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Development of a high-resolution paper-spray mass spectrometry method using street drugs for the early detection of emerging drugs in the unregulated supply†

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Adulteration of the unregulated opioid supply has contributed to increasing numbers of overdose deaths in North America. Harm-reduction drug checking has emerged as a strategy to address increasing adulteration rates by providing information about sample composition to people who use drugs. While paper-spray mass spectrometry is capable of trace detection for drug checking, the presence of newly emerging substances often goes undetected if not included in the targeted analysis method. High-resolution mass spectrometry has not been widely used in drug-checking efforts to date, but it has advanced capabilities to facilitate the detection of newly emerging substances. We present a high-resolution paper-spray mass spectrometry method developed for the detection of newly emerging compounds in the street-drug supply. The method was used to analyze a selection of opioid samples received at a drug-checking service in Victoria, British Columbia, Canada. Using this approach, newly emerging adulterants, precursors and byproducts were identified in the local street-drug supply.

1 Introduction

Over the last decade there has been a trend of increasing overdose deaths in North America, where the volatility of the unregulated drug supply has been reported as a major driving factor.[1–4] The United Nations Office on Drugs and Crime has identified over 1200 unique novel psychoactive substances internationally over the past several decades, creating great concern regarding the evolving composition of the street supply for people who use drugs.[5] The opioid supply in North America consists primarily of fentanyl, but there are large inconsistencies in the amount of fentanyl present, with a wide and variable range of adulterants added to opioid drug mixtures.[6–8] Specifically, the opioid supply is often adulterated with other sedatives, including fentanyl analogs such as carfentanil,[9] benzodiazepines,[10] veterinary sedatives such as xylazine,[11,12] and nitazenes.[13] It is suspected that adulteration occurs for several reasons, including to enhance or produce synergistic effects of the drug, or to reduce the amount of active drug necessary to achieve the desired effect.[11,14] Drug adulteration changes over time in ways that are not necessarily predictable and is considered to be a result of the various economic and law-enforcement pressures experienced by illicit drug manufacturers.[14] Adulteration results in the consumption of unknown drugs, which can cause unpredictable effects on the individual, and therefore the detection of such compounds is of great importance for people who use drugs.[15]

Harm reduction drug checking has emerged to identify drug compositions and to provide information about the drug supply to people who use drugs.[16,17] Currently, one of the limitations of drug checking is the detection of newly emerging substances, especially when they are present in low concentrations.[18,19] Most routine drug testing methods are able to identify only a limited number of compounds, resulting in newly emerging substances going undetected.[20] Some of the most commonly used drug-checking strategies include immunoassay test strips and Fourier transform infrared (FTIR)

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spectroscopy. Immunoassay test strips exist for the detection of fentanyl, benzodiazepines, and xylazine, among other drug classes, but they are unable to differentiate between the various analogs present in each class.[21–23] While FTIR has large libraries that enable the identification of a wide range of compounds, detection efforts become challenging with complex mixtures or when compounds are present at low concentrations.[24,25]

Various mass spectrometry methods have been investigated as drug-checking tools because of their ability for trace detection and quantitation. While gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are considered to be gold-standard techniques in forensic contexts, their use for drug checking is hindered by expensive equipment, the need for highly trained personnel, and long analysis times.[26–28] Various other mass spectrometry systems have been implemented for point-of-care drug analysis, notably paper-spray mass spectrometry (PS-MS),[29,30] portable GC-MS,[31] handheld high-pressure mass spectrometry (HPMS),[32] and miniature paper capillary spray ionization (PCSI) mass spectrometry.[33] While mass spectrometry methods have the advantage in sensitivity and quantitation over other drug-checking instruments, targeted methods used in routine analyses are limited to specific analytes and typically use low-resolution mass analyzers.[34,35] Such analyzers are not designed for the detection of newly emerging substances, as unit-mass resolution does not allow for a confident identification of new substances in the absence of chromatographic separation.[35] On the other hand, targeted methods require the availability of analytical reference standards, and therefore have a limited ability to keep up with the constantly evolving street-drug supply.[36]

Untargeted methods for the detection of newly emerging substances are commonly developed on LC-MS/MS instruments,[37] or other hyphenated techniques that give definitive results, but face limitations of long analysis times, highly optimized conditions, and extensive sample preparation.[38] Ambient ionization techniques offer a way to address such limitations, with rapid measurement times, little to no sample preparation and minimal operator training requirements. Such techniques have been demonstrated for drug analysis, including direct analysis in real time mass spectrometry (DART-MS)[39,40] and PS-MS.[29,30,41–43] Additionally, PS-MS has the ability to quantify trace amounts of analytes in complex matrices, which is an advantage over other ambient ionization techniques that are largely considered to be semi-quantitative.[44,45] PS-MS has been used in drug checking for quantitative analyses, but non-targeted screening in drug-checking methods with high-resolution PS-MS instruments has not been thoroughly explored.[29]

High-resolution mass spectrometry (HRMS) drug screening has emerged in response to the increasing proliferation of novel substances, and provides superior resolution and accompanying capabilities of an advanced scanning method, such as data-dependent (DDA) and data-independent (DIA) acquisition modes.[35] DIA methods fragment all precursor ions within a given mass window, which provides an abundance of data.[46] However, these data can be convoluted and it is therefore challenging to associate fragment ions with their corresponding precursors.[47,48] In contrast, DDA methods select the highest intensity ions for fragmentation. To avoid the assumption that the highest intensity ions are the most relevant, algorithms allowing for the use of exclusion lists and dynamic exclusion are beneficial for samples with large numbers of species.[49] The information obtained using HRMS allows compounds to be identified without a reference standard by obtaining the accurate mass and isotope ratio information, allowing the narrowing of possible candidates to a certain molecular formula.[37] *In silico* fragmentation algorithms can then be used to provide theoretical fragmentation patterns for molecules of interest, and the possible molecular structures associated with these fragments.[37] PS-HRMS methods have been demonstrated for drug analysis in biological matrices such as urine[50] and blood,[51] but pre-consumption testing of street drugs is limited.

