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Simultaneous determination of 40 novel psychoactive stimulants in urine by liquid chromatography-high resolution mass spectrometry and library matching

Marta Concheiro^{1,2}, Marisol Castaneto^{1,3}, Robert Kronstrand⁴, and Marilyn A. Huestis^{1,*}

¹Chemistry and Drug Metabolism, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD, USA

³Program in Toxicology, University of Maryland Baltimore, Baltimore, MD, USA

⁴Department of Forensic Genetics and Forensic Toxicology, National Board of Forensic Medicine, Linköping, Sweden Division of Drug Research, Department of Medical and Health Sciences, Linköping University, Linköping, Sweden

Abstract

The emergence of novel psychoactive substances is an ongoing challenge for analytical toxicologists. Different analogs are continuously introduced in the market to circumvent legislation and to enhance their pharmacological activity. Although detection of drugs in blood indicates recent exposure and link intoxication to the causative agent, urine is still the most preferred testing matrix in clinical and forensic settings. We developed a method for the simultaneous quantification of 8 piperazines, 4 designer amphetamines and 28 synthetic cathinones and 4 metabolites, in urine by liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS). Data were acquired in full scan and data dependent MS² mode. Compounds were quantified by precursor ion exact mass, and confirmed by product ion spectra library matching, taking into account product ions' exact mass and intensities. One-hundred μ L urine was subjected to solid phase cation exchange extraction (SOLA SCX). The chromatographic reverse-phase separation was achieved with gradient mobile phase of 0.1% formic acid in water and in acetonitrile in 20 min. The assay was linear from 2.5 or 5 to 500 μ g/L. Imprecision (n=15) was <15.4%, and accuracy (n=15) 84.2-118.5%. Extraction efficiency was 51.2-111.2%, process efficiency 57.7-104.9% and matrix effect ranged from -41.9 to 238.5% (CV<23.3%, except MDBZP CV<34%). Authentic urine specimens (n=62) were analyzed with the method that provides a comprehensive confirmation for 40 new stimulant drugs with specificity and sensitivity.

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Corresponding author Professor Dr. Dr. (h.c.) Marilyn A. Huestis, Chief, Chemistry and Drug Metabolism, Biomedical Research Center (BRC), National Institute on Drug Abuse, NIH, 251 Bayview Blvd, Suite 200 Room 05A721, Baltimore, MD 21224, mhuestis@intra.nida.nih.gov, Phone: 443-740-2524, Fax: 443-740-2823.

²Currently Department of Sciences, John Jay College of Criminal Justice, City University of New York, New York, NY, USA

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Keywords

LC-HRMS; novel psychoactive substance; synthetic cathinones; piperazines; amphetamines; urine

1. Introduction

Novel psychoactive substance (NPS) consumption grew rapidly, as “legal” alternatives to illicit drugs [1]. NPS are synthesized to circumvent drug regulations, and/or to enhance pharmacological activity. NPS are categorized based on chemical structures and psychotropic effects. The main groups are synthetic cannabinoids and stimulants, including but not limited to synthetic cathinones, piperazines and designer amphetamines. Recreational use of these new stimulants resulted in acute and even fatal toxicities [2-7].

Continuous NPS synthesis and marketing is an ongoing difficulty for toxicologists, since the majority of NPS are not detected by established analytical methods. Their constantly increasing number and the similarity in chemical structures within the groups make their detection an analytical challenge. Triple-quadrupole mass spectrometry with multiple reaction monitoring (MRM) is the most commonly adapted technique for confirmatory and quantitative drug analysis. Although MRM methods are sensitive and specific, their main drawback is that other drugs not included in the method are undetected, even if present at high concentrations. Due to dwell time and time segment restrictions, MRM methods generally include few compounds with retention time dependence. Qualitative and quantitative target MRM methods for urinary synthetic cathinones, piperazines and designer amphetamines target parent compounds [8-13]. NPS stimulants are generally consumed in high enough doses that the parent drugs are detected in urine [12].

High-resolution mass spectrometers (HRMS), Time-Of-Fight (TOF) and Orbitrap, offer several advantages for NPS detection. Depending on acquisition mode, unknown compounds can be identified retrospectively via data re-interrogation or newly emerging compounds can be easily incorporated [14-15]. Paul et al. [16] developed a urine screening method for NPS stimulants and common drugs by liquid chromatography-quadrupole-TOFMS (LC-QTOFMS) operated in data-dependent acquisition mode. However, no Orbitrap methods were published for NPS screening/confirmation with this approach. Li et al. [15] developed a urine screening method of 65 common drugs of abuse (amphetamines, cocaine, opiates, cannabinoids and hallucinogens) by LC-hybrid linear ion trap-Orbitrap MS.

We developed a sensitive and specific confirmation method for 40 NPS stimulants and 4 metabolites in urine by LC-hybrid quadrupole-Orbitrap MS (Q Exactive). With 100 µL urine, limits of quantification (LOQ) of 2.5-5 µg/L were achieved. Data were acquired in full scan and data-dependent MS² (ddMS²) mode. Compounds were determined by precursor ion exact mass and confirmed by product ion exact mass spectrum.

2. Materials and methods

2.1. Chemicals and materials

The sources of analytes and internal standards are described in Supplemental Material 1. Solid phase extraction (SPE) was performed with SOLA SCX 10 mg 1 mL cartridges (Thermo Scientific, Fremont, CA). Glacial acetic acid, formic acid, methanol, acetonitrile, water and methylene chloride were acquired from Fisher Scientific (Fair Lawn, NJ, USA). 2-propanol was purchased from Sigma (Milwaukee, WI, USA). Ammonium hydroxide 28-30%, dibasic sodium phosphate and monobasic sodium phosphate were obtained from JT Baker (Phillipsburg, NJ, USA). All solvents employed in the extraction were high-performance liquid chromatography (HPLC) grade, and LC-MS grade in the chromatographic system. Water for buffer preparation was purified-in-house by an ELGA Purelab Ultra Analytic purifier (Siemens Water Technologies, Lowell, MA, USA).

2.2. Instrumentation

LC-HRMS was performed on a Thermo Scientific NCS-3500RS UltiMate 3000 Binary Rapid system coupled to a Thermo Scientific Q Exactive Mass spectrometer (Thermo Scientific, Fremont, CA). The Ultimate 3000 system consisted of a degasser, a tertiary loading pump, a binary eluting pump, a column oven and an RS Autosampler. SPE was performed with a negative pressure manifold, and evaporation under nitrogen with a TurboVap LV® evaporator from Zymark (Hopkinton, MA, USA).

2.3. Preparation of standard solutions

Calibrators' working solutions at 0.01, 0.1 and 1g/L and combined IStd solution (Table 2) at 0.1g/L were prepared by appropriate methanolic dilution. QC working solutions, low 0.01g/L, medium 0.1g/L, and high 1g/L, were prepared in methanol from different providers or lots, when possible, than those employed for calibrators.

2.4. Specimen procedure

One-mL 0.1M phosphate buffer pH6 and 25 µL IStd solution were combined with 100 µL urine, gently vortexed and centrifuged 4,000×g at 4°C for 5 min. Supernatants were loaded onto SOLA SCX cartridges preconditioned with methanol (1 mL) and 0.1M phosphate buffer pH 6 (1 mL). Columns were washed with 1 mL 1M acetic acid and 1 mL methanol. Cartridges were dried via negative pressure at 10 psi for 5 min. The elution was performed with 1 mL 2%NH₄OH in dichloromethane/2-propanol (95:5, v/v). One-hundred µL 1%HCl in MeOH (v/v) were added to eluates before evaporation under a stream of nitrogen at 40°C. Dried samples were reconstituted in 200 µL mobile phase A (0.1% formic acid in water), vortexed briefly, and centrifuged at 4,000×g at 4°C for 5 min. Finally, supernatants were transferred into screw top autosampler vials containing 0.35 mL glass inserts.

2.5. Liquid Chromatography

Chromatographic separation was achieved with an Accucore C18 100×2.1 mm 2.6µm column (Thermo Scientific, Fremont, CA) and identically packed defender guard cartridges (10×2.1 mm, 2.6 µm). Gradient elution was performed with mobile phase A (0.1% formic

acid in water) and B (0.1% formic acid in acetonitrile) at 0.4 mL/min flow rate and at 35°C. The initial composition (2% B) was maintained for 2 min, increased from 2 to 10% B over 8 min, from 10 to 30% over 4 min, from 30 to 95% in 2 min, held at 95% for 1 min, and returned to initial conditions over 1 min. A 2 min equilibration followed, yielding a total run time of 20 min.

