

Development and optimization of coated blade spray  
- mass spectrometry for bioanalytical and  
clinical applications

by  
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## **Author's Declaration**

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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## **Abstract**

Bioanalysis is a sub-discipline of analytical chemistry that focuses on the identification and quantification of small and large molecules (*e.g.* metabolites and proteins) present in biological sample matrices (*e.g.* blood, urine, saliva, plasma, and tissue) that is concerned with measuring a biological state or condition. One of the challenges chemists face in bioanalytical applications when using conventional analytical procedures such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the inability to inject the raw sample directly into the instrument. As a result, sample preparation steps are required to isolate target analytes from the matrix components present in the sample, providing relatively clean sample extracts and reducing matrix interferences. The common limitation associated with the majority of the sample preparation techniques used in bioanalytical and clinical applications is their time-consuming nature. Thus, there is an urgent need of protocols that decrease the total analysis time. An alternative to these conventional procedures is a solid phase microextraction (SPME) based technology developed at the University of Waterloo that operates as an ambient mass spectrometry (AMS) device. Known as coated blade spray (CBS), it combines in a single device the extractive and pre-concentrative nature of SPME, with the ability to be directly coupled to mass spectrometry (MS) systems without the need for additional instrumentation (*e.g.* pumps, desorption chambers, heating systems). This thesis focuses on the ongoing development and optimization of CBS for quantitative analysis of small molecules in biofluids, as well as an assessment of the CBS platform for rapid therapeutic drug monitoring (TDM) of immunosuppressive drugs (ISDs) in whole blood. To date, this technology has been used only with Thermo Scientific mass spectrometers. Hence, the first objective was to demonstrate the suitability of this technology to effectively operate with MS instruments from different vendors (*e.g.* SCIEX and Waters). To achieve this, the experimental

parameters of the CBS technology were optimized on an AB SCIEX API 4000 triple quadrupole and Waters XEVO G2-S hybrid quadrupole time of flight (QTOF) mass spectrometers, and the benchmark analytical performance of both instruments was established. Following the identification of the optimal CBS operating parameters, these conditions were applied for the concurrent analysis of fentanyl, and five fentanyl analogs from urine and plasma using the AB SCIEX MS. Excellent figures of merit were obtained from both matrices, with limits of quantitation for all compounds at 0.5 ng/mL or lower, with less than 60 s of total analysis time per sample. In the final data chapter, the suitability of the CBS platform for routine clinical analysis of four immunosuppressive drugs (ISDs) in whole human blood was investigated, and rigorously validated, using guidelines set by the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT). The results verified that the optimized method was highly reproducible over a 10-day period, with total inter-day variation less than 5 % for all of the compounds at three different concentrations. The functional sensitivity of the method improved nearly 4 times from the preliminary proof of concept study published earlier this year by Gomez-Rios, *et al.* Likewise, a major decrease in analysis time, from 16 h to 2 h was achieved – besting the analysis times of automated immunoassays that are employed in clinical laboratories for routine analysis of ISDs. Finally, the concentrations of three immunosuppressive drugs in ~100 patient samples obtained using the CBS-MS/MS methodology were compared to the Abbot Laboratories i2000 ARCHITECT automated immunoassay system. Passing-Bablok regression analysis demonstrated significant statistical similarities between both methods. Bland-Altman plots identified minimal outliers, with some bias in the measurements that were consistent with other MS-based methods that were compared to immunoassays.

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## **Dedication**

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## List of Abbreviations

2D	two-dimensional
3D	three-dimensional
A/IS	Analyte to internal standard area ratio
AA	ammonium acetate
ACN	acetonitrile
AMS	ambient mass spectrometry
APCI	atmospheric pressure chemical ionization
CBS	coated blade spray
CE	collision energy
CMIA	chemiluminescent microparticle immunoassay
CNS	central nervous system
CXP	collision exit potential
CYCA	cyclosporine A
DART	direct analysis in real time
DBDI	dielectric barrier discharge ionization
DEA	Drug Enforcement Agency
DEP	direct electrospray probe
DESI	desorption electrospray ionization
DI	direct immersion
DMS	differential mobility spectrometry
DP	declustering potential
ECLIA	electrochemiluminescence immunoassay
ELISA	enzyme linked immunosorbent assay
EP	entrance potential
ESI	electrospray ionization
ETP	extraction time profile
EVER	everolimus
FA	formic acid

g/mol	molecular weight
GC	gas chromatography
HLB	hydrophilic-lipophilic balanced
HS	headspace
IATDMCT	International Association of Therapeutic Drug Monitoring and Clinical Toxicology
IPA	isopropanol
IS	internal standard
ISD	immunosuppressive drugs
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
LDR	linear dynamic range
LDTD	laser diode thermal desorption
LESA	liquid extraction surface analysis
LLE	liquid-liquid extraction
LOD	limit of detection
logP	octanol partitioning coefficients
LOQ	limit of quantitation
ME	matrix effects
MeOH	methanol
MM	mixed mode
MOI	microfluidic open interface
MRPL	minimum required performance limit
MS	mass spectrometry
NH <sub>4</sub> <sup>+</sup>	ammonium
NSERC	Natural Sciences and Engineering Research Council of Canada
OPP	open port probe
PAN	polyacrylonitrile
PBS	phosphate buffered saline
PD	pharmacodynamic
PESI	probe electrospray ionization
PK	pharmacokinetic

POC	point of care
PPT	protein precipitation
PS	paper spray
QC	quality control
QTOF	hybrid quadrupole time of flight
RSD	relative standard deviation
S/N	signal to noise ration
SCX	strong cation exchanger
SEM	scanning electron microscopy
SIR	sirolimus
SPE	solid phase extraction
SPME	solid phase microextraction
SRM	selected reaction monitoring
TAC	tacrolimus
TDM	therapeutic drug monitoring
TM-DART	transmission mode - direct analysis in real time
UHP	ultra-high purity
WADA	World Anti-Doping Agency
ZNSO <sub>4</sub>	zinc sulfata



# Chapter 1. Introduction

## 1.1 Bioanalysis and sample preparation

The quantitative analysis of molecules such as metabolites and biomarkers from complex biological matrices (*e.g.* urine, saliva, plasma, serum, whole blood, and tissue) is commonly referred to as bioanalysis. Bioanalysis is an integral component of pharmaceutical drug development, screening assays for prohibited drugs, identification of novel biomarkers and metabolites for disease characterization, therapeutic drug monitoring (TDM), along with many other applications.<sup>1-5</sup> The main challenge that researchers face when attempting to quantify a small molecule is the complexity, and in some cases, the variability of the sample matrix itself. All biological matrices contain numerous components such as salts, proteins, cellular debris (*e.g.* lipids and lipoproteins), and even intact cells.<sup>3,6</sup>

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the most common and widespread two-dimensional analytical technique used for analysis of biofluids due to its high sensitivity, specificity, and efficiency.<sup>1</sup> However, in almost all cases, the biological sample cannot be directly injected into the LC-MS/MS system.<sup>1</sup> To ensure reproducible and sensitive quantification, sample preparation is used to enrich, and isolate the target analyte(s) from the matrix. This ensures cleaner extracts enriched with the analyte(s) of interest and with less sample interferences.<sup>7</sup> Without adequate sample preparation, LC-MS/MS analysis is beset by a host of problems. These problems include matrix effects (ME), which lead to suppression or enhancement of analyte ion intensity, the presence of lipids, which can affect space charge density and subsequent electrospray ionization (ESI), and increased fouling of the instrument leading to instrumental downtime and higher maintenance costs.<sup>7,8</sup> Different ionization methods used with LC-MS/MS include ESI and atmospheric pressure chemical ionization (APCI). These ionization

methods are affected to different degrees by the presence of sample matrix interferences.<sup>1</sup> For example, ESI is much more susceptible to lipids and phospholipids, which enrich at the surface of electrosprayed droplets and suppress the signal of target analytes.<sup>8</sup> Conversely, APCI is affected less by the presence of lipids because there is no competition for ions to enter the gas phase. However the signal intensity is intrinsically lower than ESI, which leads to lower sensitivity for particular analytes.<sup>7,8</sup>

While there are numerous sample preparation methods employed in bioanalysis, there are several approaches that cover the majority of the conventional techniques used.<sup>3,9</sup> The simplest method is known as “dilute and shoot”, and is often used for analysis of urine.<sup>3</sup> It is the fastest approach, yet it is also the least effective.<sup>3</sup> Diluting the sample and immediately starting chromatographic separation decreases the overall matrix concentration, which ideally reduces the negative influence of the matrix. Unfortunately, there is no extraction and further enrichment of target analytes, so matrix effects can still occur, albeit in a slightly reduced capacity.<sup>10</sup> Dilute and shoot also leads to reduced sensitivity for target analytes in the sample. A sample pretreatment step approach amenable to viscous samples such as plasma and whole blood is protein precipitation (PPT).<sup>3</sup> PPT methods use a small amount of miscible organic solvent or other stressors such as heat, strong acids/bases, or various salt solutions to denature and subsequently precipitate proteins from the sample, thus releasing bound analytes.<sup>2,3</sup> After inducing precipitation, the sample is usually centrifuged to separate denatured proteins from the rest of the sample. The protein pellet is discarded, and the supernatant, which contains the target compounds, is collected and analyzed. PPT is fast and practical in some applications where protein interferences are prevalent, or when analytes are heavily bound to the matrix. However, it does not effectively separate compounds from endogenous interferences such as neutral and polar lipids.<sup>3,11</sup>

Alternatively, samples can be enriched using more intensive sample preparation techniques such as Liquid-Liquid Extraction (LLE). In principle, LLE works by mixing the aqueous biofluid with an immiscible organic solvent and selectively extracting target analytes from the matrix. Separation occurs due to analytes partitioning into the organic phase, which they prefer versus the native aqueous matrix.<sup>2</sup> While LLE is an effective method for providing clean sample extracts and reducing salt concentration, there are a number of limitations which include: 1) large sample volumes, 2) minimal extraction of polar compounds, 3) inability to extract wide range of compounds (*i.e.* unbalanced coverage), 4) large amount of consumed solvent, 5) minimal automation potential, and 6) laborious and time consuming workflows.<sup>1-3,7</sup>

Recently, a more robust sample preparation method has overtaken dilute and shoot, PPT and LLE as the most popular sample preparation method in bioanalysis: Solid Phase Extraction (SPE). SPE relies on affinity-based separation to isolate analytes of interest from the sample matrix. The sample is flowed through a sorbent – packed cartridge, and analytes with a higher affinity for the sorbent than the matrix are selectively retained within the cartridge.<sup>3</sup> Interfering matrix components with a lower affinity for the stationary phase are washed from the cartridge before target analytes are finally eluted.<sup>1,7</sup> Some of the advantages of SPE compared to LLE include: 1) increased efficiency, 2) shorter run times, 3) wider range of analyte selectivity, 4) higher recoveries, and 5) far lower solvent usage.<sup>9</sup> Furthermore, SPE has been successfully automated and can be applied on-line before chromatographic separation, allowing the sample preparation to be directly interfaced with the LC-MS/MS system for high throughput analysis.<sup>12</sup> While SPE is generally considered the gold standard in bioanalytical sample preparation, it is not without its own limitations. Clogging of the SPE cartridge due to high protein content in the sample, issues encountered with breakthrough (*i.e.* incomplete recovery of analytes when sorbent

capacity is exceeded) when analyzing large numbers of compounds or when handling large sample volumes, as well as interferences from unwanted, co-extracted compounds are some of the main concerns encountered with SPE preparation techniques for biological samples.<sup>8,13,14</sup>

## 1.2 Ambient mass spectrometry

The bioanalytical process consists of 5 stages: sampling, sample preparation, chromatographic separation, detection, and data processing. In most cases, the most time-consuming steps are sampling and sample preparation, which can take as much as 80 % of the total processing time.<sup>3</sup> Although sample preparation has proven indispensable in providing cleaner samples with less interferences – a necessity for chromatographic separation – all sample preparation methods are afflicted by the same limitation: time-consuming workflows. To address this constraint, ambient mass spectrometry (AMS) was conceived to characterize samples in their native environment.<sup>15</sup> As the name implies, ions are produced under the ambient conditions of the laboratory (*i.e.* sources are not enclosed within the instrument), with no additional sample pretreatment. There is also no chromatographic separation prior to mass spectrometric (MS) analysis, which expedites the process of sampling to introduction of analytes into the MS.<sup>15,16</sup> AMS was first conceived by Professor Graham Cook's research group at Purdue University in 2004, in the form of a revolutionary technique called desorption electrospray ionization (DESI), followed shortly afterwards by direct analysis in real time (DART) in 2005.<sup>17,18</sup> While these techniques were the first to use the “ambient” MS terminology, it was several years prior in 1999 that the direct electrospray probe (DEP) technology pioneered by Professor Jentai Shiea at the National Sun Yat-Sen University in Taiwan was reported with the same underlying principle.<sup>19</sup> The DEP device used a copper wire bent into a ring configuration to directly electrospray small volumes of sample

(< 5  $\mu$ L) into the MS under ambient conditions. In 1999, Shiea's technique was not referred to as AMS, however, in retrospect, it represents one of the first attempts of "true" ambient analysis. Since these early breakthroughs, AMS methods have garnered increased attention with techniques such as transmission mode - direct analysis in real time (TM-DART), paper spray (PS), open port probe (OPP) and microfluidic open interface (MOI), dielectric barrier discharge ionization (DBDI), probe electrospray ionization (PESI), and liquid extraction surface analysis (LESA).<sup>20-29</sup> These techniques differ significantly in terms of their instrumentation, ionization mechanisms, sample interface, detection limits, and applications. However, they all share the unifying principle of analyzing samples in their native condition (*i.e.* without sample pretreatment) by generating ions at ambient conditions.<sup>15,16</sup>

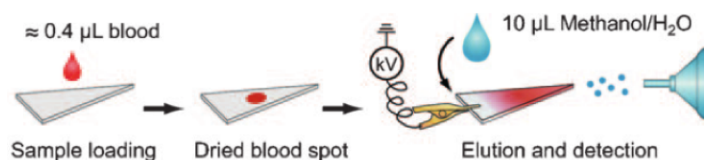
The brilliance of AMS technologies centers on rapidly decreasing the time needed to go from sampling to sample introduction into the MS. Omitting LC separation reduces dilution of the sample, and can increase sensitivity to quantify ultra-low concentration analytes (>1 ng/mL).<sup>15,16,30</sup> By analyzing the sample in its native environment, certain AMS methods are capable of acquiring the chemical and spatial information and generating a two-dimensional (2D) or three-dimensional (3D) mass spectrometric "image".<sup>31</sup> Eliminating several of the preliminary steps, AMS appears to be an excellent candidate for high throughput analysis, point of care (POC) testing or TDM.<sup>32</sup> Unfortunately, few methods have been successfully adapted due to the difficulty in automating the entire process - specifically the desorption/ionization stage - into a single, consolidated autosampler.<sup>33,34</sup> As automation/auto-sampler technologies continue to improve, this limitation will slowly be phased out.<sup>35</sup> Ultimately, the simplified workflow of direct-coupled AMS methods will make high throughput, routine analysis quite practical and feasible.

Although there are a number of advantages, there are also several disadvantages associated with AMS. Sample preparation is critical in conventional bioanalytical LC-MS/MS applications to minimize the influence of the matrix on the analysis of target analytes, thus improving the overall reproducibility of quantitative analysis at the cost of increased time. Due to the simultaneous analyte extraction and ionization steps, there is inherent irreproducibility in AMS techniques. One way to mitigate this issue is the use internal standards (*e.g.* deuterated analogues of the target analyte).<sup>36</sup> While internal standards are typically used in LC-MS/MS to ensure high precision, it is often not entirely necessary. Conversely, introduction of internal standards is critical for acceptable precision with AMS techniques to correct for sample complexity and variability.<sup>30,33,36</sup> This necessity for internal standards leads to challenges with incorporation of the internal standard into the sample, especially when analysis of small volumes of sample or whole tissue is desired.<sup>33</sup> There is also variability where the ionization occurs, resulting in irreproducible ion formation, and subsequent transport into the MS.<sup>37</sup> Furthermore, standard atmospheric ionization sources in LC-MS/MS such as ESI and APCI are enclosed within the instrument, reducing the effects of varying laboratory conditions. AMS methods are generally exposed to the open environment, where air flow currents, temperature and humidity changes can affect reproducibility by altering the ionization efficiency and the ion transmission into the MS.<sup>33,38</sup> Finally, the biggest disadvantage of AMS is the potential for ion suppression/enhancement due to the complete absence of sample preparation.<sup>15,39</sup> Analysis of biofluids using AMS methods is especially susceptible to this problem because of the high concentrations of lipids, salts and other ionic compounds present in the sample.<sup>15,40-42</sup>

### 1.3 Substrate-ESI technologies

One of the limitations of the traditional on-line ESI format is that the analyte solution must be continuously pumped into the electrospray tip (*e.g.* LC-MS/MS), in order to control the flow of analyte into the instrument.<sup>43</sup> This can be avoided by generating the charged droplets directly off a solid substrate using an applied voltage, mimicking the tip of the ESI source.<sup>15,44</sup> Several publications have reported various solid substrates as the support for ESI under ambient conditions, ranging from plant leaves to chromatography paper, sharpened wooden sticks, and metal probes.<sup>19,21,44,45</sup> Ionization from plant leaves permits direct chemical analysis of pesticides and other contaminants from the surface of the plant under ambient conditions.<sup>45</sup> Alternatively, chromatography paper is both cost – effective and readily available approach, while still providing a suitable ESI source.<sup>21</sup> The paper support can come in the form of a wipe, or a sampler exposed to the sample matrix that has been cut in a triangular shape.<sup>15,21</sup> PS, which employs the aforementioned triangular paper design, has emerged as one of the premier solid substrate-ESI techniques for the analysis of small molecules in biofluids under ambient conditions. This is due to low sample consumption ( $\leq 50 \mu\text{L}$ ), fast analyses, sufficient recoveries and no inter-device sample carryover.<sup>21</sup> The paper support is extremely inexpensive to produce, so sampler carryover is eliminated because each sample can be swabbed or pipetted onto its own paper sampler and then discarded.<sup>21</sup>

Figure 1.1 depicts a standard PS extraction workflow and ionization source.<sup>21,46</sup> Succinctly, PS begins with application of the sample onto the surface of the paper. This can be in the form of



**Figure 1.1.** PS analytical workflow for biofluids analysis.

of a biofluid spot, immersion of the paper into a aqueous sample or directly swabbing the paper onto the sampling surface.<sup>21,46,47</sup> Once the sample has been applied to the paper, the paper is dried, followed by application of organic solvent at the back edge. Capillary action pulls the solvent through the paper towards the tip, carrying dissolved analytes, and other macromolecules towards the tip. Once the electric potential has been applied, ESI occurs and a Taylor cone of aerosolized gas phase ions is generated at the apex of the paper.<sup>21,48</sup> In order to achieve sufficient ionization, a high voltage must be applied to the paper (*i.e.* 3-5 kV). The high voltage increases the rate of evaporation of solvent, leading to a shift from ESI to corona discharge as the primary source of ionization once the solvent has evaporated.<sup>15,46</sup> This shift from ESI to corona discharge increases irreproducibility, as some analytes may not be efficiently ionized during corona discharge. Additional sources of irreproducibility stem from insufficient sharpness of the tip of the paper, and the fibrous composition.<sup>37</sup> This destabilizes the electrospray, and results in multiple, smaller Taylor cones which distort the flow of ions into the MS.<sup>48</sup>

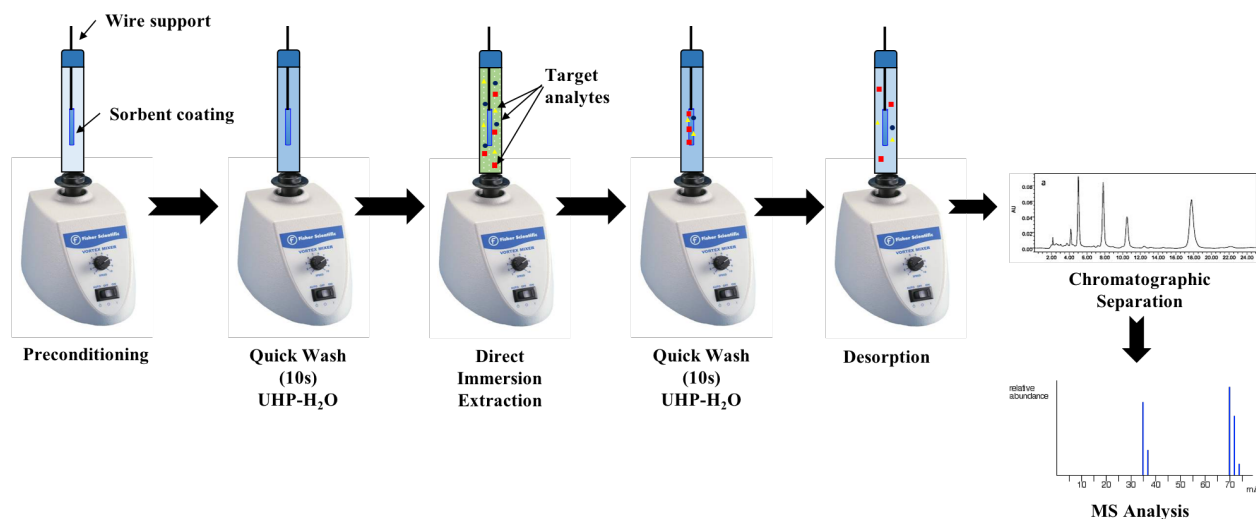
#### 1.4 Solid phase microextraction: fundamentals and direct coupling to MS

