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Plasma drug screening using paper spray mass spectrometry with integrated solid phase extraction

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Abstract

Drug overdoses have risen dramatically in recent years. We developed a simple non-targeted method using a disposable paper spray cartridge with an integrated solid phase extraction column. This method was used to screen for ~160 fentanyl analogs, synthetic cannabinoids, other synthetic drugs, and traditional drugs of abuse in over 300 authentic overdose samples collected at emergency departments in Indianapolis. A solid phase extraction step was implemented on the paper spray cartridge to enable subnanograms per milliliter synthetic drugs screening in plasma. Analysis was performed on a quadrupole orbitrap mass spectrometer using the sequential window acquisition of all theoretical fragment ion spectra approach in which tandem mass spectrometry was performed using 7 m/z isolation windows in the quadrupole. Calibration curves with isotopically labeled internal standards were constructed for 35 of the most frequently encountered synthetic and traditional illicit drugs by US toxicology labs. Additional qualitative-only drugs in a suspect screening list were also included. Limits of detection in plasma for synthetic cannabinoids ranged from 0.1 to 0.5 and 0.1 to 0.3 ng/mL for fentanyl and its analogs and between 1 and 5 ng/mL for most other drugs. Relative matrix effects were evaluated by determining the variation of the calibration slope in 10 different lots of biofluid and found to be between 3% and 20%. The method was validated on authentic overdose samples collected from two emergency departments in Indianapolis, Indiana, from suspected or known overdoses. Commonly detected synthetic drugs included fentanyl related substances, designer benzodiazepines such as flubromazolam, and the synthetic cannabinoid 5F-PB-22.

KEYWORDS
3D printing, ambient ionization, high resolution, methamphetamine, opiates

Hannah Zimmerman-Federle and Greta Ren contributed equally.

1 | INTRODUCTION

Designer drugs or new psychoactive substances (NPS) are a major public health issue[1–4]. Most NPS are chemical analogs of known drugs of abuse and are meant to elicit similar or augmented psychoactive effects[5–7]. They are sometimes sold as "legal highs" to avoid legal consequences associated with traditional illicit drugs of abuse, and when a substance is banned, a new slightly modified compound can be manufactured to avoid detection by law enforcement[3–6,8]. The number of new synthetic drugs has been rising at alarming rate, and now, there are approximately 900 synthetic compounds; these include stimulants, opiates, sedatives, and hallucinogenic compounds[1–3,5–7,9,10].

NPS have usually not been studied in humans and often have unpredictable harmful effects, including death[1–7,11–16]. NPS, such as fentanyl analogs and synthetic cannabinoids, may have higher potency than classical drugs with unwanted effects[4,16,17]. Therefore, emergency department visits due to drug use have increased as the use of NPS has increased dramatically[18–20], and the mortality rate for accidental drug poisoning is now the leading cause of injury death in the United States[1,21,22]. NPS are not detected by routine drug screens used in hospitals[16,23–25]. Often, both the patient suffering from complications related to drug use and the medical team providing their care are unaware of what drug(s) were taken[6,24,26]. Specimens can be shipped to specialized reference laboratories for testing; however, the turnaround time usually ranges from several days to weeks; therefore, nonfatal overdose samples are not routinely tested[13,23,25,27]. Furthermore, due to a lack of rapid technology to screen for new synthetic drugs, public health officials often cannot identify the involved substance behind a spike in community overdoses until the outbreak has passed[15,28].

Most common methods used for drug screening are either immunoassays (IAs) or chromatography techniques, such as liquid chromatography (LC) or gas chromatography. IAs are commonly used in a clinical setting, because they require little to no sample preparation and are capable of high throughput. Typically, they are not available for new synthetic drugs and take from months to years to develop and validate[10,29]. Additionally, their limits of detection (LODs) may be too high for more potent drugs[25,30]. There are also issues with selectivity due to synthetic drugs having similar structures[31–34]. Additionally, IAs typically only target specific groups of compounds, while many overdose samples contain multiple drugs from the same group[26].

Mass spectrometry (MS) has been proposed as an alternative to IAs, due to its higher selectivity, sensitivity, and ability to multiplex[35]. Moreover, high-resolution MS is capable of nontargeted analysis that allows new compounds to be quickly added to the method as they emerge in the dynamic synthetic drug market[28,35,36]. Chromatography methods coupled to MS, such as gas chromatography–MS or LC–MS, have excellent selectivity and sensitivity and are commonly used to detect new synthetic drugs[10,28,36–43]. Nevertheless, these assays are typically labor intensive and time-consuming, require a skilled analyst, and are rarely used for drug screening at the point of care[28]. The rapid rise in the number of synthetic drugs requires a method that is sensitive and selective, requires minimal sample preparation, and has a fast turnaround time. Ambient ionization techniques can quickly analyze complex samples with minimal sample preparation and have great potential to be used at point-of-care settings[44].

Paper spray MS (PS-MS) can perform rapid, direct analysis of samples spotted on paper or another porous substance[45,46]. A small amount of sample (5–15 μL) is placed directly onto a porous, triangular paper substrate and allowed to dry. The dried sample is positioned close to the inlet (∼4–6 mm) of an unmodified mass spectrometer. A spray solvent is applied to the back of the paper substrate; as the spray solvent wicks through, the analytes are extracted from the dried sample. A high voltage is applied, and gas-phase charged ions are directly generated from the tip via a mechanism similar to electrospray[47], and the generated ions are detected by the mass spectrometer. PS-MS has been used for a variety of applications: screening for drugs of abuse in post-mortem blood samples[48,49]; detecting pesticides in produce[50]; identifying designer drugs from herbal blends[51], blotter paper[52], and saliva[53]; analyzing fentanyl in blood and urine[54] and fentanyl analogs in urine[55]; and testing neat synthetic cannabinoids[56] and their presence in biological fluids[57]. However, the detection limits of paper spray analyses are sometimes too high for certain applications, including detection of synthetic drugs. Several studies have improved detection limits by coating the paper with various substrates (such as silica[58], polystyrene[59,60], organosilanes[61–63], and zinc oxide[64]).

