Development and Application of Solid-Phase Microextraction Probe Electrospray Ionization

by

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A thesis

presented to the University of Waterloo

in fulfilment of the

thesis requirement for the degree of

Master of Science

in

Chemistry

Waterloo, Ontario, Canada, 2020

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

Ambient ionization mass spectrometry (AIMS) is a category of mass spectrometry (MS) techniques originally characterized as using ambient ionization sources to analyze samples with little to no sample preparation and no chromatography step. This set of techniques have quickly gained popularity due to fast workflows and the ability to perform high throughput analysis. However, AIMS is prone to high matrix effects and reduced sensitivities. Solid-phase microextraction (SPME) is commonly used to mitigate these effects due to easy integration into pre-existing AIMS workflows, enabling preconcentration and extraction. Probe electrospray ionization (PESI) is a technique developed by Hiraoka and colleagues in 2007, then commercialized by Shimadzu Corporation years later. In PESI a small metal probe is dipped into the sample and immediately moved upwards, close to the inlet of a mass spectrometer to facilitate electrospray ionization (ESI). In recent years there has been a shift from using PESI for qualitative studies towards quantitative studies. With this shift in intentions, sample preparation has been incorporated into PESI workflows. The objective of this work is to incorporate SPME as a sample preparation method for PESI and develop applications for this technique. The first objective was to see if the PESI probes could be coated and to see if SPME-PESI-MS/MS could give reliable MS data. To ensure reproducibility of such small probes, intra- and inter-probe reproducibility tests by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) were conducted using drugs of abuse. These results show reproducibility of the probes with almost all relative standard deviations being ≤ 15%. Afterward, the optimal desorption solution for SPME-PESI-MS/MS was determined. It was also found that a coated PESI probe used for SPME-PESI-MS/MS could not be used for a subsequent LC-MS/MS run without extracting the sample again due to significant desorption by SPME-PESI-MS/MS. Furthermore, an application of SPME-

PESI-MS/MS to quantitate drugs of abuse from 30µL of plasma was developed. The intra-day precision of said method was under 15% for all compounds. The inter-day precision of all compounds was under 15% except for lorazepam at the 30 ng mL^{-1} validation point and oxazepam at the 90 ng mL^{-1} validation point. The accuracy of all compounds for this method was within 80-120% except for lorazepam at the 30 ng mL^{-1} validation point. The small dimensions of the coated PESI probes were then leveraged to determine the free concentration and plasma protein binding of diazepam from human plasma by SPME-PESI-MS/MS. The plasma protein binding determined by SPME-PESI-MS/MS was 99.3% which falls within literature values of 97-99% from human plasma samples spiked with 25 ng mL^{-1} of diazepam. Finally, the development of a screening method for aminoglycosides was explored with SPME-PESI-MS/MS. This was to explore the use of AIMS technologies as an alternative screening method for compounds that require conditions that are highly detrimental to MS systems (i.e. high salt concentrations or ion-pairing reagents).

Acknowledgements

I want to thank Professor Janusz Pawliszyn for the opportunity to learn and research under his guidance. I want to thank Professor Germán Sciaini and Dr. Richard Smith for taking the time to be on my MSc. Committee. I also want to thank Professor Susan Mikkelsen, the associate graduate coordinator.

I want to thank the Natural Sciences and Engineering Research Council (NSERC) and Shimadzu Corporation for the funding and resources they provided. The proposed research would not be possible without these partners. I want to thank Yohei Arao, Dr. Yuka Fujito, Dr. Tairo Ogura, and Dr. Masayuki Nishimura from Shimadzu Corporation for their continued support with this collaborative project.

I want to acknowledge the extensive help and mentorship I have received from Dr. German Augusto Gómez-Ríos, Dr. Varoon Singh, and Dr. Kanchan Sinha Roy. I also want to thank all the members of Professor Pawliszyn's research group for their support and help to develop my ideas especially Dr. Abir Khaled, Dr. Chiranjit Ghosh, Daniel Rickert, Dr. Diana Marcela Cardenas Soraca, Dominika Gruszecka, Emir Nazdrajic, Hernando Rosales, Dr. Joao Raul Belinato, Dr. Jonathan Grandy, Dr. Khaled Murtada, Mohammad Huq, and Paola Alejandra Ortiz Suárez.

Last, but not least, I want to thank my friends and family for the support they have given me.

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List of Abbreviations

ACN	Acetonitrile
AIMS	Ambient Ionization Mass Spectrometry
BQL	Below Quantifiable Level
CBS	Coated Blade Spray
DART	Direct Analysis in Real Time
DEP	Direct Electrospray Probe
DESI	Desorption Electrospray Ionization
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DpC	Di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone
DTP	Desorption Time Profile
dPESI	Dipping Probe Electrospray Ionization
ED	Equilibrium Dialysis
ESI	Electrospray Ionization
ETP	Extraction Time Profile
FA	Formic Acid
HCl	Hydrochloric Acid
HILIC	Hydrophilic Interaction Liquid Chromatography
HLB	Hydrophilic-Lipophilic Balance
H_2O	Water
IS	Internal Standard
IPA	Isopropanol
LC-MS	Liquid Chromatography Coupled to Mass Spectrometry
LC-MS/MS	Liquid Chromatography Coupled to Tandem Mass Spectrometry
LLE	Liquid-liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantitation
MeOH	Methanol

MOI	Microfluidic Open Interface
MeOH	Methanol
MS	Mass Spectrometry
NSERC	Natural Sciences and Engineering Research Council
PAN	Polyacrylonitrile
PBS	Phosphate Buffered Saline
PESI	Probe Electrospray Ionization
PPB	Plasma Protein Binding
PPT	Protein Precipitation
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RED	Rapid Equilibrium Dialysis
RSD	Relative Standard Deviation
SF-PESI	Sheath Flow Probe Electrospray Ionization
SPE	Solid Phase Extraction
SPME	Solid-Phase Microextraction
SPME-PESI	Solid-Phase Microextraction Probe Electrospray Ionization
SPME-PESI-MS/MS	Solid-Phase Microextraction Probe Electrospray Ionization Coupled to Tandem Mass Spectrometry
TDM	Therapeutic Drug Monitoring

Chapter 1 Introduction

1.1 Ambient Ionization Mass Spectrometry (AIMS)

In an analytical chemistry workflow, there are generally five major components that must be performed in sequential order: sampling, sample preparation, separation, detection, and data processing & interpretation. Generally, sample preparation is the most time consuming of the five steps with up to 80% of researchers' time spent on this step.[2] Depending on the application, the sensitivity and selectivity provided by these workflows may not justify the time taken. Minimizing or removing the sample preparation and separation steps leads to dramatically decreasing the time spent in an analytical workflow.

Ambient Ionization Mass Spectrometry (AIMS) is a category of mass spectrometry techniques which generate ions from samples under ambient conditions with little to no sample preparation and no chromatographic separation.[1] AIMS has been enabled by the development and popularization of ambient ionization techniques such as electrospray ionization (ESI), atmospheric pressure chemical ionization, and laser desorption. The lack of chromatography found in AIMS techniques allow for quick and high throughput analysis of samples when compared to traditional mass spectrometry methods that involve chromatography before MS.[3] This can be leveraged to bring rapid, real-time and relatively cheap analysis of samples on-site or in the laboratory. The number of AIMS techniques has increased rapidly from the term's inception when Takats *et al.* first published desorption electrospray ionization (DESI) in 2004.[4] AIMS is a rapidly growing field of mass spectrometry in both its adoption and the development of new techniques.

AIMS can be sorted into three categories based on desorption and ionization strategies proposed by Huang *et al.* in 2011: (1) direct ionization, (2) direct desorption/ionization, and (3)

two-step ionization.[1] Direct ionization techniques ionize analytes directly from liquid samples or droplets of the liquid sample.[1] Techniques that fall under this category include the direct electrospray probe (DEP) as shown in Figure 1.1(A).[1] DEP uses a metal ring or a metal coil that contains either an optical fiber or solid phase microextraction (SPME) fiber acting as a probe to which the liquid sample is applied; the probe subsequently undergoes ESI.[5] The probe prevents unnecessary clogging and use of syringe pumps when compared to the use of a capillary with ESI.[5] Direct desorption and ionization techniques are characterised by their use of an ambient ionization source to generate charged reactive species or metastable atoms which strike the surface of the sample leading to the desorption and ionization of the analyte.[1] DESI as shown in Figure 1.1(B) falls under this category. In DESI, a fast stream of solvent droplets charged by ESI strikes the surface of the sample at an angle to desorb and ionize analytes from the surface of the sample. The ionized analytes are then funneled into the mass spectrometer for analysis. In a two step ionization process desorption occurs first followed by ionization. The analyte is desorbed from a sample and then interacts with charged reactive species or metastable atoms generated from an ambient ionization source to form ions.[1] Direct analysis in real time (DART) is a two step ionization technique where excited gas atoms, usually helium, are used to ionize atmospheric compounds; which in turn ionizes analytes that were desorbed by evaporation or thermal desorption from the surface of a substrate. Figure 1.1(C) shows a setup of DART.[6]

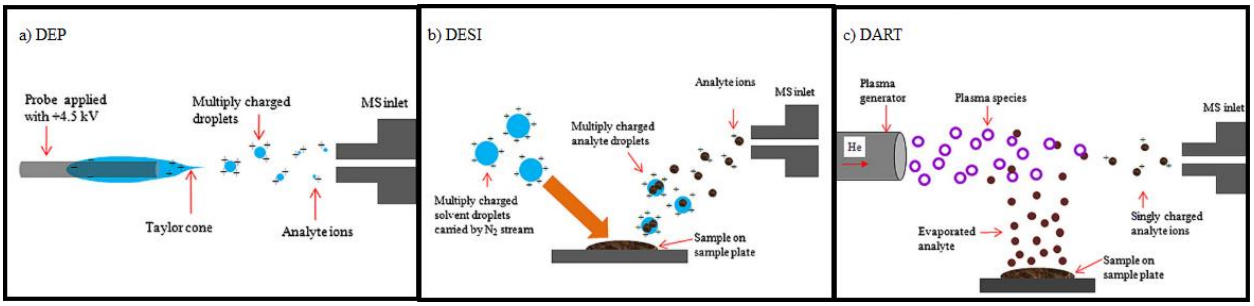

Figure 1.1 A graphical summary of AIMS techniques that fall under the different categories proposed in Huang *et al.*[1] (**A**) DEP, a direct ionization technique. (**B**) DESI, a direct desorption/ionization technique. (**C**) DART, a two-step ionization technique.

The lack of sample preparation in most AIMS techniques and the lack of chromatography has greatly reduced the total analysis time, however this set of techniques has its fair set of challenges to address and overcome. Three major challenges that AIMS techniques face are reproducibility, sensitivity, and selectivity which a perspective from Kuo *et al.* further explores.[7] One major cause of all three challenges is the complexity of the samples under analysis and its matrix effects. Matrix effects can alter the sensitivity of a method by suppressing the ionization of analytes which is particularly troublesome for ESI.[8] Additional matrix effect complications arise when using a matrix that has a large amount of variation between different samples such as whole blood.[9] Complex matrices can further reduce selectivity as matrix components may have the same mass to charge (m/z) ratio. In addition, heterogeneity within a single sample heavily influences the analytes ultimately detected and possibly quantified. The ability to analyze sample heterogeneity by AIMS is of great interest as indicated by the vast literature published on MS imaging.[10] However, applications only interested in detection and quantitation of analytes without regard to spatial information about heterogeneity within a sample can view this as a source of irreproducibility due to possible variances in sampling. As a response to these challenges there is an interest to integrate sample preparation into AIMS workflows as it reduces matrix interferents

and can concentrate analytes.[11] This in turn leads to better sensitivity and selectivity. Sample preparation techniques used must be fast and/or allow for high throughput or else the workflow would not be a practical alternative to a conventional liquid chromatography coupled to mass spectrometry (LC-MS) workflow.

1.2 Sample Preparation Techniques Used in Ambient Ionization Mass Spectrometry

Coupling sample preparation to AIMS leads to a decrease in matrix effects, as well as increases in sensitivity, linearity, and/or reproducibility. Sample preparation can be approached by modifying an already existing parameter of an AIMS technique or by introducing a new step before the AIMS technique.[11–13] Paper spray is an AIMS technique that uses paper as a substrate for sample deposition and subsequently the substrate from which ESI is generated. Paper spray is an example of an AIMS technique that has an inherent sample preparation component.[13] The cellulose fibers of paper can interact with matrix components which can alter the matrix effect and analyte sensitivity.[14] Venter *et al*. and Javanshad & Venter further explore the different sample preparation and processing options available by modifying already existing parameters of AIMS techniques.[12,13]

Tailoring sample preparation step(s) towards the combination of analyte and matrix provides increases in sensitivity, reproducibility, and lower matrix effects required for an AIMS workflow that is fit for purpose. Sample preparation steps commonly used in LC-MS workflows have been incorporated into AIMS workflows. Liquid-liquid extraction (LLE), solid phase extraction (SPE), protein precipitation (PPT) and quick, easy, cheap, effective, rugged, and safe (QuEChERS) are conventional sample preparation steps incorporated into AIMS workflows. For example, LLE with low temperature partitioning was used by Paula *et al.* to quantify benzodiazepines from beverages.[15] Another example is progesterone extracted from lake water by SPE and subsequently

quantitated by DART-MS.[16] These conventional sample preparation steps can fall into the pitfalls of being time consuming or hard to facilitate in a high throughput fashion. Both pitfalls can lead to developing a method that can not taken advantage of quick analysis times that AIMS techniques with little to no sample preparation provide nor can provide the sensitivity or selectivity that a conventional LC-MS method can provide. In addition, the use of several pieces of equipment and reagents for a sample preparation step in an AIMS workflow can lead to difficulty when attempting to implement AIMS outside of a laboratory. To mitigate these drawbacks sample preparation techniques used in AIMS workflows should be relatively quick and/or allow for high throughput sample preparation as well as use minimal equipment if attempting to implement an AIMS workflow outside of the lab.

1.3 Solid-Phase Microextraction (SPME)

SPME was introduced by Pawliszyn and can facilitate solventless sampling, sample preparation, as well as preconcentration in one step.[17] SPME is a micro-extraction technique where a small amount of extraction phase generally immobilized onto a solid support is exposed to a sample at set experimental conditions for extraction.[2] Analytes recovered by said extraction phase are ultimately desorbed for further chemical separation and detection or directly to detection.[18] Optimization of experimental conditions for successful implementation of SPME includes but is not limited to geometry of SPME devices, mode of extraction, and coating chemistry.[19] Detailed reviews on fundamentals and experimental conditions to consider when optimizing SPME protocols have been reviewed elsewhere.[2,19,20] Using a small volume of the extraction phase relative to the sample leads to only the free fraction of the analyte being extracted by SPME.[2] This non-exhaustive nature of SPME allows for monitoring chemical changes in a system with minimal disturbance and measure signals proportional to an analyte's free

concentration.[21] However, the non-exhaustive nature of SPME also requires careful consideration of which calibration method is used for quantification.[3]

Certain calibration methods are better suited for certain regimes on the extraction time profile (ETP).[22] The three regimes on an ETP are the linear regime, the kinetic regime, and the near equilibrium regime as illustrated by Figure 1.2.[22] Figure 1.2 is a typical ETP where t_{50} is the time at which 50% of the amount of analyte extracted at equilibrium is extracted. At t_{95} the amount of analyte extracted is 95% of the amount of analyte extracted at equilibrium. The traditional calibration methods: external standard calibration, standard addition calibration, and internal standard calibration can be applied in all three regimes. Other calibration methods include equilibration based calibration which is commonly used for on-site analysis where internal standard and standard addition calibration methods are not suitable.[21] In cases where equilibration of analytes is not practically feasible then kinetic calibration methods can be employed.[22]

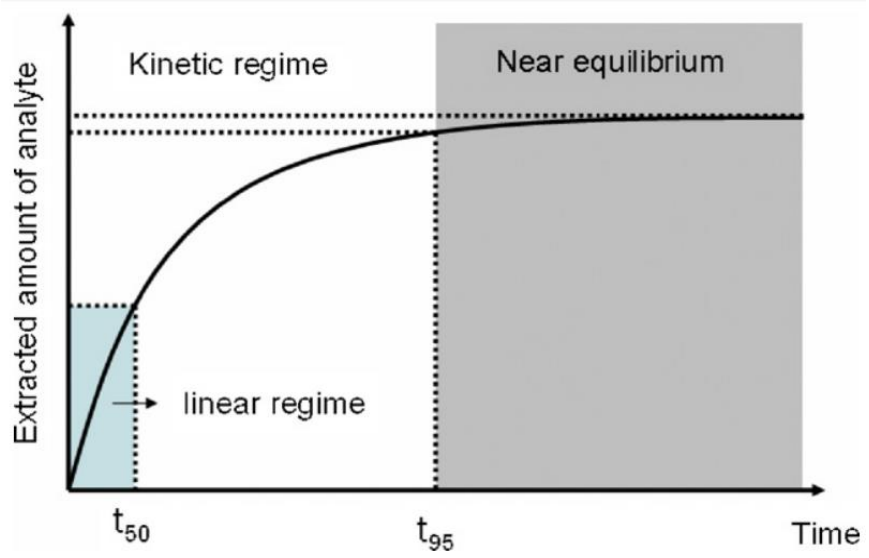

Figure 1.2 General extraction time profile (Ouyang & Pawliszyn, 2008) [22]

1.4 Solid-Phase Microextraction Coupled Directly to Mass Spectrometry

The use of SPME as a sample preparation step to enhance AIMS techniques has been proposed before the term AIMS was coined.[18] As early as 1999 Kuo & Shiea used an SPME fiber for DEP.[5] SPME allows for fast extractions, particularly when the extraction time falls in the linear calibration regime, and uses very little to no solvent. This has made SPME the most commonly integrated sample preparation technique for AIMS workflows as reported by Kuo *et al.*[7] The benefits of integrating SPME would combat the previously stated challenges of AIMS by reducing matrix effects caused by co-extraction and/or co-ionization of unwanted matrix components and improving limits of detection (LOD) by concentrating the analytes onto the SPME device. The above advantages come without compromising the speed, high throughput capabilities, and ability to apply the workflow to an environment outside of the laboratory. AIMS workflows also takes better advantage of the enrichment factor provided by SPME when compared to LC-MS workflows. This is due to the dilution found in SPME-LC-MS obtained by the desorption of SPME devices in relatively large volumes of desorption solution.[23]

To date, SPME has been integrated with several AIMS techniques including DART, DESI, coated blade spray (CBS) and microfluidic open interface (MOI).[18] SPME is incorporated in DART workflows through the use of coated thin film meshes that extract analytes which are subsequently thermally desorbed during DART.[24] In DESI workflows, SPME can be integrated using coated substrates that have extracted analytes. A stream of charged solvent droplets desorb and ionize analytes off the substrate and into the mass spectrometer.[25] MOI uses a flow isolated desorption chamber open to the ambient environment to contain desorption solution to desorb analytes from an SPME device.[26] MOI transports analytes desorbed from the desorption chamber to the ionization source given that the ionization source provides constant suction.[26] MOI has been

coupled with SPME fibers to desorb and subsequently transport analytes into the ionization source. Figure 1.3 is a schematic of MOI.[26] In CBS a sword shaped stainless steel sheet coated with a polymeric coating is used to extract a sample and subsequently used as the substrate for which ESI occurs.[27] Figure 1.4 is a workflow of CBS.[28] SPME can also be implemented in a high-throughput fashion as shown by Tascon *et at.* 2018.[29] This highlights the ability to develop high throughput SPME-MS workflows that can be used for on-site analysis.