2.6. Mass Spectrometry

The Q Exactive mass spectrometer was equipped with heated electrospray ionization source (HESI-II) and operated in the positive ionization mode. We optimized the following parameters: spray voltage 3kV, capillary temperature 350°C, heater temperature 425°C, S-lens RF level 50, sheath gas flow rate 50, auxiliary gas 13 and sweep gas 3 (manufacturer's units). Nitrogen provided spray stabilization, collision-induced dissociation in the higher energy collision dissociation (HCD) cell, and the damping gas in the C-trap. The instrument was calibrated in the positive and negative mode every 25 h. The mass spectrometer acquired a full MS and ddMS² scans. Full MS optimized acquisition parameters were: resolution 35,000 (FWHM, full width at half maximum, at m/z 200); automatic Gain Control (AGC) target 5e5; maximum injection time (IT) 120 ms; scan range 100-400 m/z and centroid spectrum data type. DdMS² optimized acquisition parameters were: resolution 17,500; AGC target 2e5; maximum IT 120 ms; loop count 10 and MSX count 1 (Top 10); isolation window in quadrupole 3 m/z ; specific normalized collision energy (NCE) for each precursor m/z in inclusion list, and centroid spectrum data type. In each cycle, a full MS event is followed by ddMS² events. In a given time, when the most abundant masses in the inclusion list (up to 10 m/z) are detected above 8.3e4 (5% underfill ratio) optimized threshold in the survey scan, a ddMS² experiment was triggered at the peak apex (2-9 s). The detected precursor ion in the inclusion list is filtered in the quadrupole (3 amu width), fragmented in HCD cell, and product ions are collected in the C-trap and analyzed in the Orbitrap. A mass tolerance of 5 ppm was employed. NCE was optimized for each compound infusing a solution of each analyte at 100 µg/L in methanol:water (50:50) at 20 µL/min.

2.7 MS² library

The in-house library contained product ion spectra for all compounds included in the method. Product ion spectrum for each compound was acquired by injecting 250 µg/L standard solution/analyte under the described LC-HRMS conditions. The library was built with LibraryManager 2.0 (Thermo Scientific, Fremont, CA) and data were processed by TraceFinder 3.1 (Thermo Scientific, Fremont, CA). The search algorithm is based upon a cross-correlation algorithm as described by K.G. Owens [17]. The product ion spectra from the 10 largest peaks are compared against the library spectra. A score of 100 is assigned to 0 mass and relative intensities differences between sample spectra and the library, while a score of 0 indicates no match. As part of the correlation analysis, the software performs an auto-correlation where the input spectrum is compared against itself, thus establishing what the maximum score is and normalize the scores for each library entry against this maximum score. The criteria for library matching were set at a score of 60 with reverse searching. Reverse searching determines if any library spectra are included in the unknown spectrum.

2.8. Identification criteria

Identification criteria included retention time (RT) within 5% of average calibrator RT, precursor ion exact m/z within 5 ppm, and library score ≥ 60 .

2.9. Method Validation

Method validation was performed following SWGTOX guidelines [18]. Calibrators and QCs were prepared by fortifying blank urine samples. Validation parameters included linearity, limits of detection (LOD), limits of quantification (LOQ), bias and imprecision, ionization suppression/enhancement, extraction efficiency, process efficiency, interferences, carryover and autosampler and short-term stability studies. Linearity (r^2) was evaluated by least squares regression with 7-8 nonzero calibrators on five days. Acceptable linearity was achieved when $r^2 \geq 0.99$ and calibrators quantified within $\pm 20\%$. LOD and LOQ were determined with decreasing concentrations of drug-fortified blank urine samples; LOD was the lowest concentration with acceptable chromatography, signal/noise ratio ≥ 3 , with analytes identified according to previously described criteria (Section 2.8). LOQ was the lowest concentrations that met LOD criteria and a signal/noise ratio ≥ 10 , and bias and imprecision within $\pm 20\%$. LOD and LOQ were evaluated in triplicate on three different days ($n=9$) in urine from 3 different sources.

Assay bias and imprecision were determined at three concentrations (low, medium and high QCs) in triplicate over five days ($n=15$). Bias was evaluated for each concentration as $100 \times \text{group mean observed concentration} / \text{known concentration}$. Acceptable bias was from 80 to 120%. Imprecision was expressed as coefficient of variation (%CV) and determined by the one-way analysis of variation (ANOVA) approach to calculate combined within-run, between-run and total imprecision. Acceptable imprecision was $< 20\%$ CV.

Ion suppression/enhancement, extraction efficiency, and process efficiency for each analyte was measured at low and high QC concentrations. Ion suppression/enhancement was assessed by comparing analyte peak areas in ten different blank samples fortified with analyte and internal standard after extraction (Set 1), to peak areas of standard solutions ($n=6$) at the same concentrations (Set 2). Standard solutions were prepared fortifying QC and IStd working solution into 1 mL elution solvent and 100 μL 1% HCl in methanol (v/v). Matrix enhancement/suppression was calculated as follows: $[(\text{mean peak area Set 1} / \text{mean peak area Set 2}) - 1] \times 100\%$. Extraction efficiency was examined by comparing analyte peak areas of five different samples fortified at low and high concentrations with internal standard before extraction (Set 3), to peak areas of Set 1. Extraction efficiency was calculated as $100 \times (\text{mean peak area Set 3} / \text{mean peak Set 1})$. Process efficiency examined the overall effect of extraction efficiency and ion suppression/enhancement on quantification of analytes, which was expressed as $100 \times (\text{mean peak area Set 3} / \text{mean peak area Set 2})$.

The selectivity of the method was evaluated with endogenous and exogenous interferences. Interferences from endogenous matrix components were investigated by analyzing urine samples from ten volunteers without the addition of internal standard. Exogenous interferences including amines (amphetamine, methamphetamine, MDA, MDMA, MDEA, phentermine), cocaine (cocaine, benzoylecgonine, ecgonine methyl ester, cocaethylene) and

benzodiazepines (alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam) were analyzed by fortifying 1 mL elution solvent and 100 μ L 1% HCl in methanol (v/v) with interferences equivalent to 1,000 μ g/L in 100 μ L urine. The mixture was evaporated to dryness and reconstituted in 200 μ L mobile phase A. Interferences were considered insignificant if analytes of interest were <LOD. Lack of carryover was demonstrated by injecting triplicate internal standard-fortified blank samples after a sample fortified at 500 μ g/L. Carryover was considered negligible if the measured concentration was <LOD.

Processed sample stability was investigated by reinjecting low and high QC extracted samples stored 48 h at 4°C on the autosampler (n=3) and calculating results against the original calibration curve. In addition, short-term stability was evaluated with urine samples fortified at low and high QC concentration and stored in 3.6 mL polypropylene cryovials for 24 h at room temperature (n=3), 72 h at 4°C (n=3) and -20°C (n=3), and after three freeze-thaw cycles (n=3). No preservatives were added. Internal standard was added to each sample just prior to extraction and processed as described. Stability was considered acceptable if QC samples quantified within $\pm 20\%$ of freshly prepared QC samples (n=3).

The developed method was applied to a quality control urine sample from UTAK (Valencia, CA, USA). The control contained butylone, ethylone, mephedrone, MDPV, methedrone and methylone at 10 μ g/L. Percent deviation to the reported nominal value was calculated as follows: $(\text{Measured value} - \text{Nominal value}) \times 100 / \text{Nominal value}$. As proof of method applicability, 62 authentic urine specimens from stimulant users were analyzed. These specimens were previously screened positive for NPS by LC-QTOFMS (Poster PA39, TIAFT meeting 2014).

3. Results

The Orbitrap showed excellent accuracy (-2.2 to 0.5 ppm error) in the determination of masses, with external calibration every 25 h (Table 1). In full scan mode, more than 20 points per peak were achieved. At the peak apex, 1-2 product ion spectra were acquired, and NCE employed for each compound are summarized in Table 1.

