Advances with Solid Substrate Spray Mass Spectrometry for Quantitative Illicit Drug Measurement

by

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B.Sc. Chemistry, California State University - Sonoma, 2019

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Abstract

The opioid crisis continues to be the leading cause of overdose and death among people who use drugs (PWUD). Harm reduction drug checking (HRDC) services aimed at preventing accidental overdose events do so by offering pre-consumption chemical measurements to help PWUD make informed decisions regarding a drug they intend to use. Conventional on-site drug checking technologies such as immunoassay test strips, colorimetry test strips, FT-IR, and portable Raman spectroscopy results do not possess adequate sensitivity for trace levels of toxic drugs, and/or the selectivity to detect newly emerging threats as they appear in the illicit drug supply. Chapter 1 of this thesis provides background information, as well as framework to why mass spectrometry (MS) is the ideal candidate for the analytical task of quantitative HRDC. Solid-substrate electrospray ionization (ESI) was developed as a rapid and direct ionization method for the qualitative and quantitative analysis of complex samples with little to no sample preparation. In this thesis, paper spray mass spectrometry (PS-MS) and paper capillary spray ionization (PCSI), two derivatives of solid-substrate ESI, were utilized for quantitative illicit drug measurements.

Conventional (quantitative) chemical analysis requires samples to be sent to centralized laboratories with the requisite supporting infrastructure, however, innovations to the field of portable MS has revolutionized the paradigm of real-time chemical analysis by bringing the laboratory to the sample, answering chemical questions when and where they are needed. In Chapter 2 of this thesis, a simple, rapid, and quantitative ambient ionization tandem mass spectrometry method was developed and implemented for the analysis of real-world illicit drug samples utilizing a miniature mass spectrometer system. The performance, characterization, and improvement of a portable/miniature mass spectrometer (based on a rectilinear quadrupole ion trap) was initially characterized using legally exempt test kit drug standards. A novel spray solvent addition system was developed to improve inter- and intra-day reproducibility, calibration linearity, and analytical sensitivity. The system was then evaluated as a potential on-site, rapid, point-of-care chemical diagnostic system to identify and quantify illicit drug analytes. Target

analytes were detected and quantified via PCSI-MS/MS for samples where conventional on-site drug checking technologies were not always effective.

In Chapter 3 of this thesis, various internal standard (ISTD) utilization strategies were evaluated with the goal of simplifying HRDC PS-MS quantitative measurements while drastically reducing waste. This was achieved by depositing ISTDs on PS-MS strips, pre- and post-sample deposition, to evaluate its quantitative performance of illicit drug analytes. Pre-sample ISTD depositions reduce disruptions in (PS-MS) HRDC services due to ISTD supply/integrity and improves turnaround time for chemical analysis; all of which reduce analytical costs (i.e., ISTD consumables, labor costs, solvent) for real world samples. Post-sample ISTD depositions offer the ability to send in-field samples to off-site laboratories for appropriate ISTD deposition. A parallel study assessed the analytical performance of delivering 1 nanogram of ISTD per paper strip by one of two methods: 1) the conventional method by “Hand”, depositing volumes with a mechanical micropipette, and 2) by “Robot” through the assistance of a robotic liquid handling system. The goal of this thesis is to promote the feasibility and wide-spread adoption of solid-substrate spray-based MS technology as an on-site HRDC tool.

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List of Abbreviations

AC	Alternating Current
AMS	Ambient Mass Spectrometry
API	Atmospheric Pressure Interface
BC	British Columbia
CID	Collision-Induced Dissociation
CEM	Chain Ejection Model
CRM	Charge Residue Model
CV %	Coefficient of Variation %
Da	Dalton
DART	Direct Analysis in Real Time
DESI	Desorption Electrospray Ionization
DAPI	Discontinuous Atmospheric Pressure Interface
DC	Direct Current
DMS	Direct Mass Spectrometry
EI	Electron Impact Ionization
ESI	Electrospray Ionization
FT-IR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography Mass Spectrometry
HPLC	High Pressure Liquid Chromatography
HRDC	Harm Reduction Drug Checking
ID	Internal Diameter
IEM	Ion Evaporation Model
ISTD(s)	Internal Standard(s)
LC-MS	Liquid Chromatography Mass Spectrometry
LIT	Linear (Quadrupole) Ion Trap
LOD	Limit of Detection
$[M-H]^-$	Deprotonated Parent Ion
$[M+H]^+$	Protonated Parent Ion
m/z	Mass to Charge Ratio

MDA	3,4 - Methylenedioxyamphetamine
MDMA	3,4 - Methylenedioxymethamphetamine
MeOH	Methanol
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NanoESI	Nanoelectrospray
ND	Not Detected
NQ	Not Quantifiable; “Fentanyl or Analogue”
OD	Outer Diameter
PCSI	Paper Capillary Spray Ionization
PS-MS	Paper Spray Mass Spectrometry
PWUD	People Who Use Drugs
QC	Quality Control
QqQ	Triple Quadrupole Mass Analyzer
Q1	1st Quadrupole in a Triple Quadrupole Mass Analyzer
q2	2nd Quadrupole in a Triple Quadrupole Mass Analyzer
Q3	3rd Quadrupole in a Triple Quadrupole Mass Analyzer
RF	Radiofrequency
RIT	Rectilinear Ion Trap
SWIFT	Stored Wave Inverse Fourier Transform
Tandem MS	Tandem Mass Spectrometry
V	Voltage
w/w %	Weight to Weight %

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Dedication

For Dad.

Thank you for your unwavering moral, emotional, and financial support in life, and especially throughout this degree. Thank you for teaching me the value of following up, the importance of being yourself, and giving me the strength to endure when I thought I couldn’t. We did it!

Chapter 1: Introduction

1.1 Harm Reduction Drug Checking

The opioid epidemic continues to have a profound impact affecting individuals, families, and communities across the globe. This crisis has resulted in devastating consequences, including skyrocketing overdose rates and an increased burden on health care systems. There are a number of strategies currently being implemented to assist people who use drugs (PWUD) such as decriminalization, naloxone programs, opioid agonist therapy, safe drug supply, safe consumption sites, and drug checking as a harm reduction service. Harm reduction drug checking (HRDC) offers PWUD access to pre-consumption chemical analysis; this approach aims to empower drug users with knowledge about the composition and potential risks associated with their substances, allowing them to make informed decisions and reduce harm. This approach not only helps prevent overdoses and adverse health effects but also fosters trust and engagement between PWUD and healthcare professionals, facilitating pathways towards further support and treatment. Overall, HRDC plays a vital role in mitigating impact of the opioid epidemic by prioritizing safety, education, and harm reduction within affected communities.

Illegally manufactured fentanyl and fentanyl analogues (fentalogs) are currently the greatest threat in the ongoing opioid crisis. PWUD are generally aware of the fentanyl contamination and polycomponent nature of illicit drugs and need HRDC technologies to provide sensitive, selective, and quantitative drug analysis to help PWUD make informed decisions and avoid an overdose event. Some of the HRDC technologies currently being implemented in British Columbia (BC) consist of colorimetric tests, drug test strips, Fourier-transform infrared spectroscopy, and Raman spectroscopy. These methods provide potentially lifesaving information but are frequently unable to detect and/or identify trace level (and newly emerging) illicit drugs. Ideally, HRDC technology should be: i) sensitive, to detect toxic substances at trace-level concentrations (i.e., < 1% w/w); ii)

selective, to determine and differentiate substances within a single sample; iii) quantitative, to determine how much of each substance is present; iv) adaptable, to address changes in drug supply; v) free from interferences (i.e., no sample carryover); vi) rapid, to provide PWUD with chemical information in a timely manner; vii) easy to use and interpret results to be effective for non-chemists; and viii) field-deployable for 'pop-up' harm reduction sites. Access to funding and resources ultimately dictate the services and drug checking technologies offered at individual harm reduction sites. A direct mass spectrometry approach called paper spray mass spectrometry (PS-MS) is an effective strategy for this analytical task[1]. PS-MS has the necessary sensitivity and selectivity to detect and quantitate illicit drug analytes with picogram detection limits[2] and requires small sample sizes (~1 mg) for quantitative analysis, a crucial factor to encourage PWUD to utilize this harm reduction strategy. Modern PS-MS systems are equipped with a (robotic) auto-sampler which permits the automation of (up to) 240 illicit drug samples, ideal for harm reduction sites with high-volume sample intake. PS-MS design incorporates separate solid substrates (i.e., filter paper) for individual (drug) samples, eliminating any sample-to-sample carryover. Turnaround time for quantitative chemical analysis is a major deciding factor on whether or not PWUD will use drug checking as a harm reduction service[3]; PS-MS analysis affords rapid chemical analyses, requiring only 5 minutes from initial drug sample intake to obtain individual quantitative drug results[4]. Recent advancements to miniature mass spectrometer systems promote the mobilization of this technology to be utilized for on-site drug checking at a reduced cost with simplified maintenance. Improvements to software allow for harm reduction personnel (i.e., not trained in MS) to access this technology without the need for a designated technician.

The goal of this thesis is to evaluate various quantitative techniques to make harm reduction drug checking logistically easier in terms of hardware (i.e., rectilinear ion trap) and workflow (i.e., pre-spotted internal standard deposition). Chapter 2 of this thesis evaluated a miniature mass spectrometer system for its ability to quantitate four target illicit drug analytes (carfentanil, fentanyl, fluorofentanyl, and etizolam) commonly observed in HRDC. Chapter 3 of this thesis utilized various internal standard deposition

strategies to improve and simplify analytical workflow for quantitative illicit drug measurements utilizing PS-MS technology.

1.2 Mass Spectrometry

Mass spectrometry (MS) is one of the most versatile technologies in modern day analytical laboratories due to its ability to separate, isolate, and detect the abundance of charged, gas phase ions. Independent of the analytical application, the goal of MS-based measurements is to identify, qualitatively or quantitatively, a compound from its constituents. MS technology accomplishes this by manipulating electric and/or magnetic fields under vacuum conditions (10^{-3}–10^{-10} torr) to sort ions before detection, ultimately yielding a mass spectrum—a two-dimensional plot depicting the analyte(s) mass-to-charge ratio (*m/z*) on the x-axis and the signal intensity on the y-axis. All mass spectrometers incorporate a sample introduction system, ion source, vacuum source, mass analyzer, detector, and a data system to record measured ions[5]. Figure 1.1 is a simplified schematic representation of a MS system.

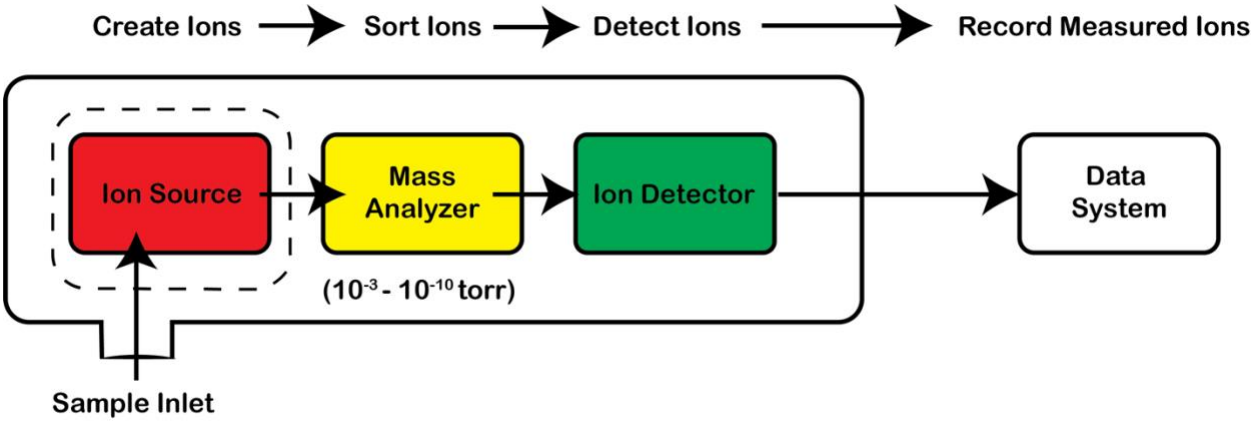

Figure 1.1: Schematic of a MS system. The dotted line around the ion source indicates that it can operate under vacuum or ambient pressure.

Regardless of the sample's state of matter, a prerequisite for MS analysis is that analytes in samples (liquid, solid, or gas) need to be transformed into isolated gas-phase ions. Analyte(s) of interest must carry a charge (positive or negative) for successful detection. Depending on the nature of the sample there are numerous methods to ionize analytes for mass analysis such as electron impact ionization (EI), chemical ionization, laser ablation methods, photoionization methods, and spray-based methods. The foundation of this thesis is based on (solid) spray-based ionization techniques and will be discussed in more detail. In the last 20 years there has been considerable advancements in MS technology due to the increasing demand for analytical sensitivity and selectivity. With smaller, and even portable instruments becoming available, MS is one of the most valuable tools in any analytical laboratory for targeted and untargeted chemical detection.

1.3 Ionization

1.3.1 Direct Mass Spectrometry

Direct mass spectrometry (DMS) is a field of analytical chemistry which affords a convenient sampling method of generating ions directly from the sample to be measured[6]. The goal of DMS is to yield instantaneous mass spectral analysis either void of sample preparation, or with a simplified sample pre-treatment process. Numerous ionization methods require a high vacuum environment to create ions, but advancements in ambient mass spectrometry (AMS) technology allow samples to be analyzed in their native environment (i.e., ambient pressure, temperature), thus propelling the field of chemical analysis forward by permitting real time chemical diagnostics. Conventionally, MS systems are coupled to a gas or liquid chromatograph (GC- or LC-MS) to provide orthogonal separation prior to mass spectrometry. This approach is often considered the 'gold standard' in terms of both sensitivity and selectivity[7]. However, GC- and LC-MS systems often require lengthy analysis times due to the incumbent sample preparation, clean up, and separation steps. All the additional requirements for these systems (i.e., external gas supplies, vacuum hardware, stable power, and wet labs for sample

preparation) inhibit mobile, in-field, point-of-care, and/or *in situ* analysis. DMS circumvents the time-consuming sample clean-up and separation steps utilized in GC-/LC-MS systems and affords rapid chemical diagnostics in a variety of applications[6]. The innovative characteristic of DMS technology is the ability to create ions in the sample's natural environment (at ambient pressure/temperature) and subsequently transfer the ions to the high vacuum manifold of the mass analyzer. There are a variety of methods to ionize molecules within DMS technology, but this thesis will focus on several spray-based ionization techniques in further detail.

The 1st published ambient ionization technique was introduced in 1973 by the Horning group, who introduced atmospheric pressure ionization[8]. This technological feat was made possible by the advent of an atmospheric pressure interface (API)[9], interfacing the sample's environment (ambient pressure; 760 torr) with the vacuum environment (10^{-3}–10^{-10} torr) of the mass analyzer. Thirty years later (2004), Dr. Graham Cooks and his research group introduced two of the most widely used DMS techniques—desorption electrospray ionization (DESI)[10] and a year later (2005) direct analysis in real time (DART)[11]. In addition to DESI & DART, the Cooks group invented paper spray mass spectrometry (PS-MS)[12], a foundation upon which this thesis is built. The molecule(s) of interest in DESI, DART, and PS-MS analysis are desorbed by exposing the sample surface to an ionizing medium[13]. The dragging force created from differential pumping carries ions from the ambient air towards the vacuum environment of the mass analyzer for subsequent detection. A myriad of DMS derivatives have risen out of emerging analytical challenges. Improvements in design and analytical performance have been continuously pursued to deliver chemical analysis through a variety of applications which conventional GC/LC-MS are not appropriate for[14–17]. Conventional chemical analysis often requires samples to be sent to centralized laboratories, however, recent portable mass spectrometer innovation grant scientists the ability to bring the lab to the sample, in-turn revolutionizing real time chemical analysis[18].

1.3.2 Electrospray Ionization

Electrospray ionization (ESI) is one of the most widely used ionization techniques and was awarded a Nobel prize (2002) for its ability to liberate gas phase ions from solution[19]. It should be noted that ESI is a method of ion transfer, rather than a pure ionization source since ions are generated in solution. The main success of ESI is transferring ions into a MS system for subsequent identification and/or structural analysis of ions ranging from small polar analytes to large biological molecules (i.e., molecular weights ranging from 100–100,000 Da[20]). This AMS technique is particularly useful for the analysis of polar analytes with functional groups that can readily accept or liberate a proton[5]. Samples may have an inherent charge, or if necessary, a charge can be imparted to the analyte(s) of interest by a charged tag[21] or by modifying solvent composition. In ESI-MS, solvent is pumped through a stainless-steel capillary which is held at a potential between 3–5 kV[22]. The applied electrostatic field distorts the meniscus into a “taylor cone” from the tip of the capillary (Figure 1.2) as the electrolytic solution is exiting the ESI capillary[5]. Figure 1.2 is a schematic representation of the mechanism for ion formation in an ESI device depicting the ejection of a jet of charged aerosol droplet that disintegrate into singly charged ions which are ultimately detected by an oppositely charged detector plate.

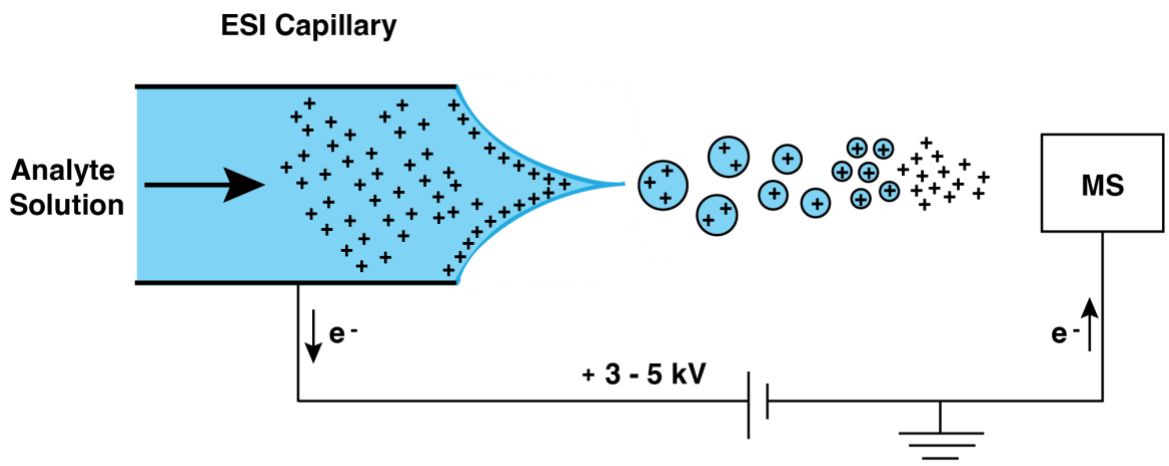

Figure 1.2: Schematic representation of the mechanism for ion formation in an ESI device depicting the ejection of a jet of charged aerosol droplets that disintegrate into singly charged ions which are ultimately detected by an oppositely charged detector plate.

There are multiple mechanisms which explain the liberation of intact gas phase ions in ESI: ion evaporation model (IEM) explains the mechanism for low molecular weight analytes, whereas charge residue model (CRM) and chain ejection model (CEM) explains the mechanisms for larger macromolecules[23]. The analytes studied in this thesis were all low molecular weight analytes (< 500 *m/z*), therefore the IEM will be explained in more detail. As charge separation occurs at the tip of the solution exiting the ESI capillary, a jet of micrometer-sized electrically charged droplets are generated. Solvent evaporation is promoted by the inclusion of a heated curtain gas or heated transfer line to produce smaller droplets. As a result, the droplet experiences an increase in charge density and ultimately, the electrostatic repulsion exceeds the surface tension of the droplet, which result in the droplet(s) rupturing. This phenomenon produces increasingly smaller subunits until the repetitive shrinking process liberates isolated charged gas-phase ions from the solution. Differential pumping and the manipulation of electronic fields assist in the transfer of these ions to the MS system. This process will be discussed in more detail (section 1.5).

ESI is considered a “soft ionization” approach because the resulting mass spectra are predominantly protonated ($[M+H]^+$) or deprotonated parent ions ($[M-H]^-$). This process starts at atmospheric pressure and incrementally proceeds into the high vacuum environment of the mass analyzer through the assistance of an API. The work presented in this thesis was accomplished using several iterations of ESI which will be discussed in further detail, however, for all subsequent ionization methods the ionization event occurs via the same mechanism (IEM). There are a number of ambient ionization techniques conceptually derived from ESI and are collectively referred to as ‘spray-based’ techniques.