A common approach for development of a non-targeted method is to optimize and evaluate the method using analytical standards, or standards spiked into a laboratory-developed matrix. Analytical standards have high levels of purity, and are most often obtained in solution form. Street drugs largely consist of chemicals synthesized in clandestine laboratories, where incomplete purification is common.[43,52] Additionally, street drugs have complex matrices, often with a variety of cutting agents and adulterants.[53] Essentially, there are any number of unknown factors that contribute to the complexities of street-drug samples that cannot be replicated in a laboratory setting. The use of street drugs in analytical method development has been proven to be successful, as it accounts for the complexities in the samples that the developed method is being aimed at.[54] Therefore, method development using street-drug samples will be more directly applicable for the detection of newly emerging adulterants in street drugs.

We present a PSMS-HRMS DDA method for the detection of newly emerging components of street drugs without the use of reference standards. To ensure that the method will be directly applicable to street-drug matrices, drug samples collected from the Substance Drug Checking Project[55] were used in the development of this method. Several newly emerging substances were detected throughout the work, highlighting the need for regular untargeted screening of the street-drug supply.

2 Methods

2.1 Materials

Acetonitrile and Optima high performance liquid chromatography (HPLC)-grade formic acid and methanol were acquired from Fisher Scientific (Ottawa, ON, Canada). Deionized water was prepared utilizing a water purification system (18.4 MΩ cm Facility Scale Reverse Osmosis/Ion Exchange Water Purification System from Applied Membranes Inc., Vista,

California, USA). VeriSpray™ sample plates for PS-MS measurements were obtained from Thermo Fisher Scientific (San Jose, CA, USA).

2.2 Sample preparation and analysis

2.2.1 Sample preparation and low-resolution analysis. The street-drug samples used for the method development had been previously collected and prepared as part of the Substance Drug Checking Project in Victoria, British Columbia, Canada. The drugs were obtained as powders or pills, where 0.5–2.1 mg of sample was weighed out and diluted in methanol to prepare a 1 mg mL^{-1} solution. To prepare samples for quantitative analysis, the drug solution was subsequently diluted in an internal standard solution, containing 17 deuterated reference compounds at 100 ng mL^{-1} in methanol, to obtain a final concentration of 6000 ng mL^{-1} solid sample. 10 μL of the solution was deposited onto a PS-MS sample strip on a VeriSpray™ sample plate (Thermo Fisher Scientific, San Jose, CA, USA) for analysis. The instrument used for the quantitative analysis on site is a TSQ Fortis™ triple quadrupole (QqQ) mass spectrometer with a VeriSpray™ paper spray ion source (Thermo Fisher Scientific, San Jose, CA, USA). The data were acquired in positive ion mode, with a spray solvent composed of acetonitrile, deionized water, and formic acid (90/9.9/0.1 v/v% acetonitrile/DI water/formic acid). The instrumental method includes a multiple-reaction monitoring (MRM) sequence for 105 targeted compounds for quantitative analysis (0–1.2 min) followed by a full scan from 50–600 *m*/*z* (1.2–2.0 min). More details of the instrument parameters and method can be found in Table S1.† This analysis method is hereafter referred to as the routine analysis method.

2.2.2 Sample preparation and high-resolution analysis. Samples used for method development on an Orbitrap Exploris 120™ mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) were prepared by diluting the 1 mg mL^{-1} street-drug solution prepared in methanol to a final concentration of 6000 ng mL^{-1}. The concentration was kept the same for the high-resolution analyses to avoid inlet fouling, and for ease of comparison with quantitative information obtained from the quantitative analyses on the QqQ instrument. 10 μL of the solution was deposited onto a PS-MS sample strip (Thermo Fisher Scientific, San Jose, CA, USA) and dried prior to analysis. An in-house-developed source simultaneously holds the paper strip in front of the inlet of the Orbitrap analogous to that described in earlier work.[30] 75 μL of spray solvent was deposited onto the PS-MS sample strip immediately before analysis. The developed PS-HRMS method is 1.1 min in duration, performed in positive ion mode with a spray voltage of 3800 V. For the initial full-scan analysis, a resolution of 120 000 was used over a scan range of 50–600 *m*/*z*. The four highest intensity precursor ions were selected in each scan, and these precursors were then dynamically excluded for 20 s to allow for the fragmentation of lower intensity ions. A ±5 ppm window was implemented for the dynamic exclusion of these compounds, and isotope peaks were also excluded. An exclusion list (provided in the ESI†) of 1942 contaminant peaks was included in the method, with a 10 ppm tolerance window. For the data-dependent MS/MS analysis, a precursor isolation window of 2 *m*/*z* was used, with a stepped high-energy collisional dissociation (HCD) of 30, 50, and 70%, normalized to the precursor mass. The resolution was set to 30 000, and data were collected in centroid mode. Further details of the instrument parameters can be found in Table S2.† The spray solvent used was a mixture of acetonitrile, deionized water, and formic acid (90/9.9/0.1 v/v% acetonitrile/water/formic acid). Isopropanol was also investigated as a spray solvent in an attempt to improve detection of poorly ionizing compounds. Further details of the method and results for the spray solvent optimization can be found in the ESI.†