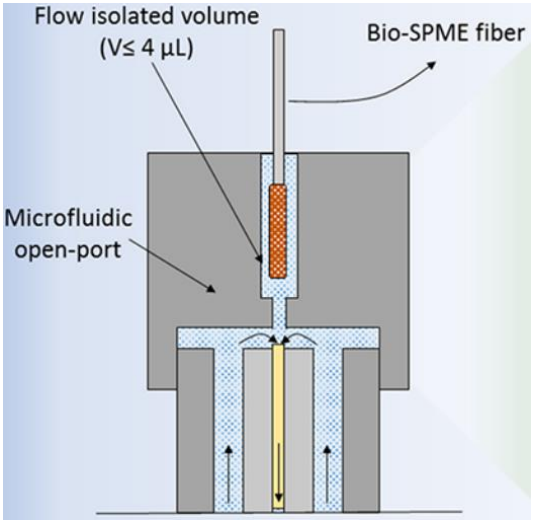

Figure 1.3 MOI schematic (Tascon *et al.*, 2018)[26]

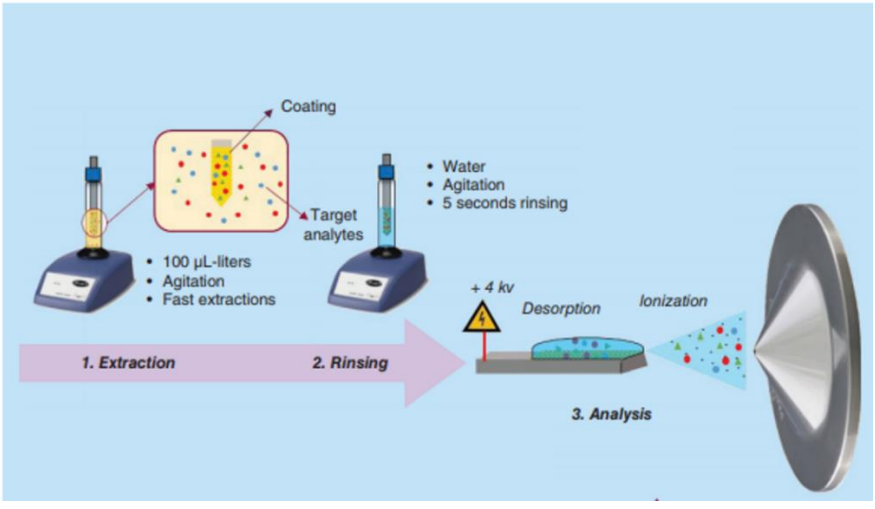

Figure 1.4 CBS workflow (Gómez-Ríos *et al.*, 2018)[28]

1.5 Probe Electrospray Ionization

Probe electrospray ionization (PESI) was introduced by Hiraoka *et al.* in 2007.[30] This technique utilizes a small untreated metal probe with a diameter in the micrometer range and a sharp tip with a diameter usually in the low micrometer or sub micrometer range.[30] The probe moves from position A in Figure 1.5(A) where the tip of the probe picks a small amount of sample upwards to position B where the probe is usually 3mm above the sampling cone of the mass spectrometer, high voltage is then applied to induce ESI as illustrated in Figure 1.5(A).[30] For a liquid sample picked by the probe it is retained as a thin liquid film by surface tension between the sample and the probe when moving from position A to position B.[30] The application of high voltage causes the formation of a Taylor cone from the thin liquid film at the probe's tip.

The picking and spraying of sample during PESI are unique amongst AIMS techniques and has led to the study of its physical parameters to gain a better understanding of the technique's fundamentals. The size of the droplets formed by ESI is dependent on the size of the probe's tip in the same manner as a capillary's size is for ESI emiters.[31] The liquid flow rate to form the droplet is dependent on the amount of liquid loaded onto the needle and the voltage applied.[31] The use of a needle rather than a capillary prevents clogging issues which is also seen in DEP.[5,30] The initial droplets formed by PESI are extremely small which in turn can decrease ion suppression which is also seen in nano-ESI.[31] Yoshimura *et al.* demonstrated that PESI picks up sample volumes in the low pL range using a stainless steel acupuncture needle with a diameter of 140µm and a tip diameter of 700nm. Yoshimura also demonstrated that the volume of sample picked during PESI is nonlinearly dependent on its surface tension and viscosity.

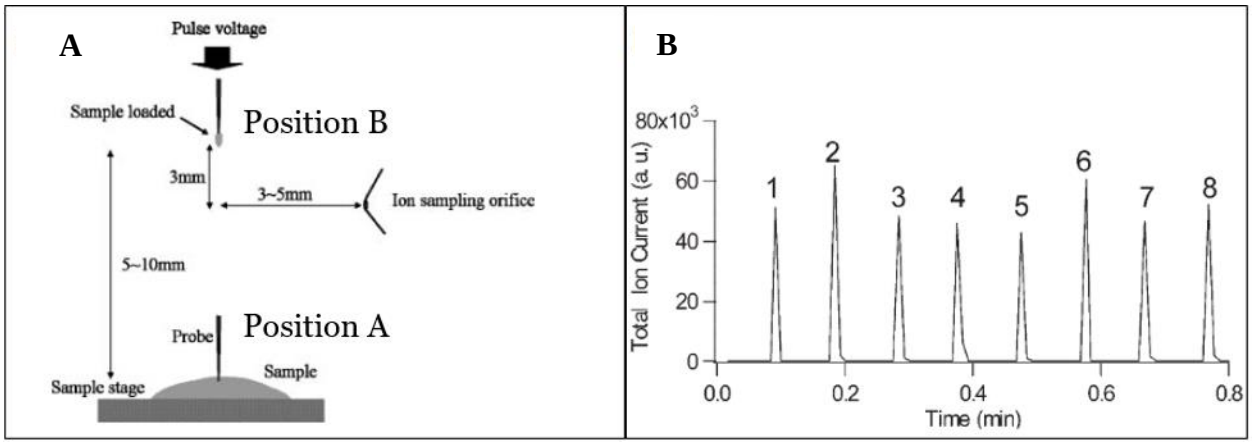

Figure 1.5 (**A**) Schematic of a PESI experiment (**B**) resulting TIC (Hiraoka *et al.*) [30]

Most PESI experiments utilizes a continuous pick and spray process where the needle continuously picks the sample and sprays it multiple times. The resulting total ion chromatograms are characteristic of Figure 1.5(B).[30] The continuous pick and spray process allows PESI to monitor chemical reactions in real time and real time analysis of living animals.[32,33] PESI can also be run such that the sample is only picked and sprayed once, this is referred to as single-shot PESI.[34]

The original configuration of PESI as highlighted in Figure 1.5(A) has two practical shortcomings; the first is that the sample cannot be dry and the second is how close the probe is to the inlet of the mass spectrometer. A variant of PESI, sheath flow PESI (SF-PESI) where the PESI probe protrudes out of a gel loading tip was designed to allow wetting of dry samples and cleaning of the needle. A syringe pump supplies solvent into the gel loading tip allowing for the probe to be wetted before picking a sample. Figure 1.6 shows a schematic of SF-PESI.[35] The biggest samples used in PESI experiments published to date are mice.[36] If samples are moderately sized then the normal PESI setup is unfeasible.[37] Literature shows attempts to remedy this by having the needle spray into an ion sampling tube to extend the distance between the needle and the mass

spectrometer.[37] The introduction of a 200 mm ion sampling tube required the use of an additional vacuum pump to obtain ion transfer efficiencies similar to that of the original PESI setup.[37] Other methods to work around the issue of sampling having to be done so close to the mass spectrometer inlet includes dipping PESI (dPESI). In dPESI the probe picks the sample offline and is allowed time for the sample picked onto the probe to dry. Afterwards the PESI procedure is done with its normal setup as shown in Figure 1.5(A) except at position A the probe picks on a wetting solvent instead of the sample.

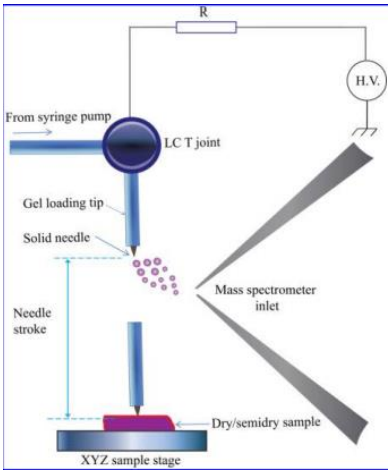

Figure 1.6 Sheath flow probe electrospray ionization (Mandal *et al.* 2013)[38]

With all its various formats, PESI has been used for various applications from the detection of compounds in food stuff, living mice, and whole blood. Quantification of analytes is underrepresented in PESI literature. A large portion of this can be attributed to matrix effects as the lack of sample preparation in most PESI applications leads to less sensitive measurements. Usui and collaborators demonstrated the quantitative capabilities of PESI by performing sample preparation before PESI analysis.[39–42] The most common sample preparation method used was a dilution with a solvent containing internal standards (IS) before a 10µL aliquot was used for PESI-MS. This dilution method is an extremely fast and simple sample preparation method allowing for

near real time analysis. However, analytes are diluted leading to a higher limit of quantitation (LOQ) compared to other sample preparation methods. This is shown by the LOQ of acetaminophen from blood serum and glyphosate from human serum being 1.56μg mL^{-1} for both cases.[40] Usui and collaborators have also quantified MT-45 from tissue via standard addition. In this case QuEChERS was applied for sample preparation.[39] Although an LOQ was not stated for this application depending on the tissue type used the lowest calibration point was either 20 ng mL^{-1} or 80 ng mL^{-1}.[39] The PESI-MS method using QuEChERS had a far lower LOQ compared to the other quantitative PESI workflows due to sample cleanup. Hisatsune *et al.* reported quantification of cyanide in 2μL of whole blood using a 10 min derivatization step before being quantitated by PESI-MS/MS. the reported was LOD of 42 ng mL^{-1}.[43] Further sensitivity increases for PESI can arise from pre-concentrating analytes from samples before MS analysis.

1.6 Solid-Phase Microextraction Probe Electrospray Ionization

SPME has been used as a sample preparation technique for PESI by Bernardo *et al.* and da Silva *et al.*[44,45] In these implementations of SPME-PESI-MS the probe is kept close to the mass spectrometer inlet and desorption solvent is applied to the probe by a system such as a syringe instead of the probe picking the desorption solvent. It should also be noted that the probes used were 500 μm in diameter for these studies. The used probes are significantly bigger than probes typically used for PESI which are 120 μm in diameter. Bernardo *et al.* was able to obtain LOQs of 2.05 ng mL^{-1} and 8.78 ng mL^{-1} for cocaine in saliva and urine, respectively.[44] In the case of da Silva *et al.*, an LOQ of 0.92 μg mL^{-1} for phorbol 12,13-diacetate extracted from *Jatropha curcas*

leaves.[45]

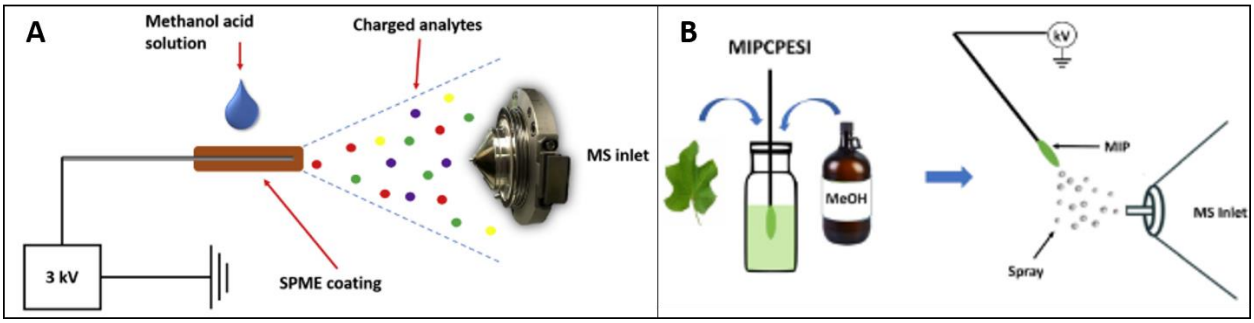

Figure 1.7 (**A**) PESI probe set up from Bernardo *et al*.[44] (**B**) PESI probes set up from da Silva *et al.*[45]

Further coupling of SPME as a sample preparation with commercial PESI probes produced by Shimadzu is of interest. Coating commercial PESI probes (120 µm in diameter, 5 mm in length, and 700 nm tip diameter) which were used in quantitative examples from section 1.5 can yield interesting applications in areas such as prenatal screening and post-mortem analysis. This is due to the possibility of leveraging the preconcentration and extraction of SPME with the small dimension of PESI to analyze samples where minimal amounts are available for analysis directly with an MS. Ultimately the goal of this body of work is to lay the foundation for the above mentioned possible applications. This was done by studying the basic characteristics of SPME-PESI coupled to tandem mass spectrometry (SPME-PESI-MS/MS), leveraging the small dimensions for plasma protein binding, and finally explore challenging compounds. In chapter 1, a study into evaluating the coating of commercial PESI probes, the characteristics of SPME-PESI-MS/MS and finally using SPME-PESI-MS/MS to quantify drugs of abuse from small volumes of plasma was conducted. In chapter 2, the small size of the coated PESI probes was leveraged to perform a proof of concept regarding determination of plasma protein binding of diazepam from

human plasma. Finally, in chapter 3, a basic exploration of screening aminoglycosides with SPME-PESI-MS/MS was attempted.

Chapter 2 Evaluation of SPME-PESI-MS/MS in Simple and Complex Matrices

2.1. Introduction

Screening and quantitating analytes at low cost and high speed has gained much attention in mass spectrometry. To meet this demand there is a focus on AIMS techniques where chromatography is not utilized before the introduction of sample to the mass spectrometer with little to no sample preparation. The lack of chromatography leads to an increase in throughput and a decrease in overall analysis time. The development of DESI and DART popularized AIMS by several aspects including throughput, speed, and reduced cost.[4,46] The number of AIMS techniques have exploded since the introduction of DESI and DART with at least 70 different techniques.[11] Other prominent techniques that have arisen include paper spray, dielectric barrier discharge, and PESI.[30,47,48]

PESI was initially reported by Hiraoka *et al.* in 2007 which consists of lowering a small conical metal probe moving downwards to be dipped into a sample followed by a rapid upwards movement to a position usually 3 mm higher than the mass spectrometer inlet for the application of high voltage and the process is repeated multiple times.[30] PESI allows for the consumption of small amounts of sample as the amount of sample adhered on to the probe is in the low pL per pick and spray cycle.[49] The use of a probe avoids clogging issues found when using capillaries and the small tip of the probe leads to smaller initial droplets being formed by conventional ESI capillaries.[31] PESI has also been shown to be more tolerate to salts when compared to conventional ESI capillaries.[31] PESI has branched out from its original configuration to alternative configurations such as sheath flow PESI and non-proximate PESI.[35,37] Literature has shown PESI

to have a wide variety of applications including rapid drug determination, metabolic profiling of live mice, and chemical profiling of tulips.[33,42,50]

Despite the large amount of interest in AIMS there is not a relatively large uptake for using AIMS in applications. A perspective by Kuo *et al.* 2020 highlights several challenges that AIMS must overcome before more mainstream usage is adopted.[7] The challenges include better reproducibility, quantification, and sensitivity. Integration of sample preparation increases sensitivity and quantification by decreasing matrix effects and/or increasing analyte concentration.[7] In selecting an appropriate sample preparation technique, it should be fast and/or allows for high throughput or else an AIMS workflow would lose much of its benefits when compared to a chromatographic counterpart. Most literature reporting on PESI focuses on qualitative studies. It is important to note that most studies reporting the use of PESI quantitatively has some level of sample preparation namely dilution with organic solvent or QueCHERs.[39–43]

One sample preparation method that is fast, allows for high throughput, and easily integrates into an AIMS workflow is SPME. Kuo *et al.* 2020 highlighted SPME as the most popularized method of sample pre-treatment for AIMS.[7] SPME was initially developed for analysis of organics in water.[51] Since then SPME has matured as a sample preparation technique to many fields from environmental to bioanalytical.[52–54] The integration of SPME to commonly used AIMS workflows such as DESI, DART, and nano-ESI.[55–57] Further introductory information of SPME based AIMS techniques can be found in a review article by Gómez-Ríos & Mirabelli.[18]

SPME was coupled with PESI by Bernardo *et al.* to extract drugs of abuse from oral fluids and urine.[44] In addition, da Silva LC *et al*. used SPME-PESI to extract phorbol esters from *Jatropha curcas* leaves.[45] These conjunctions of coated probes are large enough that the application of desorption solvent is feasible using a pipette or syringe. In this chapter the

development and application of SPME-PESI-MS/MS using commercially available PESI probes coated with polymeric material and the DPiMS-8060 interface was the focus. The probe dimensions made the application of desorption solvent not practical by syringe or pipette, therefore the repetitive pick and spray cycle method was used. The development of the probe was tested using drugs of abuse in phosphate buffered saline (PBS) and then for detecting the drugs of abuse in small volumes of plasma was developed.

2.2 Experimental

2.2.1 Chemicals and materials

LC-MS grade acetonitrile (ACN), isopropanol (IPA), methanol (MeOH), and water (H_2O) were purchased directly from Fisher Scientific (Bartlesville, OK, USA). FA, sodium chloride, potassium chloride, potassium phosphate monobasic, sodium phosphate dibasic, hydrochloric acid, HPLC grade MeOH were purchased from Sigma Aldrich (Oakville, ON, Canada). The following chemical were purchased from Sigma Aldrich (Oakville, ON, Canada) specifically for the synthesis of 1.3µm HLB particles; divinylbenzene, N-vinylpyrrolidone, and 2,2-Azobis(isobutyronitrile). The analytical standards and their deuterated analogues were purchased from Cerilliant Corporation (Round Rock, TX, USA): buprenorphine, codeine, diazepam, fentanyl, lorazepam, nordiazepam, oxazepam, propranolol, buprenorphine-d_4, codeine-d_3, diazepam-d_5, fentanyl-d_5, lorazepam-d_4, nordiazepam-d_5, and propranolol-d_7. The deuterated analogue of the standards was used for IS correction when applicable. The exception was for oxazepam where nordiazepam-d5 was used as the internal standard when applicable. Frozen, pooled gender, non- filtered human plasma with K_2EDTA as the anticoagulating agent was

purchased from Bioreclamation IVT (Westbury, NY, USA). PESI probes and sample plates were kindly donated by Shimadzu Corporation (Kyoto, Japan).

Methanolic working standards were prepared from the master standards of analytes listed in Table 2.1 with concentrations such that only a maximum of 1% organic working standard was spiked into samples of PBS or plasma. This was to prevent alterations to the matrix that can measurably affect either the equilibrium constant between the coated probe and the sample.

The HLB synthesis procedure and PBS preparation procedure from the supplemental information of Vasiljevic *et al*. was followed.[58,59] An in-house built stage equipped with a motor (MTS50/M-Z8E, 50 mm) from ThorLabs Inc. (Newton, MA, USA) was used for dip coating the PESI probes.

2.2.2 LC-MS/MS Instrumentation and Method

LC-MS/MS experiments were conducted on a Shimadzu LCMS 8060 (Kyoto, Japan) triple quadrupole mass spectrometer with Shimadzu LC-30AD liquid chromatography system (Kyoto, Japan). Detailed information on the selected reaction monitoring transitions used to quantify analytes for the LC-MS/MS experiments can be found in Table 2.1. Further experimental conditions about the LC-MS/MS method can be found in Table 2.2 and Table 2.3. The autosampler was thermostated to 4˚C and used for injection of 3µL or 6µL of PBS or plasma extracted samples, respectively. A Phenomenex Kinetex PFP column (2.1 x 100 mm x 1.7 µm particle size) was purchased directly from Phenomenex (Torrance, CA, USA) was used for separation. The column oven was thermostated to 35˚C and the flow rate used was 300 µL/min. Mobile phase A was water while mobile phase B was MeOH/ACN (v/v, 7/3) and both mobile phases contained 0.1% formic acid. The gradient was run at 10% B for 1.0 min, linearly ramped to 100% B until 7.0 min, and

held at 100% B until 9.0 min. The column returned to 10% B at 9.2 min and allowed to re-equilibrate until 11.0 min.