1.3.3 Nanoelectrospray ionization

It has been observed that reducing the diameter of the ESI capillary results in a reduction of initial droplet size[24]. This is advantageous because it not only improves ionization efficiency but allows for extremely low sample consumption which is particularly useful when dealing with expensive and/or scarce samples. This is typically performed with a pulled borosilicate glass capillary containing a nanometer sized orifice[25]. This approach, termed nanoelectrospray ionization (NanoESI), has a nanoliter/min flow rate[26] and spray is maintained using only electrostatic potential, eliminating the need for a syringe pump, in-turn simplifying the interface and workflow. Additionally, the nanoflow rate affords numerous measurement trials from just 1 mL of sample. High voltage is applied using a conductive coating on the capillary or coaxially inserting a thin conductive wire inside the capillary (as in section 2.3.2). NanoESI spray capillaries are designed for single use events, eliminating memory effects and sample-to-sample cross contamination. However, NanoESI is not a feasible HRDC strategy because assembling these capillaries is non-trivial and the fine tipped points are prone to damage. HRDC requires a simplified sampling procedure which is cost and time effective without sacrificing significant analytical performance. Chapter 2 of this thesis utilized NanoESI for method development and parameter optimization. Figure 1.3 is a schematic representation of a NanoESI interface.

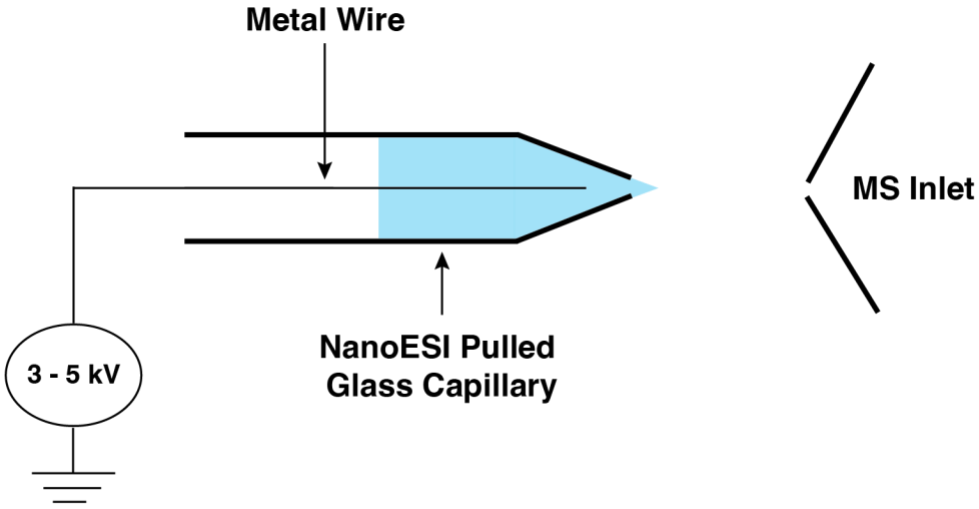

Figure 1.3: Schematic representation of NanoESI interface. The sample is incorporated into the spray solvent and injected into the NanoESI pulled glass capillary. Voltage is applied to a thin metal wire inserted coaxially into the capillary to induce a taylor cone at the tip where evaporation of solvent leads to isolated gas phase ions akin to ESI. The entire interface is positioned directly in front of the MS inlet.

1.3.4 Solid-Substrate Electrospray Ionization

Over the past 10 years, DMS technology has driven a paradigm shift in the types and scope of research questions analytical chemists can study, spurring further developments and applications. One example that has revolutionized DMS technology is solid-substrate ESI which incorporates a simplified sampling procedure for MS analysis, affording new applications of chemical diagnostics directly from a broad range of materials such as paper[27], leaves[28], wooden tips (i.e., toothpicks)[29], and coated materials[30]. Solid-substrate ESI generates ions akin to ESI (and NanoESI), however this innovation truly embodies the goal of DMS technology—to provide rapid chemical analysis with little to no sample preparation. ESI-MS requires the sample to be infused within the spray solvent through a capillary, which imparts time and infrastructure requirements for sample pre-treatment and introduces potential sample to sample carry over effects. As opposed to applying high voltage to a capillary as in ESI & NanoESI, the high voltage is applied directly to the substrate employed (i.e., paper, leaves, wooden

tips, coated materials, etc.). The chosen material either contains the analyte(s) of interest or is where a sample has been pre-deposited[31]. Solid-substrate ESI methods are a rapidly expanding field encompassing a wide range of substrates, however this thesis utilized paper and paper embedded with a capillary for sample introduction/ionization.

1.3.4.1 Paper Spray Mass Spectrometry

Paper spray mass spectrometry (PS-MS) is a relatively new DMS technique that has been heavily utilized over the last decade in a diverse range of applications, including but not limited to environmental monitoring, clinical settings, food quality, and biological analyses[29]. The key to success in PS-MS technology has been the advent of its simplified sampling procedure which takes advantage of a triangular piece of paper (i.e., filter paper) as the sampling medium. The paper substrate can be wiped onto a surface containing the analyte(s) of interest, or a small aliquot of liquid sample (≤ 10 µL) is deposited onto the paper strip[32]. Once dried, spray solvent is applied to the paper to assist in analyte extraction and ionization. Lastly, a high voltage (3–5 kV; positive or negative depending on desired analysis) is applied to the wet paper strip which ultimately yields isolated gas phase ions in a process akin to ESI[33]. One of the novel features of PS-MS technology is that ionization occurs at atmospheric pressure which significantly simplifies the interface; this feature makes it an exemplary ionization candidate for the miniaturization and mobilization of MS technologies. PS-MS technology is simple and straightforward, providing rapid and affordable chemical diagnostics with a simplified workflow. Modern PS-MS systems are equipped with advanced software which make PS-MS analysis accessible to non-chemists such as public health employees, law enforcement, harm reduction services, and medical personnel.

Paper strips are a cheap, porous solid-substrate sampling medium suitable for single use. The advantage of using filter paper as the sampling medium is not only the convenience of sampling for complex sample fluids such as whole blood, urine, or saliva, but also affords a crude (pre-analysis) chemical separation akin to paper

chromatography[34]. The paper composition and properties (i.e., thickness, pore size, solvent flow rate, and type) play a crucial role in either enhancing or impeding PS-MS ionization efficiency[27]. There have been extensive efforts and publications aimed at lowering detection limits along with improving and prolonging signal stability for complex samples. Such advancements include implementing paper pre-treatments like molecularly imprinted polymers synthesized on the surface of paper[35], hydrophilic/hydrophobic papers[36], and silver-impregnated paper for differentiating isobaric interferences[37], among others. PS-MS design is simple and straightforward, however, there are numerous factors that contribute to the successful production and detection of ions of interest. Features such as paper composition, imperfections in paper points, positioning of paper strip relative to MS inlet (angle and distance), and the solvent(s) employed all directly influence signal stability and successful chemical detection via PS-MS[33]. Imperfections in paper substrates can lead to the unwanted effect of multiple spray jets emitting from the paper, likely due to increased localized field strengths within the cellulose scaffolding of the paper strip[38]. This can result in erratic signals and irreproducible MS measurements. Figure 1.4 is a simplified schematic representation of a PS-MS interface: A) indicates the desired stable spray emitted from the tip of the paper strip from a carefully prepared PS-MS paper strip, and B) represents unwanted multiple spray jets emitting from the tip of the paper strip due to imperfect PS-MS paper strips.

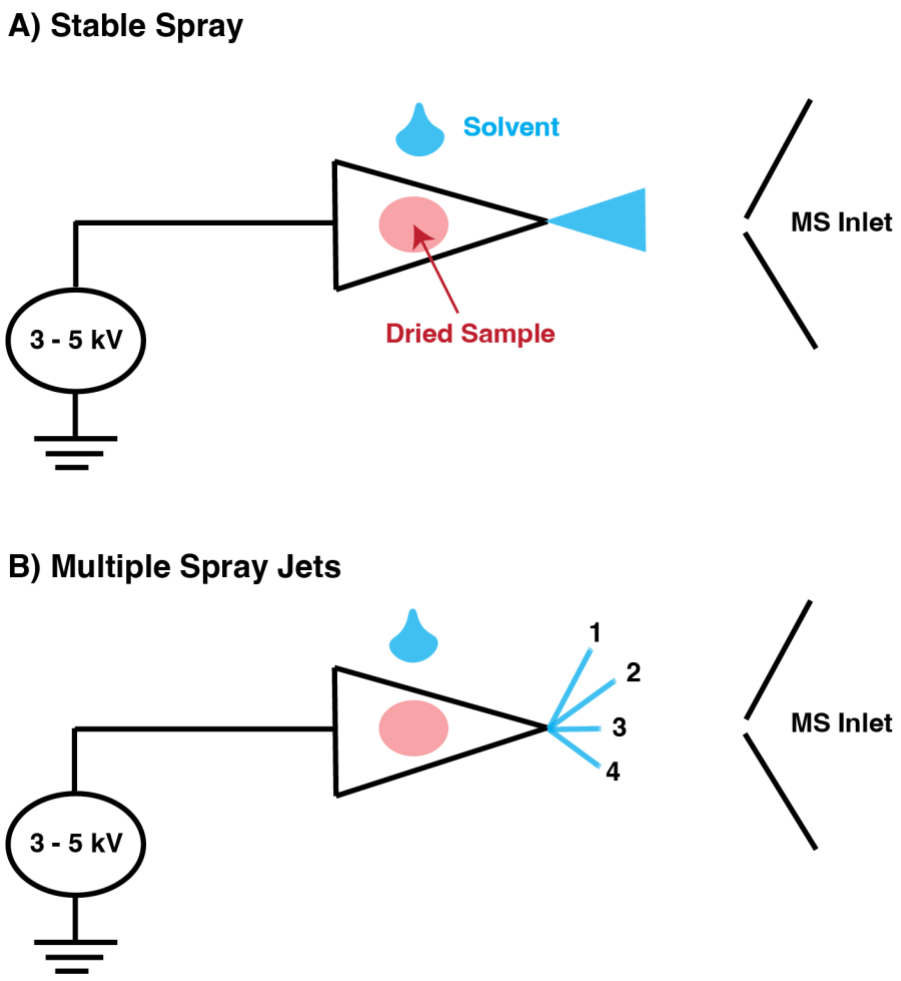

Figure 1.4: Schematic representation of PS-MS. Solvent and voltage are applied to the paper sampling substrate, which is positioned directly in front of the MS inlet. There are two depictions of PS-MS analysis: A) stable spray emitted from the tip of the paper for successful PS-MS analysis, and B) multiple spray jets due to imperfect paper strips which yield unstable spray and irreproducible MS results.

Analytes are ultimately extracted from the paper strip via solvent, therefore the delicate combination of solvent composition, solvent flow rate, and applied potential are paramount for successful PS-MS measurements[38]. The spray solvent solubilizes and extracts the analyte(s) of interest and the solvent/analyte solution wicks to the edges of the paper by capillary action[32]. Then, an applied voltage induces a taylor cone from the tip of that paper strip in the same process as ESI (and NanoESI). PS-MS has demonstrated that it is well suited for quantitative analysis because it can easily incorporate internal standards (ISTDs), either infused into the spray solvent, mixed in with

the sample, or pre-deposited on the paper strip[7]. ISTDs can be used to compensate for both matrix suppression as well as variance in paper cut and compositions[39]. Calibration curves are standard procedure for accurately quantitating analyte(s) of interest in PS-MS investigations and are constructed by choosing the concentration range in which the analyte is expected and plotting the signal intensity/area ratio of analyte to its respective internal standard as a function of analyte concentration (Figure 2.5). The sample's signal intensity/area ratio of analyte to internal standard is then plotted against analyte concentration to create a calibration curve to determine the concentration of unknown analyte(s). PS-MS is currently implemented as a HRDC tool providing PWUD with lifesaving information in BC, however, the upfront cost and infrastructure requirements have hampered the widespread application of this technology. There have been advancements in miniature MS technology as well as improving the uniformity of solid substrate sample introduction which will hopefully promote the widespread adoption of solid substrate spray-based methods as an effective (on-site) HRDC tool.

1.3.4.2 Paper Capillary Spray Ionization (PCSI)

Paper capillary spray ionization (PCSI) is a derivative of PS-MS technology which combines the advantages of PS-MS and NanoESI technologies. PCSI was developed to alleviate poor ionization efficiency which can result from non-uniform paper substrates such as inconsistent paper thickness and tip sharpness. Ren et al. demonstrated that embedding a fused silica capillary (ID: 50 μm; OD: 150 μm) into the tip of a paper substrate improved and prolonged spray stability, with the tip of the capillary acting as a NanoESI emitter[40]. PCSI retains the novel features of PS-MS such as simple sampling and rapid analysis, but couples it with the improved ionization efficiency and spray reproducibility afforded by NanoESI. PCSI is currently underexplored but may present an excellent option for high-speed and high-throughput analysis where normal PS-MS proves too imprecise/insensitive. Figure 1.5 is a simplified schematic representation of PCSI mechanism of creating ions for MS analysis. Through the wicking action of spray solvent and an applied voltage, the analyte(s) of interest are transported to the embedded

capillary, where they ultimately form a taylor cone at capillary tip. Ions are formed through the same process observed in the previously mentioned ionization methods (ESI, NanoESI, and PS-MS). PCSI was the sampling procedure employed in Chapter 2 of this thesis to ionize target analytes from illicit drug samples obtained from a harm reduction site in Victoria, BC.

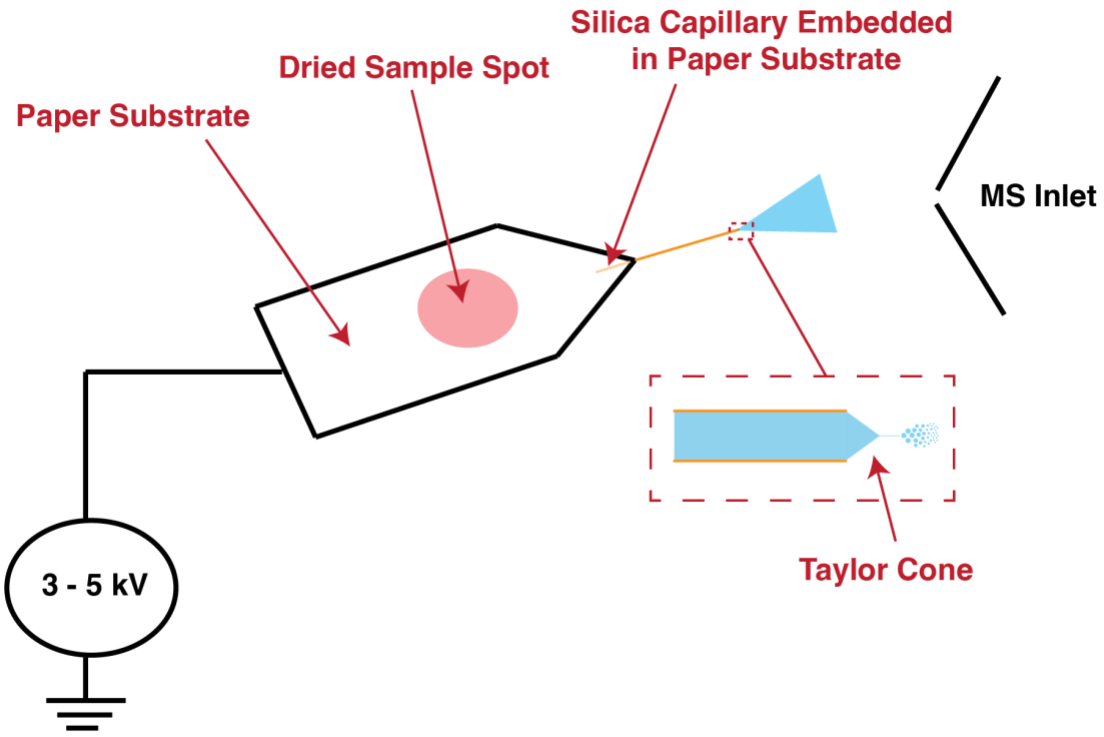

Figure 1.5: A simplified schematic of PCSI mechanism. The sample is deposited on a paper substrate with a silica capillary embedded in the tip. Once the sample is dried, spray solvent and a high voltage are applied which produce a taylor cone at the end of the capillary, as in ESI. PCSI combines the sampling advantages of PS-MS with the spray stability of NanoESI.

1.4 Tandem Mass Spectrometry

DMS has many intrinsic advantages as discussed above, however, given the lack of orthogonal separation (GC/LC), experiments must be designed carefully to ensure selective and accurate results. Tandem mass spectrometry (Tandem MS; MS/MS) is one such strategy that involves fragmentation of mass selected (precursor) ions followed by an additional MS scan of the fragments[41]. Tandem MS is the preferred strategy for quantitative DMS measurements, and the methods used in this thesis are termed "tandem-in-time" and "tandem-in-space". Tandem-in-space instruments create fragment ions from consecutive mass-analyzing processes, typically from three spatially separated mass analyzers as observed in triple quadrupole MS instruments. Tandem-in-time MS instruments utilize one mass analyzer (i.e., ion trap) to create product ions from mass-selected precursor ions[5]. Essentially, tandem-in-space instrumentation requires more hardware, whereas tandem-in-time instruments simply require more time. The data for this thesis was collected from both tandem-in-time (Chapter 2; rectilinear ion trap) and tandem-in-space (Chapter 3; triple quadrupole) MS instrumentation. The evolution of MS engineering coupled with the ingenuity of tandem MS permits the manipulation of isolated gas phase ions which will be discussed in the following sections.

The aforementioned "soft ionization" techniques (ESI, NanoESI, PS-MS, PCSI) allow chemists to analyze intact parent ions, albeit highly advantageous, this hinders the elucidation of structural information without the aid of tandem MS. Soft ionization techniques are desirable for tandem MS experiments because it keeps ions predominantly in their protonated ($[M+H]^+$) or deprotonated ($[M-H]^-$) molecular form. This is highly beneficial as there is a higher abundance of intact precursor ions to fragment. Alternatively, "hard ionization" techniques such as EI show an abundance of fragmented ions at the expense of the abundance of the precursor ion. For DMS based approaches, tandem MS is the preferred method to elucidate structural information and increase chemical specificity. It should be noted that chromatography is the most powerful separation technique, and when coupled with MS-based measurements provides legally defensible results[42]. The DMS approaches utilized in this thesis afford rapid chemical

diagnostics, however, by eliminating the time-consuming chromatography step of GC-/LC-MS systems make DMS analyses prone to isobaric (i.e., different isotopic elemental compositions yielding equal mass molecular ions) and isomeric interferences (i.e., same chemical formulae but different fragmentation patterns).

Once ions have been liberated from solution and transferred to the MS inlet, a typical tandem MS event occurs (under vacuum) in three sequential steps: 1) isolate the mass selected precursor ions (M^+), 2) apply energy to the precursor ions to induce fragmentation and 3) measure the resulting fragment ions (m^+). This process is depicted in Equation 1 as well as schematically represented in Figure 1.6.

$$M^+ \rightarrow m^+ + n \qquad \textbf{Equation 1}$$

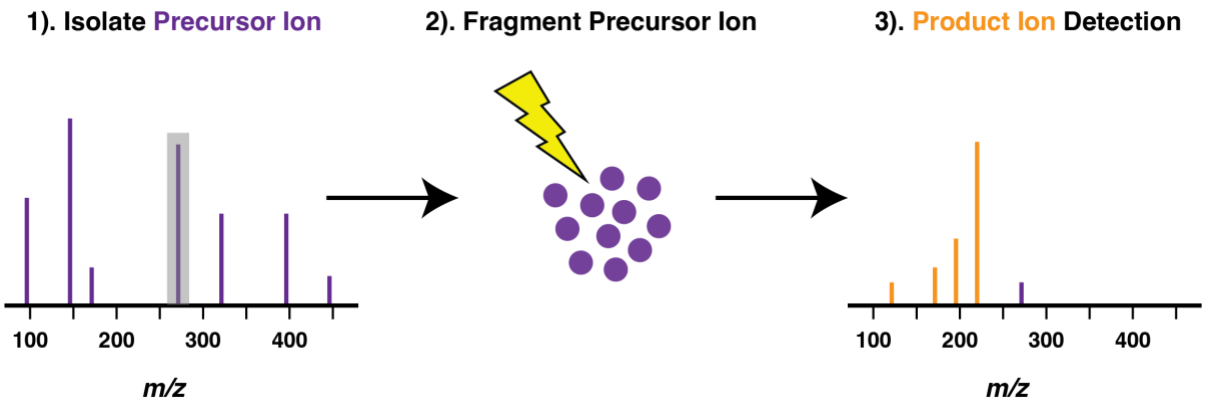

Figure 1.6: Tandem MS operation: 1) isolate mass-selected precursor ion, 2) apply energy to precursor ions to induce fragmentation, and 3) product ion detection.

The dissociation of a precursor ion produces charged (m^+) and neutral fragments (n); only charged fragments are detected because the translational motion of neutral molecules are not affected by electric and/or magnetic fields. Fragments can be subjected to additional fragmentation, and the cycle can be repeated for further analysis, referred to as MS^n (n indicates # of cycles). A fragmentation pattern refers to the characteristic set of fragment ions produced when a molecule undergoes fragmentation. The fragmentation

pattern provides valuable information about the molecular structure of the original compound (i.e., functional groups, chemical scaffolding, fragmentation pathways). When collected under reproducible conditions, fragmentation patterns can be compared to established databases or reference spectra to aid in compound identification.

