of a rapid LC-MS/MS method for direct urinalysis of designer drugs. *Drug Test Anal* 3: 496-504.
39. Penders TM, Gestring RE, Vilensky DA (2012) Intoxication delirium following use of synthetic cathinone derivatives. *Am J Drug Alcohol Abuse* 38: 616-617.
40. Macher AM, Penders TM (2012) False-positive phencyclidine immunoassay results caused by 3,4-methylenedioxypropylvalerone (MDPV). *Drug Test Anal* 5: 130-132.
41. Hofer KE, Grager B, Müller DM, Rauber-Lüthy C, Kupferschmidt H, et al. (2012) Ketamine-like effects after recreational use of methoxetamine. *Ann Emerg Med* 60: 97-99.
42. Coppola M, Mondola R (2012) Methoxetamine: from drug of abuse to rapid-acting antidepressant. *Med Hypotheses* 79: 504-507.
43. Corazza O, Schifano F, Simonato P, Fergus S, Assi S, et al. (2012) Phenomenon of new drugs on the Internet: the case of ketamine derivative methoxetamine. *Hum Psychopharmacol* 27: 145-149.
44. Zawilska JB (2014) Methoxetamine--a novel recreational drug with potent hallucinogenic properties. *Toxicol Lett* 230: 402-407.
45. Ross EA, Reisfield GM, Watson MC, Chronister CW, Goldberger BA (2012) Psychoactive "bath salts" intoxication with methylenedioxypropylvalerone. *Am J Med* 125: 854-858.
46. Winder GS, Stern N, Hosanagar A (2013) Are "bath salts" the next generation of stimulant abuse? *J Subst Abuse Treat* 44: 42-45.



47. Psychonaut Webmapping Research Group (2009) MDPV Report. London, UK, Institute of Psychiatry, King's College London.
48. Erowid Vaults (2011) 4-Methylmethcathinone/mephedrone.
49. Erowid Vaults (2011) MDPV.
50. Rosenbaum CD, Carreiro SP, Babu KM (2012) Here Today, Gone Tomorrow...and Back Again? A Review of Herbal Marijuana Alternatives (K2, Spice), Synthetic Cathinones (Bath Salts), Kratom, Salvia divinorum, Methoxetamine, and Piperazines. *J Med Toxicol* 8: 15-32.
51. Paillet-Loilier M, Cesbron A, Le Boisselier A, Bourguine J, Debruyne D (2014) Emerging drugs of abuse: current perspectives on substituted cathinones. *Subst Abuse Rehab* 5: 37-52.
52. Katz DP, Bhattacharya D, Bhattacharya S, Deruiter J, Clark CR, et al. (2014) Synthetic cathinones: "a khat and mouse game". *Toxicol Lett* 229: 349-356.
53. Lusthof KJ, Oosting R, Maes A, Verschraagen M, Dijkhuizen A, et al. (2011) A case of extreme agitation and death after the use of mephedrone in The Netherlands. *Forensic Sci Int* 206: e93-95.
54. Maskell PD, De Paoli G, Seneviratne C, Pounder DJ (2011) Mephedrone (4-methylmethcathinone)-related deaths. *J Anal Toxicol* 35: 188-191.
55. Murray BL, Murphy CM, Beuhler MC (2012) Death following recreational use of designer drug "bath salts" containing 3,4-Methylenedioxypropylvalerone (MDPV). *J Med Toxicol* 8: 69-75.
56. Wood DM, Dargan PI (2012) Mephedrone (4-methylmethcathinone): what is new in our understanding of its use and toxicity. *Prog Neuropsychopharmacol Biol Psychiatry* 39: 227-233.
57. Spiller HA, Ryan ML, Weston RG, Jansen J (2011) Clinical experience with analytic confirmation of "bath salts" and "legal highs" (synthetic cathinones) in the United States. *Clin Toxicol* 49: 499-505.
58. Borek HA, Holstege CP (2012) Hyperthermia and multiorgan failure after abuse of "bath salts" containing 3,4-methylenedioxypropylvalerone. *Ann Emerg Med* 60: 103-105.
59. Gustavsson D, Escher C (2009) Mephedrone-Internet drug which seems to have come and stay. Fatal cases in Sweden have drawn attention to previously unknown substance. *Lakartidningen* 106: 2769-2771.
60. Torrance H, Cooper G (2010) The detection of mephedrone (4-methylmethcathinone) in 4 fatalities in Scotland. *Forensic Sci Int* 202: e62-63.
61. Dickson AJ, Vorce SP, Levine B, Past MR (2010) Multiple-drug toxicity caused by the coadministration of 4-methylmethcathinone (mephedrone) and heroin. *J Anal Toxicol* 34: 162-168.
62. Bäckberg M, Lindeman E, Beck O, Helander A (2015) Characteristics of analytically confirmed 3-MMC-related intoxications from the Swedish STRIDA project. *Clin Toxicol (Phila)* 53: 46-53.
63. Kyle PB, Iverson RB, Gajagowni RG, Spencer L (2011) Illicit bath salts: not for bathing. *J Miss State Med Assoc* 52: 375-377.
64. Prosser JM, Nelson LS (2012) The toxicology of bath salts: a review of synthetic cathinones. *J Med Toxicol* 8: 33-42.
65. Ross EA, Watson M, Goldberger B (2011) "Bath salts" intoxication. *N Engl J Med* 365: 967-968.