In this paper, we describe a semiquantitative, nontargeted, PS-MS screening method to detect emerging synthetic drugs. We used a previously developed "all-in-one" paper spray cartridge with integrated solid phase extraction (SPE)[65]. The cartridge can extract and preconcentrate drugs from plasma samples, which allowed us to achieve LODs necessary to screen more potent drugs. The described method was used to analyze suspected overdose samples collected from two urban Emergency Departments.

2 | MATERIALS AND METHODS

2.1 | Chemicals and materials

HPLC grade acetonitrile, methanol, and formic acid were purchased from Fisher Scientific (Waltham, MA, USA). 25I-NBoMe, 5F-ADB, 5F-PB-22, AB-CHIMINACA, AB-CHIMINACA d4, AB-PINACA, AB-PINACA d9, ADB-FUBINACA, AM2201, AM-2201 d5, AMB-FUBINACA, APINACA, APINACA d9, cyclopropyl fentanyl, cyclopropyl fentanyl d5, FIBF, FIBF d7, MMB-CHMICA, remifentanil, THJ-2201, U-47700 d6, and XLR-11 were acquired from Cayman Chemical (Ann Arbor, MI, USA). All other analytical standards were obtained from Cerilliant (Round Rock, TX, USA). Standards were stored at −20°C. Whatman ET31 paper and gel plotting paper were bought from Whatman (Piscataway, NJ, USA). Nylon membrane filter, forty-one micrometer pore size, of was acquired from Millipore-Sigma (Burlington, MA, USA). Strata-X RP SPE was obtained from Phenomenex (Torrance, CA, USA). Top part of the cartridge was 3D printed

using a polypropylene filament using an Ultimaker 2+ extended (Geldermalsen, Gelderland, Netherlands). Human plasma with K2-EDTA anticoagulant was purchased from Innovative Research (Novi, MI, USA) and stored at −20°C.

2.2 | Standard selection

The method combined quantitative analysis of 35 compounds with nontargeted suspect screening of an additional ~160 compounds. Analytes include various classes of synthetic drugs, including fentanyl analogs, synthetic cannabinoids, synthetic cathinones (bath salts), and NBOMeS, as well as commonly encountered drugs of abuse like heroin, cocaine, methamphetamine, and controlled prescription drugs. Recent DEA Emerging Threat Reports were used for standard selection at the time of the method development. Quantitative compounds were chosen to include the drugs expected to be commonly encountered in each of the major drug classes. New drug targets were occasionally added to the suspect screening method based on reports from the DEA and other sources, while targets were sometimes removed when there were no detections both in-house and in national reports. The 35 quantitated drug targets are shown in Table 1.

2.3 | Internal standard

An internal standard (ISTD) solution was prepared by diluting 14 stable isotope labeled analogs in acetonitrile. The concentrations of each compound in the spiking solution were as follows: 1500 ng/mL of fentanyl-d5, fluoroisobutyryl fentanyl-d7, and U-4700-d6; 2000 ng/mL of AB-CHMINACA-d4, AB-FUBINACA-d4, AKB48-d9, AM-2201-d5, XLR-11-d5, and cyclopropyl fentanyl-d5; 5000 ng/mL of alprazolam-d5, cocaine-d3, etizolam d3, and heroin-d9; and 10,000 ng/mL of methamphetamine d-11. The ISTDs were assigned to the analytes by structural similarity. For compounds without a clear structural analog, the ISTD was determined empirically by generating calibration curves with each ISTD. ISTD solution was added to all samples at a 100-fold dilution before they were loaded onto the paper spray cartridge.

2.4 | Calibration and QC samples

Working solutions were prepared in acetonitrile at 10 different concentrations. Seven working solutions were used to prepare the calibration standards during method development. The remaining three working solutions were used to prepare quality control (QC) samples. All calibration and QC standards were multicomponent solutions with all drug standards together. The working solutions were stored at −20°C when not in use. To prepare calibrators and QC samples, working solutions in acetonitrile were spiked into pooled human plasma by a 50-fold dilution. Calibration curves extended to 100 times higher than the lowest calibrator for all analytes (Table 1). Calibration samples were run in duplicate, while the QC samples were run in triplicate.

2.5 | Suspected overdose samples

Authentic overdose samples were collected in collaboration with the Indiana Poison Center and the Department of Emergency Medicine's Division of Medical Toxicology at Indiana University. Plasma samples were collected from patients admitted to Emergency Departments at Eskenazi and Methodist Hospitals in Indianapolis with symptoms suggestive of drug use. Samples were collected from remnant patient blood samples collected in the ER as part of the clinical work up. Samples were collected in a BD Vacutainer K2-EDTA blood collection tube starting April 2019 to April 2022. Plasma was separated from the blood cells within 72 h of collection, and blood samples were stored in the refrigerator after standard of care analysis was completed. Once plasma was separated, it was stored at −20°C until thawed for sample preparation.

2.6 | Sample preparation

Patient samples and drug-free pooled human plasma were brought to room temperature. Calibration and QC samples were prepared by spiking drug-free plasma with working solution, and all samples (including blanks and authentic overdose samples) were spiked with a 100-fold dilution of ISTD, keeping the organic fraction below 5% to avoid protein precipitation.

2.7 | Paper spray ionization using integrated SPE

The paper spray-SPE cartridge was similar to described previously[65] and is shown in Figure 1. Three different parts were used for analysis. A cartridge with polypropylene filament was printed on a Prusa Mini+ (Prusa Research, Czech Republic) 3D printer. The SPE column was 3 mm in diameter and approximately 2 mm in height. It consisted of a 31ET paper disc on the bottom to keep the SPE powder in place, 10 mg of Strata-X RP SPE, and a nylon membrane disc on top to hold the material in place.