Fragmentation can be accomplished by a myriad of strategies, but ultimately depends on the type of mass analyzer employed. In collision-induced dissociation (CID), the ion activation method utilized in this thesis, an inert collision gas (i.e., He, N_2, Ar, ambient air) is introduced into the vacuum environment of the mass analyzer. This raises the pressure within the mass analyzer and in turn promotes bimolecular collisions between the precursor ions and the neutral gas molecules. The inelastic collisions between the ions and the neutral (collision) gas molecules results in an increase of the precursor ions internal energy. If the increase of internal energy exceeds the dissociation barrier, then precursor ions (M^+) will undergo unimolecular dissociation into their respective fragment ions (m^+)[5].

1.5 Linear Quadrupole Instrumentation

Linear quadrupole technology was awarded a Nobel prize for its mass-analyzing (and ion-trapping; section 1.5.2) capabilities[43–45] and are one of the most utilized types of MS instrumentation. Linear quadrupole systems were initially engineered to help sort and guide incoming ions for detection. However, technological advancements have allowed chemists to use quadrupole technology to perform tandem MS experiments[46]. A linear quadrupole consists of four cylindrical (or hyperbolic) metal electrodes aligned in a square configuration. The four electrodes apply fields to ions from direct current (DC) and alternating current (AC) power supplies. These electric fields manipulate the motion of ions with different *m/z* values in order to successfully traverse a linear quadrupole without striking the electrodes[5]. The periodic attraction and repulsion (in x-/y-direction) of the ions, when coupled to the correct combination of DC and AC voltages, allows a discrete *m*/*z* (or *m*/*z* range) to successfully traverse a quadrupole to a detector (i.e., mass filter). Figure

1.7 is a simple schematic representation of a linear quadrupole system and the flight path of an ion (3-D corkscrew-like motion).

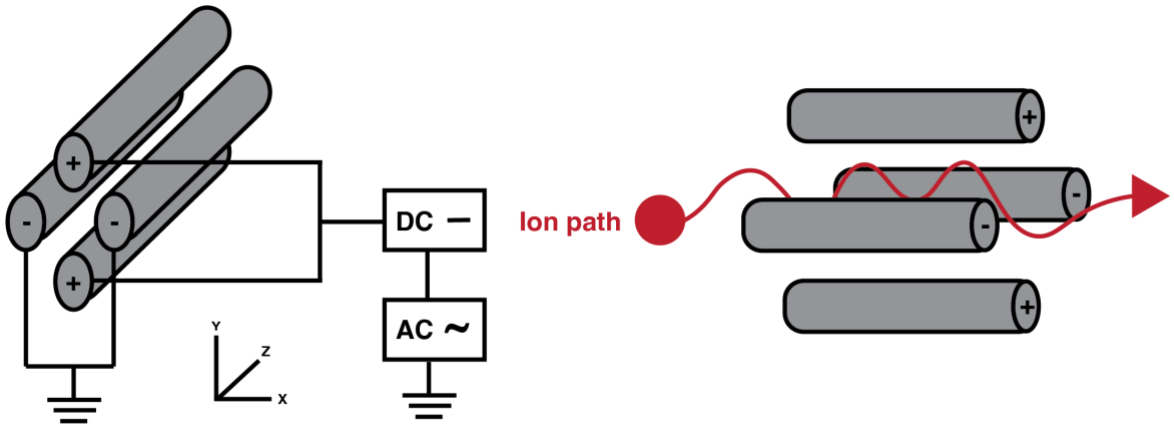

Figure 1.7: Schematic representation of a linear quadrupole system and the flight path of an ion (3-D corkscrew-like motion).

Linear quadrupoles afford a variety of applications for chemical analysis and are one of the most robust components in MS systems because they can operate as a mass filter, an ion guide, a collision cell, and an ion trap; all of which require stable x and y trajectories down the z-axis of a linear quadrupole. The stable motion of ions through the quadrupole MS electric fields can be derived from the Mathieu equations (Equation 2)[5].

$$a_x = -a_y = \frac{4\,q\,U}{m_i\,r_o^2\,\Omega^2} \qquad q_x = -q_y = \frac{2\,q\,V}{m_i\,r_o^2\,\Omega^2}$$ **Equation 2**

Plotting these dimensionless parameters, "*a*" & "*q*", results in the stability diagram for a linear quadrupole mass analyzer[47]. The variable "*a*" is related to the DC field input, and "*q*" is related to the AC field applied to the quadrupole. The resulting stability diagram depicts the regions of space for a particular *m*/*z* (or *m*/*z* range) to successfully traverse the quadrupole with stable trajectories (Figure 1.8). To operate a linear quadrupole as a

mass filter, the AC & DC fields (i.e., *a*/*q* value) corresponding to the *m*/*z* of interest are applied to the electrodes. Resolution can be controlled by adjusting the slope of the mass scan line such that it passes through the tip of the stability region, only allowing a narrow window of *m*/*z* values to pass. Generally, quadrupole mass filters are operated at nominal mass resolution (± 0.5 *m*/*z*). Figure 1.8 represents the stability diagram for a linear quadrupole when operated as a mass filter.

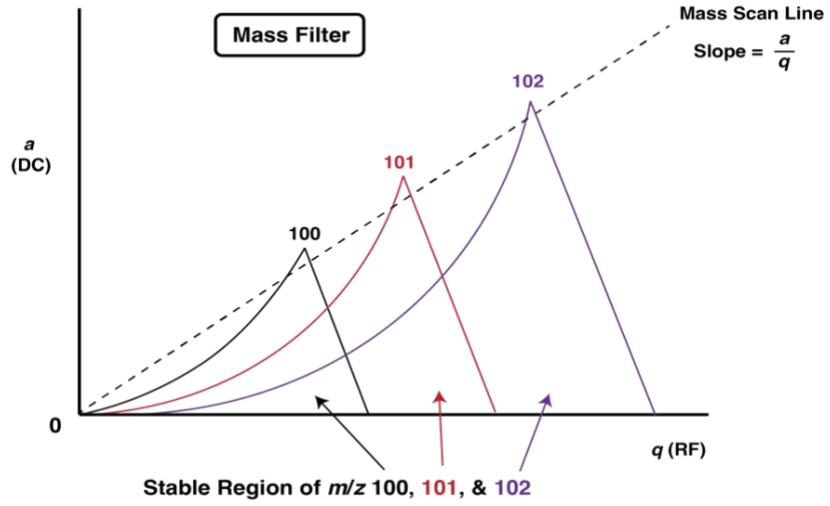

Figure 1.8: Stability diagram for a linear quadrupole when operated as a mass filter.

Alternatively, by eliminating the DC difference (*a* = 0) transforms a quadrupole into a RF-only wide band pass filter, allowing all ions to lie in the stability region to pass. RF-only quadrupoles act as an ion guide which is particularly advantageous for MS operations as it allows the transfer of ions from one component to another. Modern instruments incorporate bent quadrupole designs that act as pipelines for ions. As previously mentioned, neutral molecules are unaffected by the electric field manipulation and are subsequently removed by the high vacuum environment. Ion guides are particularly advantageous for DMS devices as they can successfully interface the atmospheric pressure ion source to the vacuum environment of the mass analyzer. Quadrupoles can operate at considerably higher pressures (10^{-3}–10^{-2} torr) which helps

concentrate ions to the center of the quadrupole fields (i.e., collisional focusing)[46]. Furthermore, linear quadrupole systems are light weight, compact, and relatively low-priced which make them exemplary candidates for miniature mass spectrometer systems without sacrificing significant analytical performance.

1.5.1 Triple Quadrupole Mass Analyzer

Triple quadrupole mass analyzers (QqQ) have become one of the most incorporated mass analyzers in conventional GC-/LC-MS systems as well as modern DMS interfaces[48]. Triple quadrupole systems are the preferred mass analyzer for advanced chemical analysis due to their excellent quantitative performance and high degree of selectivity. As previously mentioned, triple quadrupole systems are defined as tandem-in-space instrumentation because they conduct tandem MS analysis from three spatially separated linear quadrupole mass analyzers. Figure 1.9 is a simple schematic representation of a triple quadrupole system which is operated in the vacuum environment (10^{-3}–10^{-10} torr). The ion source can be operated in an ambient (760 torr) or vacuum environment.

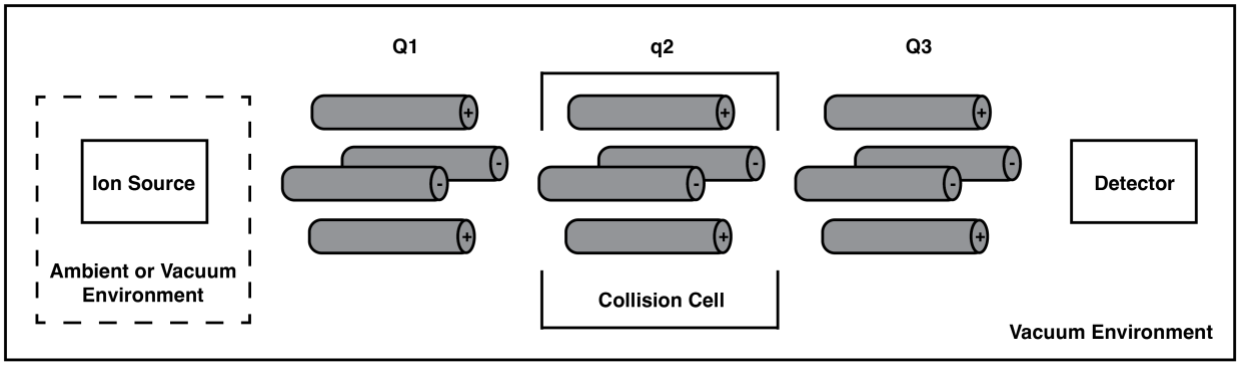

Figure 1.9: Simple schematic representation of a triple quadrupole system (QqQ) which is operated in the vacuum environment. The ion source can be operated in an ambient (760 torr) or vacuum environment (10^{-3}–10^{-10} torr).

One of the most novel features of a triple quadrupole system is that it permits multiple scan functions depending on the desired analysis such as: A) product ion scan, B) multiple reaction monitoring (MRM), C) neutral loss scan, and D) precursor ion scan. A triple quadrupole system operating in product ion scan mode utilizes quadrupole 1 (Q1) as a mass filter designed to isolate precursor ions by applying *m*/z-specific DC/AC fields. A potential difference (typically 5–50 V[49]) is applied across quadrupole 2 (q2), a RF-only collision cell, where collision gas is introduced into q2 at a pressure of 0.1–0.3 Pa[49] to promote collisions with precursor ions (i.e., CID fragmentation) to produce characteristic fragments. Collision energy (V) supplied in triple quadrupole systems (e.g., Table 3.1) is an analyte-specific value with respect to the potential difference across q2 [5]. Improvements to quadrupole systems have been implemented and modern instruments often incorporate higher order multipoles (i.e., hexapole and octapole) as q2, which improve ion-guiding capabilities and offer a wider *m/z* range[5]. Quadrupole 3 (Q3) is mass-selective (like Q1) and analyzes fragmented progeny exiting from q2. MRM performs sequential ($n \geq 2$) product ion scans and is the preferred strategy for quantitative investigations. Neutral loss scan transmits a desired range of ions from Q1 to q2 for fragmentation, and Q3 detects diagnostic fragment ions at a mass offset from Q1 corresponding to the loss of a neutral fragment during CID. In precursor ion scan mode, Q1 transmits all ions to q2 for fragmentation, where Q3 is designated to detect a specific *m*/*z* fragment. Figure 1.10 is a schematic representation of these four common scan modes utilizing a triple quadrupole system. If MS/MS is not the desired analysis, two of

the three quadrupoles can be set to RF-only and one quadrupole (Q1 or Q3) performs mass analysis, behaving as a single quadrupole for full scan analysis. A triple quadrupole system was utilized in Chapter 3 of this thesis to evaluate various ISTD deposition strategies for the quantitation of illicit drug analytes. Triple quadrupole systems are highly advantageous in terms of analytical abilities, however, requirements of a designated technician, specialized infrastructure (i.e., power, gas, solvent supplies, waste streams, venting), and the upfront cost (> $500,000) inhibit the widescale adoption of triple quadrupole systems as an on-site HRDC tool.

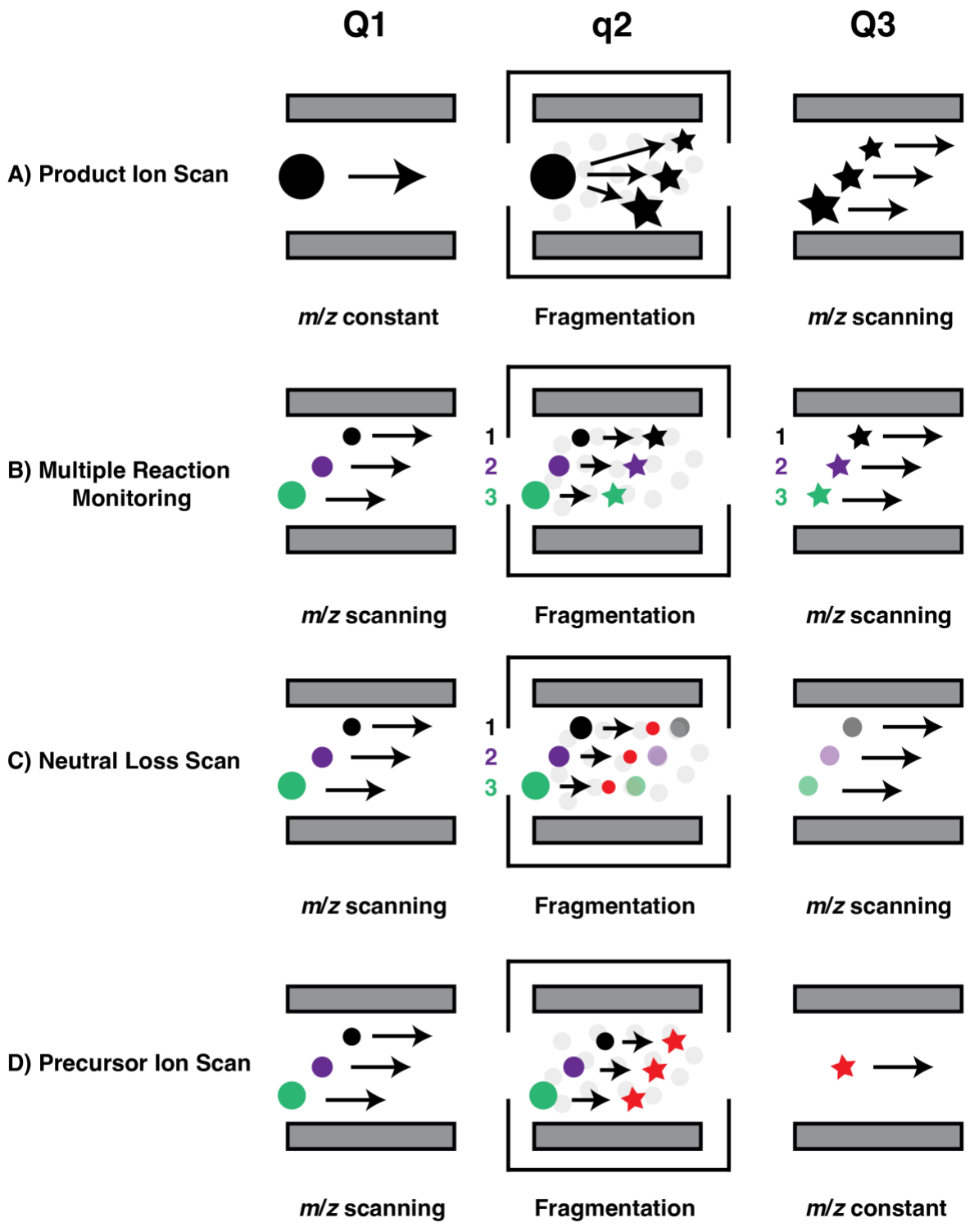

Figure 1.10: Three common scan modes performed in a triple quadrupole system: A), product ion scan, B) multiple reaction monitoring for sequential (not simultaneous) product ion scans (i.e., 1 → 2 → 3), C) neutral loss scan, and D) precursor ion scan.

1.5.2 Rectilinear Ion Trap

In addition to operating as an ion guide, collision cell, and/or mass analyzer for a beam of ions being passed through them, a linear quadrupole can function as an ion storage device, called a linear quadrupole ion trap (LIT). LITs are highly versatile tandem-in-time instruments because they perform tandem MS experiments (i.e., precursor ion selection → fragmentation → product ion detection) within the same region. LITs are often comprised of four hyperbolic electrodes arranged around a central axis, identical to a linear quadrupole, with trapping potentials placed at the end caps. It is worth noting that hyperbolic electrodes yield superior ion transmission; however, their production is costly and challenging, requiring precise machining and alignment. Electrode geometries have been continuously modified to promote simplified manufacturing, reduced cost, and the miniaturization of mass analyzing technology without sacrificing significant analytical performance. One such design improvement is a rectilinear ion trap (RIT)[50], where the quadrupole array is replaced with rectangular-shaped electrode pairs (Figure 1.11). RITs are incorporated as mass analyzers in miniature MS systems because they are physically compact, have modest vacuum requirements, and are capable of performing MS^n within a single volume. Their compatibility with atmospheric pressure ion sources has greatly extended the range of utilization for RIT technology and such devices are exemplary candidates for field applications. A miniature mass spectrometer equipped with a RIT was utilized in Chapter 2 of this thesis to quantify active components of illicit drug samples.

RITs are designed to perform all stages of ion manipulation (i.e., ion accumulation, ion storage, ion ejection) required for tandem MS analysis. Ions can be generated within an ion trap, however for the scope of this thesis ions were generated outside of the trap by PCSI and enter through the apertures in the z-electrodes (Figure 1.11). For ion storage, a trapping potential is created to prevent ions from escaping the x-, y-, and z-directions of the RIT. A slightly higher DC potential is applied to the z-electrodes to prevent ions from escaping the z-direction and AC fields are applied to the x- and y-electrodes, with one set 180 degrees out of phase with the other, to create a trapping field that confines ions in both x- and y-directions of the RIT. Typically, a buffer gas (i.e.,

helium, argon, nitrogen, or ambient air) is introduced into RITs to assist in dampening the motion of the ions towards the center. The addition of a buffer gas combined with the AC/DC trapping potentials brings the z-translational motion of ions within the trap almost to a halt[5].

The mass analysis function of a RIT is analogous to that of a linear quadrupole; therefore, the Mathieu trapping parameters (Equation 2) and the corresponding stability diagram define stable ion trajectories for a particular *m*/*z* by applying its respective AC/DC fields (*a*/*q*) (Figure 1.8). The ability to excite the motion of ions and move it away from the center of the trap is one of the most novel features of ion trap operation. There are multiple techniques to perform mass analysis within an ion trap, however, the method employed in this thesis to isolate the precursor ion (prior to fragmentation) is SWIFT (stored wave inverse Fourier transform) waveform isolation. In SWIFT applications a supplementary wide-range AC field is applied to the x/y-electrodes, excluding the secular motion frequency of the *m*/*z* (or *m*/*z* range) of interest. The supplementary AC field increases the amplitude of motion for unwanted ions, ultimately pushing them out of the stability region defined by the Mathieu trapping parameters. This allows the RIT to operate as a mass filter because as ions are pushed out of the stability region they are ultimately ejected from the trap, leaving behind only the target *m/z* value(s). During ion storage, ions can be collisionally activated with residual gas for CID fragmentation. Collision energies (V) supplied in ion trap devices (e.g., Table 2.2) are for supplementary AC frequencies that supply the ions of interest with excess energy to promote more collisions with neutral gas molecules to induce fragmentation. Resonance ejection was used for mass analysis of fragmented progeny by applying a supplementary AC field. RITs are capable of MS^n analysis where progeny ions can be stored and ejected for further product ion detection. Figure 1.11 is a simplified schematic representation of a RIT comprised of AC and DC fields where ions can either be ejected radially (slits in the x-electrodes) or axially (apertures in the z-electrodes) from the RIT.