Solid phase microextraction (SPME) is an alternative sample preparation technique that was developed by Pawliszyn and Arthur at the University of Waterloo in the early 1990s.<sup>13,49</sup> SPME was conceived to provide the perfect balance between sampling and sample preparation. This balance facilitates the extraction, and enrichment of target analytes with minimal solvent usage.<sup>50,51</sup> The general SPME device consists of a solid support coated with a sorbent; typically, a polymeric extraction phase. Common extraction phases include C18, hydrophilic-lipophilic balanced (HLB), or C18 mixed with a strong cation exchanger (SCX) – which is commonly referred to as mixed mode (MM).<sup>52</sup> The chemical composition of the coating, as well as the



geometry of the sampler are dictated by the experimental setup and chemistry of the target compounds. These parameters have been extensively studied and discussed in detail in several reviews.<sup>25,51–53</sup>

The direct immersion (DI) SPME workflow, provided in Figure 1.2, begins with a preconditioning step to prime the coating for the extraction by wetting the bonded functional groups in the SPME coating.<sup>25,54</sup> The sampler is then exposed to the sample matrix, where partitioning of analytes between the matrix and the coating begins, initiating the extraction of analytes via the free concentration in the sample.<sup>55,56</sup>



**Figure 1.2.** Classical DI-SPME workflow using a polymeric coated fiber for analysis of biofluids.

Alternatively, depending on the volatility of the analytes, the composition of the sample matrix, and the selected coating chemistry, headspace (HS) extraction methodologies can also be used.<sup>13,56</sup> HS extractions are employed when the analyte easily partitions between the sample and the gaseous volume above the liquid or solid sample (*i.e.* headspace) contained within the sampling enclosure, and when the integrity of the SPME coating is irreversibly compromised if it is placed into the sample matrix.<sup>13,52</sup> Extraction of target compounds from biofluids is generally achieved

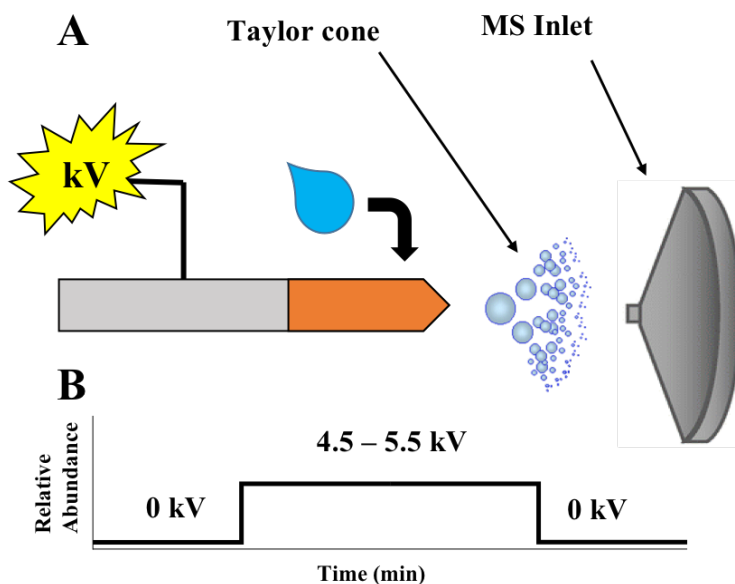
via DI of the coated sampler into the sample. Following the extraction, the SPME device is briefly washed with ultra-high purity (UHP) H<sub>2</sub>O before the analytes are desorbed from the coated sampler. In a typical SPME-Gas Chromatography (GC) – MS system, analytes can be thermally desorbed from the fiber directly into the GC injector, or desorbed into an organic solvent compatible with the GC system, followed by separation and eventual mass analysis.<sup>49,55</sup> In a standard SPME-LC-MS/MS system, extracted analytes are desorbed into an organic solvent compatible with the chromatographic method followed by separation and mass analysis.<sup>52,57,58</sup>

Inspired by other successful AMS technologies, SPME devices have been directly coupled to MS instruments under ambient conditions. Direct coupling of SPME to the MS is a practical approach to reduce analysis time and enhance limits of detection, while maintaining excellent reproducibility and instrumental lifespan. This alternative approach to AMS seeks to provide an effective compromise between sample preparation and ambient ionization. This compromise aims to shift the paradigm from truly AMS methods that employ no sample pretreatment to “enhanced” AMS methods, such that the fast and selective sample preparation/extraction capabilities of SPME can be utilized prior to MS analysis. Three general strategies for directly coupling SPME to MS for bioanalytical and clinical applications have been reported over the last decade, with examples of each given in parentheses:<sup>51,59</sup>

- A) Desorption of analytes in a chamber followed by ionization (*e.g.* OPP and MOI, DBDI, bio-SPME fibre nanoESI)
- B) Direct ionization/desorption of analytes from the SPME device (*e.g.* CBS)
- C) Simultaneous surface desorption and ionization of analytes (*e.g.* SPME-DESI, SPME-TM-DART)

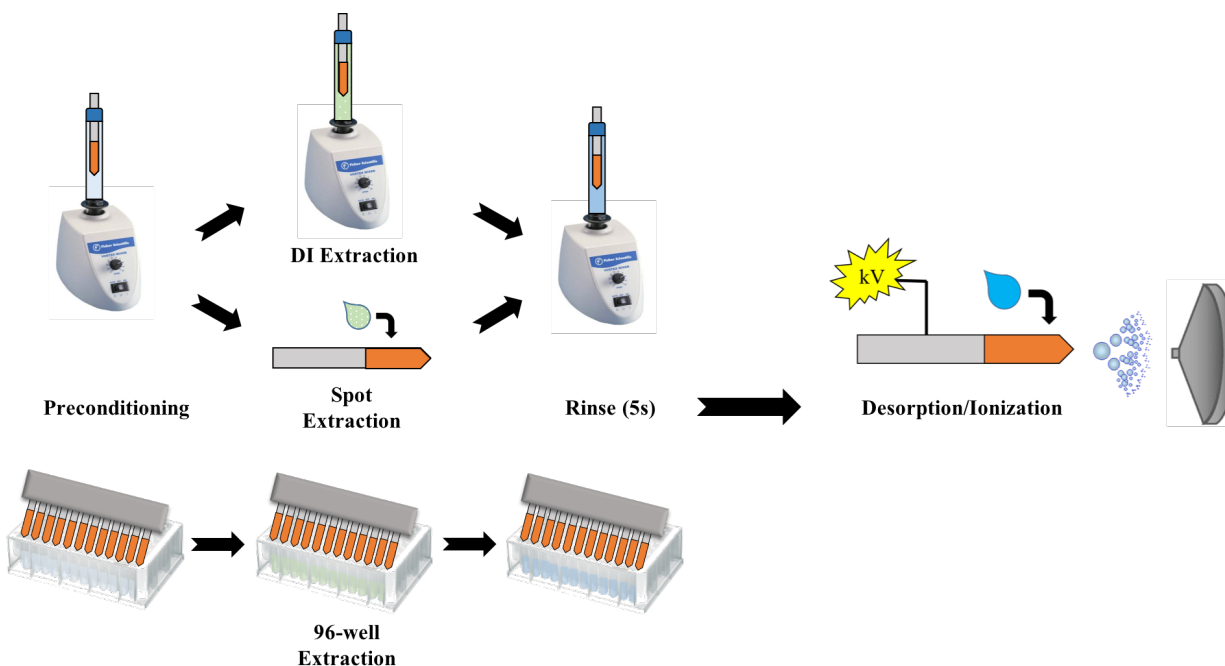
## 1.5 Coated blade spray - mass spectrometry

Coated blade spray (CBS) is one of the newest and most revolutionary SPME platforms that was conceived to be the perfect compromise between direct coupling to the MS under ambient conditions, and minimal sample preparation (see Figure 1.3).<sup>24,60</sup> Essentially, CBS consists of a stainless steel sheet, laser cut in the shape of a Roman Gladius sword that is coated with a polymeric extraction phase.<sup>24</sup> As with any ambient SPME-MS technology, analytes are extracted directly from the sample matrix onto the coated blade before being desorbed and ionized following the application of a high voltage and a small amount of organic solvent.<sup>24,60</sup> Ions are generated from the tip of the blade via the classical ESI mechanism as charged droplets or droplet clusters, exhibiting the characteristic Taylor cone.<sup>24,60</sup> The CBS technology was initially conceived to merge the rapid sample preparation abilities of SPME with a solid-substrate ESI source, thus improving on other solid-substrate ESI technologies such as PS, which offer no such selective extraction ability.



**Figure 1.3.** (A) CBS schematic. The sorbent coating is represented in orange and the blue tear drop represents the dual desorption/ionization solvent. (B) Standard “chronogram” to visualize ion signal as a function of time.

As with any DI-SPME approach, the analytical CBS workflow starts with a fast preconditioning step to “prime” the coating of the blade, as well as ensuring maximum wettability of the functional groups of the coating (see Figure 1.4).<sup>25,54</sup> The extraction follows, and depending on the matrix and the analyte concentration, can be as short as few seconds. The extraction can either occur via DI of the blade into the sample vial, in the high-throughput 96 well configuration, or by spotting a small volume of the sample matrix onto the coated area of the blade.<sup>51,60,61</sup>



**Figure 1.4.** CBS workflow with multiple extraction formats.

Depending on the level of sensitivity desired and the compounds of interest, some matrices may require longer extraction times unless additional steps are taken to improve selectivity and sensitivity, such as chemical derivatization or coatings with embedded molecular imprinted polymers or aptamers.<sup>47,62–65</sup> After the extraction, the blade is quickly rinsed to remove any matrix residues before being placed into a custom built holder/ionization interface. The blade holder ensures that the point of the blade is optimally positioned for ions to enter the MS inlet and achieve

maximum intensity.<sup>33,60</sup> A small volume of solvent (5 – 20  $\mu\text{L}$ ) is then pipetted onto the blade. The applied solvent facilitates the desorption and transfer of extracted analytes to the upper surface of the blade from the sorbent coating. After a 10 s “desorption” stage, the analytes are electrosprayed towards the MS inlet until all of the desorption solvent has been sprayed/evaporated.

CBS technology is a superior technology to other solid substrate-ESI methods because of the rigidity of the metal blade, enhanced practicality, and superior selectivity and sensitivity.<sup>60</sup> Using a sturdy metal support as opposed to paper or wood permits the precision etching of the tip to a fine point. The precise etching improves the formation and stability of the Taylor cone, ensuring a constant and reproducible spray of ions for analysis.<sup>24,66,67</sup> In comparison to PS, which uses a nonconductive, porous support, the solid metal blade acts as a conductor, ensuring a stable electric field gradient is maintained between the tip of the blade and the MS inlet throughout the entire spray event. Maintaining the stability of the electric field between the blade and MS inlet greatly improves reproducibility and efficiency of ion formation.<sup>24</sup>

The large surface area of the coating on the blade is another unique characteristic of CBS. By maximizing the surface area of the coating, while minimizing the thickness, the total extraction time can be decreased without sacrificing detection limits.<sup>13,68</sup> It was reported by Gomez-Rios *et al.* that an extraction time of 1 min for cocaine spiked into Phosphate Buffered Saline (PBS) resulted in detection limits as low as 2  $\text{pg/mL}$ <sup>24</sup>. At such short extraction times ( $t < 1$  min), the extraction takes place in the pre-equilibrium phase. During the pre-equilibrium phase, the analyte recovery is governed exclusively by analyte chemistry, convection conditions, extraction time and coating surface area.<sup>13,55</sup> If these parameters are kept constant, the only factors that influence the analyte recovery are the surface area of the coating and the intrinsic properties of the analyte of interest (*e.g.* diffusion coefficient).<sup>13,24,50,55</sup>

The most unique feature of the CBS platform is the sorbent coating on the blade, which facilitates the selective extraction and enrichment of small molecules from the sample. Solid substrate-ESI methods have no extraction capability, so macromolecules and salts deposited onto the sampler are directly ionized into the MS alongside target analytes. This leads to MEs which suppress target ion intensity, while also decreasing the lifespan of the MS.<sup>39</sup> Recent developments in coating technologies have improved the coating's affinity for compounds of interest.<sup>51,52,54,69</sup> Furthermore, new "biocompatible" sorbent coatings have been developed to facilitate *in vivo* sampling without inducing any toxic reactions in the system of study.<sup>25,51</sup>

Compared to detection limits from SPME coupled to LC MS/MS, CBS presents a significant sensitivity enhancement. Furthermore, with both sides of the blade coated, it is possible to perform one desorption/ionization event on one side of the blade, then flip the blade and perform a second elution/ionization on the other side. CBS provides an extremely practical and simple analytical platform to enrich molecules from a complex matrix prior to MS analysis with superior sensitivity and selectivity. Unlike other SPME-MS strategies, there is no additional equipment needed for analysis.<sup>60</sup> Specifically, with no capillaries or emitters necessary for CBS, the chance of clogging the emitter, which is commonly observed in nano-ESI experiments, is completely eliminated<sup>33,43</sup>.

## 1.6 Rationale for pursuing CBS

While CBS clearly has a number of promising features and applications, there is still work to be done to fully characterize the technology. CBS was conceived, and initially optimized, using a custom built blade holder system that interfaced the blade with a Thermo Scientific TSQ Vantage and TSQ Quantiva triple quadrupole instruments.<sup>24,60</sup> To expand the reach of the technology, it is

critical that further development and optimization of CBS is done to effectively interface the technology with a wide variety of MS instruments. This will undoubtedly increase the accessibility and overall usage of the technology, while further proving the robustness and applicability of CBS<sup>70</sup>.

CBS provides a tool for rapid analysis of a broad range of compounds including banned substances, pesticides, and therapeutics. The ability to tailor the polymeric sorbent coating to a specific class of chemicals, or alternatively, to a wide range of polar and non-polar compounds is one of the biggest advantages to the SPME technology. Conventional SPME devices coupled with LC-MS/MS systems (*e.g.* fiber, thin-film) or directly to the MS (*e.g.* DESI, DART, SPME-nano-ESI) have conclusively shown a wide range of applicability in the quantitative bioanalysis field.<sup>6,51,59,71</sup> It would appear that CBS also could be capable of achieving this level of analysis and achieve even lower levels of detection and quantitation. If a stable spray of ions from the blade can be maintained over the entire spray event, multi-residue analysis (*i.e.* >100 analyzed compounds) is feasible. In this type of application, the maximum number of compounds that could be analyzed would be solely dependent on the total stable spraying time, the number of scans per second on the MS (*i.e.* dwell time) and most importantly, the number of analytes extracted by the blade itself.

The speed of the entire process can enable CBS to be implemented for personalized medicine, TDM and POC treatment. To provide the highest quality of care to patients, it is of the utmost importance that the concentration of a drug or targeted metabolite in a biological sample can be rapidly and accurately quantified. Health care professionals can use CBS to effectively monitor drugs with a very narrow therapeutic range such as immunosuppressive drugs, track the spread of a chemotherapeutic as it traverses through the blood stream, monitor the level of a

biomarker or metabolite in a biofluid, or screen samples for different classes of pharmaceuticals

The thesis herein presented aims to address some of the shortcomings of the CBS-MS technology, and adapt and validate the novel methodology for quantitative analysis of several medically relevant classes of pharmaceuticals. In summary, this thesis is comprised of three distinct chapters: 1) characterizing and optimizing the CBS platform on the AB SCIEX API 4000 triple quadrupole MS and the Waters XEVO G2-S hybrid quadrupole – time of flight MS instruments, 2) applying the aforementioned optimizations from the CBS platform on the AB SCIEX API 4000 MS for the rapid, concurrent analysis of fentanyl, and five fentanyl analogs from urine and plasma, and 3) evaluating the clinical viability of CBS-MS as an alternative technique to conventional assays for the concomitant determination of four immunosuppressive drugs (ISD) in whole human blood.



## **Chapter 2. Optimization and characterization of mass spectrometric parameters for coated blade spray**

### 2.1 Introduction

To integrate the CBS technology with any mass spectrometer, the blade interface must be optimized based on the specifications of the instrument. Every MS instrument has its own design features and characteristics, with a large amount of variability between different MS vendors (*e.g.* Waters, Agilent, Shimadzu, Bruker, Thermo Scientific and AB SCIEX). With that in mind, MS manufacturers and end users require a CBS blade holder/ionization source that fits the specifications of that particular instrument and which have been rigorously optimized to maximize the analytical performance. To date, most experiments on CBS have been focused exclusively on the instrumentation of a single MS vendor. In order to demonstrate the versatility of this technology, further experimentation with other MS platforms is required. The difficulties that arise when coupling CBS with different MS vendors primarily stem from differences in the design of the front end of the instruments. Therefore, determining the optimum spatial position in which to position the blade in relation to the MS inlet is critical for ensuring the optimal ion transmission. Likewise, the presence and velocity of the laminar curtain/sweep gas flowing out of the inlet also plays a role in ensuring ion transmission, and as such, this parameter also needs to be evaluated before proceeding to future experiments.

Two instruments were interfaced with the CBS hardware platform for the first time: the AB SCIEX API-4000 Triple Quadrupole and Waters XEVO-G2-S QTOF. Due to major technical differences between the two instruments, the CBS source needed to be independently optimized to accommodate different design features. The API 4000 has several air flow currents originating from the vacuum exhaust to the right of the inlet, as well as curtain gas flowing laminarly out of

the MS inlet. During routine operation of the instrument, these gases cannot be switched off and must be accounted for during the blade position optimization process. In contrast, the XEVO G2-S MS inlet is placed orthogonal to the electrospray source, requiring an entirely different set of operating conditions to accommodate this difference. The optimal placement of the blade in relation to the inlet was also investigated on the Thermo Scientific TSQ Quantiva triple quadrupole MS, the instrument that has been the most thoroughly characterized and utilized for all of the CBS experiments to date.<sup>60</sup>

In order to identify the optimal blade position and subsequently evaluate the performance of the CBS platform on the AB SCIEX and Waters instruments, seven compounds and five deuterated internal standards were chosen for the initial validation. The selected compounds spanned a wide range of molecular weights, octanol partitioning coefficients (logP), and protein binding affinities, with the physiological properties of each compound summarized in Table 2.1. Using the optimized CBS parameters ascertained in the first portion of the work, comprehensive calibration curves spanning three orders of magnitude were constructed. These calibration curves, as well as external validation points were used to assess the accuracy, precision, linearity, and limits of quantitation/detection (LOQ/LOD) of the CBS technology on each instrument in two biological matrices (*i.e.* urine and plasma).