Table 2.1 Multiple Reaction Monitoring Parameters for Drugs of Abuse

#	Compound	Internal Standard	LogP	Precursor Ion (m/z)	Product Ion (m/z)	Q1 Pre-Bias (V)	Collision Energy	Q3 Pre-Bias (V)
1	Buprenorphine	Buprenorphine-d_4	4.98[a]	468.3	**55.1**	-11	-50	-21
1	Buprenorphine	Buprenorphine-d_4	4.98[a]	468.3	396.3	-11	-39	-30
2	Buprenorphine-d_4			472.3	**59.2**	-11	-52	-22
3	Codeine	Codeine-d_3	1.39[a]	300.2	**165.2**	-11	-42	-11
3	Codeine	Codeine-d_3	1.39 [a]	300.2	215.2	-11	-24	-15
4	Codeine-d_3			303.2	**215.1**	-11	-26	-15
5	Diazepam	Diazepam-d_5	2.82 [a]	285.0	193.1	-11	-30	-13
5	Diazepam	Diazepam-d_5	2.82 [a]	285.0	**154.1**	-11	-27	-16
6	Diazepam-d_5			290.3	198.1	-11	-32	-21
7	Fentanyl	Fentanyl-d_5	4.05 [a]	337.2	**188.3**	-10	-24	-13
7	Fentanyl	Fentanyl-d_5	4.05 [a]	337.2	105.1	-10	-38	-20
8	Fentanyl-d_5			342.3	**188.2**	-13	-24	-13
9	Lorazepam	Lorazepam-d_4	2.39 [a]	321.0	275.1	-12	-21	-20
9	Lorazepam	Lorazepam-d_4	2.39 [a]	321.0	**229.1**	-12	-29	-24
10	Lorazepam-d_4			325.2	**279.0**	-10	-24	-19
11	Nordiazepam	Nordiazepam-d_5	2.79 [a]	271.0	**140.1**	-10	-26	-25
11	Nordiazepam	Nordiazepam-d_5	2.79 [a]	271.0	165.1	-10	-27	-17
12	Nordiazepam-d_5			276.2	**213.2**	-11	-27	-15-
13	Oxazepam	Nordiazepam-d_5	2.24 [a]	286.9	**241.1**	-11	-24	-16
13	Oxazepam	Nordiazepam-d_5	2.24 [a]	286.9	269.1	-14	-16	-19
14	Propranolol	Propranolol-d_7	3.48 [a]	260.4	116.2	-10	-17	-22
14	Propranolol	Propranolol-d_7	3.48 [a]	260.4	**183.1**	-10	-18	-12
15	Propranolol-d_7			267.2	**116.2**	-10	-19	-12

[a] LogP values retrieved from Drug bank, accessed in April 2020

Note that product ions bolded are the quantitative ions. Pause time and dwell time for all compounds were both 1 msec

Table 2.2 MS conditions for LC-MS/MS

MS parameters on the LCMS-8060	
Ionization Mode	ESI
Interface voltage	4.0 kV (positive)
Interface temperature	300 °C
Desolvation line temperature	250 °C
Heating block temperature	400 °C
Nebulizing gas flow	3.0 L/min
Drying gas flow	10.0 L/min
Heating gas flow	10.0 L/min
Collision gas and pressure	Argon, 270 kPa
Dwell Time	10 ms

Table 2.3 LC Conditions

	Optimized LC conditions		
Column	Phenomenex Kinetex PFP Column 2.1 x 100 mm, 1.7 µm particle size, 100Å Torrance, CA, USA		
Mobile phase	A: water with 0.1% formic acid B: methanol/acetonitrile (v/v 7/3) with 0.1% formic acid		
Flow rate	300 µL min^{-1}		
Column temperature	35 °C		
Autosampler temperature	4 °C		
Sample injection volume	3 µL for PBS extracts 6 µL for PBS extracts		
Gradient	Time (min)	%A	%B
	0.0	90	10
	1.0	90	10
	7.0	0	100
	9.0	0	100
	9.2	90	10
	11.0	90	10

2.2.3 SPME-PESI-MS/MS Instrumentation and Method

SPME-PESI-MS/MS experiments were conducted using a Shimadzu DPiMS-8060 interface (Kyoto, Japan) and a Shimadzu LCMS 8060 (Kyoto, Japan) triple quadrupole mass spectrometer. Extensive instrumental details and optimized DPiMS-8060 interface and MS/MS parameters are provided in Tables 2.1, 2.4, and 2.5. The outage time in the sample position was 50ms, and the outage time in the ionization position was 200ms. An interface voltage of 2.3kV was applied when the probe was at the ionization position. The total acquisition time per sample was set to 0.56 min.

Table 2.4 DPiMS-8060 Parameters for PESI-MS/MS

	MS parameters on the LCMS-8060	
Extraction Mode	Top Position	-44.00 mm
	Bottom Position	-46.00 mm
	Count	1
	Probe Speed	250.00 mm/s
	Probe Acceleration	1.00 G
Analysis Mode	Ionization Position	-37.00 mm
	Outage time (Ionization Position)	200 ms
	Sample Position	-46.00 mm
	Outage time (Sample Position)	50 ms
	Probe Speed	250 mm/s
	Probe Acceleration	0.63 G

Table 2.5 Optimized MS conditions for SPME-PESI-MS/MS

MS parameters on the LCMS 8060	
Ionization Mode	ESI
Interface voltage	2.3 kV (positive)
Desolvation line temperature	250 ℃
Heating block temperature	30 ℃
Collision gas and pressure	Argon, 270 kPa
Pause Time	1 ms
Dwell Time	1 ms

2.2.4 Preparation of Coated PESI probes

A 7% (weight/volume) solution of polyacrylonitrile (PAN) in dimethylformamide (DMF) was prepared and will be referred to as the coating binder. A slurry with a composition of 9.2% 1.3μm HLB particles, 87.9% of coating binder, and 2.8% glycerol by weight was then prepared. PESI probes were etched by sonication in dilute HCl (7.4%) for 15 min. The probes were then sonicated in water for 20 min followed by sonication in LC grade MeOH for another 20 min. The etched probes were then dried in a convection oven at 125˚C and were coated the same day as the

etching. The etched PESI probes were dip-coated with the HLB-PAN slurry using an in-house built stage. The tips of the probes were coated with a length of 2 mm and dried in a convection oven at 90˚C. This coating process was repeated until a coating thickness of 6.5 µm was achieved. Prior to using the coated PSEI probes for extractions, the probes were cleaned with a MeOH/ACN/IPA (v/v/v 2/1/1) mixture for 15 min, followed by conditioning with MeOH/H_2O (v/v 1/1) for 15 min.

2.2.5 Assessment of Coated PESI Probes by LC-MS/MS using PBS

All experiments performed in this section used 300µL aliquots of PBS spiked with 10 ng mL^{-1} of standards as samples. In between extraction and desorption, the probes were rinsed with H_2O for 3s and air dried. All experiments also used 50µL of MeOH/ACN (v/v 4/1) as the desorption solution for subsequent LC-MS/MS analysis. All extractions and desorptions were static and performed at room temperature.

ETP of the coated PESI probes in PBS was constructed using the following extraction times in triplicate: 10, 30, 60, 90, and 120 min. Extraction was followed by a 3s rinse in H_2O and a 30 min desorption.

Desorption time profile (DTP) of the coated PESI probes in PBS was constructed via extraction using the optimal extraction time. Afterwards the following time points for desorption were tested in triplicate: 10, 30, 45, 60, and 75 min. Subsequently a second desorption was conducted immediately after the first with fresh desorption solvent for 75 min. The second desorption was used to assess carryover of the analyte.

Intra-probes reproducibility was tested by five cycles of extraction and desorption of coated PESI probes using optimized extraction and desorption conditions. Inter-probe

reproducibility was determined by grouping the probes from the intra-probe reproducibility test by their extraction and desorption cycle.

2.2.6 SPME-PESI-MS/MS Assessments in PBS

In this section 90 min static extractions at room temperature were performed using 300 µL PBS spiked with 10 ng mL^{-1} standards as samples. The extraction was followed by a rinse with H_2O for 3s and air drying the coated PESI probes.

The desorption solution for SPME-PESI-MS/MS was optimized by placing the dry extracted probe into the DPiMS- 8060 interface and 10 µL of desorption solution was applied to the sample plate. Finally, an SPME-PESI-MS/MS run was conducted. The desorption solutions tested varied in ratios of water to organic solvent with 0.1% formic acid. The organic solvents used were ACN, IPA, and MeOH.

To test depletion of the coated PESI probes by SPME-PESI-MS/MS three distinct scenarios were used after extraction, rinsing, and drying the coated PESI probes as follows below:

1) The coated PESI probes underwent a 30 min static desorption into 50 µL MeOH/ACN (v/v 4/1) for LC-MS/MS analysis.

2) The coated PESI probes were used for SPME-PESI-MS/MS using 10 µL of the optimized desorption solvent. Following this the coated PESI probes underwent a 30 min static desorption into 50 µL MeOH/ACN (v/v 4/1) for LC-MS/MS analysis.

3) The coated PESI probes were used for SPME-PESI-MS/MS two consecutive times using the optimal desorption solvent. Following this the coated PESI probes underwent a 30 min static desorption into 50 µL MeOH/ACN (v/v 4/1) for LC-MS/MS analysis.

2.2.7 Extraction from Small Volumes of Plasma

All plasma samples when spiked with analytes were incubated in a 4˚C refrigerator overnight to allow for adequate binding with plasma.

Extraction time profile of the coated PESI probes was conducted using aliquots of 30µL plasma spiked with 10 ng mL^{-1} of standards were statically extracted for the following time points: 10, 30, 45, 60, 75, and 90 min. Extraction was followed by a 3s rinse and a 30 min static desorption in 50µL MeOH/ACN (v/v 4/1) for LC-MS/MS analysis.

SPME-PESI-MS/MS was used to construct calibration curves by extracting 30 µL of plasma with 10 ng mL^{-1} of internal standards and the following concentrations of standards: 1, 5, 10, 25, 50, 75, 100 ng mL^{-1}. Precision and accuracy were determined by three different QC levels which were plasma spiked with the following concentrations of standards: 3, 30, and 90 ng mL^{-1}. Five replicates were used per calibration level and QC level.

2.3. Results and Discussion

2.3.1 Extraction Time Profiles and Coated PESI Probe Reproducibility in PBS

The first major objective was to investigate the reproducibility of coating the PESI probes. Before conducting intra- and inter- probe reproducibility ETP and DTP were performed to ensure adequate signal was obtained and carryover of analytes are minimized. The initial ETP attempted using agitation, however the reproducibility was poor therefore static extractions and desorptions were used. This can be due to the lack of control over the coated PESI probe contacting the sample or desorption solvent.[58] ETPs were determined by the static extraction from PBS spiked with 10 ng mL^{-1} of standards using time points ranging from 10 – 120 min. ETPs can be found in Figure 2.1 and based on these results 90 min was selected as the optimized extraction time despite the

compounds not reaching equilibrium. A 90 min static extraction was used as the optimal time as it allowed for the completion of five consecutive extraction and desorption cycles for the inter and intra probe reproducibility in a practical time frame while attaining high sensitivity. The DTP was determined by the static extraction of spiked PBS for 90 min followed by the desorption for the following time points: 10, 30, 45, 60, and 75 min. A second static desorption step was carried out to assess the carryover of analytes. Results of the desorption time profile experiment show that all analytes are desorbed quantitatively at 10 min. However, the carryover test shows that all compounds carryover percentages of 3.5% or less except propranolol and buprenorphine which had relatively high carryover percentages of 5.0 and 5.3% respectively. Therefore, a desorption time of 30 min was considered as the best desorption time, where all compounds had carryover at or under 3.2%

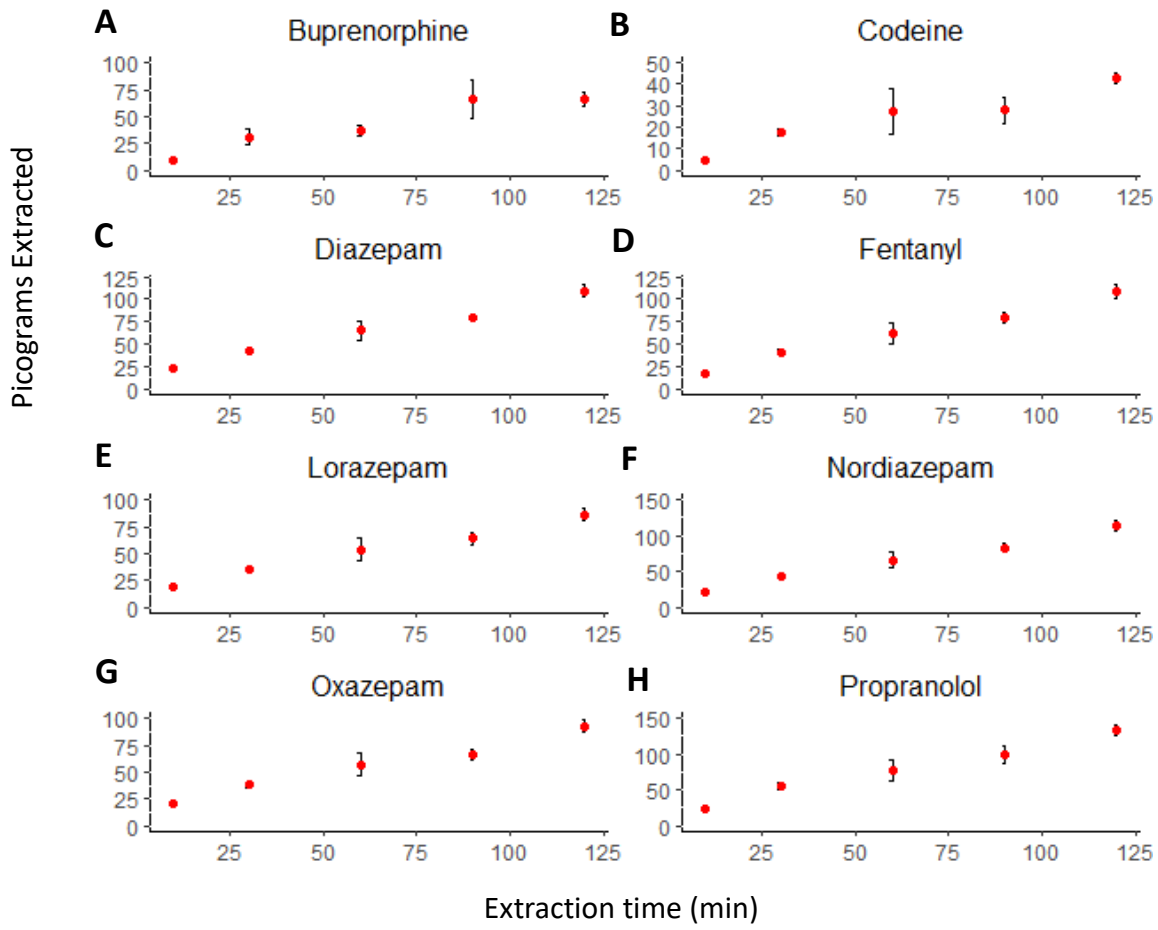

Figure 2.1 Extraction time profile of drugs of abuse in PBS (**A**) buprenorphine (**B**) codeine (**C**) diazepam (**D**) fentanyl (**E**) lorazepam (**F**) nordiazepam (**G**) oxazepam (**H**) propranolol

Intra-probe reproducibility was conducted by five consecutive extraction and desorption cycles using a 90 min extraction time and 30 min desorption time. The intra-probe reproducibility was used to observe the stability of the coated probes and the reusability of the probes. Intra-probe reproducibility was excellent as shown in Table 2.6. Good intra-probe reproducibility was demonstrated by 34 instances where relative standard deviations (RSDs) were 10% or less, 4 instances where RSDs were between 10 – 15%, and two instances where RSDs were between 15 – 20%. Inter-probe reproducibility was determined by grouping the results of the intra-probe reproducibility by the extraction and desorption cycle. The results of the inter-probe

reproducibility show good reproducibility of the etching and coating process as shown in Table 2.7. Good inter-probe reproducibility was demonstrated by 26 instances where RSDs were 10% or less, 11 instances where RSDs were between 10 – 15%, 6 instances where RSDs were between 15 – 20% and one instance where the RSD was 21%. When compared to the SPME mini tips, a similar device the inter- and intra-probe reproducibility were equal or lower than said literature values.[58] Vasiljevic et al. assessed intra-tip reproducibility for SPME mini tips by evaluating the RSDs of five extraction and desorption cycles using 200 ng mL^{-1} of diazepam, nordiazepam, oxazepam, and lorazepam[58]. Only 7 out of the 20 RSDs for the said compounds were 10% or less for the SPME-mini tips.[58] In comparison for the coated PESI probes 18 out of the 20 RSDs for the said compounds were 10% or less. For the inter-tip reproducibility test for the SPME mini tips the lowest RSD between diazepam, lorazepam, nordiazepam, or oxazepam was 18% while the highest RSD for the coated PESI probes for the said compounds was 16%.[58]

Table 2.6 Intra-probe reproducibility of coated PESI probes

	RSD (%) n = 5							
Probe Number	Buprenorphine	Codeine	Diazepam	Fentanyl	Lorazepam	Nordiazepam	Oxazepam	Propranolol
1	16	10	6	8	4	5	4	6
2	12	3	7	9	5	6	6	8
3	7	10	9	10	7	9	8	9
4	6	15	5	4	10	8	10	7
5	9	20	5	4	11	6	11	6

Table 2.7 Inter-probe reproducibility of coated PESI probes

	RSD (%) n = 5							
Extraction Cycle	Buprenorphine	Codeine	Diazepam	Fentanyl	Lorazepam	Nordiazepam	Oxazepam	Propranolol
1	7	14	7	6	7	7	6	6
2	5	20	8	7	14	10	13	8
3	15	21	14	15	15	14	16	15
4	11	7	9	11	6	9	7	9
5	7	8	3	4	4	3	4	5

2.3.2 Basic SPME-PESI-MS/MS Studies

With the extraction conditions optimized for SPME-PESI-MS/MS via the ETP done in the prior section, the desorption solvent was optimized for SPME-PESI-MS/MS. The following desorption solutions were tested: ACN/H_2O (v/v 9/1), ACN/H_2O (v/v 7/3), ACN/H_2O (v/v 1/1), IPA/H_2O (v/v 9/1), IPA/H_2O (v/v 7/3), IPA/H_2O (v/v 3/2), IPA/H_2O (v/v 1/1), IPA/H_2O (v/v 2/3), MeOH/H_2O (v/v 9/1), MeOH/H_2O (v/v 7/3), and MeOH/H_2O (v/v 1/1). All solvents listed above had 0.1%FA added as a modifier. Figure 2.2 shows the results of these experiments using normalized area counts. Area counts were normalized by dividing area counts for all desorption solvents for a particular compound by the desorption solvent that gave the highest average area count. Data for ACN/H_2O (v/v 9/1), ACN/H_2O (v/v 7/3), ACN/H_2O (v/v 1/1), and IPA: H_2O (v/v 9/1) were not included in Figure 2.2. These data points were excluded due to the inconsistency in the generation of spray events when using these desorption solvents. The desorption solvent IPA/H_2O (v/v 1/1) + 0.1% FA was chosen as the optimal desorption solvent as it gave the highest area counts for all eight compounds. Unlike other SPME based ambient mass spectrometry technologies such as CBS the organic solvent used was IPA compared to MeOH.[54] The amount of desorption solvent applied onto the coated PESI probe by the repetitive pick and spray method is heavily influenced by the surface tension and viscosity of the desorption solvent. An investigation by Yoshimura *et al*. shows a positive correlation between the increase in surface tension and viscosity with an increase of the amount of sample picked and retained onto the surface of a PESI probe.[49] The desorption solvent must also be able to sufficiently wet the coating of the SPME PESI probe to allow for adequate transfer of analytes from the coating into the desorption solvent.

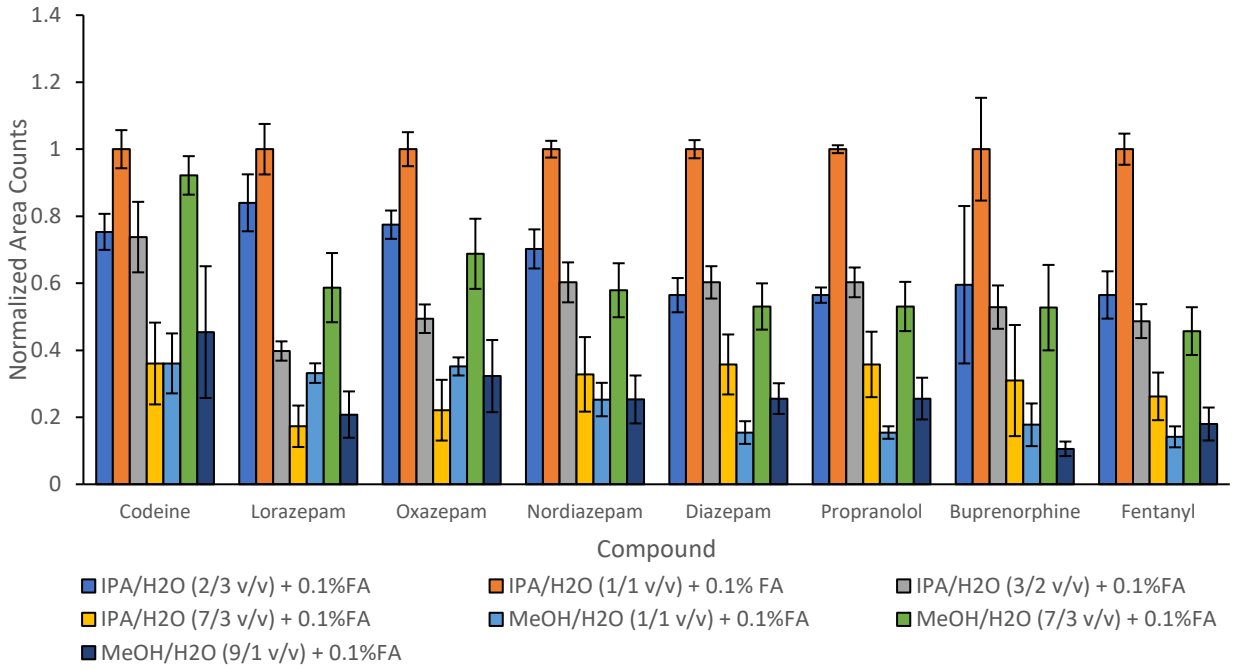

Figure 2.2 Desorption solution optimization for SPME-PESI-MS/MS.