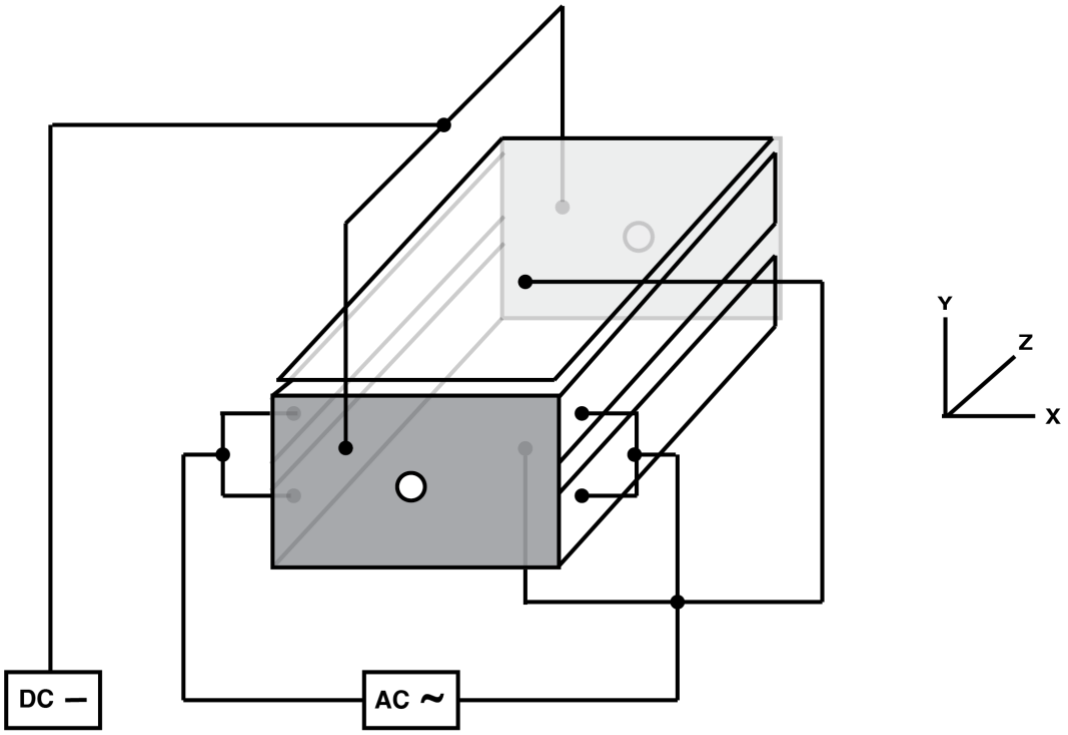

Figure 1.11 Simplified schematic representation of a rectilinear ion trap (RIT). The applied DC voltage to the z-electrodes (endcaps) create a trapping potential of z-trajectories. The x and y electrode pairs are comprised of AC (RF) fields to trap ions in the x- and y-trajectories. Ions can be ejected radially (slits in the x-electrodes) or axially (apertures in z-electrodes).

Chapter 2: Evaluation and Improvement of a Miniature Mass Spectrometer for Harm Reduction Drug Checking

Reproduced with minor changes and permission from Laxton, J-C; Monaghan, J.; Wallace, B.; Hore, D.; Wang, N.; Gill, C. *International Journal of Mass Spectrometry*, 2023, *484,* 116976. J-C. Laxton collected and analyzed the data. J-C. Laxton and C. Gill drafted the manuscript together, with intellectual and editorial contributions from all authors.

2.1 Abstract

The increasing variability of the illicit drug market has imposed serious health risks for people who use drugs (PWUD), particularly due to the increasing co-occurrence of opioids and etizolam (or other benzodiazepines) in some jurisdictions. A miniature mass spectrometer (MS) equipped with paper capillary spray ionization was evaluated for its ability to quantify fentanyl, fluorofentanyl, carfentanil, and etizolam in illicit drug samples. Prior to testing actual drug samples, inter- and intra-day reproducibility, calibration linearity, and analytical sensitivity were assessed. The development of a continuous spray solvent delivery system improved MS/MS quantitation by prolonging ionization stability. A simple, rapid, and quantitative tandem mass spectrometry method was developed and employed for the analysis of real-world illicit drug samples provided by PWUD at a harm reduction site in Victoria, BC, Canada. Limits of detection ranged from 0.001–0.24% w/w of illicit analyte in the original solid drug samples. Target analytes were detected and quantified via the miniature MS system for samples where conventional on-site drug checking technologies were not always effective. These promising results demonstrate the potential role for miniature mass spectrometer systems for on-site drug checking services that can be both sensitive to trace, toxic levels as well as adaptable/selective for new drug threats as they appear in the illicit drug supply.

2.2 Introduction

North America continues to experience record numbers of illicit drug overdoses. In 2021, more than 5,300 Canadians[51, 52] and over 100,000 Americans[53] lost their lives due to the toxic illicit drug supply. BC, Canada declared illicit drug overdose a public health emergency where rates have exceeded over 40 deaths per 100,000, with approximately 90% of accidental overdoses from 2011 to 2021 involving illegally manufactured fentanyl and/or fentanyl analogs (fentalogs)[51]. The severity of these fentanyl/fentalog related-overdoses has been exacerbated during the COVID-19 pandemic due to a number of factors such as heightened stress and anxiety, disruptions in mental health and addiction treatment, reduced harm reduction services, increased social isolation, and the ever-increasing toxicity in the illicit drug supply[54]. This ongoing public health crisis requires a multi-faceted community response[55]. A key strategy being implemented to address this problem is harm reduction, which aims to reduce harm to the drug user as well as the surrounding community by educating, destigmatizing, and providing life-saving resources to PWUD. Drug checking is increasingly viewed as one useful tool within harm reduction services responding to overdose. Drug checking involves pre-consumption chemical measurements to help PWUD and others make informed decisions regarding the use of drugs to prevent accidental overdose events[56]. Illicit drug markets are dynamic systems that evolve due to local and international demand, competition, and legislation[57]. Current chemical detection technologies used for harm reduction, coupled with the myriad of potential fentalogs (>1400[58]) has posed significant challenges for detecting and/or quantifying these analytes in illicit drug samples.

While there is no ‘gold standard’ analytical technique for harm reduction drug checking, an ideal candidate should be fast, sensitive, require small samples, as well as being selective, adaptable, quantitative, and easy to use for operators with minimal training[59, 60]. Fentanyl and benzodiazepine immunoassay test strips are rapid and sensitive, requiring minutes to return qualitative (presence/absence) results with detection limits of 0.13 µg/mL[61] and 2.5 µg/mL[62] for fentanyl and benzodiazepine, respectively. However, both are designed for urine drug tests (i.e., to detect drug

metabolites; not intended to detect the parent drugs), and test strips cannot differentiate and/or detect all of the rapidly-expanding series of fentalogs, which exhibit a wide range of toxicity (e.g., carfentanil is ~100x more potent than fentanyl[63]). Technologies such as infrared (FT-IR) & Raman spectroscopy provide semi-quantitative and selective detection, but typically cannot reliably detect and/or correctly identify trace components (< 5% w/w), which can result in a toxic dose when unexpected[64]. In the face of an increasingly potent[65] and highly variable illicit drug supply[66], PWUD are generally aware of the presence of fentanyl and other actives. Because of this, effective harm reduction drug checking requires techniques which can go beyond qualitative detection (i.e., presence/absence), and instead provide quantitative information, even at trace, potentially toxic levels (i.e., < 1% w/w).

Mass spectrometry (MS) can potentially fill this role for the quantitative analysis of illicit drugs because of its inherent sensitivity and selectivity[7]. MS is often coupled with GC- or LC-MS, providing legally defensible results[7]. However, these approaches frequently require extensive sample preparation and clean-up steps that are generally performed at centralized laboratories. Although some researchers have successfully employed off-site drug checking services for harm reduction efforts[56, 67], the complexity of utilizing chromatography-based mass spectrometry approaches generally make them ill-suited for on-site, point-of-care harm-reduction drug checking. Obstacles for effectively supporting PWUD include lengthy analysis times[3], requirements for highly trained operators, and the space and infrastructure requirements for both the instrument and any sample preparation procedures. The ongoing development of DMS-based approaches may overcome the challenges outlined above, providing timely, on-site, quantitative drug checking results for harm reduction services.

DMS strategies directly interface the mass spectrometer with samples in their native environment (e.g., atmospheric pressure and temperature)[68]. Our group has developed PS-MS based methods for the detection of fentanyl/norfentanyl in urine[39] which was later optimized to incorporate a larger suite of fentalogs in surrogate drug matrices. Borden et al. successfully piloted the use of quantitative PS-MS methodology

as an on-site, quantitative drug checking tool for harm reduction in Vancouver, Canada[4]. While the on-site use of this approach is quite promising, work-to-date has been performed using benchtop-scale triple quadrupole mass spectrometers, so both the size of the mass spectrometer and supporting infrastructure (220 Volts AC, ca. 6600 W, 24 hr cold start time) currently hampers widespread on-site use. For on-site drug checking, ideally instruments should be compact (even mobile), simple to operate, and require little or no specialized infrastructure (e.g., power, gas, solvent supplies, waste streams, venting). Miniaturizing MS systems has been extensively evaluated[68–73] and quadruple ion traps have emerged as a compelling candidate due to their relatively low manufacturing costs, inherent MS/MS capabilities, and modest vacuum requirements[74]. The recent development of the discontinuous atmospheric pressure interface (DAPI)[75] reduced the need for high vacuum pumping capacity by only allowing intermittent transport of ions into the vacuum manifold. The present work aims to evaluate a portable rectilinear ion trap MS system with a DAPI inlet for its potential use for harm-reduction drug checking. PCSI, a derivative of PS-MS, was used as an ambient ionization method because PCSI (like PS-MS) is simple to use and has no sample-to-sample carryover. The additional benefit of PCSI is its potentially improved ionization efficiency (compared to PS-MS) achieved by embedding fused silica emitter capillary into the tip of a paper sampling substrate[40].

For this proof-of-concept investigation, we targeted the quantitative detection of 4 drug components (fentanyl, fluorofentanyl, carfentanil, and etizolam) in street drug samples that have been observed in the BC illicit drug supply throughout the COVID-19 pandemic[76]. Although the analysis of other illicit drugs in real samples was not performed, it is possible to incorporate a larger suite of illicit drug analytes with this system (Table 2.2). We have refined both the utilization of PCSI based measurements with a miniature ion trap MS system and its subsequent use for the direct quantitation of targeted psychoactive substances in illicit drug samples. The system has been evaluated for inter- and intra-day reproducibility, calibration linearity, and analytical sensitivity. Our results demonstrate the use of this approach provides sufficiently sensitive and quantitative detection of fentalogs and etizolam in illicit drug samples, especially where other on-site

drug checking approaches (e.g., FT-IR and fentanyl/benzodiazepine strips) have failed to detect and/or quantify them.

2.3 Materials and Methods

2.3.1 Solvents and Standards

Fentanyl, fentanyl-d_5, fluorofentanyl, carfentanil, carfentanil-d_5, etizolam and etizolam-d_3 were purchased as legally exempt test kit analytical standards from Cerilliant Corporation (Round Rock, TX, USA). Seven-point combined calibration standards (n = 6 replicates at 0, 25, 50, 100, 250, 500, & 1000 ng/mL levels; ISTD concentration = 100 ng/mL) were gravimetrically prepared in methanol (MeOH, HPLC grade, Fisher Scientific, Ottawa, ON, Canada). Spray solvents were also prepared using HPLC grade MeOH, formic acid (FA; Fisher Scientific), and 18 MΩ·cm deionized water (Facility Scale Reverse Osmosis/Ion Exchange Water Purification System, Applied Membranes Inc., Vista, CA, USA).

2.3.2 Instrumentation

A miniature atmospheric pressure ionization mass spectrometer system equipped with a rectilinear quadrupole ion trap was used for these investigations (Model Mini ß, PURSPEC Technology (Beijing) Ltd., Beijing, China). The system dimensions are 55 cm x 24 cm x 31 cm. It weighs approximately 20 kg, consumes less than 100 W of power, operates with 115 Volts AC, and has no additional gas supply requirements[74, 77]. For optimization of ionization and MS/MS conditions, a nano-ESI platform was employed, and PCSI[40] was used for quantitative calibration and sample analysis. Figure 2.1 are images of the nano-ESI emitter (left) and the PCSI cartridge (right) employed in this study.

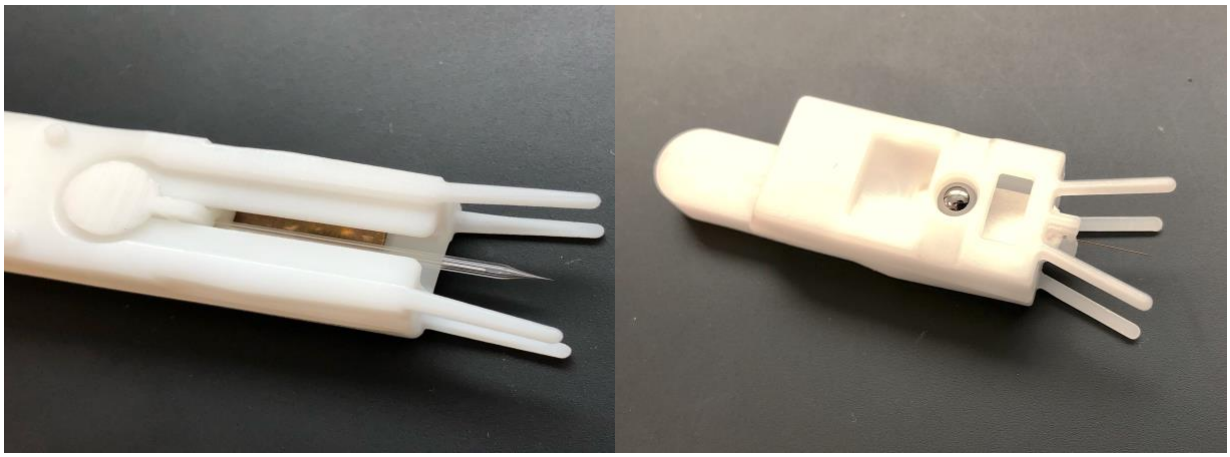

Figure 2.1: Images of the nano-spray ESI emitter (left) and PCSI cartridge (right).

Nano-ESI emitters were manufactured using a capillary micropipette puller (Model P87 Micropipette Puller, Sutter Instruments, Novato, CA, USA) and borosilicate glass capillaries (Part # BF150-86-10, Fire Polished,1.5 mm OD, 0.86 mm ID, 10 cm length, with filament, Sutter Instruments). Samples and standards were introduced to the nanospray capillaries with a gastight syringe (1.0 mL, Hamilton Company, Reno, NV, USA), and electrical contact was made with samples and standards by a platinum wire lead inserted coaxially inside the emitter capillaries. A new emitter capillary was used for each measurement, and the platinum lead was thoroughly rinsed with methanol three times between each measurement. PCSI cartridges and replacement paper capillary spray inserts were used as provided by the vendor (PCS cartridges (Model: PCS-PR) and PCS inserts, PURSPEC Technology (Beijing) Ltd., Beijing, China). When replacing the inserts, the PCSI cartridge casings were disassembled, sonicated in clean methanol for 15 minutes, and air-dried prior to paper capillary replacement. Evaluation of the washing procedure effectiveness was accomplished by verifying that blank samples showed no trace of any residual analyte(s). Samples and standards (10 µL) were deposited on the PCSI cartridges using a mechanical micropipette (Finn Pipette F2, 2–20 µL, Thermo Scientific, San Jose, CA, USA), and allowed to air dry 15 minutes before measurement.

Details regarding the base instrumental hardware of this system have been published elsewhere[74, 77, 78]. For this work, all measurements (PCSI and nano-spray ESI)

were performed in positive ionization mode (+4.5 kV), using air admitted during ion introduction (via the DAPI) for CID. In each cycle of MS analysis with DAPI, a pinch valve opens for *ca.* 20 ms, allowing ions produced in the ambient environment to be transferred into the ion trap. The pinch valve then closes to allow the vacuum pressure to drop back to millitorr range (*ca.* $1x10^{-3}$ Torr) for MS measurement[77]. Operating conditions for the miniature MS system are given in Table 2.1. For this study we utilized the major MS/MS fragment ion for quantitation. In other work we have observed minimal matrix effects for illicit drugs present in realistic surrogate drug matrices by PS-MS[79]. Software modifications to facilitate this work were developed in direct collaboration with engineers at PURSPEC Technology (PMS Client Pro Version 2.1.0.3). Mass spectral data were exported from the instrument as .csv files, and calibration curves were generated in Microsoft Excel (Excel 2019, Microsoft Office) by plotting the analyte/internal standard signal intensity ratios against analyte concentrations. Limit of detection (LOD) in methanol (ng/mL) was defined as three times the standard deviation of the zero calibrant signal divided by the slope of the calibration curve[80]. LOD as weight-to-weight percentages (w/w %) in the original solid drug sample were also determined based upon the sample dilution scheme, as these are more relevant in the context of reporting illicit drug concentrations for harm reduction.

Table 2.1: Interlaced scan sequence and MS/MS operating conditions for the miniature mass spectrometer.

Scan Sequence	Target Drug	MS/MS Transition (*m/z*)	CID Amplitude (V)
1	Fentanyl	337 → 188	2.8
2	Fentanyl-d_5	342 → 188	2.8
3	Etizolam	344 → 314	3.1
4	Etizolam-d_3	347 → 317	3.1
5	*Fluorofentanyl	355 → 188	2.8
6	Carfentanil	395 → 335	3.3
7	Carfentanil-d_5	400 → 340	3.3

*Fentanyl-d_5 used as an internal standard for fluorofentanyl.
Global operating parameters include: *Spray Voltage* = +4500 V, *injection low mass cutoff* = *m/z* 50, *CID q value* = 0.25, *scan range* = *m/z* 50–1000, 6 s per MS/MS measurement (average of 6 mass spectra) with a total scan sequence of 42 s.

A simple spray solvent delivery system was developed in-house for improved PCSI operation and is shown schematically in Figure 2.2. A gastight syringe (1.0 mL, Hamilton Company, Reno, NV, USA) and syringe pump (Fusion 100, Chemyx Inc., Stafford, TX, USA) were used to deliver solvent to the PCSI cartridges via a *ca.* 30 cm length of fused silica capillary (153 µm OD, 76 µm ID, Polymicro Technologies L.L.C., Phoenix, AZ). A small hole drilled in the PCSI cartridge held the capillary in place relative to the paper strip. The syringe pump was programmed to provide spray solvent in a prewet step of 70 µL (100 µL/min for 42 s) followed by delivery of 50 µL at 30 µL/min for 100 s to maintain consistent solvent saturation of the paper strip in the PCSI cartridge during measurements.

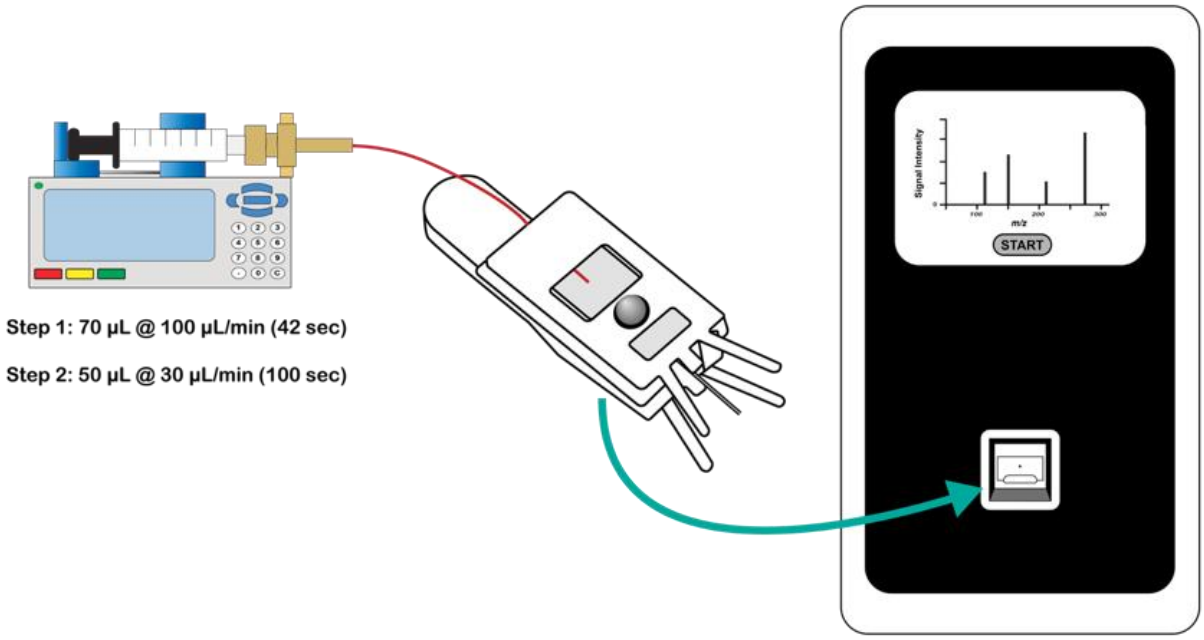

Figure 2.2: Schematic design of in-house constructed solvent delivery system for PCSI utilizing a modified PCSI cartridge. The cartridge was inserted in the miniature MS system prior to initiating spray solvent delivery. Note: Not to scale.