A 100 µL plasma aliquot was added at the top of the SPE column and allowed to passively wick through the SPE column onto the absorbent pad at the bottom of the cartridge. The sample was capped using the plastic holder and allowed to dry before analysis. For analysis, the SPE column was placed overtop the paper spray substrate, which was Whatman 31ET chromatography paper, 5 × 15 mm in size, and cut to a sharp point. A 3D printed plastic part was designed to hold the SPE column in contact with the paper. The paper tip was positioned ~5 mm away from the mass spectrometer inlet. Ninety microliters of acetonitrile with 0.1% formic acid was added to the SPE column. The solvent wicked through the SPE column onto the paper

TABLE 1 Representative analytes in method with calibration concentration range, limit of detection (LOD), and R^2 parameters.

Compound	Calibration range (ng/mL)	Internal standard	Calculated LOD (ng/mL)	R^2
25I-NBOMe	2–200	Fentanyl D_5	2.1	0.963
5F-ADB	0.2–20	AB-PINACA D_9	0.1	0.987
5F-PB-22	0.2–20	XLR-11D_5	0.2	0.905
AB-CHMINACA*	0.5–50	AB-CHMINACA D_4	0.3	0.987
AB FUBINACA*	0.5–50	XLR-11 D_5	0.5	0.966
AB-PINACA*	0.5–50	AB-PINACA D_9	0.3	0.988
Acetylfentanyl	0.2–20	Fentanyl D_5	0.1	0.984
ADB-FUBINACA	0.2–20	AM-2201 D_5	0.2	0.945
Alpha-PVP	2–200	Fentanyl D_5	1.6	0.976
Alprazolam*	2–200	Alprazolam D_5	1.4	0.984
AM-2201*	0.2–20	AM-2201 D_5	0.2	0.976
AMB-FUBINACA	0.5–50	AM-2201 D_5	0.4	0.981
APINACA*	0.5–50	APINACA D_9	1.9	0.923
Benzylpiperazine	20–2000	Fentanyl D_5	24.5	0.949
Carfentanil	0.2–20	Fentanyl D_5	0.2	0.980
Cocaine*	2–200	Cocaine D_3	0.5	0.998
Cyclopropylfentanyl*	0.5–50	Cyclopropylfentanyl D_5	0.4	0.982
Etizolam*	2–200	Etizolam D_3	1.6	0.976
Fentanyl*	0.2–20	Fentanyl D_5	0.2	0.980
4-Fluoroisobutyrfentanyl (FIBF)*	0.2–20	FIBF D_7	0.1	0.989
Furanylfentanyl	0.5–50	Fentanyl D_5	0.5	0.988
Heroin*	2–200	Heroin D^9	1.1	0.988
JWH-018	0.5–50	Am-2201 D^5	0.4	0.974
JWH-200	0.5–50	XLR-11 D^5	0.5	0.967
JWH-250	0.5–50	XLR-11 D^5	0.3	0.989
Ketamine	2–200	Cocaine D^3	1.3	0.981
LSD	2–200	Fentanyl D^5	1.3	0.984
MDPV	2–200	Fentanyl D^5	1.2	0.986
Methamphetamine*	20–2000	Methamphetamine D^{11}	35.9	0.934
Methylone	2–200	Fentanyl D^5	2.6	0.938
MMB-CHMICA	0.5–50	XLR-11 D^5	0.6	0.940
Remifentanyl	0.2–20	FIBF D^7	0.2	0.976
THJ-2201	0.5–50	AB-CHIMNACA D^4	0.5	0.964
U-47700*	0.2–20	U-47700 D^6	0.1	0.993
XLR-11*	0.5–50	XLR-11 D^5	0.3	0.989

Note: Analytes with asterisk (*) indicate a stable isotope labeled analog was used as an internal standard.

spray substrate, and a high voltage of 4.5 kV was applied to the paper, inducing an electrospray at the tip of the paper.

2.8 | MS data collection

Data were acquired on a Q-Exactive Focus orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). MS capillary inlet was set at 320°C and s-lens to 50. For automatic integration, the data processing software requires zero-intensity scans for each isolation window. Therefore, from 0 to 0.3 min, the voltage was set to 0 kV, and then, the voltage was switched to +4.5 kV from 0.3 to 2.8 min, and the voltage was set to 0 kV until the end of the run; the instrument method was 3.5 min long.

The mass spectrometer was operated in the parallel reaction monitoring (PRM) mode using a data independent screening approach with wide MS/MS isolation windows of 7.0 m/z.[66] An inclusion list consisting of 34 windows spanning *m/z* range from 150 to 420 was used to isolate and fragment drug targets (Table S1). Stepped collision energy was used to fragment as many diverse compounds as possible

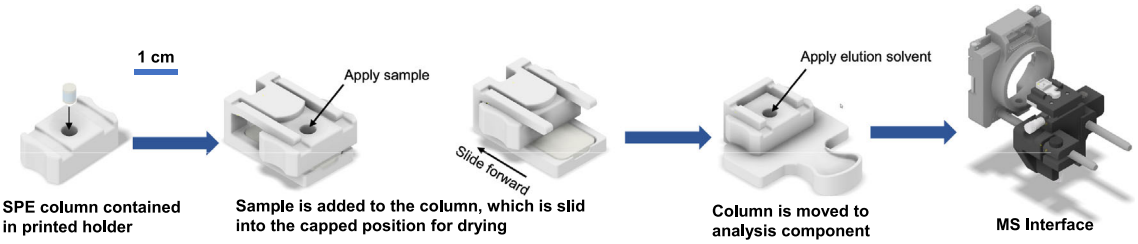

FIGURE 1 Schematic drawings showing the 3D printed parts. MS, mass spectrometry; SPE, solid phase extraction.