Figure 2.3 illustrates the difference between the electrospray patterns of SPME-PESI-MS/MS and PESI-MS/MS. The signal height is roughly constant for uncoated PESI probes (Figure 2.3(C)). The signal height for the coated PESI probes when sampling analytes spiked into the sample plate as if it were uncoated PESI probes show an increase in signal height until the 9s mark afterwards the signal height is constant (Figure 2.3(B)). The signal height decreases throughout the SPME-PESI-MS/MS run when running extracting spiked PBS (Figure 2.3(A)). The hypothesis for this decrease in Figure 2.3(A) was that significant depletion of the analytes extracted on the coated PESI probe occurred during SPME-PESI-MS/MS.

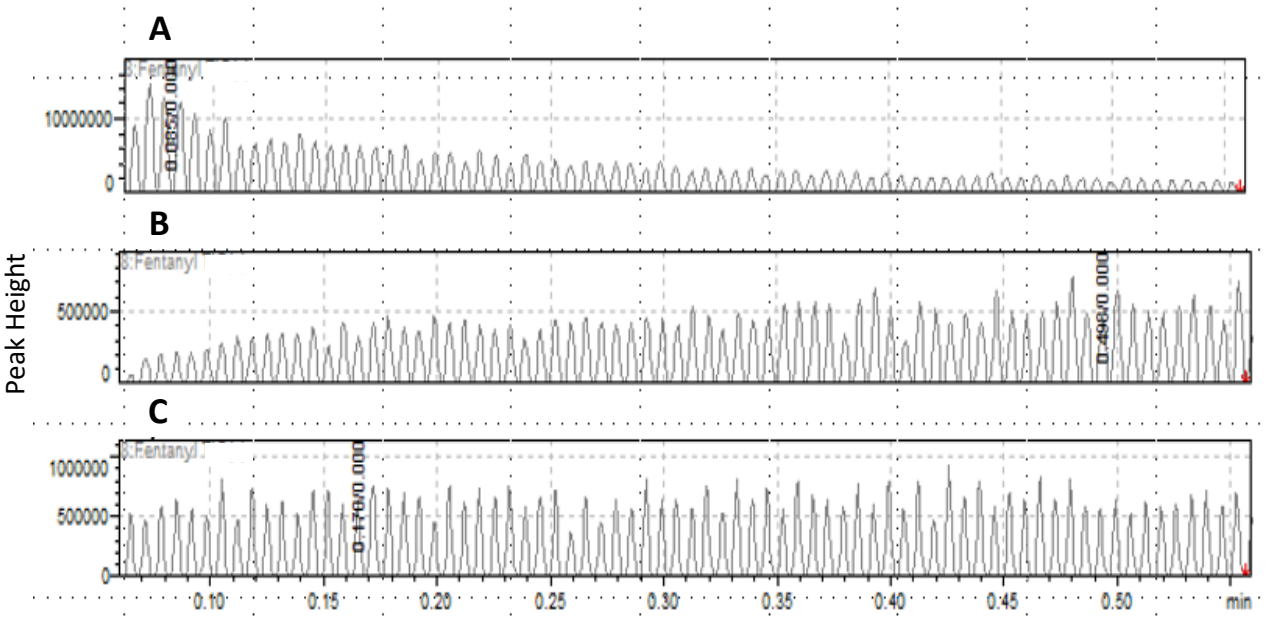

Figure 2.3 Peak heights from the selected ion monitoring of fentanyl (m/z 337.2) (**A**) A coated PESI probe statically extracted 300µL of PBS spiked with 10 ng mL^{-1} fentanyl for 90 min, followed by a 3s rinse in H_2O. Then the coated probe was desorbed using 10µL of IPA/H_2O (1/1 v/v) + 0.1% FA applied to a PESI sample plate by SPME-PESI-MS/MS (**B**) A coated PESI probe was used for PESI-MS/MS of 10µL IPA/H_2O (1/1 v/v) + 0.1% FA spiked with 10 ng mL^{-1} fentanyl applied onto a sample plate. (**C**) An unmodified PESI probe was used for PESI-MS/MS of 10µL IPA/H_2O (1/1 v/v) + 0.1% FA spiked with 10 ng mL^{-1} fentanyl applied onto a sample plate. Figure 2.3 was cropped from LabSolutions © Postrun.

This hypothesis of whether extracted coated PESI probes were significantly desorbed by SPME-PESI-MS/MS was tested by the extraction of spiked PBS sample followed by either of the three scenarios; directly desorbed for LC-MS/MS, used for one SPME-PESI-MS/MS run followed by desorption for LC-MS/MS, or used for two consecutive SPME-PESI-MS/MS runs followed by desorption for LC-MS/MS. Results from this experiment are shown in Table 2.8 expressed as depletion percentages. Depletion percentages are expressed relative to the area counts given by coated probes that were directly desorbed for LC-MS/MS without any SPME-MS/MS experiments (equation 1). One SPME-PESI-MS/MS run shows at least a 45% desorption of a given compound. Interestingly two consecutive SPME-PESI-MS/MS runs do not show a larger desorption

percentage compared to just one SPME-PESI-MS/MS experiment. The desorption percentages are relatively similar. Throughout a single SPME-PESI-MS/MS run it is most likely that the sharp decrease in signal height is due to less analytes on the coating as the run progresses. This is also shown in Table 2.9 where the decrease in area count in the second consecutive SPME-PESI-MS/MS run is expressed relative to the area count in the first SPME-PESI-MS/MS run calculated by equation 2. All analytes show at least a 77% decrease in area count for the second consecutive SPME-PESI-MS/MS run. This also means a single coated PESI probe can not be used for screening and confirmation analysis by SPME-PESI-MS/MS followed by LC-MS/MS, respectively.

Table 2.8 Desorption of Analytes from Coated Probes by SPME-PESI-MS/MS Analyzed Using LC-MS/MS

	Percentage Desorption (%) n = 5							
Number of SPME-PESI-MS/MS runs	Buprenorphine	Codeine	Diazepam	Fentanyl	Lorazepam	Nordiazepam	Oxazepam	Propranolol
1	78	68	57	57	61	54	57	46
2	77	67	65	60	67	62	65	50

$$Percent\ Desorption = \left(1 - \frac{Area\ count\ after\ SPME-PESI-\frac{MS}{MS}runs}{Area\ count\ without\ SPME-PESI-\frac{MS}{MS}run}\right) * 100\% \quad (1)$$

Table 2.9 Desorption of Analytes from Coated Probes by SPME-PESI-MS/MS Between Successive Runs

	Buprenorphine	Codeine	Diazepam	Fentanyl	Lorazepam	Nordiazepam	Oxazepam	Propranolol
Decrease of Signal (%) (n = 5)	89	77	80	81	85	80	81	81

$$Decrease\ of\ Signal = \left(1 - \frac{Area\ count\ of\ SPME-PESI-\frac{MS}{MS}run\ 2}{Area\ count\ \ of\ SPME-PESI-\frac{MS}{MS}run\ 1}\right) * 100\% \quad (2)$$

The shape of the signal for SPME-PESI-MS/MS illustrated by Figure 2.3(A) is due to a single pick and spray not being sufficient for complete desorption of analytes. Xu *et al.* described

the desorption of analytes from the coating to the desorption solvent as partitioning process where at equilibrium the equations below applies.[60]

$$Elu. = \frac{1}{1+K_{fe}\frac{V_f}{V_e}} \quad (3)$$

$$C_e = \frac{n_f}{V_e+K_{fe}V_f} \quad (4)$$

Based on equation 3 the ratio of analyte desorbed from the coated PESI probe, $Elu.$ is dictated heavily by the desorption solvent volume, Ve, when using coated PESI probes with a constant coating volume, Vf. Based on this equation the desorption of nearly all the analytes into the desorption solvent for LC-MS/MS analysis occurs due its relatively high volume of desorption solvent when compared to the coating volume. Based on the equation only a fraction of analytes are desorbed during a single pick and spray for SPME-PESI-MS/MS (equation 4). The amount of desorption solvent picked by SPME-PESI-MS/MS is probably in the same range as the amount of sample picked by PESI-MS/MS which is in the pL range.[49] This explains why each pick and spray for a coated PESI probe has observable signals for both its consecutive uses in SPME-PESI-MS/MS and signal for a subsequent LC-MS/MS run. The decrease in peak area and peak height in only a few pick and spray cycles for SPME-PESI-MS/MS can be related to the moles of analytes remaining on the coated PESI probe if the desorption volume picked is constant between pick and spray cycles as described by equation 4. Each subsequent desorption will have a lower number of moles of analyte remaining on the coated PESI probe therefore the concentration of analytes in the desorption solvent, C_e will also decrease.

2.3.3 Application of SPME-PESI-MS/MS with Plasma

SPME-PESI-MS/MS was then applied to quantitate drugs of abuse in a small volume of plasma. The purpose of using plasma was to demonstrate analyte quantitation from a complex

matrix where its constituents bind to the analytes and can cause ion suppression even if they are extracted in moderate amounts. The ETP of 30µL of spiked plasma was conducted and resulted within an optimal time of 60 min as shown in Figure 2.4 due to most compounds reaching equilibrium at this time. Afterwards, a calibration curve with seven different calibration levels with five replicates per level was constructed. Calibration curves constructed were weighted by a factor of 1/x. Table 2.10 contains factors for the line of best fit while Table 2.11 contains the other figures of merit. Figure 2.5 contains the calibration curves for the eight drugs of abuse. Intra- and inter-day precision and accuracy were assessed using three distinct levels with five replicates per levels: low, middle, and high (3, 30, 90 ng mL^{-1}). The calibration curves show linearity with R^2 values all above 0.9800 with six calibration curves showing R^2 values above 0.9900. LOQ of the drugs of abuse were calculated by determining the lowest calibration point with a signal to noise ratio of 10 or above. Nordiazepam and fentanyl had a LOQ of 1 ng mL^{-1}. Buprenorphine, codeine, diazepam, lorazepam, and propranolol had a LOQ of 5 ng mL^{-1}. Oxazepam had a LOQ of 10 ng mL^{-1}. Intraday precisions were under 15% for all compounds for concentrations above their respective LOQs. Inter-day precisions were under 15% for all compounds except for the middle level of lorazepam and the high level of oxazepam which were 16 and 27% respectively. Accuracies were between 80 – 120% for all compounds for concentrations above their respective LOQs except for the middle level of lorazepam which was 122%.

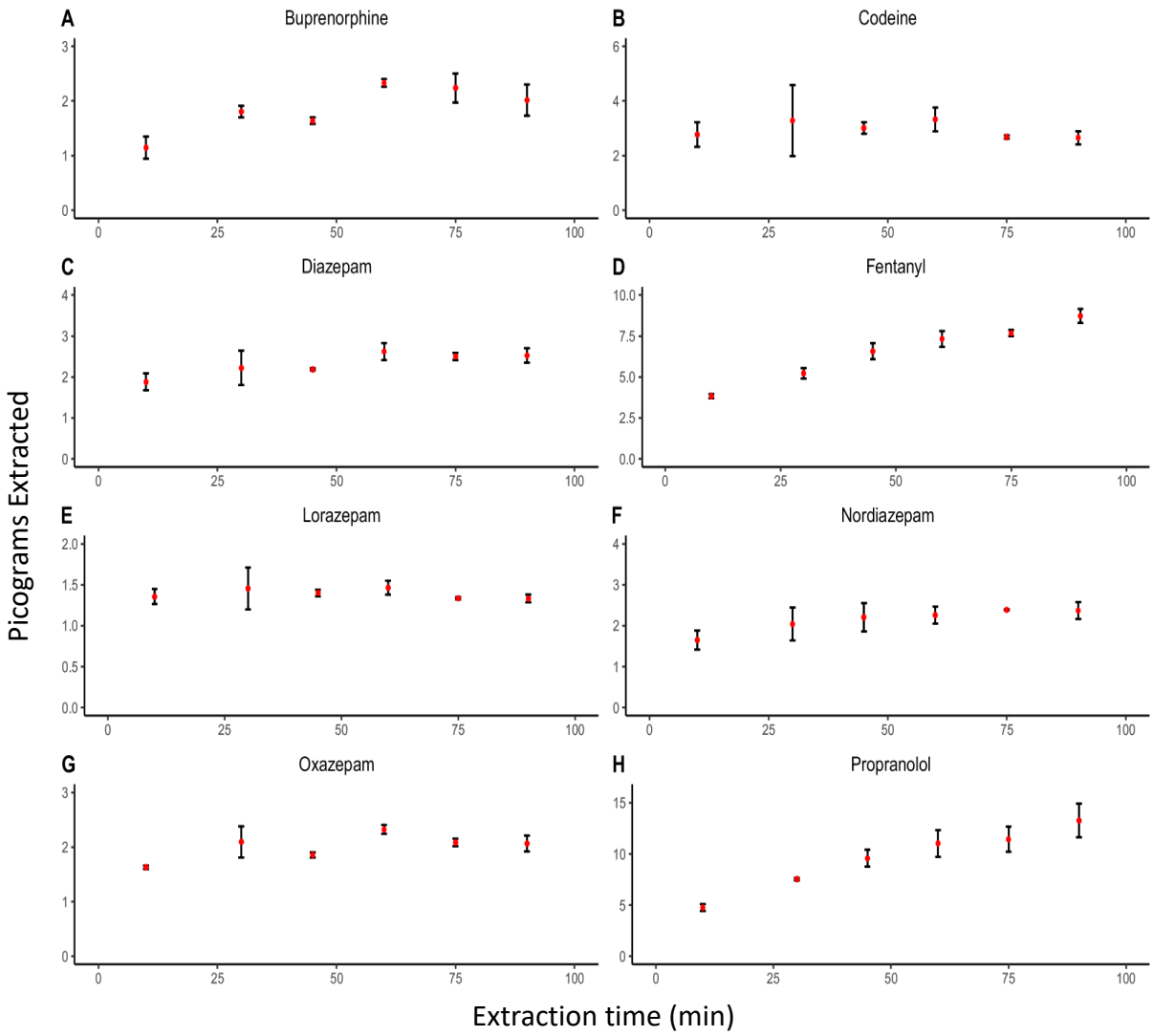

Figure 2.4 Extraction time profile of drugs of abuse from small volume plasma samples by LC-MS/MS (**A**) Buprenorphine (**B**) Codeine (**C**) Diazepam (**D**) Fentanyl (**E**) Lorazepam (**F**) Nordiazepam (**G**) Oxazepam (**H**) Propranolol

Table 2.10 Linearity of Drugs of Abuse Extracted from Small Volumes of Plasma by SPME-PESI-MS/MS

Compound	Range (ng/mL)	LOQ (ng/mL)	slope	intercept	R^2
Buprenorphine	5-100	5	0.1249	-0.0517	0.9921
Codeine	5-100	5	0.1316	-0.0790	0.9912
Diazepam	5-100	5	0.1539	-0.0716	0.9928
Fentanyl	1-100	1	0.0944	-0.0418	0.9968
Lorazepam	5-100	5	0.0172	0.0285	0.9807
Nordiazepam	1-100	1	0.2284	-0.2942	0.9818
Oxazepam	10-100	10	0.1000	-0.0004	0.9976
Propranolol	5-100	5	0.1148	-0.0418	0.9908

Table 2.11 Precision and Accuracy for Drugs of Abuse Extracted from Small Volumes of Plasma by SPME-PESI-MS/MS

Compound	Intra-day Precision (%)			Inter-day Precision (%)			Accuracy (%)		
	Low	Middle	High	Low	Middle	High	Low	Middle	High
Buprenorphine	BQL	8	9	BQL	8	12	BQL	90	103
Codeine	BQL	14	7	BQL	6	8	BQL	96	101
Diazepam	BQL	6	10	BQL	6	8	BQL	91	107
Fentanyl	4	3	2	4	3	4	93	94	105
Lorazepam	BQL	13	13	BQL	16	12	BQL	122	105
Nordiazepam	9	4	6	8	11	8	96	89	98
Oxazepam	BQL	9	11	BQL	13	27	BQL	87	99
Propranolol	BQL	3	2	BQL	3	5	BQL	93	100

BQL is below quantifiable level.
The concentrations of the low, middle, and high validation points are 3, 30, and 90 ng mL^{-1}.

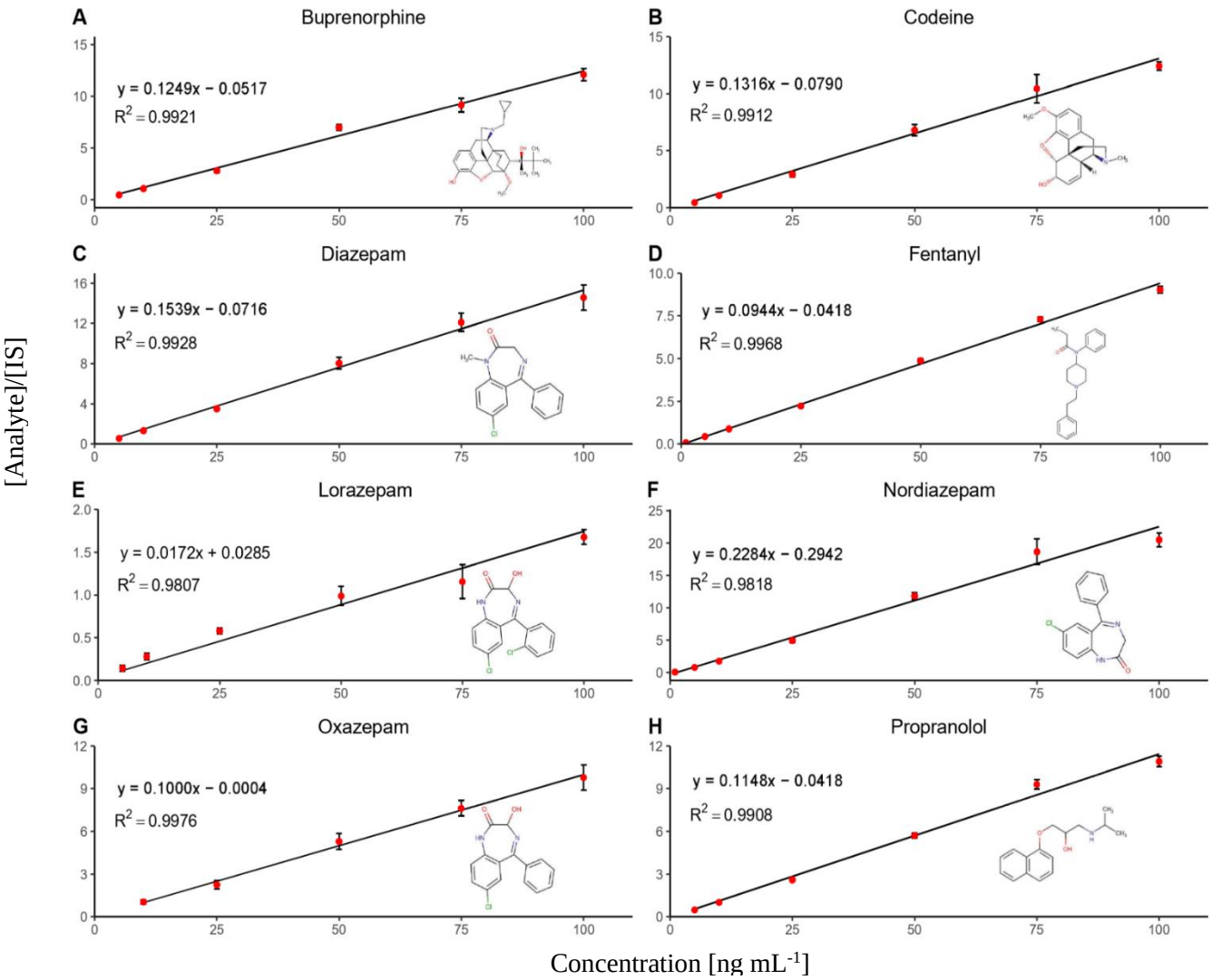

Figure 2.5 Calibration curves from extracting drugs of abuse from small samples of plasma followed by analysis by SPME-PESI-MS/MS (**A**) Buprenorphine (**B**) Codeine (**C**) Diazepam (**D**) Fentanyl (**E**) Lorazepam (**F**) Nordiazepam (**G**) Oxazepam (**H**) Propranolol

2.4. Conclusion and Future Perspective

The presented section shows the development of SPME-PESI in three major sections. The first section is proof that the coating procedure for the coated PESI probes is reproducible based on the low RSDs for intra- and inter-probe reproducibility. In second section the technique of SPME-PESI-MS/MS was further explored through three major investigations. The first

investigation explored which desorption solvent gives the best signal and the factors that influence these. The second investigation was to prove that SPME-PESI-MS/MS depletes the extracted probe and to explore why the ion chromatogram resembles the shape of a decay curve. The third investigation was to prove if SPME-PESI-MS/MS can be used to construct calibration curves. Afterwards SPME-PESI-MS/MS was used to analyze drugs of abuse from small volumes of plasma without any additional pre-treatment steps. Linearity measured by R^2 values exceeded 0.9900 for 6 of the 8 drugs of abuse and for the remaining 2 drugs of abuse R^2 values above 0.9800 was observed. LOQ obtained for fentanyl and nordiazepam were 1 ng mL^{-1}, while the LOQ obtained for buprenorphine, codeine, diazepam, lorazepam, and propranolol were 5 ng mL^{-1}, and the LOQ obtained for oxazepam was 10 ng mL^{-1}.