2.3 Exclusion list development

An exclusion list consisting of *m*/*z* values to be omitted by the method was developed to improve the detection of drug-sample components. Methanolic blank samples that were deposited on a paper strip were analyzed in full-scan mode in triplicate, and the masses of peaks with an intensity above 100 000 were added to the exclusion list (n = 2148). Illicit drug samples of five different types, namely 3,4-methylenedioxymethamphetamine (MDMA), ketamine, cocaine, a benzodiazepine (bromazolam), and a fentanyl mixture consisting of fentanyl, caffeine, and erythritol, were analyzed to identify peaks that may present as a result of sample storage or preparation. The components in these drug samples had been previously verified by analysis on a QqQ instrument. These drug samples were analyzed using the developed DDA method, which included the exclusion list of peaks from the blank sample. The aim of this strategy was to identify additional contaminants present in drug samples, where precursors selected for DDA in at least 3 of the 5 analyzed drug samples were excluded. This strategy was repeated three times, resulting in an additional 196 *m*/*z* values being added to the exclusion list. Following the removal of duplicate values, and those that shared precursor masses close to values in a developed drug precursor database, the final exclusion list contained 1942 *m*/*z* values.

2.4 DDA peak search algorithm

A database of 132 common and emerging drugs was created that included precursor masses and 1–3 fragments for a given precursor to confirm identity. Three fragments were used in most cases, and one or two fragments were used for molecules that did not have 3 major fragments in their MS/MS spectrum. Masses in this database are listed in Table S3.† It should be noted that we have assumed the presence of specific isomers for some compounds, including *ortho*-methyl fentanyl and *para*-fluoro-phenethyl-ANPP, as they have been previously detected and reported. However, this method is incapable of distinguishing between ring substitution isomers. Fragments were selected based on their relative intensities in reference spectra from mzCloud (HighChem, Slovakia), which also correspond to those collected with an Orbitrap instrument using HCD 50%. For cases where the required data did not exist in mzCloud, reference spectra were obtained from the

Center for Forensic Science Research and Education (CFSRE) monographs.[56] These spectra were collected using an LC-quadrupole time-of-flight-MS instrument at a range of 100–510 Da with a collision energy spread of 35 ± 15 eV.[56]

Instrument RAW files were parsed to the mzML format using the ThermoRawFile parser.[57] A Python script using the pymzML package was then created to parse the mzML files to obtain the various spectra and scan types. The extracted precursors and their corresponding MS/MS spectra could then be compared to values within the developed database. A tolerance window of 0.001 *m*/*z* for the selected precursors was used to tentatively identify compounds. The presence of all corresponding fragment ions within a tolerance window of 0.001 *m*/*z* in the MS/MS spectrum was used to tentatively confirm the identity of the compound. For compounds that did not have reference spectra available, *in silico* fragmentation was performed using the Mass Frontier™ software (Thermo Fisher Scientific). Briefly, the molecular structure for the compound of interest was imported into the software, and the three most abundant fragments generated as a result of *in silico* fragmentation were used to confirm the presence of the compound.

2.5 Method evaluation and identifying emerging compounds

Opioid samples (n = 65) were analyzed using the developed method to evaluate the relative detection threshold, and to investigate the detection of components of the street-drug supply outside of those detected with routine targeted analysis methods. The majority of the samples (n = 40) were collected from the Victoria, British Columbia area, while the remainder (n = 25) were collected from various locations around the province of British Columbia. Previously obtained quantitative results by PS-MS with a QqQ instrument were used to provide a relative detection threshold for the compounds analyzed.

3 Results and discussion

3.1 Evaluation of the developed method

3.1.1 Exclusion list optimization and relative detection threshold determination. Qualitative and quantitative information about the sample composition from previous drug-checking analyses was used to guide the optimization of an exclusion list and to determine relative detection limits for the PS-HRMS method. Initially, the exclusion list was developed using peaks present on blank paper spray sampling strips with intensity values above 100 000. However, upon preliminary analysis of a subset of street-drug samples (n = 18) using this method, no compounds were detected at levels below 1% w/w. These results suggested that additional *m*/*z* values should be added to the exclusion list to avoid DDA being performed on background or matrix components, allowing for the detection of lower intensity drug *m*/*z* signals. To select additional *m*/*z* values to add to the exclusion list, street-drug samples from various drug classes were analyzed to determine if any additional peaks were present from sample preparation, storage, or other sources present in street-drug samples. Street-drug samples of MDMA, ketamine, cocaine, bromazolam, and a fentanyl mixture were analyzed, and peaks selected for DDA fragmentation were compared across all five classes. Five different drug classes were analyzed to avoid the elimination of specific precursors or byproducts belonging to each drug class. The *m*/*z* values selected for fragmentation in the majority (3 of 5) of samples were added to the exclusion list. This process was repeated three times, using an updated exclusion list of the selected peaks each time.

Compounds that had quantitative information available from previous analyses were used to assess the relative method detection threshold. Using the updated exclusion list, sample components could now be detected at below 1% in the majority of cases for the analyzed samples (n = 47). Table 1 compares the detection rates for compounds above and below the detection threshold that was determined to be 1% w/w. Only compounds with previous quantitative information from routine analysis are included in Table 1. Out of the 128 sample components present across 47 samples, only four were not detected at a concentration over 1% w/w, and were therefore considered to be outliers. Two of these four components were bromazolam, where the undetected instances were at a concentration of approximately 3% w/w. The other instances included fentanyl at 2% and methamphetamine at 6%. Therefore, 1% was considered to be an appropriate approximate detection threshold for this work, with many compounds being detected below this limit consistently. Several of the studied components, including 4-anilino-*N*-phenethylpiperidine (ANPP) (n = 4), fluorofentanyl (n = 18), furanyl UF-17 (n = 1), medetomidine (n = 8), *ortho*-methylfentanyl (n = 19), and phenacetin (n = 1) were detected at all studied concentrations. Notably, no benzodiazepines were detected at below 1% w/w. However, the median concentration of bromazolam observed for samples analyzed by the Substance Drug Checking Project in 2023 was 3.6%,[58] suggesting that the 1% detection threshold is sufficient for a drug-checking context.