2.3.2 Illicit Drug Sample Collection & Preparation

Drug samples were collected as part of the Vancouver Island Drug Checking Project[81]. PWUD accessing this service provide a small amount (*ca*. 1 mg) of their drugs for testing by a variety of methods. Ethical approval for sample collection was provided by the Health Research Ethics Board at the Vancouver Island Health Authority (J2018-069). Drug samples used for this study were verified (and quantified where possible) to contain illicit drugs via FT-IR, rapid fentanyl test strips, and benzodiazepine test strips. Further discussion of potential limitations of these methods is discussed below in section 2.5. For our investigations, 1 mg/mL solution of an illicit drug sample was prepared gravimetrically (Radwag Model AS 60/220.R2 Plus 5-decimal balance, Rose Scientific, Edmonton, Alberta, Canada) in methanol, then diluted 250-fold to yield a working sample of 4000 ng/mL total drug, lowering target analyte concentrations into our calibration range. The second dilution step also introduced 100 ng/mL of isotopically labelled internal standards (fentanyl-d_5, etizolam-d_3, & carfentanil-d_5). Aliquots (10 µL) of these diluted

drug samples (and internal standards) were then spotted onto PCSI cartridges for quantitative measurement. All samples were air dried for several hours (during transport from the site) before analysis. Although only a small fraction of the diluted sample is analyzed, in cases of questionable results it is easy to make sample remeasurements. When measurement(s) are complete, the sample is disposed of in accordance with illicit drug checking legal exemption.

2.4 Results and Discussion

2.4.1 Target Analyte Optimization

Initial system optimizations were completed using the PURSPEC nano-spray ESI adapter (Figure 1) and in-house constructed emitter capillaries. Using 500 ng/mL standards prepared in methanol, MS/MS scan conditions were developed for a variety of illicit drugs/precursor and labelled internal standards ranging over multiple classes of drug analytes found in street drugs, represented in Table 2.2.

Table 2.2: Summary of optimized MS/MS instrumental scan parameters for a variety of illicit drugs/precursor and labelled internal standards ranging over multiple class of drug analytes found in street drugs. All analytes/internal standards were optimized at 500 ng/mL.

Target Analyte [500 ng/mL]	Precursor (*m/z*)	Major Product Ions (*m/z*)	*SWIFT 1 Amplitude (V)	*SWIFT 2 Amplitude (V)	CID Amplitude (V)
Methamphetamine	150	119, 91	8	3	1.28
MDA	180	163, 135, 105	8	3	2.2
MDMA	194	162, 135, 105	9	4	1.35
Ketamine	238	125, 178, 163	8	4	5
Ketamine-d_4	242	129, 211, 183	9.5	3.5	3.3
4-ANPP	281	188, 134, 105	9.5	3.5	2.4
Cocaine	304	182, 272	8	3	2.05
Alprazolam	309	281, 274, 241	9.5	3.5	2.8
Fentanyl	337	188, 239, 216	9.5	4	2.6
Fentanyl-d_5	342	188, 220	9	4	2.5
Etizolam	343	314, 307, 275	8	4	2.7
Heroin	370	328, 310, 268	9	4	2.7
Carfentanil	395	363, 335, 245	9	4	2

*SWIFT: stored waveform inverse Fourier transform
Global parameters: *Spray Voltage* = +4500 V, *Injection low mass cut off* = 50 *m/z*, *CID q value* = 0.25, *Scan range* = 50-1000 *m/z*

Four target drug analytes were chosen for this study, but as noted above, a larger suite of drugs was also examined, illustrating that the method is potentially adaptable more broadly for harm reduction drug checking. Figure 2.3 presents normalized fentanyl product ion scans (1000 ng/mL) for both ionization platforms: PCSI (top) and nano-ESI (bottom). These spectra demonstrate comparable efficiencies for the major fentanyl CID fragmentation pathway (*m/z* 337→188) with the miniature mass spectrometer, regardless of the ion source used.

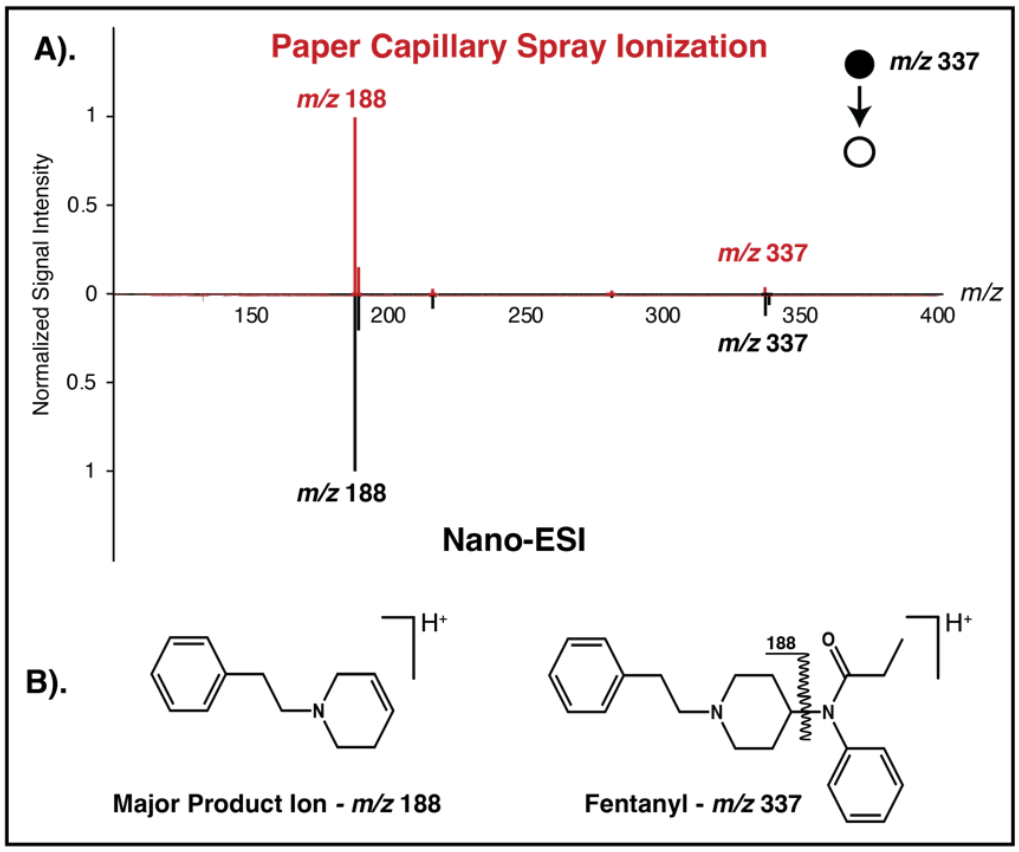

Figure 2.3: A). Fentanyl MS/MS spectra for a 1000 ng/mL standard illustrating comparable fragmentation via PCSI (top) and nano-ESI (bottom). B). Structures of protonated fentanyl ion (right) and major MS/MS product ion (left) at *m/z* 188.

2.4.2 Continuous PCSI Spray Solvent Delivery Evaluation

The commercially available PCSI cartridges provided by the vendor were designed for qualitative testing, with spray solvent delivered (4 drops: *ca*. 100 µL) via a small plastic dropper bottle. For this approach, the system was operated in a targeted, 'snapshot' mode, acquiring only single MS or MS/MS experiments (e.g., for library matching). For successful quantitative measurements, a more complex interlaced analyte/internal standard scan function was required[4]. This required software upgrades (PMS Client Pro Version 2.1.0.3) to allow multiple interlaced MS/MS scans (Table 2.1). To produce a uniform signal response through the interlaced scan sequences, we modified the PCSI cartridges to facilitate continuous spray solvent delivery by drilling a small hole through

the back side of the cartridges. A silica capillary could be inserted until it was at the midway point of the solvent well, directly in contact with the paper substrate, with the capillary secured in place by a small piece of adhesive tape. Figure 2.4 are images of the in-house constructed spray solvent delivery system (top) with the modified PCSI cartridge (bottom).

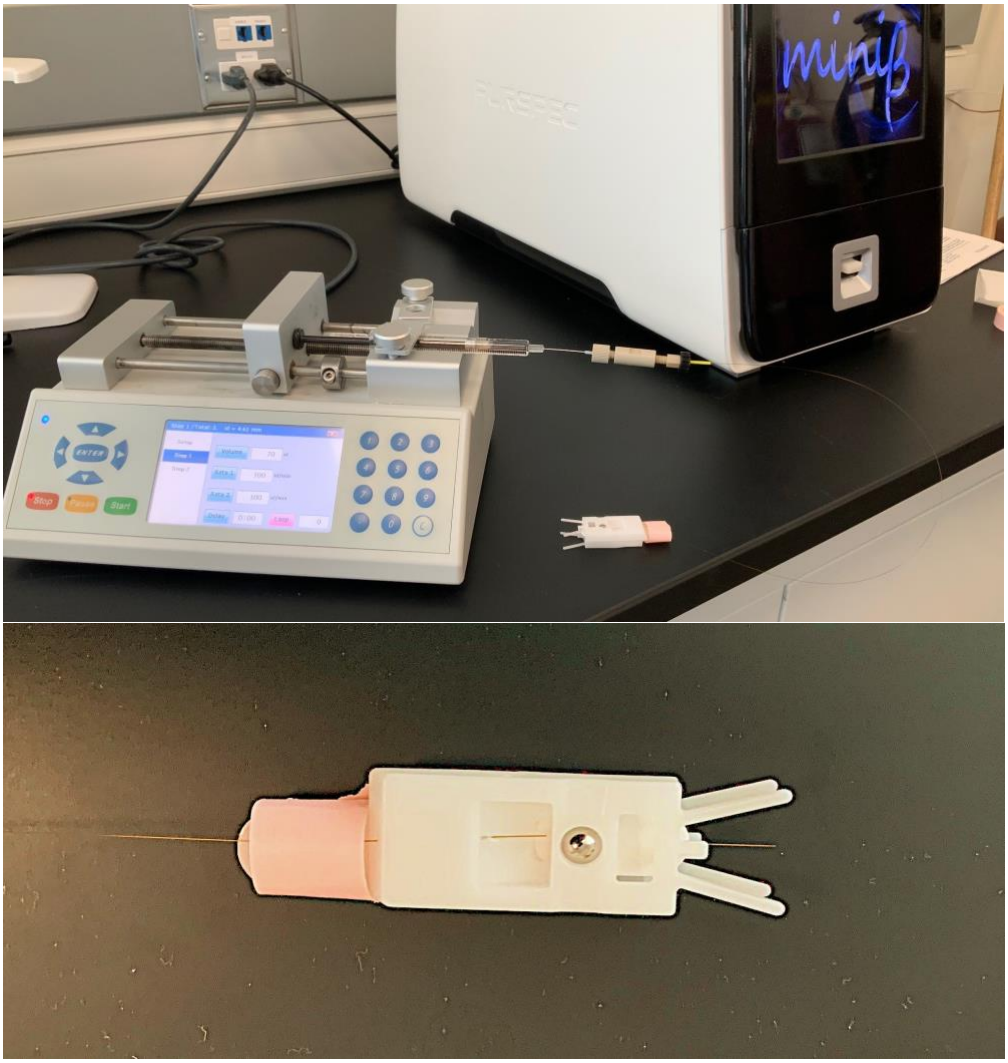

Figure 2.4: Images of in-house constructed spray solvent delivery system (top) with modified PCSI cartridge (bottom).

To evaluate PCSI signal stability with this strategy, a 10 µL aliquot of fentanyl standard (1000 ng/L) was spotted on a modified cartridge, dried, then analyzed by

continuously monitoring the fentanyl MS/MS signals for 30 replicate scans over 3 minutes (6 s per MS/MS spectral scan) with continuous methanol addition. For comparison, a control experiment was conducted using an unmodified PCSI cartridge, with four drops of methanol spray solvent deposited on the cartridge before measurement. The results of this study are presented in Figure 2.5. With one-time spray solvent addition, signals become erratic and degrade to zero as the solvent depletes from the PCSI cartridge, whereas with the continuous spray solvent delivery approach, fentanyl signals remain stable for the duration of the experiment. The improved spray stability from the continuous solvent delivery system was expected to drastically improve quantitative measurements, as 'back-to-back' scans of analyte/internal standard will be less variable & provide a more accurate signal ratio.

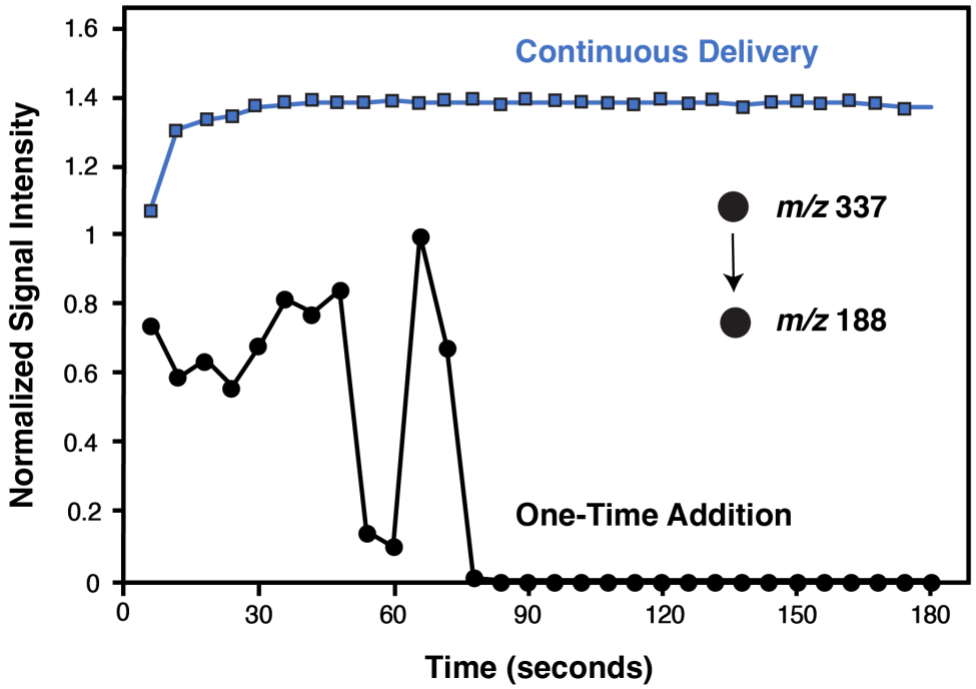

Figure 2.5: Normalized MS/MS signals (*m/z* 337 → 188) obtained by PCSI-MS/MS for a 1000 ng/mL fentanyl standard using a one-time spray solvent addition (black circles) and continuous solvent delivery (blue squares). The continuous PCSI spray solvent delivery signals are offset (+0.4) for visual clarity.

2.4.3 Spray Solvent Composition Evaluation & Quantitative Calibration

The addition of small amounts of water in spray solvent systems for ESI based ionization improves spray droplet surface tension and overall ionization stability, and the inclusion of formic acid (FA) is known to provide ionization enhancement in positive ionization modes[22]. We explored improving analytical sensitivity for the target analytes by modifying spray solvent composition. Two spray solvent systems were evaluated for their effect upon PCSI ionization: 1) 100% MeOH, and 2) 90% MeOH / 10% water / 0.1% FA.

Table 2.3 presents average calibration data for inter-day calibration (3 days) with LOD values for fentanyl, fluorofentanyl, carfentanil, and etizolam using the two spray solvent systems. We anticipated that the acidified solvent might improve overall analytical sensitivity, but the LOD and linear calibration models were slightly better for all the target fentalogs using pure methanol. Although the acidified solvent system demonstrated improved sensitivity for etizolam, we chose to use pure methanol for all subsequent work to provide maximum sensitivity for the fentalog analytes. Utilizing a mid-range carfentanil calibration standard (100 ng/mL) as a surrogate quality control sample, an acceptable CV of 2.9% was observed (n = 18, 6 replicates/day for 3 days).

Table 2.3: Average PCSI-MS/MS calibration data (0–1000 ng/mL) and sensitivity for the target drugs with two candidate spray solvent systems (100% MeOH and 90% MeOH / 10% Water / 0.1% FA).

Analyte	Solvent	Average R^2	Average Calibration Equation	LOD (ng/mL)	LOD (w/w %)
Fentanyl	Methanol	0.999	y = 0.009x + 0.038	0.92	0.023
	90/10/0.1	0.993	y = 0.007x + 0.222	1.99	0.050
Fluorofentanyl	Methanol	0.999	y = 0.008x + 0.128	0.057	0.001
	90/10/0.1	0.974	y = 0.007x + 0.264	4.93	0.12
Carfentanil	Methanol	0.999	y = 0.009x + 0.020	3.27	0.082
	90/10/0.1	0.993	y = 0.010x + 0.188	7.63	0.19
Etizolam	Methanol	0.996	y = 0.010x + 0.170	9.65	0.24
	90/10/0.1	0.993	y = 0.007x + 0.222	1.99	0.050

Calibration equations, R^2 values, and LOD presented are averaged from multi day calibrations (*n* = 3 days, Table S2). LOD expressed as ng/mL in the diluted drug solution as well as the calculated w/w % concentration of target drug in the original solid sample.

As presented in Table 2.3, acceptable linear calibrations ($R^2 > 0.99$) between 0–1000 ng/mL were obtained for all the target drugs with pure methanol spray solvent. Figure 2.6 presents average calibration curves for the target drugs. Calibration data was averaged over three days, with error bars depicting the standard deviation of each calibrant level. The relatively small amount of variation of calibrant signal levels suggests acceptable measurement reproducibility. This is consistent with previous published work utilizing PS-MS, where we have observed minimal variation in calibration slopes for variety of 'realistic' surrogate drug sample matrices such as trace fentanyl in other illicit drug mixtures[1].

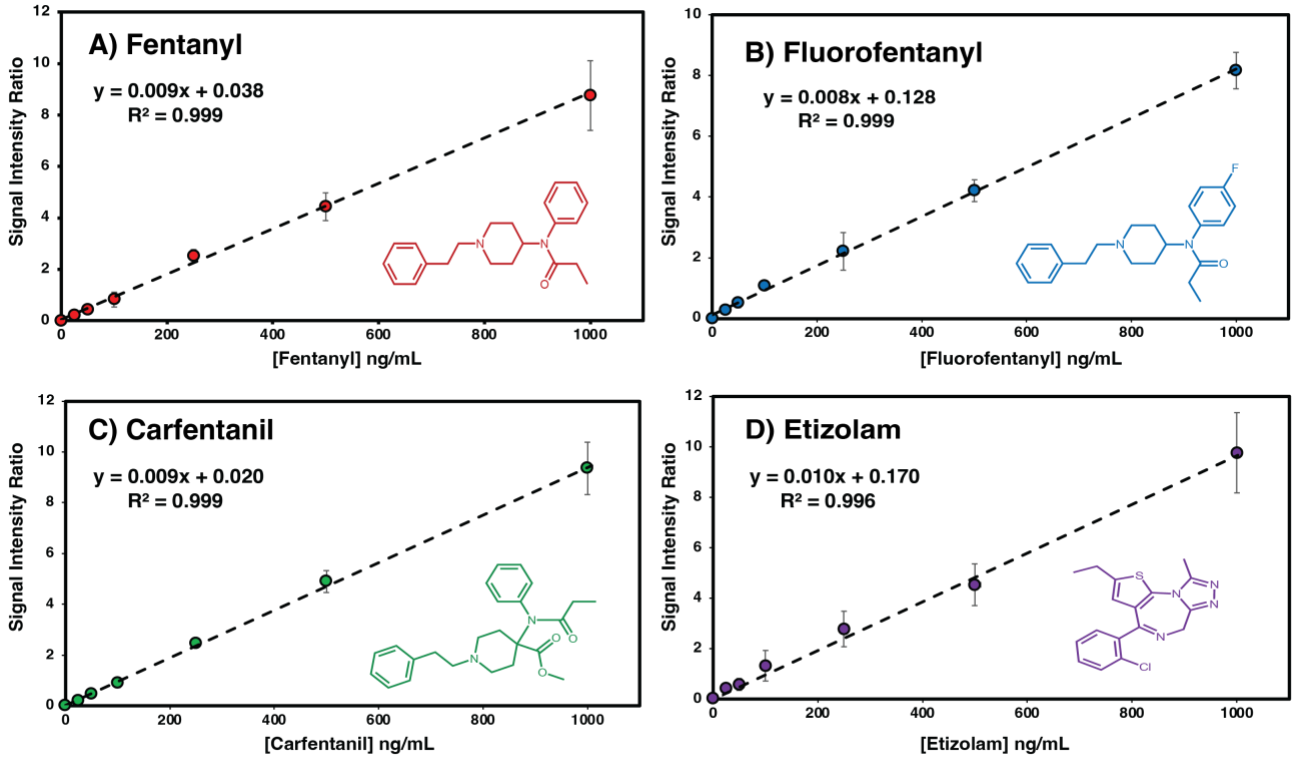

Figure 2.6: Calibration curves for the target analytes measured in this study: A) fentanyl, B) fluorofentanyl, C) carfentanil, and D) etizolam. Calibrations are the average of calibrations obtained over three days (Table 2.3).