**Table 2.1.** Summary of compounds chosen for CBS characterization and optimization.

<b>Name</b>	<b>logP</b>	<b>Molecular weight (g/mol)</b>	<b>MRPL* (ng/mL)</b>	<b>Protein Binding, %</b>
<b>Buprenorphine</b>	4.63	467.64	5	96
<b>Clenbuterol</b>	2.94	277.19	0.2	97
<b>Cocaine</b>	1.97	303.35	100	40
<b>Fentanyl</b>	4.12	336.48	2	80-85
<b>Oxycodone</b>	1.07	315.36	50	45
<b>Salbutamol</b>	0.44	239.30	100	>10
<b>Sertraline**</b>	5.06	306.23	10	98

All physiochemical data was obtained from NCBI PubChem online database (<https://pubchem.ncbi.nlm.nih.gov/>, Accessed on March 31<sup>st</sup> 2017). \*World Anti-Doping Agency (WADA) minimum required performance limits (MRPL) values shown from 2012 report.<sup>72</sup> \*\*Sertraline is not considered a banned substance by WADA standards so there is no MRPL. The United Nations Guidelines for the Forensic analysis of drugs facilitating sexual assault and other criminal acts report (2011) was used as an alternative to provide a minimum concentration.<sup>73</sup>

## 2.2 Experimental

### 2.2.1 Chemicals, reagents and materials

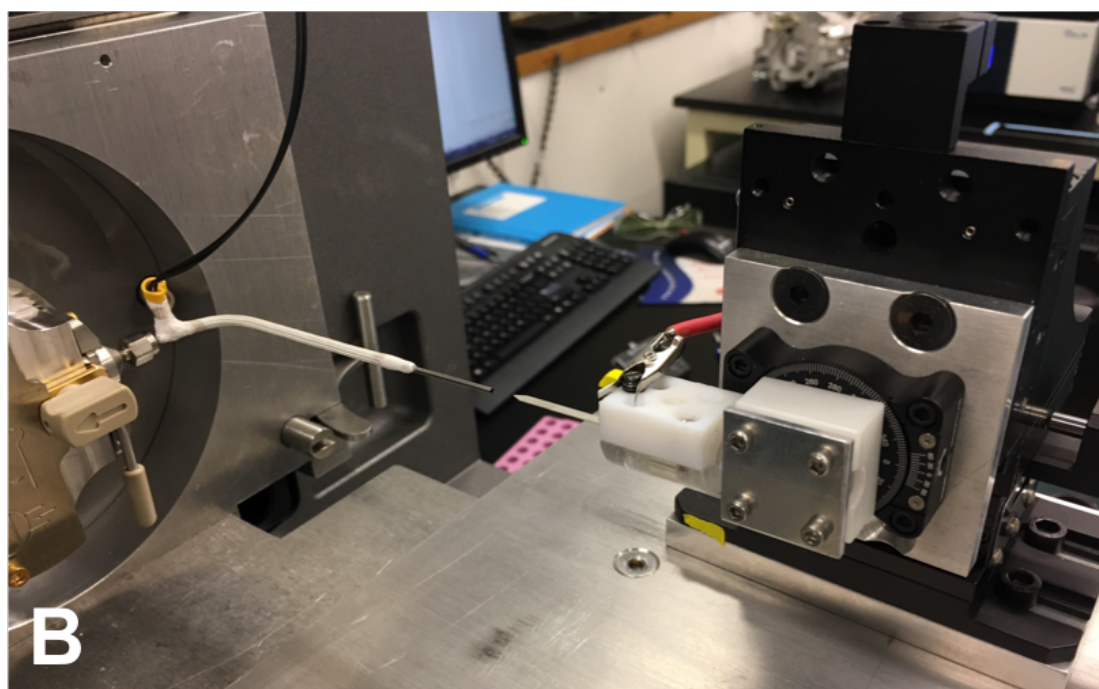
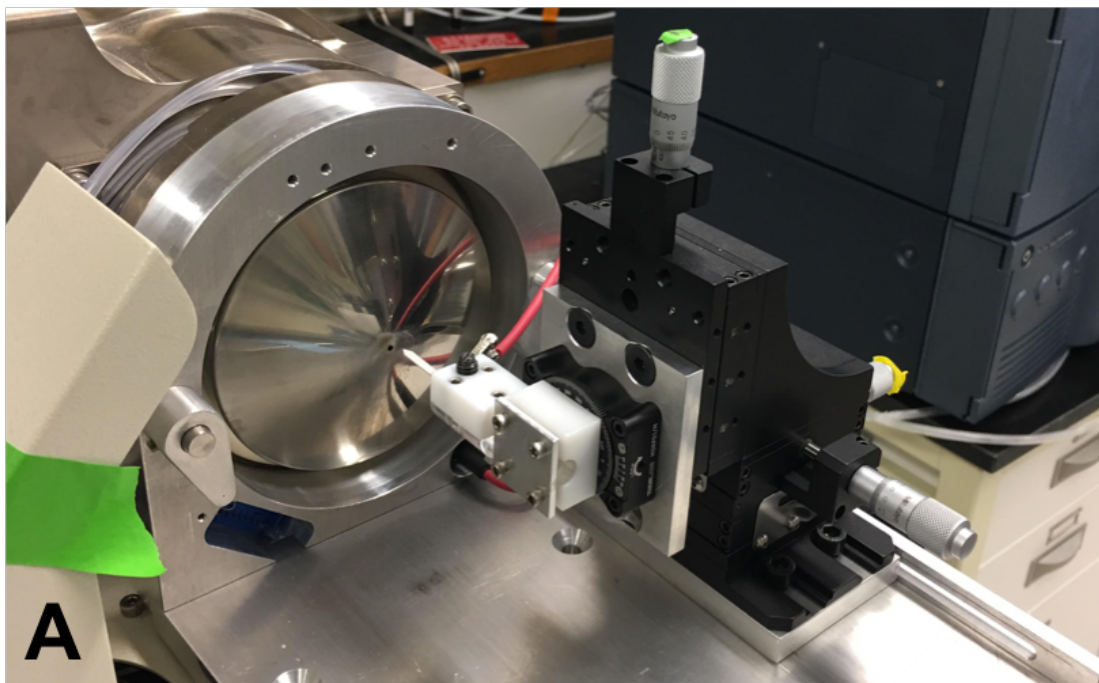
Formic acid (FA) and ammonium acetate (AA) were purchased from Sigma-Aldrich (Saint Louis, MO, USA), and LC-MS grade methanol (MeOH), acetonitrile (ACN), isopropanol (IPA) and water were purchased from Fisher Scientific (Bartlesville, OK, USA). The model compounds selected for characterization and optimization: buprenorphine, buprenorphine d<sub>4</sub>, clenbuterol, clenbuterol d<sub>9</sub>, cocaine, cocaine d<sub>3</sub>, fentanyl, fentanyl d<sub>5</sub>, oxycodone, salbutamol, sertraline, and sertraline d<sub>3</sub>, were all purchased from Cerilliant Corporation (Round Rock, TX, USA). For compounds that do not have the appropriate matched internal standard (IS), the IS that provided the best correction (*i.e.* the highest precision and accuracy) was used. Individual working stock standard solutions were prepared in MeOH at a concentration of 100 µg/mL and stored at -80°C. Human plasma (stabilized with K<sub>2</sub>-EDTA as an anti-coagulant) that had been pooled from independent healthy donors was purchased directly from BioIVT (Westbury, New York, USA), and used without any further modifications. Urine was collected and pooled from healthy donors in the Pawliszyn research group. There were no additional modifications to the urine. All biological sample collection was done with the approval of the University of Waterloo Office of Research Ethics Board. Matrix-matched calibration curves in urine and plasma were prepared by spiking the matrix and storing overnight at 4 °C. This was done to facilitate sufficient binding of the analytes with the matrix and simulate physiological binding conditions. Stainless steel blades were purchased from Shimifrez Incorporated (Concord, ON, CAN) and used for all experiments herein presented. The blades were coated with a slurry of HLB particles and polyacrylonitrile (PAN). The procedure to coat the blades was developed in the Pawliszyn research group and published recently in Scientific Reports.<sup>74</sup> The HLB particles used for the optimization and

validation of CBS platform were kindly provided by Waters Corporation. The HLB particles were 5  $\mu\text{m}$  diameter, the blade coating thickness was 10  $\mu\text{m}$ , and the blade coating length was 15 mm.

## 2.2.2 Instrumental configuration of AB SCIEX API 4000, Waters XEVO G2-S QTOF and mass spectrometric parameters

All of the experiments described in this chapter were carried out using an AB SCIEX API 4000 triple quadrupole (Concord, ON, CA), Waters XEVO G2-S QTOF (Wilmslow, UK), or Thermo Scientific TSQ Quantiva triple quadrupole MS instruments (San Jose, CA, USA). Data acquired from the API 4000 was processed using Analyst 1.6.2 (SCIEX, Concord, ON, CA). Data acquired on the XEVO G2-S QTOF was processed using Masslynx 4.1 (Waters Corporation, Wilmslow, UK). Data processing on the TSQ Quantiva was done using Thermo Scientific Trace Finder v4.1 (San Jose, CA, USA).

The blades were accurately positioned in front of the entrance of the mass spectrometer using different ionization sources built in-house by University of Waterloo Science Technical Services. The general system has been described in detail in a previous manuscript.<sup>24</sup> Modifications to the aforementioned ion source were necessary to properly accommodate the different source configurations of the MS instruments, primarily in the addition of a separate metal plate and locking pin for the XEVO G2-S, and a custom stainless-steel ring and locking pins for the API 4000. These modifications are shown in Figure 2.1 and were also built in-house University of Waterloo Technical Services. The selected reaction monitoring (SRM) transitions and optimized tuning parameters are listed in Table 2.2 and 2.3. Analysis was performed in positive ionization mode, and each analyte and deuterated IS were detected as singly charged protonated ( $\text{H}^+$ ) adducts. MS parameters used for spatial analysis of the Thermo Scientific TSQ Quantiva were the same as those used in a previous manuscript by Tascon, *et al.* in 2017.<sup>61</sup>



**Figure 2.1.** CBS ionization source interfaced with (A) AB SCIEX API 4000, and (B) Waters XEVO G2-S QTOF MS inlets.

**Table 2.2.** SRM transitions and optimized tuning parameters for the AB SCIEX API 4000.

<b>Compound</b>	<b>Precursor (<i>m/z</i>)</b>	<b>Product (<i>m/z</i>)</b>	<b>DP (V)</b>	<b>EP (V)</b>	<b>CE (V)</b>	<b>CXP (V)</b>
Buprenorphine	468.3	396.2	153.3	8.11	51.8	13.1
Buprenorphine d <sub>4</sub>	472.3	400.1	174	7.1	54.9	15.3
Clenbuterol	277.1	202.9	84.6	9.2	23	14.1
Clenbuterol d <sub>9</sub> <sup>B</sup>	286.1	203.9	59.2	8	23.9	16.5
Cocaine	304.4	182.2	96.2	9.4	28.5	12.4
Cocaine d <sub>3</sub> <sup>A</sup>	307.1	185.1	58.2	8.7	27.9	12.5
Fentanyl	337.2	188.1	95.2	9.9	31.5	13.6
Fentanyl d <sub>5</sub>	342	188.1	117.3	9.2	33.6	20
Oxycodone <sup>A,B</sup>	316.2	241.1	84.6	13.2	39.6	17.7
Salbutamol <sup>A,B</sup>	240.1	148.1	45.6	6.6	26	9.4
Sertaline	306.1	275.2	66.4	7.3	17.9	19
Sertaline d <sub>3</sub>	309.0	159.0	67.8	4.7	35.3	11.8

DP: Declustering potential; EP: Entrance potential; CE: Collision energy; CXP: Collision exit potential. <sup>A</sup> corresponds to the internal standard used for quantitation of selected compounds from urine. <sup>B</sup> corresponds to the internal standard used for quantitation of plasma.

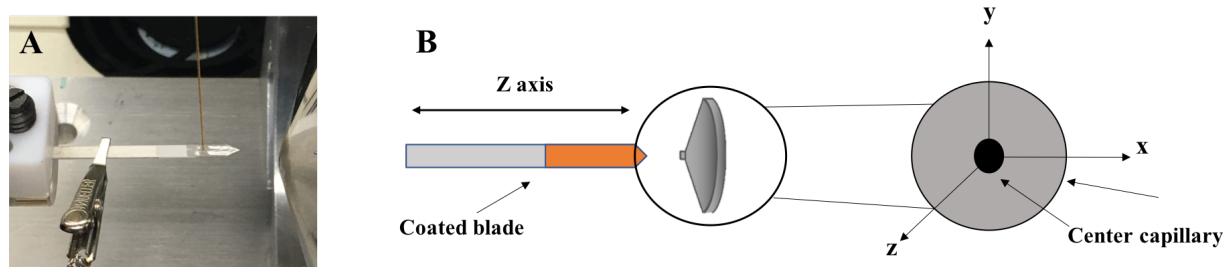
**Table 2.3.** SRM transitions and optimized tuning parameters for the Waters XEVO G2-S QTOF.

Compound	Precursor ( <i>m/z</i> )	Product ( <i>m/z</i> )	CE (V)
Buprenorphine	468.3146	396.2167	39.5
Buprenorphine d <sub>4</sub>	472.3392	400.2427	39.5
Clenbuterol	277.038	203.0138	15.5
Clenbuterol d <sub>9</sub> <sup>B</sup>	286.1467	204.0209	16.4
Cocaine	304.1563	182.1185	17.5
Cocaine d <sub>3</sub> <sup>A</sup>	307.179	185.1379	19.1
Fentanyl	337.2321	188.1456	21.5
Fentanyl d <sub>5</sub>	342.2631	188.1456	23
Oxycodone <sup>A,B</sup>	316.155	241.1179	26.5
Salbutamol	240.1607	148.0762	17.5
Sertaline	306.1614	158.9784	28
Sertaline D <sub>3</sub>	309.1006	158.9784	25.7

CE: Collision energy. <sup>A</sup> corresponds to the internal standard used for quantitation of selected compounds from urine. <sup>B</sup> corresponds to the internal standard used for quantitation of plasma.

### 2.2.3 Blade position optimization parameters

To identify the optimal blade placement in relation to the MS inlet, a constant flow of 95:5 MeOH:H<sub>2</sub>O (v/v) + 0.1% FA spiked with the selected analytes was infused onto the blade using a Hamilton auto-sampler syringe (Sigma Aldrich, Saint Louis, MO, USA) directly connected to a capillary (Figure 2.2).



**Figure 2.2.** (A) Delivery of solvent containing selected compounds onto blade. (B) Schematic depicting the *x,y,z* positioning of blade in front of the MS inlet.



Using the micro-positioning assembly on the ion source, the blade was moved in 1 mm increments in a grid pattern, while a 4.5 kV (API 4000), 5 kV (XEVO G2-S) or 5.5 kV (TSQ Quantiva) electric potential was applied to the back of the blade. The application of the high voltage induced an ESI event, and the ion signal at that particular coordinate was acquired for 30 s before integrating the area under the curve. The concentration of the selected analyte mixture used to identify the optimum position of the blade on the API 4000 and TSQ Quantiva was 10 ng/mL, and the flow delivered by the syringe pump was 10  $\mu$ L/min. The concentration of the analyte mixture used for identifying the optimum position on the XEVO G2-S was 100 ng/mL, and the flow was 7.5  $\mu$ L/min.

#### 2.2.4 Sample preparation and analytical workflow

The CBS devices used for all experiments were cleaned for 30 min with a MeOH:ACN:IPA mixture (40:40:20, v/v) before conditioning for 30 min in a MeOH:H<sub>2</sub>O solution (50:50, v/v). The CBS devices do not have to be wet prior to an extraction event. Therefore, it is practical to clean and condition a large number of devices in a batch prior to performing experiments.<sup>75</sup> The CBS workflow consisted of three stages: 1) high-throughput extraction of target analytes from 300  $\mu$ L of biofluid,<sup>61</sup> with 15 min extraction time, 1500 rpm orbital agitation at room temperature, 2) a fast H<sub>2</sub>O rinsing step (5 s) to remove loosely attached matrix constituents, and 3) MS analyses, using 12  $\mu$ L of MeOH:H<sub>2</sub>O (95:5, v/v) desorption/ionization solution spiked with 0.1% FA (FA) and 12 mM AA was pipetted onto the coating of the blade which had been placed in front of the MS inlet, facilitating desorption of analytes into the liquid phase on the surface of the coating. After 10 s of static desorption, high voltage was applied to the blade for 20 s, inducing an electrospray event that directly introduced the analytes extracted by the coated blade into the MS.

### 2.2.5 Characterization of CBS on AB SCIEX and Waters instruments

The CBS methodology was characterized in terms of accuracy, precision, linearity and sensitivity (*i.e.* limit of quantitation/detection (LOQ/LOD)). Calibration curves were prepared using pooled urine and plasma, with 12 calibration points from 0.05 – 100 ng/mL, plus a matrix blank. Three validation points (3, 30 and 90 ng/mL) were analyzed to evaluate the precision and accuracy. Four independent replicates were used to determine the imprecision of each calibration/validation point. The concentration of the IS in all calibration and validation points was 10 ng/mL. To account for experimental and instrumental variation, the ion-signal ratio of the analyte area under the curve was normalized to the area under the curve of the corresponding deuterated IS (A/Is). The LOQ was determined using the following criteria: the lowest calibration point that had signal/noise (S/N) ratio > 5, 80 – 120 % back-calculated accuracy using linear regression line of best fit, and relative standard deviation < 20 %.

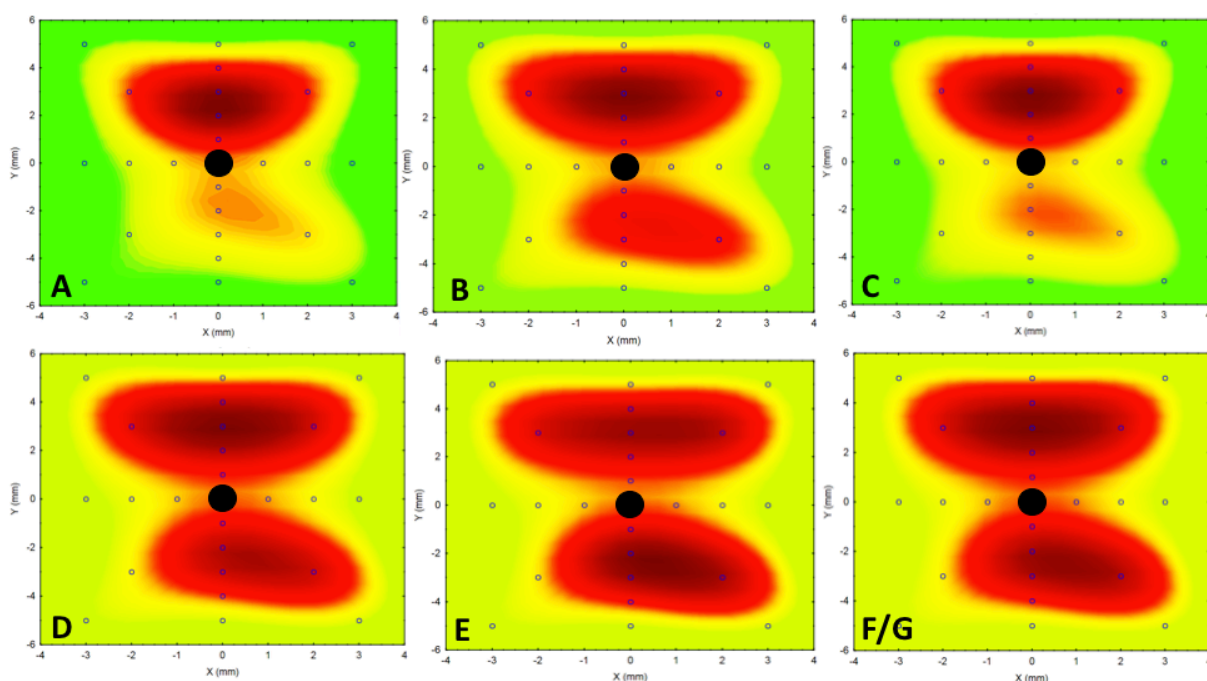
### 2.2.6 Statistical analysis

Microsoft Excel v16.14 and Tibco Statistica 13.3 basic academic bundle were used for statistical analysis.