Table 1 Detection rates for compounds with previous quantitative information present. The detection rate for each compound is compared at below and above 1% w/w, which was determined to be the detection threshold. The values in parentheses indicate the number of samples

	Detection rate		
Compound	>1%	<1%	Overall
Acetylcodeine	N/A	0 (1)	0 (1)
Acetylmorphine	1.0 (1)	0 (1)	0.5 (2)
ANPP	1.0 (3)	1.0 (1)	1.0 (4)
Bromazolam	0.83 (12)	0 (2)	0.71 (14)
Desalkygidazepam	1.0 (1)	0 (1)	0.50 (2)
Fentanyl	0.97 (32)	0.40 (5)	0.89 (37)
Flubromazepam	1.0 (2)	0 (1)	0.67 (3)
Fluorofentanyl	1.0 (10)	1.0 (8)	1.0 (18)
Furanyl UF-17	1.0 (1)	N/A	1.0 (1)
Medetomidine	1.0 (4)	1.0 (4)	1.0 (8)
Methamphetamine	0.67 (3)	N/A	0.67 (3)
ortho-Methylfentanyl	1.0 (17)	1.0 (2)	1.0 (19)
Phenacetin	1.0 (1)	N/A	1.0 (1)
Xylazine	1.0 (8)	1.0 (7)	1.0 (15)

The limited ability to detect benzodiazepines at low concentrations is suspected to be due to competitive ionization. Competitive ionization results in sample components with high proton affinities consuming most of the available charge in the ionization step, hindering the ionization of components with lower proton affinities.[53] The proton affinity of a compound refers to its ability to accept protons, which speaks to its ability to form protonated cations for positive ion mode mass spectrometry. Fig. 1 highlights the complex nature of the analyzed samples, where the majority of samples contain four or more active components, suggesting that there are multiple compounds competing for the available charge. Competitive ionization has previously been documented in drug mixtures using thermal desorption-DART-MS.[59] The proton affinities for fentanyl and several analogs has been previously reported to range from 1018 to 1078 kJ mol^{-1}.[60] While this study did not include fluorofentanyl or *ortho*-methylfentanyl, we expect new analogs to be close to or within the reported range due to their structural similarities. The proton affinities for several benzodiazepine drugs have been reported to range from 554 to 856 kJ mol^{-1}.[61] However, that study was performed prior to the emergence of many of the designer benzodiazepines that are common in the street-drug supply. While there is a larger range of proton affinity values reported for benzodiazepines due to their diverse structures, the values are lower than fentanyl analogs, and thus we expect a similar trend for the newly emerging benzodiazepines. The lower proton affinities for benzodiazepines suggest that they are subject to more competitive ionization effects, and could explain why we see lower signal intensities and a lower rate of detection for these compounds.

In an attempt to improve the signal intensity observed for benzodiazepines, isopropanol was also investigated as a spray solvent for PS-MS. While previous studies involving PS-MS for drug detection have used a mixture of acetonitrile, water, and formic acid for various drugs and metabolites in biological samples, spray-solvent optimization studies have not been demonstrated for street-drug samples prepared in methanol.[44,62,63] It has been reported that binary solvent mixtures could create challenges for PS-MS, where solvents with different evaporation rates could cause changes in solvent composition over the course of the ionization.[64] There is support in the literature for the improvement of signal intensity using isopropanol alone, which prompted the comparison of this solvent to that of the acetonitrile mixture.[64–66] However, our results showed that the acetonitrile solution resulted in superior signal intensity and repeatability for all six components in the analyzed street-drug mixture (Fig. S1†). This is likely because of improved protonation in positive ion PS-MS for acetonitrile, and the addition of formic acid for signal stability.[63,67] Therefore, the acetonitrile solution continues to be used for all analyses in this work.

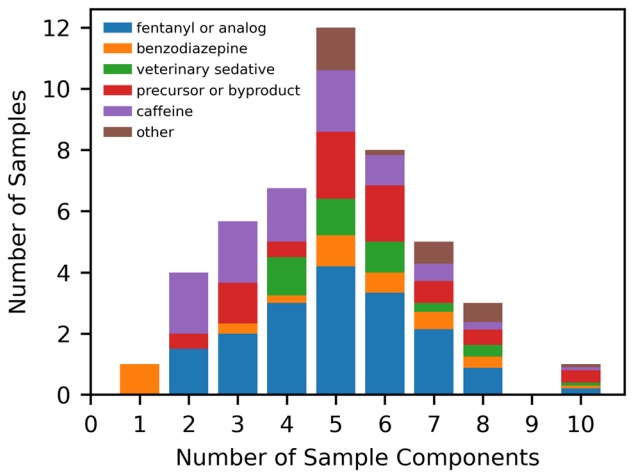

Fig. 1 A breakdown of the number of sample components for 47 samples, and the proportional makeup of drugs from each category. The number of components represents detection by either the routine quantitative analysis method at Substance Drug Checking, the developed PS-HRMS DDA method, or both. The "other" category includes methamphetamine, primidone, BTMPS, and phenacetin.