2.5 PCSI-MS/MS Illicit Drug Sample Quantitation

Spectroscopic (FT-IR) and immunoassay-based (test strips) drug checking methods are frequently unable to detect and/or identify trace level (and newly emerging) illicit drugs. For example, if a drug sample tests positive via test strip (fentanyl and/or benzodiazepine), the qualitative results inform PWUD that a 'fentanyl or analog' and/or an 'undifferentiated benzodiazepine' is present. PWUD in BC are generally aware of the fentanyl contamination and polycomponent nature of illicit drugs. However, to assess actual risks in the highly variable illicit drug supply[66], quantitative information is required. FT-IR can provide semi-quantitative results with typical detection limits of 3–5% w/w, however matrix interferents such as moisture can confound measurements, and the presence of caffeine (a common filler) can lead to further reductions in sensitivity (> 5%

w/w fentanyl detection limits). FT-IR is tethered to library matching which prevents this technology from detecting, identifying, and/or quantitating emerging threats as they enter the illicit drug supply. Effective harm reduction drug checking requires a rapid, simplified, and effective strategy for quickly returning quantitative results pertaining to a drug sample. Figure 2.7 represents the simplified workflow employed for the rapid and direct quantitative analysis of illicit drugs via a miniature mass spectrometer system.

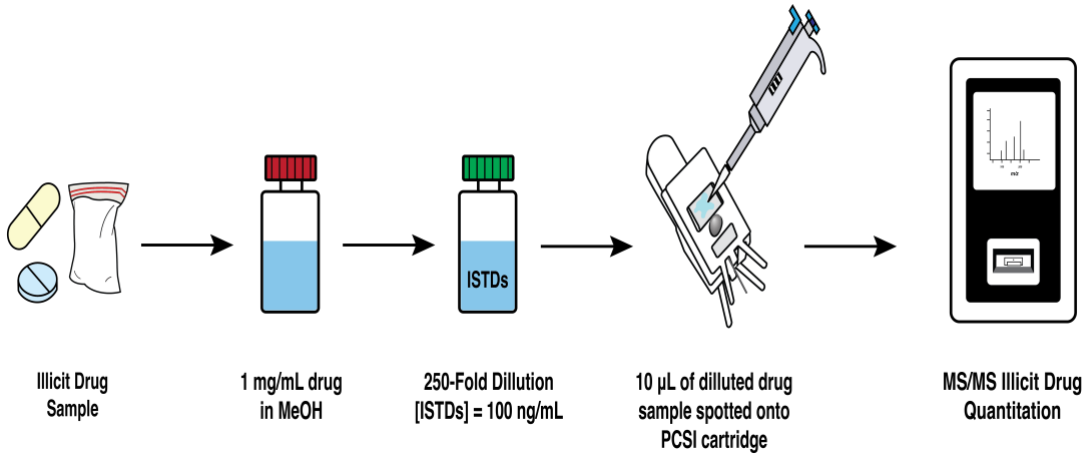

Figure 2.7: Schematic diagram representing the illicit drug testing analytical workflow for PCSI-MS analysis with a miniature mass spectrometer.

Illicit drug samples (*n* = 15) provided by PWUD in Victoria, BC were analyzed using fentanyl & benzodiazepine test strips, FT-IR, & PCSI-MS/MS using the same spray solvent (methanol) delivery system used for the calibration standards, with the results presented in Table 2.4. Test strips detected fentanyl and/or analogues in most of the samples, confirmed by quantitative analysis using PCSI-MS/MS. However, samples containing fluorofentanyl (*n* = 6; samples 3, 4, 5, 6, 7, & 11) and carfentanil (*n* = 6; samples 2, 3, 8, 9, 10, & 12) could not be distinguished by the test strips, or be detected by FT-IR. We note that if a drug sample unknowingly contains a substance that fragments the same as an internal standard, the possibility that the w/w % of a drug target could potentially be underestimated using PCSI-MS/MS. The positive identification and quantitation of high potency, trace level drug components such as carfentanil highlights the advantages of using a MS based measurement strategy to provide harm reduction advice. Simultaneous

detection and quantitation of etizolam is of particular importance for harm reduction services. The increasing frequency of the combined presence of opioids with etizolam and other benzodiazepines in the illicit drug supply[81] poses serious health risks for PWUD by reducing the efficacy of naloxone, which is used to reverse and prevent otherwise fatal opioid over-dose events. The miniature PCSI-MS/MS system successfully quantified etizolam in 12 of the 15 illicit drug samples, all of which benzodiazepine test strip and/or FT-IR methods failed to detect and/or quantify its presence (Samples 1–12, labelled in bold red in Table 2.4).

In summary, PCSI-MS/MS, because of its lower detection limits and high specificity, provides positive identification and quantitative results where the other approaches are limited to either qualitative 'presence/absence' reporting, or simply cannot detect the target drug entirely. With the sample dilution scheme employed here, the quantitative determination of target drugs up to 20% w/w in the original solid drug samples is possible. Although not performed in this investigation, analytical sensitivity can be readily adjusted by simply changing the dilution factor in the analytical workflow.

Table 2.4: Comparison of the analysis of illicit drug samples provided by PWUD in Victoria, British Columbia. Bold red indicates drug samples for which benzodiazepine test strips and/or FT-IR methods failed to detect or quantify the analyte.

Sample	Fentanyl Test Strip	Benzodiazepine Test Strip	Illicit Drug	FT-IR (w/w %)	PCSI-MS/MS (w/w %)
1	Positive	**Negative**	Fentanyl	5.3	5.6
			Fluorofentanyl	ND	ND
			Carfentanil	ND	ND
			Etizolam	**ND**	4.8
2	Positive	Positive	Fentanyl	ND	1.6
			Fluorofentanyl	ND	ND
			Carfentanil	ND	0.3
			Etizolam	**ND**	0.7
3	Positive	Positive	Fentanyl	NQ	5.2
			Fluorofentanyl	ND	1.2
			Carfentanil	ND	0.3
			Etizolam	**ND**	1.8
4	Positive	Positive	Fentanyl	25.0	2.9
			Fluorofentanyl	ND	11.9
			Carfentanil	ND	ND
			Etizolam	**ND**	12.4
5	Positive	**Negative**	Fentanyl	NQ	11.4
			Fluorofentanyl	ND	2.1
			Carfentanil	ND	ND
			Etizolam	**ND**	1.8
6	Positive	Positive	Fentanyl	ND	0.4
			Fluorofentanyl	ND	4.3
			Carfentanil	ND	ND
			Etizolam	**ND**	1.8
7	Positive	**Negative**	Fentanyl	NQ	0.06
			Fluorofentanyl	ND	2.3
			Carfentanil	ND	ND
			Etizolam	**ND**	0.8

Sample (cont'd)	Fentanyl Test Strip	Benzodiazepine Test Strip	Illicit Drug	FT-IR (w/w %)	PCSI-MS/MS (w/w %)
8	Positive	**Negative**	Fentanyl	ND	1.8
			Fluorofentanyl	ND	ND
			Carfentanil	ND	0.4
			Etizolam	**ND**	0.5
9	Positive	**Negative**	Fentanyl	NQ	3.9
			Fluorofentanyl	ND	ND
			Carfentanil	ND	0.8
			Etizolam	**ND**	0.6
10	Positive	**Negative**	Fentanyl	NQ	1.0
			Fluorofentanyl	ND	ND
			Carfentanil	ND	0.3
			Etizolam	**ND**	0.1
11	Positive	**Negative**	Fentanyl	ND	1.2
			Fluorofentanyl	NQ	6.6
			Carfentanil	ND	ND
			Etizolam	**NQ**	1.8
12	**Negative**	**Negative**	**Fentanyl**	ND	0.05
			Fluorofentanyl	ND	ND
			Carfentanil	ND	0.1
			Etizolam	**ND**	1.1
13	Negative	Negative	Fentanyl	ND	ND
			Fluorofentanyl	ND	ND
			Carfentanil	ND	ND
			Etizolam	ND	ND
14	Negative	Negative	Fentanyl	ND	ND
			Fluorofentanyl	ND	ND
			Carfentanil	ND	ND
			Etizolam	ND	ND

Sample (cont'd)	Fentanyl Test Strip	Benzodiazepine Test Strip	Illicit Drug	FT-IR (w/w %)	PCSI-MS/MS (w/w %)
15	Negative	Negative	Fentanyl	ND	ND
			Fluorofentanyl	ND	ND
			Carfentanil	ND	ND
			Etizolam	ND	ND

ND: Not detected; NQ: Not quantifiable; "fentanyl or analogue".

2.6 Conclusion

Harm reduction drug checking is showing promise as an aid in reducing the societal impact of the illicit drug overdose crisis, and the high sensitivity and specificity afforded by MS-based measurements is proving to be a useful analytical strategy. As smaller and portable mass spectrometer system development continues, it is anticipated that their role for on-site drug testing will continue to increase. This work demonstrates the use of a miniature paper capillary spray ionization tandem mass spectrometer (PCSI-MS/MS) to quantify fentanyl, fluorofentanyl, carfentanil, and etizolam in illicit drug samples. A continuous spray solvent delivery system was developed and used with PCSI to prolong ionization stability, allowing the effective use of interlaced MS/MS scan sequences for direct quantitation. The system was evaluated for inter- and intra-day reproducibility, calibration linearity, and analytical sensitivity. Limits of detection (in methanol) ranged from 0.001–0.24% w/w of illicit drug in the original solid drug samples. Target analytes were detected and quantified via PCSI-MS/MS for samples where conventional on-site drug checking technologies were not always effective.

Chapter 3: Evaluation of Internal Standard Utilization Strategies for Illicit Drug Quantitation

3.1 Introduction

There are numerous publications utilizing PS-MS technology for quantitative chemical analysis encompassing a range of analytes, including, but not limited to, trace-level organic contaminants in soil and sediment[82], biomolecules[83], therapeutic drugs[84, 85], and drugs of misuse[1, 2, 4, 39, 85–87]; all of which incorporate ISTDs to quantify target analytes. An ISTD is a molecule that is added to blanks, calibration standards, and samples at a constant level to reference against the signal intensity of the analyte(s) of interest[88]. The ideal ISTD in MS is not present in real-world samples to avoid confounding quantitative measurements, and is often the compound of interest labelled with one or more stable, heavy, isotopes (i.e., ^{2}H, ^{13}C, ^{15}N), which behave (chemically) identically to the target analyte but are readily resolved by MS. ISTDs are a requirement for the analytical task of quantitative HRDC via PS-MS technology, as it compensates for matrix interferents as well as any variability between paper substrates[39], allowing for trace-level quantitation[4]. The current PS-MS quantitative strategy utilized at a harm reduction site in Victoria, BC, dilutes an illicit drug sample into a methanolic solution containing a series of isotopically labelled ISTDs. This promotes intimate mixing of analytes and ISTDs in solution before a 10 µL aliquot of illicit drug sample (and ISTDs) is spotted onto a paper strip for PS-MS analysis, allowing ISTDs to compensate for both matrix effects and any irreproducibility during the pipetting step onto the PS-MS strip.

This ISTD approach[66] requires a relatively high quantity of expensive ISTDs to be used for each measurement, however, much of this ISTD is wasted as only a small portion (5% per replicate measurement) is deposited on paper and measured. The remaining ISTD-spiked drug solution is disposed of in accordance with illicit drug checking legal exemption protocols. This legal stipulation coupled with the typical cost of isotopically labelled ISTDs (~$200/mL; typically [100 µg/mL]) equates to discarding 95% of costly

ISTDs per illicit drug analysis. The current PS-MS HRDC quantitative strategy has provided chemical diagnostics for well over 10,000 illicit drug samples submitted by PWUD to a harm reduction site in Victoria, BC. Albeit an effective quantitative method, the current ISTD strategy requires harm reduction sites to have large stocks of ISTD solution as well as access to a designated wet lab for the storage and preparation of standards, calibrants, QC samples, and spray solvents to meet high volume testing demands. This is problematic as harm reduction sites are often in 'downtown' settings with limited temperature control (i.e., air conditioning, freezers, fume hoods), challenging safe, reproducible standard preparation and storage (i.e., due to methanol evaporation).

Conventionally, PS-MS analysis uses manual sample deposition via a mechanical micropipette. However, manual sample handling has been shown to be responsible for up to 30% of sources of analytical error[86]. Utilizing a robotic liquid handling system enhances laboratory efficiency by offering both (unattended) automation and reproducibility. A robotic liquid handling system reduce analytical costs (i.e., lower labor costs, scaled down sample and reagent volumes) while increasing sample throughput. In this chapter, alternative ISTD strategies were evaluated to replicate the current PS-MS HRDC analytical performance with less overall ISTD consumption. Here, the same quantity of ISTD (1 nanogram per ISTD) is deposited separately on each paper through various regimes of concentrations and volumes (i.e., i) 1.00 µL at a concentration of 1000 ng/mL, ii) 2.50 µL at a concentration of 400 ng/mL, iii) 5.00 µL at a concentration of 200 ng/mL, iv) 10.00 µL at a concentration of 100 ng/mL). This study evaluated the analytical performance of ISTD deposition by two different methods: 1) the conventional method by "Hand", depositing volumes with a mechanical micropipette and 2) by "Robot", through the assistance of a robotic liquid handling system. The goal of this study is to simplify the analytical workflow for quantitative HRDC while drastically reducing waste.

The work in this chapter aims to expand a pre-existing quantitative strategy[4, 64] by assessing how much analytical performance is sacrificed, if any, from pre- and post-(sample) ISTD deposition. Long-term, the aim of this work is to simplify HRDC workflows and reduce analytical costs (i.e., ISTD consumables, labor costs, solvent) for real-world

samples, thereby promoting feasibility and adoption of solid-substrate spray-based MS technology. For HRDC purposes, pre-deposited ISTDs (onto paper strips) result in a drastic reduction of ISTD consumed (proposed 15-fold reduction compared to current ISTD utilization strategy), which has the cascading effect of reducing disruptions in service due to ISTD supply/integrity and improves turn-around time for chemical analysis through a simplified workflow. For widespread adoption, pre-deposited ISTD PS-MS plates could be provided as kits (i.e., ISTDs, calibrants, QC samples) which offer a simplified workflow for high through-put PS-MS applications. Pre-spotted ISTD HRDC plates offer the ability to run daily calibrations and rapidly re-validate analytical workflow, increasing confidence in quantitative chemical measurements by accounting for day-to-day variability of PS-MS operation as well as providing an early-warning sign for degraded instrument performance. The benefits of pre-deposited ISTD(s) on PS-MS plates go beyond HRDC, the same successes can be translated to clinical applications and point-of-care services which require rapid, high-throughput, and quantitative chemical analyses. The analytical performance of depositing ISTDs, post-sample spotting, was also evaluated in this chapter, as it too offers unique analytical advantages. Post-sample ISTD depositions offer the ability to send pre-deposited samples (on PS-MS plates) to analytical laboratories for appropriate ISTD deposition and MS analysis. To the best of our knowledge, there are no publications which have thoroughly assessed ISTD utilization strategies for quantitative PS-MS investigations.

3.2 Materials and Methods

3.2.1 Solvents and Standards

Reference analytical standards were purchased as a legally exempt test kit from Cerilliant Corporation (Round Rock, TX, USA). To assess the proposed ISTD utilization strategies, six-point combined (analytes and ISTDs) calibration standards (n = 5 replicate measurements at 0, 50, 100, 250, 500, and 1000 ng/mL levels; [ISTDs] = 100 ng/mL) were gravimetrically prepared in methanol (HPLC grade, Fisher Scientific, Ottawa, ON, Canada). A test sample consisting of 8 illicit drug analytes (carfentanil, cocaine, etizolam, fentanyl, fluorofentanyl, heroin, MDMA, and MDA) was gravimetrically prepared in methanol at a target concentration of 400 ng/mL. A methanolic combined solution of ISTDs (carfentanil-d_5, cocaine-d_3, etizolam-d_3, fentanyl-d_5, heroin-d_9, MDMA-d_5, and MDA-d_5) was gravimetrically prepared at target concentrations of 100, 200, 400, and 1000 ng/mL. Spray solvents were prepared using 90% HPLC grade methanol, 0.1% formic acid (Fisher Scientific), and 10% 18 MΩ·cm deionized water (Facility Scale Reverse Osmosis/Ion Exchange Water Purification System, Applied Membranes Inc., Vista, CA, USA).

3.2.2 Instrumentation

ISTD depositions, pre- and post-sample spotting, were evaluated as a quantitative strategy on a TSQ Altis™ triple quadrupole mass spectrometer equipped with a VeriSpray™ paper spray ion source (Thermo Fisher Scientific, San Jose, CA, USA) operated in positive ion mode (+3800 V). This PS-MS system is equipped with a robotic plate loader and magazine which can autonomously analyze up to 10 VeriSpray™ plates where each plate contains 24 individual paper spray strips (i.e., up to 240 samples). ISTD depositions, pre- and post-sample spotting, were performed manually with a mechanical micropipette (Finn Pipette F2, 0.2-2 µL & 2–20 µL, Thermo Fisher Scientific, San Jose, CA, USA) as well as robotically with a robotic liquid handling system (Tri-Plus RSH

autosampler, Product Version: 2.4.60, Build Version: 2.4.20015.1623, Thermo Fisher Scientific, San Jose, CA, USA). All depositions (calibrant, ISTD, test sample) were allowed to air dry for 15 minutes before PS-MS measurement.

An additional study compared the analytical performance between two methods of small volume ISTD depositions: 1) by “Hand” (i.e., mechanical micropipette) and 2) by “Robot” (i.e., robotic liquid handling system). The robotic liquid handling system is equipped with a headspace tool, solid phase microextraction tool, liquid syringe (LS) tool, a 200 µL and 1000 µL micropipette tool. The LS syringe tool equipped with a 10 µL c-line gastight syringe (PAL3-SYH-207813, gauge 22s, θ = 6.6 mm, length = 57 mm, point style = 3, scale = 60 mm, Thermo Fisher Scientific, San Jose, CA, USA) was used to deliver 1.00, 2.50, 5.00, and 10.00 µL of a methanolic ISTD suite. The robotic liquid handling system is equipped with a washing station for the LS syringe tool and was rinsed thoroughly with methanol 20 times after every use to prevent cross contamination.

3.2.3 Analytical Workflow

For pre-sample ISTD deposition studies, step 1 of the analytical workflow required 1 ng per ISTD (7 ISTDs = 7 ng total) to be deposited per paper strip (Figure 3.1). One plate was used for each of the four volume/concentration regimes of the ISTD suite (i.e., i) 10.00 µL at a concentration of 100 ng/mL ii) 5.00 µL at a concentration of 200 ng/mL, iii) 2.50 µL at a concentration of 400 ng/mL, iv) 1.00 µL at a concentration of 1000ng/mL). For each plate (n = 24 paper strips), ISTD deposition replicates were performed by “Hand” (n = 12 replicate measurements) with a mechanical micropipette and by “Robot” (n = 12 replicate measurements) with a robotic liquid handling system in order to evaluate the analytical performance between the two methods. In step 2, 10.00 µL of the test sample was deposited by hand using a micropipette onto all 24 paper strips (mirroring the current HRDC PS-MS analytical workflow). The target concentration of the test sample was 400 ng/mL for 8 illicit drug analytes. All depositions (step 1 & 2) were air dried for 15 minutes before step 3, PS-MS/MS quantitation. These steps were repeated for all four

volume/concentration regimes (*n* = 4 plates). For post-sample ISTD deposition studies, steps 1 and 2 of the analytical workflow were performed in opposite order. The analytical workflow to quantitate drugs of misuse using the proposed ISTD utilization strategies are schematically represented in Figure 3.1.