Linearity of analyte-to-ISTD peak area ratio versus theoretical concentration was verified in urine samples from 2.5 or 5 to 500 μ g/L with $1/x$ or $1/x^2$ weighted linear regressions. The calibration model was evaluated from a set of 5 calibration curves yielding determination coefficients (r^2) above 0.95, all with residuals within $\pm 20\%$. LOD were 1-5 μ g/L, depending on the compound, and LOQ were 2.5 μ g/L for all compounds, except 2C-B-BZP, 3,4-DMMC, 5-APDB, 6-APB, DBZP and methiopropamine (5 μ g/L). Results are summarized in Table 2. Figure 1 shows the full scan total ion chromatogram (TIC) at the LOQ.

Total imprecision (<15.4%) and accuracy (84.2-118.5%) were satisfactory at all three QC concentrations (low QC 7.5 μ g/L, except 2C-B-BZP, DBZP, 5-APDB, 6-APB, methiopropamine and 3,4-DMMC 15 μ g/L; medium QC 30 μ g/L; high QC 400 μ g/L). These results are shown in Table 3. Extraction efficiencies ranged from 51.5 to 112.4%, and process efficiencies from 62.9 to 174.2%. Addition of 100 μ L 1% HCl in methanol (v/v) was necessary to avoid compound loss during evaporation. We added the lowest amount of acid

that prevented analyte loss and did not affect evaporation. Adding 50 μL yielded synthetic cathinones loss $>20\%$, and adding 150–200 μL prolonged evaporation time. Most compounds had matrix effects $<20\%$, except for 6 compounds, α -PBP, diethylcathinone, BZP and MDBZP with ion suppression from -41.9 to -25.7% , and MeOPP (22.9%) and 2C-B-BZP (238.5%) ion enhancement. In all cases CV among 10 different sources was $\text{CV}<23.3\%$, except MDBZP $\text{CV}<34\%$. Results are summarized in Table 4

Under described conditions, no interferences from any extractable endogenous urine compound ($n=10$) or from exogenous compounds were observed. No carryover was detected in any internal standard-fortified blank sample injected after a sample fortified at the upper limit of linearity ($500\text{ }\mu\text{g/L}$), either after authentic specimens at high concentrations $>500\mu\text{g/L}$.

All compounds were stable in the processed samples in the autosampler 48 h at 4°C (-18.8 to 20% difference). Short-term stability for 28 of the synthetic cathinones and metabolites was previously reported [9]. We studied short-term stability for the other 17 compounds. All showed good stability 24 h at room temperature, 72 h at 4°C and at -20°C , and after 3 freeze-thaw cycles, except MeOPP and methiopropamine (-25.2 to -21.3% difference). Results are summarized in Table 5.

Percent deviations of the UTAK quality control sample were 0 to 13% for butylone, ethylone, mephedrone, MDPV, methedrone and methylone. As proof of method, 62 authentic urine specimens were analyzed for stimulants, with 49 positive for piperazines, designer amphetamines and/or synthetic cathinones. These results are summarized in Table 6. Figure 2 shows the chromatogram of an authentic urine specimen containing synthetic cathinones. The present quantitative confirmation method confirmed the LC-QTOFMS screening results, except for one specimen that screened positive for 6-APB but was not confirmed with the present method. Up to 7 different drugs were detected in one specimen, but most were positive for 1 or 2 drugs ($n=12$ and 11 , respectively). Two drugs were detected in 11 cases, 3 and 4 drugs in 8 cases each, and 5 and 6 in 3 cases each. New non-targeted NPS were not detected by full scan screening.

4. Discussion

During method development, we evaluated different solid phase extraction procedures (mix-mode and cation exchange cartridges from different manufacturers, different washing and elution solvents, different volumes of elution solvents), and provided the procedure that yielded the best results in terms of extraction efficiency and matrix effect. The stimulant drugs included in the present method were basic compounds with a wide range of polarities. We assayed different extraction procedures targeting the highest extraction efficiency and the lowest matrix effect for as many compounds as possible. Cation exchange offered cleaner extracts than mix-mode cartridges. Washes with organic solvents other than methanol (hexane, ethyl acetate) yielded lower matrix effects but lower recoveries for polar compounds. Elution solvents with a higher percentage of organic solvents with high polarity index (2-propanol, methanol) increased matrix effects. Compared to our previous work [9], we added new drugs and drug groups (piperazines, designer amphetamines and new

synthetic cathinones); we reduced required specimen amount from 250 to 100 μ L achieving similar sensitivity and we simplified the extraction procedure reducing solvent use and time. The washing steps were simplified from 3 steps [9] to 2 (present method), and half of the elution solvent was employed (from 2 mL to 1 mL).

Although the samples were extracted by SPE, six of 40 compounds showed matrix effect; MeOPP (22.9%) and 2C-B-BZP (238.5%) ion enhancement, and α -ethylaminopentiphenone (-26.4%), BZP (-39.2%), diethylcathinone (-26.6%) and MDBZP (-41.9%) ion suppression. Among those, 2C-B-BZP (238.5%), BZP (-39.2%) and MDBZP (-41.9%) could be considered drugs with high matrix effect. Co-eluting compounds from the matrix may reduce or enhance the signal of the target analyte producing the matrix effect. This effect may be more pronounced at the beginning of a gradient (elution of salts) and towards the gradient end (elution of lipids). In the present method, BZP and MDBZP eluted early (RT 1.1 and 1.3 min, respectively) and showed ion suppression. In these cases, the short retention time may explain the ion suppression phenomenon. In the case of 2C-B-BZP, the compound eluted at 8.7 min with 10% acetonitrile + 0.1% formic acid in the middle of the gradient showing high ion enhancement. We did not determine the cause of this phenomenon, and had to optimize for simultaneous analysis of 40 analytes. The IStd employed for each compound was chosen based on RT. IStd RT was as close as possible to the analyte RT, because matrix effects change throughout the gradient. When the deuterated analog was not available, we employed the closest IStd to compensate for these effects. The same compound, 2C-B-BZP, was the only one that showed extraction efficiencies lower than 85% at low and medium QC concentrations (51.5-58%). 2C-B-BZP possess a bromine group and this could affect its polarity and therefore its extraction efficiency.

In our previously published a confirmation method for 26 synthetic cathinones and metabolites in urine by LC-HRMS, data were acquired in targeted MS² mode [9]. In targeted MS² mode, precursor ions specified in the inclusion list were selected by the quadrupole, fragmented in the HCD cell, and product ions collected in the C-trap and sent to the Orbitrap. The number of targeted MS² scan events depends directly on the number of precursor ions in the inclusion list eluting at that time. In order to achieve enough points per peak, a maximum of 9 precursor ions could be monitored in the same time window. Because of this, the number of compounds included in the method was limited and RT dependent. In the data-dependent approach, exact masses in the inclusion list monitored throughout the run trigger ddMS². The precursor ion peak was used for quantification and the product ion spectrum for confirmation. This MS method does not depend on RT and the number of compounds included in the method is unlimited. Both target-MS² and full scan+ddMS² showed comparable sensitivity (250 μ L urine, LOD 0.25-1 μ g/L, and 100 μ L urine, LOD 1-5 μ g/L, respectively) and selectivity (no endogenous or exogenous interferences) in a complex matrix such as urine. Moreover, the full scan acquisition allows later data re-interrogation to screen for unanticipated compounds. In this case, the screening is based on the precursor's exact mass and RT. These unknown compounds must be extracted and chromatographically resolved by the present method. The integration of screening and the confirmation on the same analytical instrument, may simplify the workflow in forensic and clinical laboratories saving time, costs and specimen volume.

Source parameters spray voltage, sheath gas flow, auxiliary gas and sweep gas were optimized based on mobile phase flow and analyte sensitivity. Spray voltage and the source's gas clearly affected analyte sensitivity. All these parameters affect the correct spray formation and analyte ionization. High auxiliary and sweep gas values yielded less sensitivity because they prevented the molecules to enter into the MS. S-lens RF was set at 50 and did not clearly affect analyte sensitivity. At a mass resolving power of 35,000 FWHM, compounds were quantified by accurate mass measurements of $[MH]^+$ ions, achieving more than 20 points per peak. Rajske et al [19] showed that higher resolution may increase the number of points per peak since more ions are measured with greater accuracy and more of them are identified correctly. AGC and maximum injection times were as high as possible to get the highest sensitivity but achieving at least 20 points per peak. In the present method, a mass tolerance of 5ppm was selected as a compromise between specificity and peak shape. A mass tolerance > 5ppm reduced method specificity, and narrowing the mass tolerance less than 5ppm produced peak distortion for some compounds. DdMS² was triggered if the peak intensity in full scan was above 8.3e4. This peak intensity threshold was established by examining chromatograms of blank urine samples and blank samples fortified with decreasing compound concentrations. The threshold was chosen as the limit to distinguish positive from negative samples with high sensitivity (LOD 1-5µg/L).