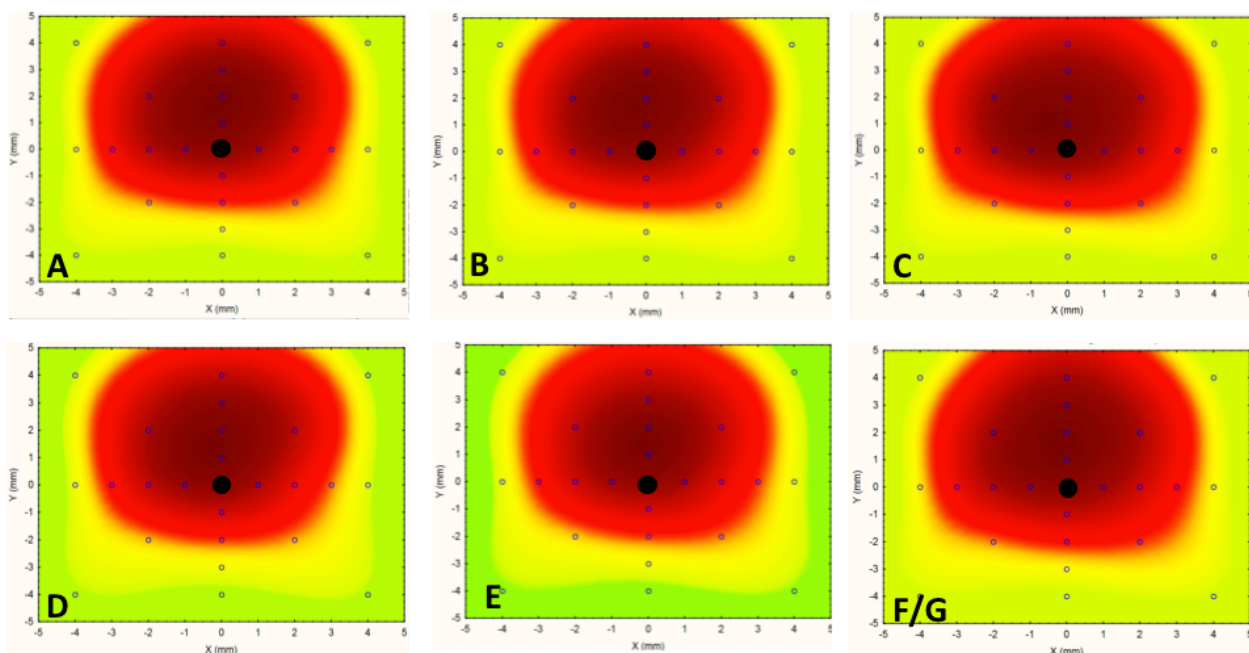
## 2.3 Results and discussion

### 2.3.1 Optimization of blade position in relation to MS inlet

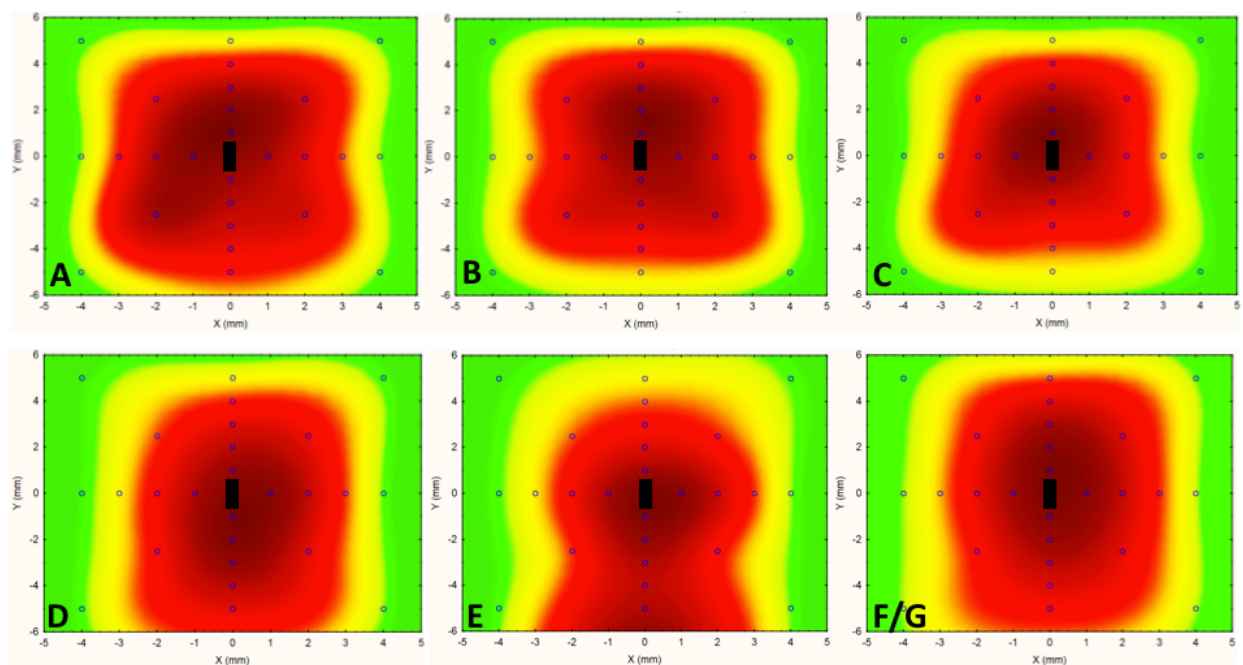
In the majority of substrate spray methodologies, there has been minimal importance placed on elucidating the optimal position of the device in front of the MS.<sup>60,76,77</sup> Furthermore, it has previously been reported that the position of the substrate spray device does not have a significant effect on the analytical performance for analysis of strongly ionized compounds such as cocaine, and so the blade is simply placed several mm back from the center of the inlet.<sup>46</sup> However, this phenomenon has never been thoroughly studied for mixtures of compounds with varying physiochemical properties. Therefore, the first stage of the CBS characterization process was to identify the position that yielded the best analytical results for the selected compounds. As shown below in Figures 2.3, 2.4 and 2.5, each instrument that was investigated had a characteristic profile that is generally conserved for all of the studied analytes.



**Figure 2.3.** Heat maps for selected compounds on AB SCIEX API 4000. The black circle represents the MS inlet (~1.55 mm diameter). Blue points represent acquisition points. (A) salbutamol. (B) clenbuterol. (C) oxycodone. (D) buprenorphine. (E) sertraline. (F) fentanyl & cocaine.



**Figure 2.4.** Heat maps for selected compounds on Waters XEVO G2-S QTOF. The black circle represents the size of the MS inlet ( $\sim 0.87$  mm diameter). Blue points represent acquisition points. (A) salbutamol. (B) clenbuterol. (C) oxycodone. (D) buprenorphine. (E) sertraline. (F) fentanyl & cocaine.



**Figure 2.5.** Heat maps for selected compounds on Thermo Scientific TSQ Quantiva. The black rectangle represents the size of the MS inlet ( $\sim 0.40$  mm x 1.6 mm). Blue points represent acquisition points. (A) salbutamol. (B) clenbuterol. (C) oxycodone. (D) buprenorphine. (E) sertraline. (F) fentanyl & cocaine.

Interestingly, the API 4000 had the strongest dependence on the blade position to achieve acceptable sensitivity and spray stability. This observation suggests that the laminar curtain gas flowing out of the inlet has a significant effect on the overall CBS performance. Out of the seven compounds that were investigated, salbutamol and oxycodone were considerably affected by the position of the CBS in regards to the MS inlet on the AB SCIEX instrument, only producing sufficient ion intensity and electrospray stability when the blade was placed several millimeters above the inlet. The other 5 compounds showed minimal preference for the blade positioned above or below the MS inlet, producing similar intensities in either position. Salbutamol and oxycodone were the most polar analytes of interest with logP values of approximately 1 or less. This position dependency may be the result of differing proton affinities between compounds; however, this hypothesis needs to be investigated further.<sup>78</sup> Ultimately, the best results were obtained when the blade is positioned ~3 mm above the inlet; this position is high enough that the curtain gas does not dry the desorption solvent or affect the electrospray stability, but low enough for a sufficient number of ions to reach the entrance.

As expected, no significant differences in terms of blade placement were observed from the analytes of interest on the XEVO G2-S and TSQ Quantiva. Both instruments displayed the highest ion intensity and spray stability when the blade was placed directly in line with the inlet, with a considerable amount of flexibility in both the *x* and *y* dimension. Since neither instrument has a laminar curtain gas that is activated during CBS experiments, this further validates the hypothesis that the presence or absence of a curtain gas is the main factor that affects the 3D placement of the blade.

The optimized blade placement and CBS conditions for the selected analytes on the AB SCIEX API 4000 were determined to be 6.7 mm back from the inlet, 3 mm above, and 4.5 kV

applied voltage. The Waters XEVO G2-S QTOF had slightly different parameters, with the optimal distance of the tip of the blade to the inlet being 6 mm, with the no offset in the x or y dimension. The optimal voltage was established to be 5 kV. Similar parameters were identified on the Thermo Scientific TSQ Quantiva, with the only difference being that the tip of the blade should be placed 8 mm from the MS inlet. The applied voltage was previously optimized in several publications, and was kept constant at 5.5 kV.<sup>60,61,79</sup>

### 2.3.2 Characterization of the CBS platform

Urine and plasma were chosen as representative matrices to assess the suitability of the API 4000 and XEVO G2-S as effective instruments to directly couple to CBS. Urine is one of the most widely available biological matrices, and the most commonly tested matrix for testing of prohibited substances by monitoring agencies such as the World Anti-Doping Agency (WADA).<sup>14</sup> The figures of merit obtained for extractions from urine using the API 4000 are shown in Table 2.4, and the results are very promising. All of the selected compounds had LOQs lower than the minimum required performance level (MRPL) set by WADA, with excellent linearity ( $R^2 > 0.9989$ ) observed as well. Accuracy values for the three validation points of all seven compounds were between 91.0–112.9%. Excellent precision was achieved, with RSD's between 1.2–14.4%. It should also be noted that using the high throughput, 96 well-plate format, total extraction and analysis time was less than 60 s per sample, demonstrating the speed, accuracy and sensitivity of the technique.<sup>61</sup>

Plasma is often used as a complement to urine for banned substance analysis to provide an alternative biological snapshot of the subject. Furthermore, plasma is a well-studied matrix that is used for the majority of pharmacokinetic (PK) and pharmacodynamic (PD) studies.<sup>80,81</sup> The

difficulty with using plasma as a biological matrix for SPME is the preferential binding of target analytes with proteins present in the matrix.<sup>56</sup> This reduces the free concentration of analytes, and thus, there is intrinsically less analyte that can be extracted by the SPME device.<sup>56</sup> Therefore, extraction conditions such as time of extraction, temperature, and agitation must be carefully optimized to ensure that maximum sensitivity is achieved when working with matrices with high protein content. As seen in Table 2.5, excellent results were obtained for analysis of the selected compounds from plasma using CBS on the API 4000. All of the compounds, with the exception of clenbuterol, had LOQs that were lower than the MRPL, with linearity ( $R^2$ ) greater than 0.994 for the selected compounds. Accuracy of the validation points was between 93.0 – 117.0 %, and precision between 0.9 – 24.3 %. In both biological matrices that were evaluated, the figures of merit for CBS were comparable to those obtained on the TSQ Quantiva, a much newer and more advanced instrument.<sup>61</sup>

This is the first instance of a SPME device being directly coupled to a hybrid QTOF MS. While these instruments cannot match the sensitivity provided by conventional triple quadrupoles, they offer a major increase in selectivity due to their high mass resolution, which can provide higher S/N ratios in particular analytical challenges.<sup>82,83</sup> In certain applications, high-resolution analysis can be incredibly useful in distinguishing between two analytes that have the same nominal mass, but different accurate mass. However, historically, the main issue with QTOF instruments was their relatively narrow dynamic range, which limited their applicability for quantitative applications.<sup>83</sup> Therefore, the main objective for coupling CBS with the Waters XEVO G2-S was to determine if a linear response could be obtained in complex biological matrices over several orders of magnitude. This would permit accurate quantitation of the selected compounds, establishing that CBS can be employed for preliminary, high resolution screening methodologies,

with confirmation of tentative positive samples with more sensitive assays such as LC-MS/MS. Furthermore, this study aims to demonstrate the feasibility of using the CBS device for untargeted analyses, where stability and reproducibility of the ion signal is critical for elucidating statistical differences between different sample groups.

The same analytical workflow used for the API 4000 was employed for the characterization of the XEVO G2-S, with the figures of merit listed in Tables 2.6 and 2.7. For the analysis of urine, all seven compounds of interest were accurately quantitated with LOQs ranging from 0.25 to 10 ng/mL. Linearity ( $R^2$ ) of the seven compounds was greater than 0.98. Three compounds achieved LOQs of < 1 ng/mL, displaying excellent accuracy (92.7 – 112.5 %) and precision (1.8 – 12.0 %) for the three validation points. The lowest concentration validation point was excluded for the compounds that had LOQs higher than 3 ng/mL; however, accuracy (83.4 – 110.9 %) and precision (2.6 – 19.4 %) were still acceptable for the two higher concentration validation points. Surprisingly, salbutamol could not be corrected using any of the deuterated internal standard due to high imprecision and inaccuracy. However, the uncorrected data was satisfactory, with RSD values less than 20 % for all calibration points and validation points.

The quantitation of the selected compounds from plasma using the XEVO G2-S also generated superb results, with five of the seven compounds reaching LOQs of at least 0.5 ng/mL, and linearity ( $R^2$ ) greater than 0.986. Accuracy and precision for compounds with < 1 ng/mL LOQs demonstrated adequate accuracy (80.3 – 117.4 %) and precision (1.4 – 16.6 %) for the three validation points that were tested. Buprenorphine and sertraline displayed poorer figures of merit, and the lowest validation point was excluded. Despite this, the accuracy (90.2 – 93.0 %) and precision (3.8 – 9.6 %) for the remaining two validation points were excellent. As reported above,



none of the deuterated IS's were capable of precisely correcting salbutamol, and therefore, no IS was used for quantitation.

As expected, the figures of merit obtained on the XEVO G2-S confirmed that the QTOF MS was less sensitive than the API 4000 triple quadrupole. However, the matrix played a significant role in the LOQs, with lower limits achieved in plasma compared to urine. Interestingly, if the traditional 10 times the S/N criterion is used to calculate the LOQs on the XEVO G2-S, it is possible to reach concentrations one order of magnitude lower for the selected analytes. This observation speaks to the high mass accuracy of the instrument – the observed noise in the matrix blanks is so low because the exact mass is used for acquisition in each SRM channel, facilitating easy detection at low concentrations. However, the loss of linearity, reflected by the decrease in accuracy and precision at the lower concentration calibration points, makes quantitation at ultra-trace levels unfeasible with the current parameters. If lower LOQs are desired, throughput could be sacrificed by increasing the extraction time and/or the sample volume, in an effort to increase the amount of analyte extracted by the device.

**Table 2.4.** Figures of merit for characterization of the CBS platform interfaced on the AB SCIEX API 4000 MS – Quantitation of selected compounds from pooled human urine.

Compound	slope	intercept	R <sup>2</sup>	LOQ (ng/mL)	Accuracy (n = 4), %			Precision (n = 4), %		
					3 ng/mL	30 ng/mL	90 ng/mL	3 ng/mL	30 ng/mL	90 ng/mL
<b>Buprenorphine</b>	0.13	0.009	0.999	0.25	91.0	93.7	102.1	8.0	6.7	3.9
<b>Clenbuterol</b>	0.19	0.009	0.999	0.1	96.7	97.6	97.4	4.4	4.1	2.0
<b>Cocaine</b>	0.09	0.000	1.000	0.1	92.8	97.2	100.3	5.7	3.8	2.5
<b>Fentanyl</b>	0.18	0.002	1.000	0.05	101.9	97.0	103.1	4.7	3.6	2.9
<b>Oxycodone</b>	0.01	0.001	0.999	0.25	112.9	98.0	95.7	11.1	12.1	11.5
<b>Salbutamol</b>	0.03	0.004	0.999	0.25	106.0	97.6	105.1	10.8	13.8	14.4
<b>Sertraline</b>	0.13	0.002	1.000	0.05	105.0	102.1	95.5	4.3	1.2	3.9

**Table 2.5.** Figures of merit for characterization of the CBS platform interfaced on the AB SCIEX API 4000 MS – Quantitation of selected compounds from pooled human plasma.

Compound	slope	intercept	R <sup>2</sup>	LOQ (ng/mL)	Accuracy (n = 4), %			Precision (n = 4), %		
					3 ng/mL	30 ng/mL	90 ng/mL	3 ng/mL	30 ng/mL	90 ng/mL
<b>Buprenorphine</b>	0.11	0.010	0.999	0.25	106.7	105.2	99.3	3.7	3.1	2.6
<b>Clenbuterol</b>	0.17	0.002	0.999	0.05	113.2	100.1	93.6	2.7	3.3	2.3
<b>Cocaine</b>	0.09	0.010	0.999	0.25	117.0	101.2	94.3	3.2	3.8	1.2
<b>Fentanyl</b>	0.18	0.007	0.999	0.25	111.1	102.4	95.5	4.2	0.9	1.2
<b>Oxycodone</b>	0.01	0.000	0.997	0.25	104.9	112.1	99.0	6.5	3.8	11.2
<b>Salbutamol</b>	0.07	-0.001	0.994	0.25	110.1	102.2	95.3	16.4	7.6	24.3
<b>Sertraline</b>	0.14	0.000	0.999	0.1	95.3	101.3	97.4	2.0	2.4	2.5

**Table 2.6.** Figures of merit for characterization of the CBS platform interfaced on the Waters XEVO G2-S QTOF MS – Quantitation of selected compounds from pooled human urine.

Compound	slope	intercept	R <sup>2</sup>	LOQ (ng/mL)	Accuracy (n = 4), %			Precision (n = 4), %		
					3 ng/mL	30 ng/mL	90 ng/mL	3 ng/mL	30 ng/mL	90 ng/mL
<b>Buprenorphine</b>	0.14	-0.54	0.994	5.00	-	85.4	83.4	-	12.6	15.1
<b>Clenbuterol</b>	0.10	0.05	0.996	0.25	98.0	107.4	97.8	4.8	4.9	12.0
<b>Cocaine</b>	0.11	-0.10	0.998	0.50	93.3	92.7	107.0	5.7	4.8	2.7
<b>Fentanyl</b>	0.09	0.11	0.994	0.25	98.2	112.5	98.1	7.1	4.4	1.8
<b>Oxycodone</b>	0.01	0.05	0.995	10.00	-	110.9	100.8	-	19.4	6.0
<b>Salbutamol*</b>	4.91	18.45	0.988	10.00	-	95.3	90.6	-	2.6	4.6
<b>Sertraline</b>	0.05	1.67	0.980	10.00	-	95.0	107.6	-	5.3	5.0

\* No internal standard correction was applied for quantitation.

**Table 2.7.** Figures of merit for characterization of the CBS platform interfaced on the Waters XEVO G2-S QTOF MS – Quantitation of selected compounds from pooled human plasma.

Compound	slope	intercept	R <sup>2</sup>	LOQ (ng/mL)	Accuracy (n = 4), %, ng/mL			Precision (n = 4), %, ng/mL		
					3 ng/mL	30 ng/mL	90 ng/mL	3 ng/mL	30 ng/mL	90 ng/mL
<b>Buprenorphine</b>	0.11	0.01	0.998	5.0	-	93.0	90.2	-	3.8	6.5
<b>Clenbuterol</b>	0.12	-0.11	0.998	0.5	86.6	98.6	100.8	10.7	5.5	5.2
<b>Cocaine</b>	0.15	-0.14	0.998	0.5	80.3	96.9	107.8	5.7	1.4	1.7
<b>Fentanyl</b>	0.09	0.10	0.991	0.5	94.7	117.4	97.6	4.5	2.2	4.1
<b>Oxycodone</b>	0.02	0.00	0.993	0.5	100.5	92.4	87.8	10.9	6.9	4.1
<b>Salbutamol*</b>	17.19	45.76	0.995	0.1	106.9	114.0	93.8	16.6	16.1	10.8
<b>Sertraline</b>	0.04	2.10	0.986	25.0	-	90.8	92.1	-	9.6	7.7

\* No internal standard correction was applied for quantitation.

## 2.4 Conclusions

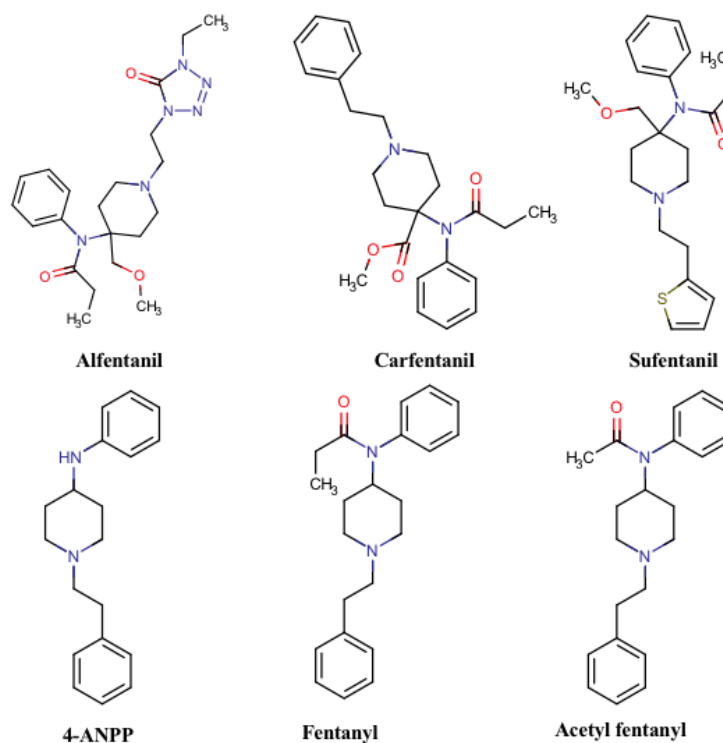
In summary, CBS is a novel SPME device that is directly coupled to the MS, permitting rapid, concomitant analysis of a wide range of compounds under ambient conditions. The main objective of this chapter was to interface the CBS technology on two instruments: the AB SCIEX API 4000 and Waters XEVO G2-S QTOF. To achieve this objective, the spatial position of the blade was identified on both instruments and compared with the instrument that has been used for the majority of CBS experiments (Thermo Scientific TSQ Quantiva), along with determination of several other CBS parameters necessary for routine operation. Following the preliminary optimization, the analytical performance of the SPME-MS technology was characterized on both instruments.

The CBS positioning experiments identified the optimal position of the blade, with each instrument exhibiting a distinct spatial profile when the blade was moved in relation to the MS inlet. An important finding to emerge from this study was that the AB SCIEX instrument was the most sensitive to the position of the blade, due to the presence of a curtain gas flowing laminarly out of the MS inlet. After completing the initial optimization, the ideal parameters were implemented to assess the analytical capabilities of CBS on each instrument. Excellent figures of merit were obtained on the AB SCIEX API 4000 MS, matching the results obtained using a more advanced instrument (Thermo Scientific TSQ Quantiva). The figures of merit for the Waters XEVO G2-S QTOF was also quite respectable, with LOQs for most of the analytes surpassing the 1 ng/mL level. Ultimately, these findings establish CBS as a versatile technology that is capable of being interfaced with a variety of different instruments, increasing the potential for future projects and applications.