3.1.2 DDA peak search algorithm. The developed search algorithm was used to quickly identify the precursors being selected for fragmentation. The performance of the algorithm was evaluated using street-drug samples of known compositions based on the results of the routine drug-checking analysis method. Precursor *m*/*z* values selected by the developed method were compared to *m*/*z* values in the database for identification of the corresponding compound. Values within a tolerance of 0.001 *m*/*z* of the true value were considered a preliminary identification, followed by the use of MS/MS data to confirm the presence of the compounds. Table 2 displays the results of the precursor selection portion of the algorithm for components in an opioid sample. In this sample, all components previously identified by the targeted QqQ analysis method were detected, along with a precursor for *ortho*-methylfentanyl, *ortho*-methyl-ANPP, which is not included in the routine analysis method. The identity of the precursor was then confirmed by the presence of characteristic fragments in the corresponding MS/MS spectrum. The database contains one, two, or three of the highest intensity fragments for each precursor. Table 2 shows which fragments were present for the precursors selected in a street-drug sample. For the displayed sample, all precursors could be confirmed by the presence of the characteristic fragments. MS/MS data for the selection of these characteristic fragments were obtained from mzCloud (HighChem, Slovakia), or where not available, from the Center for Forensic Science Research and Education (CFSRE) monographs.[56] A previous study demonstrated that data from the mzCloud database sufficiently matched in-house PS-MS data collected on an Orbitrap instrument.[51] We therefore determined the use of fragments obtained from sources in the literature would be sufficient to compare with our obtained PS-MS data. A schematic of the peak search algorithm is given in Fig. S2.†

While spectral libraries are widely used for the identification of compounds from MS/MS spectra, they are typically developed for MS/MS spectra for chromatographically separated compounds. This can create challenges when attempting to match direct mass spectrometry spectral data against refer-

Table 2 Drug precursor *m/z* values detected by the DDA method within the tolerance range of their theoretical mass (0.001 *m/z*) as identified by the algorithm for a single sample. The ppm error is also calculated and displayed. Results of the fragment search algorithm for the selected precursors are displayed, where two or three ions were used to confirm the identity of the drug, and were considered a match within a tolerance range of 0.001 *m/z*

Compound	Theoretical *m/z*	Experimental *m/z*	Error (ppm)	Ion 1	Ion 2	Ion 3
Caffeine	195.0877	195.0878	0.5126	Y	Y	N/A
Fentanyl	337.2275	337.2275	0.0000	Y	Y	Y
Medetomidine	201.1386	201.1388	0.9943	Y	Y	N/A
ortho-Methyl-ANPP	295.2169	295.2168	−0.3387	Y	Y	Y
ortho-Methylfentanyl	351.2431	351.2432	0.2847	Y	Y	Y
Xylazine	221.1107	221.1107	0.0000	Y	Y	Y

ence library spectra. Additional peaks present in the spectra resulting from the lack of chromatographic separation cause library match scores to be low, and the likelihood of identification in this manner is unreliable. Another challenge with relying on spectral libraries is that the identification of compounds is only possible if an entry exists in the database.[53] Spectra for newly emerging substances are often available online or can be predicted by *in silico* fragmentation prediction software before spectral libraries are updated. Therefore, the developed spectral matching algorithm can aid in compound identification efforts for PS-MS spectra through using fragment ion matching instead of using the entire MS/MS spectra.

Another advantage of this method is that the mass database is simple to update, and data can be reprocessed to identify compounds retrospectively. Therefore, in our approach, the aim was to optimize the instrumental method to be able to select relevant peaks, and eliminate the need for an inclusion list, allowing retrospective analysis of the data for newly emerging substances in the illicit supply when required.

3.2 Detection of substances outside of targeted methods

3.2.1 Selection of drug samples for further analysis. Data were analyzed in accordance with how this method could be implemented for on-site drug-checking services. There is currently insufficient capacity for every opioid sample to be analyzed by PS-HRMS at an off-site location. Therefore, a randomized selection of drug samples received during harm-reduction services was included in the PS-HRMS analysis. With retroactive sampling, *i.e.* after individual service users have been provided with results, the main goal at this stage is supply monitoring to identify newly emerging substances appearing in multiple samples as a new trend, and not as a single outlier. While the routine analysis method is superior in terms of its quantitative ability, there is a need to investigate the supply to identify what newly emerging compounds should be included in the routine analysis method. Thus, this sampling strategy was determined to be effective given the mentioned constraints. The samples investigated were opioid or benzodiazepine samples, which were randomly selected from August to November of 2024. Using this strategy, several compounds were detected outside of the capabilities of the targeted quantitative method, including various synthesis precursors and byproducts, which are given in Table 3. Additional substances were detected in 34% of samples, demonstrating the need for untargeted analyses in addition of routine targeted analysis methods.

3.2.2 Detection of newly emerging substances. Several newly emerging substances were identified using the developed DDA method. These included several fentanyl precursors and byproducts, primidone, and bis(2,2,6,6-tetramethyl-4-piperidyl) sebacate (BTMPS). Primidone, a prodrug of phenobarbital, is a newly emerging adulterant that has been previously detected in Alberta and Ontario.[68,69] The central nervous system depressant effects of primidone combined with other depressant drugs could increase the risk of severe or fatal toxicity.[69] Primidone was confirmed in 4 samples through the fragmentation search algorithm, with the confirming fragments highlighted in Fig. 2. The low intensity of the fragments in the spectrum could be for several reasons, including if it is present at a low concentration, information which is currently unknown, or the poor ionization efficiency of primidone in positive ionization mode. Barbiturates and barbiturate-type drugs are often analyzed using negative ion mode due to better ionization;[70,71] however, there has been some success analyzing drugs of this class using positive ionization.[72] This highlights one of the limitations of untargeted screening methods, in that the method cannot be optimized for the detection of each individual compound. However, despite such limitations, new substances including primidone were detected using the developed method. The comparatively wide precursor selection window (2 *m/z*) also contributes to the noise in the spectrum, as there are fragments from the isolation of additional precursors within that window. All precursor ions within the window for the primidone ion are displayed in Fig. S3.† However, the use of a wider isolation window can allow for greater sensitivity, and ensures a significant amount of data is collected.