Although the confirmation criteria for low resolution LC- MS² methods are well established (RT within 5% calibrators' average RT, presence of two MRM transitions and correct ion ratio), there is no consensus for confirmation criteria for high-resolution methods. According to the EU Commission Decision 2002/657/EC concerning analytical method performance for compound identification [20], a precursor ion and a product ion at high-resolution can achieve 4.5 identification points, with a minimum requirement of 4 points. In the present method, compounds are identified based on RT, presence of precursor and product ion spectrum match (score ≥ 60) with a mass tolerance of 5 ppm, fulfilling the EU Commission Decision confirmation criteria. Different score criteria (50 to 100) were evaluated. A score of 60 yielded the best results in terms of specificity and positive matches at different concentrations. The score threshold was optimized at ≥ 60 to differentiate positive from negative specimens at a wide range of concentrations. During library development, we observed that the neat concentration employed to build the library affected the library matching score. Also, we observed that compounds with less than 3 product ions yielded lower library scores. Taking all these observations into account, the optimum library score threshold was set at ≥ 60.

The probability of having an interfering isobaric ion decreases employing HRMS, with increases in mass resolution and mass accuracy; however, isobaric interferences cannot be excluded. HRMS cannot discriminate isomers with the same accurate mass and retention time; therefore, LC separation is mandatory in these cases. In the present study, several compounds with the same molecular formula were chromatographically resolved (methcathinone and normephedrone; ethylcathinone, buphedrone, 5-APB and mephedrone; buphedrone ephedrine and 4-methylephedrine; 4-MEC, pentadron and 3,4-DMMC; diethylcathinone and α-ethylaminopentiphenone; ethylone and butylone; α-PVP and 4-MPBP). However, two synthetic cathinones, MPHP and PV8, had the same molecular formula (C₁₇H₂₅NO) and retention time under the previously described chromatographic

conditions. Although different types of columns (Accucore Phenyl-X, 100×2.1 mm, 2.6 µm; Ultra biphenyl, 100×2.1 mm, 5 µm; Kinetex PFP, 100×2.1 mm, 2.6 µm; Accucore C18, 100×2.1 mm, 2.6 µm), mobile phases (water with 0.1% formic acid, 1mM ammonium formate, acetonitrile, methanol) and gradients were examined, MPHP and PV8 could not be resolved. Therefore, MPHP and PV8 could not be individually identified and quantified if both were present in the specimen.

Paul et al. [16] developed a data-dependent and scheduled LC- QTOFMS method for the simultaneous identification of 62 compounds and semi-quantification of 35 compounds in urine, including 8 synthetic cathinones, 3 piperazines and 14 amphetamines. The method required 200 µL urine and LOQ 2-20 µg/L were achieved. The present study allows the identification and quantification of 28 synthetic cathinones and 4 metabolites, 8 piperazines and 4 designer amphetamines. We also included the antidepressant trazodone because the piperazine mCPP is also a trazodone metabolite [21] and monitoring both compounds will help result interpretation. We employed 100 µL urine and LOQs were 2.5-5 µg/L.

In a previous study [9], we investigated short-term urinary synthetic cathinone stability. The pyrrolidinyl-derivatives, MDPPP, MDPBP, α-PVP, 4-MPBP and MDPV, were stable 24 h at room temperature, 72 h at 4°C and after 3 freeze-thaw cycles. We observed the same behavior for the new pyrrolidinyl-derivatives included in the method, 4-methoxy-α-PVP, α-PBP and α-PVT. All the studied piperazines were stable under the studied conditions, except MeOPP at 7.5 µg/L (up to 25.2% loss). Moreno et al [22] studied the short-term stability of 4 piperazines, BZP, TFMPP, mCPP and MeOPP, in urine. The authors did not observe any analyte loss; however, the lowest concentration studied (1,500 µg/L) was higher than in the present method (7.5 µg/L). Among designer amphetamines, only methiopropamine showed losses up to 23.1%. No data are available about the stability of this compound, and further stability studies are necessary.

As proof of concept, we analyzed 62 authentic urine specimens positive for NPS. Parent compounds were excreted in high concentrations (Table 6), suggesting that parent drugs may be the recommended target for NPS stimulants detection in urine. However, the inclusion of metabolites normephedrone and 4-MEC allowed the detection of additional positive specimens. More studies about NPS stimulants metabolite identification and their excretion profiles are required. The only specimen positive for mCPP (66.9 µg/L) was also positive for trazodone (331.4 µg/L), suggesting that the subject consumed the antidepressant trazodone, and not the piperazine drug mCPP.

5. Conclusion

We present the most comprehensive validated LC-HRMS quantitative method that targets 40 NPS analytes in 100 µL urine with high sensitivity and specificity. The full scan-ddMS² approach also offers flexibility to include additional NPS with minimal method validation steps saving laboratory time and resources. We showed that many NPS stimulants can be detected as parent compounds in urine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Simultaneous novel psychoactive substances (NPS) analysis is highly challenging.
- NPS constantly emerge with similar or completely new chemical structures.
- The method quantifies 40 urinary NPS and 4 metabolites by LC-HRMS.
- Screening and confirmation data acquired in full scan and data dependent MS².
- 100 µL urine volume achieved 2.5-5 µg/L limit of quantification (LOQ).

Fig. 1.

Full scan total ion chromatogram (TIC) of urine sample fortified at 2.5 µg/L. 1, BZP; 2, MDBZP; 3, cathinone; 4, methcathinone; 5, methiopropamine; 6, 4-fluoromethcathinone; 7, methylone; 8, ethylcathinone; 9, MeOPP; 10, pFPP; 11, α-PPP; 12, buphedrone ephedrine; 13, ethylone; 14, methedrone; 15, buphedrone; 16, normephedrone; 17, diethylcathinone; 18, 5-APDB; 19, MDPPP; 20, 4-methylephedrine; 21, butylone; 22, mephedrone; 23, 2C-B-BZP; 24, 6-APB; 25, 4-MEC; 26, 4-MEC-metabolite; 27, α-PVT; 28, MDPBP; 29, αPBP; 30, pentedrone; 31, mCPP; 32, α-ethylaminopentiophenone; 33, pentylone; 34, 3,4-DMMC; 35, α-PVP; 36, DBZP; 37, 4-MPBP; 38, MDPV; 39, 4-Cl-2,5-DMA; 40, 4-methoxy-α-PVP; 41, TFMPP; 42, pyrovalerone; 43, trazodone; 44, benzedrone; 45, MPHP; 46, PV8; 47, naphyrone.

Fig. 2.

Extracted ion chromatograms an authentic urine specimen positive for α -PPP (271.9 $\mu\text{g/L}$), α -PVP (39.1 $\mu\text{g/L}$) and MDPV (499.8 $\mu\text{g/L}$).

Table 1

Molecular formula, theoretical and measured mass of the protonated analyte, mass error (ppm), retention time (RT) and normalized collision energy (NCE) for 8 piperazines, 4 designer amphetamines, 30 synthetic cathinones and 4 metabolites, and trazodone.