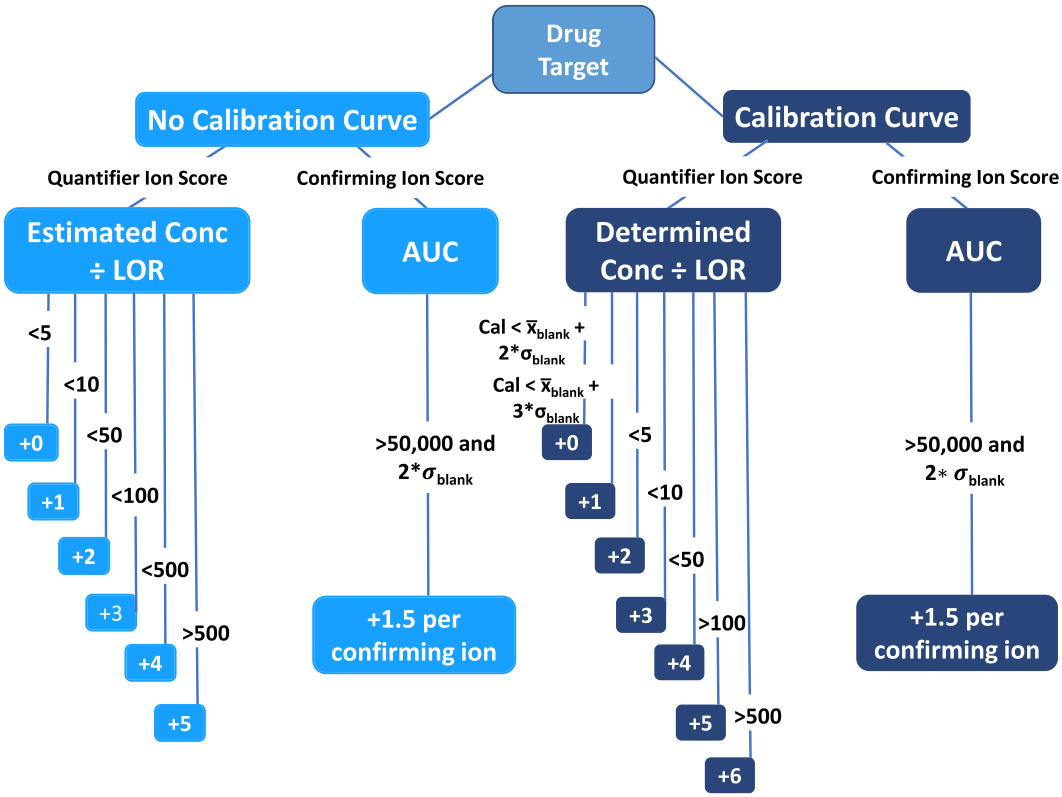

FIGURE 2 Decision tree used to assign a score for each presumed drug detection. Positive detections were taken to be a score ≥6. AUC, area under the curve; LOR, limit of reporting.

(normalized collision energy stepped through 20, 30, and 55). No full MS survey scan was performed because the QE Focus does not allow full MS scans combined with PRM scans. Resolution was set to 35,000. AGC target was 1e6, and the maximum injection time was 200 ms. Spectra were collected in profile mode.

2.9 | Data processing: Screening

All data were automatically processed using TraceFinder v. 5.1 (Thermo Fisher Scientific). An in-house library was built containing 156 compounds (Table S2). Fragment ions were determined by infusing neat standards and obtaining MS/MS spectra at normalized collision energies of 20, 30, and 55. One quantifier ion and at least two confirming ions were selected for each compound. When standards were not available, high-resolution MS/MS spectra from mzCloud (HighChem, Slovakia) were used to select quantifier and confirmatory ions. The spectra on mzCloud, like our own instrument, are collected on orbitrap mass spectrometers using HCD. The database spectra matched in-house data well enough to support the scoring approach used here. A full list of quantifier and confirming ions is shown in Table S2. Mass tolerance was set to 5 ppm. Automatic integration was used to determine the area under the curve (AUC) for quantifier and confirming ions. Concentrations were determined for the 35 analytes for which calibration curves with internal standardization were collected. For the other "screen only" compounds, concentration was roughly estimated using the calibration curve for a structurally similar drug (linked calibration curve). This estimated concentration was used in the scoring system (Figure 2) to assign a higher score to samples with higher estimated concentrations.

A unique report template was created in TraceFinder 5 which automatically assessed for the detection of each target compound and assigned a score. The algorithm for assigning the score is summarized in Figure 2. For compounds with a calibration curve, the limit of reporting (LOR) was set at the concentration of the lowest calibrant. For compounds without a calibration curve, the average blank concentration in drug-free plasma was calculated for each batch using the linked calibration curve. This was set as the LOR for the batch. To receive a score for a particular sample, a compound must have exceeded the LOR, had a quantifier ion AUC > 50,000, and had at least one detected confirming ion in addition to the quantifier ion. To pass the confirming ion check, the AUC of confirming ion must have been greater than the blank AUC + two standard deviations of the blank and also >50,000 AUC. If a compound satisfied these requirements, a score was assigned as summarized in Figure 2. A quantifier ion score was assigned from 0 to 6 based on concentration while 1.5 points were assigned per confirming ion up to a maximum of three confirming ions. Total score was the sum of the quantifier and confirming scores. If a compound had a score of ≥6 and at least one confirming ion, it was considered a positive hit for that target. To achieve a score of ≥6, drug targets had to be at least 5× higher than the lowest calibration standard. For example, fentanyl with a determined concentration of 1.2 ng/mL with two detected confirming ions would yield a score of 6 (+3 for having a concentration more than 5 but less than 10× higher than the lowest calibrator and +1.5 each for two confirming ions). A lower score threshold could be utilized if desired, but that would increase the false positive rate.

2.10 | LC–MS/MS comparison

Select samples were sent to Axis Forensic Toxicology (Indianapolis, IN) for confirmatory analysis by LC–MS/MS. Due to limited amount of sample, only one type of panel could be run per sample. An in-house LC–MS/MS method was created for additional PS-MS confirmation. Plasma samples were protein precipitated by adding 150 µL of acetonitrile to 50 µL plasma[67]. The samples were vortexed for 1 min and then centrifuged at 1500 RCF for 5 min. Samples were transferred to an autosampler vial, evaporated using nitrogen, and reconstituted in 50 µL of starting mobile phase before analysis. Control samples (1, 10, and 100 ng/mL levels) were extracted using the same sample protocol. LC–MS/MS analysis was carried out using an Ultimate 3000 HPLC system which was coupled to the Q-Exactive Orbitrap MS. A Hypersil Gold C18 held at 35°C was used for separation. The LC solvents were water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) with a 0.3 mL/min flow rate. A 10 µL sample aliquot was injected onto the column. The gradient was as follows: 0–5 min at 10% B, 5–7 min ramp to 95%, 7–7.1 min ramp back to 10% B, and finally, a hold at 10% B for 5 min. The mass spectrometer was operated in PRM mode, 4 kV spray voltage, 20 psi sheath gas, and 320°C capillary temperature.