Table 3 Summary of newly detected substances

Compound	Number of detections
BTMPS	7
F-ANPP	10
Fluoro-phenethyl-ANPP	5
ortho-Methyl-ANPP	7
Phenethyl-ANPP	1
Primidone	4
Protonitazepyne/isotonitazepyne	1

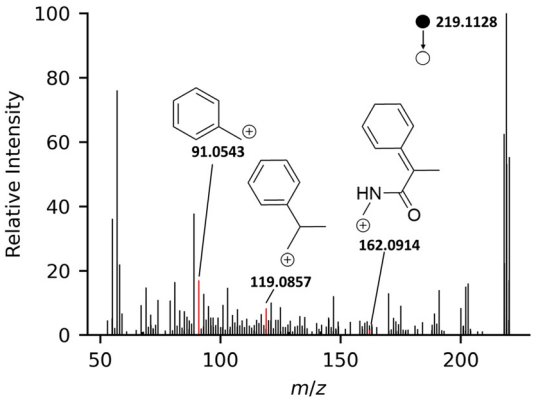

Fig. 2 High-resolution PS-MS/MS $[M + H]^+$ spectrum of primidone, with the three selected confirming ions highlighted.

BTMPS was also detected in several samples and has been identified in the unregulated drug supply in various locations across the United States.[73] BTMPS is pharmacologically active, and is widely used as a light stabilizer for plastics and in pharmaceutical products.[74,75] While this compound has been recently identified in the street-drug supply, it is difficult to identify the origin or purpose of the compound with regard to street drugs. Due to its use as a pharmaceutical stabilizer, there is a possibility that it was introduced into the street-drug supply to play a similar role.[74] BTMPS has also been studied in relation to its potential in decreasing withdrawal symptoms in rats, but such effects of the drug have not been studied on humans, and other related health harms are largely unknown.[76] The presence of BTMPS was confirmed with the PS-HRMS $[M + 2H]^{2+}$ MS/MS spectrum, as shown in Fig. 3, and is in agreement with previously published data on BTMPS.[73] The noise in the spectrum can be attributed to other ions present within the precursor isolation window, which is shown in Fig. S4.†

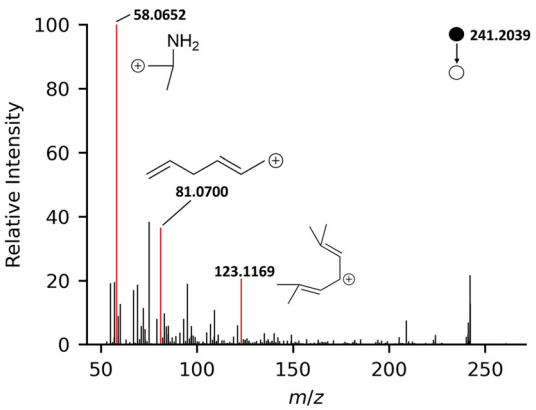

Fig. 3 High-resolution MS/MS $[M + 2H]^{2+}$ spectrum of BTMPS, with the three selected confirming ions highlighted.

The compositions of samples with BTMPS and primidone were investigated to determine if there were any trends associated with the makeup of the samples where these components are present. Table 4 lists a breakdown of the components detected for each sample in addition to the newly emerging substances. While no significant trends are apparent, all samples are complex in nature, containing drugs from 3–5 different drug classes, with 4–7 components present (Fig. 4). This highlights the importance of regular drug screening, as newly emerging substances can be present in samples that contain multiple known active components, and are not necessarily replacing known active components. Additionally, the sample compositions identified for primidone are in agreement with previous reports that indicate primidone was detected in the presence of fentanyl and/or an analog, along with medetomidine, a benzodiazepine, and/or xylazine.[69]

3.2.3 Detection of precursors and synthesis byproducts. The detection of precursors and synthesis byproducts can also play an important role in harm-reduction messaging. Detecting precursors and byproducts in low concentrations is not necessarily considered as providing the most pertinent harm-reduction information, as most of these compounds have low bioactivity compared to the drugs they are used to synthesize. Furthermore, reports suggesting harm from these compounds are limited and instead normal or weak potency has been reported.[77] However, in high concentrations, the presence of these compounds could result in acute or long-term effects, and their detection may be relevant for people who use drugs. Although not well documented, precursors may cause other unexpected effects on the individual user, including variations in taste or smell when consuming the drug. Additionally, the detection of precursors and byproducts could be used to assess the relative purity in "raw" opioid samples. The presence of precursors can also provide information regarding the composition of the sample, and may account for complex signals observed for commonly used harm-reduction techniques including FTIR and Raman spectroscopy.