Group	Compound	Molecular formula	Theoretical mass [M+H] ⁺	Measured mass [M+H] ⁺	Error (ppm)	RT (min)	NCE (%)
Piperazines	2C-B-BZP	C ₁₃ H ₁₉ BrN ₂ O ₂	315.0702	315.0699	-1.0	8.7	40
	BZP	C ₁₁ H ₁₆ N ₂	177.1386	177.1383	-1.7	1.1	45
	DBZP	C ₁₈ H ₂₂ N ₂	267.1855	267.181	-1.5	12.5	40
	mCPP	C ₁₀ H ₁₃ ClN ₂	197.0840	197.0838	-1.0	11.2	55
	MDBZP	C ₁₂ H ₁₆ N ₂ O ₂	221.1284	221.1283	-0.5	1.3	30
	MeOPP	C ₁₁ H ₁₆ N ₂ O	193.1335	193.1333	-1.0	5.5	50
	pFPP	C ₁₀ H ₁₃ FN ₂	181.1135	181.1134	-0.6	5.7	55
	TEMP	C ₁₁ H ₁₃ F ₃ N ₂	231.1103	231.1100	-1.3	13.6	55
Designer amphetamines	4-Cl-2,5-DMA	C ₁₁ H ₁₆ ClNO ₂	230.0942	230.0937	-2.2	13.3	40
	5-APDB	C ₁₁ H ₁₅ NO	178.1226	178.1225	-0.6	7.7	60
	6-APB	C ₁₁ H ₁₃ NO	176.1069	176.1066	-1.7	9.6	50
	Methiopropamine	C ₈ H ₁₃ NS	156.0841	156.0839	-1.3	4.0	35
	3,4-DMMC	C ₁₂ H ₁₇ NO	192.1382	192.1382	0.0	12.3	50
	4-Fluoromethcathinone	C ₁₀ H ₁₂ FNO	182.0975	182.0975	0.0	4.9	60
Synthetic cathinones	4-MEC	C ₁₂ H ₁₇ NO	192.1382	192.1382	0.0	10.0	50
	4-methoxy-alfa-PVP	C ₁₆ H ₂₃ NO ₂	262.1801	262.1797	-1.5	13.5	45
	4-methoxymethcathinone	C ₁₁ H ₁₅ NO ₂	194.1175	194.1175	0.0	7.0	59
	4-MPBP	C ₁₃ H ₂₁ NO	232.1695	232.1692	-1.3	12.8	55
	alfa-ethylaminopentiphenone	C ₁₃ H ₁₉ NO	206.1539	206.1538	-0.5	11.9	50
	alfa-PBP	C ₁₃ H ₂₁ NO	232.1695	232.1692	-1.3	10.9	55
	alfa-PPP	C ₁₃ H ₁₇ NO	204.1382	204.1382	0.0	6.6	58
	alfa-PVP	C ₁₃ H ₂₁ NO	232.1695	232.1692	-1.3	12.4	55
	alfa-PVT	C ₁₃ H ₁₉ NOS	238.1260	238.1256	-1.7	10.0	45
	Benzedrone	C ₁₇ H ₁₉ NO	254.1539	254.1536	-1.2	14.6	30

Group	Compound	Molecular formula	Theoretical mass [M+H] ⁺	Measured mass [M+H] ⁺	Error (ppm)	RT (min)	NCE (%)
	Buphedrone	C ₁₁ H ₁₅ NO	178.1226	178.1225	-0.6	7.1	60
	Butylone	C ₁₂ H ₁₅ NO ₃	222.1124	222.1121	-1.4	8.0	55
	Cathinone	C ₉ H ₁₁ NO	150.0913	150.0913	0.0	3.0	60
	Diethylcathinone	C ₁₃ H ₁₉ NO	206.1539	206.1538	-0.5	7.5	50
	Ethylcathinone	C ₁₁ H ₁₅ NO	178.1226	178.1225	-0.6	5.5	60
	Ethylone	C ₁₂ H ₁₅ NO ₃	222.1124	222.1121	-1.4	6.8	55
	MDPBP	C ₁₅ H ₁₉ NO ₃	262.1437	262.1434	-1.1	10.1	56
	MDPPP	C ₁₄ H ₁₇ NO ₃	248.1281	248.1278	-1.2	7.9	50
	MDPV	C ₁₆ H ₂₁ NO ₃	276.1594	276.1591	-1.1	12.9	56
	Mephedrone	C ₁₁ H ₁₃ NO	178.1226	178.1225	-0.6	8.4	60
	Methcathinone	C ₁₀ H ₁₃ NO	164.1069	164.1068	-0.6	3.9	60
	Methylone	C ₁₁ H ₁₃ NO ₃	208.0968	208.0966	-1.0	5.2	50
	MPHP	C ₁₇ H ₂₅ NO	260.2008	260.2003	-1.9	15.1	50
	Naphyrone	C ₁₉ H ₂₃ NO	282.1852	282.1851	-0.4	15.4	57
	Pentadrone	C ₁₂ H ₁₇ NO	192.1382	192.1382	0.0	10.9	50
	Pentylone	C ₁₃ H ₁₇ NO ₃	236.1281	236.1278	-1.3	11.9	50
	PV8	C ₁₇ H ₂₅ NO	260.2008	260.2003	-1.9	15.1	50
	Pyrovalerone	C ₁₆ H ₂₃ NO	246.1852	246.1850	-0.8	14.1	54
	4-MEC-metabolite	C ₁₂ H ₁₉ NO	194.1539	194.1540	0.5	9.8	58
	4-methylephedrine	C ₁₁ H ₁₇ NO	180.1382	180.1382	0.0	8.0	60
Synthetic cathinones metabolites	Normephedrone	C ₁₀ H ₁₃ NO	164.1069	164.1068	-0.6	7.4	60
	Buphedrone Ephedrine	C ₁₁ H ₁₇ NO	180.1382	180.1382	0.0	6.7	60
Antidepressant	Trazodone	C ₁₉ H ₂₂ ClN ₅ O	372.1585	372.1581	-1.1	14.2	35

Table 2

Linearity results for 8 piperazines, 4 designer amphetamine, 28 synthetic cathinones and 4 metabolites, and trazodone in urine.

Group	Compound	Linearity (µg/L)	Weighting	Intercept ±SD (n=5)	Slope ±SD (n=5)	r ² ±SD (n=5)	LOD (µg/L)	ISId
Piperazines	2C-B-BZP	5-500	1/x ²	-0.0261 ±0.0036	0.0110 ±0.0005	0.9703 ±0.0126	5	Mephedrone-d ₃
	BZP	2.5-500	1/x ²	-0.0483 ±0.0157	0.0383 ±0.0050	0.9713 ±0.0074	1	BZP-d ₇
	DBZP	5-500	1/x	0.0419 ±0.0221	0.0156 ±0.0023	0.9967 ±0.0019	5	MDPV-d ₈
	mCPP	2.5-500	1/x ²	-0.0547 ±0.1013	0.0584 ±0.0040	0.9862 ±0.0055	1	mCPP-d ₈
	MDBZP	2.5-500	1/x ²	-0.0437 ±0.0152	0.0301 ±0.0088	0.9573 ±0.0188	2.5	BZP-d ₇
	MeOPP	2.5-500	1/x ²	0.0049±0.0074	0.0229 ±0.0027	0.9500 ±0.0593	1	Methylone-d ₃
	pPPP	2.5-500	1/x ²	-0.0051 ±0.0053	0.0191 ±0.0043	0.9846 ±0.0065	2.5	Methylone-d ₃
	TFMPP	2.5-500	1/x	-0.0386 ±0.0156	0.0492 ±0.0022	0.9934 ±0.0047	1	TFMPP-d ₄
Designer amphetamines	4-Cl-2,5-DMA	2.5-500	1/x	-0.0117 ±0.0131	0.0381 ±0.0027	0.9940 ±0.0025	1	TFMPP-d ₄
	5-APDB	5-500	1/x	0.0420 ±0.0151	0.0158 ±0.0034	0.9917 ±0.0067	5	Diethylcathinone-d ₁₀
	6-APB	5-500	1/x	-0.0151 ±0.0076	0.0146 ±0.0005	0.9918 ±0.0017	5	Mephedrone-d ₃
	Methio propamine	5-500	1/x	-0.0051 ±0.0181	0.0389 ±0.0030	0.9943 ±0.0038	5	Methylone-d ₃
Synthetic cathinones	3,4-DMMC	5-500	1/x	-0.0103 ±0.0183	0.0338 ±0.0014	0.9908 ±0.0064	5	MDPV-d ₈
	4-Fluoro methcathinone	2.5-500	1/x	-0.0010 ±0.0106	0.0345 ±0.0019	0.9913 ±0.0055	2.5	Methylone-d ₃
	4-MEC	2.5-500	1/x ²	0.0101 ±0.0035	0.0457 ±0.0020	0.9907 ±0.0033	1	Mephedrone-d ₃
	4-Methoxy-α-PVP	2.5-500	1/x	0.0355 ±0.0208	0.0907 ±0.0088	0.9972 ±0.0015	1	TFMPP-d ₄
	4-Methoxy methcathinone	2.5-500	1/x ²	0.0528 ±0.0492	0.0388 ±0.0013	0.9877 ±0.0048	2.5	Ethylone-d ₅
	4-MPBP	2.5-500	1/x	-0.0210 ±0.0057	0.0407 ±0.0014	0.9885 ±0.0055	1	MDPV-d ₈
	α-Ethylamino pentiophenone	2.5-500	1/x	0.1473 ±0.0387	0.1635 ±0.0117	0.9945 ±0.0033	2.5	mCPP-d ₈
	α-PBP	2.5-500	1/x	-0.0523 ±0.0607	0.1034 ±0.0109	0.9813 ±0.0058	2.5	mCPP-d ₈
	α-PPP	2.5-500	1/x	-0.0434 ±0.0190	0.0599 ±0.0047	0.9882 ±0.0059	1	Ethylone-d ₅
	α-PVP	2.5-500	1/x	-0.0268 ±0.0049	0.0364 ±0.0019	0.9868 ±0.0077	1	MDPV-d ₈
	α-PVT	2.5-500	1/x	-0.0222 ±0.0080	0.0400 ±0.0006	0.9927 ±0.0021	1	Mephedrone-d ₃
	Benzedrone	2.5-500	1/x	-0.0022 ±0.0113	0.0287 ±0.0009	0.9939 ±0.0027	1	Naphyrone-d ₅
	Buphedrone	2.5-500	1/x ²	0.0595 ±0.0200	0.0821 ±0.0111	0.9778 ±0.0066	2.5	Diethylcathinone-d ₁₀