2.11 | IRB approval

This study was conducted under an exempt Institution Review Board (IRB) protocol reviewed by the Indiana University IRB. Samples used in this study were remnant blood samples left over from routine blood chemistry tests collected as part of standard care. Remnant blood samples were de-identified.

3 | RESULTS AND DISCUSSION

3.1 | Method optimization

Several parameters were optimized to achieve the lowest detection limits feasible for a wide variety of analytes. Most method optimization was done only with synthetic cannabinoid standards because they were found typically at low levels and were more challenging to detect compared with other potent drugs like fentanyl analogs. Two SPE materials that were water wettable, polymeric and had the ability to extract and recover wide range of analytes were tested: Strata-X RP and Oasis HLB. Optimization was limited to water-wettable materials so samples could wick through the SPE by gravity and capillary action, while polymeric materials do not require preconditioning[65]. Methanol and acetonitrile with either formic or acetic acid modifiers were tested as elution/ionization solvents. Strata-X RP with acetonitrile with 0.1% formic acid as the elution/ionization solvent gave the lowest detection limits and was used for all future experiments. In direct paper spray, sample volumes of 10 µL or less are typical. While low sample volume is advantageous for some applications, other use cases can benefit from lower detection limits. By combining SPE with paper spray, larger sample volumes decrease detection limits owing to preconcentration from the SPE[65]. This trend was observed here with plasma spiked with synthetic cannabinoids (Figure S1). A volume of 100 µL was chosen because larger volumes of authentic overdose samples may be hard to obtain. For 100 µL of spiked plasma, 10 mg of SPE sorbent had the highest signal-to-blank ratio (Figure S2), which corresponded closely to the manufacturer's recommended loading capacity.

3.2 | Semiquantitation

We opted to include semiquantitation for commonly detected drugs rather than purely qualitative screening. An estimated concentration during the screening step can help guide confirmatory analysis, especially when cost, time, or sample volume constraints preclude confirmation of every positive hit during sample screening. Calibration models were determined for the 35 analytes using spiked plasma at toxicologically relevant concentrations to enable approximate concentration determinations. LODs ranged from 0.1 to 26 ng/mL, largely dependent on the analyte. Most synthetic cannabinoids LODs were between 0.1 and 0.5 ng/mL, except for APINACA which had a higher LOD at 2 ng/mL. Fentanyl and all fentanyl analogs could be detected

at 0.5 ng/mL or lower. Most other drugs' LODs were higher, due to their hydrophilic properties with lower recovery/higher possible ion suppression. Many of the drugs with higher LODs were smaller molecules with lower specificity fragment ions, which generally correlated with higher blank signal arising from endogenous interferents, paper contaminants, and other sources of chemical background. The method was linear within the calibration range, and coefficients of determination (R^2) ranged from 0.923 to 0.998. Compounds with isotopically labeled standards tended to have better linearity, as expected.

Intraday and interday bias was below ±20% for most analytes at all three QC levels (Table S3). For interday bias, lower QC level bias ranged from −12% to 20%, medium QC bias was between −17% and 29%, and at high QC −10% to 16%. Intraday precision (Table S4) for most analytes at all levels was below 20% (with the range of 0.6–58%), and interday precision was typically below 25% (overall range 2–49%). As expected, analytes without an SIL ISTD tended to have greater variation than those with (average %CV across all interday QCs of 23.5% compared with 16.6%). Diluted QC samples yield comparable quantitation to undiluted QCs (Tables S5 and S6), showing that specimen dilution could be done when sample volume was insufficient.

Relative matrix effects of the method were assessed by constructing calibration curves in 10 different lots of plasma, which were obtained from individual donors. Relative matrix effects were determined by calculating the variability (%CV) of standard line slopes[68]. The %CV of the slopes ranged from 3% to 21% with a median value of 7.9% (Table S7). Analytes with an isotopically labeled ISTD showed significantly lower variabilities in the slope (median value of 6% vs. 10.5%). However, the calibration slopes all varied by less than 22%, which was deemed acceptable for a rapid semiquantitative screening method.

3.3 | Screening

We opted for nontargeted MS screening because targeted MS/MS places limits on the number of compounds that can be screened, and retrospective analysis cannot be performed for newly emerged drugs[28,40]. Common approaches for nontargeted methods include data-dependent acquisition, sequential window acquisition of all theoretical mass spectra (SWATH-MS)[69,70], and single-stage high-resolution MS. Data-dependent acquisition was anticipated to have poor sensitivity for this application due to the complexity of the mass spectra arising from the lack of chromatography. Likewise, single-stage MS was deemed to be of limited screening value without the additional selectivity from chromatographic separation. Therefore, we used a SWATH-MS nontargeted screening method, which allowed us to identify new compounds as they emerge without rerunning samples or changing the data acquisition method. This was achieved by using an inclusion list spanning *m/z* 147 to 432 with 7.0 m/z width MS isolation windows (Tables S1 and 1).

An MS/MS spectrum of the *m/z* 336 ± 3.5 for authentic overdose sample is shown in Figure 3a indicating detection of fentanyl. The determined concentration was 18.0 ng/mL, which is 90× higher than the lowest calibrator of 0.2 ng/mL and was assigned a quantifier ion score of 4. Three confirming ions were detected (*m/z* 134.0962, 105.0701, and 337.2265) for a confirming ion score of 4.5. The total score was 8.5 and the sample was deemed positive for fentanyl. Another spectrum is shown in Figure 3c for the *m/z* 372 ± 3.5 window. The quantifier ion for flubromazolam was found at *m/z* 343.0103 and assigned a score of 3 based off its estimated concentration of 14.7 ng/mL. Three confirming ions were detected for flubromazolam as well at *m/z* 292.1114, 237.0943, and 371.0295. The total score was therefore 7.5 and deemed positive for flubromazolam.