Further development of SPME-PESI-MS/MS can make it an ideal technique for clinical applications where minimal invasiveness is required or only extremely small sample volumes can be collected. These devices can be ideal tools for application like in-situ analysis and single cell analysis of plants and animals due to its smaller dimensions, where preconcentration by conventional sample preparation techniques is not possible due to limited sample volume. The rapid mass spectrometry determinations of extracted samples by SPME-PESI-MS/MS can make it an attractive technique in areas such as prenatal high throughput screening using multi-well parallel extraction.[53,61] The small dimensions of the coated PESI probes may lead to non depletive extraction from small volumes of sample such that free concentrations of analytes and binding constants can be determined.[61,62] The slow mass transfer kinetics can be improved by exploring sonication.[2]

Chapter 3 Determination of Plasma Protein Binding by SPME-PESI-MS/MS

3.1 Introduction

An important pharmacokinetic/pharmacodynamic parameter that is commonly quantified is plasma protein binding (PPB).[63] PPB is the binding of a drug to plasma proteins.[64] Two major constituents of human plasma known for binding pharmaceuticals in an unspecific manner are human serum albumin and α_1-acid glycoprotein. Other constituents of plasma such as lipoproteins play a role in PPB, but have not been investigated to the same depth as the aforementioned constituents in literature.[65] PPB plays a major role in determining a drug's effect on the body by influencing the free concentration of said drug which in turn influences its clearance and/or concentration available for binding with target receptors.[66] Biological differences between persons and even within the same person can shift the free concentration or PPB of a drug. [67–69] For drug with a narrow therapeutic range this shift can lead to detrimental effects which are not reflected by total concentration measurements.[70,71] In addition therapeutic agents may not have a constant PPB which is assumed when using total concentration measurements for therapeutic drug monitoring (TDM).[70] Ideally free concentration would be used in TDM due to a better correlation with a drug's therapeutic effect compared to the drug's total concentration.[71]

The quantification of PPB is commonly conducted using equilibrium dialysis (ED), and ultrafiltration. ED is considered the gold standard for PPB measurements, and is based on the partitioning of the analyte between plasma and buffer that is separated by a semi-permeable membrane.[64] Once partitioning of the analyte reaches equilibrium, the free concentration can be determined.[64] Disadvantages to ED are labour intensity, long equilibrium times for some

compounds, the possibility of Donnan effects, and the analyte possibly binding to the semipermeable membrane.[72] Alternatively, ultrafiltration uses a device containing upper and lower chambers separated by a membrane with a specific molecular cut-off to separate the unbound fraction of an analyte by centrifugation. During centrifugation, the unbound fraction passes through the membrane into the lower chamber while the bound fraction is unable to pass through the membrane, and is retained in the upper chamber.[64] Ultrafiltration has several advantages over ED, specifically that it is faster and less labour intensive than ED.[64] However, errors in the calculated PPB from ultrafiltration arise when there is nonspecific binding to the membrane, and potential leakage of plasma protein through the membrane.[65]

An alternative to ED and ultracentrifugation for measuring the PPB is SPME. SPME is a microextraction technique where analytes partition between a relatively small amount of sorbent attached to a solid support and the sample.[18] This in turn means that SPME only extract the free fraction of analytes.[22] Therefore the microextractive nature of SPME allows for the accurate determination of analyte free concentration from a sample provided the extraction is non-depletive.[22] Literature points to several examples in which SPME was used to determine either the PPB and/or free concentration of analytes.[62,73–75] Musteata, *et al*., determined the PPB of ibuprofen, warfarin, verapamil, propranolol, and caffeine with SPME.[73] Ferguson, *et al*., investigated the limitations of equilibrium dialysis by comparing PPB calculated by rapid equilibrium dialysis (RED) and SPME.[74] The results show that certain environmental chemicals used did not reach equilibrium by RED while PPB for those chemicals were determined by SPME.[74] PPB measurements by SPME can be conducted on chemicals with a wider range of physiochemical properties than RED, as reported by Ferguson *et al*.[74] The PPB of di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC), an anti-cancer drug that

chelates and has nonspecific binding to several common filtration membranes was determined by SPME by Reimerová *et al* using SPME.[75] The SPME devices in this study consisted of a silicon string support coated with C18 particles and a polydimethylsiloxane binder to avoid the nonspecific binding issues that conventional methodologies had with DpC.[75] Research has also demonstrated that a 96 well plate format of SPME can be automated to decrease labour associated with a workflow.[61]

Musteata commented that the incremental progress of free concentration measurements for TDM were in part due to the speed and affordability advantages that total concentration measurements have.[71] To increase the adoption of free concentration measurements for TDM the speed and affordability of SPME based free concentration measurements can be pushed further. One method is to use SPME based AIMS techniques as the lack of chromatography can increase speed and affordability. A recent study by Rickert *et al.* highlighted the use of CBS as a promising alternative for the TDM of the total concentration of four immunosuppressants.[54] In a similar manner SPME-PESI-MS/MS can be a promising technique for free concentration based TDM.

This chapter demonstrates a proof of concept for obtaining free concentration or PPB by SPME-PESI-MS/MS. The rationale behind using this technique is two-fold. The first reason is that other SPME based AIMS techniques for example CBS uses a much larger extraction phase which would disturb the equilibrium between the drug and the matrix.[76] The volume of sample needed for most SPME based AIMS techniques to extract negligible amounts of a therapeutic agent (under 1% recovery) is generally not practical for clinical cases.[77] In the case of SPME-PESI-MS/MS the coating is relatively small therefore less likely to deplete the target drug from the sample based on previous studies.[76] Secondly the use of nano-ESI emitters to couple small SPME fibers directly to a MS is prone to the possibility of clogging.[30] SPME-PESI-MS/MS on the other hand is not prone

to clogging. The measurement of PPB by any AIMS techniques was not reported in publication yet during the writing of this thesis.

3.2. Materials and Method

3.2.1 Chemicals and materials

LC-MS grade ACN, IPA, MeOH, and H_2O were purchased directly from Fisher Scientific (Bartlesville, OK, USA). FA, sodium chloride, potassium chloride, potassium phosphate monobasic, sodium phosphate dibasic, hydrochloric acid, HPLC grade MeOH were purchased from Sigma Aldrich (Oakville, ON, Canada). The following chemical were purchased from Sigma Aldrich (Oakville, ON, Canada) specifically for the synthesis of 1.3µm HLB particles; divinylbenzene, N-vinylpyrrolidone, and 2,2-Azobis(isobutyronitrile). Diazepam and diazepam-d_6 were purchased from Cerilliant Corporation (Round Rock, TX, USA). Frozen, pooled gender, non-filtered human plasma with K_2EDTA as the anticoagulating agent was purchased from Bioreclamation IVT (Westbury, NY, USA). PESI probes and sample plates were kindly donated by Shimadzu Corporation (Kyoto, Japan).

Methanolic working standards were prepared from the stock solutions of diazepam and diazepam-d_6. Plasma and PBS samples were spiked such that no more than 1% organic working standard was added. This was done to ensure that no alterations occurred in the matrix that can measurably affect either the equilibrium constant between the coated probe and the sample or the plasma protein binding of the analyte.[19,62] Working standards were stored in -80 ˚C. All plasma samples when spiked with analytes, and then incubated at 4 ˚C for a minimum of 12 hours to allow for adequate binding with the plasma.

The HLB synthesis procedure and PBS preparation procedure from the supplemental information of Vasiljevic *et al.* was followed.[58,59] An in-house built stage equipped with a motor (MTS50/M-Z8E, 50 mm) from ThorLabs Inc. (Newton, MA, USA) was used for dip coating the PESI probes. A VWR Thermal Shake Touch benchtop agitator was used when maintaining a temperature of 37 ˚C during static extractions. Before extractions, spiked plasma or PBS samples were heated to 37˚ for 30 min with no agitation with the benchtop agitator.

3.2.2 LC-MS/MS Instrumentation and Method

See section 2.2.2 for the LC-MS/MS instrumentation and method, the only modification is that only transitions for diazepam and diazepam-d_6 were used.

3.2.3 SPME-PESI-MS/MS Instrumentation and Method

See section 2.2.3 for the SPME-PESI-MS/MS instrumentation and method, the only modification is that only transitions for diazepam and diazepam-d_6 were used.

3.2.4 Preparation of Coated PESI probes

See section 2.2.4 for the coating procedure for the PESI probes.

3.2.5 Extraction Time Profiles

ETPs of coated PESI probes in PBS or plasma were determined by the static extraction of 1.5mL aliquots of sample spiked with 10 ng mL^{-1} of diazepam held at 37˚C for the following time points in triplicates: 10, 30, 45, 60, 75, and 90 min. Following the extraction, the probes were rinsed for 3s with H_2O, and then statically desorbed for 30 min in 50µL MeOH/ACN (v/v 4/1). The extracts were then analyzed by LC-MS/MS. PPB was calculated using the equation below

$$PPB = \left(1 - \frac{[Concentration\ from\ plasma]}{[Concentration\ from\ PBS]}\right) * 100 \quad (5)$$

3.2.6 Calibration Curves for determination of PPB

A calibration curve with IS correction and a weighting of 1/x was constructed using the following concentrations of diazepam spiked in 1.5 mL aliquots of PBS (n = 5): 0.05, 0.1, 0.25, 1, 5, 10, and 25 ng mL^{-1} using a 60 min static extraction at 37°C. Aliquots of 1.5mL plasma spiked with 25 ng mL^{-1} diazepam (n = 5) were also analyzed using the above extraction conditions. After extraction, a 3s rinse with H_2O was conducted followed by air drying. The dried probes were used for SPME-PESI-MS/MS using 10µL of IPA/H_2O (v/v 1/1) + 0.1% FA. The calibration curve constructed from PBS extracts was used to calculate the free concentration of diazepam from the plasma sample using equation (5).

3.3 Results and Discussion

3.3.1 LC-MS/MS Confirmation of PPB

Before starting any experiments with SPME-PESI-MS/MS, the capability of the coated PESI probes to measure the PPB of diazepam from human plasma was evaluated by LC-MS/MS. ETPs for PBS and plasma were constructed from the LC-MS/MS experiments as shown in Figure 3.1. Based on Figure 3.1(A), the equilibrium time for static extraction of diazepam from PBS at 37 ˚C was approximately 60 min. The equilibrium time for the static extraction of diazepam from plasma at 37 ˚C was approximately 30 min based on Figure 3.1(B). The recovery of diazepam from PBS and plasma were determined by dividing the ng extracted by the coated PESI probe at the 60 min time point calculated using an instrumental calibration curve by the ng of diazepam spiked into the sample. The recovery of diazepam from PBS and plasma at 60 min were 0.90%

and 0.015% respectively. This is under the 1% recommended recovery limit to prevent depletion of the sample.[77,78] Depletion of the analyte from the sample can result in changes to the "true" equilibrium between the free and bound concentrations of analytes in the matrix. This will ultimately lead to an incorrect determination of the PPB because the new equilibrium will not reflect the actual free concentration and PPB. The PPB of diazepam obtained by LC-MS/MS analysis was 98.4±0.5%, 98.2±0.2%, and 98.4±0.2% for the time points 60, 75, and 90 min, respectively. This was calculated by equation (3) using concentrations of extracted diazepam calculated from an instrumental calibration. When compared to literature values, there is close agreement, with reported PPBs ranging from 97 – 99% in human plasma.[64,67,79–81] Therefore capability of determining PPB by the coated PESI probes were confirmed by LC-MS/MS from these experiments. Therefore, PPB experiments by SPME-PESI-MS/MS can be conducted.

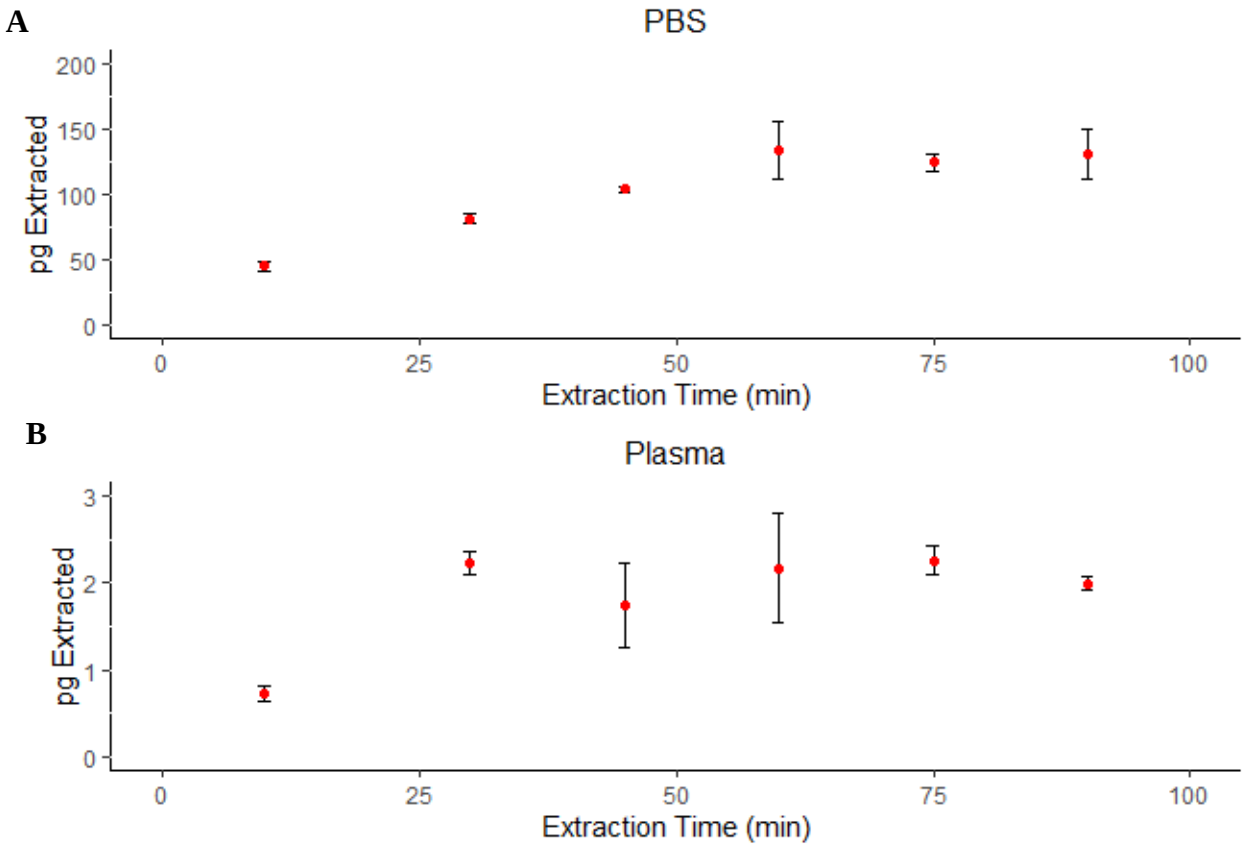

Figure 3.1 Extraction time profile of diazepam in (**A**) PBS (**B**) plasma.

3.3.2 Calibration Curve and Comparison of PPB Comparison of Values

The calibration curve constructed by SPME-PESI-MS/MS (Figure 3.2) by extracting spiked PBS with diazepam was used to calculate a PPB of 99.3 ± 0.2% which was similar to the PPB of 98.4±0.5% calculated in the previous section for the same extraction times. The calibration curve had a high linearity based on an R^2 of 0.9947 and RSDs of <14%. The concentrations of diazepam from plasma and PBS used to calculated PPB were calculated using the linear regression from Figure 3.2. The PPB calculated for diazepam by SPME-PESI-MS/MS was within the literature values of 97 – 99%.[64,67,79–81]

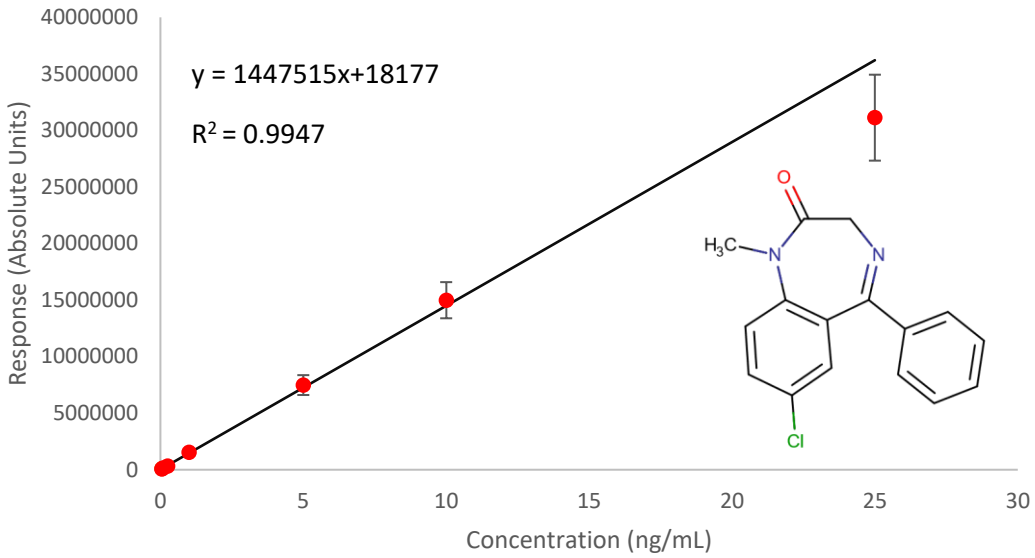

Figure 3.2 Calibration curve of diazepam extracted from PBS using SPME-PESI-MS/MS.

3.4 Conclusion and Future Perspectives

Based on the agreement of the PPB values generated by SPME-PESI-MS/MS and LC-MS/MS of 99.3±0.2% and 98.4±0.5%, respectively, and its agreement with reported literature values of 97-99%.[64,67,79–81] Therefore SPME-PESI-MS/MS has been demonstrated to be an effective tool for accurately calculating the PPB and determining the free concentration of diazepam. Furthermore, this highlights the ability to quantitate PPB by AIMS, which has not been reported yet as of the submission of this thesis. The ability to conduct AIMS workflows that can determine the free concentration, have high throughput, and have lower workflow times compared to LC-MS/MS methods provide an attractive alternative to current therapeutic drug monitoring methods that rely on determining total concentration.[71] To take the determination of PPB and free concentration from the proof of concept demonstrated in this section to a more applicable form for applications such as TDM the equilibrium time of samples should be reduced as well as coupled to portable mass spectrometry technologies. The small size of the SPME PESI probe can be

leveraged for TDM directly from relevant tissue in-vivo for more biologically significant measurements.

Chapter 4 Exploring the Screening of Aminoglycosides by SPME-PESI-MS/MS

4.1 Preamble

I would like to acknowledge the work done by Dr. Kanchan Sinha Roy to make this chapter possible, particularly the synthesize a nitrogen rich polymer for extracting aminoglycosides, help with designing several experiments conducted, and interpretation of experimental data.