## Chapter 3. Quantitative analysis of fentanyl, and five related analogs using coated blade spray – mass spectrometry

### 3.1 Introduction

Fentanyl is a potent opioid analgesic that primarily interacts with opioid  $\mu$ -receptors that are distributed throughout the central nervous system (CNS).<sup>84–86</sup> First released to the medical community in the 1960 by Dr. Janssen of Janssen pharmaceuticals in Belgium, it was marketed for chronic pain management and palliative care.<sup>85,86</sup> In recent years, more potent fentanyl analogs have been synthesized for medical and veterinary applications, with structures of some of these analogs shown below in Figure 3.1. The most powerful fentanyl analogs, such as carfentanil, have been deemed to have no medical use in humans, and have been heavily regulated and restricted as Schedule I and II compounds by the United States Drug Enforcement Agency (DEA).<sup>86</sup>



**Figure 3.1.** Structures of selected fentanyl analogs. Structures obtained from NCBI PubChem online database (<https://pubchem.ncbi.nlm.nih.gov/>, Accessed on June 12<sup>th</sup>, 2018)

Due to their potency, relative ease of access, and low cost, fentanyl and its related analogs can be easily abused, resulting in overdoses and potential fatalities. There have also been reports of recreational drugs that have been laced with these compounds, leading to accidental consumption, and adding to the growing fentanyl epidemic worldwide.<sup>86</sup> Since the late 1990's, there has been a massive spike in fatal overdoses attributed to these potent opioids, with over 16,000 deaths reported in 2013.<sup>85</sup> To mitigate this growing epidemic, it is critical that analytical methods for POC testing and harm reduction strategies are capable of detecting these compounds quickly, reliably, and sensitively, in a variety of biological matrices (*e.g.* urine, plasma or whole blood).

Before the emergence of MS/MS as the gold standard for analytical quantitation, the majority of analytical methods for analysis of fentanyl and its related analogs employed different immunoassays, followed by confirmation with GC-MS. The biggest limitation to this approach is the poor sensitivity of the immunoassays, and cross reactivity of the antibodies and between drugs and their metabolites.<sup>87</sup> More recently, LC-MS/MS methodologies have prevailed due to their increased sensitivity and selectivity for the target analytes.<sup>87</sup> Furthermore, these conventional methodologies all require sample preparation prior to analysis, with SPE being the most common approach.<sup>87</sup>

Unfortunately, the necessary sample preparation and chromatographic separation steps are time consuming and laborious, and they can sometimes require expensive reagents or specialized hardware, making conventional techniques impractical for rapid diagnostics and POC testing. Direct to MS methodologies are a superior alternative, as they improve patient care and healthcare outcomes by providing critical information faster, and more easily to frontline healthcare professionals.<sup>15,16</sup> CBS should be an ideal analytical technique for the determination of fentanyl

and fentanyl analogs from various biofluids, primarily due to its intrinsic combination of the sampling and sample preparation stages.<sup>60</sup> The practical and stream-lined analytical workflow of CBS-MS/MS is conducive for untrained personnel to operate, thus providing health care professionals with reliable, clinically relevant information.<sup>60</sup> Finally, the polymeric coating on the surface of the blade adds an increased level of selectivity that other direct to MS methods such as PS cannot match.<sup>77,88</sup>

This chapter focuses on applying the optimized CBS parameters that were identified on the AB SCIEX API 4000 MS in Chapter 2 for the concurrent analysis of fentanyl, as well as five related fentanyl analogs from human plasma and urine. The optimum extraction time for the selected compounds was determined, and the method was characterized by constructing calibration curves in both matrices to evaluate the accuracy, precision, linearity and LOQs/LODs in both matrices. Ultimately, this work serves to demonstrate CBS as a suitable high-throughput technology for rapid therapeutic diagnostics and POC applications.

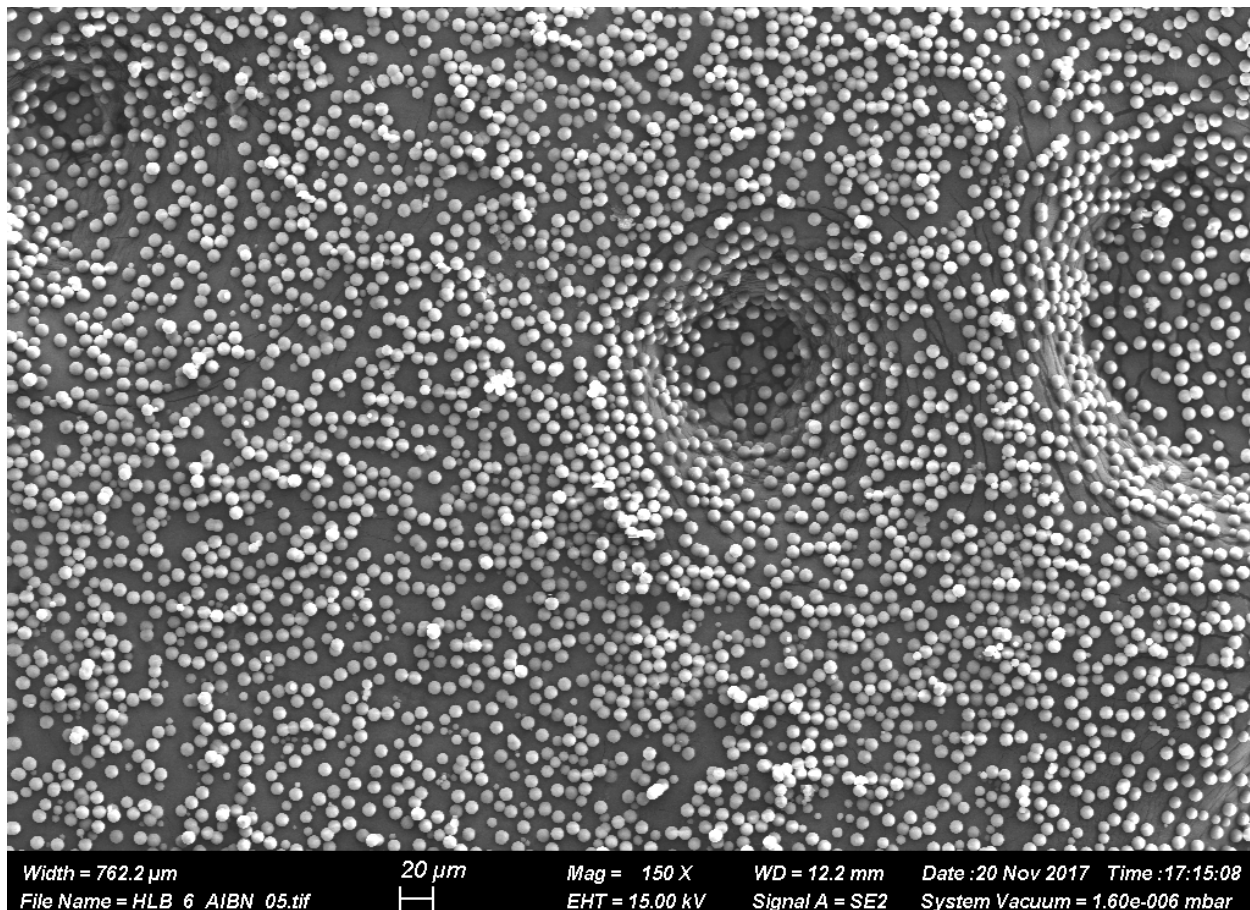
## 3.2 Experimental

### 3.2.1 Chemicals, reagents and materials

FA and AA were purchased from Sigma-Aldrich (Saint Louis, MO, USA), and LC-MS grade MeOH, ACN, IPA and water were purchased from Fisher Scientific (Bartlesville, OK, USA). Fentanyl, fentanyl d<sub>5</sub>, acetyl fentanyl, acetyl fentanyl <sup>13</sup>C<sub>6</sub>, sufentanil, sufentanil d<sub>5</sub>, carfentanil, carfentanil d<sub>5</sub>, alfentanil, and 4-Aminophenyl-1-phenethylpiperidine (4-ANPP) were all purchased from Cerilliant Corporation (Round Rock, TX, USA). For compounds that do not have the appropriate matched IS, the IS that provided the best correction (*i.e.* the highest precision and accuracy) was used. Individual working stock standard solutions were prepared in MeOH at a concentration of 100 µg/mL and stored at -80 °C. Human plasma with K<sub>2</sub>-EDTA that had been pooled from independent healthy donors was purchased directly from BioIVT (Westbury, New York, USA) and used without any further modifications. Urine was collected and pooled from healthy donors in the Pawliszyn research group. There were no additional modifications to the urine. All biological sample collection was done with the approval of the University of Waterloo Office of Research Ethics Board. Matrix-matched calibration curves, as well as samples used for constructing the extraction time profiles in urine and plasma were prepared by spiking the matrix and storing overnight at 4 °C. This was done to allow sufficient binding equilibrium of the analytes with the matrix. Stainless steel blades were purchased from Shimifrez Incorporated (Concord, ON, CAN) and used for all experiments herein presented. The blades were coated with a slurry of HLB-PAN. The HLB particles used for all experiments in this chapter were synthesized in-house by Dr. Varoon Singh.<sup>89</sup> Scanning electron microscopy (SEM) imaging was used to characterize the physical characteristics of the particles, and the average particle diameter was determined to be ~ 7.5 µm (Figure 3.2). The procedure to coat the blades was developed in the Pawliszyn group and



published recently in Scientific Reports.<sup>74</sup> The coated blades had a coating thickness of  $\sim 15 \mu\text{m}$ , and a coating length of 10 mm.



**Figure 3.2.** SEM image of in-house synthesized HLB particles.

### 3.2.2 Instrumentation

All of the experiments described in this chapter were carried out using an AB SCIEX API 4000 triple quadrupole mass spectrometer (Concord, ON, CA), implementing the optimized parameters identified in Chapter 2. Data processing was completed using Analyst 1.6.2 (SCIEX, Concord, ON, CA). The blades were accurately positioned in front of the entrance of the mass spectrometer using an ionization source built in-house by the University of Waterloo machine shop. This system has been described in detail in a previous manuscript, as well as in Chapter 2.<sup>24</sup>

The SRM transitions and optimized tuning parameters for the selected compounds are listed in Table 3.1. Analysis was performed in positive ionization mode, and each analyte and deuterated IS were detected as singly charged protonated ( $H^+$ ) adducts.

**Table 3.1.** SRM transitions and optimized tuning parameters

Compound	logP	Precursor ( <i>m/z</i> )	Product ( <i>m/z</i> )	DP (V)	EP (V)	CE (V)	CXP (V)
4-ANPP *	3.49	281.2	188.2	66.0	10.5	24.3	12.7
Acetyl fentanyl	3.11	323.2	188.2	102.2	10.0	32.7	14.1
Acetyl fentanyl $^{13}C_6$ **		329.2	188.2	90.9	11.3	30.8	17.8
Alfentanil **	2.81	417.2	165.1	95.2	8.9	35.9	17.0
Carfentanil	3.67	395.4	335.2	90.0	11.2	25.0	9.6
Carfentanil $d_5$		400.2	340.2	87.8	11.0	25.9	10.1
Fentanyl	4.05	337.3	188.3	99.1	11.2	30.7	12.4
Fentanyl $d_5$ *		342.2	188.2	98.9	10.8	32.2	14.3
Sufentanil	3.95	387.4	238.2	93.8	13.1	25.2	20.8
Sufentanil $d_5$		392.3	238.2	90.0	13.1	26.7	16.6

\* Fentanyl  $d_5$  was used as the internal standard for 4-ANPP. \*\* Acetyl fentanyl  $^{13}C_6$  was used as the IS for alfentanil. All physiochemical data was obtained from NCBI PubChem online database (<https://pubchem.ncbi.nlm.nih.gov/>, Accessed on March 31<sup>st</sup> 2017).

### 3.2.3 Sample preparation and analytical workflow

The CBS devices used for all experiments were cleaned for 30 min with a MeOH:ACN:IPA mixture (40:40:20, v/v), before conditioning for 30 min in a MeOH:H<sub>2</sub>O solution (50:50, v/v). The CBS devices do not have to be wet prior to an extraction event, therefore, it is practical to batch clean and condition a large number of devices prior to performing experiments.<sup>75</sup> The CBS workflow consisted of three stages: 1) high-throughput extraction of target analytes from 300  $\mu$ L of biofluid,<sup>61</sup> 15 or 20 min extraction time (plasma and urine, respectively), 1500 rpm orbital agitation at room temperature, 2) a fast H<sub>2</sub>O rinsing step (5 s) to remove loosely attached macromolecules, salts, and cellular debris, and 3) MS analyses, whereby 12  $\mu$ L of MeOH:H<sub>2</sub>O

(95:5, v/v) desorption/ionization solution spiked with 0.1 % FA and 12 mM AA was pipetted onto the coating of the blade which had been placed in front of the MS inlet. After 10 s of static desorption to allow extracted analytes to partition from the coating into the desorption/ionization solution on the surface, a 4.5 kV high voltage was applied to the blade for 20 s, inducing an electrospray event that directly introduced the analytes extracted by the coated blade into the MS.

#### 3.2.4 Extraction time profiles

Five extraction times between 5 – 25 mins were evaluated to identify the optimal extraction time in each matrix. The extraction time that provided the highest S/N and integrated area was chosen for the method characterization stage. Concentration of the target analytes and ISs in both matrices were kept constant at 10 ng/mL, with corresponding matrix blanks evaluated at each time point. Four individual replicates were analyzed, and the area under the curve of the spiked sample was compared to the matrix blank signal to determine the S/N at each time point.

#### 3.2.5 CBS method evaluation

The performance of the CBS method for analysis of fentanyl and 5 fentanyl analogs was evaluated in terms of accuracy, precision, linearity and sensitivity (*i.e.* LOQ and LOD). Calibration curves were prepared in pooled human urine and plasma, with nine calibration points from 0.1 – 100 ng/mL, as well as a blank. Two validation points (7.5 and 75 ng/mL) were analyzed to evaluate the precision and accuracy. Four independent replicates were used to determine the imprecision of each calibration/validation point. The concentration of the ISs in all calibration and validation points was 10 ng/mL. To account for experimental and instrumental variations, the ion-signal ratio of the analyte area under the curve was normalized to the area under the curve of the isotopically

labelled internal standard (A/Is). The LOD was estimated as the lowest concentration with S/N > 3. The LOQ was calculated using the following criteria: the lowest calibration point that had signal/noise (S/N) ratio > 5, 80 – 120 % back-calculated accuracy using linear regression line of best fit, and relative standard deviation < 20 %.

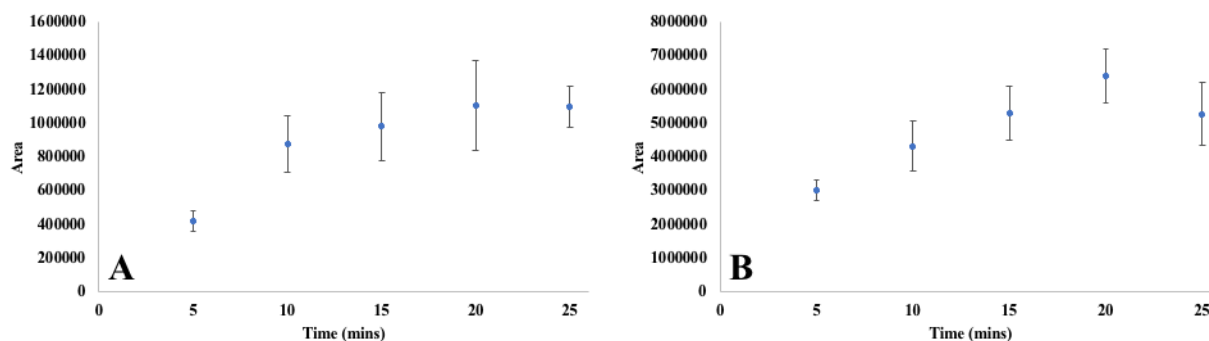
### 3.2.6 Statistical analysis

Microsoft Excel v16.14 was used for all statistical analyses.

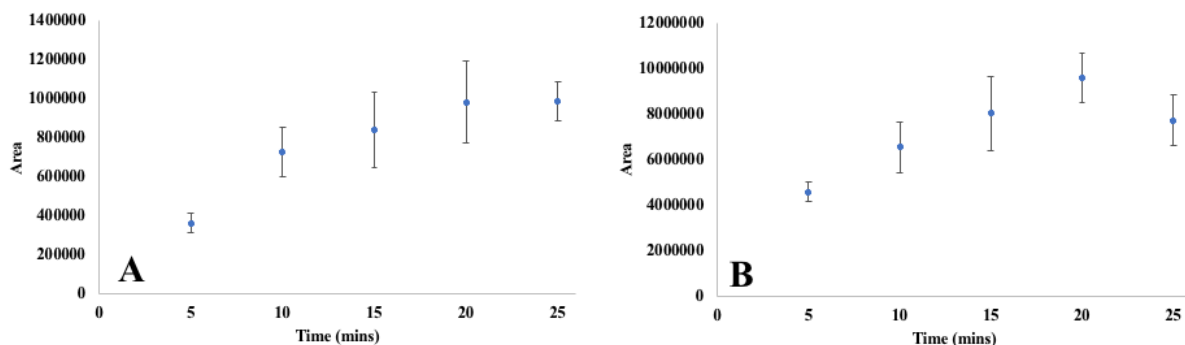
### 3.3 Results and discussion

#### 3.3.1 Determination of optimal extraction time

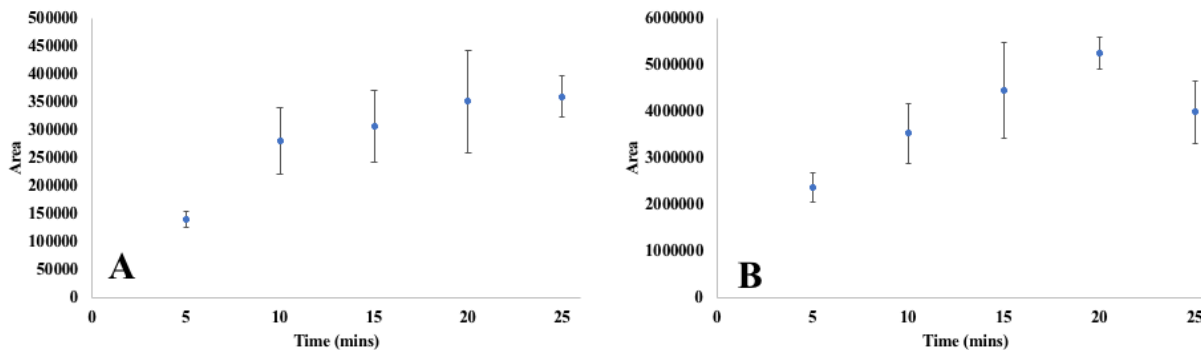
The extraction time was optimized to obtain maximum sensitivity for five of the six fentanyl analogs. Extractions from urine and plasma spiked with a 10 ng/mL mixture of the selected compounds were performed at the following time points: 5, 10, 15, 20, and 25 min. Individual extraction time profiles (ETP) for each compound in both matrices are shown below in Figures 3.3 to 3.7. The ETPs from plasma (**A**) determined that the optimal time (*i.e.* the highest signal with the lowest noise) was achieved after a 15 min extraction with 1500 rpm agitation. Likewise, the ETPs from urine (**B**) demonstrated that a longer extraction time was needed to reach the optimal conditions. In this case, a 20 min extraction with 1500 rpm was deemed to provide the best results. Beyond determining the optimum extraction time, the ETPs with corresponding matrix blanks serve a secondary purpose: to assess how the noise increases as a function of extraction time. In some instances, longer extractions may appear to extract more analyte. However, if the noise associated with the co-extraction of other substances with the same SRM transition is also increasing, then there is no improvement in the overall sensitivity of the method, and the longer extraction time serves only to decrease the throughput of the method.



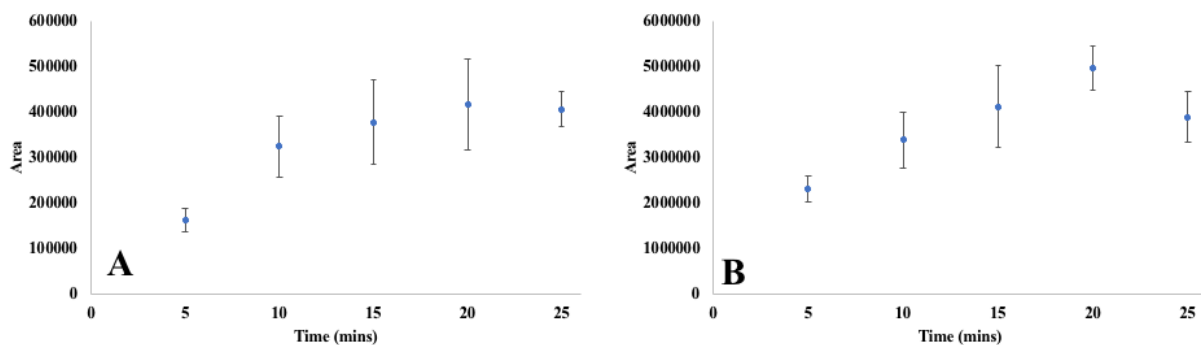
**Figure 3.3.** Extraction time profiles for acetyl fentanyl from (A) plasma and (B) urine. Four individual replicates with different CBS devices were analyzed at each time point.