3.4 | Specificity and detection challenges

In some cases, similar compounds cannot be distinguished by PS-MS. Regioisomers may not be distinguished if they yield the same fragment ions. For example, the synthetic cannabinoids 2-, 3-, 4-, and 5-Fluoro-ADB yield the same fragment ions and their MS/MS spectra are not readily distinguishable. In some cases, similar but nonisomeric compounds cannot be distinguished. This occurs when the molecular ions fall in the same SWATH-MS isolation window, the molecular ions are too low to be detected, and the fragment ions are the same. This occurs with 5F-PB-22 ($C_{23}H_{21}FN_2O_2$) and 5F-NNEI ($C_{24}H_{23}FN_2O$), which differ by 1.9793 Da but fall in the same SWATH-MS window. At the HCD energies used in the method, no molecular ion is detected and the only major fragment ions for both drugs are *m/z* 144.0444 and *m/z* 232.1132. Three samples flagged as positive for either 5F-PB-22 or 5F-NNEI were analyzed by LC–MS/MS; all three were confirmed positive for 5F-PB-22. Fentanyl and acrylfentanyl are an example of structurally similar drugs in the same SWATH-MS window that can be distinguished. Both drugs lie in the same SWATH-MS window and share the abundant fragment ions (*m/z* 188.1429 and 105.0700). However, the two drugs have multiple unique ions, including the molecular ion, that enable differentiation. Figure 4 shows extracted ion chronograms for fentanyl and acrylfentanyl from two ED patient samples, which are readily distinguished by the presence of *m/z* 337.2274 and 134.0962 (fentanyl) versus *m/z* 335.2218 and 214.1226 (acrylfentanyl).

3.5 | Comparison with LC–MS/MS

A subset of samples was sent to an independent toxicology lab to compare the performance of the method with LC–MS/MS, which is the "gold standard" for drug screening. The results are shown in Table S8. The toxicology lab reported 14 detections across the 18 samples. Twelve detections were found by both the LC–MS/MS and paper spray, two were identified only by the toxicology lab, and four were only reported by paper spray. Two of the four found only by PS-MS, 5-Fluoro ADB and JWH-018, both were estimated by PS-MS to be below the toxicology lab's cutoff. These results suggest that there is good agreement between paper spray and LC–MS/MS. Due

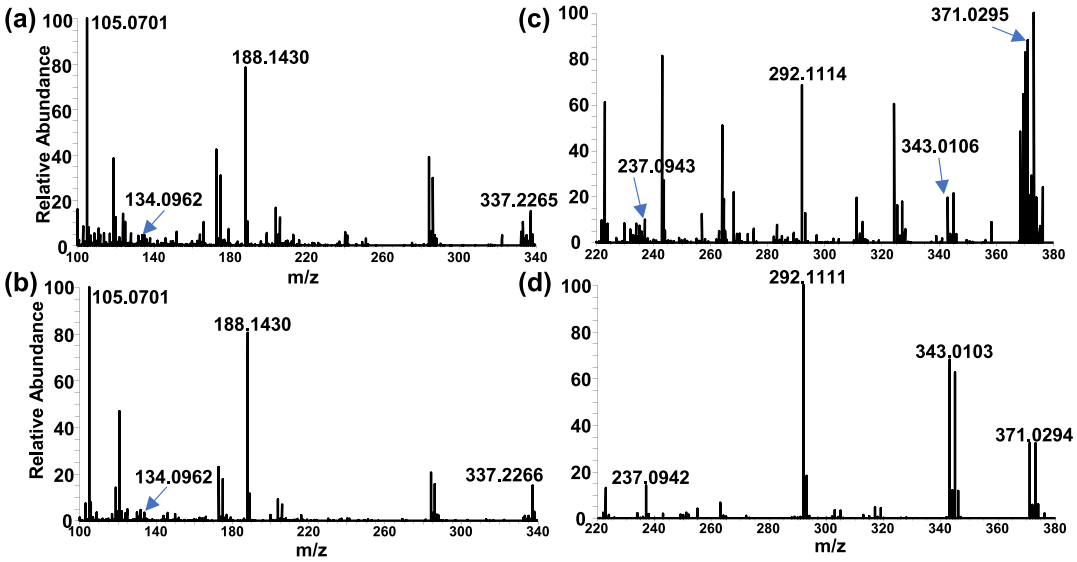

FIGURE 3 Tandem mass spectrometry spectra for channel *m/z* 336 for (a) an authentic overdose specimen and (b) a 20 ng/mL fentanyl spiked plasma calibrator. The specimen is positive for fentanyl. Tandem mass spectrometry spectra for channel *m/z* 372 for (c) an overdose specimen and (d) infusion of a neat standard of flubromazelam.

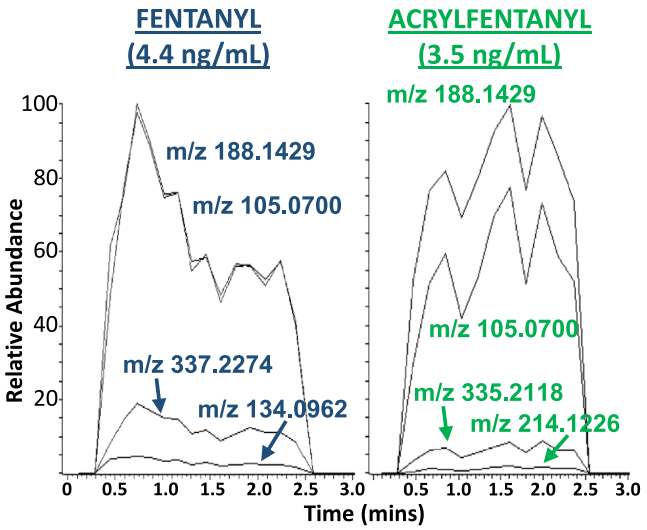

FIGURE 4 Extracted ion chronograms for two ED patient samples showing differentiation of fentanyl and acrylfentanyl, which both fall in the *m/z* 336 ± 3.5 isolation window.