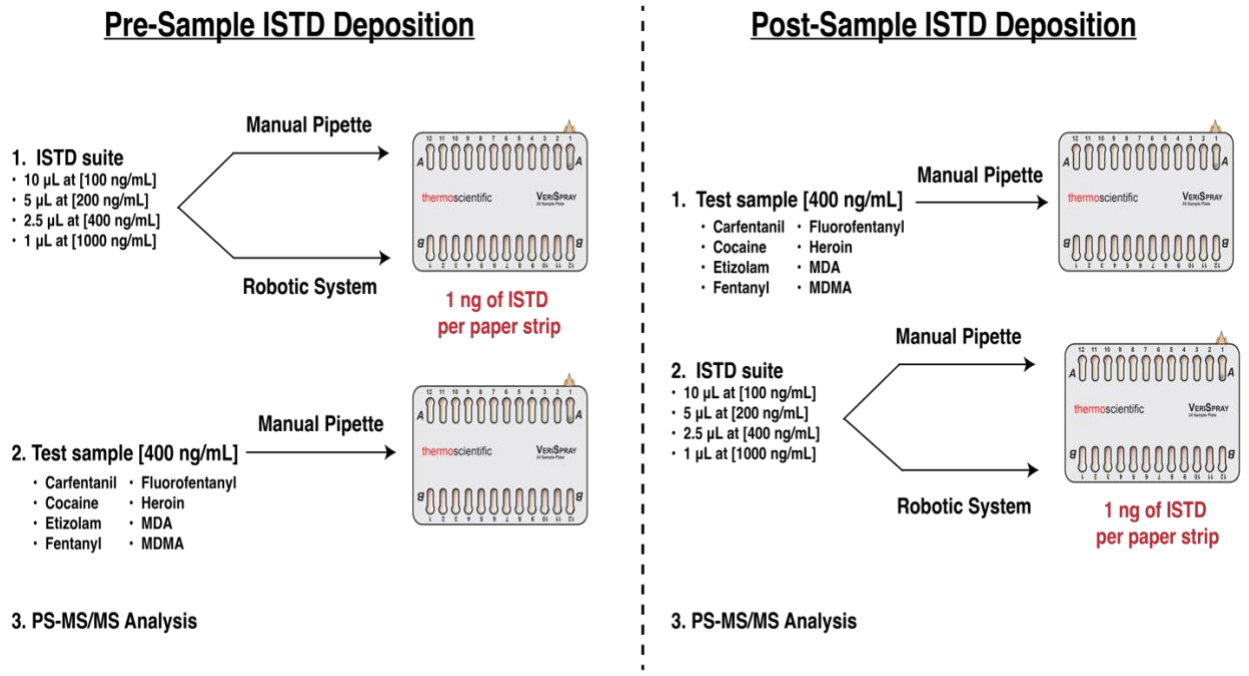

Figure 3.1: Schematic representation of the analytical workflow for pre- and post-sample ISTD deposition studies.

3.3 Results and Discussion

3.3.1 Target Analyte Optimization

MS/MS parameters were optimized using direct infusion ESI for the quantitative detection of target illicit drug analytes (*n* = 8) and their respective ISTDs (*n* = 7), represented in Table 3.1. The major product ions are listed in descending order of signal intensity and the product ion with the highest signal intensity (bold items in table 3.2) was used as the quantifier ion for that particular analyte. The signal area ratio of the analyte's quantifier ion with respect to its ISTD quantifier ion was used as the quantitative strategy.

Six-point combined calibration curves (0–1000 ng/mL) were used to quantify target illicit drug analytes. Calibrant solutions were prepared as a combined suite of illicit drug analytes and ISTDs ([ISTDs] = 100 ng/mL) to mirror the current HRDC calibration workflow [4]; 10.00 µL of calibrant solution was deposited onto paper strips. Calibrations were used to quantitatively measure a test sample which contained 8 target illicit drug analytes (all at 400 ng/mL) commonly observed in HRDC[81]. ISTDs were deposited on paper strips, pre- and post-sample deposition, through various volumes and concentrations of the ISTD suite to evaluate its quantitative analytical performance.

Table 3.1: Summary of MS/MS parameters for ISTD utilization strategies. Parameters were optimized using TraceFinder™ software. All analytes & ISTDs were optimized at 500 ng/mL using ESI in positive ion mode. Major product ions are listed in descending order of signal intensity. Bold product ions indicate the quantifier ion for each analyte.

Target Drug	**Precursor (*m/z*)**	**Major Product Ions (*m/z*)**	**Collision Energy (V)**
MDA	180.0	**163.0**, 105.0, 135.0	10.00
MDMA	194.1	**163.0**, 105.0, 135.0	12.78
Cocaine	304.2	**182.1**, 150.0, 82.0	19.41
*Fluorofentanyl	335.3	**188.1**, 105.0, 234.1	23.45
Fentanyl	337.2	**188.1**, 105.0, 216.1	22.89
Etizolam	343.2	**314.0**, 189.1, 188.1	25.27
Heroin	370.2	**268.1**, 328.1, 211.1	28.10
Carfentanil	395.2	**335.1**, 363.1, 246.1	18.14
ISTD	**Precursor (*m/z*)**	**Major Product Ions (*m/z*)**	**Collision Energy (V)**
MDA-d_5	185.1	**85.0**, 153.1, 91.0	16.83
MDMA-d_5	199.1	**165.0**, 135.0, 107.0	12.89
Cocaine-d_3	307.2	**185.1**, 153.0, 85.0	19.20
Fentanyl-d_5	342.3	**188.1**, 105.0, 221.1	23.35
Etizolam-d_5	346.2	**317.0**, 292.1, 262.0	26.13
Heroin-d_9	379.2	**272.1**, 212.1, 335.1	29.52
Carfentanil-d_5	400.3	**340.2**, 368.1, 246.1	18.29

*Fentanyl-d_5 was used as ISTD for fluorofentanyl
Global operating parameters: ESI *spray voltage* = +3800V, *ion transfer tube* = 300°C, *CID gas* = 2 mtorr,

3.3.2 ISTD Utilization Studies

The robotic liquid handling system's micropipette tool is preferred for ISTD deposition upon PS-MS plates for quantitative investigations as it can be programmed to pick-up and eject a new pipette tip for every sample, eliminating cross-contamination. However, at the time of these experiments the smallest volume micropipette tool commercially available (200 µL) was too large for the intended small volume depositions (≤ 10 µL). As a result, the (10.00 µL) liquid syringe tool was utilized for these studies. The washing station (20 methanol rinses) appeared to effectively mitigate carryover effects which suggest that a syringe might be a better tool as it requires less consumables (i.e., pipette tips). However, the results suggest that there is no significant analytical difference between the two methods of delivery ("Hand" vs. "Robot") when depositing ISTDs, pre-sample spotting. These are significant results because quantitative PS-MS HRDC is a relatively new service being offered (i.e., currently at one harm reduction site in Victoria, BC) and for the foreseeable future, depositions will be performed manually (mechanical micropipette) by graduate students and/or harm reduction staff without sacrificing significant analytical performance. The robotic liquid handling system performed significantly better than hand depositions when depositing ISTDs, post-sample spotting, evident from the acceptable percent recoveries for target analytes. The data suggest that depositing ISTDs, post-sample spotting, by hand, is not an effective quantitative approach (percent recoveries ranged from 0.89–3670%) as too much human error is introduced. The data from the 1.00 µL ISTD depositions at a concentration of 1000 ng/mL suggests that there is a lower (volume) limit to these alternative ISTD utilization strategies as average percent recovery across 8 illicit drug analytes ranged from 219.39% (pre-sample ISTD deposition) to 270.14% (post-sample ISTD deposition) when deposited by a robotic liquid handling system, and 0.89% (pre-sample ISTD deposition) to 3670% (post-sample ISTD deposition) when deposited manually. Poor quantitative performance at this volume is most likely due to the difficulty of reproducible 1.00 µL depositions, as evaporation and pipetting technique introduces too much error for quantitative investigations.

Acceptable percent recoveries in analytical chemistry range between 70–120%[89, 90]. The percent recovery of illicit drug analytes has been evaluated in a variety of different mediums with recoveries of illicit drugs in wastewater reported to be 51%– 112% by LC-MS[91], and recoveries of amphetamine-type illicit drugs by PS-MS were reported to be 74.2%–94.9% in whole blood, and 80.2%–103.6% in urine[92]. There were 16 possible combinations of pre- and post-sample ISTD depositions across four volume/concentration regimes, where ISTD depositions were delivered by two different methods (hand vs. robot), 9 of them fall within the acceptable range of percent recoveries (70–120%). Table 3.2 visually represents the quantitative performance of these alternative ISTD utilization strategies where green indicates acceptable average percent recovery across 8 illicit drug analytes, and red indicates unacceptable average percent recovery data across 8 illicit drug analytes.

Table 3.2: Visual representation of the quantitative performance for 16 combinations of pre- and post-sample ISTD deposition studies across 4 volume/concentration regimes, where ISTD depositions were delivered by two different methods ("Hand" & "Robot"); green indicates acceptable average percent recovery for 8 illicit drug analytes, red indicates average percent recovery that is out of the acceptable range.

Volume/Concentration Regime	Pre-Sample ISTD Deposition	Post-Sample ISTD Deposition
10.00 µL at 100 ng/mL	Hand 71.26 ± 11.86%	Hand 202.10 ± 15.71%
	Robot 90.30 ± 12.72%	Robot 94.16 ± 9.24%
5.00 µL at 200 ng/mL	Hand 81.72 ± 9.82%	Hand 289.17 ± 46.08%
	Robot 73.73 ± 9.08	Robot 106.62 ± 7.39%
2.50 µL at 400 ng/mL	Hand 94.42 ± 7.57%	Hand 625.43 ± 169.20%
	Robot 94.02 ± 7.85%	Robot 99.11 ± 8.00 %
1.00 µL at 1000 ng/mL	Hand 0.90 ± 1.43%	Hand 3006.32 ± 1354.39%
	Robot 219.39 ± 31.60%	Robot 279.89 ± 31.17%

Acceptable percent recovery is between 70–120%.

The best analytical performance was observed when depositing 2.50 µL of the 400 ng/mL ISTD suite, pre- and post-sample deposition. Depositing 2.50 µL of the 400 ng/mL ISTD suite, pre-sample, yielded an average percent recovery of 94.42 ± 7.57% across 8 illicit drug analytes when deposited by hand, and 94.02 ± 7.85% when deposited by a robotic liquid handling system; 2.50 µL of the 400 ng/mL ISTD suite, post-sample deposition, yielded an average percent recovery of 99.11 ± 8.00% when deposited by a

robotic liquid handling system (625 ± 169.20% when deposited by hand). These results suggests that localized depositions yield superior analytical performance. We speculate that there is less effective mixing of ISTD with sample when 'flooding' of the paper strip as observed for the 5.00 and 10.00 µL depositions, which resulted in total saturation of the paper strip. Whereas the 1.00 and 2.50 µL depositions remain localized at the point of deposition. Figure 3.2 visually represents localized ISTD deposition observed for the 1.00 and 2.50 µL depositions, as well as total saturation of the paper strip observed for the 5.00 and 10.00 µL depositions.

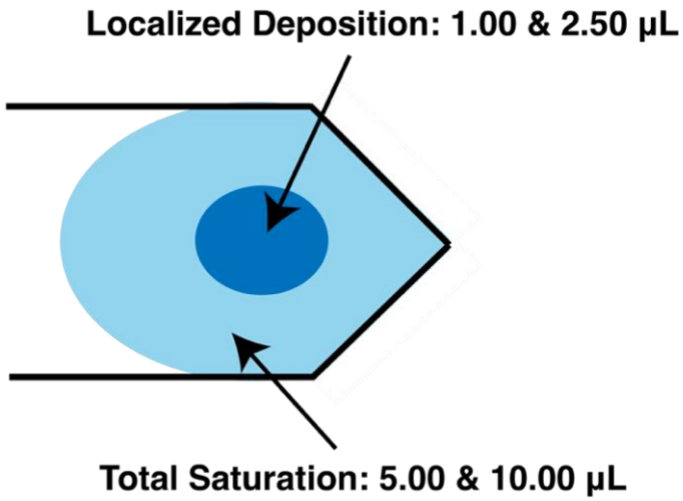

Figure 3.2: Schematic representation of 1.00, 2.50, 5.00, and 10.00 µL depositions on a paper strip for PS-MS analysis. The 5.00 & 10.00 µL depositions result in total saturation of the paper strip (light blue). The 1.00 and 2.50 µL depositions remain localized at the point of deposition (dark blue).

This body of work evaluated the analytical performance of depositing ISTDs, pre- and post-sample deposition through various concentration/volume regimes. In addition, the analytical performance of delivering (small volume) ISTDs by two different methods (i.e., "Hand" vs. "Robot") was evaluated. The results of these parallel studies when utilizing 10.00 µL at a concentration of 100 ng/mL is represented in Figure 3.3 & Table 3.3, 5.00 µL at a concentration of 200 ng/mL is represented in Figure 3.4 & Table 3.4, 2.50 µL at a concentration of 400 ng/mL is represented in Figure 3.5 & Table 3.5, and

1.00 µL at a concentration of 1000ng/mL is represented in Figure 3.6 & Table 3.6. Each figure presents the calculated concentrations for 8 illicit drug analytes with error bars depicting the standard deviation over replicate measurements (*n* = 12); a red dotted line indicates the target concentration of 400 ng/mL. Each figure is accompanied by a percent recovery table for individual illicit drug analytes as well as the average percent recovery ± standard deviation over all 8 analytes for that particular volume and concentration. The results from these studies demonstrate that the proposed quantitative strategy of pre-and post-sample ISTD depositions is an effective strategy to quantitate illicit drug analytes.

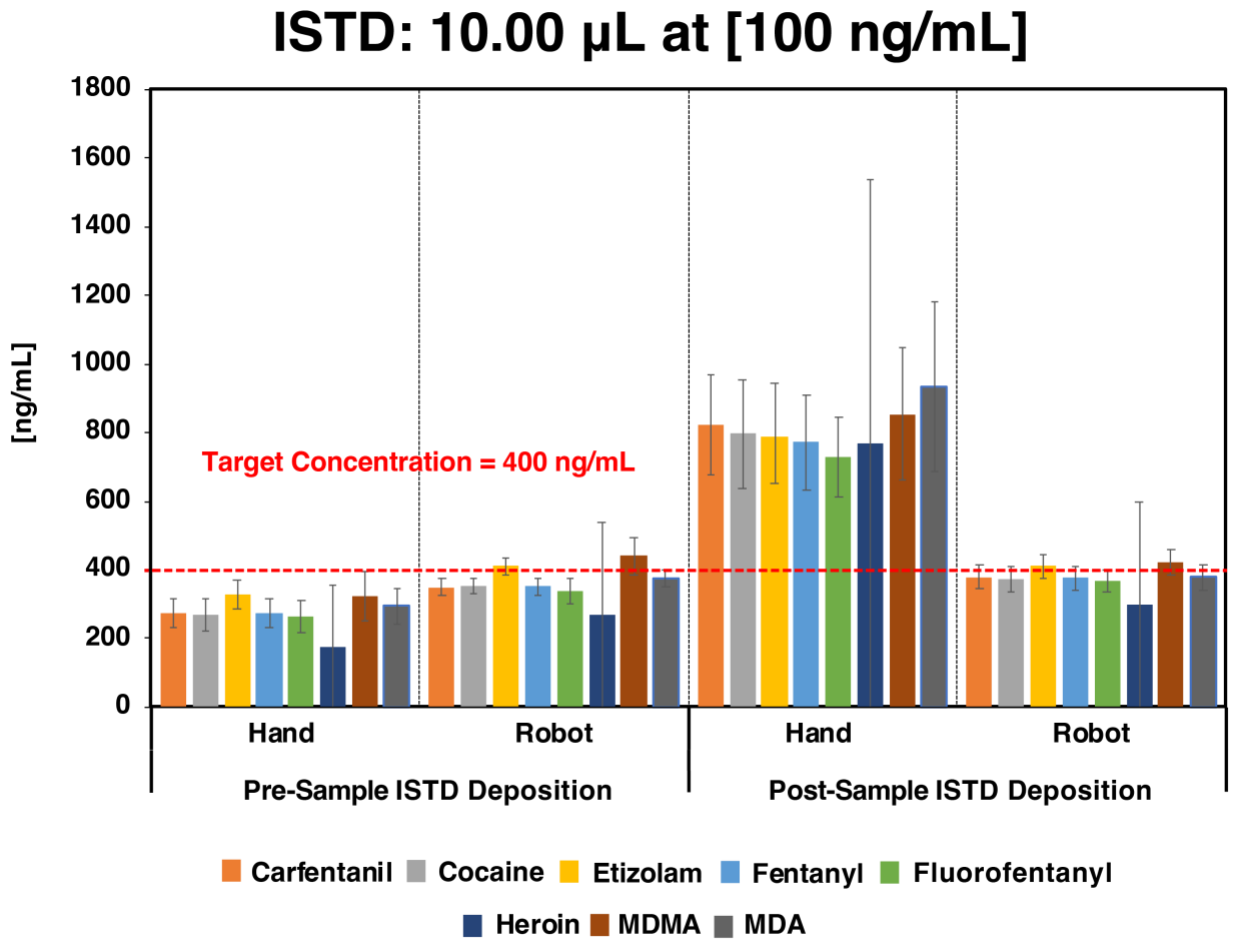

Figure 3.3: Analytical performance from depositing 10.00 µL at a concentration of 100 ng/mL of the ISTD suite, pre- and post-sample spotting. A parallel study compared the analytical performance of depositing ISTDs by "Hand" (micropipette) and by "Robot" (robotic liquid handling system). Image depicts the calculated average concentration for 8 illicit drug analytes with error bars depicting standard deviation over 12 replicates; target concentration for all analytes was 400 ng/mL.

Table 3.3: Average percent recovery ± standard deviation (*n* = 12 replicate measurements) for individual and overall illicit drug analytes utilizing 10.00 µL of a 100 ng/mL ISTD suite for pre- and post-sample ISTD deposition studies where ISTDs were delivered by "Hand" (micropipette) and by "Robot" (robotic liquid handling system).

	Percent recovery: 10.00 µL of a 100 ng/mL ISTD suite			
	Pre-Sample ISTD Deposition		**Post-Sample ISTD Deposition**	
	Hand	**Robot**	**Hand**	**Robot**
Carfentanil	**70.81 ± 7.86%**	**87.61 ± 6.74%**	**205.76 ± 37.03%**	**94.94 ± 8.71%**
Cocaine	**69.77 ± 8.80%**	**88.19 ± 5.95%**	**199.00 ± 39.55%**	**93.17 ± 8.84%**
Etizolam	**83.86 ± 8.72%**	**102.65 ± 5.74%**	**197.48 ± 37.94%**	**103.01 ± 8.32%**
Fentanyl	**70.18 ± 7.92%**	**87.72 ± 5.75%**	**192.80 ± 34.63%**	**93.87 ± 8.87%**
Fluorofentanyl	**68.16 ± 9.30%**	**84.61 ± 8.90%**	**182.57 ± 29.02%**	**92.31 ± 7.87%**
Heroin	**46.47 ± 9.17%**	**67.42 ± 6.35%**	**192.13 ± 44.09%**	**74.93 ± 8.09%**
MDA	**84.30 ± 14.61%**	**110.05 ± 13.40%**	**213.64 ± 48.10%**	**106.13 ± 9.27%**
MDMA	**76.49 ± 9.90%**	**98.05 ± 14.17%**	**233.45 ± 62.18%**	**94.90 ± 9.31%**
Avg. ± Std. Dev.	**71.26 ± 11.86%**	**90.30 ± 12.72%**	**202.10 ± 15.71%**	**94.16 ± 9.24%**

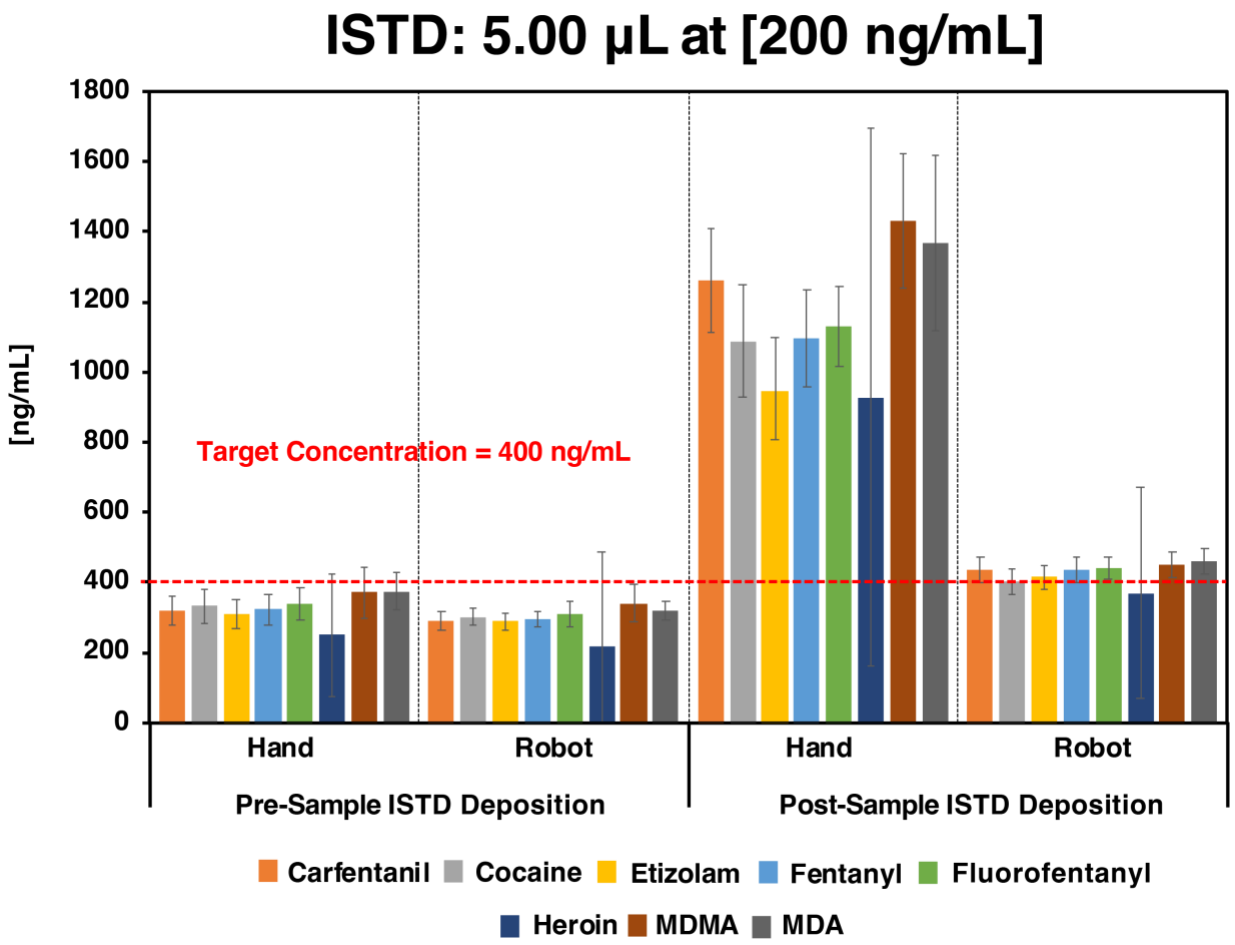

Figure 3.4: Analytical performance from depositing 5.00 µL at a concentration of 200 ng/mL of the ISTD suite, pre- and post-sample spotting. A parallel study compared the analytical performance of depositing ISTDs by "Hand" (micropipette) and by "Robot" (robotic liquid handling system). Image depicts the calculated average concentration for 8 illicit drug analytes with error bars depicting standard deviation over 12 replicates; target concentration for all analytes was 400 ng/mL.