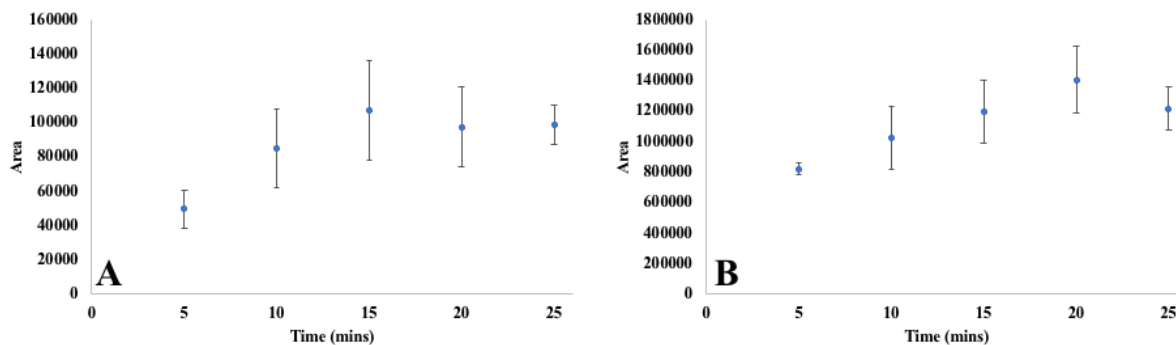
**Figure 3.4.** Extraction time profiles for fentanyl from (A) plasma and (B) urine. Four individual replicates with different CBS devices were analyzed at each time point.



**Figure 3.5.** Extraction time profiles for sufentanil from (A) plasma and (B) urine. Four individual replicates with different CBS devices were analyzed at each time point.



**Figure 3.6.** Extraction time profiles for carfentanil from (A) plasma and (B) urine. Four individual replicates with different CBS devices were analyzed at each time point.



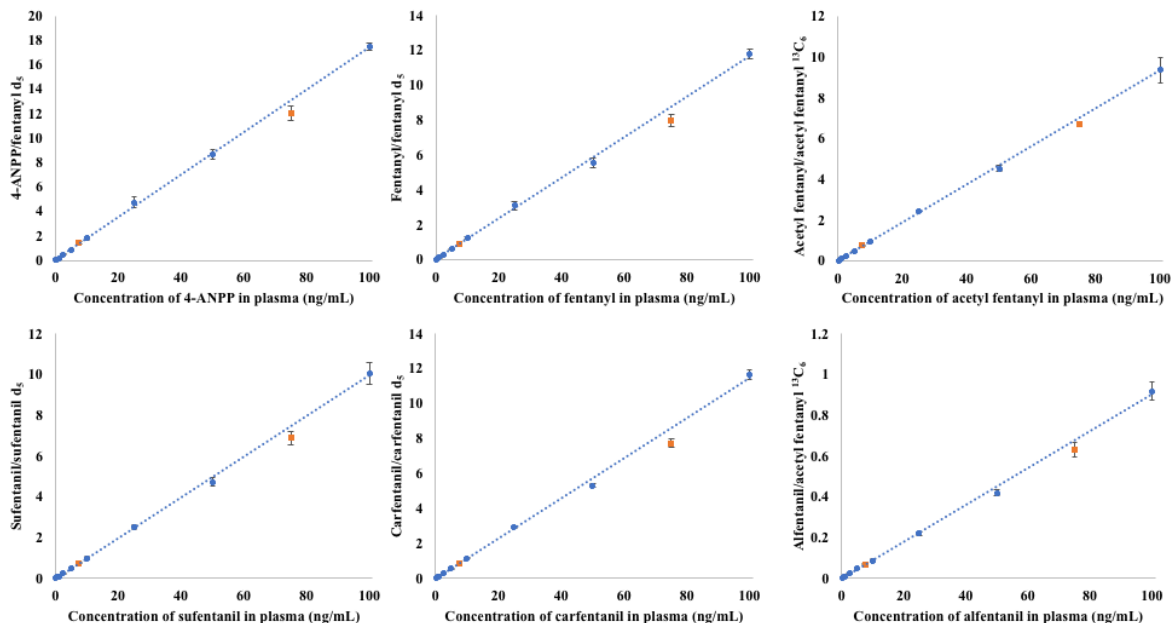
**Figure 3.7.** Extraction time profiles for alfentanil from (A) plasma and (B) urine. Four individual replicates with different CBS devices were analyzed at each time point.

### 3.3.2 Evaluation of CBS method for quantitative analysis

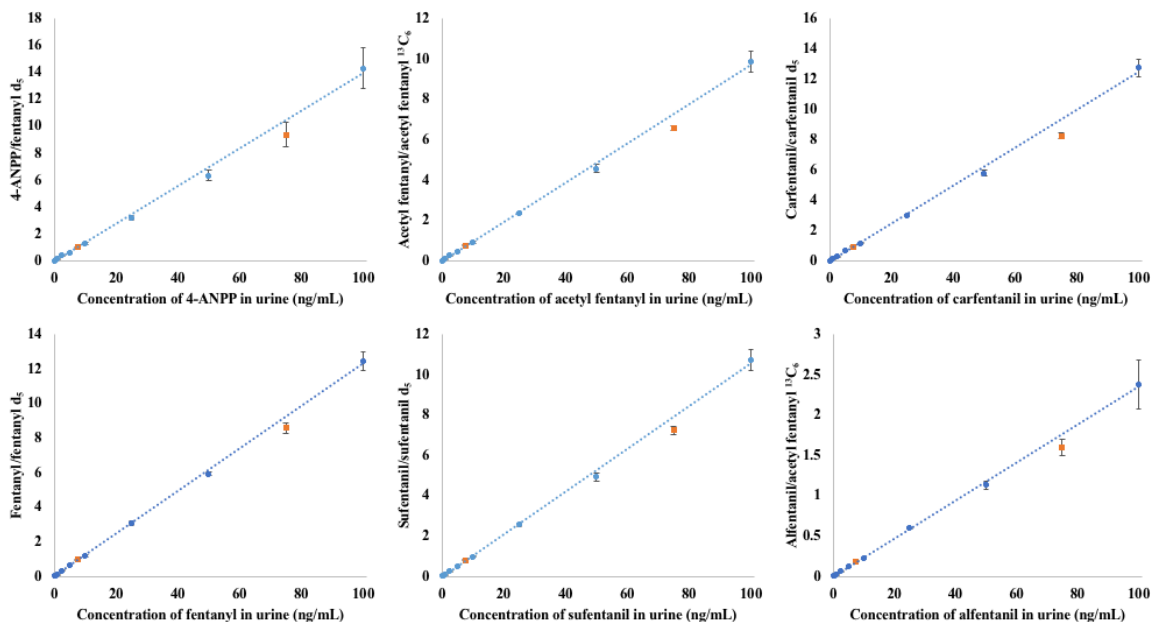
Shown below in Figure 3.8, the matrix matched calibration curves displayed excellent linearity ( $R^2 > 0.997$ ) for all six of the fentanyl analogs that were analyzed. For the quantitation of the selected analytes from plasma (Figure 3.8 (A)), very respectable figures of merit were attained, as seen in Table 3.2. The LOQs for all of the target analytes were determined to be 0.5 ng/mL. These LOQs were lower than the concentrations reported in overdose cases and fatal poisonings in whole blood, which ranged from 1.4 ng/mL to 386 ng/mL.<sup>85,90-92</sup> Precision and accuracy of the two validation points were between 97.6 – 112.3 %, and 2.7 – 13.7 % respectively. The figures of merit obtained for quantitation from human urine were also quite promising (Figure 3.8 (B)), with LOQs as low as 0.1 ng/mL for the majority of the compounds, as noted in Table 3.3. Accuracy of the two validation points ranged from 93.5 – 109.7 %, with precision between 1.2 – 7.8 %. Furthermore, using the classical  $S/N > 3$  criteria to estimate the LOD, the CBS methodology was capable of detecting some of the selected compounds in the low pg/mL range. To reach ultra-low detection limits for all of the fentanyl analogs, more advanced MS techniques such as differential mobility spectrometry (DMS) – MS or multistage  $MS^n$  are needed to decrease the background

noise from the SRM channels of carfentanil and alfentanil, effectively increasing the selectivity and S/N.

**A.**



**B.**



**Figure 3.8.** Quantitative analysis of fentanyl, and five fentanyl analogs in (A) human plasma and (B) human urine. Blue markers represent calibration points, and orange markers represent validation points. The following extraction parameters were used: 15 or 20 min extraction time from plasma and urine, respectively, 1500 rpm orbital agitation, and room temperature.



**Table 3.2.** Figures of merit for the quantitation of fentanyl, and five related fentanyl analogs from pooled human plasma

Compound	slope	intercept	R <sup>2</sup>	LOD (ng/mL)	LOQ (ng/mL)	Accuracy (n = 4), %		Precision (n = 4), %	
						7.5 ng/mL	75 ng/mL	7.5 ng/mL	75 ng/mL
<b>4-ANPP</b>	0.174	0.0597	0.999	0.012	0.5	106.5	91.7	6.1	5.1
<b>Fentanyl</b>	0.117	0.0270	0.999	0.016	0.5	102.9	91.3	5.5	4.4
<b>Acetyl fentanyl</b>	0.093	0.0132	1.000	0.006	0.5	106.6	95.8	3.9	2.0
<b>Sufentanil</b>	0.100	-0.0090	0.999	0.042	0.5	102.7	92.4	2.6	5.0
<b>Carfentanil</b>	0.115	-0.0143	0.998	0.100	0.5	101.4	90.0	9.0	3.2
<b>Alfentanil</b>	0.009	-0.0020	0.998	0.100	0.5	104.0	93.1	4.5	5.5

**Table 3.3.** Figures of merit for the quantitation of fentanyl, and five related fentanyl analogs from pooled human urine

Compound	slope	intercept	R <sup>2</sup>	LOD (ng/mL)	LOQ (ng/mL)	Accuracy (n = 4), %		Precision (n = 4), %	
						7.5 ng/mL	75 ng/mL	7.5 ng/mL	75 ng/mL
<b>4-ANPP</b>	0.141	-0.1010	0.997	0.017	0.5	87.7	81.1	5.0	9.9
<b>Fentanyl</b>	0.123	-0.0100	1.000	0.021	0.1	94.8	81.1	2.6	3.6
<b>Acetyl fentanyl</b>	0.098	-0.0300	0.999	0.042	0.1	91.7	80.5	4.5	1.5
<b>Sufentanil</b>	0.106	-0.0410	0.998	0.042	0.1	95.1	85.7	4.0	2.5
<b>Carfentanil</b>	0.126	-0.0440	0.998	0.250	0.5	98.1	87.6	4.5	2.5
<b>Alfentanil</b>	0.024	0.0000	0.999	0.188	0.5	92.3	80.6	3.1	6.6

### 3.4 Conclusions

In conclusion, the optimized CBS parameters elucidated in Chapter 2 were successfully implemented for the concomitant analysis of fentanyl, as well as five fentanyl analogs on the AB SCIEX API 4000 MS. ETPs were constructed for the target analytes in plasma and urine to determine the optimal extraction time. In plasma, the extraction time that yielded the highest S/N ratio was 15 mins, while in urine, 20 mins was deemed to be best. Following the extraction time optimization, the CBS method was characterized in terms of accuracy, precision, linearity and LOQs/LODs. In both matrices, adequate sensitivity was attained, with LOQs ranging from 0.1 – 0.5 ng/mL. In most clinical applications, 0.5 ng/mL is regarded as the minimum required LOQ. Excellent linearity, as well as accuracy and precision, were achieved for all of the compounds.

The total per sample analysis time was ~ 60 s using the high-throughput configuration, with the results clearly highlighting the capabilities of CBS to effectively quantitate fentanyl and several of its analogs in a rapid, yet highly accurate manner. Furthermore, these results also verified that HLB particles produced in-house could be used as a substitute to commercial particles. This would dramatically reduce the manufacturing cost of the devices, and consequentially the per sample cost of the analysis, without sacrificing the analytical performance. These attractive features outlined above further emphasize the applicability of CBS for POC applications, and clinical harm reduction strategies in emergency healthcare settings. Although this chapter presents a proof of concept for quantitation of fentanyl and several fentanyl analogs, the logical next step is to apply the optimized method for analysis of clinical patient samples to evaluate matrix effects and identify potential biological interferences in real samples. Furthermore, a rigorous cross validation with a gold standard LC-MS/MS method is also required to fully establish the CBS methodology as a viable alternative to conventional techniques.

## **Chapter 4. Clinical evaluation of coated blade spray – mass spectrometry for concomitant determination of four immunosuppressive drugs in whole human blood**

### 4.1 Introduction

Immunosuppressive drugs (ISDs) are prescribed to inhibit the activity of the immune system, and are administered specifically to patients who have undergone solid organ transplantation or for treatment of a variety of autoimmune conditions.<sup>93,94</sup> Despite the fact that ISDs are very effective at minimizing the rate of organ rejection, they have powerful side effects and are known to increase the susceptibility of dangerous postoperative infections. The most common ISDs prescribed for transplant patients are cyclosporine A (CYCA), tacrolimus (TAC), sirolimus (SIR), and everolimus (EVER).<sup>93,95,96</sup> These compounds can be further separated into two distinct categories: calcineurin inhibitors (CYCA and TAC), and mammalian target of rapamycin (mTOR) inhibitors (SIR and EVER).<sup>96</sup>

CYCA is a cyclic polypeptide comprised of 11 amino acids that binds to cyclophilin, forming a complex which inhibits calcineurin.<sup>96</sup> TAC is a macrolide lactone that binds to FK506 binding protein 12 (FKBP-12), forming a complex that also inhibits calcineurin.<sup>96</sup> Calcineurin functions as a serine/threonine phosphatase enzyme that plays a key role in the mammalian immune response. Inhibition of calcineurin results in decreased T-cell activation and downregulation of anti-inflammatory response related genes, specifically interleukin factor 2 (IL-2).<sup>94,96</sup> SIR (otherwise known as rapamycin), is a macrocyclic lactone that also binds to FKBP-12, however the resulting complex does not interact with calcineurin. Instead, it inhibits a protein kinase (mTOR) that functions as a key player in cell cycle progression from the G<sub>1</sub> to S phases, effectively circumventing IL-2 induced T-cell activation.<sup>96</sup> EVER is another macrocyclic lactone that is very similar, chemically and therapeutically, to SIR. The main difference between SIR and

EVER is the distinct pharmacokinetic (PK) properties of the two compounds, with EVER displaying a shorter half-life, allowing it to reach a steady state faster than SIR.<sup>96</sup>

Therapeutic Drug Monitoring (TDM) is a multidisciplinary clinical specialty used to improve patient treatment by individually quantitating and adjusting the concentration of an administered drug.<sup>5</sup> TDM, as defined by the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) “can be based on *a priori* pharmacogenetic (PG), demographic or clinical information and/or on the *a posteriori* measurement of blood concentrations of drugs (PK monitoring) and/or biomarkers (PD monitoring)”.<sup>5,97</sup> Due to a variety of factors such as narrow therapeutic range, high inter-patient PK and PG variability, and serious toxic effects if left unregulated, TDM of ISDs is critical for ensuring that the concentrations remain within the accepted target range.<sup>93,94,98,99</sup> If the ISD concentration falls into the subtherapeutic range, there is an increased risk of total organ rejection. Conversely, administration of CYCA or TAC above the recommended therapeutic dosage can lead to nephrotoxicity, hypertension and neurotoxicity, while elevated levels of SIR and EVER are associated with increased prevalence of leukopenia, thrombocytopenia and dyslipidemia.<sup>94,98,100</sup>

Historically, TDM of ISDs is performed using a variety of immunoassays, including enzyme linked immunosorbent assay (ELISA), electrochemiluminescence immunoassay (ECLIA), and chemiluminescent microparticle immunoassay (CMIA).<sup>93</sup> However, in more recent years, LC-MS/MS methods have become more established, with ~ 50 % assays for CYCA, and ~ 70 % of assays for TAC, SIR and EVER employing an LC-MS/MS system.<sup>5,101</sup> While the upfront costs of purchasing an LC-MS/MS system along with the requisite service contract are high, the low reagent and consumable costs, faster analysis times, and higher sensitivity and selectivity have

shifted the paradigm in TDM of ISDs from immunoassays towards mass spectrometric approaches.<sup>101</sup>

Despite the exceptional analytical performance afforded by modern LC-MS/MS systems, the complexity and variability of whole blood remains a challenging problem for accurate quantitation of ISDs in a clinical setting. More specifically, any analytical method used for TDM must be capable of releasing the heavily bound ISDs (*i.e.* ~ 99 % binding) from the erythrocytes that comprise 35 – 60 % of the matrix,<sup>102</sup> as well as accounting for inter-patient differences in hematocrit levels and minimizing matrix effects.<sup>75</sup> These challenges ultimately necessitate extensive sample preparation steps, with methods consisting of an SPE or LLE extraction preceded by a PPT to release the bound analytes into the free fraction of the sample.<sup>70,75,100</sup> In recent years, these tedious sample preparation protocols have been streamlined and automated by technologies such as the Phytronix Technologies Luxon™ laser diode thermal desorption (LDTD) system and the Agilent RapidFire™ SPE system, both of which can be directly interfaced with the MS.<sup>103–105</sup> Some drawbacks of these devices include the inability to monitor multiple ISDs concomitantly, the requirement for overnight incubation of samples with ISs prior to analysis, and high costs associated with the initial purchase, maintenance, and routine operation of these systems.<sup>75,104,105</sup>

The work discussed in this chapter encompasses the method development protocol, and subsequent clinical evaluation of CBS-MS/MS for concomitant analysis of four ISDs from whole human blood, building off of the original methodology published by Gómez-Ríos, *et al.* in 2018.<sup>75</sup> Moreover, using whole blood samples from patients undergoing immunosuppressant therapy at Toronto General Hospital (Toronto, ON, CAN), a statistical comparison between concentrations obtained using the optimized CBS-MS/MS method and those acquired from the Abbot Laboratories ARCHITECT™ i2000 automated CMIA is described. The aim of this work is to

assess CBS-MS/MS as a viable alternative to conventional assays for TDM of ISDs, due to its capacity to deliver total turn-around times per sample of  $< 2$  h, while exhibiting satisfactory inter-day precision, high sensitivity, and accurate quantitation to surpass the clinical criteria set by the IATDMCT.