4.2 Introduction

Aminoglycosides are a class of broad-spectrum antibiotics that inhibit protein synthesis.[82] Aminoglycosides are generally characterized by linking at least two amino sugars via a glycosidic bond with an aminocyclitol ring as shown in Figure 4.1.[83] These compounds are very hydrophilic, highly water-soluble and were among the first antibiotics to be used clinically.[82] However, their cochleotoxicity, nephrotoxicity, ototoxicity, and vestibulotoxicity led a shift away from their prescription towards less toxic antibiotics.[84–86] However, in recent years there has been a shift towards the increased prescription of aminoglycosides for clinical use due to the rise of multi-resistant microbes and a better understanding of dosage regimes for aminoglycosides.[82,87–89] While better dosage regimes help mitigate toxic side effects when used clinically, these compounds are also used in widespread practice as veterinary drugs for farm animals. Veterinary drugs are used in modern farming practices for treating infection or infection prevention.[53] Aminoglycosides as veterinary drugs when misused can be found in foodstuff at residual levels. Despite the residual levels, the toxicity of aminoglycosides may possess human risk.[83] To regulate aminoglycoside

misuse as veterinary drugs, different jurisdictions regulate the use of these compounds through regulations such as Council Directive 96/23/EC.[90]

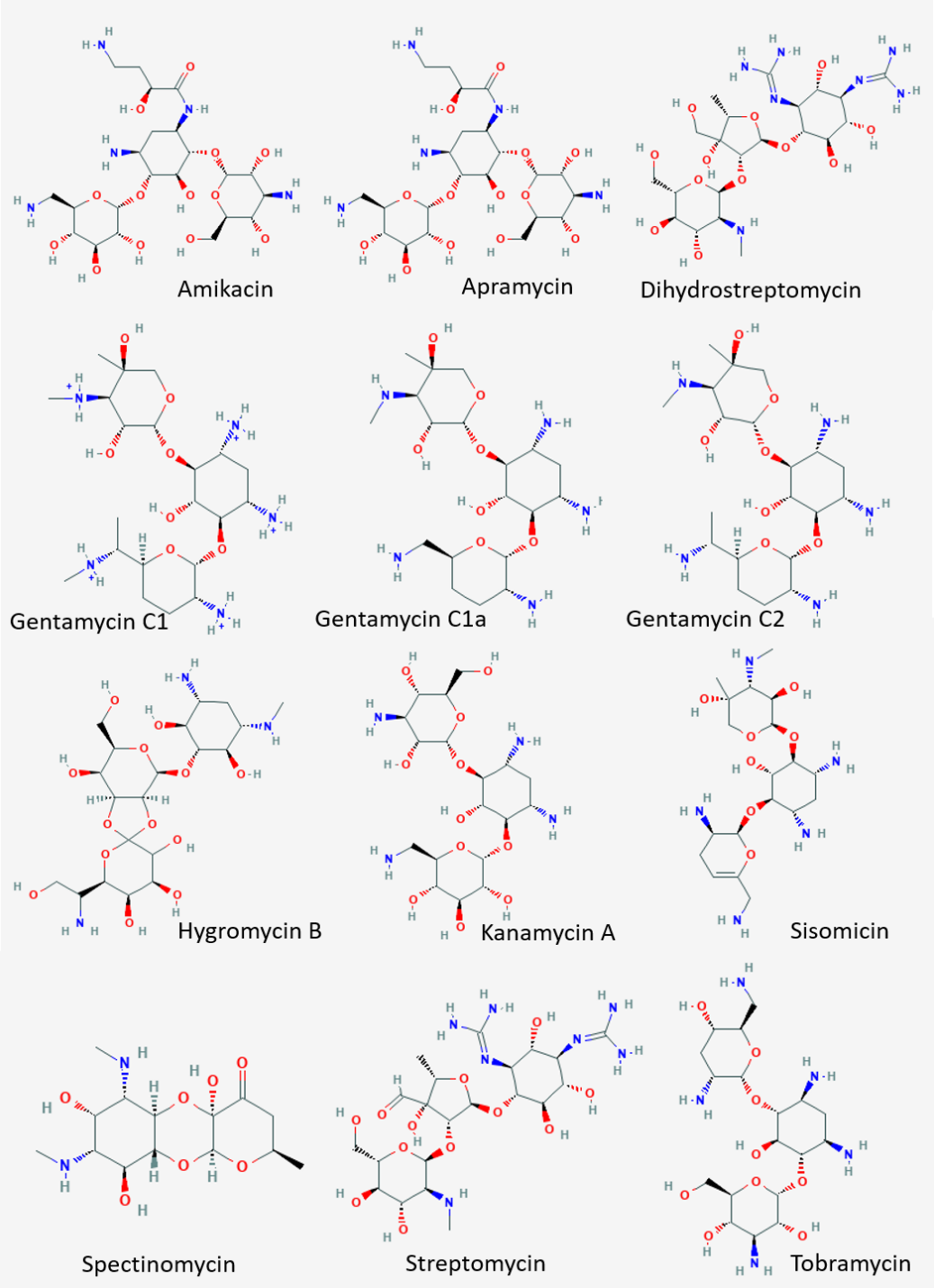

Figure 4.1 Structures of aminoglycosides of interest.

Aminoglycosides are non-metabolizable agents and thus a substantial amount of the original molecules excreted to the environment by living organisms.[83] There are several alternative sources like emissions by hospitals or pharmaceutical company, incomplete elimination in sewage treatment plants, and extensive abuse in farming and aquaculture are also available that have been released considerable amounts of aminoglycosides into the environment.[83] Their presence in the aquatic environment especially drinking water is considered a potential risk. Therefore, aminoglycosides have received great attention towards monitoring their presence in aquatic environments and wastewater effluent to ensure the quality of various types of water samples.[91–94] Monitoring the residual level of aminoglycosides in the aquatic environment particularly wastewater effluents allows for a better understand regarding the usage of aminoglycosides and the rise of aminoglycoside resistance in bacteria.[95,96] On the other hand, the monitoring of aminoglycosides in the aquatic environment is a challenging task as they are highly hydrophilic and water-soluble. The detection, monitoring, and quantification of aminoglycosides in all three areas of clinical samples, foodstuff, and water have driven the development of analytical methods that are fit for purpose.

Analytical methods giving reasonable qualitative and quantitative monitoring of multiple aminoglycosides are difficult due to the certain combination of properties they possess. The lack of a chromophore and fluorophore make certain types of detectors such as UV or fluorescence difficult without derivatization steps.[97,98] The multiple amino and hydroxyl groups that aminoglycosides contain cause high hydrophilicity which also makes it difficult to incorporate into multiresidue methods.[99–101] This was highlighted in a series of articles published by Desmarchelier and colleagues where the determination of aminoglycosides required a completely different sample preparation and LC method for proper screening compared to the other veterinary drugs.[100,102–105]

When multiple aminoglycosides are determined by LC-MS the methods tend to use either ion pairing or hydrophilic interaction liquid chromatograph (HILIC) for separation.[83] The drawbacks of using ion pairing reagents are that they are difficult to remove from the LC-MS, usually not volatile, cause ion suppression, and containment the ion source.[102,106,107] The drawbacks of using HILIC columns stem particularly from the use of zwitterionic columns where large concentrations of salt are needed in addition to FA for proper separation of aminoglycosides.[108] These drawbacks include the use of large concentrations of salt leading to salt precipitation, high organic solvent percentages which lead to poor solubility of aminoglycosides, and long equilibration times.[102,109–111] Overall the drawbacks to both ion pairing and HILIC in the case of aminoglycoside analysis leads to more maintenance of the LC-MS system and reduced operational time. To overcome these issues, AIMS can be used as a screening tool to determine the presence of aminoglycosides. The lack of chromatography and shorter workflow times leads to a screening method that is more economical than LC-MS based screening method as there is no use of high salt concentrations or ion-pairing reagents. To ensure proper sensitivity is reached sample preparation is used to preconcentrate and extract analyte.

In this chapter SPME-PESI-MS/MS was developed to qualitatively screen aminoglycosides from water. The detection of aminoglycosides by SPME techniques are sparse with only two pieces of literature that work with multiple aminoglycosides which are Chen *et al*. and Wang J. *et al*.[112,113] The hydrophilic nature of aminoglycosides demands the use of specialized coatings which Chen et al. and Wang et al have produced.[112,113] For this study, a nitrogen-rich organic polymer was used for the coating of the PESI probes, and various parameters were optimized for the screening of aminoglycosides in water.

4.3 Experimental

4.3.1 Chemicals and Method

The following LC-MS grade chemicals were purchased from Fischer Scientific (Bartlesville, OK, USA): acetone, ACN, IPA, MeOH, and H_2O. Reagent grade dimethylsufoxide (DMSO) was purchased from Sigma Aldrich. The following chemicals were purchased from Sigma Aldrich (Oakville, ON, Canada): acetic acid, FA, hydrochloric acid, LC grade MeOH, N,N-diisopropylethylamine, potassium phosphate dibasic, potassium phosphate monobasic, sodium acetate, sodium bicarbonate, sodium carbonate, and sodium hydroxide. PESI probes and sample plates were donated by Shimadzu Corporation (Kyoto, Japan).

The following solid standards were purchased directly from Sigma Aldrich (Oakville, ON, Canada): amikacin, apramycin sulfate salt, dihydrostreptomycin sulfate, hygromycin B, gentamicin sulfate salt, kanamycin A sulfate, sisomicin sulfate, spectinomycin sulfate, streptomycin sulfate, and tobramycin. Single standard master stocks of the solid standards were made in water such that the free base concentration was 2 mg mL^{-1}. The master stocks were stored at – 20 ˚C. A working stock of 100 µg mL^{-1} of aminoglycosides in water was serially diluted from the master stocks. It is noted that all standards and samples containing aminoglycosides were stored in polypropylene containers to prevent loss of aminoglycosides due to adsorption with glass.[114] Nitrogen rich polymeric material was provided by Dr. Kanchan Sinha Roy.

Acetate buffer was prepared by mixing 1.8g of sodium acetate and 4.6mL of acetic acid in 500 mL of H_2O. The pH of the acetate buffer was adjusted to pH 4 by adding additional acetic acid dropwise until the desired pH was reached. Potassium phosphate buffer (pH = 6) was prepared by mixing 2.4g of potassium phosphate dibasic and 11.8g of potassium phosphate monobasic in 500

mL of H_2O. The potassium phosphate buffer (pH = 6) was adjusted to a pH of 6 by adding 1.0M sodium hydroxide dropwise until said pH was reached. Potassium phosphate buffer (pH = 7) was prepared by mixing 9.4g of potassium phosphate dibasic and 6.3g of potassium phosphate monobasic in 500 mL of H_2O. The potassium phosphate buffer (pH = 7) was adjusted to a pH of 7 by adding 1.0M sodium hydroxide dropwise until said pH was reached. Potassium phosphate buffer (pH = 8) was prepared by mixing 16.3g of potassium phosphate dibasic and 0.89g of potassium phosphate monobasic in 500 mL of H_2O. The potassium phosphate buffer (pH = 8) was adjusted to a pH of 8 by adding 1.0M sodium hydroxide dropwise until said pH was reached. Sodium carbonate buffer was prepared by mixing 3.9g of sodium bicarbonate and 5.7g of sodium carbonate in 500mL of H_2O. The sodium carbonate buffer was adjusted to a pH of 10 by adding 1.0M sodium hydroxide dropwise until said pH was reached.

4.3.2 SPME-PESI-MS/MS Instrumentation and Method

See section 2.2.3 for the SPME-PESI-MS/MS instrumentation and method the only modification is that the MS transition table below, Table 4.1 was used instead of Table 2.1.

Table 4.1 Multiple Reaction Monitoring Parameters for Aminoglycosides

#	Compound	LogP	Precursor Ion (m/z)	Product Ion (m/z)	Q1 Pre-Bias (V)	Collision Energy	Q3 Pre-Bias (V)
1	Spectinomycin	-2.3[a]	351.2	207.2	-13	-22	-14
2	Sisomicin	-4.3[a]	448.1	254.2	-11	-24	-18
3	Gentamycin C1a	-4[a]	450.1	322.2	-11	-14	-23
4	Gentamycin C2	-4.6[b]	464.1	322.2	-11	-15	-23
5	Tobramycin	-5.8[a]	468.1	163.3	-14	-25	-17
6	Gentamycin C1	-4.1[b]	478.3	322.2	-10	-16	-23
7	Kanamycin A	-6.3[a]	485.2	163.2	-10	-25	-11
8	Hygromycin B	-6.4[a]	528.0	352.1	-20	-24	-17
9	Apramycin	-6.5[a]	540.1	217.2	-20	-26	-15
10	Streptomycin	-6.4[a]	582.0	263.2	-22	-32	-19
11	Dihydrostreptomycin	-7.3[a]	583.8	263.2	-20	-32	-19
12	Amikacin	-8.6[a]	586.1	163.2	-22	-33	-11

[a] Drug bank accessed in November 2020
[b] PubChem accessed in November 2020
Pause time and dwell time for all compounds were both 1 msec.
All compounds used $[M+H]^+$ adduct except for spectinomycin where the $[M+H_2O+H]^+$ was used

4.3.3 Preparation of Coated PESI probes

See section 2.2.4 for the coating procedure for the PESI probes. Two modifications from the method discussed in detail in Section 2.2.4 were made. First, the use of nitrogen rich polymeric material was provided by Dr. Kanchan Sinha Roy instead of the in-house synthesized 1.3µm HLB particles. Second, the coating process was repeated until a coating thickness with a radius of 11.5 µm and a length of 3 mm was achieved instead of a coating thickness of 6.5 µm and a length of 2 mm.

4.3.4 Optimization of Screening Conditions

In all optimization of screening conditions, 750µL of final sample after modifications was used for a 90 min static extraction in triplicates. Following the extraction, the probes were dried at room temperature. SPME-PESI-MS/MS was conducted using 10µL of IPA/H_2O (v/v, 1/1) + 0.1% FA as the desorption solvent for pH adjustment and matrix modification experiments. The pH of the spiked water samples were adjusted to the pHs; 4, 6, 7, 8, and 10 using the buffer salts as well as formic acid or N,N-diisopropylethylamine. The concentration of spiked aminoglycosides in the pH modified water samples were 300 ng mL^{-1} of aminoglycosides. For the matrix modification experiments, initially, water was spiked to contain 300 ng mL^{-1} of aminoglycosides. The spiked water was mixed with the following organic solvents: acetone, ACN, DMSO, IPA, and MeOH such that the final samples contained a 50/50 split of spiked water and solvent volumetrically. For further matrix modification experiments, the water sample spiked with 300 ng mL^{-1} of aminoglycosides was modified with DMSO, IPA, and MeOH in a different volumetric ratios (1/3, 1/1, and 3/1) to spiked sample.

For the desorption solvent optimization experiments, the spiked water was modified by the addition of IPA such that the final sample was 3/1 IPA/spiked water (v/v). After extraction, the different compositions of IPA/H_2O ((v/v, 4/1), (v/v, 7/3), (v/v, 3/2), and (v/v, 1/1) all containing 0.1% FA) were tested as desorption solvents to desorb the analytes from the coated PESI probes.

For the rinsing investigations, water was used as a rinsing solvent using the same matrix modification from the desorption solvent optimization. The rinsing was performed immediately after extraction for 3s. For the desorption, optimized desorption solvent was used for SPME-PESI-MS/MS analysis.

4.4 Results and Discussion

4.4.1 Effect of pH

The pH of the extraction sample is an important factor for SPME method development, especially when extracting from aqueous samples. The reason is that only undissociated or neutral analytes are extracted during the SPME process.[19] Therefore, the pH with the highest extraction efficiency for the coated probes will ensure the largest proportion of the neutral aminoglycosides, and result in the highest sensitivity. The pH of the aqueous samples spiked with aminoglycosides was adjusted over the range 4–10.

The pH modification experiments initially conducted used the following buffers; 0.2 M acetate buffer (pH = 4), 0.2 M potassium phosphate buffer (pH = 6), 0.2 M potassium phosphate buffer (pH = 7), 0.2 M potassium phosphate buffer (pH = 8), and 0.2M sodium carbonate buffer (pH = 10). These buffers were spiked with 300 ng mL^{-1} of the aminoglycosides. After the 90 min extraction, signals were not obtained therefore results were not included. This result likely means that no aminoglycosides were extracted from the buffered samples. The cause is likely that metal

ions from the buffer salt chelate with the aminoglyocsides' amino and hydroxyl groups.[111,115] This would then lead to obstruction of the active functional groups of the aminoglycosides that would be capable of interacting with the extraction phase, resulting in no extraction of the analytes which in turn shows no responses of the analytes.[115] The above experiment and associated literature referenced also concludes that the addition of the different salt concentrations is a detriment to the extraction efficiency of the aminoglycosides in an aqueous medium which is in line with literature.[112] Therefore, no experiment was examined to check the effect of salts.

Further pH experiments were performed using an organic acid or base to adjust the pH of the sample to the desired levels as inorganic buffer salts proven to be detrimental for the extraction of aminoglycosides. The pH of the spiked aqueous samples were adjusted to 4, 6, 7, 8, and 10 using FA or N,N-diisopropylethylamine accordingly. The 90 min static extractions at the adjusted pH values show significant responses for the aminoglycosides. This confirmed the detrimental effect of the inorganic salt from the buffers used in the failed pH optimization experiments. The results for the effect of varying sample pH are depicted in Figure 4.2, with the responses normalized with respect to the condition giving the highest response for an individual compound. The results show that the response of all aminoglycosides increased from a pH 4 to pH 6. Afterward, there is a slight increase in the responses at pH 7 and 8 except for apramycin. Around pH 7 to 8, most aminoglycosides are close to or above the pKa values for their amino groups.[116] Therefore, their respective neutral species populations are higher. Two notable exceptions are dihydrostreptomycin and streptomycin. These two aminoglycosides have an a five-fold increase when the pH was increased further to pH 10. All other aminoglycosides, on the other hand, have a decrease in response when shifting from a pH 8 to 10. This is most likely due to the deprotonation of the hydroxyl groups, therefore leading to a decrease in neutral species.[112] The pKa values of the

amino groups for dihydrostreptomycin and streptomycin tend to be higher compared to other aminoglycosides.[116] Based on the response a pH range between 6 to 8 is acceptable as it allows for adequate extractions of all aminoglycosides except for dihydrostreptomycin and streptomycin. Unfortunately, optimizing pH for dihydrostreptomycin and streptomycin will dramatically decrease the response for all other aminoglycosides which is not acceptable.

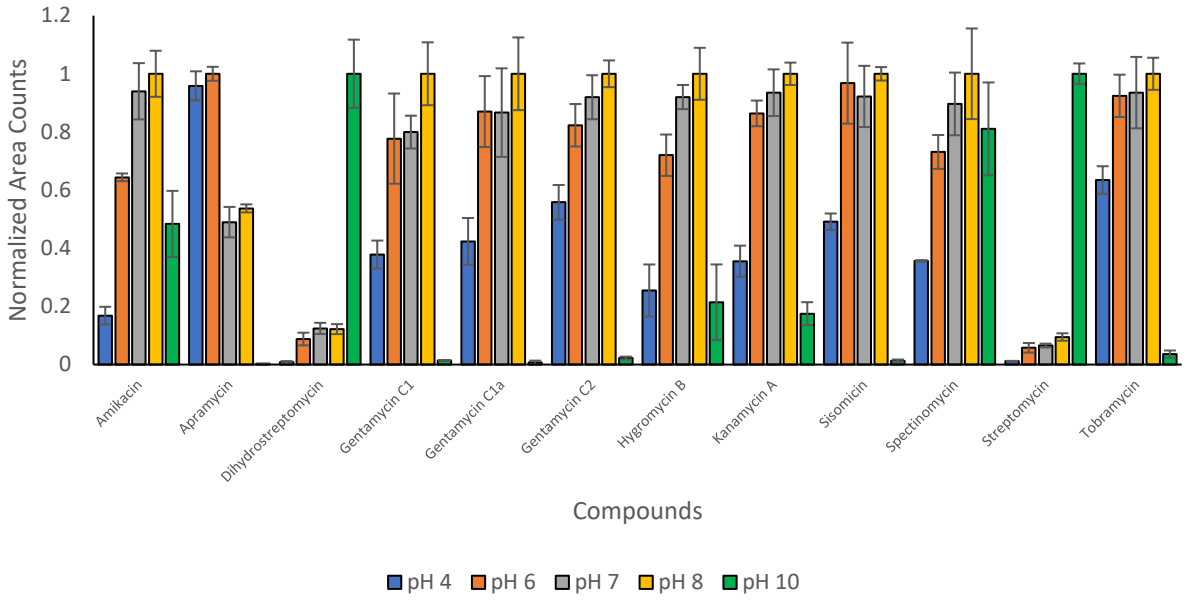

Figure 4.2 pH optimization of aminoglycoside extraction by SPME-PESI-MS/MS.

4.4.2 Effect of Organic Modifier

Results from the pH experiment suggest that pH adjustment alone is not sufficient to obtain reasonable sensitivity for all aminoglycosides from the aqueous sample. An additional parameter is required to increase responses for the aminoglycosides. Therefore, based on Wang *et al.*, who modified the sample with 50% ACN to increase extraction efficiencies, organic modifiers were introduced to modify the spiked aqueous matrix.[113]

In an aqueous matrix, the polarity and solvation effect of water will negatively affect the overall extraction efficiency of aminoglycosides. Therefore, to investigate the effect of these two factors simultaneously and to overcome these effects, different water-miscible organic solvents were used as modifiers. An equal volume of the following organic solvents; acetone, ACN, IPA, MeOH, and DMSO were mixed with equal volumes of water already spiked with 300 ng mL^{-1} of aminoglycosides. The extraction efficiency of these water-modified matrices was compared with the unmodified spiked water matrix. The results are shown in Figure 4.3. Despite having a final concentration of 150 ng mL^{-1}, the modified water matrices tend to show responses similar or higher to the unmodified water matrix which having a final concentration of 300 ng mL^{-1}.

It is evident from the Figures 4.3(A) that ACN and MeOH, both of which are polar solvents, gave a significant increase in the instrumental response for all of the analytes of interest.[117] This could be attributed to the fact that both organic modifiers lower the polarity and solvation effect of the aqueous sample (H_2O has a polarity of 10.2), ultimately enhancing the extraction efficiency.[117] To some extent, MeOH shows better responses than ACN as a modifier. The response of the MeOH modified sample with an aminoglycoside concentration of 150 ng mL^{-1} is comparable to the unmodified aqueous matrix having aminoglycoside concentration of 300 ng mL^{-1}. This indicates that extraction efficiency is enhanced either by MeOH lowering the polarity of water or by MeOH being a protic solvent and having the ability to break the solvation cage between the analytes and water molecules through hydrogen bonding.