Group	Compound	Linearity (µg/L)	Weighting	Intercept ±SD (n=5)	Slope ±SD (n=5)	r ² ±SD (n=5)	LOD (µg/L)	ISId
	Butylone	2.5-500	1/x	0.0076±0.0049	0.0305 ±0.0012	0.9952 ±0.0028	1	Butylone-d ₃
	Cathinone	2.5-500	1/x	-0.0060 ±0.0087	0.0217 ±0.0018	0.9889 ±0.0055	1	Methylone-d ₃
	Diethylcathinone	2.5-500	1/x	0.0121 ±0.0041	0.0357 ±0.0011	0.9901 ±0.0066	2.5	Diethylcathinone-d ₁₀
	Ethylcathinone	2.5-500	1/x	-0.0225 ±0.0123	0.0563 ±0.0039	0.9944 ±0.0034	1	Methylone-d ₃
	Ethylone	2.5-500	1/x	-0.0106 ±0.0212	0.0401 ±0.0025	0.9910 ±0.0041	2.5	Ethylone-d ₅
	MDPBP	2.5-500	1/x	0.0006 ±0.0142	0.0450 ±0.0027	0.9966 ±0.0022	1	Mephedrone-d ₃
	MDPPP	2.5-500	1/x ²	-0.0005 ±0.0090	0.0389 ±0.0025	0.9910 ±0.0017	1	Butylone-d ₃
	MDPV	2.5-500	1/x	0.0033 ±0.0106	0.0412 ±0.0018	0.9940 ±0.0033	1	MDPV-d ₈
	Mephedrone	2.5-500	1/x	-0.0039 ±0.0068	0.0404 ±0.0013	0.9961 ±0.0012	1	Mephedrone-d ₃
	Methcathinone	2.5-500	1/x	0.0173 ±0.0133	0.0488 ±0.0034	0.9944 ±0.0058	1	Methylone-d ₃
	Methylone	2.5-500	1/x	0.0143 ±0.0095	0.0404 ±0.0018	0.9959 ±0.0017	1	Methylone-d ₃
	Naphyrone	2.5-500	1/x	0.0017 ±0.0087	0.0325 ±0.0013	0.9946 ±0.0031	1	Naphyrone-d ₅
	Pentadone	2.5-500	1/x	0.1747 ±0.0356	0.1096 ±0.0047	0.9960 ±0.0018	2.5	mCPP-d ₈
	Pentylone	2.5-500	1/x	0.0097 ±0.0094	0.0265 ±0.0015	0.9940 ±0.0035	1	MDPV-d ₈
	Pyrovalerone	2.5-500	1/x	-0.0070 ±0.0078	0.0414 ±0.0016	0.9950 ±0.0032	1	Naphyrone-d ₅
	4-MEC metabolite	2.5-500	1/x ²	0.0195 ±0.0103	0.0424 ±0.0018	0.9867 ±0.0038	1	Mephedrone-d ₃
Synthetic cathinone metabolites	4-Methyl ephedrine	2.5-500	1/x ²	0.0143 ±0.0052	0.0264 ±0.0006	0.9859 ±0.0041	1	Butylone-d ₃
	Normephedrone	2.5-500	1/x ²	0.0159 ±0.0052	0.0339 ±0.0052	0.9737 ±0.0178	1	Diethylcathinone-d ₁₀
	Buphedrone ephedrine	2.5-500	1/x	0.0135 ±0.0053	0.0365 ±0.0016	0.9912 ±0.0037	2.5	Ethylone-d ₅
Antidepressant	Trazodone	2.5-500	1/x	0.0006 ±0.0183	0.0439 ±0.0027	0.9943 ±0.0031	1	Trazodone-d ₆

MPHP and PV8 were not included in the validation because they have the same molecular weight and they could not be chromatographically resolved.

Table 3

Pooled intra-, inter- and total imprecision and bias for 8 piperazines, 4 designer amphetamines, 28 synthetic cathinones and 4 metabolites, and trazodone in urine. Low QC (7.5 µg/L, except 2C-B-BZP, DBZP, 5-APDB, 6-APB, methiopropamine and 3,4-DMMC 15 µg/L); Medium QC (30 µg/L); High QC (400 µg/L).

Group	Compound	Pool intra-day imprecision (n=15, CV)			Inter-day imprecision (n=15, CV)			Total imprecision (n=15, CV)			Bias (n=15, %target)		
		Low	Med	High	Low	Med	High	Low	Med	High	Low	Med	High
Piperazines	2C-B-BZP	3.9	3.5	5.1	0	0	14.5	3.9	3.5	15.4	118.5	115.5	89.9
	BZP	3.9	3.3	6.7	7.6	0.9	2.6	8.6	3.4	7.2	99.1	112.2	89.5
	DBZP	8.9	4.8	7	2.9	6.1	0	9.4	7.8	7	106.9	111.4	99.5
	mCPP	7.5	7	7.6	1	9.5	7.6	7.6	11.8	10.8	106.1	102.5	100.7
	MDBZP	4.9	2.2	6	2.3	1.8	7.9	5.4	2.9	9.9	102.6	113.8	87.9
	MeOPP	8.9	7.8	3.7	5.2	0	2.6	10.4	7.8	4.5	101.5	104.1	84.2
	pPPP	9.5	10	4.1	0	0	3.1	9.5	10	5.1	100.6	103.5	92.5
	TFMPP	4.8	6.3	6.7	3.7	6.6	2.3	6.1	9.1	7.1	98	96.8	100.7
Designer amphetamines	4-Cl-2,5-DMA	5.2	6.9	8.2	3.3	6.9	0	6.1	9.8	8.2	102.8	99.4	102.6
	5-APDB	8.8	5.6	8.3	6.8	7.5	6.5	11.1	9.4	10.5	98	103.2	95.8
	6-APB	7.8	6.8	7.2	6.4	4	0	10	7.9	7.2	96.2	92.8	101.8
	Methiopropamine	8.5	4.8	7.3	6.4	3.4	5.9	10.6	5.9	9.4	108.1	106.2	103.8
	3,4-DMMC	6	6.6	6.6	7.5	9.9	0	9.6	11.9	6.6	97.9	99.7	106.8
Synthetic cathinones	4-Fluoro methcathinone	3.4	3.7	4.5	2	2.6	0	3.9	4.5	4.5	114.6	114	112.2
	4-MEC	2.8	3.7	6.9	0	1.5	1.4	2.8	3.9	7.1	116.7	116	113.1
	4-Methoxy- α -PVP	5.4	5.2	6.7	3.6	4.6	2.1	6.5	6.9	7	104	106.8	101.2
	4-Methoxy methcathinone	14.4	8	5.6	3.2	0	0	14.8	8	5.6	100.7	98.9	100.6
	4-MPBP	5.8	5.5	6.5	3.6	4.8	2.3	6.9	7.3	6.9	93.7	87.5	100.9
	α -Ethylamino pentiophenone	6.8	5.7	7.5	0	4.2	4.5	6.8	7.1	8.7	107.8	108.2	99.3
	α -PBP	7	7.2	7.6	5.2	4.4	5.5	8.7	8.5	9.3	103.3	94.8	109.3
	α -PPP	3.6	6	5.8	3.9	3.7	9.9	5.2	7.1	11.5	91.6	89.5	100.3
	α -PVP	5.1	7.2	6	4.1	5.4	3.2	6.5	8.9	6.8	96.4	88.9	106.6
	α -PVT	6.4	5.6	7.4	6.3	0	7.1	9	5.6	10.2	101.3	93.6	104.5