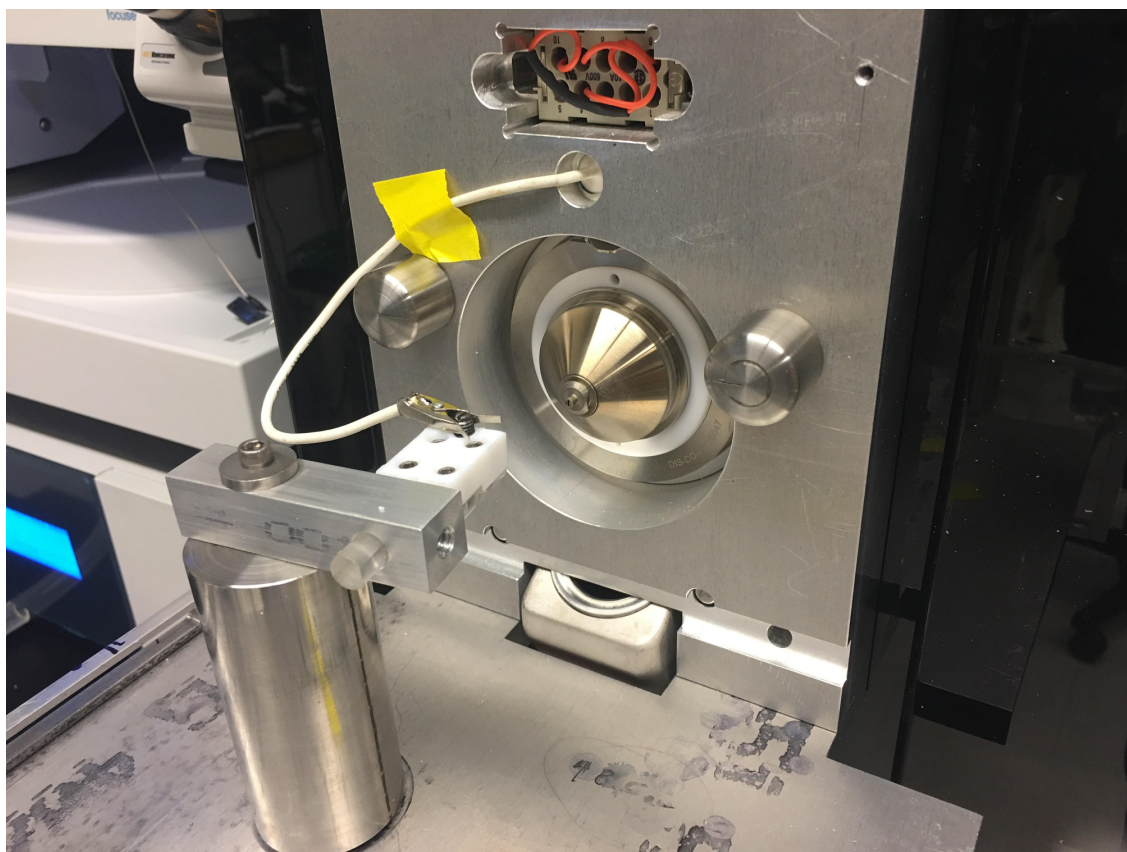
## 4.2 Experimental

### 4.2.1 Chemicals, reagents and materials

FA and AA were purchased from Sigma-Aldrich (Saint Louis, MO, USA). LC-MS grade MeOH, ACN, IPA and water were purchased from Fisher Scientific (Bartlesville, OK, USA). TAC, SIR, EVER and CYCA analytical standards were acquired from Cerilliant Corporation (Round Rock, TX, USA). Three isotopically labelled internal standards, namely EVER d<sub>4</sub>, CYCA <sup>15</sup>N<sub>11</sub> and TAC <sup>13</sup>C, d<sub>2</sub> were procured from Cerilliant Corporation (Round Rock, TX, USA). CYCA d<sub>4</sub> was acquired from Toronto Research Chemicals (Toronto, ON, CA). Individual stock standard solutions were prepared in MeOH at a concentration of 100 µg/mL and stored at -80 °C. ClinCal® whole blood immunosuppressant quality control calibrators (Blank, Levels 1 – 6) were purchased from Recipe (Munich, Germany). Liquichek™ whole blood immunosuppressant quality control calibrators (Levels 1 – 4) were purchased from Bio-Rad (Mississauga, ON, CAN). Pooled human whole blood (with K<sub>2</sub>-EDTA added as stabilizer/anti-coagulant) was purchased directly from BioIVT (Westbury, NY, USA). Residual EDTA human whole blood specimens were obtained from patients undergoing immunosuppressant therapy (CYCA – 94 patients, SIR – 97 patients, TAC – 93 patients) at the University Health Network (Toronto, ON, CAN). All blood samples that were prepared during the method development stage were spiked and stored at 4 °C overnight, prior to further sample preparation and analysis to facilitate physiological drug-erythrocyte binding conditions. HLB particles (5 µm diameter) were kindly provided by Waters Corporation (Wilmslow, UK). Stainless steel blades were purchased directly from Shimifrez Incorporated (Concord, ON, CAN) and coated using a slurry of HLB-PAN. The coating procedure used in this work was the same as the method developed and reported by Gómez-Ríos, *et al.* in 2017.<sup>74</sup>

#### 4.2.2 Instrumental configuration and mass spectrometric parameters

All of the experiments reported in this chapter were performed on a Thermo Scientific TSQ Quantiva triple quadrupole mass spectrometer (San Jose, CA, USA). Following data acquisition, data processing was done using Thermo Scientific Trace Finder v4.1 (San Jose, CA, USA). The blades were accurately positioned in front of the entrance of the TSQ Quantiva mass spectrometer using an ionization source built in-house by the University of Waterloo machine shop (Figure 4.1).



**Figure 4.1.** Custom manufactured CBS ion source directly interfaced with the TSQ Quantiva.

SRM transitions are listed in Table 4.1. Each ISD and deuterated IS were detected as singly charged ammonium ( $\text{NH}_4^+$ ) adducts.<sup>75,106</sup> Analysis was performed in positive ionization mode. Instrumental dwell time was 50 ms, and the capillary temperature was held constant at 350 °C.



**Table 4.1.** Physiochemical properties, SRM transitions and optimized MS parameters.

Compound	logP	Therapeutic range (ng/mL) <sup>70</sup>	Mass (g/mol)	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
Tacrolimus	5.59	3-15	804.03	821.5	768.4	19.86	85
Tacrolimus <sup>13</sup> C, <sub>d</sub> <sub>2</sub>			807.02	824.5	771.5	20.42	85
Sirolimus*	7.45	3-20	914.19	931.5	864.4	16.52	85
Everolimus*	7.4	3-15	958.24	975.6	908.5	16.42	87
Everolimus d <sub>4</sub> *			962.25	979.6	912.5	15.51	88
CyclosporineA	3.64	50-350	1202.64	1219.8	1202.7	16.32	96
Cyclosporine A d <sub>4</sub>			1206.64	1223.9	1206.9	11.4	97
Cyclosporine A <sup>15</sup> N <sub>11</sub>			1213.54	1231.1	1213.8	16.52	104

\*Everolimus D<sub>4</sub> was used as the matched internal standard for Sirolimus and Everolimus. All physiochemical data was obtained from NCBI PubChem online database (<https://pubchem.ncbi.nlm.nih.gov/>, Accessed on June 12<sup>th</sup>, 2017)

#### 4.2.3 Sample preparation and analytical workflow

The CBS devices used for each experiment were cleaned for 30 min with a MeOH:ACN:IPA mixture (40:40:20, v/v), before conditioning for 30 min in a MeOH:H<sub>2</sub>O solution (50:50, v/v). It is not necessary for the CBS devices to be wet prior to an extraction event, making it practical to batch clean and condition a large number of devices prior to performing experiments.<sup>75</sup> The analytical workflow originally reported by Gómez-Ríos, *et al.* and Tascon, *et al.* was used as a starting point for the experiments. Several optimizations were made to improve the inter-day stability and sensitivity of the method, while also reducing the per sample turn-around time.<sup>28,75</sup> These are addressed in detail in the Results and Discussion section. In general, the extraction protocol consisted of: 1300 µL of erythrocyte lysis solution, comprised of 0.1M Zinc sulfate (ZnSO<sub>4</sub>):ACN:H<sub>2</sub>O (60:30:10, v/v) and 1.15 ng/mL of selected ISs, was added to 200 µL whole blood. The CBS device was introduced to the mixture, and the extraction was performed. Inter-day stability and imprecision was evaluated using the following extraction parameters: 1.5 h extraction time, 1500 rpm agitation speed at room temperature. Functional sensitivity evaluation,

analysis of patient samples, and evaluation of accuracy and precision using third party calibrators was accomplished with the following extraction parameters: 1 h extraction time, 2200 rpm agitation speed and 55 °C temperature.

The CBS workflow consisted of three stages: 1) extraction of target analytes from the matrix as described above, 2) two fast H<sub>2</sub>O rinsing steps (5 s) to remove loosely attached macromolecules, salts, and cellular debris, followed by a light wipe with a Kimwipe, and 3) MS analyses, whereby 8 µL of MeOH:H<sub>2</sub>O (95:5, v/v) spiked with 0.1 % FA and 12 mM AA was pipetted onto the coating of the blade which had been placed in front of the MS inlet. After 10 s of static desorption to facilitate transport of extracted analytes from the coating to the surface of the blade, a 5.5 kV high voltage was applied to the blade for 5 s, inducing an electrospray event that directly introduced the extracted analytes into the MS.

#### 4.2.4 Inter-day stability and imprecision

To evaluate the inter-day stability and precision of the CBS methodology, extractions at three quality control (QC) levels (50, 150, and 300 ng/mL for CYCA; 2.5, 7.5, and 15 ng/mL for SIR, EVER, and TAC) were performed in triplicate. Measurements were taken once per day, for 10 days. These multi-analyte QC samples were prepared by spiking the working stock ISD standard solutions directly into the pooled whole human blood, keeping the organic solvent content < 1 %. The spiked QC calibrators were then incubated overnight at 4 °C, and on the following day, were aliquoted into 200 µL volumes to be used for the duration of the 10-day period. The details of each sample preparation strategy that was evaluated are outlined below in Results and Discussion section 4.3.1. The ion-signal ratio of the analyte area under the curve normalized to the area under the curve of the matched internal standard (A/Is) was used to account for experimental

and instrumental variations. The acceptance criterion for the total imprecision was < 10 %, based on the recommendation by the IATDMCT.<sup>70</sup>

#### 4.2.5 Functional sensitivity and quantitation

To assess sensitivity of the method, and to serve as a reference set for comparison with the single replicate calibration curves used during quantitation of real patient samples, matrix matched multi-analyte calibration curves were prepared. These calibration curves consisted of nine calibrators in quadruplicate between 2.5 – 1000 ng/mL for CYCA, and 0.25 – 100 ng/mL for SIR, EVER, and TAC, along with a blank in pooled whole human blood. The calibration curves were prepared using the same protocol outlined in 4.2.4 for the QC calibrators. Matrix-matched whole blood calibration curves were used to quantify the concentration of the ISDs in the sample. The ion-signal ratio (A/Is) of the analyte area under the curve normalized to the area under the curve of the matched internal standard accounted for experimental and instrumental variations. The same in-house QC calibrators were used to evaluate the accuracy and precision of the assay. The calibrators and QC levels were prepared with different lots to ensure method robustness and meet IATDMCT requirements.<sup>70</sup> The LOQ was determined using the following criteria: the lowest calibration point that had a S/N ratio > 5, 80 – 120 % back-calculated accuracy using the linear regression line of best fit, and relative standard deviation (RSD) < 20 %.

#### 4.2.6 Analysis of patient samples, and inter-day accuracy and precision evaluation using third party calibrators

The quantitation of ISD concentrations in anonymized patient samples was performed using a single replicate calibration curve run at the beginning of the sequence, followed by in-house QC calibrators, Bio-Rad Liquichek™ whole blood immunosuppressant quality control

calibrators (Levels 1 – 4), and Recipe ClinCal® whole blood immunosuppressant quality control calibrators (Blank, Levels 1 – 6), and then the entire set of patient samples – all with single replicate. This particular sequence of calibrators, third party QCs and samples were chosen to mimic the workflows used in routine clinical analysis. One set of patient samples was run per day for three days, either CYCA, TAC or SIR. However, the standard calibration curves, as well as both internal and external calibrators contained all of the ISDs. Therefore, the inter-day (n = 3) accuracy and precision of the Bio-Rad and Recipe QC calibrators were evaluated using the mean A/Is ratio of each level and the relative standard deviation following analysis of all of the samples. The acceptance criteria were: < 20 % RSD, and 80 – 120% back-calculated accuracy for the reported nominal concentration.

#### 4.2.7 Method comparison

Method comparison evaluation was performed using the single replicate measurement from the patient samples compared to concentrations obtained by the ARCHITECT™ i2000 automated CMIA.<sup>94</sup> CYCA patient concentrations ranged from 74.0 – 606.0 ng/mL (n = 94), SIR ranged from 1.8 – 27.6 ng/mL (n = 97), and TAC ranged from 1.8 – 16.0 ng/mL (n = 93). The slope, intercept, and correlation coefficient were calculated using the Passing-Bablok regression analysis with 95 % confidence interval. The method bias and identification of outliers were assessed using Bland-Altman plots.

#### 4.2.8 Statistical analysis

Microsoft Excel v16.14 and the XLSTAT student package v2018.4 were used for statistical analysis.

## 4.3 Results and discussion

### 4.3.1 Total inter-day imprecision

The major bottleneck in clinical analysis of real patient samples is the practical introduction of the IS to the sample during sample preparation, and subsequent incubation period that is required.<sup>107</sup> However, this requisite incubation period make total turnaround times of < 2 h nearly impossible to attain. To address this limitation, and decrease the per sample analysis time below 2 h, a novel sample preparation methodology was implemented to introduce the IS into the sample prior to extraction. Instead of spiking the IS mix directly into the whole blood and equilibrating overnight, the IS was spiked into the erythrocyte lysis solution – vastly increasing the method throughput from the original 16 h analysis time. This unconventional approach had the added benefit of decreasing experimental error, because the lysis reagent was prepared and spiked in bulk before aliquoting and storing the solution at -80 °C. Until now, the majority of published methods for TDM of ISDs have relied on specific reagents for analysis of a particular ISD, especially immunoassays such as CMIA and ECLIA, as well as other direct to MS approaches such as PS.<sup>70,94,103,108</sup> However, in the method presented herein, only one sample preparation reagent that is compatible with all four of the selected ISDs is needed for analysis. Coupled with the intrinsic advantages of SPME-MS technologies, specifically, the absence of any chromatographic separation, and combined sampling and sample preparation steps which yield a clean sample void of interfering matrix components, the innovative CBS-MS/MS technology is an efficient and robust technique for TDM of ISDs

To ensure that the ISs spiked into the lysis solution were capable of correcting variances in whole blood, three calibration curves with three replicates were prepared in different lots of blood with varying hematocrit levels. The imprecision (*i.e.* RSD) of the three calibration curve slopes

were compared, and total imprecision was determined to be < 5.0 % for CYCA, < 4.9 % for SIR, < 8.4 % for EVER, and < 3.6 % for TAC. These results demonstrated that the combined lysis solution along with the IS mixture could correct for inter-sample matrix variations and experimental error, differing from the conventional matrix matched SPME calibration methods, in which the IS must be equilibrated with the sample matrix prior to the extraction and subsequent analysis.<sup>36,102</sup>

Once it had been confirmed that spiking the ISs into the erythrocyte lysis solution was a viable alternative for introducing the ISs into the sample, the CBS-MS/MS assay stability and total imprecision over a ten-day period was evaluated. QC calibrators were prepared as outlined in section 4.2.4, and measured in triplicate, one run per day over 10 days. In total, three modified methods and one control method were investigated to determine which method would meet the requisite < 10 % total imprecision set by IATDMCT.<sup>70</sup> The experimental details are outlined below:

- 1) QC calibrators stored for first four days at 4 °C, followed by storage at -80 °C for the remaining six days. Erythrocyte lysis solution was prepared fresh each day and spiked with the IS mixture (final concentration 1.15 ng/mL).
- 2) QC calibrators were flash frozen three times, alternating between liquid N<sub>2</sub> and ice for 1 min each, before storage at -80 °C for duration of 10-day time course. Erythrocyte lysis solution was prepared fresh each day and spiked with the IS mixture (final concentration 1.15 ng/mL).
- 3) QC calibrators were flash frozen three times, alternating between liquid N<sub>2</sub> and ice for 1 min, before storage at -80 °C for duration of 10-day time course. Erythrocyte lysis solution was prepared in bulk and spiked with IS mixture (final concentration 1.15 ng/mL). 10 aliquots were made, and were individually stored at -80 °C.

4) The control method followed the protocol outlined by Gómez-Ríos, *et al.* in 2018.<sup>75</sup> The ISDs and corresponding ISs were spiked directly into the whole blood, equilibrated overnight and aliquoted, before being stored at -80 °C. Erythrocyte lysis solution was made fresh each day.

The total imprecision of each method over the 10-day period is shown below in Table 4.2. The only method that met the required < 10 % total imprecision criteria was Method 3, and consequently, this sample preparation strategy was employed for the remainder of the clinical evaluation.

**Table 4.2.** Total inter-day IS corrected imprecision for cyclosporine A (CYCA), sirolimus (SIR), Everolimus (EVER), and tacrolimus (TAC), determined by four CBS-MS/MS methods (triplicate per run, 1 run per day for 10-days).

Compound	QC Level	Method 1* RSD (%)	Method 2* RSD (%)	Method 3** RSD (%)	Control* RSD (%)
CYCA	1	29.0	48.8	4.6	49.3
	2	25.6	46.7	4.6	49.1
	3	26.8	46.8	5.1	48.8
SIR	1	10.3	8.6	2.2	13.2
	2	7.7	3.4	2.9	5.1
	3	6.4	3.2	3.4	3.3
EVER	1	9.0	4.1	3.5	5.9
	2	7.6	3.0	2.0	2.6
	3	7.7	4.2	2.2	3.2
TAC	1	20.3	14.9	2.5	2.7
	2	21.5	15.9	2.0	1.9
	3	20.4	14.5	1.6	2.4

\*Methods 1,2 and control used CYCA <sup>15</sup>N<sub>11</sub> as the matched IS. \*\* Method 3 used CYCA d<sub>4</sub> as the matched internal standard.

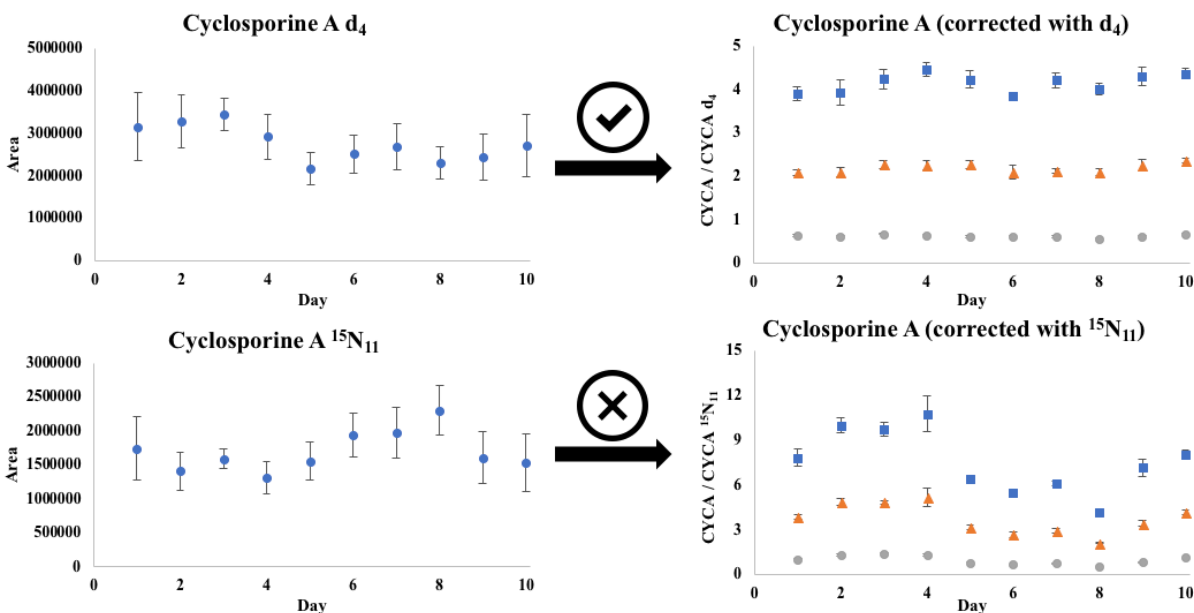
The rationale for implementing several deep-freezing cycles was to assist in lysing the erythrocytes, resulting in the heavily bound ISDs being released from cells and proteins, and effectively “normalizing” the matrix.<sup>109</sup> This was coupled with the bulk preparation and spiking of the erythrocyte lysis solution, which ultimately delivered excellent results. Surprisingly, Method 2,

which had the same deep-freezing protocol, resulted in poorer inter-day precision than Method 3 for all four ISDs. This observation suggests that bulk preparation of the lysis solution was crucial in minimizing the experimental error, as well as increasing the reliability of the measurements. This was due to the composition of the lysis solution and concentration of the IS remaining constant for all of the analyses over the 10-day period. Additionally, the low imprecision obtained for Method 3 established that the IS's were stable in the lysis solution at -80 °C, with no noticeable decrease in performance due to degradation or precipitation after prolonged storage.