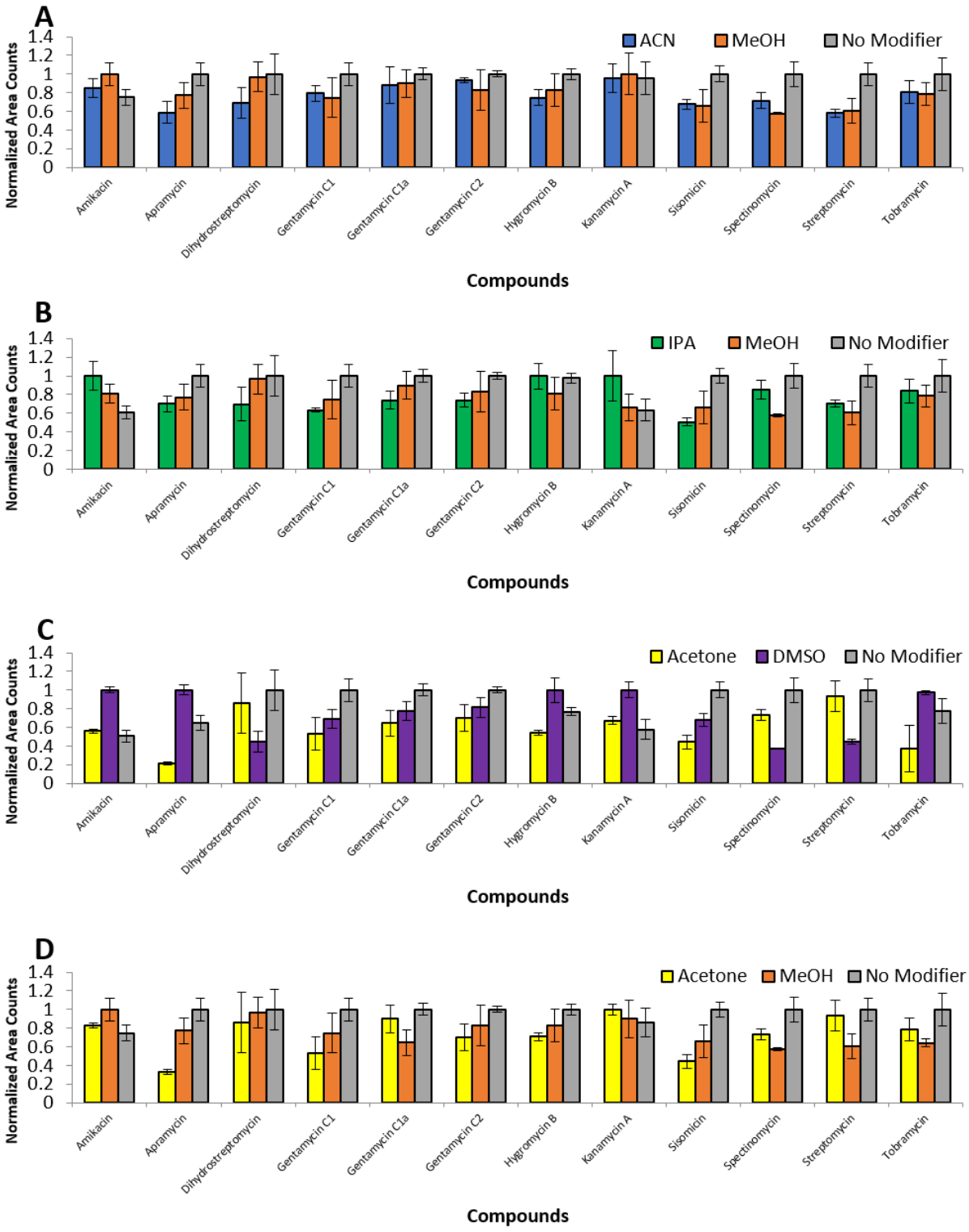

Figure 4.3 Matrix modification investigation (**A**) ACN & MeOH (**B**) IPA & MeOH (**C**) Acetone & DMSO (**D**) Acetone & MeOH

To investigate the effect of polarity and solvation closer, another protic solvent, IPA was evaluated as a potential modifier. The response between IPA and MeOH as modifiers were compared in Figure 4.3(B). The polarity index of IPA and MeOH are 3.9 and 5.1 respectively.[117] The responses of analytes show an increase when using IPA as the modifier compared to MeOH. Being a protic solvent, both modifiers have similar abilities to break the solvation cages, however the lower polarity of IPA lowers the affinity of aminoglycosides to the matrix. This, in turn, leads to a higher extraction efficiency for the IPA modified sample.

To further understand the role of modifiers, aprotic solvents like acetone, and DMSO were used. Both solvents are structurally similar, with the primary difference being the sulfur in DMSO substituted with a carbon for acetone which leads to different polarities. The polarity index of DMSO and acetone are 7.2 and 5.1 respectively.[117] The responses in Figure 4.3(C) of the analytes generally showed a significant increase when using DMSO as the modifier compared to acetone. DMSO cannot lower the polarity of the sample to the same extent as acetone, based on the polarity index. Instead, DMSO is a well-known solvent for the breaking of a hydrogen bond and solvation/hydration sphere.[118] This result indicates that DMSO enhances extraction efficiency may be primarily by breaking the hydration sphere instead of lowering the polarity.[118]

The above experimental evidence suggests both polarity and solvation/hydration have significant effects on the extraction of the aminoglycosides from water. This has further evidence by comparing analytes response when acetone or MeOH are used as modifiers in Figure 4.3(D). Both solvents have a polarity index of 5.1.[117] However, MeOH modified samples tend to show a higher response to that of acetone modified samples, despite the same effect regarding lowering polarity.

Considering the above results and discussion, it is clear that both solvation and polarity are influential factors and are simultaneously affecting the extraction of aminoglycosides from aqueous samples. Thus, selecting an appropriate matrix modifier is critical for enhancing the performance of the assay. Further optimizations were performed to obtain an optimal composition of modifiers for the screening aminoglycosides by SPME-PESI/MS/MS. These experiments investigated the response of different proportions of DMSO, IPA, and MeOH used to modify the spiked water sample, and better understand how the proportions of modifier solvent to spiked water samples would positively or negatively impact the extraction of aminoglycosides from water. It is evident from Figure 4.4 that the quantity of organic modifiers also has a significant impact on the extraction of aminoglycosides. The response of the analytes tends to increase with the quantity of organic modifiers. When the water samples were modified with 3 equivalents of organic solvents, the analytes responses tend to be higher compared to unmodified water sample (300 ng mL^{-1}) despite the concentration of the analytes being a quarter of the concentration of the unmodified samples. Based on the results in Figure 4.4 and considering the decrease in polarity and disruption of the solvation sphere around the aminoglycosides, IPA was selected as a suitable modifier over other organic solvents. Using 3 equivalents of IPA as the modifier gave the best results for dihydrostreptomycin, streptomycin, and spectinomycin which had the lowest responses of the aminoglycosides which factored heavily into this decision. DMSO was discarded as it is not being compatible with MS systems and it has a high boiling solvent, leading to long drying times before being used for SPME-PESI-MS/MS. to the MS system.

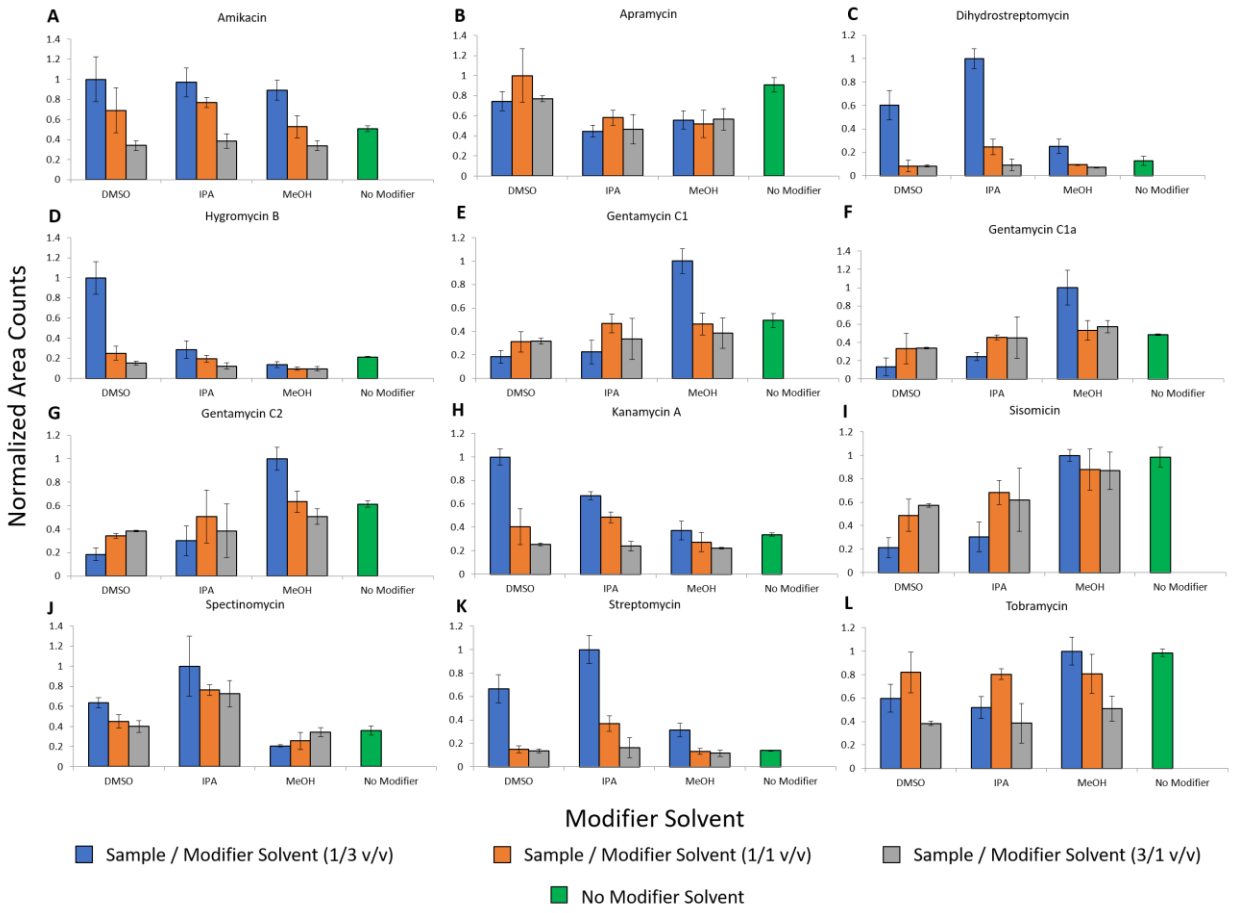

Figure 4.4 Extended Matrix Modification of Water to Enhance Aminoglycoside Extraction (**A**) Amikacin (**B**) Apramycin (**C**) Dihydrostreptomycin (**D**) Hygromycin B (**E**) Gentamycin C1 (**F**) Gentamycin C1A (**G**) Gentamycin C2 (**H**) Kanamycin A (**I**) Sisomicin (**J**) Spectinomycin (**K**) Streptomycin (**L**) Tobramycin

4.4.3 Effect of Desorption Solvent

The optimization of desorption solvent is a critical component for SPME-PESI-MS/MS as the desorption solvent must balance the following factors: optimum desorption of the analytes from the coating, the amount of desorption solvent loaded onto the coated PESI probe from the sample plate by the picking process, and the compatibility with the ESI process. Based on the results in Figure 4.5, the optimal desorption solvent was determined IPA/H_2O (v/v 3/2) + 0.1% FA. This desorption solvent gave the best response overall especially for the aminoglycosides that

tended to give lower responses. The FA plays a pivotal role for desorption as it should be noted that the extraction matrix was composed of IPA/H_2O (v/v 3/1). The mechanism of desorption hinges on the acidic desorption solvent protonating the amino groups of the aminoglycosides leading to the disruption of the interactions between the coating and analytes.

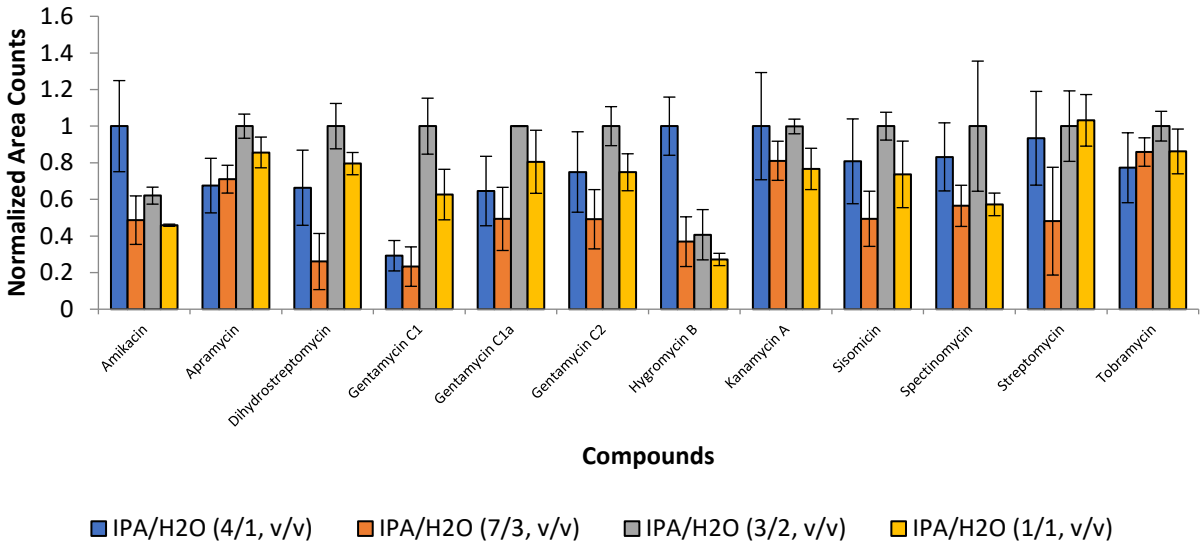

Figure 4.5 Optimization of Desorption Solvent for Aminoglycosides by SPME-PESI-MS/MS.

4.4.4 Effect of Rinsing

The effect of rinsing before the desorption/ionization step on the response produced by the SPME-PESI-MS/MS was investigated using water as rising solvent. This investigation was conducted to check whether the aminoglycosides would remain onto the coating after a rinsing step or not. The results of this investigation are compiled in Figure 4.6. The results show that rinsing with water did not show any significant decrease in signal when compared to no rinsing. This provides evidence that the aminoglycosides were strongly bound onto the coating of the coated PESI probes.

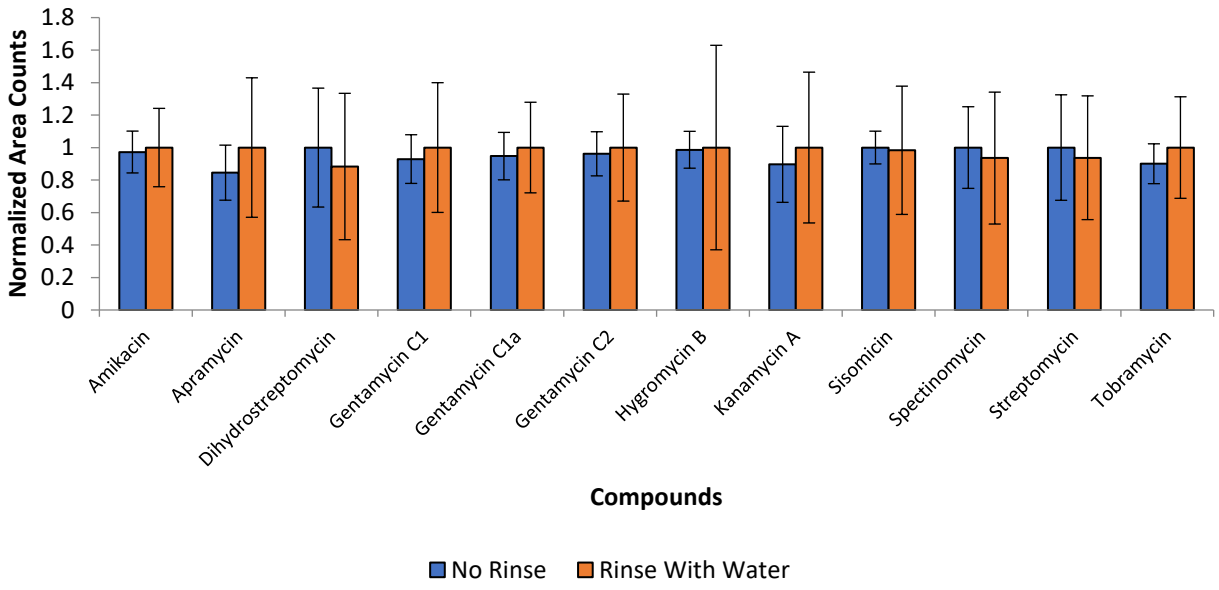

Figure 4.6 Investigation of the Effect of Rinse Solvents.

4.5 Conclusion and Future Perspectives

In this work, the qualitative screening parameters like different extraction and desorption conditions of aminoglycosides were investigated for SPME-PESI-MS/MS. It was found that a pH range between 6 – 8, with no salt, and the addition of IPA as an organic modifier gave optimal responses. The optimal desorption solvent found was IPA/H_2O (v/v 3/2) + 0.1%FA. Based on these parameters, the 12 aminoglycosides spiked in water were able to be screened. Future experiments to investigate the ability of this method to quantitate aminoglycosides include an ETP and a calibration curve. The ability to quantify these analytes by SPME-PESI-MS/MS can be characterized further with more complex matrices such as food stuffs and clinical samples. In a more broader context, the work done in this chapter demonstrates the future of SPME based technologies to target more polar compounds with novel coatings and the use of AIMS technology for screening methods in place of LC-MS based screening that use LC conditions which are not highly compatible for MS longevity.

Chapter 5 Conclusion and Looking Forward

In conclusion, SPME-PESI-MS/MS is a novel method to incorporate SPME as a sample preparation method to an already commercial PESI-MS system. Unlike previous mentions of literature using SPME-PESI, this variant of SPME-PESI uses commercial PESI probes which are 120 µm in diameter. This leads to the use of a pick and spray method for desorption and subsequently ionization. In chapter 2 several underlying aspects behind SPME-PESI-MS/MS was explored and then SPME-PESI-MS/MS was applied to 30µL plasma samples. The underlying aspects explored included the reproducibility of coating the probe, why the chromatograms of SPME-PESI-MS/MS look reminiscent of decay curves, and factors that influence the optimal desorption solvent. In chapter 3 the small dimensions of the coated probes were leveraged to perform free concentration and PPB measurements of diazepam from human plasma. Ultimately a PPB of 99.3±0.2% was derived from a sample of human plasma spiked with 25 ng mL^{-1} of diazepam. This makes it the first public reporting of AIMS technology quantitating PPB. In chapter 4 the use of SPME-PESI-MS/MS for the detection of aminoglycosides was explored by optimizing factors increasing the extraction efficiency. It was noted that controlling pH, salt concentration, and adding organic modifier are important when extracting aminoglycosides using SPME-PESI-MS/MS.

Looking forward there are many exciting aspects about SPME-PESI-MS/MS to be explored. The more basic aspects of SPME-PESI-MS/MS need to be investigated in greater depth. Different desorption solvent compositions and how their various physical properties effect the overall desorption and ionization process including tertiary mixtures as desorption solvents. Furthermore, a more in-depth study into how the desorption volume affects response should be

conducted while isolating these effects from the increased ionization efficiency from the small initial droplet size generated by PESI systems. The ability to agitate the coated probes are another important aspect to explore as orbital rotation used initially was found to be irreproducible. Expanding the use of SPME-PESI-MS/MS and AIMS in general in the arena of free concentration will be exciting and would hopefully lead to TDM that use more therapeutically relevant measurements. The exploration of extracting aminoglycosides by SPME-PESI-MS/MS hopefully provides a way forward for future alternative AIMS screening methods to current LC based screening methods that places MS systems under extremely high salt concentrations or ion pairing reagents.

References

(1) Pawliszyn, J. Solid-Phase Microextraction in Perspective. *Handb. Solid Phase Microextraction*; Elsevier, London, England. **2012**.

(2) Huang, M. Z.; Cheng, S. C.; Cho, Y. T.; Shiea, J. *Anal. Chim. Acta* **2011**, *702* (1), 1–15.

(3) Cooks, R. G.; Ouyang, Z.; Takáts, Z.; Wiseman, M. W. *Science* **2006**, *311*(5767), 1566-1570.