TABLE 2 Most commonly detected drugs October 2020 to July 2021.

Compound	Occurrence
Fentanyl	114
Methamphetamine	59
Cocaine	39
6-Monoacetylmorphine	31
Midazolam	18
Acetylfentanyl	17
Lorazepam	15
Chlordiazepoxide	14
Methadone	12
Flualprazolam	11
Flubromazolam	11
5-Fluoro AMB-PICA	10
5F-PB-22	10
Clonazepam	10
Ketamine	10
4-ANPP	9
Morphine or hydromorphone	9
Alprazolam	8
Diazepam	8
mCPP or pCPP	8

to the sample volume required by the toxicology lab, only one panel could be requested per sample. Moreover, fentanyl was on a separate panel from fentanyl-related substances. To better check the PS-MS results, we used an in-house, targeted LC–MS/MS method for the top 20 drugs detected between October 2020 and July 2021 (Table 2).

A subset of 15 patient samples were selected for confirmation analysis. Of the 43 drug detections by PS-MS, 35 were confirmed with LC–MS/MS (Table 3). Of the eight that were not confirmed by LC–MS/MS, four were ketamine. Because some of the patient samples analyzed by LC–MS/MS analysis >1 year after initial analysis with PS-MS, some of the drugs may have degraded despite storage at −20°C. Several studies have shown ketamine to be stable for 2–3 months when frozen[71,72], while another paper reported degradation after 1 month[73]. No published study has monitored stability for more than a few months. There were also 15 drugs detected by LC–MS/MS that were not found using the PS-MS method. Each of these were at

TABLE 3 Results for 15 patient samples analyzed with paper spray mass spectrometry and liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Patient sample	Paper spray MS detections	Confirmed by LC–MS/MS?	LC–MS/MS detections not found by paper spray MS
Sample 63	4-ANPP	Yes	
	Acetylfentanyl	Yes	
	Clonazepam	No	
	Fentanyl	Yes	
	Flualprazolam	Yes	
	Morphine/hydromorphone	Yes (morphine)	
Sample 69	Fentanyl	Yes	4-ANPP
	Ketamine	No	Flualprazolam
Sample 71	Cocaine	Yes	
	Fentanyl	Yes	
	Ketamine	No	
Sample 73	Fentanyl	Yes	4-ANPP
	Ketamine	No	Methamphetamine
Sample 74	Fentanyl	Yes	
	Methamphetamine	Yes	
Sample 82	Alprazolam	Yes	4-ANPP
	Clonazepam	Yes	
	Fentanyl	Yes	
Sample 83	4-ANPP	Yes	
	Fentanyl	Yes	
Sample 86	Fentanyl	Yes	4-ANPP
	Flubromazolam	Yes	
Sample 100	Methamphetamine	Yes	4-ANPP
	Fentanyl	Yes	
Sample 102	Midazolam	Yes	
	Methamphetamine	Yes	
Sample 184	Acetylfentanyl	Yes	4-ANPP
	Cocaine	Yes	5F-MDMB-PICA
			5F-PB-22
			Fentanyl
			Ketamine
			Methadone
Sample 185	Acetylfentanyl	No	4-ANPP
	Alprazolam	Yes	
	Fentanyl	Yes	
Sample 228	Clonazepam	No	
	Ketamine	No	
	Lorazepam	Yes	
Sample 231	4-ANPP	Yes	Cocaine
	Fentanyl	Yes	
	Morphine/hydromorphone	Yes (morphine)	
Sample 232	4-ANPP	Yes	
	Clonazepam	Yes	
	Cocaine	Yes	
	Fentanyl	Yes	
	Methamphetamine	Yes	
	MMB-2201	No	

low concentrations in the range of ~0.3 to 1.5 ng/mL. While the concentrations were above the PS-MS detection limit, none of them were sufficiently abundant to fulfil the detection criteria set for the PS-MS screening method. These drugs could have been flagged by the paper spray screening method if the score thresholds were lowered, thereby increasing the diagnostic sensitivity. The false positive rate would also increase, however. Overall, these results indicate that rapid screening by PS-MS corresponds well with LC–MS/MS within the established limits of the method's sensitivity and specificity. Figure S3 shows three extracted ion chromatograms of drugs detected with the PS method and confirmed with the LC–MS method.

3.6 | Analysis of emergency department overdose samples

The validated method was used to screen 320 authentic overdose samples. The median number of drugs per overdose sample was 2, with no drugs identified in 24 samples and 3 samples containing more than 9. A histogram of the number of detections is shown in Figure S4. Negative detections could be because the concentration was too low to fulfil the detection criteria or the drugs were not included in the current iteration of the data processing method. Table 2 shows the most frequently detected drugs, the top being fentanyl and methamphetamine. A total of 91 unique drugs were detected. Four of the 10 most frequently detected drugs were benzodiazepines; however, they are also commonly used in Emergency Departments. Because the samples were de-identified, it is not known if the detected drugs were used by the patient or were administered by a clinician.

Figure 5 shows the most detected drugs by class. Opioids were the most detected class of drugs, followed by stimulants, benzodiazepines, and synthetic cannabinoids. Fentanyl made up 51% of all the opioid detections, while the other 49% were from a combination of prescription opioids and heroin.