Table 3.4: Average percent recovery ± standard deviation (*n* = 12 replicate measurements) for individual and overall illicit drug analytes utilizing 5.00 µL of a 200 ng/mL ISTD suite for pre- and post-sample ISTD deposition studies where ISTDs were delivered by "Hand" (micropipette) and by "Robot" (robotic liquid handling system).

	Percent recovery: 5.00 µL of a 200 ng/mL ISTD suite			
	Pre-Sample ISTD Deposition		**Post-Sample ISTD Deposition**	
	Hand	**Robot**	**Hand**	**Robot**
Carfentanil	**79.62 ± 12.10%**	**72.83 ± 6.29%**	**315.79 ± 185.86%**	**109.01 ± 15.55%**
Cocaine	**83.20 ± 11.84%**	**75.14 ± 6.23%**	**272.12 ± 142.28%**	**100.58 ± 35.83%**
Etizolam	**77.55 ± 11.03%**	**72.11 ± 5.12%**	**236.30 ± 95.95%**	**104.23 ± 17.49%**
Fentanyl	**80.46 ± 10.81%**	**74.01 ± 6.28%**	**274.35 ± 150.08%**	**108.90 ± 15.67%**
Fluorofentanyl	**84.45 ± 12.50%**	**77.31 ± 7.85%**	**282.55 ± 151.22%**	**110.02 ± 14.48%**
Heroin	**62.21 ± 11.29%**	**53.77 ± 6.03%**	**232.09 ± 109.50%**	**92.26 ± 19.91%**
MDA	**92.79 ± 11.10%**	**84.78 ± 9.45%**	**358.15 ± 224.43%**	**112.85 ± 15.01%**
MDMA	**93.50 ± 23.76%**	**79.90 ± 7.43%**	**341.98 ± 218.66%**	**115.13 ± 20.55%**
Avg. ± Std. Dev.	**81.72 ± 9.82%**	**73.73 ± 9.08%**	**289.17 ± 46.08%**	**106.62 ± 7.39%**

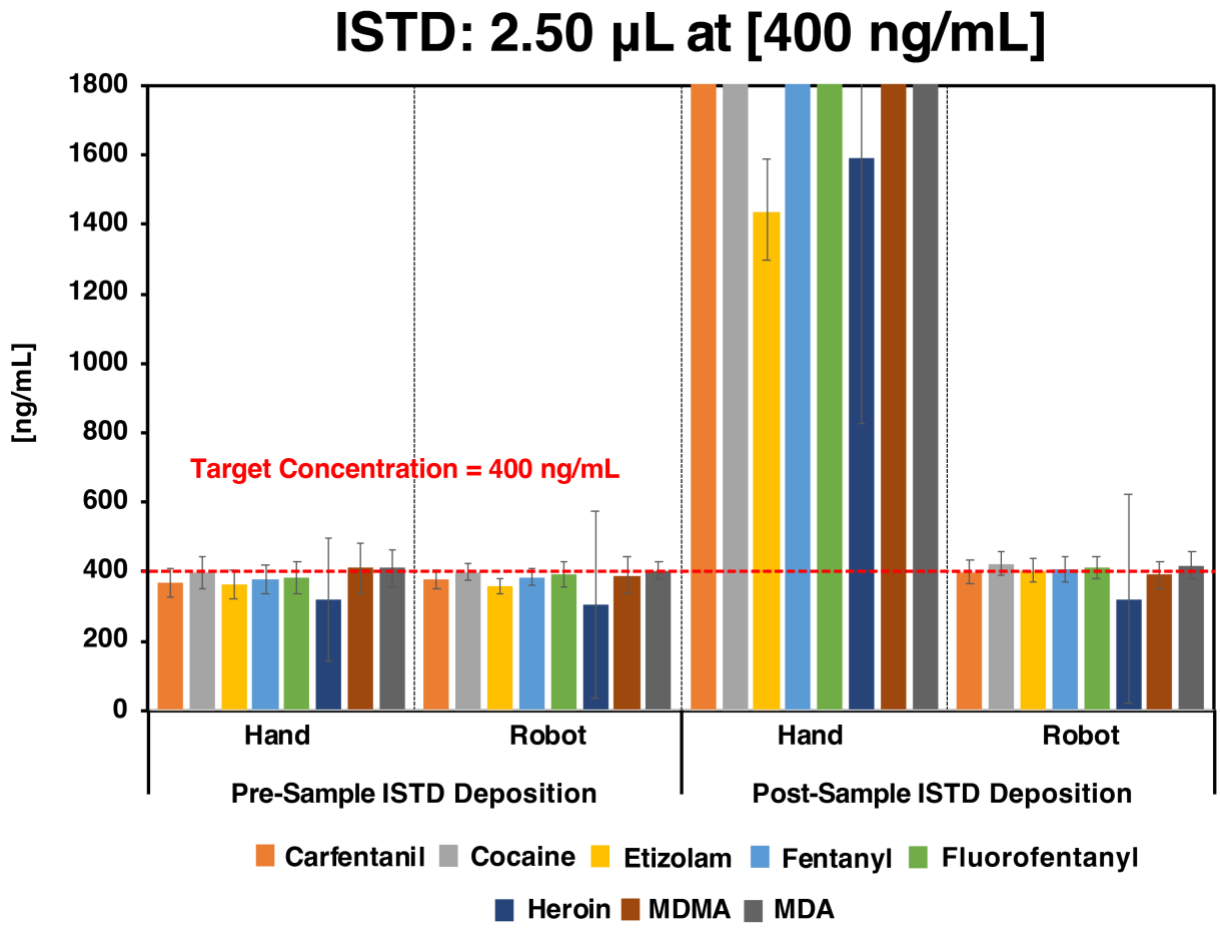

Figure 3.5: Analytical performance from depositing 2.50 µL at a concentration of 400 ng/mL of the ISTD suite, pre- and post-sample spotting. A parallel study compared the analytical performance of depositing ISTDs by "Hand" (micropipette) and by "Robot" (robotic liquid handling system). Image depicts the calculated average concentration for 8 illicit drug analytes with error bars depicting standard deviation over 12 replicates; target concentration for all analytes was 400 ng/mL. The average concentrations for 6 of 8 illicit drug analytes exceed 1800 ng/mL for post-sample ISTD deposition by hand.

Table 3.5: Average percent recovery ± standard deviation (*n* = 12 replicate measurements) for individual and overall illicit drug analytes utilizing 2.50 µL of a 400 ng/mL ISTD suite for pre- and post-sample ISTD deposition studies where ISTDs were delivered by "Hand" (micropipette) and by "Robot" (robotic liquid handling system).

	Percent recovery: 2.50 µL of a 400 ng/mL ISTD suite			
	Pre-Sample ISTD Deposition		**Post-Sample ISTD Deposition**	
	Hand	**Robot**	**Hand**	**Robot**
Carfentanil	**92.00 ± 27.24%**	**94.41 ± 14.55%**	**705.07 ± 661.40%**	**99.69 ± 37.32%**
Cocaine	**99.32 ± 29.06%**	**99.39 ± 13.84%**	**641.07 ± 594.14%**	**105.68 ± 41.79%**
Etizolam	**90.46 ± 26.88%**	**89.35 ± 14.46%**	**359.42 ± 202.76%**	**100.93 ± 42.14%**
Fentanyl	**94.00 ± 26.02%**	**96.08 ± 12.81%**	**641.84 ± 602.00 %**	**101.61 ± 37.58%**
Fluorofentanyl	**95.18 ± 26.21%**	**98.32 ± 13.34%**	**647.40 ± 605.02%**	**102.52 ± 36.17%**
Heroin	**79.42 ± 29.48%**	**76.69 ± 14.28 %**	**398.36 ± 303.40%**	**80.39 ± 40.73%**
MDA	**102.51 ± 34.89%**	**96.91 ± 15.55 %**	**749.76 ± 744.60%**	**97.47 ± 38.63%**
MDMA	**102.47 ± 35.78 %**	**101.00 ± 18.28 %**	**860.53 ± 851.07%**	**104.58 ± 49.85 %**
Avg. ± Std. Dev.	**94.42 ± 7.57%**	**94.02 ± 7.85%**	**625.43 ± 169.20%**	**99.11 ± 8.00%**

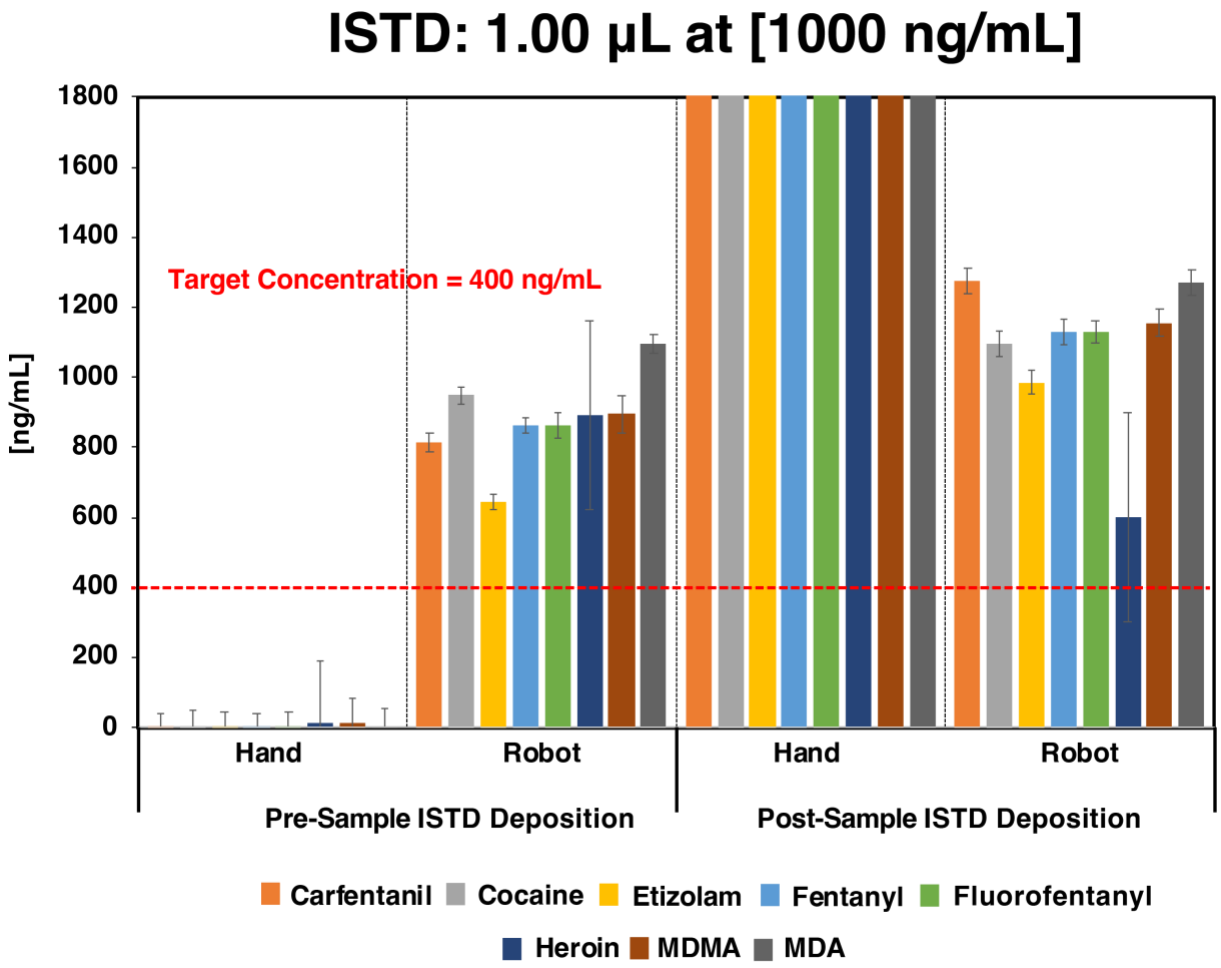

Figure 3.6: Analytical performance from depositing1.00 µL at a concentration of 1000 ng/mL of the ISTD suite, pre- and post-sample spotting. A parallel study compared the analytical performance of depositing ISTDs by “Hand” (micropipette) and by “Robot” (robotic liquid handling system). Image depicts the calculated average concentration for 8 illicit drug analytes with error bars depicting standard deviation over 12 replicates; target concentration for all analytes was 400 ng/mL. The average concentrations for all 8 illicit drug analytes exceed 1800 ng/mL for post-sample ISTD deposition by hand.

Table 3.6: Average percent recovery ± standard deviation (*n* = 12 replicate measurements) for individual and overall illicit drug analytes utilizing 1.00 µL of a 1000 ng/mL ISTD suite for pre- and post-sample ISTD deposition studies where ISTDs were delivered by "Hand" (micropipette) and by "Robot" (robotic liquid handling system).

	Percent recovery: 1.00 µL of a 1000 ng/mL ISTD suite			
	Pre-Sample ISTD Deposition		**Post-Sample ISTD Deposition**	
	Hand	**Robot**	**Hand**	**Robot**
Carfentanil	**0.22 ± 0.22%**	**203.95 ± 51.57%**	**4570.73 ± 3824.44%**	**318.82 ± 169.78%**
Cocaine	**0.21 ± 0.22%**	**237.40 ± 60.97%**	**3103.55 ± 1690.60%**	**274.17 ± 135.14%**
Etizolam	**0.22 ± 0.30%**	**161.31 ± 32.51%**	**680.41 ± 161.82%**	**246.54 ± 101.09%**
Fentanyl	**0.11 ± 0.13%**	**215.57 ± 53.53%**	**3342.28 ± 1801.50%**	**282.51 ± 143.52%**
Fluorofentanyl	**0.04 ± 0.06%**	**216.00 ± 55.21%**	**3331.29 ± 1797.12%**	**282.12 ± 140.29%**
Heroin	**3.56 ± 2.80%**	**222.76 ± 72.62%**	**1289.13 ± 550.09%**	**228.48 ± 113.01%**
MDA	**2.80 ± 2.14%**	**223.98 ± 64.07%**	**3462.41 ± 1936.70%**	**289.13 ± 166.14%**
MDMA	**0.02 ± 0.07%**	**274.11 ± 87.64%**	**4270.72 ± 2410.69%**	**317.32 ± 191.81%**
Avg. ± Std. Dev.	**0.90 ± 1.43%**	**219.39 ± 31.60%**	**3006.32 ± 1354.39%**	**279.89 ± 31.17%**

3.4 Conclusion

This body of work evaluated the analytical performance of depositing ISTDs, pre- and post-sample deposition, on PS-MS paper strips to quantitate illicit drug analytes. One nanogram per ISTD was deposited onto paper strips through four different regimes of volume and concentration (i.e., i) 10.00 µL at a concentration of 100 ng/mL, ii) 5.00 µL at a concentration of 200 ng/mL, iii) 2.50 µL at a concentration of 400 ng/mL, iv) 1.00 µL at a concentration of 1000ng/mL). Analytical performance was superior for pre- and post-sample ISTD deposition studies when depositing 2.50 µL of a 400 ng/mL ISTD suite; pre-sample ISTD depositions yielded an average percent recovery across 8 illicit drug analytes of 94.42 ± 7.57% when deposited by hand, and 94.02 ± 7.85% when deposited by a robotic liquid handling system. Post-sample ISTD depositions yielded an average percent recovery across 8 illicit drug analytes of 99.11 ± 8.00%, when deposited via a robotic liquid handling system. We suspect that at the 2.50 µL volume, ISTD deposition remains localized at the point of deposition which we speculate results in superior mixing of analytes and ISTDs (on paper) prior to measurement, as compared to the 5.00 and 10.00 µL depositions which wicked to the edges of the paper. This work suggests that localized ISTD deposition can improve quantitative performance.

In addition, a parallel study evaluated the analytical performance of depositing 1 ng per ISTD by “Hand” (i.e., mechanical micropipette) and by a “Robot” (i.e., robotic liquid handling system). There was no significant analytical difference between the two methods observed when depositing ISTDS, pre-sample spotting. However, a robotic liquid handling system significantly improved analytical performance when depositing ISTDs, post-sample deposition. A robotic liquid handling system eliminates human error and simplifies the analytical workflow for quantitative HRDC as well as high throughput quantitative PS-MS applications. Depositing ISTDs, pre- and post-sample spotting, is an effective PS-MS strategy for quantifying drugs of misuse and should be the preferred ISTD utilization strategy for HRDC as it drastically reduces ISTD consumables, simplifies the analytical workflow, and reduces cost for HRDC.

Chapter 4: Conclusion

4.1 Summary of Work

MS affords quantitative chemical analyses due to its inherent sensitivity and selectivity. The advent of DMS affords a simplified (and rapid) sampling protocol either void of sample preparation, or with a simplified sample pre-treatment process. DMS forgoes the labor intensive and time consuming step of chromatography to provide real-time chemical analysis. DMS encompasses a vast array of sampling methods, however paper (PS-MS) and paper embedded with a silica capillary (PCSI) were utilized as the sampling/ionization substrate in this thesis to quantitate target illicit drug analytes. Conventional chemical analysis requires samples to be sent to (off-site) analytical laboratories which have the supporting infrastructure (i.e., wet lab, power, gas, solvent supplies, waste streams, venting) required for MS-based measurements. However, advancements in MS technology and direct ionization have facilitated the development and application of miniature MS devices which have effectively revolutionized chemical analysis by bringing the lab to the sample's natural environment (i.e., ambient pressure/temperature) answering chemical questions when and where they are needed. Quantitative chemical analysis is a relatively new service being offered to PWUD as a harm reduction intervention, providing pre-consumption chemical measurements, allowing informed decisions regarding the substance intended for use. This body of work evaluated techniques to make quantitative HRDC logistically easier in terms of hardware (Ch. 2; miniature MS system) and analytical workflow (Ch. 3; pre-spotted ISTD deposition).

4.2 Recommendations for Future Work

A future study that would strengthen this body of work would be to evaluate the stability of ISTDs on paper (i.e., 1 day, 1 week, 1 month, 1 year) to investigate the analytical integrity and shelf-life of pre-deposited ISTDs. Additionally, investigating varying positions of (localized) ISTD deposition (i.e., front, middle, back of paper strip) and how it affects quantitative performance would strengthen the ISTD utilization studies explored in this thesis. The fate and distribution of analytes, post-deposition (i.e., localized vs. wick to the edges of the paper), is currently unexplored and could be investigated by spectroscopic or MS based imaging of analytes and internal standards after their deposition. The fate and distribution of analytes (once deposited onto paper) could help explain why localized deposition (i.e., 2.50 µL) yielded better analytical performance compared to saturating the entire paper strip (i.e., 5.00 & 10.00 µL). A future study that would broaden the applications of this work for potential clinical applications would be to evaluate the quantitative performance of pre-/post-sample ISTD deposition in a variety of different bio matrixes (i.e., urine, plasma, saliva, blood).

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