(4) Takáts, Z.; Wiseman, J. M.; Gologan, B.; Cooks, R. G. *Science* **2004**, *306* (5695), 471–473.

(5) Kuo, C. P.; Shiea, J. *Anal. Chem.* **1999**, *71* (19), 4413–4417.

(6) Gross, J. H. *Anal. Bioanal. Chem.* **2014**, *406* (1), 63–80.

(7) Kuo, T. H.; Dutkiewicz, E. P.; Pei, J.; Hsu, C. C. *Anal. Chem.* **2020**, *92* (3), 2353–2363.

(8) Shelley, J. T.; Badal, S. P.; Engelhard, C.; Hayen, H. *Anal. Bioanal. Chem.* **2018**, *410* (17), 4061–4076.

(9) Reyes-Garcés, N.; Alam, M. N.; Pawliszyn, J. *Anal. Chim. Acta* **2018**, *1001*, 40–50.

(10) Kertesz, V.; Cahill, J. F. *Anal. Bioanal. Chem.* **2020**, 1–18.

(11) Li, X.; Ma, W.; Li, H.; Ai, W.; Bai, Y.; Liu, H. *Anal. Bioanal. Chem.* **2018**, *410*(3), 715–724.

(12) Venter, A. R.; Douglass, K. A.; Shelley, J. T.; Hasman, G.; Honarvar, E. *Anal. Chem.* **2014**, *86* (1), 233–249.

(13) Javanshad, R.; Venter, A. R. *Analytical Methods* **2017**, *9*(34), 4896–4907.

(14) Frey, B. S.; Damon, D. E.; Badu-Tawiah, A. K. *Mass Spectrom. Rev.* **2020**, *39* (4), 336–370.

(15) de Paula, C.; Jurisch, M.; Piccin, E.; Augusti, R. *Drug Test. Anal.* **2018**, *10* (9), 1348–1357.

(16) Li, L.; Chen, Y.; Yang, Y.; Yang, Y.; Yang, L.; Wang, Z. *Anal. Bioanal. Chem.* **2020**, *412* (12), 2939–2947.

(17) Arthur, C. L.; Pawliszyn, J. **1990**, *62*(19), 2145-2148.

(18) Gómez-Ríos, G. A.; Mirabelli, M. F. *TrAC - Trends Anal. Chem.* **2019**, *112*, 201–211.

(19) Risticevic, S.; Lord, H.; Górecki, T.; Arthur, C. L.; Pawliszyn, J. *Nat. Protoc.* **2010**, *5* (1), 122–139.

(20) Spietelun, A.; Kloskowski, A.; Chrzanowski, W.; Namieśnik, J. *Chem. Rev.* **2012**, *113* (3), 1667–1685.

(21) Ouyang, G.; Pawliszyn, J. *J. Chromatogr. A* **2007**, *1168*, 226–235.

(22) Ouyang, G.; Pawliszyn, J. *Anal. Chim. Acta* **2008**, *627* (2), 184–197.

(23) Reyes-Garcés, N.; Gionfriddo, E.; Gómez-Ríos, G. A.; Alam, M. N.; Boyacı, E.; Bojko, B.; Singh, V.; Grandy, J.; Pawliszyn, J. *Anal. Chem.* **2017**, *90* (1), 302–360.

(24) Gómez-Ríos, G. A.; Pawliszyn, J. *Chem. Commun.* **2014**, *50* (85), 12937–12940.

(25) Kennedy, J. H.; Aurand, C.; Shirey, R.; Laughlin, B. C.; Wiseman, J. M. *Anal. Chem.* **2009**, *311* (1), 7502–7508.

(26) Tascon, M.; Alam, M. N.; Gómez-Ríos, G. A.; Pawliszyn, J. *Anal. Chem.* **2018**, *90* (4), 2631–2638.

(27) Gómez-Ríos, G. A.; Pawliszyn, J. *Angew. Chemie - Int. Ed.* **2014**, *53* (52), 14503–14507.

(28) Gómez-Ríos, G. A.; Tascon, M.; Pawliszyn, J. *Bioanalysis* **2018**, *10* (4), 257–271.

(29) Tascon, M.; Gómez-Ríos, G. A.; Reyes-Garcés, N.; Poole, J.; Boyacl, E.; Pawliszyn, J. *Anal. Chem.* **2017**, *89* (16), 8421–8428.

(30) Hiraoka, K.; Nishidate, K.; Mori, K.; Asakawa, D.; Suzuki, S. *Rapid Commun. Mass Spectrom.* **2007**, *21* (18), 3139–3144.

(31) Chen, L. C.; Nishidate, K.; Saito, Y.; Mori, K.; Asakawa, D.; Takeda, S.; Kubota, T.; Hori, H.; Hiraoka, K. *J. Phys. Chem. B* **2008**, *112* (35), 11164–11170.

(32) Yu, Z.; Chen, L. C.; Mandal, M. K.; Nonami, H.; Erra-Balsells, R.; Hiraoka, K. *J. Am. Soc. Mass Spectrom* **2012**, *23*, 728–735.

(33) Yoshimura, K.; Chen, L. C.; Yu, Z.; Hiraoka, K.; Takeda, S. *Anal. Biochem.* **2011**, *417* (2), 195–201.

(34) Mandal, M. K.; Chen, L. C.; Hiraoka, K. *J. Am. Soc. Mass Spectrom* **2011**, *22*, 1493–1500.

(35) Rahman, M. O.; Mandal, M. K.; Shida, Y.; Ninomiya, S.; Chen, L. C.; Nonami, H.; Hiraoka, K. *J. Mass Spectrom.* **2013**, *48* (7), 823–829.

(36) Zaitsu, K.; Hayashi, Y.; Murata, T.; Ohara, T.; Nakagiri, K.; Kusano, M.; Nakajima, H.; Nakajima, T.; Ishikawa, T.; Tsuchihashi, H.; Ishii, A. *Anal. Chem.* **2016**, *88* (7), 3556–3561.

(37) Yoshimura, K.; Hiraoka, K.; Johno, H.; Takeda, S.; Nakajima, M.; Chen, L. C. *Mass Spectrom.* **2014**, *3*, S0048–S0048.

(38) Iwasa, M.; Rahman, M. M.; Mandal, M. K.; Nonami, H.; Saha, S.; Shida, Y.; Ozawa, T.; Hiraoka, K. *J. Agric. Food Chem.* **2013**, *61* (33), 7889–7895.

(39) Usui, K.; Murata, T.; Fujita, Y.; Kamijo, Y.; Hanazawa, T.; Yoshizawa, T.; Funayama, M. *Drug Test. Anal.* **2018**, *10* (6), 1033–1038.

(40) Usui, K.; Kobayashi, H.; Fujita, Y.; Kubota, E.; Hanazawa, T.; Yoshizawa, T.; Kamijo, Y.; Funayama, M. *J. Food Drug Anal.* **2019**, *27* (3), 786–792.

(41) Usui, K.; Minami, E.; Fujita, Y.; Kubota, E.; Kobayashi, H.; Hanazawa, T.; Yoshizawa, T.; Kamijo, Y.; Funayama, M. *J. Pharm. Biomed. Anal.* **2019**, *174*, 175–181.

(42) Usui, K.; Minami, E.; Fujita, Y.; Kobayashi, H.; Hanazawa, T.; Kamijo, Y.; Funayama, M. *J. Pharmacol. Toxicol. Methods* **2019**, *100*, 106610

(43) Hisatsune, K.; Murata, T.; Ogata, K.; Hida, M.; Ishii, A.; Tsuchihashi, H.; Hayashi, Y.; Zaitsu, K. *ACS Omega* **2020**, *5* (36), 23351–23357.

(44) Bernardo, R. A.; da Silva, L. C.; Queiroz, M. E. C.; Vaz, B. G.; Chaves, A. R. *J. Chromatogr. A* **2019**, *1603*, 23–32.

(45) da Silva, L. C.; de Carvalho, T. C.; Pereira, I.; Marana, J. C.; Laviola, B. G.; Abdelnur, P. V.; Vaz, B. G. *J. Am. Soc. Mass Spectrom.* **2019**, *30* (10), 2051–2059.

(46) Cody, R. B.; Laramée, J. A.; Durst, H. D. *Anal. Chem.* **2005**, *77* (8), 2297–2302.

(47) Wang, H.; Liu, J.; Cooks, R. G.; Ouyang, Z. *Angew. Chemie Int. Ed.* **2010**, *49* (5), 877–880.

(48) Na, N.; Zhang, C.; Zhao, M.; Zhang, S.; Yang, C.; Fang, X.; Zhang, X. *J. Mass Spectrom.* **2007**, *42* (8), 1079–1085.

(49) Yoshimura, K.; Lee, C. C.; Asakawa, D.; Hiraoka, K.; Takeda, S. *J. Mass Spectrom.* **2009**, *44* (6), 978–985.

(50) Yu, Z.; Chen, L. C.; Suzuki, H.; Ariyada, O.; Erra-Balsells, R.; Nonami, H.; Hiraoka, K. *J. Am. Soc. Mass Spectrom.* **2009**, *20* (12), 2304–2311.

(51) Belardi, R. P.; Pawliszyn, J. B. *A Water Pollution Research Journal of Canada*; **1989**, *24*(1), 179–191

(52) Grandy, J. J.; Lashgari, M.; Heide, H. Vander; Poole, J.; Pawliszyn, J. *Environ. Pollut.* **2019**, *252*, 825–834.

(53) Khaled, A.; Gionfriddo, E.; Acquaro, V.; Singh, V.; Pawliszyn, J. *Anal. Chim. Acta* **2019**, *1056*, 34–46.

(54) Rickert, D. A.; Gómez-Ríos, G. A.; Nazdrajić, E.; Tascon, M.; Kulasingam, V.; Pawliszyn, J. B. *Anal. Bioanal. Chem.* **2020**, *412* (21), 5067–5076.

(55) D'Agostino, P. A.; Chenier, C. L.; Hancock, J. R.; Jackson Lepage, C. R. *Rapid Commun. Mass Spectrom.* **2007**, *21* (4), 543–549.

(56) Mirnaghi, F. S.; Pawliszyn, J. *Anal. Chem.* **2012**, *84* (19), 8301–8309.

(57) Hu, B.; Zheng, B.; Rickert, D.; Gómez-Ríos, G. A.; Bojko, B.; Pawliszyn, J.; Yao, Z. P. *Anal. Chim. Acta* **2019**, *1075*, 112–119.

(58) Vasiljevic, T.; Singh, V.; Pawliszyn, J. *Talanta* **2019**, *199*, 689–697.

(59) Lendor, S.; Hassani, S. A.; Boyaci, E.; Singh, V.; Womelsdorf, T.; Pawliszyn, J. *Anal. Chem.* **2019**, *91* (7), 4896–4905.

(60) Xu, J.; Liu, X.; Wang, Q.; Huang, S.; Yin, L.; Xu, J.; Liu, X.; Jiang, R.; Zhu, F.; Ouyang, G. *Anal. Chem.* **2018**, *90* (3), 1572–1577.

(61) Vuckovic, D.; Pawliszyn, J. *J. Pharm. Biomed. Anal.* **2009**, *50* (4), 550–555.

(62) Peltenburg, H.; Bosman, I. J.; Hermens, J. L. M. *J. Pharm. Biomed. Anal.* **2015**, *115*, 534–542.

(63) Lambrinidis, G.; Vallianatou, T.; Tsantili-Kakoulidou, A.. *Adv. Drug Deliv. Rev.* **2015**, *86*, 27–45.

(64) Zhang, F.; Xue, J.; Shao, J.; Jia, L. *Drug Discov. Today* **2012**, *17* (9/10), 475–485.

(65) Bohnert, T.; Gan, L. S. *J. Pharm. Sci.* **2013**, *102* (9), 2953–2994.

(66) Kratochwil, N. A.; Huber, W.; Müller, F.; Kansy, M.; Gerber, P. R. *Current Opinion in Drug Discovery and Development* **2004**, *7*(4), 507–512.

(67) Zhou, H. H.; Adedoyin, A.; Wilkinson, G. R. *Clin. Pharmacol. Ther.* **1990**, *48* (1), 10–17.

(68) Alván, G.; Bergström, K.; Borgå, O.; Iselius, L.; Pedersen, N. *Eur. J. Clin. Pharmacol.* **1983**, *25* (4), 437–441.

(69) Feely, J.; Forrest, A.; Gunn, A.; Hamilton, W.; Stevenson, I.; Crooks, J. *Clin. Pharmacol. Ther.* **1980**, *28* (6), 759–764.

(70) Musteata, F. M. *Bioanalysis* **2011**, *3* (15), 1753–1768.

(71) Musteata, F. M. *Bioanalysis* **2017**, *9*(10) 767–769.

(72) Lima, J. J.; MacKichan, J. J.; Libertin, N.; Sabino, J. *J. Pharmacokinet. Biopharm.* **1983**, *11* (5), 483–498.

(73) Musteata, F. M.; Pawliszyn, J.; Qian, M. G.; Wu, J. T.; Miwa, G. T. *J. Pharmaceutical Sciences*. **2006**, *95*(8), 1712–1722.

(74) Ferguson, K. C.; Luo, Y. S.; Rusyn, I.; Chiu, W. A. *Toxicol. Vitr.* **2019**, *60*, 245–251.

(75) Reimerová, P.; Stariat, J.; Bavlovič Piskáčková, H.; Jansová, H.; Roh, J.; Kalinowski, D. S.; Macháček, M.; Šimůnek, T.; Richardson, D. R.; Štěrbová-Kovaříková, P. *Anal. Bioanal. Chem.* **2019**, *411* (11), 2383–2394.

(76) Boyacl, E.; Bojko, B.; Reyes-Garcés, N.; Poole, J. J.; Gómez-Ríos, G. A.; Teixeira, A.; Nicol, B.; Pawliszyn, J. *Sci. Rep.* **2018**, *1167*(8), 1–10

(77) Górecki, T.; Pawliszyn, J. *Analyst* **1997,** *122*(10), 1079–1086.

(78) Heringa, M. B.; Hermens, J. L. M. *TrAC - Trends Anal. Chem.* **2003**, *22* (9), 575–587.

(79) Klotz, U.; Antonin, K. H.; Bieck, P. R. *J. Pharmacol. Exp. Ther.* **1976**, *199* (1), 67–73.

(80) Lázníček, M.; Lamka, J.; Květina, J. *Biochem. Pharmacol.* **1982**, *31* (7), 1455–1458.

(81) Davis, D.; Grossman, S.; Kitchell, B.; Shand, D.; Routledge, P. *Br. J. Clin. Pharmacol.* **1985**, *19* (2), 261–265.

(82) Krause, K. M.; Serio, A. W.; Kane, T. R.; Connolly, L. E. *Cold Spring Harb. Perspect. Med.* **2016**, *6* (6), 1–19.

(83) Farouk, F.; Azzazy, H. M. E.; Niessen, W. M. A. *Anal. Chim. Acta* **2015**, *890*, 21–43.

(84) Jiang, M.; Karasawa, T.; Steyger, P. S. *Frontiers in Cellular Neuroscience* **2017**, 11, 308.

(85) Wargo, K. A.; Edwards, J. D. *J. Pharm. Pract.* **2014**, *27* (6), 573–577.

(86) Guthrie, O. W. *Toxicology* **2008**, *249*, 91–96

(87) Durante-Mangoni, E.; Grammatikos, A.; Utili, R.; Falagas, M. E. *Int. J. Antimicrob. Agents* **2009**, *33* (3), 201–205.

(88) Stankowicz, M. S.; Ibrahim, J.; Brown, D. L. *Am. J. Heal. Pharm.* **2015**, *72* (16), 1357–1364.

(89) Becker, B.; Cooper, M. A. *ACS Chem. Biol.* **2013**, *8* (1), 105–115.

(90) *Official Journal of the European Communities* **1996,** *39*(L 125), 1–35.

(91) Felis, E.; Kalka, J.; Sochacki, A.; Kowalska, K.; Bajkacz, S.; Harnisz, M.; Korzeniewska, E. *Eur. J. Pharmacol.* **2020**, *866*, 172813.

(92) Löffler, D.; Ternes, T. A. *J. Chromatogr. A* **2003**, *1000*, 583–588.

(93) Tahrani, L.; Van Loco, J.; Mansour, H. Ben; Reyns, T. *J. Water Health* **2016**, *14* (2), 208–213.

(94) Ngigi, A. N.; Magu, M. M.; Muendo, B. M. *Environ. Monit. Assess.* **2020**, *192* (1), 1–16.

(95) An, X. L.; Su, J. Q.; Li, B.; Ouyang, W. Y.; Zhao, Y.; Chen, Q. L.; Cui, L.; Chen, H.; Gillings, M. R.; Zhang, T.; Zhu, Y. *Environ. Int.* **2018**, *117*, 146–153.

(96) Harnisz, M.; Korzeniewska, E. *Sci. Total Environ.* **2018**, *626*, 377–383.

(97) McGlinchey, T. A.; Rafter, P. A.; Regan, F.; McMahon, G. P. *Anal. Chim. Acta* **2008**, *624* (1), 1–15.

(98) Diamanti, I.; Pucciarini, L.; Moretti, S.; Sardella, R.; Giusepponi, D.; Saluti, G.; Galarini, R.; Rossi, R. *Food Chem.* **2018**, *266*, 9–16.

(99) Staub Spörri, A.; Jan, P.; Cognard, E.; Ortelli, D.; Edder, P. *Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess.* **2014**, *31* (5), 806–816.

(100) Delatour, T.; Racault, L.; Bessaire, T.; Desmarchelier, A. *Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess.* **2018**, *35* (4), 632–645.

(101) Zhao, H.; Zulkoski, J.; Mastovska, K. *J. Agric. Food Chem.* **2017**, *65*(34), 7268–7287.

(102) Savoy, M. C.; Woo, P. M.; Ulrich, P.; Tarres, A.; Mottier, P.; Desmarchelier, A. *Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess.* **2018**, *35* (4), 674–685.

(103) Bessaire, T.; Mujahid, C.; Beck, A.; Tarres, A.; Savoy, M. C.; Woo, P. M.; Mottier, P.; Desmarchelier, A. *Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess.*

2018, *35* (4), 661–673.

(104) Desmarchelier, A.; Anizan, S.; Minh Tien, M.; Savoy, M. C.; Bion, C. *Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess.* **2018**, *35* (4), 686–694.

(105) Desmarchelier, A.; Fan, K.; Minh Tien, M.; Savoy, M. C.; Tarres, A.; Fuger, D.; Goyon, A.; Bessaire, T.; Mottier, P. *Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess.* **2018**, *35* (4), 646–660.

(106) Chiaochan, C.; Koesukwiwat, U.; Yudthavorasit, S.; Leepipatpiboon, N. *Anal. Chim. Acta* **2010**, *682* (1–2), 117–129.

(107) Ianni, F.; Pucciarini, L.; Carotti, A.; Saluti, G.; Moretti, S.; Ferrone, V.; Sardella, R.; Galarini, R.; Natalini, B. *Anal. Chim. Acta* **2018**, *1044*, 174–180.

(108) Kahsay, G.; Song, H.; Van Schepdael, A.; Cabooter, D.; Adams, E. *J. Pharm. Biomed. Anal.* **2014**, *87*, 142–154.

(109) Wang, X.; Yang, S.; Li, Y.; Zhang, J.; Jin, Y.; Zhao, W.; Zhang, Y.; Huang, J.; Wang, P.; Wu, C.; Zhou, J. *J. Chromatogr. A* **2018**, *1542*, 28–36.

(110) Moreno-González, D.; Hamed, A. M.; García-Campaña, A. M.; Gámiz-Gracia, L. *Talanta* **2017**, *171*, 74–80.

(111) Yang, B.; Wang, L.; Luo, C.; Wang, X.; Sun, C. *J. AOAC Int.* **2017**, *100* (6), 1869–1878.

(112) Chen, L.; Mei, M.; Huang, X. *J. Sep. Sci.* **2017**, *40* (21), 4203–4212.

(113) Wang, J.; Zhao, Q.; Jiang, N.; Li, W.; Chen, L.; Lin, X.; Xie, Z.; You, L.; Zhang, Q. *J. Chromatogr. A* **2017**, *1485*, 24–31.

(114) Ishii, R.; Horie, M.; Chan, W.; MacNeil, J. *Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess.* **2008**, *25* (12), 1509–1519.

(115) Kaufmann, A.; Butcher, P.; Maden, K. *Anal. Chim. Acta* **2012**, *711*, 46–53.

(116) Díez, C.; Guillarme, D.; Staub Spörri, A.; Cognard, E.; Ortelli, D.; Edder, P.; Rudaz, S. *Anal. Chim. Acta* **2015**, *882*, 127–139.

(117) Snyder, L. R. *J. Chromatogr. Sci.* **1978**, *16* (6), 223–234.

(118) Roy, K. S.; Mazumder, A.; Goud, D. R.; Dubey, D. K. A *Eur. Polym. J.* **2018**, *98*, 105–115.