Group	Compound	Pool intra-day imprecision (n=15, CV)			Inter-day imprecision (n=15, CV)			Total imprecision (n=15, CV)			Bias (n=15, %target)		
		Low	Med	High	Low	Med	High	Low	Med	High	Low	Med	High
	Benzedrone	5.9	5.7	5.4	4	6.6	7	7.1	8.7	8.8	104.2	99.8	106.5
	Buphedrone	11.3	6.8	7.7	0	5.2	6.1	11.3	8.6	9.8	101.2	102.4	86.6
	Butylone	1.4	3.3	1.9	0.8	3.8	0.6	1.6	5.1	2	116	116.1	117
	Cathinone	5.1	8.5	5.4	3.5	3.8	6.9	6.2	9.3	8.7	98.8	92.2	102.3
	Diethylcathinone	3.8	3.8	1.4	0	2.2	1.6	3.8	4.4	2.1	114.4	116.7	116.6
	Ethylcathinone	1.8	3.4	3.6	0	0	2	1.8	3.4	4.1	117.2	116.7	116.8
	Ethylone	4.1	4.5	3.6	2.3	3.9	1.6	4.7	5.9	3.9	112.5	109.3	114.5
	MDPBP	4.3	5.4	6.9	2.7	0	4.3	5.1	5.4	8.1	112.1	112.2	109.7
	MDPPP	6	7.2	4	4.6	4.3	1	7.6	8.4	4.1	93	95.3	99.6
	MDPV	6	4.8	5.7	4.2	6.5	2.3	7.4	8	6.1	103.3	100.6	102.1
	Mephedrone	2.4	4	4.3	0	0	3	2.4	4	5.3	116.5	115.9	116.2
	Methcathinone	8.1	6.2	6.6	0	2.8	5.5	8.1	6.8	8.6	103.4	104.6	98.3
	Methylone	6.1	5.8	6.3	0	5.3	4.6	6.1	7.8	7.8	100.1	100.8	99.7
	Naphyrone	4.7	6.4	7.2	4	7.2	7.2	6.1	9.6	10.2	94.9	93.5	96.9
	Pentadrone	6.5	4.2	7.4	2.6	3.4	5	7.1	5.4	8.9	108	112	104.2
	Pentylone	6.6	6.5	6	5.1	10.3	2.8	8.4	12.2	6.6	102.3	100.5	99.6
Synthetic cathinone metabolites	Pyrovalerone	5.7	6.7	5.9	5	8.2	7.5	7.5	10.6	9.6	97.4	97	102.4
	4-MEC metabolite	7.1	5.8	5.8	5.7	4	7.7	9	7.1	9.6	104.9	103.2	91.7
	4-Methylephedrine	5.4	5.6	4.5	2.3	5.4	0	5.9	7.7	4.5	106.1	103.6	101.1
	Normephedrine	10.4	9.1	8	3.1	4.9	7.7	10.8	10.4	11.2	96.6	96.5	87.2
	Buphedrone ephedrine	6.2	6.3	6.4	2.9	4.1	1.5	6.8	7.6	6.6	102.5	100.4	103.1
Antidepressant	Trazodone	5	5.4	5.6	3.1	7.1	3	5.9	8.9	6.3	101.4	99.6	101.3

MPHP and PV8 were not included in the validation because they have the same molecular weight and they could not be chromatographically resolved.

Table 4

Extraction efficiency, process efficiency and matrix effect for 8 piperazines, 4 designer amphetamine, 28 synthetic cathinones and 4 metabolites, and trazodone in urine. Low QC (7.5 µg/L, except 2C-B-BZP, DBZP, 5-APDB, 6-APB, methiopropamine and 3,4-DMMC 15 µg/L); Medium QC (30 µg/L); High QC (400 µg/L).

Group	Analyte	Extraction Efficiency (n=6)			Process Efficiency (n=6)			Matrix Effect (CV) (n=10)		
		Low	Med	High	Low	Med	High	Low	Med	High
Piperazines	2C-B-BZP	51.5	58	84.9	174.2	127.8	101.6	238.5 (6)	120.4 (13.5)	19.6 (4.3)
	BZP	107.3	101.2	105.9	78.5	61.5	64.4	-26.9 (18.3)	-39.2 (23.3)	-39.2 (12.7)
	DBZP	97.4	92.2	108.9	97.5	93.1	87.5	0.2 (6.2)	1 (14)	-19.6 (5.3)
	mCPP	93.3	99.8	96.4	90.6	95.5	88.3	-2.9 (5.4)	-4.2 (12.5)	-8.4 (5.5)
	MDBZP	98.8	96.1	108.2	87.6	64	62.9	-11.3 (24.7)	-33.4 (34)	-41.9 (17.2)
	MeOPP	88.4	87	96.4	108.7	105	97.8	22.9 (12.5)	20.7 (15.9)	1.4 (8.5)
	pPPP	93.1	96.6	97	98.4	97.8	91.6	5.7 (5.5)	1.2 (15.3)	-5.5 (13.1)
Designer amphetamines	TFMPP	98.2	101.6	96.8	88.9	92.2	87.2	-9.5 (2.9)	-9.3 (13.1)	-9.9 (3.1)
	4-Cl-2,5-DMA	95.7	100.4	94.9	94.5	94.6	91	-1.3 (2)	-5.8 (13.3)	-4.1 (3.6)
	5-APDB	100.8	101.1	96.1	91.7	96.3	85.8	-9 (3.9)	-4.7 (15.1)	-10.7 (4)
	6-APB	108.6	100.7	94.3	100.1	95.4	83.6	-7.8 (3.8)	-5.2 (13.7)	-11.3 (6.3)
	Methiopropamine	96.4	100.2	96.4	101.5	100.5	96	5.3 (3.7)	0.2 (14.4)	-0.5 (3.8)
Synthetic cathinones	3,4-DMMC	93.9	97.8	93.9	102.2	101.7	91.1	8.8 (2.9)	4 (12.3)	-3 (2.9)
	4-Fluoromethcathinone	98.8	112.4	100.6	111.9	115.8	99.8	13.3 (6.5)	3 (18.6)	-0.8 (5.2)
	4-MEC	102.9	102.6	94.5	95.7	93.4	89.2	-6.9 (4)	-9 (14)	-5.7 (2.9)
	4-Methoxy- α -PVP	92.7	96.6	95.7	96.7	97.8	94.6	4.4 (3.7)	1.3 (13.2)	-1.1 (2.9)
	4-Methoxymethcathinone	96.2	100	95.5	91.4	96.4	93.7	-5 (4.1)	-3.6 (13.2)	-1.8 (3.2)
	4-MPBP	96.2	94.7	97.1	95.2	94.5	97.2	-1.1 (3.6)	-0.2 (13.3)	0.2 (4.2)
	α -Ethylamino pentiophenone	102.3	97.4	101.8	89	86.2	92.1	-12.9 (3.6)	-11.6 (14.4)	-9.5 (5.7)
	α -PBP	111.2	91.5	108.4	82.6	72.2	79.8	-25.7 (9.7)	-21 (15.3)	-26.4 (11.8)
	α -PPP	95.5	102.3	99.2	101	103.5	97.3	5.8 (3.8)	1.1 (13.1)	-1.9 (4.4)
	α -PVP	101.3	94.7	99.1	94.3	89.4	92.6	-6.9 (2.4)	-5.6 (13.5)	-6.6 (6.2)
	α -PVT	98.3	99.1	100.5	93.1	94.1	94.7	-5.3 (5.3)	-5 (13.6)	-5.8 (5.2)
	Benzedrone	99	95.8	99.5	94.6	91.6	93	-4.4 (3.4)	-4.4 (13.4)	-6.5 (7.3)