Table 4 A comparison of the original results obtained from the drug-checking site, with the unknowns identified following PS-HRMS analysis

Original results	Unknown identification
Benzodiazepine (unknown type), microcrystalline cellulose	Phenazolam
Benzodiazepine (unknown type), caffeine, erythritol, fentanyl, *ortho*-methylfentanyl, xylazine	Desalkylgidazepam
Microcrystalline cellulose, unknown	Protonitazepyne, isotonitazepyne
Caffeine, erythritol, fluorofentanyl, precursor (unknown type)	F-ANPP, fluoro-phenethyl-ANPP
Caffeine, erythritol, fluorofentanyl, precursor (unknown type)	F-ANPP, fluoro-phenethyl-ANPP
Fentanyl, precursor (unknown type)	F-ANPP, phenethyl-ANPP

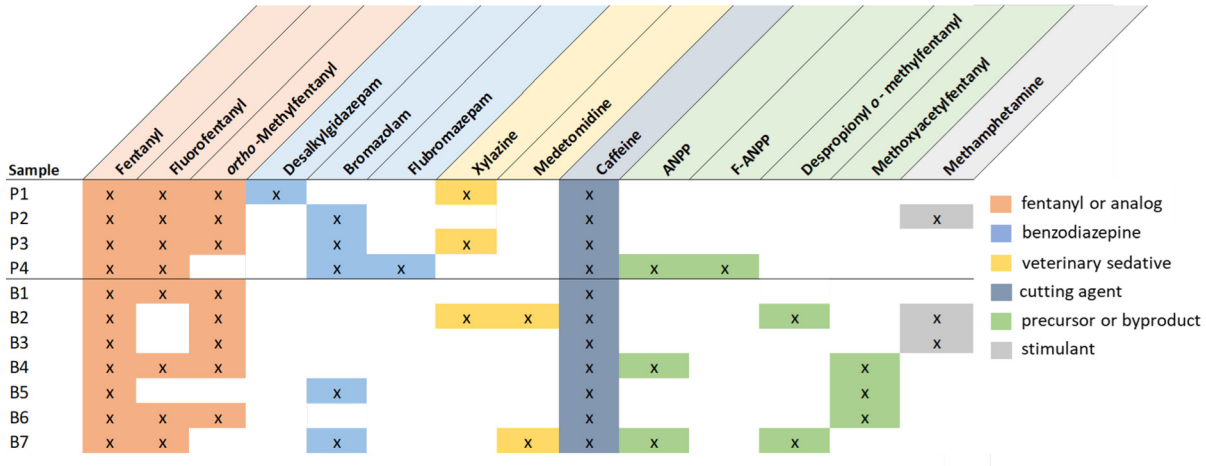

Fig. 4 Components detected in addition to primidone (P*n*) and BTMPS (B*n*) in street-drug samples.

Methoxyacetyl fentanyl, which has been identified as both a synthesis byproduct and an active analog, was detected in 14 samples in this work.[78] This drug has been identified as a synthesis byproduct in both the Gupta[79] and Siegfried synthesis[80] methods,[81] which are commonly used in clandestine laboratories as a result of their relative simplicity over the patented Janssen method.[82] All sources for detection of this compound were samples where fentanyl was also present, which suggests its presence as a synthesis byproduct over being an added adulterant.

Two analogs of ANPP were also detected. Fluoro-ANPP (F-ANPP), a fluorofentanyl precursor, was detected in 10 samples, though notably not always alongside fluorofentanyl. F-ANPP was confirmed by the presence of the selected fragments at *m*/*z* 105.0700, 134.0965, and 188.143, which are all common fragment ions for fentanyl-related compounds. The PS-HRMS/MS product spectrum for this detection is shown in Fig. S5.† *ortho*-Methyl-ANPP, an *ortho*-methylfentanyl precursor, was detected in 7 samples. This precursor was most frequently detected alongside *ortho*-methylfentanyl, except in one instance. The spectrum for *ortho*-methyl-ANPP is shown in Fig. 5. The confirming fragments for this compound are of high intensity in the spectrum, which is related to the high intensity of the *ortho*-methyl-ANPP ion in the precursor selection window (Fig. S6†).

Other emerging precursors, namely phenethyl-ANPP and fluoro-phenethyl-ANPP, were tentatively identified based on their similar structure and fragmentation pattern to ANPP and F-ANPP respectively. Phenethyl-ANPP was detected in 1 sample, and fluoro-phenethyl-ANPP was detected in 5 samples. These compounds have been suspected to result from different synthesis routes in the manufacturing of fentanyl.[83] The *in vitro* μ-opioid receptor activity of phenethyl-ANPP has been reported to be negligible, especially when compared to that of fentanyl.[83] There have been no reports on the μ-opioid receptor activity of fluoro-phenethyl-ANPP at this time, but it is suspected to be of similar activity to phenethyl-ANPP

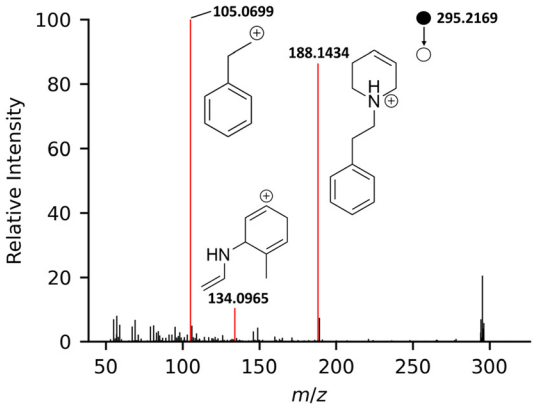

Fig. 5 High-resolution PS-MS/MS spectra for *ortho*-methyl-ANPP (*m*/*z*, 295.2169), with the three target fragment ions highlighted.

as a result of their structural similarity. While there have been several previous reports of the detection of phenethyl-ANPP in the United States,[14,83] there are limited reports regarding the detection of fluoro-phenethyl-ANPP. Fig. 6 demonstrates the similarity between the major fragments for fluoro-phenethyl-ANPP and F-ANPP, specifically at *m*/*z* 105.0700, 134.0967, and 188.1436. These fragments are shared among many 4-anilino-piperidine-type fentanyl analogs and precursors, and were identified by *in silico* fragmentation of this compound.[84] Therefore, we were able to tentatively identify the presence of this precursor in 5 samples.