One interesting observation was the major difference of CYCA  $^{15}\text{N}_{11}$  compared to CYCA  $\text{d}_4$  for accurately correcting CYCA. Methods 1, 2 and the control all used CYCA  $^{15}\text{N}_{11}$  for correction of CYCA. As seen in Table 4.2, it is apparent that the  $^{15}\text{N}_{11}$  IS was unable to correct for changes in the matrix over the 10-day time course. This would justify why the total imprecision for CYCA was significantly greater than the imprecision of the other ISDs, particularly in Methods 2 and 4. After consistently observing this result, CYCA  $\text{d}_4$  was added to the IS mixture during the evaluation of Method 3. The resultant total imprecision for CYCA decreased nearly 5 times. As seen below in Figure 4.2, a direct comparison of analysis of CYCA corrected with CYCA  $^{15}\text{N}_{11}$  and CYCA  $\text{d}_4$  illustrates the stark differences in performance between the two IS analogs. While the total imprecision of the area counts of CYCA  $^{15}\text{N}_{11}$  and CYCA  $\text{d}_4$  over 10 days were similar, 17.6 % and 15.4 % respectively, there was a clear difference in their ability to correct CYCA signal. Upon literature review, one postulation to account for these differences is “isotope effects”, a term which describes variations in physiochemical behavior, and ultimately, ionization efficiencies between labelled and unlabeled molecules.<sup>110</sup> This finding, while preliminary, suggests that the CYCA  $\text{d}_4$  mirrors the ionization of the unlabeled CYCA more similarly than CYCA  $^{15}\text{N}_{11}$ , resulting in vastly lower imprecision. Furthermore, differences in clustering of



ammonium or formate ions could also explain some of the variances between the performance of the two ISs.<sup>110</sup>

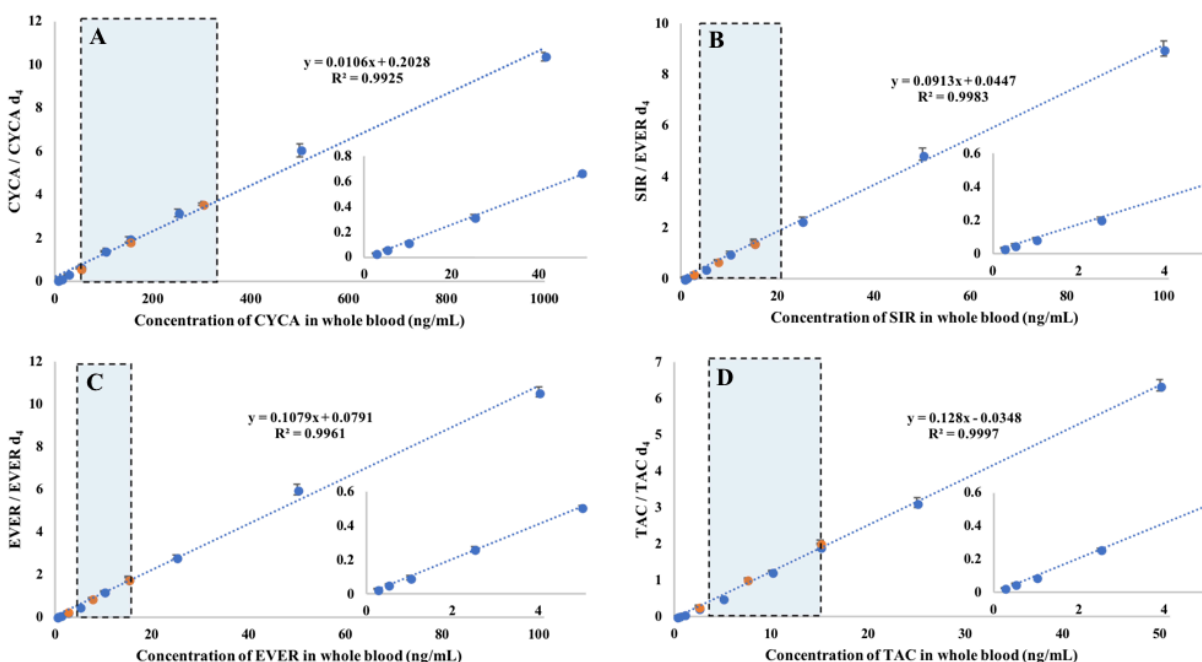


**Figure 4.2.** Comparison of performance of CYCA d<sub>4</sub> and CYCA <sup>15</sup>N<sub>11</sub> for correction of CYCA over 10-day time course using Method 3. CBS analysis consisted of the following extraction parameters: 1.5 h extraction time, 1500 rpm agitation speed at room temperature. The graphs on the left track the average area (n = 3) of each IS over 10 days. The graphs on the right illustrate the average A/Is over 10 days for both ISs. QC level 1 is marked as the grey circle, QC level 2 is marked as an orange triangle, and QC level 3 is marked as a blue square.

#### 4.3.2 Functional sensitivity and quantitation

After confirming that Method 3 met the clinical imprecision requirements recommended by the IATDMCT, the functional sensitivity of the CBS-MS/MS assay was established for each of the ISDs. In order to ensure that the LOQs were lower than the concentrations recommended for TDM, the extraction protocol was modified such that the agitation speed was increased from 1500 – 2200 rpm, and the extraction temperature was increased from room temperature (~ 20 °C) to 55°C. These changes to the protocol had the added benefit of decreasing the extraction time from 1.5 h to 1 h, which further increased the assay throughput. The data presented in Figure 4.3

displays the linear calibration curve obtained for each of the studied ISDs. The LOQs obtained using the optimized methodology were 2.5 ng/mL for CYCA, 0.5 ng/mL for SIR, 0.25 ng/mL for EVER and 0.5 ng/mL for TAC (see Table 4.3). Based on the recommendation by the IATDMCT expert consensus group, the LOQ for each ISD “should be at least one-third to one-half of the lower limit of the concentration window”, corresponding to minimum LOQs for CYCA, SIR, EVER and TAC of 20 ng/mL, 1.0 ng/mL, 1.0 ng/mL and 1.0 ng/mL, respectively.<sup>70</sup>



**Figure 4.3.** Quantitative analysis of cyclosporine A (CYCA), sirolimus (SIR), everolimus (EVER) and tacrolimus (TAC) in whole blood. The following extraction parameters were used: 1 h extraction time, 2200 rpm orbital agitation, 55 °C The shaded blue region corresponds to the typical concentrations in ISD-TDM samples.<sup>70</sup> Blue circles correspond to individual calibration points, and orange circles represent the internal QC levels.

**Table 4.3.** Figures of merit for the determination of ISDs in whole human blood.

Compound	LDR* (ng/mL)	LOQ (ng/mL)	Accuracy (n = 4), %			Precision (n = 4), %		
			QC 1	QC 2	QC 3	QC 1	QC 2	QC 3
Everolimus	0.25-100	0.25	107.7	102.2	104.9	6.3	3.8	5.2
Sirolimus	0.5-100	0.5	110.5	108.4	106.7	7.5	2.3	3.2
Tacrolimus	0.5-50	0.5	118.1	113.0	108.5	5.1	1.8	3.1
CyclosporineA	2.5-1000	2.5	89.4	101.0	106.1	2.7	1.6	1.1

\*LDR: linear dynamic range

The CBS-MS/MS method provides considerably better sensitivity than the recommended LOQs, with no compromises in terms of sample volume or throughput. Furthermore, strong linearity was obtained for each of the compounds of interest, with the linear dynamic range far exceeding the therapeutic concentration window for each ISD. Finally, as shown in Table 4.3, excellent accuracy and precision values were obtained for the in-house prepared QC calibrators, surpassing the < 10 % cut-off value set by the IATDMCT.<sup>70</sup>

#### 4.3.3 Method comparison

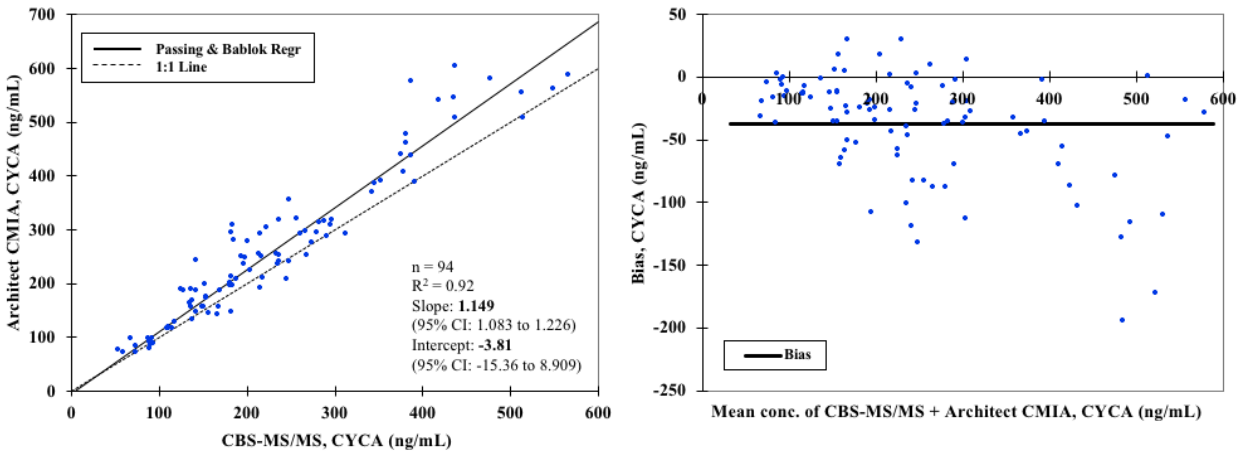
For method comparison, anonymized samples from patients undergoing immunosuppressive therapy were measured using the same method employed for the functional sensitivity evaluation. The results were then compared to concentrations obtained from the Abbot Laboratories ARCHITECT i2000 automated CMIA. The acceptance criteria were defined as a regression slope of 1.0 +/- 0.2 and an  $R^2$  of > 0.9. Figure 4.4 shows the Passing-Bablok regression and Bland-Altman analysis for CYCA, SIR and TAC. Passing-Bablok analysis is preferable for comparison of clinical methods because it does not assume that the measurement error is normally distributed and is more robust against outliers.<sup>111</sup>

CBS-MS/MS showed good agreement with the CMIA for CYCA (Figure 4.4. (A)), with a slope of 1.149 (1.083 to 1.226), intercept of -3.81 (-15.36 to 8.909) and an  $R^2 = 0.92$ . Bland-Altman analysis identified that 95.7 % (90/94 samples) fit within the 95 % confidence interval. The average bias of the measurements was determined to be -37.2 ng/mL or ~ -14.9 %. For SIR, slightly worse agreement between the two methods was observed (Figure 4.4. (B)), with slope of 1.228 (1.175 to 1.292), intercept of -0.025 (-0.607 to 0.481), and an  $R^2 = 0.95$ . The Bland-Altman analysis identified that 96.9 % (94/97 samples) fit within the 95 % confidence interval. The average

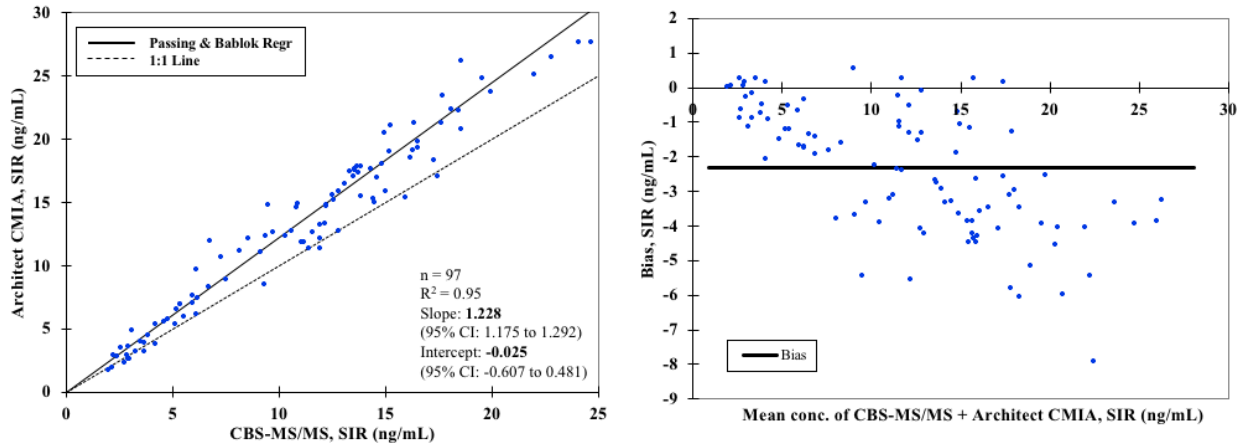
bias for CBS-MS/MS analysis of SIR was determined to be -2.32 ng/mL. The average percent bias of the method was ~ -18.6 %. Finally, analysis of TAC via CBS-MS/MS showed acceptable agreement with CMIA (Figure 4.4. (C)), with a slope of 0.951 (0.898 to 1.007), intercept of -0.870 (-1.33 to -0.387), and an  $R^2$  of 0.94. Bland-Altman analysis identified 5 outliers, with 94.6 % (88/93 samples) fitting within the 95 % confidence interval. The average bias of the measurements was determined to be 1.35 ng/mL. The average percent bias of the measurements was calculated to be 16.95 %. Overall, all three of the ISDs that were quantified using the innovative CBS-MS/MS method met the acceptance criteria, with the slope for SIR slightly exceeding the limit.

In general, a small, systematic difference between the two methods was observed for CYCA and TAC, because the 95 % confidence interval for the  $y$ -intercept of the Passing-Bablok regression analyses did not include 0. A slight proportional difference between the methods was observed for the measurements for CYCA and SIR since the 95 % confidence interval for the slope did not include 1. These observations are consistent with other comparisons reported in literature for immunoassays versus MS-based assays for ISD determination.<sup>70,93</sup> In particular, higher concentrations of ISDs determined by immunoassays can be attributed to the metabolism of the drugs in the body: the ISDs that are being monitored here are extensively metabolized, and immunoassays may not distinguish some of the metabolite artifacts from the parent compounds.<sup>93,112</sup> Furthermore, the bias in the measurements of the ISDs was also similar to other comparison studies in literature, further demonstrating the superior performance of MS-based assays over immunoassays for accurate determination of ISDs.<sup>70,93,112</sup>

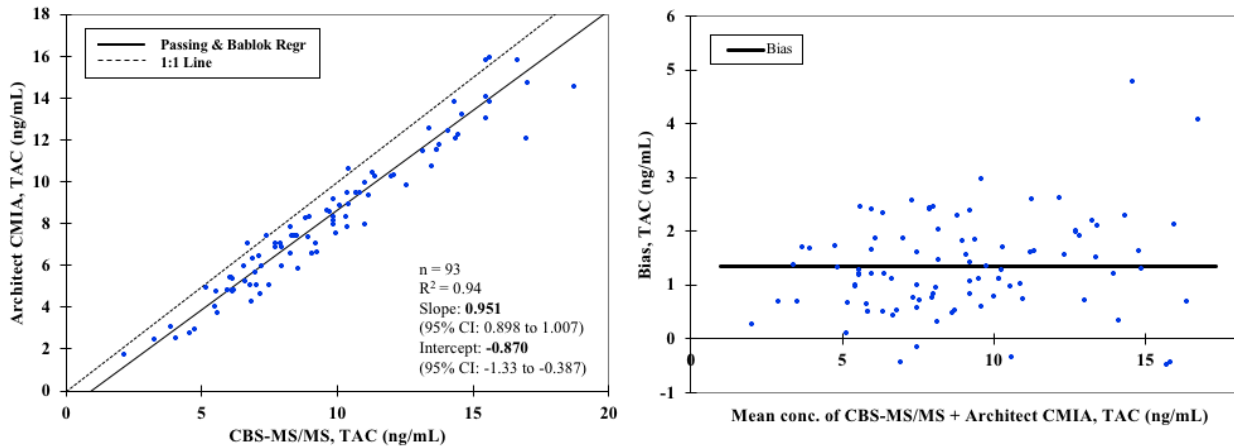
A.



B.



C.



**Figure 4.4.** Passing-Bablok regression analyses and Bland-Altman plots for (A) cyclosporine A (CYCA), (B) sirolimus (SIR), (C) tacrolimus (TAC) between CBS-MS/MS and Architect CMIA

#### 4.3.4 Inter-day accuracy and precision evaluation using third-party calibrators

Over three consecutive days, the third-party QC calibrators from Bio-Rad and Recipe were analyzed with single replicate measurements, and the inter-day precision and accuracy of the CBS-MS/MS methodology was evaluated. The results are summarized in Tables 4.4 and 4.5. Overall, excellent inter-day reproducibility was observed, with accuracy between 82.1 – 118.0 % obtained for all of the studied compounds from both sets of calibrators. Furthermore, the IADTMCT criteria of < 10 % total imprecision was obtained for almost all of the compounds and calibrators evaluated, with EVER in Recipe ClinCal® QC1 being the only exception.<sup>70</sup>

**Table 4.4.** Inter-day accuracy and precision of the CBS-MS/MS protocol using Bio-Rad Liquichek™ QC calibrators (Levels 1-4).

Compound	Accuracy (n=3), %				Precision (n=3), %			
	QC 1	QC 2	QC 3	QC 4	QC 1	QC 2	QC 3	QC 4
CYCA	84.9	101.6	107.8	108.7	2.7	8.3	7.0	4.8
SIR	94.4	99.1	93.5	N/A	2.8	8.5	6.4	N/A
TAC	103.8	91.9	87.0	86.1	3.1	3.7	2.6	2.3
EVER	N/A	118.0	94.6	107.5	N/A	5.1	1.5	1.8

**Table 4.5.** Inter-day accuracy and precision of the CBS-MS/MS protocol using Recipe ClinCal® QC calibrators (Levels 1-6).

Compound	Accuracy (n=3), %						Precision (n=3), %					
	QC 1	QC 2	QC 3	QC 4	QC 5	QC 6	QC 1	QC 2	QC 3	QC 4	QC 5	QC 6
CYCA	83.5	82.1	84.3	98.1	105.3	102.0	5.2	9.3	2.8	2.7	6.0	6.6
SIR	115.0	113.4	101.5	93.8	96.4	93.9	5.6	8.3	4.9	2.2	3.1	2.1
TAC	100.3	105.2	88.7	87.3	86.2	83.3	8.8	7.2	4.3	1.2	3.3	4.8
EVER	93.1	103.1	101.7	92.2	97.6	96.2	11.5	4.4	7.2	4.7	5.3	0.9

## 4.4 Conclusions

The method development and subsequent clinical evaluation presented in this chapter resulted in an improved method for TDM of ISDs from whole human blood using the CBS-MS/MS technology. This methodology was validated for use in a clinical setting using recommendations from the IATDMCT. By implementing an unorthodox sample preparation approach, the total per sample turnaround time of < 2 h was achieved. To the best of our knowledge, this is one of the highest throughput methods currently available for monitoring ISDs from whole blood. The optimized method demonstrated outstanding long-term stability, with total imprecision < 10 % for each of the selected ISDs over the 10-day time course. The LOQs obtained for CYCA, SIR, EVER and TAC were 2.5, 0.5, 0.25 and 0.5 ng/mL, respectfully, with excellent linearity ( $R^2 > 0.99$ ). A method comparison between CBS-MS/MS and the Abbot Architect i2000 CMIA showed good agreement between both methods for CYCA, SIR and TAC, with observed differences consistent with those presented in literature. Lastly, the inter-day precision and accuracy of CBS-MS/MS was evaluated using third party QC calibrator kits from Bio-Rad and Recipe, with results exceeding the stringent clinical guidelines.

## **Chapter 5. Summary.**

In conclusion, CBS is a novel SPME device that is directly coupled to the MS, permitting rapid and accurate determination of a wide range of compounds under ambient conditions. Initial experiments have optimized the ideal position for the blade on three MS instruments in regards to ion intensity and spray stability. The three instruments that were evaluated were the AB SCIEX API 4000 triple quadrupole, the Waters XEVO G2-S hybrid QTOF, and the Thermo Scientific TSQ Quantiva triple quadrupole. Spatial signal intensity profiling of the MS inlet revealed that the AB SCIEX instrument was the most sensitive to the blade position, whereas the other two instruments had a much larger spatial window where optimal intensity and stability were observed. Characterizing the analytical performance established that the AB SCIEX MS was capable of delivering comparable figures of merit that had been previously reported for the TSQ Quantiva for the selected compounds, in both plasma and urine. The figures of merit for the Waters QTOF were also quite promising, with respectable sensitivity obtained for the majority of the compounds that were evaluated. Ultimately, this was the first instance of an SPME device being directly coupled to a hybrid QTOF instrument, and the results obtained in these experiments will serve as a benchmark for future investigations.

Following the identification of the optimal CBS operating parameters, these conditions were applied for the concurrent analysis of fentanyl, and five fentanyl analogs from urine and plasma using the AB SCIEX MS. ETPs were used to determine the optimal extraction time in both urine and plasma for the selected compounds. The performance of the CBS method was then characterized by constructing matrix matched calibration curves in both matrices to evaluate the accuracy, precision, linearity and LOQs/LODs in both matrices. Excellent figures of merit were



obtained from both matrices, with limits of quantitation for all compounds at 0.5 ng/mL or lower, with less than 60 s of total analysis time per sample.

Finally, the clinical viability of CBS-MS as an alternative technique to conventional assays for the concomitant determination of 4 ISDs in whole human blood was investigated. This was the first instance of an SPME-MS technology being rigorously evaluated using clinical chemistry criteria recommended by the IATDMCT. The results demonstrated that the optimized method provided extremely reproducible results over a 10-day period, with total inter-day precision less than 5 % for all of the compounds at three different concentrations. The sensitivity of the method improved nearly 4 times from the preliminary proof of concept study published earlier this year, with a major increase in throughput capacity from 16 h to 2 h – besting the analysis times of automated immunoassays that are employed in clinical laboratories for routine analysis.<sup>75,94</sup> Finally, the concentrations of the selected immunosuppressive drugs in ~ 100 patient samples obtained using the CBS-MS/MS methodology were compared to the Abbot Laboratories ARCHITECT™ i2000 automated immunoassay system. Passing-Bablok regression analysis demonstrated significant statistical similarities between both methods. Bland-Altman plots identified minimal outliers, with some bias in the CBS measurements that was consistent with other MS-based methods.

In conclusion, some of the biggest limitations of the CBS platform have been addressed herein; specifically, the lack of implementation on non-Thermo Scientific instruments and evaluation of the technology for routine clinical analysis (*i.e.* TDM). The results established CBS could be interfaced with other MS vendors, with analytical figures of merit comparable to those already reported on Thermo Scientific instruments. Furthermore, it was noted that the CBS technology could also be implemented on hybrid QTOF instruments with satisfactory

performance. Lastly, a thorough clinical evaluation determined that CBS could be applied as an effective alternative methodology for determining the concentration of four immunosuppressive drugs in whole blood, with turn-around times and overall performance on par with automated immunoassays.

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