Group	Analyte	Extraction Efficiency (n=6)			Process Efficiency (n=6)			Matrix Effect (CV) (n=10)		
		Low	Med	High	Low	Med	High	Low	Med	High
	Buphedrone	97.8	98.7	98	97	94.4	89.3	-0.8 (3.7)	-4.4 (14.1)	-8.9 (4.6)
	Butylone	105.1	102.6	95.1	105.4	95	85.4	0.3 (3.4)	-7.4 (14)	-10.2 (3.6)
	Cathinone	92.6	97.4	93.4	90.1	96.6	91.3	-2.7 (5.2)	-0.8 (15.6)	-2.3 (5)
	Diethylcathinone	97.6	89.8	108.4	71.6	77.1	80.3	-26.6 (10.3)	-14.1 (16.9)	-26 (22.8)
	Ethylcathinone	99.3	99.4	98.9	99.1	100.1	96.4	-0.2 (3.4)	0.6 (13.4)	-2.5 (4.7)
	Ethylone	87.5	98	100.5	99.3	101.4	101.7	13.5 (4.7)	3.6 (12.9)	1.1 (4.6)
	MDPBP	98.2	100.6	97.5	104.6	103.4	98.6	6.5 (3.6)	2.8 (13.8)	11 (3.7)
	MDPPP	96.7	98.5	94.8	103.3	99.9	91	6.7 (3.9)	1.4 (13.8)	-3.9 (3.3)
	MDPV	95.3	97.6	96.6	98.4	99.3	95.5	3.2 (3.8)	1.7 (12.8)	-1.1 (3.1)
	Mephedrone	105	102.6	92.7	94.3	88	81.1	-10.2 (5.8)	-14.2 (13.8)	-12.5 (3.1)
	Methcathinone	96.1	95.1	94.5	101.1	98.7	93.6	5.2 (2.8)	3.7 (14)	-0.9 (5)
	Methylone	96.4	99.5	95.3	89.8	99.4	91.1	-6.9 (2.7)	-0.2 (14.8)	-4.4 (4.7)
	Naphyrone	89.6	88.3	95	91.1	89.1	91.8	1.7 (3.4)	0.9 (13.3)	-3.4 (5.8)
	Pentedrone	103.4	98.5	100.2	89.3	86	89.5	-13.6 (4.4)	-12.7 (13.5)	-10.6 (5.4)
	Pentylone	99	101	94.6	93.9	95.9	91.9	-5.2 (2.7)	-5 (14.9)	-2.9 (2.4)
Synthetic cathinone metabolites	Pyrovalerone	97.2	96.4	97.5	96.2	93.7	94.7	-1 (4.2)	-2.8 (13.4)	-2.8 (5.1)
	4-MEC metabolite	96.9	99.9	89.7	98.4	99.7	96.5	1.5 (1.5)	-0.3 (13.1)	7.6 (2.8)
	4-Methylephedrine	101.6	99.6	92.6	98.8	94.6	84.9	-2.8 (2.5)	-5 (13.7)	-8.3 (4.2)
	Normephedrone	93.6	98.5	93.9	94	99.3	85.5	0.5 (2.9)	0.9 (12.9)	-8.9 (3.3)
Antidepressant	Buphedrone ephedrine	95.8	97.3	91.7	109.3	110.1	94.3	14.1 (2.8)	13.2 (12.5)	2.9 (5.8)
	Trazodone	85.1	89.7	90.4	86.7	98.3	93.6	1.8 (3.8)	9.6 (13.7)	3.6 (4.7)

MPHP and PVS were not included in the validation because they have the same molecular weight and they could not be chromatographically resolved.

Short term stability data (% difference) of novel stimulants in urine after storage at room temperature 24h, at 4°C 72h and after 3 freeze-thaw cycles. Low QC (7.5 µg/L, except 2C-B-BZP, DBZP, 5-APDB, 6-APB, and methiopropamine 15 µg/L); Medium QC (30 µg/L); High QC (400 µg/L).

Table 5

Group	Analyte	Room Temperature 24h (n=3)		4°C 72h (n=3)		-20°C 72h (n=3)		3 freeze-thaw cycles (n=3)	
		Low	High	Low	High	Low	High	Low	High
Piperazines	2C-B-BZP	1.7	-5.5	-2.1	10.4	-2.5	-4.3	-8	2.9
	BZP	1.7	0	6.4	-3.2	4.9	-7.3	-0.4	5.4
	DBZP	-5.1	-6.7	4.2	-5	1.7	-9.8	-1.6	-5.9
	mCPP	20.2	4	19.6	1.4	18	-10.4	19.4	2.2
	MDBZP	4.8	7.6	-0.5	1.2	3.4	-2.5	6	7.8
	MeOPP	-25.1	-9.6	-21.3	-2.4	-24.3	-12.1	-25.2	5.5
Designer amphetamines	pFPP	-5.7	-12.5	-13.2	-5.3	-3.4	-13	-7.2	-3.6
	TFMPP	4.6	-3	6.2	-10.1	7.9	-17.5	4.8	-8.2
	4-Cl-2,5-DMA	8.7	4.3	8.9	0.3	10.1	-9.2	9	1
	5-APDB	-1.1	18.1	-1	11.4	-3.9	5.8	-8.4	18.8
	6-APB	18.4	16.6	11	13.2	11.9	-1.4	2.4	12.2
	Methiopropamine	-9.2	-12.3	-19.8	-23.1	-11.6	-21.7	-21.6	-17.8
Synthetic cathinones	4-Methoxy-α-PVP	6.1	5.9	4.4	2.1	8.9	-5.8	11.7	2
	α-Ethylamino pentiophenone	-6.6	-3.6	-1.9	-8.8	2.8	-12.3	-1.2	-9.7
	α-PBP	-18.3	-6.5	-8	-13.5	-4.5	-16.2	-15.2	-3.4
	α-PVT	6.7	5.4	2.6	0.8	7.6	-8.3	-0.8	-4.6
Antidepressant	Trazodone	5.2	-2.6	1.6	-7.2	7.1	-14.3	6.2	-5.6

Table 6

Authentic urine specimens (n=49) positive for piperazines, designer amphetamines and/or synthetic cathinones.

Group	Compound	N positive specimens	Range (µg/L)
Piperazines	2C-B-BZP	0	NA
	BZP	0	NA
	DBZP	0	NA
	mCPP	1	66.9
	MDBZP	0	NA
	MeOPP	0	NA
	pFPP	0	NA
	TFMPP	0	NA
Designer amphetamines	4-Cl-2,5-DMA	0	NA
	5-APDB	2	63.6 - >10,000
	6-APB	6	3.5 - >10,000
	Methiopropamine	3	30 - >10,000
Synthetic cathinones	3,4-DMMC	1	107
	4-Fluoromethcathinone	0	NA
	4-MEC	3	3.8 – 2,437
	4-methoxy-alfa-PVP	0	NA
	4-methoxymethcathinone	0	NA
	4-MPBP	0	NA
	alfa-ethylaminopentiophenone	0	NA
	alfa-PBP	0	NA
	alfa-PPP	1	271.9
	alfa-PVP	23	2.9 - >10,000
	alfa-PVT	3	5 - 724
	Benzedrone	0	NA
	Buphedrone	1	3.1
	Butylone	0	NA
	Cathinone	5	555 – 3,892
	Diethylcathinone	0	NA
	Ethylcathinone	2	3.9 – 4.4
	Ethylone	2	13.2 - 887
	MDPBP	10	4.7 – 117.5
	MDPPP	9	72.8 - 900
	MDPV	6	6 - > 10,000
	Mephedrone	11	12.4 – 3,597
	Methcathinone	3	2.7 – 6.1
	Methylone	3	13.1 – 3,759

Group	Compound	N positive specimens	Range (µg/L)
	MPHP	0	NA
	Naphyrone	0	NA
	Pentedrone	3	16.2 – 3,864
	Pentylone	2	104.7 - >10,000
	PV8	0	NA
	Pyrovalerone	0	NA
Synthetic cathinones metabolites	4-MEC-metabolite	2	3.6 – 6.1
	4-methylephedrine	6	10.7 – 332.5
	Normephedrone	12	3.8 – 8,785
	Buphedrone Ephedrine	0	NA
Antidepressant	Trazodone	1	331.4

NA: not applicable