Opioid screening in the context of emergency department patients is particularly important. Nonfatal opioid overdose-involved EMS encounters increased by 4.0% per quarter, on average, from January 2018 to March 2022, reaching 179 per 10,000 EMS encounters in Q1 of 2022[74]. Among synthetic opioids (other than methadone), fentanyl, 4-ANPP, and acetylfentanyl were the most commonly detected in this study, which is consistent with the 2020 DEA emerging threat report. Acetylfentanyl was found combined with fentanyl 59% of the time, which is consistent with published trends[75]. These three molecules together accounted for 180 of the 202 synthetic opioid (other than methadone) detections. The remainder consisted of brorphine (6), furanyl fentanyl (6), carfentanil (4), U-47700/AH-7921 (2), butyrfentanyl (1), methylfentanyl (1), cyclopropylfentanyl (1), and metonitazene (1). Brorphine is a nonfentanyl opioid first synthesized in 2018[76] that appeared in the US drug market in the second half of 2020[77], often in combination with flualprazolam and fentanyl[78]. Consistent with these findings, we detected brorphine in six patient samples from July to October 2020. All six of these samples were also positive for fentanyl and three were also positive for flualprazolam.

Drug overdose deaths involving stimulants have increased in recent years[79–81], nearly all of which involve cocaine or methamphetamine[82]. There has also been an increase in overdose deaths from combined exposure to fentanyl and stimulants. This and other evidence suggest a rise in concomitant use of both opioids and stimulants[83]. Consistent with these trends, 44 of the 66 samples positive for methamphetamine were also found to contain fentanyl (39), acetylfentanyl (2), or 6-monoacetylmorphine (3). Likewise, 28 of the 43 cocaine-positive samples were also positive for fentanyl. No other

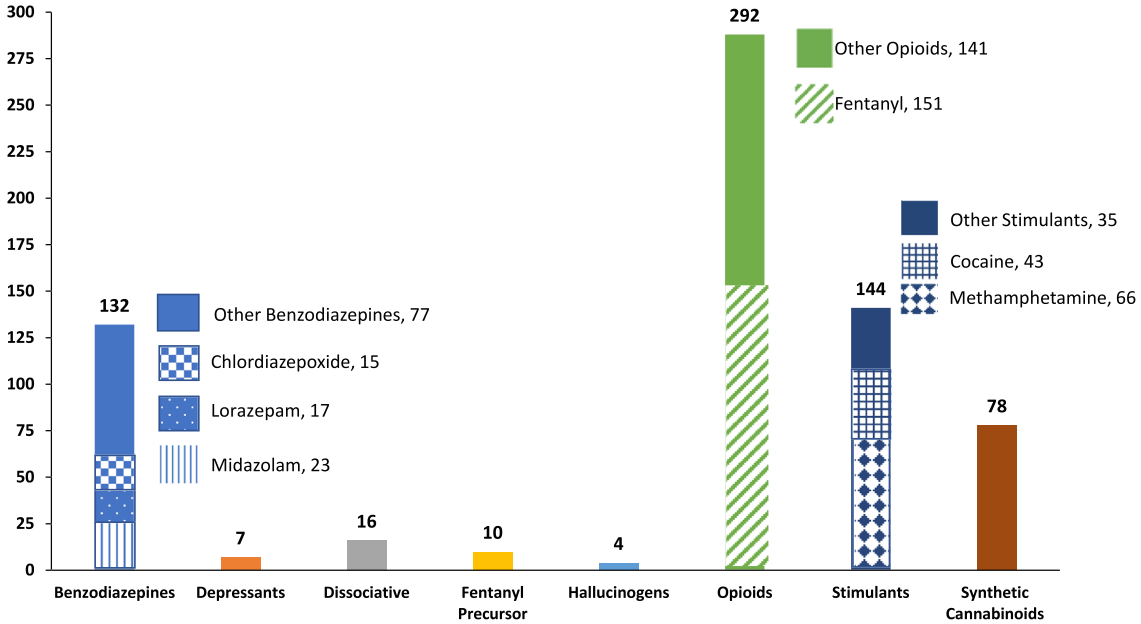

FIGURE 5 Bar chart representing the number of positives found from a specific drug class.

illicit opioid, including 6-monoacetylmorphine, was detected in combination with cocaine.

We detected designer benzodiazepines in 27 ED samples: flualprazolam (11), flubromazolam (11), pyrazolam (4), etizolam (3), and bromazolam (1). This list is consistent with the 2020 DEA Emerging Threat Report as fluaprazolam, etizolam, clonazelam, and flubromazolam were the most commonly reported to the DEA. These designer benzodiazepines were frequently found in combination with illicit opioids in this study (19/27 cases). For most cases, the opioid was fentanyl, but carfentanil, acetylfentanyl, brorphine, U-47700, and furanylfentanyl were found in combinations with designer benzos as well.

Due to the untargeted SWATH-MS data collection, the data could be analyzed retrospectively in response to emerging drug reports. Synthetic cannabinoid 4F-MDMB-BINACA was identified in seized drug casework of Center for Forensic Science Research and Education in December 2018 and was detected in several post-mortem samples, two of which were in Indiana[84]. We added the compound to the data processing method and reanalyzed previously collected data. 4F-MDMB-BINACA was detected in a previously analyzed sample. Newly identified compounds can be quickly incorporated into the screening method, without lengthy method validation procedures, and previous sample data can be reanalyzed to determine when a new synthetic drug entered the regional drug market.

4 | CONCLUSION

A semiquantitative, nontargeted drug screening method was developed with an "all-in-one" paper spray cartridge. Several parameters, such as extraction solvent, SPE sorbent, and sample volume, were investigated to improve LODs. The method has been validated and used to screen >300 authentic overdose samples collected from two urban emergency departments. The method can detect multiple classes of drugs, which is helpful given that people who use drugs frequently abuse several drugs, deliberately or not. It was also shown that a newly identified drug can be quickly added to the data processing method with no modifications to the workflow, which can help speed up the turn-around time and more accurately narrow down the time when the synthetic drug entered the market. Because there is no chromatographic separation and wider isolation windows are used, some compounds cannot be distinguished from another. In the future, coupling paper spray to ion mobility is a potential way to overcome this challenge[85,86]. Another limitation is the practicality of testing in the emergency department using high-resolution mass spectrometers, which are large and expensive and require frequent calibration. Future work will explore the use of quadrupole ion traps or triple quadrupole mass spectrometers for targeted panel drug screening.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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