3.2.4 Identification of unknown components. Several of the samples were selected based on containing "known unknowns", whether as the result of having no active components detected in pressed pills, positive results on benzodiazepine immunoassay test strips with no drugs of this class identified, or unidentifiable signals from FTIR results. The PS-HRMS DDA method was able to identify these components. The unknown sample components identified using this

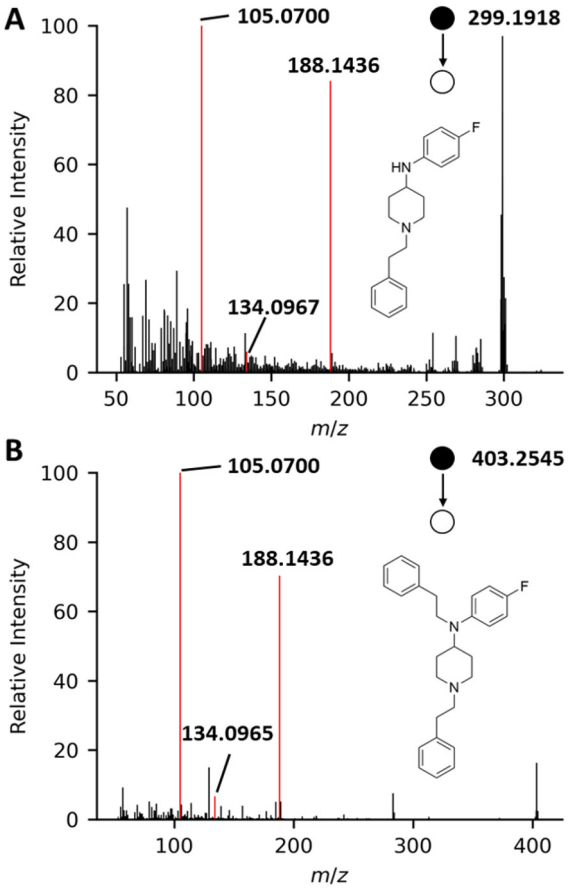

Fig. 6 High-resolution PS-MS/MS $[M + H]^+$ spectrum of (A) F-ANPP with the three selected confirming ions highlighted, and (B) fluoro-phenethyl-ANPP with three selected confirming ions highlighted.

method are listed in Table 4. It should also be noted that the detection of cutting agents such as erythritol and microcrystalline cellulose comes from the FTIR results, highlighting the benefits of using this instrument in combination with mass spectrometry.

The detection of these compounds highlights the benefits of using multiple instruments for drug checking, and how together, they allow for a more complete picture of the composition of the street-drug supply, the necessity of which has been previously reported.[34,85] Samples labeled as containing "Benzodiazepine (unknown type)" had a positive result with an immunoassay test strip, with none of the benzodiazepines in the targeted PS-MS method detected. Using the non-targeted PS-HRMS DDA method in this case allowed for the detection of benzodiazepines that were not yet included in the targeted method: phenazolam and desalkylgidazepam. Additionally, a pill with no active components detected was found to contain either of protonitazepyne or isotonitazepyne, which are isobaric and cannot be distinguished using mass spectrometry alone. There have been reports of isotonitazepyne appearing in expected oxycodone pills in Australia,[86] and protonitazepyne in drug powders in Ireland.[87] A study investigating the μ-opioid receptor activity of protonitazepyne and several other nitazenes suggests that these compounds are more potent than fentanyl.[88] However, in-depth studies of the potencies of these drugs are limited. In other cases, FTIR signals indicated a precursor is present based on partial library matching to similar precursor compounds but were unable to be confirmed as spectra for the specific precursors, F-ANPP, phenethyl-ANPP, and fluoro-phenethyl-ANPP, which are not present in the libraries. Confidence in identification can also be increased with the agreement of results from different instrumental analyses.

The PS-HRMS method developed here was able to identify newly emerging substances in samples selected at random, in addition to samples selected because an unknown component was present. This demonstrates the utility of both analyzing samples where there is an obvious unidentified component, and random selection of samples for additional trace components that may be present. The identification of newly emerging substance by the PS-HRMS method can also guide the inclusion of compounds on targeted mass spectrometry methods, allowing these substances to be routinely detected by drug-checking services. Therefore, the importance of multi-instrument drug checking is demonstrated, where the combined strengths of multiple instruments allow for a more complete understanding of the unregulated drug supply.

4 Conclusions

The developed PS-HRMS DDA method provides a solution to address a current limitation in harm-reduction drug checking regarding the detection of newly emerging substances in the street-drug supply using PS-HRMS. The simplicity and speed of such a method provides a rapid screening tool to monitor the rapidly evolving drug supply, and therefore allows further insight into the complexities of street drugs. The presented work demonstrates the usefulness of combined quantitative and qualitative mass spectrometry methods in the context of harm-reduction drug checking.

Author contributions

Conceptualization: A. M., C. G.; data collection: A. M.; methodology: A. M.; analysis and interpretation: A. M.; writing: A. M., D. K. H., C. G.; funding acquisition: B. W., D. K. H., and C. G.

Data availability

Data will be made available from the corresponding author upon reasonable request.

Conflicts of interest

There are no conflicts to declare.

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